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The pleiotropic effects of dexamethasone on breast cancer cell behaviour

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The Pleiotropic Effects of Dexamethasone on Breast Cancer Cell Behaviour

by

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Co-Authorship Declaration

I hereby declare that this dissertation incorporates material that is result of joint research, as follows: This dissertation incorporates the outcome of joint research undertaken in collaboration with Janice Tubman under the supervision of professor Lisa Porter. The collaboration is covered in Chapter 2 of the dissertation. In all cases, the key ideas, primary contributions, experimental designs, data analysis and interpretation, were performed by the author, and the contribution of co-authors was primarily through the provision of technical support in the use of the zebrafish animal model.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my dissertation, and have obtained written permission from the co-author to include the above material in my dissertation.

I certify that, with the above qualification, this dissertation, and the research to which it refers, is the product of my own work.

Abstract

Endogenous glucocorticoid (GC) steroids are lipophilic hormones secreted in response to the hypothalamic-pituitary-adrenal axis. Their anti-inflammatory and immunosuppressive potency is the basis for their frequent use in clinical applications. Dexamethasone (Dex) is a synthetic GC given to breast cancer patients to reduce emetic effects of chemotherapeutic drugs (e.g., paclitaxel (Pac)). GCs mediate their effects on cell behaviour through activation of the GC receptor (GR). Active GR regulates approximately 10% of the human genome influencing numerous physiological and developmental parameters including cell proliferation, invasion, migration, and survival. The prevalent use of Dex in breast cancer treatment is disconcerting given that little is known about its impact on breast cancer cell behaviour. We show that Dex can increase the ability of triple negative breast cancer cells to survive, migrate and invade *in vitro* as well as enhance overall metastatic properties (e.g., survival, and/or motility) *in vivo*. Moreover, there is growing evidence that the ability of Dex to promote survival extends to protection from chemotherapy-induced cell death. We show that Dex protects triple negative and luminal breast cancer cells from Pac-induced apoptosis through contrary regulation of nuclear factor kappa B (NFκB) activity. We show that Pac-activated NFκB upregulates expression of the death receptor Fas and that knock-down of NFκB abrogates Pac-induced upregulation. Thus, our data supports a role for Dex antagonizing Pac through inhibition of Pac-induced NFκB transcription of Fas.

Dedication

To:

My mom and dad, Joy and Don Crozier

My sister, Jennifer Dallaire

My wife, Adina Crozier

My children, Emma Rose, Leila Evangeline, and Levi Donovan

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List of Abbreviations and Nomenclature

#	Number
°C	degree Celsius
µg	micro gram
µl	micro liter
µM	micro molar
11β-HSD	11 beta-hydroxysteroid dehydrogenase
ACTH	adrenocorticotropic hormone
AP-1	activator protein 1
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-XL	B-cell lymphoma-extra large
BrdU	Bromodeoxyuridine
CBG	corticosteroid-binding globulin
CDK	cyclin dependent kinase
cDNA	complementary DNA
CPS	counts per second
CREB	cAMP response element-binding
CRH	corticotropin-releasing hormone
Dex	dexamethasone
DISC	death inducing signaling complex
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
DUSP1	dual specificity protein phosphatase 1
ELK-1	ETS domain-containing protein
ER	estrogen receptor
ERK	extracellular signal regulated kinase
FASLG	Fas ligand
FBS	fetal bovine serum
G2	gap2
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	glucocorticoid
GR	glucocorticoid receptor
GRE	GC response element
H	hour
HER2	human epidermal growth factor receptor 2
HRP	horseradish peroxidase
IgG	immunoglobulin G

IKKβ	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
IL-6	interleukin 6
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, α
JNK	c-Jun N-terminal kinases 1 and 2
M	Mitosis
MAP3K1	mitogen-activated protein kinase kinase kinase 1
Mg	milli gram
MHC-1	major histocompatibility complex class 1
Min	Minute
Ml	milli liter
MMP	Metalloproteinase
MMP-2	matrix metalloproteinase-2
MMP-9	matrix metallopeptidase 9
MPK-1	mitogen-activated protein kinase 1
mRNA	messenger RNA
MT	Metallothionein
NFκB	nuclear factor of kappa light polypeptide gene enhancer in B-cells
NR3C1	nuclear receptor subfamily 3, group C, member 1
Pac	Paclitaxel
PAGE	polyacrylamide gel electrophoresis
PAI-1	plasminogen activator inhibtor type 1
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
PR	progesterone receptor
PVDF	polyvinylidene fluoride
qRT	quantitiative real time
RELA	v-rel avian reticuloendotheliosis viral oncogene homolog A
RNA	ribonucleic acid
Rpm	revolutions per minute
SDS	sodium dodecyl sulphate
Sec	Second
SEM	standard deviation of the mean
SGK-1	serum/glucocorticoid regulated kinase 1
shRNA	short hairpin RNA
STAT-5	signal transducer and activator of transcription 5
TNBC	triple negative breast cancer
tPA	tissue plasminogen activator
uPA	urokinase-type plasminogen activator 1
VEGF	vascular endothelial growth factor

Chapter 1: General introduction

1.1 Breast cancer

1.1.1 Canadian breast cancer statistics

It is currently estimated (2014), that approximately two in every five Canadians will develop cancer at some point in their lives; nearly one in four Canadians will die of cancer [1]. Cancer is a collection of diseases and of the (approximate) 200,000 new diagnoses of cancer in Canada in 2014, more than half will fall into only one of four types: lung, breast, colorectal, and prostate [1]. While men have a slightly higher likelihood of developing cancer (45%) compared to women (41%), the most prevalent type of cancer in women (breast cancer) has yielded almost triple the number of potential years of life lost than has the most common cancer in men (prostate) due to the average age at which it typically presents [1]. Breast cancer is the most common type of cancer in Canadian women accounting for over 26% percent of the more than 93,000 new cases of cancer in women in 2014 [1].

1.1.2 Breast cancer metastasis

While breast cancer starts through formation of a primary tumour at a local site, it can eventually metastasize to proximal lymph nodes and/or intravasate into blood vessels traveling to distant sites [2]. It is metastasis to distant sites, rather than primary tumours, that are the greatest cause of cancer related deaths, accounting for over 90% of cancer mortality [2,3]. Nearly 15% of breast cancer patients have aggressive disease and can develop metastasis in under three years following diagnosis [2]; the most common distant sites of metastasis being liver, lung, and bone [2,3]. Metastasis of breast cancer follows a similar pattern as other solid tumours in that several key steps are necessary: 1) the ability of tumour cells to survive (elude apoptosis) and proliferate at the primary tumour site; 2)

the ability to invade surrounding tissue at the primary site; 3) the ability of the cells to migrate in the circulation or neighbouring tissue and 4) attachment and colonization at the distant site [4].

1.1.2.1 Breast cancer metastasis: survival/proliferation

Recent studies report that inhibition of apoptotic pathways are a critical characteristic of metastatic cancer cells [4], as tumour progression correlates with the loss and/or gain of function of pro- and/or anti-apoptotic markers respectively [5]. Apoptosis is the most common programmed cell death pathway in vertebrate organisms and is regulated by a number of protein ligands, receptors and proteases. In the extrinsic pathway, for example, secreted or membrane-bound Fas ligand (FasLG) binds and activates Fas receptor (Fas), which causes formation of a death inducing signaling complex (DISC) that recruits and activates caspase 8 [6]. Caspase 8 in turn activates several effector caspases including caspase 3 and caspase 7 which ultimately results in cell destruction [6]. Thus, modulators of cell survival and proliferation, e.g., inhibitors of the extrinsic pathway, whether inherent, or acquired by tumour cells, or even as an accidental by-product of medical drug treatment, could ultimately have a serious impact on the prevention or promotion of metastatic breast cancer.

1.1.2.2 Breast cancer metastasis: invasion and migration

Metastasis is an incredibly complex multi-step process that requires the ability of cells to survive and grow at the primary site, to infiltrate through the resistant barriers of surrounding tissue, as well as the ability of cells to migrate in the circulation of newly invaded tissues [2-4]. An important role for proteases that can degrade extracellular matrices and aid in intra- and extra-vasation has been well documented in these processes

[7,8]. For example, recent *in vitro* observations have identified the mutualistic role of metallothioneins (MTs) and metalloproteinases (MMPs) (e.g., MT-2A and MMP-9) in promoting cell invasion and migration in breast cancer cells [9]. Thus, modulation of mediators of invasion and/or migration, e.g., MT and MMPs, whether intrinsic, or acquired by tumour cells, or even as an accidental by-product of medical drug treatment, could ultimately have a serious impact on the prevention or promotion of metastatic breast cancer.

1.1.3 Breast cancer subtypes

Even within one disease site cancer is not a single disease, but a collection of diseases referred to as subtypes. Breast cancer subtypes can be defined very strictly by gene expression profiles [10], which yields no less than four breast cancer subtypes: luminal, normal breast-like, HER2, and basal-like [10]. Currently, classifying breast cancer patient samples at the level of gene expression profiles is proving difficult under ideal situations [11,12]. Moreover, in clinical settings it is simply not feasible, although steps to overcome existing obstacles are being pursued and developed [13]. Current practice, most common in clinics, is the classification of breast cancer samples based on the presence or absence of particular steroid hormone receptors: estrogen receptor and progesterone receptor. While the receptor status does correlate loosely with molecular sub-typing [14], exceptions exist [15].

One of the histological commonalities of the luminal, breast normal-like, HER2, and even some basal-like breast cancers is the presence of well-defined molecular targets, primarily in the form of the aforementioned protein and hormone receptors [15]. The presence of these molecular characteristics provides the basis for targeted treatment and

accounts for much of the success in treating these forms of breast cancer. Drugs that target the hormone receptors by blocking them, e.g., Tamoxifen [16], or blocking the production of hormones such as estrogen, e.g., Anastrazole, yield much success.

Approximately 15% of breast cancers, however, do not express the estrogen or progesterone receptor and do not display amplification of the HER2 gene [15]. These breast cancers are termed triple negative breast cancers (TNBCs). TNBCs are more aggressive, yield shorter survival rates, and higher percentages of relapse in TNBC patients [15]. Patients with TNBC have approximately 14% higher distant site recurrence rates, and recurrence occurs much quicker, within nearly half the mean number of years than other breast cancer subtypes [17]. Thus, metastasis, through invasion and migration and subsequent proliferation, occurs more frequently and in shorter time frames for TNBC than for other breast cancer subtypes. With over 90% percent of cancer mortality resulting from metastasis, inhibition of these aggressive cell behaviour traits is of paramount importance [18].

Currently, without specific molecular targets, TNBCs cancers are typically treated with cocktails of potent cytotoxic chemical therapies [15], including doxorubicin, cyclophosphamide, 5-flurouracil, and docetaxel, or a combination of these, to name a few. One very commonly prescribed chemotherapy drug is the potent anti-neoplastic microtubule poison paclitaxel (Pac). An unfortunate effect of Pac, however, is that it frequently causes allergic reactions in patients [19,20]. Moreover, Pac is insoluble in water and is dissolved in castor oil, marketed as Kolliphor EL/Cremophor EL, which causes hypersensitivity reactions and nausea and vomiting. Pac also causes rashes, dyspnea, hypotension, and urticaria [21]. To combat these effects, patients receiving Pac

are commonly pre-treated with the anti-inflammatory, anti-emetic synthetic glucocorticoid (GC) steroid dexamethasone (Dex) [20,22].

1.2 GCs: mechanism of action

GCs are lipophilic steroid hormones that regulate a plethora of physiological processes involved in defense, metabolism, cell survival and development [23-25]. The main GCs produced in mammals are cortisol and corticosterone [24]; cortisol predominating in humans [24]. Cortisol is produced in the zona fasciculata of the adrenal cortex. Its secretion from the adrenal cortex can be increased beyond basal levels in response to adrenocorticotropic hormone (ACTH) from the anterior pituitary. Under conditions of stress the anterior pituitary is stimulated to secrete ACTH by corticotropin-releasing hormone (CRH) from the hypothalamus [24]. The overall hypothalic-pituitary-adrenal axis is also regulated by a negative feedback loop in which increasing levels of blood cortisol inhibits the hypothalamus and/or anterior pituitary from CRH and/or ACTH secretion respectively [24]. While cortisol is a lipophilic molecule, most serum cortisol is not free to cross cell membranes, as it is bound to a carrier protein corticosteroid-binding globulin (CBG) [24]. Also important in GC activation of its target receptor is intracellular, pre-receptor metabolism. Inactive GCs (e.g., cortisone in humans) are converted to cortisol by 11 beta-hydroxysteroid dehydrogenase (11β-HSD) [24] .

Reports on the clinical use of cortisol and cortisone as therapeutics for inflammatory disease (e.g., rheumatoid arthritis, asthma, etc.) were first presented by Dr. Philip Hench and colleagues at the Seventh International Congress on Rheumatic Diseases in 1949. For the past 60 years their use has revolutionized the medical field of

treating inflammatory disease. GCs are now known to modulate, whether positively or negatively, as much as 10% of the human genome [24]. Early clinical use presented difficulties however due to the pleiotropic effects of cortisol. In the 1950's numerous groups competed to develop synthetic GCs to minimize unwanted side-effects. Some of the synthetic drugs developed during that period continue to be used regularly today; some examples include: prednisolone, prednisone, fludrocortisone, methylprednisolone, triamcinolone, paramethasone, betamethasone, and Dex [26] .

The description of the molecular mechanism of GCs to follow will not focus on the particular nuances of each synthetic derivative of cortisol. Rather, a general description of GCs mechanisms of action is described. Because the research experiments of this dissertation use Dex, any notable distinctions in molecular mechanism of action specific to Dex will be identified when and if necessary. Despite the molecular mechanism of GCs action being similar from tissue-to-tissue, the target genes and pathways affected often vary between tissues. For example, GCs cause programmed cell death in many hematopoietic cell lines, but enhance cell survival in solid tissue cell types [27].

GCs exert their effect through the cytoplasmic bound nuclear receptor NR3C1 (nuclear receptor subfamily 3, group C, member 1), also known as: the GC receptor (GR) [28]. The GR is held in a stable inactive conformation in the cytoplasm by an inhibitory complex of proteins including heat-shock proteins 90, 70 and immunophilins [29]. There is also evidence that in certain tissues, the GR interacts with the cytoskeleton when inactive [5]. Ligand bound GR can act both genomically (transactivation) and non-genomically (transrepression) [25]. Genomically the ligand-bound GR homo-dimerizes

and translocates to the nucleus where it binds to GC response elements (GREs) in the promoter region of its target genes [25]. The GR has also been reported to block access to gene promoters, out-competing other transcription factors through DNA binding. Non-genomically, the active GR can bind other transcription factors through protein-protein interaction (e.g., CREB, NFκB, AP-1, STAT-5, etc.), thus blocking their translocation to the nucleus and indirectly inhibiting the transcriptional upregulation of their respective target genes [25].

Overall Dex is a potent steroid hormone regulating numerous genes involved in a plethora of cellular pathways. Dex is commonly used as an anti-emetic in diverse chemotherapy settings including, but not limited to, treatment of ovarian cancer [30], bladder cancer [22], and colon cancer [31]. Standard clinical protocol regimens list pre-medication with Dex at a dose of 20 mg given orally 12 and 6 h prior to Pac although variance occurs in literature with some prescribing Dex 1 h prior to Pac and other multiple times/day leading up to Pac infusion. At the cellular level it is estimated that Dex concentration is approximately 1μM and Pac approximately 0.1 μM. With TNBC patients receiving Pac, and thus being pre- and co-treated with Dex, it is of clinical significance to understand what impact, if any, Dex has on breast cancer cell behaviour (Chapter 2 of this dissertation) and response to chemotherapy drugs (the efficacy of Pac treatment is addressed in Chapter 3 of this dissertation).

1.2.1 GCs and cancer cell proliferation

Reports concerning the impact of Dex on cancer cell proliferation present an unclear picture. Ambiguity on the matter may be due to any one of the three following reasons: 1) imprecise use of terminology e.g., conflating “apoptosis” and “cell cycle

arrest". For example, Chung *et al* [32] cite Frankfurt *et al* [33] and Goya *et al* [34] as being reports that prove that GCs "inhibit proliferation", where Frankfurt *et al* actually demonstrated GCs cause apoptosis of hematologic cells and Goya *et al* demonstrated GCs mediate cell cycle arrest in rat mammary cells. While both works demonstrate a role for GCs preventing cells from dividing, the mechanisms are very distinct and are only loosely related to GCs direct impact on proliferation, as naturally, dead cells do not divide. 2) Another cause for lack of certainty regarding the role of Dex in cell proliferation is that some reports may actually overreach the extent of the supporting data e.g., conflating Dex-mediated protection/cell survival with increases in proliferation. For example, a recent report on Dex-mediated increases in proliferation in MCF7 cells was supported only by cell viability assays [35]. One concern with this report is the possibility that Dex increased the survival of cells allowing them to continue dividing while non-treated cells slowed or arrested in their division. The point being that, Dex-mediated protection from cell cycle arrest is different from Dex-mediated induction of cell division. Another example is Zheng *et al's* recent working claiming that Dex induces cell proliferation in bladder cancer cells [22]. The assay used to make this claim was a cell viability assay that measures mitochondrial activity (MTT assay). For the same reason as above, these viability assays do not necessarily entail increases in proliferation. Given that corroborating data was elusive in the study (i.e., they reported no change in proliferation markers, e.g., cyclins or CDK's) their claims were tempered to a pro-survival role for Dex although the word 'proliferation' was often used interchangeably with 'survival'. Several groups have been very careful to temper their claims on the matter of Dex-induced proliferation. Pang *et al*, for example, observed increases in

tumour growth in xenografts of human breast cancer cells in mice. Without corroborating data (i.e., they observed no change in Ki-67 levels in Dex treated cells), Pang *et al* suggested that the difference in tumour sizes compared to non-Dex treated cells might be attributable to a pro-survival or anti-apoptotic role of Dex rather than a proliferative role. 3) Confusion may also be due, in part, to the tissue-specific nature of GR regulated genes. There is abundant literature showing the pro-apoptotic role of GCs in hematopoietic cells [36,37] and so GCs are often described as generally being pro-apoptotic [24]; and yet they clearly play an anti-apoptotic role in solid tumour cells [30,31]. There is no contradiction here; depending on the cell type, GCs have different effects on cell behaviour as pertains to known phenotypes (e.g., effect on apoptosis). Given that the ability to elude apoptosis and continue growing at the primary tumour site is a crucial characteristic of metastatic breast cancer cells [3], and that breast cancer patients are commonly administered Dex it is of clinical significance that the impact of Dex on these phenotypes be fully assessed.

