Stromal Cell Regulation of the Prostate Epithelial Stem Cell Niche

BY

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THESIS

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DEDICATION

I dedicate this thesis to Andrea Sejba.

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LIST OF ABBREVIATIONS

BrDU – Bromodeoxyuridine

CAF – Cancer-associated fibroblast

CSC – Cancer stem cell

DMSO – dimethylsulfoxide

E2 – 17β-Estradiol

ECM – Extracellular matrix

EDTA – ethylenediaminetetraacetic acid

ERE – Estrogen receptor-α-transfected + estradiol

ERV – Estrogen receptor-α-transfected + (ethanol) vehicle

EtOH – Ethanol

EV – Empty vector

EVV – Empty vector + (ethanol) vehicle

FBS – Fetal bovine serum

GPER – membrane-bound, G protein-coupled estrogen receptor

HSC – Hematopoietic stem cell

IL1b – Interleukin 1-b

IL-6 – Interleukin 6

LAMA5 – Laminin α5 gene

MEOH – Methanol

MSC – Mesenchymal stem cell

NGS – Normal goat serum

qPCR – quantitative real-time polymerase chain reaction

PBS – Phosphate-buffered saline

PBST – Phosphate-buffered saline with Triton X-100 detergent

PrEGM – Prostate Epithelial growth media

PrEC – Normal primary human prostate epithelial cells

PrSC – Normal primary human prostate stromal cells

PS – Prostasphere

PVDF – polyvinylidene difluoride

RIPA – Radioimmunoassay Precipitation Assay

Sca – Primary cancer-associated stromal cells derived from older donors

SCBM – Stromal cell basal medium

SCBM – Stromal cell conditioned medium

SCN – Stem cell niche

Spz – Normal primary stromal cells derived from older donors

TGFα – transforming growth factor alpha

TNFα – tumor necrosis factor alpha

UGE – urogenital sinus epithelium

UGM – urogenital sinus mesenchyme

VEGF – Vascular endothelial growth factor

I. INTRODUCTION

A. Background

1. Prostate cancer

Prostate cancer (PrCA) is the most common non-cutaneous malignancy in men in the United States and most Western developed nations[1]. More than 220,000 new cases were estimated to have been diagnosed in 2015, and more than 27,000 men were estimated to die of the disease. The clinical course of the disease is highly variable, with the cancer quickly progressing to death in some men, while remaining latent in others. This variable presentation is likely a manifestation of the molecular heterogeneity of prostate cancer, which has thus far hampered scientists' efforts to classify the disease into clinically-relevant biological classes[2]. Developing a better understanding of the molecular mechanisms that govern normal prostate biology—including elucidation of the cell or cells of origin in prostate cancer—will be a critical step toward the advancement of therapies, diagnostics and biomarker discovery.

Many of the most important mutations and pathway alterations in other cancers are also involved in prostate cancer, including p53 mutations and dysregulation of cell cycle-related genes[2]. Other alterations are more common to prostate cancer, including translocations involving the ETS family of transcription factors, and upregulation of the PI3K/Akt pathway due to PTEN deletions or mutations, which occur in nearly 70% of all prostate cancers[3,4]. Recently, efforts of The Cancer Genome Atlas (TCGA) to molecularly profile and sequence large numbers of prostate tumors, while confirming the heterogeneity of the disease, have nonetheless revealed new classes

of genes that are commonly mutated or otherwise altered, including SPOP and FOXA1 mutations[5]. Despite these continued advancements in understanding the molecular mechanisms of PrCA, one pathway remains of utmost importance—the androgen signaling pathway.

The prostate gland is an androgen-regulated tissue in normal development and in cancer. Nobel Laureate Charles Huggins first described in 1941that chemical castration induced tumor regression in prostate cancer[6]. More than 60 years later, androgen-deprivation therapy (ADT) remains the mainstay treatment for advanced or metastatic PrCA, with most men responding favorably; however, tumors invariably become resistant to ADT and progress to an advanced castration-resistant prostate cancer (CRPC) stage[7]. To date, most research has focused on the role of androgens in prostate cancer and the development therapeutics that might remain effective or prevent advancement to CRPC. Despite the development of potent new androgen receptor or androgen synthesis inhibitors such as enzalutamide and abiraterone, the ability of the disease to progress toward CRPC has persisted[8]. Therefore, it is critical that prostate cancer research continue to focus on other mechanisms which might be important in the development and progression of the disease, including pursuing a more detailed understanding of the roles of other hormones in prostate cancer. A prime candidate in this regard is estradiol, which has been well-implicated in both prostate health and disease[9].

2. The Role of Estrogen in Prostate Cancer

After the initial discovery that PrCA is an androgen-driven disease that can then be therapeutically targeted, estrogen was used as a treatment, exploiting its ability to induce chemical castration through feedback inhibition of the hypothalmopituitary axis[6]. However, estrogens are also known cancer-causing agents, with 17β-estradiol being classified as a

carcinogen by International Agency for Research on Cancer [10]. Additionally, several metabolites of estradiol have direct genotoxic effects. Although there is no conclusive evidence showing that estradiol acts as a direct mutagen, there is considerable evidence that estrogens play key roles in prostate carcinogenesis and progression[11-13]. Nevertheless, epidemiologic studies that have examined the correlation between circulating hormone levels and the incidence of prostate cancer have yielded conflicting data[14]. A meta-analysis showed that, with the exception of androstanediol glucuronide, there are no differences in circulating sex hormones between men with prostate cancer and disease-free controls[15]. However, serum estrogen levels are higher in African-Americans versus Caucasian men, which is significant since there is a nearly 2-fold higher rate of PrCA in African American men[16]. Furthermore, a wealth of literature implicates estrogen in the development and growth of the prostate gland, primarily through direct actions on ERα in the stroma and ERβ in epithelial cells[17,18]. Estradiol also indirectly influences prostate development by modulating prolactin levels via the hypothalamopituitary—gonadal axis.

3. Anatomy and function of the human prostate

The prostate is one of the male accessory sex glands and contributes an alkaline secretion containing proteolytic enzymes into the seminal plasma[19]. The parenchyma of the prostate is composed of three main epithelial cell types—a continuous sheet of polarized, secretory luminal cells, a single layer of basal cells, and rare neuroendocrine cells. These cell types exist in well-formed tubuloalveolar glands, which coalesce into 20-30 ductules that empty into the prostate sinuses, which then empty into the urethra[20].

The stroma of the human prostate is composed primarily of smooth muscle cells in the periacinar stroma, and intermixed smooth muscle cells and fibroblasts elsewhere. Throughout the stroma

there are also blood vessels, lymphatic vessels, nerves, adipose cells and circulating immune cells. The prostate stroma plays a fundamental role in the development and homeostasis of the gland. Cunha, *et al.*, published a seminal paper describing how androgen receptor positive urogenital sinus mesenchyme (UGM) is necessary and sufficient to induce formation of prostatic buds from the urogenital sinus epithelium(UGE)[21]. Thus, UGM is instructive towards the epithelium, translating cues from circulating hormones into paracrine factors that can determine lineage-specification of neighboring UGE stem cells. In the adult gland, stromal cells have been well-studied, but with limited research on stromal interactions within the normal epithelial stem cell niche.

Stromal-epithelial interactions within the cancerous prostate gland have been studied in much greater detail[22]. It is hypothesized that the malignant epithelial cells induce an altered phenotype in the neighboring stroma, which are referred to as cancer associated fibroblasts (CAFs). It should be noted, however, that *in vivo* CAFs are composed primarily of smooth muscle cells with a minor fibroblast population. Nevertheless, these CAFs have an altered transcriptional program that upregulates secretory products and engages in an exchange of factors and molecules that promote altered metabolism, survival, growth and proliferation within the tumor[23]. As with other organ systems, these stromal-epithelial interactions promote a vicious cycle at the microenvironmental level.

4. Estrogen Receptors

Three estrogen receptors are known to be expressed in the prostate, the nuclear transcription factors ERα and ERβ and the membrane-bound, G protein-coupled estrogen receptor (GPER; previously known as GPR30). Although differentiated epithelial cells primarily express ERβ and

stromal cells primarily express ERα, the expression levels and functional significance of each receptor changes throughout development. Both ERα and ERβ function as classical nuclear receptors that, upon ligand-binding, dimerize and bind to estrogen-response elements of estrogen-regulated. Based on the recruitment of either co-activators or co-repressors, the dimerized receptors then induce or inhibit gene transcription. In addition, both ERs function as mediators of membrane-initiated rapid signaling[24]. Although estradiol may have different actions on prostate stem cell through either membrane-initiated rapid signaling or nuclear-initiated genomic signaling, these distinct actions will not be a focus of this research.

5. Stem cells and the prostate stem cell niche

Stem cell fate is governed by intrinsic and extrinsic factors. Intrinsic factors control stem cell self-renewal and differentiation in a cell-autonomous manner and include intracellular signaling molecules, chromatin remodeling enzymes, transcription factors and non-coding RNA such as miRNA and piRNA[25]. Although intrinsic factors ultimately determine whether a stem cell remains quiescent, undergoes symmetric self-renewal, or differentiates via asymmetric self-renewal or committed differentiation, a network of extrinsic factors impinges upon the intrinsic network to integrate the needs of the tissue in regulating stem cell niche dynamics. Extrinsic factors include circulating hormones, locally produced paracrine factors, cellular adhesion molecules, extracellular matrix (ECM) molecules and a host of other factors. With the exception of circulating hormones, these factors are products of cells within the stem cell niche. Specific cell types within the SCN vary from tissue to tissue, but in the mouse include: mesenchymal cells, osteoblasts and osteoclasts in the HSC niche; fibroblasts and hematopoietic cells in the intestinal crypt niche; Leydig, Sertoli and vascular cells within the spermatogonial SCN[26].

The human prostate stem cell niche is incompletely understood in terms of its anatomical location, histologic characteristics, cellular constituents and hierarchical differentiation. In the adult human, the prostate stem cells reside among the basal epithelial layer of cells. There are two schools of thought on how prostate stem cells form differentiated luminal and basal epithelial cells. The first hypothesis postulates that a single multi-potent stem cell differentiates into a multipotent progenitor cell, which then differentiates into basal- or luminal-specific progenitor cells. These lineage-specific progenitor cells then give rise to the fully differentiated epithelia. The second hypothesis states that there are two separate populations of unipotent prostate stem cells—basal stem cells and luminal stem cells. Each of these divides to form lineage-specific differentiated basal and luminal cells. A diagram of both lineage models is shown in Figure 1.

There are three fundamental properties of a prostate stem cell: 1) slow growth rate / relative quiescence, 2) high replicative potential, and 3) ability to regenerate a prostate-like gland[27]. During development of the prostate gland, the stem cells undergo relatively high proliferation in order to generate sufficient progeny to form the gland. In the adult gland, however, the stem cells remain in a relatively quiescent state due to both inherent growth-control mechanisms and cues from the niche. The signaling pathways that control this growth and the decisions to enter self-renewal are both autonomous, originating from the stem cells themselves, and extrinsic, originating from progenitor cells, stromal cells, and other cells within the niche. These pathways, many of which are particularly important in mediating stromal-epithelial interactions within the stem cell niche, include the BMP/TGFβ, Wnt/βcatenin, FGF, Notch, Hedgehog and Ephrin pathways[20].

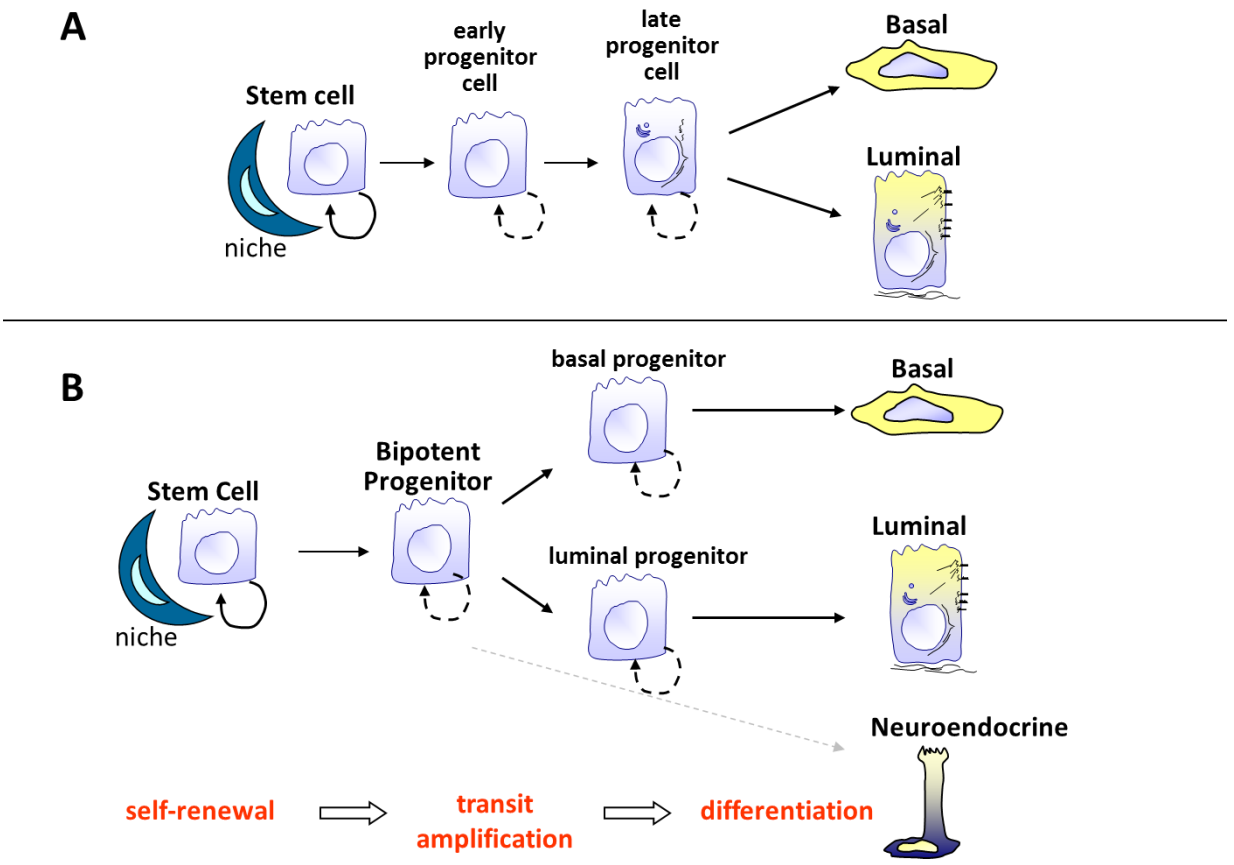


Figure 1. Two models of stem and progenitor cell lineage hierarchy in the human prostate stem cell niche.

A) The multipotent progenitor model posits that stem cells divide to give rise to a progenitor cell that is capable of differentiating into two (or three) separate lineages. B) The unipotent model posits that stem cells give rise to bipotent early progenitor cells, which then give rise to unipotent progenitor cells committed to a particular lineage.

During the past decade, the field of cancer research has become more focused on stem cells for several reasons. First is the observation that cancer cells have many of the same characteristics as stem cells: the ability to divide nearly indefinitely, to resist apoptosis, to move within a tissue or even spread into other tissues, and acquisition of altered metabolic states, among others. The second reason relates to the hypothesis that stem cells are themselves oncogenic targets and tumor-initiating cells[27,28]. Since stem cells are long-lived and quiescent, they may be susceptible to the accumulation of genetic mutations (Figure 2). Finally, the existence of cancer stem cells within most tumors is thought to play a fundamental role in tumor development and resistance to therapy.

6. Cancer Associated Fibroblasts

During tumor development and progression, malignant epithelial cells secrete factors into the microenvironment such as TGFβ and IL-6 that have the ability to transform normal stromal cells into an activated state. Although these activated stromal cells are termed cancer associated fibroblasts (CAFs), they are not necessarily fibroblasts. As discussed in Section IV, CAFs might actually arise from several different cell types other than resident normal stromal cells. The role of cancer associated fibroblasts in promoting cancer progression and metastasis is well-documented[29-31]. Through the secretion of mitogenic growth factors, cytokines and other soluble factors, CAFs can enhance growth and proliferation of tumor cells[29]. Similarly, via the secretion of extracellular matrix (ECM) constituents, as well as ECM-modifying matrix metalloproteases, CAFs are able to alter the mechanical and biological properties of the ECM[32]. This, in turn, can elicit diverse responses from tumor cells, such as perturbations in cell cycle regulation via integrin-mediated signaling or escape into the microvasculature aided by a degraded ECM[29].

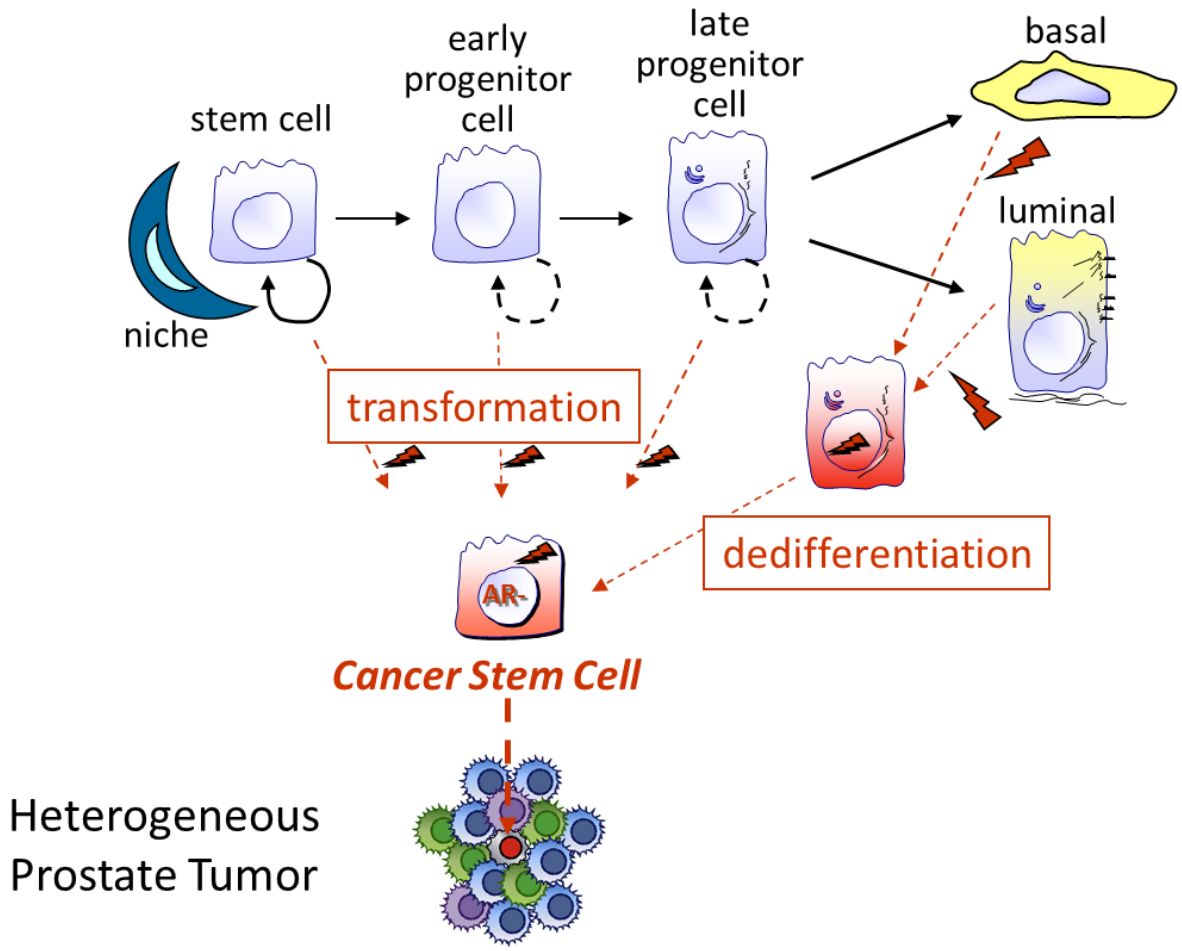


Figure 2. Model of stem and progenitor cell transformation within the prostate stem cell niche.

Cancer stem cells may arise from transformation of long-lived epithelial stem and progenitor cells. Alternatively, differentiated basal, luminal or neuroendocrine (not shown) cells may undergo transformation followed by dedifferentiation into a cancer stem cell.

These interactions are not unidirectional. Indeed, tumor cells secrete factors that are known to maintain CAFs in an activated state. Furthermore, CAFs and tumor cells are known to engage in reciprocal exchange of metabolic intermediates[33]. It is important to note, however, that some studies suggest that the pro-tumorigenic effects of CAFs are stage-dependent in certain cancers, with CAFs inhibiting tumor growth in very early stages[29]. On the other hand, some studies have provided evidence that CAFs can promote or even mediate carcinogenesis. In a tissue recombination model, human CAFs engrafted with initiated human epithelial cells induced prostate intraepithelial neoplasia (PIN)-like lesions, while normal stromal cells did not induce PIN-like lesions[34]. Furthermore, prostate carcinoma can be induced in a hormonal carcinogenesis model with testosterone and estrogen, whereby human BPH-1 cells that do not express AR or ERs are engrafted into mice along with rat urogenital sinus mesenchyme, which does express AR and ERs[35]. Under such a model, hormonal carcinogenesis is thought to be mediated through stromal hormone receptors. Whether or not these models of carcinogenesis involve stromal or CAF effects on stem or progenitor cells is unknown. Regarding CAF effects on cancers progression, however, there is strong evidence suggests that CAFs exert part of their pro-tumorigenic effects by engaging with cancer stem cells (CSC)[23,36].

B. Hypothesis and Specific Aims

The molecular mechanisms underlying developmental morphogenesis have long been suspected to also play important roles in cancer. Stem cells, being fundamental to both of these processes, can be the cells-of-origin in those cancers whose pathogeneses involve subversion of developmental signaling pathways. Therefore, cells and signaling pathways that regulate stem cells during development are likely to also play a role in carcinogenesis.

Seminal work by Cunha in the 1970's showed that the prostate stroma is essential for normal androgenic development of the prostate gland, including epithelial growth and differentiation[21]. Thus, it stands to reason that key aspects of stromal regulation of epithelial development are mediated through control of the epithelial stem cells, but this has yet to be proven. Given the compelling evidence that stem cells are preferential cells-of-origin in many cancers, it is essential that the regulatory mechanisms governing normal prostate stem cell homeostasis are elucidated so that this knowledge can be exploited towards development of novel treatments for prostate cancer growth regulation.

Recent evidence from our laboratory suggests that estrogens are key modulators of prostate epithelial stem and progenitor cells. Furthermore, it has been shown by our laboratory and others that estrogens can act as carcinogens in rodent and human models of prostate cancer[11,13]. When these two facts are considered along with the well-documented role of estrogen receptor signaling in prostate stromal cells, an intriguing model emerges whereby aberrant stromal estrogenic control of epithelial stem cells may contribute to prostate carcinogenesis. To test the hypothesis that **prostate stromal cells secrete paracrine factors that regulate epithelial stem and progenitor cells in both normal and cancer niches, and that 17β-estradiol modulates the secretion of these factors**, the following specific aims were proposed for this thesis work:

1. Elucidate how stromal cells modulate prostate stem and progenitor cell homeostasis within the normal adult human stem cell niche.

2. Determine how estrogen signaling modulates the stromal-epithelial signaling axis within the normal adult human stem cell niche.

3. Delineate the role of stromal cells in the prostate cancer stem cell niche.

AIM 1: Elucidate how stromal cells modulate prostate stem and progenitor cell self-renewal, proliferation, lineage specification and differentiation within the *normal* adult human stem cell niche.

An *in vitro* prostasphere (PS) system allowed culture of enriched primary human prostate stem and progenitor cells. By culturing PS with primary human stromal cells (PrSC)—either via direct co-culture or with conditioned media—epithelial stem cell niche interaction with stromal cells were modeled. The stromal effects on PS growth and stem/progenitor cell self-renewal were monitored by established growth and differentiation assays and specialized stem cell assays. The signaling mechanisms mediating these effects were identified by bioinformatics analysis of stromal gene expression and by ELISA-based assays for secreted-factors.

AIM 2: Determine how estrogen signaling modulates the stromal-epithelial signaling axis within the *normal* adult human stem cell niche.

The indirect, stromal-mediated effects of estradiol on PS growth and stem/progenitor cell self-renewal were monitored by established growth and differentiation assays and specialized stem cell assays. The paracrine signaling pathways that mediate these estrogenic effects between stromal and epithelial stem-progenitor cells were identified by bioinformatics analysis of stromal gene expression and by ELISA-based assays for secreted-factors. Normal human prostate cells—including the stromal WPMY-1 cell line, primary human prostate stromal cells (PrSC), and the epithelial stem cell line WPE-stem— were used to selectively up- or down-regulate ERα using lentiviral gene expression constructs.

AIM 3: Delineate the role of stromal estrogen signaling in the prostate *cancer* stem cell niche.

Although adenocarcinoma of the prostate is, by definition, only a malignancy of the epithelial cells, the associated stromal cells can be activated into cancer associated fibroblasts (CAFs) that support growth of the tumor. Therefore, primary human PrSC or primary human CAFs were co-cultured with either normal PS or PS derived from prostate cancer cell lines to determine if estrogen signaling through CAFs plays a fundamental role in tumor support, progression, or reprogramming of adjacent tissues within the *malignant* stem cell microenvironment.

This work was performed using patient- and donor-derived primary cells in order to maximize the translational relevance of this work, while at the same time reducing artifacts often introduced by monoclonal, immortalized cell lines.

II. MATERIALS AND METHODS

A. *In vitro* modeling of stromal-epithelial interactions within the prostate stem cell niche

1. Culture of primary prostate epithelial and stromal cells, and established cell lines

Normal primary human prostate stromal cells (PrSC) from two separate young, disease-free donors were acquired from Lonza Group. PrSC were cultured in Lonza Stromal Cell Basal Medium with manufacturer-supplied supplements: 5% fetal bovine serum, hFGF-B, human recombinant insulin, gentamicin-amphotericin. Normal primary human prostate epithelial cells (PrEC) from three separate young, disease-free donors were also acquired from Lonza Group. PrEC were cultured in ProstaLife Prostate Epithelial growth media (PrEGM; Life Technologies) with manufacturer-supplied supplements: L-glutamine (6mM), extract-P (0.4%), epinephrine (1.0 μM), recombinant human transforming growth factor alpha (TGFα; 0.5 ng/mL), hydrocortisone hemisuccinate (100 ng/mL), recombinant human insulin (5 μg/mL), apo-transferrin (5 μg/mL). PrEC from individual donors were first expanded, next all three donors' cells were pooled, aliquoted and frozen for use in experiments.

Primary cancer associated fibroblasts (CAF) were obtained from Dr. Donna Peehl at Stanford University and Dr. Larisa Nonn at the University of Illinois at Chicago. CAFs were derived from resected primary tumors with a range of Gleason scores. Primary peripheral zone stromal cells (Spz), taken from pathologist-verified tumor free regions of prostate cancer biopsies, were also provide by Dr. Larisa Nonn. All primary cells were used at low passage (<10).

The stem and progenitor fraction of PrEC was enriched using the prostasphere (PS) culture

system, which utilizes a 3-dimensional matrigel matrix to select for the growth of cells able to survive in anchorage-independent conditions[37]. Reduced growth factor matrigel was obtained from BD Life Sciences and combined 1:1 with PrEGM to form slurry that solidifies into a 3D matrix at 37° C. Pooled PrEC were mixed with the matrigel/PrEGM slurry and plated into rings at the bottom of cell culture plate wells at a density of either 25,000 cells/250 µL slurry in 24-well plates or 50,000/500 µL slurry in 12-well plates. After allowing the matrix to solidify at 37° C, either 500 µL or 1000 µL of PrEGM was added to each well of 12- or 24-well plates, respectively. Every two days half of the media was refreshed and PS cultures were taken to 5, 7, 14 or 30 days for different assays.

The WPMY-1 stromal cell line was obtained from ATCC (Bethesda, MD) and grown in DMEM supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin[38]. For assays that measured estrogenic effects within this cell line (i.e., estradiol-stimulated gene expression microarray) phenol red-free DMEM was used.

All cells were cultured using standard sterile technique in a laminar flow biosafety cabinet and otherwise kept in a humidified incubator at 37° C with 5% CO_2. Cells were passaged in phenol-red-free 0.05% Trypsin/ethylenediaminetetraacetic acid (EDTA) or 0.0025% Trypsin/EDTA. Cells were cryopreserved in their basal medium supplemented with 10% dimethylsulfoxide (DMSO) and 30% FBS (Fetal Bovine Serum, certified, US origin 16000-036). After resuspension of cells in cryopreservation media and placement into Corning cryovials , isopropanol-filled canisters were utilized to achieve a rate-controlled freezing of cells overnight in a -80° C freezer, after which cells were stored in liquid nitrogen.

2. Co-culture of primary prostate epithelial and stromal cells

Prostate stromal-epithelial interactions were modeled *in vitro* by: 1) direct co-culture of both cell types, 2) co-culture using cell-culture plate inserts and 3) conditioned media approaches (detailed in next section). Direct-contact co-culture of both cell types was achieved by first plating stromal cells in their primary media at 10,000 cells/1.9cm^2 and allowing them to establish for 24-48 hours (cell-type dependent). Media was then washed to remove traces of serum and PS were plated in a layer of matrigel directly on top of stromal cells. Non-contact co-culture was achieved by first plating 5,000-10,000 stromal cells in Millipore 0.8 micron cell culture inserts in 24-well plates. Once stromal cells reached 70% confluence, SCBM was replaced with serum-free media and inserts containing stromal cells were transferred to plates containing freshly seeded 3D PS cultures in matrigel. The co-cultures were cultured in PrEGM for 5, 7, 14 or 30 days. Controls containing PS only were grown along with co-cultures. Inserts were replaced with fresh stromal cells every 4-5 days in order to minimize changes induced by confluence.

3. Conditioned media

Since both stromal and epithelial prostate cells express ERs, assays of direct co-culture experiments cannot discriminate between the effects of E2 acting though one cell type versus the other and synergistic effects involving both cell types. Therefore, conditioned media from EtOH- or E2-treated PrSCs was used as treatment during PS culture. Stromal cells were grown to 70% confluence and fresh media containing either EtOH control or 10nM E2 was then added. After 72 hours the media was removed and charcoal-stripped to remove all E2 and other lipophilic hormones present. Media was then aliquoted and frozen for future use, either non-concentrated or after concentration using a Millipore Amicon Ultra-15 Centrifugal Filtration Unit. PS were cultured to day 5, day 7 or day 14 and treated with varying concentrations of conditioned media derived from either EtOH or E2-treated PrSC. Preliminary experiments were

performed to compare the effects of concentrated media vs. non-concentrated media and the conditions yielding maximum stromal influence on PS gene expression via qPCR were obtained by concentrating the media 40-fold and using it at a final effective concentration of 1.875x.

4. Prostasphere count & size analysis

Prostaspheres cultured for 5, 7, 14 or 30 days were isolated from matrigel with dispase and placed into chamber slides for image acquisition on an EVOS inverted microscope. Using proprietary MATLAB-based image analysis software, the number and size of each PS can be measured. Raw data was exported into Microsoft Excel for analysis of PS size and number.

5. BrdU proliferation assay for assessment of progenitor cell amplification

A 2-hour BrdU proliferation assay was used to assess progenitor cell proliferation in day 5 PS cultures. On day 5, PS were dispersed from matrigel using dispase, and placed into 4 or 8 well chamber slides with fresh PrEGM + 1 μM BrdU for 2 hours. The media was then replaced with PrEGM containing no BrdU and PS were left to attach overnight in an incubator. Once attached, PS were fixed and immunostained for BrdU as described in the following section. All cells were imaged using a fluorescent microscope and images were manually scored for percentage of BrdU-positive nuclei.

6. BrdU label-retention assay for assessment of stem cell self-renewal

A long-term BrdU label-retention assay was used to assess symmetric stem cell self-rewnewal[39]. PrEC were grown in 2D culture for 5-6 days with 1 μM bromodeoxyuridine (BrdU), a thymidine analog that incorporates into DNA during cell division. After 5-6 days, cells were transferred to 3D culture and PS were grown for a 5-14 day wash-out period in the absence of BrdU. During

this time, rapidly dividing progenitor cells and differentiating cells dilute out the BrdU label. Only stem cells, which are relatively quiescent with limited symmetric or asymmetric cell division, retain high levels of the BrdU label. After the 5-14 day culture period, PS are dispersed from matrigel using the dispase enzyme mixture and plated overnight to attach on chamber slides. Immunocytochemistry was then performed (using methanol fixative as detailed in a later section) with an anti-BrdU antibody to assess for and quantify the BrdU-retaining stem cells.

7. Immunocytochemistry, immunofluorescent microscopy and confocal microscopy

Unless otherwise noted, all immunocytochemistry was performed using the following protocol. Cells were allowed to attach onto 4 or 8 well cell-culture-treated chamber slides, media was aspirated and cells were washed quickly with ice-cold PBS before fixation with ice-cold MeOH at -20° C for 20 minutes. After fixation slides were washed three times with room temperature PBS and then blocked with PBS + 0.0025% Triton X-100 + 5% normal goat serum (NGS) for 30 minutes at room temperature. Cells were incubated with primary antibodies overnight in 4° C in PBS + 0.00125% Triton X-100 + 2.5% NGS. For BrdU immunostaining, the protocol was modified by the addition of the following steps before blocking in PBST: nuclear DNA was denatured with 1M HCl for 20 30 minutes at room temperature, followed by three five minute washes in PBS.

8. RNA isolation and quantitative real-time polymerase chain reaction

For gene expression analysis, RNA was isolated from cells using either the Qiagen RNeasy RNA isolation kit, or a modified protocol utilizing Trizol then phenol-chloroform extraction followed by purification using the Quiagen RNeasy kit. RNA concentrations were measured with a Nanodrop spectrophotometer and 1 µg of RNA was utilized for cDNA synthesis using an iScript

First Strand reverse transcription kit. Generally, 15-25 ng of cDNA per reaction was utilized for quantitative real-time polymerase chain reaction (qPCR) on a Bio-Rad CFX96 thermal cycler. Technical duplicates were ran in all qPCR reactions and internal housekeeping genes were utilized to normalize gene expression. Raw fluorescence data was analyzed using the ddCT method and Ct thresholds were calculated automatically using the Bio-Rad CFX Manager software.

