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**13. ABSTRACT** ***(Maximum 200 Words)***
The polypeptide heregulin has been shown to reverse the transformed phenotype of certain cultured breast cancer cell lines and hence convert them to more normal, milk-producing cells. In contrast, heregulin is reported to induce proliferation of other breast cancer cell lines. These diverse effects of heregulin are mediated by receptors in the Erb/HER family, which function as coreceptor complexes. How the different signaling pathways activated by ErbB family receptors can elicit alternatively the differentiation or proliferation of breast cancer cells was investigated in the previous year of funding. Our experiments addressed the mechanism of activation of mitogen-activated protein kinase (MAPK) by hergulin coreceptors. As one read-out of MAPK activation, we monitored the nuclear translocation of the activated enzyme by confocal immunofluorescence microscopy. In some breast cancer cell lines (e.g. MCF-7). MAPK immunostaining showed a clear translocation from cytoplasm to nucleus in response to heregulin. In other cells (e.g. SK-BR-3), MAPK was constitutively located in the nucleus, which indicated a persistent activation of this signaling pathway. Interestingly, we observed that inhibitors of the phosphoinositide (PI) 3-kinase signaling pathway blocked the nuclear translocation of MAPK. Our findings indicate a point of significant cross-talk between two major signaling pathways activated by hergulin receptors.

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John J Holland 5/4/00

PI - Signature Date

for Huaming Tan

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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.

