The Regulation of RANK and RANKL mRNA Expression through Activation of the JAK2/STAT5a Pathway in Human Breast Cancer Cell Lines ©

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A Thesis Submitted in Partial Fulfillment of the Requirements of the Degree of

Masters in Applied Bioscience

In

The Faculty of Science

Applied Bioscience MSc.

University of Ontario Institute of Technology

Sept 2009

Abstract

The receptor activator of nuclear factor-κB (RANK) and its ligand, RANKL has have been implicated as an important link between breast cancer and metastasis to bone because of their ability to activate intracellular signal cascades leading to altered cancer cell behaviour and bone breakdown. The JAK/STAT5a cell signaling pathway is also crucial to breast biology and is involved in transcriptional regulation of many genes. The objective of this study is to determine if RANKL mRNA expression is regulated through the JAK/STAT5a pathway by stimulating human breast cancer cell lines, MCF-7 and MDA-MB-231, with prolactin (PRL), epidermal growth factor (EGF) and heregulin-beta1 (HRG-β1), all known to activate STAT5a and play a role in breast cancer progression. This study shows that RANKL expression is upregulated by PRL, EGF and HRG-β1, and that PRL and HRG-β1 regulate transcription through the JAK/STAT5a pathway.

Keywords: RANKL, STAT5, breast cancer, prolactin, epidermal growth factor, heregulin

Acknowledgments:

First, I would like to deeply thank my supervisor, Dr. Holly Jones-Taggart. Thank you for always being there with support and encouragement in both my research and writing. She has truly made my time here a more positive experience and pushed me to achieve my greatest. I definitely would not have been able to complete this degree without her guidance and assistance.

A great thanks to the rest of my committee, Dr. Otto Sanchez and Julia Green-Johnson, for your positivity and advice with regards to my research. You were always happy and willing to listen and to help me succeed.

The fellow graduate students have always been a great source of support and have brought light to the everyday stresses of research. They have created a supportive environment and we have become close friends. They have made my academic career far more enjoyable.

Lastly, I wish to thank my family and friends for all their love and support. They have always been a source of comfort and encouragement. They have always believed in my strength and potential and gave me great confidence to be successful.

List of Abbreviations:

ATCC – American Type Culture Collection

BSA – bovine serum albumin

DMSO – dimethyl sulfoxide

ECL – enhanced chemiluminesent

EDTA - ethylenediaminetetraacetic acid

EGF – epidermal growth factor

EGFR – epidermal growth factor receptor

EMEM – Eagle's minimal essential media

ER – estrogen receptor

FBS – fetal bovine serum

$GAPDH_3$ – glyceraldehyde 3-phosphatase dehydrogenase

GAS – gamma interferon activation site

GRB-2 – growth factor receptor-bound protein 2

HER – human epidermal growth factor receptor

HRG – heregulin

IGF-2 – insulin-like growth factor-2

IGFR – insulin-like growth factor receptor

IL – inerleukin

iNOS – inducible nitrous oxide synthase

JAK2 – Janus-activated kinase 2

MAPK – mitogen-actived protein kinase

MGF – mammary gland factor

MMP-9 – matrix metalloproteinase 9

NDF – neu differentiation factor

NF-κB – nuclear factor kappa B

NRG – neuregulin

ODF – osteoclast differentiation factor

OPG – osteoprotegerin

p/s – penicillin/streptomycin

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PI3K – phosphatidylinostol-3 kinase

PLC-γ - phospholipase C gamma

PR – progesterone receptor

PRE – prolactin-response element

PRL – prolactin

PRLR – prolactin receptor

PTHrP -parathyroid hormone-related protein

PVDF – polyvinylidene fluoride

RANK – receptor activator of nuclear factor kappa B

RANKL – receptor activator of nuclear factor kappa B ligand

SDS – sodium dodecyl sulfate

SH2 – src homology 2

SOS – son of sevenless

STAT – signal transducer and activator of transcription

TGF-α – transforming growth factor alpha

TNF – tumor necrosis factor

TNFR – tumor necrosis factor receptor

TRAF – tumor necrosis factor receptor-associated factors

WAP – whey acidic protein

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1.1 – Receptor Activator of Nuclear Factor-κB and Its Ligand

1.1.1 – RANK

The Receptor Activator of Nuclear Factor κB (RANK - also known as tumor necrosis factor-related activation-induced cytokine (TRANCE) and osteoclast differentiation factor (ODF)) is a member of the tumor necrosis factor (TNF) cell surface receptor superfamily. This receptor is involved in the activation of the transcription factor Nuclear Factor Kappa-B (NF- κB), and thus controls transcription of its target genes via an intracellular cascade of signals (Wei et al, 2001).

The RANK protein is a transmembrane receptor that consists of 383 amino acids and contains a highly conserved cysteine extracellular domain and a relatively long intracellular domain unique to other TNF receptors (Darnay et al, 1998). RANK mRNA is highly expressed in bone marrow derived osteoclasts and mature osteoclast progenitors including bone cells. RANK protein is typically expressed on cells of the macrophage/monocytic lineage, T cells, B cells, dentritic cells, and fibroblasts (Anderson et al, 1997; Hsu et al, 1999). Studies prove that RANK is expressed in mammary epithelial cells, but not stromal cells (Srivastava et al, 2003)

1.1.2 – RANKL

The receptor activator of nuclear factor-κB ligand (RANKL) binds the RANK receptor initiating a signal transduction pathway. The *rankl* gene resides on the human

chromosome 13q14 (Anderson et al, 1997) and spans approximately 36 kb of genomic DNA and comprises six exons. The gene also exists as two other isoforms: hRANKL2 and hRANKL3. These isoforms are translated at the downstream in-frame start codons (Suzuki et al, 2004) and both lack the N-terminus intracellular domain. The hRANKL3 isoform also lacks a transmembrane domain, resulting in a soluble form of RANKL. All three isoforms contain identical C-terminal extracellular domains.

The RANKL protein is highly conserved with human and mouse sharing 87% homology (Lacey et al, 1998). It also shares 30% homology to TNF-related apoptosis – inducing ligand (TRAIL) (Lacey et al, 1998) and 20% homology to Fas ligand (Anderson et al, 1997). The RANKL protein also exists in three forms: a 45 kDa membrane-associated protein, a 31 kDa soluble protein by proteolytic cleavage or as a 39.5 kDa soluble protein following expression of the hRANKL3 isoform (Ikeda et al, 2001). RANKL mRNA is expressed in fewer tissues, but at high levels in bone and bone marrow, with some expression in thymus, spleen, fetal liver, and Peyer's patches (Simonet et al, 1997). Its expression is induced in breast epithelial cells only during pregnancy and lactation (Fata et al, 2000).

1.1.3 – The NF-κB Signaling Pathway

NF-κB is a transcriptional regulator present in mammalian cells, where it regulates genes involved in apoptosis, cell cycle control, immune responses, and many other cell events (Dolcet et al, 2005). This regulator is activated by two separate pathways, the canonical pathway and alternative pathway, each of which leads to nuclear translocation and the activation of specific genes (Dolcet et al, 2005).

The RANK-RANKL pair exerts their effects through the alternative signal transduction pathway that causes activation of NF-κB (Cao et al, 2001). Although the exact mechanism of this transduction pathway is not fully elucidated, there has been considerable progress in unfolding this pathway, especially in osteoclasts and osteoclast precursor cells. RANKL, either membrane-bound or soluble forms, bind to the RANK receptor ultimately causing activation of the catalytic subunit IKKα (Cao et al, 2001). When RANKL binds with its receptor, the RANK receptor interacts with subunits referred to as TNF-receptor associated factors (TRAFs). TRAF molecules are cytoplasmic adaptor proteins that mediate the signaling events for specific members of the TNFR family. There are currently six known TRAF proteins all containing an N-terminal zinc RING finger, multiple zinc fingers, and C-terminal TRAF-N and TRAF-C domains. After ligand binding, RANK can interact with a variety of TRAF proteins (1, 2, 3, 5 and 6) (Hsu et al, 1999), however TRAF6 appears to be the most critical for RANK signaling (Mizukami et al, 2002).

Recent studies have shown activation of NF-κB to be linked to several types of cancer (Karin et al, 2002). In many tumour cells, genes involved in NF- κB activation are deregulated, causing it to be constantly activated (Dolcet et al, 2005). NF- κB is believed to play many roles in normal cell cycle progression, including promoting the expression of cyclins, important cell cycle machinery. NF- κB promotes expression of cyclins D1 (Guttridge et al, 1999), D2 (Hinz et al, 1999), D3 (Hinz et al, 2001), and E (Hsia et al, 2002), and c-myc (Duyao et al, 1992). In particular, cyclin D1 expression has proven to be involved in mammary gland development and breast carcinogenesis (Yu et al, 2001).

1.2 - Functions of the RANK/RANKL Pathway

1.2.1 – Role in Bone Remodeling and Bone Disease

The RANK/RANKL pathway has emerged as an important pathway in bone remodeling. Bone is constantly degraded and reformed, at a consistent rate through the actions of osteoblasts and osteoclasts. These two cell types have opposite functions in bone remodeling and skeletal homeostasis. Osteoblasts originate from mesenchymal stem cells and differentiate into mature, matrix-secreting osteoblasts. Osteoblasts are responsible for the rebuilding of bone by secreting extracellular matrix proteins, which over time become mineralized to form functional bone. Osteoclasts are multinucleated giant cells involved in degrading bone matrix and originate from hematopietic cells. Degradation is accomplished by the osteoclast attaching to the surface of the cell and creating a sealed off environment in to which it will secrete proteolytic enzymes, removing osteoid and exposing the bone mineral. The osteoclast then releases H^+ into the space, creating pores in the bone.

The cycle of bone remodeling is a tightly regulated process, involving several stages. The process of osteoclast maturation and bone resorption is tightly regulated by three main factors: the RANK receptor, its ligand, RANKL, and a decoy receptor osteoprotegerin (OPG). RANK is expressed by most multinucleated osteoclasts (Burgess et al, 1999), and RANKL mRNA is expressed in high levels by bone osteoblasts (Simonet et al, 1997). RANKL binds specifically to the RANK receptor on osteoclast or osteoclast precursor cells and activation of this pathway by RANKL binding results in osteoclast maturation (Malywankar et al, 2000; Lacey et al, 1998), increased osteoclast activity (Lacey et al, 1998), and inhibition of osteoclast apoptosis (Fuller et al, 1998).

Studies have specifically shown RANKL to affect bone resorption by inducing osteoclasts to undergo a rearrangement of actin to form rings and perform multiple cycles of bone resorption (Burgess et al, 1999). See Figure 1.

OPG acts as a decoy receptor, decreasing the effects of RANK-RANKL signaling by competitively binding to RANKL. OPG is a RANK homolog and functions by binding to RANKL on osteoblast/stromal cells and thus inhibiting RANK-RANKL interaction and osteoclast activity (Figure 1). OPG mRNA is expressed in a large number of tissues including lung, kidney, heart, liver, stomach, intestine, brain and spinal cord, thyroid gland, and bone (Simonet et al, 1997; Yasuda et al, 1998).

Knockout studies in mice have been the major contributor to knowledge about the functions of the RANK/RANKL pathway in bone. Studies in which mice are rendered null for the *rankl* gene, mice have been shown to suffer osteopetrosis, stunted growth and a defect in tooth eruption due to failed osteoclast development (Simonet et al, 1997; Kong et al, 1999a). RANKL -/- osteoblasts were not able to support normal osteoclastogenesis, and therefore mice showed decreased osteoclast production and severely impaired bone remodeling (Kong et al, 1999a).

Figure 1: The activation of osteoclast cells through the binding of RANKL to the RANK receptor. OPG binds RANKL as a decoy receptor, inhibiting the binding of RANK

1.2.2 – Role in Immunology

Along with being expressed in bone cells, RANK and RANKL are also expressed in activated T cells, B cells, and dendritic cells (Anderson et al, 1997; Kong et al, 1999b). This information led to the discovery of the role of this pathway in survival of dendritic cells, dendritic-T-cell interactions (Page and Miossec, 2005), organogenesis and lymphocyte differentiation (Wong et al, 1999). A study by Kong et al (1999b), found that co-culturing activated CD4+ T cells and bone marrow cells induced osteoclastogenesis, which could be blocked by the addition of OPG. Soluble RANKL produced by T lymphocytes is able to induce functional osteoclasts (Kong et al, 1999b). This indicates that there is a clear link between activated T lymphocytes and osteoclastogenesis, thus linking bone destruction and immune disorders, such as rheumatoid arthritis (Kong et al, 1999b). RANKL knock-out mice are immuno-compromised and lack Peyer's patches.

1.2.3 – Role in Lobular-Alveolar Development

The human breast undergoes many stages of development, growth and differentiation. The mammary gland is composed of epithelial cells forming a branched ductal system during puberty and a lobuloalveolar compartment that develops during pregnancy (Henninghausen and Robinson, 2001). The ducts constantly branch into smaller and smaller ductules, eventually terminating in lobules. These lobules consist of alveoli, which in turn consist of secretory epithelial cells that undergo functional differentiation (Henninghausen and Robinson, 2001). This complete structure allows for lactation in the mammary gland after pregnancy (Henninghausen and Robinson, 2001).

A new role for RANK/RANKL pathway emerged from the study of mice rendered null for RANK and RANKL through genetic ablation. Along with defects in osteoclast development and immune dysfunction, mice also lacked lobulo-alveolar structures during pregnancy, eliminating lactation (Simonet et al, 1997; Fata et al, 2000). Loss of these structures was due to lack of proliferation and differentiation of the alveolar buds into functional mammary gland, but mice actually exhibited normal mammary development at earlier stages, such as ductal side-branching and formation of the initial alveolar buds (Fata et al, 2000). Fata et al (2000) found that RANKL induced lobulo-alveolar development in mice by stimulating proliferation of alveolar bud epithelia. Delivery of RANKL into the mammary tissue of pregnant RANKL-/- mice restored the lobulo-alveolar differentiation and milk production (Fata et al, 2000).

The RANK-RANKL pathway was the first system identified that is connected to both breast development and bone destruction, providing clues to the prevalence of osteoporosis in women and the frequent occurrence of metastasis of breast cancer to bone. Thus, the RANK/RANKL system became the first molecular link between breast and bone.

1.2.4 – Role in Breast Cancer Progression and Metastasis

RANKL has recently been studied for its role in breast cancer progression. Not only is RANKL required for the formation of lobulo-alveolar structures in the breast during pregnancy, but it is also been shown to cause an increase in proliferation and decrease in apoptosis of mammary epithelial cells when over-expressed in transgenic mice (Gonzalez-Suarez et al, 2007). Other studies report an increase in motility in breast

cancer cell lines (Jones et al, 2006) and other carcinomas (Mikami et al, 2009) with recombinant RANKL protein stimulation and also report that blockade of RANK/RANKL signaling slows metastasis of cancer to bone (Canon et al, 2008).

Metastasis to bone is the most common outcome of advanced breast cancer, and is what most often causes lethality. In fact, approximately 80% of patients with advanced breast cancer suffer from bone metastases (Lau et al, 2009). For this reason, researchers have been attempting to understand the mechanism of preferential bone metastases. After understanding the role of the RANK/RANKL system in osteolysis and osteoclast maturation and its connection to female hormones and involvement in proliferation of breast tissue, it is clear that this system exposes an important link between breast and bone.

It has been shown that RANKL plays an important role in the aspects of breast cancer progression by affecting proliferation, apoptosis and motility; but what causes the large amount of breast metastasis to bone? Metastasis requires several important steps, in order for a tumor to leave its primary site and sustain in a secondary location in the body. The metastatic process begins with proliferation of the primary tumor and invasion through adjacent tissues and basement membranes. Once the tumor invades blood vessels or lymphatic channels, tumor cells can detach from the primary tumor site and travel through the blood or lymphatic fluid to a secondary organ. Upon arrival at the secondary tissue, the tumor extravasates into the surrounding tissue and continues to proliferate. All of these steps are highly regulated by the expression or inhibition of particular genes giving the tumor cells these metastatic phenotypes.

The outcome of bone metastases depends on the microenvironment and the interactions of the tumor and host cells. Stephen Paget (1889) was the first to introduce the concept of the 'seed and soil' theory, which describes the interaction of breast tumor cells with bone. The bone microenvironment is a very fertile "soil" for seeding circulating cancer cells. Bone turnover provides an environment rich in factors for cancer growth. As mentioned previously, RANKL is a regulator in osteoclastogenesis and ultimately bone degradation, which releases cytokines and growth factors. In breast cancer, bone metastases are predominately osteolytic, meaning bone destruction is caused by osteoclast stimulation and not by the direct effects of the cancer cells on bone (Yoneda et al, 1994). The mechanism of osteolytic metastasis has not been fully elucidated, but there are many factors that are slowly being unraveled by researchers. In metatastic human breast cancer, parathyroid hormone-related protein (PTHrP) has so far been shown to be the main player. PTHrP is a key mediator for osteoclast activation and is expressed in human osteolytic breast cancer cells *in vivo* (Bryden et al, 2002). Release of PTHrP and interleukins (IL) 1, 6, and 11 by tumor cells stimulate production of RANKL and decrease OPG in stromal and osteoblast cells (Thomas et al, 1999). Conversely, expression of these proteins did not cause an upregulation of RANKL in breast cancer cells themselves, indicating that the tumor cells have an indirect effect on osteoclast activation (Thomas et al, 1999). However, some studies do show that RANKL expression in breast cancer cells is upregulated by co-culture with osteoblasts (Schubert et al, 2008). An increase of RANKL in osteoblast and stromal cells will ultimately lead to an increase in bone resorption and the formation of osteolytic lesions. Bone resorption in turn, causes the release of growth factors that facilitate tumor cell growth and survival,

completing a feedback loop. RANKL can also stimulate angiogenesis (Kim et al, 2003) and endothelial cell survival (Kim et al, 2002), which suggests a role in supporting vascularization in bone metastases.