1.2.2 GCs and cancer cell invasion and migration

Two other characteristics essential to metastasis of breast cancer cells is their propensity to invade and migrate. The subject matter of Dex and cancer cell invasion and migration has been reported on in several tissue types. Shiratsuchi *et al* show that activator protein 1 (AP-1) transcriptionally upregulates urokinase-type plasminogen activator 1 (uPA) in human squamous cell carcinoma cell lines [38], as part of its program to degrade extra-cellular matrix and promote cell motility. AP-1 is a known target of active GR transrepression [39] and uPA is also known to be suppressed by Dex, not only as demonstrated by Shiratsuchi *et al*, but also in other tissues [40]. In short, Dex

antagonizes an important mediator of invasion in these cells. Other studies pertaining to Dex regulation of invasion and migration such as Piette *et al's* study in glioma cell lines (U373 MG) implicate a role for Dex-mediated inhibition of extracellular signal regulated kinase 1/2 (ERK1/2) through transcriptional regulation of dual specificity protein phosphatase 1 (DUSP1) [41]. DUSP1 inactivates ERK1/2 by dephosphorylation resulting in decreased proliferation, migration and invasion [41]. Zheng *et al* also report decreases in cellular invasion in bladder cancer cells following treatment with Dex [22]. It is of clinical significance to determine what impact Dex might have on breast cancer cell invasiveness and migratory capacity. If Dex increases these negative characteristics of breast cancer then alterations in current clinical protocol are warranted. If, on the other hand, as reported in the aforementioned tissue cell lines, Dex inhibits invasion and migration, elucidating the mechanisms by which Dex mediates its effects could provide better therapeutic strategies.

1.2.3 GCs and cancer cell survival

Although, as mentioned above, GCs (including Dex) are commonly thought of as pro-apoptotic [24], there is significant data showing their pro-survival role in solid tumour cells [30]. Numerous studies in diverse tissues and organisms support a pro-survival/anti-apoptotic role for Dex. For example, human and rat hepatocytes [42], through B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma-extra large (Bcl-XL) signaling, are protected from spontaneous apoptosis; human mammary epithelial cells are protected from serum withdrawal through upregulation of serum and glucocorticoid kinase 1 (SGK-1) signaling; and kidney cells are protected from puromycin aminonucleoside-

induced apoptosis through contrary regulation of Bcl-2 associated X protein (Bax) and p53 signaling [43]; all through Dex treatment.

Most relevant to the current work, and of clinical significance, is that apart from protection against natural stressors and serum withdrawal conditions, increasing data demonstrates that Dex can mediate protection against drug-induced apoptosis; in particular against chemotherapy-induced apoptosis. Early studies in mice suggested that the protective role of Dex could be a beneficial byproduct of using GCs in chemotherapy, as Dex was reported to protect bone marrow cells from unwanted cytotoxic effects of chemotherapy [44]. In that study, the chemotherapy drug Dex protected against was the pyrimidine analog 5-fluorouracil [44]. Huang *et al* also reported GC mediated protection from another group of chemotherapy agents, namely, the *Vinca* alkaloids vincristine and vinblastine [45]. In their study GCs protected breast cancer BCap 37 and epidermoid tumour KB cells against *Vinca* alkaloid induced apoptosis, although the GC used was Triamcinalone acetonide (TA) and not Dex specifically [45].

At least two pathways have been identified as playing putative roles in Dex-mediated rescue from Pac-induced cell death in breast cancer: DUSP1 and IκBα/NFκB signaling. Wu *et al* demonstrated through microarray analysis that early response genes, including SGK-1 and DUSP1 were upregulated by treatment with Dex, and that these genes could aid in mediating resistance to Pac [46] whether through ectopic expression or through their Dex-induced upregulation. Knockdown of these proteins resulted in a decrease of Dex's ability to rescue the cells from Pac-induced apoptosis. Wu *et al* later proposed a pathway in which Dex causes transcriptional upregulation of DUSP1 through GR; DUSP1 activity leads to dephosphorylation and inactivation of ERK 1/2 and c-Jun

N-terminal kinases 1 and 2 (JNK 1/2) [47]. They observed a correlative decrease in ETS domain-containing protein (ELK1) phosphorylation (a downstream target of ERK 1/2 and JNK 1/2) [47]. Loss of activation of ELK1 correlated with decreases in Pac-induced activation of tissue plasminogen activator (tPA) [47]. Downstream targets of Pac-activated tPA, however were not elucidated [47].

The IκBα/NFκB signaling pathway has also been identified as a putative participant in GC-mediated rescue of ovarian, epidermoid and breast cancer cells from Pac-induced apoptosis. Most of the studies examining the role of GC mediated rescue for Pac and IκBα/NFκB signaling used the aforementioned synthetic GC TA [48] and not Dex, but activation of GR was responsible for the GC-mediated effects nonetheless. Fan *et al* had previously demonstrated that TA could rescue BCap 37, ovarian (OV2008), DDT_1 MF2 smooth muscle cells, which are a leiomyosarcoma cell line derived from hamster ductus deferens, as well as Rat breast cancer cell lines (Con8 and 8RUV7) from Pac-induced cell death [49,50]. Moreover, at that time, elucidation of Pac's diverse mechanisms of action was developing. Fan suggested that Pac could induce apoptosis in one of three ways: 1) indirectly through activation of mitotic arrest. Microtubule stabilization by Pac would cause cells in G2/M arrest to activate cell programmed death pathways [51]. 2) Indirectly through microtubule stabilization and apart from mitotic arrest; since microtubule dynamic instability is essential for normal functions in a cell, Fan proposed that stabilization of microtubules by Pac could activate death pathways [51]. 3) Directly though gene regulation; Fan proposed that since GCs do not interfere with Pac-induced microtubule stabilization, yet antagonize Pac-induced apoptosis, perhaps Dex mediated its effects through inhibition of an essential component of Pac's

program [51]. At the time of Fan's work, novel reports of NFκB as a potential mediator of apoptosis [52,53] were being published.

Since NFκB was known to be inhibited by GC/GR signaling, Fan's group assessed what impact Dex might have on Pac-regulation of NFκB signaling. Huang *et al* were able to demonstrate that certain components of the upstream regulatory pathways of NFκB were affected by Pac [48,54]. Moreover they were able to demonstrate that the most likely point of antagonism between Dex and Pac, as pertains to NFκB signaling was in Dex's ability to transcriptionally upregulate IκBα, the protein inhibitor of NFκB that keeps NFκB sequestered in the cytoplasm [48,54]. Further upstream, and unaffected by Dex, Pac treatment of BCap37 and OV2008 cells caused increases in mitogen-activated protein kinase kinase kinase 1 (MAP3K1) transcripts [48,54]. MAP3K1 causes phosphorylation and activation of IKKβ, which Fan showed to cause phosphorylation of IκBα. Phosphorylation of IκBα results in its degradation and the subsequent release of NFκB for nuclear translocation. These phenotypes were not demonstrated with Dex, nor was it reported if these phenotypes held true for other breast cancer cell lines. Moreover, the downstream targets for Pac-activated NFκB were not identified, leaving questions as to whether or not, and how, NFκB was directly part of the apoptotic program of Pac [48,54].

1.3 Summary

Breast cancer is the most prevalent form of cancer in Canadian women; approximately 14 women die each day in Canada from the disease. It is metastasis of breast cancer to distant sites that is the leading cause of cancer mortality and not the disease at the local primary tumour site. Metastasis of breast cancer, as with other solid

tumour metastasis, requires several key stages and characteristics of tumour cells: 1) the ability of tumour cells to survive (elude apoptosis) and proliferate at the primary tumour site; 2) the ability to invade surrounding tissue at primary site; 3) the ability of the cells to migrate in circulation or neighbouring tissue and 4) attachment and colonization of distant site. Chemotherapy is a potent tool for treating breast cancer. It is primarily used for treating the most aggressive breast cancers, namely TNBCs, however it is also used for late stage aggressive luminal cancers and luminal cancers that have developed resistance to conventional therapies. One of the most commonly used chemotherapy drugs is Pac. Because Pac causes hypersensitivity reactions and emetic reactions in patients, clinicians administer the synthetic anti-emetic steroid Dex. As a GC, Dex can regulate a plethora of genes through activation of GR. These genes are implicated in numerous developmental and physiological processes including, but not limited to, cell survival, proliferation, invasion and migration. Because Dex is administered in chemotherapy regimens, it is of clinical significance to assess what impact, if any, Dex treatment might have on cancer cell behaviour. Moreover, it is of clinical significance to assess what impact Dex might have on the efficacy of Pac in killing breast cancer cells. Elucidating the molecular mechanisms of Dex-mediated protection from Pac could help us tailor current clinical regimens and provide insight into Pac-mediated cancer cell death, thus providing direction for developing more effective treatments for breast cancer patients.

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Chapter 2: Dexamethasone augments metastatic characteristics of breast cancer cells *in vitro* and *in vivo*

2.1 Introduction

Breast cancer is a heterogeneous disease that clinicians classify into subtypes based on the histological presentation or absence of protein receptors, e.g., estrogen receptor (ER), progesterone receptor (PR), and epidermal growth factor receptor HER2/neu [1,2]. These receptors serve as molecular targets for many conventional anti-cancer therapies, e.g., Tamoxifen and Trastuzumab. Cancers that present ER and PR are generally classified into the luminal subtype, while those lacking these receptors are termed basal breast cancers. One subclass of basal breast cancer is termed triple negative breast cancer (TNBC) as they lack expression of the ER, PR, and amplification of HER2. TNBCs, representing approximately 15% of breast cancers, are one of the most aggressive forms of the disease and are among the most difficult to treat as they lack the aforementioned molecular targets [3]. To deal with these, clinicians commonly rely on cytotoxic drugs such as paclitaxel (Pac) [3].

Pac is a microtubule stabilizing agent that causes dividing cells, whether cancerous or not, to arrest in metaphase of mitosis [4,5]. Downstream of microtubule stabilization, or even through gene regulation independent of microtubule stabilization, Pac can cause apoptosis [6-9]. Pac is a very effective chemotherapeutic agent that has saved countless cancer patient's lives over the past twenty years [10]. Unfortunately Pac, and in many cases the vehicle in which it is dissolved (Kolliphor EL), causes unwanted side-effects. To lessen and even prevent many of these side-effects, glucocorticoids (GCs), e.g., dexamethasone (Dex), are administered in advance of chemotherapy [11].

Dex mediates its anti-emetic effects through the glucocorticoid receptor (GR). Active GR can regulate gene expression of approximately 10% of the human genome

[12]. Active GR is involved in the development and regulation of a plethora of physiological processes including, but not limited to: inflammation, blood pressure, sensitivity to catecholamines, neuronal and glial cell activity, brain, breast, and bone development, homeostasis of body temperature, as well as carbohydrate, protein, and lipid metabolism [12]. Moreover, GCs can affect the cellular process of division, survival, apoptosis, migration and invasion [13-15]; critical processes also implicated in metastasis of cancer.

In general, most breast cancer related deaths are due to metastatic spread of the disease and not the primary tumour itself [16]. For metastasis to occur, no less than three important cellular characteristics are needed: 1) the ability to survive (elude apoptosis) and grow/proliferate at the primary tumour site; 2) the ability to invade through boundaries at the primary site, and 3) the ability to move or migrate, whether in circulation or within neigbouring tissues [17].

Given that Dex, a potent modulator of cell behaviour, is administered to TNBC patients hours, and, in some cases, multiple times per day in advance of chemotherapy, we sought to examine what impact Dex might have on breast cancer cell behaviour focusing primarily on TNBC cells [18]. Patients with luminal cancers that have developed resistance to conventional therapies or present with aggressive forms of the disease may also receive Pac, and therefore Dex, hence we also analyzed luminal cancer cell behaviour following Dex treatment.

We found that GR levels correlated to breast cancer subtypes with TNBCs showing the highest expression on average, and the luminal breast cancers showing

relatively low expression. We also found that treatment of breast cancers cells with Dex increased overall cell numbers, invasiveness, and migratory capacity, compared to non-treated cells, and that TNBCs demonstrated the most pronounced phenotypes in response to Dex.

2.2 Material and methods

Cell culture

Human breast cancer cells MCF7 (HTB22; gift from Tiffany Seagroves; University of Tennessee: Health Science Center); MDA-MB-231 (HTB26; ATCC), Hs578t (HTB126; ATCC), and MDA-MB-468 (HTB132; ATCC) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; D5796; Sigma). T47D cells (HTB-133; ATCC) were cultured in RPMI-1640 Medium (R8758; Sigma) with 2 units/ml of insulin (I-5500; Sigma). SK-BR-3 cells (HTB-30; ATCC) were cultured in McCoy's 5A Medium (30-2007; ATCC). All cells were supplemented with 10% fetal bovine serum (FBS; F1051; Sigma) and 1% Penicillin and Streptomycin and were maintained in an atmosphere of 5% CO_2 at 37˚C. For passaging, seeding, and quantification of cell numbers, cells were collected with 0.25% trypsin and counted using the TC10™ Automated Cell Counter from BioRad (Catalog #145-0010). Cell media was changed from complete media to serum-free media 24-hours prior to treatment. Serum-free media was replaced with media containing charcoal-treated FBS (10%) during the treatment stage.

Compounds and antibodies

The following antibodies were used at a dilution of 1:1000: Actin (MAB1501R; Chemicon) and GR-α (Cat. # 3626-1; Epitomics). Secondary antibodies used were HRP-conjugated anti-mouse IgG (A9917; Sigma) and anti-rabbit IgG (A0545: Sigma). Charcol (C6241; Sigma), Paclitaxel (T7402; Sigma), Dexamethasone (DN1187; BioBasic), and RU-486 (Mifepristone; M8056; Sigma).

Immuno-blotting

Samples were lysed with 0.1% NP40 buffer supplemented with Leupeptin (10 µg/ml; 103476-89-7; BioBasic), Aprotinin (10 µg/ml; A3428; Sigma), and PMSF (1 mM; DB0425; BioBasic). Samples were analyzed by 10% SDS-PAGE and then transferred to a PVDF membrane. Primary antibodies were applied and incubated over night at 4°C at dilutions specified above. Proteins were detected via treatment with Perkin-Elmer Enhanced Chemiluminscence reagent/ECL Western Gel Substrate (NEL10S, Perkin Elmer and quantified using FlourChem HD2 software (AlphaInnotech; Perkin Elmer).

Apoptotic assays

Caspase 3/7-glo assay (Promega; G8090) was used to measure the apoptotic state of treated cells. 24 h post-treatment cells were collected via trypsinization and lysed. 50 µl of Caspase-Glo® 3/7 reagent was added in each well of a white-walled 96-well plate containing 50 µl of lysis buffer as blank, negative control cell lysates, or treated cell lysates with the final concentration of 1 µg/µl. Contents were gently mixed in the wells using a plate shaker at 300-500 rpm for 30 sec. Cell lysates were incubated at room temperature for 30 min and the luminescence of each sample was measured using Wallac Victor 1420 plate reader.

Migration assay

Cells were seeded (1×10^5) in 500 µL of serum-free media in Falcon Cell Culture Inserts (353182; Becton-dickinson) in the wells of a 12 well cell culture plate with 1 ml of complete media (serum-free control). Cells were treated with ethanol (vehicle control) or different concentrations of Dex (0.1 µM, 1 µM, 10 µM, 50 µM, or 100 µM) and

incubated for 24 h. Following treatment, the inserts were carefully taken out, cells that did not migrate through the pores and therefore remained on the upper side of the filter membrane were gently removed, and the migrated cells were quickly stained with 400 µL of 1% crystal violet in 2% ethanol for 10 min. The inserts were then merged in water to remove excess crystal violet and air-dried. Different views of the cells attached to the membrane were imaged using a Leica microscope. The crystal violet was then released with extraction buffer and the absorption of the samples was measured at 590 nm using a Wallac Victor 1420 plate reader.

Invasion assay

Prior to seeding, cell culture inserts were coated with 100 µl of Cultrex BME (3433; Trevigen), diluted to 5 mg/ml, for 4 h at 37°C to gel. Cells were then seeded (1 x 10^5) in 500 µL of serum-free media in inserts in the wells of a 12 well cell culture plate with 1 ml of complete media (serum-free control). Cells were treated with ethanol (vehicle control) or different concentrations of Dex (0.1 µM, 1 µM, 10 µM) and incubated for 24 h. Following treatment, the inserts were carefully taken out, cells that did not migrate through the pores and therefore remained on the upper side of the filter membrane were gently removed, and the migrated cells were quickly stained with 400 µL of 1% crystal violet in 2% ethanol for 10 min. The inserts were then merged in water to remove excess crystal violet and air-dried. Different views of the cells attached to the membrane were imaged using a Leica microscope. The crystal violet was then released with extraction buffer and the absorption of the samples was measured at 590 nm using a Wallac Victor 1420 plate reader.

Animal care and handling

Wild-type Zebrafish (*Danio rerio)* were handled in compliance with local animal care regulations and standard protocols of Canada. Adult fish were kept at 28.5°C and bred according to protocols available in the Zebrafish Book [19].

Implantation procedure, treatment, and imaging

Zebrafish eggs were collected after fertilization and kept in E3 embryo media (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 10^{-5}% Methylene Blue) at 28°C in an incubator until ready to inject. Before injection 200,000 cells in 200 µl of serum-free media were labeled with 1 µL of DiD (red) (Vybrant; V-22887; Invitrogen) at 37°C for 20 min. Cells were washed with 200 µl of serum-free media twice and resuspended in 20 µl of serum-free media, kept at 37°C for 20 min, and placed on ice until ready to inject. 48 h post-fertilization (hpf) the embryos were dechorionated with fine tip forceps and anesthetised with 0.168 mg/ml of Tricaine (MS-222; Sigma). 50-100 labeled cells/9.2 nl were loaded into glass capillary needles and injected into the yolk sac of each embryo using a Nanoject II (FSSP9706473; Fisher Scientific). After injection, embryos were placed in E3 embryo media and 1 h post-implantation (hpi) were examined using a Leica fluorescence stereomicroscope to exclude any embryo with cells outside of the implantation area. Following injection, zebrafish were transferred to 96-well plates, with one zebrafish per well. Dex was diluted to a final concentration of 1 µM in fish water and added to each well of the treatment fish 1 hpi. 24 hpi and 24 h post-treatment (hpt) the fish were anesthetized with 0.168 mg/ml Tricaine in a 96-well plate, with one embryo per well. The embryos were imaged using a Leica fluorescence microscope.

