# TGFβ-Dependent ROS Regulates a Local Endocrine Communication Network Between Stromal and Epithelial Cells in the Prostate

By

**Melanie Jean Grubisha**

BS, Biology and Chemistry, Carlow University, 2005

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This dissertation was presented

By

Melanie Jean Grubisha

It was defended on

November 1, 2011

And approved by

Patrick Pagano, PhD

Committee Chair

Professor of Pharmacology

Guillermo Romero, PhD

Professor of Pharmacology

William LaFramboise, PhD

Professor of Pathology

Daniel Johnson, PhD

Professor of Pharmacology

Richard Steinman, MD, PhD

Professor of Medicine/Professor of Pharmacology

Donald DeFranco, PhD

Dissertation Advisor

Professor of Pharmacology, University of Pittsburgh School of Medicine

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The development of prostate cancer (PCa) can be considered a co-evolution of both the epithelial and stromal cells; indeed, the latter develop their own unique gene signature during cancer progression that has potential predictive value in determining a patient's outcome. Cancer-associated "reactive" stroma is characterized by heterogeneity in Transforming Growth Factor β (TGFβ) signaling, transdifferentiation into a myofibroblast phenotype by stromal fibroblasts, and an increased production of reactive oxygen species (ROS). In this study, we sought to examine the basis for PCa cell response to reactive prostate stromal cells (i.e. myofibroblasts) *in vitro.* Specifically, we have shown that human prostate derived fibroblastic (i.e. PS30) and myofibroblastic (i.e. WPMY-1) cell lines and primary stromal cells have the capacity to inhibit DU145 PCa cell motility in co-culture through the production of a precursor ligand for estrogen receptor β (ERβ). Activating the ERβ pathway in adjacent DU145 cells leads to induction of the cell adhesion molecule E-cadherin and a subsequent reduction in cell motility. However, an increased responsiveness to TGF-β1 in WPMY-1 cells triggers induction of COX-2 expression and elevated ROS production, which ultimately raises extracellular $H_2O_2$. $H_2O_2$ derived from WPMY-1 cells acts in a paracrine manner to decrease the recruitment of ERβ to the E-cadherin promoter in co-cultured DU145 cells, as revealed by chromatin immunoprecipitation assays. shRNA knockdown of COX-2 in WPMY-1 cells abolishes the TGF-β1-induced ROS production and restores the inhibitory effects of myofibroblasts on DU145 cell motility in co-culture. Therefore, despite their "reactive" stroma phenotype, limiting the TGFβ-driven ROS production in WPMY-1 cells restores their inherent capacity to

limit tumor progression through a local endocrine network targeting ERβ in adjacent PCa cells. Our results imply that controlling the redox status of the local milieu may offer a route for utilizing inherent regulatory mechanisms to limit cancer cell motility, ultimately acting to reduce its spread and dissemination.

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# PREFACE

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# 1.0 Introduction

Despite the broad range of current available therapies, prostate cancer (PCa) remains a major source of morbidity and mortality among men. It is second only to lung cancer in tumor-associated deaths among males [1]. Public health officials predict a dramatic increase in the incidence of prostate cancer in the coming years, underscoring the need for more effective therapies [2].

In order to develop improved targeted therapies, a more thorough understanding of the molecular pathogenesis of prostate cancer is necessary. First-line therapy for most localized prostate cancer cases is radical prostatectomy, in which the entire prostate gland is surgically removed. However, recurrent disease or delayed diagnosis are generally widespread and are therefore targeted by systemic androgen-deprivation therapy. While initially effective, androgen-deprivation ultimately leads to androgen-independent disease, at which point therapy becomes palliative. The entire process of recurrence, initial androgen dependence, and ultimately androgen-independence speaks of an intricate molecular interplay of various factors that cause a continual evolution in the phenotype of the disease. Understanding these molecular pathways at any given point can provide a druggable target for specific therapy set to disrupt the continuum of disease.

In this project, I sought to understand the molecular mechanisms underlying advanced, androgen-independent disease. It is at this point, after the failure of all current therapies, that the disease is the least well understood and the current therapeutic modalities are the least effective.

## 1.1 The Prostate Gland: Structure and Function

### 1.1.1 Normal Growth and Development

The male reproductive tract is formed from the urogenital sinus (UGS) in response to fetal androgens [3]. The production of testosterone stimulates the budding of prostate epithelium from the UGS, which expresses high levels of androgen receptor (AR) during that development period [4]. By the

11th week of development, in response to high levels of circulating testosterone, the mesenchymal cells begin to differentiate into the smooth muscle and fibroblasts that form the stroma [5, 6]. The adult prostate maintains AR expression, and signaling through the AR regulates new growth to replace the constant turnover of cells in the organ. In early adulthood, the testes are significant contributors of testosterone and subsequent AR activation in the prostate. In older men, adrenal synthesis of DHEA and its subsequent conversion to the potent androgen metabolite dihydrotestosterone (DHT) is the major source of AR ligand [7, 8].

In the normal adult prostate, there is a glandular epithelial component and a fibromuscular stroma. The epithelial cells lining the glands express AR and are the source of prostate specific antigen (PSA), which is currently a biomarker for prostate health. They are arranged into a neatly delineated squamous basal layer and columnar secretory layer. The stromal compartment comprises both fibroblasts and smooth muscle cells, with a predominance of the latter, arranged into an organized orientation of fibers [9]. In 1981, McNeal proposed the concept of a zonal anatomy of the adult prostate, partitioning it into 4 zones based on their location and embryonic structure of origin: the peripheral , central, transition, and peri-urethral zones [10]. The former 2 zones comprise the glandular part of the prostate and account for ~95% of its total size. This zoning anatomy is currently still used to describe where prostate cancers are located, with the greatest percentage of neoplasias occurring in the large peripheral zone [4].

Figure 1 shows H&E stained microscopic slides of normal and cancerous prostate anatomy (http://library.med.utah.edu/WebPath/TUTORIAL/PROSTATE/PROSTATE.html#4). The normal specimen demonstrates a clearly defined epithelial-lined gland surrounded by an organized fibromuscular stroma, whereas the PCa specimen shows a loss of normal glandular architecture, a loss of structure in the basal and secretory epithelia, and infiltrating immune cells (stained red).

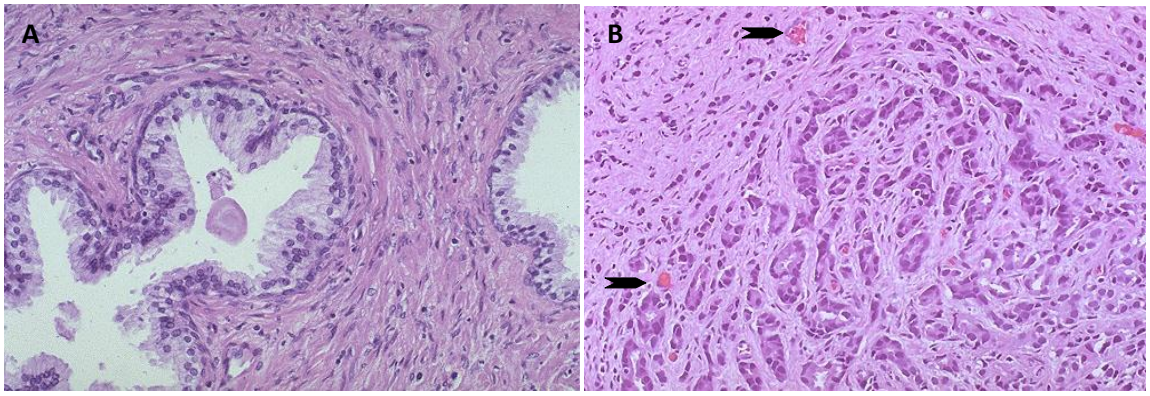



**Figure 1. Tissue staining of normal and cancerous specimens from excised human prostate samples.**

A. H&E stained microscopic slide of normal prostate gland demonstrating normal gland architecture in the left portion of the specimen. A squamous basal epithelial layer separates the secretory columnar epithelium from a fibromuscular stroma.
B. H&E stained microscopic slide of a poorly differentiated prostate carcinoma. Note the loss of normal glands separated by areas of organized stroma. The glands appear smaller and have lost the basal epithelial layer. Red staining indicates infiltration by immune cells (arrowheads).

### 1.1.2 PIN and Carcinogenesis

Most prostate cancers are glandular/epithelial in nature and are therefore classified as adenocarcinomas. Histologically, a precursor to PCa is prostatic intraepithelial neoplasia (PIN), characterized by a thickening of the epithelial layer and a loss of distinct basal and secretory layering. Since PIN retains the basal membrane separating the epithelium from the stroma, it is not considered cancerous. However, high-grade PIN is considered a cancerous precursor [4].

Most prostate cancers are multi-focal, meaning they have multiple locations within the gland and are often separated by areas of normal prostate anatomy. In order to determine the extent to which the prostate is affected in a particular patient, 12-site needle biopsies are obtained and scored. A pathologist reviews each biopsy sample, and a number of cancer-containing out of total samples

obtained is expressed: e.g. 7/12. Within the cancerous specimens, a modified Gleason grading system is employed. A pathologist grades the extent of architectural disarray 1-4, with 4 being complete loss of structure, and will assign a number representing the most commonly observed number and another representing the most severe loss of differentiation. The grades are then added together to give a final score: e.g. Gleason score 2 + 4 = 6 [11].

While many prostate cancers are developed late in life, are slow growing, and remain organ-confined for the extent of the patient's life, others are aggressive and may spread rapidly. Partin tables have been developed to predict the probability of a patient having organ-confined disease based upon 3 parameters at diagnosis: clinical stage, biopsy Gleason score, and serum PSA level [12, 13]. These models are clinically useful in determining a patient's risk of developing aggressive or advanced disease, and may help guide the clinician in his/her treatment paradigm. A fast-growing or aggressive tumor will initially break free of the prostate tissue capsule, at which point it begins with a local spread to nearby lymph nodes and urinary tract structures. Eventually PCa will metastasize to distal organs, most often forming osteoblastic lesions in the skeleton, though many cases of dural PCa metastases have been reported [14, 15]. A patient's risk of relapsing into this late-stage disease is inversely related to the presence of extraprostatic extension, seminal vesicle extension, positive lymph nodes, or positive margins at surgery [16].

While PCa is diagnosed on biopsy by a loss of tissue architecture and the presence of abnormal cells, on a molecular level it is marked by genetic alterations and a decrease in apoptosis, which ultimately lead to this observed cellular dysplasia and tissue disorganization [4]. Deregulation of androgen signaling is a molecular hallmark of advanced disease, and will be discussed in further detail in the subsequent section.

## 1.2 The role of androgens in prostatic carcinoma

### 1.2.1 Nuclear receptor structure and function

Nuclear receptors are a superfamily of receptors that modulate transcription of various genes. The focus herein will be on a subgroup of nuclear receptors, the steroid hormone receptors (SRs). When bound to their respective ligand, the steroid hormone receptors translocate from the cytoplasm to the nucleus and initiate alterations in transcription of specific target genes. They contain a ligand-binding domain (LBD) and a DNA-binding domain (DBD), allowing them to display ligand-dependent activation and controlled regulation of specific target genes [17]. Emerging insight into nuclear translocation has revealed that SRs possess both nuclear localization and nuclear export sequences and are capable of nucleocytoplasmic shuttling. It is thought that nuclear export is one way in which gene transcription is terminated [18]. SRs have distinct functions in both the cytoplasm and the nucleus, with the latter being activity as a transcription factor involving direct DNA binding and transcriptional complex recruitment at specific target genes [19]. In the cytoplasm, SRs are bound by heat-shock proteins (HSP) and are in monomeric form. Upon ligand binding, the HSP are shed and the receptors translocate to the nucleus [20] . When part of an active transcriptional complex, SRs exist in homo- or heterodimers. While this dimerization can occur in either the cytoplasm or the nucleus, it is a necessary component for transcriptional activity. Tissue-specificity of nuclear receptor action is regulated by expression of various coactivators and corepressors, and different target genes can be differentially activated or repressed depending upon the specific composition of the transcriptional complex [21]. Figure 2 shows a diagram of nuclear receptor activation and target gene regulation.

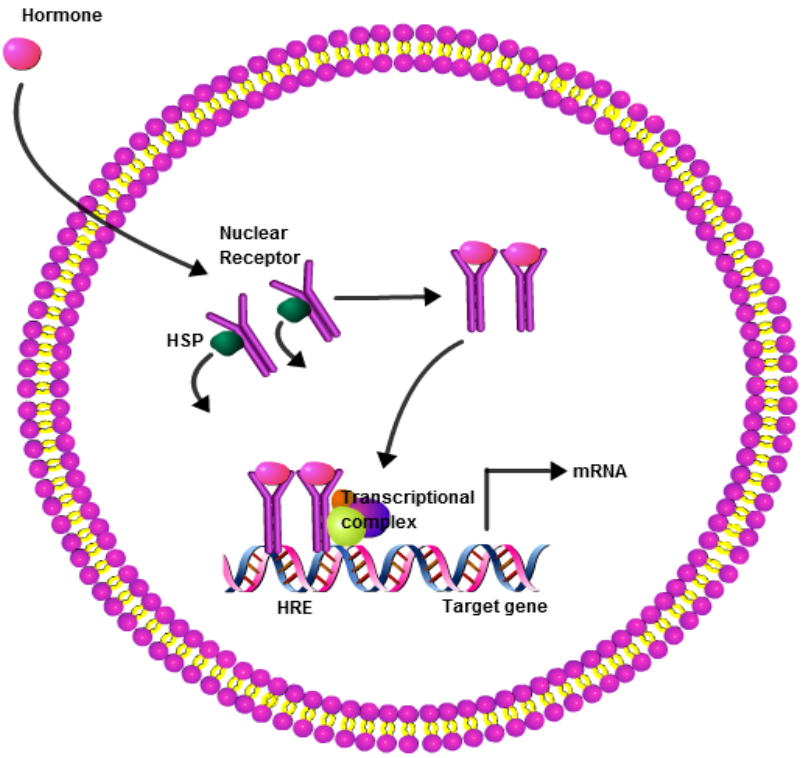



**Figure 2. A simplified schematic of nuclear receptor signaling.** Upon entering the cell, hormone is able to bind its specific nuclear receptor. This induces shedding of heat shock proteins (HSP) and allows the receptors to homo- or heterodimerize. The dimerized receptors then recognize a specific hormone response element (HRE) in the promoter region of a target gene. Recruitment of appropriate transcriptional machinery, including coactivators, polymerases, etc., will then initiate gene transcription.

Currently there are 48 members of the human nuclear receptor superfamily [22], but our focus here will be on the subset of endocrine nuclear receptors, primarily the androgen receptor (AR) and estrogen receptor subtype beta (ERβ). Due to the highly conserved structure of nuclear hormone receptors, endogenous hormone ligands are often capable of binding multiple receptor subtypes; however, ligands often display differential binding affinities for the various receptors, and thus at physiologic concentrations specificity is achieved due to preferential binding of the ligand to the receptor for which it has the lowest Kd [23].

Additionally, steroidogenesis is an intricate and tightly controlled process. Differential enzyme expression in various tissues allows metabolism that either activates or inactivates various steroid hormone precursors into ligands that will preferentially bind the nuclear hormone receptor of importance in that tissue. In the prostate, for example, the enzyme 5α-reductase is highly expressed. 5α-reductase converts testosterone into dihydrotestosterone (DHT), a ligand much more potent for the AR than testosterone [24, 25]. Unlike testosterone, DHT is unable to be converted to estradiol by the enzyme aromatase [26], thus expression of 5α-reductase ensures adequate activation of AR in the prostate while preventing the production of ligands for the estrogen receptor (ER).

The androgen receptor is highly expressed in androgen-responsive tissues, such as those found in the male reproductive organs. During development and again during puberty, circulating levels of androgens are extremely high as the primary and secondary sex organs develop and mature [27, 28]. In the adult male prostate, androgens regulate the normal cycle of cell growth, maturation, and death [8]. As evidence of AR activity being intimately involved in prostate growth, a target gene of AR, prostate specific antigen (PSA), is currently used as a screening marker for hyperproliferative diseases of the prostate (PIN, PCa, and benign prostatic hyperplasia, BPH) [29]. The following section will address the complicated biphasic role of AR in prostate cancer in more detail.

### 1.2.2 Androgen dependence and deregulation

As previously mentioned, in the adult prostate AR signaling is vital to the maintenance of normal growth and function. Prostate epithelial cells are undergoing a constant low level of turnover, and androgen signaling is crucial for the stimulation of new cell growth and development. Due to this growth and developmental function of AR, it is to be expected that in prostate cancer an abundance of AR signaling underlies the overgrowth of epithelial cells seen in carcinogenesis. Initially, the abundance of AR activity is detected by increased serum levels of PSA, an AR target gene. During this early stage of

disease, multiple factors can contribute to the hyperactivity of AR. Mutations within the AR gene can lead to a more promiscuous receptor which can be activated in the absence of ligand, or by additional growth factors such as EGF and IGF-1 [30-34]. Increased expression of AR-specific co-activators and/or alterations in post-translational modifications can also be the source of aberrant AR signaling in early prostate cancer [35].

While initial therapy for PCa is removal of the organ, recurrent disease is a common problem. Without a localized target tissue, the recurrent cancer cells, which are still dependent upon androgen signaling for continued growth, are typically targeted through systemic androgen deprivation [36]. This chemical castration is achieved by administration of either a GnRH agonist, a 5α-reductase inhibitor, or a specific AR-antagonist [37, 38]. The former two lead to an overall decrease in endogenous production of androgens, whereas the latter targets the ability of circulating androgens to effectively activate the AR. This chemical castration is initially effective, but after prolonged androgen deprivation eventually the metastatic disease becomes castration resistant and unresponsive to this therapeutic approach [39]. Castration-resistant disease, however, is still dependent upon androgen receptor signaling; it is the therapeutic approach to ablating androgen activation that loses efficacy, as aberrant AR activation resumes despite the modalities employed for chemical castration [40]. Patients with CRPC still exhibit activation of AR target genes (e.g. PSA), thus demonstrating that AR is still active in the course of the disease despite a therapeutic attempt to remove potent circulating androgens.

Recent research has further complicated the role of AR in PCa through an intricate series of highly specific AR-knockouts in mice. While its results still remain a hot area of debate, a detailed study by Niu et al. has shown that AR in the prostatic epithelium is tumor suppressive, whereas AR in the prostatic stroma promotes invasion by a cancerous epithelium [41]. Interestingly, additional follow-up of these results led to the hypothesis that one of the mechanisms through which AR alters epithelial

invasive characteristics is through modulation of TGFβ signaling. A subset of TGFβ responsive genes, including COX-2 (which will be addressed in more detail in a subsequent section), are not upregulated following TGFβ treatment when AR is stably re-expressed in PC3 PCa cells [41]. These TGFβ responsive genes act to promote tumor aggressiveness, thus inhibiting their induction via re-expression of AR acts in a tumor-suppressive manner. Androgen signaling, even in castration-resistant disease, appears to be a two-edged sword. Clearly, studies such as these would argue against global androgen deprivation therapy, again underscoring the need for a more thorough understanding of prostate cancer growth and progression to develop more targeted and efficacious therapies.

### 1.2.3 Estrogen receptor in the prostate

Estrogen receptor (ER) is similar to AR in conserved nuclear receptor structure and function, but its natural ligands are estrogenic in nature, such as estradiol. Estrogens can be synthesized from adrenal androgens in peripheral tissues through the action of the enzyme aromatase. As men age, their circulating levels of testosterone naturally decline, but the stable expression of aromatase ensures that estrogen levels remain constant [42]. Thus, in the ageing male population, the role of estrogen as a regulatory steroid within hormone-dependent tissues such as the prostate becomes increasingly important. Additionally, laser capture microdissection of normal human prostate tissue has identified aromatase expression in normal stromal cells; in PCa, malignantly transformed cells also acquire aromatase expression [43]. This expression pattern ensures local synthesis of estrogens within the prostate, highlighting their importance in prostate biology.

ER exists in 2 subtypes designated as ERα and ERβ. Unlike AR, whose activation is associated with prostate growth, signaling through ER appears to be more complex, lending in large part to differential activities of the ER subtypes. ERα activation in the prostate is associated with three distinct responses: aberrant proliferation, inflammation, and cancer [42]. Knockout of aromatase in a mouse

model (ArKO) leads to lifelong increased levels of androgens, yet the mice fail to develop prostate cancer [44]. Administration of synthetic estrogens early in development, however, leads to abnormal prostate biology later in life in these same ArKO mice [45]. These studies suggest that it is not a singular steroid hormone pathway that is responsible for growth and maintenance of the prostate, but rather that it may be the balance between androgenic and estrogenic signaling that underlies steroid signaling in prostate biology.

In the prostate, the prevailing ER subtype is ERβ [46, 47]. Knockout mice have been generated that are deficient only in the ERβ subtype, and extensive studies performed with these models have shown that while ERα mediates estrogen-induced prostatic inflammation and pathologies, ERβ may confer a beneficial effect in maintenance of normal homeostasis [42]. Supporting epidemiological evidence also exists, as men who consume higher dietary intakes of phytoestrogens (weakly estrogenic compounds that exhibit a significantly higher affinity for ERβ over ERα) exhibit a lower incidence of PCa [48, 49].

A unique feature of ERβ is its sensitivity to oxidation. This reportedly high redox sensitivity of ERβ may be important in tumors or tissues exposed to chronic inflammation (to be discussed in detail in a later section). In particular, chronic inflammation leads to increased levels of reactive oxygen species (ROS) and subsequent development of an oxidizing milieu. Keeping this in mind, the oxidation-sensitive motifs in ERβ may be of particular relevance in PCa. Kumar et al. showed that oxidative stress in PCa cells is required for an aggressive phenotype, and that PCa cells are capable of generating high levels of ROS [50]. Thus, ERβ in the prostate could potentially be susceptible to oxidation through inherent generation of ROS.

Zinc-finger motifs are a common feature among the nuclear receptor family of transcription factors [51]. In ERβ, the first zinc-finger is necessary for DNA binding and the second zinc-finger is

responsible for receptor dimerization, an essential step for stabilization of DNA binding at the promoter [51, 52]. With a high number of cysteine residues, these motifs are particularly susceptible to oxidation. Oxidation at a cysteine residue results in the loss of 2 protons and subsequent formation of a disulfide bond, and this structural change induces alterations in the protein structure. Specifically, ERβ oxidation occurs primarily in the 2$^{nd}$ zinc finger motif, and the resulting conformational change in protein structure de-stabilizes and ultimately prevents DNA binding [52, 53]. Electromobility shift assays (EMSA) have demonstrated a loss of DNA-binding when ERβ is subjected to oxidation by $H_2O_2$ [52]. This redox-sensitivity has been extensively studied in breast cancer, and it has been shown that up to one-third of untreated ER-positive breast cancer tumors have structurally altered ER that is unable to bind its cognate DNA sequence at its target genes [54]. This effect is reversible with addition of reducing agents, and *in vitro* studies have isolated breast cancer tumors with non-functional ER and restored DNA binding activity through vigorous treatment with the thiol reducing agent, DTT [55].

Activation of ERβ by estradiol is suppresses prostate epithelial cell growth, and activation of the receptor by androgen metabolites inhibits cell motility by increasing cell adhesion [56, 57]. Specifically, ERβ activation by androgen metabolites increases expression of E-cadherin, an epithelial marker associated with cell adhesion [58]. Loss of cell adhesion and increased motility are hallmarks of carcinomas, thus ERβ could potentially serve to limit carcinogenesis in the prostate by maintaining adequate expression of E-cadherin and hence inhibiting the generation of a more motile phenotype.

Moreover, recent work has shown that androgen metabolites can be potent activators of the ERβ subtype, and in the prostate these androgen derivatives act as the endogenous ERβ ligands [46, 59]. Specifically, 2 DHT metabolites, 5α-androstane-3α,17β-adiol (3α-Adiol) and 5α-androstane-3β,17β-adiol (3β-Adiol), have been shown to be potent ERβ ligands [58, 59]. Activation of ERβ in the prostate is ensured by local tissue expression of the enzymes AKR1C2 and AKR1C3, which are responsible for the

metabolism of DHT into 3α-Adiol and 3β-Adiol, respectively [60, 61]. The importance of these metabolites as ERβ ligands is underscored by the fact that the local concentration of 3β-Adiol in the prostate is one hundred fold higher than that of estradiol [56]. Interestingly, these steroid-converting enzymes are of growing interest in prostate cancer. Polymorphisms in these enzymes have been correlated with prostate cancer risk [62], and recent endeavors to identify small molecule modulators have been undertaken using modern high-throughput screening techniques [63].

More recent work has demonstrated another ERβ-dependent signaling pathway active in prostate cancer which may serve to limit tumor aggressiveness. Epithelial-to-mesenchymal transition (EMT) is a crucial early step in the acquisition of a more motile and invasive phenotype, hence permitting cancer cells to migrate and invade surrounding tissues. ERβ activation downregulates TGFβ and hypoxia induced EMT through regulation of Vascular Endothelial Growth Factor (VEGF) promoter [56]. An inverse relationship exists between ERβ expression and the progression of prostate cancer to a high Gleason grade [56]; thus, signaling through ERβ appears to be one mechanism through which prostate growth is tightly regulated. Hence, this pathway presents a putative target for therapeutic intervention. Figure 3 shows the major steroidogenic and steroid metabolizing pathways active in the prostate.