1.3 – Signal Transducer and Activator of Transcription Pathway

1.3.1 – STAT proteins

Signal transducers and activators of transcription (STATs) are transcriptional regulators involved in many biological processes, including cell growth, differentiation and survival. Seven STAT proteins have been identified: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 (Copeland et al, 1995). Each of the STAT proteins contains several conserved domains, which tend to be present in transcription factors. These domains include heptad leucine repeats, a helix-turn-helix motif and Src Homology (SH)- 2 and -3 domains. The SH2 domain is involved in the specific binding to a sequence motif flanking a tyrosine phosphorylated residue, while the SH3 domain is likely involved in targeting of signaling components to specific subcellular locations.

These STAT proteins are activated through signal transduction pathways and become phosphorylated on tyrosine residues. After the protein is activated by phosphorylation, it forms homomeric and heteromeric dimers by an SH2 phosphotyrosine interaction, and then enters the nucleus where it will regulate transcription.

1.3.2 – STAT5a Pathway in Mammary Gland Development

The STAT5 protein was first discovered in the mouse mammary gland and termed a “mammary gland factor (MGF)” (Schmitt-Ney et al, 1991). It was soon discovered that

this protein shared significant sequence homology with the STAT family and was later termed “STAT5” (Gouilleux et al, 1994; Wakao et al, 1994). Another isoform of the STAT5 protein was discovered in the mammary gland shortly following and was subsequently termed “STAT5b”, where the original STAT5 protein was termed “STAT5a”. These two compounds are highly similar, sharing 96% homology (Liu et al, 1995). Stat5a transduces its signal through interaction with Janus-activated kinase (JAK)-2. Binding of a ligand to the receptor allows for receptor dimerization bringing two JAK2 molecules in close proximity and allowing them to phosphorylate tyrosine residues on themselves and the receptor. Other STAT5a/b proteins are able to recognize these tyrosine motifs through their SH2 domains and are recruited to docking sites. This causes rapid phosphorylation of a conserved tyrosine residue on the C-terminus of Stat5a by JAK2. Phosphorylation of Tyr694 on the Stat5a molecule causes it to be active and homo- or heterodimerize and translocate to the nucleus.

Stat5a is involved in mammary gland development, specifically in pregnancy during lactation. Phosphorylation of Stat5a and Stat5b is very low in the mammary tissue of virgin mice and during early pregnancy, however levels rise drastically after day 14 of pregnancy in mice (Lui et al, 1996), indicating that Stat5a phosphorylation may be a critical step in the terminal differentiation of mammary secretory epithelium. Stat5a null mice exhibit decreased lobuloalveolar development and are unable to lactate after their pregnancy (Liu et al, 1997).

The importance of STAT5a in the development of secretory lobuloalveolar structures relies mainly on its ability to regulate gene expression. Most notably, STAT5a plays a major role in the expression of the milk-secreting proteins (Liu et al, 1997).

Along with milk proteins, STAT5a is thought to regulate genes involved in proliferation and cell cycle progression; however these mechanisms are far less recognized. Cyclin D1 is an important cell cycle regulator that is thought to be regulated by STAT5a, as mice deficient in cyclin D1 exhibit similar phenotypes to STAT5a knock-out mice (Fantl et al, 1995; Sicinski et al, 1995). It is hypothesized that cyclin D1 is either upregulated directly by STAT5a through activation of its promoter (Brockman and Schuler, 2005) or indirectly through the activation of insulin-like growth factor-2 (IGF-2) (Brisken et al, 2002). Thus, STAT5a plays a major role in proliferation and development of the breast during pregnancy.

1.3.4 –STAT5a Pathway in Breast Cancer

Although the JAK2-STAT5a pathway is involved in normal development of the mammary gland and breast, it is also being investigated for its role in cancer progression. STAT5a is involved in normal cellular processes that involve accelerated cell proliferation (Iavnilovitch et al, 2002) and inhibition of cell apoptosis (Cui et al, 2004), therefore it is possible that disruption of STAT5a regulation may be a cause of cancer progression and initiation. This may happen through mutations enhancing the transactivation potential, altered activity of STAT5 regulators, or through expression of interacting proteins.

Studies of STAT5a in cancer initiated in transgenic mice expressing TGF-α in their mammary glands, show development of mammary hyperplasia and tumors, in which the onset could be delayed by the inactivation of STAT5a. Mice with decreased levels of STAT5a due to mutations displayed fewer adenocarcinoma tumors and the tumors were

smaller in size (Ren et al, 2002). This was the first bit of evidence that linked STAT5a to mammary-tumor initiation and progression.

In cases of human breast cancer, studies have shown conflicting results when it comes to the involvement of STAT5a in initiation, progression, and metastasis. A study completed by Cotarla et al (2004), indicated that STAT5a was tyrosine-phosphorylated and nuclear localized in 76% of invasive breast adenocarcinomas examined. This study also observed a positive correlation between tumor differentiation and active STAT5a. Conversely, in a larger study with more than 1100 breast cancer specimens, active STAT5a was associated with a favorable prognosis (Nevalainen et al, 2004). Active STAT5a was associated with an increased overall patient survival, and loss of activated STAT5a in primary tumors of patients with lymph node-negative breast cancer was associated with an increased risk of death from breast cancer (Nevalainen et al, 2004). Other studies confirm that STAT5 protein levels in estrogen receptor (ER)-positive breast cancer were correlated with favorable prognosis and improved response to antiestrogenic therapy (Yamashita et al, 2006). Also, levels of active STAT5a were generally reduced in breast cancers when compared to healthy luminal breast epithelial cells (Bratthauer et al, 2006), however, these levels were retained in more differentiated, secretory breast cancer (Strauss et al, 2006). More studies will need to be conducted in order to determine how STAT5 is implicated in breast cancer, although present evidence clearly shows that it plays an important role.

1.3.5 – Regulation of RANKL Transcription by STAT

RANKL transcription is regulated by STAT proteins, due to their ability to bind to and induce transcription through the prolactin-response elements (PRE) in the

promoter region of the *rankl* gene (Standke et al, 1994; Wakao et al, 1994). RANKL has been shown to be upregulated in mouse mammary epithelial cells through the induction of the JAK2-STAT5a pathway by prolactin (Srivastava et al, 2003). However, regulation of RANK/RANKL has not been determined in human breast epithelial or breast cancer cells.

1.4 – Prolactin

1.4.1 - Prolactin and the Prolactin Receptor

Prolactin (PRL) is a polypeptide hormone primarily synthesized and secreted by lactotrope cells in the anterior pituitary gland, although it is also produced in other glands, including the breast, deciduas, and parts of the central nervous system and immune system. The prolactin receptor (PRLR) belongs to the cytokine hematopoietic family of receptors and contains three specific domains: an extracellular ligand-binding domain, a hydrophobic transmembrane domain, and an intracellular cytoplasmic domain. The cytoplasmic domain includes two conserved motifs: box 1, a proline rich domain involved in protein-protein interactions, and box 2, which contains no known consensus sequence, but plays a crucial role in signal transduction.

There are three forms of PRLRs present: long, intermediate and short, which differ in the length of their cytoplasmic domains. The intermediate form results from a deletion mutation of the long form, and lack 198 amino acids in its cytoplasmic region. The long and intermediate forms have both been recognized in mammary and human carcinomas and are both able to induce differentiation as well as mitogenic effects.

1.4.2 – Induction of JAK2-STAT5a pathway by prolactin

The most well known activator of the JAK2-STAT5a pathway in mammary gland development is PRL. Binding of PRL to its receptor initiates homodimerization of the receptor, followed by phosphorylation of JAK2 (Rui et al, 1994). JAK2 then phosphorylates the PRLR and the STAT protein docks at the tyrosine residues of the PRLR that were phosphorylated. The activated JAK2 then causes phosphorylation of the tyrosine residue - 694 (Tyr-694) on the STAT5a protein (Gouilleux et al, 1994). Phosphorylated STAT5a forms homo and hetero-dimers and is transported to the nucleus, where it regulates the transcription of many genes. Figure 2 illustrates this.

PRL has been shown to induce STAT5a activation in many cell lines, including human breast cancer cells. Canbay et al (1997) demonstrated that when T47-C breast carcinoma cells were stimulated with PRL, JAK2 was phosphorylated in a time- and dose-dependent manner. The same study showed a similar effect in MCF-7, ZR-75-1 and MDA-MB-231 cells, but to a lesser extent. In breast cancer cell lines, JAK2 phosphorylation by PRL has caused ErbB2 phosphorylation, stimulating the Ras-mitogen activated protein kinase (MAPK) pathway, leading to unrestricted proliferation (Yamauchi et al, 2000).

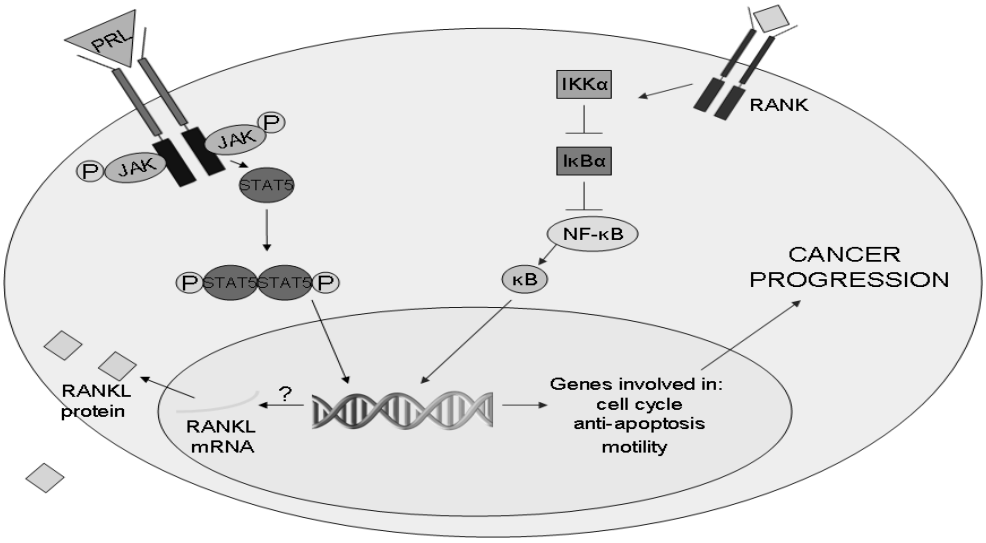

Figure 2: The induction of the JAK2-STAT5a pathway by prolactin and its proposed effect on the transcriptional regulation of RANKL.

1.4.3 - Prolactin in Breast Development

It has already been stated that STAT5a plays an important role in breast development, and that PRL activates the JAK2-STAT5a pathway. Therefore, the main role of PRL is the development of alveolar buds in the breast during pregnancy, and the stimulation of postpartum lactogenesis by driving expression of milk producing proteins, such as whey acidic protein (WAP) (Andres et al, 1987) and β-lactoglobulin (Whitelaw, 1995).

Evidence of the role of PRL in mammary gland development and lactation come largely from studies with organ cultures and cell lines. Many *in vivo* studies involve the generation of mice from which the genes encoding the PRLR, Stat5a and Stat5b have been inactivated. In these studies, PRLR -/- mice displayed normal ductal elongation during puberty, however secondary side-branching does not occur and ducts become more distended as the mice age (Ormandy et al, 1997; Brisken et al, 1999). Female mice

expressing only one PRLR allele failed to lactate after their first pregnancy due to reduced mammary gland development (Ormandy et al, 1997). This indicates that epithelial cell proliferation during pregnancy is dependent on sufficient PRLR, which cannot be obtained with only one allele. In PRLR -/- mice epithelium is unable to proliferate to form lobuloalveoli (Brisken et al, 1999), similar to the phenotype seen in STAT5a -/- mutants. PRLR -/- mothers fail to lactate and mammary glands show formation of lobules, but they are unable to form an open lumen or secrete milk (Ormandy et al, 1997; Brisken et al, 1999).

PRL also drives expression of RANKL through the JAK2-Stat5a pathway in mammary epithelial cells (Srivastava et al, 2003). Using dominant negative mutants for JAK2 and STAT5a, these researchers discovered that there is a clear link between the PRL response and STAT5a activation. This study found gamma interferon activation site (GAS) elements in the promoter region of the RANKL gene, which they believe is how the PRL-response was conferred (Srivastava et al, 2003). The finding of this paper leads to the conclusion that PRL activates the JAK2-STAT5a pathway to activate RANKL expression in mammary epithelial cells. Activation of the RANK/RANKL pathway through STAT5a therefore initiates the formation of lobuloalveolar structures in lactating mice, supported by similar phenotypes seen by STAT5a -/- and RANKL -/- mutants.

1.4.4 - Prolactin and Breast Cancer

Over time many studies have found a positive link between PRL and mammary gland carcinogenesis in mice. *In vivo* studies in transgenic and knockout mice are consistent with a role for PRL in mammary carcinogenesis. Transgenic mice expressing

PRL from the metallothionein promoter develop mammary tumors (Wennbo et al, 1997) and normal mice that are administered ovine PRL daily show an increase in the incidence of mammary tumorgenesis (Boot et al, 1962). A study by Yanai and Nagasawa (1972) shows that mammary tumors secrete PRL and that administration of a drug shown to reduce PRL causes a drastic reduction in the incidence of mammary tumors. PRL is produced by transgenic mammary epithelial tumor cells in culture and is able to promote lobuloalveolar development, indicating that PRL may be acting in an autocrine manner to promote proliferation and carcinogenesis (Wennbo et al, 1997).

In humans, the role of PRL in breast cancer is far less established and relies mostly on correlation. PRLR expression has been found in over 70% of breast biopsy samples by specific biniding assays (Codegone et al, 1981; Peyrat et al, 1981; Bonneterre et al, 1982). In breast cancer surgical samples, cancerous tissues contained significantly more mRNA for the PRLR than did adjacent, noninvolved tissue from the same patient (Clevenger et al, 1995). Serum prolactin is detected in 60-85% of breast cancer biopsies (Purnell et al, 1982; Agarwal et al, 1989), however there is no clear correlation between levels of PRL and PRLR content or the etiology or prognosis of the disease (DePlacido et al, 1990). Holtkamp et al. (1984) reported elevated levels of PRL in 44% of patients with metastatic breast cancer and several other studies have claimed that basal serum levels were higher in women who were at risk of familial breast cancer (Love et al, 1991). Most significantly, a study by Vonderhaar (1998) shows that PRL is synthesized by MCF-7 and T47Dco human breast cancer cells and acts in an autocrine manner to stimulate cell proliferation. This indicates that breast cancer cells are stimulating their own growth

through the secretion of PRL and that PRL can be directly correlated to the progression of human breast cancer.

1.5 – Epidermal Growth Factor

1.5.1 – Epidermal Growth Factor and Family Receptors

The epidermal growth factor receptor family, also known as the ErbB protein family, consists of four structurally related receptor tyrosine kinases: erbB-1 (also known as EGFR), erbB-2 (also known as Human Epidermal growth factor Receptor 2 (HER2)), erbB-3 (HER3), erbB4 (HER4). Epidermal growth factor (EGF) binds to EGFR, however this receptor has many other functional ligands including transforming growth factor (TGF)-α (Garrett et al, 2002). EGF only binds to EGFR, but the other erbB receptors bind a variety of other growth factor ligands that initiate tyrosine kinase cascades.

1.5.2 – EGF and Signal Transduction

Along with PRL, epidermal growth factor (EGF) can also activate STATs to elicit transcriptional regulation of many genes. Binding of EGF or other ligands to the EGFR can cause STAT proteins to bind to the receptor, becoming activated through the SH2 domain (Silvennoinen et al, 1993). EGFR can phosphorylate STAT1, STAT3 and STAT5 proteins on Y701, Y705, and Y694 residues respectively (Rane and Reddy, 2002). Although STATs can bind directly to the EGFR, JAKs are required to provide maximum STAT activation. After phosphorylation, STAT proteins dimerize and

translocate to the nucleus where they can bind to a variety of target genes or other regulatory proteins c-fos and c-jun, which regulate transcription (Leeman et al, 2006).

Not only does the EGFR mediate signals directly through STAT proteins, it can also indirectly activate STAT proteins through alternative pathways. For instance, EGF is involved in the signaling through phospholipase C-γ (PLC-γ) (Wahl et al, 1990) and phosphatidylinostol-3 kinase (PI3K) (Bjorge et al, 1990). EGF can also activate the MAPK pathway, which regulates cell proliferation and survival (Scaltriti and Baselga, 2006). In this pathway, growth factor receptor-bound protein 2 (GRB2) and son of sevenless (SOS) complex bind to EGFR and phosphorylate Ras (Scaltriti and Baselga, 2006). Binding of EGF to the EGFR can also cause nuclear translocation of the entire complex and direct transcriptional regulation of genes (Lin et al, 2001). For instance, EGFR functions as a transcription co-factor that interacts with and activates the cyclin D1 gene, a promoter of cell proliferation (Lin et al, 2001). The inducible nitrous oxide synthase (iNOS) gene has recently been discovered as another target of EGFR signaling (Lo et al, 2005).

1.5.3 – EGF and Mammary Gland Development

Evidence found in the studies of mice suggest that it is signaling through EGFR and ErbB-2 that are responsible for ductal development of the pubescent gland, while signaling though ErbB-3 and ErbB-4 is important for alveolar morphogenesis and lactation. In egfr-/- mutants, mice lack ductal development and show very few proliferating cells in the stroma or epithelial ducts, indicating EGF is involved in the ductal morphogenesis of the mammary gland (Wiensen et al, 1999). These same mice however, showed normal development of lobuloalveolar structures during pregnancy,

since the mammary glands were still competent to respond to other hormonal signals (such as prolactin) to elicit a lactational phenotype (Wiensen et al, 1999). EGF is expressed in virgin and pregnant mice, as well as 10-day lactating mice and is localized in the inner layers of the terminal end bud and in ductal cells of the mammary epithelium (Snedeker et al, 1991). Studies have detected only EGFR and ErbB-2 receptors in the developing virgin gland, while all four ErbB receptors were expressed and maximally phosphorylated during pregnancy and lactation (Schroeder and Lee, 1998; Sebastian et al, 1998).

1.5.4 – EGF and Breast Cancer

A clear tie lies between EGF and breast cancer, as it is also linked to STAT5a activation and has been shown to activate NF-κB, both of which are correlated to breast cancer progression. The role of EGF in breast cancer first came to light when an experiment showed that overexpression of EGFR in cells in the continuous presence of EGF caused the cells to become transformed (Velu et al, 1987).

