REGULATION OF ESTROGEN RECEPTOR-ALPHA MEDIATED GENE EXPRESSION AND ENDOCRINE RESISTANCE THROUGH ESTROGEN RECEPTOR-ALPHA PHOSPHORYLATION AND MICRO-RNA IN BREAST CANCER

BY

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DISSERTATION

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ABSTRACT

Estrogens are associated with the development and progression of breast cancer in addition to their role in normal reproductive physiology, and estrogen receptors (ER) mediate the actions of estrogen in target tissues by regulating the expression of numerous biologically important target genes. The progression of human breast cancer and the development of resistance to endocrine therapies are thought to be associated with ERα phosphorylation. We generated multiple combinations of ER phospho-mutants, at residues serine 104, 106, 118, 167, 236, and 305, and examined their impact on receptor half-life, the agonist and antagonist balance of selective estrogen receptor modulators (SERMs) and selective estrogen receptor downregulators (SERDs), the regulation of ER transcriptional activity, and stimulation of cell proliferation in response to estradiol and SERMs/SERD. We showed that changes in ERα affecting the phosphorylation status of the receptor greatly impact receptor function and differential SERM and SERD modulated cellular responses that could contribute to resistance to endocrine therapies in breast cancer. We also studied the regulation of microRNAs (miRNAs) by estradiol and growth factors through ERα and extracellular signal-regulated kinase 2 (ERK2) in order to understand their physiological impact on breast cancer. We identified nine miRNA-encoding genes harboring overlapping ERα and ERK2 binding sites close to their transcription start sites, which require ERα and ERK2 for transcriptional induction as well as estradiol-mediated miRNA regulation. We then identified TP63, a target of miR-101, miR-190 and miR-196a2, and showed that TP63 plays an important role in estradiol- or growth factor-mediated cellular response in breast cancer cells (MCF-7 and MDA-MB-231) by increasing tumor cell growth and *in vitro* invasion mainly controlled by miR-196a2 action. These results suggest a

tumor-suppressive role of miR-196a2 in regulating TP63 expression and the aggressive behavior of breast cancers.

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CHAPTER 1

Introduction

1.1 Estrogen Receptors

Estrogens are the major hormones regulating female reproductive functions and actions in the breast and uterus. Estrogen plays key roles in reproductive physiology and development but also exerts central effects in breast and endometrial cancers [1]. Estrogen functions are mediated through two estrogen receptors, ERα and ERβ, which differ in their tissue distribution and physiological functions [2]. The estrogen receptors (ER) are members of the nuclear receptor (NR) superfamily of ligand-activated DNA-binding transcription factors [3].

The estrogen receptor, like other members of the NR superfamily, contains several functional domains [4]. The N-terminal portion is largely unstructured and contains the so called ligand-independent activation function (AF-1) [5]. AF-1 activity can be modulated by the action of intracellular signaling pathways via post-translational modifications. In response to ligand binding, AF-1 synergizes with the ligand-dependent activation function (AF-2) which resides in the C-terminal ligand binding domain (LBD) [6, 7]. The LBD contains 12 alpha-helices (H1-H12) which undergo conformational changes upon ligand binding [8]. In particular, the rearrangement of helix 12 results in a docking surface for interaction with coactivator proteins [9-11] such as the p160 family (SRC-1/NcoA1; SRC-2/TIF2/GRIP-1; SRC-3/pCIP/RAC3/ACTR/AIB-1/TRAM-1) and the HATs p300/CBP [12]. The position of helix 12 also determines whether or not a ligand behaves as an agonist or an antagonist [10, 13, 14]. The central region of the receptor contains the DNA-binding domain (DBD) with two alpha-helices and two type-II zinc fingers that are involved in recognition and binding to the cognate response

elements (EREs) or DNA [15, 16] and also form surfaces involved in receptor dimerization [17, 18].

1.2 Molecular Mechanisms of Estrogen Action

ER functions primarily as a ligand-dependent transcription factor by regulating the expression of its target genes through direct genomic activity [19]. Regulation of ERα target genes in many different tissues has been identified thanks to recent technological advances which allowed the analysis of global changes in gene expression and genome-wide DNA interaction sites (e.g. cDNA microarrays, ChIP-chip, ChIP-Seq or ChIP-PET) [20]. In the absence of ligand, the ER resides mostly in the nucleus as an inactive apoprotein bound to molecular chaperones. Upon ligand binding, ER is released from the chaperones, dimerizes and binds to response elements within the regulatory regions of target genes [21]. Recent genome-wide studies showed that many ER binding sites do not contain a consensus ERE motif [22-24] and it has been shown that ERs can also tether to DNA interacting with transcription factors such as stimulating protein 1 (SP1) [25], and activating protein 1 (AP1) [26] in order to mediate estrogen-regulated gene expression. Once on DNA, ER acts as a nucleation site for coregulators which induce histone modifications and stabilize the basal transcriptional machinery leading to the formation of a competent transcription initiation complex [27, 28]. The strength and duration of the signal is controlled by the clearance of ER via the ubiquitin-proteasome pathway [29].

ERα activities can also exert rapid stimulatory effects on a variety of cytosolic signal transduction pathways [21, 30-32]. This rapid nongenomic activity occurs outside the nucleus and is independent of gene transcription. Membrane-associated or cytoplasmic ER couples with components of signaling complexes such as receptor tyrosine kinases (EGFR, IGFR, HER2

receptor), Src or G proteins triggering the activities of downstream signaling pathways [33-35]. These cytoplasmic signals can then be transduced to the nucleus where they result in post-translational modifications on ER itself or other transcription factors and cofactors thus affecting target gene expression [36].

1.3 Phosphorylation of ERα and Kinase Signaling

Post-translational modifications (PTMs) are a major mechanism by which protein function can be regulated. Transient modifications such as phosphorylation, sumoylation, acetylation, ubiquitination and methylation are essential for the regulation of protein function. Proteins can be modified combinatorially at multiple sites increasing the complexity and adding another layer of functional control [37]. The important roles of PTMs in nuclear receptor (NR) biology have been proposed by multiple studies which suggested that these modifications can provide a potential explanation for cell- and gene-specific regulation by steroid receptors. They can affect receptor stability, ligand binding affinity, subcellular localization and protein-protein interactions [38, 39]. The transcriptional activity of steroid nuclear receptors is mainly governed by ligand binding, but post-translational modifications serve as integrating signal from intracellular pathways.

The best characterized PTM that affects NR transcriptional activities is phosphorylation [40]. ERα possesses multiple phosphorylation sites mainly scattered along the N-terminal region (Ser-104/106/118/167) and DBD (Ser-236). These sites have been shown to be targets of multiple kinases. It has been demonstrated that ERα phosphorylation on serine residues is also enhanced in response to E2 or growth factors in a number of different cell types [41-44]. Ser-104, Ser-106 and Ser-118 were initially identified as phosphorylation sites by comparison of

phosphopeptide patterns generated by wild type and mutant ERs [42]. These three sites are proline-directed protein kinase consensus sequences, and they are targeted upon activation of the MAPK pathway by EGF or estradiol [45, 46]. Also the cyclin-A-cyclin dependent kinase 2 (Cdk2) complex has been shown to phosphorylate Ser-104 and Ser-106 [47]. However, as inhibition of MAPK was not able to prevent the estradiol-induced phosphorylation on Ser-118, the presence of other kinases regulating Ser-118 phosphorylation has been proposed [48, 49]. Ser-167 is targeted by p90 ribosomal S6 kinase (Rsk) after activation of the MAPK pathway [46] or by casein kinase II and AKT, at least *in vitro* [50-53]. Ser-236 and Ser-305 are located in the DBD and the LBD, respectively, and are targets of different kinase pathways. *In vitro*, Protein kinase A (PKA) can phosphorylate Ser-236 [54], while p21-activated kinase-1 (Pak1) directly phosphorylates Ser-305 [55]. It has been reported that Tyr-537 can also be phosphorylated in MCF-7 cells but not in response to E2 [56, 57]. *In vitro*, Tyr-537 can be phosphorylated by src tyrosine kinases [57].

1.4 Estrogen Receptor and Breast Cancer

In addition to their roles in normal reproductive physiology, estrogens are also associated with the development and progression of breast cancer [58]. A majority (70%) of primary human breast cancers is ERα positive, which correlates with better prognosis [59]. As ERα plays the major role in breast cancer development and progression, clinical targeting of the receptor by antiestrogens has become the most important treatment option for women with ER-positive breast cancer [60]. One of the selective estrogen receptor down-regulators (SERDs), ICI 182,780 blocks ERα action by mediating receptor degradation in estrogen-target tissues such as the breast, uterus and bone [61]. However, completely abrogating estrogenic activity in all tissues is

not desirable because it can lead to loss of bone density [62]. Thus, selective estrogen-receptor modulators (SERMs) which selectively inhibit or stimulate ER action in selected target tissues would serve as a more optimal treatment option. The most widely used SERM, tamoxifen is a partial agonist/antagonist which blocks estrogen action by binding to ER in breast cancers but acts as an agonist in uterus [63]. Unfortunately many patients fail to respond to initial drug therapy primarily due to lack of expression of ERα (intrinsic resistance) and responsive patients also develop resistance to SERM therapy following prolonged exposure to tamoxifen (acquired resistance) [64, 65]. Therefore, a greater understanding on the mechanisms that cause endocrine resistance is in high demand.

1.5 ER/HER2 Pathway Crosstalk and Endocrine Resistance

The progression of human breast cancer and the development of resistance to endocrine therapies are thought to be associated with alterations of multiple pathways which are also involved in ERα phosphorylation [66, 67]. Overexpression of growth factor receptors such as the epidermal growth factor receptor (EGFR) and the human epidermal growth factor receptor 2 (HER2) has been associated with resistance to endocrine therapy [68, 69]. The activation of downstream effectors through signaling pathways can phosphorylate ERα and its coactivators such as AIB1, leading to the abnormal regulation of ER target gene expression [70-72].

There are several clinical reports showing that ERα site-specific phospho-status correlates with patient survival and endocrine resistance in breast cancer. ERα phosphorylated at Ser-118 could be a possible biomarker of responsiveness to endocrine therapy as patients with higher levels of phospho-Ser-118 have better disease-free and overall survival than phospho-Ser-118 poor tumors [73, 74]. Another group showed the association between recurrence-free

survival after tamoxifen treatment and ERαS118-P expression in 239 premenopausal patients with breast cancer. They found a benefit from adjuvant tamoxfien among patients whose tumors had high ERαS118-P expression [75]. Phosphorylation of ERα Ser-167 is also predictive of response to endocrine therapy and could serve as a prognostic marker for metastatic breast cancer [76], while also contributing to tamoxifen resistance [77]. More recent studies demonstrated that the combined phosphorylation status of ERα affects survival in ER-positive breast cancer and endocrine therapy resistance. Yamashita *et al.* (2008) showed that the combination of low phosphorylation of ERα Ser-118 and high phosphorylation of ERα Ser-167 was associated with improved disease-free and overall survival and better response to tamoxifen treatment in ER-positive breast cancer [78].

1.6 MicroRNAs, key post-transcriptional regulators of gene expression

MicroRNAs (miRNAs) are a class of 21-23 nucleotide-long noncoding RNAs that modulate gene expression by post-transcriptional repression [79-81]. miRNAs have emerged as an important focus of research in molecular biology following the initial identification of two small noncoding RNAs, *lin-4* and *let-7*, and their regulatory roles in timing of the nematode worm *Caenorhabditis elegans* development [82-84]. Many more have since been found in animals, plants and fungi, reflected with 677 human miRNA sequences detailed as of September 2008 in the Miranda database (www.microrna.org), and bioinformatics predictions indicate that mammalian miRNAs can regulate ~30% of all protein-coding genes [80, 85]. miRNAs can affect both the translation and stability of mRNAs by their sequence complementarity to the 3' UTR of the mRNAs of target genes in the cytoplasm [80]. However, additional functions of miRNAs are

possible, for they could regulate pre-mRNA processing in the nucleus or act as chaperones modifying mRNA structure or modulating mRNA-protein interactions [86].

1.7 miRNA biogenesis and target recognition

miRNAs are processed from precursor transcripts (pri-miRNAs), which are either transcribed from independent miRNA genes or are portions of introns of protein-coding RNA polymerase II transcripts. Each pri-miRNA contains one or more hairpin structures that are recognized and processed by the RNase III type endonuclease Drosha and its partner, DGGR8 (DiGeorge syndrome critical region gene 8) [86]. The Drosha-DGGR8 complex generates a 70-nucleotide stem loop known as pre-miRNA, which is transported to the cytoplasm by exportin 5 [79, 81, 87, 88]. The pre-miRNA is recognized by Dicer, another RNase III type endonuclease complexed with TRBP (TAR RNA-binding protein), to yield a 21-nucleotide miRNA duplex with a protruding 2-nucleotide 3' end. One strand is then selected to function as a mature miRNA (generally, the strand with 5' terminus), and the other strand is degraded [79, 87, 89, 90]. The mature miRNA is loaded into the RNA-induced silencing complex (RISC), which contains Argonaute (AGO) proteins and the single-stranded miRNA. In mammals, four AGO proteins (AGO1 to AGO4) function in the miRNA repression by RNA interference (RNAi) or by repressing protein synthesis when tethered to the mRNA 3' UTR [88, 91, 92]. Apart from AGOs, miRNA-ribonucleoprotein (RNP) complexs (miRNPs) can contain other proteins that function as regulatory factors or effectors mediating the inhibitory function of miRNPs [88-90]. Mature miRNA allows the RISC to recognize target mRNAs through partial sequence complementarity with its target. Most miRNA-binding sites in metazoan mRNAs reside in the 3' UTR and are usually present in multiple copies in order for effective repression of translation [93], but

miRNAs also exert their repressive function through interaction with the binding sites in the 5' UTR or coding regions of target mRNAs [94]. One rule for miRNA-target recognition is a contiguous and perfect base pairing of the miRNA 5' region nucleotides 2-8 (the seed region), which nucleates the miRNA-mRNA interaction [95, 96]. GU pairs or mismatches in the seed region greatly affect repression, whereas an A residue across position 1 of the miRNA and an AU-rich composition across from position 9 improve the site efficiency [96]. Bulges or mismatches must be present in the central region of the miRNA-mRNA duplex to prevent the AGO-mediated endonucleolytic cleavage of mRNA, while there must be reasonable complementarity to the miRNA 3' half to stabilize the interaction [95, 97]. For long 3' UTRs, a position that is not too far away from the poly(A) tail or the termination codon can make the 3' UTR regions less structured and hence more accessible to miRNP recognition [97, 98], affecting miRNA-mediated repression [99].

1.8 Mechanisms of post-transcriptional regulation by miRNAs

The effects of miRNAs on protein synthesis can result from mRNA destabilization or translational repression. The studies carried out using HeLa cells and reporter mRNAs that had multiple binding sites for miRNAs in their 3' UTR showed that the 7-methylguanosine (m^7G) cap at the 5' UTR of mRNA is essential for translational repression [100, 101]. Also, some factors bound at the 3' UTR exert their inhibitory effect on translational repression by recruiting proteins that either interfere with the eukaryotic translation initiation factors (eIFs), eIF4E-eIF4G interaction or bind directly to the cap preventing the assembly of the 40S ribosome initiation complex [102-104]. The miRNA-mediated deadenylation of a poly(A) tail contributed to the repression of m^7G-capped mRNAs by disrupting the eIF4F-mediated mRNA circularization *in*

vitro [105, 106]. An alternative mechanism of miRNA action besides preventing the synergy between the 5' cap and 3' poly(A) tail, is translational repression by preventing 60S ribosomal subunit joining to the 40S initiation complex through the interaction between RISC and eIF6 [107]. The repression can also occur at post-initiation phases of translation by stalling or slowing down of elongating ribosomes [108-110]. miRNA can destabilize mRNAs and change the transcript levels by binding of miRNPs to mRNA 3' UTR, which can induce deadenylation and decay of target mRNAs [111-113]. It is not known what determines whether an mRNA undergoes the miRNA-mediated degradation or translational-repression pathway. However, accessory proteins bound to the 3'UTR or structural subtleties of miRNA-mRNA duplexes are suggested to be involved in the process [114, 115].

1.9 miRNAs and signal transduction

Despite great advances, the physiological functions of miRNAs and their target gene regulation in cells and organisms are still largely undiscovered. Due to the numerous putative targets of each miRNA and the presence of miRNAs as families of redundant genes as well as their often rather modest effects on protein output [116], it has been challenging to predict and experimentally prove the biological impact of individual miRNAs [80, 117]. Different cell types respond differently to a signal according to their interpretation, and the unique set of miRNAs in each cell type can serve in such context-dependent gene expression. Signal transduction pathways are ideal candidates for miRNA-mediated regulation as signaling complexes exert dose-dependent responses to the environmental inputs, which is amenable to the multi-gene regulatory nature of miRNAs for quantitative fluctuations [118, 119]. For example, Nodal, a morphogen that induces the germ layers in early vertebrate embryos, is regulated by miR-15 and

miR-16 to create the asymmetry by targeting the Nodal receptor activin receptor type 2A (ACVR2A) [120]. The miR-23b cluster has been shown to target three Smads (SMAD3, SMAD4 and SMAD5), inhibiting the TGFβ-mediated anti-proliferative response in liver stem cells [121]. miR-21 also serves as a general enhancer of receptor tyrosine kinase (RTK) signaling by targeting PTEN and Sprouty [122, 123], perhaps explaining its frequent up-regulation in various human tumors including breast cancer [124]. miRNAs can also serve as mediators of crosstalk between signaling pathways. For example, miR-192 enables positive crosstalk between TGFβ and AKT signaling [125]. Recent studies have reported a type of reciprocal regulation between miRNAs and their targets. Reciprocal inhibition between the transcription factors zinc finger E-box-binding homeobox 1 (ZEB1), ZEB2 and the miR-200 family regulates the switch between epithelial and mesenchymal states by TGFβ signaling in stem cells [126, 127]. Signaling networks can also regulate the processing of miRNA precursor. TGFβ and BMP signaling affects the expression of mature miR-21 by Smads associating with the Drosha complex and promoting the processing of pri-miR-21 [128].

