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TITLE: A Non-Nuclear Role of the Estrogen Receptor Alpha in the Regulation of Cell-Cell Interactions

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14. ABSTRACT Proliferation and metastasis of many breast cancers depend on the steroid hormone estrogen. The actions of estrogens are mediated by the estrogen receptors ER α and ER β . These hormone-regulated transcription factors translate the presence of estrogen into changes in gene expression. According to new findings, these receptors also act outside of the nucleus and are often found associated with the plasma membrane. In contrast to their roles in regulating cell proliferation, very little is known about the mechanisms by which estrogens promote metastasis. It has been suggested that estrogens aid this process by changing the expression of cell adhesion proteins, such as E-cadherin. However, results in our laboratory have opened the possibility that disruption of cell adhesions by estrogens involves the direct interaction of ER with cell adhesion proteins. The goal of this grant is to explore this possibility. If true, this mechanism would represent a novel example of a non-nuclear activity of the estrogen receptor, steer ongoing studies on the role of estrogens in the regulation of cellular adhesions into a new direction, and open new venues for the prevention, diagnosis and therapy of breast cancer.					
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Introduction

Proliferation and the formation of metastases by many breast cancers depend on the steroid hormone estrogen. The actions of estrogens are mediated by the estrogen receptors ER α and ER β . These receptors function as hormone-regulated transcription factors that translate the presence of estrogen into changes in gene expression. New findings suggest that these receptors can also act outside of the nucleus and are often found associated with the plasma membrane. For example, it has been shown that proliferation of the human breast cancer cell line MCF7 depends on the interaction of cytosolic ER α with the Rous sarcoma virus kinase (Src) and the phosphatidyl inositol 3-kinase (PI3K) (1).

In contrast to their roles in regulating cell proliferation, very little is known about the mechanisms by which estrogens promote the formation of metastases. It has been suggested that estrogens aid this process by changing the expression of cell adhesion proteins, such as E-cadherin (2). Recent results in our laboratory have opened the possibility for a different mechanism. While studying the functional interactions of ER α with other gene regulatory proteins, we found that ER α interacts with α - and β -catenin (Fig. 1). In addition to regulating gene expression, β -catenin forms a heterocomplex with α -catenin that links the transmembrane protein E-cadherin to the cytoskeleton. These interactions are essential for the formation of stable E-cadherin-dependent adherens junctions, which mediate cell-cell interactions. By mapping the interaction of ER α with α - and β -catenin, we identified that ER binds close to the structurally characterized heterodimerization domains of α - and β -catenin (3). Moreover, studies by others have demonstrated that ER α is present at the cell membrane of MCF7 cells, a tumorigenic mammary epithelial cell line, whose cellular adhesions are remodeled by estrogens (4,5).