In murine studies, a casual relationship is represented by the amount of nuclear accumulation of EGFR and cell proliferation and DNA synthesis (Marti et al, 1991; Marti et al, 1995; Lin et al, 2001). Research shows that EGFR can regulate autocrine and paracrine growth signals *in vitro* (Ennis et al, 1989; Valverius et al, 1990).

EGFR is overexpressed in many human cancers including breast, prostate, ovarian, liver, bladder, stomach, lung, colon, esophagus and larynx (Khazaie et al, 1993). In a study by Kondapaka (1997), EGF induced proliferation of metastatic and non-metastatic breast cancer cells and induced expression of matrix metalloproteinase 9 (MMP-9). This enzyme has been linked to tumor invasion and metastasis because of its

ability to hydrolyze a variety of extracellular matrix proteins. Many studies on human isolated tissues have shown nuclear localized EGFR were correlated with a poor clinical outcome in breast cancer (Klijn et al, 1992; Seshadri et al, 1996). As mentioned, nuclear EGFR mediates transcriptional activation of the cyclin D1 gene, a positive regulator in cell proliferation (Lin et al, 2001), creating yet another positive link to breast cancer progression.

1.6 – Heregulin Beta-1

1.6.1 - Heregulin Family

Heregulin (HRG), also known as neu differentiation factor (NDF), belongs to the Type I class neuregulin 1 (NRG1). Neuregulins are a family of structurally related proteins that are actually a part of the EGF family and are involved in growth and differentiation of epithelial, glial and muscle cells in culture. There are several forms of HRG: HRG-α, HRGβ1, HRGβ2, HRGβ3, and HRGγ. HRGs actually contain an EGF-domain, which shares homology with the EGF protein. This domain is involved in HRG ligand binding to erbB3 and erbB4 receptors (Jones et al, 1998). These receptors can form homo- or heterodimers that can mediate the STAT5a pathway, similar to EGFR.

1.6.2 – Heregulin β-1 Signalling in Mammary Gland Development

HRG is an important factor in pathways involved in mammary gland development and its expression is highly regulated. HRG-α is expressed in mammary mesenchyme adjacent to lobuloalveolar structures and is only expressed at the onset of pregnancy (Yang et al, 1995). During pregnancy, HRG-α and -β function to induce lobuloalveolar

formation and to produce required milk proteins (Yang et al, 1995). Similar to TNF-α, both HRG-α and HRG-β induce epithelial branching and differentiation into lobuloalveolar structures and stimulate the accumulation of luminal secretory products, such as beta-casein, an essential milk protein *in vivo* (Jones et al, 1996). HRG-β1 acts through the STAT5 pathway, as it is known to bind to and activate erbB3 and erbB4 receptors (Jones et al, 1998; Puricelli et al, 2002). In the mammary gland, erbB3 and erbB4 cause a signaling cascade that activates STAT5a phosphorylation leading to lobuloalveolar development (Jones et al, 1999). Mice that lack HRG show decrease beta-casein production, as well as reduced alveolar proliferation and differentiation (Li et al, 2002). This indicates that HRG, similar to PRL, is involved in the differentiation of mammary epithelial cells to milk-secreting lobuloalveolar structures (Jones et al, 1996).

1.6.3 – Heregulin β-1 in Breast Cancer

Although HRG is involved in the normal development of the mammary glands during pregnancy, studies have also linked HRG to breast cancer progression. HRG-β1 is linked to the STAT5a pathway, and can act as a mitogen, differentiation factor and transforming agent, raising questions on its effect in human breast cancer. Studies have found that in cells over-expressing erbB2, HRG stimulation increases motility and invasion (DeCorte et al, 1994; Hijazi et al, 2000). However, another paper reports that HRG-β1 causes the inhibition of proliferation and migration in highly metastatic breast cancer cells (Puricelli et al, 2002). The same study also showed that HRG-β1 increased the amount of phosphotyrosine STAT5, indicating HRG acts through this pathway in human breast cancer cells (Puricelli et al, 2002). In two different breast cancer cell lines,

MCF-7 and SKBr3, HRG-β1 causes an upregulation of MMP-9, which plays a role in invasion and angiogenesis of tumors (Yao et al, 2001).

1.7 – Breast Cancer Cell Lines Used in this Study

The ability to study cancer cells at the cellular or tissue level is beneficial because it allows for researchers to study mechanisms in greater detail, without having to worry about the difficulties of working with *in vivo* models. Cell culture allows for an in depth study of molecular signaling pathways and gene regulation in human cells, without having to use an animal model.

Cell lines derived from human breast tissue can be investigated for hormone and growth factor-mediated effects on RANK and RANKL gene expression. This study will use two commonly used cell lines MCF-7 cells and MDA-MB-231 cells. Both lines are human breast epithelial adenocarcinoma cells derived from a pleural effusion from a breast cancer patient. The difference in these cell lines is that MDA-MB-231 cells are derived from a more progressed state of breast cancer than MCF-7 cells and therefore have slight differences in morphology and rate of proliferation. MCF-7 cells retain several characteristics of differentiated breast epithelium and therefore in general contain more of the same cell receptors. For instance, MCF-7 cells are known to contain high amounts of ERs (Brooks et al, 1973), while MDA-MB-231 cells are estrogen receptor-negative. MCF-7 cells also contain high levels of prolactin receptor (PRLR), transforming growth factor alpha-receptor (TGF-αR), insulin-like growth factor-1 receptor (IGFR-1), progesterone receptor (PR), and some others. MCF-7 cells also contain low amounts of epidermal growth factor receptor (EGFR) and erbB2 (HER2).

MDA-MB-231 cells have a fairly different receptor profile, only containing EGFR, TGF-αR, IGFR-1 and low levels of erbB2 (HER2). Differences in receptor profiling and cell morphology, will allow the two cell lines to react separately to various stimuli.

1.8 – Research Objectives:

1.8.1 – Hormonal Regulation of RANKL Gene Expression

The overall objective of our research is to determine the mechanism of regulation of the expression of RANK/RANKL system in human breast cancer cell lines. Although there is evidence that the RANK/RANKL system is involved in breast cancer progression and metastasis, it is not known under what circumstances (i.e. by which extracellular stimuli) the system is regulated. We propose to determine the molecular mechanisms driving RANK and RANKL gene expression by studying mRNA levels in cancer cells stimulated with specific hormones and growth factors. This research will focus on factors that are known to activate the Stat5a pathway, since this pathway is important in breast development and promotion of breast cancer and have been shown to regulate RANKL in mouse mammary epithelial cells (Srivastava et al, 2003). The first objective of this research is to investigate the effects of the hormones prolactin and HRG-β1 and growth factor EGF, on the expression of RANKL mRNA in MCF-7 and MDA-MB-231 cell lines. Since induction of RANKL by hormones and growth factors has not been shown in human breast cancer, we hypothesize that stimulation by these hormones and growth factors that act through the STAT5a pathway will cause upregulation of RANKL.

After first testing the effects of PRL stimulation on RANKL in human breast cancers, the next focus was on other selected growth factors known to promote breast cancer progression via Stat5a. These growth factors include heregulin (HRG-β1) and epidermal growth factor (EGF).

1.8.2 – Modulation of Stat5a

The STAT5a pathway is involved in the regulation of many genes and has been linked to breast cancer progression. RANKL is not normally expressed in breast epithelial cells or breast cancer cell lines, but induced expression has been linked to migration, proliferation and reduced apoptosis in mouse mammary epithelial cells. If the specific growth factors or hormones induce RANKL expression, the next objective of this study is to determine whether it was regulated through the JAK2/STAT5a pathway. Using the selected growth factors or hormones that induce expression of RANKL, and a specific JAK inhibitor, studies can be used to examine the relationship between STAT5a activation and RANKL mRNA expression. This study will provide information that will add to the understanding of the regulation of RANKL in breast cancer and provide a link between the STAT5a pathway and RANK/RANKL expression in human breast cancer.

2 - Materials and Methods

2.1 – Growth of Cell Lines

Human breast carcinoma cell lines, MCF-7's (American Type Culture Collection (ATCC)) were maintained in Eagle's Minimal Essential Media (EMEM) with Earle's salts and 2mM L-glutamine (ATCC) supplemented with 10% fetal bovine serum (FBS) (Hyclone), 0.1% penicillin/streptomycin (p/s) (Hyclone) and 0.01mg/mL insulin (Sigma Aldrich). These cells were incubated at 37^{o}C with 5% CO_2. MDA-MB-231 cells (ATCC) were grown in L-15 Leibovitz's media (Hyclone) with 2mM L-glutamine (Hyclone) supplemented with 10% FBS and 0.1% p/s. They were grown at 37^{o}C, in the absence of CO_2. Human intestinal epithelial, HT-29 cells were also purchased from ATCC. These cells were maintained in RPMI-1640 medium (Hyclone) supplemented with 10% FBS and 0.1% p/s and incubated at 37^{o}C at 5% CO_2.

All cell lines were adherent cultures, grown in 75 cm^{2}Corning cell culture flasks with 10mL of culture media. Media was replaced every two days and cells were passaged after confluency reached 70-75%. Cells were liberated using 1mL 0.5% Trypsin/EDTA (Hyclone). First, media was aspirated and cells were washed three times with 1X PBS. One mL of Trypsin/EDTA was added to the 75 cm^2 flask and incubated for 2 minutes at 37^{o}C. Once cells were in suspension, they were rinsed with 10 mL of cell culture media and placed in a 50 mL Falcon tube. Cells were spun at 1200 x g for 5 minutes and the pellet was resuspended in 5 mL media. One hundred μL were stained with a ½ dilution of trypan-blue (MP Biomedicals, Inc.) and counted using a hemocytometer. After counting, one hundred thousand cells were added to each new culture flask. Cells were usually passaged every 7-10 days. For long-term storage, 9 x

10^6 cells diluted in 900µL EMEM were added to 100µL dimethyl-sulfoxide (DMSO) and 300µL FBS. Cells were frozen in cryovials (Fisher Scientific) and slow-frozen at -80°C in a Nalgene "Mr. Frosty" Cryo 1°C Freezing Container (Fisher Scientific) overnight and then transferred to liquid nitrogen.

2.2 – Stimulation of MCF-7 and MDA-MB-231 cells with PRL, HRG-β1 and EGF

All hormones and growth factors were purchased from Sigma Aldrich. PRL powder was reconstituted in 1mg/mL bovine serum albumin (BSA) (Invitrogen), 4 mM HCl solution (Sigma Aldrich) to a concentration of 50mg/mL. The hormone was further diluted in serum-free EMEM media to a final concentration of 2.5mg/mL or 100nM, a concentration used in similar stimulation studies (Rui et al, 1994; Kirken et al, 1997). EGF was reconstituted in dH_20 to a concentration of 100µM, and further diluted in serum-free EMEM media to a final concentration of 10nM. HRG-β1 was resuspended in dH_20 to a concentration of 5µg/mL and then further diluted to 20ng/mL in serum-free medium (Yao et al, 2001; Puricelli et al, 2002).

For experimentation, cells were grown in 6-well plates with 2mL media in the appropriate conditions (as stated above). Experiments began when cells were 80-90% confluent (approximately 500,000 cells per well). Media was removed and replaced with 1 mL serum-free media supplemented with the previously noted concentrations of PRL, EGF, or HRG-1. RNA and protein samples were isolated at various time-points in order to identify the optimal length of stimulation for signal transduction, transcription and translation of genes. Cells were stimulated for 15, 30 and 60 minute incubation periods at 37°C at 5% CO_2. After stimulation, media was removed and cells were washed once with 1X phosphate buffered saline (PBS). Prior to RNA isolation, 2mL of EMEM media was

added to cells and cells were left to incubate for 24, 48, or 72 hours and RNA was isolated after this time. Protein was isolated directly after stimulation, since only phosphorylation of STAT5a was being investigated.

2.3 – Crystal Violet Assay

Proliferation of MCF-7 cells in the presence of PRL, EGF, and HRG-β1 was measured using a crystal violet assay. In a 96-well plate, 5000 cells were added to 195µL of EMEM media containing 100nM PRL, 10nM EGF, 20ng/mL HRG-β1, or without growth factor or hormones. Cells were incubated at 37^{o}C in 5% CO_2 for 2-5 days and the crystal violet assay was carried out every 24 hours. Experiments were set up for 5 days, and all experiments were done in triplicate.

After each incubation period, the crystal violet assay was performed. Media was removed from all wells by inverting the dish and gently blotting on absorbent pads. Wells were washed once with 200µL of 1X PBS and solution was removed by blotting on absorbent pads. Fifty µL of 0.5% Crystal Violet Solution (Fisher Scientific) was added to each well and gently agitated for 10 minutes. The dye was removed with reverse-osmosis water, until running clean and wells were left to dry for 30 minutes. Cells were solubilized in 100µL DMSO solution by shaking for 10 minutes on low-speed. Absorbance of the solution was read at 570nm. A standard curve of absorbance vs. cell number was generated using SigmaPlot to compare absorbance to estimate proliferation. SigmaPlot was used to generate linear regressions and used to generate p values using an unpaired t-test.

2.4 - Detection of mRNA

2.4.1 – RNA Isolation and cDNA Synthesis

Total RNA was isolated from MCF-7 and MDA-MB-231 cells using two methods: filtration and phenol-chloroform extraction. RNA was isolated from approximately 5-10 million cells. RNA was isolated 24 hours, 48 hours and 72 hours after stimulation with PRL, EGF and HRG-β1.

The first method used the GenElute Mammalian RNA Isolation Kit (Sigma Aldrich). Cells were isolated using 0.5% trypsin/EDTA and lysed in solution. The cell lysate was filtered through a GenElute filtration column by spinning at 12, 000 x g for two minutes. The filtered lysate was washed with 70% ethanol and this solution was filtered through the GenElute binding column by centrifugation. The column was washed with Wash Solution I by centrifuging at 12, 000 x g for 15 seconds. It was then washed twice with Wash Solution II, again centrifuging at 12, 000 x g for 15 seconds. The RNA was then eluted using fifty μL of elution solution and centrifuging at 12, 000 x g for 1 minute.

The second method used TRIreagent also purchased from Sigma Aldrich. Cells were lysed by adding 1 mL TriReagent to the wells and incubating for 2 minutes. The lysate was then pipetted to a microfuge tube and 200 μL of chloroform was added. The solution was incubated for 2-3 minutes at room temperature before being spun at 12, 000 x g for 15 minutes at 4^{o}C. RNA was isolated from the upper aqueous layer by precipitation with 70% isopropyl alcohol. The RNA was spun at 12, 000 x g for 10 minutes and the pellet was washed with 75% ethanol. RNA was resuspended in 20 μL sterile DNAse/RNAse-free water by incubating at 65^{o}C for 10 minutes.

One unit of RNAse inhibitor was added to all RNA solutions to prevent degradation. Samples were then converted to cDNA or stored at -80^{o}C. RNA was reverse-transcribed to cDNA using Superscript II RT enzyme and oligo(dT)$_{12-18}$ primers (Invitrogen). Two µL of isolated RNA was combined with 25µg/mL oligo(dT)$_{12-18}$ primers and 0.5mM dNTPs. The reaction was heated to 65^{o}C in order for primers to anneal to the template mRNA strand. The reaction was chilled on ice before 1X First Strand Buffer and 10mM DTT were added. The cDNA synthesis was carried out at 42^{o}C for fifty minutes using 200U Superscript II reverse transcriptase (RT). The reverse transcriptase was inactivated by heating at 70^{o}C for fifteen minutes. cDNA samples were stored at -20^{o}C.

2.4.2 – Polymerase Chain Reaction (PCR)

GAPDH, RANK and RANKL transcripts were amplified from constructed cDNA using PCR. All PCR primers were designed based on previous research and purchased from Integrated DNA Technologies (IDT). PCR reactions were carried out using a Thermocycler Px2 (ThermoElectron) and GoTaq Hotstart Polymerase (Promega). GoTaq was activated with an initial incubation of 95^{o}C for 2 minutes. One microlitre of cDNA was used as template for each amplification reaction.

Glyceraldehyde 3-phosphatase dehydrogenase (GAPDH$_3$), a common housekeeping gene, was used as a genomic control for possible contamination of cDNA samples and as a positive PCR control. Since the PCR amplification was not quantitative, this gene was only used as a control for genomic contamination. The following primers were used for amplification: 5'-ACCACAGTCCATGCCATCAC-3' (sense) and 5'-TCCACCACCCTGTTGCTGTA-3' (antisense), amplifying 555bp from

genomic DNA, and 451bp from cDNA. These primers span an intron, which accounts for larger amplicon size from genomic DNA (Yan et al, 2006). The reaction mixture included 120µM dNTPs, 10pmol primers, 4mM MgCl2 and 1.25U Taq. Following the initial activation was thirty-five cycles of denaturing at 94^{o}C for 30 seconds, primer annealing at 60^{o}C for 30 seconds and extension at 72^{o}C for 30 seconds. Following these cycles, there was a final annealing reaction of 72^{o}C for 10 minutes.

The following RANK primers were used to produce a 452bp fragment: 5'-GGGAAAGCACTCACAGCTAATTTG-3' (sense) and GCACTGGCTTAAACTGTCATTCTCC-3' (antisense) and were described in Jones et al (2006). This reaction was completed using a final concentration of 120µM dNTPs, 2mM MgCl, 10pmol primers, and 1U Taq. The reaction was also started with an initial incubation at 95^{o}C for 2 minutes, followed by thirty-five cycles of 94^{o}C for 30 seconds, 57^{o}C for 30 seconds, and 72^{o}C for 30 seconds, ending with a final extension at 72^{o}C for 10 minutes.

