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MOLECULAR CHARACTERIZATION OF ESTROGEN RECEPTOR BETA VARIANTS; CANCER CELL PROLIFERATION AND INVASION

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To my parents and my son Weillison Hsu

献给我敬爱的父母，外婆和儿子

ABSTRACT

Estrogen plays crucial roles in the pathogenesis of breast cancer. Most of the known effects of estrogen signaling are mediated by estrogen receptors (ERs), ERα and ERβ. ERα is explored for breast cancer molecular classification and is a target of endocrine therapy. The discovery of the second ER (ERβ) including its variants led to a need for re-evaluation of the biology of estrogen. This thesis aims to characterize molecular aspects of ERβ variants and provide knowledge to elucidate roles of ERβ variants in tumorigenesis with focus on breast cancer.

In **PAPER I**, we determined the frequency of a novel human ERβ isoform, human ERβ548 (hERβ548), which had been demonstrated to display different functional characteristics than wild-type ERβ, in several populations including African (n = 96), Caucasian (n = 100), and Asian (n = 128) subjects. We did not detect any alleles that correspond to hERβ548 in these samples or in additional samples of heterogeneous origin. This study concluded, for the first time, that hERβ548 is not a common variant in Africans, Caucasians, or Asians.

In **PAPER II**, we identified five novel polymorphisms in the ERβ gene in an African population. Two of these variants, I3V and V320G were expected to change the amino acid sequence of the ERβ protein. Compared to the wild-type ERβ, the V320G variant showed significantly decreased maximal transcriptional activity in the ERE mediated reporter assay. A pull-down assay and surface plasmon resonance analysis revealed that the decreased transcriptional activity of the novel ERβ variant hERβV320G was associated with weaker interaction with a co-factor, TIF2.

In **PAPER III**, we assayed the interaction of several known ligands with mouse ERβ1 (mERβ1) and mouse ERβins (mERβ2). A significant difference in ligand binding properties was observed. Our results suggest that ligand selectivity and co-activator recruitment of ERβ isoforms constitute additional levels of specificity that influence the transcriptional response in estrogen target cells in mouse.

In **PAPER IV**, 202 clinical patient specimens, different non-small cell lung cancer (NSCLC) cell lines and transgenic mouse models were used to investigate the role of the EGFR signaling pathway for tumorigenesis of NSCLC. We showed that activation of the EGFR pathway or hypoxia could promote cell invasion but not survival. Furthermore, we demonstrated that the HIF-1α/MET axis is involved in both EGFR and hypoxia induced signaling pathways, leading to cancer cell invasiveness.

In **PAPER V**, a breast cancer cell line BT549 that endogenously expresses the hERβ variant hERβ2 in the absence of ERα and wild-type ERβ was used to study the effects of hERβ2 signaling on breast cancer cell behavior and associated molecular mechanisms. Our data indicate that hERβ2 promotes proliferation and invasion in this cell line. A total of 263 genes were identified as hERβ2-upregulated genes and 662 identified as hERβ2-downregulated genes. hERβ2-regulated genes were involved in cell morphology, DNA replication and repair, cell death and survival. Based on our data, we hypothesize that effects of hERβ2 on proliferation and invasion were mediated via repression of prolyl hydroxylase 3 (PHD3) gene expression and induction of protein levels of the hypoxia induced factor 1 (HIF-1α) and MET.

In conclusion, the studies presented in this thesis contribute to the knowledge of the function of ERβ variants, and give additional insight into the molecular mechanisms underlying cancer cell proliferation and invasion.

LIST OF PUBLICATIONS

1. **Xu L**, Pan-Hammarström Q, Försti A, Hemminki K, Hammarström L, Labuda D, Gustafsson JA, Dahlman-Wright K. *Human estrogen receptor beta 548 is not a common variant in three distinct populations.* Endocrinology. 2003 Aug; 144(8):3541-6.

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3. Zhao C, Toresson G, **Xu L**, Koehler KF, Gustafsson JA, Dahlman-Wright K. *Mouse estrogen receptor beta isoforms exhibit differences in ligand selectivity and coactivator recruitment.* Biochemistry. 2005 Jun 7;44(22):7936-44.

4. **Xu L,** Nilsson MB, Saintigny P, Cascone T, Herynk MH, Du Z, Nikolinakos PG, Yang Y, Prudkin L, Liu D, Lee JJ, Johnson FM, Wong KK, Girard L, Gazdar AF, Minna JD, Kurie JM, Wistuba II, Heymach JV. *Epidermal growth factor receptor regulates MET levels and invasiveness through hypoxia-inducible factor-1alpha in non-small cell lung cancer cells.* Oncogene. 2010 May 6;29(18):2616-27.

5. **Xu L**, Zhao C, Dey P, Ström A, Gustafsson JA, Dahlman-Wright K. *Estrogen receptor beta2 induces proliferation and invasion of breast cancer cells; association with regulation of prolyl hydroxylase 3, hypoxia induced factor 1 alpha and MET.* Manuscript

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List of abbreviations

AF	Activation Function
AP-1	Activator Protein 1
AR	Androgen Receptor
CK5/6	Cytokeratins 5 and 6
DBD	DNA binding domain
E2	17β-estradiol
EGFR	Epidermal Growth Factor Receptor
ERα	Estrogen Receptor α
ERβ	Estrogen Receptor β
ERE	Estrogen Response Element
EMT	Epithelial-to-Mesenchymal Transition
HER2	Human Epidermal Growth Factor Receptor 2
HGF	Hepatocyte Growth Factor
HIF-1α	Hypoxia Induced Factor 1α
HPLC	High-Performance Liquid Chromatography
IPA	Ingenuity Pathway Analysis
LBD	Ligand Binding Domain
NR	Nuclear Receptor
PHD	Prolyl hydroxylase domain-containing proteins
PR	Progesterone Receptor
SERM	Selective Estrogen Receptor Modulator
siRNA	Small Interfering RNA
SNP	Single Nucleotide Polymorphism
Sp1	Specificity Protein 1
TKI	Tyrosine-Kinase Inhibitor

1 INTRODUCTION

1.1 Estrogen receptors

Estrogen plays crucial roles in many tissues in the body, and may be involved in the pathogenesis of many endocrine related diseases, such as breast cancer, uterine cancer, prostate cancer, autoimmune diseases, osteoporosis and metabolic disorders. Most of the effects of estrogen are mediated by the estrogen receptors (ERs). For a long time, only one ER was thought to be the receptor that is responsible for mediating the effects of estrogen. This receptor is now called ERα. However, in 1996, another ER, now named ERβ, was reported.

ERs belong to the nuclear receptor (NR) superfamily[1, 2], which includes receptors for glucocorticoids, mineralocorticoids, progesterone, androgens, estrogens, thyroid hormones, vitamin D and retinoic acid. Upon ligand dependent or independent activation, these receptors form dimers and bind to their corresponding response element in the regulatory region of their target genes, thus regulating transcription [3]. For ERs, the specific sequences of DNA are known as estrogen response elements (EREs) [4]. The NRs include four functional domains. The N-terminal A/B domain contributes a ligand-independent transcriptional activation function (AF-1), a site involved in co-activator binding and transcriptional activation of target genes. AF-1 is very active in ERα but has lower activity in ERβ [5]. The DNA binding domain (DBD; sometimes referred to as the C-region) contains two zinc fingers and mediates sequence specific DNA binding and contributes to receptor dimerization. The DBD is linked to the ligand-binding domain (LBD or E/F domain) by the D domain or hinge region that is less well conserved and characterized. The LBD binds various ligands and is also involved in receptor dimerization, nuclear translocation, co-factor binding, and transactivation of target gene expression. An activation function 2 (AF-2) localized within this domain constitutes a ligand-dependent transactivation function.

ERα and ERβ are encoded by separate genes. ERβ has an amino acid identity of 96% to that of ERα in the DBD, which suggests that ERβ would recognize and bind to specific DNA sequences similarly to ERα. The LBD is much less homologous between ERβ and ERα, only 59%, also the ligand binding pockets of the two receptors are different in structure, indicating the possibility of a different ligand spectrum for ERβ versus ERα [6]. The N-terminal AF-1 and C-terminal AF-2 domains are not well conserved, suggesting that the proteins interacting with ERβ for its transcriptional activation functions may be considerably different from those interacting with ERα.

The discovery of the second ER, ERβ, led to a need for re-evaluation of the biology of estrogen. While the physiological functions of ERβ are still being investigated, important insight has been gained, both with regard to its molecular mechanisms of action and its role in physiology and disease. At ERE sites, ERβ has weaker transcriptional activity than ERα [7-10]. Additionally, there are important DNA binding sites for ERs, other than EREs where ERβ shows different, sometimes even opposite, effects to those of ERα, e.g. at AP-1 and Sp1 sites [11-14].

The phenotypes displayed by the ERβ knockout (BERKO) animal model, such as atretic ovary, hyperplastic prostate and neuronal degeneration, suggest that ERβ plays crucial physiological roles. In human, ERα and ERβ show distinct tissue distribution. Altered expression of ERβ has been found in several kinds of cancer tissues, such as breast cancer, prostate cancer and colon cancer, in which ERβ expression is often decreased and sometimes re-gained when the cancer progresses to late stage.

Both ERs are widely expressed in different tissues. Notable differences in tissue and cellular distribution between ERα and ERβ are shown in Table 1.

Table 1 Distribution of ERα and ERβ in human tissues and cells [15-18]

	ERα	ERβ
Adipose tissue	±	±
Adrenal	+	−
Bladder	−	+
Bone	+	+
Bone marrow	−	+
Brain	+	+
Colon	−	+
Endometrium	+	+
Epididymus	−	+
Fallopian tube	−	+
Gastrointestinal tract	−	+
Heart	+	+
Kidney	+	+
Liver	+	−
Lung	−	+
Muscle	−	−
Breast	+	+
Ovarian granulosa cells	+	++
Ovarian theca cells	++	+
Pancreatic cancer	+	+
Pituitary gland	−	+
Prostate	±	++
Small intestine	−	+
Testes	±	+
Thymus	−	+
Uterus	++	+
Vagina	+	−
Vascular endothelium	−	+

1.2 ER ligands

17β-estradiol (E2) is the primary ligand for ER and is the predominant form of estrogen hormone in premenopausal women. Estrone (E1) and estriol (E3) are two other common ligands with estrogenic activity. They have weaker agonist effects than E2 and are found predominantly in postmenopausal women and pregnant women, respectively.

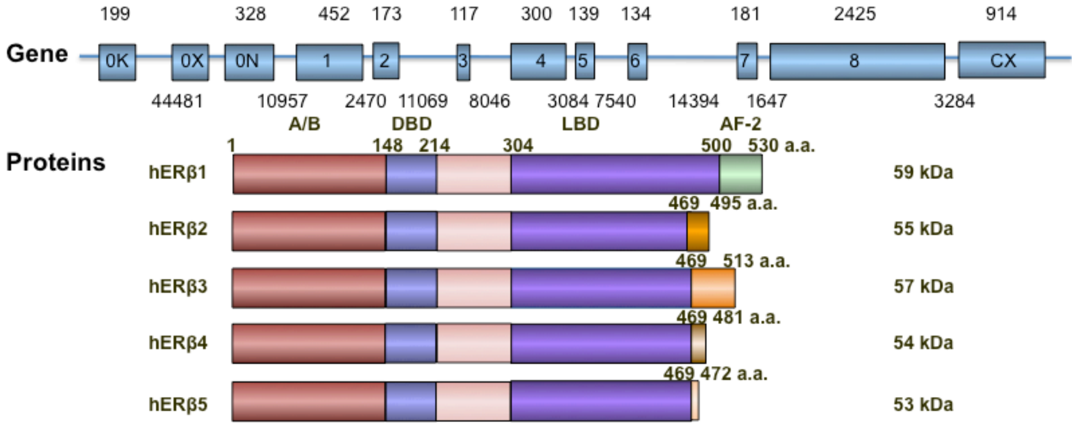
Selective estrogen receptor modulators (SERMs) refer to a group of compounds that act as either an estrogen agonist or antagonist dependent on the cell type and tissue. The commonly used SERMs in breast cancer are tamoxifen, roloxifene and toremifene. Three mechanisms have been proposed to account for the mixed antagonist/agonist effect of SERMs on ERs: (1) distinct ER conformation upon ligand binding; (2) differential expression and binding to the ER of coregulatory proteins; and (3) differential expression of ERα and/or ERβ subtypes in a given target tissue [19]. Fulvestrant/ICI 182,780 is a selective estrogen receptor down-regulator (SERD), a complete ER antagonist. It works by binding to ER and inhibition of its activity by nuclear export and degradation.

ER subtype-selective ligands have been developed. Propyl pyrazole triol (PPT) is a well-characterized synthetic ERα agonist, with a 410-fold relative binding affinity for ERα versus ERβ [20]. 2,3-bis (4-hydroxyphenyl)-propionitrile (DPN) is a well-characterized synthetic ERβ agonist, with a 70–300-fold selectivity for ERβ compared to ERα [21-23]. Phyto-estrogens (plant-derived) SERMs have steroid structures and estrogen-like properties. Genistein is a phytoestrogen, which has a greater binding affinity for ERβ than for ERα [23].

1.3 ERβ isoforms

Like many other genes, ERβ is expressed as different isoforms, the functions of which need to be addressed, in order to fully understand the physiological functions of ERβ. Endogenously expressed ER variants may contribute to the diversity of E2 actions in different tissues. Multiple ERβ isoforms exist as a result of either deletion of one or more coding exons, alternative splicing of the last coding exons, or alternative usage of untranslated exons in the 5’ region. To date, five full-length isoforms, designated ERβ1-5, have been reported in human (Figure 1). The full-length ERβ1 mRNA is translated from 8 exons, encoding 530 amino acids, while the ERβ2-5 transcripts share identical sequences with ERβ1 from exon 1 to exon 7, but have unique sequences in place of exon 8. Functional studies have shown that ERβ2 can form heterodimers with ERα and ERβ1 on ERE and negatively regulate

the transcriptional activity of ERα, but not of ERβ1. ERβ4 and β5 can heterodimerize with ERβ1 and enhance its transcriptional activation function in a ligand-dependent manner [24]. The expression of ERβ3 appears to be restricted to the testis [25]. The properties of these ERβ variants are shown in Table 2.



hERβ 453aaLANLLMLLSHVRHARSNKGMEHLLNMKCKNVVPVYDLLLEMLNAHVLRGCKSSITGSEXSPAEDSKSKEGSQNPQSQ
hERβ2 453aaLANLLMLLSHVRHARAEKASQTLTSFGMKMETLLPEATMEQ
hERβ3 453aaLANLLMLLSHVRHASSLSLSWRLFMLREASCHGVRQTPGGAHMSVSRSRSFEAQQPRE
hERβ4 453aaLANLLMLLSHVRHARWGEKQFIHLKLS
hERβ5 453aaLANLLMLLSHVRHAYAP

Figure 1. Structural and sequence comparisons for the human ERβ gene and protein. *For the gene, exons are indicated with boxes and introns with lines. The numbers above each box designate the size of the exons (bp); the numbers below each line indicates the size of the respective introns (bp). For the proteins, numbers indicate the amino acids positions in each protein and kDa. Functional domains are marked above the protein structure. ERβ variants are formed from alternative splicing of the last coding exon. Amino acid sequences of the unique C terminuses are listed at the bottom.*

Table 2 Properties of human ERβ variants

Isoform	ERβ1	ERβ2	ERβ3	ERβ4	ERβ5
E2 binding[7, 24, 26]	+	–	+	±	±
ERE binding[1, 27]	+	±	NA	+	±
Dimer/ERα[1, 8, 25]	+	+	+	+	+
Dimer/ERβ1[1, 24]	+	+	+	+	+
Homodimers[24]	+	–	NA	-	-
Inhibits ERα function [8]	+	+	+	NA	+
Enhance ERβ function*[1]		–	NA	+	+
Nuclear localization	+	+	NA	+	+
Cancers[28-33]	CC, BC, TC, OC	BC, PC, EC, CC,GC, OC, TC			BC, OC

* Enhance ERβ1-induced transactivation in a ligand-dependent manner
+, interaction; -, no interaction; NA, information not available; BC, breast carcinoma; PC, prostate carcinoma; EC, endometrioid carcinoma; CC, colorectal carcinoma; GC, gastric adenocarcinoma; OC, ovary carcinoma; TC, thyroid cancer.

More specifically, the following ERβ isoforms have been characterized in this thesis.

1.3.1 Potential N-terminus extended variant, human ERβ548

Human ERβ (hERβ) cDNA was cloned as a protein of 485 amino acids in length in 1996 [7, 8]. In 1998, the N-terminus of hERβ was extended with 45 extra amino acids. This protein is now referred to as hERβ1 [1, 2]. hERβ1 has been considered as the full length hERβ. However, a hERβ cDNA that corresponds to a protein of 548 amino acids was cloned from human testis cDNA. It was suggested that a genomic polymorphism corresponding to an insertion of an extra A-T base pair places an upstream ATG in frame with the rest of the coding sequence. The authors speculate that this might represent a polymorphism in human populations [34]. However, our study of African, Caucasian and Asian populations failed to support that human ERβ548 exists [35].

1.3.2 **hERβ2**

The most studied variant of hERβ, hERβ2, is the result of alternative splicing, exchanging exon 8 for an alternative exon which has 26 unique amino acids. hERβ2 lacks the AF-2 core region and has undetectable affinity for ligands. It acts as a dominant-negative inhibitor of ERα [1]. It has been shown that hERβ2 heterodimerizes with ERα and inhibits ligand induced ERα transcriptional activity by inducing proteasome-dependent degradation of ERα [36]. hERβ2 was also shown to inhibit recruitment of ERα to estrogen-responsive promoters, leading to suppression of the expression of ERα regulated genes including *CCND1* and *CDKN1A*.

Evidences suggest that in contrast to hERβ1, hERβ2 has proliferative characteristics and is associated with cell proliferation and invasion and aggressive phenotype [37]. The expression of hERβ2 is widespread and has been reported to have higher expression in cancer tissue compared to normal tissue for breast, colorectal, prostate and non-small cell lung cancer [28, 33, 38, 39].

1.3.3 **rERβ2 (rERβins)**

In rodents, many isoforms have been reported. The most well studied isoform of rodent ERβ is rodent ERβins (rERβins), which has an 18 amino acid insertion in the LBD region. Interestingly, rERβins is expressed in most tissues of rat and mouse. Furthermore, it has markedly reduced ligand binding, requiring 100 to 1000 fold higher E2 concentrations for maximal activation compared to wild-type ERβ and, at physiological E2 concentrations (with the exception of the ovary where E2 concentrations are higher), it can suppress E2 dependent ERα and ERβ mediated activation of gene expression [40-42]. It is interesting to speculate that rERβins is designed to activate gene expression only at the high concentrations of E2 present in the ovary. Alternatively, it might be designed to respond to other ligands.

1.4 Expression of ER variants in association with diseases

ERβ expression has been found in normal breast tissue and breast cancer. In normal breast, ERα exists in epithelial cells while ERβ is found in both epithelial and stromal cells. ER expression changes during breast cancer progression. ERα levels increase while ERβ levels decrease in breast cancer compared to normal breast, and ERβ level is negatively correlated with aggressive clinical features of breast cancer. In breast cancer expressing both ERs, estrogen could act via ERβ to moderate ERα driven proliferation.

Increased expression of ERβ2 correlated with the level of tumor inflammation and grade, respectively, in breast cancer [43]. Several studies have indicated that ERβ2 is associated with poor clinical outcome and poor survival in human cancers. Furthermore, ERβ2 expression correlates with aggressive phenotypical features. In prostate cancer, ERβ2 was shown to correlate with poor prognosis and could promote cancer cell proliferation and invasion [37, 38]. In contrast, one research group reported decreased expression of ERβ2 in colon cancer compared with the normal colon tissue [30].

1.4.1 **ERβ1 expression in correlation to clinical features**

Many studies indicated that ERβ1 expression is a predictor of favorable outcome. Generally higher levels of ERβ1 expression were associated with longer overall survival and disease free survival [44-46], better response to anti-estrogen therapy such as tamoxifen [47, 48], lower grade, negative lymph-node status and smaller tumor size [49-51].

Twenty nine out of 43 studies showed that ERβ1 is highly expressed in normal tissues relative to tumors [28, 31, 52-55] and that lower ERβ1 expression in tumors is associated with poor differentiation, invasive properties, metastasis and high stage of tumor [33, 43, 56-59]. Consistent with these findings, other studies showed that higher expression of ERβ1 is associated with low biological aggressiveness,

favorable clinical outcomes, better response to endocrine therapy and better survival [15, 29, 44, 46, 48, 50, 51, 60-67]. However, nine studies did not find any association between ERβ1 expression and pathology parameters, major clinical outcomes or cancer stage [15, 68-75].

Although it seems ERβ1 is a biomarker for favorable clinical outcomes, some studies indicated that the ERβ1 expression level is higher in tumors compared to normal tissues [76]. One study of 167 invasive breast cancers from postmenopausal women showed that high ERβ1 expression was associated with elevated cell proliferation and not correlated with clinical outcomes in the absent of ERα [71]. Moreover, ERβ1 was found to be positively correlated with aggressive clinical features in a study of 926 breast cancer patients [77]. Another study showed the enhanced nuclear and cytoplasmic ERβ1 expression was associated with advanced stage in colon cancer [76]. ERβ1 expression was also shown to correlate with poor cell differentiation and poor overall survival in a Chinese population [78]. Furthermore, few studies showed that ERβ1 promotes cell proliferation in ERα-negative breast cancers [71, 79]. Thus, at present the relation of ERβ1 to clinicopathological parameters is unclear and more studies are needed to conclude physiological function of ERβ1 in breast cancer.

The function of ERβ1 can be different when it is expressed alone compared to when it is co-expressed with other ERs. Additionally, the function of cytoplasmic ERβ isoforms may differ from nuclear ERβ isoforms. Furthermore, ERβ isoforms have been shown to exert tissue-selective functions and may also connect with hormonal status [80].

1.4.2 **ERβ2 expression in correlation to clinical features**

Many recent studies explored the function of ERβ2 in cancer. 22 of the above 41 studies also examined ERβ2 expression. These studies showed ERβ2 differently correlated to clinical features compared with ERβ1. Five out of 22 studies did not find a correlation between ERβ2 expression and clinical outcomes or survival. Ten

studies showed that ERβ2 correlated to better outcomes. Seven studies showed that ERβ2 was highly expressed in tumor, and its expression was increased in advanced stages of tumors, associated with invasiveness, metastasis and poor survival [15, 29, 33, 43, 48, 53, 64, 73, 81] .

Lymphocytes express high levels of ERβ2, which may contribute to the high expression level of ERβ2 in tumors with inflammation [43]. The cellular localization of ERβ isoforms determines tumor outcomes. Thus nuclear ERβ2 expression is associated with good outcome whereas cytoplasmic ERβ2 expression is associated with worse overall survival [75].

Most of the studies on the expression of ERβ2 have been analyzed at the mRNA or protein levels, in the latter case based on immunohistochemistry (IHC). Different ERβ2 antibodies may influence the results. All these have to be taken into account when comparing results from different studies. At present the relation of ERβ2 to clinical features is still unclear and more studies are needed to make conclusions about the contribution of ERβ2 expression in cancer.

1.4.3 **ERβ SNPs in association with diseases**

Around 720 single nucleotide polymorphisms (SNPs) have been identified in the ERβ gene [82]. They locate to the 5'UTR, promoter region, introns, 3'UTR, and coding regions. SNPs in coding regions and regulatory regions may affect gene and/or protein function and SNPs in the 3'UTR may contribute to translatability and mRNA transcript stability [83, 84]. rs4986938, rs928554 and rs1256049 are frequent ERβ polymorphisms. None of these SNPs change the amino acid sequence of the ERβ protein [85-87]. Polymorphisms in ERβ have been correlated to breast cancer [85-88], ovulatory dysfunctions [89], bone mineral density [90], hypertension [91], bulimic disease [88] and androgen levels [92].

1.5 Breast cancer molecular subtypes

Breast cancer is a common cause of cancer death among women. It is a disease with different morphological features and clinical behaviors, and 5 subtypes (Luminal A, Luminal B, normal breast like, human epidermal growth factor receptor 2 (HER2) enriched and basal-like breast cancer) have been defined according to microarray gene profiling based classification [93] (Table 3). Approximately 60-80% of basal-like breast cancers and 71% of claudin low breast cancer correspond to triple negative breast cancers (TNBCs), which are characterized by a lack of expression of ER, progesterone receptor (PR) and HER2. TNBC, which constitutes 15–25% of all breast cancers, is a cancer with aggressive phenotypes and the worst prognosis among breast cancer subtypes. It affects mostly in younger age or premenopausal groups and the incident rate is high in African American [94]. TNBC lacks effective targeted therapies. Furthermore, TNBC lacks any known predictive biomarkers and often develop distant metastasis in tissues such as brain and lung.

Table 3 Molecular classification of breast cancer

Subtypes [95]	**Markers**				**Incidence rate** [94, 95]	**Prognosis**	**Therapy**
	ER	**PR**	**HER2**	**Other markers**			
Luminal A	+	+	−	Low Ki67	40-62%	Good prognosis, less invasiveness	Hormonal therapy
Luminal B	+	±	±	HER2− with high Ki67	9-20%	Moderate	Hormonal, chemotherapy, HER2 blockade
Basal-like	−	−	−	CK5/6+ and/or EGFR+	8-20%	Poor	Chemotherapy
HER2+	−	−	+	?	4-16%	Poor-moderate	Chemotherapy, HER2 blockade
Normal like	−	?	?	?	6-10%	Moderate	?
Claudin low	−	−	−	Enrichment for EMT markers, immune response genes and cancer stem cell-like	7-14%	Poor	?

1.5.1 Therapeutic treatment of breast cancer

Many factors can influence the effect of therapeutic treatment of breast cancer such as the type of breast cancer, the stage of the cancer, the status of ER, PR and HER2, patient age, menopausal status, family breast cancer history and the status of ER variants.

The main types of treatment for breast cancer are surgery, radiation therapy, chemotherapy, hormone therapy and targeted therapy. Among them, surgery and radiation therapy are local therapies, which treat the tumor at the local site without affecting the whole body. Chemotherapy, hormone therapy and targeted therapy are systemic therapies, which will affect cancer cells in the whole body. Sometimes breast cancer patients also need neoadjuvant therapy before surgery to make the tumor shrink and allow for a less extensive surgery and/or adjuvant therapy after traditional treatments in order to prevent cancer cells coming back.

Hormonal therapy is targeted to inhibition of estrogen production and ER action (Figure 2). It is used as an adjuvant therapy in ERα-positive breast cancers. Tamoxifen is the oldest, most well-known and prescribed SERM, which can interfere with estrogen binding to ERs in the breast. Aromatase inhibitors, e.g., anastrozole, work by inhibiting estrogen synthesis. An aromatase inhibitor is the hormonal therapy to start with for postmenopausal women. Pure antiestrogen such as fulvestrant can also be used to block estrogen signaling pathways. Small molecules that block co-factor binding or inhibit ERα binding to ERE are considered as novel ER inhibitors with significant clinical potential [96, 97].

70% of ERα-positive breast cancer patients response to hormonal therapy. However, 30-40% of patients initially responding to hormonal therapy eventually relapse as a significant fraction of breast cancers develop endocrine therapy resistance. Identifying key regulators and pathways involved in ER signaling in resistant breast cancer would provide opportunities for a new generation of therapeutic targets in breast cancer.

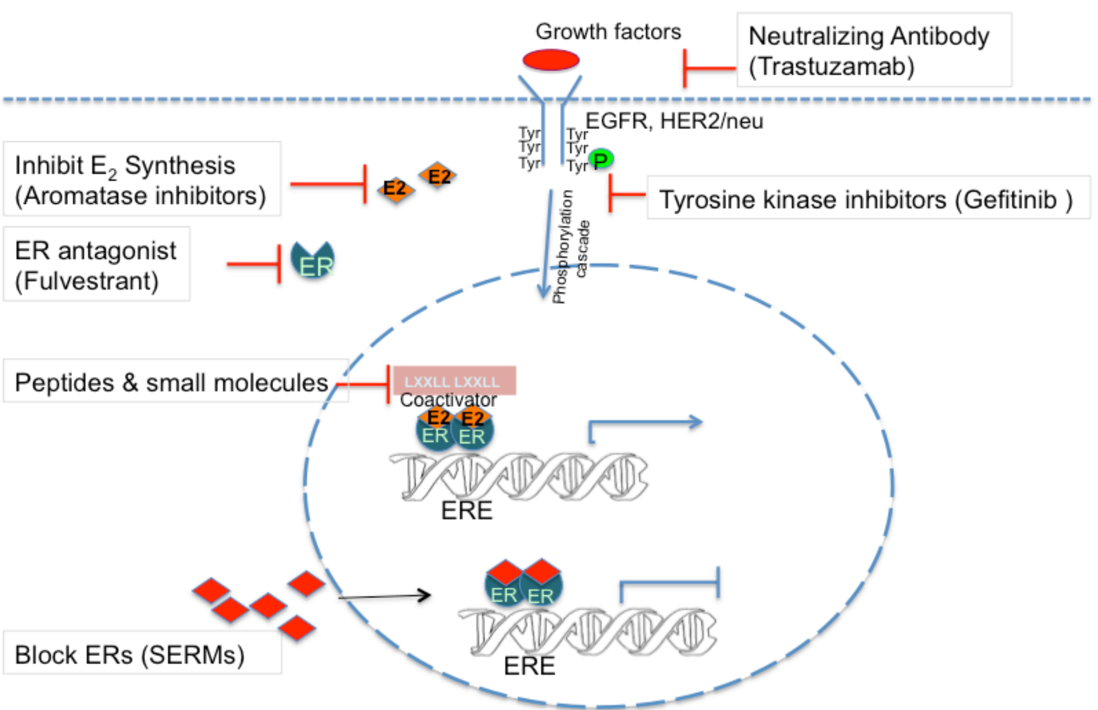


Figure 2. Therapeutic strategies targeting ER action in breast cancer. *One treatment option is to block ER action by using selective estrogen receptor modulators (SERMs) such as tamoxifen to compete with estrogens for binding to ER. Fulvestrant/ICI 182,780 is a complete ER antagonist, which binds to the ER and promotes its degradation. Other forms of therapy include using aromatase inhibitors to inhibit the synthesis of estrogen production, using small molecules and peptides to block co-activator binding to liganded ER, using monoclonal antibodies against HER2 such as trastuzimab or targeting tyrosine kinases directly by tyrosine kinase inhibitors such as gefitinib to inhibit non-ligand activated ER action.*

1.6 ER signaling pathways

ERs are transcription factors including two transcriptional activation domains. The classical ER signaling occurs through ligand binding to the LBD of the receptor and induction of ligand specific conformational changes in the ER protein. The receptors form dimers and bind to DNA at EREs through their DBD. Most of the known estrogen derived effects are mediated via this direct interaction of ERs with DNA. Interaction of ERs with DNA is followed by recruitment of co-activators, leading to the induction of chromatin remodeling and increased transcription of estrogen targeted genes.

The non-classical genomic ER signaling pathway is mediated by the tethering of ER to DNA through protein-protein interactions with other transcription factors such as

AP-1 and Sp1, a so-called tethering mechanism (Figure 3). This pathway of ER action is also called the indirect ER signaling pathway. Furthermore, ER can be activated in a ligand independent way. Growth factor signaling or stimulation of other signaling pathways leads to activation of kinases that can phosphorylate the dimerized intracellular ERs, which subsequently active target genes and trigger downstream signal transduction cascades even in the absence of ligand.

ER signaling can also occur through a non-genomic pathway, which refers to that ER can regulate gene expression without binding directly to DNA. This action has been shown to involve the activation of downstream cascades such as PKC, PKA, and MAPK via membrane-localized ERs. In addition, an orphan G protein-coupled receptor (GPR30) in the cell membrane has been reported to mediate nongenomic ER signaling. The activities of GPR30 in response to estrogen were shown to be mediated through its ability to induce expression of ERα36, a novel variant of ERα, which in turn acted as an extranuclear ER to mediate nongenomic estrogen signaling [98].