All image analysis was completed using ImageJ software and was adapted from a previously described method for animal bio-imaging assays [20]. The image sequence for each embryo was imported into ImageJ and the image was rotated for each embryo so that the injection sites would be aligned. The images were converted to a 32-bit gray-scale and the threshold was adjusted to eliminate background pixels. The injection sites were chosen as the midpoint of the yolk sacs. Using the measure function, the exact coordinates for the injection site were measured. The Analyze particle tool was then used to record the coordinates of each labeled cell foci within the entire embryo. The coordinates of each tumour foci were corrected to the injection site coordinates using the formula: $(X_{foci}-X_{origin}, Y_{origin}-Y_{foci})$. For each focus the distance travelled from the injection site was calculated using the formula: $\sqrt{(X_{corrected}^2+Y_{corrected}^2)}$. The cumulative distance (CD) of all foci was calculated per embryo and averaged within an experimental group to determine mean CD.

Statistical analysis

Student *t* test was employed using Statistica software. All results are expressed as mean.

2.3 Results

2.3.1 TNBCs express higher levels of GR.

TNBC patients are treated with the anti-emetic steroid Dex 1, 6, or 12 h or even multiple times per day immediately in advance of, or, in some cases, for days leading up to, chemotherapeutic treatment with Pac [21,22]. Dex mediates its effects through the GR. We therefore assessed the relative levels of GR across a panel of breast cancer cell lines. Three were of the TNBC subtype (MDA-MB-231, Hs578t, MDA-MB-231), and three were of the luminal subtype classification (MCF7, SK-BR-3, T47D) (Fig. 2.1). The highest levels of expression were presented by the TNBC cells line with the highly aggressive MDA-MB-231 cells displaying the highest levels, followed by the Hs578t cells and the MDA-MB-468 cells respectively (Fig. 2.1, upper panel). The highest level of expression in the luminal cell lines was the MCF7s, with SK-BR-3 and T47D showing much lower expression, respectively (Fig. 2.1, upper panel).

Figure 2.1

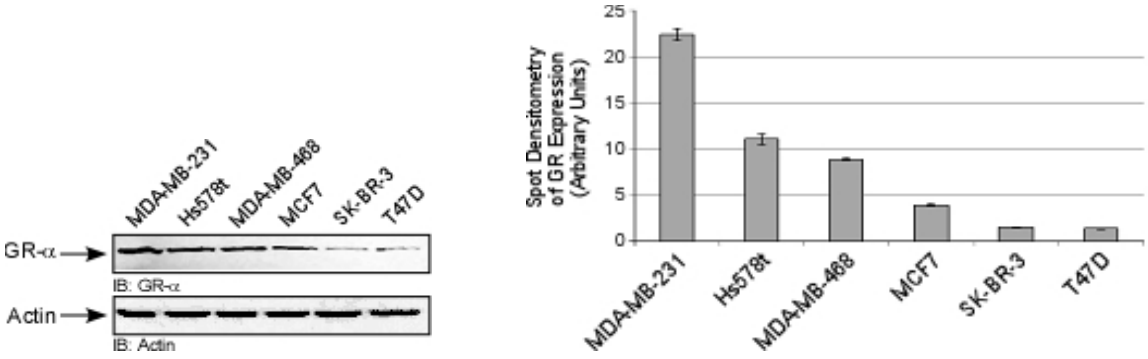


Figure 2.1 Relative expression of GR in TNBC and luminal breast cancer cell lines.

TNBC cell lines MDA-MB-231, Hs578t, and MDA-MB-468 and luminal breast cancer cell lines MCF7, SK-BR-3 and T47D were lysed and subjected to Western blotting. Endogenous GR-α levels were measured by immune-blotting. Densitometry analysis of three separate experiments, indicating GR protein levels normalized to Actin, (lower panel) is represented as mean ± SEM.

2.3.2 Dex increases cell numbers of breast cancer cells *in vitro*.

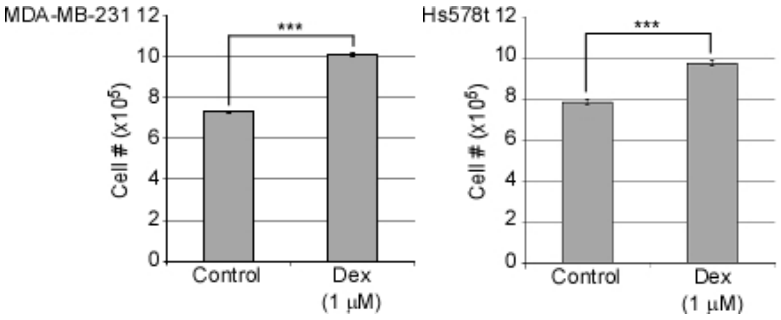
The ability of breast cancer cells to survive at the primary tumour site and to be able to grow and proliferate is of critical significance for metastasis [17]. To assess whether or not Dex affects the survival and growth of breast cancer cells, we treated breast cancer cells representing both TNBC (MDA-MB-231 and Hs578t), and luminal subtypes (MCF 7, SK-BR-3, and T47D) with vehicle (control), Dex (1 μM) for 24 h to mimic clinical protocol (Fig. 2.2). The two TNBC cell lines, MDA-MB-231 and Hs578t, showed the greatest total cell number at 38% and 24% differential between Dex-treated and control cells (Figs. 2.2A and 2C). The highest differential between Dex-treated and control cells amongst the luminal breast cancer cell lines occurred in MCF7s with 22% difference. (Figs. 2.2B and 2C); the MCF7s have the highest GR expression amongst the luminal cell lines (Fig. 2.1). The SK-BR-3 and T47D cell lines displayed the smallest difference in cell number between treated and control cells at 8% and 7% differential respectively (Figs. 2.2B and 2C). These findings correlate with GR protein levels (Fig. 2.1).

To assess whether the differential in cell number between Dex-treated and control cells was due to proliferative activity or anti-apoptotic activity, we analyzed caspase 3 and 7 activity under the same conditions using the TNBC cell lines (MDA-MB-468 and Hs578t) and the luminal (SK-BR-3 and T47D) cell lines (Figs. 2.2D and 2E). All four cell lines showed decreases in caspase 3 and 7 activity indicating that Dex-mediated difference in cell number between Dex-treated and control cells may be a result of a Dex-induced pro-survival and not necessarily increases in proliferation. The cell lines expressing the highest levels of GR also demonstrated greater sensitivity to Dex-

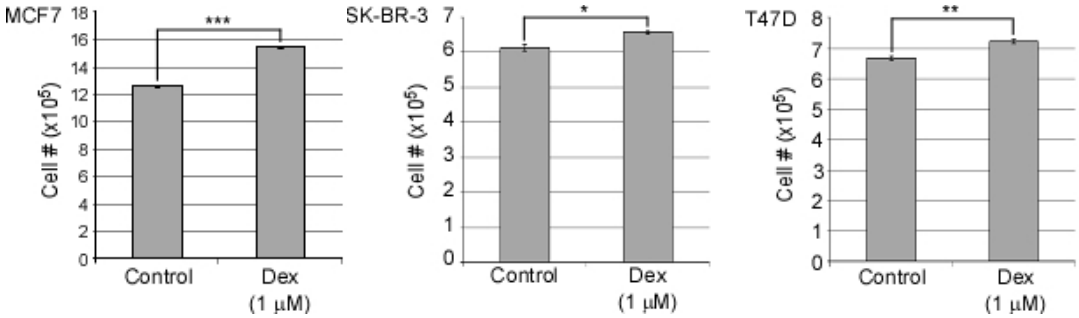
mediated inhibition of the caspases. The Hs578t cells showed 60.7% decrease in caspase 3 and 7 activity. The luminal cell lines SK-BR-3 and T47D displayed 30.9% and 34.4%, respectively.

Figure 2.2

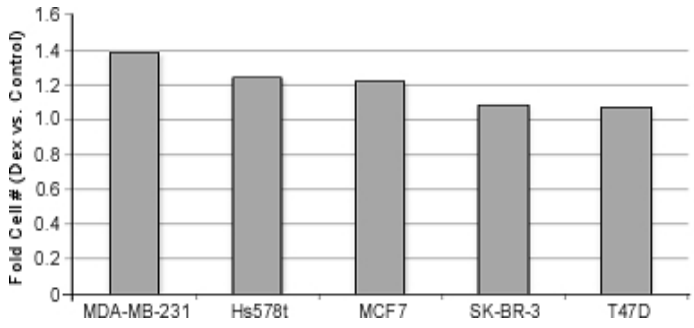
2.2 A.



2.2B.



2.2C.



2.2D. **2.2E.**

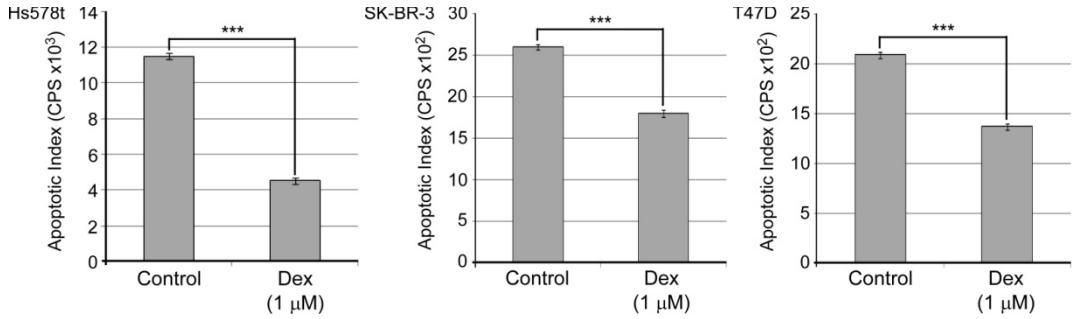


Figure 2.2 Impact of Dex on total cell number 24 h post-treatment.

A. TNBC cell lines MDA-MB-231 and Hs578t and **B.** luminal breast cancer cell lines MCF7, SK-BR-3 and T47D were treated with vehicle (control) or Dex for 24 h. Cells were collected and counted for total cell number. **C.** Graphic representation of the fold-change in Dex-treated cells relative to vehicle of each respective cell line. **D.** The TNBC cell line Hs578t and **E.** luminal breast cancer cell lines SK-BR-3, and T47D were treated for 24 h with vehicle (control) or Dex for 24 h. Cells were collected and lysed. Luciferase activities were measured using equal amounts of cell lysate mixed with Caspase 3/7 Glo reagent and luminescence was quantified by spectrophotometry.

Graphs show the mean value of at least three experiments, each performed in triplicate, upon which statistical analysis was performed; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

2.3.3 Dex increases migration of TNBC cell lines *in vitro*.

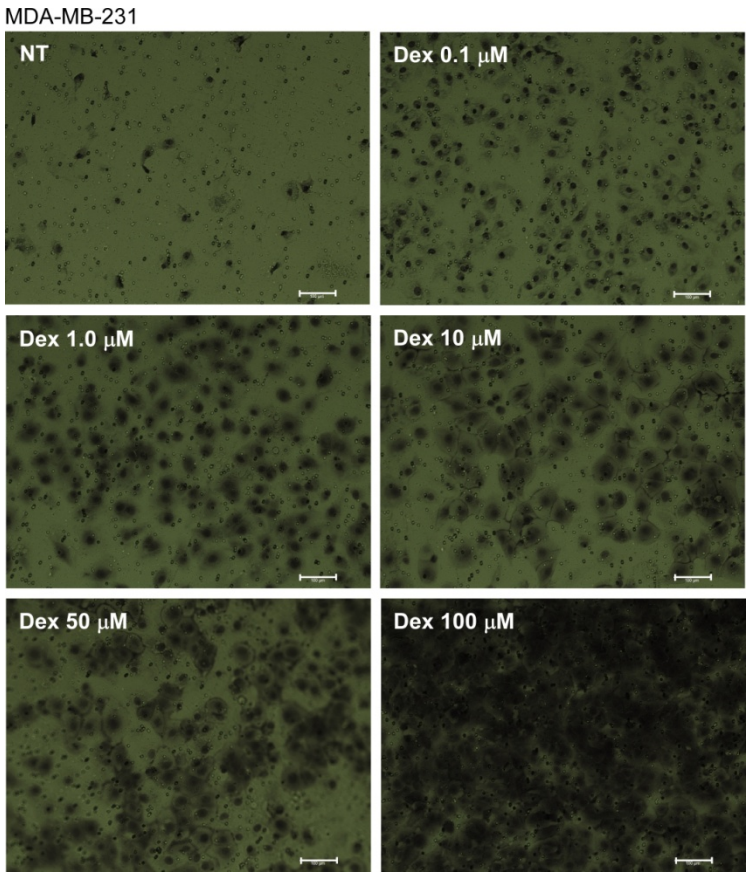
The migratory properties of a cell are a key parameter in determining metastatic capacity. To assess whether Dex could alter the migration of breast cancer cell lines *in vitro,* we performed cyto-select transwell assays. TNBC cells MDA-MB-231s were treated with vehicle (control) or a dose range of Dex and cell migration was measured by microscopy (Fig. 2.3A; upper panels) and quantified by spectrophotometry (Fig. 2.3A, lower panel). A statistically significant increase in migration was observed compared to control cells in the MDA-MB-231 cell line. We repeated the migration assay for the TNBC cell line MDA-MB-468 with vehicle (control) and with Dex (1 μM). We observed a statistically significant increase in migration (Fig. 2.3B). The luminal breast cancer cell line T47D did not display an increase in migration following Dex treatment (Fig. 2.3C; image not available).

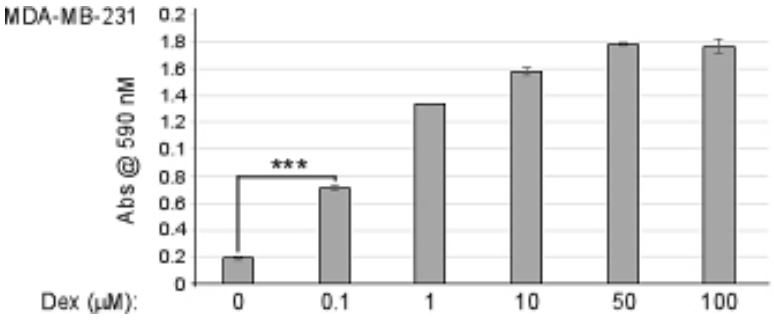
To ensure that differences in migration of Dex-treated cells compared to control cells were not due to Dex-induced proliferation or enhanced survival in the serum-free conditions, MDA-MB-231 and MDA-MB-468 cells were seeded in serum-free media on tissue culture plates and grown with vehicle (control) or Dex (1 μM) for 24 h (the length of the entire migration assay). There was no statistically significant change in cell number between treated and control cells in these media conditions (Fig. 2.3D and 3E). Thus, it is unlikely that proliferation effects can account for the migration phenotype. To ensure that the same would hold true for cells that were in complete media for the duration of the assay (i.e., putative cells that migrated immediately after seeding), MDA-MB-231 and MDA-MB-468 cells were grown with vehicle (control) or Dex in serum-free media and then the media was replaced with complete media for an additional 24 h. No statistically

significant change in cell number was observed (Fig. 2.3D and 3E). Thus, it is unlikely that Dex-treated cells that migrated had any proliferative advantage over migratory control cells once in complete media (as both would be in complete media) during the assay timeframe. When MDA-MB-231 cells were left for an additional 24 h (total time in complete media = 48 h), a statistically significant difference in cell number was observed for Dex-treated cells suggesting a Dex-mediated proliferative or pro-survival advantage (Fig. 2.3D).

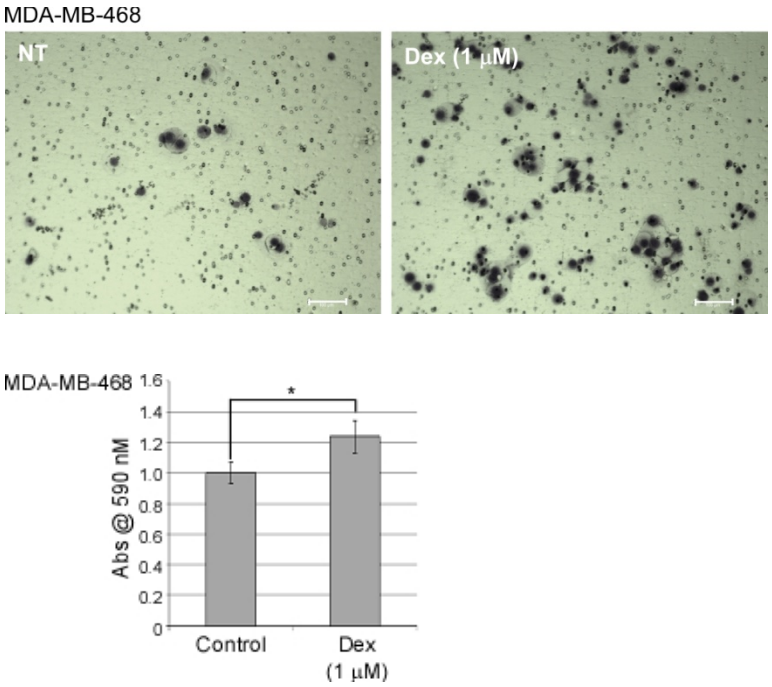
Figure 2.3

2.3A.

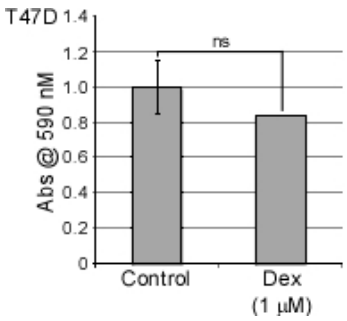




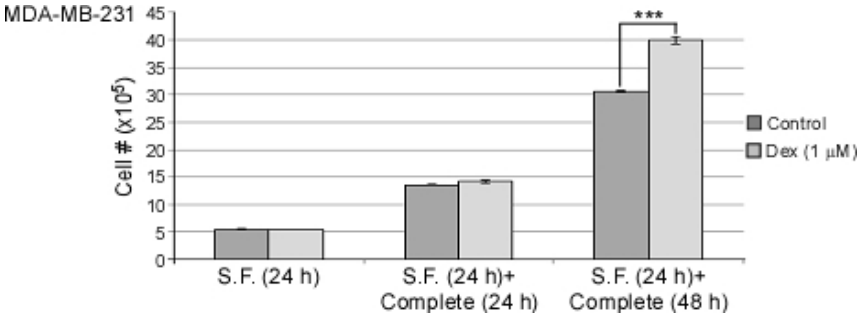
2.3B.



2.3C.



2.3D.



2.3E.

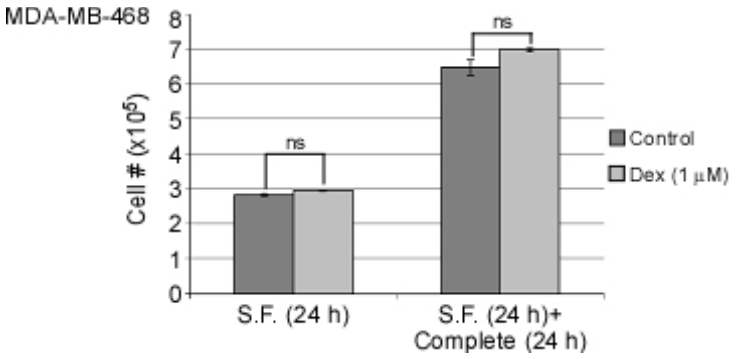


Figure 2.3 Impact of Dex on migration of triple negative and luminal breast cancer cell lines *in vitro*.