9. Cytokine Array

A Biorad Bio-plex Human Cytokine Type I Array was used to simultaneously measure secreted protein levels of six inflammatory cytokines (IL-6, VEGF, TNFα, IL10, IL1b, IL-17) in conditioned culture medium from the following stromal cells and cell lines: PrSC expressing pLVX/empty vector or ERα, WPMY-1 expressing pLVX/empty vector or ERα, primary CAF, primary Spz. All cells were established in their primary culture medium until 70% confluent, then media was replaced with MCDB 105 + bFGF + insulin + 5% charcoal-stripped FBS +/- 10nM E2 for 72 hours. Conditioned culture media was harvested, spun down at 10,000 G at 4° C for 10 minutes to remove debris, aliquoted and frozen until assayed. Cells were next trypsinized and counted on a hemocytometer. A Bio-Rad Bio-Plex flow cytometer was utilized to measure protein levels in samples and these were compared to a standard curve of known concentration of all analytes. Protein levels were normalized to their respective cell number counts and then expressed as percentage of vehicle control.

10. Western Blot

Analysis of protein expression was accomplished by standard western blotting techniques. After washing with ice cold PBS, cells were lysed in RIPA buffer containing 150 mM NaCl, 0.1%

Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 50 mM Tris-HCl, pH 8.0 and a protease inhibitor cocktail (X). Cells were scraped off with a plastic cell scraper while on ice and the lysates where vortexed and placed under constant agitation for 30 minutes in 4° C. The lysates were centrifuged for 20 minutes at 12,000 G at 4° C, after which the supernatant was aspirated and used for downstream processing. Protein concentration of each sample was determined using a Pierce BCA assay kit in a 96-well plate format and absorbance was read at 562 nm using a Gen5 plate reader (X). Microsoft Excel was utilized to interpolate sample concentrations based on a quadratic curvilinear regression of the standards. The appropriate amount of 4x Lammeli loading buffer (8% SDS, 20% β-mercaptoethanol, 40% glycerol, 0.008% bromophenol blue, 0.25 M Tris-HCl) was added to the samples, which were boiled at 95° C for 5 minutes. Denatured samples in loading buffer were frozen at -20° C or used immediately, and 15-30ug of denatured protein sample was loaded into an ExpressPlus precast gradient SDS-PAGE gel and ran for 1-2 hours at 100 V in a Bio-Rad Protean Electrophoresis module. Gels were next transferred overnight onto a MeOH-activated PVDF membrane. Ponceau Red dye was used to asses total protein transfer prior to antibody staining. Membranes were blocked for 1 hour in a blocking buffer of 10% dry milk reconstituted in PBST. Primary antibodies were added into PBST + 5% milk and incubated on the membrane overnight at 4° C. Membranes were washed with PBST for 15 minutes three times and HRP-conjugated secondary antibodies were added in blocking buffer. Membranes were again washed in three times in PBST and chemiluminescent signals were generated using Pierce Signal Detection Kit (X), followed by standard film development using a darkroom developer.

11. Molecular Biology and Lentiviral induction of Estrogen Receptor-α expression

A lentiviral expression system (Addgene, Cambridge, MA) was utilized to stably introduce either the pLVX/empty vector plasmid or the pLVX/ERα overexpression construct into WPMY-1 stromal cell line and two separate patient-derived PrSC primary cell cultures. The ERα gene was PCR amplified from human PrECs and cloned into the pLVX plasmid using a Gisbson Assembly kit (New England Biolabs (Ipswich, MA). HEK293T cells were utilized to generate virus using a third generation lentiviral system.

12. siRNA

Small-interfering RNA experiments were performed with the Lipofectamine 2000 Reagent kit (Thermo-Fisher). Cells were plated so that they would be ~70% confluent on the day of the experiment.

B. *In silico* and bioinformatic analyses

1. Illumina gene expression microarray

All stromal cells were grown to 70% confluence and treated +/- 10nM E2 in charcoal-stripped media for 24 hours. RNA was extracted using Quiagen RNeasy as described previously. RNA integrity was analyzed on an Agilent Bio-Analyzer at the University of Chicago Genomics Core Facility. RIN scores were 10 for all microarray samples utilized, with the exception of four samples that all had sufficiently high RIN numbers ≥ 8. Illumina HT-12 BeadArray microarrays were utilized through the University of Chicago Genomics Core Facility.

2. Microarray analysis and differential gene expression analysis using R, lumi

Microarray data was analyzed in the R statistical environment using the *lumi* package (Figure 3) as downloaded from Bioconductor (www.bioconductor.org). The BeadStudio output file was

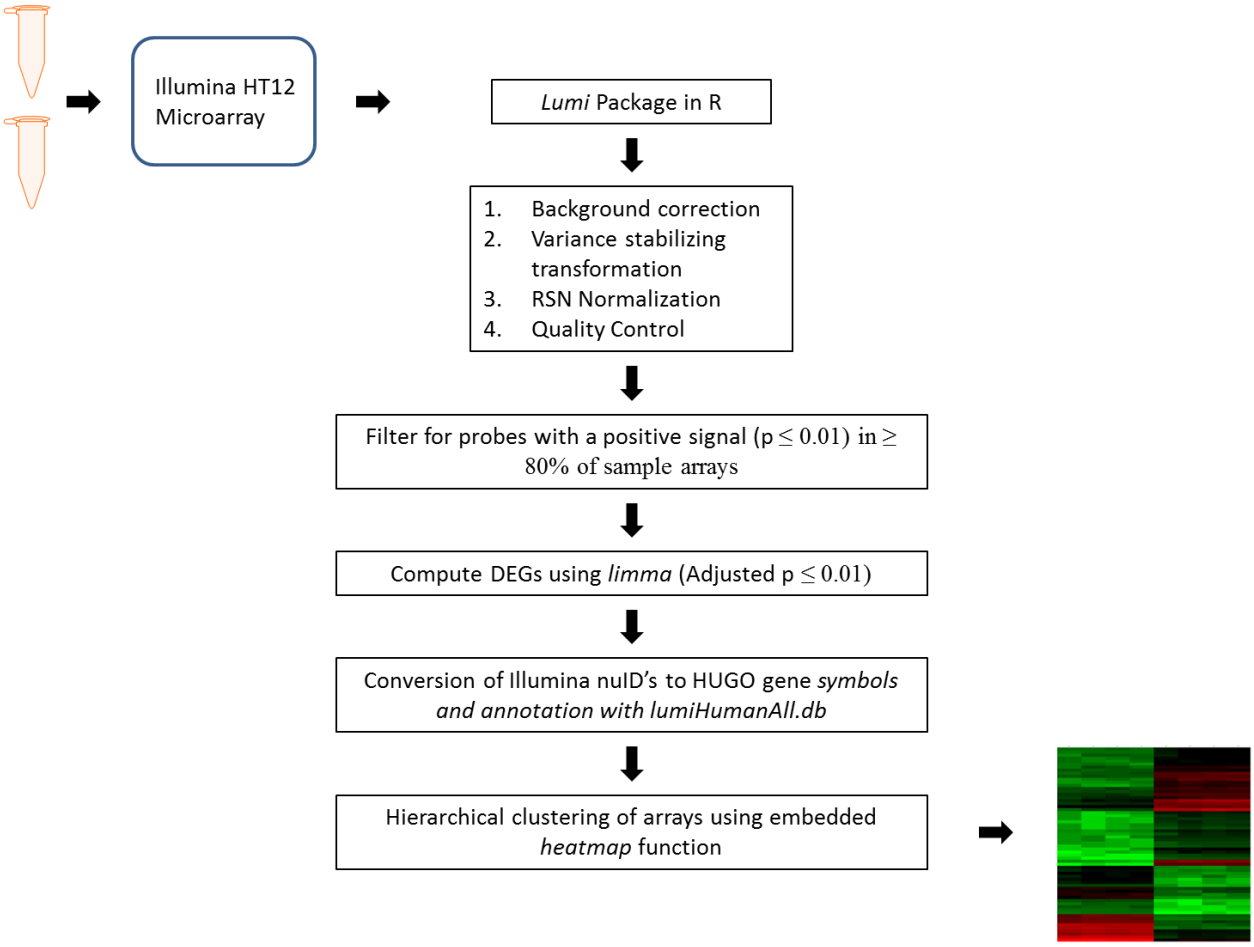


Figure 3. Microarray data analysis workflow.

loaded into *lumi* and background corrected with the lumiB function. Variance-stabilizing transformation was performed with the lumiT function. Due to its robust performance on bead array data, robust spline normalization was chosen and implemented via the lumiN function. Quality control was then performed before filtering for probes with a positive signal ($p \leq 0.01$) in $\geq 80\%$ of sample arrays. After using the *limma* function to fit a linear model, differentially expressed genes (DEGs) were compiled using an adjusted p-value threshold of $p \leq 0.01$. Conversion of Illumina nuID's to HUGO gene symbols was performed with subsequent annotation with the *lumiHumanAll.db* database. Hierarchical clustering of arrays was performed using the embedded heatmap function. Background-corrected, variance-stabilized and RSN-normalized data was then output to tab-delimited files for additional analyses using the BRB-ArrayTools program developed by Dr. Richard Simon[40].

3. Gene Set Enrichment Analysis and Leading Edge Analysis

The Broad Institute's implementation of the Gene Set Enrichment Analysis (GSEA) algorithm was utilized to analyze whole-genome expression datasets. Gene set permutation was utilized to assess for statistical significance in enrichment score, since all comparisons involved datasets with fewer than 7 samples per phenotype. Leading edge analyses were also performed using the Broad GSEA program. Gene set enrichments were manually chosen for use in leading edge analyses.

4. Survival Analysis Using Gene Signatures

The Cancer Genome Atlas (TCGA) database was accessed via the cBioPortal website at www.cbioportal.org. Survival analyses were performed by querying for gene alterations involving copy number gain and/or mRNA expression z-score ≥ 2, which roughly correlates to 2

standard deviations from the mean expression derived from all diploid samples. Additional survival analyses were performed using the Survival Risk Prediction tool within BRB-ArrayTools using a penalized Cox regression model.

C. Statistical Analysis

All statistical analyses were performed in GraphPad Prism. One- and two-way analyses of variance (ANOVA) were performed with Tukey post-hoc comparison tests. Student's t-test was used for comparison of two sample means. One-tailed t-tests were used as appropriate according to hypotheses. For analysis of high-dimensional datasets where multiple comparisons were performed, either Benjamini corrections or false-discovery rates (FDR) ≤ 0.05 were utilized. The exception was in correction for Gene Set Enrichment Algorithm results, where FDR ≤ 0.25 was used, as is conventional for this algorithm.

III. EFFECTS OF NORMAL STROMA ON THE BENIGN STEM CELL NICHE

A. Abstract

The location and cellular constituents of the human prostate stem cell niche are unknown. In rodents, however, the SCN is thought to exist in the basal cell layer of the proximal prostatic ducts[41,42]. In the rat, these proximal ducts are surrounded by stromal smooth muscle cells, which produce gradients of TGF-β that decrease toward the distal ducts[43]. Given that the stroma is known to play a fundamental role in prostate morphogenesis, that there is robust evidence of its localization near the SCN, and that it secretes morphogens known to be active in the SCN of other tissues, it is very likely that stromal cells also play a key role in the human prostate SCN[44]. To test the hypothesis that stromal cells modulate prostate stem and progenitor cell self-renewal, proliferation, lineage specification and differentiation within the adult human *normal* stem cell niche, an *in vitro* co-culture system was utilized to model stromal-epithelial interactions within the SCN.

Using the *in vitro* prostasphere (PS) system, enriched primary human prostate stem and progenitor cells were co-cultured with normal primary stromal cells or the normal stromal cell line WPMY-1. Long-term BrdU label-retaining assays demonstrated an increase in the number of label-retaining stem cells per PS when co-cultured with stromal cells, suggesting that stromal cells increase symmetric stem cell self-renewal. Progenitor cell proliferation assays showed that stromal cell co-culture delays the period of transient amplification of progenitor cells, likely a secondary effect of an early shift towards symmetric stem cell self-renewal. Differentiation assays indicated that stromal cells alter lineage commitment within PS, shifting progenitor cells

towards a basal phenotype and away from a luminal cell fate.

These data suggest a model of the human prostate stem cell niche whereby stromal cells secrete factors that restrain differentiation of epithelial stem cells and shift the lineage commitment of their progeny (Figure 4). Such a model has clear implications in the setting of prostate cancer, a hallmark of which is a shift in lineage commitment of cancer cells toward a luminal phenotype. A better understanding of the normal signaling axes that govern stem cell self-renewal and progenitor lineage commitment may shed light on novel targets to prevent prostate carcinogenesis or limit disease progression.

B. Results

1. Model Overview

Since extrinsic stem cell regulatory factors can involve long range endocrine signaling, paracrine mechanisms or cellular adhesion-mediated pathways, distinct co-culture models were utilized to enable distinction between effects due to unidirectional vs. bidirectional signaling, as well as those due to paracrine signaling vs. cell-cell or cell-matrix mediated signaling. Three basic approaches were utilized: direct contact co-culture, co-culture separated by culture plate inserts, and a conditioned media approach (Figure 5). The conditioned media approach allowed discrimination of effects due to unidirectional signaling from stromal cells, versus effects of bidirectional signaling in co-culture models. Additionally, this approach allowed for the concentration of stromal-derived factors, which provided insight into the potency of stromal-derived factors. Direct contact co-culture enabled assessment of signaling mechanisms involving ultra-short distances, ECM deposition or remodeling, or even direct cell-cell contact, versus insert-separated co-culture that only allowed for paracrine signaling. Inserts with 0.8 μm pores were utilized in order to allow free passage of small soluble cytokines and growth factors while

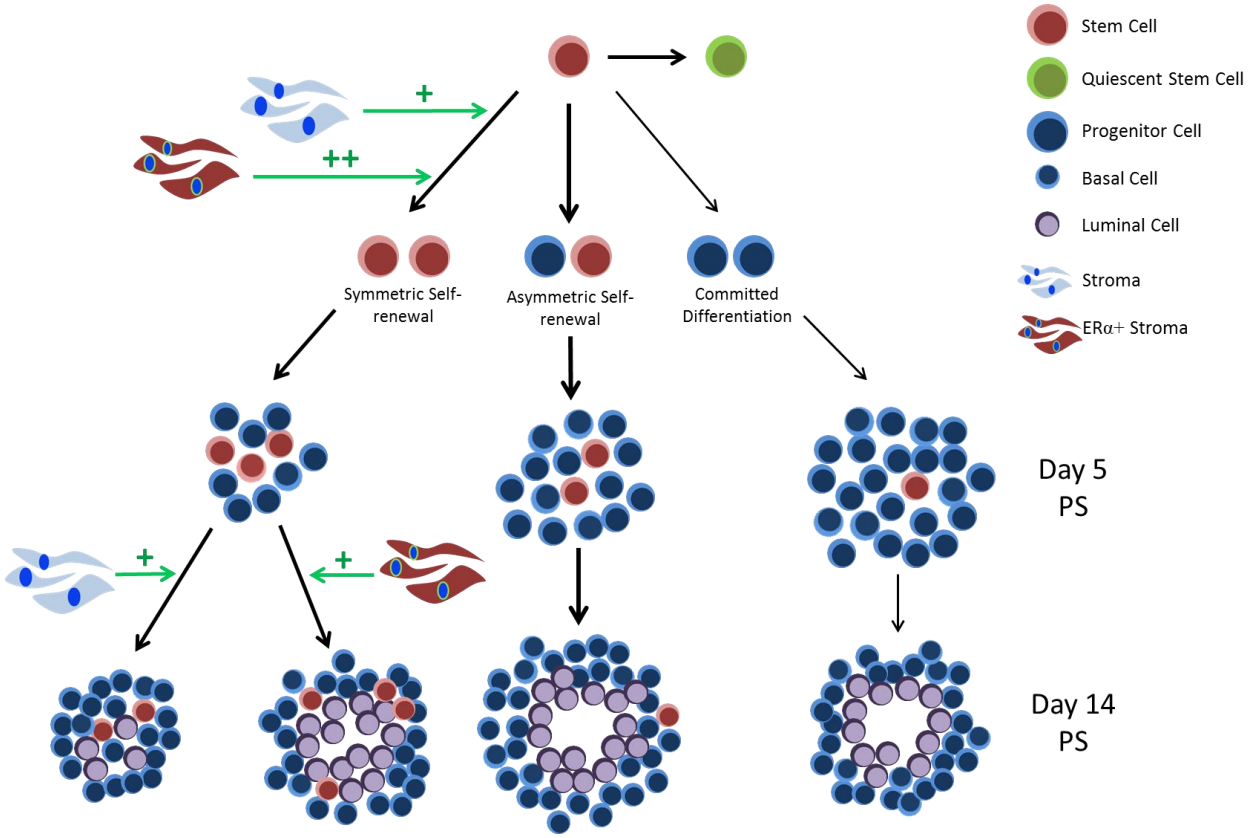


Figure 4. Model of stromal cell regulation of the prostate epithelial stem cell niche.

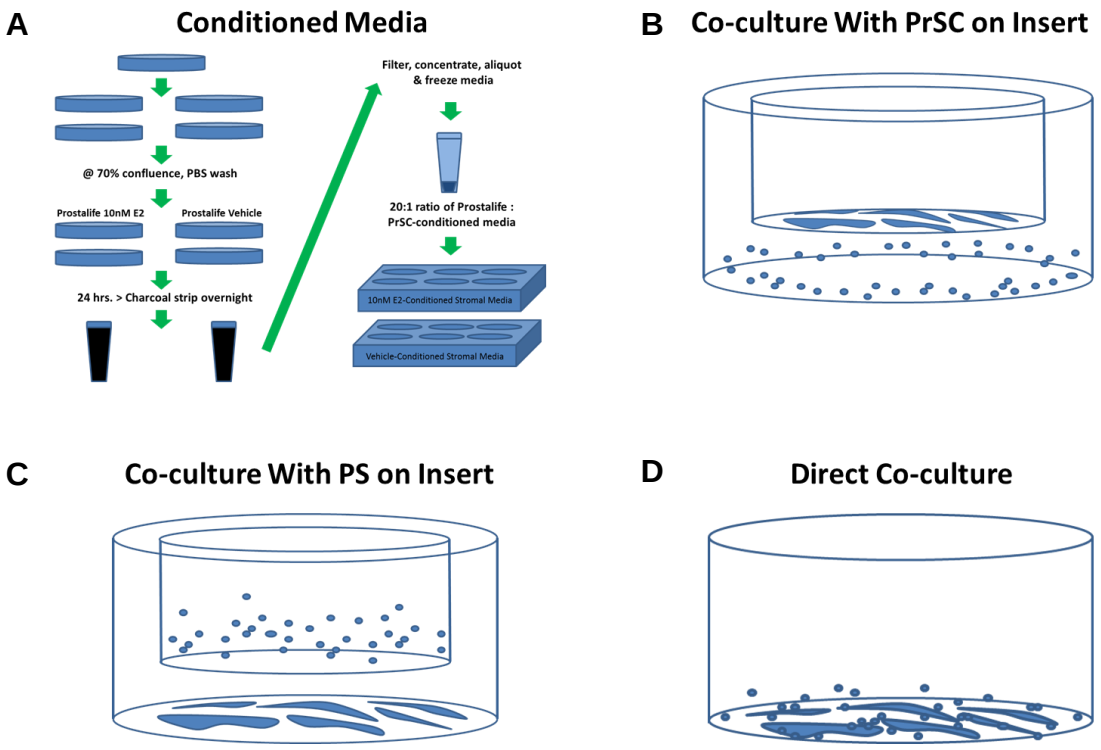


Figure 5. Co-culture models utilized in this study.

A) Conditioned media; B) Co-culture with stromal cells grown on the insert and 3D PS grown in matrigel on the bottom of the well; C) Co-culture with 3D PS grown on the insert and stromal cells grown on the bottom of the well; D) Direct-contact co-culture with stromal cells seeded first on the bottom of the well, then 3D PS grown in matrigel directly on top of the stromal cells.

prohibiting cellular migration as well as passage of very large macromolecules, such as ECM polymers.

2. Stromal cells increase symmetric stem cell self-renewal in an *in vitro* model of the benign prostate stem cell niche

Prostaspheres derived from BrdU-labeled pooled primary PrEC were cultured for 5 days with or without benign Spz stromal cells derived from two separate donors. After 5 days, long-term BrdU label-retaining cells were stained via immunocytochemistry stained assess stem cell self-renewal. The average number of BrdU label-retaining cells was two-fold higher in PS cultured with stromal cells (Figure 6), suggesting that the Spz cells secrete factors that increase symmetric self-renewal within epithelial stem cells.

Since the stroma represents an admixture of fibroblasts, smooth muscle and several other types of cells and since patient-derived primary cells can exhibit marked phenotypic heterogeneity when transferred to *in vitro* conditions, two additional co-culture models were utilized to test if these initial results could be replicated with different stromal cell populations across different time points. The benign myofibroblastic stromal cell line WPMY-1 was co-cultured directly with BrdU-labeled, primary PrEC-derived PS for 7 days, after which a label-retaining assay was performed. The average number of label-retaining epithelial cells was higher in PS co-cultured with WPMY-1 cells (data not shown), albeit the fold-change compared to control PS was less than that in D5 PS co-cultured with primary Spz cells.

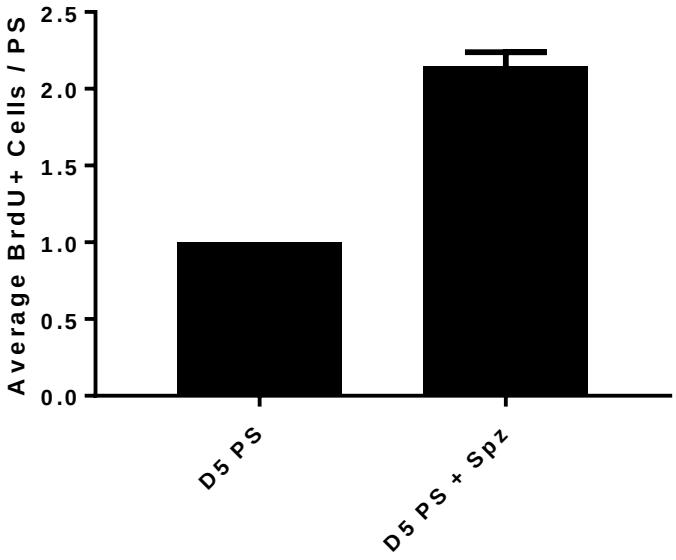


Figure 6. Effect of Spz stromal cell co-culture on stem cell self-renewal in D5 PS.

Co-culture with primary Spz stromal cells increases symmetric stem cell self-renewal in D5 PS. D5 PS n=1; D5 PS + Spz n=2. Thus, tests for significance were not performed.

A third model utilized primary PS co-cultured for 14 days with primary PrSC derived from young, disease-free donors. This experimental model was designed to correlate multiple end-points, including gene expression analysis of differentiation markers (data presented in a later section), in addition to stem cell self-renewal assays. Therefore, cell culture plate inserts were utilized to allow for pure separation and analysis of each cellular compartment. Label-retaining assays results showed a significant increase in BrdU-positive cells in PS co-cultured with PrSC, compared to control PS (Figure 7).

3. Stromal cells modulate progenitor cell proliferation within an *in vitro* model of the benign prostate stem cell niche

Although prostaspheres are mixtures of stem and progenitor cells, the vast majority of cells at early time points are progenitor cells. Thus, assays measuring total cell proliferation within day 5 or day 7 PS are indicative of progenitor cell proliferation. A direct co-culture model combining D5 or D7 PS with WPMY-1 stromal cells was utilized to assess stromal influences on progenitor cell proliferation. Two different assays were utilized to assess progenitor cell proliferation: a short-term BrdU proliferation assay and PS size measurement. The 2 hour BrdU proliferation assay performed on D5 PS demonstrated a 1.5-fold increase in proliferation of progenitor cells when cultured with stromal cells, compared to those cultured without stroma (Figure 8). In contrast, spheroid size measurement of D7 PS showed that co-culture with stromal cells decreased PS size, suggesting a reduction in progenitor cell proliferation (Figure 9).

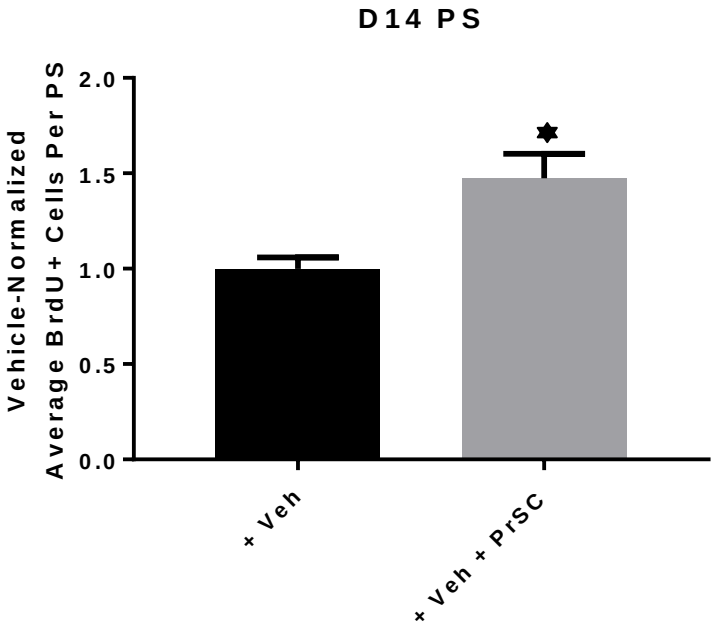


Figure 7. Effect of co-culture with PrSC stromal cells on stem cell self-renewal in D14 PS.

Co-culture with normal PrSC significantly increases the average number of BrdU-positive stem cells per PS over 14 day culture. For both conditions n=3. Two-tailed t-test p=0.0221

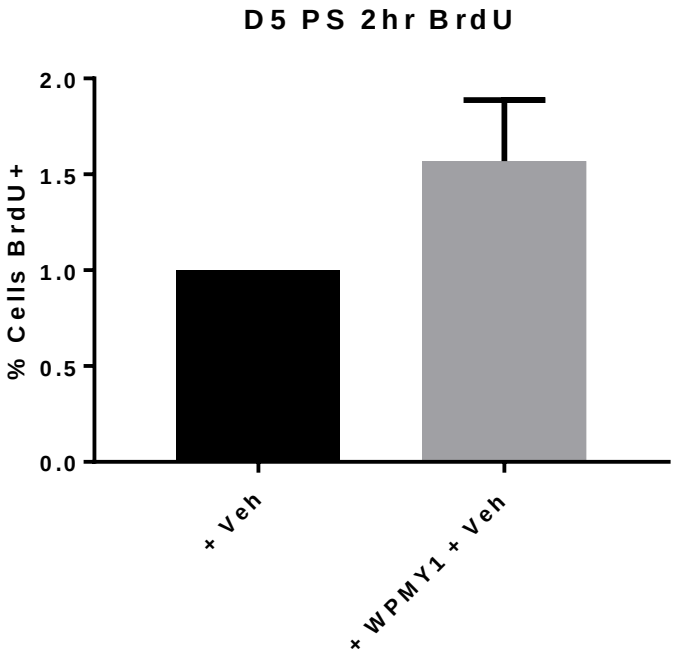


Figure 8. Effect of WPMY-1 stromal cell co-culture on progenitor cell proliferation in D5 PS.

WPMY-1/EV stromal cells increase progenitor cell proliferation via 2-hour BrdU proliferation assay with D5 PS. Co-culture with stromal cells increases percentage of PS progenitor cells incorporating BrdU by 50%. Veh n=1; + WPMY1 + Veh n=2. Thus, tests for significance were not performed.

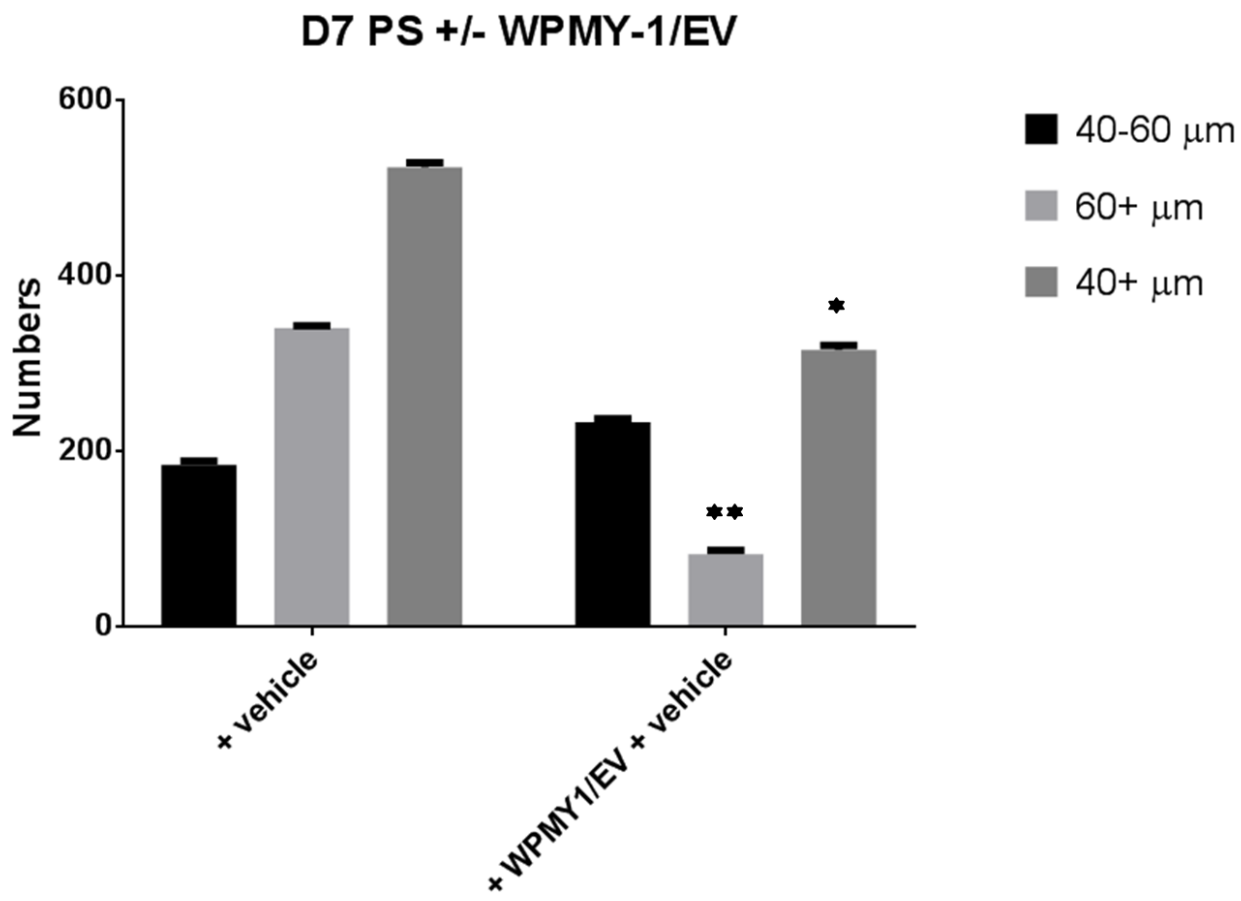


Figure 9. Size Distribution of D7 PS co-cultured with WPMY-1 cells

Spheroid size measurement of D7 PS showed that co-culture with stromal cells decreased PS size, suggesting a reduction in progenitor cell proliferation. Two-tailed t-test, n=3 for all conditions. *$p<0.05$, **$p<0.01$

4. Stromal cells alter lineage specification of differentiated epithelial cells within an *in vitro* model of the benign prostate stem cell niche

A 14 day co-culture model using PS and primary PrSC permitted analysis of differentiation and lineage commitment concurrent with stem and progenitor cell assays. Cultures were established with PrSC seeded on hanging culture plate inserts, permitting diffusion of soluble factors while allowing pure RNA isolation from the epithelial or stromal compartments. After 14 days of culture, PS grown with PrSC displayed a less-developed double-layered morphology compared to control PS (Figure 10). Since the double-layered morphology is thought to correlate with the development of distinct outer basal and inner luminal epithelial layers, RT-qPCR analysis of four differentiation genes was performed. Gene expression of basal epithelial markers p63 and Hoxb13 were both increased in PS with co-culture of PrSC, with the latter being statistically significant (Figure 11). Expression of luminal markers CK8 and NKX3.1 were both decreased with co-culture of stromal cells, with CK8 being statistically significant (Figure 11).