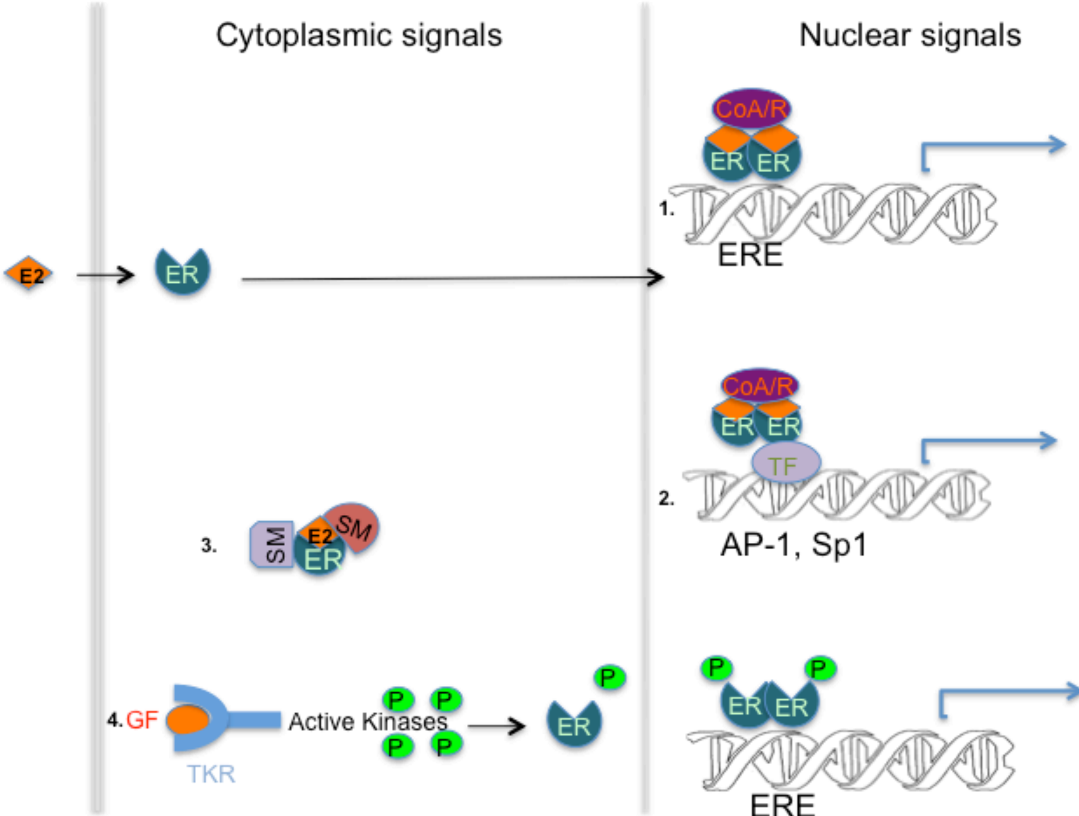


Figure 3. Schematic models illustrating ER signaling pathways [99]. ***1, 2****, and* ***4*** *illustrate the genomic action in the nucleus, while* ***3*** *illustrates the non-genomic action in the cytoplasm.* ***1****. Estrogen binds to ERs. Liganded ERs form dimers and bind directly to estrogen response elements (EREs) in target genes in the nucleus.* ***2.*** *Ligand/ER complexes tether to other transcription factors, bound to their response elements (RE) in target genes, to activate the transcription of non-ERE containing genes.* ***3.*** *Ligand activated membrane bound ERs in complex with other factors functions as 'second messengers' (SMs) act to activate non-genomic signaling cascades.* ***4.*** *Growth factor (GF) activated protein kinase cascades phosphorylate and activate ERs leading to binding to EREs in target genes in the nucleus, resulting in ligand independent activation.*

1.7 Signaling pathways in cancer biology

Cancer is the result of cell growth and division out of control starting to invade neighboring tissues and metastasize. Proliferation potential, cell growth out of control (insensitivity to growth inhibitory signals), apoptotic escape, limitless replicative potential, angiogenesis and invasion/metastasis are the six hallmarks in cancer progression [100]. Many complex signal transduction processes are involved

in cell growth and survival. Disruption of those signaling pathways may cause cancer. Over 40 pathways have been demonstrated to relate to cancer biology including epidermal growth factor receptor (EGFR) signaling, PI3K/AKT signaling, Ras/Raf/Mek signaling and MET signaling. Some of these signaling pathways will be discussed further below.

In epithelial cancers such as breast cancer, metastasis is thought to occur start with epithelial-to-mesenchymal transition (EMT). EMT is a process of loss of epithelial phenotypes and gain of new migratory and invasive growth phenotypes by cytoskeleton rearrangements and cellular adhesion, structure and morphology alternation. Epithelial markers on the cell surface such as E-cadherin or integrins are replaced with mesenchymal markers, such as vimentin, N-cadherin, or fibronectin. During the process of EMT, cells lose their characteristic epithelial traits and instead gain migratory potential and detach from the basal membrane [101].

1.7.1 **ER signaling in tumorigenesis**

Evidence from the clinic, cell line based *in vitro* models and *in vivo* animal models revealed that estrogen and ERs contribute to tumorigenesis, including breast, uterine, colorectal, prostate, lung, pancreatic and ovarian cancers [102-108]. Aberrant estrogen signaling results in disruption of the cell cycle, apoptosis and DNA repair, which are implicated in cancer initiation and progression and may also influence the response to cancer therapy [109, 110]. E2 was reported to enhance ovarian cancer migration through ERα mediated EMT. Snail, Slug and E-cadherin were identified as transcriptional targets of ER signaling in EMT [111].

Many studies indicated that ER subtypes play different roles in tumorigenesis and in response to cancer therapy. ERβ1 acts as a tumor suppressor in cancer biology [103]. Activated ERβ1 signaling by introducing ERβ1 or its agonists has anti-proliferative effects. Recent evidence showed that overexpression of ERβ1 exerts tumor repressive functions in human malignant pleural mesothelioma via EGFR inactivation and affects the response to Gefitinib, an EGFR inhibitor [112]. ERβ1 knockdown in ERβ1 positive cells confers a more invasive phenotype, increases

anchorage independent proliferation and elevates the constitutive activation of EGFR-coupled signal transduction pathways [112]. In ERα-positive breast cancer cells, ERβ1 expression was found to reduce Akt activation through down regulation of HER2/HER3 signaling. On the contrary, in one study of prostate cancer cells, ERβ2 was shown to increase proliferation and up-regulate factors known to be involved in bone metastasis, whereas ERβ1 inhibited these parameters [37].

In prostate cancer, ERβ1 represses basal-like breast cancer EMT transition by destabilizing EGFR [113]. In breast cancer, loss of TBK1 was reported to drive induction of EMT through down regulating ERα expression in ERα-positive cancer. Furthermore, ERβ1 was reported to inhibit EMT and invasion in TNBC *in vitro* or *in vivo* in a zebrafish model [113]. ERβ1 inhibits EMT through up-regulation of miR-200a/b/429 and the subsequent repression of ZEB1 and SIP1, which in turn leads to increased expression of E-cadherin [113].

ERα seems to contribute to tumor progression primarily by having a mitogenic role to stimulate cell proliferation. ERα promotes breast cancer cell proliferation both *in vivo* and *in vitro* by increasing the expression of MYC, cyclin D1 (CCND1) to facilitate cell cycle progression [114-116]. In the absence of ligand, MAPK- and PI3K-driven phosphorylation can enhance ERα transcriptional activity and induce breast cancer cell proliferation [117, 118]. ERα also plays a role in EMT. In breast cancer cells, ERα interacts with AIB1 (also known as SRC-3), which could bind to the ERα-binding site on the Snial1 promoter, resulting in increased expression of Snail1 and the subsequent repression of E-cadherin [119, 120].

Recent studies also found that ERα extranuclear signaling is involved in breast cancer cell migration and metastasis through activation of kinase cascades. For examples, ERα extranuclear signaling promotes stimulation of the Src kinase, MAPK, PI3K, and protein kinase C pathways in the cytosol. Many of these kinases activated by ERα extranuclear signaling have been shown to be implicated in breast cancer metastasis [121, 122]. However, the molecular mechanisms by which extranuclear ER exerts its function remain unclear.

1.7.2 **EGFR**

The epidermal growth factor receptor (EGFR, HER-1, c-*erb*B-1) is a subfamily of four closely related receptor tyrosine kinases (RTKs): EGFR (ErbB-1), HER2/c-neu (ErbB-2), Her 3 (ErbB-3) and Her 4 (ErbB-4). These four transmembrane growth factor receptors share similarities in structure and function. The HER2 gene is amplified and HER2 is overexpressed in 25% to 30% of breast cancers, increasing the aggressiveness of the tumor [123]. Lack of response to endocrine therapy, together with increased metastasis and poor survival, has been shown to be associated with over-expression of EGFR and HER2 in clinical breast cancer [124, 125].

There are two major binding ligands, epidermal growth factor (EGF) and transforming growth factor-alpha (TGF-α) that can activate EGFR. Ligand binding to EGFR results in receptor homo- or hetero-dimerization (with one of the HER family of receptor tyrosine kinases) followed by autophosphorylation of the tyrosine kinase domain. Phosphorylated tyrosine residues serve as binding sites for the recruitment of signal transducers and activators of intracellular substrates. The Ras/Raf/MAPK and PI3K/Akt pathways are the major signaling routes for the HER family, including EGFR. These pathways control several important biologic processes, including cellular proliferation, invasiveness, angiogenesis and inhibition of apoptosis [126-128]. EGFR signaling pathways are shown in Figure 4.

Genetic mutations or gene amplifications in RTKs and their downstream factors result in aberrant cell signaling often leading to cancer cell growth and/or resistance to cancer therapies [129, 130]. The well-studied RTKs mutation is the EGFR-activating mutation in non-small cell lung cancer (NSCLC). Certain mutations in the EGFR tyrosine kinase domain including an amino acid substitution at exon 21 (L858R) and in-frame deletions in exon 19 were found to be predictors of clinical response to EGFR tyrosine-kinase inhibitors (TKIs). EGFR-activating mutations represent a subset of NSCLC patients and are predictors of EGFR TKI treatments [131].

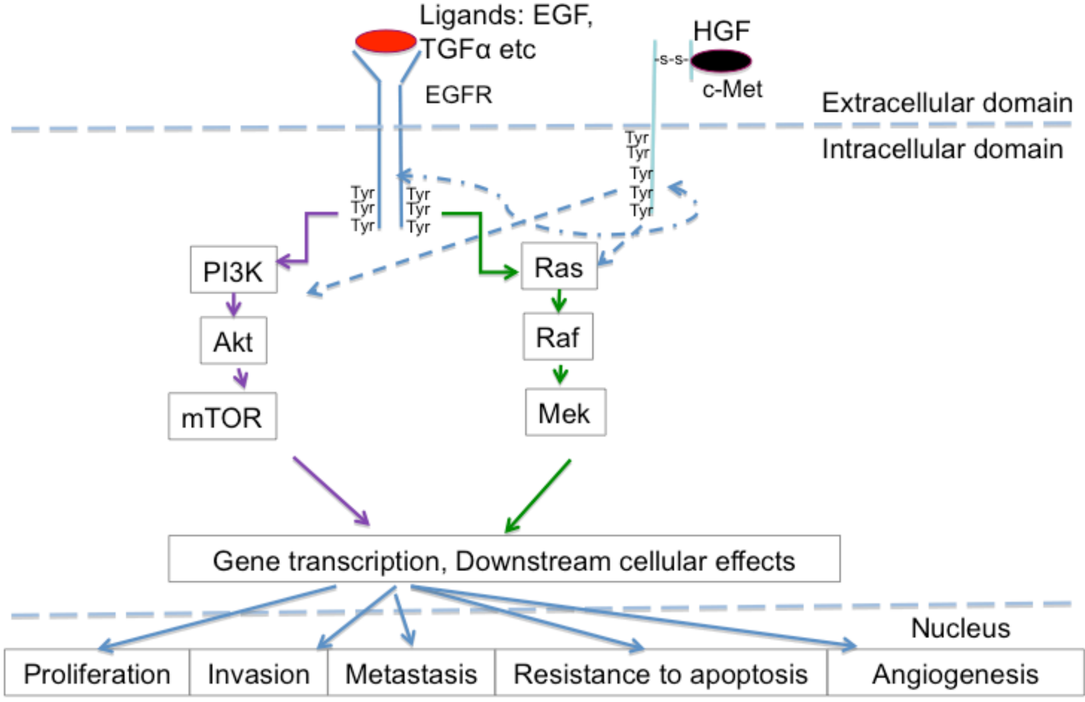


Figure 4. EGFR and MET cell signaling pathways. *EGFR activates several major downstream signaling pathways, including Ras–Raf–Mek and the pathway consisting of phosphoinositide 3-kinase (PI3K), Akt, and mammalian target of rapamycin (mTOR), which in turn may have an effect on proliferation, survival, invasiveness, metastasis, and tumor angiogenesis. These pathways may also be modulated by other receptor tyrosine kinases, such as the HGF/MET signaling pathway.*

1.7.3 **MET**

The MET oncogene, encoding for the tyrosine kinase receptor for hepatocyte growth factor (HGF) is over expressed in various cancer cells. The MET tyrosine-kinase receptor is an established mediator of cancer cell invasiveness. MET can increase the viability of cancer cells [132]. Cross talk between the EGFR and MET has been identified in several tumor types, with HGF being able to transactivate EGFR and conversely EGFR ligands activating MET. EGFR inhibitors have been shown to attenuate HGF-mediated proliferation, migration and invasion of several breast cancer cell lines *in vitro*. HGF and/or MET expression increase with tumor progression and each is independently associated with poor prognosis. HGF/MET expression gradually increases during breast cancer progression from normal breast, carcinoma *in situ* to invasive carcinoma [133], suggesting their involvement in the malignant progression in breast cancer. Tumor hypoxic areas show MET overexpression [134]. The HGF/MET pathway is important in basal-like breast

cancer and is considered as a strong candidate target for treating premalignant basal-like lesions [135]. MET is also associated with invasion and metastasis in breast cancer. Its expression and activation correlate with tumor hypoxia and HIF-1α activation [136]. When MET is inhibited, hypoxia induced invasive growth was prevented [126, 134]. Previous findings also showed that MET levels can be regulated by HIF-1α [126].

1.7.4 **HIF-1α**

Tumors are surrounded by a low-oxygen environment, making the hypoxia-induced factor HIF-1 signaling important for cell survival. HIF-1 is a heterodimeric protein consisting of two subunits, HIF-1α and HIF-1β. HIF-1α, which forms a DNA-binding heterodimer with the constitutively expressed HIF-1β subunit, is stabilized and activated under hypoxia. Upon activation, HIF-1 binds to the hypoxia response elements of target genes. The activity of HIF-1 is regulated through the stabilization and activation of HIF-1α.

HIF-1α modulates the expression of 1-5% of human genes, including genes involved in glycolysis, cell cycle control, proliferation, invasion, angiogenesis and metastasis [137]. Aberrant signaling via pathways such as Ras-MAPK, Src, or PI3K/mTOR increases HIF-1α expression under normoxic and hypoxic conditions. PI3K/AKT activation was also reported to increase HIF-1α stability through mTOR, particularly in breast cancer [137]. HIF-1α is associated with poor prognosis and therapy resistance in cancer and is also a major determinant of invasion and metastasis in a wide variety of tumor types [138].

1.7.5 **PHDs**

Prolyl hydroxylases domain-containing proteins (PHDs) regulate the appropriate balance of HIF-1α protein at the post-translation level. PHDs are oxygen sensors that can target two proline residues (p402 and p564) in two -Leu-X-X-Leu-Ala-Pro sequences in the oxygen-dependent degradation domain of HIF-1α in the presence

of oxygen. The hydroxylated HIF-1α can be recognized by the tumor suppressor von Hippel-Lindau (pVHL) protein, followed by polyubiquitination by the VHL E3 ubiquitin ligase, which targets proteins for degradation by the proteasome. β2-adrenergic receptor, which is a prototypic G protein-coupled receptor, was identified as a new hydroxylation substrate of PHD3 [139]. The discovery of this new target helps us to better understand the functionality of PHDs and the cellular response to oxygen.

In pancreatic cancer, PHD2 exerts tumor-suppressive acitivity [140]. In breast cancer, high PHD2 expression has been shown to be associated with better survival [141]. Similarly high PHD3 expression is correlated with good clinical prognosis markers such as lower tumor grade, smaller tumor size and lower proliferation [141]. PHD3 may also be a critical regulator of apoptosis in sympathetic neural development and breast cancer [141, 142]. In pancreatic cancer, high PHD2 expression is associated with lymph node negativity and PHD2 can suppress angiogenic cytokines to inhibit angiogenesis mediated tumor growth and invasion [140]. Interestingly, ERβ was reported to sustain epithelial differentiation by promoting PHD2 expression via direct binding to an ERE in the 5′ UTR of the PHD2 gene in prostate cancer cell line [143].

2 AIMS OF THE THESIS

ERβ is expressed as different isoforms, the functions of which need to be addressed, in order to fully understand the physiological functions of ERβ. The overall aim of this thesis was to clarify functionality and mechanisms of the ERβ variants, focusing on breast cancer.

The specific aims were:

PAPER I To investigate the frequency of the reported hERβ548 variant in human populations.

PAPER II To identify and characterize hERβ variants in an African American population.

PAPER III To characterize the function of mERβ2.

PAPER IV To investigate the mechanisms of EGFR signaling involved in cell invasiveness in NSCLC.

PAPER V To investigate the effects of hERβ2 on breast cancer cell proliferation and invasion, including characterization of pathways that may contribute to the observed phenotypes with specific focus on the PHDs and HIF-1α pathways.

3 METHODOLOGICAL CONSIDERATIONS

3.1 WAVE^{TM} technology

WAVE^{TM} is a high-performance liquid chromatography (HPLC) based technology to detect mutations, based on the resolution of hetero duplexes and homo duplexes by HPLC. Individuals who are heterozygous for a mutation or polymorphism have a 1:1 ratio of wild-type and mutant DNA. A mixture of hetero- and homo-duplexes is formed when PCR products are annealed by heating to 95°C and cooling slowly. DNA from individuals who are homozygous for a mutant allele is detected by the addition of wild-type DNA. Thus, each sample is analyzed both in the presence and absence of a wild-type PCR product. The ability of the WAVE method to resolve hetero-duplex DNA from homo-duplex DNA in minutes makes it a powerful tool in the field of mutation detection. We used this technology **in *PAPER I*** to assay a suggested polymorphism in the 5' UTR of hERβ that would generate an upstream ATG in frame with the rest of the coding sequence to generate a longer form of hERβ, hERβ548. We also used this technology **in *PAPER II*** to identify SNPs in hERβ in an African population. In the latter study, samples with aberrant HPLC profiles were subjected to DNA sequencing and compared with the published genomic sequence of the hERβ gene.

3.2 Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR, real-Time PCR) is a widely used sensitive method for accurate quantification of mRNA at low throughput. It includes double-stranded DNA-binding dyes for detection of amplified DNA (SYBR Green dye) or fluorescent dye labeled probe methods for detection of amplified DNA (TaqMan probes). **In *PAPER IV***, in order to get accurate results, we used the TaqMan probe method, which has both high specificity and reproducibility. The primary disadvantage of the SYBR Green dye method is that it detects all double-stranded DNA, including non-specific reaction products. **In**

PAPER V**,** we compared both methods for some experiments and got similar results and then mainly used the SYBR Green method in this study.

In qPCR, the accumulation of specific amplified PCR products in "real time" during PCR amplification was detected. The first cycle at which point when the fluorescent signal is above the background signal is called the "Ct" or threshold cycle, which is used to quantify the number of substrates present in the initial template quantity. We used comparative CT method to calculate the relative fold change. The amount of target, normalized to an endogenous reference and relative to a control, is given by: $2^{-\Delta\Delta CT}$.

ΔCT = CT target − CT endogenous house keeping

$\Delta\Delta CT$ = ΔCT test sample – ΔCT control sample

3.3 Gene expression microarray analysis

DNA microarray is a technology that can monitor global gene expression on a single array giving researchers the opportunity to get a better picture of the interactions among thousands of genes simultaneously.

Two Affymetrix expression array types were used in our studies. **In *PAPER IV***, we used the Affymetrix GeneChip Human Genome U133A (HG-U133A) array which contains approximately 45,000 probe sets representing more than 39,000 transcripts derived from approximately 33,000 well-substantiated human genes. This array was used to perform global gene expression analysis on 53 gene arrays representing 53 NSCLC cell lines.

In *PAPER V*, we used the Affymetrix Human Gene 1.1 ST arrays, which contain probes for 33299 gene sequences to identify global target genes for hERβ2. A cut-off fold of 1.5 and p value < 0.05 were used to define regulated genes. The total regulated genes were loaded and analyzed using Ingenuity Pathway Analysis software (IPA) (Ingenuity), a bioinformatic tool for network, functional and pathway analysis. Pathway analysis identifies specific biological processes. Genes

were ranked and mapped to networks in IPA. Important networks related to biological function are given and scored. The most significantly regulated genes were shown and classified upon molecular and cellular function.

3.4 Cell lines

Several immortalized cell lines from different tissues have been used in this thesis. Cell lines are considered as standard *in vitro* model systems due to their ease of cultivation and manipulation.

In *PAPER IV*, NSCLC cell lines H3255, H1975, H1993, and HCC827, A549 and Calu-6 were used. H3255, H1975 and HCC827 represent cell lines bearing EGFR-activating mutations. The HCC827 cell line is from a pulmonary adenocarcinoma, which harbors an in-frame E746 - A750 deletion in exon 19 in the EGFR tyrosine kinase domain. H3255 was initiated from malignant cells isolated from the pleural effusion. It carries a L858R point mutation in exon 21 in the EGFR tyrosine kinase domain. H1975 was isolated from a lung adenocarcinoma with an L858R EGFR activating mutation and a T790M mutation in exon 20, which made it resistant to EGFR TKIs. A549 is a human pulmonary adenocarcinoma epithelial cell line, with wild-type EGFR and a KRAS mutation. It was used as a cell line with non-EGFR mutation in the study. Calu6 is a human pulmonary adenocarcinoma epithelial cell line, which is wild-type for EGFR. H1993 is derived from a metastatic site of a female pulmonary adenocarcinoma patient with MET gene amplification. NIH-3T3 cells expressing wild-type EGFR or EGFR bearing the L858R mutation or the deletion mutant ΔL747-S752del [144] were obtained from Dr. Jeffrey Engelman (Dana-Farber Cancer Institute).

In *PAPER V*, the breast cancer cell line BT549 was used. BT549 is of epithelial origin and derived from invasive ductal carcinoma. BT549 harbors a p53 R249S mutation and pTEN mutation.

3.5 Generation of artificial mutations

Mutagenesis is the process by which the genetic information is changed. It is an important technique in the laboratory to examine the effects of mutations. In this thesis, we use the QuickChange^{TM} XL Site-Directed mutagenesis kit (Stratagene, La Jolla, CA) to generate several artificial mutations for further functional studies. The sequences of the artificial mutations were confirmed by DNA sequencing. **In *PAPER I***, in order to validate the method for screening the novel N terminus extended isoform in different populations, hERβ548 plasmid that has the reported extra nucleotide of the ERβ gene was generated from hERβ530. **In *PAPER II***, In order to study the function of ERβ SNPs existed in African samples, hERβ105AèG and hERβ1057TèG plasmids were generated from hERβ530. **In *PAPER IV***, HIF-1α mutant with proline to alanine in two positions 402 and 564 (HIF-1α P402A; P564A) were generated from the wild-type HIF-1α. This form is stabilized in normoxia because of the loss of VHL-mediated polyubiquitination and subsequent degradation. Also stable cell lines expressing wild-type EGFR or EGFR bearing the L858R mutation or the deletion mutant ΔL747-S752del were used.

3.6 Small interfering RNA (siRNA)

SiRNA is also known as silencing RNA or short interfering RNA. It is a double-stranded RNA, about 20-25 base pairs long, which interferes with the expression of genes having the complementary nucleotide sequence. SiRNAs correspond to short double-stranded RNAs with phosphorylated 5' ends and hydroxylated 3' ends with two overhanging nucleotides.

siRNAs are important tools for validating gene function. siRNAs can be introduced into the cell by transient transfection. **In *PAPER IV***, commercial available siGENOME Non-Targeting siRNA pool against EGFR, MET, HIF-1α and scramble control were introduced by transient transfection to study the effect of EGFR signaling and MET-HIF-1α axis in EGFR or hypoxia induced NSCLC cell invasiveness. **In *PAPER V***, multiple siRNAs targeting hERβ2 and a scramble

control were introduced into a TNBC cell line BT549 to study the function of hERβ2.

3.7 Cell proliferation assay

The MTT assay is a common used colorimetric assay for determining the number of viable cells by measuring the cellular metabolic activity via NAD(P)H-dependent cellular oxidoreductase enzymes that reduce the tetrazolium dye, MTT, to its insoluble form formazan, giving a purple color. When cells are proliferating, the dye accumulates. Cell viability is determined by measuring the absorbance at a certain wavelength. MTS and WST assays use alternative dyes to the MTT Assays.
In this thesis, two cell proliferation assay kits were used. **In *PAPER IV***, we detected the effect of EGFR inhibitor and MET inhibitor on cell proliferation of NSCLC cell lines A549, HCC827 and H1993. We used the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit, which is based on MTS and an electron mediator reagent. The electron mediator reagent together with MTS yields a stable solution. **In *PAPER V***, we determined the effect of hERβ2 on cell proliferation in a TNBC cell line BT549. We used the WST-1 kit, which contains water-soluble tetrazolium salts combined with electron coupling to form a water-soluble formazan. The insoluble formazan accumulated outside cells, which decreases the toxicity to cells.

3.8 Cell invasiveness

The ability of cancer cells to invade is directly correlated with tumor metastatic potential. The BD BioCoat™ Matrigel Invasion Chamber is a low throughput, efficient quantitative measurement for evaluating invasion of tumor cells. In this thesis, BD BioCoat™ Growth Factor Reduced Matrigel™ Invasion Chambers were used. 2.5×10^4 cells were seeded in the upper chamber with 0% FBS media and complete media containing 10% FBS was added to the lower chamber. After 24 h, cells in the upper chamber were removed by scraping. Cells that migrated to the lower chamber were stained and counted.

4 RESULTS AND DISCUSSION

4.1 PAPER I

HUMAN ESTROGEN RECEPTOR BETA 548 IS NOT A COMMON VARIANT IN THREE DISTINCT POPULATIONS

This study concluded for the first time that hERβ548 is not a common variant in Africans, Caucasians, or Asians.

Several isoforms of ERβ have been reported, including variants with different N-terminal ends. In rodents, two in-frame initiation codons (ATGs) are used to produce proteins of 530 and 549 amino acids, respectively. In humans, the upstream ATG was out of frame in all clones reported, until human clones with an extra A-T base pair placing an upstream ATG in frame with the rest of the coding sequence were reported. The authors suggested that this could represent a novel polymorphism in the ERβ gene. Because the suggested longer human ERβ548 (hERβ548) and the previously identified hERβ530 display different functional characteristics *in vitro*, it is of interest to determine if this variant constitutes a polymorphism in human populations.

We determined the frequency of this novel isoform in several populations including African (n = 96), Caucasian (n = 100), and Asian (n = 128) subjects using denaturing HPLC. We did not detect any alleles that correspond to hERβ548 in these samples or in additional samples of heterogeneous origin.

We concluded that hERβ548 is not a common variant in Africans, Caucasians, or Asians.

4.2 PAPER II

IDENTIFICATION OF A FUNCTIONAL VARIANT OF ESTROGEN RECEOTOR BETA IN AN AFRICAN POPULATION

The aim of this study was to identify and characterize ERβ variants in an African America population.

We identified five novel polymorphisms in the ERβ gene in an African population. Two of these variants I3V and V320G are expected to change the amino acid sequence of the ERβ protein. The I3V mutation displayed no differences with regard to transcriptional activity in a reporter assay, as compared with the wild-type receptor. The V320G mutation, however, showed significantly decreased maximal transcriptional activity in a reporter assay, although its binding affinity for E2 was not affected. A pull-down assay indicated that the interaction of full-length TIF2 with hERβV320G was weaker than with hERβwt. Moreover, surface plasmon resonance analysis revealed reduced interaction of the hERβV320G variant with the NR box I and II modules of TIF2.

These results indicate that the decreased transcriptional activity of the novel ERβ variant, hERβV320G, is due to the weaker interaction with a co-factor TIF2. This novel polymorphism could provide a tool for human genetic studies of diseases in the African population.

4.3 PAPER III

MOUSE ESTROGEN RECEPTOR BETA ISOFORMS EXHIBIT DIFFERENCES IN LIGAND SELECTIVITY AND COACTIVATOR RECRUITMENT

Mouse ERβ1 (mERβ1) corresponds to the wild-type mERβ while the mouse ERβ2 (mERβ2) is an alternative splice variant with 18 amino acid insertions in the LBD. In this study, we have assayed the interaction of several known ligands with mouse ERβ1 and mouse ERβ2 for the first time.

Our studies showed that mERβ1 and mERβ2 display differences with regard to ligand binding. The binding affinity of E2 was mERβ1 selective (14-fold) while binding affinity of raloxifene was mERβ2 selective (8-fold). In order to reach maximal transcriptional activation, mERβ2 required 10-fold greater E2 concentrations compared to mERβ1, whereas raloxifene was more potent in antagonizing E2-induced gene expression via mERβ2 than mERβ1. Furthermore, mERβ2 showed significantly decreased E2-induced maximal transcriptional activity as compared to mERβ1. A pull-down assay and surface plasmon resonance analysis indicate that decreased E2-induced transcriptional activity of mERβ2 is associated with reduced interaction with both TIF2 and RAP250 co-activators compared to mERβ1.

These results suggest that ligand selectivity and co-activator recruitment of ERβ isoforms constitute additional levels of specificity that influence the transcriptional response in estrogen target cells in mouse.

When novel SERMs are tested in animal studies, the isoform dependent ligand selectivity in animals needs to be considered.

4.4 PAPER IV

EPIDERMAL GROWTH FACTOR RECEPTOR REGULATES MET LEVELS AND INVASIVENESS THROUGH HYPOXIA-INDUCIBLE FACOTR-1ALPHA IN NON-SMALL CELL LUNG CANCER CELLS

NSCLC is the leading cause of cancer-related mortality in the United States. The five-year survival rate can be lower than 2% for patients with distant stage. EGFR plays an important role in cell survival, cell proliferation, invasion and angiogenesis in NSCLC. A subtype of NSCLC patients carrying mutations in the EGFR tyrosine kinase domain, which make the EGFR auto activated and sensitive to EGFR TKIs. EGFR-activating mutations become a predictor marker of clinical response to EGFR TKIs. The aim of this study was to identify the signaling pathway involved

in the invasiveness of NSCLC.

In this study, we analyzed 202 clinical patient specimens, different NSCLC cell lines and transgenic mouse models to investigate the EGFR signaling pathway in relation to tumorigenesis of NSCLC. Our results showed that EGFR-activating mutations are associated with elevated HIF-1α and MET levels in NSCLC cell lines. An activated EGFR pathway or hypoxia can promote cell invasion, which is associated with the increased expression of p-MET, MET, and HIF-1α. The invasiveness is MET dependent, and could be diminished by MET inhibitors or siRNA. These findings establish that, in the absence of *MET* amplification, EGFR signaling can regulate MET levels through HIF-1α and that MET is a key downstream mediator of EGFR-induced invasiveness in EGFR-dependent NSCLC cells.

Our results indicated that EGFR- and/or hypoxia-induced tumorigenic effects in NSCLC are mediated through promoting cell invasion, which can be regulated via HIF-1α/MET axis. MET is the key downstream mediator of cell invasion in NSCLC cells. HIF-1α/MET axis involved in both EGFR and hypoxia induced signaling pathways suggests that there might be an overlap in the mechanisms that EGFR and hypoxia promote malignant feature and therapeutic resistance.

Targeting of the MET pathway together with EGFR pathway may further block the tumor invasion beyond the effect of EGFR inhibition alone, may also prevent the possible resistance through MET amplification.

4.5 PAPER V

ESTROGEN RECEPTOR BETA1 INDUCES PROLIFERATION AND INVASION OF BREAST CANCER CELLS; ASSOCIATION WITH REGULATION OF PROLYL HYDROXYLASE 3, HYPOXIA INDUCED FACTOR 1 ALPHA AND MET

Many studies indicated that ERβ variant hERβ2 is expressed at higher levels than

hERβ1 in breast cancer and that hERβ2 expression is associated with aggressive phenotypes in various cancers. We determine the phenotypes and molecular mechanisms of hERβ2, independent of ERα and hERβ1, in breast cancer cells.

The TNBC cell line BT549 was used as model system. hERβ2 levels were modulated by transient overexpression or knockdown by siRNAs. Cell proliferation and invasion were assayed by the WST-1 cell proliferation assay kit and growth factor reduced BD Matrigel™ invasion chamber, respectively. GeneChip® Human Gene 1.1 ST Affymetrix microarrays were used to assay global gene expression. hERβ2 regulation of mRNA and protein levels of selected genes was investigated by qPCR and western blot analysis, respectively.

