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| <b>13. ABSTRACT (Maximum 200 Words)</b><br><p>Breast cancer is a heterogenous disease, wherein the disregulation of numerous signaling pathways can cooperate to facilitate aberrant cell growth. We endeavored to understand how Brk, a soluble PTK overexpressed in nearly two-thirds of breast tumors, affects the development and progression of breast cancer. We studied Brk effects on cell viability by expressing GFP-Brk fusion proteins in breast cancer cells, and analyzed Annexin-V staining, indicative of apoptotic cell death, by flow cytometry. We found that Brk expression could increase the percentage of apoptotic cell death. Furthermore, we found that Brk inhibits the important cell survival molecule Akt, preventing Akt phosphorylation of the FOXO family member FKHR. In a separate line of investigation, we found that prolonged treatment of T47D breast cancer cells results in a biphasic pattern of Erk activation; the latent, sustained MAPK activation by progestins requires EGFR and MMP signaling, suggesting a positive, autocrine feedback mechanism of regulation. Significantly, progestin stimulation of breast cancer cell proliferation is sensitive to inhibitors of MAPK and EGFR signaling. By studying fundamental aspects of signal transduction in breast cancer, we hope to gain a better understanding of the molecular mechanisms of disease, thus facilitating a more informed approach to drug design.</p> |                                                                 |                                                                |                                                                                       |                                 |
| <b>14. SUBJECT TERMS</b><br>Tyrosine kinase, Src, oncogene, apoptosis                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |                                                                 |                                                                |                                                                                       | <b>15. NUMBER OF PAGES</b><br>8 |
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## INTRODUCTION:

Breast cancer is a heterogeneous disease; the disruption of normal control mechanisms safeguarding signaling pathways can result in the development and progression of tumor growth. A major challenge facing investigators today is to better understand the distinguishing features within the subtypes of the disease. Two-thirds of breast cancers are hormone dependent, wherein tumor cell growth is driven by the female steroid hormones estrogen and progesterone; ten percent of breast cancers are genetically linked (ex. BRCA1,2 mutations), and the remainder represent a cooperation of multiple dysregulated signaling pathways, including MAPKs, ErbB receptor signaling, IGF receptor signaling, etc. Protein tyrosine kinases (PTKs), such as the ErbB and IGF receptors, normally perform important roles for the regulation of cell differentiation and proliferation; however, a majority of invasive breast carcinomas reportedly possess elevated PTK enzyme activity, indicative of a poor prognosis (Hennipman *et al.*, 1989). Breast tumor kinase (Brk) is a soluble PTK overexpressed in two-thirds of breast tumors, yet undetectable in normal breast tissue (Mitchell *et al.*, 1994; Barker *et al.*, 1997). Currently, very little is known about the function of Brk and the advantage conferred by Brk expression on malignant cell growth and survival. The objective of the studies originally proposed was to determine the regulatory effects of Brk on both apoptosis and proliferation in normal breast epithelium compared to breast cancer cells.

## BODY:

**Specific Aim 1:** *Determine the effects of Brk overexpression on the viability of normal vs tumor cell lines that do not endogenously express Brk.* Cell viability experiments were conducted in three Brk-null cell lines, HEK 293, MDA-MB-435, and BT-549. The cell lines were transiently transfected by FuGene (Roche) with GFP-tagged Brk or GFP alone. After 48 hrs expression, live cells were collected with trypsin, and incubated with Annexin-V-PI stain. Cells were then analyzed by flow cytometry, and live, GFP-positive cells were gated on to selectively study the effects of the expressed Brk, then analyzed for Annexin V staining. A significant, albeit modest, fraction of Brk-expressing, BT-549 breast cancer cells exhibited a positive staining for the Annexin V marker of apoptosis. No difference in the fraction of apoptotic cells was seen in the MDA-MB-435 nor HEK293 cell lines expressing either GFP-Brk or GFP alone.

The findings that Brk expression in BT-549 cells increased cell death was included in a manuscript *submitted for publication* in May, 2004, of which I was a contributing second-author. In addition, I conducted experiments to test the effect of Brk expression on Akt activity, an important mediator of cell survival. For these studies I examined the effects of Brk expression on the ability of Akt to phosphorylate its substrate, FKHR, a member of the FOXO family of transcription factors. Phosphorylation of FKHR by Akt enables FKHR export from the nucleus and subsequent cytoplasmic degradation, thus preventing FKHR from promoting the transcription of apoptotic genes, such as *FasL*. We found that Brk overexpression decreases FKHR phosphorylation, consistent with Brk repression of Akt function.

