Roles of the ER- α 36-EGFR/HER2 Positive Regulatory Loops in Tamoxifen Resistance

Li Yin[#] and Zhao-Yi Wang^{#*}

Departments of Medical Microbiology and Immunology, Creighton University
Medical School, 2500 California Plaza, Omaha, NE, USA;

#Current address: Beijing Shenogen Pharma Group, Building B, No. 29, life science park road, Beijing, China 102206.

**Correspondence to* Zhao-Yi Wang, Beijing Shenogen Pharma Group, Building B, No. 29, life science park road, Beijing, China 102206.
E. Mail: charlie.wang@shenogen.com

Short Title: The ER- α 36-EGFR/HER2 loops in tamoxifen resistance.

Abstract

Tamoxifen provided a successful treatment for ER-positive breast cancer for the past four decades. However, most breast tumors are eventually resistant to tamoxifen therapy. Extensive researches were conducted to understand the molecular mechanisms involved in tamoxifen resistance, and have revealed that multiple signaling molecules and pathways such as EGFR and HER2 are involved in tamoxifen resistance. Currently, the mechanisms by which tamoxifen sensitive breast cancer cells acquire these signaling pathways and develop tamoxifen resistance have not been elucidated. The identification of ER- α 36, a variant of ER- α , that is able to mediate agonist activity of tamoxifen provided great insights into the underlying mechanisms of tamoxifen resistance. In this review, we will discuss the biological function and the possible underlying mechanisms of ER- α 36 in tamoxifen resistance and specifically illustrate a novel cross-talk mechanism; positive regulatory loops between the ER- α 36 and EGFR/HER2 in tamoxifen resistance. The function and the underlying mechanisms of ER- α 36 in tamoxifen resistance of the breast cancer stem/progenitor cells will also be discussed. Finally, we will postulate a novel approach to restore tamoxifen sensitivity in tamoxifen resistant breast cancer cells.

Key Words: ER- α 36, EGFR, HER2, breast cancer, tamoxifen resistance.

1. Introduction

Endocrine therapy using tamoxifen is currently the most effective treatment for advanced ER-positive breast cancer. Tamoxifen acts through the estrogen receptor (ER) pathway, which has been proven to reduce relapse, death rates and the risk of contralateral breast cancer. However, patients often develop resistance to tamoxifen, which limits its effectiveness [1-3]. Many researches were conducted to understand the molecular pathways involved in tamoxifen resistance and have revealed that multiple signaling molecules and pathways such as EGFR and HER2 are involved in tamoxifen resistance [4-6]. All of these pathways bypass the requirement of estrogen signaling for growth of ER-positive breast cancer cells and render breast cancer cells tamoxifen resistant.

Previously, our laboratory identified and cloned a variant of ER- α , ER- α 36, which has a molecular weight of 36-kDa [7, 8]. The transcript of ER- α 36 is initiated from a previously unidentified promoter in the first intron of the ER- α gene [9]. This ER- α differs from the original 66 kDa ER- α (ER- α 66) because it lacks both transcriptional activation domains (AF-1 and AF-2) but retains the DNA-binding and dimerization domains, and partial ligand-binding domain [8]. ER- α 36 is mainly localized near the plasma membrane and mediates rapid estrogen signaling [8]. A retrospective study of breast cancer patients revealed that about 40% cases of total human breast cancer patients were positive for ER- α 36; about 40% cases ER-positive breast cancer co-expressed ER- α 66 and ER- α 36 and about 40% cases of ER-negative breast cancer that lacked ER- α 66 expression were positive for ER- α 36 [10]. The breast cancer patients with tumors expressing high levels of ER- α 36 less benefited from tamoxifen therapy than those with low levels of ER- α 36 expression did, and ER- α 36 expression is significantly associated with the HER2 expression [10]. These results indicate that enhanced level of ER- α 36 expression in breast cancer cells is one of the mechanisms involved tamoxifen resistance.

In this review, we will specifically illustrate a novel cross-talk mechanism; positive regulatory loops between ER- α 36 and EGFR/HER2 in tamoxifen resistance.

