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13. ABSTRACT (Maximum 200 Words)

Heregulin (HRG) is a growth factor that activates *erbB-2-3-4* receptors. We have generated a novel model of tumor progression from a hormone-dependent to a hormone-independent phenotype by introducing HRG into breast cancer cells. We now would like to investigate the mechanism by which HRG induces tumor progression. Our working hypothesis is that expression of HRG induces an uncontrolled mitogen-activated protein kinase (MAPK) cascade producing unbalanced growth promoting genes. **The proposed studies aim to determine whether blocking MAPK activation, cells revert to become hormone-dependent and antiestrogen responsive.**

During the first year of funding, we maintained the timeline outlined in the statement of work: a) Generated a *Ras* dominant negative (N17) regulated expression vector; b) Performed transfections into a number of MCF-7/HRG cells and isolated specific drug-resistant clones; c) Partially characterized these clones; and d) Initiated the construction of the MAPK mutant. The major findings of our work are that expression of N17 in MCF-7/HRG cells results in reversion from an anchorage-independent to anchorage-dependent phenotype. Moreover, when analyzing the response to estradiol, MCF-7/HRG/N17 cells regained hormonal response to a level of the wild type MCF-7 cells. **This data demonstrates activation of the MAPK via the HRG pathway promotes an aggressive hormone-independent phenotype.**

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TABLE OF CONTENTS

Front Cover	1
Standard Form (SF) 298, Report Documentation Page.....	2
Table of Contents	3
Introduction	4
Body	5
Key Research Accomplishments.....	11
Reportable Outcomes	12
Conclusions	12
References	13
Appendices	n/a

INTRODUCTION

Clinical studies have shown that the *erbB-2* oncogene product, when overexpressed, correlates with tamoxifen resistance in estrogen receptor positive (ER+) breast cancer specimens. The response rate to tamoxifen in the metastatic setting varies from 50-75% in ER positive patients. In patients whose tumors overexpress *erbB-2* in the context of the ER receptor, this response rate decreases to 17%. Although the presence of ER is employed to predict the hormone dependency of a tumor, the relationship with response to endocrine therapy is not absolute (not all ER+ patients respond to endocrine therapy). Significant levels of ER have been detected in over 60% of human breast cancers, but at best only two-thirds of these ER positive tumors respond to endocrine therapy. Why this should occur is unclear. However, our experimental studies have demonstrated a relationship between the ER and *erbB-2* signaling pathways. For example, it has been shown that estradiol down regulates *erbB-2* in overexpressing cells and that ER is required for this to occur. Potentiation of breast cancer cell growth by either the ER or *erbB*-pathway may make cells less amenable to anti-proliferative strategies directed to the alternative pathway.

It has been shown that inhibition of Ras-dependent signaling and the oncogenic function of Ras by farnesyl transferase inhibitors and/or blockers of Ras membrane anchorage are limited due to alternative prenylation of Ras. It has been shown that inhibition of the Ras-dependent Raf1-MAPK cascade is activated by S-farnesylthiosalicylic acid, which affects Ras membrane association but not Ras farnesylation. These data and our preliminary studies lead us to further hypothesize that *ras* activity contributes to the acquisition of an aggressive hormone-independent breast cancer phenotype. To assess this hypothesis, we employed a compound that inhibits ras anchorage to the membrane named FTS. This reagent represents a new class of Ras antagonists that may be useful for the inhibition of various types of oncogenic Ras isoforms independently of their prenylation.

We have generated a unique breast cancer tumor progression model and are equipped to design and evaluate ways to revert the progressive phenotype. We showed that MCF-7 cells, which are ER-positive, progressed to a more aggressive phenotype and rendered tumorigenic and metastatic *in vivo* merely by transfecting them with HRG. It may therefore be possible to inhibit both the uncontrolled cell proliferation and the metastatic properties of breast cancer cells by blocking either HRG or the MAPK pathway. We predict that cells can bypass their normal estrogenic requirements, *if* they develop an alternative escape mechanism. One alternative pathway appears to be the *erbB*-receptors pathway.

