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14. ABSTRACT

We have generated ER-negative breast cancer cell lines (C4-12) that express ERα only in the cytoplasm (cERα - deletion of the nuclear localization sequence) to characterize the putative cytoplasmic functions of ERα. As expected, estrogen stimulation of C4-12-cERα didn't stimulate genomic ER activity. However, estrogen also failed to increase growth of these cells and didn't stimulate non-genomic ER signaling via ERK1/2 or Akt. Interestingly, however, we found that estradiol stimulated cERα was degraded by the proteasome (challenging the notion that transcription and degradation are linked), and also found that cERα was completely resistant to ICI182780-mediated degradation. We have generated C4-12 cells expressing ERα exclusively in the plasma membrane (chimeric protein of rhodopsin fused to full length ERα and including the NLS) and found that estrogen is able to rapidly activate ERK1/2 in these cells. Rho-ER is degraded by estrogen and ICI, directly implicating the NLS of ER in ICI-mediated degradation. These studies do not support a major role for cytoplasmic ER in non-genomic ER signaling, however they clearly illustrate significant differences between estrogen and ICI-mediated ERα degradation.

15. SUBJECT TERMS
estrogen receptor alpha, breast cancer, cyclin D1, IRS-1

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Table of Contents

INTRODUCTION

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

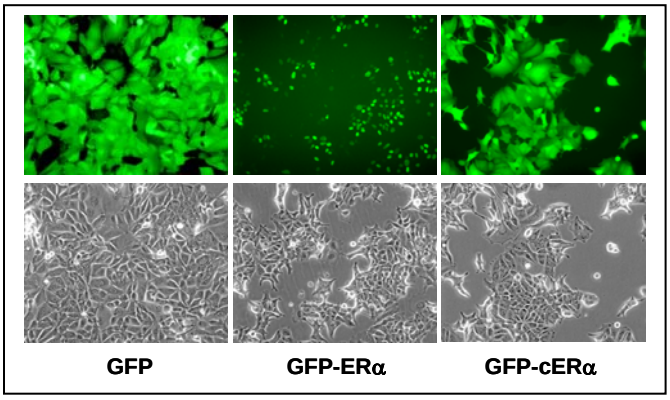
BODY

Summary of the project

This is a final progress report. In the first year of the project we made significant progress, having generated stable transfectants that express ERα in the cytoplasm (cERα) 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 cERα (although we did generate interesting data regarding the ability of estrogen to cause downregulation of cERα, but the inability of ICI182780 to do this). This suggests that either these cells were not suitable for studying rapid estrogen effects or that perhaps the ERα needed to be in or near the plasma membrane to signal in this manner. In the second year we struggled to create an ER variant that would reside in the plasma membrane. We suspected that the small localization tag (CAAX from k-ras), placed on the C-terminus of ERα was folded within the protein and inaccessible for attachment to the plasma membrane. Supporting this, we created a fusion protein with an N-terminal myristylation sequence from Akt and found that this protein did show some plasma membrane localization (~15% of the total protein), however, again the majority of the protein remained ctoplasmic. In this third year we were able to target the ER to the plasma membrane by using a chimeric fusion protein consisting of rhodopsin and ERα. We 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. The most important aspect of these studies is the finding that the cytoplasmic and plasma membrane bound ER are degraded by ER ligands in a unique manner, and we believe that these fusion proteins will give us a unique tool to study ER degradation further.

Task 1: To create and characterize ERα-negative MCF-7 cells (C4-12) that stably express GFP tagged membrane ERα (mERα), cytoplasmic ERα (cERα), or wild type (wtERα) (Months 1-12):

i) Stably transfect ERα-negative MCF-7 (C4-12) cells with GFP, GFP-wtERα, GFP-mERα and GFP-cERα, and select cell lines that have low and high levels of the receptor (Months 1-6).



We stably transfected C4-12 cells with GFP, GFP-wtERα, and GFP-cERα. We isolated multiple clones and using immunofluoresence microscopy shown that the cERα was indeed expressed in the cytoplasm (compared to wt ERα which is mainly nuclear) (Figure 1). GFP alone is expressed all over the cell.

