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Functions of Membrane-Localized Estrogen Receptor (Alpha)

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13. ABSTRACT (Maximum 200 Words) We previously developed a cell line system in which exogenous expression of estrogen receptor alpha (ER α) in an ER α -negative cell line resulted in ER α -mediated signaling and proliferation. We previously reported generation of a cell lines that expressed ER α only in the cytoplasm (c ER α) to characterize the putative cytoplasmic (non-genomic) function of ER α . However, while we found that cER α was not able to stimulate genomic ER action, and found interesting differences in estrogen-mediated downregulation of cER α , we were unable to show that this receptor could activate short-term non-genomic signaling. Since last year we have now started studying a membrane-targeted ER α (rhodopsin fused to ER α). We have generated stable cells expressing this receptor and show that this form of Era is exclusively localized to the plasma membrane, and also estrogen is able to rapidly activate ERK1/2 in these cells. We have now also generated MCF-7 or MCF-7/HER2 cells overexpressing either cER α or rho- ER α and are currently examining the effect of this receptor on hormone response in these cells. We will finish the study using a one-year no cost extension.				
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INTRODUCTION

Since the discovery of membrane estrogen receptor α (ER α) more than 20 years ago, reports on this form of ER α signaling have continued to be documented and have recently received increasing attention. However, this field remains very controversial with nuclear ER α action being studied in much greater detail and becoming much better understood. The IDEA of this proposal is to create a novel and unique model of breast cancer cells that express only cytoplasmic or membrane estrogen receptor (and not nuclear ER α) and then compare and contrast ER α action to cells that express no ER α or wild-type ER α . We are in a unique situation to perform this, as we have recently shown for the first time that ER can be functionally expressed and regulate proliferation in an ER α -ve breast cancer cell line (C4-12).

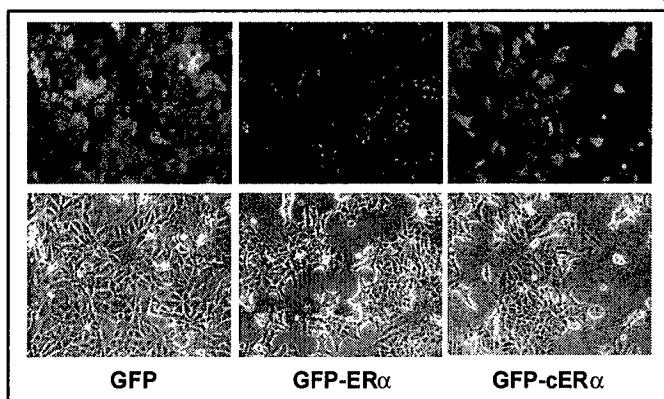
BODY

Summary

This progress report is for year 3 of the project. This was supposed to be a final report for the project, however, due to the generation of many exciting models at the end of the project period, we have requested and had approved a one-year no cost extension. In the first year of the project we made significant progress, having generated stable transfectants that express ER α in the cytoplasm (cER α) and having performed a preliminary characterization of these cells. However, as described in the body of the report in the second year, we were unable to identify any increase in rapid estrogen signaling in these cells that expressed the cER α (although we did generate interesting data regarding the ability of estrogen to cause downregulation of cER α , but the inability of ICI182780 to do this). This suggests that either these cells are not suitable for studying rapid estrogen effects or that perhaps the ER α needed to be in or near the plasma membrane to signal in this manner. In the second year we also struggled to create an ER variant that would reside in the plasma membrane. We expect that the small localization tag (myr or CAAX), placed on the N or C-terminus of ER α is folded within the protein and inaccessible for attachment to the plasma membrane. However, in this third year we were able to target the ER to the plasma membrane by using a fusion protein consisting of rhodopsin and ER α . We have stably expressed this construct in C4-12 cells and find that it is expressed exclusively in the plasma membrane. Furthermore, we have proven our hypothesis by showing that this receptor can now respond to short – term (minutes) estrogen by increased ERK1/2 phosphorylation. We have also generated stable transfectants of MCF-7 or MCF-7 cells overexpressing HER2 (MCF-7/HER2), and will use these to study the effect of plasma membrane ER on hormone action and anti-estrogen resistance.