1.10 miRNAs and breast cancer

The association of miRNAs in cancer biology is of great interest in regards to their regulatory functions in proliferation, differentiation and apoptosis [124, 129, 130]. Because of miRNAs' broad influence over diverse genetic pathways, the alteration in miRNA expression is likely to be pleiotropic and contribute to disease, including cancer [131, 132]. miRNA expression correlates with various cancers, and these are thought to function as both tumor suppressors and oncogenes [131]. High-throughput miRNA expression profiling in breast cancer cell lines and tissues identified a large set of miRNAs expressed at different levels compared to the normal

breast [133-135]. miRNA signatures predicting the expression levels of the estrogen, progesterone and HER2/*neu* receptors which characterize different breast cancer phenotypes have also been identified to elucidate the role for these miRNAs in disease classification of breast cancer and also serving as a prognostic biomarker [136]. In the study by Iorio *et al.* (2005), miR-125b, miR-145, miR-21, and miR-155 were significantly deregulated, and the expression of those miRNAs were correlated with specific breast cancer pathologic features such as estrogen and progesterone receptor expression, tumor stage or proliferation index [135]. Another group also identified four miRNAs (miR-7, miR-128a, miR-210 and miR-516-3p) associated with aggressiveness of lymph node-negative, estrogen receptor-positive human breast cancer [137]. Epigenetic mechanisms have also been proposed to play a role in the regulation of miRNA expression in breast cancer [138, 139]. Five miRNAs (miR-9-1, miR-124a3, miR-148, miR-152 and miR-663) showed hypermethylation and inactivation from 71 primary human breast cancer specimens [138]. Also, a functional genetic variant in the mature region of miR-196a2 and its potential oncogenic role in breast tumorigenesis was identified by functional analysis [140].

More interesting, several studies have reported the regulation between miRNAs and estrogen receptor alpha (ERα) signaling and its impact on endocrine resistance in breast cancer. miRNA-221/222 expression was up-regulated in ERα-negative breast cancer and conferred tamoxifen resistance by targeting ERα and the cell cycle inhibitor $p27^{Kip1}$ [141, 142]. miR-206 expression was down-regulated in ERα-positive breast cancer and repressed ERα mRNA and protein synthesis [143, 144]. It was also revealed that estradiol can regulate miRNA expression (i.e. miR-21) and modulate target gene expression in breast cancer cells [145, 146]. More profound understanding of the association of miRNAs in regulation of specific gene sets and

signaling pathways, and further pathological processes in breast cancer biology will definitely provide insight into the underlying mechanisms of oncogenesis as well as putative treatment strategies using miRNA therapies [131]. In the studies described below in Chapter 3, we have focused on miRNAs regulating tumor protein p63 (TP63) and several other proteins because of their likely important roles in controlling the phenotypic properties of breast cancer cells.

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CHAPTER 2

Phosphorylation Status of Estrogen Receptor alpha and Endocrine Resistance in Breast Cancer

2.1 Abstract

The estrogen receptor alpha (ER) contains multiple serine residues capable of undergoing post-translational modification by phosphorylation. In order to understand the role of phosphorylation status in affecting the response of receptor to the natural hormone estradiol, the selective estrogen receptor modulator, tamoxifen, and the selective estrogen receptor down-regulator, ICI182,780 (Fulvestrant), we generated multiple combinations of ER phospho-mutants, at residues serine 104, 106, 118, 167, 236, and 305, and examined their impact on receptor half-life, the agonist and antagonist balance of SERMs and SERDs, the regulation of ER transcriptional activity, and stimulation of cell proliferation in response to estradiol and SERMs/SERD. ERα mutants were generated by substituting serine residues with either alanine or glutamic acid, and of the sixteen mutants screened, half were selected for further analysis. The mutant receptors were generated into U2OS osteosarcoma-tetracycline regulated- ERα stable cell lines for characterization. These phospho-ER mutant receptors were also expressed in MCF-7 breast cancer cells with concomitant knock-down of endogenous ERα. Receptors with changes at Ser-118 and Ser-167 showed altered responses to the antiestrogens tamoxifen and ICI182,780, i.e. strong agonistic stimulation and weak estrogen antagonistic activity of tamoxifen and ICI182,780 on gene regulation and differential stimulation of cell proliferation. Other mutant ERs showed increased protein stability in the presence of estradiol or ICI182,780. Hence, changes in ERα affecting the phosphorylation status of the receptor greatly impact

receptor function and differential SERM and SERD modulated cellular responses that could contribute to resistance to endocrine therapies in breast cancer.

2.2 Introduction

The estrogen receptor (ER) is a member of the nuclear receptor (NR) superfamily of transcription factors that, upon hormone binding, mediates the actions of estrogen in target tissues by regulating the expression of numerous biologically important target genes [1]. The transcriptional activity of ER is fine tuned by a variety of coregulators that are recruited by ER to DNA regulatory elements. These coregulators can be divided into co-activators and co-repressors according to their stimulatory or inhibitory actions, respectively, on the transcription of target genes [2, 3].

Post-translational modifications (PTMs) are a major mechanism by which protein function can be regulated. Transient modifications such as phosphorylation, sumoylation, acetylation, ubiquitination and methylation are essential for the regulation of protein function. Proteins can be modified combinatorially at multiple sites increasing the complexity and adding another layer of functional control [4]. The important roles of PTMs in NR biology have been proposed by multiple studies which suggested that these modifications can provide a potential explanation for cell- and gene-specific regulation by steroid receptors. They can affect receptor stability, ligand binding affinity, subcellular localization and protein-protein interactions [5, 6]. The transcriptional activity of steroid nuclear receptors is mainly governed by ligand binding, but post-translational modifications can also serve to integrate signaling from intracellular pathways. The best characterized PTM that affects ER transcriptional activities is phosphorylation. ERα is phosphorylated by different kinases such as c-Src, PKA, MAPK and Akt, impacting on its ligand

binding affinity, coregulator binding and transcriptional activity [7]. An additional layer of complexity is added by the regulation of coregulator proteins via PTMs which are required to fine tune NRs activity. Moreover, an interplay between different PTMs has been suggested [8, 9].

Several amino acid residues in ERα can be subjected to phosphorylation (e.g. Ser, Thr and Tyr) in response to estradiol or growth factors via activation of intracellular signaling pathways [10-14]. The estrogen receptor, like other members of the NR superfamily, contains several functional domains [15]. The N-terminal domain is largely unstructured and contains the so called ligand-independent activation function (AF-1) [16]. AF-1 activity can be modulated by the action of intracellular signaling pathways via post-translational modifications. The central region of the receptor contains the DNA-binding domain (DBD) with two alpha-helices and two type-II zinc fingers that are involved in recognition and binding to the cognate response elements (EREs) or DNA [17, 18] and also form surfaces involved in dimerization [19, 20]. ERα possesses multiple phosphorylation sites mainly scattered along the N-terminal region (Ser-104/106/118/167) and DBD (Ser-236). These sites have been shown to be targets of multiple kinases. Phosphorylation of ERα modulates the action of the receptor by altering or fine-tuning its compatibility with other proteins or DNA, and may impact on the outcome of breast cancer treatments.

In addition to their role in normal reproductive physiology, estrogens are also associated with the development and progression of breast cancer [21]. As ERα plays a major role in breast cancer development and progression, clinical targeting of the receptor by antiestrogens has become the most important treatment option for women with ER-positive breast cancer [22]. Tamoxifen, the most widely used selective estrogen-receptor modulator (SERM) is a partial

agonist/antagonist which blocks estrogen action by binding to ER in breast cancers but acts as an agonist in uterus [23]. Unfortunately, many patients fail to respond to initial drug therapy primarily due to lack of expression of ERα (intrinsic resistance) and responsive patients also often develop resistance to SERM therapy following prolonged exposure to tamoxifen (acquired resistance) [24, 25]. The progression of human breast cancer and the development of resistance to endocrine therapies are thought to be associated with alterations of multiple pathways which are also involved in ERα phosphorylation [26, 27]. Overexpression of growth factor receptors such as the epidermal growth factor receptor (EGFR) and the human epidermal growth factor receptor 2 (HER2) has been associated with resistance to endocrine therapy [28, 29]. There are also several clinical reports showing that ERα site-specific phospho-status correlates with patient survival and endocrine resistance in breast cancer [27]. ERα phosphorylated at Ser-118 could be a possible biomarker of responsiveness to endocrine therapy as patients with higher levels of phospho-Ser-118 have a better disease free and overall survival than phospho-Ser-118 poor tumors [30-33]. Phosphorylation of ERα at Ser-167 is also predictive of response to endocrine therapy and could serve as a prognostic marker for metastatic breast cancer [34, 35]. More recent studies demonstrated that the combination of the phosphorylation status of ERα affects survival in ER-positive breast cancer and endocrine therapy resistance. Yamashita *et al.* (2008) showed that the combination of low phosphorylation of ERα Ser-118 and high phosphorylation of ERα Ser-167 was associated with improved disease-free and overall survival and better response to tamoxifen treatment in ER-positive breast cancer [36]. Breast tumors with an ERα mutation at Lys-303 (K303R) exhibit decreased sensitivity to tamoxifen and increased resistance to an aromatase inhibitor when coupled with phosphorylation of Ser-305 which appears to confer increasing ligand-independent activity of the mutant receptor [37, 38].

In this study, we focused on how the combinatorial phosphorylation status of ERα impacts receptor function and its response to SERMs and SERDs. For this purpose we generated ERα phospho-mutants with combinatorial mutations at known phospho-serine sites. We first used ER-negative osteosarcoma U2OS cells to characterize several ERα phospho-mutants. In two different systems (U2OS and MCF-7 cells), receptors with opposite changes at Ser-118 and Ser-167 (e.g. 118A/167E vs. 118E/167A) were examined and found to exhibit altered responses to 4-hydroxytamoxifen (Tam) and ICI 182,780 (ICI) on gene regulation and stimulation of cell proliferation. These results correlate well with the tamoxifen resistance observed in breast tumors with high phosphorylation at Ser-118 and low phosphorylation at Ser-167 of ERα [36]. We also detected mutants with altered affinity for E2 or SERMs and mutants that were not down-regulated by ICI. Thus, this study demonstrates that changes in the combinatorial phosphorylation status of ER greatly impact receptor function and show differential SERM and SERD activity that could contribute to resistance to endocrine therapies in breast cancer.

2.3 Materials and Methods

Cell culture and Generation of U2OS-ERα stable cell lines

MCF-7 (human breast cancer), U2OS (human osteosarcoma) and Hec-1 (human endometrial adenocarcinoma) cells were maintained as previously described [39-41]. Four days before ligand treatment, cells were switched to treatment media (phenol red-free MEM [Sigma] containing 5 % charcoal dextran-treated calf serum). U2OS cells were stably infected with the lentivirus delievering tetracycline repressor and ERα wild type (Wt) or mutants and grown as previously described [40]. U2OS-ERα stable cells were seeded 2 days prior to induction of ER expression with tetracycline (Invitrogen). Induction was for 48 hr prior to ligand treatments. The

amount of tetracycline used was adjusted to induce the expression of flag-ERα Wt and mutants to comparable levels (250 – 1000 ng/μl).

Mutagenesis, viral system generation and luciferase reporter assays

Site-directed mutagenesis was performed on pCMV5-Flag-ERα Wt plasmid using the QuickChange II kit (Stratagene) according to the manufacturer's instructions. The pCMV5-Flag-ERα Wt plasmid was previously created in the lab by inserting a FLAG epitope at the N-terminus of ERα. Multiple rounds of site-directed mutagenesis were performed to generate the desired combinatorial mutations. All plasmids were sequenced to confirm the introduction of the desired mutations. pCMV5-Flag-ERα Wt and mutant plasmids were then subcloned into the Gateway® entry vector pENTR™/TEV/D-TOPO®, followed by the generation of lentivirus expression vectors pLenti4/TO/V5-DEST-ERα Wt or mutants. Lentiviral-delivered ERα Wt and mutants were generated using a ViraPower T-REx Lentiviral Expression System according to the manufacturer's instructions (Invitrogen). Lentivirus was produced by transfecting the 293FT producer cell line with the expression constructs pLenti4/TO/V5-DEST-ERα Wt or mutants. Recombinant adenovirus (Ad) pAdEasy-1 carrying Wt or selected mutant ERα were generated and prepared as described (Stratagene) [42]. Virus was concentrated up to 10^{12} effective plaque-forming units/ml, using CsCl gradients. Hec-1 cells were seeded in 24-well plates 24 hr before transfection and transfected with 500 ng of 2X-ERE-pS2-Luc, 100 ng of pCMV-β-gal and 5 ng of pCMV5-Flag-ERα (Wt or mutants) using Lipofectin-transfection method (Invitrogen, Carlsbad, CA). Four hours after transfection, cells were treated with 0.1 % ethanol or E2 for 24 hr. Cell extracts were then prepared and luciferase assays were performed using the Luciferase Assay System according to the manufacturer's instructions (Promega).

Chromatin Immunoprecipitation

U2OS-ERα or MCF-7 cells were treated with 0.1 % ethanol, 10 nM E2 or 100 nM Tam for 45 min. ChIP assays were performed essentially as described before [43]. Antibodies used were: ERα (HC-20) and p300 (N-15) from Santa Cruz Biotechnology.

Ligand binding and RBA assays

ERα Wt or mutants were transfected in 293FT cells and nuclear extracts were prepared in protein extraction buffer (20 mM HEPES pH 8, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10 % glycerol and protease inhibitor cocktail), diluted with binding buffer (50 mM Tris pH 7.5, 10 % glycerol, pH 7.4) and then incubated in duplicate with a range of ^{3}H-E2 concentrations alone or with 100-fold excess unlabeled E2 for 3 hr on ice. Aliquots were used to determine the total ^{3}H-E_2 in the sample. Hydroxylapatite slurry was added and incubated for an additional 15 min on ice. The slurry was washed twice and its radioactivity then determined by scintillation counting. Relative binding affinities were determined by competitive radiometric binding assays using 10 nM ^{3}H-E_2 as tracer, using methods previously described [44, 45]. Incubations were done at 0 °C for 18–24 hr, and hydroxylapatite was used to absorb bound receptor-ligand complex. The binding affinities are expressed as relative binding affinity (RBA) values, where the RBA of estradiol is 100 %.

Western Immunoblotting and In-Cell Western assays

Whole cell extracts were prepared in lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 % NP-40, 1 % SDS, 5 % glycerol, and protease inhibitor cocktail). Western blot analysis of

whole cell extracts followed common protocols and specific antibodies were used for ERα (HC-20, Santa Cruz Biotechnology) and β-actin (AC-15, Sigma). ERα expression in U2OS stable cell lines was quantified using infrared-based In-Cell Western assay system as described by the manufacturer (Li-Cor Biosciences).

RT-PCR and Real-time Quantitative PCR

Total RNA from U2OS-ERα stable cells and MCF-7 cells was isolated using TRIzol® (Invitrogen) following manufacturer's instruction. RNA samples were reverse transcribed by M-MuLV Reverse Transcriptase (New England Biolabs) in 20 μl and subsequently diluted to 500 μl with sterile water. Real-time PCR was performed to quantify gene expression levels and ChIP samples. Each real-time PCR reaction contained 4 μl of diluted cDNA or ChIP sample, 5 μl of 2X SYBR Green PCR Master Mix (Applied Biosystems. Foster City, CA), 0.5 μl of 1.25 μM forward and reverse primers and 0.5 μl of ddH2O. The real-time PCR was carried out in an ABI prism 7900 HT Sequence Detection System (Applied Biosystems. Foster City, CA) for 40 cycles (95 °C for 15 sec and 60 °C for 1 min) after an initial 10 min incubation at 95 °C. The fold change in expression of each gene was calculated as described previously, with 36B4 as an internal control [46].

siRNA transfection and adenovirus (Ad) infection

To knock down endogenous ERα in MCF-7 cells, siRNA duplexes against the 3' UTR region of ERα were transfected at a final concentration of 5 nM using DharmaFECT transfection reagent (Dharmacon, Lafayett,CO) as per manufacturer's recommendations 72 hr before harvest. The following sequences were used: *siERα* sense: 5'-

rGrUrArArUrArCrCrArGrCrUrArArArGrCrCrArArArCrAAT-3'; *siERα* antisense: 5'-rArUrUrGrUrUrUrGrGrCrUrUrUrArGrCrUrGrGrUrArUrUrArCrArU-3'; *siNT (siGENOME Non-Targeting siRNA #2)* as a negative control. The cells were then infected with control adenovirus (CMV) or Adenoviruses expressing ERα-Wt or mutants for 64 hr before harvest. Conditions were used to express comparable amounts of adenovirus-carried ERs to those of endogenous ERα in MCF-7 cells.

Proliferation assays

MCF-7 cells expressing AdERα-Wt or mutants Ae or Ea followed by siERα transfection as described above were seeded at a concentration of 1,000 cells/well in 100 μl of culture medium and treated with 0.1 % ethanol, 10 nM E2, 100 nM Tam or 100 nM ICI. Cell proliferation was assessed using a WST-1 kit according to the manufacturer's instructions (Roche Applied Science). The absorbance of the samples was analyzed using a microplate ELISA reader at 450 nm.

2.4 Results

Generation and Characterization of ERα phospho-mutant.

To address the combinatorial role of phosphorylation at multiple serine sites on ER activity, we designed mutant ERs that mimic specific phosphorylation states. We introduced the combinatorial changes at known ER phospho-sites, namely Ser-104, 106, 118, 167, 236, and 305. We mutated each site into an alanine to mimic lack of phosphorylation, or into a glutamic acid to mimic a constitutively phosphorylated state. To reduce the number of possible combinations, we grouped the first three functionally related [47-49] serine sites (targeted by the

serine/proline-directed protein kinases), Ser-104, 106, 118, into a single coordinated mutant (e.g. all three Ala or Glu). The 16 mutant ERs that were generated thus contained coordinate changes at Site I (the three MAPK sites, Ser-104, 106, 118), plus individual changes at Site II (Ser-167), Site III (Ser-236), and Site IV (Ser-305) (Figure 2.1A). In order to characterize these ER mutants, we first transfected ER negative HEC-1 (and U2OS) cells and, by Western blot analysis, we found that all the ER mutants were expressed similarly indicating that there was no apparent effect on the protein synthesis by the combinations of the mutations (Figure 2.1B). We then transiently transfected ERα mutants in HEC-1 (and U2OS) cells together with 2xERE reporter gene construct (2xERE-pS2-luciferase) to check if the ER mutants showed different transactivation responses (Figure 2.1C). The most striking observation was the fact that the alternation of activity occurs, in most cases, in pairs of mutant ERs. This is consistent with an overall high activity when Site III (Ser-236) is mutated to generate A and low activity when Site III is mutated to generate E. By contrast, changing Site IV (Ser-305) to either E or A had no effect on their transcriptional activity, as evident from the equivalent activity of adjacent pairs of ER mutants. The effect of Site II depended on the status of Site I. When Site I (Ser-104, 106, 118) was mutated into an A, the only effects on transcriptional activity was determined by Site III, where activity alternates between wild type (A) and essentially null (E). By contrast, when Site I was mutated into an E, Site II showed major effects on receptor activity (alternating between superactive and null), provided that Site III was mutated as As. When Site III is a glutamic acid, however, the pronounced modulatory effect of Site II was eliminated, always resulting in receptors with low activity.

Generation of U2OS-ERα stable cell lines and tetracycline-induced ERα expression.

