Thomas Jefferson University

Annual Progress Report: 2008 Formula Grant

Reporting Period

July 1, 2009 – June 30, 2010

Formula Grant Overview

The Thomas Jefferson University received $3,455,597 in formula funds for the grant award period January 1, 2009 through December 31, 2012. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Role and Regulation of Focal Adhesion Kinase in Melanoma - Malignant melanoma is the deadliest form of skin cancer. Currently, the spreading of melanoma, known as metastasis, is only preventable by early detection and surgical excision of primary tumors. Therefore, it is critical that we understand the mechanisms underlying melanoma initiation and progression. The depth of invasion of a melanoma into the skin (dermis) is a determinant of treatment outcome. A gene, labeled B-RAF, is mutated in two-thirds of melanomas. This project has identified a target of the B-RAF gene known as focal adhesion kinase (FAK). This study will test whether B-RAF regulates FAK, which in turn promotes melanoma cell invasion into the dermis. Ultimately, the focus of this project is to identify why melanomas display invasive growth and, in doing so, identify novel targets for therapeutic intervention.

Anticipated Duration of Project

1/1/2009 - 12/31/2012

Project Overview

Malignant melanoma is the deadliest form of skin cancer and is poorly treatable. Depth of melanoma invasion into the dermis is used as a clinical determinant for prognosis. The long-term goal is to determine mechanisms underlying invasive growth of human melanomas. Mutations in the B-RAF gene are found in 50-70% of melanomas, and mutant B-RAF activation of the MEK-ERK1/2 pathway is required for invasion and resistance to apoptosis. Additionally, signaling from interaction with the surrounding extracellular matrix promotes invasion. Our preliminary data show that B-RAF-MEK-ERK1/2 signaling controls the phosphorylation of focal adhesion kinase (FAK) at serine 910. FAK localizes to sites of cell interaction with its extracellular matrix. Since these interactions regulate cell invasion, the goal of this project is to determine the role of serine 910 phosphorylation of FAK in melanoma cell invasion.

In Specific Aim 1, the hypothesis that phosphorylation of FAK at serine 910 regulates FAK localization and turnover in focal adhesions, and cellular migration and invasion, will be tested. Experiments will utilize live cell imaging, cell migration and invasion assays. Analysis of cellular properties in complex multi-cell type systems is critical to mimic the *in vivo* situation. Thus, in Specific Aim 2, experiments will determine the requirement of FAK in invasive melanoma growth in a 3-D human skin reconstruct system. Further studies will test the role of serine 910 phosphorylation of FAK in this system. Overall, the study is designed to identify new mechanisms underlying B-RAF regulation of invasive properties in melanoma.

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Expected Research Outcomes and Benefits

At the completion of the project, we expect to have identified a protein that plays a critical role in melanoma cell invasion and understand what regulates its properties. This is a key question, since invasive melanoma is associated with a poor patient prognosis. Ultimately, this work could provide avenues to develop new therapeutic strategies for the treatment of melanoma. These avenues could be in combination with existing treatments, and may be particularly important in patients who develop resistance to initial treatments. Furthermore, a focus of this project is to discover a marker that will serve to identify particularly invasive, and thus aggressive, forms of melanoma.

Summary of Research Completed

Mutations within the serine kinase, BRAF, occur in approximately two-thirds of melanomas. The majority of mutations in BRAF lead to hyper-activation of the MEK-ERK1/2 pathway. In vitro and in vivo xenograft studies demonstrating a requirement for BRAF in the proliferation, survival, and invasion of mutant BRAF melanoma cells have formed the pre-clinical basis for BRAF inhibitors in clinical trials. Indeed, RAF/MEK inhibitor trials are underway and phase I results with a BRAF inhibitor, PLX4032/RG7204, were highly encouraging. Two thirds of mutant BRAF melanoma patients treated with PLX4032 exhibited objective responses: 2 patients displayed complete response and 18 patients displayed partial responses following 15 day treatments. However, non-mutant BRAF melanoma patients did not respond.

The goal of this project is to determine effects of mutant B-RAF-dependent phosphorylation of the focal adhesion kinase (FAK) in the malignant properties of melanoma cells. We have demonstrated in preliminary data that phosphorylation of FAK at serine 910 is dependent on active B-RAF signaling in human melanoma cells. In our continuing efforts to determine the role of serine phosphorylation in focal adhesion kinase (FAK) localization, we analyzed serine 910 phosphorylation in mutant NRAS melanoma cells. NRAS is mutated in approximately 20% of melanomas and these mutations are mutually exclusive from BRAF mutations. For these studies we utilized PLX4720, the pre-clinical analog of PLX4032. PLX4720 treatment enhanced FAK serine 910 phosphorylation in mutant N-RAS melanoma cells, whereas no effect on tyrosine 397 phosphorylation was detected (Figure 1). Consistent with effects on ERK1/2, the MEK inhibitor, U0126, decreased FAK serine 910 phosphorylation.

These findings prompted us to analyze the effects of PLX4720 further upstream in the pathway. Consistent with prior reports, the B-RAF inhibitor, PLX4720, and the MEK inhibitor, U0126, effectively blocked MEK and ERK1/2 activation in mutant B-RAF harboring vertical growth and metastatic melanoma cell lines, A375, WM793, WM983A and 1205Lu (Figure 2A). By contrast, treatment of mutant N-RAS melanoma cell lines, Sbcl2, WM1361A, WM1366, and WM1346, resulted in a dramatic increase in MEK and ERK1/2 phosphorylation (Figure 2B). PLX4720 treatment also enhanced MEK and ERK1/2 phosphorylation in WM3211, which is wild-type for both B-RAF and N-RAS (Figure 2C). In these cells, the enhanced MEK and ERK1/2 phosphorylation may be promoted by autocrine growth factor activation of RAS. Similar to mutant B-RAF melanomas, ERK1/2 phosphorylation was inhibited by the MEK inhibitor, U0126, in non-mutant B-RAF cells (Figures 2B and C). By contrast, treatment of primary human melanocytes with PLX4720 did not affect MEK-ERK1/2 signaling (Figure 2C).

To determine if the PLX4720-induced MEK/ERK1/2 signaling lead to actin rearrangement, PLX4720-treated mutant NRAS WM1361A cells were stained with TRITC-phalloidin and analyzed by immunofluorescence. Compared to DMSO and U0126-treated cells, PLX4720 treatment in WM1361A cells led to a decrease in actin stress fiber formation (Figure. 3A). Since both FAK and actin rearrangement are involved with cell migration/invasion, PLX4720-treated Sbcl2 cells were assayed for cell migration in Boyden chambers and invasion through Matrigel. Compared to DMSO and U0126 treated cells, PLX4720-treated cells displayed an increase in cell migration and invasion (Figure 3B). Together, our data indicate that treatment of mutant N-RAS with PLX4720 results in a rapid induction of the MEK/ERK1/2 pathway that leads to phosphorylation of serine 910 of FAK and actin cytoskeleton rearrangement that results in an increase in cell migration/invasion.

FAK signaling is also involved in the disassembly of focal adhesions and actin cytoskeleton rearrangement and cell migration/invasion. In previous reports, we have outlined the generation of erase and replace systems in which we can inhibit the expression of endogenous FAK, while simultaneously expressing exogenous FAK proteins with the addition of doxycycline. The cell lines generated exhibit tight regulation and only express exogenous FAK and the shRNA for FAK upon the addition of doxycycline. They also express similar levels of FAK (GFP tagged FAK wild-type, non-phosphorylatable GFP-FAK S910A and phosphomimetic GFP-FAK S910E). We will utilize these reagents to determine the role of serine 910 FAK phosphorylation in mutant BRAF melanoma cells and mutant NRAS melanoma cells treated with PLX4720.

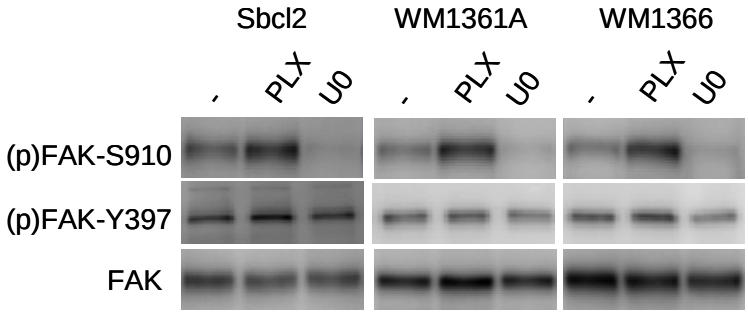

Figure 1. PLX4720-induced MEK-ERK1/2 hyperactivation regulates FAK serine 910 phosphorylation. Sbcl2, WM1361A and WM1366 cell lines were treated with DMSO (-),1 µM PLX4720 or 10 µM U0126 for 16 h. Lysates were analyzed by Western blotting using antibodies for (p)FAK-S910 (Biosource Int., Camarillo, CA), (p)FAK-Y397 (BD Transduction, San Jose, CA), and total FAK (Cell Signaling Technology).

