SILIBININ EXERTS CANCER CHEMOPREVENTIVE EFFICACY VIA 
TARGETING PROSTATE CANCER CELL AND 
CANCER-ASSOCIATED FIBROBLAST INTERACTION 

By 
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Prostate cancer (PCA) kills thousands in the US each year despite massive investment and success in improving early detection and treatment. Importantly, even successful treatment is still associated with persistent and often highly disruptive adverse health effects on the patient. As a consequence, the development of alternative treatment regimens like chemoprevention remains of great interest. Chemoprevention is intended for long-term and continuous use to prevent or halt disease even in individuals with no outward sign of disease. A developing tumor and its surrounding tumor microenvironment (TME) can be together thought of as a nascent organ, with specific components carrying out distinct functions. To study these interactions, we developed a cell culture system that would isolate the secretions of PCA cells and cancer associated fibroblasts (CAFs) to identify their effects on TME elements which might support PCA progression. This system would allow us in parallel to study the capacity of the natural product silibinin to target these interactions, which was selected on the basis of its well established anticancer properties. We first used this system to study the effects of PCA cells on human fibroblasts, the most numerous cells in the TME. We found that exposure to PCA secreted TGFβ2 could activate naïve fibroblasts, which could be inhibited by silibinin. PCA cells could alter fibroblasts into a phenotype similar to CAFs isolated from patients with clinically confirmed PCA. These fibroblasts have been transformed by close residence to a developing tumor into a
constitutively activated phenotype, the presence of which is associated with poor patient prognosis. Consistent with these reports, our second set of studies revealed that CAFs could induce an aggressive phenotype in treated prostate epithelial and PCA cells that could be inhibited by silibinin. We found that these responses were a result of secretion of MCP-1. Interestingly, we noted a significant reduction of immune cell recruitment in animals treated with silibinin which was associated with a marked reduction in tumor growth. Thus, PCA cells can recruit normal fibroblasts, turning them into CAFs, which in turn promotes immune cell recruitment. These effects together may serve to promote tumor growth and expansion resulting in a continuing cycle ultimately serving to support PCA progression.

The form and content of this abstract are approved. I recommend its publication.

Approved: Rajesh Agarwal
I dedicate this work to my family,

It’s been a long road...
ACKNOWLEDGEMENTS

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<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>ARCaP_M</td>
<td>Androgen repressed metastatic human prostate cancer</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>CAFs</td>
<td>Cancer associated fibroblasts</td>
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<td>CCM</td>
<td>Control conditioned media</td>
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<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
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<tr>
<td>CDKI</td>
<td>Cyclin dependent kinase inhibitor</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>Chk2</td>
<td>Checkpoint kinase-2</td>
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<tr>
<td>CMC</td>
<td>Carboxymethylcellulose</td>
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<tr>
<td>CTL</td>
<td>Cytotoxic T-cell</td>
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<tr>
<td>CXCL1</td>
<td>(C-X-C motif) ligand 1</td>
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<td>DAB</td>
<td>3,3′-diaminobenzidine</td>
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<td>DAPI</td>
<td>4’,6’-diamidino-2-phenyindole</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>DRE</td>
<td>Digital rectal examinations</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
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<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
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<tr>
<td>ERK1/2</td>
<td>Extracellular signal-regulated kinases 1/2</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>---------</td>
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<tr>
<td>FAP</td>
<td>Fibroblast activation protein</td>
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<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
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<td>Grape seed extract</td>
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<td>Glutathione</td>
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<td>Hepatocyte growth factor</td>
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<td>IGFBP-3</td>
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<td>Insulin-like growth factor receptor</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>IKKα</td>
<td>IκB kinase α</td>
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<td>Interleukin-13</td>
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<td>IL-1ra</td>
<td>Interleukin-1 receptor antagonist</td>
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<td>IL-6</td>
<td>Interleukin-6</td>
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<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<td>IκBα</td>
<td>Inhibitor of κBα</td>
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<td>JNK1/2</td>
<td>c-Jun N-terminal kinases 1/2</td>
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<td>LD50</td>
<td>50% lethal dose</td>
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<td>Description</td>
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<tr>
<td>LRP6</td>
<td>Low density lipoprotein receptor-related protein-6 (LRP6)</td>
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<tr>
<td>Ly6g</td>
<td>Lymphocyte antigen 6 complex, locus G</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein-1</td>
</tr>
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<td>MDSC</td>
<td>Myeloid derived stem cell</td>
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<tr>
<td>MIF-1</td>
<td>Macrophage migration inhibitory factor-1</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
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<tr>
<td>PCA</td>
<td>Prostate cancer</td>
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<tr>
<td>PDEF</td>
<td>Prostate epithelium-derived Ets transcription factor</td>
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<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>Platelet endothelial cell adhesion molecule-1</td>
</tr>
<tr>
<td>PIN</td>
<td>Prostatic intraepithelial neoplasia</td>
</tr>
<tr>
<td>PrSCs</td>
<td>Prostate stromal cells</td>
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<tr>
<td>PSA</td>
<td>Prostate specific antigen</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SBCM</td>
<td>Silibinin conditioned media</td>
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<tr>
<td>SCGM</td>
<td>Stromal cell growth medium</td>
</tr>
<tr>
<td>SDF-1</td>
<td>Stromal cell-derived factor-1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>SELECT</td>
<td>Selenium and vitamin E cancer prevention trial</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>STAT-3</td>
<td>Signal transducer and activator of transcription-3</td>
</tr>
<tr>
<td>STR</td>
<td>Short tandem repeat</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumor associated macrophage</td>
</tr>
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<td>TGF-β</td>
<td>Transforming growth factor-β</td>
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<td>TGFβ1</td>
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<td>Transforming growth factor-α</td>
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<td>TME</td>
<td>Tumor microenvironment</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
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<tr>
<td>TRAMP</td>
<td>Transgenic adenocarcinoma of the mouse prostate</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>VEGF-A</td>
<td>Vascular endothelial growth factor-A</td>
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<tr>
<td>VEGFR2</td>
<td>Vascular endothelial growth factor receptor 2</td>
</tr>
<tr>
<td>VITAL</td>
<td>VITamins and lifestyle</td>
</tr>
<tr>
<td>ZEB1</td>
<td>Zinc finger E-box-binding homeobox 1</td>
</tr>
<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
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CHAPTER I
BACKGROUND

Prostate Cancer

Prostate cancer (PCA) is the most commonly diagnosed cancer in American men, and the second highest cancer related mortality with an estimated 220,800 new cases and 27,540 deaths in 2015 alone (1). This pattern is repeated in most other Western nations; worldwide PCA is the second most commonly diagnosed cancer in men with incidence expected to grow further (2). Risk factors for PCA include age, race/ethnicity, family history of the disease, and inflammation. PCA is generally diagnosed in older men with the average age of diagnosis being in the mid 60’s having lowered from 70 years old just a few decades ago (3). In terms of race/ethnicity, African American men are more likely to develop PCA as compared to Caucasian men and more likely to suffer mortality as a result (4). Having a 1st degree relative diagnosed with PCA doubles the risk of PCA, two such relatives is associated with a fivefold increase in risk for PCA as compared to men with no family history of the disease (5). The presence of inflammation in prostate biopsies has also been associated with a near doubling of PCA risk (6). Massive investment and notable success in improving early detection of PCA (which ironically might be partially responsible for the high incidence rates of PCA reported in developed countries) has resulted in an increase in the treatment of PCA as early and localized disease rather than as more advanced forms (7). In fact, the reduction in age of diagnosis might very well be a consequence of these efforts. However, studies have revealed that despite this notable improvement in PCA detection and the associated
high incidence rates as a result, they still fail to fully express the magnitude of PCA prevalence in the population.

As mentioned previously, based on diagnostic evidence, advanced age is considered a key risk factor for PCA. With an average age of diagnosis in the mid 60’s and the traditional paradigm of cancer being a multistage sequence based on stochastic genetic disturbances (and thus could be expected to be a lengthy and haphazard process) lends credence to age being an important risk factor. Young men then would not be expected to develop the disease in any significant numbers. In contravention of this clinical evidence and the prevailing wisdom that arose from it, an autopsy study of prostates taken from 152 patients who died of other causes and had no previous diagnosis of PCA, revealed that by their 30’s, 27% of men had already developed PCA (8). Further 9% of men still in their 20’s were found to already have the PCA precursor, prostatic intraepithelial neoplasia (PIN) (8). In another autopsy study investigating 1,327 men of Chinese (Hong Kong and Singapore), Swedish, German, Jamaican, Israeli, and Ugandan nationality, 30% of men in their 50s were found to have PCA, which increased to 80% of men who had entered their 70s (9). Consistent with established PCA racial/ethnic risk factors, Chinese men exhibited lower latent PCA rates as compared to Swedish, German, and Jamaican men. In an alternative analysis, similar evidence for undetected PCA was provided, with perhaps more troubling implications. In the PCA Prevention Trial, 18,882 men were followed for seven years. Of these men, 2,950 exhibited clinically normal results from both annual prostate specific antigen (PSA) tests as well as digital rectal examinations (DRE) throughout the seven years of the study. Importantly, the PSA and DRE exams are the hallmark early detection assays for
PCA. Despite seven years with a clean bill of health, 449 of the 2,950 (12%) men would be later diagnosed with PCA when prostate biopsies were taken at the end of the study (10). Even these numbers may undercut the prevalence of PCA in the population as a sextant biopsy was used in this study in place of a more thorough and extensive biopsy regimen. This reveals that even after years of extensive investment in awareness and screening efforts to improve early detection of PCA, the standard diagnostic tools for PCA can still fail to detect the disease in patients for up to seven years (if not longer). These are especially troubling findings as undiagnosed PCA could progress to metastasis prior to discovery and this is a critical threshold for patient survival. Treatment of early, local PCA can have a nearly 100% 5-year survival rate whereas the rate following treatment of metastatic disease drops precipitously to just 28% (11).

While patient survival and health are the most important measures of success in addressing PCA, achieving high rates of early detection along with aggressive treatment of PCA have attendant costs. From increasing importance we will start with the financial costs. Just focusing on the US, and just including the direct health care costs of treating PCA, an estimated $11.85 billion was spent in 2010 alone (12). As large a number as that is, it is only expected to grow as a consequence of improved detection protocols, development of more advanced treatments, and the aging demographics of America along with increased life expectancy (as age is a risk factor for the development of PCA). As an example, during the period between 2002 and 2005, the cost of treating PCA increased by more than $350 million (13). Furthermore, these direct costs do not incorporate secondary financial losses as a result of PCA, such as lost worker productivity (of both the patient and caregivers). Following these relatively easy to
quantify costs is the cost to patient well-being even following successful treatment with traditional interventions. These include urinary incontinence, bowel issues, and impotence. Following 127 patients three years after a radical prostatectomy, a study found that 58% of these patients still reported varying degrees of urinary dysfunction and 27% reported bowel issues (14). These findings are especially troubling not only for their years long duration, but also that these numbers are so high even after the study was normalized by only reporting patients with normal function prior to treatment. Perhaps more distressing, 94% reported some level of sexual dysfunction, with roughly 2/3’s of them being listed as severe. Another, larger study of 1291 patients similarly focused on following patients after radical prostatectomy reported that two years later, nearly half of patients reported urinary issues ranging from complete lack of control to frequent or occasional leakage (15). In this study, sexual dysfunction was found to be lower, but still affecting about 60% of patients (15). In the previously described study following patients for three years, patients treated with radiation were also investigated. Here, urinary issues were reduced, with this being reported by only 17% of patients, but it was associated with a marked increase in bowel dysfunction, reported in 66% of patients (14). Sexual dysfunction remained a problem for 74% of patients. These studies, as informative as they are on the troubling prevalence of serious adverse effects in patients following PCA treatment, still do not fully encapsulate the physical and mental burden of disease diagnosis, treatment, and recovery. In light of that reality and the huge (and growing) costs of proactive PCA screening and aggressive treatment, the United States Preventive Services Task Forces has recommended against regular PSA screening in asymptomatic men, which is intended to lower costs and incidence of
aggressive treatment, which together may not have actually translated to a significant improvement in patient mortality (16, 17) and simply have increased costs and otherwise lowered patient quality of life though this position is somewhat controversial (18). This failure again, may be a result of a failure to fully capture PCA prevalence with currently used diagnostic screening assays. As a consequence of these findings, there is continuing and growing interest in developing alternative interventions that might adopt preventative measures intended to reduce risk of PCA rather than focusing on aggressive treatment.

**Chemoprevention**

One alternative method for PCA intervention has been conceived under a broader umbrella of cancer chemoprevention. Cancer chemoprevention is a treatment regime where chemical agents are administered to provide long-term, perhaps indefinite, reduction of cancer risk. These agents should ideally be well-tolerated, have broad efficacy, orally bioavailable, and be inexpensive (19, 20). This philosophy is based on the recognition of cancer as a multistage process progressing over the prolonged period of time necessary to accumulate the multitude of dysfunctions (resistance to apoptosis, uncontrolled growth and invasion, etc.) needed both in the tumor cells as well as the cellular microenvironment they reside in. This period of time and the potential cellular/molecular gatekeepers that must be overcome in cancer development provides an opportunity to stunt or eliminate cancerous cells at the earliest stages of development or even at localized lesions. This treatment regime is especially relevant for PCA as it is a disease that generally progresses relatively slowly, but as previously mentioned, premalignant lesions can be found in men in their 20’s and PCA might be expected to
be found in the majority of men by a certain age (9). This broad umbrella can be further subdivided based on intended target and treatment population into primary, secondary, and tertiary cancer chemoprevention (Figure 1.1) (21, 22). Primary chemoprevention is intended to address the very earliest stage of cancer progression, at the level of initiated cells, inhibiting progression to premalignant lesions. This is ideal as it would have the broadest impact, reducing incidence of disease, concomitant treatment costs, and adverse effects in patients, while reducing mortality. This then would be intended for asymptomatic patients or even in men with no detectable PCA so as to limit the development of disease in the first place. Primary chemoprevention would be intended to be used by the broadest section of the population for the longest period especially in high risk populations. As previously mentioned, major demographic risk factors for PCA include age and race/ethnicity and thus individuals in these risk categories would be most appropriate for inclusion in this preemptive treatment regime. Secondary chemoprevention is concentrated on situations where initiated cells have already progressed into forming a premalignant tumor. Here, secondary chemoprevention is focused on the arrest and ideally, eradication of premalignant tumors to prevent their transformation into malignant cells. This regime would be intended for patients with detectable but not yet benign tumors but might also be used for the remainder of a patient’s life to slow or reverse PCA progression. Tertiary chemoprevention is applicable where tumors have progressed to malignancy; and here, tertiary chemoprevention is intended to operate on two separate but related elements. Primarily, it is intended to restrict a primary tumor to localized disease at the site of development. This is a critical stage in most cancers, but as mentioned previously, especially so, for
PCA where patient 5-year survival drops nearly 72% upon progression from localized to metastatic disease. Secondly, it is intended to prevent recurrence of the disease in the face of successful intervention, another critical stage in patient survival. This last factor may then require extended use, and similar to primary chemoprevention potentially be provided in the absence of any overt signs of disease as a preemptive measure. In pursuit of these goals, several promising agents have been investigated.