We hypothesize that there is compensation between the alternative signaling pathways. Thus, blocking one receptor pathway will result in the re-activation of the other. Clinically, that is in patients that are positive for both ER and *erbB*-, treatment with tamoxifen may result in increased proliferation through the *erbB*- signal transduction pathway. Conversely, interrupting the *erbB*- growth pathway with signaling blockers, ligand blockers or by other mechanisms may enhance proliferation through the ER system, and thereby restoring antiestrogen sensitivity.

To assess the mechanism by which HRG promotes this aggressive phenotype, we first determined the involvement of the *ras*-signaling cascade in this process. We observed an increased in activated *ras* in the HRG transfected MCF-7 cells (MCF-7/HRG) when compared to the control MCF-7 cells. Furthermore, a marked increase in activation of mitogen-activated protein kinase (MAPK) was seen in the MCF-7/HRG cells. The proposed studies will provide a better understanding of the pathway by which cells acquire a hormone-independent phenotype and will help us to design targeted therapies for this particular population of breast cancer patients.

BODY

The goal of the research outlined in this proposal is to extend ongoing studies. The following experiments are designed to shed light on the biological and molecular mechanisms by which HRG mediates and/or induces cellular transformation of breast cancer cells to a more invasive and hormone-independent phenotype. The original technical objectives were as follows:

- Task 1: To determine whether changes upstream of MAPK activity play a role in HRG induction of hormone-independent breast cancer phenotype.** We will determine if Ras activation is necessary or sufficient to block HRG action. These studies will be accomplished by transfecting a dominant negative Ras mutant (N17) in a Tet-regulated expression vector into MCF-7/HRG cells. Following the transfection, drug resistant clones will be isolated and characterized. This characterization includes determining the ability of these cells to become anchorage- as well as hormone-dependent.
- Task 2: To determine the direct effect of MAPK activity, using dominant-negative MAPK mutants.** These studies will be accomplished by transfecting a dominant negative MAPK mutant in a Tet-regulated expression vector into MCF-7/HRG cells. Following the transfection, drug resistant clones will be isolated and characterized. Characterization includes determining the ability of these cells to become anchorage-dependent as well as hormone-dependent.
- Task 3: To determine the ability of specific pharmacological kinase inhibitors (PD98059- MEK1 inhibitor and PD158780- HRG/*erbB*- inhibitor) to restore hormonal responsiveness of the HRG transfected cells.** These studies will be performed *in vitro* using MCF-7/HRG cells. Other inhibitors could be used as they become available.

STATEMENT OF WORK:

ACCOMPLISHED OBJECTIVES FROM TASKS 1-3:

STATEMENT OF WORK

Task 1: To determine whether changes upstream of MAPK activity play a role in HRG induction of hormone-independent breast cancer phenotype. We will determine if Ras activation is necessary or sufficient to block HRG action. These studies will be accomplished by transfecting a dominant negative Ras mutant (N17) in a retroviral expression vector into MCF-7/HRG cells. Following the transfection, drug resistant clones will be isolated and characterized. This characterization includes determining the ability of these cells to become anchorage- as well as hormone-dependent.

Because of technical difficulties with the Tet-regulated expression vector system as well as many failures because of leakage of expression in the non-regulated cells, we decided to take a different approach and performed the experiments using a retroviral vector and an isolated population of cells rather than cloning.

Task 1: Months 8-16: Biochemical and biological characterization of isolated clones: Anchorage-dependent and -independent growth assays. Assays will be performed in the presence or absence of estradiol. Biochemical characterization: measurements of *erbB*-receptor tyrosine phosphorylation and MAPK and *Ras* activity. To begin the *in vivo* experiments.

