

Award Number: W81XWH-04-1-0392

TITLE: cSrc and Her2 Signaling Pathways Cooperate with Estrogen to Promote ER Phosphorylation, Ubiquitination and Proteolysis in ER Negative Breast Cancers

PRINCIPAL INVESTIGATOR: Isabel Chu, Ph.D.

CONTRACTING ORGANIZATION: University of Miami School of Medicine  
Miami, Fl 33136

REPORT DATE: March 2007

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

# REPORT DOCUMENTATION PAGE

*Form Approved*  
*OMB No. 0704-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

<b>1. REPORT DATE</b> 01-03-2007			<b>2. REPORT TYPE</b> Annual Summary		<b>3. DATES COVERED</b> 27 Feb 2004 – 26 Feb 2007	
<b>4. TITLE AND SUBTITLE</b>  cSrc and Her2 Signaling Pathways Cooperate with Estrogen to Promote ER Phosphorylation, Ubiquitination and Proteolysis in ER Negative Breast Cancers					<b>5a. CONTRACT NUMBER</b>	
					<b>5b. GRANT NUMBER</b> W81XWH-04-1-0392	
					<b>5c. PROGRAM ELEMENT NUMBER</b>	
<b>6. AUTHOR(S)</b>  Isabel Chu, Ph.D.  Email: ichu@med.miami.edu					<b>5d. PROJECT NUMBER</b>	
					<b>5e. TASK NUMBER</b>	
					<b>5f. WORK UNIT NUMBER</b>	
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b>  University of Miami School of Medicine Miami, Fl 33136					<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012						
<b>10. SPONSOR/MONITOR'S ACRONYM(S)</b>					<b>11. SPONSOR/MONITOR'S REPORT NUMBER(S)</b>	
<b>13. SUPPLEMENTARY NOTES</b> Original contains colored plates: ALL DTIC reproductions will be in black and white.						
<b>14. ABSTRACT</b> Breast cancer is the most frequent cancer in women. On third of new breast cancers do not express estrogen receptor $\alpha$ (ER) protein and these have a worse prognosis than ER positive breast cancers. The ER is a ligand activated transcription factor. Estrogen:ER binding stimulates rapid Src activation that feeds back to phosphorylate the ER and increases its transcriptional activity. Estrogen binding to the ER rapidly activates ubiquitin-dependent ER proteolysis which in turn regulates ER activity. The data that I present here suggest that Src activates ER proteolysis. Src inhibition impaired estrogen stimulated ER ubiquitylation and proteolysis. The weakly ER positive, MDA-MB-361 and ER negative, BT-20 breast cancer lines both have high Src activity. ER was increased by estrogen deprivation, proteasome inhibition. Src inhibitors impaired ER ubiquitylation and degradation both in vivo and in vitro. Src levels or activity were increased in primary ER negative breast cancers compared to ER positive. We proposed that Src, when recruited by ligand dependent or independent ER activation, leads to ER or co-activator phosphorylation to regulate ubiquitin-dependent ER degradation. Some ER negative breast cancers are estrogen responsive: they express ER mRNA but ER protein levels are undetectable due to accelerated Src mediated ER proteolysis. Oncogenic RTK and Src activation may alter phosphorylation of the ER or of key co-regulators to activate both ER proteolysis and ER target gene transcription. The elucidation of mechanisms underlying ER loss in ER negative breast cancer may indicate why these cancers are so clinically aggressive. Pathways identified may yield new targets for molecular based therapies for this treatment-resistant form of breast cancer.						
<b>15. SUBJECT TERMS</b> cSrc, estrogen receptor, Receptor Tyrosine Kinase (RTK), signal transduction, Phosphorylation						
<b>16. SECURITY CLASSIFICATION OF:</b>				<b>17. LIMITATION OF ABSTRACT</b>	<b>18. NUMBER OF PAGES</b>	<b>19a. NAME OF RESPONSIBLE PERSON</b> USAMRMC
<b>a. REPORT</b> U	<b>b. ABSTRACT</b> U	<b>c. THIS PAGE</b> U	<b>19b. TELEPHONE NUMBER</b> (include area code)			

## Table of Contents

<b>Introduction.....</b>	<b>4</b>
<b>Body.....</b>	<b>4</b>
<b>Key Research Accomplishments.....</b>	<b>12</b>
<b>Reportable Outcomes.....</b>	<b>13</b>
<b>Conclusions.....</b>	<b>14</b>
<b>References.....</b>	<b>15</b>
<b>Appendix I.....</b>	<b>17</b>
<b>Appendix II.....</b>	

## INTRODUCTION

Breast cancer is the most frequent cancer and the second most common cause of death from cancer in women. One third of new breast cancers do not express estrogen receptor  $\alpha$  (ER) protein and these have a worse prognosis than ER positive breast cancers (McClelland et al., 1986). The lack of ER protein cannot be explained by genetic changes or lack of gene expression (Ferguson and Davidson, 1997). Using Real-Time PCR, we showed that 200 ER negative and 50 ER positive breast cancers all express ER mRNA. ER is a ligand activated transcription factor. Estrogen binding to ER stimulates rapid activation of Src and signaling pathways (Song et al., 2004) that affect cell proliferation and feedback to phosphorylate ER and increases its transcriptional activity. ER can also be phosphorylated and activated in a ligand independent manner by mitogenic signaling (Kato et al., 1995; Bunone et al., 1996).

Activation of many transcription factors is linked to their proteolysis. Estrogen binding to ER rapidly activates ubiquitin-dependent ER proteolysis which in turn regulates and limits ER activity (Lonard et al., 2000). The ubiquitin-dependent proteasome may not only activate ER-dependent transcription by regulating co-activator binding but also limit transactivation by degrading the receptor. Many steroid hormone co-activators are also ubiquitin ligases and may regulate both receptor activation and proteolysis. Ubiquitylation involves ubiquitin transfer to an ubiquitin-conjugating enzyme by an ubiquitin-activating enzyme. Ubiquitin ligase binding is often triggered by substrate phosphorylation. Many ubiquitin ligases recognize and bind specifically to appropriately phosphorylated substrate proteins to facilitate their ubiquitylation and proteasomal degradation.

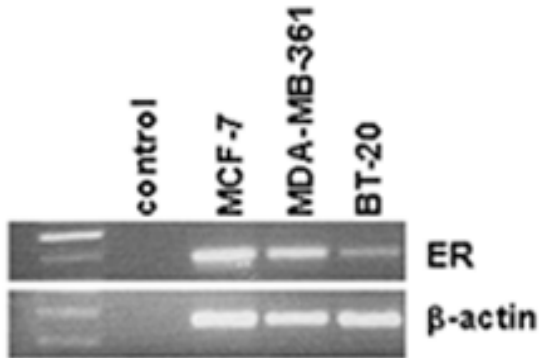
This final report summarizes my findings indicating that estrogen or growth factor signaling leads to Src activation and increased ER ubiquitination. I present new evidence suggesting a direct role of cSrc in mediating ER ubiquitination and proteasomal degradation.

## BODY

During my first year, I investigated the role of cSrc on ER degradation by identifying a potential phosphorylation site. I observed that mutations of Tyrosine 537 to Alanine (Y537A-ER) is more stable and is not degraded in response to estrogen treatment. However, Y537 to phenylalanine (Y537F-ER) was less stable and the steady state level in response to estrogen was decreased. These findings suggest that mutations at this site are critical structurally influencing stability. For this reason, during the next two years, I concentrated in finding the difference between Src phosphotyrosinated ER vs. non-phosphorylated ER rather than working with mutations of Y537-ER since these mutants could result in altered ubiquitination due to structural reasons instead of lack of a phosphate group. A summary of the approaches of my proposed tasks to elucidate the mechanisms involved in ER degradation in response to estrogen stimulation are described below.

**Inhibition of Src increases ER protein levels in breast cancer lines with activated cSrc.**

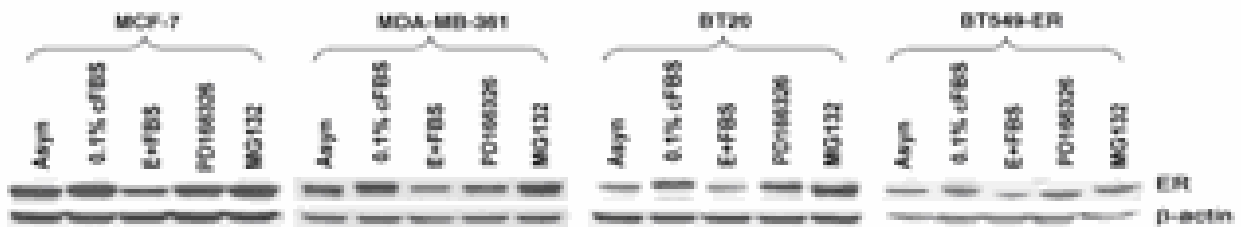
The breast cancer cell line, BT-20 shows both cSrc and EGFR activation while ErbB2 and cSrc are activated in MDA-MB-361(Belsches-Jablonski et al., 2001). BT-20 has been characterized as ER negative, and ER levels are reduced in MDA-MB-361. I detected ER mRNA in MCF-7, BT-20 and MDA-MB-361 (Figure 1).



**Figure 1. Breast cancer lines overexpressing cSrc have reduced ER mRNA.** ER mRNA was assayed in MCF-7, MDA-MB-361 and BT-20 by RT-PCR. Reaction products were resolved and visualized by Gel Doc.

We observed increased Src kinase activity in MDA-MB-361 and BT-20 and decreased ER protein levels in these breast cancer cell lines (Figure 4A, appendix 1) suggesting a correlation between high Src activity and decreased ER protein levels. As in MCF-7, ER accumulated in both BT-20 and MDA-MB-361 following serum and estrogen deprivation or proteasome inhibition (Figure 2). The Src inhibitor PP1 accumulated ER levels in MCF-7 but not in MDA-MB-361 or BT-20 (data not shown). This could be due to the toxicity of PP1 in these Src over expressing cell lines. Thus, I used a less toxic Src inhibitor, PD166326. When this inhibitor was used, we observed abrogation of estrogen dependent ER degradation in MCF-7, MDA-MB-361 and BT-20 (Figure 2). For BT-20, blots were exposed longer and more protein (125 µg vs 20 µg/lane) was loaded than for ER + lines.

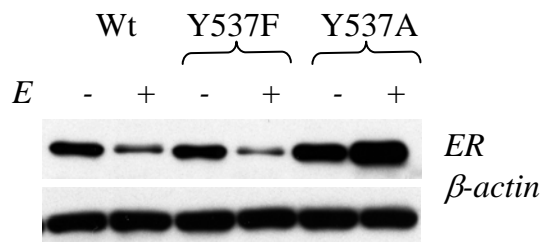
To confirm these findings I created a stable cell line expressing ER. The ER negative BT549 does not express any detectable ER and has elevated Src activation (data not shown). BT-549 expressing ER, ER-BT549, also responded to estrogen for ER degradation. Furthermore, treatment of ER-BT549 with the Src inhibitor prior to estrogen stimulation partially abrogated estrogen dependent ER degradation (Figure 2). Thus, Src appears to activate estrogen stimulated ER proteolysis in both ER+ and ER- breast lines.



**Figure 2** Estrogen activates ER proteolysis (detected by reduced ER 6 h after estrogen addition) in MCF-7, MDA-MB-361, BT-20 and ER-BT549. Re-addition of estrogen to growth factor and estrogen-deprived cells reduced ER levels and this was impaired by pre-treatment with proteasome inhibitor (MG132) or Src inhibitor PD166326.

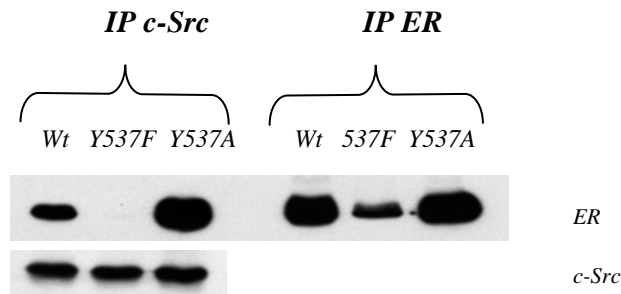
### Critical role of an intact Y537 residue for ER activity and degradation

The tyrosine 537 residue in ER (Y537-ER) has been shown to be phosphorylated by Src *in vitro* (Arnold et al., 1995). We previously observed that mutation of Y537-ER to an alanine (Y537A-ER) was non-responsive to estrogen mediated ER degradation. To investigate if that was the case with the phenylalanine mutant (Y537F-ER), I constructed the Y537F-ER mutant. The F residue has a benzyl ring and resembles the non-phosphomimetic residue of the tyrosine residue. Thus, I transfected WT-, Y537F- and Y537A-ER into MCF-7. I confirmed that ER-Y537A does not get degraded after estrogen stimulation (Figure 3). However, I observed that the Y537F-ER mutant was still degraded after estrogen addition (Figure 3). It is possible that an intact tyrosine residue at that position is very critically structurally for proper ER response to estrogen. Thus, we observed a difference in ER stability when that site is mutated to A or F. Furthermore, Katzellenbogen group has found that mutations of Tyr 537 to different residues affect ER transcriptional activity (Weis et al., 1996). Taken together, these results offer strong evidence of the importance of that site in ER mediated transcriptional regulation and degradation.



**Figure 3.** MCF-7 cells were transfected with WT, Y37F, Y537A-ER plasmids and then arrested for 48 hrs by estradiol depletion. Estradiol was added for 6 hrs and cell lysates collected for Western blot for ER or  $\beta$ -actin

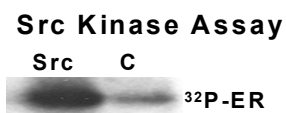
In order to determine if the Y537 site in ER is structurally critical, we investigated by immunoprecipitation the binding affinities of Src to WT-ER vs mutant-ER (Y537F or Y537A). We observed that Src bound more strongly in Y537A-ER mutant, followed by WT-ER and with the weakest and almost non-detectable interaction with Y537F-ER mutant (figure 4). These results suggest that the region at Y537-ER is very important in mediating the interaction with Src. Therefore, it is possible that mutations at this site influence the structure of ER in such a way that it affects Src's affinity to ER. Thus, Y537 seems to play a role not only in degradation, transcription but also in Src's affinity to ER.



**Figure 4.** MCF-7 cells were transfected with WT- Y537F, or Y537A-ER and then immunoprecipitated with an Src or ER antibody. ER and Src were assayed by Western blot.

### Src phosphorylates ER *in vitro*

Ubiquitin mediated proteolysis is often triggered by a substrate phosphorylation event that promotes substrate interaction with its ubiquitin ligase. We proposed that Src once activated through cross-talk and recruitment by ligand-activated ER or through oncogenic activation by RTK signaling in cancer, Src in turn mediates the phosphorylation of the ER and/or key co-regulators to facilitate ER interaction with a number of potential ubiquitin ligases. In order to confirm our previous findings suggesting that Src can phosphorylate ER, I performed an ER *in vitro* kinase assay. We observed that incubation with Src promotes ER phosphorylation as detected by <sup>32</sup>P-ATP (Figure 5).



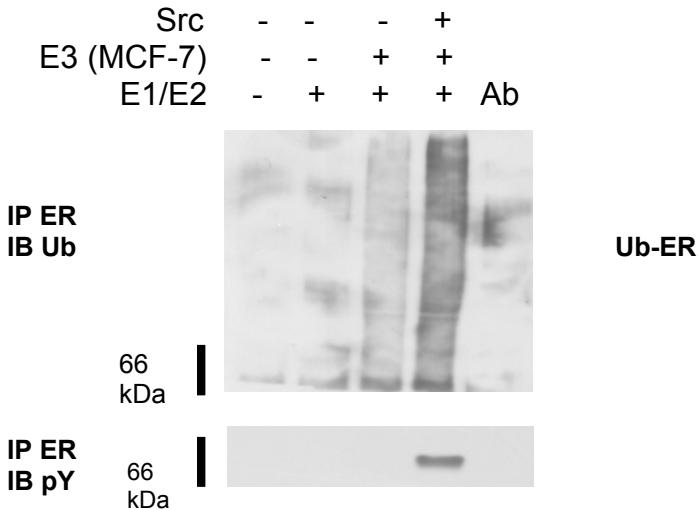
**Figure 5. Src phosphorylates the ER *in vitro*.** Recombinant ER was reacted with recombinant Src (Cell Signaling) under kinase assay conditions for 30 min, resolved by SDS-PAGE, the gel dried and autoradiographed. In the control lane (C), recombinant Src was pre-mixed with Src inhibitor, PP1, prior to the kinase reaction.

I also attempted to identify a phosphotyrosylated site in ER using a tyrosine specific antibody *in vivo*. However, I could not detect phosphotyrosylated ER when I immunoprecipitated ER from MCF-7. This could be due to the limitations in the experimental techniques or because this site is very unstable and thus, the difficulty in its detection *in vivo*

### Src enhances ER ubiquitylation and degradation *in vitro*

To further investigate how Src may regulate the ubiquitylation and degradation of ER, we assayed these effects in an entirely *in vitro* system using purified recombinant ER, ubiquitin, ubiquitin activating enzyme (E1), ubiquitin conjugating enzymes (Ubc) and different sources of putative E3 ubiquitin ligases. I used baculovirus expressed recombinant ER and purified recombinant HA-tagged ubiquitin and ubiquitin activating enzyme (UBA). I used cell lysate as a source of E3 ligase. Recombinant ER was mixed with ubiquitin, UBA, and MCF-7 lysate as the source of E3 ligase and incubated as described (Carrano et al., 1999). ER was precipitated and immunoblotted. We observed increase ER ubiquitylation when cells were incubated with Src in addition to

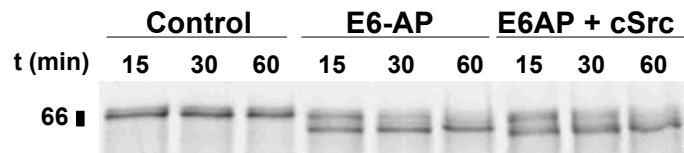
E1, E2 and E3. Our findings indicate that in an *in vitro* system, Src can promote increased ER ubiquitination suggesting a direct involvement of Src in ER ubiquitination. (Figure 6). Furthermore we observed that ER was phosphotyrosinated when probed with a pY antibody (Figure 6). Overall these findings suggest that Src dependent ER phosphorylation increases ER ubiquitination *in vitro*.



**Figure 6 . Src increases ER ubiquitination in vitro.**

Recombinant ER was incubated with E1/E2 and MCF-7 lysate as source of E3 with or without Src and the reactions were allowed to proceed for 30 min. a) Ubiquitinated ER was detected using a Ubiquitin specific antibody and b) pY ER was detected after ER Immunoprecipitation and Probing with a pY antibody.

E6AP is a member of the HECT family of ubiquitin ligases. It was shown to act as a co-activator for a number of nuclear hormone receptors, including the ER. Previous findings have shown that addition of E6AP can promote partial proteolytic ER degradation (Gao et al., 2005). When *in vitro* transcribed and translated ER (IVT ER) is incubated with ubiquitin, UBA and Ubch7 and an ATP source, the addition of E6AP accelerates the appearance of a faster migrating ER. In similar types of assays, we tested whether pre-incubation of the ER with Src would accelerate the E6AP mediated partial proteolysis of the ER. Addition of recombinant Src had no effect on E6AP mediated ER degradation (Figure 7).

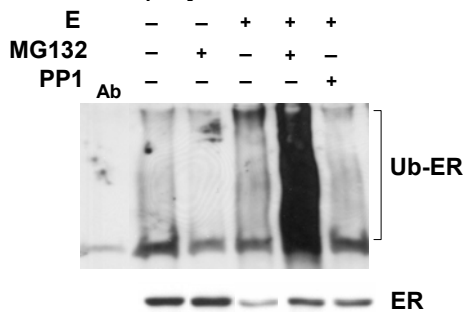


**Figure 7. Src does not activate E6AP-mediated ER proteolysis in vitro.** IVT-ER was subjected to *in vitro* ubiquitin mediated degradation following the method of Nawaz et al(Gao et al., 2005). Addition of E6AP activated ER proteolytic cleavage (faster mobility forms, middle panel) but this was unchanged by incubation of the ER with Src (E6AP+Src). Control (left) had no added ubiquitin ligase.

ER proteolysis may be mediated by a number of potential different E3 ligases *in vivo*. While E6AP mediated ER degradation was not stimulated by Src, Src may modulate the action of different E3 ligases on the ER.

## Src inhibition impairs ER ubiquitylation

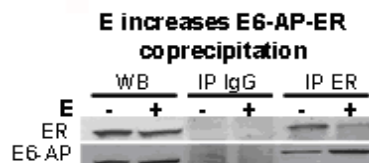
To test more *in vivo* how Src inhibition impaired ER proteolysis, we investigated how Src inhibitor drugs affected the ligand stimulated ubiquitylation of ER. MCF-7 cells were estrogen deprived and cells were treated with complete medium and estrogen and lysed 6 h later. Some cells were treated with the proteasome inhibitor, MG132, at the time of addition of estradiol to the medium and others were treated with the Src inhibitor PP1. The ER was immunoprecipitated and complexes resolved and immunoblotted with anti-ubiquitin antibody, then stripped and re-probed with ER antibody. The ER levels were highest following estrogen deprivation and a modest degree of ubiquitylation was observed after proteasome inhibition of these cells. ER levels fell abruptly with addition of estradiol. While MG132 prevented the loss of ER protein levels following estrogen stimulation, detection of ubiquitylated ER was enhanced (Figure 8). In contrast, estrogen treatment together with Src inhibitor impaired ER ubiquitylation despite its effect to prevent ER degradation. Thus, confirming that Src activity may be required to facilitate the ubiquitylation of ER.



**Figure 8. Src inhibition prevents estrogen stimulated ER-ubiquitylation.** MCF-7 cells were estrogen deprived for 48 h followed by addition of estradiol with or without the proteasome inhibitor MG132 or the Src inhibitor, PP1. Cells were lysed, ER immunoprecipitated and complexes resolved by SDS-PAGE and blotted with anti-ubiquitin antibody (top). While proteasome inhibition increased the detection of ubiquitylated ER in estrogen stimulated cells, Src inhibitor abolished detection of ER-ubiquitylation. ER immunoblot (bottom) shows that both PP1 and MG132 impaired estrogen stimulated proteolysis.

## The E3 ubiquitin-protein ligase, E6-AP promotes ER $\alpha$ degradation

E6-AP is expressed in both nucleus and cytoplasm and was first identified as an E3 ligase for p53(Hatakeyama et al., 1997; Huibregtse et al., 1993). Previous findings indicate that E6-AP is not only a coactivator (Nawaz et al., 1999), but also an ubiquitin ligase for the ER. Upon estrogen treatment, we observed increased interaction of E6AP with ER *in vivo* and an estrogen dependent ER degradation(Figure 9). These results support the notion that E6-AP acts as an E3 ligase to promote ER $\alpha$  degradation in response to estrogen treatment through the ubiquitin-proteasome pathway.