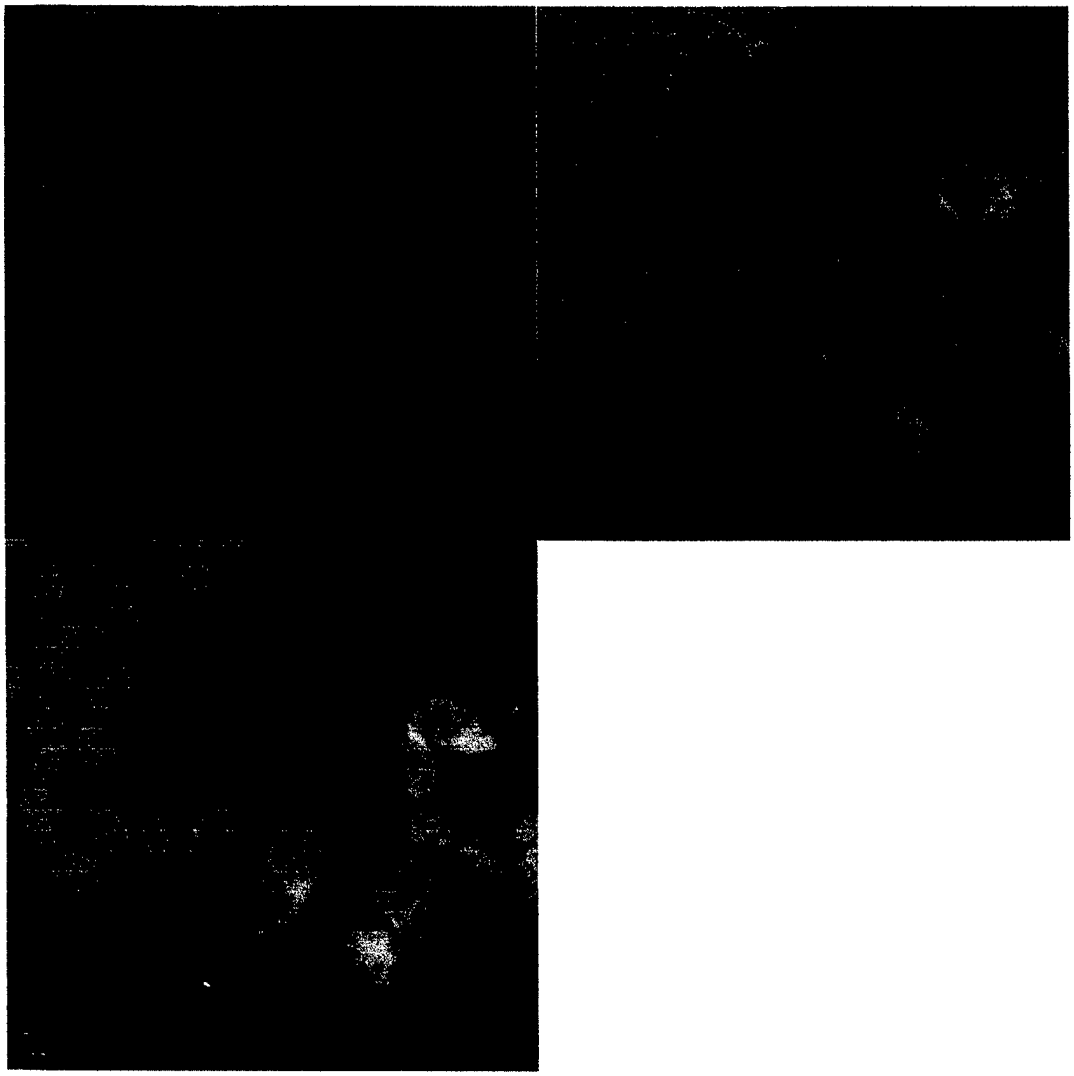
## BODY

In the previous year of funding, we undertook an examination of two signaling pathways activated by heregulin coreceptors: the mitogen-activated protein kinase (MAPK) and phosphoinositide (PI) 3-kinase signaling pathways. Our previous studies had shown that PI 3-kinase plays a dominant role as a mediator of cellular transformation by the ErbB2/ErbB3 coreceptor as assessed by *in vitro* assays of anchorage-independent growth. However, we considered that these two signaling pathways, both inherently mitogenic, might cooperate in the transformation of cancerous cells in other contexts. We began by assessing the ability of heregulin to stimulate MAPK activation in breast cancer cell lines. As a means of detecting MAPK activation and its ensuing nuclear translocation, we employed laser confocal immunofluorescence microscopy with a MAPK-specific antibody (see Figure 1). Upon optimization of the MAPK immunostaining protocol, we could observe in one breast cancer cell line (i.e. MCF-7) a clear translocation of MAPK from the cytoplasm to the nucleus upon stimulation with heregulin (see Figure 2). In another cell line (i.e. SK-BR-3), MAPK was seen to be constitutively localized in the nucleus (see Figure 6). As the overexpression and constitutive activation of ErbB family receptors has been documented in SK-BR-3 cells (14), these data support the conclusion that overexpression of ErbB receptor proteins and the subsequent constitutive activation of their downstream signaling targets is one mechanism for the enhanced proliferation of breast cancer cells.

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 element of the MAPK signaling cascade, can also directly interact with and activate PI 3-kinase (15). In contrast, the protein serine/threonine kinase Akt, a downstream target of PI 3-kinase, has been recently shown to phosphorylate and inhibit Raf, an upstream kinase in the MAPK cascade (16). As the ErbB2/ErbB3 coreceptor appears to be unlikely designed to activate both MAPK and PI 3-kinase pathways, we wished to examine how these signaling pathways might functionally interact in this context of this coreceptor system. Our preliminary *in vitro* studies of the anchorage-independent growth of ErbB2/ErbB3-expressing fibroblasts stimulated with heregulin showed

