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entitled

Glucocorticoid Receptor beta Increases the Migration of Human Urothelial Carcinoma Cells

by

Lucien R. McBeth

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Master of Science Degree in Biomedical Sciences

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An Abstract of

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Urothelial carcinoma is one of the most prevalent cancers encountered in the country, and recent investigations demonstrate the important role of glucocorticoid signaling in the disease. We have shown that an alternate isoform of the glucocorticoid receptor (GR), GRβ, causes migration of human urothelial carcinoma cells. We begin with a literature review of the role of GRβ and another nuclear receptor, the androgen receptor, in urothelial carcinoma. Next, we investigate the role of GRβ in the migration of human urothelial carcinoma cells in two transitional human uroepithelial carcinoma cells, UMUC-3 and T24. We found that the T24 cells have higher GRβ expression compared to the UMUC-3, and that both cell lines had a similar GRα level. Interestingly, the higher GRβ expression was correlated with enhanced migration rates, which was reduced with GRβ inhibition. In-silico analysis of the 3’ untranslated region (3’UTR) of human GRβ revealed a potential micro-RNA (miRNA) binding site for miR33a, miR144, and miR181. Therefore, we cloned the 3’UTR of human GRβ and mutated the miRNA binding sites, which showed that miR144 positively regulates GRβ expression. In addition, miR144 and
GRβ expression were increased during migration of uroepithelial carcinoma cells. Therefore, we constructed a peptide nucleic acid (PNA) conjugated to a cell penetrating-peptide (CPP) that we termed Sweet-P to inhibit the miR144 binding site in the 3’UTR of human GRβ. Furthermore, Sweet-P decreased GRβ expression and, as a result, inhibited migration of uroepithelial carcinoma cells, demonstrating its potential as a therapeutic. We then complete the thesis with a discussion of the potential for Sweet-P in cancer therapy and other GRβ-related diseases, which may serve as the first anti-GRβ drug.
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List of Abbreviations

ANOVA ........................ Analysis of variance
AR ............................. Androgen receptor
ARE ............................ Androgen-response element
BCG ............................ Bacillus calmette guerin
CDDP .......................... cis-diamminedichloroplatinum
CIS ............................. Carcinoma in situ
COX-2 .......................... Cyclooxygenase-2
CPP ............................. Cell-penetrating peptide
Dex ............................. Dexamethasone
EGF ............................. Epidermal growth factor
EGFR ........................... Epidermal growth factor receptor
EMT ............................. Epithelial-mesenchymal transition
ER ............................. Estrogen receptor
FBS ............................. Fetal bovine serum
FKBP51 ........................ FK506 binding protein 51
FSCN1 .......................... Fascin actin-bundling protein 1
GC ............................. Glucocorticoid
GILZ .......................... Glucocorticoid-induced leucine zipper
GR ............................. Glucocorticoid receptor
HSP ............................. Heat shock protein
INF-α ........................... Interferon-α
IGFR-1 .......................... Insulin-like growth factor receptor 1
IL-6 ............................. Interleukin-6
IRS ............................. Insulin receptor substrate
M1 ............................... Pro-inflammatory type 1 macrophage
M2 ............................... Anti-inflammatory type 2 macrophage
MEM ............................. Minimum essential medium
MHC ............................ Major histocompatibility complex
mRNA .......................... messenger ribonucleic acid
miRNA .......................... micro ribonucleic acid
mTOR ........................... Mammalian target of rapamycin
NF-κB .......................... Nuclear factor kappa-light-chain-enhancer of activated B cells
NRP ............................. Neuropilin
PI3K ........................... Phosphoinositide 3-kinase
PNA......................Peptide nucleic acid
PTEN..........................Phosphatase and tensin homolog deleted on chromosome 10
S.E.............................Standard error
shRNA........................Short hairpin ribonucleic acid
SPARC......................Secreted protein acidic and rich in cysteine
RT-PCR......................Real-time polymerase chain reaction
TNFα........................Tumor necrosis factor alpha
UTR............................Untranslated region
VEGF.........................Vascular endothelial growth factor
Veh............................Vehicle
List of Symbols

\[ \alpha \] ................. Alpha
\[ \beta \] ...................... Beta
\[ \kappa \] ...................... Kappa
Chapter 1

Introduction

Urothelial carcinoma is the 4th most common cancer in men, and the 5th most common cancer overall (Society 2015). While urothelial carcinoma is not an especially lethal disease (12.3% 10-year mortality rate), it does have a high rate of recurrence (74.3%) and progression (33.7%) within ten years (Chamie et al. 2013). Due to the high probability of recurrence and progression of urothelial carcinoma, there is a need for surveillance and frequent treatment to prevent progression. These factors together posit bladder cancer as one of the most costly cancers for lifetime treatment (Sievert et al. 2009). Currently, there are no treatments that prevent urothelial carcinomas or their recurrences. However, glucocorticoids (GC) have been widely used as co-medication in patients with advanced urothelial carcinomas due to their activation of anti-inflammatory and apoptosis pathways (Dietrich et al. 2009). GCs bind to the GC receptor (GR), whose expression is reduced in bladder cancer tumors compared to normal cells (Zheng et al. 2012, Ishiguro, Kawahara, Zheng, Netto, et al. 2014). Ishiguro et al. showed that GR expression was higher in benign compared to tumor tissues, low-grade compared to high-grade tumors, and in non-muscle invasive compared to muscle-invasive tumors. In addition, low GR expression was correlated with tumor recurrence, and lower Kaplan-Meier progression-free survival. GR
expression was also shown to be a strong independent predictor for non-muscle invasive tumor recurrence.

**Figure 1-1. Human bladder tissue anatomy and staging of urothelial carcinoma.**
The human bladder is comprised of four tissue layers: transitional uroepithelium (inner lining), lamina propria (connective tissue), detrusor muscle (muscle layer), and adventitia (fat around bladder). Urothelial Carcinoma is staged depending upon the degree of muscle invasion and metastasis. Stage I consists of a tumor that has not invaded the muscle (A). Stage II consists of tumor that has invaded the muscle (B), while Stage III consists of a tumor that has invaded through the muscle layer and into the adventitia or reproductive organs (C). Stage IV consists of a tumor that is found in more than one lymph node or has metastasized to the pelvic wall or beyond (D).
*Image adapted from National Cancer Institute: http://www.cancer.gov/types/bladder/patient/bladder-treatment-pdq#section/109*

The human bladder is composed of four tissue distinct layers: the uroepithelium, lamina propria, muscular layer (or detrusor muscle), and the adventitia (*Figure 1-1*) (Winder et al. 2014). The bladder serves its function as a luminal space that holds
concentrated compounds and metabolites that will be excreted from the body in to urine. This places the uroepithelial tissue at a unique position in that it is exposed to higher concentrations of carcinogens than is often observed elsewhere in the body. Because of this, it is not surprising that the majority of urothelial carcinomas encountered in the developed world arise from the uroepithelium. Within urothelial carcinoma, there are three clinicopathological forms: papillar, solid/nodular, and carcinoma in situ (CIS). Tumors are pathologically staged based upon their degree of muscle invasion/metastasis (Figure 1-1). Stage I tumors have not invaded the muscle layer (Figure 1-1 A). Stage II tumors have invaded the muscle layer, and Stage III tumors have invaded through the muscle layer into the adventitia or reproductive organs. Stage IV tumors are those that are found in two or more lymph nodes or have metastasized to the pelvic wall or beyond. The current grading system for urothelial carcinomas is defined as either low or high-grade, depending on histological findings and pathological staging. Low-grade tumors generally have a favorable prognosis but a high rate of recurrence (60-80%) (Herr et al. 1995). High-grade tumors are more aggressive and much more likely to invade the muscle layer and metastasize (Sievert et al. 2009). Due to the differing prognoses, there are different treatment regimens for the two grades of urothelial carcinoma.

Low grade urothelial carcinoma treatment can be difficult due to the high recurrence rates. Common treatment methods include tumor resection with adjuvant Mitomycin C (a chemotherapeutic treatment) or Bacillus Calmette Guerin (BCG, a biologic treatment) to prevent recurrences. Superficial high-grade (noninvasive) urothelial carcinomas are also treated with BCG. Muscle invasive high-grade urothelial carcinomas
require more radical treatment, including neoadjuvant chemotherapy combined with cystoprostatectomy or radiation therapy.

Urothelial carcinomas commonly occur in men over 65 years old, and can arise from a variety of causes, including both environmental/lifestyle and iatrogenic factors. Cigarette smoking causes 50% of urothelial carcinomas in men, and 35% of urothelial carcinomas in women (Zeegers et al. 2000). This causal relationship is unique due to the fact that smoking associated cancers are more common in the respiratory system. The uniqueness is likely due to the exposure of the uroepithelium to carcinogens from cigarette smoking being concentrated in the urine to be excreted from the body. Similar exposure to carcinogens from environmental or occupational sources include aromatic amines and polycyclic aromatic hydrocarbons used in manufacturing (Boffetta, Jourenkova, and Gustavsson 1997). In addition, certain urinary tract infections caused by Schistosoma haematobium have been shown to be associated with urothelial carcinoma (Cheever 1978). Iatrogenic factors include the exposure of the uroepithelial to acrolein, a metabolite of cyclophosphamide commonly used to treat cancer patients, and therapeutic pelvic radiation (Kiuchi et al. 2009, Boice et al. 1988). All of these factors induce inflammation in the bladder, which is thought to be a precursor to the development of urothelial carcinoma.