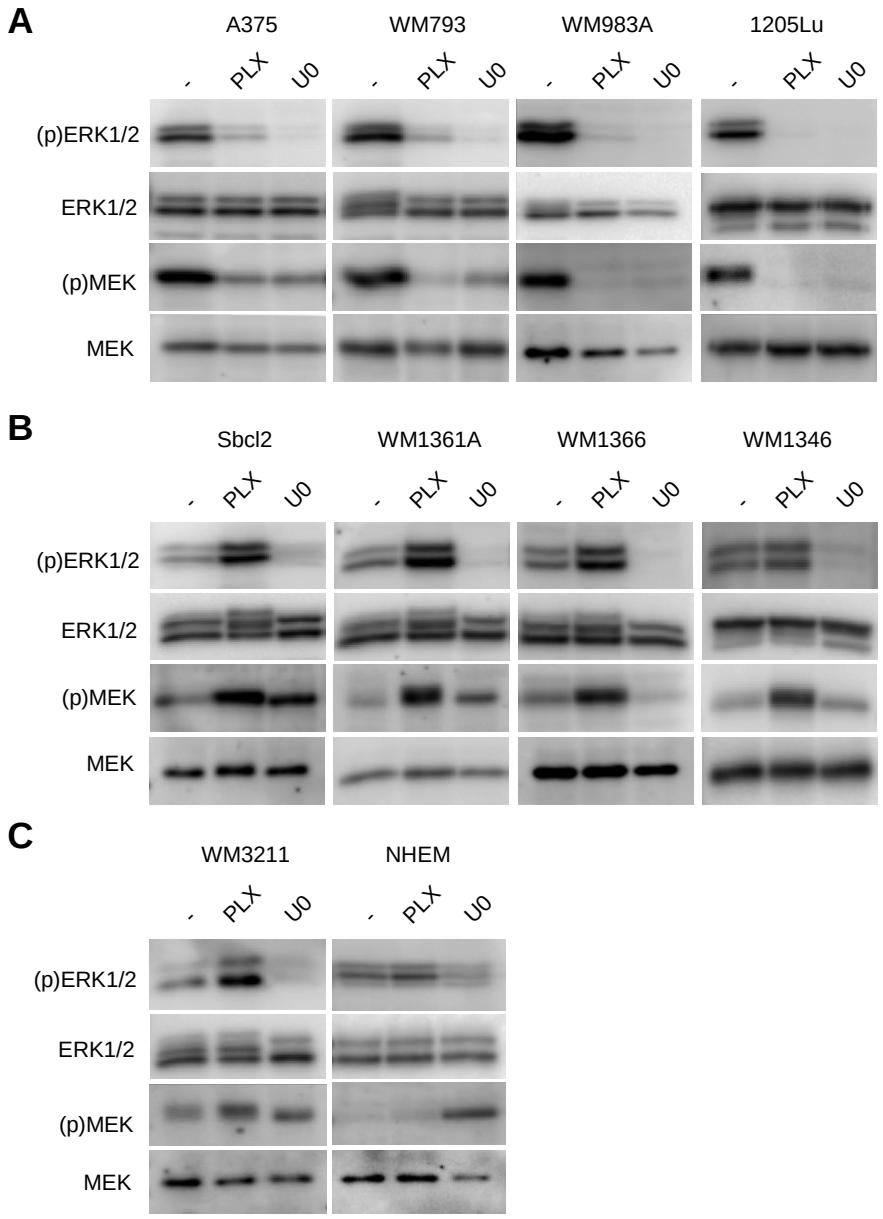

Figure 2. Treatment of mutant N-RAS melanoma cell lines induces MEK-ERK1/2 signaling. (A) Mutant B-RAF cell lines A375, WM793,WM983A and 1205Lu (all B-RAF^{V600E}); (B) Mutant N-RAS cell lines Sbcl2 (N-RAS$^{Q61K/R/L}$), WM1361A, WM1366, and WM1346; and (C) wild-type B-RAF/N-RAS WM3211 cells and neonatal human melanocytes (NHEM) were cultured, as previously described. All cell lines were treated with DMSO (-), 1 µM PLX4720 (PLX) [Plexxikon] and 10 µM U0126 (U0) [Cell Signaling Technology, Danvers, MA] for 16 h. Lysates were analyzed by Western blotting as previously described using a Versadoc Imaging system (BioRad, Hercules CA). Antibodies for ERK1/2 (Santa Cruz Biotechnology); MEK1, phospho-ERK (Thr^{202}/Tyr^{204}) [(p)ERK1/2], phospho-MEK1/2 (Ser^{217}/Ser^{221}) [(p)MEK] (Cell Signaling Technology).

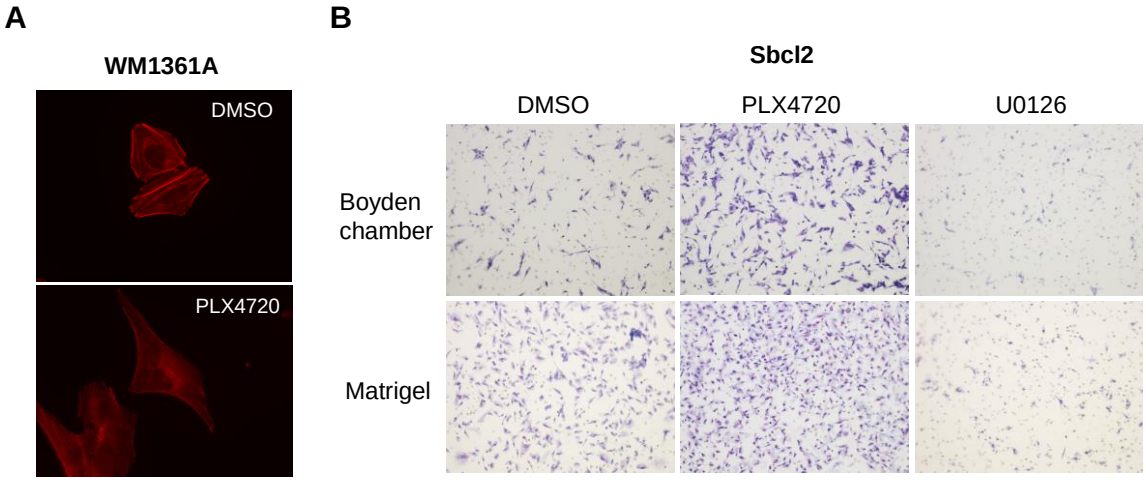

Figure 3. PLX4720 induces actin cytoskeletal re-arrangements and enhances migration and invasion in mutant NRAS melanoma cells. (A) Mutant NRAS WM1361A cells were treated with DMSO, PLX4720 and U0126 for 16 hours. Cells were fixed and stained with TRITC-phalloidin and analyzed by immunofluorescence. (B) Mutant NRAS Sbcl2 cells were treated with DMSO, PLX4720 and U0126 for 16 hours. Cells were placed in Boyden chambers with and without Matrigel. Cells were incubated for 16 hours prior to fixing and staining.

Publications resulting from this work:

Kaplan, F.M., Mastrangelo, M.J. and Aplin, A.E. (2010) The wrath of RAFs: rogue behavior of B-RAF kinase inhibitors. *J. Investigative Dermatol.* In press.

Kaplan, F., Shao, Y., Mayberry, M. and Aplin, A.E. (2010) Hyperactivation of MEK-ERK1/2 signaling and resistance to apoptosis induced by PLX4720 in mutant N-RAS melanoma cells. *Oncogene* Revised manuscript submitted.

Research Project 2: Project Title and Purpose

Prolactin and Growth Factor Signaling in Breast Cancer - Progression of breast cancer from early solitary lesions to advanced metastatic disease requires loss of cellular differentiation and epithelial-to-mesenchymal transition (EMT). While extensive efforts have been devoted to understanding the factors that allow this transition to occur in breast cancer, little work has focused on factors that prevent EMT, or on molecular strategies to reverse EMT for use in differentiation therapy. The long-range goal of this laboratory is to identify molecular mechanisms that govern growth and cellular differentiation of human breast cancer to improve therapies, and to use this knowledge to improve drug responsiveness of individual tumors, so that more effective, personalized medical treatment can be realized for cancer patients.

Anticipated Duration of Project

1/1/2009 - 12/31/2012

Project Overview

The central hypothesis is that epidermal growth factor receptor signaling inhibits the prolactin receptor (PRLR)-Jak2-Stat5 pathway in breast cancer, which in turn promotes epithelial-to-mesenchymal transition (EMT) and loss of estrogen receptor (ER) expression.

Aim #1 Determine whether the PRLR-Jak2-Stat5 pathway in human breast cancer is inhibited by EMT-promoting growth factors in the tumor microenvironment. The hypothesis is that paracrine growth factor receptor activation by epidermal growth factor (EGF) ligands inhibits the differentiation-promoting PRL-Jak2-Stat5 pathway in breast cancer, through a mechanism that involves Erk1/2 phosphorylation of Jak2 on the inhibitory Ser523 residue. We will *a)* determine whether experimental EGFR/Her2-mediated signaling inactivates PRL-induced Stat5 activation in breast cancer lines through Jak2 inhibition, due to activation of either Erk1/2, Jnk and/or Akt pathways, and *b)* analyze whether signaling by EGF receptors and hyperactivation of Erk1/2, Jnk and/or Akt kinases is associated with inactivation of Stat5 in two large sets of human breast cancer specimens (n=850).

Aim #2 Determine whether Stat5a activation is sufficient to overcome dedifferentiation, EMT, and loss of ERα induced by EGFR/Her2 in human breast cancer. The hypothesis is that EGFR/Her2 inhibition of the PRLR-Jak2-Stat5 pathway is required for EGFR/Her2-induced EMT, cell-cycle progression, and loss of ERα expression. We will determine *a)* whether Stat5 reactivation overcomes EGFR/Her2-induced EMT, and *b)* whether Stat5 reactivation restores ERα expression in ERα-negative, EGFR/Her2-positive breast cancer *in vitro* and *in vivo*.

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Expected Research Outcomes and Benefits

More than one million new cases of breast cancer are diagnosed each year worldwide, and an estimated 370,000 women die each year. The majority of breast cancer fatalities are caused by metastasis (spreading to other organs) of the primary tumor. Identifying the molecular changes that lead to metastatic progression is critical for developing better strategies to prevent spreading and treat advanced breast cancer. While it is generally accepted that loss of differentiation and

epithelial-to-mesenchymal transition (EMT) are required for breast cancer metastasis, efforts to reverse de-differentiation of breast cancer have been limited.

The Stat5 protein is a suppressor of human breast cancer dedifferentiation, invasion, and progression. Studies are also designed to determine whether pharmacologic inhibition of EGFR/ErbB2 will restore prolactin (PRL)-Jak2-Stat5a/b signaling in breast cancer *in vitro* and *in vivo*. This is important because PRL is a key activator of Stat5 in normal and malignant breast epithelia. If our hypotheses are correct, EGFR/Her2 inhibition combined with Stat5 hyperactivation (e.g., by prolactin or Stat5-ptpase inhibitors) plus antiestrogens may be effective against aggressive ERα-negative, EGFR/Her-2 positive breast cancer.

Furthermore, combined biomarker analyses of Stat5, ERα and EGFR/Her-2 are expected to improve diagnostic classification of human breast cancer and prediction of response to targeted therapies. More effective personalized medicine for breast cancer patients could be a result. Finally, the scientific environment at the Kimmel Cancer Center at Thomas Jefferson University is highly collaborative and the possibilities for translational research are outstanding, so that if successful, the project can move forward with clinical trials.

Summary of Research Completed

We are pleased to report solid progress during year 2 of this project.

Novel Progress related to Aim 1 showing that Prolactin Inhibits BCL6 Expression in Breast Cancer through a Stat5a-Dependent Mechanism

Research related to Aim 2 completed during this fiscal year, has resulted in novel discoveries. We have shown that prolactin inhibits BCL6 expression in breast cancer through a Stat5a-dependent mechanism. BCL6 is a promoter of epithelial-to-mesenchymal transition (EMT), and aggressive breast cancer. BCL6 is a transcriptional repressor that recognizes DNA target sequences similar to those recognized by signal transducers and activators of transcriptions 5 (Stat5). BCL6 disrupts differentiation of breast epithelia, is downregulated during lactation, and is upregulated in poorly differentiated breast cancer.

In contrast, Stat5a mediates prolactin-induced differentiation of mammary epithelia, and loss of Stat5 signaling in human breast cancer is associated with undifferentiated histology and poor prognosis. Here, we identify the mammary cell growth factor prolactin as a potent suppressor of BCL6 protein expression in human breast cancer through a mechanism that requires Stat5a, but not prolactin-activated Stat5b, MEK-ERK, or PI3KAKT pathways. Prolactin rapidly suppressed BCL6 mRNA in T47D, MCF7, ZR75.1, and SKBr3 breast cancer cell lines, followed by prolonged reduction of BCL6 protein levels within 3 hours. Prolactin suppression of BCL6 was enhanced by overexpression of Stat5a but not Stat5b, was mimicked by constitutively active Stat5a, but did not require the transactivation domain of Stat5a.

Stat5 chromatin immunoprecipitation demonstrated physical interaction with a BCL6 gene regulatory region, and BCL6 transcript repression required histone deacetylase activity based on sensitivity to trichostatin A. Functionally, BCL6 overexpression disrupted prolactin induction of

Stat5 reporter genes. Prolactin suppression of BCL6 was extended to xenotransplant tumors in nude mice *in vivo* and to freshly isolated human breast cancer explants *ex vivo*. Quantitative immunohistochemistry revealed elevated BCL6 in high-grade and metastatic breast cancer compared with ductal carcinoma *in situ* (DCIS) and nonmalignant breast, and cellular BCL6 protein levels correlated negatively with nuclear Stat5a ($r = -0.52$; $P < 0.001$), but not with Stat5b. Loss of prolactin-Stat5a signaling and concomitant upregulation of BCL6 may represent a regulatory switch facilitating undifferentiated histology and poor prognosis of breast cancer.

Novel progress that relates to Aim 1 is data showing that Stat5 status predicts prognosis and response to anti-estrogen therapy.

Purpose: To investigate nuclear localized and tyrosine phosphorylated Stat5 (Nuc-pYStat5) as a marker of prognosis in node-negative breast cancer and as a predictor of response to anti-estrogen therapy.

