CRIPTO-1 AND AMPHIREGULIN PRODUCTION IN TRANSFORMED MAMMARY EPITHELIAL CELLS GROWN ON HYDROXYAPATITE SCAFFOLDING

By

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The members of the Committee appointed to examine the thesis of Brena Baze Holman find it satisfactory and recommend that it be accepted.

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CRIPTO-1 AND AMPHIREGULIN PRODUCTION IN TRANSFORMED MAMMARY EPITHELIAL CELLS GROWN ON HYDROXYAPATITE SCAFFOLDING

Abstract

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The leading cancer in women is breast cancer making up 26 % of new cancer cases. Early detection of this disease is commonly linked to calcifications within the breast and typically relates to aggressive forms. Advanced stages of breast cancer metastasize to bone forming secondary lesions. This is believed to be a result of breast cancer cells forming bone-like proteins responsible for attachment and calcification typical in osteoblast cells. In addition, transformed mammary epithelial cells overproduce cripto-1 (CR) and amphiregulin (AR) proteins, which causes the cells to become more aggressive. Three cell lines representing nontransformed cells, aggressive cancerous cells, and less aggressive cancerous cells were used to identify possible correlations between CR, AR and bone-like calcifications. These cells were grown on hydroxyapatite scaffolding (a major component of bone) or plastic and studied for rate of attachment, proliferation, and production of CR and AR. It is shown here that CR increases secretion and overall production when grown on hydroxyapatite scaffolding. Also, the molecular weight of the protein identified as CR was much heavier compared to the known molecular weights. The rate of attachment was

not altered when the cells were grown on hydroxyapatite but the growth of all three cell lines was delayed. Although AR did not have any significant differences it still provides a possible role in mammary tumorogenesis. In addition these results provide insight to the relationship of hydroxyapatite and CR production and their role in transformation of mammary epithelial cells.

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Dedication

I dedicate this collection of work to my parents for their kindness, understanding and constant funding to my kitchen.

Chapter 1

Seed and Soil from Breast to Bone: no real dirt involved

Breast cancer research has been increasing and changing pace to become assertive in trying to abolish the disease. One of the major areas of interest in breast cancer research has been the role of microcalcification in tumorogenesis as a tool in predicting malignancy. Mammography has been an essential part of identifying non-palpable breast carcinomas because the image catches the calcification leading to biopsy for further analysis [1]. In this chapter mammary development and tumor progression will be described. In addition a description of cancer associated proteins and the similarity between tumorogenic cells and bone-derived cells will also be provided.

Development of the mammary gland is controlled by several hormones that regulate mammary stem cell determination and overall function. Mammary gland research has focused on estrogen, progesterone and several members of the epidermal growth factor (EGF) family of proteins. These hormones act on both mammary stem cells and the differentiated cell population to create an environment in which the mammary gland is able to be modified through puberty, pregnancy, lactation, and involution [2].

A stem cell population has the ability to self renew as well as give rise to a population of progenitors and then tissue specific cells. In the mammary gland, stem cells are present throughout development, pregnancy, and involution [3].

Mammary gland stem cells primarily reside at the terminal end buds where the majority of changes during female mammary development occur. The most undifferentiated stem cells are negative for estrogen receptor and are known to repopulate the mammary gland through self-renewal to maintain a constant population of stem cells. They give rise to progenitor cells which further differentiate into one of the three specific cell types of the mammary gland [2; 4; 5]. It is in the later stages of differentiation at which progenitor cells into the gain ability to express isoforms of estrogen receptor α [3]. During periods of non-pregnancy, stem cells remain quiescent, juxtapose to the sites of the mammary system. The mammary stem cell population is dependent upon signaling from estrogen, progesterone and prolactin to become activated for proliferation and differentiation.

The mammary gland is primarily composed of three differentiated cell types: ductal, alveolar and myoepithelial cells [3; 5]. Also present in the gland are fibroblasts, adipocytes, and other cell types important to the structure of the mammary gland but not affecting gland function as shown in Figure 1 [6]. Mammary gland development begins as an extension of the epithelium in embryonic stages. This occurs via invaginations and tubular formation which requires a series of proliferative and apoptotic events initiated by maternal hormones. These remodeling events cease once maternal hormones are removed after birth [5]. Further maturation of the mammary gland is delayed until puberty when the female begins cycling, thus producing the necessary estrogen, progesterone and prolactin among other essential proteins. In turn, these

hormones affect the mammary gland stem cells, primarily at the terminal end bud which will eventually be responsible for morphogenesis during pregnancy and lactation [3; 7]. The morphogenic changes in the mammary gland are important in alveolar development. Once the cells are fully developed they are able to produce and secrete milk. During involution of the mammary gland, apoptosis of the differentiated cells occurs leaving, a population of stem cells to replenish the mammary gland in response to another pregnancy cycle[8].

It is the stem cell population that is believed to be responsible for the development of most breast cancers [2; 5; 7]. Theoretically, the self renewing stem cell population accumulates cancer causing mutations while in its quiescent state and once activated by hormone stimulation tumor formation may begin [9]. Activated cells rapidly divide but due to mutation lack the ability to form organized structures, thus forming a mammary tumor. Breast cancer (mammary tumors) can arise as several distinctive types including in situ or invasive carcinomas of the ductal or lobular cells. In situ cancers only affect the site of tumor development (ie: just ductal cells) and if detected in a timely manner a lumpectomy is an optimal decision for the patient. However, invasive carcinomas, although initially formed in ductal or lobular cells, spread through barriers laid out by the basal lamina. The aggressive spreading of these cells allows tumor formation within the entire breast. A mastectomy is typically required for complete removal of the invasive carcinomas.

In the United States, breast cancer is the most prevalent cancer in women with an estimated 178,000 new cases each year. Additionally it ranks second for

mortality with approximately 40,000 deaths each year [10]. It is estimated that in a given 5 year period there are 4.4 million women that will be alive and in remission or will be diagnosed with breast cancer and are in the process of fighting the disease [11; 12; 13]. Survival rates have only increased as a result of continued research to better understand the disease. Analysis of tumors for gene mutations, receptor status, and protein production has allowed patients, with the help of their physician, to design the best treatment options for their particular situation.

cripto-1, amphiregulin, transforming growth factor-alpha, and estrogen receptor are proteins that help regulate growth and development of the mammary gland[14; 15]. These proteins are also associated with tumor formation and invasiveness when overexpressed [11; 12; 16; 17]. These growth factor-like proteins and receptors may interact in cells via several different mechanisms to cause rapid activation, proliferation, or differentiation [18]. While normal cells require an array of nutrients and interaction with the environment to maintain viability; most cancer cells are distinct in that they are able to detach, become free floating, and evade anoikis. Cancer-associated proteins, such as cripto-1, are capable of transforming a normal cell into a cancerous cell through endogenous receptor binding or exogenously through autocrine, juxtacrine or paracrine activity allowing the transformed cells to proliferate and remain sustainable in suspension [14].

