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Award Number: DAMD17-99-1-9298

TITLE: A Novel Approach to Increase Breast Cancer Cell  
Chemosensitivity: Disruption of the Anti-Apoptotic Function of  
Translation Factor eIF4E

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REPORT DATE: October 2001

TYPE OF REPORT: Annual

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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20020719 096

# 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

<b>1. AGENCY USE ONLY (Leave blank)</b>		<b>2. REPORT DATE</b> October 2001	<b>3. REPORT TYPE AND DATES COVERED</b> Annual (1 Oct 00 - 30 Sep 01)	
<b>4. TITLE AND SUBTITLE</b> A Novel Approach to Increase Breast Cancer Cell Chemosensitivity: Disruption of the Anti-Apoptotic Function of Translation Factor eIF4E			<b>5. FUNDING NUMBERS</b> DAMD17-99-1-9298	
<b>6. AUTHOR(S)</b> Vitaly A. Polunovsky, Ph.D.				
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> University of Minnesota Minneapolis, Minnesota 55415-1226  E-Mail: polun001@tc.umn.edu			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b>				
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited				<b>12b. DISTRIBUTION CODE</b>
<b>13. ABSTRACT (Maximum 200 Words)</b> In this report we present data in support of <b>Aim 2</b> of our project. Utilizing human breast carcinoma cell lines as an in vitro model for naturally occurring malignancy, we demonstrated that enforced overexpression of translation factor eIF4E in human mammary epithelial cells (HMECs) confers to them a transformed phenotype and protects against apoptotic death. In accord with this, suppression of eIF4E-driven translation by ectopic expression translational repressor 4E-BP1 stimulated apoptosis and abrogated chemoresistance breast carcinoma cells in a manner dependent on the phosphorylation status of ectopic 4E-BP1. Our data show that targeted disruption of cap-dependent translational machinery selectively sensitizes breast carcinoma cells to the subset anti-cancer drugs that includes camptothecin and doxorubicin. These drugs are considered as promising candidates for collaboration with anti-eIF4E pre-treatments in suppression of breast tumor growth. Based on these data, we plan to employ novel pharmacological approaches including disruption of eIF4E-to-cap association by phosphoramidated analogues of the 5' methylguanosine cap of mRNA.				
<b>14. SUBJECT TERMS</b> Breast Cancer, translational control, apoptosis, chemotherapy agents			<b>15. NUMBER OF PAGES</b> 13	
			<b>16. PRICE CODE</b>	
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited	

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## Introduction

Eukaryotic translation initiation factor 4E (eIF4E) is the mRNA cap binding protein which functions during translation of cellular mRNAs possessing the 5' cap structure. Overexpressed eIF4E suppresses oncogene-dependent apoptosis, causes malignant transformation and leads to multi-drug resistance. The function of eIF4E is negatively regulated by members of the family of translational repressors, the eIF4E-binding proteins 4E-BPs. When hypophosphorylated, 4E-BPs block cap-dependent translation by sequestering eIF4E in a translationally inactive complex. Upon hyperphosphorylation in response to hormones or growth factors, the 4E-BPs and eIF4E dissociate allowing eIF4E to form an active translation initiation complex. Previously, we demonstrated that 4E-BP1 sensitizes fibroblasts to apoptosis and suppresses Ras-dependent tumorigenicity in a manner strictly dependent on its ability to sequester eIF4E from a translationally active complex with eIF4G. The objective of this awarded project is to experimentally test the idea that targeted disruption of the anti-apoptotic function of eIF4E can sensitize breast carcinoma cells to therapeutic doses of a non-genotoxic cytostatic agent such as lovastatin and/or to low concentrations of genotoxic agents. We also propose to develop treatment strategies which will include disruption of the anti-apoptotic function of eIF4E in a combination with treatments with non-toxic doses of lovastatin and conventional anti-neoplastic agents. Here we present data in support of Aim 1 (Task 1) of our project.