Based on these findings, we chose five ERα mutants for further characterization. We then generated tetracycline inducible stable U2OS-ERα Wt or mutant cell lines. The chosen ERα mutants and their abbreviations are shown in Figure 2.2A. Different amounts of tetracycline were used to obtain comparable level of expression of Wt and mutant ERs in each stable cell line, and this was confirmed by Western blot analysis (Figure 2.2B).

The Ee mutant requires a high concentration of E2 to respond to ligands.

We first wanted to check if phospho-mutant ERs showed alterations in sensitivity to E2. For this purpose we conducted E2 dose response studies in HEC-1 cells using a 2xERE reporter gene construct. As shown in Figure 2.3A, we could subdivide the ER mutants into 3 groups; group 1 was comprised of a single mutant (Ee) which showed a right-shift in the dose-response curve indicating less potency but more efficacy than Wt in transient transactivation activity. Phosphorylation at Ser-236 which is mimicked by an glutamic acid substitution at Site III, has been reported to alter receptor dimerization and DNA binding [50], and we also saw that mutants containing a glutamic acid at that position lacked transcriptional activity (group 3). Group 2 was comprised of the mutants containing an alanine at Site III showing the similar pattern of dose responses with that of Wt. We then checked if similar responses were occurring also in the U2OS-ERα Wt or mutants stable cell lines using either a 2xERE luciferase reporter assay (data not shown) or quantitative PCR analysis measuring the induction of the endogenous pS2 mRNA (Figure 2.3B). Also in these experiments, the ERα-Ee showed a right-shifted curve of E2 response in both assays as 1 μM E2 was able to fully restore E2 responsiveness; while the mutant Eaea, that presumably has a defect in DNA binding due to the glutamic acid substitution at site III, did not show any activity even at high concentration of E2. We then checked, by ChIP assay,

if ERα recruitment to the pS2 promoter (-300 bp) also followed the same pattern as gene expression, and we found it to be the case as we could detect recovery of ER recruitment at high E2 concentration for the Ee mutant and no recruitment for the Eaea mutant (Figure 2.3C). We also determined if the protein half-life was different between ERα-Wt and Ee, and thus we performed a tetracycline-removal experiment and found that, for ERα Wt, the receptor half-life was decreased by E2 treatment from 5 hr to 3 hr independently of E2 concentration whereas only the high concentration of E2 (1 μM) was able to induce E2-mediated protein degradation of mutant Ee receptor (Figure 2.3D). Because of the differences in receptor degradation and transcriptional activity, we examined if the ER mutants would bind E2 or other ligands with altered affinity. Because mutation of Site IV (Ser-305) had no effect on ER transcriptional activity in reporter gene assays, we examined E2 binding of the eight ER mutants that contained an alanine at Site IV. As shown in Table 1, generally the changes in E2 binding affinity were relatively small, ranging, compared to wild type ERα, from ca. 2-fold higher to a 4-fold lower affinity. The Ee mutant showed the lowest estradiol binding affinity with a K_d that was 4 fold higher than the wild type receptor. This might at least partially explain the need for higher E2 concentration in order to elicit full transcriptional response of the Ee mutant. Relative binding affinities (RBAs) of 4-hydroxytamoxifen (Tam) and ICI 182,780 (ICI) were also analyzed, and only the mutants AAAEAA (Ae) and EEEEEA showed statistically different Tam and ICI RBAs (Table 2.1).

The ERα-Ee mutant shows altered transcriptional response to Tam and ICI, and reduced ICI-mediated protein degradation.

To understand the effects of SERMs/SERD on the mutant receptor Ee, the mRNA induction of well-known E2-mediated ERα target genes GREB1 and PDZK1 was measured with quantitative PCR in a comparison between U2OS-ERα Wt and Ee cells. Agonistic and antagonistic effects of Tam and ICI were tested by treating the cells with Tam or ICI with or without E2. As the mutant Ee were shown to have lower E2 binding affinity, 1 μM E2 was treated to elicit the E2-induced gene expression in U2OS-ERα Ee cells. The agonistic effects of Tam were also seen only with the higher concentration (5 μM) in ERα-Ee cells, although the Tam RBA of the mutant Ee was not significantly different from that of Wt. In ERα-Ee cells, Tam and ICI had weaker antagonistic effects on E2-induced transcriptional activation than in ERα-Wt cells. However, Tam agonistic effects were carried to levels comparable to Wt except that the Ee mutant needed higher concentration of Tam to be effective (Figure 2.4A). Notably, ICI-induced antagonism of E2 action was highly suppressed in ERα-Ee cells, driving the question whether ICI-induced ERα degradation is affected or suppressed in the Ee mutant. ERα protein levels were, therefore, compared after 4 hr treatment of E2, Tam or ICI in U2OS stable cells expressing ERα-Wt or Ee. Lesser degradation of ERα protein mediated by ICI with or without E2 was detected in ERα-Ee cells, and appeared to follow the reduction of ICI-induced antagonism on the Ee mutant ER (Figure 2.4B).

Differential antagonistic responses to Tam and ICI in ERα-Ae and Ea mutants.

Because of increasing evidence from clinical studies that the phospho-status of ERα is highly correlated with patients' response to endocrine therapies in breast tumors [32, 36, 51, 52], we studied the effects of SERMs/SERD using the ERα phospho-mutants Ae and Ea, which have opposite combinations of phosphorylation mutations at Sites 1 and II ,mimicking the

phosphorylation status observed by Yamashita *et al*. in primary tumors [36]. We first treated U2OS-ERα Wt, Ae, and Ea stable cell lines with 10 nM E2 alone or in combination with 100 nM Tam or 100 nM ICI and checked their effect on known ERα target genes by QPCR. The ERα-Ae mutant showed ca. 50% of Wt receptor activity in terms of E2 transcription and ER recruitment at both pS2 and GREB1 genes. In this mutant cell line, Tam acted as a full antagonist thus losing any partial agonistic effect. No effect was seen on ICI antagonistic effect. In contrast, the Ea mutant displayed a very strong Tam agonist response and Tam failed to subdue E2 action, both by QPCR and ChIP assays (Figure 2.5).

ERα phospho-mutants Ae and Ea elicit differential antagonism by Tam in MCF-7 cells.

Because of the differential responses to Tam by the ERα phospho-mutants in the U2OS stable cell lines, we wanted to confirm this finding in MCF-7 cells which is a widely used ERα positive cell model to study breast cancer biology and endocrine sensitivity and resistance. Because MCF-7 cells express endogenous ERα, we devised an siRNA approach by targeting the 3' UTR of ERα, which is missing from the ERα sequence used to generate phospho-mutants in the adenoviralvector expression system. Using this method, we were able to knock-down endogenous ERα by siRNA transfection in MCF-7 cells, and successfully re-express ERα Wt, Ae and Ea mutants using adenoviral infection. As shown in Figure 2.6A, we were able to express Wt and mutant ERs to levels similar to the endogenous ERα in MCF-7 cells.

We then assessed regulation of gene expression and recruitment of ERα and cofactors by E2 and Tam treatment in MCF-7 cells. MCF-7 cells were transfected with non-targeting (NT) or ERα 3'-UTR siRNAs, and then transduced with or without adenoviruses carrying ERα-Wt or mutants, followed by E2 and Tam treatment for 24 hr. We confirmed that the responses with

ligand treatment in adenovirus-carrying cells were exerted by ectopically introduced ERs by comparing with negative control samples (NT or siERα without adERα). As shown in the experiments with U2OS-ERα Ae and Ea cells, MCF-7 cells expressing ERα-Ea showed higher pS2 and GREB1 mRNA induction by Tam without altering E2 agonistic effects compared to siERα+Wt cells (Figure 2.6B). We then analyzed ERα recruitment to the pS2 ER-binding site in the promoter region. Similarly to what we observed in U2OS-ERα stable cell lines (Figure 2.5), Tam elicited a full agonistic response on ERα recruitment in the Ea mutant compared to the Ae and Wt receptors (Figure 2.6C). Chromatin recruitment of cofactors that are known to interact with ERα (e.g. SRC-3 or p300) was also examined in an effort to explain the differential tamoxifen-mediated responses in gene expression between two phospho-mutant ERs. The ERα coactivator SRC-3 did not show any differential pattern of recruitment to the ER-binding sites by E2 or Tam in the mutant ERα expressing cells compared to Wt (data not shown). However, the histone acetyltransferase p300 was recruited to the pS2 promoter region by the mutant Ea at an increased level compared to ERα-Wt after Tam treatment, providing a possible mechanistic explanation to the increased tamoxifen agonism of the ERα phospho-mutant Ea (Figure 2.6D).

Increased Tamoxifen-induced cell growth of MCF-7 cells expressing the ERα-Ee mutant.

To examine the role of the Ea mutant in Tam agonism, we performed cell proliferation assays in MCF-7 cells expressing exogenous ERα Wt or mutants Ae or Ea treated 10 nM E2, 100 nM Tam, 100 nM ICI or Tam plus ICI for four days. As shown in Figure 2.7, Tam stimulated the growth of Ea cells more, compared to the Wt or Ae cells, whereas E2-mediated growth was similar for all the three ER types. These results strongly suggest that the phospho-combination of ERα mimicking high phosphorylation at Ser-118 and low phosphorylation at

Ser-167, which is exemplified by the ERα-Ea mutant, could be a cause of endocrine resistance due to diminished antiestrogenic capabilities accompanied with an increased cofactor recruitment to E2-responsive genes, resulting in the aberrant cell proliferation in response to tamoxifen.

2.5 Discussion

ERα is a key transcription factor that controls breast cancer biology. In fact, the majority (70 %) of primary human breast cancers are ERα positive, and ERα expression in breast tumors is usually an indication of good prognosis [53]. Even though endocrine therapy with selective estrogen-receptor modulators (SERMs) or selective estrogen-receptor downregulators (SERDs) are very important treatment options for women with ER-positive breast cancer, the development of resistance to endocrine therapies is also prominent and results in the progression of the disease [24, 25]. Overexpression of tyrosine kinases such as HER2 or EGFR is thought to contribute to the development of endocrine resistance [28, 29], and the activation of downstream signaling effectors results in phosphorylation of ERα and its cofactors leading to the abnormal regulation of ERα target gene expression [27, 54].

In this study, we developed a systematic approach to study the impact of the combinatorial effect of phosphorylation sites on ERα activity, by generating a series of ERα phospho-mutants and analyzed them in both ER-negative (U2OS) and -positive cell lines (MCF-7). We generated sixteen ERα phospho-mutants by site-directed mutagenesis altering Ser-104, 106,118, 167, 236 and 305 either to an alanine, to mimic lack of phosphorylation, or a glutamic acid, to mimic constitutively phosphorylated state. By using reporter gene assays, we identified different groups of receptor mutants; group1 was comprised of a single mutant (Ee) which showed a right-shift in the dose-response curve indicating less potency but more efficacy than

Wt in both transient transactivation activity and pS2 endogenous gene expression, accompanied with ER recruitment only at high E2 concentration to the pS2 ER-binding site. Group 2 was comprised of the mutants containing an alanine at Site III showing the similar pattern of E2 dose responses with that of Wt. We saw that mutants containing a glutamic acid at Site III lack transcription activity as well as ER recruitment (group 3) presumably due to the hindered receptor dimerization and DNA binding. We also found that the Wt receptor half-life was decreased by E2 treatment independently of the concentration whereas only the high concentration of E2 (1 μM) was able to induce E2-mediated protein degradation of mutant Ee receptor. In fact, the Ee mutant showed lower estradiol binding affinity than the Wt receptor, explaining the need for higher E2 concentration in order to elicit full transcriptional response of the Ee mutant.

We examined agonistic and antagonistic effects of SERMs/SERD on the ER phospho-mutants, and saw that ICI-induced antagonism of E2 action was highly suppressed in ERα-Ee cells, and this corresponded with less degradation of ERα protein mediated by ICI with or without E2 in ERα-Ee cells. Because the combination of high S118P/low S167P was shown to be more resistant to tamoxifen in primary breast cancer [36], we focused on the mutants ERα-Ae and ERα-Ea which mimic the phosphorylation status in their mutation. Tam acted as a full antagonist, thus losing any partial agonistic effect on the Ae mutant. This observation might be providing an explanatory mechanism on a molecular level of the better response to endocrine therapy in patients with breast tumors showing low phosphorylation at Ser-118 and high phosphorylation at Ser-167 of ERα. In contrast, the Ea mutant mimicking high S118P/low S167P displayed a very strong Tam agonist response which failed to subdue E2 action, both in terms of E2 transcription and ER chromatin recruitment. Similarly to what we observed in

U2OS-ERα stable cell lines, MCF-7 cells expressing ERα-Ea showed higher pS2 and GREB1 mRNA induction by Tam without altering E2 agonistic effects compared to siERα+Wt cells, and Tam also exerted a full agonistic response on ERα recruitment. The role of cofactors in altering the responses to SERMs through their dynamic interaction with ERα is suggested in several cancer environments such as uterus carcinoma or osteoblastic cell lines [55, 56]. The histone acetyltransferase p300, which is a known ERα cofactor, was recruited to the pS2 promoter region by the Ea mutant at an increased level compared to ERα-Wt after Tam treatment, demonstrating the increased tamoxifen agonisim of the Ea mutant. This result provides an example of how the altered tamoxifen response of the ERα phospho-mutants is regulated by the preferential use of cofactors according to the phospho-code of ERα. We also observed an increased tamoxifen-induced growth of MCF-7 cells expressing the ERα-Ee mutant, confirming the diminished antiestrogenic capabilities of tamoxifen on the Ea mutant receptor, accompanied with an increased cofactor recruitment by Tam. These observations strongly suggest that the phospho-combination of ERα mimicking high phosphorylation at Ser-118 and low phosphorylation at Ser-167, which is exemplified by the ERα-Ea mutant, could be a cause of endocrine resistance and, hence, contribute to the aggressiveness and recurrence of breast cancer.

In this study, we have demonstrated that post-transcriptionally modified ERα exhibited altered gene regulation, affinity for ligands and stimulation of cell proliferation according to their phospho-code. Also the regulation of ERα activity resulted from the combination of PTMs on multiple residues. In conclusion, the changes in the combinatorial phosphorylation status of ER greatly impact receptor function and show differential SERM and SERD activity that could contribute to resistance to endocrine therapies in breast cancer. Understanding the potential mechanism of differential SERMs/SERD-modulated cellular responses driven by the phospho-

status of ERα could give new strategies in endocrine therapy for many different types of breast tumors in terms of their ERα phosphorylation status.

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2.7 Figures and Tables

Figure 2.1

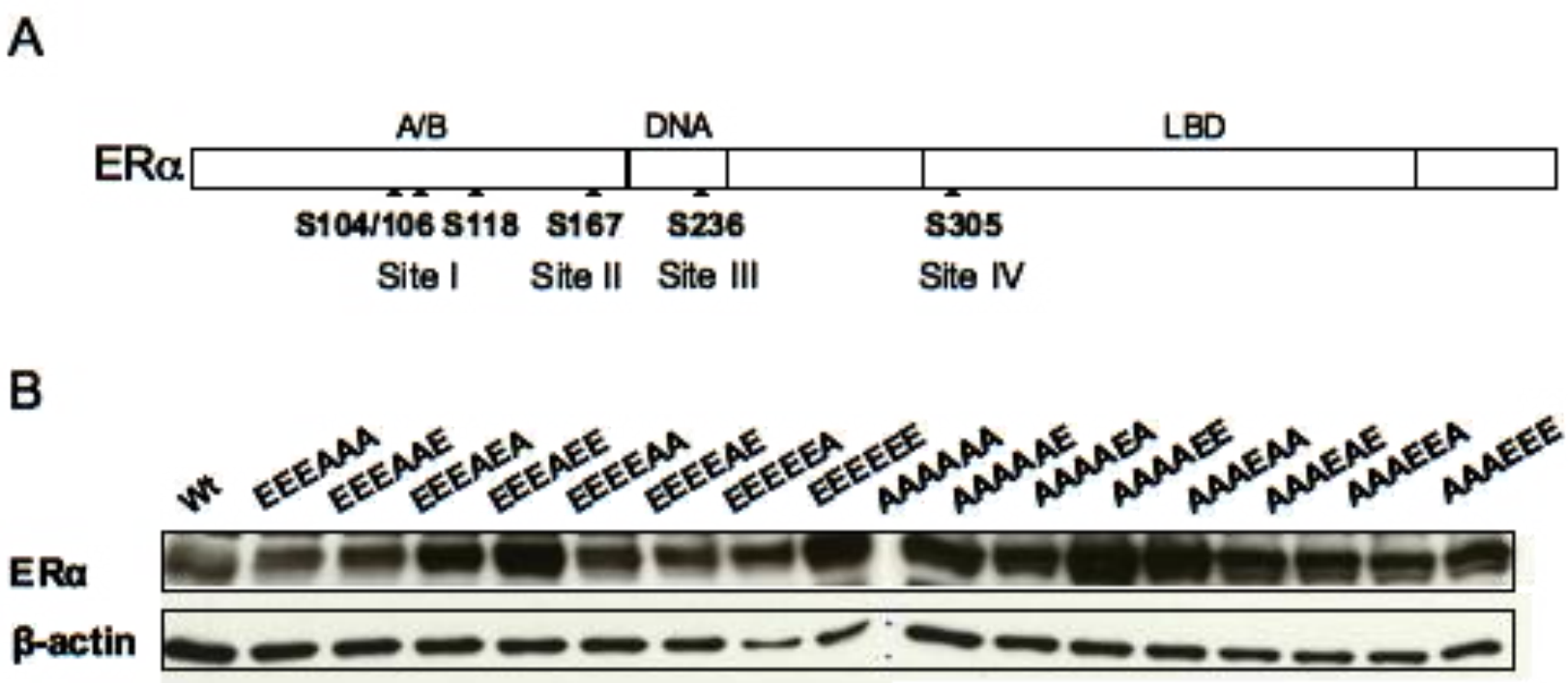

Figure. 2.1 (cont.)

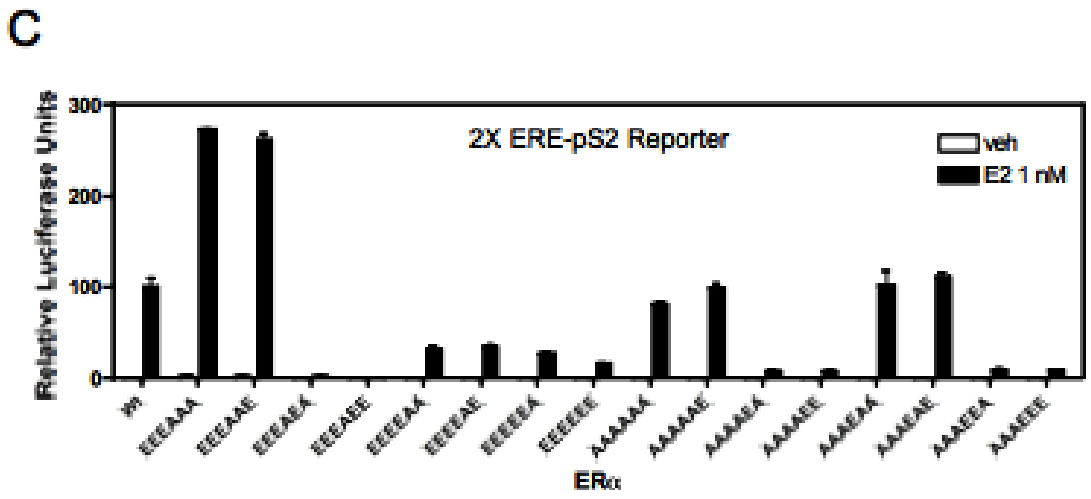

Figure 2.1 Generation and characterization of ER phospho-mutants.