Figure 1.1 Schematic for the tiers of chemoprevention.
Chemoprevention Studies

Given the qualities ideally represented in any potential chemopreventive agent, such as low toxicity and cost, many of the attempts to develop these chemical entities have focused on natural products and their derivatives. Many of these agents have been selected on the basis of their historical medicinal use, correlation with reduced cancer risk in population studies, or their unique set of chemical properties. Pomegranate juice has been investigated as an anti-PCA agent on the basis of its high concentration of antioxidant phytochemicals, particularly polyphenolic ellagitannins. This was investigated in a phase II study involving 46 patients for 13 months following surgery or radiotherapy (23). The study was limited to patients that still had detectable PSA but had Gleason scores ≤7 which is the upper limit score for PCA that is considered successfully treatable. It was found that daily treatment with 8 oz of pomegranate juice elicited no adverse effects but significantly increased the time it took for PSA levels to double (15 months vs 54 months). Tomatoes (Solanum lycopersicum) contain large amounts of lycopene, a carotenoid with strong antioxidant properties and thus consumption of tomato has been investigated as a PCA chemopreventive therapy. A prospective cohort study was conducted for six years with 47,894 subjects that were initially clear of any diagnosis of cancer and had their dietary intake of food following for a 1 year period (24). It was found that consumption of tomatoes, tomato sauce, and pizza significantly reduced risk of PCA and consumption was inversely associated with PCA risk. Cruciferous vegetables contain a host of compounds, among them glucosinolates and phytoalexins which are both reactive sets of compounds used by plants as defense mechanisms against pests and infection. The capacity of these
vegetables to act as a PCA protective agent was investigated in a multicenter, multiethnic case-control study involving 3,237 patients with histologically confirmed PCA (25). Consumption of cruciferous vegetables was found to be inversely associated with PCA risk, particularly for advanced cancer. This finding has been supported by a review of 12 epidemiological studies revealing a modest correlation of high intake of cruciferous vegetables with reduced PCA risk (26). Moderate wine consumption has been associated with several health benefits. As such it has also been investigated as an anti-PCA agent. One derivative, grape seed extract (GSE) is a potent antioxidant and as a result of the concentrated flavonoids and proanthocyanidins it contains (27). On this basis, GSE was investigated in the VITamins And Lifestyle (VITAL) study of 35,239 men. It was found that GSE supplementation significantly lowered PCA risk in treated men (28). In regards to agents identified based on correlation with reduced cancer risk in population studies, Asian populations have been found to have low levels of PCA incidence. Thus, traditional foodstuffs have been investigated for their suitability as chemopreventive agents. One such item is green tea (brewed from *Camellia sinensis*) which is enriched in flavonoids such as the antioxidant polyphenolic catechins (29, 30). Several studies have shown that green tea intake is inversely related to PCA risk, resulting in both lower incidence and progression (30-32). Another foodstuff that has been investigated is soybeans (*Glycine max*) which contain a complex mixture of several antioxidant isoflavones. Like with green tea, soy consumption has been associated with an inverse risk of PCA (33, 34).

Zinc is an essential mineral that is found to be highly enriched in normal prostatic tissues which is significantly reduced in PCA tumors (35). This mineral serves critical
roles in a plethora of cellular functions among them being DNA repair and maintenance of the immune system. On the basis of these properties, zinc was investigated in a population based cohort study of 525 Swedish men with a previous diagnosis of PCA. These men were followed for a mean of 6.4 years and questioned in regard to zinc intake. It was found that high zinc intake was associated with a reduction in PCA related mortality particularly in localized disease (36). Selenium is an essential micronutrient that is a critical cofactor in the reduction of antioxidant enzymes. Vitamin E is a group of plant derived lipid soluble antioxidants which are used for many metabolic processes, but in this context used to scavenge free radicals and protect cellular membranes from oxidation. On the basis of promising in vitro studies (37-39) and preliminary clinical trials (40-42) revealing potential anti-PCA properties in these nutrients, they were investigated in the Phase III Selenium and Vitamin E Cancer Prevention Trial (SELECT). The effect of selenium and vitamin E both separately and combined on the incidence of PCA was to be examined. This was intended to be a comprehensive investigation involving more than 35,000 patients that would be followed up to 12 years (43, 44) but was halted as there was no observed improvement in PCA incidence and in fact revealed an increase in incidence of PCA in patients given vitamin E vs placebo (44-46). This reveals the difficulty in translating promising preliminary findings into clinically useful interventions for PCA chemoprevention. Certain pharmacological agents have also been investigated for suitability as PCA chemopreventive agents. As inflammation is a risk factor for many cancers and PCA specifically (47, 48), the use of non-steroidal anti-inflammatory drugs (NSAIDs) has been assayed. Efforts to study one commonly used NSAID, aspirin, have had similarly conflicting reports as the SELECT
study. A study of 5,955 men with localized PCA as well showed that aspirin use reduced PCA mortality (49). However, a study of 70,144 men found that short-term aspirin use did not reduce PCA risk but long-term use did (50) whereas another study of 47,882 men showed that aspirin did not reduce PCA incidence but instead reduced incidence of metastasis (a critical stage as previously mentioned) (51). In contrast, a population-based case-control study of 1,001 PCA patients revealed aspirin significantly reduced PCA risk, but not disease aggressiveness (52). Finally, a study of 90,100 men followed for up to 32 years, it was found that up to six aspirin a day were required to produce modest PCA chemopreventive effect (53). Similar contradictory results can be found for other agents such as ibuprofen. As such there remains a need for further investigations into the development of chemopreventive agents.

**Silibinin**

**Background**

Silibinin is one such natural product that has been investigated for chemopreventive efficacy. This agent is an approximate equimolar mixture of two diastereomers, silybin A and silybin B. It is the main active ingredient of silymarin, a standardized extract milk thistle (*Silybum marianum*; Asteraceae) seeds. Silibinin is the most abundant constituent of silymarin, though it does contain several related flavonolignans such as dihydrosilybin, isosilybin, silychristin, and silydianin. Milk thistle originates from the Mediterranean where it has been used for millennia as a remedy for a host of ailments centered on the liver, gall bladder and kidneys. This speaks to silibinin’s relatively low costs to administer and toxicity, ideal characteristics of potential chemopreventive agents. In fact, in studies where animals were injected intravenously with silymarin, a
50% lethal dose (LD50) required extremely large doses. Mice tolerated 400-1050 mg/kg, rats 385-920 mg/kg, and rabbits and dogs 140-300 mg/kg (54-56). Similarly, oral delivery required over 10 g/kg to achieve toxicity (54-56). In recent years, milk thistle has been used to treat hepatic injury resulting from bile duct inflammation, cirrhosis, fatty liver, mushroom toxicity, and viral hepatitis (57). This efficacy might be a consequence of silibinin’s potent antioxidant properties derived from being a flavonoid, which have been noted for their antioxidant potential. Flavonoids have been reported to inhibit inflammation, neoplasia, and hepatic injury among other disorders (58). Silibinin specifically has been shown to support cellular antioxidant mechanisms like glutathione (GSH) and superoxide dismutase (SOD) by scavenging free radicals and reactive oxygen species (ROS) (59, 60). This may then prevent lipid peroxidation and subsequent cell death by eliminating or reducing the oxidative stress resulting from hepatic injury, toxicity, and disease. It is these and other chemical properties that have led to its investigation as a cancer chemopreventive agent.

Unfortunately, as a flavonolignan, silibinin is poorly water soluble. In addition, polyphenolic compounds are generally dependent on deprotonation of their hydroxyl groups to become water soluble, a state that is severely inhibited in the acidic conditions of the stomach and upper small intestine. Thus, silibinin generally requires encapsulation or other modification for effective human administration. To assay 60 minutes, with a half-life of 57-127 minutes (61). Silibinin reached a peak of 2.5 ± 0.4 µg/g tissue. Sulfate and beta glucuronidate conjugated-silibinin peaked at 1 hour, with a half-life of 45 to 94 minutes (61). To improve bioavailability, silibinin was put into a mixture with phosphatidylcholine, a major component of biological membranes and
notable for having an amphipathic character (having both a hydrophobic tail section and a hydrophilic head section). This creates a molecular interaction where phosphatidylcholine’s hydrophobic lipid tails can freely associate with each other (or in this case the hydrophobic silibinin) while allowing their hydrophilic choline heads to coordinate with water, ultimately increasing the effective solubility of silibinin in water. This mixture was developed as a commercial formulation called Siliphos® and is approximately 30% silibinin by weight. To address toxicity and bioavailability, a human trial involving 13 patients with advanced PCA was conducted. This silybin-phytosome was administered 3 times daily. An initial dose of 2.5 g/day was gradually increased to 20 g/day in 4-week intervals. Consistent with silibinin’s low toxicity in animal studies, adverse events generally consisted of grade 1-2 hyperbilirubinemia (9-13 patients) with one case of grade 3 toxicity. Thirteen grams of silybin-phytosome across 3 daily treatments were found to be well-tolerated and showed good plasma bioavailability (62). To identify achievable prostate tissue levels of silibinin in patients, a subsequent trial involving 12 patients with localized PCA that were to be treated by prostatectomy was conducted. Six patients were given the previously identified well-tolerated treatment regime while another six remained as controls. Treatment length varied from 14-31 days with a mean of 20 days. This trial showed silibinin reached a mean serum blood level of 19.7 μM after 1 hr; however, its level in prostate tissue was extremely low (496.6 pmol/g). Again, toxicity was found to be low, though following surgery, one grade 4 thromboembolic event was reported (63).
Anti PCA properties of silibinin

Silibinin targets dysregulated cell signaling in PCA.

At a stage from which all other cancer associated dysfunction might be expected to derive from, silibinin has been shown to disrupt several key molecular pathways critical to PCA development and progression. Silibinin treatment abrogated constitutively activated signal transducer and activator of transcription-3 (STAT-3) in human PCA DU-145 cells (64) and disrupted epidermal growth factor receptor (EGFR) signaling in both DU-145 and LNCaP cells (65, 66), insulin-like growth factor receptor (IGFR) signaling in PC3 cells (67), Wnt/β-catenin signaling in both PC3 and DU-145 cells (68),
nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling in DU-145 (69, 70), and androgen receptor (AR) signaling in LNCaP cells. This last effect was through both direct action by reducing nuclear localization of the AR (71) and indirect action by downregulating the AR co-activator, the prostate epithelium-derived Ets transcription factor (PDEF) (72, 73). Silibinin-mediated disruption of epidermal growth factor (EGF) signaling in PCA cells resulted in a decrease in secreted transforming growth factor-α (TGF-α) and was associated with a reduction in mitogen-activated protein kinase (MAPK) activity, both extracellular signal-regulated kinases 1/2 (ERK1/2) and c-Jun N-terminal kinases 1/2 (JNK1/2) (66). In contrast, disruption of IGFR signaling appeared to be a result of dose dependent induction of mRNA (and subsequent increase in protein levels in PC3 conditioned media) for insulin-like growth factor-binding protein-3 (IGFBP-3) (67). This effect was also found in vivo, where silibinin fed mice exhibited higher levels of both circulating and tumor resident IGFBP-3 which was associated with more differentiated tumors (74-76). Silibinin-mediated disruption of the Wnt/β-catenin pathway appeared to result from a comprehensive inhibition of the co-receptor, the low density lipoprotein receptor-related protein-6 (LRP6) at the levels of promoter, mRNA, protein expression, as well as phosphorylation (68). In terms of NF-κB, silibinin treatment comprehensively altered signaling, reducing constitutive activation in DU-145 cells, decreasing IκB kinase α (IKKα) kinase activity, subsequently reducing the ratio of phospho- inhibitor of κBα (IκBα) to total IκBα, which served to reduce the translocation of p50 and p65 NF-κB subunits to the nucleus (69).
Silibinin inhibits PCA proliferation.

As a consequence of silibinin’s capacity to alter signaling of these proliferative molecular pathways, it might be expected that silibinin can inhibit PCA proliferation and this was confirmed in multiple studies using several different models (77-80). Further, in mice fed silibinin, tumor growth was reduced in both xenograft implants as well as spontaneously developed prostate tumors in transgenic mice (75, 76, 81-83). These effects were found to be a result of potent silibinin mediated cell cycle arrest (77).

Silibinin modulated the cyclins–cyclin dependent kinases (CDKs)–cyclin dependent kinase inhibitors (CDKIs) pathway at several points, inducing G1 arrest in PCA cells. Effects include: decreasing expression of cyclin D1, cyclin D3, cyclin E, CDK4, CDK6 and CDK2, decreasing kinase activity of CDK2 and CDK4, increasing levels of CDKIs Kip1/p27 and Cip1/p21, and sequestering cyclin D1 and CDK2 in the cytoplasm (76, 77, 79, 84, 85). In addition, silibinin treatment markedly increased retinoblastoma (Rb) protein levels, specifically hypophosphorylated Rb/p107 and Rb2/p130. This was in conjunction with a marked decrease in the protein levels of the transcription factors, E2F3, E2F4 and E2F5. These silibinin dependent activities together serve to inhibit cell cycle progression (79, 84).

Intriguingly, silibinin treatment also induced G2-M arrest in PCA cells (79). This phenomenon was achieved through action on the checkpoint kinase-2 (Chk2)–Cdc25C–Cdc2/cyclin B1 pathway. Here, silibinin reduced protein levels of cyclin A, cyclin B1, both total and phosphorylated Cdc2, Cdc25B, and Cdc25C phosphatases while reducing inhibiting Cdc2 kinase activity (77, 86, 87). Furthermore, silibinin mediated inhibition of Cdc25C phosphatases along with increased Chk-2 phosphorylation resulted in the
translocation of nuclear Cdc25C to the cytoplasm as a result of now elevated phosphorylation (77). This last effect was accompanied by an increased binding with 14-3-3 protein. In addition, silibinin treatment reduced telomerase activity in LNCaP cells and DNA topoisomerase IIα activity in DU145 cells (72, 88). Perhaps relevant to instances of tertiary PCA chemoprevention, mitoxantrone and doxorubicin synergized with silibinin treatment to inhibit PCA cell proliferation (86, 89) and cisplatin and carboplatin synergized with silibinin to induce G2-M arrest which appeared to operate through reduction in Cdc2, cyclin B1 and Cdc25c (90).

**Silibinin induces apoptosis in PCA cells.**

Silibinin has also been shown to induce apoptosis in DU-145, H-7, I-8, and I-26 PCA cell lines cells *in vitro* and PC3 and DU-145 cells *in vivo* (64, 75, 76, 90, 91). The H-7, I-8, and I-26 PCA cells were derived from rats that had PCA induced by N-methyl-N-nitrosourea-testosterone protocols (91). This phenomenon appears to operate by decreasing Bcl-2 and survivin levels, activating caspases (caspase 3, 9, and 7), releasing cytochrome c from PCA mitochondria, leading to apoptosis (64, 75, 76, 90). In this modality, mitoxantrone, doxorubicin, cisplatin, and carboplatin were again found to synergize their effects with silibinin (86, 89, 90).