In this study we show that hERβ2 is the dominant ER isoform in the BT549 cell line and promotes proliferation and invasion of this cell line. A total of 263 genes were identified as hERβ2-upregulated genes and 662 identified as hERβ2-downregulated genes. hERβ2-regulated genes were involved in cell morphology, DNA replication and repair, cell death and survival. We show that hERβ2 represses PHD3 gene expression and induces protein levels of HIF-1α and MET.

We concluded that hERβ2 promotes cell proliferation and invasion of BT549 breast cancer cells. The invasive phenotype could potentially be mediated through transcriptional repression of PHD3, followed by up-regulation of the HIF1α-MET pathway.

5 GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

5.1 ERβ SNPs in African Americans and disease susceptibility

Compared to white Americans, African-Americans have poor outcome of certain diseases such as breast cancer and prostate cancer. The mortality rate of breast cancer is three times higher in African American than other populations. Notably, the prevalence of TNBC is highest in premenopausal African American women [145]. It was reported that 39% of premenopausal African American breast cancer patients was diagnosed with TNBC [146]. The prevalence of TNBC among non-African American is around 15% and the difference was not seen in other breast cancer subtype groups [146]. We speculate that African-specific genetic variants may be associated with the susceptibility to specific subtypes of cancer. One recent report found that the SNP rs10069690 in the TERT gene was significantly associated with TNBC in an African ancestry population [147]. We are one of the very few groups studying polymorphisms of ERβ genes in African populations. We screened the ERβ gene in an African population for polymorphisms. The identified polymorphism, particularly a functional polymorphism, would constitute important tools for further disease association studies in this population. Future studies should address the frequency of the two identified polymorphisms that change the amino acid sequence of the ERβ protein in a much larger cohort. It would also be interesting to investigate if these SNPs are present, and their frequency, in other populations. Finally, further functional studies of these variants could involve generating mice strains with the corresponding SNPs in ERβ.

5.2 Rodent ERβ2; considerations when performing animal studies using ER agonists and antagonists

Our studies showed that the binding affinity of E2 was mERβ1 selective while raloxifene was mERβ2 selective. mERβ2 required 10-fold greater E2 concentrations compared to mERβ1 to reach the maximal transactivity, whereas raloxifene was more potent in antagonizing E2-induced gene expression via mERβ2 than mERβ1.

Mouse and rat are commonly used animal models to study ER function *in vivo*. Furthermore, disease models in mouse and rat are used to evaluate the effect of compounds with estrogenic or anti-estrogenic properties to improve the disease condition. As humans do not have mERβ2, this isoform-dependent ligand selectivity, reported for the two rodent ERβs in this study, needs to be considered when novel compounds are tested in animal studies. A further complication arises from the fact that the relative levels of mERβ1 and mERβ2 likely vary between tissues. The development of mice strains lacking expression of mERβ2 would be an important development. However, humanized mice, in which the mouse ERβ LBD was replaced by human ERβ LBD showed embryo lethality (Per Antonson, personal communication).

5.3 Estrogen signaling, cell proliferation and invasion

Estrogens promotes epithelial cell proliferation in both normal and neoplastic breast [148] and ER signaling is associated with tumorigenesis, metastasis and therapeutic resistance.

In our study we showed that hERβ2 is the predominant endogenously expressed ER in a TNBC cell line BT549 and promotes cell proliferation and invasion in this cell line. We demonstrate that the invasive phenotype associated with hERβ2 expression could potentially be via repressing expression of PHD3 followed by up-regulation of the HIF-1α-MET pathway or direct regulation of MET.
EGFR and HIF-1α-MET signalling pathways are known to play important roles in tumorigenesis and therapeutic resistance. Recently PHD3 has been shown to exert

tumor-protective functions and to inhibit tumor growth in pancreatic cancer. Our study contributes to the understanding of the molecular mechanism of hERβ2 regulation of cell proliferation and invasion. Future studies should focus on how hERβ2 regulates PHD3 and ultimately cell proliferation and invasion. It will be important to identify additional cell lines expressing hERβ2 in the absence of other ERs to investigate the generality of the observations, both TNBC cell lines and cell lines of different origin, including regulation of PHD3 and the HIF-1a-MET pathway. Furthermore, it will be important to pursue similar studies in cell lines expressing hERβ2 in the presence of ERα and/or hERβ1. The correlation of hERβ2 with PHD3 or components of the HIF-1α-MET pathway in clinical samples will provide evidence for the clinical relevance of our findings. Although, in this thesis, we focus on hERβ2 regulation of PHD3 and the HIF-1α-MET pathway, other potential mechanisms should be explored. These could be derived from a more careful examination of the global gene expression profiling data already obtained. Furthermore, the global gene expression profiling data is derived from a single time point following inhibition of hERβ2 with siRNA. Performing global gene expression profiling at additional time points following siRNA transfection might reveal alternative potential targets responsible for the observed phenotypes. Such studies could also include identification hERβ2 regulated miRNAs and other non-coding RNAs. Finally, although hERβ2 does not bind tested ER ligands, it is possible that compounds that inhibit its function or target genes can be identified, thus providing potential therapeutic agents for breast cancers that express hERβ2.

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Human Estrogen Receptor β 548 Is Not a Common Variant in Three Distinct Populations

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Several isoforms of estrogen receptor (ER) β (also known as NR3A2) have been reported, including variants with different N-terminal ends. In rodents, two in-frame initiation codons (ATGs) are used to produce proteins of 530 and 549 amino acids, respectively. In humans, the upstream ATG is out of frame in all clones reported, until recently, when human clones with an extra A-T base pair placing the upstream ATG in frame were reported. The authors suggested that this could represent a novel polymorphism in the ERβ gene. Because human ERβ548 (hERβ548) and hERβ530 display different functional characteristics *in vitro*, it is of interest to determine if this variant constitutes a polymorphism in human populations. We therefore determined the frequency of this novel isoform in several populations including African (n = 96), Caucasian (n = 100), and Asian (n = 128) subjects using denaturing HPLC. We did not detect any alleles that correspond to hERβ548 in these samples or in additional samples of heterogeneous origin. It is concluded that hERβ548 is not a common variant in Africans, Caucasians, or Asians. (*Endocrinology* 144: 3541–3546, 2003)

MOST OF THE effects of estrogen are mediated by estrogen receptors (ERs). ERs belong to the steroid hormone receptor gene superfamily of ligand-activated transcription factors.

For many years, one ER was thought to mediate all cellular effects of estrogen. This receptor is now referred to as ERα (NR3A1). However, in 1995 another ER, named ERβ (NR3A2), was cloned from rat prostate (1). Several isoforms of ERβ have subsequently been reported, including variants with differing N-terminal ends. The ERβ gene cloned from rat prostate (1) encodes a protein of 485 amino acids. Three years later, a rat prostate ERβ cDNA sequence was submitted to GenBank, which differs from the initial sequence by the addition of one nucleotide upstream of the start codon. The extra nucleotide removes the in-frame stop codon upstream of the start codon initially reported (1), resulting in a cDNA that encodes 64 additional amino acids at the N terminus (2). This form is now referred to as rERβ549 and is considered to be the long form or full-length rodent ERβ.

The first human ERβ (hERβ) cloned encompassed 477 amino acids (3). The N terminus of hERβ has since then been extended. Ogawa *et al.* (4) cloned a longer hERβ that has been considered as the full-length ERβ, consisting of 530 amino acids, hERβ530. There is an initiation codon (ATG) at a similar position in human clones as that encoding the full-length rodent rERβ549 (Fig. 1, A and B). However, in all human clones originally reported, this ATG is out of frame with the rest of the coding sequence.

Recently, an N-terminally extended hERβ variant, corresponding to hERβ548, was cloned from human testis cDNA and genomic DNA (5). An additional A-T base pair shifts the out of frame ATG to be in frame (Fig 1C). In the following, the allele representing the extra A-T base pair and expected to encode hERβ548, is referred to as the +A allele. Interestingly, hERβ548 appears more robust than hERβ530 with regard to transcriptional activation via an estrogen response element in response to 17β-estradiol. Furthermore, both tamoxifen and raloxifen showed significant agonist activity via hERβ548, which was not observed via hERβ530. The authors suggested that this extra nucleotide might represent a polymorphism. If this is true, it is of obvious interest to determine the frequency of hERβ548 in different populations.

Materials and Methods

Samples

Blood samples were taken from blood donors for the following population groups: Africans (n = 96, Gambian, from Banjul) and Asians (n = 128 Han Chinese, from Beijing). The Caucasian samples were from Finland (n = 100, which included 50 breast cancer patients).

Information for additional samples analyzed from diverse origins is shown in Table 1. Studies were approved by ethical committees.

PCR

Primer ERβ-5′ untranslated region (UTR) 5′: TTATACTTGCCCACGAATCTTT and primer ERβ-5′UTR3′: CTTGCTTCACACCAGGGACTCT were used to amplify part of hERβ exon 1. PCR amplifications were performed in a total volume of 25 μl containing 250 μM deoxynucleotide triphosphates, 10–50 ng of template DNA, 0.5 μM each of primers, 1.25 U AmpliTaq Gold DNA polymerase (PE Applied Biosystems, Foster City, CA), in 1× reaction buffer [10 mM Tris HCl (pH 8.3); 50 mM KCl; and 2.5 mM $MgCl_2$]. PCR amplification was carried out at 94 C for 10 min and then cycled 35 times at 94 C for 30 sec, 57 C for 30 sec and 72 C for 45 sec, followed by 10 min at 72 C.

Abbreviations: DHPLC, Denaturing HPLC; hERβ, human ERβ; UTR, untranslated region.

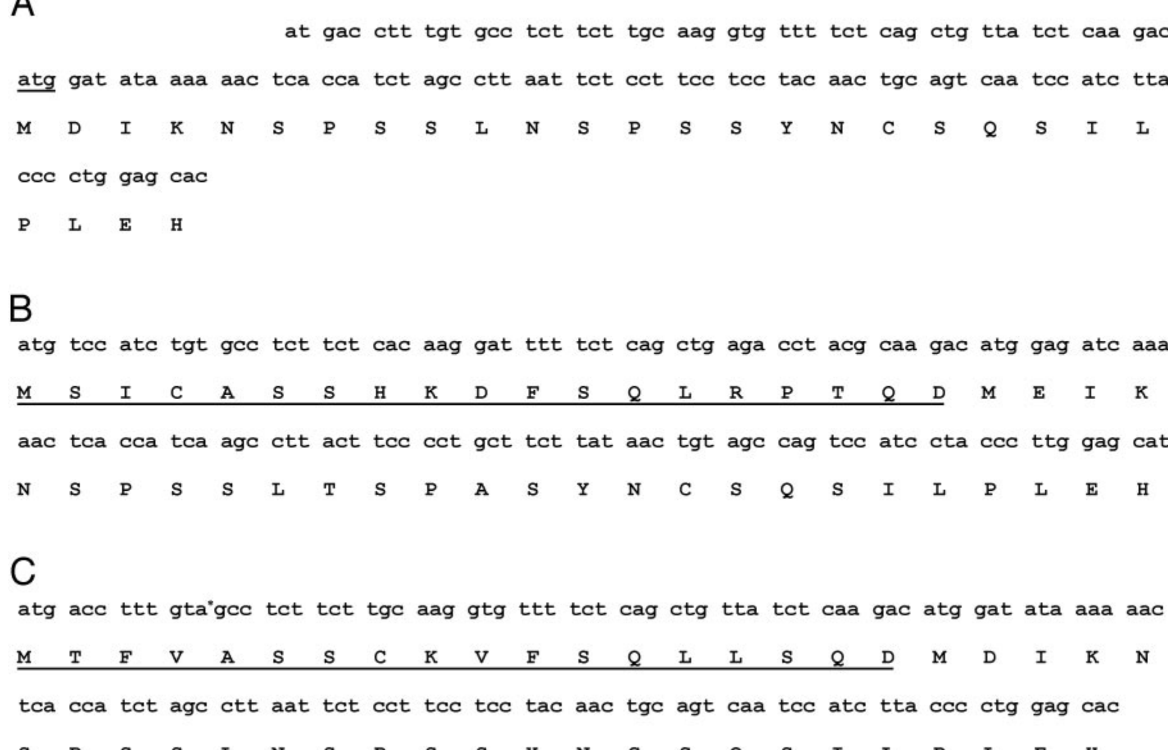


FIG. 1. Nucleotide and deduced amino acid sequences of the N-terminal region of hERβ. The amino acids sequence is given in the one letter code. A, The sequence that encodes hERβ530. The upstream ATG is out of frame with the rest of the coding region. The GenBank accession no. is AB006590. B, The sequence that encodes full-length mouse ERβ549. The additional 19 amino acids that are specific to mERβ549 are *underlined*. The GenBank accession no. is AF067422. C, The sequence that encodes hERβ548. The extra A that places the upstream ATG in frame with the rest of the coding sequence is marked by *. The additional 18 amino acids that are specific to hERβ548 are *underlined*. The GenBank accession no. is AX029400.

TABLE 1. Wave Fragment Analysis System analysis of diverse-origin samples that were negative for hERβ548

Genomic DNA	Number of chromosomes analyzed (2n)
Diverse geographical samples[a]	
Mixed European descent	88
Near East and North Africa	94
Caucasian subjects with female infertility	130
cDNA	Number of samples analyzed (n)
English subjects with breast cancer	6
Human testis Marathon-ready cDNA (CLONTECH)	1

[a] DNA samples of European, Near-Eastern, and North-African origin, representing a variety of regions and populations, were obtained on nonnominative basis from consenting adults providing information about their ethnic, linguistic, and geographic origins or were purchased from Coriell Institute for Medical Research (Camden, NJ).

Generation of an artificial hERβ548 clone

A standard ERβ530 plasmid contains the 5′UTR where the ATG encoding hERβ548 is out of frame. An artificial hERβ548 plasmid that has the reported extra nucleotide of the ERβ gene that generates hERβ548 (Ref. 5; and Fig. 2B) was created from the standard hERβ530 plasmid using the QuikChange XL Site-Directed mutagenesis kit (Stratagene, La Jolla, CA). The sequences of these two clones were confirmed by DNA sequencing. DNA fragments amplified by PCR (using the same pair of primers as used in amplifying genomic DNA) from these plasmids served as controls throughout the experiments and are named hERβ530st and hERβ548art, respectively. The sequences of these PCR products were confirmed by DNA sequencing.

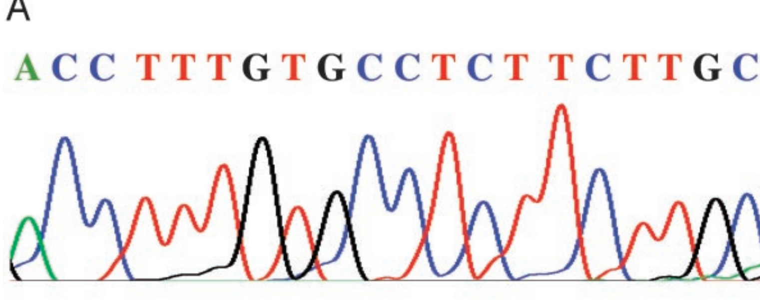
Denaturing HPLC (DHPLC)

Samples were denatured at 95 C and then cooled to 25 C over 45 min to enable the formation of heteroduplexes. Samples were analyzed with DHPLC using a Wave Fragment Analysis System (Transgenomics, Omaha, NE) and DNASep Column as described (6) using the suggested temperature 58 C.

Results

Generation of an artificial clone encoding hERβ548

hERβ530 has been considered to be the full-length hERβ. The sequences of the N-terminal region of the hERβ gene including that generating the recently reported hERβ548 are shown in Fig. 1. The sequence of a plasmid encoding hERβ530 is shown in Fig. 2A. This plasmid and subsequent PCR products generated from it are referred to as hERβ530st. An artificial plasmid encoding hERβ548 was generated including the extra A-T base pair as reported (5). This plasmid and subsequent PCR products generated from it are referred to as hERβ548art.



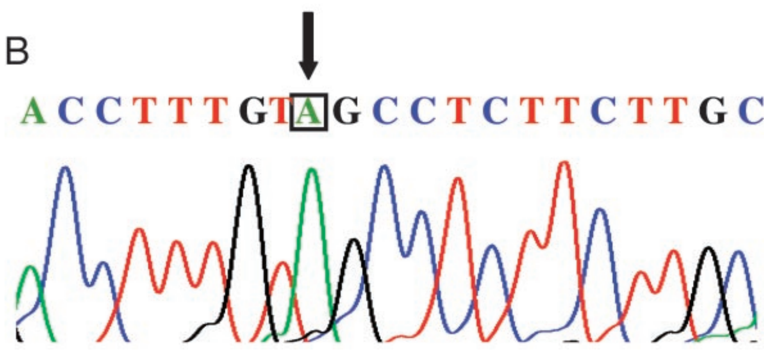


FIG. 2. DNA sequence flanking the artificial extra A nucleotide producing the + A allele. A, Sequence of hERβ530st. B, Sequence of hERβ548art, which was created by mutagenesis. The artificially inserted A (*boxed*) is indicated by an *arrow*.

Validation of DHPLC for detection of the +A allele

DHPLC is based on the differential adsorption of homo- and hetero-duplexes to a hydrophobic matrix on a chromatographic column. Amplified products with a mismatch will form hetero-duplexes that have decreased interaction with the matrix and will be eluted earlier than the normal homo-duplexes. Figure 3 shows that the +A allele can be detected with DHPLC using the employed conditions. To detect possible homozygotes for the +A allele, PCR products from analyzed individuals were mixed with the hERβ530st. To detect heterozygotes for the +A allele, samples were analyzed without mixing. The sensitivity of DHPLC in detecting the presence of an extra nucleotide was evaluated with PCR fragments derived from hERβ 530st and hERβ 548art, respectively. When the amounts of hERβ530st and hERβ548art differ less than 10-fold, the +A allele can be detected (Fig. 3). This shows that it is not absolutely critical for the analysis that the amounts of target and hERβ530st PCR products, respectively, are identical.

Screening of genomic DNA from different populations

In total, 96, 100, and 128 DNA samples from African, Caucasian, and Asian subjects, respectively, were screened by DHPLC. Figure 4A shows, from the top, representative DHPLC profiles obtained from analysis of genomic DNA from Caucasian (n = 100), African (n = 96), and Asian (n = 128) populations, analyzed after mixing with hERβ530st. Similar profiles were obtained when samples were analyzed without prior mixing with hERβ530st. No heteroduplex peak, indicative of the presence of hERβ548art, could be identified in any of the samples. The presence of hERβ548 would have been seen as a heterduplex as shown in Fig. 4B where the samples were mixed with hERβ548art. This figure also shows that the hERβ530 variant can be detected in these samples.

In addition, we have also screened a number of additional samples for the hERβ548 variant. These data are summarized in Table 1. We have analyzed genomic DNA from individuals from a very diverse geographical sampling and from individuals with syndromes related to infertility. cDNA has been analyzed from breast cancer patients and from a commercial source. We did not identify hERβ548 in these samples.

From these results, we conclude that the human 548-amino-acid ERβ does not represent a common allele.

Discussion

Knowing that a single gene might generate several protein products, researchers need to address an additional level of complexity in understanding the function of any gene and its encoded protein product. The ERβ gene is an example of a gene from which several protein products are derived. This occurs through alternative RNA splicing (7–19) or through the utilization of different translation start codons in the 5′ flanking region, generating several N-terminally variable ERβ proteins. In this report, we focus on the frequency of the recently reported hERβ 548 isoform (5). We developed a DHPLC assay for screening of the +A allele and showed that it was robust with regard to detection of samples heterozy-

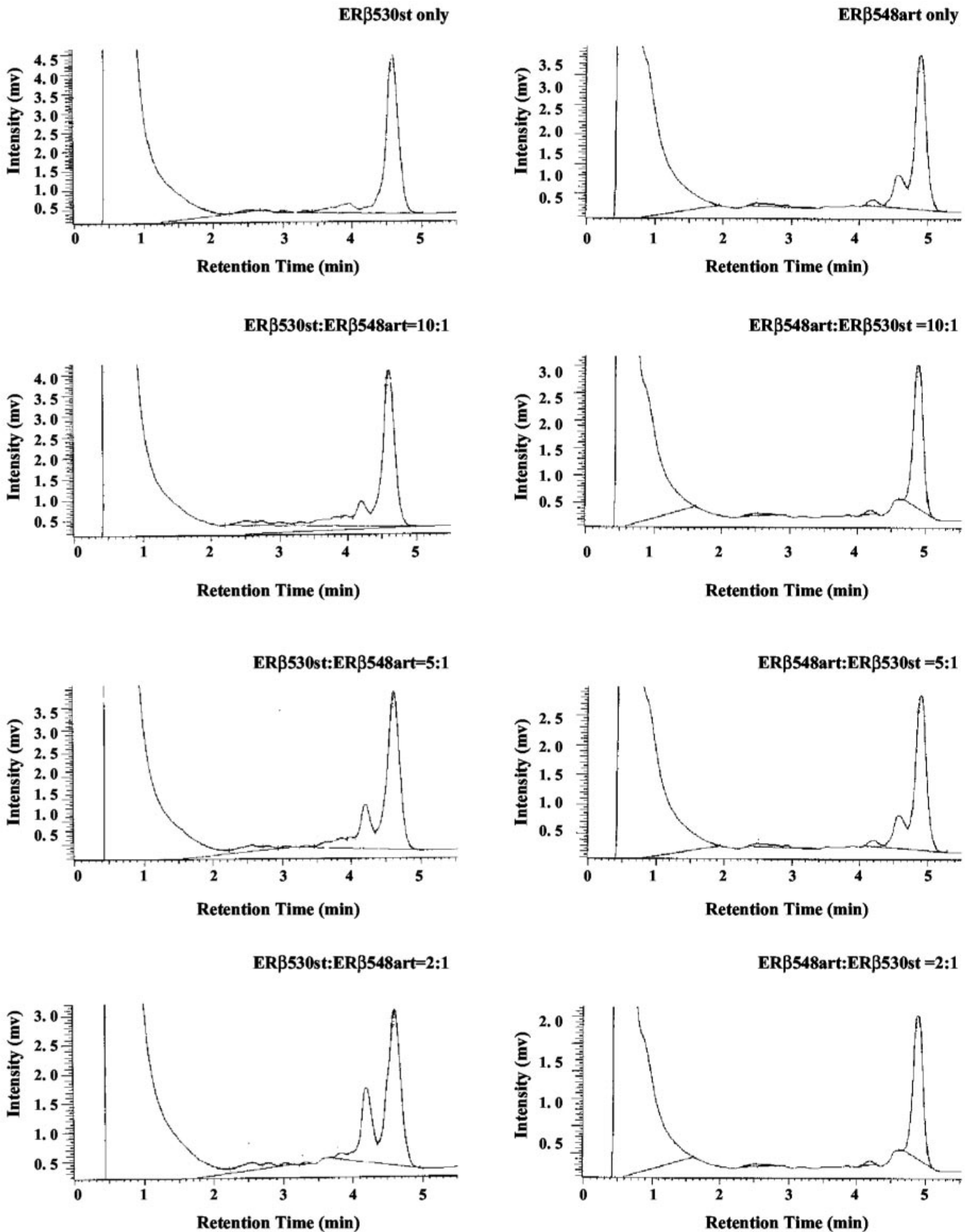


FIG. 3. DHPLC profiles obtained from a wide range of ratios between ERβ530st and ERβ548art. When the ratio is no less than 1:10, the heteroduplex peak could be detected.

gous and homozygous for the +A allele. This report focuses on the screening of samples from 128 Asian, 96 African, and 100 Caucasian individuals for the +A allele encoding hERβ 548. Notably and surprisingly, we did not identify any single allele corresponding to the +A allele. Moreover, analysis of further samples of different origin did not reveal a single +A allele. The reason why we did not detect hERβ548 in human testis Marathon-ready cDNA (CLONTECH Laboratories, Inc., Palo Alto, CA), where it was identified in Ref. 5, is presently unclear. The lot number is not indicated in Ref. 5. Without knowledge about the lot number, CLONTECH Laboratories, Inc. cannot track if the lots are derived from

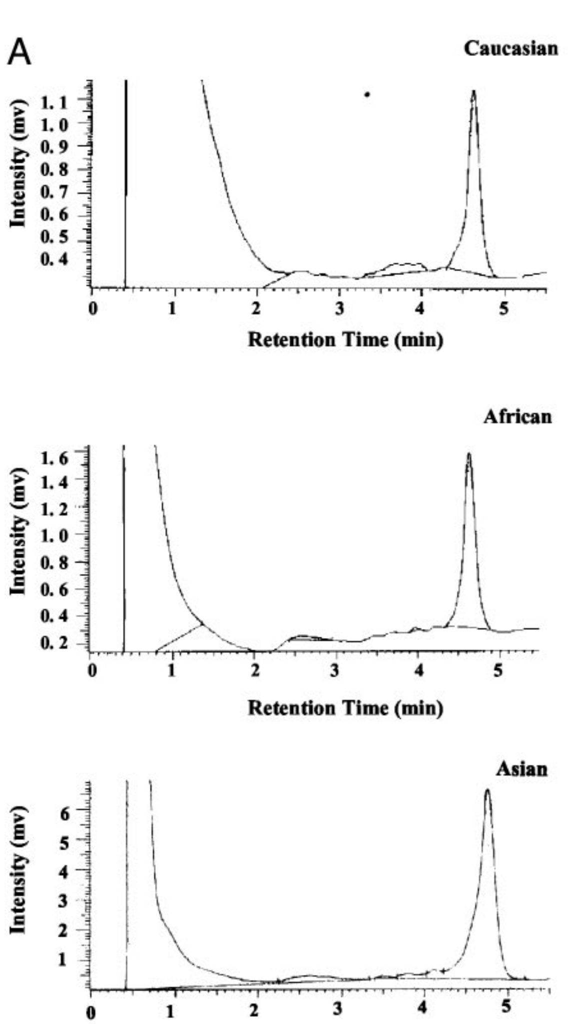


FIG. 4. A, Shows, from the *top*, representative DHPLC profiles obtained from analysis of genomic DNA from Caucasian (n = 100), African (n = 96) and Asian (n = 128) populations. No heteroduplex peak was found. B, Shows that the hERβ530 can be detected in this assay by mixing with hERβ548art. Shown is one representative analysis of 12.

identical RNA sources (information from CLONTECH Laboratories, Inc.).

In this paper, we demonstrate that a potential polymorphic ERβ variant encoding hERβ548 is, if it at all exists, a rare variant in African, Caucasian, and Asian populations. However, there is still the interesting possibility that this allele could exist in special populations and/or that it could be specifically associated with certain syndromes.

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Identification of a functional variant of estrogen receptor beta in an African population

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In this study, we identified five novel polymorphisms in the estrogen receptor beta (ERβ) gene in an African population. Interestingly, two of these variants are expected to change the amino acid sequence of the ERβ protein. These changes correspond to an isoleucine to valine substitution at amino acid position 3 (I3V) and a valine to glycine substitution at position 320 (V320G), respectively. The functional consequences of these amino acid substitutions were determined in different *in vitro* assays. The I3V mutation displayed no differences with regard to transcriptional activity in a reporter assay, as compared with the wild-type receptor. The V320G mutation, however, showed significantly decreased maximal transcriptional activity in a reporter assay, although its binding affinity for 17β-estradiol was not affected. A pull-down assay indicated that the interaction of full-length TIF2 with hERβV320G was weaker than with hERβwt. Moreover, surface plasmon resonance analysis revealed reduced interaction of the V320G ERβ variant with the NR box I and II modules of TIF2. To our knowledge, this represents the first identification of a functional polymorphism in the ERβ gene. This novel polymorphism provides a tool for human genetic studies of diseases in the African population.

Introduction

Estrogen receptors (ERs) belong to the steroid/retinoid receptor gene superfamily, which contains the receptors for glucocorticoids, mineralocorticoids, progesterone, androgen, thyroid hormone, vitamin D and retinoic acid. As a family, its members share some structural and functional similarities including four functional domains. From the N-terminus to the C-terminus of the receptor molecule, these are: the A/B region that contributes to the transcriptional activation function; the C-region, or the DNA-binding domain, that harbors the DNA-binding function mediating specific DNA binding; the hinge region followed by the ligand-binding domain (the LBD or the E/F domain). The LBD harbors the ligand-binding pocket as well as sites for co-factor binding, transactivation, nuclear localization and interactions with heat shock proteins (1). Upon ligand-dependent or -independent activation, these receptors form dimers and modulate transcription by binding to their corresponding hormone response elements (for example ERE, estrogen response element) in the promoter region of target genes (2). There are two estrogen receptors, ERα and ERβ. These two receptors show high homology, particularly in the DNA-binding domain. The receptors are expressed in a distinct but sometimes over-lapping mode and display functional similarities as well as differences, sometimes even opposite actions (3).

Abbreviations: ER, estrogen receptor; ERE, estrogen response element; LBD, ligand-binding domain; SPR, surface plasmon resonance.

Polymorphisms in ER genes, the major mediators of estrogen signaling, are associated with some endocrine related disorders. Polymorphisms in ERα are associated with breast cancer (4–6), endometrial cancer (7), lupus nephritis (8), menstrual disorder (9), Alzheimer's disease (10), osteoporosis (11) and coronary artery disease (12). Polymorphisms in the ERβ gene have been correlated to other pathological states as compared with ERα polymorphisms, such as ovulatory dysfunctions (9), hypertension (13), bone mineral density (14) and androgen levels (15). No data are available regarding polymorphisms in ER genes in African populations.

Several genetic differences have been described between African Americans and white Americans, which may account for the higher incidence of certain diseases in the former population. For example, estrogen metabolism appears to vary according to race, with a higher ratio of inactive:active metabolites in whites compared with blacks (16). Polymorphisms in some steroid hormone nuclear receptors have been shown to correlate with race related endocrine diseases. For example, short CAG repeat lengths in the androgen receptor gene were found in African Americans and possibly associated with a higher stage of prostate cancer (17). Polymorphisms in the vitamin D receptor gene are associated with bone mass differences between African Americans and white Americans (18).

In this investigation we screened the ERβ gene in an African population for polymorphisms. Any identified polymorphism, particularly a functional polymorphism, would constitute important tools for further association with diseases.

Materials and methods

Samples

Nigerian healthy blood donors ($n = 96$ from Banjul, Gambian) were included in the study. Genomic DNA was isolated using standard phenol–chloroform extraction followed by ethanol precipitation. The studies were approved by the ethical committee of the Karolinska Institute.

PCR

PCR amplifications were performed in a total volume of 25 μl containing 200 μM dNTPs, 10–50 ng of template DNA, 0.4 μM each of primers, 1.25 U *Taq* DNA polymerase (Roche, Mannheim, Germany), in 1× reaction buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$). Primer sequences to amplify the ERβ gene were designed based on the published GenBank ERβ gene sequence NT_025892 (Table I). PCR amplification was carried out at 94°C for 10 min followed by 35 cycles (94°C for 30 s, 57–60°C for 30 s and 72°C for 45 s), and finally 10 min at 72°C.

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Table I. Primers for PCR amplification of the ERβ gene fragments

Exon	Primer sequences	Product size
5′ UTR	Forward 5′ TTATACTTGCCCACGAATCTTT 3′ Reverse 5′ CTTGCTTCACACCAGGGACTCT 3′	419 bp
1	Forward 5′ CTTAATTCTCCTTCCTCCTAC 3′ Reverse 5′ GTGATTTGAGAAATGGCTAGC 3′	387 bp
2	Forward 5′ GCTTTGCTGTATCAGATTTCCGGG 3′ Reverse 5′ ATTTCTGCCAAGTCATCTCTGC 3′	407 bp
3	Forward 5′ TGGCTTTGTACCTGTACTGGTCAT 3′ Reverse 5′ GCCAAAATCTGCCTCCCATAATC 3′	473 bp
4	Forward 5′ GTCGTTGGTTTTGCTAGTACGG 3′ Reverse 5′ CCAGCTGAGGACCTGTTAAATA-TCTAGGC 3′	567 bp
5	Forward 5′ GTTGCGCAGCTTAACTTCAAAGT-TTTCTTC 3′ Reverse 5′ TGAAGGAGCTGATGCTATCATC 3′	456 bp
6	Forward 5′ GTTTCCTGAAGCTATGTTCCT 3′ Reverse 5′ CGCTAGTTGTAGAAACAGCAT 3′	242 bp
7	Forward 5′ TGCATTAGGCCAGGCTTCTCTTCT 3′ Reverse 5′ GTGCCCATCTTTGCTTACAGGTG 3′	562 bp
8	Forward 5′ GTAGACTGGCTCTGAGCAAAGA-GAGCC 3′ Reverse 5′ CCAAGCCTGCCATCACCAAATGAG 3′	405 bp
cx	Forward 5′ GAGCAAACCAGCTTAAAGGCCC 3′ Reverse 5′ CTCATGGGTGAGACATCTGCAAGC 3′	473 bp

Denaturing high-performance liquid chromatography (DHPLC)

PCR products from amplification of genomic DNA from all individuals were analysed using DHPLC on a WAVE DNA Fragment Analysis System (Transgenomic, Cheshire, UK) and DNASep Column as described (19) using the temperature suggested by the WAVEMAKER™ Software package (Transgenomic, Crewe, UK). Prior to DHPLC analysis, PCR products were denatured at 95°C for 5 min and then cooled to 25°C over 45 min to enable the formation of heteroduplexes. Aliquots of 5 μl were automatically loaded on the DNAsep column for heteroduplex analysis.

