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14. ABSTRACT
Estrogen regulates the proliferation and development of tissues expressing estrogen receptors and is implicated as a risk factor for the development of breast cancer. One third of new breast cancers do not express estrogen receptor α (ER) protein and these have a worse prognosis than ER positive breast cancers. Here we investigated how mechanisms underlying the reduced ER protein in ER negative cancers may be linked to their aggressive behavior. Estrogen binding to the ER rapidly stimulates ubiquitin-dependent ER proteolysis which in turn regulates ER activity. Our data suggest that Src activates ER proteolysis. Src can phosphorylated ER in vitro. Src transfection accelerated ER proteolysis in MCF-7 cells. The Src inhibitor, PP1, impaired estrogen stimulated ER ubiquitylation and proteolysis in vivo and in vitro. The weakly ER positive, MDA-MB361 and ER negative, BT-20 breast cancer lines both have highly activated Src and decreased ER half-life. Thus, these data provide a direct link between Src activation and ER proteasomal degradation and supports a model whereby Src many phosphorylate ER, resulting in increased ubiquitination and proteolysis.

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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 α (ER) protein and these have a worse prognosis than ER positive breast cancers[1]. The lack of ER protein cannot be explained by genetic changes or lack of gene expression[2]. Using Real-Time PCR, our lab showed that 200 ER negative and 50 ER positive breast cancers all express ER mRNA. The discordance between ER mRNA and protein status, the large range and overlap of ER mRNA concentrations in both ER positive and ER negative cancers indicate important post-transcriptional ER regulation. The ER is a ligand activated transcription factor. Estrogen binding to the ER stimulates rapid activation of Src and signaling pathways[4] that affect cell proliferation and feedback to phosphorylate the ER and increases its transcriptional activity. The ER can also be phosphorylated and activated in a ligand independent manner by mitogenic signaling [5,6].

Estrogen binding to the ER rapidly activates ubiquitin-dependent ER proteolysis which in turn regulates and limits ER activity[7]. 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 ER 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.

Our preliminary data suggest that Src activation contributes to ER proteolysis. Src transfection accelerated ER proteolysis, reduced ER t1/2 and levels. Her2 and Src overexpression together further reduced ER levels. Src inhibition impaired estrogen stimulated ER ubiquitylation and proteolysis. The weakly ER positive, MDA-MB361 and ER negative, BT-20 breast cancer lines both have highly activated Src. ER protein was detected but unstable and was increased by estrogen deprivation, proteasome inhibition and by Src inhibitors in both lines. Thus, ER levels in *both* ER positive and negative lines are affected by estrogen. Moreover, Src activity was increased in primary ER negative breast cancers compared to ER positive.

This progress report summarizes our findings indicating that estrogen or growth factor signaling leads to Src activation and increased ER ubiquitination. We present new evidence suggesting a direct role of cSrc in mediating ER ubiquitination and proteasomal degradation. Furthermore, we observed ER phosphotyrosilation when mixed with cSrc.

BODY

During my second year, I used different approaches to address my proposed tasks. In the first year we 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, we concentrated our efforts in finding the difference between Src phosphotyrosilated 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 will be described below.

Src phosphorylates ER in vitro.

Ubiquitin mediated proteolysis is often triggered by a substrate phosphorylation event that promotes substrate interaction with its ubiquitin ligase. Our hypothesis is 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. ER phosphorylation at Y537 is required for binding of Src to the ER[8] and mutation of this site modulates ER transcriptional activity. ER has only one tyrosine site, at Y537, and we confirmed that Src can phosphorylate ER *in vitro*[9] (see Fig1)

Fig 1. 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.

Src enhances ER ubiquitylation and degradation in vitro.

In this Task, we proposed experiments that may confirm that Src inhibition impairs ligand stimulated ER ubiquitylation and provide data to indicate if Src is implicated either directly through ER phosphorylation or potentially indirectly in ER proteolysis. To further investigate how Src may regulate the ubiquitylation and degradation of the 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 difference sources of putative E3 ubiquitin ligases.

