Molecular Mechanisms Driving Chemokine-mediated Prostate Myofibroblast Phenoconversion

by

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To my parents, Ana G. Nieves González and José A. Rodríguez Donate
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Abstract

Benign prostate hyperplasia (BPH), a proliferative condition of the prostate common in aging in men, and smooth muscle hypercontractility are associated with urinary voiding dysfunction (LUTD) manifest as Lower Urinary Tract Symptoms (LUTS). However, work from our laboratory has revealed that tissue fibrosis may also be an important and previously unrecognized pathobiology contributing to LUTD. Tissue fibrosis arises from the differentiation of fibroblasts or other progenitor cells into myofibroblasts, which then produce and secrete collagens and fibronectins that augment and remodel the extracellular matrix (ECM). In this dissertation we report that diet-induced obesity and associated tissue inflammation, can drive prostatic fibrosis and LUTS in an aging accelerated mouse model. We also report that, although myofibroblast differentiation is commonly associated with activation of the TGFβ/TGFβRI axis, pro-inflammatory chemokine CXCL12/CXCR4-mediated signaling can promote myofibroblast phenoconversion through non-canonical EGFR-coupled pathways that do not depend on TGFβ/TGFβRI axis activation or Smad signaling. Furthermore, we show through RNAsq approaches that CXCL12/CXCR4 and TGFβ/TGFRI induce largely similar transcriptional
signatures during prostate myofibroblast phenoconversion. One key difference, however, is that CXCL12/CXCR4 axis activation induced the gene transcription and protein expression of cullins, which play key roles in collagen export. Therefore, activation of the CXCL12/CXCR4 axis may promote fibrosis both through increased collagen production and collagen transport into the ECM. Taken altogether, the data presented in this dissertation provides solid evidence in support of fibrosis as one of the contributors to LUTS, and provides detailed analysis on how pro-inflammatory proteins secreted by prostate fibroblasts promote myofibroblasts phenoconversion. This information may be crucial for the development of novel therapies and potential biomarkers for prostatic fibrosis and treatment of LUTS.
CHAPTER 1

Introduction

1.1 Prostatic fibrosis, lower urinary tract symptoms, and BPH

Lower urinary tract symptoms (LUTS) are a costly and potentially critical medical problem for millions of ageing men. This spectrum disorder encompasses symptoms such as weak stream, nocturia, and sensations of incomplete emptying and intermittent or hesitant urination, all of which are indicative of lower urinary tract dysfunction (LUTD). If left untreated or treated ineffectively, LUTD can progress to bladder dysfunction, which can lead to urinary retention, recurrent UTI, bladder calculi, and, eventually, renal impairment.\textsuperscript{1-5} LUTD is often, although not always, concomitant with BPH—a proliferative but nonmalignant enlargement of the prostate.

Surgical ablation of prostate tissue and medical approaches to targeting androgen activity (for example, 5α-reductase inhibitors) or smooth muscle contractility (for example, α-adrenergic receptor antagonists) can be utilized to manage LUTD. In the USA, there has been a steady decline in the use of surgical transurethral prostatectomy (TURP) over the past 10 years, as well
as a steady increase in the use of minimally invasive technologies (MIST), particularly laser vaporization.\textsuperscript{6,7} In a recent study of 1,645 hospital patients with LUTS, the 4-year retreatment rate for laser ablation was 8.3\% compared with 12.8\% for TURP.\textsuperscript{8} A community-based study reported significant improvements in LUTS after treatment with either TURP or laser vaporization, but not with 5α-reductase inhibitors or α-adrenergic receptor antagonists (which only stabilized LUTS).\textsuperscript{9}

However, other studies have shown a significant improvement in LUTS in men treated with α-adrenergic receptor antagonists or 5α-reductase inhibitors (both alone and in combination). For example, the Medical Therapy of Prostatic Symptoms (MTOPS) study showed that monotherapy with either doxazosin (an α-adrenergic receptor antagonist) or finasteride (a 5α-reductase inhibitor) resulted in significant improvements (reductions of ≥4 points) in American Urological Association Symptom Scores (AUASS) for LUTS associated with BPH, with a particularly pronounced effect with combination therapy (reductions of >7 points).\textsuperscript{10,11} Clinical progression of BPH (≥4 point increase in AUASS) was evident at 4 years in 4.6\% (36 of 786) of men given combination therapy, 8.5\% (65 of 768) of men given finasteride, 7.3\% (55 of 756) of men given doxazosin, and 13.2\% (97 of 737) of men given placebo,\textsuperscript{10} suggesting that, although generally effective, medications that target androgen receptor activity or smooth muscle contraction do not target all of the mechanisms that contribute towards LUTS. Although both surgical and medical approaches can improve urinary flow, such treatments are not effective for all men, can produce adverse effects that result in discontinuation of the therapeutic regimen, and do not abrogate the risk for disease progression.\textsuperscript{1} Pathobiology other than androgen-mediated proliferation and smooth muscle dysfunction might, therefore, contribute to the development and progression of LUTD (Figure 1).
Figure 1.1 | Prostatic pathobiologies that contribute towards lower urinary tract dysfunction. The prostate consists of ductal glands surrounded by fibromuscular stroma, which, in turn, surrounds the prostatic urethra. a | The ductal glands and fibromuscular stroma can separately or concurrently hyperproliferate, producing prostatic enlargement and urethral obstruction. BPH can be medically managed by 5α-reductase inhibitors, which prevent the conversion of testosterone to its active form, dihydrotestosterone, leading to reduced levels of available dihydrotestosterone and prostate tissue proliferation. b | Myofibroblast phenoconversion and accumulation—and consequent ECM deposition in periurethral prostatic tissue (and possibly also adjacent tissues)—causes tissue stiffness and reduced urethral compliance in men with lower urinary tract symptoms. c | Smooth muscle within the prostate can exhibit dysfunctional contractility with consequent urinary voiding dysfunction, which can be medically managed with α-adrenergic receptor antagonists (which relax smooth muscle). Abbreviation: ECM, extracellular matrix.
1.2 Inflammation and LUTD

1.2.1 Prostatic inflammatory infiltrate

Inflammatory infiltrates are very commonly observed in prostate tissue specimens from men with BPH and LUTS, and comprise about 70% T lymphocytes, 15% B cells, and 15% macrophages, as well as a smaller subpopulation of mast cells.\textsuperscript{10–12} Resident T-lymphocyte populations in prostate tissues actively secrete a diverse array of chemokines into the surrounding microenvironment. Immunohistochemical studies examining the histopathology of BPH have reported the presence of inflammatory infiltrate containing leucocytes associated with acute or chronic inflammation (or both).\textsuperscript{12–14} Neutrophilic or lymphocytic infiltrates were identified in 90% of transurethral resections of the prostate (TURP) specimens from 80 patients with BPH or LUTS but no history of prostatitis or prostatic infection.\textsuperscript{12} Chronic inflammatory infiltrate was also detected in 30–60% of 1,197 randomly selected men with BPH or LUTS recruited to the MTOPS study. Patients with chronic inflammatory infiltrate had larger prostate volumes and were more likely to experience clinical progression and acute urinary retention than those with no evidence of inflammation.\textsuperscript{13} A prospective study of 167 autopsied prostates identified 93 glands that harbored evidence of BPH; 75% of these glands contained inflammatory infiltrate (predominantly associated with chronic inflammation) compared with 50% of glands without signs of BPH and 55% of glands with evidence of cancer (Box 1).\textsuperscript{14}

1.2.2 Prostatitis and UTI

Another source of inflammation and inflammatory damage to the lower urinary tract is prostatitis. Several epidemiological studies have demonstrated an association between prostatitis and subsequent development of LUTD. Data from the Health Professionals Follow-up Study
showed a significant association between history of gonorrhoeal infection or young-onset (aged <30 years) prostatitis and later development of LUTS. The Olmsted County Men’s Health Study showed that men with physician-diagnosed prostatitis were significantly more likely to develop prostatism, BPH, LUTS, or an enlarged prostate \((P < 0.0001)\), receive treatment for BPH or LUTS \((P < 0.0001)\), and develop acute urinary retention \((P = 0.01)\) than those without a physician diagnosis of prostatitis. Moreover, even after adjusting for age and number of baseline physician visits, men with physician-diagnosed prostatitis were also more likely to receive subsequent treatment for BPH or LUTS. Combined data from five studies involving a total of 10,617 men suggest that men reporting a history of prostatitis have a substantially increased risk of developing BPH, LUTS, and prostate cancer. UTIs are also associated with male LUTD. In one study of 208 patients with bacteriuria, 54% were diagnosed with UTIs and these patients demonstrated voiding dysfunction manifested by higher rates of dysuria \((P = 0.0001)\), urgency \((P = 0.0001)\), and frequency \((P = 0.0001; \text{Box 1})\).

1.2.3 Inflammation associated with ageing

Prostatic inflammation also results from the normal process of ageing. Significantly greater concentrations of interleukin-8 and the closely related C-X-C motif chemokine (CXCL) 5 are secreted by stromal fibroblasts cultured from the prostates of older men compared with younger men. Moreover, the secretion of interleukin-8, CXCL5, CXCL1, CXCL6, and CXCL12 by ageing prostate stroma induces proliferative responses from both epithelial and stromal prostate cells \textit{in vitro}. A likely source of CXCLs is senescent cells within the prostate tissue microenvironment. Many types of mammalian cells—with the exception of cells in continually renewing tissues originating from particular types of stem cells—become growth-arrested (senescent) over time. By definition, senescent cells are nonreplicative. Cells become senescent
when their chromosomal telomeres become too short to permit further DNA synthesis and cell division. Such cells have effectively reached replicative exhaustion and entered replicative senescence. Cells might also become senescent under conditions of stress, which often results in DNA damage and growth arrest. Although these cells have not reached their Hayflick limit, they are nonreplicative and have entered cellular senescence. Many studies have shown that senescent cells accumulate with age in vivo. Studies have shown that senescing fibroblastic and epithelial cells secrete a medley of inflammation-associated proteins, including interleukins and chemokines (CC-type and C-X-C motif). The Senescence Associated Secretory Profiles (SASPs) identified in these two studies were remarkably similar and corroborated with those previously identified for senescent prostate stromal fibroblasts and cells isolated from ageing and enlarged human prostates.

Other studies have shown that myofibroblast-rich reactive stroma characterizes hyperplastic, dysplastic, and neoplastic-associated prostatic stroma. BPH nodules exhibit elevated epithelial CXCL8 immunoreactivity (commonly associated with reactive stroma). CXCL8 induces the differentiation of fibroblasts to myofibroblasts, and overexpression of a keratinocyte-derived chemokine (the mouse homologue of CXCL8) in mouse prostatic epithelium can produce hyperplastic prostate epithelial acini (typically associated with a periacinar reactive stroma). In addition, CXCL5, CXCL8, and CXCL12 promote the transition of normal prostate fibroblasts to myofibroblasts in vitro. Taken together, these data suggest that an ageing inflammatory microenvironment might be conducive to myofibroblast accumulation and tissue fibrosis in the prostate. Thus, in the absence of comorbid disease processes, ‘normal’ ageing processes might suffice to promote fibrotic changes in lower urinary tract tissue architecture and consequent obstructive voiding symptoms (Box 1).
1.2.4 Inflammation associated with diabetes

A fourth source of inflammation that can potentially affect the lower urinary tract is type 2 diabetes mellitus (T2DM). Data from several recent epidemiological studies suggest that LUTD occurs more frequently among men with T2DM than in healthy controls. Among 9,856 men with clinically diagnosed BPH, the presence of diabetes mellitus (13% prevalence) was associated with increased severity of LUTS, affecting voiding function more than storage function. Patients with BPH and T2DM had a significantly higher baseline International Prostate Symptoms Score (IPSS; 20.5 ± 0.2) and a significantly lower maximal urinary flow rate (Qmax; 10.4 ± 0.2) than those with BPH but without T2DM (18.6 ± 0.1 and 11.5 ± 0.1, respectively; \( P < 0.001 \)). Indeed, it has been hypothesized that T2DM and LUTD share an underlying inflammatory pathogenesis as many important cytokines involved in inflammation are associated with both conditions. An association between T2DM with LUTD initiation and progression has been biologically confirmed in animal models. Rabbits fed a high-fat diet (HFD) exhibited metabolic syndrome, as evidenced by hyperglycaemia and glucose intolerance, increased serum triglycerides and cholesterol levels, and increased mean arterial pressure (MAP) and visceral fat tissue (VAT). These rabbits also developed bladder alterations (including fibrosis, hypoxia, and low-grade inflammation) in conjunction with reduced bladder compliance. Thus, a HFD was associated with metabolic syndrome, T2DM, inflammation, and urinary voiding dysfunction. Similar studies showed that HFD-fed SAMP6 and AKR/J mice developed diet-induced obesity and T2DM concurrently with increased VAT, prostatic inflammation, prostatic and urethral tissue fibrosis, and urinary voiding dysfunction. Taken together, these studies show that lower urinary tract inflammation is epidemiologically and biologically linked to tissue fibrosis and
LUTD (Box 1).

1.3 Prostatic fibrosis

Tissue inflammation caused by ageing, infection, and other inflammatory disease processes is epidemiologically associated with the subsequent development of tissue fibrosis in multiple organ systems, leading to conditions such as pancreatic dysfunction,43,44 chronic obstructive pulmonary diseases,45,46 cirrhotic nonalcoholic fatty liver disease,47,48 and Crohn’s disease.49–51 Mechanistically, fibrosis occurs downstream of inflammation (Figure 2), and can be considered as an inflammation-initiated, aberrant wound-healing process that is characterized by myofibroblast accumulation, collagen deposition, extracellular matrix (ECM) remodelling, and increased tissue stiffness.36,51–54 Tissue fibrosis impairs organ function by replacing normal tissue with highly collagenized scar tissue, increasing tissue stiffness (thereby reducing tissue elasticity and compliance), disrupting or ablating normal tissue innervation and vasculature.
Figure 1.2 | Contribution of inflammation and fibrosis to lower urinary tract dysfunction (LUTD). UTI, prostatitis, ageing, and type 2 diabetes mellitus are all sources of tissue inflammation that promotes fibrosis in the lower urinary tract. Periurethral tissue fibrosis, stromal or epithelial prostatic proliferation, and smooth muscle dysfunction can, alone or in combination, promote male LUTD. These three pathobiologies can occur concurrently in the same prostate gland.
1.3.1 Wound-activated ECM remodelling

Fibrosis can be regarded as an errant wound-healing process characterized by the activation and accumulation of myofibroblasts, which are transiently produced in many tissues as part of the normal wound response. Several cell types, including fibroblasts, pericytes, fibrocytes, and mesenchymal cells, might be capable of differentiating into myofibroblasts. The common hallmarks of myofibroblast differentiation are expression of α-smooth muscle actin (α-SMA) and collagen type I, which is a large component of myofibroblast-secreted ECM. Myofibroblasts expressing α-SMA form focal adhesions to the surrounding collagen-augmented ECM, and contraction of these myofibroblasts provides the mechanical force needed for wound contracture and closure. Subsequent wound closure reduces the mechanical load on the myofibroblasts—potentially sensed by α-SMA, which is thought to be a mechanosensor protein—leading to dissolution of focal adhesions, the disassembly of α-SMA, and eventual myofibroblast apoptosis and cell death. If wound closure does not occur, myofibroblasts do not receive the mechanical signal to undergo apoptosis and continue to accumulate and deposit ECM, thereby replacing normal tissue with fibrotic tissue.

Prostate stromal fibroblasts can be induced to express fibrosis-associated collagen 1 and 3 and α-SMA, and to undergo complete functional myofibroblast phenoconversion in response to exposure to the canonical profibrotic protein TGF-β1 or the CXCLs CXCL5, CXCL8, and CXCL12 (even in the absence of exogenous TGF-β1). Moreover, CXCL12-mediated myofibroblast phenoconversion can be completely abrogated by inhibition of the CXCL12 receptor CXCR4. These findings suggest that CXCLs, which comprise inflammatory proteins known to be highly expressed in the ageing prostate, can efficiently and completely mediate myofibroblast phenoconversion and might, therefore, promote the fibrotic changes in prostate tissue.
tissue architecture that are associated with the development and progression of male LUTD.\textsuperscript{36}

1.3.2 Periurethral ECM deposition and fibrosis

A recent study of periurethral prostate tissues from 28 men used uniaxial load–unload mechanical testing to determine the mechanical stiffness of these tissues. Corresponding tissue sections were digitally imaged and colour-segmented using a programme within MATLAB that separates and quantifies colour elements from images of tissues stained with Masson’s trichrome, permitting quantitation of blue-stained areas corresponding to extracellular collagen. Periurethral prostate tissues from men with LUTS (>8 AUASI points) were significantly stiffer ($P = 0.0016$; Pearson correlation [r] = 0.82) and demonstrated significantly greater collagen content ($P = 0.0038$; r = 0.60) and lower glandularity than tissues from men without LUTS (<7 AUASI points). In addition, histological inflammation was more pronounced in tissues with greater stiffness from patients reporting moderate or severe LUTS. When combined, these findings suggest that periurethral ECM deposition and fibrosis reduces urethral flexibility and compliance, thereby contributing to urinary obstructive symptoms and LUTS (Figure 3).\textsuperscript{56}
Figure 1.3 | Myofibroblast phenoconversion and the initiation of fibrosis in the prostate. 