2. Early events of the rapid estrogen signaling mediated by ER- α 36

It becomes clear now that estrogens elicit two signaling pathways; the first requires hours to days to result in transcriptional changes of target genes and is known as genomic, classic or nuclear signaling pathway while the second occurs in seconds to minutes after estrogen treatment and usually initiates at the plasma membrane. Thus, the second signaling pathway is also known as a non-classic, non-genomic, extra-nuclear or rapid signaling pathway that is mediated by estrogen-binding proteins associated with the plasma membrane and employs various cytoplasmic signaling proteins, growth factor receptors and G-protein-coupled-receptor signaling pathways [11-14].

Laboratory evidence indicated that both ER- α and ER- β are involved in the rapid estrogen signaling; transient transfection of the nuclear ER- α cDNA into ER-“null” cells leads to expression of both membrane and nuclear ER- α [15]. Thus, it is postulated that

the membrane-associated ER- α is derived from the same transcript as the nuclear ER- α . However, experimental evidence also suggests that more than one rapid signaling pathway is associated with estrogen action. Results from several laboratories using the membrane-impermeable compound, E2 β conjugated with bovine serum albumin (E2 β -BSA), indicated the existence of two functionally distinct pathways: one sensitive to anti-estrogens and one resistant [16-18]. These findings suggest that another membrane-based ER may exist since all known ERs are sensitive to anti-estrogen inhibition.

Previously, Razandi et al., reported that HB-EGF released by matrix metalloproteinases (MMP)-2 and -9 that are activated by the G protein-coupled receptors via the Src signaling is responsible for estrogen-induced activation of the EGFR and the MAPK/ERK in MCF7 cells [19]. Later, Song et al., reported that IGF-1R is also involved in the rapid estrogen signaling of MCF7 cells [20]. Previously, our group reported that ER- α 36 physically interacts with the EGFR and HER2 in breast cancer cells [21, 22]. After E2 β treatment, ER- α 36 was gradually dissociated from the EGFR and associated with Src and Shc, suggesting that ER- α 36 might dynamically change its partners with the EGFR/Src/Shc complex during the rapid estrogen signaling [21]. Recently, our group further reported that E2 β induced the MAPK/ERK activation in MCF7 cells expressing high levels of recombinant ER- α 36 while ER- α 36 expression knocked-down totally abolished the rapid estrogen signaling in MCF7 cells that still retained ER- α 66 expression [23]. Using neutralizing antibodies and different chemical inhibitors, we found that that Src, MMPs, HB-EGF, AREG, IGF-1R, EGFR and HER2 are all involved in the rapid estrogen signaling mediated by ER- α 36 in ER-positive breast cancer MCF7 cells [23, Figure 1]. Thus, ER- α 36 is the estrogen receptor that mediates rapid estrogen signaling that employs the receptors, IGF-1R, EGFR and HER2. Estrogen induces a coordinated expression of ER- α 36, EGFR and HER2 in MCF7 cells [23], which facilitates the ER- α 36-mediated rapid estrogen signaling. Taken together, the ER- α 36-mediated rapid estrogen signaling is a synchronized process that requires a number of growth factors and their receptors, consistent with the previous view that estrogens acts as growth factors [24, 25].

3. The ER- α 36 and the EGFR/HER2 positive regulatory loops

The EGFR is essential for ductal morphogenesis during normal mammary gland development [26] and its overexpression either at the gene or protein levels is well documented in breast cancer [27]. It has been reported that the cross-communication between EGFR and ER- α 66 leads to serine phosphorylation of ER- α 66 and ligand-independent activation of the ER- α 66-mediated transcription [28, 29]. Conversely, the EGF signaling is strongly enhanced by the ER- α 66 in ER-positive MCF7 cells and anti-estrogen ICI 182, 780 was able to block the EGF signaling [30]. The bidirectional signaling between the EGFR and ER- α 66 has been extensively reviewed before [31].

HER2 is amplified and/or overexpressed in approximately 15-20% of breast cancers [32, 33]. HER2-overexpressing breast cancer patients exhibit a poor prognosis because of

a high incidence of metastases, disease progression, and resistance to current endocrine therapy regimens in the tumors co-expressing ER- α 66 [33-35].