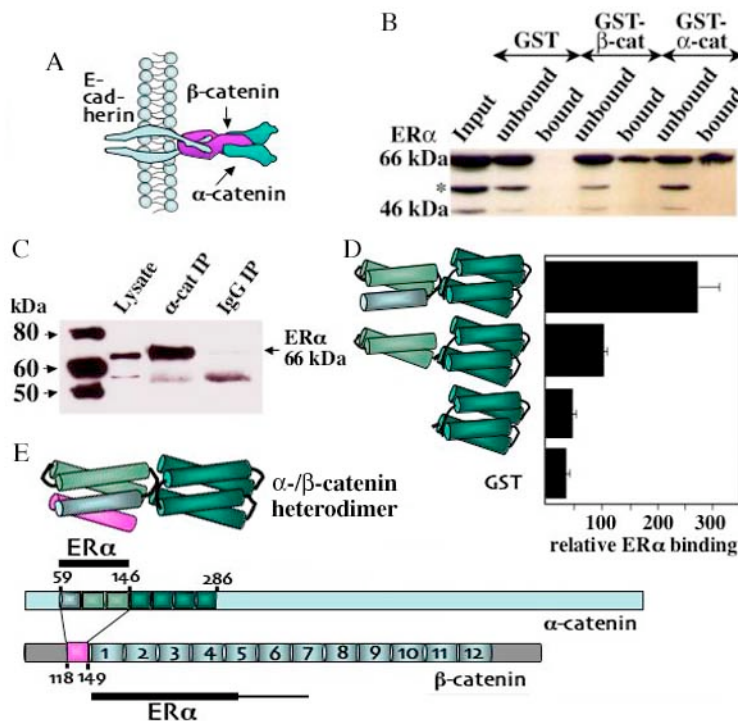


Fig. 1 Interaction of ER α with α - and β -catenin. A. Composition of E-cadherin-dependent adherens junctions. B. Interaction of ER α (66 kDa and 46 kDa isoforms) of MCF7 cell lysates with recombinantly expressed and purified glutathione S-transferase β -catenin and α -catenin (amino acids 90-286) fusion proteins bound to glutathione agarose. Bound ER α was monitored by immunoblot analysis. * unspecific band recognized by the ER α antibody. These interactions are not hormone-dependent (data not shown). C. Co-immunoprecipitation of endogenous ER α 66 kDa and α -catenin in MCF7 lysates. D. GST pulldown assay monitoring the interaction of *in vitro* expressed and 35 S-labeled ER α 66 kDa and GST fusions of the α -catenin fragments 55-286, 90-286 and 146-286. E. Map of the interactions of ER α with α - and β -catenin. ER α binds to the α -/ β -catenin heterodimerization domain.

Based on these findings we hypothesized that disruption of adherens junctions by estrogens involves the interaction of ER α with α - and β -catenin. If true, this would represent a novel example of a non-nuclear activity of the estrogen receptor and steer ongoing studies on the role of estrogens in the remodeling of cellular adhesions into a new direction.

Research Accomplishments

Objective 1

Is membrane localization and interaction of ER α with α - and β -catenin necessary for the ability of 17 β -estradiol (E2) to disrupt adherens junctions in MCF7 cells?

To investigate whether changes in the expression of ER α alter MCF7 cell-cell interactions, we stably integrated expression vectors for either ER α , ER α 46 kDa or the ER α mutant S518A (S518 is the human homologous residue to mouse S522) into MCF7 cells. Due to an alternative start site, ER α is expressed as a 66 kDa and 46 kDa form that lacks the N-terminal activation domain (AF1) (6). Through heterodimerization, ER α 46 kDa suppresses the transcriptional activity of ER α 66 kDa (6). Moreover, the N-terminus of the 46 kDa variant can be lipid-modified resulting in an enhanced recruitment of ER α 46 kDa to the plasma membrane (7). In contrast to ER α 46 kDa, the transcriptional response of the ER α mutant S518A has been shown to be comparable to that of ER α 66 kDa (8). However, this mutant has a dominant-negative effect on the membrane localization of ER α .

Construction and initial characterization of MCF7 lines - We completed the cloning and initial expression tests of the integration expression vectors for these various constructs. For each construct, we characterized 10 lines with respect to their cell morphology, growth, expression of the transgene and hormone responsiveness. Examples of these characterizations for the lines we finally selected are shown in Fig. 2. We also attempted the integration of the α -catenin 90-286, and β -catenin 141-286 fragments, which interact with ER α (Fig. 2). These fragments contain the α -/ β -heterodimerization surface that mediates the interaction of α - and β -catenin in adherens junction complexes. However, MCF7 lines that overexpress α -catenin 90-286 or β -catenin 141-286 were heterogeneous, growth impaired and tend to apoptose suggesting that these fragments likely affect several aspects of β -catenin signaling. Therefore, we decided not to continue with these fragments.