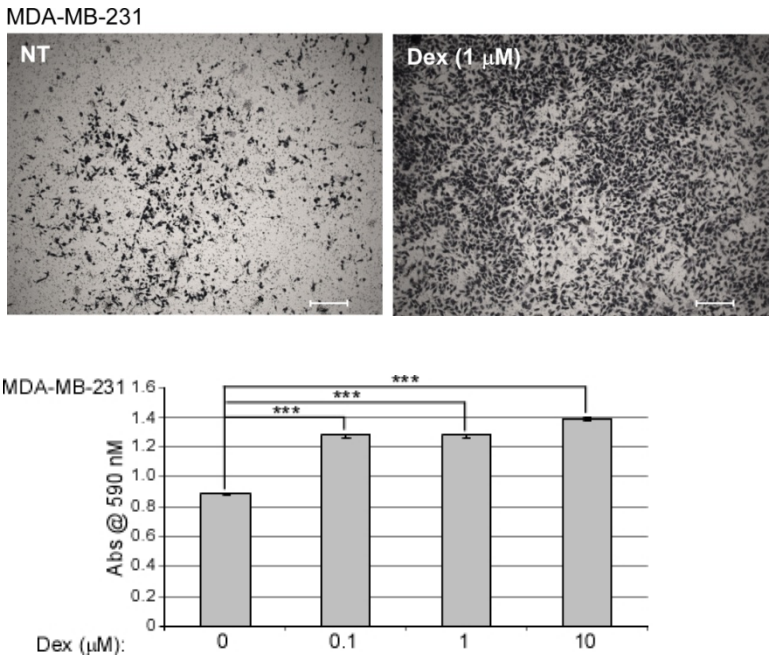
A-C TNBC cell lines MDA-MB-231 (A) or MDA-MB-468 (B) or luminal breast cancer cell line T47D (C) were seeded ($1x10^5$) in serum-free media in cyto-select migration chambers placed in complete media and treated with vehicle (control) or with Dex (dose indicated on X axis) for 24 h. Migration of the crystal violet stained cells through the membrane pores was visualized by microscopy (upper panels). Crystal violet-stained cells were extracted and quantified by measuring the absorbance at 590 nM (lower panels). Scale bars represent 100 μM. **D.** MDA-MB-231 cells were grown on tissue culture plates for 24 h with Dex (1 μM) or vehicle control in serum-free media and collected for count of total cell number or media was replaced with complete media for an additional 24 h or 48 h. Cells were collected and counted. **E.** MDA-MB-468 cells were grown on tissue culture plates for 24 h with vehicle control or Dex (1 μM) in serum-free media and collected for count of total cell number or media was replaced with complete media for an additional 24 h.

2.3.4 Dex increases invasiveness of TNBC cell lines *in vitro*.

Previous work in bladder cancer cell lines demonstrated that Dex decreased cell invasion [14]. To assess the effect of Dex on breast cancer cell lines *in vitro,* we used a cyto-select transwell invasion assay in which the chamber wells were coated with a collagen based extra cellular matrix. We treated MDA-MB-231 breast cancer cells with vehicle (control) or with increasing concentrations (0.1-10 μM) of Dex. 24 h post-treatment cells that had migrated through the pores toward complete media were stained with crystal violet and imaged (Fig. 2.4A). We observed an increase in invasiveness with increases in Dex concentration. Cells were then lysed and analyzed by spectrophotometry to accurately quantify invasiveness compared to control (Fig. 2.4A, lower panel). Dex caused statistically significant increase in cell number of the highly invasive MDA-MB-231 (Fig. 2.4B). We also assessed the impact of Dex on invasiveness in Hs578t cells and observed statistically significant increases as well (Fig. 2.4C).

Figure 2.4

2.4A.



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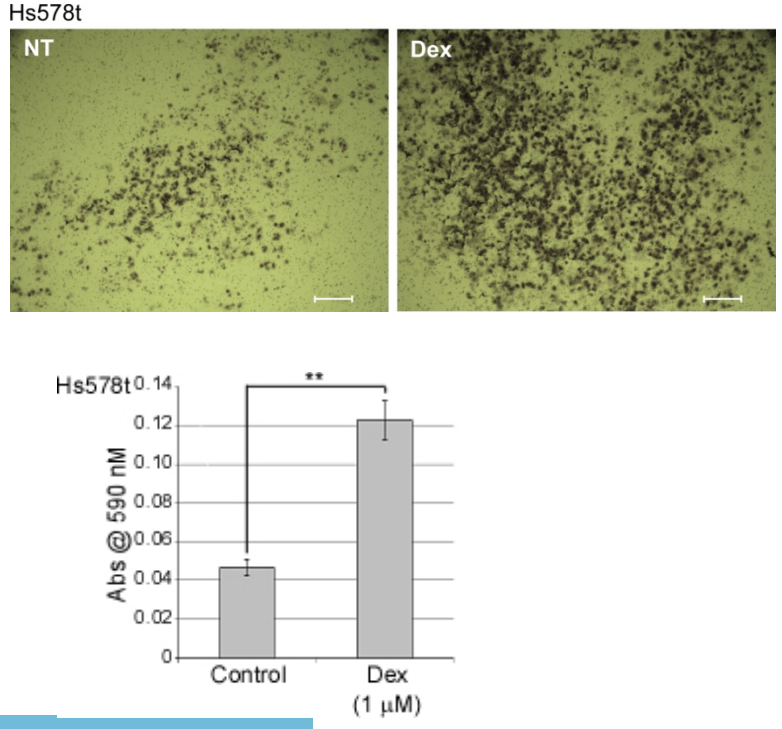


Figure 2.4 Dex increases invasiveness of TNBC cell lines *in vitro*.

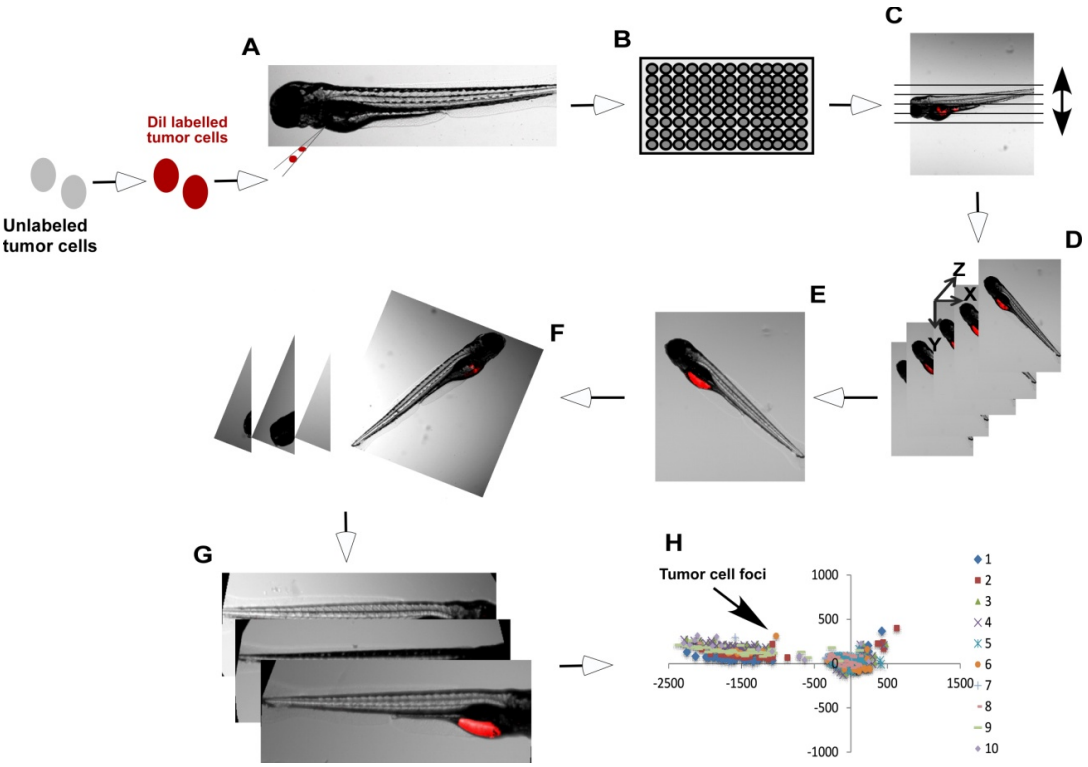
A. MDA-MB-231 and **B.** Hs578t were seeded in cultrex coated cyto-select migration chambers and treated with vehicle (control) or with Dex (at the indicated concentration for 24 h). Cells were stained with crystal violet and images were taken with Leica microscope (upper panels). Crystal violet-stained cells were extracted and quantified by spectrophotometry at absorbance of 590 nM (lower panels). The absorption data from which they were averaged are presented as the means ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

2.3.5 Dex increases metastatic properties of TNBC cell lines *in vivo*.

To examine the effect of Dex on breast cancer cell behaviour *in vivo* DiD labeled MDA-MB-231 cells were injected into the yolk sack of zebrafish. Zebrafish were grown in water with and without Dex. 24 h post treatment (hpt) the fish were anesthetized and imaged using a Leica fluorescence microscope.

As expected of the highly aggressive MDA-MB-231 cells, numerous cells were found re-localized in the tail vein of the control zebrafish. The addition of Dex to the fish water, however, resulted in a statistically significant increase in the number of re-localized cells compared to controls (Fig.3.5J). Moreover, the average cumulative distance (measured in μm) traveled by the Dex-treated fish was statistically significant compared to control fish (Fig. 3.5K)

Figure 2.5



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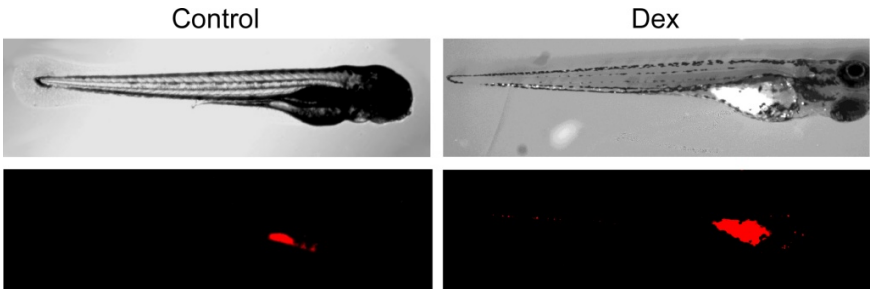

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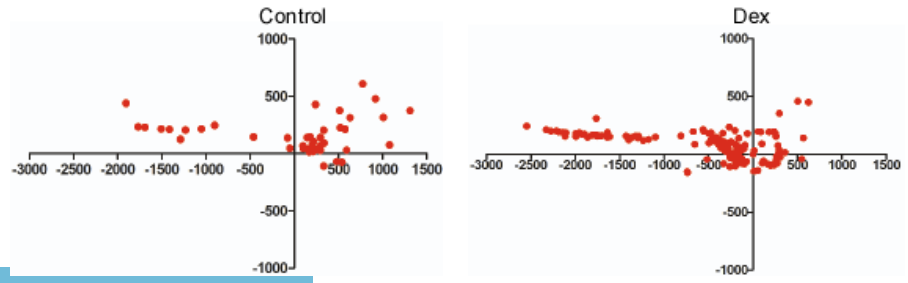

2.5K.

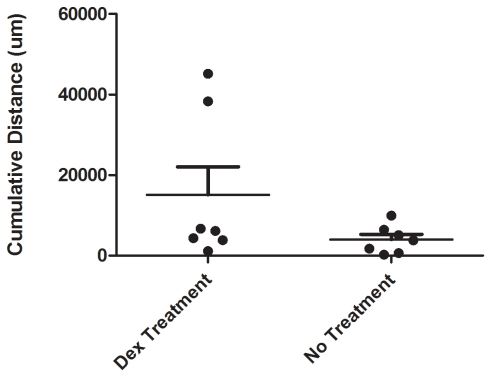

Figure 2.5 Dex augments metastatic characteristics of TNBC cells in zebrafish model.

A. Cells were labeled with a fluorescent marker and implanted into 48 h post-fertilization (hpf) embryos. **B.** Embryos were treated in a 96-well plate with Dex 1 h post-injection (hpi). **C.** Embryos were anesthetized and imaged with a fluorescent microscope 24 h post-treatment. **D.** Multiple Z plane images were taken of each embryo and **E.** composite images were made using ImageJ software. **F.** All composite images were gathered, **G.** aligned to a specific orientation, and **H.** analyzed to determine tumour foci position relative to injection site (0.0 of graph). Each data series represents one fish. Axes represent the distance (μm) from the injection site. (I) Representative brightfield and fluorescent images taken 24 hpi of MDA-MB-231 cells injected into 48 hpf embryos and treated with or without Dex from 1 hpi to 24 hpi. **J.** Graphs of tumour coordinate 24 hpi and 24 h post treatment. **K.** Graph of cumulative distance (CD) from injection site for tumour foci. Each point represents CD of one embryo and line represents mean CD.

2.4 Discussion

30% of breast cancer patients develop metastatic breast cancer for which there is no cure [23]. Metastatic breast cancer cells present several key characteristic, namely 1) the ability to survive by eluding apoptosis and continuing to grow and proliferate at the primary tumour site; 2) the ability to invade through surrounding tissue; 3) the ability to migrate in circulation or within neighbouring tissues and 4) colonization at distant sites [23,24]. These characteristics may be intrinsic or acquired through mutation [24]. Alternatively these characteristics could potentially be augmented by drug treatment. Thus, the effect of clinical therapies and adjuvant drugs on breast cancer cell characteristics is of clinical significance to progression of metastasis.

In chemotherapy regimens, Pac is administered to TNBC patients and luminal breast cancer patients with late stage, aggressive breast cancer and those that display resistance to conventional therapies. Pac, despite being a very effective chemotherapy, causes allergic and hypersensitivity reactions as well as nausea and vomiting in patients [4,25]. Dex is administered to breast cancer patients in advance and along with Pac to combat these effects [25,26].

We demonstrate that administration of Dex increases cell number, migratory capacity and invasiveness within 24 h post-treatment relative to vehicle-treated control cells *in vitro* (Figs. 2.2-2.4). Previous reports claim that Dex increases cell proliferation in solid cancer cells [14,15]. These previous studies used only cell viability assays and no counts were performed. While we did perform cell counts, neither our data, nor those reports, excludes the possibility that differences in Dex-treated cell number versus control cell number are due to increased survival and not increased proliferation. At most we

show that Dex allowed cells to continue dividing better than those that did not receive Dex (Fig. 2.2A and 2B). Thus, differences in cell number compared to control could be attributable to enhanced cell survival with Dex. Zheng *et al* also report that their claims of increased proliferation were not corroborated by increases in the proliferative markers, cyclins and CDKs [14]. Pang *et al* also interpret data concerning Dex-mediated increases in tumour size that did not show increased expression of Ki-67 as most likely being due to an increase in survival [27]. Furthermore, and consistent with these findings, we demonstrate that Dex decreased activity of caspases 3 and 7 compared to control cells (Fig. 2.2D and 2E). These differences in caspase activity may account for variance in the cell number of Dex-treated cells compared to control populations. Further analysis with BrdU incorporation or Ki-67 expression could give insight into this matter of concern.

We also demonstrate that Dex-treated breast cancer cells have increased motility as evident in migration and invasion assays (Figs. 2.3 and 2.4). One alternate explanation to our data that must be considered however is that the Dex-treated cells were surviving and/or proliferating faster producing more cells on the pre-migration and pre-invasion side of the chambers compared to control chambers. Thus, even if equal percentages of cells migrated or invaded thereafter, the Dex-treated chambers would have more cells to migrate or invade and a selective advantage over control chambers. Another alternative explanation to our observation is that while Dex would confer no advantage in cell number pre-migration or pre-invasion, Dex treated cells would be 'primed' for increased proliferation or survival compared to control cells once they reached the complete media post-migration or post-invasion. In this model equal numbers of cells exist in both the control and Dex-treated cell chambers pre-migration or pre-invasion and equal numbers

of cells migrate or invade. Once these cells reach complete media, however, the Dex treated cells proliferate sooner and thus account for differences in the assay. We demonstrate that neither of these explanations can account for the observed data in the conditions and brief timeframe that the experiment takes place (Figs. 2.3D and 3E). Given longer periods of time, however, we show that the latter explanation could be true and further supports our earlier report that Dex enhances survival and/or proliferation in breast cancer cells (Fig. 2.3D).

Our *in vivo* data supports Dex's role in enhancing one, if not more, characteristics of metastatic breast cancer cells. Which of the Dex-mediated effects i.e., proliferation/survival, migration or invasion is most essential for this phenotype is currently unknown (Fig. 2.5). Given that Dex affects proliferation/survival of the MDA-MB-231 breast cancer cells it is possible that cells proliferate more in the yolk sack and hence more cells were present to invade and migrate to distant sites, properties MDA-MB-231 cells possess intrinsically. Also, it may be that these highly invasive cells exited the yolk sack with equal efficiency compared to non-treated fish, but Dex mediates proliferation or survival of invasive cells thus affecting total cell number at the time of quantification. Consistent with this explanation, Zheng *et al* ascribed increases in tumour mass in mice xenografts of Dex treated mice to increased survival of tumour cells resulting in larger tumours [14]. Another possibility is that Dex increased the invasive properties or the migratory properties but had no impact of survival or proliferation in the fish. This explanation would conflict with Zheng *et al's* report that no invasion or migration happened in xenografts of bladder cancer cells in Dex-treated mice [14]. It would also conflict with several groups' *in vitro* and *in vivo* work. A search of the

literature shows several reports on Dex as reducing migration and invasion [13,14,28,29]. These studies are not in breast cancer cell lines and most are *in vitro*. For example, Hayashido *et al* report that Dex causes down-regulation of uPA and increases in plasminogen activator inhibitor type 1 (PAI-1) resulting in decreased invasion through collagen type 1 gel in squamous cell carcinoma [13]. Shiratsuchi *et al* also show Dex decreases invasion in squamous cell carcinoma through inhibition of uPA signaling, effectively blocking epidermal growth factor-induced invasion [29]. *In vitro* and *in vivo* work by Zheng *et al* show that Dex down-regulates expression of invasion-related genes (MMP-2/MMP-9, IL-6, VEGF) and also reduced the development of bloody ascites in xenograft mice, an indicator of metastasis [14]. GR activity is tissue specific and further research into the mechanisms by which Dex causes increases in migration and invasion may provide insight into the differences compared to these aforementioned studies.

