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14. ABSTRACT -The tumor microenvironment plays a critical role in supporting cancer cells particularly as they disengage from limitations on their growth and motility imposed by surrounding nonreactive stromal cells. We show here that stromal-derived androgenic precursors are metabolized by DU145 human prostate cancer (PCa) cells to generate ligands for ERβ, which acts to limit their motility through transcriptional regulation of E-cadherin. While primary human PCa-associated fibroblasts and the human WPMY-1 reactive prostate stromal cell line maintain this inherent ERβ-dependent motility inhibitor activity, they are subverted by TGF-β1 pro-oxidant signals derived from co-cultured DU145 PCa cells. Specifically, stromal-produced H_2O_2, which requires Cox-2, acts as a second paracrine factor to inhibit ERβ activity in adjacent DU145 cells. ChIP analysis reveals that ERβ recruitment to the E-cadherin promoter is inhibited when H_2O_2 is present. Both neutralization of H_2O_2 with catalase and prevention of its production by silencing Cox-2 expression in stromal cells restore the motility-suppression activity of stromal-derived ERβ ligand precursors. These data suggest that reactive stromal cells may still have a capacity to limit cancer cell motility through a local endocrine network but must be protected from pro-oxidant signals triggered by cancer cell derived TGF-β1 to exhibit this cancer suppressive function.

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Introduction:

Prostate cancer is a leading cause of morbidity and mortality amongst westernized nations. However, a unique paradigm exists with this disease where many men will die with prostate cancer, while others will die from prostate cancer. We have yet to determine what contributes to a more aggressive form of disease and, once metastatic disease is present, few treatment options are available and within 2-3 yrs treatment generally becomes palliative. In this work, we sought to better understand the pathophysiology of this advanced, metastatic disease when our current treatments are ineffective. Specifically, we looked at the stromal microenvironment as a potential regulator of cancer cell motility.

Body:

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. Please see appended manuscript for a detailed description of this work (appendix A).

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.

Key Research Accomplishments:

- Identification of an inherent motility suppression network involving prostate stromal cells and PCa cells
- Generation of a stromal cell line with lentiviral-mediated Cox-2 knockdown
- Demonstration of loss of ERβ function secondary to oxidation

Reportable outcomes:

1. Publication of a data-driven manuscript

A local paracrine and endocrine network involving TGFβ, Cox-2, ROS, and estrogen receptor β influences reactive stromal cell regulation of **prostate** cancer cell motility.
Grubisha MJ, Cifuentes ME, Hammes SR, Defranco DB.
Mol Endocrinol. 2012 Jun;26(6):940-54. doi: 10.1210/me.2011-1371. Epub 2012 May 16.

2. Acquisition of doctor of philosophy degree from University of Pittsburgh, granted November 1, 2011

Conclusion:

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. 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. 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 but, more importantly, DHEA's role is primarily a precursor to either estrogenic or androgenic steroids. 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. 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. 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; 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. 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. 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. 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. 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.

Appendix A

Motility suppression by reactive human prostate stromal cells is subverted by cancer cell derived TGFβ

Abbreviated title: Local endocrine network suppresses motility

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Abstract

The tumor microenvironment plays a critical role in supporting cancer cells particularly as they disengage from limitations on their growth and motility imposed by surrounding nonreactive stromal cells. We show here that stromal-derived androgenic precursors are metabolized by DU145 human prostate cancer (PCa) cells to generate ligands for ERβ, which acts to limit their motility through transcriptional regulation of E-cadherin. While primary human PCa-associated fibroblasts and the human WPMY-1 reactive prostate stromal cell line maintain this inherent ERβ-dependent motility inhibitor activity, they are subverted by TGF-β1 pro-oxidant signals derived from co-cultured DU145 PCa cells. Specifically, stromal-produced H_2O_2, which requires Cox-2, acts as a second paracrine factor to inhibit ERβ activity in adjacent DU145 cells. ChIP analysis reveals that ERβ recruitment to the E-cadherin promoter is inhibited when H_2O_2 is present. Both neutralization of H_2O_2 with catalase and prevention of its production by silencing Cox-2 expression in stromal cells restore the motility-suppression activity of stromal-derived ERβ ligand precursors. These data suggest that reactive stromal cells may still have a capacity to limit cancer cell motility through a local endocrine network but must be protected from pro-oxidant signals triggered by cancer cell derived TGF-β1 to exhibit this cancer suppressive function.

Introduction

Since Paget first proposed his "seed and soil" hypothesis in 1889 [1], increasing attention has been paid to the tumor microenvironment for its role in tumor initiation, development, and progression. Furthermore, tissue recombination experiments with mixed prostate stromal/epithelial cell xenografts first revealed that transformation of epithelial cells is also accompanied by a "transdifferentiation" of fibroblasts that generates cells (i.e. cancer associated fibroblasts (CAFs) or reactive stroma) that either promote or are permissive to the ultimate formation of a cancerous lesion with metastatic potential [2-4].

One key signaling mediator in the transdifferentiation of fibroblasts into a "reactive" stromal phenotype is the cytokine Transforming Growth Factor β (TGFβ), most commonly the TGF-β1 isoform. The resulting change in fibroblasts to a myofibroblast phenotype is defined by a co-expression of both fibroblastic and smooth muscle markers, such as vimentin and smooth muscle α-actin, respectively [5]. TGFβ's role in tumor development and progression extends beyond its effects on stroma, but its impact on cancer cells is complex. Specifically, TGFβ is tumor suppressive during early phases of cancer development but switches to tumor promoting once the cancer is well established [6, 7]. A number of unique components of the TGFβ signaling pathway have been identified that appear to impact the execution of its diverse actions in cancer progression. Many components of the TGFβ signaling pathway are altered in cancers, and in some cases these changes are directly correlated with tumor grade, prognosis, or patient outcome [8-12].

In addition to aberrant TGFβ signaling, much attention has recently been paid to the role of chronic inflammation in the development of prostate cancer [13, 14]. Several characteristics of chronic inflammation are increased, such as infiltration by inflammatory cells (i.e. macrophages and leukocytes), induction of pro-inflammatory enzymes such as Cox-2, and production of reactive oxygen and reactive nitrogen species (ROS and RNS, respectively) [13]. These events are not mutually exclusive, with increased infiltration and induction of Cox-2 leading to some of the increased oxidative stress observed in chronic inflammation. Cox-2 is an inducible isoform of an enzyme responsible for catalyzing the conversion of arachidonic acid to bicyclic peroxides [15]. ROS are byproducts of this reaction, and thus Cox-2 can be a significant source of ROS in some cells [16-18]. ROS production by cancer cells is

necessary for an aggressive phenotype, and highly migratory and invasive PCa cell lines produce significantly more H_2O_2 than their noncancerous or less aggressive counterparts [19].