BIOLOGICAL CHARACTERIZATION OF THE N17 EXPRESSING CELLS *IN VITRO* USING ANCHORAGE-DEPENDENT AND -INDEPENDENT GROWTH ASSAYS:

We determined that MCF-7/HRG cells grow in an anchorage-independent fashion in the absence of estradiol. In contrast, the control cells (MCF-7/V1) grow exclusively in the presence of estradiol. Analysis of the N17-transfected MCF-7/HRG cells (MCF-7/HRG/N17) resulted in inhibition of anchorage-independent growth, as compared with control MCF-7/HRG cells. Moreover, when analyzing the response to estradiol, MCF-7/HRG/N17 cells regained the hormonal response to a level typical of the wild type MCF-7 cells. The ability of the dominant negative Ras mutant (N17) to abolish HRG induction of the anti-estrogen resistant phenotype will confirm the requirement of Ras in the induction of a Tam-resistant phenotype. We have use V12, an active mutant of Ras as control.

We have recently isolated cells that were infected with the N17 retroviral vector. These cells are currently been characterized biologically *in vitro* and future studies will be performed *in vivo*.

Task 2: Months 12-16: Biochemical characterization: measurements of *erbB*-receptor tyrosine phosphorylation and MAPK and Ras activity.

Task 2: Months 20-28: To characterize the biological activity of the specific pharmacological agents in vitro using all of the transfected and control cell lines: Measurements of erbB-receptor tyrosine phosphorylation, MAPK activity. To biologically characterize the kinase inhibitors: Anchorage-dependent and -independent growth assays. Assays will be performed in the presence or absence of estradiol. To continue with the biological characterization of transfected cells in vivo.