Task 1: To create and characterize ER α -negative MCF-7 cells (C4-12) that stably express GFP tagged membrane ER α (mER α), cytoplasmic ER α (cER α), or wild type (wtER α) (Months 1-12):

i) *Stably transfect ER α -negative MCF-7 (C4-12) cells with GFP, GFP-wtER α , GFP-mER α and GFP-cER α , and select cell lines that have low and high levels of the receptor (Months 1-6).*



We have stably transfected C4-12 cells with GFP, GFP-wtER α , and GFP-cER α . We have isolated multiple clones and using immunofluorescence microscopy shown that the cER α is indeed expressed in the cytoplasm (compared to wt ER α which is mainly nuclear) (Figure 1). GFP alone is expressed all over the cell.

Figure 1: Stable expression of GFP-cER α in C4-12 cells. cER α was generated by deletion of the nls

(245-270aa) in ER α . Stable clones of GFP, GFP-wtER α , and GFP-cER α were obtained and visualized by fluorescence microscopy (top panels) or by phase contrast (lower panels). GFP was widely distributed over the cell. In contrast, wt-ER α was exclusively nuclear; however, cER α was again widely distributed over the cell and did not show nuclear localization.

We have confirmed that GFP-cER α does not show nuclear localization by biochemical fractionation (data not shown) and confocal microscopy (Figure 2).

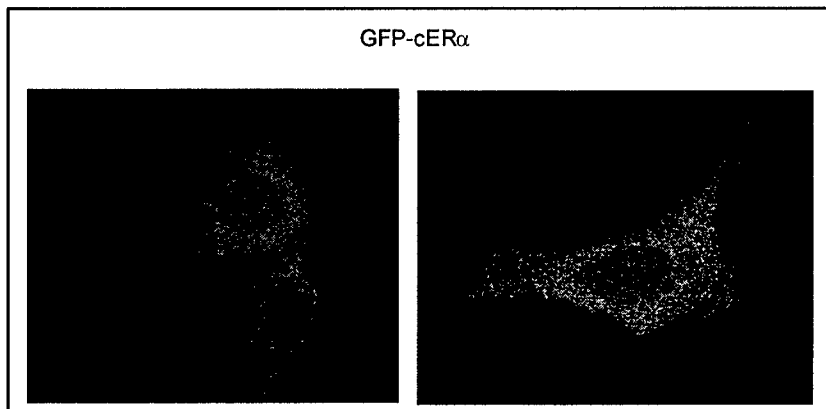


Figure 2: GFP-cER α is exclusively cytoplasmic. C4-12 cells stable expressing GFP-cER α were stained with propidium iodide (red) and then examined by confocal microscopy. The GFP signal shows that the cER α is exclusively cytoplasmic.

A setback came however, when we tried to express mER α , which was not targeted to the membrane (data not shown). Tagging of ER α with either C or N-terminal membrane signals does not send ER α to the membrane. This is probably due to folding of the protein making the tag inaccessible. We have therefore entered into collaboration with Dr Wang from Johns Hopkins University. He generated an ER α construct that consists of rhodopsin linked to ER α . Rhodopsin is membrane bound and so directs ER α to the plasma membrane (Xu Y *Mol Endocrinol* 2004 Jan, 18:86-96.) We found the rho-ER α to be in the endoplasmic reticulum following transient transfection with high concentrations of DNA (data not shown), however, lower amounts of DNA caused the rho-ER α to give only a plasma membrane signal (Figure 3).

