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13. ABSTRACT *(Maximum 200 Words)*
The polypeptide heregulin is involved in normal mammary development and controls the proliferation of human breast cancer cells. These effects of heregulin are mediated by receptors in the ErbB/HER family. How ErbB family receptors function to control breast cancer cell proliferation was investigated in the previous year. Our research elaborated upon our discovery of a cross-communication between two distinct signaling pathways activated by heregulin receptors. Specifically, we had observed that the phosphoinositide (PI) 3-kinase signaling pathway was necessary for mitogen-activated protein kinase (MAPK) signaling, i.e. the appearance of activated MAPK in the cell nucleus. This novel effect whereby two well-studied growth-promoting pathways cooperate in signaling to the nucleus could be of major significance in breast cancer growth control. Two crucial questions about this signaling interaction were addressed: (1) whether the involvement of PI 3-kinase in MAPK activation could be observed in a cell line not of breast cancer origin, and (2) at which step of the MAPK signaling cascade was PI 3-kinase signaling involved. We determined that the affect of PI 3-kinase occurred in a non-transformed fibroblast cell line, and that the involvement of PI 3-kinase was subsequent to MAPK activation, possibly in the MAPK nuclear translocation event itself.

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INTRODUCTION

Breast cancer cells have been observed to express abnormally high levels of receptor proteins in the ErbB family, which includes the EGF receptor, ErbB2, ErbB3 and ErbB4 (also designated as HER1-HER4, respectively) (**1-4**). High levels of EGF receptor and ErbB2 expression in tumor cells have been considered indicators of poor prognosis (**5**). Given that these receptors activate mitogenic signaling pathways, it is possible that they play a role in the abnormal proliferation of breast cancer cells. The polypeptide heregulin (**6**) is secreted from breast cancer cells (**7**), and has been shown to activate ErbB2, ErbB3 and ErbB4 receptor proteins (**8-11**). Whereas ErbB4 can respond to heregulin independently, ErbB2 and ErbB3 have been shown to function together as a coreceptor for heregulin (**12**). With the discovery of heregulin (originally designated as Neu differentiation factor) came the observation that this factor could induce the re-differentiation of certain cultured breast cancer cell lines, specifically the cell lines MDA-MB-453 and AU-565 (**13**). Hence, in response to heregulin, these breast cancer cells show a flatter morphology, the presence of lipid droplets, and elevated levels of the milk protein casein. The observation that heregulin can alternatively induce either the proliferation or the re-differentiation of breast cancer cells raises numerous questions about the mechanisms by which this ErbB receptor ligand activates cellular responses. Presumably, clarifying these cellular control mechanisms would lead to a better understanding of breast cancer development, which in turn could lead to the discovery of novel therapeutic or prophylactic measures. Multiple signaling pathways are engaged by ErbB family receptors in response to heregulin. The focus of the proposed research is to identify those signaling pathways that alternatively elicit either the proliferation or differentiation of breast cancer cells.

BODY

In the progress report of last year, we described our previous studies of heregulin/ErbB receptor signaling in the context of cultured breast cancer cell lines, and our discovery of a novel interaction between the mitogen-activated protein kinase (MAPK) and phosphoinositide (PI) 3-kinase signaling pathways. Specifically, we had observed that a blockade of PI 3-kinase signaling with either of two pharmacologic inhibitors resulted in a failure of MAPK to translocate to the nucleus upon stimulation of cellular ErbB receptors with heregulin. This apparent interaction between two key pathways known to control cellular proliferation could be of much significance to the study of breast cancer, and of other cancers in which these pathways are believed to be involved. In the previous funding period, we sought to address two important questions concerning the interaction of the PI 3-kinase and MAPK pathways: (1) Is the involvement of PI 3-kinase in the heregulin-dependent nuclear translocation of activated MAPK a phenomenon restricted to breast cancer cell lines?, and (2) at which step of the Ras/MAPK signaling cascade is PI 3-kinase involved? We obtained at least partial answers to each of these questions.