Healthy prostate tissue is comprised of diverse cell types, including fibrocytes, fibroblasts, epithelial cells, and endothelial cells, as well as others not shown here (for example, neurons and leucocytes). 

Many of these cell types can act as precursor cells that undergo myofibroblast phenoconversion upon exposure to profibrotic stimuli. Myofibroblasts accumulate and deposit excessive ECM, which replaces normal tissue with stiff noncompliant fibrotic tissue. 

Abbreviation: ECM, extracellular matrix.
1.4 Therapeutic targeting of fibrosis

1.4.1 Standard of care

Several clinical trials have shown that patients who undergo radiation therapy to the prostate—a procedure that can induce tissue fibrosis or urethral strictures—demonstrate significant reductions in LUTS after treatment with α-adrenergic receptor antagonists. Animal studies have shown that the ventral prostates of rats treated with the α-adrenergic receptor agonist phenylephrine are affected by interstitial fibrosis, inflammation, neoangiogenesis, and de novo synthesis of collagen (suggestive of a desmoplastic reaction). Conversely, other studies have shown that the ventral prostates of adult Wistar rats treated with the α-adrenergic receptor antagonist doxazosin contain increased levels of collagen and collagen fibrils compared with untreated controls. A-adrenergic receptor antagonists are known to target vascular and smooth muscle cells in the lower urinary tract. However, myofibroblasts, like smooth muscle cells, are contractile, and further work is required to determine whether myofibroblasts respond to α-adrenergic receptor antagonists in a similar manner to smooth muscle cells in the lower urinary tract.

Several studies have documented histological changes in prostate tissue architecture—including increased levels of inflammatory infiltrate and fibrosis—after androgen deprivation therapy (ADT). Studies to examine prostate tissues from men treated with short-term or long-term 5α-reductase inhibitor therapy for BPH or LUTS have not yet been conducted. However, given the association between tissue fibrosis and LUTD, such studies are warranted. Were ADT to be associated with myofibroblast phenoconversion and fibrosis in nonmalignant prostate tissues, such treatment might actually contribute to the progression of BPH and LUTS in some men.
1.4.2 Antifibrotic agents

If fibrosis is a pathobiology that contributes to LUTD, then antifibrotic therapeutic agents might be efficacious for treating men with LUTD, especially men who don’t respond to 5α-reductase inhibitors or α-adrenergic receptor antagonists. Surgical ablation of periurethral prostate tissues via conventional resection or MIST approaches is likely to ablate both proliferative and fibrotic tissues contributing to LUTD, thereby producing symptom relief. However, fibrosis is a recurrent condition and tissues from sequential resections should be examined in order to determine whether resection provides durable symptom relief for men with periurethral fibrosis. Several humanized antibody or small molecule inhibitor antifibrotic therapeutic agents are currently in preclinical or clinical trials for conditions such as idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, renal fibrosis, hepatic fibrosis, cardiac fibrosis, dermal fibrosis, and other fibrotic diseases. For example, pirfenidone, which targets TGF-β, is approved for the treatment of idiopathic pulmonary fibrosis in Japan, the European Union, and the US. Therapeutics designed to inhibit the activities of particular inflammatory proteins, such as TNF-α (etanercept), interleukin-13 (QAX576), and CCL2 (CNTO-888), are in phase II clinical trials. These novel agents are designed to interfere with the activities of particular proteins that promote myofibroblast phenoconversion or ECM production, including TGF-β1, connective tissue growth factor, lysyl oxidase, interleukins, CC-type chemokines, integrins, and signalling proteins (for example, JNK and Jak2). Some of these agents might prove useful for treating fibrosis-promoted LUTD in men who have failed current standard-of-care therapeutic or surgical ablation approaches. Treatment modalities do not need to be systemic as targeted modalities can be efficiently delivered to tissues of the lower urinary tract via instillation into the
bladder or injection into the prostate (‘reverse’ biopsy).

In summary, tissue inflammation resulting from ageing, infection, or other inflammatory disease processes (for example, T2DM) is associated with the subsequent development of tissue fibrosis in the prostate. Periurethral prostate tissue fibrosis is, in turn, associated with LUTD, suggesting that fibrosis might be a previously unrecognized pathobiology that contributes to LUTD. Thus, antifibrotic therapeutic agents should be considered as a new approach to efficaciously treating men with LUTD, especially those who don’t experience durable responses to 5α-reductase inhibitors, α-adrenergic receptor antagonists, or surgical ablation.

1.5 Potential novel therapeutic targets

Tissue fibrosis is caused by the unregulated proliferation and differentiation of myofibroblasts, which is usually achieved through TGF-β1. TGF-β1 belongs to the TGF-β family of cytokines, small secreted proteins that are important for intercellular communication and biological processes such as cellular differentiation, growth and survival. TGF-β1 drives the differentiation of several cell types to profibrotic myofibroblast through the activation of Smad kinases and upregulation the expression of extracellular matrix components, such as collagen and fibronectins, and contractile proteins like α-smooth muscle actin, which is required during the wound healing process. However, aberrant regulation in TGF-β1 production and secretion can lead to the development of several pathological conditions, fibrotic diseases being among them, by promoting myofibroblast proliferation, differentiation and survival. Surprisingly, recent data has shown that pro-inflammatory CXC-type chemokines can drive myofibroblast differentiation as well as TGF-β1. It has also been documented that in cancer activated breast cancer myofibroblasts CXCL12, a CXC-type chemokine, is required for autocrine regulation of the TGF-β1 signaling pathway and maintaining myofibroblasts phenotype. Several other studies...
have shown that CXCL12 is involved in the development of fibrosis in several organs and injury models. This data, generated from our group and others, lead us to focus on the mechanism of action and potential role of CXC-type chemokines, specifically CXCL12, in prostatic fibrosis as a potential therapeutic target.

1.5.1 CXC-type Chemokines

CXC-type chemokines are a family of small, secreted proteins that share to conserved cystines separated by one not conserved amino acid and signal through G-coupled membrane receptors. CXC-type chemokines are chemo-attractants and pro-inflammatory proteins. These two properties of this family of protein provide them with a crucial rule in the migration and homing of cells to specific tissues, including but not limited to cancer, angiogenesis and embryogenesis.

One of the most studied members of this super family of secreted proteins is CXCL12 and it’s receptor CXCR4. CXCR4 binds exclusively to CXCL12 and it’s documented to be overexpressed in a wide range of cancers including ovarian, breast and prostate cancer. This interaction between CXCL12 and CXCR4 has been shown to have a crucial role in site-specific metastasis as CXCR4 expressing metastatic cancer cells migrate to CXCL12-rich areas such as the bone marrow and lymph nodes. The CXCL12/CXCR4 axis is also involved in tumor growth as documented in animal studies involving the use of AMD3100, a small molecule inhibitor of the CXCL12/CXCR4 axis, and how inhibition of this axis resulted in reduced tumor growth. Therefore, understanding the signaling mechanisms used by this protein, and family of proteins, can help us better understand how not only disease progression, but pro-fibrotic events as previously discussed are achieved and how they can be potentially blocked.

1.5.2 CXCL12 signaling mechanisms
CXCR4 is a seven member transmembrane G-couple protein receptor (GPCR) that only binds to the CXC-type chemokine CXCL12. Upon binding of CXCL12 to CXCR4, the GPCR complex is released and signaling occurs via the β and γ subunits of the complex. Upon ligand binding, rapid activation of MAPK (MEK/Erk) and Akt signaling pathways occurs to promote biological processes such as cell migration, proliferation and survival. We have reported that in prostate epithelial cells this ligand-receptor induces a robust transcriptional response, and this ligand-receptor signaling cascades relies on the transactivation of EGFR via the action of ADAM metalloproteases. However, reports from the last decade have shown that CXCL12 can also affect cell differentiation and proliferation by interacting with another receptor, CXCR7. CXCR7 had been long thought to be a negative regulator of CXCL12 signaling by acting as a decoy receptor, especially since it binds to CXCL12 with an affinity close to ten times higher than CXCR4. But recent data suggest that CXCL12/CXCR7 plays a role during embryogenesis as evidence by studies in zebrafish where it was shown that CXCR7 is important for the CXCL12/CXCR4-directed cell migration during embryogenesis. Furthermore, signaling studies in HEK-293 cells showed that, when over-expressed, a fraction of the available CXCR7 creates heterodimers with CXCR4. These heterodimers showed stronger CXCL12-driven signaling when compared to CXCR4 by itself, demonstrating the complexity of the signaling events exerted by this chemokine. However, based on several reports, and for simplicity’s sake, we will focus the last section to the CXCL12/CXCR4 axis role in tissue fibrosis.

1.5.3 CXCL12/CXCR4 axis in tissue fibrosis

The origin of fibrosis in pathological conditions is not always understood; myofibroblasts can be derived from resident or circulating fibroblasts and fibrocytes that are attracted to affected area by the interaction chemokines are their receptors. Diseases such as idiopathic pulmonary fibrosis
(IPF) are characterized by an increase in numbers of fibroblasts\textsuperscript{53,55} and fibrocytes in the injured area\textsuperscript{74}. As previously stated, this cell migration heavily relies in trafficking control by chemokines, and one of the most heavily studied ones is CXCL12. It has been reported that using small molecule inhibitors such as AMD3100, an establish CXCR4 inhibitor; can block the migration of fibroblasts and fibrocytes in a bleomycin-induced pulmonary fibrosis mouse model\textsuperscript{74,75}. Additionally, treatment with MSX-122, a novel CXCR4 antagonist, in a radiation-induced pulmonary fibrosis mouse model showed significant reduction in the development of pulmonary fibrosis\textsuperscript{76}. These animal-based studies show the potential that targeting CXCL12/CXCR4 has on treating pulmonary fibrosis. However, our group reported that in vitro, using immortalized and primary fibroblasts, CXCL12 can drive the differentiation of prostate myofibroblasts in the absence of any other stimuli. This differentiation process was shown to be CXCR4, not CXCR7, dependent as evidenced in small molecule inhibitor studies\textsuperscript{36}. Taken together, these reports show the CXCL12/CXCR4 axis as an attractive target with potential therapeutic value. The data presented in this dissertation will further validate this hypothesis and provide enough data to develop projects that could test the importance of CXCL12, and other chemokines, in the development and progression of prostatic fibrosis.
1.6 References

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CHAPTER 2

Obesity-Induced Diabetes and Lower Urinary Tract Fibrosis Promote Urinary Voiding Dysfunction in a Mouse Model

2.1 Abstract

Progressive aging- and inflammation-associated fibrosis effectively remolds the extracellular matrix (ECM) to increase prostate tissue stiffness and reduce urethral flexibility, resulting in urinary flow obstruction and lower urinary tract symptoms (LUTS). In the current study, we sought to test whether senescence-accelerated mouse prone (SAMP) 6 mice, which were reported to develop prostatic fibrosis, would also develop LUTS, and whether these symptoms would be exacerbated by diet-induced obesity and concurrent Type 2 Diabetes Mellitus (T2DM). To accomplish this, SAMP6 and AKR/J background strain mice were fed regular mouse chow, low fat diet chow, or high fat diet chow for 8 months, then subjected to glucose tolerance tests, assessed for plasma insulin levels, evaluated for urinary voiding function, and assessed for lower urinary tract fibrosis. The results of these studies show that SAMP6 mice and AKR/J background strain mice develop diet-induced obesity and T2DM concurrent with
urinary voiding dysfunction. Moreover, urinary voiding dysfunction was more severe in SAMP6 than AKR/J mice and was associated with pronounced prostatic and urethral tissue fibrosis. Taken together, these studies suggest that obesity, T2DM, lower urinary tract fibrosis, and urinary voiding dysfunction are inextricably and biologically linked.

2.2 Introduction

Benign prostatic hyperplasia (BPH) is one of the most common benign proliferative conditions associated with aging in men. BPH is a chronic, progressive disease of the prostate which conservatively affects 30–35% of men aged 60 or older and results in a significantly negative impact on quality of life. This negative impact is due to various co-morbidities that develop concurrently with BPH that collectively produce lower urinary tract symptoms (LUTS) characteristic of lower urinary tract dysfunction, or LUTD. LUTD is itself a progressive disorder that is manifesting as urgency, nocturia, urinary frequency, weak urinary stream, and incomplete bladder emptying. Without effective treatment, LUTD can lead to bladder outlet obstruction (BOO) and subsequent bladder wall hypertrophy, increased bladder mass, and bladder dysfunction manifest as acute urinary retention, recurrent urinary tract infections, bladder stones, and, eventually, renal dysfunction. Type 2 Diabetes Mellitus (T2DM), a chronic disorder of carbohydrate, fat, and protein metabolism, is an important cause of morbidity and mortality in the US. Like BPH/LUTD, T2DM is associated with older age, as well as with obesity, a family history of diabetes, a history of gestational diabetes, impaired glucose metabolism, physical inactivity, and race/ethnicity. The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) reports that T2DM currently affects 25.8 million people of all ages, or 8.3%, of the entire US population. In 2010, the most recent year for which there is data, 10.9 million
(26.9%) of all US residents aged 65 years or older had T2DM, and 1.9 million people aged 20 years or older were newly diagnosed with T2DM\textsuperscript{6}. There is growing evidence that LUTD and T2DM are linked etiologically and biologically. Diet-induced obesity has been identified as a risk factor for both T2DM and LUTS in men\textsuperscript{7,8}. Conversely, reversion of obesity through weight loss is linked with reduction of symptoms associated with diabetes and LUTS\textsuperscript{9}. Several epidemiologic studies that have examined the association between LUTS and diabetes also suggest that LUTS may occur more frequently among men with diabetes, with an estimated 25\% to nearly twofold increased risk of LUTS in men with diabetes\textsuperscript{10-13}. In addition, among 9,856 men with clinically diagnosed BPH, the presence of diabetes (13\%) was associated with increased LUTS severity, affecting voiding more than storage function. Patients with BPH and diabetes had a significantly higher baseline International Prostate Symptoms Score (IPSS) and a significantly lower maximal urinary flow rate (Qmax) that those without diabetes (both P < 0.001)\textsuperscript{12}. A possible explanation for an association between BPH and diabetes beyond that explained by comorbidity due to age is that the two disease states can cause at least partially similar urological symptoms. However, the underlying biological mechanisms producing these symptoms may be different. Dib et al. studies 50 consecutive diabetic patients, 23 (46\%) of whom had concurrent BOO symptoms based on pressure/flow urodynamic measurements. However, no significant differences in IPSS or prostate volume were apparent between diabetic patients with BOO compared to those without obstructive symptoms. These data suggest little, if any, association between BOO in diabetic patients with prostate enlargement and LUTS\textsuperscript{14}.

Similarly, a multiethnic community based study demonstrated positive associations between diabetes and irritative LUTS and nocturia, but not between diabetes across measures more specific to BPH (i.e., prostate volume, PSA, and peak urinary flow rate)\textsuperscript{15}. Taken together,
these studies suggest that the presence of diabetes may be less related to prostate growth and more related to the dynamic components of lower urinary tract function. We have previously reported that progressive aging- and inflammation-associated fibrosis effectively remolds the extracellular matrix (ECM) to increase prostate tissue stiffness and reduce urethral flexibility, resulting in urinary flow obstruction and LUTS\textsuperscript{16}. These observations suggest that tissue fibrosis adversely changes the dynamic of lower urinary tract function. In the current study, we sought to test these associations in a mouse model of accelerated aging subjected to diet-induced obesity and T2DM. Specifically, the studies reported here were designed to test whether senescence-accelerated mouse prone (SAMP) 6 mice, which were reported to develop prostatic fibrosis\textsuperscript{17-20}, would also develop LUTS, and whether these symptoms would be exacerbated by diet-induced obesity and concurrent T2DM. The results of these studies show that SAMP6 mice and AKR/J background strain mice develop diet-induced obesity and T2DM concurrent with urinary voiding dysfunction. Moreover, urinary voiding dysfunction is more severe in SAMP6 mice and is associated with pronounced prostatic and urethral fibrosis. Taken together, these studies suggest that obesity, T2DM, lower urinary tract fibrosis, and urinary voiding dysfunction are inextricably and biologically linked.