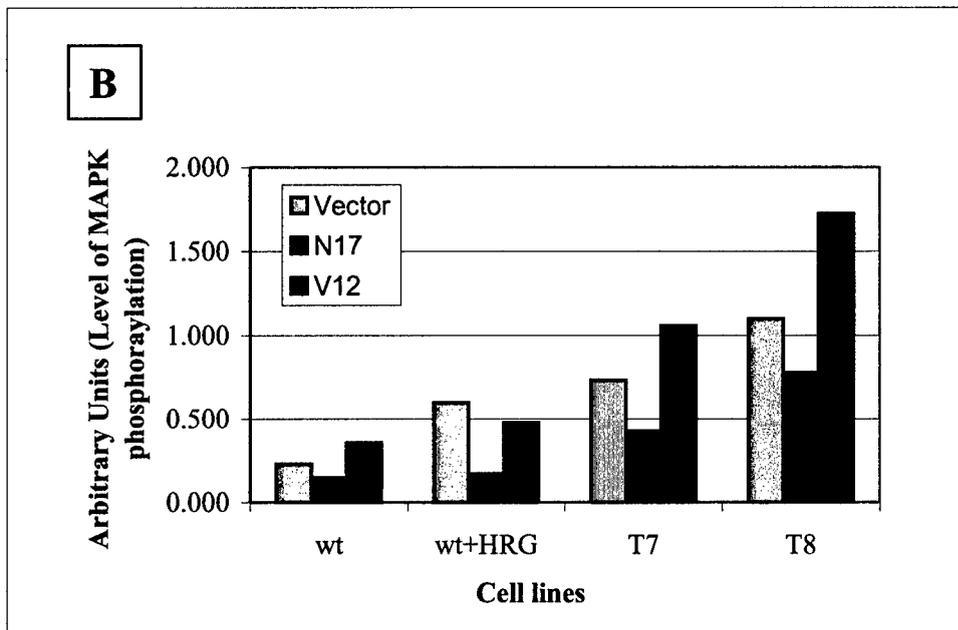
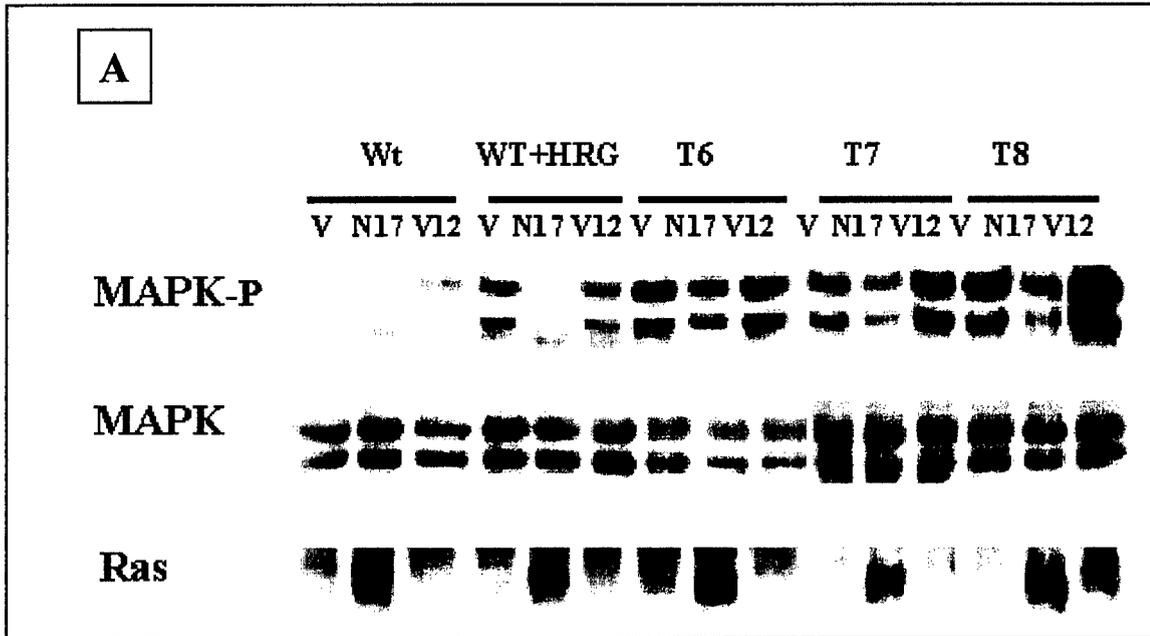
BIOLOGICHEMICAL CHARACTERIZATION OF THE N17 EXPRESSING CELLS

- 1. Expression of N17 in MCF-7/HRG/N17 cells.**
- 2. Effect on MAPK expression and phosphorylation.**

After the infection of MCF-7/HRG cells with the N17 dominant negative mutant, we determine the level of N17 expression and its influence on MAPK expression and activity. Cells were grown in serum free conditions and lysates were made and assayed on Western blot analysis. As can be seen in figure 1A, all the cells containing the dominant negative mutant of Ras, N17, showed a decrease in phosphorylated MAPK (Top panel). The levels of MAPK protein were unchanged (middle panel) and the expression level of Ras was increased in all the transfected clones (lower panel). The results were quantified and represented in Figure 1B.

These studies demonstrated that we have successfully infected the MCF-7/HRG cells with the dominant-negative mutant N17 and that the introduction of this mutant promoted a decreased in activated, phosphorylated MAPK.

Figure 1A-B: Inhibition of MAPK activity by Dominant Negative Mutant of Ras, N17



wt= Wild type MCF-7 cells, T6, T7, T8 = MCF-7/HRG cells, wt+HRG= wild type cells treated with Heregulin (HRG), N17= dominant negative Ras mutant, V12=activated ras mutant

Task 1: Months 16-26:

To continue the Characterization of the N17 transfected cells *in vitro* and *in vivo*: animal experiments will be performed in the presence or absence of estradiol and antiestrogens Tamoxifen and ICI 182,780.