We first attempted to determine if this signaling interaction occurred in a non-transformed fibroblast cell line, NIH-3T3, with which we had characterized various aspects of ErbB receptor signaling. In particular, we examined heregulin-dependent MAPK activation in the context of NIH-3T3 cells expressing endogenous ErbB2 and recombinant ErbB3 proteins (NIH-3T3-B3 cells). We had previously documented that this cell line shows heregulin-dependent MAPK activation and DNA synthesis (**14**). As a means of detecting MAPK activation and its ensuing nuclear translocation, we employed laser confocal immunofluorescence microscopy with a MAPK-specific antibody. Figure 1 shows the typical heregulin-dependent MAPK translocation seen in NIH-3T3-B3 cells. Note that in the control serum-deprived cells MAPK appeared to be excluded from the nucleus, but in cells stimulated with heregulin, cytoplasmic MAPK immunostaining was decreased as nuclear immunostaining became prominent. This heregulin-dependent nuclear translocation of MAPK in NIH-3T3-B3 cells was reminiscent of that we previously observed in the MCF7 breast cancer cell line. Figure 2 shows the effects of both MAPK kinase (MAPKK or MEK-1)-specific (PD98059) and PI 3-kinase-specific (wortmannin, LY294002) inhibitors on heregulin-

dependent MAPK translocation in NIH-3T3-B3 cells. As expected, inhibition of MAPKK blocked the nuclear translocation of MAPK, presumably because inactive, nonphosphorylated MAPK is retained in the cytoplasm via its interaction with MAPKK (**15**). However, inhibition of PI 3-kinase with either wortmannin or LY294002 also blocked heregulin-dependent nuclear translocation of MAPK. Thus, just as we had observed in our prior studies of MCF7 cells, the activity of PI 3-kinase appeared to be required for the nuclear accumulation of MAPK. The observation of this signaling interaction in a non-transformed fibroblast cell line suggests that this phenomenon might occur generally in the context of ErbB receptor signaling, and is not restricted to certain breast cancer cell lines. Of course, it remains to be determined if this interaction would be observed in the context of signaling by other growth factors that activate both MAPK and PI 3-kinase signaling events.

Between the MAPK and PI 3-kinase signaling pathways there exist several potential avenues for transmodulation that could result in alterations in the signaling of the individual pathways. For example, it is well known that H-Ras, which is a key upstream element of the MAPK signaling cascade, can also directly interact with and activate PI 3-kinase (**16**). In contrast, the protein serine/threonine kinase Akt, a downstream target of PI 3-kinase, has been recently shown to phosphorylate and inhibit Raf, a kinase in the Ras/Raf/MAPKK/MAPK cascade (**17**). The effect of PI 3-kinase inhibitors on the nuclear translocation of MAPK described above suggested a role for PI 3-kinase or one of its downstream signaling targets in either MAPK activation or the subsequent nuclear translocation event. To further clarify the mechanism of PI 3-kinase involvement, we first sought to determine if PI 3-kinase was necessary for activation of MAPK catalytic activity. To this end, MAPK activities in lysates of MCF7 cells were assayed, with cells being either non-stimulated (control) or stimulated with heregulin in the presence or absence of MAPKK (U0126) or PI 3-kinase (wortmannin, LY294002) inhibitors (Figure 3). As anticipated, the MAPKK inhibitor significantly attenuated the activation of MAPK by heregulin. In contrast, we observed no attenuation of heregulin-stimulated MAPK activity by the PI 3-kinase inhibitors. Thus, we reasoned that the observed effect of PI 3-kinase inhibition on the nuclear translocation of MAPK must reflect an event subsequent to MAPK activation by the upstream kinase MAPKK. Hence, it appeared that PI 3-kinase activity was not necessary for signaling events upstream of MAPK activation, including the sequential activation of Ras, Raf and MAPPK, but was necessary for an ensuing process, perhaps the nuclear translocation of activated MAPK or the nuclear retention of MAPK. The effect of the various inhibitors on MAPK activation was also examined in the context of SK-BR-3 cells, in which we had previously observed a constitutive nuclear localization of MAPK and its reversal by either MAPKK or PI 3-kinase inhibitors. Here the MAPKK inhibitor PD98059 was shown to block MAPK activation, whereas PI 3-kinase inhibitors again had no effect. We note that, consistent with our observed constitutive nuclear localization of MAPK in SK-BR-3 cells, the enzyme appeared also to be constitutively activated.

