Anti-Fibrotic Properties of Mesenchymal Stem Cells in Partial Bladder Outlet Obstruction

By

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Abstract

Partial Bladder Outlet Obstruction (pBOO) is characterized by an initial inflammatory response which progresses to smooth muscle hypertrophy, and fibrosis. The resulting high urine storage pressure significantly damages the bladder wall and poses a risk of renal failure. pBOO is characterized by exaggerated stretch, hydrodynamic pressure, and inflammation which cause significant damage to the bladder wall. Several studies have implicated hypoxia in its pathophysiology. However, the isolated progressive effect of hypoxia on bladder cells is not yet defined. As well, current treatment modalities of pBOO are crude and carry high risk of morbidity. Mesenchymal stem cells (MSCs) are undifferentiated multipotent adult cells with reparative, immunomodulatory, anti-inflammatory, and anti-fibrotic capacities. MSCs therapy is an emerging paradigm with several reported experimental successes. However its mechanism of action is not well understood.

The experiments of this thesis were designed to investigate the single stress effects of hypoxia on smooth muscle cells of the bladder and the anti-fibrotic effects of bone marrow-derived MSCs on hypoxia-induced pathways. The in-vitro mechanisms were confirmed in whole animal models.

Sub-confluent normal human bladder smooth muscle cells (hbSMC) were cultured in 3% O_2 tension for 2, 24, 48, and 72 h. Then, this experiment was repeated with either the direct or indirect co-culture with bone marrow derived MSCs. High pore density transwells were used for indirect co-cultures. Total RNA, cellular proteins, and secreted proteins were used for gene expression analysis, immunoblotting, and ELISA, respectively. Twenty Sprague Dawley rats were randomly assigned into 5 groups: unobstructed controls, pBOO for 2 weeks, pBOO for 4

weeks, pBOO + MSCs for 2 weeks, and pBOO + MSCs for 4 weeks. pBOO was surgically induced followed by intravenous injection of MSCs. Endpoint urodynamics was performed and bladder tissues harvested for analysis. RT-PCR and immunohistochemistry were performed to study gene and protein expression of major inflammatory and pro-fibrotic markers.

Transcription of hypoxia-inducible factor (HIF) 1α and HIF2α were transiently induced after 2 h of hypoxia ($p < 0.05$), whereas HIF3 was upregulated after 72 h ($p < 0.005$). HIF1 and HIF3α proteins were significantly induced after 2 and 72 h, respectively. VEGF mRNA increased significantly after 24 and 72 h ($p < 0.005$). The inflammatory cytokines, TGFβ1 (protein and mRNA), IL 1β, 1L6, and TNFα (mRNA) demonstrated a time-dependent increased expression. Furthermore, the anti-inflammatory cytokine IL-10 was downregulated after 72 h ($p < 0.05$). Evidence of smooth muscle cell dedifferentiation included increased αSMA, VIMENTIN, and DESMIN. Evidence of pro-fibrotic changes included increased CTGF, SMAD 2, and SMAD 3 as well as COLLAGENS I, II, III, and IV, FIBRONECTIN, AGGRECAN, and TIMP 1 transcripts ($p < 0.05$). Total Collagen proteins also increased time-dependently ($p < 0.05$).

Both the direct and indirect MSCs co-cultures inhibited > 50% of hypoxia-induced TGFβ1 and IL-6 expression ($p < 0.005$) in a HIF-independent manner. Also, both MSCs co-culture techniques induced > 200% increase in IL-10 protein ($p < 0.005$) and inhibited hypoxia-induced αSMA, collagen I and III transcripts as well as total collagen proteins ($p < 0.0001$). Contrastingly, the hypoxia-induced IL-1β and TNFα were inhibited by only the direct co-cultures ($p < 0.05$). The induction of pBOO resulted in an upregulation of TGFβ1, SMAD2/3, HIF1α, HIF3α, VEGF, TNFα, mTOR, p70S6K, COL I, and COL III expression in a time dependent manner. This was coupled with a downregulation of IL-10 expression. Increased bladder

collagen deposition was directly related to the duration of pBOO and there was an associated high urine storage pressure. Two weeks after therapy, MSCs immunomodulatory effect (defined by reduced TNFα, increased IL-10 and VEGF) was most predominant. Significant downregulation of the pro-fibrotic genes occurred 4 weeks after therapy. End filling pressure, hypertrophy and fibrosis were significantly reduced after MSCs therapy ($p<0.05$).

Together, the studies described in this thesis demonstrate that hypoxia induced significant inflammatory and fibrotic effects in bladder smooth muscle cells similar to pBOO. Furthermore, this work has elucidated the ability of MSCs to target inflammatory and pro-fibrotic pathways to prevent bladder deterioration due to pBOO. The outcomes of this study are promising and lay an important foundation for the prevention and potential treatment of end stage fibrotic bladder with mesenchymal stem cells.

Preface

This thesis is an original work by Bridget Wiafe. The research project, of which this thesis is a part, received ethical approval from the University of Alberta's Research Ethics Office. The Animal Care and Use, Health Sciences Committee (ACUC) provided ethical approval for the experimental use of rats, Project Name "treatment of rat models of pBOO with Mesenchymal Stem Cells", No: AUP00001759. Date: June 6, 2016. A waiver of informed consent was obtained for research involving discarded human tissue.

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Dedication

This thesis is dedicated to the God of Host, the El-Elyon, and the way maker whose divine inspiration, guidance and provision has brought me through.

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List of Abbreviations

α: alpha

α-MEM: alpha-minimal essential medium

αSMA: alpha smooth muscle actin

ANOVA: analysis of variance

β: beta

β-actin: beta-actin

BMNC: bone marrow-derived mononucleated cell

CD: cluster of differentiation

cDNA: complementary deoxyribonucleic acid

CIC: Clean Intermittent Catheterization

CO_2: Carbon dioxide

Col: Collagen

CT: Cycle Threshold

CTGF: connective tissue growth factor

DAPI: 4',6-diamidino-2-phenylindole

DMEM: Dulbecco's modified Eagle's medium

ECM: extracellular matrix

EMT: epithelial to mesenchymal transition

EPO: erythropoietin

FBS: fetal bovine serum

FGF-2: fibroblast growth factor-two, basic fibroblast growth factor

H: Hour

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIF: Hypoxia inducible factor

HLA: human leukocyte antigen

HRE: Hypoxia response element

IHC: Immunohistochemistry

IL: interleukin

MRI: Magnetic resonance imaging

mRNA: messenger ribonucleic acid

MSCs: mesenchymal stem cells

MTOR: mechanistic target of rapamycin

N_2: Nitrogen

NCBI: National Center for Biotechnology Information

O_2: Oxygen

P70 S6K: Ribosomal protein S6 kinase beta-1

pBOO: partial bladder outlet obstruction

PBS: phosphate-buffered saline

PDGF: platelet-derived growth factor

PUV: Posterior urethral valve

RNA: ribonucleic acid

RT-qPCR: reverse-transcription quantitative real-time polymerase chain reaction

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEM: standard error of the mean

SLRP: small leucine-rich repeat proteoglycan

SMAD: Small mothers against decapentaplegic gene

T150: Tissue-culture flask (surface area of 150 cm^2)

TGF-β: transforming growth factor-beta

TIMP: Tissue inhibitor of metalloproteinase

TNFα: Tumor necrosis factor alpha

VEGF: Vascular endothelial growth factor

Chapter 1

Introduction

Bridget Wiafe

1.1. Thesis synopsis

Partial bladder outlet obstruction (pBOO) results from a myriad of conditions that anatomically or functionally impede the flow of urine. It affects an estimate of 1.1 billion people worldwide [1]. In the elderly, the prevalence of pBOO correlates with age and over 90% of men 90 years and above are affected with this condition [2]. Pediatric urology clinics are also inundated with children suffering from abnormal bladders. It commonly presents with varying severity in the newborn with renal failure, spina bifida, and posterior urethral valves [3, 4]. Thus, pBOO is common in school aged children resulting in incontinence and urinary tract infections. It is an inflammatory and fibrotic condition that is associated with high pressure urine storage and can induce significant irreversible damage to the bladder. If untreated, this compromises the bladder's function and increases the risk of urinary tract infections, incontinence, hydronephrosis and renal failure [5]. Bladder deterioration is fundamentally driven by hypoxia, whereby the constant contraction of bladder smooth muscles and elevation of intra-vesical pressure results in the compression of capillaries and decreased blood flow to the bladder wall [6]. However, the hypoxia-driven pathophysiology of the bladder is not yet elucidated.

Current clinical management is cumbersome, remains crude, and carries significant risk of morbidity with minimal efficacy. Clean intermittent self-catheterization in combination with anticholinergic drugs such as oxybutynin is the golden standard for treatment [7]. However, intermittent catheterization is burdensome and associated with high risks of urethral trauma, urinary tract infections and lack of self-esteem [8]. Also, oxybutynin and other bladder relaxants are associated with serious side effects and lack of long term efficacy [9]. Moreover, for patients who develop a low capacity, high pressure, poorly compliant bladder, surgical bladder augmentation using autologous intestinal tissue may be beneficial. However, this intervention is associated with major metabolic disturbances, recurrent urinary tract infections and long term graft survival issues [10].

Mesenchymal stem cells (MSCs) are adult multipotent cells with immuno-modulatory, reparative, anti-inflammatory and anti-fibrotic capacities. Stem cell therapy has shown promising experimental outcomes in the treatment of several tissue injury and fibrotic conditions [11]. However, its mechanisms of action are not well understood. Also, despite a relatively positive experimental outcome, not much work is done in the area of modulating the inflammatory and pro-fibrotic profile of pBOO.

The objective of this thesis is to elucidate the role of hypoxia in the pathophysiology of pBOO and to provide insight into the anti-fibrotic effects of mesenchymal stem cells in pBOO. The thesis will commence with an introduction into bladder anatomy and physiology, partial bladder outlet obstruction, causes, diagnosis and treatment. Hypoxia, Regenerative medicine and stem cell therapy will also be covered under the introduction. This will be followed by a review of the current applications and potential of stem cell therapy in urology. The subsequent chapters

will report a number of in vitro and in vivo experiments designed to investigate specific variables of hypoxia and mesenchymal stem cell therapy of the bladder.

1.2 The Urinary Bladder Anatomy

The urinary bladder is an elastic, muscular and water-tight organ that stores urine excreted by the kidneys. It has a broad body that tapers at the base to form the bladder neck. This opens into the urethra that drains urine out of the body. Sphincter muscles found at the bladder neck and urethra contract to prevent urine leakage. The bladder has an inner mucosal lining of stratified epithelia called the urothelium. Beneath is a submucosal layer called the lamina propria which contains nerves and blood vessels. Underlying this stratum is a three-layered coat of smooth muscles called the detrusor muscle. The outer layer is comprised of peritoneum serosa [12] (Figure 1.1 &1.2).

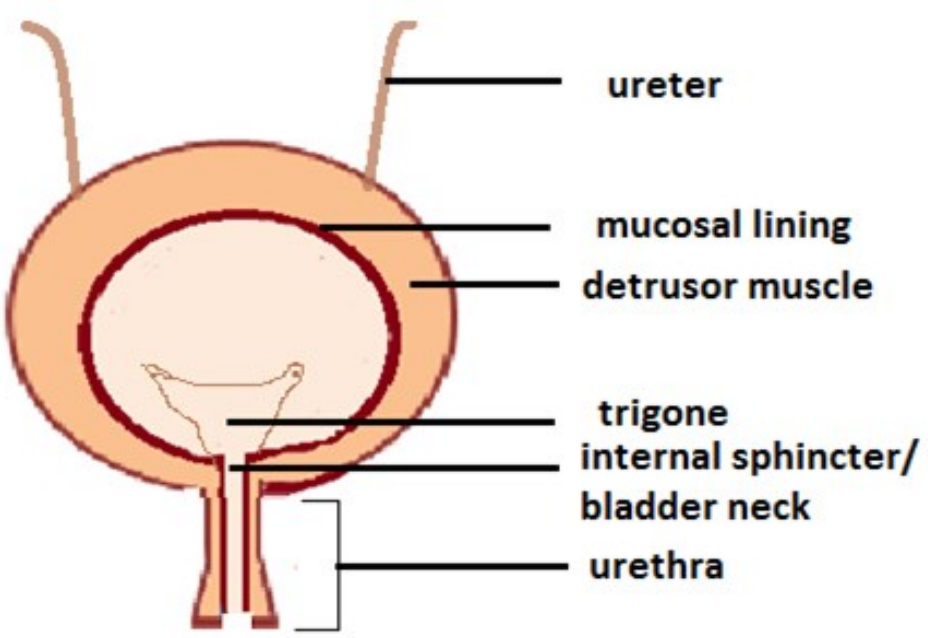

Figure 1.1 Schematic drawing illustrating the anatomical parts of the urinary bladder

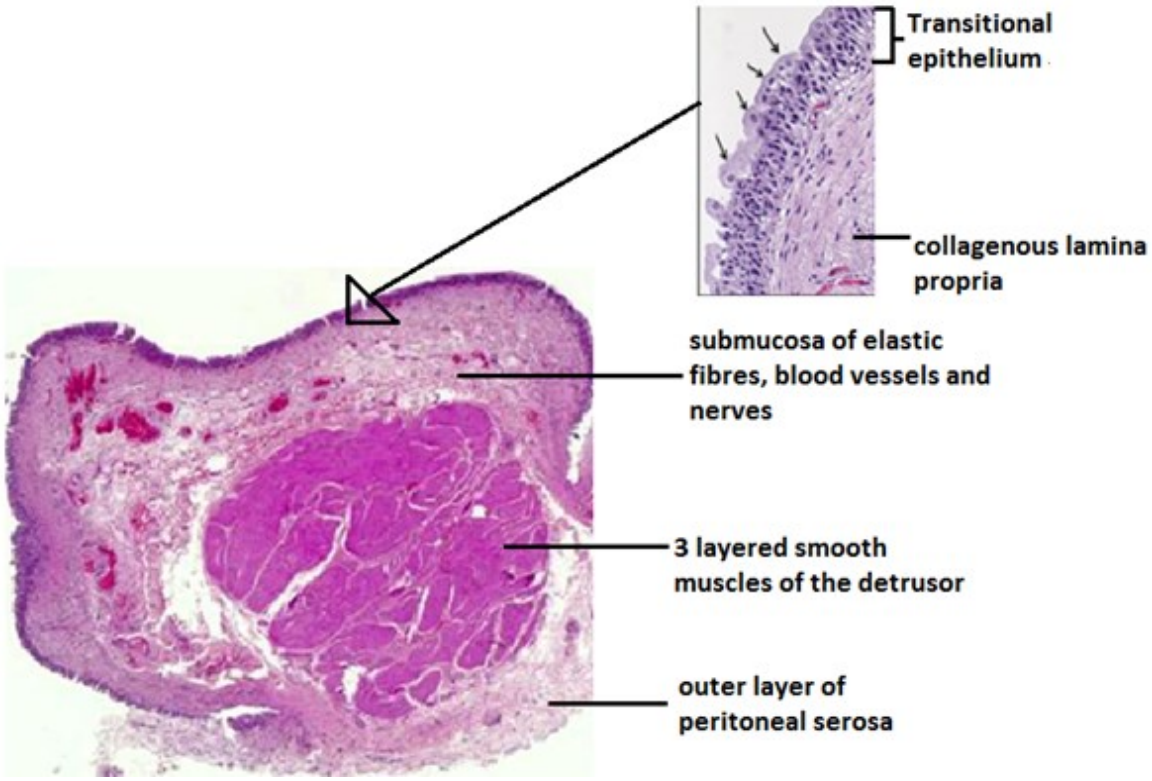

Figure 1. 2. Histology of the urinary bladder. Haematoxylin and Eosin stained longitudinal section of a human bladder biopsy. The umbrella cells of the mucosa are indicated by black arrows in the high power section.

1.3 Bladder Physiology

Complex co-ordination between the central nervous system, the detrusor and sphincter muscles is required for the bladder to perform its primary tasks of storing and expelling urine. Sympathetic nerves are found mainly on the bladder neck and urethra that controls internal sphincter tone required for urine storage. Skeletal muscle and the somatic nervous system regulate an external sphincter with voluntary activity. Normal urine storage requires the sphincters to be potently contracted and highly resistant to urine flow, with a relaxed detrusor muscle. The elastic properties of the detrusor also contribute to a very compliant organ, whereby large volumes of urine are stored at low pressures [13]. Bladder compliance is a measure of the change in volume per unit increase in pressure during filling [14].

Bladder emptying requires relaxation of these sphincters, and a coordinated contraction of the detrusor muscle, mediated via the parasympathetic system. The pressure in the bladder should only rise minimally during voiding, ensuring minimal stress to the organ [13].

1.4. Partial Bladder Outlet Obstruction (pBOO)

pBOO is a condition of anatomical or functional impedance to the flow of urine. It was first described by the Scottish surgeon, John Hunter in 1978 as "the disease of the bladder arising from obstruction and its consequence is an increased irritability, by which the bladder admits of little distension, because quick its action and thick its coats'' [15]. Anatomic obstruction may occur secondary to benign prostate hyperplasia (BPH), posterior urethral valve, or urethral stricture diseases. However, functional obstruction can occur with an impairment of the nervous system, whereby the normal sphincter is prevented from relaxation. This is exacerbated if

denervation also results in excess contractility of the detrusor. pBOO remains a significant source of morbidity in urology, increasing the risk of urinary incontinence, urinary tract infections, and renal failure [16].

Clinically, pBOO presents with Lower Urinary Tract Symptoms (LUTS) which include urgency, frequency of urination, nocturia, urinary retention, hesitancy, decreased force of stream, and urinary dribbling after voiding [16].

pBOO is a progressive disease that is characterized by elevated urine storage pressure and bladder wall remodeling [17]. Chronic exposure of the bladder wall to increased voiding resistance and high pressures, irreversibly damages its muscle and interstitial connective tissue. This compromises the ability of the bladder to store urine at low pressures, resulting in a low volume, high pressure, and poorly compliant bladder. The increased pressure results in urinary incontinence if the pressure in the bladder exceeds the resistive capability of the sphincter. Hydronephrosis and renal failure may result if the pressure in the bladder exceeds the pressure in the ureter and renal collecting system, preventing urine flow from the kidney. This increased renal pressure prevents tubular and glomerular function and places the patient at risk for hydronephrosis and renal failure [18].

Partial bladder outlet obstruction, whether acute or chronic, can result in contractile failure which is characterized by the inability to void or acute urinary retention. The bladder compensates for the deteriorating detrusor functions by rapidly increasing bladder mass. Thus, bladder muscle hypertrophy is often the initial compensatory response to the resistance to urine outflow. This is thought to increase the contractile force needed to expel urine against the increased resistance. However, decompensation occurs with time and it is marked by a low

energy, poorly contractile bladder with increased collagen and connective tissue deposition [18]. Essentially, these alterations have a negative impact on blood flow to the bladder wall. Thus, even though the normal bladder is exposed to transient ischemia during the filling and voiding stages, this is exaggerated in the obstructed bladder [19, 20].

Mechanical stretch, hydrodynamic pressure, inflammation and hypoxia are the factors believed to be composite mediators in the pathophysiology of pBOO. However, much data is derived from the hypothesis that mechanical stretch is a primary source of inflammation and the subsequent tissue damage sets the stage for fibrosis [21]. This is not surprising, as the end-result of an unrelieved obstruction can be devastating, as seen by the unremitting progression to the end-stage, fibrotic, poorly compliant, high-pressure bladder.

1.4.1 Etiological Causes of pBOO

1.4.1.1 Benign Prostatic Hyperplasia (BPH)

BPH is the non-malignant proliferation of the cellular mass coupled with an increase in smooth muscle tone of the prostate. Thus, the pathophysiology of BPH comprise of two main mechanisms:

1. The static mechanism in which there is compression of the urethra due to increased prostatic volume. This stems from the proliferation of glandular epithelium, connective tissue and smooth muscles of the prostate.

2. The dynamic mechanism which describes the resultant increase in smooth muscle activity and prevents appropriate relaxation and reduction in resistance to urine flow [22].