(A) Schematics of the location of serine sites in ERα that have been mutated in this study.
(B) Wild type (Wt) and mutant ERs were transfected into HEC-1 cells. Whole cell extracts were prepared and analyzed by Western immunoblotting with ERα-specific antibody. β-actin was used as a loading control.
(C) HEC-1 cells were transfected with pCMV5-Flag-ERα (Wt or mutants) and 2xERE-pS2-luciferase reporter gene. Luciferase assay activity was measured after 24 hr treatment with 0.1 % ethanol vehicle (veh) or 1 nM E2, and normalized to β-galactosidase. Data are expressed as relative luciferase units (RLU) with ERα wild type set as 100 ± SD of triplicate samples.

Figure 2.2

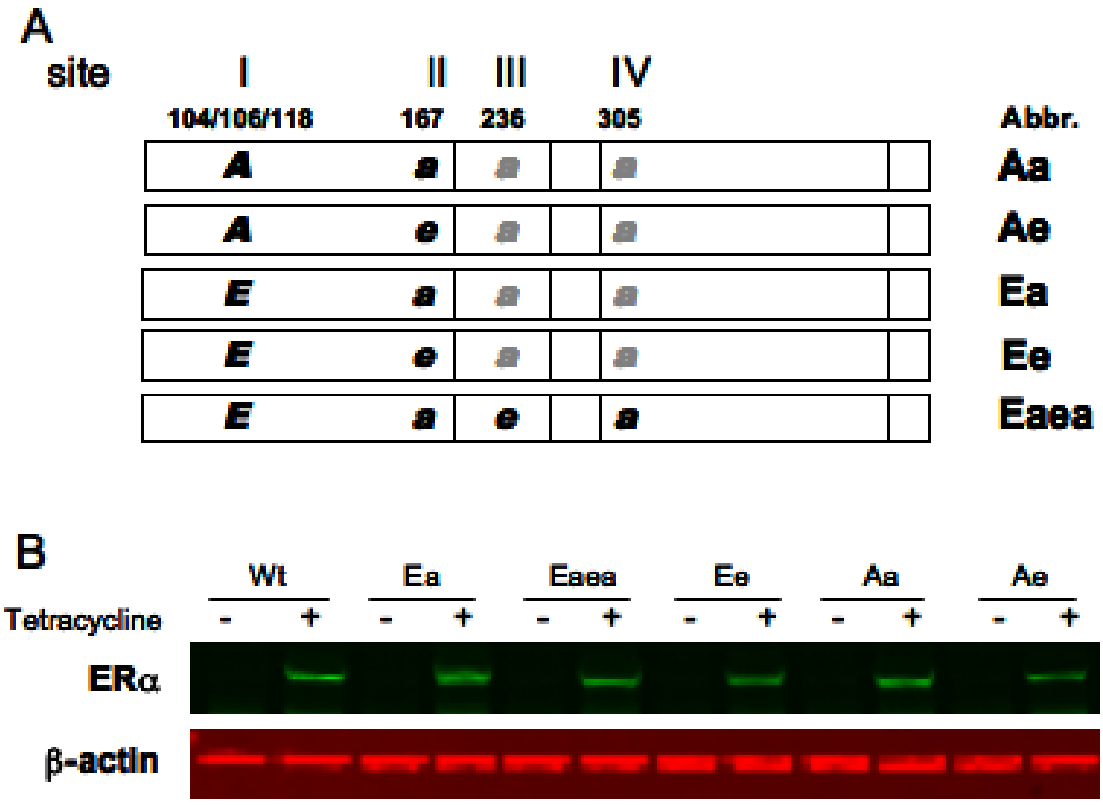

Figure 2.2 Generation of U2OS-tetracycline regulated-ERα stable cell lines.

(A) Mutations were made at Ser-104/106/118 (site I) and at the other sites (site II, III and IV, respectively) by site-directed mutagenesis. Mutations introduced at site I are represented as A (three alanines) or E (three glutamic acids), and those at the other sites are represented as a (alanine) or e (glutamic acid). Abbreviations for the mutants are the combination of site I and II, except for the mutant Eaea.
(B) Induction of ER protein expression in U2OS-ERα stable cell lines by tetracycline was verified by western blotting analysis with ERα-specific antibody. β-actin was used as a loading control.

Figure 2.3

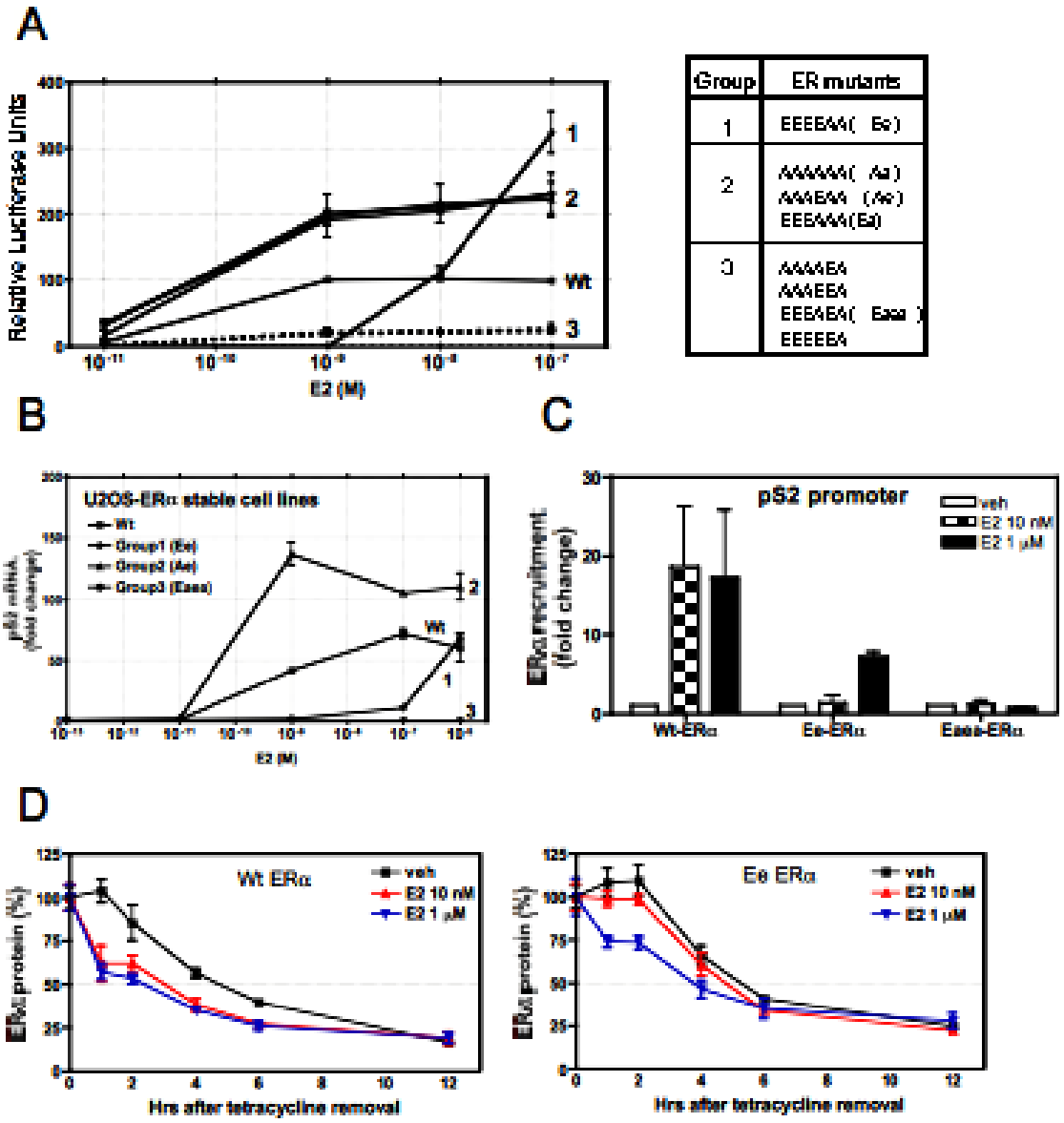

Figure 2.3 Transactivation activity of ERα Wt and mutants in transient or stable expression systems, and E2-mediated gene expression, ERα recruitment and receptor turnover of ERα-Ee.

(A) HEC-1 cells were transfected with pCMV5-Flag-ERα (Wt or mutants) constructs. Receptor transcriptional activities were measured by luciferase assay after 24 hr treatment with five concentrations of E2 (10^{-11}, 10^{-10}, 10^{-9}, 10^{-8} and 10^{-7} M), and were normalized to β-galactosidase. Data are expressed as relative luciferase units (RLU) with ERα Wt 1 nM E2 set as 100 ± SD of

Figure 2.3 (cont.)

triplicate samples. The ERα phospho-mutants are categorized into three groups (Group 1, 2 and 3) based on their E2 dose response patterns.
(B) Transcription level of endogenous pS2 gene in U2OS-ERα stable cell lines (Wt, Ee, Ae, and Eaea) was assessed by QPCR after 24 hr treatment with E2 in concentrations indicated following ERα induction by tetracycline for 24 hr. Data represent average fold change ± SD of triplicate samples.
(C) ERα recruitment to the pS2 ER-binding site was measured by qPCR after 45 min treatment with 0.1 % ethanol vehicle (veh) or E2 (10 nM or 1 mM) following ERα induction by tetracycline for 48 hrs. Data are expressed as fold change normalized to wild type veh ± SD of triplicate samples.
(D) ERα protein levels were measured by In-Cell Western assay at indicated times after tetracycline removal by changing media containing 0.1 % ethanol vehicle (veh) or E2 (10 nM or 1 mM). ERα Wt or Ee expression in U2OS-ERα stable cells was induced by tetracycline for 48 hrs prior to the media change. Data are expressed as ERα level at 0 hr set as 100 ± SD of triplicate samples.

Figure 2.4

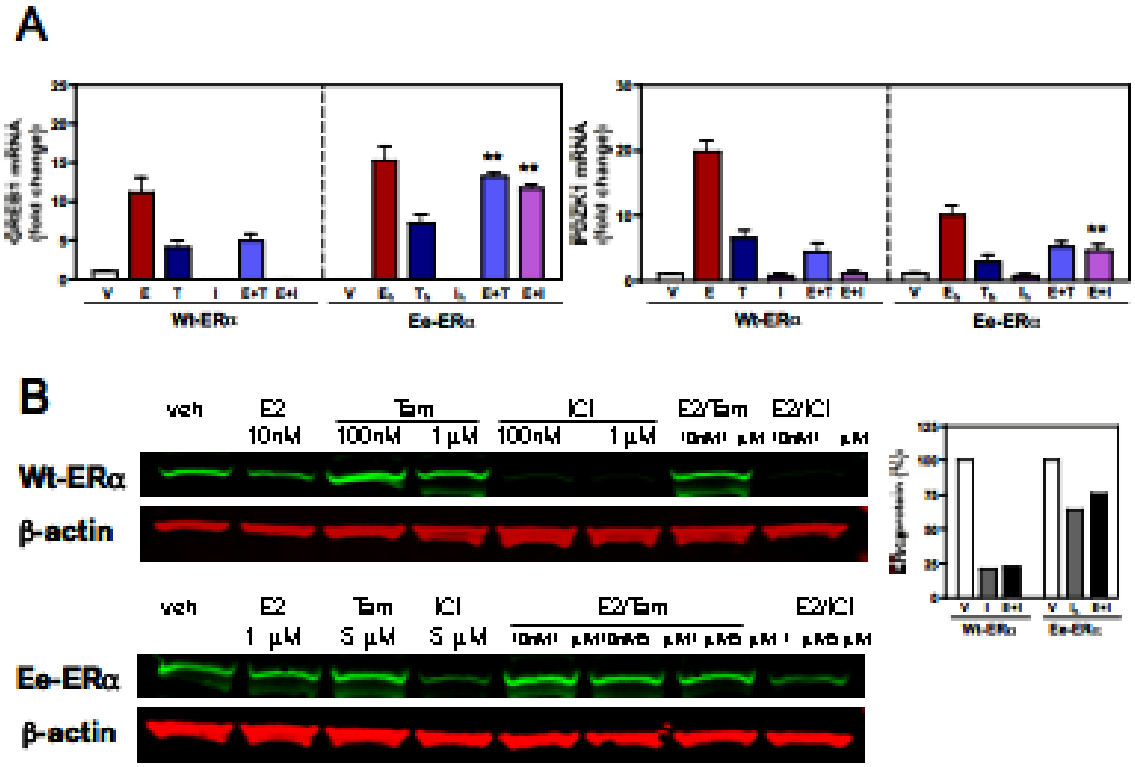

Figure 2.4 The ERα-Ee mutant shows altered mRNA induction in response to Tam and ICI and reduced ICI-mediated protein degradation.

(A) Induction of GREB1 and PDZK1 genes in U2OS-ERα Wt or Ee stable cell lines was assessed by qPCR after 24 hr treatment with 0.1 % ethanol vehicle (V), 10 nM E2 (E), 1 mM E2 (E_h), 100 nM Tam (T), 5 mM Tam (T_h), 100 nM ICI (I) or 5 mM ICI (I_h) following ERα induction by tetracycline for 24 hrs. Data represent average fold change ± SD of triplicate samples. ***P*-value < 0.01 compared to Wt.
(B) ERα protein levels in U2OS-ERα Wt or Ee stable cells after 24 hr treatment with 0.1 % ethanol vehicle (veh), E2, Tam or ICI of indicated final concentrations following ERα induction by tetracycline for 24 hr. β-actin was used as a loading control. The quantification of ERα protein is represented as veh set as 100 %.

Figure 2.5

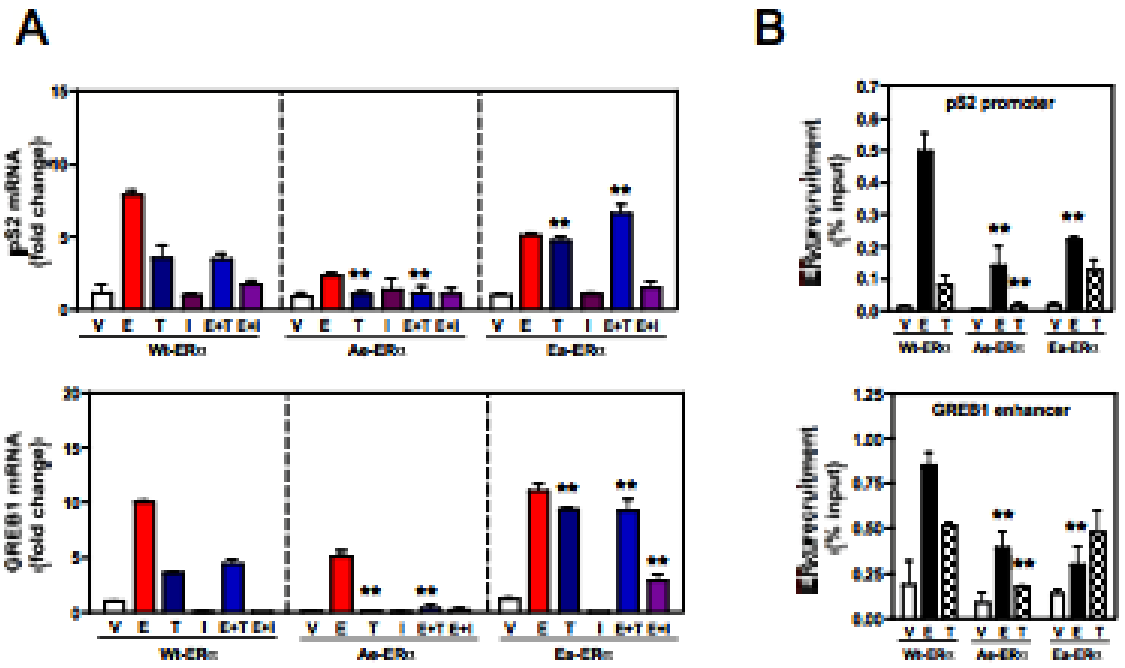

Figure 2.5 The ERα-Ae and -Ea mutants show differential antagonistic response to Tam and ICI.

(A) Induction of pS2 and GREB1 genes in U2OS-ERα Wt, Ae or Ea stable cell lines was assessed by QPCR after 24 hr treatment with 0.1 % ethanol vehicle (V), 10 nM E2 (E), 100 nM Tam (T) or 100 nM ICI (I) following ERα induction by tetracycline for 24 hr. Data represent average fold change ± SD of triplicate samples and normalized to Wt vehicle. **P-value < 0.01 compared to Wt.
(B) Differential ERα recruitment to the pS2 and GREB1 ER-binding sites in U2OS-ERα Wt, Ae or Ea stable cell lines was measured by QPCR after 45 min ligand treatment with 0.1 % ethanol vehicle (V), 10 nM E2 (E) or 100 nM Tam (T) following ERα induction by tetracycline for 48 hr. Data are expressed as percentage of input ± SD of two separate experiments. **P-value < 0.01 compared to Wt.