In conclusion, Dex impacts breast cancer cell behaviour as pertains to survival/proliferation, invasion and migratory capacity. Given the common usage of Dex in treating breast cancer patients with the most severe forms of breast cancer further research into elucidating the molecular mechanisms driving these effects and the impact of Dex on overall breast cancer outcomes is warranted.

2.5 Acknowledgements

To Espanta Jalili, thank you for all of your guidance in experiment design, execution, as well as edits of the text. We acknowledge Tiffany Seagroves for her gracious donation of the MCF7 cell line. We acknowledge Janice Tubman for her work with the zebrafish injections and quantification of data. Funding for this work was provided by Seeds 4 Hope.

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Chapter 3: Paclitaxel-induced transcriptional regulation of Fas signaling pathway is antagonized by dexamethasone

3.1 Introduction

Glucocorticoids (GCs) regulate numerous physiological parameters and are involved in the development and maintenance of many tissues [1-3]. GCs can act both genomically and non-genomically. Genomically, GCs activate the glucocorticoid receptor (GR) which translocates to the nucleus where, upon binding DNA, it can activate or repress the expression of hundreds of genes [2]. The genomic effect of active GR is also tissue specific [2]. GCs can activate apoptosis in hematopoietic cells [2,4,5], yet they have been shown to inhibit apoptosis in solid tumour cells [6]. While the sensitivity of hematopoietic cells to GCs serves as the basis of synthetic GC use in treatment of leukemias [5,7], the protective role of GCs in solid tumours has been reported in ovarian, epidermoid, muscle, and breast cancer cells [6,8,9]. The clinical significance of the pleiotropic and tissue-specific impact of GCs is that beyond their use as part of the chemotherapy proper in leukemias, synthetic GCs (e.g., dexamethasone (Dex)) are also administered to breast cancer patients as antimetics. As such, Dex efficiently reduces nausea and vomiting as well as suppresses hypersensitivity reactions induced by chemotherapy drugs (e.g., paclitaxel (Pac)) and the vehicles in which they are delivered (e.g., Koliphor EL). Disconcerting is the growing body of data warning of the protective role of GCs in solid tumours [8,10-12]. Despite such evidence, to date there have been no clinical trials examining the effect of Dex on tumour response or the efficacy of Pac [6].

Pac is a microtubule-stabilizing agent that causes metaphase arrest in mitotic cells [13]. Pac also activates cell death programs both downstream of microtubule stabilization as well as independent of microtubule stabilizing effects [14]. Previous reports have identified Pac-activated mediators of apoptosis, which happen to also be contrarily

regulated by Dex (e.g., SGK-1, DUSP-1, IκBα) [6,15-18]. The mechanisms by which Pac modulates these pathways have been partially elucidated, but there remains much to be explained; especially as pertains to the putative targets of these pathways. For example, Huang *et al* reported on regulation of the transcription factor NFκB through IκBα degradation, and activation of other upstream regulators of NFκB, as a mediator of Pac-induced apoptosis in human breast BCap37, human ovarian OV2008, and human epidermoid tumour KB cells [18]. Nevertheless, the specific downstream targets of Pac-activated NFκB and how these might regulate Pac-induced apoptosis remains unclear.

In clinical practice, breast cancers are often identified histologically based on the presence, absence, or levels of particular receptors that serve as markers for targeted therapy (e.g., estrogen, progesterone, or HER2). Tumours that present with the estrogen receptor (often termed luminal), for example, can be treated with Tamoxifen or other estrogen antagonists. Breast cancers that do not express the estrogen or progesterone receptor, or do not display amplification of HER2 are termed triple-negative breast cancers (TNBCs) and are very aggressive with low overall survival rates [19]. These cancers are treated with potent cytotoxic drugs (e.g., Pac). Pac may also be used on non-TNBCs that are late stage aggressive luminal breast cancers and/or other luminal breast cancers that have become resistant to traditional therapies. Because Pac serves as a last line of defense against the most aggressive forms of breast cancer, ensuring optimal conditions of efficacy is of paramount therapeutic importance. Elucidating the molecular pathways of Dex-mediated protection against Pac will not only help us better understand how Pac kills cancer cells, but might provide insight into sensitizing breast cancers to chemotherapy by identification of putative targets.

In clinical settings Dex is administered at least one hour prior to Pac. We show that both TNBCs and luminal breast cancers respond to Pac, and that Dex rescues these cells from Pac-induced apoptosis (most significantly in TNBCs). We show in several of the most Pac-sensitive cell lines that Pac activates NFκB. Furthermore, we show that NFκB is an important mediator of Pac-induced apoptosis and a target of Dex-mediated rescue from Pac-induced apoptosis. We then searched for potential NFκB-regulated transcripts. We report here that both Fas ligand (FASLG) and Fas receptor (FAS) are upregulated by Pac. These transcripts are contrarily regulated by Dex and Pac-induced transcriptional regulation of Fas is antagonized by Dex. We present a critical component of the protective role of Dex in Pac-mediated apoptosis.

3.2 Materials and methods

Cell culture

Human breast cancer cells MCF7 (HTB22; gift from Tiffany Seagroves; University of Tennessee: Health Science Center); MDA-MB-231 (HTB26; ATCC), Hs578t (HTB126; ATCC), and MDA-MB-468 (HTB132; ATCC) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; D5796; Sigma). T47D cells (HTB-133; ATCC) were cultured in RPMI-1640 Medium (R8758; Sigma) with 2 units/ml of insulin (I-5500; Sigma). SK-BR-3 cells (HTB-30; ATCC) were cultured in McCoy's 5A Medium (30-2007; ATCC). All cells were supplemented with 10% fetal bovine serum (FBS; F1051; Sigma) and 1% Penicillin and Streptomycin and were maintained in an atmosphere of 5% CO_2 at 37˚C. For passaging, seeding, and quantification of cell numbers, cells were collected with 0.25% trypsin and counted using the TC10™ Automated Cell Counter from BioRad (Catalog #145-0010). Cell media was changed from complete media to serum-free media 24-hours prior to treatment. Serum-free media was replaced with media containing charcoal-treated FBS (10%) during the treatment stage.

Plasmids

3X-MHC-luc NFκB plasmid was a generous gift from Christine Pratt (Ottawa). The sh Scramble-pLKO.1-puro was constructed by using the scrambled sequence; CCTAAGGTTAAGTCGCCCTCG. The gene-specific shRNA expression plasmids were constructed using synthetic oligonucleotides targeting NFκB1,

GATGACATCCAGATTCGATTT, and RELA, CCTGAGGCTATAACTCGCCTA, which were cloned in the pLKO.1-puro plasmid (8453; Addgene).

Transfection

Cells were transiently transfected using jetPRIME (CA89129-922; VWR). Total of 3 µg plasmid DNA was diluted into 200 µl jetPRIME buffer. After vortexing the mix, 4 µl jetPRIME was added and vortexed. Reaction was incubated for 10 min at room temperature. The transfection mix was added drop-wise into the medium. Cells were incubated at 37˚C for at least 24 h.

Infection

MDA-MB-468 cells were infected with virus:culture media ratio of 1:1, supplemented with 8 µg/ml polybrene and incubated for 8 h. Cells were then recovered for 24 h in their relevant culture media prior to addition of puromycin (1 µg per ml of media) for stable cell line generation. 10 days after selection, colonies were picked for expansion and screening for expression.

Compounds and antibodies

The following antibodies were used at a dilution of 1:1000 dilution: Actin (MAB1501R; Chemicon), p105/p50 (1559; Epitomics), p65 (1546; Epitomics), and Fas (5709-1; Epitomics). Secondary antibodies used were HRP-conjugated anti-mouse IgG (A9917; Sigma) and anti-rabbit IgG (A0545: Sigma). Charcoal (C6241; Sigma), Paclitaxel (T7402; Sigma), Dexamethasone (DN1187; BioBasic), and RU-486 (Mifepristone; M8056; Sigma).

Immuno-blotting

Samples were lysed with 0.1% NP40 buffer supplemented with Leupeptin (10 µg/ml; 103476-89-7; BioBasic), Aprotinin (10 µg/ml; A3428; Sigma), and PMSF (1 mM; DB0425; BioBasic). Samples were analyzed by 10% SDS-PAGE and then transferred to a PVDF membrane. Primary antibodies were applied and incubated over night at 4°C at dilutions specified above. Proteins were detected via treatment with Perkin-Elmer Enhanced Chemiluminscence reagent/ECL Western Gel Substrate (NEL10S, Perkin Elmer and quantified using FlourChem HD2 software (AlphaInnotech; Perkin Elmer).

Luciferase assays

Cells were harvested 24 h post-transfection with luciferase constructs and mixed with Bright-glo reagent (E2610; Promega), at 1:1 ratio. Luminescence spectra of the samples were measured using a Wallac Victor 1420 plate reader (PerkinElmer 3TM-1420).

Apoptotic assays

Caspase 3/7-glo assay (Promega; G8090) was used to measure the apoptotic state of treated cells. 24 h post-treatment cells were collected via trypsinization and lysed. 50 µl of Caspase-Glo® 3/7 reagent was added in each well of a white-walled 96-well plate containing 50 µl of lysis buffer as blank, negative control cell lysates, or treated cell lysates with the final concentration of 1 µg/µl. Contents were gently mixed in the wells using a plate shaker at 300-500 rpm for 30 sec. Cell lysates were incubated at room temperature for 30 min and the luminescence of each sample was measured using Wallac Victor 1420 plate reader.

qRT-PCR analysis

24 h post-treatment, cells were collected via trypsinization and total cell RNA was extracted using RNeasy Plus Mini Kit (Qiagen; 74134). RNA was converted to first strand cDNA using SuperScript™ II Reverse Transcriptase (18064; Invitrogen). Relative quantities of mRNA expression were quantified using the following oligos: 5'-CCCATTTAACAGGCAAGTCCAA-3' and 5'-AAGTACAGCCCAGTTTCATTGATCA-3' for FASLG and 5'-ATCTAACTTGGGGTGGCTTTGTC-3' and 5'-ATTTATTGCCACTGTTTCAGGATTT-3' for FAS and analyzed using ViiA 7 software 1.1 (ABI Viia7 Real Time PCR System). Cells were collected following respective treatment and their total RNA was subjected to real time PCR. Expression levels were normalized to GAPDH and presented as fold relative to the control.

Statistical analysis

Student *t* test was employed using Statistica software. All results are expressed as mean ± SEM and differences were considered significant at p values of <0.05.

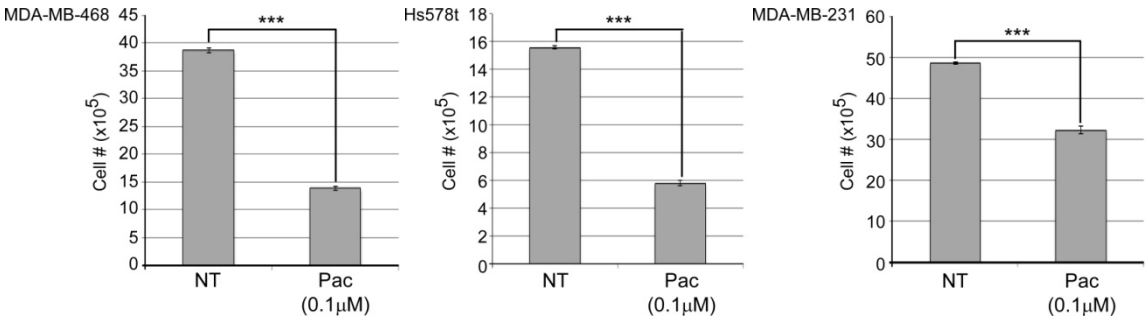
3.3 Results

3.3.1 Both ER+ luminal and TNBC cells are sensitive to Pac *in vitro*.

Pac treatment is reserved primarily for patients with TNBC in clinical settings. Patients with luminal breast cancers may also receive Pac if late diagnosis shows an aggressive phenotype or if the patient exhibits resistance to more conventional therapies. To assess the sensitivity of breast cancer cells to Pac we treated six breast cancer cell lines, representing both luminal and TNBC subtypes of breast cancer (luminal: MCF 7, SK-BR-3, T47D; TNBC: Hs578t, MDA-MB-231, MDA-MB-468), with Pac (0.1 μM). Cells were then collected and counted via trypan blue exclusion. Each cell line showed statistically significant reduction in total viable cell number (Fig. 3.1). The TNBCs showed, on average, the greatest statistically significant reduction in viable cell numbers to Pac (Fig. 3.1A) when compared to the luminal breast cancers (Fig. 3.1B). The most sensitive cell lines, as measured by percentage of decrease in viable cell number, were the MDA-MB-468 (64% decrease), and Hs578t cell lines (62.8% decrease) (Fig. 3.1C).

Figure 3.1

3.1A.



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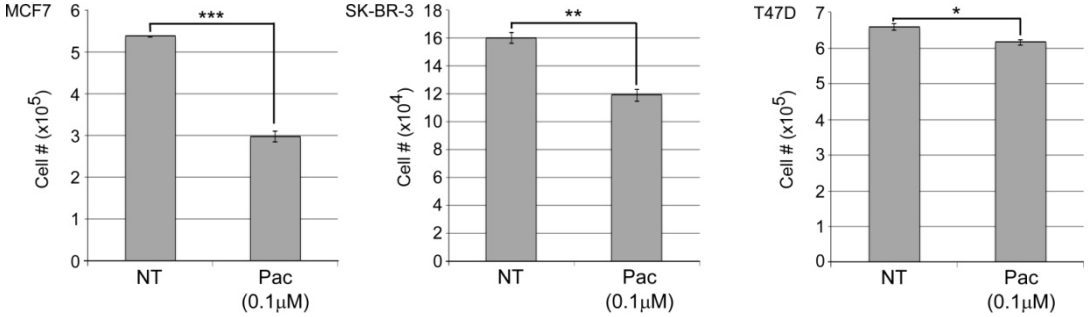

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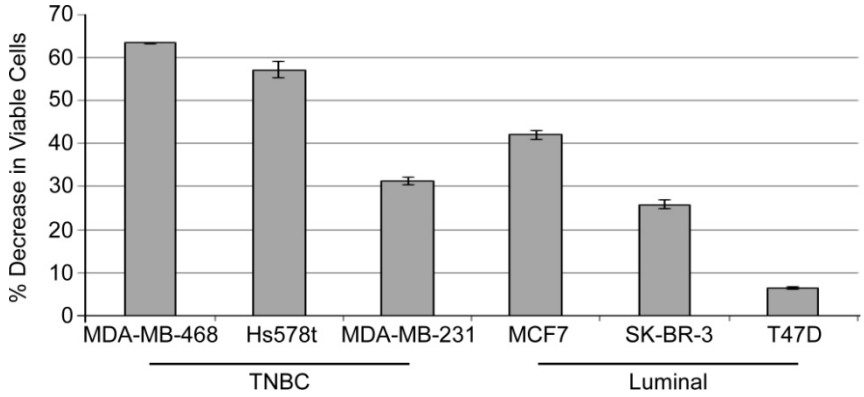


Figure 3.1 TNBC and luminal breast cancer cell line sensitivity to Pac.

A. TNBC cell lines MDA-MB-468, Hs578t, and MDA-MB-231 cells were treated for 24 h with vehicle (NT) or Pac and collected for cell count of total live cell number. **B.** Luminal breast cancer cell lines MCF7, SK-BR-3 and T47D cells were treated with Pac or NT for 24 h and collected for cell count of total live cells. **C.** Percentage of decrease in average of total cell numbers of Pac treated breast cancer cells compared to average number of vehicle-treated control numbers.

All the experiments were performed at least three times and represented as mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

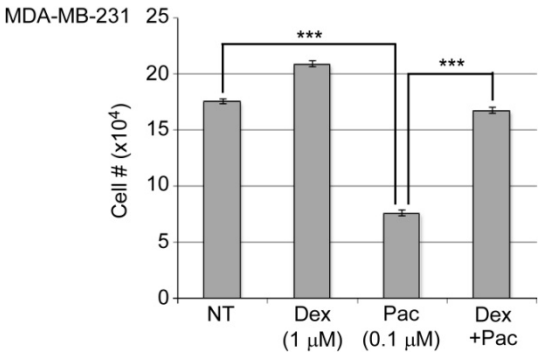
3.3.2 Dex treatment enhances cell survival in Pac-treated breast cancer through inhibition of Pac-induced apoptosis.

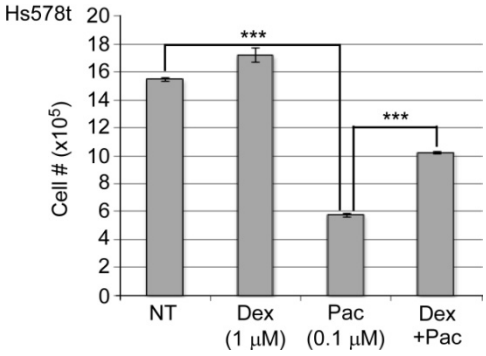
In the clinic, breast cancer patients receive Dex at least 1 h prior to receiving Pac; we similarly treated six breast cancer cell lines (two representatives of TNBCs and three of luminals) to assess the impact of Dex pre-treatment on the efficacy of Pac. For the three TNBC cell lines pre-treatment with Dex produced statistically very significant rescue of total viable cell number (Figs. 3.2A and 2C). For the three luminal cell lines, the results were mixed (Fig. 3.2B). The rescue effect of Dex was most significant for the MCF-7 cells (Fig. 3.2B, left panel), which were also the most Pac sensitive of the luminal cell lines (Fig. 3.1C). The SK-BR-3 and T47D luminal cell lines displayed no statistically significant rescue in total cell number with Dex (Fig. 3.2B, bottom panels). To ensure that the Dex-mediated rescue was downstream of GR activation we treated MDA-MB-468 cells with the GR antagonist Mifepristone (RU-486) (Fig. 3.2C). GR inhibition by RU-486 completely mitigated any Dex-mediated rescue from Pac (Fig. 3.2C).

To further assess the means by which Dex mediates its protection from Pac, we performed an apoptotic assay examining caspase 3 and 7 activity in the two most Pac-sensitive, MDA-MB-468 and Hs578t, and least Pac-sensitive, SK-BR-3 and T47D, cell lines (Fig. 3.2D). In all four cell lines, pre-treatment with Dex 1 h in advance of Pac yielded statistically very high rescue from Pac-induced apoptosis (Fig. 3.2D).