Distinctively, BPH-associated hyperplasia occurs in the transitional zone of the prostate whereas in prostate cancer, disease initiates from the proximal peripheral region [23]. Even though the specific mechanism regulating cellular proliferation in BPH is largely unknown, there is evidence suggesting the involvement of complex mechanisms like chronic inflammation and abnormal wound healing processes. Factors such as age, diabetes, obesity and inflammation are known to increase the risk of BPH. The incidence of pBOO correlates with age: the prevalence of BPH in younger men is 5% and the prevalence doubles by age thirty. 60% of men between 60 to 70 years are affected with BPH and the prevalence increases to 80% in men 70 years and above and 90% in men 90 years and above [24].

This condition is diagnosed through a combination of clinical symptoms, urodynamic studies, and cystoscopy, as no major allele with high penetrance for BPH has been identified. However, there are various reports suggesting polymorphism associations of some low-penetrant genes [25]. BPH is thought to be hereditary when it occurs early in life. Studies show that close relatives of men with early onset of BPH (<67 years) have a 66% lifetime risk of having prostatectomy due to BPH as compared to 17% risk in the control group [26]. BPH is genetically heterogeneous: Apart from early onset, familial BPH is distinctive from the sporadic type by having larger prostate volumes and increased risk of having prostatectomy [27].

1.4.1.2 Spina Bifida

Spina bifida is one of the most serious congenital defects compatible with survival. It is caused by failure of the spinal mesoderm to develop properly leading to abnormal closure of the neural

tube during embryogenesis. This can result in severe nerve and spinal cord injury. Consequently, there is significant morbidity across multiple organ systems [28].

The prevalence of spina bifida worldwide is about 1 in every 1000 births and 0.86 in every 1000 births in Canada [29]. Risk factors such as folate deficiency, maternal use of anticonvulsants, obesity, and a high blood glucose level during early pregnancy predisposes the developing embryo to spina bifida. Also, the high recurrence rate of spina bifida among siblings of people with the condition provides evidence for the genetic involvement of spina bifida. Polymorphisms and association of major genes involved in the folate metabolism pathway has been studied with the aim of identifying some genetic markers for the disease. However, to date, no gene with complete penetrance has been identified [30]. The association of the preconception and early pregnancy maternal fortification of folic acid with significant reduction in neural tube defects is a major breakthrough in this area.

However, with spina Bifida, the urinary tract becomes a primary source of morbidity and embarrassment. This defect is characterized by a hyperactive bladder and closed sphincter, therefore, a fixed Partial Bladder Outlet Obstruction. Consequently, urinary incontinence and urinary tract infections are very common. Furthermore, if left untreated, the sustained elevated bladder pressures may lead to permanent bladder damage and kidney failure [31].

Current clinical interventions include intermittent urethral catheterization, which is our only means of bypassing the fixed urethral resistance and ensuring complete bladder emptying. The empty bladder ensures a low-pressure system. This is extremely burdensome and increases risk of infection. Taking drugs such as antimuscarinics and aggressive medical therapy is used to target the parasympathetic synapses and reduce detrusor contractility. This further reduces the

risk of elevated bladder pressures by maximizing bladder compliance. However, if these measures fail, the bladder must be surgically enlarged to ensure adequate storage volumes and pressures, and this is most commonly done with an intestinal patch. This surgical bladder augmentation is associated with serious morbidity and potential mortality [32].

1.4.1.3 Posterior Urethral Valves (PUV)

This is characterized by abnormal fetal development which results in the presence of a membranous fold in the male posterior urethra. This obstruction results in a low capacity high-pressure hypercontractile bladder which can be apparent pre-nataly. Even though PUV is a rare condition that occurs in about 1 in 4000 to 7000 births, it is associated with high rates of mortality and morbidity. PUV is the commonest cause of urinary tract obstruction in newborns and accounts for up to 17% of all pediatric cases of end stage renal failure [33]. Reports on the genetic regulation of the disease are scanty but there is evidence to support the possibility of familial recurrence. Also, some genetic mutations have been identified [34]. Presently, the implementation of prenatal screening and diagnostic techniques coupled with intensive neonatal care has improved mortality rate significantly [35].

1.4.1.4 Urethral Stricture Disease

This is a condition in which progressive scarring along the segments of the urethra causes a narrowing of its lumen. The epithelial lining of the cavernous tissue is most affected with the increasing ECM deposition. Over time, the highly elastic normal columnar epithelium is replaced with stratified squamous epithelium which lacks the ability to accommodate the normal urethral

hydrodynamic pressure. Hence there is exacerbation of tissue damage due to the high pressures leading to increased fibrosis in the urethral lumen [36]. This results in obstructive symptoms, incontinence and recurrent UTI.

The commonest causes of urethral strictures are iatrogenic in nature. A third of all strictures occur in the penile urethra whereas a half of narrowing occur along the bulbar urethral. Strictures in both segments of the urethra have also been reported [37]. Risk factors for urethral stricture disease include male gender, infection of the urethra, inflammation, trauma and radiation therapy. Urethral stricture tends to affect more elderly men (>65 years) and the prevalence is around 0.6% in this population [38]. Straddle and urethral injuries mainly from instrumentation, as well as radical prostatectomy increases the risks of developing urethral stricture disease [39]. However, genetic factors are unknown [40]. A combination of retrograde urethrography and voiding cystourethrography are useful diagnostic tools for determining the site, span, and severity of the stricture [39].

1.4.2 Diagnosis of pBOO

Patients usually present with LUTS and are initially assessed for risk factors of pBOO. However, pBOO is diagnosed by urodynamics, which allows a direct measurement of intravesical pressures with a catheter placed in the bladder [41]. Urodynamic studies are functional studies that measure filling pressures and the ability to void completely. The study has two phases: 1. the invasive cystometrogram measures parameters of the filling phase and 2. The non-invasive voiding pressure flow study. Typically, the bladder is filled slowly with a saline solution via a catheter, which also contains a pressure monitor. During the voiding phase of the test, the rate of

urinary flow is recorded. Subtracting the intraabdominal pressure, acquired with a rectal pressure monitor, from the intravesical pressure, gives detrusor contractility. These parameters provide useful information for the diagnosis of bladder outlet resistance [42]. Evidence of LUTS is quantified as a measure of severity, prognosis and response to therapy in pBOO. Postvoid volume of 200ml or more is a symptom of deteriorating bladder function and a risk factor for recurrent urinary tract infections and acute renal failure [43].

Video urodynamic assessment (VUA) adds radiographic details of the bladder and urethra, and is usually performed to provide specific information such as the presence of anatomical anomalies such as urethral valves, strictures, lesions, stones, etc. Residual urine, vesicoureteral reflux, and detrusor-sphincter dysynergia can also be detected using VUA [44].

1.4.3 Clinical Treatment of pBOO

Depending on the cause and severity, management of pBOO may involve medication or surgery to reduce the obstruction, or catheterization and, medication to reduce the stress on the bladder. Failure of the above results in major bladder reconstructive surgery.

1.4.3.1 Medical Relief of pBOO

Drugs such as alpha-adrenoceptor antagonists (α-blockers), 5a reductase inhibitors and anti-cholinergics have shown to be beneficial in improving urodynamic parameters secondary to prostatic hypertrophy [45]. The focus of this treatment is to reduce prostatic volume and muscle tone. Thus, 5α-reductase inhibitors and α-adrenergic antagonists are first line treatment options [46]. 5α-reductase inhibitors target the static component of the BPH by preventing the

conversion of testosterone to dihydrotestosterone and reduce the stromal volume of the prostate, increases urethral diameter and decreases resistance to urine flow. Common examples include finasteride and dutasteride and these have been known to be effective in reducing prostatic volume of mostly patients with enlarged prostates [47]. α-adrenergic antagonists on the other hand inhibits alpha adrenergic stimulations and thereby reduces bladder neck tone. Tamsulosin, terazosin, alfuzosin, and doxazosin are common examples of α-adrenergic antagonists [48].

1.4.3.2 Surgical Relief of pBOO

In cases of prostatic hypertrophy, urethral stricture, and posterior urethral valves, the anatomic obstruction can be relieved with simple and effective surgical procedures. This includes trans-urethral resection and urethroplasty. However, stricture recurrence and prostatic regrowth are common. Furthermore, despite relief of the obstruction, if the detrusor has already been significantly damaged, then bladder decompensation may be permanent, and the patient may still suffer from elevated bladder pressures and its sequela [49].

1.4.3.3 Clean Intermittent Catheterization (CIC)

Catheterization is the process by which the bladder is drained of urine through an inserted hollow tube. CIC is an indispensable first line intervention for bladder conditions in which complete voiding is unattainable. This is done to prevent the buildup of high urine storage pressure that has detrimental effects on the kidneys. Catheterization also helps to reduce the incidence of storage-related problems such as urinary frequency and urgency, incontinence, bladder stones and persistent UTI [8]. Clean intermittent self-catheterization (in which the patient himself

inserts and removes catheter) has been promoted as a gold standard for the management of significant urinary retention and voiding dysfunction [50]. However, regardless of the benefit, catheterizing 4-6 times daily can be a bothersome practice, that can potentially increase the risk of acquiring infections, urethral trauma, and is associated with stigmatization, embarrassment and decreased self-confidence [8].

Consistent and effective emptying of the bladder is required to prevent over-filling. If the bladder is unable to empty, the pressure in the bladder remains elevated. This, in turn, contributes to bladder wall stretch and potential constriction of blood vessels. Both of these are significant stressors and result in a generalized inflammatory response [50].

1.4.3.4 Therapeutic Management of pBOO

Therapeutic management of the bladder comprise mainly of bladder relaxant medications. Anticholinergic medications competitively inhibit the binding of acetylcholine to the muscarinic receptors on the detrusor and bladder mucosa and thereby prevent involuntary contraction during urine storage [51]. Anticholinergic drugs include darifenacin, fesoteridine, oxybutynin, tolterodine, trospium chloride and solifenacin. However, the most frequently used is Oxybutynin, a bladder smooth-muscle relaxant, which improves bladder urodynamics by the inhibition of detrusor hypertonicity and hyperreflexia. This eliminates high-pressure involuntary detrusor contractions and urine leakage and also prevents high-pressure bladder storage and high-pressure voiding. Decreasing bladder contractility is also of fundamental importance on reducing detrusor stress. The early and aggressive use of Oxybutynin and clean intermittent catheterization has

been proven to decrease the deterioration of the bladder to a fibrotic state requiring major surgical reconstruction [52].

The clinical use of intravesical botulinum toxin as a second line therapy is increasing in popularity. The neurotoxin is derived from Clostridium botulinum and it is capable of blocking the pre-synaptic nerve terminal release of acetylcholine. Even though, the botulin toxin is not tolerated by all patients, significant improvements in bladder function have been reported [53]. However, the effect is not permanent, and patients require injection via cystoscopy every 3-6 months, and eventually tachyphylaxis occurs where the drug is no longer effective. This likely coincides with the ultimate deterioration of the bladder, where the hypertrophied smooth muscle has deteriorated, and the bladder has lost its elastic properties [54].

The management of bladder outlet obstruction is saddled with the burden of ineffectiveness of long term anticholinergics and muscarinic antagonists as first line treatment [55]. Coupled with their unpleasant side effects such as dry throat, lips, nose and skin, constipation, tachycardia (increased heart rate), blurred vision, and increased body temperature [9], there is therefore, a need to intensify research to find a better first line treatment option.

1.4.3.5 Augmentation Cystoplasty

Augmentation cystoplasty is the most effective therapy for the bladder that has progressed beyond smooth muscle hypertrophy and has developed into a small capacity, high pressure bladder secondary to fibrosis. This end-stage bladder is the least compliant and subsequent small capacity volumes result in the complete inability to store urine at safe pressures. Augmentation cystoplasty is a surgical technique that makes use of autograft of intestinal tissues to improve

bladder compliance and function. Despite satisfactory reports of improved bladder capacity, augmentation cystoplasty is associated with serious side effects such as thromboembolism, metabolic disturbances, incontinence, bacteriuria, urinary tract stones, malignancy, etc. [56].

1.4.4 Experimental Evaluation and Treatment of pBOO

Many authors have been fervently exploring the molecular pathways by which pBOO has its effect on the bladder, and many more have been searching for improved treatment options. In-vivo studies have highlighted the role of inflammatory and fibrotic pathways. Furthermore, multiple well-designed animal models have provided great insight into whole organ deterioration and treatment options.

1.4.4.1 Molecular Changes in pBOO

Both experimental and clinical studies have reported short and long-term effects of partial bladder outlet obstruction [17, 57-64]. This has led to the identification of numerous genes and pathways as causative factors. These include, hypoxia inducible factor (HIF), Transforming Growth Factor Beta (TGF-β), Sma and Mad Related Family (SMAD) [57,58], connective tissue growth factor (CTGF), platelet derived growth factor (PDGF) [17], mammalian target of rapamycin (mTOR) [60] and others such as VEGF, Light and Heavy chain caldesmon isoforms, IL-1B, collagens, Rho-kinase, protein kinase C.

The TGF-β-SMAD pathway is well known for extracellular matrix production and in pBOO, inhibition of this pathway resulted in a significant reduction in fibrosis [57]. mTOR is a master regulator of cell cycle progression and visceral smooth muscle cell differentiation. Repression of this protein by rapamycin effectively attenuated obstruction-induced hypertrophy

and collagen deposition [59]. Caldesmon is an actin-associated protein that regulates smooth muscle contraction. It has two isoforms; the non-muscle type is of a light molecular weight (l-cad) and the muscle-specific type is of a heavy molecular weight (h-cad). Obstruction-induced bladder decompensation is associated with increased expression of the non-muscle specific l-cad so that the ratio of h-cad to l-cad reduces significantly during long periods of decompensation [60]. Rho-kinase and protein kinase C are regulatory proteins of bladder contractility. Rho-kinase isoforms are upregulated in the compensatory phase of pBOO [61] whereas protein kinase C is downregulated in pBOO [62].

Further characterization of the bladder's response to obstruction confirmed that the pathophysiology is secondary to mechanisms used throughout other organ systems. We were the first to describe the role of small leucine-rich proteoglycans (SLRP) in the bladder after pBOO [63]. These molecules are integral to proper collagen assembly and function. As per skin and other organs, Decorin is down-regulated and Biglycan is up-regulated with the progression of fibrosis after the inflammatory cascade [64]. There was also further evidence that ischemia plays a predominant role, with an increase in HIF-1alpha associated with activation of the Ras homolog transforming protein member A (Rho) and RhoA Kinase (ROCK) pathways. Increased SPRP levels confirmed that the pathway to fibrosis is initiated early, likely with the onset of inflammation.

1.5 Hypoxia

Reduction in oxygen tension which is termed hypoxia, plays essential roles in the pathophysiology many conditions. In urology, hypoxia has been found to correlate with the incidence of lower urinary tract symptoms in elderly patients [65]. Also, several studies have identified hypoxia in obstructed bladder tissues, suggesting an important role in the pathophysiology of pBOO. It is hypothesized that the increased pressure secondary to pBOO results in compression of the blood vessels. Also, the increased muscular contractility occlude bladder capillaries. The detrusor smooth muscle is extremely sensitive to hypoxia, especially with regards to its integrity and function; hypoxia inhibits proliferation of bladder smooth muscle cells via increasing cell cycle inhibition [66]. Animal studies have also shown that pBOO results in high production of reactive oxygen species and mitochondrial lipid peroxidation [67]; similar effects were observed when bladder smooth muscle cells were exposed to hypoxia [68]. The multi-stress effects of stretch, inflammation, hydrodynamic pressure and hypoxia resulted in sequential events of inflammation, hypertrophy and fibrosis [17]. However, the isolated contribution of hypoxia in these pBOO-driven events has not yet been established.

1.5.1 Cellular Response to Hypoxia

The ability of many cell types to sense and respond to reduced oxygen tensions is essential for their survival. Reduction in cellular and systematic oxygen tensions induce a complex cascade of response pathways that attempts to compensate for the loss and restore oxygen supply to cells. The Hypoxia Inducible Factor (HIF) is the major mediator of oxygen homeostasis and the master regulator of the protective responses [69]. HIF is a sequence-specific DNA-binding protein that

regulates the transcriptional response to hypoxia and forms part of the basic helix-loop-helix (bHLH-PAS) superfamily [70].

HIF plays an important role both physiologically and pathologically. Major physiologic roles include promoting angiogenesis during a) embryogenic development, b) cell proliferation and differentiation d) wound healing. Pathologically, the transcription factor is implicated in cancers and fibrotic conditions where it has been associated with aberrant angiogenesis that is supportive of cancer growth as well as excessive extracellular matrix production in fibrosis [70].

The HIF family is characterized by a heterodimeric structure and consists of three known members; HIF 1, 2 and 3 [71]. Structurally, each member consists of a stable and uniformly distributed β-subunit and an oxygen-degradable, hypoxia-inducible α subunit. HIF α is mainly regulated post-translationally so that during normal oxygen supply, it is constantly degraded by hydroxylation-dependent proteosomal polyubiquitination. Proline hydroxylation of HIF α which occurs during normoxia is catalyzed by prolyl hydroxylase (PHD) and the cofactors Fe2+ and α-ketoglutarate (α-KG) in an oxygen dependent reaction. This process recruits the tumour suppressor protein called von Hippel‑Lindau (vHL) which in turn attracts the 2-E3-ubiquitin-ligase complex to initiate the degradation process [69].

In contrast, during hypoxia, the α subunit is stabilized via prolyl hydroxylase inhibition. This terminates the rapid breakdown and HIF α is able to reach detectable levels in tissues. The accumulating HIF α translocate into the nucleus and subsequently dimerizes with the β-subunit forming an α-β complex that is a transcription factor for stimulating the expression of hypoxia-response genes. These genes encode for numerous cytoprotective proteins to combat the

deleterious effects of hypoxia. One key protective protein is the Vascular Endothelial Growth Factor (VEGF), a major angiogenic factor responsible for stimulating vascular growth [70].

Both heterodimerization and DNA binding is mediated by the bHLH-PAS domain of HIF [71].

1.5.2 Differential Expression of HIF in Tissues

HIF 1α levels are often used as a marker for HIF transcriptional activity in many tissues [72]. HIF 1α and HIF 2α proteins are similar in structure and sometimes redundant in cellular functions. However, HIF 2α is only expressed in a limited number of tissues and cell types. HIF 3α is the latest discovery in the HIF family; its expression is different from the other two and it has many splice variants that can competitively inhibit HIF 1 and 2α. The cellular response of HIF 3α is observed as either complementary or suppressive to HIF 1 and 2α depending on the studies [73]. Both HIF 1 and 3α have numerous common target genes. Luciferase reporter assay and chromatin immunoprecipitation studies have shown that HIF 3α contains a functional hypoxia response element to which HIF 1α binds to enhance the expression of HIF 3α [74]. However, other studies have shown an upregulation of HIF 3α in hypoxia when HIF 1 and 2α levels were unaltered [75].

1.5.3 Hypoxia Response Genes

These are genes that have the nucleotide sequence 5'-RCGTG-3' called the hypoxia response element (HRE) in their promoters or enhancer regions, to which the HIF heterodimer binds in order to induce transcription. R in the HRE nucleotide sequence denotes either an adenine (A) or Guanine (G) [76]. This cis-acting DNA binding sequence was first identified in 1991 by

Semenza et al in the 3' Flanking Region of the erythropoietin (EPO) [77]. The HRE was reported to be essential for the transcriptional activation of the EPO gene during hypoxia. Subsequently, HIF was found to regulate the expression of more than 100 genes that have HRE [78]. These genes mainly encode for proteins that mediate adaptive physiological processes such as angiogenesis, erythropoiesis, autophagy, apoptosis, anaerobic metabolism, etc. Common examples of genes with HRE include EPO, vascular endothelial growth factor (VEGF), Lysyl Oxidase, Protein kinase C-binding protein 1, glucose-transporter 1 (GLUT1), Lactate dehydrogenase A (LDHA), aldolase A (ALDA), phosphoglycerate kinase 1 (PGK1) , etc. [72, 64]. Connective tissue growth factor (CTGF) which is implicated in fibrotic conditions is also a proven HIF-Target gene [79].