RANKL was amplified using the following primers: 5'-ACTGGATCCGGATCAGGATG-3' (sense) and 5'-AGCTGCGAAGGGGCACATGA-3' (anti-sense), amplifying a 386bp fragment. These primers were previously used to amplify RANKL in HT-29 cell lines (Vidal et al, 2003). In this experiment, HT-29 cells were used as a control for RANKL expression. The amplification of this fragment included final concentrations of 120µM dNTPs, 5pmol primers, 2mM MgCl2, and 1.25U Taq. This reaction included thirty-five cycles of 94^{o}C for 45 seconds, 60^{o}C for 1 minute 30 seconds, 72^{o}C for 45 seconds, following the initial 95^{o}C for 2 minutes. The cycle was ended with a final extension of 72^{o}C for 7 minutes.

Table 1: Summary of the primers and conditions used to amplify each gene and the length products produced by PCR amplification

Product	*Primers (5' → 3')*	*cDNA Amplicon*	*Genomic DNA Amplicon*	*Annealing Temperature*
GAPDH	Sense: ACCACAGTCCATGCCATCAC Anti-sense: TCCACCACCCTGTTGCTGTA	451 bp	555 bp	60°C
RANK	Sense: GGGAAAGCACTCACAGCTAATTTG Anti-sense: GCACTGGCTTAAACTGTCATTCTCC	452 bp	7307 bp	57°C
RANKL	Sense: ACTGGATCCGGATCAGGATG Anti-sense: AGCTGCGAAGGGGCACATGA	389 bp	556 bp	60°C

2.4.3 – Electrophoresis and Sequencing of PCR Products

The size of each PCR product was determined by electrophoresis through a 1.5% agarose gel in 1X TAE buffer at 100V for approximately one hour for adequate separation. Gels were stained with 0.5ng/mL ethidium bromide for twenty-five minutes and visualized under UV using a FluorChem™ SP (Alpha Innotech) gel-dock. DNA fragments were excised from the agarose gel and purified using the PureLink Gel DNA Extraction Kit purchased from Invitrogen. The agarose was dissolved by incubation in Gel Solublization Buffer at 50°C for fifteen minutes, mixing by inversion every three minutes. The dissolved gel mixture was loaded onto a PureLink™ Clean-up Spin Column inside a Wash Tube. This was centrifuged at 10 000 x g for one minute. The

column was then washed with Wash Buffer containing ethanol and centrifuged for 10 000 x g for one minute. After the flow-through liquid was removed, the column was dried by a final centrifugation at 13 000 x g for three minutes. The DNA was eluted from the column by adding fifty microlitres Elution Buffer and incubating for one minute at room temperature and centrifuging at 13 000 x g for one minute. The purified DNA was stored at -20^{o}C.

Purified PCR products were sent to Nanuq McGill University and Genome Quebec Innovation Center (Montreal, QC) for further purification and sequencing. Sequencing was completed with $GAPDH_3$, RANK and RANKL primers described above.

2.5 Protein Analysis

2.5.1 – Protein Isolation

Protein was isolated from all cell lines using RIPA buffer containing 50mM Tris-HCl (pH 7.4), 150mM sodium chloride (NaCl) (Sigma), 10mM ethylenediaminetetraacetic acid (EDTA), 0.1% Triton X-100 (Sigma), 1% NaDeoxycholate (Sigma), and 0.1% sodium dodecyl sulfate (SDS) (BioRad). Cells were grown in a 6-well plate and grown to 80-100% confluency. Cells were lysed with 100µL of RIPA buffer and kept on ice with gentle agitation for fifteen minutes. The cell lysate was vortexed for one minute and kept on ice for ten minutes. The DNA and cellular debris was sheared using a 22-gauge needle, then solutions were centrifuged at 13, 000 rpm for twenty minutes at 4^{o}C. The supernatant containing the protein mixture was quantified using a Bradford Assay. For the Bradford Assay, a standard curve was created using dilutions of BSA ranging from 0 – 1 mg/mL in dH_20. Samples were diluted 50 fold

with Bradford Reagent and incubated at room temperature for ten minutes. Absorbance was read at 595nm using Synergy HT plate reader (Bio-Tek, Fisher). Protein concentrations were calculated using the BSA standard curve. Protein was stored at -20^{o}C in 30µg aliquots in 1X Laemmli buffer containing 30% glycerol (Sigma), 187.5mM Tris-HCl (pH 6.8), 6% SDS, 0.009% bromophenol blue (Sigma), and 5% beta-mercaptoethanol (BioRad).

2.5.2 - Western Blot Analysis

Thirty micrograms of protein were separated on an acrylamide gel composed of an 8% resolving gel and 5% stacking gel. The gel was run at 50V in Electrode buffer containing 14.4% glycine powder (MP Biomedicals, Inc) and 30.3% Tris-base (Sigma). Gels were run in the BioRad Mini-PROTEAN® Tetra Cell. The samples were run at a constant voltage of 50V until the protein samples reached the resolving gel, when the voltage was increased to 100V until the samples reached the end of the gel. Correct loading and running of the protein gels were determined by staining with Brilliant Blue G Concentrate (Sigma) for 30 minutes, followed by destaining with 50% methanol (Sigma), 10% acetic acid (Sigma) solution with gentle agitation. The destaining solution was changed several times, until the background stain was washed away and protein bands were clearly visible.

Protein was transferred from the acrylamide gel to a polyvinylidene fluoride (PVDF) membrane (BioRad) using the BioRad Mini Trans-Blot® Electrophoretic Transfer Cell. The transfer was completed at 4^{o}C overnight at 30V in transfer buffer containing 1X Electrode Buffer, 20% methanol and 0.01% SDS. Transfer was confirmed by staining the membrane with 0.1% Ponceau S (w/v) in 5% (v/v) acetic acid (Sigma) for

fifteen minutes and washing the background with distilled water. After transfer was confirmed, the membrane was washed with Wash Buffer, containing 1X TBS and 0.1% tween (Sigma), until the stain was removed.

2.5.3 - Detection of STAT5a and phospho-STAT5a Protein

Antibodies for STAT5a, phospho-STAT5a and beta-actin were purchased from SantaCruz Biotechnology. STAT5a (sc1081), p-STAT5a (sc-101806) and beta-actin (sc-130657) are rabbit monoclonal antibodies that bind to 94kDa, 94kDa, and 43kDa peptides respectively. A secondary goat anti-rabbit IgG, horseradish peroxidase (HRP)-conjugated antibody was used for detection of the primary antibody. Antibody detection was accomplished using Enhanced Chemiluminescent (ECL) substrate (Pierce).

To detect unphosphorylated STAT5a, the PVDF membrane containing transferred protein was incubated with 0.2µg/mL STAT5a (L-20) antibody in a Petri-dish for one hour at room temperature with gentle agitation. To detect for phospho-STAT5a and beta-actin, membranes were incubated overnight at 4^{o}C with 0.1µg/mL p-STAT5a and beta-actin (R-22) antibody.

After incubation with the primary antibody, the membrane was washed three times with Wash Buffer in a Petri-dish, for ten minutes each. The membrane was then incubated with 0.08µg/mL secondary antibodies for one hour at room temperature, and washed again for three consecutive washes. Luminescence was completed using Thermo Scientific Pierce Enhanced Chemiluminescent (ECL) Western Blotting Substrate. After wash solution was removed, equal quantities of Detection Solution #1 and #2 were poured over the membrane and left for five minutes at room temperature in the absence of light. The membrane was wrapped in a single layer of plastic wrap then exposed to

autoradiography film (Fuji) for two minutes in an autoradiography cassette purchased from Fischer Scientific. After exposure, the film was developed using the Kodak X-OMAT 1000A Processor.

2.6 – Inhibition Studies

Tyrphostin AG490 is a JAK2-specific inhibitor used to block the STAT5a pathway. AG490 powder was purchased from Sigma Aldrich and reconstituted in DMSO to a concentration of 50mM and stored at -80^{o}C in the absence of light. AG490 was further diluted with PRL, EGF, or HRG-β1 in serum-free EMEM media. Stimulation assays were carried out as described above with the presence of 50μM AG-490. After stimulation for 15, 30 and 60 minutes, media containing stimulant and inhibitor was aspirated and cells were washed with 1X PBS. Protein and RNA was isolated as described previously. Expression of the GAPDH, RANK, and RANKL genes and STAT-5a and p-STAT5a proteins were detected using the previously described methods.

3 - Results

3.1 - MCF-7 cells Express RANK, but not RANKL

RT-PCR was conducted using isolated RNA from MCF-7 cells. PCR results in Figure 3 show that this cell line expresses RANK mRNA (452bp), while it does not express RANKL (327bp) under unstimulated conditions. Human $GAPDH_3$ expression was used as a control to indicate genomic contamination of RNA samples and as a positive control for RT-PCR procedures. HT-29 cells were used as a positive control for RANKL expression. PCR products were excised from the agarose gel, purified and sent for sequencing in order to further identify the products as RANK and RANKL. The sequence information obtained from McGill University and Genome Quebec Innovation Center for the RANK and RANKL can be seen in Figure 4 A and 5A, respectively. A BLAST search of the proposed RANK sequence (Figure 2B) shows that this product was in fact the human tumor necrosis factor receptor superfamily, member 11a (also known as RANK) mRNA. The sequence alignment was highly similar, showing 98% identity, with 0 gaps. The BLAST search with the RANKL sequence isolated from HT-29 cells also confirmed the fact that the sequence was in fact human tumor necrosis factor (ligand) superfamily, member 11 (also known as RANKL) mRNA (Figure 3B). This alignment showed 100% identity, with 0 gaps. Figure 6 shows PCR amplified $GAPDH_3$ in genomic DNA isolated from MCF-7 and MDA-MB-231cells and shows two amplified bands, as compared to its detection in cDNA, showing only one band. Primers were designed to span an intron in genomic DNA, so PCR would generate a larger band with genomic DNA than with cDNA.

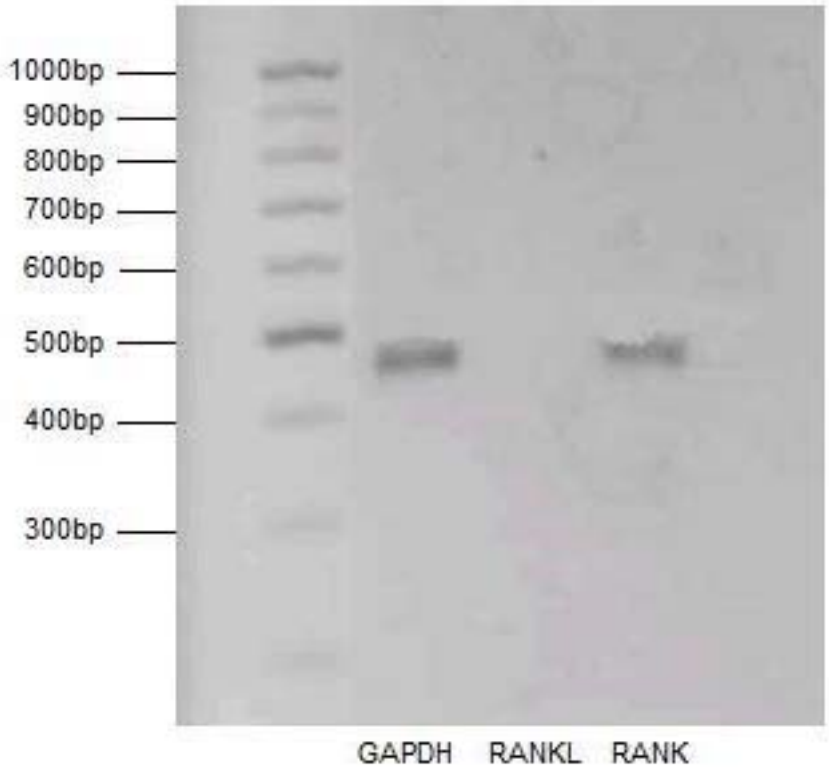

Figure 3: RT-PCR products showing GAPDH (451bp), RANK (452bp) and the absence of RANKL (329bp) in unstimulated MCF-7 cells.

1 aagcactcac agctaatttg tggcactgga tcaatgaggc ttgtggccgc ctaagtggag
61 ataaggagtc ctcaggtgac agttgtgtca gtacacacac ggcaaacttt ggtcagcagg
121 gagcatgtga aggtgtctta ctgctgactc tggaggagaa gacatttcca gaagatatgt
181 gctacccaga tcaaggtggt gtctgtcagg gcacatgtgt aggaggtggt ccctacgcac
241 aaggcgaaga tgccaggatg ctctcatNNN NNagcaagac cgagatagag gaagacagct
301 tcagacagat gcccacagaa gatgaataca tggacaggcc ctcccagccc acagaccag

A

>ref|NM_003839.2| Homo sapiens tumor necrosis factor receptor superfamily, member 11a, NFKB activator (TNFRSF11A), mRNA
Length=3133

GENE ID: 8792 TNFRSF11A | tumor necrosis factor receptor superfamily, member 11a, NFKB activator [Homo sapiens] (Over 10 PubMed links)

Score = 649 bits (351), Expect = 0.0
Identities = 356/361 (98%), Gaps = 0/361 (0%)
Strand=Plus/Minus

```
Query  57    CTGGTCTGTGGGCTGGGAGGGCCTGTCCATGTATTCATCTTCTGTGGGCATCTGTCTGAA  116
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Query  177   TGCGTAGGGACCACCTCCTACACATGTGCCCTGACAGACACCACCTTGATCTGGGTAGCA  236
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             |
Sbjct  755   C  755
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B

Figure 4: A -Sequence of purified RANK PCR fragment isolated from MCF-7 cells. Sequencing was done at the McGill University and Genome Quebec Innovation Center; **B** - BLASTn alignment of isolated RANK sequence (Query) with Homo sapiens tumor necrosis factor receptor superfamily, member 11a, NF-κB activator (RANK) mRNA (Subject)

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A

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>ref|NM_033012.2| Homo sapiens tumor necrosis factor (ligand) superfamily, member
11 (TNFSF11), transcript variant 2, mRNA
Length=1931

 Score =  597 bits (323),  Expect = 1e-167
 Identities = 323/323 (100%), Gaps = 0/323 (0%)
 Strand=Plus/Plus

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             |||||||||||||||||||||||
Sbjct  1124  CCTGGTCATGTGCCCCTTCGCAG  1146
```

B

Figure 5: **A** - Sequence of purified RANK PCR fragment isolated from MCF-7 cells. Sequencing was done at the McGill University and Genome Quebec Innovation Center; **B** -Nucleuotide BLAST alignment of isolated RANKL (Query) with Homo sapiens tumor necrosis factor (ligand) superfamily, member 11 (RANKL), transcript variant 2, mRNA (Subject)

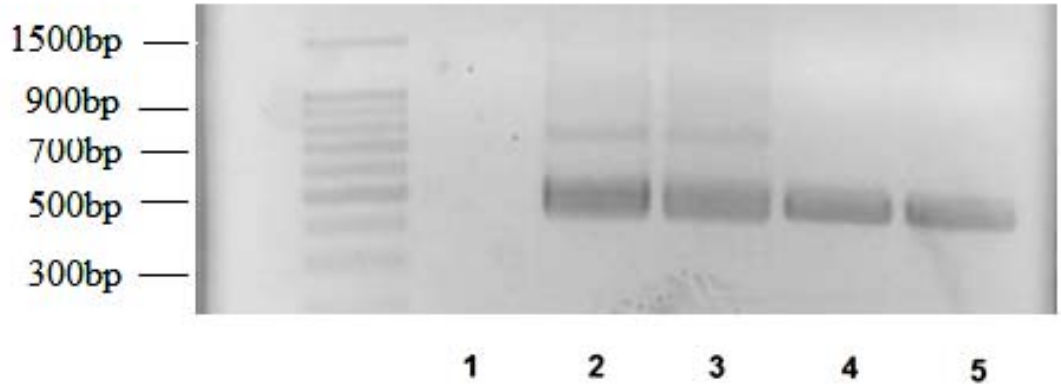


Figure 6: RT-PCR products of $GAPDH_3$ showing the differences in amplicon size of genomic (555bp) and complementary (451bp) DNA isolated from MCF-7 and MDA-MB-231 cell lines. Lane 1: Blank (water control), 2: MDA-MB-231 genomic DNA, 3: MCF-7 genomic DNA, 4: MDA-MB-231 cDNA, 5: MCF-7 cDNA

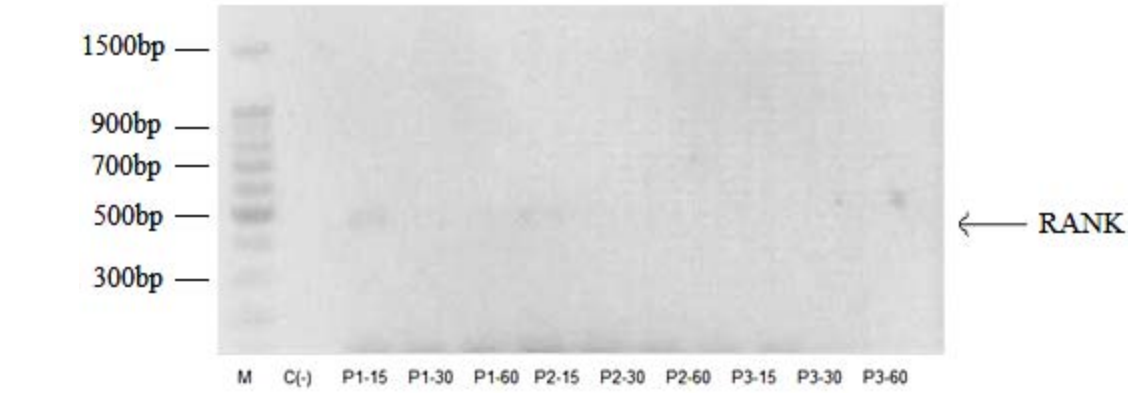
3.2 - RANKL Expression is Upregulated, while RANK Expression Diminishes in Response to PRL, EGF, and HRG-β1 in MCF-7 cells

Since RT-PCR of cDNA isolated from unstimulated MCF-7 cells demonstrated no RANKL expression, cells were stimulated with PRL, EGF, and HRG-β1 for varying amount of time in order to observe if RANKL expression could be induced. MCF-7 cells were stimulated with growth factors or hormones for 15, 30 and 60 minutes, and RNA was isolated after 24, 48 and 72 hours for all treatments. In cells stimulated with 100nM PRL, RANKL is induced in all stimulation and isolation time-points (Figure 7B). Looking at Figure 7C, there appears to be no cDNA amplification at the 60 minute stimulation were RNA was isolated at 72 hours. This particular time-point did not show $GAPDH_3$ expression (Figure 7C). RANK does not appear to be expressed in any PRL-stimulated samples, except for detection after 15 minutes of exposure, where RNA was isolated at 24 or 48 hours (Figure 7A).