Materials and Methods: Levels of Nuc-pYStat5 were analyzed in five archival materials of breast cancer by traditional diaminobenzidine-chromogen immunostaining, and pathologist scoring in whole tissue sections or by immunofluorescence and automated quantitative analysis (AQUA) in tissue microarrays.

Results: Nuc-pYStat5 was an independent marker of prognosis in node-negative breast cancer when adjusted for other common pathology parameters in multivariate analyses, both by standard chromogen detection with pathologist scoring of whole tissue sections (Material I; n=301) and by quantitative immunofluorescence of tissue microarrays (Material II; n=158). Corresponding results were achieved by two distinct monoclonal antibodies. Breast cancer progression Material III (n=130) revealed frequent loss of Nuc-pYStat5 in invasive carcinoma compared to normal breast epithelia or DCIS, and general loss of Nuc-pYStat5 in lymph node metastases. In Material II loss of Nuc-pYStat5 was associated with increased risk of adjuvant anti-estrogen therapy failure, and subsequent analysis of independent Materials IV (n=166) and IV (n=65) supported this notion. AQUA quantification identified a cohort of patients with low Nuc-pYStat5 who were at especially elevated risk for failure of adjuvant anti-estrogen therapy (multivariate hazard ratio=7.9, 95% CI=2.5-25.0, $p<0.001$).

Conclusions: Nuc-pYStat5 is an independent prognostic marker in node-negative breast cancer. Furthermore, if confirmed in prospective studies, Nuc-pYStat5 may become useful as an independent predictive marker of response to adjuvant anti-estrogen therapy regardless of lymph node status.

Key Research Accomplishments

Novel progress showing that prolactin inhibits expression of the protooncogene, BCL6, in breast cancer through a Stat5a-dependent mechanism.

Novel progress demonstrating a key predictive role of Stat5 status in breast cancer to antiestrogen therapy as well as a prognostic factor.

Research Project 3: Project Title and Purpose

Stat5 and ErbB2 in Prostate Cancer - Organ-confined primary prostate cancer is typically treated by surgery, radiation, hormone therapy, or different combinations of these three treatment modalities, depending on the age and operability of the patient. If prostate cancer has already invaded the neighboring tissues or metastasized to distant sites by the time of the diagnosis, the main treatment options include radiation, hormone therapy and/or chemotherapy (docetaxel) combined with (neo) adjuvant therapies. However, the existing pharmacological therapies for prostate cancer only provide a temporary relief of the symptoms and the cancer growth, while the hormone-refractory form of prostate cancer develops. Moreover, no effective therapies for metastatic prostate cancer currently exist. In this project, the molecular basis of why positive activation status of transcription factor Stat5a/b may provide a biomarker for sensitivity of prostate cancer to ErbB2-inhibitors as a therapy will be examined.

Anticipated Duration of Project

1/1/2009 – 12/31/2012

Project Overview

In this project, the researcher will test the following central hypothesis: Active transcription factor Stat5a/b mediates resistance of prostate cancer to (neo) adjuvant therapies such as ErbB2 inhibition. Preliminary data demonstrates that active Stat5a/b transcriptionally synergizes with wild-type and mutated androgen receptor (AR) in prostate cancer cells, where Stat5a/b increases transcriptional activity of AR. AR, in turn, increases transcriptional activity of Stat5a/b. Second, we show that epidermal growth factor (EGF) activates Stat5a/b in prostate cancer cells, and, importantly, inhibition of ErbB2 protein expression blocks phosphorylation of Stat5a/b. At the completion of this project it is expected to be established whether activation of Stat5a/b would identify those prostate cancers that respond to ErbB2 inhibition by growth suppression, and whether active Stat5a/b combined with phosphorylated ErbB2 provides a better prognostic indicator of poor clinical outcome than active Stat5a/b alone.

Aim 1) Determine whether ErbB2 activates Stat5a/b in a set of human prostate cancer cell lines, and whether ErbB2 inhibition leads to apoptosis of human prostate cancer cells and decreases prostate xenograft tumor growth through inhibition of Stat5a/b.

Aim 2) Determine whether Stat5a/b is the mediator of the synergetic interaction between ErbB2 and AR in prostate cancer cells.

Aim 3) Determine whether active Stat5a/b combined with phosphorylated ErbB2 provides a better prognostic indicator of poor clinical outcome than active Stat5a/b alone.

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Expected Research Outcomes and Benefits

The concept of Stat5a/b as a determinant of therapeutic response of prostate cancer to ErbB2 inhibition is entirely novel. Previous preclinical studies on the role of ErbB2 in the growth of prostate cancer cells have been relatively unsystematic and have yielded conflicting results. The results of clinical trials on ErbB2 inhibitors in prostate cancer have been disappointing. This may be due to clinical trials having focused on advanced prostate cancer only. Second, the trials have not included any selection methods for patients in terms of ErbB2 positivity or activation status of other key growth regulating kinase pathways of prostate cancer. These studies provide the unique opportunity to determine the role of ErbB2 and ErbB2 inhibitors for prostate cancer growth in preclinical models of prostate cancer.

Summary of Research Completed

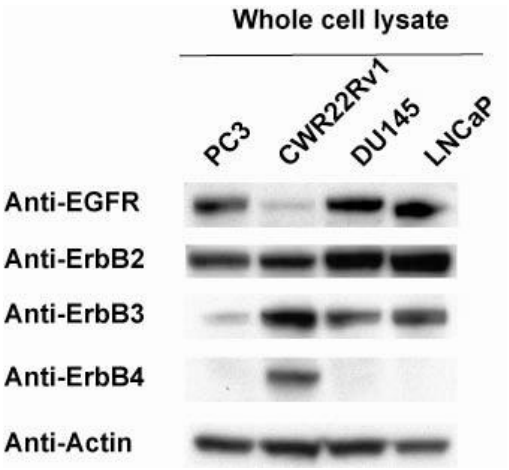
This current project tests the following central hypothesis: Active transcription factor Stat5a/b determines the response of prostate cancer to hormone therapy, radiation therapy and (neo)adjuvant therapies such as ErbB2 inhibition.

ErbB1, 2, 3 and 4 are transmembrane tyrosine kinase receptors consisting of an extracellular binding domain, a transmembrane segment and (except for ErbB3) a functional intracellular tyrosine kinase domain. The tyrosine kinase domains are activated by both homo- and heterodimerization, generally induced by ligand binding. In contrast to the three other HER receptors, the extracellular domain of ErbB2 can adopt a fixed conformation resembling a ligand-activated state, permitting it to dimerize in the absence of a ligand. ErbB2 (185 kDa) is the preferred dimerization partner for the other ErbB family members. Despite the fact that ErbB2 gene amplification is uncommon in prostate cancer, ErbB2 protein is over-expressed in prostate cancer, and the molecular mechanisms underlying the protein over-expression are unclear. The spontaneous dimerization partner of ErbB2 or the signaling pathways activated by ErbB2 in human prostate cancer cell lines or in clinical prostate cancers have not yet been identified. Moreover, ErbB2 is known to induce transcriptional activity of AR in human prostate cancer cells, but the signaling pathway that mediates this effect is so far unidentified. The clinical trials of prostate cancer using Tratuzumab (ERbB2/ErbB2-inhibitor) or Pertuzumab (inhibitor of ErbB2 dimerization with other members of the Erb-family) have not indicated objective benefits for tumor growth. It is important to note that the failure of these trials may have been because these clinical trials did not include any methods of patient selection in terms of activation status of key protein kinase signaling pathways (MAPK, Akt, Stat5). The applicants

propose that Stat5a/b activation in prostate cancer is a prerequisite for a favorable therapeutic response to ErbB2 inhibitors.

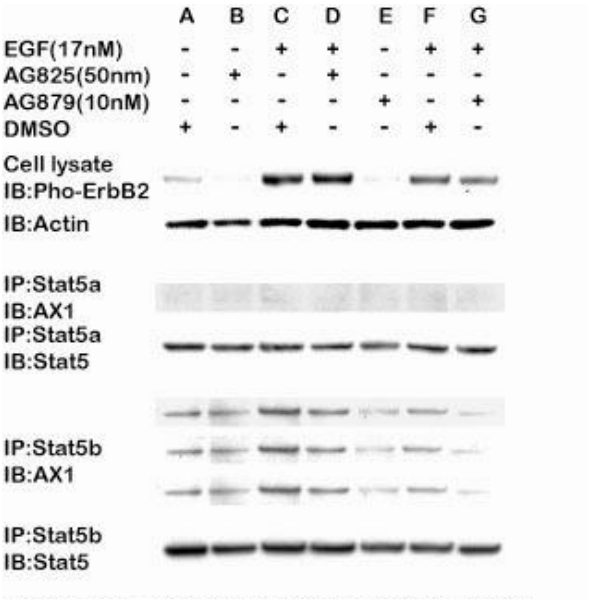
In the current reporting period, the applicants demonstrate the expression levels of EGFR, ErbB2, ErbB3 and ErbB4 in PC-3, CWR22Rv1, DU145 and LNCaP human prostate cancer cell lines (Fig. 1). The applicants further show that AG825, which is a potent and selective inhibitor of HER2 (*neu*/ErbB2; IC_{50} = 0.35 uM) relative to HER1 (IC_{50} = 19 uM) autophosphorylation, attenuates phosphorylation of Stat5b in DU145 cells (Fig. 2), Stat5a and Stat5b in LNCaP cells (Fig. 3), and Stat5a in CWR22Rv1 cells (Fig. 4). Next, the applicants cloned shRNA against ErbB2 to a lentiviral vector and show that ErbB2 inhibition by shRNA inhibits Stat5a/b phosphorylation in CWR22Rv1 human prostate cancer cells. Most importantly, inhibition of ErbB2 resulted in significant decrease in CWR22Rv1 human prostate cancer cell viability (Fig. 5 and 6).

Fig.1.



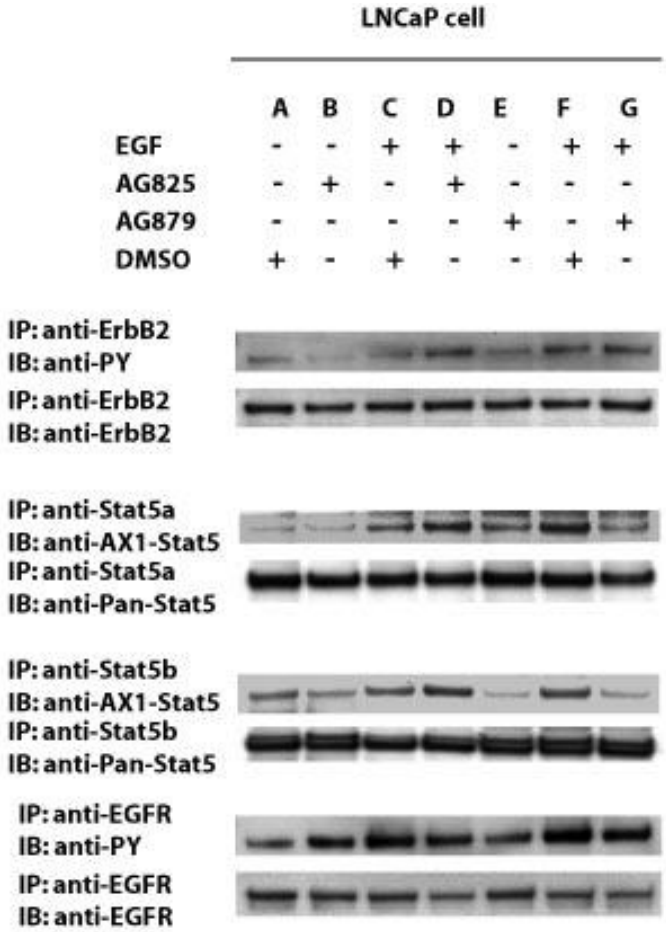
The expression of the members of the EGF receptor family in human prostate cancer cells (Fig.1.). EGFR is highly expressed in PC3, DU145 and LNCaP cells, while the expression of EGFR is weak in CWR22Rv cells. ErbB2 proteins are expressed in PC3, CWR22Rv, DU145 and LNCaP cells. In addition, ErbB3 is highly expressed in CWR22Rv, DU145 and LNCaP cells. Finally, ErbB4 is only expressed in CWR22Rv cells but not in PC3, DU145, or LNCaP human prostate cancer cells.