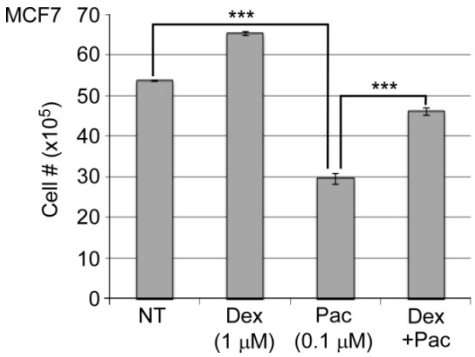
Figure 3.2

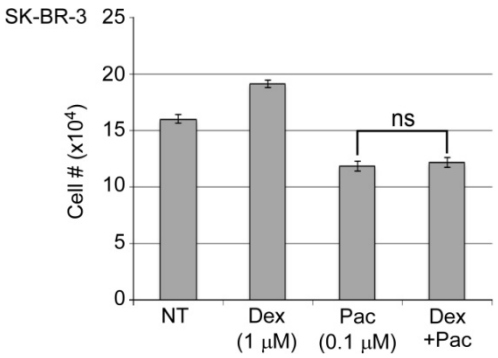
3.2A.

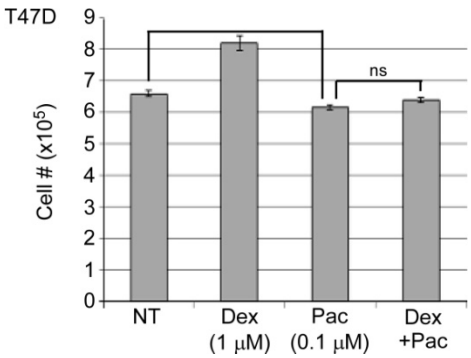




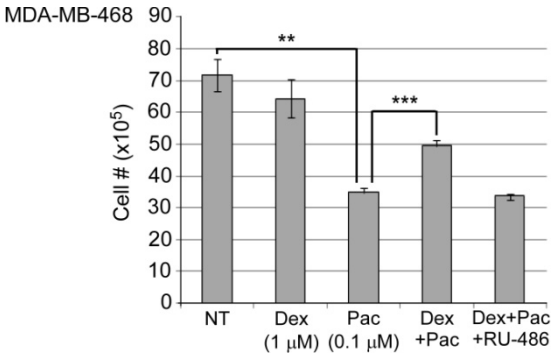
3.2B.







3.2C.



3.2D.

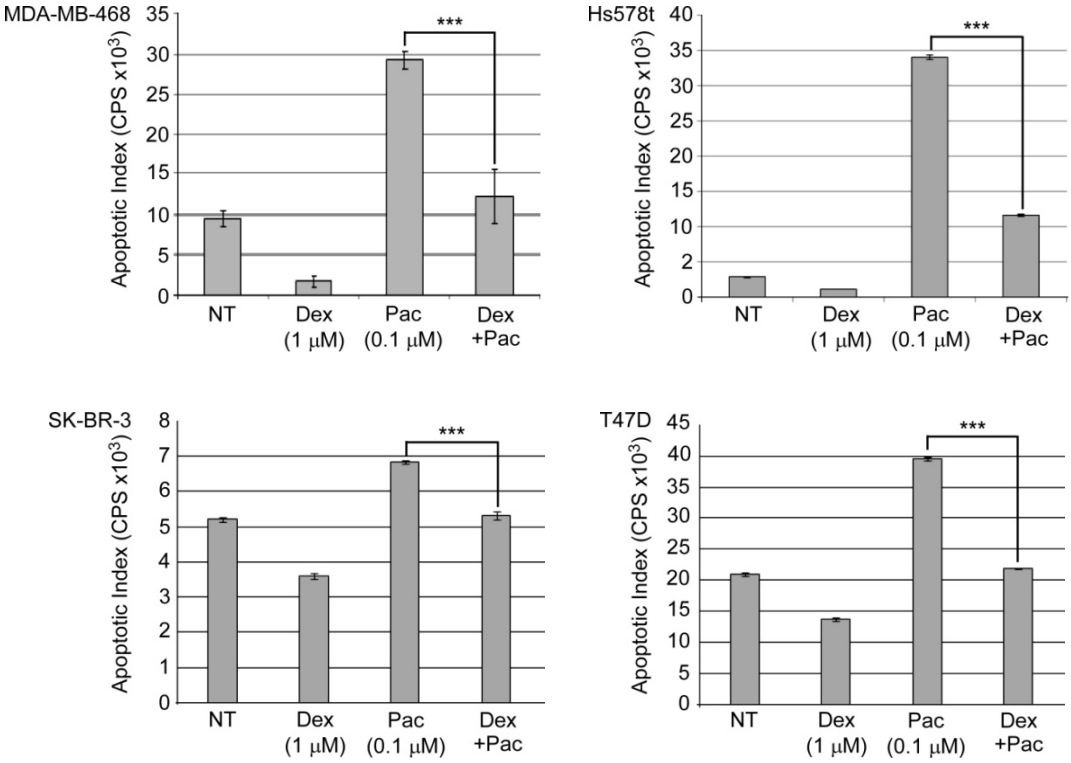


Figure 3.2 Pro-survival and anti-apoptotic role of Dex from Pac in TNBC and luminal breast cancer cell lines.

A. and **B.** TNBC cell lines, MDA-MB-231 and Hs578t, and luminal breast cancer cell lines, MCF7, SK-BR-3, and T47D were treated for 24 h with vehicle (NT), Dex, Pac, or co-treated with Dex and Pac (Dex 1 h prior to Pac). Cells were then counted for total cell number. **C.** MDA-MB-468 cells were treated for 24 h with vehicle, Dex, Pac, or co-treated with Dex and Pac (Dex 1 h prior to Pac) with or without RU-486. Cells were then counted for total cell number. **D.** TNBC cell lines MDA-MB-231 and Hs578t (upper panels) and luminal breast cancer cell lines SK-BR-3 and T47D (lower panels) were treated for 24 h with vehicle, Dex, Pac, or a combination of Dex and Pac (Dex 1 h prior to Pac). Cells were collected and lysed. Luciferase activity was measured using equal amounts of cell lysate mixed with Caspase 3/7 Glo buffer and luminescence was measured by spectrophotometry.

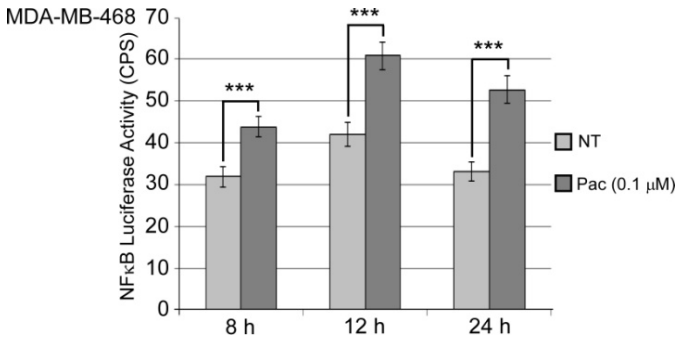
Graphs show the mean value of at least three experiments, each performed in triplicate, upon which statistical analysis was performed; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

3.3.3 Contrary regulation of NFκB by Pac and Dex.

Previous reports demonstrated that upstream regulators of NFκB signaling were affected upon Pac-treatment [17,18]. GCs, including Dex, are potent inhibitors of NFκB inflammatory signaling, hence we sought to determine if NFκB was an important mediator of Pac-induced apoptosis in these cell lines, and if Dex-mediated rescue of Pac-treated breast cancer cells was caused by inhibition of NFκB. To determine if Dex-mediated rescue from Pac-induced apoptosis is via NFκB, a construct bearing three copies of the promoter for the major histocompatibility complex class 1 ((MHC-1), a known transcriptional target of NFκB) upstream of a gene for luciferase was transiently transfected into MDA-MB-468 cells. 24 h following transfection, cells were treated with or without Pac and collected at 8, 12, and 24 h (Fig. 3.3A). Cell lysates were analyzed by spectrophotometry for overall luminescence. Pac-treated cells showed statistically significant increases in luciferase activity at all three time points demonstrating increased Pac-induced activation of the NFκB transcription factor (Fig. 3.3A). To assess the impact of Dex-treatment and co-treatment of Dex and Pac, MDA-MB-468 and MDA-MB-231 cells were transiently transfected with the same construct as above. Cells were treated for 24 h with either vehicle (NT), with Dex, Pac, or with co-treatment of Dex and Pac (Dex 1 h prior to Pac). Cells were then collected and lysates were measured for overall luminescence (Fig. 3.3B). For both MDA-MB-468 and MDA-MB-231 cells, Pac increased luminescence indicating an enhanced activation of NFκB. Importantly, 1 h pre-treatment with Dex completely antagonized Pac-induced NFκB activity (Fig. 3.3B).

Figure 3.3

3.3A.



3.3B.

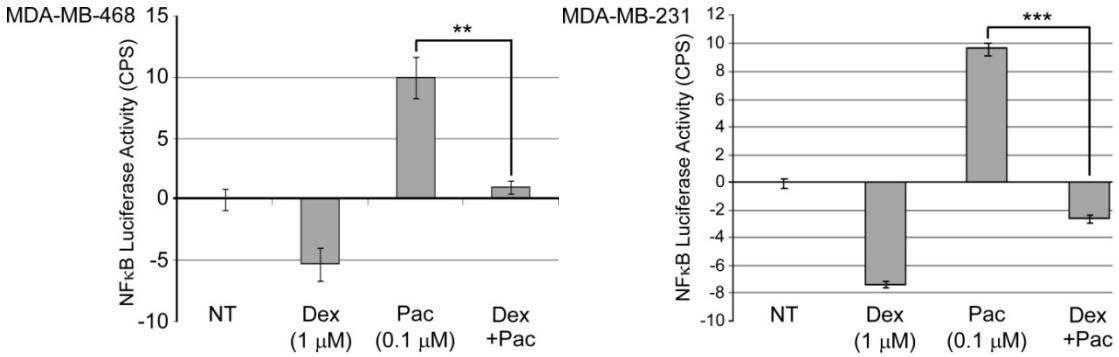


Figure 3.3 Pac upregulates activity of NFκB and is antagonized by Dex.

A. MDA-MB-468 cells were transiently transfected with 3X-MHC-luc NFκB reporter construct. Cells were then treated with the vehicle or Pac for 8, 12, and 24 h, and collected and lysed. Cell lysates were mixed with Bright-glo assay buffer and luminescence was measured by spectrophotometry. **B.** MDA-MB-468 and MDA-MB-231 cells were transiently transfected with 3X-MHC-luc NFκB reporter construct for 24 h. Cells were then treated with vehicle (NT), Dex, Pac, or co-treated with Dex and Pac

(Dex 1 h prior to Pac) for an additional 24 h, collected and lysed. Cell lysates were mixed with Bright-glo assay buffer and luminescence was measured by spectrophotometry.

Represented data are mean ± SEM of three individual experiments, each performed in triplicate. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

3.3.4 Knock-down of NFκB subunits desensitizes breast cancer cells to Pac and diminishes Dex-mediated rescue from Pac.

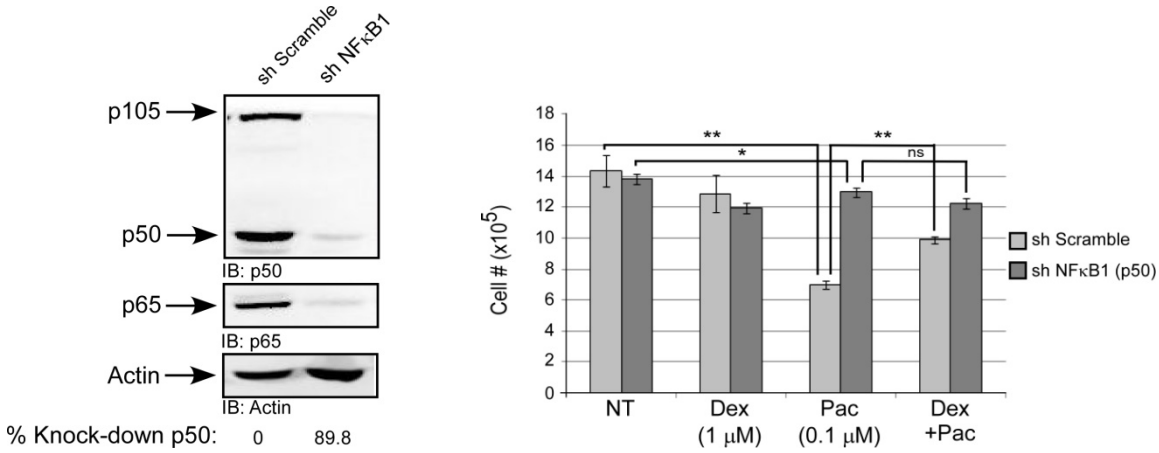
NFκB1 transcript produces p105, which is then cleaved to p50 before binding to the RELA gene product p65 to form the canonical NFκB transcription factor complex. To assess the essentiality of NFκB as a mediator of both Pac-induced apoptosis and Dex-mediated rescue from Pac, we knocked down both NFκB1 (p50) and RELA (p65) subunits of NFκB through lentiviral infection of shRNA constructs. Successful knock-down of NFκB1 was observed in stably infected MDA-MB-468 cells displaying nearly 90% decrease in p50 protein levels (Fig. 4A). Interestingly, knock-down of NFκB1 resulted in significant decrease of p65 protein as well (Fig. 3.4A; left panel). Control and NFκB1 knock-down cells were treated for 24 h with vehicle (NT), Dex, Pac, as well as co-treatment with Dex and Pac (Dex 1 h prior to Pac), and total cell number of each sample was assessed (Fig. 3.4A; right panel). Knock-down of NFκB1 significantly reduced Pac-mediated apoptosis showing only a 6.21% decrease ($p=0.0487$) in viable cell number compared to over a 50% reduction ($p=0.0021$) in control cells. Dex rescued over 40% ($p=0.001$) of control cells, while the rescue was completely lost in the NFκB1 knock-down cells.

Just as for NFκB, stably infected MDA-MB-468 cells were generated for knock-down of RELA. The knock-down of RELA proved highly successful resulting in 87.9% decrease of p65 levels (Fig. 3.4B; left panel). MDA-MB-468 cells infected with sh Scramble or sh RELA were treated for 24 h with vehicle or with Dex, Pac, or Dex plus Pac (Dex 1 h prior to Pac), and total cell number for each sample was assessed (Fig. 3.4B; right panel). Unlike cells depleted of p50 protein, knock-down of RELA did not

alter the ability of Pac to decrease cell number as much as knockdown of p50 did. Importantly, as seen in NFκB1 knock-down cells, knock-down of RELA abrogated Dex-induced protection, showing only a slight decrease in total cell number of 8% compared to Pac only cells (Fig. 3.4B; right panel). NFκB appears to play an important role in both Pac-induced decrease in viable cells and Dex-mediated rescue of cells from Pac. The most notable changes in cellular response to Pac and Dex were observed in the sh NFκB1 cell line, which may be attributed to the significant decrease in both subunits being knocked down (Fig. 3.4A). To assess the impact of knock-down of NFκB1 and RELA on Pac-induced apoptosis and Dex-mediated rescue for Pac we performed an apoptotic assay examining caspase 3 and 7 activity levels (Fig. 3.4C). Both sh NFκB1 and sh RELA cell lines showed statistically significant decrease in sensitivity to Pac-induced apoptosis.

Figure 3.4

3.4A.



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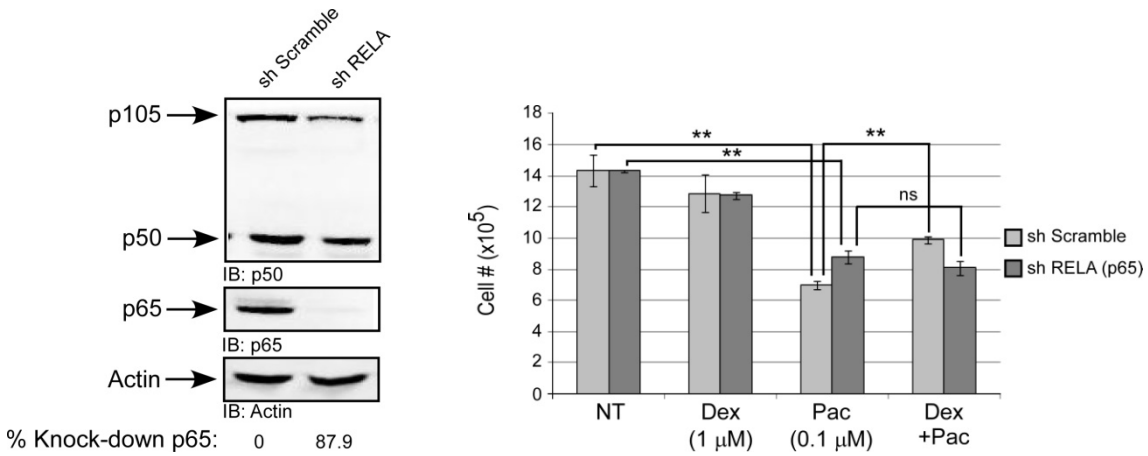

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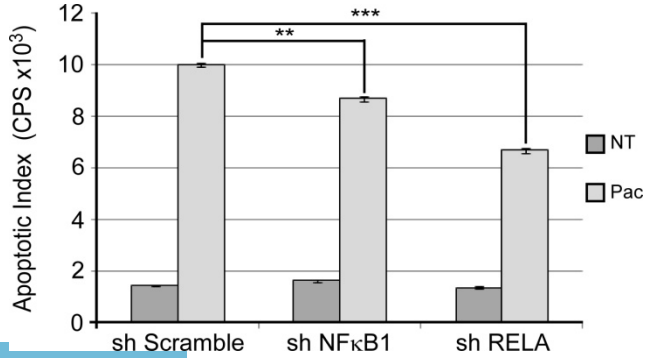


Figure 3.4 Essentiality of NFκB for Pac sensitivity and Dex-mediated rescue of breast cancer cells.

A. MDA-MB-468 cells were stably infected with sh Scramble control or sh NFκB1 construct, and lysates were subjected to immuno-blotting to monitor for p50 and p65 protein levels (left panel). The membrane was probed for p105/p50, p65 and Actin as a loading control. The stable cells were also treated for 24 h with vehicle (NT), Dex, Pac, or co-treated with Dex and Pac (Dex 1 h prior to Pac). Cells were then collected and counted for total cell number (right panel). **B.** Lysates from MDA-MB-468 cells stably infected with a sh Scramble, as the control, or sh RELA were subjected to immuno-blotting to monitor for p105/p50 and p65 protein levels (left panel). The same membrane was used to probe for Actin to ensure equal protein loading. Percentage of knock-down was calculated by densitometry. The stable lines were also treated for 24 h with vehicle (NT), Dex, Pac, or co-treated with Dex and Pac (Dex 1 h prior to Pac). Cells were then collected and counted for total cell number (right panel). **C.** Stable sh Scramble, sh NFκB1, and sh RELA cells were treated with vehicle (NT) or with Pac for 24 h. Cells were collected and lysed. Luciferase activities of the lysates were measured using equal amounts of cell lysate mixed with Caspase 3/7 Glo buffer and luminescence was measured by spectrophotometry.