Effect of N17 on the growth of MCF-7/HRG cells in anchorage-dependent and -independent assays: Preliminary data obtained in the laboratory demonstrates that the anchorage-dependent growth of the N17 infected MCF-7/HRG cells does not change as compared to the non-infected cells. However, it appears that the estrogen responsiveness is restored in anchorage-dependent growth assays. **These data confirms our hypothesis that activation of the MAPK signaling pathway is involved in the ability of HRG expressing cells to become hormone-independent.**

Task 2: To verify the direct effect of MAPK activity, using dominant-negative MAPK mutants (Δ MAPK)

Task 2: Months 10-16: Cloning the dominant negative Δ MAPK mutant into an expression vector. Transfection of a dominant negative MAPK mutant (Δ MAPK), cloning of single clones expressing Δ MAPK regulated by Tetracycline.

CLONING THE Δ MAPK cDNA INTO AN EXPRESSION VECTOR: During the course of the last year we have generated the expression vectors. We have introduced into the cells the vector expressing the tetracycline promoter. After confirming the chosen expression system was successfully expressed we are in the position to transfect the Δ MAPK mutant into the Tet-regulated expression vector (Invitrogen). Insert-less vectors will be transfected as controls.

Unfortunately, the vectors we had used failed to be regulated by Tet, therefore we had to take a different approach and decided to use retroviral vector containing a Δ MAPK cDNA. We are currently in the process of determining the biochemical and biological characterization of isolated clones. Biochemical characterization: Measurements of *erbB*-receptor tyrosine phosphorylation, MAPK activity. Biological characterization: Anchorage-dependent and -independent growth assays. Assays will be performed in the presence or absence of estradiol. We will further continue with the biological characterization of transfected cells *in vivo*.

Task 2: Months 16-22: Continue with the biochemical and biological characterization of isolated clones. Biochemical characterization: Measurements of *erbB*-receptor tyrosine phosphorylation, MAPK activity. Biological characterization: Anchorage-dependent and -independent growth assays. Assays will be performed in the presence or absence of estradiol. To continue with the biological characterization of transfected cells *in vivo*.

We have already introduced the retroviral vector containing the dominant negative mutant of MAPK and we are currently in the process of characterizing the cellular properties of the cells as well as the activation of the *erbB*- receptors. We will complete these studies in the next few months. The delay in the performance of these experiments has been because of the unsatisfactory transfection and cloning of the cells when the experiments were performed using a regulated promoter expression vector.

Task 3: To determine the ability of specific pharmacological kinase inhibitors (PD98059-MEK1 inhibitor and PD158780- HRG/*erbB*- inhibitor) to restore hormonal responsiveness of the HRG transfected cells. These studies will be performed *in vitro* using MCF-7/HRG cells.