These experiments showed that PI 3-kinase inhibitors do not block the activation of MAPK per se, but instead interfere with its movement and/or retention in the nucleus. Nuclear translocation of MAPK is a crucial event in MAPK signaling, as the ultimate targets of MAPK are believed to be nuclear transcription factors. Indeed, nuclear translocation of MAPK has been shown to be necessary for MAPK-dependent gene transcription and cell cycle entry (**18**). *A priori* the requirement of PI 3-kinase signaling activity for the appropriate nuclear disposition of activated MAPK could reflect an effect on one of several processes including (1) release of activated MAPK from cytoplasmic anchoring proteins such as MAPKK (**15**), (2) the nuclear import event, which is presumed to be dependent-upon nucleo/cytoplasmic transport machinery (**19, 20**), (3) retention of activated MAPK in the nucleus via specific anchoring proteins (**21, 22**), and (4) the nuclear export event. Continued investigation of the mechanism of MAPK nuclear translocation and the apparent involvement of the PI 3-kinase signaling pathway could yield important new insights into the mechanism of breast cancer cell growth control. Such investigations will be pursued in the next and final year of the funded research (see revised Statement of Work below).

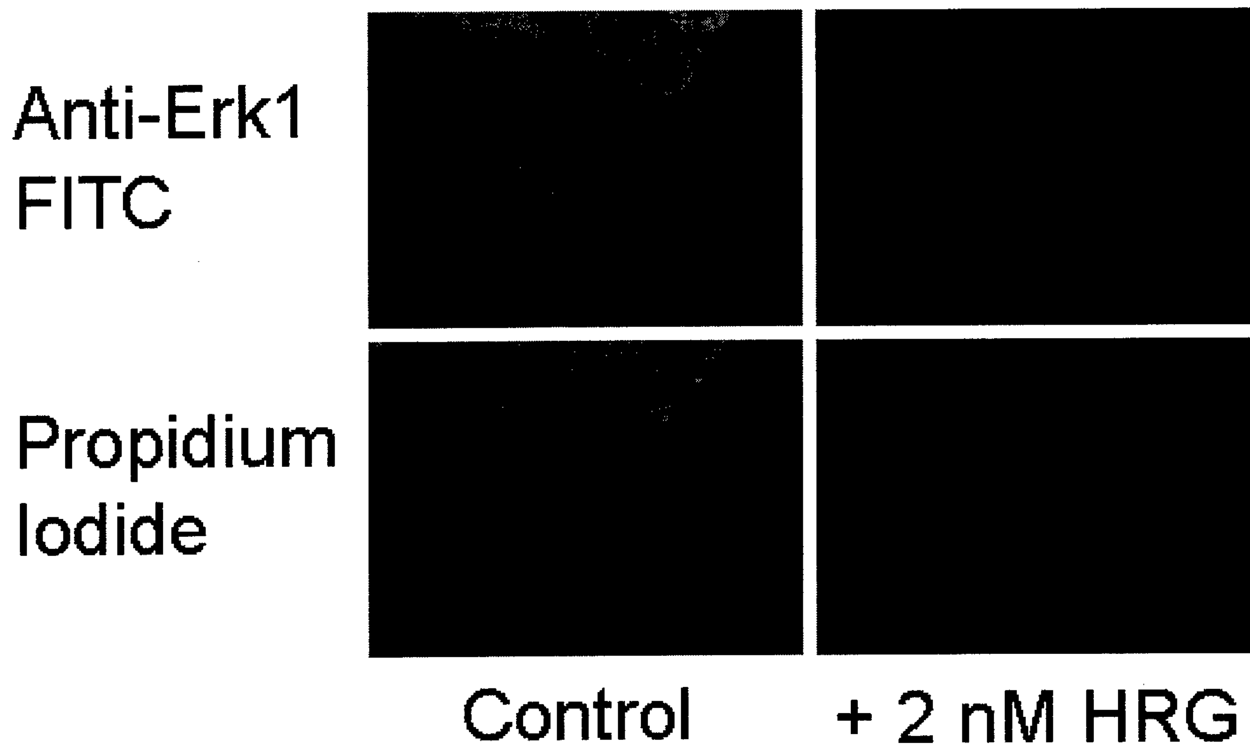

Figure 1. Heregulin-dependent nuclear translocation of MAPK in NIH-3T3-B3 cells. NIH-3T3 fibroblasts expressing endogenous ErbB2 and recombinant ErbB3 proteins (NIH-3T3-B3 cells) (**14**) were cultured on glass coverslips, then serum-deprived overnight. Cells were subsequently stimulated for 1 h at 37 C with 2 nM heregulin-β1 (HRG) or vehicle (Control) as indicated, and then subjected to immunostaining with MAPK antibody (anti-Erk1) and FITC-conjugated secondary antibody. MAPK localization was assessed by laser confocal microscopy (green staining, upper panels). Nuclei were stained with propidium iodide (red-orange staining, lower panels).