Glucocorticoids are anti-inflammatory and apoptotic, which occurs by binding to and activating the hormone-binding isoform of GR, GRα. Upon ligand binding, GRα acts as a transcription factor that increases transcription of genes such as IκBα and COX-2 (to inhibit inflammation), p27 and p21 (cell cycle arrest proteins), and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (pro-apoptotic protein) (Deroo and Archer 2001, Rogatsky, Trowbridge, and Garabedian 1997, Yemelyanov et al. 2007, Ni et al. 2011,
Lim et al. 2014). In contrast, the alternate GR isoform, GRβ, has been shown to act as an inhibitor of GRα (Hinds et al. 2010, Stechschulte et al. 2014). GRβ is encoded by the same gene as GRα, but results from an alternate splicing event that includes a different exon 9 and thus results in a smaller protein that lacks the ligand binding domain for GCs (Hinds et al. 2010). Most investigations of GRβ have been shown to inhibit GRα activity by dimerizing with GRα to interrupt its gene transcription regulation. Recent work has shown that GRβ may have an independent function from GRα that augments growth factor induced proliferation. Increasing GRβ expression into mouse cell lines decreased PTEN expression (an inhibitor of the PI3K/AKT pathway) and increased AKT1 phosphorylation and cell proliferation (Stechschulte et al. 2014). Also, in glioblastoma cells, GRβ has been shown to interact with TCF-4 to regulate the β-catenin/Wnt pathway to induce proliferation (Wang et al. 2015). In addition, the loss of GRβ reduced proliferation of LNCaP-ARA70β, RC165, and DU145 prostate cancer cells lines, indicating that GRβ plays a role in the growth pathway (Ligr et al. 2012). GRβ has been associated with a variety of immunological diseases such as ulcerative colitis, asthma, and chronic sinusitis (Lewis-Tuffin and Cidlowski 2006, Yin et al. 2013, Leung et al. 1998, Zhang et al. 2007), as well as cancer cell migration and proliferation in glioblastoma (Yin et al. 2013) and leukemia (Longui et al. 2000). GR is involved in urothelial carcinoma, but the role remains to be elucidated (McBeth et al. 2015). Long-term GC treatment can increase the subsequent risk of urothelial carcinoma, possibly through immunosuppression (Dietrich et al. 2009, McBeth et al. 2015) or by causing GC resistance through elevated GRβ (McBeth et al. 2015, John et al. 2016). In addition, while GCs can suppress urothelial carcinoma invasion, they can also induce proliferation (Ishiguro, Kawahara, Zheng, Kashiwagi, et al. 2014,
Zheng et al. 2012). These confounding results could be due to GRβ activity, but this needs to be investigated.

Due to the high costs and rates of recurrence and progression of urothelial carcinoma, a therapy to prevent muscle invasion and metastasis could be greatly beneficial to patients and the medical community. Because of the previous data that shows GRβ’s involvement in the migration of glioblastoma cells and the known involvement (role yet to be deciphered) of GR in urothelial carcinoma, we investigated the role of GRβ role in urothelial carcinoma. We hypothesized that GRβ would be expressed higher in bladder cancer cells that are more migratory and proliferative. Through this work, we developed the first anti-GRβ molecule to inhibit expression. These studies open a potential new therapy for the prevention of urothelial carcinoma progression through tumor cell migration, but also for a method to inhibit GRβ-related diseases.
Chapter 2

Involvement of the Androgen and Glucocorticoid Receptors in Bladder Cancer

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Abstract

Bladder cancer is encountered worldwide having been associated with a host of environmental and lifestyle risk factors. The disease has a male to female prevalence of 3:1. This disparity has raised the possibility of the androgen receptor (AR) pathway being involved in the genesis of the disease; indeed, research has shown that AR is involved in and is likely a driver of bladder cancer. Similarly, an inflammatory response has been implicated as a major player in bladder carcinogenesis. Consistent with this concept, recent work on anti-inflammatory glucocorticoid signaling points to a pathway that may impact bladder cancer. The glucocorticoid receptor (GR)-α isoform has an important role in suppressing inflammatory processes, which may be attenuated by AR in the development of bladder cancer. In addition, a GR isoform that is inhibitory to GRα, GRβ, is pro-inflammatory and has been shown to induce cancer growth. In this manuscript, we review the evidence of inflammatory mediators, and the relationship of AR and GR isoforms as they relate to the propensity for bladder cancer.
Introduction

Bladder cancer is the sixth most common cancer in the United States [30]. It was predicted that there would be approximately 75,000 new cases and 16,000 deaths in 2014. There is a recognized predilection for males with an incidence ratio of 3:1. Urothelial carcinoma, arising in the mucosa of the bladder, accounts for the majority of cases encountered in the United States. There are three clinicopathologic forms of urothelial carcinomas: papillary, solid/nodular and carcinoma in situ (CIS). Grading of these cancers is currently defined as either low or high grade depending upon standard histological findings. Patients with low-grade tumors generally have a favorable prognosis; however, the risk of recurrence is high (60–80%) [9]. High grade tumors are much more aggressive with a proclivity for invasion and metastasis [5]. CIS by definition is high grade and its presence in combination with papillary or solid cancers can alter treatment paradigms and prognosis. Pathological staging is based on depth of bladder wall invasion. High grade and bladder wall muscle invasion are associated with poorer outcomes [4].

Recent investigations have shown that inflammation and pro-inflammatory cytokine production are correlated with advanced cases of cancer, and may be indicators of a poor prognosis [31]. Pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNFα) and interleukin-6 (IL-6), lead to an inflammatory state that stimulates tumor growth [32]. There is evidence that an isoform of the glucocorticoid receptor (GR), GRβ, which is inhibitory to glucocorticoid action, increased by inflammation, and may lead to cancer growth [2, 33]. Further studies have shown that GRβ may also enhance androgen receptor (AR) induced growth in prostate cancer cells [2]. However, the relationship of AR and GRβ has not been established. The importance of anti-inflammatory glucocorticoids
in the management of bladder cancer is only now becoming understood. Herein, we will
discuss the roles of the androgen and glucocorticoid nuclear receptor signaling pathways
as they relate to inflammation and bladder cancer.

Factors Leading to Bladder Cancer

Inflammatory pathways and agents that cause inflammation are associated with
bladder cancer, which include certain types of infections, environmental/lifestyle factors,
and iatrogenic factors.

Inflammation from urinary tract infections caused by *Schistosoma haematobium*,
which is a digenetic trematode found in Africa and the Middle East, are associated with a
separate type of bladder cancer, squamous cell bladder cancer (also known as bilharzial
bladder cancer) [12]. Heavy egg deposits in the bladder mucosa and submucosa occur
during the acute phase of *S. haematobium* infection in humans [12, 34]. The eggs act as a
mechanical irritant to the bladder epithelium, inducing chronic inflammatory lesions thus
priming the bladder for inflammation and carcinogenesis [35]. There is a 5.6:1 male
prevalence for the egg-induced schistoma bladder cancers (4.3:1 incidence in males for
non-egg schistoma), which is greater than any other bladder squamous cell carcinoma in
patients from Egypt [36]. This may be due to a higher male susceptibility, or more exposure
of males to causative agents. Interestingly, the loss of the Y chromosome was observed in
7 of 17 (41%) male cases studied with *S. haematobium* induced bilharzial bladder cancer
[37], indicating a unique male pathway in squamous cell bladder cancer development.
However, there have been no investigations in patients with *S. haematobium* infection on
the involvement of the androgen or glucocorticoid receptors.
Both lifestyle factors and environmental agents have been causally related to the development of bladder cancer. Interestingly, there is a male propensity in bladder cancer caused by cigarette smoking, which is estimated to contribute to 50% of cases in men and 35% in women [10]. Regular cystitis is positively associated with bladder cancer risk, and may be due to chronic inflammation from carcinogens in the urine of patients that smoke [38]. Environmental or occupational exposure to various chemical carcinogens, such as aromatic amines and polycyclic aromatic hydrocarbons used in the production of aluminum, coal gasification, roofing and carbon black manufacturing, are agents known to potentially induce bladder cancer [11, 39], which is most likely through induction of inflammation and chronic cystitis in bladder. There is a separation in the amount of occupational chemical exposure in men versus women [40], which may indicate a predisposition of bladder cancer in males. However, the effect of chemical carcinogens on AR signaling activity in bladder is unknown.

Iatrogenic factors that cause bladder cancer include chemotherapeutic agents and radiation. Cyclophosphamide is widely used in a variety of clinical scenarios, which can form metabolites that can contribute to the development of bladder cancer [41]. The inactive metabolite of cyclophosphamide, acrolein, is excreted into urine which induces inflammation of the bladder leading to hemorrhagic cystitis [13]. Bladder epithelial damage occurs because of a reduction of endogenous glutathione and generation of free radicals that initiate lipid peroxidation and other cell damage. There have been no differences found in the treatment of cyclophosphamide and bladder cancer between males and females [42, 43]. In addition, treatment of rats with cyclophosphamide in males showed no significant difference in respect to male reproductive organ weights, serum
testosterone, luteinizing hormone or follicle-stimulating hormone, epididymal sperm counts or fertility [43]. However, cyclophosphamide has been shown to penetrate the male reproductive tract and can be transmitted sexually to a female partner, which may affect progeny outcome [42]. Other iatrogenic factors, such as chronic low-dose radiation, may also lead to bladder cancer through oxidative stress and a reduction in DNA repair by an increase of nitric oxide and reactive oxygen species [44-46]. Therapeutic pelvic radiation used for abnormal uterine bleeding, ovarian, cervical and prostate cancer is associated with an increase in bladder cancer risk [14]. Altogether, iatrogenic factors insult the bladder, causing inflammation, resulting in DNA damage and mucosal aberrations leading to bladder cancer. However, there is no correlation for sexual prevalence that has been observed.

**Current Therapies**

Treatment of localized bladder cancer can vary from simple fulguration to multimodal therapy including radical extirpative therapy. A number of treatment paradigms exist depending on the clinical situation. Low-grade papillary tumors are handled frequently with simple electrodessication. Inflammation has been shown to play a role in bladder cancer and therapies that are immunomodulators have proven useful in treatment. Adjuvant intravesical therapy with either chemotherapeutic agents such as Mitomycin C or a biologic such as Bacillus Calmette Guerin (BCG) may be employed to prevent recurrences [47]. BCG has proved useful in the management of CIS and superficial high-grade papillary (noninvasive) cancers. BCG is commonly used to prevent bladder cancer recurrence after transurethral resection of the bladder tumor [9, 48, 49]. For high-
grade lesions, treatment is based on the depth of invasion. Muscle invasion usually leads to a much more radical treatment including neoadjuvant chemotherapy combined with surgical removal of the bladder or radiation therapy.

Inflammatory Pathways and Bladder Cancer

BCG is a weakened vaccine strain of bovine tuberculosis from *Mycobacterium bovis* that functions as an immunotherapy to redirect the immune system to clear bladder cancer cells (reviewed in [50]). It has been shown that internalization of BCG by urothelial cells enhanced the expression of the major histocompatibility complex (MHC) class II and cluster of differentiation 1 (CD1) proteins [51]; thus, indicating that endothelial cells can present more antigens of BCG infection and likely tumor presence. However, up to 40% of patients fail to respond to immunotherapy [52]. In males, BCG treatment has been associated with relatively rare complications of penile edema and meatal ulceration [53], as well as epididymous-orchitis [54, 55]. BCG treatment has been shown to be detrimental to healthy sperm development in young men following therapy [56]. In addition, intratesticular injection of BCG in dogs caused a severe granulomatous reaction with widespread degeneration of the tubules, resulting in azoospermia [57]. However, the response to BCG treatment in men and women has shown similar results.