**Silibinin inhibits PCA invasion and metastasis.**

As previously mentioned, the transition from local to metastatic disease is a critical threshold for PCA in terms of patient mortality. In multiple studies, silibinin was found to promote an epithelial phenotype in PCA cells, serving to inhibit their metastasis (70, 82, 83, 92). Silibinin treated PC3, PC3MM3 and C4-2B cells exhibited elevated E-cadherin levels with concomitant reduction in migratory and invasive capacity (92).
This activity was dependent on the downregulation of regulatory molecules for epithelial to mesenchymal transition (EMT) such as Slug, Snail, phospho-Akt (ser^{473}), nuclear β-catenin, phospho-Src (tyr^{419}) and Hakai (92). The increase in E-cadherin expression by silibinin in PCA cells was also found in vivo, where silibinin treated transgenic adenocarcinoma of the mouse prostate (TRAMP) mice also exhibited decreased levels of matrix metalloproteinases (MMPs), Snail, fibronectin, and vimentin which together was associated with a reduction in PCA metastasis (82, 83). In silibinin treated androgen repressed metastatic human prostate cancer (ARCaPM) cells, there was decreased expression of the transcription factors zinc finger E-box-binding homeobox 1 (ZEB1) and Slug which are both regulators of EMT. Consistent with that activity, these cells exhibited a reduction of EMT markers, vimentin and MMP-2, which in turn corresponded to a dose- and time- dependent reduction in cellular motility, migration, and invasion (70, 93). Silibinin was also found to reduce expression of MMP-9 in several PCA cell lines (82, 83).

**Silibinin inhibits PCA angiogenesis.**

Finally, silibinin can target PCA angiogenesis, a stage critical for both PCA growth and progression in the primary tumor as well as for successful colonization of distant organs and tissues following metastasis. Silibinin treatment decreased vascular endothelial growth factor (VEGF) expression which correlated with decreased microvessel density found in PCA tumors from several different models (75, 76, 82). In TRAMP mice, silibinin feeding reduced expression of angiogenesis markers such as platelet endothelial cell adhesion molecule-1 (PECAM1)/CD-31, VEGF, vascular endothelial growth factor receptor 2 (VEGFR2), hypoxia-inducible factor-1α (HIF-1α),
and inducible nitric oxide synthase (iNOS) (83). These effects of silibinin were associated with alterations in metabolic profile of PCA tumors in TRAMP mice. Treated mice exhibited increased glucose and citrate levels with concomitant decrease in lactate, cholesterol, and phosphatidylcholine levels (94). These findings were supported in vitro where silibinin treated LNCaP and PC3 PCA cells exhibited reduced synthesis of HIF-1α both constitutively as well as when induced by hypoxia (95). This is a critical effect as hypoxia-induced HIF-1α expression is a key promoter of angiogenesis in solid tumors via enhancing the secretion of several pro-angiogenic factors (96, 97). Importantly, these phenomena involve a notable silibinin effect on non-tumor cells which reside near developing PCA cells (endothelial cells, fibroblasts, immune cells etc.) and incorporate elements of the tumor microenvironment.

**Tumor Microenvironment**

**Background**

The tumor microenvironment (TME) encompasses all the cellular and non-cellular components in close proximity with a resident tumor (98). The TME, when considered at all, was classically seen as a passive element to be colonized by an encroaching mass of cancer cells. The cancerous lesion was viewed as the active agent driving progression. The TME then, could be reductively seen as merely a set of molecular and cellular barriers to be overcome as a tumor progresses along the multistage process of cancer development (99). In time and as further insights into cancer initiation and progression have been gained, the TME has been reassessed as a far more active participant to carcinogenesis than previously believed. For example, chronic inflammation has been associated with an increased cancer risk in the same tissues (100, 101), suggesting the
activity and condition of normal tissues can influence the development of cancer. In addition, the existence of cancer in situ, particularly of ‘occult’ tumors that do not otherwise produce signs of disease in patients (as was found in autopsy studies of men with no prior diagnosis of PCA) (8, 102) is evidence that the dysfunctional activities of cancerous cells alone may not be sufficient to drive their growth and progression. In fact, the TME has been found to contribute to and support many of the hallmark properties of cancer such as promoting angiogenesis, inducing proliferative signals, initiating invasion and metastasis, and inhibiting apoptosis and growth suppressors (100, 103-106) and is now considered a critically important engine driving cancer progression.

The TME is a complex arena in which there are many players. In this context, a tumor and its surrounding TME could be considered a distinct organ composed of many specialized components with varied responsibilities that all support the continued viability and progression of a developing cancerous lesion. These components develop over time through a multifaceted set of interactions between each other and the rest of the organism (107). The TME includes, but is not limited to, cellular components such as resident and recruited fibroblasts and immune cells as well as the interstitial extracellular matrix (ECM) and a host of signaling molecules. These agents form a complex web of communication, transmitting from either cancerous cells of the tumor as well as the cellular components of the TME. These signals in turn may be received by many different audiences, inducing autocrine signaling in the same cells, or inducing paracrine signaling between distinct elements of the TME or between the tumor and TME, or even inducing activity in cells at distant regions otherwise unrelated to the
tumor site such as the preparation of pre-metastatic niches prior to their subsequent colonization by metastasizing cancer cells (100).

One particular set of signaling molecules found to be relevant to TME signaling and tumor progression are members of the transforming growth factor β (TGFβ) family. TGFβ is generally considered a tumor suppressive agent, at least in normal cells and early in carcinogenesis as it maintains cell differentiation, exhibits strong cytostatic effects, and even induces apoptosis in many cell types (108). This protective effect is lost at some point in tumor development and TGFβ signaling becomes adapted by a tumor to support cancer progression. In fact, TGFβ secretion is often upregulated in cancer cells, and found to promote PCA tumorigenesis (109). This change is likely caused through various mutations along the receptor signaling pathway of cancer cells, but also through cancer/TME interactions to bypass the cytostatic effects of TGFβ and instead marshal its other properties to become cancer promoting, driving EMT and proliferation (110, 111). This is both through direct action of TGFβ which induces loss of polarity and adhesion in cancer cells promoting EMT, as well as through secondary effects by promoting the remodeling of the ECM and silencing of immune surveillance through action on TME elements such as nearby resident fibroblasts and immune cells.

**Cancer Associated Fibroblasts**

Fibroblasts are a heterogeneous group of mesenchymal cells, and are by far the most numerous cellular component of the TME (112). They are normally quiescent, serving to maintain tissue integrity through the secretion of ECM molecules (principally collagen). However, in response to stimulating cues following disruption of connective tissue, fibroblasts are activated, initiating a wound repair program. These activators
include platelet derived growth factor (PDGF), thrombin, interleukin-1β (IL-1β), IL-6, IL-13, IL-33, and most importantly, TGFβ (113-115). The members of the TGFβ molecular family are the prototypical profibrotic agents (113, 114) and their inhibition markedly reduces fibroblast activation (116, 117). In their activated state, fibroblasts become proliferative and capable of chemotaxis to sites of injury. Upon differentiation into myofibroblasts, they develop contractile properties to physically remodel the ECM in efforts to seal a wound (118). This can be identified as a marked upregulation in the concentration and organization of α-smooth muscle actin (α-SMA), along with the expression of the intermediate filament vimentin and the fibroblast activation protein (FAP). In addition, activated fibroblasts exhibit drastically enhanced secretion of ECM molecules as well as TGFβ1, IL-1β, IL-33, and CXC and CC chemokines (113, 114, 119, 120). The secreted ECM molecules and chemokines activate and direct immune cells to the site of injury. As TGFβ1, IL-1β, and IL-33 were also involved in the initial stimulation of these fibroblasts, activated fibroblasts create a positive feedback loop promoting their own replication and further recruitment. This is an important facet of wound repair as this process is necessarily time dependent to ensure the rapid recovery of structural integrity in the organism and thus must be able to quickly propagate initial activating signals. In turn, these signals can be expected to quickly cease upon successful repair of damage to the connective tissue resulting in the return to quiescence of local fibroblasts. In fact, myofibroblasts are only transiently present in wounds (121). However, in instances where the inflammation is chronic, fibroblasts become inappropriately activated driving their continuous and reciprocal activation. This damages native tissues and leads to the accumulated deposition of poorly integrated
ECM which is structurally unsound and biologically less useful, resulting in pathological fibrosis. Ultimately, this then leads to a loss of normal function at the site of chronic inflammation.

It is this condition which critically promotes the processes involving fibroblasts found in close proximity to a developing cancer. Normally, fibroblasts might be expected to restrict the expansion of a growing tumor through their efforts to maintain tissue integrity and ECM homeostasis in the face of cancer cell growth and tumor secreted proteases. However, cancer has been appropriately described as a wound that does not heal (122-124). Thus, fibroblasts in close proximity to a tumor become activated for extended periods by stimulatory cues (either as a result of an underlying inflammatory condition as previously mentioned which are associated with many cancers or directly mediated by cancerous cells themselves). These fibroblasts in close proximity to a tumor are then “educated” over time, gaining a new phenotype, and becoming cancer associated fibroblasts (CAFs) (125). They gain the abilities of their wound healing brethren, but these are now in service of driving greater dysfunction rather than eliminating it. Furthermore, unlike the induction of wound healing myofibroblasts, the activation of CAFs is both constitutive and irreversible, no longer requiring further stimulation nor a chronic inflammatory environment to maintain (126, 127). Here, their contractile ability is used to physically disrupt ECM making it vulnerable to (and thus promoting) invasion by infiltrating cancer cells (128-130). It has also been shown that this physical disruption of ECM can liberate latent TGFβ1 stored in ECM driving both CAF as well as cancer cell proliferation (131). In addition, the collagen they now deposit is akin to that found in scar formation, no longer effective as
containment for a tumor but instead acting as a scaffold for the infiltration of cells (132). Further, in addition to those agents previously reported to be secreted by wound healing fibroblasts, CAFs have been found to secrete VEGF, fibroblast growth factors (FGF), hepatocyte growth factor, insulin-like growth factor-1 & 2 (IGF-1 & -2), and stromal cell-derived factor-1 (SDF-1/CXCL12) (113, 114, 119, 120, 133-135). These molecules together can support the cancer related dysfunctions that have been associated with the TME overall. They have been found to support cancer cell proliferation, angiogenesis, and invasion and metastasis (133-142). More troublingly, along with these other activities, CAFs are not subject to the apoptotic regulation that typically follows fibroblast activation (143, 144) which normally serves to eliminate activated myofibroblasts preventing their persistent residence and accumulation in the absence of continuing stimulation (i.e. once the wound has healed). Instead, CAFs are continually viable and proliferating, while simultaneously initiating autocrine feedback loops by secreting fibroblast activating agents (such as TGFβ), exacerbating their contribution to TME dysfunction over time. Finally, CAF secretion of CXC and CC chemokines along with various cytokines, together serves to recruit and activate infiltrating immune cells. The importance of these phenomena collectively is made evident as the presence of CAFs is associated with poor prognosis in patients in several different cancer types (128, 145, 146).

**Immune Cells**

Immune cells are a highly heterogeneous population that exists to protect the body from infection and to foreign bodies and cellular detritus (such as those created at the site of infection or in a wound). They are released from bone marrow and differentiate
from hematopoietic stem cells into broad categories listed here in order of decreasing circulating percentage: granulocyte, lymphocyte, and monocyte. These cells along with their various subtypes and terminal differentiations have different specialties and thus play different immune roles. As a whole, immune cells operate to detect, halt, and clear infections. They are capable of a variety of activities including phagocytosis, secretion of cytotoxic ROS and reactive nitrogen species (RNS), and secretion of a host of cytokines and chemokines (including several related to fibroblast activation such as IL-1β) (147, 148). In regards to tumor development, under normal conditions, leukocytes help to maintain immune surveillance, serving to eliminate or stunt tumor growth (101). However, as mentioned, under the dysregulated conditions of chronic inflammation, the resultant leukocyte recruitment has been associated with an increase in cancer risk (100, 101). For example, gastric infection by *Helicobacter pylori* results in mucosal inflammation, recruiting lymphocytes and neutrophils. Unfortunately, if this immune response fails to clear the disease, their activity instead works to create a persistent inflammatory state in the gastric lining. This development has been associated with a 2-3 fold increase in risk for gastric cancer (149). Likewise, inflammatory bowel diseases such as ulcerative colitis and Crohn’s disease are a consequence of autoimmune dysfunctions resulting in chronic immune cell activation and recruitment to the gastric tract. This has been associated with a 5-7 fold increase in risk of developing colorectal cancer (150). In the specific case of prostate cancer, presence of inflammation in biopsy cores taken from the prostate correlated to a 78% increase in risk for prostate cancer compared to those with no sign of inflammation (6). In fact, chronic inflammation and concomitant leukocyte recruitment has been shown to promote mutagenesis and
oncogene activation, resulting in tumor cell proliferation and metastasis while rescuing cancer cells from apoptosis (103). These effects appear to be driven by leukocyte-derived cytokines, chemokines, eicosanoids, and reactive oxygen species (ROS) (151).

Even in the absence of an underlying chronic inflammatory state, immune cells have been found to promote cancerous growth through several pathways. They can directly induce proliferation by secreting various growth promoting factors such as chemokines. In addition, immune cells are known to secrete a host of proteinases with a collectively broad range of targets. These can serve to directly remove growth inhibitory signals in cancerous cells caused by their adhesion to surrounding ECM (152) as well as enzymatically liberate compounds such as EGF, FGFs, heparins, histamine, interleukins, TGFβ, and tumor necrosis factor-α (TNF-α) from ECM-bound stores (153). These proteinase-related effects have been associated with promoting cancer cell invasion and metastasis (154) and along with macrophage secretion of VEGF-A have been implicated in promoting angiogenesis revealing a notable interaction by immune cells on other TME elements (103). Immune cells can also promote tumor cell survival (155), and in a paradoxical twist a subtype of macrophages called tumor associated macrophages (TAMs) recruit a specific subtype of T-cells (Tregs) which suppress cytotoxic T-cell (CTL) activity (156). Another subtype of monocytes, the so called myeloid derived stem cells (MDSCs), in turn promote the generation of Tregs (157) which further suppresses immune clearance of tumor cells. In addition, TGFβ is known to inhibit immune surveillance by natural killer (NK) and CTL cells (121). This would further critically interfere with the capacity of the immune system to eliminate the tumor
even in situations where cancerous cells were successfully detected and marked for elimination by other components of the immune system.