Figure 2.6

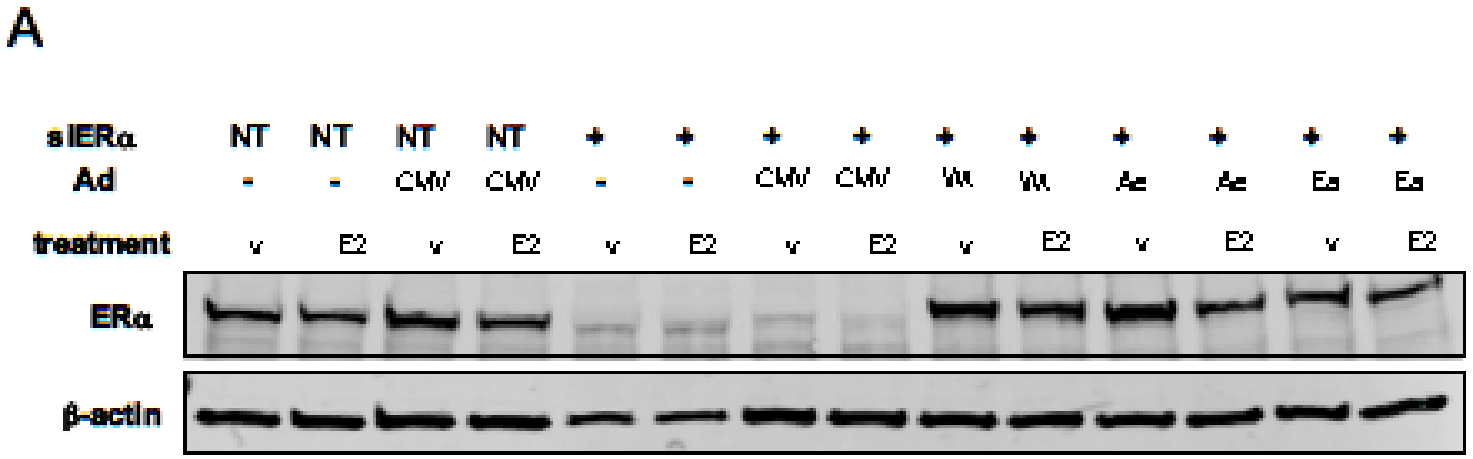

Figure 2.6 (cont.)

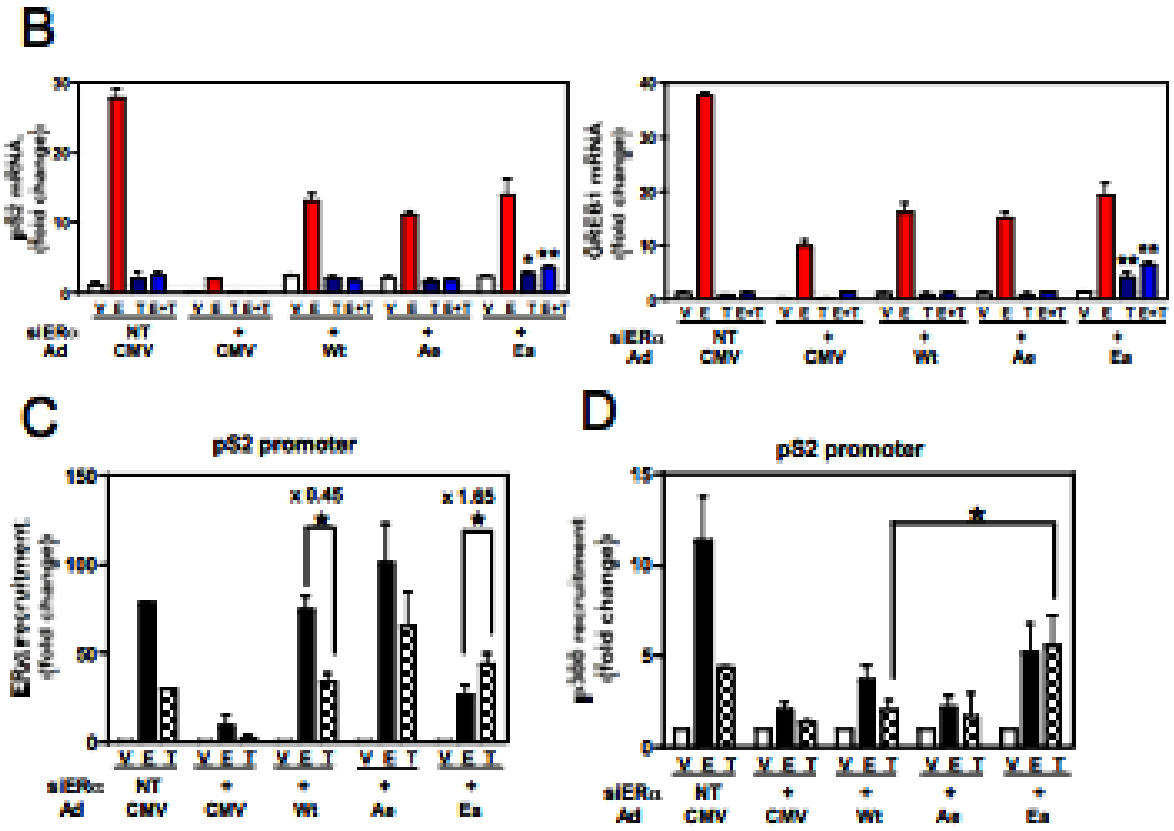

Figure 2.6 ERα-Ae and -Ea elicit differential antagonistic effects by Tam in MCF-7 cells.

(A) MCF-7 cells were transfected with 5 nM non-targeting control siRNA (NT) or ERα 3'-UTR siRNA for 72 hr then transduced with control adenovirus (CMV) or ERα adenoviruses (Wt, Ae or Ea) for 64 hr. Whole cell extracts were prepared and analyzed by western immunoblotting with ERα-specific antibody in order to verify the expression of AdERα-Wt and mutants after knock-down of endogenous ERα. β-actin was used as a loading control.
(B) The induction of pS2 and GREB1 genes after 0.1 % ethanol vehicle (V), 10 nM E2 (E), 1 mM Tam (T) or E2 plus Tam (E+T) treatment for 24 hr was assessed in MCF-7 cells by qPCR following the same procedure of siRNA transfection and adenovirus transduction as described above. Data represent average fold change ± SD of triplicate samples and normalized to NT CMV vehicle. *P-value < 0.05 compared to Wt. **P-value < 0.01 compared to Wt.
(C) Differential ERα recruitment and (D) p300 recruitment to the pS2 ER-binding site were measured by QPCR after 45 min treatment with 0.1 % ethanol vehicle (V), 10 nM E2 (E), 100 nM Tam (T). Data are expressed as fold change ± SD of three separate experiments normalized to each corresponding vehicle. * P-value < 0.05.

Figure 2.7

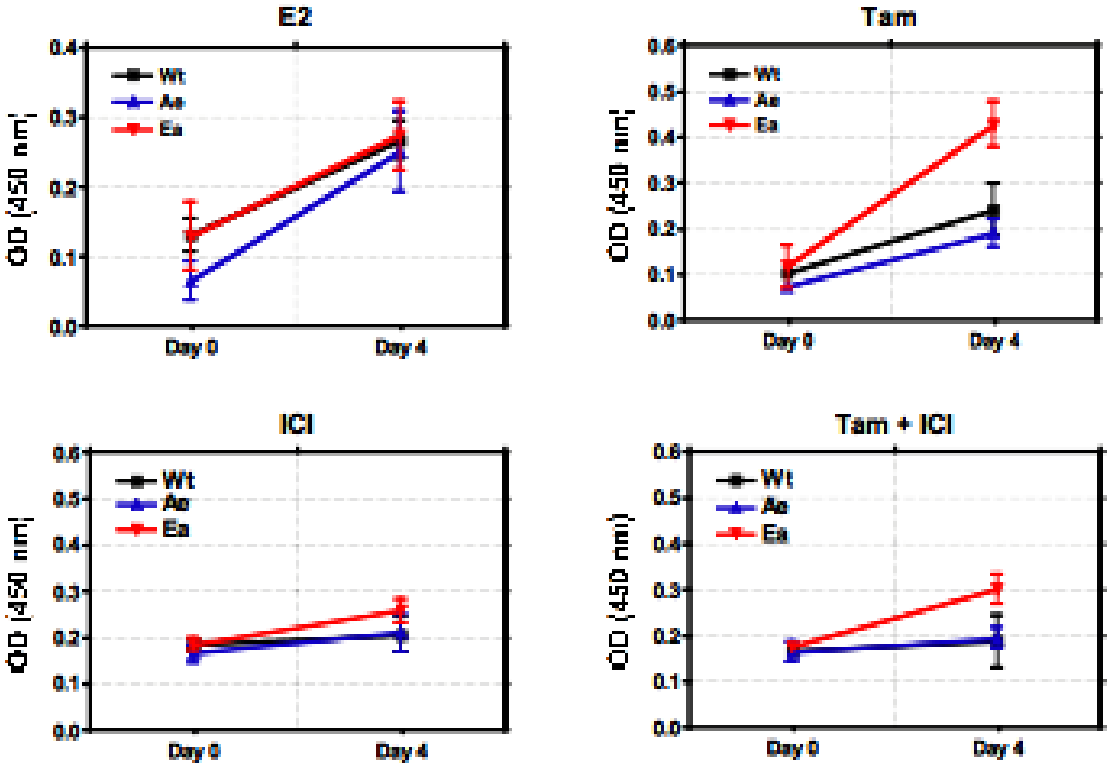

Figure 2.7 Increased growth of MCF-7 cells expressing the ERα-Ee mutant in response to tamoxifen, but little change in response to E2 or ICI.

MCF-7 cells expressing AdERα-Wt, Ae or Ea were treated with 10 nM E2, 100 nM Tam, 100 nM ICI or Tam plus ICI for 4 days. Cell proliferation was assessed using WTS-1 kit as described in Materials and Methods. The data represent the means ± SD of four determinations.

Table 2.1

ERα	Wt	EEEAAA	EEEAEA	EEEEAA	EEEEEA	AAAAAA	AAAAEA	AAAEAA	AAAEEA
E2 binding K_d (nM)	0.85	0.8	2.5**	3.18**	0.26	0.43	0.37	0.62	0.58
RBA E2 (%)	100	100	100	100	100	100	100	100	100
RBA Tam (%)	122	180	165.5	169.5	170	132	144	207*	160
RBA ICI (%)	28.9	32.7	40	28.0	60.5**	32.4	47.0	36.1*	46.9

* *P*-value < 0.05 compared to Wt.
** *P*-value < 0.01 compared to Wt.

Table 2.1 (ccont.)

Table 2.1 Estradiol (E2) binding affinity ($K_{d)}$ of ERα Wt and eight phospho-mutants and their relative binding affinities for Tam and ICI.

HEK 293FT cells were transfected with 8 μg of pCMV5-Flag-ERα wild type or mutant constructs. Nuclear extracts were prepared and incubated with a range of ^{3}H-E2 concentrations alone or with 100-fold excess unlabeled E2 for 3 hrs. Aliquots were used to determine the total ^{3}H-E2 in the sample. Hydroxylapatite slurry was added, and its radioactivity then determined by scintillation counting. K_d is measured as a constant for dissociation. Relative binding affinities were determined by competitive radiometric binding assays using 10 nM ^{3}H-E_2 as tracer. The binding affinities are expressed as relative binding affinity (RBA) values, where the RBA of estradiol is set as 100 %. The data are from two independently processed assays.

CHAPTER 3

Regulation of ERα-mediated Gene and Protein Expression by Estradiol and Growth Factors through Alterations in miRNAs

3.1 Abstract

The association of miRNAs in cancer biology is of great interest, especially in regards to their regulatory functions in proliferation, differentiation and survival of tumor cells. We investigated the regulatory role of miRNAs in breast cancer cells, focusing on their impact on ERα-mediated gene expression by estradiol (E2) and growth factors. The aim of this project is to study the regulation of miRNAs by E2 and growth factors through ERα and ERK2, and to understand the physiological impact of select miRNAs on breast cancer by target gene regulation (i.e. TP63). We identified nine miRNA-encoding genes harboring overlapping ERα and ERK2 binding sites in a 50 Kb window around their transcription start sites in MCF-7 cells. ERα and ERK2 were shown to directly bind to the overlapping binding sites near the E2-upregulated miRNAs (miR-135a2, miR-196a2, miR-101 and miR-190) and to be required for transcriptional induction of these miRNAs as well as for E2-mediated miRNA regulation. We also identified TP63, a target gene of miR-101, miR-190 and miR-196a2, and showed that TP63 plays an important role in E2- or growth factor-mediated cellular response in breast cancer cells by increasing tumor cell growth and *in vitro* invasion mainly controlled by miR-196a2 action. We observed that the expression of ΔNp63α was correlated inversely with ERα levels in several breast cancer cell lines, and showed that *in vitro* invasion and tumor growth properties were reversed by enforced miR-196a2 expression in ERα negative MDA-MB-231 cells. These results imply that the regulation of miR-196a2 by ERα and/or ERK2 signaling in breast cancer is

associated with different molecular subtypes of breast cancer, possibly mediated through differential TP63 expression affecting tumor cell growth and invasion ability. The findings suggest a potential tumor-suppressive treatment strategy to alleviate the aggressive behavior and poor prognosis of the ERα-negative basal-like breast cancer subtype by manipulating the miR-196a2-TP63 circuit in these breast cancer cells.

3.2 Introduction

MicroRNAs (miRNAs) are a class of 21-23 nucleotide-long noncoding RNAs that modulate gene expression by post-transcriptional repression [1-3]. miRNAs have emerged as an important focus of research in molecular biology following the initial identification of two small noncoding RNAs, *lin-4* and *let-7*, and their regulatory roles in timing of the nematode worm *Caenorhabditis elegans* development [4-6]. Many more have since been found in animals, plants and fungi, reflected with 677 human miRNA sequences detailed as of September 2008 in the Miranda database (www.microrna.org), and bioinformatics predictions indicate that mammalian miRNAs can regulate ~30% of all protein-coding genes [2, 7]. miRNAs can affect both the translation and stability of mRNAs by their sequence complementarity to the 3' UTR of the target genes in the cytoplasm [2, 8]. However, additional functions of miRNAs are possible, for they could regulate pre-mRNA processing in the nucleus or act as chaperones modifying mRNA structure or modulating mRNA-protein interactions [9].

The association of miRNAs in cancer biology is of great interest because of their regulatory functions in proliferation, differentiation and apoptosis [10-12]. Because of miRNAs' broad influence over diverse genetic pathways, the alteration in miRNA expression is likely to be pleiotropic and contribute to disease, including cancer [13, 14]. miRNA expression correlates

with various cancers, and these are thought to function as both tumor suppressors and oncogenes [13]. High-throughput miRNA expression profiling in breast cancer cell lines and tissues identified a large set of miRNAs expressed at different levels compared to the normal breast [15-17]. miRNA signatures predicting the expression levels of the estrogen, progesterone and HER2/*neu* receptors, which characterize different breast cancer phenotypes, have also been identified to elucidate the role for these miRNAs in disease classification of breast cancer and also serving as a prognostic biomarker [18]. In the study by Iorio *et al.* (2005), miR-125b, miR-145, miR-21, and miR-155 were significantly deregulated, and the expression of those miRNAs were correlated with specific breast cancer pathologic features such as estrogen and progesterone receptor expression, tumor stage or proliferation index [17]. Another group also identified four miRNAs (miR-7, miR-128a, miR-210 and miR-516-3p) associated with aggressiveness of lymph node-negative, estrogen receptor-positive human breast cancer [19]. Also, a functional genetic variant in the mature region of miR-196a2 and its potential oncogenic role in breast tumorigenesis was identified by functional analysis [20]. More interesting, many studies have reported the regulation between miRNAs and estrogen receptor alpha (ERα) signaling and its impact on endocrine resistance in breast cancer. miRNA-221/222 expression was up-regulated in ERα-negative breast cancer and conferred tamoxifen resistance by targeting ERα and the cell cycle inhibitor $p27^{Kip1}$ [21, 22]. miR-206 expression was down-regulated in ERα-positive breast cancer and repressed ERα mRNA and protein synthesis [23, 24]. It was also revealed that estradiol can regulate miRNA expression (i.e. miR-21) and modulate target gene expression in breast cancer cells [25, 26]. The ratio of the miRNA level to its target transcript was also taken into consideration and showed a correlation with breast cancer cell migration and metastasis by the miR-196 family and HOXC8 [27].

TP63, a member of p53 tumor-suppressor gene family acts at least in part as oncogenic and tumor suppressive genes in human cancer [28-31], and plays roles in tumor growth, apoptosis and metastasis of human cancer [28, 31, 32]. The translational products of TP63 are crucial for the maintenance of a stem cell population in the human epithelium [33] and are necessary for the normal development of all epithelial tissues [34], including mammary glands [35, 36]. Among six isoforms of TP63, ΔNp63α, that lacks the transactivating N-terminal region, is the predominant form expressed in many carcinomas, which promotes tumor growth and inhibits apoptosis [37, 38]. TP63 is found in a subset of highly aggressive ER negative breast cancers that represent a basal and myoepithelial phenotype and have a poor clinical outcome [39, 40]. Overexpression of ΔNp63α is also reported to induce a stem cell phenotype in MCF-7 breast cancer cells through the Notch pathway [41]. In this study, we have focused on miR-196a2 regulating TP63 and several other proteins because of their likely important roles in the tumor progression and hormone regulation of breast cancer cells.

3.3 Materials and Methods

Cell Culture, RNA Extraction, and Real Time PCR Analysis

MCF-7 and MDA-MB-231 breast cancer cells were routinely maintained in MEM (Sigma-Aldrich Corp., St. Louis, MO) supplemented with 5% calf serum (HyClone, Logan, UT) or in L-15 (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), respectively. Four days before E2 treatment, cells were switched to phenol red-free MEM containing 5% charcoal-dextran-treated calf serum or phenol red-free L-15 containing 10% charcoal-dextran-treated fetal bovine serum, respectively. Medium was changed on day 2 and 4 of culture, and then cells were transfected with 20 nM of siGENOME Ctrl, ERα or ERK2

using Dharmafect. After 48 hours of transfection, cells were treated for 24h with 0.1% ethanol or 10 nM E2. Cells were also treated with 10 ng/ml EGF (Sigma-Aldrich corp.) for the time course experiment. After treatments, total RNA was isolated, reverse-transcribed and analyzed by real-time PCR as described previously [42]. For the analysis of miRNA expression total RNA was isolated as previously described, reverse transcribed using the primers for each miRNA (Applied Biosystems) and analyzed by real-time PCR using TAQMAN chemistry and primers from Applied biosystems.

MicroRNA Array

Total RNA was isolated using Trizol reagent. Then miRNA was enriched using RT^2 qPCR-grade miRNA isolation kit according to manufacturer's instructions. Two hundred nanograms of enriched small RNA were converted into cDNA using RT^2 miRNA First strand kit. The cDNAs were mixed with 2 × RT2 SYBR Green PCR Master Mix (SABiosciences) and dispersed into 384-well Human Genome miRNA PCR Array (MAH-3200E, SABiosciences) with 10 μl/well reaction volume. The PCR array contained a panel of primer sets for 376 most abundantly expressed and best characterized human miRNAs, four small RNAs as the internal controls and four quality controls. The real-time qRT-PCR was performed on a 7900 real-time PCR system (Applied Biosystems Inc., Foster, CA) with following cycling parameters: 95 °C for 10 mins, then 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. SYBR Green fluorescence was recorded from every well during the annealing step of each cycle.