To further examine how stromal cells direct lineage commitment in differentiating progenitor cells, PS co-cultured directly with WPMY-1 cells for 14 days were fixed, sectioned and immunohistochemically stained for basal and luminal differentiation markers. PS grown with WPMY-1 cells were smaller and lacked a distinctive lumen, consistent with D7 assays and the D14 co-cultures with PrSC, respectively. PS co-cultured with WPMY-1 cells also displayed markedly-reduced levels of a luminal epithelial marker, via quantified fluorescence intensity of CK8/18 immunostaining (Figure 12). Unexpectedly, immunofluorescent staining of the basal epithelial marker CK14 was also markedly reduced in PS co-cultured with WPMY-1 cells (Figure 12), although this was a different basal marker than p63 which was used as a gene

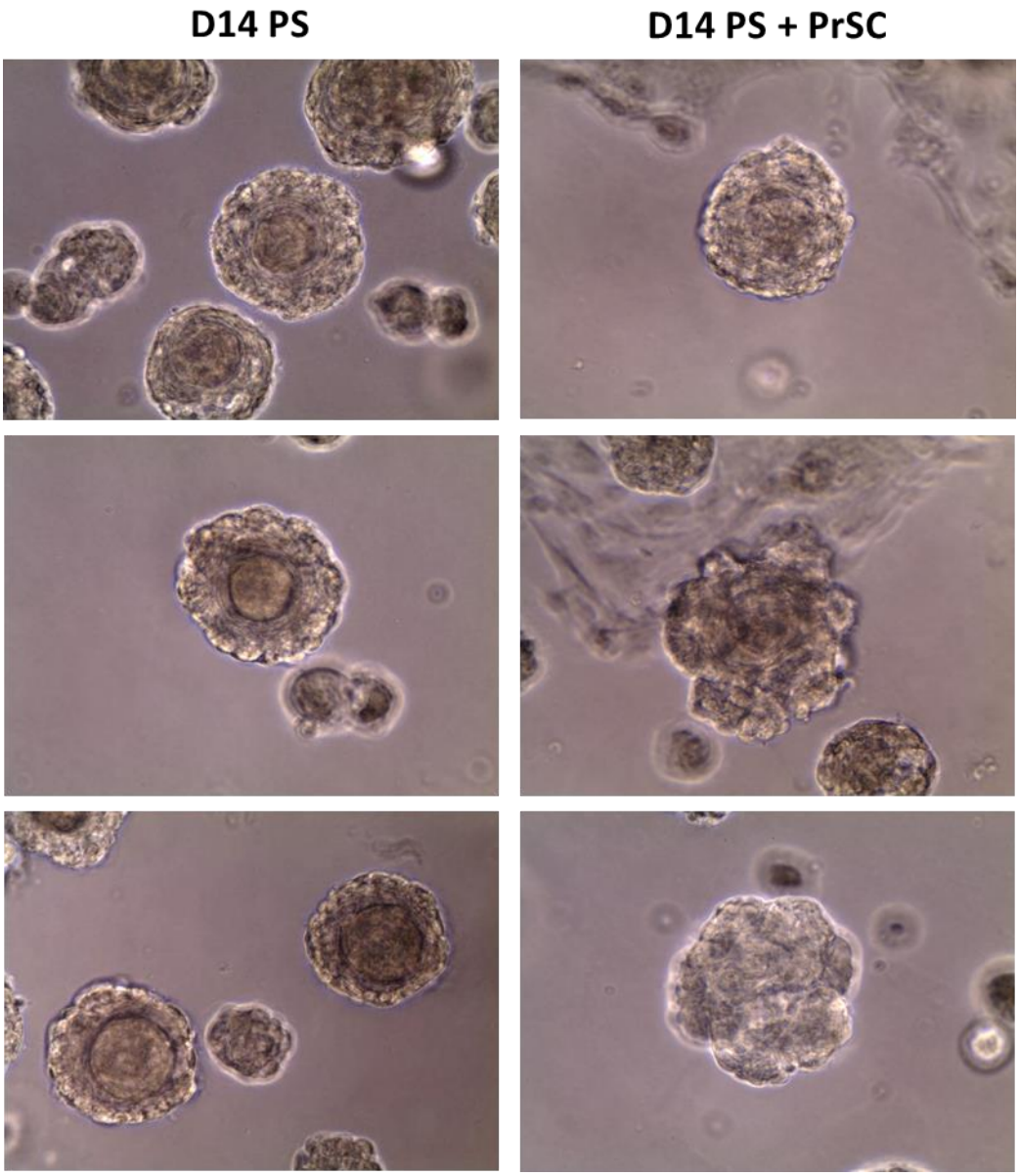


Figure 10. Co-culture with PrSC Alters Morphology of D14 PS.

Prostaspheres were cultured for 14 days in 3D matrigel with or without PrSC on inserts and treated with vehicle or 10nM E2.

Morphology of PS at D14 reveals loss of distinct double layer when cultured with PrSC, suggesting loss of luminal cell formation.

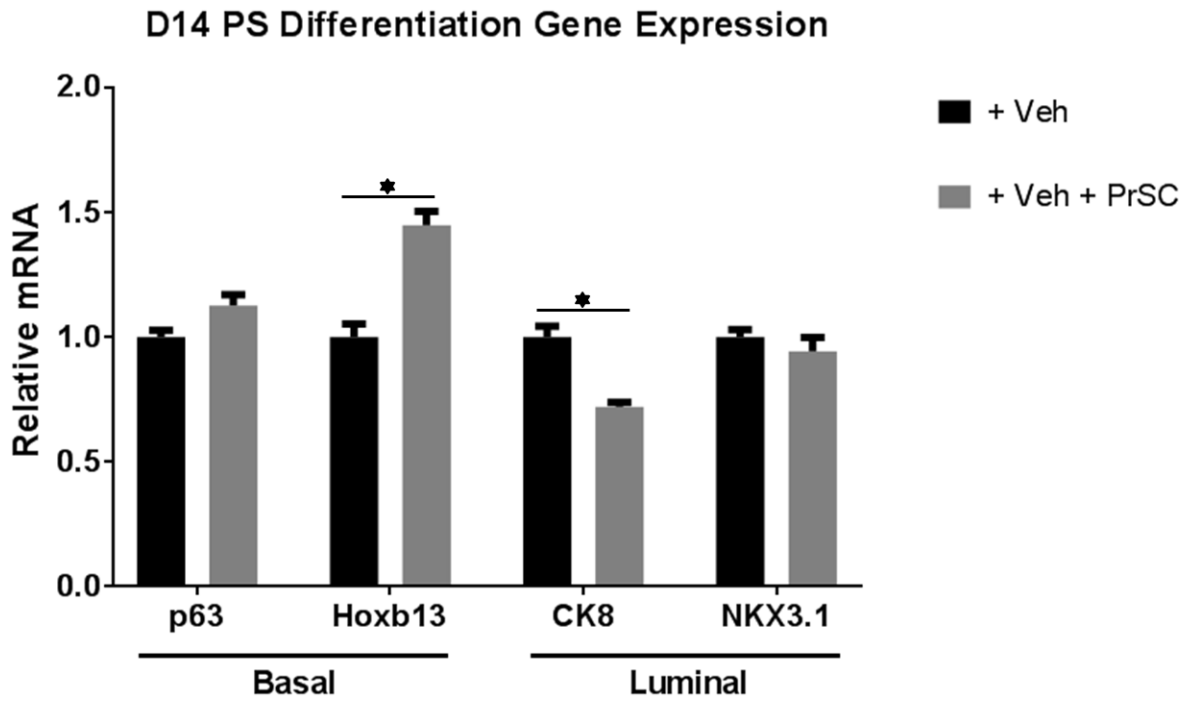


Figure 11. Effect of co-culture with PrSC stromal cells on gene expression of basal and luminal differentiation markers in D14 PS.

Co-culture with PrSC delays PS expression of luminal and increases expression of basal differentiation genes. Prostaspheres were cultured for 14 days in 3D matrigel with or without PrSC on inserts and treated with vehicle or 10nM E2. RT-pPCR gene expression analysis showed increase in the basal epithelial markers p63 and HOXb13 with E2 and PrSC co-culture, while the luminal markers CK8 and NKX3.1 were down-regulated in the presence of PrSC co-culture. These results suggest that PrSCs can influence lineage specification of progenitor cells. One-way ANOVA; n=3; *p <0.05

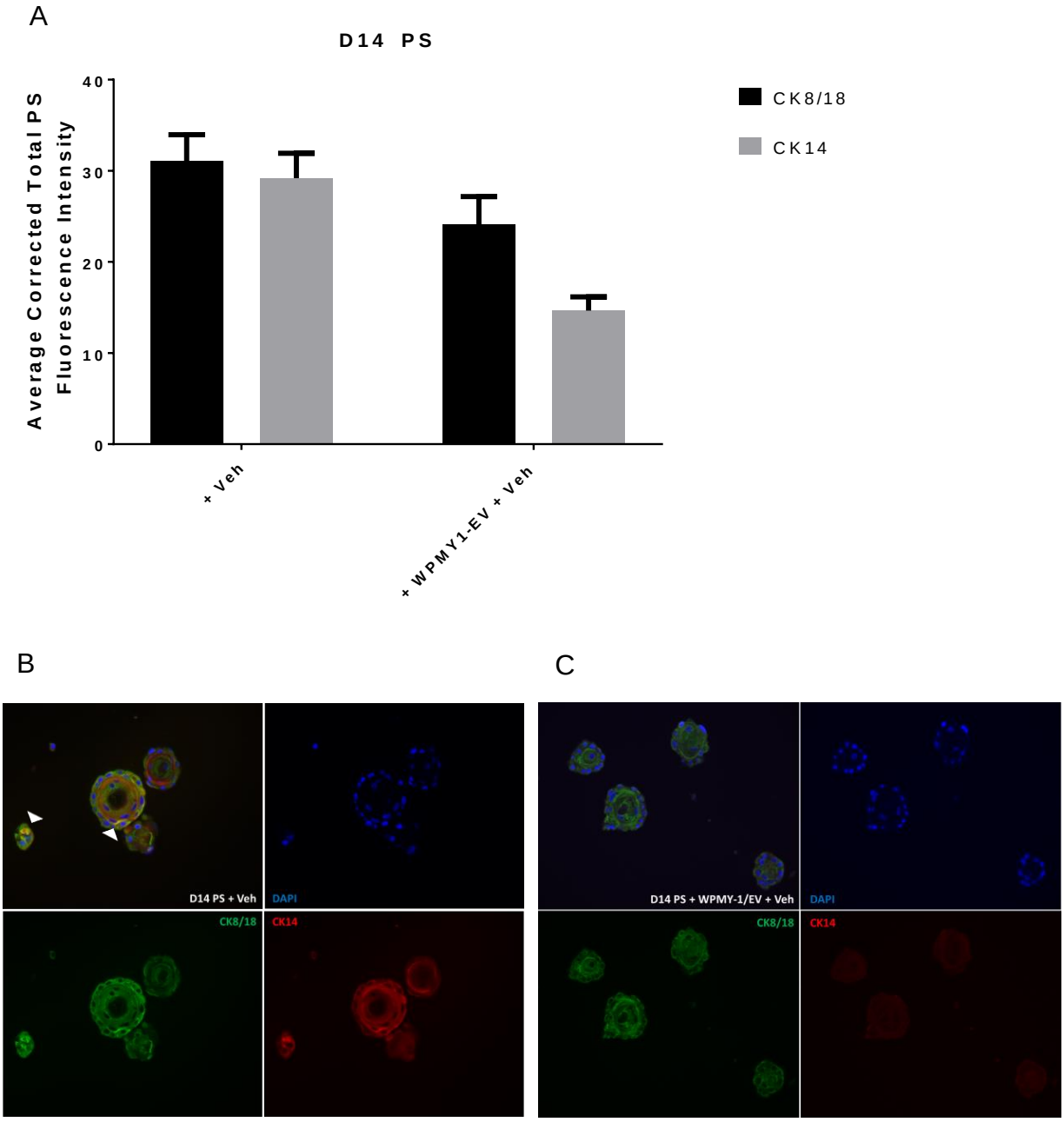


Figure. 12 Basal and luminal cytokeratin expression in D14 PS co-cultured with WPMY-1 cells.

PS co-cultured for 14 days with WPMY-1 cells display markedly-reduced levels of both luminal and epithelial markers. Luminal CK8/18 is in green, basal CK14 is in red. A) Quantification of immunofluorescent (IF) intensity. B) D14 PS co-cultured without stromal cells. Arrowheads show double-positive cells. C) PS co-cultured for 14 days with WPMY-1 stromal cells. The IF intensities of ≥ 20 PS for each condition were averaged. No test for significance was performed.

expression marker of basal cells. PS grown with WPMY-1 cells also had markedly reduced numbers of cells co-expressing luminal CK8/18 and basal CK14 markers (Figure 12, arrowheads).

C. Discussion

A critical unresolved issue in cancer research is identification of the cell of origin in different cancers. Stem and progenitor cells are excellent candidates due to their longevity, epigenetic malleability and high proliferative capacity. In the prostate gland, the cell-of-origin question is not merely of academic interest, as tumors with basal vs. luminal origin have been shown to have distinct molecular phenotypes that are prognostic of patient outcomes[45]. Efforts to identify the prostate cancer cell of origin through lineage tracing studies, *ex vivo* cell culture and tissue engraftment assays are ongoing, but thus far have yielded inconclusive results[42,46-50]. Should a consistent lineage hierarchy model emerge, it will still be critically important to develop an understanding of the normal prostate stem cell niche using a human primary cell model. Furthermore, any therapeutic efforts to prevent or treat cancers arising from stem or progenitor cells would involve modulation of the cellular signaling pathways responsible for regulating the stem cell niche.

The current study sought to develop an *in vitro* model of the normal stem cell niche using primary human cells. The focus on patient and donor specimens tremendously enhanced the translational relevance of this work, while at the same time reducing artefactual noise often introduced by cell lines. The model focused on elucidating the regulatory role of stromal cells, since stromal-epithelial interactions have well-documented roles in both prostate morphogenesis

and cancer progression. Using the now well-established prostasphere model as a source of enriched primary stem and progenitor cells, both primary patient-derived and cell line stromal cells were used in a complement of co-culture models to study stromal effects on stem cell self-renewal, progenitor cell proliferation and epithelial lineage commitment. The data from these studies support a model whereby stromal cells support epithelial stem cell symmetric self-renewal, thereby increasing stem cell numbers. In the PS system, this delays rapid progenitor cell amplification, which can only be initiated after asymmetric self-renewal, and delays bilayer differentiation (Figure 13).

Long term BrdU label-retaining assays from D5 PS co-cultured with multiple patient-derived Spz (Figure 6**)** suggest that benign stroma increases stem cell symmetric self-renewal in PS. This data is consistent with results from other models, whereby WPMY-1 or PrSC cells co-cultured with PS for 7 or 14 days, respectively, also increased BrdU label-retaining cells (Figure 7). These sustained effects using multiple stromal cell models suggest that the observed phenomena represent a fundamental regulatory effect of stroma on the stem cell niche. Furthermore, data from these stem cell assays and data from progenitor cell proliferation assays are consistent with a model where stromal cells restrain, but do not altogether prohibit stem cell differentiation within the stem cell niche.

Two different assays were used to measure progenitor cell proliferation—a 2-hour BrdU proliferation assay and PS size measurement. The 2-hour BrdU proliferation assay represents the proliferation rate at a single point in time (or a brief 2-hour window in time). The PS size measurement assay, however, represents an integrated measurement of progenitor cell

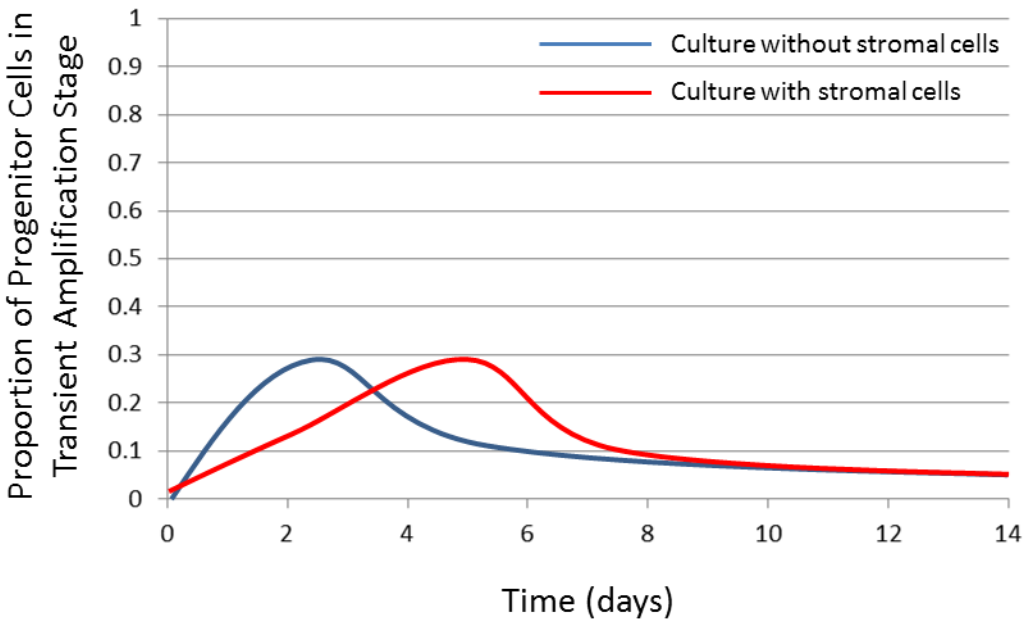


Figure 13. Proposed model of how stromal cells alter proliferation dynamics within the stem cell niche.

In PS cultured with stromal cells, the peak fraction of progenitor cells in the transient amplification stage is delayed due to increased symmetric stem cell self-renewal, which delays generation of progenitor cells since these cells can only arise through asymmetric stem cell self-renewal.

proliferation up to a specific point in time, D7 PS in this case. The fact that D7 PS co-cultured with WPMY-1 stromal cells are significantly smaller in size than control PS grown without stromal cells indicates that total progenitor cell proliferation is decreased over 7 days of co-culture. On the other hand, data from 2-hour BrdU proliferation assays in D5 PS shows that spheroids co-cultured with stromal cells have a higher percentage of cells incorporating BrdU. This suggests that stromal cells alter the proliferation dynamics within the stem cell niche by delaying the period of rapid, transient amplification of progenitor cells.

An increase in active progenitor cell proliferation at D5 and a decrease in total progenitor cell proliferation up to D7 are consistent with the observation that co-culture of PS with stromal cells increases symmetric stem cell self-renewal. Since progenitor cells can only arise after asymmetric division of stem cells, even a slight increase in the ratio of symmetric-to-asymmetric self-renewal could yield large differences in the number of progenitor cells in D5 PS. These stromal effects on progenitor cell number and proliferation could be mediated through stem cells only, or could be due to distinct effects on stem and progenitor cells.

To further support the notion that stromal effects on epithelial progenitor cells are mediated through their stem cell precursors, size differences between control and co-cultured D14 PS are reduced compared to D7 PS, although this may be assay- and stromal cell-type-dependent. Nevertheless, this suggests that at least in some models, if given enough time, spheroids co-cultured with stromal cells will eventually attain similar size to control PS (Data not shown). Taken by themselves, the data from stem cell self-renewal and progenitor cell proliferation

assays would be consistent with a model whereby stromal cells effects on the epithelial SCN are primarily through regulation of stem cell numbers, with only minor effects on progenitor cells.

By day 14, many progenitor cells within a PS have terminally differentiated into basal or luminal cells and most spheroids take on a double-layered morphology. These characteristics are confirmed with gene expression analysis, which show robust mRNA levels of basal and luminal differentiation genes. Co-culture of PrSC with PS altered lineage commitment at D14, as evidenced by morphological differences and altered differentiation gene expression. The loss of the double-layered morphology and a trend towards increase in basal gene expression markers and concomitant decrease in luminal gene expression markers suggests that PrSC promote a shift toward basal cell lineage. Alternatively, PrSC might merely slow the entire lineage development and differentiation processes, by virtue of enhancing stem cell symmetric self-renewal. It should be noted that this latter scenario would depend on a hierarchical model whereby luminal cells either develop from basal progenitors or develop only after the basal compartment has matured.

Results from a direct-contact 14 day co-culture model using WPMY-1 stromal cells provide complimentary results to the D14 PrSC model. The reduced diameter or altogether loss of luminal structures in IHC-stained fixed sections of PS co-cultured with WPMY-1 cells correlates strongly to the visually apparent loss of luminal layers in D14 PS cultured with PrSC. On the other hand, immunostaining of these fixed PS with luminal CK8/18 and basal CK14 markers yielded discrepant and unexpected results. Although the quantified intensity of luminal CK8/18 immunostaining was reduced in WPMY-1 co-cultured PS, correlating to the significant reduction of CK8 gene expression in PrSC co-cultured PS, the intensity of basal CK14 immunostaining

was reduced even more markedly, an opposite effect of the trend towards increase in basal gene markers in the PrSC model. This discrepancy might be easily explained by the fact that RNA levels do not always correlate with protein levels; however, the strong morphological similarities between PS in both models suggests that there is indeed a true stromal effect on epithelial lineage commitment.

One possibility that would reconcile this discrepancy is that very early basal progenitors might express p63, but not yet basal cytokeratins such as CK14. Indeed, p63+ basal cells are known to harbor stem-cell populations that give rise to many epithelial tissues, including all stratified epithelia, and p63-null mice do not develop a prostate [51-53]. Lineage tracing studies of CK14-positive prostate basal cells have demonstrated that while all CK14+ cells expressed p63, not all p63+ basal cells expressed CK14, hinting that p63+ expression may occur earlier in the lineage hierarchy than other basal cytokeratins[54]. Furthermore, multiple lineage tracing and histological studies have found a small fraction of basal cells that co-express luminal markers (e.g., p63/CK18 or CK5/CK8) suggesting the existence of an intermediate progenitor status of cells that express both basal and luminal markers[45,54,55]. Notably, D14 PS grown without WPMY-1 cells clearly have a minor population of cells that co-express basal and luminal cytokeratins (Figure 12, arrowheads); however, PS co-cultured with WPMY-1 cells have no evidence of co-staining intermediate cells. It is possible that stromal co-culture restrains D14 stem and progenitor cell differentiation to a pre-intermediate cell lineage state. Although the present studies do not provide an exact hierarchical map of how stromal cells modulate lineage commitment within the prostate SCN, it is clear that there are distinct alterations in lineage commitment due to stromal influences. Others have argued that since nearly all prostate cancers

display a luminal phenotype, dysregulation of epithelial differentiation must be a necessary step in carcinogenesis with a basal cell of origin[50]. Especially considering that one hallmark of cancer is dysregulated differentiation, this is a persuasive argument. Therefore, the fact that prostate stromal cells can modify the lineage fate of stem and progenitor cells, which are considered likely cells of origin in prostate cancer, presents an intriguing possibility that stromal-epithelial interactions within the SCN might be subverted in carcinogenesis.

Collectively, the present results represent some of the first evidence from primary human tissues that stromal cells regulate the epithelial stem cell niche within the benign prostate. The proposed model serves as a framework for studying the role of estrogen signaling in the benign stem cell niche and how cancer associated fibroblasts affect the normal and cancer SCN.

IV. THE ROLE OF ESTROGEN SIGNALLING IN STROMAL REGULATION OF THE BENIGN STEM CELL NICHE

A. Abstract

Prostate epithelial stem and progenitor cells express estrogen receptors ERα and ERβ and are direct targets of estrogen, which influences their self-renewal and proliferative activities. These cells reside within a stem cell niche that in turn influences their fate decisions. In the prostate, this niche includes neighboring prostate stromal cells that express ERα in vivo and mediate hormonal actions in the prostate. Given the evidence presented in section III that stromal cells regulate the benign prostate epithelial stem cell niche, we herein sought to determine whether estrogen signally plays a role in modulating stromal-epithelial interactions within the prostate stem cell niche.

Using the *in vitro* prostasphere (PS) system, enriched primary human prostate stem and progenitor cells were cultured using several methods: 1) culture with conditioned media from E2-stimulated prostate stromal cells, 2) co-culture with primary normal human prostate stromal cells, or 3) co-culture with the normal human stromal cell line WPMY-1, stably transfected with human-ERα or empty vector. Conditioned media experiments demonstrated that E2-stimulated prostate stromal cells secrete factors that increase gene expression of several stem cell factors. Long-term BrdU label-retention assays demonstrated that E2 and stromal cells both increase symmetric stem cell self-renewal, perhaps through distinct mechanisms. Progenitor cell proliferation assays showed that robust expression of ERα in stromal cells can act in a ligand-independent manner to modulate the stromal cell influence on progenitor cell proliferation

dynamics. Differentiation assays indicated that E2 and stromal cells coordinately—and perhaps cooperatively—increase basal lineage commitment within PS, but have opposite effects on luminal lineage commitment. The signaling mechanisms mediating these effects were investigated by bioinformatics analysis of a stromal gene expression microarray and by ELISA-based assays for secreted-factors. Several factors, including laminin-α5 and IL-6, were investigated for their potential role in mediating the described stromal effects on epithelial stem and progenitor cells.

Together, these data suggest that stromal cells influence epithelial stem and progenitor cells through both ER- and non-ER-mediated mechanisms. Although the relative importance of these mechanisms might be difficult to ascertain via *in vitro* model systems, it is likely, based on the data presented, that non-estrogenic stromal influences dominate over the estrogenic influences. Furthermore, stromal ERα-mediated influences on the SCN, particularly concerning progenitor cell proliferation and lineage commitment, operate in both ligand-dependent and ligand-independent mechanisms.

B. Results

1. Model Overview

The two culture systems described in section III—direct contact co-culture and co-culture separated by culture plate inserts—were used to investigate how estrogen modulates the stromal-epithelial signaling axis in the stem cell niche. Since co-culture models cannot readily distinguish between estrogen's direct effects on epithelial stem and progenitor cells versus stromal-mediated effects, a conditioned media approach was also utilized (Figure 5). This approach allowed for the concentration of stromal-derived factors, which provided insight into the potency of these secreted factors.

Primary human cell cultures were utilized to maximize the translational relevance of this work, however, a major caveat of the use of primary cells is that they can quickly lose their steroid hormone receptor expression when cultured *in vitro*. Indeed, expression levels of ERα—the major ER expressed in human prostate stroma—ranged from undetectable to low or moderate in the various primary patient-derived stromal cultures that were utilized (Table 1). Therefore, a lentiviral vector system was employed to exogenously express ERα in the human WPMY-1 prostate stromal cell line and in two patient-derived primary prostate stromal cell cultures, PrSC1 and PrSC2. Validation of ERα protein expression is shown in Figure 14.

2. Estradiol-stimulated stromal cell conditioned medium increases stemness gene expression in day 5 prostaspheres

To determine if E2 modulates stromal-derived extrinsic stem cell regulatory factors, primary PrSC were treated with ethanol vehicle or 10nM E2 for 72 hours, after which conditioned media was charcoal-stripped, concentrated and added to PS cultures. After 5 days of culture with concentrated vehicle- or E2-stimulated SCCM, with renewal of half of the media and SCCM every 48 hours, PS total RNA was isolated and expression of multiple stemness genes was analyzed via RT-qPCR (Figure 15). Expression of the core stem cell genes *Nanog* and *Sox2* was significantly increased by E2-stimulated SCCM. Four other stemness genes known to be important in prostate stem cells—*Oct4*, *TBX3*, *ABCG2* and *CD49f*—all trended towards an increase with E2-stimulated SCCM, although the differences were not statistically significant. The coordinated increase in these critical stemness genes demonstrates that estradiol, acting through stromal ERs, can stimulate the release of stromal factors that serve as extrinsic stem cell regulatory molecules.

	ERα	ERβ	GPER
PrSC (1&2)	-	++	+++
WPMY-1	+	++	+++
CAF	- / + / ++	++	?
Spz	- / + / ++	++	?
Sca	- / + / ++	++	?
WPMY-1/ERα	++++	++	+++
PrSC/ERα	++++	++	?

Table 1. Estrogen receptor expression in stromal cells.

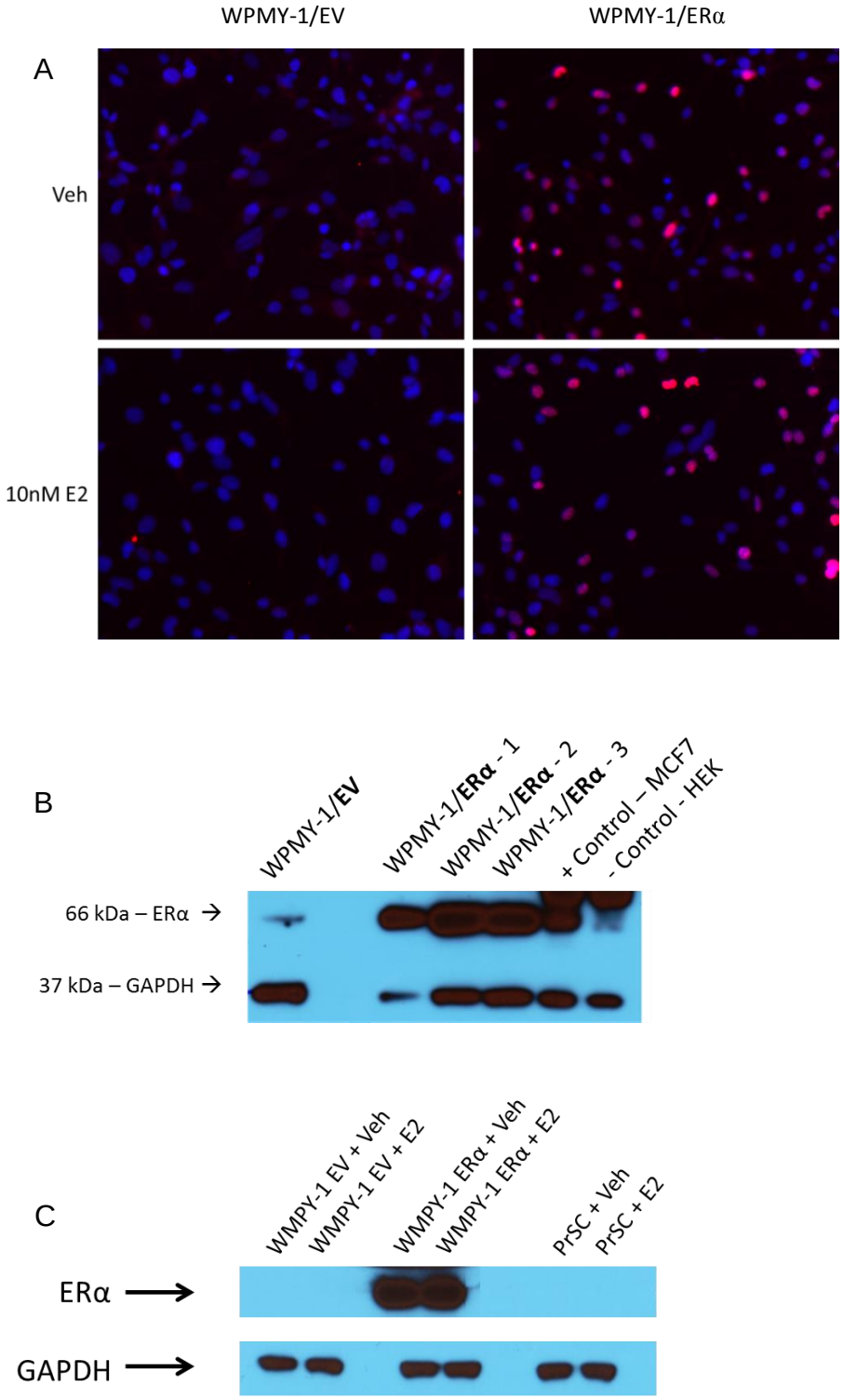


Figure 14. Validation of ERα Expression in WPMY-1/ERα and PrSC2/ERα.

A) ICC of WPMY-1/EV/ERα cells; ERα = red, blue = DAPI. B) Western blot of early passage WPMY-1/ERα cells, HEK as negative control for ERα, MCF7 as positive control for ERα. C) Western blot for ERα in PrSC1 cells and later passage WPMY-1/ERα, demonstrating that PrSC do not express ERα in vitro and the ERα-expression construct results in stable expression.

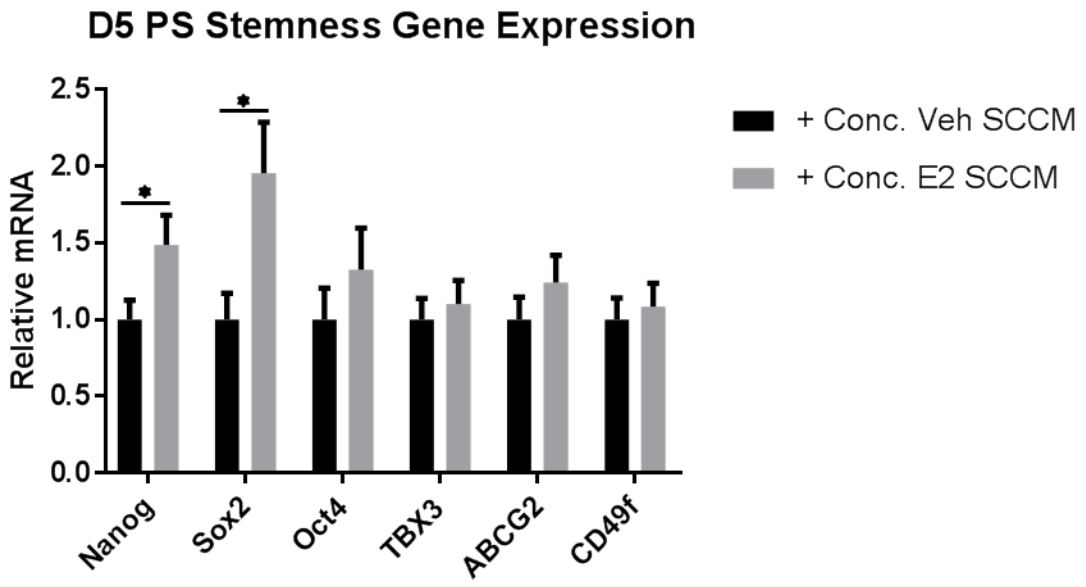


Figure 15. Stemness gene expression in D5 PS treated with E2-stimulated stromal cell conditioned media.

E2-stimulated stromal cell conditioned media increases stemness gene expression in D5 PS**.** D5 PS + Concentrated (40x concentrated; 1.875x final concentration in culture) stromal cell conditioned media

N=4. One-tailed t-test *$p<0.05$

3. The effects of E2 and stromal co-culture on stem cell self-renewal are additive, not synergistic

Since increases in stemness gene expression were measured in whole PS, which are a mixture of stem and progenitor cells, it was important to utilize the BrdU label-retaining assay to more accurately measure stromal-mediated estrogen effects on stem cell self-renewal. To that end, the same co-culture experiments introduced in Section III were utilized to determine if stromal co-culture and E2 treatment resulted in stimulatory effects on stem cell self-renewal.

BrdU-labeled PS co-cultured with or without two separate patient-derived benign Spz stromal were treated with ethanol control or 10nM E2 for 5 days. Stem cell self-renewal was assessed via the BrdU label-retaining assay (Figure 16). Compared to vehicle control, E2 treatment yielded a 1.68-fold increase in the average number of BrdU label-retaining cells per PS, while stromal co-culture yield a 2.15-fold increase. The combined effect of E2 treatment and stromal co-culture was a 2.29-fold increase in the average BrdU label retaining cells per PS. The additive nature of these effects suggests that they may represent two biologically distinct exogenous factors that promote symmetric stem cell self-renewal within PS.