In a 1994 study, 68 tissue samples of cancerous tissue and 23 non-involved breast tissue samples were examined for presence of cripto-1 (CR) and

amphiregulin (AR). Of the carcinoma samples, 82 % were positive for CR, 77 % were positive for AR. Of the non-involved breast tissue samples only 13 % and 43 % respectively were positive for CR and AR staining [19]. Immunocytochemical analysis for both these proteins shows significantly increased production within carcinoma samples and only minor production in the non-involved breast tissue. The increased production of both these proteins in cancerous versus non-cancerous tissues suggests their expression may be correlated to breast cancer [19].

Amphiregulin (AR) is a glycoprotein and a member of the EGF-cripto FRL1 cryptic (EGF-CFC) gene family with the conserved EGF-like motif and cysteine rich domain (known as the CFC region). It was first identified in conditioned media of MCF-7 carcinoma cells treated with phorbol 12-myristate 13-acetate (PMA), a potent tumor promoter [19]. AR is able to inhibit the growth of A431 cells but a stimulatory response was seen in human forearm fibroblasts; both in a dose dependant manner [16]. AR was found to be ineffective in stimulating proliferation of SK-BR-3, a breast cancer cell line but was effective in stimulating proliferation and survival in MDA-MB-468 cells [20; 21]. AR is able to stimulate tyrosine phosphorylation through the EGF-receptor and $p185^{erbB2}$ [22; 23]. It appears that some breast and skin cancer cell lines are either inhibited or remain unstimulated by AR treatment. However, AR has a significant involvement in the survival and proliferation of nontransformed MCF-10A, MDA-MB-468 breast carcinoma, and SK-OV-3 ovarian carcinoma cells.

Cripto-1 (CR), also known as tertocarcinoma-derived growth factor, is also a part of the EGF-CFC gene family and contains the conserved EGF-like motif, the CFC region, and a short hydrophobic carboxy terminus [22]. CR protein is approximately 20 kDa but is heavily modified through post-translational modification and therefore can be found at [11; 23].. Its expression is significantly increased in the epithelial mammary cells of pregnant and lactating females compared to epithelial mammary cells from virgins or involuted females [22]. CR binds to its novel receptor that is not activated by other EGF-related peptides and enhances tyrosine transphosphorylation of erbB-4 and fibroblast growth factor receptor-1 without direct binding [24; 25]. The enhanced tyrosine kinase activation leads to proliferation and cell survival [12; 16; 22]. Overexpression and increased production of CR leads to increased invasion ability across matrigel as well as a resistance to anoikis as shown by decreased levels of fragmented DNA [26]. A combination of increased cell survival, proliferation, and resistance to anoikis leads to greater ability for cells overexpressing CR to metastasize thereby producing a more aggressive phenotype.

In order for metastasis to occur, degradation of the microenvironment is required, thus releasing cells and allowing them to become freely suspended. This provides an opportunity to enter the blood stream, allowing migration to other sites [27]. Primary cells require interaction with the microenvironment to maintain viability. When these cells are under stress they typically undergo apoptosis but in some cases they remain viable and are now immortalized as a

response to surviving the stress. Immortalized cells can continue to obtain cancer causing mutations leading to tumor formation. Furthermore, these cells can gain ability to migrate and invade primary tissue sites or enter the circulatory system.

One cause of breast cancer metastasis is the large production and secretion of matrix metalloproteinases (MMPs) from transformed mammary epithelial cells. MMPs are zinc-dependant zymogens that require enzymatic cleavage (post-translational modification) to become active. In normal tissue, active MMP's play a large role in remodeling of the extracellular matrix (ECM) which cells depend on for both autocrine and juxtacrine communication. However, cancerous cells produce increased amounts of MMP's creating more degredation of the ECM increasing the area in which cells can proliferate. The increased production of MMP's from breast cancer cells leads to tumor formation and angiogenesis. MMP's are not only involved in the process of ECM degradation but also in the regulation of cytokines, growth factors, and receptors allowing cancer cells to become highly proliferative and invasive [28]. MMP's in transformed mammary epithelial cells stimulate growth factors that cause angiogenesis, increasing the chance for cancer cells to enter circulation. Cancer cells expressing high levels of cancer related proteins, such as CR, are able to avoid anoikis, providing a larger window of time to find a location that is rich in nutrients compatible with survival. These cells are also released in large aregates that enhance the ability of secondary tumor formation [29; 30]. The combination of MMP's and overexpression of cancer-related proteins allows cancerous cells to form self-sustaining tumors, migrate into the circulatory system, and form secondary

tumor sites. This concept is the “seed and soil” hypothesis [28; 31; 32]. It is believed that the cancer cells are the seeds that are planted into soil or another tissue site that is compatible with cancer cell growth. Many breast cancers produce proteins found primarily in bone, a major site of secondary tumor formation derived from breast cancers is bone tissue [33]. This leads to severe pain in advanced stages of the disease as well as increased mortality rates as in the case of prostate and lung cancers [32; 34].

With breast cancer being a disease that has increased incidence with age, bone density can be an issue in advanced stages of breast cancer with bone metastasis. Bone is comprised of approximately 20-30 % collagen, which provides strength and flexibility, and 70-80 % hydroxyapatite giving rigidity and support to the bone structure [33]. In normal healthy individuals bone is in constant turnover to maintain important structure and functionality. Osteolysis is caused by osteoclasts which secrete potent enzymes (ie: MMP’s) that degrade the minerals of bone and release soluble calcium phosphates and other bone moiety into the blood stream. The released calcium phosphates take part in important signaling events that regulate growth of all cells but more importantly the stimulation of osteoblasts. In normal turnover osteoblasts enter sites where bone resorption has occurred and begin to produce and secrete calcium phosphorous. Once secreted it eventually becomes precipitated and transformed into hydroxyapatite, the major component of bone. As women age, a significant reduction in estrogen occurs resulting in decreased osteoblast stimulation, reduced bone rebuilding and increased bone resorption leading to osteoporosis.

Within the bone matrix there are several proteins that contribute to the attachment of osteoclasts and osteoblasts. These cells produce and secrete integrin $\alpha_v\beta3$, vitronectin, and most importantly, bone sialoprotein (BSP), osteonectin (ON), and osteopontin (OP). BSP, ON and OP are involved with bone mineralization [33]. Breast cancer cells utilize these proteins causing microcalcifications in mammary tumors [35; 36; 37]. Breast cancer cells produce and secrete these bone proteins in their primary site and are partially the cause of poor survivability of patients due to the strong interactions made with the bone microenvironment when metastasis occurs [36]. Additionally, the cancer cells also inhibit activity of osteblasts decreasing chances for bone recovery [38].