1.5.4 Regulation of HIF Function

Beyond oxygen levels, the transcription and translation of HIF α is regulated by various signal transduction pathways [80]. The expression of HIF α like other proteins is regulated by the phosphatidyl inositol-4,5-bisphosphate-3-kinase (PI3K) pathway through its target protein kinase B (Akt) and the downstream effector mammalian target of rapamycin (mTOR). This pathway is inhibited by the presence of a functional Phosphatase and tensin homolog (PTEN). The PI3K/AKT/mTOR pathway together with the activation of RAS/ERK lead to HIF α transcription and translation. The resulting activation of the mitogen-activated protein kinase (MAPK)/ERK signaling transactivates and stabilizes HIF α [81]. As well, hypoxia can phosphorylate MAPK which is essential for HIF α stabilization, cytoplasmic accumulation and nuclear translocation [82]. Importantly, the regulatory relationship between the PI3K/mTOR/MAPK/ERK pathways and HIF may explain why these pathways together with HIF are commonly implicated in most

inflammatory and fibrotic conditions. In these pathological states, HIF is able to stimulate adaptive survival processes that maintain oxygen homeostasis and metabolic equilibrium [82].

As a protective role, functional p53 tumor suppressor protein induces degradation of abnormally high HIF levels [83]. Thus, in summary, HIF function can be activated by the loss of function of tumor suppressors such as VHL, PTEN and p53 and the gain of function of genes that encode for mTOR, PI3K, RAS, AKT. The binding of Heat shock protein 90 (Hsp90) to HIF α is known to induce stability to the alpha conformation against the von Hippel Lindau-mediated degradation [80].

1.5.5. Role of Hypoxia in Inflammation and Fibrosis

The level of oxygenation is a critical factor in the microenvironment of tissue injury. The induction of HIF in acute injury has beneficial effects that help the tissue to adapt to hypoxia and stimulate restorative angiogenic growth. However, in most chronic inflammatory conditions, the HIF pathway is known to drive tissue fibrosis [84].

In many clinical conditions, hypoxia is known to promote pro-inflammatory and pro-fibrotic mediators and cytokines; Transforming Growth Factor Beta (TGFβ) is a master regulator of cell growth, proliferation, differentiation, and the Epithelial-Mesenchymal Transition (EMT). TGFβ stimulation involves two pathways known as the canonical (Smad related) and non-canonical (non smad) which ultimately lead to fibrosis. Some of the downstream pro-fibrotic effects of TGFβ Stimulation include epithelial to mesenchymal transition, activation of myofibroblast, and Connective Tissue Transforming Growth Factor (CTGF), as well as inhibition of extracellular matrix breakdown [85].

At the molecular level, both hypoxia and inflammation are regulated by complex inter-related pathways. Hypoxia is known to induce the nuclear translocation and functional activity of nuclear factor-κB (NFκB), which is the master regulator of inflammatory pathways. The NFkB binds to and stabilizes HIF expression in the inflammatory milieu [86]. Moreover, HIF and NF-kB have some common target genes and hypoxia can trigger the production of certain cytokines that promote inflammation (pro-inflammatory cytokines) [87].

Two key intermediary processes involved in increased collagen production are an upregulation of tissue inhibitors of metalloproteases (TIMP) and an activation of myofibroblasts [88-90]. HIF does not only augment extracellular matrix protein production but also, there is evidence suggesting that it directly contributes to fibrosis by promoting the expression of profibrotic factors like the tissue inhibitors of metalloproteases (TIMP) [90]. Moreover, hypoxia, as well as inflammation, TGFB and PDGF can increase extracellular matrix production via Myofibroblast activation [17, 90].

1.6 In-Vivo Induction of pBOO and Therapeutic Response

Our laboratory has successfully characterized the progression of bladder deterioration after pBOO and demonstrated multiple effective therapies [17, 91, and 92]. After 2 weeks of pBOO the bladders demonstrated a massive up-regulation in the transcription and production of many inflammatory cytokines involved in multiple pathways. Prolonged obstruction demonstrated that the inflammatory response is replaced with a compensatory smooth muscle hypertrophy evident by microscopy and associated with significant increases in bladder pressures and capacity. Further periods of pBOO, up to 8 weeks, demonstrated a complete loss of normal tissue

architecture and replacement with dense, inelastic collagen. This results in further increases in pressures, but a decrease in capacity [17]. This temporal model closely replicates the clinical scenario, whereby the pBOO results in initial LUTS, which progresses to incontinence, and eventually an end-stage bladder which is a threat to normal renal function.

We were able to demonstrate that out current medical therapy effectively reduces bladder pressures. We chose to study oxybutynin, the gold standard for decreasing bladder contractility and reducing storage pressures. Our model was able to demonstrate improved urodynamic parameters, as well as a decrease in bladder weight and thickness. Histology confirmed a minimization of the expected smooth muscle hypertrophy expected after pBOO. Furthermore, the first improvements were seen with oxybutynin, via detrusor contractility. However, the treatment with oxybutynin did not seem to decrease inflammatory and fibrotic mediators, such as: TGFβ, HIF-1α, CTGF, or mTOR [92]. We hypothesized that although our current gold-standard demonstrated efficacy, the crude upstream inhibition at the muscular level did not seem to affect the inflammatory milieu. This may explain its lack of long-term efficacy and reinforced our interest in an anti-inflammatory / anti-fibrotic intervention.

1.7. Introduction to Regenerative Medicine

Regenerative medicine is an interdisciplinary innovative technology in medicine that uses cells, proteins and genes to replace, restore and/or rebuild human tissues and organs. First published in 1992, Leland Kaiser was the first to express hope that regenerative medicine will revolutionize the treatment of chronic diseases [93]. However, William Hazeltine is believed to have publicized regenerative medicine in 2000 to 2003. This led to high public interest in the

therapeutic potential of regenerative medicine to solve serious health challenges such as organ failure, cardiovascular diseases, hematological disorders, degenerative disorders, brain and spinal cord damage. Importantly, this innovation is expected to provide a solution to the donor organ shortages and tissue rejection that confronts contemporary transplantation [94]. Stem cells play a key role in this therapy and the commonly researched areas of this field include: a) the injection of stem cells into circulation or directly into a dysfunctional tissue or organ in order to stimulate healing and regeneration. b) The use of cells and biocompatible materials to engineer new tissues and organs for transplant [95].

Remarkably, after years of hope and high expectations, regenerative medicine is yet to bring about most of the anticipated therapeutic transformations. Therefore there is the need to intensify research to overcome any challenges and fully benefit from this innovation.

1.8. Stem Cell Therapy

Stem cells are a unique collection of unspecialized cells in the body that have multi-lineage differentiation capacities, are self-renewing and able to modulate immune responses. When transplanted, they home in to injured tissues, are anti-inflammatory, anti-fibrotic, and stimulate blood vessel formation. These are some of the features that have generated excitement for their curative and reparative use. Some proposed mechanisms by which stem cells effect their therapeutic roles include cell-to-cell contact between stem cells and host cells, trans differentiation, where stem cells differentiate into a specific cell type to replace damaged cells of an organ, and paracrine effects. The paracrine effect describes the ability of stem cells to produce

concomitant amounts of bioactive factors that exerts regenerative effects on injured tissues. These secretory factors are capable of modulating recipient's immune responses [96].

In the last decade, paracrine signaling has emerged as an important mechanism, playing a primary role in stem cell therapy. Media in which stem cells have been cultured, called stem cell-conditioned media is known to contain stem cell-secreted factors that have anti-inflammatory, anti-scarring, angiogenic, healing and regenerative effects in various disease models [97, 98].

Microvesicles (spherical cell membrane fragments that contain genes, lipids, proteins and micro RNAs) derived from stem cells have also been shown to have therapeutic and reparative value. The microvesicle-paracrine signaling is currently attracting attention due to the ability of microvesicles to transfer their bioactive content to recipient cells. This leads to modulation of gene expression, inducing significant functional and phenotypic changes in the recipient cells [99]. Research into the injection of stem cells, stem cell-conditioned media, and stem cell microvesicles have produced promising results in both human and animal studies (99, 100). The advantages that MSCs-derived microvesicles have over the systemic transplant of whole stem cells is their nano-sizes reducing the chance of clogging and compromising the circulatory system. Also, microvesicles are relatively stable with repeated freezing and thawing [101]. However, the major setbacks to the use of these MSCs-exosomes has been lack of proper organ targeting when systemically injected coupled with challenges in scaling up production to generate adequate quantities for research and clinical use [102]. There are three main types of stem cells and they are: embryonic stem cells, induced pluripotent stem cells, and adult stem cells.

1.8.1 Human Embryonic Stem Cells

These cells have the highest proliferative capacity and can differentiate into the three germ layers of the body; endoderm, ectoderm and the mesoderm. They are derived from the inner cellular mass of a pre-implanted embryo that is 3 to 5 days old. Thus, they have the capacity to form the over 200 different types of cells of the human body [103]. The emerging field of regenerative medicine was motivated by the isolation of these embryonic stem cells and discovery of their ability to differentiate into different cell types of the body (pluripotency). However, research into the possibility of using these cells to treat chronic diseases has been hampered by a strong ethical battle over the need to destroy the life of a human embryo in order to acquire these cells. Later, this field was revamped by the possibility of obtaining these cells from the extra embryos produced during in vitro fertilization treatments [104].

1.8.2. Induced Pluripotent Cells

These are a novel class of adult differentiated cells reprogramed into embryonic stem cells by the introduction of four embryonic regulatory proteins (Oct3/4, Sox2, c-Myc, and Klf4) [105]. This discovery in 2006 was hailed by the scientific community as a direct solution to the moral issue surrounding the acquisition of human embryonic stem cells. The authors, Takahashi and Yamanaka received prestigious awards such as the Shaw Prize (2008), Nobel Prize in Physiology or Medicine (2012), Millennium Technology Prize (2012), and Breakthrough Prize in Life Sciences (2013) for their novel discovery. However, after a decade of their discovery, the oncogenic transformation potential of these cells have limited excitement for their therapeutic use.

1.8.3. Adult Stem Cells

These are undifferentiated adult cells that are derived from the blood (hematopoietic) and other mesenchymal sources such adipose tissue, bone marrow, skin, urine, gum, placenta, umbilical cord, and skeletal muscles. These hematopoietic and mesenchymal stem cells have a wide spectrum of application in regenerative medicine. Their broad tissue distribution, ease of isolation, ability to induce immune response coupled with the ease of expansion and reduced oncogenic transformation abilities have made mesenchymal stem cells the most frequently used in clinical trials [106].

1.8.3.1 Mesenchymal Stem Cells

Even though Mesenchymal stem cells were first isolated in the 60s, credit is given to the work of Friedenstein and colleagues in 1976 who isolated and described them as plastic-adherent clonal cells [107]. Since then several researches using these cells have emerged. Subsequent work revealed that MSCs are self-renewing multipotent cells and are able to differentiate into the mesoderm-derived cells [108]. MSCs are characterized by the expression and absence of several surface markers. To ensure uniformity in definition, the International Society for Cellular Therapy proposed minimal criteria for defining human MSCs as: 1. they must be plastic-adherent in standard culture conditions. 2. MSCs must express the cluster of differentiation (CD) markers such as CD105, CD73 and CD90, coupled with the absence of CD14, CD11b, CD19, CD34, CD45,CD79 alpha and HLA-DR surface antigens. 3. MSCs must have the ability to differentiate into osteoblasts, adipocytes and chondroblasts in vitro under the appropriate stimulating conditions [109]. However, all these characterization criteria do not uniquely distinguish MSCs: in culture, MSCs are identified as fibroblast-like cells and share most of the above characteristics

with fibroblasts. In addition studies have shown that fibroblasts also have the ability to modulate macrophage responses in vitro [110]. Therefore, there remains a crucial need to identify unique phenotypic criteria to redefine mesenchymal stem cells.

Even though MSCs are derived from several tissues, bone-marrow derived MSCs have been most studied. Despite their low frequency in the bone marrow (0.001-0.01%), MSCs are easily isolated from the bone marrow under local anesthesia and expanded in culture while maintaining their therapeutic value [111]. When transplanted, bone marrow-derived MSCs have a remarkable ability to home in to sites of tissue injury to promote tissue repair [112]. The ability of these cells to secrete an array of bioactive substances play beneficial roles in tissue injury. This trophic activity is thought to create the needed environment that stimulates a regenerative response and reduces injury area [111].

1.9. Rational, Aims and Objectives of Study

The high number of people affected by pBOO, the resulting bladder deterioration, lack of an effective therapy as well as the increased morbidity associated with current treatment modalities were the motivating factors for this study. PBOO affects over 1.1 billion people worldwide including both children and adults [1]. It is a progressive condition with multifactorial etiological causatives. Current available treatment lack long term efficacy and has failed to effectively target disease pathways [9]. In order to make significant contribution to this field, we prioritized the need to understand the underlying molecular mechanisms of this condition.

Increased bladder contractility, constriction of blood vessel and reduction of blood flow to the obstructed bladder are significant events that occur in pBOO [6]. Consequently, hypoxia was

identified in the obstructed bladder tissue. However, its exact role in disease pathology was not known.

The benefits of mesenchymal stem cell therapy has been elucidated in various disease models. However, the mechanisms underlying its therapeutic effects is not well understood. Also the ability of stem cells to inhibit hypoxia-induced inflammatory and fibrotic mechanisms is not yet known. Therefore, the experiments of this thesis were designed to answer these research questions.

The specific objectives of this study were to:

1. Elucidate the isolated effects of progressive hypoxia on urinary bladder cells:
    a) Explore the link between hypoxia and inflammation, EMT, smooth muscle dedifferentiation, pro-fibrosis and extracellular matrix synthesis.
    b) Define hypoxia regulation of the bladder at the molecular level; explore the link between the different HIF α subtypes and progressive hypoxia.

2. Investigate the ability of MSCs to inhibit hypoxia signaling pathways
    a) Explore the anti-fibrotic effects of MSCs in vitro using established hypoxia model;
    b) Investigate the role of MSCs paracrine and cell-cell mechanisms of action in the inhibition of inflammatory and fibrotic pathways.

3. Investigate the ability of MSCs to inhibit pBOO-induced fibrotic pathways;
    a) Confirm in vitro mechanisms in animal models of pBOO;

b) Investigate the anti-fibrotic effects of MSCs in the pBOO pathology, taking a look at multiple targeted pathways

c) Explore the ability of MSCs to preserve bladder integrity and function after pBOO.

Chapter 2

Stem Cell Therapy: Current Applications and Potential for Urology

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2.1 Overview

Stem cell therapy holds the potential to revolutionize the treatment of a number of chronic conditions. Its ability to homme to injured sites of the body, stimulate angiogenesis, tissue regeneration, immunomodulation, anti-inflammatory and anti-fibrotic factors have attracted their use in the treatment of many conditions. Urology has registered one of the highest shown some significant experimental successes using stem cell therapy. However, the rate of clinical

applications is comparatively lower. This review takes a look at our efforts so far and what needs to be done in order to maximize the clinical benefit we can derive from stem cells.

2.2 Introduction to Stem Cells

Stem cells refer to a unique cell type with multiple unique characteristics including: unlimited expansion, self-renewal and a multipotent differentiation capacity. These characteristics have resulted in enormous excitement for their potential regenerative, reparative and immunomodulatory exploitation in the treatment of a number of disease conditions at both experimental and clinical levels. Stem cells are derived from three main sources: Human Embryonic stem cells are derived from the core mass of 4 to 5 day old human embryos. They have very high proliferative potential and can differentiate into an endoderm, mesoderm or an ectoderm [1]. However, the embryo-destroying nature of its acquisition raises one of the fiercest ethical issues in the history of medicine and therefore, limiting its potential clinical use.

It was to overcome these moral issues, that the award winning research by Takahashi and Yamanaka in 2006 was able to reprogram adult differentiated cells into embryonic stem cells by overexpressing some four transcription factors. This was referred to as induced pluripotent stem cells [2]. Since its discovery, critics have questioned the safety of its clinical application as its implantation into nude mice induced teratoma formation. Thus not much has been made of this novel discovery. A third category, called adult stem cells is derived from tissues of the body, commonly, the blood (hematopoietic) and other mesenchymal tissues including bone marrow, skeletal muscles, skin, brain and adipose. These mesenchymal stem cells (MSCs) have been

found to have a wide spectrum of applications in the areas of therapeutics, regenerative medicine and tissue engineering [3].

2.3 Mechanism of Action of Stem Cells

The specific mechanism of action of stem cells has not yet been clearly defined. However, some proposed mechanisms include cell-cell contact, paracrine signaling, cell fusion, differentiation into a specific cell type, neovascularization or a combination of these mechanisms [4]. The paracrine effect has attracted more attention in the past 5 years. This is because stem cells actively secret cytokines, chemokines and growth factors that affect their microenvironment [5]. Moreover, it has been established that the differentiation pattern and the specific participation of stem cells in terms of tissue repair, maintenance and generation depends on its niche. The immediate environment of the stem cells including the surrounding cells and the trophic factors secreted by the stem cells act together to create unique actions and capabilities dependent on that particular milieu [6].

2.4 Therapeutic Use of Stem Cells

Stem cells have a broad range of applicability including the following the following areas:

2.4.1 Stem Cell Therapy in Autoimmune, Inflammatory and Hematopoietic Diseases

All over the world, stem cell clinical exploits seem to be increasing by the day. Commonly, the transplantation of hematopoietic stem cells from a healthy donor has been beneficial in the treatment of leukemia and other cancers of the blood. Umbilical cord blood-derived stem cells are also approved by the US FDA for treating hemato-immunopathologies [7]. After 20 years of intensive research and successful phases of clinical trials, Health Canada also approved the use

of mesenchymal stem cells called prochymal in the treatment of children with steroid-resistant graft vs host disease. In this therapy, stem cells derived from healthy donors are expanded in culture, frozen until needed and administered intravenously. Successes achieved with this treatment since 2012 have motivated clinical trials of prochymal in the treatment of diabetes type 1, cardiovascular and crohn's diseases [8].

2.4.2 Clinical Application in Fibrotic Conditions

The ability of mesenchymal stem cells (MSCs) to migrate to injured site or a diseased organ in the body, promote tissue repair and inhibit inflammation has motivated their use in the treatment of multiple fibrotic conditions including myocardial infarction, pulmonary, hepatic and renal fibrosis. A safety trial that tested the use of allogenic laboratory-expanded bone marrow-derived MSCs in 53 patients after myocardial infarction found the administration of MSCs to be safe and also effective in reducing arrhythmic events and improving pulmonary function. It was also shown to be effective in increasing left ventricular ejection fraction after 3 months of treatment [9]. In another study, intracoronary administration of MSCs after acute myocardial infarction resulted in enhanced myocardial function and perfusion with no records of arrhythmias and deaths within 3 months in human subjects [8]. Similarly, several other studies have also reported the effectiveness of MSCs in improving various cardiovascular parameters in patients with myocardial defects [10-14]. The most outstanding of these is the recent reports by Williams et al [10] that reported that there is remodelling of cardiac structure and improvement in function after the transendocardial injection of MSCs in myocardial infarction patients who had developed chronic ischemic cardiomyopathy.

In the trials of liver fibrosis, numerous clinical studies have reported the effectiveness of MSCs in reducing liver fibrosis and improving liver function [15-23]. Nonetheless, stem cell therapy of pulmonary fibrosis has seen very little of translation. The first two human studies were primarily designed to test the safety of MSCs in pulmonary fibrotic patients [24, 25]. Even though the study achieved this primary objective, it was quite disappointing that there was no improvement in lung and respiratory function after 6 months of MSC administration. However, these studies were limited by a small sample size and the lack of placebo control groups. Currently; there is an ongoing large-sample sized multicentric non-randomized clinical trial to test the efficacy of MSCs in pulmonary fibrosis that hopes to achieve clinically relevant results.

Fibrosis is marked by an increase in production and deposition of extracellular matrix proteins of which collagen is predominant, and a reduction in their breakdown. The administration of MSCs has been effective in reducing collagen components of a variety of fibrosis models [26-33]. This is thought to be mediated by the secretion of anti-inflammatory cytokines, including IL-6 and IL-10. Stem cells are also known to inhibit many inflammatory/profibrotic markers such as TNFα, IFNα/γ, TGFβ, etc. [34, 35]. The increased production of matrix metalloproteases and the downregulation of tissue inhibitors of metalloproteases have also been proposed as a possible mechanism by which stem cells reverse fibrosis [36].