2.3 Experimental Procedures

**Mouse Strains and Maintenance.** Two related mouse strains were used in these studies: SAMP6 and the AKR/J background strain. Colonies were established from eight females and four males per strain purchased at 6 weeks of age (Harlan Laboratories, Indianapolis, IN). At 6–8 weeks of age, 25 mice each SAMP6 and AKR/J were fed the following diets: high fat diet (HFD) containing 60% calories from fat, 20% protein, and 20% carbohydrates (58Y1, Test Diet,
Richmond, IN); low fat diet (LFD) containing 10.2% calories from fat, 18.3% protein, and 71.5% carbohydrates (58Y2, Test Diet, Richmond, IN), or regular diet (RD) grain-based mouse chow containing 13.50% calories from fat, 28.50% protein, and 58% carbohydrates (#5001, Lab Diet, St. Louis, MO). The mice were fed daily with fresh high, low fat or regular chow (5 g/day) for 8 months. Mice were housed in the Unit for Animal Laboratory Medicine (ULAM) facility at the University of Michigan under enriched conditions at a constant temperature (22–23°C) with a 12/12 hr light/dark cycle and optimal humidity and free access to tap water and food ad libitum. The body weights of all mice were measured once during the first week of the month for eight consecutive months. All animal procedures were performed using protocols approved by the University Committee on Use and Care of Animals (UCUCA).

**Oral Glucose Tolerance Test.** Oral glucose tolerance tests (OGTTs) were carried out just prior to the termination of the study. OGTTs were measured in 10 mice each fed HFD or LFD from each mouse strain (a total of 40 mice). The OGTTs were performed in semi-fasted mice, for example, on the day preceding the test; mice fasted for 5 hr, starting at 8:30 am. Glucose 50% was given around 1:30 pm via oral gavage at 2.0 g/kg. Blood samples were collected prior to and after the gavage at the time of 0, 15, 30, 60, and 120 min after via tail vein bleeding. The oral glucose load was given by gavage via a gastric tube connected to a syringe to ensure accurate dosing. Blood glucose concentration was measured at all-time points using a glucometer (Acucheck, Roche).

**Plasma Insulin Measurement.** Blood samples were collected in tubes containing EDTA to avoid clotting and placed on ice until they were centrifuged at 3,000 rpm and frozen at -80°C until plasma content measurements were conducted. Plasma levels of insulin were determined using Millipore rat/mouse insulin using a commercially available insulin ELISA kit [12]. Animals
were restrained repeatedly for less than a minute each time while blood samples were collected.

**Determination of Urinary Voiding Function.** The urinary voiding function of all of mice was measured once during the first week of the month for eight consecutive months. The urinary voiding function was determined using a filter paper assay, as previously described with some modification [21]. For these studies, all mice in a single cage (4–5 mice) were placed into a urine-monitoring cage. The bottom of this cage was fitted with a wire mesh beneath which was Whatman Grade 3 filter paper. The mice were left in the monitoring cage for 5 hr, after which the filter paper was collected. Urine, which had soaked into the filter paper, was strongly fluorescent under UV light and was easily visualized under those conditions. The pattern of urine spots was independently determined by two different observers (M. G.-K. and J. R.-N.) using a modification of previously described methods [21]. Each voiding pattern was given a grade of 1–5 depending upon the size and number of urine spots such that a score of 1 indicated the observation of single, large urine spots (no voiding dysfunction) and a score of 5 indicated the observation of many small, scattered urine spots (obstructive voiding dysfunction). The data from the two independent observers was averaged and statistically analyzed.

**Tissue Processing and Histological Evaluation.** Mouse lower urinary tracts were harvested at 10 months of age (after 8 months of feeding with RD, HFD, or LFD). Male mice were euthanized by CO2 asphyxiation in accordance with institutional guidelines. Lower urinary tracts consisting of bladder, prostatic and pelvic urethra, prostate and seminal vesicles were resected en bloc and placed in ice cold saline. Peripheral adipose tissue was removed and distal prostate lobe aspects were teased apart to liberate each from its attachments to other prostate lobes, seminal vesicle, and urethra. To preserve proximodistal orientation of seminal vesicle and prostate ducts, lower urinary tract tissues were fixed intact by orienting them on their lateral
surfaces, sandwiching them between histology sponges and fixing them overnight at 48°C in 10% neutral buffered formalin. Histology sponges were removed and tissues were dehydrated through an ethanol series, cleared in xylene and infiltrated with paraffin. Tissue sections were subjected to hematoxylin/eosin (H/E) staining and assessed for histopathology or subjected to Masson’s Trichrome staining to assess collagen content. Prostate wet weight was not measured because of inherent variability in prostate morphology in the different strains.

**Collagen Content.** Collagen content was determined using the methodology described previously. Briefly, Masson’s Trichrome stained tissue sections were digitally imaged using a PathScan Enabler IV and color segmented using a subprogram within MATLAB (R2010a; MathWorks, Natick, MA) that separates and quantifies color elements from trichrome images, permitting quantitation of blue-stained areas corresponding to extracellular collagen. Using the area of a single tissue section as the denominator, this approach provides a means to calculate the portion of this area that is made up by mature extracellular collagen I (the numerator), hence, the percentage of extracellular collagen type I (numerator/denominator \( \times 100 \)) in that tissue section. By extension, this method also permits calculation of the percentage of the entire piece of tissue that is made up of mature extracellular collagen I.

**Statistical Analysis.** Averages and standard deviations were calculated and compared using two-tailed Students’ t-tests. In all tests, \( P < 0.05 \) was considered statistically significant.

### 2.4 Results

**High Fat Diet-Fed Mice Develop Obesity-Induced Type 2 Diabetes Mellitus**

Both SAMP6 (Fig. 1A,C) and AKR/J (Fig. 1B,D) mice demonstrated cumulative weight gains of
1–2 g and 5–7 g when fed a RD or LFD over an 8-month period, respectively. In contrast, SAMP6 (Fig. 1A,C) and AKR/J (Fig. 1B,D) mice almost doubled in body weight, from ~30 g at the initiation of a HFD to a total body weight of 50–60 g (cumulative weight gain of 20–30 g), when fed a HFD over an 8-month period. At the end of 8 months, SAMP6 and AKR/J mice fed continuously either the LFD or HFD were subjected to 2 hr OGTTs. The results of the OGTTs showed that SAMP6 HFD-fed mice demonstrated the highest blood glucose levels, which peaked at 420 mg/dl at 40 min, followed by SAMP6 LFD-fed mice (300 mg/dl glucose), AKR/J HFD-fed mice (220 mg/dl) and AKR/J LFD-fed mice (200 mg/dl) (Fig. 2A). The average areas under the curve (AUCs) demonstrated significantly higher blood glucose levels for SAMP6 HFD-fed compared to LFD-fed mice (P < 0.0003), and AKR/J HFD-fed compared to LFD fed mice (P < 0.03) (Fig. 2B). The AUCs also showed that blood glucose levels were significantly higher for SAMP6 HFD-fed compared to AKR/J HFD-fed (P < 0.001) and for SAMP6 LFD-fed compared to AKR/J LFD-fed (P < 0.04) mice. Plasma insulin levels were determined concurrent with the blood glucose levels. The results of these studies showed that plasma insulin levels were significantly higher for SAMP6 compared to AKR/J HFD-fed mice (P < 0.001), for SAMP6 HFD-fed compared to LFD-fed mice (P < 0.001) and for AKR/J HFD-fed compared to LFD-fed mice (P < 0.002) (Fig. 2C,D). Taken together, these data suggest that both SAMP6 and AKR/J HFD-fed mice developed hyperglycemia and insulin resistance (hyperinsulinemia) consistent with T2DM concurrent with diet-induced obesity.
Figure 2.1 High fat diet-induced obesity in SAMP6 and AKR/J Mice. The graphs depict the cumulative weight gain of SAMP6 (A) and AKR/J (B) mice fed regular mouse chow diet (RD), low fat diet (LFD) or high fat diet (HFD) for 8 months. Mice from both strains fed RD or LFD gained 1–5 g over the 8-month period whereas those fed HFD virtually doubled in weight and gained 20–30 g over the same period. Photographs depict representative SAMP6 (C) and AKR/J (D) mice after 8 months on LFD or HFD diets as indicated.
Figure 2.2 SAMP6 and AKR/J mice HFD-fed mice develop hyperglycemia and insulin resistance consistent with T2DM. A: At the end of 8 months, SAMP6 and AKR/J mice fed continuously either the LFD or HFD were subjected to 2 hr oral glucose tolerance tests (OGTTs). SAMP6 HFD-fed mice demonstrated the highest blood glucose levels, which peaked at 420 mg/dl at 40 min, followed by SAMP6 LFD-fed mice (300 mg/dl glucose), AKR/J HFD-fed mice (220 mg/dl), and AKR/J LFD-fed mice (200 mg/dl). B) The average areas under the curve (AUCs) demonstrated significantly higher blood glucose levels for SAMP6 HFD-fed compared to LFD-fed mice (P < 0.0003), and AKR/J HFD-fed compared to LFD-fed mice (P < 0.03). The AUCs also showed that blood glucose levels were significantly higher for SAMP6 HFD-fed compared to AKR/J HFD-fed (P < 0.001) and for SAMP6 LFD-fed compared to AKR/J LFD-fed (P < 0.04) mice. C: Plasma insulin levels were determined concurrent with the blood glucose levels. D: Plasma insulin levels were significantly higher for SAMP6 compared to AKR/J HFD-fed mice (P < 0.001), for SAMP6 HFD-fed compared to LFD-fed mice (P < 0.001) and for AKR/J HFD-fed compared to LFD-fed mice (P < 0.002).
High Fat Diet-Fed Mice Develop Urinary Voiding Dysfunction

Several epidemiologic studies have linked LUTS with T2DM\(^9\,^{12}\). Therefore, we examined both HFD-fed and LFD-fed mice for urinary voiding function. For these studies, all mice in a single cage (4–5 mice) were placed into a urine monitoring cage for 5 hr and the pattern of urine spots on the filter paper visualized under UV light. The pattern of urine spots was assessed using a modification of previously described methods by two different observers and the results averaged and statistically analyzed. As shown in Figure 3A, the urinary voiding pattern was graded on a scale of 1–5 depending upon the size and number of urine spots such that a score of 1 indicated the observation of single, large urine spots (no voiding dysfunction) and a score of 5 indicated the observation of many small, scattered urine spots (obstructive voiding dysfunction). Urine voiding patterns were assessed for SAMP6 and AKR/J mice after 7 and 8 months of continuous feeding on RD, LFD, or HFD. Both SAMP6 and AKR/J mice demonstrated normal urinary voiding patterns after 7 or 8 months continuous RD-feeding. SAMP6 and AKR/J mice demonstrated similar though slightly abnormal urinary voiding patterns suggestive of mild obstruction at 7 and 8 months continuous LFD-feeding. Both SAMP6 and AKR/J mice demonstrated abnormal urinary voiding patterns suggestive of moderate obstruction for AKR/J mice and moderate/severe obstruction for SAMP6 mice at 7 and 8 months continuous HFD-feeding. The urine voiding scores for both SAMP6 and AKR/J HFD-fed mice were significantly worse than for LFD-fed (P < 0.008) and RD-fed mice (P < 0.001). Further, SAMP6 urinary voiding dysfunction was significantly worse (P < 0.001) than that of AKR/J mice at both 7 and 8 months continuous HFD-feeding (Fig. 3B). In addition to dermatitis, both SAMP6 and AKR/J HFD-fed mice exhibited acute
Figure 2.3 High fat diet-fed mice develop urinary voiding dysfunction. A) The urinary voiding pattern was graded on a scale of 1^5 depending upon the size and number of urine spots such that a score of 1 indicated the observation of single, large urine spots (no voiding dysfunction) and a score of 5 indicated the observation of many small, scattered urine spots (obstructive voiding dysfunction). B: Graph depicts 95% confidence intervals for urine voiding patterns for SAMP6 (black) and AKR/J (grapy) mice after 7 (diamond) or 8 (square) months of continuous feeding on RD, LFD, or HFD diets. Both SAMP6 and AKR/J mice demonstrated normal urinary voiding patterns after 7 or 8 months continuous RD-feeding. SAMP6 and AKR/J mice demonstrated similar though slightly abnormal urinary voiding patterns suggestive of mild obstruction at 7 and 8 months continuous LFD-feeding. Both SAMP6 and AKR/J mice demonstrated abnormal urinary voiding patterns suggestive of moderate obstruction for AKR/J mice and moderate/severe obstruction for SAMP6 mice at 7 and 8 months continuous HFD-feeding that was significantly worse than LFD-fed (P < 0.008) and RD-fed mice (P < 0.001). Further, SAMP6 urinary voiding dysfunction was significantly worse (P < 0.001) than that of AKR/J mice at both 7 and 8 months continuous HFD-feeding.
urinary retention and were unable to void urine, requiring euthanasia (Fig. 4A,B). The results of these studies demonstrate that continuous feeding on a HFD was associated with the acquisition of urinary voiding dysfunction for both SAMP6 and AKR/J mice, but the level of dysfunction was most severe for SAMP6 HFD-fed mice.

**HFD-Fed Mice Exhibit Lower Urinary Tract Fibrosis**

Previous studies from our group showed that the peri-urethral prostate tissues from men with LUTS were significantly stiffer (P < 0.0016, r 1/4 0.82) and demonstrated significantly higher collagen content (P < 0.0038, r 1/4 0.60) and lower glandularity than those from men without LUTS. These observations suggested that ECM deposition and fibrosis contribute to urinary obstructive symptoms and LUTS. Therefore, we examined the lower urinary tract tissues of LFD-fed and HFD-fed mice for histopathological evidence of fibrosis. Masson’s Trichrome stained tissue sections of fixed and embedded mouse lower urinary tracts (Fig. 5A) were digitally imaged and color segmented using a subprogram within MATLAB that separates and quantifies color elements from images of stained tissues, permitting quantitation of blue-stained areas corresponding to extracellular collagen. Because previous studies had suggested that SAMP6 mice exhibited fibrosis of the dorsal lobe we specifically focused on analyzing the collagen content of the dorsal lobe. Although the urethral region presented as a sagittal, rather than the desired coronal, cross-sectional area in these particular preparations of the lower urinary tract, these were also analyzed for collagen content. As shown in Figure 5B, the prostatic dorsal lobes of SAMP6 (P < 0.001) and AKR/J (P < 0.01) HFD-fed mice demonstrated 2–4 higher collagen content than those of LFD-fed
Figure 2.4 High fat diet-fed mice develop urinary retention and dermatitis. The majority of SAMP6 and AKR/J HFD-fed mice develop urinary retention (A) evident as inflated bladders (B, arrow, HFD mouse) concurrent with urinary voiding dysfunction. A smaller number of HFD-fed mice also developed chronic dermatitis (A). Epididymal fat pads are notably enlarged in SAMP6 HFD-fed mice (B, asterisks).
Figure 2.5 HFD-fed mice exhibit lower urinary tract fibrosis. A) Masson’s Trichrome stained tissue sections of fixed and embedded mouse lower urinary tracts were digitally imaged and color segmented using a subprogram within MATLAB that separates and quantifies color elements from images of Masson’s Trichrome stained tissues, permitting quantitation of blue-stained areas corresponding to extracellular collagen [16,23]. B) The percent collagen content of the prostatic dorsal lobes of SAMP6 (P < 0.001) and AKR/J (P < 0.01) HFD-fed mice demonstrated 2^4x higher collagen levels than those of LFD-fed mice (P < 0.001). Similarly, the urethral regions of SAMP6 (P < 0.001) and AKR/J (P < 0.001) HFD-fed mice (P < 0.003) demonstrated 2^4x higher collagen levels than those of LFD-fed mice. C) Photomicrographs (20x,i,iii;40x, ii, iv) of serial hematoxylin/eosin stained sections to those analyzed for collagen content demonstrated peri-glandular prostatic fibrosis(red arrows) in SAMP6 HFD-fed mouse #68 (i, ii) and milder focal fibroplasia/stromal expansion (yellow arrows) in SAMP6 LFD-fed mouse #80 (iii, iv). These differences in peri-glandular fibrosis were more evident in SAMP6 HFD-fed than LFD-fed mice but did not reach, statistical significance (P ••• 0.10). D: Photomicrographs (4x,i,iii; 20x, ii, iv) of SAMP6 LFD-fed mice (i, ii) mice demonstrate significantly higher levels of brown adipose tissue (BAT) than white adipose tissue (WAT) compared to HFD-fed mice (iii, iv) (P ••• 0.005). Insets in ii and iv are at 40x.Tissues are shown from SAMP6 LFD-fed mouse #68 and HFD-fed mouse#80.
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TABLE 2.1 Histopathological Characteristics of Murine Prostates
mice (P < 0.001). Similarly, the urethral regions of SAMP6 (P < 0.001) and AKR/J (P < 0.001) HFD-fed mice (P < 0.003) demonstrated 2–4 higher collagen levels than those of LFD-fed mice. Histopathological analysis of serial H/E stained sections to those analyzed for collagen content demonstrated peri-glandular prostatic fibrosis in SAMP6 HFD-fed mice (Fig. 5C, i and ii) and milder focal fibroplasia in SAMP6 LFD-fed mice (Fig. 5C, iii and iv). These differences in peri-glandular fibrosis were more evident in SAMP6 HFD-fed than LFD-fed mice, and these differences trended towards, but did not reach, statistical significance (P 1/4 0.10) (Table I).

