AD__________________

Award Number: DAMD17-02-1-0485

TITLE: Role of E-Cadherin Homophilic Contacts in the Inhibition of Cell Growth of Primary Breast Cells

PRINCIPAL INVESTIGATOR: Mirna Perez-Moreno, Ph.D.
Doctor Elaine Fuchs

CONTRACTING ORGANIZATION: The Rockefeller University
New York, New York 10021

REPORT DATE: August 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2004	Annual Summary (17 Jul 03 - 16 Jul 04)

4. TITLE AND SUBTITLE
Role of E-Cadherin Homophilic Contacts in the Inhibition of Cell Growth of Primary Breast Cells

5. FUNDING NUMBERS
DAMD17-02-1-0485

6. AUTHOR(S)
Mirna Perez-Moreno, Ph.D.
Doctor Elaine Fuchs

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Rockefeller University
New York, New York 10021

E-Mail: perezmm@rockefeller.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT	12b. DISTRIBUTION CODE
Approved for Public Release; Distribution Unlimited	

13. ABSTRACT (Maximum 200 Words)

At present, it remains obscure whether E-cadherin directly transfers growth inhibitory signals to the cells, or if other types of molecular cell interactions indirectly influenced by the establishment of cadherin mediated cell contacts, are responsible for contact inhibition of growth. In this study I selectively activate the formation of E-cadherin homophilic adhesive bonds, using a specific recombinant protein to engage E-cadherin molecules at the cell surface of primary epithelial cells. My previous data demonstrated that E-cadherin is capable of transducing a growth inhibitory signal independent of other cell-cell interactions. Moreover, my previous data also shows that this event is not mediated through a Wnt/β-catenin signaling antagonism. This has directed me to analyze the relationship between the association of $p120^{ctn}$ with E-cadherin and the inhibition of cell growth. We have successfully generated E-cadherinΔ120 transgenic mice, and p120 conditional KO mice in skin and mammary epithelia to study the consequences of the loss of the interaction of p120 with E-cadherin in vivo. In addition using a yeast-two hybrid screen I have identified novel interacting partners of p120ctn that can be potentially involved in transducing growth inhibitory signals to the cells.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Cell-cell interactions, regulation of cell proliferation, cell cycle, cell signaling, tumor suppressor gene	7
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

AWARD NUMBER: DAMD17-02-1-0485
TITLE: Role of E-cadherin homophilic contacts in the inhibition of cell growth of primary breast cells.
PRINCIPAL INVESTIGATOR: Mirna A. Perez-Moreno, Ph.D.
Postdoctoral Fellowship Award-Department of Defense USA

INTRODUCTION

Cadherins are the primary regulators of the overall state of epithelial cell contact and facilitate the establishment of other kind of cellular interactions. Cadherins have been postulated to be responsible for the phenomenon of contact inhibition of cell proliferation, but their exact roles in contact inhibition and possible mechanisms underlying their effect on cell growth are poorly understood.

Until now it has not been clear if E-cadherin by itself is able to transfer growth inhibitory signals to the cells or if the participation of other factors, that are indirectly influenced by the establishment of cadherin mediated cell contacts, are responsible for cell growth inhibition.

In this work, I am studying the direct role of E-cadherin in the generation of cell growth inhibitory signals to clarify this important issue. This will open new avenues to understand the normal behavior of the cells, and the mechanisms that could provoke the aberrant signals that induce aggressive cancer. Normal epithelial cells are being used in this study, in isolated manner to ensure the adhesion contacts are only mediated by E-cadherin. The results obtained will be the basis to identify new elements that are associated with tumor aggressiveness that could be useful for the design of directed therapeutic approaches.

BODY:

The statement of work for this grant is included below for reference. Our previous data have shown that the engagement of E-cadherin in homophilic adhesive bonds is capable of transducing a growth inhibitory signal independent of other cell-cell interactions (Task 1). Moreover, our previous data also shows that this event is not mediated through a Wnt/β-catenin signaling antagonism (Task 3). Thus, is likely that other cytoplasmic mediators of cadherin function may be responsible for the cell growth inhibition effect triggered by the specific engagement of E-cadherin.