As with all tissues, the prostate possess inherent mechanisms for maintenance of homeostasis. In the normal prostate, growth factors such as TGFβ act to limit cell proliferation and maintain normal prostate size [20]. Additionally, the prostate is a steroid-dependent organ and relies heavily on a delicate balance of pro- and anti-proliferative signals stemming from various steroid hormones. Androgen biosynthesis and signaling plays a particularly prominent role in both normal and cancerous prostate biology, but there is increasing recognition for the importance of estrogen action in prostate biology [21]. Work done by Risbridger and colleagues highlights the importance of intraprostatic estrogen synthesis by aromatic conversion of androgens, and a series of knockout studies has implicated the ERα subtype in prostatic inflammation and malignancy [22]. ERβ signaling, on the other hand, acts to suppress prostatic growth and PCa cell motility [23]. The endogenous ERβ ligands in prostatic tissue are the androgen metabolites 3α-Adiol and 3β-Adiol, and their importance is underscored by high expression levels of the aldo keto reductase (AKR1C) enzymes, which serve to convert androgenic precursors into the Adiols within the prostate tissue [24, 25].

In this study, we sought to examine the basis for PCa cell response to reactive prostate stromal cells *in* vitro. Our results established a role for cancer cell-derived TGF-β1, acting in a paracrine signaling network via COX-2 dependent ROS production in neighboring stromal cells, to support aggressive PCa cell motility *in vitro.* However, we uncovered an inherent motility suppressive activity present in reactive stromal cells that we identified as an androgenic precursor to an ERβ ligand. Thus, despite their reactive phenotype, these cells are not genetically reprogrammed to fully support cancer progression but rather maintain an intrinsic capacity to limit cancer cell motility. Cancer cells subvert this innate suppression through enhanced production of short-acting mediators, namely ROS, in the surrounding microenvironment.

Results

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

We have used a modified scratch assay to examine the impact of stromal cell derived factors on PCa cell motility *in vitro*. This assay has been widely used and is well established to represent the classical "wound healing" migratory response in cells without the focus on chemotaxis seen in Boyden chambers [26]. We used this assay to assess motility of the androgen-independent DU145 human PCa cell line when co-cultured with human prostate stromal cells. Importantly, in the co-culture system that we employ stromal and PCa cells are not in direct contact but share a common growth medium.

Figure 1a displays the results of a scratch assay with DU145 cells co-cultured with either established human stromal cell lines (WPMY-1 and PS30), or with primary stromal cultures isolated from PCa tissue (PC116118 and PC115116). DU145 cells plated at ~90% confluence can close a constant diameter wound approximately 22% within 24hrs in low serum media. To exclude the influence of proliferation on wound healing, the doubling time of DU145 cells under the same low-serum conditions was determined to be approximately 40 hrs. While the motility of these cells is not significantly affected when co-cultured with CAFs or WPMY-1 cells, their movement is significantly reduced upon co-culture with PS30 cells. WPMY-1 cells exhibit a robust myofibroblast or "reactive" phenotype in contrast to the fibroblastic phenotype of PS30 cells. We therefore examined TGFβ signaling components in the two cell lines since it is an important contributor to the reactive stromal phenotype in the prostate [27]. Transient transfection of both the WPMY-1 and PS30 cell lines with 3TP-lux, a Smad binding element luciferase reporter construct, demonstrated that the WPMY-1 line has a significantly more robust response to exogenous TGF-β1 than PS30 cells (Fig 1b). Additionally, WPMY-1 cells express significantly higher levels of Smad proteins as indicated by Western blot analysis (Fig 1c). Thus unlike PS-30 cells, WPMY-1 cells are TGF-ß1 responsive and therefore susceptible in the co-cultures we employ to TGF-ß1 produced by DU145 cells [28].

To uncover the role of TGFβ signaling in modulating stromal cell regulation of PCa cell motility, we used an interfering TGF-β1 antibody in co-culture assays. As shown in Figure 1d, inhibition of TGFβ signaling

did not affect the 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 attain 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. As a direct test of this hypothesis, we isolated conditioned media (CM) from WPMY-1 cells grown overnight in 1% serum-containing media with or without exogenous TGF-β1 (5 ng/mL) and added this CM to freshly wounded naïve DU145 cells. Surprisingly, CM from WPMY-1 cells treated with exogenous TGF-β1 significantly inhibited DU145 motility (Fig 1e). Therefore, either TGF-β1 is necessary but not sufficient to block SMIF activity in WPMY-1 cells, or the effect of TGF-β1 on SMIF is mediated through a short-lived molecule.

Increased motility is a characteristic of epithelial-to-mesenchymal transition (EMT), and one hallmark sign of EMT is loss of the epithelial cell adhesion molecule E-cadherin. To assess whether the effect of CM on DU145 motility could be through induction of E-cadherin expression, we treated naïve DU145 cells with either control or WPMY-1 CM and then subjected the cells to western blot analysis for E-cadherin. As Figure 1f shows, CM significantly enhanced expression of E-cadherin, consistent with an increase in cell adhesion and thus a decrease in cell motility.

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

Hydrogen peroxide is one ROS ultimately generated in response to TGF-β1 that can participate in local paracrine signaling. 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 accumulation. As Figure 2a shows, a 3h TGF-β1 treatment significantly increased production of H_2O_2 at both 2 and 5 ng/mL doses. Thus, H_2O_2 presents 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 impermeable enzyme that metabolizes H_2O_2 to H_2O and O_2. We again included the primary stromal cultures to confirm that H_2O_2 was responsible for the permissive effect seen in the CAF co-cultures as well. As Figure 2b shows, while addition of catalase did not alter the highly motile phenotype of the DU145 cells, it reversed the permissive effect of CAFs and restored the activity of SMIF when added to DU145/myofibroblast and DU145/CAF co-cultures. Therefore, extracellular H_2O_2 is necessary to override SMIF activity 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.

Cox-2 is necessary for H_2O_2 Generation in WPMY-1 cells

TGF-β1 is an important component of stromal-epithelial cross-talk and is necessary for the transdifferentiation of stromal fibroblasts into a myofibroblastic reactive stroma phenotype [5, 27]. The mobilization of oxidant signaling pathways is utilized by TGF-β1 to influence paracrine communication. For example, TGF-β1 induces the expression of enzymes such as Cox-2 and some NOX isoforms that generate ROS. In fact, 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 subsequently abolished the Cox-2 mRNA induction in co-cultured WPMY-1 cells (Fig 3a).

To determine to what extent stromal-derived Cox-2 is involved in ROS generation that impacts cancer cell motility in co-cultures, we first stably expressed a Cox-2 shRNA (SH4) in WPMY-1 cells using a recombinant lentiviral vector along with control cells expressing a scrambled shRNA sequence (Scr). As basal levels of Cox-2 mRNA are very low in WPMY-1 cells, the efficiency of Cox-2 mRNA knockdown was analyzed via qRT-PCR following treatment of WPMY-1 or SH4 cells with 5 ng/mL TGF-β1. As Figure 3b shows, TGF-β1 induction of Cox-2 mRNA was reduced 65% in SH4 cells relative to Scr. Since extracellular H_2O_2 is necessary for the 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 [17], SH4 cells were subjected to an endpoint Amplex Red assay identical to the one performed on WPMY-1 cells in Fig 2a. As Fig 3c shows, TGF-β1 is unable to produce a significant

increase in H_2O_2 levels in WPMY-1 cells lacking inducible Cox-2, suggesting that Cox-2 is at least partially responsible for the TGF-β1 induced increase in H_2O_2 production.