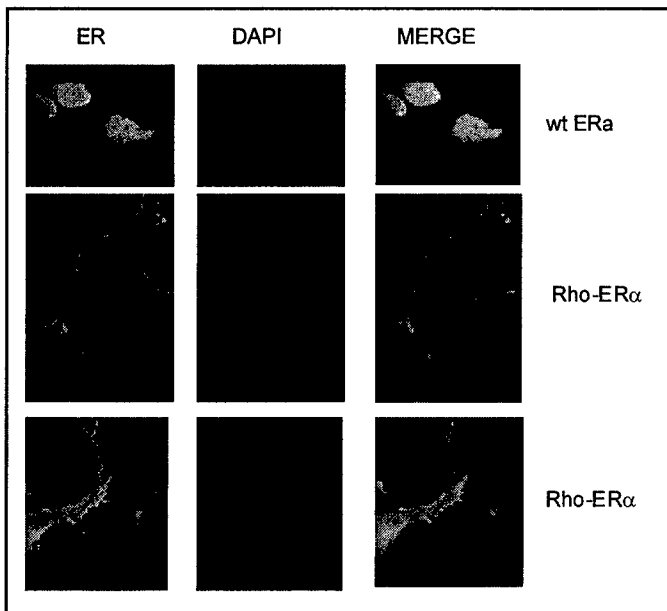


Figure 3: Localization of rho-ER α . 293 cells were transiently transfected with wt-ER α or rho-ER α and then ER α detected by immunostaining with ER α antibody (6F11, neomarkers) and detected by anti-mouse antibodies conjugated to alexa488 (left panels). The nucleus was identified by staining with DAPI (middle panels). A merged image is also shown (right panels). As expected, wtER α was entirely nuclear, whereas the rhoER α construct showed exclusive cytoplasmic or membrane staining. We have not confirmed that this is solely membrane staining by confocal microscopy; however, the staining is clearly very different to cER α , and looks very similar to GFP that has a membrane tag on it (data not shown)

In this year (year 3) we have generated several stable transfectants that express rho-ER α (Figure 4). Figure 4 shows immunofluorescence for ER α (red) or a nuclear stain (DAPI – blue) on MCF-7 cells or

C4-12 cells stably expressing rho- ER α . This figure clearly shows that MCF-7 cells express mainly nuclear ER with overlay of both red and blue signal. In contrast C4-12 cells show no nuclear ER (nucleus is only blue) but the ER is now exclusively localized to the plasma membrane. Figure 4B shows an immunoblot of C4-12 cells (left panel) and MCF-7 cells that were screened for expression of various forms of ER (this is an example, we screened over 400 colonies and generated more than 4 clones per construct – GFP-wtER, GFP-cER, and rho- ER α). The highlighted lanes with numbers represents 1) C4-12 rho ER α , 2) MCF-7 wGFP-wtER, 3) MCF-7 GFP-cER, 4) MCF-7HER2 GFP-cER, 5) MCF-7 rho-ER α , 6) MCF-7 rho- ER α). The tagging of GFP or rho makes it appear with a higher molecular weight. The rho-ER often appears as multiple bands probably due to its insertion in the plasma membrane and resulting posttranslational modifications of the rhodopsin protein. This immunoblot is only shown as an example of the tools we now have to use.