Samples with aberrant HPLC profiles were subjected to DNA sequencing using ABI Prism® BigDye™ Terminator Cycle Sequence Ready Reaction Kit (Applied Biosystems) and compared with the published genomic sequence of the ERβ gene. In certain cases, Restriction Fragment Length Polymorphism analysis was used to confirm polymorphisms. *Fok*I was used to score 105A→G, *Rsa*I was used to score 1082G→A, *Alu*I was used to score1730G→A and *Van*91I was used to score 1057T→G.

Generation of human ERβ plasmids containing the 105A→G or 1057T→G mutations

A wild-type pSG5-hERβ plasmid was a gift from Dr Michel Tujague at the Department of Biosciences, Karolinska Institute. hERβ plasmids that incorporate the identified amino acid changes of the ERβ gene were created from the wild-type hERβ530 plasmid using the QuickChange™ XL Site-Directed mutagenesis kit (Stratagene, La Jolla, CA) according to the instruction manual. DNA sequencing confirmed the sequences of the mutant clones. The resulting plasmids are named hERβ105A→G and hERβ1057T→G, respectively.

Transient transfection assays and western blot analysis

HEK293 cells were cultured in a 1:1 mixture of Ham's Nutrient mixture F12 (Invitrogen) and DMEM (Invitrogen) supplemented with 5% FBS and 100 U penicillin/ml and 100 mg streptomycin/ml. For transfection, the cells were seeded at a density of 1×10^4 cells/well in 96-well plates and co-transfected with 2× ERE TK luciferase reporter plasmid (0.4 μg) (20) and the respective ER expression vectors (0.016 μg). A pRL-TK control plasmid, which contains a Renilla luciferase gene, was included to control for differences in transfection efficiencies. The medium was replaced with a phenol red-free mixture of F12 and DMEM containing 5% dextran-coated charcoal-treated FBS and 100 U penicillin/ml and 100 μg streptomycin/ml upon transfection. 17β-Estradiol (0.1, 1, 10, 100 nM) or vehicle (in 0.1% ethanol) was added just after transfection. The cells were harvested 24 h after transfection and luciferase activities were determined using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

Western blotting was done according to the protocol as described (21). ERβ was detected with an ERβ LBD rabbit polyclonal antibody produced by us as described previously (22). As a positive control, the recombinant human ERβ530 protein purchased from Panvera (Madison, WI) was used.

Cloning and expression of hERβwt and hERβ1057T→G LBDs

The LBDs (R254 to Q530) were obtained by PCR using full-length cDNAs as templates and primers that contained appropriate restriction sites. hERβwt and hERβ1057T→G were cloned into pET15b (Novagen, Madison, WI) to generate proteins with N-terminal His-tags. The sequences of all constructs were verified by DNA sequencing.

Cultures (500 ml) of the *Escherichia coli* strain BL21, transformed with the appropriate expression plasmids, were cultivated overnight in LB supplemented with 100 μg/ml of ampicillin at 37°C. When the OD600 reached 1.0, IPTG was added to a final concentration of 1 mM and incubation continued for 3 h at 25°C. The cells were pelleted and the supernatant was discarded. The pellet was suspended in 5 ml (one-tenth of the culture volume) of extraction buffer [complete EDTA-free, Roche Diagnostics, Germany, 0.3 M NaCl, 20 mM Tris (pH 8.0), 0.01 mg/ml DNase, 0.01 mg/ml RNase, 10 mM $MgCl_2$, 0.25 mg/ml lysozyme, 1 mM β-ME and protease inhibitor cocktail tablet]. The samples were sonicated for 4 min at 50% duty (total sonication time 2 min). The homogenate was centrifuged at 13 000 *g* for 20 min at 4°C. The supernatant was applied to a TALON metal affinity column (Clontech Laboratories, Palo Alto, CA). Fractions containing the purified protein were dialyzed against 20 mM Tris (pH 8), 150 mM NaCl, 1 mM DTT and frozen at −80°C. The purified protein was >95% pure as determined from Coomassie stained SDS–PAGE gels. The protein concentrations were measured using the Coomassie Protein Assay Kit (Pierce, IL) according to the manufacturer's instructions.

Scintillation proximity assay

The assay was performed in 96-well microplates (PerkinElmer Life Sciences, MA). Polyvinyltoluene copper-loaded his-tag beads were purchased from Amersham. The reaction mixture (60 μl/well) containing assay buffer (1 mM EDTA, 0.9 M KH_2PO_4, 0.1 M K_2HPO_4, 20 mM Na_2MoO_4 and 0.05% monothioglycerol), beads (30 μg/well) and purified ERβ LBD (final concentration of 20 nM) was incubated at 4°C for at least 1 h. For saturation ligand-binding analysis, a sample of various concentrations of [^{3}H]17β-estradiol (S.A. = 95 Ci/mmol) in the presence or absence of a 300-fold excess of unlabeled 17β-estradiol was then added. The assay plates were sealed, allowed to settle overnight and subsequently counted on a Wallac 1450 micro-β counter. The dissociation constant (K_d) was calculated as the free concentration of radioligand at half-maximal specific binding by fitting data to the Hill equation and by linear Scatchard transformation (23). Curve fitting was done in Prism (GraphPad Software).

For ligand competition studies, purified ERβ LBDs (20 nM) were incubated overnight at 4°C with a range of test compound concentrations. A final concentration of 1.5 nM [^{3}H]17β-estradiol (30 μl/well) was used. The ligands were tested three times with similar results. Curve fitting was performed using Prism (GraphPad Software) and the IC_{50}s determined. IC_{50} values were converted to K_i using the Cheng-Prusoff equation, $K_i = IC_{50}/(1 + D/K_d)$, where D is the concentration of the radioligand (24).

Pull-down assay

For pull-down assays, purified His-tagged ERβ LBDs (100 μg) were bound to 60 μl of Talon resin and then equilibrated in 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 10% glycerol and 0.5% NP-40 (equilibration buffer). The gel slurry was then divided into two equal aliquots and to each tube, 2.5 ml of *in vitro* translated, ^{35}S-labeled (TNT coupled reticulocyte lysate system, Promega), full-length TIF2 was added in a total volume of 150 μl equilibration buffer containing 1.5% BSA. Estradiol or vehicle (ethanol) was added as indicated. As control, TIF2 was mixed with Talon gel without bound ERβ. All samples were incubated for 2 h with gentle shaking at 4°C. After washing three times with equilibration buffer, bound proteins were eluted with SDS–PAGE sample buffer and separated on a 12% polyacrylamide gel. The gel was stained with Coomassie Blue, followed by determination of ^{35}S using a PhosphoImager instrument.

Surface plasmon resonance (SPR) analysis

All SPR measurements were performed on a BIAcore 2000 instrument (BIAcore AB, Uppsala, Sweden). All experiments were performed at 25°C, and at a flow rate of 5 μl/min. Research grade streptavidin sensor chips were obtained from BIAcore AB. The streptavidin chips were first treated with three 1-min pulses of 50 mM NaOH and 1 M NaCl at a flow rate of 5 μl/min. N-terminally biotinylated peptides (>95% purity) were purchased from Interactiva (Germany). The human TIF2 L*XX*LL peptide sequences were as follows: Box 1 (residues 636–649), KGQTKLLQLLTTKS and Box 2 (residues 685–698), EKHKILHRLLQDSS. Peptides were immobilized on individual surfaces to 200 RU responses. Samples of purified hERβwt-LBD or hERβV320G-LBD in the presence or absence of estradiol were then injected over each surface. After injection stop, the surfaces were washed with buffer to monitor the dissociation phase. The buffer used was 50 mM Tris–HCl, pH 7.4,

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150 mM NaCl, 1 mM EDTA, 0.05% Tween 20. For the kinetic measurements, various concentrations of hERβwt-LBD and hERβV320G-LBD (from 0.25 to 2 μM) were injected over the chip surfaces. The BIAevaluation software version 3.1 was used for evaluation. Different binding models (different rate equations) were tested in the global curve fitting procedure, and the model best describing the experimental data was a 1:1 binding with drifting baseline model. The apparent K_d values are calculated as described (25).

Results

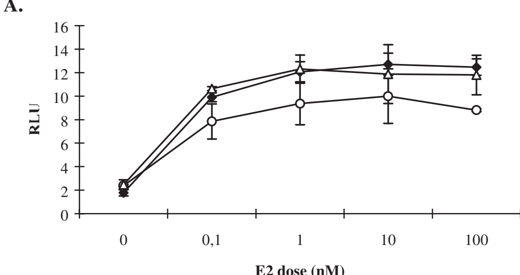
ERβ polymorphism screening in an African population

We screened genomic DNA from 96 Nigerians to identify polymorphisms in the ERβ gene in an African population. Analysis of the coding exons and flanking intron sequences revealed several known but also novel variants. The results are summarized in Table II. Three variants (1082G→A, 1505-4A→G and 1730A→G) that have been reported previously in Caucasian populations (26,27) were also found in the African population but with different frequencies. None of these polymorphisms change the amino acid sequence of the ERβ protein. Five novel polymorphisms in the coding region of the ERβ gene were found in the African population. Interestingly, as shown in Table II, two of these novel polymorphisms change the amino acid sequence of the ERβ protein. The novel amino acid changes are 105A→G [changing amino acid 3, isoleucine (I)→valine (V)] in exon 1 and 1057T→G [changing amino acid 320, valine (V)→glycine (G)] in exon 5. These two ERβ variants are, in the following, referred to as hERβI3V and hERβV320G, respectively.

hERβV320G shows reduced transcriptional activation in a transactivation assay

To test if the identified receptor variants displayed any differences compared with the wild-type receptor with regard to transcriptional activation, a reporter assay was used. We generated plasmids expressing either hERβI3V or hERβV320G under control of the SV40 promoter. The transcriptional activities of the variants were compared with that of the wild-type ERβ using an ERE-luciferase reporter system. No differences with regard to transcriptional activation were observed for the hERβI3V variant (Figure 1A). However, hERβV320G showed significantly decreased maximal transcriptional activity compared with wild-type ERβ (Figure 1A). To confirm equivalent ERβ expression, extracts from HEK293 cells transfected with equal amounts of the expression vectors for hERβwt, hERβV320G, or hERβI3V were separated by SDS–PAGE and analysed for ERβ expression by western blot (Figure 1B). This analysis shows that the different ERβ derivatives are expressed at similar levels. The observed reduction in maximal transcriptional activity could imply that hERβV320G is defective for interactions with co-factors. Interestingly, as shown in

A.



B.

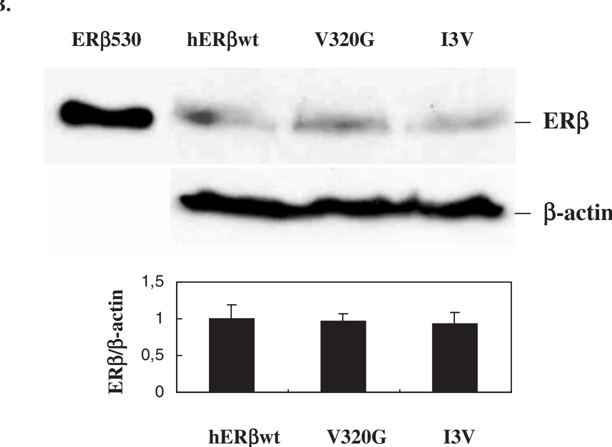


Fig. 1. hERβV320G has decreased maximal transcriptional activity compared with hERβwt or hERβI3V. (**A**) Transcriptional activity of ERβ wild-type and variant proteins assayed on 2× ERE TK luciferase reporter. HEK293 cells were transfected with the 2× ERE TK luciferase reporter plasmid and expression plasmids encoding wild-type (◆) or mutated-type ERβ (hERβI3V = Δ, hERβV320G = ◯), respectively. Cells were treated with vehicle (ethanol) or indicated concentrations of 17β-estradiol. Values represent the mean ± SD of three independent experiments. (**B**) Western blot of identical amounts (150 μg protein) of whole cell extracts from HEK293 cells transfected with equal amounts of the expression vectors for hERβwt, hERβV320G or hERβI3V. Recombinant human ERβ530 protein was used as positive control. The blot was stripped and probed for β-actin. The bands were quantified by densitometric scanning and the amount of ERβ normalized to β-actin. Data are the value (in pixels) for ERβ divided by the value (in pixels) for β-actin and normalized to the hERβwt expression level, which was set to 1. The bar graph shows the mean ± SD of the three separate experiments.

Table II. Polymorphisms in the ERβ gene in an African population

	Nucleotide[a]	Amino acid	Frequency			
			Reported (26,27) (Caucasian)		African (n = 96)	
			Heterozygotes	Homozygotes	Heterozygotes	Homozygotes
Exon 1	105A→G	3I→V			0.052 (n = 5)	0
	143C→T	Silent			0.042 (n = 4)	0
Exon 2	566A→T	Silent			0.01 (n = 1)	0
Exon 5	1057T→G	320V→G			0.031 (n = 3)	0
	1100T→G	Silent			0.01 (n = 1)	0
	1082G→A	Silent	0.03–0.16	0.004	0.33 (n = 32)	0.02 (n = 2)
Exon 8-	1505-4A→G		0.06–0.16	0.004	0.32 (n = 31)	0.02 (n = 2)
3′ UTR	1730A→G		0.43–0.52	0.11–0.13	0.25 (n = 24)	0.07 (n = 7)

[a]See ref. (49) for numbering of genomic sequences.

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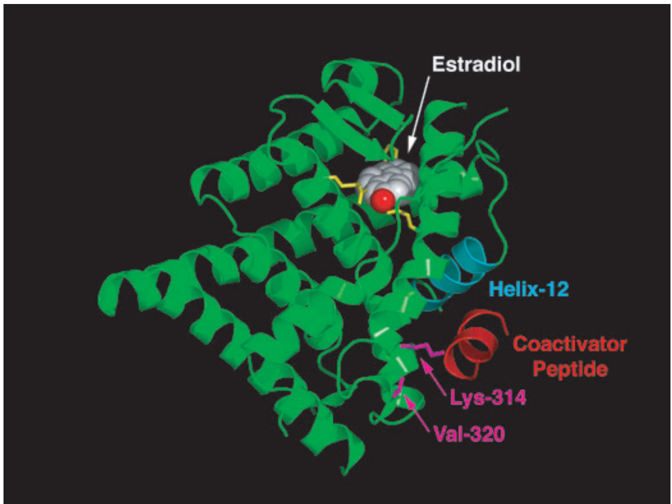


Fig. 2. Modeled structure of human ER-β LBD complexed with 17β-estradiol and the TIF2 co-activator peptide [PBD accession number 1QKM (29) and human ER-α complexed with 17β-estradiol and the TIF2 co-activator peptide (1GWR) (50)]. The protein is depicted by a green ribbon/tube while the ligand estradiol is represented by white and red spheres (carbon and oxygen atoms, respectively). Helix-12 and the TIF2 co-activator peptide are depicted in cyan and red, respectively. The positions of the Lys-314 and Val-320 sidechains are shown as magenta tubes. This figure was produced using the PyMOL program (http://www.pymol.org).

Figure 2, the amino acid changed in hERβV320G is located on the surface of the receptor protein, quite distant from the ligand-binding pocket.

hERβwt and hERβV320G bind ligands with similar affinity

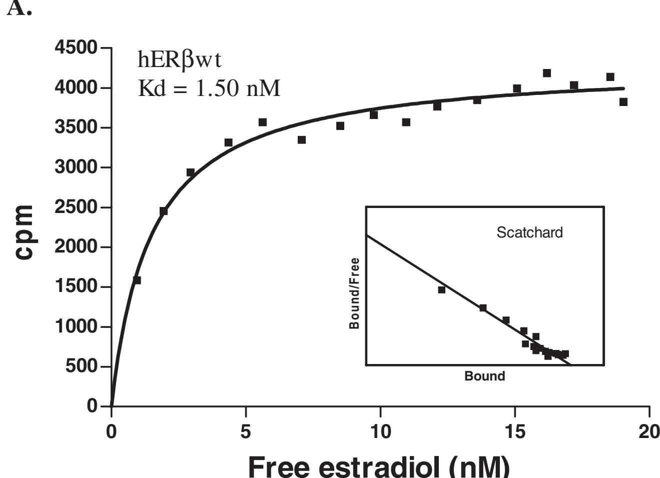
To facilitate a biochemical characterization of hERβV320G, and particularly to compare it to hERβwt, the LBDs of these two proteins were expressed in *E.coli* and the recombinant proteins were purified. In Figure 3A and B, the results of saturation ligand-binding assays with tritiated estradiol are shown. The measured K_d values were 1.50 nM for hERβwt wt and 1.79 nM for hERβV320G (Figure 3). Affinities for estradiol, tamoxifen and raloxifen, as determined in a competition assay, were also similar for hERβwt and hERβV320G. Thus, the K_is of estradiol, tamoxifen and raloxifen for hERβwt were 0.25, 5.45 and 9.45 nM and for hERβV320G they were 0.33, 5.33 and 10.11 nM, respectively. This is consistent with the position of the variant amino acid distant from the ligand-binding pocket (Figure 2).

hERβV320G shows reduced co-factor interaction

Having demonstrated that hERβV320G binds ligands with similar affinity as hERβwt, yet shows significantly reduced maximal transactivation in a reporter assay, we hypothesized that hERβV320G is defective for co-factor interactions. This is consistent with the position of the V320G amino acid change, on the surface of the receptor protein in a region postulated to be involved in co-factor interactions (Figure 2). To test this hypothesis, we performed pull-down assays using purified His-tagged ERβ LBDs and ^{35}S-labeled full-length TIF2 in the presence or absence of estradiol. As shown in Figure 4, TIF2 bound to hERβwt and hERβV320G in a ligand-dependent manner. Furthermore, the interaction of TIF2 with hERβV320G was weaker than with hERβwt.

To more quantitatively examine TIF2 interaction to hERβV320G and hERβwt, we used the SPR analysis, where

A.



B.

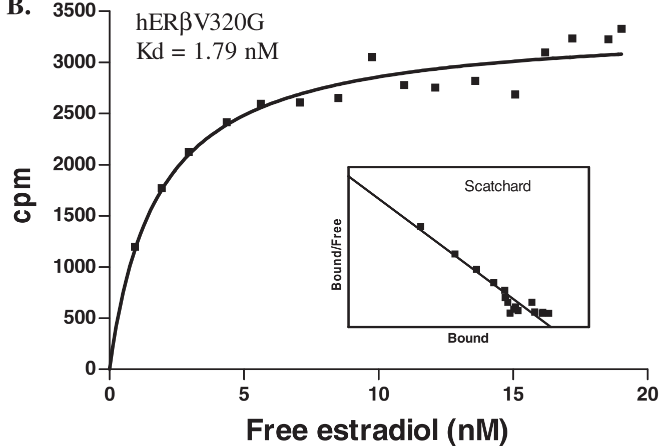


Fig. 3. Saturation and Scatchard analyses for [^{3}H]17β-estradiol binding to hERβwt and hERβV320G. hERβwt and hERβV320G LBDs (20 nM) were incubated with increasing concentrations (0–20 nM) of [^{3}H]17β-estradiol at 4°C overnight. The inset shows linear transformation of the data by Scatchard analysis.

	Input	1	2	3	4	5	6
E2		−	+	−	+	−	+

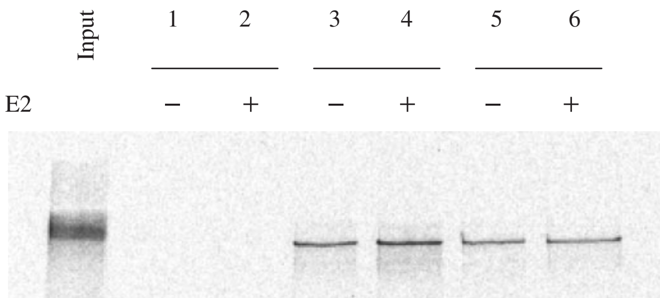


Fig. 4. Analysis of TIF2 intercation with hERβwt and hERβV320G by pull-down assay. *In vitro* translated TIF2 was incubated with hERβwt (lanes 4 and 5) and hERβV320G (lanes 6 and 7), with or without estradiol (E2) as indicated. Non-specific binding of TIF2 to Talon beads is shown in lanes 2 and 3. Input corresponds to 0.5 μl of the *in vitro* translation mixture. The experiment was repeated three times with similar results.

biotinylated 14mer peptides containing NR box motifs (L*XX*LL) from TIF2 were captured via streptavidin to the chip surface. Figure 5 illustrates the binding of hERβV320G and hERβwt in the presence or absence of estradiol to the NR

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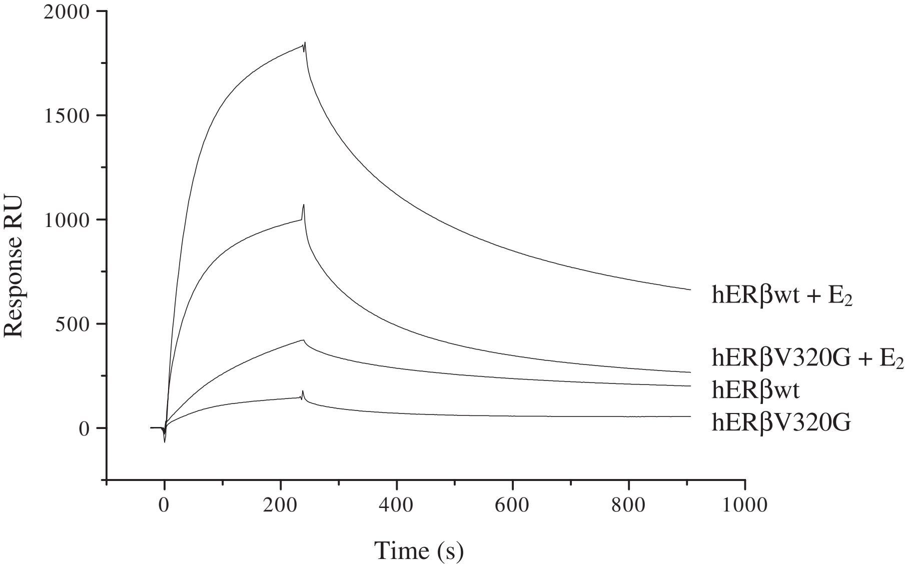

Fig. 5. SPR analyses of hERβwt and hERβV320G binding to TIF2. The real-time binding sensogram shows injection of 1 μM hERβwt and 1 μM hERβV320G in the presence or absence of estradiol over a surface immobilized with 200 RU of TIF2 NR box1.

box of TIF2. The result showed that binding of estradiol to ERβ enhances the affinity of ERβ-peptide interaction. Binding studies were made for the different TIF2 NR box peptides using five to six different concentrations of hERβwt-LBD or hERβV320G-LBD unliganded or liganded with estradiol ranging from 0.25 to 2 μM. Affinity determination analysis was then performed using BIAevaluation software. The curve fitting analysis showed that the model best describing the experimental data was a 1:1 binding with drifting baseline model. The K_d for binding to TIF2 box1 was 0.23 ± 0.04 μM (mean ± SD) and 0.52 ± 0.05 μM and K_d for binding to TIF2 box2 was 0.39 ± 0.1 μM and 0.79 ± 0.1 μM for hERβwt-LBD and hERβV320G-LBD liganded with estradiol, respectively. The determined affinities show that hERβV320G displays reduced affinity for TIF2 as compared with that of wild-type ERβ. Reduced affinity for TIF2 was also seen with unliganded hERβV320G-LBD as compared with unliganded hERβwt-LBD (data not shown).

Discussion

In this paper we identify five novel polymorphisms in the hERβ gene in an African population. Interestingly, two of these polymorphisms are expected to change the corresponding amino acid of the encoded ERβ protein. These polymorphisms have not been identified in any of the published reports that describe the characterization of polymorphisms in the ERβ gene in both Caucasians (n = 502) (27) and Asians (n = 184) (28). In Africans, the variant protein hERβV320G, encoded by one of these polymorphic ERβ genes, displays lower maximal transcriptional activity compared with wild-type ERβ. To our knowledge, this represents the first example of a functional polymorphism in the ERβ gene.

The hERβV320G change results in the amino acid substitution of valine for glycine in helix 4 of the LBD. Helix 4 does not participate directly in binding of the ligand (29). However, it forms part of the surface that interacts with co-activators (30). The hERβV320G side chain is in close proximity to K314, which forms half of the charge-clamp of the co-activator-binding pocket. Therefore, the hERβV320G may perturb the conformation of K314, which in turn perturbs cofactor interactions. Consistent with this, we did not find any changes in ligand-binding affinity of hERβV320G. However, this variant displays decreased interaction with the co-activator TIF2. We propose that reduced co-factor interaction is the cause for the observed reduced maximal transactivation.

Several endocrine-related diseases show population-based differences in terms of disease incidence and/or disease progression but the reason for this racial difference is unclear. When compared with white American women, African American women have a higher incidence of breast cancer before the age of 40 years, and the prognosis after a diagnosed breast cancer is reported to be poorer at all ages (31). Studies have suggested that higher plasma levels of insulin-like growth factor-1 (IGF-1) may account for African American women having higher risk of premenopausal breast cancer than white women and may also be an important determinant of breast cancer risk in postmenopausal women (32–34). A prolonged exposure to estrogens has been repeatedly found to be associated with an increased risk for breast cancer. Racial disparities in conditions such as postmenopausal obesity, early menarche and late menopause that all increase endogenous levels of estrogens may put an African American woman at a greater risk for breast cancer (35). African American men present a higher stage of prostate cancer and a worse outcome of this disease than non-African American men (36). The increased frequency of prostate cancer among African American men has been attributed to either greater plasma levels of testosterone (37) or an increased dihydrotestosterone/testosterone ratio (38) in this population. Previous studies also revealed that the plasma level of IGF-1 might be a predictor of prostate cancer risk (39,40). Some of these racial differences could be attributed to population differences in genetic polymorphisms. Polymorphisms involving genes coding for the androgen receptor, phase I/II enzymes and vitamin D receptor have been associated with an increased risk of prostate cancer (41). Polymorphisms that are associated with breast cancer risk have been identified in the genes involved in a wide variety of functions including steroid hormone metabolism,

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detoxification of environmental carcinogens and tumor suppressor genes (42).

In this study, we have identified one novel polymorphism in the ERβ gene in an African population that showed decreased transcriptional activity. Dysregulation of ERβ expression has been reported to be associated with progressions of breast and prostate cancer (43–46), indicating that ERβ functions as a tumor suppressor gene. The hyperplastic prostate (47) and dedifferentiated mammary gland (48) phenotypes displayed by ERβ knockout mice support a role for ERβ in control of mammary and prostate growth. Further association studies are required to determine whether this polymorphism is involved in the increased incidence of prostate and breast cancers in Africans.

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Mouse Estrogen Receptor β Isoforms Exhibit Differences in Ligand Selectivity and Coactivator Recruitment

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ABSTRACT: Estrogens exert their physiological effects through two estrogen receptor (ER) subtypes, ERα and ERβ. In mouse, the cloning of an alternative splice variant of the wild-type ERβ (mERβ1), mERβ2, which contains an 18 amino acid insertion in the ligand binding domain, contributed an additional level of complexity to estrogen signaling. In this study we have assayed the interaction of several known ligands with mERβ1 and mERβ2. The binding affinity of estradiol was 14-fold higher for mERβ1 than for mERβ2. In contrast, raloxifene was dramatically (8-fold) mERβ2 selective. The selectivity for mERβ2 was abolished when the 2-arylbenzothiophene core of the raloxifene molecule was tested for binding affinity, demonstrating that the 3-aroyl side chain of raloxifene plays an important role in contributing to its mERβ2 selectivity. The opposite isoform selectivity found for estradiol and raloxifene in our ligand binding assay was also reflected in the transactivation assay system. That is, mERβ2 required 10-fold greater estradiol concentrations for maximal activation compared to mERβ1, whereas raloxifene was more potent in antagonizing estradiol-induced gene expression via mERβ2 than mERβ1. The raloxifene core behaved as a pure agonist. Furthermore, mERβ2 showed significantly decreased estradiol-induced maximal transcriptional activity as compared to mERβ1. A pull-down assay indicated that the interactions of TIF2 and RAP250 with mERβ2 were weaker than with mERβ1. To assess TIF2 and RAP250 interactions with ERs more quantitatively, we examined the interaction of LXXLL containing peptides derived from TIF2 and RAP250 with mERβ1 and mERβ2 using surface plasmon resonance analysis. Our results indicate that mERβ2 interacts with both coactivators with lower affinity, which may explain its reduced transcriptional activity. Taken together, these results suggest that ligand selectivity and coactivator recruitment of the ERβ isoforms constitute additional levels of specificity that influence the transcriptional response in estrogen target cells.

Estrogens exert their physiological effects through two estrogen receptor (ER)[1] subtypes, ERα and ERβ, that belong to a large family of transcription factors, the nuclear receptor family (*1*). ERα and ERβ contain characteristic sequence motifs associated with transactivation, DNA binding, and hormone binding (*2*). They share high homology within the DNA binding domain and modest homology within the ligand binding domain (LBD). The LBD is multifunctional and, in addition to harboring the ligand binding pocket, encompasses regions for receptor dimerization and ligand-dependent (AF-2) transactivation (*3*). Hormone binding to the ER LBD induces a conformational change in the receptor that initiates a series of events that culminate in the activation or repression of responsive genes (*4*). Although the precise mechanism by which ER affects gene transcription remains to be determined, it appears to be mediated, at least in the case of AF-2 activation, via nuclear receptor coregulators that are recruited by the DNA-bound receptor (*5*).

To date, a number of ERβ mRNA isoforms, generated by alternative mRNA splicing, have been described in human, mouse, and rat. In mouse, the originally described wild-type form has been named mERβ1 (*6*). mERβ2 is an alternative splice variant where an additional exon is spliced in-frame between exons 5 and 6 to generate a protein with an 18 amino acid insertion in the LBD (*7*). The inserted amino acid sequence shares significant (16 out of 18 amino acids) homology with the respective insert in the rat ERβ2 (rERβ2) isoform (*8*). It was found that mERβ1 and mERβ2 are expressed at similar relative levels in some tissues such as the ovary and lung. However, in a range of tissues such as liver, pancreas, gut, and bone, mERβ2 mRNA is more abundant (*7, 8*). In ligand binding assays ERβ2 binds estradiol with a lower affinity than ERβ1 (*9*−*12*). In a reporter assay, ERβ2 shows lower transcriptional activity than ERβ1 and acts as a negative regulator when it is coexpressed with ERα or ERβ1 (*12*). The physiological role of rodent ERβ2 remains to be determined. It was shown that rERβ2 protein was upregulated during the lactation period, suggesting that rERβ2 may play a role in silencing of ERα function during lactation in rat mammary gland (*13*).

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[1] Abbreviations: ER, estrogen receptor; LBD, ligand binding domain; SPR, surface plasmon resonance.

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Ligand-dependent interaction of nuclear receptors and coactivators is a critical step in nuclear receptor-mediated transcriptional regulation. The most studied coactivators for nuclear receptors belong to the steroid receptor coactivator 1 (SRC-1) family, which contains three related members, referred to as SRC-1, SRC-2/GRIP1/TIF2, and SRC-3/p/CIP/RAC3/ACTR/AIB1/TRAM1 (*14*). Critical for the function of these coactivators is the central nuclear receptor interaction domain consisting of three equally spaced conserved LXXLL motifs, also called nuclear receptor boxes. Nuclear receptor-activating protein 250 (RAP250), also called ASC-2, PRIP, TRBP, and NRC, was recently cloned and described as a novel nuclear receptor coactivator consisting of two LXXLL motifs, of which only the N-terminal motif interacts with nuclear receptors in general (*15*).

In this paper we address the characteristics of two mouse ERβ isoforms, mERβ1 and mERβ2, with regard to ligand selectivity and transcriptional activation.

EXPERIMENTAL PROCEDURES

Materials. 17β-Estradiol, 4-OH-tamoxifen, genistein, and raloxifene were from Sigma-Aldrich Sweden AB. ICI-182,-780 was obtained from Tocris, Inc. The raloxifene core [2-(4-hydroxyphenyl)benzo[*b*]thiophen-6-ol] was prepared at Karo Bio AB (Sweden) according to the method of Jones et al. (*16*). The radioligand [^{3}H]-17β-estradiol was purchased from PerkinElmer Life Sciences Inc.