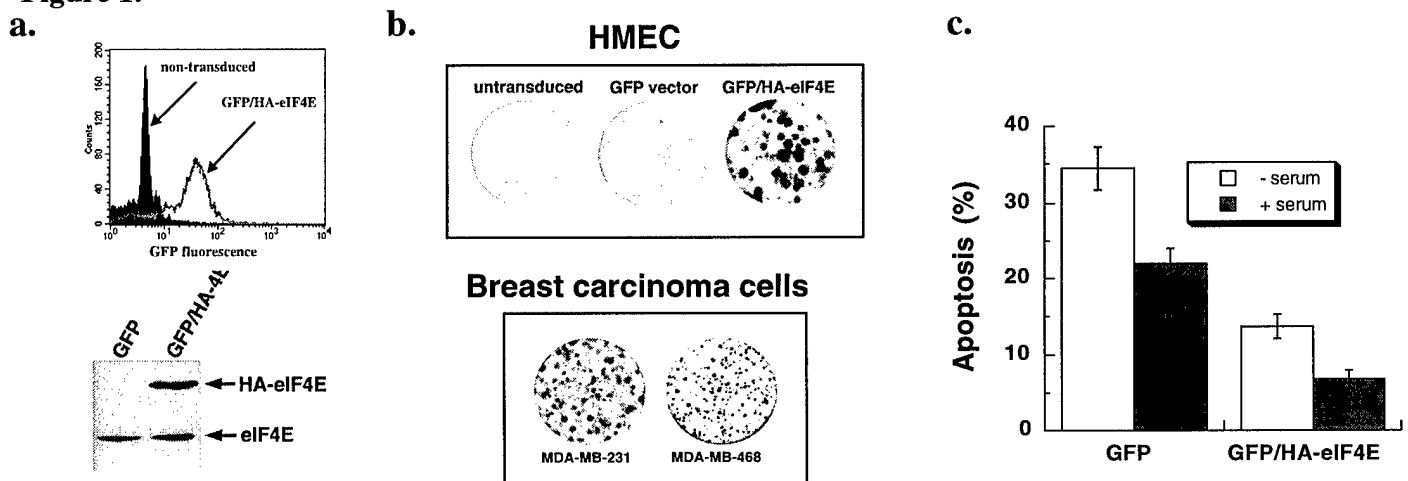
**Aim 2. *In vitro*, develop a novel protocol(s) for breast carcinoma treatment that will include pharmacological inhibitors of eIF4E activity .**

- A. Determine conditions for anti-eIF4E pre-treatment(s) to sensitize breast cancer cells to drug-induced apoptosis.
- B. Develop optimal regimens for treatment of pre-sensitized breast cancer cells by lovastatin alone and in combination with genotoxic cytostatic agents

**Experimental Data in Support of Aim 2:**

1. Enforced overexpression of eIF4E converts human mammary epithelial cells (HMECs) into transformed cells that are resistant to apoptosis. Our reported data in support of Aim 1 suggest that cells from breast carcinoma lines function in a translationally active state (Annual Report 2000). To detect whether anti-eIF4E interventions are sufficiently potent to decrease viability and tumorigenicity of breast cancer cells, we first aimed to detect whether gain of eIF4E function in HMECs confers to them a transformed phenotype and protects against apoptotic death. Overexpressed eIF4E was shown to transform immortalized rodent fibroblasts (Lazaris-Karatzas et al., 1990) and increases their resistance to apoptosis (Polunovsky et al., 1996; Tan et al., 2000). It is however unclear whether increased overexpressed eIF4E is able to transform breast epithelial cells and rescue from apoptotic death. To examine the effects of abnormally overexpressed eIF4E on growth and viability of non-transformed mammary epithelial cells, immortalized HMECs (line 184-A1, Berkeley National Laboratory) were infected with replication-defective retrovirus generated from the MSCV-M1GR1 retroviral construct containing sequences encoding GFP and HA-tagged human eIF4E. GFP-positive cells were sorted by using FACScan (Becton Dickinson) and 400 transduced or non-transduced HMECs were plated into 60 mm wells of 6-well clusters to detect their ability to form transformed foci in vitro. The same amounts of breast carcinoma cells were plated into 60 mm wells as a control for malignant cell growth.