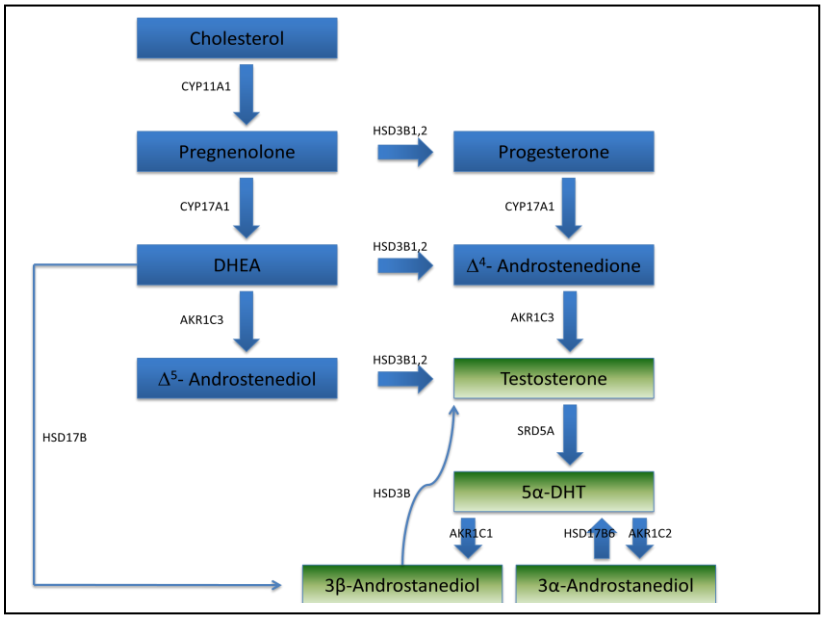


**Figure 3. A schematic of steroid metabolism in the prostate**. The enzymatic profile of the prostate tissue allows *de novo* steroid synthesis as well as conversion among various steroids and steroid precursors. The main steroids and metabolites active in the prostate are represented in green.

## 1.3 The tumor microenvironment

### 1.3.1 Tumor-stroma co-evolution

The importance of the microenvironment in the fate and development of carcinomas is well recognized. Elegantly designed co-culture experiments by Gerry Cunha and collaborators demonstrated that, in the prostate, signaling from the microenvironment could "instruct" an initiated epithelial phenotype to become cancerous. Initiated epithelial cells (immortalized but non-tumorigenic) failed to form tumors in mice either alone or when co-transplanted with normal prostatic fibroblasts, but when co-transplanted with cancer-associated fibroblasts (CAFs) the mice underwent significant tumor development [64]. A later study designed by Petersen and Bissell showed that a normal stromal microenvironment could revert breast cancer cells back to a near normal phenotype when grown together in 3D co-culture [65]. It is well established that while it is the epithelial cells of the prostate that actually develop into cancer, the surrounding stromal fibroblasts co-evolve alongside this transformation and eventually differentiate into an altered form known as "myofibroblasts," otherwise referred to as CAFs. In addition to CAFs, the tumor microenvironment recruits inflammatory and immune cells, displays an altered production of and response to various growth factors and signaling molecules, and collectively develops into a "reactive" stroma [66].

Under normal conditions in a healthy prostate, the stroma acts as a balance to keep the epithelium in check, secreting factors that limit cell proliferation, migration, etc. [65, 67]. However, upon malignant transformation, the transdifferentiated CAFs lose their ability to restrain the epithelium and

instead acquire a permissive and/or supportive role in cancer progression. This reactive stroma evolves its own unique signature, including increased fibroblast growth factor (FGF2), connective tissue growth factor (cTGF), vimentin, smooth-muscle α-actin, and tenascin levels [68]. Current work is investigating the usefulness of the stromal signature in predicting both the initial development of prostate cancer as well as biochemical-free recurrence for patients following localized therapy [9, 68]. While the usefulness of the stromal signature as a clinical diagnostic tool is indeed interesting, it is perhaps more promising to attempt to understand the initial drivers of this stromal transdifferentiation. Targeting the tumor microenvironment could provide a unique and specific alternative therapy and could potentially utilize the body's own defenses to control the cancer proliferation and spread.

Co-evolution of the stroma surrounding the tumor in the prostate is intimately related to three key factors: hypoxia, oxidative stress, and autophagy/mitophagy [69]. The focus here will be primarily oxidative stress. *In vitro* co-culture experiments involving human breast cancer cells and stromal fibroblasts demonstrated that cancer cells induce oxidative stress in adjacent fibroblasts, as measured by an increased production of reactive oxygen species (ROS) and DNA damage [70, 71]. In response to this increased production of ROS, neighboring cancer cells exhibit antioxidant defense responses, including increased production of the antioxidant enzyme peroxiredoxin1 and the anti-apoptotic protein TIGAR [69]. However, while the cancer cells demonstrate pro-survival responses, increased local ROS production by the stroma appear to prevail in oxidative stress, which is a function of the balance between ROS production and its catabolism by antioxidant enzymes and nutrients. A complex area of research, per se, the intricacies of oxidative stress will not be explained in detail here. Suffice it to say that substantially elevated ROS levels result in cellular glutathione depletion whose effects are widely deleterious. Germane to this discussion, it is reported that oxidative stress instigates additional DNA damage, thus contributing to increased genomic instability in both the cancer cells and the surrounding

stroma [72, 73]. Figure 4 below is a simplified diagram of the oxidative stress model of tumor-stroma co-evolution.

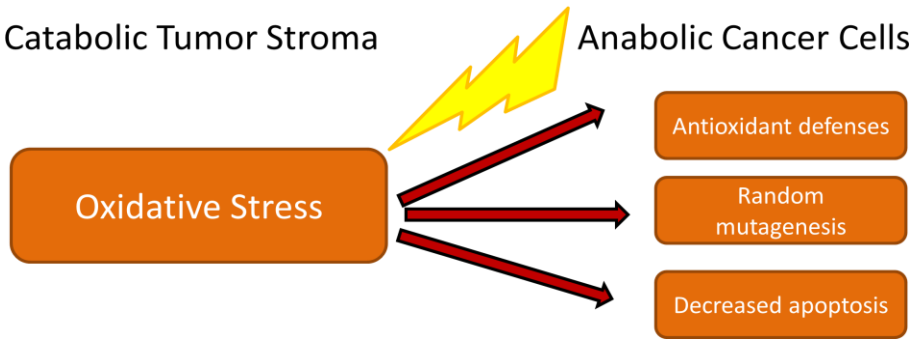



**Figure 4. Oxidative stress model of tumor-stroma co-evolution.** Cancer cells induce oxidative stress in adjacent stromal cells. This increases ROS production by the stroma, and the ROS species act as paracrine mediators to induce antioxidant defenses, random mutagenesis, and ultimately decreased apoptosis in neighboring cancer cells. It is postulated that elevated ROS production outweighs cancer cell antioxidant defenses leading to overall oxidative stress. This eventually leads to a shift in net energy usage, wherein the stroma becomes catabolic and the released nutrients are recycled to "feed" the anabolic cancer cells.

Corroboration of this oxidative stress-driven model of tumor-stroma co-evolution lies in experiments demonstrating that oxidative stress response genes, such as Hypoxia Inducible Factor 1 alpha (HIF1α) and Nuclear Factor kappa B (NFκB), are activated in CAFs [71]. Additionally, activating both HIF1α and NFκB in stromal fibroblasts was sufficient to confer a reactive, myofibroblastic phenotype, further underscoring the important role that oxidative stress plays in transforming a normal prostatic stroma into a cancer-supportive microenvironment [69].

### 1.3.2 Transforming Growth Factor β

One key signaling mediator in the transdifferentiation of a stromal cell into a reactive cancer associated fibroblast (CAF) is the cytokine Transforming Growth Factor β (TGFβ) [66, 74]. TGFβ is a 25

kDa homodimeric secreted protein that binds to a set of heterodimeric receptors, initiating a signaling cascade that ultimately results in alterations in transcription of TGFβ responsive genes [75]. Figure 5 shows a simplified schematic of canonical, Smad-dependent TGFβ signaling. An alternative Smad-independent pathway also exists, generally understood to signal through ERK and p38 MAPK activation [76]. TGFβ is an active player in wound healing, tissue repair, and, as will be discussed in detail, cancer initiation and progression [65, 77, 78]. TGFβ has been shown to induce the phenotypic and morphologic change in the stromal microenvironment of the prostate known as transdifferentiation [66]. The resulting change in fibroblasts to a myofibroblast phenotype is defined by a co-expression of both fibroblastic and smooth muscle markers such as smooth muscle α-actin expression, tenascin, and vimentin [66, 79, 80], and it carries with it alterations in the stromal cells' interactions with the cancerous epithelial cells [67]. In addition to mediating transdifferentiation of fibroblasts, TGFβ also induces the expression of several key pro-cancerous genes involved in cell adhesion, angiogenesis, and redox signaling [81].

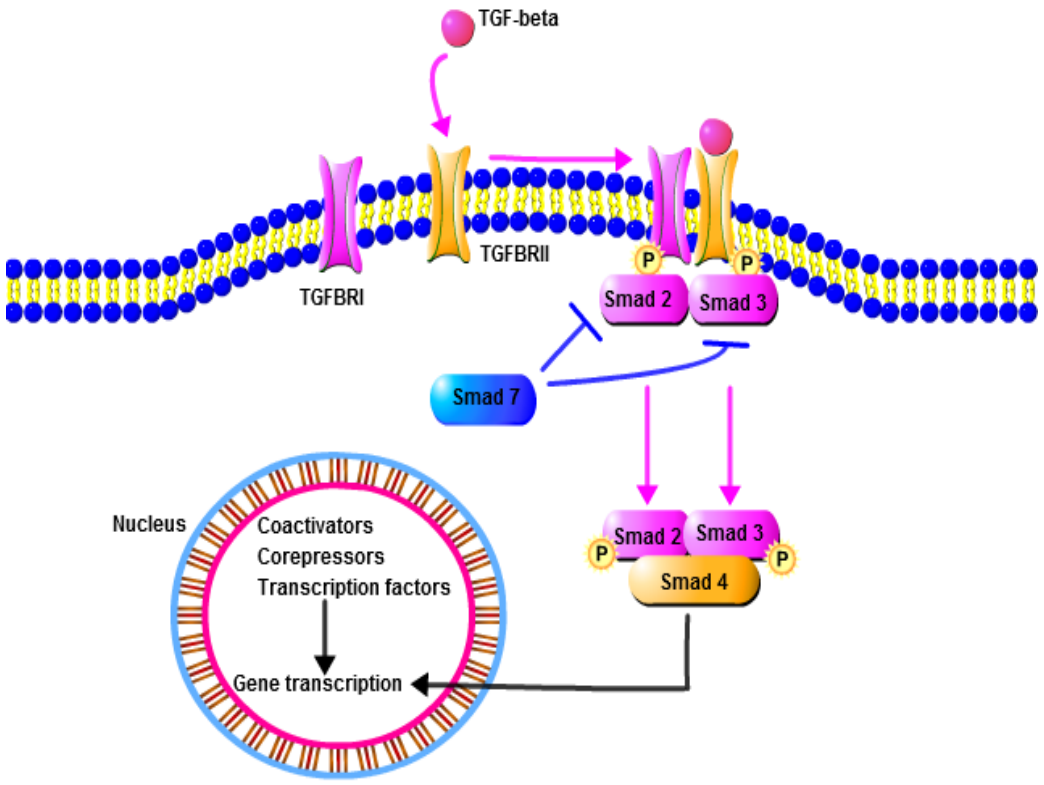



**Figure 5. A simplified schematic of Smad-dependent TGFβ signaling.** TGFβ binds to TGFβRII, which then heterodimerizes with TGFβRI. Following receptor cross-phosphorylation, receptor-associated Smads (Smad 2 and Smad 3) are phosphorylated, complex with the common Smad 4, and translocate to the nucleus. The Smad complex recognizes Smad binding elements in the promoter regions of target genes and, upon DNA binding, recruits transcriptional machinery to initiate gene transcription.

TGFβ is unique among growth factors involved in prostate cancer because it appears to have a biphasic effect. In normal prostate and early carcinogenesis, TGFβ exerts a growth inhibitory effect. Treatment of prostatic epithelium with TGFβ prevents the cells from transitioning from G1 to S phase, and it is thought that in the normal prostate TGFβ is, in part, responsible for maintaining normal prostate size [75]. In some human cancers, loss of functional TGFβ signaling contributes to carcinogenesis. This has been demonstrated through loss of function mutations in TGFβ receptors, downregulation of Smad proteins responsible for propagating TGFβ signaling, overexpression of

negative regulators of the TGFβ pathway, and alterations in expression levels of co-activators and co-repressors [77, 82-85]. These observations would lead to the conclusion that TGFβ is capable of suppressing tumorigenicity, and a primary therapeutic goal would be to restore adequate TGFβ signaling.

Paradoxically, however, tissue staining from both human and orthotopic rat models of PCa has shown a significantly higher level of TGFβ in cancerous versus normal tissue [75, 86], and cell lines derived from human prostate cancer, such as PC3 and DU145 lines, are known to produce relatively large amounts of the TGF-β1 isoform [87]. This has been demonstrated by both quantitation of mRNA transcript levels of TGF-β1 as well as direct measurement of secreted TGF-β1 by enzyme-linked immunosorbant assay (ELISA) [87]. Additionally, overexpression of TGFβ in prostate cancer correlates with increased risk of metastasis and worse prognosis [86], and staining for the phosphorylated form of Smad2 (indicative of active canonical TGFβ pathway activation) is highest in CAFs immediately surrounding cancerous foci in samples taken from prostate biopsy specimens [82]. While normal prostatic epithelium is growth suppressed by TGFβ, cancerous cells are immune to its growth inhibiting effects, thus allowing them to overproduce the cytokine yet evade any autocrine effects it may have on their growth [75]. As such, much attention has been directed to the effects of locally produced TGFβ on the prostatic stroma. As previously mentioned, TGFβ is sufficient to induce transdifferentiation of prostatic fibroblasts into reactive myofibroblasts. New research has outlined the concept of "stromal heterogeneity" in TGFβ signaling. Some stromal cells lose the ability to respond to local TGFβ, usually through loss of the TGFβ type II receptor (TGFβRII), while others maintain an intact TGFβ response pathway. With the loss of TGFβ response in some cells, the local production of TGFβ increases, thus having a more profound effect on those cells in which TGFβ signaling remains intact [82, 88]. This innovative concept succinctly explains the paradoxical observation that while advanced PCa is associated with a loss of TGFβ signaling pathway components, the local production of TGFβ is

significantly increased and there are focal points of increased TGFβ signaling, as evidenced by elevated phospho-Smad2 staining in CAFs surrounding tumor foci [82].

Taken together, these data suggest a complex role for TGFβ in the initiation and progression of PCa. Rather than target TGFβ signaling directly, which has been undertaken with disappointing clinical results thus far [89], downstream targets of the pathway should be carefully elucidated and more directly targeted.

## 1.4 Inflammation

### 1.4.1 Cancer as a wound that never heals

When a tissue is injured, the immediate response by the body is activation of inflammatory pathways. This inflammation serves multiple key functions: recruitment of immune cells to fight off potential infectious agents, removal of damaged cells and debris, stimulation of migration of cells into the wounded area, re-vascularization within the wound, and promotion of proliferation of the newly migrated cells to restore tissue integrity. Coordination of all of these processes is a tightly regulated mechanism, and in normal wound healing these pro-inflammatory signals are offset by anti-inflammatory signals following successful repair of the wound. If the inflammation is not terminated, but instead enters a chronic state of unbalanced pro-inflammatory signaling, detrimental sequelae may result. Many human diseases, ranging from rheumatoid arthritis to obesity, are linked to chronically high levels of pro-inflammatory mediators. A current cancer model suggests that cancerous tissue may also posses this imbalance of pro- and anti-inflammatory signals, and in many respects cancer behaves simply as a wound that never heals [90].

The first step in wound repair is recruitment and activation of immune cells. This is accomplished through release of chemokines, which are cytokine growth factors that have a specific chemoattractant property for circulating immune cells. Once activated, the immune cells migrate out of

circulation and enter the wounded tissue. Binding of antigen to various toll-like receptors (TLRs) expressed on the immune cells activates a signaling cascade that leads to the production of inflammatory cytokines, such as tumor necrosis factor alpha (TNFα) and interleukins 1β and 6 (IL-1β and IL-6, respectively) [91, 92]. Following the propagation of pro-inflammatory signals, the immune cells then begin the processes of fighting off infection and removing dead or wounded cells and cellular debris. Immune cells can sterilize the wounded area through production of reactive oxygen and nitrogen species (ROS and RNS, respectively) and various proteases such as plasminogen and matrix metalloproteinases (MMPs) [93, 94]. These reactive species induce fatal DNA damage to engulfed bacteria, and the proteases serve to degrade the extracellular matrix to facilitate cell migration for a later step of wound repair. However, in cancerous tissue there is a distinct lack of bacterial presence, yet the production of ROS, RNS, and proteases continues to occur as if the tumor were in a chronically inflamed state [78]. Rather than supporting a healing process, these mediators can be damaging and actually prolong the state of inflammation with a feed-forward mechanism. As the tumor milieu becomes more reactive as a result of increased immune cell secreted factors, it initiates a state of injury and the body responds by further increasing production of inflammatory mediators. The ROS and RNS produced in the milieu can further propagate the cancer by initiating additional DNA mutations in the nearby cancer cells, and some studies have suggested that cancer cells posses a more sensitive stress response to ROS than their non-transformed counterparts [95].

In addition to extravasation of immune cells in response to chemotactic signals released by the wounded tissue, uninjured cells must also become motile and migrate into the wounded area to aid in restoration of tissue integrity. In normal wound healing, epithelial cells surrounding a wound acquire a more mesenchymal phenotype through an incomplete version of the process of epithelial to mesenchymal transition (EMT). This more mesenchymal phenotype is characterized by a loss of cell-cell adhesion, which directly increases their motility and allows them to migrate into the wound to restore

the epithelial layer [78, 90, 96]. During EMT, epithelial cells will lose expression of the epithelial marker E-cadherin and will begin to express markers of a mesenchymal lineage, such as N-cadherin, thrombospondin, vimentin, etc. In addition, upregulation of β1 integrins permits the binding of the cells to the ECM to allow migration into the wound [96].

In normal wound healing, however, this loss of E-cadherin and subsequent increase in motility is a transient process, and following re-epithelialization of the wound the migrated cells revert back to a normal epithelial phenotype [97]. Cancerous cells also undergo EMT and gain the mesenchymal properties associated with the transition such as loss of cell-cell adhesion, increased migratory capacity, increased invasion, etc [98]. Unlike normal wound healing, however, carcinoma cells do not receive the signal to revert back to a normal epithelial phenotype. They fail to re-express E-cadherin, and their ability to metastasize is promoted by enhanced migration and invasion into nearby blood and lymphatic vessels for hematogenous and lymphatic spread. The importance of E-cadherin expression in metastatic capacity was demonstrated by Onder et al., who showed that HMLER, a tumorigenic but non-metastatic breast cancer cell line, can become invasive and highly metastatic following specific shRNA knockdown of E-cadherin [99].

As the migrating cells enter the wound and begin to proliferate to restore tissue integrity, they need a steady supply of oxygen and nutrients to fuel the process. Angiogenesis is the process of sprouting new blood vessels from already existing ones, and it is crucial to the wound healing process. Arguably the most important player in the angiogenic process is Vascular Endothelial Growth Factor (VEGF), and its expression can be activated through multiple pathways, including TGFβ signaling as well as in response to hypoxia through regulation by HIF1α [56]. A common feature between cancer and wound healing is increased expression of VEGF; just as newly forming granulation tissue needs a blood supply, growing tumor tissue is also limited by the extent of its available blood supply [100]. *In vivo*

studies demonstrated the importance of VEGF in wound repair through specific deletion of the VEGF gene in a mouse model of skin carcinogenesis. Following deletion of VEGF, deficient wound repair was observed; interestingly, the mice also demonstrated a resistance to the development of chemically induced skin cancer [101]. In contrast, overexpression of VEGF in mice accelerated the development of skin carcinoma [102]. In both animal models and human cancer tissues, it has been shown that VEGF is upregulated at both the mRNA and protein levels [103, 104]. During wound repair, there is a careful balance of pro- and anti-angiogenic factors at work. While new blood vessels are needed, the scales are tipped towards the pro-angiogenic factors. Following completion of the repair, however, the anti-angiogenic factors, such as Thrombospondin-1 (TSP1) and the chemokine IP10, inhibit further blood vessel formation and act to arrest the process. In cancerous tissues, it is thought that angiogenesis proceeds uninhibited by both a continuous overexpression of pro-angiogenic factors, such as VEGF, as well as a disparate underexpression of anti-angiogenic factors [104]. Understanding the mechanisms by which angiogenesis occurs in cancer, as well as how the balance between pro- and anti-angiogenic factors is determined, may present viable targets for therapeutic intervention in the treatment of cancer.

### 1.4.2 COX-2: An inducible mediator of inflammation

One of the hallmark enzymes involved in inflammation is cyclooxygenase-2 (COX-2), an inducible isoform of an enzyme family responsible for the biosynthesis of prostaglandins and other prostanoids. COX-2 catalyzes the oxidation of arachidonic acid (AA) in a stereospecific manner into active mediators of inflammation, such as prostaglandin E2 (PGE2) and thromboxane (TXA) [105]. Figure 6 shows a simple schematic of arachidonic acid metabolism. During the 2-step oxidation process of AA, ROS are generated as a byproduct [106]. In primary cortical neurons, superoxide generated by COX-2 is responsible for increasing neuronal susceptibility to iron toxicity. This increase in toxicity can be reversed by co-addition of antioxidants or by pharmacological inhibition of COX-2 [107]. In the prostate, ROS is known to be

actively involved in stimulating EMT in cancer cells, and recent work by Giannoni et al. has shown that COX-2 is the primary source of ROS during CAF-mediated EMT in PCa cell lines. Again, this effect can be inhibited by either co-administration of antioxidants or by selective inhibition of COX-2 [108]. While oxidative stress signaling is clearly underlying these effects, in general antioxidants are less effective in blocking or reversing prostate pathology than inhibition of ROS generation at the source [109].

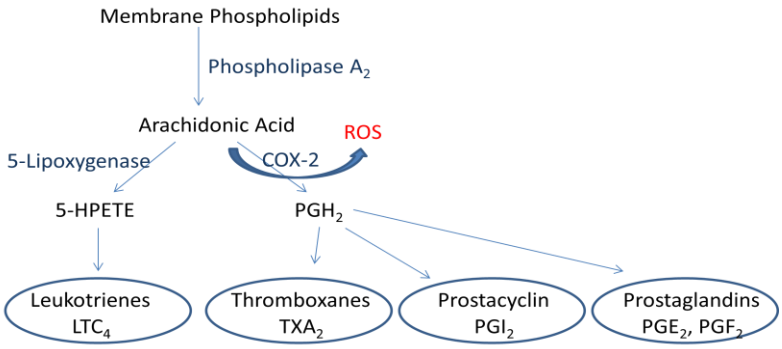



**Figure 6. Schematic of arachidonic acid metabolism.** Membrane phospholipids are liberated from the plasma membrane via the action of phospholipase $A_2$ to provide arachidonic acid (AA). AA can be metabolized either by 5-lipoxygenase or cyclooxygenase (COX). As a byproduct of the enzymatic activity of COX-2, ROS are produced.

Constitutively high levels of COX-2 are linked to tumorigenesis, decreased apoptosis, and cell-cycle dysregulation, thus underscoring the importance of tightly controlling COX-2 expression and activity [106]. Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and ibuprofen are pharmacological inhibitors of the COX enzymes, and given the inducible nature of COX-2 in inflammation, specific COX-2 inhibitors have since been developed and approved for use [110]. There is epidemiological evidence to suggest that long-term NSAID use decreases the risk of colon cancer [111], but in prostate cancer results remain inconclusive [112, 113].

*In vitro* analysis of PCa cell lines demonstrates a persistently elevated level of COX-2 expression, and this has been verified by tissue staining of PCa specimens showing elevated COX-2 presence in cancerous versus normal samples [86, 106]. Treatment of PC3 tumor-bearing mice with a selective COX-2 inhibitor significantly reduces the tumor burden, indicating that high COX-2 expression by cancer cells may support continued proliferation [106]. Interestingly, however, it is not just COX-2 in the cancer cells that is necessary for its role in carcinogenesis. Xenografts of Lewis lung carcinoma cells into COX-2 null mice are unable to form tumors despite the presence of COX-2 in the cancer cells themselves. It was later demonstrated that stromal COX-2 is necessary for the production of the potent angiogenic factor, VEGF, and thus is an essential component to tumorigenesis [114]. Studies done on laryngeal squamous cell carcinoma have correlated stromal COX-2 expression with a worse cancer grade [115]. Clearly, COX-2 plays a vital role in the development and progression of cancer. Specifically, stromal COX-2 is necessary for tumorigenesis and, as such, presents a viable target for therapeutic intervention.