In a first instance we established *in vitro* ubiquitylation assays for recombinant ER as described [10]. We have ER produced in bacculovirus in the lab. We also have expressed and purified recombinant HA-tagged ubiquitin, ubiquitin activating enzyme (UBA) and different Ubcs in E coli. In preliminary experiments, cell lysate will be used as a source of E3 ligase. UbcH5, UbcH7, Ubc8, Ubc3 and Ubc4 have been kindly provided by my colleague, Dr. Zafar Nawaz. Recombinant ER were mixed with ubiquitin, UBA, different Ubcs, and different sources of E3 ligase and incubated as described[11]. At intervals, the ER will be precipitated and resolved and immunoblotted. The decay of ER levels over time and the extent of ER-ubiquitylation were detected by immunoblotting with anti-ER and anti-ubiquitin or anti-HA antibodies respectively. We observed increase ER ubiquitilation 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. (see Fig. 2A). Furthermore we observed that ER was phosphotyrosilated when probed with a pY antibody (Fig. 2b). Overall these findings suggest that Src dependent ER phosphorylation increases ubiquitination *in vitro.*

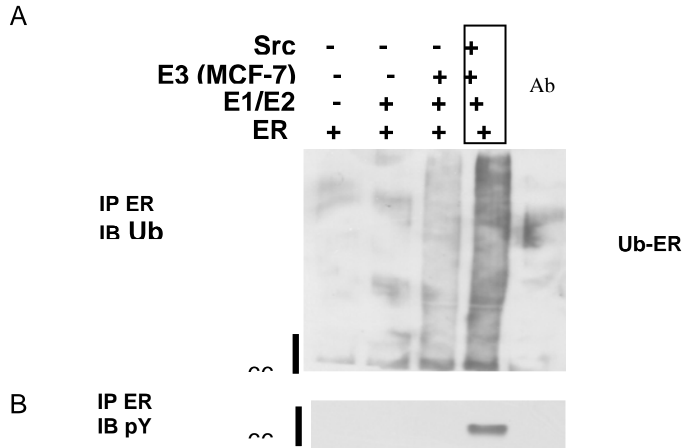

Fig2 . Src increases ER ubiquitination in vitro. Recombinant ER was incubated with E1/E2 and MCF-7 lysate as source of E3 with or without Src and the reactions were allowed to proceed for 30 min. a) Ubiquitinated ER was detected using a Ubiquitin specific antibody and b) pY ER was detected after ER Immunoprecipitation 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. The Nawaz lab has shown that addition of E6AP can promote partial proteolytic ER degradation [3]. 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 (see Fig 3).

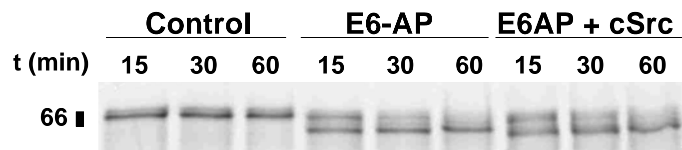

Fig 3. Src does not activate E6AP-mediated ER proteolysis in vitro. IVT-ER was subjected to *in vitro* ubiquitin mediated degradation following the method of Nawaz et al[3]. Addition of E6AP activated ER proteolytic cleavage (faster mobility forms, middle panel) but this was unchanged by incubation of 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. Thus, we will explore if Src can influence degradation through other coactivators or E3 ligases. The ubiquitin ligases MDM2[12] and BRCA-1[13] have been shown to act as ER co-activators. In addition,

estrogen has been shown to stimulate binding of MDM2 to the ERE of the pS2 promoter in CHIP assays [14]. Moreover, a sequence search of over 70 putative F-box components of SCF-type multiprotein ubiquitin ligases [15] revealed several F-box proteins that contain one or more LXXLL motifs. The co-activator LXXLL motif interacts with the hydrophobic groove of the agonist bound ER within its ligand binding domain. Thus, SCF-type ubiquitin F-box protein-ligases that bear an LXXLL could be implicated both as ER co-activators and in ligand stimulated ER proteolysis. In the following, we will test effects of Src on *in vitro* ER ubiquitylation and proteolysis using different sources of ubiquitin ligase.