**Figure 1.**



**Figure 1. Introduction of HA-eIF4E into HMECs stimulates colony formation and rescues from apoptosis.**

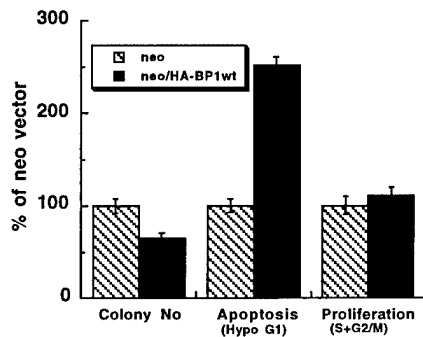
(a) GFP fluorescence (top) and steady state levels of endogenous and HA-tagged eIF4E (bottom) in infected and non-infected HMECs. (b) Photo of representative wells at 20th day after plating. (c) Flow cytometric analysis apoptotic frequencies in HA-eIF4E expressing or mock transduced HMECs in the presence or absence of serum

About 95 % of sorted cells expressed HA-tagged exogenous eIF4E (Figure 1a). The data of Figures 1b and 1c show that enforced overexpression of eIF4E confers non-transformed HMECs the ability to form transformed foci in vitro (b) and that increased eIF4E can substitute for serum survival factors in preventing spontaneous apoptosis (c). These results suggests that **introduction of exogenous eIF4E confers HMECs some aspects of transformed phenotype including the ability to form colonies in vitro and increased self-sufficiency for suppression of intrinsic apoptotic machinery.**

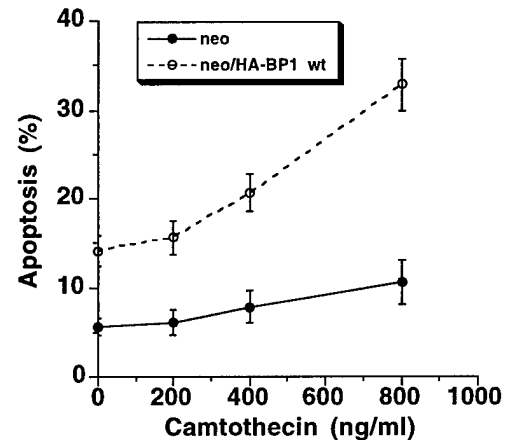
2. Translational repressor 4E-BP1 suppresses colony formation in MDA-MB-231 breast carcinoma cells and cooperates with camptothecin in inducing apoptotic death. The function of eIF4E is inhibited by members of the family of translational repressors, the eIF4E binding proteins (4E-BPs, also known as PHAS) (Gingras et al., 2000a). When hypophosphorylated, 4E-BPs compete with eIF4G for binding to eIF4E and sequester eIF4E in a non-functional complex. Upon hyperphosphorylation, 4E-BPs dissociate from the complex with eIF4E allowing it to form an active translation initiation complex. To detect whether 4E-BP1 decreases viability and chemoresistance of breast cancer cells, MDA-MB-231 breast carcinoma cells were transfected with a vector pACTAG-2 engineered to encode a *neo* resistance gene cassette and haemagglutinin (HA) tagged human wt 4E-BP1 (Gingras et al., 1999b). Transfected cells were incubated in the presence of G418 and numbers of neomycin resistant colonies were scored after three weeks.

**Figure 2.**

**a.**



**b.**



**Figure 2.** Ectopic 4E-BP1 decreases viability and chemoresistance in MDA-MB-231 breast carcinoma cells. (a) Quantitative analysis of colony forming ability, apoptosis, and cell proliferation in transfected cells. (b) Dose-dependent effect of camptothecin on apoptosis.