Epithelial hyperplasia was more evident in AKR/J HFD-fed and LFD-fed mice than SAMP6 mice, but the observed differences between diets (for either strain) were not significant (Table I). The anterior prostates appeared normal in both HFD-fed and LFD-fed SAMP6 and AKR/J mice, as did the seminal vesicles though some mild epithelial hyperplasia was observed for AKR/J (not shown). Notably, significantly higher levels of brown adipose tissue (BAT) than white adipose tissue (WAT) was evident in SAMP6 LFD-fed compared to HFD-fed mice (P 1/4 0.005) (Table I) (Fig. 5D). A similar trend was noted for AKR/J LFD-fed compared to HFD-fed mice but these differences did not reach significance (Table I).

2.5 Discussion

Previous studies from our research group showed that lower urinary tract fibrosis is associated with moderate/severe LUTS in American men\(^{16}\). The studies reported here now show that SAMP6 mice and AKR/J background strain mice develop diet-induced obesity and T2DM concurrent with urinary voiding dysfunction and pronounced lower urinary tract fibrosis. Taken together, these studies suggest that obesity, T2DM, lower urinary tract fibrosis, and urinary voiding dysfunction are inextricably and biologically linked. Both the human and mouse studies
point to inflammation as a common biological link between lower urinary tract fibrosis, LUTD, obesity, and T2DM. Several studies have shown that male LUTD is associated with inflammation of the prostate, evident as prostatic inflammatory infiltrate and/or prostatitis. Immunohistochemical studies examining the histopathology of BPH have reported the presence of pervasive inflammatory infiltrate comprising leukocytes associated with acute inflammation, chronic inflammation, or both. Inflammatory cells comprising neutrophillic or lymphocytic infiltrates were identified in 90% of transurethral resections of the prostate (TURP) specimens from 80 patients diagnosed with BPH/LUTS but no history of prostatitis or prostatic infection. Chronic inflammatory infiltrate was also detected in 30–60% of 1,197 randomly selected men with BPH/ LUTS as part of the Medical Therapy of Prostatic Symptoms (MTOPS) study. Patients with chronic inflammatory infiltrate had larger prostate volumes and demonstrated significantly more clinical progression and acute urinary retention than those with no evidence of inflammation [25,26]. A study that prospectively analyzed 167 autopsied prostates identified 93 glands harboring BPH/LUTS, and 75% of these demonstrated inflammatory infiltrate (predominantly chronic inflammation) compared to 50% of those without BPH/LUTS and 55% of those with evidence of cancer. As originally reported by Theyer et al. and Steiner et al. and recently summarized by Kramer et al. [30], inflammatory infiltrates are very commonly observed in BPH/LUTS specimens and comprise 70% T-lymphocytes, 15% B cells, and 15% macrophages, as well as mast cells. These studies also showed that resident T-lymphocyte populations in prostate tissues actively secrete a diverse array of chemokines into the surrounding microenvironment, including those known to strongly induce myofibroblast differentiation and therefore potentiate fibrosis. Studies from our own laboratory have shown that the aging human prostate is characterized by an inflammatory tissue microenvironment.
CXCL8 (IL-8) and a closely related CXC-type chemokine, CXCL5 (ENA-78), are secreted at significantly higher levels by stromal fibroblasts cultured from the prostates of older compared to younger men\textsuperscript{31-33}. Moreover, these and additional chemokines (CXCL1, CXCL6, CXCL12) secreted by aging prostate stroma induced proliferative responses from both epithelial and stromal prostate cells in vitro\textsuperscript{32,34,35}. Studies published by the Rowley laboratory have shown that myofibroblast-rich ‘‘reactive stroma’’ characterizes hyperplastic, dysplastic, and neoplastic-associated prostatic stroma\textsuperscript{36,37}, that BPH nodules exhibited elevated epithelial CXCL8 immunoreactivity associated with reactive stroma\textsuperscript{37}, that CXCL8 was sufficient for induction of a fibroblast to myofibroblast transition\textsuperscript{37}, and that over-expression of KC, the mouse homologue of CXCL8, in mouse prostatic epithelium was sufficient to produce hyperplastic prostate epithelial acini associated with a periacinar reactive stroma\textsuperscript{38}.

In addition to low-level, but persistent, chronic inflammation and aging-associated inflammatory changes in the tissue microenvironment, obesity likely contributes significantly to inflammatory changes in adjacent tissues. Obesity-mediated inflammatory changes in the tissue microenvironment, or ‘‘metainflammation,’’\textsuperscript{39} include those produced by adipocytes and resident macrophages in WAT. In addition to triglyceride and lipid storage, WAT adipocytes secrete a medley of endocrine and paracrine factors, collectively termed adipokines, which include inflammatory mediators such as interleukins, CC- and CXC-type chemokines, and TGF-\(\beta\)\textsuperscript{40,41}. As reported here, significantly higher levels of WAT were evident for HFD-fed compared to LFD-fed mice in association with lower urinary tract fibrosis and urinary voiding dysfunction. The level and composition of inflammatory adipokines is altered in obesity-associated WAT\textsuperscript{41}, and some of these are known to activate the IKK\(b\)/NF\(\kappa\)B pathway in adipocytes, hepatocytes, and associated macrophages. NF-\(\kappa\)B is a powerful transcription factor that mediates the
expression of multiple genes encoding inflammatory mediators, including several interleukins, CC- and CXC-type chemokines, and TGF-b\textsuperscript{42,43}. TGF-b is a well-known profibrotic protein that promotes myofibroblast phenoconversion and tissue fibrosis\textsuperscript{44,45}. A recent study reported that adipose tissue from obese subjects contained increased areas of fibrosis, which correlated inversely with insulin and positively with macrophage number, compared to adipose tissue from lean subjects. Although macrophages in crownlike structures (CLS) were more abundant in obese adipose tissue, the majority of macrophages were associated with fibrosis and were not organized in CLS. Macrophages in CLS were predominantly M1, but most other macrophages, particularly those in fibrotic areas, were M2. Moreover, TGF-b was more abundant in M2 macrophages and was further increased by coculture with adipocytes. Downstream effectors of TGF-b, such as plasminogen activator inhibitor-1, collagen VI, and phosphorylated Smad, were increased in macrophages and adipocytes. Thus, adipose tissue of insulin-resistant humans demonstrated increased fibrosis, M2 macrophage abundance, and TGF-b activity\textsuperscript{46}. Recent studies from our research group show that several CXC-type chemokines, notably CXCL5, CXCL8, and CXCL12, can mediate myofibroblast phenoconversion and accumulation in the absence of TGF-b\textsuperscript{47}. Taken together, these studies suggest that inflammatory changes in the tissue microenvironment brought about through low level but chronic infiltration by the innate immune system, aging, and/or obesity may be sufficient to promote myofibroblast phenoconversion, accumulation, and fibrosis in affected tissues, including those of the lower urinary tract.

The studies reported here utilized the SAMP mouse model, which was developed through inbreeding of AKR/J mice as a model of spontaneous senescence in mice. The aging pattern in this model is considered to be due to an accelerated senescence rather than to premature aging or
An early study noted that the most striking histological changes in SAMP6 mouse prostates were stromal hyperplasia and inflammation in the dorsal prostate at age 15 months\textsuperscript{19}. Moreover, regular mouse chow-fed SAMP6 mice exhibit higher levels of plasma glucose, triglyceride, insulin, and leptin levels and hepatic lipid levels, but lower levels of plasma glucagon and adiponectin, than age-matched AKR/J mice\textsuperscript{20}. These observations suggest that SAMP6 mice may be predisposed to developing hyperglycemia and insulin resistance consistent with T2DM. Indeed, the results of the studies reported here support this conclusion and show that HFD-fed SAMP6 mice exhibited higher levels of hyperglycemia and insulin resistance concurrent with worse lower urinary tract fibrosis and dysfunction compared to HFD-fed AKR/J mice.

In summary, the studies reported here demonstrate the acquisition of urinary voiding dysfunction concurrent with obesity-induced T2DM and lower urinary tract fibrosis in a mouse model. This model recapitulates several epidemiological studies of human populations that associate obesity, metabolic syndrome, and T2DM with urinary voiding dysfunction. Therefore, further studies using these mouse models may provide the means to delineate the cellular mechanisms that biologically couple lower urinary tract fibrosis and urinary voiding dysfunction with obesity-induced T2DM.

2.6 Disclosure

In this study I participated in the generation of data for the following aspects of the project along with Dr. Merhnaz Gharae-Kermani: mouse colony maintenance (breeding and feeding special diets), dissection of mice at the end of the study, image analysis for trichrome stained prostate and bladder paraffin embedded slides, and voiding image scoring and analysis.
2.7 References

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CHAPTER 3

CXCL12/CXCR4 Axis Activation Mediates Prostate Myofibroblast Phenoconversion Through Non-Canonical EGFR/MEK/ERK Signaling.

3.1 Abstract

Benign prostate hyperplasia (BPH), an enlargement of the prostate common in aging in men, is associated with urinary voiding dysfunction manifest as Lower Urinary Tract Symptoms (LUTS). Although inflammation and abnormal muscle smooth contractions are known to play key roles in the development of LUTS, tissue fibrosis may also be an important and previously unrecognized contributing factor. Tissue fibrosis arises from the unregulated differentiation of fibroblasts or other precursor cell types into myofibroblasts, which is usually accomplished by activation of the TGFβ/TGFβR axis. Previously we reported that the CXC-type chemokines, CXCL5, CXCL8 and CXCL12, which are up-regulated in the aging in the prostate, can drive this differentiation process as well in the absence of TGFβ. Based on this data we sought to elucidate the molecular mechanisms employed by CXCL12, and its receptor CXCR4, during prostate myofibroblast phenoconversion. The results of these studies suggest that CXCL12/CXCR4-
mediated signaling events in prostate myofibroblast phenoconversion may proceed through non-canonical pathways that do not depend on TGFβ/TGFβR axis activation or Smad signaling. Here we report that CXCL12/CXCR4 axis activation promotes signaling through the EGFR and downstream MEK/ERK and PI3K/Akt pathways during myofibroblast phenoconversion, but not through TGFβ/TGFβR and downstream Smad signaling, in prostate fibroblasts undergoing myofibroblast phenoconversion. We document that EGFR transactivation is required for CXCL12 signaling and expression of genes associate with myofibroblast phenoconversion (α-SMA, COL1a1). Our study successfully identified TGFβ/TGFβR-independent molecular mechanisms that promote CXCL12/CXCR4-induced myofibroblast phenoconversion. This information may be crucial for the development of novel therapies and potential biomarkers for prostatic fibrosis.

3.2 Introduction

Benign prostate hyperplasia (BPH) is an aging related prostatic enlargement that affects the majority of older men1. Complications can arise from BPH that affect the quality of life of patients. Some of this complications, or symptoms, include nocturia, incomplete voiding of the bladder, weak stream, and are collective known as lower urinary tract symptoms (LUTS) 1-3. LUTS is treated with a wide range of pharmaceutical agents that target two aspects of LUTS: hyperplasia and smooth muscle dysfunction4,5. 5-alpha-reductase inhibitors, which treat hyperplasia, target the androgen receptor to reduce the size of the prostate by inhibiting cell proliferation and therefore decreasing pressure in the segment of the urethra that goes through the prostate. Alpha-1-adrenergic receptor antagonists target smooth muscle function to relax the muscular tissue surrounding the urethra, allowing better urine flow in these patients6,7. However,
these therapeutic approaches are not always effective, suggesting that additional pathobiologies may contribute to LUTS in aging men\textsuperscript{5,8}.

Recent data from several groups, including our own, has shown that inflammation plays a role in the development of collagen deposition and consequent tissue remodeling associated with fibrosis in multiple organs, including the prostate\textsuperscript{9-13}. Fibrosis is the result of unregulated tissue repair\textsuperscript{14,15}. Tissue repair involves several stages that include inflammation, the recruitment immune, vascular, and stromal cell types, extracellular matrix deposition and tissue remodeling. As part of this process, resident fibroblasts and other progenitor cells respond to inflammatory signals through proliferation and phenoconversion to a myofibroblast phenotype\textsuperscript{11}. These newly formed myofibroblasts, in turn, deposit the extracellular matrix that promotes wound closure. Upon wound repair, myofibroblasts either migrate out of the wound area or undergo apoptosis\textsuperscript{16}. If myofibroblasts abnormally persist, excess ECM is deposited, resulting in scarring and tissue stiffening\textsuperscript{17}.

Inflammatory infiltrate is commonly observed in the prostate during aging, and is especially notable in enlarged glands\textsuperscript{9,18}. In addition, our group has previously shown that pro-inflammatory proteins are secreted by aging prostate fibroblasts, particularly CXC-type chemokines\textsuperscript{19,20}. We have shown that CXCL5, CXCL8 and CXCL12 can promote the phenoconversion of prostate fibroblasts to myofibroblasts\textsuperscript{21}. However, the molecular mechanisms underlying these CXC-type chemokine-mediated phenoconversion events were not known. In this manuscript, we examined whether CXCL12/CXCR4-mediated myofibroblast phenoconversion was coupled to canonical TGFβ/TGFβR signaling. The results of these studies demonstrate that CXCL12/CXCR4-mediated myofibroblast phenoconversion is accomplished through non-canonical MEK/Erk signaling pathways. This finding is significant because it shows
that multiple signaling pathways may require targeting in order to develop effective anti-fibrotic agents for use in the lower urinary tract.

3.3 Experimental Procedures

**Cell culture and treatments.** N1 prostate fibroblasts and primary prostate fibroblasts were grown in 5% HIE culture media (Ham’s F-12, 5% FBS, Insulin [5 µg/mL], EGF [10 ng/mL], Hydrocortisone [1 µg/mL], Fungizone [0.5 µg/mL], Gentamicin [0.05 mg/mL]). Prior to treatment, cells were serum starved overnight using SF HIE (Ham’s F12, EGF [50 ng/mL], 0.1% BSA, Insulin [5 µg/mL], Transferrin [5 µg/mL], 50 µM sodium selenite, 10 uM 3,3’, 5-triiodo-L-thyronine, Hydrocortisone [1 µg/mL], Fungizone [0.5 µg/mL], Gentamicin [0.05 mg/mL]) Fibroblasts were then treated with 100pM of human CXCL12 (R&D Systems) or 50 ng/ml EGF, or 0.01% BSA vehicle, and/or with 4 ng/mL TGFβ (R&D Systems) or 20nM citrate vehicle, and collected at the desired time points. For inhibitor treatments, fibroblasts were treated with chemical inhibitors 2 hours prior to CXCL12 treatment. The chemical inhibitors used were the following: AMD3100 (25 uM, Sigma-Aldrich), SB431542 (500 nM, Sigma-Aldrich), AG1478 (500 nM, Invitrogen), U0126 (10 uM, Sigma-Aldrich) & Wortmannin (1uM, Sigma-Aldrich).