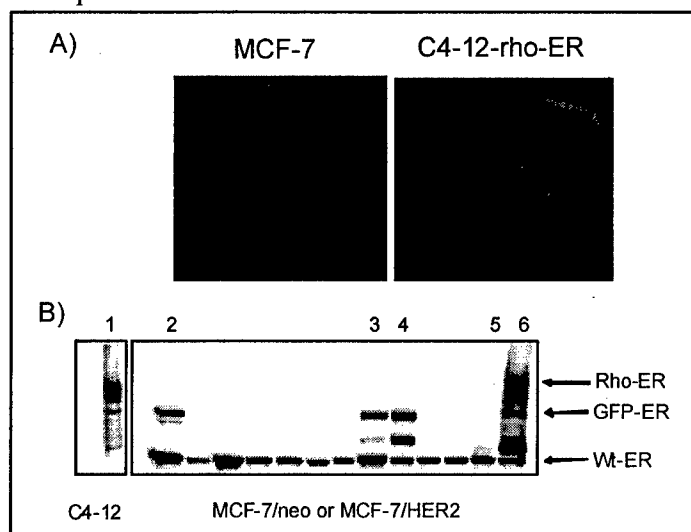


Figure 4: Stable expression of rho-ER in C4-12, MCF-7 and MCF-7/HER2 cells. A) Immunofluorescence using antibodies against ER (red) and a DAPI as a nuclear stain (blue). Note the nuclear ER in MCF-7 (left) but the lack of ER in the nucleus of C4-12-rhoER and the presence of a strong plasma membrane localization. B) Representative immunoblot (from 400 screened clones) for various forms of ER. Immunoblot is with an ER antibody. Lane 21 shows C4-12 cells positive for rho-ER (increased molecular weight due to fusion with rhodopsin). 2 is wt-GFP-ER. 3 and 4 are GFP-cER (with an unknown lower species). 5 and 6 are rho-ER. Note the multiple isoforms of rho-ER which we believe maybe due to the posttranslational (probably glycosylation) of rhodopsin.

ii) Use biochemical fractionation and confocal microscopy to determine whether mER α and cER α are expressed only in the membrane and cytoplasm respectively, and test whether mER α and cER α are capable of binding estradiol (E2) and tamoxifen (Tam) (Months 4-8).

Figure 1 confirms that cER α is only expressed in the cytoplasm, while wt-ER α is mainly nuclear. Figure 3 shows that rho-ER α is mainly membranous by transient transfection, but is exclusively associated with the plasma-membrane upon stable transfection (Figure 4).

We have not tested directly whether cER α can bind E2 or tam, however an indirect measure is the ability of E2 to downregulate the receptor (which occurs after E2 binding). We found that cER α is degraded following E2 stimulation, suggesting in an indirect way that this receptor can bind E2. In one preliminary experiment we have found that rho- ER α is not degraded by estrogen. This highlights the interesting ability of cER α to be degraded by estrogen. This also argues against the literature suggesting a link between ER transcriptional activity and degradation – something which has been refuted by multiple recent publications.

iii) Examine whether mER α or cER α associate with membrane or cytoplasmic structures (e.g. clathrin-coated pits) (Months 7-12).

We have not yet performed these assays. This will now be done on the rho ER α stable transfectants.

Task 2: To compare and contrast the effects of estrogen in C4-12-cER α , mER α and wtER α cells (Months 12-24):

i) Analyze the effect of short (mins) and long-term (hours) E2 stimulation on localization, movement, and degradation of the different GFP-ER α variants (Months 12-16).

We have not examined localization and movement, but we have found that the cER α can be degraded by E2 (Figure 5).

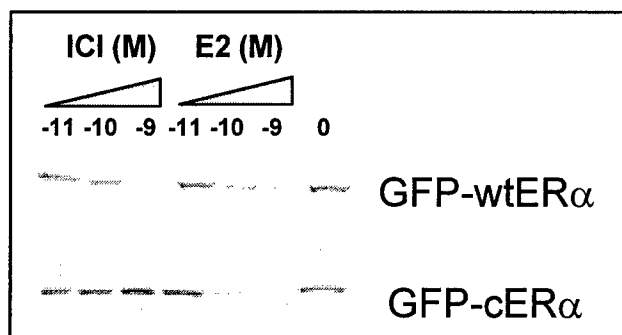


Figure 5: cER α is completely resistant to antiestrogen ICI 182780-mediated degradation. C4-12wt-ER α and cER α stable transfectants were starved in serum-free overnight and then treated for 8 hours with increasing concentrations of estradiol (E2) or ICI182780 (ICI). As expected, wtER α protein levels were reduced upon exposure to both E2 and ICI. This effect was blocked with the proteasome inhibitor (lactacystin 10 μ M) (data not shown). Of note, ICI required a 10-fold higher excess, which has previously been noted by others. In contrast to wtER α , cER α proteins levels decreased upon

exposure to E2, but were not affected by any concentration of ICI.