Plasmids, Transient Transfection Assays, and Western Blot Analysis. The pSG5-mERβ1 expression plasmid was a gift from Dr. K. Pettersson at the Department of Biosciences, Karolinska Institutet. This expression vector includes the cDNA that encodes the full-length murine ERβ (549 aa) (*17*). The murine ERβ2 expression plasmid (pSG5-mERβ2) was constructed by replacement of the murine ERβ1 cDNA sequences spanning nucleotides 841−1200 (GenBank, U81451) with the corresponding region of the murine ERβ2 generated by RT-PCR, using *Sac*I/*Bst*BI restriction sites. The sequences of the plasmids were verified by DNA sequencing.

HEK293 cells were cultured in a 1:1 mixture of Ham's nutrient mixture F12 (Invitrogen) and DMEM (Invitrogen) supplemented with 5% FBS, 100 units of penicillin/mL, and 100 μg of streptomycin/mL. For transfection, cells were seeded at a density of 5×10^4 cells/well in 24-well plates and cotransfected with 2 × ERE TK luciferase reporter plasmid (0.8 μg) together with pSG5-mERβ1 or pSG5-mERβ2 (0.016 μg) expression plasmids. A pRL-TK control plasmid, which contains a *Renilla* luciferase gene, was included to control for differences in transfection efficiencies. Transfections using the Superfect reagent (Qiagen) were performed according to the manufacturer's protocol. The medium was replaced with a phenol red-free mixture of F12 and DMEM containing 5% dextran-coated charcoal-treated FBS, 100 units of penicillin/mL, and 100 μg of streptomycin/mL upon transfection. Estradiol, raloxifene, the raloxifene core, or vehicle (in 0.1% ethanol) was added immediately after transfection. The cells were harvested 24 h after transfection, and luciferase activities were determined using the dual luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Western blotting was done according to the protocol as described (*13*). ERβ was detected with an ERβ LBD rabbit polyclonal antibody produced by us as described previously (*18*).

Cloning and Expression of mERβ1 and mERβ2 Ligand Binding Domains. The LBDs of murine ERβ1 (R209 to Q485, GenBank, U81451) and the corresponding region of murine ERβ2, containing the 18 aa insertion, were generated by PCR using pSG5-mERβ1 and pSG5-mERβ2 as templates, respectively, and primers that contained appropriate restriction sites. The LBDs of mERβ1 and mERβ2 were cloned into pET15b (Novagen, Madison, WI) to generate proteins with N-terminal His tags. The sequences of the constructs were verified by DNA sequencing.

Cultures (500 mL) of the *Escherichia coli* strain BL21, transformed with the appropriate expression plasmids, were cultivated overnight in LB supplemented with 100 μg/mL ampicillin at 37 °C. When the OD_{600} reached 1.0, IPTG was added to a final concentration of 1 mM and incubation continued for 3 h at 25 °C. The cells were pelleted, and the supernatant was discarded. The pellet was suspended in 5 mL of extraction buffer (0.3 M NaCl, 20 mM Tris-HCl, pH 8.0, 0.01 mg/mL DNase, 0.01 mg/mL RNase, 10 mM $MgCl_2$, 0.25 mg/mL lysozyme, 1 mM β-mercaptoethanol, and protease inhibitor cocktail tablet). The samples were sonicated for 4 min at 50% duty (total sonication time 2 min). The homogenate was centrifuged at 20000*g* for 20 min at 4 °C. The supernatant was applied to a TALON metal affinity column (Clontech Laboratories, Inc., Palo Alto, CA). Fractions containing the purified protein were dialyzed against 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1 mM DTT and frozen at −80 °C. The purified protein was more than 95% pure as determined from Coomassie-stained SDS−PAGE gels. The protein concentrations were measured using the Coomassie protein assay kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

Scintillation Proximity Assay (SPA). The assay was performed in 96-well microplates (PerkinElmer Life Sciences Inc., Boston, MA). Polyvinyltoluene (PVT) copper-loaded His-tag beads were purchased from Amersham Corp. The reaction mixture (60 μL per well) containing assay buffer (1 mM EDTA, 0.9 M KH_2PO_4, 0.1 M K_2HPO_4, 20 mM Na_2MoO_4, and 0.05% monothioglycerol), beads (30 μg/well), and purified ERβ LBD (final concentration of 5 nM) was incubated at 4 °C for at least 1 h. The concentration of active receptors that are able to bind ligand was 0.6 nM, calculated from B_{max} (the maximal density of receptor sites). For saturation ligand binding analysis, a sample of various concentrations of [^{3}H]-17β-estradiol (SA = 95 Ci/mmol) in the presence or absence of a 300-fold excess of unlabeled 17β-estradiol was then added. The assay plates were sealed, allowed to settle overnight, and subsequently counted on a Wallac 1450 micro-β-counter. The dissociation constant (K_d) was calculated as the free concentration of radioligand at half-maximal specific binding by fitting data to the Hill equation and by linear Scatchard transformation (*19*). Curve fitting was done in Prism (GraphPad Software Inc.).

For ligand competition studies, purified ERβ LBDs (5 nM) were incubated overnight at 4 °C with a range of test compound concentrations. A final concentration of 1.5 nM [^{3}H]-17β-estradiol (30 μL per well) was used. The ligands were tested three times with similar results. Curve fitting was performed using Prism (GraphPad Software Inc.), and the IC_{50}s were determined. IC_{50} values were converted to K_i

using the Cheng−Prusoff equation, $K_i = IC_{50}/(1 + D/K_d)$, where D is the concentration of the radioligand (*20*).

Surface Plasmon Resonance (SPR) Analysis for Raloxifene Binding. SPR analyses were performed according to the protocol as described (*21*). Research grade CM5 sensor chips were obtained from BIAcore AB. Penta-His antibody (Qiagen) surfaces were prepared using standard amine coupling procedures.

Plasmids and Pull-Down Assay. TIF2 and RAP250 for in vitro translation were expressed from the previously described plasmids, pSG5Gal4-RAP250 (aa 819−1096) (*15*) containing the first LXXLL motif and pBKCMV-TIF2 (*22*) containing full-length TIF2, respectively.

For pull-down assays, purified His-tagged ERβ LBDs (100 μg) were bound to 60 μL of Talon resin and then equilibrated in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 10% glycerol, and 0.5% NP-40 (equilibration buffer). The gel slurry was then divided into two equal aliquots, and to each tube was added 2.5 μL of in vitro translated, ^{35}S-labeled (TNT coupled reticulocyte lysate system, Promega), TIF2 or RAP250 in a total volume of 150 μL of equilibration buffer containing 1.5% BSA. Estradiol or vehicle (ethanol) was added as indicated. As control, TIF2 or RAP250 was mixed with Talon gel without bound ERβ. All samples were incubated for 2 h with gentle shaking at 4 °C. After washing three times with equilibration buffer, bound proteins were eluted with SDS−PAGE sample buffer and separated on a 12% polyacrylamide gel. The gel was stained with Coomassie blue, followed by determination of ^{35}S using a Phosphoimager instrument.

Surface Plasmon Resonance (SPR) Analysis for Coactivator Binding. All SPR measurements were performed on a BIAcore 2000 instrument (BIAcore AB, Uppsala, Sweden). All experiments were performed at 25 °C and at a flow rate of 5 μL/min. Research grade streptavidin sensor chips were obtained from BIAcore AB. The streptavidin chips were first treated with three 1-min pulses of 50 mM NaOH and 1 M NaCl at a flow rate of 5 μL/min. N-Terminally biotinylated peptides (>95% purity) were purchased from Interactiva (Germany). LXXLL peptide sequences were as follows: TIF2 Box1 (residues 636−649), KGQTKLLQLLTTKS; TIF2 Box2 (residues 685−698), EKHKILHRLLQDSS; TIF2 Box3 (residues 742−755), KENALLRYLLDKDD; RAP250 Box1 (residues 882−895), LTSPLLVNLLQSDI. Peptides were immobilized on individual surfaces to 200 RU responses, and a non-LXXLL peptide was immobilized on a control surface for on-line reference subtraction. Samples of purified mERβ1 LBD or mERβ2 LBD were then injected over each surface. After injection stopped, the surfaces were washed with buffer to monitor the dissociation phase. The buffer used was 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.05% Tween 20. For the kinetic measurements, various concentrations of mERβ1 LBD or mERβ2 LBD (from 20 to 100 nM) were injected over the chip surfaces. The BIAevaluation software version 3.1 was used for evaluation. Different binding models (different rate equations) were tested in the global curve fitting procedure, and the model best describing the experimental data was a conformational change model. The apparent K_D values are calculated as described (*23*).

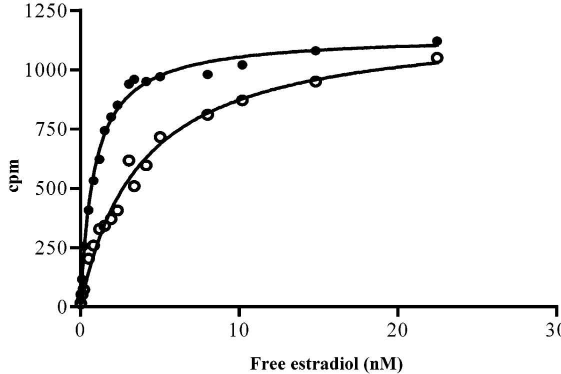


FIGURE 1: Saturation analyses for [^{3}H]-17β-estradiol binding to mERβ1 and mERβ2. mERβ1 (●) and mERβ2 (○) LBDs were incubated with increasing concentrations (0−30 nM) of [^{3}H]-17β-estradiol at 4 °C overnight. Nonlinear regression in Prism (GraphPad Software) of the data gives a K_d of 0.8 nM for mERβ1 and a K_d of 4.2 nM for mERβ2.

RESULTS

mERβ1 and mERβ2 Display Isoform Selectivity with Regard to Ligand Binding. The C-terminal regions containing the LBD of mERβ1 and mERβ2 were expressed in *E. coli*, and the recombinant proteins were purified. Ligand binding to purified proteins was measured using a scintillation proximity assay. In Figure 1, the results of saturation ligand binding assays with tritiated estradiol are shown. The measured K_d values were 0.8 nM for mERβ1 and 5-fold higher (K_d = 4.2 nM) for mERβ2. We next compared the binding affinities of mERβ1 and mERβ2 to a set of ER ligands using a competition assay. Table 1 shows the K_is of these compounds. As expected, the selectivity of estradiol was higher for mERβ1 than for mERβ2 (14-fold). Interestingly, raloxifene was dramatically (8-fold) mERβ2 selective. When the raloxifene core was tested in the ligand binding assays, the selectivity for mERβ2 was abolished, confirming that the selectivity for mERβ2 was due to, at least in part, the 3-aroyl side chain of raloxifene. No striking differences in the binding affinities for 4-OH-tamoxifen, ICI-182,780, or genistein between mERβ1 and mERβ2 could be detected. These data indicate that ER ligands exhibit distinct selectivity for mouse ERβ isoforms.

To further confirm the higher binding affinity of mERβ2 compared to mERβ1 for raloxifene, we used SPR analysis to directly assay binding affinities. Figure 2 depicts the binding responses obtained for the concentration series of raloxifene injected across the mERβ1 or mERβ2 surface. It is clear from a visual inspection of the data that raloxifene dissociates from the mERβ1 faster than the mERβ2 isoform. The measured K_D values were 0.31 μM for mERβ1 and 10-fold lower (K_D = 0.03 μM) for mERβ2, confirming that raloxifene has a higher affinity for mERβ2 as compared to mERβ1.

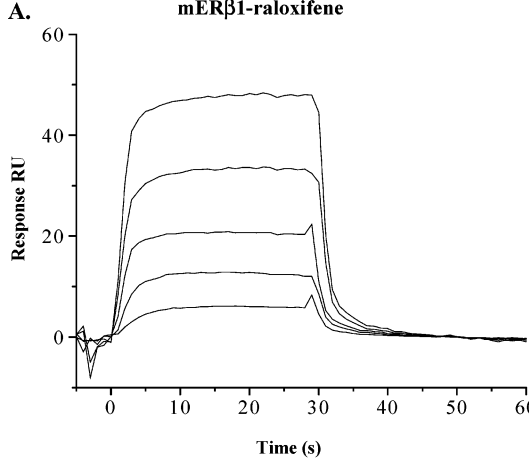
mERβ1 and mERβ2 Isoform Selectivity Is Maintained in Transcriptional Regulation. Estradiol and raloxifene exhibited different isoform selectivity with regard to ligand binding for the mouse ERβ. Next, we examined how this selectivity was translated into modulation of transcriptional activity. Estradiol induced a concentration-dependent activation of mERβ1 and mERβ2 from a 2 × ERE driven reporter gene in transiently transfected HEK293 cells (Figure 3A). Notably, mERβ1 had higher estradiol-induced activity than mERβ2.

Table 1: Binding Affinity of Selected Ligands for mERβ1 and mERβ2

Compound	Structure	Ki (nM)	
		mERβ1	mERβ2
17-β-Estradiol		0.15	2.13
Raloxifene		23.31	3.02
Raloxifene core		3.42	20.40
4-OH-tamoxifen		1.50	3.03
ICI-182,780		56.20	45.92
Genistein		2.82	8.13

Thus, maximal transcriptional activation by mERβ1 was observed at 1 nM estradiol, whereas maximal transactivation by mERβ2 was observed at 10 nM estradiol, at only 60% of the maximal level seen with mERβ1. Raloxifene showed only antagonist activity on both mERβ1 and mERβ2. As shown in Figure 3B, raloxifene was more potent in antagonizing estradiol-induced gene expression with mERβ2 than with mERβ1, which is in agreement with its higher relative binding affinity to mERβ2 than to mERβ1. In contrast to raloxifene, the raloxifene core behaved as a pure agonist for both mERβ1 and mERβ2 (Figure 3C). To confirm equivalent ERβ expression, extracts from HEK293 cells transfected with equal amounts of the expression vectors for mERβ1 or mERβ2 used in transient transfection assays were separated by SDS−PAGE and analyzed for ERβ expression by Western blot (Figure 3D). This analysis shows that mERβ1 and mERβ2 are expressed at similar levels.

mERβ2 Has Reduced Interaction with Coactivators. As shown in Figure 3A, mERβ2 displayed significantly decreased estradiol-induced maximal transcriptional activity as compared to mERβ1. Because the interaction of ER with coactivators is believed to determine the magnitude of transcriptional activity of the receptor, we compared the affinities of mERβ1 and mERβ2 to the coactivators TIF2 and RAP250. We performed pull-down assays using purified His-tagged ERβ LBDs and ^{35}S-labeled TIF2 or RAP250 in the presence or absence of estradiol. As shown in Figure 4, the binding of TIF2 and RAP250 to mERβ1 and mERβ2 appeared to be enhanced by the presence of estradiol.



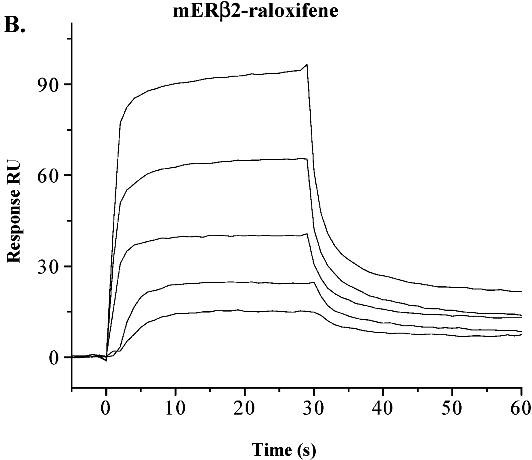


FIGURE 2: Kinetic analysis of raloxifene binding to mERβ1 and mERβ2. Raloxifene was injected at concentrations of 0.03125, 0.0625, 0.125, 0.25, and 0.5 μM over captured mERβ1 (A) and mERβ2 (B). The data were best described using a simple 1:1 interaction model, yielding a K_D of 0.31 μM for mERβ1 and a K_D of 0.03 μM for mERβ2.

Furthermore, the interactions of TIF2 and RAP250 with mERβ2 were weaker than with mERβ1.

To examine interactions of TIF2 and RAP250 with ERs more quantitatively, we used SPR analysis, where biotinylated 14-mer peptides containing NR-box motifs (LXXLL) from TIF2 or RAP250 were captured via streptavidin to the chip surface. Figure 5A demonstrates overlaid sensorgrams of injections of unliganded mERβ2 or mERβ2 liganded with estradiol, 4-OH-tamoxifen, genistein, ICI-182,780, or raloxifene assayed for binding to the TIF2 NR-Box2 peptide. These results indicate that binding of ER agonists, estradiol and genistein, to mERβ2 enhances the mERβ2−peptide interaction, whereas 4-OH-tamoxifen, ICI-182,780, and raloxifene decrease the affinity. Similar differences in binding, depending on ligand status, were seen with mERβ1 and also with RAP250 interaction (data not shown). These data suggest that conformational changes, induced by ligand binding, impose different affinities for receptor−coactivator interactions. In Figure 5B, the concentration-dependent association of estradiol-bound mERβ2 to RAP250 NR-Box1 or TIF2 NR-Box2 is shown together with the best calculated fit. Similar binding studies between mERβ1 and all the different TIF2 and RAP250 NR-box peptides were also performed (data not shown). Using BIAevaluation software, the affinity of receptor−peptide interaction was determined. The best

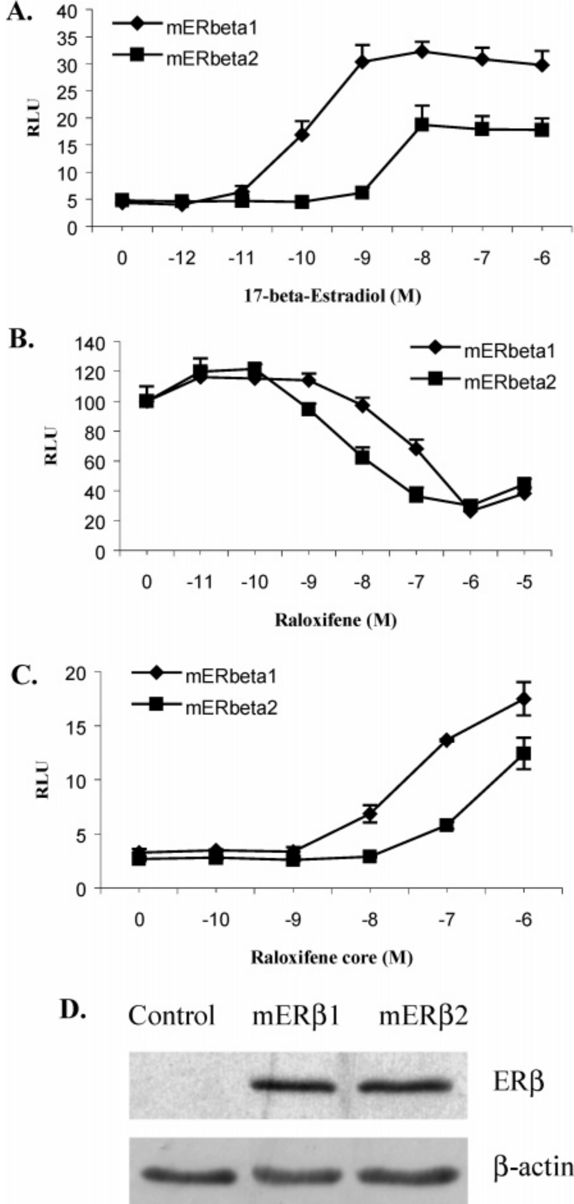


FIGURE 3: Transcriptional activity of mERβ1 and mERβ2 proteins assayed on 2 × ERE TK luciferase reporter. HEK293 cells were transfected with the 2 × ERE TK luciferase reporter plasmid and expression plasmids encoding mERβ1 or mERβ2. (A) Cells were treated with vehicle (ethanol) or the indicated concentrations of 17β-estradiol. (B) Antagonist potency of raloxifene for mERβ1 and mERβ2 in the presence of 10 nM 17β-estradiol. Cells were treated with vehicle (ethanol) or the indicated concentrations of raloxifene. Values obtained from cells treated with only 10 nM 17β-estradiol were arbitrarily set to 100. (C) Cells were treated with vehicle (ethanol) or the indicated concentrations of the raloxifene core. Values represent the mean ± SD of three independent experiments. (D) Western blot analysis of ERβ and β-actin. Identical amounts (100 μg of protein) of whole cell extracts from nontransfected control HEK293 cells or cells transfected with equal amounts of expression vectors for mERβ1 and mERβ2, respectively, were analyzed. The bands were quantified by densitometric scanning, and the amount of ERβ was normalized to β-actin.

fit for all peptide−ER interactions tested was obtained using a conformational change model. The apparent affinities listed in Table 2 show that the TIF2 peptides have higher affinity for mERβ1 and mERβ2 than the RAP250 peptide. The data also show that both TIF2 and RAP250 have binding preferences for mERβ1. In comparison to mERβ1, the lower affinity of mERβ2 to coactivators may account for its reduced maximal transcriptional activity.

DISCUSSION

Since 1997, when a new isoform of the rat ERβ gene named ERβ2 was identified (*8*), only a few studies have addressed the function of this variant (*9*−*12*). It has been shown that ERβ2 binds estradiol with a lower affinity than ERβ1. ERβ2 can form heterodimers with ERβ1 as well as with ERα. Further, transient coexpression of ERβ2 and ERα or ERβ1 in cell lines results in ERβ2-induced reduction of ERα and ERβ1 activity. In this paper, we have addressed the effect of ligand binding selectivity and ligand-induced recruitment of coactivators on the transcriptional activation profiles of mouse ERβ1 and ERβ2.

It is predicted that the 18 amino acid insertion of ERβ2 lies within helix 6 of the receptor (*24*). Given that the insertion is right after the β-sheet that contains Phe-356, which makes direct contact with the ligands, and in close proximity to helix 5, known to have direct interactions with coactivators, the inserted amino acids might affect both ligand binding and coactivator interactions. We have compared the binding affinities of mERβ1 and mERβ2 to a set of ER ligands. In accordance with previous studies, our results show that the binding affinity of estradiol for mERβ1 was much higher than for mERβ2. On the contrary, raloxifene was found to be dramatically mERβ2 selective. The differences found for estradiol and raloxifene in our ligand binding assay were also reflected in the transactivation assay. This observation suggests that raloxifene could act as a potent antagonist of ERβ2 actions and that this differential raloxifene sensitivity may represent a way to dissect the physiological importance of ERβ2. Furthermore, we demonstrated that the raloxifene core behaved as an agonist, showing that the selective antagonist potency of raloxifene for mERβ2 is due to its 3-aroyl side chain. Raloxifene but not 4-OH-tamoxifen or ICI has been shown to be a higher affinity ligand and a more potent estrogen antagonist for human ERα than ERβ (*25*). Therefore, the mERβ2 binding selectivity for these three ligands more closely resembles that of ERα than of mERβ1. In the present study, we also observed that mERβ2 showed significantly decreased estradiol-induced maximal transcriptional activity than mERβ1. We therefore evaluated the affinities of mERβ1 and mERβ2 for coactivators TIF2 and RAP250. Our results indicate that mERβ2 interacts with both coactivators with lower affinity than mERβ1, which may explain why the transcriptional activity of mERβ2 is lower than that of mERβ1.

A detailed understanding of why raloxifene is selective for mERβ2 over mERβ1 is not possible since no crystallographic structure of the ERβ2 isoform is currently available. However, a comparison of the existing raloxifene structures complexed with hERα (*26*) (PDB accession code 1ERR) and rERβ1 (*27*) (PDB accession code 1QKN) is instructive. As mentioned above, the raloxifene binding characteristics of mERβ2 more closely resemble ERα than ERβ1. As shown in Figure 6A, Phe-322 in the ERβ/raloxifene crystallographic structure is pointed toward the ligand forcing the 2-aryl group of raloxifene upward. As a consequence, the hydrogen bond between His-430 and the 4′-hydroxyl group of raloxifene is weakened (O···H−N angle

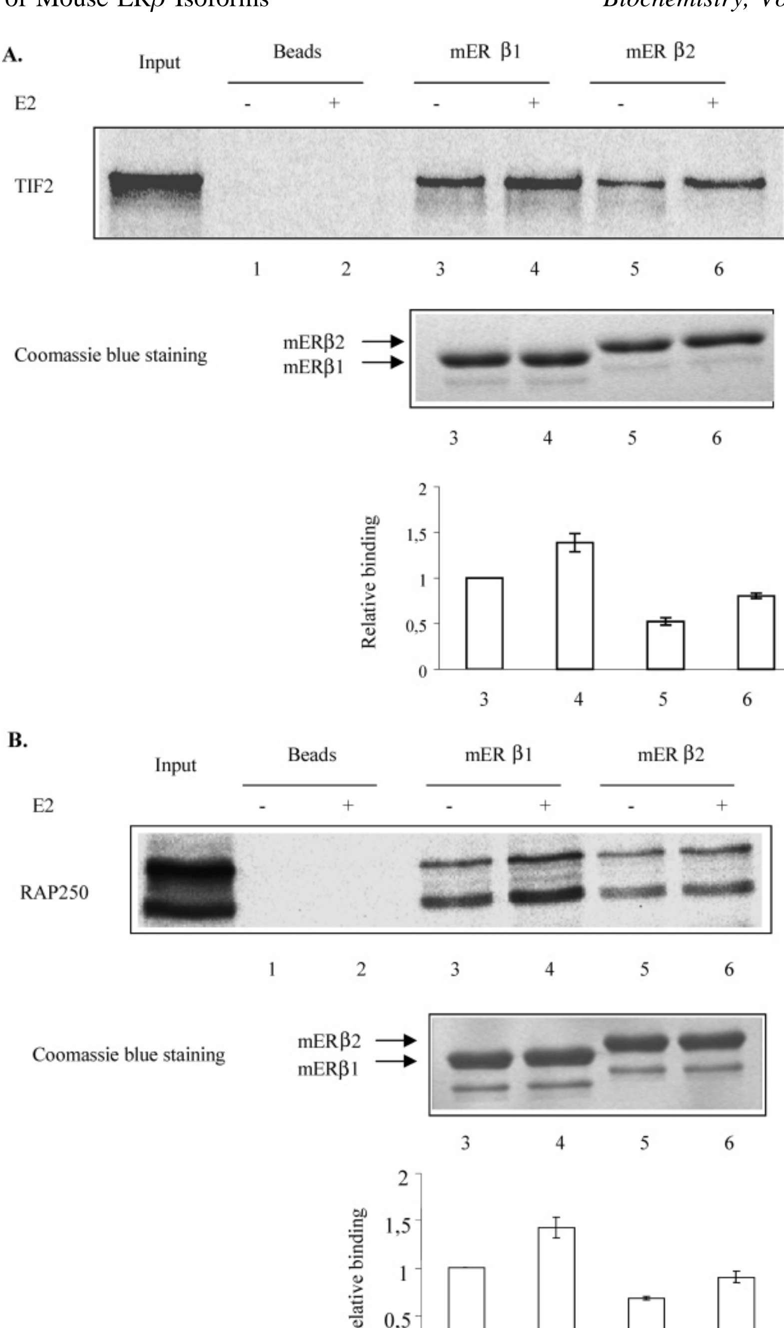
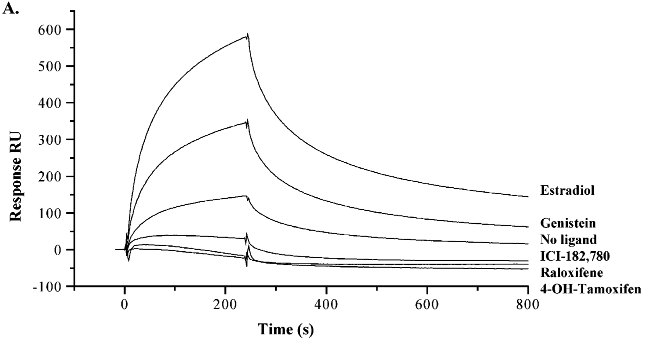
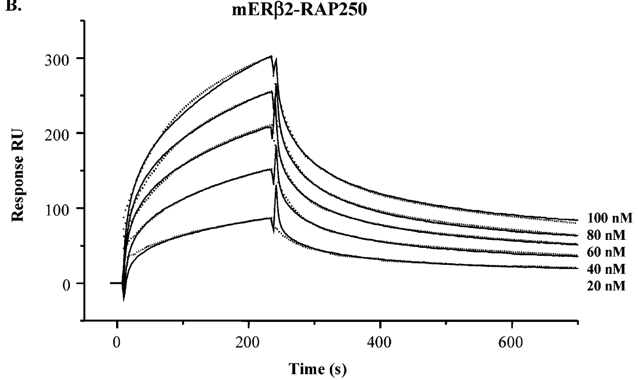


FIGURE 4: Analysis of TIF2 (A) and RAP250 (B) interactions with mERβ1 and mERβ2 by pull-down assay. In vitro translated TIF2 or RAP250 was incubated with mERβ1 (lanes 3 and 4) and mERβ2 (lanes 5 and 6), with or without estradiol (E2) as indicated. Nonspecific binding of TIF2 or RAP250 to Talon beads is shown in lanes 1 and 2. Input corresponds to 0.5 μL of the in vitro translation mixture. The amount of loaded mERβ1 (lanes 3 and 4) and mERβ2 (lanes 5 and 6) was examined by Coomassie blue staining (middle panel). The bar graph shows the relative binding of mERβ1 (lanes 3 and 4) and mERβ2 (lanes 5 and 6) to TIF2 or RAP250 compared to the amount of loaded protein, as determined by densitometric scanning. The relative binding of mERβ1 to TIF2 or RAP250 in the absence of estradiol was set to 1. Nonspecific binding of TIF2 or RAP250 to a 6 × His control protein, which does not interact with coactivators, could not be detected using the experimental conditions employed (data not shown).

= 145° vs 180° for an ideal hydrogen bond). In contrast, the corresponding Phe-425 is pointed away from the ligand in the ERα crystallographic structure, allowing more flexibility for positioning of the ligand in the binding cavity, and consequently the hydrogen bond between His-524 and the 4′-hydroxyl group is stronger (O···H−N angle = 165° in 1ERR). Hence the conformation of Phe-322 is an important determinant of the affinity of the various ER isoforms for raloxifene. Now turning to ERβ2, the insert between Arg-364 and Asp-365 on helix 6 is very close to Phe-322 (Figure 6A) and therefore could easily influence the preferred conformation of this phenylalanine residue. The ERβ2 insert may perturb the conformation of Phe-322 so that it behaves more like in ERα, making more room for the 2-aryl group of raloxifene so that in turn the 4′-hydroxyl group can form a stronger hydrogen bond to His-430. This would account for the increased affinity of raloxifene for ERβ2. The explanation for why raloxifene but not the raloxifene core is ERβ2 selective can be understood by comparing the crystallographic structure of ERα complexed





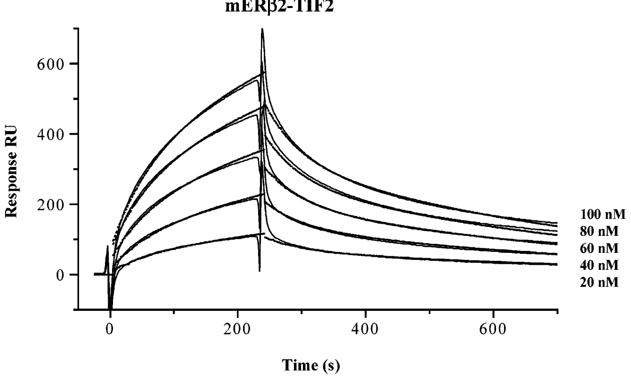


FIGURE 5: SPR analysis of LXXLL peptide binding to mERβ2. (A) Overlaid sensorgrams showing injections of 100 nM unliganded mERβ2 and mERβ2 liganded with estradiol, genistein, ICI-182,-780, raloxifene, or 4-OH-tamoxifen, respectively, over a surface captured with 200 RU of TIF2 NR-Box2 peptide. Ligand concentrations of 1 μM were used. (B) Overlaid sensorgrams showing injections of mERβ2 liganded with 1 μM estradiol at protein concentrations of 20, 40, 60, 80, and 100 nM over a surface captured with 200 RU of RAP250 NR-Box1 peptide or TIF2 NR-Box2 (solid lines) and best calculated fit using a conformational change model (dotted lines).

with the raloxifene core (PDB accession code 1GWQ) (*28*) to the two raloxifene structures, 1ERR and 1QKN (see Figure 6B). The raloxifene core binds to ER in a flipped orientation relative to raloxifene. In this binding mode, the 6-hydroxyl group of the raloxifene core forms a strong hydrogen bond to His-524 (O···H−N angle = 179°). Also, in this flipped orientation, the distance between the raloxifene core and Phe-425 is larger so that it is less sensitive to the conformation of this phenylalanine residue. In contrast, the 3-aroyl side chain forces the benzothiophene moiety of raloxifene to bind in a flipped orientation relative to the raloxifene core and pushes the 2-aryl group toward Phe-322.