**Western Blotting**-Following the desired treatment, cells were collected in protease inhibitor cocktail (PIC)-containing PBS. Cells were lysed in Radioimmunoprecipitation assay (RIPA) Buffer (10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl). Protein quantification was carried out using Bio-Rad OneStep Bradford reagent and an Elx800 Microplate Reader (Bio-Tek) with Gen5 software. Protein lysates were prepared for electrophoresis using 4X Lithium Dodecyl Sulfate (LDS) Sample Buffer and β-ME and run in 8% Tris-Gly SDS precast gels (Life Technologies), then transferred to nitrocellulose membranes (Thermo Scientific) using the Pierce G2 blotter. Membranes were blocked using a 5% Milk TBS-T solution for one hour. Primary antibody
incubation was performed using a 5% BSA TBS-T (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.6) solution for EGFR (#2232), Y1068 pEGFR (#2234), Akt (#9272), S473 pAkt (#9271), Smad3 (#9523), S423/425 pSmad3 (#9520), Erk 1/2 (#9102), T202/Y204 pErk (#9101), Y416 pSrc (#2101), Src (#2108), TGFβRI (#3712), GAPDH (#2118) from Cell Signaling Technologies, actin (#SC1615) from Santa Cruz Biotechnology, and CXCR4 (#ab2074) from AbCam antibodies. Secondary antibody incubations using Horse Radish Peroxidase, HRP, Conjugated anti-rabbit [1:5,000 dilution, Cell Signaling] or fluorescent anti-mouse/rabbit [1:50,000 dilution, LiCor Systems]) were performed for 1 hour at room temperature. Membranes were washed twice with TBS-T and scanned using the Odyssey CLx and e-Digit (LI-COR), for the detection of fluorescent and HRP-conjugated antibodies respectively. Immunoblots were quantified and analyzed using the ImageStudio software suite (LI-COR).

**RNA Extraction and Gene Expression Analysis.** N1 and primary prostate fibroblast cells were treated as above and subjected to RNA extraction using Trizol reagent (Invitrogen, Carlsbad, CA). Purified RNA was treated with DNase and qRT-PCR analysis was performed using a QuantStudio 12K Flex Real-Time PCR System, reagents and software (Applied Biosystems, Carlsbad, CA). For all experiments, 1 μg of RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). qPCR was performed using Assays on Demand (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Reactions were performed in triplicate, including no template controls and amplification of an endogenous control transcript, Larger Ribosomal Protein (RPLPO) to assess template concentration and loading precision. Results were analyzed for integrated and precision using the QuantStudio 12K Flex Software. Cycle numbers to threshold were calculated by subtracting averaged control from averaged experimental values and collagen 1α1 (COL1α1), α-
smooth muscle actin (ACTA2) and TGFβ (TGFβ1) transcript levels were normalized to those of RPLPO using the Pfaffl method. Gene-specific assays were Hs0016400_m1 for COL1α1, Hs00909449_m1 for ACTA2, Hs00998130 for_m1 for TGFβ1, and Hs99999902_m1 for RPLPO (Applied Biosystems, Carlsbad, CA).

**Zymography analysis of MMP-2 activation.** Zymograms were performed using Novex® Zymogram Gelatin Gels using the XCell SureLock™ Mini-Cell (Invitrogen) following manufacturer's procedures. N1 prostate fibroblasts were seeded at 65-70% confluency, washed with PBS and switched to SF HIE media for 24 hrs before being treated with 0.01% BSA vehicle or 100pM CXCL12 for 60 minutes. Cell lysates were collected using RIPA buffer (pH 8.0) and 15 ug of protein were loaded for each sample in 10% Gelatin Gels. After electrophoresis, gels were incubated in Zymogram Renaturing Buffer (Invitrogen) for 30 minutes at room temperature with gentle agitation. After incubation, the gels were transferred to Zymogram Developing Buffer (Invitrogen) and equilibrated for 30 minutes at room temperature with gentle agitation. Once equilibrated, fresh Developing Buffer was added to the gels and incubated at 37°C overnight. Gels were stained using SimplyBlue™ Safestain (Invitrogen) following manufacturer’s procedure. Gels were scanned using the Odyssey CLx Imager (LI-COR) and mean area over the cover for the lack of pixel was measured using IMAGEJ (http://rsbweb.nih.gov/ij/index.html).

**Immunofluorescence.** N1 prostate fibroblasts were plated on chamber slides coated with 10ug/ml fibronectin (Sigma-Aldrich, St. Louis, MO). Cells were washed with phosphate buffered saline (PBS), and then switched to SF HIE media for 24 hr. The cells were then treated with 0.1% BSA in PBS (vehicle) or 100pM CXCL12 for 48 hours at 37°C in a 5% CO2 incubator in the absence or presence of small molecule inhibitors or antibodies as described
above. Primary antibodies were diluted in blocking solution and included 1:200 dilution FITC-conjugated mouse monoclonal anti-α-smooth muscle actin (aSMA), and 1:100 dilution biotin-conjugated rabbit polyclonal anti-collagen type 1 (COL1) (Rockland Immunochemicals, Gilbertsville, PA). Cells were counterstained for 5 min with 1 mg/ml DAPI (Molecular Probes, Eugene, OR) in Tris-Buffered Saline/Tween 20, washed three times for 5 min each with TBST, and mounted in an Aqua-mount (Lerner Laboratories, PA). Photomicrographs were taken on an Olympus BX51 fluorescence microscope. PE-Cy 5 streptavidin (BD Pharmingen San Diego, CA) or anti-mouse Alexa 488 or Alexa 555 (Invitrogen, Carlsbad, CA) secondary antibodies were used at 1:2000 dilutions. Control mouse IgG2a (Sigma-Aldrich, St. Louis, MO) and rabbit IgG biotin conjugate (Rockland Immunochemicals, Gilbertsville, PA) were used at 1:2000 dilution. Fluorescent images at 40x were digitally captured using an Olympus BX51 photomicroscope with mercury bulb and Olympic filter cubes U-MU (dichroic mirror DM400, excitation filter BP330–385, barrier filter BA420), U- MWB (dichroic mirror DM500, excitation filter BP450–480, barrier filter BA515) and U-MSWB (dichroic mirror DM570, excitation filter BP510–550, barrier filter BA590).

**Statistical Analysis.** Averages and standard deviations were calculated and compared using Student’s t-tests. In all tests, p<.05 was considered statistically significant.
3.4 Results

**CXCL12/CXCR4 axis-mediated myofibroblast phenoconversion is not coupled to Smad3 phosphorylation.**

We have previously shown that both CXCL12 and TGFβ mediate the myofibroblast phenoconversion of prostate fibroblasts\(^{21}\). Many studies have shown that activation of the TGFβ/TGFβR axis promotes immediate Smad3 phosphorylation during myofibroblast phenoconversion\(^{14,24-26}\). Therefore, we first examined whether activation of the CXCL12/CXCR4 axis also promoted Smad3 phosphorylation during myofibroblast phenoconversion. To test this, N1 immortalized\(^{22}\) or SFT1\(^{23}\) primary prostate fibroblasts were treated in SF HIE media with either 100pM CXCL12 or 4ng/ml TGFβ. The results of these studies showed that activation of the CXCL12/CXCR4 axis rapidly but phosphorylated the EGFR at tyrosine 1068 as well as downstream MAPK/Erk and PI3K/Akt signaling pathways, but did not promote Smad3 phosphorylation, in both N1 (Fig 1A) and SFT1 (Fig 1B) cells. In contrast, TGFβ treatment strongly induced Smad3 phosphorylation as expected, but not that of EGFR or Erk in both cell cultures (Fig 1 A, B). It is important to note that TGFβ treatment weakly activated Erk signaling at 5 minutes post-treatment though this effect was greatly reduced compared to CXCL12 treatment. Taken together, these data indicate that the CXCL12/CXCR4 axis promotes signaling through Smad-independent, non-canonical EGFR and downstream MAPK/Erk and PI3K/Akt pathways in the absence of TGFβ in both N1 and primary prostate fibroblasts. This clearly indicates that CXCL12 and TGFβ stimulate different intracellular signaling mechanisms to promote myofibroblast phenoconversion.
**Figure 3.1** CXCL12/CXCR4 axis-mediated myofibroblast phenoconversion is not coupled to Smad3 phosphorylation. N1 fibroblasts (A) and primary prostate fibroblasts (B) were treated in defined serum-free Ham’s media with CXCL12 (100pM), or 0.01% BSA vehicle, and TGFβ (4 ng/mL) or 20mM citric acid vehicle. In both types of cells EGFR, Akt and Erk1/2 were phosphorylated upon CXCL12 treatment. TGFβ treatment activated Smad-mediated signaling and transient Erk1/2 phosphorylation, but not EGFR or Akt phosphorylation. Total antibodies for each kinase, as well as GAPDH and actin, were used as loading control. Protein molecular weight in kilodaltons is indicted by arrows.
CXCL12/CXCR4-mediated signaling and myofibroblast phenoconversion requires EGFR transactivation.

Previously our group had shown that intracellular signaling downstream of CXCL12/CXCR4 axis activation requires transactivation of the EGFR in prostate epithelial cells\textsuperscript{27}. In order to determine whether EGFR transactivation is required for CXCL12/CXCR4-mediated signaling in prostate fibroblasts, N1 cells were pre-treated with inhibitors that block CXCR4 activation (AMD3100, AMD); EGFR auto-phosphorylation (AG1478, AG), MEK activation (U0126, UO), P13K activation (Wortmannin, Wrt) or TGF\(\beta\)RI activation (SB431542, SB). Untreated cells demonstrate little or no basal phosphorylation of Akt, Smad3, or ERK (Figure 2A). When treated with EGF, N1 cells demonstrate low Akt and Smad3 phosphorylation but robust ERK phosphorylation (Figure 2A). In contrast, N1 cells treated with TGF\(\beta\) demonstrated robust Smad3 phosphorylation but no Akt or ERK phosphorylation (Figure 2A). When treated with 100pM CXCL12, N1 cells evinced elevated levels of EGFR and ERK phosphorylation (Figure 1B). However, EGFR phosphorylation was not evident in cells pre-treated with AMD or AG prior to CXCL12 treatment (Figure 2B). Moreover, neither ERK or Akt phosphorylation were evident in cells pretreated with U0 or Wrt prior to CXCL12 treatment (Figure 2B) These data suggest that activation of the CXCL12/CXCR4 axis promotes EGFR transactivation, and that this transactivation is necessary for downstream intracellular signaling in prostate fibroblasts. In contrast, N1 cells pre-treated with SB431542, small molecule inhibitor of the TGF\(\beta\)RI, showed uninterrupted CXCL12/CXCR4 axis signaling, suggesting that inhibition of the TGF\(\beta\)/TGF\(\beta\)RI was not coupled to CXCL12/CXCR4 axis signaling.
Figure 3.2 CXCL12/CXCR4-mediated signaling and myofibroblast phenoconversion requires EGFR transactivation (A) N1 fibroblasts were pre-treated with chemical inhibitors of CXCR4 (25 μM AMD3100), EGFR (500 nM AG1478), MEK/ERK (10 μM U0126), P13K (1μM Wortmannin [WORT]), and TGFβRI (500 nM SB431542) or with 50 ng/ml EGF or 4ng/ml TGFβ for three hours. Very little basal phosphorylation was observed though treatment with U0126 promoted higher levels of basal pAkt. (B) N1 fibroblasts were pre-treated with chemical inhibitors of CXCR4 (25 μM AMD3100), EGFR (500 nM AG1478), MEK/ERK (10 μM U0126), P13K (1μM Wortmannin [WORT]), and TGFβRI (500 nM SB431542) for two hours prior to CXCL12 treatment. Treatment with the EGFR inhibitor AG1478, totally ablated the phosphorylation and activation of downstream targets. Inhibition of TGFβRI activation with SB431542 had no effect of CXCL12 signaling.
Based on these results we next examined the potential functional effects of EGFR inhibition on CXCL12/CXCR4-mediated transcription of genes that encode smooth muscle actin (ACTA2) or collagen 1 (COL1α1), both of which are transcribed during myofibroblast phenoconversion. Pre-treatment with AG1478 prior to CXCL12 treatment completely inhibited CXCL12/CXCR4 axis-mediated intracellular signaling activation as observed by the lack of EGFR, Erk, or Akt phosphorylation (Figure 3A). As observed previously, activation of the CXCL12/CXCR4 axis did not promote Smad3 phosphorylation (Figure 3A). Quantitative RT-PCR studies showed that activation of the CXCL12/CXCR4 axis promoted transcription of both ACTA2 and COL1α1 at levels significantly higher than vehicle-cells (Figure 3B). Pre-treatment with the EGFR inhibitor, AG1478 ablated CXCL12/CXCR4 axis-induced transcription of both genes (Figure 3B).
Figure 3.3 Activation of the CXCL12/CXCR4 and TGFβ/TGFβR axes independently promote ACTA2 and COL1A1 gene expression (A) N1 fibroblasts were treated with vehicle (DMSO) or EGFR inhibitor (AG1478, 500 nM) prior to CXCL12 treatment. Phosphorylation of EGFR, Akt, Smad and Erk1/2 were assessed via western blot. (B) qRT-PCR analysis of myofibroblast marker expression after CXCL12 (100pM) treatment in the presence or absence of AG1478 (500 nM). Expression levels of α-smooth muscle actin (ACTA2) and collagen 1α1 (COL1α1) were analyzed over the course of 24 hours of treatment. * = p-value < 0.05. Error bars, SE. Treatment with AG1478 reduced or ablated the CXCL12/CXCR4-mediated stimulation of both the ACTA2 and COL1α1 genes.
**MMP and Src activation are associated with CXCL12/CXCR4-mediated EGFR transactivation.**

The EGFR can be phosphorylated through extracellular means through Src-mediated MMP/ADAM activation which catalyzes EGF-type ligand shedding, or through intracellular means via Src-mediated phosphorylation of Tyr845 within the EGFR tyrosine kinase domain. Previously our group reported that CXCL12/CXCR4 axis-mediated transactivation of EGFR was coupled to activation of Matrix Metalloproteinase (MMP) and A Disintegrin and Metalloproteinase (ADAM) proteins which, in turn, catalyzed shedding of the EGFR ligand, amphiregulin, in prostate epithelial cells. Therefore, we pursued Zymogram analysis to determine whether CXCL12/CXCR4 axis-mediated transactivation of EGFR in prostate fibroblasts might also depend upon metalloproteinase activation. The results of these studies showed that whole cell lysates demonstrated depletion of pro-MM2 and accumulation of activated MMP2 in CXCL12-treated compared to vehicle-cells (Figure 4A). Western blot analysis of N1 fibroblasts pre-treated with BB-94, a pan-inhibitor of MMPs and ADAMs, prior to CXCL12 treatment demonstrated dramatically decreased levels of EGFR and ERK phosphorylation compared to non-pretreated cells (Figure 4B). Further western blot studies demonstrated phosphorylation of Src at Y416 and of EGFR at Y845 subsequent to treatment of N1 cells with 100pM CXCL12. Taken together, these results clearly indicate that CXCL12/CXCR4 axis-mediated phosphorylation of Src contributes to EGFR transactivation through direct phosphorylation at Y845 and through MMP-2 activation. These data also suggest that Src activation and downstream MMP/ADAM activation may provide novel therapeutic targets to inhibit CXCL12/CXCR4 driven profibrotic stimuli.
Figure 3.4 The CXCL12/CXCR4 axes mediates EGFR transactivation through both external and internal cellular mechanisms. (A) Zymography of MMP-2 upon CXCL12 (100pM), or 0.01% BSA/PBS vehicle treatment (upper panel) was analyzed using ImageJ to quantify pixel intensity and visualize early MMP-2 activation (lower panel). Pro-MMP-2 levels decrease while activated MMP-2 levels increase consequent to CXCL12 treatment. Data shown is representative of replicate experiments. (B) N1 fibroblasts were treated with BB-94 (500 nM), a pan-inhibitor of MMPs and ADAMs, prior to CXCL12 treatment. CXCL12/CXCR4 axis-mediated signaling was analyzed via western blot for phosphorylation of EGFR and MEK/Erk. Total antibodies for each kinase and actin were used as loading control. Both EGFR and ERK phosphorylation are greatly inhibited by BB-94, suggesting that CXCL12/CXCR4 axis-mediated MMP-2 activation is an extracellular mechanism for EGFR phosphorylation. (C) N1 fibroblasts were treated with 100pm CXCL12 demonstrate phosphorylation of Src and EGFR at Y845, suggesting that CXCL12/CXCR4 axis-mediated Src activation is an intracellular mechanism for EGFR phosphorylation.
The CXCL12/CXCR4 and TGFβ/TGFβRI axes function independently to promote myofibroblast phenoconversion.