Next, we sought to determine if ablation 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 and other fibroblast cells [29]. As shown in Figure 3d, 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 necessary for TGF-β1 inducible H_2O_2 in WPMY-1 cells, we included catalase (1500 units/mL) in co-culture scratch assays. As shown in Fig 3d, 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) maintain the ability to limit DU145 motility in co-culture. Additionally, stromal Cox-2 is necessary for the H_2O_2 production that is responsible for limiting the activity of SMIF.

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

To confirm that H_2O_2 could alter the activity of SMIF, we added varying physiologic concentrations of H_2O_2 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, H_2O_2 addition eliminated the motility inhibitory activity of the WPMY-1 CM (Fig 4a). Furthermore, H_2O_2 reverses the inhibitory effect of the SH4 cells on DU145 motility in co-culture (Fig 4b). 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 this metabolite of Cox-2 is unlikely to impact SMIF activity.

Our 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 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 4c 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 (see Fig 4a). These results imply that SMIF is not being oxidized, but rather that H_2O_2 is acting directly on DU145 cells to impact their response to SMIF.

The motility inhibitory activity of WPMY-1 cells acts via ERβ in DU145 cells

For an initial assessment of the identity of SMIF produced by WPMY-1 cells, we used a column fractionation technique 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 5a 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.

In lieu of further purification of the CM 5 kDa and below fraction, we assessed candidate low molecular weight inhibitors of cancer cell motility. Two androgen derivatives, 5α-androstane-3α,17β-adiol (3α-Adiol) and 5α-androstane-3β,17β-adiol (3β-Adiol) have been found to increase cancer cell adhesion and decrease cell motility in an ERβ-dependent manner [30, 31]. These androgen metabolites do not bind AR but are potent ligands for ERβ [31]. DU145 cells express the ERβ isoform but no detectable ERα [30-32]. Therefore, to determine if this pathway was responsible for oxidant-dependent inhibition of motility in co-culture, we examined the impact of H_2O_2 on the motility inhibitory activity of exogenous 3β-Adiol (10^{-6} M).

Consistent with previously published reports, 3β-Adiol suppressed DU145 cell motility [30, 31]. However, this inhibitory effect is reversed by H_2O_2 (Figure 5b). To further establish the role of ERβ in regulating the motility response of DU145 cells to WPMY-1 generated factors, we added 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) to the wound healing assays. As Figure 5c shows, addition of both

tamoxifen and PHTPP reverses the motility inhibitory effect of SMIF found in WPMY-1 CM. Thus, the WPMY-1 produced SMIF acts on ERβ in DU145 cells to limit their motility.

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 [31]. We have already shown that WPMY-1 cell CM induces E-cadherin expression in DU145 cells but, importantly, we 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 mRNA expression. As Figure 5d shows, WPMY-1 cell CM significantly increased E-cadherin expression, which is lost when H_2O_2 (10 μM) is present.

SMIF-Induced Activity of ERβ is Oxidation-Sensitive in DU145 Cells

H_2O_2 could interfere with ERβ action at multiple levels to limit the induction of E-cadherin brought about by stromal cell derived ERβ ligands or ligand precursors. For example, when DU145 cells are treated with H_2O_2, nuclear retention of ERβ is decreased (Figure 6a). While this is observed in both control and CM conditions, 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 decrease ERβ action at relevant targets at least in part by limiting its nuclear retention.

To determine if this decreased nuclear retention of ERβ in the presence of H_2O_2 was associated with a concomitant decrease in transcriptional activity, we next performed chromatin immunoprecipitation (ChIP) to quantify promoter occupancy by ERβ on the E-cadherin gene. Unpublished data from the lab of Benita Katzenellenbogen identified multiple ERβ binding sites both upstream of and within the promoter region of E-cadherin. Using data kindly provided by the Katzenellenbogen laboratory, we designed primer sequences around several of these ERβ binding sites. As Figure 6b shows, under control conditions there is minimal promoter occupancy, and this remains unaffected by the presence of H_2O_2. When CM containing active SMIF is added to DU145 cells, a significant increase in promoter occupancy is seen at two distinct ERβ binding sites within the E-cadherin gene. Importantly, this increased promoter occupancy is reversed when

H_2O_2 is present. This is in accordance with previously shown qRT-PCR data demonstrating a reduced induction of E-cadherin mRNA transcripts following addition of H_2O_2 (see Figure 5d).

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) [33]. DU145 cells express AKR1C enzymes and are capable of catalyzing redox reactions at the C17 position of steroid hormones [34, 35]. Previous work has shown that the androgenic precursor DHEA can be metabolized to ERβ ligands through the activity of these AKR1C enzymes; Specifically, the AKR1C3 subtype can convert DHEA directly to the highly potent androstene Δ5-3β-Adiol, and additional enzymatic steps involving the activity of 5α-reductase can lead to the production of the androstane, 3β-Adiol [36]. Thus, we hypothesized that SMIF was the androgenic precursor DHEA that the DU145 cells were metabolizing to an ERβ ligand. To test this hypothesis, we used the known AKR1C1-3 inhibitor flufenamic acid (FA) as well as the 5α-reductase inhibitor dutasteride [37]. As Figure 7a shows, DHEA inhibits DU145 motility. This inhibition is lost when FA is present, but not when dutasteride is added. Collectively, these data suggest that DHEA is being directly converted to the highly potent Δ5-3β-Adiol. The androstene Δ5-3β-Adiol, rather than the androstane 3β-Adiol, is responsible for the motility-suppressive effects of DHEA since dutasteride failed to reverse the suppressive effect of DHEA on DU145 motility. Since DU145 cells are AR negative, this suggests that DHEA is exerting its effects via a 3β-Adiol metabolite acting on ERβ. Similarly, FA reverses the inhibitory activity of CM, suggesting that DHEA derived from the CM is being metabolized to Δ5-3β-Adiol through an AKR1C-dependent pathway (Figure 7b).

To further confirm that WPMY-1 stromal cells produce DHEA as the androgenic precursor to Δ5-3β-Adiol production, we measured both testosterone and DHEA in stromal cell CM. As Table 1 shows, testosterone levels were undetectable in CM, but DHEA levels were significantly higher in CM versus control samples. While absolute levels of DHEA in CM were low, constitutive production by the stroma would likely be sufficient to maintain persistent ERβ activation through conversion to Δ5-3β-Adiol.

Given these data, we propose a working model to describe the bidirectional communication between the stromal and epithelial compartments with respect to the microenvironment's role in cancer cell motility.

Figures 7c and 7d indicate 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 to an ERβ ligand (i.e. DHEA) that is a potent inhibitor of PCa cell motility. However, 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. H_2O_2 produced in a Cox-2 dependent manner in stromal cells blocks the motility inhibitory effect of an ERβ ligand produced in cancer cells from stromal-derived DHEA.