## 1.5 Goals of Dissertation

The goals of this project are all directed at furthering our understanding of how the stromal microenvironment influences prostate cancer cell motility. I hypothesize that the prostatic stroma, irrespective of its activation state, maintains an intrinsic ability to limit cancer cell motility. However, cancer cells subvert this effect by increasing ROS production in neighboring myofibroblasts. In testing this hypothesis, I have pursued 3 main goals:

1. To determine how a differential stromal response to locally produced TGFβ determines the redox status of the microenvironment
2. To understand how the redox status of the local milieu directly affects the cancer cells' ability to respond to stromal-produced paracrine factors

3. To identify the mediator responsible for the inherent ability of reactive prostate stromal cells to limit PCa cell motility in the absence of oxidative stress signaling

The delicate interplay of all of these factors undoubtedly contributes to the complicated nature of therapy-resistant prostate cancer. To date, no one has suggested that a phenotypically transdifferentiated stroma maintains its inherent inhibition of PCa cell motility; ***this project addresses the novel concept that the prostatic stroma, irrespective of its "activation state," maintains an intrinsic capacity to limit cancer cell motility through a local endocrine network . Adjacent cancer cells however, subvert this network through enhancement of ROS generation in the stromal cells. Thus, future therapeutic interventions should consider the gene set signature of both the cancer and surrounding microenvironment, as the balance of paracrine mediators between the two may ultimately determine the potency and efficacy of a drug for the treatment of PCa.***

# 2.0 MATERIALS AND METHODS

## 2.1 Chemicals and Reagents

Recombinant human TGF-β1 and TGF-β1 neutralizing antibody were purchased from R&D systems (Minneapolis. MN) and were reconstituted according to the manufacturer's protocol. PGE2 and catalase were purchased from Sigma-Aldrich (St. Louis, MO). Amicon Ultra-4 centrifugal filter devices for fractionating conditioned media were purchased from Millipore (Billerica, MA). 4-hydroxytamoxifen and PHTPP were purchased from Tocris Bioscience (Ellisville, MO). 3β-Adiol was purchased from Sigma-Aldrich (St. Louis, MO). Antibodies against ERβ were purchased from Millipore (Billerica, MA), Genetex (Irvine, CA), and Calbiochem (San Diego, CA). An additional ERβ antibody was a gift from the lab of Benita Katzenellenbogen (University of Illinois, Urbana) [116]. Antibodies against E-cadherin, Smad 2/3 and Smad 4 were purchased from Cell Signaling Technology (Danvers, MA). Fluorophore-tagged secondary antibody was purchased from Molecular Probes (Eugene, OR). Antibody against β-actin and secondary HRP-conjugated antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). HTS Transwell 24-well plate inserts (3.0 μm pore size) were purchased from Corning Incorporated (Corning, NY).

## 2.2 Cell culture

WPMY-1, PS30, and DU145 cell lines are commercially available and were purchased from American Type Culture Collection (Rockville, MD). SH4 cells were generated as described below. Primary cultures PC116118 and PC115116 were obtained from radical prostatectomy specimens through UPCI. The cells were maintained in monolayer at 37$^{o}$C in a 5% $CO_2$ incubator in RPMI-1640 supplemented with 5% FBS and 1% penicillin/streptomycin. Cells were routinely passaged at a confluence of ~90%.

## 2.3 Transient Transfection

Cells were transfected using Lipofectamine LTX with Plus Reagent (Invitrogen, Carlsbad, CA). WPMY-1 and PS30 cells were plated at ~70% confluency in a 12-well plate and grown overnight in antibiotic-free RPMI-1640 media with 5% FBS. The following day, per well amounts of 0.5 µg 3TP-lux, 0.1 µg Renilla-luc, and 0.5 µL Plus reagent were incubated in opti-mem for 5 min. 1.5 µL LTX reagent was added, and the complex was incubated at room temperature for 30 min. The mixture was then added to the cells dropwise and incubated overnight. The following day cells were serum-starved for ~2h followed by treatment with TGF-β1 (0, 1, 2 ,5, 10 ng/mL). Cells were lysed in passive lysis buffer (Promega, Madison, WI), and the dual-injector luminometer Lumat LB 9507 (Berthold Technologies, Wildbad, Germany) was used to record both firefly and Renilla luciferase relative light unit (RLU) values. Firefly luciferase values were normalized to Renilla.

## 2.4 Western blotting

Whole cell lysates were prepared by lysing WPMY-1 cells in RIPA buffer (50mM Tris,150 mM NaCl, 0.1 % SDS, 0.5% Na Deoxycholate, 1 % Triton X 100). 15 µg total protein was run on a 10% acrylamide gel and transferred to a nitrocellulose membrane using a Transblot SD Semi-Dry transfer apparatus (Bio-Rad, Hercules, CA). Membranes were blocked 1h room temperature in 5% non-fat dry milk in PBS containing 0.1% tween (PBS-T). The indicated antibody was added to a solution of 5% BSA/PBS-T in a concentration of 1:1000 and incubated overnight at $4^{o}$ with gentle rocking. Membranes were washed for 5 min 3x in PBS-T and then incubated with HRP-conjugated secondary antibody in a concentration of 1:3000 for 30 min at room temp. Membranes were washed in PBS-T an additional 3 times, and enhanced chemiluminescence reagents were used to detect the HRP signal.

Fractionation of lysates into nuclear and cytoplasmic fractions was carried out using according to the following protocol. Briefly, cells were trypsinzed and centrifuged at 1000 rpm for 4 min. The pellet was

washed once with PBS and re-pelleted. It was re-suspended in buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, protease inhibitors) and incubated on ice for 5 min. Cells were lysed using a pre-chilled Dounce homogenizer. Dounced cells were centrifuged at 1000 rpm for 4 min to pellet nuclei, and the supernatant was retained as the cytoplasmic fraction. The nuclear pellet was resuspended in S1 solution (0.25M sucrose, 10 mM $MgCl_2$, protease inhibitors), and this was layered over an equal volume of S3 solution (0.88M sucrose, 0.5 mM $MgCl_2$, protease inhibitors). The suspension was centrifuged at 3500 rpm for 10 min at 4$^{o}$C. To prepare the cytoplasmic lysate, 1/5 total volume of 5X RIPA buffer was added and the samples were centrifuged at 3500 rpm for 10 min at 4$^{o}$C. The nuclear pellet was resuspended in 1X RIPA buffer and centrifuged at 3500 rpm for 10 min at 4$^{o}$C. The separate nuclear and cytoplasmic fractions were then subjected to a western blot as detailed above.

## 2.5 Immunofluorescent staining

DU145 cells were plated on coverslips at ~50% confluency and grown overnight. The following day the media was changed to either fresh 1% serum media or WPMY-1 CM and the cells were grown an additional 24h. Cells were then washed once in PBS and fixed to the slides using a 4% paraformaldehyde solution, followed by permeabilization with PBS/Triton-X (0.1%). Two additional PBS washes were performed, and then the cells were blocked using Superblock (ScyTec Laboratories, Logan, UT). Cells were washed twice with PBS and then incubated overnight at 4$^{o}$ with E-cadherin antibody in PBS (1:1000). The following day additional PBS washes were performed, a FITC-conjugated secondary antibody (1:300) and DAPI nuclear stain (1:10,000) were added, and coverslips were mounted on slides using Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

## 2.6 Generation of stable knockdown lines

A lentivirus set containing 5 unique shRNA sequences specific for Cox-2 (Homo sapiens prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (PTGS2)) was purchased

from UPCI Lentiviral Core Facility (Pittsburgh, PA). WPMY-1 cells were seeded at 50% confluency in Optimem and grown overnight. The following day the media was replaced with fresh Optimem containing lentivirus and a final concentration of 8μg/mL polybrene. Cells were incubated ~18h at 37$^{o}$C, and the following day the media was replaced with fresh RPMI-1640 containing 10% FBS. On day 3 after the infection, cells were passaged through a selection media containing 2 μg/mL puromycin for 5 days. Viable cells were expanded and a quantitative real-time PCR experiment was performed to confirm knockdown of COX-2 mRNA. The cell population demonstrating the greatest viability and knockdown were named SH4.

## 2.7 Indirect co-culture wound healing assay

Prostate stromal cells were plated in 6-well dishes at a density of 3X10$^{5}$ cells/well and grown overnight. Prostate epithelial tumor cells DU145 were seeded on coverslips in 6-well plates at a density of 2.5X10$^{5}$ cells/well and grown overnight. The following day, the stromal cells were placed in serum free media (RPMI-1640 + 1%pen/strep) for ~2 hrs. The epithelial cells were scratched using a 200 μL pipette tip and the coverslip was then transferred cell-side up to the stromal cell containing well. The media was immediately replaced with RPMI-1640 + 1% FBS + 1% pen/strep and, when indicated, catalase was used at a final amount of 1500 units/mL. The wound was imaged at time zero. The co-culture system was incubated and the same areas were then imaged at time 24 hrs. The denuded zone was measured at time zero and again at time 24 hrs, and the percent wound closure was calculated by subtracting the 0 hr wound size from the 24 hr wound size and multiplying by 100. TGF-β1 neutralizing antibody was used at a final concentration of 10 μg/mL.

## 2.8 Conditioned Media

Stromal cells were plated at the same density as the indirect co-culture assay and grown overnight. The following day they were serum starved for ~2 hrs and then the media was replaced with fresh media

containing 1% serum. Where indicated, exogenous TGF-β1 was added when indicated at a final concentration of 5 ng/mL. The cells were incubated overnight and the media was collected the following day, centrifuged at 1500xg for 3 minutes, and stored at -20$^{o}$C. Prior to column fractionation, media was syringe filtered. The ultrafiltrate was diluted into fresh serum-free media, and the lower filtrate was used without any additional dilutions.

Conditioned media was thawed and placed on freshly wounded naïve DU145 cells, and the wounds were imaged at time zero and time 24h. Wound closure was calculated as described above. Where indicated, $H_2O_2$ was added to conditioned media at final concentrations of 5, 10, and 20 µM prior to its use in the wound healing assay.

## 2.9 RNA isolation, reverse transcription, and real-time PCR

WPMY-1 prostate stromal cells were plated in 6-well plates at a density of 2.5x10$^5$ cells/well and were grown overnight. The following day they were placed in serum-free media and were serum starved for ~2 hrs. TGF-β1 (0, 2, 5, 10 ng/mL) was then added and the cells were incubated overnight. WPMY-1 cells from co-culture were grown in the lower chamber of a transwell system and co-cultured in 1% FBS RPMI-1640 media for 24 hrs with a DU145 containing insert. The following day the media was removed, the cells were washed in sterile 1x PBS, and harvested in 500 µL cold Trizol. RNA was extracted using the RNeasy kit from Qiagen (Valencia, CA) and was quantified on the Nanodrop ND-1000. cDNA was synthesized using the iScript kit from Bio-Rad (Hercules, CA) according to the kit protocol, and the final product was diluted to a total volume of 100µL using nuclease-free water. Quantitative real-time PCR was performed using the iTaq Sybr green kit from Bio-Rad (Hercules, CA). Table 1 shows the primer sequences that were used. Samples were run through an initial denaturation step of 95$^{o}$ for 10 min followed by 40 cycles of 95$^{o}$ for 30s, 55$^{o}$ for 1 min, and 72$^{o}$ for 1 min. Relative expression was determined using the comparative Ct method.

**Table 1. Primer sequences used in qRT-PCR**

| Gene | Forward | Reverse |
|---|---|---|
| Cox-2 | 5'-ATCACAGGCTTCCATTGACC-3' | 5'-CAGGATAGAGCTCCACAGCA-3' |
| E-cadherin | 5'-TGAAGGTGACAGAGCCTCTGGAT-3' | 5'-TGGGTGAATTCGGGCTTGTT-3' |
| GAPDH | 5'-TTGCCATCAATGACCCCTTCA-3' | 5'-CGCCCCACTTGATTTTGGA-3' |

## 2.10 Chromatin Immunoprecipitation (ChIP)

DU145 cells were grown to ~80% confluence on 15cm plates and treated for 18h with either control or WPMY-1 CM -/+ $H_2O_2$ (10μM). The following day, media was replaced with fresh complete growth medium (5% FBS) containing 1% formaldehyde for cross-linking. Cells were incubated at 37$^o$ for 20 min, followed by addition of glycine and an additional 10 min incubation at room temperature. Cells were washed once in 1x PBS, then collected in ice-cold 1x PBS containing protease inhibitors. Cells were pelleted at 2000 rpm for 10 minutes at 4$^o$. PBS was discarded and cells were resuspended in lysis buffer containing protease inhibitors. Following a 15 min incubation on ice, cells were sonicated at maximum setting using a bioruptor with 30 sec on/off pulses for 10 min. Cells were incubated on ice 5 min, and an additional 5 min of sonication was performed. A DNA gel was run to ensure adequate shearing of the chromatin to approximately 1 kb fragments. Sheared chromatin was diluted in ChIP dilution buffer containing protease inhibitors to a final concentration of 250 μg/mL. Antibodies were linked to anti-mouse IgG magnetic beads in low-salt immune complex buffer for 6 hrs at 4$^o$. Anti-ERβ antibodies were used at a final concentration of 2 μg/mL. During antibody linking, chromatin samples were precleared using fresh magnetic beads, and a portion of this precleared sample was used as input sample. The

remaining precleared chromatin was then incubated overnight at 4$^{o}$ with the antibody-linked beads. The following day, beads were washed for 15 min 1x each with low salt immune complex buffer, high salt immune complex buffer, LiCl immune complex buffer, and 1x TE buffer. After the final wash, beads were resuspended in 150 µL TE for a final rinse before addition of 400 µL elution buffer. Proteinase K (5 µL) was added to each sample and they were incubated overnight at 65$^{o}$. The following day DNA was extracted using phenol:chloroform:isoamyl alcohol (25:24:1). Glycogen (2 µL) and sodium acetate (35 µL) were added and samples were vortexed, followed by addition of 800 µL ice-cold ethanol. Samples were incubated at -20$^{o}$ 1-2 hrs and then centrifuged at 13,000 rpm for 60 min at 4$^{o}$. Pellet was washed once with 70% EtOH, respun, and pellet was allowed to air dry on ice for 2 hrs. Pellet was resuspended in 30 µL RNase-free water, and the DNA was then subjected to quantitative real-time PCR analysis (described above). Table 2 below lists the primers used for 3 different ERβ binding sites within the E-cadherin promoter. RT-PCR results were calculated using the $\Delta\Delta C_t$ method and are presented as assay site IP fold enrichment.

**Table 2. Primer sequences for ERβ binding sites within the E-cadherin promoter**

| Site | Forward | Reverse |
|---|---|---|
| 1 | 5'-GACCTGAGACCTTTGGCCCCTA-3' | 5'-TATCTCCTCTTGGCGAACTTGG-3' |
| 2 | 5'-CAATCAGCGGTACGGGGGGCGG-3' | 5'-GGTTCTTTCCAGCATTTATCCT-3' |
| 3 | 5'-CCGGTTCCATCTACCTTTCCCC-3' | 5'-GAACCAATGACCCGACACCGAA-3' |

## 2.11 Measurement of ROS Production

WPMY-1 cells were plated at a density of 3000 cells/well in black-walled clear bottom 96-well tissue culture plates (Greiner Bio-One, Radnor, PA) in phenol-red free RPMI-1640 containing 5% FBS. The

following day, cells were serum starved for ~90 min and TGFβ was added in fresh serum-free media. The cells were incubated for 3h and $H_2O_2$ production was measured using an Amplex Red Enzyme Assay (Invitrogen, Carlsbad, CA).

## 2.12 Imaging and Densitometric Analysis

All images were obtained on an Olympus IX71 inverted microscope (Olympus, Center Valley, PA). Western blot films were electronically scanned and the images were analyzed using Image J software (open sourced through the NIH).

## 2.13 Statistical Analysis

Two-sample comparisons were performed using the Student *t* test. Multiple comparisons were performed using a one-way ANOVA followed by the Tukey test or a two-way ANOVA followed by a Bonferroni posttest. All data are represented $\pm$ SEM and are representative of ≥ 3 independent biological replicates. *p* values < .05 are considered significant.

# 3.0 RESULTS

## 3.1 TGF-β1 signaling in reactive human prostate stromal cells overrides their inherent motility inhibitory activity towards co-cultured PCa cells

It has been previously established that co-culturing non-cancerous immortalized prostate epithelial cells with cancer-associated fibroblasts (CAFs) leads to malignant transformation and tumor development in a xenograft mouse model [3, 64, 117]. To test the impact of indirect co-culture with CAFs on the motility of a prostate cancer cell line, I used a modified wound healing assay as previously described [118]. It is important to note that in this assay the stromal and cancer cells are not in direct contact, but they do share a common growth medium.

In addition to using 2 primary CAF cultures (PC116118 and PC115116), I also chose 2 established and well-characterized prostate stromal cell lines. The WPMY-1 line is myofibroblastic and thus imitates a CAF-like phenotype, whereas the PS30 line is derived from the immortalization of normal prostatic fibroblasts. The cancer cell line that I chose for my experiments is the DU145, which is a highly aggressive and motile cell line derived from a distant metastatic site. Of particular interest is that the DU145 line overproduces TGF-β1, yet itself has become immune to the growth suppressive effects TGF-β1 exerts on normal prostatic epithelium.

As Figure 7 demonstrates, both primary CAFs and the myofibroblastic cell line WPMY-1 are permissive of DU145 motility in indirect co-culture. In low-serum containing conditions, DU145 cells growing at near confluence can close a constant diameter wound approximately 22% in 24 hrs. This wound closure is not significantly altered by co-culture with either CAFs or WPMY-1 cells, but it is significantly inhibited by the presence of PS30 cells. To exclude the influence of proliferation on this wound closing effect, the doubling time of DU145 cells under these same low-serum conditions was

determined to be approximately 40 hrs. Thus, at 24 hrs the most significant contributor to wound closure is the movement of existing cells into the denuded zone.

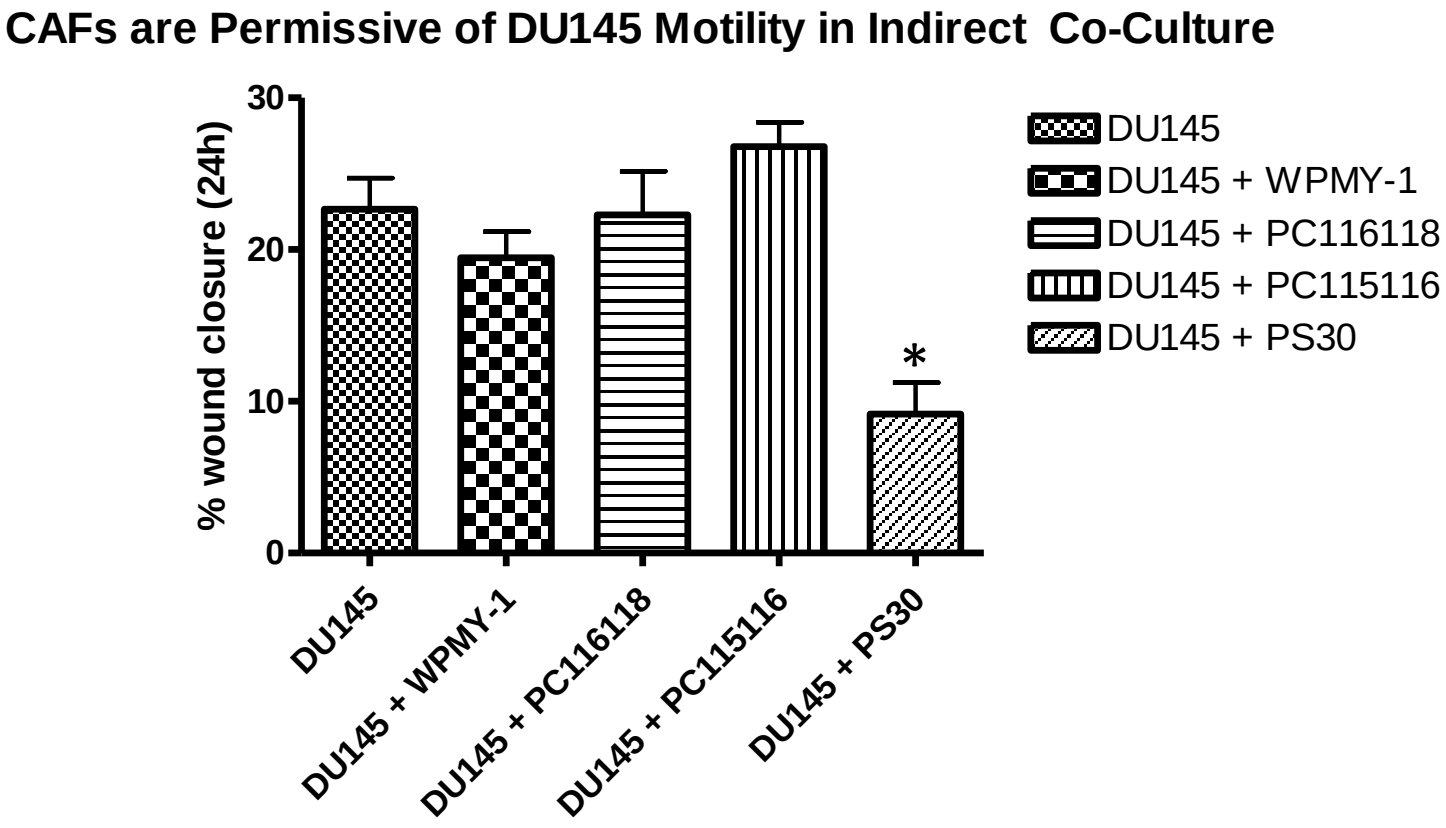



**Figure 7**. **Indirect co-culture with CAFs and WPMY-1 cells is permissive of inherent DU145 motility**

In a modified wound healing assay, DU145 motility is unaffected by the presence of CAFs (PC116118 and PC115116) or WPMY-1 cells, but is significantly reduced by PS30 cells. Data represent the mean ± SEM from 3 independent experiments, each repeated in technical triplicate. A one-way ANOVA followed by Tukey's multiple comparison test was performed.*p<.05 compared to respective DU145 control.

Since DU145 cells secrete high levels of active TGF-β1, I hypothesized that the discrepancy of myofibroblastic and fibroblastic stromal cells on DU145 motility was due to a differential response to locally produced TGF-β1. To assess if there was an inherent difference in response to TGF-β1, the WPMY-1 and PS30 lines were employed as models to represent a myofibroblast and a fibroblast, respectively, and a measurement of the transcriptional activity of a TGFβ-response element (3TP-lux) following stimulation with exogenous TGF-β1 was performed. WPMY-1 and PS30 cells were transiently co-transfected with the Smad-binding element luciferase construct (3TP-lux) and Renilla luciferase (Renilla-luc). The cells were then treated with various concentrations of TGF-β1, and cell extracts were analyzed for luciferase activity. Figure 8 demonstrates that at all concentrations of TGF-β1, WPMY-1 cells exhibit a significantly more robust transcriptional response than the PS30 cells.

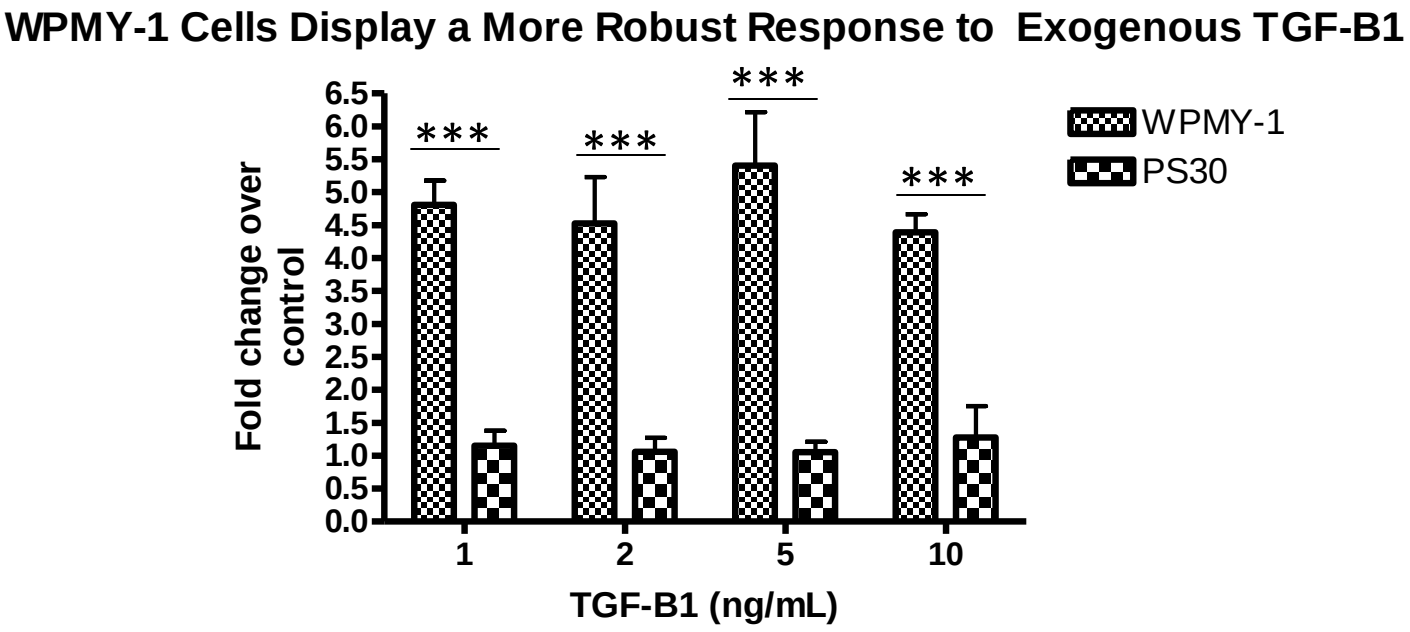
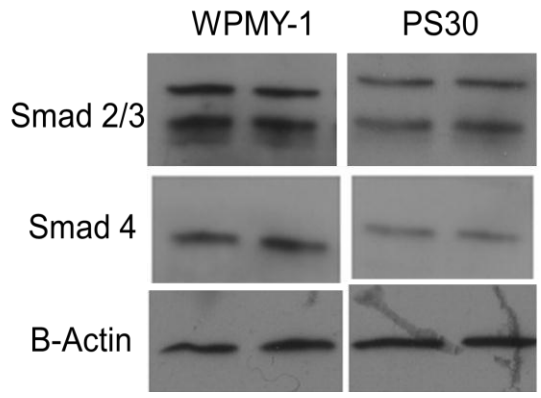



**Figure 8**. **WPMY-1 cells exhibit a more robust transcriptional response to exogenous TGF-β1 treatment.**

WPMY-1 and PS30 cells were transiently co-transfected with the 3TP-lux-luciferase (luc) reporter and Renilla-luc overnight, then subjected to a 6h TGF-β1 treatment the following day (0, 1 , 2, 5 10 ng/mL). Cells were lysed and firefly luc activity was measured and normalized to Renilla luc activity. Data represent the mean ± SEM of 3 independent experiments, each performed in triplicate. A two-way ANOVA followed by Bonferroni posttest was performed. ***p<.001

To further test whether WPMY-1 cells possess a more active TGFβ response pathway, a western blot analysis of whole cell lysates was performed to determine the level of Smad proteins in WPMY-1 and PS30 cells. Figure 9a is a representative blot of 2 independent samples from each cell type. Densitometric analysis of the blots reveals that WPMY-1 cells contain significantly more Smad 3 and Smad 4 protein than PS30 cells (Figure 9b). This is in accordance with the observation that WPMY-1 cells have a more robust response to TGF-β1.



