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The Role of Cortisol in Breast Cancer Initiation and Progression

By Jenna C. Ritchie

A Thesis
Submitted to the Faculty of Graduate Studies
through Biological Sciences
in Partial Fulfillment of the Requirements for
the Degree of Master of Science at the
University of Windsor

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ABSTRACT

A great deal of evidence is accumulating that stress may affect cancer progression and patient survival, yet the mechanisms for this association are poorly understood. Cortisol is the primary stress hormone where several clinical studies have demonstrated that cortisol levels positively correlate with high mortality rate and recurrence of cancer. This study focuses on resolving the mechanism of cortisol in breast cancer initiation and progression. Our data demonstrates that stimulation with cortisol directly enhances breast cancer cell numbers and increases the rate that cells exit from G1 phase of the cell cycle. Furthermore, we demonstrate that cortisol enhances breast cancer cell migration as measured by the ability to repair a wound as well as cell migration towards a chemoattractant. Microarray analysis has revealed numerous interesting targets for these effects. This approach to studying tumourigenesis may provide targets for drug design that will improve current treatment strategies.

Dedication

I dedicate this thesis to myself because I am the one who put in all the work!

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List of Abbreviations

11β- HSD: 11β-hydroxysteroid dehydrogenase

ACTH: Adrenocorticotropic hormone

AF-1: Activation Function 1

AF-2: Activation Function 2

AP-1: Activator Protein 1

AR: Androgen Receptor

AVP: Arginine vasopressin

CBG: Corticosteroid-binding-globulin

CDK: Cyclin dependent kinase

CKI: Cyclin kinase inhibitor

CRH: Corticotrophin-releasing hormone

DBD: DNA binding domain

DMSO: Dimethyl sulfoxide

Dex: Dexamethasone

ER: Oestrogen Receptor

GR: Glucocorticoid Receptor

GRE: Glucocorticoid regulatory element

HPA: Hypothalamic- pituitary- adrenal

Hsp: Heat shock protein

LBD: Ligand Binding Domain

MR: Mineralcorticoid Receptor

MTT: Thiazolyl Blue Tetrazolium Bromide

NF-kB: Nuclear Factor- kappa B

NTD: amino terminal domain

PR: Progesterone Receptor

Q-RT-PCR: Quantitative Real Time Polymerase Chain Reaction

RU-486: Mifepristone

TAM: Tamoxifen

TF: Transcription Factors

INTRODUCTION:

Cortisol and Cancer: Clinical Evidence

A great deal of evidence is accumulating that stress and other behavioural factors may affect cancer progression and patient survival. Cortisol measurements are thought to be a dependable method in assessing an individual's physiological stress. However, studies looking at social support surrounding the patient and levels of cortisol remain elusive among clinical data (Turner-Cobb, Sephton, Koopman, Blake-Mortimer, & Spiegel, 2000).

It has been shown that as high as 70% of patients with breast cancer show constantly high levels of cortisol (Turner-Cobb *et al.*, 2000). Several clinical studies have demonstrated that cortisol levels correlate positively with cancer severity (Abercrombie, Giese-Davis, Sephton, Epel, Turner-Cobb, and Spiegel, 2004) and mortality rate and recurrence of breast cancer (Sephton, Sapolsky, Kraemer, and Spiegel, 2000). Sephton *et al.* (2000) reported that flatter slopes of cortisol were linked to fatigue amongst breast cancer patients and even predicted shorter life-span, and was completely independent of other prognostic factors. Ambercrombie *et al.* (2004) studied women with metastatic breast cancer and found similar results to Sephton *et al.* (2000) such that women with metastatic breast cancer were more likely to show flatter cortisol slopes compared to the healthy control population. Ambercrombie *et al.* (2004) postulated that such abnormalities could be a result of the Hypothalamic- Pituitary- Adrenal (HPA) feedback system malfunction, hypersensitivity towards stress, inability to inactivate cortisol, or even sleep irregularities.

Kagaya, Okamura, Takebayashir, Akechi, Morinobu, Yamawaki, & Uchitomi (2003) studied psychological problems and cortisol concentrations in patients with breast cancer throughout their treatment. Participants were assessed for a variety of disorders including, but not exclusive to depression-dejection, and tension-anxiety. Kagaya *et al.* (2003) found 35% of participants had elevated cortisol levels throughout the course of their treatment, but this was not dependent upon the psychiatric disorders found within group. Since the heightened levels of cortisol did not appear to be correlated with the psychiatric disorders, Kagaya *et al.* (2003) hypothesized that this was related to the breast cancer itself.

Chronic stress may result in a more sensitive HPA response to stress over-time. Spiegel, Giese-Davis. Taylor, & Kraemer (2006) looked at the 'pathobiology' of specific cortisol concentrations taken throughout the day in women with metastatic breast cancer. Spiegel et al. (2006) hypothesized that flatter cortisol levels would be associated with an inability to suppress cortisol assessed through a dexamethasone (DEX) suppression test. hyperactivity of Adrenocorticotrophin Hormone (ACTH), as well as a higher sensitivity to stress. Participants included metastatic breast cancer patients undergoing treatment. Cortisol was measured through saliva samples taken on 2 successive days at 5 specific time points throughout the day. waking, 30 minutes after waking, noon, 5 and 9 pm. Spiegel *et al.* (2006) found an increasing daytime cortisol slope with increased cortisol concentrations 30 minutes post waking and this trend continued and was highly associated with flatter cortisol slopes throughout the day. Furthermore, participants who had a higher level of cortisol consistently throughout the day did not reveal changes during the administered stress measures. Flatter day time cortisol slopes were also

correlated with HPA failure to suppress cortisol levels via the DEX suppression test suggesting dysfunction of the negative feedback system within the HPA.

Turner-Cobb *et al.* (2000) predicted that the higher the quality and quantity of social support given would be more correlated to normal endocrine levels of cortisol. The researchers hypothesized that a greater social network and better quality of support would correlate with lower cortisol levels, and that greater social networks and quality of support would correlate with a steeper diurnal slope of cortisol. Participants with metastatic breast cancer were assigned at random to either the psychosocial treatment or the educational control group. All participants were required to complete baseline questionnaires that assess perceived social support, the quality of social support, as well as measures assessing perceived available as well as quality of support prior to treatment. Participants were asked to collect saliva samples at 8am, 12pm, 5pm, and 9pm on 3 successive days. Turner-Cobb *et al.* (2000) found a significant negative correlation with positive appraisal, belonging, and support with respect to cortisol levels. Turner-Cobb *et al.*, (2000) found no support for their hypothesis for slope of cortisol with respect to quantity, but did find statistical significance for quality of support received. These results are also comparable to other studies measuring other endocrine and immune responses where social support was highly correlated with proper immune functioning (Turner-Cobb *et al.*, 2000).

Despite multiple studies consistently reporting similar findings, there are studies that cannot replicate them or found different results altogether. Recently, Palesh, Butler, Koopman, Giese-Davis, Carlson, & Spiegel (2007) suggested that women who have

endured stressful or traumatic events, or perceive these events to be particularly stressful or traumatic in their lifetime have a decreased ability to resist tumour formation. Although finding some significant difference in cortisol levels when comparing cancer patients and controls with respect to perceived psychological-social support, Palesh *et al.* (2007) could not find any significant differences between participants who experienced or perceived only stressful events compared to those who only experienced or perceived traumatic ones. Because there were no differences found between traumatic and stressful events and cortisol levels, these results do not clearly link breast cancer and cortisol.

Vedhara, Tuin stra, Miles, Sanderman, & Ranchor (2006) compared breast cancer patients with healthy controls using 4 different indices that are widely used amongst the scientific community: $AUC_{g,}$ AUC_{i}, EPQR-S, and HADS in regards to studying breast cancer and the possible role that cortisol may or may not have. The first 2 are indices highly used to measure cortisol whereas the latter 2 are indices that are used to measure distress. Participants were required to take numerous saliva samples at specific times on 2 successive days that were then analyzed for cortisol levels using a variety of methods. It was revealed that the reliability between measurements was very low, producing low validity. Furthermore, Vadhera *et al.* (2006) could not find distinct differences when comparing breast cancer patients to normal controls. Interestingly, Nunes, Rodriguez, da Silva Hoffmann, Luz, Filho, Miller, & Bauer (2007) compared breast cancer patients receiving 'radiotherapy' as well as receiving information and treatment for stress relief to breast cancer patients only receiving 'radiotherapy treatment. Within a 24 day period Nunes *et al.* (2007) found that relaxation techniques helped to lower psychological

problems such as stress, anxiety, and depression but found cortisol levels to not be dependent on such therapeutic aids.

Cortisol and Cancer: Clinical data limitations

Despite the advancement in clinical research, data remains controversial making conclusions difficult to infer and producing low reliability between studies. The majority of the problems in regards to clinical data in relation to support systems and cortisol are due to methodology. Variables such as how cortisol samples were collected, the time of day cortisol levels were assessed at, what type of support was given, participant differences, in addition to integrating compounding variables such as other medications the patient may be taking all are contributing factors to why the data is not clear. Additional caveats to the current *in vivo* clinical studies conducted on this topic include time discrepancy allotted for testing; the majority of studies collect data for less than or up to a year making it hard to generalize long-term information. There are also confounding issues inherent to dealing with human populations; for support studies even if participants receive identical support, the support given may not be perceived as such by each individual. Many of the studies rely on the participants' ability to measure their own cortisol levels making researchers highly dependent on the participant's willingness, accuracy and ability to accurately record data. Cortisol is also extremely tissue-specific and can have various outcomes depending on where it is located within the body (Ashwell, Lu, and Vacchio, 2000) making any specific conclusions to the role of cortisol in breast cancer very non-specific. It is therefore, extremely important to determine the

cellular effects of cortisol and to clearly resolve the underlying molecular mechanisms of cortisol in both normal breast growth and development as well as in breast tumorigenesis.

Physiological Role of Cortisol in a Healthy Individual:

The effects of cortisol throughout the body range tremendously, many are important for maintaining homeostasis as well as preparing the organism to react and manage physical and psychological stress. Stress can be defined as many things including, but not exclusive to exercise, emotional disturbances, trauma, haemorrhage and fever (Ashwell, Lu, & Vacchio, 2000). Cortisol promotes carbohydrate and protein disassembly as well as exerts numerous, complicated effects on fat distribution as well as breakdown. Cortisol is imperatively involved with immune and inflammatory processes; which are crucial for many actions involved with body immune defence mechanisms. It is within these defence mechanisms that it can be observed that cortisol functions to protect the host from stress whereby halting the pathophysiological responses that the body undergoes in the event of tissue damage and inflammation. Cortisol, in healthy cells is an anti-inflammatory causing apoptosis through the mitochrondrial pathway requiring Apaf-1 and caspase-9. Cortisol is capable of indirectly increasing blood pressure by affecting the tissue-sensitivity to catecholamines. Its synthesis also affects bone formation, and can display both positive and negative effects on cellular proliferation and cell death. Other effects consist of mood and behavioural changes; decrease/increase in food intake, body temperature, sensitivity towards pain as well as problems with neuroendocrine functioning (Buckingham, 2006).

Cortisol Synthesis:

Cortisol is the principal glucocorticoid in man. Cortisol is released into the blood stream in a pulsatile nature and concentration increases or decreases; which is reflective of the time of day. Cortisol levels are approximately 3-5 times higher in the early morning hours prior to waking and are lowest early in the sleep cycle. These concentrations can spike in a matter of moments in response to physical or psychological stress. This 'stress-response' will override the normal circadian function of cortisol and will vary depending on the severity and longevity of the stressor (Buckingham, 2006).

Cortisol synthesis first commences within the hypothalamus with the secretion of corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) by the parvocellular neurons within the paraventricular nucleus. Traveling through the hypophyseal-portal blood vessels, CRH and AVP reach and bind to the type 1 corticotrophin-releasing hormone receptor and the type 1b vasopressin receptor, respectively, of the anterior pituitary gland. This causes the release of adrenocorticotrophic hormone (ACTH) from the corticotrophin producing cells into blood circulation (Buckingham, 2006). ACTH circulates throughout the body and binds to type 2 melanocortin receptors which trigger the synthesis of corticosterone/cortisol within the zona fasciculate of the adrenal gland (Buckingham, 2006). Cortisol synthesis begins with the cleavage of the side chain of cholesterol by enzyme p450scc which creates pregnenolone. Pregnenolone undergoes hydroxylation at carbon 17 by cytochrome enzyme p450c17, leading to 2 potential pathways for cortisol synthesis: the inactive form cortisone, and the active form cortisol. Within the endoplasmic reticulum, pregnenolone undergoes another hydroxylation by cytochrome enzyme p450c21 creating 11-deoxycorticosterone, the inactive form of cortisol. 11-deoxycorticosterone is converted

into active cortisol by p450c11 within the mitochondria (Ashwell *et al.*, 2000). Because cortisol is lipophilic, it diffuses instantaneously through diffusion into the blood stream as part of a protein complex consisting of the high-capacity, but low affinity protein albumin as well as the low-capacity high affinity protein, corticosteroid-binding globulin (CBG) (Ashwell *et al.*, 2000). This in turn, acts as a negative feedback system whereby cortisol, once in the bloodstream, reaches the hypothalamus and pituitary glands and suppresses the production of CRH, AVP, and ACTH. The sensitivity of this negative feedback system is extremely dependent on the levels of cortisol already present in the bloodstream (Buckingham, 2006).

Ninety-five percent of cortisol in the bloodstream is bound to CBG and the remaining 5% of 'free cortisol' is able to diffuse through the membrane to exert cortisol-mediated effects. The amount of free cortisol capable of binding to the glucocorticoid receptor (GR) at any given time in a healthy individual ranges from 0.5-1 nM, but can decrease approximately 20-30% within the early stages of sleep. However, in cases of intense physical stress, such as inflamed tissue, local serine proteases are capable of releasing the bound cortisol from the CBG, thereby increasing free cortisol levels, in some cases this is found to exceed levels higher than 50 μM (Buckingham, 2006). Variations in cortisol secretion depend on concentrations already present within the bloodstream, the time of day, and any perceived stressors occurring, either physical and or psychological. Cortisol levels over the course of the day ranges anywhere from 3 to 10 times higher the basal concentration (Ashwell *et al.*, 2000).

To date, 2 unique isoforms 11β-hydroxysteroid dehydrogenase (HSD), with differing actions have been found: HSD type 1 and HSD- type 2. Type 2 is found to be co-expressed with the mineralcorticoid receptor (MR) in the kidneys, parotid and sweat glands, as well as smooth muscle tissue. It has also been found within the developing brain as well as the placenta, and is thought to act as a protective agent against the possibly harmful effects of cortisol surplus (Buckingham, 2006). 11β-HSD1's role is to stimulate inactive cortisone to its active counterpart, cortisol. 11β-HSD1 is found predominately within the liver, adipose tissues and its regulation is sensitive to numerous factors including cortisol, stress, and cytokines. It has been shown to be capable of bidirectional function *in vitro* acting as both a dehydrogenase and reductase (Buckingham, 2006). 11β-HSD1 is found where the GR is abundant. but it demonstrates low affinity for cortisol. It has been suggested that this reflects that the main role of 11β-HSD1 is to generate and increase the local amount of 'free cortisol' where cortisol is critical in endocrine functioning (Buckingham, 2006). The number of GR present within normal and cancerous breast tissue is extremely correlated with the enzyme 11β-HSD1.