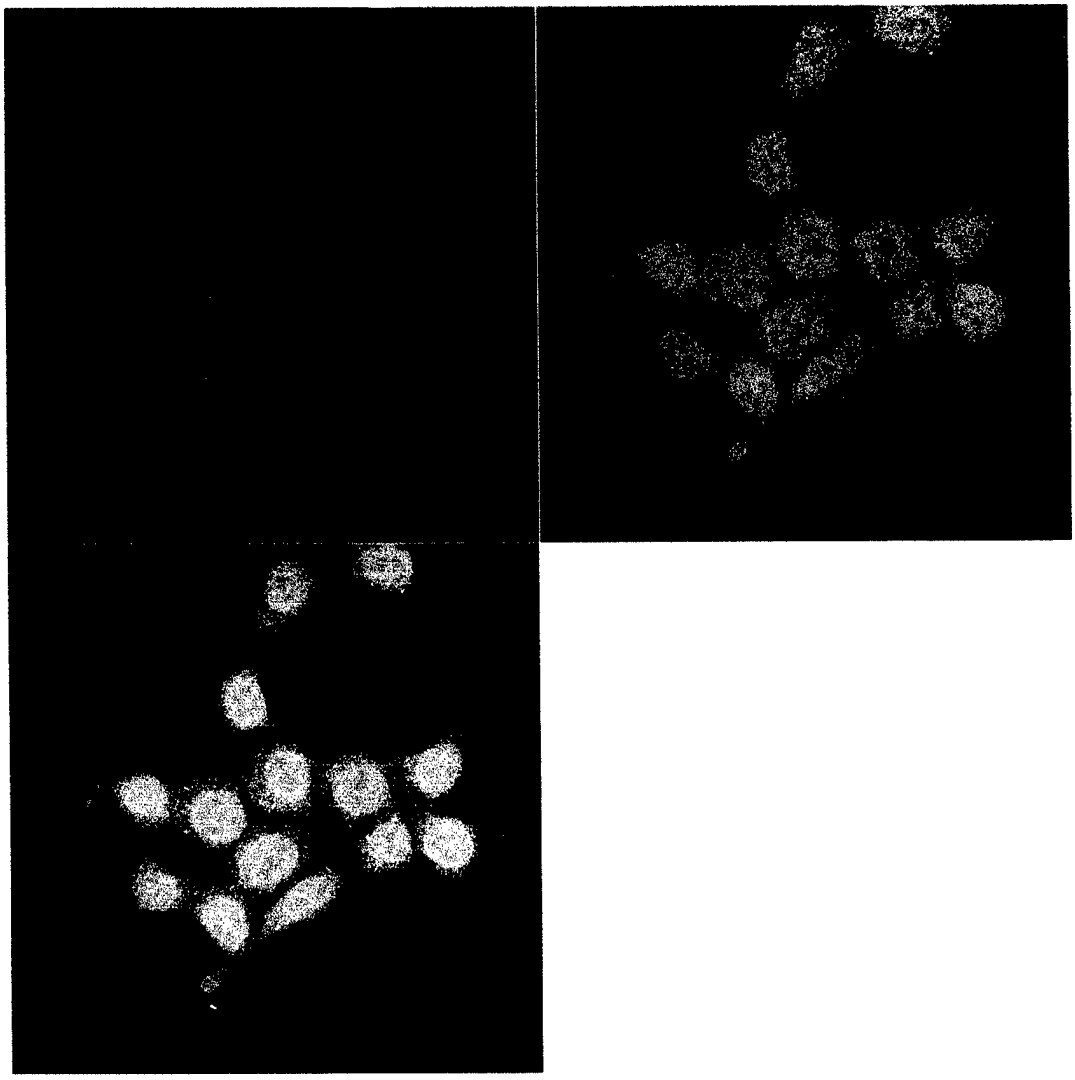
that the PI 3-kinase signaling pathway was dominant. However, in assays of DNA synthesis (mitogenesis) both pathways contributed significantly (Vijapurkar and Koland, unpublished data). We now sought to determine whether in the context of human breast cancer cells expressing ErbB family heregulin receptors the MAPK and PI 3-kinase signaling pathways might functionally interact. Again, MAPK activation and nuclear translocation was assayed by immunofluorescence microscopy. Again, in the MCF-7 cell line, we observed a heregulin-dependent nuclear translocation of MAPK. The possible involvement of PI 3-kinase in MAPK nuclear translocation was examined with the use of the PI 3-kinase inhibitors wortmannin and LY294002. The role of the upstream MAPK kinase (MEK1) was indicated by application of a specific MEK1 inhibitor, PD98059. As expected, the MEK1 inhibitor completely blocked the heregulin-dependent translocation of MAPK in MCF-7 cells (see Figure 5). Quite unexpectedly, the PI 3-kinase inhibitors also blocked heregulin-dependent MAPK translocation in these cells (see Figures 3 and 4). In SK-BR-3 cells, MAPK nuclear translocation was again seen to be constitutive and thus not heregulin-dependent, but the MEK1 and PI 3-kinase inhibitors all blocked the translocation of MAPK (data not shown). Hence, while in the case of SK-BR-3 cells the constitutive activation of ErbB receptors leads to the constitutive localization of MAPK in the nucleus, in these cells too, the blockade of PI 3-kinase activity interfered with MAPK transit to the nucleus.