As mentioned earlier, cancer has been described as a wound that does not heal (122-124). These findings together suggest a cycle where one stage involves leukocytes being continuously drawn to the TME of a resident tumor, whether as a result of an underlying chronically inflamed state that may have initiated the cancer or as a result of this persistent “wound” that fails to resolve itself. Once these leukocytes arrive, the key effectors for cell clearance (NK and CTL cells) are suppressed which prevents their ability to address tumor cells. However, it does not prevent recruited leukocytes from secreting free radicals, inflammatory agents, and other signaling molecules in fruitless efforts to address the activating conditions that first recruited them and it is these that are then then adapted by other elements of the TME as well as cancer cells themselves to contribute to tumor progression.
CHAPTER II

SILIBININ PREVENTS PROSTATE CANCER CELL-MEDIATED DIFFERENTIATION OF NAÏVE FIBROBLASTS INTO CANCER-ASSOCIATED FIBROBLAST PHENOTYPE BY TARGETING TGFβ2

Introduction

Cancer is a heterogeneous disease encompassing myriad components: genetic susceptibility in the host, the degree and nature of exposure to environmental insults, the accumulation of these insults both systemically and locally at a developing tumor, and finally, the interactions of the otherwise normal cells and tissues in close contact with the cancerous lesion (139, 140, 158-166). This last set of interactions include the TME or stroma, the total sum of ECM components, soluble signaling factors, and neighboring noncancerous cells that interface with a tumor (134, 139-142). The TME is now recognized as an integral component to carcinogenesis that directly contributes to the development of malignancy (134, 142, 167). Thus, targeting this array of interactions, which if left unchecked, serves to support a progressive cancerous lesion, is considered a promising translational cancer preventive strategy. While the development of such a preventive strategy is of obvious importance in the case of all cancers, it is doubly so for prostate cancer (PCA) because of the high burden of this disease.

PCA is the most common cancer diagnosed in American men, and despite major investment in early detection, remains the second leading cause of cancer-associated mortality. The high mortality results partially from the fact that about 75% of patients diagnosed with metastatic disease die within 5 years (7). Furthermore, while the elimination of a tumor is of primary concern, even in the face of successful treatment, there is a significant probability for disrupting the quality of life for the patient, ranging from incontinence to impotence which may persist for years (14, 15). Thus the development of novel early preventive measures remains a high priority. In pursuit of developing such interventions, research and clinical efforts to date have focused on the dysfunctional activity of cancerous cells in a tumor. Only recently recognized is the contribution of otherwise normal healthy cells interfacing with a developing tumor. In this regard, neighboring fibroblasts have been suggested as a key cellular component of the PCA stroma, and their activity promotes tumor growth, invasiveness, metastasis, and angiogenesis (139-141). In fact, recent findings suggest that developing lesions directly act on nearby noncancerous stromal cells in a positive feedback loop to promote tumor development (125). A potential mechanism by which this phenomenon may be sustained is through PCA cell secretion of transforming growth factor β2 (TGFβ2), which has numerous tissue specific roles, but most relevant in this milieu, it signals the differentiation of normal fibroblasts into myofibroblasts, capable of remodeling the extracellular matrix and allowing cancer cells to infiltrate nearby tissues (136-138). Therefore, targeting TGFβ2 expression in PCA cells as well as the differentiation of normal fibroblasts into CAFs using non-toxic agents could be an attractive approach to prevent PCA progression.
Silibinin is a component of milk thistle (*Silybum marianum*; Asteraceae) seed extract and extensively used as a hepatoprotective intervention in both acute and chronic ailments (57). More recently, silibinin has shown broad spectrum efficacy against PCA. It disrupts important signaling pathways necessary for PCA progression (64, 66, 69), inhibits proliferation while inducing apoptosis in PCA cells (64, 75, 91), inhibits EMT reducing PCA cell migration, invasion, and metastasis (70, 83, 92), as well as angiogenesis (75). However, silibinin’s potential to abrogate the interactions of PCA cells with stromal cells remains to be determined. To address this possibility, we first developed *in vitro* protocols to recapitulate the activation/ transformation of naïve fibroblasts by aggressive PCA cells. For these studies, normal human prostate stromal cells (PrSCs) were used as a model for naïve fibroblasts. We generated PCA cell conditioned media capable of altering the phenotype of PrSCs into a myofibroblastic one. In the current study, this phenotype was found to be similar to that of CAF cells isolated from a resection of clinically confirmed PCA and thus was labeled a CAF-like phenotype. Parallel to this effort, we also examined if silibinin, whether through direct action on stromal cells or through indirect action altering PCA cell-conditioned media, could ameliorate this PCA-mediated differentiation of fibroblasts, and thus halt an important step for carcinogenesis. Finally, we examined the direct effect of silibinin on CAF cells in an effort to determine if fibroblasts that have already become constitutively active in an aggressive phenotype can be rescued by silibinin intervention. Herein, our findings indicate that in addition to its widely reported anti-PCA properties, silibinin also inhibits a more recently acknowledged avenue for cancer progression, namely cancer cell-mediated recruitment of stromal fibroblasts.
Materials and Methods

Cell Lines & Reagents

Human PCA PC3, DU-145, LNCaP and 22Rv1 cells were from ATCC. C4-2B cells were from ViroMed Laboratories. PCA cell lines were tested and authenticated by DNA profiling for polymorphic short tandem repeat (STR) markers at University of Colorado Cancer Center DNA Sequencing & Analysis Core. RPMI 1640 media, other cell culture materials, TGFβ1 ELISA kit, and CAS-Block were from Invitrogen. PrSCs, stromal cell growth medium (SCGM), and Bullet-kits were from Lonza. Prostate CAFs were from a prostatectomy specimen removed at Wake Forest University (142). Pathology of specimen was verified by two board-certified pathologists (AC and JS). No patient identifiers were retained and use of discarded tissue was not considered human subjects research by Wake Forest University IRB. DAPI and silibinin were from Sigma, IL-6 from Cell Signaling, and TGFβ1, TGFβ2, and goat IgG isotype control antibody were obtained from Gibco. TGFβ2 ELISA kit and TGFβ2-neutralizing antibody were obtained from R&D, antibodies to IL-6, α-SMA and FAP (fibroblast activation protein) from Abcam, antibody to vimentin and HRP conjugated streptavidin from Santa Cruz, 3,3′-diaminobenzidine (DAB) peroxidase substrate kit from Vector Labs, biotinylated antibodies and mouse IgG's from DAKO, and transwell invasion chambers from BD Biosciences.

Cell Culture & Conditioned Media

DU-145 and PC3 cells were cultured in RPMI 1640 with 10% heat inactivated FBS and 100 U/ml penicillin G and 100 μg/ml streptomycin sulfate under standard conditions. LNCaP, C4-2B, and 22Rv1 cells were cultured in RPMI 1640 with 10% FBS
and 100 U/ml penicillin G and 100 µg/ml streptomycin sulfate. PrSCs were cultured in SCGM with Bullet-kits. Prostate CAF cells were cultured in MCDB105 medium with 10% FBS and 100 U/ml penicillin G and 100 µg/ml streptomycin sulfate. Silibinin stock was in dimethyl sulfoxide (DMSO), with equal DMSO (not to exceed 0.1%, v/v) in each treatment. TGFβ1 and TGFβ2 were activated by citric acid prior to use. PCA cells were incubated in media for 72 hrs, washed twice, followed by media supplemented with 0.5% serum for 48 hrs. This media was then collected as respective cell line control conditioned media (CCM). Similarly, silibinin conditioned media (SBCM) was collected from PCA cells incubated with 30, 60 or 90 µM silibinin for 72 hrs, washed twice, followed by media supplemented with 0.5% serum (without silibinin) for 48 hrs and labeled as SBCM30, SBCM60 and SBCM90.

**Cell Viability & Immunoblotting**

PrSCs (3 x 10^4 cells per well) were seeded and treated as indicated, and then cell number and cell death determined by Trypan blue using a haemocytometer as described previously (168). Total cell lysates were prepared in non-denaturing lysis buffer and immunoblotting performed as described earlier (168). Bands were scanned with Adobe Photoshop 6.0 and the mean density of each band was analyzed by the Scion Image program.

**Confocal Imaging**

PrSCs were grown on cover slips and incubated in basal media, CCM in presence or absence of silibinin (30-90 µM doses), SBCM, TGFβ1 or TGFβ2 (1-10 ng/mL), and control or TGFβ2 neutralizing antibodies (0.4 µg/mL). Except where noted, cells were treated for 24 hrs, then fixed in 3.7% formaldehyde overnight at 4ºC, permeabilized
with 0.1% Triton X-100 for 15 min and thereafter blocking was done under 5% serum condition. Cells were washed with PBS containing 0.2% Tween and incubated with anti-α-SMA antibody and 4’,6’-Diamidino-2-phenylindole (DAPI) for 30 min. Cell images were captured at 1500x magnification on a Nikon inverted confocal microscope using 688/405 nm laser wavelengths to detect α-SMA (green) and DAPI (blue) emissions, respectively.

**Immunohistochemistry**

Paraffin-embedded PC3 tumor tissue sections from a PC3 xenograft study (76) were used to determine the *in vivo* effect of silibinin administration on the levels of TGFβ2, α-SMA, vimentin and FAP by IHC as described before (76, 169). Briefly, sections were incubated with anti-TGFβ2 (15 μg/mL dilutions), anti-α-SMA (1:75 dilutions), anti-vimentin (1:75 dilutions), and anti-FAP (1:50 dilutions) antibodies, followed by a specific biotinylated secondary antibody (1:50 dilutions), and then conjugated HRP streptavidin and DAB working solution, and counterstained with hematoxylin. Stained sections were analyzed by Zeiss Axioscope 2 microscope and images captured by AxioCam MrC5 camera at 400x magnifications. Immunoreactivity (represented by brown staining) was scored as 0+ (no staining), 1+ (weak staining), 2+ (moderate staining), 3+ (strong staining), 4+ (very strong staining).

**ELISA Assays**

For the quantification of TGFβ1, CCM as well as SBCM 30, 60, 90 were collected from PC3 cells as previously described in Methods. These samples were then processed and assayed according to the provided protocols in a TGFβ1 ELISA kit. Regression curves from known standards were used to quantify the resultant O.D.’s as
concentrations of TGFβ1. For the quantification of TGFβ2, CCM and SBCM30, 60 and 90 were collected from LNCaP, 22Rv1, C4-2B, DU-145, and PC3 cells as previously described in Methods. These samples were then processed and assayed according to manufacturer’s protocols in a TGFβ2 ELISA kit. Regression curves from known standards were used to quantify the resultant O.D.’s as concentrations of TGFβ2.

**Invasion Assay**

The invasion assay was performed using invasion chambers as per vendor’s protocol. The bottom chambers of a Transwell insert were filled with SCGM with 5% FBS and the top chambers were seeded with 50,000 PrSCs (previously treated for 24 hrs according to stated conditions) per well in SCGM (with 0.5% FBS). After 18 hrs of incubation, cells on the top surface of the membrane (non-invasive cells) were scraped with a cotton swab and cells spreading on the bottom sides of the membrane (invasive cells) were fixed, stained, and mounted. Images were captured using Cannon Power Shot A640 camera on Zeiss inverted microscope and total number of invasive cells was counted and percentage of cell invasion was calculated.

**Statistical Analysis**

Statistical analysis was performed using Graphpad Prism software. Data was analyzed using t-test, one-way or two-way ANOVA (where appropriate) followed by Newman-Keuls or Bonferroni post-hoc tests respectively, and a statistically significant difference was considered to be at P<0.05.
Results

Media Conditioned by PCA Cells has the Capacity to Induce a CAF-like Phenotype in PrSCs

Experiments were undertaken to optimize both the protocols for conditioning media with PCA cells and for using this conditioned media to potently activate PrSCs. An additional criterion of interest was the capacity to assay the inhibition of this response by silibinin. Thus, media conditioned by PCγ cells in the absence and the presence of silibinin was denoted as CCM and SBCM, respectively. Based on the work of Giannoni et al (125), reporting that IL-6 secreted by PCA cells induces a CAF-like phenotype in naïve fibroblasts, we first focused on the accumulation of IL-6 in media as a measure of potency for conditioned media. Not surprisingly, data revealed that the concentration of IL-6 in media conditioned by PC3 cells increased over time (Figure 2.1A). It was also found that at 72 hrs, silibinin had the capacity to dose-dependently decrease IL-6 levels in PC3 conditioned media. Thus, future experiments were designed for 72 hrs incubation time (with or without silibinin) to produce conditioned media. Extensive reports have delineated the inhibitory effects of silibinin directly on PCA cells (64, 66, 69, 70, 75, 83, 91, 92). Thus, silibinin treatment of PC3 cells has the potential to directly inhibit their capacity to activate PrSCs. In addition, since high serum levels found in supplemented growth media may obfuscate both the activation and the inhibition of subsequently assayed cellular responses, we elected to follow the 72 hrs incubation period with multiple washes followed by incubation of PC3 cells with low serum (0.5%) growth media. This was designed to eliminate both contaminating silibinin as well as other activating agents found in growth media.
Figure 2.1 Establishing the parameters for collecting PCA cell conditioned media and PrSC activation. (A) PC3 were treated with silibinin at the indicated concentration or DMSO vehicle for the indicated times. Media was collected, concentrated, and analyzed for IL-6 by Western blotting. (B) PC3 were treated with silibinin at the indicated concentrations or DMSO vehicle for 72 hrs, and then incubated in low serum growth media for the indicated times. Media was collected, concentrated, and analyzed for IL-6 expression. (C- D) PC3 control conditioned media (CCM) and silibinin treated-PC3 conditioned media (SBCM) were collected as detailed in the Methods. Subsequently, PrSCS were exposed to PrSC basal media (basal), CCM or SBCM and analyzed for α-SMA (C) and vimentin (D) expression after 24 hrs. 50/50, 75/25, 100/0 denotes percentage conditioned media/basal PrSC growth media, respectively. β-actin was used as a loading control. Densitometric data are listed below each band as a fold change over their respective controls, N.D. denotes not detected.

Having identified the treatment time, we next sought to identify the time required for PC3 cells to effectively condition medium for further studies. IL-6 was not detectable in fresh low serum media, but was detectable after 6 hrs of conditioning (Figure 2.1B). PC3 cells treated with silibinin (60 µM) and then washed, exhibited a
significant decrease in IL-6 levels by 48 hrs, and thus, this time was used in future studies as the time in which PC3 cells (treated or untreated with silibinin) would condition media to examine its effect on stromal cells. Having now identified the parameters by which CCM/SBCM would be generated, we next sought to identify the concentration of CCM to PrSC media that would most potently activate PrSCs. We found that 100% CCM induced the expression of the CAF-like markers, α-SMA and vimentin, in PrSCs, which, compared to CCM, was modestly reduced in SBCM-treated PrSCs (Figure 2.1C and 2.1D). Thus, 100% CCM and SBCM was used in all future experiments.