This is an important result given that a number of groups have proposed that E2-mediated degradation of ER α is linked to transcription. cER α is a variant ER that cant activate transcription, thus the degradation of cER by E2 represents a new paradigm for E2-mediated degradation of ER. Interestingly, while wt-ER α is degraded by antiestrogens such as ICI182780, the cER α is not degraded significantly by ICI182780 suggesting that this is a nuclear mediated event and that E2 and ICI degradation mechanisms are distinct. We have found that membrane localization of rho-ER α also inhibits degradation.

ii) Examine whether ER-responsive genes (e.g. TGF α , PgR, cathepsin D, pS2, IRS-1, cyclin D1) are induced by E2 and inhibited by Tam (Months 15-20) by the different GFP-ER α variants.

We have found that cER α is incapable of inducing expression of genes such as IRS-1, IGF-IR, and cyclin D1 (Figure 6). This is consistent with it not being able to activate gene transcription in an ERE-luc reporter assay (data not shown).

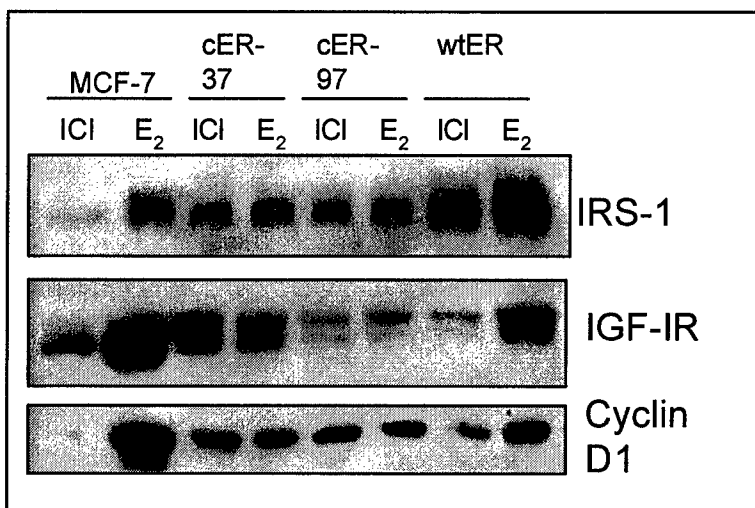


Figure 6 cER α doesn't confer estrogen-induction of ER-responsive genes. MCF-7, C4-12cER α and C4-12wtER α cells were starved in serum-free medium overnight and then treated with either antiestrogen (ICI, 10-9M) or estrogen (E2, 10-9M) for 24 hrs. Cells were lysed in 5% SDS and immunoblotted with antibodies to insulin receptor substrate-1 (IRS-1), insulin-like growth factor receptor 1 (IGF-IR) or cyclin D1. As expected, all 3 genes were induced by estrogen in MCF-7 cells and also in C4-12 cells expressing wtER α . In contrast, two stable clones of C4-12ER α did not show estrogen regulation of IRS-1, IGF-IR or cyclin D1.

In a preliminary result with the C4-12rho ER α we have found that this receptor is also unable to confer estrogen induction of IRS-1. This is not surprising given its attachment to the plasma membrane.

iii) Determine whether E2 stimulation results in an increase in S-phase and cell proliferation in C4-12-cER α and mER α compared to C4-12wtER α (Months 18-24).

We have found that E2 stimulation is able to increase S-phase in wt- ER α cells, but is unable to have an effect in cER α cells (Figure 7), consistent with this variant not inducing gene transcription (Figure 5). This is despite the fact that the cER α can clearly bind E2 and be degraded.