The effect of PI 3-kinase inhibitors on the nuclear translocation of MAPK suggests a role for PI 3-kinase or its downstream signaling targets in MAPK activation or the subsequent nuclear translocation event. Our preliminary experiments have shown that PI 3-kinase inhibitors do not block the activation of MAPK per se, but instead interfere with its movement to the nucleus. Nuclear translocation of MAPK is a crucial event in MAPK signaling, as the ultimate targets of MAPK activation are believed to be nuclear transcription factors. 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.



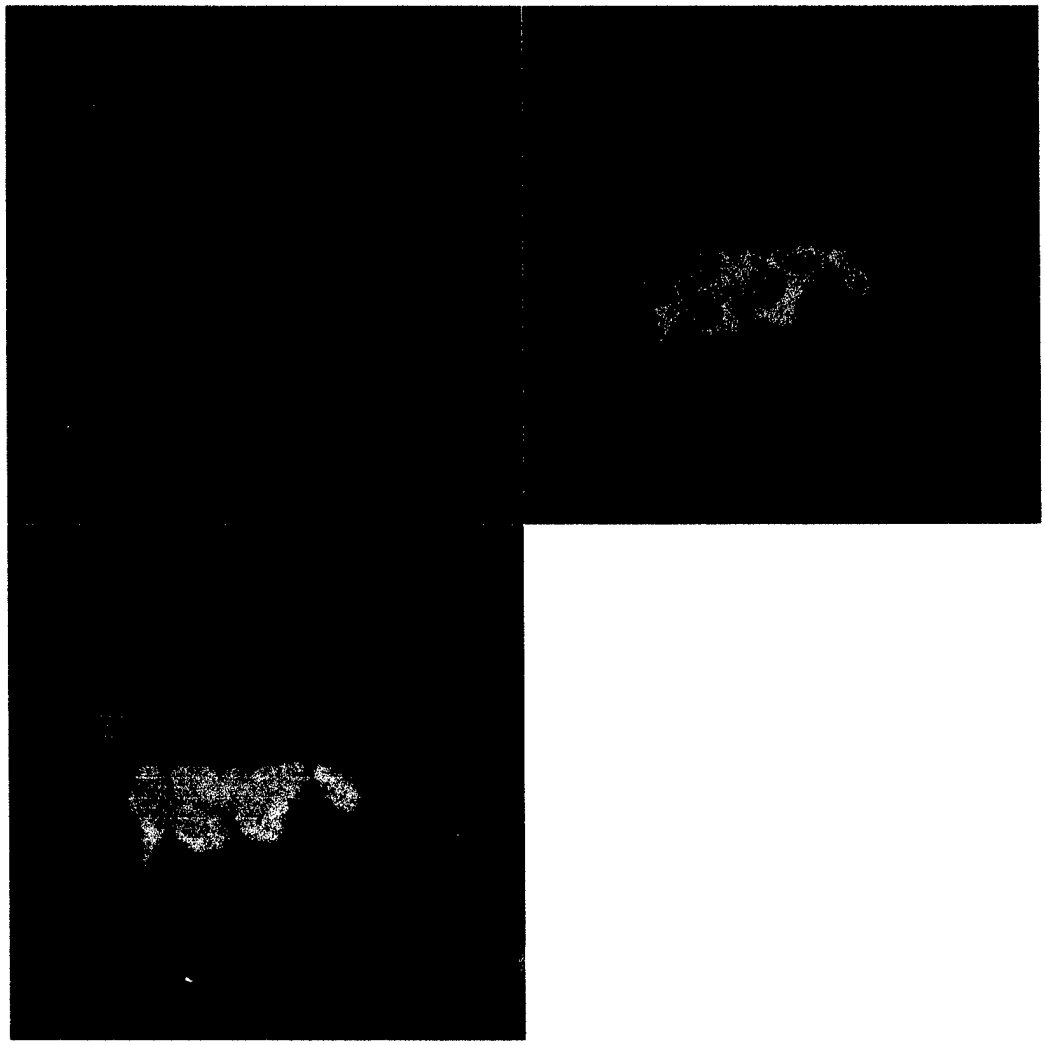
**Control**

**Figure 1. MAPK Localization in MCF-7 Breast Cancer Cells.** MCF-7 breast cancer cells were cultured on glass coverslips, then without addition of inhibitors or prior stimulation with heregulin were subjected to immunostaining with MAPK antibody (anti-Erk1) and FITC-conjugated secondary antibody. MAPK localization was assessed by laser confocal microscopy (green staining, upper right panel). Nuclei were stained with propidium iodide (red-orange staining, upper left panel). Lower panel is a merged image of MAPK and propidium iodide staining.