Fig.2.



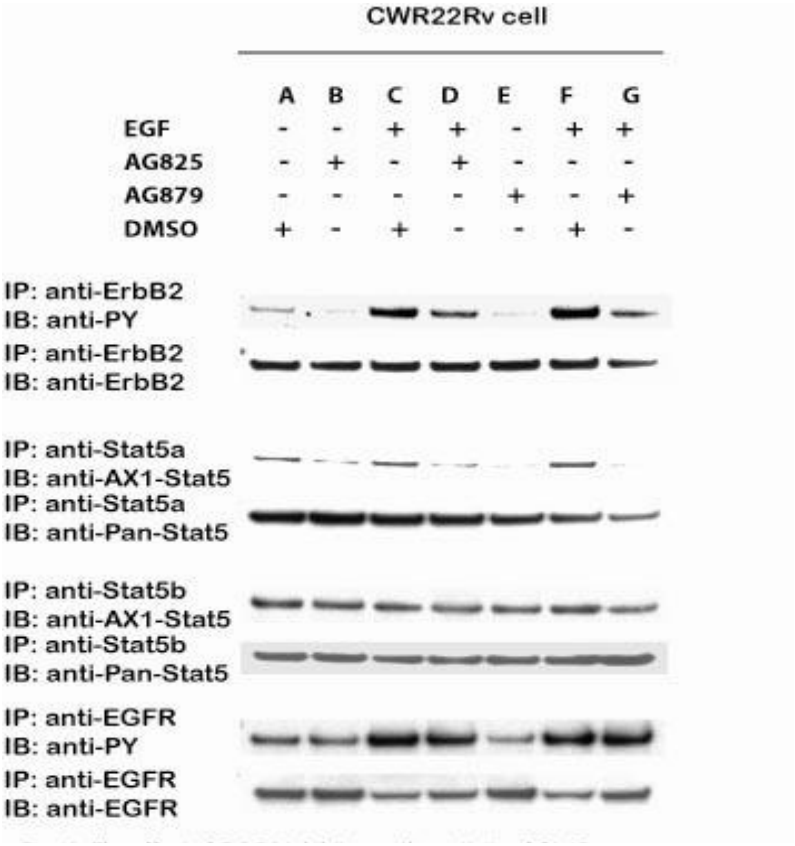
<u>The pharmacological ErbB2 inhibitors block the activation of Stat5a/b in DU145 human prostate cancer cells (Fig. 2).</u> DU145 human prostate cancer cells were exponentially grown and treated with the ErbB2 inhibitors AG825 or AG879 at 50 nM or 10 nM concentrations, respectively, for 48 h. The cells were harvested and Stat5a and Stat5b were immunoprecipitated and immunoblotted with anti-phospho-Stat5a/b polyclonal antibodies. In addition, ErbB2 levels and phosphor-ErbB2 levels were analyzed from the whole cell lysates by Western blotting.

Fig. 3.



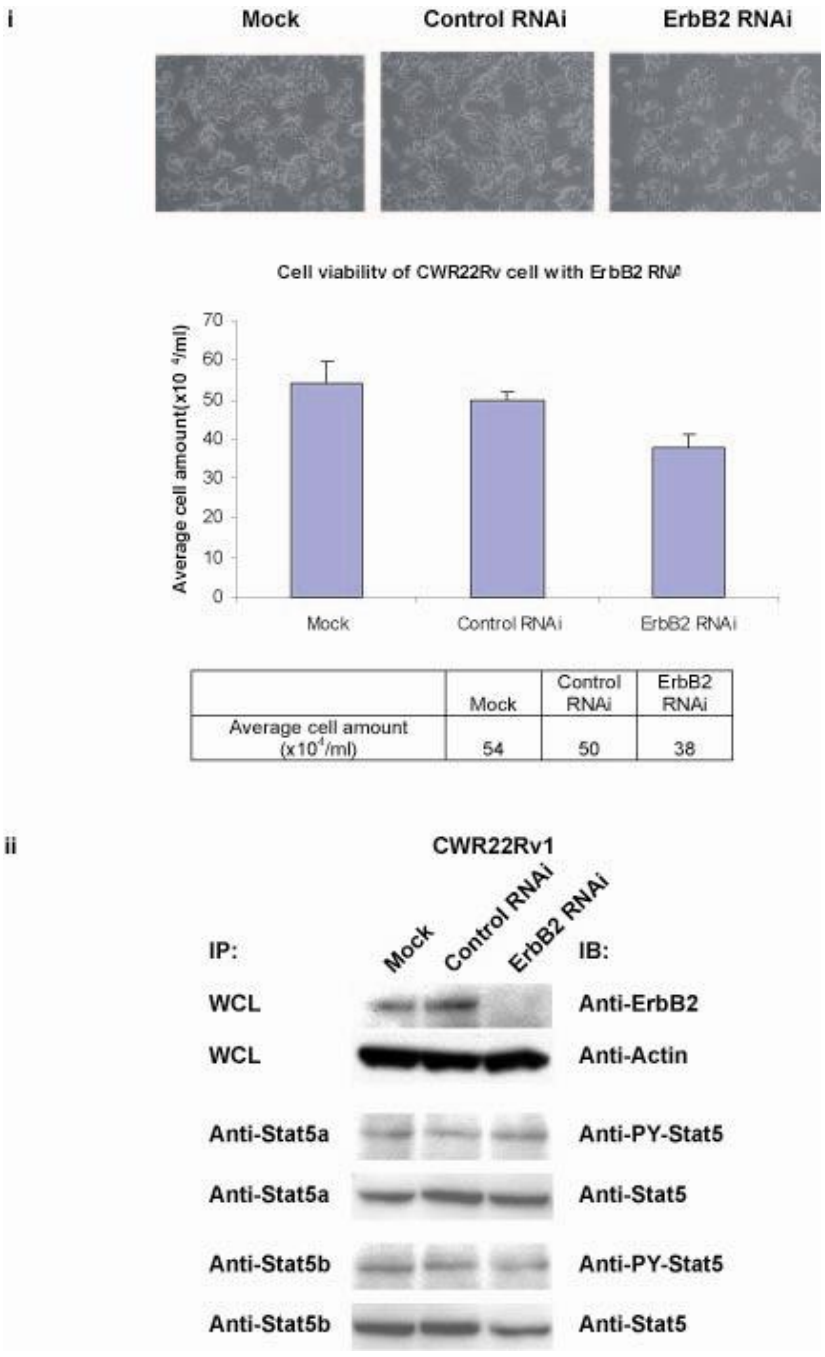
<u>The pharmacological ErbB2 inhibitors block the activation of Stat5a/b in LNCap human prostate cancer cells (Fig. 3).</u> LNCaP human prostate cancer cels were exponentially grown and treated with the ErbB2 inhibitors AG825 or AG879 at 50 nM or 10 nM concentrations, respectively, for 48 h. The cells were harvested and Stat5a and Stat5b were immunoprepcipitated and immunoblotted with anti-phospho-Stat5a/b polyclonal antibodies. In addition, ErbB2 levels and phosphor-ErbB2 levels were analyzed from the whole cell lysates by Western blotting.

Fig .4.



<u>The pharmacological ErbB2 inhibitors block the activation of Stat5a/b in CWR22Rv1 human prostate cancer cells (Fig. 4).</u> CWR22Rv1 human prostate cancer cels were exponentially grown and treated with the ErbB2 inhibitors AG825 or AG879 at 50 nM or 10 nM concentrations, respectively, for 48 h. The cells were harvested and Stat5a and Stat5b were immunoprepcipitated and immunoblotted with anti-phospho-Stat5a/b polyclonal antibodies. In addition, ErbB2 levels and phospho-ErbB2 levels were analyzed from the whole cell lysates by Western blotting.

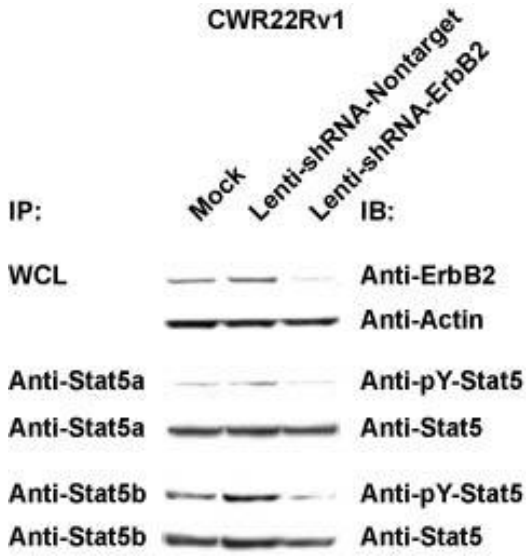
Fig. 5.



	Mock	Control RNAi	ErbB2 RNAi
Average cell amount ($x10^4$/ml)	54	50	38

<u>Inhibition of ErbB2 by lentiviral expression of ErbB2 shRNA induces death of CWR22Rv human prostate cancer cells (Fig. 5).</u> CWR22Rv1 human prostate cancer cels were exponentially grown and treated with the ErbB2shRNA lentivirus for 72 h. The cell viability was determined by the MTT assay and cell counting.

Fig. 6.



When ErbB2 expression was inhibited by lentiviral expression of ErbB2 shRNA in CWR22Rv1 cells, activation of Stat5a/b was efficiently achived (Fig. 6). CWR22Rv1 human prostate cancer cels were exponentially grown and treated with the ErbB2shRNA lentivirus for 72 h. Stat5a/Stat5b were immunoprecipitated and blotted with anti-Stat5a/b phosphor mAb.

Research Project 4: Project Title and Purpose

Targeting the IGF-I Receptor in Cancer - This project proposes to determine conditions which will maximize the therapeutic effect of antibodies to the insulin growth factor I (IGF-I) receptor (a receptor for a growth factor) in cancerous tumors. Many cancers (including lung, colon and breast cancers), have already shown susceptibility to this treatment, and these antibodies are currently being used in clinical trials. Clinicians are now anxious to know which human tumors are most sensitive to the antibodies to the IGF-I receptor. There are intra- and extra-cellular factors that condition the response of tumor cells to treatment and the purpose of this project is to identify these conditions.

Anticipated Duration of Project

1/1/2009 - 12/31/2012

Project Overview

Specific Aim # 1: IRS-1 Acetylation and its Effect on IRS-1 Function. This aim focuses on the IGF-1 receptor (IGF-IR) and its docking protein, insulin receptor substrate I (IRS-1), and their role in colon cancer. The hypothesis is that IRS-1 is the key molecule in the role of the IGF-IR

in cancer, and could possibly be a biomarker for sensitivity of colon cancer cells to antibodies to the IGF-IR.