In order to determine whether the CXCL12/CXCR4 and TGFβ/TGFβRI axes functioned together, in parallel, or independently to promote myofibroblast phenoconversion, we performed siRNA-mediated knockdowns of TGFβRI and CXCR4 to determine whether CXCR4 alone or both CXCR4 and TGFβRI were required for CXCL12-mediated myofibroblast phenoconversion. N1 cells knocked down for the CXCR4 receptor then treated with CXCL12 demonstrated greatly reduced EGFR, Akt and Erk1/2 phosphorylation when compared to control scramble siRNA transfected cells (Figure 5A). However, N1 cells knocked down for the TGFβRI receptor demonstrated robust phosphorylation of EGFR, Akt and Erk1/2 compared to control scramble siRNA transfected cells (Figure 5B). These results indicate that CXCL12-mediated intracellular signaling is coupled only to CXCR4, and does not require TGFβRI. To further test this finding, N1 cells knocked down for CXCR4 or TGFβRI were treated with CXCL12 and monitored for ACTA2 and COL1α1 gene expression. As shown in Figure 5C, CXCR4 knock-down, but not TGFβRI knock-down cells, demonstrated significantly reduced ACTA2 and COL1α1 transcript levels compared to control scramble siRNA transfected cells in response to treatment with CXCL12. Conversely, cells knocked down for TGFβRI, but not CXCR4, demonstrated significantly reduced ACTA2 and COL1α1 transcript levels compared to control scramble siRNA transfected cells in response to treatment with TGFβ (Figure 5D).
Figure 3.5 The CXCL12/CXCR4 and TGFβ/TGFβRI axes function independently to promote myofibroblast phenoconversion. N1 fibroblasts were transfected with 20nM Scramble, anti-CXCR4 (A), and anti-TGFβRI (B) siRNAs for 24 hours and treated with CXCL12 (100pM) for 1 hour. CXCR4 partial knockdown (A) reduced the CXCL12-mediated activation of EGFR, Akt and Erk1/2. However, TGFβRI knockdown (B) had no effect in the CXCL12-mediated activation of EGFR, Akt and Erk1/2. siRNA-mediated knockdown were validated using antibodies against target receptor. Total antibodies for each kinase and actin were used as loading control. N1 fibroblasts were transfected with 20nM Scramble, anti-CXCR4, and anti-TGFβRI siRNAs for 24 hours and treated with CXCL12 (100 pM) (C) and TGFβ (4 ng/mL) (D) for 24 hours. qRT-PCR analysis of α-smooth muscle actin (ACTA2), collagen 1α1 (COL1α1) and TGFβ (TGFβ 1) was performed. * = p-Value <0.05, ** = p-Value < 0.001. Error bars, SE.
Based on these findings, immunofluorescence studies were performed to examine potential axes cross talk at the protein level. As shown in Figure 6, N1 cells treated with CXCL12 demonstrate co-expression of alpha smooth muscle actin (αSMA) and collagen 1 (COL1) proteins and the acquisition of a stellate morphology consistent with myofibroblast phenoconversion. Co-expression was ablated in cells pre-treated with the MEK inhibitor U0126; the CXCR4 inhibitor AMD3100; the PI3K inhibitor Wortmannin (WORT), and the EGFR inhibitor AG1478 (Figure 6). In contrast, cells pre-treated with either the TGFβR1 small molecule inhibitor A-83-01 or an anti-TGFβR1 monoclonal antibody then treated with CXCL12 demonstrated robust αSMA and COL1α1 co-expression and myofibroblast morphology (Figure 6). Taken together, the siRNA and immunofluorescence data demonstrate that the CXCL12/CXCR4 axis promotes the transcription and expression of genes and proteins associated with myofibroblast phenoconversion in the absence of TGFβ or the TGFβ/TGFβR signaling axis. This suggests a novel and non-canonical mechanism by which a pro-inflammatory chemokine promotes myofibroblast phenoconversion. As shown schematically in Figure 7, our data shows that CXCL12/CXCR4 axis activation stimulates Src phosphorylation and MMP/ADAM protein activation that promotes transactivation of the EGFR and downstream MEK/Erk and PI3K/Akt signaling cascades. The activation of these cascades leads to the expression of myofibroblast markers such as ACTA2 and COL1α1, coincident with the phenoconversion of prostate fibroblasts to myofibroblasts. These events occur in the complete absence of TGFβ and are not coupled to activation of the TGFβ/TGFβR axis. Therefore, the CXCL12/CXCR4 and TGFβ/TGFβR axes function independently to promote prostate myofibroblast phenoconversion.
Figure 3.6 Inhibition TGFβ and TGFβRII-mediated signaling does not affect CXCL12-mediated myofibroblast phenoconversion. Immunofluorescence analysis of N1 fibroblasts left untreated (CXCL12) or treated with 100pM CXCL12 (CXCL12) for 48 hours in the absence or presence of MEK/ERK (10 uM U0126), CXCR4 (250 uM AMD3100), EGFR (250 uM AG1478), P13K (1uM Wortmannin [WORT]), ALK-5 (TGFβRII)(20uM A-83-01) small molecular inhibitors or an antibody against TGFβRII (200 ng/ml TGFβ MAb). Figure depicts photomicrographs merging separate images of cells stained for α-smooth muscle actin (green) or collagen 1 (red) proteins or DAPI (blue nuclear stain); orange color indicates α-smooth muscle actin and collagen 1 colocalization.
Figure 3.7 Proposed mechanism of action used by CXCL12 to drive myofibroblast phenoconversion. Upon binding to CXCR4, CXCL12 promotes the activation of MMPs, and potentially Src, leading to the transactivation of EGFR. Active EGFR then activates Akt and MEK/Erk signaling pathways to promote myofibroblast phenoconversion. CXCL12-mediated phenoconversion acts independently of TGFβR/Smad-mediated myofibroblast phenoconversion.
3.5 Discussion

It has been documented previously that EGFR transactivation plays a role in the fibroblast to myofibroblast differentiation of lung fibroblasts through lipid raft-bound CD44 and TGFβ-mediated signaling\(^\text{30}\). However, we observed that EGFR transactivation in prostate fibroblasts by the CXCL12/CXCR4 axis occurred in the absence of TGFβ. We report in this study that activation of the CXCL12/CXCR4 axis leads to a rapid phosphorylation of Src, EGFR, and downstream MEK/Erk and PI3K/Akt signaling pathways, and EGFR transactivation is essential for the expression of myofibroblast markers at the RNA and protein (summarized in Figure 7). Moreover, EGFR transactivation may occur through both extracellular, ADAM/MMP-mediated mechanisms as well as through intracellular phosphorylation of the Y845 residue, as previously observed in prostate epithelial cells\(^\text{31}\).

It was recently reported that TGFβ and CXCL12 work together in an autocrine fashion to promote the differentiation of mammary stromal myofibroblasts\(^\text{24}\). However, our results show no crosstalk between TGFβ and CXCL12 in prostate fibroblasts. Small molecule inhibition of TGFβRI signaling neither augmented, nor ablated activation of the EGFR or downstream MEK/Erk and PI3K/Akt signaling cascades by CXCL12. More importantly, siRNA-mediated knockdown of CXCR4 and/or TGFβRI had no effect in the activation and downstream gene expression induction by the other receptor by its respective ligand. Knock down or inhibition of TGFβRI had no effect in the CXCL12/CXCR4 axis-mediated expression of myofibroblast markers at the RNA level (Figure 5) and did not inhibit myofibroblast phenoconversion (Figure 6). These results clearly indicate that CXCL12 and TGFβ promote prostate myofibroblast phenoconversion independently, through mechanisms that do not crosstalk or overlap.
Our study provides further insight into prostatic fibrosis, which is a potential pathobiology of benign prostatic disease contributing to lower urinary tract symptoms (LUTS) \(^8,32\). Other diseases such as idiopathic pulmonary fibrosis, pancreatic dysfunction and cirrhotic nonalcoholic fatty liver disease include fibrosis as a major component of their pathobiologies \(^{16,33}\). Our group has documented the presence of peri-urethral tissue fibrosis in human \(^{34}\) and mouse tissues associated with lower urinary tract dysfunction \(^{13}\), and has shown that myofibroblast phenoconversion can be promoted by pro-inflammatory CXC-type chemokines as well as TGFβ \(^{21}\). The data presented in the current study extends these findings and elucidates the signaling mechanisms through which activation of CXCR4 by CXCL12 induces a TGFβ / TGFβR-independent transactivation of EGFR by MMP/ADAM proteases, which in turn leads to downstream signaling by MEK/Erk and PI3K/Akt to induce myofibroblast marker expression and phenoconversion (Figure 7). Thus, CXCL12/CXCR4 axis activation may provide an attractive molecular target for novel therapeutics to treat prostatic fibrosis and LUTS.
3.6 References


CHAPTER 4

Whole Transcriptome Sequencing and Target Hits Validation in CXCL12 and TGFβ
treated N1 fibroblasts

4.1 Introduction

Our studies have shown that, through non-cannonical signaling pathways, the CXCL12/CXCR4 axis can drive the \textit{in vitro} phenoconversion of prostate fibroblasts to myofibroblasts. This data complements our previous studies showing that diet-induced inflammation leads to LUTS-like symptoms in mice and the development of prostatic fibrosis\textsuperscript{1}, as well as our study into tissue dynamics and the positive correlation between stiffness, and collagen deposition, and symptom severity in patients\textsuperscript{2}. In this dissertation we have presented so far that the inflammatory chemokine CXCL12, which is up-regulated by aging prostate fibroblasts\textsuperscript{3,4}, achieves phenoconversion of prostate fibroblasts into myofibroblasts just as well as TGFβ, which is considered the main driving force of tissue fibrosis. TGFβ / TGFβ R achieves myofibroblast phenoconverion through Smad signaling, while the CXCL12/CXCR4 axis rely on the transactivation of EGFR and subsequent activation of MEK/Erk and Akt signaling pathways.
Although both molecules begin phenoconversion through different mechanisms, the end result is the same when it comes to myofibroblast phenoconversion as evidence by expression of myofibroblasts markers and functional assays. These results lead us to hypothesize that CXCL12/CXCR4 and TGFβ/ TGFβR activation must share a substantial amount of their transcriptional footprint in order to promote phenoconversion of prostate myofibroblasts. In order to test this hypothesis we used next-generation sequencing techniques to perform whole transcriptome sequencing, known as RNASEq, of CXCL12 and TGFβ treated prostate fibroblast in order to identify similarities between the treatments. RNASEq allows us to look, not only at our genes of interest such as COL1A1 and ACTA2, but at all the genes that are been regulated by the treatments when compared to vehicle controls. The goal of this study is to verify whether or not there is a shared transcriptional signature between treatments, and identify unique transcriptional signatures that could help us differentiate between treatments.

4.2 Experimental Procedures

**Cell culture and chemokine treatment.** N1 prostate fibroblasts were grown in 5% HIE culture media (Ham’s F-12, 5% FBS, Insulin [5 µg/mL], EGF [10 ng/mL], Hydrocortisone [1 µg/mL], Fungizone [0.5 µg/mL], Gentamicin [0.05 mg/mL]). Prior to treatment, cells were serum starved overnight using SF HIE (Ham’s F12, EGF [50 ng/mL], 0.1% BSA, Insulin [5 µg/mL], Transferrin [5 µg/mL], 50 µM sodium selenite, 10 uM 3,3’,5-triiodo-L-thyronine, Hydrocortisone [1 µg/mL], Fungizone [0.5 µg/mL], Gentamicin [0.05 mg/mL]). Fibroblasts were then treated with 100pM of human CXCL12 (R&D Systems), or 0.01% BSA vehicle, and/or with 4 ng/mL TGFβ (R&D Systems) or 20nM citrate vehicle, and RNA collected at 12 hours post-treatment.
**RNA Extraction and Gene Expression Analysis.** N1 and primary prostate fibroblast cells were treated as above and subjected to RNA extraction using Trizol reagent (Invitrogen, Carlsbad, CA). Purified RNA was analyzed for concentration, sizing and quality control using the 2100 Bioanalyzer (Agilent Technologies). RNA samples were then handled over to the Genomics Core of the Center for Personalized Cancer Therapy (University of Massachusetts, Boston) where RNASeq was carried out using the Illumina 2500 Ultra High-Throughput Sequencer.

**Data Analysis.** RNASeq data analysis was carried out with the help of Todd Riley, professor of Bioinformatics at the University of Massachusetts, Boston and Diego Almanza, undergraduate student in Biology at the University of Massachusetts, Boston. Aligned sequences were analyzed with EdgeR R Package (Bioconductor) for differential gene expression, and with Pathview (Bioconductor) for pathway data integration and visualization.

**Western blot analysis-target validation.** Following treatment with CXCL12, TGFβ, or vehicle for 12 hours, N1 cells were collected in protease inhibitor cocktail (PIC)-containing PBS. Cells were lysed in Radioimmunoprecipitation assay (RIPA) Buffer (10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl). Protein quantification was carried out using Bio-Rad OneStep Bradford reagent and an Elx800 Microplate Reader (Bio-Tek) with Gen5 software. Protein lysates were prepared for electrophoresis using 4X Lithium Dodecyl Sulfate (LDS) Sample Buffer and β-ME and run in 8% Tris-Gly SDS precast gels (Life Technologies), then transferred to nitrocellulose membranes (Thermo Scientific) using the Pierce G2 blotter. Membranes were blocked using a 5% Milk TBS-T solution for one hour. Primary antibody incubation was performed using a 5% BSA TBS-T (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.6) solution for CUL1 (#2232), CUL3 (#2234), CUL4A (#9272), KLHL12 (#9271), and GAPHD (#9523) from Cell Signaling.
Secondary antibody incubations using Horse Radish Peroxidase, HRP, Conjugated anti-rabbit [1:5,000 dilution, Cell Signaling] were performed for 1 hour at room temperature. Membranes were washed twice with TBS-T and scanned using the Odyssey CLx and c-Digit (LI-COR). ImmunobLOTS were quantified and analyzed using the ImageStudio software suite (LI-COR).

**RNA extraction and gene expression analysis.** N1 cells were treated as above and subjected to RNA extraction using Trizol reagent (Invitrogen, Carlsbad, CA). Purified RNA was treated with DNase and qRT-PCR analysis was performed using a QuantStudio 12K Flex Real-Time PCR System, reagents and software (Applied Biosystems, Carlsbad, CA). For all experiments, 1 ug of RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). qPCR was performed using Assays on Demand (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Reactions were performed in triplicate, including no template controls and amplification of an endogenous control transcript, Larger Ribosomal Protein (RPLPO) to assess template concentration and loading precision. Results were analyzed for integrated and precision using the QuantStudio 12K Flex Software. Cycle numbers to threshold were calculated by subtracting averaged control from averaged experimental values and cullin 1 (CUL1), cullin 3 (CUL3), cullin 4A (CUL4A), and cullin 4B (CUL4B) transcript levels were normalized to those of RPLPO using the Pfaffl method. Gene-specific assays were Hs01117001_m1 for CUL1, Hs00180183_m1 for CUL3, Hs00757716_m1 for CUL4A, Hs00186086 for CUL4B and Hs99999902_m1 for RPLPO (Applied Biosystems, Carlsbad, CA).

**Collagen secretion analysis.** N1 fibroblasts were treated as described above for 72 hours and cell lysates were collected in RIPA buffer. Enzyme-linked immunosorbent analysis was carried out using Chondrex Human Collagen 1 (#6021) and Collagen II (#6018) following
manufacturers procedures. Sample reading was carried out was using the Elx800 Microplate Reader (Bio-Tek) with Gen5 software.