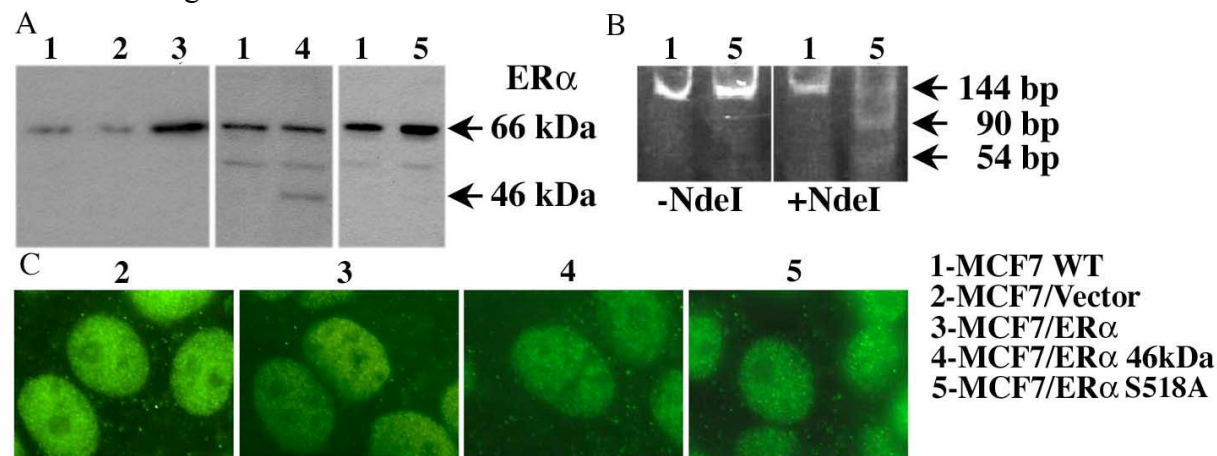


Fig. 2 Basic characterization of MCF7 lines

A. Expression of the transgenes monitored by immunoblot analysis using an ER α specific antibody. B. Since WT ER α and the ER α S518A mutant have similar molecular weights, expression of the mutant was monitored by cleaving amplified ER α cDNA with a restriction enzyme that only recognizes ER α S518A DNA. C. ER α expression in these MCF7 lines monitored by indirect immunofluorescence. Like in MCF7 WT, in the stable MCF7 lines the majority of ER α is nuclear.

Overall growth behavior of MCF7 lines - MCF7 lines containing either an empty expression vector or vectors for ER α , ER α 46 kDa or the ER α mutant S518A displayed striking differences in their growth behavior (Fig. 3). While the MCF7/vector cells grew similar to WT MCF7 cells, MCF7/ER α lines tended to form tight clusters. MCF7/ER α 46 kDa cells grew primarily as monolayer, whereas MCF7/ER α 66 kDa cells formed multilayered clusters and displayed a morphology that is similar to WT MCF7 cells treated with E2. In contrast, MCF7/ER α S518A cells were only loosely associated with one another and apoptosed upon reaching confluence. The phenotypes of these cells support our hypothesis that ER α might have a direct effect on the formation of cell adhesions. We are in the process of repeating these analyses in the absence and presence of 17 β -estradiol and to obtain high-resolution pictures of cell protrusions formed by these lines.

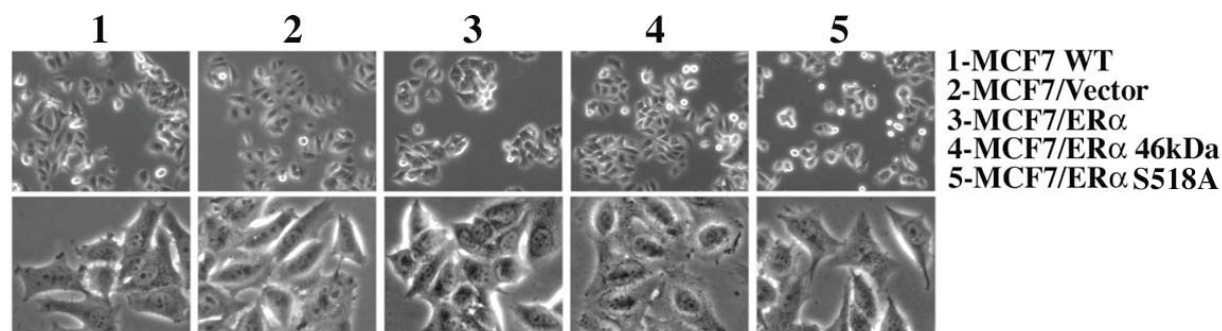


Fig. 3 Subconfluent growth pattern of MCF7 lines containing either an empty expression vector or vectors for ER α , ER α 46 kDa or the ER α mutant S518A.

ER α membrane localization - To monitor the membrane localization of ER α in MCF7 cells, we developed an experimental strategy to reliably prepare membranes from MCF7 cells that are not contaminated by cytosolic proteins (Fig. 4A). MCF7 fractionation experiments performed according to this protocol revealed that in MCF7 WT cells, about 5% of the total ER α is associated with membranes (Fig. 4B). This fraction does not seem to change in the presence of E2. We are in the process of repeating these experiments with the stable cell lines. Preliminary indirect immunofluorescence analyses are consistent with the proposed dominant-negative effect of the ER α S518A mutant on ER α cytosolic and membrane localization (Fig. 1C).