Previously, we discovered the existence of the cross-regulatory loops between ER- α 36 and the EGFR/HER2 [21, 22]; ER- α 36 positively regulates EGFR and HER2 expression while the EGFR and HER2 up-regulate the promoter activity of ER- α 36 through an Ap1 binding site located in the 5' flanking sequence of ER- α 36 gene. We further revealed that ER- α 36 stabilized EGFR protein and ER- α 36-mediated estrogen signaling induced the HER2 promoter activity [21, 22]. Recently, Li et al. reported that transfection of an ER- α 36 expression vector into MCF7 cells induced the EGFR expression [36]. We found that estrogen treatment increased the steady state levels of EGFR and HER2 proteins in a pattern similar to ER- α 36 and the dual kinase inhibitor lapatinib diminished estrogen-mediated induction of ER- α 36 [37]. Thus, the fact that both EGFR and HER2 are involved in the rapid estrogen signaling indicates that coordinated induction of EGFR, HER2 and ER- α 36 expression by estrogen is a pre-requisite for the rapid estrogen signaling mediated by ER- α 36.

A significant correlation between ER- α 36 and HER2 expression has been reported in breast cancer patients [10]. Co-expression of ER- α 36 and HER2 is also detected in ER-negative apocrine breast cancer [38]. In addition, a correlation of ER- α 36 and EGFR expression has been found in endometrial cancer patients [39]. Thus, it is possible that the ER- α 36 and the EGFR/HER2 positive regulatory loops play important roles in the initiation and progression of estrogen-related human tumors.

4. ER- α 36 and tamoxifen resistance

Despite the significant anti-tumor activities of tamoxifen, most breast tumors are eventually resistant to tamoxifen therapy. Essentially, two forms of tamoxifen resistance occur: *de novo* and acquired resistance [2, 3, 40]. About 40-50% ER-positive tumors are already resistant to tamoxifen by the time of diagnosis; the *de novo* resistance. However, the underlying mechanisms of the *de novo* resistance in these ER-positive tumors are largely unknown [2, 3]. In addition, most initially responsive breast tumors gradually acquire tamoxifen resistance. Many researches were conducted to understand the molecular pathways involved in acquired tamoxifen resistance and have revealed that multiple signaling molecules and pathways such as EGFR and HER2 are involved [4-6]. Gained signaling through these pathways often bypasses the requirement of estrogen signaling for growth of ER-positive breast cancer cells.

Previously, we found that antiestrogens tamoxifen and ICI 182, 780 failed to block ER- α 36-mediated rapid estrogen signaling [8, 41]. Recently, we also found that ER- α 36 mediated agonist activities of tamoxifen such as activation of the MAPK/ERK and the PI3K/AKT signaling pathways in ER-negative breast cancer cells and endometrial cancer cells that express high levels of endogenous ER- α 36 [42, 43]. In cells expressing ER- α 36, the effects of tamoxifen exhibited a non-monotonic, or biphasic dose response curve;

tamoxifen at low concentrations, elicited a mitogenic signaling pathway to stimulate cell proliferation while at high concentrations, tamoxifen inhibited cell growth [42], consistent with the previous findings that some breast cancers may be initially growth inhibited by tamoxifen, and later become dependent on tamoxifen for proliferation [44, 45].

Recently, we found that tamoxifen induced ER- α 36 expression in tamoxifen sensitive breast cancer MCF7 cells, and ER- α 36 is then able to mediate agonist activity of tamoxifen [46]. In addition, knockdown of the ER- α 36 expression in tamoxifen resistant breast cancer cells restored tamoxifen sensitivity in these cells [47]. Thus, the gained ER- α 36 expression is one of the underlying mechanisms of acquired tamoxifen resistance.

5. The ER- α 36 and EGFR/HER2 positive regulatory loops in tamoxifen resistance

Both experimental and clinical evidence have indicated that the HER2 and EGFR signaling pathways are involved in tamoxifen resistance. Laboratory evidence has shown that estrogen-dependent MCF7 cells that over-express HER2 are rendered breast cancer cells tamoxifen resistant [48]. Hence the HER2 pathway has been extensively investigated for its contribution towards development of tamoxifen resistance and now HER2 has been proposed as a potential marker of tamoxifen sensitivity.