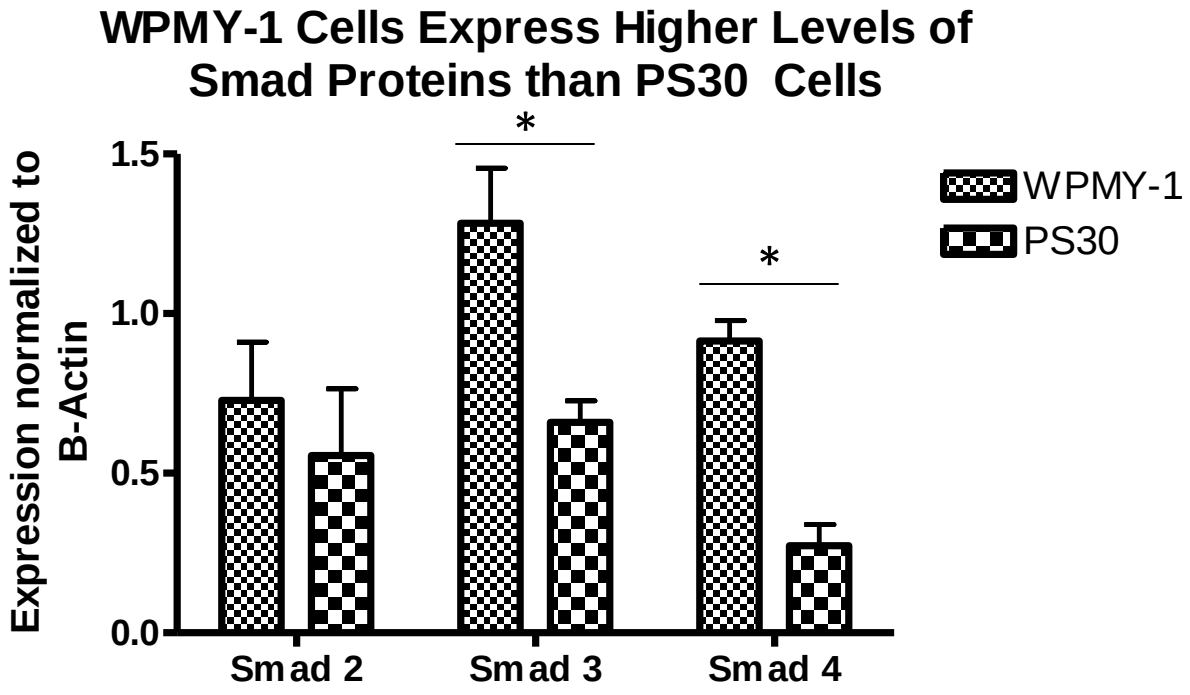
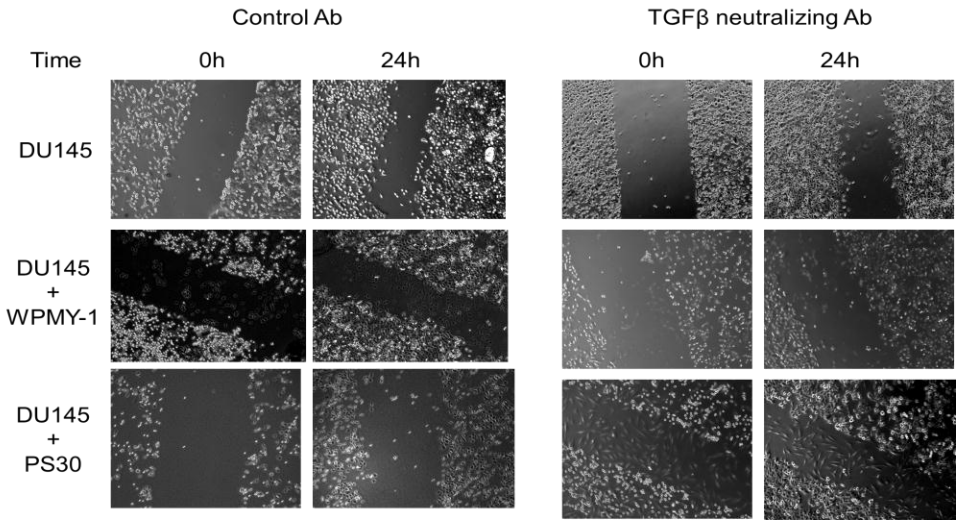




**Figure 9**. **WPMY-1 cells express higher levels of Smads 3&4**

Untreated WPMY-1 and PS30 cells were lysed in cold RIPA buffer and subjected to western blot analysis for Smads 2,3, and 4. (A) A representative blot is shown, and (B) a graphical display of densitometric analysis representing the mean ± SEM of 3 independent experiments is presented. A 2-way ANOVA followed by a Bonferroni posttest was performed. *p<.05

To uncover the role of TGFβ signaling in modulating stromal cell regulation of PCa cell motility, an interfering TGF-β1 antibody was used in co-culture assays. The upper panel (Figure 10a) shows representative images from the wound healing assay taken at zero and twenty-four hours. Quantitation of multiple repeats is shown in graphical form in Figure 10b, and these results demonstrate that inhibition of TGFβ signaling did not affect the inherent motility of DU145 cells but uncovered an inherent motility inhibitory activity of the WPMY-1 cells. The motility inhibitory activity of the PS30 cells was not affected by the TGF-β1 neutralizing antibody. These results suggest that while TGF-β1 does not affect DU145 cell movement, it is required for reactive stromal cells to obtain their permissive effect on cancer cell motility. Additionally, these data suggest that while both reactive and non-reactive prostate stromal cells produce an inherent cancer cell motility inhibitory factor (hereafter referred to as stromal-derived motility inhibitory factor, SMIF), reactive stroma respond to TGF-β1 produced by cancer cells to limit either the production or activity of SMIF.



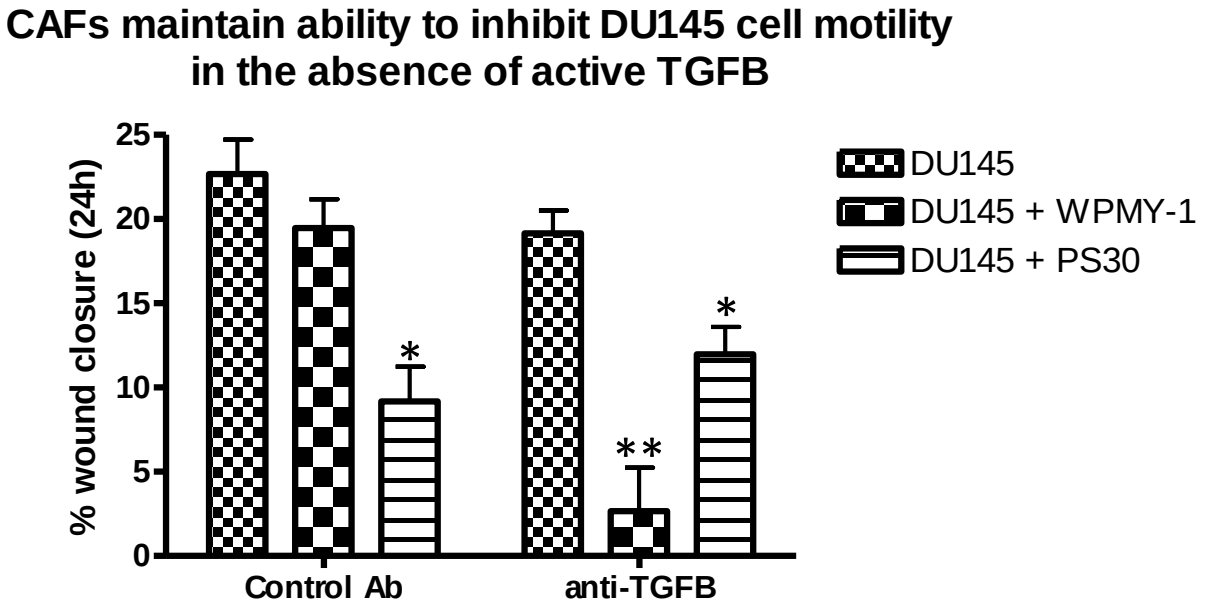




**Figure 10. Locally produced TGF-β1 is necessary for the permissive effect of WPMY-1 cells on DU145 motility.**

WPMY-1 cells are permissive of the highly motile phenotype of DU145 PCa cells; PS30 cells inhibit DU145 motility in co-culture. Addition of a TGFβ1-neutralizing antibody blocks the permissive effects of co-cultured WPMY-1 cells on PCa cell motility, but does not affect motility when added to DU145 cells alone or in co-culture with PS30 cells. (A) The upper panel is representative images from the modified wound healing assay. (B) Data represent the mean ± SEM from 4 independent experiments, each repeated in technical triplicate. A one-way ANOVA followed by Tukey's multiple comparison test was performed.*p<.05 compared to respective DU145 control. ** p< .01 relative to DU145 treated with TGFβ neutralizing antibody.

As a direct test to determine if TGF-β1 limits the production of SMIF, conditioned media (CM) was isolated from WPMY-1 cells grown overnight in 1% serum-containing media with or without exogenous TGF-β1 (5 ng/mL) and added to freshly wounded naïve DU145 cells. Surprisingly, CM from WPMY-1 cells treated with exogenous TGF-β1 significantly inhibited DU145 motility (Figure 11). Thus, TGF-β1 does not inhibit the production of SMIF, but rather blocks its activity when present in co-culture. Because the inhibitory activity is transferrable in CM, the effect of TGF-β1 on SMIF activity is likely mediated through a short-lived molecule.

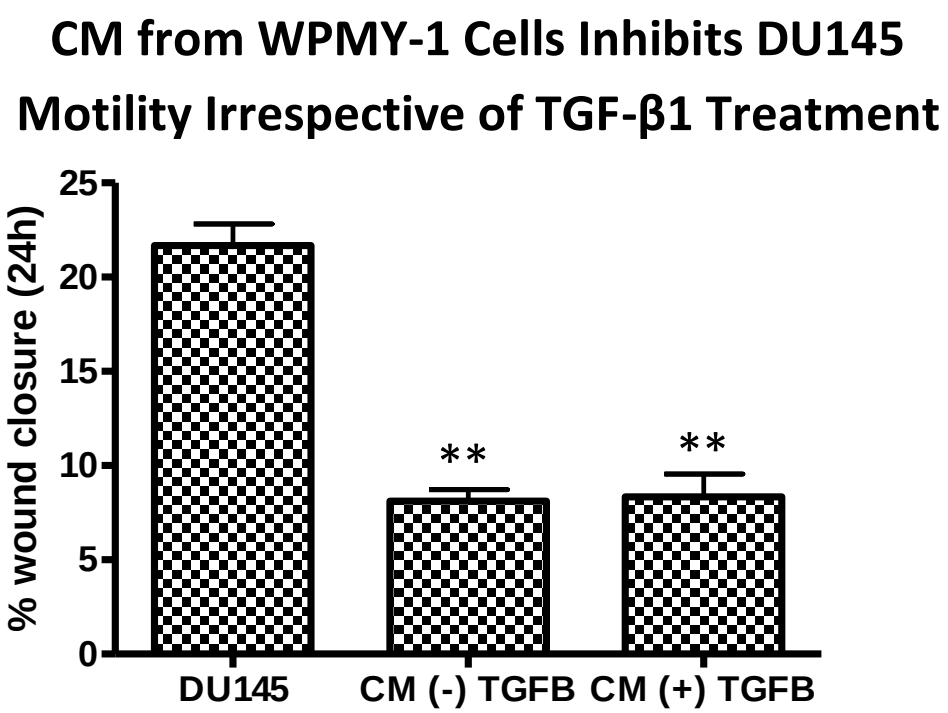
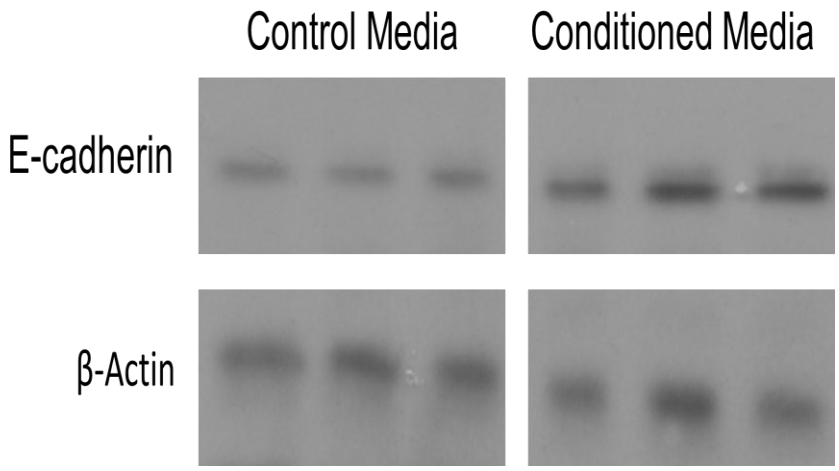



**Figure 11. Inhibition of DU145 motility is transferrable in WPMY-1 CM irrespective of TGF-β1 treatment**

WPMY-1 cell CM was obtained following overnight growth in 1% serum media -/+ TGF-β1 (5 ng/mL). Naïve DU145 cells were wounded and media replaced with WPMY-1 CM. Wound closure over 24h was determined. Data represent the mean ± SEM from 3 independent experiments, each repeated in technical triplicate. A one-way ANOVA followed by Tukey's multiple comparison test was performed. ** $p < .01$ compared to control media

As an additional test of the effect of CM on DU145 motility, naïve DU145 cells were treated with either control or WPMY-1 CM and then subjected to Western blot analysis for the adhesion molecule E-cadherin. PCa cells undergo full or partial epithelial-to-mesenchymal transition (EMT) and thus lose expression of the epithelial marker E-cadherin. A loss of E-cadherin is associated with a decrease in cell adhesion and a subsequent increase in motility. As Figure 12 shows, CM significantly enhanced expression of E-cadherin, consistent with partial reversal of EMT and hence a decrease in cell motility. Additionally, immunoflourescence staining for E-cadherin was also performed. Figure 13 displays representative images from immunofluorescent staining showing increased E-cadherin staining along the cell membrane in DU145 cells following overnight incubation with CM.



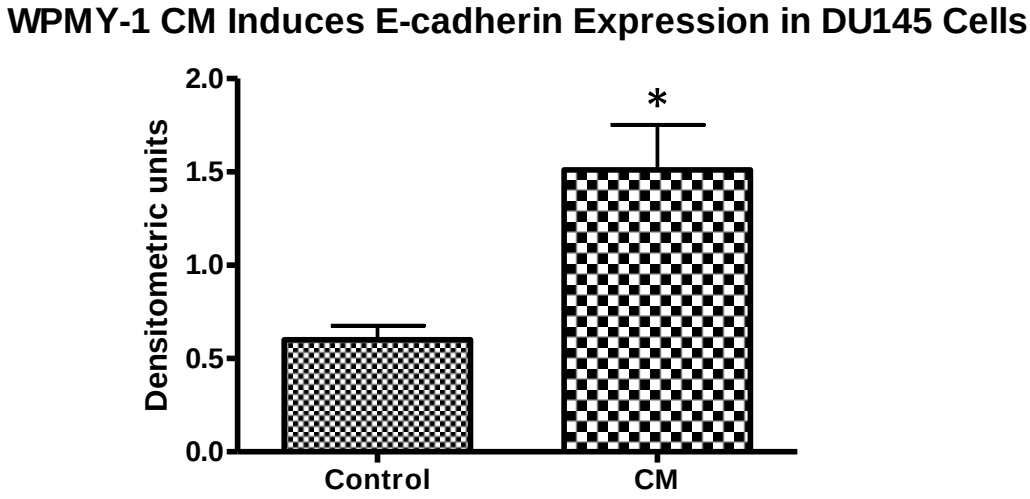




**Figure 12**. **WPMY-1 CM induces E-cadherin expression in DU145 cells**

Western blot of whole cell lysate from DU145 cells cultured in WPMY-1 CM for 24 hrs reveals a significant increase in protein levels of the cell adhesion molecule E-cadherin. (A) The upper panel is a blot representative of 3 biological replicates (B) A graphical summary of the densitometric analysis of E-cadherin expression normalized to β-actin used as a loading control. Bars represent the mean of 3 independent experiments ± SEM. A t-test was performed to determine significance. *p<.05

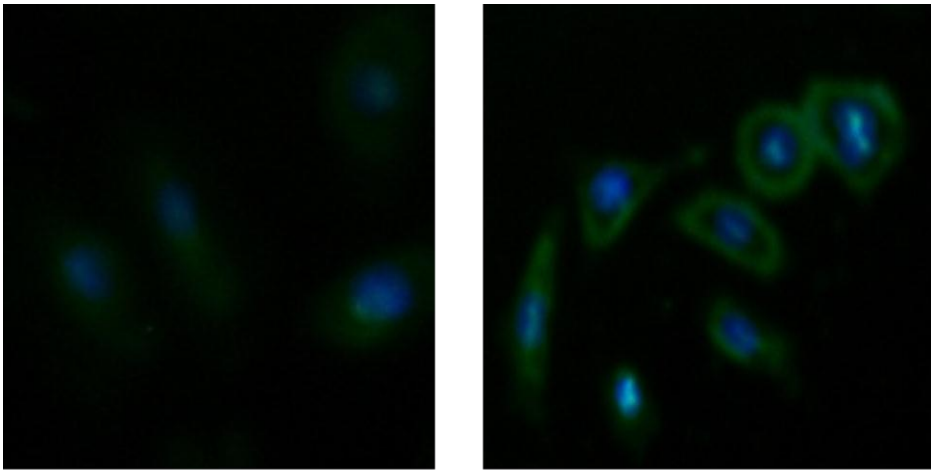



**Figure 13. WPMY-1 CM induces E-cadherin staining in DU145 cells.**

DU145 cells were grown in WPMY-1 CM for 24 hrs, fixed in 4% paraformaldehyde, and stained for E-cadherin. DAPI was used as a nuclear stain, and DAPI and FITC fluorescence were recorded and overlayed. Control cells display a diffuse cytoplasmic pattern for E-cadherin, while CM treatment results in a more intense cytoplasmic staining for E-cadherin which is concentrated at the cell membrane, consistent with increased adhesion.

## 3.2 ROS generated following TGF-β1 signaling overrides the inherent motility inhibitory activity of WPMY-1 cells

ROS are known to be active mediators of the TGFβ-driven transdifferentiation into myofibroblasts [119], and recent work has shown that expression of NADPH oxidase 4 (NOX4), an $H_2O_2$-generating enzyme, is necessary for the apoptotic effects of TGF-β1 on pulmonary epithelial cells [120]. Clearly TGFβ and ROS production are intimately related; thus, I next turned my focus to investigating ROS as a potential short-lived mediator of the TGFβ-dependent effect on SMIF activity in co-culture. Hydrogen peroxide is one ROS ultimately generated in response to TGF-β1 that can participate in local paracrine signaling. While $H_2O_2$ is not typically considered a very short-lived molecule, antioxidant defenses within the cancer cells could potentially neutralize $H_2O_2$ if it is not constituitively being produced; thus, CM may contain small amounts of $H_2O_2$, but in the absence of chronic production as in co-culture, the $H_2O_2$ is neutralized before it exerts a measurable effect on the cancer cells. To examine whether TGF-β1 activates an oxidant signaling pathway in WPMY-1 cells, an Amplex Red endpoint assay was performed to measure $H_2O_2$ production. As Figure 14 shows, a 3h TGF-β1 treatment significantly increased production of $H_2O_2$ at both 2 and 5 ng/mL doses.

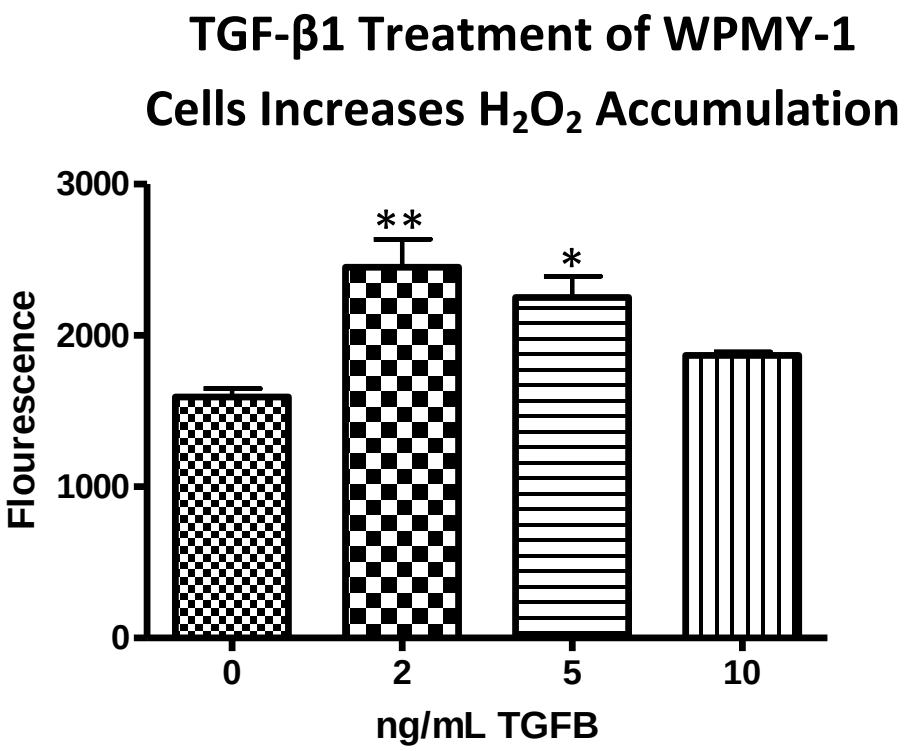



**Figure 14. Addition of TGF-β1 to WPMY-1 cells increases $H_2O_2$ production.**

In response to exogenous TGF-β1, WPMY-1 cells produce increased levels of $H_2O_2$ as measured by an endpoint Amplex Red assay. WPMY-1 cells were serum starved for 90 min before addition of TGF-β1 in fresh serum free media. Cells were incubated with the TGFβ for 3h, Amplex Red was added to cell cultures, and an endpoint fluorescence reading was recorded at 1h. Data represent mean from 3 biological replicates $\pm$ SEM. A one-way ANOVA followed by Tukey's multiple comparison test was performed. * $p<.05$, ** $p< .01$ relative to untreated WPMY-1 control

A TGF-β1-dependent increase in $H_2O_2$ suggests that it may be a viable candidate for the non-transferrable inhibitor of SMIF. Therefore, wound healing assays were performed with the addition of catalase (1500 units/mL), a cell impermeant enzyme that metabolizes $H_2O_2$ to $H_2O$ and $O_2$. I again included the primary stromal cultures to confirm that $H_2O_2$ was responsible for the permissive effect of the CAF phenotype. As Figure 15 shows, while addition of catalase did not alter the inherent highly motile phenotype of the DU145 cells, it reversed the permissive effect of WPMY-1 and CAFs and restored the activity of SMIF when added to DU145/CAF and DU145/WPMY-1 co-cultures. Therefore, extracellular $H_2O_2$ is necessary for WPMY-1 and CAFs to override the inherent activity of SMIF and support the efficient motility of co-cultured DU145 cells. Importantly, extracellular $H_2O_2$ does not regulate the inherent motility capacity of DU145 cells.