Discussion

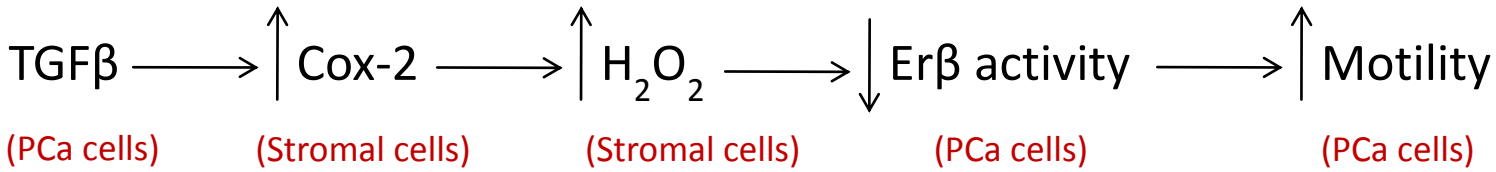
The development of PCa can be considered a co-evolution of both the epithelial and stromal cells; indeed, the latter develop their own unique gene signature during cancer progression that has potential predictive value in determining a patient's outcome [10]. Among some of the key features of a reactive stroma are heterogeneity in TGFβ signaling, acquisition of a myofibroblastic phenotype similar to that seen in normal wound healing, and an increase in oxidative stress as shown by elevated production of ROS [5, 19, 38]. The work presented herein, however, suggest that despite these alterations even reactive, cancer-associated stromal cells possess the inherent capacity to limit PCa cell motility through the production of a precursor to an ERβ ligand (i.e. DHEA). The loss of inhibition over cancer cell motility is determined not by an irreversible adaptation preventing production of DHEA by the stromal cell, but rather an alteration in the redox status of the surrounding milieu resulting from increased TGF-β1-dependent ROS production. By providing TGF-β1, cancer cells not only drive stromal cell transdifferentiation but subvert a local endocrine signaling network utilizing stromal cell steroid precursors that would otherwise limit their ability to migrate and invade surrounding tissue. Our results are consistent with clinical studies that establish a role for ERβ in preventing EMT and maintaining a lower grade of PCa. Tissue staining from PCa specimens demonstrates that high grade PCA shows a concurrent loss of both ERβ and E-cadherin [24]. Furthermore, an increased risk of PCa has been correlated with genetic mutations within the AKR1C family of enzymes responsible for metabolizing DHEA and other androgenic precursors into potent ERβ ligands [39, 40]. Importantly, we have demonstrated that the stromal cells, irrespective of their reactive phenotype, maintain the ability to produce a precursor to an ERβ ligand that acts to limit cancer cell motility. This is seen in both a myofibroblastic cell line (WPMY-1) as well as primary cultures of CAFs isolated from prostatectomy specimens (PC115116 and PC116118). The disruption of PCa ERβ signaling that we observe is not due to a genetic or epigenetic alteration, but rather is the result of increased H_2O_2 production by surrounding stromal cells that ultimately inhibits ERβ activity in adjacent PCa cells. This decrease in responsiveness is seen at both the biological and molecular level, as measured by a loss of inhibition in wound healing assays in which H_2O_2 is present (either exogenous or endogenously produced) and a failure of ERβ-dependent E-cadherin induction in DU145 cells when H_2O_2 is added concomitantly with the myofibroblast cell CM. ERβ has been shown previously to be sensitive to oxidation, which occurs

primarily from modifications in redox-sensitive cysteine motifs in the 2nd zinc finger. The resulting conformational change de-stabilizes ERβ and ultimately prevents its DNA binding [41, 42]. We observed a defect in nuclear retention of ERβ and a subsequent loss of induction of E-cadherin in DU145 cells exposed to H_2O_2 contained in stromal cell CM. These effects on ERβ signaling that impact the response to stromal derived ERβ ligands (or ligand precursors) could be due to direct oxidation of the receptor and/or other components of its signaling pathway.

We have identified stromal Cox-2 as a necessary component in the generation of H_2O_2 responsible for loss of ERβ signaling in the DU145 cells and, as such, a driving factor in the loss of motility inhibition by a myofibroblastic stroma. This is in accordance with previous work which shows that Cox-2 is overexpressed in primary PCa with metastatic potential. Of particular relevance is the work done by Rao et al. showing an inverse relationship between Cox-2 and E-cadherin expression in PCa tissue, which coincides with our model with describes an induction of stromal Cox-2 ultimately leading to decreased E-cadherin expression in adjacent PCa cells. Our model describing a bidirectional paracrine communication network further underscores the importance of considering the biological impact of Cox-2 in cells that comprise the tumor microenvironment and not just cancer cells themselves. In fact, a recent study demonstrated that overexpression of Cox-2 in the stroma of laryngeal squamous cell carcinoma specimens correlated with a worse tumor grade, suggesting an important role for Cox-2 in the cancer microenvironment [43]. Cox-2 is also overexpressed in the surrounding stroma in neoplastic and cancerous prostate tissue [44]. Finally, *in vivo* experiments with lung carcinoma cells showed that Cox-2 expressing tumor cells were unable to grow in a Cox-2 -/- host, highlighting the importance of stromal Cox-2 in cancer growth and progression [45]. Our results add to this growing interest in stromal-derived ROS signaling by identifying the consequences of a Cox-2 dependent signaling mediator (H_2O_2) on cancer cell response to a steroid metabolite produced within their microenvironment that would normally have the capacity to limit cancer cell motility.

The androgenic precursor, DHEA, is constitutively produced by the stroma and is subsequently metabolized by the cancer cell to the androgen derivative Δ5-3β-Adiol. While it is unable to bind AR, it is a potent activator of ERβ [30, 32]. Activation of ERβ by this Adiol induces E-cadherin expression and subsequently increases cell adhesion, ultimately leading to a decrease in motility [31]. The loss of motility inhibition in DU145 cells when H_2O_2 is present in either shared or conditioned media suggests that the

sensitivity of this cellular response is due to a redox sensitivity of this endocrine communication network. Since addition of H_2O_2 can inhibit exogenous Adiol action, it is unlikely that H_2O_2 influences DHEA metabolism in PCa cells, but rather that it acts directly on ERβ and thus results in subsequent loss of Adiol action. Blocking the production or action of the second paracrine factor, H_2O_2, at any point in the cascade, restores the cancer cells' ability to respond to locally produced DHEA/Δ5-3β-Adiol in an ERβ-dependent manner. A simple schematic describing the interrelation of TGFβ, oxidative stress, and motility in bidirectional communication between PCa and stromal cells is presented below.



In summary, we have highlighted the importance of oxidative stress within the local milieu with respect to permitting DU145 cancer cell motility despite constitutive production of DHEA by stromal cells. Interestingly, DU145 cells do not intrinsically respond to either TGF-β1 or H_2O_2, but use both molecules to favorably alter their microenvironment to allow their movement and spread to proceed unimpaired. Elucidation of this complex paracrine/local endocrine signaling interaction between cancer cells and their surrounding stroma provides multiple points for therapeutic intervention, and offers evidence to support the rational design of a new treatment paradigm for advanced aggressive prostate cancer. For example, FDA-approved Cox-2 inhibitors could be combined with additional antioxidants to ensure a reduction in local ROS production, thus restoring the activity of ERβ in the cancer cells to respond to locally produced ligands.

