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PRINCIPAL INVESTIGATOR: Mina J. Bissell, Ph.D.

CONTRACTING ORGANIZATION: Lawrence Berkeley Laboratory  
Berkeley, CA 94720

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## **INTRODUCTION**

The goal of this project is to provide undergraduate trainees with exposure to areas of breast cancer research that focus on the role of microenvironment in mammary gland biology and neoplasia. It is only recently that this area of research has been recognized as playing a crucial role in the regulation of tissue specificity and it is now of particular interest to determine how misregulation of microenvironmental cues contributes to the development and progression of breast cancer. Trainees in this project benefit from working in a program that investigates the intersection of hormones, growth factors, and extracellular matrix (ECM) signaling and remodeling during mammary gland morphogenesis, differentiation, and carcinogenesis. The unifying hypothesis for the scientific programs is that the unit of function in higher organisms is not the cells but the tissue itself, and that the processes related to its function are mediated by the connections between the components of the microenvironment (hormones, growth factors, and ECM) via their receptors to signals that change gene expression. Our investigations of breast cancer development and progression are based on the viewpoint that cancer cells follow these same basic principles, and that cancer prevention or changes in the cancer phenotype are possible through modification of environmental signals. In this program, undergraduate trainees have frequent interaction with mentors and with advanced postdoctoral fellows, and will prepare reports to be presented in organized, biweekly progress meetings. At the end of the program, the undergraduates will present their work in a formal setting to all the preceptors. Additionally, each participating undergraduate will be subsequently tracked and their career paths will be followed to determine how many of them pursue postgraduate research in breast cancer or related medical fields. Together, this program will enrich the undergraduate trainees through interaction with a dynamic research environment.

## **BODY**

In this section, research accomplishments of individual undergraduates will be summarized, with an emphasis on the forward direction of the projects.

**Connie Chen**

**Mina Bissell Lab**

**Supervisor: Derek Radisky**

**Project Title: Molecular Determinants of Epimorphin Branching Morphogenesis**

### **Abstract:**

Identification of the signals controlling the development of the mammary gland epithelial ductal system has been an intensive topic of investigation. This process can be modeled in 3-dimensional culture systems in which mammary epithelial cells are grown in collagen-I gels. Previous investigations have identified epimorphin (EPM) as an essential morphogen for mammary gland development through branching and luminal morphogenesis. EPM is the extracellular form of the intracellular protein syntaxin-2, a member of the syntaxin family of vesicle fusion mediators. EPM is produced only in the

stromal compartment of the mammary gland, but binds to nearly all cultured luminal epithelial cells, suggesting the presence of a cell surface receptor on luminal epithelial cells. In this study, we identify the domain of EPM responsible for directing branching activity in the 3D collagen assay. Using the structure of the highly homologous syntaxin-1A (which shows no activity as a mammary epithelial morphogen) as a basis for a homology model of EPM, we selected a region of EPM that we predict to be the active domain, identified four amino acids that are distinct between EPM and syntaxin-1A, and performed site-specific mutagenesis of syntaxin-1A to change all four to those that are found in EPM. We found that this hybrid product (which we have named HS 1→2) reproduced the activity of EPM in the 3D collagen assay. By creating an active morphogen from an inactive template, we have identified the active domain of EPM.

### **Introduction:**

Branching morphogenesis is an essential process for development of mature, functional mammary glands. During puberty, mammary gland end buds proliferate and invade the mesenchymal fat pad, establishing a network of ductal branches. During pregnancy, the epithelium continues to grow, developing additional lobuloalveolar structures (Radisky et al., 2003). *In vivo*, these developmental processes are dependent on communication between the stroma and the epithelium; these interactions direct branching morphogenesis, for tubule initiation and extension, as well as luminal morphogenesis, for formation of secretory acini. Branching morphogenesis can further be divided into two distinct processes (Wiseman and Werb, 2002), as primary branches form from separation of the advancing terminal end buds, while secondary and tertiary branches emerge from mature ducts. Both types of branching morphogenesis is dependent on an epithelial-to-mesenchymal transition, because as the epithelium branches, the stroma must undergo remodeling to make room for and provide support to the developing epithelial tubes (Metzger et al., 1999).

While several types of growth factors can stimulate mammary epithelial cell proliferation, epimorphin is a required morphogen to provide direction and orientation to the developing structures (Hirai et al., 1998). Previous studies have shown that matrix metalloproteinases (MMPs) are needed for mammary branching morphogenesis induced by growth factors and epimorphin (Simian et al., 2001). Epimorphin-treated mammary epithelial cells were found to increase levels of MMP-2, MMP-3, and MMP-9 (Simian et al., 2001) and inhibition of the MMPs completely blocked the branching activity (Hirai et al., 2001). These studies show epimorphin branching morphogenesis is mediated through MMPs and the pathway connecting epimorphin and implicate this effector is an important target for investigation. In the mammary gland, epimorphin is present at the surface of both stromal fibroblasts and myoepithelial cells and plays a large role in mammary gland morphogenesis. *In vitro* experiments have shown that epimorphin directs distinct morphogenic pathways in mammary epithelial cells cultured in 3D collagen. Depending orientation of presentation, epimorphin stimulates strikingly different morphogenic processes. Polar presentation of epimorphin to the outer surface of cell clusters lead to branching morphogenesis. However, the presentation of epimorphin around every cell (apolar) in the cluster results in luminal morphogenesis where a large central lumen forms in cystic structures (Hirai et al., 1998).

Although epimorphin was first identified as a morphogen through the blocking of its activity using an epimorphin monoclonal antibody (Hirai et al., 1992; Gumbiner, 1992), the same molecule was later identified as the extracellular form of syntaxin-2, a functionally distinct molecule and a member of the syntaxin family of proteins that function in vesicle fusion (Bennett et al., 1993; Pelham, 1993). Although epimorphin has yet to be crystallized, syntaxin structure has been extensively studied through nuclear magnetic resonance (NMR) and crystallography. Different members of the syntaxin family are highly homologous, and are likely to be structurally similar. The common characteristic that defines the syntaxin family of proteins is a conserved C terminal region that generally precedes a transmembrane domain. This C-terminal region also known as the SNARE domain is believed to be directly involved in membrane fusion. Through NMR spectroscopy studies, residues 27-146 of syntaxins consist of an independently folded domain with a three-helix bundle structure. The interface between the second and third helices forms a long groove that exhibits substantial residue conservation between different syntaxins. This groove is an ideal binding site for a helix of another protein that can pack against it completing the four-helix bundle. For syntaxin, Synaptobrevin/VAMP and SNAP-25, form a tight complex with the SNARE domain that is known as the core complex or the SNARE complex. Formation of this complex is believed to provide the driving force for fusion of vesicles with the plasma membranes (Fernandez et al., 1998).