**Figure 9. E6-AP regulates ER levels.** Estrogen stimulation of E-deprived cells decreased ER levels but increased E6-AP co-IP with ER.

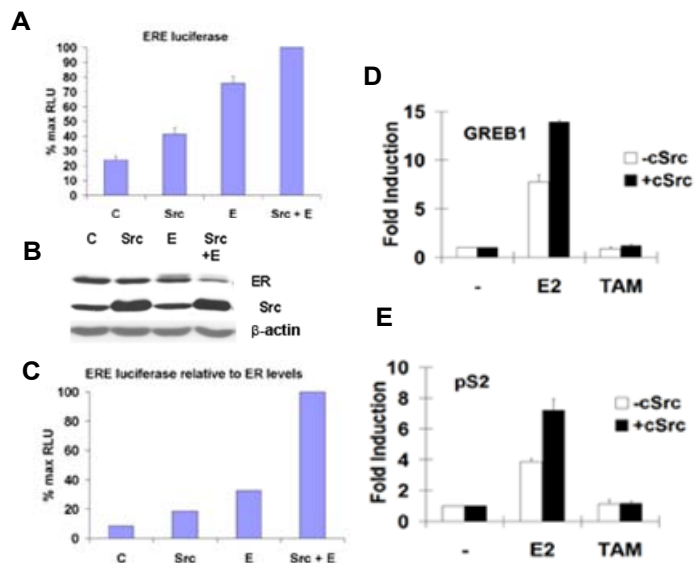
## Src and estrogen cooperate to stimulate ER transcriptional activity

For many transcriptional factors, activation of transcriptional activity is linked to proteolysis of the transcription factor, thereby limiting the extent of gene induction (Tansey, 2001). Since Src transfection and induction both stimulated ER proteolysis, we investigated effects of Src with and without estrogen on ER transcriptional activity. MCF-7 cells were transfected with 500 ng estrogen response element (ERE) luciferase, with or without active Src. Cells were treated with 10  $\mu$ M and/or estrogen for 4 h before lysate collection and then luciferase activity was measured to determine ER transcriptional activity.

In proliferating cells, addition of estrogen or Src transfection each increased ERE luciferase activity within 4 hrs (Figure 10A). Each treatment alone modestly reduced ER while ER levels fell more notably after both Src transfection together with estrogen (Figure 10B). When ERE luciferase activity relative to available ER was measured (correcting for the reduced ER level at 4 h), Src transfection and estrogen stimulation had more than additive effects (Figure 10C).

Effects of Src on estrogen activation of cellular ER target genes, pS2 and GREB1 was assayed by Q-RT-PCR. For this I developed stable transfected MCF-7 cells that expressed active Src. These Src-MCF-7 cells were estrogen deprived for two days and Src was induced or not within the last 16 hrs of starvation. (Figure 10D and E).

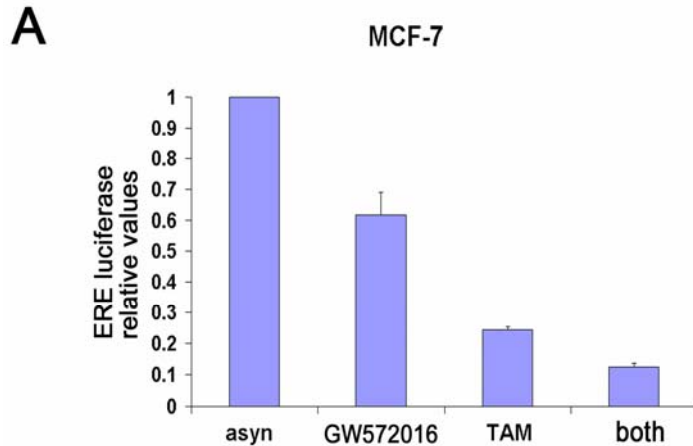
Within 3 h of estrogen addition, GREB1 and pS2 mRNA levels increased by 8 and 4 fold respectively compared to baseline without estrogen. With prior Src induction at 3 hrs after estrogen, GREB1 and pS2 levels were 14 and 7 fold higher than in estrogen starved cells. These findings suggest that the effect of Src on expression of GREB1 and pS2 genes is mediated by ER and Src increases the ER action on these GREB1 and pS2.



**Figure 10. Src cooperates with estrogen to activate ER transcriptional activity.** MCF-7 were transfected with an ERE luciferase reporter (2xERE) and either with a vector encoding activated Src (Src-Y530F) or empty vector control. **A** ERE luciferase activity increased after 10 nM 17- $\beta$  estradiol (E) for 4 h and 4 h after Src-Y530F transfection. ERE activity increased further with E and Src transfection together **B** ER levels and Src before (C) and 4 h after Src transfection, E treatment or both. **C** Graph shows relative luciferase activity taking into account the reduced levels of ER at 4 h. **D & E** MCFpINDSrc2 were estrogen and serum starved for 48 hours. Src was induced or not by the addition of ponesterone A for 16 hours and then cells were treated with either estrogen (E2) or Tamoxifen (TAM) and either GREB1 (**D**) or pS2 (**E**) gene expression assayed by Q-RT-PCR. Src had no effect on these genes in the presence of TAM indicating Src effects are ER dependent.

**GW572016, an ErbB1 and ErbB2 inhibitor, inhibited ER transcriptional activity, increased p27 levels, decreased cyclin E-Cdk2 activity resulting in cell cycle arrest**

Since we observed that Src transfection increased ER transcriptional activity, in order to determine if the opposite was also true, we used GW572016 which is an inhibitor of ErbB1 and ErbB2. Treatment of GW572016 alone reduced ER transcriptional activity by 38% in MCF-7 cells. Treatment of GW572016 in combination with tamoxifen resulted in a cooperative reduction in ER activity in MCF-7 cells (Figure 11). Treatment for 48 hrs with GW572016 also resulted in increased p27 protein, decreased cyclin E-Cdk2 activity and promotion of cell cycle arrest. More detailed effects of the ErbB1/2 inhibitor on ER positive breast cancer cell lines are described in appendix 2. Thus inhibition of RTK signaling not only results in inhibition of ER transcriptional activity but also in cell cycle arrest in cooperation with the antiestrogen, tamoxifen in MCF-7 cells.



**Figure 11.** MCF-7 cells were transfected with the luciferase reporter gene under the control of an ERE promoter sequence. Cells were then treated with GW572016 with or without tamoxifen for 48 hrs and then collected for luciferase activity.

## KEY RESEARCH ACCOMPLISHMENTS

Demonstrated that cSrc over expression cooperates with estrogen to stimulate ER degradation. Transfection of cSrc into MCF-7 resulted in a reduction of ER levels. Similarly over expression of ErbB2 reduced ER levels in MCF-7 clones.

Demonstrated that inhibition of cSrc using PD166326 resulted in abrogation of estrogen stimulated ER degradation in MCF-7, BT-20, MDA-MB-361 and BT549-ER

There is increased Src activity in cell lines with reduced ER levels. The EGFR over expressing cell line, BT-20, and the ErbB2 over expressing cell line, MDA-MB-361 have greater Src activity compared to MCF-7 as assayed through a Src kinase assay and have reduced ER levels compared to MCF-7

Demonstrated that Y537A-ER is a very stable protein compared to WT-ER and does not respond to estrogen dependent ER degradation. However, Y537F-ER was more sensitive to estrogen dependent ER degradation.

Demonstrated different binding affinities of ER to Src with mutations at Y537

Demonstrated that purified recombinant Src can phosphorylate purified recombinant ER *in vitro*.

Demonstrated that Src can increase ER ubiquitination in a reconstituted *in vitro* system containing E1/E2 and MCF-7 lysate as a source of E3 ligase suggesting a direct role of Src in E mediated Src ubiquitination.

In an *in vitro* system determined that Src had no effect on E6AP mediated ER degradation, suggesting that Src increased ER ubiquitination is independent of E6AP. Thus, Src may influence increased ubiquitination mediated through a different E3 ligase.

Demonstrated that inhibition of Src using the Src inhibitor PP1 decreased estrogen dependent ER transcriptional activity through decreased mRNA of ER products and Src transfection and estrogen cooperate to stimulate ERE transcriptional activity. These results give further evidence that activation of ER transcriptional activity is coupled to ER proteolysis.

Demonstrated that inhibition of EGFR/ErbB2 upon treatment with GW572016 resulted in decreased ERE luciferase activity, increased p27, increased p27 bound to cyclin E/Cdk2 and in decreased cyclin E/Cdk2 kinase activity.

## REPORTABLE OUTCOMES

### MANUSCRIPTS

**Isabel Chu**, Angel Arnaout, Sophie Loiseau, Jun Sun, Arun Seth, Chris McMahon, Kathy Chun, Bryan Hennessy, Gordon Mills and Joyce Slingerland. Src promotes estrogen dependent ER $\alpha$  proteolysis in human breast cancer. Journal of Clinical investigation. Accepted with minor revisions. 2007

**Isabel Chu**, Kimberly Blackwell, Susie Chen and Joyce Slingerland. 2005. The dual ErbB1/ErbB2 inhibitor, Lapatinib (GW572016), cooperates with Tamoxifen to Inhibit Both Cell Proliferation and Estrogen Dependent Gene Expression in Antiestrogen-Resistant Breast Cancer. Cancer Research, 65: 18-25.

### POSTER AND ORAL PRESENTATIONS

Isabel Chu, Cheng Keat-Tan, Ludger Hengst and Joyce Slingerland. P27 phosphorylation by Src regulates inhibition of cyclin E- Cdk2. Cold Spring Harbor cell cycle meeting, May 17-21, 2006, New York.

Isabel Chu, Sophie Loiseau, and Joyce Slingerland. Src cooperates with Estrogen To Activate Ligand Dependent Era Proteolysis in Human Breast Cancer. Annual UM/Sylvester Cancer Research Poster Session, Miami, Florida, May 19, 2005

Isabel Chu and Joyce Slingerland. The dual ErbB1/ErbB2 inhibitor, Lapatinib (GW572016), cooperates with Tamoxifen to Inhibit Both Cell Proliferation and Estrogen Dependent Gene Expression in Antiestrogen-Resistant Breast Cancer. Era of Hope 2005 Department of Defense Breast Cancer Research Program Meeting, Pennsylvania Convention Center, Philadelphia, Pennsylvania- June 8-11, 2005

cSrc and Her2 cooperate with estrogen to activate ligand dependent ER $\alpha$  proteolysis implications for therapy of ER negative breast cancer, Isabel Chu, Jun Sun, Angel Arnaout and Joyce Slingerland. Keystone symposia, February-March, 2004

### DEGREE

Obtained PhD from the work described above and I have already accepted a position for a postdoctoral fellow at Dr. Jeffrey Green, NCI, Maryland. I will start my new position in June, 2007.

## CONCLUSION

One third of breast cancers lack detectable ER protein and have a worse prognosis than ER positive breast cancers. This report describes my findings suggesting a novel mechanism whereby ER protein loss in ER negative cancers may be a consequence of accelerated ER degradation. This may be linked to their aggressive behavior. Estrogen binding to the ER rapidly activates ubiquitin-dependent ER proteolysis which in turn regulates ER activity. We show that Src induction accelerates ER proteolysis and Src inhibitors impaired estrogen stimulated ER ubiquitylation and degradation. The weakly ER positive, MDA-MB361 and ER negative, BT-20 breast cancer lines both have highly activated Src. ER was increased by estrogen deprivation, proteasome inhibition and by Src inhibitors in these lines. Src accelerated ER ubiquitylation and proteasomal degradation *in vitro*. Src activity was increased in primary ER negative breast cancers compared to ER positive. It is possible that ER activation by estrogen or growth factor signaling leads to Src mediated ER or co-activator phosphorylation events that regulate ER-co-activator action and ubiquitin-dependent ER degradation. In a subset of ER negative breast cancers, oncogenic Src activation may alter phosphorylation of the ER or of key co-regulators to activate both ER proteolysis and ER dependent transcription.

My findings give us a better understanding in the link between two “non-genomic” consequences of ER activation: cross-talk with Src, and ER proteolysis, with the regulation of ER driven gene expression. In addition to its effects to promote breast cancer proliferation and survival, oncogenic Src activation may activate ER proteolysis in breast cancers. The elucidation of mechanisms underlying ER loss in ER negative breast cancer may indicate why these cancers have such an aggressive clinical course. Pathways identified may yield new targets for molecular based therapies for this particularly treatment-resistant form of breast cancer.

## Reference List

- Arnold,S.F., Obourn,J.D., Jaffe,H., and Notides,A.C. (1995). Phosphorylation of the human estrogen receptor on tyrosine 537 in vivo and by Src family tyrosine kinases in vitro. *Molecular Endocrinology* 9, 24-33.
- Belsches-Jablonski,A.P., Biscardi,J.S., Peavy,D.R., Tice,D.A., Romney,D.A., and Parsons,S.J. (2001). Src family kinases and HER2 interactions in human breast cancer cell growth and survival. *Oncogene* 20, 1465-1475.
- Bunone,G., Briand,P.A., Miksicek,R.J., and Picard,D. (1996). Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *EMBO J* 15, 2174-2183.
- Carrano,A.C., Eytan,E., Hershko,A., and Pagano,M. (1999). SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. *Nature Cell Biol.* 1, 193-199.
- Ferguson,A.T. and Davidson,N.E. (1997). Regulation of estrogen receptor alpha function in breast cancer. *Critical Reviews in Oncogenesis* 8, 29-46.
- Gao,X., Mohsin,S.K., Gatalica,Z., Fu,G., Sharma,P., and Nawaz,Z. (2005). Decreased expression of e6-associated protein in breast and prostate carcinomas. *Endocrinology* 146, 1707-1712.
- Hatakeyama,S., Jensen,J.P., and Weissman,A.M. (1997). Subcellular localization and ubiquitin-conjugating enzyme (E2) interactions of mammalian HECT family ubiquitin protein ligases  
1. *J. Biol. Chem.* 272, 15085-15092.
- Huibregtse,J.M., Scheffner,M., and Howley,P.M. (1993). Localization of the E6-AP regions that direct human papillomavirus E6 binding, association with p53, and ubiquitination of associated proteins  
1. *Mol. Cell Biol.* 13, 4918-4927.
- Kato,S., Endoh,H., Masuhiro,Y., Kitamoto,T., Uchiyama,S., Sasaki,H., Masushige,S., Gotoh,Y., Nishida,E., Kawashima,H., Metzger,D., and Chambon (1995). Activation of the estrogen receptor through phosphorylation by mitogen- activated protein kinase. *Science* 270, 1491-1494.
- Lonard,D.M., Nawaz,Z., Smith,C.L., and O'Malley,B.W. (2000). The 26S proteasome is required for estrogen receptor-alpha and coactivator turnover and for efficient estrogen receptor-alpha transactivation. *Molecular Cell* 5, 939-948.
- McClelland,R.A., Barger,U., Miller,L.S., Powles,T.J., and Coombes,R.C. (1986). Immunocytochemical assay for estrogen receptor in patients with breast cancer: relationship to a biochemical assay and to outcome of therapy. *Journal of Clinical Oncology* 4, 1171-1176.
- Nawaz,Z., Lonard,D.M., Smith,C.L., Lev-Lehman,E., Tsai,S.Y., Tsai,M.J., and O'Malley,B.W. (1999). The Angelman syndrome-associated protein, E6-AP, is a coactivator for the nuclear hormone receptor superfamily. *Molecular and Cellular Biology* 19, 1182-1189.
- Song,R.X., Barnes,C.J., Zhang,Z.G., Bao,Y.D., Kumar,R., and Santen,R.J. (2004). The role of Shc and insulin-like growth factor 1 receptor in mediating the translocation of estrogen receptor alpha to the plasma membrane. *Proceedings of the National Academy of Sciences of the United States of America* 101, 2076-2081.
- Tansey,W.P. (2001). Transcriptional activation: risky business. *Genes & Dev.* 15, 1045-1050.

Weis,K.E., Ekena,K., Thomas,J.A., Lazenec,G., and Katzenellenbogen,B.S. (1996). Constitutively active human estrogen receptors containing amino acid substitutions for tyrosine 537 in the receptor protein. *Mol. Endocrinol.* *10*, 1388-1398.

## **APPENDIX I**

## Src promotes estrogen dependent ER $\alpha$ proteolysis in human breast cancer

ISABEL CHU<sup>1,2</sup>, ANGEL ARNAOUT<sup>2</sup>, SOPHIE LOISEAU, JUN SUN<sup>1</sup>, ARUN SETH<sup>2</sup>, CHRIS MCMAHON<sup>2</sup>, KATHY CHUN<sup>3</sup>, BRYAN HENNESSEY<sup>4</sup>, GORDON B. MILLS<sup>4</sup> AND JOYCE SLINGERLAND<sup>1,5</sup>

*<sup>1</sup>Braman Family Breast Cancer Institute, Sylvester Comprehensive Cancer Center, University of Miami Miller School of Medicine, Miami, Florida, , Departments of <sup>2</sup>Medical Biophysics and <sup>3</sup>Surgery, University of Toronto, Toronto, Ontario, <sup>4</sup>Department of Systems Biology, MD Anderson Cancer Center, Houston, Texas*

Corresponding author:

Joyce Slingerland MD FRCP(C) PhD  
Director, Braman Family Breast Cancer Institute  
University of Miami Miller School of Medicine  
Batchelor Building, 4th Floor rm 419  
1580 N.W. 10th Avenue  
Miami, FL 33136  
phone: 305-243-7265  
fax: 305-243-4787  
email:jslingerland@med.miami.edu

**Nonstandard abbreviations used:** ER, estrogen receptor  $\alpha$ ; ERE, estrogen response element; AF, activation function; h-PBGD, human porphobilinogen deaminase; LBA, ligand binding assay; LLnL, N-Acetyl-Leu-Leu-Norleucinal; cFBS, charcoal stripped FBS; RPPA, reverse phase tissue lysate array; HR, hormone receptor; PR, progesterone receptor; TF, transcription factors; PA, ponasterone A.

Running Title: Src promotes ligand activated ER $\alpha$  proteolysis

## **Abstract**

Estrogen drives both transcriptional activation and proteolysis of estrogen receptor  $\alpha$ . Here we observed that ER mRNA is expressed with variable and overlapping levels in all of 200 ER negative and 50 ER positive primary breast cancers pointing to important post-transcriptional ER regulation. Our data indicate that Src cooperates with estrogen to activate ER proteolysis. Inducible Src stimulated ligand activated ER transcriptional activity and reduced ER t1/2. Src levels correlated inversely with ER levels in primary breast cancers. ER negative primary breast cancers and cell lines showed increased Src levels and/or activity compared to ER positive cancers and lines. ER protein was less stable in ER negative lines. In both ER positive and negative lines, proteasome inhibitors increased ER levels. Src inhibition impaired ligand activated ER ubiquitylation and increased ER levels. Src increased ER-ubiquitylation and degradation *in vitro*. These data provide a novel link between Src activation and the ER proteolysis and support a model whereby cross talk between liganded ER and Src may drive ER transcriptional activity and target ER for ubiquitin dependent proteolysis. Oncogenic cSrc activation may not only promote mitogenic signaling to augment proliferation, but also contribute to estrogen activated ER loss in a subset of ER negative breast cancers, altering prognosis and response to therapy.

## Introduction

Estrogen regulates the proliferation and development of tissues expressing estrogen receptors and is a risk factor for breast cancer development. One third of new breast cancers lack detectable ER $\alpha$  protein and have a worse prognosis than ER $\alpha$  positive (ER+) breast cancers (1). ER $\alpha$  negative (ER-) breast cancers do not respond to hormone response modifiers like tamoxifen (2) and often show *de novo* or acquired resistance to chemotherapy (1). While there are two forms of ER, ER $\alpha$  and ER $\beta$  (3-5), considerably more is known about the role of ER $\alpha$  in human breast cancer. This study investigates ER $\alpha$  exclusively and ER refers hereafter to ER $\alpha$ . While estrogen is mitogenic for cultured ER+ breast cancer lines, ER- breast cancer lines proliferate in the absence of estrogen and ER- breast cancers are generally believed to be estrogen independent.

Factors responsible for the ER- status of breast cancers remain largely unknown. *ER* gene deletions, rearrangements and point mutations are too uncommon to account for the ER- phenotype (6;7). ER promoter hypermethylation has been observed a minority (up to 25%) of ER- breast carcinomas (6). In three early non-quantitative studies, ER mRNA was detected in a majority (67-71%) of 64 primary ER- cancers (8-10) indicating post-transcriptional or post-translational control of ER levels in human breast cancers. Transcriptional profiling has demonstrated ER mRNA is detected but variably reduced in ER- compared with ER+ cancers (11-13). The distinct gene expression profiles of ER+ and ER- cancers have led to the hypothesis that these two tumor groups arise from different tissues of origin, with ER-/Her2- tumors derived from

the basal epithelium while ER<sup>+</sup> cancers reflect a luminal epithelial origin (14;15). The present data shed further light on mechanisms regulating ER levels.

The ER is a 66 kDa protein nuclear hormone receptor transcription factor (16). Upon ligand binding, ER dimerizes and associates with coactivators and chromatin remodeling factors, to activate transcription of estrogen response element (ERE) containing genes (17). ER contains two transcription activation functions (AF): AF-1 and AF-2. AF-1 can be phosphorylated and activated in a ligand independent manner following growth factor stimulation, while AF-2 is activated by ligand stimulated changes in ER conformation(18;19). The ER phosphorylation state affects coactivator binding and ER-DNA binding affinity.

In addition to transcriptional activation, ligand-ER binding rapidly activates cross talk with mitogenic signaling kinases (for review see(20;21)). Estrogen-ER binding promotes a rapid and transient ER:cSrc interaction, binding to Shc and Ras-mitogen activated protein kinase (MAPK) signaling (22-25). In some cell types, estrogen stimulates tripartite ER, cSrc and PI3K complex formation, leading to PKB/AKT and MAPK activation (26). Signaling kinases activated by liganded ER not only activate mitogenic cascades, they can also phosphorylate the ER and its coactivators generating a feed forward loop that augments ER transcriptional activity (20;21;27).

The ER can also be phosphorylated and activated in a ligand independent manner in response to peptide growth factors including IGF-I(28), TGF- $\alpha$ (29) and EGF(30;31) that activate PKB and MAPK signaling pathways resulting in ER phosphorylation and ER-dependent transcription. Phosphorylation of amino terminal (30;32;33) and C-terminal (34-36) sites on the ER increase ER transcriptional activity.

Estrogen binding to the ER rapidly stimulates ER ubiquitylation and proteolysis (37-39). Unliganded ER is very stable, with  $t_{1/2}$  of up to 5 days (37). Upon ligand binding, the ER  $t_{1/2}$  drops dramatically to 3-5 h (37;39). The detection of ubiquitinated ER *in vivo* in uterine tissue (37) and the finding that proteasome inhibition abrogates estrogen stimulated ER loss confirms an *in vivo* role for proteasome-mediated ER degradation in regulating ER levels (38;39).