In summary, breast cancer cells produce bone-related proteins and several proteins of the EGF gene family. Breast cancer metastasizes primarily to bone but the relationship between the bone microenvironment and proteins such as CR and AR are not well known. Since mineralization occurs in primary mammary tumors it is important to understand the effects mineralization has on cell growth at the level of protein production. Treating breast cancer cells with hydroxyapatite particles increases production of MMP's enhances cancer cell proliferation shown by increased thymadine uptake [39]. This gives a better understanding of how breast cancer cells migrate from primary tumors. Although hydroxyapatite increases thymadine uptake, effects on proliferation have not been well defined. It is proposed in this research to investigate cripto-1 and amphiregulin changes in fast progressing breast cancer cells, slow progressing cancer cells and non-transformed mammary epithelial cells exposed

to hydroxyapatite scaffolds. This will, in part, define the role of hydroxyapatite in progressing cell transformation and increasing mitogenic responses of cellular milieu.

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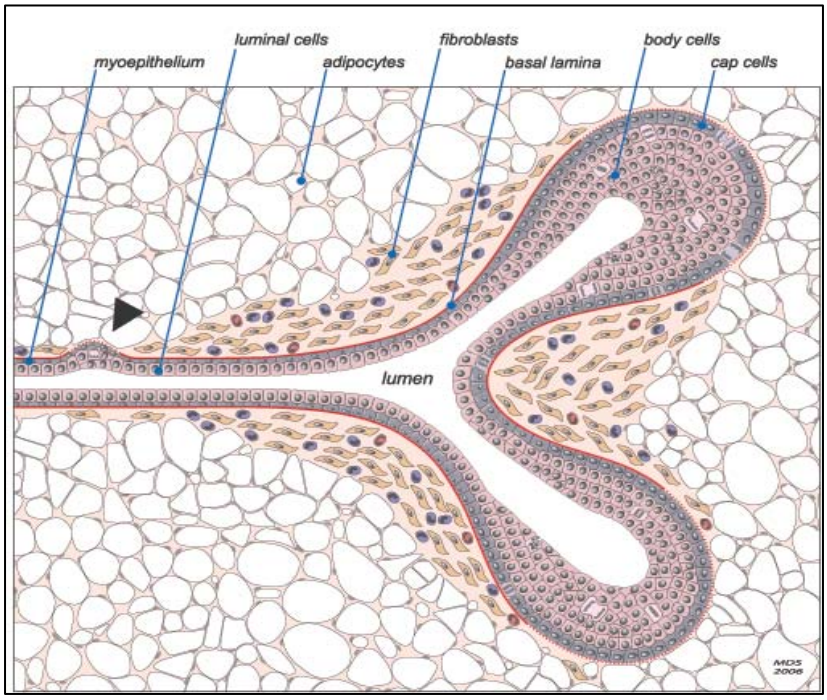

Figure 1 Schematic diagram depicting the major features of a bifurcating terminal end buds (TEB). Notable features include the considerable proliferative activity (mitoses) within the TEBs, the single layer of TEB cap cells and multilayered pre-luminal body cells, the characteristic presence of a fibroblast and collagen-rich strobal collar surrounding the neck of the bifurcating TEB, and its conspicuous absence beyond the invading distal cap of each new TEB. An increased number of macrophages and eosinophils is also shown. Although there is no evidence that normal ductal cells ever cross the basal lamina, thinning of the basement membrane (dashed lines) at the leading edge of the invading ducts may reflect partial enzymatic degredation and/or incomplete de novo synthesis of the basal lamina (derived from Visvader et al. 2006).

Chapter 2

Cripto-1 and amphiregulin production in transformed mammary epithelial cells exposed to hydroxyapatite scaffolding

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Abstract

Cripto-1 (CR) and amphiregulin are proteins regularly produced during mammary gland development, and become overexpressed in breast cancer cells. As a result of breast cancer metastases primarily targeting bone, there is an interest in studying how bone-like material can change the progression of breast cancer cells. (+)SA, (-)SA and CL-S1 cell lines were used to assess morphology, rate of attachment. proliferation, and protein production when grown on hydroxyapatite (HA). Growth rates were reduced in all cell lines grown on HA compared to control surfaces. Exposure to HA caused (+)SA, (-)SA and CL-S1 cell lines to secrete modified CR products. Cell lysates from CL-S1 cells grown on HA had comparable production of CR to transformed cell lines, (+)SA and (-)SA. These results suggest that the malignancy of breast cancer is strongly influenced by tumor calcification leading to a increased production of CR.

Key Words Cripto-1, Amphiregulin, hydroxyapatite, cell transformation, breast cancer, metastasis

Introduction

Breast cancer is a high risk disease in women with an estimated 178,000 new cases each year [1]. Additionally, mortality as a result of breast cancer ranks second with approximately 40,000 deaths each year [2]. Two proteins of the epidermal growth factor gene family, cripto-1 (CR) and amphiregulin (AR) are commonly upregulated in the disease. They act as mitogens on cancerous and nontransformed cells [3; 4; 5; 6]. These proteins have important roles in the development of the mammary gland, especially during pregnancy and lactation. When mammary epithelial cells genetically transform, it is more likely that CR and AR become enhanced and are correlated with cell invasiveness [5; 6; 7].

It has been reported previously that cancer cells can be released as multiple cell aggregates from tumors at a rate of more than 10 million cells per day[8]. These aggregates are the cause secondary tumor formation, or metastasis. Metastatic breast cancer to secondary sites is the leading cause of death in patients. Microcalcification is common in breast cancer and is one of the leading detection methods through mammography [9; 10]. Forty to sixty percent of progressed stages of breast cancer metastasize to bone causing secondary tumor formation [11; 12]. The composition of bone is 70-80 % hydroxyapatite and 20-30 % collagen produced by osteoblast cells that express osteopontin, osteonectin and bone sialoprotein matrix components through integrin binding [13]. This is believed to be a result of transformed mammary epithelial cells that produce bone matrix components having the ability to form strong interactions with bone matrix components [14; 15].

In a previous study, the use of hydroxyapatite (HA) particles,have been able to promote significant mitogenesis and caused an increased production of matrix metalloproteinases (MMP-2 and MMP-9) in MCF-7 and human mammary epithelial cells (HMEC) [16]. The production of MMPs correlates the induction of cell detachment from their anchoring point leading to possible malignancy. HA material has also shown ability to increase proliferative responses in MCF-7, Hs578T and HMEC [16]. It is hypothesized that this may be caused by altering one or more of the mitogenic proteins upregulated in breast cancer.