Deletion analyses have shown that the functional domains of epimorphin and syntaxin can be distinguished (Hirai et al., 1998, Weimbs et al., 1997). Mammary luminal epithelial cells respond to epimorphin but do not express it, and are surrounded by epimorphin expressing mesenchymal cell populations, demonstrating the existence of epimorphin receptors on the outside of the luminal epithelial cells, the properties of which can be investigated using cell adhesion assays. Studies have shown that the cells attached to the three alpha helical region (H12) of recombinant epimorphin but not to the region containing the SNARE helix and transmembrane domain (H3). Domain minimization of epimorphin activity was also tested using branching assays, where SCp2 (mammary epithelial cells) were embedded in collagen-1 in the presence or absence of recombinant epimorphin. The H12 region was shown to be sufficient to induce branching morphogenesis while the H3 was not (Hirai et al., 1998). Thus, the SNARE domain is dispensible for epimorphin cell binding and morphogenesis activity; in contrast, deletion analyses of syntaxin proteins have suggested that the SNARE domain is sufficient by itself to mediate vesicle fusion (Weimbs et al., 1997)

While the three helix domain of epimorphin is active by itself to mediate cell binding and morphogenesis, numerous biochemical and structural studies have shown that this structure prefers to bind to a fourth alpha helix to form a four-helix bundle. These observations are suggestive of a hypothetical fourth helix, perhaps on the epimorphin receptor, that may bind to epimorphin in the cleft between the second and third helix. If so, then this cleft is predicted to be the critical morphogenic determinant of epimorphin; it is the objective of this study to evaluate this prediction.

## **Materials and Methods:**

### *Cells*

The functionally normal and nontumorigenic mouse mammary epithelial cell line, SCp2, was maintained in growth medium (DME/F12 supplemented with 2% FBS, 5ug/ml, insulin and 50 ug/ml gentamicin).

#### *Generation of Recombinant Epimorphin*

Expression constructs were generated by PCR amplification using cDNA for mouse EPM or human syntaxin-1 as template and were cloned into a pET27 expression vector, and subsequent site-directed mutageneses was performed using the Stratagene Quickchange PCR-based mutagenesis kit. Recombinant epimorphin (HS EPM) is identical to endogenous epimorphin except that it lacks the N-terminal 26 amino acids, as well as the linker, SNARE helix, and transmembrane domains. Recombinant syntaxin-1a (HS 1A) is derived from the three-helix bundle domain of Syntaxin 1a (homologous to HS EPM). Recombinant HS1→2 is derived from HS 1A with the proposed active site residues mutated to the ones in Syntaxin-2. The proteins were tagged with six histidine residues and expressed in *Escherichia coli*. The *E. coli* was lysed with BugBuster reagent supplemented with protease inhibitors and benzonase and purified over Ni columns. For use in cell culture, recombinant proteins were dialyzed against 1X PBS and filtered under sterile conditions. Proteins were diluted to a concentration of 0.76 mg/ml using 1X PBS.

#### *3D Cell culture*

The cell clusters were prepared as follows: agarose was heated in DME/F12 (final 2%) and 1 ml of the solution was added to each well of 24-well plates. After the agarose gelled, 1.5ml of growth medium was added to each well and incubated for 1 hr at 37° C in a CO<sub>2</sub> incubator. This medium was then discarded and SCp2 cells suspended in 500ul of growth medium containing 1,000U of desoxyribonuclease I (DNase I) were added on top of the agarose gel and incubated at 37° C with gentle rotation (100rpm) for 24 hr, which yielded rounded and well packed cell clusters. Unclustered single cells were removed by centrifugation and the clusters were then washed three times with DME/F12. Cell clusters were embedded in type I collagen gels. Acid-soluble collagen (Cellagen) was mixed gently on ice (8 vol) with 1 vol of 10X DME/F12 and 1 vol of 0.1N NaOH. 100 µl of the collagen solution was added into each well of a 48 well plate, which was then incubated at 37° C to allow for polymerization of the basal collagen layer. The cell clusters were suspended in growth medium and 10 µl of the suspension (24-40 clusters) was mixed with 10 µl of laminin, 10 µl of protein solution and 70 µl of the collagen solution, and poured onto the basal collagen layer and placed at 37° C for gelation. After gelation of the collagen, 200 µl of growth medium containing 10% protein solution and 50 ng/ml EGF (epidermal growth factor) was added on top. Cultures were maintained at 37° C in tissue culture incubators and liquid media was changed every other day.

#### **Results:**

##### *Development of homology model*

Key residues in the predicted functional domain were identified by homology modeling and visualization using the DeepView Swiss-Pdb Viewer. A model structure of epimorphin was created by threading the sequence of epimorphin into the published crystal structure of syntaxin-1a. In the closed conformation structure of syntaxin-1a, the SNARE helix is binding as a fourth helix in the cleft between the 2<sup>nd</sup> and 3<sup>rd</sup> alpha-

helices; accordingly, the SNARE helix was used as a model for a hypothetical helix that binds to the three alpha helical region of epimorphin. Together, these were used as a model to identify the amino acid residues of the epimorphin three-helix bundle with side chains pointing towards the proposed hypothetical fourth helix.

### *Morphogenesis*

Of the residues in the homology model of epimorphin that were predicted to be in contact with the hypothetical fourth helix, four were found to be different between syntaxin-1a and epimorphin. To create the HS1→2 recombinant protein, these four residues were changed on the syntaxin-1a template to those found in syntaxin-2 (epimorphin), using site-specific mutagenesis with the Stratagene Quik Change Kit. In helix 2, arginine90 was mutated to a methionine and aspartate102 was mutated to a glutamate; in helix 3, phenylalanine142 was mutated to a tyrosine and serine146 was mutated to a cysteine.