As an alternative approach, long-term BrdU-labeled PS were cultured for 14 days in the presence or absence of PrSC on inserts and treated with vehicle or 10nM E2 (Figure 17). Compared to vehicle-treated control PS, treatment with E2 yielded a modest, non-significant 1.15-fold increase in the average number of label-retaining cells per PS. As previously shown, co-culture with PrSC for 14 days significantly increased BrdU label-retaining cell per PS. Likewise, the combination of E2 treatment and co-culture with PrSC significantly increased the number of label-retaining stem cells compared to both vehicle- and E2-treated PS alone. While there was a

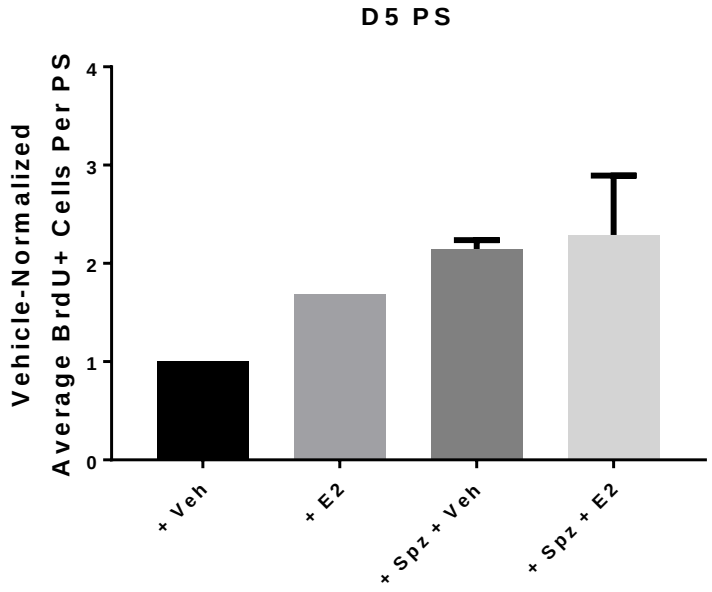


Figure 16. Effect of E2 and Spz stromal cell co-culture on stem cell self-renewal in D5 PS.

BrdU label-retention assay in D5 PS +/- Spz stromal cells. Veh and E2, n=1. Spz + Veh and Spz + E2 N=2. No tests for significance were performed.

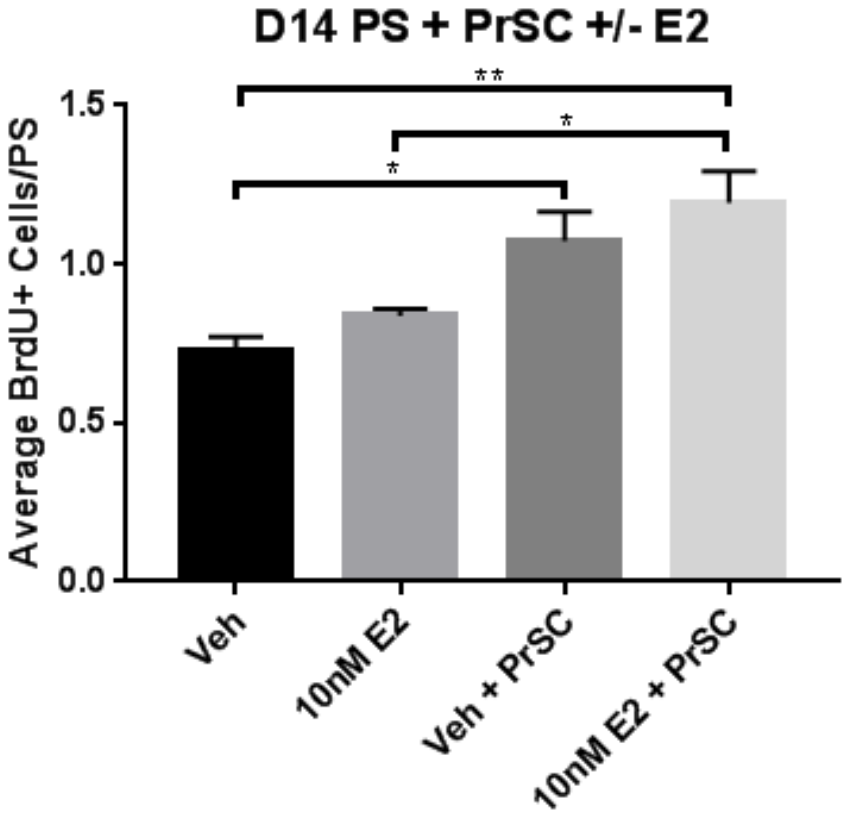


Figure 17. Effect of E2 and PrSC stromal cell co-culture on stem cell self-renewal in D14 PS.

Co-culture with PrSC increases the average number of BrdU-positive stem cells per PS in D14 PS, suggesting that stromal cells increase symmetric stem cell self-renewal. The effects of E2 and stromal cells on increasing symmetric self-renewal appear additive. N=3. One-way ANOVA. *$p<0.05$, **$p<0.01$.

modest increase in labeled stem cells with E2 + PrSC compared to PS co-cultured with PrSC + vehicle, this was not significant. In fact, two-way ANOVA confirmed that the effects of E2 treatment and stromal co-culture were exactly additive of the individual E2 or PrSC co-culture effects alone.

Data from conditioned media experiments suggested that E2 stimulates production of stromal-derived factors which can increase stemness gene expression in D5 PS. Conversely, data from stem cell self-renewal assays in D5 and D14 co-cultures suggested that estrogenic effects on stem self-renewal may not be mediated through stromal cells. In order to rectify these data and more robustly interrogate the role of stromal ER-mediated estrogenic effects on the stem cell niche, the benign prostate stromal cell line WPMY-1 was next utilized to over-expression ERα via a lentiviral expression construct.

Day 7 PS were cultured with no stromal cells, or in direct contact with either WPMY-1/Empty Vector (EV), WPMY-1/ ERα or PrSC, all with or without 10nM E2 (Figure 18). Co-culture with either WPMY-1/EV or PrSC resulted in a ~1.25-fold increase in BrdU-positive cells per D7 PS, compared to control PS grown alone, as shown in Section III. Furthermore, as shown by the Prins Laboratory, treatment with E2 alone resulted in a 1.35-fold increase in label-retaining cells[56]. Combination of E2 treatment plus stromal co-culture with either WPMY-1/EV or PrSC in the PS assay produced a 2.12- to 2.22-fold increase in label-retaining cells, suggesting possible synergistic effects between stromal cells and E2 on increasing stem cell self-renewal. Interestingly, when PS were co-cultured with WPMY-1/ ERα + vehicle, a potential ligand-independent effect on stem cell self-renewal was seen due to stromal ERα over-expression alone,

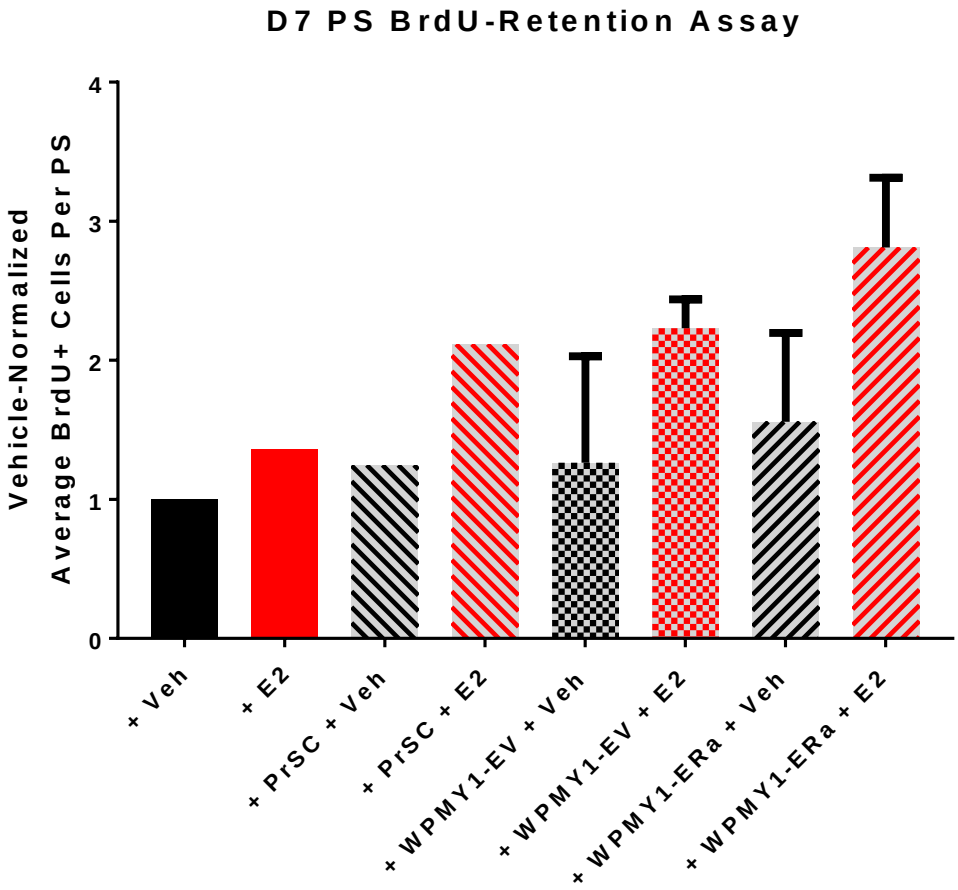


Figure 18. Effect of E2, stromal cell co-culture and ERα stromal cell co-culture on stem cell self-renewal in D7 PS.

D7 PS co-cultures with WPMY-1/EV, WPMY-1/ERα or PrSC2. N=1, except for conditions with error bars, which are N=2. Error bars = SEM. No tests for significance were performed.

as compared to WPMY-1/EV plus vehicle. Most notably, the addition of E2 to co-cultures of PS with WPMY-1/ERα cells resulted in the highest increase in BrdU label retaining cells, suggesting that E2 acting through stromal ERα has a synergistic effect on augmenting stem cell self-renewal.

Taken together, the data from all experimental approaches indicates that stromal-mediated E2 actions within the SCN increase stem cell self-renewal and the expression of stemness genes within a PS admixture of stem and progenitor cells. Discrepant results between the BrdU label-retention numbers per PS in the two separate approaches may reflect a number of variables, including: 1) different culture periods of 7 days when a stromal E2 effect was observed, and 14 days when the stromal-E2 effect was no longer apparent; 2) use of primary stromal cells with little or no ERα remaining versus the human WPMY-1 stromal cell line with either low basal or lentivirus-stabilized ERα expression; 3) co-culture of PrSC using an insert yielding data showing no PrSC-E2 effects and direct contact co-culture with PrSC-E2 yielding observed effects on PS stem cell self-renewal.

4. ERα expression in WPMY-1 cells increases progenitor cell proliferation in D5 and D7 PS over spheres grown with empty vector WPMY-1

The WPMY-1/ERα cells were next used to assess whether estrogen signaling modulates stromal regulation of epithelial progenitor cell proliferation. PS were cultured in direct contact with WPMY-1/EV or WPMY-1/ERα for 5 days with ethanol vehicle or 10nM E2, after which a 2 hour BrdU-pulse proliferation assay was performed (Figure 19). Co-culture of PS with WPMY-1 cells, with or without ERα expression, increased progenitor cell proliferation over PS cultured

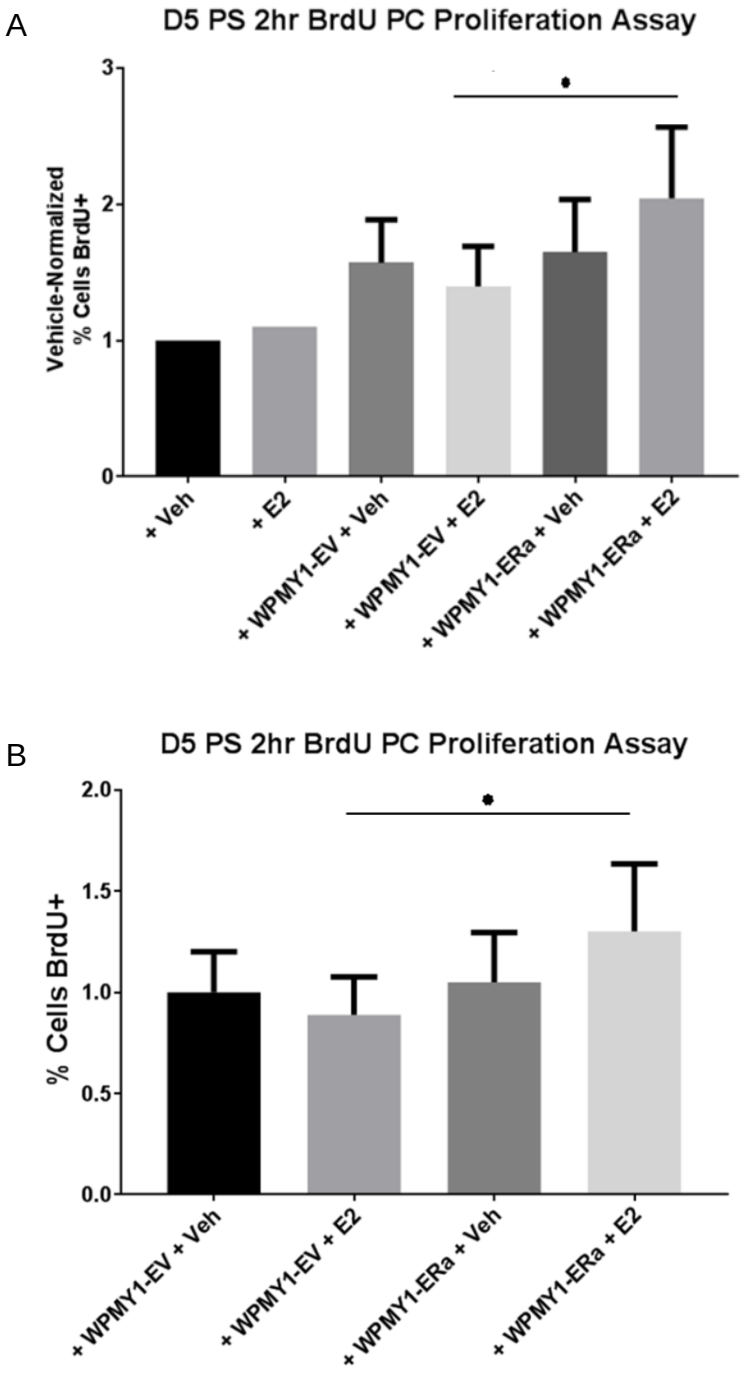


Figure 19. Effect of E2 and stromal-ERα co-culture on progenitor cell proliferation in D5 PS.

WPMY-1/ERα stromal cells promote progenitor cell proliferation in a ligand-independent manner. A. Vehicle and E2 treated PS as reference; N=4 for treatments with error bars, N=1 for Vehicle and E2 treated PS. B. Co-cultures alone, N=4. *$p<0.05$

alone. While addition of E2 to WPMY-1/EV co-cultures did not alter progenitor cell proliferation over vehicle-treated co-cultures, E2 treatment with WPMY-1/ERα cells increased progenitor cell proliferation when compared to WPMY-1/EV cells with E2. Together, this suggests that E2 can act through ERα in stromal cells to augment progenitor cell proliferation.

Further evidence suggesting that stromal ERα modulates the dynamics of progenitor cell proliferation was obtained by measuring the size of D7 PS co-cultured with vehicle- or E2-treated WPMY-1/EV or WPMY-1/ERα cells (Figure 20). Measurements of PS diameter, a rough read-out of progenitor cell numbers and thus overall proliferative events, showed co-culture with WPMY-1/ERα stromal cells significantly increased the size of PS compared to WPMY-1/EV co-cultures, implicating a ligand-independent effect of stromal ERα expression on progenitor cell proliferation. When E2 was added to the cultures, there was no effect in EV cells, whereas E2 in the ERα-expressing stromal cells increased PS size compared to WPMY-1/EV with E2. Taken together, the present results suggest that ERα in stromal cells can augment epithelial progenitor cell proliferation in a ligand-dependent and ligand-independent manner.

5. Estrogen signaling alters stromal cell influences on epithelial lineage specification and differentiation within an *in vitro* model of the benign prostate stem cell niche

Data from gene expression and immunohistochemistry assays in section III suggested that stromal cells may alter differentiation dynamics and lineage specification of epithelial stem and progenitor cells, delaying luminal cell differentiation and either maintaining or possibly increasing early basal progenitor cell status (Figures 10-12). The same assays were next utilized to investigate how stromal estrogen signaling modulates these effects. Gene expression analysis

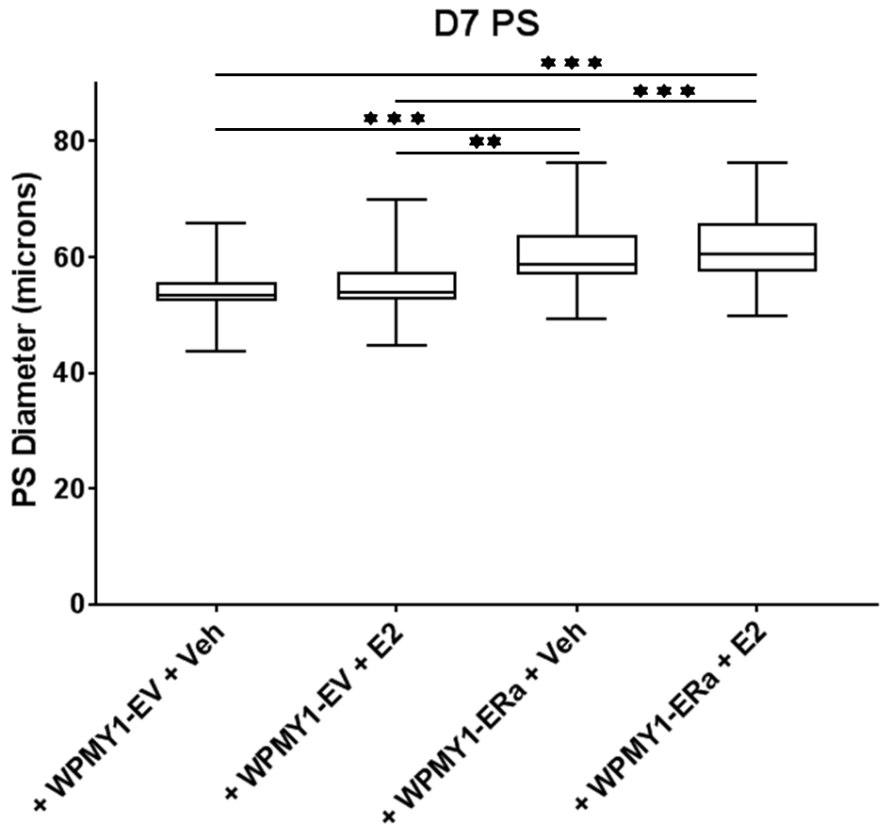


Figure 20. Effect of ERα overexpression on size of D7 PS co-cultured with empty vector or ERα WPMY-1 cells +/- E2.

Overexpression of ERα in WPMY-1 significantly increases day 7 PS size compared to co-cultures with empty vector WPMY-1 cells. PS were grown for 7 days in matrigel directly on 2D WPMY-1 cells and co-cultures were treated with vehicle or 10nM E2. Overexpression of ERα in WPMY-1 significantly increased day 7 PS size compared to co-cultures with empty vector WPMY-1 cells suggesting a ligand-independent effect of stromal ERα on progenitor cell amplification.

Boxes represent middle quartiles of data; bars represent upper and lower quartiles.
n=9, *** adjusted P Value < 0.001, ** adjusted P Value = 0.01

of D14 PS co-cultured with PrSC +/- 10nM E2 demonstrated that E2 and stromal cells both affect basal and luminal cell gene expression (Figure 21). Estradiol treatment alone significantly increased both basal and luminal markers, *Hoxb13* and *CK8*, respectively, while *p63* and *NKX3.1* both trended upward. The combined effects of E2 treatment and stromal co-culture resulted in additive effects on basal cell *p63* and *Hoxb13* gene expression, both being significantly increased over vehicle control PS. In contrast, stromal co-culture without E2 suppressed luminal cell *CK8* and *NKX3.1* gene expression. It is noteworthy that E2 treatment of stromal-PS co-cultures was not able to overcome the effect of stromal cells in decreasing luminal *CK8* and *NKX3.1* gene expression, suggesting that stromal influences are dominant.

The WPMY-1/ERα cell line was next utilized to more robustly evaluate stromal-ER signaling effects on epithelial lineage specification. Using the same IHC assay as described in section III, D14 PS treated +/- 10nM E2 and co-cultured with or without WPMY-1/EV or WPMY-1/ERα were immunofluorescently stained for basal and luminal cytokeratins (Figure 22 & 23). Quantification of fluorescent intensity (Figure 22) revealed no substantial effects of E2 alone on either basal or luminal cytokeratin expression. Likewise, E2 treatment of PS co-cultured with WPMY-1/EV did not yield differences in cytokeratin protein expression over the previously noted suppressive effects on CK14 with WPMY-1 cell co-cultures. Stable expression of stromal ERα, however, did substantially restore basal and luminal cytokeratin expression to near control levels, an effect which was ligand independent. Interestingly, the addition of E2 to WPMY-1/ERα co-cultures tended to augment the restoration of luminal cytokeratin expression towards control PS levels, while abrogating the restoration of basal cytokeratin expression.

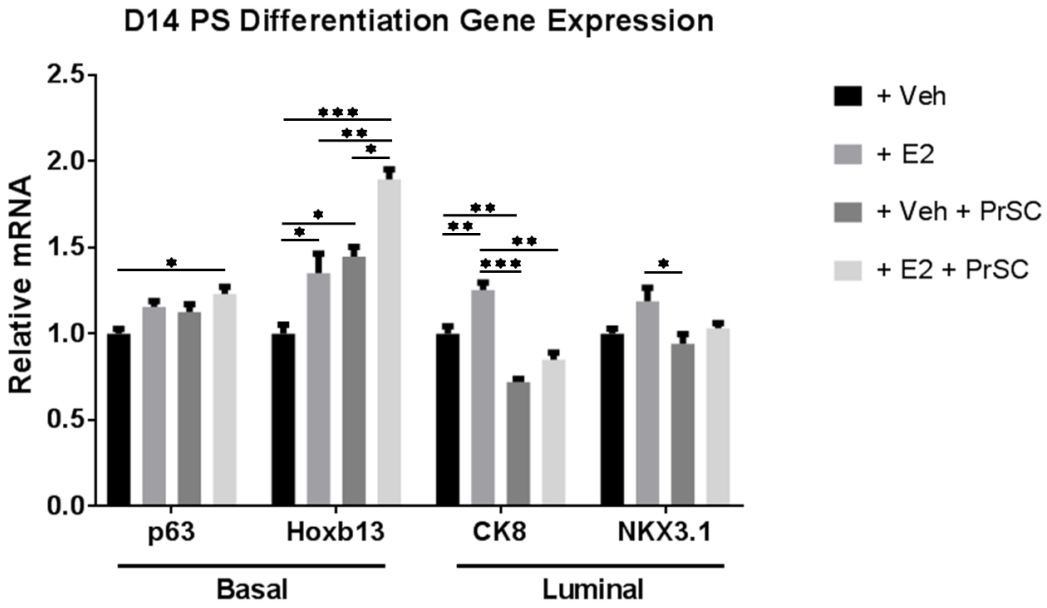


Figure 21. Differentiation marker gene expression in D14 PS co-cultured with PrSC +/- 10nM E2.

Both E2 and stromal cells both affect basal and luminal cell gene expression. N=3, *** adjusted P Value < 0.001, ** adjusted P Value = 0.01, * adjusted P Value = 0.05

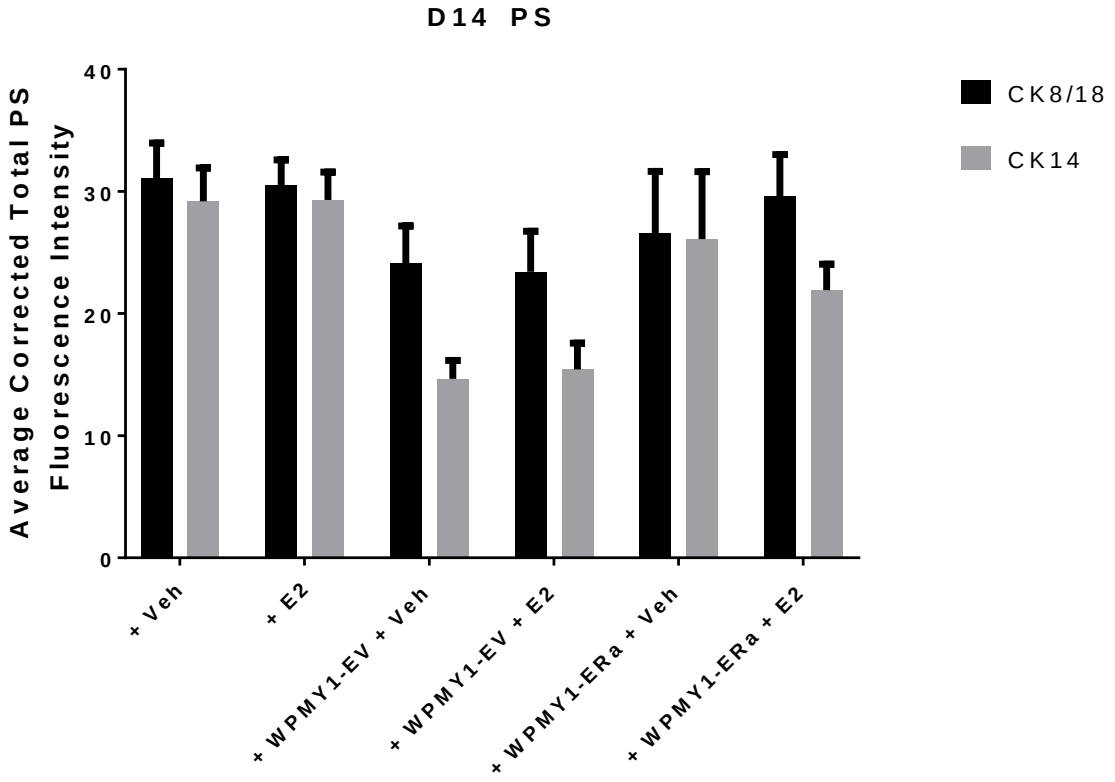


Figure 22. Quantification of fluorescent staining for basal and luminal cytokeratins in D14 PS +/- WPMY/EV/ERα

PS co-cultured for 14 days with WPMY-1/EV/ERα cells +/- E2 display altered levels of both luminal and epithelial markers. Quantification of immunofluorescent (IF) intensity; the IF intensities of ≥ 20 PS for each condition were averaged. No test for significance was performed.

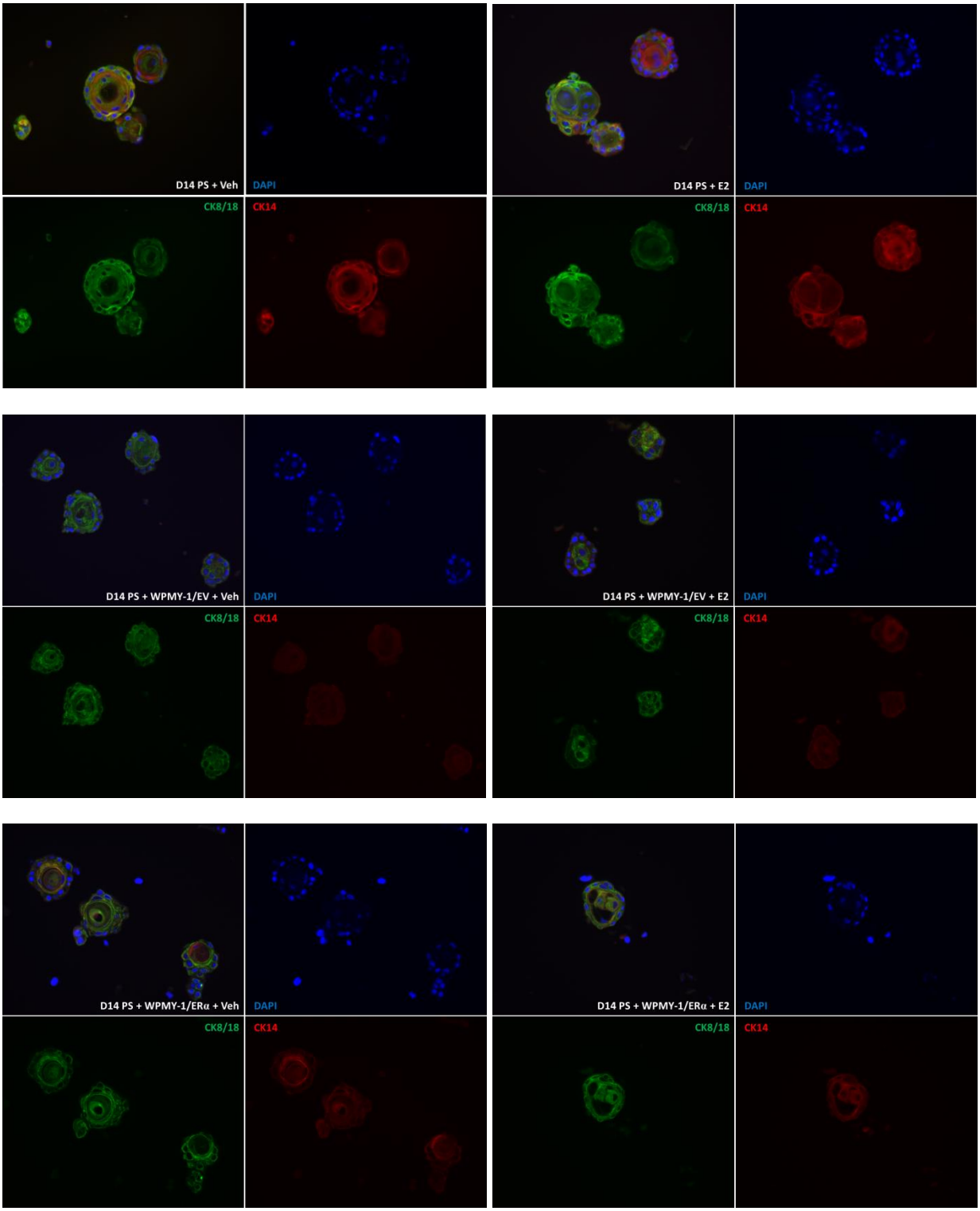


Figure 23. Immunofluorescent staining for basal and luminal cytokeratins in D14 PS +/- WPMY/EV/ERα.

PS co-cultured for 14 days with WPMY-1; luminal CK8/18 is in green, basal CK14 is in red.

6. Whole-genome expression analysis of WPMY-1/EV/ERα cells identifies extracellular matrix remodeling, immune effector and metabolic pathways as potential mediators of stromal-ER influences on the epithelial stem cell niche

Given the consistent evidence that stromal cells alters epithelial stem and progenitor cell homeostasis and that stromal-ERα modulates these effects, an Illumina gene expression microarray was utilized to identify candidate genes and signaling pathways potentially responsible for these effects. WPMY-1/EV and WPMY-1/ERα stromal cells were treated with or without 10nM estradiol for 24 hours, after which total RNA was isolated and used in downstream gene expression microarray analysis. Upon bioinformatics analysis of microarray results with a false discovery rate p-value threshold set at 0.001, 858 genes were found to be differentially expressed between WPMY-1/EV and WPMY-1/ERα stromal cells, with limited differences noted by the addition of E2 (Figure 24). The clustered heatmap of these genes, upon manual inspection, showed that major coordinately altered groups of genes include: IGF-binding proteins, glycoproteins, extracellular matrix-associated proteins and secreted factors, EGF-associated and nutrient-responsive genes, and immune regulatory genes. Gene ontology analysis of genes over-expressed in WPMY-1/ERα confirmed that three major biological themes were upregulated in WPMY-1/ERα cells: extracellular matrix remodeling, immune regulatory and glucose metabolic processes (Figure 25).

To identify individual E2- or ERα-regulated genes that may be mediating stromal effects on epithelial stem and progenitor cells, the list of 858 differentially regulated genes was reduced to those that encode for secreted factors, and in turn these genes were analyzed according to whether or not they were upregulated by E2 or stromal-ERα (Figure 26). Using this rigorous

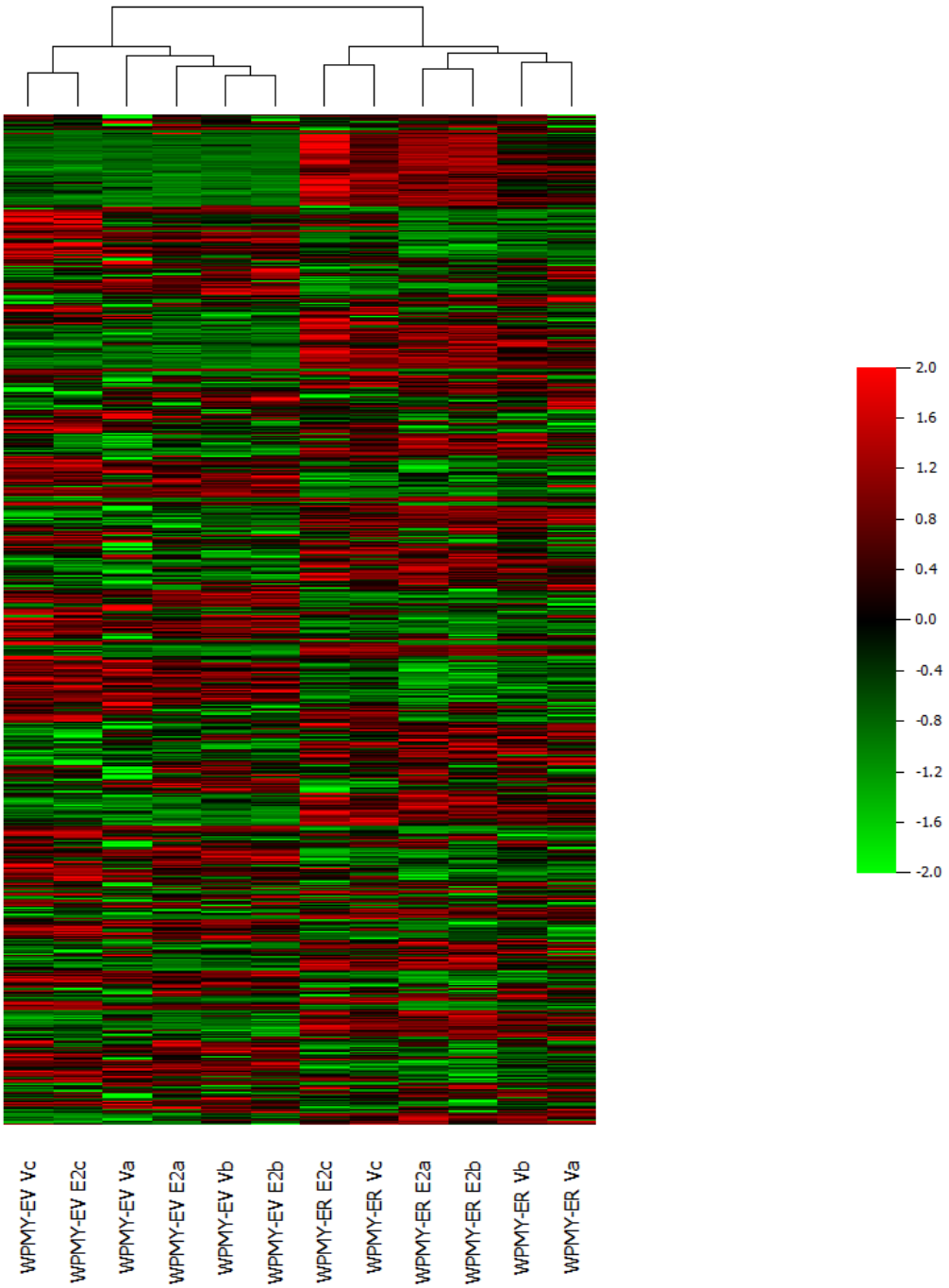


Figure 24. Global hierarchical clustering heatmap of WPMY/ERα vs. WPMY/EV Microarrays.