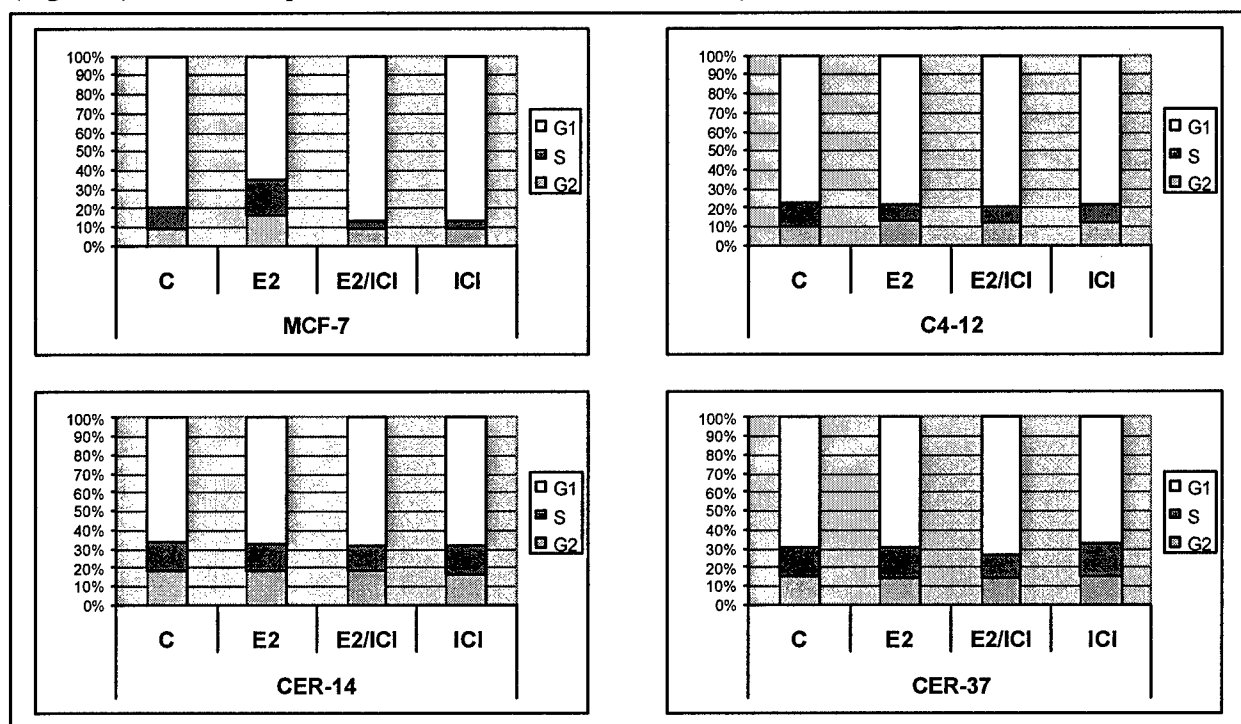
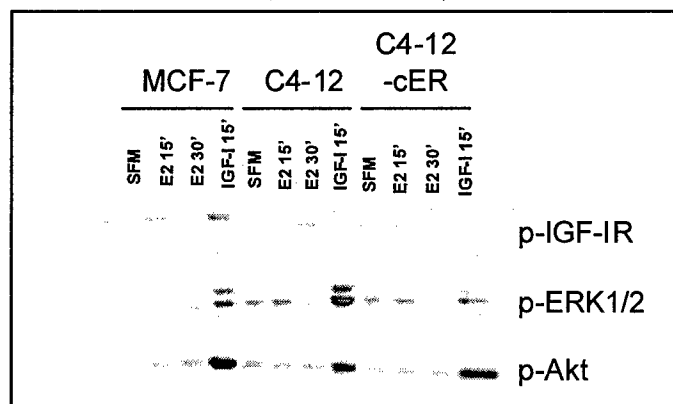


Figure 7: cER α does not confer estrogen stimulated S-phase entry. MCF-7, C4-12, and C4-12 cells expressing cER α were starved in serum-free medium overnight, and then stimulated with estradiol (1nM) or ICI (100nM) or the combination for 16hrs. Cells were then fixed in alcohol, stained with propidium iodide and FACS analysis performed. MCF-7 cells stimulated with estradiol showed an induction in S-phase fraction (red) and also an increase in cells entering G2/M (blue). These changes were completely blocked by ICI. In contrast, ER α negative C4-12 cells, or the cER α expressing cells showed no changes in response to E2 or ICI.

We will now be performing these experiments in the C4-12rho ER α to see whether the transient induction of ERK1/2 phosphorylation that we see with estrogen stimulation can confer a proliferative advantage.