The biological significance of the existence of two main ERβ isoforms in rat and mouse is presently unclear. Given

Table 2: Apparent Dissociation Constants and Rate Constants for Interactions between TIF2 or RAP250 NR-Box Peptides and Estradiol-Bound mERβ1 and mERβ2[a]

	TIF2			RAP250
mERβ1	Box1	Box2	Box3	Box1
K_D (nM)	7.3 ± 2.9	4.2 ± 0.8	12.7 ± 4.1	41.5 ± 6.9
$k_{a1} \times 10^4$ ($M^{-1} s^{-1}$)	12.1 ± 1.0	35.3 ± 2.3	15.1 ± 0.6	10.9 ± 0.3
$k_{d1} \times 10^{-3}$ (s^{-1})	3.4 ± 0.6	3.6 ± 0.8	3.3 ± 0.9	6.1 ± 0.1
$k_{a2} \times 10^{-3}$ (s^{-1})	1.9 ± 0.1	1.2 ± 0.2	1.8 ± 0.2	2.3 ± 0.1
$k_{d2} \times 10^{-3}$ (s^{-1})	0.5 ± 0.1	0.5 ± 0.1	1.0 ± 0.3	1.7 ± 0.2

	TIF2			RAP250
mERβ2	Box1	Box2	Box3	Box1
K_D (nM)	66.3 ± 23.2	63.7 ± 21.2	120.8 ± 30.5	272.3 ± 82.4
$k_{a1} \times 10^4$ ($M^{-1} s^{-1}$)	4.6 ± 1.2	4.5 ± 0.6	3.4 ± 0.3	6.0 ± 0.8
$k_{d1} \times 10^{-3}$ (s^{-1})	6.2 ± 0.8	5.1 ± 0.6	5.6 ± 1.0	32.3 ± 2.6
$k_{a2} \times 10^{-3}$ (s^{-1})	2.0 ± 0.2	1.8 ± 0.1	1.5 ± 0.1	4.5 ± 1.0
$k_{d2} \times 10^{-3}$ (s^{-1})	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.2	2.3 ± 0.4

[a] The apparent K_D values were obtained from SPR-generated data using the curve fitting analysis program BIAevaluation. The data were best described using a conformational change model according to equation

$$A + B \underset{k_{d1}}{\overset{k_{a1}}{\rightleftharpoons}} AB \underset{k_{d2}}{\overset{k_{a2}}{\rightleftharpoons}} AB^*$$

The receptor (A) first forms an unstable complex (AB) with the peptide (B) and then undergoes a conformational change that leads to a more stable complex (AB*). $K_D = (k_{d1}/k_{a1})(k_{d2}/k_{a2})$. Values represent the mean ± SD of three independent experiments.

that ERβ2 suppressed the ERα-mediated transcriptional response, one possibility is that ERβ2 acts as a negative regulatory partner of ERα under specific physiological conditions. It has been demonstrated that ERβ1 acts as modulator of ERα-mediated gene transcription in mouse uterus (*29*). Furthermore, it was found that the ERβ2 protein is upregulated and the colocalization of ERβ2 with ERα is increased during the lactation period in rat mammary gland, indicating a possible role of ERβ2 as a dominant repressor of ERα (*13*). Our study and those of others demonstrate that the two receptor isoforms show differences in response to estradiol and other ligands. It is thus also possible that regulation of receptor isoform expression could govern the tissue effects of specific estrogenic agents. The other possibility is that ERβ2 provides a cellular mechanism to respond to elevated estradiol levels, e.g., achieved in the ovary during pregnancy or the periovulatory phase, higher than those required to fully activate ERα and ERβ1. In addition, it cannot be excluded that there exist phyto- or xenoestrogens that act preferentially at ERβ2. ERβ2 may also have other functions. For example, it was shown that, in the presence of estradiol or raloxifene, ERβ2 can activate the transforming growth factor β promoter as efficiently as ERα and ERβ1 (*11*).

In mouse and rat, the tissue distribution and/or the relative levels of ERβ1 and ERβ2 mRNA seem to be quite different, that is, high expression in liver, pancreas, uterus, breast, and brain for ERβ2 and in nervous system for ERβ1 (*30*),

A.

B.

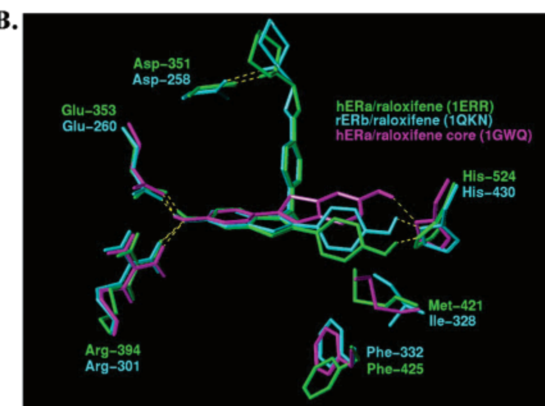


FIGURE 6: (A) An overlay of hER/raloxifene (green, PDB accession code 1ERR) and rERβ1/raloxifene (cyan, 1QKN) crystallographic structures. Critical amino acid side chains (green = ERα and cyan = ERβ) and raloxifene ligand atoms (white = carbon, red = oxygen, blue = nitrogen, and yellow = sulfur) are depicted as tubes. Hydrogen-bonding interactions between the ligands and receptors are shown as dashed yellow lines. The conformation of Phe-425 (ERα)/Phe-322 (ERβ1) affects the positioning of the 2-aryl group of raloxifene and consequently the strength of the hydrogen bond between His-524 (ERα)/His-430 (ERβ1) of the receptor and the 4′-hydroxyl group of the ligand. The close proximity of the 18 aa insert between Arg-319 and Asp-320 in ERβ2 to Phe-322 may influence the preferred conformation of Phe-322, providing a possible explanation for the selectivity of raloxifene for ERβ2 vs ERβ1 (see text for further discussion). This figure was produced using the PyMOL program (*34*). (B) An overlay of hERα/raloxifene (green, PDB accession code 1ERR), rERβ1/raloxifene (cyan, 1QKN), and the hERα/raloxifene core (magenta, 1GWQ) crystallographic structures.

implying a specific mechanism regulating expression of one or the other splice variant. In humans, tissue-specific expression of the ERα gene has been shown to be regulated by multiple promoters (*31*). Further studies, including cloning and characterization of putative promoters, should help to elucidate the regulatory mechanisms of ERβ isoform expression. Interestingly, the expression of ERβ2 mRNA has been demonstrated in various human cancer cell lines (*9*). A variant of ERα, analogous to ERβ2, which contains an in-frame insertion between exons 5 and 6 that encodes an additional 23 amino acids in the LBD has been reported in human breast cancers (*32*). In breast, raloxifene acts as a classical antiestrogen to inhibit the growth of mammary carcinoma (*33*). Our findings that raloxifene binds preferentially to ERβ2 imply that the varying expression of ERβ isoforms under certain conditions might have important pharmacological implications.

Selective estrogen receptor modulators (SERMs) are developed to display either agonist or antagonist activity in a tissue-selective manner and are likely to provide novel and improved therapeutic strategies. Although the mechanisms by which these ligands accomplish tissue-selective activity remain to be fully elucidated, multiple ERs provide opportunities for the synthesis of receptor-selective ligands as one class of SERMs. However, isoform-dependent ligand selectivity, reported for the two rodent ERβs in this study, needs to be considered when novel compounds are tested in animal studies.

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ORIGINAL ARTICLE

Epidermal growth factor receptor regulates MET levels and invasiveness through hypoxia-inducible factor-1α in non-small cell lung cancer cells

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Recent studies have established that amplification of the *MET* proto-oncogene can cause resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) in non-small cell lung cancer (NSCLC) cell lines with EGFR-activating mutations. The role of non-amplified *MET* in EGFR-dependent signaling before TKI resistance, however, is not well understood. Using NSCLC cell lines and transgenic models, we demonstrate here that EGFR activation by either mutation or ligand binding increases MET gene expression and protein levels. Our analysis of 202 NSCLC patient specimens was consistent with these observations: levels of MET were significantly higher in NSCLC with *EGFR* mutations than in NSCLC with wild-type EGFR. EGFR regulation of MET levels in cell lines occurred through the hypoxia-inducible factor (HIF)-1α pathway in a hypoxia-independent manner. This regulation was lost, however, after *MET* gene amplification or overexpression of a constitutively active form of HIF-1α. EGFR- and hypoxia-induced invasiveness of NSCLC cells, but not cell survival, were found to be MET dependent. These findings establish that, absent *MET* amplification, EGFR signaling can regulate MET levels through HIF-1α and that MET is a key downstream mediator of EGFR-induced invasiveness in EGFR-dependent NSCLC cells.
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Keywords: EGFR; MET; non-small cell lung cancer; HIF-1α; invasiveness

Introduction

Non-small cell lung cancer (NSCLC) is the leading cause of cancer death in the United States. Epidermal growth factor receptor (EGFR)-activating mutations have been described in a subset of NSCLC patients, and activated EGFR is known to influence tumor cell survival, proliferation, angiogenesis, and invasiveness (Lynch *et al*., 2004; Paez *et al*., 2004; Pao *et al*., 2004; Janne *et al*., 2005; Pao and Miller, 2005; Ciardiello and Tortora, 2008). EGFR tyrosine kinase inhibitors (TKIs) such as erlotinib and gefitinib are clinically active in 10–20% of NSCLC patients (Fukuoka *et al*., 2003; Kris *et al*., 2003; Shepherd *et al*., 2005; Thatcher *et al*., 2005). Activating mutations within the *EGFR* tyrosine kinase domain including an amino acid substitution at exon 21 (L858R) and in-frame deletions in exon 19 were found to be predictors of clinical response to EGFR TKIs (Lynch *et al*., 2004; Paez *et al*., 2004; Pao *et al*., 2004).

Recent evidence suggests that in NSCLC cells activating EGFR mutations or amplification of the *MET* proto-oncogene caused acquired resistance to EGFR TKIs by driving activation of the PI3K pathway (Engelman *et al*., 2007). The role of MET in EGFR-dependent signaling before the emergence of TKI resistance is not well understood; however, *MET* is regulated by hypoxia and hypoxia-inducible factor-1α (HIF-1α) and is thought to contribute to invasive tumor growth (Pennacchietti *et al*., 2003). The MET protein is a receptor tyrosine kinases whose activation can cause malignant transformation and tumorigenesis (Cooper *et al*., 1986; Park *et al*., 1987; Stabile *et al*., 2004). Upon ligand binding, MET activates downstream signaling molecules including PI3K, Src, and signal transducer and activator of transcription-3 (Rosario and Birchmeier, 2003), triggering the key metastatic steps of cell dissociation (Qiao *et al*., 2002), migration (Yi *et al*., 1998), and invasion (Bredin *et al*., 2003). MET is overexpressed in multiple malignancies and is associated with aggressive disease (Peruzzi and Bottaro, 2006). In

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NSCLC, MET levels are elevated in resected tumors compared with normal tissue (Liu and Tsao, 1993; Ichimura *et al*., 1996; Olivero *et al*., 1996), and high expression of the MET ligand hepatocyte growth factor (HGF) is associated with aggressive disease and a poor prognosis (Siegfried *et al*., 1998).

Recent studies have suggested a link between EGFR signaling and MET. Expression and phosphorylation of EGFR and MET correlate in multiple malignancies (Weinberger *et al*., 2005). Aberrant EGFR activation results in elevated MET phosphorylation in thyroid carcinoma cells (Bergstrom *et al*., 2000). EGFR function has been implicated in HGF-induced hepatocyte proliferation (Scheving *et al*., 2002) and is required for MET-mediated colon cancer cell invasiveness (Pai *et al*., 2003). Recent studies of phosphoprotein networks reveal an association between EGFR and MET activation (Huang *et al*., 2007; Guo *et al*., 2008), and have reported direct crosstalk between EGFR and the MET (Jo *et al*., 2000; Huang *et al*., 2007).

A plausible link between the EGFR and MET pathways is HIF-1, which has two subunits (HIF-1α and HIF-1β), and is known to contribute to tumor cell motility and invasiveness. EGF has been shown to modulate HIF-1α levels in prostate, breast, and lung cancer cell lines (Zhong *et al*., 2000; Phillips *et al*., 2005; Peng *et al*., 2006), and positive correlations between EGFR and HIF-1α expression have been observed in NSCLC (Hirami *et al*., 2004; Swinson *et al*., 2004).

Here, we have used clinical specimens, transgenic mouse models, and cell lines to investigate the hypothesis that EGFR signaling may regulate MET levels through HIF-1α but that *MET* amplification, which occurs in EGFR TKI resistance, would uncouple MET levels from EGFR regulation. We hypothesized further that EGFR-induced invasiveness, like hypoxia-induced invasiveness, is mediated downstream at least in part by the HIF-1α/MET axis.

Results

EGFR-activating mutations are associated with elevated levels of MET in NSCLC clinical samples

To investigate a possible association between EGFR activation and MET in clinical specimens, we evaluated MET levels by immunohistochemistry and assessed *EGFR* mutations in 202 human NSCLC clinical specimens. Out of 202 samples, 22 had detectable *EGFR* mutations. Specimens were immunostained for MET and scored based on an intensity score (0, 1, 2, or 3) and an extension percentage. The final score was the product of these two values. The mean score for MET expression was 39.46 ± 64.52. Therefore, a score of 40 was considered the cutoff for classifying low and high levels of MET expression. The mean MET expression score was significantly higher in specimens with mutated *EGFR* (73.64 ± 70.68) than in specimens with WT *EGFR* (48.72 ± 71.72; $P = 0.04$; Figure 1a). Furthermore, 37% of NSCLC tumors with WT *EGFR* expressed high levels of membranous MET, whereas 68% of NSCLC tumors with mutated *EGFR* expressed high levels of membranous MET ($P = 0.005$; Figure 1b). Among adenocarcinomas with EGFR-activating mutations, we did not observe any association between EGFR expression and survival. However, considering the small sample size, no definitive conclusions can be drawn.

EGFR activation modulates MET expression in transgenic murine models of NSCLC

We investigated whether a similar association between EGFR-activating mutations and MET expression occurred in murine models of NSCLC. We used transgenic mice with lung tumors driven by lung-specific mutated K-RAS or activating EGFR mutation (Forsythe *et al*., 1996; Johnson *et al*., 2001). Lung tumor sections were immunostained for MET and scored as described above. *K-RAS*-driven lesions had an average score of 6.75, whereas tumors with *EGFR*-activating mutations had an average staining score of 40.65 (Figure 1c; $P < 0.001$). Treatment of mice bearing EGFR-driven lung tumors with the EGFR TKI erlotinib (50 mg/kg/day) for 48 h abolished MET, providing evidence that MET levels were regulated by EGFR activation.

EGFR-activating mutations are associated with elevated HIF-1α and MET levels in NSCLC cell lines

Given our finding that tumors with *EGFR* mutations exhibit higher MET expression, we investigated MET regulation by EGFR and its role in EGFR-mediated NSCLC invasiveness. We evaluated *MET* RNA levels in NSCLC cell lines by performing gene expression analysis on gene arrays of 53 previously characterized NSCLC lines (eight lines with mutated EGFR) (GEO 4824) (Zhou *et al*., 2006). *MET* RNA levels were significantly higher in *EGFR*-mutated cell lines than in NSCLC cell lines expressing WT *EGFR* (Figure 2a; $P = 0.002$); however, *MET* expression levels in cell lines with *K-RAS* mutations were not significantly different compared with cell lines with WT *K-RAS*. Moreover, we observed a significant association between *EGFR* gene copy number (>4 copies using RT–PCR) and levels of *MET* expression ($P = 0.03$, Figure 2b).

We evaluated MET protein levels in NSCLC with or without *EGFR*-activating mutations and observed constitutive EGFR phosphorylation in cell lines with mutated *EGFR*, which was associated with increased phosphorylated MET (p-MET) and MET expression (Figure 2c). Cell lines with *EGFR*-activating mutations were positive for HIF-1α expression in normoxia. HCC827 cells, which exhibited the most robust expression of p-EGFR, produced the highest levels of HIF-1α, p-MET, and MET. Western data are supported by ELISA analysis showing higher levels of p-EGFR, p-MET, and HIF-1α in cell lines with EGFR-activating mutations compared with cells with WT *EGFR* (Figures 2d–f).

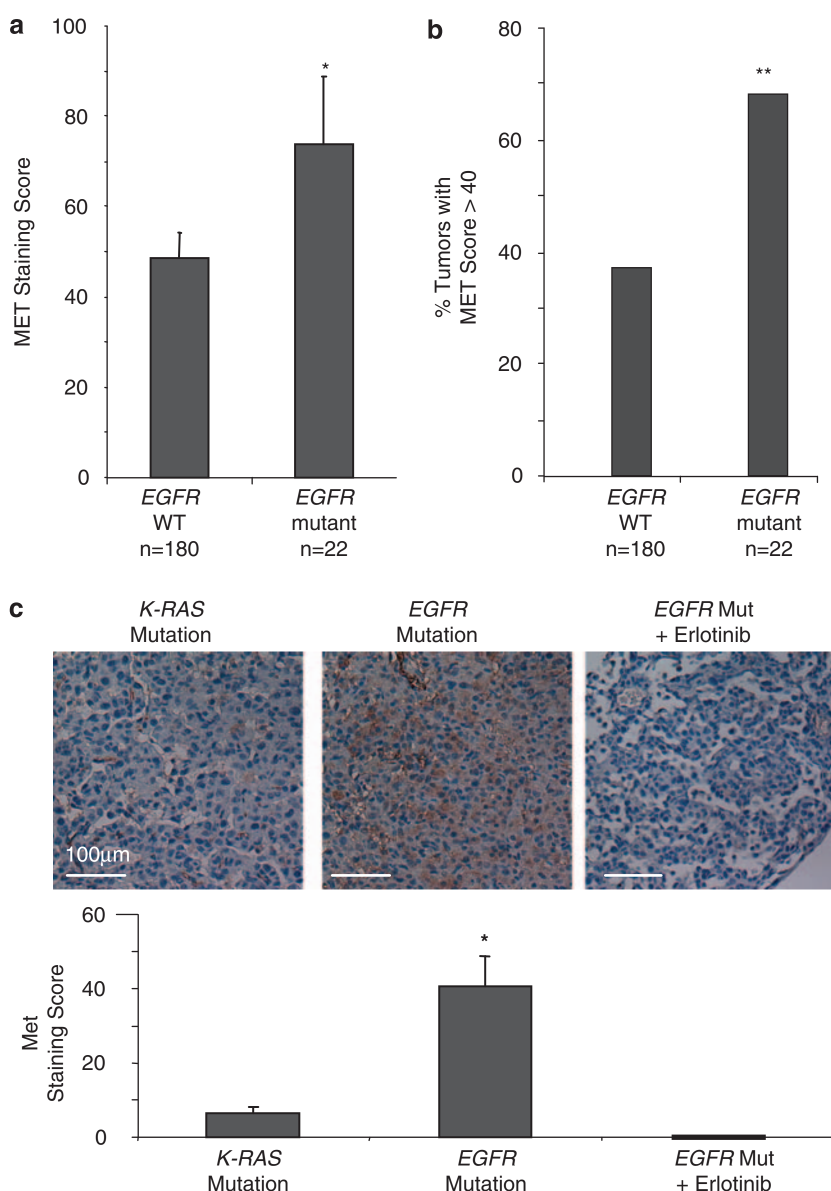

Figure 1 Elevated MET and HGF expression correlates with *EGFR*-activating mutations in NSCLC tumor samples. NSCLC clinical specimens ($n = 202$) were immunostained with anti-MET ab and scored (**a**). *EGFR*-activating mutations correlated with elevated levels of MET. Bars, s.e.m.; $*P<0.05$. (**b**) Data are presented as the percentage of tumors with high MET expression; $**P<0.005$. (**c**). Murine lung tumors driven by *K-RAS* or *EGFR*-activating mutations were immunostained with anti-MET ab, and positive staining was quantified. Weak or negative MET staining was observed in K-RAS-driven tumors, whereas tumors with *EGFR*-activating mutations exhibited elevated MET expression. Erlotinib treatment diminished MET expression. Representative images are shown. Columns, mean score; bars, s.e.m. $*P<0.001$.

Activated EGFR modulates p-MET, MET, and HIF-1α
We treated HCC827 cells with 1 μM of erlotinib for 12 h and evaluated p-MET, MET, and HIF-1α levels. Erlotinib reduced p-MET and MET protein (Figure 3a). EGFR inhibition resulted in diminished HIF-1α levels. p-MET, MET, and p-EGFR were further analyzed by ELISA assay (Figure 3b). Consistant with data obtained by western blot, erlotinib decreased p-EGFR ($P = 0.009$), p-MET ($P = 0.1$), and MET ($P = 0.001$) levels. As HIF-1α is known to regulate *MET* transcription, we determined whether mutated *EGFR* would regulate *MET* mRNA levels. We treated HCC827 cells with or without erlotinib (1 μM) for 12 h and collected RNA for RT–PCR to evaluate changes in *MET* mRNA relative to *GAPDH* RNA. Inhibition of EGFR activity resulted in approximately a 50% decrease in *MET* RNA compared with control levels (Figure 3c).

To further show that EGFR signaling modulates HIF-1α and MET protein expression, we transfected HCC827 cells with control siRNA and EGFR-, HIF-1α-, and MET-targeting siRNA. Knockdown of EGFR decreased p-MET, MET, and HIF-1α levels. HIF-1α-targeting siRNA did not alter EGFR expression but reduced MET expression and activation, whereas MET

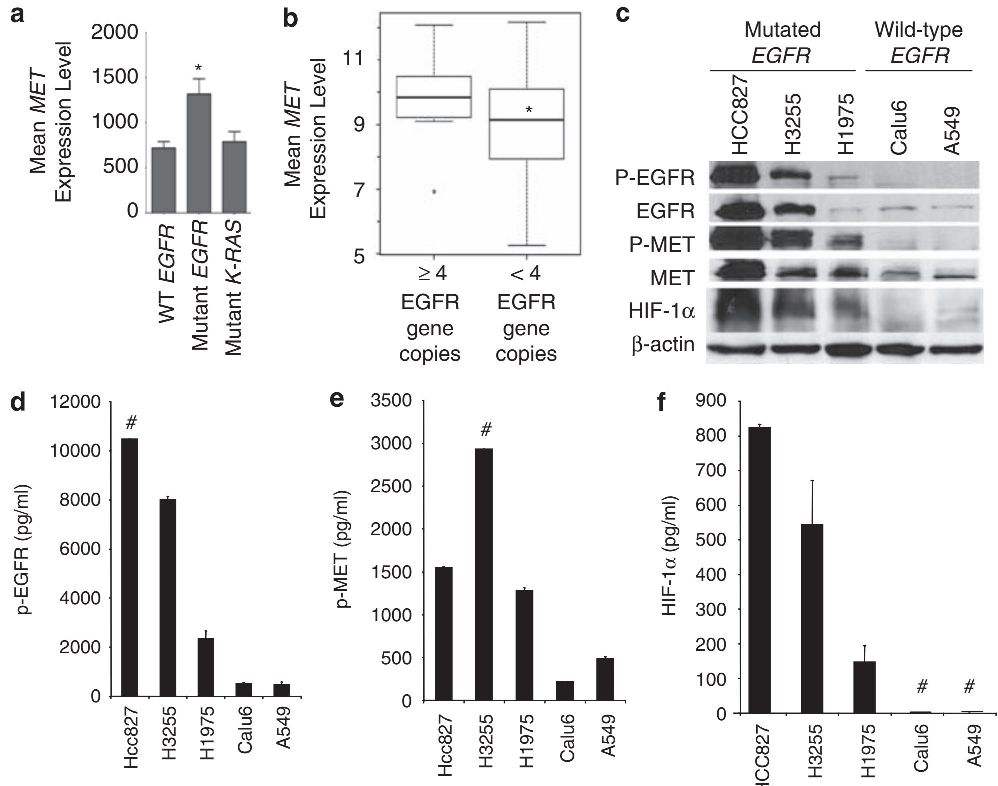

Figure 2 *EGFR*-activating mutations are associated with elevated MET and HIF-1α levels in NSCLC cell lines. (**a**) Gene expression analysis was performed on gene arrays of 53 NSCLC lines. *MET* expression was elevated in NSCLC cell lines harboring *EGFR*-activating mutations; $*P=0.002$. (**b**) MET expression in 53 NSCLC cell lines with high EGFR gene copy number (>4 copies) vs low copy number (<4 copies); $*P=0.03$. (**c**) Western blot was used to evaluate pEGFR, EGFR, p-MET, MET, and HIF-1α expression in NSCLC cell lines expressing WT EGFR or mutationally activated EGFR. The presence of *EGFR*-activating mutations was associated with increased levels of p-MET, MET, and HIF-1α. (**d–f**) ELISA assay was used to analyze levels of p-EGFR (**d**), p-MET (**e**), and HIF-1α (**f**) in NSCLC cell lines expressing WT EGFR or mutationally activated EGFR. # indicates samples that were out of range.

siRNA reduced MET but not EGFR or HIF-1α levels (Figure 3d), indicating that HIF-1α and MET are downstream of EGFR. Similar results were obtained by HIF-1α ELISA assay. siRNA directed against EGFR but not MET decreased HIF-1α levels ($P=0.009$; Figure 3e).

MET amplification has been described in a subset of NSCLC patients (Zhao *et al*., 2005; Engelman *et al*., 2007). To determine whether *MET* amplification would result in MET expression that was independent of EGFR, we treated H1993 NSCLC cells, which harbor an amplified *MET* allele (Engelman *et al*., 2007; Lutterbach *et al*., 2007), with erlotinib, and evaluated p-EGFR, EGFR, p-MET and MET levels. In contrast to the EGFR-dependent cell lines tested, pharmacological inhibition of EGFR did not diminish MET expression in this cell line (Figure 3f).

Previous studies suggested that activated EGFR can directly induce phosphorylation of MET (Bergstrom *et al*., 2000; Jo *et al*., 2000). To evaluate the effect of EGFR activation on MET in NSCLC, we stimulated A549 cells with EGF with or without erlotinib. Phosphorylated EGFR was detected 30 min after ligand stimulation, and EGFR activation was inhibited with erlotinib (Figure 3g). EGFR levels decreased 12 h after the addition of EGF, which may have been a result of receptor internalization. EGF stimulation triggered rises in p-MET levels at 30 min, suggesting that EGFR directly activated MET. p-MET levels remained detectable 6 h after EGF stimulation. Prolonged exposure (24 h) to EGF resulted in increased levels of MET protein (Figure 3h).

EGFR-mediated invasion of NSCLC cells is MET dependent

To show that MET activation increases invasiveness in NSCLC and that this can be abrogated with the MET TKI, PHA-665752, we treated A549 and HCC827 tumor cells with the MET ligand HGF alone or with PHA-66752. Cell invasion was measured using Matrigel-coated Boyden chambers. In both cell lines, HGF stimulation resulted in a significant increase in invasiveness, and this was inhibited with the addition of PHA-665752 ($P<0.05$; Figure 4a). As EGFR activation has been shown to modulate tumor cell invasion in multiple

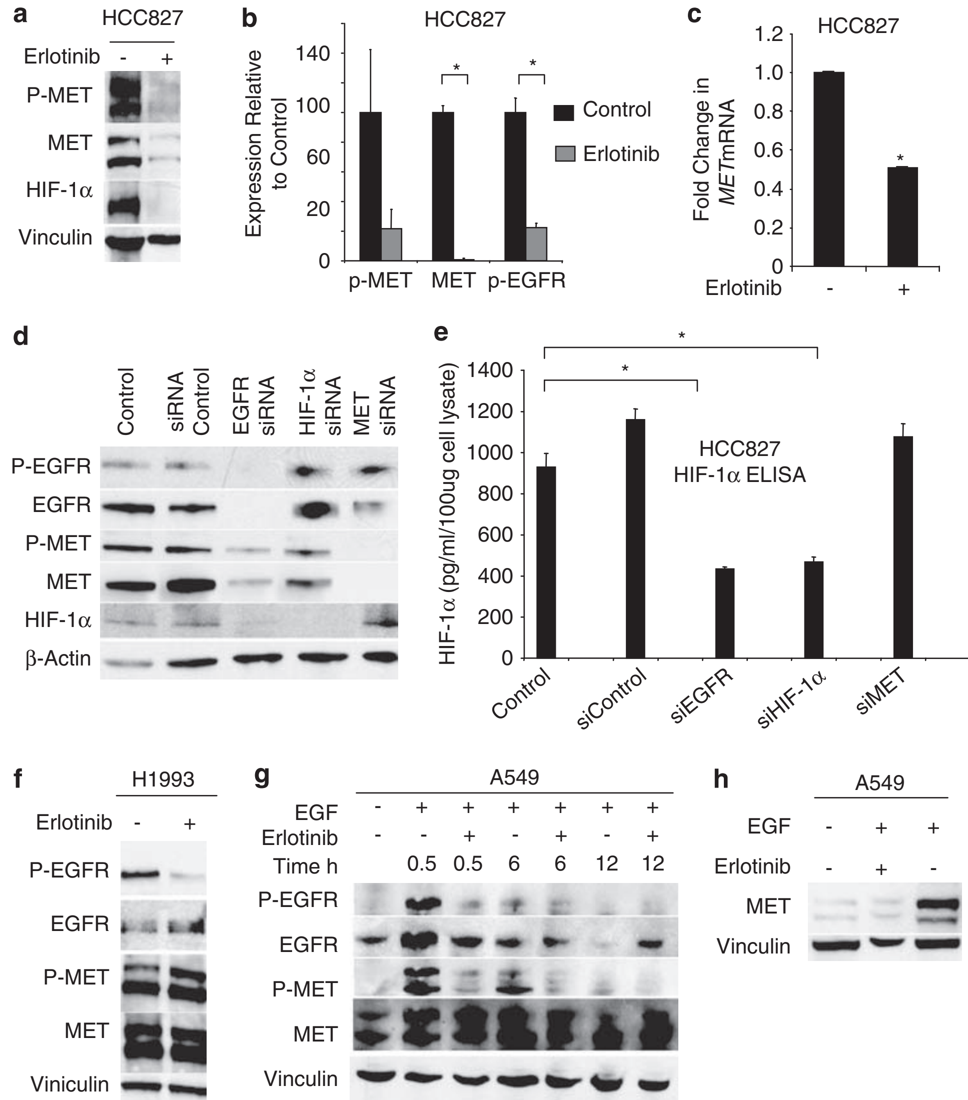

Figure 3 EGFR activation modulates levels of MET and p-MET in NSCLC cell lines. (**a**) HCC827 cells were treated with 1 μM erlotinib for 12 h. Protein lysates were collected after 8 h, and p-MET, MET, and HIF-1α levels were evaluated by western blot. (**b**) HCC827 cells were treated with 1 μM erlotinib for 12 h, and levels of p-MET, MET, and p-EGFR were evaluated by ELISA assay. *$P<0.01$. (**c**) HCC827 cells were treated with 1 μM erlotinib for 12 h, and *MET* mRNA levels were evaluated by RT–PCR. Inhibition of EGFR activation decreased *MET* RNA. Bars, s.d.; *$P<0.001$. (**d**) HCC827 cells were transfected with siRNA oligonucleotides directed against EGFR, HIF-1α, MET, and non-targeting control siRNA. After 72 h, protein lysates were collected and western blot was performed. (**e**) HIF-1α levels are decreased in HCC827 cells transfected with siRNA-targeting EGFR and HIF-1α as determined by ELISA. *$P<0.05$. (**f**) H1993 cells, which have an amplified *MET* allele, were treated with 1 μM erlotinib for 12 h, and p-EGFR, EGFR, p-MET and MET expression were evaluated by immunoblot. (**g**) A549 cells were serum starved for 12 h and treated with EGF (60 ng/ml) with or without 1 μM erlotinib. Protein lysates were collected at the indicated times, and EGFR and MET activation were evaluated by immunoblot. (**h**) A549 cells were serum starved for 12 h and then stimulated with 60 ng/ml EGF with or without 1 μM erlotinib for 24 h.

cell types including NSCLC (Hamada *et al.*, 1995; Damstrup *et al.*, 1998), we investigated whether EGFR activation's effect on tumor cell invasion is MET mediated. We stimulated A549 cells with EGF alone or with erlotinib or the MET TKI, PHA-665752. EGF induced a twofold increase in cell invasion compared with control ($P<0.05$; Figure 4b). The addition of erlotinib or PHA-665752 reduced the number of invading cells to control levels, indicating that EGFR-driven cell invasion is MET dependent. In a similar experiment using HCC827 cells, in which EGFR is constitutively activated, EGF stimulation did not increase tumor cell invasiveness compared with control levels; however, pharmacological inhibition of EGFR or MET activation significantly reduced the number of invading cells (Figure 4c; $P<0.05$).