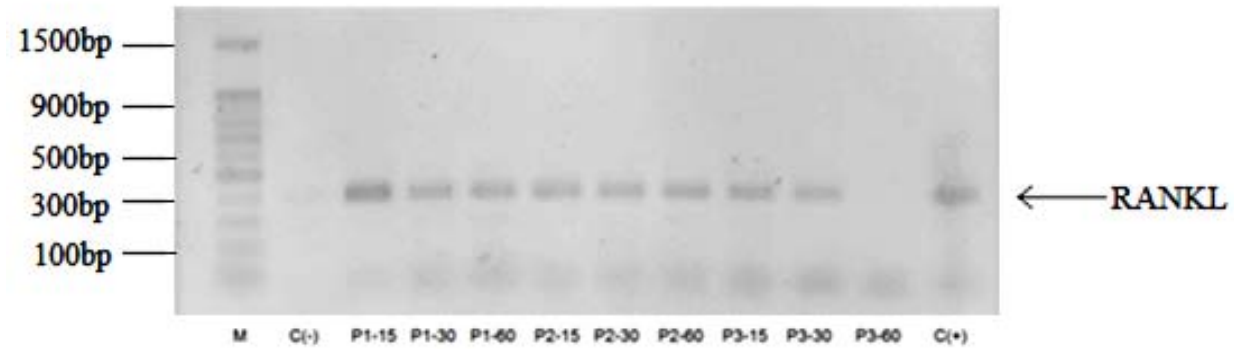
MCF-7 cells stimulated with 10nM EGF revealed RANKL expression in all samples and time-points, even after 60 minute stimulation and RNA isolation at 72 hours, although this band is very faint (Figure 8A). These stimulated cells also showed no RANK expression in any samples (Figure 8B), while $GAPDH_3$ was seen in all samples (Figure 8C).

HRG-β1-stimulated MCF-7 cells had RANKL expression in all cDNA samples except cells stimulated for 30 minutes, RNA isolated after 24 hours, and those stimulated for 60 minutes, with RNA isolated after 72 hours; however, since these samples did not express $GAPDH_3$ they should not be included in this analysis of induced RANKL expression (Figure 9A and 9C). No cDNA samples from MCF-7 cells stimulated with 20ng/mL HRG-β1 displayed RANK expression (Figure 9B).

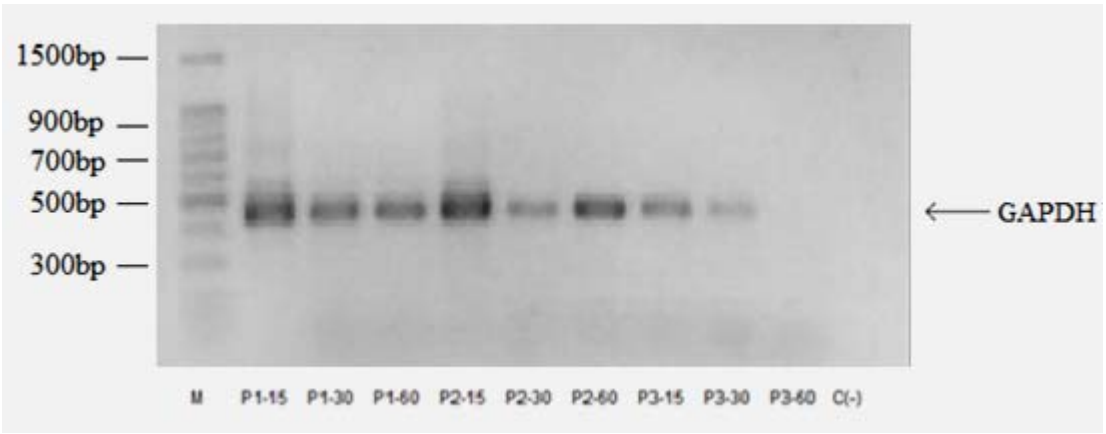
Figure 10 shows that RANK was expressed in unstimulated MCF-7 cells and not in cells stimulated with 100nM PRL in a single PCR reaction.



A

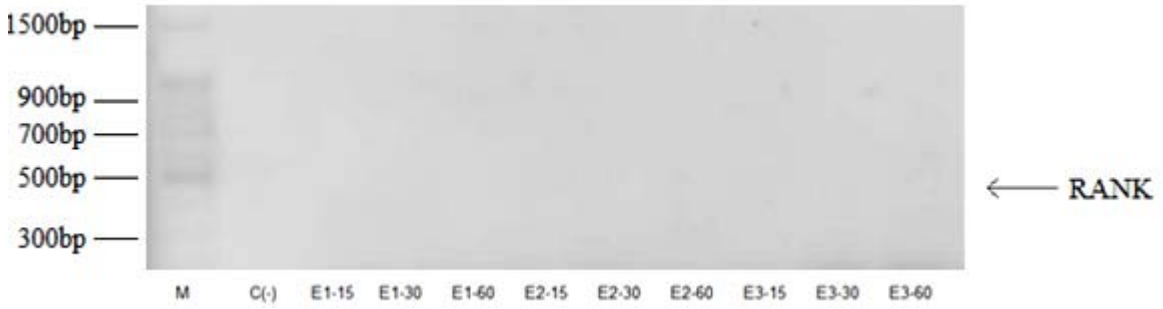


B



C

Figure 7:RT –PCR showing RANK PCR product (**A**), the presence of RANKL (**B**) and GAPDH (**C**) expression in MCF-7 cells after 24 (P1), 48 (P2), and 72 (P3) hours after stimulation with 100 nM PRL for 15, 30 and 60 minutes. C(-) indicates a negative control in which cDNA was not added to the PCR reaction.



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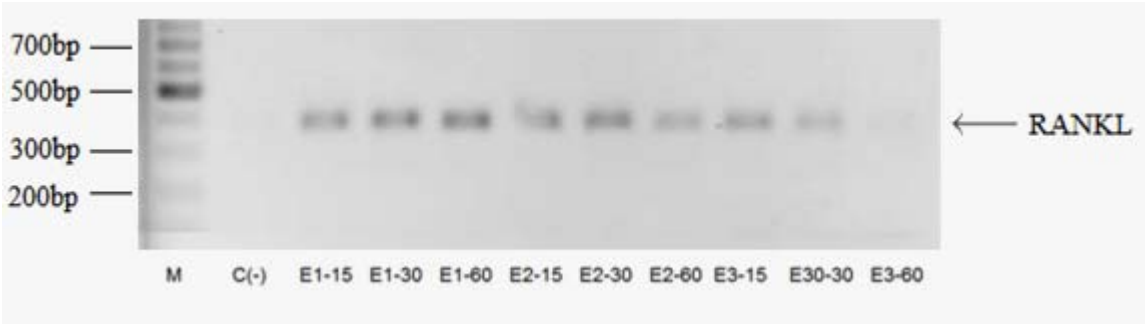
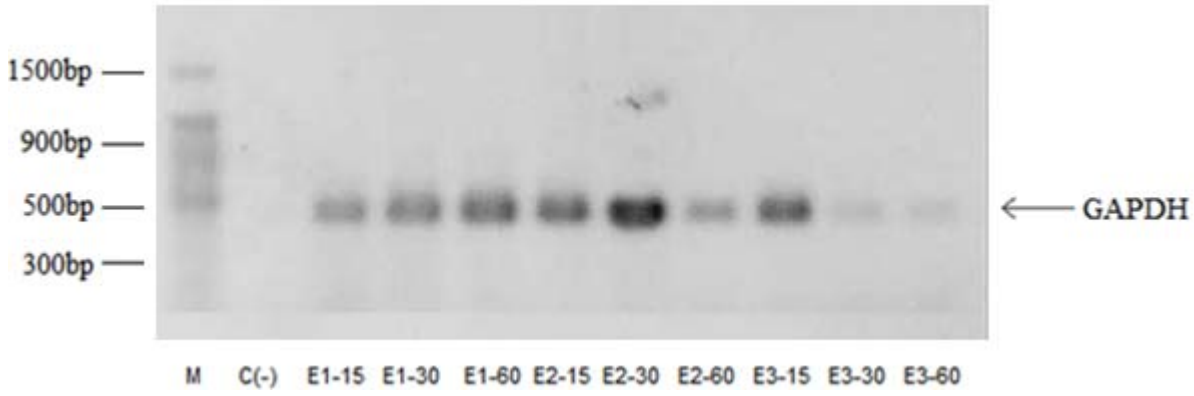
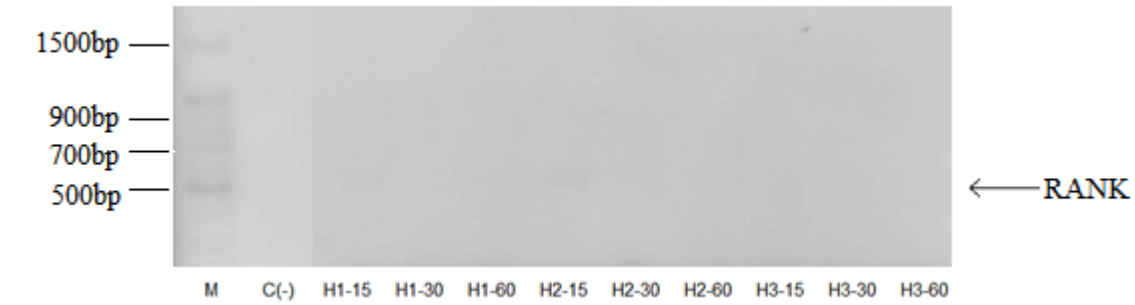


B

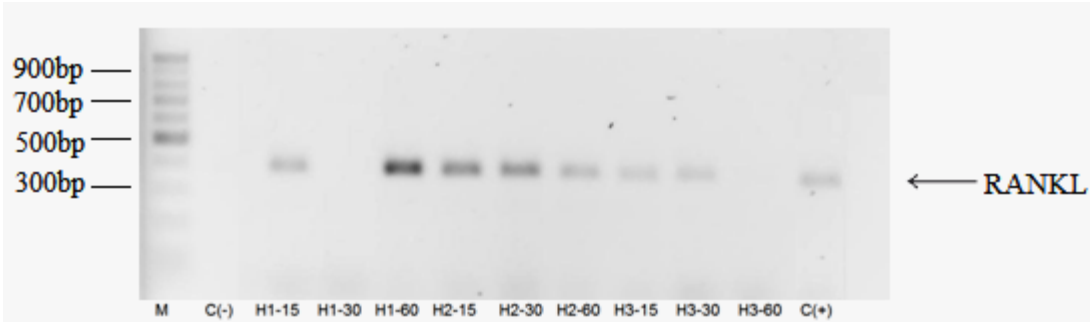


C

Figure 8:RT-PCR showing absence of RANK (**A**), the presence of RANKL (**B**) and GAPDH (**C**) expression in MCF-7 cells after 24 (E1), 48 (E2), and 72 (E3) hours after stimulation with 10 nM EGF for 15, 30 and 60 minutes. C(-) indicates a negative control in which cDNA was not added to the PCR reaction.



A



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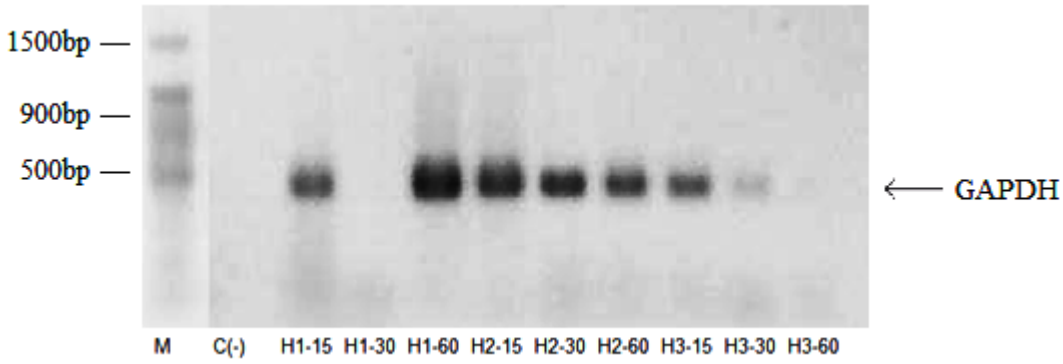


C

Figure 9:RT-PCR showing RANK PCR product (**A**) presence of RANKL (**B**) and GAPDH (**C**) expression in MCF-7 cells after 24 (H1), 48 (H2), and 72 (H3) hours after stimulation with 20 ng/mL HRG for 15, 30 and 60 minutes. C(-) indicates a negative control in which cDNA was not added to the PCR reaction.

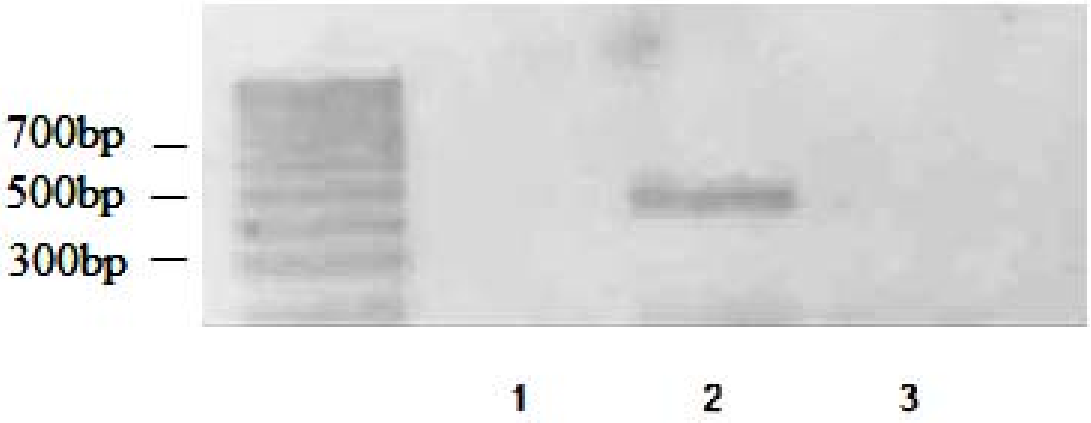


Figure 10: RT-PCR showing presence or absence of RANK in stimulated and unstimulated MCF-7 cells. 1 – cDNA created from MCF-7 cells stimulated with 100nM PRL for 15 minutes, 2 – cDNA created from unstimulated MCF-7 cells, 3 – PCR product minus cDNA.

3.3 - STAT-5a is Phosphorylated in Response to PRL, EGF and HRG-1 in MCF-7 cells

After showing that RANKL can be upregulated by stimulation with PRL, EGF, and HRG-β1, protein isolation and western blot analysis was used to determine if these hormones and growth factors were acting through the STAT5a pathway to stimulate expression. As shown in Figure 11 and 12, protein isolated from unstimulated MDA-MB-231 and MCF-7 cells, respectively, show STAT5a protein expression (92kDa), but not p-STAT5a (94kDa).

MCF-7 cells stimulated with PRL for 15 minutes showed little expression of p-STAT5a, while those stimulated for 30 or 60 minutes displayed higher levels of p-STAT5a expression (Figure 13A). In protein isolated from all cells stimulated with PRL, STAT5a was no longer visible (Figure 13B). At all time points of stimulation, cells grown in EGF showed p-STAT5a expression, as well as reduced levels of STAT5a (Figure 13A and B). After growth with HRG-β1, cells show p-STAT5a, but STAT5a protein was not detected with early stimulation time points (Figure 13A and B).