Recently, our group has reported another level of cross-talk mechanism between the growth factor and estrogen signaling pathways; positive regulatory loops between ER- α 36 and EGFR/HER2 in breast cancer cells [21, 22]. Recently, we found that tamoxifen induced ER- α 36, EGFR and HER2 expression in tamoxifen sensitive MCF7 cells and tamoxifen failed to induce EGFR and HER2 expression in MCF7 cells with knocked-down level of ER- α 36 [46]. In addition, MCF7/TAM cells selected by long-term cultivation in the presence of tamoxifen also expressed elevated levels of endogenous ER- α 36, EGFR and HER2, and knockdown of ER- α 36 expression in MCF7/TAM cells reduced EGFR and HER2 expression [47]. MCF7 cells with forced expression of ER- α 36 also expressed increased levels of EGFR and HER2 [47]. These results are consistent with the previous findings that ER- α 36 is required for both EGFR membrane localization and E2 β -mediated stimulation of EGFR expression in TCam-2 cells [49]. The EGFR and HER2 dual kinase inhibitor Lapatinib blocked tamoxifen induction of ER- α 36 expression [46]. Thus, the gained expression of the ER- α 36 and EGFR/HER2 positive regulatory loops is one of the mechanisms by which tamoxifen sensitive breast cancer cells acquire the growth factor signaling pathways and develop acquired tamoxifen resistance. These positive regulatory loops bypass the requirement of estrogen signaling for growth and elevated level of ER- α 36 also mediates agonist activity of tamoxifen.

6. Disruption of the ER- α 36 and EGFR/HER2 positive regulatory loops restores tamoxifen sensitivity.

As described above, the ER- α 36-EGFR/HER2 positive regulatory loops are one of the mechanisms by which tamoxifen sensitive breast cancer cells gained expression of the

growth factor receptors during tamoxifen treatment. Enhanced expression of EGFR, HER2 and ER- α 36 was found in tamoxifen resistant MCF7 cells [47]. Thus, it is possible that disruption of the ER- α 36 and EGFR/HER2 positive regulatory loops may restore tamoxifen sensitivity in tamoxifen-resistant cells.

In preclinical studies, a combination of trastuzumab and tamoxifen treatment has been demonstrated to result in synergistic growth inhibition of HER2 expressing breast cancer cells [50, 51]. The dual kinase inhibitor, Lapatinib, also has been shown to cooperate with tamoxifen to inhibit cell proliferation and estrogen-dependent gene expression in tamoxifen-resistant breast cancer cells [52]. It thus was proposed that combination of growth factor and estrogen receptor targeted therapies may provide a novel and effective approach to treatment of tamoxifen resistant breast cancer.

Recently, we discovered that knock-down of ER- α 36 expression downregulated both HER2 and EGFR expression in HER2-overexpressing BT474 cells and tamoxifen resistant MCF7 cells, and restored tamoxifen sensitivity in these cells [47, 53]. In addition, inhibition of both the EGFR and HER2 signaling pathways with the dual kinase inhibitor Lapatinib disrupted the positive regulatory loops, downregulated ER- α 36 expression and restored tamoxifen sensitivity [46], which provided a novel molecular mechanism to the function of the dual kinase inhibitor Lapatinib; disruption of the ER- α 36-EGFR/HER2 positive regulatory loops.

Previously, we found that the potent ER- α 66 disruptor ICI 182, 780 failed to degrade ER- α 36 due to the lacking of the critical Helix 12 in the C-terminal of ER- α 36 protein [41]. Recently, we reported that a falconoid, Brousoflavonol B (5, 7, 3', 4'-Tetrahydroxy-3-methoxy- 6,8-diprenylflavone) purified from the bark of *Broussonetia papyrifera* was able to down-regulate ER- α 36 expression and inhibited proliferation of ER-positive and -negative breast cancer cells [54, 55]. Brousoflavonol B was also able to disrupt the ER- α 36-EGFR/HER2 positive regulatory loops; downregulated ER- α 36, HER2 and EGFR, which restored tamoxifen sensitivity in tamoxifen resistant breast cancer cells [46, 53]. Taken together, disruption of the ER- α 36 and EGFR/HER2 positive regulatory loops through either inhibition of the ER- α 36-mediated signaling or the EGFR/HER2 signaling may provide novel approaches to restore tamoxifen sensitivity in tamoxifen resistant breast cancer cells.

7. Perspective

Accumulating experimental and clinical evidence indicate that breast cancer arises from mammary stem/progenitor cell populations [56]. Recently, we reported that ER-positive breast cancer stem/progenitor cells express high levels of the ER- α 36 and EGFR/HER2 positive regulatory loops, and knock-down of ER- α 36 expression reduced the stem/progenitor cell population in breast cancer cells [22, 46, 57]. Thus, the ER- α 36-mediated rapid estrogen signaling and the ER- α 36 and EGFR/HER2 positive regulatory

loops play important roles in maintenance and regulation of breast cancer stem/progenitor cells.