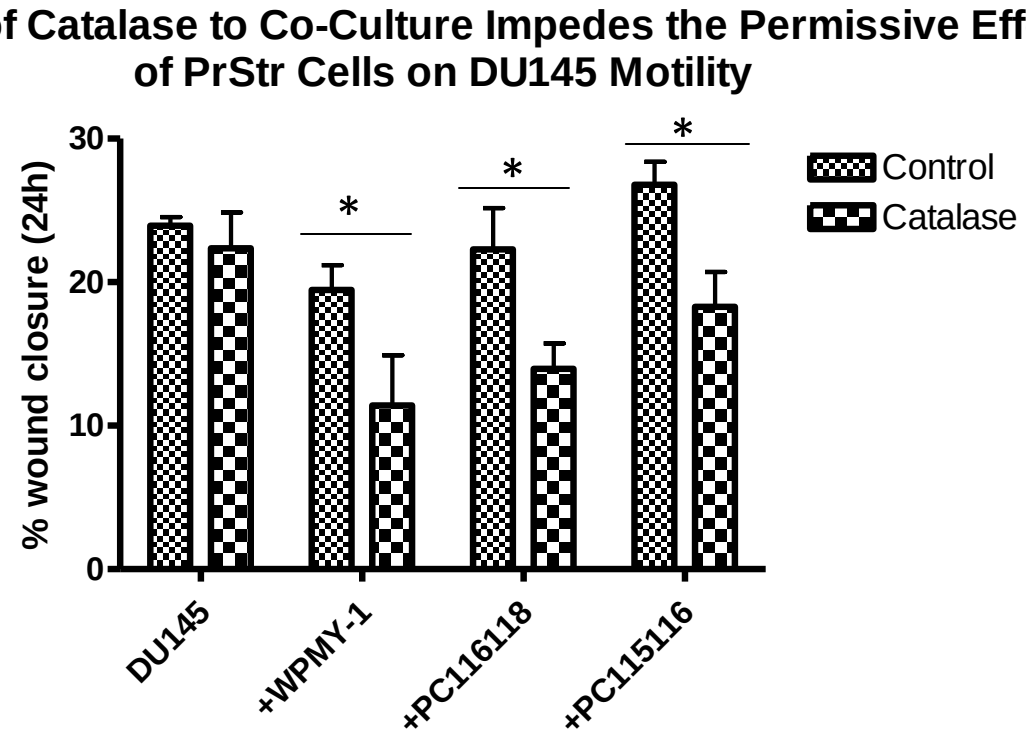



**Figure 15. Addition of catalase to co-cultures reverses the permissive role of CAFs and WPMY-1 cells on DU145 motility**

The modified wound healing assay was performed with the addition of 1500 units of catalase per 1 mL of media. Data are representative of 3 independent experiments ± SEM. A one-way ANOVA followed by Tukey's multiple comparison test was performed. * $p < 0.05$ comparing control co-culture to co-culture with the addition of catalase.

Since PS30 cells are less responsive to TGF-β1, it can be inferred that they lack the increased ROS production following TGF-β1 stimulation that is seen in the myofibroblastic WPMY-1 cells. The addition of catalase neutralized $H_2O_2$ in the CAF and myofibroblastic co-cultures and led to a restoration of SMIF activity. Thus, to further confirm a role for extracellular $H_2O_2$ as a negative regulator of SMIF activity, exogenous $H_2O_2$ (10 μM) was added to DU145/PS30 co-culture to test whether the SMIF activity was inhibited upon introduction of oxidative stress signaling. As expected, addition of $H_2O_2$ to DU145/PS30 co-culture significantly increased the motile capacity of the DU145 cells when compared to untreated co-culture conditions (Figure 16). This again suggests a crucial role for extracellular $H_2O_2$ in inhibiting the effect of SMIF on DU145 motility in co-culture.

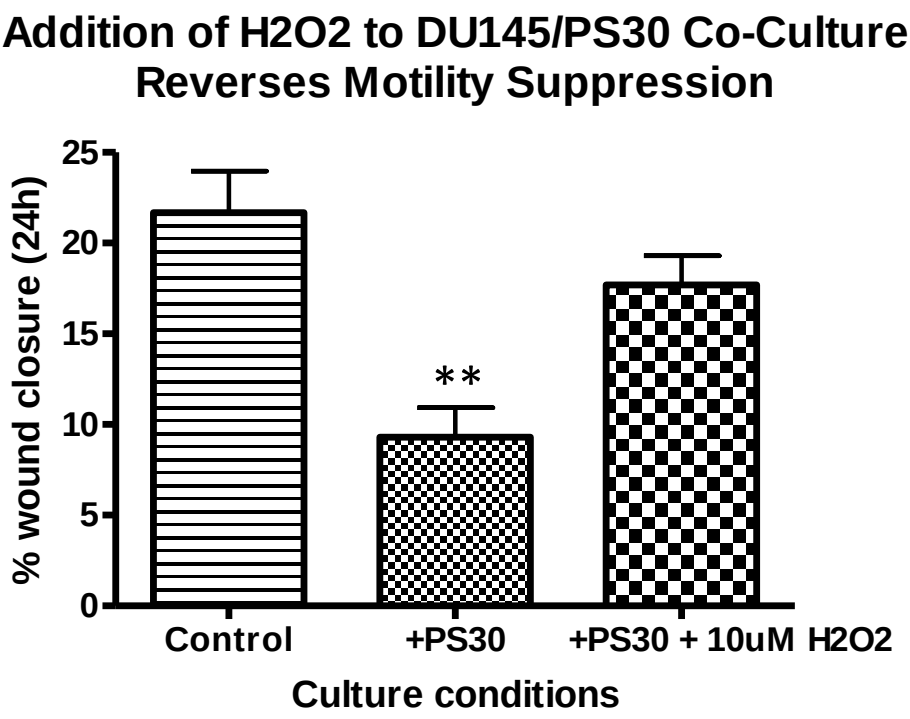



**Figure 16. Addition of $H_2O_2$ to DU145/PS30 co-culture reverses motility suppression of DU145 cells**

DU145 cells were co-cultured with PS30 cells as described previously under either control or $H_2O_2$-containing (10 μM) conditions. A modified wound healing assay was performed and wound closure over 24 hrs was calculated. Data represent mean ± SEM of 3 independent experiments. A one-way ANOVA followed by Tukey's multiple comparison test was performed. ** p<.01

## 3.3 Cox-2 is a predominant source of ROS in WPMY-1 cells

Having confirmed the role of both TGF-β1 and $H_2O_2$ in modulating the activity of SMIF in co-culture, I next sought to identify a source of ROS that is downstream of TGFβ signaling. Using a wound healing assay to assess biological function of SMIF, it was apposite to consider a variation of the normal wound healing process as underlying the observed effects thus far. In aberrant wound healing, excessive TGFβ is present [121]. Thus, in the wound healing assay, I hypothesized that the mobilization of redox signaling pathways is utilized by TGF-β1 to influence paracrine communication. That is, TGF-β1 induces the expression of enzymes such as COX-2 and some NOX isoforms that generate ROS. In DU145/WPMY-1 co-cultures, COX-2 mRNA was induced in WPMY-1 cells as revealed by quantitative real-time PCR. TGF-β1's role in this induction was confirmed by inclusion of a TGF-β1 neutralizing antibody, which abolished the COX-2 mRNA induction in co-cultured WPMY-1 cells (Figure 17).

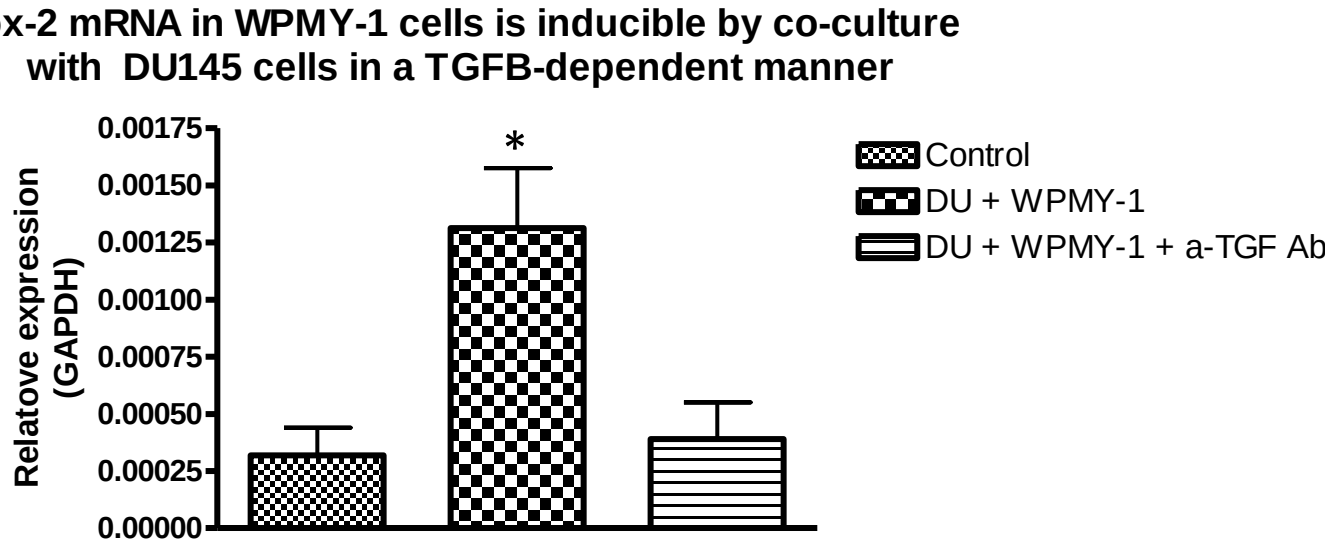



**Figure 17. Co-culture with DU145 cells increases levels of COX-2 mRNA transcript in WPMY-1 cells**

A transwell insert containing DU145 cells was placed into a chamber containing serum-starved WPMY-1 cells, and the media was replaced with fresh 1% serum-containing RPMI -/+ a TGFβ neutralizing antibody. Control cells were incubated with an empty insert. The cells were co-cultured for 24h and the WPMY-1 cells from the lower chamber were collected in Trizol and subject to reverse transcription and quantitative real-time PCR for COX-2 transcript levels. Data represent the mean ± SEM from 3 independent experiments. A one-way ANOVA followed by Tukey's multiple comparison test was performed.* $p < .05$ relative to control and TGFβ neutralizing antibody.

To determine to what extent stromal-derived COX-2 is involved in producing ROS that have an impact on cancer cell motility in co-cultures, a pharmacologic selective COX-2 inhibitor, NS398, was used in co-cultures. However, NS398 significantly decreased inherent DU145 motility due to the expression of COX-2 in the DU145 cells (data not shown). Thus, this approach proved ineffective at isolating the specific role of stromal COX-2. In order to understand the contribution of stromal COX-2, a recombinant lentiviral vector system expressing a specific COX-2 shRNA (SH4) sequence was used to generate a stable WPMY-1 variant line lacking inducible COX-2. A control vector expressing a scrambled shRNA (Scr) sequence was also stably expressed in a different variant line. This approach allowed for the selective knockdown of COX-2 in stromal cells in co-culture while leaving DU145 COX-2 unaffected. As basal levels of COX-2 mRNA are very low in WPMY-1 cells, the efficiency of the knockdown was analyzed via qRT-PCR following treatment of WPMY-1 or SH4 cells with 5 ng/mL TGF-β1. As Figure 18 shows, TGF-β1 induction of COX-2 mRNA was reduced 65% in SH4 cells relative to WPMY-1.

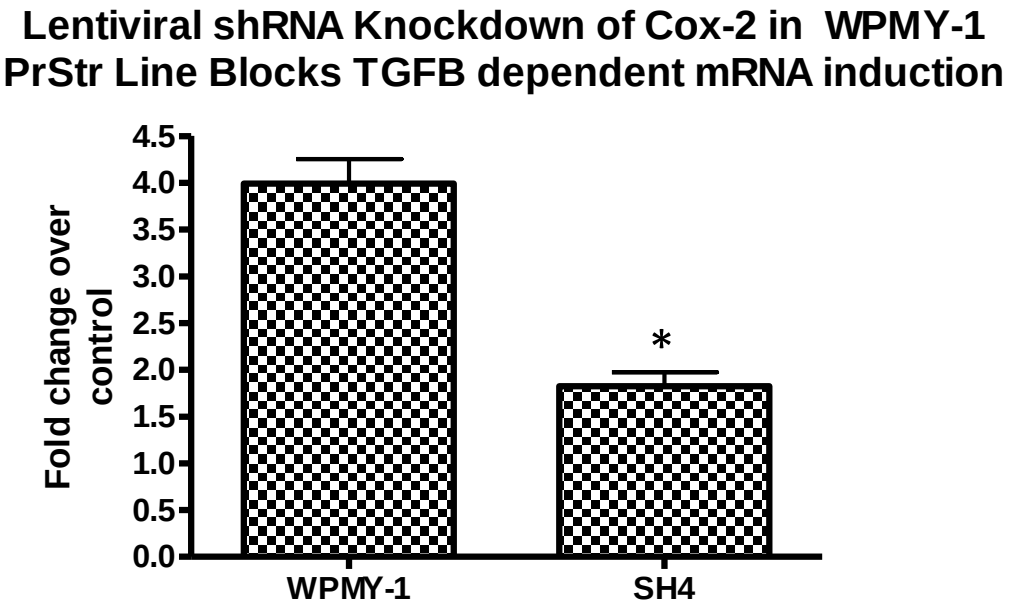



**Figure 18. Stable knockdown of COX-2 in WPMY-1 cells**

A lentiviral vector expressing shRNA directed against COX-2 (SH4) was used to stably infect WPMY-1 cells. Following selection through puromycin and clonal expansion, the resulting lines were subjected to a 24h treatment with TGFβ in serum-free media (5 ng/mL) and COX-2 mRNA levels determined by quantitative RT-PCR. Fold change over untreated controls was determined. Data represent 3 independent experiments ± SEM. A one-way ANOVA followed by Tukey's multiple comparison test was performed. *p<.05

Since extracellular $H_2O_2$ is necessary for inhibition of SMIF activity, we sought to determine if COX-2 plays a significant role in the generation of $H_2O_2$. Given that previous work has shown that COX-2 can be a significant source of ROS generation [107], SH4 cells were subjected to an endpoint Amplex Red assay identical to the one performed on WPMY-1 cells in Figure 14. As Figure 19 shows, TGF-β1 is unable to produce a significant increase in $H_2O_2$ production in WPMY-1 cells lacking inducible COX-2. This is in contrast to the wild-type WPMY-1 cells, which respond to TGF-β1 with a significant increase in $H_2O_2$ production (see Figure 14). This suggests that COX-2 is at least partially responsible for the TGF-β1 induced increase in $H_2O_2$ production.

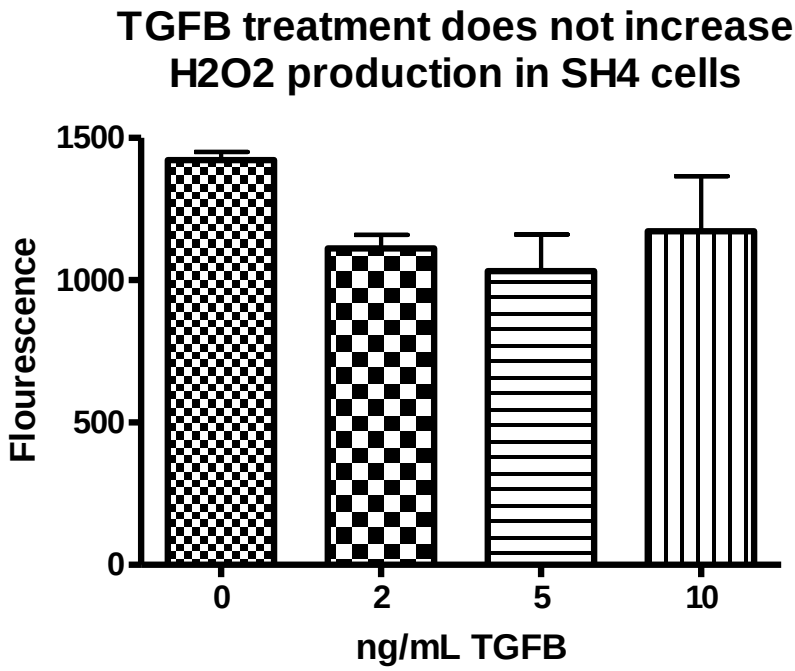



**Figure 19. TGF-β1 does not induce $H_2O_2$ production in SH4 cells**

In response to exogenous TGF-β1, SH4 cells lack an increase in $H_2O_2$ production as measured by an endpoint Amplex Red assay. SH4 cells were serum starved for 90 min before addition of TGF-β1 in fresh serum free media. Cells were incubated with the TGFβ for 3h, Amplex Red was added to co-cultures, and an endpoint reading was recorded at 1h. Data represent mean from 3 biological replicates ± SEM.

Of interest next was to determine if knockdown of COX-2 altered the TGF-β1-dependent inhibition of SMIF in WPMY-1 cells. This is of particular relevance since other ROS-generating enzymes are responsive to TGF-β1 in WPMY-1 cells. As shown in Figure 20, DU145 motility is inhibited when co-cultured with SH4 cells. This is in contrast to WPMY-1 and Scr cells, which are permissive for DU145 motility in co-culture. Importantly, the degree of motility inhibition between DU145/WPMY-1 co-culture with catalase and DU145/SH4 co-culture is not significantly different, indicating that ablation of COX-2 is sufficient to reduce $H_2O_2$ levels to an extent that restores SMIF activity. To further confirm that stromal COX-2 is a significant source of TGF-β1 inducible $H_2O_2$ in WPMY-1 cells, catalase (1500 units/mL) was included in co-culture scratch assays. Figure 20 shows that the addition of catalase does not further accentuate the inhibitory activity of SH4 cells on DU145 motility in co-cultures. Therefore, WPMY-1 cells in which TGF-β1 induction of COX-2 expression and subsequent $H_2O_2$ production is limited (SH4 cells) acquire the characteristics of non-reactive stroma to limit DU145 motility in co-culture. Additionally, COX-2 is the predominant source of TGF-β1 inducible $H_2O_2$ in WPMY-1 cells that is responsible for limiting the activity of SMIF.

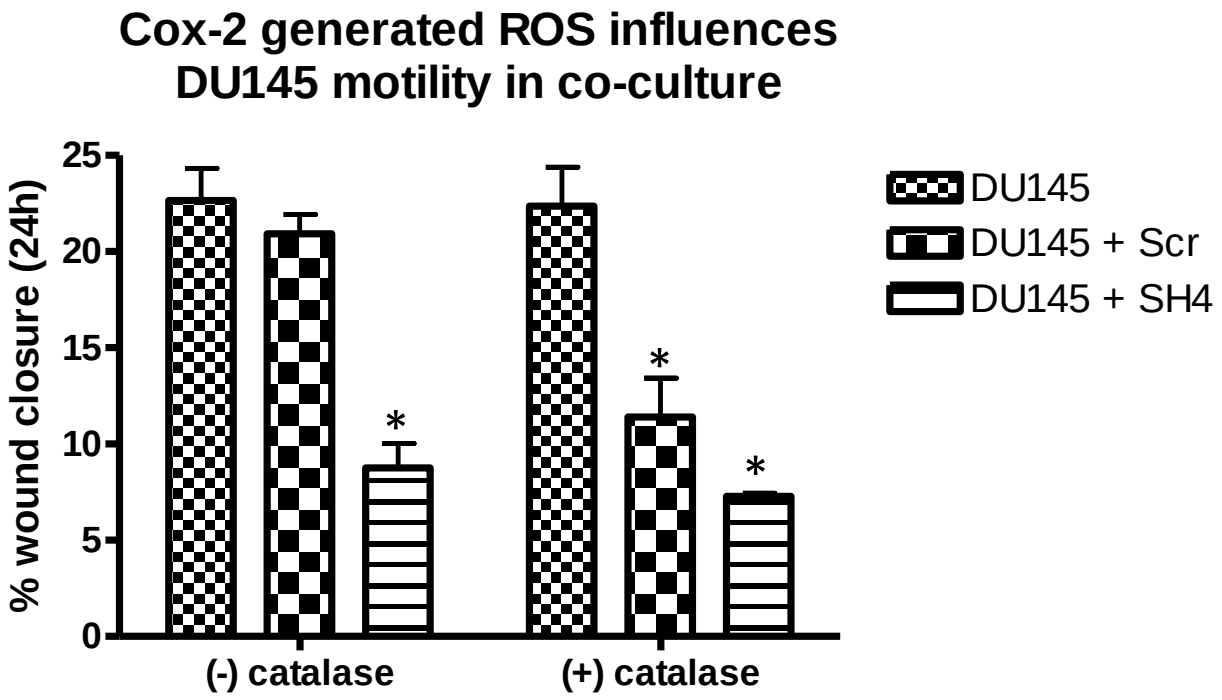



**Figure 20. COX-2 is the predominant source of $H_2O_2$ responsible for WPMY-1 cells' permissive effect on DU145 motility**

Addition of catalase to co-cultures reverses the permissive role of Scr cells in DU145 motility but does not further accentuate the motility inhibition offered by the SH4 cells. The modified wound healing assay was performed with the addition of 1500 units of catalase per 1 mL of media. A one-way ANOVA followed by Tukey's multiple comparison test was performed. * $p < .05$ relative to appropriate DU145 control

## 3.4 Hydrogen peroxide produced by COX-2 in WPMY-1 cells modulates the response of DU145 cells to SMIF

In the absence of chronic TGF-β1 stimulation in co-culture with DU145 cells, WPMY-1 cells produce an inhibitor of cancer cell motility (SMIF). This inhibitor is transferrable in conditioned media and accumulates when COX-2 is ablated in WPMY-1 cells. Thus, I hypothesized that stromal COX-2 was generating $H_2O_2$, and this was in turn modulating the effect of SMIF. To test whether $H_2O_2$ could alter the activity of SMIF, varying concentrations of $H_2O_2$ were added to CM from WPMY-1 cells. While a single bolus of $H_2O_2$ (5, 10, and 20 μM) does not significantly influence DU145 motility alone, $H_2O_2$ addition concentration-dependently reversed the motility inhibitory activity of the WPMY-1 CM (Figure 21).

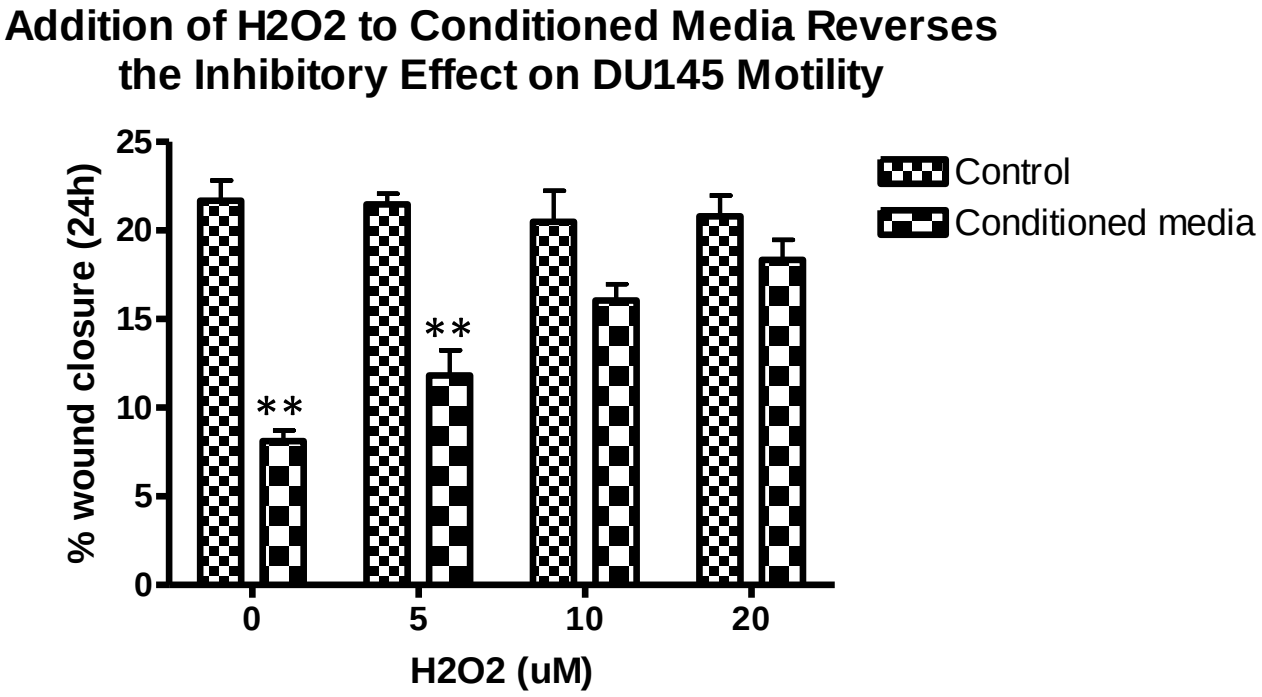



**Figure 21. Addition of $H_2O_2$ to CM inhibits the activity of SMIF on DU145 motility**

Conditioned media loses its inhibitory effect on DU145 motility at concentrations of $H_2O_2 \geq 10$ µM. CM was generated as previously described from WPMY-1 cells. Naïve DU145 cells were wounded and the media was replaced with CM to which varying amounts of $H_2O_2$ had been added. Data represent the mean of 6 independent experiments $\pm$ SEM. A one-way ANOVA followed by Tukey's multiple comparison test was performed. **p<.01 relative to appropriate control media

To further examine whether it is $H_2O_2$ generated by stromal COX-2 is indeed necessary for inhibition of SMIF activity, exogenous $H_2O_2$ was added to DU145/SH4 co-cultures. Figure 22 shows that the inhibitory effect of SH4 cells on DU145 motility is lost when $H_2O_2$ is re-introduced to co-cultures. This suggests that TGFβ-dependent induction of COX-2 is necessary for $H_2O_2$ production in co-culture to limit the activity of SMIF. Additionally, when the major prostatic COX-2 metabolite $PGE_2$ was added to co-cultures, no significant change in motility was observed (data not shown), suggesting that COX-2-derived $PGE_2$ is not involved in SMIF activity.