To prevent tumors, macrophages must migrate in the area surrounding the tumor [58]. The exact role that macrophages play depends on which subtype they belong to, as there are pro- and anti-inflammatory types [58] (reviewed in [59, 60]). Insults that induce bladder inflammation without host-derived secreted protein acidic and rich in cysteine (SPARC) cause activation of pro-inflammatory macrophages and nuclear factor kappa-
light-chain-enhancer of activated B cells (NF-κB) [61, 62], which enhances growth of bladder cells. However, pro-inflammatory macrophages can also regulate cancer growth. Type 1 (pro-inflammatory) macrophages co-cultured with human bladder cancer cells arrested cancer cell growth, and increased TNFα expression and phosphoinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway activity when compared to cancer cells grown alone and cancer cells co-cultured with type 2 (anti-inflammatory) macrophages [63].

AR can be pro-inflammatory by inducing expression of genes such as TNFα [64], which can enhance immune cell invasion and may contribute to chronic inflammation (Figure 1). Several proteins, including the AR, is found in both bladder stromal cells as well as the urothelium, are known to contribute to bladder cancer growth [65, 66]. The effect of GRβ on AR guided pro-inflammatory pathways in bladder cancer remains unknown. However, Ligr et al. recently showed that GRβ can increase AR regulated growth in prostate cancer cells [2]. Future studies on the relationship of GRβ and AR would strengthen our understanding and if they work in conjunction to inhibit the anti-inflammatory actions of glucocorticoids.
The use of non-steroidal anti-inflammatory drugs is inversely associated with bladder cancer due to their inhibition of the cyclooxegenase-2 (COX-2) inflammatory pathway [67]. Overexpression of COX-2 is associated with proliferation, angiogenesis and dysregulation of apoptosis in bladder cancer cells and is up-regulated in bladder epithelial cancer [68-70]. Interferon-α (IFN-α) decreased expression of COX-1 and increased COX-2 in bladder cancer cells, suggesting that IFN-α plays a role in COX-2 up-regulation in urothelial cancer cells [70]. Glucocorticoids are inhibitors of COX-2 expression (Figure 2) [19], suggesting that they may be useful for inhibition of bladder cancer. Glucocorticoids also have a beneficial anti-inflammatory response by increasing IκBα, an inhibitor of pro-inflammatory NF-κB [15].
Glucocorticoids and Bladder Cancer

Glucocorticoids are commonly used drugs for treatment of inflammatory and autoimmune disorders. GR is expressed as two alternate major isoforms, GRα and GRβ [1, 20, 22, 71]. Glucocorticoids control anti-inflammatory cellular processes by binding to and activating GRα. Anti-proliferative properties of glucocorticoids are mediated through GRα, which is a hormone-activated transcription factor [72, 73] that increases cell cycle arrest proteins p27 and p21 [16, 17] as well as the apoptotic-gene phosphatase and tensin homolog deleted on chromosome 10 (PTEN) [18]. In contrast to GRα, GRβ lacks part of the ligand-binding domain, helix 12, of the GR protein and cannot bind glucocorticoids [20]. Although the function of GRβ is not well understood, it has been shown that GRβ acts as an inhibitor to GRα [1, 20, 74-76]. GRβ is induced by inflammatory pathways such as TNFα and NF-κB [33], suggesting that it may have a paramount role in inflammation that is associated with bladder cancer (Figure 2). The inhibitory role of GRβ on glucocorticoid action in the immune system has related it to a variety of immunological diseases, such as: ulcerative colitis, asthma, and chronic sinusitis [22, 24-26, 77]. Now, it is also being observed that GRβ may regulate proliferation as well as cancer growth in
glioblastoma [77] and leukemia [26]. Potentially, this may occur through the ability of GRβ to augment a chronic inflammatory state by inhibition of GRα and glucocorticoid action.

It has been shown that GR plays a role in bladder cancer, although the precise mechanism and which isoform of the receptor is unknown. GR expression tends to be weaker in bladder cancer tumors than in normal cells, and strong GR expression tends to be correlated with a better prognosis [7, 8]. Glucocorticoids have been widely used as co-medication in patients with advanced bladder cancer [6]. However, recent studies have raised the possibility of an increased risk of bladder cancer from systemic use of glucocorticoids. Recent investigations have demonstrated that glucocorticoids (e.g. corticosterone, dexamethasone, prednisone) suppress bladder cancer cell invasion, while dexamethasone may induce proliferation via inhibiting apoptosis [29, 78]. However, these effects may be mediated by GRβ, which has been shown to exert a stimulatory effect on proliferation [20], possibly by increasing inflammation in bladder by inhibition of GRα. Glucocorticoids are known to interfere with the transcriptional activity of several immune related transcription factors, including NF-κB [7]. It has been shown that GRα can directly function as a co-repressor of NF-κB. Additionally, the synthetic glucocorticoid dexamethasone, inactivates NF-κB and down regulates NF-κB-dependent cytokine IL-6, which may be a central mechanism involved in GR–mediated inhibition of bladder cancer cell invasion [7]. We have shown that dexamethasone can increase the expression of GRβ in mouse embryonic fibroblast cells [20]. Interestingly, constant exposure of glucocorticoids in patients leads to elevated GRβ and glucocorticoid resistance, which is due to decreased affinity for GCs and increased total GR proteins [74, 79], suggesting a chronic glucocorticoid resistant state. Furthermore, GRβ can regulate growth through
suppression of PTEN, enhancing PI3Kinase/AKT induced proliferation [1]. Suppression of GRβ by siRNA inhibited growth of AR positive prostate cancer cells [2]. This suggests that GRβ may positively affect AR signaling activity, and that chronic glucocorticoid treatment in males could result in activation of the GRβ/AR axis leading to bladder cancer (Figure 3).

Several glucocorticoids have been used clinically as cytotoxic agents, predominantly for hematologic malignancies [80]. Evidence suggests a glucocorticoid-induced resistance to cytotoxic effects of the anti-neoplastic drug cis-diamminedichloroplatinum (CDDP), the most effective agent currently used against urothelial carcinoma [81]. A glucocorticoid is often used as co-medication in the standard chemotherapy regimens for bladder cancer, due to its protective factor against toxic chemotherapy drugs. However, prolonged systemic use of glucocorticoids has been shown to increase the subsequent risk of bladder cancer, possibly due to immunosuppression [6]
or long-term induction of GRβ causing glucocorticoid resistance leading to inflammation. However, the exact mechanism remains unknown.

**Androgens and Bladder Cancer**

In males, the AR has been shown to play a key role in prostate cancer genesis and progression [82]. However, the role of AR in bladder cancer and the proclivity for males has only recently drawn attention [83, 84]. AR is a ligand-inducible transcription factor that regulates the expression of several genes (Figure 1) [85-87]. AR ligands, the principal being the predominately male hormone, testosterone, enter the target cell and bind to AR directly or after conversion to 5α-dihydrotestosterone (DHT). The ligand-AR complex induces a conformational change in AR, resulting in release of the heat shock proteins (HSPs) and translocation of the complex from the cytoplasm to the nucleus [88]. After translocation, activated AR binds to DNA at androgen-response elements in promoters and recruits additional proteins, leading to specific transcriptional activation or repression of target genes [89]. Several human bladder cancer cell lines have been found to express AR [90-94]. Additionally, AR expression has been detected in human bladder cancer obtained after surgical removal [95-99]. The role of AR in normal bladder is unclear [100].

The role of AR in prostate has been more defined. Investigations have shown that ligand-independent activation of the AR pathway occurs in prostate cancer, which can be enhanced by epidermal growth factor (EGF) through the signal transduction pathway [86]. Connecting AR in prostate and bladder cancers, dysregulation of the epidermal growth factor receptor (EGFR) is associated with bladder cancer [101], suggesting that these cancer pathways may be interconnected. It has been shown that AR can increase expression
and activity of EGFR and a protein encoded by the ERBB2 (also known as Her2) gene [101], implying androgen-mediated bladder cancer tumorigenesis and clinical progression via the regulation of the EGFR/ERBB2 pathways. The separation of these pathways in males and females is unknown. However, it may be overly activated in males because of the increased level of androgens.

Earlier investigators have shown that a variety of AR gene alterations are important in the development of bladder cancer, such as allelic loss and gene mutation. This could explain some of the differences between male and female tumors. Allelic loss of the AR locus has been found in cases of muscle-invasive bladder tumors, but not in the adjacent non-neoplastic tissue [102]. Additionally, mRNAs from two human bladder cancer cell lines have revealed AR sequences with short CAG repeat lengths, suggesting altered mRNA sequences of the AR gene could contribute to bladder cancer [93]. Demonstrating the susceptibility of males, bladder cancer tumors implanted in rats that were treated with androgenic hormones grew more rapidly than rats treated with estrogenic hormones [103]. This was also supported by two studies in mice using AR knockout animals, which indicated a critical role of androgen signaling in bladder carcinogenesis [91, 104]. It is therefore suggested that androgenic hormones stimulate bladder tumor growth, whereas estrogenic hormones may do the opposite (or at least do not stimulate). However, there is evidence of AR induced bladder cancer in females. A study of transitional cell carcinoma showed that AR is expressed in women patients and found that 30% of the bladder cancer tumors are AR positive, and that non-tumor tissue may also express AR [105]. In addition, the same relationship between AR level and pathological stage was found in men and women.
The estrogen receptor (ER) β has been shown to be highly expressed in bladder cancer, with elevated ERβ expression being correlated with increased bladder cancer stage [106, 107]. In addition, it has been shown that ERβ selective anti-estrogen drug, raloxifin, causes bladder cancer cells to undergo apoptosis [108]. This shows that the use of anti-estrogen therapy may be useful in treating bladder cancer; however, ERβ has not been shown as of yet to be a driver of bladder cancer in a similar manner as AR. ERα has been shown to interact with GR in breast, where this isoform is dominant [109]. However, no work has been done showing an ERβ and GR interaction.