The possible involvement of breast cancer stem/progenitor cells in tamoxifen resistance has been proposed [58] and demonstrated [59]. Recently, we reported that ER-positive breast cancer stem/progenitor cells were relatively more resistant to tamoxifen than the bulk cells [60]. We found that tamoxifen treatment enriched breast cancer stem/progenitor cells and also stimulated proliferation of breast cancer progenitor/intermediate cells as well as self-renewal of breast cancer stem cells [60]. We also found that tamoxifen resistant MCF7 cells contain high percentage of cancer stem/progenitor cells [60]. Thus, our results suggested that high level of the ER- α 36 and EGFR/HER2 positive regulator loops in ER-positive breast cancer stem/progenitor cells is one of the mechanisms by which the ER-positive breast cancer stem/progenitor cells are relatively more resistant to tamoxifen than the bulk cells. Again, disruption of these regulatory loops with Lapatinib or Broussonol B was able to sensitize these breast cancer stem/progenitor cells to tamoxifen [46].

A better understanding of how ER-positive breast cancer cells that drives breast tumor resistance to tamoxifen therapy is an essential step to advance our knowledge of breast cancer and to improve management. Our current results provided strong evidence to support an important role of the ER- α 36 and EGFR/HER2 positive regulatory loops in tamoxifen resistance and provided a rational for development of therapeutic approaches to overcome tamoxifen resistance by targeting the ER- α 36 and EGFR/HER2 positive regulatory loops.

Acknowledgements

This work was supported by Department of Defense grant DAMD 11-1-0497 and by Nebraska Tobacco Settlement Biomedical Research Program Award LB595 to Z.Y. Wang.

References:

1. Macgregor JJ, Jordan V.G. Basic guide to the mechanisms of antiestrogens action. *Pharmacological Review* 1998; 50: 151-196.
2. Clarke R, Leonessa F, Welch, JN, Skaar TC. Cellular and molecular pharmacology of antiestrogens action and resistance. *Pharmacological Review* 2001; 53: 25-71.
3. Clarke R, Liu MC, Bouker KB, Gu ZP, Lee RY, Zhu YL, Shaar TC, Gomez B, O'Brien K, Wang Y, Hilakivi-Clarke LA. Antiestrogen resistance in breast cancer and the role of estrogen receptor signaling. *Oncogene* 2003; 22: 7316-7339.
4. Normanno N, Di Maio M, De Maio E, De Luca A, Matteis Ad, Giordano A, Perrone F. Mechanisms of endocrine resistance and novel therapeutic strategies in breast cancer. *Endocrine-Related Cancer* 2005; 12: 721-747.
5. Dorssers L C J, van der Flier S, Brinkman A, van Agthoven T, Veldscholte J, Berns EMJJ, Klijn JGM, Beex LVAM, Foekens JA. Tamoxifen Resistance in Breast Cancer: Elucidating Mechanisms. *Drugs* 2001; 61: 1721-1733.
6. Osborne CK, Schiff R. Mechanisms of endocrine resistance in breast cancer. *Ann Rev. Med* 2011; 62: 233-247.
7. Wang ZY, Zhang XT, Shen P, Loggie BW, Chang YC, Deuel TF. Identification, cloning and expression of human estrogen receptor- α 36, a novel variant of human estrogen receptor- α . *Biochem. Biophys. Res. Commun* 2005; 336: 1023-1027.
8. Wang ZY, Zhang XT, Shen P, Loggie BW, Chang YC, Deuel TF. A variant of estrogen receptor- α , hER- α 36: transduction of estrogen and anti-estrogen dependent membrane-initiated mitogenic signaling. *Proc. Natl. Acad. Sci. U.S.A.* 2006; 103: 9063-9068.
9. Zou Y, Ding L, Coleman M, Wang ZY. Estrogen receptor- α (ER- α) suppresses expression of its variant ER- α 36. *FESEB Letters* 2009; 583: 1368–1374.
10. Shi L, Dong B, Li ZW, Lu YW, Ouyang T, Li JF, Wang TF, Fan ZQ, Fan T, Lin BY, Wang ZY, Xie YT. Expression of ER- α 36, a novel variant of estrogen receptor- α , and resistance to tamoxifen treatment in breast cancer. *J. Clinical Oncology* 2009; 27: 3423-3429.
11. Driggers PH, Segars JH. Estrogen action and cytoplasmic signaling pathways. Part II: the role of growth factors and phosphorylation in estrogen signaling. *Trends Endocrinol Metab* 2002; 13: 422-427.
12. Segars JH, Driggers PH. Estrogen action and cytoplasmic signaling cascades. Part I: membrane-associated signaling complexes. *Trends Endocrinol Metab* 2002; 13: 349-354.
13. Kelly MJ, Levin ER. Rapid actions of plasma membrane estrogen receptors. *Trends Endocrinol Metab* 2001; 12: 152-156.
14. Hammes SR, Levin ER. Extranuclear steroid receptors: nature and actions. *Endocr. Rev* 2007; 28: 726-741.
15. Razandi M, Pedram A, Greene GL, Levin ER. Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ER- α and ER- β expressed in Chinese hamster ovary cells. *Mol Endocrinol* 1999; 13: 307-319.
16. Russell KS, Haynes MP, Sinha D, Clerisme E, Bender JR. Human vascular endothelial cells contain membrane binding sites for estradiol, which mediate rapid intracellular signaling. *Proc Natl Acad Sci U S A.* 2000; 97: 5930-5935.