Bars indicate SEM of at least three individual experiments. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

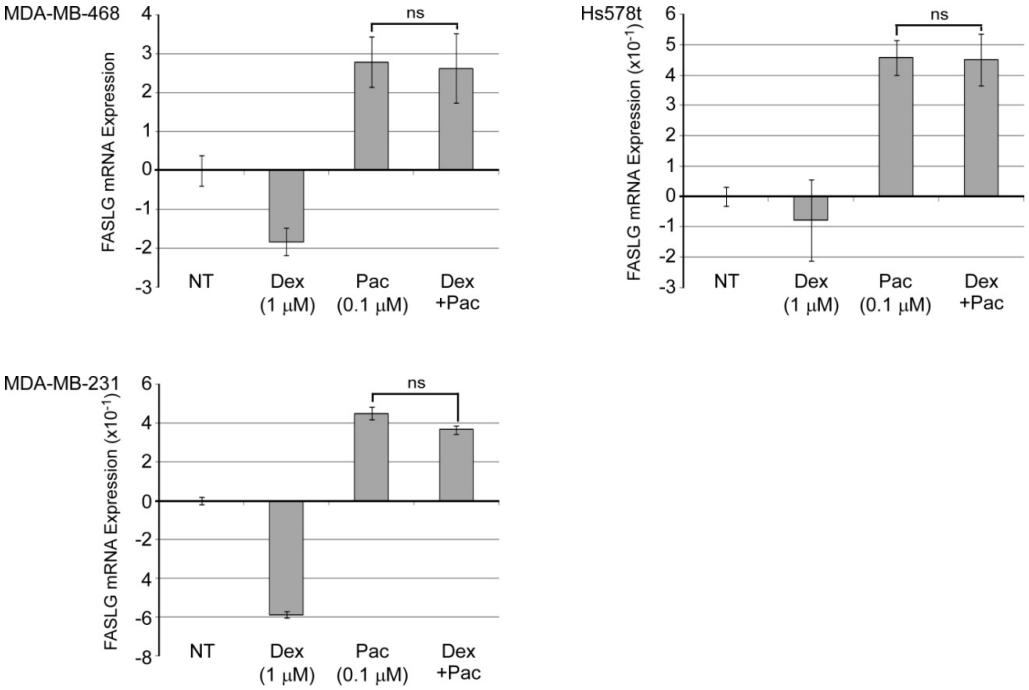
3.3.5 Contrary transcriptional regulation of FASLG and FAS by Pac and Dex.

NFκB regulates more than one hundred and fifty genes. Previous reports in T lymphocytes identified binding sites for NFκB in the promoters of pro-apoptotic FASLG and FAS, which are the genes for the Fas ligand and Fas receptor respectfully [20,21]. To search out a putative target of Pac-activated NFκB and mechanism by which Dex antagonizes Pac we analyzed changes in FASLG mRNA levels 24 h post-treatment with vehicle (NT), or with Dex, or Pac, or with co-treatment with Dex and Pac (Dex 1 h prior to Pac) for MDA-MB-468, Hs578t and MDA-MB-231 cells (Fig. 3.5A). Pac increased transcriptional expression of FASLG in all three cell lines. And, while Dex alone showed decreases in FASLG expression levels for all three cell lines, it was not able to decrease Pac-induced FASLG expression to any statistically significant degree (MDA-MB-468 ($p=0.8948$), Hs578t ($p=0.9504$), MDA-MB-231 ($p=0.1013$)). We repeated the experiment analyzing changes in mRNA for FAS (Fig. 3.5B). For all three cell lines Pac-induced increases in FAS expression, and Dex-treated cells displayed an overall decrease in FAS expression. Unlike for FASLG, however, pre-treatment with Dex was able to override Pac-induced FAS expression in a statistically significant manner (MDA-MB-468 ($p=0.0170$), Hs578t ($p =0.0075$), MDA-MB-231 ($p=0.02$)) (Fig. 5B). To assess potential changes in Fas receptor protein with and without Dex, Pac, and Dex plus Pac (Dex 1 h prior to Pac) for Hs578t western blot analysis was conducted (Fig. 3.5C). The results showed a clear increase in Pac-induced expression of Fas receptor, which was abrogated by addition of Dex. Changes in were quantified by densitometry showing a high statistical significance ($p=<0.0001$) between vehicle control and Pac-treated cellular

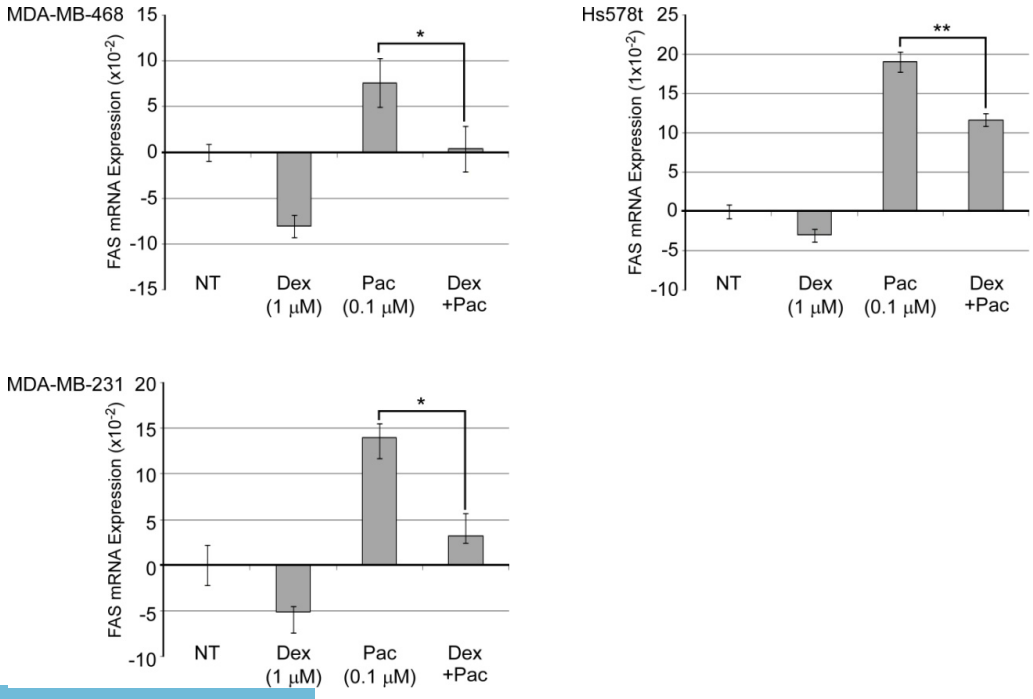
levels of Fas (Fig. 3.5C; right panel). Dex also abrogated Pac-induced expression of Fas to a statistically very significant degree ($p=<0.0001$).

Figure 3.5

3.5A.



3.5B.



3.5C.

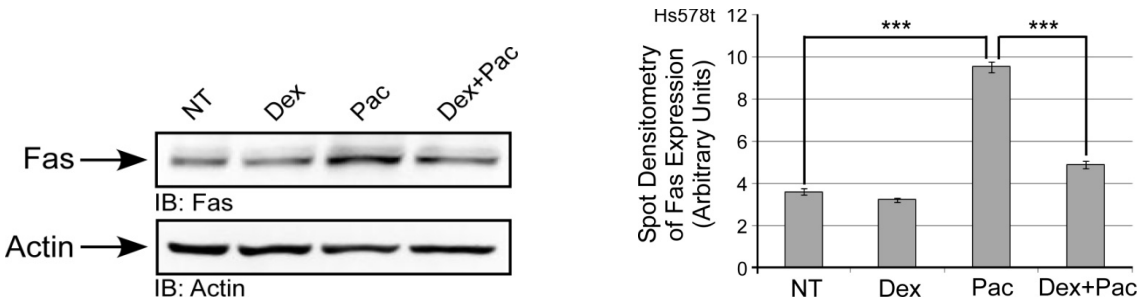


Figure 3.5 FASLG and FAS are upregulated by Pac and down-regulated by Dex.

A. and **B.** TNBC breast cancer cell lines MDA-MB-468, Hs578t, and MDA-MB-231 cells were treated with vehicle (NT), Dex, Pac, or co-treated Dex and Pac (Dex 1 h prior to Pac) for 24 h. Quantitative real-time PCR analysis of FASLG and FAS mRNA are expressed as relative fold change compared to control (NT) following normalization to GAPDH. **C.** Hs578t cells were treated with vehicle (NT), Dex, Pac, or co-treated with Dex and Pac (Dex 1 h prior to Pac) for 24 h to be examined for Fas levels by subjecting the whole-cell lysates to SDS-PAGE. Densitometry of n=3 is shown in the right panel.

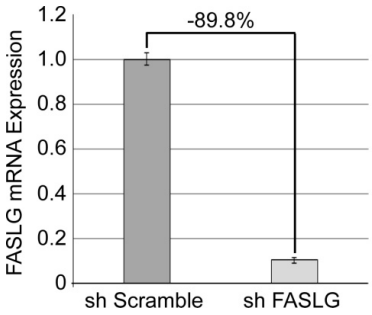
Represented data are mean ± SEM of at least three individual experiments. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

3.3.6 Knock-down of FASLG desensitizes breast cancer cells to Pac-induced apoptosis.

FasLG is a transmembrane protein that binds Fas receptor to initiate cellular death pathways [22]. To assess the significance of FasLG/Fas receptor signaling in mediation of Pac-induced apoptosis and Dex-mediated rescue of breast cancer cells from Pac we stably infected MDA-MB-468 cells with a sh FASLG construct. The extent of FASLG knock-down was measured by quantitative real time PCR (qRT) of FASLG mRNA levels. Knock-down of FASLG was observed at 89.8% decrease in mRNA levels compared to the sh Scramble control infected parental cell line (Fig. 3.6A). Knock-down of FASLG significantly impaired the ability of Pac to decrease total viable cell numbers (14.6% decrease in total cell number) compared to sh Scramble parental cells (51.3% decrease in total cell number) (Fig. 3.6B). Moreover, the ability of Dex to rescue cells from Pac is greatly mitigated by the knock-down of FASLG. Dex increased total cell number when pre-administered to Pac-treated cells by 41.2% compared to a rescue of only 5.22% in sh FASLG cells (Fig. 3.6B).

Figure 3.6

3.6A.



3.6B.

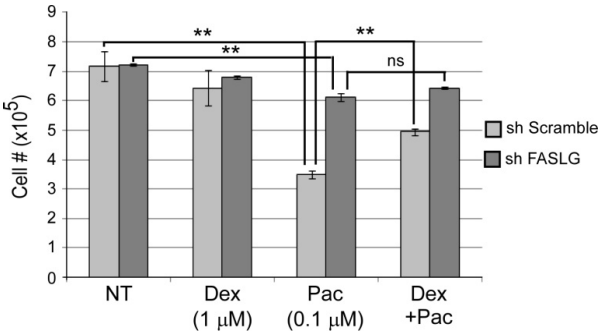


Figure 3.6 Essentiality of FasLG/Fas signaling in sensitivity of breast cancer cells to Pac.

A. Expression of FASLG in MDA-MB-468 cells stably infected with sh FASLG was analyzed using real-time PCR. The relative expression level of FASLG in the knock-down sample is calculated as relative fold change compared to control. **B.** Stable sh Scramble or sh FASLG cells were treated for 24 h with vehicle, Dex, Pac, or co-treated with Dex and Pac. Cells were collected and counted for total cell number**.** Data are represented as mean value ± SEM of at least three individual experiments. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

3.3.7 Fas receptor transcription is downstream of Pac-activated NFκB

While both FASLG and FAS expression increased following Pac treatment (Figs. 3.5A and 5B) Dex was unable to suppress FASLG expression during co-treatment with Pac suggesting the possibility of different mediators of Pac-induced transcriptional regulation for each gene. Moreover, the inhibition of NFκB activity by Dex (Fig. 3.3B) does not necessitate that Dex down-regulates FAS by inhibition of NFκB. To ensure FAS transcriptional regulation by Pac is directed through activation of NFκB we treated sh Scramble, sh p50 and sh p65 cells with and without Pac for 24 h. Cells were collected and mRNA for FAS was quantified by qRT (Fig. 7). The sh Scramble stable cells show a significant increase in FAS expression ($p=0.0031$) (Fig. 3.7). The knock-down of p50 protein, which also significantly decreased p65 levels (Fig. 3.4A), resulted in much less expression of FAS following Pac treatment with no significant change in Pac-treated sh NFκB cells relative to the non-treated control ($p=0.2687$) (Fig. 3.7). By knock-down of the p65 protein, FAS levels did not significantly increase following Pac treatment compared to vehicle (NT) controls ($p=0.2646$) (Fig. 3.7).

Figure 3.7

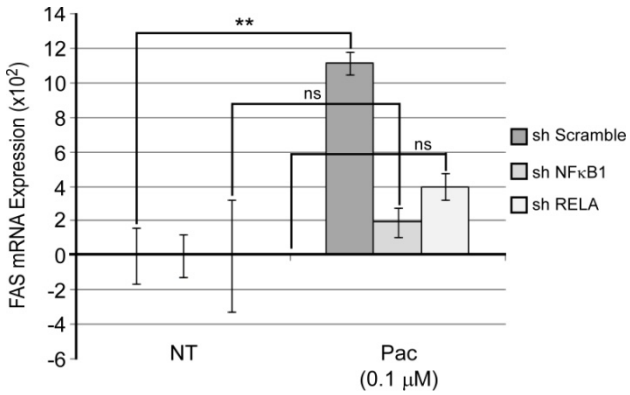


Figure 3.7 NFκB is essential for Pac-mediated transcriptional upregulation of FAS.

Sh Scramble, sh p50, and sh p65 cells were treated with the vehicle or Pac for 24 h. Cells were collected and their total RNA was subjected to real time PCR. Expression levels of FAS were normalized to GAPDH and presented as fold relative to the control.

Represented data are mean ± SEM of three individual experiments. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

3.4 Discussion

Pac is a potent anti-cancer drug often reserved for TNBCs and as a last line of defense against late stage luminal cancers or those that display resistance to traditional therapies. Side-effects of Pac, and the vehicle in which it is dissolved (Kolliphor EL), are counteracted by administration of synthetic GCs such as Dex. Dex is often given hours before chemotherapy, and often administered even multiple times per day. Despite the tremendous efficacy of Pac as an anti-cancer drug, there are increasing reports indicating that co-treatment with Dex antagonizes Pac in several solid tumour cell lines [23-25].

To elucidate the effect of Dex pre-treatment on Pac-induced cell death in breast cancer cells, and the underlying mechanism, we established an *in vitro* set-up in which Dex was administrated to cells 1 h prior to Pac [6]. This setting not only imitates the most common clinical application of Dex, but also shows the extent of its protective role even when given once immediately prior to chemotherapy. Our observations in this study support that Dex pre-treatment antagonizes Pac-induced cell death by abrogation of FAS/FASLG expression via the NFκB transcription factor. Initially, we resolve the sensitivity of various breast cancer cell lines, both TNBCs (MDA-MB-468, Hs578t, and MDA-MB-231) and luminal (MCF 7, SK-BR-3, and T47D) to Pac alone (Fig. 3.1). While all showed sensitivity to Pac, on average, the TNBCs were much more responsive than the luminal cell lines. Our data now supports that the observed differential resistance between TNBCs and luminal breast cancer cell lines may be due to altered regulation of NFκB in luminal cells, as was previously reported [18]. We show that sensitivity of each respective cell line to Pac alone was matched by their likelihood for Dex-mediated rescue from Pac, demonstrated by statistically significant increases in viable cell number of

TNBCs (Figs. 3.2A and 2B). Our data also supports that sensitivity to Dex-mediated rescue from Pac may be due levels of GR being highest in the TNBCs, making TNBCs more primed for Dex-mediated action. Therefore, we hypothesized that pre-treatment of TNBCs with the GR antagonist mifepristone (RU-486) should decrease the likelihood of Dex-mediated rescue from Pac (Fig. 3.2C). When tested, addition of RU-486 to co-treated Pac and Dex MDA-MB-468 cells significantly reversed the Dex-mediated effects, which corroborates reports that pre-medication of RU-486 with GCs can be an alternative solution to block the proliferative/survival effects of Dex and increasing Pac-induced apoptosis in TNBC patients [26]. As suggested by others, Dex treated cells generally showed increase in cell number (Figs. 3.2A and 2B), however, we show that Dex-mediated rescue from Pac could primarily be mediated through inhibition of Pac-induced apoptosis (Fig. 3.2D).

GCs are known to exhibit adverse pleiotropic effects in a cell-type dependent manner [2]. While the precise mechanism of GC-induced resistance to Pac is a developing story, it may be associated with multiple pathways depending on the cell type [27]. Regardless, our data supports that Dex-mediated resistance to Pac is mediated through, at least in part, the control of gene expression [28]. Regulators of NFκB, in particular IκBα, have been shown to play a part in biological activities such as apoptosis, and to also be regulated by Pac [18]. Here, we successfully show that Pac promoted NFκB activation in different breast cancer cell lines, and that this activation was dampened by pre-treatment with Dex (Fig. 3.3). Our data supports the claim that the antagonistic effects of Dex and Pac might be through their oppositional regulation of the NFκB pathway. Knock-down of NFκB subunits, p50 and p65, desensitized the cells to

Pac-mediated apoptosis (Fig. 3.4). While Pac treatment reduced cell viability by more than 50% in control cells, stable knockdown of p50 and p65 we resulted in diminished sensitivity to Pac with decreases of only 6.21% and 38.6% respectively. It is not entirely surprising that Pac continues to cause some level of apoptosis even with substantial knock-down of NFκB given that it is a potent cytotoxic drug activating numerous cellular responses that directly or indirectly activate various programmed cell death responses. Our data supports NFκB being among the list of potent mediators of Pac-induced apoptosis.

While NFκB transcriptionally regulates a number of genes involved in cell survival signaling, it also regulates two prominent activators of apoptosis: FASLG and FAS [29]. In this study, expression of both genes, FASLG and FAS, were down-regulated by Dex and upregulated by Pac (Fig. 3.5). However, only Pac-induced upregulation of FAS was significantly obstructed by Dex in co-treatment conditions. Given our finding that FasLG/Fas signaling is a potential mediator of Pac-induced apoptosis in TNBCs, we knocked down FASLG. Cell counts following treatment with Dex and Pac showed nearly 50% reduction in viable cell number whereas knock-down of FASLG exhibited only 14.6% decrease in total viable cell number (Fig. 3.7B). Thus, the ability of Dex to antagonize Pac requires activity of the FasLG/Fas pathway. We also found that knock-down of NFκB subunits attenuated FAS expression to the point that no statistically significant increase in expression occurred (Fig. 3.7), which strongly supports that the transcriptional regulation of Pac-induced FAS is a mediated through NFκB.