### *Branching activity and Cell Adhesion*

If the proposed active site of the protein carried key residues required for epimorphin activity, it was expected that HS EPM would be active in the branching assay, HS Syn1a would be inactive, and HS1→2 would regain branching activity. Using these recombinant proteins in branching assays, SCp2 cells were embedded in collagen I and incubated with 5% serum + GI plus EGF and 10% volume of recombinant protein solution. Untreated cells, or cells treated either with growth factors or EPM alone (but not both) showed no branching morphogenesis. However, cells simultaneously treated with growth factors and epimorphin did exhibit branching activity, depending upon the form of epimorphin that was used. After 10d, the cells incubated with HS EPM and EGF exhibited abundant branching, while the corresponding HS Syn1a-treated cultures showed only small stubby branches. The recombinant protein HS1→2 was found to be active, beginning to stimulate branching after 4 d, demonstrating that our mutations had conferred morphogenic activity upon an inactive template.

### **Discussion:**

#### *Structural insights can be used in functional studies of homologous proteins*

In this study, we used information from the crystal structure of the homologous protein syntaxin-1a to develop a hypothesis about the function of epimorphin, and to target amino acids predicted to be involved in the epimorphin morphogenesis domain. Use of this strategy relied upon the fact that epimorphin and syntaxin have very distinct functions and functional domains, but are encoded by the same gene. These distinct functions are reflective of the different localizations of the epimorphin/syntaxin-2 protein: epimorphin is localized to the extracellular surface of the plasma membrane, while syntaxin is localized to the cytoplasmic face. Epimorphin is also unusual in that the protein has no canonical exocytosis-targeting signal sequence, and as such, appears to cross membrane without transiting through the endoplasmic reticulum and golgi apparatus. Other examples of proteins that exit cells without using the classical protein secretion route include fibroblast growth factors 1 and 2 and interleukin-1B (Siders et al., 1995), which also lack signal sequences but which have well-characterized functions outside of the cell. Epimorphin/syntaxin-2 can be contrasted from these proteins in that it

appears to have completely distinct functions depending upon its membrane orientation. Intracellularly, syntaxin-2 mediates membrane fusion, while extracellularly, epimorphin acts as a morphogen. Epimorphin/syntaxin-2 is not unique in its topologically distinct functionalities, as it is one of a subcategory of proteins that lack signal sequences but also have distinct extracellular and intracellular functions. Other examples of these proteins include phosphohexose isomerase, autocrine motility factor, RHAMM/CD168, Galectin-1, HMGB1/amphoterin, tissue transglutaminase, and thioredoxin/ADF (Radisky et al., 2003). Many of these proteins have been studied structurally in one topological/functional context, so our strategy to identify functional domains of epimorphin through structural analysis of the homologous syntaxin-1a could also be applied to other members of the family of proteins with distinct extracellular and intracellular functions.

The discovery of the active site of epimorphin may also have applicability towards investigations that provide insight into mechanisms involved in the development of breast cancer. The transcription factor CCAAT/enhancer binding protein  $\beta$  (C/EBP $\beta$ ) regulates the expression of many genes involved in proliferation and terminal differentiation. C/EBP $\beta$  has been shown to be important for normal mammary gland morphogenesis and epithelial cell fate determination (Robinson et al., 1998). C/EBP $\beta$  functions as a homo- or heteromeric dimer of its two constituent isoforms: LAP (liver activating protein) and LIP (liver inhibiting protein) (Descombes and Schibler, 1991; Buck et al., 1994). LAP and LIP are derived from alternative translation initiation from the a single mRNA transcript with LAP being the full-length form containing a transactivating, dimerization, and DNA-binding domains, while LIP is a truncated form lacking the transactivating domain. LAP and LIP are mutually antagonistic, and alterations in the relative ratio of expression of the LAP and LIP can lead to dramatic alterations in expression levels of of genes signaling either differentiation or proliferation. The two isoforms are crucial for mammary gland development and are also known to be overexpressed in breast cancer. Investigations of WAP-LIP mice, which express increased LIP selectively in mammary epithelial cells, show that LIP can induce epithelial proliferation and the formation of mammary hyperplasias and that a LIP-initiated growth cascade may be susceptible to additional oncogenic hits, which could lead to the initiation and progression of neoplasia (Zahnow et al., 2001). Intriguingly, epimorphin is the only known regulator of C/EBP $\beta$  (Hirai et al., 2001), and it has been found to increase the overall levels of C/EBP $\beta$  and to increase the ratio of LIP to LAP in both culture and in the WAP-EPM transgenic mouse (Hirai et al., 2001), which also shows significantly elevated rates of malignancy (J.L.Bascom *et al.*, manuscript in preparation). The investigation of the mechanism of how epimorphin controls the levels of the protein isoforms necessitates the identification of the epimorphin receptor which has yet to be found. The results described here detail the identification of the epimorphin active site, a key step in the path towards discovery of the receptor. Additionally, the identification of the binding cleft may be useful for creating an  $\alpha$ -helix designed to bind to the active site tight enough to prevent the binding of epimorphin to its receptor and therefore blocking the effects that epimorphin binding. Given that epimorphin acts to increase the ratio of LIP to LAP, which signals for increased malignancy, inhibiting epimorphin binding could have potential as a cancer preventative or therapeutic agent.

