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Award Number: DAMD17-99-1-9169

TITLE: The Involvement of Human Cyr61 in Heregulin Induction of Breast Tumor Progression

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REPORT DATE: August 2000

TYPE OF REPORT: Annual Summary

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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20010102 145

DTIC QUALITY INSPECTED 4

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)

2. REPORT DATE
August 2000

3. REPORT TYPE AND DATES COVERED
Annual Summary (1 Aug 99 - 31 Jul 00)

4. TITLE AND SUBTITLE
The Involvement of Human Cyr61 in Heregulin Induction of Breast Tumor Progression

5. FUNDING NUMBERS
DAMD17-99-1-9169

6. AUTHOR(S)
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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
This report contains colored photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Cyr61, a member of a newly identified CCN gene family, was isolated and identified by differential expression between estrogen receptor (ER)-positive and ER-negative breast cancer cells. Cyr61 is a ligand for the integrin $\alpha v\beta 3$, which is involved in tumorigenesis and angiogenesis (formation of new blood vessels). We showed previously that expression of Cyr61 in HRG-transfected MCF-7 cells is greatly increased compared to parental MCF-7 cells. We also showed that Cyr61 is expressed in all the invasive, metastatic, HRG-expressing, and ER-negative breast cancer cell lines. Moreover, Cyr61 was detected in about 30% of invasive human breast tumor biopsies. Most significantly, an anti-Cyr61 blocking antibody abolishes the invasiveness and migration of HRG-expressing breast cancer cells *in vitro*. To understand the role of Cyr61 in breast cancer progression, the human Cyr61 cDNA was introduced to ER-negative, HRG-negative breast cancer cells. In addition, Cyr61-expressing cells showed a growth advantage in serum deplated conditions. The preliminary results suggest that Cyr61 is sufficient to promote estrogen independence and anti-estrogen resistance of breast cancer cells. Further *in vitro* and *in vivo* characterization of Cyr61-expressing breast cancer cells will demonstrate whether Cyr61 is a key regulator or not in breast cancer growth and progression.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
11

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

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Introduction

Most estrogen (E2)-dependent and anti-estrogen responsive breast tumors spontaneously progress to an E2-independent and anti-estrogen-resistant phenotype, becoming deadly metastatic diseases. The mechanism by which breast cancer appears to progress from an E2-dependent to an E2-independent phenotype is still under investigation. We have shown that expression of heregulin (HRG), an activator of *erbB*-2/3/4 receptor signaling pathways, is closely associated with an invasive breast cancer phenotype. Furthermore, we demonstrated that HRG induces breast cancer progression, as determined by loss of estrogen receptor (ER) function and response, tumorigenicity, invasion, and metastasis. It is possible that HRG induces activation of the *erbB* signaling pathways, leading to regulation of downstream genes that regulate and control cancer progression. With this in mind, we isolated and identified the human homologue of a mouse immediate-early response gene, Cyr61, differentially expressed in ER-negative, HRG-positive breast cancer cells. Cyr61 is a secreted cysteine-rich protein that is associated with the cell surface and the extracellular matrix (ECM). Cyr61 mediates cell adhesion, migration, and angiogenesis. We have shown that Cyr61 is co-expressed with HRG in all the metastatic breast cancer cell lines tested, its expression is inversely correlated with ER expression, and it is associated with HRG-induced breast cancer chemomigration and metastasis, possibly through interactions with the $\alpha v\beta 3$ integrin receptor. Furthermore, we have established that Cyr61 was expressed in about 30% of invasive breast cancer tumor biopsies, implying a possible role of Cyr61 in breast cancer progression. The goal of the proposed studies is to determine the involvement of Cyr61 in breast cancer progression. I will test whether expression of Cyr61 is necessary and/or sufficient for HRG-induction of breast cancer progression, whether blocking of Cyr61 expression reverts aggressive phenotypes of breast cancer, and whether modulation of Cyr61 expression affects integrin signaling, leading to the acquisition of breast cancer progression.

Body

Task 1 in the proposed study is to determine whether expression of Cyr61 is necessary and/or sufficient to confer E2-independent and anti-estrogen resistant phenotypes on ER-positive breast epithelial cells in months 1-12. A constitutive expression vector for human Cyr61 cDNA (pBK.CMV/Cyr61) was generated and confirmed by restriction enzymatic mapping and sequencing analysis. The Cyr61 expression construct or the control vector were co-transfected with pcDNA3.1/zeo, which carries a zeocine-resistant gene, into an ER^+, HRG^- breast cancer cell line, MCF-7, by electroporation. Stable clones were selected in the presence of zeocin for 3 weeks. Ten vector control clones and twenty-five Cyr61-transefected clones were isolated.