2.4.3 Tissue Engineering Use of Stem Cells

Injuries, cancer, inflammation, dysfunction, aging, etc. are some examples of conditions that may require tissue or organ transplant. The scarcity of organ donors cannot be overemphasized and

tissue engineering using stem cells is thought to have the potential to overcome this problem. Excellent studies by Adesida et al have highlighted the importance of stem cells in cartilage and meniscus regeneration [37-41]. Even though so far, the bulk of applications of stem cells in tissue engineering remain preclinical, there are some clinical successes attained with regenerative stem cell work. Patients with severe alveolar ridge atrophy who received tissue engineered bone made of autogenously sourced, lab-expanded mesenchymal stem cells and platelet-enriched serum developed a regenerative and functional bone that improved mastication [42]. Other clinical studies have also reported the improvement of bone structure and function by transplanting stem cell tissue-engineered bone into patients with a variety of bone-related pathologies [43-46].

2.5 Potential Use of Stem Cells in the Urinary Tract

The existing and potential clinical applicability of stem cells in urology are categorized below:

2.5.1 Renal Fibrosis

Acute, chronic and end stage kidney failure are major health concerns. Complicated by the lack of nephrogenesis and the low regenerative capacity of the adult kidneys [124], injured nephrons are not replaced leading to a progressive loss of function, scarring and fibrosis. MSCs possess significant immunomodulatory effects, promote tissue healing and regeneration, prevent apoptosis, and are pro-angiogenic [47, 48]. Experimentally, stem cell therapy of a variety of kidney injury models has been shown to be effective in reducing renal fibrosis [49-56]. By putting together 71 preclinical stem cell-based studies of kidney injuries using meta-analysis,

Papazova and his colleagues reported that the intravenous or renal artery injection of bone-derived mesenchymal stem cells was effective in treating chronic kidney diseases [57]. However, we are still at the early stages of clinical applications. Clinical trials carried out to test the ability of stem cells to suppress immune-response and aid regeneration in kidney transplant recipients was successful [58, 59]. It has also been reported in a clinical trial that the administration of allogeneic stem cells reduces the risk of kidney failure in patients undergoing open heart surgery [60]. Invariably, initiating cell based therapies at an early stage of renal diseases will have enormous benefits of improving the tissue repairing capacity of the kidneys so as to reduce the risk of disease progression. Since ischemia and reperfusion play major roles in renal damage, the introduction of stem cells is likely to stimulate angiogenic and mitotic pathways which are both important for damage repair.

2.5.2 Bladder Obstruction

The fact that partial bladder outlet obstruction (pBOO) causes significant fibrosis and tissue remodelling cannot be overemphasized. Our lab has demonstrated that bladder outlet obstruction progresses from inflammation through hyperthrophy to fibrosis [61]. Even though bladder obstruction is a major urological problem with debilitating consequences, minimal contemporary progress has been made in its management. Despite the scarcity of such studies, work specifically with stem cells has been encouraging: Using the same pBOO model, our lab demonstrated that a short term administration of MSCs causes' significant reduction in hypoxia and inflammatory markers whiles improving urodynamics. [62]. Woo et al have also demonstrated the effectiveness of MSCs in reducing hypoxia and fibrosis in mice models of pBOO [63]. Subsequently, Lee et al also reported the ability of MSCs to reduce the high collagen

and TGFB levels induced by pBOO to normal levels after its administration in rat models [64]. Comparatively, looking at how much work has gone into the preclinical studies of MSCs in cardiovascular and kidney diseases, there is indeed a big gap in our knowledge of MSC therapy in pBOO; the different sources, routes of administration and doses of MSC therapy in pBOO have not yet been studied. More importantly, the paracrine effects and other mechanisms of action are yet to be elucidated.

2.5.3 Urinary Incontinence

Stress urinary incontinence (SUI) and overactive bladder (OB) are major public health problems that negatively affect the quality of life of many worldwide. The urethral sphincter muscles play crucial roles in urinary continence. Thus, efforts in stem cell therapy have focused on regenerating the urethral sphincter. Animal studies of cell therapy of SUI have been very promising with improvements in both histology and function: Administration of MSCs resulted in a functional increase in urethral muscles, elastin and connective tissue [65-67]. However, results obtained from clinical studies have not yet demonstrated substantial successes. Four different clinical trials that examined the efficacy of intrasphincteric injection of MSCs in men and women with SUI achieved moderate results with a few reports of significant improvements in continence [68-71]. The largest study was conducted in 222 men with postprostatectomy urinary incontinence. 54% of the subjects responded to MSC therapy whiles only 12% of this number ultimately became continent [72]. In a recent study of 5 patients with SUI, transurethral injections of autologous adipose stem cells combined with bovine gel and saline, resulted in a negative cough test in only one subject when bladder was saline-filled to a 500ml capacity. Three more subjects achieved a negative cough test after one year of treatment leading the authors to

describe the efficacy of treatment as suboptimal [73]. The most encouraging report from the cell based therapies of SUI is a recent report of a two year trial that evaluated the transurethral injection of autologous, lab-expanded skeletal muscle-derived MSC in 16 SUI patients. This study achieved a success rate of 75% with 50% completely recovering continence [74].

2.5.4 Tissue Engineering

Urology has remained at the forefront of tissue engineering and regenerative medicine, and has seen applications in the kidney, urethra, and the bladder. However, the use of stem cells in regenerating urological organs is still at the experimental stages.

In attempts to tissue engineer the bladder, different types of materials have been tested for compatibility. These range from acellular matrices of bladder and small intestine submucosa, synthetic polymers as well as collagen and alginate [75]. In a clinical study in 1998, an implantation of collagen scaffold seeded with laboratory expanded autologous urothelial and bladder smooth muscle cells, covered with fibrin glue and omentum was able to replace the diseased bladder and improved urodynamics and bladder compliance [76]. This gives hope that the use of MSCs and scaffold to regenerate a functional bladder is feasible given all the advantages of stem cells over somatic cells.

Engineering a completely functional kidney for transplant may be an extremely complex and ambitious project, given the multiple functions of the kidneys, the complex architecture of the nephrons and tubules as well as the risk of tissue rejection. Nonetheless, a renal assist device made up of donor renal tubular cells performed reabsorption in renal disease patients when used

in combination with dialysis [77]. This, indicating that, in tissue engineering the kidney, focusing on a single function at a time may be beneficial.

Urethral stricture is surgically corrected (urethroplasty) by an autograft of buccal mucosa, penile or scrotal tissues. Challenges with the current procedures may be the vexation and tissue damage that may result from obtaining the graft [78] as well as the tendency of long term implanted graft deterioration [79]. Urethral tissue engineering techniques that use cadaveric bladder matrix or synthetic collagen matrix have been tested in patients with hypospadias and those with strictures [79-81]. Results obtained from these studies were comparable with those obtained from the existing urethroplasty procedures. Therefore, it has been proposed that to achieve better urethral reconstruction, bone marrow MSC-seeded bladder acellular matrix should be considered as it gave a 100% success rate in rats [82].

Factors that are crucial in tissue engineering are the scaffold, seeded cells as well as cytokines, growth factors and extracellular matrix that drives angiogenesis, vascularization and neurogenesis of the regenerated organ [83, 84]. Since stem cells secret their own growth promoting cytokines and are able to stimulate vascularization and nerve development, this presents an opportunity for their exploration in tissue engineering urological organs. The discovery that stem cells can be derived from excreted urine has been received with much enthusiasm in the field of urology. Apart from the simplicity and non-invasiveness of their acquisition, they are able to differentiate into bladder urothelia and smooth muscle phenotypes. More importantly, the in vivo multilineage differentiation ability of these stem cells was safely demonstrated in nude mice without the formation of a teratoma [85]. Thus, it is our hope that the full potential of these urine derived stem cells will be exploited in the treatment of urological

conditions.

2.6 Paracrine Effects of Stem Cells on Urologic Factors

Hypoxia is a well-known factor in the pathophysiology of a number of urologic conditions including renal fibrosis, bladder obstruction, overactive bladder, unilateral ureteral obstruction, cancers, etc. In models of these diseases, several cytokines and chemokines are produced by the hypoxic tissue which attracts MSCs to the site of injury. Proinflammatory cytokines are essential in stimulating the angiogenic, lineage-specific differentiation, proliferative and immunomodulatory functions of stem cells. Interleukin (IL) 1 α and β, Tumor Necrotic Factor (TNF) α and interferon ɣ are examples of proinflammatory markers that play roles in kidney fibrosis, cancers, bladder obstruction and other urologic diseases [86-89]. Recent studies in our lab showed that hypoxia treatment of normal bladder smooth muscle cells results in a time-dependent increase in endogenous TNF α levels. Interestingly, all these stated cytokines have been found to stimulate the immunomodulatory effects of bone marrow-derived MSCs [90, 91]. IL receptor agonist and IL 6 secreted by MSCs inhibit macrophage-activated TNFα secretion and neutrophil apoptosis respectively [92, 93]. This suggests therefore, that stem cell application has a huge therapeutic potential in the field of urology.

2.7 Future Directions

It is obvious that MSC has enormous potential in urologic care. Although tissue engineering and renal repair aspects are the best studied, we believe that their application in pBOO is substantial. The paracrine immunomodulatory effects of these cells should be able to

significantly reduce the inflammation and hypoxia seen after pBOO, and effectively prevent secondary decompensation. This would revolutionize the treatment of patients with spina bifida, posterior urethral valves, and prostatic hypertrophy. The volume of work done on stem cells in renal diseases is very inspiring. This must guide and motivate us to apply stem cells therapeutically. Moreover, much more work is required for stem cell therapy in pBOO and urinary incontinence to be successful.

Chapter 3

Hypoxia-increased expression of genes involved in inflammation, dedifferentiation, pro-fibrosis, and extracellular matrix remodeling of human bladder smooth muscle cells

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3.1 Introduction

Partial bladder outlet obstruction (pBOO) occurs as a sequela of benign prostate hyperplasia, posterior urethral valve disease, urethral stricture disease, cancer, and neural tube defects. The resistance to voiding initially results in a compensatory response, with bladder smooth muscle hypertrophy. However, excessive, prolonged exposure can lead to decompensation [1]. A myriad of factors including stretch, hypoxia, hydrodynamic pressure, and/or inflammation are involved in the etiology of bladder damage. Also, in the aged population, hypoxia has been known to correlate with increased urological disorders [2]. In the early development of pBOO, there is a sequential process of inflammation, hypertrophy, and eventually culminating in fibrosis [3]. Further in its development, the obstructed hypercontractile bladder is exposed to more frequent yet prolonged cycles of ischemia during the filling and voiding stages [4]. Even though many studies have identified hypoxia in the muscle layer of the obstructed bladder, the effects of hypoxia as a sole stressor on bladder injury remain unknown.

The ability of many cell types to sense and respond to reduced oxygen tensions is essential for their survival. The major protein central to mediating this protective response is the hypoxia-inducible factor (HIF) [5]. The HIF family is characterized by a heterodimeric structure and consists of three known members: HIF1, HIF2, and HIF3. Structurally, each member consists of a stable and uniformly distributed β-subunit and an oxygen-sensitive, hypoxia-inducible α-subunit. During hypoxia, the α-subunit is stabilized via prolyl hydroxylase inhibition and subsequently dimerizes with the β-subunit. The α-β dimer is a transcription factor for stimulating the transcription of key genes. These genes encode for numerous cytoprotective proteins to combat the deleterious effects of hypoxia [5]. One key protective protein is the vascular

endothelial growth factor (VEGF), a major angiogenic factor responsible for stimulating vascular growth.

In many clinical conditions, hypoxia is known to promote pro-inflammatory mediators and cytokines; transforming growth factor beta (TGFβ) is a master regulator of cell growth, proliferation, differentiation, and the epithelial-mesenchymal transition (EMT). Stimulation of the TGFβ-SMAD pathway results in the downstream activation of connective tissue transforming growth factor (CTGF); this inevitably leads to increased collagen deposition. Not only does TGFβ act independently to augment extracellular matrix production, exposure to hypoxia has similar effects at the level of transcription and translation [6, 7]. Two key intermediary processes involved in increased collagen production are an upregulation of tissue inhibitors of metalloproteases (TIMP) and an activation of myofibroblasts [8-10].

In order to understand the mechanisms involved in the development of pBOO, we hypothesized that exposing normal bladder smooth muscle cells to hypoxia would stimulate inflammation and induce a fibrotic phenotype.

3.2 Materials and Methods

Established normal human bladder smooth cells were purchased from ScienCell Research Laboratories (Carlsbad, CA) in 2015, and their smooth muscle phenotype was confirmed by their culture characteristics and the expression of the smooth muscle markers; h-caldesmon, desmin, α smooth muscle actin (αSMA) as well as the absence of l-caldesmon as previously established [11, 12]. They were cultured in complete smooth muscle medium containing smooth muscle growth factors (1×), fetal bovine serum (10%), and 1 × 1000 U/ml penicillin and 1000 mg/ml

streptomycin from ScienCell Research Laboratories. Cells were cultured in humidified air at 37°C with 5% CO_2. Exposure to hypoxia Cells between passages 3 and 10 were used for this experiment. 2×10^6 cells/100-mm culture dish were maintained at normoxic conditions of 21% O2 in humidified air, 5% CO_2, 74%N_2 at 37°C; hypoxic conditions were defined as a gas mixture of 3% O_2, 5% CO_2, 92% N_2 at 37°C for 2, 24, 48, or 72 h, maintained in a Thermo Scientific series II water jacket CO2 incubator (Waltham, MA). For controls, equal numbers of cells were maintained under normoxic conditions for an equivalent length of time.

3.2.1 Quantitative real-time PCR

Immediately at the end of the specified period of hypoxic or normoxic culture of cells, spent medium was frozen, cells were washed with cold PBS and lysed, and RNA was extracted using RNeasy Mini kit (Qiagen, Valencia CA). One microgram of total RNA sample was reverse transcribed using Quantitect Reverse Transcription kit (Qiagen). Real-time PCR reaction was set up using the Kapa Sybr Fast qPCR Kit (Kapa Biosystems, Boston, MA) and specific oligo-dt primer sets for genes of interest (Table 1). An initial enzyme activation at 95°C for 3 min and subsequent 40 cycles of denaturing (95°C, 2 s) and annealing/extension (60°C, 30 s) were all carried out in a Bio-Rad CFX96 real-time system (Kallang, Singapore).Gene expression of hypoxic cultured samples relative to its normoxic cultured control were normalized to an average of two endogenous controls, β actin and 18S, using the comparative Ct method and expressed as $2-\Delta\Delta ct$.

Table 3.1 List of human primer sequences used for the gene expression studies

Target Gene	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')
HIF 1α	TTC ACC TGA GCC TAA TAGTCC	CAA GTC TAA ATC TGT GTCCTG
HIF 2α	CAA CCT CAA GTC AGC CACCT	TGC TGG ATT GGT TCA CAC
HIF 3α	TTC TCC TTG CGC ATGAAGAGTACG	TCT GCG CAG GTG GCT TGTAGG
CTGF	CAA GGG CCT CTT CTG TGACT	ACG TGC ACT GGT ACT TGCAG
VEGF	CTA CCT CCA CCA TGC CAAGT	GCA GTA GCT GCG CTG ATAGA
TGFβ	TGGAAGTGGATCCACGCGCCCAAGG	GCAGGAGCGCACGATCATGTTGGAC
αSMA	CCG ACC GAA TGC AGA AGGA	ACAGAGTATTTGCGCTCCGAA
TNFα	CTT CTC CTT CCT GAT CGTGG	GCT GGT TAT CTC TCA GCTCCA
Collagen I	CAG CCG CTT CAC CTA CAGC	TTTTGTATTCAATCACTGTCT TGCC
Collagen II	CTGGCTCCCAACACTGCCAACGTC	TCCTTTGGGTTTGCAACGGATTCT
Collagen III	TGAAAGGACACAGAGGCTTCG	GCA CCA TTC TTA CCA GGCTC
Collagen IV	CAG CCA GAC CATTCAGATCC	TGG CGC ACT TCT AAA CTCCT
Aggrecan	TGA GGA GGG CTG GAA CAATACC	GGA GGT GCT AAT TGCAGGGAACA
Fibronectin	GGA GAA TTC AAG TGT GACCCTCA	TGC CAC TGT TCT CCT ACGTGG
18S	CGG CTA CAT CCA AGG AA	GCT GGA ATT ACC GCG GCT
Interleukin 1β	ACA GAT GAA GTG CTC CTT CCA	GTC GGA GAT TCG TAG CTG GAT
Interleukin 6	TGGTCTTTTGGAGTTTGAGGTA	AGGTTTCTGACCAGAAGAAGGA
Interleukin 10	CCCTGGGTGAGAAGCTGAAG	CACTGCCTTGCTCTTATTTTCACA
Vimentin	GACAATGCGTCTCTGGCACGTCTT	TCCTCCGCCTCCTGCAGGTTCTT
B actin	AAGCCACCCCACTTCTCTCTAA	AATGCTATCACCTCCCCTGTGT
Smad 2	GTTCCTGCCTTTGCTGAGAC	TCTCTTTGCCAGGAATGCTT
Smad 3	TGCTGGTGACTGGATAGCAG	CTCCTTGGAAGGTGCTGAAG
TIMP 1	GACGGCCTTCTGCAATTCC	GTATAAGGTGGTCTGGTTGACTTCTG
Desmin	AAGATGGCCTTGGATGTGGA	GTTGATCCTGCTCTCCTCGC

3.2.2 Western Blot Analysis

Hypoxic or normoxic incubated cells were washed with cold PBS and lysed in a RIPA-protease inhibitor cocktail on ice. Protein concentration of lysates was determined using Pierce™ BCA Protein Assay Kit (Waltham, MA) and denatured at 98°C for 5 min in a sodium dodecyl sulphate

(SDS)-loading buffer. Forty-microgram protein volumes were separated by 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE). Transferred proteins on nitrocellulose membranes were blocked with 5% non-fat milk in TBS 0.05% Tween 20. Primary antibody incubation was done overnight at 4°C using anti-human HIF1α (1:2000, #AF1935), HIF2α (1:1000, #AF2997) (R&D Systems), HIF3α (whole serum, PCRP-HIF3A-1B1, IOWA DSHB, Iowa City, IA), vimentin (1:1000, #5741), and tubulin (1:10,000, #2144) (Cell Signalling Boston, MA). Protein bands were developed by incubation in secondary antibodies conjugated to horseradish peroxidase (Cell Signalling #7076, Santa Cruz #sc2768) and visualized using SuperSignal chemiluminescence substrate (Thermo Scientific).

3.2.3 ELISA for TGFβ and Total Collagen

Secreted TGFβ1 in culture media was determined using Quantikine ELISA from R&D Systems (Minneapolis, MN) as previously described [13]. Briefly, latent TGFβ1 in 100 µl of harvested culture media was activated and total concentration was determined by immunoreaction to pre-coated monoclonal human TGFβ1 using the quantitative sandwich enzyme immunoassay technique according to manufacturers' instructions.

Total synthesized collagen was determined using Chondrex hydroxyproline assay kit (WA). Samples were hydrolyzed with concentrated HCl at 120°C for 24 h, and hydroxyproline levels were estimated from a subsequent chromogenic reaction. Total collagen level in each sample was calculated as the percentage of hydroxyproline divided by 13.5 as already described [14]. All ELISA tests were carried out in duplicates for n = 3.

3.2.4 Statistical Analysis

Data was analyzed using GraphPad prism 6.0 software (GraphPad Prism Inc., La Jolla, CA). Differences between groups were assessed using one-way analysis of variance

(ANOVA) followed by Dunnett's multiple comparison test. All values were expressed as mean ± standard error; $p < 0.05$ was considered statistically significant.

3.3 Results

3.3.1 Hypoxia-inducible Factor and Pro-angiogenic Response:

The incubation of normal bladder smooth muscle cells in 3% hypoxia induced immediate and significant effects. Levels of HIF1α and HIF2α rose by 2.3- and 2.7- fold, respectively, by 2 h hypoxia ($p < 0.05$ for both), but there were no increases in transcript levels at subsequent times. HIF3α transcript levels did not exhibit any increase until 48 h at which point values rose by 2.6-fold ($p < 0.05$); this positive effect was further amplified by 5.8-fold following 72h ($p < 0.005$). HIF1 and HIF3α proteins were significantly upregulated only after 2 and 72 h, respectively, whereas HIF2α protein demonstrated only a slight increase after 24 h. The pattern of VEGF expression paralleled that of HIF1α and HIF2α. VEGF levels increase by 5-fold after 2 h; by 24 h, expression had increased by a factor 10.4 relative to control ($p < 0.005$). Despite a transient decline in values by 48 h, there was a 16.2-fold increase after 72 h.