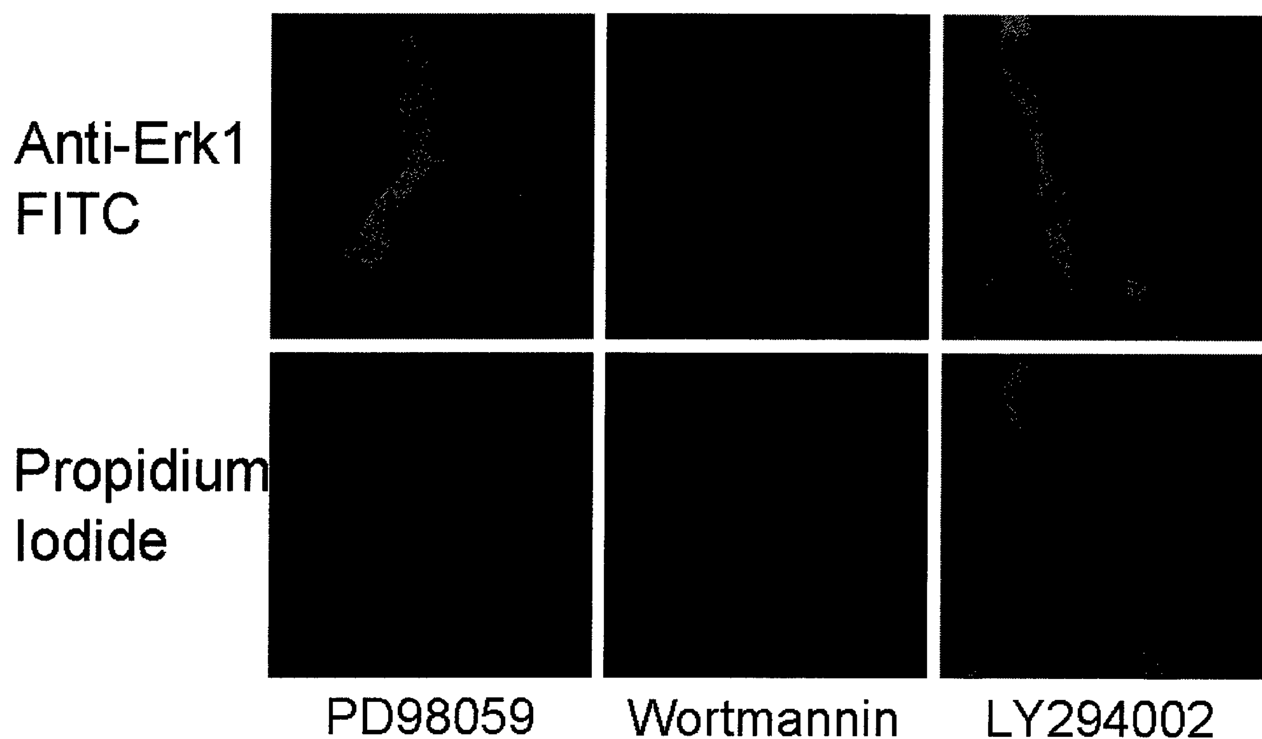

Figure 2. Effect of MAPKK and PI 3-kinase inhibitors on heregulin-stimulated nuclear translocation of MAPK in NIH-3T3-B3 cells. NIH-3T3-B3 cells were cultured on glass coverslips, serum-deprived overnight, then preincubated with either 20 μM PD98059 (MAPKK/MEK-1 inhibitor), 1 μM wortmannin (nonspecific PI 3-kinase inhibitor), or 30 μM LY294002 (PI 3-kinase inhibitor) for 20 min at 37 C. Cells were then stimulated with 2 nM heregulin-β1 for 1 h at 37 C and immunostained with MAPK antibody (see Figure 1 legend). MAPK localization (green staining, upper panels). Nuclear staining (red-orange staining, lower panels).