Total RNA was isolated and purified from both vector- and Cyr61-transfected clones, and expression of Cyr61 mRNA was analyzed by RNAse protection assays in months 6-7. The representative results were shown in Figure 1. Cyr61 expression was observed in 21 out of 25 clones at various levels. Expression of Cyr61 is about 5- to 20-fold increased as compared to the vector control clones, which express undetectable or very low levels of Cyr61 mRNA, even in the presence of regular FBS media. Expression of the Cyr61 protein in the stable lines is currently underway. Since Cyr61 is a secreted protein, to examine the expression of the Cyr61 protein, conditioned media (250 ml) have been collected from individual clones, and will be purified by heparin column chromatography, and analyzed by Western blotting using a specific anti-Cyr61 rabbit polyclonal antibody.

In vitro characterization of Cyr61-transfectants has been initiated during months 9-12. To examine E2 dependence and anti-estrogen resistance of Cyr61-expressing MCF-7 clones, anchorage-dependent and anchorage-independent growth assays were performed. MCF-7/Cyr61 cells were cultured in charcoal-stripped fetal bovine serum (FBS) media for 4 days and treated in the presence of estrogen, anti-estrogens (tamoxifen and ICI), and combinations of estrogen and

either anti-estrogen. As shown in Figures 2 and 3, wild type- or vector-transfected MCF-7 was dependent on estrogen for growth and was sensitive to anti-estrogen treatment. However, MCF-7/Cyr61 cells became E2-independent and anti-estrogen-resistant for growth in both anchorage-dependent and anchorage-independent growth assays.

Further *in vitro* characterization of Cyr61-expressing clones, including Boyden chamber assay, matrigel outgrowth assay, and steroid hormone receptor assay, zymogram assays of metallomatrix protease, will be performed. Clones that show changes of migration and invasiveness *in vitro* will be inoculated into athymic nude mice to examine tumor formation and metastatic potential *in vivo.*

The original attempt to use tetracycline-inducible expression system for controlling expression level of Cyr61 was tested but without much success. Since MCF-7 cells expressing various levels of Cyr61, at least, at the RNA level, were obtained using a constitutive expression vector, it is possible to compare phenotypic changes among different Cyr61-expressing clones.

Next task in the proposal is to determine whether blockage of Cyr61 expression restores E2-responsiveness and/or results in loss of metastatic phenotypes of aggressive breast cancer cells in months 10-36. Three different approaches were proposed to block Cyr61 expression. The progress is described as following: (1) A constitutive expression vector for anti-sense Cyr61 cDNA (pcDNA3.1/zeo/AS-Cyr61) was constructed and will be introduced to ER-, HRG+, invasive, and metastatic breast cancer cell lines, such as MDA-MB-231, MDA-MB-435, and MCF-7/HRG (clones T2, T7, and T8). (2) The anti-sense Cyr61 oligonucleotides were synthesized and will be tested in cultures. (3) Oligonucleotides to generate ribozymes specific to Cyr61 were designed. The ribozymes will be constructed and characterized once the oligonucleotides are synthesized.

This first year training period of the DOD BCRP post-doctoral traineeship has allowed me to develop and master essential skills and techniques in endocrinology, biochemistry, cell biology, and molecular biology required for cancer research. It is evident in the research accomplishments described above. In addition, it has offered me with tremendous opportunities not only to establish extensive collaborations with breast cancer researchers, but also to interact with breast cancer advocates and survivors to guide our research direction timely to meet their needs. With the support from DOD, the proposed project should enhance our understanding at both the cellular and molecular levels of breast cancer progression, and should help developing Cyr61-targeted therapies to halt tumor progression and/or metastasis.

Figures

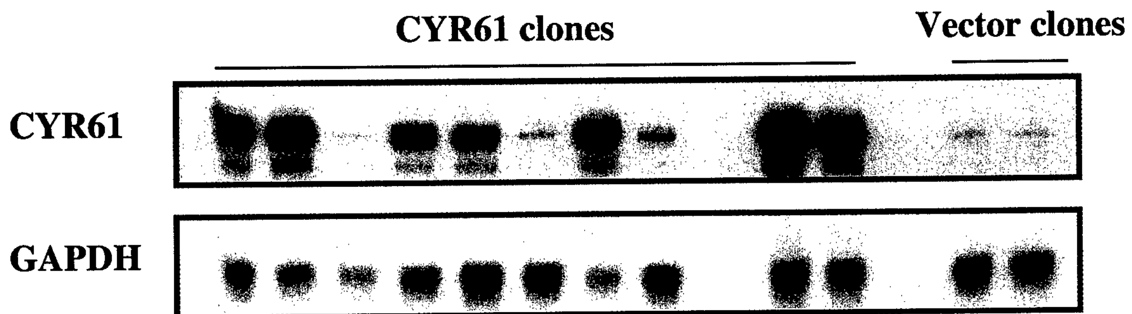

Figure 1. Expression of Cyr61 mRNA in MCF-7 clones by RNase protection assay. Total RNA was isolated from vector- and Cyr61-transfected MCF-7 cells. Thirty micrograms of total RNA was used for RNase protection assay using a specific anit-sense Cyr61 riboprobe. GAPDH is a house keeping gene and used as an internal control for RNA loading.