To further elucidate the mechanism by which EGFR-activating mutations drive tumor cell invasion, we transfected HCC827 cells with EGFR-, HIF-1α-, or MET-targeting siRNA and evaluated cell invasion. Knockdown of EGFR, HIF-1α, or MET resulted in decreased invasive capacity (Figure 4d; $P<0.001$),

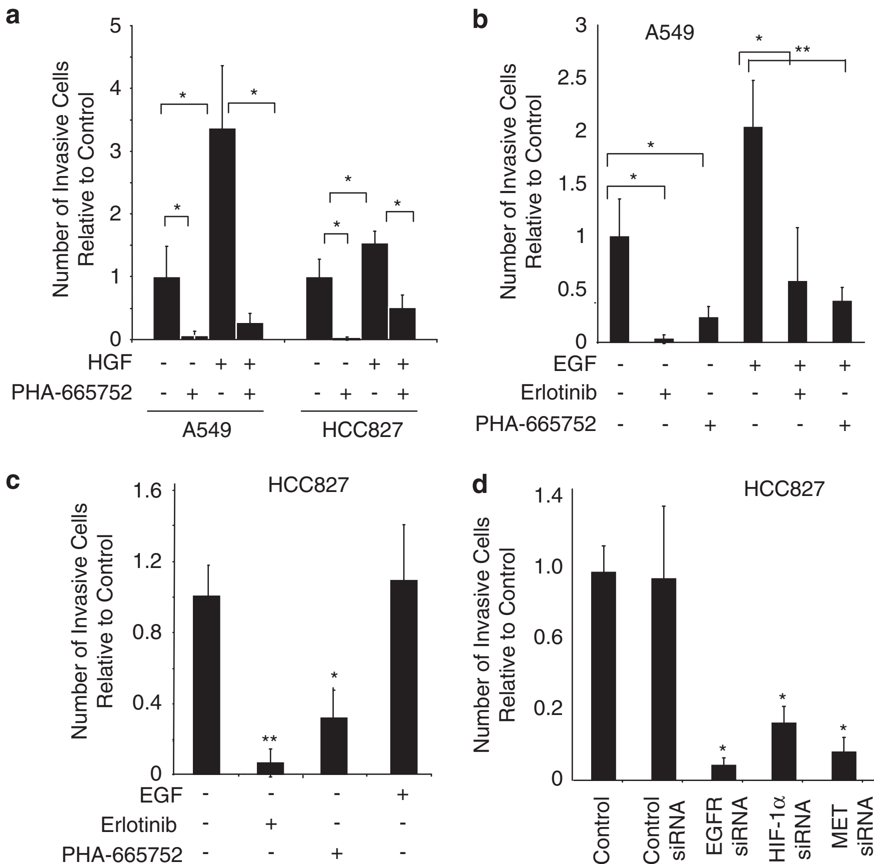

Figure 4 MET is required for EGFR-driven invasiveness of NSCLC cell lines. (**a**) A549 and HCC827 cells were seeded onto Matrigel-coated invasion chambers. Cells were treated with HGF (40 ng/ml) with or without PHA-663225 (1 μM) and incubated for 48 h. Migrating cells were quantified by bright-field microscopy. Bars, % s.d.; $*P<0.05$. (**b**) A549 cells were seeded onto Matrigel-coated invasion chambers. Cells were treated with EGF (60 ng/ml), erlotinib (1 μM), or PHA-663225 (1 μM) and incubated for 24 h. EGF significantly enhanced cell invasion, and EGFR and MET inhibitors abrogated EGF-induced invasion. Bars, % s.d.; $*P<0.05$; $**P<0.001$. (**c**) HCC827 cells were treated with EGF, erlotinib, or PHA-663225 and tumor cell invasion was evaluated by Boyden chamber assay. Bars, % s.d.; $*P<0.05$; $**P<0.001$. (**d**) HCC8827 cells were transfected with control siRNA or siRNA-targeting EGFR, HIF-1α, or MET, and tumor cell invasion was evaluated by Boyden chamber assay. Bars, % s.d.; $*P<0.001$.

whereas control siRNA did not affect invasive capacity. To confirm that decreases in the number of invasive cells after erlotinib or siRNA treatment was indeed do to changes in the invasive capacity of tumor cells and not because of changes in cell viability or proliferation, we separately performed a similar study in which the number of invasive cells was normalized to the number of cells that did not invade through the chamber (Supplemental Figure 1). These data were in agreement with the findings shown above.

To determine whether MET inhibition impacted cellular growth, we conducted MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assays in the presence of increasing concentrations of erlotinib or PHA-665752. HCC827 cells were sensitive to the EGFR inhibitor erlotinib (IC_{50} <10 nM), whereas H1993 and A549 cells were less sensitive (Supplemental Figure 2). None of the three cell lines were sensitive to PHA-665752 at concentrations as high as 10 μM. This suggested that invasiveness but not cell survival was MET dependent in these cell lines.

Hypoxia-induced invasiveness of NSCLC cells is MET dependent

Previous studies show that hypoxia increases the invasive capacity of tumor cells (Cuvier *et al.*, 1997). Therefore, we investigated whether MET activation mediates hypoxia-induced invasiveness of tumor cells. A549 cells were plated in Matrigel-coated Boyden chambers and incubated in normoxia (21% oxygen) or hypoxia (0.1% oxygen) with or without erlotinib or PHA-665752. Hypoxia enhanced tumor cell invasion more than 2.5-fold ($P<0.001$). Erlotinib caused a moderate decrease in tumor cell invasiveness, whereas MET inhibition reduced hypoxia-induced tumor cell invasion to levels below baseline (Figure 5a; $P<0.001$).

To examine the role of HIF-1α in this pathway, we stably transfected A549 cells with a variant form of HIF-1α (HA- HIF-1α P402A;P564A) that is constitutively stabilized in normoxia because of proline to alanine substitutions at the VHL binding site critical for HIF-1α polyubiquitination and degradation (Masson *et al.*, 2001; Hu *et al.*, 2003; Kim *et al.*, 2006). Expression of the HIF-1α variant augmented MET production

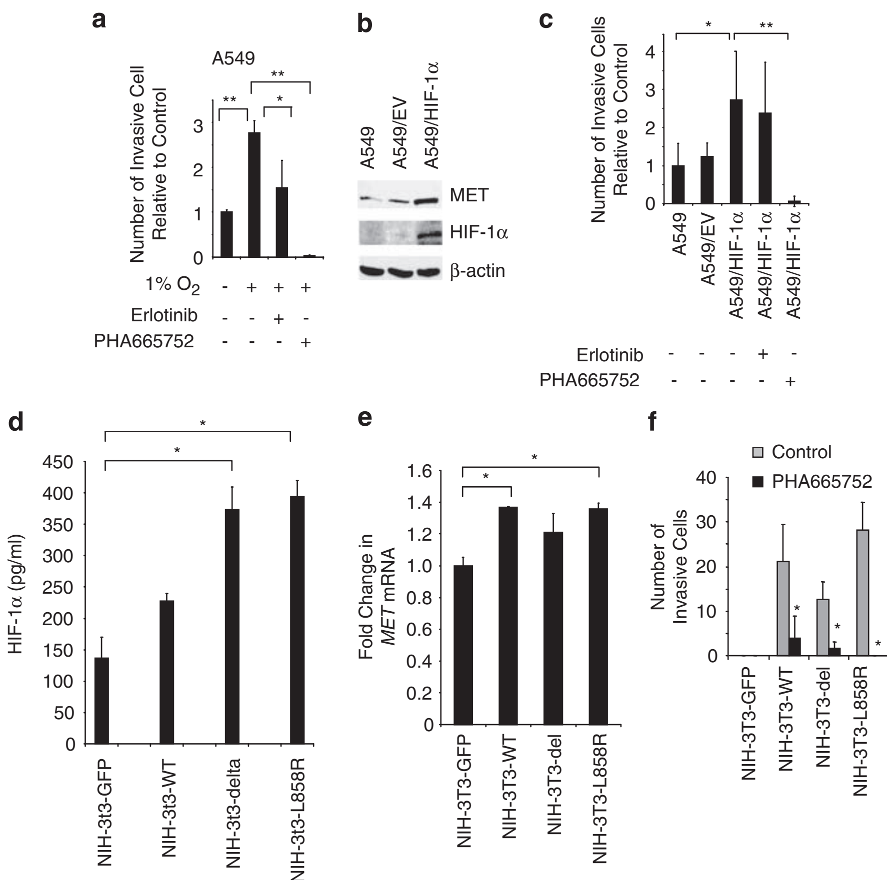

Figure 5 Hypoxia-induced tumor cell invasion is MET dependent. (**a**) A549 cells were seeded onto growth factor-reduced Matrigel-coated invasion chambers and incubated in normoxia or hypoxic (1% O_2) conditions with or without erlotinib (1 μM) or PHA-663225 (1 μM) for 24 h, and the number of invading cells was quantified. Hypoxia-induced invasion was MET dependent but EGFR independent. Bars, % s.d.; *$P<0.05$; **$P<0.001$. (**b**) A549 cells were transfected to overexpress a degradation-resistant HIF-1α variant (HA- HIF-1α P402A;P564A), as shown by immunoblot. Increased HIF-1α was associated with enhanced MET expression. (**c**) A549 cells and A549 cells transfected with empty vector (EV) or plasmid containing the HIF1α mutant were evaluated for changes in invasive capacity in media with or without erlotinib (1 μM) or PHA-663225 (1 μM). After 24 h the number of invading cells was quantified. Bars, % s.d.; *$P<0.05$; **$P=0.006$. (**d**) NIH-3T3 cells expressing GFP, WT EGFR, or EGFR bearing the L858R mutation or the deletion mutant *ΔL747-S752del* were evaluated for HIF-1α levels by ELISA. *$P<0.05$. (**e**) MET mRNA expression was evaluated by RT–PCR in NIH-3T3 cells expressing WT EGFR and EGFR-activating mutations. *$P<0.05$. (**f**) NIH-3T3 cells expressing GFP, WT EGFR, or EGFR bearing the L858R mutation or the deletion mutant *ΔL747-S752del* were allowed to migrate through Matrigel-coated invasion chambers for 24 h with or without PHA-663225 (1 μM). Bars, s.d.; *$P<0.05$.

(Figure 5b) and was associated with enhanced tumor cell invasion ($P<0.05$; Figure 5c). PHA-665752, but not erlotinib, reduced the number of invading cells to below baseline values ($P<0.05$). These findings were consistent with MET being a downstream mediator of HIF-1α-mediated invasiveness in thse cells.

We examined the impact of WT and mutated EGFR, and the role of MET, on invasiveness in NIH-3T3 cells. NIH-3T3 cells were stably transfected with control GFP plasmid, WT *EGFR* or *EGFR* bearing the L858R mutation or the deletion mutant ΔL747-5752del. A greater than twofold increase in HIF-1α levels was observed in cells bearing the ΔL747-5752del deletion ($P=0.02$) or the L858R mutation ($P=0.01$) compared with GFP transfected controls (Figure 5d). RT–PCR analysis revealed that NIH-3T3 cells expressing L858R expressed increased *MET* mRNA levels compared with GFP transfected controls ($P=0.02$; Figure 5e). NIH-3T3 cells stably transfected with control GFP plasmid, WT *EGFR*, or *EGFR* bearing the L858R mutation or the deletion mutant ΔL747-5752del were allowed to invade Matrigel-coated Boyden chambers with or without PHA-66752. Cells expressing WT or activated EGFR had enhanced invasive capacity compared with cells transfected with GFP vector, and inhibition of MET significantly reduced the number of invasive cells to near baseline values (Figure 5f).

Discussion

This study offers new insights into the mechanisms by which EGFR mediates its tumorigenic effects and provides new evidence that the HIF-1α/MET axis is critical to regulating invasiveness induced not only by hypoxia but by EGFR as well, thus illustrating the convergence of two pathways known to drive invasive

tumor growth. In NSCLC cells, we showed that EGFR activation, by either EGFR kinase mutations or ligand binding, increased MET levels through a hypoxia-independent mechanism involving expression of HIF-1α. MET was uncoupled from EGFR regulation, however, in a cell line with *MET* amplification, a finding consistent with the recently described role of *MET* amplification in EGFR TKI resistance (Engelman *et al*., 2007). Overexpression of a constitutively active form of HIF-1α also abrogated the regulation of MET levels by EGFR. Therefore, though this study shows that EGFR signaling can regulate MET levels and that MET can be downstream mediator of EGFR-induced invasiveness, it also suggests that there are ways by which this pathway may be bypassed.

We initially investigated MET levels in tumor specimens from 202 NSCLC patients by immunohistochemistry and observed increased levels of MET in tumors with *EGFR*-activating mutations compared with tumors with WT *EGFR*. Consistent with these findings, we observed elevated levels of MET in a previously described transgenic murine model of NSCLC with lung-specific expression of an *EGFR*-activating mutation. MET levels decreased significantly after treatment with the EGFR inhibitor erlotinib. We also observed that NSCLC cell lines expressing mutated *EGFR* exhibited elevated *MET* gene expression and protein levels compared with cells with WT *EGFR*, and these levels could be reduced by pharmacologically inhibiting EGFR or with siRNA directed against EGFR. The addition of an EGFR inhibitor decreased *MET* mRNA, indicating that in NSCLC cells, *EGFR*-activating mutations augment MET expression at the transcriptional level. Collectively, these results provide evidence that activated EGFR has a critical role in regulating MET expression in NSCLC tumor cells.

MET amplification has been described in the setting of gastric cancers (Smolen *et al*., 2006) and NSCLC (Engelman *et al*., 2007). Engelman *et al*. (2007) reported that among lung cancers with *EGFR*-activating mutations, *MET* amplification occurred in 22% of tumors that developed resistance to gefitinib or erlotinib. Overall, *MET* amplification occurs in only 4% of all NSCLC cases (Zhao *et al*., 2005), whereas high levels of MET and p-MET are detectable in 36 and 21% of NSCLC cases, respectively (Nakamura *et al*., 2007). MET expression also has been shown to be regulated by hypoxia (Pennacchietti *et al*., 2003). Here, we found evidence that EGFR is a key regulator of MET levels in cells without *MET* amplification, and that this occurs through hypoxia-independent regulation of HIF-1α. By contrast, in H1993 cells bearing MET gene amplification, EGFR blockade did not result in a reduction in *MET* levels, suggesting that *MET* amplification resulted in an uncoupling of MET protein levels from EGFR regulation.

Ligand-induced phosphorylation of EGFR has been shown to induce rises in HIF-1α in a cell type-specific manner. We observed that HIF-1α levels were elevated in NSCLC cell lines bearing *EGFR* mutations even in the absence of added ligand, and that treatment with an EGFR inhibitor diminished HIF-1α expression. Although this study did not specifically address the regulation of angiogenic factors such as VEGF, these findings are consistent with recent studies that EGFR regulates angiogenic factors, at least in part, through HIF-1α-dependent mechanisms (Swinson *et al*., 2004; Luwor *et al*., 2005; Pore *et al*., 2006; Swinson and O'Byrne, 2006). Studies designed to elucidate the mechanism by which EGFR-activating mutations regulate HIF-1α levels are ongoing.

In addition to enhancing MET levels, EGFR activation resulted in increases in MET receptor phosphorylation within 30 min of EGF stimulation, an effect blocked by erlotinib. Similar observations have been made with other cell types (Bergstrom *et al*., 2000; Jo *et al*., 2000); these and other published data support the idea that EGFR may directly phosphorylate MET (Bergstrom *et al*., 2000; Jo *et al*., 2000). Hypoxia is a known regulator of HGF, presumably through HIF-1α (Ide *et al*., 2006). It is feasible that EGFR-activating mutations promote HGF production through HIF-1α. Collectively, these data suggest that EGFR/HIF-1α activation may not only regulate MET levels, but may also impact MET signal transduction through other mechanisms.

We investigated the consequences of EGFR-regulated MET. We observed that the invasiveness (Figure 4) but not survival (Supplemental Figure 2) of NSCLC cells bearing EGFR-activating mutations was MET dependent, as pharmacological inhibition or siRNA directed against MET abrogated cell invasion. Invasiveness and MET levels were reduced by siRNA knockdown of HIF-1α, whereas EGFR levels were unaffected; indicating that MET is downstream of HIF-1α and EGFR. We observed that EGF- and hypoxia-induced invasiveness were both MET dependent, and that heterologous expression of a constitutively active form of HIF-1α induced invasiveness that was independent of EGF stimulation but remained MET dependent (Figure 5). Furthermore, heterologous expression of wild-type or mutated EGFR in NIH 3T3 fibroblasts increased invasiveness in an MET-dependent manner, providing further evidence that EGFR-mediated invasiveness is mediated at least in part by MET. These results support a model in which either hypoxia or EGFR activation can drive invasiveness by converging on a common HIF/MET pathway, which appears to be separable from EGFR-induced survival and proliferation. *MET* amplification appears to provide one route for circumventing this pathway. Others will likely emerge, and these findings do not exclude the likelihood that other pathways contribute to the invasive phenotype.

Our findings that EGFR can regulate MET levels through hypoxia-independent regulation of HIF-1α, and that MET is a downstream mediator of both EGFR- and hypoxia-induced invasivenss, have important clinical and biological implications. Even for tumors thought to be primarily driven by the EGFR pathway (that is with activating EGFR mutations), targeting of the MET pathway in combination with EGFR blockade may further reduce tumor invasiveness

beyond the effect of EGFR inhibition alone, in addition to the previously noted potential benefit of preventing the emergence of resistance through MET amplification (Engelman *et al.*, 2007). It also raises the question of whether other tyrosine kinases may regulate MET in a similar manner in NSCLC and other diseases. Finally, the observation that the EGFR and hypoxia converge on the HIF-1α/MET axis suggests that there may be additional overlap in the mechanisms by which EGFR and hypoxia promote malignant behavior and therapeutic resistance.

Materials and methods

Cell lines
Drs John Minna and Adi Gazdar (UT Southwestern Medical School, Dallas, TX, USA) provided H3255, H1975, H1993, and HCC827 cells. A549 and Calu-6 were obtained from the ATCC (Rockville, MD, USA). NIH-3T3 cells expressing WT *EGFR* or *EGFR* bearing the L858R mutation or the deletion mutant ΔL747-S752del (Shimamura *et al.*, 2005) were obtained from Dr Jeffrey Engelman (Dana-Farber Cancer Institute) and were maintained in 10% FBS DMEM containing 1 mg/ml puromycin.

Mice
Animals were treated in accordance with the guidelines of the US Department of Agriculture and the NIH. *Kras*^{LA1} mice (Johnson *et al.*, 2001) were obtained from Dr Tyler Jacks (Massachusetts Institute of Technology, Cambridge, MA, USA). *CCSP-rtTA* transgenic mice (obtained from J Whitsett at The University of Cincinnati, Cincinnati, OH, USA) were bred with *Tet-op-hEGFR L858R-Luc* to yield mice with lung tumors driven by EGFR activation (Ji *et al.*, 2006). At 6 months of age, lungs were collected. Animals bearing EGFR-driven lung tumors were treated with vehicle (1% Tween-80; Sigma-Aldrich, St Louis, MO, USA), or erlotinib (50 mg/kg/day) by gavage for 48 h.

Gene expression analysis
Affymetrix GeneChip Human Genome U133A (HG-U133A) was used to perform gene expression analysis on 53 gene arrays of NSCLC cell lines prepared by John Minna and colleagues (UT Southwestern, Dallas, TX, USA; (Zhou *et al.*, 2006). CEL-type data files were obtained from NCBI-GEO dataset GSE4824 (NCBI-GEO, 2007). CHIP (2007) software (http://biosun1.harvard.edu/complab/dchip/) was used to generate probe-level gene expression, median intensity, percentage of probe set outliers, and percentage of single probe outliers (Lin *et al.*, 2004). Information files, including the HG-U133A gene information files and Chip Description Files, were downloaded from the Affymetrix web site. CEL and other data files were extracted. Array images were inspected for contamination and bad hybridization. Normalization was performed using the invariant-set normalization method (Li and Hung Wong, 2001). Model-based expression and background subtraction using the 5th percentile of region (perfect match only) was completed by checking for single, array, and probe outliers. In the array analysis and clustering, array outliers were treated as missing values and no log transformation was performed. Comparison within dCHIP of the WT *EGFR* vs mutated *EGFR* groups using a more than 1.2-fold change in gene expression, a 90% confidence interval for fold change, and a 90% present call yielded one probeset for *MET* (203510_at). Data were further analyzed using GraphPad software (version 5, GraphPad Software Inc., La Jolla, CA, USA).

Detection of HIF-1α, MET, and EGFR
Protein lysates were extracted using RIPA buffer (50mM Tris–HCl, pH 7.4, 150mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS, and protease inhibitors. Protein (60 μg) was used for western blotting. Antibodies against EGFR (Y1068, Cell Signaling Technology Inc., Danvers, MA, USA), EGFR (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) MET (Santa Cruz), HIF-1α (Pharmingen, San Diego, CA, USA), β-actin (Sigma), and vinculin (Sigma) were used. HIF-1α, p-EGFR, MET, and p-MET ELISAs were obtained from R&D systems (Minneapolis, MN, USA).

RNA isolation and RT–PCR
HCC827 cells were treated with complete media with or without 1 μM erlotinib for 12 h. Total RNA was extracted using Trizol (Life Technologies, Carlsbad, CA, USA), and purified with the RNeasy kit (Qiagen, Hilden, Germany). We used SuperScriptTM III RNase H-Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) to convert RNA into cDNA. The oligonucleotide primers used were published previously (Shimazaki *et al.*, 2003).

Plasmids and transfections
HIF-1α cDNA (OriGene Technologies Inc., Rockville, MD, USA) was subcloned into the pcDNA3.1 vector with a flag tagged in the N-terminal, and the HIF-1α mutant with proline to alanine substitutions positions 402 and 564 (HA- HIF-1α P402A;P564A), which are known VHL-binding sites, was constructed as described (Kim *et al.*, 2006). This form is stabilized in normoxia because of the loss of VHL-mediated polyubiquitination and subsequent degradation (Masson *et al.*, 2001; Hu *et al.*, 2003). For siRNA transfections, HCC827 cells were transfected with siRNA targetting EGFR, MET, HIF-1α and control siRNA at a final concentration of 100 nM using Dharmafect 1 transfection reagent (Dharmacon, Lafayette, CO, USA). Protein was isoloated after 72 h. Transfected cells were plated for invasion assays after 48 h.

Invasion assay
We seeded 2.5×10^4 cells in the upper chamber of 24-well BD Biocoat growth factor reduced Matrigel invasion chambers (8.0 μm pore, Becton Dickinson, Bedford, MA, USA) with 0% FBS media and added media containing 10% FBS to the lower chamber. After 24 h, cells in the upper chamber were removed by scraping. Cells that migrated to the lower chamber were stained and counted using bright-field microscopy under a low-power ($\times 40$) objective. PHA-663225 was obtained from Pfizer (New York, NY, USA).

Clinical specimens
Tissue specimens from 202 surgically resected lung carcinomas were obtained from the Lung Cancer Specialized Program of Research Excellence (SPORE) Tissue Bank at The University of Texas MD Anderson Cancer Center (Houston, TX, USA). Two hundred and two specimens had known EGFR status. Microarrays for each specimen were created with three cores from formalin-fixed, paraffin-embedded blocks. All specimens were of pathologic TNM stages I–IV according to the revised International System for Staging Lung Cancer (Beadsmoore and Screaton, 2003).

Immunohistochemistry staining
Using paraffin-embedded tissue sections, antigen retrieval was performed by steaming in citrate buffer (pH 6.0). Endogenous peroxidases were blocked using 3% H_2O_2. After protein blocking, slides were incubated with anti-HGFR (1:50; R&D Systems), washed, and incubated with a Universal LSAB + Kit/HRP, visualization kit (DakoCytomation, Carpinteria, CA, USA). For tumor sections from transgenic animals, antigen retrieval and blocking was performed as above. Slides were incubated in 1:100 anti-mouse MET antibodies (Santa Cruz) and then in secondary antibody (Jackson Research Laboratories, Bar Harbor, ME, USA). NSCLC specimens were used as positive controls for MET staining. As a negative control, we followed the above procedure omitting the primary antibodies. For quantification, each specimen was evaluated using an intensity score (0, 1, 2, or 3) and an extension percentage (Yang *et al.*, 2008). The final staining score was the product of these two values. An average from the three cores was obtained for each specimen.

Statistics
Student's *t*-tests were performed using two-tailed tests with unequal variance for Gaussian distributed data. For statistical analysis of clinical specimens, Wilcoxon rank-sum tests were used when comparing continuous variables between mutation groups. To correlate mutation and other discrete covariates, we used a chi-square test or Fisher's exact test. Two-sided *P*-values $\leqslant 0.05$ were considered significant.

Accession numbers
SNP and CGH raw data are available in the Gene Expression Omnibus (GEO) database: GEO accession GSE4824 (http://www.ncbi.nlm.nih.gov/geo/).

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)

Estrogen receptor beta2 induces proliferation and invasion of breast cancer cells; association with regulation of prolyl hydroxylase 3, hypoxia induced factor 1 alpha and MET

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Keywords: ERβ2, proliferation, invasion, PHD3, gene expression profile

ABSTRACT

BACKGROUND: Estrogens exert physiological effects through two estrogen receptor (ER) subtypes, ERα and ERβ, which belong to the nuclear receptor family of ligand-activated transcription factors. The human ERβ variant ERβ2 is expressed at higher levels than ERβ1 in many breast cancer cell lines and breast tumours. Increasing evidence supports the hypothesis that ERβ2, in contrast to ERβ1, is associated with aggressive phenotypes of various cancers. In this manuscript we determine phenotypes and molecular mechanisms of ERβ2, independent of ERα and ERβ1, in breast cancer cells.

METHODS: The triple negative breast cancer (TNBC) cell line BT549 was used as model system. ERβ2 levels were modulated by transient overexpression or knockdown by small interfering RNA. Cell proliferation and invasion were assayed by the WST-1 cell proliferation assay kit and growth factor reduced BD Matrigel™ invasion chamber, respectively. GeneChip® Human Gene 1.1 ST Affymetrix microarrays were used to assay global gene expression. ERβ2 regulation of mRNA and protein levels of selected genes was investigated by qPCR and western blot analysis, respectively.

RESULTS: In this study we show that ERβ2 is the dominant ER isoform in the BT549 cell line and promotes proliferation and invasion of this cell line. A total of 263 genes were identified as ERβ2-upregulated genes and 662 identified as ERβ2-downregulated genes. ERβ2-regulated genes were involved in cell morphology, DNA replication and repair, cell death and survival. We show that ERβ2 represses prolyl hydroxylase 3 (PHD3) gene expression and induces protein levels of the hypoxia induced factor 1 (HIF-1α) and MET.

CONCLUSION: ERβ2 promotes cell proliferation and invasion of BT549 breast cancer cells. The invasive phenotype could potentially be mediated through transcriptional repression of PHD3, followed by up-regulation of the HIF1α-MET pathway.

INTRODUCTION

Breast cancer is a common cause of cancer death among women. Estrogen plays crucial roles in breast cancer with most of the effects of estrogen mediated by estrogen receptors (ERs). For a long time, only one ER was thought to be the receptor that is responsible for mediating the effects of estrogen. This receptor is now called ERα [1]. However, in 1996, another ER, now named ERβ, was discovered [2]. Both receptors belong to the nuclear receptor superfamily [3, 4]. Like many other genes, ERβ is expressed as different isoforms, the functions of which need to be further studied in order to understand the physiological functions of ERβ. ERβ1 is considered as the full length ERβ. The most studied variant of ERβ, ERβ2, is the result of alternative splicing, the last 61 amino acids of ERβ1 being replaced by 26 unique amino acids from an alternative exon. ERβ2 lacks the intact ligand binding domain and activation function 2 (AF-2) core region. Since ERβ2 has an intact DNA-binding domain and an intact N-terminal domain, including the AF-1 domain, ERβ2 could be directly involved in gene regulation.

Several lines of evidence suggest that ERβ2 is associated with proliferation and invasion of cancer cells. This is in contrast to ERβ1, which is suggested to have anti-proliferative characteristics [5]. In breast, ovarian, prostate and non-small cell lung cancer, it was reported that the expression of ERβ2 is widespread and higher in cancer tissue compared to normal tissue [6-8]**.** Furthermore it was found that the expression of ERβ2 correlates with aggressive phenotypical features in breast, prostate and colon cancer [6, 9, 10]. Negative ERβ2 status is significantly correlated to longer overall survival time in case of the luminal subtype of breast cancer [10]. ERβ2 was correlated with poor prognosis and could promote cancer cell proliferation and invasion of prostate cancer [6, 11]. In line with the differential role of ERβ1

and ERβ2 with regard to cancer cell proliferation and invasion, ERβ2 increased proliferation and up-regulated factors involved in bone metastasis while ERβ1 inhibited these factors [5].

HIF-1α is a major determinant of invasion and metastasis in a wide variety of tumor types. Its expression is regulated through modifications at the posttranslational level. In the presence of oxygen, three prolyl hydroxylase enzymes (PHD), PHD1, PHD2 and PHD3, can hydroxylate two proline residues (p402 and p564) of HIF-1α in the oxygen-dependent degradation domain. Hydroxylated HIF-1α can by recognized by the tumour suppressor von Hippel-Lindau protein, which targets HIF-1α for degradation [12]. Tumour hypoxic areas show MET overexpression [13]. When MET is inhibited, hypoxia induced invasive growth was prevented [14]. The MET tyrosine-kinase receptor is an established mediator of cancer cell invasiveness. MET can increase the viability of cancer cells [14]. Previous findings showed that HIF-1α can regulate MET levels, with MET being an established mediator of cancer cell invasiveness [15].

In this study, we focus on the role of ERβ2 for breast cancer cell proliferation and invasion using the BT549 cell line as a model that expresses endogenous ERβ2 but no ERα or ERβ1. We demonstrate that ERβ2 promotes cell proliferation and invasion in this model. We investigate ERβ2 dependent global effects on gene expression and suggest a model by which ERβ2 affects invasiveness of cells.

MATERIALS AND METHODS

RNA isolation and cDNA synthesis

Total RNA was extracted using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. cDNA was synthesized from 1 μg of total RNA with Taqman Reverse Transcription reagents according to the standard protocol (Roche).

qPCR

Real-time PCR was performed with the SYBR Green I dye master mix (Applied Biosystems, Foster City, CA). qPCR reactions were performed using a 7500 Fast Real-Time PCR System (Applied Biosystems) applying the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 50 sec. mRNA expression levels were normalized relative to GAPDH or 36B4 internal control.

Plasmids and transfection

The ERβ2 cDNA was subcloned into the pcDNA3.1 vector with a flag tag at the N-terminus. For ERβ2 siRNA transfection, BT549 cells were transfected with siRNA targeting ERβ2 with a sequence 5'-GAAUGAAGAUGGAGACUCUUU-3' (Thermo) or control siRNA (Sigma) at a final concentration of 100 nM using INTERFERin™ siRNA transfection reagent (Polyplus). For ERβ2 cDNA transfection, BT549 cells were transfected with pcDNA3-flag-ERβ2 or control pcDNA3-EV using lipofectamine®2000 according to manufacturer's instructions (Invitrogen).

Western blot analysis and ELISA assay

Protein lysates were extracted using RIPA buffer (Sigma Aldrich). 30 mg of protein was used for western blot and ELISA analysis. Antibodies against MET (Santa Cruz Biotechnology

Inc., Santa Cruz, CA, USA), HIF-1α (Pharmingen, San Diego, CA, USA) and β-actin (Sigma) were used. HIF-1α ELISA was performed using a kit from R&D systems (Minneapolis, MN, USA).

Immunohistochemistry staining

Immunohistochemistry staining was performed as described previously [11].

Cell proliferation assay

1000 cells per well were seeded onto 96-well plates. Cell proliferation was measured using a WST-1 kit (Roche Applied Science).

Invasion assay

24 hours after the transfection, approximately 2.5×10^4 cells were seeded in the upper chamber of 24-well BD Biocoat growth factor reduced Matrigel invasion chambers (8.0 μm pore, Becton Dickinson, Bedford, MA, USA) with 0% FBS media. Medium containing 10% FBS was added to the lower chamber. For ERβ2 siRNA and control siRNA transfection, cells in the upper chamber were removed by scraping after 30h; for ERβ2 cDNA and control transfection, cells in the upper chamber were removed after 24h. Cells that migrated to the lower chamber were fixed, stained and counted under a microscope.