17. Chen Z, Yuhanna IS, Galcheva-Gargova Z, Karas RH, Mendelsohn ME, Shaul PW. Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. *J Clin. Invest.* 1999; 103: 401-406.
18. Watters JJ, Campbell JS, Cunningham MJ, Krebs EG, Dorsa DM. Rapid membrane effects of steroids in neuroblastoma cells: effects of estrogen on mitogen activated protein kinase signaling cascade and c-fos immediate early gene transcription. *Endocrinology* 1997; 138: 4030-4033.
19. Razandi, M., Pedram, A., Park, ST., Levin, ER. Proximal events in signaling by plasma membrane estrogen receptors. *J Biol Chem* 2003; 278: 2701-2712.
20. Song RX, Zhang Z, Chen Y, Bao Y, Santen RJ. Estrogen signaling via a linear pathway involving insulin-like growth factor I receptor, matrix metalloproteinases, and epidermal growth factor receptor to activate mitogen-activated protein kinase in MCF-7 breast cancer cells. *Endocrinology* 2007; 148: 4091-4101.
21. Zhang XT, Kang LG, Ding L, Vranic S, Gatalica Z, Wang ZY. A positive feedback loop of ER-alpha36/EGFR promotes malignant growth of ER-negative breast cancer cells. *Oncogene* 2001; 30: 770-780.
22. Kang L, Guo YM, Zhang XT, Wang ZY. A positive cross-regulation of HER2 and ER-alpha36 controls ALDH1 positive breast cancer cells. *J Steroid Biochem Mol Biol* 2011; 127: 262-268.
23. Zhang XT, Deng H, Wang ZY. Estrogen activation of the mitogen-activated protein kinase is mediated by ER-alpha36 in ER-positive breast cancer cells. *Journal of Steroid Biochemistry & Molecular Biology* 2014; 143: 434-443.
24. Migliaccio A, Castoria G, Di Domenico M, de Falco A, Bilancio A, Lombardi M, Bottero D, Varricchio L, Nanayakkara M, Rotondi A, Auricchio F. Sex steroid hormones act as growth factors. *J Steroid Biochem Mol Biol* 2002; 83: 31-35.
25. Shupnik MA. Crosstalk between steroid receptors and the c-Src-receptor tyrosine kinase pathways: implications for cell proliferation. *Oncogene* 2004; 23: 7979-7989.
26. Troyer KL, Lee DC. Regulation of mouse mammary gland development and tumorigenesis by the ERBB signaling network. *J Mammary Gland Biol. Neoplasia.* 2001; 6:7-21.
27. Kumar R, Wang RA. Protein kinases in mammary gland development and cancer. *Microsc Res Tech* 2002; 59: 49-57.
28. Bunone G, Briand PA, Miksicek RJ, Picard D. Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *EMBO J.* 1996; 15: 2174-2183.
29. Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D, Chambon P. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* 1995; 270: 1491-1494.
30. Migliaccio A, Castoria G, Di Domenico M, Ciociola A, Lombardi M, De Falco A, Nanayakkara M, Bottero D, De Stasio R, Varricchio L, Auricchio F. Crosstalk between EGFR and extranuclear steroid receptors. *Ann N Y Acad Sci* 2006; 1089: 194-200.
31. Levin ER. Bidirectional signaling between the estrogen receptor and the epidermal growth factor receptor. *Mol. Endocrinol* 2003; 17: 309-317.