Figure 1: Stable expression of GFP-cERα in C4-12 cells. cERα was generated by deletion of the nls (245-270aa) in ERα. Stable clones of GFP, GFP-wtERα, and GFP-cERα 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-ERα was exclusively nuclear; however, cERα was again widely distributed over the cell and did not show nuclear localization.

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

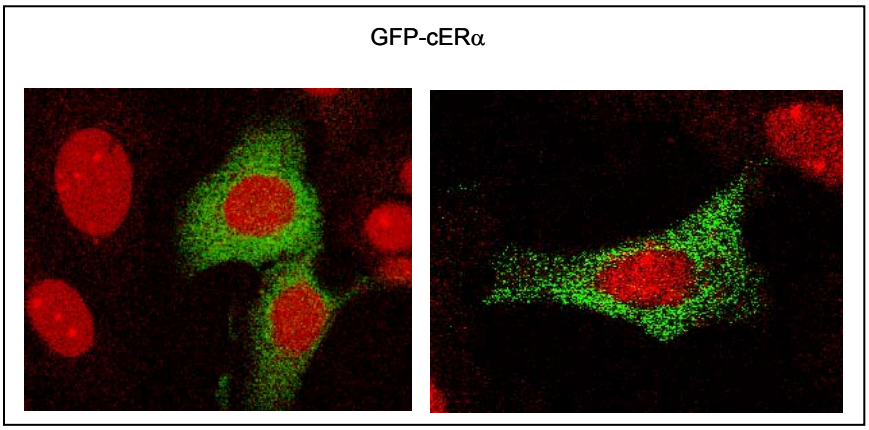


Figure 2: GFP-cERα is exclusively cytoplasmic. C4-12 cells stable expressing GFP-cERα were stained with propridium iodide (red) and then examined by confocal microscopy. The GFP signal shows that the cERα is exclusively cytoplasmic.

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

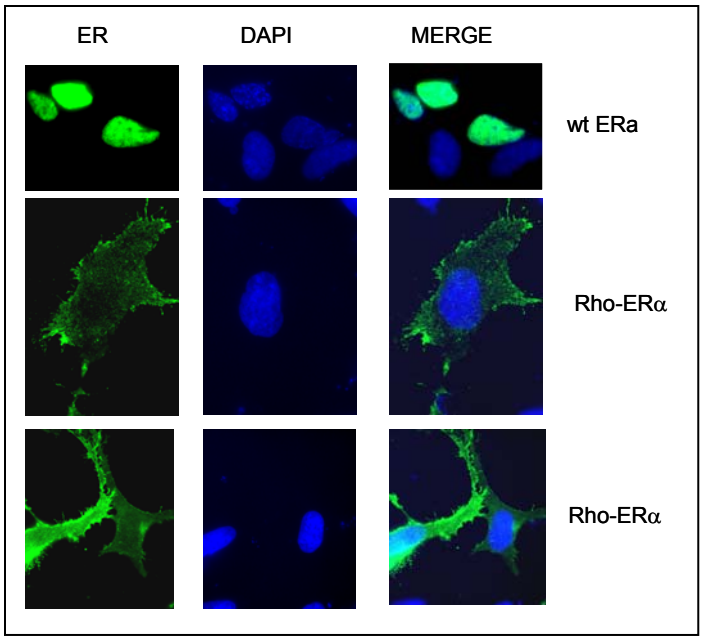


Figure 3: Localization of rho-ERα. 293 cells were transiently transfected with wt-ERα or rho-ERα and then ERα detected by immunostaining with ERα antibody (6F11, neomarkers) and detected by anti-mouse antibodies conjugated to alexa488 (left panels). The nucleus was identified by staining with DAPI (middle panels). A merged image is also shown (right panels). As expected, wtERα was entirely nuclear, whereas the rhoERα construct showed exclusive cytoplasmic or membrane staining.