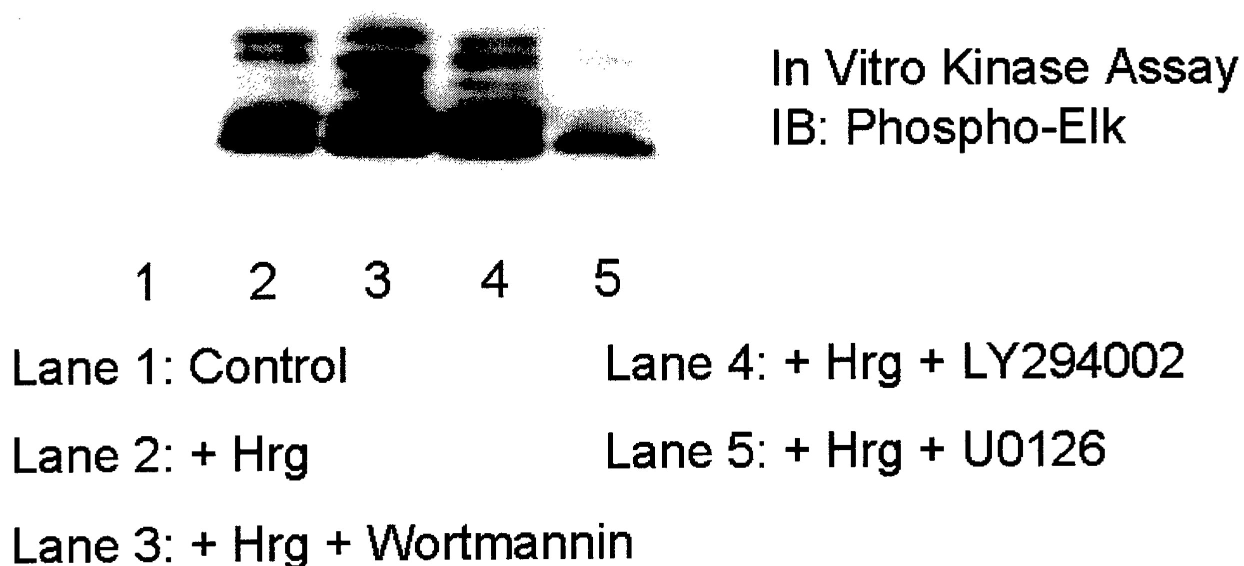

Figure 3. Effect of MAPKK and PI 3-kinase inhibitors on heregulin-dependent MAPK activation in MCF7 cells. MCF7 breast cancer cells were serum-deprived overnight, then stimulated for 15 min at 37 C with 2 nM heregulin-β1 (+Hrg) or vehicle (Control). Some cells were incubated for 20 min at 37 in the presence of 1 μM wortmannin (nonspecific PI 3-kinase inhibitor), 30 μM LY294002 (PI 3-kinase inhibitor), or 20 μM U0126 (MAPKK/MEK-1 inhibitor) prior to heregulin stimulation. After stimulation, cells lysates were assayed for MAPK activity by an immune-complex kinase assay (MAPK Kinase Assay Kit, New England BioLabs). The heterogeneous bands in the immunoblot indicate the phosphorylation of the MAPK substrate Elk.