Materials and Methods

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, flufenamic acid, and catalase were purchased from Sigma-Aldrich (St. Louis, MO) and were reconstituted according to the manufacturer's instructions. 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). DHEA was purchased from Steraloids (Newport, RI). 3β-Adiol was purchased from Sigma-Aldrich (St. Louis, MO). Dutasteride was kindly provided by Zhou Wang of the UPCI. Antibodies against E-cadherin, Smad 2/3 and Smad 4 were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against ERβ were purchased from Millipore (Billerica, MA) and obtained from the lab of Benita Katzenellenbogen. Antibody against β-actin and secondary HRP-conjugated antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture

WPMY-1, PS30, PC3, 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 from the lab of William LaFramboise. The cells were maintained in monolayer at 37^0 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%.

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 a

dual-injector luminometer was used to record both firefly and Renilla luciferase relative light unit (RLU) values. Firefly luciferase values were normalized to Renilla.

Western blotting

Whole cell lysates were prepared by lysing WPMY-1 cells in RIPA buffer. 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 4^0 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 and room temp. Membranes were washed in PBS-T an additional 3 times, and enhanced chemiluminescence reagents were used to detect the HRP signal.

Nuclear and cytoplasmic fractionation was performed following the publicly available Lammond Lab protocol (http://www.lamondlab.com/pdf/CellFractionation.pdf). The nuclear and cytoplasmic fractions were then treated as previously detailed for the whole cell lysate protocol.

Knockdown assays

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 37^0 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.

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.

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% charcoal-stripped phenol-red free 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 -20^0 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.

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 2 shows the primer sequences that were used. Samples were run through an initial denaturation step of 95^0 for 10 min followed by 40 cycles of 95^0 for 30s, 55^0 for 1 min, and 72^0 for 1 min. Relative expression was determined using the comparative Ct method.

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 TGF-β1 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).

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 37^0 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 4^0 . 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 4^0 . 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 4^0 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 65^0 . 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 -20^0 1-2 hrs and then centrifuged at 13,000 rpm for 60 min at 4^0 . 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 3**Error! Reference source not found.** below lists the primers used for 2 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.

DHEA ELISA

Concentration of DHEA (pg/mL) in WPMY-1 CM was determined using the DHEA ELISA kit from Abnova (Taipei City, Taiwan) according to the manufacturer's protocol. Samples and standards were incubated with antibody overnight at 4º C. OD was read at 405 nm using a SpectraMax microplate reader.

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 ± SEM and are representative of ≥ 3 independent biological replicates. *p* values < .05 are considered significant.

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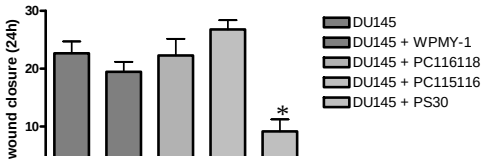
Figure 1. Paracrine signaling modulates cancer cell motility.
(A) In indirect co-culture, DU145 motility is unaffected by the presence of CAFs or WPMY-1 cells, but is significantly reduced by PS30 cells. Data represent the mean ± SEM from 3 independent experiments, each repeated in technical triplicate. A one-way ANOVA followed by Tukey's multiple comparison test was performed.*p<.05 compared to respective DU145 control.
(B) WPMY-1 and PS30 cells were transiently co-transfected with the 3TP-lux-luciferase (luc) and Renilla-luc reporter plasmids overnight, then subjected to a 6h TGF-β1 treatment the following day (0, 1 , 2, 5 10 ng/mL. 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
(C) WPMY-1 cells express higher levels of Smads 3&4. A representative blot is shown, and 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
(D) The left panel is representative images from the modified wound healing assay, the right panel is a graphical display of all replicates. 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.
(E) 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
(F) DU145 cells were treated for 24h with either 1% serum-containing media (control) or WPMY-1 CM, followed by western blot analysis for E-cadherin expression. The left panel shows an image from 3 independent samples; the right panel is a graphical representation of the normalized densitometry. A t-test was performed for pairwise comparison. * p< .05

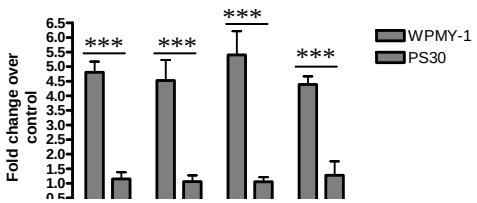
A.

Myofibroblasts and CAFs are Permissive of DU145 Motility

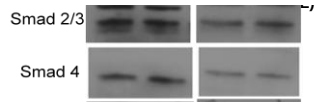


B.

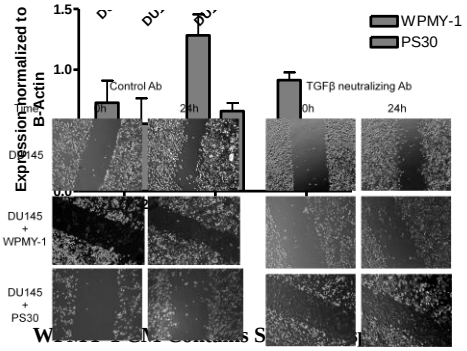
WPMY-1 Cells Possess a More Robust Response to Exogenous TGF-β1



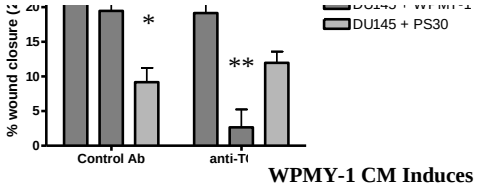
C. **WPMY-1 Cells Express Higher Smad Protein Levels**



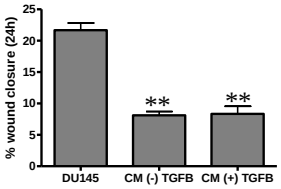
D.



Myofibroblasts Maintain an Inherent Motility Suppression in the Absence of TGF-β1



E. **WPMY-1 CM Contains Suppressive Factor(s) Exogenous TGF-β1 Treatment**



F.

WPMY-1 CM Induces E-cadherin Expression in DU145 Cells

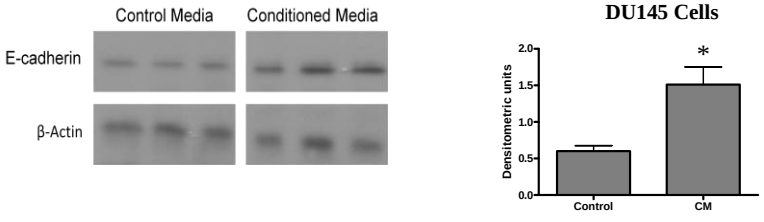
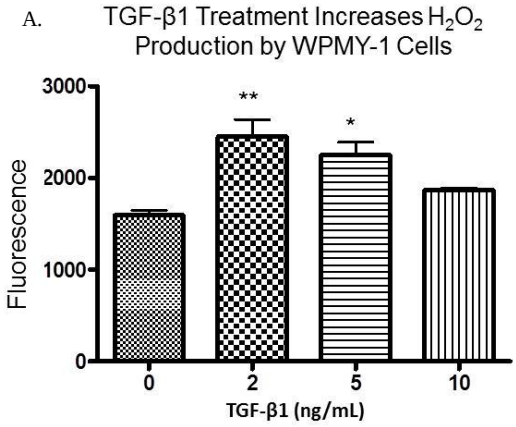


Figure 2. TGF-β1 treatment triggers H_2O_2 production in reactive stromal cells, which can limit SMIF activity. (A) In response to exogenous TGF-β1, WPMY-1 cells produce increased levels of H_2O_2 as measured by an endpoint Amplex Red assay. WPMY-1 cells were serum starved for 90 min before addition 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 reading was recorded at 1h. Data represent mean from 3 biological replicates ± SEM. A one-way ANOVA followed by Tukey's multiple comparison test was performed.* $p<.05$, ** $p< .01$ relative to untreated WPMY-1 control
(B) 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.* $p< .05$ comparing control co-culture to co-culture with the addition of catalase.