E-cadherin function depends on its association with the cytoplasmic proteins known as catenins (α-,β-/plakoglobin, and $p120^{ctn}$). Since my previous findings demonstrated that E-cadherin transduce a growth inhibitory signal to the cells through a Wnt/β-catenin signaling independent mechanism, this has directed me to analyze the relationship between the association of $p120^{ctn}$ with E-cadherin and the inhibition of cell growth (Task 2).

$p120^{ctn}$ is frequently altered and/or lost in tumors of the colon, bladder, stomach, breast, prostate, lung and pancreas (Thoreson and Reynolds, 2002). In a study of invasive ductal carcinomas, p120 was completely lost in 10% of cases (Dillon et al., 1998). A second report on invasive breast carcinomas showed p120 loss in 10% of cases, 58% demonstrated heterogeneous expression and only 5% showed cytoplasmic staining (Nakopoulou et al., 2002). Recent identification and characterization of a p120-deficient colorectal cell line showed that p120 deficiency appears to result in strongly reduced levels of E-cadherin, which in turn leads to loosely organized cells that fail to maintain epithelial morphology (Ireton et al., 2002). Restoring p120 rescues the epithelial phenotype, and it has been postulated that that p120 may function as a tumor suppressor through its ability to stabilize and/or regulate E-cadherin (Davis et al, 2003).

In this work different approaches are being used to analyze the role of p120ctn in the inhibition of cell growth mediated by E-cadherin engagement. During this period of funding mouse genetics has been used to generate a conditional knock out in mammary epithelia and skin. This has provided an excellent opportunity to examine exclusively the consequences of p120 loss in vivo. The laboratory of Dr. Elaine Fuchs lab has a lot of expertise utilizing these kinds of approaches. This work is being carried out in collaboration with Dr. Albert Reynolds (Vanderbilt University, Nashville, Tennessee, USA).

In addition, transgenic mice expressing a mutant E-cadherin that contains point mutations that block its interaction with $p120^{ctn}$ have also been generated. The expression of this construct is being driven under the control of K14 promoter. This construct is expressed in both skin and mammary epithelia. In the last year of funding we have accomplished the most difficult step of obtaining these mice. There were several delays associated with problems with MSV infection in the p120ctn conditional KO mice and poor breeding conditions at Rockefeller University. These have now been successfully resolved and overall the project is in good shape and there are no major problems to report aside from the fact has taken longer we anticipated, but this kind of problems are relatively normal issues associated with this kind of work.

Initially I will be focusing in analyzing the consequences of the loss p120^{ctn} interaction with E-cadherin and the total loss of p120^{ctn} in skin. This will help me to assess the importance of this protein on an otherwise wild-type background, and to directly test the significance of the correlation between loss of this protein and characteristics typically associated with cancer. Based on the previous role for p120ctn in the stabilization of cadherin molecules we expect to observed noticeable phenotypic defects such as intense damage in skin. Changes in cell-cell junctions upon p120ctn ablation are anticipated and possible defects on hair follicle development are also expected. I will also analyze if there are any defects in epidermal differentiation and cellular hyperproliferation displayed in the epidermis. For the *in vitro* studies, cells will be isolated from skin and mammary epithelia from both p120^{ctn} knock out mice and E-cadherin-? p120 mice. Similar studies as those described in my previous annual report, such as measuring the percentage of cells in S-phase and cell proliferation after the specific engagement of E-cadherin on the cell surface, will be carried out to analyze if p120^{ctn} is responsible for cell growth inhibition. If the engagement of E cadherin in a homophilic adhesive bond is capable of transducing a growth inhibitory signal in these cells these results will suggest that p120^{ctn} could be responsible for mediating E-cadherin effect on cell contact inhibition. Further investigation will be needed to clarify the biological mechanism by which p120^{ctn} transduces the E-cadherin growth inhibitory signal.
For these purposes, a yeast two hybrid screen of newborn mouse skin cDNA was performed using two different regions of the p120^{ctn} molecules as baits (Matchmaker two-hybrid system 3, Clontech). One comprises the N-terminus of p120^{ctn} (1-480 aa) and the second one comprises the C-terminus (480-980aa) of the p120^{ctn} protein. Previously known interaction partners, including E-cadherin and Kaiso were identified. In addition we identified novel partners of p120^{ctn} that can be potentially involved in transducing growth inhibitory signals, among them the transcription factor AP2γ, and the Guanine nucleotide binding protein, alpha stimulating (Gα) were identified. To address whether these interactions are physiological relevant, p120^{ctn} null cells will be used to perform different assays in vitro.
The results that will be obtained using these different approaches will be invaluable for understanding the role of p120^{ctn} in cancer, and we are optimistic that our experiments will result in important progress in understanding the mechanisms underlying how E-cadherin engagement in homophilic adhesive bonds exerts its effects on cell growth.