Global hierarchical clustering heatmap of WPMY/ERα vs. WPMY/EV +/- 10nM E2 for 24 hours. Samples cluster into two main clades according to ERα expression.

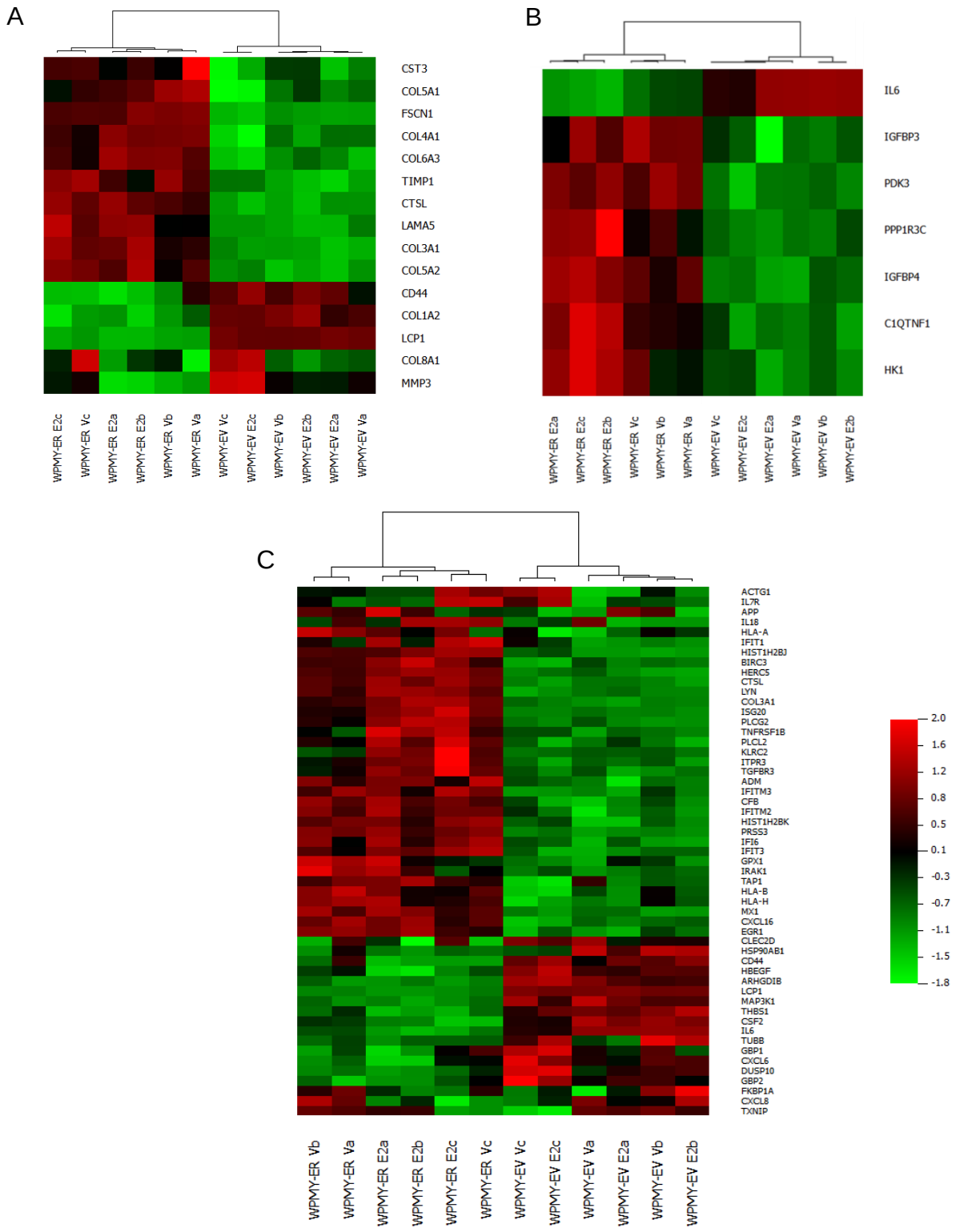
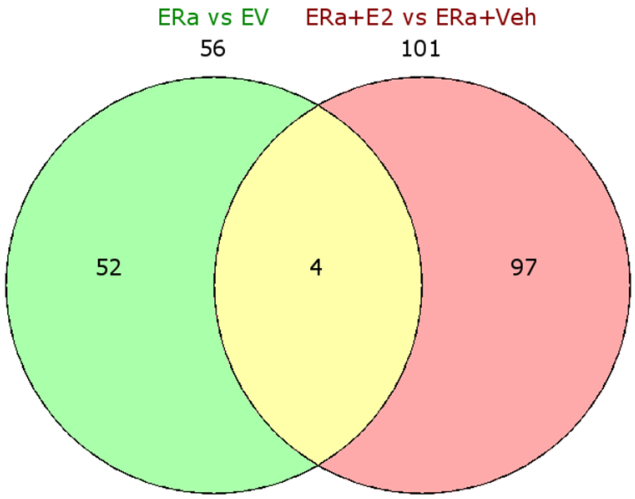


Figure 25. Heatmaps showing upregulation in WPMY/ERα of Gene Ontology Categories.

A) Extracellular Matrix Disassembly, B) Glucose Metabolic Processes and C) Immune Effector Processes



Secreted Factor Genes Upregulated in *both* [ERα vs. EV] & [ERα+E2 vs. ERα+Veh]	
Gene Symbol	Gene Name
LAMA5	laminin, alpha 5
C1QTNF1	C1q and tumor necrosis factor related protein 1
GPC1	glypican 1
SVEP1	sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1

Figure 26. Identification of genes that are upregulated by both ERα expression and E2 treatment in WPMY-1/ERα and WPMY-1/EV cells.

The Venn diagram shows the overlap (yellow) in genes that were upregulated in WPMY-1/ERα vs. WPMY-1/EV (green) and WPMY-1/ERα + E2 vs. WPMY-1/ERα + Veh (red). The four genes that overlap are listed in the table.

criteria four genes—*LAMA5*, *C1QTNF1*, *GPC1* and *SVEP*—were identified that were upregulated by stromal-ERα and further increased when stimulated with E2.

7. Laminin α5

Of the four estrogen-regulated secreted factor genes, *LAMA5* had the strongest empirical and literature-based evidence of involvement in stem cell biology and was therefore interrogated first. Prostaspheres were grown for 5 days in co-culture with WPMY-1/EV/ERα cells that were transfected with either scrambled siRNA or siRNA targeting *LAMA5* RNA. While measurements of PS size and number again confirmed a marked increase with ERα overexpression, there were no substantial differences between PS grown with scramble siRNA versus siLAMA5 transfected stromal cells (Figure 27). Furthermore, BrdU label retaining assays revealed no evidence of differences in stem cell self-renewal between these two treatments (data not shown). Of the four laminin trimers that contain laminin-α5 as a subunit, laminin-511 has been demonstrated to be a sufficient substrate to maintain growth of mouse embryonic stem cells[57-59]. Therefore, recombinant E8-fragments, which comprise the receptor-binding domain of the laminin-511 trimer, were tested for their ability to replace matrigel in a ultra-low attachment PS assay. PrEC were seeded in wells of an ultra-low attachment 96-well plate with either matrigel slurry, 5% matrigel, or a dilution range of E8-fragments. After 14 days of culture, the positive control wells with either 3D matrigel or 5% matrigel contained well-formed PS, while the wells containing E8 fragments contained no PS (Figure 28). Finally, PrEC were grown in PuraMatrix, a synthetic peptide hydrogel matrix, supplemented with a dilution range of recombinant E8 fragments. No PS growth was observed in any condition (data not shown). Together, these results suggest that LAMA5 or laminin-511

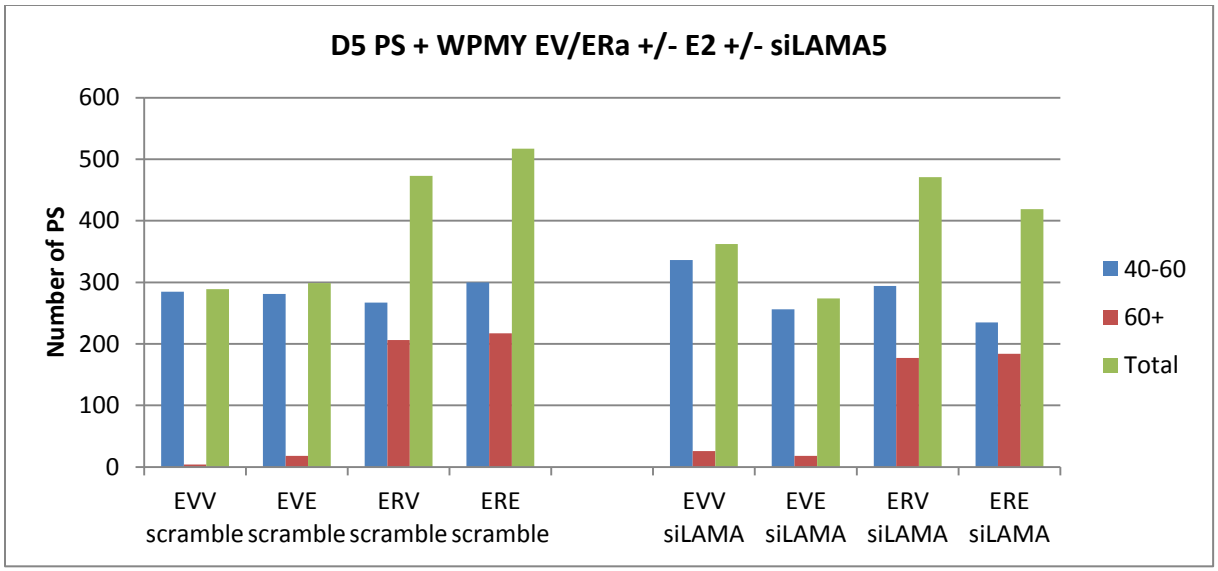


Figure 27. Effect of LAMA5 knockdown in WPMY-1/ERα and WPMY-1/EV cells on size distribution of D5 PS.

PS cultured for 5 days with WPMY-1/ERα or WPMY-1/EV +/- E2 +/- siRNA against the *LAMA5* gene product. The diameter of PS was measured and the total number of PS in each category of diameter is represented by the blue, red and green bars.

	PrEC in 3D Matrigel	PrEC + 5% Matrigel	PrEC + 1.5µg/mL E8	PrEC + 1.0µg/mL E8	PrEC + 0.5µg/mL E8	PrEC + 0.25µg/mL E8	PrEC + 0.125µg/mL E8	PrEC + 0.05µg/mL E8	PrEC + 0.025µg/mL E8	PrEC + Media Alone
PS formed after 14 days?	+++	++	-	-	-	-	-	-	-	-

Figure 28. Recombinant Laminin 511 E8 Fragments do not substitute for matrigel in ultra-low attachment spheroid formation assay.

PS were cultured in 96 well plates with either prostate epithelial cells (PrEC) plus media alone, PrEC in 3D matrigel slurry with media, PrEC in media plus 5% matrigel, or PrEC in media plus a dosage range of Laminin 511 E8 fragments. No PS formed in wells with any concentration of Laminin 511 E8 fragments, indicating that the E8 fragments do not substitute for matrigel in this experimental setup.

alone are insufficient to replicate the stromal-ERα effects on the stem & progenitor cell niche. Nonetheless, they do not rule out that in combination with other necessary factors, they may be important niche components.

8. Interleukin 6

Immune regulation was one of the three major biological pathways modulated by stromal ERα. The *IL-6* gene, which was one of the most down-regulated genes by stromal ERα, has been implicated by a wealth of literature as being important in both stem cell biology and cancer, including prostate cancer. Therefore, IL-6 was investigated as a possible mediator of stromal-ERα effects on prostate stem and progenitor cells. A Bioplex cytokine array was utilized to measure the protein levels of IL-6 secreted from two different stromal-ERα expression constructs, WPMY-1/EV/ERα stable cell line and PrSC2/EV/ERα transient primary culture cells (Figure 29). Both cell types were treated with vehicle or 10nM E2 for 24 hours and the cell supernatant was analyzed for levels of secreted IL-6 protein. Unexpectedly, IL-6 protein levels were increased by exogenous expression of ERα in WPMY-1 cells, an effect that was ligand-independent. Although this increase was not statistically significant, it nonetheless did not correlate to the ERα-associated reduction in *IL6* mRNA level from the gene expression microarray. The PrSC2/ERα cells, however, did show a significant reduction in the secreted IL-6 protein level by virtue of ERα expression alone. Treatment of these PrSC2/ERα cells with E2 resulted in an additional significant reduction in IL-6 protein secretion, suggesting that stromal ERα does indeed decrease IL-6 secretion in a ligand-dependent manner.

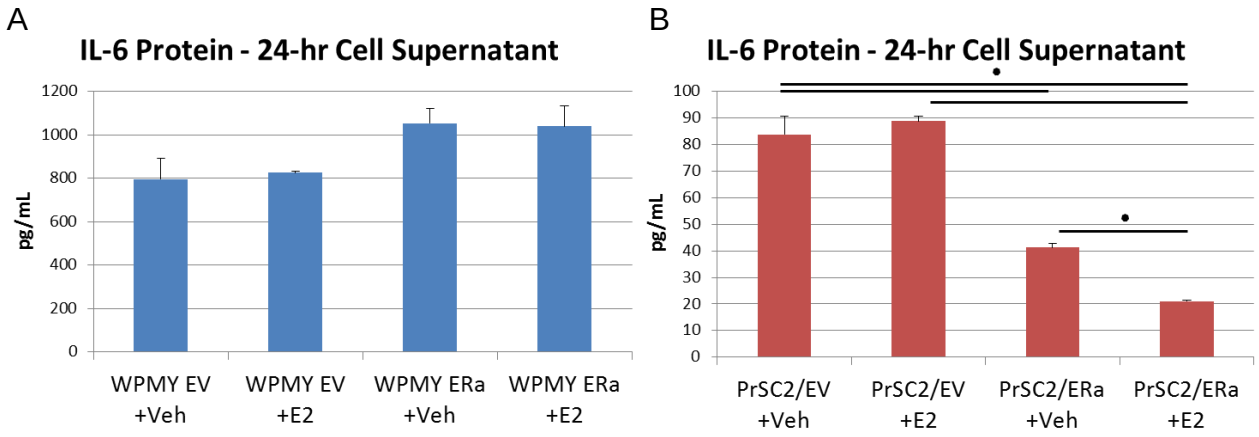


Figure 29. IL-6 protein measurements from Bioplex Cytokine Array.

IL-6 protein level in 72-hr cell culture supernatants from Bioplex Cytokine Array for A) WPMY-1/ERα or WPMY-1/EV cells +/- 10nM E2; B) PrSC2/ERα or PrSC2/EV cells +/- 10nM E2

The role of IL-6 in PS formation was assessed by adding increasing concentrations of recombinant IL-6 to PS cultured with WPMY-1/ERα stromal cells for 7 days. Assessment of PS size and number after 7 days demonstrated a dose-dependent effect of IL-6 on PS formation in co-cultures with WPMY-1/ERα cells; however, no IL-6 effect was observed on control PS grown without stromal cells (Figure 30).

C. Discussion

The prostate gland is an androgen-regulated tissue in both normal development and homeostasis, as well as in cancer. Estrogen, however, is also known to play an important role in these processes. In rat models, early life estrogen exposure causes squamous metaplasia and disrupts branching morphogenesis[60,61]. Early exposures also cause a developmental estrogenization effect through persistent epigenetic alterations that predispose animals to hyperplasia, inflammation, prostatic intraepithelial neoplasia, and hormonally-driven carcinogenesis later in life[60,62]. Furthermore, estradiol can drive prostate carcinogenesis in an androgen-supplemented, chimeric humanized mouse model of prostate cancer[13]. Clearly, the prostate gland is regulated by estrogen in both health and disease.

Many of estrogen's effects on the prostate have been attributed to ER expression in the epithelium, which variably expresses ERα and ERβ, while others have been attributed to ER expression in the stroma, which expresses mainly ERα *in vivo*. Previous work from this laboratory established that prostate stem and progenitor express robust levels of ERα, ERβ and GPER and are direct targets of estradiol, which increases stem cell self-renewal and progenitor cell proliferation[13]. Given the evidence presented in this thesis that that stromal cells modulate

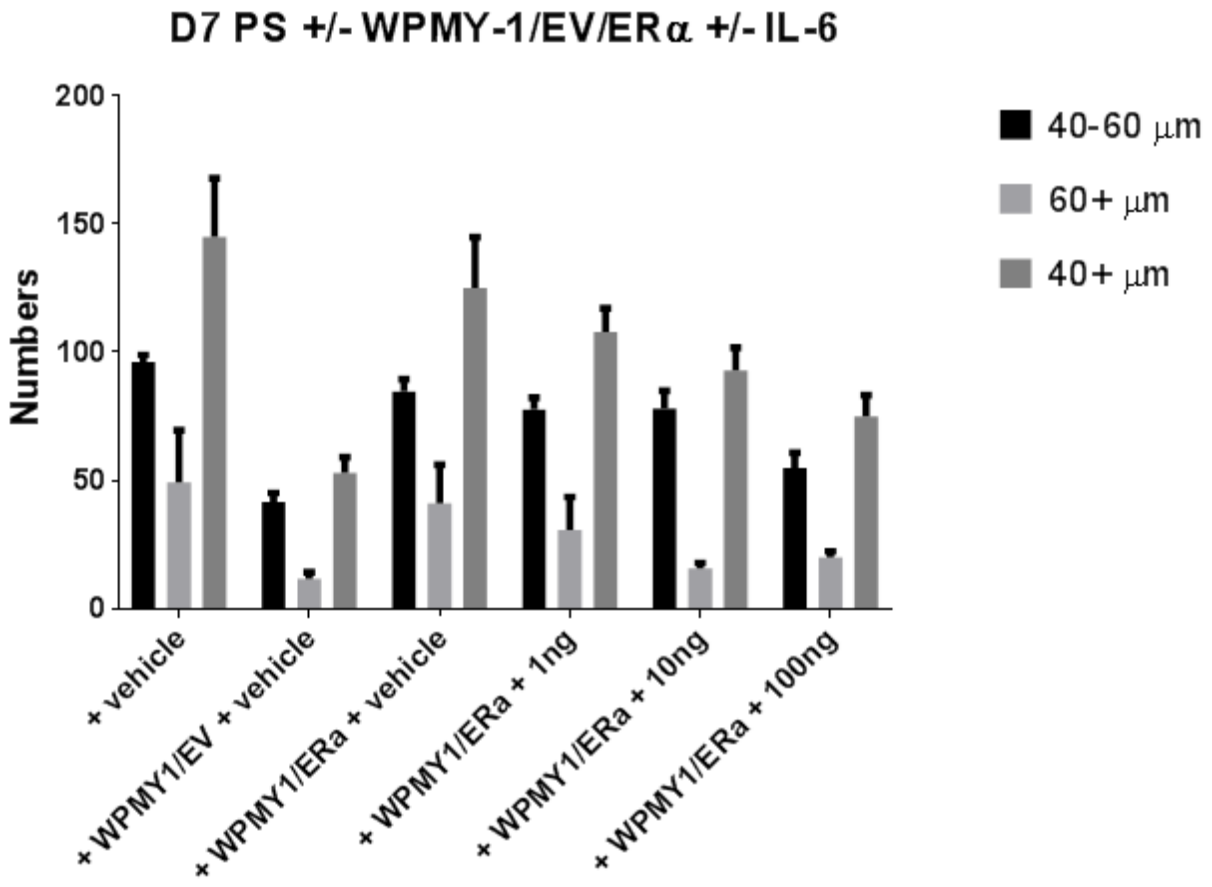


Figure 30. Effect of IL-6 treatment in overcoming pro-proliferative effects of stromal-ERα on PS size in D7 PS.

D7 PS +/- WPMY-1/EV/ERα stromal cells + 0, 1, 10 or 100ng IL-6. Error bars represent SEM between 3 technical replicates. No tests for significance were performed.

the epithelial stem cell niche, and given the fact that both of these cell types are known to express ERs *in vivo*, it is highly plausible that estrogen regulates the stem cell niche not only through direct effects on epithelial stem and progenitor cells, but also through indirect effects via stromal ER. Indeed, in tissues such as the hematopoietic system, in which E2 is known to modulate stem cell self-renewal and differentiation, stromal cells have been shown to regulate extrinsic stem cell regulatory factors in an estrogen-dependent manner[63,64]. Thus, the present study sought to identify stromal-mediated E2-regulation of extrinsic stem cell regulatory factors within the prostate epithelial stem cell niche. We hypothesized that estradiol stimulates the secretion of stromal cell paracrine signaling factors that have activity on nearby stem or progenitor cells.

Initial studies measuring gene expression of stem cell factors in PS treated with E2-stimulated stromal cell conditioned media demonstrated that E2 does indeed have indirect, stromal-mediated actions on stem cell gene expression. Although human prostate stem cells have not yet been reliably isolated and characterized, considerable evidence exists from genomic studies, sphere-forming assays and embryonic stem cell studies that the stemness genes selected for these assays are important in prostate stem cell biology[65-67]. The genes included four transcription factors central to pluripotency—*Nanog*, *Sox2*, *Oct4* and *Tbx3*—and two well-characterized genes encoding stem cell surface markers—*ABCG2* and *CD49f* (integrin-α6). Although all six genes showed a coordinated increase in PS treated with E2-stimulated SCCM from primary stromal cells, these increases were modest and only the core stemness factors *Nanog* and *Sox2* were significantly increased. This was not surprising, however, considering that even in D5 PS, the majority of cells are proliferating progenitor cells, with the stem cells being a very minor

population. Another important aspect of this data is that the conditioned media had to be concentrated 40-fold (with subsequent dilution into PrEGM yielding an effective concentration of 1.875-fold) to produce effects on PS stemness gene expression; without concentration, no differences were seen between vehicle- and E2-stimulated SCCM (data not shown). The need to concentrate the SCCM might suggest that the factors responsible are secreted by stromal cells at low concentrations, which would be consistent with paracrine factors that signal at short distances within a defined physiological niche.

Gene-level upregulation of stem cell factors provides strong evidence of stromal-ER-mediated estrogenic effects on epithelial stem cells, but this assay nonetheless measures mRNA from a mixture of stem and progenitor cells. Therefore, the more sensitive BrdU label-retaining assay was used to measure stem cell self-renewal at the level of individual cells. Prostaspheres were co-cultured with primary stromal cells +/- 10nM E2 for either 5 or 14 days. At both time points, E2-treated PS had increased numbers of BrdU label-retaining cells, indicating that E2 increases symmetric stem cell self-renewal. Likewise, in both D5 and D14 PS, combination of E2 treatment with stromal cell co-culture yielded increases in the number of label-retaining cells that were essentially additive effects of the separate stromal and E2 influences.

The additive effects of E2 and stromal co-culture suggested that E2 might not signal through stromal-ER to induce secretion of exogenous stem cell factors. Although this stands in contrast to results of the SCCM gene expression experiments, several interpretations might reconcile these discrepancies. First, estrogenic and non-estrogenic stromal-secreted extrinsic factors could potentially converge on the same intrinsic pathway within stem cells. In such a scenario, the two

signals might be redundant. Alternatively, since there are temporal and biological limitations on the instances of stem cell self-renewal that can occur in a given population of cells over a finite period of time, the magnitude of the combined stromal and E2 effects might represent the maximal rate of stem-cell self-renewal. Finally, there exists the possibility that E2 and stromal cells do indeed act synergistically to increase stem cell self-renewal, but these effects are obscured by limitations of *in vitro* modelling.

Evidence of synergistic, stromal-ER-mediated effects of E2 in increasing symmetric stem cell self-renewal differed between the two patient-derived Spz cultures used in the D5 PS co-culture BrdU label-retaining assays. When only the results from co-culture with the Spz3 primary stromal strain are considered, possible synergy between E2 and stromal co-culture can be observed. Furthermore, levels of ERα as assessed by ICC, was higher in the Spz3 stromal cells compared to the Spz2 cells (Figure 31), suggesting high expression of ERα protein might be necessary to observe evidence of indirect, stromal-ERα-mediated actions of E2 on stem cells. In order to test this hypothesis, the WPMY-1 stromal cell line, which expresses low ERα protein *in vitro*, was utilized to induce exogenous expression of ERα via a lentiviral vector.

Direct contact co-culture of PS with WPMY-1/ERα cells +/- 10nM E2 for 7 days yielded an apparent ligand-independent effect of stromal-ERα on increasing symmetric stem cell renewal via the BrdU label-retaining assay. Addition of E2 to co-cultures with WPMY-1/ERα cells demonstrated a possible synergistic effect between stromal co-culture and E2 treatment. Interestingly, addition of E2 to WPMY-1/EV co-cultures also resulted in a combined effect that might be interpreted as a synergistic response; however, high assay variability precluded a

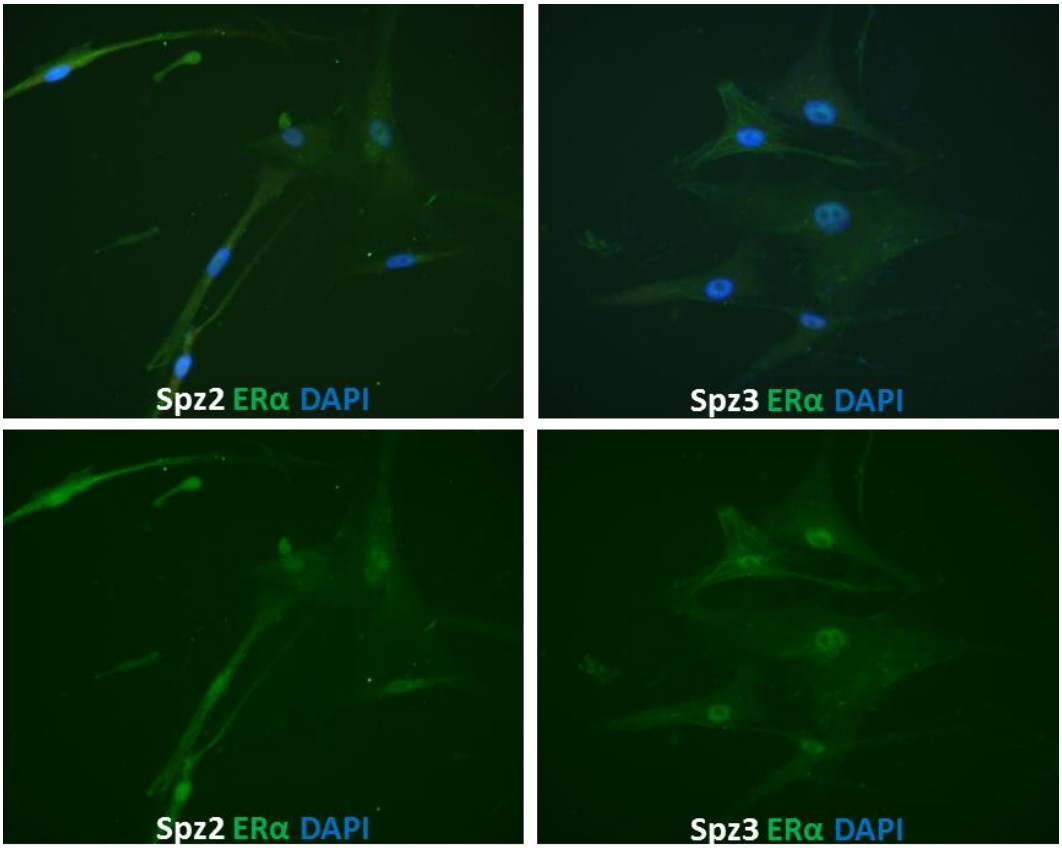


Figure 31. ERα expression via ICC on two primary patient-derived Spz stromal cell cultures.

The Spz3 stromal cells have stronger nuclear ERα signal.

reliable conclusion. Regardless, this data set provides conclusive evidence of stromal-mediated E2 effects on the SCN. Furthermore, the possibility that stromal-ERα has ligand-independent activity within the SCN is an important observation.

Additional evidence of ligand-independent stromal-ERα actions was seen in progenitor cell proliferation assays. The same assays that were used in section III —the 2-hour BrdU proliferation assay of D5 PS and measurement of D7 PS size—were herein used to assess the effects of stromal-ERα on progenitor cell proliferation. The effect of E2 treatment alone had no effect on epithelial progenitor cell proliferation in either the D5 or D7 assays. Likewise, combined treatment of E2 with WPMY-1/EV cells yielded no discernable changes compared to vehicle-treated co-cultures. Furthermore, co-culture with WPMY-1/ERα yielded no change versus empty vector stromal cells in the D5 PS 2-hour BrdU proliferation assay. However, treatment of WPMY-1/ERα co-cultures with E2 yielded a significant increase in proliferating progenitor cells versus the WMPY-1/EV E2-treated co-cultures, suggesting that stromal-ERα conveys estrogenic effects on progenitor cell proliferation in early spheroid development. While the final size of D7 PS was consistently and significantly larger in PS co-cultured with WPMY-1/ERα cells compared to WPMY-1/EV, no E2 effect was seen on that parameter. In summary, co-culture with WPMY-1/ERα cells produced evidence of ligand-dependent effects on progenitor cell proliferation in D5 PS and ligand-independent effects on size of D7 PS. Thus, expression of ERα clearly alters the stromal influence on proliferation dynamics within the stem cell niche.

A critically important finding from these progenitor cell proliferation studies is that the phenotype of larger PS with WPMY-1/ERα co-culture required direct-contact of the stromal cells and matrigel slurry. When PS and stromal cells were separated via an insert, no difference in PS size was seen between WPMY-1/ERα and WPMY-1/EV. This suggests that factors responsible for enhancing progenitor cell proliferation act over short distances, are concentration-dependent, or potentially involve cell-cell or cell-matrix interactions. Many of the molecular mechanisms involved in morphological development share these properties[68]. Morphogens can induce polarized responses in target cells based on their concentration, i.e. high or low concentration can induce differentiation of target cells into one of two lineages[69]. Morphogens are secreted in gradients—their concentration at a target cell is a function of distance between the source cell and target cell. The proximity-dependent effects of WPMY-1/ERα cells on increasing PS size compared to empty vector stromal cells is consistent with the properties of a morphogen. These effects are also consistent with the requirements of cell-matrix interactions, which often involve presentation or cleavage of growth factors or morphogens.

Assessment of how estrogen signaling impacts the previously described stromal influences on progenitor cell lineage commitment and differentiation was achieved using D14 co-cultures with both primary PrSC and WPMY-1/ERα stromal cells +/- E2. Day 14 PS treated with 10nM E2 alone showed significant increases in both basal and luminal differentiation markers at the mRNA level, with all gene transcripts increasing and *Hoxb13* and *CK8* being significant. The combination of E2 treatment and PrSC co-culture yielded significant, seemingly additive effects on expression of both basal markers *p63* and *Hoxb13*, suggesting that both E2 and stromal cells promote basal lineage specification. Contrary to the basal lineage markers, the luminal markers

were oppositely regulated by E2 and stromal cells, with E2 increasing luminal gene expression and stromal cells decreasing expression. Of note, the stromal effects on decreasing luminal gene expression seemed to be dominant over the E2-mediated increases, suggesting that stromal-derived factors are more commanding extrinsic regulatory factors within the SCN than estrogen.

The notion that stromal-derived factors are more potent than E2 in regulating the SCN was also borne out in the results of the IHC immunostaining assay for cytokeratin markers in D14 PS co-cultured with WPMY-1/EV/ERα cells. This assay demonstrated that E2 had no effect on basal or luminal cytokeratin expression in either PS grown alone or co-cultured with WPMY-1/EV stromal cells. As described in section III, however, co-culture with stromal cells strongly decreased expression of both luminal and basal cytokeratins, hinting that stromal cells secreted factors that are more potent than E2 in terms of their ability to alter lineage specification. Importantly, co-culture with WPMY-1/ERα markedly restored basal and luminal cytokeratin levels toward those of control PS. This was yet again an example of ligand-independent effects of stromal-ERα. When these WPMY-1/ERα co-cultures were treated with E2, there was modest evidence of a more pronounced restoration of luminal CK8/18 expression, but a less robust restoration of basal CK14 expression, hinting at ligand-dependent stromal-ERα effects as well. Perhaps the most striking observation from these two D14 differentiation and lineage commitment assays is that stromal-ER signaling has opposite effects on basal/luminal lineage specification than non-estrogenic stromal influences. Combined E2 treatment and co-culture with PrSC, which express nearly undetectable levels of ERα, demonstrated a reduction of luminal gene expression compared to control PS grown with or without E2. Conversely, when PS were co-cultured with WPMY-1/ERα cells and E2, luminal cytokeratin expression was

unchanged compared to control PS +/- E2, and it was increased compared to WPMY-1/EV +/- E2.