4.3 Results

**CXCL12 & TGF-β have similar transcriptomes**

Although CXCL12 and TGFβ signal through different molecular mechanisms to drive myofibroblast pheconversion, the transcriptional signature for both molecules is relatively similar. We focused our RNASeq analysis to genes with over 100 alignments, or counts, that had a p-Value < 0.001 to identify upregulated/downregulated genes. For CXCL12, we identified 9,029 genes that were differentially regulated when compared to vehicle control. The differentially expressed genes belong to a wide range of process including: anti-viral response, DNA repair, cell cycle progression, and Golgi transport. For TGFβ, we identified 8,683 genes that were differentially regulated when compared to vehicle control. The differentially expressed genes, although as diverse as those identified in CXCL12, heavily focused on ECM components, assembly and disassembly. Interestingly, CXCL12 and TGFβ shared many of the genes that are differentially upregulated by both molecules, as evidenced in Table 3.1. By looking at the top 25 upregulated genes for each molecule, we identified (highlighted in red) 11 genes that are shared by both molecules upon treatment to prostate fibroblasts. However, although both molecules share wide ranges of genes, TGFβ has an overall more robust transcriptional response. This is evident at two different levels: the fold change of the top hit gene (13.91 FC for CXCL12, 100.29 FC for TGFβ), and at the shared genes expression fold change (i.e. RSAD2, 10.55 FC for CXCL12, 20.21 for FC TGFβ). The same trend was observed in the top 25 genes that are differentially upregulated between each treatment (genes that are upregulated with CXCL12 compared to genes upregulated by TGFβ, and vice versa). In Table 3.2 we can observe that
genes that are upregulated by TGFβ over CXCL12 exhibit a higher fold change than those of CXCL12 over TGFβ. Only one of the genes upregulated by CXCL12 (FABP4) over TGFβ is within the range of fold changes observed in the TGFβ group. Interestingly, out of the top 25 differentially upregulated genes by TGFβ over CXCL12, 8 of them are directly related to ECM assembly and remodeling (Table 4.2). This shows the robust, and biased, transcriptional response that TGFβ is known for to promote fibrosis and myofibroblast differentiation.
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In order to address whether or not CXCL12 and TGFβ truly have similar transcriptional response we had to work around the robust TGFβ-mediated transcriptional response since it masked any transcriptional changes by CXCL12 when compared to each other. To achieve this, we used the Pathview R package to integrate our gene expression data into pathways of interest and visualize how each molecule is affecting the expression of the members of pathways of interest to our study. In addition to pathway data integration for visualization, Pathview’s data normalization algorithm considers the number of counts, for experimental and reference genes, and the depth of sequencing. This normalization algorithm bypasses any masking by TGFβ over CXCL12-treated cells. Pathview analysis of normalized sequencing data showed that CXCL12 and TGFβ have almost identical transcriptional signatures for the regulation of ECM-related proteins and their membrane bound receptors (Figure 4.1, 4.2). Upregulation of genes from the collagen, laminin, and fibronectin family of ECM proteins is evident for both CXCL12 and TGFβ. As previously stated, collagen is an essential component of the ECM secreted by myofibroblast during differentiation and tissue repair, and it interacts with fibronectin and laminin to assemble a complex network of fibers that are required for proper myofibroblast function\(^8,9\). In addition to ECM components, we observed that both molecules upregulate the expression of key members of the integrin family of proteins that are essential ECM binding partners for myofibroblast function. Integrins \(\alpha1,2,4,7\) and \(\beta1\) are required for ECM interaction and maintenance of phenotype\(^9\), and these are all upregulated by both molecules based on our Pathview analysis.
Figure 4.1 Pathview analysis for ECM components and membrane bound interacting partners for CXCL12 treated fibroblasts. Arbitrary gene expression unit were assigned from -1 to 1, and these are color coded; green correspond to down regulated genes, red corresponds to upregulated genes when compared to vehicle treated control, gray areas represent no significant change between treatments, white indicates no data available from the imported sequencing files. Color coding represents the average of three independent experiments. Important components for myofibroblast phenoconversion, such as collagen, lamininn and fibronectin, are upregulated by CXCL12 in prostate fibroblasts.
Figure 4.2 Pathview analysis for ECM components and membrane bound interacting partners for TGFβ treated fibroblasts. Arbitrary gene expression unit were assigned from -1 to 1, and these are color coded: green correspond to down regulated genes, red corresponds to upregulated genes when compared to vehicle treated control. Gray areas represent no significant change between treatments, white indicates no data available from the imported sequencing files. Color coding represents the average of three independent experiments. Important components for myofibroblast phenoconversion, such as collagen, lamininn and fibronectin, exhibit robust upregulation by TGFβ, as well as essential integrins for ECM anchoring and signaling in prostate fibroblasts.
Overall this data analysis shows that, although CXCL12 and TGFβ work through completely different pathways to promote prostate myofibroblasts phenoconversion, the overall transcriptional signature for both molecules in terms of ECM-related genes is similar. However, when we conducted the same analysis but instead of comparing each treatment to the vehicle control, we compared CXCL12 to TGFβ, we confirmed once again that the transcriptional response by TGFβ is more robust than that of CXCL12. As it can be observed in Figure 4.3, except for Laminin, all the ECM and Integrin members are at lower expression levels in the CXCL12 samples than TGFβ. This is not surprising as it has been heavily documented that TGFβ strongly induces pro-fibrotic genes expression\textsuperscript{10}. Previously we have documented this as well when we compared the level of expression of the genes collagen 1α1 and α-smooth muscle actin between CXCL12 and TGFβ treated prostate fibroblasts\textsuperscript{4}. This data confirms that CXCL12 and TGFβ have, at different levels, the same transcriptional signature. This discovery is of great importance to our study since it further proves that CXCL12 and TGFβ, in prostate fibroblasts, have the same end effect, or result, in terms of myofibroblasts and cell differentiation. Although this data shows that CXCL12 and TGFβ share similar transcriptional signature, it is our goal to identify CXCL12 unique pathways or mechanisms. In the next section we will address the validation of one mechanism that seems to be unique to CXCL12-driven myofibroblast phenoconversion.
Figure 4.3 Pathview analysis for ECM components and membrane bound interacting partners for TGFβ and CXCL12 treated fibroblasts. Arbitrary gene expression unit were assigned from -1 to 1, and these are color coded. This figure represents CXCL12-mediated gene expression, over TGFβ-mediated expression. It is observed that, although CXCL12 upregulates the same ECM and integrin members as TGFβ, when compared to TGFβ the expression of these genes is much lower for CXCL12. This data analysis shows the difference in gene expression intensity by both molecules.
Figure 4.4 Pathview analysis for Cullin-RING ubiquitin protein ligases for TGFβ and CXCL12 treated fibroblasts. CXCL12 and TGFβ upregulate cullins 1-5, and the BTB-containing binding partner for cullin 3. CXCL12 over TGFβ analysis revealed that CXCL12 induces stronger expression of the RING finger member RBX1 and cullin 3 recognizing BTB-containing protein.
Figure 4.5 Cell morphology analysis for N1 fibroblasts treated with vehicle (0.1% BSA), CXCL12 or TGFβ for 24 hours. Fibroblasts showed higher amounts of ribosome-like structure after CXCL12 treatment when compared to vehicle and TGFβ treated cells. Representative cells, containing high numbers of these vesicles are indicated with black arrow heads.
Validation of a subset of genes reveals upregulation of collagen export by CXCL12

After identifying and analyzing the top 500 genes for each treatment, we identified a group of proteins that are significantly upregulated by CXCL12 and TGFβ that are related to myofibroblasts differentiation and ECM deposition. Pathview analysis of the cullin RING ubiquitin ligase family of proteins showed upregulation by both CXCL12 and TGFβ (Figure 4.4). When expression of these proteins by CXCL12 was analyzed over TGFβ it was discovered that cullin proteins are upregulated at the same level by both treatments. However expression of the cullin adapter RBX1 and cullin 3 interacting domain BTB was upregulated at a higher level in CXCL12 treated samples than TGFβ. Cullin proteins are involved in ER-Golgi vesicles trafficking, are cullin 3 is required for the export of collagen from the ER to the extracellular space by assembling ribosome-looking COPII vesicles\(^{11}\). Automatically these proteins became on interest for our study since they might hint at a mechanistic difference between the treatments, especially since it has been observed that CXCL12 treated fibroblasts exhibit higher amounts of ribosome-like vesicles as early as 24 hours post treatment (Figure 4.5). In order to validate, and confirm, that CXCL12 is promoting a higher gene expression of cullin-RING related than TGFβ. We conducted RT-PCR analysis of RNA extracts from cells treated with both molecules. RT-PCR analysis of early time points (0, 4, 8, 24 hours post treatment) of CXCL12 and TGFβ treated N1 fibroblasts yielded results as expected from the cell morphology analysis. The expression levels for cullin 1, 3, 4A and 4B were higher at 4 and 8 hours-post CXCL12 treatment when compared to TGFβ (Figure 4.6). Cullin 1, 3, 4A and 4b were also analyzed at the protein level via western blot and had higher expression levels in CXCL12-treated cells were evident at 24 and 72 hours post treatment (Figure 4.7). This data shows that, although the RNASeq analysis showed that CXCL12 and TGFβ upregulate cullin RING proteins, the expression dynamic for
each treatment is different and CXCL12 shows higher cullin protein accumulation at later time points.

**CXCL12 promotes higher collagen export than TGFβ in prostate fibroblasts**

Upon western blot analysis it was confirmed that all cullin proteins analyzed are upregulated upon CXCL12 treatment but not TGFβ. Quantification of the western blots revealed that Cullin 4A had 4-5 fold higher levels of protein expression for the CXCL12 treated cells when compared to TGFβ treated cells. Cullin 3, specifically, binds to the BTB-containing adaptor protein KLHL12 to aid in the formation of the COPII vesicles\(^{11}\). These results indicate the CXCL12 could potentially be preferentially upregulating the collagen transport system instead of collagen production. Therefore we conducted ELISA experiments using cell lysates to quantify the amount of collagen 1 and 2 secreted by both treatments. Preliminary ELISA results show a robust upregulation of collagen 1 and 2 export by CXCL12 when compared to TGFβ and vehicle. The concentration of collagen 1 in lysates from cells treated with CXCL12 was 8 fold higher than TGFβ, and for collagen 2 the concentration was 2 fold TGFβ. Altogether this data shows that CXCL12 is upregulating the collagen transport machinery and promoting higher collagen export than TGFβ.
Figure 4.6 Activation of the CXCL12/CXCR4 promotes early induction of CUL1, 3, 4 A/B. Expression of CUL 3 and 4 A/B was significantly higher at 8 hours with CXCL12 compared to TGFβ treated cells. Expression of CUL 1, 3 and 4A was significantly higher on TGFβ treated fibroblasts. * = p-value < 0.05, ** = p-value < 0.01. Error bars, SE.
Figure 4.7 Western blot analysis of cullin proteins revealed that CXCL12 treated fibroblasts upregulates cullin 1, 3 and 4 over vehicle and TGFβ treatments. Western blot quantification showed up to 5 fold higher amount of cullins 3 and 4 in CXCL12 over TGFβ treatment.
Figure 4.8 Collagen content analysis reveals higher collagen export by CXCL12. N1 fibroblasts were treated with CXCL12 (100pM), TGFβ (4ng/mL), or vehicle (0.1% BSA) for 72 hours. Cell lysates were collected to be analyzed via ELISA for collagen content. The data shows increased export of collagen 1 & 2 by CXCL12 when compared to TGFβ and vehicle sample.
4.4 Discussion

Collagen is one of the main components of the ECM that myofibroblasts rely to promote fibrosis and tissue architecture remodeling\textsuperscript{8,12}. Mature collagen fibers are secreted from the ER into the ECM by a complex mechanism that involves the creation of COPII vesicles from the ER to the Golgi apparatus, and then to the cellular membrane. The formation of COPII vesicles requires the assembly of seven-protein member that are integral for the structure and function on this vesicles. The COPII vesicles complex is made up of members of the Sec family of proteins (Sec 13, 23, 24 31) and Cullin-RING members CUL3 and KLHL12\textsuperscript{11}. Our analysis of the RNASeq data for the members of the Cullin-RING COPII vesicles resulted in higher transcript levels for Cullin proteins for both CXCL12 and TGFβ over vehicle control. These results were consistent with the fact that collagen is being secreted in our prostate fibroblasts with either CXCL12 or TGFβ, however the changes in gene expression were not significantly different between treatments over vehicle treatment. Upon closer look at the cell morphology during CXCL12 and TGFβ treatments we observed striking morphological differences between treated cells. Prior to total phenoconversion, it was observed at 24 hours that CXCL12 treated cells exhibited more ribosomal-looking granules inside them than TGFβ, and vehicle control cells. Some of the TGFβ treated cells exhibited the same type of vesicle formation, but not at the same degree, or number, as CXCL12 treated samples (Figure 4.5, denoted by black arrow heads). COPII vesicles usually have “ribosome-like” structures, which led us to believe that CXCL12 treated fibroblasts were producing more of this vesicles compared to TGFβ. The fact that more vesicles were observed in CXCL12 treated samples than TGFβ, and higher cullin proteins levels were observed as well, led us to hypothesize that CXCL12 is exploiting the collagen transport...
system to promote myofibroblasts differentiation. In fact, ELISA experiments confirmed this by showing higher collagen content being secreted by CXCL12 than TGFβ-treated fibroblasts.

From analyzing the entire transcriptome of fibroblasts treated with CXCL12 and TGFβ, to validating some of the target genes from the sequencing analysis, we have discovered two things that support our hypothesis. CXCL12 and TGFβ have very similar transcriptional responses in prostate fibroblasts (Figures 4.1, 4.2), indicating that indeed these proteins are potentially upregulating a similar set of pro-fibrotic genes. However, western blot validation of a subset of target genes revealed a mechanism that is not shared by both molecules. Although preliminary, our data suggests that CXCL12 is promoting the upregulation of all the collagen secretion machinery to achieve ECM deposition and myofibroblast phenoconversion. More experiments are necessary to fully dissect this mechanism and to know, whether or not, CXCL12 is indeed promoting myofibroblast phenoconversion through novel mechanisms that differ from TGFβ. This could lead us one step closer to understanding how this secreted chemokines are promoting prostatic fibrosis and providing potential novel therapeutic target for it.
4.5 References


CHAPTER 5
Conclusion and Future Directions

5.1 Conclusion

Prostatic fibrosis is an overlooked and understudied pathobiology of lower urinary tract diseases (LUTD). In this dissertation, and outside studies, we provide strong evidence supporting the idea that fibrosis plays a pivotal role in the development and progression of LUTD. First, we showed that a western diet that induced diabetes promotes the development of LUTD and prostatic fibrosis in an aging-accelerated mouse model\(^1\). This was reflected in the urine spot assays, which showed inability of high fat-fed mice to void their bladders, and trichrome staining analysis, that showed increased collagen content in high fat-fed mice as well. The increase in collagen content in the LUTD suffering mice correlates with previous reports from our group that highly symptomatic patients demonstrated stiffer per-urethral tissue when mechanically tested. It has been documented that high fat diet induces inflammatory effects in different organs, and perhaps this is the same case in our mouse model\(^2-4\). High fat fed mice in our studies could have been suffering from increase inflammatory infiltrate in the that promoted the development of tissue
fibrosis\textsuperscript{1}. However there are several questions that need to be addressed in order to fully validate this animal model. For example, since our mouse studies were based on a “western diet” and are trying to mimic human eating habits; how can exercise affect the development of LUTD and prostatic fibrosis? If we revert the weight gain in our mice, do we also revert, or stopped, the development of LUTD and fibrosis? Answering these questions could provide the foundation to a non drug-based therapeutic approach to combat diet-induced LUTD. Especially since our mice are prone to aging faster, our mouse model system is completely capable of addressing these issues. These are interesting question that should be address in order to have a better understanding of the potential physiological applications of our mouse-based studies.

We then moved on to the mechanisms used by the CXCL12/CXCR4 axis to promote prostate myofibroblast phenoconversion. As previously discussed, myofibroblast are the cell type responsible for the development of tissue fibrosis. This project developed from a previous report from our group that showed how several different CXC-type chemokines can drive prostate myofibroblast phenoconversion. We focused on CXCL12 because it was the chemokine with the most robust effects in differentiating prostate myofibroblasts in our previous study\textsuperscript{5}. CXCL12 proved to be a very interesting protein to focus on since it signals through non-canonical pathways, compared to TGF\(\beta\), to achieve myofibroblast phenoconversion. The model was tested through the use of small molecule inhibitors and siRNA-mediated knockdowns, and proved that CXCL12/CXCR4 promote myofibroblast phenoconversion by transactivating EGFR and not signaling through canonical Smads. This data is very attractive since it not only further validate our previous report of CXC-type chemokine and prostate myofibroblasts, but it provides an alternative route to achieve myofibroblast phenoconversion. However, the previous study from our group reported two other chemokines, CXCL5 and CXCL8, to be also capable of driving
prostate myofibroblast phenoconversion. These two chemokines are also heavily secreted by aging fibroblasts. This poses the question, are all of these chemokines acting through the same molecular mechanisms to promote phenoconversion? If so, can new therapies be developed to target the activation of these pathways in the prostate and prevent fibrosis development? However, this could only be addressed once the mechanisms used by these two other chemokines are uncovered. Knowing these molecular mechanisms will prove to be crucial in developing new therapies since we are aware that all these chemokines are upregulated in the prostate. And it would be unrealistic, based on the fact that all three can promote myofibroblast phenoconversion, to propose that one chemokine is acting in isolation and not orchestrated with the others. CXCL5 and CXCL8 both bind to the closely related receptors CXCR1 and CXCR2.