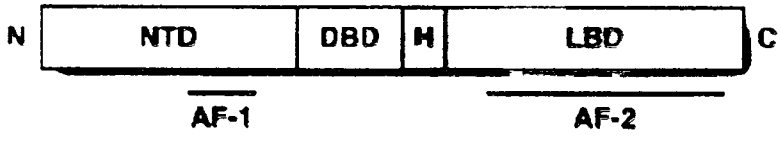
Steroid Receptor Family

There are 48 members within the nuclear receptor superfamily. Within this family are many sub-groups. Members of the steroid superfamily include the GR, MR, progesterone receptor (PR), 2 oestrogen receptors (ER), and androgen receptor (AR) (Faus and Haendler, 2006). All steroid receptors share common characteristics such as a highly conserved centrally localized zinc-finger DNA binding domain (DBD), a less conserved carboxyl-terminal ligand binding domain (LBD) (Heitzer, Wolf, Sanchez,

Witchel, & DeFranco, 2007). The amino terminal domain (NTD) differs between steroid receptors except to the abundant negatively charged acidic amino acids. This region is often referred to as the activation function 1 (AF-1) and functions in transcriptional regulation that is not necessarily independent of ligand binding (Heitzer *et al.*. 2007).

GR Gene and Protein Structure:

The GR gene contains 9 exons capable of alternative splicing, which allows for variations of the GR to be observed. The isoforms most commonly studied, due to their relative abundance, are GRα, and GRβ (Lu and Cidlowski, 2006). These isoforms differ in their C-terminal region beginning at amino acid 727 where GRα has an additional 50 amino acids, but GRβ only has 15. these residues share no apparent identity. It is known that glucocorticoids predominately arbitrate their effects via the GRα. The GRα protein is a single 777 amino- acid structure that resides within the cytoplasm of the cell bound to heat shock proteins (hsps) 90, 70. 50, and 20 (Buckingham, 2006). This maintains the integrity of the GR by facilitating proper folding as well as preventing the GR from binding to the glucocorticoid regulatory elements (GREs) in the absence of cortisol (Heitzer *et al.*, 2007) GRs can be found in almost every cell type within the human body. and comprise an approximate 200 to 30, 000 per cell where the affinity for cortisol around 30nM (Adcock, Caramori, and Ito, 2006). The GR is divided into 4 distinct sections as illustrated below:



(Heitzer *et al.*, 2007)

The N-terminal AF-1 region, containing imperative transactivational functions. This region has been shown to interact directly with basal transcriptional machinery as well as other cofactors that contribute in transcriptional regulation; moreover, interference of AF-1 decreases reporter gene expression (Heitzer *et al.*. 2007). There is also an important DNA binding domain (DBD) comprised of 2 zinc fingers and a hinge region housing the nuclear localization signal as well as the ligand binding domain (LBD) with supplementary transactivation functions. The LBD, also known as AF-2, constitutes 12 alpha-helices that go through broad conformation changes post ligand binding (Faus and Haendler, 2006). The LBD remains hidden within the GR while it is associated with hsp90, and contains sequences for protein-protein interaction with hsp90. Unlike AF-1, AF-2 is completely dependent on glucocorticoid binding; which allows AF-2 to undergo a conformational change enabling co-activators or co-repressors to contribute in transcriptional activation. Furthermore, AF-2 can act in unison with AF-1 to facilitate transcriptional activity (Heitzer *et al.*, 2007).

The most impressive structure within the GR is the C-terminal α-helix, which folds over enclosing the hydrophobic pocket where the ligand has bound. This folding allows for cofactors to recognize the active GR complex which is essential for transcription machinery to be recruited for the activation of target genes (Faus and Haendler, 2006).

As referred to above, it is this region that differs between GRα and GRβ. Due to these differences, GRα is activated via ligand binding while GRβ is not capable of ligand binding, hence glucocorticoids demonstrate their effects through the GRα (Moutsatsou

and Papavassiliou, 2008). In addition to differences in the mechanism of activation between these two receptor subtypes, these receptors are distributed differently. Both are expressed throughout the body, but GRα is expressed at high levels while GRβ is expressed at very low levels. QRT-PCR as well as western blot examination revealed that it is the GRα, and not GRβ that is expressed in the breast (Moutsatsou and Papavassiliou, 2008).

GR Signalling:

Glucocorticoids bind to the GR via passive diffusion through the cellular membrane due to their 'lipophilicity', however, it has been reported that even glucocorticoids might actually use a membrane transporter in order to move into the cytoplasm of the cell (Tasker, Di, Malcher-Lopes, 2006). The GR is reliant on 3 factors that are essential for GR activation: the amount of cortisol available for binding, the receptor, as well as the recruitment of other transcription factors and proteins. Functioning as a monomer, the GR can bind and inhibit proinflammatory transcription factors (TFs) such as NF-kB and AP-1, nongenomically (Adcock *et al.*, 2006). As a homodimer, the GR complex is capable of shuttling into the nucleus and binding to the specific DNA elements, GREs within the promoter regions specific to proinflammatory gene targets thereby blocking gene expression.

Genomic activity of the GR requires a 'conformational change' upon binding cortisol whereby the hsps are removed (Moutsatsou and Papavassiliou, 2008). In addition to binding GRE elements directly, the active GR complex can also associate and interact with other DNA bound transcription factors to regulate transcriptional effects (Heitzer *et*

al., 2007). A proper GR-DNA complex with other proteins regulates the communication of other co-activator complexes on specific GR target promoters. The GR cannot recruit all cofactors that are necessary around the target promoters, but with the aid of steroid coactivators that are recruited by the GR, this enables the recruitment of other coactivators as well as chromatin remodelling complexes to form the transcription initiation complex allowing for local chromatin remodelling (Ito, Chung, & Adcock, 2006).

GR-GRE recruitment can result in an increase or a decrease of GRE-dependent gene transcription (Moutsatsou and Papavassiliou, 2008). Furthermore, corticosteroid binding to the GR transcriptionally activates the expression of ribonucleases as well mRNA destabilizing proteins which target anti-inflammatory genes, thereby indirectly regulating the expression of genes. From a therapeutic standpoint however, the most well studied action of the glucocorticoids is to repress the transcription of cytokines and chemokine genes implemented in inflammation (Adcock *et al.*, 2006). GR activation also results in activation of many genes implicated in metabolic homeostasis such as raising blood glucose levels, gluconeogenesis, as well as the recruitment of amino and fatty acids (Heitzer *et al.*, 2007).

GR and Receptor Cross-talk

Evidence has shown that GR signalling engages in considerable cross-talk and promiscuity between the other steroid receptors, oestrogen and progesterone. Glucocorticoids and progestins bind to their specific receptor, however, Fryer, Nordeen, & Archer (1998) demonstrated that anti-progestins which inhibit the cell from utilizing or

synthesizing progesterone, inhibited GR-mediated transcription in breast cancer cells. Furthermore, Wan and Nordeen (2003) revealed that glucocorticoid- like effects of progesterone have been shown in some tissues, while progesterone-like effects of glucocorticoids in other tissues have also been observed (Moutsatsou & Papavassiliou, 2008). Breast cancer cells expressing PR, but not the GR were utilized to demonstrate that cortisol mimicked the effects of progesterone by significantly inhibiting growth, cell spreading and focal adhesions (Leo, Guo, Woon, Aw, and Lin, 2004). In contrast, PR-negative but GR-positive breast cancer cells, DEX, a synthetic of cortisol, resulted in an increase of focal adhesion and cellular proliferation (Moutsatsou & Papavassiliou, 2008). Given that GCs and progestins are used in breast cancer treatment strategies, having a clear understanding of the molecular biology mediating these effects in both PR-positive and PR-negative breast cancers is essential.

As mentioned above, GR can interact with NF-kB nongenomically; this may play an essential role in mediating the effects of GR in breast cancer cells. NF-kB becomes activated through TNF-α, which increases gene transcription of anti-apoptotic properties, but this may lead to cell sensitivity of TNF-α induced cellular death. TNF-α can activate both apoptosis as well as cell survival depending on signalling activation (Moutsatsou & Papavassiliou, 2008). Ozes, Mayo, Gustin, Pfeffer, and Donner (1999) have shown that an activated NF-kB through cellular survival genes PI3K or Akt suppress TNF-α induced cellular death in the human breast cancer cell line, MCF7. The importance of GR interactions with NF-kB in breast cancer cells is not well explored.

Cortisol and Cell Cycle Progression

GR-signalling exerts many effects including: cell growth arrest, apoptosis, as well as differentiation to name a few. The underlying mechanisms involving these effects are of high interest to the research field. Cell division occurs through 2 general stages: DNA replication (S phase) and division of the replicated DNA into 2 daughter cells (mitosis; M phase). Before DNA replication can occur there are growth phases the cell must go through in order to ensure that the cell has sufficient nutrients and is without damage prior to proceeding into S phase, this is referred to as Gap 1 (G1) of the cell cycle (Vermeulen, Bockstaele, and Berneman, 2003). Cells are in this phase of the cell cycle for the longest period of time, and the majority of all asynchronous cells will be found at this stage. Should the environment be inadequate to support DNA synthesis the cell will enter a resting state, referred to as G0 or quiescence, where it will remain until conditions are favourable. However, if conditions are favourable and the cell commits to DNA replication, thereby passing a G1-S restriction point, the cell cannot enter G0 at this stage and DNA synthesis will ensue. Following completion of S phase the cell enters Gap2 (G2) of the cell cycle, a stage where the cell prepares for mitosis and ensures that DNA synthesis has been successfully completed without error prior to entry into mitosis (Vermeulen *et al.*, 2003). These phase transitions are controlled by important catalytic proteins known as cyclin-dependent kinases (CDKs), which are ubiquitously expressed but are activated at specific times during the cell cycle to transition a cell through each specific phase. A total of 9 CDKs are known, but only 4 are characterized to function as described to regulate the cell cycle: CDK 2, 4 and 6 function during G1; CDK2 is also functional during S phase, and CDK1 is the sole CDK regulating movement through G2 and M phase in mammals. The regulatory proteins that activate CDKs during specific cell

cycle phases have been coined cyclins. Cyclin D1, 2, and 3 bind to both CDK4 and 6 allowing the cell to enter into G1. Cyclin D is a unique cyclin in that the protein levels do not rise and fall with the cell cycle instead it is synthesized when growth factors are present. Cyclin E binds to CDK2 which allows the cell to transition from G1 into S phase; it is within this phase that cyclin A binds to CDK2, and is required for the cell to successfully transition into G2. It is within the end of G2 phase and the beginning of the M phase where cyclin A binds to CDK1 to trigger the cell into M phase. Mitosis proceeds with the binding of cyclin B to CDK1 and movement of this complex into the nucleus to phosphorylate substrates required for the stages of mitosis and cytokinesis (Vermeulen *et al.*, 2003).

CDK function can be inhibited by proteins known as CDK inhibitors (CKI) that bind either to the CDK directly, rendering the CDK incapable of interacting with its specific cyclin, or they bind to the CDK-cyclin complex resulting in inactivation of the complex (Vermeulen *et al.*, 2003). These inhibitors are classified into 2 primary families: the INK4 and Cip/Kip families. The INK4 family consists of $p15^{INK4B}$, 16^{INK4A}, 18^{INK4C}, and 19^{INK4D} that target inactivation of CDK4 and 6 preventing cyclin D binding within the G1 phase of the cell cycle. The Cip/Kip family consists of $p21^{CIP1}$, 27^{KIP1}, and 57^{KIP2} that target inactivation of CDK-cyclin complexes. Hsu and Defranco (1995) have inferred that GR signalling might be involved in cell cycle regulation. The researchers found that GR signalling stimulated transition from G1 through S phase and they postulated that the cell cycle is more sensitive to glucocorticoids in G1/S than any other phase of the cell cycle. However, Abel, Wochnik, Ruegg, Holsboer and Rein (2002) have suggested that GR

signalling is indeed fully operational throughout the entire cell cycle, however due to chromatin condensation GR signalling becomes repressed within G2/M phase.

Cortisol and Microarray Analysis

The invention of the microarray has revealed some remarkable and abundant genes that are regulated by glucocorticoids (Buckingham, 2006). However, the use of microarrays has created many new questions, and has answered few. As expected, microarray studies have targeted genes involved in inflammation, immunity, cellular proliferation, cellular death, differentiation, as well as endocrine function (Buckingham, 2006). Interestingly, there are numerous genes that cannot be placed within these categories or their function is still unknown. Adding a further complication, microarray studies have also revealed that different cells types, differences in cortisol concentration and formulation and timing of delivery all reveal differences in the regulated genes (Buckingham, 2006). Kauppi, Jakob, Famegardh, *et al.* (2003) utilized a microarray with a lung epithelial cell line treated with DEX and in the presence of cyclohexamide to isolate genes transcriptionally regulated by cortisol. This study demonstrated that DEX can up-regulate 108 genes as well as downregulating 73 after only 6 hours treatment. Many of the other regulated genes were categorized under proliferation, apoptosis, inflammation, as well as surfactant synthesis. At the very least, microarray studies have demonstrated how remarkable and complex the system of glucocorticoid action truly is; with the ability to orchestrate a wide range of control through the physiological as well as the pathological system (Buckingham, 2006).

Normal Breast Development; Links to Carcinogenesis.

The mammary gland is a unique organ in that it undergoes the majority of development post-embryonically. At puberty (approximately day 21 in the mouse) the adult mammary stem cells undergo a burst of proliferation, as well as degrading of the extra cellular matrix (ECM) to form the rudimentary ductal tree. Surges of hormones at the onset of pregnancy induce yet another wave of proliferation to establish the alveolar epithelium necessary to produce milk proteins during lactation. Mid-pregnancy, approximately day 10 pregnancy, the gland begins preparing to undergo functional differentiation by altering the morphology of the gland as well as initiating gene expression necessary to synthesize milk proteins. Upon the birth of the pups the gland ceases to proliferate and the alveoli begin to secrete milk. Genes regulating proliferation represent potential targets that may be aberrantly regulated in breast cancer to support uncontrolled proliferation. Similarly, genes regulating differentiation might represent tumour suppressor genes that may be mutated or down-regulated in carcinogenesis. Indeed, understanding normal breast development has revealed critical regulators of specific forms of breast cancer such as c-Myc (Butt, Caldon, McNeil, Swarbrick, Musgrove, & Sutherland, 2008), ras (McCubrey, Steelman, Chappell, Abrams, Wong , Chang, Lehmann, Terrian, Milella, Tafuri, Stivala, Libra, Basecke, Evangelisti , Martelli, & Franklin, 2007) and ErbB2/Neu (Murphy, Millar , & Lee, 2005). It is known that abnormal morphology of the ECM is highly prevalent in metastatic breast cancers (Lee, Hwang, Mead, and Ip, 2001).

Little is known about cortisol and normal breast development. The literature suggests that glucocorticoids promote expression of lactating proteins, casein and lactalbumin (Ono and Oka, 1980). Interestingly, looking at 11β-HSD, the enzyme

responsible for inactivating glucocorticoids, levels were high in both virgin and pregnant rat mammary cells, but 11β-HSD ability to inactivate glucocorticoids was decreased by 75% during lactation (Quirk, Slattery, and Funder, 1990). It has also been shown that cortisol promotes morphology changes in the normal mammary epithelial cells (MECs) such as 'alveolar and multilobular branching' (Darcy, et al., 1995). The essential GR-responsive genes responding to cortisol during normal development have not been isolated.