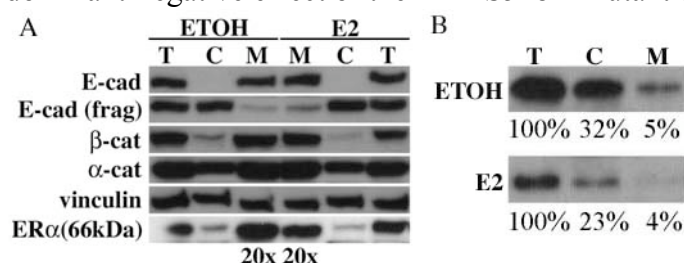


Fig. 4 Membrane localization of ER α in MCF7
A. Abundance of ER α and adherens junction proteins in total lysate (T), or cytosol (C) and membrane (M) fractions of MCF7 cells that had been treated with either vehicle (ETOH) or 1 nM E2 for 3 days. Proteins were identified by immunoblot analysis. Please note that membrane fractions are 20x more concentrated than total cell lysate or cytosolic cell fractions. B. Quantitative analysis ER α (66 kDa) in cytosol and membrane fractions of MCF7 cells. Cells were treated as in A.

Interaction of ER α with E-cadherin adherens junction complexes in MCF7 cells - To determine whether ER α interacts with E-cadherin adherens junction complexes we investigated by indirect immunofluorescence whether ER α co-localizes with E-cadherin or β -catenin. In the absence of hormone, staining for E-cadherin/ β -catenin and ER α did not overlap, suggesting that ER α does not interact with these junctions (Fig. 5). Treatment of MCF7 cells with 17 β -estradiol (E2) for three days resulted in a dispersion of junction complexes and an increase in ER α and E-cadherin/ β -catenin co-localization. However, due to the disperse pattern of ER α and E-cadherin/ β -catenin, overlap in the staining for these proteins could be fortuitous. Moreover, thus far, we have been unable to detect ER α in co-immunoprecipitated E-cadherin adherens junction complexes (Fig. 6). We are in the process of completing these studies with the stable MCF7 lines.

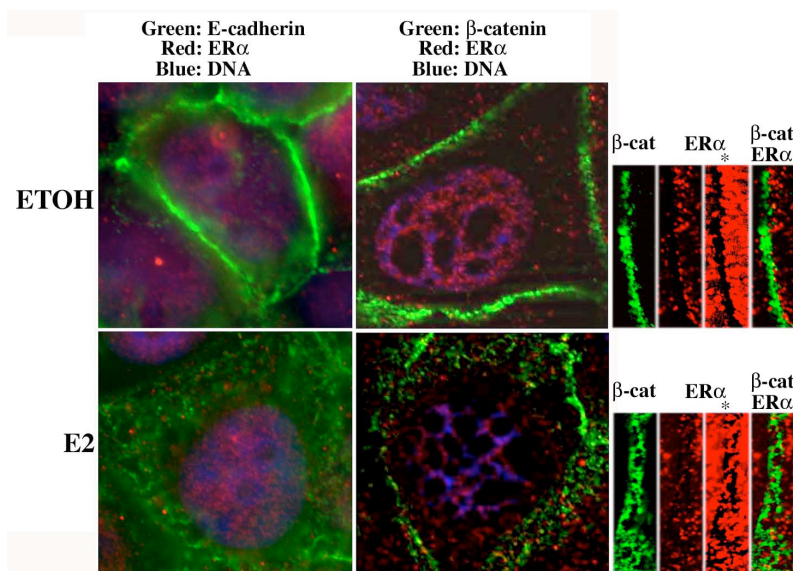


Fig. 5 Analysis of potential co-localization of ER α and E-cadherin/ β -catenin in MCF7 cells by indirect immunofluorescence. MCF7 cells were treated with either vehicle (ETOH) or 1 nM E2 for four days. Antibodies were fluorescently labeled as indicated. DNA is DAPI-stained. For a membrane fraction of vehicle and E2-treated cells β -catenin, ER α and β -catenin/ER α staining is shown separately. "*", enhanced images.

Objective 2

Does E2 remodel adherens junctions by interfering with the interactions between α - and β -catenin?