- 32 Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987; 235: 177-182.
- 33 Chia S, Norris B, Speers C, Cheang M, Gilks B, Grown AM, Huntsman D, Olivotto IA, Nielsen TO, Gelmon K. Human epidermal growth factor receptor 2 overexpression as a prognostic factor in a large tissue microarray series of node-negative breast cancers. *J Clin Oncol* 2008; 26: 5697-5704.
- 34 Wright C, Angus B, Nicholson S, Sainsbury JR, Cairns J, Gullick WJ, Kelly P, Harris CH. Expression of c-erbB-2 oncoprotein: a prognostic indicator in human breast cancer. *Cancer Res* 1989; 49: 2087-2090.
- 35 Pegram MD, Pauletti G, Slamon DJ. HER-2/neu as a predictive marker of response to breast cancer therapy. *Breast Cancer Res Treat* 1998; 52: 65-77.
36. Li G, Zhang J, Jin K, He K, Zheng Y, Xu X, Wang H, Wang H, Li Z, Yu X, Teng X, Cao J, Teng L. Estrogen receptor- α 36 is involved in development of Acquired Tamoxifen Resistance via regulating the growth status switch in breast cancer cells. *Molecular Oncology* 2013; 7: 611-624.
37. Zhang, X. T., Deng H., Wang Z. Y. Estrogen activation of the mitogen-activated Protein kinase is mediated by ER- α 36 in ER-positive breast cancer cells. *Journal of Steroid Biochemistry and Molecular Biology* 2014; 143: 434-443.
38. Vranic S, Gatalica, Z, Deng H, Frkovic-Grazio S, Lee MJ, Olga Gurjeva O, Wang, ZY. ER-alpha36, a novel isoform of ER-alpha66, is commonly over-expressed in apocrine and adenoid cystic carcinomas of the breast. *J of Clinical Pathology* 2011; 64: 54-57.
- 39 Tu BB, Lin SL, Yan LY, Wang Z-Y, Sun QY, Qiao J. ER-alpha36, a novel variant of estrogen receptor alpha, is involved in EGFR-related carcinogenesis in endometrial cancer. *Am J Obstet Gynecol* 2011; 205: 227: e1-6.
- 40 Ring A, Dowsett M. Mechanisms of tamoxifen resistance. *Endocr Relat Cancer* 2004; 11: 643-658.
- 41 Kang LG, Wang ZY. Breast cancer cell growth inhibition by phenethyl isothiocyanate is associated with down-regulation of oestrogen receptor-alpha36. *J Cell Mol Med*. 2010; 14: 1485- 1493.
- 42 Zhang XT, Ding L, Kang LG, Wang Z-Y. Estrogen receptor alpha36 mediates mitogenic antiestrogen signaling in ER-negative breast cancer cells. *PLoS ONE* 2012; 7: e30174.
- 43 Lin SL, Yan LY, Zhang XT, Yuan J, Li M, Qiao J, Wang ZY, Sun QY. ER alpha36, a variant of ER-alpha, promotes tamoxifen agonist action in endometrial cancer cells via the MAPK/ERK and PI3K/Akt pathways. *PLoS One* 2010; 5: e9013.
- 44 Osborne CK, Coronado EB, Robinson JP. Human breast cancer in the athymic nude mouse: cytostatic effects of long-term antiestrogen therapy. *Eur J Cancer Clin Oncol*. 1987; 23: 1189-1196.
- 45 Gottardis MM, Jordan VC. Development of tamoxifen stimulated growth of MCF-7 tumors in athymic mice after a long-term antiestrogen administration. *Cancer Res* 1988; 48: 5183-5187.