**Silibinin Directly Inhibits CCM-mediated Activation of PrSCs as well as Constitutive Activation in CAFs**

We next sought to identify if silibinin could directly inhibit PrSC activation by CCM, and found that indeed silibinin (30-90 μM) dose-dependently inhibits CCM-induced α-SMA expression in PrSCs (Figure 2.2A). Interestingly, we also found that even high levels of recombinant IL-6 could not replicate the degree of α-SMA induction which was found in CCM-treated PrSCs, but TGFβ1 could (Figure 2.2A). This is consistent with the capacity of TGFβ1 to induce differentiation and α-SMA expression in fibroblasts (170). Thus, we next attempted to replicate α-SMA expression induced by CCM with increasing concentrations of TGFβ1. This was found to roughly correspond to slightly less than 2.5 ng/mL TGFβ1 (Figure 2.2B).
Figure 2.2 Effect of silibinin on PC3 conditioned media induced transformation of PrSCs, and on constitutive and TGFβ1-induced α-SMA expression in CAFs. (A) PrSCs were exposed to basal media (basal), CCM ± silibinin (0, 30, 60, 90 μM), IL-6 (50 ng/mL), or TGFβ1 (10 ng/mL) and analyzed for α-SMA after 24 hrs. (B) PrSCs were exposed to basal media (basal), TGFβ1 at indicated concentrations, or CCM, and analyzed for α-SMA after 24 hrs. β-actin was used as a loading control. Densitometric data are listed below each band as a fold change over their respective controls. (C) PrSCs were exposed to basal media (basal), CCM + silibinin (SB, 90 μM), or TGFβ1 (10 ng/mL) + silibinin (90 μM) for 24 hrs; prostate CAF cells were exposed to CAF basal media (basal) + silibinin (90 μM) or TGFβ1 (10 ng/mL) + silibinin (90 μM) for 24 hrs and probed for α-SMA (green) with α-SMA antibody and DNA with DAPI (blue). Magnification was 1500x.

While Western blot analysis identified CCM-mediated upregulation of α-SMA expression in PrSCs, immunofluorescent analysis confirmed an increase in the organization of α-SMA in both CCM- as well as TGFβ1-treated PrSCs (Figure 2.2C). Consistent with Western analysis, silibinin abrogated both CCM- and TGFβ1-mediated activation of α-SMA to baseline levels (Figure 2.2C). A sample with no
primary antibody was used as a control for potential autofluorescence (Figure 2.2C). We further examined the direct effect of silibinin on CAFs, human prostate fibroblasts isolated from clinical resections of PCA. These prostate CAFs exhibited strong baseline expression and organization of α-SMA that was further enhanced by TGFβ1, and both constitutive and TGFβ1-induced α-SMA expression was abrogated by silibinin (Figure 2.2C).

**Silibinin Inhibits CCM-mediated Invasion in PrSCs and does not Induce Cell Death in CAFs**

Given the cellular function of α-SMA, we next sought to determine if the reduction in α-SMA expression and organization translated to a reduction in cell motility of PrSCs. To accomplish this, we performed an invasion assay. PrSCs were incubated for 24 hrs in either low serum growth media or CCM in the presence or absence of silibinin. Following incubation, cells were seeded onto invasion chambers and allowed to migrate. As shown in Figure 2.3A, the presence of CCM induced migration of PrSCs which was significantly decreased by silibinin. To confirm that this silibinin-mediated inhibition of α-SMA expression as well as PrSCs motility was not a consequence of cellular death, we performed a cell viability assay where a high dose of silibinin silibinin (90 µM) treatment was used. This resulted in only a slightly lower cell count and higher cell death as controls, but these were not found to be statistically significant (Figure 2.3B).
Figure 2.3 Effect of silibinin on PC3 conditioned media induced invasion in PrSCs, and on CAFs viability. (A) PrSCs were exposed to basal media (basal) or CCM ± silibinin (90 µM) and then seeded on Transwells after 24 hrs. PrSCs were allowed to invade through matrigel for 18 hrs and then invasive cells were counted. Data shown in bar diagram represent mean ± SEM of three samples for each group (**, p<0.01, *, p<0.05). (B) Prostate CAF cells were incubated in CAF basal media (basal) + silibinin (90 µM) or treated with TGFβ1 (10 ng/mL) + silibinin (90 µM) for 24 hrs. At the end, total cell number and dead cell percentage was determined. Data shown in bar diagram represent mean ± SEM of three samples for each group.

**TGFβ2 is a Major Component of CCM**

To more precisely determine the concentration of TGFβ1 in CCM as well as to determine if the inhibition seen in α-SMA and vimentin expression by SBCM was a consequence of a direct reduction in TGFβ1 secretion by PCA cells following silibinin treatment, we assayed conditioned media with a TGFβ1 ELISA. Surprisingly, we found that in CCM as well as in SBCM 30, 60 and 90, the expression of TGFβ1 was very low (roughly 1 pg/mL), in fact near the detection limit of the assay (Figure 2.4A). Based on the robust activation of PrSCs elicited by CCM despite the minimal levels of detected TGFβ1, we next assayed for another TGFβ isoform i.e. TGFβ2 in CCM. Here we
detected more than 4 ng/mL of TGFβ2 in CCM which was dose-dependently decreased by silibinin (Figure 2.4B). Another PCA cell line, DU-145, also secreted substantial amounts of TGFβ2, though to a lower degree as compared to PC3 cells, which was also dose-dependently decreased by silibinin (Figure 2.4B). In other PCA cell lines LNCaP, C4-2B and 22Rv1, TGFβ2 expression measured by ELISA was either undetectable or extremely low (Figure 2.4B). As a control for further studies, we also confirmed that the TGFβ2-mediated increase in the expression of α-SMA in PrSCs could be inhibited by TGFβ2 specific antibody (Figure 2.4C).

**Both Silibinin and TGFβ2 Neutralizing Antibody Inhibit TGFβ2- as well as CCM-Mediated Expression of α-SMA in PrSCs**

We next sought to confirm the role of TGFβ2 in CCM-mediated activation of PrSCs. We found that neutralizing antibody to TGFβ2 could abrogate TGFβ2-mediated upregulation of α-SMA as detected by immunofluorescence (Figure 2.5A, a-d). This effect was also found in the relatively modest activation by SBCM, as well as the more robust α-SMA activation induced in SBCM fortified with exogenous TGFβ2, which was also abrogated by TGFβ2 neutralizing antibody (Figure 2.5A, e-h). In turn, silibinin was found to inhibit both TGFβ2- and CCM-mediated upregulation of α-SMA, though not if it was applied concurrently with the activating cues (Figure 2.5A, i-p). The efficacy of the TGFβ2 neutralizing antibody was confirmed through Western blot analyses (Figure 2.5B). Time course expression of α-SMA in response to TGFB2 expression as well as silibinin inhibitory dose response were also assayed to verify immunofluorescence data.
Figure 2.4 TGFβ isotypes expression in PCA cells, and TGFβ2 role in inducing α-SMA in prostate fibroblasts. (A) TGFβ1 expression was analyzed by ELISA in CCM and SBCM 30, 60, and 90 from human PCA PCα cells as detailed in Methods. Regression curve used to quantify results is also shown. Data shown in the bar diagram represent mean ± SEM of three samples for each group. (B) TGFβ2 expression was analyzed by ELISA in CCM and SBCM 30, 60, and 90 from the listed human PCA cell lines. Regression curve used to quantify results is also shown. Data shown in bar diagram represent mean ± SEM of three samples for each group, (**, p<0.001) (C) PrSCs were incubated in the presence or the absence of TGFβ2 and TGFβ2 neutralizing antibody at indicated concentrations for 24 hrs. Thereafter, PrSCs were collected and analyzed for α-SMA expression by Western blotting. β-actin was used as a loading control. Densitometry data are listed below each band as a fold change over their respective controls.
Figure 2.5 Contribution of TGFβ2 to PC3 conditioned media-caused induction of α-SMA in PrSCs. (A) PrSCs were exposed to basal media (basal), CCM, or SBCM, in presence or absence of silibinin and/or TGFβ2 at indicated concentrations. In specific cases, 2 hrs treated PrSCs were analyzed or washed and then incubated with basal media or media supplemented with silibinin or TGFβ2 for an additional 22 hrs. After a total of 24 hrs, cells were analyzed for α-SMA either by (A) immunofluorescence or (B-C) Western blot analysis. β-actin was used as a loading control. Densitometric data are listed below each band as a fold change over their respective controls.
Silibinin Inhibits TGFβ2 Expression *In Vivo*, Corresponding to a Reduction in Activated Fibroblast Biomarkers

While promising, our studies to this point were conducted in cell culture, and it remained to be determined if silibinin' effects as well as the correlation of PCA cells with dysfunction of host tissues could be replicated in a milieu closer to a clinical setting. To this end, we sought to extend our system into an animal model. We had recently completed a study involving the injection of PC3 tumor cells into mice and thus we employed these tissues to address this next avenue of investigation; notably, we have previously reported that prolonged feeding of mice with silibinin (0.5%, w/w in diet) strongly inhibits the growth of PC3 tumors in nude mice (76).

Consistent with our model highlighting the relevance of otherwise normal tissues that happen to interface with an incipient lesion, we report that immunohistochemical staining for TGFβ2 and CAF-like biomarkers α-SMA, vimentin, and FAP exhibited strong localization to the periphery of tumor sections. Also importantly, PC3 tumor tissues from silibinin-fed mice exhibited significantly reduced TGFβ2 expression (Figure 2.6A) and this phenomenon also corroborated with significant decreases in α-SMA and vimentin whereas there was a more modest reduction in FAP expression (Figure 2.6B-D) together indicating a reduction in activation and recruitment of native fibroblasts to regions of cancerous growth.
Figure 2.6 Effect of silibinin feeding on the expression of TGFβ2 and CAF-like biomarkers in PC3 tumors. PC3 tumor tissues were analyzed by IHC to determine silibinin’s effects on the expression of (A) TGFβ2, (B) α-SMA, (C) vimentin, and (D) FAP. Immunoreactivity (represented by brown staining) of these biomarkers was scored as 0+ (no staining), 1+ (weak staining), 2+ (moderate staining), 3+ (strong staining), and 4+ (very strong staining). Data shown in bar diagram represent mean ± SEM of three to five samples for each group. SB, silibinin.
Discussion

Studies focused on elucidating the cellular dysfunction and disrupted signaling pathways involved in carcinogenesis have done much to identify and characterize novel avenues for the detection, management, and treatment of various cancers. These efforts have also enumerated a host of agents both exogenous and endogenous that lend momentum to the progression of these diseases. They have to date generally focused on the gain or loss of gene function within cancer cells that provide the tumor as a whole the means to escape growth regulation and host immunity (158-162, 164-166). The potential for contribution by tissues adjacent to the developing tumor has only recently emerged, but has gained a great deal of interest. In regards to this latter aspect, the series of reciprocal interactions in a developing cancerous lesion and the stroma surrounding it have become recognized as a major component for malignancy. This becomes more relevant with the interpretation of a tumor as a chronically persistent wound. This injury then drives inflammation, which has been well-documented as associated with cancers in general and PCA particularly (122-124). This inflammation in turn drives a runaway wound healing program, chronically recruiting and activating nearby fibroblasts, altering them into a myofibroblastic or CAF-like phenotype competent to remodel the ECM and allow for cancer cell migration, invasion, and metastasis, as well as angiogenesis (134, 139-142). This is as a consequence of the physiological properties of myofibroblasts and their primary role in wound healing. Through extensive expression and organization of α-SMA, they exhibit potent contractile properties allowing for the gross manipulation of ECM microstructure to close a wound, or in the case of a tumor, to inadvertently disrupt the ECM to facilitate cancer cell EMT (129,
This phenomenon was confirmed in our study where exposure to PCA conditioned media amplified both the expression and organization of α-SMA in what were initially human prostate fibroblasts. This translated to a marked induction of invasiveness in PrSCs as a consequence of exposure to CCM.

As a result of this chronic inflammation and the interplay of several different cell types, a host of signaling molecules have been presented as potential contributors to this effect. We first assayed for the effect of IL-6, a well-established inflammatory mediator (125), and then of TGFβ1, reported to contribute to carcinogenesis in several tumor models, including PCA (170-173). However, we conducted the majority of our work focusing on TGFβ2, on the basis of its capacity to induce α-SMA as well as analysis of conditioned media by ELISA. The specific role of TGFβ2 in mediating the effects of conditioned media on PrSCs was confirmed by specific neutralizing antibody. As mentioned, TGFβ family expression is found to be elevated in many hyperplastic disorders, among them carcinomas (136). Yet, in normal epithelial tissues, it is associated with promoting apoptosis and inhibiting proliferation. This property reverses as cancer advances, TGFβ appearing to lose the capacity to maintain homeostasis and instead promoting proliferation and EMT (136-138). This may be a consequence of interplay between TGFβ and other signaling factors within the stroma. Here we show that the addition of TGFβ (1 or 2) elicited a similar effect as that elicited by CCM, both serving to increase the expression and organization of α-SMA in PrSCs. Interestingly, PCA cells in culture expressed significantly higher amounts of TGFβ2 compared to TGFβ1, which was strongly decreased by silibinin treatment. In future studies, we will
further analyze the mechanism through which silibinin targets TGFβ expression in PCA cells.

Overall, we report here that silibinin inhibits the capacity of PCA cells to induce a CAF-like phenotype in PrSCs both by direct intervention of PrSCs but also indirectly by acting on PCA cells' capacity to secrete TGFβ2 as summarized in Figure 2.7. This direct intervention was found in cases where TGFβ2 was exogenously added as a positive control, consistent with a report where silibinin treatment inhibited trans-differentiation of human tenon fibroblasts by TGFβ1 (174), as well as in CAF cells that constitutively expressed markers for activated fibroblasts (summarized in Figure 2.6). This is further in line with extensive data supporting silibinin's potent inhibition of an array of PCA properties: altering deregulated cell signaling (64, 66, 69), inhibiting proliferation (64, 75, 91), EMT (70, 83, 92), and angiogenesis (75). Our findings lend support to the notion that in addition to these well-established features, silibinin might also target the newly characterized interaction between PCA cells and their surrounding microenvironment, critical for the development and progression of a malignant tumor and as such opens the possibility for use of this agent as more than a potential therapeutic but also a prophylaxis, preventing an incipient lesion from gaining the foothold required to expand beyond its current microenvironment. In this regard, it is important to highlight that silibinin has entered phase I/II clinical trial in PCA patients, and completed dose-escalation studies show that the concentrations of silibinin employed in our present cell culture studies are those which are physiologically achievable in the serum of PCA patients (62), which further signifies the translational potential of our findings in the present study to prevent and manage PCA clinically.
Figure 2.7 Schematic for the induction of a CAF-like phenotype by PCA cells and potential target for intervention by silibinin. Silibinin treatment inhibited PCA cells induced transformation of prostate stromal cells into a CAF-like phenotype via targeting TGFβ2 expression. Silibinin treatment also directly decreased α-SMA expression in CAF cells.
CHAPTER III
SILIBININ INHIBITS CANCER ASSOCIATED FIBROBLASTS-INDUCED GROWTH AND INVASIVENESS IN PROSTATE CANCER CELLS VIA DECREASING MONOCYTE CHEMOTACTIC PROTEIN-1 LEVEL AND IMMUNE CELLS RECRUITMENT IN THE TUMOR MICROENVIRONMENT

Introduction

Classically, the tumor microenvironment has been seen as a passive bystander in carcinogenesis; the growing tumor was viewed as the active player, progressively overcoming molecular and cellular barriers through the various stages of cancer development (99). In contrast to this traditional view, the tumor microenvironment has recently been recognized as an active participant in the development of a tumor (100, 105, 106) contributing to many of the hallmark properties of cancer such as promoting angiogenesis and proliferative signals while inhibiting apoptosis and growth suppressors, along with other dysfunctional elements (103, 104). The tumor microenvironment may in fact be a critical and necessary element to cancer progression and understanding this component would provide new targets for novel treatments necessary to address the growing burden of cancer worldwide (175-177). Within the tumor microenvironment, fibroblasts represent the most numerous cellular element

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They are normally quiescent, but can be activated in the case of an injury to differentiate into myofibroblasts. It is this last activity combined with their large number that highlights their importance as components of the tumor microenvironment. These fibroblasts can be thought of as cancer-associated cells residing in close proximity with the expanding borders of a growing tumor. Most importantly, these CAFs are irreversibly and constitutively activated and unlike the myofibroblasts operating “normally” in response to a wound, they are not subject to programmed cell death allowing for their continued activity. In fact, their presence has been associated with poor prognosis in several cancer types.