Task 3: To determine whether previously reported short-term (minutes) E2-mediated effects are observed in C4-12-cER α or mER α cells (Months 24-36):

i) Perform coimmunoprecipitation and colocalization to determine if mER α and cER α can bind p85 and activate PI3K (Months 24-30).



Despite preliminary evidence that cER α was able to associate with p85, subsequent experiments failed to confirm an association with either p85 or IGF-IR. In addition we have been unable to show that cER can enhance short-term mediated activation of ERK1/2 or Akt by estradiol (Figure 8).

Figure 8: cER α does not allow short-term estrogen signaling in C4-12 cells. MCF-7, C4-12 and C4-

12cER α cells were starved in SFM overnight and then stimulate with estradiol (1nM, 15 or 30mins) or IGF-I (10nM, 15mins). Cells were lysed and immunoblotted for p-IGF-IR, pERK1/2 and pAkt. MCF-7 cells did not show a response to estradiol at 15 or 30mins. Similar results were also seen in both C4-12 cells. As a positive control, all 3 cell lines responded to IGF-I.

We do not know whether this result simply shows that cER α is not able to activate these pathways in these cells, or whether the specific system we chose is not suitable for detection of short-term estrogen effects. However, we were able for the first time to detect rapid estrogen signaling in the stable transfectants of C4-12 cells expressing rho-ER (Figure 9). In this experiments (repeated three times) we were able to show that 15mins of estrogen exposure caused an increase in p-ERK1/2 that was equivalent to the positive control (15 mins exposure to IGF-I). However the induction was always transient and decreased after 30 mins (similar to that seen with growth factors).

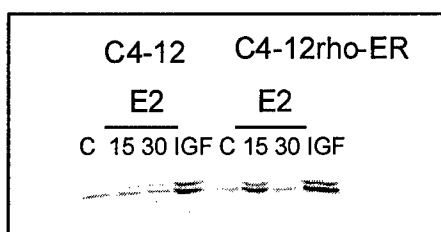


Figure 9: Rapid phosphorylation of ERK1/2 in response to short-term estrogen in C4-12-rho-ER. C4-12 and C4-12rho-ER were starved overnight in serum free medium and then stimulated with estradiol (1nM) for 15 or 30 minutes. As a positive control for ERK1/2 activation, cells were stimulated with IGF-I (10nM) for 15 mins). Cells were lysed and immunoblotted with an antibody to pERK1/2. Total ERK1/2 levels were unchanged (data not shown).

We would like to now examine the interaction with Her2 signaling, which has been shown by our collaborators Drs Schiff and Osborne to be important for rapid ER signaling in MCF-7 cells (Figure 10)

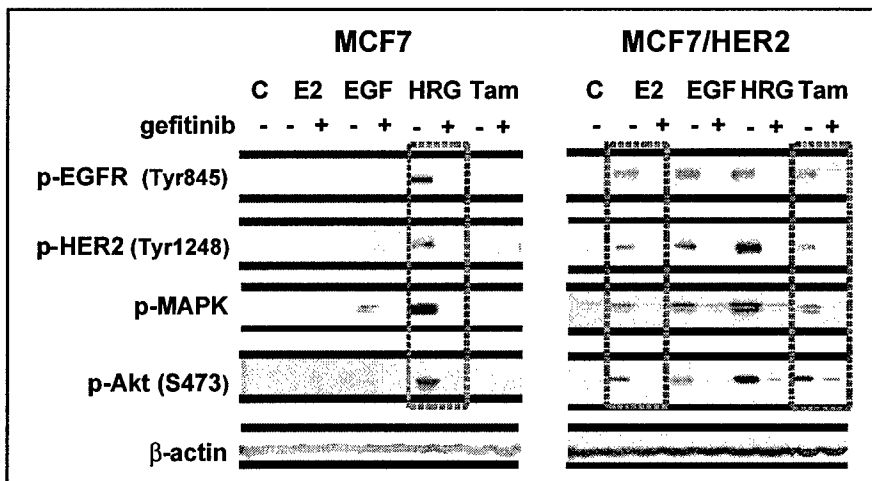


Figure 10: Rapid estrogen signaling in MCF-7 cells that overexpress HER-2. MCF-7 and MCF-7 cells stably overexpressing HER-2. Cells were starved overnight in serum-free medium and then stimulated for 15 minutes with estradiol (1nM), epidermal growth factor (EGF, 100nM), heregulin (HRG, 100nM) or tamoxifen (Tam, 100nM). These incubations were performed in the presence or absence of the EGFR kinase inhibitor Iressa (gefitinib). Cells were then lysed and immunoblotted for phospho-EGFR,