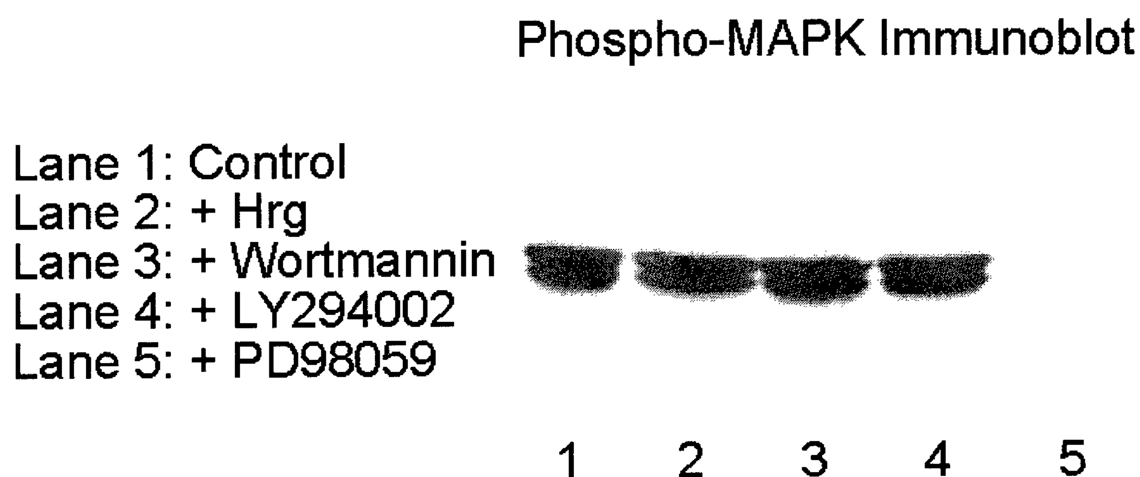

Figure 4. Effect of MAPKK and PI 3-kinase inhibitors on MAPK activation in SK-BR-3 cells. MCF7 breast cancer cells were serum-deprived overnight, then stimulated for 15 min at 37 C with 2 nM heregulin-β1 (+Hrg) or vehicle (Control). Some cells were incubated for 20 min at 37 in the presence of 1 μM wortmannin (nonspecific PI 3-kinase inhibitor), 30 μM LY294002 (PI 3-kinase inhibitor), or 20 μM PD98059 (MAPKK/MEK-1 inhibitor) as indicated. After the various treatments, cells lysates were assayed for MAPK phosphorylation by immunoblotting with a phospho-MAPK antibody (Santa Cruz Biotechnology).

KEY RESEARCH ACCOMPLISHMENTS

1. Demonstration that the heregulin-dependent nuclear translocation of MAPK in NIH-3T3 fibroblasts requires the activity of PI 3-kinase. This finding generalizes our previous observation of this phenomenon in cultured breast cancer cells to a nontransformed cell line.

2. Demonstration that PI 3-kinase inhibitors block neither the heregulin-dependent activation of MAPK in MCF7 cells nor the constitutive activation of MAPK seen in SK-BR-3 cells. This important result indicates that the requirement for PI 3-kinase in MAPK signaling occurs at a step subsequent to MAPK activation, possibly in the nuclear translocation event itself.

REPORTABLE OUTCOMES

None.

CONCLUSIONS

In the previous year of funding we have further explored a novel signaling interaction between the PI 3-kinase and MAPK pathways, specifically, a requirement for PI 3-kinase activity in the nuclear translocation of MAPK that occurs subsequent to its activation. Initially observed in studies of cultured breast cancer cells, we extended our observation of this phenomenon to cultured NIH-3T3 fibroblasts, a classic model cell line for growth factor signaling studies. Given that this phenomenon occurs not only in breast cancer cells, we can now determine if it is limited to ErbB receptor signaling systems or occurs more generally in the context of growth factor receptor systems. Secondly, by testing the effect of PI 3-kinase inhibitors on cellular MAPK activity, we determined that the effect of PI 3-kinase on MAPK nuclear translocation occurs at a step subsequent to MAPK activation, possibly the nuclear translocation event itself. Given that both the MAPK and PI 3-kinase signaling pathways have long-acknowledged roles in cancerous transformation, our observation of a cooperation of these two signaling pathways could represent a significant advancement in the study of cancer cell growth control. It is hoped that our future studies of this novel signaling interaction will identify the exact mechanism by which PI 3-kinase is involved in the nuclear translocation of the activated MAPK.

REFERENCES

1. Kraus, M. H., W. Issing, T. Miki, N. C. Popescu, and S. A. Aaronson. 1989. Isolation and characterization of *ERBB3*, a third member of the *ERBB*/epidermal growth factor receptor family: evidence for overexpression in a subset of human mammary tumors. Proc. Natl. Acad. Sci. USA **86:**9193-9197.

2. Slamon, D. J., W. Godolphin, L. A. Jones, J. A. Holt, S. G. Wong, D. E. Keith, W. J. Levin, S. G. Stuart, J. Udove, A. Ullrich, and M. F. Press. 1989. Studies of the HER-2/*neu* Proto-oncogene in Human Breast and Ovarian Cancer. Science **244:**707-712.