Targeting of AR may potentially be a good therapy for bladder cancer in males. An effective prostate cancer treatment is chemical castration using luteinizing hormone-releasing hormone analogues to ablate testicular androgens or use of anti-androgens (e.g. flutamide), which block androgen signaling at the level of the AR. Typically, anti-androgens are used in early stage prostate cancer. While this therapy is successful at first, the hormonal therapy often fails and patients relapse with “castrate-resistant” prostate cancer. This resistance comes from the selection of cells that bypass androgen requirement by mechanisms including AR gene mutation or receptor amplification [110-114]. Additionally, it has been found that dihydrotestosterone up-regulates ERBB2 in androgen-receptor positive bladder cancer cells [115]. The communication between the AR and EGFR pathways may play a role in the male prevalence in bladder cancer. While it is known that AR positively correlates with an increased risk of developing prostate cancer, it is unknown whether anti-androgens can have an effect on bladder cancer.
Sexual Dimorphism in Cancer Aggressiveness

Males have been shown to develop more high-grade bladder cancer tumors in comparison to females (55.7% males vs 42.0% females) as well as a greater percentage of invasive tumors (26.5% males vs 22.0% females) [101]. Recent research has shown that androgens and AR can induce epithelial-mesenchymal transition (EMT) which is often seen as an indicator for metastasis [116, 117]. The aggressiveness of tumors is derived from oxygen and other nutrients that cancer cells use to induce local neovascularization. Vascular endothelial growth factor (VEGF) is a potent endothelial cell mitogen that stimulates proliferation, migration and tube formation leading to angiogenic growth of new blood vessels and is essential during development [118]. Neuropilin (NRP)-1 and homologue NRP-2 are co-receptors that enhance responses to several growth factors, such as VEGF, and mediators under physiological and pathological conditions [119-122]. NRPs and VEGF receptors are constitutently expressed on normal bladder epithelial cells and have been shown to be upregulated in an animal model of chronically inflamed cells, indicating neovascularization [123]. NRPs can regulate the cancer-induced vascular and inflammatory responses. Glucocorticoids have been shown to suppress NRP [124] and VEGF expression [29, 125]. Androgens, on the other hand, increase VEGF expression [126]. In males, GRβ may enhance neovascularization potential through inhibition of GRα, as well as activation of AR and inflammatory pathways that increase VEGF and NRP. We have shown that GRβ does enhance activity of the PI3-kinase and Akt cascade by suppression of PTEN (Figure 3) [127], a known inhibitor of growth and tumor suppressor gene [1]. Ultimately, this may lead to the inflammatory processes that can lead to the progression of bladder cancer in males.
Conclusions

Insight into the cellular biology of the bladder cancer disease process offers the opportunity to develop innovative and more targeted therapies. The androgen and the glucocorticoid receptors are both members of the steroid receptor superfamily, and appear to offer promise as therapeutic targets for enhanced treatment paradigms. Much research remains to be performed in order to define the roles of glucocorticoids, anti-androgens, and the GR isoforms in the management of bladder cancer. A continuing understanding of the roles of AR and other molecules, such as GRβ, that may directly or indirectly regulate androgens may help reveal better strategies for the management of bladder cancer in males. Additionally, androgen ablation therapy may prove to be a useful for treatment in males with bladder cancer. A further understanding of the molecular signaling pathways that cause the predilection for males will aid in the advancement of bladder cancer therapy.

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References:


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Chapter 3

Glucocorticoid receptor beta increases migration of human bladder cancer cells

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Abstract

Bladder cancer is observed worldwide having been associated with a host of environmental and lifestyle risk factors. Recent investigations on anti-inflammatory glucocorticoid signaling point to a pathway that may impact bladder cancer. Here we show an inverse effect on the glucocorticoid receptor (GR) isoform signaling that may lead to bladder cancer. We found similar GRα expression levels in the transitional uroepithelial cancer cell lines T24 and UMUC-3. However, the T24 cells showed a significant (p<0.05) increased expression of GRβ compared to UMUC-3, which also correlated with higher migration rates. Knockdown of GRβ in the T24 cells resulted in a decreased migration rate. Mutational analysis of the 3’ untranslated region (UTR) of human GRβ revealed that miR144 might positively regulate expression. Indeed, overexpression of miR144 increased GRβ by 3.8 fold. In addition, miR144 and GRβ were upregulated during migration. We used a peptide nucleic acid conjugated to a cell penetrating-peptide (Sweet-P) to block the binding site for miR144 in the 3’UTR of GRβ. Sweet-P effectively prevented miR144 actions and decreased GRβ expression, as well as the migration of the T24 human bladder cancer cells. Therefore, GRβ may have a significant role in bladder cancer, and possibly serve as a therapeutic target for the disease.
Introduction

Bladder cancer was the fourth most prevalent cancer in men, and fifth overall in 2015 [1]. Recently, glucocorticoids (GCs) have been used in bladder cancer for their protective properties against the toxic effects of chemotherapy [4]. GCs may cause resistance to cisplatin, which is a treatment commonly used for bladder cancer [6, 25]. The GC receptor (GR) is expressed as different isoforms, GRα and GRβ, which are a result of alternative splicing of a single gene [18-20, 28, 29]. GCs bind and activate the ligand-binding GR isoform, GRα, which is a transcription factor that increases genes involved in cell cycle arrest and apoptosis [14-16, 30, 31]. GRβ lacks the ligand-binding domain for GCs [18, 29], and has been shown to be inhibitory to GRα [18, 19, 32-34]. A higher total GR expression has been correlated with a better prognosis in bladder cancer [6, 35]. However, the specific roles of GRα or GRβ in bladder cancer are unknown.

Recent work has shown a conundrum, in that GCs can suppress bladder cancer invasion, but also induce proliferation [27, 35]. GCs are commonly used to inhibit growth in hematological cancers [24] and solid tumors [36]. Long-term GC treatment can increase the risk of bladder cancer, possibly through immunosuppression [4, 25], or by causing GC resistance through elevated GRβ [25, 29]. The later remains to be elucidated. We have recently shown that GRβ can suppress the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) expression and increase Akt1 guided proliferation [19]. Furthermore, GRβ has been shown to be involved in the migratory process of astrocytes and the development of glioblastoma [21]. Longui et al. showed that the effectiveness of GCs in patients was reduced with a lower GRα/GRβ ratio [24]. Factors that regulate the expression of GRα or GRβ may influence the response to GCs, and possibly mediate
growth. GC resistance in sepsis has been shown to be affected by microRNA 124 (miR124), which down-regulated GRα, causing increased immune cell growth [37].

It has been previously demonstrated that a naturally occurring mutation in the AUUA motif of the 3’ untranslated region (UTR) of GRα and GRβ results in increased mRNA stability and protein expression [38]. Targeting of the 3’ UTR of genes by miRNAs may alter mRNA stability, which has been recently recognized to be involved in processes that regulate cancer development or progression [39-41]. Some miRNAs have been proposed as biomarkers to detect and predict the severity of bladder cancer [42-45]. The miRNAs that may regulate bladder cancer proliferation may be of importance, which was shown by miR125b targeting of the E2F3 transcription factor [46], a tumor suppressor that regulates the cell cycle. Furthermore, miR145 and miR133a decreased bladder cancer aggressiveness by targeting fascin actin-bundling protein 1 (FSCN1) [47], which binds β-catenin to increase motility and invasion. Higher-grade bladder tumors have been shown to express elevated miR144 [48], which has also been shown to promote cell proliferation in nasopharyngeal carcinoma [49]. However, the involvement of miRNAs and their regulation of GRα or GRβ in bladder cancer development or progression are unknown.

In this investigation, we show that GRβ enhanced migration of human bladder cancer cells. We found three potential miRNA target sites in the 3’ UTR of human GRβ and show that miR144 positively affected human GRβ expression. Additionally, we show that blocking the binding site of miR144 in the 3’ UTR of human GRβ inhibited expression, and, as a result, decreased migration of bladder cancer cells.
Results

GRβ & GRα in Human Bladder Cancer Cells

We have previously shown that GRβ is involved in regulating cellular pathways that are known to be involved in cancer [50]. However, the analysis was performed in non-cancerous mouse fibroblast and 3T3-L1 cells. To examine the two GR isoforms in human bladder cancer, we assayed their expression in two transitional uroepithelial cancer cell lines, UMUC3 and T24. As shown by immunofluorescence staining and mRNA expression, we found that the T24 cell line had a higher expression of GRβ compared to the UMUC-3 (Figures 1A & B). GRα had similar levels by immunofluorescence and mRNA in the T24 cells. To determine if human bladder cancer cells that have higher GRβ expression are more migratory, we conducted a wound-healing migration assay. The T24 cells with higher GRβ expression had a significantly (ANOVA p<0.0001) faster migration compared to the UMUC-3 (Figure 1C & D). To show the effect of GRβ in the T24 cell line we established a stable cell line with an shRNA lentivirus targeting human GRβ (Figure 2A). The knockdown of GRβ expression in the T24 cells (64% reduction) (Figure 2B) resulted in a significant (ANOVA: p<0.001) decrease in migration (Figure 2C).
Figure 3-1. GRβ and GRα expression and migration in UMUC3 and T24 human bladder cancer cells. GRβ and GRα expression was measured by immunofluorescence (A). Secondary antibodies (labeling GRα or GRβ) are shown in green, DAPI (nuclei labeling) are shown in blue, and a bright field images are shown in gray (scale bar = 25 μm). GRβ and GRα mRNA expression measured by Real-Time PCR (B). *, p<0.05; **, p<0.01 (versus UMUC3) (±S.E.; n=3). Migration was measured in UMUC3 and T24 cells at 5, 10, 15, 20, and 25 hours post wounding as the fold-changed wound width remaining (C). Images of the migration assay at 0 and 15 hours post wounding, with wound edges marked at T0 (black solid line) and T15 (white dashed line). ANOVA p<0.0001; Bonferoni comparisons ****, p<0.0001 (versus UMUC3) (±S.E.; n=6).
We have previously shown that insulin increased GRβ mRNA and protein expression in cells [18, 50] and livers of mice [18]. To determine the effect of insulin or dexamethasone (Dex) on GR isoform localization and expression, we treated the T24 and UMUC-3 bladder cancer cells for 30 minutes and labeled with human GRα or GRβ antibodies for immunofluorescence staining. Insulin treatment significantly increased GRβ expression in the T24 (p<0.01) and UMUC-3 (p<0.0001) cells (Figure 3A & C). However, there was no difference observed in GRβ expression or localization in the human bladder
cancer cells with Dex treatment, even though we have previously shown that GCs increased GRβ in normal mouse cells [18]. As for localization, GRβ was higher in the nucleus in the UMUC-3 with insulin, but not with Dex. There was no change in GRβ localization with insulin or Dex in the T24 cells. The GRα expression was significantly decreased (p<0.001) by insulin in the T24 cells, but no effect was observed in the UMUC-3 (Figure 3B & D). Dex treatment increased GRα protein in both cell lines, as well as translocation from the cytoplasm (control) to the nucleus (Dex). These results may indicate a pro-growth pathway that involves the induction of GRβ and inhibition of GRα for proliferation or migration.
GRα Controlled Gene Transcription in Human Bladder Cancer Cells