Specific Aim # 2: Role of IRS-1 in oncogenesis and its relationship to the EGFR. A problem that confronts clinicians when using antibodies to growth factor receptors in cancer therapy is the ability to identify tumors that are particularly sensitive to targeting. This project will focus on why certain tumors become resistant to therapy with receptor antibodies. This is well illustrated in antibodies to the epidermal growth factor receptor (EGFR) that are currently used in clinical practice. This aim will study the problem of resistance to therapy in breast and lung cancer cells, based on the hypothesis that IRS-1 plays a major role in resistance. In addition, we will study the role of Stat proteins in cancer in relation to the interaction with IRS-1.

The objectives will be accomplished using mass spectrometry, and molecular and cell biology methodologies with which this laboratory has over 20 years of experience. Levels of IRS-1 can be modulated by down-regulating it or by increasing it through ectopic expression.

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Expected Research Outcomes and Benefits

The outcome of this project is to provide new criteria for the treatment of human cancer with antibodies to the IGF-I receptor. The ongoing clinical trials have indicated that antibodies against the IGF-I receptor are effective against various forms of cancer, and Phase II clinical trials are now being carried out at several institutions in cancers of the lung, breast, and colon, brain tumors and bone sarcomas. No cancer treatment is effective against all forms of cancer, and resistance to treatment is unfortunately a frequent outcome with almost all therapies. The benefits of this research will allow clinicians to apply the specific therapy that will yield the best results when using antibodies to the IGF-IR. The project will also define the mechanisms of resistance of cancer cells to therapeutic interventions. This is an exquisitely translational research project, with basic science exploring modalities to be directly applied in clinical venues.

Summary of Research Completed

We have continued our research focused on optimizing the treatment of human cancers by the targeting of the insulin-like growth factor receptor-1 (IGF-IR). Our approach has followed two

different avenues of investigation. The first approach has been to study how the IGF-IR relates to other oncogenes and tumor suppressors. We have shown that the insulin receptor substrate-1 (IRS-1), a docking protein for the IGF-IR, interacts directly (demonstrated by immuno-precipitation) with the oncogene c-met, the receptor for hepatocyte growth factor (HGF). IRS-1 and c-met do not only interact, but they de-stabilize each other. When IRS-1 is highly expressed, c-met is down-regulated, and when c-met is highly expressed, IRS-1 is down-regulated.

This surprising finding (two oncogenes down-regulating each other) has an important implication. c-met promotes motility (its ligand HGF was originally described as scatter factor for its ability to increase cell motility), while IRS-1 is known to inhibit motility. This finding may explain a paradox of IRS-1 expression. The literature shows unambiguously that IRS-1 is strongly mitogenic, anti-differentiating and anti-apoptotic. Yet, IRS-1 levels are often decreased, not in primary cancers, but in metastatic cancers. We and others, aware of the fact that IRS-1 inhibits cell motility, proposed that IRS-1 is decreased in metastatic human cancers (especially breast cancer metastases) because cells need motility to metastasize. The decrease in IRS-1 expression would be compensated by an increase in the oncogene c-met.

These results could have an immediate impact on future approaches to therapy. In the absence of IRS-1, the IGF-IR sends a differentiation signal; only when IRS-1 is well expressed, the IGF-IR sends a mitogenic, transforming signal. Targeting the IGF-IR in metastatic tumors (where IRS-1 is decreased or absent) could actually have an adverse effect, because it would attenuate the pro-differentiation signal originating from the IGF-IR. Our finding, available on line at the web site of the Journal of Cellular Physiology, was well documented, thanks to the wealth of cell lines and reagents we have accumulated in the more than 20 years we have dedicated to the study of the role of the IGF-IR in cancer. We have used as controls cells that do not express IRS-1 or c-met, or the IGF-IR, and the result is a solid result. The 2nd implication of this finding is more theoretical. Why would 2 oncogenes antagonize each other? We are pursuing an answer to this question and a clue may be found in another result we have obtained.

This second result has been the product of our collaboration with the laboratory of Dr. Richard Pestell, of the Kimmel Cancer Center, where they have shown repeatedly that DACH1 acts as a tumor suppressor in breast and prostate cells. DACH1 is the human homologous of Dachsund, a Drosophila gene required for the development of eye and limb. Clinical and experimental evidence have established DACH1 as a tumor suppressor, and we became interested in the possible role that the IGF-IR may have on DACH1 expression. Somewhat surprisingly, we have found that IGF-1 actually increases the expression of DACH1, under conditions in which IGF-1 is mitogenic. In this case, instead of two oncogenes antagonizing each other, we may have 2 growth factors antagonizing each other. We are actively pursuing this paradoxical finding.

Finally, we have continued our studies on microRNA 145, a tumor suppressor microRNA (miR) that targets the IGF-IR and IRS-1. We have discovered an unusual aspect of miR145 by isolating a genomic clone that, transfected into cells, expresses a mature miR145. The clone has two highly conserved sequences (from armadillo to man), one on the 5' side of the pre-miR sequence, the other on the 3' side of the pre-miR sequence. When the highly conserved sequence on the 3' side of the pre-miR is deleted, mature miR145 expression is abrogated. Deletion of the highly conserved sequence on the 5' side has no effect. Although this is an unabashedly basic

science finding, one should remember tat miRs are now becoming possible players in a clinical setting. Previous research has shown that an injected tumor suppressor miR actually inhibited tumor growth in a xenotransplant in mice. In addition, miRs can now be detected in human plasma, and a recent publication has shown an increase in plasma levels of a specific miR in human prostate cancer cases. miR145 is an established tumor suppressor and may play a significant role in human cancers.

Research Project 5: Project Title and Purpose

The Role of MicroRNA Gene Expression in Therapy Resistance of Human Breast Cancer - This year breast cancer will kill over 40,000 women in the United States, and there will be over 210,000 new cases, according to statistics from the American Cancer Society and Centers for Disease Control. Since the risk of developing breast cancer increases with age, the prevalence of this disease can be expected to increase as the overall population continues to age. However, studies on the underlying genesis and progression of breast cancer, utilizing the latest techniques of molecular biology, offer hope of finding more effective treatments. Studies over the last two years have demonstrated that the presence of "stem cells" which are found within human breast cancer, contribute to therapy resistance. This project will focus on a class of genes called the microRNA (miRNA) found in breast cancer stem cells, and their role in therapy resistance and breast cancer metastasis.

Anticipated Duration of Project

1/1/2009 - 12/31/2012

Project Overview

The long term goal of this project is to obtain important information about the molecular events associated with breast cancer, and develop new molecular targets with which to improve the treatment. Metastatic breast cancer is incurable and lethal: its treatment at present is only palliative, intended to relieve pain and suffering, but not to cure the disease. In order to understand metastatic breast cancer this project will identify new molecular targets for its treatment, by isolating cells involved in the progression from the primary tumor to the metastatic tumor, and determine the changes in miRNA gene expression that accompany this progression.

The specific aims and methods of the project are:

1) Isolate the cellular intermediates in the progression from the primary breast tumor to the metastatic tumor and determine the changes in miRNA gene expression that accompany this progression. Based upon evidence that circulating tumor cells (CTCs) found in the peripheral blood are linked to the spread of breast cancer, a combined screening called the CellSearch system will be used to identify CTCs from patients with metastatic disease.
2) Elucidate the changes in miRNA expression in breast cancer associated with the development of resistance to tamoxifen and chemotherapeutic agents therapy. The miRNA expression profiles in tamoxifen-resistant tumors and cell lines selected in culture will be determined. These

will be compared to the miRNA expression profiles in their tamoxifen-sensitive counterparts as either tumors or the parental cell lines in culture.
3) Elucidate the miRNA signatures regulating breast cancer stem cells. The migration of these mammary stem cells in response to stem cell factor (SCF) will be examined. To determine the specificity of the cells enriched by epitope markers in SCF, complementary approaches will be used: 1. slide populations (SP) analysis; 2. epitope staining for Sca1 and other putative progenitor cell markers; 3. BrdU labeling retention; and 4. heterotypic transplantation.

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Expected Research Outcomes and Benefits

One of the keys for improving current modes of cancer treatment is identifying which specific genes are being expressed in tumors. Using this approach could lead to the development of treatments tailored to the unique gene expression patterns in breast cancer patients. This study will focus on the patterns of miRNA expression in relation to the interplay of cellular factors affecting the growth of breast cancer cells, on the changes in miRNA expression associated with the most lethal stage of breast cancer, namely metastasis, and on the changes in miRNA expression that are associated with resistance to chemotherapy.

Historically, it has been both time-consuming and technically difficult to screen large numbers of tumors for cancer-specific gene expression. To overcome these problems, a powerful tool for the study of gene expression called microarray analysis was employed. Essentially, a custom "biochip" that combines tens of thousands of gene probes on a single glass slide was created by researchers here at the Kimmel Cancer Center. It has enabled efficient screening for the expression of thousands and thousands of genes in a broad range of human tumors (referred to below as "expression profiling"). Knowing which of the hundreds of miRNA genes are preferentially expressed in various cancers could help to improve diagnosis and lead to innovative therapies for those cancers.

Summary of Research Completed

This project period focued on Aim 3- Elucidate the miRNA signatures regulating breast cancer stem cells. The migration of these mammary stem cells in response to stem cell factor (SCF) were examined. To determine the specificity of the cells enriched by epitope markers in SCF,

complementary approaches were used: 1. SP analysis; 2. epitope staining for Sca1 and other putative progenitor cell markers; 3. BrdU labeling retention; 4. heterotypic transplantation.

In order to examine the cyclin D1 and Rb dependent micro RNA molecular circuitry and their contribution to BTIC (breast cancer stem cells), we first examined the mirna circuitry regulated by pRB. cyclin $D1^{-/-}$ and $Rb^{-/-}$ mice were intercrossed to develop cyclin $D1^{-/-}$, $Rb^{-/-}$, and cyclin $D1^{-/-}$ and $Rb^{-/-}$ fibroblasts. Molecular genotyping demonstrated the deletion of the cyclin D1 gene in cyclin $D1^{-/-}$ mice and the loss of cyclin D1 protein abundance (Figure 1B). In the cyclin $D1^{-/-}/Rb^{-/-}$ and cyclin D1/Rb protein was abolished (Figure 1B). View of those cell sizes was not significantly different between the genotypes. The *cyclin* $D1^{-/-}$ cells were significantly reduced in proliferation rate. The $Rb^{-/-}$ were increased in proliferation and cyclin D1/Rb double knockout showed no significant change in proliferation (Figure 1B). Genome-wide expression profiling, micro RNA profiling was conducted on the mouse embryo fibroblasts.

Twenty miRNA were selectively altered in abundance in the cyclin $D1^{-/-}$, six miRNA were selectively altered in the $Rb^{-/-}$, and thirteen miRNA were lost in both $DR^{-/-}$ and $Rb^{-/-}$ cells (Figure 2A). MIR-16B and 24 were reduced in *cyclin* $D1^{-/-}$ cells (Figure 2E). Blot analysis confirmed mIR-16 was unaltered in the $pRB^{-/-}$ but reduced in the *cyclin* $D1^{-/-}$ (Figure 2C) and re-expression of cyclin D1 in cyclin $D1^{-/-}$ MEFs restored MIR-16 abundance (Figure 2D). MIR-24 abundance was reduced in the cyclin $D1^{-/-}$ cells (Figure 2F). The $Rb^{-/-}$ cells showed a reduction in MIR-221 (Figure 3A). Re-expression of the Rb induced MIR-221 (data not shown). Cyclin D1a expression did not affect MIR-221 consistent with the original micro RNA analysis (Figure 2A).