HER-2, Akt and ERK1/2 (MAPK). The left panel shows that estradiol or tam is unable to stimulate short-term signaling in MCF-7 cells, which can only be seen with heregulin (red box). In stark contrast, estradiol and tamoxifen were able to activate and phosphorylate all signaling intermediates in MCF-7/HER2 cells, and this was completely inhibited by EGFR blockade. We will therefore use these cells to better understand the role of cER α and rhoER α in this response. Figure kindly provided by Drs Osborne and Schiff, Breast Center, Baylor College of Medicine.

We have therefore stably expressed GFP-wtER, GFP-cER, and rho ER α in MCF-7 and MCF-7/HER2 cells and are examining rapid estrogen signaling and anti-estrogen resistance in collaboration with Drs Rachel Schiff and C. Kent Osborne. A first preliminary experiment showed little effect of the receptor on ERK1/2 signaling, however we couldn't repeat previous results from Dr Schiff in parental cells that weren't transfected (Fig 9). Therefore we have given the cells to her lab and she is repeating the experiment using their conditions.

ii) *Examine whether E2-stimulation of C4-12-mER α and cER α cells results in mobilization of intracellular Ca²⁺ and activation of PKC (Months 30-34).*

Not yet performed

iii) *Determine whether E2 can induce an anti-apoptotic response in C4-12-mER α and cER α cells (Months 32-36).*

Not yet performed

KEY RESEARCH ACCOMPLISHMENTS

- Generation of C4-12 cells that express ER α only in the cytoplasm (C4-12- cER α).
- Evidence that cER α is degraded by estrogen but not by anti-estrogen
- Generation of C4-12 cells expressing rho- ER α .
- Evidence that rho- ER α is able to respond to short-term estrogen stimulation by enhancing ERK1/2 phosphorylation
- Generation of MCF-7 or MCF-7/HER2 cells stably expressing GFP-wtER, GFP-cER, or rho-ER.

REPORTABLE OUTCOMES

Development of stable cell lines:

C4-12-GFP

C4-12-GFP-wtER

C4-12-GFP-cER α

C4-12-rhoER

MCF-7-GFP-wtER

MCF-7-GFP-cER

MCF-7-rhoER

MCF-7/HER2-GFP-wtER

MCF-7/HER2-GFP-cER

MCF-7/HER2-rhoER

CONCLUSIONS

This project will use a unique cell line model (C4-12) to test if cytoplasmic (cER α) or membrane targeted ER α (mER α) can perform signaling and mediated proliferation. This research is critical, as several recent studies have suggested that cER α or mER α is important, and pathologists only analyze nuclear ER α , which might misclassify a number of breast cancer patients. We have generated cells that express ER α only in the cytoplasm. We find that this receptor can't activate gene transcription or proliferation, despite the fact that the receptor is degraded by E2 and thus presumably can bind E2. However, this receptor is unable to stimulate short term estrogen events. We have also expressed ER in the plasma membrane and find that this receptor can respond rapidly to estrogen by enhancing ERK1/2 phosphorylation. We are currently examining the effect of this receptor in MCF-7 or MCF-7/HER2 cells. Understanding any potential role of cER α or mER α is critical for the complete understanding of estrogen action and targeting in breast cancer.

PRESENTATION

2004 "Expression of non-nuclear ER in ER-negative breast cancer cells doesn't confer estrogen-stimulated growth". 14th Annual Breast Cancer Think Tank Meeting, St Kitts.