A.



B. **Catalase Restores SMIF Activity in Co-Cultures**

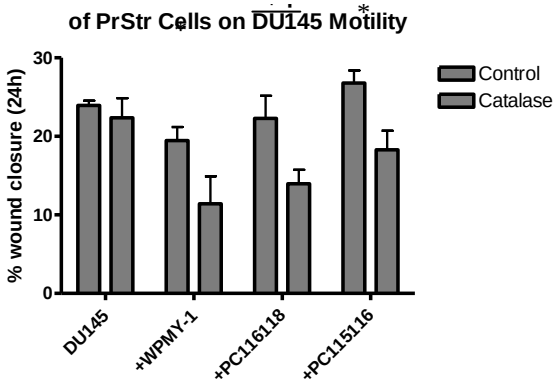


Figure 3. TGFβ-inducible Cox-2 in stromal cells generates the H_2O_2 necessary to limit SMIF activity and permit PCa cell motility in co-cultures.

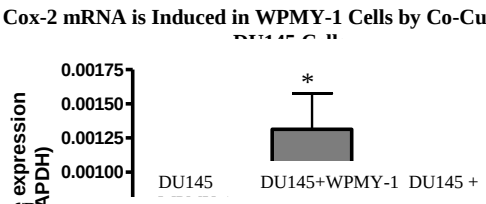
(A) A transwell insert containing DU145 cells was placed into a chamber containing serum-starved WPMY-1 cells, and the media was replaced with fresh 1% serum-containing RPMI -/+ a TGF-β1 neutralizing antibody. The cells were co-cultured for 24h and the Cox-2 levels in WPMY-1 cells were determined by qRT-PCR. Data represent the mean ± SEM from 3 independent experiments. A one-way ANOVA followed by Tukey's multiple comparison test was performed.* $p < .05$

(B) A lentiviral vector expressing shRNA either scrambled (Scr) or directed against Cox-2 (SH4) was used to stably infect WPMY-1 cells. The resulting lines were subjected to a 24h treatment with TGF-β1 in serum-free media (5 ng/mL) and Cox-2 mRNA levels determined by qRT-PCR. Data represent 3 independent experiments ± SEM. A one-way ANOVA followed by Tukey's multiple comparison test was performed. *$p<.05$

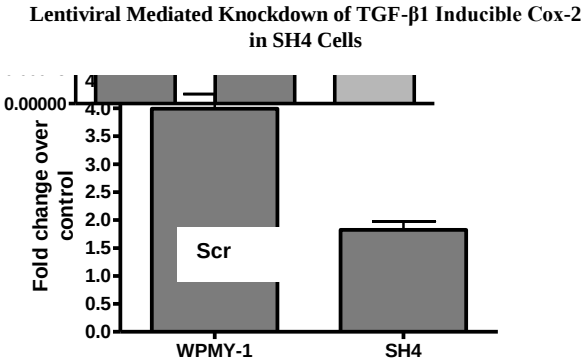
(C) SH4 cells were serum starved for 90 min before addition of TGF-β1 in fresh serum free media. Cells were incubated with the TGFβ for 3h, Amplex Red was added to co-cultures, and an endpoint reading was recorded at 1h. Data represent mean from 3 biological replicates ± SEM.

(D) 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

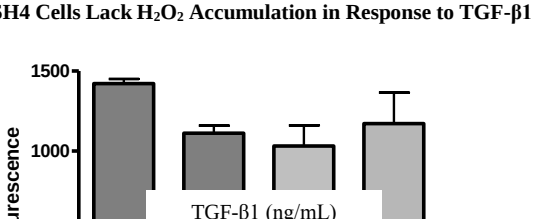
A.


Cox-2 mRNA is Induced in WPMY-1 Cells by Co-Culture with
Relatove expression (GAPDH)
0.00175
0.00150
0.00125
0.00100
*
DU145
DU145+WPMY-1
DU145 +

B.


Lentiviral Mediated Knockdown of TGF-β1 Inducible Cox-2 in SH4 Cells
Fold change over control
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Scr
WPMY-1
SH4

C.


SH4 Cells Lack H_2O_2 Accumulation in Response to TGF-β1
1500
1000
TGF-ß1 (ng/mL)

D.

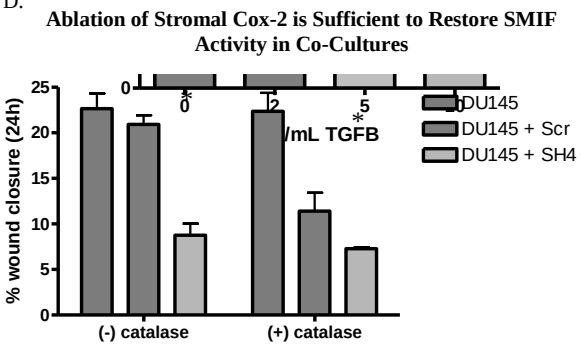
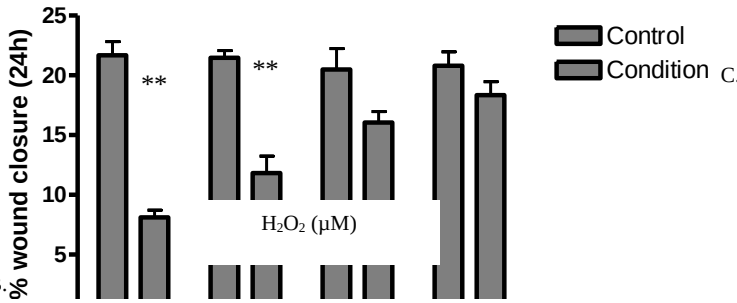

Ablation of Stromal Cox-2 is Sufficient to Restore SMIF Activity in Co-Cultures
% wound closure (24h)
25
20
15
10
5
0
*
*
/mL TGFB
DU145
DU145 + Scr
DU145 + SH4
(-) catalase
(+) catalase

Figure 4. Cox-2-dependent H_2O_2 generation in WPMY-1 cells inhibits DU145 cell response to SMIF.
(A) 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 ± SEM. A one-way ANOVA followed by Tukey's multiple comparison test was performed. **p<.01 relative to appropriate control media
(B) Addition of H_2O_2 (10 μM) to the DU145/SH4 co-culture reverses the motility inhibition observed 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
(C) 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

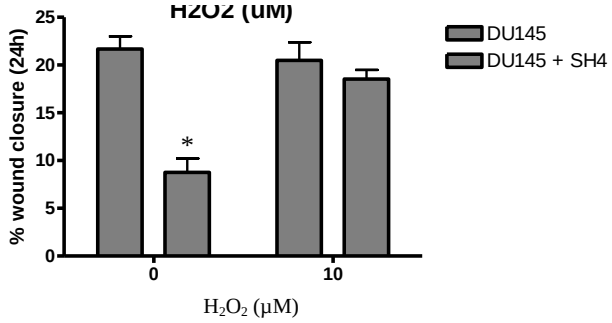
A.