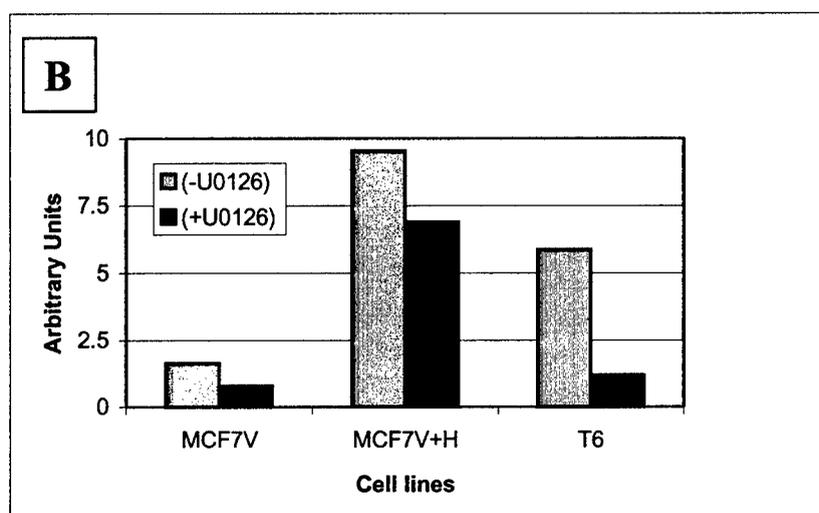
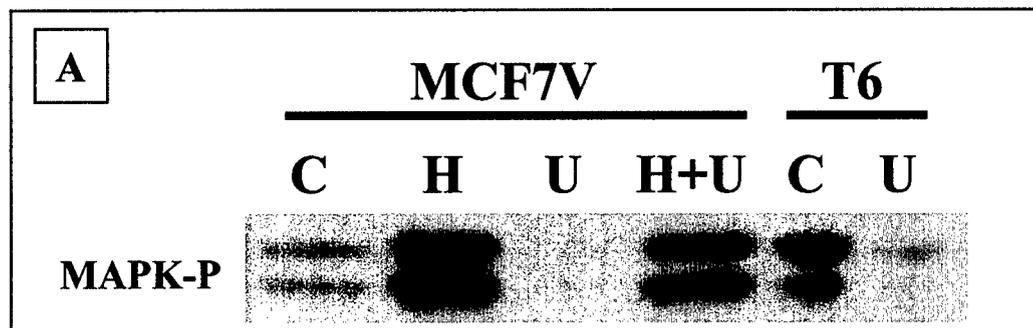
Task 3: Months 20-28: To characterize the biological activity of the specific pharmacological agents *in vitro* using all of the transfected and control cell lines using kinase inhibitors.

In the process of selecting specific MEK-1 inhibitors, we tried a number of inhibitors that are commercially available. After evaluating a series of inhibitors, we decided that the U0126 kinase inhibitor was the most adequate for our cell system and we have used it in all of the studies described below.

Effect of a MEK-1 inhibitor on the constitutive activation of MAPK in MCF-7/HRG cell:

Our goal was to determine the ability of specific pharmacological kinase inhibitors of MEK to inhibit activation of MAPK and restore their restore hormonal responsiveness. As can be seen in Figure 2 A-B, a MEK-1 inhibitor, U0126, was able to block the activation of MAPK when exogenously induced by HRG (H) as well as the constitutive MAPK phosphorylation (Figure 2A) Quantification of these data is demonstrated in Figure 2B.

Figure 2 A-B: MEK-1 Inhibitor, U0126, Inhibits Heregulin Induced activation of MAPK



C=Control, H=HRG, U=U0126, T6= MCF-7/HRG cells and MCFV=MCF-7 vector cells.

TASKS REMAINING TO BE PERFORMED DURING THE NEXT YEAR FROM INITIAL STATEMENT OF WORK

- Continue with the biological characterization of the dominant negatives N17 and MAPK. Assays will be performed in the presence or absence of estradiol. To continue with the biological characterization of transfected cells *in vivo*.
- To continue the characterization of the Δ MAPK transfected cells *in vivo*: animal experiments will be performed in the presence or absence of estradiol, tamoxifen and IC1 182,780.

KEY RESEARCH ACCOMPLISHMENTS

- The dominant negative Ras mutant was successfully introduced into a retroviral expression vector and it was used to infect breast cancer cells that overexpress HRG.

- Isolated cultures of HRG expressing cells (T6, T7, and T8) containing the dominant negative mutant of Ras (N17) and cultures containing a negative and a positive control (V12).
- Demonstrated that the infection with Ras was successful since Ras was highly expressed in the cells containing the retroviral vector containing N17. No expression was seen in the negative control cells, which contained the retroviral vector alone.
- Demonstrated that expression of N17 caused a decrease in activated MAPK and had no effect on the levels of MAPK protein.
- Demonstrate that a MEK-1 inhibitor was able to block HRG induction of MAPK activation in breast cancer cells as well as the decrease in phosphorylated MAPK in the HRG transfected cells.
- Demonstrated that blockage of Ras activation reverts HRG expressing cells from estrogen-independent to -dependent, therefore protecting breast cancer cells from becoming estrogen-independent.
- Introduced the dominant negative Δ MAPK mutant into a retroviral vector, infected cells and isolated expressing colonies.