In year 3 we generated several stable transfectants that expressed rho-ERα (Figure 4). Figure 4 shows immunofluoresence for ERα (red) or a nuclear stain (DAPI – blue) on MCF-7 cells or C4-12 cells stably expressing rho- ERα. 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 (red signal). 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- ERα). The highlighted lanes with numbers represents 1) C4-12 rho ERα, 2) MCF-7 wGFP-wtER, 3) MCF-7 GFP-cER, 4) MCF-7HER2 GFP-cER, 5) MCF-7 rho- ERα, 6) MCF-7 rho-ERα). 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 it insertion in the plasma membrane and resulting posttranslational modifications of the rhodopsin protein. This immunoblot is only showed as an example of the tools we have used.

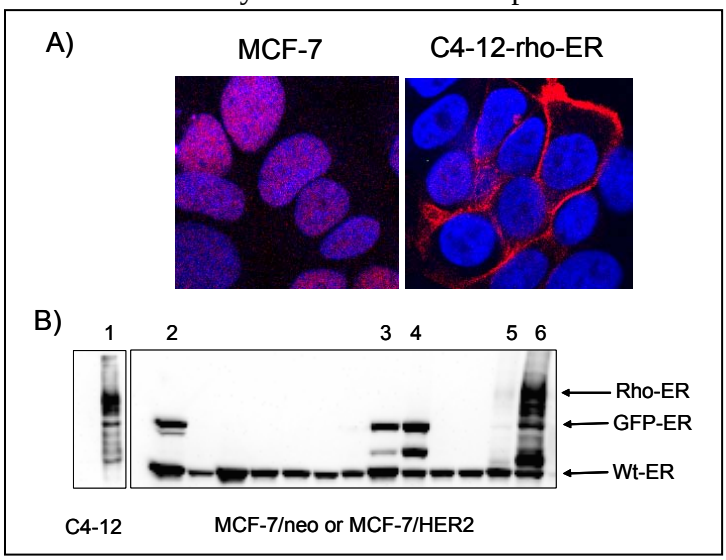


Figure 4: Stable expression of rho-ER in C4-12, MCF-7 and MCF-7/HER2 cells. A) Immunofluoresence using antibodies against ER (red) and a DAPI as a nuclear stain (blue). Note the nuclear ER in MCF-7 (left) but the lack of ER in the nucleus of C4-12-rhoER and the presence of a strong plasma membrane localization. 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 mERα and cERα are expressed only in the membrane and cytoplasm respectively, and test whether mERα and cERα are capable of binding estradiol (E2) and tamoxifen (Tam) (Months (4-8).

Figure 1 confirms that cERα is only expressed in the cytoplasm, while wt-ERα is mainly nuclear. Figure 3 shows that rho-ERα 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 cERα 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 cERα and rho-ER are degraded following E2 stimulation, suggesting in an indirect way that this receptor can bind E2. This highlights the interesting ability of cERα 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 mERα or cERα associate with membrane or cytoplasmic structures (e.g. clathirin-coated pits) (Months 7-12).

Not performed.

Task 2: To compare and contrast the effects of estrogen in C4-12-cERα, mERα and wtERα 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-ERα variants (Months 12-16).

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

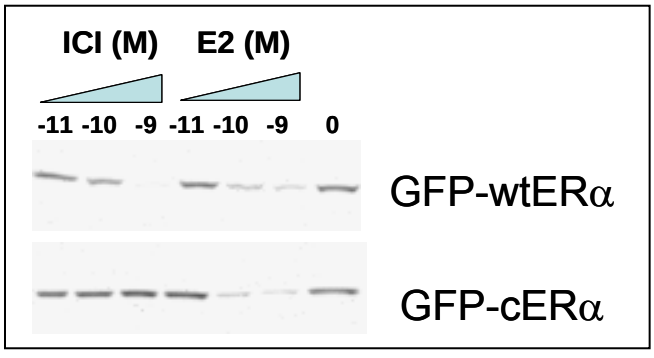


Figure 5: cERα is completely resistant to antiestrogen ICI 182780-mediated degradation. C4-12wt-ERα and cERα stable transfectants were starved in serum-free overnight and then treated for 8 hours with increasing concentrations of estradiol (E2) or ICI182780 (ICI). As expected, wtERα protein levels were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