The role of the GR isoforms in human bladder cancer is unknown, especially the gene regulator activity of GRα. To determine the GRα-induced gene activity in human bladder cancer cells, we treated the T24 and UMUC-3 cells with Dex for 2 hours in hormone-free dialyzed serum. To test genes that are directly regulated by GRα, we measured mRNA expression of known controlled genes FK506 binding protein 51

Figure 3-3. Dexamethasone and insulin treatment in T24 and UMUC-3 cells. GRβ and GRα expression and location was measured using immunofluorescence with control (vehicle treatment), dexamethasone, or insulin treatments (A, B, C, D). Cells were seeded onto coverslips in media containing 10% dialyzed FBS for 24 hours before treating. Cells were treated with 100 nM insulin, 100 nM dexamethasone, or vehicle for 30 minutes. Secondary antibodies (labeling of human GRα or GRβ) are shown in green, DAPI (nuclei labeling) are shown in blue, a merge of the green and blue images are shown in panel 3 to represent localization, and a bright field image is shown in gray (scale bar = 25 μm). Data are represented as fold change compared to control. **, p<0.01; ***, p<0.001; ****, p<0.0001 (versus control) (±S.E.; n=3).
(FKBP51), glucocorticoid-induced leucine zipper (GILZ), and p21 (Figure 4). The UMUC3 cell line was more responsive to the Dex treatment on FKBP51, GILZ and p21 mRNA expression, likely due to the lower expression of GRβ leading to less GRα inhibition. The VEGFA expression was not different in the T24 cells, but was significantly (p<0.01) deceased by Dex treatment in the UMUC-3 cells. The GRα expression was significantly (p<0.05) lower in the T24 cells, but Dex treatment did not change the mRNA expression in UMUC-3 or T24 cells. The GRβ mRNA expression did not change with Dex treatments. However, it should be noted that the levels of GRβ and GRα mRNA are different than in Figure 1B, which is mostly likely due to the use of hormone-free serum for the glucocorticoid treatment.
GRβ expression is controlled by miRNAs

To assay miRNA control of GRβ expression, we used the in-silico prediction software Targetscan (version 6.2) to find miRNAs that may bind to the 3’-UTR of human GRβ (Supplemental Figure 1) [51-53]. Three miRNAs were predicted to bind the 3’UTR of human GRβ (miR33a, miR181a/b/c/d, and miR144). To determine which of the predicted miRNAs may regulate human GRβ expression, we used the pMirTarget vector with the 3’ UTR of human GRβ (pMirTarget 3’ UTR hGRβ) inserted after the luciferase reporter gene, which is under the control of the IRES promoter (Figure 5A). Next, we
mutated the predicted binding sites to all adenines (Supplemental Figure 2) to determine the potential of the miRNA on human GRβ expression. We transfected the UMUC-3 and T24 cells with the pMirTarget 3’ UTR hGRβ mutants and measured luciferase activity (Figure 5B). Mutational analysis of the miR144 binding site resulted in a decrease of 77% (UMUC-3) and 81% (T24) in the reporter expression, indicating that miR144 may enhance human GRβ expression. Interestingly, mutation of the miR181 site also decreased of luciferase in pMirTarget 3’ UTR hGRβ, but this was not observed in the UMUC-3. Total RNA was extracted from the UMUC-3 and T24 cells to measure the miRNA expression that may target the 3’ UTR of human GRβ (Figure 5C). Interestingly, miR33a, miR144, miR181a, miR181b, miR181c, and miR181d were all increased in the T24 cells. Next, we wanted to determine if miR33a, miR144, miR181a, miR181b, miR181c, or miR181d changed during a scratch assay and if this affected the human GRβ or GRα expression. A scratch (wounding) assay of the T24 cell line showed that miR144 (4 fold) and GRβ (3.2 fold) were both increased (Figures 5D & 5E). The miR33a, miR181a, miR181b, miR181c, and miR181d were unchanged during the scratch (wounding) assay. Interestingly, GRα mRNA expression was also unchanged with the scratch (wounding) assay. To show that miR144 specifically regulates human GRβ, we overexpressed a plasmid containing the precursor of human miR144 in pCMV-MIR or empty vector. The miR144 containing pCMV-MIR vector resulted in a 184-fold increase in miR144 expression compared to the empty vector (p=0.002). The overexpression of miR144 resulted in a significant (p<0.05) increase in human GRβ expression (3.8 fold), while not changing GRα (Figure 5F).
Figure 3-5. The human 3’UTR of GRβ is regulated by miR144. The pMirTarget vector containing the 3’UTR of human GRβ was cloned into a luciferase reporter gene (3’UTR GRβ-Luc) (A). The T24 and UMUC-3 bladder cancer cells were transfected with the 3’UTR GRβ-Luc expression construct with mutation in the miRNA binding site for miR181, miR144, or miR33a and was measured by a luciferase assay, and normalized to renilla (B). ***, p<0.001; ****, p<0.0001 (versus WT) (±S.E.; n=6). The miRNA expression in the UMUC3 and T24 cells was measured using Real-Time PCR (C). *, p<0.05; **, p<0.01 (versus UMUC3) (±S.E.; n=3). Total RNA was harvested at the time of wounding and 3 hours after from the T24 cells in media containing 10% dialyzed FBS to determine the expression during migration assay for miRNA expression (D). *, p<0.05 (versus T0) (±S.E.; n=3), and for mRNA expression of GRβ and GRα expression (E). ***, p<0.001 (versus T0) (±S.E.; n=3). A plasmid containing the human miR144 in the pCMV-MIR vector was transfected in the T24 cells to show how miR144 overexpression affected the expression of GRβ and GRα as measured by Real-time PCR (F). *, p<0.05 (versus T24 Vector) (±S.E.; n=3).
Dexamethasone control of migration and miRNA expression

Next, we investigated the effect of Dex on migration and regulation of miRNA expression. Dex treatment was performed 30 minutes before scratch assay (wounding) of the T24 bladder cancer cells. The Dex treatment significantly (ANOVA: p<0.001) decreased migration, while there was no affect in the UMUC-3 cells (Figure 6A). Dex treatment decreased expression of miR144, miR181a, and miR181c in the T24 cells, but not in the UMUC-3 cells (Figure 6B). Insulin did not significantly change expression of miR33a, miR144, miR181a, miR181b, miR181c, or miR181d in the T24 cells. However, insulin did suppress miR181a expression in the UMUC-3 cells.
Drug targeting the miR144 enhancement of GRβ

To inhibit the binding of miR144 to the 3’UTR of GRβ, we developed a peptide nucleic acid (PNA) conjugated with a cell-penetrating peptide (CPP) (Sweet-P) targeting the site (Figure 7A). A dose dependence response curve indicated that Sweet-P
significantly (p<0.05) decreased GRβ mRNA expression in the T24 human bladder cancer at 1.0 nM, 10 nM, 50 nM, and 100 nM (Figure 7B). To confirm our endogenous gene finding, we transfected the T24 bladder cancer cells with the pMirTarget 3’ UTR hGRβ construct and treated with Sweet-P for 48 hours (Figure 7C). The luciferase expression of the pMirTarget 3’ UTR hGRβ construct was significantly (p<0.001) reduced at 0.1 nM, 1.0 nM, and 10 nM. Sweet-P (10nM) reduced GRβ protein expression, but had no effect on GRα (Figure 7D). Furthermore, Sweet-P significantly increased GRα activity with dexamethasone treatment by enhancing expression of FKBP51 (p<0.05) and decreasing a known GRα regulated gene, tumor necrosis factor α (TNFα) (Figure 7E). To show that Sweet-P specifically targets miR144-binding site in the 3’UTR of hGRβ, we measured protein expression by immunohistochemistry of two known miR144 targets that have been shown to be suppressed, PTEN [49] and the mammalian target of rapamycin (mTOR) [54]. The results show that mTOR expression is unaffected by Sweet-P (10nM), and PTEN expression was significantly (p<0.0001) increased with treatment. To determine if Sweet-P could inhibit migration, we treated the T24 bladder cancer cells with 10 nM Sweet-P during a scratch (wounding) assay. The results show that Sweet-P significantly (ANOVA: p<0.001) inhibited migration of the T24 bladder cancer cells during migration (Figure 7D).
Figure 3-7. Blocking the miR144 binding site in the 3’UTR of human GRβ by Sweet-P inhibits expression and cell migration. A peptide nucleic acid (PNA) conjugated to a cell penetrating peptide (CPP) (Sweet-P) was designed to bind to the miR144 binding site in the 3’UTR of human GRβ mRNA (A). GRβ expression in T24 cells was measured at increasing doses of Sweet-P (0, 0.1, 1.0, 10, 50, and 100 nM) for 48 hours after and human GRβ mRNA was measured by Real-time PCR (B). ANOVA p<0.01; Dunnett’s comparisons *, p<0.05; **, p<0.01 (versus 0nM) (±S.E.; n=6). (cont.)
Discussion

This is the first study to show that GRβ is enhanced during the migration of human bladder cancer cells. Suppression of GRβ by lentiviral shRNA decreased the migration of T24 cells. Yin et al. showed that GRβ increases the migration of astrocytes and brain cancer cells (glioblastoma) [21]. Several other studies have also demonstrated that GRβ is elevated in cancers and inflammatory diseases, which leads to increased growth [24, 55-60]. GCs have been shown to inhibit migration and proliferation of cancer cells in medulloblastoma [61], osteosarcomas [62, 63], A549 human lung cancer cells [64], as well as other lung cancer cells: squamous cell carcinoma lines (EPLC-32M1 and NCI-H157), large-cell carcinoma cell line (LCLC-97TM1) and a cell line from mesothelioma (MSTO-211H) [65]. Increasing GRβ may provide a state of GC resistance that reduces their ability to inhibit growth and migration. Bombesin has been shown to induce resistance to GCs by induction of GRβ in human prostate cancer cells [57]. We showed in this study that the
T24 human bladder cancer cells had a reduced response to GCs compared to the UMUC-3, which may be due to elevated GRβ. The migratory potential of T24 cells, but not the UMUC3 cells, was inhibited by dexamethasone. Dexamethasone has been previously shown to inhibit the invasion of bladder cancer cells, including the UMUC-3 [35]. In this study, we show that dexamethasone treatment inhibited miR144, which we have shown is a positive regulator of GRβ and is increased during migration. The inhibition of miR144 may be a potential mechanism that migration was reduced by dexamethasone in the T24 cells.