Surprisingly, re-expression of cyclin D1b induced MIR-221 abundance (Figure 3A). The presence of cyclin D1a and cyclin D1b in the cyclin $D1^{-/-}$ cells was confirmed by the detection of the flag epitope of the retroviral expression vectors (data not shown). The finding that cyclin D1a was unable to induce MIR-221 further but that cyclin D1b was, led us to examine further, the molecular mechanisms governing MIR-221 abundance. Cyclin D1a and cyclin D1b differ by the carboxyl terminal, alternately-spliced region (Figure 3B). The cyclin D1a and cyclin D1b isoforms differ in their ability to induce the expression of $p27^{KIP1}$ [1]. In order to determine whether the induction of p27 by cyclin D1b was responsible for differential induction of 221, the relative abundance of p27 was assessed in the cyclin $D1^{-/-}$ MEFs transduced with cyclin D1a or cyclin D1b flag tagged retroviral former expression vectors (Figure 3C).

Cyclin D1a induced p27 although cyclin D1 failed to induce p27, as previously shown (Figure 3C). The time of course of induction was concordant using the kinetics with which cyclin D1b induced MIR-221 (Figure 3D). Cyclin D1b induction after 6 hours occurred concurrently with induction of MIR-222 and MIR-222. A survey of breast cancer cell lines demonstrated the inverse relationship between MR-221 and p27 abundance. The p27 3′UTR contains a mIR-221/222 binding site (Figure 3F). Luciferase reporter genes encoding the p27 3′UTR and a point mutation of MIR-221 binding site demonstrated the induction of the relatively higher activity of the p27 3′UTR containing the mutation of the MIR-221 binding site compared with the wild-type sequence (Figure 3G). Collectively, these studies demonstrate that the p27 3′UTR contains a site of repression via MIR-221. These studies are consistent with the model in which pRb induces MIR-221 and cyclin D1b induces MIR-221 via p27. This pathway functions independently of cyclin D1a.

In order to determine the functional significance of increased MIR-221 expression, MIR-221 transduced and the MDA-MB-231 cells were analyzed in migration assays (Figure 4A). The MDA-MB-231 cells expressing MIR-221 and anti-murine MIR-221 and MIR-222 demonstrated the reduction in MIR-221 expression by the anti-MIR-221. The anti-MIR-221 or anti-MIR-222 reduced the rate of wound closure consistent with the role of MIR-221 to enhance cell migration (Figure 3C)

Methods Cyclin D1 and pRb heterozygote mice were intercrossed to derive MEFs. Human breast cancer specimens and matching normal breast tissue samples (frozen tissues) were provided by Dr. McCue at Thomas Jefferson Hospital. All the procedures were approved by the Institutional Review Board (IRB) of Thomas Jefferson University. $pMSCV_{puro}$ vector was used to express miR-17/20 cluster as described previously. Wild type and mutated *p27* 3′ UTR sequences were inserted into the *Xba*I - *Fse*I site immediately downstream of the stop codon in the pGL3 control Firefly Luciferase reporter vector**.** MCF-7 cells were transduced with the $pMSCV_{puro}$ vector expressing each miRNA cluster or vector control as described previously. The same number of miR-overexpressing cells and control cells were plated at equal seeding densities.

After attachment to the plates, cells were washed twice with PBS and overlaid with phenol red free DMEM without serum and allowed to grow for another 24 hours before collecting the medium (supernatant) from the cultures. The collected medium was centrifuged at 2000 x g for 10 min and filtered through a 0.2 µm membrane to remove cellular debris and intact cells to yield conditioned medium. For proliferation assays the absorbance value for each well was corrected by subtracting the reading at 570 nm from the reading at 405 nm**.** Data are presented as mean ± SEM. Student's t-test was used for analysis, only $P < 0.05$ was considered significant.

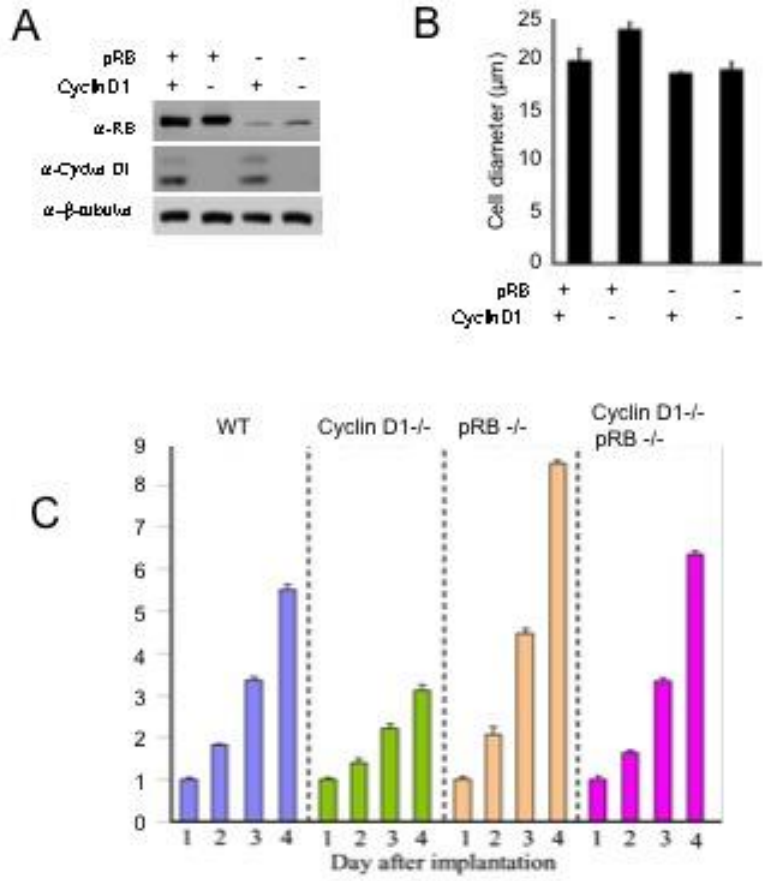

Figure 1 Cyclin D1$^{-/-}$ proliferative defect is restored by the Rb deletion. (A) Western blot analysis of pRB$^{-/-}$, cyclin D1$^{-/-}$, and pRb/cyclin D1$^{-/-}$ MEFs. (B) Cell diameter of MEFs derived from three distinct transgenic phenotypes with comparison to wild-type MEFs and (C) Cellular proliferation assays of the MEFs for each genotype.

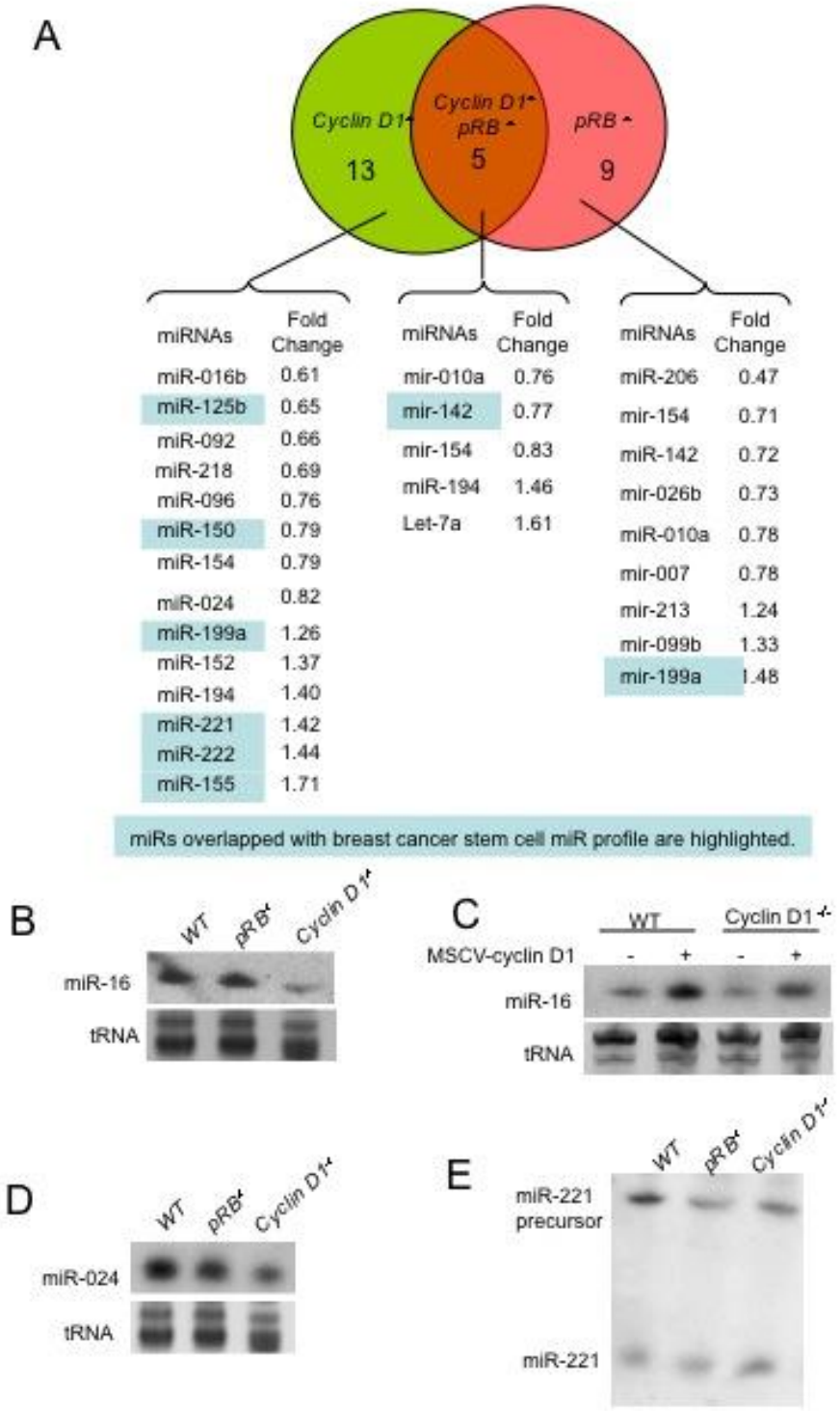

Figure 2 Cyclin D1 vs. pRB micro RNA expression profiling. Micro RNAs derive from cyclin $D1^{-/-}$, $pRB^{-/-}$ or cyclin $D1^{-}$, $pRb^{-/-}$. MEFs were compared and are shown within the Venn Diagram. (A) In blue are micro RNAs shown previously to play a role in regulating stem cell function. (B) The northern blot analysis of mit16 abundance in the MEFs as indicated, and (C) Upon transduction with a retroviral expression vector encoding cyclin D1. (D) Northern blot analysis for MIR024 in each genotype, and (E) mIR-221