In this study, morphological characteristics of cancer cells were not compromised. However, the structural morphology of nontransformed CL-S1 cells appeared to develop characteristics similar to cells that have undergone transformation. We also observed the attachment and proliferation of transformed versus nontransformed cells grown on HA. All cell lines (CL-S1, (-)SA and (+)SA) demonstrated reduced attachment and cellular proliferation was significantly delayed when grown on HA. The CL-S1 cell line is a nontransformed mammary epithelial cell derived from a subpopulation of BALB/c mice containing mammary outgrowths. The (+)SA and (-)SA cell lines were also derived from the same BALB/c mice, but from the outgrowths. The difference between the two cell lines is their growth properties in soft agar (SA).

In addition, HA effects on production of CR and AR were surveyed in each of the cell lines. Western blotting of cell lysates and conditioned medium did not have detectable amounts of AR. Varying degrees of CR is detectable in both cell

lysates and conditioned medium from all three cell lines exposed to HA compared to controls. Novel modified CR products were identified and induced to be expressed in CL-S1 cells when exposed to HA. This study shows that mammary tumor interaction with HA plays a role in cell transformation through upregulation of CR and leads to increased metastasis to bone.

Materials and methods

Hydroxyapatite preparation. Hydroxyapatite discs were prepared with 0.5 g of HA powder (Berkeley Advanced Biomaterials, Inc., CA) in a compression mold with a diameter of 12.7 mm. Discs were formed by uniaxial pressing for 5 min. Following formation, the samples were sintered at 1300°C for 3 h for best densification and mechanical stability. Samples were autoclaved for *in vitro* studies in phosphate buffered saline (PBS)

Cell Culture. Cancer cell lines (+)SA and (-)SA were developed from outgrowths of the mammary fat pad in BALB/c mice and characterized using soft agar growth analysis [17]. Nontransformed Cl-S1 cells are a subline that maintained a more epithelial morphology developed also from the mammary fat pads of BALB/c mice [18]. were used to understand the growth of cancer on bone-like surfaces. All cell lines were grown in McCoy's 5A medium supplemented with 2.2 g/L sodium bicarbonate, 5 % bovine calf serum (BCS, Hyclone), 5 % fetal bovine serum (FBS, Hyclone), 0.1 g/L penicillin, and 0.1 g/L streptomycin (serum containing media). Protein experiments were subsequently grown in Breninski's defined medium (BDM) containing McCoy's 5 A medium, 10

μg/ml insulin, 5 μg/ml transferrin, 10 ng/ml EGF and 1 μg/ml hydrocortisone. Cultures were maintained in a humidified incubator at 37°C and 5 % carbon dioxide. Cells were removed from culture plates using PBS/EDTA containing 2.5 % trypsin and counted on a hemocytometer. Cells were plated in tissue culture treated, polystyrene, 48-well plates (greiner bio-one, Monroe, CA) at a density of 500 nuclei/mm^2. The bottom of each HA treatment wells was coated with high-pressure stopcock grease (Dow Corning, Midland, MI) to prevent cells from attaching to the plastic and to form a base for the HA samples. Samples were used for either time point studies or protein analysis.

Antibodies. Rabbit polycolonal antibody to mouse cripto-1 (Novus Biologicals Inc, Littleton, CO) was used to recognize the N-terminal portion of murine cripto for western blotting. Amphiregulin polyclonal antibody (Chemicon International, Temecula, CA) was used to detect the secreted portion of human amphiregulin in western blotting and has no cross-reactivity with the other EGF-related peptides. Actin antibody (Sigma, St. Louis, MO) was used as a cell lysate loading control for western blots.

Fixation and Protein Collection. Time point studies were stopped with 4 % paraformaldahyde in 0.1 M PBS at 0.25 h, 0.5 h, 3.0 h, 6.0 h, 10 h, 24 h, 48 h, and 72 h followed by staining with propidium iodide for visualization of nuclei. Protein analysis studies were grown for 5 days in serum containing media on experimental plates. At day 5, experimental wells were rinsed 3 times with PBS, and replaced with BDM. After two days of incubation conditioned media was removed and concentrated in Centriplus YM-3 Centrifugal filter units with a

nominal molecular weight limit of 3kDa (Millipore, Billerica, MA). Twelve wells per treatment were trypsinized and aliquoted to obtain a single cell suspension. Each treatment was equalized to the lowest number collected and lysed for western blot analysis.

SDS-PAGE and western blotting. A Bradford protein assay (BioRad, Hercules, CA) was used to quantify the amount of protein present in the conditioned media and cell lysates. A 12 % gel was used under denaturing conditions follwed by electrophoretic transfer to polyvinylidene difluoride (PVDF) membranes. Antibody incubations were performed in 5 % (w/v) non-fat milk in 0.1 mol/L tris base, pH 7.5, 0.15 mol/L NaCl, and 0.05 % (v/v) Tween-20. Positive antibody reactivity was detected using appropriate secondary antibody obtained from Sigma-Aldrich (St. Louis, MO) using enhanced chemiluminescence reagents (Amersham) followed by exposure to X-ray film (Pierce). Proteins were quantified by densitometric analysis of the film with Quantity One software (Bio-Rad).

Microscopy. Time point experiments fixed and stained with propidium iodide at 0.25 h, 0.5 h, 1.5 h, 3 h, 6 h, 10 h, 24 h, 48 h, 72 h, and 96 h. Five images of each well were obtained using the Bio Rad confocal in the Franchesci Electron Microscopy Center. The images were counted for fluorescently stained nuclei using ImageJ [19]. Analysis was done in Microsoft Excel to obtain an average number of cells per visual field and correlated back to total cells/well. Scanning electron microscopy (SEM) was prepared with a paraformaldehyde fixative followed by osmium tetroxide post-fix and gold sputter coating.

Statistical Analysis Significant differences were determined using standard error of the mean.

Results

We have currently investigated HA effects on changing the attachment, proliferation and protein production in mouse-derived transformed mammary epithelial cells. Nine different time points were fixed and stained with propidium iodide to observe the attachment and proliferation of all three cell lines exposed to HA or plastic surfaces. The first 6 time points were in the first 24 h and displayed a typical increasing attachment with time (Figure 1a). Overall, there was a reduced attachment at 24 h across all cell lines exposed to either HA or plastic (Figure 1). All lines grown on HA, had delayed initiation into proliferation compared to the plastic exposed cells. Only (+)SA cells reached growth comparable to the cell lines grown on plastic, but did not reach a final growth comparable to control surfaces (Figure 1b). The (-)SA and CL-S1 cells were significantly reduced in proliferative capabilities when plated on the HA and did not reach any significant growth from their attachment. The (+)SA and (-)SA cell lines had comparable growth by 96 h when exposed to HA and CL-S1 was only comparable to (-)SA but not (+)SA.