Importantly, the degradation of ERα by E2 was blocked by the 26S proteasome inhibitor lactacystin (Figure 6) and MG132 (data not shown). This is an important result given that a number of groups have proposed that E2-mediated degradation of ERα is linked to transcription. cERα 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. More importantly, while wt-ERα was degraded by antiestrogens such as ICI182780, the cERα was completely resistant to degradation by ICI182780.

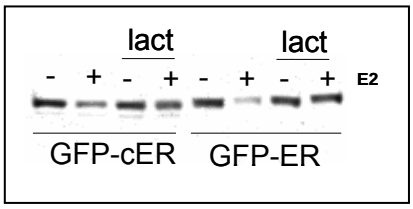


Figure 6: E2-mediated degradation of both wt and cERa is blocked by proteasome inhibition. C4-12-wt-ER and C4-12-cER cells were preincubated with lactacystin (10mM) for 30 mins and then incubated with or without E2 (1nM) for 6 hrs. Cells were lysed and immunoblotted for Era.

We next examined the ability of E2 and ICI to cause degradation of rho-ER that was tethered to the plasma membrane. Unlike cER, we found that rhoER was degraded by both E2 and ICI. This was despite the fact that rho-ER was unable to stimulate gene expression (similar to cER) (see next section).

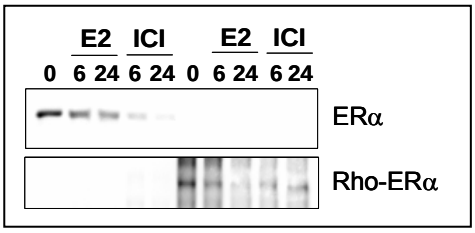
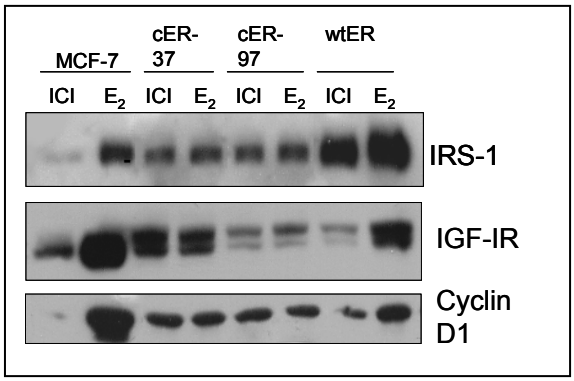


Figure 7: E2 and ICI-mediated degradation of wt-ER and rho-ER. MCF-7 cells and C4-12rho-ER were incubated with vehicle, E2 (1nM) or ICI (1nM) for 6 or 24 hrs, then lysed and immunoblotted for ER.

Given that the rho-ER construct is a fusion protein containing full-length ER including the NLS, the lack of ICI-mediated degradation of cER would see to be due to its lack of the NLS. We will study this further using chimeric fusions proteins specifically focusing on the NLS.

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



We have found that cERα 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 8: cERα doesn't confer estrogen-induction of ER-responsive genes. MCF-7, C4-12cERα and C4-12wtERα 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 wtERα. In contrast, two stable clones of C4-12ERα did not show estrogen regulation of IRS-1, IGF-IR or cyclin D1.

Similar to the cER result, we found that rho-ER is incapable of stimulating an estrogen-regualted gene IRS-1 (Figure 9)

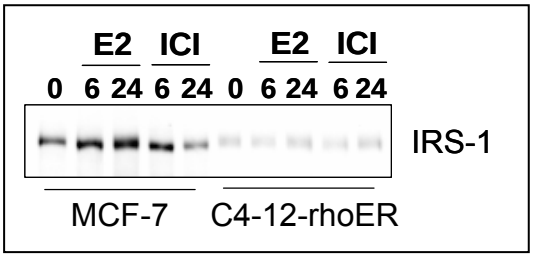


Figure 9: rho-ER fails to affect IRS-1 levels in response to E2 or ICI. MCF-7 cells and C4-12rho-ER were incubated with vehicle, E2 (1nM) or ICI (1nM) for 6 or 24 hrs, then lysed and immunoblotted for ER.

iii) Determine whether E2 stimulation results in an increase in S-phase and cell proliferation in C4-12-cERα and mERα compared to C4-12wtERα (Months 18-24).