Anti-miR, Pre-miR or TP63 miScript Target Protector Transfection

MCF-7 and MDA-MB-231 breast cancer cells were transfected with 20 nM of Anti-miR-196a* (Ambion), Pre-miR-196a* (Ambion) or negative controls (Anti-miR or Pre-miR) using Dharmafect in order to knock-down or overexpress miR-196a2, respectively. After 48 hours of transfection, cells were treated for 24h with 0.1% ethanol or 10 nM E2. For the target protector assay, cells were transfected with 100 nM TP63 miScript target protector or negative control protector using Dharmafect. After 48 h of transfection, cells were treated for 24h with 0.1 % ethanol or 10 nM E2, and subjected to RNA isolation for gene expression analysis.

Proliferation, Soft Agar Colony Formation and Invasion Assays

MCF-7 or MDA-MB-231 cells were transfected with 20 nM Anti-miR-196a* or Pre-miR-196a*, as described above, and then were seeded at a concentration of 1,000 cells/well in 100 μl of culture medium and treated with 0.1% ethanol or 10 nM E2. Cell proliferation was assessed using a WST-1 kit according to the manufacturer's instructions (Roche Applied Science). The absorbance of the samples was analyzed using a microplate ELISA reader at 450 nm. Invasion assays were performed 24 h posttransfection using BDBioCoat MATRIGEL invasion chambers (BD Biosciences) with 10% fetal bovine serum as chemoattractant. Cells (2.5×10^4 cells/well) were plated into the chambers and allowed to invade for 24 h. The remaining cells in the chambers were removed with cotton swabs and the invading cells on the lower surface of the chambers were stained with Wright-Giemsa staining solution. The number of invading cells was calculated by counting three different fields under a phase-contrast microscope. For the soft agar colony formation assay, cells (5×10^3 cells/well) were seeded in 0.35% agar and cultured for 10 days at 37°C under 5% CO_2. Dishes were stained with 0.05% crystal violet, and colonies were counted in the entire dish.

Chromatin Immunoprecipitation (ChIP) Assays and Western Blot Analysis

MCF-7 cells were treated with 0.1% ethanol or 10 nM E2 for 45 min or 2 hr. ChIP assays were performed essentially as described before [43]. Antibodies used were: ERα (HC-20) and ERK2 (D-2) from Santa Cruz Biotechnology. Whole cell extracts were prepared in lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 % NP-40, 1 % SDS, 5 % glycerol, and protease inhibitor cocktail). Western blot analysis of whole cell extracts followed common protocols and specific antibodies were used for ERα (HC-20, Santa Cruz Biotechnology), ERK2 (D-2, Santa Cruz Biotechnology), SPRY1 (H-120, Santa Cruz Biotechnology), TP63 (ab53039, Abcam) and β-actin (AC-15, Sigma).

3.4 Results

Identification of miRNAs harboring overlapping ERα and ERK2 binding sites in MCF-7 cells

We mapped ERα and ERK2 binding sites to regions that contain noncoding RNAs (ncRNAs) to investigate possible collaboration between ERα and ERK2 in miRNA regulation, by utilizing a ChIP-on-chip microarray analysis of ERα and ERK2 binding with E2 treatment in MCF-7 cells [44]. From the genome wide ChIP-on-chip analysis of ERα and ERK2 binding sites, using a 50 Kb window around the transcription start site (TSS) of annotated ncRNAs in the human genome, we identified 10 ncRNAs (miR-101, miR-938, miR-196a2, miR-615-3p, miR-135a2, miR-190, miR-190b, miR-21, miR944 and miR-1208) harboring both ERα and ERK2 binding sites after E2 treatment (Figure 3.1A), and 9 of 10 miRNA-encoding genes (except for miR-190b) having overlapping ERα and ERK2 binding sites. We used an miRNA microarray to evaluate miRNA expression profiles and identified the miRNAs that are up-regulated (miR-

196a2, miR-615-3p, miR-135a2 and miR-944) or down-regulated (miR-190b and miR-21) by E2. ERα or ERK2 knock-down reduced miRNA expression and blocked E2 regulation of miRNAs that harbor both ERα and ERK2 binding sites (Figure 3.1B). Among the 10 miRNAs, we chose for further investigation two E2-upregulated miRNAs, miR-135a2 and miR-196a2, that were highly up-regulated by E2 and harbored overlapping ERα and ERK2 binding sites close to the TSS (within a 10 kb window) (Figure 3.1C).

miRNA target gene prediction

The microRNA databases and target prediction tools MicroCosm Targets (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/), PicTar (http://pictar.mdc-berlin.de/) and TargetScan (http://www.targetscan.org/index.html) were used to identify potential miRNA targets. Based on the observation that miR-135a2 and miR-196a2 expression was up-regulated by E2 and down-regulated by ERα or ERK2 knock-down, we utilized the microarray gene expression data after E2 treatment or ERα/ERK2 knock-down (done previously in the lab by Z. Madak-Erdogan) to narrow down the potential miRNA target genes to focus on in this study (Figure 3.1D). Among the genes that came up in the miRNA target prediction tools, we verified the target genes of miR-135a2 and miR-196a2 that were up-regulated in their mRNA levels after ERα or ERK2 knock-down and down-regulated after 10 nM E2 treatment for 24 h in MCF-7 cells by quantitative RT-PCR.

Regulation of miRNA and target gene expression by ERα and ERK2

In order to verify the impact of ERα and ERK2 on the expression of the miRNAs, we performed ERα and ERK2 knock-down experiments in MCF-7 cells. ERα or ERK2 depletion

decreased the expression of miR-135a2 and miR-196a2 genes and blocked E2-mediated regulation (Figure 3.2A). By ChIP assay, the involvement of ERα and ERK2 in expression of the miRNAs as well as in transcriptional activation was confirmed by assessing the recruitment of ERα, ERK2 and RNA polymerase II to the binding sites and TSS regions of miR-135a2 and miR-196a2. ERα and ERK2 were both recruited to the miR-135a2 binding site and one of the miR-196a2 binding sites (4101) at 45 min of E2 treatment and showed a decrease by 2 h (Figure 3.2B). The recruitment of active RNA Polymerase II (pSer5-polII) was also examined by ChIP assay at 45 min of E2 treatment in MCF-7 cells, and supported transcriptional activation on the TSS of each miRNA (Figure 3.2C). We identified miR-135a2 target genes, GHR and MAP3K3, and miR-196a2 target genes, SPRY1 and TP63, to show down-regulated mRNA levels with 24 h of E2 treatment (Figure 3.2D). They also had increased basal level expression and loss of E2-mediated repression upon knock-down of ERα or ERK2. The protein levels of SPRY1 and TP63 (ΔNp63α and ΔNp63γ, isoforms of TP63) were down-regulated by E2 and up-regulated by knock-down of ERα or ERK2, confirming the regulation of the target genes by miR-196a2 at the translational level as well as at the mRNA level (Figure 3.2E).

Regulation of miR-196a2 and target gene expression by E2 and EGF

To study the regulatory role of ERα in collaboration with ERK2 on altering miRNA target gene expression, we conducted E2 and EGF time course experiments in MCF-7 cells. The expression level of miR-196a2 was up-regulated ca. 7-fold by E2 over the 24-h period. miR-196a2 RNA was also up-regulated by EGF at the early time point (6 h) and then returned to the basal level (Figure 3.3A). Both E2 and EGF treatment reduced the mRNA level of the target genes, SPRY1 and TP63, and E2 had a greater impact on repressing both of these target genes

compared to EGF (Figure 3.3B). The protein level of SPRY1 was decreased over time by E2 and also by EGF at the later time point (24 h). The protein expression of ΔNp63γ was also down-regulated by E2 and EGF, confirming the effect of altered miRNA expression also at the translational level (Figure 3.3C).

TP63 targeted by miR-101, miR-190 and miR-196a2

The miR-196a2 target gene TP63 is also targeted by miR-101 and miR-190 (Figure 3.4A). Because we found these miRNAs (miR-101, miR-190 and miR-196a2) to be up-regulated by E2 and to have overlapping ERα and ERK2 binding sites, TP63 seems to be one of the major target genes that is regulated by E2 and potential growth factors such as EGF in MCF-7 cells. Besides TP63, we found eight additional common target genes of miR-101, miR-190 and miR-196a2 by using miRNA target gene prediction tools –ASXL1, NEUROD1, IGF1, BBS2, C18orf37, TFAP2A, MCTS1 and MGAT4A (Figure 3.4B), and verified ERα or ERK2-mediated regulation of these target transcripts (Figure 3.4C).

Knock-down of miR-196a2 decreases TP63 expression and increases cell growth in MCF-7 cells

In order to verify the effect of miRNAs on regulation of target gene expression, antisense inhibition of miR-196a2 expression by using Anti-miR oligos was conducted in MCF-7 cells (Figure 3.5A). Treatment with Anti-miR-196a* increased the basal expression of target genes, TP63 and SPRY1 (Figure 3.5B). In squamous epithelium, the pro-apoptotic gene IGFBP3 is transcriptionally repressed by ΔNp63α [38]. We observed that IGFBP3 was down-regulated by knock-down of miR-196a2 (Figure 3.5C), which implies the potential suppression of apoptosis

and increased cell proliferation by TP63 in MCF-7 cells. Therefore, we next investigated the effect of miR-196a2 knock-down on breast cancer cell growth by conducting proliferation assays. Inhibition of miR-196a2 enhanced cell proliferation with or without E2 treatment by day 4 (Figure 3.5D), supporting the oncogenic role of TP63 regulated by miR-196a2 in breast cancer.

Overexpression of miR-196a2 increases TP63 expression and decreases growth of MCF-7 cells

In order to confirm the impact of up-regulated miRNAs on target transcripts, overexpression of miR-196a2 was performed using Pre-miR-196a* oligos in MCF-7 cells (Figure 3.6A). Enforced miR-196a2 expression inhibited TP63 expression but the effect on SPRY1 expression levels was minimal (Figure 3.6B). The sensitivity of each transcript toward multiple miRNAs depends on various factors such as complimentarity of sequences or copy numbers of miRNA binding sites on each 3' UTR [45-47]. Overexpression of miR-196a2 increased the basal level of IGFBP3 mRNA (Figure 3.6C), suggesting that down-regulated TP63 may cause cell growth repression. In subsequent cell proliferation assay experiments, we observed decreased cell growth with or without E2 treatment (Figure 3.6D).

miR-196a2 directly targets TP63 in MCF-7 cells

The biological activity of miRNAs is primarily mediated by interaction with matching recognition sequences in the 3' UTRs of target genes and by translational repression. To determine whether TP63 is a direct target of miR-196a2, we utilized TP63 miScript target protector sequence which selectively recognizes miR-196a* target sequences only on the TP63 3' UTR, thereby blocking interaction between miR-196a2 and TP63 target mRNA. Transfection

of TP63 protector oligos increased basal TP63 mRNA levels and recovered down-regulated TP63 expression by Pre-miR-196a* in MCF-7 cells (Figure 3.7A). Immunoblotting with TP63 antibody showed that ΔNp63γ was significantly down-regulated by miR-196a2 overexpression and it was partially recovered by cotransfection with TP63 protector (Figure 3.7B). The effect of TP63 protector on inhibition of miR-196a2 interaction with TP63 mRNA also resulted in enhanced proliferation in MCF-7 cells and enhancement of proliferation was also observed with miR-196a* cotransfection (Figure 3.7C).

TP63-mediated regulation of cell growth and invasion in MDA-MB-231 ERα negative breast cancer cells by miR-196a2 overexpression

TP63 is one of the Basal-like breast cancer markers, along with cytokeratins (CK5/6, CK14, CK17) and EGFR [29, 30]. To address whether the expression of TP63 and miR-196a2 is correlated with ERα and ERK2 levels, we examined the levels of TP63, miR-196a2, ERα and ERK2 in several ERα positive and negative cell lines (Figure 3.8A). Different isoforms of TP63, ΔNp63γ and ΔNp63α were detected in ERα positive cells (MCF-7, T47D, ZR75-1 and MDA-MB-231 ER+) and ER negative cells (MDA-MB-231 and MDA-MB-453; Figure 3.8A). ΔNp63α, that lacks the transactivating N-terminal region, is the predominant form expressed in many carcinomas [37, 38], which may contribute to a basal and myoepithelial phenotype in highly aggressive ER negative breast cancers with a poor clinical outcome [39, 40]. Of note, ΔNp63α expression was reduced with stable ERα expression in MDA-MB-231 cells when the ΔNp63γ level was increased. This switch of TP63 isoform expression suggests that the expression of ΔNp63α is ERα-dependent and may take part in developing the more aggressive tumorigenic phenotypes of ERα-negative breast cancers [41].

Expression of miR-196a2 was higher in ERα-positive cell lines compared to ERα-negative cells lines, except for MDA-MB-453 cells (Figure 3.8B). Interestingly, the level of miR-196a2 increased ca. 10 fold with stable ERα expression in MDA-MB-231 cells when the ΔNp63α level was decreased. We overexpressed miR-196a2 using Pre-miR-196a* oligos in ER-negative MDA-MB-231 cells (Figure 3.8C). In subsequent cell proliferation assay experiments, overexpression of miR-196a2 decreased cell growth (Figure 3.8D). We next investigated the effect of miR-196a2 on invasive properties of breast cancer cells by analyzing *in vitro* invasion using Matrigel invasion chambers. Enforced miR-196a2 expression inhibited *in vitro* invasion of MDA-MB-231 cells (Figure 3.8E). To examine the effect of miR-196a2 on tumor growth, soft agar colony formation assays were performed with miR-196a2 overexpression in MDA-MB-231 cells. After 8 days of incubation, the colony numbers and colony size were greatly reduced with Pre-miR-196a* transfection compared to control (Figure 3.8F), suggesting a potent tumor suppressive role of miR-196a2 through TP63 regulation in breast cancer cells.

3.5 Discussion

The involvement of miRNAs in human cancer has become a rising interest in the cancer research field. miRNA was introduced as an additional layer in the gene expression regulatory system at the post-transcriptional level by destabilizing target mRNAs using RNA interference mechanism and at the translational level by repressing the translation process. High-throughput miRNA expression profiling in breast cancer cell lines and tissues identified a large set of miRNAs expressed at different levels compared to the normal breast [15-17]. In this study, we have focused on elucidating the regulatory role of ERα and ERK2 in miRNA expression and understanding its physiological impact on breast cancer by target gene regulation. Using a

microarray analysis of ERα and ERK2 binding upon E2 treatment in MCF-7 cells, we identified 10 miRNAs that are harboring both ERα and ERK2 binding sites within a 50 Kb window around the TSS of annotated ncRNAs in the human genome. Nine out of ten miRNAs had overlapping ERα and ERK2 binding sites, implying a possible collaborative action between ERα and ERK2 in miRNA regulation (Figure 3.1). In addition, we identified TP63, a target gene of miR-101, miR-190 and miR-196a2, and showed that TP63 may be important for E2- or growth factor-mediated cellular response in breast cancer cells, by increasing tumor cell growth or *in vitro* invasion mainly controlled by miR-196a2 action.

ERα and ERK2 directly bind to the overlapping binding sites near the E2-upregulated miRNAs (miR-135a2, miR-196a2, miR-101 and miR-190) and are required for transcriptional induction of these miRNAs as well as E2-mediated miRNA regulation. Loss of miRNA expression by depletion of ERα or ERK2 induced an increase of the miRNA target gene basal expression levels (Figure 3.2). This shows that ERα and ERK2 serve as transcription factors not only regulating transcription of primary protein-encoding target genes but that they also control expression of groups of miRNAs to potently coordinate the functional outcome mediated through miRNA- targeted genes.

Although miRNAs may target multiple genes pertinent to tumor cell growth, the tumor suppressive role of miR-196a2 is apparently mainly associated with TP63 because miR-196a2 significantly inhibits TP63 expression and cell proliferation (Figure 3.7). TP63, one of the Basal-like breast cancer biomarkers [29, 30], is targeted by three E2-regulated miRNAs, miR-101, miR-190 and miR-196a2. It is common that a group of miRNAs target the same gene or a group of genes that have similar physiological functions. This allows maintenance of a fine-tuned control of phenotypic properties that are attributed to multiple genes, by regulating different

groups of miRNAs depending on the various external inputs for signal transduction in the cell. ΔNp63, a predominantly expressed TP63 isoform in cancer cells, is amino-terminally truncated and can oppose the transactivation capabilities of the full-length protein [28, 31]. The ability of ΔNp63α to enhance proliferation and suppress apoptosis suggests a possible contribution of TP63 in tumorigenesis and breast cancer progression [37, 38, 41]. Particularly, an increase of ΔNp63α expression upon ERα knock-down suggests an inverse relationship between ERα and TP63, accounting for the opposite breast cancer phenotypes of ERα positive luminal type and triple-negative basal-like breast cancers. It is also reported that EGFR/MAPK signaling can induce a switch in ERα positive luminal-A type MCF-7 breast cancer cells to an ERα negative, basal-like phenotype by modulating miR-206 that represses ERα activity [48].

We showed that miR-196a2 is deeply involved in breast cancer proliferation as the knockdown of miR-196a2 promoted cell growth with decreased levels of an apoptotic marker and TP63 target gene, IGFBP3 in MCF-7 cells (Figure 3.5). The importance of miR-196a2 in tumor cell growth is further supported by the observation that both TP63 and MCF-7 cell proliferation were greatly reduced by overexpresion of miR-196a2 (Figure 3.6). Li *et al.* (2010) showed that miR-196 family suppressed *in vitro* invasion and *in vivo* spontaneous metastasis of breast cancer cells through inhibiting HOXC8 expression [27]. It was of great interest for us to examine whether overexpression of miR-196a2 might be able to alleviate the aggressive behavior and poor prognosis of Basal-like breast cancer subtype by down-regulating TP63 in ERα negative breast cancer cells. Of note, the expression of ΔNp63α was correlated inversely with ERα levels in several breast cancer cell lines (Figure 3.8). Relative levels of miR-196a2 were mainly dependent on ERα expression rather than ERK2 levels, implying that ERK2 action on miRNA expression is secondary to ERα activity [44]. Interestingly, MDA-MB-453 cells had

a comparable amount of miR-196a2 with MCF-7 cells suggesting that ERK2 may act through other signaling pathways to foster miRNA expression without ERα in this cell line. We also showed that *in vitro* invasion and tumor growth properties were reversed by enforced miR-196a2 expression in ERα negative MDA-MB-231 cells (Figure 3.8). More studies are yet to be done to manifest a potential tumor-suppressive treatment strategy of using the miR-196a2-TP63 circuit in ERα negative breast cancer. However, the role of miR-196a2 on TP63 regulation seems to be remarkable on account of its E2 responsiveness and ERα and/or ERK2-dependent expression in breast cancer cells.