The results revealed decreased capacity to form colonies in cells transfected with 4E-BP1 as compared to the cells transfected with the empty neo vector (Fig. 2a). The reduction of colony formation observed could have resulted from inhibition of cell cycle transit, activation of cell death, or both. Flow cytometric analysis of HA-4E-BP1 transfected cells revealed no changes in the S + G2/M fraction of cycling cells, whereas the fraction of hypodiploid apoptotic cells was significantly increased (2b). 4E-BP1 cooperated with anti-tumor agents camptothecin (Fig 2c) and doxorubicin (data not shown) in induction of apoptosis. However, it was only marginally effective in promoting the proapoptotic function of lovastatin or paclitaxel. **Thus, these data show that enforced overexpression of wild type 4E-BP1 in breast cancer cells is associated with increased cell susceptibility to apoptosis. They also suggest that 4E-BP1-induced repression of cap-dependent translation selectively sensitizes breast cancer cells to anti-cancer drugs and that camptothecin and doxorubicin are most promising partners of anti-eIF4E interventions in anti-tumor therapy.**

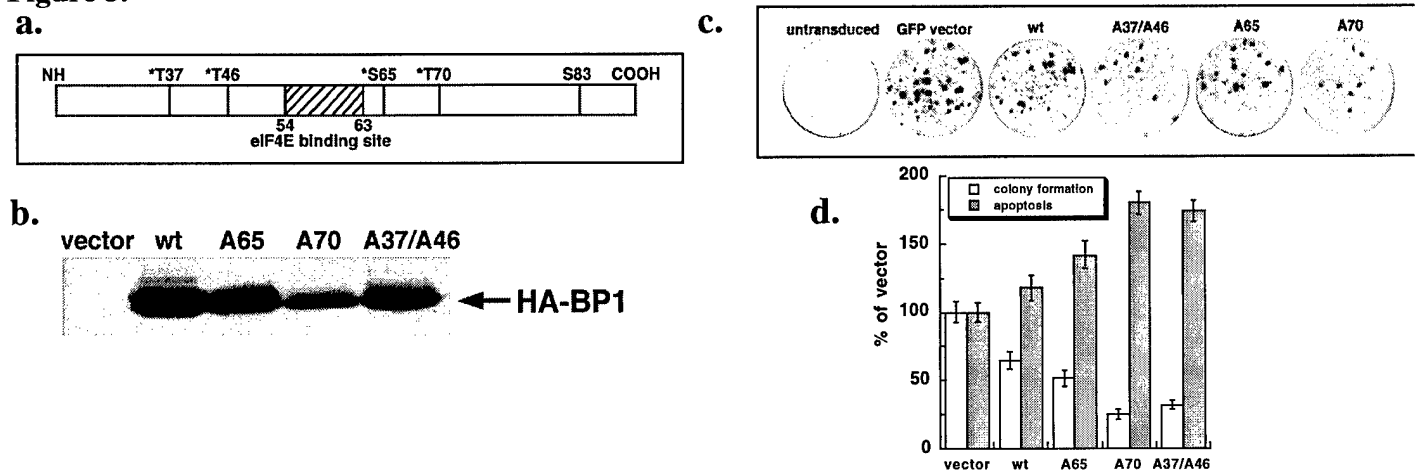
3. Dephosphorylation of 4E-BP1 as an important condition for optimization of the anti-eIF4E pre-treatment. Results of our experiments show that breast carcinoma cells reveal enhanced degree of 4E-BP1 phosphorylation compared to normal HMECs (DOD Grant Annual Report 2000). Based on this, we proposed that the 4E-BP1-mediated negative control of cap-dependent translation is relaxed or impaired in course of breast tumorigenesis. We recently demonstrated (Polunovsky et al., 2000) that enforced dephosphorylation of 4E-BP1 in Ras transformed fibroblasts significantly increases its proapoptotic function. We therefore aimed to examine whether the phosphorylation status of 4E-BP1 governs its proapoptotic potency in breast cancer cells. Hypophosphorylation of 4E-BP1 can be accomplished in at least two ways (Raught et al., 2000): (i) pharmacologically, by treatment with rapamycin, which blocks the final stage in the kinase cascade leading to phosphorylation of 4E-BP1; (ii) genetically, by mutating the 4E-BP1 phosphorylation sites followed by ectopic expression of mutants in tested cells.