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

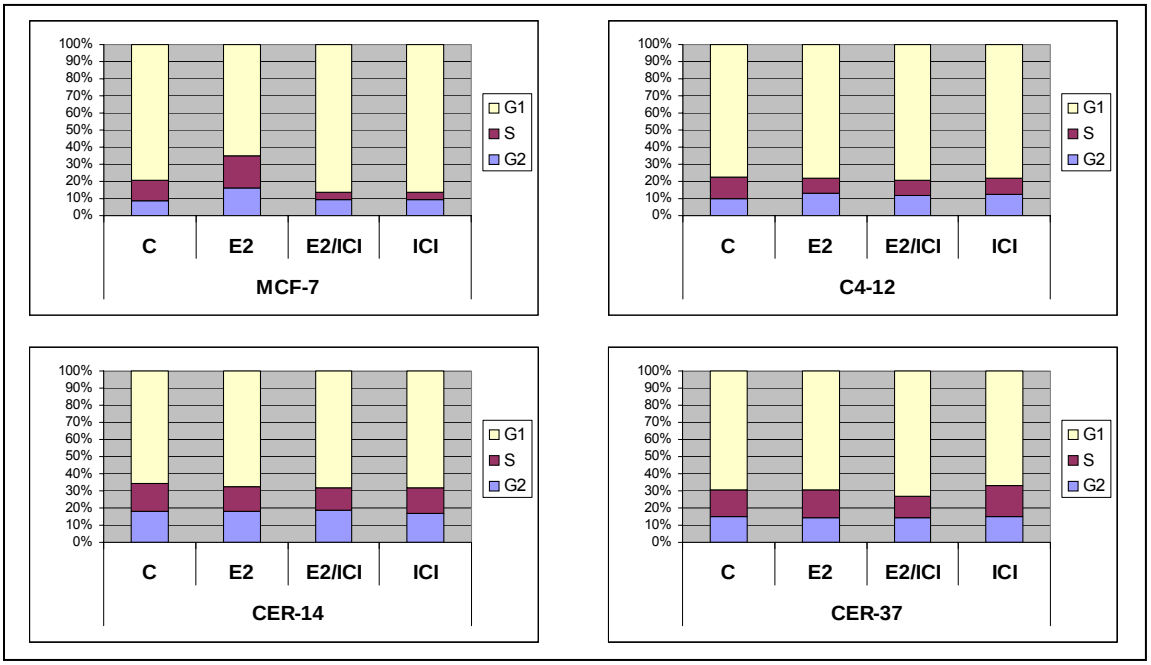


Figure 10: cERα does not confer estrogen stimulated S-phase entry. MCF-7, C4-12, and C4-12 cells expressing cERα were starved in serum-free medium overnight, and then stimulated with estradiol (1nm) or ICI (100nM) or the combination for 16hrs. Cells were then fixed in alcohol, stained with propridium 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, ERα negative C4-12 cells, or the cERα expressing cells showed no changes in response to E2 or ICI.

We have not performed these experiments in the C4-12rho ERα to see whether the transient induction of ERK1/2 phosphorylation that we see with estrogen stimulation (Figure 12) can confer a proliferative advantage**.**

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

i) Perform coimmunoprecipiation and colocalization to determine if mERα and cERα can bind p85 and activate PI3K (Months 24-30).

Despite preliminary evidence that cERα 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 11).