Does binding of ER α to α - and β -catenin impair the formation of the α - and β -catenin heterocomplex?

Our initial finding that ER α interacts with the α -/ β -catenin heterodimerization domain suggested that ER α affects the formation of E-cadherin adherens junctions by regulating the interactions between α - and β -catenin. Support for this model was provided by observations in endothelial cells where E2 seems to transiently impair the interaction between α - and β -catenin (9). To determine whether E2 influences the interactions between α - and β -catenin in MCF7 cells, we co-immunoprecipitated E-cadherin, α - and β -catenin from MCF7 cells that were treated with either vehicle or E2 for various times. The results from these studies, however, did not reveal any significant affects of E2 on the interactions between E-cadherin complexes.

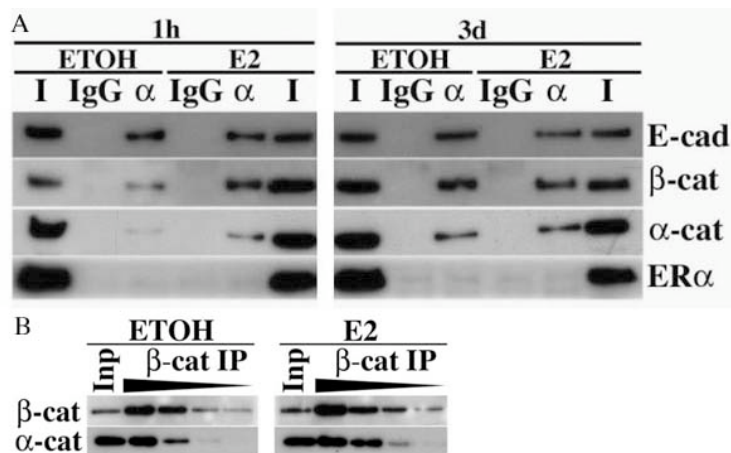


Fig. 6 Analysis of E-cadherin-dependent adherens junctions in the absence and presence of E2.

A. MCF7 cells were treated with either vehicle (ETOH) or 1 nM E2 for 1 or 3 days. E-cadherin-dependent adherens junctions were immunoprecipitated and probed with antibodies against E-cadherin, β -catenin, α -catenin and ER α . I, input; IgG, non-specific antibody, α , E-cadherin-specific antibody. B. Co-immunoprecipitation of α -/ β -catenin in the presence of various concentrations of MCF7 cell lysates. MCF7 cells were treated with either vehicle or 1 nM E2 for 1h. Proteins were immunoprecipitated with a β -catenin-specific antibody. Immunoprecipitated complexes were probed with antibodies against α -catenin and β -catenin.

ER α interacts with α -catenin homodimers - In addition to the membrane-associated α -catenin that is part of adherens junctions, in MCF7 cells a substantial portion of α -catenin is also present in the cytosol and in the nucleus (Fig. 4 A,B). Structural and biochemical studies have shown that α -catenin homodimerization and α -/ β -catenin heterodimerization compete with each other (3). In both cases dimerization is mediated by the formation of a four-helix bundle (3). In the case of the α -catenin homodimer this bundle is formed by the α -catenin α -helices H1 and H2 (amino acids 90-146), whereas in the case of the α -/ β -heterodimer the α -catenin α -helices H0, H1 and H2 (amino acids 59-146) are complemented by a β -catenin α -helix (amino acids 118-149) (Fig. 7A). To determine whether ER α interacts with the α -catenin homodimer or the α -/ β -heterodimer, we purified these dimers and monitored their interaction with *in vitro* expressed ER α . As shown in figure 7A, ER α interacted preferably with the α -catenin homodimer.