Understanding the aberrant regulation of miRNAs by E2 and growth factors in breast cancer cells is of great significance, since those signaling pathways are heavily involved in the development and progression of breast tumors as well as in the responsiveness of breast cancer patients to endocrine therapies. Many studies have already reported the involvement of miRNAs in ERα signaling and their impact on endocrine resistance in breast cancer [21-26]. In this study, we suggest that the regulation of miR-196a2 by ERα and/or ERK2 signaling in breast cancer may contribute to divergent physiological properties and clinical outcomes of different subtypes of breast tumors, those that are ERα-positive versus those that are ERα-negative, possibly mediated through differential TP63 expression affecting tumor cell growth and invasion ability. Our study suggests that, in addition to relying on breast cancer subtype marker expression, looking into the levels of miRNAs that are regulated by ERα or growth factor signaling should be considered to make an accurate prognosis of breast cancer. Understanding potential mechanisms of miRNA-modulated cellular responses driven by ERα and cooperative growth factor signaling could offer new strategies in breast cancer therapy for many different subtypes of breast tumors in accordance with their miRNA signatures.

3.6 References

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3.7 Figures and Tables

Figure 3.1

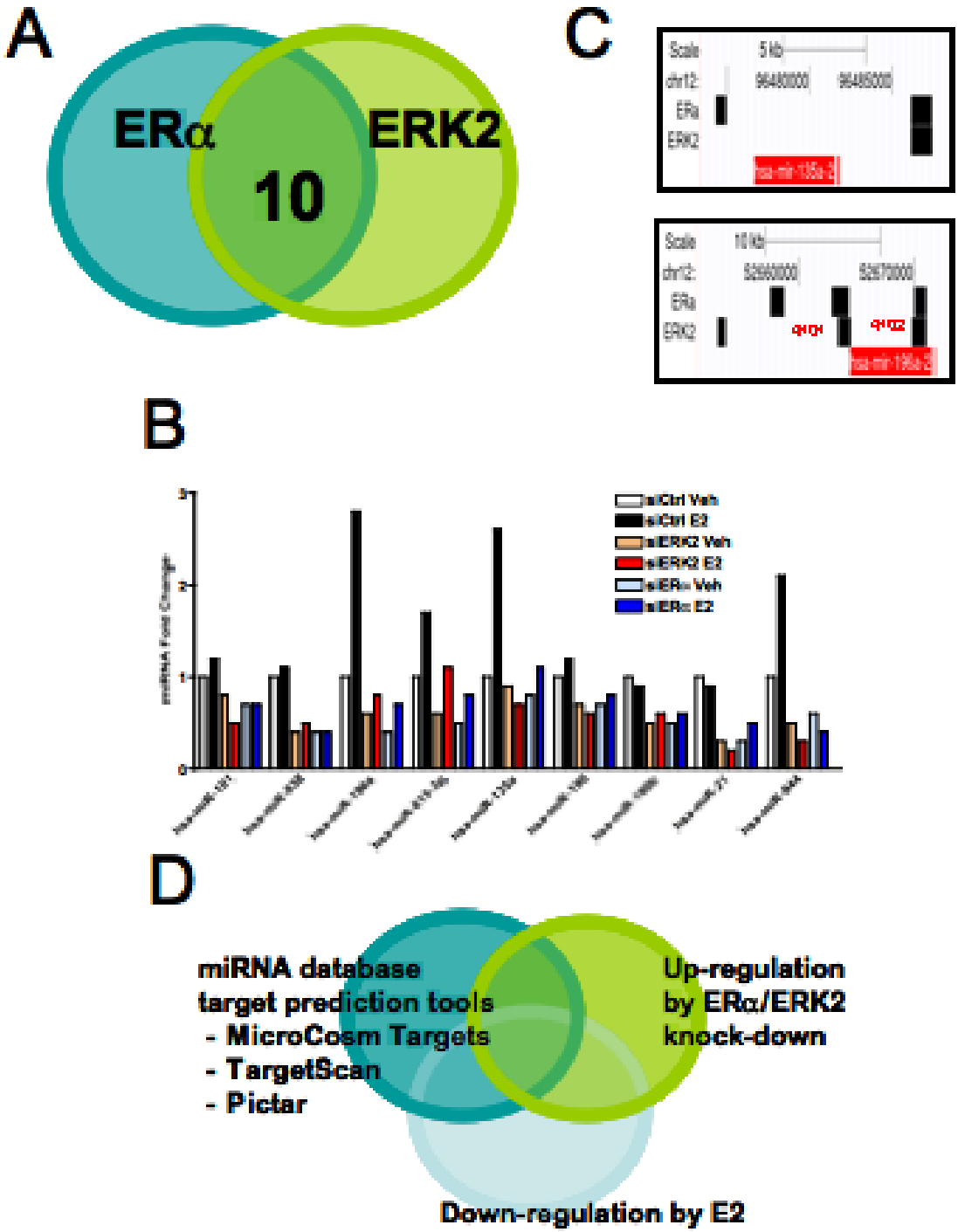

Figure 3.1 (cont.)

Figure 3.1 Association of ERα and ERK2 binding sites with miRNA promoters

(A) Venn diagram showing the number of miRNAs (10) that harbor both ERα and ERK2 binding sites within a 50 kb window around the transcription start site. 9 of the 10 have overlapping ERα and ERK2 binding sites.
(B) Impact of ERα or ERK2 knock-down on the expression of each miRNA. MCF-7 cells were transfected with siCtrl, siERα or siERK2 and treated with 0.1% ethanol (veh) or 10 nM E2 for 6 h. Expression levels of each miRNA were determined by the miRNA microarray analysis.
(C) ERα and ERK2 binding sites near miR-135a2 and miR-196a2 genes.
(D) Overview of approach for prediction of potential miRNA target genes by utilizing target prediction tools and our microarray gene expression data.

Figure 3.2

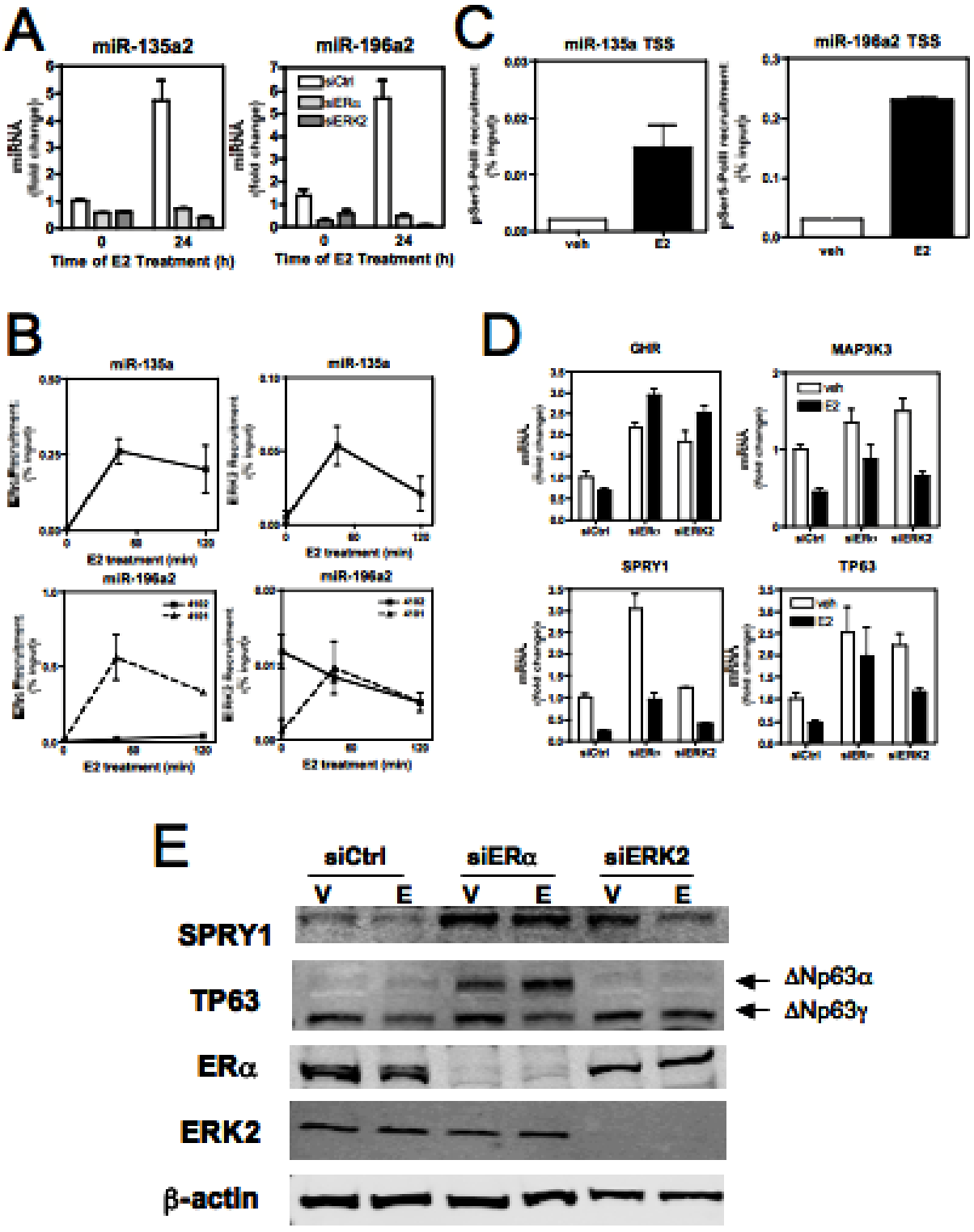
A
miR-135a2
miR-196a2
miRNA (fold change)
siCtrl
siERα
siERK2
0
24
Time of E2 Treatment (h)
C
miR-135a TSS
miR-196a2 TSS
pSer5-Pol II recruitment (% input)
veh
E2
B
miR-135a
miR-196a2
E2 treatment (min)
D
QHR
MAP3K3
SPRY1
TP63
veh
E2
E
siCtrl
siERα
siERK2
V
E
SPRY1
TP63
ERα
ERK2
β-actin
ΔNp63α
ΔNp63γ

Figure 3.2 (cont.)

Figure 3.2 Regulation of miRNAs and target gene expression by ERα and ERK2 in MCF-7 cells

(A) Impact of ERα or ERK2 knock-down on the expression of miR-135a2 and miR-196a2. MCF-7 cells were transfected with siCtrl, siERα or siERK2 and treated with 0.1% ethanol (veh) or 10 nM E2 for 0 or 24 h. Total RNA was harvested and subjected to RT-PCR using specific primers for each miRNA. Expression levels of each miRNA were determined using Taqman probe based quantitative PCR.
(B) ERα and ERK2 recruitment to the overlapping binding sites of miR-135a2 and miR-196a2 with 0.1% ethanol (veh) or 10 nM E2 treatment for 45 min. miR-196a2 has two binding sites (4101 and 4102) labeled according to the ERα binding site number.
(C) Phospho-serine5 RNA Polymerase II recruitment to the transcription start site (TSS) of miR-135a2 and miR-196a2 with 0.1% ethanol (veh) or 10 nM E2 treatment for 45 min.
(D) Impact of ERα or ERK2 knock-down on expression of miR-135a2 target mRNAs (GHR and MAP3K3), and miR-196a2 target mRNAs (SPRY1 and TP63). MCF-7 cells were transfected with siCtrl, siERα or siERK2 for 48 h then treated with 0.1% ethanol (veh) or 10 nM E2 for 24h.
(E) Impact of ERα or ERK2 knock-down on the miR-196a2 target genes, SPRY1 and TP63 protein levels by western blot analysis. β-actin was used as a loading control.

Figure 3.3

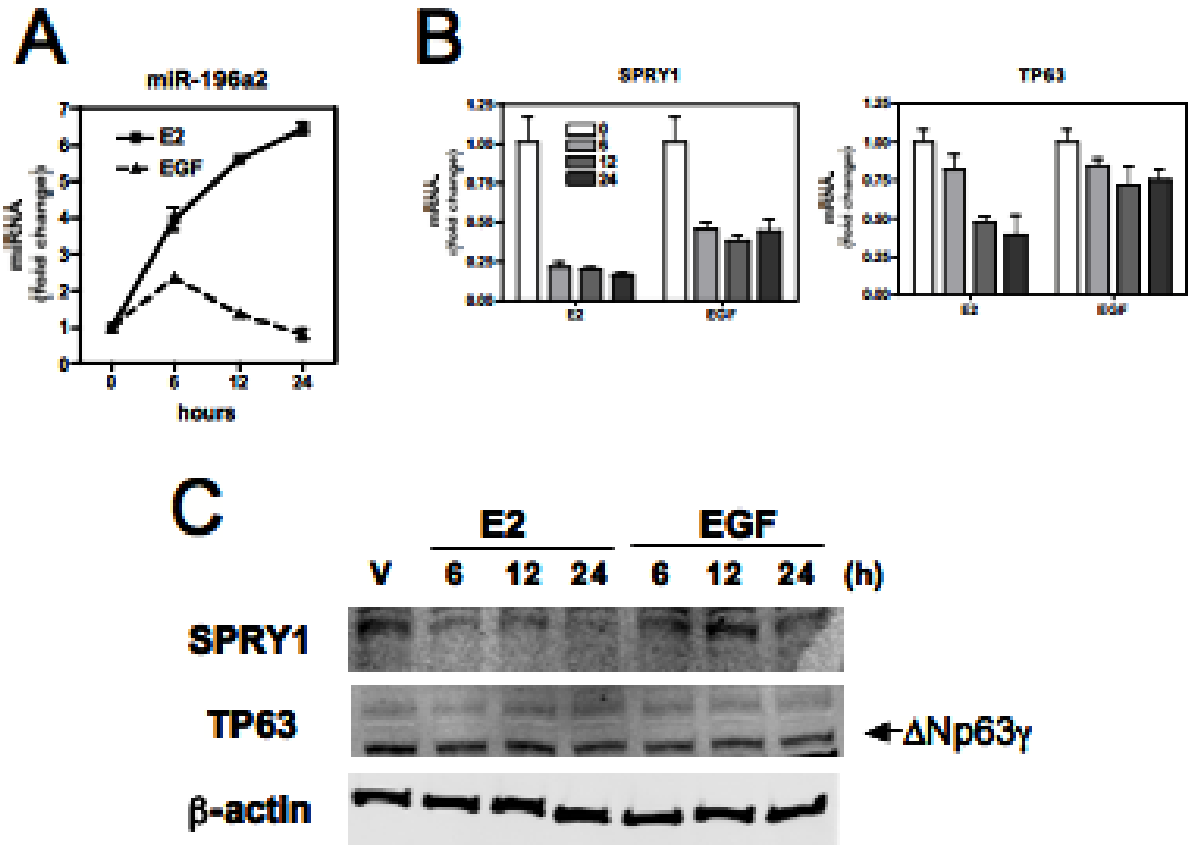

Figure 3.3 Regulation of miR-196a2 and target gene expression by E2 and EGF in MCF-7 cells

(A) Time-course of expression of miR-196a2 by E2 or EGF. MCF-7 cells were treated with 10 nM E2 or 10 ng/ml EGF for up to 24 h. Total RNA was harvested and subjected to RT-PCR using specific primers for each miRNA. Expression levels of each miRNA were determined using Taqman probe based quantitative PCR.
(B) Regulation of the mRNA expression of miR-196a2 target genes with 10 nM E2 or 10 ng/ml EGF treatment for 0, 6, 12 and 24 h.
(C) Regulation of the protein expression of miR-196a2 target genes by E2 or EGF was analyzed by western blot analysis. β-actin was used as a loading control.

Figure 3.4

A

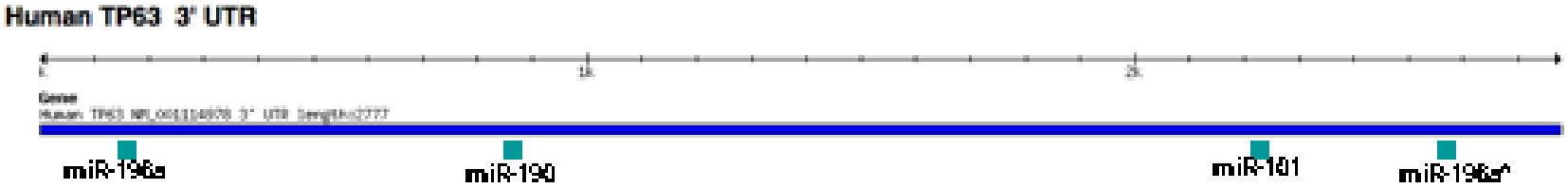

Figure 3.4 (cont.)

B

TP73L	TP63 tumor protein p63
ASXL1	additional sex combs like 1
NEUROD1	NEUROD1
IGF1	insulin-like growth factor 1
BBS2	Bardet-Biedl syndrome 2
C18orf37	INO80C, INO80 complex subunit C
TFAP2A	transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha)
MCTS1	malignant T cell amplified sequence 1
MGAT4A	mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme A

C

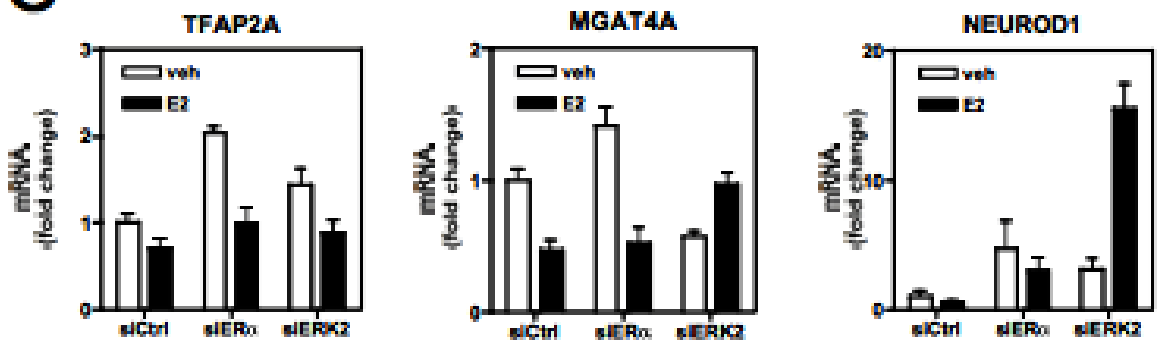

Figure 3.4 TP63 targeting by miR-101, miR-190 and miR-196a2

(A) Schematic of the 3' UTR of the TP63 gene and miRNAs targeting site.
(B) List of genes targeted by miR-101, miR-190 and miR-196a2.
(C) Impact of ERα or ERK2 knock-down on expression of common target mRNAs of miR-101, miR-190 and miR-196a2. MCF-7 cells were transfected with siCtrl, siERα or siERK2 for 48 h then treated with 0.1% ethanol (veh) or 10 nM E2 for 24 h.