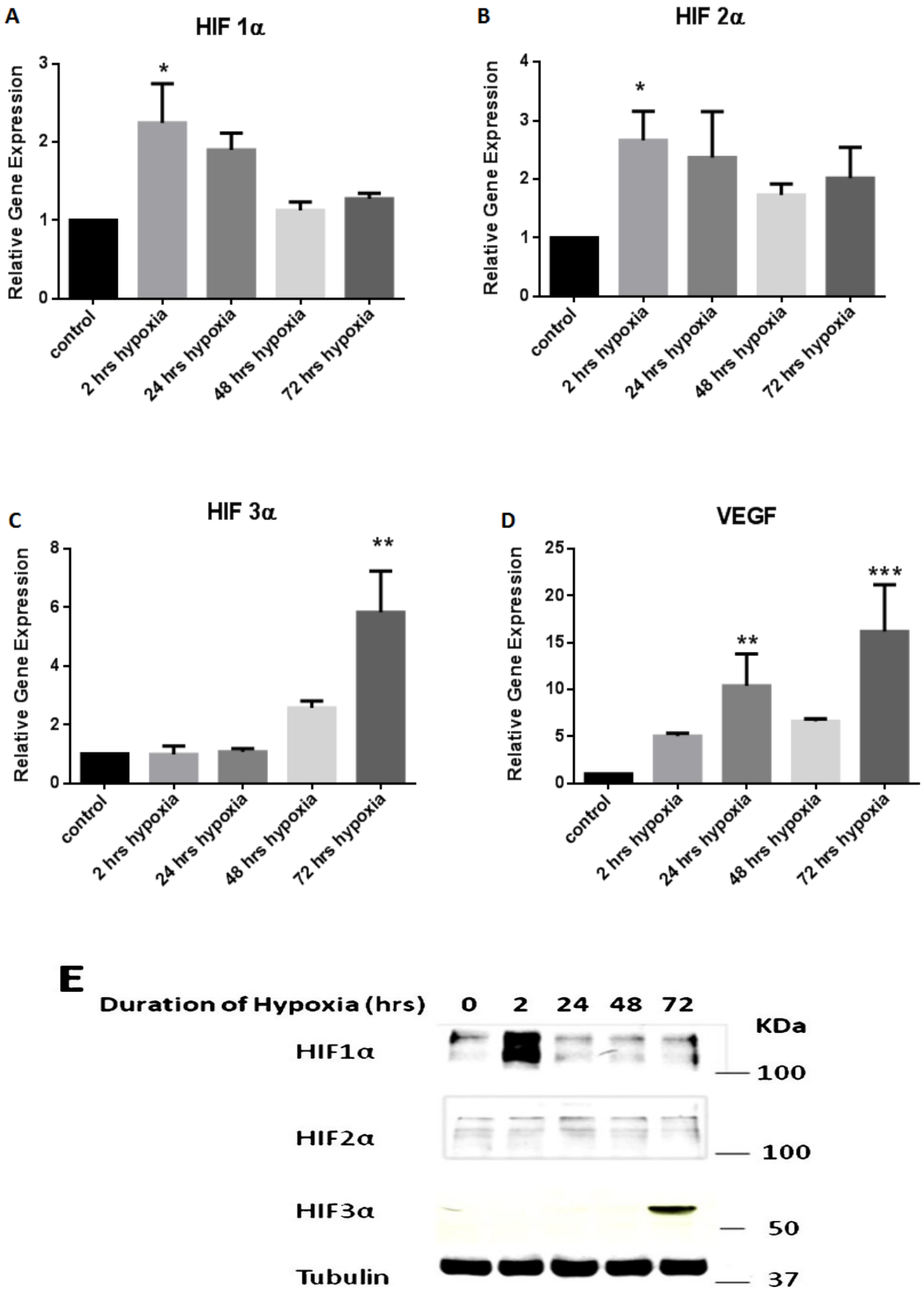
A
HIF 1α
Relative Gene Expression
*
control
2 hrs hypoxia
24 hrs hypoxia
48 hrs hypoxia
72 hrs hypoxia
B
HIF 2α
Relative Gene Expression
*
control
2 hrs hypoxia
24 hrs hypoxia
48 hrs hypoxia
72 hrs hypoxia
C
HIF 3α
Relative Gene Expression
**
control
2 hrs hypoxia
24 hrs hypoxia
48 hrs hypoxia
72 hrs hypoxia
D
VEGF
Relative Gene Expression
**
***
control
2 hrs hypoxia
24 hrs hypoxia
48 hrs hypoxia
72 hrs hypoxia
E
Duration of Hypoxia (hrs)
0
2
24
48
72
KDa
HIF1α
100
HIF2α
100
HIF3α
50
Tubulin
37

Figure 3.1 Hypoxia-inducible factor and pro-angiogenic genes increased due to hypoxia. (A, B) Human bladder smooth muscle cells responded to hypoxia by transient upregulation of transcription of HIF 1 and 2 α. (C) upregulation of HIF3α transcripts after 48 and 72 h of hypoxia. (D) Hypoxia induces transcription of VEGF after 24 and 72 h of hypoxia. Graph represents mean ± SEM and n = 5. One-way ANOVA followed by Dunnet's multiple comparison was used to compare each hypoxic group with its corresponding normoxic controls (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$). Normoxic controls were assigned an expression of 1. (E) Western blot for the HIF proteins demonstrated upregulation of HIF 1α protein (~116 kDa) after 2 h, slight increase in HIF2α (~115 kDa) after 24 h, and a significant increase in HIF3α (~70 kDa) after 72 h of hypoxia. Tubulin protein bands were used to show the equality of loaded total proteins of each sample.

3.3.2 Inflammatory Cytokines

TGF-B1 transcripts increased 2.7- fold over 48 h ($p < 0.05$) and by a total 4.4-fold after 72 h ($p < 0.005$) (Fig. 2). TGFβ1 protein levels reflected the increases in gene expression; between 24 and 72 h, there was a time-dependent increase of 27% ($p < 0.005$), 28% ($p < 0.05$), and 55% ($p < 0.005$), respectively. TNFα transcripts increased to 2.7-fold after 48 h ($p < 0.005$), and by 3.2-fold after 72 h ($p < 0.005$). IL 1β gene expression also increased by 3.2-fold after 72 h ($p < 0.005$), whereas IL-6 expression increased 8.9- fold by 24 h ($p < 0.005$). At 72 h, fold increases had reached 12.6 after 72 h ($p < 0.005$). Transcript levels of the anti-inflammatory cytokine, IL-10 exhibited a consistent decline; at the completion of the time, course levels had dropped to 44% relative to control ($p < 0.005$).

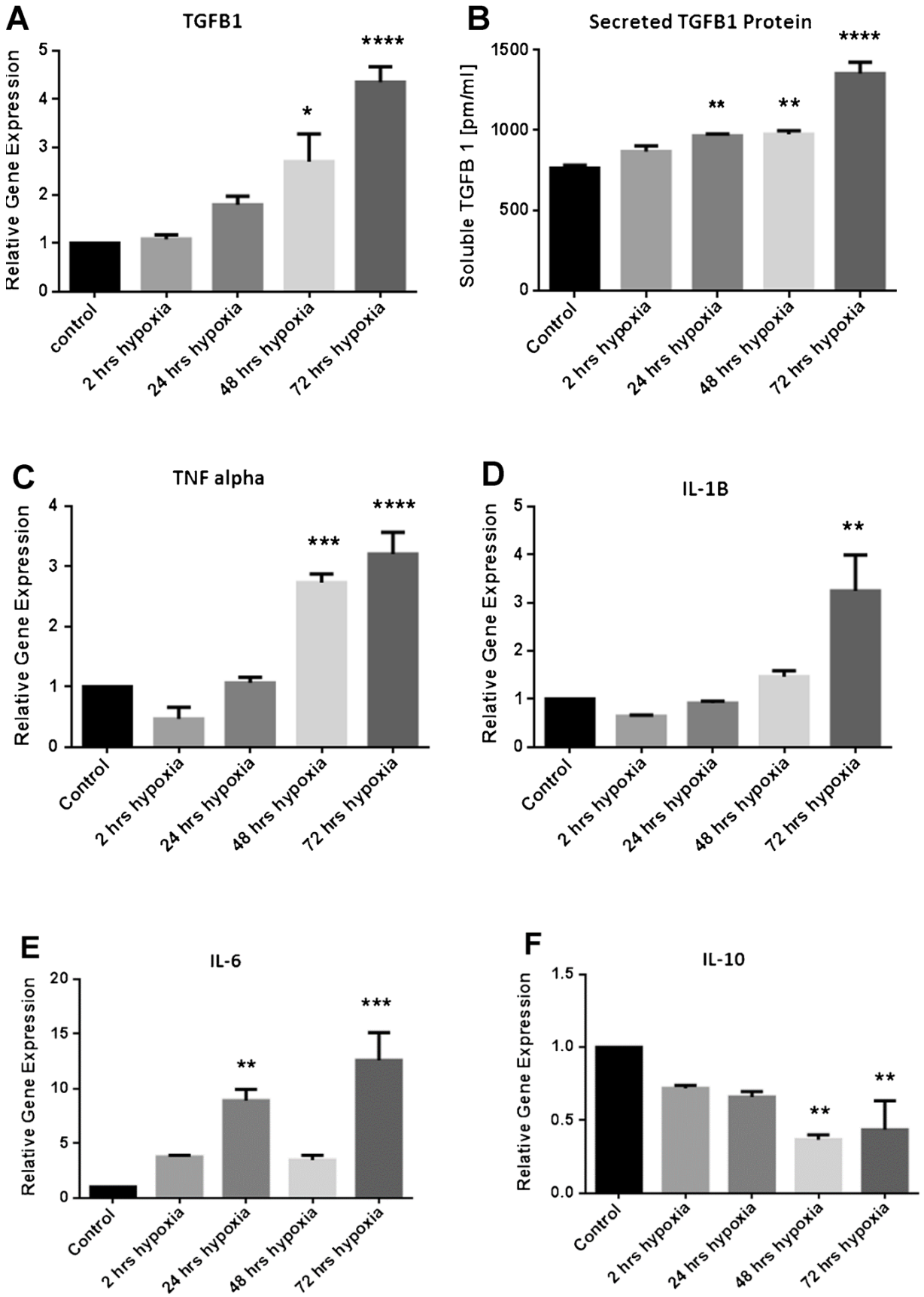
A
TGFB1
Relative Gene Expression
control
2 hrs hypoxia
24 hrs hypoxia
48 hrs hypoxia
72 hrs hypoxia
B
Secreted TGFB1 Protein
Soluble TGFB 1 [pm/ml]
Control
2 hrs hypoxia
24 hrs hypoxia
48 hrs hypoxia
72 hrs hypoxia
C
TNF alpha
Relative Gene Expression
Control
2 hrs hypoxia
24 hrs hypoxia
48 hrs hypoxia
72 hrs hypoxia
D
IL-1B
Relative Gene Expression
Control
2 hrs hypoxia
24 hrs hypoxia
48 hrs hypoxia
72 hrs hypoxia
E
IL-6
Relative Gene Expression
Control
2 hrs hypoxia
24 hrs hypoxia
48 hrs hypoxia
72 hrs hypoxia
F
IL-10
Relative Gene Expression
Control
2 hrs hypoxia
24 hrs hypoxia
48 hrs hypoxia
72 hrs hypoxia

Figure 3.2 Hypoxia increased inflammatory cytokine production. (A, B) Hypoxia-induced increased TGFβ1 transcription and translation. Total TGFβ1 protein in bladder smooth muscle cell culture medium after hypoxic incubation was activated and measured by ELISA. (C) TNF α, (D) interleukin 1β, and (E) interleukin 6 transcripts were upregulated during hypoxia. (F) Downregulation of interleukin 10 transcription during hypoxic stress (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, ****$p < 0.0001$, represents comparison between hypoxic groups and the normoxic controls).

3.3.3 Smooth Muscle Dedifferentiation, EMT, and Pro-fibrotic Response

Since smooth muscle dedifferentiation is associated with increased cell migration and high extracellular matrix production similar to what occurs in myofibroblast activation and EMT, we assessed it using the expression of αSMA, vimentin, and desmin (Fig. 3). A pro-fibrotic response was also assessed via an upregulation of three key genes, Smad 2, Smad 3, and CTGF. The human detrusor smooth muscle cells responded to hypoxia by increasing the transcription of αSMA in a time-dependent manner. A 5.6-fold increase ($p < 0.05$) in expression was recorded after 48 h followed by a 7.5-fold increase ($p < 0.005$) over normoxic controls. Both vimentin and desmin transcript levels increased 5-fold after 72 h ($p < 0.0005$) while vimentin protein showed a progressive increase with increasing duration of hypoxia. Expression of Smad 2 increased by 3.6-fold after 48 h ($p < 0.05$), and both Smad 2 and 3 increased by 5-fold after 72 h ($p < 0.005$ for both). CTGF increased by 2.7 ($p < 0.05$) after 72 h of hypoxic culture.

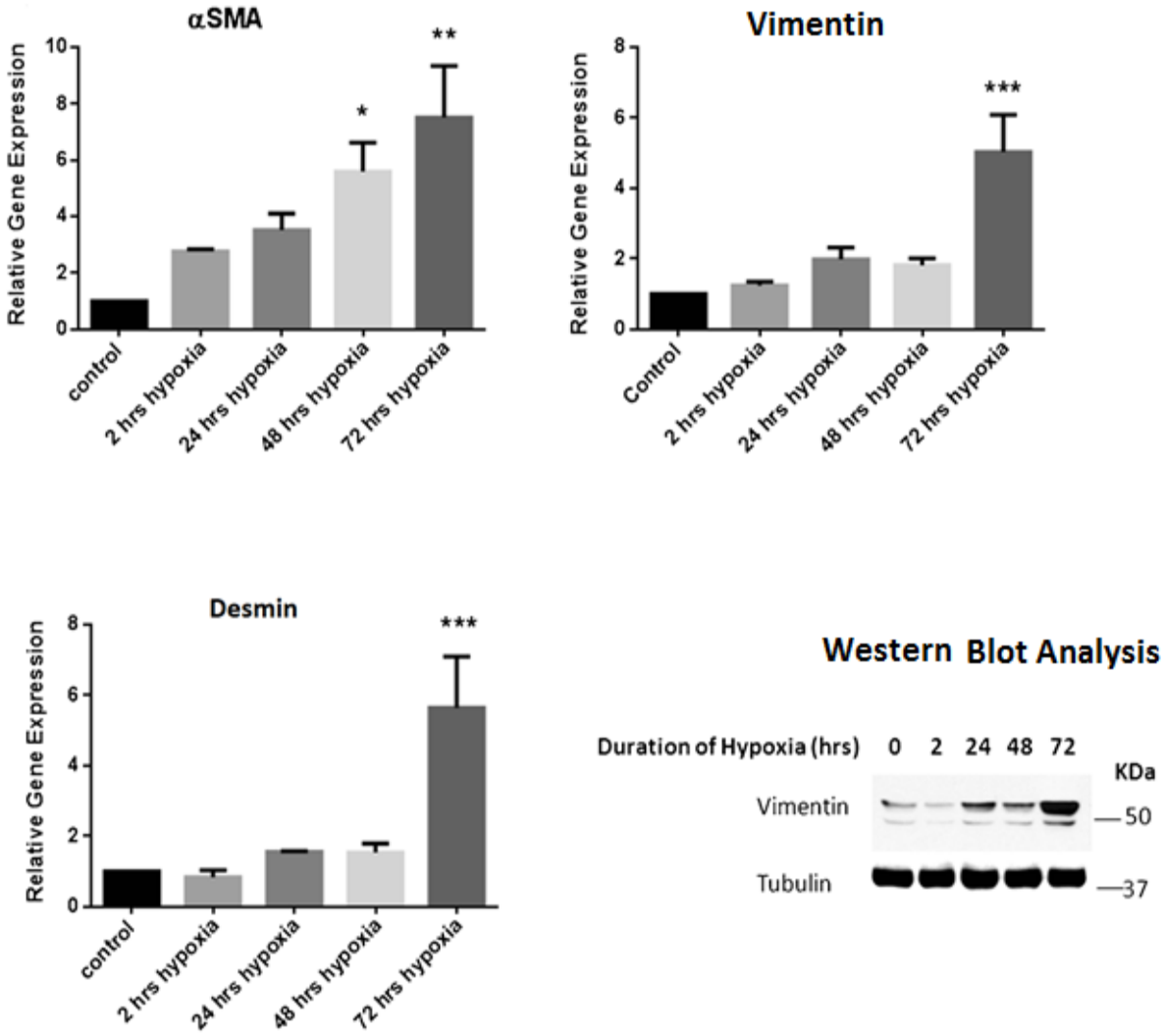

Figure 3.3 Hypoxia-induced smooth muscle cell dedifferentiation. Increased gene expression of αSMA, vimentin, and desmin were indicative of dedifferentiation of normal smooth muscle cells during hypoxia. Western blot analysis of vimentin show increasing protein with increasing duration of hypoxia. Distinct bands for vimentin were detected at ~57 kDa. The equality of loaded total protein for each time point is shown by the tubulin bands

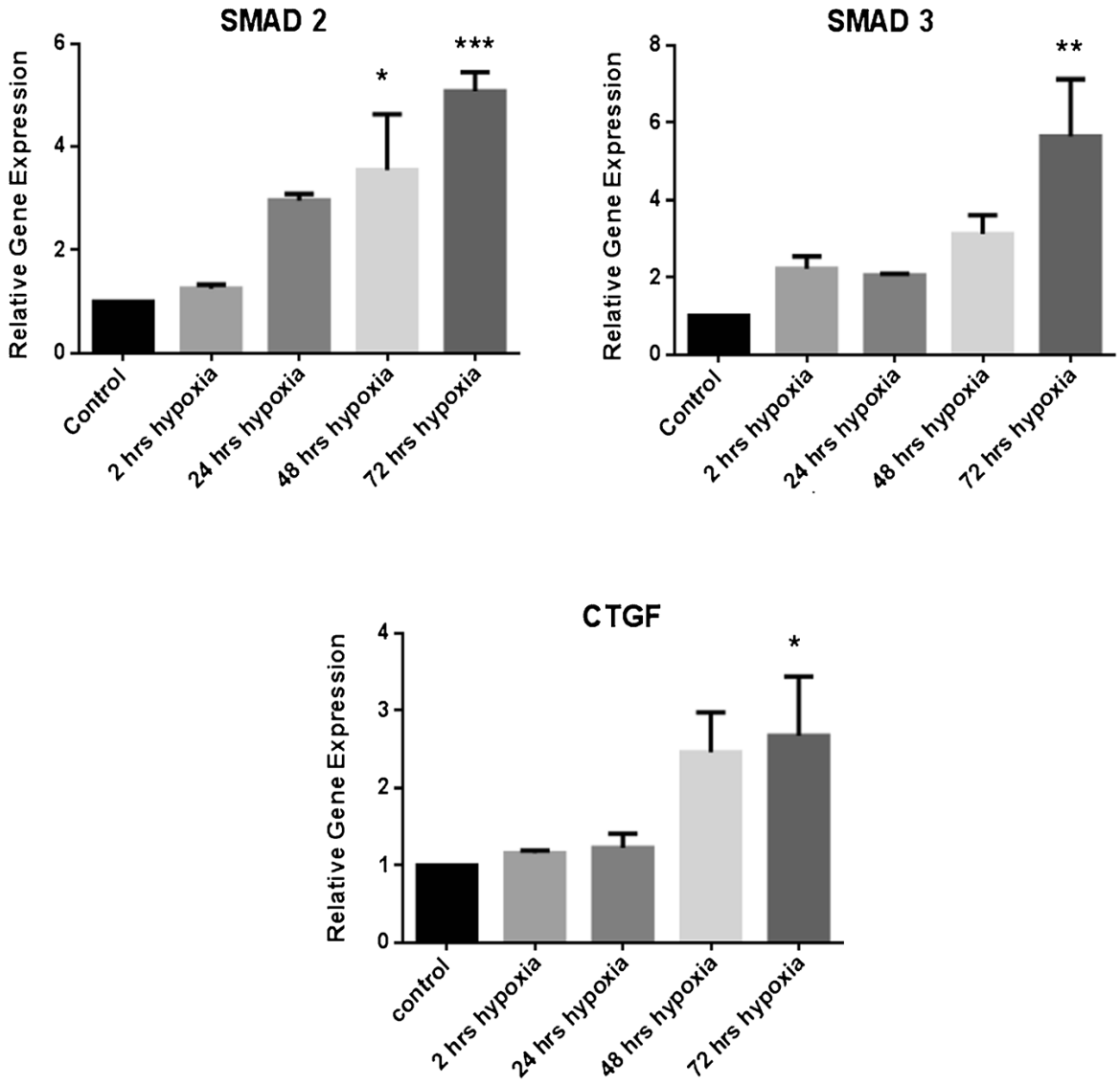

Figure 3.4 pro-fibrotic response genes upregulated by hypoxia. Pro-fibrotic genes; SMAD 2, SMAD 3, and CTGF were upregulated after 72 h hypoxia (n = 5). **P<0.005, *p<0.05 compared to normoxia controls

3.3.4 Extracellular Matrix Production and Breakdown

Collagens I, II, III and IV, fibronectin, and aggrecan are key components in fibrosis; protein and transcript levels were measured in hbSMC during hypoxia. TIMP 1 was also assessed as a

measure of inhibition to the breakdown of these fibrotic proteins. Collagen I transcripts exhibited a consistent increase over the entire time course, with a 3.4-fold increase after 2 h eventually reaching a maximum fold increase of 12 by 72 h ($p < 0.05$ and $p < 0.005$, respectively). Collagen II transcript levels showed a 4-fold increase by 72 h ($p < 0.005$); collagen III exhibited a similar increase, although a 5-fold increase was evident at 48 and 72 h ($p < 0.05$). Collagen IV transcripts rose by almost 8-fold following 72 h hypoxia ($p < 0.005$). Total secreted collagen remained at control values until 24 h at which point levels rose by 100% ($p < 0.0001$); values remained consistently elevated during prolong hypoxia.