CXCR1 and CXCR2 recognize chemokines that contain an ELR amino acid domain next to the CXC motif, and both receptors (and chemokines) play major roles in cancer progression and metastasis. Since these two receptors are closely related, and both chemokines can bind it, we hypothesize that CXCL5 and CXCL8 will have very similar signaling mechanisms potentially through the MAPK and p38 signaling pathways. However, it is not clear whether these two chemokines are capable, or depend on, of transactivating EGFR upon binding to their receptors.

If we happen to uncover that all of these chemokines shared the same signaling mechanisms in prostate fibroblasts, which would allow easier therapeutic targeting of chemokine-mediated prostatic fibrosis.

Our whole transcriptome sequencing revealed that CXCL12 and TGFβ have similar transcriptional signatures both at the overall top hit and myofibroblast-related genes. This data was expected as both molecules efficiently drive myofibroblast phenoconversion. Still, more in-depth and network analysis is needed to fully grasp the potential applications of this data.
However, preliminary analysis of the sequencing data led us to examine the cullin ubiquitin ligase-based ER-Golgi collagen transport. We decided to validate target cullin proteins for their role in collagen export expecting to see upregulation by both CXCL12 and TGFβ since this transport system is essential for collagen deposition in the ECM\textsuperscript{14}. Our experiments showed that CXCL12 treatment of fibroblasts induces a high expression of cullin proteins compared to TGFβ treatment. More surprising, ELISA analysis showed higher collagen export by CXCL12 as well, complementing the western blot data. This data has led to hypothesis several scenarios on how CXCL12 and TGFβ have similar and different mechanisms to achieve myofibroblast phenoconversion. For example, based on this preliminary data we can argue that CXCL12 is upregulating the collagen transport system to achieve ECM deposition rather than increasing collagen production. This hypothesis could provide an explanation as to why we don’t observe robust induction of collagen transcript levels compared to TGFβ, but we do observe collagen deposition by CXCL12. But in order to test this hypothesis the cullin 3-mediated collagen transport should be somehow inhibited in CXCL12 treated cells and observe whether phenoconversion is achieved on prostate fibroblasts. This mechanistic difference, in addition to the non-canonical signaling observed by CXCL12, poses as a very attractive model for novel ways in which pro-inflammatory proteins can drive cell differentiation to promote tissue fibrosis. I have focused the discussion of the data presented in this dissertation to prostatic fibrosis. However, since these chemokines are involved in many pathological conditions in several organs it is important to see whether our proposed mechanisms are unique to the prostate or shared among organs. It has been reported that the CXCL12/CXCR4 axis is involved in the development of fibrosis in the lung and heart\textsuperscript{15-17}. This leads us to believe that the pro-fibrotic effects observed by CXCL12 in our studies are not unique to the prostate. However, these reports
do not provide any mechanistic details and therefore we can’t say that the CXCL12/CXCR4 axis is acting through the same molecular mechanisms as in prostate fibroblasts. More detailed studies in different organ systems are needed to address this question.

All together the data generated in all of the projects presented in this dissertation provide us with enough ground to address all of these questions and future directions. With our aging mouse model, immortalized prostate cell lines and access to robust bioinformatics analysis tools future projects in will be able to identify the signaling mechanisms of other chemokines and validate our novel pro-fibrotic mechanisms.

5.2 Future Directions

CXCL12 role in our obese mouse model

As shown in chapter 2, our senescence accelerated mouse prone 6 (SAMP6) model develops lower urinary tract dysfunction (LUTD) as a consequence of being on a high fat diet\(^1\). Furthermore, we showed that the development of LUTD correlated with higher collagen content in the urethra and prostate of these mice. However we haven’t identified the potential cause of the manifestation of these pathobiologies. One candidate molecule is chemokine CXCL12 since this pro-inflammatory protein has been heavily studied by our group, and is the main focus of chapter 3 in this thesis, and we have shown that it can drive the phenoconversion of myofibroblasts in the absence of the canonical TGFβ. CXCL12 is upregulated in aging prostate fibroblasts\(^18\) and it could be playing a role in the diet-induced inflammation-mediated prostatic fibrosis. In order to address this we would have to analysis the prostate-specific secretome in high fat and low fat fed mice in order to identified secreted proteins, like CXCL12, that might be present in the prostate of our mice. Recently a group in Spain reported a mass-spectrometry based analysis to look at the fat-derived secretome in human tissues\(^19\). The technique, called
Comparison of Isotope-Labeled Amino acid Incorporation Rates (CILAIR), utilized adipose tissue explants that were analyzed after 3 days in culture for their secretome. We can follow a similar protocol with our mice by explanting and culturing prostate tissue and analyzing the secretome of these culture cells.

However, if we identified CXCL12 as being secreted by the explanted tissue that doesn’t necessarily mean that CXCL12 is involved in the development of prostatic fibrosis in these mice. But once and if identified, we could carry experiments where we locally inhibit CXCL12 action with the use of chemical inhibitors (such as AMD3100 or Chalcone-4) and see whether or not inhibition of CXCL12 in the high fat-fed mice leads to reduction of collagen content and reduction in the development of LUTD. These sets of experiments will compliment the already published studies from our group looking at CXCL12 in human aging fibroblasts and the first diet-induced LUTD development study that is described in chapter 2.

**CXCL12 and fibrocyte recruitment to the prostate**

CXCL12 is very interesting target in prostatic fibrosis for several reasons. Our group has shown that this chemokine is up regulated by aging fibroblasts, and in the absence of TGFβ it can drive the differentiation of prostate myofibroblasts. We also showed, in chapter 3, that CXCL12 achieves this TGFβ-independent differentiation through the use of non-canonical signaling pathways that are not used by TGFβ in our prostate fibroblasts. However, CXCL12 has been documented to play a pivotal role in the development and progression of idiopathic pulmonary fibrosis (IPF) \(^{17}\). Studies using small molecule inhibitors for the CXCL12/CXCR4 in IPF-induced mice have shown that lung secretion of CXCL12 is responsible for the recruitment of CXCR4 expressing fibrocytes\(^{20,21}\). This recruitment of fibrocytes to the lung by CXCL12 provides an attractive therapeutic target for IPF.
In our system we have observed that CXCL12 can drive myofibroblast phenoconversion in vitro, but it isn't clear whether CXCL12 is driving phenoconversion or recruiting fibrocytes to promote prostatic fibrosis. To determine this, we could conduct mouse-based experiments to test this hypothesis in the prostate. One way that we could address this is by carrying out some adoptive cell transfer experiment in our diet-induced LUTD mouse model. Fibrocytes could be isolated from the bone marrow of healthy mice via flow cytometry and tagged with a fluorescent protein for tracking. Fluorescently tagged (GFP), either constantly expressed or under the control of a doxycycline inducible promoter, fibrocytes could be injected into the blood stream of our mice during high and low fat diet periods. Mouse prostate tissue can then be analyze via immunohistochemistry for the presence of GFP within the fibrotic tissue. This would indicate that the CXCL12 secreted by the mouse prostatic tissue is recruiting the fluorescently tagged fibrocytes. However, tissue should also be staining for mature fibrocytes and quantify how many of the mature myofibroblasts are also GFP positive. This would give us an idea of how the origin of the myofibroblast driving prostatic fibrosis since we would be able to differentiate between resident fibroblasts/fibrocytes and circulating fibrocytes to identify the population of cells responsible for the development of prostatic fibrosis.

However, the reverse experimental set-up can be proposed in order to look for outside recruitment of fibrocytes without the need of having to inject mice with tagged cells. In order to look for the recruitment of circulating fibrocytes we could generate SAMP6 mice that express GFP under the control of ARR2-Probasin promoter, a modified rat prostate specific promoter\textsuperscript{22}. The mice would then be fed high or low fat diets in order to promote diet-induced LUTD. Prostatic tissue can be collected and analyzed via immunofluorescence and immunohistochemistry for the presence of myofibroblasts. The ratio between fluorescently-
tagged and non-fluorescent myofibroblasts will let us know how many of the active myofibroblasts involved in the development of prostatic fibrosis came from circulating cells and not resident cells. In addition to that, the diet can be complimented with localized small molecule inhibitor treatment for the CXCL12/CXCR4 axis in order to address the role of this chemokine in the recruitment of circulating cells during prostatic fibrosis development. Addressing the cell source for the myofibroblasts is a crucial step in understanding the development of LUTD in order to look for potential therapeutic targets. The proposed designs here focus on CXCL12 since this chemokine is the focus of this dissertation.

**Potential role of complement activation in prostatic fibrosis**

Our lab has focused on dissecting the role of CXC-type chemokines (CXCL5, 8, 12) in promoting myofibroblast phenoconversion since these proteins were identified as being heavily expressed by aging prostate fibroblasts when compared to young fibroblasts in culture using an Affimetrix gene expression analysis system\(^\text{18}\). However, these chemokines were not the top expressed secreted proteins in the study; these proteins were preceded by C3 and CD55, which are two components of the complement pathway. This is important to keep in mind since our chemokines are not acting in isolation in our system and could be impacted by these complement pathway members. The complement pathway is a group of pathways that are part of our immunity programs\(^\text{23}\). The complement pathway has been associated with a wide range of pathobiologies that include infertility, atherosclerosis and, more importantly, fibrosis\(^\text{23-25}\). The complement pathway could be activated in three different ways: the classical activation pathway is initiated by antigen-bound antibodies, the alternative pathway is initiated by the presence of an infectious agent and the mannose-binding lectin pathway is initiated by the binding of mannose-binding lectin proteases to carbohydrates in the surface of bacteria. However, an important
common component of these three activation pathways is that all of them converge on C3, which is the complement component that was observed to be upregulated in prostate aging fibroblasts. Activation of the complement pathway triggers the recruitment of neutrophils and monocytes to fight the foreign object present in the system, hence triggering inflammation. Interestingly, the other complement member upregulated by aging prostate fibroblasts, CD55, is a negative regulator of complement activation. It appears that aging prostate fibrosis are triggering both, pro-inflammatory complement activation and regulation. However, it has been documented that pro-inflammatory complement components, such C3, can enhance CXCL12/CXCR4-mediated chemotaxis. This relationship between complement activation and CXCL12 function is of special interest since complement activation has been heavily documented to be involved in the development of liver fibrosis. It would be interest to address whether or not complement activation is involved in the development of prostatic fibrosis by either enhancing CXCL12 secretion or signaling, or by enhancing CXCL12’s recruitment of circulating fibrocytes by increasing inflammation in the prostate. But first, experiments must be carried out looking at complement components and their activation in human and mouse prostatic tissue to validate its presence at the organ level and potential relevance.

Our group has already some preliminary data from our obese mouse model that supports the idea of studying the role of complement in prostatic fibrosis. Tissue from the prostate, liver, heart and spleen, of mice that followed the same diet as the one described in chapter 2 were collected and analyzed for collagen content as described. RNA extracted from the livers of these mice, which showed higher collagen content in their tissue and fat accumulation for those that were in the high fat diet, was sequenced via RNASeq and also analyzed via Nanostring analysis for gene expression. The preliminary data from these experiments showed that most of the
components of the complement pathway were upregulated in the livers of high fat fed mice. The data is not shown here as it is not part of this project, and hasn’t been fully analyzed, but points toward complement potentially having a role in inflammation-driven prostatic fibrosis. However, this preliminary data only validates the role of complement activation in liver disease, similar gene expression profiling should be carried out in mouse prostatic tissue to address whether complement upregulation is also observed.

The role of CUL3 in CXCL12-mediated myofibroblast phenoconversion

Another important set of proteins that might be involved in chemokine mediated prostatic fibrosis is the cullin-RING protein ubiquitin ligases discussed in chapter 4, specifically CUL3. Protein ubiquitination is often involved in proteasome-directed protein degradation, however protein ubiquitination can have roles other than protein degradation\(^\text{14}\). For example, protein ubiquitination is involved in gene expression regulation. Ubiquitination of histones alters the chromosomal structure to allow access to different enzymes and transcription factors to bind to the DNA and activate or repress transcription, therefore acting as a modulator of gene expression. Ubiquitination is also involved in direct cellular localization of proteins. More importantly for this dissertation, protein ubiquitination can be involved in protein trafficking, as it is the case for CUL3. As previously discussed, CUL3 is part of the BTB- CUL3-RBX1 complex that is responsible for the maturation of ER-Golgi vesicles and collagen transport by regulating the size of COPII vesicles, which carry collagen from the ER to the cellular membrane\(^\text{14}\). We showed in chapter 4 that CXCL12 treatment of prostate fibroblasts upregulated the presence of ribosome-like vesicles and the levels of CUL3 gene and protein expression when compared to vehicle and TGFB treated fibroblasts. CUL3 protein levels increase correlated with increased COL1 and COL2 export in CXCL12 treated cells. These results hint at the hypothesis...
that CXCL12 might be achieving myofibroblast phenoconversion by increasing the collagen export machinery, rather than collagen itself. But this hypothesis needs to be further tested in order to understand the reason behind this CUL3 increase. The first step would be to prove that the increase in CUL3 is indeed due to the activation of the CXCL12/CXCR4 axis with the use of chemical inhibitors such as AMD3100. Secondly, the importance of CUL3 presence in CXCL12 and TGFB should be addressed by either knockdown CUL3 via use of siRNA or the CRISPR/Cas system. We hypothesize that CUL3 is required for phenoconversion since it is essential for the export of collagen from the ER. However, it is also important to address the importance of CUL3 activity but unfortunately there are no specific small molecule inhibitors available for CUL3. Instead we could take a cullin pan-inhibition approach by using MLN-4924, a reported pan-inhibitor of cullin proteins. Addressing CUL3 role in prostatic myofibroblast phenoconversion could provide valuable mechanistic details that could potentially have therapeutic value. These experiments could also provide deeper insight into non-canonical promotion of fibrosis in other organs as well.

**Targeting CXC-type chemokines**

The main focus of this dissertation has been the CXC-type chemokine CXCL12 and its mechanism of action to promote myofibroblast phenoconversion. However, it is known that CXCL12 is not the only CXC-type chemokine that can achieve this process. CXCL5 and CXCL8 were reported by our group to be capable of promoting such phenoconversion. CXCL5 and CXCL8 share the same set of GPCR receptors, CXCR1 and CXCR2, and can signal through both\(^7,8,10\). It is important for therapeutic value to identify the pathways responsible for CXCL5 and CXCL8 mediated myofibroblast phenoconversion. These CXC-type chemokines pose as attractive targets for prostate anti-fibrotic therapy since potential, just as CXCL12, these
chemokines signal and carry out their pro-fibrotic effect through non-canonical signaling. The rationale behind focusing on these chemokines for targeting prostatic fibrosis is based on the fact that targeting TGFβ is detrimental for organ development and health. TGFβ is involved in many important cellular processes that are not limited to fibrosis, and its inhibition or removal can be lethal\textsuperscript{28}. Of interest to our studies would be to see whether these two other chemokines also signal through EGFR as CXCL12 does. Knowing that both of these chemokines signal through GPCRs it is entirely possible that both of them might be able to signal through EGFR. An orchestrated movement by these chemokines to signal through EGFR will provide a valuable therapeutic target for treating LUTD, especially since there are small molecule inhibitors reported to be effective at treating EGFR-mediated pulmonary fibrosis. More importantly, knowing that CXCL12 signaling also promotes cells proliferation, and this signaling occurs through EGFR; EGFR signaling targeting in the prostate could potentially serve as a double therapeutic by addressing both, fibrosis and BPH. However, in order to consider this approach it is important, as previously stated, to identify the signaling mechanisms used by other up-regulated chemokines.
5.3 References