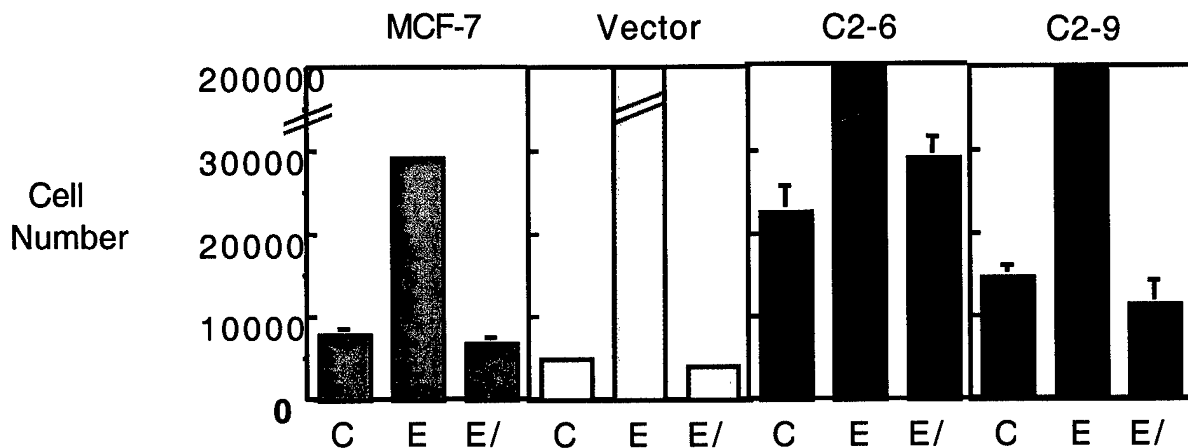

Figure 2. Expression of Cyr61 results in loss of estrogen dependence and anti-estrogen sensitivity in MCF-7 cells. Vector- and Cyr61-transfected MCF-7 as well as wild-type MCF-7 cells were pre-treated with charcoal-stripped FBS media for 4 days and plated (5,000 cells/well) in triplicate in 24-well plates. Cells were treated in the presence of estrogen or the combination of estrogen and anti-estrogen (ICI). Cell number was counted at day 10. Two representative clones were shown. Similar results were obtained from at least three experiments.

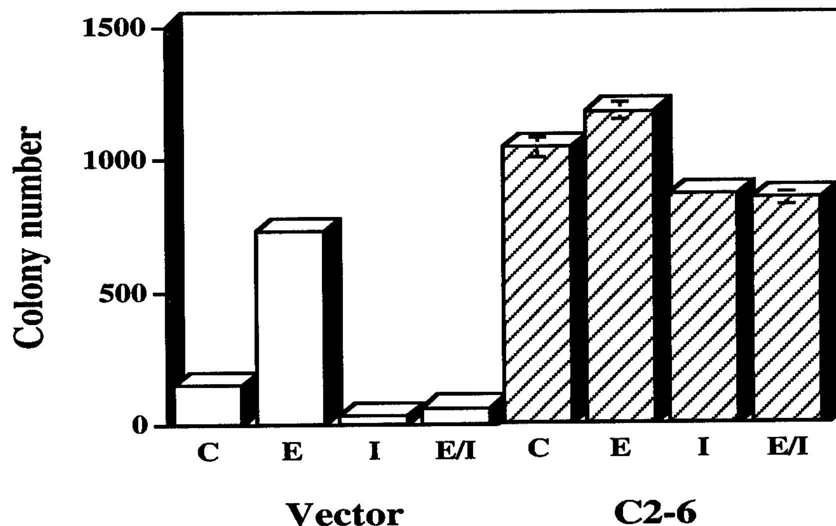

Figure 3. Expression of Cyr61 induces estrogen-independent and anti-estrogen-resistant colony formation of MCF-7 cells. Vector- and Cyr61-transfected MCF-7 cells were pre-treated with charcoal-stripped FBS media for 4 days. Cells (20,000 cells/well) in 0.35% agar solution were plated onto a 0.6% of bottom agar layer in triplicate in 6-well plates with treatments as indicated. C, control; E, estrogen (10^{-9}M); I, ICI (10^{-7}M); and E/I, combination of estrogen and ICI. Colony number was counted at day 21. Data of one representative clone, C2-6, were shown here. Similar results were obtained from at least four different experiments.