ER ubiquitination and proteasome activity are intimately linked to ER dependent transcriptional activation (40;41). Ligand binding activates both ER dependent transcription and ER ubiquitination (40). Proteasome inhibitors and mutations that inhibit coactivator binding both abrogate ligand mediated ER proteolysis and ERE transcriptional activity (41). Different ligands stimulate ER proteolysis to different degrees (42) and ubiquitin ligases BRCA1 (43), MDM2 (44) and E6AP (45) can all stimulate estrogen induced transcriptional activity.

The 60 kDa cSrc tyrosine kinase regulates cellular proliferation, motility, and tumor metastasis (46). Increased levels or activity of cSrc have been observed in primary breast cancers (47) but an association with ER levels has not been reported. Here we demonstrate that both ER+ and ER- primary human breast carcinomas express ER mRNA. Crosstalk between liganded ER and Src appears to promote proteasomal degradation of the ER. Src inhibition impaired ligand-activated ER ubiquitylation and ER proteolysis, while cSrc induction shortened the ER  $t_{1/2}$ . Src induction also increased ER-driven transcription. ER negative breast cancer specimens and cell lines showed elevated cSrc levels or activity compared to ER positive tumors and ER proteolysis was increased in ER-negative cell lines. Src stimulated both ER ubiquitylation and

proteasome dependent ER degradation *in vitro*. These observations and the inverse correlation between cSrc and ER levels in primary breast cancers suggest that Src may promote cell proliferation by stimulating transcription coupled ER degradation in human breast cancers.

## Results

*ER negative breast cancers express ER mRNA.* Tumor ER mRNA was quantitated by comparing real time RT-PCR (QPCR) crossing point values to a standard curve generated from serial dilutions of ER cDNA plasmid (not shown). Quantitation of housekeeping gene human porphobilinogen deaminase (h-PBGD) expression demonstrated similar mRNA quality in both ER+ and ER- breast cancers. ER protein levels were determined in a single clinical reference lab by ligand binding assay (LBA). ER mRNA was detected in all of 50 ER+ and 200 ER- cancers (Figure 1A, representative samples). The scatter plot of ER mRNA values (Figure 1B) and graphed distribution (Figure 1C) show considerable variability and overlap in ER mRNA concentrations in ER+ and ER- tumors. The mean ER mRNA concentration in ER+ cancers was  $1.14 \times 10^3$  (range  $1.02 \times 10^{-1}$  to  $1.19 \times 10^4$ ) fmol/ $\mu$ g RNA and was  $1.27 \times 10^3$  (range of  $4.55 \times 10^{-2}$  to  $3.56 \times 10^4$ ) fmol/ $\mu$ g in ER- cancers. While the lowest and highest ER mRNA concentrations were similar and the mean ER mRNA values did not differ significantly between the ER+ and ER- cancers ( $p > 0.50$ ), the modal ER mRNA value in the ER- tumors was approximately one log lower than in the ER+ cancers. When ER protein concentrations were graphed versus mRNA values, there was no clear relationship between ER protein and mRNA levels for either ER+ or ER- cancers (Figures 1D and 1E).

*Serum growth factors synergize with estrogen to activate ER proteolysis.* As a baseline for further study, we showed that estradiol added to estrogen-deprived MCF-7

cells stimulated a rapid reduction of ER protein that was impaired by proteasome inhibition with N-Acetyl-Leu-Leu-Norleucinal (LLnL) (Figure 2A). The ER protein  $t_{1/2}$  was >24 h in estrogen depleted MCF-7. Within 6 h after addition of estrogen, the ER  $t_{1/2}$  fell to 5 h (Figure 2B). A significant reduction in ER  $t_{1/2}$  was also noted within 1 h of ligand addition (not shown).

Cross talk of Src, PI3K and receptor tyrosine kinases with liganded ER leads to ER phosphorylation and activation of ER transcriptional activity (21;28). To determine if ER/signaling cross talk may also modulate ligand activated ER proteolysis, we tested if addition of growth factors would affect estrogen stimulated ER loss. MCF-7 was growth factor and estrogen deprived in 0.1% charcoal stripped FBS (cFBS) for 48 h. Addition of estrogen together with either 5% FBS reduced ER levels more rapidly than observed with estrogen alone (Figure 2C). Growth factor stimulation with 5% cFBS without added estrogen was not sufficient to trigger ER proteolysis. Thus, serum growth factors may activate signaling kinases to promote estrogen activated ER proteolysis.

*Src promotes estrogen stimulated ER degradation.* Liganded ER binds cSrc leading to cSrc activation (22). Treatment of MCF-7 with the Src inhibitor, PP1, caused a dose dependent accumulation of ER over 48 h (data not shown). PP1 also impaired the fall in ER levels observed when estrogen and growth factor starved cells were transferred to serum together with estradiol (Figure 2D). Thus, cSrc may promote ligand activated ER proteolysis. Transfection of activated Src (PCI-Src Y530F) reduced ER levels. The ER  $t_{1/2}$  fell from 14h in asynchronously proliferating MCF-7 to 9h at 24 h after cSrc transfection (Figure 2E).

To assay further the effect of Src on ER stability, two different MCF-7 derivatives were generated to inducibly express activated Src-Y350F. MCFpINDSrc2 cells were deprived of growth factors for 48 h. Src was induced by treatment with ponasterone A (PA) for 24 hours prior to addition of estradiol (left panel Figure 2F). Induction of activated Src did not reduce ER levels in the absence of estrogen. However, within 3 hours of estradiol addition, ER levels were markedly lower in Src induced cells (right, Figure 2F), and ER t<sub>1/2</sub> was reduced to 2.6h in cells stimulated by estrogen together with Src induction compared to an ER t<sub>1/2</sub> of 8.1 h in cells treated with estrogen alone (Figure 2G). Treatment with PA alone did not reduce the ER t<sub>1/2</sub> (not shown). At 24 hrs after Src induction, quantitative real time PCR (QPCR) showed a modest increase in ER mRNA expression compared to uninduced cells, thus the more rapid ER protein loss in estrogen treated, Src induced cells was not due to reduced ER mRNA expression. Data in a second Src inducible cell line also confirmed these findings (data not shown). Thus Src appears to cooperate with estrogen to stimulate ER proteolysis. Proteasome inhibition partially reversed the effects of expression of activated Src (not shown) compatible with the effects being post translational.

*Src promotes ligand activated ER transcriptional activity.* Activation of many transcriptional factors is linked to factor proteolysis (48). Since Src clearly contributes to ER proteolysis, we assayed effects of Src on ER transcriptional activity. In cells grown in the presence of full serum and estrogen, addition of 10<sup>-8</sup>M estradiol or Src transfection each reproducibly increased ERE luciferase activity within 4 hours, albeit less notably with Src alone (Figure 3A). Estrogen together with Src transfection

increased ERE luciferase activity beyond that induced by estrogen alone. Src transfection and estradiol stimulation also decreased ER levels beyond that seen with estrogen alone (Figure 3B). When ERE luciferase activity relative to available ER was measured (correcting for the reduced ER level at 4 h), Src transfection and estrogen stimulation had more than additive effects (Figure 3C).

Effects of Src on estrogen activation of cellular ER target genes, pS2 and GREB1 was assayed by QPCR. MCFpINDSrc2 cells were estrogen deprived for two days and Src was induced or not within the last 16 hours of starvation. Within 3 h of estrogen addition, GREB1 and pS2 mRNA levels increased by 8 and 4 fold respectively, compared to 14 and 7 fold higher than baseline with prior Src induction. Src induction alone did not activate GREB1 or pS2 (not shown). Neither gene was activated by Tamoxifen, with or without Src induction. Thus the effect of Src on these two genes was ER-mediated and Src increased the transcriptional potency of the ER on these two ER target genes (Figures 3D and 3E).

*MEK and PI3K are not sufficient to promote ligand mediated ER proteolysis.* MEK inhibition of asynchronous MCF-7 with U0126 for 48 h reduced ER levels (Supplemental Figure 1A). In estrogen deprived MCF-7, MEK inhibition prior to estrogen addition led to a greater loss of ER (Supplemental Figure 1B) and a shorter ER  $t_{1/2}$  (not shown) than with estrogen alone. Thus, in these assay conditions, MEK effectors appear to oppose ligand stimulated ER proteolysis.

Treatment with the PI3K inhibitor, LY294002, did not affect ER levels in asynchronous MCF-7 (Supplemental Figure 1C). In estrogen deprived MCF-7, PI3K

inhibition prior to estrogen repletion inhibited PKB phosphorylation and cell cycle progression, but did not affect estrogen-mediated ER loss (Supplemental Figure 1,D and E). Thus, estrogen stimulated ER proteolysis does not require PI3K/PKB action or cell cycle entry.

*Reduced ER protein levels and stability in breast cancer lines with activated cSrc.*

The BT-20 breast cancer line shows both cSrc and EGFR activation, while Her2 and cSrc are activated in MDA-MB-361 (49). ER mRNA was detected in MCF-7, BT-20 and MDA-MB-361 by non-quantitative RT-PCR (data not shown). Although BT-20 has been characterized as ER negative, low but detectable ER protein was present on ER immunoprecipitation from 1 mg cell lysate (Figure 4A). cSrc kinase activities were increased (Figure 4B), while ER levels and  $t_{1/2}$  were reduced in BT-20 and MDA-MB-361 compared to MCF-7(Figure 4C). The calculated ER  $t_{1/2}$  was 14 h in asynchronous MCF-7, 9 h in MDA-MB-361 and 5 h in BT-20.

To further assay effects of Src and proteasome function on ER levels in ER negative breast cancer lines, the BT549 line was transfected with ER to generate the stable line: BT549-ER. Src activity was elevated and the ER  $t_{1/2}$  was 3.9 hrs in asynchronous BT549-ER (not shown). In both ER+ (MCF-7 and MDA-MB-361) and ER- (BT-20 and BT549-ER) lines, ER levels increased with estrogen deprivation. Estrogen stimulated ER loss (E+FBS) was impaired by proteasome inhibition with MG132 and also by Src inhibition with PD166326 (Figure 4D). For BT-20, blots were exposed longer and more protein was loaded than for ER + lines. Thus Src appears to activate estrogen stimulated ER proteolysis in both ER+ and ER- breast lines.

*Src inhibition impairs estrogen stimulated ER-ubiquitylation in vivo.* To test the effect of Src inhibition on ligand driven ER-ubiquitylation, MCF-7 cells were estrogen deprived and then stimulated with estrogen with or without prior addition of MG132 or the Src inhibitor PP1. The ER was immunoprecipitated from equal amounts of protein lysate, complexes resolved and immunoblotted with anti-ubiquitin antibody, then stripped and re-probed for total ER. ER levels were maximal in estrogen deprived cells. Although ER levels were reduced 6 h after estrogen stimulation, detection of ER-ubiquitylation (Ub-ER, bottom panel) was modestly increased. When estrogen deprived cells were treated with estrogen and proteasome inhibitor (MG132), the ER protein level remained elevated (top) and ubiquitylated ER level was readily detected (bottom, Ub-ER, Figure 5A). In contrast, while Src inhibition with PP1 prevented estrogen-stimulated loss of the ER protein and ER protein levels remained high (ER protein level shown in lane 4, top Figure 5A) and ER ubiquitylation was minimal (bottom, Ub-ER). Thus, Src inhibition impaired ligand activated ER-ubiquitylation, and prevented ligand mediated loss of ER.

*Src activates ER ubiquitylation and degradation in vitro.* To assay the effect of Src on ER ubiquitylation and degradation *in vitro*, recombinant ER was pretreated with Src kinase or mock treated and then equal amounts of ER were reacted with recombinant ubiquitin, ubiquitin-activating enzyme (E1), UbcH7 (E2) and E3 ligase supplied from asynchronous MCF-7 cell lysate. ER was then precipitated, resolved and ubiquitylated ER detected by immunoblotting with anti-ubiquitin Ab. In these assays, little ER

degradation occurred. ER-tyrosine phosphorylation was detected only in Src treated samples. ER-ubiquitylation was enhanced by pre-treatment of the ER with Src kinase (Figure 5B).

For ER degradation, assay conditions were modified as described in Experimental procedures. Recombinant ER was either pre-treated or not with Src kinase as above and then treated with the E1, E2 and E3 mixture with or without addition of 26S proteasome fraction. ER degradation was minimal in assays with either Src or 26S alone. When Src-pre-treated ER was incubated with E1, E2, E3 together with 26S proteasome, ER was completely degraded (Figure 5C).

*cSrc is activated in ER negative primary breast cancers.* cSrc kinase activity was assayed in lysates from 18 ER- and 22 ER+ primary human breast cancers. The ER status determined at diagnosis by ligand binding assay was verified by ER immunoblotting.  $\beta$ -actin blotting verified equal loading and equal protein in cSrc kinase assays. Elevated cSrc activity was observed in 78% (14/18) of ER- breast cancers. In contrast, only 18% (4/22) of ER+ tumors showed Src activity above non-specific antibody controls (representative tumor data in Figure 6A).

*Src levels and ER are inversely correlated in primary human breast cancers.* To extend data above, ER and Src protein expression were quantitated by reverse phase tissue lysate array (RPPA) in 101 primary breast cancers using validated monospecific antibodies demonstrated to reflect western blotting results with multiple tumor samples providing a high throughput quantitative analysis (50). Of ninety eight tumors in which

the hormone receptor (HR) status was known, sixty eight of were classified as positive for ER and/or progesterone receptor (PR) by IHC in pathology evaluation at diagnosis. ER quantified by RPPA was significantly higher in pathologic ER-positive breast cancers ( $p=9.6 \times 10^{-9}$ ), as expected. Src protein was significantly higher in pathologic ER and PR-negative ( $p=0.03$ ) than in hormone receptor-positive tumors (ER or PR positive) cancers. The distribution of Src values in HR positive and negative cancers is graphed in Figure 6B. When 68 HR positive tumors were compared with 23 'triple receptor-negative' tumors (negative for ER and PR by IHC and for HER2 by FISH), Src levels were highest in triple receptor-negative tumors ( $p=0.02$ ). In all tumors, quantified ER and Src expression were inversely correlated (correlation coefficient 0.26;  $p=0.008$ , see graph Figure 6C). In the subset of 68 hormone receptor-positive tumors, there was also a statistically significant inverse correlation between quantified expression of ER and Src ( $CC=0.30$ ;  $p=0.01$ ).

## Discussion

ER- breast cancers have distinct gene expression profiles and are clinically more aggressive than ER+ cancers (12). The present study supports the hypothesis that in at least a subset of ER negative breast cancers, Src activation may drive estrogen dependent ER proteolysis. ER gene alterations are too infrequent to explain the lack of detectable ER protein in up to one third of breast cancers (6;7). Early studies indicated that up to 60-70% of ER- tumors express ER mRNA (8-10). More sensitive QPCR demonstrated ER mRNA in all of 52 ER- primary breast cancers (51). In the present analysis, all of 200 ER- breast cancers expressed ER mRNA with considerable variability and overlap in values in ER+ and ER- cancers. While mean ER mRNA concentrations did not differ significantly between ER+ and ER- cancers, the modal distribution of ER mRNA concentrations was lower in ER negative cancers. This is consistent with microarray studies that compared individual tumor ER mRNA to reference cRNA pooled from ER+ and ER- tumors (11) or to the average signal from all tumors (12;13) to reveal lower average ER gene expression in ER- versus ER+ cancers. The variability in ER mRNA levels and the discordance observed between ER mRNA and protein in both tumor types, point to important post-transcriptional controls of ER levels.

Up to one third of primary breast cancers show *HER2/erbB-2* amplification and a similar proportion have increased EGFR expression. Both are strongly associated with an ER- status (52;53). Transfection of either *EGFR* or activated *Her2* can reduce ER levels in MCF-7 cells and this has been attributed to MAPK activation (54). However,

both of these receptors activate Src. In breast cancer cells, cSrc binds phosphorylated Her2 or EGFR promoting synergistic activation to stimulate breast cancer cell proliferation and survival (49). Indeed Src inhibitors impair Her2 and EGFR driven mitogenesis (49;55). Src is also transiently recruited to and activated by estrogen bound ER, leading to MAPK activation (22-25).

Src can phosphorylate ER *in vitro* (56; 57). ER phosphorylation by Src increases its affinity for estrogen (27), and may also affect ER-coactivator binding and transcriptional activity (58;59). The present study indicates that Src can drive expression of certain ER target genes suggesting the presence of an important feed forward signaling loop involving estrogen, the ER and Src.

Cross talk between liganded ER and Src may not only regulate ER transcriptional activity, but also activate ER proteolysis. Inhibition of cellular Src impaired estrogen mediated ER ubiquitylation and ER loss. Induced Src expression increased *pS2* and *GREB1* expression and ligand activated ER proteolysis. In breast cancer lines, increased Src activity correlated with a shortened ER  $t_{1/2}$ . In both ER+ breast lines and in ER- BT-20 and BT549-ER, proteasome inhibition increased ER protein levels. Moreover, in both ER+ and ER- lines, estrogen withdrawal increased ER levels, and estrogen-stimulated ER loss was impaired by Src inhibition.

Src kinase assays showed cSrc activation in a majority of primary ER- tumors in a relatively small primary tumor set. In a larger group of over 100 primary breast cancers, Src protein levels correlated inversely with ER in both ER+ and ER- tumors as assayed by sensitive RPPA. ER- cancers had higher Src levels than ER+, and this inverse statistical association was stronger in the subset of triple negative tumors compared to

ER positive. These findings are consistent with our recent analysis of over 700 primary cancers in which ER negative status correlated significantly ( $p < 0.001$ ) with Src activation detected by immunohistochemical staining for Y416 phosphorylated Src (A. Arnaout and JMS, manuscript in review). Although our data indicate that Src contributes to ER regulation in breast cancers, there are clearly a number of tumors with high Src levels that retain ER protein and tumors with low ER levels that do not have high Src levels or activity. Thus, additional Src independent mechanisms may regulate ER protein levels. Tumors with very low ER mRNA levels may reflect ER promoter methylation (6).

Src appears to promote the ubiquitylation of ER since Src inhibition impaired cellular ER-ubiquitylation and proteolysis *in vivo*. Moreover ER phosphorylation by Src increased both ER ubiquitylation and 26S proteasome mediated ER degradation *in vitro*. These data support a model in which liganded ER recruits cSrc or cSrc dependent kinases leading to phosphorylation events that facilitate ER binding to coactivators/components of the proteolytic machinery. Ligand and Src activated ER-ubiquitylation may be linked to transcriptional activation of a subset of ER-regulated genes. While our *in vitro* data support a direct effect, with Src phosphorylation of ER promoting its ubiquitin dependent degradation, ligand activated coactivator phosphorylation may also regulate ER degradation and transcriptional activity. SRC-3/AIB1 proteolysis accompanies estrogen stimulated ER activation (60). How specific Src-dependent ER and/or coactivator phosphorylation events modulate the profile of coactivator binding, ERE selection and ER proteolysis will require further investigation.

A recent report suggests that Src-mediated tyrosine phosphorylation may also regulate androgen receptor function (61).

Signaling pathways that activate many transcription factors (TF), including c-Jun, c-Myc and E2F-1 also trigger their ubiquitin dependent degradation (48), thereby limiting transactivator function. Ubiquitylation is required for transcriptional activity of certain TFs (48;62). TF ubiquitylation may influence coactivator/repressor binding (48) with coactivators subsequently enhancing ubiquitylation of certain transcription factors (63). Ligand mediated proteolysis regulates turnover of most nuclear receptors (reviewed in (64)). Several ER coactivators are also known to be ubiquitin ligases (43-45) or proteasome subunits (65).

In some (40;41) but not all models (66;67), proteasome inhibition decreases estrogen-ER-transcriptional activity despite an increase in ER abundance. ER mutations that impair co-activator binding abrogate ligand-stimulated ER degradation (41). Thus, co-activator binding may regulate not only transcriptional activity but also ligand mediated ER degradation. ER cycles on and off the ERE (40;68). Ligand increases the duration of ER-ERE binding and modifies ubiquitin ligase binding (40). Proteasome inhibition has been shown to dissociate ubiquitin-bound ER from ERE motifs and reduce ER transcriptional activity. Thus, for a subset of ER-driven genes, ER ubiquitylation and transcription may be closely linked.

Cell type and promoter specific differences affect how ER proteolysis influences target gene expression (66;67). In one study, proteasome inhibition increased expression of cellular *pS2* and *cathepsin D* but decreased *prolactin* expression (67). In certain promoter contexts, ligand activated ER may escape ubiquitylation and

proteasomal degradation and yet remain functional. While proteolytic degradation of the ER after ERE firing may allow re-loading of the promoter, ubiquitylation and proteasomal degradation may potentially serve a more global role in regulating the abundance and overall activity of the ER. Moreover, constitutive ER activation could potentially lead to reduced ER levels, due to constitutive ER proteolysis.

ER phosphorylation by different signaling pathways could theoretically promote recruitment of different coactivators or ubiquitin pathway components, changing both the profile of ER targets expressed and the rate of ligand stimulated ER proteolysis in different tissues. During breast cancer progression, Src activation may alter coactivator binding, shifting ER transcriptional targets to profiles that promote oncogenic change.

ER- breast cell lines are considered estrogen insensitive since they do not require estrogen for growth. This and the clinical observation that ER- breast cancers do not respond to antiestrogen therapies (2) have led to the belief that ER- tumors are estrogen independent. Our data raise the concern that at least a subset of ER- breast cancers, particularly those with oncogenic Src activation, may indeed be responsive to estrogen *in vivo*. Constitutive ER proteolysis in at least a subset of ER- cancers may not reflect extinguished ER-dependent transcription, but rather a shift to constitutive activation of different ER transcriptional targets. The therapeutic implications of this work are potentially very significant and warrant further investigation.

## Methods

*Breast cancers used for ER mRNA quantitation.* Cryopreserved primary invasive human breast cancers were obtained from the tumor repository of the Sunnybrook Health Sciences Center clinical ER quantitation reference lab with approval from the hospital Ethics Review Board. One expert clinical biochemist performed all ER cytosolic ligand binding assays (LBA) (69). Concordance of ER LBA with ER immunohistochemistry was verified in 40 tumors.

*RNA extraction and ER mRNA quantitation.* mRNA was extracted from 300 macrodissected carcinomas (100gm) using TRIZOL per manufacturer (Molecular Research Center, Cincinnati, OH). All RNAs were visualized on ethidium gels. 250 tumor RNA samples with OD 260/280 >1.3 and <2.1 from 50 ER positive cancers (ER by LBA >30 fmol/mg protein) and 200 ER negative cancers (<10 fmol/mg protein) were analysed. QPCR of human porphobilinogen deaminase (h-PBGD) expression using the LightCycler h-PBGD Housekeeping Gene Kit (Roche) primer/hybridization mixture demonstrated similar expression and equal RNA quality in both groups (Student's t test).