In this context, we have previously identified the capacity of PCA cells to alter the phenotype of naïve human fibroblasts into one similar to CAFs isolated from clinical resections of PCA patients. In other words, PCA cells could “educate” nearby healthy fibroblasts to acquire a CAF-phenotype. In the present study, we now turned to investigate the capacity of CAFs to alter the phenotype of PCA cells. We sought to identify if CAFs could induce PCA growth and progression as well as elucidate the molecular mechanism underlying our observations. Towards these goals, we used PCA patient’s CAFs to formulate CAF control conditioned media (labeled as CAF CCM). This provided a means to replicate the transmission of activating signals from CAFs to nearby cells, while also allowing for the specific treatment of CAFs in isolation from downstream presentation to PCA cells. In parallel, we investigated whether we could target CAFs-induced activity in PCA cells with the natural compound silibinin, which has shown broad anti-cancer efficacy in a host of cancer models, as well as specific anti-PCA properties, inhibiting growth, angiogenesis, invasion, metastasis, while promoting
apoptosis (64, 69, 75, 92, 179-183). CAFs were treated with silibinin, and these cells were also allowed to condition media which was labeled SBCM. In addition, we investigated whether silibinin could directly interfere with CAF-mediated activation of PCA cells. We also examined the potential of CAF-PCA cell activation (as well as its inhibition by silibinin) within mouse models to recreate the clinically relevant conditions of the tumor microenvironment found in an organism. This would also allow for the introduction of immune cells to our PCA tumor model. This was an important consideration as the chronic inflammation associated with dysregulated leukocyte recruitment to tumors has been linked to cancer progression (100, 101). Our results indicate that CAFs do in fact have the capacity to activate PCA cells which we found to be dependent on monocyte chemotactic protein-1 (MCP-1) and this effect could be inhibited by silibinin treatment of either CAF or PCA cells. Consistent with the defined role of MCP-1 in leukocyte recruitment, in our mouse models we identified significant immune cell recruitment and fibroblast activation which were inhibited by silibinin treatment.

**Materials & Methods**

**Reagents & Cell Culture**

All the cell lines and DMEM media were obtained from the American Type Culture Collection (Manassas, VA). RPMI1640, Keratinocyte Serum Free Medium, and other cell culture materials were from Invitrogen Corporation (Gaithersberg, MD). MCDB105 media and dehydroisoandrosterone was obtained from Sigma Aldrich (St. Louis, MO). Bovine insulin was purchased from Gemini Bio-Products (West Sacramento, CA). NuSerum was purchased from BD (Corning, NY). CAFs were isolated from clinical
samples extracted from patients with PCA and generously provided by Dr. Scott D. Cramer as described earlier (178). All other reagents were obtained in their commercially available highest purity grade.

The RWPE-1 cells were used as representatives of human prostatic epithelial cells at the earliest stage of initiation as they were isolated from healthy adult humans and simply transfected with HPV-18 papilloma virus. These cells were in turn serially exposed to N-methyl-N-nitrosurea to develop the increasingly tumorigenic WPE-1 NA-22 and WPE-1 NB-14 cell lines, representing progressing tumors (184). PC3 cells were used as they are a highly metastatic cell line derived from advanced PCA cells that have metastasized to bone (185). CAF, as a function of their direct recovery from clinical resections from PCA patients were representative of the constitutively activated fibroblasts residing near an advanced, aggressive tumor (142). All cells were cultured at 37°C in a humidified 5% CO₂ incubator. PC3 cells were cultured in RPMI1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS) and 100 U/ml penicillin G and 100 µg/ml streptomycin sulfate. CAFs were cultured in MCDB105 media supplemented with 10% FBS and 100 µg/mL gentamycin. RWPE-1, WPE-1 NA-22, and WPE-1 NB-14 cells were cultured in Keratinocyte Serum Free Medium + Bullet Kit Supplement. TRAMPC1 cells were cultured in high glucose DMEM supplemented with 5% FBS and 5% Nu-Serum along with bovine insulin (0.005 mg/mL) and dehydroisoandrosterone (10 nM). Silibinin stock solution was prepared in DMSO. An equal amount of DMSO (vehicle) was present in each treatment, including control; DMSO concentration did not exceed 0.1% (v/v) in any treatment.
**Conditioned Media**

Control conditioned media (CCM) and silibinin-treatment conditioned media (SBCM) was formulated by incubating CAFs in MCDB105 media (10% FBS) with DMSO vehicle or silibinin (90 µM) respectively. After 72 hrs, this media was removed and cells washed twice with PBS. Cells were then incubated for 48 hrs in low serum MCDB105 media (0.5% FBS). This conditioned media was then collected and labelled ‘CCM’ or ‘SBCM’ based on initial treatment. In each case, cells were trypsinized, collected, and counted to normalize conditioned media volume against cell number.

**Immunofluorescence Confocal Microscopy**

Cells were grown on cover slips and incubated in basal media or CCM in the presence or absence of silibinin (50 µM) or SBCM. Cells were treated for 24 hrs, then fixed in 3.7% formaldehyde for 30 min and thereafter blocking was done under 5% serum condition. Cells were then incubated with anti-E-cadherin primary antibody (3195S, Cell Signaling, Danvers, MA) in 0.1% Triton X-100 overnight at 4°C and then washed with PBS (3x). Cells were then incubated with Alexa Fluor 555-tagged secondary antibody (red) from Molecular Probes (A21422, Eugene, OR) along with DAPI (blue) for 1 hr. Cell images were captured at 600x magnification on a Nikon inverted confocal microscope using 561/405 nm laser wavelengths to detect E-cadherin (red) and DAPI (blue) emissions, respectively. Fluorescence intensity was quantified using Image J software.
**Invasion Assay**

Invasion assay was performed using Trans-well chambers from BD (Corning, NY) as per vendor’s protocol. Briefly, the bottom chambers were filled with CCM, SBCM, or media with 0.5% FBS where indicated, and the top chambers were seeded with 100,000 cells (WPE-1 NA-22 and WPE-1 NB-14) or 25,000 cells (PC3) per well in Keratinocyte or RPMI media (with 0.5% FBS) respectively. After 18 hrs of incubation, cells on the top surface of the membrane (non-invasive cells) were removed with a cotton swab and the cells spreading on the bottom sides of the membrane (invasive cells) were fixed, stained, and mounted. Images were captured using a Cannon Power Shot A640 camera on a Zeiss inverted microscope and the total number of invasive cells was counted.

**Clonogenic Assay**

Cells were cultured on 6-well plates (5×10² per well). Every 48 hrs, fresh media and appropriate treatment was added as indicated. At the end of the 8\textsuperscript{th} day, cells were washed twice with ice cold PBS, fixed with a mixture of methanol and glacial acetic acid (3:1) for 10 minutes and then stained with 1% crystal violet for 15 minutes followed by washing with deionized water. For quantification purposes, the entire well was analyzed and colonies with more than 50 cells were scored and counted on a Zeiss inverted microscope. Photomicrographs were captured using a Canon Power Shot digital camera.
**Cytokine, Chemokine & ELISA Assays**

CCM and SBCM were analyzed by cytokine array and MCP-1 ELISA (both from R&D, Minneapolis, MN) per vendor’s protocols. Autoradiography films from the cytokine/chemokine array were visualized with ECL solution for the indicated times. For the MCP-1 ELISA, regression curves from known standards were used to quantify the resultant O.D.’s as concentrations of MCP-1.

**Real-Time PCR**

CAFs were incubated with vehicle control (DMSO) or silibinin (90 µM) for the indicated times and cells were trypsinized, collected as a pellet, and lysed with Buffer RLT provided with the RNasey Mini kit (Qiagen, Germantown, MD). Total RNA was then isolated per vendor’s protocols, with concentration and integrity confirmed by Nanodrop. Genomic DNA was then eliminated and 1st strand complementary DNA (cDNA) prepared using RT-PCR 1st strand kit (Qiagen, Germantown, MD). Human MCP-1 primers (Qiagen, Germantown, MD): Forward primer (5’-aagatctcagtgcagaggctcg-)’, Reverse primer (5’-ttgcttgtccaggtggtccat-’), and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers: Forward (5’-CCCCTGGCCAAGGTAC-’), Reverse primer (5’-ACAGCCTTGGCAGGCAG-’) were used for semi-quantitative RT-PCR analysis. PCR mix was prepared with RT² SYBR Green qPCR Mastermix (Qiagen, Germantown, MD) per vendor’s protocols. Amplification parameters were: initial denaturation for 10 min at 90°C; 40 cycles of denaturation for 1 min at 90°C, annealing for 15s at 60°C and extension for 30 s at 72°C; and final extension for 5 min at 60°C. The relative quantification of gene expression between control and silibinin treated
samples was achieved by normalization against endogenous GAPDH using the ΔΔC\text{T} method with data analyzed using manufacturer provided software.

**Electrophoretic Mobility Shift & Supershift Assay**

CAF cells were incubated with DMSO or silibinin (90 µM) for the indicated times, lysed, and nuclear lysates isolated. These were then processed as previously described (186). Briefly, NF-κB and activator protein-1 (AP-1) specific oligonucleotides (3.5 pmol) were end-labeled with γ\text{-}^{32}\text{P-ATP (3000 Ci/mmol at 10 mCi/ml) using T4 polynucleotide kinase in 10x kinase buffer per vendor’s protocols (Promega, Madison, WI). Labeled double-stranded oligo probe was separated from free γ\text{-}^{32}\text{P ATP using a G-25 Sephadex column. The consensus sequences of the oligonucleotides used were: 5’-AGT TGA GGG GAC TTT CCC AGG C-3’ and 3’-TCA ACT CCC CTG AAA GGG TCC G-5’ for NF-κB; 5’-CGC TTG ATG AGT CAG CCG GAA-3’ and 3’-GCG AAC TAC TCA GTC GGC CTT-5’ for AP-1. To begin, 8 µg protein from nuclear extracts was incubated with 5x gel shift binding buffer [20% glycerol, 5 mM MgCl}_2, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 50 mM Tris–HCl and 0.25 mg/ml poly (dI-dC) poly (dI-dC)] and then with \textsuperscript{32}P-end-labeled consensus oligonucleotide for 20 min at 37°C. DNA–protein complexes thus formed were resolved on 6% DNA retardation gels (Invitrogen, Gaithersburg, MD). For the supershift assay, samples were incubated with anti-p65 (sc-109), anti-p50 (sc-1191), anti-c-Jun (sc-1694), or anti-c-Fos (sc-52) antibody all from Santa Cruz Biotechnology (Dallas, TX) before the addition of \textsuperscript{32}P end-labeled NF-κB or AP-1 oligo. DNA-protein or DNA-protein-antibody complexes thus formed were resolved on 6% DNA retardation gels. To check the
specificity of DNA binding, labeled probe sample was also run together with other samples. In each case, the gel was dried and bands were visualized by autoradiography.

**TRAMPC1 Allograft**

Animal care and treatments were in accordance with approved Institutional guidelines and IACUC approved protocol. Male C57Bl/6 mice were injected with $2.5 \times 10^6$ TRAMPC1 cells subcutaneously on each flank in media and Matrigel in a 1:1 ratio. Mice were orally gavaged six times a week (once daily) with silibinin (200 mg/kg body weight) or vehicle carboxymethylcellulose (CMC, 0.5% w/v). Animals were permitted free access to food and water. Food consumption and animal body weight were recorded weekly, and animals were monitored daily for general health. Once tumor allograft growth commenced, tumor sizes were measured twice weekly using digital caliper and tumor volume was calculated by the formula: $0.5236 L_1(L_2)^2$, where $L_1$ is long diameter, and $L_2$ is short diameter. At the end, mice were sacrificed, tumors collected and analyzed.

**Immunohistochemistry**

Paraffin-embedded TRAMPC1 allograft tissue sections as well as archived TRAMP PCA tissue sections from our earlier completed study (83) were incubated with anti-MCP-1 (ab25124, 10 µg/mL), anti-F4/80 (ab6640, 1:75), anti-CD3 (ab16669, 1:75), and anti-lymphocyte antigen 6 complex, locus G (Ly6g) (ab25377, 1:50) antibodies. In addition, TRAMPC1 allograft tissue sections were also incubated with anti-osteocalcin (sc-30044, 1:100), anti-collagen I (ab34710, 5 µg/mL), anti-vimentin (sc-7557, 1:100), anti-fibroblast activated protein (ab53066, 1:100), anti-α-SMA (ab7817, 1:50), and anti-TGFβ2 (AF-302-NA, 10 µg/mL). All antibodies were obtained from Abcam.
(Cambridge, MA), except anti-osteocalcin and anti-vimentin from Santa Cruz Biotechnology (Dallas, TX) and anti-TGFβ2 from R&D (Minneapolis, MN). This was followed by incubation with appropriate biotinylated secondary antibody (Dako, Carpinteria, CA, 1:250 dilutions), and then conjugated HRP streptavidin and DAB working solution, and counterstained with hematoxylin. Stained sections were analyzed by Zeiss Axioskope 2 microscope and images captured by AxioCam MrC5 camera at 400x magnifications. Immunoreactivity (represented by brown staining) was scored as 0+ (no staining), 1+ (weak staining), 2+ (moderate staining), 3+ (strong staining), 4+ (very strong staining).

**Statistical Analysis**

Statistical analysis was performed using Graphpad Prism software. Data was analyzed using t-test, one-way or two-way ANOVA (where appropriate) followed by Newman-Keuls or Bonferroni post-hoc tests respectively, and a statistically significant difference was considered to be at p<0.05.