The model described thus far suggests that stromal ERα expression augments the overall effect of stromal cells in promoting stem cell self-renewal. Whether this effect is ligand-dependent or ligand-independent might be a function of stromal cell lineage or subtype, which were not interrogated in this study. In contrast to effects on stem cells, the evidence presented suggests that stromal ERα expression might promote distinct and even opposite influences on progenitor cell differentiation and lineage specification, compared to non-ER-expressing stromal cells. There are conflicting reports in the literature on the expression of ERα in prostate stromal cells, both in primary human tissues and in *in vivo* and *in vitro* animal models[18,70,71]. One obvious explanation for these inconsistencies is that stromal populations within the prostate are heterogeneous, with ERα expression being dependent on stromal cell subtype, anatomical microenvironment, or disease state. If ERα-positive and ERα-negative stromal cells do in fact have distinct effects on their regulation of the epithelial SCN, this might have important implications in prostate development, homeostasis and both benign and malignant disease states.

Interrogation of the molecular and transcriptional differences between ERα-positive and ERα-negative stromal cells was achieved via an Illumina gene expression microarray. The top gene ontological categories that were enriched in WPMY-1/ERα cells included ECM deposition and remodeling, immune regulation, and metabolism. At the molecular level, several of the ERα-upregulated genes included members of the insulin-like growth factor binding protein (IFGBP) family and proteins involved in regulation of epidermal growth factor (EGF) signaling. These

changes are consistent with finding from a study by Vitkus, *et al.*, wherein rat PS-1 cells that were transduced with ERα displayed significantly higher gene expression of *IGF-1*, *IGFBP5* and *EGF* [72]. Interestingly, the authors had also found that the BPH-1 epithelial cell line grew significantly faster in conditioned media from the PS-1/ERα cells than PS-1/empty vector cells, providing a strong corollary to findings from the present study wherein WPMY-1/ERα cells increased progenitor cell proliferation in D5 PS compared to empty vector co-cultures. Although the Vitkus study did not involve enriched stem and progenitor cells, the prospect that proteins such as IGFBPs play important roles in stromal regulation of the epithelial SCN is supported by evidence from the Tang group, which reported that basal cells enriched for stem cell gene signatures also expressed high mRNA levels of IGFBP genes[65]. Proliferative effects of stromal-ERα have also been reported in setting of prostate cancer, where CAFs overexpressing ERα were shown to increase the growth of multiple epithelial cancer cell lines[71]. In a separate component of the Vitkus study, mice with ERα knocked out specifically in smooth muscle stromal cells were found to have significantly reduced collagen deposition in prostate basement membranes, again correlating to the present study's findings that ERα up-regulates collagens and ECM deposition-related genes. Taken as a whole, the enrichments found in the present study's microarray analysis seem to correlate well with findings from other studies that have interrogated the role of ERα in stromal cells.

Since ERα expression in stromal cells seemed to operate in both ligand-dependent and ligand-independent manners, genes that were commonly upregulated by both ERα expression alone and E2 treatment were given highest priority for follow-up. After comparing WPMY-1/ERα vs. WPMY-1/EV and WPMY-1/EV + 10nM E2 vs. WPMY-1/EV + vehicle, four genes were found

to be commonly upregulated: *LAMA5*, *GPC1*, *C1QTNF1* and *SVEP1*. Interestingly, the SVEP1 protein contains an EGF domain, GPC1 binds morphogens such as BPMs and FGFs, and *LAMA5* encodes for the α5 subunit of the ECM protein laminin.

The laminin-α5 protein product of the *LAMA5* gene was interrogated first due to an overwhelming amount of literature suggesting its importance in prostate stem cell biology. Laminins are large heterotrimeric ECM proteins composed of α, β and γ subunits and are responsible for mediating cell-matrix interactions by virtue of binding to heterodimer integrin receptors[73]. Laminins 511 and 521, two isoforms containing the laminin-α5 subunit, were shown to bind both alpha-6/beta-1 and alpha-6/beta-4 integrins[74]. Integrins in general have been shown to play important roles in stem cell biology, mediating signals from the ECM that serve to maintain pluripotency[68,75]. Integrin alpha-6, also known as CD49f, has been proposed as a marker of both murine and human prostate stem cells[76-78]. In murine prostate stem cells, integrin alpha-6 is a likely component of the focal adhesion complex that binds to laminins present in matrigel[37]. This notion that a putative prostate stem cell surface protein binds to laminins in matrigel is supported by a report demonstrating that laminin is necessary but not sufficient for spheroid culture of prostate stem cells[37]. In a mass spectroscopic analysis of proteins expressed in ground-state pluripotency, laminin-α5 was found to be upregulated in pluripotent mouse ESC compared to those stimulated to differentiate[79]. Embryonic stem cells (ESC) can be maintained in an undifferentiated state by growing them on a feeder layer of mouse embryonic fibroblasts (MEF)[80,81]. Specific factors, such as leukemia inhibitory factor and laminin-α5, have been identified as sufficient replacements of MEF in their ability to maintain ESC in an undifferentiated state[82,83].

Multiple approaches were taken to investigate the possible role of laminin-α5 in mediating the observed effects of stromal cells on the epithelial SCN. The first approach used siRNA-mediated knock-down of *LAMA5* in WPMY-1/EV/ERα stromal cell co-cultures. This approach did not yield significant differences in PS size or number—two readouts of progenitor cell proliferation and stem cell self-renewal, respectively—between empty vector and ERα co-cultures. Although a 50% knockdown of *LAMA5* mRNA was sustained for at least 3 days of the 5 day co-culture period, this might not have been adequate to significantly decrease stromal secretion of laminin-α5 protein, which is a very high molecular weight protein complexed in a stable heterotrimeric form and likely has a long half-life. Subsequent assays focused on trying to establish a role of laminin-α5 in prostate stem cell self-renewal. The ligand-binding domain of laminin-α5 is available in recombinant E8 fragments, which have been shown to support growth and self-renewal of hESC in MEF feeder-free conditions[84]. Prostaspheres grown in regular 3D matrigel culture showed no differences with or without supplementation with these recombinant E8 fragments. Matrigel is essentially a mixture of bioactive ECM proteins, including many laminins[85]. Thus, if laminin-α5 is indeed involved in prostate stem cell self-renewal it is not surprising that supplementation with recombinant E8 fragments had no effect in a culture system that is already saturated with laminins. To avoid the use of matrigel, two additional approaches were taken. The peptide hydrogel PuraMatrix was utilized as a replacement for matrigel. PrECs were grown with either PuraMatrix alone or PuraMatrix supplemented with E8 fragments, but neither condition yielded PS after 7 days of culture. Finally, PS were grown in ultra-low attachment plates with 5% matrigel or a range of concentration of E8 fragments, but only 5% matrigel permitted spheroid growth. Since it has been shown previously that laminins are necessary but not sufficient for PS formation, either the E8 fragment does not have proper

biological activity or there are other essential laminins or factors that are not present in the various culture conditions utilized.

Since immune regulation was one of the top deregulated biological pathways in WPMY-1/ERα and IL-6 was one of the most down-regulated genes in these cells, efforts were next focused toward interrogating the role of this cytokine in stromal regulation of the SCN. Although it has well documented roles in numerous cancers, IL-6 is also thought to play important roles in normal stem cell biology. The IL-6 superfamily member leukemia inhibitory factor (LIF) plays a fundamental role in maintenance of ESC pluripotency, and both factors signal through dimerized receptors that contain a Gp130 subunit[86,87]. Portillo-Lara, *et al.*, found that IL-6 mRNA was upregulated in the $CD133^+$ cancer stem cell population from multiple prostate cancer cell lines[88]. This population of cells had enhanced sphere-forming capacity, as well as co-enrichment of several other genes suspected of being involved in prostate stem cells, such as *Nanog*, *Sox2*, and *ESR1*. Kroon, *et al.*, showed that both benign prostate stem-like cells and cancer stem cells express IL-6 receptor (IL-6R) and have activated phosphor-STAT3 signaling downstream of this receptor[89]. Importantly, the authors demonstrated that blockade of down-stream IL-6R signaling pathways suppressed colony formation using primary cancer stem cells derived from high Gleason grade tumors. Within the hematopoietic stem cell niche, stromal cells such as mesenchymal stem cells (MSC) and osteoblasts are known to regulate hematopoietic stem cells (HSC) via IL-6 and other factors such as Jagged-1 and GM-CSF[90,91]. Qiu *et al.* have demonstrated that E2 can signal through ERs on MSC to indirectly modulate HSC proliferation[92]. Furthermore, Bernad, *et al.*, demonstrated that IL-6 plays important roles in regulation of both HSC self-renewal and progenitor cell proliferation in mice[93]. Finally, high

levels of IL-6 secreted by human foreskin fibroblasts have been purported to usurp the need for LIF in maintenance of mouse ESC via signaling through the JAK/STAT pathway[94].

Two separate experimental model systems used in this laboratory provided evidence of a correlation between *IL6* gene expression and proliferative capacity of prostate stem and progenitor cells (Figure 32). Evidence from this study demonstrated that *IL6* gene expression was down-regulated by ERα expression in WPMY-1 cells compared to empty vector, which was correlated to larger PS in D7 co-cultures with ERα vs. empty vector stroma. A separate study from this laboratory demonstrated a correlation between high *IL6* gene expression and smaller prostate organoid size with inorganic arsenic treatment (Figure 32). Therefore, given the evidence in the literature that IL-6 regulates stem cell self-renewal, it was hypothesized that IL-6 binds to IL-6R on prostate stem cells and inhibits asymmetric self-renewal. Selective inhibition of asymmetric self-renewal would reduce the total number of rapidly proliferating progenitor cells and would be consistent with the evidence from label-retention assays that stromal cells increase symmetric self-renewal.

Secretion of IL-6 was measured in both WPMY-1 and PrSC2 stromal cells that expressed ERα or empty vector. Despite the fact that mRNA levels were strongly suppressed by ERα in WPMY-1 cells, no statistically significant difference in protein level was seen between WPMY-1/EV and WPMY-1/ERα, regardless of E2 treatment. In primary PrSC2 cells, however, ERα expression and E2 stimulation both significantly suppressed IL-6 secretion. Co-culture assays with WPMY-1/ERα or WPMY-1/EV cells demonstrated that addition of recombinant IL-6 at to PS + WPMY-

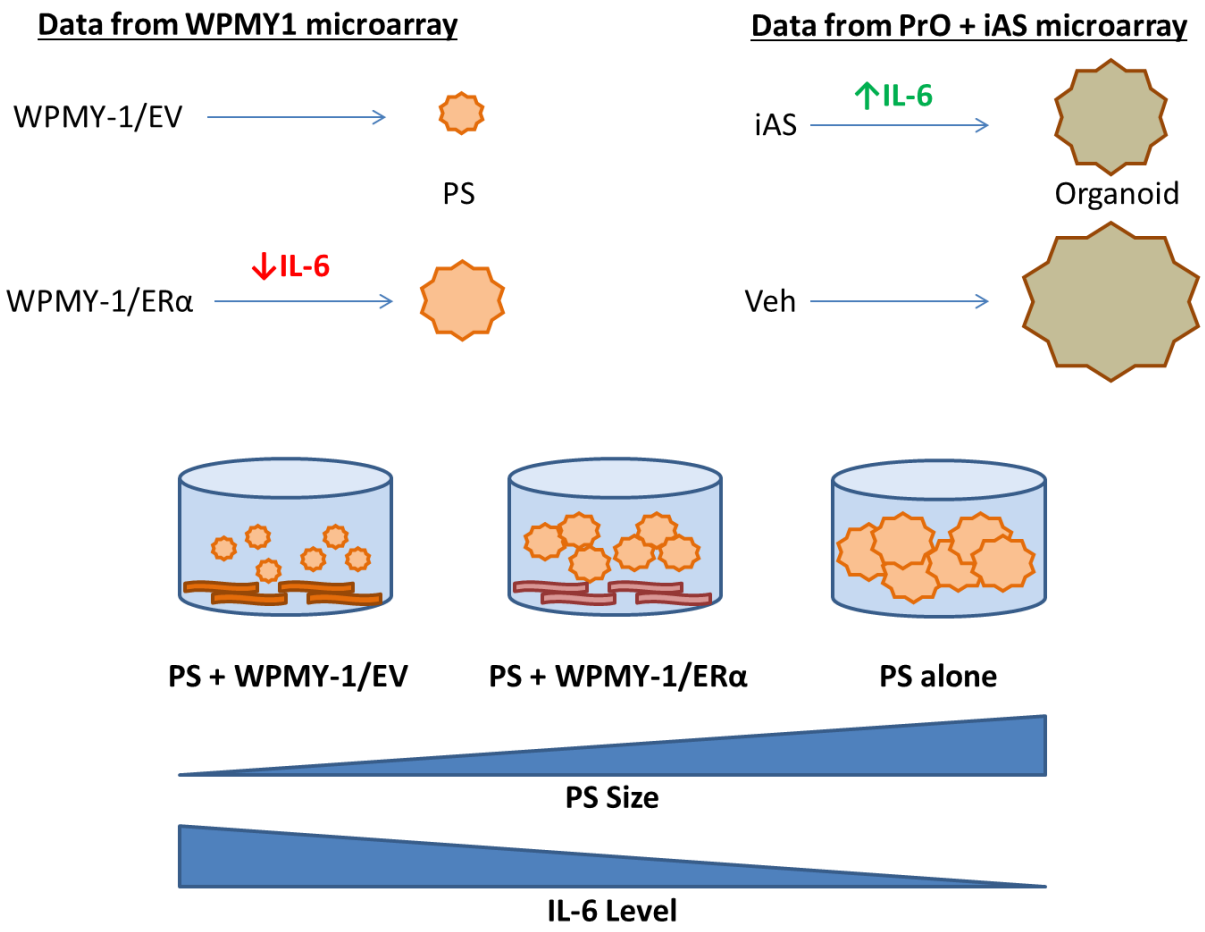


Figure 32. Observed correlations between *IL6* gene expression and size of PS or prostate organoid cultures in two separate model systems.

Low *IL6* gene expression in ERα stromal cell correlated with larger PS in co-culture with these cells. High *IL6* gene expression correlated with smaller prostate organoid size with inorganic arsenic treatment.

1/ERα cells had a dose-dependent effect in decreasing the side and total number of PS. Addition of IL-6 to co-cultures partially abrogated the effects of stromal-ERα and shifted the size of the PS closer to that of PS co-cultured with empty vector stromal cells, which had significantly higher IL-6 mRNA than WPMY-1/ERα cells. When PS were co-cultured without stromal cells, however, addition of IL-6 had no effect, suggesting that the signaling mechanism involved might require additional stromal-secreted factors. Alternatively, IL-6 might signal in an autocrine manner through stromal cell IL-6 receptor to induce the secretion of a separate extrinsic niche factor. These preliminary data suggest that IL-6 may play a role in ERα-driven stromal effects on the SCN. Additional experiments will need to be performed to confirm these preliminary data and to more robustly test the mechanism by which IL-6 might regulate prostate stem or progenitor cells. Furthermore, it will be important to perform the same co-culture experiments with PrSC2 cells to see if IL-6 supplementation can overcome any effects conferred on PS formation by PrSC2/ERα cells or E2 treatment.

As the stem cell field continues to mature, a cogent picture is beginning to emerge regarding the role of hormonal influences on stem cell niches. The results of the present study are paralleled by recent work in the bone marrow hematopoietic stem cell niche. Illing, *et al.*, described direct and indirect actions of E2 on hematopoietic stem cell homeostasis in both *in vitro* and *in vivo* assays[64]. Their *in vitro* studies demonstrated that preadipose bone stromal cell feeder layers pretreated with E2 supported higher numbers HSC colony growth. Furthermore, *in vivo* studies utilizing CSFU-labeled HSC showed higher engraftment of HSCs injected into E2-treated irradiated mice. Based upon gene expression analysis of the preadipose stromal cells, the authors

concluded that E2-induced changes in cellular adhesion molecules might be partially responsible for the indirect stromal-mediated effects of estrogen on HSC numbers.

The present studies provide the first evidence using primary human cells that estrogen signaling modulates stromal regulation of the prostate epithelial stem cell niche. Within these assays, evidence consistently suggests that stem cell self-renewal and progenitor cell proliferation are more robustly modulated in co-culture conditions where the stromal cells and PS are in close proximity to each other. Both the broad gene ontological differences and individual differentially regulated genes between empty vector and ERα stroma suggested that ECM proteins, morphogens and immune regulatory molecules are responsible for the altered SCN-regulatory effects of stromal-ERα. The ligand-independent nature of some of these stromal-ERα effects is important and has implications for the means and degree by which circulating hormones or endocrine-disrupting chemicals might influence stromal-epithelial interactions within the SCN. Ultimately, the present findings may provide critical insights into the role of estrogen signaling in the development and progression of prostate cancer.

V. EFFECTS OF CANCER-ASSOCIATED STROMA ON THE NORMAL STEM CELL NICHE & THE ROLE OF ESTROGEN RECEPTOR ALPHA IN MEDIATING ACTIVATION OF BENIGN STROMA —*Preliminary Observations*

A. Abstract

Cancer-associated fibroblasts (CAFs) arise due to reciprocal interactions between malignant tumor epithelial cells and nearby benign stromal cells. CAFs have been shown to promote tumor development, progression and metastasis in multiple organ systems[29]. In tissues such as the prostate, breast and lung, the pro-tumorigenic properties of CAFs have been attributed, in part, to their interactions with cancer stem cells (CSC)[95-97]. In the prostate cancer literature, there are conflicting reports as to whether ERα expression in CAFs enhances or attenuates these pro-tumorigenic effects[71,98]. The evidence presented in this thesis suggests that normal prostate stromal cells regulate benign epithelial stem and progenitor cells, and that stromal-ERα can modulate these interactions in ligand-dependent and –independent manners. Since CSC and benign stem cells share many molecular and functional similarities, we hypothesized that prostate CAFs exert many of the same influences on epithelial stem cells—both benign and malignant—and that CAF ERα expression promotes a tumorigenic response. Both *in vitro* and *in silico* approaches were utilized to assess whether CAFs regulate epithelial stem cells and to predict the impact of stromal ERα expression on prostate cancer clinical outcomes.

The effects of CAFs on benign human epithelial stem and progenitor cells, while present, could not be distinguished from those of benign stromal cells; however, more work is warranted.

Despite a lack of robust evidence that CAFs distinctly influence the benign stem cell niche, several remarkable associations were observed between stromal ERα expression, the CAF phenotype, and stromal contributions toward poor clinical outcome in prostate cancer. Although still preliminary, evidence from gene expression microarray analyses suggests that CAFs are more estrogen-responsive than benign Spz stromal cells. Furthermore, gene signatures derived from these estrogen-responsive CAFs and distinct gene signatures derived from two stable ERα-expressing stromal cell cultures, WPMY-1/ERα and PrSC/ERα, can be used to dichotomize patients within the Cancer Genome Atlas (TCGA) Prostate Adenocarcinoma database into two groups with significant differences in disease-free survival. These findings may have important implications regarding the impact and predictive value of stromal ERα expression on clinical outcome in prostate cancer.

B. Results

1. Model Overview

Interactions between CAFs and benign stem and progenitor cells were modeled using insert-separated co-culture of PS and three separate pairs of patient-matched primary CAF and Spz cells. The *in silico* analyses utilized Illumina gene expression microarray data for 24 hour vehicle or 10nM E2 treated cultures of the following stromal cells: 1) 3 separate patient- or age-matched pairs of primary Spz and CAF stromal cultures; 2) two additional non-matched primary CAF cultures; 3) three technical replicates of primary young, disease-free donor-derived PrSC stromal cells that were transfected with either empty vector or ERα; 4) three technical replicates of the WPMY-1 human stromal cell line that were transfected with either empty vector or ERα. All microarray data was analyzed in the R statistical environment, and follow-up analyses were

performed using the Broad Institute's implementation of the Gene Set Enrichment Algorithm (GSEA). Other publicly-available bioinformatics resources were utilized as described in later sections.

2. Cancer Associated Fibroblasts and Benign Stromal Cells Have Similar Effects on the Epithelial Stem Cell Niche

To determine if CAFs have the capacity to modulate stem cell self-renewal and progenitor cell proliferation within the benign stem cell niche, and to determine if this capacity is qualitatively or quantitatively different than that of benign stromal cells, the previously-described PS and stromal cell co-culture assay was utilized with primary PrEC, Spz and CAFs. Pooled primary PrEC were cultured in the 3D PS assay either alone or in insert-separated co-culture with one of three separate pairs of patient-matched primary CAF and Spz cells: Spz1/Sca1, Spz2/Sca2 or Spz3/Sca3. After 7 days, the size and number of PS was measured (Figure 33). Since PS size at day 7 is a rough measure of progenitor cell proliferation and the total number of PS is a rough measure of stem cell activity—*vis-à-vis* sphere-forming capacity—this assay served as readout for CAF effects on both stem and progenitor cells. A significant increase in both the total number (≥40μ diameter) and fractions of small (40-80μ diameter) and large (>80μ diameter) PS was seen with E2 treatment alone, an effect which has been previously observed by this laboratory[56]. When PS were co-cultured with Spz +/- 10nM E2, no significant differences with control PS + vehicle were seen across any metric. In terms of the total number of PS, co-culture with Spz cells +/- E2 abrogated the effect of E2 on PS cultured alone, suggesting as previously noted that stromal influences on PS growth dynamics are dominant over the direct effects of E2 on stem and progenitor cells. The effect of co-culture with CAFs +/- E2 was indistinguishable

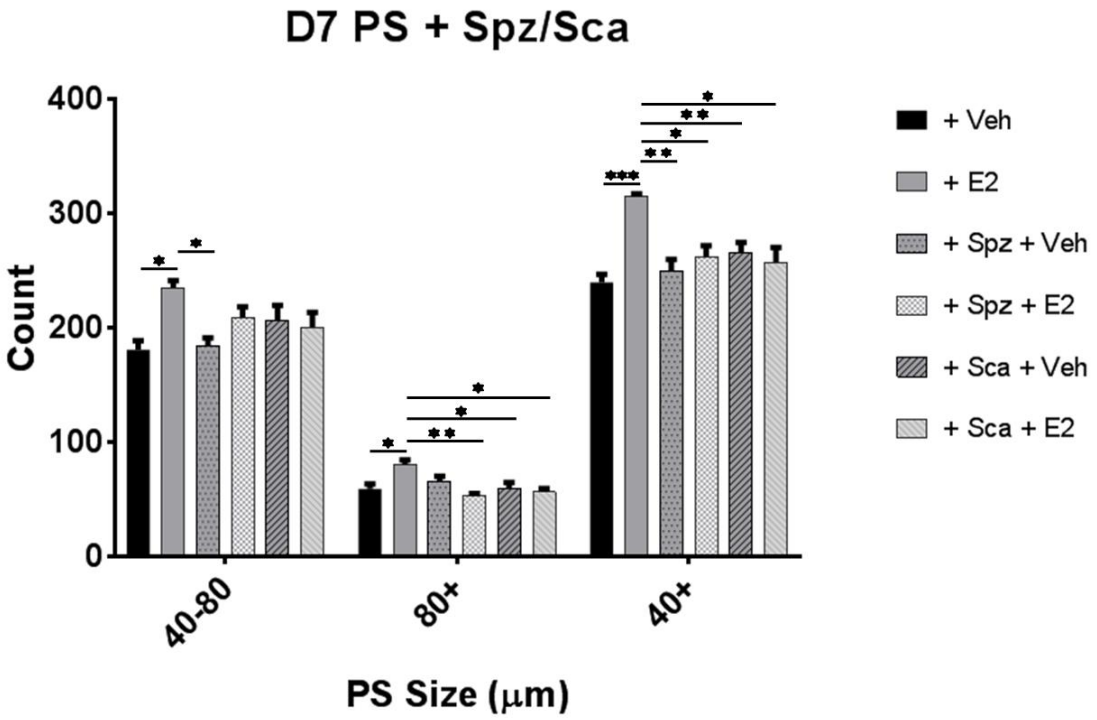


Figure 33. Effect of CAF and Spz co-culture on D7 PS size and number.

Three separate primary patient-derived matched pairs of Spz/Sca cells were used in co-culture with PS formed from pooled primary PrEC. n=3, Two-way ANOVA with post-hoc comparisons against PS + Veh. ***P<0.001, **P<0.01, *P<0.05,

from that of benign Spz co-culture, in that no significant differences were seen between CAF or Spz co-culture and vehicle-treated control PS.

Assessment of CAF effects on stem cell self-renewal was performed with a label retention assay that utilized the cytoplasmic dye CSFE substituted for BrdU. PrEC were labeled with CSFE for 30 minutes, during which time the dye was taken up into the cell and metabolized into a membrane-impermeable form. These primary cells were then cultured in 3D to form PS, either alone or with Sca2 or Spz2 +/- 10nM E2 for 7 days using insert-separated co-culture. The average number of label-retaining cells per PS was increased by 33% in PS cultured alone with E2 compared to vehicle control (Figure 34). No substantial increase in label-retaining PS was seen in either the Spz co-cultured PS (7% increase) or CAF co-cultured (8% increase) PS over vehicle PS cultured alone. When stromal co-culture was combined with E2, however, a similar ~30% increase in the average number of label-retaining stem cells was seen for both CAF and Spz co-cultured PS as seen for PS cultured in E2.

3. Benign Primary Stromal Cells That Stably-Express ERα Share Morphological and Molecular Characteristics With Cancer-Associated Fibroblasts

Lentiviral induction of ERα into two primary normal stromal cultures, PrSC-1 and PrSC2, resulted in a profound morphological change from a smooth muscle-like spindle shape to a fibroblastic-like stellate shape (Figure 35). Notably, these morphological changes occurred in both primary cultures, which were derived from different donors, and occurred in virtually all cells immediately after the first passage during selection for puromycin resistance. Since this phenotypic shift is classically associated with alteration of normal fibroblasts into an activated

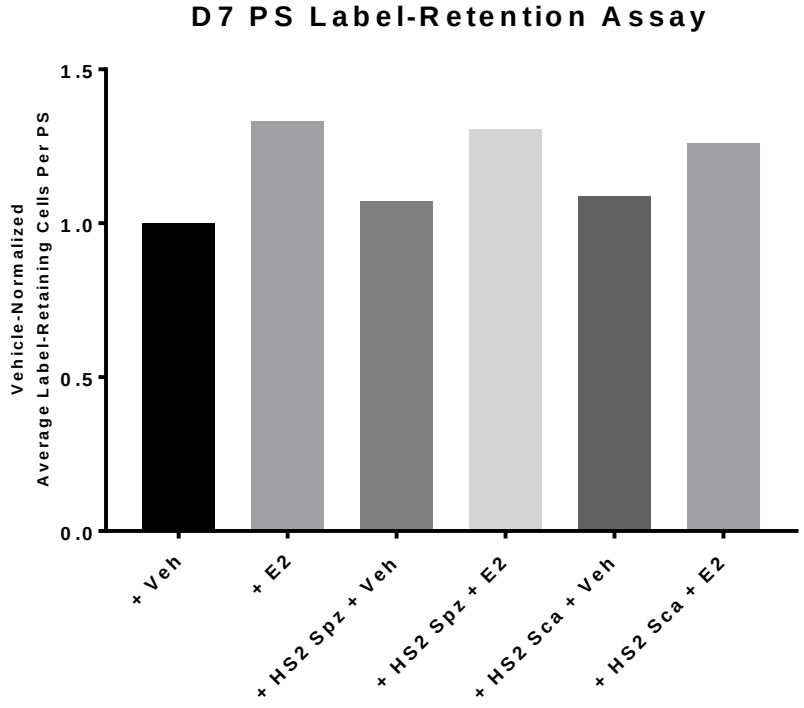


Figure 34. Effect of CAF and Spz co-culture on CSFE label-retaining cells in D7 PS.

Matched primary patient-derived Spz/Sca cells were used in co-culture with PS formed from CSFE-labeled pooled primary PrEC. n=1, this no test for significance was performed.

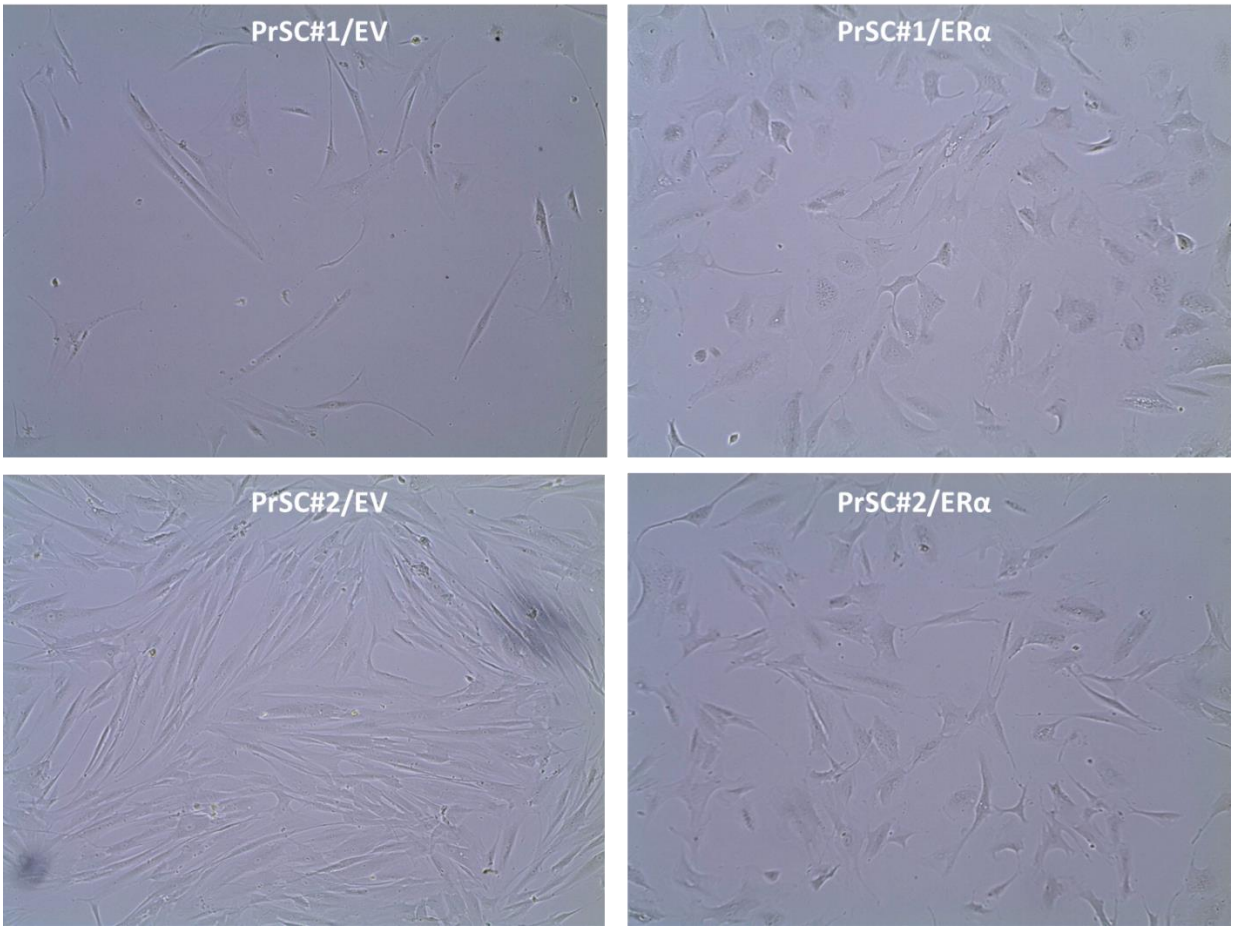


Figure 35. ERα expression in primary PrSC stromal cells induces a morphological change toward myofibroblastic-like phenotype.

PrSC1 & PrSC2 transduced with empty vector (left panels) and transduced with ERα (right panels).

state, which includes cancer-activated and inflammation-activated fibroblasts, both ERα-expressing primary cell cultures and their empty vector controls were stained for characteristic markers of activated CAFs, α-smooth muscle actin (αSMA) and vimentin. As positive and negative controls, the patient-matched primary benign and CAF cultures Spz2 and Sca2 were also stained for these markers. The Sca2 cells stained strongly for αSMA, although the individual cells in this culture displayed a binary expression pattern, indicative of heterogeneity that is common in primary cultures (Figure 36). As expected, none of the benign Spz2 cells were positive for αSMA. The PrSC2/ERα cells stained positive for both αSMA and vimentin, while the empty vector PrSC2 cells were negative for both markers at the same exposure times. The cells were co-stained for ERα as verification of lentivirus induction, but no correlation was observed between intensity of nuclear ERα staining and αSMA-positivity. Notably, both the empty vector and ERα PrSC-1 cells were negative for αSMA and vimentin (data not shown).

4. Benign Stromal Cells Stably-Expressing ERα Share Gene Expression Patterns with CAFs

The phenotypic similarities between PrSC2/ERα cells and CAFs were further explored by comparing gene expression signatures between these cells. Gene expression microarray analyses were performed on the following pairs of stromal cells that were treated with vehicle or 10nM E2 for 24 hours: primary PrSC2/ERα and PrSC2/EV cells; 3 pairs of patient- or age-matched primary CAF and benign Spz cells; and the WPMY-1/ERα and WPMY-1/EV human stromal cell lines. Since hierarchical clustering was not informative as to the hypothesized relationship between ERα-stromal cells and CAFs, an approach was taken to computationally assess up- and down-regulated biological pathways based on global gene expression profiles.