QPCR reactions used the LightCycler System (Roche Molecular Biochemicals) and the QuantiTect SYBR Green RT-PCR kit (Qiagen Inc.). For primers see Supplemental data. A standard curve for ER mRNA quantitation was generated using serial dilutions of full-length human ER cDNA plasmid, PCMV5hER- $\alpha$  (provided by B. Katzenellenbogen). MCF-7 ER mRNA was quantitated using the PCMV5hER- $\alpha$  plasmid standard curve. MCF-7 mRNA was run as positive control in all tumor ER

mRNA QPCR reactions. Tumor ER mRNA values ranged from 10 fg/ $\mu$ l to 1  $\mu$ g/ $\mu$ l. Melting curve analysis ensured exclusion of primer dimers from each analysis. ER mRNA concentrations in ER + and - cancers were compared by Student's *t*-test.

*Sequencing of ER cDNA PCR product.* All tumor ER PCR products were visualized by gel electrophoresis. For a subset, PCR amplified ER cDNA was gel extracted with QIAquick Gel Extraction Kit (Qiagen) and 10 ng DNA sequenced using 3.2 pmol of each ER sequencing primer, Terminator Reaction Mix (ABI Prism BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit) and the ABI Prism 3100 Genetic Analyser.

*Cell culture.* MCF-7 was grown in 5% FBS and estradiol was depleted in 5% charcoal-stripped FBS (cFBS) for 48 h as in (70). Depletion of both growth factors and estradiol used transfer to 0.1% cFBS for 48 h. The ER- BT-20 and BT549 and the weakly ER+ MDA-MB-361 (provided by S. Parsons) were grown in DMEM (49). The identity of ER+ and ER- lines was confirmed by karyotyping. To assay effects of growth factors on ER levels, 10nM estrogen with or without 5% FBS or 5% cFBS alone was added to MCF-7 that had been estrogen and growth factor-depleted for 48 h and ER levels assayed 1 to 6 hours later.

*Plasmids and transfection and generation of inducible Src MCF-7 lines.* Activated human cSrc vector, PCI-Src Y530F (from D. Fujita University of Calgary) or

empty PCI (10  $\mu$ g) was transfected into MCF-7 using lipofectamine PLUSTM (GIBCO). BT549 was transfected with PCMV5hER- $\alpha$  and stable lines cloned.

*Construction of MCF-7 lines with inducible Src expression.* Src Y530F cDNA was cloned into pIND and transfected into MCF-7 with an integrated pVgRXR vector (Invitrogen). Src was induced with 2  $\mu$ M ponasterone A (PA). MCFpINDSrc2 had high Src induction 8-24 h after PA. This line was estrogen deprived as above for 72 hours and 2  $\mu$ M PA added or not for the last 10 h of estrogen deprivation. Cells were then transferred to 0.1% cFBS and 10nM estradiol was added for 6h and ER  $t_{1/2}$  assayed by cycloheximide chase (see below).

*Flow cytometric analysis.* Brdu pulse labeling and flow cytometric analysis were as described (70).

*Antibodies.* The ER $\alpha$  mAb H222, was supplied by G. Greene (U. of Chicago); ER antibody HC-20 and anti-ubiquitin antibody, P4D1 were from Santa Cruz; and anti-Src mAb GD11 was from Upstate Biotechnology. Antibodies to MAPK, phosphorylated MAPK (P-MAPK), total PKB, activated PKB (PKB-P) and anti-phosphotyrosine antibody P-tyr-102 were from Cell Signaling and to  $\beta$ -actin from Sigma.

*Immunoblotting and cycloheximide chase.* Cells were lysed in ice cold D/RB buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, 2.5 mM EGTA pH 8.0, 10% glycerol, 10 mM  $\beta$ -glycerophosphate, 1 mM NaF, 0.1% Tween-20, 1 mM PMSF,

0.1 mM Na<sub>2</sub>VO<sub>4</sub>, 0.5 mM DTT, and 0.02 mg/ml each of aprotinin, leupepsin and pepstatin). Protein was quantitated by Bradford. Westerns used 20-100 μg protein per lane. The ER *t*<sub>1/2</sub> was determined by cycloheximide (CHX) chase with 100 μg CHX added at t=0. Cells were lysed at times indicated and ER blotted. ER protein was quantitated by densitometry of 3 experiments with Molecular Dynamics Imaging system and Image Quant software.

*Effects of MEK and PI3K inhibition on ER stability.* To assay effects of MEK or PI3K inhibition on ER levels, increasing concentrations of UO126 (Promega) (0.1-10 μM) or LY294002 (Promega) (0.5-8 μM) were added to asynchronous MCF-7 for 48 h prior to immunoblotting or flow cytometry. Estradiol and growth factor depleted MCF-7 were treated with either 10 μM UO126 or 8 μM LY294002 for 30 min prior to stimulation with 17β-estradiol for 6 h, followed by immunoblotting and flow cytometry.

*Cellular Src kinase assays.* Cell lines or primary human breast cancers were lysed in ice cold NP40 lysis buffer (70) with added 0.1 mM Na<sub>2</sub>VO<sub>4</sub> and 1 mM EDTA pH 8.0. Src was precipitated from 200 μg lysate and Src kinase assayed as described (71).

*ERE luciferase assays.* MCF-7 cells were transfected with 500 ng of plasmid bearing 2 tandem ERE (2 x ERE luc), 50 ng phRL-TK luc and 100 ng cSrc-Y530F using lipofectamine/plus per manufacturer (Gibco). Cells were treated with 10 μM PP1 and/or 10nM estrogen for 4 h prior to luciferase assays using dual-luciferase reporter assays (Promega) and Beckman Coulter LD 400.

*Q-RT-PCR of ER target genes pS2 and GREB1.* MCFpINDSrc2 was maintained in 5% cFBS for 2 days before adding 2  $\mu$ M Ponasterone A for 16 hrs to induce Src. The cells with and without Src induction were then treated with either 10 nM E2 or 100 nM tamoxifen for 3 hrs. Total RNA was isolated using TRIzol® per (Invitrogen, Carlsbad, CA). cDNA synthesis used 1  $\mu$ g total RNA with iScript cDNA kit (Bio-Rad, Hercules, CA). QPCR used icyclerIQ PCR detection system (Bio-Rad, Hercules, CA) with 10 ng cDNA sample in iQ SyberGreen supermix (Bio-Rad, Hercules, CA). PCR conditions and primers are described in Supplemental data.

*Detection of ER ubiquitylation in vivo.* MCF-7 were starved in 0.1% cFBS for 48 hrs and then either maintained in 0.1% cFBS or transferred to 5% cFBS medium with 10 nM estradiol with or without 10  $\mu$ M PP1 or 10  $\mu$ M MG132. 6 h after estrogen addition, cells were lysed in 50 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 2 mM EDTA, 50 mM NaF, 1% NP40, 1% SDS and boiled for 10 min and then centrifuged for 1 min at 12,000 rpm at 22°C. Supernatant protein was quantitated and ER and  $\beta$ -actin assayed by Western. To detect ubiquitylated ER, ER was immunoprecipitated from 500  $\mu$ g lysate, resolved by SDS-PAGE and transferred to nitrocellulose (Biorad). The membrane was boiled in transfer buffer for 10 min and immunoblotted with antibodies against ER, or ubiquitin.

*In vitro ER ubiquitylation assay.* Ubiquitylation assays used 40 ng recombinant ER (Sigma), GST-ubiquitin-activating enzyme (E1), GST-ubiquitin-conjugating enzyme UbcH7 (E2), MCF-7 lysate (50 $\mu$ g) as E3 source and an energy regenerating solution

from Boston Biochem, in 7.4 mM Hepes, pH 7.4, 5 mM KCl, 1.5 mM MgCl<sub>2</sub> for 60 min at 37 °C. Prior to ubiquitylation assays, recombinant ER was either incubated with 10 ng recombinant Src kinase (Upstate) or mock-treated for 5 min at 30°C in 7.4 mM Hepes pH 7.4, 5 mM KCl, 1.5 mM MgCl<sub>2</sub>. ER was precipitated, complexes resolved and transferred to nitrocellulose (Biorad, 0.45 μm). The membrane was boiled for 10 min and ER and ubiquitylated ER (Ub-ER) detected as above.

*In vitro ER degradation assay.* ED degradation assays used E1, E2 and E3 as above, with the following modifications. To catalyze *in vitro* degradation of ER, 50 nM of 26S proteasome fraction (Boston Biochem) was added for 30 min at 37 °C in 7.4 mM Hepes pH 7.4, 5 mM KCl, 1.5 mM MgCl<sub>2</sub>, with 1 mM DTT. ER was assayed by Western.

*Reverse phase tissue lysate array (RPPA).* 101 fresh frozen primary breast tumors from the M. D. Anderson Cancer Center Breast Tissue Tumor Bank were macrodissected and lysed as described (50), boiled in 1% SDS and protein-rich supernatants were serially diluted using a Tecan liquid handling robot. A robotic GeneTAC arrayer (Genomic Solutions) created arrays of six twofold serial dilutions for each tumor lysate on nitrocellulose-coated glass slides (FAST Slides, Schleicher & Schuell). Arrayed slides were probed with estrogen receptor alpha antibody (NeoMarkers), Src (Upstate), and the signal amplified using a DakoCytomation (Carpinteria, CA) catalyzed system. A secondary antibody (anti-rabbit) was used as a starting point for signal amplification. All samples were normalized for protein loading as

in (50;72). NCSS/PASS (Kaysville, Utah) software was used for two sided t tests and canonical correlation. ER and/or progesterone receptor (PR) were assayed by IHC in pathology evaluation at diagnosis.

## **Acknowledgements**

We thank G. Greene for mAb H222, Don Fujita for PCI-Src Y530F, and S. Parsons for cell lines. IC was supported by a U of Toronto Medical Oncology Post-Graduate Award and by DOD grant W81XWH-04-0392. AA was supported by a Canadian Breast Cancer Foundation Physician Fellowship. JMS is supported by the Braman Family Breast Cancer Institute of UM Sylvester Comprehensive Cancer Center.

## References

1. Jordan, V.C. 1995. Studies on the estrogen receptor in breast cancer - 20 years as a target for the treatment and prevention of cancer. *Breast Cancer Res Treat* **36**:267-285.
2. Clarke, M., Collins, R., Davies, C., Godwin, J., Gray, R., and Peto, R. 1998. Tamoxifen for early breast cancer: An overview of the randomised trials. *Lancet* **351**:1451-1467.
3. Green, S., Walter, P., Greene, G., Krust, A., Goffin, C., Jensen, E., Scrace, G., Waterfield, M., and Chambon, P. 1986. Cloning of the human oestrogen receptor cDNA. *J. Steroid Biochem.* **24**:77-83.
4. Kuiper, G.G., Enmark, E., Peltö-Huikko, M., Nilsson, S., and Gustafsson, J.A. 1996. Cloning of a novel receptor expressed in rat prostate and ovary. *Proc. Natl. Acad. Sci U. S. A.* **93**:5925-5930.
5. Mosselman, S., Polman, J., and Dijkema, R. 1996. ER-beta: identification and characterization of a novel human estrogen receptor. *FEBS Lett.* **392**:49-53.
6. Ferguson, A.T., and Davidson, N.E. 1997. Regulation of estrogen receptor alpha function in breast cancer. *Critical Reviews in Oncogenesis* **8**:29-46.
7. Roodi, N., Bailey, L.R., Kao, W.Y., Verrier, C.S., Yee, C.J., Dupong, W.D., and Parl, F.F. 1995. Estrogen receptor gene analysis in estrogen receptor-positive and receptor-negative primary breast cancer. *J Natl Cancer Inst* **87**:446-451.
8. Carmeci, C., deConinck, E.C., Lawton, T., Bloch, D.A., and Weigel, R.J. 1997. Analysis of estrogen receptor messenger RNA in breast carcinomas from archival specimens is predictive of tumor biology. *Am J Pathol* **150**:1563-1570.
9. Henry, J.A., Nicholson, S., Farndon, J.R., Westley, B.R., and May, F.E.B. 1988. Measurement of oestrogen receptor mRNA levels in human breast tumours. *Br J Cancer* **58**:600-605.
10. Garcia, T., Lehrer, S., Bloomer, W.D., and Schachter, B. 1988. A variant estrogen receptor messenger ribonucleic acid is associated with reduced levels of estrogen binding in human mammary tumors. *Molecular Endocrinology* **2**:785-791.
11. 't Veer, L.J., Dai, H., van de Vijver, M.J., He, Y.D., Hart, A.A., Mao, M., Peterse, H.L., van der, K.K., Marton, M.J., Witteveen, A.T. et al 2002. Gene expression profiling predicts clinical outcome of breast cancer. *Nature* **415**:530-536.
12. Gruvberger, S., Ringner, M., Chen, Y., Panavally, S., Saal, L.H., Borg, A., Ferno, M., Peterson, C., and Meltzer, P.S. 2001. Estrogen receptor status in breast cancer is associated with remarkably distinct gene expression patterns. *Cancer Res* **61**:5979-5984.
13. West, M., Blanchette, C., Dressman, H., Huang, E., Ishida, S., Spang, R., Zuzan, H., Olson, J.A., Jr., Marks, J.R., and Nevins, J.R. 2001. Predicting the clinical status of human breast cancer by using gene expression profiles. *Proc Natl Acad Sci U. S. A* **98**:11462-11467.
14. Perou, C.M., Sorlie, T., Eisen, M.B., van de, R.M., Jeffrey, S.S., Rees, C.A., Pollack, J.R., Ross, D.T., Johnsen, H., Akslén, L.A. et al 2000. Molecular portraits of human breast tumours. *Nature* **406**:747-752.

15. Sorlie, T., Perou, C.M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M.B., van de, R.M., Jeffrey, S.S. et al 2001. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U. S. A* **98**:10869-10874.
16. Nilsson, S., and Gustafsson, J.A. 2000. Estrogen receptor transcription and transactivation: Basic aspects of estrogen action. *Breast Cancer Res* **2**:360-366.
17. Klinge, C.M. 2000. Estrogen receptor interaction with co-activators and co-repressors. *Steroids* **65**:227-251.
18. Tora, L., White, J., Brou, C., Tasset, D., Webster, N., Scheer, E., and Chambon, P. 1989. The human estrogen receptor has two independent nonacidic transcriptional activation functions. *Cell* **59**:477-487.
19. Danielian, P.S., White, R., Lees, J.A., and Parker, M.G. 1992. Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. *EMBO J* **11**:1025-1033.
20. Hall, J.M., Couse, J.F., and Korach, K.S. 2001. The multifaceted mechanisms of estradiol and estrogen receptor signaling. *J Biol Chem* **276**:36869-36872.
21. Coleman, K.M., and Smith, C.L. 2001. Intracellular signaling pathways: nongenomic actions of estrogens and ligand-independent activation of estrogen receptors. *Front Biosci* **6**:D1379-D1391.
22. Migliaccio, A., DiDomenico, M., Castona, C., DeFalco, A., Bontempo, P., Nola, E., and Auricchio, F. 1996. Tyrosine kinases/p21ras/MAP-kinase pathway activation by estradiol receptor complex in MCF-7 cells. *EMBO J* **15**:1292-300.
23. Song, R.X., Barnes, C.J., Zhang, Z.G., Bao, Y.D., Kumar, R., and Santen, R.J. 2004. The role of Shc and insulin-like growth factor 1 receptor in mediating the translocation of estrogen receptor to the plasma membrane. *Proceedings of the National Academy of Sciences of the United States of America* **101**:2076-2081.
24. Migliaccio, A., Castoria, G., Di Domenico, M., de Falco, A., Bilancio, A., Lombardi, M., Barone, M.V., Ametrano, D., Zannini, M.S., Abbondanza, C. et al 2000. Steroid-induced androgen receptor-oestradiol receptor beta-*Src* complex triggers prostate cancer cell proliferation. *EMBO J* **19**:5406-5417.
25. Wong, C.W., McNally, C., Nickbarg, E., Komm, B.S., and Cheskis, B.J. 2002. Estrogen receptor-interacting protein that modulates its nongenomic activity-crosstalk with *Src*/*Erk* phosphorylation cascade. *Proc Natl. Acad. Sci. U. S. A* **99**:14783-14788.
26. Castoria, G., Migliaccio, A., Bilancio, A., Di Domenico, M., de Falco, A., Lombardi, M., Fiorentino, R., Varricchio, L., Barone, M.V., and Auricchio, F. 2001. PI3-kinase in concert with *Src* promotes the S-phase entry of oestradiol-stimulated MCF-7 cells. *EMBO J* **20**:6050-6059.
27. Likhite, V.S., Stossi, F., Kim, K., Katzenellenbogen, B.S., and Katzenellenbogen, J.A. 2006. Kinase-Specific Phosphorylation of the Estrogen Receptor Changes Receptor Interactions with Ligand, DNA, and Coregulators Associated with Alterations in Estrogen and Tamoxifen Activity  
1. *Mol. Endocrinol.*
28. Aronica, S.M., and Katzenellenbogen, B.S. 1993. Stimulation of estrogen receptor-mediated transcription and alteration in the phosphorylation state of the rat uterine estrogen receptor by estrogen, cyclic adenosine monophosphate, and insulin-like growth factor-I. *Mol Endocrinol* **7**:743-752.

29. Bunone,G., Briand,P.A., Miksicek,R.J., and Picard,D. 1996. Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *EMBO J* **15**:2174-2183.
30. Kato,S., Endoh,H., Masuhiro,Y., Kitamoto,T., Uchiyama,S., Sasaki,H., Masushige,S., Gotoh,Y., Nishida,E., Kawashima,H. et al 1995. Activation of the estrogen receptor through phosphorylation by mitogen- activated protein kinase. *Science* **270**:1491-1494.
31. Ignar-Trowbridge,D.M., Nelson,K.G., Bidwell,M.C., Curtis,S.W., Washburn,T.F., McLachlan,J.A., and Korach,K.S. 1992. Coupling of dual signaling pathways: epidermal growth factor action involves the estrogen receptor. *Proc. Natl. Acad. Sci. U. S. A.* **89**:4658-4662.
32. Simoncini,T., Hafezi-Moghadam,A., Brazil,D.P., Ley,K., Chin,W.W., and Liao,J.K. 2000. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature* **407**:538-541.
33. Joel,P.B., Smith,J., Sturgill,T.W., Fisher,T.L., Blenis,J., and Lannigan,D.A. 1998. pp90rsk1 regulates estrogen receptor-mediated transcription through phosphorylation of Ser-167. *Mol Cell Biol.* **18**:1978-1984.
34. Yudt,M.R., Vorojeikina,D., Zhong,L., Skafar,D.F., Sasson,S., Gasiewicz,T.A., and Notides,A.C. 1999. Function of estrogen receptor tyrosine 537 in hormone binding, DNA binding, and transactivation. *Biochem J* **38**:14146-14156.
35. Arnold,S.F., Melamed,M., Vorojeikina,D.P., Notides,A.C., and Sasson,S. 1997. Estradiol-binding mechanism and binding capacity of the human estrogen receptor is regulated by tyrosine phosphorylation. *Mol. Endocrinol.* **11**:48-53.
36. Weis,K.E., Ekena,K., Thomas,J.A., Lazennec,G., and Katzenellenbogen,B.S. 1996. Constitutively active human estrogen receptors containing amino acid substitutions for tyrosine 537 in the receptor protein. *Mol. Endocrinol.* **10**:1388-1398.
37. Nirmala,P.B., and Thampan,R.V. 1995. Ubiquitination of the rat uterine estrogen receptor: Dependence on estradiol. *Biochemical and Biophysical Research Communications* **213**:24-31.
38. Nawaz,Z., Lonard,D.M., Dennis,A.P., Smith,C.L., and O'Malley,B.W. 1999. Proteasome-dependent degradation of the human estrogen receptor. *Proc Natl Acad Sci Usa* **96**:1858-1862.
39. Alarid,E.T., Bakopoulos,N., and Solodin,N. 1999. Proteasome-mediated proteolysis of estrogen receptor: A novel component in autologous Down-regulation. *Molecular Endocrinology* **13**:1522-1534.
40. Reid,G., Hubner,M.R., Metivier,R., Brand,H., Denger,S., Manu,D., Beaudouin,J., Ellenberg,J., and Gannon,F. 2003. Cyclic, proteasome-mediated turnover of unliganded and liganded ERalpha on responsive promoters is an integral feature of estrogen signaling. *Mol. Cell* **11**:695-707.
41. Lonard,D.M., Nawaz,Z., Smith,C.L., and O'Malley,B.W. 2000. The 26S proteasome is required for estrogen receptor-alpha and coactivator turnover and for efficient estrogen receptor-alpha transactivation. *Molecular Cell* **5**:939-948.
42. Wijayaratne,A.L., and McDonnell,D.P. 2001. The human estrogen receptor-alpha is a ubiquitinated protein whose stability is affected differentially by agonists, antagonists, and selective estrogen receptor modulators. *J Biol. Chem.* **276**:35684-35692.

43. Fan,S., Wang,J., Yuan,R., Ma,Y., Meng,Q., Erdos,M.R., Pestell,R.G., Yuan,F., Auborn,K.J., Goldberg,I.D. et al 1999. BRCA1 inhibition of estrogen receptor signaling in transfected cells. *Science* **284**:1354-1356.
44. Saji,S., Okumura,N., Eguchi,H., Nakashima,S., Suzuki,A., Toi,M., Nozawa,Y., Saji,S., and Hayashi,S. 2001. MDM2 enhances the function of estrogen receptor alpha in human breast cancer cells. *Biochemical and Biophysical Research Communications* **281**:259-265.
45. Nawaz,Z., Lonard,D.M., Smith,C.L., Lev-Lehman,E., Tsai,S.Y., Tsai,M.J., and O'Malley,B.W. 1999. The Angelman syndrome-associated protein, E6-AP, is a coactivator for the nuclear hormone receptor superfamily. *Molecular and Cellular Biology* **19**:1182-1189.
46. Thomas,S.M., and Brugge,J.S. 1997. Cellular functions regulated by Src family kinases. *Annu Rev Cell Dev Biol* **13**:513-609.
47. Rosen,N., Bolen,J.B., Schwartz,A.M., Cohen,P., DeSeau,V., and Israel,M.A. 1986. Analysis of pp60c-src protein kinase activity in human tumor cell lines and tissues. *J Biol Chem* **261**:13754-13759.
48. Tansey,W.P. 2001. Transcriptional activation: risky business. *Genes & Dev.* **15**:1045-1050.
49. Belsches-Jablonski,A.P., Biscardi,J.S., Peavy,D.R., Tice,D.A., Romney,D.A., and Parsons,S.J. 2001. Src family kinases and HER2 interactions in human breast cancer cell growth and survival. *Oncogene* **20**:1465-1475.
50. Sheehan,K.M., Calvert,V.S., Kay,E.W., Lu,Y., Fishman,D., Espina,V., Aquino,J., Speer,R., Araujo,R., Mills,G.B. et al 2005. Use of reverse phase protein microarrays and reference standard development for molecular network analysis of metastatic ovarian carcinoma 1. *Mol. Cell Proteomics.* **4**:346-355.
51. Iwao,K., Miyoshi,Y., Egawa,C., Ikeda,N., Tsukamoto,F., and Noguchi,S. 2000. Quantitative analysis of estrogen receptor-alpha and -beta messenger RNA expression in breast carcinoma by real-time polymerase chain reaction. *Cancer* **89**:1732-1738.
52. Pegram,M.D., Pauletti,G., and Slamon,D.J. 1998. HER-2/neu as a predictive marker of response to breast cancer therapy. *Breast Cancer Research and Treatment* **52**:65-77.
53. Nicholson,S., Wright,C., Sainsbury,J.R., Halcrow,P., Kelly,P., Angus,B., Farndon,J.R., and Harris,A.L. 1990. Epidermal growth factor receptor (EGFR) as a marker for poor prognosis in node-negative breast cancer patients: neu and tamoxifen failure. *Journal of Steroid Biochemistry and Molecular Biology* **37**:811-814.
54. Oh,A.S., Lorant,L.A., Holloway,J.N., Miller,D.L., Kern,F.G., and El-Ashry,D. 2001. Hyperactivation of MAPK induces loss of ERalpha expression in breast cancer cells. *Mol Endocrinol* **15**:1344-1359.
55. Biscardi,J.S., Maa,M.C., Tice,D.A., Cox,M.E., Leu,T.H., and Parsons,S.J. 1999. c-Src-mediated phosphorylation of the epidermal growth factor receptor on Tyr845 and Tyr1101 is associated with modulation of receptor function. *J Biol Chem* **274**:8335-8343.
56. Arnold,S.F., Obourn,J.D., Jaffe,H., and Notides,A.C. 1995. Phosphorylation of the human estrogen receptor on tyrosine 537 in vivo and by Src family tyrosine kinases in vitro. *Molecular Endocrinology* **9**:24-33.