**3A. Rapamycin do not activates apoptosis in MDA-MB-231.** In sharp contrast to findings obtained in experiments with mouse fibroblasts (Polunovsky et al., 2000, Lee et al, submitted), rapamycin partially

suppressed 4E-BP1 phosphorylation but did not stimulate drug-induced apoptosis in breast carcinoma cell lines with diverse genetic background (data not shown). Moreover, rapamycin was unable to potentiate the pro-apoptotic effect of ectopically overexpressed wild type or mutant 4E-BP1. **These results indicate that in contrast to transformed fibroblasts, breast carcinoma cells are resistant to rapamycin-induced metabolic alterations leading to apoptosis.**

**3B. Mutating phosphorylation sites increases the pro-apoptotic potency of exogenous 4E-BP1.** To comprehensively examine the impact of 4E-BP1 phosphorylation state on growth of malignant cells, we quantified the colony forming efficiency of MDA-MB-231 breast carcinoma cells transfected with a neomycin resistance cassette bearing sequences for wild type 4E-BP1 or Ser/Thr ((S/T) to Ala (A) mutants at the phosphorylation sites indicated (Fig.3a). We employed three mutant forms of 4E-BP1: one double mutant (A37/A46) to eliminate phosphorylation N-terminal to the eIF4E binding site, and two single mutants A65 and A70 at phosphorylation sites C-terminal to the eIF4E binding site. To detect any potential experimental bias resulting from systematic differences in ectopic wild type or mutant 4E-BP1 expression due to self repression (i.e. negative feedback from the ectopic protein itself leading to decreased translation of ectopic and/or endogenous 4E-BP1 despite identical gene transfer efficiency), cells were transiently transfected in parallel with a pACTAneo vector encoding wild type or mutant HA-tagged 4E-BP1 using identical procedures in four independent experiments. Immunoblot analysis of 4E-BP1 expression showed similarity for endogenous 4E-BP1 and all forms of HA-tagged 4E-BP1 except cells transfected with A70. For this mutant, endogenous and exogenous 4E-BP1 were reproducibly expressed at a lower level (Fig. 3b). Replication of this experiment employing a retroviral gene transfer procedure led to similar results (not shown), confirming the repressive potency of the A70 mutant for MDA-MB-231 cells.

**Figure 3.**



**Figure 3. 4E-BP1 suppresses MDA-MB-231 breast cancer cell colony formation capacity in a manner dependent on its phosphorylation status.** (a) Shown are positions of the four serine/threonine sites of 4E-BP1 mutated to alanine (designated by asterisks) relative to the eIF4E-binding region. (b) Expression of exogenous 4E-BP1 in MDA-MB-231 cells transfected with wild type or mutant 4E-BP1. (c) Photo of breast cell colony formation. (d) Colony formation abilities and frequency of apoptosis in transfected cells.

Measurements of cap-dependent and IRES-driven translational activities (performed by using a bicistronic luciferase reporter construct, demonstrated reduction of cap-dependent translation rates in cells ectopically overexpressing HA-4E-BP1 (data not shown). Mutating phosphorylated sites increased an ability of 4E-BP1 to repress translation with 75-85% inhibition of cap-dependent translation by the A70 and double A37/A46 mutants. As shown at the Fig. 3, expression of both wild type and mutant 4E-BP1 reduced MDA-MB-231 colony formation efficiency, ranging from 20% fewer colonies in response to introduction of wild type 4E-BP1, to 75-80 % reduction of colony number after transfer of A70 or A37/A46 mutants. **Together, these results indicate that mutating phosphorylation sites increases the pro-apoptotic function of 4E-BP1 and that the rank-order of proapoptotic potency of each 4E-BP1 phosphorylation site mutant matches its potency in repressing cap-dependent protein synthesis..**

## Discussion

1. Previously we found that both the apoptotic and translational machinery are activated in all tested breast carcinomas when compared to non-transformed breast epithelial cells (DOD Annual Report 2000). Here we show that enforced overexpression of translational factor eIF4E induces some aspects of transformation in immortalized human mammary epithelial cells and increases their resistance to spontaneous and drug-induced apoptosis. Together, these findings confirmed proof of principle: breast cancer cells acquire metabolic alterations leading to increased cap-dependent translation to oppose transformation-related activation of their intrinsic apoptotic program. They also demonstrated that genetic alterations leading to activation of the cap-dependent translational apparatus can cooperate with other cancer-associated genetic changes in transformation of primary human mammary epithelial cells into drug resistant tumor cells.