**Results**

**Silibinin Treatment Reversed CAF CCM-mediated Reduction in E-cadherin Expression in Prostate Epithelial and PCA Cells**

The presence of CAFs has been implicated in poor prognosis for patients of several types of cancer (128, 145, 146). Thus, we sought to identify whether CAFs play a direct role in PCA progression by inducing an invasive or aggressive phenotype in prostate epithelial and PCA cells. In parallel, we sought to identify the capacity of silibinin to target any CAF-induced activities in these cells. To accomplish these dual goals, we created a system by which CAFs would condition media which could then be applied to
prostate epithelial or PCA cells. For the purposes of these studies, media conditioned with CAF in the absence and presence of silibinin will be denoted simply as control conditioned media (CCM) and silibinin conditioned media (SBCM), respectively.

Our initial probe for invasive potential was the quantification of E-cadherin expression by immunofluorescence. E-cadherin, is a well-established marker for epithelial cells (187), and conversely, useful as an inverse measure of their mesenchymal nature (which can also be directly characterized by assaying mesenchymal markers such as N-cadherin, fibronectin, and vimentin). We found that exposure to CCM for 24 hrs reduced E-cadherin expression by 40% (p<0.05) as compared to basal controls in the minimally transformed prostatic epithelial cell line RWPE-1 (Figure 3.1A), by 50% in the transformed and tumorigenic cell line WPE-1 NB-14 (p<0.05) (Figure 3.1B), and by 21% in the PCA cell line PC3 (p<0.05) (Figure 3.1C), suggesting CAFs support aggressive phenotypes in prostate/PCA cells. Addition of silibinin (CCM + SB, 50 μM) was able to at least partially recover this decrease in E-cadherin expression, significantly (p<0.05) increasing E-cadherin levels over CCM treatment in all three cell types (Figure 3.1A-3.1C). SBCM treatment, in turn, elicited a less dramatic reduction in E-cadherin expression as compared to CCM in RWPE-1 and NB-14 cells, but not in PC3 cells (Figure 3.1A-3.1C). Both these findings support the notion that silibinin can inhibit the capacity of CAFs to induce a more mesenchymal phenotype on prostate/PC3 cells.
Figure 3.1 Characterizing the capacity of CCM to reduce E-cadherin expression. (A) RPWE-1, (B) WPE-1 NB-14, and (C) PC3 cells were incubated in basal media, CAF CCM or SBCM in the presence or absence of silibinin (50 µM). After 24 hrs, cells were analyzed for E-cadherin level (red) by immunofluorescence. Nuclei were stained with DAPI (blue). Immunofluorescence was quantified and normalized against cell number with data representing mean ± SEM for ten randomly selected fields (*, ** p<0.05 from basal and CCM respectively).
Silibinin Treatment Reversed CAF CCM-mediated Increased Invasiveness and Clonogenicity in Prostate Epithelial and PCA Cells

To investigate whether these changes in E-cadherin levels corresponded with functional changes in invasive phenotype, we next performed an invasion assay. Basal media, CCM or SBCM were placed in the bottom wells of an invasion chamber. PCA cells were seeded in the top chamber and the cells allowed to invade through a matrigel layer. CCM elicited markedly increased invasiveness in all the observed cell lines (2.5-fold, 2.8-fold, and 11.2-fold in WPE-1 NA-22, WPE-1 NA14 and PC3 cells, respectively) over basal controls, which was significantly (p<0.05) reduced in SBCM treated cells (Figure 3.2A) confirming that CAFs can induce a significant invasive potential in PCA cells which can be inhibited by silibinin. Clonogenicity is another measure of PCA aggressiveness. To identify whether CAFs can enhance colony formation, we performed a clonogenic assay wherein cells were seeded at very low density (~55 cells/cm²) and grown for 8 days in basal media, SBCM or CCM in the presence or absence of silibinin (50 μM). To prevent nutrient depletion, media was changed every two days along with fresh treatment. We found that CCM significantly (p<0.05) elevated clone formation over basal controls in RWPE-1 by 26% (Figure 3.2B) and WPE-1 NB-14 cells by 41% (Figure 3.2C) but not in PC3 cells (Figure 3.2D). PC3 cells as a model of aggressive PCA might be expected to be a saturating level of colony formation that is insensitive to further activation. SBCM elicited colony formation roughly equivalent to basal controls, and colony formation was completely inhibited by direct application of silibinin. Thus, silibinin can act both indirectly on colony formation by inhibiting CAF-mediated clonogenicity as well as directly by inhibiting proliferation.
Figure 3.2 Investigating the capacity of CAF CCM to increase the invasiveness and clonogenicity of PCA cells. (A) Basal media, CAF CCM or SBCM were placed in the bottom wells of Transwell invasion chambers, and WPE-1 NA-22, WPE-1 NB-14, and PC3 cells were seeded onto the top chamber. Cells were allowed to invade for 18 hrs and then fixed and imaged. Cells were quantified across five randomly selected fields at 400x magnification with three replicates (*, ** p<0.05 from basal and CCM respectively). (B) RWPE-1, (C) WPE-1 NB-14, and (D) PC3 cells were incubated in basal media, CAF CCM, SBCM or CCM with silibinin (50 µM) for 8 days. Cells were then fixed and imaged for colony formation. Colonies consisting of more than 50 cells were counted. Data shown in bar diagram represent mean ± SEM of three samples for each group, (* and **, p<0.05).
Silibinin Treatment Inhibited CAF CCM-Induced PCA Invasiveness by Targeting MCP-1

Having identified the capacity of CAFs to promote invasion and clone formation, we next sought to identify what factors secreted by these cells could be responsible for these observed changes in PCA cells phenotype. We also sought to identify if the inhibitory effect of silibinin was a result of inhibiting secretion of these molecular agents. Thus, we collected CCM and SBCM from CAFs and performed a cytokine/chemokine array. We found that CAFs potently secreted MCP-1 far in excess of other molecules (Figure 3.3A, CCM panels), which was followed distantly by chemokine (C-X-C motif) ligand 1 (CXCL1) and macrophage migration inhibitory factor (MIF-1). Expression for all three molecules was reduced with treatment of CAFs by silibinin (Figure 3.3A, SBCM panels). The fold difference in signal between CCM and SBCM of these and other detected molecules were tabulated in Figure 3.3B showing a nearly 12-fold change in signal for MCP-1 between CCM and SBCM, along with a more than 3-fold change in CXCL1 and MIF. Other less expressed molecules that were decreased by silibinin treatment are C5/C5a, IL-1ra, and IL-13 (Figure 3.3B). Some of the detected molecules that increased following silibinin treatment were CD154, granulocyte macrophage colony stimulating factor (GM-CSF), and IL-6 (Figure 3.3B). Given its high expression, MCP-1 was further quantified by ELISA revealing that CCM contained ~3 ng of MCP-1 and SBCM ~1 ng (Figure 3.3C).
Based on these results, we elected to focus on the contribution of MCP-1 to the observed differences in phenotype between CCM and SBCM treated PCA cells, which might be related to its capacity to induce chemotaxis in immune cells through the activation of G-protein-coupled receptors (in this case CCR2 principally) marshaling adhesion molecules and glycosaminoglycans (188).

Figure 3.3 Characterizing the role of MCP-1 in CAF CCM mediated PCA cell invasiveness. Figure continued on next page.
To specifically confirm the contribution of MCP-1, we again performed an invasion assay using PC3 cells. Consistent with previous results, we found a marked induction of invasion in the presence of CCM over basal controls (Figure 3.3D). In addition, basal media supplemented with exogenous MCP-1 to levels found in our MCP-1 ELISA of CCM (3 ng) elicited almost as much invasion in PC3 cells as CCM itself. SBCM, consistent with its lower levels of MCP-1 (1 ng) elicited lower levels of invasion, and
consistent with our previous finding, basal media supplemented with similar levels of MCP-1 (1 ng) as SBCM elicited almost as much invasion. Importantly, SBCM supplemented with exogenous MCP-1 to the level of CCM (1 ng from SBCM, 2 ng exogenously added, 3ng total) induced almost as much invasiveness as CCM. To further confirm the role of MCP-1, we performed our invasion assay with the addition of specific neutralizing antibodies to MCP-1 (Figure 3.3E). We report a significant reduction (p<0.05) in PC3 invasion upon addition of these antibodies confirming the role of MCP-1 in CAF mediated invasion (Figure 3.3E). Furthermore, addition of silibinin (50 μM) directly, nearly completely abrogated PC3 invasion, confirming silibinin’s capacity to inhibit PCA response to CAF-mediated invasion.

**Silibinin Treatment Reduced MCP-1 Transcription via Inhibiting the DNA-binding of NF-κB and AP-1 Transcription Factors**

Based on these findings, we sought to elucidate the mechanism by which silibinin inhibited CAF secretion of MCP-1. We first sought to identify if the reduced concentration of MCP-1 found in SBCM vs CCM was a direct result of reduced synthesis of MCP-1 by silibinin treatment. We observed a 2-fold (p<0.05) decrease in MCP-1 mRNA in silibinin treated CAF cells vs control by 12 hrs which reached a maximum of a 4-fold reduction by 48 hrs (Figure 3.4A). To further characterize the mechanism of silibinin-mediated inhibition of MCP-1 expression, we next analyzed the signaling in CAFs upstream of MCP-1 transcription. Accordingly, we collected nuclear lysates of control and silibinin treated CAF cells and analyzed them by electrophoretic mobility shift assay (EMSA) and supershift assay for NF-κB and AP-1, known transcription regulators of MCP-1 (189-191). EMSA is intended to reveal protein
binding to radiolabeled oligonucleotides specific for the given nuclear transcription factors. Binding is detected as a retardation of the progress of these oligonucleotides through an electrophoretic gel as the protein/DNA complex is larger than free probe and thus travels slower. Here, our EMSA assay revealed a notable reduction in nuclear NF-κB and AP-1 in silibinin treated (90 μM) CAF vs control (Figure 3.4B and 3.4C). For the supershift assay, specific antibodies for the indicated transcription factors were added along with specific radiolabeled oligonucleotides to prior to electrophoretic separation. These would form a complex with recognized molecules of interest contained in the nuclear lysates and further retard their progress through the gel (forming an antibody/protein/DNA complex) creating the namesake supershift. Our supershift assay reveals that p50 and c-jun were much more significant components of the total NF-κB and AP-1 signals, respectively (Figure 3.4D). The significance of this particular combination of molecules is not clear as most reports indicate the putative p50/p65 heterodimer as critical for MCP-1 gene activation, though the contribution of c-jun has been well documented (189, 192, 193).

**Silibinin Feeding Reduced TRAMPC1 Allograft Growth via Decreasing MCP-1, CAFs Activation, and Immune Cells Recruitment**

Spurred by these findings, we elected to examine this model of PCA progression *in vivo*, taking prostatic tumor TRAMPC1 cells derived from TRAMP mice and injecting them into each flank of syngeneic C57Bl/6 mice. Importantly, this would provide an immune competent background on which to further investigate the contribution of MCP-1 on immune cell recruitment as well as the effect of silibinin on this interaction. Mice were treated by oral gavage 6 times a week with vehicle control (CMC) or silibinin
(200 mg/kg). We found that at the end of the study both tumor volume and mass were significantly (p<0.05) reduced by silibinin treatment over control (55% and 68% respectively) (Figure 3.5A-3.5B). Tumor growth and silibinin treatment had no effect on mouse weight throughout the study (Figure 3.5C). Allografts were then collected and processed by IHC (Figure 3.5D). Consistent with our in vitro data, MCP-1 accumulation was significantly (p<0.05) reduced as compared to control, and consistent with its normally ascribed immune function (188), this corresponded to a reduction in infiltration by macrophages (F4/80). In addition, we also noted a significant (p<0.05) reduction in neutrophils (Ly6g), but not T-cells (CD3) in silibinin treated mice as compared to controls, which is also consistent with data shown in Figure 3.3B revealing a marked reduction in SBCM of CXCL1, a potent neutrophil chemokine (194). Interestingly, upon sacrifice and tumor collection, we found that these tumors were quite hard, perhaps a sign of fibrosis or even bone formation. Thus, we analyzed them for the presence of collagen I, and the bone precursor molecule, osteocalcin. We noted an extensive amount of both in tumors of control mice that was significantly (p<0.05) reduced in silibinin treated mice, suggesting decreased CAF and osteoblast activity. To further confirm the presence of cancer associated fibroblasts we assayed for the presence of CAF markers, α-SMA, FAP, and vimentin, as well as TGFβ2 a molecule we previously investigated as an activator of naïve human fibroblasts (178). We found extensive amounts of all four molecules in tumors from control mice, which were significantly (p<0.05) reduced in silibinin treated mice.
Figure 3.4 Silibinin inhibits MCP-1 transcript level as well as DNA binding of transcriptional regulators of MCP-1 in CAFs. CAFs were incubated in basal media in the presence or absence of silibinin (90 µM) for the indicated times. (A) At the end of each time-point cells were collected and RNA isolated. Real-time PCR was performed to measure silibinin effect on MCP-1 mRNA as detailed in Methods. Data was normalized against GAPDH. (**, p<0.01) (B-D) Nuclear lysates were collected and analyzed by EMSA and supershift assay to define silibinin (90 µM) effect on MCP-1 transcriptional regulators NF-κB and AP-1 after 24-72 hrs of treatment.
Figure 3.5 Silibinin feeding inhibits TRAMPC1 allograft growth in C57Bl/6 mice via targeting immune cells recruitment, bone precursor molecules and activated fibroblast markers.
Figure 3.5 Silibinin feeding inhibits TRAMPC1 allograft growth in C57Bl/6 mice via targeting immune cells recruitment, bone precursor molecules and activated fibroblast markers. Male C57Bl/6 mice were injected subcutaneously with 2.5 x 10^6 million TRAMPC1 cells in each flank and treated six times a week (once daily) with silibinin (200 mg/kg body weight) or vehicle (0.5% CMC) for 66 days. (A) Tumor volume was measured as described in Methods. (B) Tumor mass was determined upon mouse sacrifice. (C) Mouse weights were measured weekly (*, p>0.05; **, p>0.01). (D) Allografts were collected, sectioned, and analyzed by IHC for MCP-1, F4/80, Ly6g, CD3, Osteocalcin, Collagen I, α-SMA, FAP, Vimentin, and TGFβ-2. Data shown in bar diagrams represent mean ± SEM of immunoreactivity scores for ten randomly selected fields from three samples for each group.