Fibronectin transcripts showed a consistent increase from 1.9- to 3.9-fold; $p < 0.005$ for values between 24 and 72 h. Both aggrecan and TIMP 1 had similar patterns of expression; 2 h exposure to hypoxia induced a 3-fold increase in mRNA ($p < 0.05$). Levels dropped to control levels by 24 h and then after 72 h, both aggrecan and TIMP 1 rose again by 4.6- and 2.6-fold, respectively ($p < 0.005$ for both).

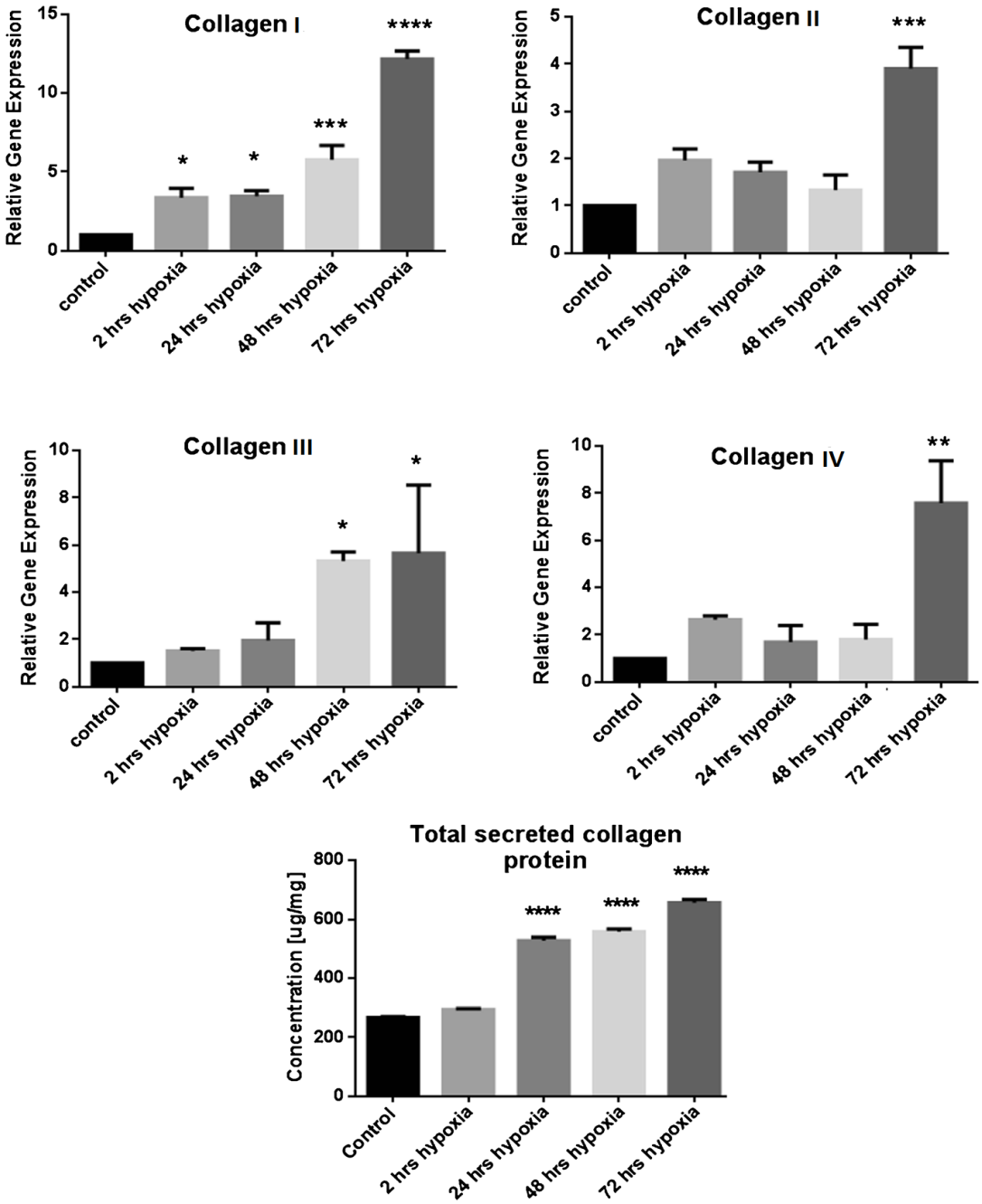

Figure 3.5. Upregulation of total collagen and collagen subtypes by hypoxia. Human bladder smooth muscle cells responded to hypoxia by the exponential increase in transcript levels of the collagen subtypes as well as total collagen. Early upregulation of collagen I and III transcripts preceded a later increase of collagen II and IV. Newly synthesized collagen in media was estimated from the total concentration of hydroxyproline.

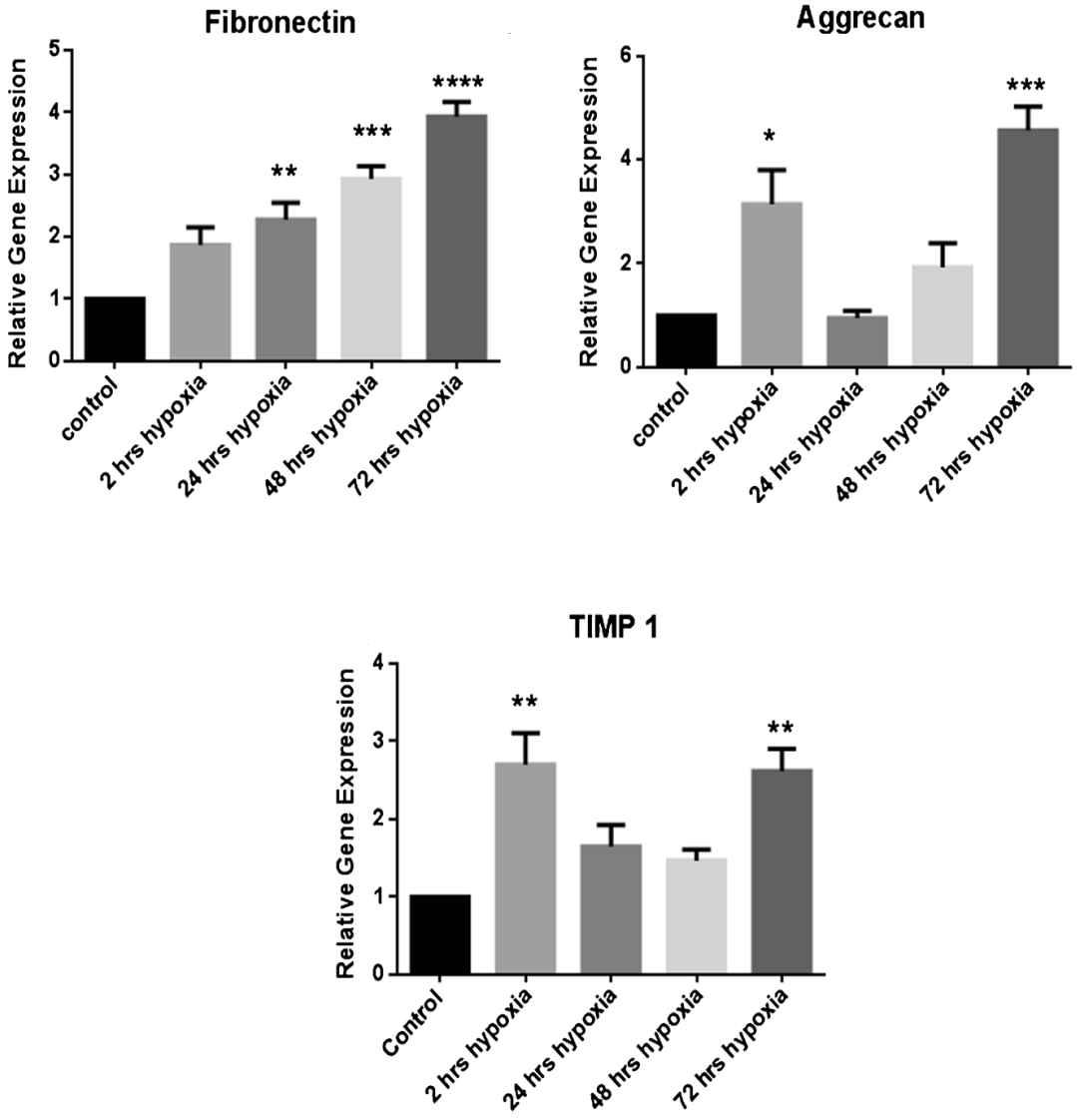

Figure 3.6 Hypoxia-induced extracellular matrix production and inhibition of breakdown

Transcript levels of fibronectin, aggrecan, and TIMP 1 were upregulated when bladder smooth muscles were incubated in hypoxia. **$p < 0.005$, ***$p < 0.0005$, ****$p < 0.0001$, represents comparison between hypoxic groups and the normoxic control).

3.4 Discussion

Bladder outlet obstruction is a serious urological condition that can lead to irreversible bladder and renal damage [1]. Animal studies have shown that pBOO results in a significant decrease in blood flow to the bladder muscle layer which correlates with contractile dysfunction [15] and increased collagen production [3]. The initiation and progression of pBOO is associated with an increased expression of pro-inflammatory, pro-fibrotic, and angiogenic genes, namely HIF, TGFβ, VEGF, αSMA, CTGF, TNFα, IL 1β, and collagen. However, these responses are due to a combined multicomponent stress involving stretching, increased hydrodynamic pressure, inflammation, and hypoxia. Other studies have delineated the role of stretching, mechanical deformation, inflammation, and elevated hydrodynamic pressure as single stressors [16, 17]. However, the exact role of hypoxia in the progression of this pathological process has not yet been investigated. Therefore, this study was designed to test the hypothesis that normal bladder smooth muscle cells exposed to hypoxia, without the confounding effects of stretching or contracting forces, will lead to an inflammatory response and a pro-fibrotic phenotype.

3.4.1 HIF Expression Pattern in Hypoxia and Pro-angiogenic Response

The importance of the HIF transcriptional factor to cell survival has been well-established [5]. In our study, the transcription and translation of both HIF1 and HIF2α were transiently upregulated in response to short-term hypoxia (2–24 h). This is consistent with findings from other cells [18, 19]. Meanwhile, some previous studies on cells from the cerebral cortex, hippocampus, lung, heart, and Chinese hamster ovary cells have reported that the transcription of HIF1 and HIF2α is not affected by hypoxia [20] and is exclusively under post-translational regulation. In our study, HIF1α upregulation during the early phase of hypoxia (2 h) may be an emergency response

mechanism that ensures stable levels of HIFα proteins in order to ensure that an immediate cytoprotective response is mounted. The increase in VEGF mRNA after 24 h is consistent with the proven relationship involving a HIF-mediated pro-angiogenic effect.

As far as we are aware, our study is the first to document the involvement of HIF3α in human bladder cells with a primary role in cytoprotective responses to prolong hypoxia. Interestingly, the role of HIF3α in mediating responses to hypoxia is not ubiquitous; HIF3α has been found to be tissue and organ-specific. In the previous study examining the involvement of HIF3α in hypoxia in multiple rodent organs, HIF3α expression was elevated in rat brain, lung, and heart tissues with no effect on HIF1α and HIF2α. This finding suggests that there may be a protective role mediated by HIF3α during hypoxia that is independent of HIF1α and HIF2α [20]. In our study, HIF3α genes and protein were significantly expressed after 72 h of hypoxia when HIF1 and HIF2α proteins had resumed normoxic control levels. These results are congruous with a role of HIF3α that is complementary to that of HIF1α and HIF2α. This purported mechanism provides an explanation of a biphasic upregulation of VEGF where VEGF transcripts had increased again following 72 h when only HIF3α was the sole upstream factor. Conversely, the inverse relationship between HIF1α and HIF2α with HIF3 has been suggested to be the result of HIF3α-mediated suppression of HIF1α and HIF2α expression [21].

3.4.2 Hypoxia-induced Inflammatory Cytokine Production

The major regulatory role of TGFβ in fibrosis makes it a very important marker in fibrotic diseases. In our study using human bladder smooth muscle cells as an in vitro model, hypoxia

stimulated TGFβ1 expression at both mRNA and protein levels in a time-dependent manner; this is consistent with a previous study using human hepatic stellate cells [19]. Of more physiologic relevance, TGF β1 expression has been found to increase in whole animal models of pBOO [22].

Hypoxia also induced the expression TNFα, IL 1β, and IL 6 which are all part of the acute phase proteins secreted in response to inflammation. However, expression of IL 10 was downregulated in a time-dependent pattern. This is consistent with the study of trophoblast cells of pre-eclamptic pregnancies that reported a similar pattern of reduced IL 10 and increased IL 6 when exposed to hypoxia [23]. Data from our current study demonstrate that isolated hypoxic stress can produce a robust inflammatory response. Prior studies focusing specifically on pBOO have attributed the inflammatory response to contractility, bladder remodeling, and functional deterioration [3, 22, and 24]. In our results, the initial robust IL6 response we detected was most likely responsible for the direct inhibition of TNFα transcription over the first 24 h. The first incidence of TNFα upregulation occurred after 48 h, which corresponded to a marked decline in IL 6. Thus, our findings are consistent with a previously established inhibitory effect of IL 6 on both TNFα and IL 1β expression [25].

Unexpectedly, TNFα and IL 1β mRNA levels increased significantly after 72 h when IL6 remained elevated. This suggests that there may be other important factors and mediators of interplay here; IL 10 is typically referred to as an anti-inflammatory (and anti-fibrotic) cytokine where suppression of IL 10 levels has pro-inflammatory sequelae [26]. We found a time-dependent decline in IL 10 transcripts, with values reaching significance after 48– 72 h. Despite an initial inhibitory effect of IL 6 on TNF over the first 24 h, it is possible that the secondary protracted effect was governed by the loss of IL 10. Whether the biphasic response of TNFα

throughout extended periods of hypoxia is regulated by the ratio of IL 10/IL 6 as opposed to absolute transcript levels remains to be established.

3.4.3 Smooth Muscle Cell Dedifferentiation, EMT, and Pro-fibrotic Response

In our study, the epithelial to mesenchymal transition and the smooth muscle cell dedifferentiation was profound. A pro-fibrotic response involving TGF β transcription and translation was evident at the early stage (<24 h) followed by a progressive increase in αSMA throughout the entire period of hypoxia; this is congruous with the findings in proximal tubular cells [27]. Later stage development of pro-fibrotic response was evidenced by increases in desmin, a proven index of late stage fibrosis. Importantly, our results strongly suggest a relationship between prolong hypoxia, HIF3α, and EMT (using vimentin as a marker) in bladder smooth muscle cells. Exploring this area further may have therapeutic potentials in obstructive bladder conditions.

Increased synthesis and decreased breakdown of extracellular matrix proteins are the hallmark events characteristic of fibrosis. The pro-fibrotic response of human bladder smooth muscle cells in our study was pronounced; total collagen protein increased by 100%. Synthesis of the four central matrix components in our model was the direct result of an early and progressive upregulation of collagens I and III. Collagens I and III are the key interstitial collagens most commonly found in obstructive bladder fibrosis [3, 28]. The significance of the later stage increases in collagens II and IV transcription may be related to a secondary mechanism of matrix formation following initial pro-fibrotic events. TGFβ also plays a key role in fibronectin synthesis at transcriptional and translational levels; fibronectin is an essential

substrate for αSMA synthesis [29]. From our results, both the fibronectin gene and secreted TGFβ1 protein were significantly upregulated after 24 h hypoxia and further increased throughout prolonged exposure. αSMA expression was only increased after 48 h, likely secondary to increase in both fibronectin and TGFβ1.

Although our data indicate that there is a concerted cellular effort resulting in an overall increase in multiple metabolic pathway of fibrosis. The marked increase in expression of TIMP 1 reaffirms the ability of hypoxia to induce and sustain fibrotic pathways via multiple regulatory control mechanisms. Our data demonstrating the involvement of two opposing mechanisms, matrix synthesis, and proteolytic inhibition are consistent with those of a previous study focusing on human renal fibroblasts cultured in low oxygen tensions [30].

The data presented in this study provides strong evidence for the mechanistic detail as follows; Exposure of the bladder smooth muscle cells to hypoxia led to an upregulation of HIF. Increases in HIF transcription triggered the observed increase in Smad 2 and Smad 3 transcription presumably via the formation of a HIF-SMAD complex as has been previously established [31]. Subsequent increases in Smad proteins provided greater amounts of substrate for TGF β1-mediated phosphorylation. This activation of Smad2/3 promoted the formation of the trimeric protein complex responsible for CTGF expression which, in turn, stimulated the increase in secreted matrix components (collagen I-IV, fibronectin, and aggrecan). In reference to pBOO, these events are fundamental to the pathological development of fibrosis and inhibition of the TGFβ-SMAD pathway in pBOO results in a significant reduction in fibrosis [28].

3.5 Conclusion

This study has provided overwhelming evidence that hypoxia exposure, as a single isolated stress without the confounding effects of stretching and hydrodynamic pressures, can induce fibrotic phenotype in smooth muscle cells mechanistically via inflammation, smooth muscle de-differentiation, and a pro-fibrotic switch in normal human bladder smooth muscle cells characterized by increased ECM production. This work gives foundational understanding of hypoxia-driven bladder deterioration which we hope will eventually translate into improved clinical outcomes.

Chapter 4

Mesenchymal stem cells inhibit hypoxia-induced inflammatory and fibrotic pathways in bladder smooth muscle cells

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4.1 Introduction

Hypoxia is known to play a significant role in the pathophysiology of several conditions including Partial Bladder Outlet Obstruction (pBOO), renal, cardiovascular and respiratory diseases [1]. pBOO represents a condition whereby increased resistance to urine outflow results in increased muscular contractility, hypertrophy and a decreased mucosal perfusion. Some

characteristic features include increased urine storage pressure, high hydrodynamic pressure and exaggerated resistance inducing significant damage to bladder tissues [2]. Ultimately, it may lead to a fibrotic bladder state where the normal trilaminar smooth muscle architecture is replaced by a non-compliant, disorganized collagenous state.