57. Arnold,S.F., Obourn,J.D., Yudt,M.R., Carter,T.H., and Notides,A.C. 1995. In vivo and in vitro phosphorylation of the human estrogen receptor. *J Steroid Biochem Mol Biol* **42**:159-171.
58. Shah,Y.M., and Rowan,B.G. 2005. The Src kinase pathway promotes tamoxifen agonist action in Ishikawa endometrial cells through phosphorylation-dependent stabilization of estrogen receptor (alpha) promoter interaction and elevated steroid receptor coactivator 1 activity  
1. *Mol. Endocrinol.* **19**:732-748.
59. Sisci,D., Aquila,S., Middea,E., Gentile,M., Maggiolini,M., Mastroianni,F., Montanaro,D., and Ando,S. 2004. Fibronectin and type IV collagen activate ERalpha AF-1 by c-Src pathway: effect on breast cancer cell motility  
1. *Oncogene* **23**:8920-8930.
60. Shao,W., Keeton,E.K., McDonnell,D.P., and Brown,M. 2004. Coactivator AIB1 links estrogen receptor transcriptional activity and stability. *Proc. Natl. Acad. Sci. U. S. A* **101**:11599-11604.
61. Guo,Z., Dai,B., Jiang,T., Xu,K., Xie,Y., Kim,O., Nesheiwat,I., Kong,X., Melamed,J., Handratta,V.D. et al 2006. Regulation of androgen receptor activity by tyrosine phosphorylation  
1. *Cancer Cell* **10**:309-319.
62. Salghetti,S.E., Caudy,A.A., Chenoweth,J.G., and Tansey,W.P. 2001. Regulation of transcriptional activation domain function by ubiquitin. *Science* **293**:1651-1653.
63. Fukuchi,M., Imamura,T., Chiba,T., Ebisawa,T., Kawabata,M., Tanaka,K., and Miyazono,K. 2001. Ligand-dependent degradation of Smad3 by a ubiquitin ligase complex of ROC1 and associated proteins. *Mol Biol Cell* **12**:1431-1443.
64. Nawaz,Z., and O'Malley,B.W. 2004. Urban renewal in the nucleus: is protein turnover by proteasomes absolutely required for nuclear receptor-regulated transcription? *Mol. Endocrinol.* **18**:493-499.
65. von Baur,E., Zechel,C., Heery,D., Heine,M.J.S., Garnier,J.M., Vivat,V., Le Douarin,B., Gronemeyer,H., Chambon,P., and Losson,R. 1996. Differential ligand-dependent interactions between the AF-2 activating domain of nuclear receptors and the putative transcriptional intermediary factors mSUG1 and TIF1. *The EMBO J* **15**:110-124.
66. Alarid,E.T., Preisler-Mashek,M.T., and Solodin,N.M. 2003. Thyroid hormone is an inhibitor of estrogen-induced degradation of estrogen receptor-alpha protein: estrogen-dependent proteolysis is not essential for receptor transactivation function in the pituitary. *Endocrinology* **144**:3469-3476.
67. Fan,M., Nakshatri,H., and Nephew,K.P. 2004. Inhibiting proteasomal proteolysis sustains estrogen receptor-alpha activation. *Mol. Endocrinol.* **18**:2603-2615.
68. Shang,Y., Hu,X., DiRenzo,J., Lazar,M.A., and Brown,M. 2000. Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. *Cell* **103**:843-852.
69. Hassapoglidou,S., Diamandis,E.P., and Sutherland,D.J.A. 1993. Quantification of p53 protein in tumor cell lines, breast tissue extracts and serum with time-resolved immunofluorometry. *Oncogene* **8**:1501-1509.
70. Cariou,S., Donovan,J.C., Flanagan,W.M., Milic,A., Bhattacharya,N., and Slingerland,J.M. 2000. Down-regulation of p21WAF1/CIP1 or p27Kip1 abrogates antiestrogen-mediated cell cycle arrest in human breast cancer cells. *Proc. Natl. Acad. Sci. U. S. A* **97**:9042-9046.

71. Egan,C., Pang,A., Durda,D., Cheng,H.C., Wang,J.H., and Fujita,D.J. 1999. Activation of Src in human breast tumor cell lines: elevated levels of phosphotyrosine phosphatase activity that preferentially recognizes the Src carboxy terminal negative regulatory tyrosine 530. *Oncogene* **18**:1227-1237.
72. Tibes,R., Qiu,Y., Lu,Y., Hennessy,B., Andreeff,M., Mills,G.B., and Kornblau,S.M. 2006. Reverse phase protein array: validation of a novel proteomic technology and utility for analysis of primary leukemia specimens and hematopoietic stem cells  
1. *Mol. Cancer Ther.* **5**:2512-2521.

## Figure Legends

### Figure 1

ER- and ER+ human breast cancers express ER mRNA. **(A)** PCR of ER and hPBGD from breast tumors. C= no mRNA control. **(B)** Scatter-plot of ER mRNA concentrations (means shown by horizontal line). **(C)** Frequency of ER mRNA concentrations rounded to the nearest logarithm value. **(D)** ER mRNA (fm/mg total RNA) plotted versus ER protein by LBA (fm/mg cytosolic protein) in the same human breast cancers.

### Figure 2

Src promotes estrogen-dependent ER degradation. **(A)** ER before and 6 h after estradiol (E) added to E-depleted MCF-7 with or without proteasome inhibitor, LLnL.  $\beta$ -actin shows equal loading. **(B)** The ER  $t_{1/2}$  assayed in E-depleted cells and at 6 h after E addition by cycloheximide (CHX) chase. Densitometric analysis of 3 CHX chase experiments (mean  $\pm$  SEM). **(C)** Cells were grown in 0.1% cFBS for 48 h then treated with estrogen alone (+E), 5% cFBS +E, or 5% cFBS alone. ER and  $\beta$ -actin were assayed 6 h later. **(D)** Serum and E deprived MCF-7 were transferred to 5% FBS plus E, with or without added Src inhibitor PP1 and ER assayed 6h later. **(E and F)** MCF-7 was transfected with Src-Y530F (Src) or empty vector (mock) and 24 h later **(E)** ER and Src assayed or **(F)** ER  $t_{1/2}$  assayed by CHX chase (mean  $\pm$  SEM). **(G and H)** MCFpINCSrc2 line was E depleted for 48 hrs and Src induced (+) or not (-) for 16 hrs prior to addition of E. **(G)** Src at time of E addition (left), and ER and  $\beta$ -actin before and 3 h after E was added (right). **(H)** CHX pulse chase, starting 3h after E addition.

### Figure 3

Src promotes estrogen dependent ER transcriptional activity. **(A-C)** MCF-7 was transfected with ERE luciferase reporter and either Src-Y530F (Src) or empty vector (C) and then stimulated with E. **(A)** ERE luciferase activity and **(B)** ER and Src levels before (C) and 4 hours after Src transfection, E or both. **(C)** Relative ERE luciferase activity corrected for ER level. **(D and E)** MCFpINDSrc2 was E depleted for 48 hrs and Src induced (+) or not (-) for 16 hrs prior to addition of E or E plus tamoxifen (TAM). QPCR of cellular **(D)** GREB1 and **(E)** pS2.

### Figure 4

Evidence for estrogen regulation of ER levels in ER+ and ER- breast cancer lines. **(A)** ER detected by immunoblotting ER precipitates from 1 mg cell lysate of asynchronous ER+ MCF-7 and MDA-MB-361 and ER- BT-20. **(B)** cSrc activity in asynchronous MCF-7, MDA-MB-361 and BT-20. **(C)** ER  $t_{1/2}$  in asynchronous cells, assayed by CHX chase, calculated from 3 independent assays (mean +/- SEM). **(D)** After 48h serum and E deprivation in 0.1% cFBS, MCF-7, MDA-MB-361, BT-20 and BT549-ER were stimulated with E + 5%FBS with or without prior addition of MG132 or Src inhibitor PD166326 and ER assayed 6 h later.

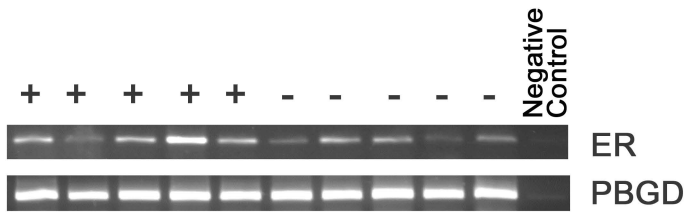
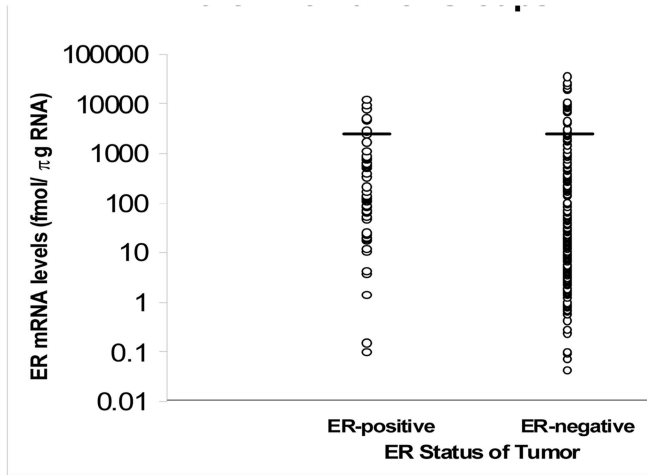
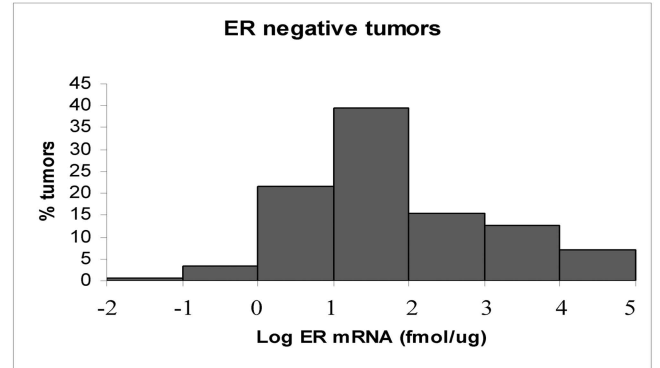
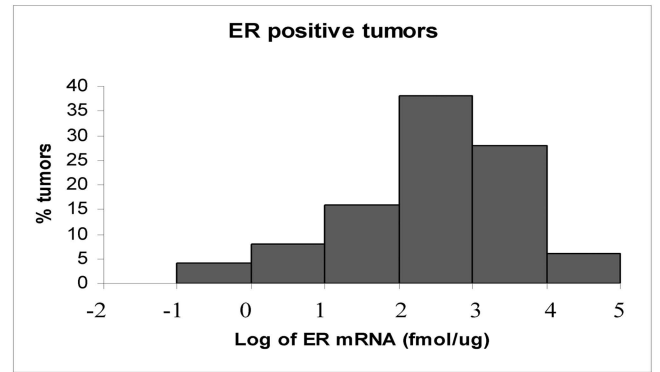
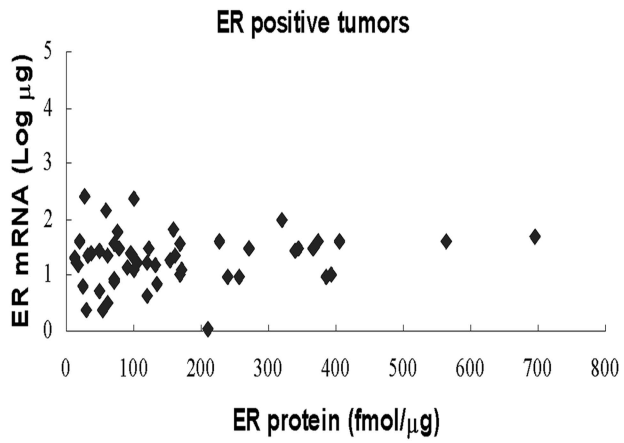
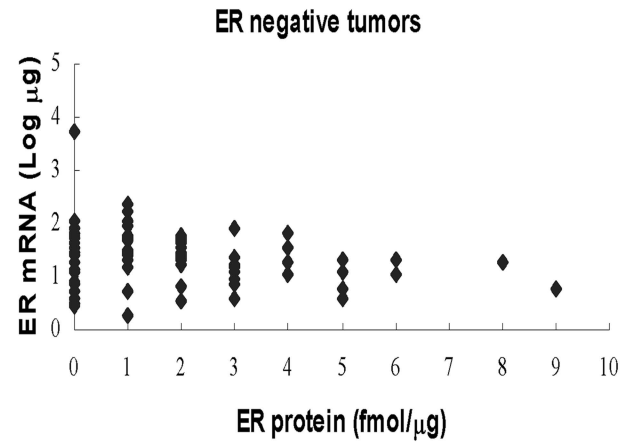
### Figure 5

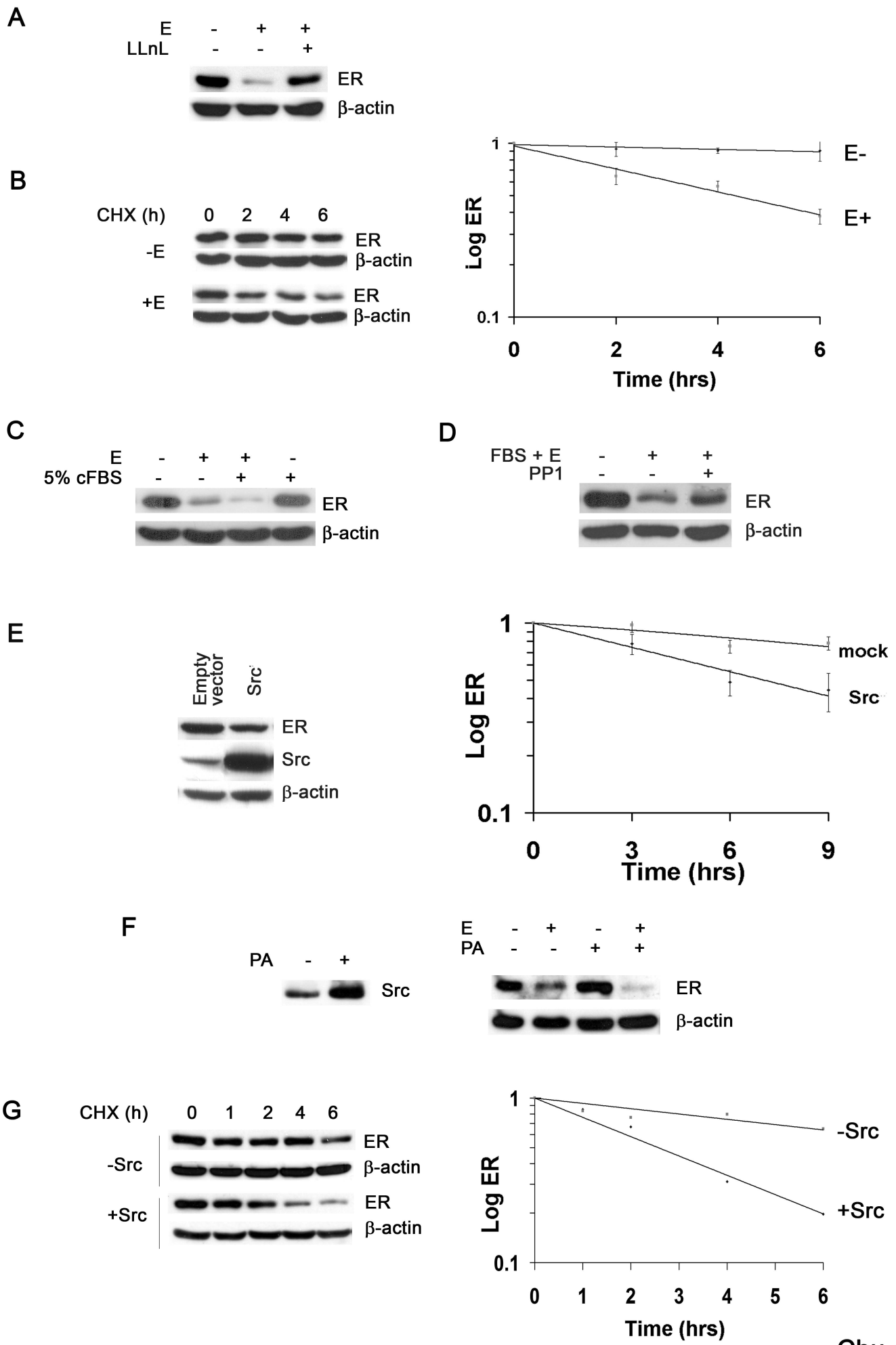
Src stimulates ER ubiquitylation and degradation *in vivo* and *in vitro*. **(A)** Serum and E-deprived MCF-7 were treated with E and 5% cFBS, with or without prior addition of MG132 or PP1, for 6 hr and ER levels were assayed.  $\beta$ -actin blotting confirms equal

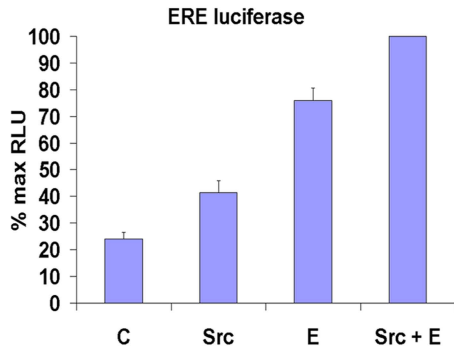
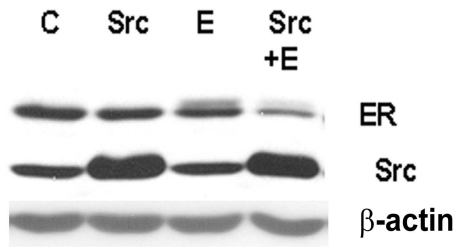
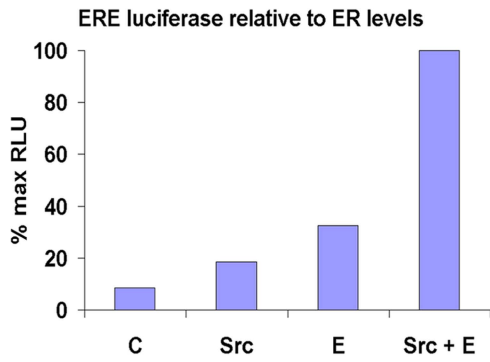
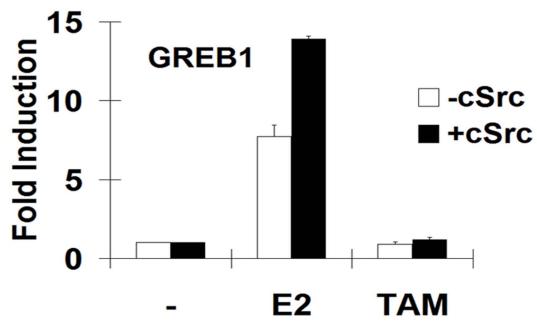
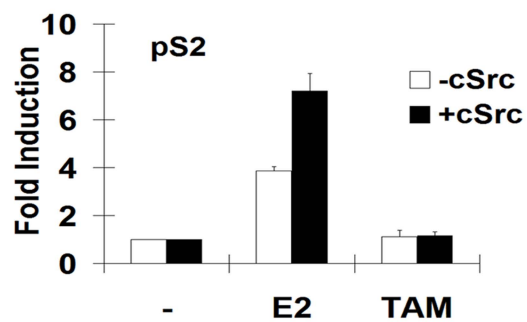
loading. ER was precipitated, ER complexes resolved and ubiquitylated ER detected with anti-ubiquitin antibody. **(B)** For *in vitro* ER ubiquitylation, recombinant ER protein was reacted with E1, E2, with or without E3, and with or without prior ER reaction with Src kinase, as described for 60 min. ER immunoprecipitates were resolved and blotted with anti-ubiquitin or anti-phosphotyrosine antibodies. The membrane was stripped and reprobed for ER. **(C)** *In vitro* degradation of recombinant ER used E1, E2 and E3 with or without prior incubation with Src and/or addition of 26S proteasome fraction as described in Experimental procedures.