Cortisol and Breast Cancer: Molecular Evidence

The GR has been found in a large number of primary human breast cancers. Abberant GR expression is seen in breast cancer stroma, particularly at more serious stages of tumour development (Moutsatsou and Papavassiliou, 2008). Lien et al., (2006) looked at GR expression levels using immunohistochemistry in 400 human breast tissue samples ranging from normal tissue, to invasive lesions. Studies indicate that approximately 20% of breast cancer profiles show the primary cause is due to mutations in BRCA1 and 2, TP53, PTEN, and ATM (Rosman, Kaklamani, Pasche, 2007) with an additional number of genes contributing to the overall survival of the patient (Oldenburg, Meijers-Heijboer, Cornelisse, Devilee, 2007). Psychosocial as well as psychological stress have also been correlated as causative factors (Bleiker and van der Ploeg, 1999). Previous work performed by Holden, Pakula, & Mooney (1998) revealed prolonged stress increased the amount of TNF in a variety of organ carcinomas. Another theory has been suggested whereby stress increases the chance of breast cancer as well as the chance of overcoming this disease by decreasing the ability of a cell to respond to DNA damage or to even apoptose. Numerous studies in mice as well as humans reveal significant

differences in a cell's ability to repair DNA damage or to undergo apoptosis while under stress compared to cells not under stressful conditions (Glaser, Thorn, Tarr. Kiecoit-Glaser, D'Ambrosio, 1985; Fischman, Pero, & Kelly, 1996; Cohen, Marshall, Cheng, Agarwal, Wei, 2000; Amsterdam and Sasson, 2002). To date, there is no model put forth that explains these findings. It is possible to consider a model where cortisol may change the ability of proteins that are responsible and pivotal in DNA repair and apoptosis to be hindered. Antonova and Mueller (2008) revealed that BRCA1 within the mouse healthy mammary gland cell line, EPH4, was down-regulated by cortisone, the active form of cortisol (270nM). These authors suggest that these observations could shed light on a molecular pathway for experimental manipulation to study possible alterations in DNA repair mechanisms and cell survival during stressful times. By enabling the tumour suppressor role of BRCA1, cortisone may affect the ability of cells to preserve genomic stability and may even assist the cells' alterations to reveal carcinogenic properties. Previous reports have shown that expression levels of BRCA1 increase during cellular proliferation in the mammary gland (Gudas, Li, Nguyen, Jensen, Rauscher, Cowon, 1996) Antonova and Mueller (2008) were curious if cell proliferation hinders the normal repressive effects of cortisone on BRCA1 promoter activity and found that cortisone treated cells completely repressed the BRCA1 promoter. It is also known that oestrogen enhances the BRCA1 expression in proliferating cells (Romagnolo, Annab, Thompson, Risinger, Terry, Barrett, & Afshari, 1998). To establish whether oestrogen-induced BRCA1 expression prevents the repressive capabilities of cortisone, Antonova and Mueller (2008) treated EPH4-L6 cells with 10nM oestrogen with and without cortisone at a concentration of 1μg/mL. Predictably, oestrogen increased expression of BRCA1

promoter without cortisone. With the addition of cortisone, the BRCA1 promoter was repressed despite oestrogen treatment. This group concluded that cortisone can abolish the promoter activity of BRCA1 by oestrogen.

This Masters project focuses on the underlying molecular mechanisms of cortisol's potential role in breast cancer initiation and progression. Given all clinical and molecular evidence described we hypothesized that cortisol functions through the glucocorticoid receptor to mediate effects on cell growth, adhesion and migration properties in both normal and cancerous breast cells. Specific aims of this project were to determine the effects of cortisol on cell proliferation, as well as migration and invasion, in a panel of cancerous and normal breast cells and to evaluate the pathway of cortisol's mediated function in invasive breast cancerous cell lines.

METHODOLOGY:

Cell Culture.

Human mammary breast cancer cells, MCF7 (gift from Tiffany Seagroves, U. of Tennessee), HTB-26 and HTB-132 (graciously donated by Cindy Zahnow, John Hopkins), normal mouse epithelial cells, HC11 (graciously donated by Carrie Shermanko, U. of Calgary), and normal human breast cells HTB-125 (ATCC) were maintained in RMPI-1640 medium (Sigma) or Dulbecco's Modified Medium (DMEM) (HTB-125, HTB-26, -132) containing 2mM L-glutamine (Sigma), penicillin (Invitrogen), streptomycin (Invitrogen) and were cultured in a 5% CO_2, 37°C environment. MCF7 cells were supplemented with 10% (vol/vol) new born calf serum (Sigma) and HTB-26, HTB-132, HC11, HTB-125 cells were supplemented with 10% fetal bovine serum. HC11 cells

were supplemented with 10ng/mL epidermal growth factor (Invitrogen). Additionally, HC11 cells were supplemented with 5ug/ml insulin (Sigma). Normal human breast cell line MCF-10A (graciously donated by Tiffany Seagroves, U of Tennessee) were maintained in DMEM/F12 medium supplemented with 5% horse serum, 10μg/mL hydrocortisone, 10μg/mL insulin, 20ng/mL epidermal growth factor. Receptor status for MCF7, HC11 and MCF-10A GR, ER, and PR positive. Cell lines HTB-26,HTB-132, and HTB-125 are ER negative, GR and PR positive.

Reagents.

Hydrocortisone, 98% (Cortisol, Sigma) was dissolved in Dimethyl sulfoxide, 99.5% (DMSO, Sigma) at 0.362g/10mls for stock solution. Further dilutions included a 1:1000 ratio stock to cell culture media to obtain working experimental concentrations within the range of cortisol levels observed from *in vivo* data. Experimental control, DMSO, was used per volume comparison for cortisol.

Tamoxifen (TAM, Sigma) stock solution was previously dissolved to a 10 mM concentration, and diluted to obtain various experimental concentrations. Mifepristone minimum, 98% (RU486, Sigma) was dissolved into a 1M stock solution at 0.42959g/1 ml DMSO. Trypan Blue Solution 0.4% (Sigma # T8154) was used for proliferation assays. Thiazolyl Blue Tetrazolium Bromide (MTT), 98% (Sigma #M2128) was dissolved in PBS at 5mg/mL and used in proliferation assays. Extraction buffer for MTT consisted of 50mL N, N- Dimethylformamide, 99% (Sigma), 10g of Sodium Dodecyl Sulfate (SDS), 2.5mL glacial acetic acid, 2.5mL HCl, 30mL H20, adjusted to ph of 7.4.

Cell Proliferation.

Cells were removed via trypsinization, and a 50 μL sample of cells was diluted 1:2 using a Trypan Blue stock solution. A Bright-Line hemacytometer (Fisher Scientific) was used to obtain an estimated average of available amount of cells present. Calculation for estimated number of cells used is: # of cells counted/ 4 = XX. (XX)(2 x10^4)(# mL used to remove cells) (Peraino and Eisler. 1973). A total of 3 separate samples from each cell line utilized were counted to ensure highest accuracy, and an average from these 3 separate counts was used in calculating cellular numbers per ml of media for the experimental plates. Cells were plated at 4x10^6 per 3ml of serum-filled media per 60mm tissue-culture plate, and allowed to adhere to ensure normal cellular function. Cortisol was added 24 hours post-seeding with dose concentrations ranging from 0.1 μM to 80 μM. A DMSO vehicle control was utilized using the highest μl volume, and a no treatment control was also incorporated. Experimental plates were incubated and cells were trypsinized at specific time points 0, 2, 4, 6, 12, and 24 hr post-treatment. To obtain statistical data each treatment rendered 3 experimental plates (N= 108). Upon trypsinization, cells were collected and 3 samples from each experimental plate were counted using a blind control with a hemacytometer in triplicate fashion for each time point (N= 54). Tetrazolium Assay (MTT assay) was utilized as a control from the trypan blue exclusion assay. Cells were removed and counted as explained above, and place into a 96 well plate at 5 x10^4 per well. Cells were allowed to adhere to ensure normal cellular functioning. Cortisol was added 24 hours post-seeding with dose concentrations ranging from 0.1 μM to 80 μM. A DMSO vehicle control was utilized using the highest μl volume, and a no treatment control was also incorporated. Each dose was performed in triplicate for statistical analysis. Experimental wells were incubated where 20μL/well of

MTT was added and allowed to incubate for 4 hours at 37°C. Following MTT incubation, 100 μL extraction buffer was added to each experimental wells, wrapped in tin foil and allowed to incubate at 37°C over-night. Data was quantified using the Victor$_3$ 1420 Multi-label Counter (Perkin Elmer) using a 590 nm wavelength filter set.

Migration and Wound healing Assays.

HTB-26 and HC11 cells were removed via trypsinization, counted as described previously, and suspended in a 1x10^6 cellular solution. Migration was assessed using the Cell Migration Assay Cytoselect 24 well (8μm, Colorimetric) (Cell Biolabs, Inc.) following the protocol suggested by the manufacturer with a 24 hr incubation time. Data was quantified using the Victor$_3$ 1420 Multi-label Counter (Perkin Elmer) using a 590nm wavelength filter set.

Wound healing assays were performed where HTB-26 and HC11 cells were grown to confluence on gridded tissue culture plates. Wounds were created with the aid of a pipette tip scratched against the surface. Cortisol at various concentrations or the appropriate DMSO control was added and cells were allowed to incubate for 24 hours. Observations were recorded using the microscope software minisee 1.0.9.37 (Scoptek) in addition to being quantified with the counting of open grids at various time points, 0, 3, 6, 12, and 24 hours.

Synchronization and Cell Cycle Analysis.

MCF7 cells were trypsinized from culture plates, and counted as described previously. Cells were plated at 8x10^4 per 10cm tissue-culture plate and allowed a 24 hr time period for adherence followed by serum-starvation for 24 hours to synchronize the cell

population in G0. Following release into serum media containing 70 ng nocodazole and either 40 μM cortisol or DMSO control, cells were specific time points, 0, 2, 4, 6, 12, and 24 hours. At each time point 100 μl of a 1 mL collected sample was added to 400 μl of cold PBS and 50 μL of a 500 mg/mL propidium iodide stock solution and placed into glass cuvettes for cell cycle analysis via flow cytometry. Data was collected using a Beckman Coulter FC500 (Biology Dept.; U of Windsor) and cell cycle profiles were analyzed using CPX Beckman Coulter FC500 software.

Receptor Inhibition Assay.

MCF7 cells were trypsinized, counted and plated, as described above, onto a 6 well tissue culture plate at 1 x 10^5 per 1.5 mls of media per well and allowed 24 hr to adhere. Various concentrations and combinations of Tamoxifen and RU486 were added to the experimental wells in the presence of 40 μM cortisol or a DMSO control to the MCF7 cell line and incubated for 12 hours. Cells were trypsinized and counted in triplicate for statistical analysis. The receptor inhibition assay was performed in triplicate for statistical analysis.

siRNA Transfection.

HTB-26 cells were trypsinized, counted and plated as described previously onto a 6-well tissue culture plate at 2 x 10^5 per 2 mls of serum-filled media per well and allowed to grow to 80% confluency. Transfection of HTB-26 cells were carried out using an optimized protocol based on that previously outlined by Ehrhardt, Schmoke, Matzke, Knoblauch, Will, Wixler, and Ludwig *et al.* (2006). In brief, the transfection reagent Polyethylenimine (branched PEI) (Sigma-Aldrich; 408727) was used in solution at 1

mg/mL, neutralized with HCl, and sterile filtered. 10 μL of NaCl (150 mM) was combined with 6 μL of PEI stock in tube 1 and 10 μL NaCl (150 mM) and siRNA against the GR (2 μg) (Santacruz; sc-35505) in tube 2. Scrambled siRNA (Sigma, sc-37007) was used as control (2 μg/well). Each tube was incubated at room temperature for 10 minutes and then combined and incubated for an additional 10 min. at room temperature. The transfection reagents were then added to each well drop-wise and incubated for 12 hrs. upon which cortisol (40 μM) or DMSO control was added. 12 hours. Post-treatment with cortisol or DMSO cells were removed via trypsinization and a 50 μL sample was used for cell proliferation assessment utilizing the trypan blue exclusion assay as described above. The remainder of the sample was used for total RNA extraction and converted to cDNA as described below. RT-PCR was performed to confirm GR knockdown as described below.

RT-PCR.

Total RNA was extracted using RNeasy Plus (Qiagen). RNA quality and quantity were analyzed using the RNA 6000 Nano Assay and 2100 Bioanalyzer (Agilent). For endpoint RT-PCR, reverse transcription was carried out using Superscript ™ II Reverse Transcriptase (Invitrogen). Taq PCR (Qiagen) was used for all PCR reactions. All primers were designed using the program Primer Express, and were generated (Sigma) to span exon-exon junctions: Human GR (hGR) forward primer (A201) 5'-CAGCATGCCGCTATCGAAA- 3`, reverse primer (A202) 5`-TAGTGGCCTGCTGAATTCCTT- 3' Human GAPDH (hGAPDH) forward primer (A087) 5'- GCACCGTCAAGGCTGAGAAC- 3', reverse primer (A088) 5'-GGATCTCGCTCCTGGAAGATG-3' Human GAPDH was used as control. PCR cycles

were optimized to ensure that end-point measures were on a log-phase scale, in all blots shown 27 amplification cycles were used. All PCR products were separated on 8% polyacrylamide gel electrophoresis. Gels were visualized on an Alpha Innotech HD2 Gel Documentation System after ethidium bromide staining for 15 minutes using AlphaEaseFC Software.

Q-RT-PCR was carried out using primer/probe sets from ABI; hFRYL (Hs01102973_m1) and endogenous control 18S (Hs99999901_s1). For assessment of the gene FRYL in breast cancer lines, mRNA and cDNA were extracted, as described above, cDNA was added to a specified well in a 96-well Optical Reaction Plate (code 128) (ABI). Assessment of the gene FRYL in breast cancer tissue was performed using Human Breast Cancer TissueScan Real-Time plates (BCRT501; OriGene). In both cases, taqman gene expression master mixes as well as the primer/probe sets were used according to the manufacturer's instruction (ABI). Real Time PCR was performed according to the specification and recommendations from ABI. Q-RT-PCR was done in triplicate. and fold expression values (RQ) were calculated against the normalized control.

Microarray.

HTB-26 cells were plated onto 10 cm plates at 8x10^6 per 8 mls of serum-filled media per plate and allowed to adhere for 24 hours. Cortisol or DMSO control was added and allowed to incubate for 0 and 12 hours. Upon time-point completion, cells were trypsinized and total RNA was extracted using the RNeasy Plus Kit (Qiagen). RNA quality and quantity were analyzed using the RNA 6000 Nano Assay and 2100 Bioanalyzer (Agilent). RNA was packed in dry ice and shipped to the University Health Network microarray facility (Toronto, Ontario, Canada). RNA was converted into cDNA

where cortisol and DMSO control treatment cDNA was labelled with Cyanine dyes (Cy5 and Cy3 dye, respectively) (Amersham Biosciences). Hybridization of both cDNA treatments to a 27K human gene array was then performed on the Advalytix SlideBooster™ (Advalytix, Germany) utilizing DIG Easy Hyb hybridization solution. Flip dye ratio was used as an internal control. The 27K human array chip was scanned on the Agilent G2565BA scanner and quantified with ArrayVision v.8.0 (Imaging Research Inc.). Data was analysed using the LOWESS method of normalization and then through SAM (Statistical Analysis of Microarrays). Gene targets were initially selected based on gene up or down regulation with a minimum of a 6 log increase or decrease. Further screening of gene targets included criteria fitting novelty, relation to breast cancer, and relation to cancer in general.

RESULTS:

Cortisol Stimulates Proliferation in Subsets of Human Breast Cells.