2. In accord with our expectations, targeted disruption of the cap-dependent translational complex by ectopic overexpression of the translational repressor 4E-BP1 stimulates apoptosis and abrogates chemoresistance in breast carcinoma cells harboring diverse oncogenic alteration. In addition, expression of 4E-BP1 phosphorylation site mutants potently activates apoptosis in a phosphorylation-site specific manner which parallels repression of cap-dependent translation. These results demonstrate a close connection between cellular functions controlled by 4E-BP1 and the regulation of apoptosis.

3. Contrary to our expectations, rapamycin neither stimulate apoptosis nor potentiate 4E-BP1- or drug-induced cell death. Molecular bases of the differential effect of rapamycin of transformed fibroblasts and breast cancer cells remain to be clarified. Most importantly from a therapeutic point of view, these data suggest that, at least in breast carcinomas, pharmacological normalization of the upregulated apoptotic machinery can be achieved more effectively by disrupting association of eIF4E with capped mRNAs than by interfering with the eIF4E-to-eIF4G binding. Based on these data, we plan to employ novel pharmacological approaches including disruption of eIF4E-to-cap association by phosphoramidated analogues of the 5' methylguanosine cap of mRNA. These compounds will be synthesized in the laboratory of Dr. Wagner (Pharmacy School, University of Minnesota) in course of the collaborative work supported by the NIH/NCI grant 1 UO1 CA 91220-01 (V. Polunovsky PI, 2001-2005).

4. To develop optimal regimens for treatment of pre-sensitized breast cancer cells, we explored the role of overexpressed 4E-BP1 in apoptosis induced by a variety of cytostatic agents. We found that both wild type and hypophosphorylated 4E-BP1 sensitize breast carcinoma cells to the topoisomerase I inhibitor camptothecin and conventional anti-tumor antibiotic doxorubicin. Conversely, only marginal effects were observed when lovastatin and paclitaxel were applied. These data show that targeted disruption of cap-dependent translational machinery selectively sensitize breast carcinoma cells to a subset of anti-cancer drugs, and that doxorubicin and the topoisomerase I inhibitors are promising candidates for collaboration with anti-eIF4E pre-treatments in suppression of breast tumor growth.

## **Key Research Accomplishments**

- Based on our findings, we argue that genetic alterations leading to activation of the cap-dependent translational apparatus confer mammary epithelial cells some aspects of malignant transformation. Based on this conclusion, we plan to experimentally test a hypothesis that translation initiation factors eIF4E and/or eIF4G can cooperate with other cancer-associated genetic changes in transformation of primary human mammary epithelial cells into drug resistant tumor cells.
- Our results suggest that transfer of genes encoding translational repressor 4E-BP1 or its phosphorylation site mutant forms chemosensitize breast cancer cells to safe doses of some conventional anti-cancer drugs, including doxorubicin and the topoisomerase I inhibitors. These findings establish prerequisites for development of optimal regimens for treatment of pre-sensitized breast cancer cells by safe doses of conventional anti-tumor agents.
- Our data show that in contrast to transformed fibroblasts, pharmacological inhibition of the 4E-BP1 phosphorylation signaling by rapamycin does not significantly stimulate apoptosis in breast carcinomas. Based on these data, we plan to employ novel pharmacological approaches including disruption of eIF4E-to-cap association by phosphoramidated analogues of the 5' methylguanosine cap of mRNA. These compounds will be synthesized in the laboratory of Dr. Wagner (Pharmacy School, University of Minnesota) in course of the collaborative work supported by the NIH/NCI grant 1 UO1 CA 91220-01 (V. Polunovsky PI, 2001-2005).

## Reportable Outcomes

### • Manuscripts, Abstracts, Presentations

#### Manuscript:

1. Avdulov S, Shunan Li, Peterson M, Bitterman P, and **Polunovsky VA**,. Translation control of tumorigenesis and viability in human mammary epithelial cells: Anti-apoptotic function of the initiation factor eIF4E. Cancer Res., (In preparation).