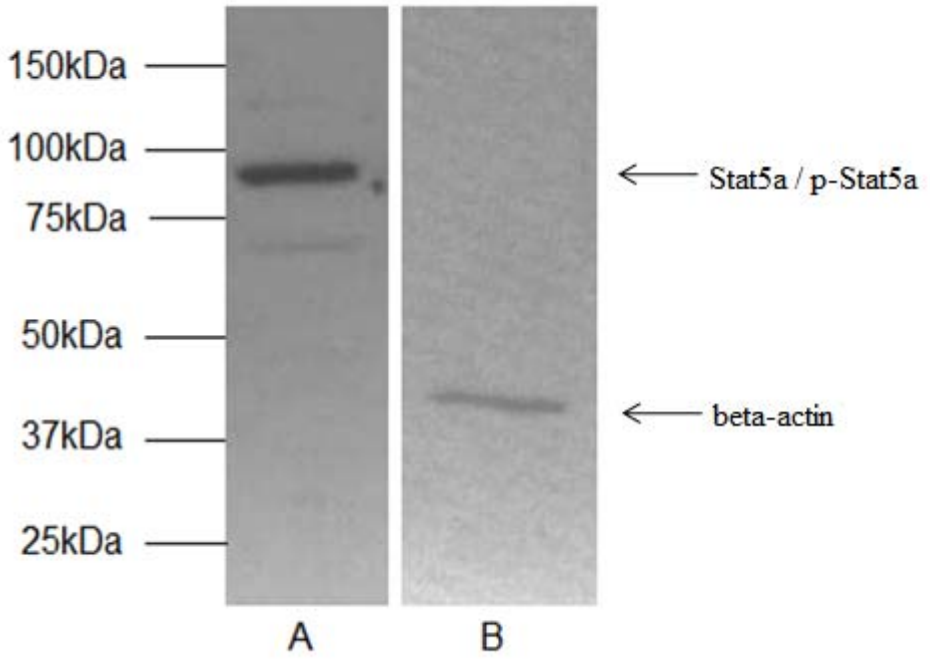


Figure 11: Western blot analysis of whole protein samples isolated from unstimulated MDA-MB-231 cells. **A** – Membrane probed with Stat5a antibodies (94kDa); **B** – Membrane probed with p-Stat5a (expected size – 94kDa) and β-actin (43kDa)

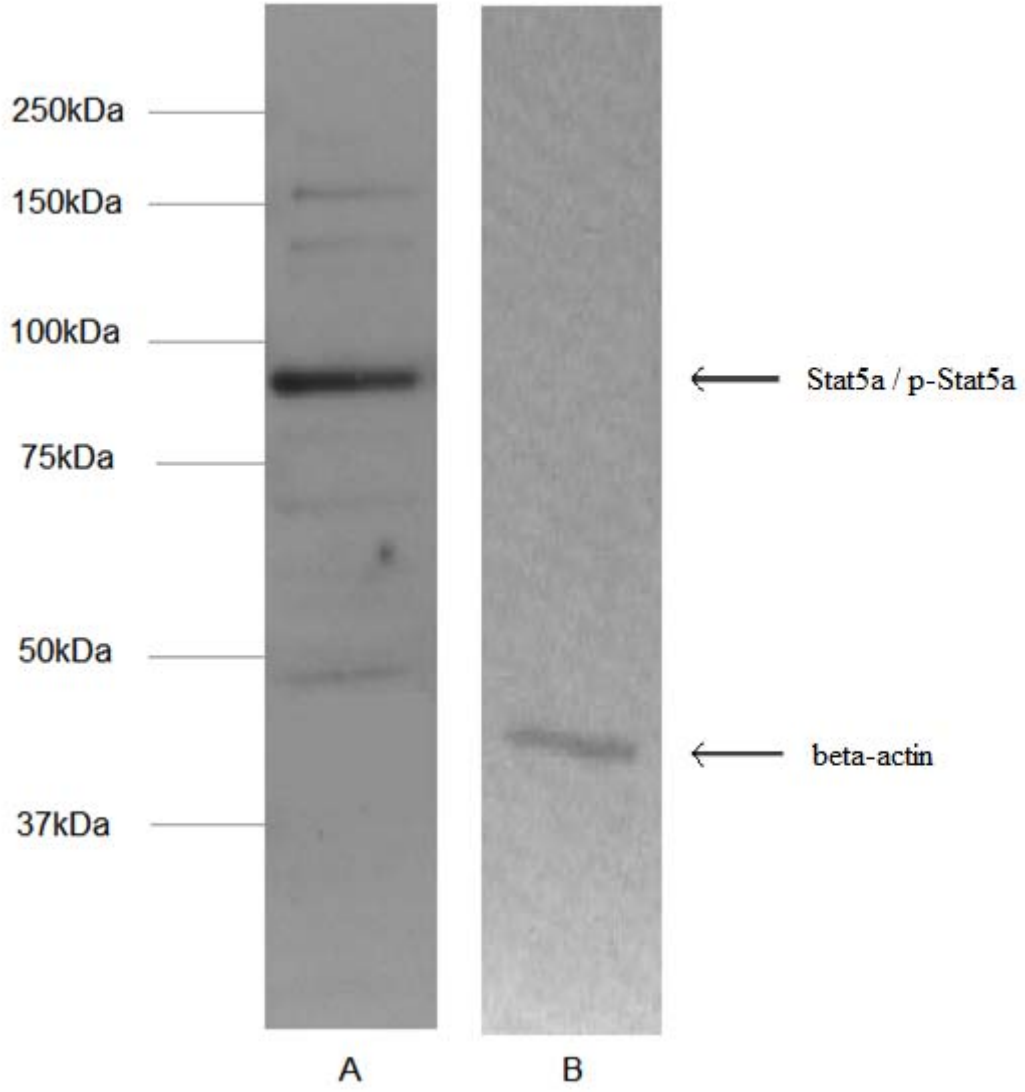
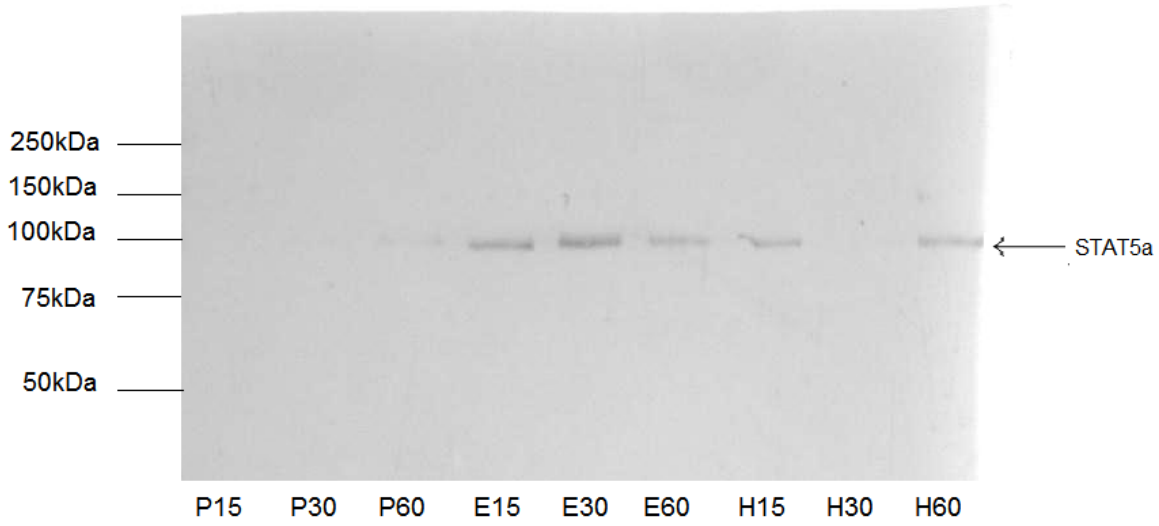


Figure 12: Western blot analysis of whole protein samples isolated from unstimulated MCF-7 cells. **A** – Membrane probed with Stat5a antibodies (94kDa); **B** – Membrane probed with p-Stat5a (expected size – 94kDa) and β-actin (43kDa)



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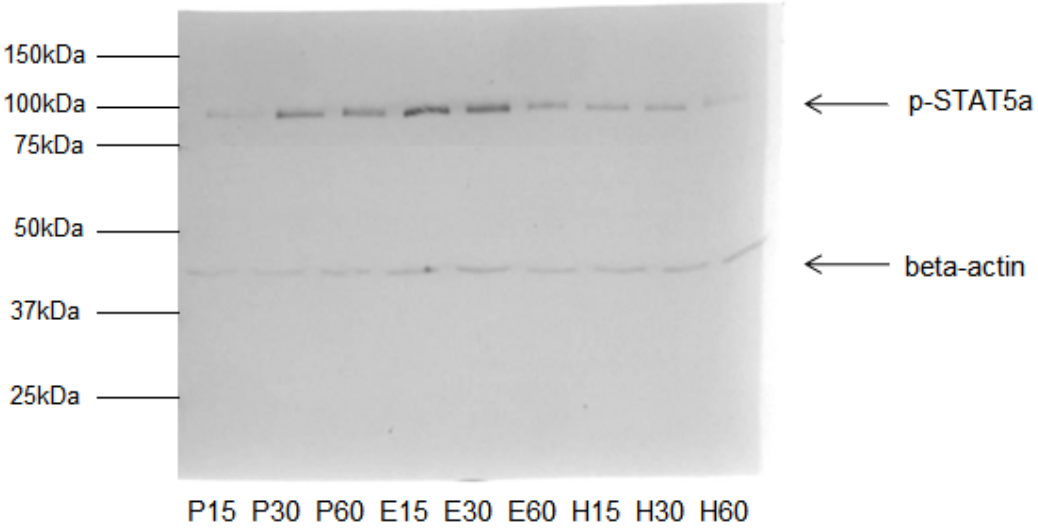

B

Figure 13: Western blot analysis of whole protein isolated from MCF-7 cells after exposure to 100nM PRL (P), 10nM EGF (E) or 20 ng/mL HRG-β1 (H) for 15, 30 or 60 minutes. **A** – Membrane probed with Stat5a antibody (94kDa); **B** – Membrane probed with p-Stat5a (94kDa) and β-actin (43kDa)

3.4 - Inhibition of STAT5a Phosphorylation Causes Inhibition of RANKL Expression in MCF-7 cells treated with PRL and HRG-β1

Cells were also incubated for 15 minutes with both AG490 inhibitor (to block STAT5 phosphorylation) in addition to hormones/growth factors and RNA was isolated after 24 hours to determine RANKL expression. Cells incubated with both the inhibitor and PRL or HRG-β1 no longer showed RANKL expression, however cells incubated with EGF along with the inhibitor still exhibited detectable levels of RANKL mRNA. RANK was still not expressed after incubation with both the inhibitor and various hormones/growth factors. See Figure 14.

Tyrophostin AG490 is a JAK2 inhibitor, used here to inhibit the phosphorylation and activation of STAT-5a. Figure 15A and B confirms the inhibitor activity by showing that cells incubated with the AG490 inhibitor and hormones/growth factors for 30 minutes no longer had detectable p-STAT5a protein and STAT5a was detectable.

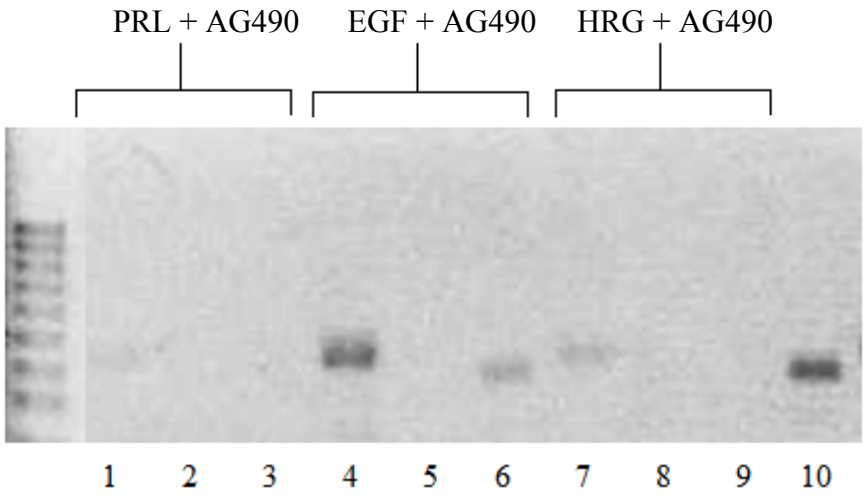

Figure 14:RT-PCR of $GAPDH_3$, RANK, and RANKL in MCF-7 cells stimulated with PRL, EGF, and HRG-β1 in the presence of 100 μM Tyrophostin AG490 inhibitor. Lanes 1, 4, 7 – $GAPDH_3$ expression; Lanes 2, 5, 8 – RANK expression; Lanes 3, 6, 9 – RANKL expression; Lane 10 – RANKL expression in HT-29 cells (positive control)

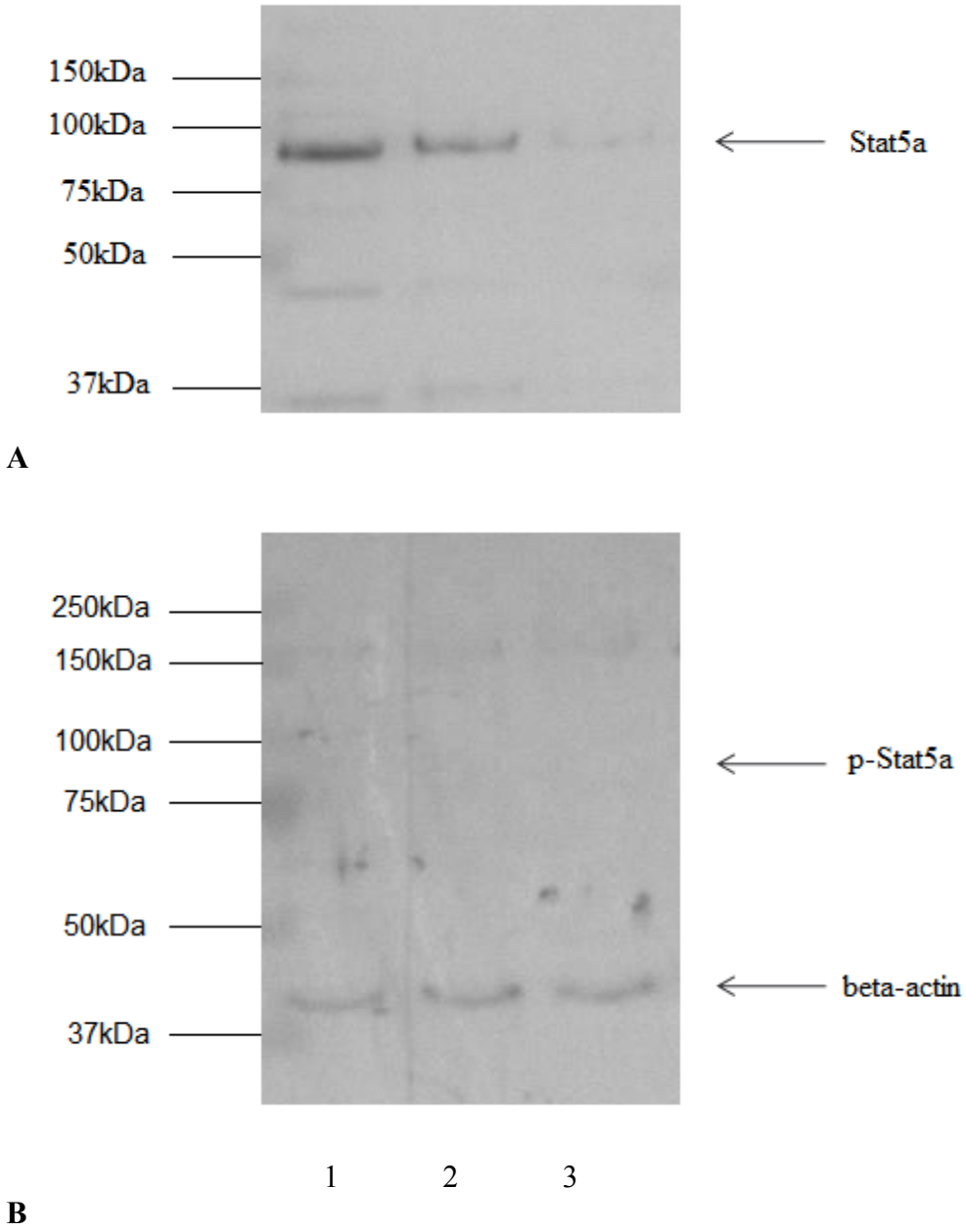

Figure 15: Western Blot Analysis of whole protein isolated from MCF-7 cells treated with AG490 inhibitor and PRL, EGF, or HRG-β1. **A** – Stat5a expression (94kDa); **B** – Membrane probed with p-Stat5a (expected size – 94kDa) and β-actin (43kDa). Lanes: 1 - 100nM PRL, 2- 10nM EGF, and 3 – 20ng/mL HRG-β1

3.5 - The Effects of PRL, EGF, and HRG-b1 on Proliferation in MCF-7 and MDA-MB-231 cells

Proliferation of MDA-MB-231 and MCF-7 cells untreated, and treated with PRL, EGF and HRG-β1, was determined using crystal violet staining and reading absorbance at 570nm. Standard curves were created to relate absorbance values to actual cell number. A linear standard curve was created for each cell line with cell numbers ranging from 5000 to 30 000 cells. The standard curve for MDA-MB-231 cells produced a linear relation of $y = 4.01913*10^{-5}x + 0.1446$ (Figure 16), while the curve for MCF-7 cells produced a relation of $y = 7.94945*10^{-5}x + 0.2233$ (Figure 17), where x represents the number of cells and y represents the absorbance at 570nm.

In MDA-MB-341 cells, there was no significant difference between the numbers of cells after 72 hours for untreated and cells treated with PRL, HRG-β1 and EGF (Figure 18, 19, 20). After 96 hours, cells stimulated with 100nM PRL did show a significant increase in cell number compared to the control sample, p=0.009542808 (Figure 18). In MCF-7 cells, there was no significant difference between the numbers of cells from 0 to 120 hours for untreated cells and for those treated with PRL, EGF, or HRG-β1 (Figure 21, 22, 23).

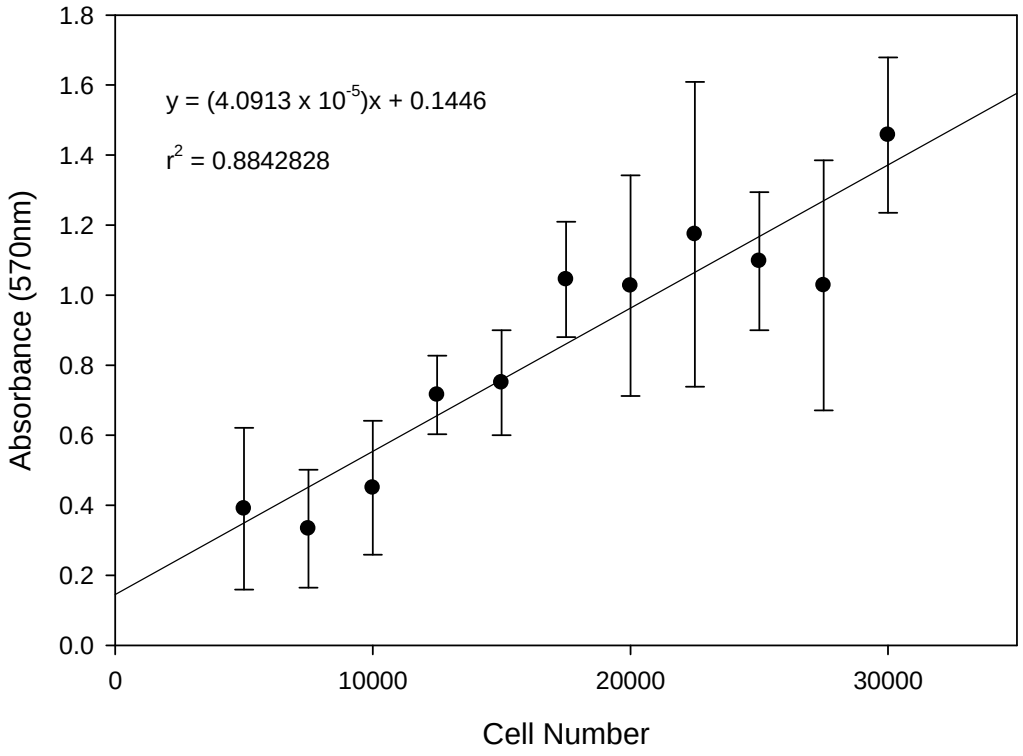

Figure 16: Standard curve relating the number of MDA-MB-231 cells to the absorbance (570nm) read from the crystal violet assay (n=5).