Western blot analysis was used to study internal and external protein changes in transformed and nontransformed cells grown on HA. CR protein was found at 28 kDa in similar amounts in all three cell lines exposed to plastic and HA (Figure 2a). More interesting were the additional modified CR products

found at 76, 60, and 58 kDA when exposed to the HA scaffolding. The nontransformed CL-S1 line had an increased production of the 76 and 58 kDa form of CR when exposed to the HA. The increased production of CR when grown on HA is comparable to that of the transformed (-)SA and (+)SA lines grown on plastic (Figure 2b). CL-S1 cells when grown on plastic surfaces did not produce the 76 kDa product. Both (+)SA and (-)SA cell lines produced more modified products compared to the nontransformed CL-S1 experiments.

There were no loading controls for the conditioned media experiments, however, the Bradford protein assay was used to load equal amounts of protein for each group. This method was also used in measuring protein from cell lysates and the actin loading control verified equal loading. The secretion of CR into the conditioned media was clear in all cell lines exposed to either plastic or HA (Figure 3a). However, exposing each cell line to HA caused a dramatic increase of secreted CR at ~90 kDa and ~65 kDA compared to all cell lines grown on plastic secreting only the ~90 kDa product (Figure 3b). The more aggressive (+)SA cell line secreted much more CR when grown on HA in comparison to (-)SA and nontransformed CL-S1 cell lines. Amphiregulin was not detectable through western blot in conditioned media or cell lysates.

To examine morphological changes caused by growing the cells on HA, SEM was used to obtain images of the cells interacting with their environment. Transformed mammary epithelial cell lines retain typical morphological characteristics when exposed to HA scaffolds. Breast cancer cell lines, (+)SA and (-)SA, have the typical cobblestone appearance on both the plastic and HA

(Figure 4a-d). These cells flatten and form a sheet of cells that have the ability to overlap into large clusters. (+)SA cells are not contact inhibited and form tight connections between the membranes for juxtacrine signaling. (+)SA cells clustered in groups more tightly on the HA scaffolds with some degree of overlapping. These cells appeared more sheet like on the plastic and membrane boundaries were difficult to distinguish. (-)SA cells did not change in appearance indicating that the cells were not compromised by the change in surfaces. They continued to form clear connections with the surfaces through filopodia. Nontransformed CL-S1 cells retained the ability to spread out and maintain an even distribution. They have a fibroblastic appearance on the plastic but their growth on HA causes them to become spheroid (Figure 4e, f). They still produce filopodia attachments to the HA scaffolding but their attachment appears less robust. The cells still appear to have the ability to replicate on the HA material as Figure 3c shows two instances of cells undergoing potential cytokenesis.

Discussion

Hydroxyapatite has been used in numerous *in vitro* studies; from looking at its ability to stimulate mitogenesis in cells to being used in bone implant studies. Here we further explored its effects as a compatible material for attachment, its mitogenic properties and its ability to stimulate protein synthesis. The upregulation of CR may play a role in mammary tumor formation and metastasis.

The percent of attachment in all three cell lines used was low (~30 % in most cases) suggesting the cells did not attach well to either the HA or plastic surfaces. The HA had significantly less attachment by 24 h further suggesting that HA was not an environment cells could adhere to quickly. Decreased attachment in both cases could be due to the fact that there is no true basement membrane for the cells to adhere. Cells require a basement membrane for secure attachment to surfaces. Collagen, fibronectin and laminin are examples of basement membrane components that enable cells to bind to the RGD sequence components which are important in signaling events. Cell attachment to surfaces can affect motility, actin mobilization, as well as protein synthesis as seen in these experiments. The slow growth rate is a possible result if the cells require the synthesis of a basement membrane to initiate signaling events that lead to growth. In addition, the nontransformed cell line is probably least compatible with the HA surface because it is unlikely these cells produce bone sialoprotein, osteonectin or osteopontin as the cancerous cell lines do. If a collagen containing basement membrane was provided as a thin coating over the scaffolds, the cells would be more compatible with the microenvironment, leading to a different rate of attachment and and possibly proliferation.

Once the cells attach to either of the surfaces they begin typical cellular processes, including protein synthesis, proliferation, and/or differentiation depending on their external environment. Here we see an increased production of CR occurring in the transformed cell lines (+)SA and (-)SA when grown on HA. The HA also caused CL-S1 cells to begin producing the larger 76 kDa CR protein

and increased the production of 60 kDa CR. Growing the nontransformed cells on HA resulted in a transformation of the CL-S1 cells that would otherwise be producing different post-translationally modified forms of CR as shown in the control. Again, typically CR can be found at 24, 28, and 36 kDA and less commonly at 16 and 60 kDa. Here we find several variations of CR at 58 and76 kDa in the cell lysates and 65 and 90 kDa in conditioned media.

In certain environments cells interact with neighboring cells through use of specific receptors , as well as growth factor-like proteins that will be secreted for autocrine and juxtacrine receptor binding. Exposure to the HA material initiated the nontransformed CL-S1 cell line to produce modified products similar to that of transformed cell lines signifying possible cell transformation induced by HA. Secretion of CR was increased dramatically in (+)SA cells compared to (-)SA and CL-S1 cells suggesting the aggressiveness of this particular cell line can be contributed to CR and can be further enhanced in the presence of HA material.

Cancer cells form aggregates in suspension which result in increased likelihood to form metastatic cancers [8]. We see in all three lines grown on HA scaffolds that cells have a higher tendency for cell clustering, less prevalent in the CL-S1 line. Cells that have become transformed change morphologically and typically appear rounded rather than flattened on substratum. The rounded shape of the CL-S1 cells grown on HA still had filopodia present indicating attachment. Morphology, however, resembled cells that had undergone transformation.

To further explore the effects of HA on transformed and nontransformed cells, intracellular proteins should be analyzed for additional pathways independent of CR. The use of mass spectrometry would allow identification of the varying CR products that resulted when exposed to HA scaffolding. We would like to determine if HA is sufficient to induce CR mediated proliferation and survival and how this process is initiated.