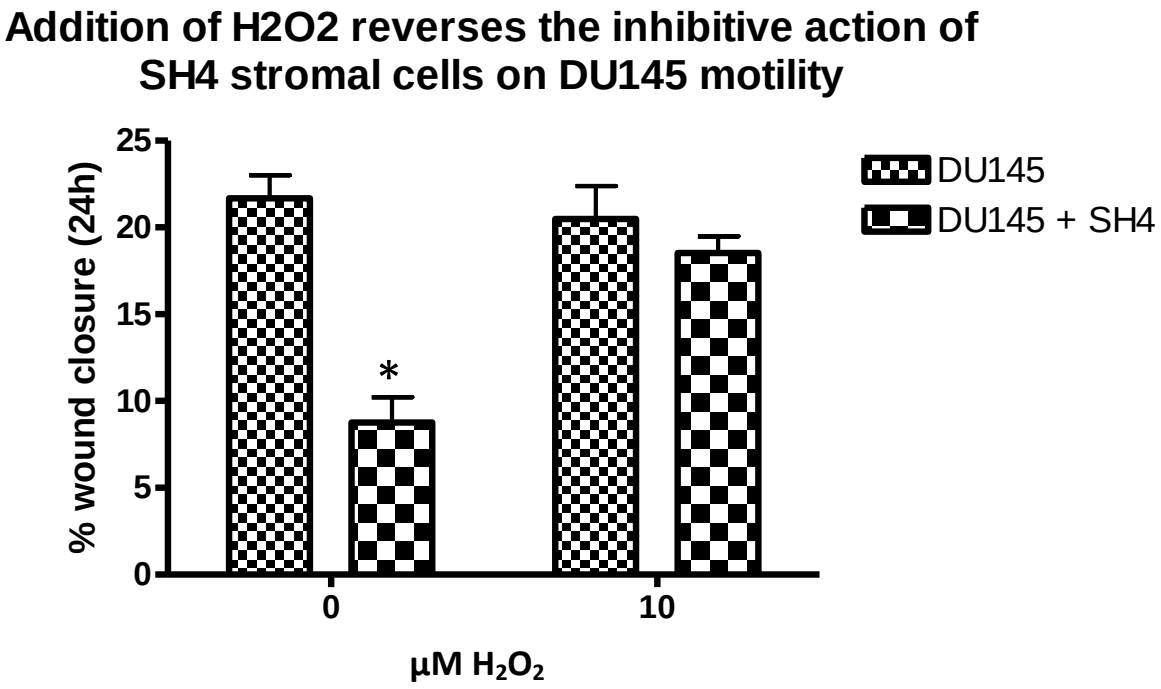



**Figure 22**. **$H_2O_2$ generated by stromal COX-2 is necessary for inhibition of SMIF in co-culture**

Addition of $H_2O_2$ (10 µM) to the DU145/SH4 co-culture reverses the motility inhibition seen under basal conditions. A modified wound healing assay as previously described was carried out with and without the addition of $H_2O_2$. Data represent the results of 4 independent experiments ± SEM. A one-way ANOVA followed by Tukey's multiple comparison test was performed. * $p<.05$ relative to appropriate DU145 control

These results suggest that either SMIF is being oxidatively modified by $H_2O_2$, or that $H_2O_2$ produced by WPMY-1 cells is influencing the response of the DU145 cells to the constitutively produced SMIF. To address this issue, CM was pre-treated with 10 μM $H_2O_2$ for 3h to allow potential oxidation of SMIF. Prior to treating wounded naïve DU145 cells with the CM, catalase (1500 units/mL) was added to neutralize the remaining $H_2O_2$. This was to ensure that SMIF had the opportunity to be oxidatively modified without exposing the DU145 cells to $H_2O_2$. As Figure 23 shows, pre-treatment of the WPMY-1 CM with $H_2O_2$ did not alter its inhibitory effect on DU145 cell motility. As shown previously, continuous exposure of DU145 cells to CM and $H_2O_2$ reversed the motility suppression effect of CM alone. These results imply that the WPMY-1 cell produced SMIF is not being oxidized, but rather that $H_2O_2$ is acting directly on the DU145 cells' response to SMIF.

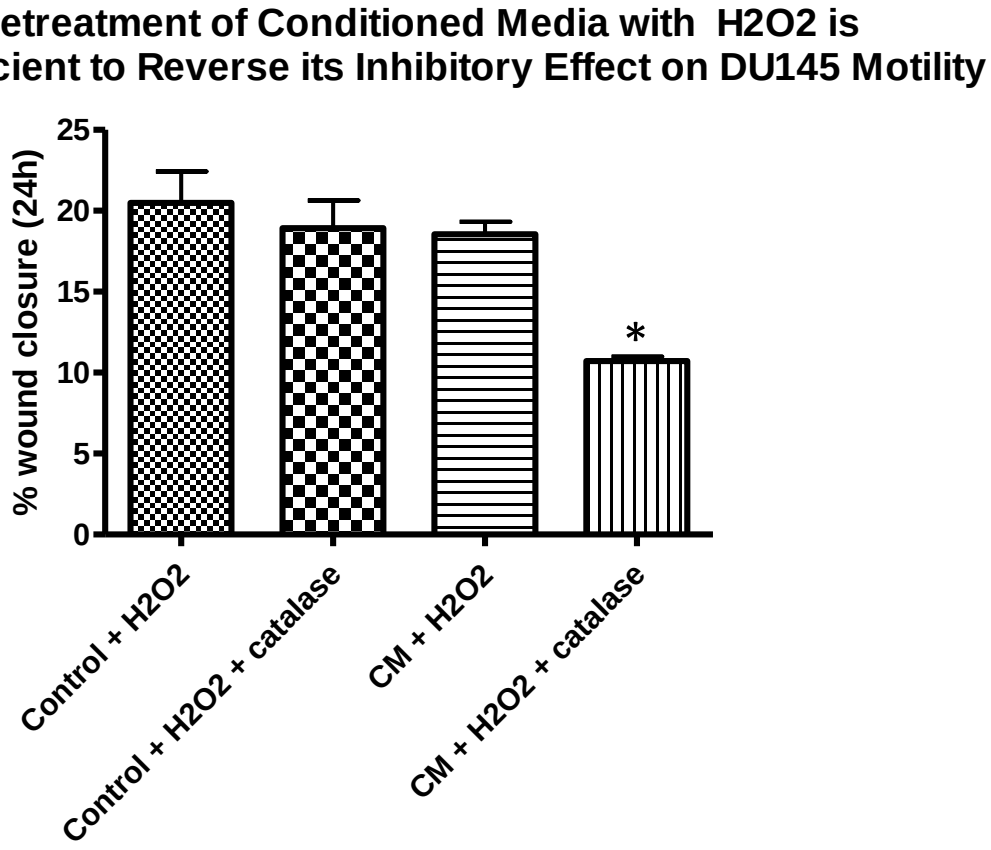



**Figure 23. $H_2O_2$ acts directly on the DU145 cells to inhibit their response to SMIF**

CM was incubated with 10 µM $H_2O_2$ for 3h. Following this pretreatment, a portion of the media was then treated with catalase (1500 units/mL) and the 2 different treatment groups were added to wounded naïve DU145 cells. Data represent 3 independent experiments ± SEM. A one-way ANOVA followed by Tukey's multiple comparison test was performed. * $p<.05$ compared to all other conditions

## 3.5 The motility inhibitory activity of WPMY-1 cells acts via ERβ in DU145 cells to modulate their motility

The current data suggest that SMIF is constitutively produced by the stroma, regardless of a myofibroblastic phenotype. Rather, it is a loss of DU145 responsiveness to the action of SMIF as a result of local $H_2O_2$ production from an increased TGFβ responsiveness leading to an inflammatory milieu. The next step was to elucidate the identity of SMIF and further understand how $H_2O_2$ was preventing its action. For an initial assessment of the identity of SMIF produced by WPMY-1 cells, a column fractionation technique was used to separate the components in the CM into high (>5 kD) and low (<5 kD) molecular weight fractions. These separate fractions were then applied to naïve DU145 cells and their motility measured. As Figure 24 shows, the low molecular weight fraction retains the inhibitory activity present in intact WPMY-1 cell CM on DU145 cell motility. The high molecular weight fraction is permissive for DU145 motility and re-addition of the low MW fraction again restores the motility suppression observed with complete CM.

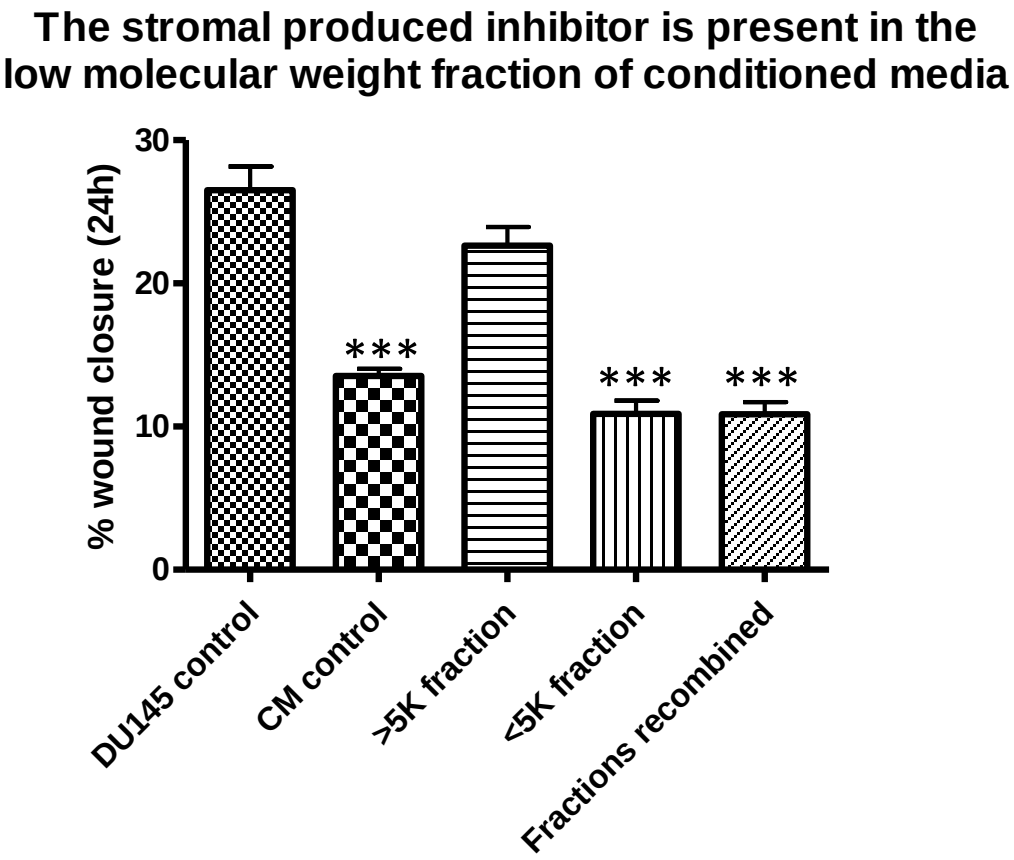



**Figure 24. SMIF is present in the low molecular weight fraction of CM**

WPMY-1 CM was fractionated into high and low molecular weight fractions, and each fraction was tested independently for biological activity. The low molecular weight fraction (pore size <5kD) retains inhibitory effects on DU145 motility. Data are representative of 4 independent biological replicates ± SEM. A one-way ANOVA followed by Tukey's multiple comparison test was performed, ***p<.001 compared to both DU145 control and the high molecular weight fraction

In lieu of further purification of the CM 5 kDa and below fraction, we postulated that 2 candidate potential low molecular weight inhibitors of cancer cell motility were involved. Two androgen derivatives, 5α-androstane-3α,17β-adiol (3α-Adiol) and 5α-androstane-3β,17β-adiol (3β-Adiol), whose molecular weights are both <5kD, have been found to increase cancer cell adhesion and decrease cell motility in an ERβ-dependent manner [58, 59]. These androgen metabolites do not bind AR but are potent ligands for ERβ [58]. DU145 cells express the ERβ isoform but no detectable ERα [46, 58, 59]. Therefore, to determine if this pathway was responsible for the oxidant-dependent inhibition of motility in co-culture, an examination of the impact of $H_2O_2$ on the motility inhibitory activity of exogenous 3β-Adiol ($10^{-6}$ M) was performed. Consistent with previously published reports, 3β-Adiol suppressed DU145 cell motility [58, 59]. Intriguingly, this inhibitory effect was reversed by $H_2O_2$ (Figure 25).

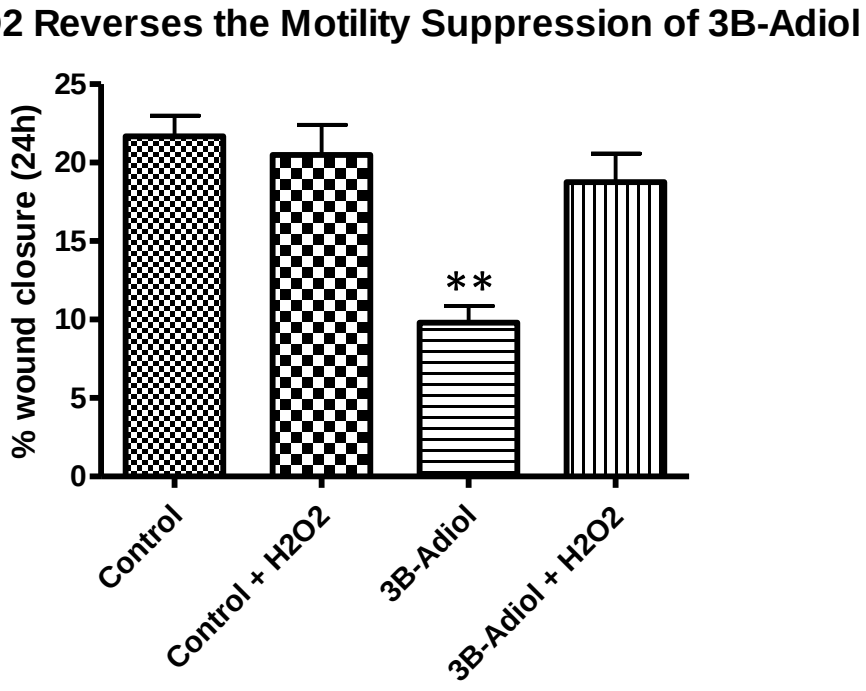



**Figure 25. 3β-Adiol inhibits DU145 motility in an oxidation-sensitive manner**

Addition of exogenous 3β-Adiol ($10^{-6}$ M) is able to significantly inhibit DU145 motility. This effect is reversed when 10 μM $H_2O_2$ is added to the media. Data are representative of 4 independent biological replicates ± SEM. A one-way ANOVA followed by Tukey's multiple comparison test was performed. **p<.01 compared to all other conditions

To further investigate the role of ERβ in regulating the motility response of DU145 cells to WPMY-1 generated factors, tamoxifen, which acts as an ERβ antagonist, and the selective ERβ antagonist 4-[2-Phenyl-5,7-*bis*(trifluoromethyl)pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol (PHTPP) were added to the wound healing assays. As Figure 26 shows, addition of both tamoxifen and PHTPP reverses the motility inhibitory effect of WPMY-1 CM. Thus, these data corroborate that the WPMY-1 produced SMIF acts on ERβ in DU145 cells to limit their motility.

**Use of the SERM Tamoxifen and selective ER-beta antagonist PHTPP reverses the motility suppression of stromal conditioned media on DU145 cancer cells**

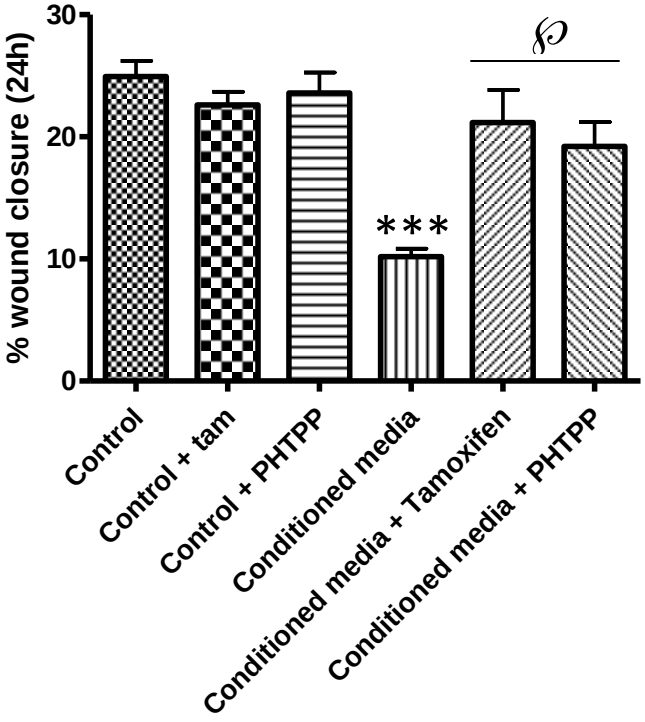



**Figure 26. SMIF acts on DU145 cells through ERβ**

Naïve DU145 cells were wounded and treated with either control or WPMY-1 CM with 4-hydroxytamoxifen ($10^{-7}$ M) or PHTPP (0.1 μM). Results are representative of 4 independent biological replicates ± SEM. A one-way ANOVA followed by Tukey's multiple comparison test was performed. ***p<.001 compared to conditions containing control media ℘ p<.05 compared to CM

Previous work has shown E-cadherin to be a downstream target of ERβ signaling that is responsible for the decrease in motility seen with 3β-Adiol treatment [58]. I have already shown that WPMY-1 cell CM induces E-cadherin expression in DU145 cells but, importantly, I sought to determine if the addition of $H_2O_2$ to CM would reverse this effect. DU145 cells were treated with either control or CM -/+ $H_2O_2$ for 18 hrs, and qRT-PCR analysis was performed for E-cadherin expression. As Figure 27 shows, WPMY-1 cell CM significantly increased E-cadherin expression, which is lost when $H_2O_2$ (10 μM) is present.

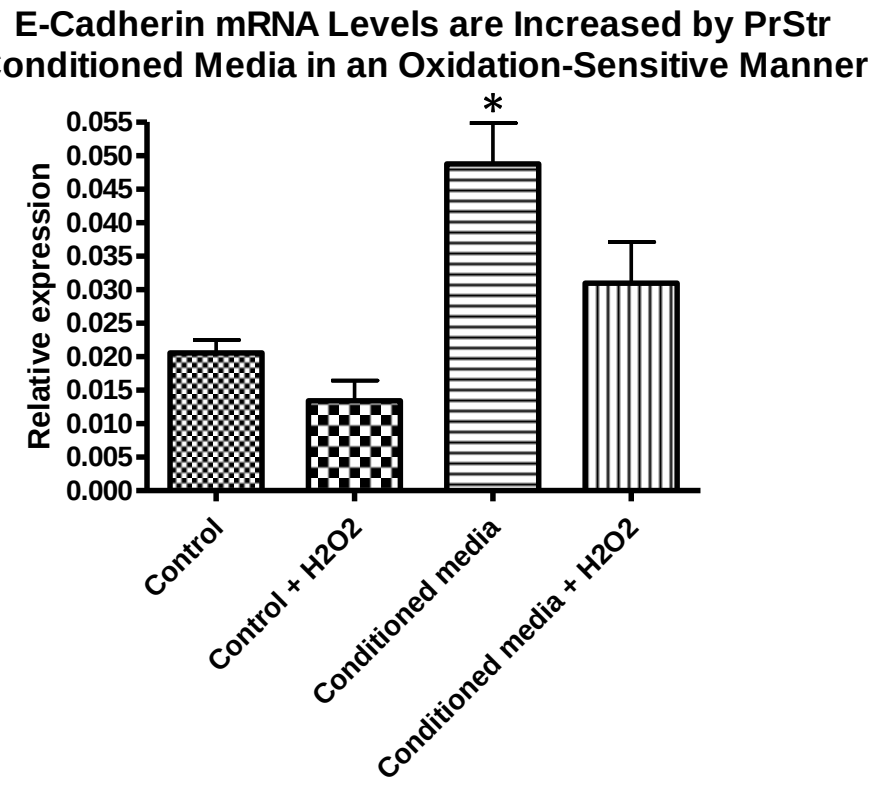



**Figure 27. Treatment with CM induces E-cadherin mRNA in DU145 cells in an oxidation-sensitive manner**

Naïve DU145 cells were treated for 18h with either control 1% serum media or WPMY-1 CM, -/+ 10 μM $H_2O_2$. The cells were then harvested in Trizol and subjected to quantitative real-time PCR for E-cadherin transcript levels. Results are indicative of 3 independent samples and are displayed ± SEM. A one-way ANOVA followed by Tukey's multiple comparison test was performed. *p<.05 relative to all other conditions

Various steps in ERβ signaling pathway were examined in order to uncover the mechanisms of $H_2O_2$ inhibition of E-cadherin mRNA expression. While most SR expression levels are decreased by chronic treatment with ligand, it has been suggested that estradiol (E2) positively regulates both ERα and ERβ expression [95]. Furthermore, ERα and ERβ expression have both been shown to be altered by oxidative stress, including administration of $H_2O_2$ to MCF-7 cells in culture [95]. Thus, a reasonable explanation for the loss of E-cadherin induction under $H_2O_2$-containing conditions was a downregulation of ERβ expression. To test this, DU145 cells were grown for 24h in either control or WPMY-1 CM -/+ $H_2O_2$ (10 μM) followed by immunoblotting of whole cell lysate for ERβ. However, as shown in Figure 28, $H_2O_2$ did not alter ERβ levels. Interestingly, WPMY-1 CM treatment of DU145 cells led to an increase in ERβ levels, which may contribute to efficient induction of E-cadherin.

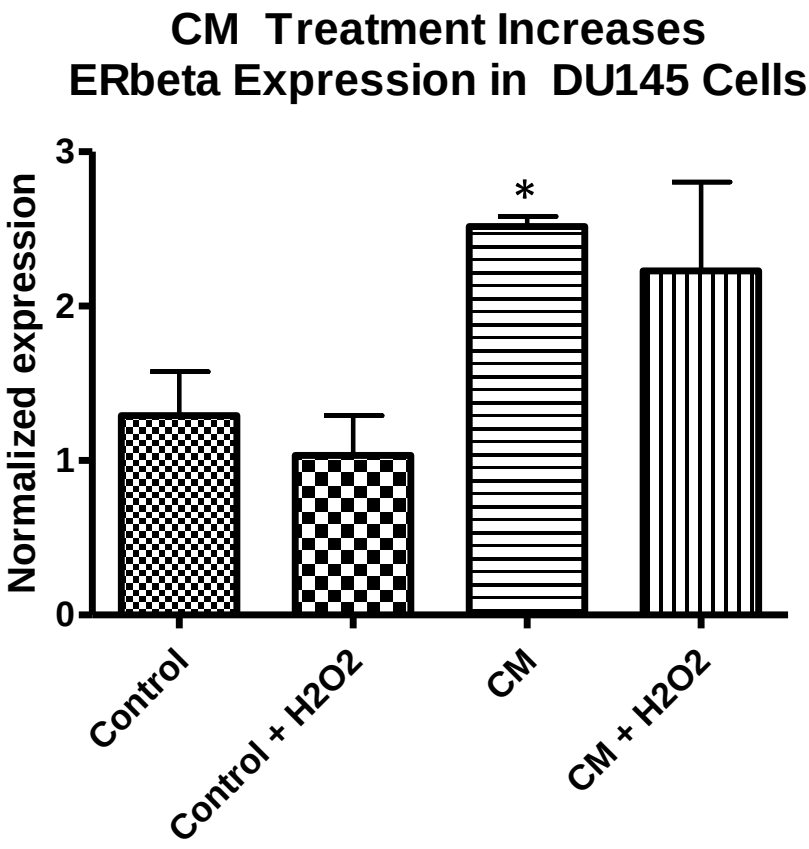



**Figure 28. CM treatment increases ERβ expression in DU145 cells.**

DU145 cells were grown for 24h in either control or WPMY-1 conditioned media -/+ $H_2O_2$ (10 μM). Cells were then lysed in RIPA buffer and whole cell lysate was subjected to western blot analysis for ERβ. The blot was stripped and re-probed for β-actin to serve as a loading control. Bars represent mean normalized densitometric values from 3 independent experiments. A one-way ANOVA followed by Dunnett's multiple comparison test was performed. All values were compared to the control. * $p<.05$

ERβ has the capacity to shuttle between both the nucleus and the cytoplasm. While nuclear ERβ localization does not necessarily indicate activity, ERβ acting directly as a DNA-binding transcription factor must be nuclear. Thus, a decreased nuclear retention of ERβ is indirectly indicative of less transcriptional activity. Although $H_2O_2$ did not alter ERβ expression, it did reduce its nuclear retention as reflected in a reduced nuclear/cytoplasmic ratio in biochemical fractionation experiments (Figure 29). While this is seen in both control and CM conditions, the biological relevance of the effect of $H_2O_2$ is only seen when the ERβ signaling pathway is activated by an ERβ ligand in the CM. Thus, $H_2O_2$ treatment alone does not affect DU145 motility, but in the presence of 3β-Adiol (from CM) treatment with $H_2O_2$ is sufficient to downregulate ERβ signaling by decreasing nuclear retention of the receptor.