Silibinin Feeding of TRAMP Mice Inhibited MCP-1 Expression in Prostate Tumors Which Corresponded to a Reduction in Immune Cell Infiltration

To further confirm our findings in a tumor system that closely follows clinical presentation of PCA in humans, we assayed archived samples taken from a previously completed study (83) performed in the transgenic adenocarcinoma of the mouse prostate (TRAMP) mouse model. This model is both immune competent and most importantly spontaneously forms prostate tumors unlike most mouse models. In this study, TRAMP mice (at age 12 weeks) were given control or 1% silibinin supplemented diet for 8 weeks whereupon they were sacrificed at 20 weeks of age. Prostate tumors from TRAMP mice were collected and analyzed. Silibinin fed TRAMP mice exhibited significant less pathological score and disease advancement (in terms of PIN, adenocarcinoma and metastasis) compared to control which corresponded to a reduction in biomarkers for proliferation, angiogenesis and invasion (31). In the present study, TRAMP prostate tissues were further analyzed by IHC for MCP-1 and immune cells biomarkers. Consistent with our other results, silibinin treatment significantly reduced the MCP-1 expression (Figure 3.6A), which was associated with a reduction in F4/80 (Figure 3.6B) and Ly6g (Figure 3.6C) but no significant change in CD3 expression (Figure 3.6D).
Figure 3.6 Effect of silibinin feeding on MCP-1 expression and immune cell recruitment to sites of PCA in TRAMP mice. Prostate tissues from control or silibinin fed TRAMP mice were analyzed by IHC for (A) MCP-1, (B) F4/80, (C) Ly6g and (D) CD3. Data shown in bar diagrams represent mean ± SEM of immunoreactivity scores for ten randomly selected fields from three samples for each group.
Discussion

The tumor microenvironment plays a central role at each stage of carcinogenesis. Both the stromal fibroblasts surrounding a tumor and the immune cells infiltrating a tumor might be expected to act as tumor suppressors. Stromal fibroblasts encapsulate and physically constrain the growth of a tumor while leukocytes maintain immune surveillance, eliminating or stunting the growing tumor (101). There appears to be a stage where these previously tumor suppressive elements transition into becoming tumor permissive, and even tumor supportive. The development of CAF has been recently recognized as an indicator of poor prognosis in cancer patients (128, 145, 146), and efforts have been made to characterize the actions and mechanisms through which they support tumor progression. Likewise, the contribution of an inflammatory background associated with chronic leukocyte recruitment to tumor progression has been well established (100, 101). Therefore, these cellular components of the tumor microenvironment offer novel opportunities to prevent and/or treat cancer.

In a previous study, we identified the capacity of PCA cells to induce a CAF-like phenotype in naïve fibroblasts (178). In the present study, we found that CAFs could increase the clonogenicity and invasiveness of PCA cells. This comes together as a cycle whereby PCA cells recruit/induce CAFs to support PCA invasiveness, which promotes their expansion into new tissues where they can again recruit/induce more CAFs, ultimately increasing the pool of PCA cells that escape cellular barriers to begin metastasis, a critical stage in cancer related mortality. In addition, we identified that MCP-1 is the principal agent driving this response, though the collective contributions of other chemokines such as CXCL1 and MIF that were also detected in CAF CCM
cannot be discounted. In fact, while MCP-1 neutralizing antibody dramatically inhibited CCM/MCP-1 mediated invasion in PC3 cells, it failed to completely abrogate this effect providing some evidence for such a contribution. Still, a critical role for MCP-1 can be identified based on its shown effects, its relatively high concentration compared to other assayed chemokines, as well as reports from earlier studies showing that that MCP-1 supports PCA growth at least partially through phosphoinositide-3-kinase activity (which is also relevant to invasiveness) (195, 196). Consistent with the typically assigned role of MCP-1 and other chemokines, we found that immune cells were recruited to both TRAMPC1 allografts in C57Bl/6 mice as well as prostate tumors in TRAMP mice. Interestingly, in our previous study we found that PCA cells secreted TGFβ2. TGFβ isoforms have typically been identified as tumor suppressive. However, in a situation similar to that of cancer associated immune cells and fibroblasts described previously, TGFβ becomes adapted by aggressive cancer cells to instead induce tumor cell EMT (110). Additionally, and in light of the present work perhaps most importantly, TGFβ also serves to inhibit immune surveillance by NK and CTL cells (121). This then implies a multifaceted activity whereby PCA cells secrete TGFβ isotypes to recruit/induce CAFs, that in turn secrete MCP-1 and other chemokines to recruit immune cells, after which resident TGFβ inhibits CTL and NK activity while allowing for other immune cell mediated activities (particularly the establishment of a chronic inflammatory background along with immunosuppression) that have been reported to support tumor cell proliferation and metastasis while reducing apoptosis (103).

In addition to these findings, we report that application of the flavanolignan, silibinin, inhibited CAF-mediated promotion of invasiveness in PCA cells as well as
inhibited tumor growth, fibroblast activation, and immune cell recruitment in vivo. Our results were consistent with silibinin’s established effects directly inhibiting EMT, migration, and invasion in PCA cells (70, 83, 92) as well as actions related to the tumor microenvironment such as inhibiting angiogenesis (75). In this context, we had previously investigated the ability of silibinin to target PCA-mediated alteration of naïve human prostate fibroblasts into a CAF-like phenotype, finding that silibinin could indeed target this early dysfunction of the cellular microenvironment brought on by PCA cells (178). This promotes a broad role for silibinin regarding the prostate tumor microenvironment. It can target the upstream event of an incipient lesion recruiting fibroblasts to drive further dysfunction. It can inhibit the downstream event of these now constitutively activated fibroblasts acting to promote PCA cell invasion. It can also prevent CAFs ability to promote immune cell infiltration, while also inhibiting tumor angiogenesis. These effects are in addition to silibinin’s well-documented ability to directly rescue dysfunctional cell signaling in PCA (64, 66, 69), reducing proliferation and promoting apoptosis (64, 75, 91). These results together point to the potential of silibinin to serve as a novel agent in PCA management via targeting cancer cells, tumor microenvironment components, as well as interactions between the two.

PCA remains a pressing issue, for in spite of notable investment and success in improving early detection, it remains the second leading cause for cancer-related mortality among American men (197). Additionally, autopsy studies reveal a significant incidence of undetected PCA in patients (8) and notable amounts in patients much younger than typically expected, suggesting these diagnostic efforts may still fall short of completely effective screening. Together, these data highlight two related needs: the
development of novel agents targeting aspects of the disease that may currently be unaddressed and the development of agents that can serve as long-term prophylaxis prior to any specific diagnosis of PCA. Importantly, silibinin is a natural product, taken for millennia as the main active ingredient in milk thistle for treatment of several disorders. As such, it has been shown to be well-tolerated and of low toxicity, preferable properties for any long-term intervention, and perhaps even making silibinin suitable for use prior to diagnosis of PCA. To that end, and in light of silibinin’s ability to target both PCA cells directly as well as to inhibit the dysfunctional activities within the tumor microenvironment that have been identified as critical for the support and progression of a tumor, silibinin has been investigated in phase I/II clinical trials in PCA patients (62, 63). Herein, dose-escalation studies confirm that the concentrations of silibinin used in our cell culture studies were physiologically achievable in the plasma of PCA patients (62).

For the purposes of the present study, there remains a concern that silibinin treatment reduced the size of tumors, and thus any observed changes in marker levels might be related to changes in tumor volume rather than specific inhibition of CAF activity and/or immune cells recruitment. As a consequence, an experiment where tumors are collected at sequential times (and thus predicted to be of increasing sizes) might be needed to address this concern. Alternatively, tumors could be allowed to grow to a certain size and then silibinin treatment could be initiated. Further studies will also be needed to specifically address the contribution of MCP-1 in vivo, particularly in regards to tumor growth and immune cell recruitment. These will involve the use of MCP-1 KO mice and syngeneic mouse CAF and TRAMPC1 cells. These mice provide a background
incapable of secreting MCP-1 whereas injected CAFs would be expected to provide this agent with the TRAMPC1 cells being the tumorigenic cell line used in the present study. Taken together, we identified a role for CAFs in mediating PCA dysfunction through the secretion of MCP-1 and identified the capacity of silibinin to inhibit this phenomenon, both as a direct action on CAF secretion of MCP-1 as well as directly inhibiting PCA activity in response to exposure to CAF conditioned media.
CHAPTER IV

SUMMARY

Prostate cancer remains the second leading cause of cancer-related mortality among American men despite massive investment and notable success in improving early detection (197). This situation might be expected to worsen with the US population expected to grow older and age being a major risk factor for PCA. In addition, autopsy and comprehensive biopsy studies reveal that this success in improving PCA diagnosis may not fully screen for PCA as it can still be found in a significant fraction of men with no prior diagnosis of PCA (8). This is especially troubling as PCA that has not been successfully detected at an early stage may advance to metastasis where patient survival drops significantly. This highlights the need for the development of novel agents to treat PCA, particularly with the ability to be administered as long-term prophylaxis prior to any specific diagnosis of PCA. Numerous agents have been investigated to fulfill these goals based on their historical medicinal use, correlation with reduced risk in population studies, their unique chemical properties, or their role in biological systems. One such compound that has been investigated for its potential to be developed into an effective long-term PCA intervention is the natural compound, silibinin. This agent has been used for millennia as a natural remedy for several ailments and more recently used as a clinically prescribed hepatoprotective agent. Thus, it has significant evidence for being well-tolerated with low or no toxicity. Relevant to PCA intervention, silibinin has been shown to inhibit several hallmark characteristics of cancer development such as uncontrolled proliferation, invasion and metastasis, induced angiogenesis, and evasion of apoptosis (64, 66, 69, 70, 75, 83, 91, 92). However, the
critical involvement of normal tissues and cells in close contact with a developing tumor (collectively referred to as the tumor microenvironment) has recently been recognized. In fact, these interactions have been reported to support cancer cell migration, invasion, and metastasis, as well as angiogenesis (134, 139-142). While these components might at first work to restrain a growing tumor, their activity becomes adapted to support PCA growth and progression (101). The TME has many components, but of chief importance are the stromal fibroblasts that represent the most numerous cell type in the TME, as well as recruited immune cells. Both of these are associated with cancer progression and worse prognosis (100, 101, 128, 145, 146). In regards to fibroblasts, this is likely a consequence of the role of activated fibroblasts in wound healing and the recognition of a developing tumor as a source of inflammation that cannot be resolved. Furthermore the ECM and assorted secreted molecules conduct signals between all these elements.

To analyze this relationship and assay for the capacity of silibin in to target this complex set of cellular relationships, we developed a cell culture model wherein PCA cells could condition media by secreting various factors into growth media. This conditioned media was then presented to naïve human fibroblasts to identify its effect on these cells as well as to identify any role for silibinin in inhibiting this interaction. This process was split into two parts to allow for the specific treatment of PCA cells separate from downstream exposure to naïve PrSCs. In this context we identified that PCA CCM could activate naïve PrSC into a phenotype reminiscent of the constitutively activated phenotype found in cancer associated fibroblasts, increasing expression and organization of the myofibroblast marker α-SMA. We found this effect to be dependent on TGFβ2 secretion. These CAFs were isolated from clinical resections from PCA
patients and were representative of fibroblasts that are transformed following prolonged exposure to a developing tumor. In parallel, we found that treatment with silibinin could inhibit the capacity of PCA cells to activate downstream PrSCs both by indirect action in decreasing PCA cell secretion of TGFβ2 as well as by direct action on PrSCs inhibiting their response to TGFβ2. This effect was replicated in vivo in samples taken from a previously completed study (76) where PC3 cells were injected into nude mice and it was found that silibinin feeding reduced markers for fibroblast activation as well as TGFβ2.

Having identified the ability of PCA cells to activate naïve human fibroblasts through the secretion of soluble TGFβ2, and the capacity of silibinin to inhibit this response, we elected to investigate the capacity of our clinically derived CAF cells to mediate PCA aggressiveness, again with an interest in silibinin’s potential to inhibit this response. We recreated our system of conditioned media, here using CAF cells to condition media to now be provided to PCA cells. This system was again a two part system. Incubation of CAFs with vehicle control or silibinin was conducted in the first stage. Following multiple washes to eliminate vehicle control or silibinin, CAF cells were incubated in low serum growth media (.5% FBS) in the second stage. It is this second stage that was collected as CAF CCM or SBCM respectively. This protocol was designed to differentiate silibinin treatment between the CAF and the PCA cell lines. In these studies, we identified that CAF cells potently secreted MCP-1 which specifically enhanced invasiveness of PCA cells exposed to CAF CCM. We found that silibinin could inhibit CAF cells ability to secrete MCP-1 by inhibiting its synthesis at the level of DNA binding of MCP-1 transcription factors; which translated to a reduction in
MCP-1 accumulation in CAF conditioned media as well as subsequent induction of invasiveness in downstream PCA cells. Further, direct application of silibinin on PCA cells, consistent with previously published reports, also significantly inhibited invasion. We replicated these findings in two mouse models, showing that silibinin feeding reduced PCA tumor growth and expression of MCP-1 which was in turn associated with a reduction in expression of markers of fibroblast activation. Furthermore, given the role of MCP-1 in mediating immune cell recruitment, we assayed for the recruitment of immune cells in these samples. Interestingly, we found that silibinin feeding of mice significantly inhibited expression of markers for macrophages and neutrophils in their tumors. These silibinin mediated actions have been schematically summarized in Figure 4.1.

The effects of silibinin treatment on PCA/TME interactions might be predicted to be mediated through several modalities as they appear to operate on multiple different mechanisms. As previously mentioned, we found a reduction in MCP-1 mRNA in silibinin treated CAFs which was associated with their impaired capacity to secrete MCP-1 into media for downstream exposure to PCA and immune cells. This appeared to be a result of reduction in nuclear translocation of NFκB and AP-1 transcription factors. This effect of silibinin has been reported in both PCA and other cancer related models involving treatment with silibinin (69, 186, 198-201). In that vein, inhibition of α-SMA expression by silibinin might also operate through inhibition of AP-1 as this has been reported to be involved in downstream TGFβ signaling as well as α-SMA synthesis specifically (202, 203). Similarly, silibinin’s well-documented cytostatic effects on various cell cycle regulators (cyclins, CDK’s, CDKI’s, etc.) reducing their
protein levels and kinase activities, might explain the reduction in clonogenicity of PCA cells treated with silibinin (75-79, 82, 84, 85). Silibinin mediated reduction of CCM induced invasion in both PrSC’s and PCA cells appeared to operate (in the case of PCA) through both a reduction in MCP-1 secretion as mentioned, and through direct action on PrSC’s and PCA cells which has been reported in other cancer models (82, 83, 94, 204). Importantly these reported effects were associated with an increase in E-cadherin levels, which we also found. These effects were reported to be a consequence of silibinin’s downregulation of E-cadherin regulatory molecules Slug, Snail, phospho-Akt(ser\textsuperscript{473}), nuclear β-catenin, phospho-Src(tyr\textsuperscript{419}) and Hakai (92) which might similarly be the mechanism in our models.

Thus, PCA cells may then recruit/induce a CAF phenotype in nearby fibroblasts which in turn support PCA invasion where they can encounter new sources of fibroblasts to serially replicate this cycle feeding the growth and progression of a nascent tumor into an aggressive, invasive form. Our studies reveal that in addition to silibinin’s well characterized anti-PCA effects, it has the capacity to potently interfere with PCA/TME interactions that have been reported to be critical for PCA progression. Taken together, this provides significant evidence for the ability of silibinin to operate as an effective prophylaxis for PCA by targeting cancerous cells directly, the surrounding tumor microenvironment, and the web of communications between these two elements.
Figure 4.1 Schematic for the cyclical interactions of PCA cells and surrounding TME elements.
REFERENCES


APPENDIX A

PUBLICATIONS RESULTING FROM THESIS