**Grace Marily Cruz**

**Satyabrata Nandi Lab**

**Supervisor: Rajkumar Lakshmanaswamy**

**Project Title: Comparison of the Rates of Apoptosis in Mammary Epithelial Cells at Different Stages of the Estrous Cycle in Hormone-Protected Lewis Rats**

**Abstract:**

Apoptosis is the most common form of eukaryotic cell death. It is evident in mammary gland development and regionally, at each stage of the estrous cycle. Apoptosis is important in setting a counterbalance against periodic proliferations, which, if left unmediated may result in carcinogenesis. It has been a known fact that early pregnancy confers protection against breast cancer in women. The same phenomenon has been observed in rats and mice. Experimental evidence has shown that short-term hormone treatment with pregnancy level estrogen is highly effective in decreasing the risk for mammary carcinogenesis. This protective effect has not been fully understood. However, it is hypothesized that the rates of apoptosis are altered in protected compared to unprotected animals. The purpose of this study was to look at the differences in the rates of apoptosis (Vakkala et al., 1999) at different stages of the estrous cycle and (Andres and Strange, 1999) between the normal mammary glands of protected and unprotected animals to determine whether or not the rates of apoptosis are significantly altered in protected animals. Determination of rates of apoptosis was done using the Fragment End Labeling (FragEL) Method and Immunocytochemical Staining. Data quantification using computerized means indicated that there is approximately a two-fold increase in apoptosis during the metestrous stage in protected animals. No significant alteration in apoptosis was found at other stages of the estrous cycle and between the different treatment groups.

**Introduction:**

Apoptosis is the most common form of eukaryotic cell death. It is a highly regulated cellular process that leads to the destruction of individual cells that occurs in response to various cell death signals. Morphologically, apoptosis is characterized by nuclear shrinkage and pyknosis, which eventually leads to nuclear fragmentation and phagocytosis of apoptotic cells. DNA fragmentation, its end result can be detected biochemically by DNA electrophoresis (Vakkala et al., 1999).

As a physiological process in normal cells, apoptosis is important in maintaining tissue homeostasis, which is achieved through the critical balance of cell proliferation and cell death. Apoptosis sets a counterbalance against periodic proliferations, which, if left unmediated may result in carcinogenesis. In the mammary gland, the presence of apoptotic mammary epithelial cells (MEC) is evident at each stage of the estrous cycle and during post-lactational mammary gland involution (Andres and Strange, 1999). In the process of post-lactational involution cell death is very evident and involves the whole mammary gland. During other developmental stages, however cell death is observed to be focal (Andres and Strange, 1999; Strange et al, 2001). This regional occurrence of apoptotic cells at different stages of mammary gland development supports the idea of a

balance between proliferating, differentiating, and apoptotic cells as a result of cyclic hormonal control (Schedin et al., 2000).

The balance between cell proliferation and cell death occurs periodically in tissues such as the uterus and the mammary gland. The physiological changes that accompany mammary gland development, from initial development to pregnancy and weaning are characterized by the dynamic interaction between multiple hormones, primarily the ovarian hormones, progesterone and estrogen (Medh and Thompson, 2000). During pregnancy, the high levels of estrogen promote proliferation and differentiation of the mammary ducts, in preparation for lactation. Cessation of lactation presents a lack of lactogenic hormone stimulation, which is what triggers apoptosis.

In the absence of pregnancy, the menstrual cycle also presents this dynamic hormonal activity. Different stages characterize specific physiological occurrences and thus, different hormone levels to maintain tissue homeostasis. Low ovarian hormones make mammary epithelial cells susceptible to cell death. Thus, during the late luteal stage, when circulating progesterone levels are dramatically decreased, apoptosis is at its peak. This is also evident morphologically through lobular regression and epithelial sloughing during menstruation (Schedin et al., 2000). Similar to human breast tissue development, rat MEC proliferation, differentiation and apoptosis is dependent on the cyclic hormonal control of the estrous cycle.

The proestrous stage, which is equivalent to the follicular phase of the menstrual cycle, is characterized by high estrogen levels. Ovulation is the defining event of the estrous stage, which is parallel to the luteal phase of the menstrual cycle. Hormone levels dramatically decline in metestrus and diestrus. This cyclic pattern of circulating hormone levels coincides with that observed in humans, and thus, the balance of apoptosis and cell proliferation is still maintained (Schedin et al., 2000; Medh and Thompson, 2000). The significance of the preservation of this balance is exemplified by the incidence of cancer; that is, carcinogenesis results in a disruption of this balance where there is a higher rate of proliferation than apoptosis at any particular cycle.

It has been a known fact that early pregnancy confers protection against breast cancer in women. Women who have undergone a full term pregnancy before the age of twenty have a 50% reduced risk of breast cancer, compared to nulliparous women (MacMahon et al., 1970). The same phenomenon of parity protection against mammary carcinogenesis is also observed in rats. The mechanism for this protective effect has not been fully elucidated, although it has been thought to be related to the increases in hormone levels during pregnancy that cause persistent changes in the systemic hormonal environment in parous females (Lakshmanaswamy et al., 2001). Experimental evidence has shown that short-term hormone treatment with pregnancy level estrogen, or estrogen and progesterone is highly effective in decreasing the risk for mammary carcinogenesis. It is hypothesized that the rates of apoptosis is altered in protected compared to unprotected animals. It is not certain how the rates of apoptosis are altered, whether or not the animals given protective hormone treatment necessarily have a higher rate of apoptosis.

This investigation was undertaken as a baseline study on the effects on the rates of apoptosis at different stages of the estrous cycle in normal mammary glands. Moreover, it will examine the persistent effects of protective and unprotective estrogen treatments on the rates of apoptosis in MEC. If the rates of apoptosis are indeed found to be altered with

respect to particular treatments of estrogen and with particular stages in the estrous cycle, we can potentially offer a plausible explanation for the protective effect of pregnancy levels of estrogen against mammary carcinogenesis. Furthermore, this finding will also provide a basis for the development of preventative measures against breast cancer in humans.

## **Materials and methods:**

### *Animals*

Seven-week old virgin Lewis rats were purchased from Harlan Sprague-Dawley (Indianapolis and San Diego). The rats were housed in a temperature-controlled room with 12-h light/dark schedule. They were fed food (Teklad 8640; Teklad, Madison, WI) and water ad libitum. All of the procedures followed University of California Animal Care and Use Committee guidelines.

### *Hormone Treatment*

Hormone treatments were started 2 weeks after the rats were purchased. The hormones were packed in individual silastic capsules (size 0.078 inch i.d. x 0.125 inch o.d., 2 cm in length; Baxter Health Care, Mundelein, IL). All doses of Estradiol-17- $\beta$  (Sigma) were packed in the silastic capsules in a cellulose matrix. Control animals received empty silastic capsules. All silastic capsules were dorsally implanted s.c. All capsules were primed before implantation by soaking in media 199 (GIBCO) overnight at 37 °C.