STATEMENT OF WORK

Task 1. Analyze whether the engagement of E-cadherin in a homophilic adhesive bond is capable of transducing a growth inhibitory signal. (Months 1-6)

a. Purify the functionally active human E-cadherin protein from CHO transfected cells, as a chimeric protein with the Ig Fc domain at the C-terminus of the E-cadherin ectodomain.

b. Using beads coated with the functionally active Fc-E-cadherin, cover the surface of primary breast cells grown at subconfluence to avoid cell-cell interactions. Initially cells grown under growth stimulatory conditions will be analyzed. Assays will be performed to ascertain whether E-cadherin homophilic contacts generate a growth inhibitory signal, such as proliferation assays and the determination of the cells entered into the S-phase by BrdU labeling. Beads coated with other surface proteins or antibodies directed against them such as N-CAM, class I HLA proteins, Fc receptor, or Na^+/K^+ ATPase β subunit will be included as controls for ligand specificity.

Task 2. Identify the regions of E-cadherin cytoplasmic domain that are responsible for cell growth inhibition. (Months 18-36)

a. A series of plasmids expressing chimeric or mutant proteins containing different regions of the E-cadherin cytoplasmic domain will be used. The fact that the adhesive function of E-cadherin depends on its association with the cytoplasmic proteins known as catenins α–, β–/plakoglobin, and p120^{ctn} catenin) will direct us to analyze, as a first step, the relationship between the association of these proteins and the inhibition of cell growth mediated by E-cadherin. $p120^{ctn}$ and β–catenin bind directly to E-cadherin. α – catenin, as well, acts as bridge connecting E-cadherin to β – catenin, which in turn associates with actin filaments, directly o via α – actinin or vinculin proteins.

b. Initially, I will use some constructs lacking the β– catenin binding site, including a construct that lacks the complete β–catenin binding site and an E-cadherin-α catenin fusion construct deleted in the βcatenin binding region of both proteins. This is because of β–catenin, in addition to its role in cell adhesion, has also a role as a mediator of the Wnt transduction pathway, being imported into the nucleus with LEF/TCF transcription factors modulating the expression of certain genes. The fact that some of these genes are implicated in cell growth control raises the possibility that E-cadherin could transduce signals to the nucleus through Wnt/β–catenin signaling.

c. To express the human E-cadherin cDNA constructs, breast cell lines that lack E - cadherin expression will be used to avoid the difficulties that primary cells exhibit to transfection and to avoid the participation of the endogenous E-cadherin protein. The MDA-MBA-231 breast cancer cell line is a good candidate because it exhibits a reproducible growth response to expression of wild type E-cadherin. Transfected and drug selected clones will be tested for their capacity to express E-cadherin mutant proteins and for its functional adhesive activity using cell adhesion assays.

d. A similar approach as described in the *Task 1*, part b. will be used to determine the cytoplasmic regions of E-cadherin responsible for cell growth inhibition.

e. If our data suggest that the β–catenin binding region of the E-cadherin cytoplasmic domain contains the information to inhibit cell growth, the following steps will used to analyze the β–catenin signaling activity in this event (see *Task 3*). A failure to observe any involvement of the β–catenin binding region in the inhibition of cell growth mediated by E-cadherin homophilic contacts, will suggest that other E-cadherin binding proteins such as $p120^{ctn}$ are involved. To investigate this possibility, a mutant construct that contains a point mutation that block the interaction of E-cadherin with $p120^{ctn}$ would be used. If the $p120^{ctn}$ protein seems not to be involved in growth inhibition, we will conclude that E-cadherin probably interacts with other growth related signals in the cells. A detailed description of the analysis of such other signals is beyond the scope of this proposal, but some directions could include the analysis of the roles of the MAPK, PKC or Ras pathways.