#### Abstracts:

1. **Polunovsky VA**,. Avdulov S, Shunan Li, Peterson M, Gingras A-C, Sonenberg N, and Bitterman P. Translation control of malignancy: Translation repressor 4E-BP1 activates apoptosis in breast cancer cells in a manner dependent on its phosphorylation status. Abstract was selected for oral presentation at AACR Annual Meeting,, March 24-28, New Orleans, LA , 2001

#### Presentations:

1. **Polunovsky VA**,. Avdulov S, Shunan Li, Peterson M, Gingras A-C, Sonenberg N, and Bitterman P. Translation control of malignancy: Translation repressor 4E-BP1 activates apoptosis in breast cancer cells in a manner dependent on its phosphorylation status. AACR Annual Meeting,, March 24-28, New Orleans, LA , 2001
2. Avdulov SV, Li S, Peterson M, Gingras A-C, Sonenberg N, Bitterman P, **Polunovsky VA**. Translational control of acquired chemoresistance in breast cancer cells: anti-apoptotic function of the activated cap-dependent translational apparatus. International Meeting in Sardinia: "New Targets in Molecular Carcinogenesis." September 23-26, 2001

### • Patents

Not available

### • Degrees obtained that are supported by this research

Not available

### • Informatics such as databases and animal models, etc

Not available

### • Funding applied for based on work supported by this award

#### Active:

#### National Institutes of Health

Translational Apparatus as a Target for Cancer Drug Discovery (V. Polunovsky PI)

RFA CA-00-002

04/01/2001-03/31/200

\$350,000 Direct cost/year, 40% effort

Pending:

Department of Defense

Cap-dependent Translational Control of Human Breast Carcinogenesis and Tumor Cell  
Chemoresistance

Proposal Category: Idea

09/01/2002 – 08/31/2005

\$140,000 Direct cost/year, 40% effort

• **Employment or research opportunities applied for/or received....**

Not available

## Conclusions

In this report we present experimental data in support of Aim 2 of our project: *In vitro*, develop a novel protocol(s) for breast carcinoma treatment that will include pharmacological inhibitors of eIF4E activity. Our results clearly show that enforced overexpression of translational factor eIF4E induces some aspects of transformation in immortalized human mammary epithelial cells and increases their resistance to spontaneous and drug-induced apoptosis. In line with these observations, targeted disruption of the cap-dependent translational complex by ectopic overexpression of the translational repressor 4E-BP1 stimulates apoptosis and abrogates chemoresistance in breast carcinoma cells harboring diverse oncogenic alteration. In addition, expression of 4E-BP1 phosphorylation site mutants potently activates apoptosis in a phosphorylation-site specific manner, which parallels repression of cap-dependent translation. Together, these findings confirmed proof of principle: breast cancer cells acquire metabolic alterations leading to increased cap-dependent translation to oppose transformation-related activation of their intrinsic apoptotic program. They also demonstrated that genetic alterations leading to activation of the cap-dependent translational apparatus can cooperate with other cancer-associated genetic changes in transformation of primary human mammary epithelial cells into drug resistant tumor cells. We also found that that targeted disruption of cap-dependent translational machinery selectively sensitizes breast carcinoma cells to a limited subset of anti-cancer drugs including doxorubicin and the topoisomerase I inhibitors, which are considered as promising candidates for anti-eIF4E adjuvant therapy. Contrary to our expectations, pharmacological suppression of 4E-BP1 phosphorylation by rapamycin neither stimulates apoptosis nor potentiates 4E-BP1- or drug-induced cell death. Based on these data, we plan to employ a novel pharmacological approaches including disruption of eIF4E-to-cap association by phosphoramidated analogues of the 5' methylguanosine cap of mRNA. These compounds will be synthesized in the laboratory of Dr. Wagner (Pharmacy School, University of Minnesota) in course of the collaborative work supported by the NIH/NCI grant 1 UO1 CA 91220-01 (V. Polunovsky PI, 2001-2005).

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