### *Effect of Different Doses of E on Prevention of Mammary Carcinogenesis*

When the rats were 9 weeks of age, they were divided into 3 groups, each consisting of 12 rats and receiving one of the following treatments: (i) control, (ii) 10  $\mu$ g of Estradiol-17- $\beta$ , and (iii) 200  $\mu$ g of Estradiol-17- $\beta$ . Each treatment was continued for 2 weeks (until the rats were 11 weeks old) and at the end of the treatment, the silastic capsules were removed from the animals.

### *Identification of Estrous Stage*

Vaginal smears were taken to identify at which stage of the estrous cycle the rats were in. Each stage was identified by the types of cells present in the smears as observed under a light microscope. Vaginal smears from proestrous stage rats were predominantly epithelial cells, while the presence of predominantly cornified epithelial cells and some small circular lymphocytes marked the estrous stage. Metestrous was identified as determined by the presence of polymorphonuclear cells or the presence of all three prominent vaginal cell types (cornified epithelial cells, ovoid cells, and polymorphonuclear cells) in the vaginal lavages, while the diestrous stage was identified as having mostly lymphocytes and some epithelial cells. Animals were euthanized across stage of estrous to assure that the tissue pooled from estrous-stage rats was obtained from animals with the same age distribution.

### *Fragment End Labeling (FragEL) Assay for Detecting Fragmented DNA in Apoptotic MECs*

To detect apoptotic cells, *in situ* labeling of the 3' ends of the DNA fragments generated by apoptosis-associated endonucleases was used. Fragment end labeling was performed using the Oncogene BrdUTP-FragEL DNA Fragmentation Detection Kit (Oncogene, cat #QIA80). The 3' ends are labeled using a molecular biology-based, end-labeling, histochemical technique. Incorporation of bromo-deoxyuridine triphosphate (BrdUTP) using terminal deoxynucleotidyl transferase (TdT) occurs more readily than addition of biotin, fluorescein, or digoxigenin-modified nucleotides. An easier to detect signal results by the increase in the number of modified nucleotides on the apoptotic DNA fragments. Control slides were also provided with the kit.

#### *Immunocytochemical (ICC) Staining*

Five  $\mu\text{m}$  sections were cut from histological samples (mammary gland, vagina, uterus, and intestine) embedded in paraffin, and were fixed on microscope slides (two sections of 4 to 5 tissues per section). The slides were incubated at 37 °C overnight and then put in a 56 °C oven to melt the wax. Slides were dewaxed in histoclear and rehydrated in graded alcohol. 100  $\mu\text{l}$  of Proteinase K (20  $\mu\text{g}/\text{ml}$  in 10 mM Tris buffer, pH 8) was applied to each section. Endogenous peroxidase activity was consumed by applying 3%  $\text{H}_2\text{O}_2$  in methanol for 5 minutes.

For equilibration, 100  $\mu\text{l}$  of Reaction Buffer was applied to the test sections. The labeling mixture (10 $\mu\text{l}$  5X Reaction Mix, 8  $\mu\text{l}$  BrdUTP, 0.75 $\mu\text{l}$  TdT Enzyme, and 32.25 d $\text{H}_2\text{O}$ ) was applied to every test section as prescribed by the product protocol, and incubated for 1.5 hours at 37 °C. Nonspecific binding was blocked using Block buffer (Oncogene, cat # QIA80). The primary antibody, a biotinylated monoclonal antibody which recognizes BrdUTP incorporation (20X anti-BrdUTP-biotin, diluted 1:20 in block buffer) was applied to the test sections and incubated at room temperature for 1 hour. The peroxidase streptavidin 200X conjugate was then added (diluted 1:200 in block buffer) and incubated for 30 minutes.

Color was developed by diaminobenzidine (DAB, prepared by adding 1 ml tap water, 1 DAB tablet, and 1  $\text{H}_2\text{O}_2$ /urea tablet (Oncogene, QIA80)), after which, the sections were counterstained with hematoxylin and mounted with Permount. Negative control stainings were carried out using 1X Reaction Buffer instead of the labeling mixture. Positive Controls were carried out by the addition of DNase I in buffer (50 mM Tris buffer + 10 mM  $\text{MgCl}_2$  + 1% BSA) before peroxidase activity suppression.

#### *Data Quantification and Analysis*

Cells were counted using a light microscope and a tap counter. Between 1000 and 1200 total cells were counted for each of the three mammary glands (control, 10  $\mu\text{g}$  E, and 200  $\mu\text{g}$  E) in a section. Positive (apoptotic) cells were brown, while negative (non-apoptotic) cells were blue. Apoptotic cells were defined if the whole nuclear area was labeled positive. Careful scrutiny was also taken to make sure that only apoptotic mammary epithelial cells (MEC) and not myoepithelial cells (elongated with pointed edges, as opposed to spherical MEC) were counted. The percentage of positive cells was calculated to determine the rate of apoptosis of each mammary gland. Manual counts were confirmed using computer quantification methods. For this project, the ACT-1 Program for Nikon Camera and the Image Pro Plus (IPP) Program were utilized.

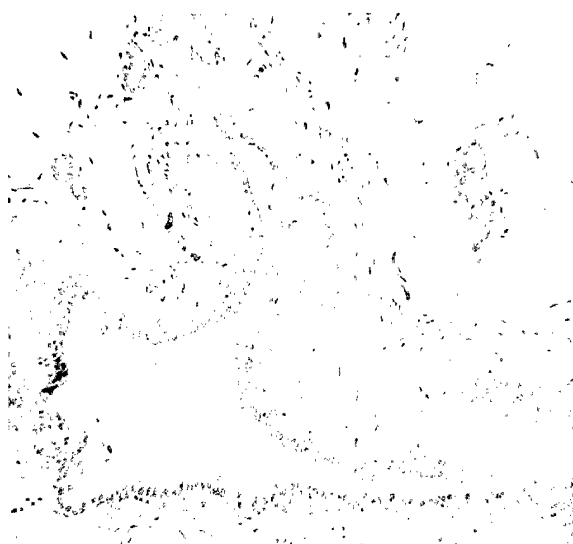
**Results:**

**Table 1. Rates of Apoptosis according to Stage in Estrous Cycle with respect to Different Hormone Treatments**

Treatment	Stage of Estrous Cycle			
	Proestrus	Estrus	Metestrus	Diestrus
Control	14.5%	12.0%	11.8%	9.1%
10 $\mu$ g of E	27.9%	9.0%	14.0%	13.2%
200 $\mu$ g of E	24.8%	8.3%	24.2%	8.7%