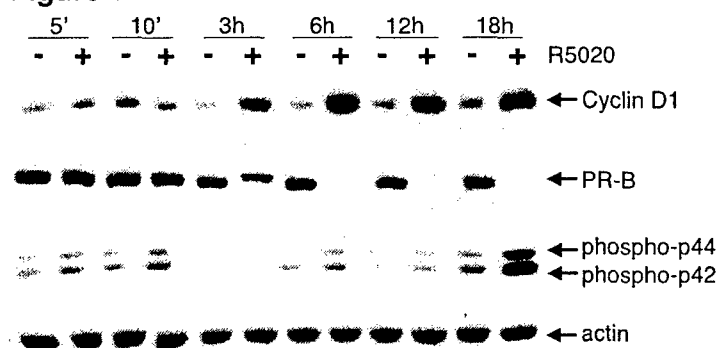
Further investigation of Brk's dual roles in proliferation and apoptosis stalled due to significant difficulties with reproducibility. When I began my graduate studies in Dr. Lange's laboratory, I worked on both Brk, as well as a side project involving PR cross-

talk with MAPK signaling pathways. Below I describe my experience with the PR project.

### PR activation of Erk signal transduction:

The research activities conducted in Dr. Langes laboratory focus on two subjects: 1.) Characterization of Brk signaling in breast cancer cells; and 2.) Progesterone receptor (PR) regulation by cross-talk with growth factor receptor signaling pathways. The projects are united in their objective to gain an understanding the contribution of aberrant regulation of signaling pathways to breast tumor cell growth. During the course of my studies, I became interested in the ability of PR to function as both a ligand-dependent transcription factor, as well as an activator of intracellular signaling pathways. As such, I conducted a few pilot experiments to examine Erk activation in breast cancer cells treated with progestins. In addition to the previously reported rapid and transient activation of Src and downstream Erks 1 and 2, we discovered that a second period of sustained Erk activation is induced at latent timepoints of progestin treatment (Figure 1). Numerous studies have demonstrated the importance of Erk signal strength and duration on the determination of appropriate cellular response to distinct extracellular signals. Further, the mechanism by which progestins induce proliferation of breast cancer cells is largely unknown. Therefore, we were very excited to learn more about the novel ability of progestins to stimulate a biphasic, temporal activation pattern of the Erk signaling pathway.

**Figure 1**



**Figure 1: Progestin treatment induces a biphasic activation of p42/p44 MAPKs in breast cancer cells.** T47D-YB cells were grown in steroid-free media for 48 hours, then treated with or without R5020 for the indicated timepoints. Cells were collected in RIPA and Western blotted for expression of cyclin D1, PR-B, or activated (phospho) forms of p42/p44 MAPKs. Actin blot included as a control for protein loading.

The initial phase of p42/p44 MAPK activation induced in response to PR ligand-binding is rapid and transient (Boonyaratanakornkit *et al.*, 2001; Migliaccio *et al.*, 1998), and the second or latent wave of p42/p44 signaling begins around 6 hours, peaks from 18-24 hours, and is sustained as long as 72 hours (Fig. 1). PR is classically defined as a ligand-activated transcription factor. Therefore, we undertook studies to examine the mechanism by which PR could activate a cytoplasmic signaling cascade even after PR protein has been degraded (Fig. 1). We found that latent p42/p44 MAPK activation is sensitive to inhibitors of transcription and translation, but may not require the classical function of the PR as an activator of transcription, as demonstrated by the ability of both a transcriptionally defective PR (S294A), and the PR transcriptional antagonist RU486 to activate p42/p44 MAPKs at latent time points. Instead, latent activation of p42/p44 MAPK signaling appears to be dependent upon the ability of liganded PR to bind Src and initiate the rapid early phase of cellular signaling. This finding is further supported