Characterization of pBOO in animal models has shown that, there is an initial inflammatory phase that sets the stage for the subsequent hypertrophic and fibrotic events [3]. Irreversible morphological, biochemical and functional alterations of the bladder due to pBOO have also been reported. These transformations are believed to be caused by a combination of factors that includes mechanical stretch, hydrodynamic pressure, inflammation and hypoxia. With the exception of hypoxia, the roles of all of these factors as single stressors have been investigated [4, 5]. However, although, several studies have identified hypoxia in obstructed bladder tissues [3, 6-8], the effects of hypoxia as a single stressor was yet to be examined in isolation. We have demonstrated that bladder smooth muscle cells (bSMCs) incubated under hypoxic conditions is sufficient to incite an inflammatory cascade, and a subsequent pro-fibrotic response. These included an increase of the hypoxia inducible factor alpha subunits (HIFα), VEGF, TGFβ1, IL-1B, IL-6, TNFα, a decrease of IL-10 transcripts, as well as increased total collagen production [9].

Mesenchymal stem cells (MSCs) are multipotent adult cells that have multilineage differentiation and immunomodulatory capacities [10]. This has resulted in extensive study and their use in tissue repair and regeneration across various inflammatory and fibrotic conditions. We were able to demonstrate the short term effectiveness of MSCs in ameliorating inflammatory factors in an animal model of pBOO [11]. Other studies using animal models of pBOO have reported the effectiveness of MSCs in improving urodynamic and molecular parameters [12, 13].

With regards to stem cell therapy, many mechanisms of action have been proposed; that include cell-to-cell contact, paracrine communication, and cell type specific differentiation [14]. Despite the huge potential of cell therapy in the treatment of pBOO, its exact mechanism is not clearly understood. We hypothesized that the co-culture of bSMCs with MSCs in a reduced oxygen tension will mitigate the inflammatory and pro-fibrotic cascade. Furthermore, to elucidate the mechanisms of action; both direct and indirect co-culture techniques were used.

4.2 Materials and Methods

4.2.1 Bladder Smooth Muscle Cells

Human bSMCs purchased from ScienCell Research Laboratories (Carlsbad, CA) were cultured in smooth muscle growth medium supplemented with 10% fetal bovine serum, 1X smooth muscle growth factors, 1X 1000 U/ml penicillin and 1000 mg/ml streptomycin from ScienCell Research Laboratories (CA). The phenotype of the cells was confirmed by culture characteristics coupled with the expression of bladder smooth muscle markers; high molecular weight caldesmon, desmin, α smooth muscle actin (αSMA) as well as the absence of light molecule weight caldesmon as previously established [9, 15, 16] (figure 4.1).

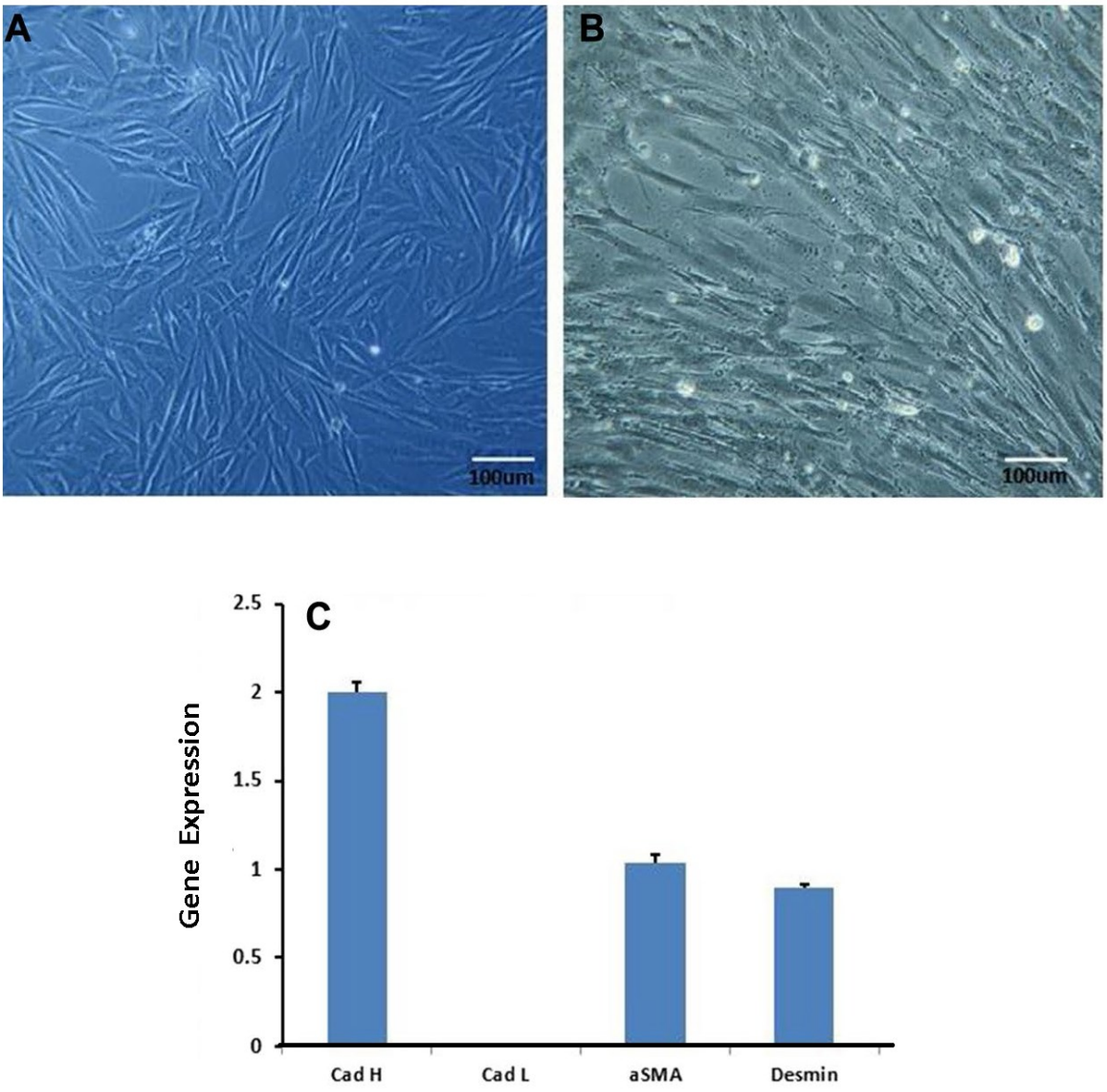

Figure 4.1 Culture micrographs and characterization of cells. A. Normal human bladder smooth muscle cell culture at passage 4 showing the typical spindle-shaped elongated cells. The formation of cellular masses of parallel arranged cells became noticeable after day 3 of culture. Note also the characteristic "hill" and "valley" growth pattern. B. Isolated human bone marrow-derived mesenchymal stem cell culture at passage 2, showing plastic-adherent spindle-shaped fibroblast- like cells. Micrographs were taken with an inverted microscope using ×20 objective lens. C. Bladder smooth muscle cell characterization data showing the expression of heavy molecular weight caldesmon (Cad H), αSMA and desmin. The smooth muscle cells lacked the expression of light molecular weight caldesmon (Cad L).

4.2.2 Human Bone Marrow Derived Mesenchymal Stem Cells

MSCs were isolated from Bone marrow aspirates of surgically discarded material obtained from the iliac crest of 6 donors. These were obtained by approval and a waiver of informed consent of the ethics committee of the University of Alberta (Edmonton, Canada). MSCs were isolated and expanded as previously reported [17]. Briefly, bone marrow mononuclear cells (BMMCs) were isolated from the aspirates using Histopaque-1077 (Sigma-Aldrich Canada Co, Ontario, Canada). Then, 15 million BMMCs were cultured in 150 cm^2 tissue culture flask. Culture medium was alpha-minimal essential medium (α-MEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 88.5 units/mL penicillin- streptomycin, 0.26 g/mL L-glutamine, 8.8 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 885 x 10−6 mol/L sodium pyruvate (Life Technologies, Burlington, Canada), and 5 ng/mL fibroblast growth factor-2 (FGF-2) (Neuromics, Edina, MN). After 7 days, culture medium on the adherent nucleated cells was changed and the culture was expanded to passage 2. The mesenchymal stem cell phenotype of the cells was confirmed by the positive expression of markers: CD151, CD105, CD90, CD73, CD44 and the absence of CD34 and CD14 using flow cytometry [17] (figure 4.1).

4.2.3 Direct and Indirect Co-culture Procedures

To examine the effects of MSCs co-culture on hypoxia-induced inflammatory and fibrotic pathways, 6- well plates were seeded with 6 $x10^5$ human bladder Smooth Muscle Cells (SMCs) with either the direct or indirect addition of 3 x 10^5 MSCs. Indirect co-cultures were set up in a transwell system in which the SMCs and MSCs were physically separated by a high pore density (0.4um) transwell inserts (Becton Dickinson, New Jersey, USA). This limited interaction between the two cell types to the diffusion of soluble factors across the membranes. Cells were

incubated in hypoxia defined as 3% O_2 tension, 5% CO_2, and 92% N_2 at 37^oC for 72 hours. Normoxia control conditions were also defined as 21% O_2, 5% CO_2, and 74% N_2 at 37^oC for 72 hours.

4.2.4 Reverse Transcription Real Time-PCR

At the end of the specified incubation period, cells were transferred on ice before the subsequent procedures. Culture medium was immediately harvested and stored at -80^oC and total RNA was extracted using RNeasy Mini kit (Qiagen, CA, USA). The Quantitect Reverse Transcription kit (Qiagen, CA, USA) was used for first strand complementary DNA (cDNA) production. Quantitative real time PCR was carried out in a Biorad CFX96 Real time system (Kallang, Singapore) using Kapa Sybr Fast qPCR Kit (Kapa Biosystems, Boston, USA), cDNA samples, and oligo-dt primers specific for target genes published in table 4.

Forty cycles of denaturing at 95°C (2 sec) and annealing/extension at 60°C (30 sec) followed initial 3 min enzyme activation at 95°C. Gene expression of the experimental groups relative to the normoxia incubated SMCs controls were normalized using 2 endogenous controls; beta actin and 18S.

Table 4.1 List of human primer sequences used for the gene expression studies

Target Gene	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')
HIF 1α	TTC ACC TGA GCC TAA TAGTCC	CAA GTC TAA ATC TGT GTCCTG
HIF 3α	TTC TCC TTG CGC ATGAAGAGTACG	TCT GCG CAG GTG GCT TGTAGG
VEGF	CTA CCT CCA CCA TGC CAAGT	GCA GTA GCT GCG CTG ATAGA
TGFβ	TGGAAGTGGATCCACGCGCCCAAGG	GCAGGAGCGCACGATCATGTTGGAC
αSMA	CCG ACC GAA TGC AGA AGGA	ACAGAGTATTTGCGCTCCGAA
TNFα	CTT CTC CTT CCT GAT CGTGG	GCT GGT TAT CTC TCA GCTCCA
Collagen 1	CAG CCG CTT CAC CTA CAGC	TTTTGTATTCAATCACTGTCT TGCC
Collagen 3	TGAAAGGACACAGAGGCTTCG	GCA CCA TTC TTA CCA GGCTC
18S	CGG CTA CAT CCA AGG AA	GCT GGA ATT ACC GCG GCT
Interleukin 1β	ACA GAT GAA GTG CTC CTT CCA	GTC GGA GAT TCG TAG CTG GAT
Interleukin 6	TGGTCTTTTGGAGTTTGAGGTA	AGGTTTCTGACCAGAAGAAGGA
Interleukin 10	CCCTGGGTGAGAAGCTGAAG	CACTGCCTTGCTCTTATTTTCACA
B actin	AAGCCACCCCACTTCTCTCTAA	AATGCTATCACCTCCCCTGTGT

4.2.5 Cytokine Measurement

The multiplex assays (U-PLEX) for biomarker group 1 (Meso Scale Diagnostics, Rockville, MD) enabled the concurrent measurement of protein levels of cytokines and growth factors in harvested culture media. Briefly, each specific biotinylated antibody of cytokines of interest were coupled to a linker, pooled and used to coat the high electrode multi-spot microplates. Samples were run alongside a serially diluted multi-calibrator standard. These standards consisted of a cocktail of cytokines and growth factor proteins of known concentrations. Following incubation

in a composite of SULFO-TAG labeled detection antibodies, quantities of analytes in samples were determined on a Sector Imager 6000 Plate Reader (Meso Scale Diagnostics).

4.2.6 Sirius Red Collagen Detection Assay

Total soluble collagen secreted into culture media by cells was determined using the Sirius red technique. Manufacturer's instructions for the Sirius Red Collagen Detection kit (Chondrex, Inc, Redmond, WA) were followed. Briefly, collagen content in samples and in a serially diluted standards were precipitated by the addition of Sirius red solution, collected, purified and then resolubilized. Optical densities of the pure collagen samples were read at 520nm using a microplate reader.

4.2.7 Statistical Analysis

Graphpad prism 6.0 (Graphpad Prism INC, CA, USA) was used to analyze data. Results represent data from at least three independent experiments which are presented as mean ± SEM. One way analysis of variance (ANOVA) and the Student's T test with Bonferroni adjustments were used to evaluate differences between groups. P value of <0.05 was accepted as statistically significant.

4.3 Results

4.3.1 Pro- and Anti- inflammatory Cytokines

Co-culture of bSMCs with MSCs gave varying effects on cytokines expression; Hypoxic incubation of a monoculture of bSMCs increased TGFβ1 transcript levels by 4.6 fold ($p<0.0001$). However, transcript levels were reduced by more than 50% in both the direct and transwell co-cultures with MSCs ($p<0.005$ for both). Similarly, IL-6 transcripts increased by 6.1 fold in hypoxia-incubated bSMCs. Nonetheless, both direct and indirect co-culture with MSCs under hypoxia resulted in a substantial reduction to baseline normoxic control levels ($p<0.05$ for both). IL-6 protein in the hypoxia-incubated bSMCs monoculture was 24% higher than normoxic controls ($p<0.005$). Levels were significantly reduced by 27% and 24% in the direct and transwell co-cultures ($p<0.005$ for both) respectively (figure 4.2A).

Transcript levels of IL-10, the anti-inflammatory and anti-fibrotic cytokine was downregulated by 0.25 fold in the hypoxic bSMCs monoculture ($p<0.05$). Nonetheless, there was an increase of 2.3 and 3.25 fold in the direct ($p<0.050$) and indirect ($p<0.005$) co-cultures respectively. Likewise, the hypoxia incubation of bSMCs also resulted in a 69% reduction in IL-10 protein ($p<0.005$). Nevertheless, both the direct and transwell co-culture techniques resulted in an upregulation of 241% and 252% respectively above the hypoxia-incubated bSMCs levels ($p<0.005$ for both, figure 4.2B).

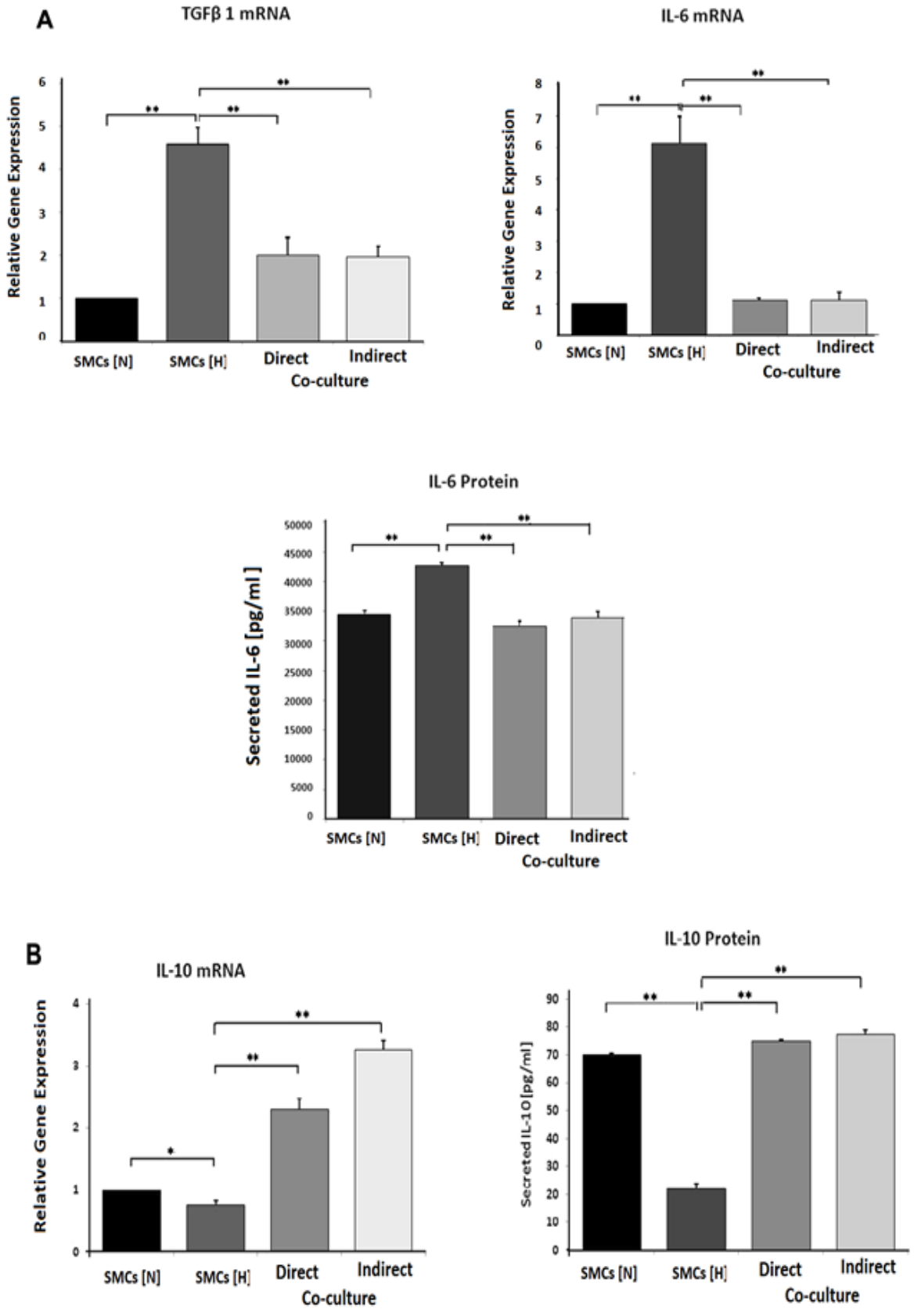
A
TGFβ 1 mRNA
Relative Gene Expression
SMCs [N]
SMCs [H]
Direct
Indirect
Co-culture
IL-6 mRNA
IL-6 Protein
Secreted IL-6 [pg/ml]
B
IL-10 mRNA
IL-10 Protein
Secreted IL-10 [pg/ml]

Figure 4.2 MSC co-culture significantly inhibited inflammatory response to hypoxia and increased anti-inflammatory cytokine production: A. both direct and indirect co-cultures were effective in inhibiting TGFβ1 and IL-6 expression under hypoxia. B. bladder smooth muscle cells (bSMCs) responded to hypoxia by a downregulation of IL-10. Both the direct and indirect MSCs co-culture induced a significant increase in the level of this cytokine. SMC [N]: bSMC monoculture controls incubated in normoxia (21% O2). SMC [H]: bSMC monoculture incubated in hypoxia. One-way ANOVA and the Student's t test with Bonferroni corrections were used to statistically evaluate the differences between the hypoxia-incubated SMC monoculture and the co-culture groups (*$p < 0.05$, **$p < 0.005$).

4.3.2 Differential expression TNF α and IL-1β MSCs co-cultures

Both TNFα and IL-1β gave similar effects where transcript levels rose by 3.8 and 3.0 fold respectively in bSMCs monoculture exposed to 72 hours of hypoxia ($p<0.005$ for both). The direct co-culture with MSCs reduced transcript levels by approximately 50% ($p<0.005$) for both genes. Transcript levels of both cytokines remained elevated in the indirect co-cultures. TNFα protein levels were 61.6 ± 1.2 pg/ml in hypoxia-incubated bSMCs cultures whiles levels in normoxia-incubated controls was 37.5 ± 5.0 pg/ml ($p<0.005$). Level of this protein was reduced in the direct co-cultures to 30.6±3.8 pg/ml ($p<0.005$) but remained elevated in transwell cultures at 59.8 ±6.7 pg/ml compared to levels in hypoxic bSMCs. IL-1β protein was 122.1±4.4pg/ml in normoxic bSMCs and increased to 152.7±2.24pg/ml in hypoxic bSMCs ($p<0.05$). A direct co-culture with MSCs reduced protein levels to 93.10±5.02pg/ml ($p<0.005$) but levels increased in the transwell cultures to 212.2±22.56pg/ml ($p<0.005$, figure 4.3).