Apoptotic mammary epithelial cells were evident in all stages of the estrous cycle. The regional occurrence of apoptosis at all stages (Figure 1) and at different treatments was evident, as observed in the light microscopy analysis of the different mammary glands. Among the control animals, apoptosis was found to be highest at the proestrous stage and lowest at the diestrous stage (Table 1). The rates of apoptosis were significantly altered in the protected group (animals treated with short-term high pregnancy level of estrogen, 200  $\mu$ g of E) as opposed to the unprotected group (animals treated with short-term, non-pregnancy level of estrogen, 10  $\mu$ g of E); however, no significant alteration was found in the rates of apoptosis with respect to the different stages of the estrous cycle. Moreover, it was observed that there is approximately a two-fold increase in the rate of apoptosis at the metestrous stage, in protected animals compared to the unprotected ones (Figure 2). Although the percentage of apoptotic mammary epithelial cells varied widely between treatments within a single stage, no significant alteration in the rates of apoptosis was found between different treatments at any of the other stages in the estrous cycle.

**Figure 1. Evidence of the Regional Occurrence of Apoptosis at Each Stage of the Estrous Cycle**



Within a single mammary duct, there are positive (brown-stained cells, apoptotic) and negative (purple-stained, non-apoptotic) mammary epithelial cells, not so far away from each other. This is consistent with previous observations from other studies that apoptosis appears regionally at each stage of the estrous cycle.

*200X picture of a normal mammary duct in the Diestrous Stage (control)*

**Figure 2. A Comparison Between the Protected and Unprotected Mammary Glands at the Metestrous Stage of the Estrous Cycle**



Unprotected Mammary Gland  
(10 $\mu$ g of E) at Metestrous  
200X Magnification

Protected Mammary Gland  
(200 $\mu$ g of E) at Metestrous  
200X Magnification

It is evident from the above pictures that there are more apoptotic MECs (brown-stained) in the mammary gland given the short-term high pregnancy level estrogen treatment than in the mammary gland given the short-term non-pregnancy level estrogen treatment. The increase in apoptosis in the protected group may be a plausible explanation for the hormone-induced protection conferred by short-term hormone treatments.

#### **Discussion:**

Tissue homeostasis is achieved through the critical balance of cell proliferation and cell death. Apoptosis has been shown to act as a counterbalance to cell proliferation and therefore is a critical factor in tissue homeostasis (Sabourin et al., 1994). Studies have shown that there is an increase in the frequency of proliferation during the later stages of the menstrual cycle in humans (Masters et al., 1977; Meyer, 1977). This progressive proliferation, if left unmediated may result to carcinogenesis in the normal mammary gland unless a certain degree of cell deletion occurs (Ferguson and Anderson, 1981). Thus, the significance of apoptosis as a physiological process could not be stressed more.

In this study, the rate of apoptosis in the metestrous stage was found to be increased by approximately two-fold in the protected compared to the unprotected and control groups. The increase in apoptosis may be a plausible explanation for the protection that is conferred by short-term pregnancy level estradiol treatment in these rats. The rate of apoptosis in the unprotected group of the metestrous stage was not significantly different from the control group in the same stage (see Table 1).

An increase in apoptosis means that there is not as much proliferation occurring at a specific period of time. This coincides with the critical balance that is maintained for tissue homeostasis. In the course of mammary gland development, the terminal end buds, which are the sites of high proliferation, are the structures that are most susceptible to carcinogenesis (Russo and Russo, 1996). Countering this high proliferation may decrease the frequency of transformation and thus, would also decrease the likelihood of carcinogenesis. A decrease in transformation would also explain why short-term pregnancy level hormone treatment inhibits latent cancers from developing into frank cancers (Guzman et al., 1999).

Although there was no significant alteration in the rates of apoptosis across stages of the estrous cycle, a significant increase in apoptosis found in the protected group of the metestrous stage was enough to lead to the idea that an alteration in the rate of apoptosis is a plausible explanation for the protective effect that is conferred by short-term pregnancy level hormone treatment.

### **Conclusion:**

Based on the findings of this study, it can be concluded that there is a two-fold increase in the rate of apoptosis in the protected animals compared to the unprotected and control animals in the metestrous stage. This is sufficient evidence to suggest that this increase in cell death may be one of the reasons for the protective effect that is conferred by early pregnancy, as confirmed by the administration of short-term pregnancy level hormone treatments, to mimic this effect. Moreover, increase in apoptosis in the protected group may be a preventative mechanism that inhibits the development of latent tumors into frank cancers; although its exact mechanism has yet to be investigated.

**Kim Dang**

**Mina Bissell Lab**

**Supervisor: Katrin Lorenz**

**Project Title: Signaling Pathway Analysis in HMT-3522 Human Breast Epithelial Cells**

### **Introduction:**

We are using the phenotypically normal mammary epithelial cell (MEC) line S1, and its tumorigenic progeny, T4, as a model system to study pathways potentially involved in the progression of breast cancer. Gene expression profiles obtained via microarray analysis of S1 and T4 cells in three-dimensional (3D) culture have identified several differentially expressed genes. Among these genes are those that code for proteins involved in cell growth, cell proliferation, structure, adhesion, and cell survival; we are currently focusing on epidermal growth factor receptor (EGFR),  $\beta$ 1-integrin, and dual specificity phosphatase (DUSP), all of which are linked via the mitogen-activated protein kinase (MAPK) signaling pathway. Previous studies have shown that inhibition of EGFR,  $\beta$ 1-integrin, and MAPK in T4 cell lines have led to growth arrest and restoration of cell polarity. Our aim is to characterize the expression patterns of EGFR,  $\beta$ 1-integrin, and DUSP in T4 and S1 MEC on both the transcription and translation level to identify the level at which they are regulated under normal and/or inhibitory conditions.