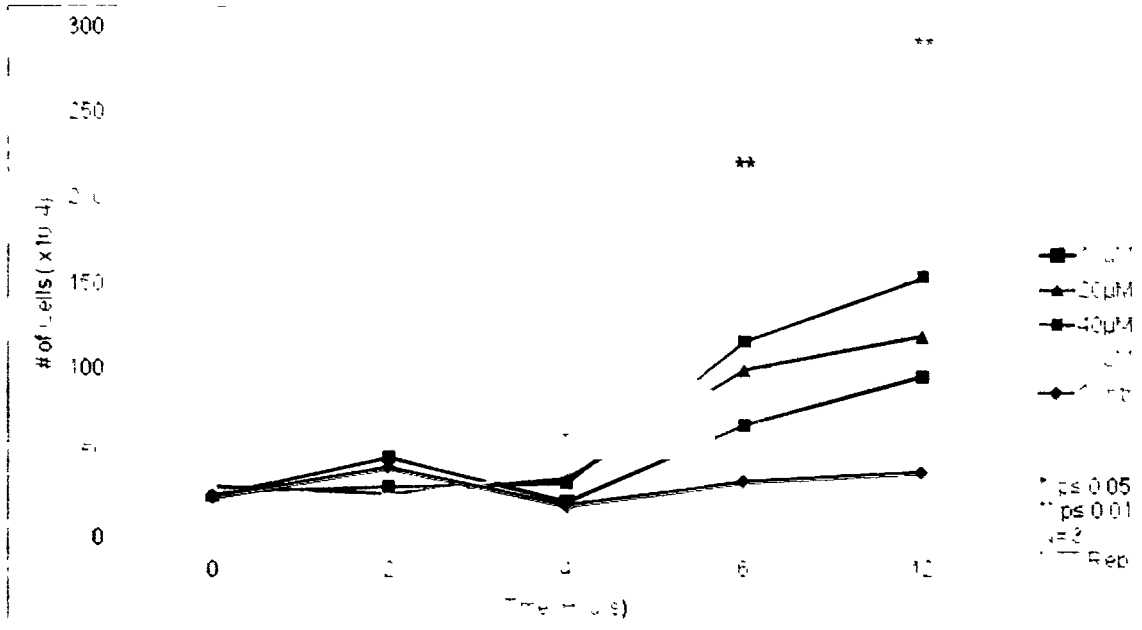
Utilizing various cell lines trypan blue exclusion (Figure **1A**, **D**, and **E**) and MTT assays (Figure **1B**, and **C**) were performed using cortisol or the vehicle control, DMSO. Increased proliferation was seen in the human cancer cell line MCF7 as early as 4 hours with statistical significance reached consistently for all doses post 4 hours ($p \leq 0.05$) (Figure **1A**). Cell proliferation was seen in a dose responsive manner with 2.5 fold increase cell numbers by 12 hours for 80μM. Cortisol stimulates highly invasive human breast cancer cell lines HTB-26 (Figure **1B**) with statistical significance with the 10 and 20 μM concentrations ($p \leq 0.01$), as well as with the invasive breast cancer cell line HTB-132 ($p \leq 0.01$) (Figure **1C**). In Figure **1E** cortisol treatment of the non-cancerous mouse mammary cell line HC11, having a p53 mutation, did not show increased proliferation within the early time points, but did significantly proliferate by time points 12 and 24 hr ($p \leq 0.01$). These results were not found with respect to the non-cancerous human cell line HTB-125 (Figure **1D**) where significance was not reached consistently throughout each time point. Collectively, this data demonstrates that cortisol enhances breast cancer cell numbers and may render susceptibility to breast cells harbouring mutations in p53.

Figure 1. Cortisol Stimulates Proliferation in Subsets of Human Breast Cells.

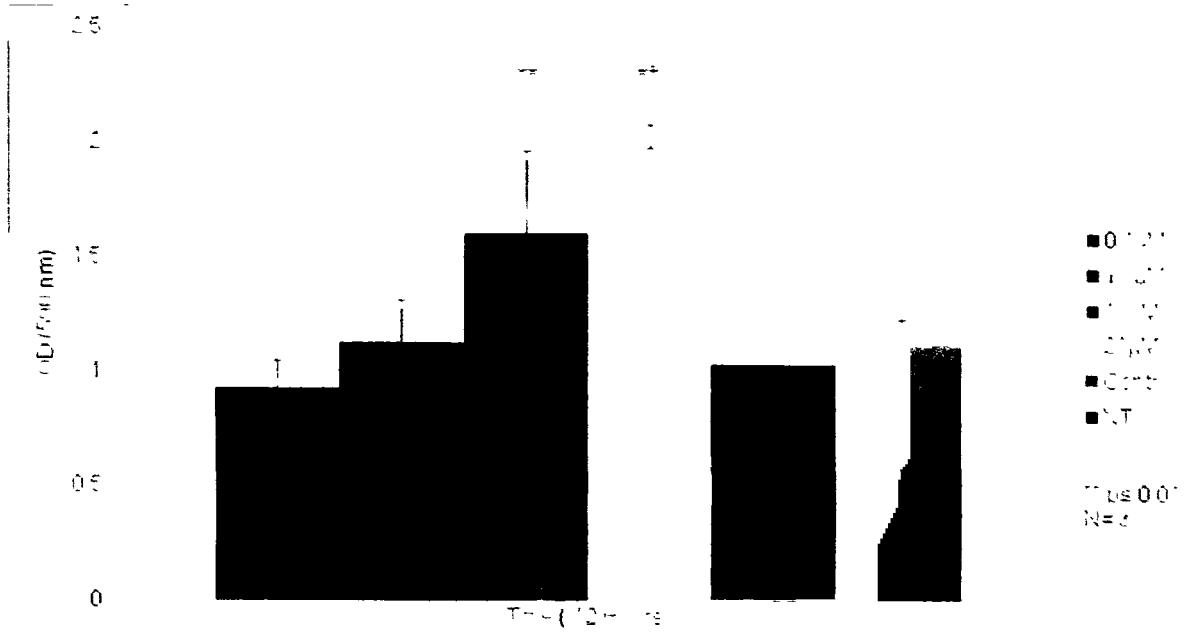
(A) Trypan blue analysis using MCF7 cells was performed in triplicate. Coloured lines represent proliferation over time with various concentrations of cortisol shown as 10μM (■), 20μM (▲), 40μM (■), 80μM ( ), or Control (◆). Control represents the vehicle that cortisol was brought into solution, DMSO. Time was measured in hours as shown on the X-axis, and number of cells counted is reflected on the Y-axis. Results were verified using MTT analysis (MTT Replicated) (MTT Rep.).

(B) Proliferation of human invasive breast cancer cells, HTB-26 was studied using a MTT assay performed in triplicate. Coloured bars represent proliferation with various concentrations of cortisol shown in the legend as 0.1μM (■), 1.0μM (■), 10μM (■), 20μM ( ), Control (■), and NT (■). Control represents DMSO treated cells; No treatment (NT) represents cells lacking treatment with either cortisol or DMSO. Time was measured in hours, and proliferation was measured by absorbance at 590nm as shown on the X and Y axis, respectively.

1A



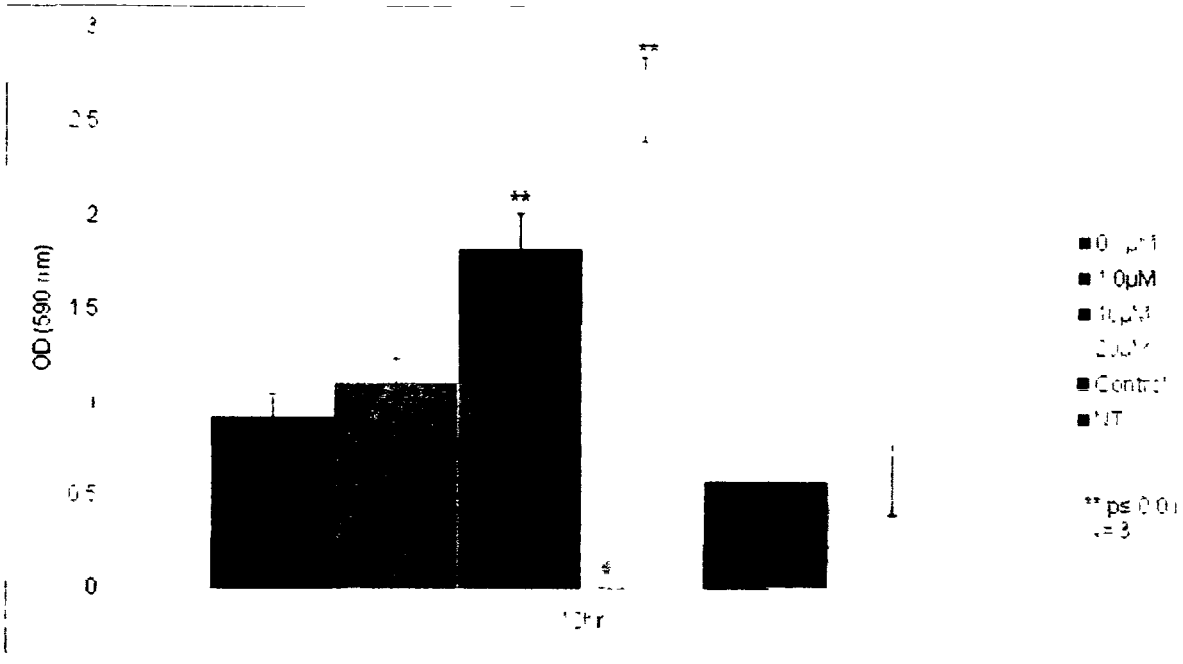
1B



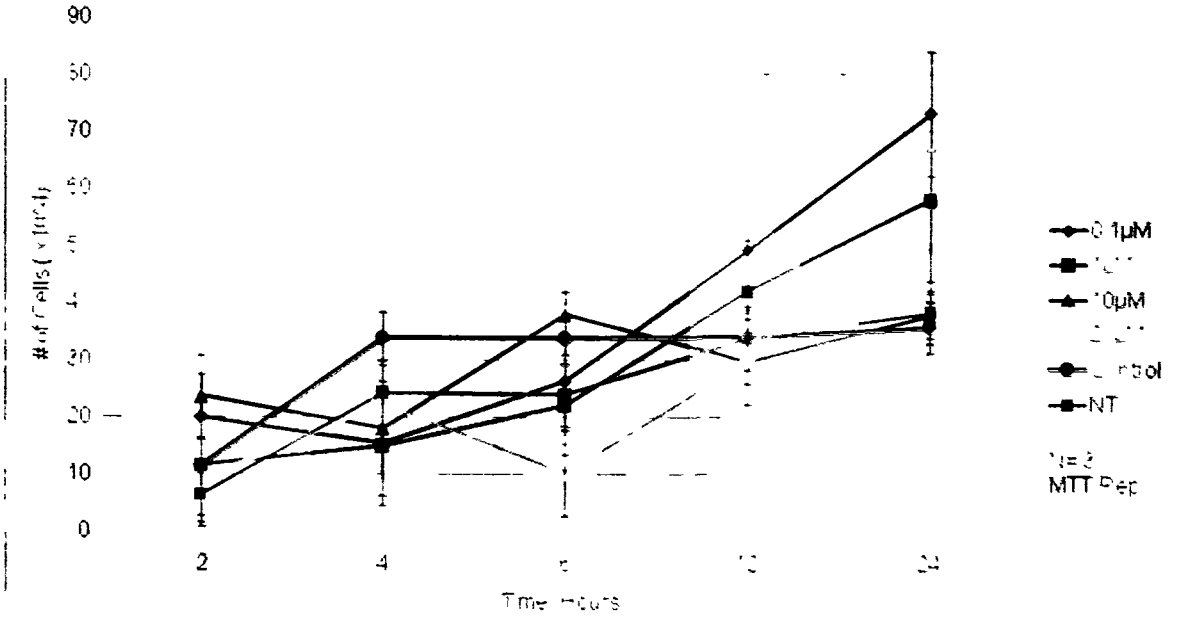
(**C**) Proliferation of human invasive breast cancer cells HTB-132 measured by MTT assay, performed in triplicate. Coloured bars represent proliferation with various concentrations of cortisol shown as 0.1μM (■). 1.0μM (■), 10μM (■), 20μM ( ), Control (■), and NT (■). Control represents cells treated with DMSO; NT represents cells without treatment. Time was measured in hours, and proliferation was measured by absorbance at 590nm as shown on the X and Y axis, respectively.

(**D**) Human breast cell line HTB-125 utilizing a trypan blue exclusion assay, performed in triplicate and was replicated by MTT analysis. Coloured lines represent cellular proliferation over time with various concentrations of cortisol shown as 0.1μM (■), 1μM (■), 10μM ( ), 20μM (■), Control (■), or NT (■). Control represents the vehicle that cortisol was brought into solution, DMSO whereas NT represents cells untreated. Time was measured in hours as shown on the X axis, and number of cells counted is reflected on the Y axis.

1C

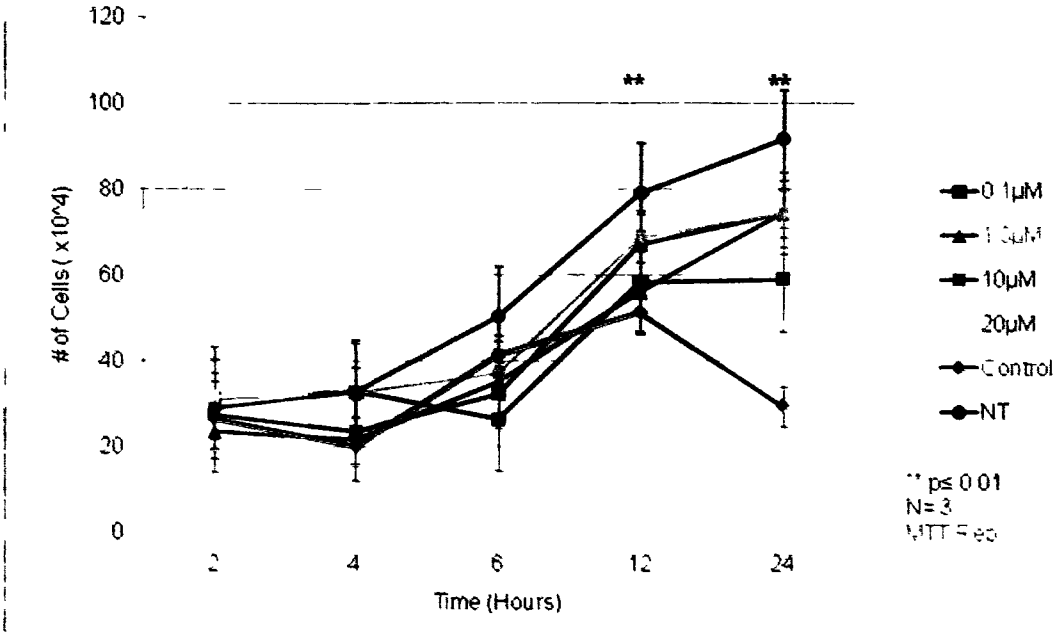

OD (590 nm)
2.5
2
1.5
0.5
0
**
**
0 µM
10µM
Control
NT
** p≤ 0.01

1D


90
70
30
20
10
0
2
4
0.1µM
10µM
NT

(E) Normal mouse mammary cell line HC11 utilizing a trypan blue exclusion assay, performed in triplicate and was MTT replicated. Coloured lines represent cellular proliferation over time with various concentrations of cortisol shown in the legend as 0.1μM (■), 1μM (■), 10μM (■), 20μM ( ) Control (■), or NT (■). Control represents DMSO treated cells and NT represents untreated cells. Time was measured in hours as shown on the X axis, and number of cells counted is reflected on the Y axis. For all sections statistical analysis was performed using a 2-tailed T-Test assuming equal variance with standard deviations used to represent error bars. Statistical significance represents probability (p). Results were not considered significant if a p value was more than 0.05.