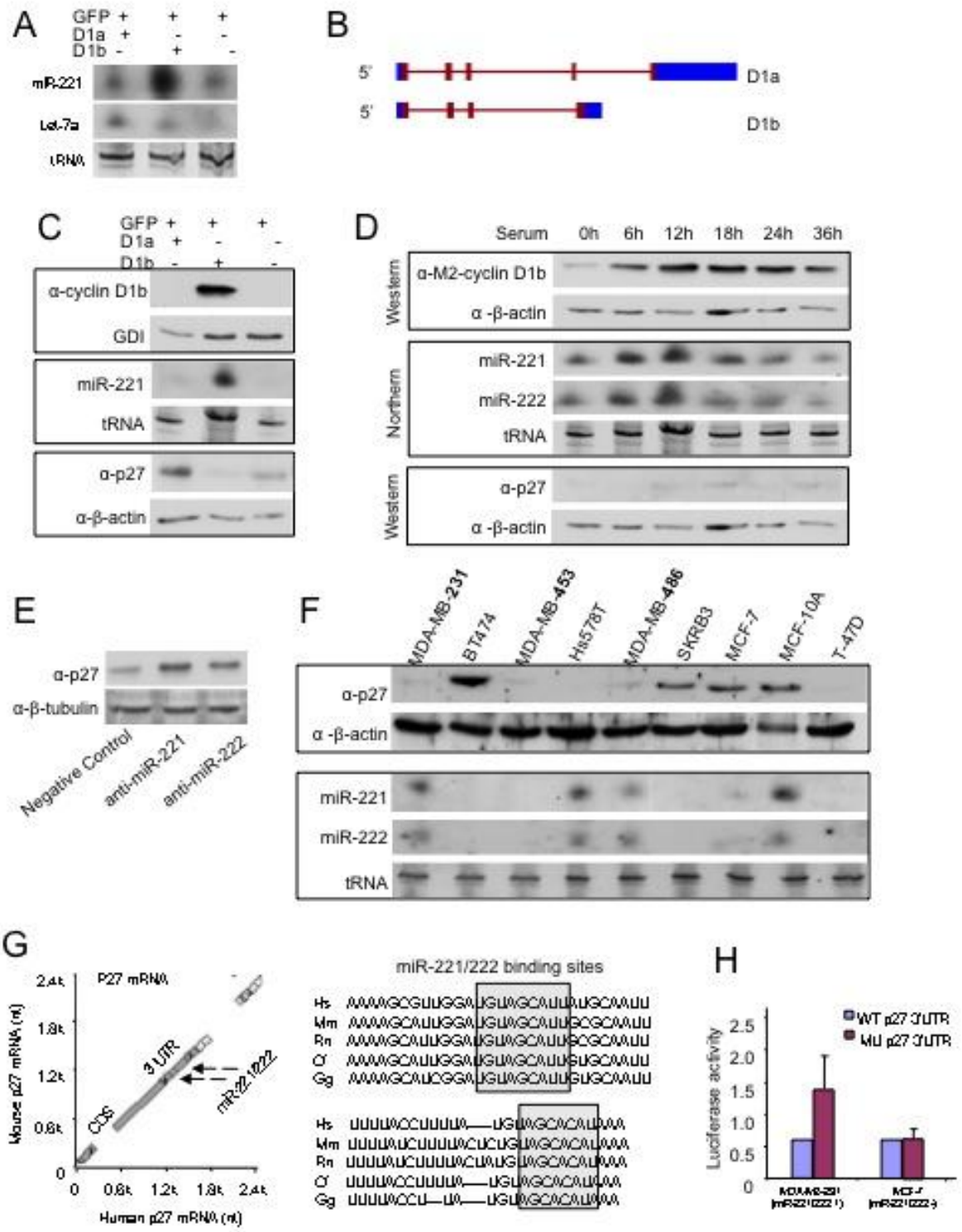

Figure 3. MIR-221 abundance in cyclin D1$^{-/-}$ cells rescued with either cyclin D1a or cyclin D1b expression vector. (A) The schematic representation of the cyclin D1a and (B) cyclin D1b protein (C) Relative abundance of MIR-221 and p27 in cyclin D1$^{-/-}$ MEFs transduced with either cyclin D1a or cyclin D1b expression vector. (D) Relative abundance of MIR-221/MIR-222. Upon serum reduction of cyclin D1$^{-/-}$ MEFs transduced with a M2 flagged epitope tagged cyclin D1b expression vector. Relative abundance of p27 is shown by Western blot. (E) MEFs transduced with either anti-MIR-221 or anti-MIR-222 with Western blot analysis for p27, beta turbulent shown as loading control. (F) Relative abundance of MIR-221/MIR-222 in breast cancer cell lines. (G) p27 3′ UTR of mouse and human with MIR-221/MIR-222 binding site,and (H) luciferase activity of the p27 UTR either wt or mutant of the mir222 binding site, in presence of coexpressed mir as indicated in the figure. Data are mean + SEM for N> 5 separate experiments.

Research Project 6: Project Title and Purpose

Mechanisms for Metastasis Suppression through Kisspeptin Regulation of the Microenvironment
Metastasis (spreading) is an unfavorable milestone in the clinical course of cancer. The ability of tumor cells to spread from the primary site and invade normal tissues requires a specific set of properties enabling them to proliferate and migrate. It is also critical that these malignant cells have a supportive niche within which they can expand. The KiSS1 gene was discovered based on its ability to block metastasis of human cells in mouse models of melanoma. Current evidence suggests that the product of this gene is secreted by tumor cells and prevents the migrating cells from developing a niche. These experiments, which will elucidate the mechanisms of action, may provide novel, non-toxic approaches for the treatment and prevention of tumor metastasis.

Anticipated Duration of Project

1/1/2009 - 12/31/2012

Project Overview

Metastasis is a grim milestone in the majority of cancers. The spread of tumor cells to secondary sites requires a cascade of properties that enable them to invade normal tissues, migrate into blood vessels, and proliferate while detached from other cells. Also required is a receptive, supportive environment that can be induced by the tumor cells through tissue remodeling, and stromal cells to provide a microenvironment that fosters growth. The cellular elements of this microenvironment include inflammatory cells, endothelial cells, fibroblasts, and myofibroblasts. The latter two populations have been designated carcinoma-associated fibroblasts, and these cells have been found to promote malignant properties. The stroma of metastatic tumors have highly similar gene expression patterns to that of wound healing, and is therefore associated with the "wound-response gene signature" of metastasis and poor prognosis.

The KiSS1 gene encodes a secreted protein that is processed to peptides (kisspeptins: KP). KP was discovered to be absent from metastatic tumors, and able to suppress metastasis in model systems. While KPs are ligands for GPR54, a G protein coupled receptor, the mechanism for metastasis suppression has not been elucidated, and the cells that express GPR54 have not been identified. Since KP expression can inhibit pulmonary metastasis from tumor cells that lack GPR54 expression, it is likely that a paracrine mechanism is active. It has been demonstrated that low levels of GPR54 are expressed by fibroblast cell lines. The objective of this project is to elucidate the paracrine mechanism responsible for metastasis suppression by kisspeptins, and to identify GPR54+ target cells responsible for these effects. The hypothesis of the project is that KP secretion decreases the formation of activated stromal fibroblasts and myofibroblasts. This hypothesis will be tested in mouse wound healing (Specific Aim 1) and pulmonary fibrosis (Specific Aim 2) models to determine whether administration of KPs or novel downsized analogs, can block the formation of the fibroblastic microenvironment.

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Expected Research Outcomes and Benefits

Despite advances in chemotherapy, the metastatic spread of cancer is still associated with a poor prognosis, and few effective treatments are available. Kisspeptins (KPs) are ligands (bonding agents) for a G protein coupled receptor, which is the class of targets for about one-third of drugs. Thus, the demonstration that KPs are effective anti-metastatic agents will open the door to a new type of therapy. Prior to the pursuit of a therapeutic role for KPs, it is critical to develop an understanding of the precise mechanisms for suppression of metastasis, which to date has been elusive. The available data indicates that KPs do not directly inhibit the spread of tumor cells. Instead, there is evidence that they control the local environment for migrating tumor cells and prohibit the formation of a supportive niche. Thus, it is possible that a therapy based on KPs would block the ability of tumor cells to establish a receptive environment.

One approach to determining the mechanism for metastasis suppression by KPs, is to study a similar environment and identify its targets that express the corresponding receptor (GPR54). Since there is evidence that GPR54 is expressed in higher levels by lung fibroblasts, we will characterize the effects of KPs to identify relevant targets and mechanisms in pulmonary fibrosis models and in wound healing using mice. This project will also provide insight into the effects of KPs on regenerative processes while maintaining relevance to tumor therapy, because the wound healing environment has extensive molecular similarity to that of malignant tumors. The targeting of drugs to block host responses instead of tumor functions has the potential advantages of decreased toxicity and decreased emergence of resistant tumor cells.

Summary of Research Completed

The research conducted over the past year focused on defining the role of the metastasis suppressor KISS1 and its receptor GPR54, in tumor cell metastasis and more specifically in the tissue remodeling occurring during invasion of the metastatic site. Since it is believed that common mechanisms of tissue remodeling exist in metastasis and other physiological or physiopathological processes, such as wound healing and lung fibrosis, mouse models of wound healing and lung fibrosis were chosen to evaluate the influence of KISS1 and GPR54.

A new synthetic compound was used as an agonist of GPR54, in the focus on wound healing. The decapeptide KP10 derived from the normal processing of KISS1 is the most active physiological agonist of GPR54, but its sensitivity to proteolytic degradation by matrix metalloproteinases makes it very short-lived in vivo. We previously used TOM80, a pentapeptide derived from KP10, with increased stability as an agonist of GPR54. A new compound (FTM145), which was recently obtained by our collaborator Dr. Fujii (University of Kyoto), is also derived from KP10. This modified pentapeptide was shown to have a very high resistance to proteases with an in vitro half-life in mouse serum over 24 hours, compared to less than 1 hour and about 2 hours for KP10 and TOM80, respectively. FTM145 was first fully characterized in vitro for its activity as an agonist of GPR54. FTM145 was dissolved at 5 mM in 1:1 PBS: DMSO, diluted to 500 uM in PBS, sterile filtered (0.2 um) and finally diluted in sterile PBS to the working concentration of 50 uM. The biological activity of this preparation was tested against the similarly prepared reference solution of KP10. HEK-293 cells transfected with human GPR54 were seeded overnight into tissue culture dishes containing complete medium, starved for 16 hours and exposed for 5 minutes to concentrations of KP10 or TOM80 ranging from 1 nM to 100 nM. Whole cell lysates were made in SDS lysis buffer. Since ERK MAP kinase was shown to be a signaling pathway activated upon GPR54 stimulation, phosphorylation of ERK was assessed by western blotting using antibodies to total ERK and phospho-ERK detected by secondary antibodies labeled with infrared dyes. An infrared scanner (LI-COR) was used to analyze the membranes. Results show that KP10 and TOM80 activate GPR54 with an identical potency.

We then conducted experiments to evaluate the effect of KP10 and FTM145 on wound healing as well as the expression of GPR54 in the healing skin. In the previous experiment, we observed that male mice would frequently fight and inflict additional wounds to each other, thus possibly introducing some artifacts in the data. As a consequence, we chose to work exclusively with females for the subsequent experiments. We also previously noticed an elevation of GPR54 at an early time point during healing (7 days after wounding) but not when healing was complete (12 days). In the next experiments, we chose to study samples after 2 days (inflammatory phase) and 5 days after wounding. C57BL/6J mice (9 weeks old, females) were anesthetized with pentobarbital and received a full thickness skin excision performed with a 6 mm biopsy punch in the lumbar area. The excised skin was immediately snap frozen in liquid nitrogen for RNA studies (T=0). The mice were immediately divided in 3 groups of 12 animals each, receiving i.p. injections of either PBS (control group), KP10 or FTM145 (50 uM each, 100 to 150 uL depending on the weight of the animal).

The concentration and volumes of compound used were chosen based on an expected peak concentration of about 1 uM in the extracellular compartment. The animals were injected with PBS or the compounds at the time of the skin incision and every day thereafter. The size of the wounds was measured at the time of the sacrifice of the animals. Half of the mice (6 in each group) were sacrificed after 2 days. Since the wounds had not started to close at that time point, the wound bed and the skin in a 2 mm radius around the wound were carefully excised with scissors. The tissues were cut with a scalpel in 2 identical halves: one was fixed for 24 hours in PBS-4% paraformaldehyde for histological studies, the other was placed in a 1.5 mL microtube and snap frozen in liquid nitrogen for RNA studies. The remaining mice were sacrificed 5 days after wounding and skin tissues centered on the wounded area were obtained using a 6 mm skin

punch and processed in an identical fashion. A contraleteral punch was performed as a control and was also snap frozen.