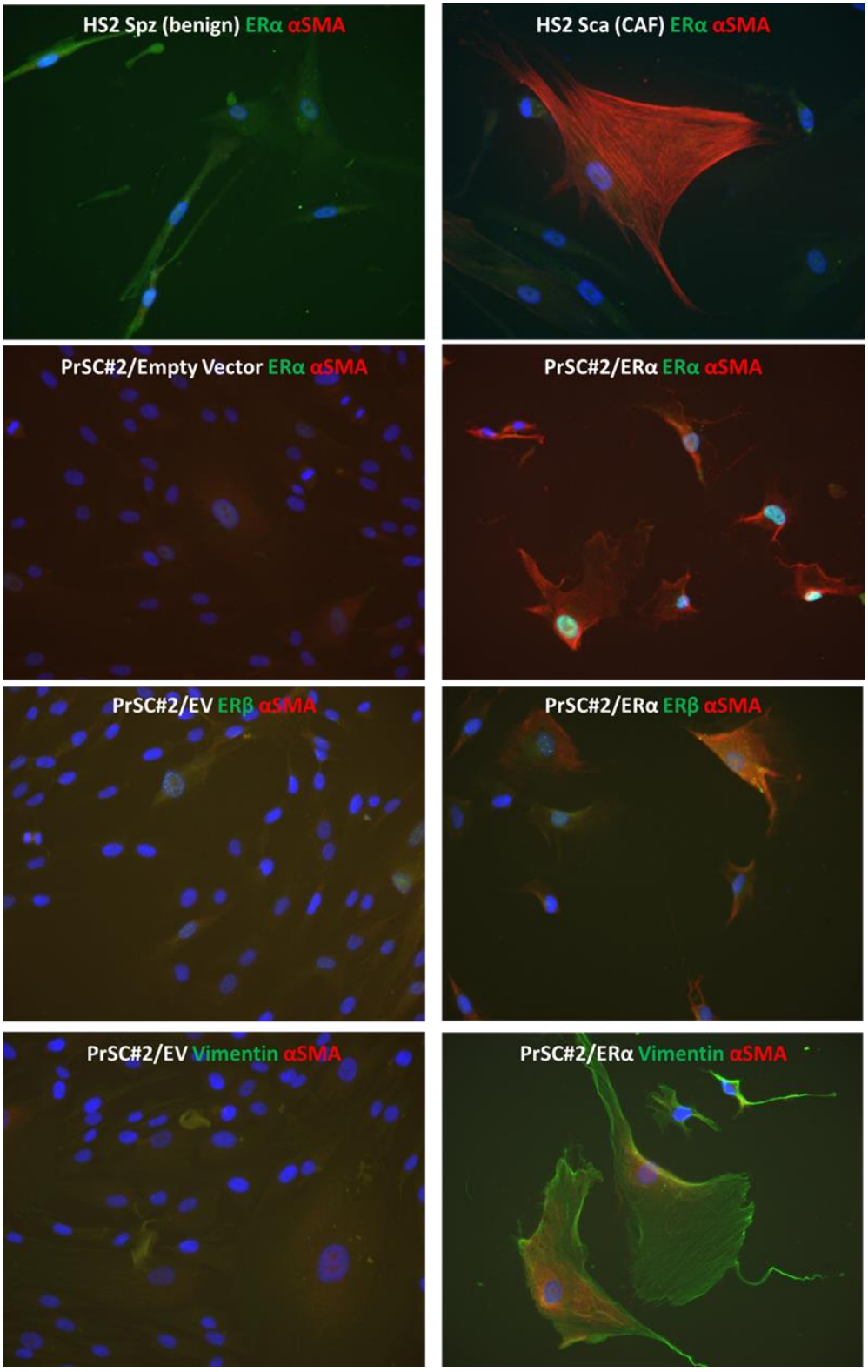


Figure 36. ICC for ERα, αSMA, Vimentin and ERβ in Spz2, Sca2, PrSC2/EV, PrSC2/ERα.

The Broad Institute's implementation of the Gene Set Enrichment Analysis (GSEA) algorithm was utilized to identify pathway enrichment between three separate comparisons: 1) E2-treated CAFs versus E2-treated Spz cells, 2) E2-treated PrSC/ERα versus E2-treated PrSC/EV, and 3) E2-treated WPMY-1/ERα versus E2-treated WPMY-1/EV[99]. The GSEA algorithm was employed using the Broad Hallmarks gene set to assess for similarly-regulated gene networks between CAFs and ERα-expressing benign stromal cells. Results demonstrated striking similarities between up-regulated and down-regulated gene sets in E2-treated CAFs and ERα-stromal cells, versus their respective comparisons of E2-treated Spz and empty vector stromal cells (Figure 37). Several biological pathways that are classically associated with CAFs—TGF-β signaling, glycolysis, angiogenesis, hypoxia response and IL-6 signaling—were found to indeed be upregulated in the CAFs versus the Spz cells in this study, confirming the ontology of these primary cells. The ERα-stromal cells, compared to their respective empty vector controls, displayed near-identical up- or down-regulation of these pathways (Figure 37, B and C). To identify the core set of genes responsible for driving the similarities between CAFs and ERα stromal cells, a leading edge analysis was performed on 6 of the Hallmark gene sets that were commonly upregulated amongst the three comparisons: TGF-β signaling, glycolysis, hypoxia response, IL-6 signaling, early estrogen response, late estrogen response. The top 30 genes, in terms of membership within the 6 queried gene sets, are listed for each leading edge analysis (Figure 38). A total of 104 genes were responsible for driving the upregulation of these 6 biological pathways in CAFs versus Spz; likewise, 191 genes drove the differences in WPMY-1/ERα versus EV and 218 genes drove the differences between PrSC2/ERα and EV (Figure 39). A common core of 25 genes was present in the gene lists for each of the three leading edge analyses (Table 2; Figure 39). The Broad Hallmark gene sets are designed to provide more

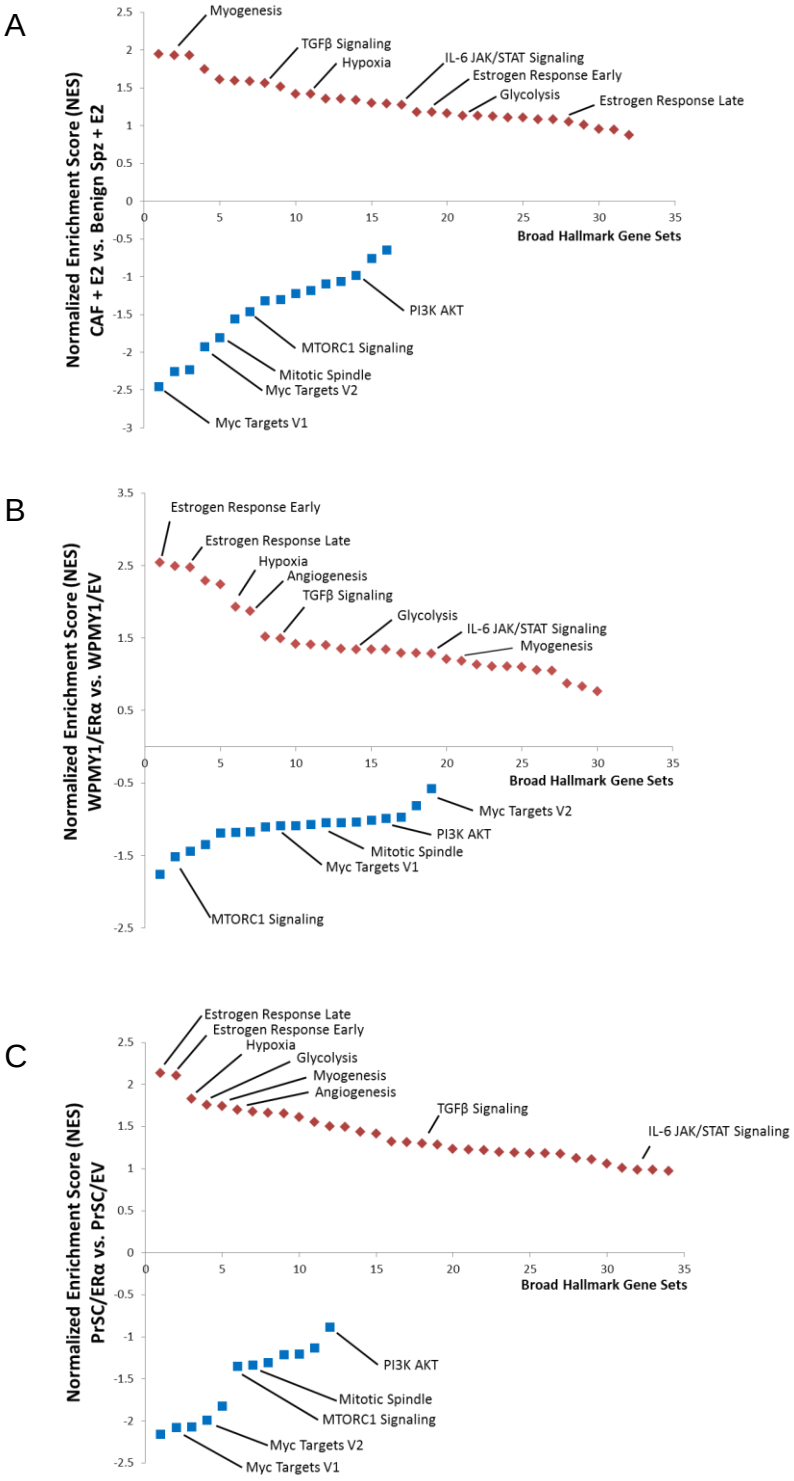


Figure 37. Nominal enrichment scores for Broad Hallmark Gene Set Enrichment Analysis of CAF vs. Spz, WPMY-1/ERα vs. EV, PrSC2/ERα vs. EV.

GSEA for Broad Hallmarks gene sets in A) CAF vs. Spz; B) WPMY-1/ERα vs. WPMY-1/EV; C) PrSC2/ERα vs. PrSC2/EV. All cells treated with 10nM E2 for 24 hours.

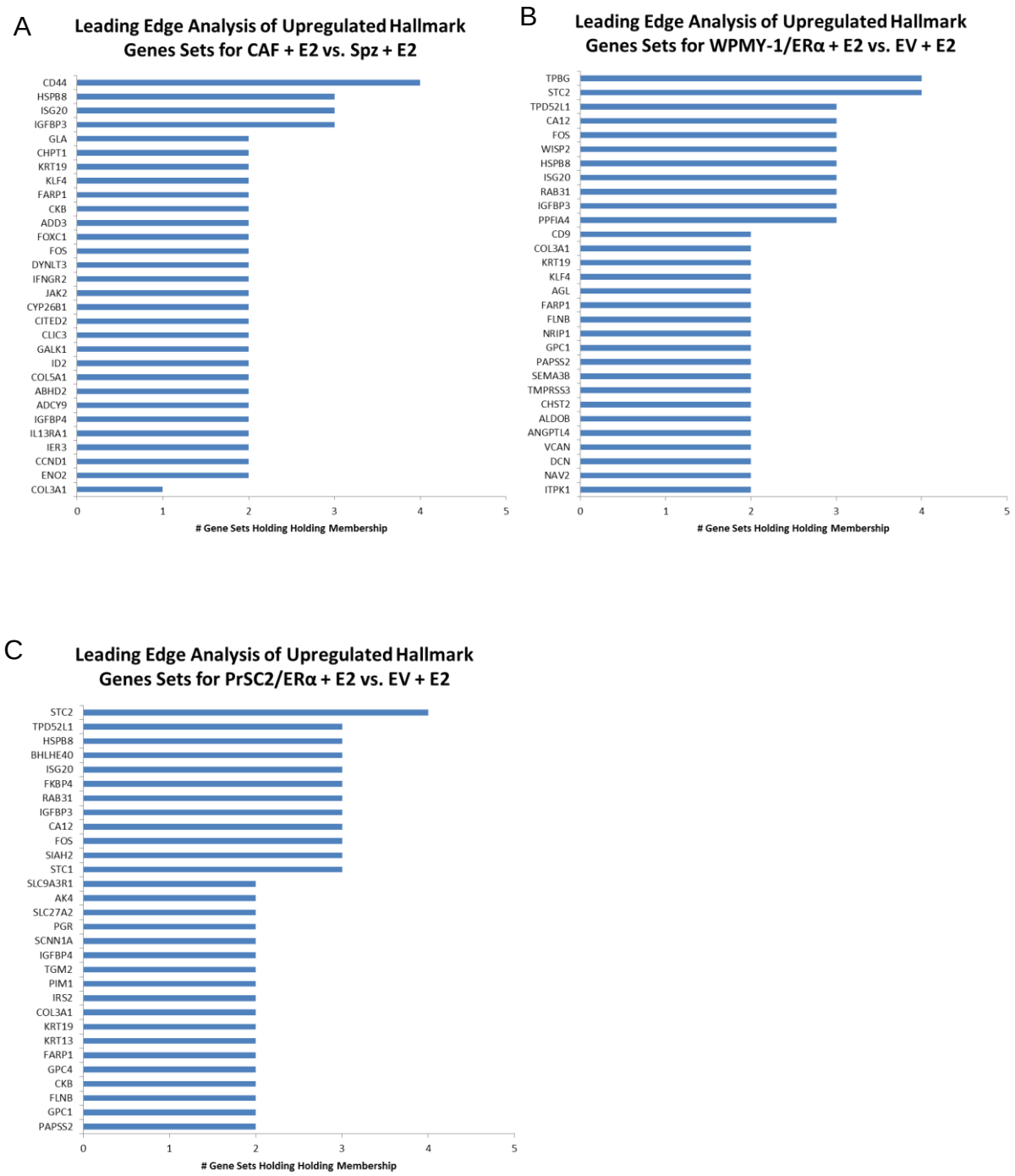


Figure 38. Leading Edge Analysis.

Top 30 leading edge genes with the highest membership amongst queried Hallmark gene sets for each of the 3 comparisons: A) CAF vs. Spz; B) WPMY-1/ERα vs. WPMY-1/EV; C) PrSC2/ERα vs. PrSC2/EV.

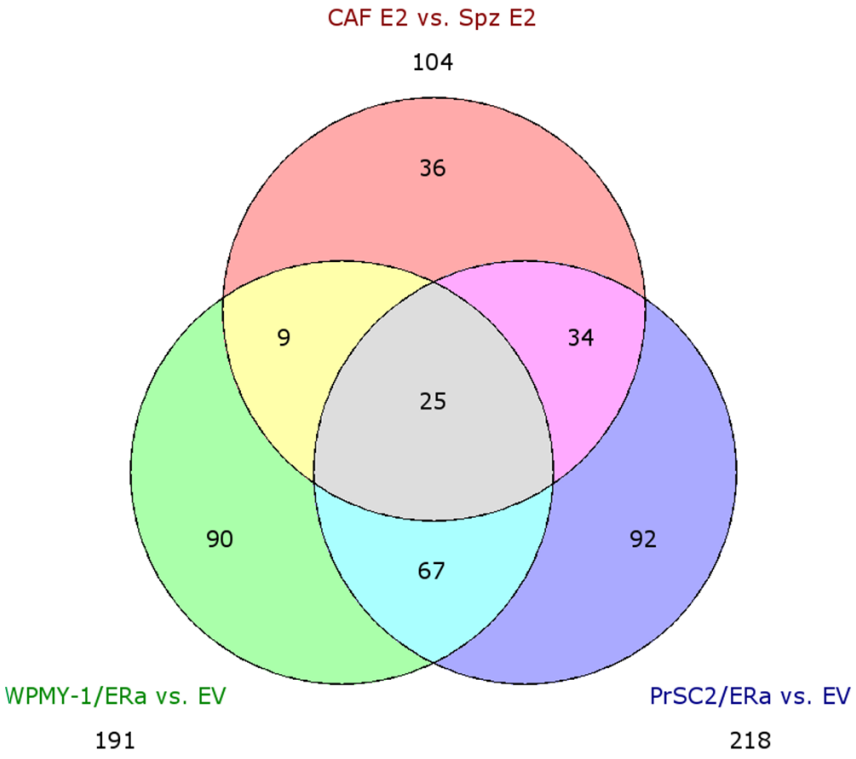


Figure 39. Venn diagram of leading edge genes for 6 commonly upregulated pathways amongst the 3 stromal cell comparisons.

Venn Diagram of leading edge genes for 6 commonly upregulated pathways amongst the 3 comparisons. Twenty-five genes are common to all 3 groups.

Gene	Description
A2M	alpha-2-macroglobulin
ALDH3A2	aldehyde dehydrogenase 3 family member A2
ANG	angiogenin
CAPN5	calpain 5
COL3A1	collagen type III alpha 1 chain
COL5A1	collagen type V alpha 1 chain
CYP26B1	cytochrome P450 family 26 subfamily B member 1
DUSP1	dual specificity phosphatase 1
DUSP1	dual specificity phosphatase 12
ENG	endoglin
FARP1	FERM, ARH/RhoGEF and pleckstrin domain protein 1
FOS	Fos proto-oncogene, AP-1 transcription factor subunit
FOXO4	forkhead box O4
GADD45B	growth arrest and DNA damage inducible beta
GJA1	gap junction protein alpha 1
HES1	hes family bHLH transcription factor 1
HES1	hes family bHLH transcription factor 1
HES1	chromosome 21 open reading frame 33
HSPB8	heat shock protein family B (small) member 8
HSPB8	heat shock protein family B (small) member 8
ID1	inhibitor of DNA binding 1, HLH protein
ID2	inhibitor of DNA binding 2, HLH protein
ID3	inhibitor of DNA binding 3, HLH protein
IGFBP3	insulin like growth factor binding protein 3
IGFBP4	insulin like growth factor binding protein 4

Table 2. Genes common to the leading edge analyses for select coordinately up-regulated Hallmark gene sets.

robust gene enrichment analyses by reducing the redundancy across queried gene sets, however, they accomplish this at the expense of discrimination between closely related but distinct biological pathways. Therefore, the 25 genes that were common to the three leading edge analyses were subjected to functional annotation using the Database for Annotation, Visualization and Integrated Discovery (DAVID) tool from the National Institute of Allergy and Infectious Disease. Pathways that were significantly enriched (Benjamini false discovery rate < 0.05) in the list of 25 genes included growth factor binding, heart development, TGFβ signaling pathway, wound response, and response to hormone stimulus (Table 3). Many of these pathways were not surprising, given the Hallmark gene sets from which they were derived, however, the two highest enriched pathways, growth factor binding and heart development, provided intriguing insight into precisely what the hypothesized shared phenotype between CAFs and ERα stroma might entail.

5. Gene Signatures of CAFs and ERα Stromal Cells Have Predictive Value in Clinical Prostate Cancer Datasets

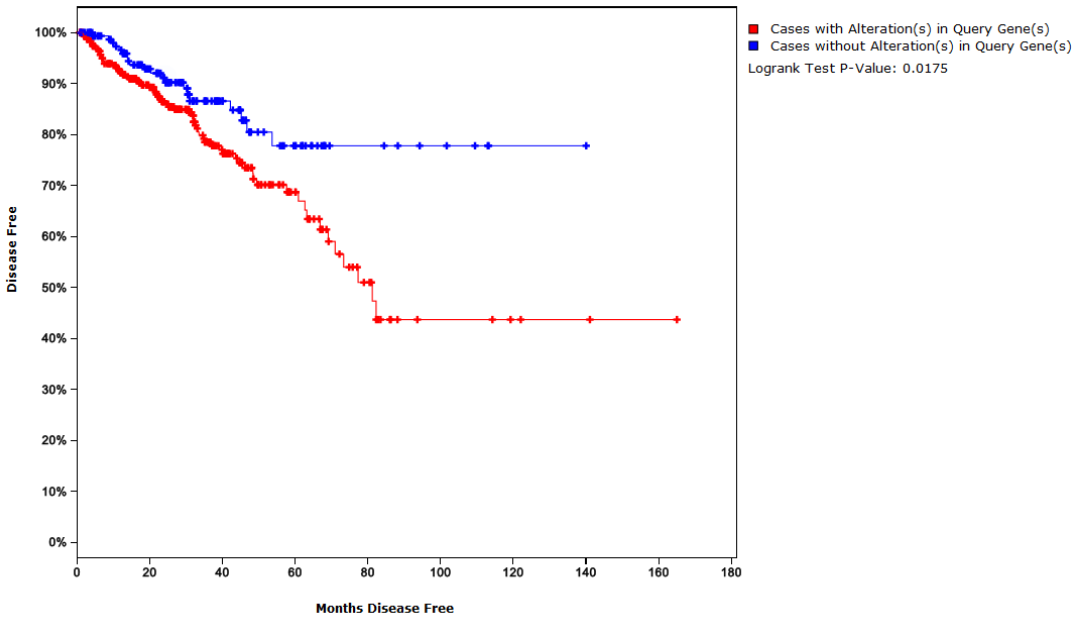
Since CAFs have been shown to enhance disease progression and impact clinical outcome in multiple cancers, the clinical relevance of the shared gene expression patterns between CAFs and ERα stromal cells was investigated. The TCGA Prostate Adenocarcinoma dataset was utilized to determine if upregulation of genes within the 25-gene signature of common leading edge genes affects clinical outcome. When patients were dichotomized into two groups—those with alterations involving upregulation, gain or amplification at the mRNA- or gene-level of any of the genes in the 25-gene signature, and those with no alterations—a statistically significant decrease in disease-free survival was observed for those men with upregulation of these genes

Term	Category	Gene Count	% of total genes	P-Value	Benjamini FDR
growth factor binding	GOTERM_MF_FAT	7	28	2.20E-08	3.00E-06
heart development	GOTERM_BP_FAT	7	28	1.20E-06	7.90E-04
response to organic substance	GOTERM_BP_FAT	10	40	1.40E-06	4.50E-04
transforming growth factor beta receptor signaling pathway	GOTERM_BP_FAT	4	16	1.40E-04	3.00E-02
negative regulation of macromolecule metabolic process	GOTERM_BP_FAT	8	32	1.50E-04	2.50E-02
response to wounding	GOTERM_BP_FAT	7	28	2.00E-04	2.60E-02
response to hormone stimulus	GOTERM_BP_FAT	6	24	3.20E-04	3.40E-02
domain:Helix-loop-helix motif	UP_SEQ_FEATURE	4	16	3.80E-04	3.70E-02
response to endogenous stimulus	GOTERM_BP_FAT	6	24	5.10E-04	4.60E-02
Basic helix-loop-helix dimerization region bHLH	INTERPRO	4	16	5.70E-04	4.90E-02
transmembrane receptor protein serine/threonine kinase signaling pathway	GOTERM_BP_FAT	4	16	6.80E-04	5.40E-02
blood vessel development	GOTERM_BP_FAT	5	20	7.10E-04	5.00E-02
compositionally biased region:Ser/Thr-rich	UP_SEQ_FEATURE	3	12	7.30E-04	3.60E-02
extracellular region part	GOTERM_CC_FAT	7	28	7.30E-04	8.20E-02
response to protein stimulus	GOTERM_BP_FAT	4	16	7.60E-04	4.80E-02
vasculature development	GOTERM_BP_FAT	5	20	7.80E-04	4.50E-02
71.Id_proteins_G0-to-S_cell_cycle	BBID	3	12	9.30E-04	6.50E-03
negative regulation of transcription from RNA polymerase II promoter	GOTERM_BP_FAT	5	20	9.60E-04	5.10E-02
HLH	SMART	4	16	1.20E-03	2.60E-02

Table 3. DAVID Functional Annotation of 25 Genes common to 3 leading edge analyses.

(Figure 40). To validate these findings in a different dataset, a publicly available gene expression microarray of prostate cancer specimens from men in the Swedish Watchful Waiting cohort was used[100]. Since this dataset includes a microarray that only measured 6100 genes—compared to genome-wide coverage for the microarrays utilized in the present studies—not all of the genes from the leading edge 25-gene signature could be tested. Nonetheless, a penalized cox regression model utilizing 21 of the genes demonstrated a Kaplan-Meier curve that was suggestive of a decrease in disease-free survival for patients within this cohort who were in the 95th percentile risk group, but had an insignificant log-rank p-value=0.14 (Figure 41).

Since this 25-gene signature was derived in a manner that was inherently biased towards gene-expression characteristics of CAFs—i.e., the 6 commonly upregulated Hallmark gene sets were chosen specifically due to their association with a CAF phenotype—a second methodology was utilized that was agnostic to any similarities between ERα stromal cells and CAFs. Differential gene expression between each of the same three pairs of stromal cells—CAF vs. Spz, WPMY-1/ERα vs. EV, PrSC/ERα vs. EV—was computed in the R statistical computing environment. After thresholding for a false discovery q-value < 0.05, the top 25 differentially expressed genes (DEGs) were selected for CAFs, PrSC/ERα, and WPMY-1/ERα (Table 4). Surprisingly, there was very little overlap between these genes (Figure 42). Using the same approach described earlier, the TCGA prostate cancer dataset was sequentially queried for the top 25 genes from each of the three individual sets of DEGs. The two gene sets from the PrSC2/ERα and the WPMY-1/ERα cells were associated with significant decreases in disease-free survival for those men who over-expressed any combination of the genes (Figure 43). The 25 genes upregulated in CAF + E2 versus Spz + E2 yielded a Kaplan-Meier curve that was highly suggestive of a



	#total cases	#cases relapsed	median months disease free
Cases with Alteration(s) in Query Gene(s)	**318**	**69**	**81.24**
Cases without Alteration(s) in Query Gene(s)	**166**	**20**	**NA**

Figure 40. Gene- or mRNA-level upregulation of the leading edge 25-gene signature confers significant disadvantage in disease-free survival to biochemical recurrence.

Kaplan-Meier curve for all 499 patients in the Cancer Genome Atlas cohort who over-expressed any combination of the 25-gene signature from Table 2. Those patients who over-express genes in this 25-gene signature have significant disease-free survival disadvantage. Log-rank p-value between risk groups = 0.0175.

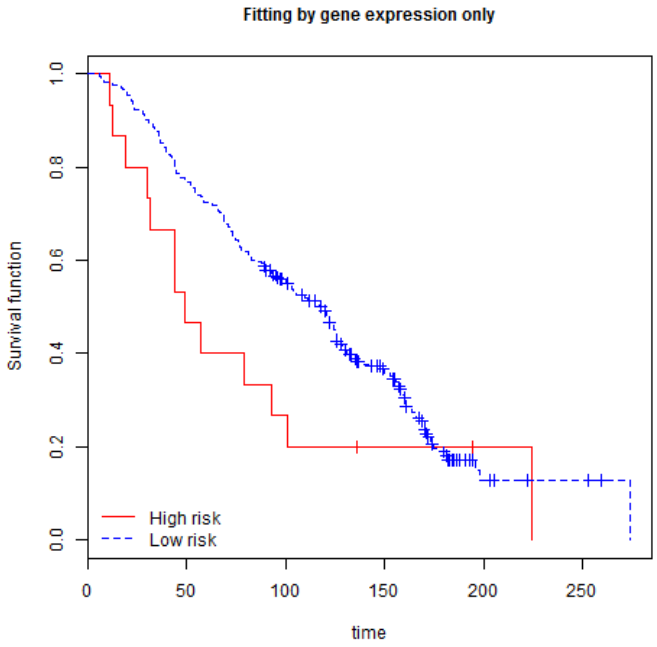


Figure 41. Kaplan-Meier curve for patients in the Swedish Watchful Waiting cohort.

Kaplan-Meier curve for patients in the Swedish Watchful Waiting cohort who over-expressed 21 genes (of the leading edge 25-gene signature) at the 95th percentile prognostic index. A trend towards a decreased disease-free survival is observed in those men who overexpressed these genes at the mRNA level (high risk group) compared to those who did not (low risk). Log-rank p-value between risk groups based on 100 permutations = 0.14.

CAF + E2 vs. Spz + E2	WPMY-1/ERα + E2 vs. WPMY-1/EV + E2	PrSC2/ERα + E2 vs. PrSC2/EV + E2	GSE26910 CAF vs. Benign
ACO1	ACKR3	ACPP	AGR2
ACSL3	BHLHE40	C1QTNF1	ASPN
ARID5B	**CA12**	**CA12**	CRISPLD1
ARSB	COL3A1	CRYAB	CTHRC1
ATAD1	COL5A2	CYP1B1	GPR160
ATG12	COL6A3	DDIT4L	**GREM1**
ATP9A	CTSL	**GREM1**	HIST1H1C
CASC4	EIF1AY	HSD11B1	HMGN2P46
CERK	GFRA2	IGFBP3	KLK2
CTNNA1	HIST2H2AA3	LOXL4	KLK3
CYB5R3	HIST2H2AA4	LUM	LINC01207
DBT	HIST2H2AC	MARCKSL1	LTF
ECM1	IFI6	MGP	MAL2
EGFLAM	IGFBP4	NNAT	NPY
ERAP2	IGFBP6	PFKFB3	OR51E2
EYA2	ISG20	PLOD2	PLA1A
FAM45A	MATN2	**PRSS23**	PRAC1
FUCA2	MX1	PTGS2	RSPO3
ID2	MXRA5	**SERPINB6**	SFRP4
IGFBP2	**PRSS23**	SLC51B	SLC38A11
ITGB1BP1	RPS4Y1	SRGN	TARP
ITGB5	**SERPINB6**	STC2	THBS4
ITPRIP	SERPINE2	TGM2	TMSB15A
LAMP1	SGK1	TSKU	TRPM8
PTEN	ZMIZ1	VCAN	TSPAN1

Table 4. Top 25 DEG for each pairwise comparison. Genes are sorted in alphabetical order.

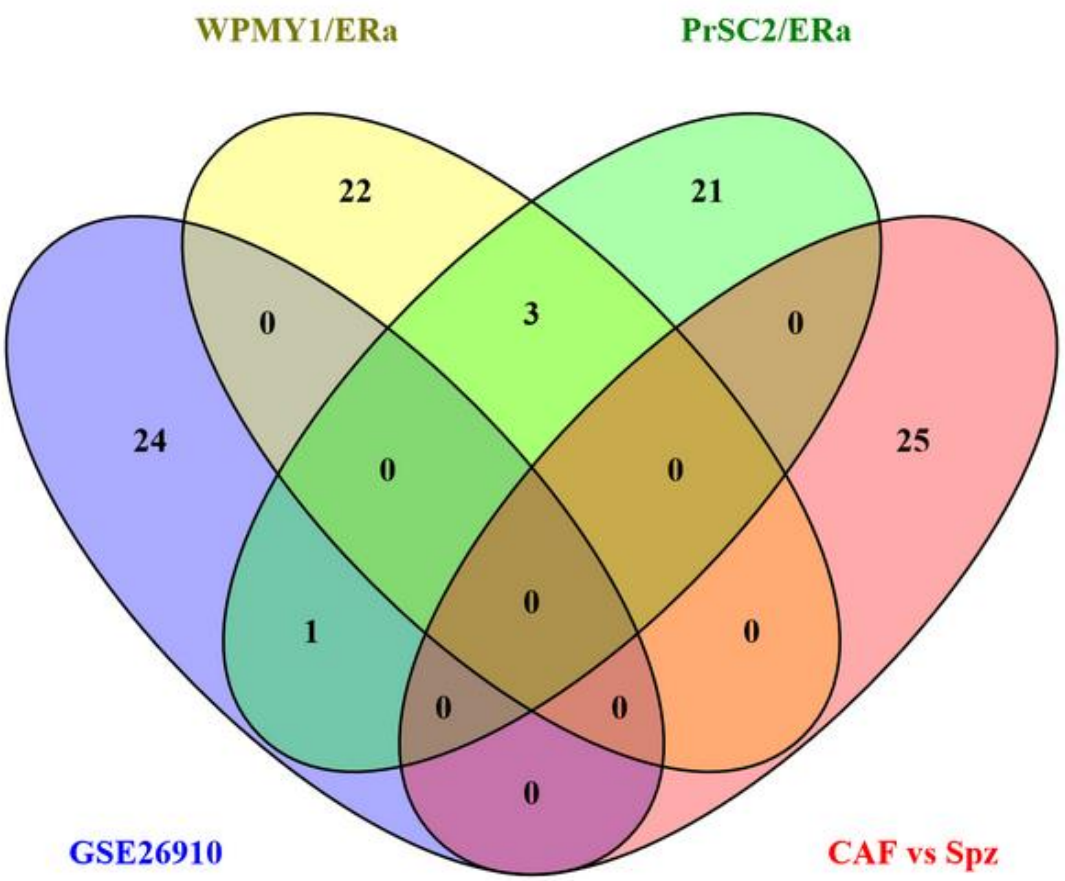


Figure 42. Overlap between top 25 DEGs lists.

Venn diagram showing overlap of genes listed in Table 4, representing the top 25 differentially-regulated genes in each of the comparisons: CAF + E2 vs. Spz + E2; WPMY-1/ERα + E2 vs. WPMY-1/EV + E2; PrSC2/ERα + E2 vs. PrSC2/EV + E2; CAF vs. Benign from publicly-available microarray data from GSE26910.

decrease in disease-free survival, but with a log-rank p-value of 0.0540 it did not meet the cutoff for significance (Figure 43). Nevertheless, the hypothesized relationship between ERα stromal cells and CAFs was strengthened by these findings.

Since primary cells tend to lose expression of steroid hormone receptors *in vitro*, the estrogen responsiveness of the CAFs and Spz cells utilized in this study might have been under-represented. Therefore, a separate *in silico* approach, which took advantage of publicly available microarray gene expression data derived from fresh-frozen, laser-capture micro-dissected benign and cancer-associated prostate stroma, was utilized in an attempt to recapitulate the findings from the *in vitro* gene expression profiling experiments. The dataset from Planche, *et al.*, which contained gene expression data for 6 pairs of patient-matched normal and cancer-associated stroma, was downloaded and DEGs were determined as previously described[101]. The top 25 genes that were overexpressed in CAFs from the Planche dataset were used in the same survival analysis pipeline as the previous gene sets. Similar to the survival analysis using CAFs + E2 vs. Spz + E2, the genes overexpressed in CAFs compared to benign stroma suggested a reduced disease-free survival time within the TCGA prostate cancer dataset, but the log-rank p-value was not significant.