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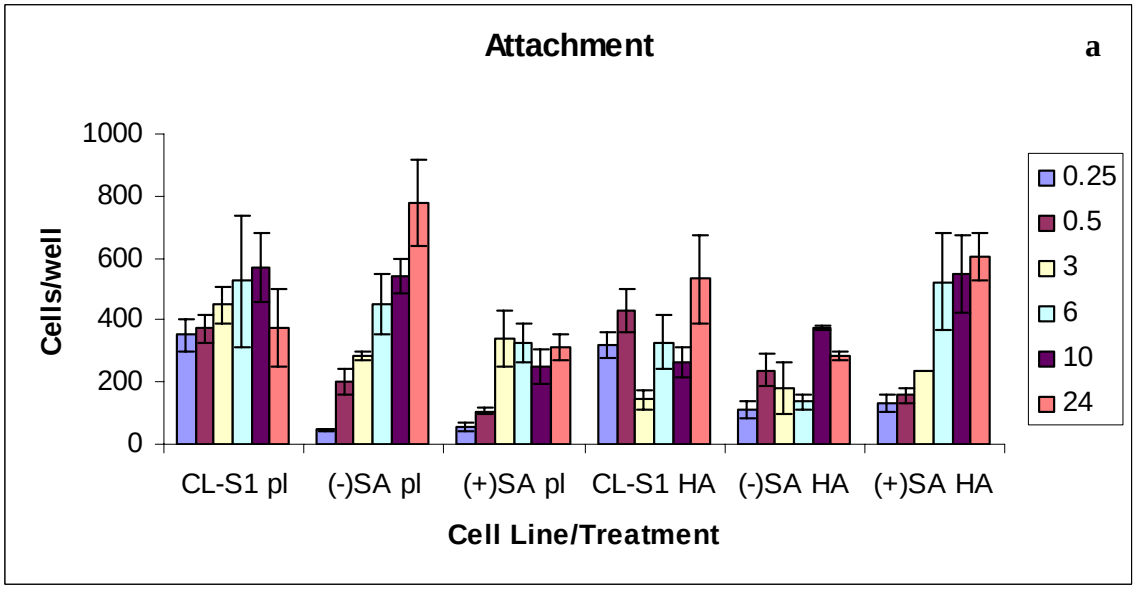

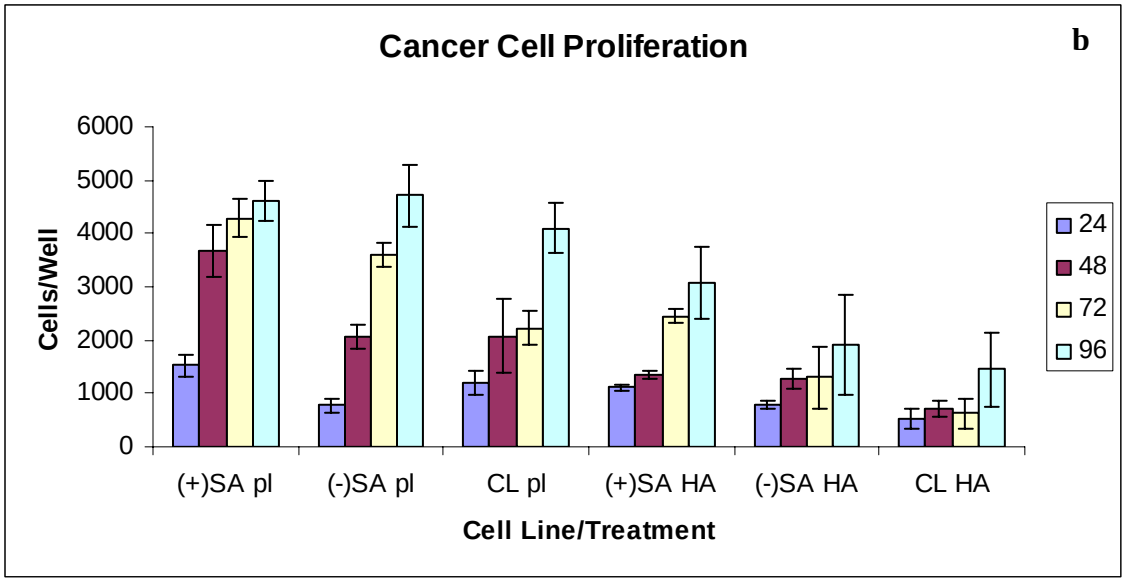

Figure 1 Cell counting averages for attachment (a) and proliferation data (b) for each cell line at each of the time points mentioned in Materials and Methods. Data are presented as means ± SEM. The graphs are representative to averages of 5 fields/well with three replicates per time point.