1E


120
100
80
60
40
20
0
# of Cells (x10^4)
**
**
2
4
6
12
24
Time (Hours)
0.1µM
1.0µM
10µM
20µM
Control
NT
** p≤ 0.01
N= 3

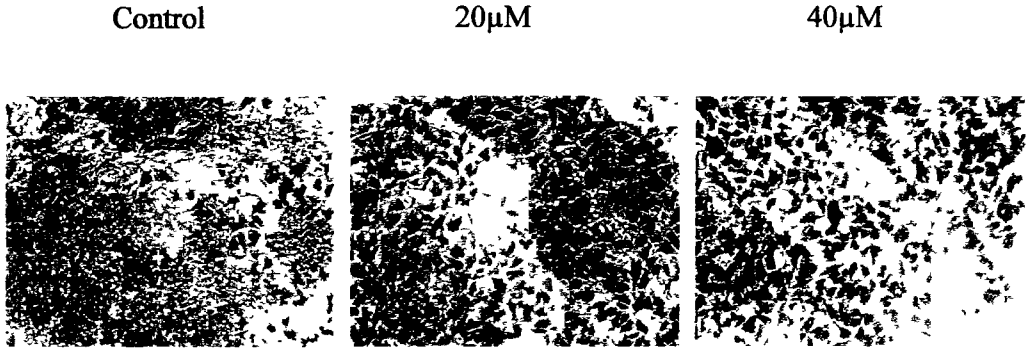
Cortisol Stimulates Migration in Human Breast Cancerous Cells.

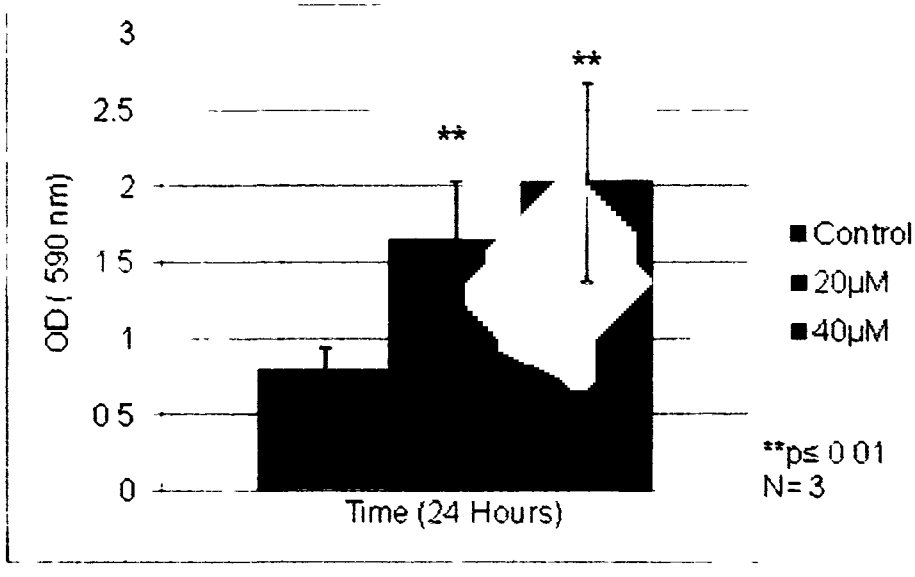
To study the effects of cortisol on breast cell migration a Cytoselect™ 24-Well Cell Migration Assay (Cell Biolabs, CBA-100) assays were utilized. Figure 2 demonstrates that cortisol stimulates migration towards a chemoattractant significantly at both 10 μM and 20 μM concentrations ($p \leq 0.01$) in the invasive human breast cancer cell line HTB-26 cells but failed to significantly aid in migration of the normal mouse mammary HC11 cell line (Figure **2A & B**). Wound healing assays demonstrated similar results where cortisol concentrations ranging from 10- 80 μM stimulated migration, or closing of a wound, in the human invasive breast cancer HTB-26 cell line (Figure **2C**). As early as 3 hours post wound infliction HTB-26 cells treated with cortisol (80μM) revealed migration capabilities reaching significance ($p \leq 0.01$). Statistical significance was continuously met at 6, 12 and 24 hours ($p \leq 0.05$, and $p \leq 0.01$). By the end of the time course HTB-26 cells treated with cortisol stimulated migration, or closed the wound compared to the DMSO control (80 μM) as well as the no treatment (NT) control. However, wound healing of the normal mouse mammary cell line HC11 treated with cortisol at various concentrations (10-80 μM) revealed no statistical significance compared to the vehicle control (80 μM) as well as the NT control. Reaching statistical significance ($p \leq 0.01$) at 24 hours the vehicle control and NT control were able to migrate and close the wound, but not the cortisol treated cells (Figure **2D**). Collectively, these data demonstrate that cortisol aids the migration of cancerous breast cells as measured by the ability move towards a chemoattractant as well as in the ability to repair a wound.

Figure 2. Cortisol Stimulates Migration in Human Breast Cancerous Cells.

(A) Migration assays were performed using cortisol at 20 μM and 40 μM and control, DMSO, in HTB-26 cells. Purple staining indicates cells which have migrated through the polycarbonate barrier (upper panels); the coloured bar graph reflects statistical analysis of staining experiments carried out in triplicate with Control (■), 20 μM (■), and 40 μM (■) (lower panels). Control represents a DMSO vehicle control. Time was measured in hours, and proliferation was measured by absorbance at 590 nm as shown on the X and Y axis, respectively.

2A

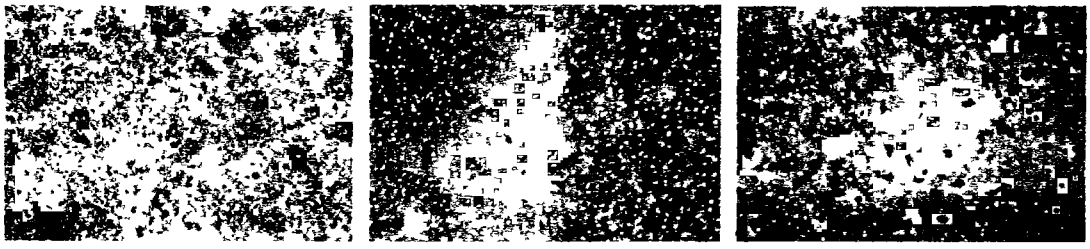

Control
20µM
40µM

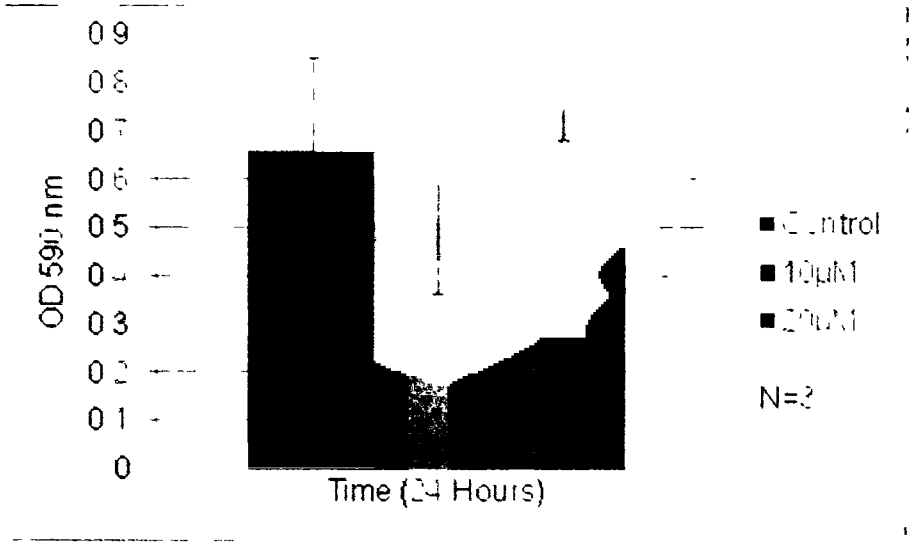

3
2.5
2
1.5
1
0.5
0
OD(590 nm)
**
**
Time (24 Hours)
Control
20µM
40µM
**p≤ 0.01
N= 3

(B) Migration assays were performed using cortisol and vehicular control in HC11 cells. Purple staining indicates cells which have migrated through the polycarbonate barrier (upper panels); coloured bar graphs reflect statistical analysis of staining experiments carried out in triplicate with Control (■), 20 μM (■), and 40 μM (■) (lower panels). Control represents the DMSO vehicle control. Time was measured in hours, and proliferation was measured by absorbance at 590 nm as shown on the X and Y axis, respectively.

2B

Control 10μM 20μM

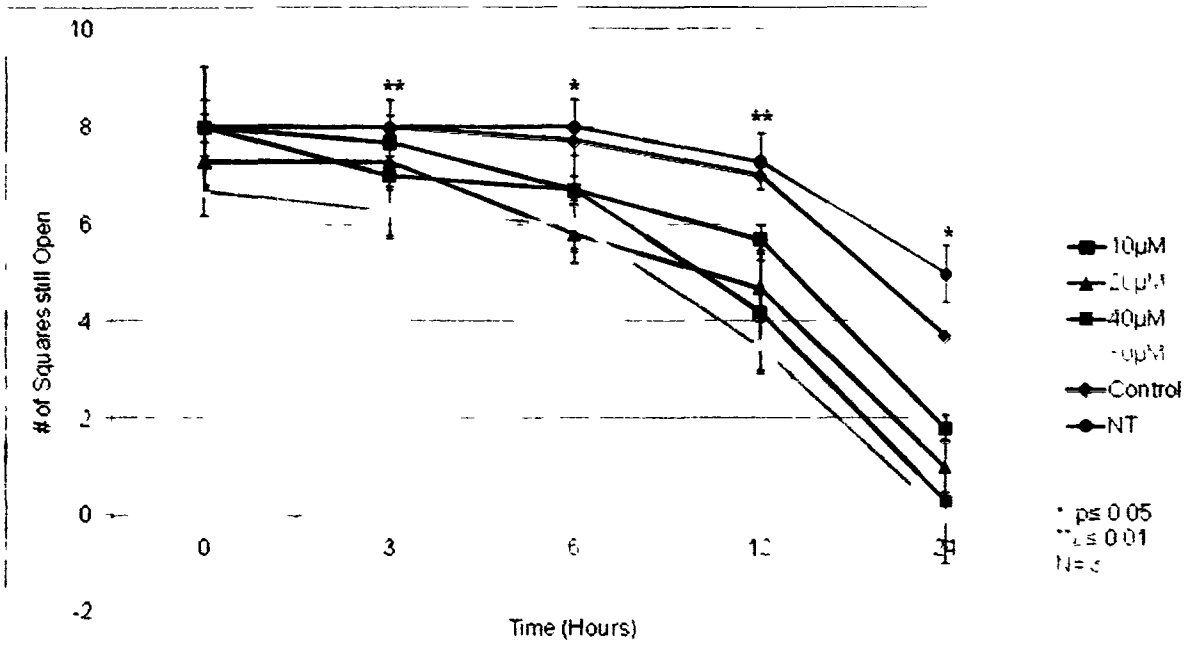




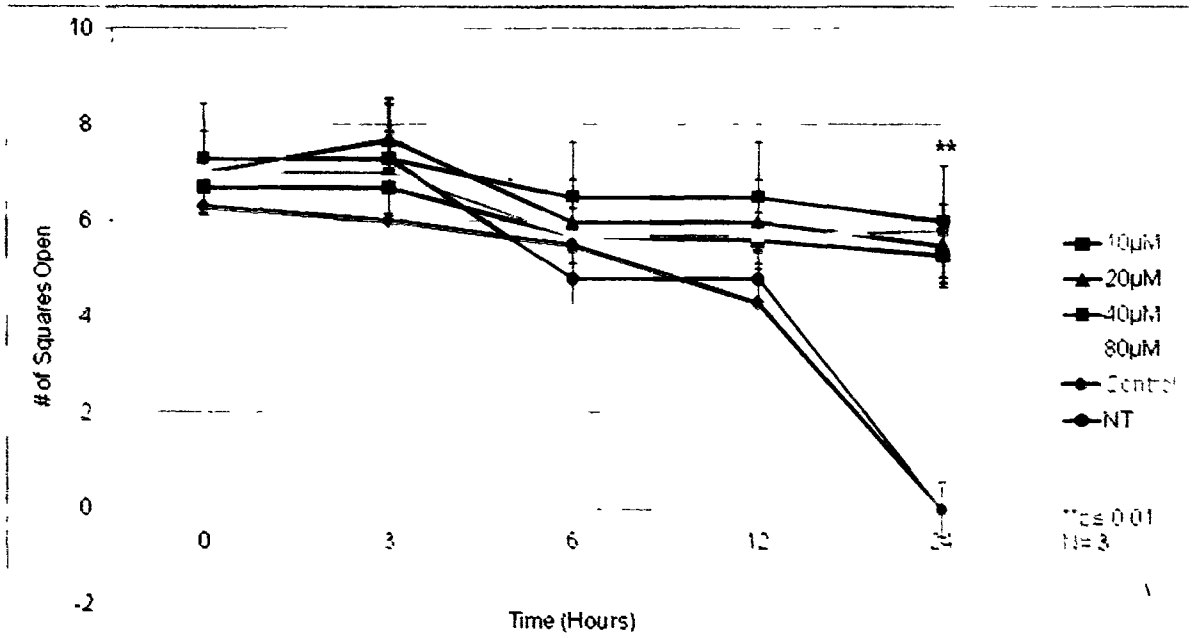
(**C**) Wound healing was performed utilizing HTB-26 cell line treated with cortisol concentrations of 10μM (■), 20μM (■), 40μM (■), 80μM ( ), Control (■), and NT (■) and was performed in triplicate. Control represents a DMSO vehicle control and NT represents cells untreated. Line graphs reflect migration of HTB-26 cells to close a wound over time and how many grids the cells migrated as shown on the X and Y axis, respectively. Visual observations were taken using the microscope software minisee 1.0.9.37. Visual observations were quantified by super-imposing a control image consisting of 1mm by 1 mm grids. Number of cells that migrated across to close the wound was counted.

(**D**) Wound healing was performed utilizing HC11 cell line treated with cortisol concentrations of 10μM (■), 20μM (■), 40μM (■), 80μM ( ), Control (■), and NT (■) and was performed in triplicate. Control represents a DMSO vehicle control and NT represents cells. Line graphs reflect migration of HC11 cells to close a wound over time and how many grids the cells migrated as shown on the X and Y axis, respectively. Visual observations were taken using the microscope software minisee 1.0.9.37 Visual observations were quantified by super-imposing a control image consisting of 1 mm by 1mm grids. Number of cells that migrated across to close the wound was counted. In all panels, errors bars reflect standard deviation with statistical significance assessed using a 2-tailed T-Test assuming equal variance with probability (p). Results were not considered significant if a p value was more than 0.05.