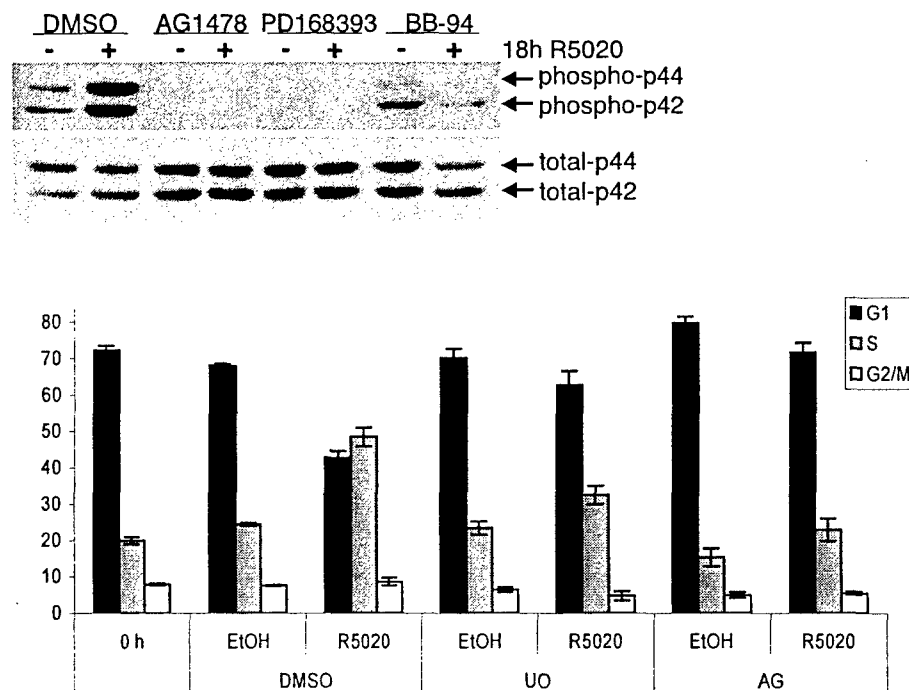
by the ability of the Src tyrosine kinase inhibitor, PP2, to prevent both the early and the late phases of p42/p44 MAPK activation by progestins.

Early activation of p42/p44 MAPKs by PR-ligand binding contributes to PR function by providing positive feedback, via direct phosphorylation of the liganded receptor. This event mediates PR nuclear localization and increased transcriptional activation of endogenous target genes. Additionally, activation of MAPKs by liganded PR can influence gene expression independently of PR transcription, such as cyclin D1 and CDK2. S294A mutant PR-B is a weak transcription factor, unable to be activated by cross-talk signals from activated MAPKs, but still capable of inducing proliferation in response to 18 hours of progestin treatment, as well as regulation of cyclin D1 over a 72 hour time course of R5020. As such, at six hours of R5020 treatment, the S294A has a delayed increase of cyclin D1 upregulation, with a magnitude similar to levels in cells treated with the antiprogestin RU486. When PR is unable to be phosphorylated by MAPKs, its function as a transcription factor (S294A) is greatly impaired, and the regulation of cyclin D1 is completely dependent upon p42/p44 MAPKs. Thus, p42/p44 MAPK activity modifies both PR function as a transcription factor, and additionally contributes to regulation of progestin-induced target genes that lack canonical PREs, such as cyclin D1 and CDK2, in a manner independent of the PR transcriptional activity.

Both early and late phases of p42/p44 MAPK activation were found to be sensitive to inhibition of EGFR tyrosine kinase. We focused our investigation here on the late phase of activation, but future studies will examine whether EGFR becomes rapidly phosphorylated in response to PR-ligand binding, and furthermore whether rapid cellular signaling is mediated by EGFR extracellular ligand shedding, or complex formation with Shc and Src, analogous to the mechanism employed by rapid, estrogen induced ER/Shc/Src binding to IGF-1R.