In summary, Dex significantly diminishes Pac-induced apoptosis in breast cancer cell lines of luminal and TNBCs subtypes. Inhibition is most significant for the TNBCs

for which Pac is a primary therapeutic agent. Our data adds to the mechanistic information available describing this phenomenon (Fig. 3.8). We show that Pac and Dex contrarily regulate NFκB and Dex is able to abrogate Pac-mediated activation of NFκB. Furthermore, knock-down of the subunits of canonical NFκB (p50/p65) densensitizes TNBCs to Pac supporting that NFκB signaling plays a critical role in Pac-mediated apoptosis in these cell lines. While Pac and Dex contrarily regulate gene expression of FASLG and FAS, only FAS expression is overridden by Dex under co-treatment conditions with Pac and is corroborated at the protein level. We show that FasLG/Fas signaling is a critical component of Pac-induced apoptosis in these cells as knock-down of FASLG desensitizes cells to Pac. Finally, Pac-mediated regulation of Fas is through NFκB as knock-down of the NFκB subunits mitigates Pac's ability to upregulate FAS. Collectively this work adds to the body of growing knowledge that GCs such as Dex override breast cancer responses to chemotherapy. These data may have important implications for patient outcomes in the clinic and need to be further explored in clinical trials.

Figure 3.8

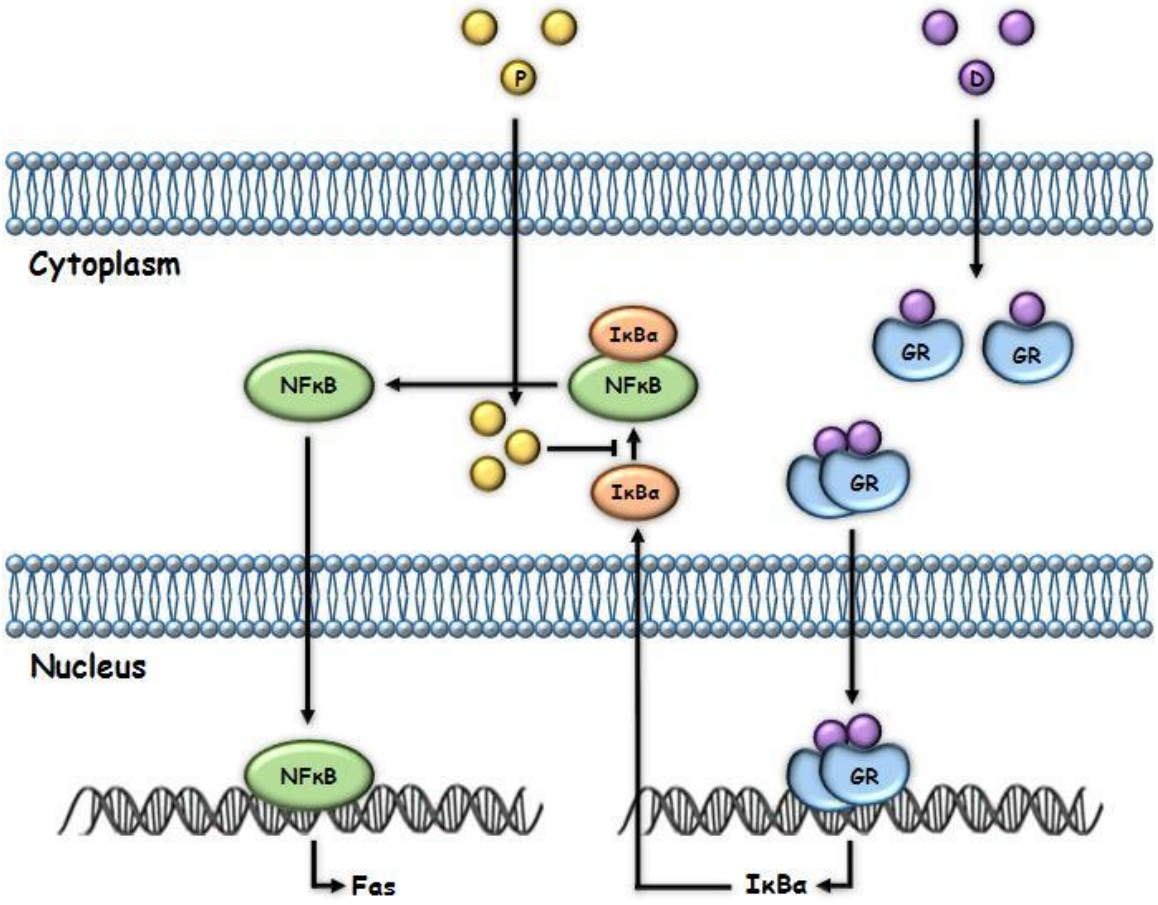


Figure 3.8 Dex and Pac contrarily regulate Fas signaling through NFκB regulation.

A general depiction of Pac and Dex regulation of Fas expression through the NFκB signaling pathway. See text for detailed explanation of pathway. D: Dex; P: Pac.

3.5 Acknowledgments

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Chapter 4: General discussion and future directions

4.1 Overview

Breast cancer is the most prevalent form of cancer to affect Canadian women with approximately 24,000 new cases each year [1]. Approximately 30% of women with breast cancer will develop metastatic disease for which there is no cure [2,3]. Progression to a metastatic state from the primary tumor site requires tumor cells to possess unique characteristics allowing them to 1) elude apoptosis and to grow and proliferate at the primary tumor site; 2) invade through existing tissue membrane boundaries; 3) migrate in circulation, or within neighbouring tissues, and 4) attach and colonize at a distant site [4]. These characteristics may be inherent in certain subpopulations of breast cancer cells, they may be acquired through mutation(s), or drug treatment of cancer cells may promote these characteristics [3].

Breast cancers are categorized histologically into luminal and triple negative (TNBCs) subtypes based on hormone receptor status [5-7]. The absence of these hormone receptors in TNBCs creates difficulty in clinical treatment. TNBC patients, along with late stage and hormone-resistant luminal breast cancer patients, commonly receive paclitaxel (Pac) [8,9]. Pac and the vehicle it is dissolved in, however, frequently cause hypersensitivity, nausea, vomiting, and allergic reactions in patients [10-12]. To counteract these reactions, the synthetic glucocorticoid dexamethasone (Dex) is administered hours and sometimes days in advance of Pac. Dex is a potent activator of the glucocorticoid receptor (GR) which regulates either negatively or positively approximately 10% of the human genome [13]. Given the pleiotropic effects of Dex in numerous tissues, we assessed the impact of Dex both *in vitro* and *in vivo* on breast cancer survival/proliferation, migration and invasion.

We demonstrate that administration of Dex increases cell number within 24 h post-treatment relative to vehicle-treated control cells *in vitro* (Figs. 2.2A and 2B). Sensitivity to Dex-mediated increases in cell number correlates with breast cancer subtype. It remains unclear, however, as to whether or not Dex-mediated effects are due to increased proliferation, survival or a combination of both. We demonstrate, using apoptosis assays, that Dex downregulated the activity of apoptotic proteases Caspase 3 and 7 compared to control and in co-treatment conditions with Pac compared to Pac alone (Figs. 2.2D and 2E). It remains to be determined how much of the decrease in caspase activity accounts for the difference in total live cell number of the Dex-treated population versus control population. Nevertheless this decrease in caspase activity is consistent with a Dex-induced pro-survival role. As the question at hand was to determine whether or not Dex could enhance a critical characteristic of metastatic breast cancer cells (i.e. survival and growth), we demonstrate in the affirmative that it does. Clarification on the matter of Dex-mediated cell survival versus Dex-mediated proliferation could be produced by performing bromodeoxyuridine (BrdU) incorporation assays; alternatively, antigen Ki-67 expression could be measured to see if changes in proliferation are occurring. Also, further corroboration of Dex-mediated increases in cell number could be produced by treating cells with vehicle control, Dex alone and or Dex + RU-486 supporting the conclusion that active GR is responsible for the Dex-mediated effects.

In regards to *in vitro* increases in cell migration and invasion we show that Dex causes overall cell motility as migration through cyto-select chambers and invasion through collagen coated chambers greatly increased following Dex treatment (Figs. 2.3 and 2.4). A legitimate concern is that apparent increases in migration and invasion might

be false positives due to increases in overall cell number. We performed experiments to address two possible alternative explanations for the observed migration and invasion phenotype, in particular: 1) whether Dex-treated cells could proliferate or survive better in the serum-free conditions over the 24 h period of the assay or 2) that Dex-treated cells had proliferative pathways activated such that when they migrated or invaded to complete growth media conditions they had a proliferative advantage (e.g., a head start) compared to those cells that migrated or invaded in control chambers. To test the first alternate explanation Dex effects on overall cell number were tested in the serum-free conditions (mimicking pre-migration side of chamber) over the full 24 h period mimicking the length of the migration and invasion assays (Figs. 2.3D and 3E). The Dex treated chambers did not have more cells in them to migrate compared to vehicle control cells, hence enhanced proliferation of the cells in the pre-migration chamber did not explain the migration/invasion assay results. To test the second alternative explanation, we grew cells in serum-free conditions with vehicle or with Dex for 24 h and then replaced their media with complete growth media lacking Dex (mimicking a condition in which the cells had migrated or invaded). Dex-treated cells did not show an increase cell number in complete media within 24 h of changing media from serum-free to complete (Fig. 2.3D and 3E). We did observe, however, increases in MDA-MB-231 cell number compared to control populations if Dex-treated cells were allowed more time (48 h) in growth factor positive conditions (Fig. 2.3D). Thus, it would appear that not only does Dex increase cell motility, but Dex-treated cells that invade or migrate eventually either proliferate faster, or survive better following movement, than non-Dex-treated cells.

One other matter of concern to address is how do we explain having observed increases in cell number in Dex-treated cells compared to vehicle controls cells earlier and then claim such does not occur in the same time-period during the migration and invasion assays? Increases in cell number demonstrated earlier (Fig. 2.2) were in complete media conditions in which growth factors are present throughout the experiment compared to the serum-free conditions of the migration and invasion assays in which growth factors are only available to cells that cross the membrane. Thus, it is true that Dex enhances cell numbers compared to vehicle-treated control, but this phenotype is not observed in the early stages of the migration/invasion assays which last for a short period of time and are conducted in serum-free conditions. Overall, the implication for breast cancer patients is that multiple metastatic characteristics could be enhanced by Dex treatment in clinic.

Our data also demonstrates that Dex mediates overall metastatic properties of human breast cancer cells *in vivo* using a zebrafish model (Fig. 2.5). We demonstrate that Dex-treated fish have more cancer cells at greater distances from initial injection sites than control fish. Which cell behaviour is responsible for this physiological effect needs to be carefully dissected. It is possible that increased proliferation or survival in the yolk sack of Dex treated fish could increase the likelihood of increased cells at distant sites. Alternatively, increased migration or invasion could also explain the increased number of distant site cells in Dex treated fish. Increases in proliferation or survival of inherently invasive or migratory cells after invasion from the yolk sack could also explain the difference between Dex-treated and control fish. Nonetheless, the phenotype of increased

numbers of viable cells at distant sites holds true for Dex-treated fish relative to control fish. These observations warrant further research into the matter of concern at hand.

Some might argue that the impact of Dex on the proliferation, or invasiveness, or migratory capacity of breast cancer cells is of no concern as these patients ultimately receive the potent cytotoxic drug Pac. We sought to further previous work on the topic of Pac-resistance in Dex-treated breast cancer cells. We sought to assess the prevalence of this phenotype in breast cancer cells lines representative of TNBC and luminal breast cancers, and to elucidate a mechanism by which Dex might mediate its pro-survival role against Pac.

We demonstrate that Dex does protect breast cancer cells from Pac, in particular from Pac-induced apoptosis. We also find that overall TNBCs were more sensitive to Pac than luminal cell types, encouraging given that TNBCs are highly dependent on Pac for treatment. However, Dex induced a strong rescue in both subtypes as demonstrated by inhibition of caspase 3 and 7 activity. One anomaly with the luminal cell lines (SK-BR-3 and T47D) was that Dex's rescue from Pac activation of caspases 3 and 7 (Fig. 3.2D) was inconspicuous in the total live cell number (Fig 3.2B). This phenotype may suggest an alternative means of Pac-induced death that does not involve activation of caspase 3 and 7.

Previous work had demonstrated that NFκB signaling could play an important role in Pac-induced apoptosis. An upstream regulator of NFκB, MAP3K1, is transcriptionally upregulated following Pac treatment [14,15]. MAP3K1 phosphorylates IKKβ1 resulting in phosphorylation and subsequent degradation of IκBa [14,15].

Unphosphorylated IκBα sequesters NFκB in the cytoplasm. Huang *et al* show that transcriptional upregulation of IκBα is important in mediation of Dex-inhibition of Pac. We identify the downstream target of Pac-induced NFκB as Fas receptor (Fas). While both Fas ligand (FasLG) and Fas are transcriptionally upregulated following Pac treatment, and both are transcriptionally down-regulated by Dex-alone treatment, only Pac-induced Fas transcription was antagonized following co-treatment of Dex and Pac. Knockdown of the p50 and p65 subunits of NFκB diminished Pac transcriptional upregulation of Fas, putting Fas signaling downstream of Pac-activated NFκB. Thus, the overall pathway would be as follows in the absence of Dex: Pac, through an unknown mediator causes transcriptional upregulation of MAP3K1. MAP3K1 phosphorylates and activates IKKβ1. Active IKKβ1 phosphorylates IκBα and marks it for ubiquitination and degradation by the 26S proteasome complex. Following degradation of IκBα NFκB translocates to the nucleus where it binds promoter element in the FAS gene. Upregulation of FAS (and FASLG by another Pac-mediated pathway), results in eventual activation of the extrinsic pathway of apoptosis. In the presence of Dex transcriptional upregulation of IκBα could saturate IKKβ1 activity leaving residual IκBα protein to sequester a portion of NFκB complexes in the cytoplasm. Pac's activation of NFκB would be partially attenuated by the diminished NFκB transcriptional activity and subsequent diminished upregulation of the extrinsic pathway mediator Fas.

Overall our work shows that the administration of Dex to breast cancer patients could increase negative characteristics of metastatic breast cancer cells even within the early hours of treatment. Given that Dex also protects breast cancer cells from Pac-induced apoptosis it may be that not only a greater number of breast cancer cells survive

chemotherapy, but also that those cells are made more dangerous in the process. Nevertheless, there remains work to be done to better understand these reported effects of Dex on breast cancer cell behaviour. Such future studies could include both immediate *in* vitro and *in vivo* laboratory work as well as longer term clinical trials.

4.2 Future Directions

Several short-term immediate *in vitro* assays could provide clarity concerning the impact of Dex on breast cancer cell behaviour. Repeating the cell counts, as well as the migration and invasion assays with the addition of a Dex + RU-486 treatment would falsify or corroborate the role of the GR in mediating the reported effects. Moreover, since previous reports of Dex's impact on these same phenotypes in other cell lines differed in response, when compared to the breast cancer cell lines use herein, the addition of other such cell lines (e.g., bladder cancer cell lines) could serve as positive controls against which we could compare our reported findings [16]. Dosing and timing of Dex and/or Pac treatment were consistently conducted for all experiments with Dex being administered at 1 μM and given one hour in advance of Pac (0.1 mM) during co-treatment experiments. These doses and timings were based on previous reports in the literature as to being the most clinically relevant [17]. For several experiments (primarily those involving Dex alone) numerous concentrations of Dex were used although we strove to ensure that for each cell line Dex (1 μM) was a common treatment. It could prove valuable to use various concentrations of Dex and Pac as well as alternate timings of delivery to determine if the impact of Dex on Pac is dose/timing dependent. Such data could be useful for altering clinical protocols so as to maximize the efficiency of Pac while minimizes the pro-survival role of the anti-emetic Dex. Furthermore, breast cancer

patients do not receive Paclitaxel alone but rather a cocktail of antineoplastic drugs. Replicating the order, timing and dosages of these drugs in the presence and absence of Dex would demonstrate the potency or impotence of Dex to antagonize chemotherapy on these breast cancer cell lines.

In terms of the *in vivo* assays performed herein, other model organisms such as a mouse model could strengthen the results observed in the zebrafish. For example, breast cancer cells (TNBC, e.g., MDA-MB-231 and luminal, e.g., MCF 7) could be transplanted for xenograft studies into the mammary fat pad of female severe compromised immune-deficient mice (SCID). Once tumours form and reach approximately 200 mm^3 the mice could be treated with vehicle, Dex or Dex and RU-486. The longest and shortest diameters of the tumours would be measured by calipers several times a week and tumour volume calculated. There are several advantages and disadvantages to using the mouse model compared to the zebrafish model. Similar to the zebrafish (which lack an immune system up to day 14), the SCID mice lack an immune system [18]. The absence of the immune system gives the technical advantage of establishing cancer cells without immune system-mediated detection and destruction. In mice the absence of the immune system makes interpretation of the relevance of the observed data more difficult for cancer in humans with an intact immune system, where as the zebrafish immune system develops at maturity. The mouse model provides the advantage of being a mammalian model system with a mammary gland, allowing for orthotopic injections, however, there remain differences between mouse mammary glands and human mammary glands. For example, human cells are not as well adapted for the mouse mammary environment. A recent report shows that breast cancer metastasis to bone in which the target organ is of

human origin, the cancer cells preferentially migrate and colonize the bone of human origin, thus exhibiting selective preference [19].

Despite reports of the Dex's ability to antagonize Pac, to date there have been no clinical trial studies to compare the anti-cancer efficiency of Pac in the presence and absence of Dex. Such a trial would be of high clinical relevance to patients. Currently all patients receiving Pac are co-treated with Dex, with some patients receiving several doses of Dex prior to chemotherapy treatment. Dex is administered to block the undesired emetic effects of Pac but many of these effects are due to the delivery vehicle Pac is dissolved in, namely castor oil [10]. Alternate forms of Pac, Abraxane, now exist where the drug is fused to serum albumin and not dissolved in castor oil, lowering the need for anti-emetics. Clinical trials using traditional Pac and Abraxane in the presence and absence of Dex could address both the differences as to the efficiency of the alternate delivery systems and whether or not Dex is even needed with Abraxane. Clinical trials could yield both early results, such as overall tumour response (as measured by changes in tumour volume) as well as long-term result data, e.g., disease-free state, etc. Our data, and that of others, strongly support the need for a direct clinical trial to be performed; Abraxane provides a very nice alternative that could be used and studied in the presence and absence of gluocorticoids for this purpose.

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