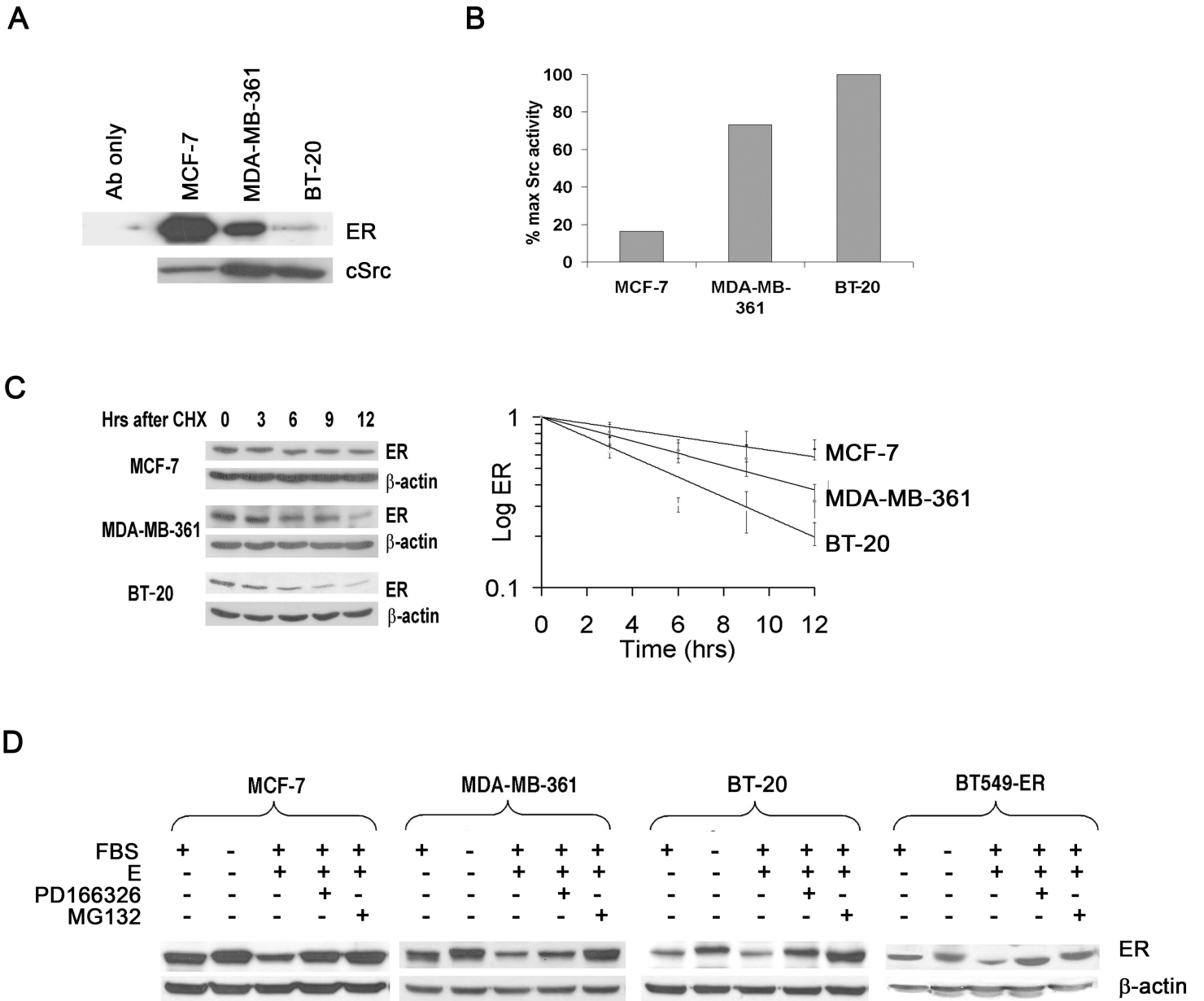
## Figure 6

cSrc activity and/or levels are elevated in ER- primary breast cancers. **(A)** Cryopreserved breast tumors were lysed, ER status verified by Western and cSrc kinase activity assayed.  $\beta$ -actin showed equal loading. **(B and C)** ER and Src protein levels were quantitated by RPPA in 101 primary breast cancers as in Experimental procedures. **(B)** Histogram distribution of log Src protein levels rounded to nearest log for HR+ (ER and/or PR positive) and HR- (ER and PR negative) cancers. **(C)** Dot plot of Src and ER protein values in all cancers.

**A****B****C****D****E**

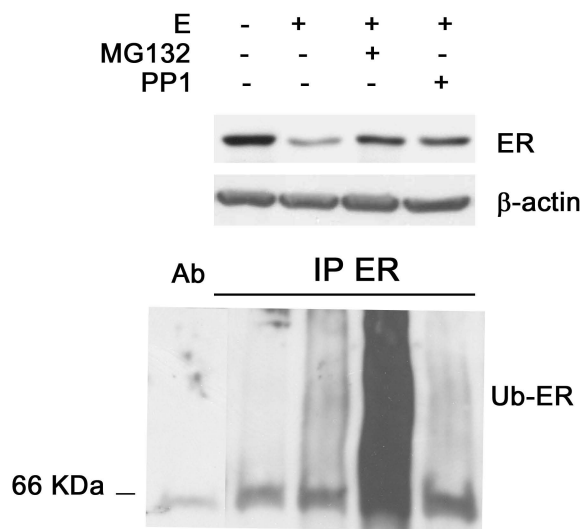


**A****B****C****D****E**

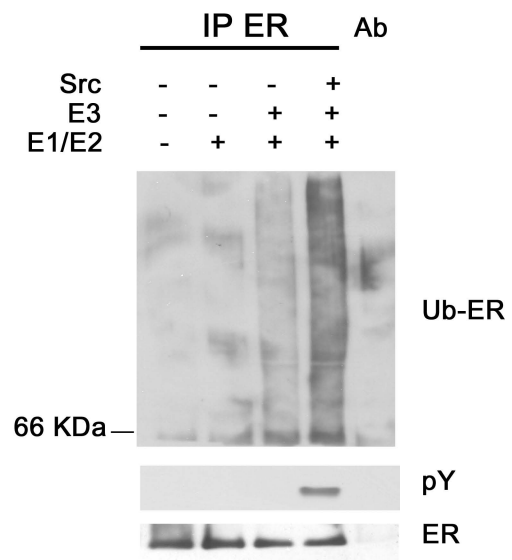


Chu et al Fig4

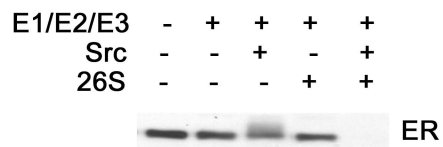
**A**



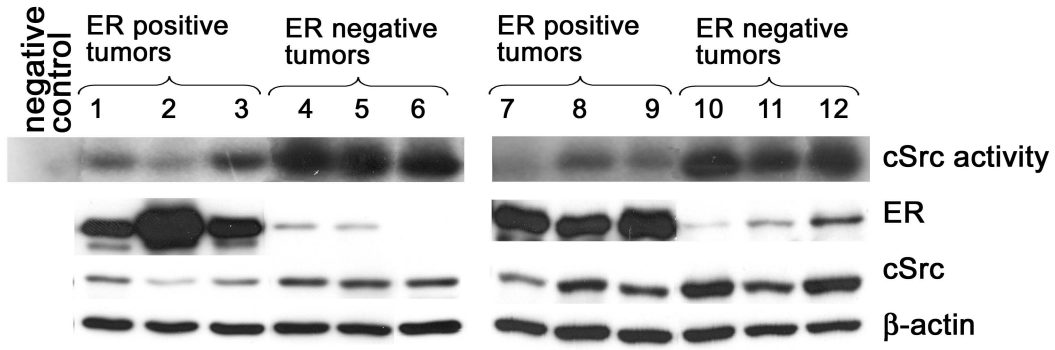
**B**



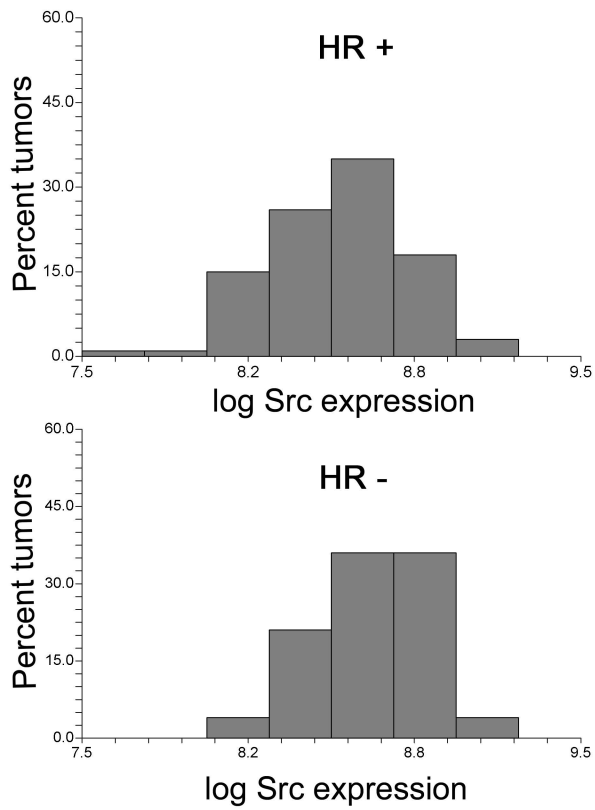
**C**



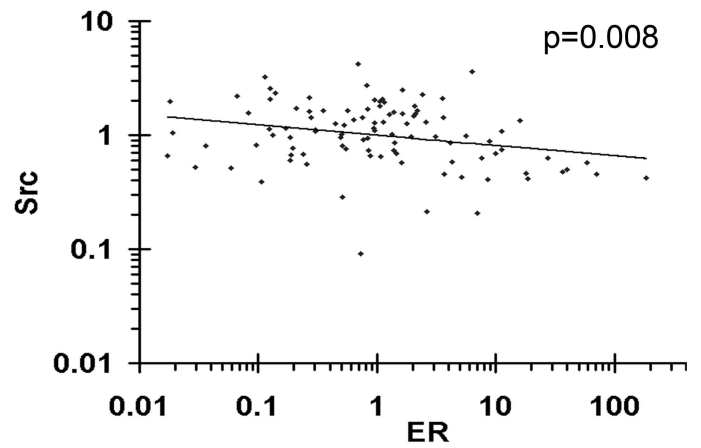
**A**



**B**



**C**



## APPENDIX II

# The Dual ErbB1/ErbB2 Inhibitor, Lapatinib (GW572016), Cooperates with Tamoxifen to Inhibit Both Cell Proliferation- and Estrogen-Dependent Gene Expression in Antiestrogen-Resistant Breast Cancer

Isabel Chu,<sup>1,3</sup> Kimberly Blackwell,<sup>4</sup> Susie Chen,<sup>1</sup> and Joyce Slingerland<sup>1,2</sup>

<sup>1</sup>The Braman Breast Cancer Institute, UM Sylvester Comprehensive Cancer Center and <sup>2</sup>Department of Biochemistry and Molecular Biology, University of Miami School of Medicine, Miami, Florida; <sup>3</sup>Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada; and <sup>4</sup>Division of Medical Oncology, Department of Medicine, Duke University Comprehensive Cancer Center, Durham, North Carolina

## Abstract

Effective treatment of estrogen receptor (ER)-positive breast cancers with tamoxifen is often curtailed by the development of drug resistance. Antiestrogen-resistant breast cancers often show increased expression of the epidermal growth factor receptor family members, ErbB1 and ErbB2. Tamoxifen activates the cyclin-dependent kinase inhibitor, p27 to mediate G<sub>1</sub> arrest. ErbB2 or ErbB1 overexpression can abrogate tamoxifen sensitivity in breast cancer lines through both reduction in p27 levels and inhibition of its function. Here we show that the dual ErbB1/ErbB2 inhibitor, lapatinib (GW572016), can restore tamoxifen sensitivity in ER-positive, tamoxifen-resistant breast cancer models. Treatment of MCF-7<sup>Pr</sup>, T-47D, and ZR-75 cells with lapatinib or tamoxifen alone caused an incomplete cell cycle arrest. Treatment with both drugs led to a more rapid and profound cell cycle arrest in all three lines. Mitogen-activated protein kinase and protein kinase B were inhibited by lapatinib. The two drugs together caused a greater reduction of cyclin D1 and a greater p27 increase and cyclin E-cdk2 inhibition than observed with either drug alone. In addition to inhibiting mitogenic signaling and cell cycle progression, lapatinib inhibited estrogen-stimulated ER transcriptional activity and cooperated with tamoxifen to further reduce ER-dependent transcription. Lapatinib in combination with tamoxifen effectively inhibited the growth of tamoxifen-resistant ErbB2 overexpressing MCF-7 mammary tumor xenografts. These data provide strong preclinical data to support clinical trials of ErbB1/ErbB2 inhibitors in combination with tamoxifen in the treatment of human breast cancer. (Cancer Res 2005; 65(1): 18-25)

## Introduction

The epidermal growth factor receptor or ErbB family of receptor tyrosine kinases consists of four members including epidermal growth factor receptor (also called Her1 or ErbB1), Her2 (ErbB2 or neu), Her3 (ErbB3), and Her4 (ErbB4). Upon ligand binding, ErbB family members homodimerize and heterodimerize resulting in the phosphorylation of their intracellular kinase domains (1). Once ErbB1 and ErbB2 are activated, the phosphotyrosylated sites in their

Src-homology 2 (SH2) domains serve as docking sites for adaptor proteins such as Shc, Grb2, and Sos resulting in the activation of the Ras/Raf/mitogen-activated protein kinase (MAPK) kinase (MEK)/MAPK and PI3K/protein kinase B (PKB) pathways and promotion of proliferation and mitogenesis (1, 2). The *ErbB2* gene is amplified and overexpressed in up to 30% of primary breast cancers (3) and this is associated with poor patient prognosis (4, 5). ErbB1 is also overexpressed in up to 30% of primary invasive breast cancers and this is correlated with reduced overall survival, proliferation, and higher metastatic potential (6, 7). Inhibition of ErbB1 signaling reduces both ErbB1 and ErbB2 activity and delays tumorigenesis in MMTV/Neu mice (8). The cooperative activation of proliferative pathways by these two receptors has stimulated the development of a number of small molecule inhibitors of members of the ErbB family for use as anticancer agents.

Upon estrogen binding, estrogen receptor  $\alpha$  (ER $\alpha$ ) recruits and activates Src leading to activation of Shc, MEK/MAPK, and PI3K/PKB (9). Activation of these signaling kinases has a dual effect on cell proliferation: it modulates cell cycle regulators to stimulate cell cycle progression (10, 11), and these activated kinases phosphorylate ER $\alpha$  to positively regulate its transcriptional activity (12, 13). Liganded ER dimerizes and associates with coactivators resulting in transcriptional activation of estrogen-responsive genes (14). Newly diagnosed ER-positive breast cancers are commonly treated with the antiestrogen tamoxifen. Tamoxifen competes with estrogen for ER binding, leading to inhibition of cell cycle progression and G<sub>1</sub> arrest (10, 11). However, tamoxifen treatment is often limited by the development of resistance and disease relapse (15). In ER-positive primary breast cancers, overexpression of both ErbB1 and ErbB2 is associated with resistance to tamoxifen therapy (7, 16). Overexpression of *ErbB2* in MCF-7 causes a loss of sensitivity to tamoxifen (17, 18). Moreover, MCF-7 cells grown in the continuous presence of tamoxifen not only develop tamoxifen resistance but also show elevated total and phosphorylated ErbB1 and ErbB2 (19).

In ER-positive breast cancer cells, estrogens recruit quiescent cells into cell cycle and stimulate G<sub>1</sub> cell cycle progression. Tamoxifen causes G<sub>0</sub>/G<sub>1</sub> cell cycle arrest. Cell cycle progression is governed by a family of cyclin-dependent kinase (cdk) that are regulated by cyclin binding, by cdk phosphorylation, and by association with specific cdk inhibitors (20). In quiescent cells, levels of the kinase inhibitor protein, p27, are elevated and p27 binds tightly and inhibits cyclin E-cdk2. In normal and malignant mammary epithelial cells, estrogens and growth factors stimulate p27 phosphorylation and loss of p27 from cyclin E-cdk2 complexes, with resulting cyclin E-cdk2 activation and p27 proteolysis promoting S-phase entry and cell cycle progression (21, 22). We have shown that p27 is essential

**Note:** Isabel Chu and Kimberly Blackwell contributed equally to this work.

**Request for reprints:** J. Slingerland, UM Sylvester Comprehensive Cancer Center, 1475 N.W. 12<sup>th</sup> Ave., (D8-4), Miami FL 33136. Phone: 305-243-7265; E-mail: jslingerland@med.miami.edu or K. Blackwell, Box 3893DUMC, Durham NC 27710. Phone: 919-681-2901; E-mail: black034@mc.duke.edu.

©2005 American Association for Cancer Research.

for G<sub>1</sub> arrest by tamoxifen (22). Moreover, transfection of constitutively activated MEK caused tamoxifen resistance through changes in p27 phosphorylation and a loss of its inhibitory function (23). MEK inhibition restored responsiveness to tamoxifen in ErbB2 or MEK overexpressing MCF-7 models (23).

Considerable attention has been directed to the development of therapeutic inhibitors of ErbB1 and ErbB2 for breast cancer treatment (24). Trastuzumab is a humanized anti-ErbB2 antibody that is approved for the treatment of *ErbB2* amplified metastatic breast cancer. Several new quinazoline drugs that target ErbB1 are under investigation in clinical trials in cancer patients. However, cooperative activation of different ErbB family members through heterodimerization could circumvent the therapeutic efficacy of inhibition of a single receptor. Thus, small molecule inhibitors that inhibit both ErbB1 and ErbB2 would be therapeutically advantageous. Because both ErbB1 and ErbB2 activate MEK/MAPK leading to loss of p27 function and tamoxifen resistance, we investigated the potential for a reversible dual inhibitor of both ErbB1 and ErbB2, lapatinib (GW572016), to restore tamoxifen-mediated cell cycle arrest and to overcome tamoxifen-resistant breast tumor growth. Lapatinib and tamoxifen together led to a more profound cell cycle arrest than tamoxifen alone through an increase in p27 levels, increased p27 binding to and inhibition of cyclin E-cdk2 and down-regulation of ER transcriptional activity.

## Materials and Methods

**Cell Culture.** MCF-7 were grown in Improved Modified Eagle's medium (option Zn<sup>2+</sup>) supplemented with 5% fetal bovine serum and insulin as in ref. (11). ZR-75 (provided by P. Darbre, Cell and Molecular Biology, University of Reading, Berkshire, UK) and T-47D (American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 10% fetal bovine serum and 1 mmol/L sodium pyruvate. Relatively early passage MCF-7 cells were initially obtained from the Osborne Lab. These cells were initially very sensitive to G<sub>1</sub> arrest by 4-hydroxy-tamoxifen (4-OH-TAM), with a reduction in % S-phase cells from 40% to 2% to 4% and an increase in the % G<sub>0</sub>/G<sub>1</sub> cells to over 90% within 48 hours of drug treatment (22). Within 30 to 40 passages in the above media, a gradual loss of tamoxifen responsiveness was noted. The variant of MCF-7 used in these studies, MCF-7<sup>Pr</sup>, had been cultured for >50 passages and had acquired partial resistance to tamoxifen compared with our earlier passage MCF-7. This line retained estrogen dependence for proliferation.

**Cell Cycle Effects of Tamoxifen and Lapatinib.** MCF-7<sup>Pr</sup>, ZR-75 and T-47D were treated with 10 nmol/L 4-OH-TAM (Sigma, St. Louis, MO) or 10 μmol/L lapatinib (GW572016; Glaxo SmithKline, Research Triangle Park, NJ) or both. Asynchronously proliferating untreated and drug treated cells were collected 48 hours later for flow cytometry and lysates were prepared at the same times for immunoblotting. MCF-7<sup>Pr</sup> cells were synchronized in quiescence by estrogen deprivation for 48 hours as described in (11). Cells were stimulated to reenter the cell cycle by addition of 10 nmol/L 17-β-estradiol and then recovered for protein and cell cycle analysis.

**Flow Cytometric Analysis.** Cells were pulse-labeled with 10 μmol/L bromodeoxyuridine for 2 hours and then fixed and stained with anti-bromodeoxyuridine-conjugated FITC (Becton Dickinson, Franklin Lakes, NJ) and propidium iodide. Cell cycle analysis was carried out using a Becton Dickinson FACScan, with Quest software as described in ref. (11).

**Antibodies.** Antibodies against MAPK, phosphorylated, activated MAPK (MAPK-P), PKB and phosphorylated activated PKB (PKB-P) were obtained from New England Biolabs (Beverly, MA), ErbB1 and ErbB2 antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA) and β-actin from Sigma. p27 antibody was obtained from Transduction Laboratories (Lexington, KY), p21 antibody from Santa Cruz; anti-PSTAIR was used for detection of cdk2 in cyclin E immunoprecipitates; cyclin E1 monoclonal antibodies E12 and E172 were from E. Harlow (Mass. General, MA; ref. 25).

**Immunoblotting and Immunoprecipitation.** Cells were lysed in ice cold 0.1% or 1% NP40 lysis buffer. Protein was quantitated by Bradford analysis and Western analysis of cyclin E, p27, p21, cdk2, MAPK, PKB, MAPK-P, and PKB-P was carried out as described in ref. (23). Equal protein loading was confirmed by probing for β-actin.

Cyclin E was immunoprecipitated from 200 μg of protein lysate with anti-cyclin E monoclonal antibody 172. Cyclin E-associated proteins were detected by immunoblotting and cyclin E-cdk2 activity was assayed. Briefly, for kinase assays immunoprecipitated cyclin E was incubated with [<sup>32</sup>P]-ATP (Amersham, Piscataway, NJ) and histone H1 (Roche, Indianapolis, IN) at 30 °C and radioactivity in histone H1 substrate was visualized by autoradiography.

**Luciferase Assays.** Cells were seeded in 24-well plates and transfected with a plasmid encoding the firefly luciferase gene driven by a promoter bearing two-tandem estrogen response elements (2xERE<sub>Luc</sub>) and a cytomegalovirus-driven *Renilla* luciferase construct (phRL-TK-luc) using lipofectamine PLUS (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. Cells were treated with 1 nmol/L 4-OH-TAM and 5 mol/L lapatinib for 24 hours before recovery for luciferase assays. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega, San Luis Obispo, CA) and a dual-channel luminometer from Thermo LabSystems (Needham, MA).

**Growth Inhibition of Tamoxifen-Resistant MCF7 Tumor Xenografts.** Ovariectomized athymic BALB/c nude mice were purchased from Taconic (Germantown, NY) and used for the xenograft studies. Tamoxifen-resistant MCF-7 tumors (MCF-TAMR) were derived by serial passage of tumor xenografts into animals treated with both tamoxifen (5 mg s.c. continuous release 60-day pellets; Innovative Research, Sarasota, FL) and estradiol (0.36 mg s.c. continuous release 60-day pellets, Innovative Research). After at least six serial xenograft passages of tumor that sustained growth in the presence of tamoxifen and estradiol, tumors were designated tamoxifen-resistant. Twenty-seven nude mice with MCF-TAMR established xenografts (median size at initiation of treatment was 40.0 mm<sup>3</sup>) were randomized to either placebo (vehicle) or treatment with lapatinib (100 mg/kg daily) by oral gavage. All mice continued to receive estradiol and tamoxifen treatment. Tamoxifen treatment was maintained in all animals to maintain selection pressure for continued resistance and because tumors regress when the tamoxifen is withdrawn. Tumors were measured thrice per week and tumor volume was determined using the formula: (length × width<sup>2</sup>) × (π/6). The primary objective in the xenograft study was the comparison of time with reach five times the initial tumor volume between the tamoxifen plus placebo-treated MCF-TAMR tumors and the tamoxifen plus lapatinib-treated MCF-TAMR tumors. Tumor lysates were collected from 2nd passage for tamoxifen-sensitive MCF-7 tumors and from 10th passage for MCF-TAMR tumors.

**Statistical Analysis.** Effects of drug treatment (lapatinib, tamoxifen or both) were compared with the % S phase of asynchronous cells in three repeat experiments. The % S-phase means for the four conditions were compared by ANOVA followed by mean pairwise comparison using Bonferroni multiple comparison approach. *P* < 0.05 was set as the criteria for statistical significance.