2C


10
8
6
4
2
0
-2
# of Squares still Open
0
3
6
12
24
Time (Hours)
**
*
**
*
10μM
20μM
40μM
80μM
Control
NT
* p≤ 0.05
** p≤ 0.01
N=3

2D

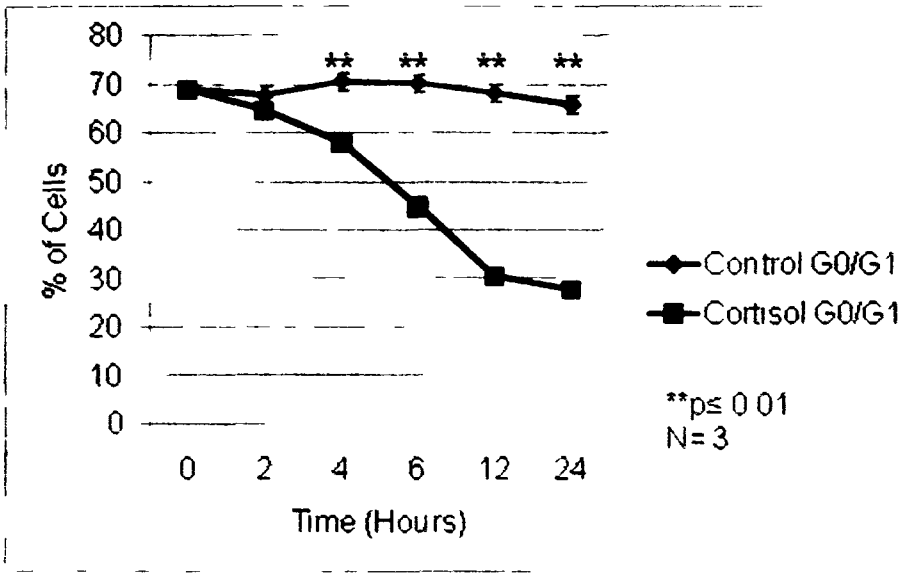

10
8
6
4
2
0
-2
# of Squares Open
0
3
6
12
24
Time (Hours)
**
10μM
20μM
40μM
80μM
Control
NT
** p≤ 0.01
N=3

Cortisol Stimulates Human Breast Cancerous Cells Throughout the Cell Cycle.

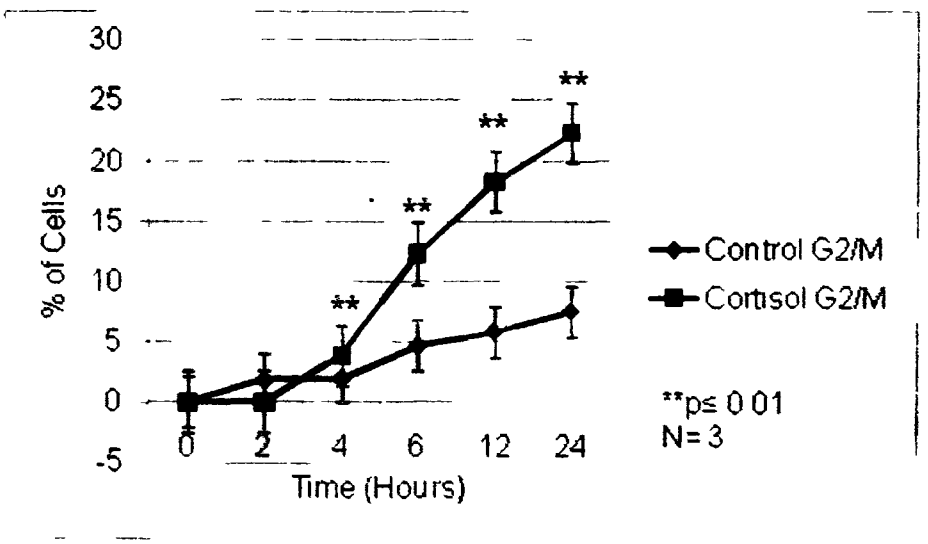
To determine whether cortisol effects were reflecting changes at one specific point in the cell division cycle, cells were monitored over a time course following cortisol treatment by flow cytometry. As seen in Figure **3** cortisol treated cells stimulated the human breast cancer MCF7 cell line continuously and significantly faster out of GO/G1 ($p \leq 0.01$) as early as 4 hours post treatment compared to the vehicular control. By 24 hrs. post-treatment cortisol treated cells revealed a 40% reduction of cells in G0/G1 phase of the cell cycle over control (Figure **3A**). Similarly, cortisol treated MCF7 cells continually and significantly ($p \leq 0.01$) increased the rate at which cells entered into G2/M phase of the cell cycle as compared to vehicle control (Figure **3B**). By 24 hrs post-treatment cortisol treated cells reveal a 23% increase in the G2/M cell population. Both cell populations had approximately 20% of the cells in a sub-G0 population indicative of apoptosis following treatement. For the cortisol treated cells an additional polyploidy population was noted that was not included in this analysis. This data supports that cortisol enhances breast cancer cell progression through the cell cycle.

Figure 3. Cortisol Stimulates Human Breast Cancerous Cells Throughout the Cell Cycle. **(A)** Cell cycle analysis utilizing flow cytometry was performed on MCF7 cells treated with 40 μM (■) or DMSO control (■) in triplicate. Line graph represents MCF7 cells exiting G0/G1 phase of the cell cycle. Time was measured in hours as shown on the X-axis, and percent of cells assessed is reflected on the Y-axis. **(B)** Cell cycle analysis utilizing flow cytometry was performed on MCF7 cells treated with 40 μM (■) or DMSO control (■). Line graph represents MCF7 cells entering G2/M phase of the cell cycle. Time was measured in hours as shown on the X-axis, and percent of cells assessed is reflected on the Y-axis. For both panels, statistical analysis was performed using a 2-tailed T-Test assuming equal variance with standard deviations used to represent error bars. Statistical significance represents probability (p). Results were not considered significant if a p value was more than 0.05.

3A


80
70
60
50
40
30
20
10
0
% of Cells
**
**
**
**
0
2
4
6
12
24
Time (Hours)
Control G0/G1
Cortisol G0/G1
**p≤ 0 01
N= 3

3B


30
25
20
15
10
5
0
-5
% of Cells
**
**
**
**
0
2
4
6
12
24
Time (Hours)
Control G2/M
Cortisol G2/M
**p≤ 0 01
N= 3

Cortisol Effects Are Mediated through Glucocorticoid Receptor Signalling.

It is widely accepted that cortisol-mediated effects may be mediated through the GR or via cross talk with other steroid receptors. To determine the role of these other receptors on GR-mediated proliferation in breast cells we utilized pharmacological and knockdown approaches. Utilizing the human breast cancer cell line MCF7, which are ER, PR and GR positive, increasing concentrations of the GR/PR inhibitor, RU486 show remarkable significant decreases ($p \leq 0.01$) in cell proliferation in the presence of cortisol where increasing concentrations of the ER inhibitor, TAM did not have any effect on cellular proliferation in the presence of cortisol (Figure **4A**). This data demonstrates that cortisol is not mediating specific proliferative effects through the ER. To further analyze the contribution of the GR to these proliferative effects the GR was knocked down in the human invasive breast cancer cell line HTB-26, which are ER negative but and PR and GR positive (Figure **4B**). This was accomplished using specific siRNA or a scrambled siRNA control and proliferation was assessed in the presence of cortisol or vehicle control. RT-PCR confirmed partial, but significant knock-down on the GR ($p \leq 0.01$) compared to siRNA control through denso-spot analysis (Figure **4C**). GAPDH was used as a loading control and was not significantly different in siRNA against the GR and siRNA control ($p = 0.1822$). These results demonstrate that knockdown of the GR significantly decreased ($p \leq 0.01$) cortisol's proliferative effects compared to the control. confirming cortisol's effects are mediated through the GR (Figure **4B**).

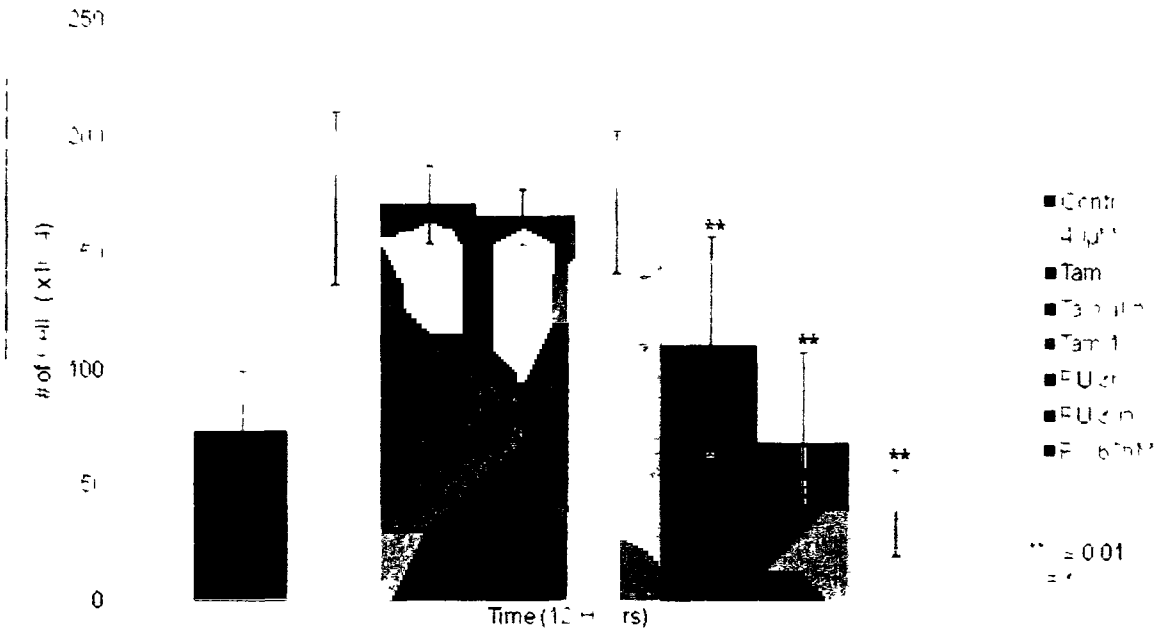
Figure 4. Cortisol Effects Are Mediated through Glucocorticoid Receptor Signalling.

(A) TAM at 1 nm (■), 10 nm (■), and 100 nm (■) were utilized as an oestrogen receptor inhibitor as well as RU486 at concentration of 3 nm (■), 30 nm (■), and 60 nm (■), a glucocorticoid and progesterone receptor inhibitor were utilized in the presence of cortisol at 40 μM ( ) or DMSO control (■) using the MCF7 cells. The DMSO control was utilized for comparison against receptor inhibition to assess whether proliferation was significant. Bar graph represents cellular proliferation in the presence of a receptor inhibitor, and was assess using a trypan blue exclusion assay. Time was measured in hours as shown on the X-axis, and number of cells counted is reflected on the Y-axis. Statistical analysis reflects triplicate counts.

(B) Inhibition of the GR was done utilizing siRNA against the GR in the HTB-26 cell line and was performed in triplicate. Bar graph represents cellular proliferation in the presence of the siRNA against the GR (■), siRNA scrambled (■), and DMSO control

(■). The vehicle control was utilized for comparison against siRNA against the GR as well as the siScrambled to assess whether proliferation was still significant in the siScrambled control. Cell proliferation was assessed using a trypan blue exclusion assay. Time was measured in hours as shown on the X-axis, and number of cells counted is reflected on the Y-axis.

4A

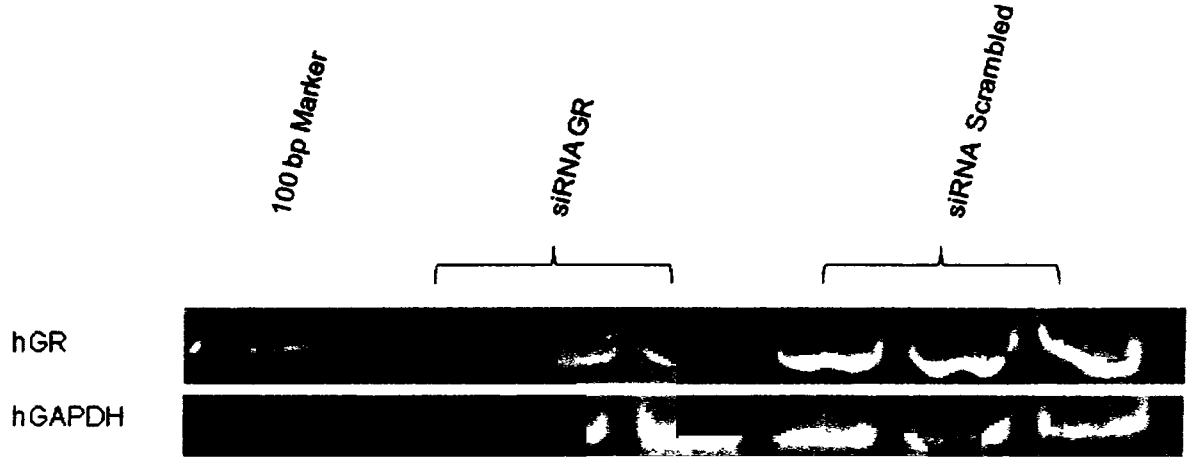

250
100
50
0
**
**
**
Tam
Time (12 H rs)
** ≤ 0.01

4B


120
100
60
40
20
0
**
siScrambled
siGP
Control
** p≤ 0.01
N= 3

(C) RT-PCR was performed utilizing cDNA generated from the HTB-26 treated cells from the siRNA against the GR and the siScrambled performed in triplicate. Human GR is shown in the upper panel as hGR where hGAPDH represented loading control, human GAPDH in the lower panel. Expression fold values were assessed using denso spot analysis. For all panels, statistical analysis was performed using a 2-tailed T-Test assuming equal variance with statistical significance represents probability (p). Results were not considered significant if a p value was more than 0.05.

4C



Identification of Cortisol-Mediated Gene Expression in Invasive Human Breast Cancer Cells.

There are a number of microarray studies currently conducted using DEX treatment, as well as several using cortisol treatment however there is no existing data for invasive human breast cancer cells. Our data reflect that cell lines such as the HTB-26 respond most drastically with regard to proliferation and migration in response to cortisol. Importantly, these cells are ER independent and therefore allow us to study the gene expression effects of cortisol in the absence of cross talk from the ER. HTB-26 cells were treated with cortisol at 40 μM, or a DMSO control and RNA was sent to the UHN facility at the University of Toronto, Canada. Synthesis of cDNA was performed and hybridized to a 27K human array chip, and performed in triplicate. SAM analysis revealed a total of 624 significantly down-regulated genes, and 398 significantly up-regulated genes were revealed for a total of 1,022 potential gene targets (**A** & **B**). All potential gene targets were assessed and selected based on set criteria. In total, 24 significantly down-regulated (**A**) and 14 significantly up-regulated gene targets (**B**) were selected for further analysis. Selection criterion was based on a minimal expression fold log value of 6 followed by further selection for those targets exhibiting novelty, relation to breast cancer, and relation to cancer in general.

Table 1. Cortisol Influences Up-Regulated Gene Expression in Various Genes.

Represents genes that were significantly down-regulated by cortisol at 40 μM compared to the DMSO control. Expression fold value represents a log value of cortisol treated HTB-26 cells where expected expression fold value represents a log value of vehicular control treated HTB-26 cells. Log values represent the exponent value with a base of 2, such as gene Ovo-like 1 (Drosophilia) expression value is $2^{-16.52595}$ Microarray gene expression was performed in triplicate and statistical analysis was assessed via SAM. Data was not considered significant if the log value was below 6.

Table 1

Name of Gene Target	Expression Fold Value	Expected Expression Fold Value
Ovo-like 1(Drosophila)	-16.52595	0.48045823
Protein tyrosine phosphatase, non-receptor type 21	-16.340612	0.45944917
RAP2B, member of RAS oncogene family	-16.18119	0.46842855
Glutamate receptor interacting protein 1	-15.769973	0.5296949
Mitogen-activated protein kinase kinase kinase 7	-14.718367	0.4430995
Tetraspanin 8	-14.269757	0.44092378
NLR family, pyrin domain containing 1	-14.00973	0.4729342
Glypican 4	-12.848505	0.4675239
Integrator complex subunit 6	-11.160135	0.43560275
P21 (CDKN1A)-activated kinase 2	-11.058357	0.6534912
Leucine rich repeat containing 61	-10.76905	-0.4869869
B-cell CLL/lymphoma 10	-10.52266	0.43166247
Killer cell lectin-like receptor subfamily C, member 2	-10.456471	0.4410412
Cyclin-dependent kinase 3	-10.409155	0.4648322
HSPB (heat shock 27kDa) associated protein 1	-10.202217	0.56796604
Growth arrest-specific 2	-9.612179	0.44288394
BH3 interacting domain death agonist	-9.232355	0.50470066
DNA-damage-inducible transcript 4	-9.009577	0.4942023
Kruppel-like factor 12	-8.8060055	1.7626563
Tumor necrosis factor (ligand) superfamily, member 8	-8.57361	0.4619426
Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	-7.837138	0.44549263
Inhibitor of growth family, member 1	-6.747879	0.4268193
Jun D proto-oncogene	-6.6851487	1.1614074
Fibroblast growth factor 7 (keratinocyte growth factor)	-6.2941537	1.8904877

Table 2. Cortisol Influences Down-Regulated Gene Expression in Various Genes.