A role of ER α in F-actin cytoskeleton remodeling? - Until very recently it was believed that the role of α -catenin in adherens junctions is to link E-cadherin/ β -catenin to the cytoskeleton through direct or indirect interactions with F-actin. However, new results demonstrated that adherens junction complexes are not directly connected to the cytoskeleton (10). Rather it appears that increasing concentrations of adherens junction complexes inhibit the branching of F-actin fibers in the vicinity of these junctions and to promote the formation of parallel F-actin fiber bundles. According to a current model, this switch in F-actin polymerization is mediated by cytosolic α -catenin homodimers, which bind F-actin and prevent branching of these fibers by the Arp2/3 complex (Fig. 7B). Formation of these homodimers is mediated by the recruitment of α -catenin by E-cadherin/ β -catenin, which enables the establishment of junction-associated equilibrium between membrane-associated α -/ β -catenin heterodimers and cytosolic α -catenin homodimers (11). This model suggests that the interaction of ER α with α -catenin may interfere with the α -catenin-mediated change in F-actin polymerization. To test whether this is the case, we phalloidin-stained the F-actin cytoskeleton in MCF7 cells that were treated with either vehicle or E2 for 4 days. Consistent with an effect of E2 on F-actin polymerization, in E2-treated cells parallel F-actin fibers were frequently disrupted and branched F-actin fibers appear

to be more frequent (Fig. 7C). We are in the process to analyze whether E2 disrupts the interaction of α -catenin with F-actin. If this is the case, we will investigate whether these effects are mediated by cytosolic or membrane-associated ER α .

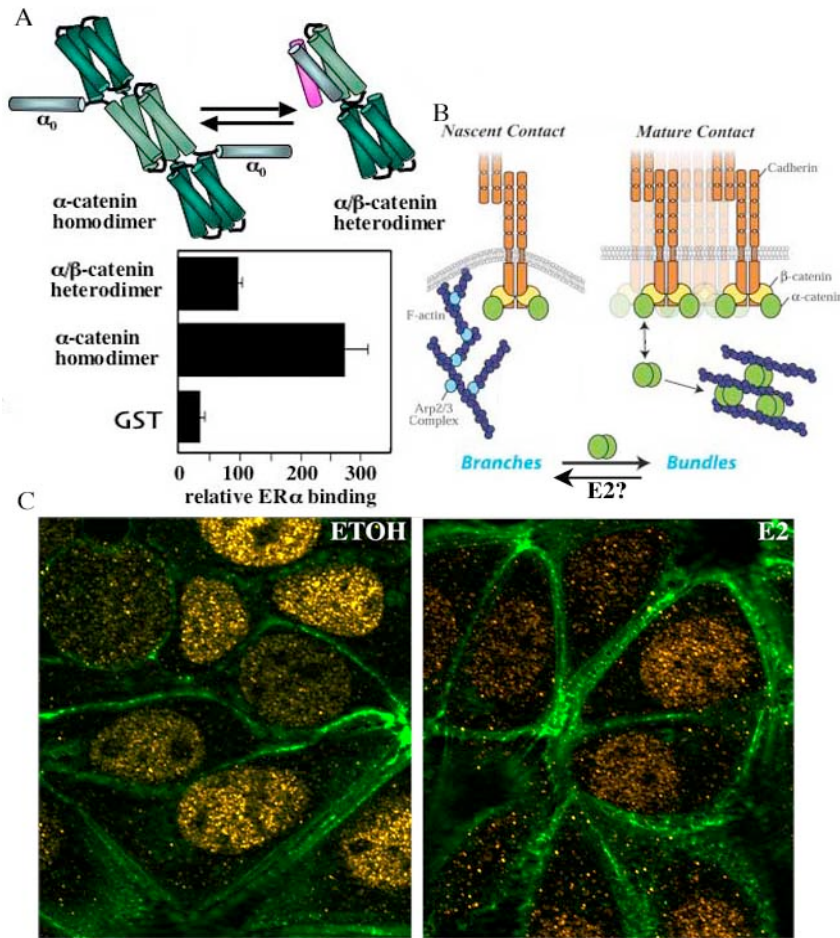


Fig. 7 Role of E-cadherin-dependent adherens junctions in F-actin remodeling
A. GST-pull-down experiment comparing the interaction of ER α with GST or GST-fused α -catenin homodimers and α - β -catenin heterodimers. the α -helix of β -catenin that interacts with α -catenin is shown in pink. **B.** Model for the adherens junction-mediated changes in F-actin polymerization according to (11). A high concentration of adherens junctions establishes a local equilibrium between α -catenin homodimers and α - β -catenin heterodimers. The α -catenin homodimers bind F-actin and prevent the branching of F-actin fibers by the Arp2/3 complex. **C.** ER α and F-actin localization in MCF7 cells that have been treated with either vehicle (ETOH) or 1 nM E2 for four days. F-actin was stained with phalloidin, ER α by indirect immunofluorescence using an ER α -specific antibody.