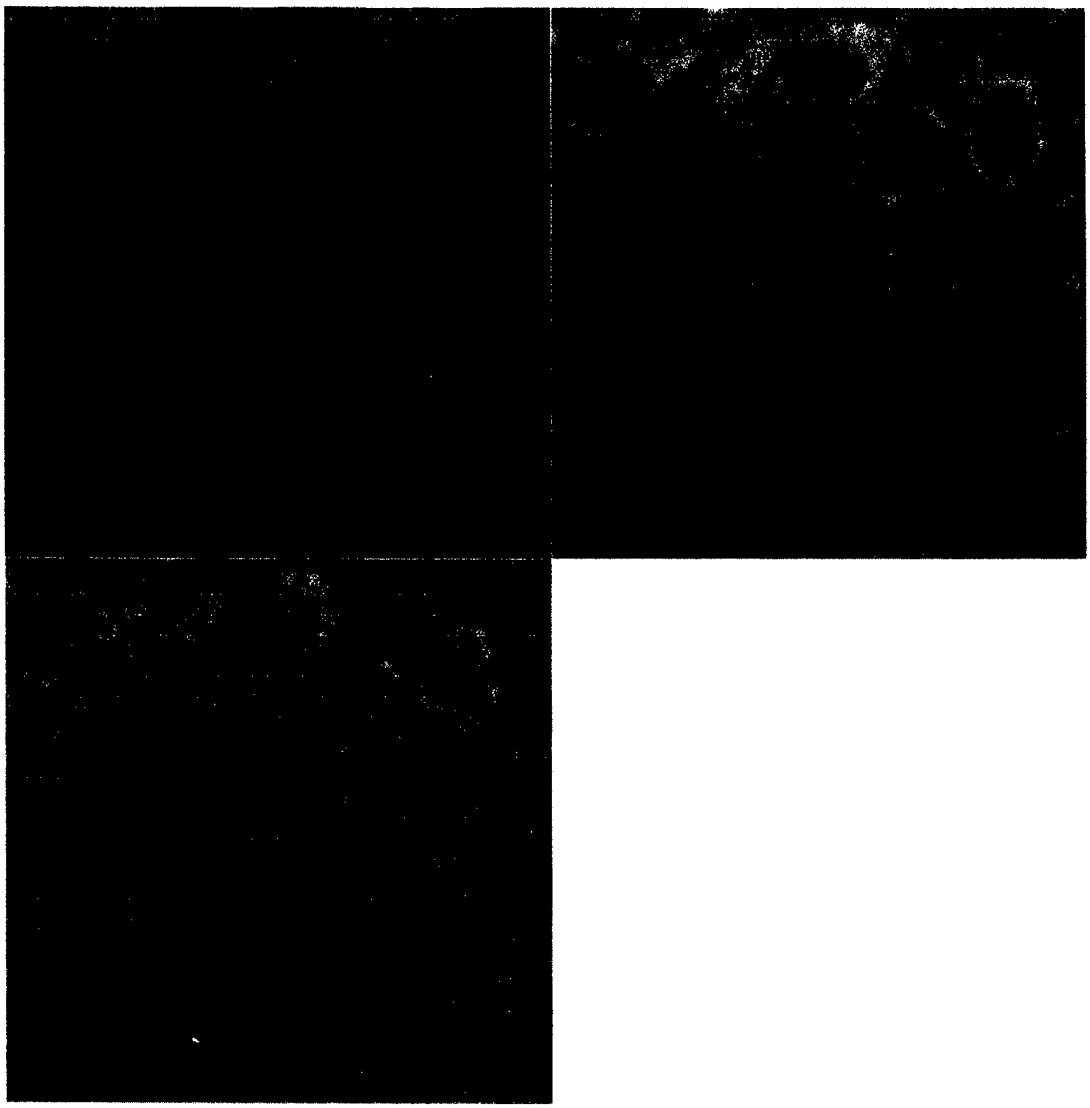
**+2 nM Heregulin**

**Figure 2. Heregulin-stimulated Nuclear Translocation of MAPK in MCF-7 Breast Cancer Cells.** MCF-7 breast cancer cells were cultured on glass coverslips, then without addition of inhibitors were stimulated with 2 nM heregulin-β1 for 1 h at 37 C. Cells were subjected to immunostaining with MAPK antibody (see Figure 1 legend). MAPK localization (green staining, upper right panel). Nuclear staining (red-orange staining, upper left panel). Merged image of MAPK and nuclear staining (lower panel).