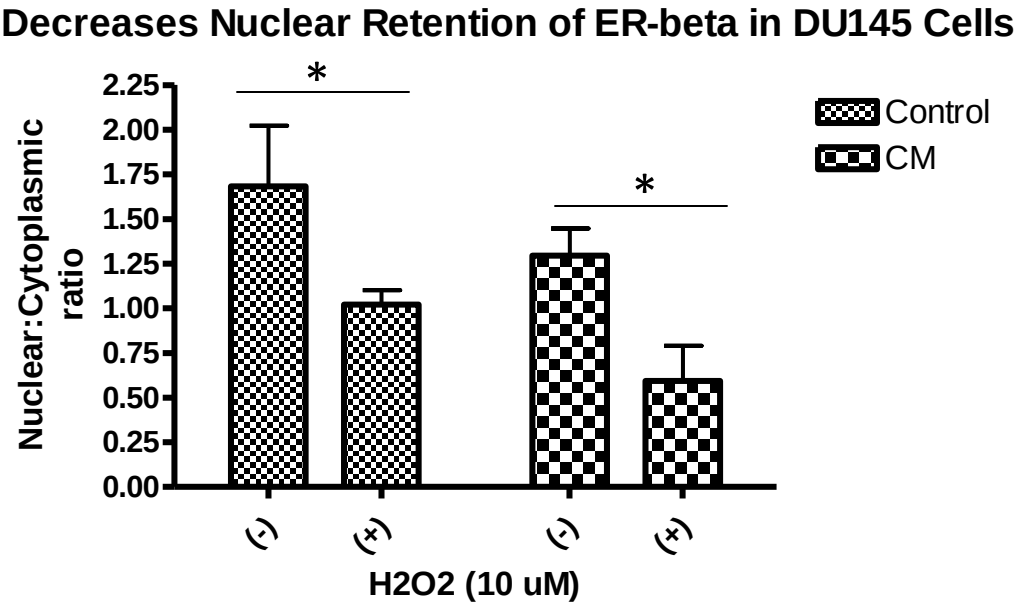



**Figure 29. $H_2O_2$ decreases nuclear retention of ERβ in DU145 cells.**

Nuclear and cytoplasmic lysates were prepared from DU145 cells and subjected to western blot analysis for ERβ protein. Results are displayed as densitometric analysis from 3 independent blots. A 2-way ANOVA followed by a Bonferroni posttest was performed. *p<.05

Previous work has shown that ERβ is sensitive to oxidation and, as a result, exhibits destabilized DNA binding. To test if $H_2O_2$ was capable of destabilizing DNA binding of ERβ at its target gene E-cadherin, a chromatin immunoprecipitation (ChIP) assay followed by quantitative RT-PCR was performed (Figure 30). Preliminary results demonstrate that there is a significant reduction in occupancy by ERβ at the E-cadherin promoter. Three different previously defined ERβ binding sites were investigated (unpublished data from the lab of Benita Katzenellenbogen), and all 3 demonstrated decreased ERβ binding in cells subjected to $H_2O_2$. Importantly, this is consistent with previously shown data demonstrating a decreased nuclear retention of ERβ in DU145 cells subjected to $H_2O_2$ (Figure 29).

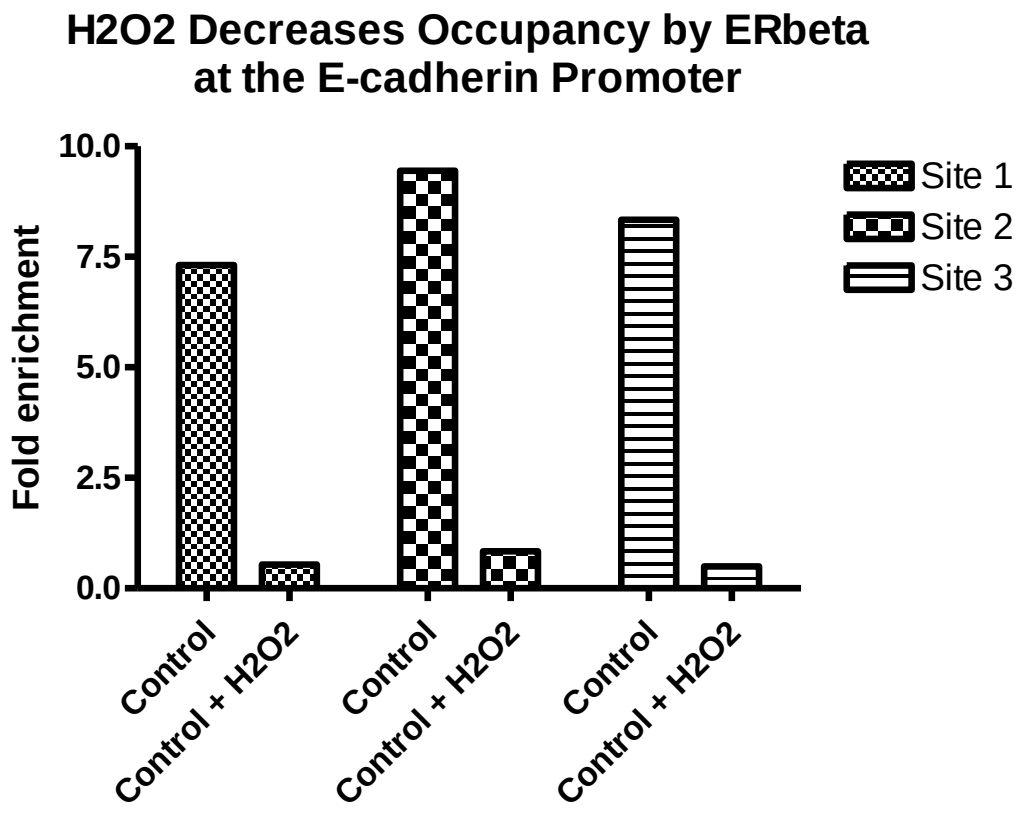



**Figure 30. $H_2O_2$ decreases occupancy by ERβ at the E-cadherin promoter.**

DU145 cells were treated for 18h with 1% charcoal-stripped FBS phenol-red free medium -/+ $H_2O_2$ (10μM). An ERβ pulldown followed by deproteination and quantitative RT-PCR using 3 different primer sets for previously determined ERβ binding sites within the E-cadherin promoter region was performed. Results are preliminary and are representative of a single experiment. qRT-PCR was performed in technical duplicate.

## 3.6 SMIF is an androgenic precursor to an ERβ ligand

3β-Adiol can be synthesized from a number of androgenic precursors through the enzymatic pathway involving 17β-hydroxysteroid dehydrogenases (17βHSD), also known as aldo-keto reductases (AKR1C) [7]. DU145 cells express AKR1C enzymes and are capable of catalyzing redox reactions at the C17 position of steroid hormones [122, 123]. Previous work has shown that the androgenic precursor DHEA can be metabolized to 3β-Adiol through the activity of these AKR1C enzymes; specifically, the AKR1C3 subtype can convert DHEA directly to 3β-Adiol [124]. Thus, to test if SMIF was an androgenic precursor such as DHEA that the DU145 cells were metabolizing to an ERβ ligand, I used the known AKR1C1-3 inhibitor flufenamic acid (FA) [125]. As Figure 31 shows, exogenous DHEA inhibits DU145 motility, but this inhibition is lost when FA is present. This indicates that DHEA is capable of inhibiting DU145 motility, but it is not a direct effect of DHEA and rather requires metabolism through an AKR1C pathway. Additionally, DU145 cells are AR negative. This further suggests that DHEA is exerting its effects via a metabolite acting on ERβ rather than through a direct androgen effect. Similarly, FA reverses the inhibitory activity of CM, suggesting that DHEA or a similar precursor derived from the CM is being metabolized to an ERβ ligand through an AKR1C-dependent pathway.

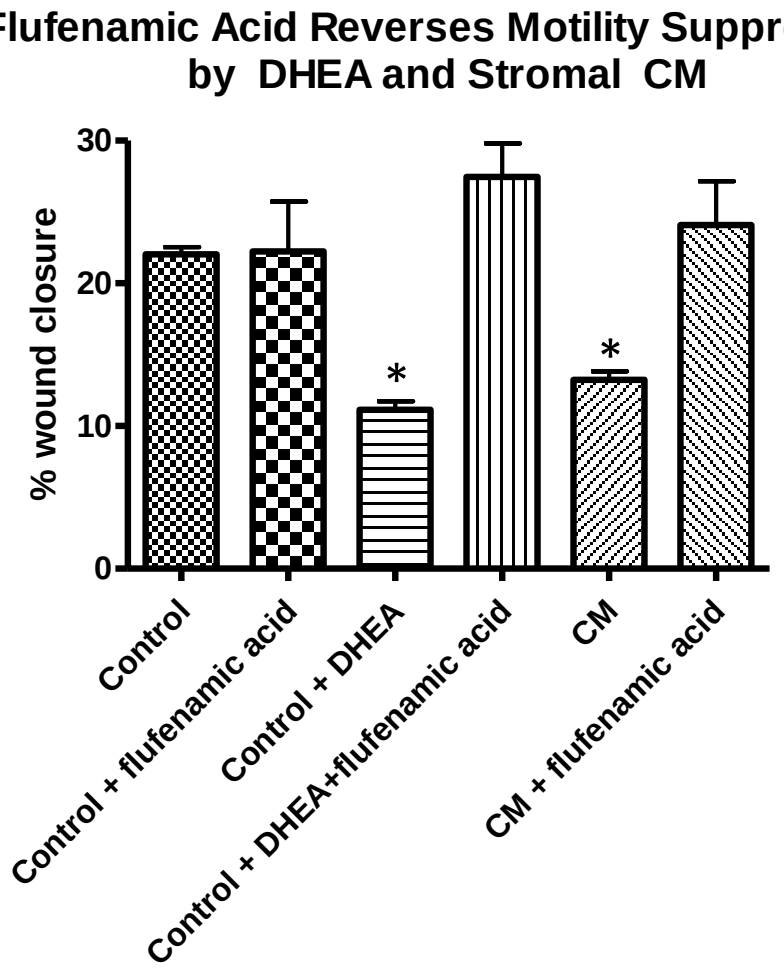



**Figure 31. SMIF is an androgenic precursor metabolized by the DU145 cells to an ERβ ligand.**

Flufenamic acid blocks the inhibitory activity of DHEA and SMIF. Wound healing assays were performed on DU145 cells in either control or CM using flufenamic acid (50 μM) and DHEA (100 nM) where indicated. Results are representative of 4 independent experiments, each done in technical quadruplicate. A one-way ANOVA followed by Tukey's multiple comparison test was performed. * $p < .05$

The inhibitory effect of CM is lost upon addition of $H_2O_2$; consequently, addition of $H_2O_2$ to DHEA-treated DU145 cells was predicted to reverse the motility suppression, consistent with the hypothesis that DHEA is a candidate as the previously-defined SMIF. Addition of $H_2O_2$ could potentially inhibit either metabolism of DHEA into ERβ ligands, or it could act directly on ERβ and thus simply block its activity. Previous experiments demonstrated that the motility inhibition exerted by exogenous 3β-Adiol is reversible upon addition of $H_2O_2$ (see Figure 25), suggesting that the action of $H_2O_2$ is at the level of ERβ and not simply an alteration in AKR1C metabolizing capacity. To confirm that SMIF could be DHEA, exogenous $H_2O_2$ was added to a wound healing assay performed on naïve DU145 cells. Figure 32 demonstrates that addition of $H_2O_2$ is capable of reversing the effect of exogenous DHEA in a manner similar to that seen when $H_2O_2$ is added to CM (see Figure 21). These data suggest that DHEA is a likely candidate for the identity of SMIF, and $H_2O_2$ serves to inhibit the action of DHEA metabolites by acting directly on ERβ in the DU145 cells.

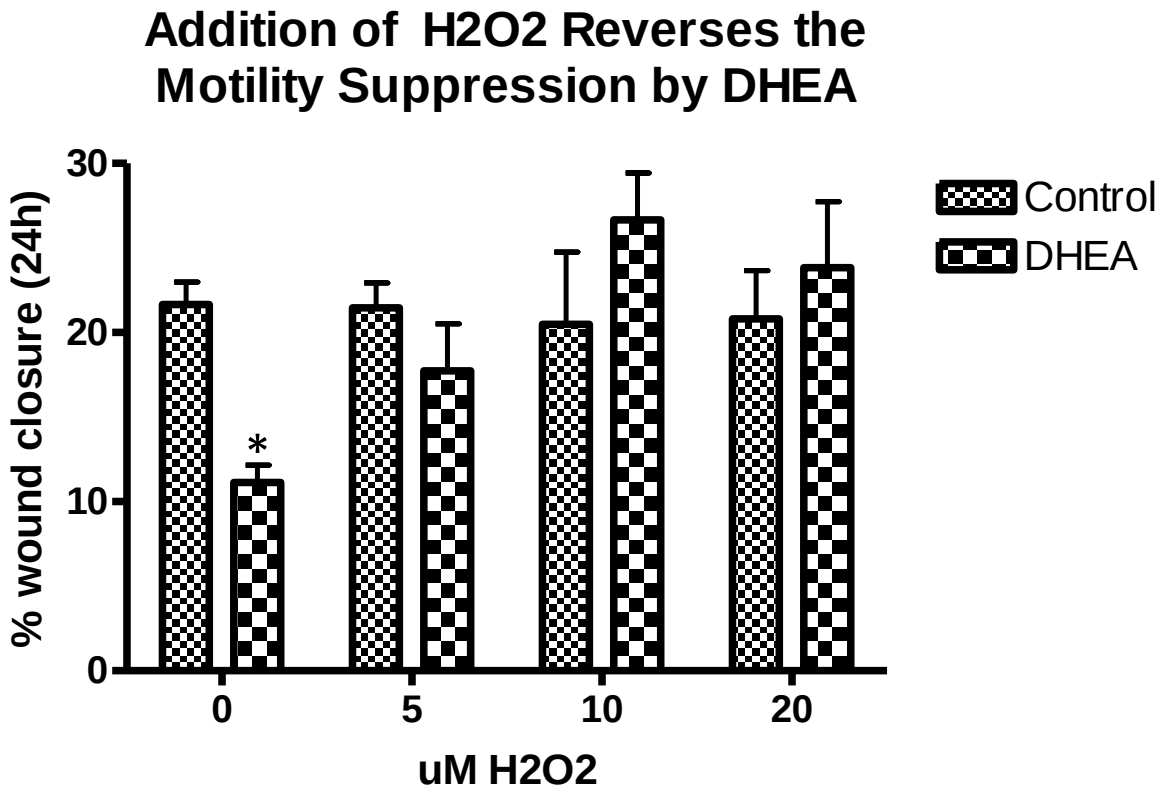



**Figure 32. Addition of $H_2O_2$ reverses the motility suppression by DHEA.**

Naïve DU145 cells were wounded and treated with either vehicle (control) or DHEA (100nM) under increasing concentrations of $H_2O_2$. Results are representative of 4 independent experiments, each done in technical quadruplicate. Bars represent the mean $\pm$ SEM. A 2-way ANOVA followed by a Bonferroni posttest was performed. * $p<.05$ relative to all other conditions

Given these data, a working model was developed to describe the bidirectional communication between the stromal and epithelial compartments with respect to the microenvironment's role in cancer cell motility. Figure 33 and Figure 34 are cartoon diagrams indicating the major points of interest in the stromal/epithelial communication cascade: First, irrespective of their state of "reactivity", prostate stromal cells have the capacity to produce a precursor (potentially DHEA) to an ERβ ligand that is a potent inhibitor of PCa cell motility. Furthermore, locally produced TGF-β1 by PCa cells induces a pro-inflammatory and pro-oxidant milieu in TGF-β1 hyper-responsive stromal cells, leading to upregulation of Cox-2. Finally, $H_2O_2$ produced by Cox-2 blocks the effect of 3β-Adiol derived from stromal DHEA by affecting its target ERβ.

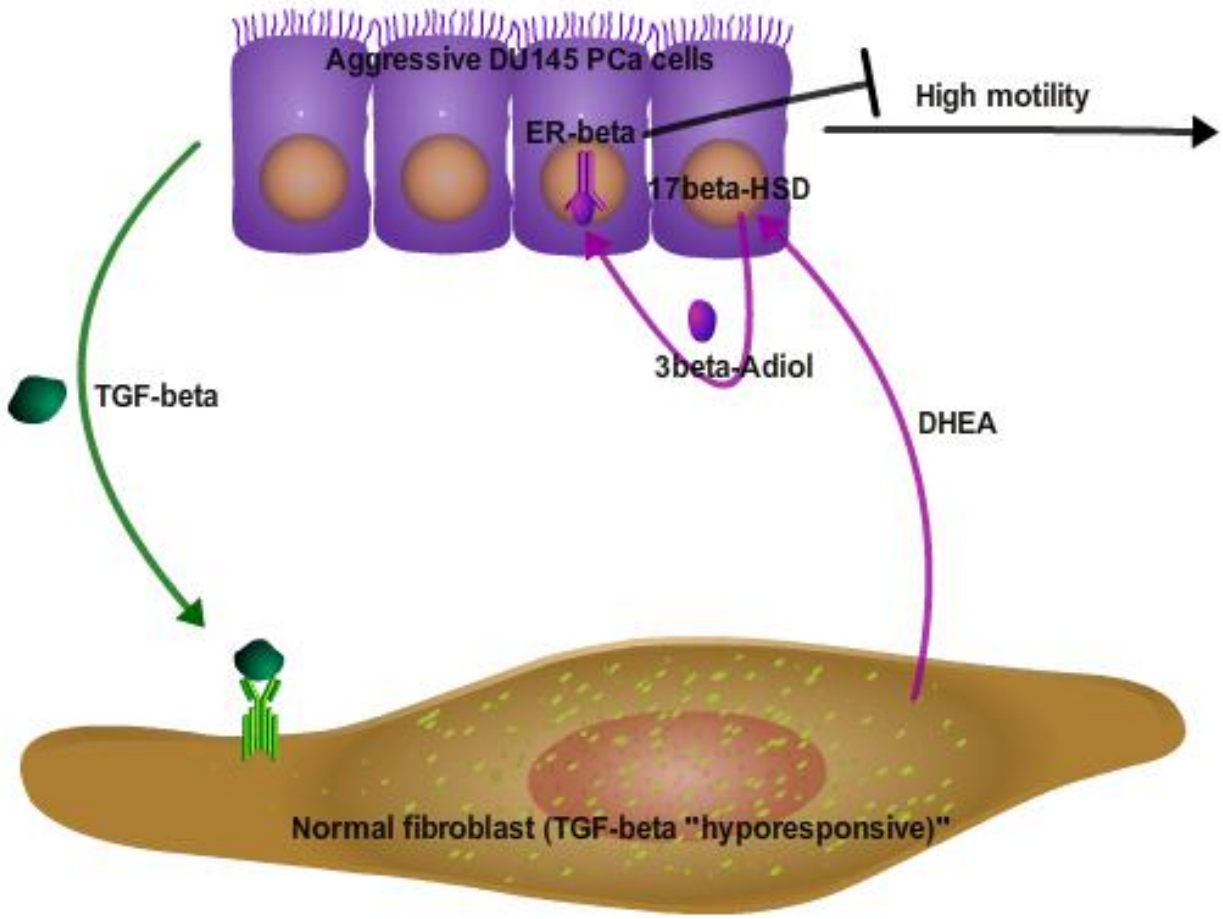



**Figure 33. Stromal fibroblasts exert an inherent capacity to suppress PCa cell motility.**

In a normal fibroblast, DHEA is constitutively produced and secreted. It is metabolized by AKR1C enzymes in the DU145 cells into potent ERβ ligands, which then act to limit motility.

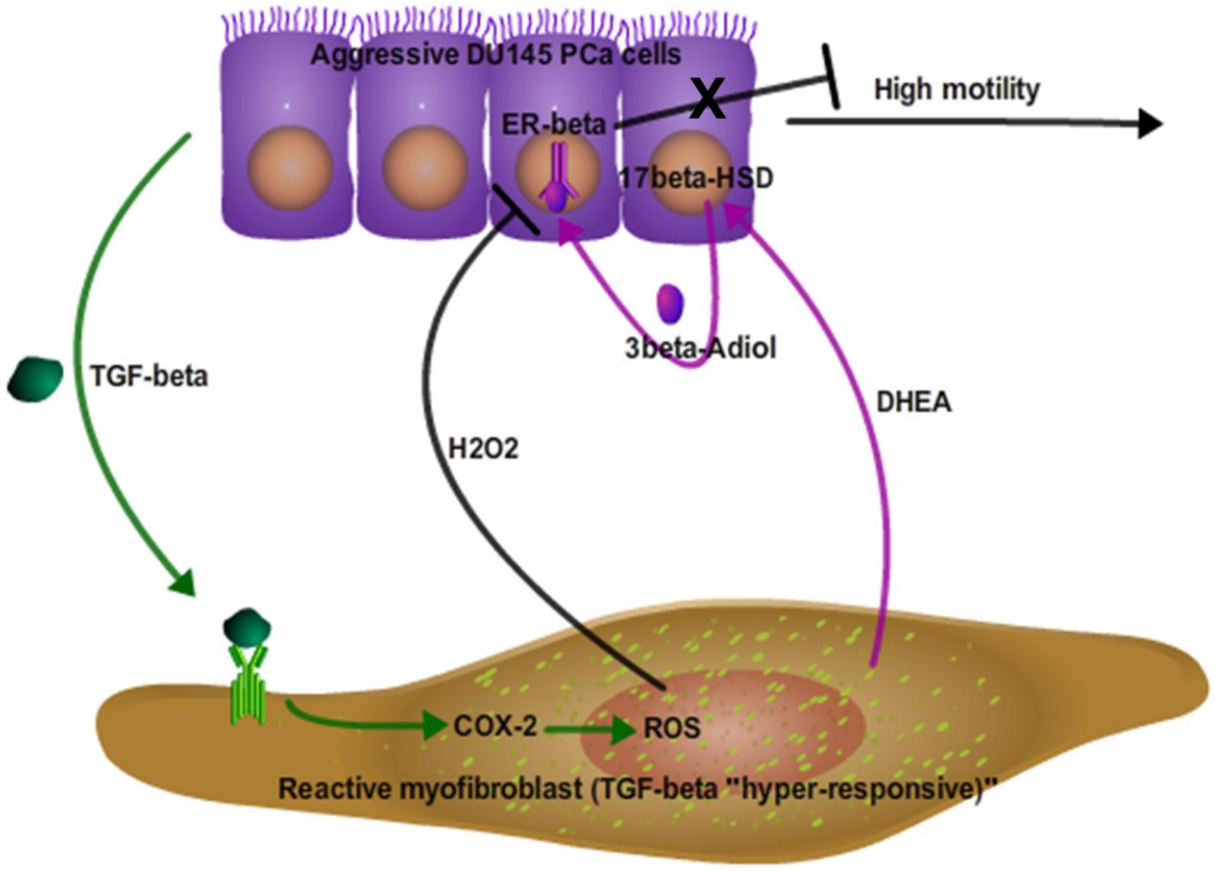



**Figure 34. Stromal myofibroblasts alter the redox status of the local milieu and thus lose their inherent capacity to limit PCa cell motility.**

In reactive myofibroblasts, locally produced TGF-β1 stimulates the production of ROS which is secreted in the form of $H_2O_2$. The $H_2O_2$ acts as a second paracrine factor to limit the transcriptional activity of ERβ, thus alleviating its inhibitory effect on DU145 motility.

# 4.0 Discussion

## 4.1 Summary of findings

The work presented here demonstrates that reactive stromal cells retain an inherent regulatory control over cancer cell motility through secreted paracrine factors. Specifically, stromal cells secrete an androgen metabolite (DHEA?), that is a precursor for a selective ERβ ligand. Activation of ERβ in cancer cells following conversion of the stromal produced ligand increases E-cadherin expression and thus inhibits cell motility. However, ERβ activity in the cancer cells is reduced following its oxidation by $H_2O_2$, which is produced by an ROS-generating system in TGFβ treated stromal cells.

Both CAFs and the myofibroblastic WPMY-1 cells employ extracellular $H_2O_2$ as a paracrine mediator responsible for producing a permissive effect on DU145 cancer cell motility in co-culture. Inhibition of this $H_2O_2$ restores their inherent ability to limit DU145 motility within this same co-culture. COX-2 is the chief producer of local $H_2O_2$ by WPMY-1 cells, and specific ablation of COX-2 using an shRNA expressing lentivirus is sufficient to restore the inhibitory action of WPMY-1 cells in co-culture.

The key stimulatory factor driving the upregulation of stromal COX-2 was determined to be TGF-β1 produced by the DU145 cells. Inhibition of TGF-β1 in co-culture is sufficient to restore WPMY-1 cells' inherent ability to suppress DU145 motility. While TGF-β1 is constitutively produced, an enhanced TGFβ response pathway in the WPMY-1 cells leads to an induction of COX-2 and subsequent $H_2O_2$ production. The data uniquely demonstrate that it is not a loss of function in the WPMY-1 cells that is responsible for their permissive effect on DU145 motility, but rather it is an increased responsiveness within the TGFβ signaling pathway that leads to the production of an additional paracrine factor ($H_2O_2$) that acts directly on the DU145 cells to inhibit the effect of the constitutively produced DHEA/3β-Adiol.

Figure 35 is a simple schematic summarizing the bidirectional paracrine communications between the cancer cells and the nearby stromal cells, ultimately resulting in an increased motile capacity.

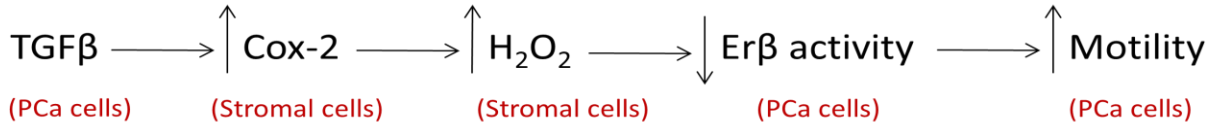



**Figure 35. A simple schematic of bidirectional paracrine communications between PCa cells and nearby stromal cells.**

Overproduction of TGF-β1 by PCa cells induces COX-2 expression and subsequent $H_2O_2$ production in nearby stromal cells. $H_2O_2$ acts as paracrine mediator to decrease ERβ activity in the PCa cells, subsequently alleviating its anti-motility effects

## 4.2 Differential responsiveness to locally produced TGF-β1

TGFβ signaling has long been regarded as a "double-edged sword" in cancer biology; early in carcinogenesis TGFβ is growth suppressive, but as the disease progresses TGFβ becomes cancer promoting [77]. Tissue staining of prostatic adenocarcinomas shows enhanced phospho-Smad2 staining in the stroma, suggesting that the TGFβ cascade is hyperactive [82]. Additionally, staining for TGF-β1 has shown to be significantly higher in PCa specimens [126].