Addition of H_2O_2 to CM Inhibits SMIF Activity



B.

H_2O_2 Addition Inhibits SMIF Activity in SH4 Co-Cultures



C.

SMIF Maintains Activity Despite H_2O_2 Pretreatment

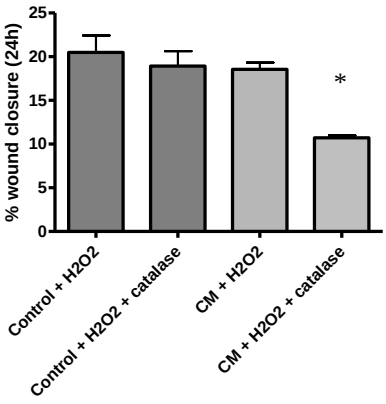


Figure 5. SMIF from WPMY-1 CM blocks DU145 motility in an ERβ-dependent manner

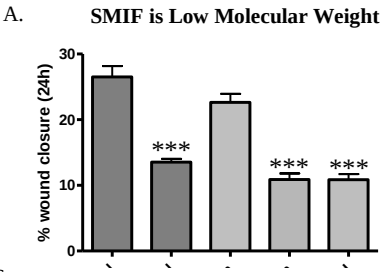
(A) WPMY-1 CM was fractionated into high and low molecular weight fractions, and each fraction was tested independently for biological activity. The low molecular weight fraction (pore size <5kD) retains inhibitory effects on DU145 motility. Data are representative of 4 independent biological replicates ± 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

(B) Addition of exogenous 3β-Adiol (10^{-6} M) is able to significantly inhibit DU145 motility. This 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

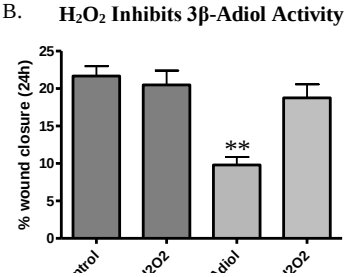
(C) Naïve DU145 cells were wounded and treated with either control or WPMY-1 CM with 4-hydroxytamoxifen (10^{-7} M) or PHTPP (0.1 μM). Results are representative of 4 independent biological replicates ± SEM. A one-way ANOVA followed by Tukey's multiple comparison test was performed. ***p<.001 compared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

(D) Naïve DU145 cells were treated for 18h with either control or WPMY-1 CM, -/+ 10 μM H_2O_2. The cells were harvested in Trizol and subjected to qRT- 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

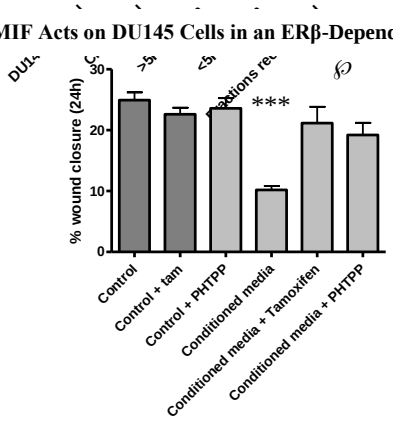
A.


SMIF is Low Molecular Weight
% wound closure (24h)
30
20
10
0
***
***
***

B.


H2O2 Inhibits 3β-Adiol Activity
% wound closure (24h)
25
20
15
10
5
0
**

C.


SMIF Acts on DU145 Cells in an ERβ-Dependent Manner
% wound closure (24h)
30
20
10
0
***
Control
Control + tam
Control + PHTPP
Conditioned media
Conditioned media + Tamoxifen
Conditioned media + PHTPP

D.

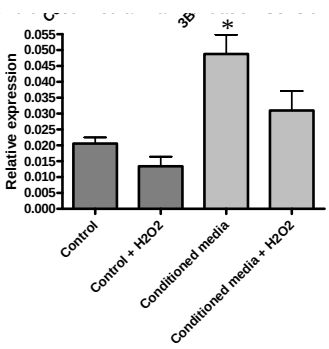
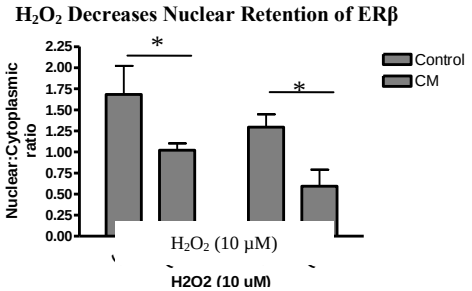

SMIF Induces E-cadherin in a Redox-Sensitive
Relative expression
0.055
0.050
0.045
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
*
Control
Control + H2O2
Conditioned media
Conditioned media + H2O2

Figure 6. Oxidized ERβ exhibits less DNA binding at the E-cadherin promoter

(A) 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

(B) DU145 cells were grown in either control or CM -/+ H_2O_2 (10 μM) for 18h. ChIP using ERβ antibodies was performed, and qRT-PCR analysis for previously defined ERβ binding sites within the E-cadherin promoter region revealed a significant increase in promoter occupancy in the presence of CM. This increase was reversed when H_2O_2 was present in CM. Results are representative of 3 independent experiments. A one-way ANOVA followed by Tukey multiple comparison test was performed. *p<.05 compared to all other conditions at that promoter site

A.


H2O2 Decreases Nuclear Retention of ERβ
Nuclear:Cytoplasmic ratio
2.25
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
*
Control
CM
H2O2 (10 µM)

B.