REPORTABLE OUTCOMES

- Development of cell lines.
- Development of animal models.
- A MEK-1 inhibitor can specifically block activation of MAPK in cells expressing HRG and can induce cell growth inhibition.

CONCLUSIONS

The inverse correlation between HRG and ER expression in breast cancer cell lines prompts us to hypothesize that HRG triggers a cascade of events that lead to a hormone-independent phenotype. Therefore, we transfected HRG cDNA into an ER-positive breast cancer cell line MCF-7. The first observation after transfection was that the *erbB*-receptor signaling pathway appears constitutively activated, as shown by receptor phosphorylation and higher basal levels of MAPK activity. In addition, under estrogen-deprived conditions, the doubling time for the MCF-7/HRG cells was significantly shorter than the control cells (MCF-7/wild type or MCF-7/Vector). Moreover, estradiol (E₂) did not induce the proliferation of MCF-7/HRG cells, in contrast to control cells that were clearly stimulated. Interestingly, the MCF-7/HRG cells were unresponsive to estradiol in an anchorage-dependent fashion and also grew in an anchorage-independent fashion in the absence of Estradiol. In contrast, the control MCF-7 cells were totally dependent upon estradiol stimulation for anchorage-independent growth. MCF-7/HRG cells were hormone-independent and anti-estrogen resistant *in vivo*. The tumors developed by MCF-7/HRG cells in the presence of Tam were larger than those developed in its absence. Control cells (MCF-7/V) did not form tumors in the absence of E₂ and were tumorigenic exclusively in the presence of estradiol and sensitive to Tam, as predicted.

To decide whether the acquisition of the hormone-independent phenotype was a result of the loss of ER expression and/or ER function, we determined both the level of ER expression and the modulation of progesterone receptor (PgR) expression by estradiol. The level of ER in MCF-7/HRG was lower than the control cells and, more importantly, E₂ did not regulate the level of PgR expression. Our initial results imply that the HRG-transfected cells have lost ER function. Thus it seems that constitutive expression of HRG not only down-regulates ER but also blocks its E₂-mediated activation, abolishing the induction of PgR by E₂.

We hypothesized that increased activation of the *Ras-Raf-MAPK* pathway by HRG would result in transactivation of the ER receptor, thereby losing ER function. Therefore, it may be that blocking these signaling cascade cells will revert the hormone-independent phenotype induced by HRG. To validate our hypothesis we generated a *Ras* dominant negative (N17) retroviral expression vector and performed an infection on parental MCF-7 and MCF-7/HRG transfected cells (T6, T7, and T8 cells).

We have already determined that MCF-7/HRG cells grow in an anchorage-independent fashion in the absence of estradiol. In contrast, the control cells (MCF-7/V1) grow exclusively in the presence of estradiol. Analysis of the pool population of the N17-infected MCF-7/HRG cells (MCF-7/HRG/N17) resulted in the apparent inhibition of anchorage-independent growth, as contrasted with control MCF-7/HRG cells. We have also demonstrated that the introduction of N17 into the MCF-7/HRG cells promoted a significant decrease in phosphorylation of MAPK, implying that MAPK activity plays a fundamental role in the growth of MCF-7/HRG cells. In addition, we have also constructed similar vectors containing the dominant negative MAPK and performed similar studies, which are currently in progress.

Moreover, we have determined that a pharmacological inhibitor of MEK-1, was capable of blocking the activation of MAPK in cells exogenously treated with HRG as well as in the HRG transfected MCF-7 cells.

Our future studies are aimed at defining the function of these important signal transducers in breast cancer cells, especially how they control and modulate responses to growth-inducing factors in vivo. Interference with the action of specific MAPKs will provide new intervention strategies to halt breast cancer progression.

REFERENCES

Not Applicable.