Represents genes that were significantly up-regulated by cortisol at 40 μM compared to the vehicle control, DMSO. Expression fold value represents a log value of cortisol treated HTB-26 cells where expected expression fold value represents a log value of vehicular control treated HTB-26 cells. Log values represent the exponent value with a base of 2, such as gene Metallothionein 2A expression value is $2^{22.425978}$ Statistical analysis was assessed utilizing SAM. Data was not considered significant is the log value was below 6.

Table 2

Name of Gene Target	Expression Fold Value	Expected Expression Fold Value
Metallothionein 2A	22.425978	-0.14324372
Metallothionein 1F	13.396568	-3.9294183
Metallothionein 1G	12.301996	0.31089145
Growth factor independent 1 transcription repressor	10.780789	2.0504496
DENN/MADD domain containing 3	10.189645	0.815617
Transcription factor 7-like 2 (T-cell specific, HMG-box)	10.01574	0.97176766
Aryl-hydrocarbon receptor repressor	9.644095	1.5719044
Metallothionein 1E	8.898076	-1.0625384
Ras homolog gene family, member Q	8.528412	1.2853632
Protocadherin 8	7.5462537	0.58093745
BCL2/adenovirus E1B 19kDa interacting protein 1	7.1896663	1.521623
Dickkopf homolog 1 (Xenopus laevis)	6.850006	2.9487922
Mitogen-activated protein kinase 8 interacting protein 3	6.7509437	0.65893143
Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	6.6810217	1.6156677
Cyclin B2	6.386154	1.9802139
Mitogen-activated protein kinase-activated protein kinase 3	6.104611	1.114327

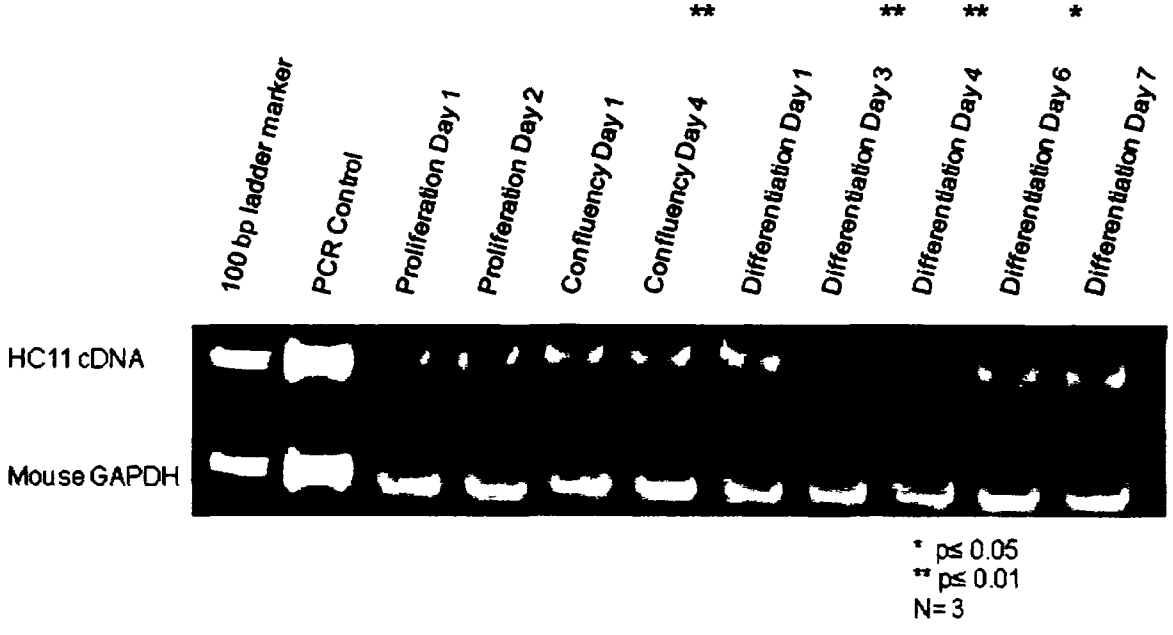
FRYL Gene Expression in Normal Mammary Development and Breast Cancer Progression.

As part of a pilot microarray study utilizing a separate cell line a number of interesting, unique genes were isolated which differed from those selected through the HTB-26 array (data not shown). One of these targets was the FRYL gene, found to be 5 fold elevated when treated with 20 μM cortisol for 12 hrs. Following up on this potential target a differentiation time course was conducted using normal mouse mammary cell line HC11 looking at FRYL expression levels utilizing RT-PCR followed by denso spot analysis revealed a slight, but significant ($p \leq 0.01$, confluency day 4) up-regulation just prior differentiation. During differentiation FRYL was found to be significantly down-regulated through differentiation days 3-6 ($p \leq 0.01$, days 3 & 4; $p \leq 0.05$, day 6) (Figure **5A**). Denso spot analysis revealed no significant difference for loading control, mGAPDH. RT-PCR was performed in triplicate with denso spot analysis for statistical purposes. Q-RT-PCR was then carried out to assess FRYL expression among a panel of human breast cancer cell lines MCF7, HTB-26, and HTB-132 as well as the normal human breast cell line MCF10-A treated with cortisol, a DMSO control, or no treatment (0 hr) (Figure **5B**). This study revealed a significant log fold increase of 7 ($p \leq 0.01$) in the cortisol treated human breast cancer cell line HTB-132, cells containing a p53 mutation. Further analysis of the potential role of this gene in human breast cancers was conducted using Q-RT-PCR analysis of a human breast cancer cDNA array (Figure **5C**). This work revealed FRYL to be up-regulated in the breast cancer stages 1 and 3A.

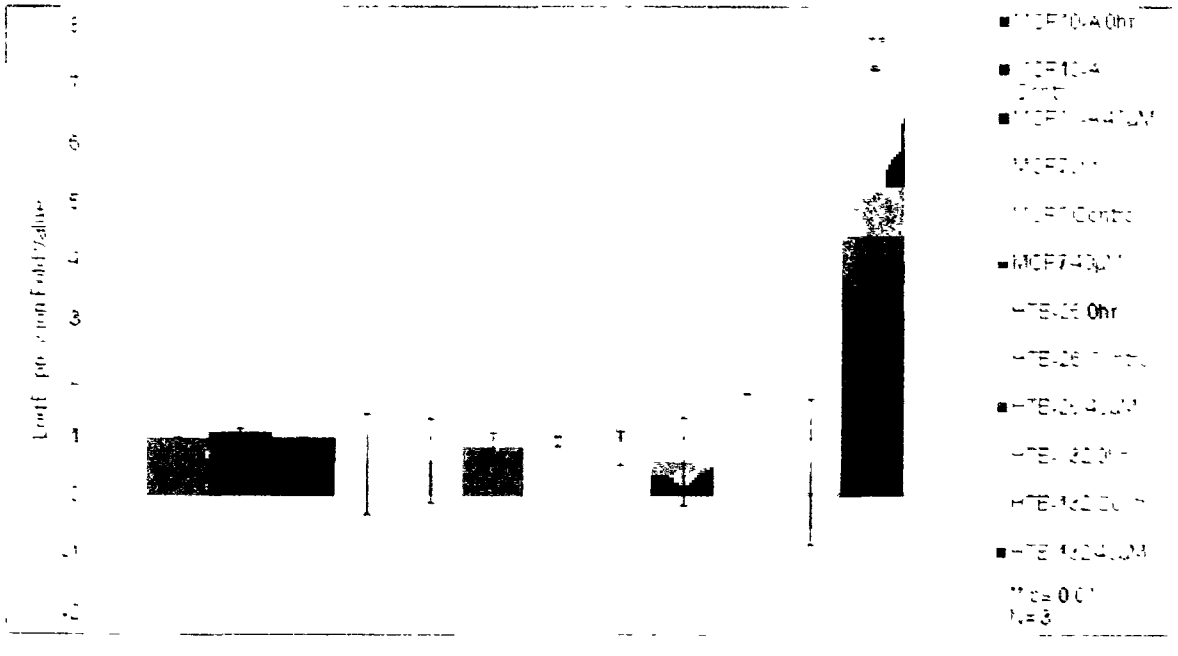
Figure 5. FRYL Gene Expression in Normal Mammary Development and Breast Cancer Progression.

(**A**) HC11 cDNA assessing FRYL expression was performed utilizing RT-PCR. Upper panel reflects mouse FRYL (mFRYL) expression levels throughout the differentiation time course. Lower panel reflects mouse GAPDH (mGAPDH) utilized as a loading control. cDNA assessing FRYL expression was performed in triplicate with expression fold values assessed using denso spot analysis. Statistical significance was determined using a 2-tailed T-Test assuming equal variance. Statistical significance represents probability (p). Results were not considered significant if a p value was more than 0.05. (**B**) Q-RT-PCR assessed FRYL expression in cell lines MCF-10A (■), MCF7 (■), HTB-26 (■), and HTB-132 (■). Coloured bars represent the various cell lines used where variation of colour shading reflect specific treatments for each cell such that 0 hr represents no treatment, DMSO control, and cortisol at 40 μM. The Y axis reflects fold expression. Q-RT-PCR was performed in triplicate including a loading control, 18s. Raw values were normalized to MCF-10A 0 hr with statistical analysis performed using a 2-tailed T-Test assuming equal variance with standard deviations used as error bars. Statistical significance reflects experiment in triplicate where probability (p) is shown. Results were not considered significant if a p value was more than 0.05.

5A

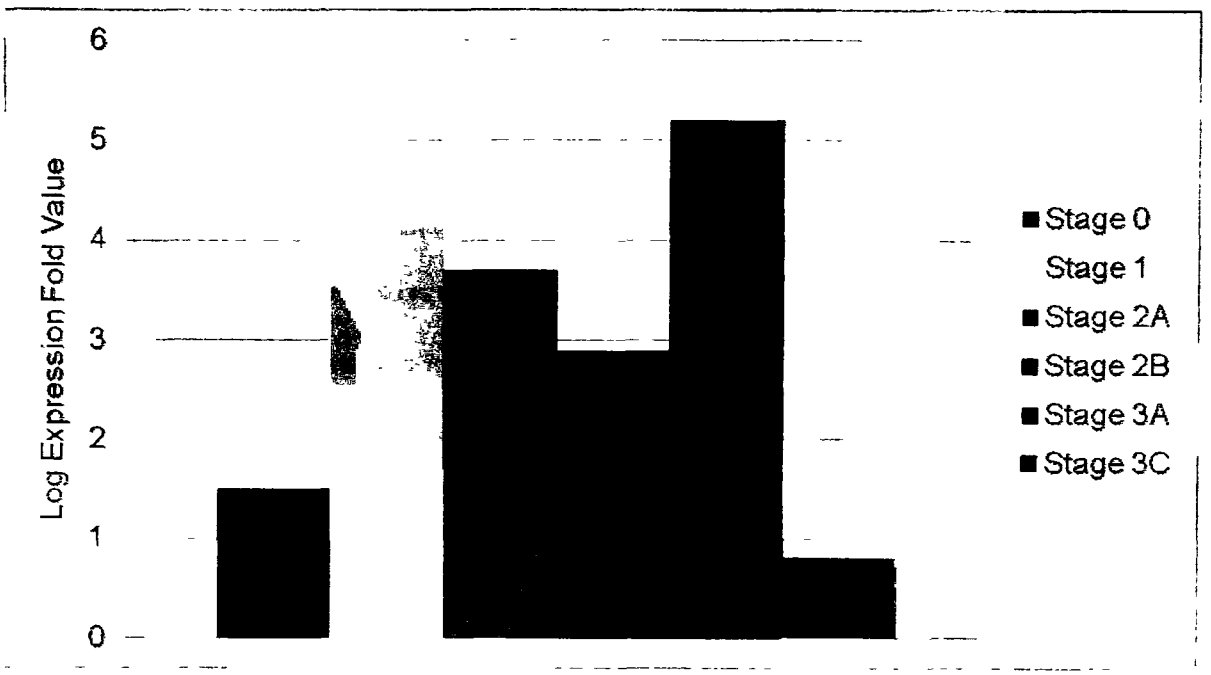

**
**
**
*
100 bp ladder marker
PCR Control
Proliferation Day 1
Proliferation Day 2
Confluency Day 1
Confluency Day 4
Differentiation Day 1
Differentiation Day 3
Differentiation Day 4
Differentiation Day 6
Differentiation Day 7
HC11 cDNA
Mouse GAPDH
* p≤ 0.05
** p≤ 0.01
N= 3

5B



(C) Q-RT-PCR assessing FRYL expression in various breast cancer stages was performed utilizing a human breast cancer tissue panel array. Coloured bars represent various stages of breast cancer development at stage 0 (■), 1 ( ), 2A (■), 2B (■) 3A (■) and 3C (■) with the Y axis reflecting fold expression of FRYL. Raw values were normalized to a stage 0 breast tissue sample, and breast cancer tissue samples were normalized utilizing β-actin by Origene.

5C


Log Expression Fold Value
6
5
4
3
2
1
0
Stage 0
Stage 1
Stage 2A
Stage 2B
Stage 3A
Stage 3C

DISCUSSION:

This project focuses on the molecular mechanisms of cortisol's potential role in breast cancer initiation and progression. It was hypothesized that cortisol functions through the glucocorticoid receptor to mediate effects on cell growth, adhesion and migration properties in both normal and cancerous breast cells. Specific aims of this project were to determine the effects of cortisol on: cell proliferation in normal and cancerous breast cells as well as migration and invasion in cancerous breast cells, and the pathway of cortisol's mediated function in breast cancerous cell lines.

We demonstrate that stimulation with physiological levels of cortisol directly enhances breast cancer cell numbers compared to normal breast cells. The human breast cancer cell line MCF7 (Figure 1A) revealed that cortisol increased proliferation as early as 4 hrs and continued to significantly stimulate proliferation ($p \leq 0.05$) throughout the time course compared to the normal human breast cell line HTB-125 (Figure 1D), as well as the normal mouse mammary cell line HC11 (Figure 1E). It was revealed that at later time points, 12 and 24 hours, HC11 cells treated with cortisol were able to reach significant proliferation values compared to control. Although considered "normal" the HC11 cell line does have a p53 mutation which increases the probability that over time this cell line will become cancerous. Furthermore, it was demonstrated that both human invasive cancer cell lines HTB-26 and HTB-132 were significantly stimulated by cortisol. Despite showing similar proliferation patterns, when comparing the HTB-26 line to the HTB-132 line, the -132 line showed more significant cellular proliferation at the 20 μM ($p \leq 0.0198$) stimulated by cortisol. One of the differences between these 2 invasive cell

lines is that the HTB-132 cell line has a mutated p53 whereas HTB-26 has normal p53 expression. These data support that p53 status may confer sensitivity to cortisol-mediated proliferative effects; while interesting and potentially significant clinically this hypothesis requires further investigation.