46. Yin L, Zhang XT, Bian XW, Guo YM, Wang ZY. Disruption of the ER- α 36-EGFR/HER2 positive regulatory loops restores tamoxifen sensitivity in tamoxifen resistance breast cancer cells. *PLoS ONE* 2014; 9: e107369.
47. Zhang XT, Wang ZY. Estrogen receptor- α variant, ER- α 36, is involved in tamoxifen resistance and estrogen hypersensitivity. *Endocrinology* 2013; 154: 1990-1998
48. Benz CC, Scott GK, Sarup JC, Johnson RM, Tripathy D, Coronado E, Shepard HM, Osborne CK. Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with her2/neu. *Breast Cancer Res Treat* 1992; 24: 85-95.
49. Wallacides A, Chesnel A, Ajj H, Chillet M, Flament S, Dumond H. Estrogens promote proliferation of the seminoma-like TCam-2 cell line through a GPER-dependent ER α 36 induction. *Mol Cell Endocrinol* 2012; 350: 61-71.
50. Kurokawa H, Arteaga CL. Inhibition of erbB receptor (HER) tyrosine kinases as a strategy to abrogate antiestrogen resistance in human breast cancer. *Clin Cancer Res* 2001; 7: 4436s-4442s; discussion 4411s-4412s.
51. Wang CX, Koay DC, Edwards A, Lu Z, Mor G, Ocal IT, DiGiovanna MP: In vitro and in vivo effects of combination of trastuzumab (Herceptin) and tamoxifen in breast cancer. *Breast Cancer Res Treat* 2005; 92: 251-263.
52. Chu I, Blackwell K, Chen S, Slingerland J. The dual ErbB1 and ErbB2 inhibitor, Lapatinib (GW572016), cooperates with tamoxifen to inhibit both cell proliferation and estrogen-dependent gene expression in antiestrogen-resistant breast cancer. *Cancer Res* 2005; 65: 18-25.
53. Yin L, Pan XH, Zhang XT, Guo YM, Wang ZY, GongYQ, WangML. Downregulation of ER- α 36 expression sensitizes HER2 overexpressing breast cancer cells to tamoxifen. *Am J Cancer Res* 2015; 5:530-544.
54. Guo MX, Wang ML, Zhang XY, Deng H, Wang ZY. Brousoflavonol B restricts growth of ER-negative breast cancer stem-like cells. *AntiCancer Research* 2013; 33:1873-1880.
55. Guo MX, Wang ML, Zhang XT, Deng H, Wang ZY. A novel anticancer agent Brousoflavonol B Downregulates ER- α 36 expression and inhibits growth of ER-negative breast cancer MDA-MB-231 cells. *European Journal of Pharmacology* 2013; 714: 56-64.
56. Oliveira, LR, Jeffrey SS, Ribeiro-Silva A. Stem cells in human breast cancer. *Histol. Histopathol.* 2010; 25: 371-385.
57. Deng H, Zhang XT, Wang ML, Zheng HY, Liu LJ, Wang ZY. ER- α 36-mediated rapid estrogen signaling positively regulates ER-positive breast cancer stem/progenitor Cells. *PLoS ONE* 2014; e88034.
58. O'Brien CS, Howell SJ, Farnie G, Clarke RB. Resistance to endocrine therapy: are breast cancer stem cells culprits. *J. Mammary Gland Biol Neoplasia* 2009, 14: 45-54.
59. Piva M, Domenici G, Iriondo O, Rábano M, Simões BM, Comaills V, Barredo I, López-Ruiz JA, Zabalza I, Kypta R. Sox2 promotes tamoxifen resistance in breast cancer cells. *EMBO Mol Med.* 2013; 5: 1-14.
60. Deng H, Yin L, Zhang XT, Liu LJ, Wang ML, Wang ZY. ER- α variant ER- α 36 mediates antiestrogen resistance in ER-positive breast cancer stem/progenitor cells. *J Steroid Biochem Mol Biol* 2014; 144: 417-426.

Figure 1. The signaling pathways of ER-α36-mediated rapid estrogen signaling and the ER-α36-EGFR/HER2 positive regulatory loops. ER-α36 mediates E2β-stimulated release of HB-EGF presumably through GPCR. HB-EGF then activates EGFR, HER2 and signaling proteins such as c-Src as well as adaptor proteins such as Shc, which mediates the rapid estrogen signaling via the PI3K/AKT and MAPK/ERK signaling pathways. ER-α36-mediated rapid estrogen signaling also involves direct activation of the STAT3/5 through the EGFR/Src. ER-α36-mediated estrogen and tamoxifen signaling enhances EGFR protein stability presumably through physical interaction between ER-α36 and EGFR while the EGFR signaling pathway up-regulates the promoter activity of ER-α36 through an Ap1 binding site in the 5' flanking sequence of ER-α36 gene. ER-α36-mediated signaling also positively regulates HER2 transcription while the HER2 signaling up-regulates the promoter activity of ER-α36.