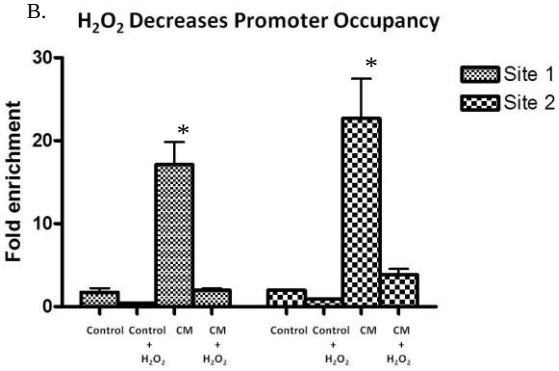
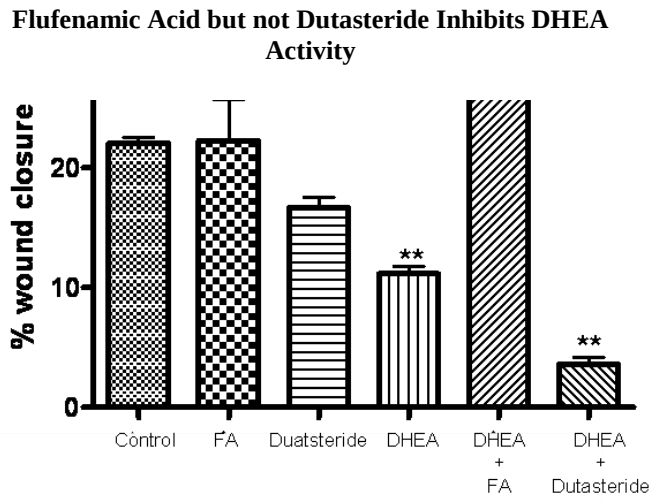
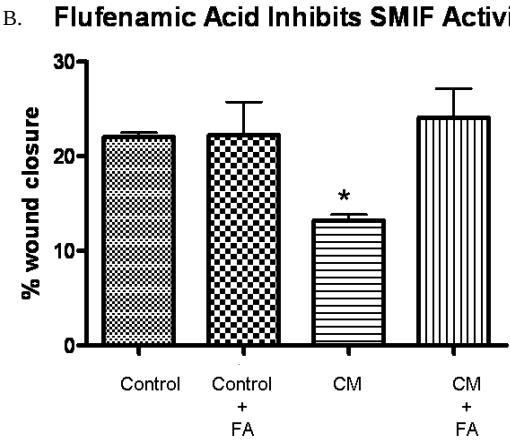

H2O2 Decreases Promoter Occupancy
Fold enrichment
30
20
10
0
*
*
Site 1
Site 2
Control
Control + H2O2
CM
CM + H2O2
Control
Control + H2O2
CM
CM + H2O2

Figure 7. SMIF is an androgenic precursor metabolized by the DU145 cells to an ERβ ligand.
(A) Flufenamic acid, but not dutasteride, blocks the inhibitory activity of DHEA. Wound healing assays were performed on DU145 cells with either and DHEA (100 nM), flufenamic acid (50 μM), or dutasteride (1 μM) where indicated. Results are representative of 4 independent experiments, each done in technical quadruplicate. A one-way ANOVA followed by Dunnett's multiple comparison test using untreated DU145 cells as the control column was performed. ** $p < .01$
(B) Flufenamic acid inhibits SMIF activity in CM. Wound healing assays were performed on DU145 cells treated with CM ± FA (50 μM). Results are representative of 4 independent experiments, each done in technical quadruplicate. A one-way AONVA followed by Tukey's multiple comparison test was performed. * $p<.05$
(C) In a normal fibroblast, DHEA is constitutively produced and secreted. It is metabolized by 17βHSD enzymes in the DU145 cells into potent ERβ ligands, which then act to limit motility.
(D) 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.

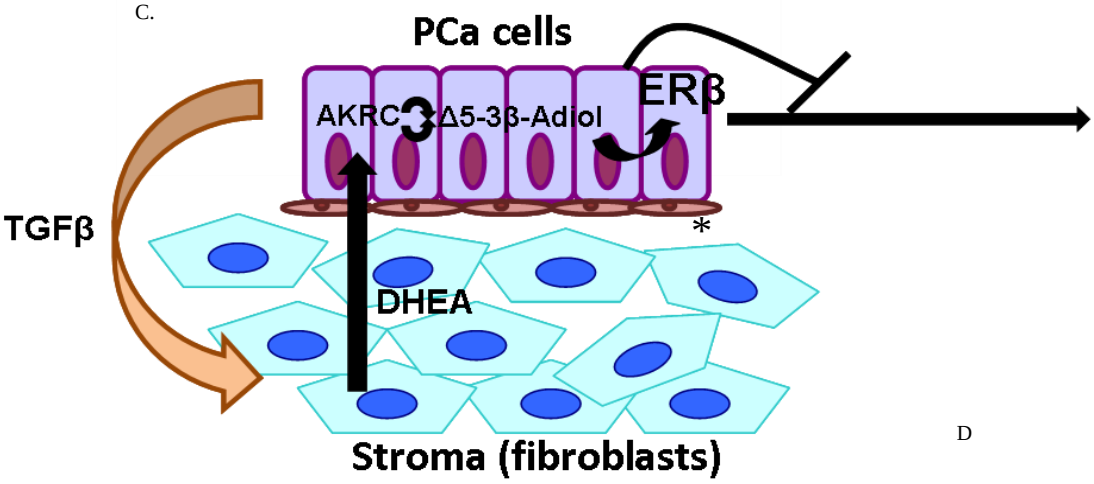
A


Flufenamic Acid but not Dutasteride Inhibits DHEA Activity
% wound closure
0
10
20
**
**
Control
FA
Duatsteride
DHEA
DHEA + FA
DHEA + Dutasteride

B.


Flufenamic Acid Inhibits SMIF Activi
% wound closure
0
10
20
30
*
Control
Control + FA
CM
CM + FA

C.


PCa cells
AKRC3
Δ5-3β-Adiol
ERβ
*
TGFβ
DHEA
Stroma (fibroblasts)

D

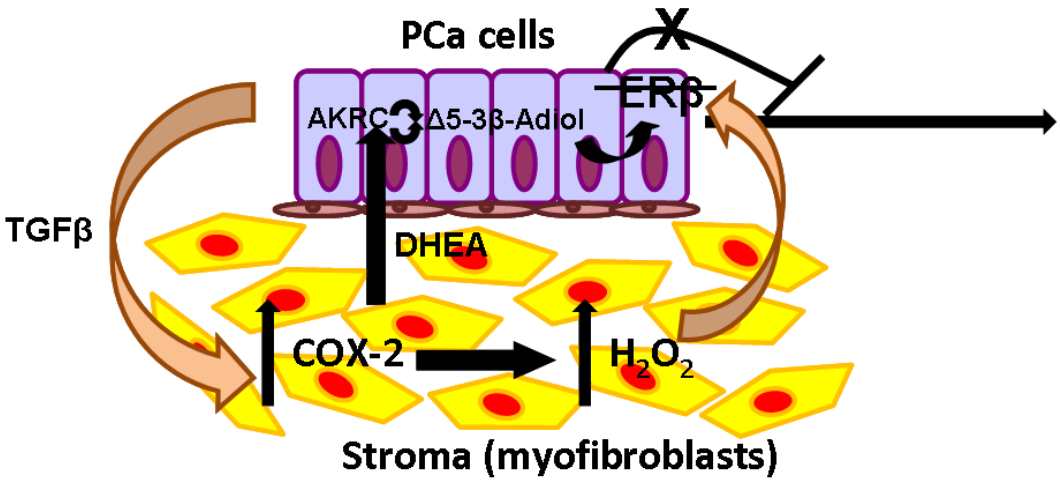

PCa cells
X
AKRC3
Δ5-3β-Adiol
ERβ
TGFβ
DHEA
COX-2
H2O2
Stroma (myofibroblasts)

Table 1. Testosterone and DHEA in WPMY-1 CM

Table 2. Primer sequences for Real-time 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’