Appendices

Key research accomplishments

1. A constitutive expression vector for human Cyr61 cDNA was generated.
2. Stable lines of MCF-7 cells expressing Cyr61 (designated as MCF-7/Cyr61) were established.
3. Cyr61 expression in the stable clones was confirmed by RNAse protection assay.
4. Initial *in vitro* characterization of MCF-7/Cyr61 clones was performed, including anchorage-dependent growth assays, and anchorage-independent soft agar colony formation assays.
5. A constitutive expression vector for anti-sense Cyr61 cDNA was made.
6. Anti-sense Cyr61 oligonucleotides were synthesized.

Abstracts/Presentations

1. **Tsai, M.-S.** 2000. Involvement of Cyr61, a ligand for an integrin, in breast cancer progression. Department of Cell and Molecular Biology, Lawrence Berkeley National Laboratory. (oral presentation)

1. **Tsai, M.-S.**, Bogart, D., and Lupu, R. 2000. Expression of Cyr61, a ligand for integrin $\alpha v\beta 3$, modulates breast cancer progression. Abstract of the 91st Annual Meeting of the American Association for Cancer Research, San Francisco, CA. (oral presentation)

Department of Cell and Molecular Biology, Lawrence Berkeley National Laboratory. (oral presentation)

Title: Involvement of Cyr61, a ligand for an integrin, in breast cancer progression.

Tsai, M.-S. and Lupu, R.

Abstract:

In searching for differentially expressed genes between estrogen receptor (ER)-positive (ER^+), non-metastatic and ER^-, metastatic breast cancer cells, the human homologue of a murine immediate-early gene, Cyr61, was isolated and identified. Cyr61 is a ligand for the $\alpha v\beta 3$ integrin receptor. We demonstrated that expression of Cyr61 in HRG-transfected MCF-7 cells is greatly increased (5- to 20-fold) compared to parental MCF-7 cells, in which Cyr61 is nearly undetectable. Most recently, we have shown that Cyr61 is expressed in all the invasive, metastatic, HRG- expressing and ER^- breast cancer cell lines and highly expressed in about 30% of invasive human breast tumor biopsies tested. Most significantly, an anti-Cyr61 blocking antibody abolishes the invasiveness and migration of HRG-expressing breast cancer cell lines and HRG-transfected MCF-7 *in vitro.* We also showed that HRG-transfected MCF-7 expresses higher levels of the $\alpha v\beta 3$ integrin than parental MCF-7, indicating that these cells acquire angiogenic characteristics. Coincidently, the $\alpha v\beta 3$ integrin has been shown to be an angiogenic integrin and a prognostic indicator for breast carcinomas. In conclusion, our results suggest that Cyr61 may be involved in HRG-induced breast cancer progression.

91st Annual Meeting of the American Association for Cancer Research, San Francisco, CA. (oral presentation)

Abstract number: #463

Title: Expression of Cyr61, a ligand for integrin αvβ3, modulates breast cancer progression. Tsai, M.-S., Bogart, D., and Lupu, R. Lawrence Berkeley National Laboratory, University of California, Berkeley, CA 94720.

Abstract:

We have previously identified the human homologue of a murine immediate-early gene, Cyr61, which is differentially expressed in the invasive, metastatic, estrogen receptor-negative (ER-), heregulin-overexpressing breast cancer cells and in some breast tumors. Cyr61, a ligand for the αvβ3 integrin, has been shown to mediate cell adhesion, migration, extracellular matrix signaling, and neovascularization. We have demonstrated that blocking of Cyr61 inhibited chemoinvasiveness and chemomigration of aggressive breast cancer cells. To investigate whether Cyr61 plays a role in breast cancer progression, we introduced the human Cyr61 cDNA into the ER+ MCF-7 cells. We demonstrated that expression of Cyr61 results in the progression of MCF-7 cells from an estrogen-dependent to –independent phenotype. The Cyr61-expressing MCF-7 cells (MCF-7/Cyr61) showed estrogen dependent. Moreover, MCF-7/Cyr61 cells showed a growth advantage in serum depleted conditions. A 5-10 fold increase in cell proliferation was observed in MCF-7/Cyr61 as compared to vector control cells. Furthermore, the Cyr61 clones exhibited a characteristic invasive outgrowth pattern in Matrigel. In contrast, the Matrigel outgrowth of vector control cells was rarely apparent even in the presence of estrogen. Overall, these in vitro studies suggest that Cyr61 may be a key regulator of breast tumor growth and progression, perhaps through activation of the integrin pathway. Our results should provide new insights into the role of Cyr61 and the mechanism by which breast cancer cells acquire the aggressive, hormone-independent phenotypes.