## Results

**Lapatinib and Tamoxifen Cooperate to Inhibit Cell Cycle Progression.** We observed a progressive development of resistance to cell cycle arrest by tamoxifen with long-term passage (*n* > 50) of MCF-7. Treatment (48 hours) of early passage MCF-7 with 4-OH-TAM caused G<sub>1</sub> arrest, with a decrease in the % S phase from 37% to 2% and an increase in the % cells with 2N DNA content to over 90% (22). In late passage MCF-7 (MCF-7<sup>Pr</sup>), treatment with 4-OH-TAM for 48 hours failed to cause complete cell cycle arrest: the % S-phase cells decreased from 40% to only 16% in MCF-7<sup>Pr</sup>. Lapatinib treatment (10 μmol/L) reduced the % S from 40% to 25% with a concomitant increase in the G<sub>1</sub> fraction. With both 4-OH-TAM

and lapatinib together, the % S phase of MCF-7<sup>Pr</sup> fell dramatically to 2% as compared with each treatment alone ( $P < 0.05$ ; Figs. 1A and 2). Two other ER-positive lines, T-47D obtained from American Type Culture Collection, and ZR-75 obtained from the Darbre lab, did not undergo complete G<sub>1</sub> arrest in response to 48 hours of 4-OH-TAM, exhibiting a degree of tamoxifen resistance similar to that of MCF-7<sup>Pr</sup>. Treatment of asynchronous T-47D cultures with either lapatinib or 4-OH-TAM alone, caused a partial arrest with the % S-phase cells falling from 25% to 13% or 17%, respectively, whereas combined treatment with lapatinib and 4-OH-TAM reduced the % S-phase cells to 2% ( $P < 0.05$ ; Figs. 1A and 2). A similar pattern was observed in ZR-75 cells (Figs. 1A and 2). Thus, ErbB1/ErbB2 inhibition and ER blockade can cooperate to inhibit cell cycle progression, with the two drugs together having at least additive effects on cell cycle progression. It is noteworthy that we did not observe increased ErbB1 or ErbB2 expression in MCF-7<sup>Pr</sup> compared with parental MCF-7 cells (Fig. 1C). Thus, the partial 4-OH-TAM-resistant phenotype observed in MCF-7<sup>Pr</sup> does not arise from elevated ErbB1 or ErbB2 expression. Furthermore, we observed that MCF-7<sup>Pr</sup>, T-47D, and ZR-75 expressed barely

detectable ErbB1 protein levels compared with ErbB1 overexpressing BT-20 cells and relatively low levels of ErbB2 compared with ErbB2 overexpressing MDA-MB-361 (Fig. 1C).

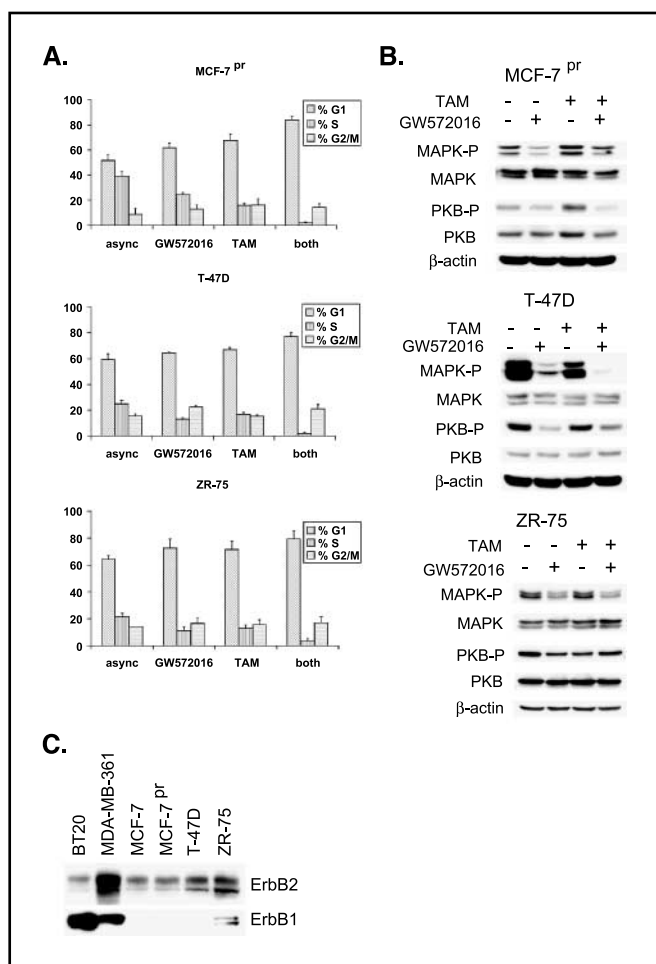
#### Effects of Lapatinib and Tamoxifen on MAPK and PKB.

Lapatinib has been shown to effectively inhibit ErbB1 and ErbB2 (26, 27). Both ErbB1 and ErbB2 activate the PI3K/PKB and Ras/Raf/MAPK pathways (1). Inhibition of either MEK or PI3K results in cell cycle arrest in MCF-7 cells (23, 28). Furthermore, constitutive MEK activation in MCF-7 has been shown to confer tamoxifen resistance (23, 29). In MCF-7<sup>Pr</sup>, 48 hours treatment with 10  $\mu\text{mol/L}$  lapatinib decreased the activating phosphorylation of both MAPK and PKB as shown by immunoblotting for MAPK-P and PKB-P, whereas total MAPK and PKB levels were unchanged (Fig. 1B). 4-OH-TAM stimulated MAPK activity in MCF-7 as shown previously (30). Whereas this may reflect a partial estrogen agonistic effect of 4-OH-TAM in MCF-7, certain mammary cell types can show sustained MAPK activation when arrested by inhibitory cytokines and by differentiation. Treatment with lapatinib and 4-OH-TAM together inhibited MAPK and PKB activities more notably than either drug alone. Note that unlike MCF-7<sup>Pr</sup>, in which 4-OH-TAM stimulated MAPK activity, in T-47D and ZR-75, 4-OH-TAM partially inhibited MAPK. Lapatinib inhibited both MAPK and PKB when added alone. MAPK-P was more profoundly inhibited by both drugs together in T-47D but not in ZR-75 (Fig. 1B). These data underline the heterogeneity in the response of MAPK to epidermal growth factor receptor family and ER blockade among ER-positive breast cancer lines. Thus, inhibition of MAPK phosphorylation may not be the best surrogate end point for growth arrest by receptor tyrosine kinase inhibitors.

**Lapatinib and Tamoxifen Effects on p27 and Cyclin E-cdk2 Inhibition.** Our flow cytometry data showed that lapatinib and 4-OH-TAM can act together to arrest cells in G<sub>1</sub>. Because both MEK and PI3K regulate p27 levels and function in breast cells (23, 29, 31), we assayed drug effects on p27 and other cell cycle regulators, p27 binding to cyclin E and cyclin E-cdk2 activity. In MCF-7<sup>Pr</sup>, treatment with either lapatinib or 4-OH-TAM alone modestly reduced cyclin D1 levels by less than 2-fold, whereas treatment with both inhibitors resulted in a synergistic 5-fold reduction in cyclin D1 levels (Fig. 2A). The two drugs together also had a more profound effect on cyclin D1 levels than did either drug alone in T-47D and ZR-75 (Fig. 2B and C). p27 levels increased by 1.4- to 2-fold with either drug alone and by ~2.5- to 5-fold in cells treated with both, with less dramatic increases observed in T-47D.

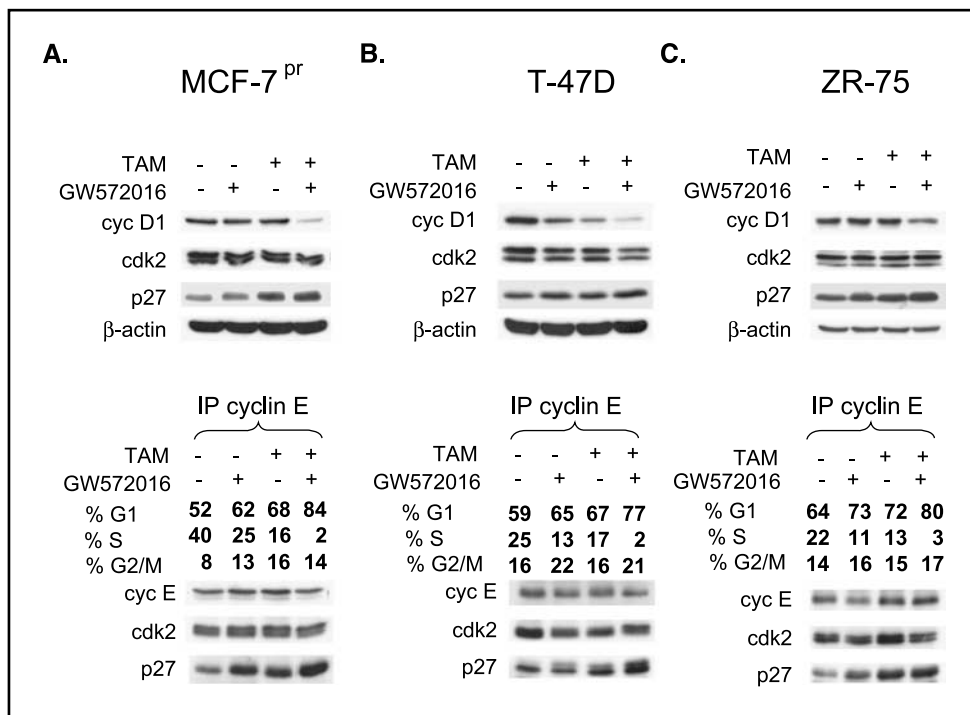
When asynchronous cells were treated with drug for 48 hours, immunoprecipitation followed by Western blotting showed that p27 bound to cyclin E-cdk2 increased by 2.0-fold in MCF-7<sup>Pr</sup> treated with lapatinib and by 1.8-fold following 4-OH-TAM alone (Fig. 2A). Treatment with lapatinib and 4-OH-TAM resulted in a 5-fold increase in p27 bound to cyclin E-cdk2. Similar effects were observed in T-47D and ZR-75 (Fig. 2B and C). The increased p27 binding to cyclin E-cdk2 mediated cyclin E-cdk2 inhibition (Fig. 3). Lapatinib or 4-OH-TAM treatment each partly inhibited cyclin E-cdk2, with more profound kinase inhibition following treatment with both drugs together (representative data for ZR-75 in Fig. 3).

**Lapatinib and Tamoxifen Inhibit Estrogen-Dependent G<sub>1</sub>-S Progression.** Although they exhibit partial tamoxifen resistance, our MCF-7<sup>Pr</sup> cells are estrogen-dependent for proliferation. Stimulation of estrogen-deprived quiescent MCF-7<sup>Pr</sup> cells with estrogen for 18 hours activated MAPK (Fig. 4A). Moreover, it stimulated cell cycle reentry with 54% of cells in S phase after 18 hours of estradiol treatment (Fig. 4B). Consistent with prior data (11), estrogen

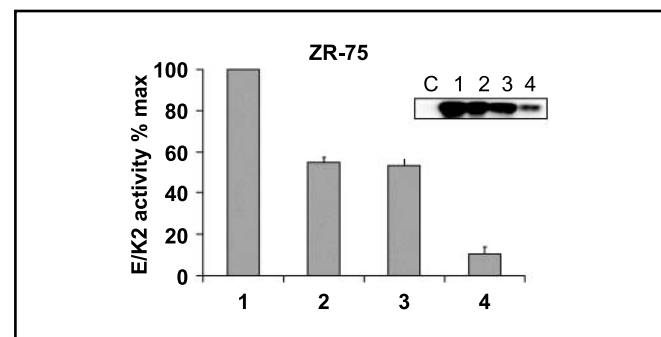


**Figure 1.** Lapatinib and tamoxifen cooperate to induce G<sub>1</sub> arrest in ER-positive breast cancer lines. **A**, asynchronous MCF-7<sup>Pr</sup>, T-47D, and ZR-75 cultures were treated with 10  $\mu\text{mol/L}$  lapatinib (GW572016), 10 nmol/L 4-OH-TAM or both inhibitors for 48 hours before flow cytometric analysis. Bars, SE. **B**, Western analyses for MAPK-P, MAPK, PKB, P-PKB, and  $\beta$ -actin used cells treated as in **A**. **C**, Western blot analysis for ErbB1 and ErbB2 in asynchronous BT-20, MDA-MB-361, early passage MCF-7 and MCF-7<sup>Pr</sup>, T-47D, and ZR-75.

**Figure 2.** Lapatinib and tamoxifen reduce cyclin D1, increase p27 and increase cyclin E-cdk2 bound p27. Cells were treated with lapatinib (GW572016) and 4-OH-TAM for 48 hours and cell lysates were collected as in Fig 1. In A, B, and C, cyclin D1, cdk2 and p27 protein levels and cyclin E complexes were assayed by immunoblotting in MCF-7<sup>Pr</sup>, T-47D, and ZR-75, respectively.  $\beta$ -Actin as a loading control. Cyclin E immunoprecipitates were resolved by SDS-PAGE and associated proteins detected by blotting using the indicated antibodies. Flow cytometric data are shown for cells recovered 48 hours after each treatment condition.



reduced p27 levels (Fig. 4A), notably decreased cyclin E-bound p27 (Fig. 4B) and activated cyclin E-cdk2. These effects were partially abrogated by treatment with either 4-OH-TAM or lapatinib alone (Fig. 4A-C). Treatment with both drugs together prevented the loss of p27 and its release from cyclin E-cdk2 and completely inhibited cyclin E-cdk2 activation and cell cycle reentry (Fig. 4A-C). Taken together, these findings suggest that activation of ErbB1/ErbB2 following estrogen-ER binding is required for estrogen-dependent G<sub>1</sub>-to-S phase cell cycle progression. Moreover, they suggest that therapeutic ER blockade and receptor tyrosine kinase inhibition may have greater efficacy in abrogating estrogen-stimulated breast cancer cell proliferation than that observed with either treatment modality alone.



**Figure 3.** Lapatinib and tamoxifen cooperate to inhibit cyclin E-cdk2. ZR-75 were treated with lapatinib (GW572016), 4-OH-TAM or both for 48 hours as in Figs. 1 and 2. Cell lysates were collected for cyclin E immunoprecipitation and cyclin E-cdk2 activity was assayed using histone H1 as substrate. Kinase reactions were resolved and radioactivity quantitated by scintillation counting and graphed as a percent maximum. *Inset*, autoradiographed histone H1 bands. C, immunoglobulin G control; 1, no treatment; 2, lapatinib; 3, 4-OH-TAM; 4, lapatinib and 4-OH-TAM. Bars, SE.

**Tamoxifen and Lapatinib Inhibit ER Transcriptional Activity.** For many years, the therapeutic effect of tamoxifen was thought to result from its inhibition of estrogen-dependent ER transcriptional activity. However, liganded ER is also known to bind and activate Src, PI3K, and MAPK, leading to activation of mitogenic signaling and cell cycle progression. MAPK once activated following estrogen stimulation can in turn phosphorylate the ER in a manner that activates its transcriptional activity (12, 13). Here, we show that the therapeutic efficacy of the dual ErbB1/ErbB2 inhibitor, lapatinib (GW572016) results not only from inhibition of signaling via MAPK and PKB but also involves inhibition of estrogen ER transcriptional activity in ER-positive breast cancer cells.

We assayed ER transcriptional activity using an ERE reporter construct (2xERE<sub>luc</sub>). Treatment with lapatinib alone reduced ER transcriptional activity by 38% and 39% in MCF-7<sup>Pr</sup> and ZR-75, respectively (Fig. 5A and B). 4-OH-TAM reduced ERE-luciferase activities by 76% and 71% for MCF-7<sup>Pr</sup> and ZR-75, respectively. Treatment with both drugs resulted in a further reduction in ER activity in both cell lines. Thus, in addition to antiproliferative effects resulting from inhibition of mitogenic signaling and G<sub>1</sub> cyclin-cdks, lapatinib can work together with 4-OH-TAM to inhibit estrogen activated ER transcriptional activity.

**Lapatinib Inhibits Growth of Tamoxifen-Resistant Breast Cancer Xenograft Tumors.** We next tested the ability of lapatinib to inhibit growth or cause regression of tamoxifen-resistant MCF-TAMR xenografts *in vivo*. In contrast to the parental MCF-7 line and early xenograft tumors, late passage serial explant xenograft MCF-TAMR tumors are not only resistant to growth inhibition by tamoxifen, they are growth stimulated by tamoxifen (32). The MCF-TAMR xenograft tumors require estradiol for tumor establishment and growth, and all ovariectomized animals were treated with estradiol implants throughout these experiments.

Treatment of MCF-TAMR tumor xenografts with 4-OH-TAM and lapatinib significantly inhibited tumor growth when compared with 4-OH-TAM treatment alone (Fig. 6B). Because these tumors regress when 4-OH-TAM is withdrawn (32), the effect of lapatinib alone was not tested. No toxicity due to lapatinib was seen in any of the 13 animals treated with lapatinib and 4-OH-TAM. There were three complete tumor regressions (23%) in the lapatinib/4-OH-TAM treated group compared with continued growth of all 14 4-OH-TAM-treated xenografts in control animals. There was a significant delay in the time to reach a 5-fold increase in the initial tumor volume in lapatinib/4-OH-TAM-treated animals versus 4-OH-TAM alone (median, 38 versus 25 days; Wilcoxon sum-rank test;  $P = 0.004$ ). The increase in tumor volume over time (tumor volume day 35 – tumor volume day 0/tumor volume day 0) was significantly reduced in lapatinib/4-OH-TAM treated tumors versus 4-OH-TAM alone (4.5% median volume change versus 9.9%; Wilcoxon sum-rank test,  $P = 0.0026$ ). Consistent with previous findings, ErbB2 and ErbB1 levels were increased in the MCF-TAMR tumor samples compared with MCF-7 cells (Fig. 6A).

## Discussion

Tamoxifen has been widely used for breast cancer prevention, for treatment of breast cancer in the adjuvant setting to prevent recurrence after tumor removal, and for metastatic disease (15). Tamoxifen resistance presents a major clinical problem. The mechanisms whereby ER-positive breast cancers develop resistance to tamoxifen are not fully understood. However, there is considerable evidence from cell lines and mouse models that constitutive activation of receptor tyrosine kinase pathways, including those downstream of ErbB1 and ErbB2 can induce tamoxifen resistance (16–19, 23).

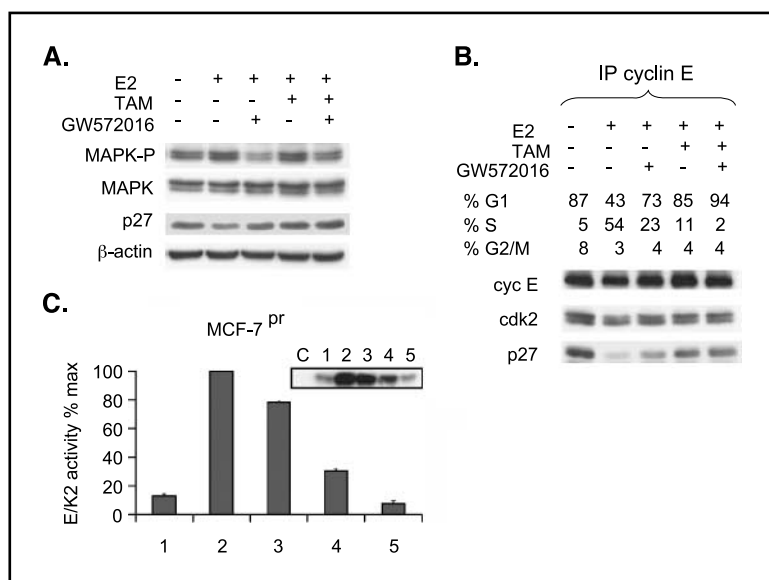
Lapatinib is a potent inhibitor of ErbB1 and ErbB2 signaling. It binds to the catalytic domain of ErbB1 and ErbB2 and inhibits autophosphorylation of the receptors (26, 27). Lapatinib has been shown to be effective as an anticancer agent in both preclinical models (26, 27) and in Phase I/II trials in cancer patients (33). The present study was undertaken to assay the potential efficacy of

lapatinib to restore tamoxifen-mediated growth arrest in breast cancer cell lines and xenograft tumors that have developed tamoxifen resistance. Tamoxifen has a cytostatic effect on breast cancer growth and causes G<sub>1</sub> cell cycle arrest in ER-positive breast cancer cells (10). Because prior work from our group and others has shown that tamoxifen-dependent G<sub>1</sub> arrest requires intact kinase inhibitor protein function (11, 34), we assayed the effects of these agents on p27 and its target cdk2.

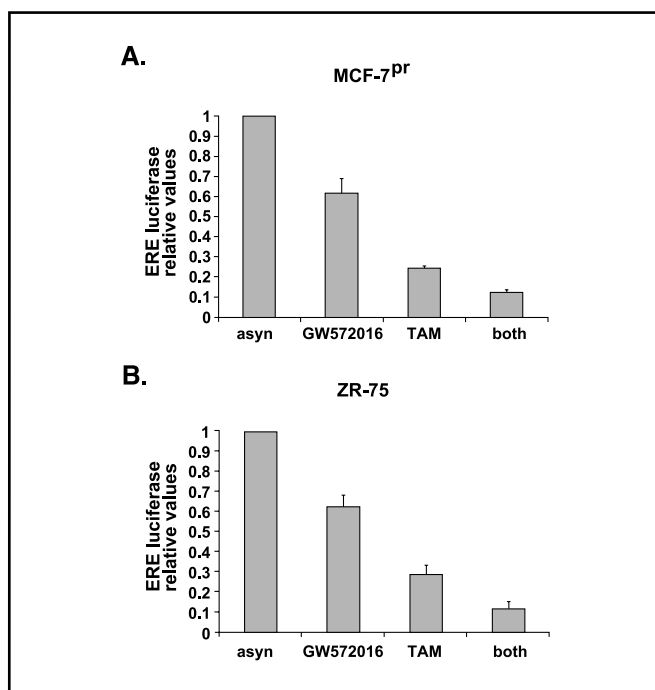
Although p27 is strongly expressed in quiescent mammary epithelial cells, p27 levels are reduced in up to 60% of primary human breast cancers and this has been correlated with poor patient prognosis (35, 36). p27 is required for antiestrogen-mediated cell cycle arrest and deregulation of p27 in *ErbB2* transfected cells is causally linked to antiestrogen resistance (11, 23, 37, 38). Lapatinib, when added to 4-OH-TAM effectively inhibited ErbB-dependent MAPK and PKB activation, and increased the binding and inhibition of cyclin E-cdk2 by p27 more effectively than either drug alone. It is noteworthy that the increase in p27 binding to cyclin E-cdk2 following lapatinib and 4-OH-TAM was consistently higher in all three cell lines than the up-regulation of p27 protein levels. Thus, ErbB1 and ErbB2 signaling seems to alter the affinity of p27 for its target cdk2. This is consistent with earlier work showing that p27 redistribution onto cdk2 complexes preceded the increase in p27 levels in SKBR3 and BT474 cells after ErbB2 inhibition (38).