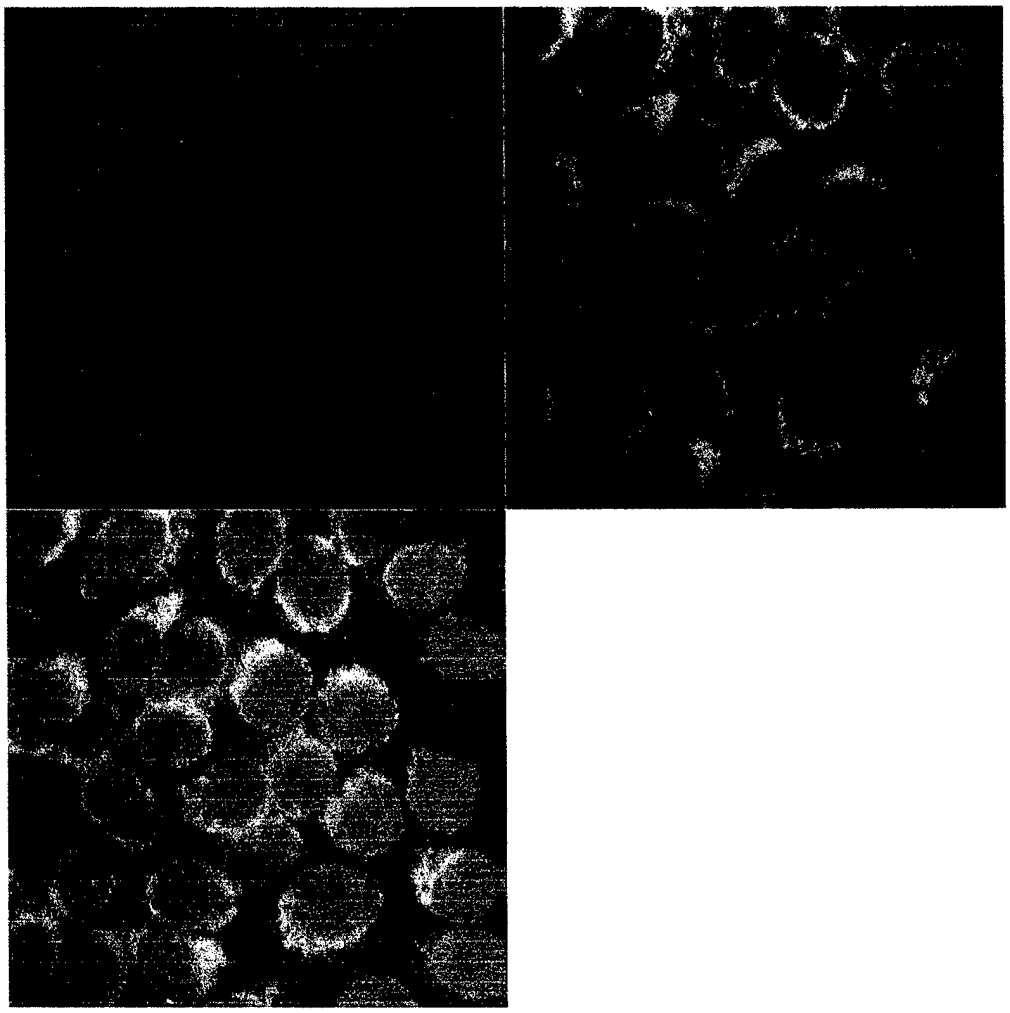
**+1 μM Wortmannin, +2 nM Heregulin**

**Figure 3. Effect of the PI 3-kinase Inhibitor Wortmannin on Heregulin-stimulated Nuclear Translocation of MAPK in MCF-7 Breast Cancer Cells.** MCF-7 breast cancer cells were cultured on glass coverslips, then preincubated with 1 μM wortmannin for 20 min at 37 C. Cells were 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 right panel). Nuclear staining (red-orange staining, upper left panel). Merged image of MAPK and nuclear staining (lower panel).



**+30 μM LY294002, +2 nM Heregulin**

**Figure 4. Effect of the PI 3-kinase Inhibitor LY294002 on Heregulin-stimulated Nuclear Translocation of MAPK in MCF-7 Breast Cancer Cells.** MCF-7 breast cancer cells were cultured on glass coverslips, then preincubated with 30 μM LY294002 for 20 min at 37 C. Cells were 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 right panel). Nuclear staining (red-orange staining, upper left panel). Merged image of MAPK and nuclear staining (lower panel).



**+20 μM PD98059, +2 nM Heregulin**

**Figure 5. Effect of the MEK1 Inhibitor PD98059 on Heregulin-stimulated Nuclear Translocation of MAPK in MCF-7 Breast Cancer Cells.** MCF-7 breast cancer cells were cultured on glass coverslips, then preincubated with 20 μM PD98059 for 20 min at 37 C. Cells were 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 right panel). Nuclear staining (red-orange staining, upper left panel). Merged image of MAPK and nuclear staining (lower panel).