Gene Expression Microarray Analysis

Total RNA from three biological replicates were hybridized to Affymetrix Human Gene 1.1 ST arrays, which contain probes for 33299 gene sequences. Experimental steps such as probe synthesis, hybridization and scanning were done according to the Affymetrix protocol (www.affymetrix.com). Pre-processing for background correction/normalization was

performed in the Affymetrix Expression Console using the Robust Multichip Average (RMA) method[16]. Two-tailed Student's t-test was used to derive p-values, and the false discovery rates were estimated using the q-value. A cut-off fold change of at least 1.5 and p value < 0.05 were used to define differentially regulated genes. The q-value, representing the false discovery rate, was less or equal to 0.1.

To derive genes as input for pathway analysis the following cut-offs were applied; fold change of ≥ 2, p value < 0.05 and q value ≤ 0.1. The Ingenuity Pathway Analysis (IPA) software (www.ingenuity.com) was used for this analysis. The entire IPA core analyses was performed based on information in the Ingenuity Pathway Knowledge Base (IPKB), which derives information from known relationships of molecules, functions and interactions of genes published in the literature.

IPA analysis identified pathways that were most significantly changed upon ERβ2 knock down. Associated network functions and molecular functions are two of the categories used for enrichment testing within IPA. Biological functions were ranked according to the significance of that function to the network.

Fisher's exact test was used to derive the *p*-values in IPA.

Statistics

Student's *t*-tests were performed using two-tailed tests with unequal variance.

RESULTS

The TNBC cell line BT549 expresses high levels of ERβ2

We screened the expression of ERβ2 in a panel of 8 breast cancer cell lines, including 2 ER-positive luminal (MCF-7 and MDA-MB-175), 3 HER2-positive (SK-BR-3, HCC1569, and MDA-MB-453) and 3 TNBC (MDA-MB-231, BT549 and Hs578T) cell lines. The ERβ2

mRNA was highly expressed in the 2 TNBC cell lines, BT549 and MDA-MB-231. The highest level of endogenous ERβ2 expression was observed in the BT549 cell line (Figure 1). The mRNA expression levels of ERβ1 and ERα in this cell line is undetectable (data not shown).

Depletion of ERβ2 inhibits cellular proliferation and invasion *in vitro*

We used siRNA to silence ERβ2 expression. Successful knockdown of ERβ2 was confirmed at both the mRNA and protein levels (Figures 2A and 2B). Furthermore, this experiment revealed that ERβ2 is mainly localized to the cytoplasm of cells (Figure 2B, Left panel). Interestingly, ERβ2 knockdown inhibited cell proliferation of BT549 cells (Figure 2C). Moreover, ERβ2 silencing decreased the number of invading cells per 20 × field from 34.6 ± 9.0 in the control siRNA group to 13.4 ± 5.5 in the ERβ2 siRNA treated group (Figure 2D) ($p < 0.05$).

ERβ2 overexpression confers a more proliferative and invasive phenotype *in vitro*

To further confirm the effect of ERβ2 on cellular proliferation and invasion, we investigated these phenotypes after exogenous ERβ2 overexpression. Successful overexpression of ERβ2 was confirmed by western blot analysis (Figure 3A). Importantly, ERβ2 overexpression promoted cell proliferation in BT549 cells (Figure 3B). Additionally, BT549 cells overexpressing ERβ2 displayed a more invasive phenotype with 55.0 ± 5.0 cells migrating through the chamber compared to 9.2 ± 2.7 cells for the control cells (Figure 3C) ($p < 0.001$). These results further support the link between ERβ2 expression and the cellular proliferation and invasion.

ERβ2 influences global gene expression

To gain insight into ERβ2 regulated transcriptional networks, we determined gene expression profiles for BT549 cells upon ERβ2 knockdown. We identified 662 genes, applying a false discovery rate of less or equal to 0.1, as up regulated (fold change equal or greater than 1.5, $p < 0.05$) while the expression of 263 genes was repressed (fold change equal or less than 1.5, $p < 0.05$) upon ERβ2 knockdown. Network analysis revealed the top three ranked networks regulated by endogenous ERβ2 in BT549 cells as cell morphology, DNA replication and repair, cell death and survival and cell morphology (Table 1A). Molecular and cellular functional classification analysis in IPA shows how the alterations of gene expression were predicted to disrupt various molecular and cellular functions. For our data set, the top 5 highlighted molecular and cellular functions were cell cycle, cell death and survival, morphology, development and organization, which were represented by 4 to 8 genes with *p*-values between 1.94×10^{-3}–4.57×10^{-2} and 1.94×10^{-3}–3.63×10^{-2} after ERβ2 knockdown (Table 1B).

Consistent with the anti-proliferative effect of ERβ2 depletion, Cyclin E/A (*CCNE2/CCNA2*) were down regulated by 1.3 and 1.4 fold, respectively, with a concomitant increase in the expression of p21WAF1/CIP1 (*CDKN1A*) by 1.5 fold.

Validation of gene expression profiling data by qPCR

qPCR analysis was performed to confirm changed expression of 6 genes (HOXA7, GAB1, RECK, TGFB3, E2F2 and PHD3) identified as being regulated by ERβ2 in the gene expression profiling analysis. qPCR results were consistent with the microarray data, thus demonstrating the accuracy of the array approach (Figure 4).

ERβ2 promotes BT549 cell invasion potentially through repressing PHD3 followed by up-regulation of the HIF-1α-MET pathway

One of the genes, the expression of which was up-regulated, by knockdown of ERβ2 was PHD3 (Figure 5A, Left Panel). Importantly, the PHD3 mRNA level was reduced by overexpression of ERβ2 (Figure 5A, Right Panel), further establishing a link between ERβ2 and PHD3. As PHD3 has been shown to regulate HIF-1α stability [17], we next determined if the HIF-1α - MET pathway was regulated by ERβ2 as the HIF-1α - MET pathway has been shown to mediate cellular invasion in other systems [15]. Interestingly, knockdown of ERβ2 decreased HIF-1α levels while overexpression of ERβ2 increased HIF-1α and MET levels (Figure 5B). Changes in HIF-1α protein levels were further confirmed by ELISA (Figure 5C).

DISCUSSION

Breast cancer is a disease with different morphological features and clinical behaviours, and 6 intrinsic subtypes (Luminal A, Luminal B, normal breast like, HER2 enriched, basal-like breast cancer and Claudin low breast cancer) have been defined according to microarray based classification [18]. Approximately 60-80% of basal-like breast cancers correspond to TNBC, which are characterized by a lack of expression of ERα, progesterone receptor (PR) and HER2 overexpression. In our present study, we used BT549 due to the high endogenous ERβ2 expression. BT549 is a TNBC cell line. TNBC constitutes 10–25% of all breast cancers, has aggressive phenotypes and displays the worst prognosis among breast cancer subtypes. It affects mostly younger age groups and there is a lack of effective targeted therapies. TNBC lacks predictive biomarkers and often develop distant metastases in distant tissues such as brain and lung. In the present study, we demonstrate that ERβ2 is highly expressed in TNBC cells and promotes cell proliferation and invasion, which is in line with

the aggressive behavior of TNBC.

Estrogen signalling via ERs has been linked to the promotion of breast cancer. While ERα has been shown to generally promote proliferation in breast cancer, ERβ1 has been suggested to display anti-proliferative properties including in breast cancer [19]. Less is known about the role of the ERβ2 variant in breast cancer. One reason for this lack of knowledge is that ERβ2 is only expressed in humans and primates and but not in rodents, making it difficult to study it in the context of an animal model. The focus of this study was to elucidate the role of ERβ2 in breast cancer cells. Furthermore, since cross talk between ERα and ERβ1 and ERβ2 signalling has been demonstrated, we focused on the role of ERβ2 expressed alone.

Expression of ERβ variants in breast cancer has been analyzed at the mRNA or protein level in several clinical materials. However, the extent to which breast tumours express ERβ2 alone is still unclear. Studies of ERβ1 and ERβ2 in cell lines have been limited by difficulties in identifying cell lines that express endogenous ERβ. In this study we identify a breast cancer cell line that exclusively expresses endogenous ERβ2.

The data shown in this study relies on a single siRNA against ERβ2. However, we observed the opposite phenotypes in a system where ERβ2 was overexpressed, supporting the connection between ERβ2 and proliferation and invasion. Furthermore, effects on PHD3 mRNA levels as well as HIF-1α protein levels were opposite between ERβ2 siRNA treated and ERβ2 overexpressing cells, strengthening their connection to ERβ2 signalling. For MET, the effect of knock down of ERβ2 was less clear. In addition, transfection of two siRNA oligos from Sigma targeting both ERβ1 and ERβ2 inhibited proliferation and invasion of BT549 cells compared to transfection with control siRNA (data not shown). As the mRNA level of ERβ1 is undetectable in this cell line (data not shown), those siRNA oligos should target ERβ2 in this system.

We used gene expression profiling to approach the molecular mechanisms associated with the proliferative and invasive properties of ERβ2 and identified changes in pathways and molecular classes associated with cancer, cell cycle, cell survival and cell death compatible with a role of ERβ2 in cellular proliferation and invasion. Our data shows that endogenous ERβ2 is mainly localised to the cytoplasm of BT549 cells (Figure 2B, Left panel). This suggests that ERβ2 effects on gene regulation are indirect. How ERβ2 exerts its effects in BT549 cells, and other cell lines where ERα and ERβ1 are not expressed, remain to be determined. In systems where ERβ2 is co-expressed with ERα we have shown that ERβ2 induces proteasome-dependent degradation of ERα, presumably through the formation of ERβ2/ERα heterodimers [20]. In this case ERβ2-mediated degradation of ERα is one mechanism whereby expression of ERβ2 inhibits recruitment of ERα to the estrogen-responsive promoters, leading to suppression of ERα-regulated gene expression. We are only aware of one published study addressing changes in gene expression profiles in response to expression of ERβ2 alone [21]. In this study, Hs578T TNBC cells were engineered to express ERβ2. This study reported that expression of ERβ2 had no effects on gene expression [21]. Furthermore, we have observed that ERβ2 does not modulate gene expression in HEK293 cells engineered to stably express ERβ2 alone (unpublished data).

Pathway analysis can serve as a biological verification of non-random biological differences, especially if the results are supported by an experimental hypothesis and additional data. Additionally, based on IPA analysis, new hypothesis can be derived. Our IPA analysis showed that ERβ2 is involved in cell morphology, cell cycle, cell death and survival. In general, the gene expression profiling revealed changed expression of a very limited number of genes that could be causative for the observed phenotypes (data not shown). However, as our studies focused on a snapshot of gene expression, it is possible that we missed genes, the

changed expression of which, influence the phenotype. Future studies should address changes in gene expression at additional time points following knock down of ERβ2. ERβ2 promoted cell proliferation could be caused by the observed down-regulation of *CCNE2/CCNA2* and up-regulation of *CDKN1A*. Knockdown of ERβ2 also down regulates the Wilms tumor-1 (WT-1) mRNA which is a transcription factor that plays an important role in cellular development, cell survival and angiogenesis [22].

In this study we propose that the invasive phenotype associated with ERβ2 expression could potentially be via repressing of PHD3 followed by up-regulation of the HIF-1α-MET pathway or by directl regulation of MET （Figure 6). In line with this, PHD3 has been shown to exert tumour-protective functions and inhibits tumour growth in pancreatic cancer [23]. In a recent study, Thomas et al shows that ERβ1 represses basal-like breast cancer epithelial to mesenchymal transition by destabilizing epidermal growth factor receptor (EGFR) where the mesenchymal state represents a state of invasion [24]. In our system, we observe that ERβ2 increases EGFR protein levels (data not shown). Since the HIF-1α-MET pathway is known to be regulated by EGFR, this might represent an alternative mechanism for ERβ2 regulation of the HIF-1α-MET pathway and subsequent invasion [15].

In summary, we demonstrate that ERβ2 promotes cell proliferation and invasion when expressed in the absence of ERβ1 and ERα. To further our understanding of the clinical relevance of ERβ2, the role of ERβ2 needs to be examined in human breast cancer specimens. It will be important to generate mouse models that express ERβ2 in different tissues. Furthermore, although ERβ2 does not bind tested ER ligands, it is possible that compounds that inhibit its function or target genes can be identified, thus providing potential therapeutic agents for breast cancers that express ERβ2.

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FIGURE LEGENDS

Figure 1. The TNBC cell line BT549 has the highest endogenous expression of ERβ2
Analysis of ERβ2 mRNA levels in ER+/PR+/HER2- breast cancer cell lines (MCF-7 and MDA-MB-175), ER-/PR-/HER2+ breast cancer cell lines (SK-BR-3, HCC1569 and MDA-MB-453) and TNBC cell lines (MDA-MB-231, BT549 and Hs578T) by qPCR. mRNA levels are normalized to GAPDH and mRNA levels are presented as means ± of relative fold with SD, with one corresponding to expression level in MCF-7 cells.

Figure 2. Depletion of ERβ2 inhibits cellular proliferation and invasion. (A) ERβ2 siRNA down-regulates ERβ2 mRNA in BT549 cells. ERβ2 mRNA levels were determined by qPCR after transfection with control siRNA or siRNAs against ERβ2. Data are normalized to 36B4 and shown as relative fold compared to siControl with SD.*$p < 0.05$. (B) ERβ2 siRNA down-regulates ERβ2 protein in BT549 cells. Immunohistochemistry staining showing that the ERβ2 is located to the cytoplasm in BT549 cells and that the level is reduced after transfection with siRNA targeting ERβ2. (C) ERβ2 depletion reduces proliferation in BT549 cells. BT549 cells were transfected with siRNA against ERβ2 or control siRNA. WST-1 assays of cell metabolic activity as a measure of cellular proliferation were carried out at the indicated time points after siRNA transfection. Data are shown as means with SD. **$p<0.01$, ***$p < 0.001$. Experiments were repeated twice. One representative experiment in shown. (D) ERβ2 depletion reduces invasion in BT549 cells. BT549 cells were transfected with siRNA against ERβ2 or control siRNA, and cell invasion was evaluated by the BD Biocoat growth factor reduced Matrigel invasion chambers assay. Data represent means with SD. *$p < 0.05$. Experiments were repeated twice. One representative experiment in shown.

Figure 3. ERβ2 overexpression confers a more proliferative and invasive phenotype *in vitro*. (A) Western blot analysis showing increased protein levels of ERβ2 after transient transfection with flag tagged ERβ2. ERβ2 was detected by an anti-flag antibody. β-actin was used as a loading control. The arrow indicates the position of the overexpressed ERβ2 protein. (B) ERβ2 overexpression promotes cell proliferation in BT549 cells. WST-1 assays of cell metabolic activity as a measure of cell proliferation were carried out at the indicated time points after transfection of flag tagged ERβ2 or empty vector, respectively. Ratio of absorbance to day2 is calculated. Data are shown as means of relative absorbance with SD. *$p < 0.05$, **$p<0.01$. Experiments were repeated twice. One representative experiment in shown. (C) ERβ2 overexpression promotes cell invasion in BT549 cells BT549 cells were transfected with flag tagged ERβ2 or empty vector, and cell invasion was evaluated by BD Biocoat growth factor reduced Matrigel invasion chambers assay. Data represent means with SD. ***$p < 0.001$. Experiments were repeated twice. One representative experiment in shown.

Figure 4. Validation of gene expression profiling data by qPCR

Real-time PCR analysis for a subset of genes from the same RNA as was used for the microarray analysis. mRNA levels are normalized to 36B4 and mRNA levels are presented as means ± of relative fold with SD, relative to control siRNA-treated cells. Fold changes derived from microarray analysis is presented as numbers below the bars.

Figure 5. ERβ2 modulates levels of PHD3, HIF1α and MET in BT549 cells. (A) Top panel, BT549 cells were transfected with siRNA against ERβ2 or control siRNA, RNA was collected after 48h, and PHD3 levels were determined by qPCR assay, mRNA levels are normalized to 36B4, **$P<0.005$. Bottom panel, BT549 cells were transfected with

pcDNA3-flag tagged ERβ2 or an empty control vector. RNA was collected after 48h, and PHD3 levels were determined by qPCR assay, mRNA levels are normalized to 36B4. (B) BT549 cells were transfected with siRNA against ERβ2, a control siRNA, pcDNA3-flag tagged ERβ2 or an empty control vector. Protein lysates were collected and MET, and HIF-1α levels were evaluated by western blot analysis. β actin was probed as a loading control. (C) BT549 cells were transfected with siRNA against ERβ2, a non-targeting control siRNA, pcDNA3-flag tagged ERβ2 or an empty control vector. Protein lysates were collected and HIF-1α levels were evaluated by ELISA assay. ** $P<0.005$.

Figure 6. Proposed model of ERβ2 promoting cell proliferation and invasion in BT549

ERβ2 abrogates the expression of PHD3. We hypothesize that the phenotypes associated with ERβ2 expression could potentially be via repressing of PHD3 followed by up-regulation of the HIF-1α-MET pathway or direct up-regulation of MET.

Table 1A Changed networks after knockdown of ERβ2 in BT549 cells

Top Networks		
ID	Associated Network Functions	Score
1	Cell Morphology, DNA Replication, Recombination and Repair, Developmental Disorder	32
2	Cancer, Reproductive System Disease, Cell Dearth and Survival	24
3	Cell Morphology, Dermatological Diseases and Conditions, Developmental Disorder	24

Table 1B Changed molecular functions after knockdown of ERβ2 in BT549 cells

Name	***p*-value**	**#Molecules**
Cell Cycle	1.94E-03-4.01E-02	5
Cell Death and Survival	1.94E-03-4.46E-02	5
Cell Morphology	1.94E-03-4.57E-02	8
Cellular Development and Organization	1.94E-03-3.63-02	7
Cellular Development	1.94E-03-3.79-02	4

Figure 1

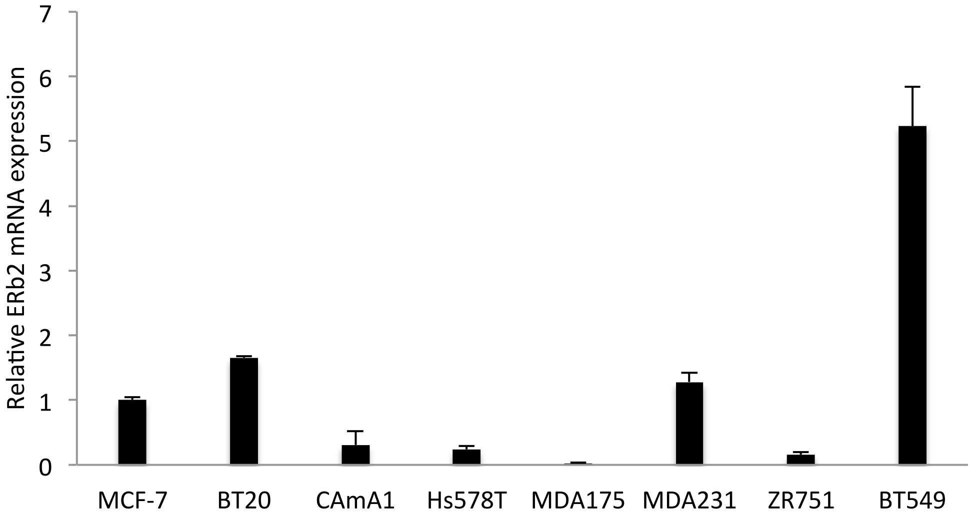

Figure 2

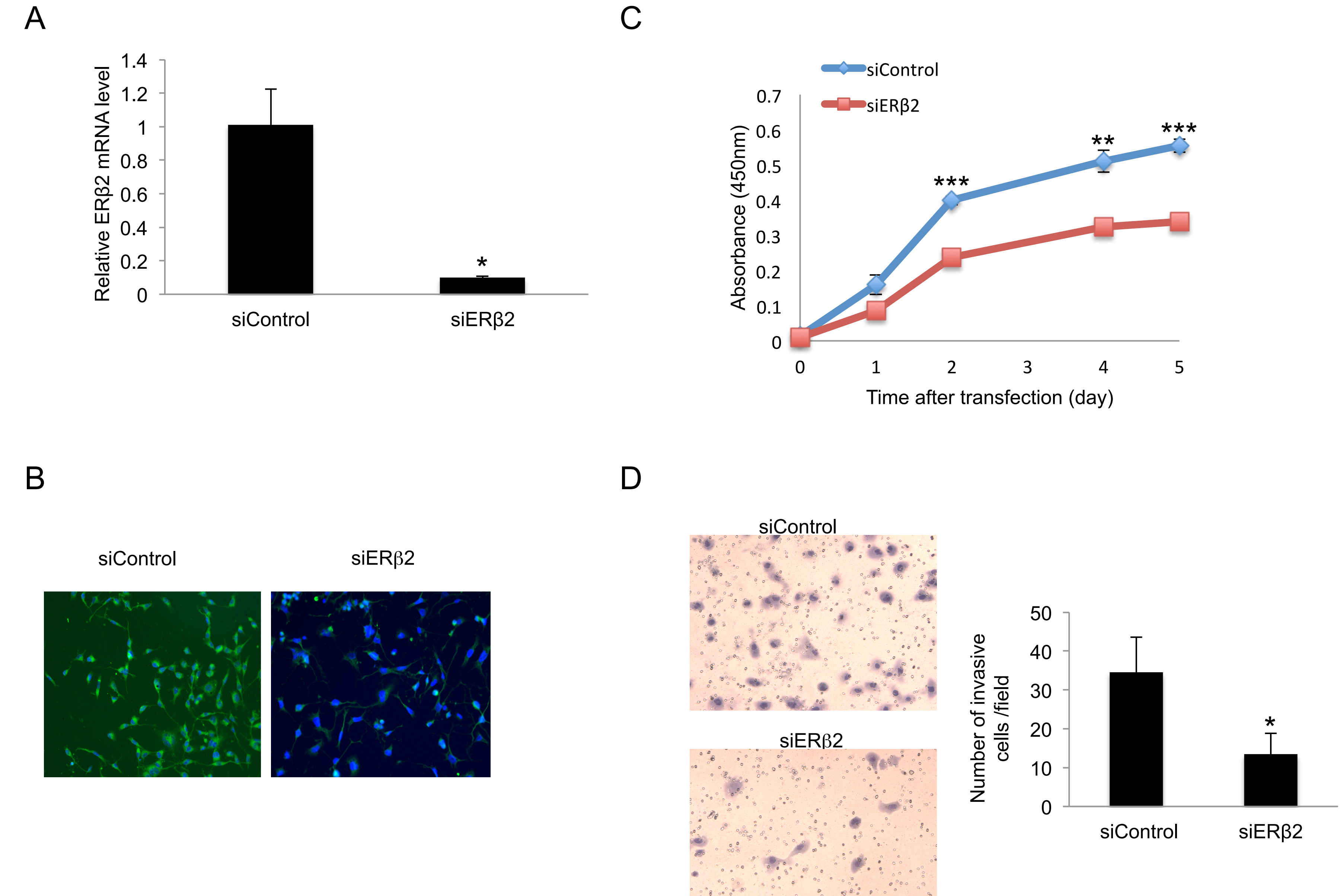

Figure 3

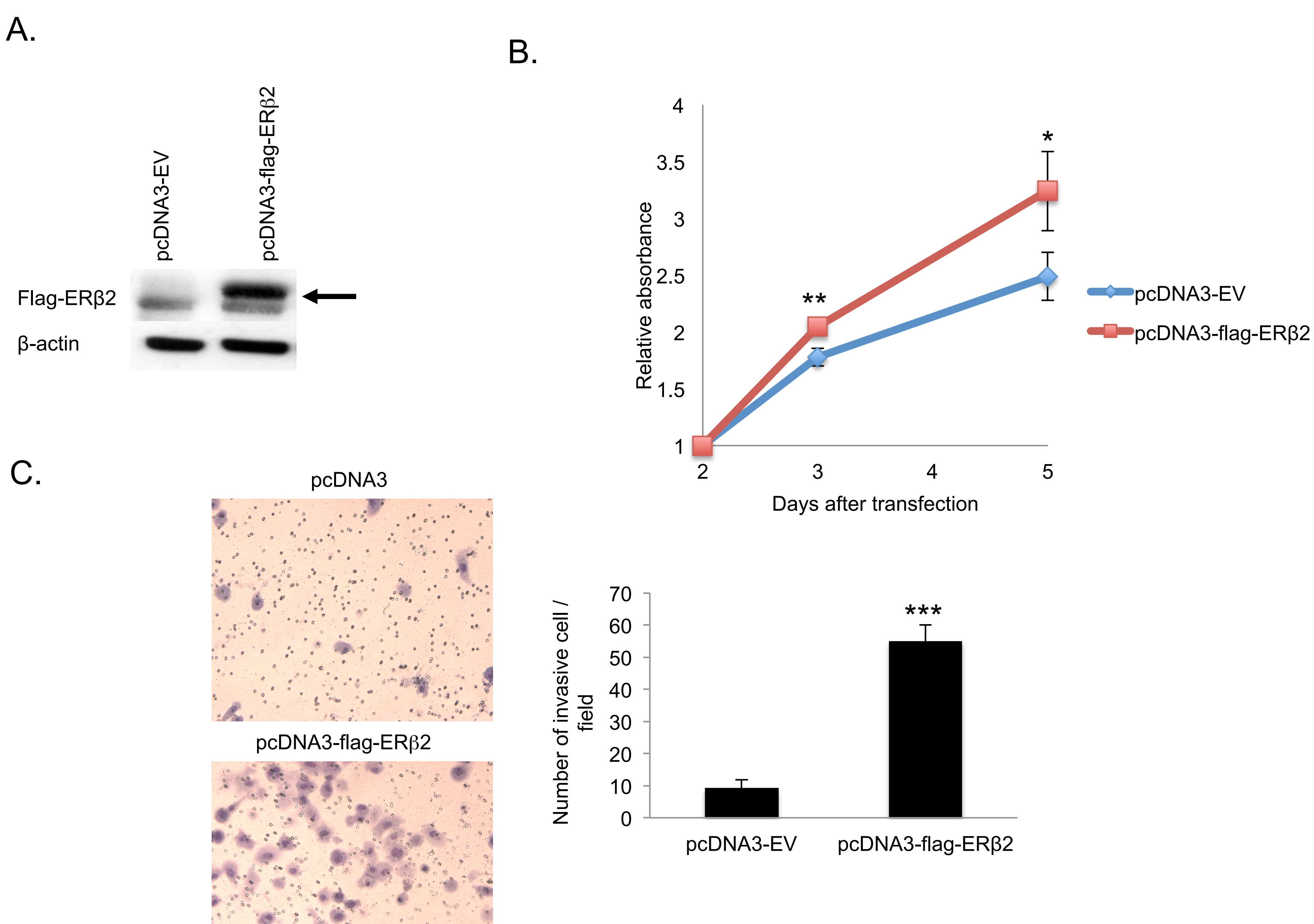

Figure 4

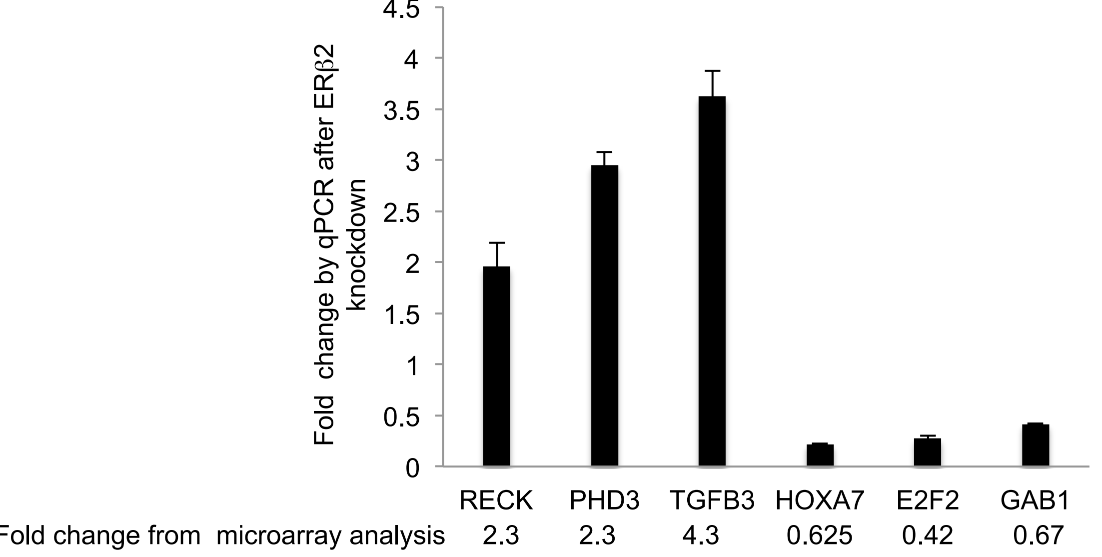

Figure 5

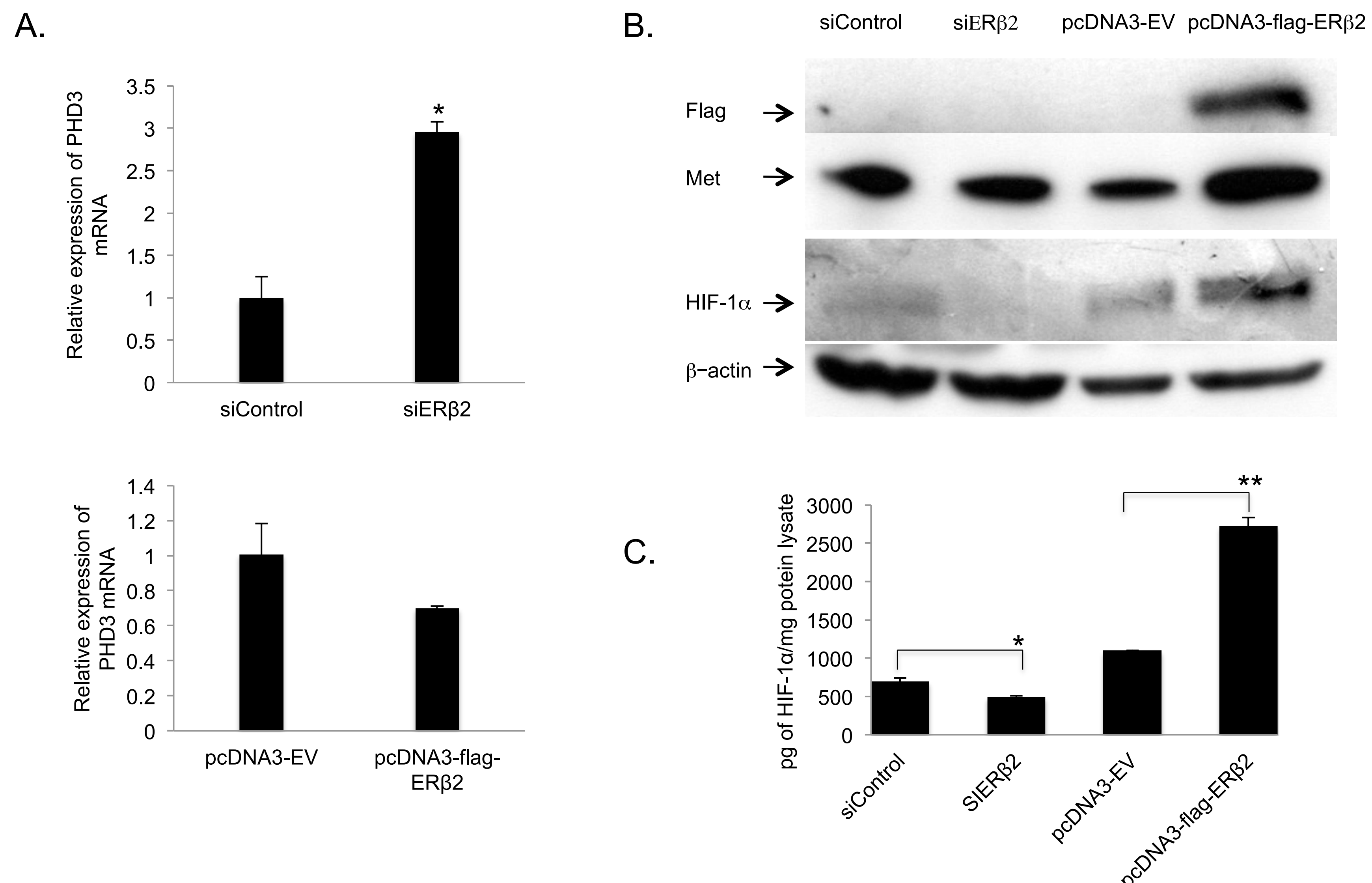

Figure 6