Both MEK/MAPK and PKB alter p27 phosphorylation and oppose the cdk inhibitory effects of p27 (23, 31, 37, 39). Transfection of activated MEK reduces the cyclin E-cdk2 inhibitory activity of p27 (23) and activates p27 proteolysis (23, 38). Moreover, constitutive PKB activation shifts p27 into the cytoplasm away from nuclear cdk targets (31, 37, 39). PI3K/PKB-dependent p27 phosphorylation also increases p27 assembly into cyclin D1-cdk4 complexes and a loss of p27 binding to cyclin E-cdk2.<sup>5</sup> Thus, lapatinib has the potential to oppose p27 proteolysis, and to reverse the aberrant cytoplasmic sequestration of p27 seen in many cancers by causing p27 redistribution from cyclin D1-cdk4 to cyclin E-cdk2 complexes.

One of the cell cycle effectors most dramatically affected by the combination treatment with lapatinib and 4-OH-TAM was cyclin D1. Cyclin D1 is a transcriptional target of the ER (40), and in some but not all studies, its overexpression in ER-positive breast



**Figure 4.** Effects of ER blockade and ErbB1/ErbB2 inhibition on estrogen-stimulated cell cycle entry. Estrogen-deprived, quiescent MCF-7<sup>Pr</sup> were treated with lapatinib (GW572016) and/or 4-OH-TAM 30 minutes before stimulation with estradiol as described in Materials and Methods. Eighteen hours after estradiol addition, cells were harvested for protein and flow cytometric analysis. **A**, MAPK-P, MAPK, p27, and β-actin were assayed by Western blot. **B**, cell cycle profiles following different treatments. Cyclin E immunoprecipitates were resolved, transferred to membrane and associated proteins detected by immunoblotting. **2C**, cyclin E-cdk2 activity was assayed and results graphed as % maximum. *Autorad inset*, radioactivity in histone H1 for nonspecific immunoglobulin G control (C) and cyclin E immunoprecipitates following different drug treatments indicated (1-5).



**Figure 5.** Both lapatinib and tamoxifen inhibit ER transcriptional activity. Asynchronous growing (A) MCF-7<sup>Pr</sup> and (B) ZR-75 were transfected with 2xERELuc for 24 hours before treatment with DMSO control, 5  $\mu$ M/L lapatinib (GW572016), 1 nmol/L 4-OH-TAM or both drugs together for an additional 24 hours. Columns, mean of three independent experiments; bars, SE. Fold inhibition of ERE luciferase activity in untreated asynchronous control cells (asyn).

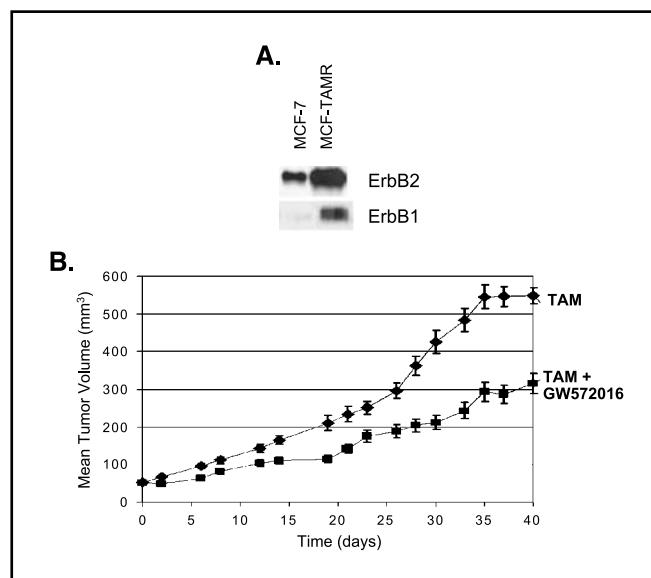
cancers has been associated with poor prognosis (41, 42). Cyclin D1 deficient mice are resistant to breast cancers induced by transgenic *ErbB2/neu* (43) suggesting a critical role of cyclin D1 in *ErbB2* mediated tumorigenesis. Furthermore, overexpression of cyclin D1 can mediate antiestrogen resistance in breast cancer cell lines (44). ER blockade and ErbB1/ErbB2 inhibition reduced cyclin D1 levels most notably in MCF-7<sup>Pr</sup> and in T-47D. This may reflect both transcriptional repression (40) and a loss of cyclin D1 stability, because PKB inhibition leads to cyclin D1 proteolysis (45, 46). The reduced effect of lapatinib on PKB in ZR-75 may in part account for the less dramatic loss of cyclin D1 in ZR-75 than in MCF-7<sup>Pr</sup> and T-47D.

The effects of lapatinib and 4-OH-TAM on MAPK and PKB activities varied within the three cell lines tested. Inhibition of MAPK and PKB by both drugs together was not much greater than that achieved with lapatinib alone in MCF-7<sup>Pr</sup> and ZR-75, yet cell cycle inhibition was significantly greater with both drugs together. It is noteworthy that in a recent Phase I clinical trial of this agent, MAPK and PKB inhibition was not always correlated with tumor response and may not be the most useful surrogate end point (47). Other cellular pathways in addition to MAPK and PKB need to be inhibited to achieve maximal growth arrest.

Although the antiproliferative effects of ErbB1/ErbB2 inhibitors are thought to result from inhibition of mitogenic signaling and effects on cell cycle regulators, our data suggest that in ER-positive cancers, they also inhibit estrogen stimulated ER transcriptional

activity. Lapatinib and 4-OH-TAM both inhibited ER-dependent transcription. Lapatinib treatment alone resulted in 40% reduction in ERE luciferase activity. The repression of ERE-dependent transcription was even more profound in 4-OH-TAM and lapatinib-treated cells. This reduction could in part result from inhibition of ErbB1/2 by lapatinib, reducing input to MAPK and PKB. Estrogen binding to the ER activates Src and leads to Shc, Ras and MAPK and PKB activation (48–50). Both MAPK and PKB once activated can phosphorylate the ER at specific sites to increase its transcriptional activity (12, 49, 51, 52). The ER can also be activated in a ligand-independent manner through oncogenic receptor tyrosine kinase activation (53, 54). Inhibition of signaling cross-talk and ER phosphorylation is an additional mechanism whereby by lapatinib may cooperate with 4-OH-TAM to impair both estrogen-dependent and ligand-independent ER transcriptional activity. Although there is evidence that insulin-like growth factor-I receptor can cross-talk with liganded ER, lapatinib does not inhibit the insulin-like growth factor-I receptor either *in vitro* or in cell-based assays (data not shown). Thus, lapatinib-mediated restoration of growth arrest in these tamoxifen-resistant models does not likely involve insulin-like growth factor-I receptor inhibition.

In some breast cancers and in MCF-7/HER2-18 cells, tamoxifen resistance may arise through altered effects of tamoxifen on ER activated transcription. In these cells, tamoxifen-bound ER may recruit coactivators to the ERE rather than corepressors as is observed in sensitive cells (55). Shou et al. (55) recently showed that pretreatment with the pure ErbB1 inhibitor, gefitinib, restored corepressor binding to the 4-OH-TAM-bound ER at ERE response elements in the PS2 promoter in MCF-7/HER2-18 cells. These findings suggest that ErbB1/ErbB2 signaling may modulate ER phosphorylation and conformation leading to altered ER-coactivator/corepressor complexes formation.



**Figure 6.** Lapatinib inhibits the growth of tamoxifen-resistant mammary tumor xenografts. A, Western blot analysis for ErbB1 and ErbB2 of tumor lysates from MCF-7 and MCF-TAMR tumors. B, tamoxifen-resistant tumor xenografts were implanted into the flanks of nude mice and continuously grown with tamoxifen and estrogen pellets. When the tumor volumes reached 40 mm<sup>3</sup>, 13 mice were treated with lapatinib (GW572016) as described in Materials and Methods, and the remaining 14 animals were maintained as controls. All mice were maintained on tamoxifen. Points, mean tumor volumes.

<sup>5</sup> J. Liang and J. Slingerland, manuscript in preparation.

In many cancer-derived lines, higher basal levels of ErbB2 or ErbB1 are associated with a greater dependence on these receptors for cell survival and proliferation. The antitumor effects of ErbB1 or ErbB2 inhibitors have been mostly assayed in lines with constitutive ErbB1 or ErbB2 activation (8, 19, 29, 37, 56). Preclinical and clinical studies of ErbB1 inhibitors show the greatest efficacy in cells with increased expression or activation of the ErbB family member targeted by the drug (24, 57). Indeed, cells with the highest ErbB2 or ErbB1 activation are most sensitive to lapatinib (26, 27). These data raise the concern that breast and other cancers that do not have activated ErbB1 or ErbB2 will not be optimally responsive to these drugs.

We and others have shown that MCF-7<sup>PR</sup>, T-47D, and ZR75 lines do not have *ErbB2* amplification and have low ErbB1 and ErbB2 protein levels (58, 59). In these lines, lapatinib alone caused only partial cell cycle blockade. It is noteworthy in this regard that in tamoxifen-sensitive MCF-7 xenografts, lapatinib alone has minimal antitumor efficacy.<sup>6</sup> Previous studies have shown that ErbB2 or ErbB1 inhibitors can facilitate the antiproliferative effects of tamoxifen (29, 56). Here we show that lapatinib together with tamoxifen has significant antitumor activity *in vivo* in tamoxifen-resistant MCF-TAMR xenografts that have elevated ErbB2 expression and a modest increase in ErbB1. Moreover, in three independent ER-positive cell lines with *low* ErbB1 and ErbB2 levels, whereas lapatinib had only modest antiproliferative activity when used alone, 4-OH-TAM and lapatinib effectively blocked cell cycle progression. These data show the potential efficacy of tamoxifen together with lapatinib in treatment of ER-positive

breast cancers that do not have high intrinsic activities of ErbB2 or ErbB1. The cell cycle inhibitory function of p27 and inhibition of ER-dependent transcription were both enhanced by the combination of lapatinib and 4-OH-TAM in MCF-7<sup>PR</sup>, T-47D, and ZR-75.

Our mouse xenograft data and data from breast cell lines indicate that the combination of lapatinib and tamoxifen has the potential to abrogate tamoxifen resistance or delay its development in ER-positive breast cancer in the metastatic and adjuvant settings. Moreover, because the combination of these two drugs was more effective than either alone in the context of low basal ErbB1/ErbB2 in the cell culture studies presented here, lapatinib in combination with tamoxifen may be beneficial in patients with ER-positive breast cancer irrespective of the ErbB1/ErbB2 status of the tumor. Clinical trials to investigate and optimize combinations of tamoxifen and receptor tyrosine kinase inhibitors, such as lapatinib, are clearly warranted.

## Acknowledgments

Received 4/27/2004; revised 10/22/2004; accepted 10/27/2004.

**Grant support:** DOD Breast Cancer Program Pre-doctoral fellowship award (I. Chu), Burroughs Wellcome Clinician Scientist Award in Translational Research (J.M. Slingerland), and Braman Breast Cancer Institute of the UM Sylvester Comprehensive Cancer Center (J.M. Slingerland).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Neil Spector for helpful conversations and critical review of the manuscript. We acknowledge assistance with experiments from G. Goodwin.

<sup>6</sup> N. Spector, unpublished results.

## References

- Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol* 2001;2:127–37.
- Jorissen RN, Walker F, Pouliot N, Garrett TP, Ward CW, Burgess AW. Epidermal growth factor receptor: mechanisms of activation and signalling. *Exp Cell Res* 2003;284:31–53.
- 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;235:177–82.
- Slamon DJ, Godolphin W, Jones LA, et al. Studies of the *Her-2/neu* proto-oncogene in human breast and ovarian cancer. *Science* 1989;244:707–12.
- Borg A, Tandon AK, Sigurdsson H, et al. *Her-2/neu* amplification predicts poor survival in node-positive breast cancer. *Cancer Res* 1990;50:4332–7.
- Tsutsui S, Ohno S, Murakami S, Hachitanda Y, Oda S. Prognostic value of epidermal growth factor receptor (EGFR) and its relationship to the estrogen receptor status in 1029 patients with breast cancer. *Breast Cancer Res Treat* 2002;71:67–75.
- Nicholson S, Wright C, Sainsbury JR, et al. Epidermal growth factor receptor (EGFR) as a marker for poor prognosis in node-negative breast cancer patients: *neu* and tamoxifen failure. *J Steroid Biochem Mol Biol* 1990;37:811–4.
- Lenferink AEG, Simpson Jf, Shawver LK, Coffey RJ, Forbes JT, Arteaga CL. Blockade of the epidermal growth factor receptor tyrosine kinase suppresses tumorigenesis in MMTV/Neu plus MMTV/TGF- $\alpha$  bigenic mice. *Proc Natl Acad Sci U S A* 2000;97:9609–14.
- Coleman KM, Smith CL. Intracellular signaling pathways: nongenomic actions of estrogens and ligand-independent activation of estrogen receptors. *Front Biosci* 2001;6:D1379–91.
- Musgrove EA, Sutherland RL. Cell cycle control by steroid hormones. *Cancer Biol* 1994;5:381–9.
- Cariou S, Donovan JC, Flanagan WM, Milic A, Bhattacharya N, Slingerland JM. Down-regulation of p21WAF1/CIP1 or p27Kip1 abrogates antiestrogen-mediated cell cycle arrest in human breast cancer cells. *Proc Natl Acad Sci U S A* 2000;97:9042–6.
- Kato S, Endoh H, Masuhiro Y, et al. Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* 1995;270:1491–4.
- Le Goff PL, Montano MM, Schodin DJ, Katzenellenbogen BS. Phosphorylation of the human estrogen receptor. *J Biol Chem* 1994;269:4458–66.
- Klinge CM. Estrogen receptor interaction with co-activators and co-repressors. *Steroids* 2000;65:227–51.
- Jordan VC. Studies on the estrogen receptor in breast cancer: 20 years as a target for the treatment and prevention of cancer. *Breast Cancer Res Treat* 1995;36:267–85.
- De Placido S, Carlomagno C, De Laurentiis M, Bianco AR. c-erbB2 expression predicts tamoxifen efficacy in breast cancer patients. *Breast Cancer Res Treat* 1998;52:55–64.
- Benz CC, Scott GK, Sarup JC, et al. Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with *HER2/neu*. *Breast Cancer Res Treat* 1992;24:85–95.
- Pietras RJ, Arboleda J, Reese DM, et al. *HER-2* tyrosine kinase pathway targets estrogen receptor and promotes hormone-independent growth in human breast cancer cells. *Oncogene* 1995;10:2435–46.
- Knowlden JM, Hutcheson IR, Jones HE, et al. Elevated levels of epidermal growth factor receptor/c-erbB2 heterodimers mediate an autocrine growth regulatory pathway in tamoxifen-resistant MCF-7 cells. *Endocrinology* 2003;144:1032–44.
- Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G<sub>1</sub>-phase progression. *Genes Dev* 1999;13:1501–12.
- Slingerland J, Pagano M. Regulation of the cdk inhibitor p27 and its deregulation in cancer. *J Cell Physiol* 2000;183:10–7.
- Cariou S, Catzavelos C, Slingerland JM. Prognostic implications of expression of the cell cycle inhibitor protein p27<sup>Kip1</sup>. *Breast Cancer Res Treat* 1998;52:29–41.
- Donovan JC, Milic A, Slingerland JM. Constitutive MEK/MAPK activation leads to p27Kip1 deregulation and antiestrogen resistance in human breast cancer cells. *J Biol Chem* 2001;276:40888–95.
- Arteaga CL. ErbB-targeted therapeutic approaches in human cancer. *Exp Cell Res* 2003;284:122–30.
- Lees E, Faha B, Dulic V, Reed SI, Harlow E. Cyclin E/cdk2 and cyclin A/cdk2 kinases associate with p107 and E2F in a temporally distinct manner. *Genes Dev* 1992;6:1874–85.
- Xia WL, Mullin RJ, Keith BR, et al. Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. *Oncogene* 2002;21:6255–63.
- Rusnak DW, Lackey K, Affleck K, et al. The effects of the novel, reversible epidermal growth factor receptor/Erbb-2 tyrosine kinase inhibitor, GW2016, on the growth of human normal and tumor-derived cell lines *in vitro* and *in vivo*. *Mol Cancer Ther* 2001;1:85–94.

28. Castoria G, Migliaccio A, Bilancio A, et al. PI3-kinase in concert with Src promotes the S-phase entry of oestradiol-stimulated MCF-7 cells. *EMBO J* 2001;20:6050-9.
29. Kurokawa H, Arteaga CL. ErbB (HER) receptors can abrogate antiestrogen action in human breast cancer by multiple signaling mechanisms. *Clin Cancer Res* 2003;9:511-55.
30. Rabenoelina F, Semlali A, Duchesne M-J, Freiss G, Pons M, Badia E. Effect of prolonged hydroxytamoxifen treatment of MCF-7 cells on mitogen activated kinase cascade. *Int J Cancer* 2002;98:698-706.
31. Liang J, Zubovitz J, Petrocelli T, et al. PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G<sub>1</sub> arrest. *Nat Med* 2002;8:1153-60.
32. Connor CE, Norris JD, Broadwater G, et al. Circumventing tamoxifen resistance in breast cancers using antiestrogens that induce unique conformational changes in the estrogen receptor. *Cancer Res* 2001;61:2917-22.
33. Blackwell KL, Kaplan EH, Franco SX, et al. A phase II, open-label, multicenter study of GW572016 in patients with trastuzumab-refractory metastatic breast cancer [abstract 3006]. *Proc Am Soc Clin Oncol* 2004;23:196.
34. Carroll JS, Prall OW, Musgrove EA, Sutherland RL. A pure estrogen antagonist inhibits cyclin E-Cdk2 activity in MCF-7 breast cancer cells and induces accumulation of p130-E2F4 complexes characteristic of quiescence. *J Biol Chem* 2000;275:38221-9.
35. Catzavelos C, Bhattacharya N, Ung YC, et al. Decreased levels of the cell-cycle inhibitor p27Kip1 protein: prognostic implications in primary breast cancer. *Nat Med* 1997;3:227-30.
36. Porter PL, Malone KE, Heagerty PJ, et al. Expression of cell cycle regulators p27kip1 and cyclin E, alone and in combination, correlate with survival in young breast cancer patients. *Nat Med* 1997;3:222-5.
37. Yakes FM, Chinratanalab W, Ritter CA, King W, Seelig S, Arteaga CL. Herceptin-induced inhibition of phosphatidylinositol-3 kinase and Akt is required for antibody-mediated effects on p27, cyclin D1, and antitumor action. *Cancer Res* 2002;62:4132-41.
38. Lane HA, Beuvink I, Motoyama AB, Daly JM, Neve RM, Hynes NE. ErbB2 potentiates breast tumor proliferation through modulation of p27(Kip1)-Cdk2 complex formation receptor overexpression does not determine growth dependency. *Mol Cell Biol* 2000;20:3210-23.
39. Viglietto G, Motti ML, Bruni P, et al. Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27(Kip1) by PKB/Akt-mediated phosphorylation in breast cancer. *Nat Med* 2002;8:1136-44.
40. Sabbah M, Courilleau D, Mester J, Redeuilh G. Estrogen induction of the cyclin D1 promoter: involvement of a cAMP response-like element. *Proc Natl Acad Sci U S A* 1999;96:11217-22.
41. Kenny FS, Hui R, Musgrove EA, et al. Overexpression of cyclin D1 messenger RNA predicts for poor prognosis in estrogen receptor-positive breast cancer. *Clin Cancer Res* 1999;5:2069-76.
42. Alle KM, Henshall SM, Field AS, Sutherland RL. Cyclin D1 protein is overexpressed in hyperplasia and intraductal carcinoma of the breast. *Clin Cancer Res* 1998;4:847-54.
43. Yu Q, Geng Y, Sicinski P. Specific protection against breast cancers by cyclin D1 ablation. *Nature* 2001;411:1017-21.
44. Wilkine NR, Prall OW, Musgrove EA, Sutherland RL. Inducible overexpression of cyclin D1 in breast cancer cells reverses the growth-inhibitory effects of antiestrogens. *Clin Cancer Res* 1997;3:849-54.
45. Diehl JA, Zindy F, Sherr CJ. Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. *Genes Dev* 1997;11:957-72.
46. Diehl JA, Cheng M, Roussel MF, Sherr CJ. Glycogen synthase kinase-3 $\beta$  regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev* 1998;12:3499-511.
47. Dees EC, Burris H, Hurwitz H, et al. Clinical summary of 67 heavily pre-treated patients with metastatic breast carcinomas treated with GW572016 in phase Ib study [abstract 3188]. *Proc Am Soc Clin Oncol* 2004;24:1.
48. Migliaccio A, Piccolo D, Castoria G, et al. Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. *EMBO J* 1998;17:2008-18.
49. Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, Liao JK. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature* 2000;407:538-41.
50. Song RX, Barnes CJ, Zhang ZG, Bao YD, Kumar R, Santen RJ. The role of Shc and insulin-like growth factor 1 receptor in mediating the translocation of estrogen receptor  $\alpha$  to the plasma membrane. *Proc Natl Acad Sci U S A* 2004;101:2076-81.
51. 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;15:2174-83.
52. Campbell RA, Bhat-Nakshatri P, Patel NM, Constantinidou D, Ali S, Nakshatri H. Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor  $\alpha$ : a new model for anti-estrogen resistance. *J Biol Chem* 2001;276:9817-24.
53. Denton RR, Koszewski NJ, Notides AC. Estrogen receptor phosphorylation. Hormonal dependence and consequence on specific DNA binding. *J Biol Chem* 1992;267:7263-8.
54. Ignar-Trowbridge DM, Teng CT, Ross KA, Parker MG, Korach KS, McLachlan JA. Peptide growth factors elicit estrogen receptor-dependent transcriptional activation of an estrogen-responsive element. *Mol Endocrinol* 1993;7:992-8.
55. Shou J, Massarweh S, Osborne CK, et al. Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/*neu* cross-talk in ER/HER2-positive breast cancer. *J Natl Cancer Inst* 2004;96:926-35.
56. Kurokawa H, Lenferink AE, Simpson Jf, et al. Inhibition of HER2/*neu* (erbB-2) and mitogen-activated protein kinases enhances tamoxifen action against HER2-overexpressing, tamoxifen-resistant breast cancer cells. *Cancer Res* 2000;60:5887-94.
57. Bundred NJ, Chan K, Anderson NG. Studies of epidermal growth factor receptor inhibition in breast cancer. *Endocr Relat Cancer* 2001;8:183-9.
58. Belsches-Jablonski AP, Biscardi JS, Peavy DR, Tice DA, Romney DA, Parsons SJ. Src family kinases and HER2 interactions in human breast cancer cell growth and survival. *Oncogene* 2001;20:1465-75.
59. deFazio A, Chiew YE, Sini RL, Janes PW, Sutherland RL. Expression of c-erbB receptors, heregulin and oestrogen receptor in human breast cell lines. *Int J Cancer* 2000;87(4):487-98.