The model employed herein coincides perfectly with these observations: the DU145 PCa cells secrete relatively high levels of active TGF-β1, and the WPMY-1 cells demonstrate an enhanced responsiveness. WPMY-1 cells show a greater transcriptional response to exogenous TGF-β1 than their more fibroblastic PS30 counterparts. Furthermore, the WPMY-1 cells express significantly higher levels of several Smad proteins, which are mediators of the canonical TGFβ signaling pathway. Both the elevated transcriptional activity and the increased expression of Smad proteins suggests that WPMY-1

cells have acquired an increased responsiveness to TGFβ. Recent work has shown that TGFβ receptor (TGFβR) heterogeneity in stromal cells is necessary for promotion of carcinogenesis [82, 88]. While some stromal cells lose TGFβR expression, the cells which retain active TGFβ signaling demonstrate an enhanced responsiveness. This enhanced responsiveness leads to the negative effect of TGFβ signaling in cancer progression, as unchecked signaling leads to the "wound that never heals" phenotype. The WPMY-1 cells demonstrate the detrimental effect of enhanced TGFβ signaling on cancer cell motility.

While TGFβ is well established as the responsible factor for transdifferentiation of prostatic fibroblasts into myofibroblasts [66], this project has shown it maintains crucial effects beyond inducing this initial phenotypic change. An enhanced responsiveness to TGF-β1 in the WPMY-1 cells results in the chronic activation of components of the wound healing cascade, most importantly the stimulation of COX-2 expression. The importance of this is underscored when a TGF-β1 neutralizing antibody is added to DU145/WPMY-1 co-culture and a dramatic inhibition of DU145 motility is observed. The novelty lies in the capability of the WPMY-1 cells to inhibit DU145 cell motility in the absence of active TGF-β1. This shows that myofibroblasts retain the ability to impose inherent control over cancer cell motility, but this effect is negated through the production of a second paracrine factor produced downstream of TGFβ signaling.

In the model of stromal heterogeneity, loss of TGFβ signaling in some of the stromal cells leads to a subsequent overproduction of TGF-β1 by the neighboring cancer cells in an effort to favorably modulate the microenvironment [82, 88, 126]. Thus, in stromal cells maintaining intact TGFβ signaling, abundant TGF-β1 is present to ensure a robust activation of the TGFβ pathway. Potent activation of the TGFβ pathway in stromal cells results in a permissive effect on cancer cell motility, as seen when DU145 cells are co-cultured with WPMY-1 cells. This permissive effect allows the cancer to move and infiltrate unrestrained, eventually leading to invasion of surrounding tissues and distant metastases.

## 4.3 COX-2 in the prostate stroma

In WPMY-1 cells, TGF-β1 induces expression of the pro-inflammatory enzyme COX-2. As a byproduct of its enzymatic activity, COX-2 generates $H_2O_2$, which acts a second paracrine factor to inhibit ERβ activity in the cancer cells.

COX-2 is a multi-faceted enzyme whose expression is generally labile and transient. In some diseases such as PCa, however, consistently high levels of COX-2 are associated with worse prognosis [86, 106]. It is important to understand the expression profile of COX-2 more completely, since the cancer cells themselves often overexpress COX-2. Addition of selective pharmacologic inhibitors of COX-2 has been shown to decrease cancer cell growth, induce apoptosis, and decrease tumor size in animal models [106]. Interestingly, it is not simply COX-2 expression by the cancer cells that is responsible for increased aggressiveness. A xenograft of Lewis lung carcinoma cells (positively expressing COX-2) was unable to form a tumor in a COX-2 -/- host, suggesting that stromal COX-2 is necessary for tumor development. Upon further investigation, it was found that the COX-2 -/- fibroblasts were unable to secrete VEGF, an obligatory requirement for tumor formation [114]. Similarly, DU145 cells secrete TGF-β1 which increases expression of stromal COX-2 in WPMY-1 cells and hence the production of $H_2O_2$, thus granting them reprieve from the motility-suppressive effects otherwise imposed. Figure 36 is from a study done in 2010 by Richardsen et al. showing that high expression of COX-2 in the stroma correlates with decreased disease-specific survival [86]. These clinical data are in accordance with the work presented herein which demonstrates that upregulation of stromal COX-2 can inhibit the motility suppression normally offered by the surrounding stroma through inactivation of ERβ in the cancer cells.

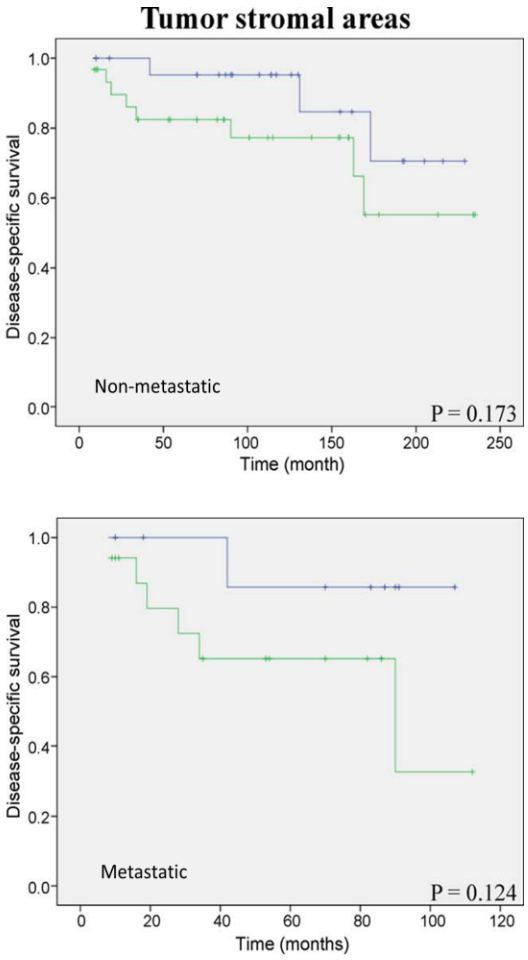



**Figure 36. High stromal COX-2 staining correlates with decreased survival in patients with primary PCa.**

In both non-metastatic and metastatic primary PCa, high expression (green line) of COX-2 trends with a shorter disease-specific survival time.

Adapted from *E. Richardsen at al./Cancer Epidemiology 34 (2010) 316-322*

In addition to COX-2 expression alone as a predictor of survival, another study showed that COX-2 staining is inversely related to E-cadherin expression in neighboring cancer cells [127]. Loss of E-cadherin expression is associated with EMT and subsequently with increased migration and invasion [128]; similarly, increased stromal COX-2 staining is associated with a poorer prognosis in PCa and others [115, 129]. The data presented herein demonstrate a molecular basis for this observation. Induction of

COX-2 increases $H_2O_2$ production by stromal cells, which acts directly on ERβ in cancer cells both by preventing its DNA binding and decreasing its nuclear retention. ERβ is thus unable to adequately bind and activate the E-cadherin promoter, hence a loss of E-cadherin expression. Since even a myofibroblast is capable of secreting a precursor leading to the production of potent ERβ ligands, use of a selective COX-2 inhibitor could potentially restore the expression of E-cadherin and subsequent inherent motility suppression endogenously offered by the stroma.

Clinical trials with NSAIDs (non-selective COX inhibitors) have had ambiguous results in PCa [112, 113]. In a simplified system such as a 2 cell indirect co-culture, it may be an oversimplification to assume that the redox status of the milieu is controlled solely by ROS produced during COX-2 enzymatic activity. However, the data presented in this work clearly validate the role of extracellular $H_2O_2$ as a crucial paracrine factor affecting the cancer cells. Rather than use selective COX-2 inhibitors alone for cancer treatment, it would logically follow to use a combination therapy approach of a COX-2 inhibitor and an antioxidant. Inhibiting COX-2 would significantly decrease the inflammatory nature of the microenvironment, and an antioxidant would further inhibit any ROS produced by redundant or parallel pathways.

## 4.4 Intraprostatic steroid metabolism and estrogen signaling

As men age, serum testosterone levels naturally decline and the main source of androgens shifts to adrenal DHEA [7]. However, a concomitant equilibrium of estrogen levels leads to decrease in the testosterone to estrogen ratio (T:E) [43]. This observation has led to extensive work studying the role of estrogens in the prostate. Aromatase is the enzyme responsible for aromatic conversion of androgens to estrogens, and high local expression of aromatase within the prostate ensures adequate intraprostatic estrogen synthesis. Work by Gail Risbridger et al. demonstrated that an aromatase-knockout mouse

model is unable to form PCa despite chronically elevated levels of circulating androgens, thus highlighting the crucial role for estrogenic signaling in abnormal prostate pathology [43].

However, ERβ activation within the prostate is due largely to androgen metabolites rather than estrogens [56]. Quantitative real-time PCR of both PCa cell lines and patient-derived tumor samples revealed elevated levels of the enzyme AKR1C3, which is responsible for the conversion of the androgenic precursor DHEA into the ERβ ligand 3β-Adiol [61]. Additionally, the prostate expresses AKR1C1 and AKR1C2, both of which catalyze the conversion of the potent androgen DHT into the ERβ ligands 3β-Adiol and 3α-Adiol, respectively [63]. Analysis of PCa specimens revealed limited expression of enzymes responsible for *de novo* steroidogenesis, indicating that the conversion of androgenic precursors is the predominant method of controlling the balance in androgenic and estrogenic signaling pathways [61].

Consistent with this concept of intraprostatic steroid metabolism and estrogenic signaling, the data presented herein demonstrate that DU145 PCa cells are capable of metabolizing locally produced DHEA into potent ERβ ligands. Inhibition of AKR1C1-3 with flufenamic acid abolished the motility-suppressive effects of both exogenous DHEA and WPMY-1 CM, indicating that metabolism through an AKR1C-dependent pathway is necessary for DHEA to exert its inhibitory effects. Additionally, addition of an ERβ antagonist to WPMY-1 CM blocked its inhibitory effects on DU145 motility, indicating that its inhibition is through an ERβ-dependent mechanism. Constitutive production of DHEA or other selective ERβ ligand precursor by the stroma may serve to inherently limit cancer cell motility by increasing cell adhesion through induction of E-cadherin. Upon transdifferentiation into myofibroblasts, stromal cells gain additional characteristics that lead to further paracrine signals to the cancer cells, but they maintain the ability to produce DHEA as a paracrine mediator capable of inhibiting motility. Perturbations in this DHEA/3β-Adiol/ERβ pathway could potentially increase the risk of PCa development by allowing excess

motility and loss of E-cadherin expression in epithelial cells. Consistent with this prediction, several studies have shown an increased risk of PCa development in men with genetic polymorphisms in AKR1C enzymes that render them less active [62, 130]. Future therapeutic paradigms should take into account the retained ability of the stroma to inhibit cancer cell motility through an ERβ-dependent pathway, and should focus on creating a microenvironment favorable to maintenance of ERβ activity by controlling the redox status of the local milieu.

Epidemiological support for ERβ's role in negatively regulating PCa is seen in tissue staining done by Mak et al. which demonstrates that E-cadherin expression is directly correlated with ERβ expression in PCa [56]. Loss of E-cadherin is a known marker for EMT, which is associated with loss of cell adhesion and a subsequent increase in cell motility [131]; thus, loss of ERβ expression is directly associated with increased motility and aggressiveness. In fact, ERβ expression is inversely related to the progression of PCa to a high Gleason grade [56]. Figure 37 below is adapted from work performed by Mak et al. and shows tissue staining from normal and Gleason grade 3 and 5 PCa specimens. Loss of ERβ coincides with a loss of E-cadherin expression and is seen in the higher Gleason grade sample; these data are consistent with the work presented herein which demonstrates that activation of ERβ signaling in DU145 PCa cells via a paracrine mediator in CM induces expression of E-cadherin.

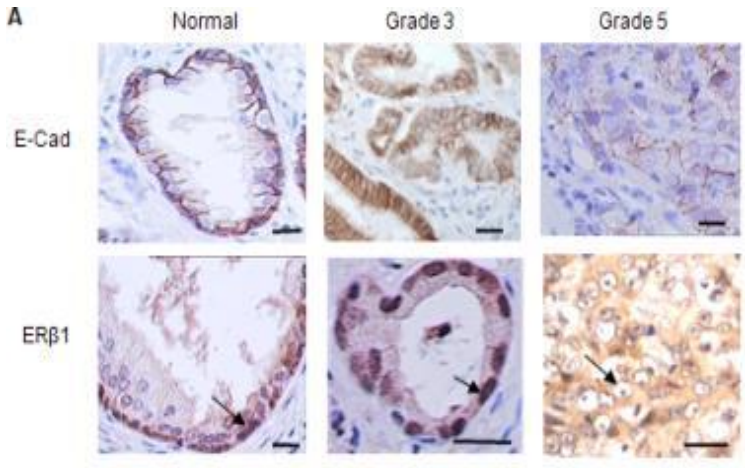



**Figure 37. ERβ expression is directly correlated with E-cadherin expression.**

Tissue staining of specimens from either normal or cancerous prostate. In normal and low Gleason grade PCa ERβ is localized to the nucleus and E-cadherin expression is seen throughout the epithelium. In Gleason grade 5, ERβ staining is absent and E-cadherin expression is lost.

Adapted from *P. Mak at al./Cancer Cell 17 (2010) 319-332*

## 4.5 Redox-sensitivity of ERβ

Previous work has shown ERβ to be susceptible to oxidation at various cysteine residues within one or both of its zinc fingers [52, 53]. A significant number of patients with breast cancer who fail to respond to the anti-estrogen tamoxifen have been shown to have structural defects in their ER which prevents it from stably binding DNA [54]. Importantly, transient exposure to oxidants results in a reversible oxidation of ERβ, but chronic exposure to free radicals and/or ROS ultimately leads to an irreversible alkylation and permanent loss of DNA binding [52]. Additionally, work done in the breast cancer cell line MCF-7 revealed that oxidative stress, specifically $H_2O_2$, was able to alter expression levels of ERβ [95]. These data expound upon the sensitivity of ERβ activity to the local redox status and highlight the importance of limiting chronic ROS production.

Similarly, the results presented in this work demonstrate that ERβ is unable to activate its target gene E-cadherin in DU145 cells when $H_2O_2$ is present in the growth media, either from local production by the stromal cells or through exogenous addition. $H_2O_2$ pretreatment of the CM followed by neutralization was unable to obtain a permissive effect on DU145 motility in a wound healing assay, indicating that the $H_2O_2$ was acting directly on the DU145 cells. Inclusion of an ERβ antagonist abolishes the inhibitory effect of CM, showing that ERβ is the target of SMIF in the DU145 cells. The known potent redox-sensitivity of ERβ thus explains why the addition of $H_2O_2$ is capable of negating the motility-inhibitory activity of CM.

In the co-culture model employed herein, ERβ in the DU145 cells could be directly oxidatively modified by $H_2O_2$ produced by the WPMY-1 cells and thus exhibit decreased DNA binding at the E-cadherin promoter. Preliminary data demonstrates decreased occupancy by ERβ at the E-cadherin promoter in the presence of $H_2O_2$, which also coincides with the observation that $H_2O_2$ treatment decreases nuclear retention of ERβ. This decreased promoter binding would result in decreased transcription of E-cadherin and a subsequent permission of cell motility, both of which were shown to occur. $H_2O_2$ did not affect protein levels of ERβ in the DU145 cells, thus suggesting that oxidative stress signaling is affecting ERβ activity directly and not simply regulating its expression. In addition to direct effects on ERβ binding at the E-cadherin promoter, $H_2O_2$ could be acting to alter the composition of the transcriptional complex that is recruited. Work done by Saijo et al. showed that the transcriptional complex recruited by ERβ differs when the activating ligand is Adiol as opposed to estradiol [124], suggesting that differential coactivator or corepressor recruitment is another potential mechanism for distinct actions of ERβ.

While the mechanism by which $H_2O_2$ decreases ERβ activity in the DU145 cells remains yet to be elucidated, the overall impact of the redox-sensitivity of ERβ is clear. Increased $H_2O_2$ leads to a decrease

in ERβ activity and a subsequent reduction in E-cadherin; both decreased ERβ and E-cadherin are directly correlated with progression of PCa to a worse Gleason grade, underscoring the importance of maintaining adequate ERβ function in the prostate [56]. The work presented herein suggest that neutralizing ROS/ $H_2O_2$ production by the stroma can serve as one mechanism by which ERβ activity remains unimpaired.

## 4.6 Clinical Implications

The overarching theme demonstrated by this work is the crucial role oxidative stress plays in altering steroid receptor signaling within the cancerous prostate. The role of the tumor microenvironment can change from inhibitive to permissive simply by paracrine signals generated by the cancer cells in an effort to escape intrinsic control. Cancer cells are elusive to many therapies due to an unstable genetic composition, making selective targeting a difficult therapeutic task. Thus, development of therapeutic paradigms that capitalize on the intrinsic regulatory mechanisms of the microenvironment could be a crucial step in targeting the molecular mechanisms underlying growth and dissemination of adjacent cancer cells.

This project identifies an inherent capacity of myofibroblasts and CAFs to limit PCa cell motility given an inhibition in oxidative stress signaling within the local milieu. Constitutive production of DHEA or other androgenic precursor by the stroma leads to generation of ERβ ligands in an AKR1C-dependnent pathway within the cancer cells, and these ligands serve to activate ERβ and subsequently increase E-cadherin expression, ultimately leading to a decrease in cell motility. Pharmacologic inhibitors of COX-2 are available, and they could serve to limit production of ROS within the stroma. Additionally, antioxidants could potentially be administered to further decrease the oxidative stress placed on the PCa cells and thus permit intact ERβ signaling. Notably, this work demonstrated that even aggressive PCa cells maintain the inherent molecular machinery to produce potent ERβ ligands given an adequate

precursor, and they retain the ability to revert to a less motile phenotype in response to ERβ signaling. This could justify the development of synthetic ERβ ligands to activate the protective mechanisms incurred by this pathway. Halogen-substituted indazoles have been shown to exhibit anti-inflammatory activity in microglia through an ERβ-dependent pathway *in vivo* [124], underscoring the viability of synthetic ERβ ligands as potential future therapeutic agents.

## 4.7 Future Directions

The benefit of DHEA metabolism in the prostate remains an area of debate; a recent review concluded that in normal prostate, DHEA has no deleterious effects, but in the diseased prostate a reactive stromal component leads to altered DHEA metabolism and increased androgenic signaling [132]. Specifically, it has been shown in an *in* vitro co-culture model that TGF-β1 induced transdifferentiation of stromal cells leads to increased androgenicity of DHEA in the cancer cells, as measured by increased PSA secretion [132]. DHEA can have direct effects on ERβ as demonstrated in a binding assay which showed a higher affinity of DHEA for ERβ as opposed to either ERα or AR [133] but, more importantly, DHEA's role is primarily a precursor to either estrogenic or androgenic steroids [134]. Based on the work presented here, I would hypothesize that it is not an alteration in DHEA metabolism that leads to increased androgenic signaling in the presence of a reactive stroma, but rather it is the loss of ERβ activity that leads to a subsequent increase in AR action through loss of competition.

Advanced technologies such as ChIP-on-chip and ChIP-seq have allowed the identification of *cis*-acting targets (i.e. DNA binding sites) by *trans*-acting factors (i.e. transcription factors, in this case steroid receptors). The unique signature of a specific transcription factor on a genome-wide scale is thus referred to as its cistrome, which collectively identifies all of the target genes possessing direct binding sites for the specified transcription factor [137]. Work recently published from Myles Brown et al. used a ChIP-seq approach to show a unique AR cistrome in ER(-) as compared to ER(+) breast cancer [135]. This

is consistent with the hypothesis that increased DHEA androgenicity in PCa cells when co-cultured with reactive stroma could be due to a differential activation of AR target genes following loss of ERβ activity. Additionally, it has recently been shown that cross-talk between multiple transcription factors occurs preceding activation of some target genes [136]; by extension, it can be hypothesized that inactivation of ERβ via oxidation could disrupt cross-talk between it and AR and thus tip the balance in the direction of AR and androgenic signaling. This concept of steroid-receptor cross-talk and the resulting AR transcriptional imprint that differs as a result of loss of activity of ERβ is an area that remains largely unexplored as of yet. While castrate-resistant prostate cancer (CRPC) is defined by a lack of response to androgen deprivation, the PCa cells actually retain AR-dependence but their AR becomes more promiscuous and is activated by additional growth factors [30-34]. Using this model of CRPC, which represents advanced disease, one could seek to understand whether a loss of functional ERβ contributes to a different genomic signature of AR and thus leads to androgenic signaling in the absence of potent androgens. Specifically, I would determine if the AR cistrome differs in CRPC cells in response to both androgens and ERβ ligand precursors when ERβ is non-functional or absent. I would predict that in the absence of functional ERβ, DHEA or a similar precursor still undergoes the same metabolic conversions, but AR activity is enhanced due to a lack of competition from ERβ.

Furthermore, while Adiol is known to bind ERβ with a much higher affinity than either ERα or AR, in the absence of functional ERβ any Adiol produced has the potential to bind another available steroid receptor. This could lead to activation of pro-inflammatory pathways (ERα activation) and increased migration and proliferation (AR activation) simply by disrupting the balance of steroid receptor competition and cross-talk within the prostate. Using an RNA-seq approach, I would investigate the activation of both ERα and AR target genes in response to DHEA in CRPC cells in which ERβ has been selectively knocked down, hypothesizing that in the absence of functional ERβ both ERα and AR activity is increased. RNA-seq would allow gene expression profiling by measuring mRNA levels,

thus providing a powerful tool for analyzing the expression of AR and ERα target genes in CRPC cells lacking functional ERβ. The depth of information gained from this approach, however, will necessitate further analysis using pathway mapping tools, but I would predict to see an increase in inflammatory genes (stemming from increased ERα activation) as well as genes involved in cell proliferation, migration, and invasion (resulting from increased AR activation).

Additional future work might aim to better identify the mechanisms by which oxidative stress limits ERβ activity. Specific experiments such as chromatin immunoprecipitation (ChIP) have already produced preliminary data, and would be repeated to more confidently characterize the DNA-binding affinity of normal and oxidized ERβ at the E-cadherin promoter. Furthermore, a Western blot for oxidized proteins could be utilized to determine to what extent ERβ is oxidized in DU145 cells following exposure to physiological levels of $H_2O_2$ [138]. Additional experiments can be designed to determine if the oxidation of ERβ under these circumstances is reversible, and if ERβ activity can be restored following treatment of DU145 cells with either antioxidants or thiol-reducing agents.

Recent work has begun to uncover the importance of chromatin remodeling in gene activation, as elegantly shown by Susanne Mandrup et al. [136]. This group identified unique transcriptional "hotspots" (defined as co-occupancy by 2 or more transcription factors) that vary at different time points during adipogenesis. Building upon this same theory, studies would be designed to investigate ERβ activity at the E-cadherin gene at various time points. Moreover, the composition of various co-activator and co-repressor complexes that are recruited to the E-cadherin promoter along with ERβ following activation of the receptor by an androgen metabolite would be determined. Differential recruitment within the transcriptional complex could potentially underlie the different effects seen when ERβ signaling is activated under oxidative stress conditions, and this could again provide valuable information for the identification of future therapeutic targets.

Additionally, *in vivo* studies looking at the role of oxidative stress and ERβ in cancer cell motility will play an important role in further understanding the clinical implications of this work. A xenograft of WPMY-1/DU145 cells implanted under the renal capsule in a mouse model would provide the most simplistic method for studying paracrine interactions influencing cell motility *in vivo*. The sub-renal capsule model provides a method for easily measuring migration of PCa cells into surrounding normal tissue, and the near proximity of ample vasculature provides a method for hematogenous spread by more aggressive PCa cells. Using this model, I would treat with titrating doses of either a selective COX-2 inhibitor or antioxidants and upon sacrificing the animal I would look for evidence of increased motility (spread into adjacent structures or distant metastases) and decreased cell adhesion (E-cadherin staining within the implanted tumor). I predict that the co-administration of COX-2 inhibitors or antioxidants would significantly decrease the spread of the tumor and lead to an appreciable increase in E-cadherin expression. Importantly, I would isolate ERβ from tumor samples and assay its DNA-binding activity, hypothesizing that ERβ isolated from tumors in the absence of COX-2 inhibition or antioxidants would exhibit less DNA binding affinity due to oxidation. This *in vivo* work could highlight the importance of limiting oxidative stress within the prostate in order to take advantage of an inherent regulatory mechanism offered by the adjacent stromal cells regardless of their state of transdifferentiation.

The role of estrogens in the prostate is an emerging field displaying great therapeutic potential. However, past experience of using androgen-deprivation therapy has shown that targeting only one steroid receptor loses efficacy in a short period of time. It is likely to be an oversimplification, then, to suggest targeting only ERβ for limiting cell motility in PCa. Much of the future work proposed here suggests critical work for understanding the delicate balance between AR and ERβ in PCa. Over 20% of human cancers are associated with chronic inflammation, with prostate cancer often following the trend [132]. The redox-sensitivity of ERβ leaves it susceptible to a decrease in activity, perhaps favoring signaling through AR-dependent pathways. A better understanding of ERβ activation in the prostate,

coupled with the knowledge of how it affects AR, could potentially lead to highly specific therapies that intervene at key points to intercept multiple signaling pathways and thus provide greater clinical success.

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