As shown by the mutation in the 3’UTR of human GRβ reporter, and plasmid overexpression, miR144 is a positive regulator of human GRβ expression and not GRα. Iwaya et al. showed that miR144 downregulated mTOR, a regulator of cellular growth and metabolism, and the loss of miR144 leads to the progression of colorectal cancer [54]. Zhang et al. showed that miR144 downregulated PTEN expression [49], a tumor suppressor gene that regulates many cellular functions including cell proliferation. Guo et al. showed that miR144 inhibits bladder cancer proliferation by targeting the enhancer the zeste homolog 2 (EZH2), a downstream regulator of the Wnt/β-catenin pathway that mediates growth [48]. However, the effects of miR144 on migration were not tested. There are a plethora of targets for miRNAs, and the specific blockade of miR144 binding to the 3’UTR of GRβ by Sweet-P resulted in decreased GRβ mRNA expression and 3’UTR GRβ-luc reporter assay. The effect of Sweet-P was specific for the 3’UTR of human GRβ, as mTOR and PTEN, which are known to be suppressed by miR144, were not lower but PTEN was significantly higher. We have recently shown that GRβ binds to the PTEN promoter to inhibit expression [50], and therefore the suppression of GRβ by Sweet-P
treatment caused derepression of PTEN. Sweet-P did suppress GRβ protein expression, but did not change GRα. However, the GRα activity was increased with Sweet-P treatment. Moreover, the downregulation of GRβ by Sweet-P inhibited migration of bladder cancer cells, indicating that it may serve as a potential therapy for bladder cancer. The inhibitory effect of dexamethasone on the migration of the T24 cells supports that GRα is a suppressor of bladder cancer, which is also shown by Sweet-P enhancing GRα activity and suppressing migration of the bladder cancer cells. Dexamethasone inhibition of VEGF-A supports that GCs may inhibit bladder cancer, VEGF-A levels were found to be greater in higher-grade urothelial tumors [66]. Dexamethasone decreased miR144 and migration of the T24 cells, which indicates that suppression of miR144 levels may also reduce GRβ expression. However, two-hour dexamethasone treatment in hormone-free serum did not affect GRβ mRNA, or 30-minute treatment did not change the protein. Glucocorticoids may alter GRβ expression with longer treatment. We showed in mouse fibroblast that GRβ increased with dexamethasone treatment [18], but no change was observed in mouse C2C12 myoblast [67]. During migration, dexamethasone suppression of miR144 may have a larger impact on GRβ expression.

The effect of insulin on enhancing GRβ expression and inhibiting GRα suggests that it may increase the risk of bladder cancer. However, insulin did not increase miR144 expression with an acute two-hour treatment, which suggests that insulin may enhance GRβ levels by a different mechanism. The effect of insulin on cancer cell migration rate has not been studied, but the insulin-like growth factor receptor I (IGFR-I) has been shown to promote invasion of bladder cancer cells through an Akt and mitogen activated protein kinase (MAPK) dependent mechanism [68]. There has been no correlation found for
bladder cancer in insulin-resistant type II diabetics [69], which suggests that insulin may not have a role. However, IGF-I may signal to GRβ to increase bladder cancer invasion, but the effect of IGF-I on GRβ expression has not been investigated. Most likely the increase of miR144 during T24 migration and its enhancement of GRβ expression are mediated in a non-insulin dependent manner. Two drugs for the treatment of type II diabetes, rosiglitazone and pioglitazone, have been shown to induce bladder cancer [70, 71], but their effect on miR144 or GRβ expression is unknown. Recent studies have revealed that there is a 3:1 incidence in men compared to women for bladder cancer, which may be mediated by the androgen receptor (AR) [25]. Presumably, there may be an interaction between AR and GRβ in prostate cancer. Ligr et al. showed that suppression of GRβ in LNCaP, RC165, and DU145 human prostate cancer cells inhibited growth [72]. However, the signaling involvement of GRβ and AR in bladder cancer has not been investigated.

In conclusion, GRβ mediates bladder cancer migration and may serve as a target for therapy. The 3’ UTR of GRβ is enhanced by miR144 during human bladder cancer migration. Blocking the interaction of miR144 with the 3’ UTR of GRβ by Sweet-P slowed bladder cancer migration. The antagonism of human GRβ by Sweet-P, drug interaction, or gene targeting may serve as a potential treatment for bladder cancer.

**Materials & Methods**

**Cell Lines and Culture**

The human uroepithelial carcinoma cell lines UMUC-3 and T24 (ATCC) were routinely cultured and maintained in Minimum Essential Medium (MEM) containing 10%
fetal bovine serum (FBS) with 1% antibiotic-antimycotic. Cells were maintained at 37°C and 5% CO2. Media was changed to MEM containing 10% dialyzed-FBS with 1% antibiotic-antimycotic 24 hours before hormone treatments.

RNA Extraction for mRNA Quantification and Real-Time PCR Analysis

Total RNA was extracted from cell cultures using the 5-Prime PerfectPure RNA Cell Kit (Fisher Scientific Company, LLC). Total RNA was read on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PCR amplification of the cDNA was performed by quantitative real-time PCR using TrueAmp SYBR Green qPCR SuperMix (Smart Bioscience). The thermocycling protocol consisted of 10 min at 95°C, 40 cycles of 15 sec at 95°C, 30 sec at 60°C, and 20 sec at 72°C and finished with a melting curve ranging from 60–95°C to allow distinction of specific products. Normalization was performed in separate reactions with primers to GAPDH.

RNA Extraction for miRNA Quantification and Real-Time PCR Analysis

Total RNA was extracted from cell cultures using the miRNeasy Mini Kit (Qiagen). Total RNA was read on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and cDNA was synthesized using the miScript II RT Kit (Qiagen). PCR amplification of the cDNA was performed by quantitative real-time PCR using miScript SYBR Green PCR Kit (Qiagen). The thermocycling protocol consisted of 15 min at 95°C, 40 cycles of 15 sec at 94°C, 30 sec at 55°C, and 30 sec at 70°C and finished with a melting
curve ranging from 60–95°C to allow distinction of specific products. The miScript Primer Assay primers were purchased from Qiagen. Normalization was performed in separate reactions with primers to Hs_RNU6-2_11, At_U19_1, and Hs_SNORD61_11.

**Immunoflourescence and Microscopy**

Samples were imaged using a Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems, Bannockburn, IL) equipped with conventional solid state and a Ti-sapphire tunable multiphoton laser (Coherent, Santa Clara, CA). Images were acquired in the XYZ plane in 1 µm steps with a 63X oil objective (NA 1.40). Images were acquired with the LAS AF software in sequential scan mode. AlexaFluor488 was excited at 488 nm with collection at 500-558 nm and DAPI was excited with the multi-photon (MP) laser tuned to 790 nm with collection at 420-500 nm. Images are 2D projections of the image stack as labeled. Three images were taken per slide and ImageJ software was used to measure the immunofluorescence of each cell (average 40 cells) in the images.

**Migration Assay**

Cells were seeded on a 6-well plate and grown for 24 hours until a monolayer of 90% confluent cells were obtained. A scratch wound in the cell monolayer was introduced using a sterile pipette tip. Images were taken at the time of wounding, and every 5 hours thereafter. Migration was measured as the fold change of the width of the wound remaining. To determine miRNA and mRNA expression during the scratch (wounding assay), we collected total RNA using the miRNeasy Mini Kit (Qiagen) (described above) at 0 and 3 hours after the migration.
**Generation of Lentiviral Constructs**

To establish a T24 cell line that has hGRβ stably knocked down, the pGFP-C-shLenti plasmid containing either GRβ shRNA (CCAGAAAGCACATCTCACACATTAATCTG) or scrambled shRNA (Origene) was packaged into a lentiviral construct using the Lenti-vpak Packaging Kit (Origene) by transfection in 293-GP2 cells. The supernatants were harvested and the cell debris was removed by filtration through 0.45 μM filter. The supernatant was used to infect T24 cells after addition of polybrene (10 ug/ml, Sigma Chemical Co., St. Louis, MO) to establish cell lines with stable expression of hGRβ shRNA (T24 GRβ KD) or expressing scrambled shRNA (T24 Scramble). After 72 h the cells were initially selected using Puromycin (10 μg/mL). Cells were then secondarily selected by sorting through flow cytometry for GFP by the Flow Cytometry Core Facility at the University of Toledo Health Science Campus.

**Transient Transfection**

For transient transfection cells were plated on a 6-well or 12-well dish in MEM containing 10% FBS. Cells were washed with OPTI-MEM and transfected using GeneFect (Alkali Scientific, Inc.), according to the manufacturer’s protocol. OPTI-MEM was removed after 12h and MEM containing 10% FBS was added.

**Promoter Reporter Assays**

The expression vector pMirTarget containing the 3’UTR of hGRβ (hGRβ 3’UTR-luc) was purchased from (Origene). The binding sites were mutated using the QuikChange Lightning Multi Site-Directed Mutagenesis Kits (Agilent Technologies). Successful
mutations were confirmed through sequencing by Operon MWG. Cells were seeded onto a 12-well plate and grown overnight. Transient transfection was performed as described above. To determine the effect of Sweet-P on the T24 bladder cancer cells, we treated 24 h after transfection for 48 hours post transfection, 3’UTR GRβ-Luc WT or mutant expression was measured by luciferase, and pRL-CMV Renilla reporter for normalization to transfection efficiency, using the Promega dual luciferase assay system (Promega, Madison, WI).