Cell migration and invasion are key components to metastatic breast cancer (Sliva, 2004). Metastatic cancers are known to have reduced levels of 'metastasis suppressor genes' (Moutsatsou and Papavassiliou, 2008) and drug targets are currently under development as part of novel breast cancer treatment strategies (MacDonald, Freije, Stracke, Manrow, & Steeg, 1996). Recently, Ouatas, Halverson, and Steeg, (2002) demonstrated that DEX stimulates expression of Nm23-H1 and Nm23-H2; two genes known to be highly expressed in metastatic breast cancer. Ouatas *et al.* (2002) also showed that this increased expression level was through a GRE-dependent transcriptional mechanism. High levels of cortisol have been found in patients with adrenal, lung, and bone metastasis (Palesh *et al.*, 2007). Patients with metastatic breast cancer show higher, flatter cortisol rates in addition to decreased survival rates (Spiegel *et al.*, 2006). Furthermore, it has been shown that glucocorticoid supplements significantly increase metastasis, *in vivo* (Herr and Buchler, 2006; Herr and Marme, 2005).

Cortisol levels have been known to increase invasiveness in ovarian cancer (Sood *et al.*, 2006), but little is known about cortisol and invasiveness in general. Given the previous literature surrounding cortisol and metastasis we felt it important to assess cortisol's potential effects on migration and invasion. Utilizing a migration assay, it was shown that cortisol significantly ($p \leq 0.01$) enhances breast cancerous cell migration of the

invasive human breast cancer cell line HTB- 26 (Figure 2A) as measured by the ability to migrate towards a chemoattractant compared to normal mammary mouse cell line, HC11 (Figure 2B). Invasion was measured by wound healing assays measuring the ability to invade an open space, or repair a wound. Cortisol stimulated the human invasive breast cancer cell line HTB-26 (Figure 2C) to repair the wound compared to control whereas cortisol did not stimulate the normal mammary mouse cell line HC11 (Figure 2D). This supports the proliferation studies to suggest that cortisol has differential effects on cells of varying growth status. 'Normal' cells with intact checkpoints appear to prevent cortisol effects for the limited time course tested, whether these results would differ if experiments were carried out over a longer period of time remains to be determined. The effects of cortisol on breast cancer cells is not only important for the physiological relevance reflecting the potential adverse results of a stress, but clinically these results are important since they can help to reconcile the studies utilizing DEX. DEX treatment has revealed both positive and negative effects on breast cell growth (van den Berg, Claffie, Boylan, McKillen, Lynch, & McKibben, 1996; Ryde, Nicholls, & Dowsett, 1992, respectively). Although a synthetic of cortisol, DEX is structurally different from cortisol such that it has fluorine off of carbon 9, and is considered to be 20-30 times more potent than cortisol (Zoorob, and Cender, 1998). Considering cortisol is known to produce different effects throughout the body, and at different physiological concentrations, the higher potency of DEX relative to cortisol that the observations made with DEX are not an accurate interpretation or even assumption that cortisol would behave similarly. This holds tremendous implications for clinical treatment with respect to breast cancer.

Cell cycle analysis revealed cortisol significantly increases the rate that cells exit from G1 phase of the cell cycle as early as 4hrs ($p \leq 0.01$), and continues to stimulate the cell significantly throughout the cell cycle into G2/M as early as 2 hrs ($p \leq 0.01$). It has been shown that many breast cancer cells over-express G1/S cyclins such as cyclin E (Barton, Akli, & Keyomarsi, 2006) at the mRNA and protein levels (Keyomarsi, Conte, Toyofuku, & Fox, 1995). It is known that cyclin E has different isoforms, one of which is a "post-translational cleavage" of the full-length cyclin E into a lower molecular weight (LMW) and reveals a more active protein than the full-length cyclin E. These altered cyclin E proteins are unique to tumour cells in addition to being associated with stages of breast cancer (Barton *et al.*, 2006). Keyomarsi *et al.* (2002) assessed cyclin E levels in breast cancer tumour samples from 395 women to examine if there was a correlation of cyclin E with these breast tumours. Their results demonstrate that both the full length and shorter length sequence of cyclin E were present: they concluded that cyclin E is a very strong predictor of cancer survival through breast cancer stages 1 to 3. Furthermore, Matsumoto and Maller (2004) demonstrated that this short isoforms version of cyclin E is functional and over-expressing this isoforms in normal cells stimulate them to move into S phase and even G2/M phase of the cell cycle. Given cortisol's ability to stimulate cancerous cells out of G0/G1 and into S phase future project objectives include assessing cortisol's possible interaction with cyclin E. Protein analysis looking at cell cycle regulators such as cyclin E and D would be valuable to address specific areas within the cell cycle cortisol is mediating its effects. Further work includes looking into the CDKs 2, 4 and 6, as well as the CDKIs. These future project objectives will help to narrow where specifically cortisol is interacting within the cell cycle, and with what partners.

Interestingly, Dr. Porter pulled out numerous proteins in a yeast 2 hybrid screen (data unpublished) but did not pull out cyclin E. Future work includes looking at the different isoforms of cyclin E with cortisol to investigate if 1 or more isoforms interact with cortisol as this would help to explain the observed transition through G1/S. Furthermore, cyclin B did come up in the yeast 2 hybrid screen; which is consistent with a larger population of cells in G2/M (Wang, Fu, Mani, Wadler, Senderowicz, & Pestell, 2001).

Glucocorticoids are known to interact with multiple steroid receptors and demonstrate steroid cross-talk capability (Moutsatsou and Papavassiliou, 2008). Thus it was imperative to determine which steroid receptor was mediating cortisol's effects through. Receptor inhibition using TAM revealed that cortisol is working through either the PR or GR, or both, and not the ER (Figure 4A). Selective knockdown using siRNA against the GR was utilized to determine whether the effects observed were indeed mediated through the GR. Cortisol's effects are working through the GR: a trypan blue exclusion assay demonstrates a significance decrease ($p \leq 0.01$) in proliferation compared to the siRNA control (Figure 4B), and was now no longer significant compared to the vechicle control ($p=0.284$). Although the siRNA against the GR only partially knocked-down the receptor (Figure 4C) denso spot analysis confirmed a significant decrease in GR levels ($p \leq 0.01$) whereas all GAPDH levels did not significantly differ ($p= 0.1822$). Statistical analysis comparing proliferation numbers with all RU486 concentrations against the proliferation number for the siRNA against the GR revealed no significance difference ($p= 0.0624$; $p= 0.279$; $p= 0.935$, respective to RU486 concentration) thus confirming that cortisol-mediated proliferation effects on the cell lines tested is solely interacting through the GR.

Cortisol is known to target numerous genes revealing genomic and non-genomic effects (Buckingham, 2006). However, which genes are affected and by what degree is highly dependent upon where cortisol is in the body (Moutsatsou and Papavasiliou, 2008). Microarray analysis has revealed numerous putative targets that could explain the effects of cortisol observed in these cancerous cell lines. Many interesting genes were found to be significantly down-regulated. Protein tyrosine phosphatase, non receptor 21 is known to be involved in several cell processes such as growth, cell cycle, and being linked to oncogenesis (Liu, Kulp, Sugimoto, Jiang, Chang, & Li, 2002). Kruppel-like factor 12 is also known to be involved in carcinogenesis (Tong, Czerwenka, Heinze, Ryffel, Schuster. Witt, Leodolter, & Zeillinger, 2006). Tetraspanin 8 is also in cellular processes such as development, and has been known to be linked to several cancers (Carloni, Mazzocca, & Ravichandran, 2004). The NLR family. pyrin domain containing 1 are known to be highly involved in apoptosis (Franchi and Nuriez, 2008). Another gene found to be down-regulated that is involved in apoptosis is the Inhibitor of growth Family, Member 1 and has also been shown to be down-regulated in numerous cancers (Hara et al., 2003) as well as being involved in the p53 signalling pathway. In addition to this, protein BH3 interacting domain death agonist is also involved in apoptosis (Mebratu, Dickey, Evans, & Tesfaigzi, 2008). Integrator complex subunit 6 is thought to be a potential tumour suppressor (Hata and Nakayama, 2007). A very interesting potential gene of interest is p21 (CDKN1A)- activated kinase 2 which is an important gene encoding for a CDKI, and helps in cell cycle development in the G1 phase. This gene is dependent upon p53, and helps with cell cycle cessation in the event of DNA damage (Roy, Singh, Agarwal, Siriwardana, Schafani, & Agarwal, 2008). The Leucine rich repeat

containing 61 gene is part of a family that is known to be down-regulated in breast cancer (De Souza Santos, Bessa, Netto, & Nagi, 2008).

Several significantly up-regulated genes were also selected such as metallothionein (MT) 2A, 1G and 1F Although little is known about 1G; there are multiple reports focus on 2A and 1F and their link to breast cancer. MT-2A expression levels have been shown to be positively correlated with increase cancer cell numbers (Jin, Chow, & Tan, 2002) in addition to being associated with increased mortality rates among breast cancer patients (Jin, Huang, Tan, & Bay, 2004). Abdel-Mageed and Agrawal (1997) also demonstrated that the breast cancer cell line, MCF7, when over-expressed with MT-2A demonstrated increase in proliferation. Another exciting gene target is Growth Factor Independent 1 Transcription Repressor which encodes for a transcriptional repressor protein and is known to play a role in oncogenesis (Person, Li, Duan, Benson, Wechsler, Papadaki, Eliopoulos, Bertolone, Nakamoto, Papayannopoulou, Grimes, & Horwitz, 2003). Another interesting up-regulated gene target is protocadherin 8 which is known to be involved in cellular adhesion. Cellular adhesion is also known to be a key component to invasiveness (Yu, Koujak, Nagase, Li, Su, Wang, Keniry, Memeo, Rojtman, Mansukhani, Hibshoosh, Tycko, & Parsons, 2008).

Future project directions include primer design for all selected targets. Cell line HTB-26 will be counted, and seeded as described previously, treated with cortisol or the appropriate control, and allowed to incubate for 12 hours. Following incubation, cells with be lysed, RNA extracted and cDNA synthesized. Confirmation of gene targets will include RT-PCR with cDNA from the HTB-26 experimental cells. Upon confirmation,

gene targets will be assessed to see if they are essential or sufficient for cortisol mediated proliferation through selective knock-down using shRNA contructs expressed in the pSHAG1 plasmid as well as over-expression of the gene target by shuttling the cDNA into pLENTI4, both of these constructs are gateway plasmids that allow the option of recombining into a lenti-viral vector. Upon successful cloning into the knock-down vector pSHAG1 and over-expression vector pLENTI-4, any cell line can be infected and effects on cell proliferation, migration, gene expression and cell cycle progression will be performed.

The human gene fury (FRYL), originally described in drosophila, is known to be involved in ensuring the polarized cell extensions remain intact. FRYL is also thought to be involved in controlling the actin cytoskeleton, and may even work to activate transcription (The Universal Protein Resource (UniProt)). It is highly conserved which is thought to be indicative of gross selective pressures to sustain the region that is essential for protein function (Hayette, Cornillet-Lefebvre, Tigaud, Struski, Forissier, Berchet, Doll, Gillot, Brahim, Delabesse, Magaud, & Rimokh, 2005). Its homolog among yeast and *C. elegans* have been identified as being imperative for the proper functioning of the Ndr family of AGC kinases; which are known to be crucial regulators of the cell cycle as well as morphogenesis (He, Fang, Emoto, Jan, & Adler, 2005; Tamaskovic, Bichsel, & Hemmings, 2003). Although FRYL in humans remains elusive, it is most commonly associated with myeloid lymphatic leukemia, or mixed lineage leukemia (MLL) as a fusion partner (Hayette *et al.*, 2005). Three case studies looking at MLL have reported reciprocal chromosomal translocations between the MLL gene and the FRYL gene, 2 of which presented cases of breast cancer. Hayette *et al.* (2005) were the first to identify

FRYL as a novel fusion partner with the MLL gene. They reported a case study of a 67-year old female who was previously diagnosed with breast adenocarcinoma, and then was diagnosed as suffering from 'therapy-related acute lymphoblastic leukemia (ALL)' Sait, Claydon, Conroy, Nowak, Barcos, and Baer (2007) reported a case of a 49-year old female who was originally diagnosed with acute lymphocytic lymphoma, but then re-diagnosed a year later with multifocal invasive ductal carcinoma which was negative for ER and the PR.

Denso-spot analysis revealed a slight increase in gene expression from proliferation day 1 to confluency day 4 with statistical significance found for confluency day 4 ($p \leq 0.01$) and then was down-regulated during the differentiation stages with statistical significance being met for differencial days 3, 4, and 6 ($p \leq 0.01$ for days 3 and 4; $p \leq 0.05$ for day 6) of the differentiation time course and not during the proliferative stages of HC11 cell line suggesting (Figure 6A) that in normal development, FRYL levels remain constant up until differentiation where it becomes down-regulated. Quantitative Real-Time PCR analysis revealed FRYL to be significantly up-regulated ($p \leq 0.01$) in the invasive breast cancer cell line HTB-132 compared to all other breast cancer cell lines as well as the normal human breast cell line MCF10-A (Figure 6B). As stated earlier, invasive cell line HTB-132 harbour a p53 mutation. Interestingly, Wiederschain, Kawai, Shilatifard, and Yuan (2005) demonstrated that many of the MLL fusion proteins, such as FRYL, actually are capable of preventing p53 activity even when the fusion proteins were expressed at low levels; which is thought to possible explain chemotherapy resistance. Barlev, Liu, Chehab, Mansfield, Harris, Halazonetis, and Berger (2001) have even implicated MLL fusion partners, such as FRYL, are directly linked to changing normal

bone marrow cells into leukemias among animal models. Assessing the potential implications of FRYL in human breast cancer using the cDNA array in Figure 6C revealed interesting preliminary data where FRYL appears to be low during stage 0 of breast cancer then increases expression nearly 3 fold in stage 1 then showing slight decline in stages 2A and B, only to increase 2 fold in the early phases of stage 3. This could potentially indicate that early expression of this protein is implicated in initiation of specific forms of breast cancer. To date, no link between FRYL and breast cancer have been eluded, but the association between p53 and FRYL warrants further investigation. The preliminary data shown here suggests that FRYL is down-regulated in normal mammary development up until differentiation, but is severely up-regulated in the invasive cell line HTB-132 with a p53 mutation. Because the other cancer cell lines do not have this p53 mutation perhaps wild-type p53 prevents inappropriate expression of FRYL and thereby preventing potentially adverse effects mediated by this event. This association requires further investigation.

Our data demonstrates that stimulation with physiological levels of cortisol directly enhances breast cancer cell numbers and increases the rate that cells exit from G1 phase of the cell cycle. Furthermore, we have demonstrated that cortisol enhances breast cancer cell migration as measured by the ability to repair a wound as well as cell migration towards a chemoattractant. Microarray analysis has revealed a number of very interesting putative targets for these effects. We have begun confirmation of these selected targets utilizing RT-PCR. Furthermore, from a previous microarray study we have identified a potential novel gene, FRYL, which could be interacting with p53. To date, FRYL has not been linked to breast cancer, and could be an important breast cancer marker.

This unique approach to the study of tumourigenesis will play an important role in the understanding an individual's risk of breast cancer and may reveal novel biomarkers that ultimately lead to earlier diagnosis of breast cancer. Additionally, these pathways may provide targets for drug design that will improve current treatment strategies after diagnosis. Future work includes assessing these potential gene targets for their importance of these identified targets to determine if they are essential or sufficient for cortisol mediated proliferation. Collectively, this work holds tremendous promise for improving the treatment, long-term remission and quality of life for patients with breast cancer.

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