Key Research Accomplishments

Objective 1:

Is membrane localization and interaction of ER α with α - and β -catenin necessary for the ability of 17 β -estradiol (E2) to disrupt adherens junctions in MCF7 cells? (Total: 9 months)

Task 1: Stable integration of ER α S518A (mouse ER α S522A), α -catenin 90-286 and β -catenin 141-286 into MCF7 cells

- Clone ER α , ER α S518A, α -catenin 90-286 and β -catenin 141-286 into mammalian expression/ integration vector under control of an inducible promoter. Confirm constructs by sequencing. (3 weeks)

Completed

- Transfect MCF7 cells with expression/ integration plasmids for ER α , ER α S518A, α -catenin 90-286, and β -catenin 141-286 and monitor expression of these proteins using Western blot analysis. This step is to confirm that these constructs express the respective proteins. (3 weeks)

Completed

- Transfect MCF7 cells with expression/ integration vectors for ER α , ER α S518A, α -catenin 90-286, and β -catenin 141-286. Select clones that are resistant to the selective antibiotic (geneticin). Isolate and propagate several clones per construct. (2 months)

Completed

- Identify clones for further functional analysis: Characterize several clones per line with respect to cell morphology, growth, expression of the transgene and hormone responsiveness using immunofluorescence, Western blot analysis of cell lysates, and reporter assays. (2 months)
We completed the analysis of cell morphology, growth, expression of the transgene and hormone responsiveness of the MCF7 lines containing either an empty expression vector or integrated expression vectors for ER α , ER α 46 kDa or the ER α mutant S518A using indirect immunofluorescence and immunoblot analysis of cell lysates. Instead of the proposed reporter assays, we are in the process of analyzing the hormone response of endogenous ER target genes using real time PCR. Consistent with our hypothesis that ER α might have a direct effect on the formation of cell adhesions, these cell lines displayed striking differences in their growth behavior. Moreover, we constructed lines that overexpress α -catenin 90-286 or β -catenin 141-286. However, these lines were heterogeneous and growth-impaired indicating that these fragments affect other aspects of β -catenin signaling. Therefore, we decided to not continue with these fragments.

Task 2: Does expression of ER α , ER α S518A, α -catenin 90-286, and β -catenin 141-286 affect ER α membrane localization and the ability of 17 β -estradiol (E2) to impair adherens junction formation in MCF7 cells?

- Monitor ER α plasma membrane localization in MCF7 cells and in the stable MCF7 lines prepared above in the absence or presence of E2 using immunofluorescence and Western blot analysis of cell fractions. (1.5 month)
We established a protocol for the quantitative analysis of ER α membrane localization in MCF7 cells and monitored ER α membrane localization in WT-MCF7 cells in the absence and presence of E2. We found that about 5% of ER α is associated with membranes both in the absence and presence of E2. We are repeating these experiments with the stable MCF7 lines.
- Monitor formation of adherens junction and association of ER α with adherens junction proteins in MCF7 cells via co-localization of E-cadherin, α -catenin, and ER α using immunofluorescence. Verify transgene-dependent changes in the formation of adherens junctions and ER α recruitment using co-immunoprecipitations and quantitative Western blot analysis. (2 months)
We monitored the localization of ER α , E-cadherin and β -catenin in double-stained MCF7 cells that have been treated either with vehicle or E2 for various times (1h- several days). These experiments did not provide convincing evidence for colocalization of ER α with E-cadherin adherens junctions. Moreover, in these experiments ER α did not co-immunoprecipitate with E-cadherin or β -catenin in amounts that we were able to detect. We are in the process of repeating these experiments with the stable cell lines.

Objective 2:

Does E2 remodel adherens junctions by interfering with the interactions between α - and β -catenin? Does binding of ER α to α - and β -catenin impair the formation of the α - and β -catenin heterocomplex? (Total: 3 months)

Task 3: Monitor the interaction between α - and β -catenin in MCF7 cells in the absence and presence of E2 using co-immunoprecipitations and quantitative Western blot analysis. (1 month)

Completed. We developed a co-immunoprecipitation protocol for the quantitative analysis of E-cadherin-dependent adherens junctions in MCF7 cells. The results from these studies indicated that in WT MCF7 cells E2 does affect neither the amount nor the composition of these junctions.