The fixed fragments were then embedded in paraffin and sections were stained with hematoxylin and eosin and observed by microscopy. The frozen fractions were used for total RNA extraction using a mIRvana kit (Ambion). Purified samples were quantified on a Nanodrop (Thermo) and analyzed on a 2100 Bioanalyzer (Agilent) to verify the integrity of the RNA. All RNAs were found to be of high quality with an integrity number above 8. After reverse transcription (Sigma), samples were analyzed for GPR54 content by q-PCR as described previously using a SYBR green-based method (QIAGEN) for detection of the PCR products. The q-PCR reactions were performed on a Stratagene MX3005P real-time thermocycler (95°C initial denaturation for 15 min, followed by 40 cycles consisting of 94°C denaturation, 45 sec, 60°C annealing, 45 sec, 72°C elongation, 45 sec) and analyzed using MxPro software (Stratagene). The nature of the amplification products was tested by performing a melting curve at the end of the amplification cycles. Quantitative detection of GAPDH was performed in parallel and all GPR54 data were normalized to GAPDH for the same sample.

The size of the wound appeared to be independent of the treatment received at either time point (Figure 1). Examination of the FFPE sections stained by H&E did not reveal any obvious difference between groups, with similar granulation reactions and initial re-epithelialization. The data of qRT-PCR do not indicate a major influence of KP10 or FTM145 on the expression of GPR54 in the wounded area at day 5 but a relatively small but significant increase at 2 days post-wounding appears with KP10 and FTM145 but not in the PBS group compared to the T=0 samples (Figure 2 and Figure 3). However, qRT-PCR analysis confirmed an increase of GPR54 expression at 5 days compared to day 0 and day 2, irrespectively of the treatment received. This increase was specific for the wounded area as the tissues contralateral punches exhibit levels of GPR54 identical to the T=0 samples. This increase of expression of GPR54 was highly significant in all 3 groups (Figure 4). These data indicate either an up-regulation of GPR54 in cells residing in the skin before wounding or, more likely, the expression of GPR54 by cells recruited to the wounded area during the healing process.

A detailed analysis of the histology of the FFPE sections was performed in order to attempt to identify the cells recruited to the wound and identify the possible differences induced by the GPR54 agonists. Antibodies to alpha smooth muscle actin and collagen type I (for identification of myofibroblasts), von Willebrand Factor and CD31 (endothelial cells), F4/80 (macrophages) and CD3 (T lymphocytes) were used to stain FFPE sections by immunohistochemistry. However, the Ventana used for processing of the slides yielded a high background and optimization of the staining is required.

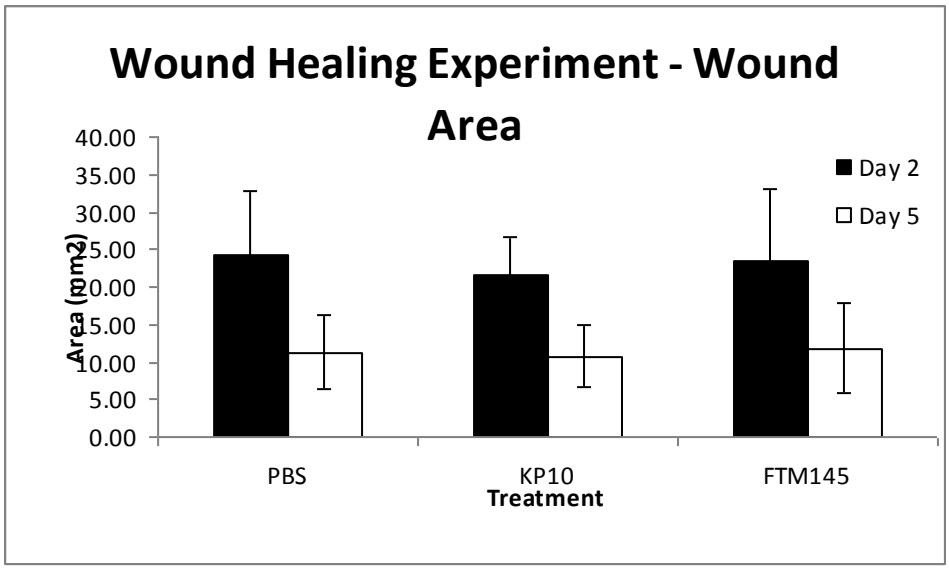

Figure 1: Measurement of wound area.

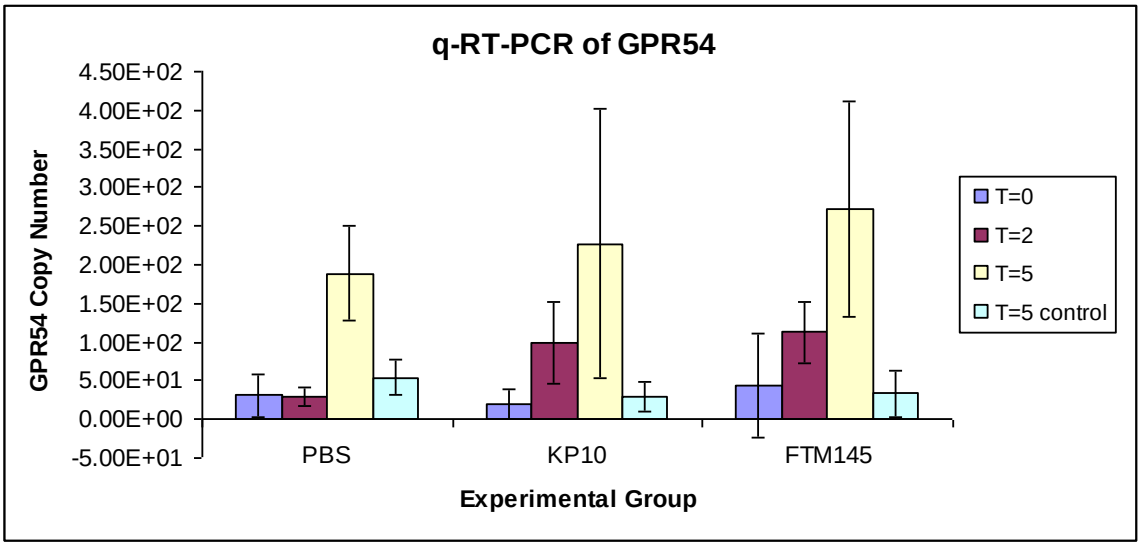

Figure 2: Summary of the quantification of GPR54 by quantitative PCR in the excised wounds.

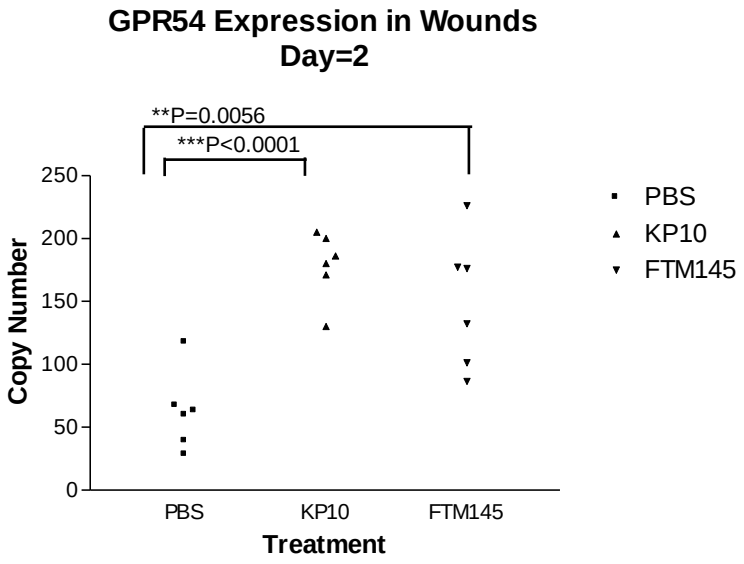

Figure 3: Treatment with KP10 and FTM145 increases the expression of GPR54 in the wounds as assessed by q-RT-PCR.

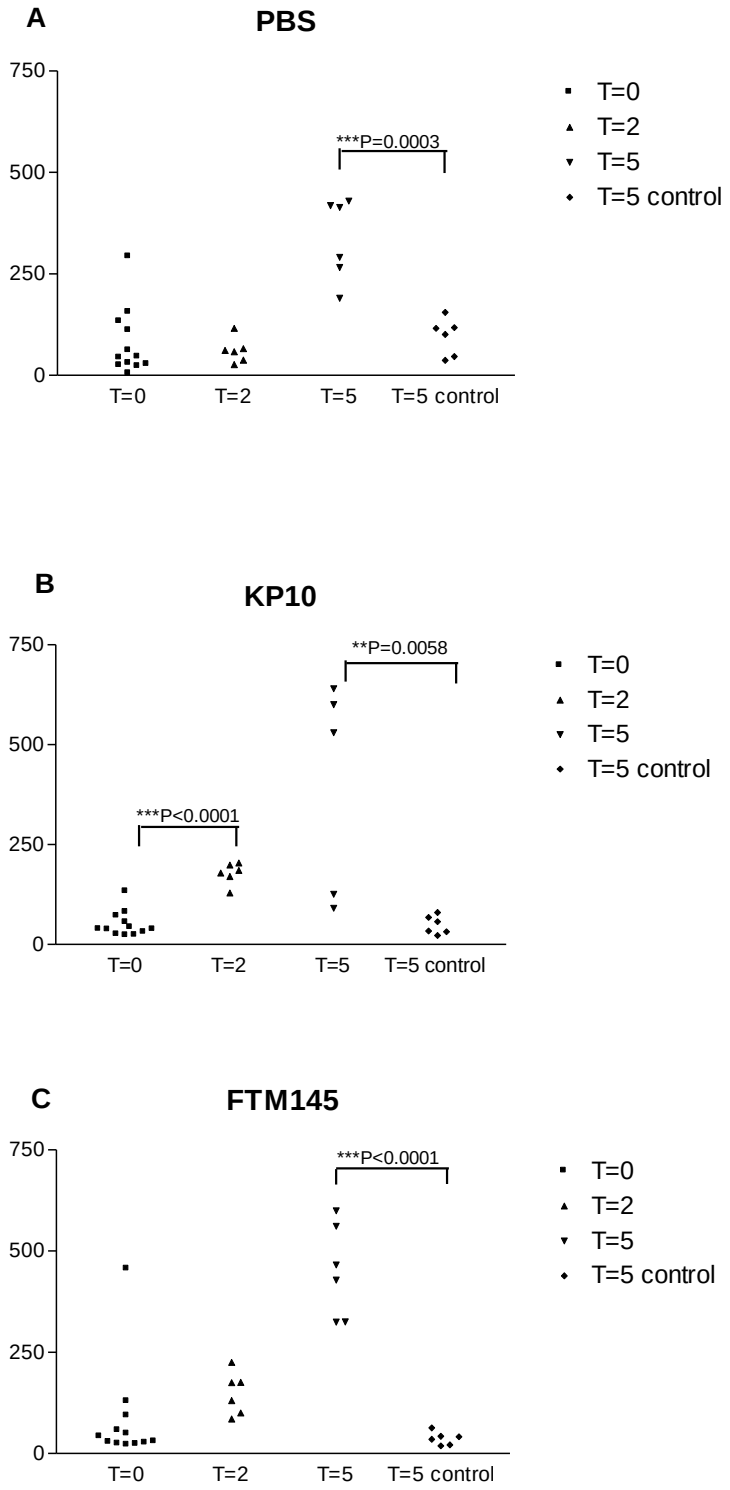

Figure 4: Statistical analysis of the data of quantification of GPR54 by qRT-PCR.