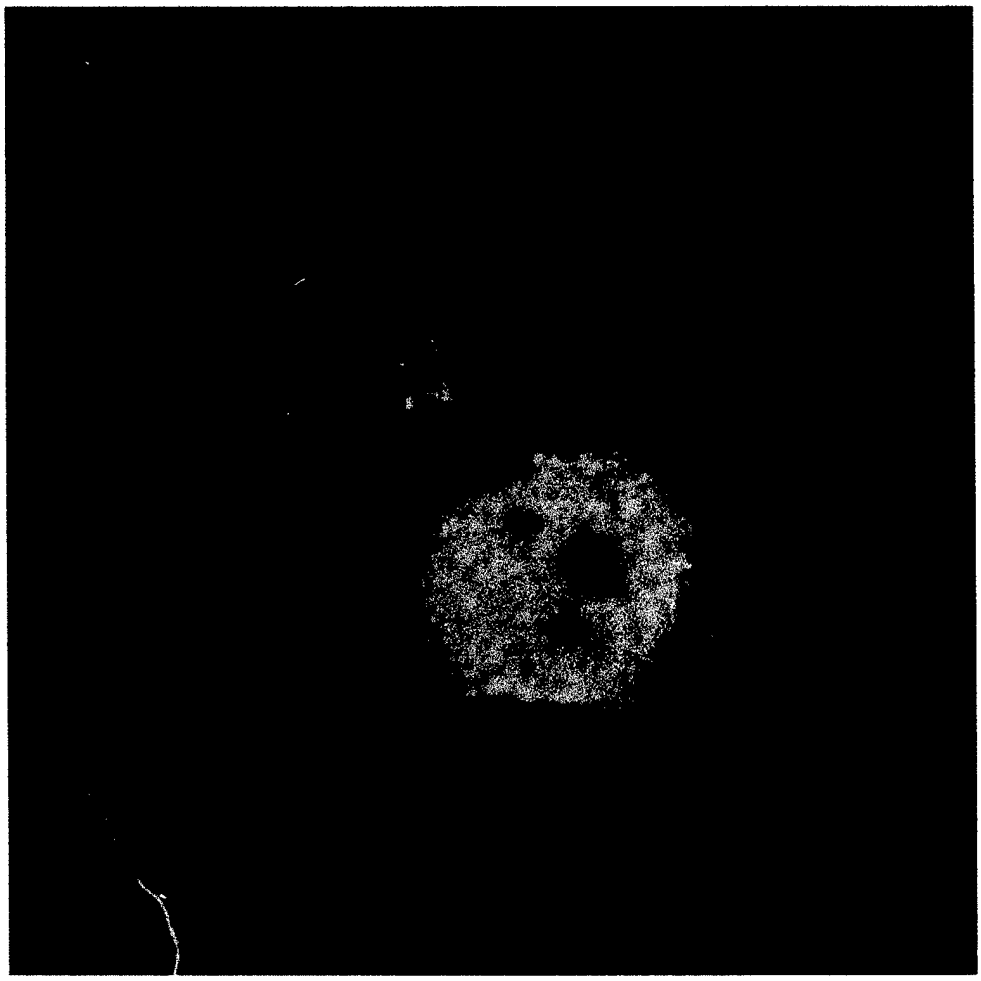


**Figure 6. Constitutive MAPK Nuclear Localization in SK-BR-3 Breast Cancer Cells.** SK-BR-3 breast cancer cells were cultured on glass coverslips and without prior stimulation or treatment with inhibitors subjected to immunostaining with MAPK antibody (see Figure 1 legend). MAPK showed constitutive nuclear localization.

## KEY RESEARCH ACCOMPLISHMENTS

1. Demonstration of the constitutive nuclear localization of mitogen-activated protein kinase (MAPK) in some cultured breast cancer cell lines.
2. Identification of an apparent role for phosphoinositide 3-kinase in the nuclear translocation of the MAPK signaling enzyme.

## REPORTABLE OUTCOMES

None.

## CONCLUSIONS

Our studies in the previous year of funding led to two significant conclusions regarding the signaling mechanisms of ErbB family receptors in human breast cancer cells. First, in some breast cancer cell lines (e.g. SK-BR-3 cells), MAPK appears to be constitutively activated and localized in the nucleus. This constitutive MAPK activation may be a consequence of the aberrant expression and activation of ErbB receptors that occurs in this and a subset of other breast cancer cell lines, and might contribute to the proliferation and metastasis of such cells. Further studies will determine whether aberrant ErbB receptor expression is indeed responsible for the constitutive MAPK activation observed in SK-BR-3 cells. Second, we have apparently identified a novel role for PI 3-kinase in the nuclear translocation of MAPK that occurs upon activation of the MAPK signaling pathway. This finding might explain how the multiple signaling pathways activated by ErbB family receptors cooperate in mitogenic signal transduction or in cellular transformation. Subsequent studies will determine whether this novel interplay between PI 3-kinase and MAPK signaling pathways occurs in other breast cancer cell lines or in response to activation of growth factor receptors in general. Finally, we intend to clarify the mechanism(s) by which PI 3-kinase can promote nuclear translocation of the MAPK signaling enzyme.

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