We will also observe the response of S1 and MCF12A, another phenotypically normal MEC line, to inhibitor treatments to determine whether the resulting reorganization of cell shape, polarity, and proliferative status are general responses or a specific for tumorigenic MEC lines.

**Techniques employed:**

*Quantitative PCR.* Total RNA was extracted from non-malignant S1 cells, tumorigenic T4 cells, and T4 cells treated with various inhibitors. Complimentary DNAs were prepared by random hexamer-primed reverse transcription (RT), and as a negative control, no reverse transcriptase reactions were run. For PCR, a set of primers specifically corresponding to the gene of interest was tested to find optimal annealing temperature; the linear range of amplification was determined, as was the optimal 18S:competimer ratio. Once these were complete, PCR of all samples was performed.

*Immunostaining.* Cells were fixed onto slides with either paraformaldehyde or methanol-acetone, nonspecific binding sites were blocked with goat serum, and primary staining was followed by incubation with FITC- or Texas Red-conjugated secondary antibodies. DAPI was used to stain nuclear DNA, and fluorescence microscopy was used to visualize the slide.

*Western analysis.* Protein lysates were obtained and gels were run and transferred according to standard procedures. Standardization was performed using Ponceau staining of the blot, and bands were visualized using anti-HRP and chemiluminescence.

**Cindy Pham**

**Caroline Kane Lab**

**Supervisor: Rachel Fish**

**Project Title: Role of TFIIS Transcriptional Elongation Factor**

**Introduction:**

The transcriptional machinery is highly conserved from yeast to man, and TFIIS is one example of a transcriptional elongation factor. TFIIS stimulates the cleavage and readthrough activities of RNA polymerase II, and contains a protein motif known as a YEATS domain, which is also found in Taf14, another transcriptional factor with homologues in mammalian cells. Taf14 is also believed to play a role in transcription because it has been biochemically isolated from a number of nuclear complexes, many of which have a role in transcription, either in chromatin remodeling or in directly stimulating transcriptional activation. The gene encoding Taf14 has been found to genetically interact with PPR2, the gene encoding TFIIS. When both genes are disrupted, there is cell death known as synthetic lethality. Homologous recombination between AF9 and ENL and transcript elongation factors has been related to myeloid leukemogenesis, which is related to misregulated transcription. Accordingly, a study on how TFIIS interacts with Taf14 may provide insight into how misregulated transcription occurs in cancer cells, and yeast is an ideal model for studying these genetic interactions.

**Projects pursued:**

*Mutational analysis of Taf14.* Previous work in Caroline Kane's lab has shown by deletional analysis that the N-terminus of TFIIS is required for viability of a Taf14 disruption strain. This interaction has been previously characterized from the viewpoint of TFIIS; here, double disruption strains will be used to identify which region(s) of Taf14 are important for interaction with TFIIS. This will be done by designing deletion constructs of Taf14 and using constructs that have point mutations. It is hypothesized that the YEATS domain may be important for the TAF14 and TFIIS interaction, because the YEATS domain is so widely conserved in yeast and human orthologues of these proteins.

*Reconstitution of TAF14 and TFIIS interaction.* This project investigates whether cells disrupted for TAF14 and encoding only TFIIS with a Myc tag at the C-terminus (PPR2-Myc) are viable. It is hypothesized that the Myc tag at the C-terminus will interfere with the function of the third domain of TFIIS, as this domain has been shown previously to be required in stimulating the transcription cleavage and readthrough activities of RNA polymerase II.

*Investigation of myc-PPR2 mutants.* Cells with the genomic integration of pp42 with a myc tag at the C-terminus will be generated, and assessed for production of a functional TFIIS protein. Yeast cells that lack functional TFIIS manifest sensitivity to the nucleotide-depleting drug, 6-azauracil. This sensitivity has led to the model that sensitivity to 6-azauracil is indicative of a defect in transcriptional elongation. It is hypothesized that the Myc tag at the C-terminus will interfere with the important third domain of TFIIS, rendering TFIIS non-functional and the cells sensitive to 6-azauracil. If this hypothesis is correct, then it can be determined whether transforming wild-type TFIIS can rescue the 6-azauracil sensitivity.

**Adia Scrubb and Yalda Afshar**

**Mary Helen Barcellos-Hoff Lab**

**Supervisor: Michael Jobling**

**Project Title: Human Fibroblasts and p53 Response**

**Rationale:**

The protein TGF $\beta$  has been found to activate p53 phosphorylation upon DNA damage caused by IR in mouse epithelial cells. p53 is a protein that plays an important role in cell cycle arrest and apoptosis of cells with DNA damage. In mouse epithelial cells, it has been shown that a decrease in activated TGF $\beta$  results in a decrease in p53 phosphorylation. In mouse fibroblasts, a decrease in TGF $\beta$  results in an increase of p53 phosphorylation. p53 has the ability to differentiate between tumor and normal cells; accordingly, the p53 response has the potential to be used as a cancer treatment.

**Hypothesis:**

Mouse fibroblasts and epithelial cells show different, though related, increased p53 responses to decreased TGF $\beta$ . We predict that human fibroblasts will show a similar increased p53 response to decreased TGF $\beta$ .

**Relation to Breast Cancer:**

Ionizing radiation causes direct DNA damage or DNA damage by free radicals. Activation of TGF $\beta$  by free radicals stimulates binding to cell receptors, initiating a chain of responses leading to p53 phosphorylation, and thereby affecting cell fate for apoptosis vs cell cycle arrest.