Task 3. To identify whether the growth inhibitory signal mediated by E-cadherin homophilic contacts is mediated through Wnt/β–catenin signaling antagonism, the following experiments will be carried out: (Months 6-18)

a. Determine if the attachment of the E-cadherin in homophilic contacts influence the Wnt/β–catenin signaling activity. It is known that as a final step of the Wnt pathway, β–catenin interacts with LEF/TCF-type transcription factors, being imported into the nucleus activating the transcription of certain genes. To measure the signaling activity of β–catenin, a b- catenin/TCF dependent reporter gene assays (Top/Fop-flash reporter assay) will be used. Breast cell lines will be used for this experiment to avoid the difficulties that primary cells exhibit to transfection.

b. Determine if the growth inhibitory signal of E-cadherin mediated contacts is reverted by constitutively active forms of TCF. As control for specificity wild type or dominant negative forms of TCF will be co-transfected and its effect on the E-cadherin mediated cell growth inhibition will be tested.

c. If there appears to be no involvement of β – catenin signaling activity in the inhibition of cell growth, but the growth inhibitory activity remains associated with the β–catenin binding region of E-cadherin, it is possible that other proteins that bind to the cytoplasmic tail of E-cadherin via β – catenin, such as α-catenin, are responsible for this event. To assess this possibility, α – catenin negative cell lines will be used, such as the MDA-MB-468 breast cancer cell line, which maintains the expression of E-cadherin and β–catenin proteins. After the reintroduction of α – catenin, a similar approach as described in the *Task 1*, part b. will be used to determine the involvement of α – catenin in the cell growth inhibition mediated by E-cadherin contacts. A series of plasmids expressing mutant α – catenin proteins (including a construct that lacks the β–catenin binding site, a construct that lacks the actin binding site and a construct that lacks both protein binding sites) will be used as a control.

KEY RESEARCH ACCOMPLISHMENTS

- Generation of both E-cadherinΔp120 transgenic mice, and p120 conditional KO mice in skin and mammary epithelia.
- Identification of novel interacting partners of p120ctn that can be potentially involved in transducing growth inhibitory signals such as the transcription factor AP2γ and the Guanine nucleotide binding protein, alpha stimulating (Gα).

REPORTABLE OUTCOMES and CONCLUSIONS

Even though I do not have yet a reportable outcome in the form of a completed manuscript, we now have generated different mice models that will allow us to test whether p120^{ctn} is involved in the inhibition of cell growth mediated by E-cadherin homophilic contacts. We are now in an excellent position to complete the experiments necessary to understand the role of p120ctn in this process and the results obtained in the next period of funding will result in important progress in understanding how cadherins are responsible for the phenomenon of contact inhibition of cell proliferation.

REFERENCES

Davis, M., Ireton, I. and Reynolds, A. 2003. A core function for p120-catenin in cadherin turnover. J Cell Biol *163*, 525-534.

Dillon, D.A., D'Aquila, T., Reynolds, A.B., Fearon, E.R., Rimm, D.L. 1998. The expression of p120ctn protein in breast cancer is independent of alpha- and beta-catenin and E-cadherin. Am J Pathol *152*, 75-82.

Ireton, R.C., Davis, M., van Hengel, J., Mariner, D., Barnes, D., Barnes, K., Thoreson, M., Anastasiadis, P., Matrisian, L., Bundy, L., Sealy, L., Gilbert, B., van Roy, F., Reynolds, A. 2002. A novel role for p120 catenin in E-cadherin function. J Cell Biol *159*, 465-476.

Nakopoulou, L., Gakiopoulou-Givalou, H., Karayiannakis, A.J., Giannopoulou, I., Keramopoulos, A., Davaris, P., Pignatelli, M. 2002. Abnormal alpha-catenin expression in invasive breast cancer correlates with poor patient survival. Histopathology *40*, 536-546.

Thoreson, M.A. and Reynolds, A.B. (2002). Altered expression of the catenin p120 in human cancer: implications for tumor progression. Differentiation *70*, 583-589.