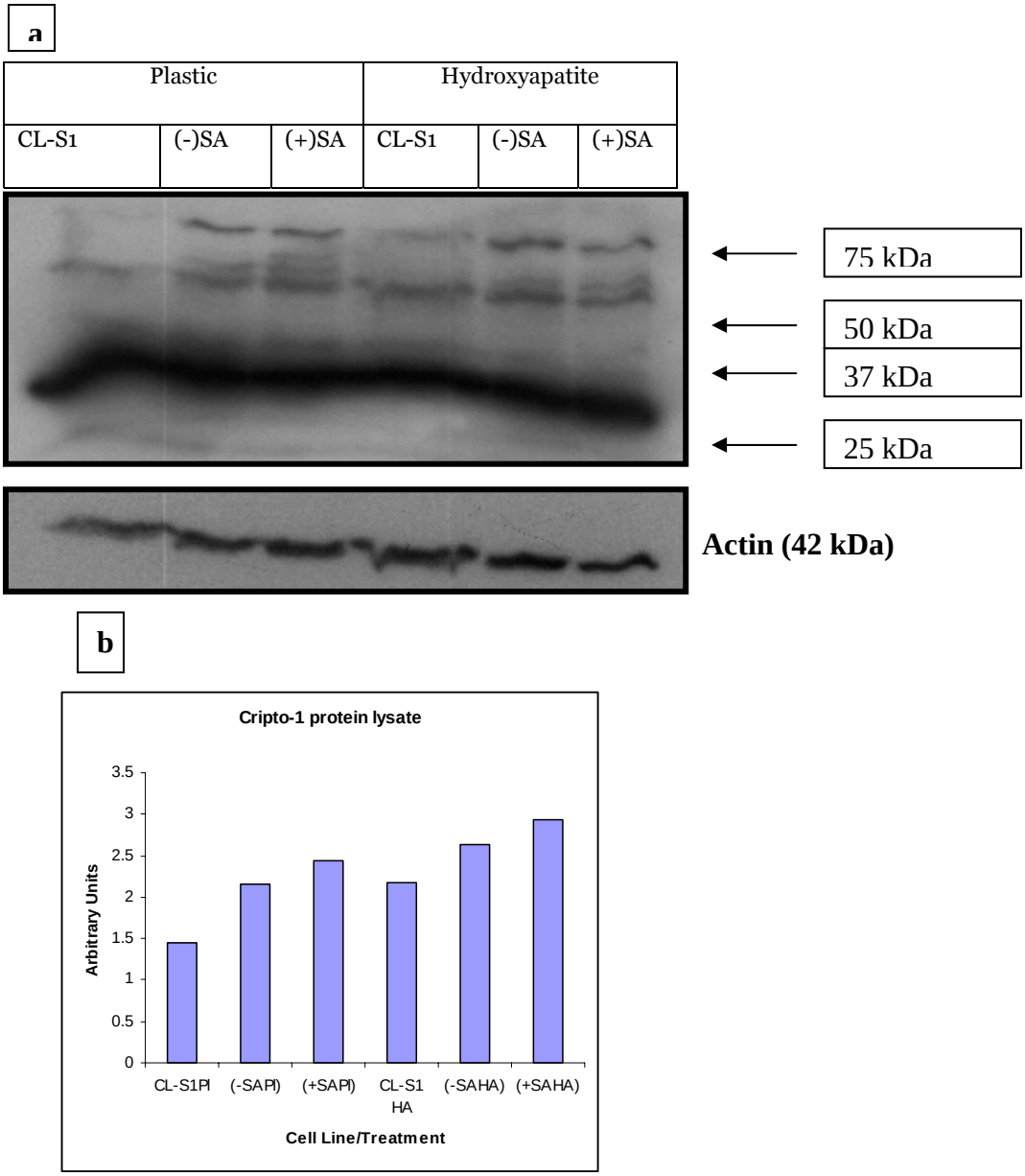

Figure 2 Western blots for CR in the cell lysates of three representative mammary epithelial cell lines exposed to plastic or HA. Five μg total protein for each cell line was probed for CR and actin (A). Data is representative of CR density adjusted to background and normalized to actin (B). Molecular weights were determined using a pre-stained Bio-Rad molecular weight ladder.

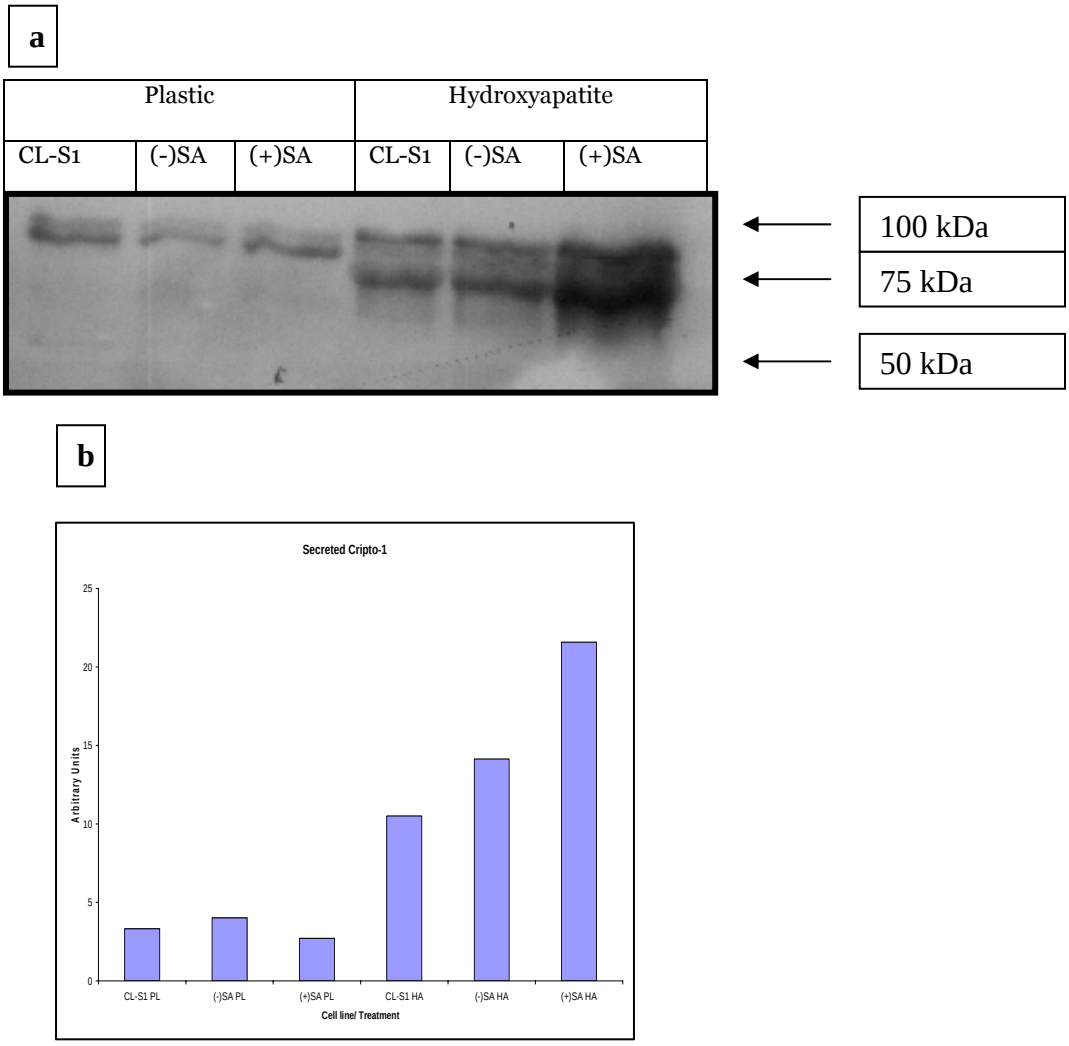

Figure 3 Western blots for CR in the conditioned media of three representative mammary epithelial cell lines exposed to plastic or HA. Five μg total protein for each cell line was probed for CR (A). Data is representative of CR density adjusted to background (B). No loading control was used. Molecular weights were determined using a pre-stained Bio-Rad molecular weight ladder.