**Christiane Abouzeid**

**Mina Bissell Lab**

**Supervisor: Paraic Kenny**

**Project Title: Determining Differential TGF $\alpha$  expression in S1 and T4 cells**

**Background:**

Previous studies in the lab indicate that specific ligands of the Epidermal Growth Factor Receptor family (which includes 7 different ligands), such as Amphiregulin, are present in varying amounts in normal (S1) and malignant (T4-2) cell lines. While S1 cells require EGF for proliferation, serial passage in the absence of EGF resulted in an outgrowth population (T4-2), which is malignant in vivo. The S1 cells were obtained by a reduction mammoplasty. Focusing specifically on another member of the EGFR family, TGF-alpha, this project set out to determine whether there are differing levels of TGF-alpha expression in S1 and T4-2 cell lines. TGF-alpha is sorted to the basolateral surface of polarized epithelial cells and is rapidly consumed by EGFRs confined to the basolateral surface once cleaved by TACE/ADAM 17, a membrane-bound protease. The hope here was to obtain a clear, reproducible result of expression. Once this result is established, the role of TGF-alpha in the survival of each cell line will be further examined. Then plasmids will be constructed, which will allow the possibility of inducing overexpression of TGF-alpha, as well as other ligands. After that, the specific effect that TGF-alpha induces will be examined; perhaps TGF-alpha will induce morphologically malignant characteristics in S1 cells? Also, the addition of antisense TGF-alpha may be added to T4-2 cells in an attempt to revert them to a more normal phenotype. However, prior to these experiments, the relative quantities of TGF-alpha and other EGFR ligands, such as Amphiregulin, that S1 and T4-2 cells contain will be important to determine. In addition to determining TGF-alpha expression, I will also be making more TGF-alpha from the RT-PCR product confirming presence which will be incorporated into the plasmids once they are produced.

**Results and Relevance:**

By repeated trials with RT-PCR due to varying results, finally a reproducible result of an overexpression of TGF-alpha in T4-2 cells was obtained by RT-PCR. RT-PCR at various cycles proved that TGF-alpha overexpression in the T4-2 v. the S1 cells was visible at 30 and 35 cycles. Additionally, I have constructed pBM, a plasmid which will be used to insert TGF-alpha into S1 cells. While determining overexpression is just one step of the process, it was crucial to confirm it in order to proceed with our experiments. In studying the manner TGF-alpha behaves in normal versus malignant cell lines, one is able to better understand the critical part of a pathway that may lead to

uncontrolled cell growth. With this knowledge, one might glean possible therapeutic benefits that function in inhibiting the upregulation of TGF-alpha and thus manipulate the morphology of potentially invasive cancer cells.

**Karla Galvez**

**Mina Bissell Lab**

**Supervisors: Jordi Alcaraz and Ren Xu**

**Project Title: Mechanochemical Signaling for  $\beta$ -Casein Expression**

Previous research in Mina Bissell's laboratory has found that both physical and chemical signals are required for induction of transcription of the milk protein  $\beta$ -casein in mouse mammary epithelial cells; that in addition to chemical signals from the extracellular matrix and hormones, the cells also required physical alterations of cell shape in order to functionally differentiate. Here, we have explored the relationship between chemical and physical inputs of cell function by subjecting EpH4 mouse mammary epithelial cells cultured in drip conditions to different levels of strain, and monitoring the induced changes in  $\beta$ -casein transcription and translation by RT-PCR and western blot. As a preliminary study, we measured the rate of induction of  $\beta$ -casein in cells cultured on the flexible substrata in differentiation medium (pI) or in differentiation medium to which EHS has been added. We found that the cells rapidly expressed  $\beta$ -casein when exposed to EHS, and that sustained incubation led to induction of  $\beta$ -casein even in cultures to which EHS had not been added (Figure 3). Future studies will involve dynamic and static stretching experiments.

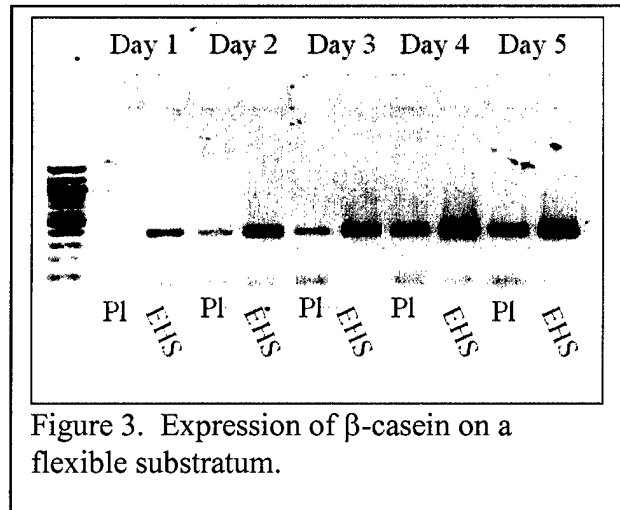


Figure 3. Expression of  $\beta$ -casein on a flexible substratum.

### KEY RESEARCH ACCOMPLISHMENTS

- **Connie Chen** (Bissell laboratory) identified the activity domain of epimorphin, a mammary morphogen involved in branching morphogenesis
- **Grace Marily Cruz** (Nandi laboratory) demonstrated that estrogen administration protects against apoptosis during metestrous stage of the estrous cycle.
- **Kim Dang** (Bissell laboratory) identified expression differences of key signaling molecules in normal and malignant human breast cells.

- **Cindy Pham** (Kane laboratory) used genetics techniques to dissect the synthetic lethality phenotype between PPR2 and TAF14.
- **Adia Scrubb** and **Yalda Afshar** (Barcellos-Hoff laboratory) demonstrated that human fibroblasts show an increased p53 response to decreased TGF $\beta$  levels.
- **Christiane Abouzeid** (Bissell lab) showed that TGF $\alpha$  is differentially expressed between normal and malignant human breast cells.
- **Karla Galvez** (Bissell lab) found that physical alterations of mammary epithelial cell morphology (by stretching on a flexible substratum) led to increased production of  $\beta$ -casein.

## REPORTABLE OUTCOMES

**Connie Chen**, **Grace Cruz**, and **Yalda Afshar** graduated with B.S. degrees, and used their research in their honors theses. **Cindy Pham** presented her work in a poster at the 43rd Annual Meeting of the American Society for Cell Biology. As of yet, no publications or patents have derived from this work.

## CONCLUSIONS

All of the preceptors agreed that the undergraduates were highly productive and enthusiastic about their research, and most of the summer trainees stayed on in their training labs for the remainder of the academic year, either as research associates or on a volunteer basis. Several manuscripts are currently in preparation that contain experimental results produced by the undergraduate trainees. Furthermore, the first year of the program was very beneficial for fine-tuning the procedures, and the second year of undergraduate trainees will benefit from a clearer set of guidelines, as a result.

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