The gene *ESR1*, which encodes for ERα, was significantly upregulated in CAFs in the Planche dataset, although it was not one of the top 25 overexpressed genes. Nevertheless, this was additional support for the hypothesized relationship between CAFs and stromal-ERα. To ascertain if estrogen signaling was indeed upregulated in the CAFs from this dataset, as opposed to merely message-level upregulation of *ESR1*, additional bioinformatics analysis algorithms

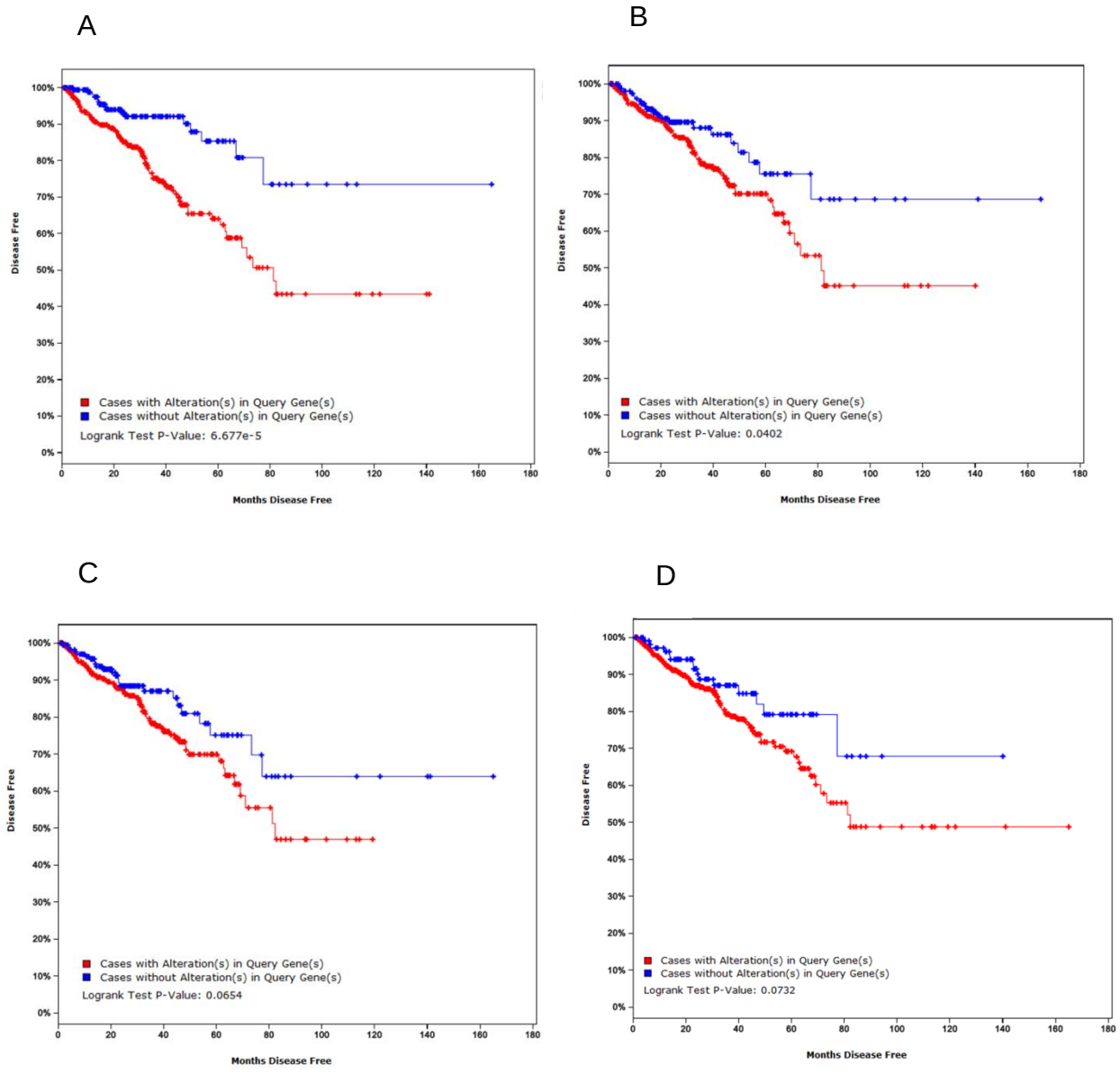


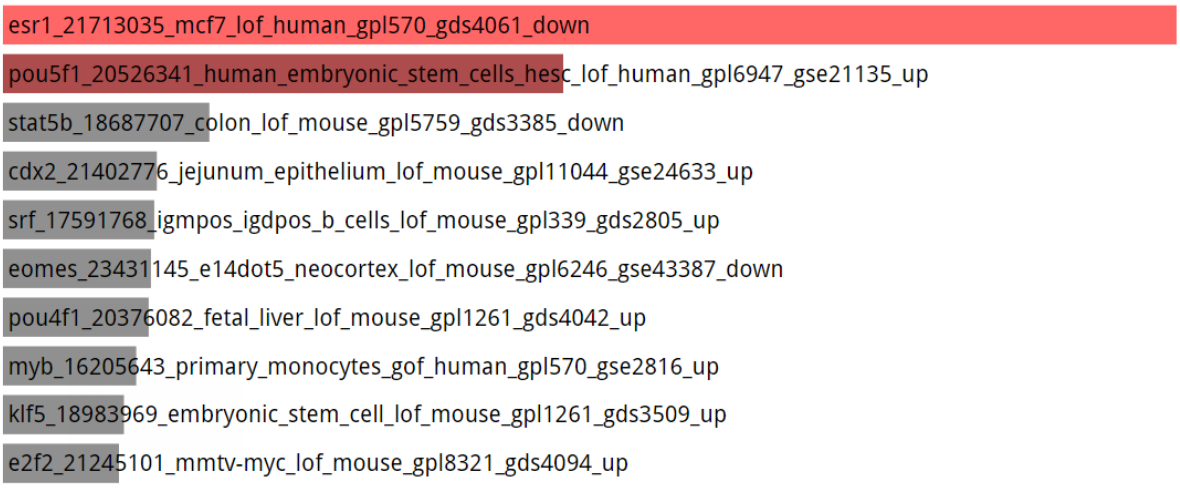
Figure 43. Kaplan Meier curves of survival analyses performed on TCGA dataset using 4 different DEG lists from various Stromal ERα/EV and CAF/Spz models compared.

A) Top 25 genes overexpressed in WPMY-1/ERα vs /EV confer significant disease-free survival disadvantage for high-expressers in TCGA. B) Top 25 genes overexpressed in PrSC2/ERα vs /EV confer significant disease-free survival disadvantage for high-expressers in TCGA. C) Top 25 genes overexpressed in CAF + E2 vs Spz + E2 suggests possible disease-free survival disadvantage for high-expressers in TCGA. D) Top 25 genes overexpressed in CAF vs. benign stroma from GSE26910 suggests possible disease-free survival disadvantage for high-expressers in TCGA.

were employed. The top 500 overexpressed genes in CAFs versus benign stromal cells from the Planche dataset were queried through the *Enrichr* web portal against a suite of curated microarray and RNA-seq gene expression libraries. One of these libraries is a compendium of all publicly available datasets for which global gene expression was measured in mammalian model systems upon experimentally-induced transcription factors loss-of-function (TF-LOF). When the top 500 overexpressed genes in CAFs versus Spz were queried against this TF-LOF library, the top hit was a dataset containing genes down-regulated after siRNA-silencing of *ESR1* in MCF-7 human breast cancer cells (Figure 44). Furthermore, a query against a library of datasets derived from ligand-treated cells or animals demonstrated statistically significant over-representation of genes from several studies in which mice were treated with E2 (Figure 45). Thus, *in silico* analysis of an independent microarray dataset derived from pure LCM-captured primary stromal cells provided further evidence of a relationship between CAF gene signatures and stromal-ERα gene signatures.

C. Discussion

The concept that carcinogenesis is normal development gone awry was first proposed over one hundred years ago by two French biologists, Lobstein and Recamier[102]. Years later, in the 1970's, an American physician-scientist named G. Barry Pierce reintroduced this concept, along with a wealth of experimental evidence supporting his hypothesis that carcinogenesis arises in part due to dysregulation of developmental processes[103]. In the past two decades, as the stem cell biology field has matured, it has become increasingly clear that stem and progenitor cells are likely cells-of-origin for many cancers, thus corroborating Dr. Pierce's proposed relationships between normal development and carcinogenesis[104]. The provocative findings in the present



	Name	P-value	Adjusted p-value	Z-score	Combined score
1	esr1_21713035_mcf7_lof_human_gpl570_gds4061_down	0.006355	1	-2	10.14
2	pou5f1_20526341_human_embryonic_stem_cells_hesc_lof_human_gpl6947_gse21135_up	0.02152	1	-1.39	5.32
3	stat5b_18687707_colon_lof_mouse_gpl5759_gds3385_down	0.1259	1	-1.23	2.54
4	cdx2_21402776_jejunum_epithelium_lof_mouse_gpl11044_gse24633_up	0.3266	1	-1.9	2.13
5	srf_17591768_igmpos_igdpos_b_cells_lof_mouse_gpl339_gds2805_up	0.361	1	-2.07	2.11
6	eomes_23431145_e14dot5_neocortex_lof_mouse_gpl6246_gse43387_down	0.3251	1	-1.85	2.08
7	pou4f1_20376082_fetal_liver_lof_mouse_gpl1261_gds4042_up	0.3746	1	-2.1	2.07
8	myb_16205643_primary_monocytes_gof_human_gpl570_gse2816_up	0.3013	1	-1.64	1.97
9	klf5_18983969_embryonic_stem_cell_lof_mouse_gpl1261_gds3509_up	0.3531	1	-1.8	1.87
10	e2f2_21245101_mmtv-myc_lof_mouse_gpl8321_gds4094_up	0.3763	1	-1.88	1.83

Figure 44. Results of an Enrichr analysis comparing the top 500 overexpressed genes in CAFs vs. benign stromal cells from the Planche dataset against a library of gene sets from TF-LOF experiments.

Top figure: the *ESR1* gene, which codes for ERα protein, is significantly overexpressed in CAFs vs. benign stroma in the Planche dataset, suggesting a role of ERα in CAF biology.

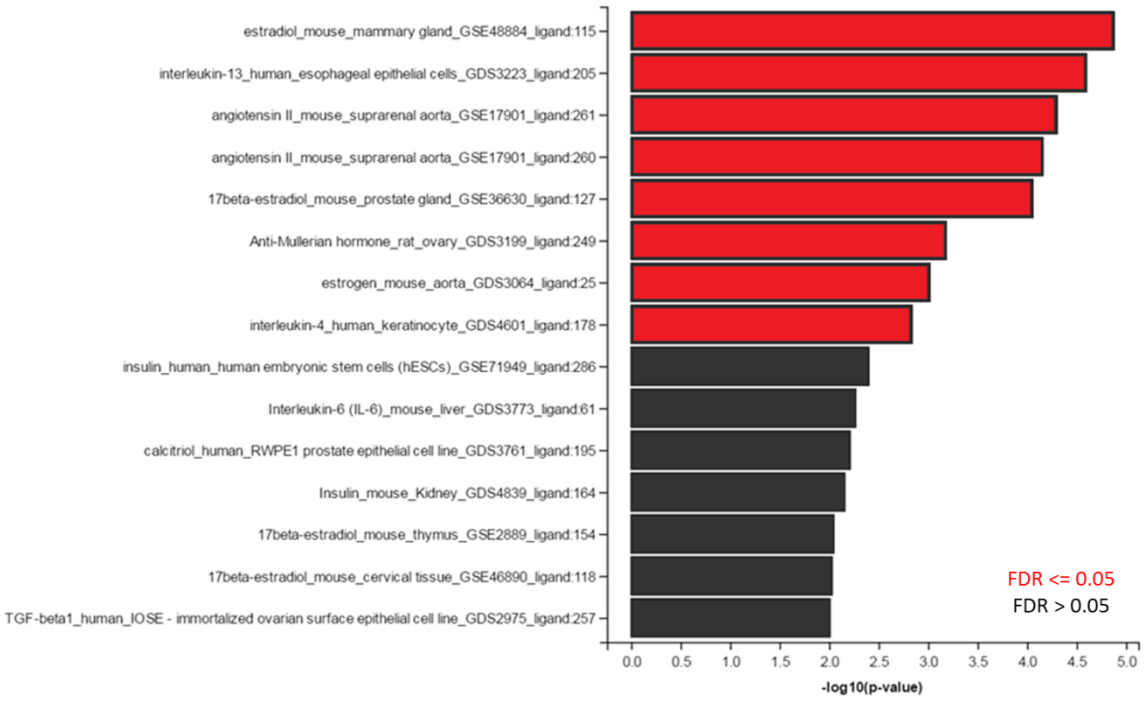


Figure 45. Gene Set Enrichment Analysis through Enrichr of CAF vs. Benign Prostate Stroma from GSE26910 and using the library Ligand_Perturbations_from_GEO_up.

Gene Set Enrichment Analysis through Enrichr of CAF vs. Benign Prostate Stroma from GSE26910 and using the library Ligand_Perturbations_from_GEO_up. CAF-upregulated genes from GSE26910 are enriched for genes which are targets of estradiol in murine models.

study, linking the CAF phenotype to a stromal-ERα phenotype, can be considered in the context of this paradigm, where carcinogenesis usurps normal development processes. It is well-established that the stroma is an essential mediator of hormonal influences on prostate development[21,105]. Furthermore, there is rational evidence suggesting that stromal ERs and AR can mediate hormonal carcinogenesis[34]. Thus, it stands to reason that part of the pro-tumorigenic effects of CAFs, at least in prostate cancer, might involve a dysregulated stromal-ERα signaling axis. Furthermore, it is possible that dysregulation of ERα signaling in normal stromal cells might contribute to carcinogenesis. The findings of the present study provide tantalizing evidence supporting such notions.

The capacity of CAFs to influence early stem and progenitor cells, both benign and malignant, is of paramount importance. There are multiple studies, particularly in the prostate cancer field, that detail the ability of CAFs to regulate cancer stem cells (CSC)[71,97,106,107]. Giannoni, *et al.*, found that CAF-secreted MMPs can upregulate EMT in CSC, increasing expression of stemness cell-surface markers such as CD44 and promoting sphere-forming capacity in these CSC[106]. Interestingly, the capacity to influence CSCs was also demonstrated by normal stromal cells stimulated with TGFβ or IL-6, the latter molecule being secreted by cancer cells[106]. Surprisingly, despite a wealth of evidence that CAFs can modulate stemness in CSC, little is known about their ability to influence the normal stem cell niche.

Although more in-depth studies are necessary before any definitive conclusions can be made, the limited evidence presented in this study regarding CAF influences on the benign SCN can be interpreted as CAFs having equivalent effects as benign stromal cells on epithelial stem cell self-

renewal and progenitor cell proliferation. These preliminary results were unexpected, given the multitude of data demonstrating that CAFs increase stemness properties in CSC[22,23,107]. It must be noted that the label-retaining assay utilized in these CAF studies employed the cytoplasmic marker CSFE, which is less-sensitive than BrdU, a marker that is incorporated into the DNA. Nevertheless, given the evidence previously presented in this study on stromal cell regulation of the benign stem cell niche, and given the known ability of CAFs to regulate CSCs, promote tumor progression and perhaps even mediate carcinogenesis, it seems highly likely that CAFs could influence the benign stem cell niche. Such a hypothesis seems rational, considering data regarding the role of stromal-ERα in the benign niche and the unexpected finding of a relationship between a CAF phenotype and a stromal-ERα phenotype.

Stabilized expression of ERα in two different normal primary stromal cultures resulted in morphological shifts resembling those of activated fibroblasts or CAFs. In one of these cultures, the PrSC2/ERα cells, increased protein-level expression of αSMA and vimentin supported the idea that stabilized expression of ERα induced a CAF-like phenotype. Subsequent gene expression analyses in the PrSC2/ERα cells revealed that multiple genes involved in the TGFβ signaling pathway were upregulated compared to empty vector PrSC2 cells. One possible explanation for the striking morphological shift of these ERα stromal cells is that upregulation of an autocrine TGFβ signaling pathway resulted in "self-activation." A study by Untergasser, *et al.*, demonstrated that treatment of primary PrSC stromal cells with TGFβ resulted in a fibroblast-to-myofibroblast transdifferentiation, similar to what was observed with ERα induction in PrSC in this study[108]. Additionally, TGFβ-treated PrSC from the Untergasser study showed upregulation of several genes that were also seen to be upregulated by ERα in PrSC in

this study, most importantly *Igfbp3*. Furthermore, the authors noted that TGFβ-activated PrSC took on a senescent state, which is often associated with the CAF phenotype. Gene expression analyses of PrSC2/ERα versus PrSC/EV cells suggested that ERα expression also induces a senescent phenotype. Although the two different PrSC cultures displayed a similar morphological shift suggesting a fibroblast-to-myofibroblast transdifferentiation, only the PrSC2 culture demonstrated upregulation of αSMA and vimentin at the protein level. This might be explained by the heterogeneity of primary cells, but an alternative explanation is also possible. The activation of stromal cells into a myofibroblastic state occurs normally in the wound healing response; but these activated myofibroblasts exist only temporarily, undergoing apoptosis upon resolution of the tissue injury[109]. Due to poor growth after lentiviral induction of ERα, the PrSC1 cells were growth in culture for an extra week and passaged one additional time before ICC staining for αSMA and vimentin, compared to the PrSC2 cells which were only passaged twice before ICC staining. It is certainly possible that ERα-induced stromal activation is a temporary phenomenon, and PrSC1 cells might have begun reversion to their original state before ICC staining was performed. Regardless, these issues would need to be more rigorously investigated before any definitive conclusions can be drawn.

Gene expression profiling of ERα-expressing stromal cells and multiple CAF and Spz cultures was performed to enable a more rigorous comparison between the observed ERα stromal phenotype and that of CAFs. Surprisingly, hierarchical clustering revealed no major sample groupings according to either E2 treatment or CAF/Spz phenotype in the stromal cells (Figure 46). Notably, the Spz1 and Sca1 stromal cultures, which were derived from the same patient, were clustered separately into two of the four major clades in the dendrogram. This observation

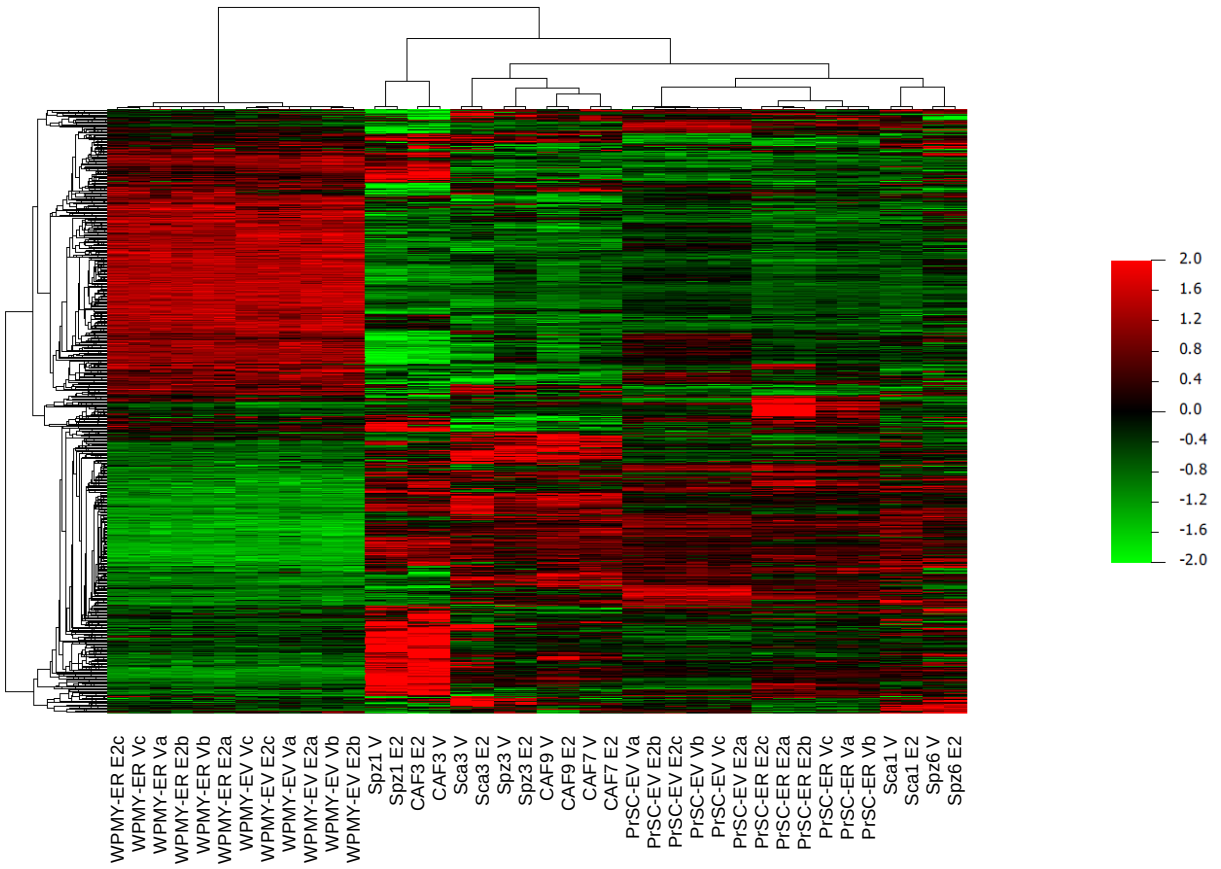


Figure 46. Heatmap of all microarray datasets used in this study.

Heatmap of Global Gene Expression for all microarray datasets used in this study. Genes were centered and scaled and clustered using 1 minus correlation and average linkage.

was particularly interesting due to the somewhat recent appreciation that stromal cells—particularly cancer-associated stromal cells—have multiple origins. In the case of the prostate, CAFs are thought to arise from either the activation of resident stromal cells or the recruitment of bone-marrow-derived mesenchymal stem cells. There is also limited evidence that some CAFs arise from the transdifferentiation of endothelial cells, pericytes or even epithelial cells[29,110]. As a future study, it would be interesting to interrogate whether ERα expression in CAFs correlates to their cell-of-origin.

The hypothesis that stromal ERα expression induces a CAF-like phenotype was most strongly corroborated by results of GSEA analyses using the Broad Hallmark gene set library. As expected, the CAFs when compared to benign Spz stromal cells demonstrated upregulation of biological pathways such as TGF-β signaling, glycolysis, angiogenesis, hypoxia response and IL-6 signaling. Furthermore, they had concomitant down-regulation of pathways involved in cellular proliferation, such as Myc target expression, mitotic spindle processes, mTOR pathway activation and AKT signaling. This suggested that the primary CAFs utilized in these studies were indeed of a senescent phenotype. The fact that two separate stromal-ERα expression models displayed nearly identical up- or down-regulation of these CAF-associated pathways is very strong evidence of a link between ERα expression and induction of an activated-stroma phenotype.

A leading edge analysis of genes responsible for driving the upregulation of the shared phenotype between CAFs and ERα stromal cells yielded a set of 25 genes that were common to the individual leading edge gene sets for each of the three comparisons of CAFs vs. Spz,

PrSC2/ERα vs. EV and WPMY-1/ERα vs. EV. This 25 gene signature was demonstrated to have clinical relevance upon dichotomization of 491 prostate cancer patients from the TCGA into two groups based on either high or low expression of these, whereby the high-expressing group had significantly shorter disease free survival as measured by prostate-specific antigen (PSA) biochemical recurrence. Additional analyses were performed to demonstrate that the clinical relevance of the shared gene set is not merely a reflection of a pro-tumorigenic gene signature derived from CAFs. Rather, genes that were overexpressed in stromal cells due to ERα expression also conferred a survival disadvantage when over-expressed in patients in the TCGA dataset.

The methodology utilized in these survival analyses relies on the assumption that a gene signature derived from stromal cells is relevant to gene expression data from the TCGA. Although it is accepted that the majority of the signal in the TCGA RNA-seq data is from tumor epithelial cells, the samples submitted to the TCGA are neither laser-capture microdissected nor FACS-sorted and thus are a mixture of epithelial and stroma cells. This is further confirmed by the PrCA sample histology provided for each patient within the TCGA PrCA database. Various studies have established that gene expression signals from multiple cell types exist within datasets such as the TCGA database[111,112]. The objective of these analyses was primarily to determine biological relevance of the 25-gene signatures, not to derive a predictive biomarker gene panel, and the methodology utilized served this purpose.

Examination of the various derived gene signatures demonstrated increased expression of several genes that have been biologically implicated in stromal-epithelial interactions in both normal

developmental and cancer settings. Within the 25-gene signature derived from the leading edge analysis, three different inhibitor-of-DNA binding (Id) proteins were present: *Id1*, *Id2* and *Id3*. The *Id2* gene was also in the list of the top 25 over-expressed genes in E2-treated CAFs vs. E2-treated Spz cells. The Id family of transcription factor proteins is named for the role of these proteins in inhibiting premature differentiation of embryonic stem cells. A recent study demonstrated that TGFβ signaling acts to induce differentiation of hESC via downregulation of *Id1*[113]. Interestingly, the authors found that TGFβ suppressed *Id1* gene expression in undifferentiated hESC, but increased *Id1* expression in differentiated ESC. Using a murine pulmonary metastasis model, Gao *et al.* demonstrated that expression of Id1 in bone marrow-derived endothelial progenitor cells (EPCs) is necessary for the growth of micro-metastases into macro-metastases[114]. Additionally, tumor cells induce the expression of Id1in EPCs[114]. Furthermore, other inhibitor of DNA binding proteins are crucial for angiogenesis, as tumor growth was found to be impaired in $Id1^{+/-}$ $Id3^{-/-}$ mice[115]. Thus, these Id proteins are relevant to both normal development and cancer, and seem to be regulated by TGFβ signaling.

Another set of genes that were observed to be upregulated in analyses of both ERα stromal cells compared to empty vector cells and in the analysis of common leading edge genes between CAF and ERα stromal cells is the IGFBP family of genes. In the 25-gene signature from the leading edge analysis, *IGFBP3* and *IGFBP4* were upregulated, while *IGFBP2*, *IGFBP3* and *IGFBP4* were respectively upregulated in CAFs vs Spz, WPMY-1/ERα vs. EV, and PrSC2/ERα vs. EV. This family of proteins is involved in binding and presentation of insulin-like growth factor 1 (IGF-1) and IGF-2. One study investigating a putative stem cell gene expression signature of prostate basal cells found mRNA levels of IGFBP proteins to be upregulated in these cells[65].

Furthermore, unpublished work from this laboratory suggests that the IGF-1 receptor, IGF-1R, plays an important role in regulation of adult human prostate epithelial stem cell self-renewal.

Comparative gene expression analyses have yielded compelling evidence that ERα expression in stromal cells confers a phenotype similar to CAFs, or otherwise activated stromal cells. The data presented thus far also indicate that ERα modulation of TGFβ signaling—perhaps via engagement of an autocrine signaling loop—might be mechanistically involved in the observed induction of an activated myofibroblast phenotype. Furthermore, the evidence suggests that ERα expression might be regulating multiple signaling pathways that have previously been implicated in either morphological development or regulation of adult stem cells. Finally, the upregulated genes that are responsible for driving these biological pathways in ERα stromal cells and CAFs have been shown to have clinical relevance in human prostate cancer.

The present work has provided provocative evidence linking a stromal ERα phenotype to molecular mechanisms involved in normal tissue development. The idea that these mechanisms then have biological significance in prostate cancer progression reflects the longstanding link between cancer and development.

VII. SIGNIFICANCE AND FUTURE DIRECTIONS

The data herein presented represent the first evidence using primary human tissues that stromal cells regulate epithelial stem cell self-renewal and progenitor cell proliferation within the benign prostate stem cell niche. Furthermore, these stromal-epithelial interactions within the niche are subject to modulation by estrogen signaling through stromal-ERα, which can act in both ligand-dependent and ligand-independent mechanisms. The capacity of stromal cells to increase symmetric stem cell self-renewal, and the capability of stromal-ERα to potentiate this effect, has important implications for prostate development, adult tissue homeostasis and especially for cancer.

Accumulating evidence suggests that stem cells are preferential cells-of-origin in carcinogenesis. Thus, many scientists have hypothesized that any biological influence that increases stem cell numbers could potentially contribute to cancer risk. Furthermore, a convergence of many decades of research within the fields of environmental toxicology, epidemiology and stem and developmental biology have begun to shed light on the longstanding hypothesis of the developmental basis of adult disease. Sufficient evidence now exists to support the notion that exogenous environmental influences, such as endocrine disrupting chemicals, can epigenetically alter developing tissues, likely via actions on stem and progenitor cells, and predispose tissues to disease later in life. The present work, although conducted using adult tissues, describes a physiological model that could potentially mediate pro-carcinogenic influences of endocrine disrupting chemicals through stromal-ERα-directed effects on epithelial stem and progenitor cells.

Preliminary evidence from this work also supports the provocative notion that changes in ERα expression can initiate stromal cell activation toward a myofibroblastic or CAF-like state. Since activated fibroblasts are known to have pro-inflammatory and otherwise deleterious effects on benign epithelial cells, a process such as ERα-induced stromal activation could provide a mode of initiating epithelial disease through the stroma within an adult. Furthermore, ERα-induced stromal activation toward a CAF-like phenotype has obvious implications in prostate cancer development and progression.

A fundamental goal of future studies must be to elucidate the molecular mediators of stroma cell influences on stem and progenitor cells. The present study suggests the possibility that the extracellular matrix may be critical in mediating these influences, perhaps in relation to its ability to sequester and spatially organize the availability of morphogens such as TGFβ, which is implicated in many aspects of this work. As this laboratory and others continue to work toward isolating and characterizing prostate epithelial stem cells, it is likely that the mediators of stromal-epithelial interactions within the SCN will become apparent. Furthermore, additional work is warranted in elucidating how CAFs regulate the benign and cancer stem cell niches. Such studies will serve to strengthen the translational impact of the present work.

Future studies should also be directed toward understanding the potential role of ERα expression in activation of stromal cells. It would be interesting to interrogate whether ERα expression in CAFs correlates to their cell- or tissue-of-origin. This might have important implications for prostate cancer metastasis, which is certainly influenced by organ tropism.

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Primary Stromal Cell Cultures					
De-identified Patient / Line	**Type**	**Original Passage**	**Grade**	**Age**	**Race**
CAF1	CAF	5	3+3	58	-
CAF2	CAF	5	3+3	63	-
CAF3	CAF	4	4+4	61	EA
CAF4	CAF	4	3+3	53	-
CAF5	CAF	5	4+3	46	-
CAF6	CAF	3	5+4	60	-
CAF7	CAF	5	4+4	68	-
CAF8	CAF	4	4+4	58	EA
CAF9	CAF	4	3+4	49	-
Spz3	Spz	3	-	63	AA
Spz1	Spz	3	-	51	AA
Sca1	CAF	3	-	51	AA
Spz2	Spz	3	-	-	-
Sca2	CAF	3	-	-	-
Sca3	CAF	2	-	-	AA
Spz4	Spz	5	-	61	EA
Spz5	Spz	5	-	58	EA
Spz6	Spz	5	-	-	H

Table 5. De-identified primary patient-derived stromal cell cultures utilized. CAF = cancer associated fibroblast, Spz = benign peripheral zone stroma.

VITA

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	Lammers, A.R., **Gauntner, T.D**. Mechanics of torque generation duringquadrupedal arboreal locomotion. Journal of Biomechanics. 2008; 41(11): 2388-95.
ABSTRACTS & PRESENTATIONS	Stable ERα-Expressing Benign Primary Prostate Stromal Cells Share Morphological Features, Molecular Characteristics and a Clinically-Predictive Gene Expression Signature With Cancer-Associated Fibroblasts Abstract & Poster Presentation at the Society for Basic Urologic Research Fall Symposium, Scottsdale, AZ, 2016
	Differential Actions of Estrogen Receptor α and β via Non-genomic Signaling in Human Prostate Stem-Progenitor Cells Abstract & Poster Presentation at the Society for Basic Urologic Research Fall Symposium, Scottsdale, AZ, 2016

Human Prostate Stromal Cells Enhance Epithelial Stem Cell Self-renewal and Progenitor Cell Proliferation
Poster Presentation at the Society for Basic Urologic Research Fall Symposium,
Fort Lauderdale, FL, 2015

Modeling Stromal-Epithelial Interactions to Assess Homeostatic and Carcinogenic Influences of 17β-Estradiol on the Prostate Stem Cell Niche
Poster Presentation at the ASCI/AAP Joint Meeting,
Chicago, IL, April 2015

Distinct Actions of ERα and ERβ in Human Prostate Stem and Progenitor Cell Self-Renewal and Differentiation
Poster Presentation at the Endocrine Society Annual Meeting
Chicago, IL, June 2014

Synthesis and Evaluation of Pyrrolidine Derivatives as CCR1 Antagonists for *in Vitro* Inhibition of Multiple Myeloma
Abstracts of Papers of the American Chemical Society
Washington, DC, August 2012

CCR1 Antagonists Inhibit Osteoclastogenesis
Poster presentation at the International Conference on Cancer-Induced Bone Disease
Chicago, IL, November 2011

Evaluation of CCR1 Antagonists in Multiple Myeloma and Osteolytic Bone Disease
Poster presentation at the American Osteopathic Association Medical Conference
Orlando, FL, October 2011

Evaluating Allosteric Modulators of CCR1 for Multiple Myeloma
Poster presentation at the Gordon Research Conference in Medicinal Chemistry
Colby-Sawyer College, New London, NH, August 2011

Development of a HaloTag Receptor to Study Chemokine Receptor CCR1 Internalization
Poster presentation at Discovery on Target Meeting
Cambridge Healthtech Institute, Boston, MA, November 2010

Determination of Polycyclic Aromatic Hydrocarbons in Parking Lot Seal Coat
Poster presentation at student research symposium

The Ohio State University, Columbus, OH, June 2008

Center of Pressure and Torque in the Manus and Pes During Quadrupedal Arboreal Locomotion
Poster presentation at student research symposium
Cleveland State University, Cleveland, OH, August 2007

TEACHING

Guest Lecturer, "Cancer Stem Cells" Graduate Pathology Course,
Department of Pathology, College of Medicine,
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Laboratory Teaching Assistant, Physiology for Clinical Services
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PROFESSIONAL EXPERIENCE

Systems Analyst, Ernst & Young, LLP,
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Business Systems Analyst, Anthem Benefit Administrators,
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Hazard Claims Analyst, Safeguard Properties,
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VOLUNTEER

Medical Volunteer
RESTCare Clinic, Chicago, IL 2010-2014

HIV Testing Counselor
The Free Medical Clinic of Greater Cleveland,
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Patient Transporter
Lakewood Hospital, Lakewood, OH, 2007-2008

Tutor, Open Doors Youth After-School Program
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HONORS & AWARDS

Travel Award
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First Prize Award—Student Research Competition for poster "Evaluation of CCR1 Antagonists in Multiple Myeloma and Osteolytic Bone Disease"
American Osteopathic Association Research Conference

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Scaife Family Foundation Scholarship
National Rural Institute on Alcohol and Drug Abuse
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Kenneth A. Suarez Summer Research Fellowship
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Student Researcher of the Year Award
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Dean's List (All Semesters)
Cleveland State University, 2007-2009

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PROFESSIONAL MEMBERSHIP

Society for Basic Urological Research, *Member-in-Training*

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American Physician Scientists Association

International Bone & Mineral Society, *Member-in-Training*

Student Osteopathic Medical Association

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