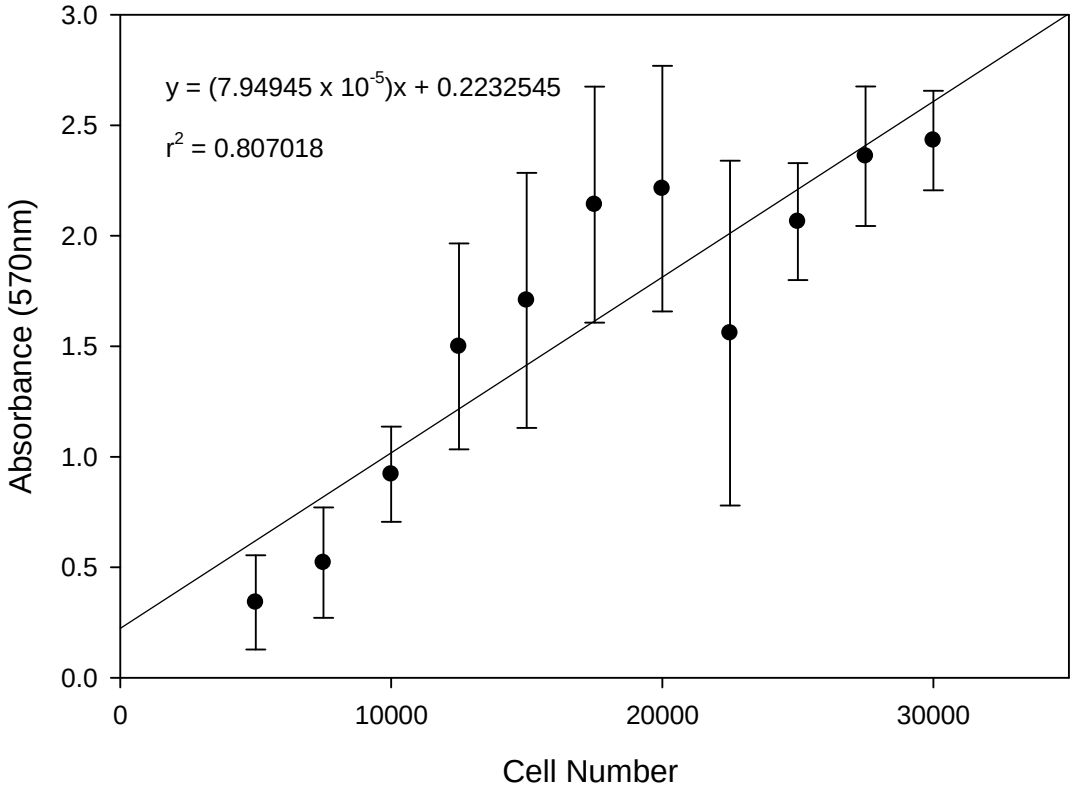

Figure 17: Standard curve relating the number of MCF-7 cells to the absorbance (570nm) read from the crystal violet assay (n=5)

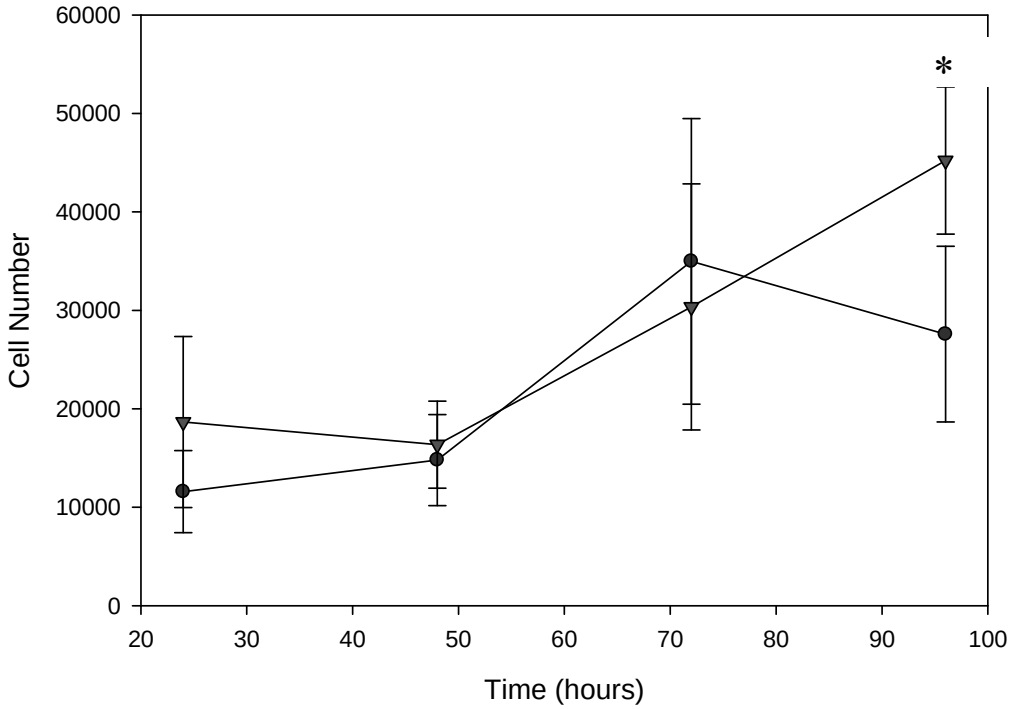

Figure 18: Proliferation of MDA-MB-231 cells over a span of four days (96 hours) in untreated control cells —●— as compared to cells treated with 100nM PRL —▼—; * indicates a p-value < 0.05; (n= 5)

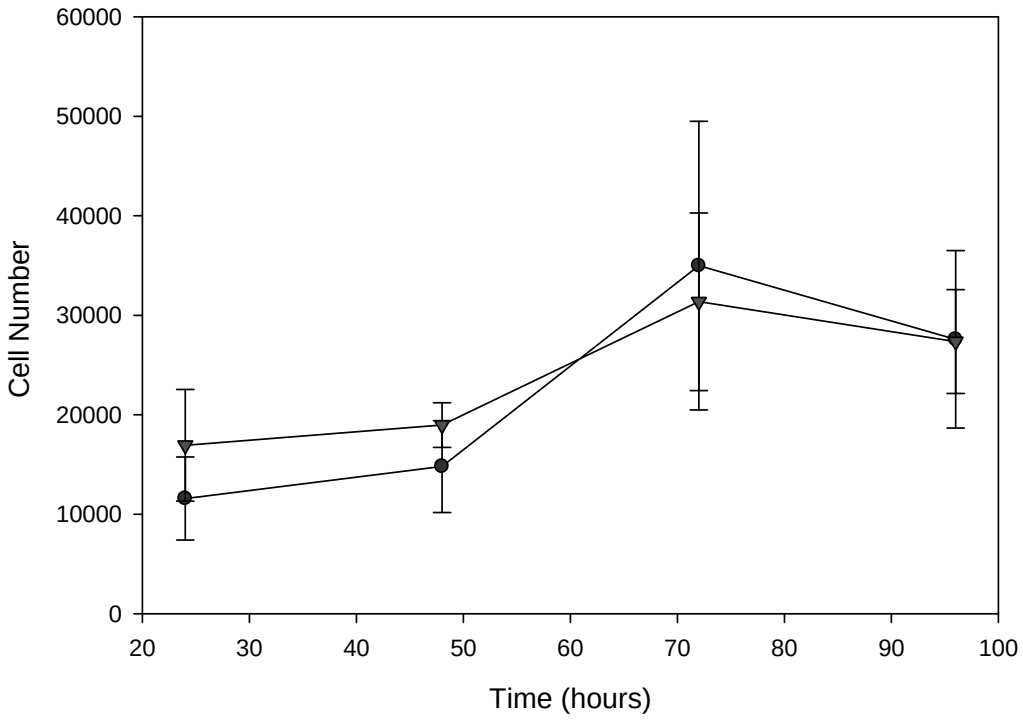

Figure 19: Proliferation of MDA-MB-231 cells over a span of four days (96 hours) in untreated control cells as compared to cells treated with 10nM EGF ; (n= 5)

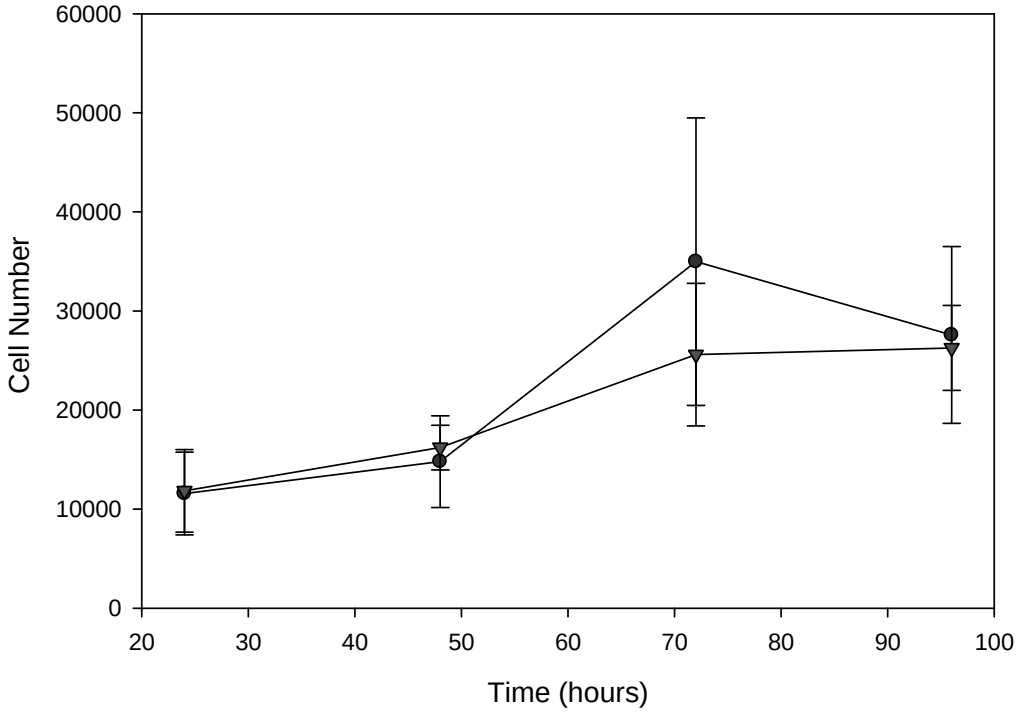

Figure 20: Proliferation of MDA-MB-231 cells over a span of four days (96 hours) in untreated control cells as compared to cells treated with 20ng/mL HRG ; (n= 5)

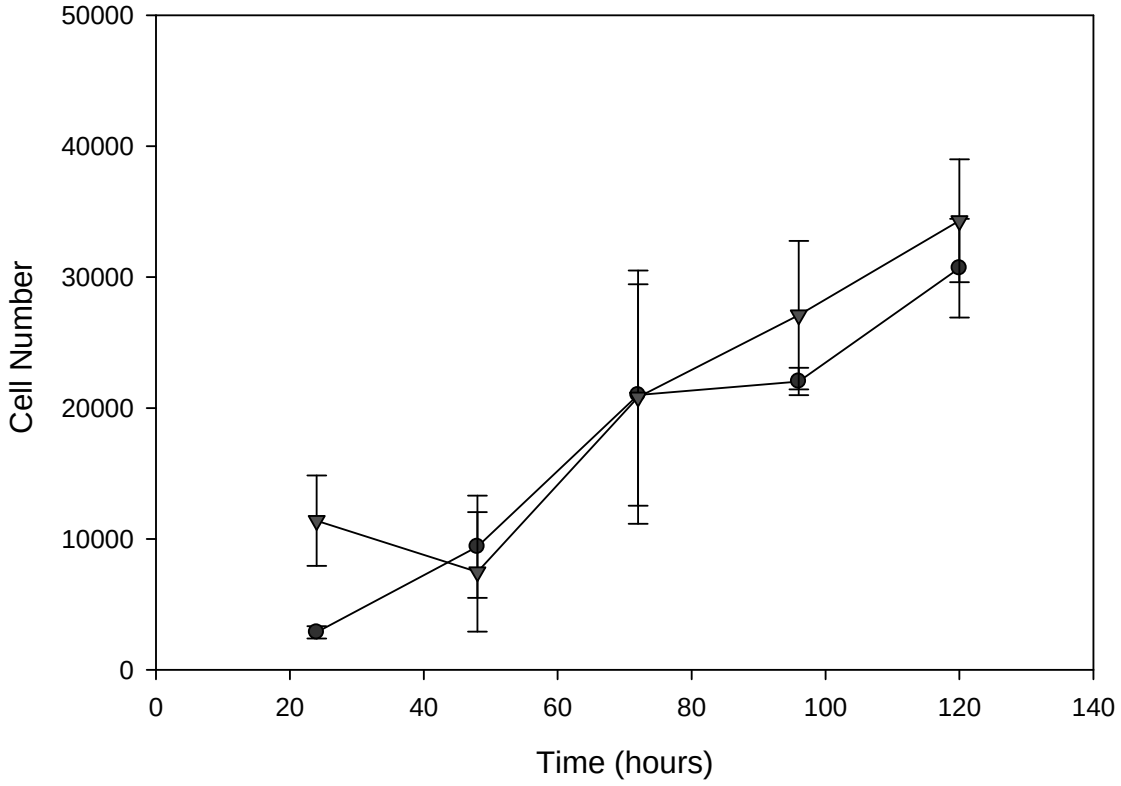

Figure 21: Proliferation of MCF-7 cells over a span of four days (96 hours) in untreated control cells —●— as compared to cells treated with 100nM PRL —▼—; (n= 5)

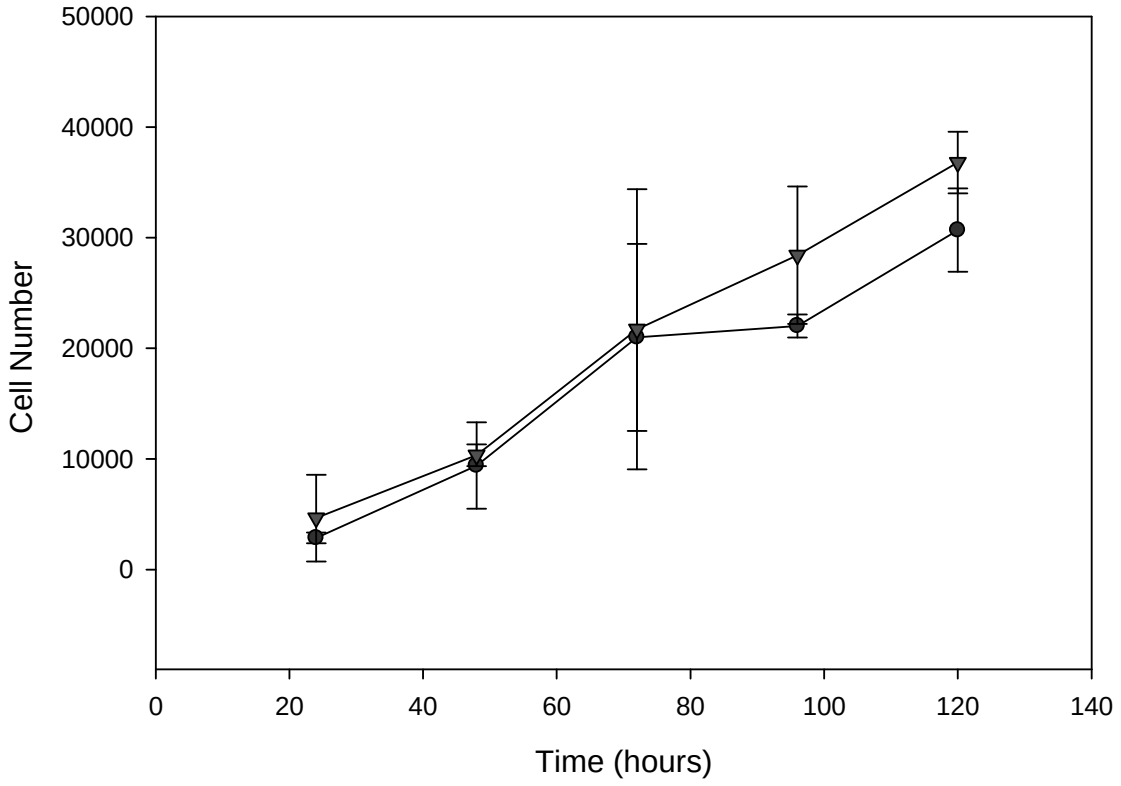

Figure 22: Proliferation of MCF-7 cells over a span of four days (96 hours) in untreated control cells as compared to cells treated with 10nM EGF ; (n= 5)