The second phase of p42/p44 MAPK activation is dependent upon signaling through the EGFR; 18 hours of progestin treatment increased the levels of activated EGFR, and inhibition of EGFR tyrosine kinase with two different agents, AG1478 and PD168393, blocked latent activation of MAPKs by progestin (Fig 2). The sensitivity of latent activation of MAPKs to broad inhibition of MMPs (BB-94) is suggestive of a mechanism of autocrine feedback mediated by extracellular EGFR ligand shedding and subsequent transactivation EGFR-MAPK pathway (Fig. 2). Progestins have been reported to regulate the expression of numerous growth factors, including EGF, TGF- $\alpha$ , and IGF-II, as well as ErbB receptors and insulin receptors; but the purpose of progestin regulation of autocrine agents and any connection to proliferation has remained elusive (Shi, *et al.*, 1994). From the results presented here, we suggest that the production of autocrine ligands may be responsible for the biphasic activation of p42/p44 MAPKs, and EGFR transactivation is required for the proliferative effects of progestins. Important future experiments will be aimed at the identification of the EGFR autocrine ligand(s) induced by progestins, and whether insulin or IGF signaling is involved in the ability of progestins to mediate cell proliferation and/or survival.

**Figure 2**



**Figure 2: Evidence of EGFR autocrine loop involvement in proliferative action of progestins. A. EGFR and MMP inhibitors block latent MAPK activation by progestins.** T47D-YB cells were grown in media supplemented with serum depleted of steroid hormones for 48h. Prior to addition of R5020 (10 nM), cells were incubated for 30' in the presence of DMSO vehicle control, EGFR inhibitors AG1478 (1  $\mu$ M) or PD168393 (10  $\mu$ M), or broad MMP inhibitor BB-94 (10  $\mu$ M). Lysates were collected after 18h R5020 treatment, and subjected to SDS-PAGE. Western blotting for phospho-p42/p44 indicates that EGFR inhibitors as well as the MMP inhibitor blocked activation of MAPK at 18h of progestin treatment. **B. Proliferative action of progestin is partially blocked by inhibitors of p42/p44 MAPKs and EGFR tyrosine kinase.** T47D-YB cells were plated in triplicate and grown in the absence of hormones for 48 hours. Cells were pretreated for 30' with DMSO vehicle control or inhibitors, U0126 (20 $\mu$ M) or AG1478 (10 $\mu$ M), prior to addition of R5020 or EtOH vehicle control. After 18h of progestin treatment, cells were collected with trypsin, subjected to EtOH fixation, and stained with propidium iodide. DNA content was measured by flow cytometry. Bars indicate percentage of cells in indicated phase of the cell cycle: G<sub>1</sub> (black), S (grey), G<sub>2</sub>/M (white).

## KEY RESEARCH ACCOMPLISHMENTS

Study of Brk's function as a potential inhibitor of the Akt cell survival signaling pathway and role in promotion of apoptosis.

Investigation of the function of PR as an activator of Erk signaling pathway.

Demonstration of the role of Erk and EGFR signaling in progestin-stimulation of breast cancer cell proliferation.

## REPORTABLE OUTCOMES:

Abstract: **E. Faivre**, P. Zhang, D. Fitzsimmons, C. Lange. Endocrine Society Annual Research Conference, June 2003.

Zhang, P., **Faivre, E.**, Olsen, A., Skildum, A., Fitzsimmons, D., and **Lange, C.A.** Regulated Association of Breast Tumor Kinase (Brk) with Akt Signaling Complexes. *Manuscript Submitted.*

Skildum A., **Faivre. E.**, Lange, C.A.. "Progestin and Mitogen Activated Protein Kinase-dependent changes in cell cycle regulation in breast cancer cells."  
*Manuscript in preparation.*

### **CONCLUSIONS:**

The disruption of signaling pathway integrity and specificity is a major mechanism contributing to the dysregulation of breast tumor cell growth. Brk is a soluble tyrosine kinase whose function is yet unknown, but may perform a dual roles in the stimulation of breast cancer cell proliferation coupled with a "failsafe" function, directing aberrantly proliferating cells toward apoptotic pathways. Here we report that Brk can inhibit Akt phosphorylation of FKHR, and overexpression in a Brk null breast cancer cell line facilitates an increase in apoptotic cells. Due to difficulties with reproducibility on the Brk project, we began experiments with a related breast cancer signal transduction project studying the role of PR as an activator of the mitogenic Erk signaling cascade. Significantly, we found that treatment of breast cancer cells with a single dose of progestin results in a PR-dependent biphasic, temporal pattern of Erk activation. Further, the ability of progestins to stimulate proliferation of breast cancer cells is sensitive to inhibition of Erk and EGFR signaling, suggesting an important mechanism of cross-talk between steroid hormone and growth factor receptors in the regulation of breast cancer cell growth.

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