Task 4: Determine whether ER α and β -catenin compete for binding to α -catenin using *in vitro* interaction measurements.

- Clone, express and purify a GST: α -catenin 1-286His₆ fusion protein. Confirm constructs by sequencing. This α -catenin fragment can interact with both, β -catenin and ER α . (2 weeks).

Completed. Since the GST: α -catenin 1-286 His₆ fusion protein was prone to proteolysis, we also cloned and purified GST: α -catenin 59-286 His₆, which is more stable. This fragment contains the complete α -catenin homodomain. We also cloned and purified a GST: β -catenin 118-149: α -catenin 90-286 His₆ fusion protein, which represents the α -/ β -catenin heterodimer.

- Analyze the ability of full length ER α (purchased from Invitrogen) and β -catenin (already purified) to compete the interaction of GST: α -catenin 1-286His₆ with β -catenin or ER α , respectively, using quantitative GST-pulldown assays. These experiments will be performed in the absence and presence of E2. (1.5 months)

By comparing the interactions of ER α with the purified α -/ β -catenin heterodimer and α -catenin homodimer, we found that ER α interacts preferably with the α -catenin homodimer. This result suggested that instead of interfering with the formation of E-cadherin adherens junction complexes, ER α might regulate the ability of α -catenin homodimers to induce the formation of parallel F-actin bundles. Supporting this hypothesis we found that E2-treated MCF7 cells have significant fewer parallel F-actin bundles than untreated cells. We are in the process of analyzing whether ER α affects the interaction of α -catenin with F-actin.

Reportable Outcomes

Abstract:

J. Jacobson, L. Schwarcz, M. Lib-Myagkov, and Beatrice Darimont,
Regulation of cell adhesions by estrogen receptor alpha
Gordon Conference "Signaling by Adhesion Receptors", South Hadley, MA
June 25-30, 2006

Poster Presentations:

J. Jacobson, L. Schwarcz, M. Lib-Myagkov, B. Darimont
Regulation of cell adhesions by estrogen in human MCF7 breast cancer cells
Westcoast "Comparative Endocrinology" Meeting, Newport, USA.
March 23-25, 2006

J. Jacobson, L. Schwarcz, M. Lib-Myagkov, B. Darimont
Regulation of cell adhesions by estrogen receptor alpha
Gordon Conference "Signaling by Adhesion Receptors", South Hadley, MA
June 25-30, 2006

Research Seminar:

J. Jacobson, M. Lib-Myagkov, L. Schwarcz, T. Takayama, B. Darimont
Linking steroid and beta-catenin signaling
Hospital of Special Surgery, Cornell University, New York
September 09, 2006

Experience/Training:

Doctoral training: Jana Jacobson
Research experience: Sachiko Takayama, Lin Fang

Conclusions

Proliferation and the formation of metastases by many breast cancers depend on the steroid hormone estrogen whose actions are by the estrogen receptors ER α and ER β . Very little is known about the mechanisms by which estrogen promotes the formation of metastases. Recent results in our laboratory suggested the possibility that ER α might regulate the formation of E-cadherin-dependent adherens junctions by interacting with the cell adhesion proteins β -catenin and α -catenin. Consistent with this hypothesis, we found that in human MCF7 breast cancer cells ER α can be membrane-associated. Moreover, our preliminary analysis of stable MCF7 lines, which overexpress ER α mutants that can alter the association of ER α with mem-

branes, indicated that these lines display different cell-cell interactions. However, analysis of E-cadherin-dependent adherens junctions by indirect immunofluorescence and co-immunoprecipitation approaches argue against a direct remodeling of these junctional complexes by ER α . Instead our results suggest that the interaction of ER α with α -catenin homodimers may affect the switch between branched F-actin fibers and parallel F-actin bundles, which is associated with the formation of stable cell-cell interactions. We are in the process of determining whether binding of ER α to α -catenin alters the interaction of α -catenin homodimers with F-actin and whether this mechanism involves membrane-associated ER α . In summary, although our initial mechanism is likely not true, thus far our results are supportive of a novel non-nuclear activity of ER α in the regulation of cellular adhesions. We are in the process of summarizing a part of our results for publication.

Due to personnel issues we were unable to pursue the proposed studies until about half a year ago. We are grateful for the granted no-cost extension of this grant and will complete this project within the given time frame.

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