**miRNA Overexpression**

The cloning vector pCMV-MIR containing the miR144 sequence was purchased from Origene. Cells were seeded on a 6-well plate, and transient transfection of the plasmid was completed as above. After 48 hours-post transfection, RNA was harvested as described above.

**Targeting of the human GRβ mRNA**

A peptide nucleic acid (PNA) conjugated to a cell penetrating peptide (CPP) targeting the miR144 binding site in the 3’UTR of the human GRβ (Sweet-P) was designed using PANAGENE website (http://www.panagene.com). The Sweet-P sequence targeted the miR144 binding site in the 3’ UTR of human GRβ (Supplemental Figure 2). All PNAs were attached to an O Linker and a modified TAT protein (VQRKRQKLMP) for delivery into the cell (CPP). Treatment with Sweet-P was performed for 48 hrs before the cells were analyzed.
**Statistical Analysis**

Data were analyzed with Prism 5 (GraphPad Software, San Diego, CA) using analysis of variance combined with Tukey’s post-test to compare pairs of group means or unpaired t tests. Additionally, two-way ANOVA was utilized in multiple comparisons, and followed by either the Bonferroni or Dunnet post hoc analyses to identify interactions. p values of 0.05 or smaller were considered statistically significant.

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Supplemental Figures

**Supplemental Figure 3-S1. In-silico analysis of the 3’UTR of human GRβ.** The human GRβ 3’UTR analysis of potential miRNA binding sites using human TargetScan (version 6.2) software.

**3’UTR of human GRβ (with TGA stop codon)**

\[
\text{TGA} \overline{ttt} \text{cat} \underline{ccc} \text{aaca} \text{aactt} \underline{ggc} \text{gct} \text{taa} \underline{aaaaaat} \text{aga} \text{act} \text{taat} \underline{gaa} \text{a} \underline{aga} \text{at} \underline{tatg} \text{tcct}
\]

**Supplemental Figure 3-S2. The 3’UTR of human GRβ with the mutation of the binding sites for miRNAs.** The sequence is shown, with the miR181a, b, c, & d binding site highlighted in green, miR144 binding site highlighted in blue, and the miR33a binding site highlighted in red.
Chapter 4

Sweet-P inhibition of glucocorticoid receptor β as a potential cancer therapy

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Abstract

The need for the development of new cancer therapies and push for the design of new targeting techniques is on the rise, and would be useful for cancers that are resistant to current drug treatments. The understanding of the genome has significantly advanced cancer therapy, as well as prevention and earlier detection. This research highlight discusses a potential new type of cancer-targeting molecule, Sweet-P, which is the first of its kind. Sweet-P specifically targets the microRNA-144 binding site in the 3’ untranslated region (3’ UTR) of the human glucocorticoid receptor β (GRβ), which has been demonstrated to increase expression. GRβ has been shown to be highly expressed in cells from solid tumors of uroepithelial carcinomas, gliomas, osteosarcomas, and hepatocellular carcinomas, as well as in liquid tumors cells from leukemia patients. In non-cancerous diseases, GRβ has been shown to be highly expressed in glucocorticoid-resistant asthma. These maladies brought the need for the development of the Sweet-P anti-GRβ molecule. Sweet-P was shown to repress the migration of bladder cancer cells, and may serve as a new therapeutic for GRβ-related diseases.
In our recent work, published in Oncotarget, we have discovered that the glucocorticoid receptor β (GRβ) causes migration (movement) of human bladder cancer cells \[^{1}\]. Bladder cancer is the 4\(^{th}\) most common cancer in men, and the 5\(^{th}\) most overall \[^{2}\]. Almost three-quarters of bladder cancer patients may have a recurrence, and one-third experience progression, causing the need for constant lifelong surveillance and treatment \[^{3}\]. The long-term therapy results in bladder cancer being the most costly cancer for lifetime regimen \[^{4}\], which brings the need for new and better treatments. Because we showed that GRβ plays a role in bladder cancer migration, we set out to construct the first anti-GRβ molecule, which we termed Sweet-P, with the goal of providing a potential new therapy. Sweet-P was designed as a peptide nucleic acid (PNA), conjugated to the Trans-Activator of Transcription (TAT) protein from HIV (for cellular delivery) to specifically target the 3’ untranslated region (3’ UTR) of human GRβ. Sweet-P functions by specifically blocking the microRNA-144 (miR-144) binding site in the 3’UTR of human GRβ (Figure 1), which we showed increases expression. Furthermore, Sweet-P and shRNA suppression of GRβ in human bladder cancer cells attenuated migration \[^{1}\].
The gene that codes for GR in humans is found on the q arm of chromosome 5 \[5, 6\], and is a single GR gene that is alternative spliced to give rise to at least five isoforms \(\alpha\), \(\beta\), \(\gamma\), A, and P \[5, 7-9\]. GR\(\alpha\) and GR\(\beta\) have been the most investigated isoforms. GR\(\alpha\) is identical to GR\(\beta\) from exons 2-8 and is distinguished by alternative splicing of exon 9 in humans resulting in the differing of the C-terminus \[10\]. GR\(\alpha\) contains an additional fifty amino acids derived from the proximal portion of exon 9 that constructs helix 12 for ligand binding. GR\(\beta\) does not have the capacity to bind glucocorticoids because of an additional fifteen amino acids derived from the distal portion of exon 9 that causes a degenerate helix 12 \[5, 9, 11, 12\]. The alternative splicing mechanism in humans is different than in mouse \[11\], rat \[13\], and zebrafish \[14\], but in these species that GR\(\beta\) has been identified, GR\(\alpha\) and GR\(\beta\) are identical through exon 8 with an addition of an alternatively spliced intron 8. In humans, the 3’ UTR of GR\(\beta\) and GR\(\alpha\) are different \[10\] and are targeted differently by miRNAs. For instance, miR-144 increased GR\(\beta\) but had no effect on GR\(\alpha\) expression in human bladder cancer cells \[11\]. However, GC resistance in sepsis is influenced by miR-124, which
downregulated GRα \textsuperscript{[15]}. The effect of miR-124 on GRβ is unknown and miRNAs that target GRβ or GRα are very limited.

GRβ has been shown to antagonize GRα, which has been demonstrated to be due to the competition with GRα for glucocorticoid response elements (GREs)/coregulators, coactivator squelching through the transactivation domain, and through inactive α/β dimers that bind in the nucleus \textsuperscript{[6, 11, 16, 17]}. Therefore, increasing GRβ levels can lead to a GC-resistant state that allows for an elevation of proinflammatory cytokines and transcription factors \textsuperscript{[10, 11, 18-20]}. The ratio of GRα:GRβ is a critical factor in GC disease states \textsuperscript{[10, 17, 18, 20]}. A high GRα:GRβ ratio can be indicative of a GC-sensitive state while a low ratio would be considered GC-resistant \textsuperscript{[18]}. Importantly, Sweet-P inhibition of GRβ increased the responsiveness to GCs \textsuperscript{[1]}, which indicates that it may reverse GRβ induced GC-resistant diseases. Also, GRβ has recently been shown to have positive and negative GRα independent transcriptional activity \textsuperscript{[6, 12]}. We recently demonstrated that mouse GRβ specifically binds to the promoter of phosphatase and tensin homolog (PTEN), which increased Akt1 guided proliferation \textsuperscript{[21]}. We also showed that Sweet-P inhibition of human GRβ increased PTEN expression in bladder cancer cells \textsuperscript{[1]} (Figure 1). There may be other GRβ-specific gene targets that are increased in cancer, and microarray or RNA-seq studies would help strengthen our understanding of the involvement of GRβ in cancer. However, this work is yet to be done.

Sweet-P may have several clinical applications as GRβ has been shown to be involved in other cancer types. For example, treatment with GCs as a first line therapy in acute lymphoblastic leukemia (ALL) is effective due to its ability to arrest cell growth and trigger apoptosis. Unfortunately, resistance to therapeutic GCs is common, which has been
attributed to increased levels of GRβ or decreased GRα [22]. The GRβ interaction with β-catenin and transcription factor-4 (TCF-4) was shown to positively regulate astrocyte activity, leading to increased proliferation [23, 24]. This observation further supports our previous finding of GRβ stimulation of growth [21], albeit via Akt1 activation and PTEN inhibition. Also, GRβ was shown to increase migration of glioblastoma cells [25]. However, the interaction of miR-144 with the GRβ 3’UTR in glioblastoma or ALL is unknown. In LNCaP-ARA70β prostate cancer cells, which express increased levels of GRβ, Ligr et al. reported increased cellular growth and proliferation [26]. Furthermore, treatment with methotrexate in peripheral mononuclear and lymphocyte cells resulted in decreased GRβ expression, thus increasing GC sensitivity [27]. Additionally, Piotrowska et al. demonstrated in Hut-78 and Raji B-lymphoma cells, MCF-7 breast cancer cells, and HT-29 colon carcinoma cells that known growth inhibitors trichostatin, sodium butyrate, and 5-Aza-20-deoxycytidine treatment suppressed GRβ and enhanced GRα with an increase in GC sensitivity [28, 29]. However, miR-144 levels, proliferation, or migration were not assessed in these studies. Nevertheless, these observations indicate the necessity of developing an anti-GRβ therapy to specifically target GRβ-related cancers.

In non-cancerous diseases, the resistance to GCs due to high levels of GRβ have been reported, and Sweet-P can potentially be used as a novel therapy. Most clinically relevant is the anti-inflammatory and immunosuppressant effects of GCs, which have been shown to decrease levels of cytokines, chemokines, and vasoactive agents. GCs reduce the movement of leukocytes to inflamed areas, and the function of immunocompetent cells [8]. In mice, increased GC levels induce apoptosis in thymocytes [30]. Because of the anti-inflammatory effects of GCs, they are commonly prescribed to asthma patients. Many
studies have demonstrated an elevated expression of GRβ and GC-insensitivity in the airways of asthma patients \cite{31-34}. Christodoulopoulos et al. showed that approximately 8% of cells in large and 2% of cells in small airways of patients were GRβ positive. However, mild asthma patients had an increase of 14% (7 fold) in GRβ positive cells in the small airways, but no change in expression in large airways. Alarmingly, in fatal asthmatic patients, the airways showed a dramatic increase in GRβ positive cells to 21% (2.5 fold) in large and 35% (17 fold) in small airways \cite{31}. Hamid et al. reported an increased number of GRβ immunoreactive inflammatory cells in the airway T-cells of GC-resistance patients when compared to GC-sensitive or healthy patients \cite{33}. In tuberculin-driven cutaneous inflammatory lesions of patients with GC-resistance asthma, increased number of cells expressing GRβ was also reported \cite{34}. Furthermore, Goleva et al. demonstrated in bronchoalveolar lavage macrophages that GC-insensitive asthmatics have elevated GRβ mRNA and protein levels in comparison to GC-sensitive patients \cite{32}. Of most interest, the authors reported enhancement of dexamethasone-induced GRα transactivation in GC-insensitive asthmatics after RNAi silencing of GRβ. As such, Sweet-P suppression of GRβ may serve useful for GC-insensitive asthmatic patients.