3. Lovekin, C., I. O. Ellis, A. Locker, J. Robertson, J. Bell, R. Nicholson, W. J. Gullick, C. W. Elston, and R. W. Blamey. 1991. c-erbB-2 oncoprotein expression in primary and advanced breast cancer. Br. J. Cancer **63:**439-443.

4. Gullick, W. J. 1990. 4. The role of the epidermal growth factor receptor and the c-erbB-2 protein in breast cancer. Int. J. Cancer **46:**55-61.

5. Jain, S., M. I. Filipe, W. J. Gullick, J. Linehan, and R. W. Morris. 1991. c-erbB-2 proto-oncogene expression and its relationship to survival in gastric carcinoma: An immunohistochemical study on archival material. Int. J. Cancer **48:**668-671.

6. Ben-Baruch, N., and Y. Yarden. 1994. Neu differentiation factors: A family of alternatively spliced neuronal and mesenchymal factors. Proc. Soc. Exp. Biol .Med. **206:**221-227.

7. Holmes, W. E., M. X. Sliwkowski, R. W. Akita, W. J. Henzel, J. Lee, J. W. Park, D. Yansura, N. Abadi, H. Raab, G. D. Lewis, H. M. Shepard, W. J. Kuang, W. I. Wood, D. V. Goeddel, and R. L. Vandlen. 1992. Identification of heregulin, a specific activator of $p185^{erbB2}$. Science **256:**1205-1210.

8. Plowman, G. D., J. M. Green, J. M. Culouscou, G. W. Carlton, V. M. Rothwell, and S. Buckley. 1993. Heregulin induces tyrosine phosphorylation of $HER4/p180^{erbB4}$. Nature **366:**473-475.

9. Carraway, K. L., III, and L. C. Cantley. 1994. A neu acquaintance for ErbB3 and ErbB4: A role for receptor heterodimerization in growth signaling. Cell **78:**5-8.

10. Carraway, K. L., III, M. X. Sliwkowski, R. Akita, J. V. Platko, P. M. Guy, A. Nuijens, A. J. Diamonti, R. L. Vandlen, L. C. Cantley, and R. A. Cerione. 1994. The *erbB3* gene product is a receptor for heregulin. J. Biol. Chem. **269:**14303-14306.

11. Soltoff, S. P., K. L. Carraway, III, S. A. Prigent, W. G. Gullick, and L. C. Cantley. 1994. ErbB3 is involved in activation of phosphatidylinositol 3-kinase by epidermal growth factor. Mol. Cell. Biol. **14:**3550-3558.

12. Sliwkowski, M. X., G. Schaefer, R. W. Akita, J. A. Lofgren, V. D. Fitzpatrick, A. Nuijens, B. M. Fendly, R. A. Cerione, R. L. Vandlen, and K. L. Carraway, III. 1994. Coexpression of *erb*B2 and *erb*B3 proteins reconstitutes a high affinity receptor for heregulin. J. Biol. Chem. **269:**14661-14665.

13. Wen, D., E. Peles, R. Cupples, S. V. Suggs, S. S. Bacus, Y. Luo, G. Trail, S. Hu, S. M. Silbiger, R. B. Levy, R. A. Koski, H. S. Lu, and Y. Yarden. 1992. Neu differentiation factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. Cell **69:**559-572.

14. Vijapurkar, U., K. Cheng, and J. G. Koland. 1998. Mutation of a Shc binding site tyrosine residue in ErbB3/HER3 blocks heregulin-dependent activation of mitogen-activated protein kinase. J. Biol. Chem. **273:**20996-1002.

15. Fukuda, M., Y. Gotoh, and E. Nishida. 1997. Interaction of MAP kinase with MAP kinase kinase: its possible role in the control of nucleocytoplasmic transport of MAP kinase. EMBO J. **16:**1901-8.

16. Rodriguez-Viciana, P., P. H. Warne, R. Dhand, B. Vanhaesebroeck, I. Gout, M. J. Fry, M. D. Waterfield, and J. Downward. 1994. Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature **370:**527-32.

17. Zimmermann, S., and K. Moelling. 1999. Phosphorylation and regulation of Raf by Akt (protein kinase B). Science **286:**1741-4.