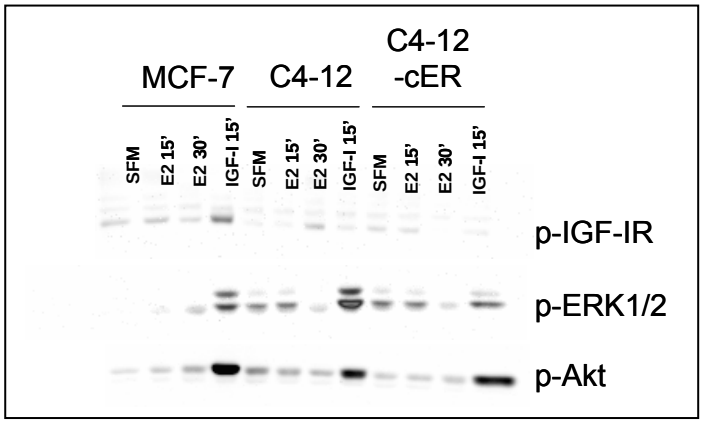


Figure 11: cERα does not allow short-term estrogen signaling in C4-12 cells. MCF-7, C4-12 and C4-12cERα cells were starved in SFM overnight and then stimulate with estradiol (1nm, 15 or 30mins) or IGF-I (10nM, 15mins). Cells were lysed and immunoblotted for p-IGF-IR, p-ERK1/2 and p-Akt. MCF-7 cells did 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 cERα 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 12). 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).

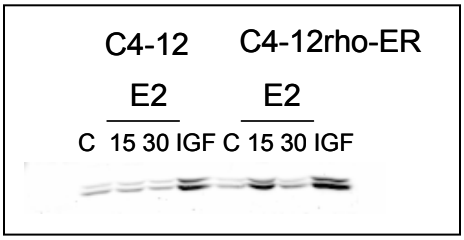


Figure 12: Rapid phosphorylation of ERK1/2 in response to short-term estrogen in C4-12-rho-ER. C4-12 and C4-12rho-ER were starved overnight in serum free medium and then stimulated with estradiol (1nM) for 15 or 30 minutes. As a positive control for ERK1/2 activation, cells were stimulated with IGF-I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

ii) Examine whether E2-stimulation of C4-12-mERα and cERα cells results in mobilization of intracellular Ca^{2+} and activation of PKC (Months 30-34).

Not performed

iii) Determine whether E2 can induce an anti-apoptotic response in C4-12-mERα and cERα cells (Months 32-36).

Not performed

KEY RESEARCH ACCOMPLISHMENTS

- Generation of C4-12 cells that express ERα only in the cytoplasm (C4-12-cERα).
- Evidence that cERα is degraded by estrogen but not by anti-estrogen
- Generation of C4-12 cells expressing rho- ERα.
- Evidence that rho- ERα 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-cERα
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 used a unique cell line model (C4-12) to test if cytoplasmic (cERα) or membrane targeted ERα (mERα) can perform signaling and mediated proliferation. This research is critical, as several recent studies have suggested that cERα or mERα is important, and pathologists only analyze nuclear ERα, which might misclassify a number of breast cancer patients. We have generated cells that express ERα 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. Importantly this receptor is alos degraded by estrogen again challenging the notion of a link between estrogen mediated

degradation of the receptor and proteasome degradation. Understanding any potential role of cERα or mERα is critical for the complete understanding of estrogen action and targeting in breast cancer.

ORAL 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.

POSTER PRESENTATION

2005 "TARGETED CYTOPLASMIC LOCALIZATION OF ERALPHA IN C4-12 CELLS REVEALS NOVEL INSIGHT INTO LIGAND-STIMULATED PROTEASOME-MEDIATED DEGRADATION OF ERALPHA BUT DOESN'T SHOW NON-GENOMIC SIGNALING
Adrian V. Lee, Curtis Thorne, Ping Zhang, Zawaunyka Lazard, and Steffi Oesterreich
4th Era of Hope Meeting

MANUSCRIPT

Novel insights in ligand-dependent degradation of ER using subcellular localization mutant receptors. In preparation for the Journal of Biological Chemistry