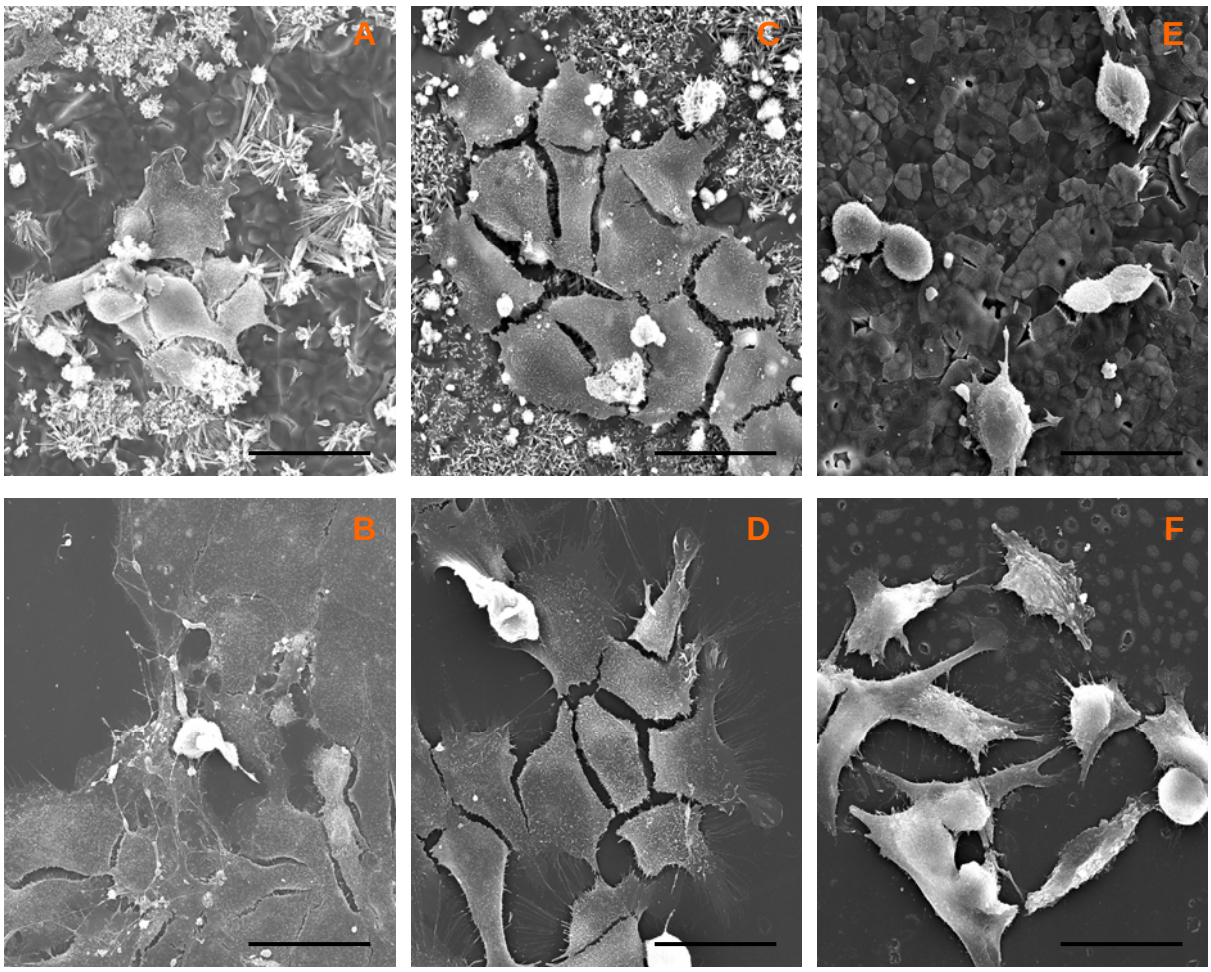

Figure 4 Scanning electron micrographs of +SA, -SA and CL-S1 cell lines grown on hydroxyapatite (upper panels A, C, and E) or plastic (lower panels B, D, and F). +SA (A & B) and –SA (C & D) cell lines maintain a cobble stone appearance (cell clustering) characteristic of cancer cells on both hydroxyapatite and plastic. CL-S1 cells become more rounded on hydroxyapatite (E) than on the plastic (F) and both are more spread out, with exception to mitosis.

Chapter 3

Summary and Conclusion

There are several mechanisms that must be considered when venturing into the field of mammary tumorogenesis. In this study we focused on cripto-1 (CR) and amphiregulin (AR) changes in mammary cell responses to hydroxyapatite (HA), the calcium based molecule that makes up 70-80 % of bone. HA has continued to show its dramatic role in mammary tumorogenesis. HA crystals are found in both benign and malignant cancers but are the main form found in invasive ductal carcinomas [1]. Although use of microcalcifications as a diagnostic tool is still inconsistent it is clear that HA has a significant effect on the progression of the disease through its ability to affect mitogenesis, and protein production [2; 3]. In this study, the response nontransformed mammary epithelial cells had to the HA material showed a significant change in both morphological characteristics and CR protein production. We continued to see its ability to affect both transformed and nontransformed mammary epithelial cells through post-translational modification of CR.

Although nontransformed cells produce CR there was a change in the modified cripto-1 constructs as shown through western blot analysis. These additional constructs may play a role in mammary epithelial cell transformation. To further test the effects of HA on cell transformation knocking down CR from the system would further describe the effects to be direct or indirect.

Although AR was not detectable through western blot analysis it should not be ruled out as a possible regulator in mammary epithelial transformation. AR has been shown to play a role in transforming immortalized human and mouse mammary epithelial cells [4]. Proliferation of HMECs increased in the presence of AR, whereas, tumoral cell lines did not have enhanced proliferation [4; 5]. Tumoral cell lines did have an enhanced production of plasminogen activator which led to increased invasiveness [5]. It should be further analyzed at the gene level to see if transcriptional changes are occurring due to the exposure to HA scaffolding.

Transformed mammary epithelial cells produce bone-sialoprotein, osteonectin and osteopontin which are all key components in the calcification process [6; 7; 8]. In addition, these cells decrease osteoblast activity allowing tumor cell invasion which leads to poor survivability [9; 10]. The results of this study indicate that attachment and proliferation of transformed mammary epithelial cells is low. However, it has been shown in other lab groups, that HA is mitogenic [3]. The other studies used HA particles as a treatment to cells that were already adhered. Here we looked at the ability of these cells to adhere as an example of metastasis. This suggests that attachment may be compromised and thusly resulting in the delay of proliferation. Several studies show that CR has direct effects on tumorogenesis on mammary epithelial cells [11; 12]. Here we see further that HA affects protein synthesis and can be compared back to calcification of mammary tumors and increased malignancy.

Since the bone microenvironment is the primary site of breast cancer metastases, it can also be hypothesized that the HA component is driving transformed mammary epithelial cells to up-regulate cripto-1 production. This would promote cell survival and proliferation in their own population as well as transformation of neighboring nontransformed mammary epithelial cells.

The use of HA in mammary tumor studies continues to prove a useful tool in understanding its biological characteristics. Further studies looking into intracellular pathways that lead to identification of receptor interaction with the HA would give insight as to how CR production is influenced. In addition, the novel modified CR products should be cloned and studied for their effects on cell proliferation and transformation. Mass spectrometry would be a useful tool in identifying the different forms of CR that occur due to post-translational modification. This technique would allow further detail on if the change in molecular weights is due to glycosylation or sumoylation. In conclusion, we know HA still functions as an important part of the development of tumorogenesis in the breast and the involvement of CR and AR in this process could provide more insight into the mechanism of action.

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