Our work highlights miR-144’s role in inducing migration of bladder cancer cells via GRβ; however, miR-144 has been demonstrated to play roles, both positive and negative, in many other forms of cancers and diseases. For example, miR-144 has been shown to contribute to the pathogenesis of Alzheimer’s disease through the downregulation of ADAM10 \cite{35} but is essential for proper erythropoiesis by downregulating RAB14 \cite{36}. Also, miR-144 has been shown to promote nasopharyngeal carcinoma through the downregulation of PTEN, a regulator of the PI3K/AKT pathway \cite{37}, and induce breast
cancer and hepatocarcinoma cell proliferation through the downregulation of Runx1, a tumor-suppressor gene \[^{38}\]. Interestingly, estrogen treatment (E2) in SkBr3 breast cancer and HepG2 hepatocarcinoma cells increased the expression of miR-144 through the PI3K/ERK/Elk1 transduction pathway \[^{39}\], which may serve as a positive activator of GRβ. Solakidi et al. showed in HepG2 and SaOS-2 cells that GRβ and ERα were localized mainly in the nucleus, particularly concentrated in nuclear structures which suggest a direct involvement of GRβ and ERα in nucleolar-related processes \[^{40}\]. However, the interaction of ERα and miR-144 signaling to increase GRβ activity has not been studied. In contrast to oncogenic properties of miR-144, the loss of miR-144 expression has been shown to be related to the progression of colorectal cancer through the derepression of mTOR, a cell growth and metabolism regulator \[^{41}\]. However, because the decrease in miR-144 expression leads to colorectal cancer progression, GRβ may not have an involvement. Also, miR-144 has been shown to inhibit the migration, invasion, and proliferation of carcinomas such as rectal cancer \[^{42}\] and osteosarcoma \[^{43}\], which was attributed to the downregulation of ROCK1 \[^{42}\]. High levels of GRβ was shown in SaOS-2 osteosarcoma cells, which suggest that miR-144 and GRβ signaling may be differentially regulated in bone cancer. Similarly, miR-144 inhibited migration and proliferation of hepatocarcinoma cells by the downregulation of AKT3 \[^{44}\] and non-squamous cell lung carcinoma through the downregulation of ZFX \[^{45}\]. Due to the diverse targeting of many different genes, inhibiting the global function of miR-144 during cancer therapy could be detrimental through off-target effects, and result in the de-repression of oncogenes. The specificity of Sweet-P blocking only the interaction of miR-144 with the 3’UTR of GRβ (Figure 1) to suppress
cancer cell migration may be particularly useful due to the presentation of fewer side effects.

In conclusion, our discovery of Sweet-P targeting GRβ in bladder cancer sheds light on a novel drug therapy that specifically targets a gene known to cause growth, proliferation, migration, and GC hormonal therapy resistance. At this point, we have shown that the Sweet-P molecule suppresses GRβ in bladder cancer. In addition, we have shown that Sweet-P only targets GRβ and not other miR-144 regulated genes. More importantly, Sweet-P inhibits the ability of cancer cells to migrate. We will also be testing the effect of the Sweet-P molecule on other types of cancer. Essentially, Sweet-P may be used as a treatment option for several different carcinomas where GRβ is highly expressed including bladder, prostate, lung, or glioblastoma, as well as for liquid tumors such as in leukemia. Sweet-P can be beneficial for non-cancerous diseases also, such as asthma and GC-insensitive disease states caused by increased GRβ. Thus, Sweet-P serves as the first anti-GRβ molecule that may provide a new therapy.

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Chapter 5

Discussion & Conclusions

We were successfully able to demonstrate that GRβ increases the migration of urothelial carcinoma cells, and could be used as a target to inhibit movement. Males do have a 3:1 predilection for the development of bladder cancer, compared to females, which suggest that the androgen receptor (AR) may also be involved. GRβ and AR may work in corroboration as pro-inflammatory mediators that function to increase the progression of bladder cancer. However, more work remains to be done to further our understanding of GRβ and AR in urothelial carcinoma.

Glucocorticoids (GCs) are commonly prescribed to patients undergoing treatment for urothelial carcinoma to reduce inflammation. In addition, GCs have been shown to suppress urothelial carcinoma cell proliferation and invasion; however, recent work has demonstrated that dexamethasone (Dex) treatment can stimulate proliferation (Ishiguro, Kawahara, Zheng, Kashiwagi, et al. 2014, Zheng et al. 2012) and long-term GC treatment increases the risk for urothelial carcinoma (Dietrich et al. 2009). This brings into question whether GCs should be given to patients and what are the mechanisms that lead to these confounding results. One of the proposed mechanisms is that GRβ, which was not investigated in their study, may induce proliferation overtime by causing GC resistance.
GCs increase GRβ expression (Hinds et al. 2010), and could be the reason for Dex-induced proliferation of uroepithelial carcinoma cells. Therefore, GRβ inhibition, such as through Sweet-P treatment, may sensitize to GCs carried out by GRα and solidify GC use in the treatment of urothelial carcinoma.

There has been shown to be increased incidence of bladder cancer in males compared to females (Chapter 2), which may suggest that the AR may be involved. Ligr et al. showed that GRβ suppression by siRNA reduced growth of prostate cancer cells (Ligr et al. 2012). However, the role of GRβ on AR activity is yet to be established. The role of AR in urothelial carcinoma is an emerging area of study, but the role remains unclear (Litman et al. 2007, McBeth et al. 2015). The AR pathway has been shown to be enhanced by epidermal growth factor (EGF) signaling in prostate cancer (Miyamoto, Messing, and Chang 2004), and EGFR dysregulation is associated with urothelial carcinoma (Zheng et al. 2011); indicating that many of the same roles of AR in prostate cancer, where it has been extensively researched, may also be involved in urothelial carcinoma. However, the relationship of GRβ and AR was not investigated in our current experiments, and should be investigated in future work.

Macrophages can be grouped into two different groupings, the classically activated pro-inflammatory macrophages (M1) and the alternatively activated anti-inflammatory macrophages (M2). These two populations are defined by their production of different cytokines, with M1 producing TNFα and iNOS, while M2 provide Arg1 and FIZZ1 (Biswas and Mantovani 2010, Solinas et al. 2009). Interestingly, TNFα, an M1 associated cytokine, has been shown to induce GRβ expression (Webster et al. 2001, Hinds et al. 2010). Co-culturing T24 cells with M1 macrophages inhibited proliferation, but failed to
induce apoptosis and induced invasion (Dufresne et al. 2011). This failure to induce apoptosis while simultaneously causing invasion could be due to the M1 macrophages producing TNFα, which results in increased GRβ expression. Based on the data that we present here, an increase in GRβ could be the reason for the increased invasion, and it should be investigated. It would be interesting to determine if the same co-culturing experiment conducted on cells expressing lower GRβ, such as UMUC-3 or T24 GRβ KD, or the treatment with Sweet-P could cause more inhibition of proliferation, the induction of apoptosis, or the suppression of invasion. GRβ and its pro-inflammatory effects could shift the macrophage population towards M1. One of the proposed mechanisms for BCG treatment effectiveness is the transition of the macrophage population from pro-M1 to anti-M2 inflammation. BCG may inhibit GRβ expression, which results in a shift to a higher M2 population. Thus, suppressing GRβ expression or activity.

As previously discussed, smoking is thought to account for 50% of male and 35% of female urothelial carcinomas (Zeegers et al. 2000). The mechanism is not known, but GRβ could play a role. It has been shown that cigarette smokers have a 2.2-fold less GRα:GRβ ratio in their peripheral blood monocytes (Livingston et al. 2004), which may be due to an increase in GRβ expression or a decrease in GRα expression. The authors hypothesized that this change in GR isoform ratio was due to the exposure of cigarette smoke causing production of pro-inflammatory cytokines, which have been shown to increase GRβ expression. Due to our data showing that GRβ plays a role in urothelial carcinoma (Chapter 3), and the relationship of cigarette smoking, we extend our hypothesis to propose that the increased pro-inflammatory cytokines produced in the response to
cigarette carcinogens concentrated in the urine could induce GRβ expression in the bladder, thus predisposing these patients to urothelial carcinoma.

A similar line of thought could be applied to other causes of urothelial carcinomas. The exposure of transitional uroepithelial cells to concentrated amounts of carcinogens, such as those discussed previously in Chapter 2 that environmental, occupational, or iatrogenic sources, or the mechanical irritation due to the deposit of Schistosoma haematobium ovum, cause an increase in the production of pro-inflammatory cytokines. These pro-inflammatory cytokines then increase GRβ expression, which predisposes the patient to urothelial carcinoma. These hypotheses should be tested in order to better understand the bladder cancer and improve prevention.

In conclusion, this work is the first to demonstrate the role of GRβ in urothelial carcinoma, and that it causes migration of human cancer cells. In addition, we have shown that GRβ can serve as a drug target to prevent migration of urothelial carcinoma cells (Chapter 3). Furthermore, we have developed the anti-GRβ Sweet-P molecule to prevent migration of cancer cells (Chapter 3 & 4). Also, Sweet-P could potentially serve as a treatment for the other diseases and conditions where GRβ plays a role. Sweet-P inhibition of GRβ and enhancement of the GC response may be useful for cancers such as urothelial carcinoma, glioblastoma, and leukemia, as well as a therapeutic for conditions such as asthma, Crohn’s disease, and ulcerative colitis. (Hamid et al. 1999, Goleva et al. 2006, Honda et al. 2000, Towers et al. 2005). These studies have lead to the development of the first anti-GRβ molecule that may be useful for several GRβ-related diseases.
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