18. Brunet, A., D. Roux, P. Lenormand, S. Dowd, S. Keyse, and J. Pouyssegur. 1999. Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. EMBO J. **18:**664-74.

19. Reiser, V., G. Ammerer, and H. Ruis. 1999. Nucleocytoplasmic traffic of MAP kinases. Gene Expression **7:**247-54.

20. Ferrigno, P., F. Posas, D. Koepp, H. Saito, and P. A. Silver. 1998. Regulated nucleo/cytoplasmic exchange of HOG1 MAPK requires the importin beta homologs NMD5 and XPO1. EMBO J. **17:**5606-14.

21. Yang, S. H., P. R. Yates, A. J. Whitmarsh, R. J. Davis, and A. D. Sharrocks. 1998. The Elk-1 ETS-domain transcription factor contains a mitogen-activated protein kinase targeting motif. Mol. Cell. Biol. **18:**710-20.

22. Lenormand, P., J. M. Brondello, A. Brunet, and J. Pouyssegur. 1998. Growth factor-induced p42/p44 MAPK nuclear translocation and retention requires both MAPK activation and neosynthesis of nuclear anchoring proteins. J. Cell Biol. **142:**625-33.

REVISED STATEMENT OF WORK

The review of the previous progress report recommended that we submit a revised statement of work, to indicate how the detailed aims of the project have been altered since the project was originally described. We note that while the vagaries of experimental work have led to some revision of our experimental approach, the overall goal of the project remains the same, i.e. to understand how heregulin/ErbB receptor systems function to control the differentiation and proliferation of breast cancer cells. Our discovery in the first year of the project of an interaction between the PI 3-kinase and MAPK signaling pathways has led to a focusing of our experimental efforts upon the exact mechanism of this novel signaling interaction.

STATEMENT OF WORK

Task 1. Investigation of potential interaction between MAPK and PI 3-kinase signaling pathways

- A. Construction of MAPK-green fluorescent protein (GFP) fusion protein expression vector (months 1-3)
- B. Characterization of the MAPK-GFP construct as a reporter of the subcellular localization of MAPK by fluorescence microscopy (months 4-6)
- C. Optimization of immunostaining methods for localization of MAPK in breast cancer cells by fluorescence microscopy (months 7-9)
- D. Observation of heregulin-dependent MAPK nuclear translocation and the effect of MAPKK and PI 3-kinase inhibitors (months 10-12)

Task 2. Investigating the role of PI 3-kinase in the activation and nuclear translocation of MAPK

- A. Optimization of immunostaining methods for localization of MAPK in NIH-3T3 and NIH-3T3-B3 cells (months 13-14)
- B. Observation of heregulin-dependent MAPK nuclear translocation and the effect of MAPKK and PI 3-kinase inhibitors in NIH-3T3-B3 cells (months 15-16)
- C. Development of MAPK activity assays (month 17)
- D. Assaying the effects of MAPKK and PI 3-kinase inhibitors on the activation of MAPK in MCF7, SK-BR-3 and NIH-3T3-B3 cells (months 18-19)
- E. Observe MAPK nuclear translocation in NIH-3T3 cell lines expressing mutant forms of the ErbB3 receptor (months 20-24)

Task 3. Characterizing effects of altered MAPK subcellular localization on transcriptional activation by MAPK

- A. Develop a luciferase reporter system for measurement of the transcriptional activity of a MAPK-responsive promoter (months 25-27)
- B. Characterize the effects of MAPKK and PI 3-kinase inhibitors on MAPK-responsive gene transcription (months 28-30)
- C. Utilize a Gal4-Elk fusion protein construct as an assay of phosphorylation of the MAPK substrate and nuclear transcription factor Elk (months 31-32)

Task 4. Investigating roles of distinct PI 3-kinase signaling pathway components in MAPK nuclear translocation

- A. Develop methods for transient expression of an epitope-tagged MAPK protein and for assaying the activation state and subcellular localization of the expressed protein (months 33-34)
- B. Characterize the roles of PI 3-kinase signaling pathway components (Akt kinase; PTEN phosphatidylinositol phosphatase) by transient expression of their constitutively active and dominant-interfering mutant forms with epitope-tagged MAPK (months 35-36)