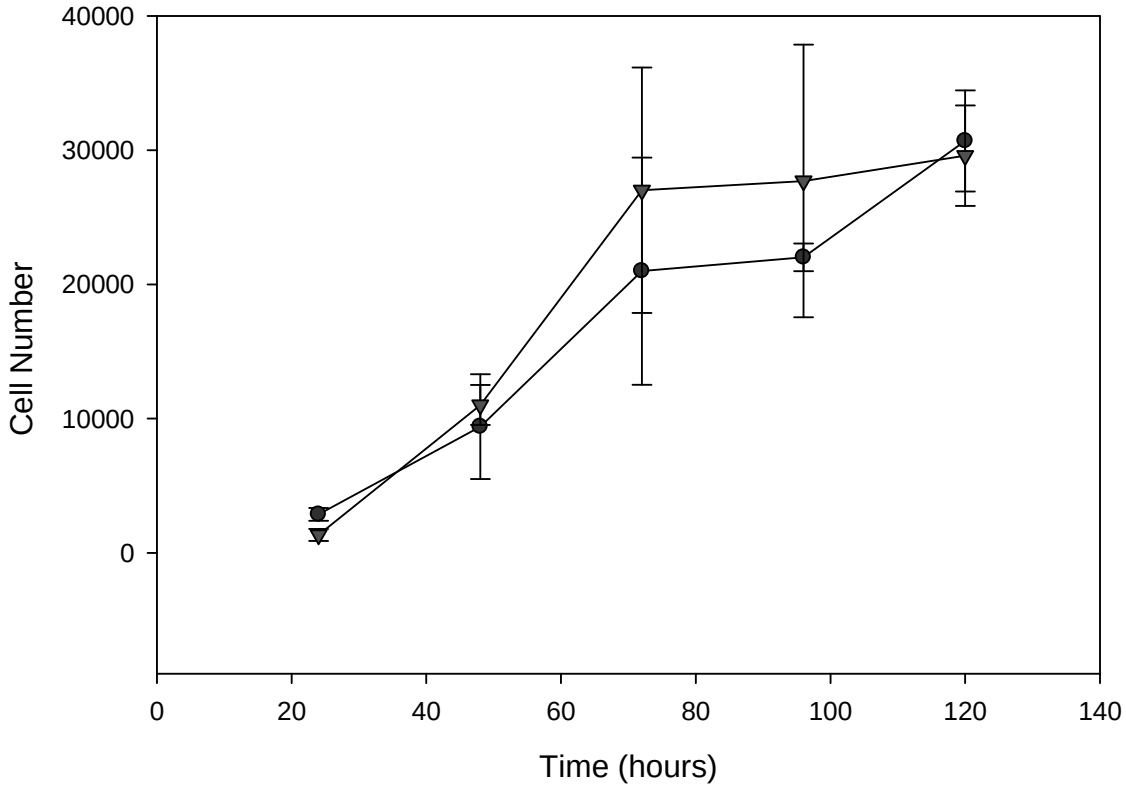

Figure 23: Proliferation of MCF-7 cells over a span of four days (96 hours) in untreated control cells —●— as compared to cells treated with 20ng/mL HRG —▼— ; (n= 5)

4 - Discussion

4. 1 - RANK and RANKL Expression in Human Breast Cancer Cells

In normal breast epithelial cells, RANK is constitutively expressed throughout development, while RANKL is only expressed during the stages of pregnancy and lactation. It is at this point that cells are rapidly dividing and forming lobuloalveolar structures. Previous studies have shown that RANK, but not RANKL mRNA is expressed in many human breast carcinoma cells. One study in particular indicated that although RANK is expressed in normal breast epithelial cells, as well as tumor cells invading bone, RANKL is only expressed in metastatic cells (Bhatia et al, 2005). Another study showed by quantitative RT-PCR, that RANKL is weakly expressed in tissues isolated from human breast tumors (Reinholz et al, 2002).

As shown in Figure 3, MCF-7 cells grown in their regular culture medium expressed RANK mRNA and but not RANKL mRNA, consistent with previous research. Purified RT-PCR amplicon were sequenced and confirmed identity of human RANK and RANKL (Figure 4 and 5).

4.2 – Effects of PRL on MCF-7 and MDA-MB-231 cells

4.2.1 - PRL Induces RANKL Expression through the JAK2-STAT5a Pathway in MCF-7 cells

Although RANKL is not normally expressed in MCF-7 or MDA-MB-231 cells, when MCF-7 cells are treated with 100nM PRL, RANKL mRNA expression is induced (see Figure 7B). RANKL mRNA was detectable after 15, 30 and 60 minutes of incubation with PRL. The one-hour treatment sample did not appear to have any isolated

cDNA, as the RT-PCR control, $GAPDH_3$, was not amplified (see Figure 7C). This may have been a problem with RNA isolation, or with the synthesis of complementary DNA. However, it is clear that RANKL was upregulated with the addition of PRL to the culture media. This process appears to be regulated through the Stat5a pathway, as addition of PRL to the growth culture media induced STAT5a to become phosphorylated (see Figure 13). AG490 is a specific inhibitor of JAK2 phosphorylation, thereby inhibiting the subsequent phosphorylation of STAT5a. When STAT5a phosphorylation is blocked with a JAK2 inhibitor, PRL was no longer seen to induce RANKL expression, therefore linking the expression of RANKL directly to phosphorylation of STAT5a (see Figure 14). Activation of the STAT5a by PRL is a well-characterized event in mammary epithelial cells (Srivastava et al, 2003) and human breast cells. Our work is consistent with Srivastava et al (2003), which showed that RANKL could be induced by PRL through the STAT5a pathway in mouse mammary epithelial cells. In human breast cancer cells, including both MCF-7 and MDA-MB-231 cells, PRL was also able to activate STAT5a through JAK2 phosphorylation. Our research shows that PRL induced activation of JAK2 and phosphorylation of STAT5a and that this activation is responsible for the transcriptional regulation of the RANKL gene.

4.2.2 - PRL stimulates proliferation in MDA-MB-231 cells, but not MCF-7 cells

Prolactin acts to stimulate proliferation and differentiation of terminal end buds into milk-secreting lobuloalveolar structures. PRL is distinguished as a mitogen and is known to activate proliferation in murine mammary and human breast epithelial cells. Therefore, it was expected that human breast cancer cells expressing a receptor to PRL would have a higher rate of proliferation when treated. After four days of exposure to

100nM PRL, only MDA-MB-231 cells showed a significant increase in cell number when compared to the control study. This finding is interesting given the fact that MDA-MB-231 cells do not actually possess the PRLR; therefore PRL is stimulating proliferation through another unknown mechanism.

On the other hand, MCF-7 cells do possess the PRLR and in fact, one study conducted by Ginsberg and Vanderhaar (1995) revealed that PRL exposure causes proliferation in MCF-7 human breast cancer cells. This same study showed that MCF-7 cells might actually synthesize and secrete PRL in preconditioned media. Our experiment showed that after all four days, MCF-7 cells did not show a significant difference in cell number with PRL (see Figure 21), however more replicates of this experiment are required before further conclusions can be drawn.

Since the effects of PRL on cell number was only seen after the fourth day in MDA-MB-231 cells, it is likely that further proliferation studies with longer PRL exposure, more time points taken and even more alternative methodologies may reveal different results in both cell lines.

4.3 – The Effects of EGF on MCF-7 and MDA-MB-231 cells

4.3.1 - EGF Induces RANKL Expression through the JAK2-STAT5a and other pathways in MCF-7 cells

EGF acts through the EGFR to induce signals through a variety of signal transduction pathways. In this study, stimulation of MCF-7 cells with 10nM EGF showed an induction in RANKL transcription after only 15 minutes of stimulation time (Figure 8B). In a study by Biswas et al (2000), human breast cancer cell lines treated with EGF showed activation of NF-κB. Since our study shows that EGF causes an

increase in RANKL transcription, it is possible that the activation of NF-κB in human cancer cells was due to the activation of the RANK-RANKL pathway.

In unstimulated MCF-7 cell lines, there was no p-STAT5a present (see Figure 12), however addition of EGF to the culture medium not only induced cells to expressed RANKL, but p-STAT5a was also isolated from whole protein lysates. To connect the STAT5a activation to RANKL expression, JAK2 activity was inhibited with AG490. In this case, RANKL was still being transcribed in MCF-7 cells stimulated with EGF. This indicates that although EGF may be activating the JAK2-STAT5a pathway to induce STAT5a phosphorylation, there must be one or more pathways also activated which are causing RANKL induction. EGF not only activates STAT5a through JAK-activation, but it is also known to activate a variety of other pathways, including the Ras/MAPK pathway (Scaltriti and Baselga, 2006), PLC-γ (Wahl et al, 1990), and PI3K (Bjorge et al, 1990). Although JAK2 phosphorylation is required for STAT5a activation in most cases, EGFR can also directly phosphorylate STAT proteins, only requiring JAK2 for maximal activation. One study actually showed that EGFR activates STAT1, 3 and 5 via Src in human breast cancer cells (Olayioye et al, 1999). Olayioye et al (1999) also indicated that EGFR-Src activation of STAT5a cause phosphorylation of tyrosine residues other thanY694, possibly allowing these phosphorylated STAT5a proteins to effect different downstream targets. This means that AG490 inhibition may not have been sufficient to obstruct all STAT5 activation by EGF. Our studies indicate that after incubation with the AG490 JAK2 inhibitor, STAT5a Y694 phosphorylation as detected by the specific sc-101806phospho-STAT5a (Tyr694) antibody is diminished. Since STAT5a phosphorylation on the Y694 residue is dependent on JAK2, it is likely that EGF acts

through an alternative pathway to induce RANKL expression. Further studies in which these pathways can be specifically inhibited will have to be conducted in order to determine the exact mechanisms responsible for the EGF-regulation of RANKL transcription in breast cancer cells.

4.3.2 – EGF does not show stimulation of proliferation in human breast cancer cells

EGF is responsible for the proliferation of ductal epithelial cells in mammary gland development (Wiesen et al, 1999). In breast cancer, there is a strong correlation between the amount of EGFR and cell proliferation (Marti et al, 1991). Therefore, it was expected that EGF would increase the rate of proliferation in human breast cancer cells in this study. Since MDA-MB-231 cells contain higher levels of EGFR, the effect in these cells would be expected to be greater than in MCF-7 cells. However, in the experiment shown, EGF did not cause a significant increase in proliferation (see Figure 22). This is in contrast to a previous study indicated that stimulation of MCF-7 cells with EGF had a significant increase in proliferation with EGF concentrations as low as 0.01ng/mL (Osborne et al, 1980).

4.4 – The Effects of HRG-β1 on MCF-7 and MDA-MB-231 cells

4.4.1 - HRG-β1 Induces RANKL Expression through the JAK2-STAT5a Pathway in MCF-7 cells

Similar to PRL and EGF, HRG-β1 is also involved in mammary gland development during pregnancy and is responsible for the formation of lobuloalveolar structures (Yang et al, 1990). HRG binds to erbB3 and erbB4 causing activation of STAT5a in mammary epithelial cells (Jones et al, 1998). Therefore it was hypothesized

that HRG-β1 would mediate expression of the RANKL gene. In this study, HRG-β1 caused MCF-7 cells to express RANKL mRNA. Expression of RANKL mRNA was seen after only fifteen minutes of exposure to 20ng/mL HRG-β1 (see Figure 9B). This means that HRG is upregulating the expression of RANKL mRNA expression in MCF-7 human breast cancer cells.

When JAK2 phosphorylation was inhibited using AG490 in HRG-stimulated MCF-7 cells, RANKL mRNA expression was no longer seen (see Figure 14). This indicates that similar to PRL, HRG-β1 causes JAK2 phosphorylation, which causes phosphorylation and activation of STAT5a and subsequently promotion of the RANKL gene. Therefore this inhibition study proves our hypothesis that HRG stimulates RANKL mRNA expression through activation of the JAK2-STAT5a pathway in MCF-7 human breast cancer cells.

4.4.2 - HRG-β1 stimulation does not result in increased proliferation of human breast cancer cells

In this study, HRG-1 did not induce proliferation in MDA-MB-231 (see Figure 20) or MCF-7 (see Figure 23) human breast cancer cell lines. Treated cells displayed cell numbers that were not statistically different than untreated cells, throughout the entire four-day period. Although MCF-7 cells contain both the erbB3 and erbB4 receptors, MDA-MB-231 cells only contain EGFR and low levels of erbB2 (Ferger et al, 1998), which do not convey the HRG-response. Therefore, if we were only assuming that HRG is only binding erbB3 and erbB4, we would only expect MCF-7 cells and not MDA-MB-231 cells to elicit increased cell growth due to STAT5a activation.

Previous studies have shown HRG to increase cell proliferation in HC11 mammary epithelial cells (Marte et al, 1995) and in vivo, creating ductal branching in mice (Jones et al, 1996). Proliferation was also increased through HRG stimulation in MCF-7 (Lewis et al, 1996; Hutcheson et al, 2007), T47D and many other human breast cancer cell lines (Lewis et al, 1996). Studies on invasive, metastatic breast cancers show conflicting results, indicating a decrease in proliferation due to HRG-stimulation. Puricelli et al (2002) and Xu et al (1992) have shown that HRG can actually inhibit proliferation in metastatic mammary tumor cells and breast cancer cells, respectively.

4.5 - RANK Expression is diminished in MCF-7 cells After Addition of PRL, EGF and HRG-β1

Although this study was intended to determine the regulation of RANKL gene expression, we have also determined that the addition of PRL, EGF and HRG-1 not only induces RANKL expression, each also caused a decrease in RANK mRNA expression. In all treatments, RANK expression was diminished and was not revived after addition of the AG490 inhibitor (see Figure 14). This indicates that the JAK2 activation of the STAT5a pathway is not the reason for this change in expression. No previous research has studied the effects of these factors on RANK mRNA expression; therefore it is unclear what specifically may be causing this response. As shown in Figure 10, RT-PCR of RANK cDNA from stimulated and unstimulated cells show this effect to be specific to the addition of hormones and growth factors to the media. Previous studies has shown RANK is expressed in normal and cancerous breast cells. Srivastava et al (2003) showed that RANKL was upregulated by PRL in mammary epithelial cells, but RANK expression was not determined after stimulation. No other studies look at the effects of

PRL, EGF, or HRG-1 on the expression of RANK mRNA. It is possible that the compounds are acting through alternative pathways to regulate the expression of this gene.

4.7 - Therapeutic Implications

Many studies have recognized thea link between breast and bone metastases to be the RANK-RANKL pathway. Since stimulation of the RANK-RANKL pathway is believed to cause an increase in metastasis of breast cancer to bone, clinical studies are currently underway to examine how this pathway can be regulated to control the spread of cancer. Under normal physiology, OPG acts to inhibit the RANK-RANKLpathway in osteoclasts, thereby regulating the process of bone remodeling. Given that OPG acts in this way, it is hypothesized that this molecule would limit breast-to-bone metastases. In fact, studies show that mice treated with recombinant OPG show decreased osteolytic destruction and tumor burden in bone (Morony et al, 2001). Recombinant OPG has been used in clinical trials (Body et al, 2003) and was shown to suppress bone resorption; however, more trials are needed to determine the effects on metastases and long term implications. Other inhibitory methods, including neutralizing antibodies specific for RANK, are being investigated. Our studies attempt to determine the regulation of the RANKL gene, which could act as an alternative target for inhibiting breast-to-bone metastases.

4.8 - Future Directions

In this study RANKL mRNA transcripts are used as an indication of RANKL expression. Ideally protein expression would also be studied in order to obtain an accurate impression of RANKL expression. However, currently there has been little success with antibodies designed to bind RANK or RANKL, likely because these proteins are involved in T-cell activation and immune function. Although some antibodies are available there has been limited success. Identifying the induction of RANKL mRNA still allows us to identify how transcription of the gene is regulated. In order to determine if the RANKL protein is actually upregulated, an option for future research would involve directly tagging the RANK receptor. If the RANK protein itself is manufactured with a fluorescent tag, it is possible to get an indication of RANKL protein expression, by looking at protein-protein interactions on the cell surface.

The effects of hormones and growth factors can also be examined using cells over-expressing the RANK receptor.

Proliferation assays did not show significant differences in cell number between treated and untreated samples for MCF-7 cells, and the only increase in growth was seen with PRL stimulation in MDA-MB-231 cell lines. Since previous research contradicts our results, further experiments will need to be conducted to prove validity. Taking more time-points for results, and increasing the length of the study may give a more accuracy to our results. It would also be beneficial to try different methods of measuring cell proliferation in order to confirm our results. Crystal violet binds DNA and thereby measures the relative number of cells; therefore, troubles may arise when binucleated cells are present. There are many other cell viability and proliferation assays available

that measure metabolic activity, for example, WST-1, MTT and XTT assays. Other available methods include measuring the incorporation of radioactive molecules in DNA synthesis and measuring fluorescence. An example of this type of method includes 5-bromo-2-deoxy-uridine labeling. Therefore, given the wide variety of proliferation assays available, it would be beneficial to explore various methods to confirm the results of this study.

Although these experiments indicated that EGF could activate phosphorylation of STAT5a, it was not solely this pathway that was involved in RANKL induction. In order to elucidate the mechanism of RANKL expression, further studies will need to be done targeting other pathways activated by EGF.

Lastly, hormone and growth factor effects on RANK and RANKL mRNA expression could be further investigated using more quantitative methods such as real-time PCR. Our study only focuses on induction or complete removal of gene expression, therefore it would be beneficial to quantify these results. As mentioned, protein studies would also give a better indication of RANK and RANKL expression within the cell.

This study gives insight to the regulation of RANK and RANKL expression in human breast cancer, providing the foundation for understanding the mechanism of RANKL activation and its impact on breast cancer progression and metastases.

5 – Conclusion

This study was conducted to determine the regulation of RANKL mRNA expression in human breast cancer cell lines. Both RANKL and STAT5a play an important role in development of normal mammary tissue, and we attempted to establish this same link in breast cancer cell lines using growth factors and hormones known to activate STAT5a. Three important STAT5a activators were used: PRL, EGF and HRG-1, all which have important roles in lobuloalveolar formation and are also implicated in breast cancer progression. We confirmed that both MDA-MB-231 and MCF-7 cells express RANK mRNA, and neither express RANKL under normal culture conditions. In MCF-7 human breast cancer cells, all treatments were able to induce RANKL expression. Both PRL and HRG-1 induced RANKL mRNA expression through activation of the STAT5a pathway, while it appears EGF induced expression through an alternative pathway. Intriguingly, RANK expression appears to be downregulated by these same factors and further studies may elucidate this mechanism. Results are too preliminary to conclude on the ability of these hormones and growth factors to promote proliferation in these cell lines.

Overall this investigation provides evidence for STAT5a-induced RANKL mRNA expression in human breast cancer. The study indicates that several factors may be regulating RANK and RANKL expression in breast cancer cells, which may lead to further cancer progression and links to metastasis to bone. This is the first study that shows RANKL mRNA induction in human breast cancer cells. More studies will need to

be conducted to determine the further mechanisms of RANKL induction, its role in breast cancer progression, and links to bone metastases.

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