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 the 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 (Fig 4). 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 the ER.

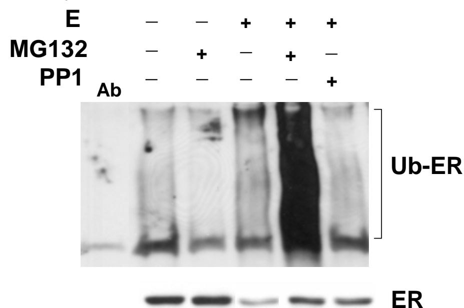

Fig. 4 Src inhibition prevents estrogen stimulated ER-ubiquitylation. MCF-7 cells were estrogen deprived for 48 h followed by addition of estradiol with or without the proteasome inhibitor MG132 or the Src inhibitor, PP1. Cells were lysed, ER immunoprecipitated and complexes resolved by SDS-PAGE and 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.

Src inhibition partially abrogates E and serum dependent ER degradation

Further data supporting a role for Src in estrogen stimulated ER proteolysis in ER negative breast cancer was obtained as follows. The ER negative line BT549 did not show detectable ER protein expression even with scaled up immunoblots. Src activity was increased in this line to a greater extent than in BT-20. To test the effect of Src on ER proteolysis in BT549, we transfected this line with an expression vector for ERα and selected for stable clones. The half-life of ER was very rapid in these cells (not shown).

In our first year we observed that PP1 did not result in increased ER levels in MDA-MB-361 or BT20, since PP1 was toxic in these cell lines possibly due to non-specific inhibition of other kinases. We used the Src specific inhibitor, PD166326. ER levels increased following treatment with either proteasome inhibitors or Src inhibitors (Fig 5).

F

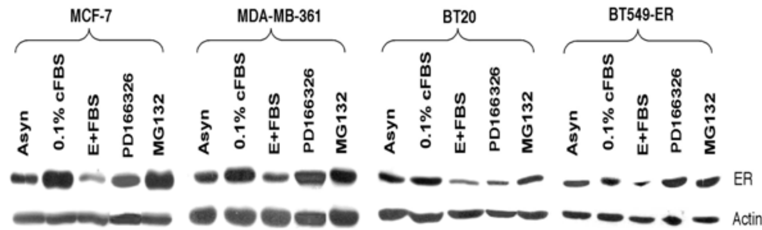

Fig 5 Estrogen activates ER proteolysis (detected by reduced ER 6 h after estrogen addition) in MCF-7, MDA-MB-361, BT-20 and ER-BT549. Re-addition of estrogen to growth factor and estrogen-deprived cells reduced ER levels and this was impaired by pre-treatment with proteasome inhibitor (MG132) or Src inhibitor PD166326.

KEY RESEARCH ACCOMPLISHMENTS

Demonstrated that purified recombinant Src can phosphorylate purified recombinant ER in vitro. We also observed phosphotyrosilated ER when ER was immunoprecipitated and probed for a phosphotyrosine specific antibody

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.

We observed that inhibition of Src using PP1 resulted in impaired ER ubiquitination *in vivo* suggesting that active Src is required for efficient ER ubiquitination in MCF-7 cells.

Src inhibition using the inhibitor PD166 326 resulted in partial abrogation of E and serum dependent ER degradation.

REPORTABLE OUTCOMES

Manuscripts

1. Article

   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.

2. Poster presentations

   Sophie Loiseau, Isabel Chu 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

CONCLUSION

One third of new breast cancers do not express estrogen receptor α (ER) protein and these have a worse prognosis than ER positive breast cancers. This grant investigates how mechanisms underlying the reduced ER protein in ER negative cancers may be linked to their aggressive behavior. Estrogen binding to the ER rapidly activates ubiquitin-dependent ER proteolysis which in turn regulates ER activity. Our data suggest that Src activates ER proteolysis. Src transfection accelerated ER proteolysis. Src inhibition impaired estrogen stimulated ER ubiquitylation and proteolysis. 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 both lines. Src activity was increased in primary ER negative breast cancers compared to ER positive. Our findings will elucidate 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.

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