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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

We have recently developed a cell line system in which exogenous expression of estrogen receptor alpha (ERα) in an ERα-negative cell line results in ERα -mediated signaling and proliferation. We proposed in this project to express ERα mutants and use this system to define ERα action in breast cancer. We have generated breast cancer cells lines that express ERα only in the cytoplasm to characterize the putative cytoplasmic (non-genomic) function of ERα. We have found that the cytoplasmic ERα can bind estrogen and is down-regulated, similar to wild-type ERα. However, the cytoplasmic ERα can't activate gene transcription (due to its inability to enter the nucleus), and also can't stimulate cell cycle progression or proliferation. Consistent with the cytoplasmic ERα not activating gene transcription or cell cycle progression, cytoplasmic ERα is not able to induce the estrogen-regulated genes cyclin D1 or IRS-1. We are now determining whether the cytoplasmic ERα is able to interact with cytoplasmic signaling intermediates and confer non-genomic signaling, and also whether localization of ERα specifically to the membrane can enhance the non-genomic actions of ERα

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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 is to create a novel and unique model of breast cancer cells that express 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 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 ERα -ve breast cancer cell line (C4-12).

BODY

Introduction

We have made significant progress on this project, having generated stable transfectants that express ERα in the cytoplasm (cERα) and having performed a preliminary characterization of these cells. Specific results are detailed below in relation to the original S.O.W.

Despite the progress, we have had two major setbacks. First, the C4-12 cells that we use as a model are supposed to be ERα-negative (which is critical for the project); however, they often re-express ERα which makes interpretation of the results impossible. A problem is that we do not know why C4-12 cells are ERα -negative, and thus we can't control their ability to spontaneously re-express ERα. We have tried to grow the cells under different conditions, but this has not helped. We have carried on with the project, analyzing the cells at every time point to confirm that they are ERα -negative. The project is still feasible, however the progress is delayed by the continual screening and the discarding of cells which become spontaneously ERα -positive. We continue on using the cells as they are a unique resource; there is no other ERα -negative cell line in which ERα can be reexpressed and growth measured. Thus we will continue to work with this model and if we can find out why ERα is spontaneously expressed perhaps in the future we can control the ERα levels in this cell line.

Our second setback was the inability of our membrane-targeted ERα to be localized to the membrane. We expect that the localization tag, which is on the C-terminus of ERα is folded within the protein and inaccessible for attachment to the plasma membrane. We have therefore started to reclone the ERα downstream of an N-terminal myristylation tag and we will examine whether this is targeted to the membrane. Despite this setback we have continued characterization of our cytoplasmic ERα variant as described below.

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 have stably transfected C4-12 cells with GFP, GFP-wtERα, and GFP-cERα and GFP-mERα. We have isolated multiple clones and using immunofluoresence microscopy shown that the cERα is indeed expressed in the cytoplasm (compared to wtERa which is mainly nuclear). GFP alone is expressed all over the cell. We have confirmed these localization results using biochemical fractionation.

A setback came when we expressed the mERα, which was not targeted to the membrane. We do not know why this is, but it may be due to the membrane tag being on the c-terminus of the protein and not being accessible for insertion into the plasma membrane. We have therefore altered the cloning

strategy and are cloning a myristylation tag onto the N-terminus of ERα to see if this is targeted to the membrane.

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).

As stated in i), we have used confocal microscopy and biochemical fractionation to confirm that the cERα is only expressed in the cytoplasm, while wt-ERα is mainly nuclear. These techniques also indicated that the mERα did not target to the membrane and led us to change the strategy for cloning and targeting mERα to the membrane.

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 is degraded following E2 stimulation, suggesting in an indirect way that this receptor can bind E2. This assay however does not tell us the relative binding affinity of this receptor which can only come through ligand binding assays.

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. 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. Interestingly, while wt- ERα is degraded by antiestrogens such as faslodex, the cERα is not degraded significantly by faslodex suggesting that this is a nuclear mediated event and that E2 and faslodex degradation mechanisms are distinct. We are very interested in these results and will be placing a major emphasis on trying to understand these differences as we may have created a new model for understanding degradation of ER. We are also interested if membrane localization would completely inhibit degradation.

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 and cyclin D1. This is consistent with it not being able to activate gene transcription. We are analyzing more downstream genes, and will compare these results with the mERα cells once they are generated.

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, consistent with this variant not inducing gene transcription. This is despite

the fact that the cERα can clearly bind E2 and be degraded. We feel that if we can show that this receptor can mediate short term E2 responses such as activation of MAPK and Akt then this would be evidence that this short term E2 action does not lead to proliferation.

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).

Preliminary results suggest that the cERα can bind p85. These results are being confirmed, and the resulting effects on pAkt measured.

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 antiestrogen

REPORTABLE OUTCOMES

Development of cell lines:
C4-12-GFP
C4-12-GFP-cERα

CONCLUSIONS

This project will use 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 cell 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. If we can show that this receptor is able to perform the short-term effects on signal transduction that other group have shown, then this research would call into question the relevance of these other studies.
Understanding any potential role of cERα or mERα is critical for the complete understanding of estrogen action and targeting in breast cancer.

REFERENCES

None