Figure 3.5

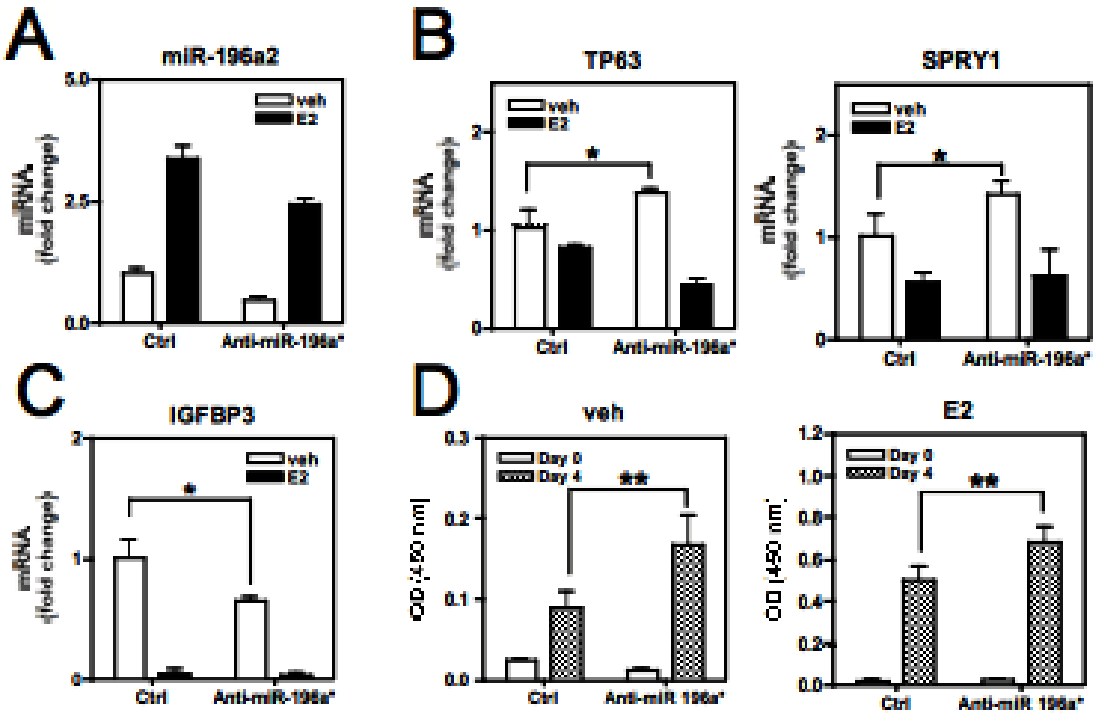

Figure 3.5 Knock-down of miR-196a2 increases expression of TP63 and SPRY1 and cell proliferation in MCF-7 cells

(A) Knock-down of miR-196a2 in MCF-7 cells. Cells were transfected with Anti-miR-196a* or negative control (Ctrl) and treated with 0.1% ethanol (veh) or 10 nM E2 for 24 h. Total RNA was harvested and subjected to RT-PCR using specific primers for each miRNA. Expression levels of each miRNA were determined using Taqman probe based quantitative PCR.
(B) Impact of miR-196a2 knock-down on the expression of miR-196a2 target genes, TP63 and SPRY1 with 0.1% ethanol (veh) or 10 nM E2 treatment for 24 h. * p-value < 0.05
(C) Knock-down of miR-196a2 reduces the expression of an apoptotic marker gene, IGFBP3. •p-value < 0.05
(D) Impact of miR-196a2 knock-down on MCF-7 cell proliferation. Cells were transfected with 20 nM Anti-miR-196a* and then were seeded at a concentration of 1,000 cells/well in 100 ml of culture medium and treated with 0.1% ethanol or 10 nM E2 for 4 d. Cell proliferation was assessed using a WST-1 kit. ** p-value < 0.01

Figure 3.6

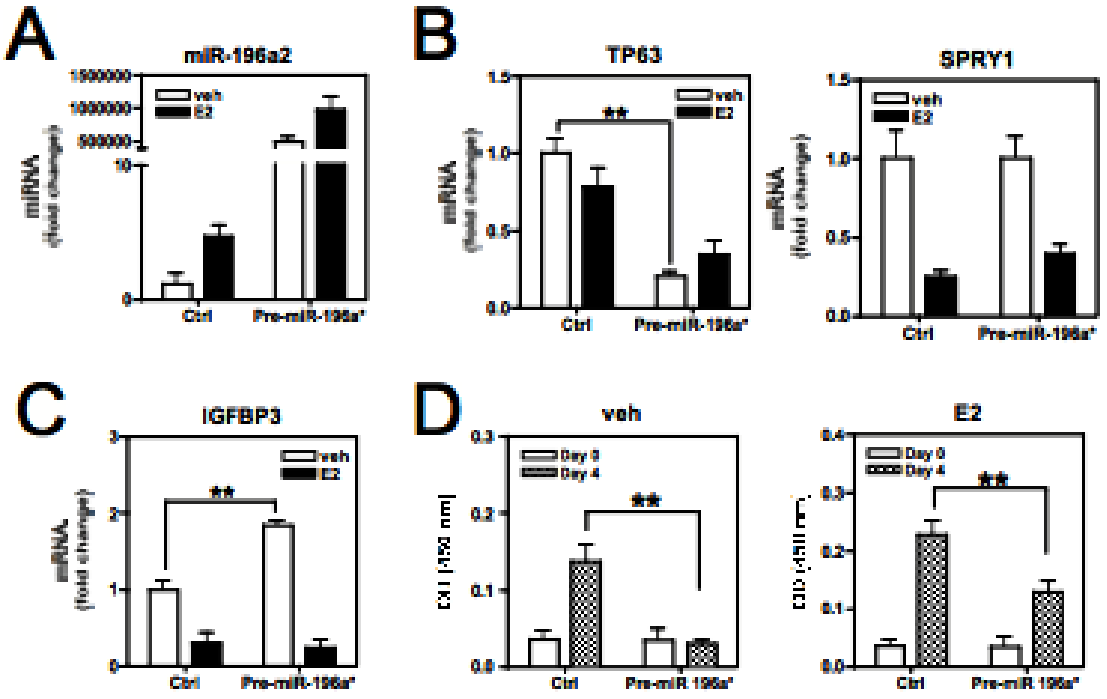

Figure 3.6 Overexpression of miR-196a2 decreases expression of TP63 and cell proliferation in MCF-7 cells

(A) Overexpression of miR-196a2 in MCF-7 cells. Cells were transfected with Pre-miR-196a* or negative control (Ctrl) and treated with 0.1% ethanol (veh) or 10 nM E2 for 24 h. Total RNA was harvested and subjected to RT-PCR using specific primers for each miRNA. Expression levels of each miRNA were determined using Taqman probe based quantitative PCR.
(B) Impact of miR-196a2 overexpression on the expression of miR-196a2 target genes, TP63 and SPRY1 with 0.1% ethanol (veh) or 10 nM E2 treatment for 24 h. ** *p*-value < 0.01
(C) Overexpression of miR-196a2 increases the expression of an apoptotic marker gene, IGFBP3. ** *p*-value < 0.01
(D) Impact of miR-196a2 overexpression on MCF-7 cell proliferation. Cells were transfected with 20 nM Pre-miR-196a* and then were seeded at a concentration of 1,000 cells/well in 100 ml of culture medium and treated with 0.1% ethanol or 10 nM E2 for 4 d. Cell proliferation was assessed using a WST-1 kit. ** *p*-value < 0.01

Figure 3.7

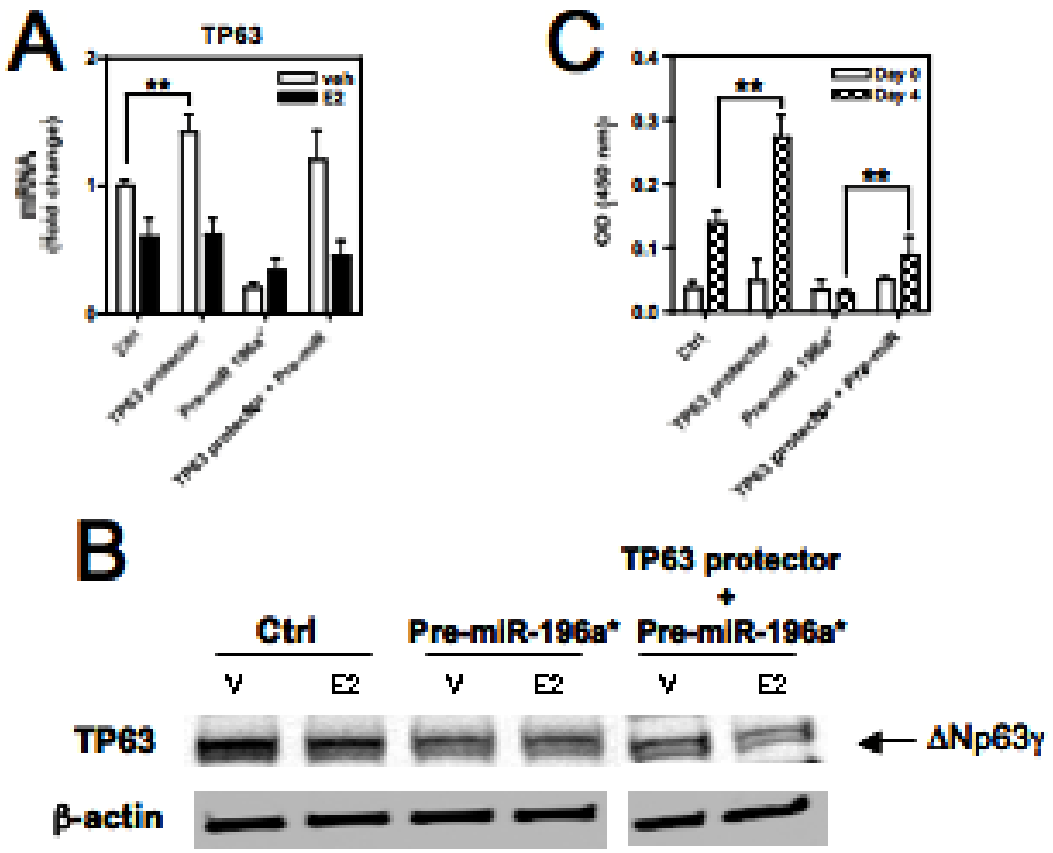

Figure 3.7 miR-196a2 inhibits TP63 expression and cell proliferation in MCF-7 cells

(A) Impact of TP63 target protector on TP63 mRNA expression. MCF-7 cells were transfected with 100 nM TP63 miScript target protector or negative control protector (Ctrl) with or without 10 nM Pre-miR-196a*. After 48 hr transfection, cells were treated with 0.1% ethanol (veh) or 10 nM E2 for 24 hr and then subjected to RNA isolation for gene expression analysis. ** *p*-value < 0.01

(B) ΔNp63δγ expression after Pre-miR-196a* transfection with or without TP63 target protector. β-actin was used as a loading control.

(C) Impact of TP63 target protector on MCF-7 cell proliferation. Cells were transfected with 100 nM TP63 miScript target protector or negative control protector (Ctrl) with or without 10 nM Pre-miR-196a*. Cells were then seeded at a concentration of 1,000 cells/well in 100 ml of culture medium and treated with 0.1% ethanol or 10 nM E2 for 4 d. Cell proliferation was assessed using a WST-1 kit. ** *p*-value < 0.01

Figure 3.8

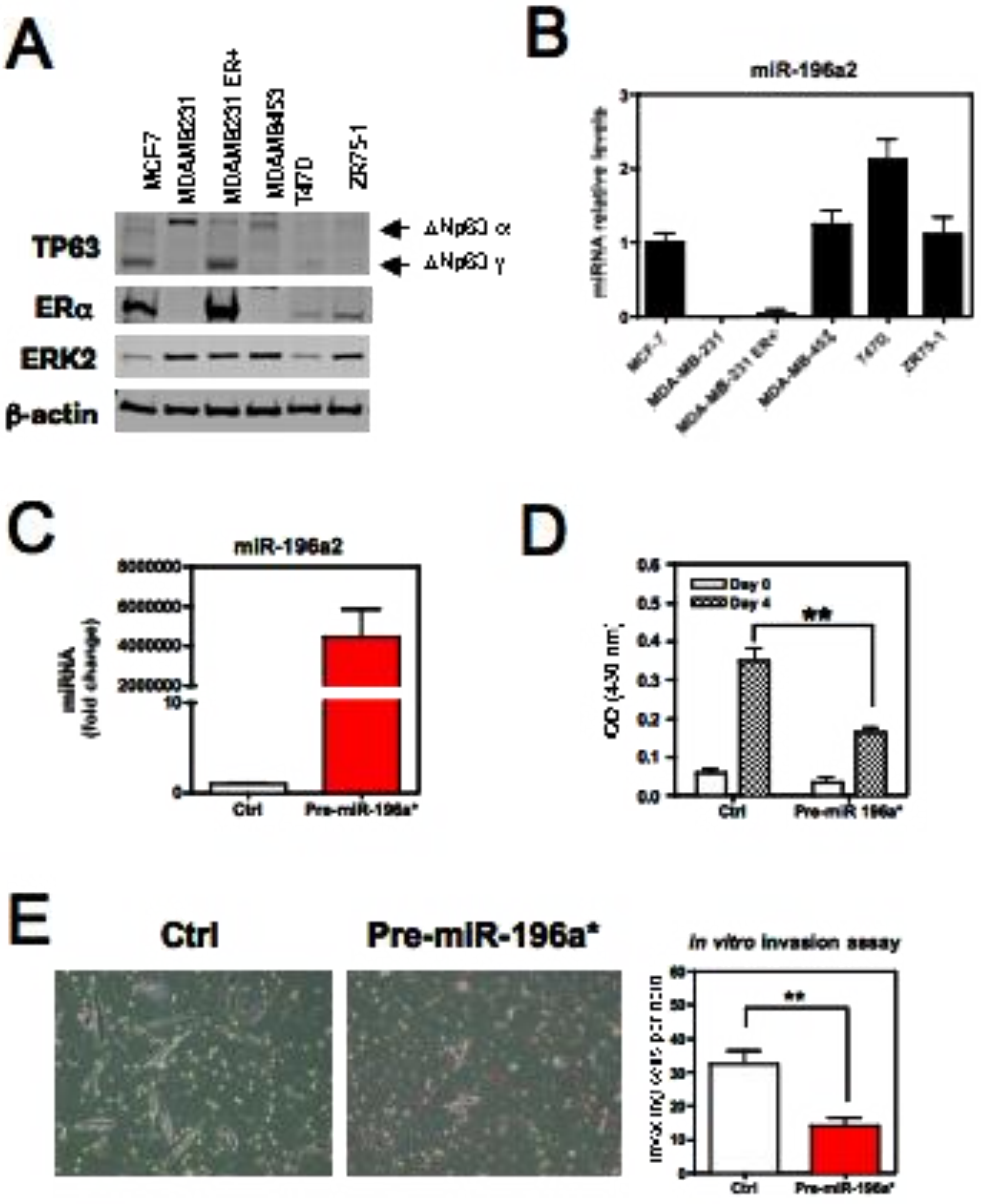
A
MCF7
MDAMB231
MDAMB231 ER+
MDAMB453
T47D
ZR75-1
TP63
ERα
ERK2
β-actin
ΔNp63 α
ΔNp63 γ
B
miR-196a2
C
miR-196a2
miRNA (fold change)
Ctrl
Pre-miR-196a*
D
Day 0
Day 4
**
Ctrl
Pre-miR 196a*
E
Ctrl
Pre-miR-196a*
In vitro invasion assay
**
Ctrl
Pre-miR-196a*

Figure 3.8 (cont.)

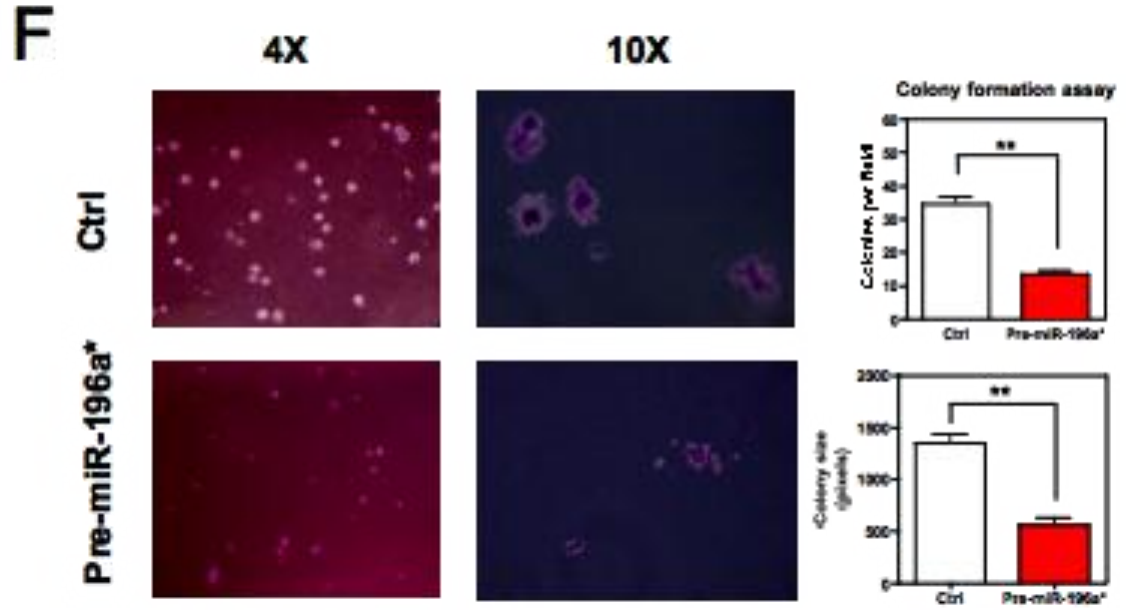

Figure 3.8 TP63 expression in breast cancer cell lines and TP63-mediated regulation of cell growth and invasion by miR-196a2 overexpression in MDA-MB-231 cells

(A) TP63 protein expression in ERα-positive and -negative breast cancer cell lines. β-actin was used as a loading control.
(B) Relative levels of miR-196a2 in breast cancer cell lines.
(C) Overexpression of miR-196a2 in MDA-MB-231 cells. Cells were transfected with Pre-miR-196a* or negative control (Ctrl). Total RNA was harvested and subjected to RT-PCR using specific primers for each miRNA. Expression levels of each miRNA were determined using Taqman probe based quantitative PCR.
(D) Impact of miR-196a2 overexpression on MDA-MB-231 cell proliferation. Cells were transfected with 20 nM Pre-miR-196a* and then were seeded at a concentration of 1,000 cells/well in 100 ml of culture medium. Cell proliferation was assessed using a WST-1 kit. ** *p*-value < 0.01
(E) MDA-MB-231 cells were transfected with Pre-miR-196a* or negative control (Ctrl) then analyzed for *in vitro* invasion assay as described in Materials and Methods. ** *p*-value < 0.01
(F) MDA-MB-231 cells were transfected with Pre-miR-196a* or negative control (Ctrl) then analyzed for soft agar colony formation assay as described in Materials and Methods. Colony numbers per field were counted. Colony size was measured using ImageJ software. ** *p*-value < 0.01