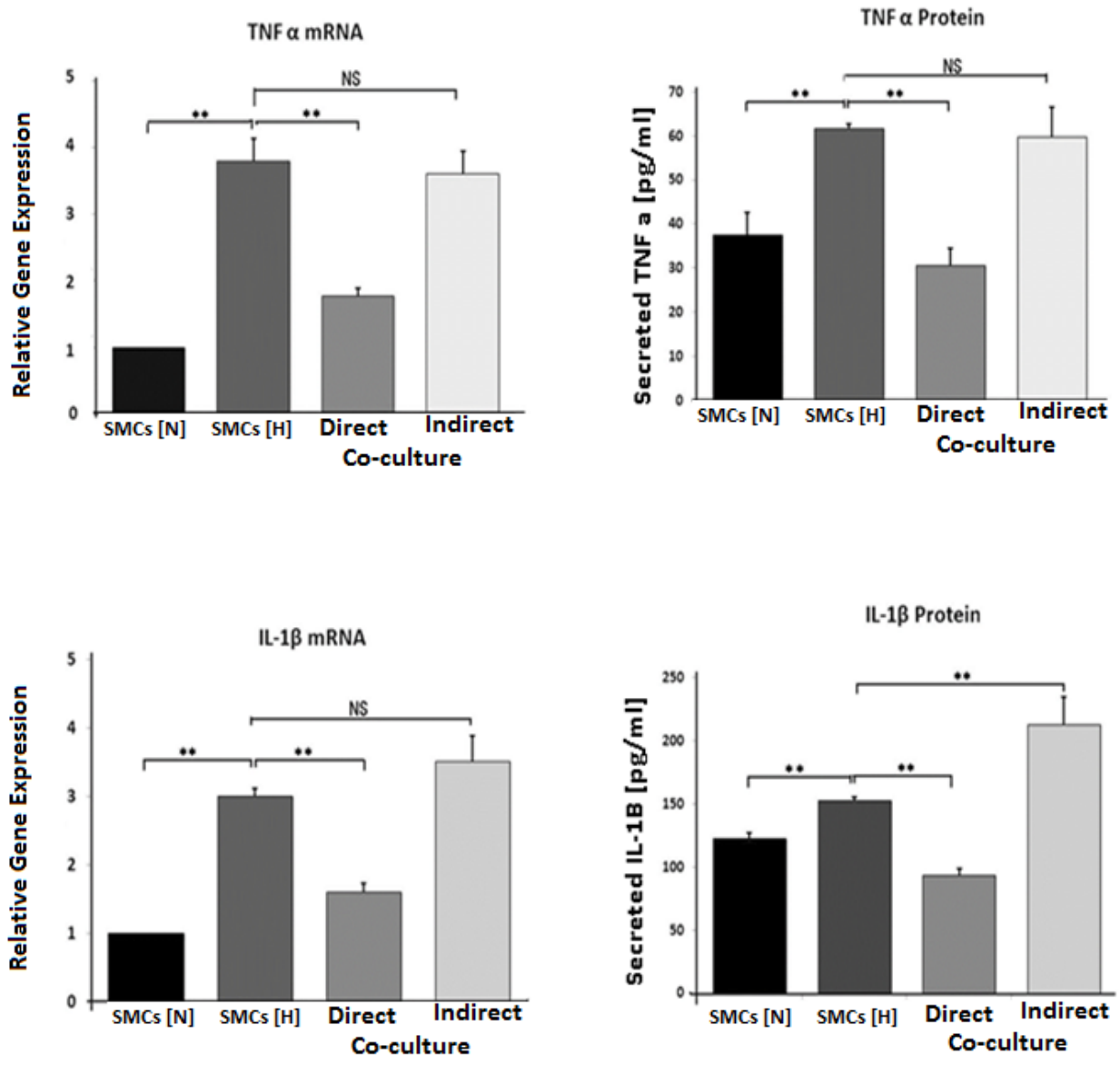

Figure 4.3 Pro-inflammatory cytokines that were only reduced by the direct co-culture technique and not the indirect: to determine if MSCs co-culture inhibits hypoxia-induced inflammatory cytokine expression, MSC- bSMC co-culture was compared with bSMCs monoculture similarly cultured under hypoxic conditions: hypoxia-induced TNFα and IL-1β levels remained elevated in transwells, whereas levels were significantly mitigated in the direct MSC- bSMC co-cultures. Results represent data from at least three independent experiments which are presented as mean (± SEM).

4.3.3 Pro‑fibrotic genes expression in MSCs co-culture

αSMA transcripts increased by 5.2-fold when bSMCs were cultured under hypoxia ($p < 0.005$) and both the direct and indirect co-culture techniques reduced levels to 1.4- and 1.2-fold, respectively ($p < 0.005$ for both). Both collagen 1 and 3 mRNA showed a similar pattern of expression in cultures; there was a 9.9-fold increase in collagen 1 transcripts when bSMCs were incubated in hypoxia ($p < 0.005$) and both the direct and indirect co-culture techniques induced a 78 and 72% decrease in transcript levels, respectively ($p < 0.005$ for both). Collagen 3 transcripts which increased by 4.5-fold in the hypoxic bSMCs monoculture ($p < 0.005$) were reduced by 84% when co-cultured directly with MSCs. The indirect co-culture also induced a decrease of 74% in transcript levels ($p < 0.005$ for both). Total collagen protein secreted into media was 120.4 ± 4.89 pg/ml in the normoxic bSMCs control, but increased significantly to 269.6 ± 36.15 pg/ml when bSMCs were incubated under hypoxia ($p < 0.005$). However, levels of this protein were reduced to 116.2 ± 17.48 pg/ml in the direct co-culture and to 140.9 ± 27.8 pg/ml in the indirect co-culture ($p < 005$ for both, figure 4.4).

4.3.4 Hypoxic and Pro-angiogenic Response

culture of bSMCs in 3% hypoxia for 72 hours did not significantly increase HIF 1α transcript but resulted in a 4.5 fold increase in the transcript levels of HIF 3α ($p<0.005$). Notwithstanding, both HIF 1 and 3α transcript levels remained unaltered by co-culturing with MSCs under hypoxic conditions. Similarly, VEGF transcripts increased significantly by 7.9 fold after 72 hours of hypoxic incubation of the bSMCs monoculture ($p<0.005$). Co-culturing with MSCs did not significantly affect the elevated levels of this transcript. VEGF protein increased by 35.7% when

bSMCs were incubated in hypoxia ($p<0.005$) and remained significantly unchanged when co-cultured either directly or indirectly with MSCs under hypoxic conditions (figure 4.5).

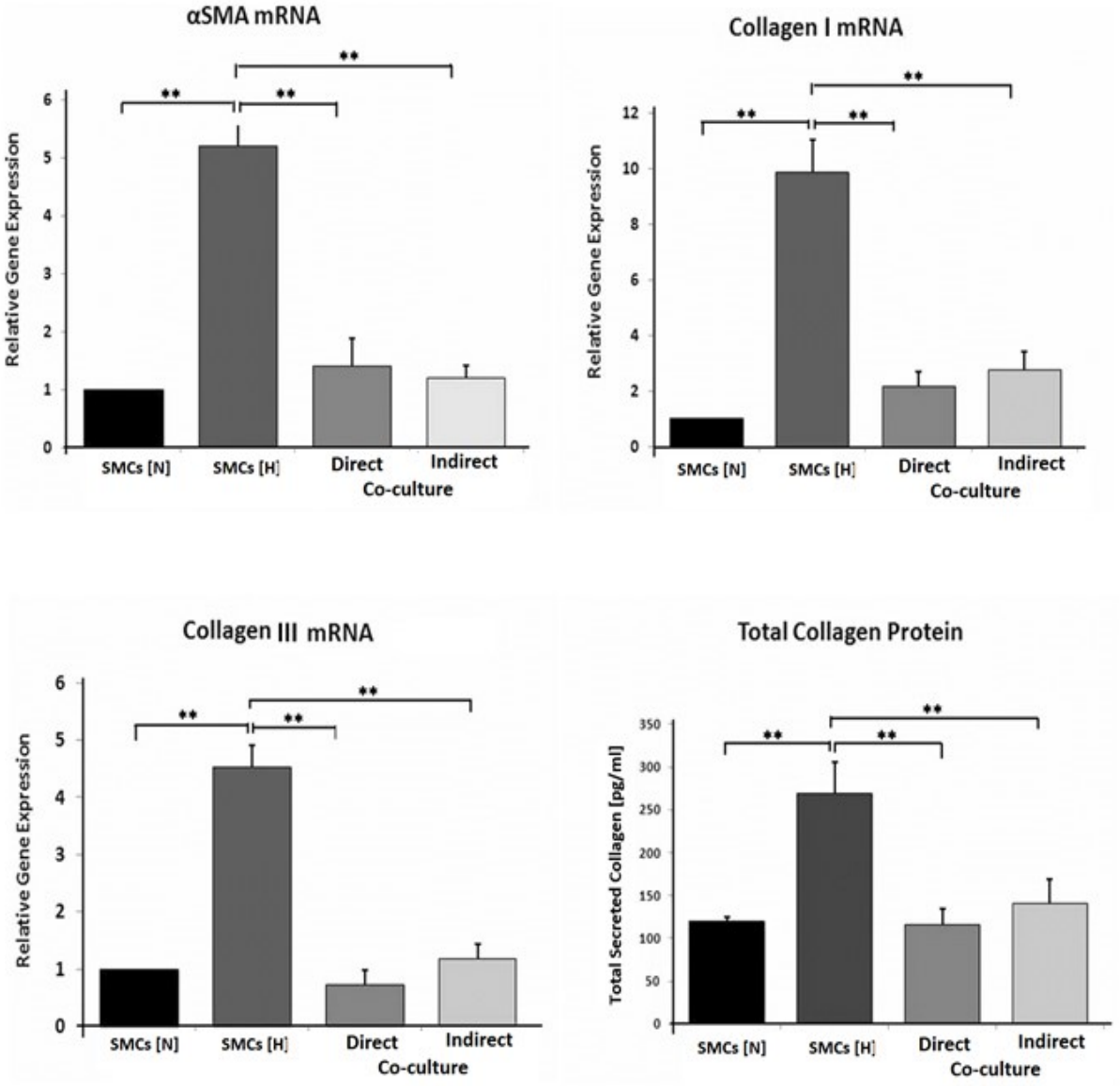

Figure 4.4 Co-culture with MSCs inhibited pro-fibrotic gene expression: hypoxia-induced αSMA, collagen I and III mRNA levels were reduced by both co-culture techniques. Total soluble collagen in culture medium measured by the Sirius Red technique was significantly reduced in both the direct and indirect MSCs co-cultures compared to the hypoxic hBSMC.

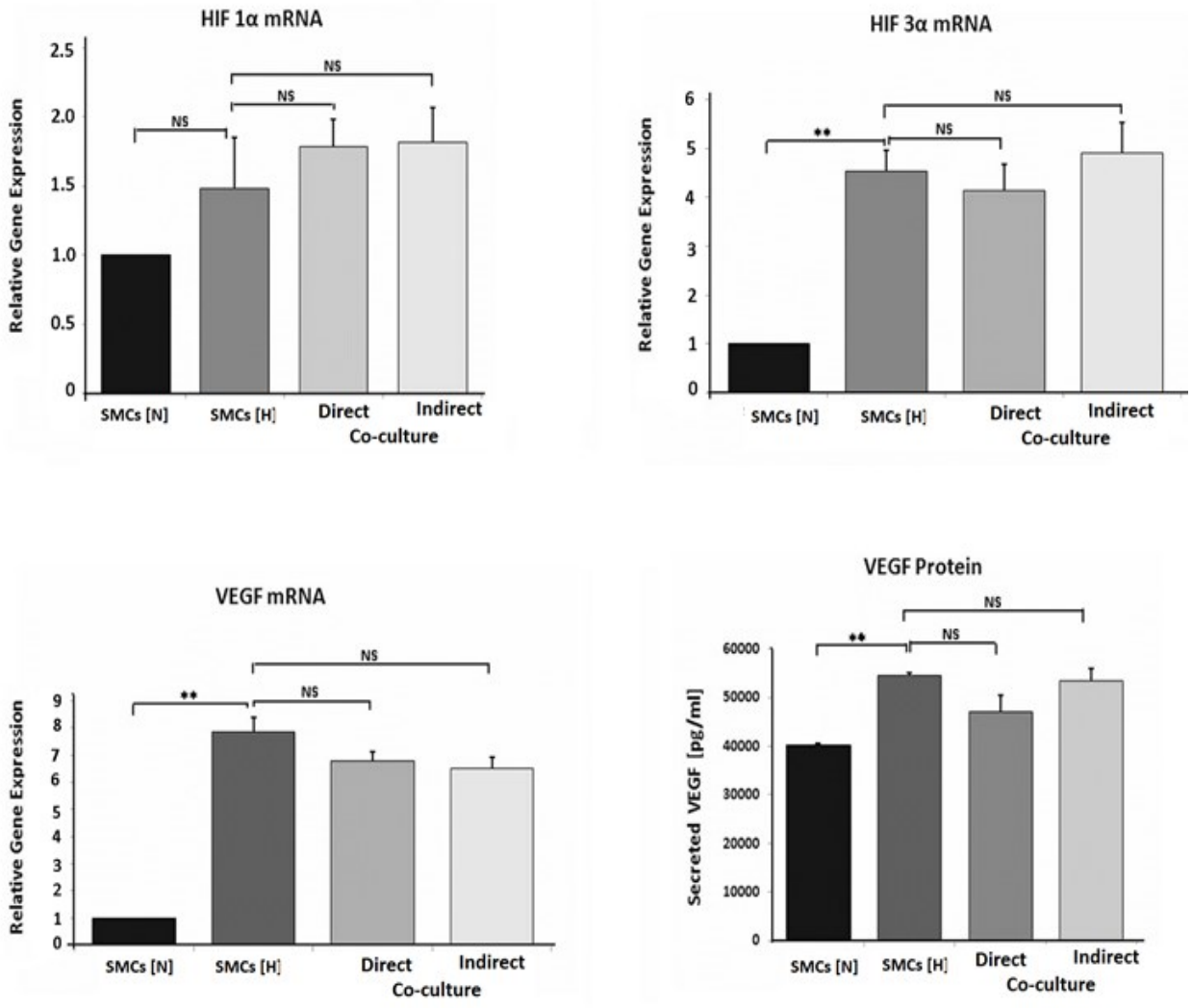

Figure 4. 5 Unaltered HIF Expression and proangiogenic response by MSCs co-culture in hypoxia: co-culture with MSCs did not significantly affect the expression of HIF 1α, 3α, and VEGF. Bladder smooth muscle cells (bSMCs) were co-cultured with mesenchymal stem cells (MSCs) in a ratio of 2:1 under hypoxia (3% O_2 tension)

4.4 Discussion

4.4.1 Inflammatory Cytokine Inhibition by MSCs

The paracrine immunomodulatory property of stem cells has gained much attention in the last decade especially with regards to the treatment of inflammatory and fibrotic conditions like pBOO. Therefore, the current experiments were designed to investigate the underlying molecular mechanisms. Even though the transforming growth factor-β1 (TGFβ1) is required for normal cellular growth and differentiation, it is also a known potent mediator of fibrosis. This cytokine is implicated in disease processes such as epithelial-mesenchymal transition, myofibroblast activation, epithelial cell apoptosis, and extracellular matrix production [18]. As a result, numerous studies have investigated strategies for inhibiting TGFβ expression, signaling pathways, and function in order to reduce fibrosis. Physiologically, elevated levels of TGFβ1 correlated with the stage of bladder obstruction in patients and in animal models of pBOO. As well, its inhibition led to a significant reduction in fibrosis [19-22]. In the current study, the efficient downregulation of TGFβ1 expression by both co-culture techniques reflects the anti-fibrotic nature of MSCs when co-cultured directly or indirectly. A similar effect was seen with the downregulation of IL-6, a pro-inflammatory cytokine. Mechanistically, TGFβ1 has been proposed to have regulatory effects on IL-6 [23]. Thus, the reduced expression of TGFβ 1 in the co-cultures may be important for the inhibition of IL-6 expression.

IL-1β and TNFα are pro-inflammatory cytokines that are expressed in common chronic pathologies such as rheumatoid arthritis, diabetes, myocardial infarction and inflammatory lung diseases. The binding of these cytokines to their respective receptors set the stage for further inflammatory events leading to tissue damage [24]. As a therapeutic mechanism, MSCs are known to express IL-1 receptor agonist (1L8 1Ra), a cytokine which binds to the IL-1 receptor

thereby, competitively inhibiting IL-1β [25]. Interestingly, in our study, both cytokines showed the same pattern of expression in culture where levels were only reduced in the direct co-cultures and not in the indirect method of culture. This strongly suggests that the MSCs-specific paracrine action alone was not sufficient to immune-modulate these two cytokines but the physical MSCs to bSMCs contact was also required. Thus, providing evidence to support the report that the paracrine-mediated immunosuppressive effects of stem cells are further enhanced if direct cell–cell contact between stem cells and other cells are allowed [26]. However, admittedly, our inability to analyze only the smooth muscle cell population of the direct co-culture could also account for the difference in cytokine expression between the direct and indirect co-cultures.

4.4.2 Anti-inflammatory Cytokine Upregulation in MSCs Co-culture

IL-10 is a potent anti-fibrotic and immunomodulatory cytokine secreted in response to elevated levels of inflammatory cytokines such as IL-β, TNFα and interferon (IFN) [22, 28]. In our study, the exposure of bSMCs to hypoxia substantially reduced IL-10 levels. However, its elevated levels in the MSCs co-cultures are an important part of this study. With the high levels of IL-β and TNFα in the transwell co-cultures, it was not surprising that we found higher IL-10 transcript levels in the transwell co-cultures than the direct co-culture system. This data supports the anti-inflammatory, anti-fibrotic and immunomodulatory nature of MSCs. Overall, our results is consistent with the study by Choi et al who reported that MSCs co-culture with macrophages inhibited lipopolysacharide-induced expression of IL-6 and IL-1β and increased IL-4-induced macrophage expression of IL-10 [29]. Aggarwal et al also found increased IL-10 and decreased TNFα levels when MSCs were co-cultured with immune cells [30].

4.4.3 Pro-fibrotic Gene inhibition by MSCs Co-culture

Studies by our group and others have shown that collagen 1 and 3 are important collagen subtypes commonly upregulated in the obstructed and fibrotic bladder [3, 9, 21]. Essentially, the reduction in TGFβ1 expression coupled with the upregulation of IL-10 in both the direct and indirect co-culture systems may have accounted for the reduced collagen levels in the MSCs co-cultures. Thus, despite the high levels of the inflammatory cytokines IL-β and TNFα in the indirect/transwell co-cultures, the potent anti-fibrotic effect of MSCs significantly inhibited hypoxia- induced total collagen levels. Therefore, from this outcome, we theorize that the therapeutic use of MSCs-conditioned media to treat hypoxic and fibrotic conditions may be equally effective as the physical injection of mesenchymal stem cells.

4.4.4 Hypoxia and Angiogenic Response Unaltered by MSCs co-culture

The hypoxia inducible transcriptional factor (HIF) is important in stimulating the transcription of cytoprotective genes under conditions of low oxygen tension [1]. All the three known members of the HIF family; HIF 1, 2, and 3 have been identified in bSMCs [9]. Our current finding is consistent with our previous studies that showed that the oxygen-sensitive alpha subunit, HIF 3α transcription increased in response to prolonged hypoxia (72 hours). However, HIF 1α response was immediate and transient increasing only after 2 hours of hypoxia. This study did not determine HIF 2α response because its activity appeared redundant in bSMCs from our previous findings. In the current study, it was interesting to observe that the HIF expression of the bSMCs was not significantly affected by co-culturing with mesenchymal stem cells under reduced oxygen tension. The unaltered VEGF expression, a proven hypoxia-response gene, provides evidence that HIF function is unaffected by the MSCs co-culture. Since the HIF cellular system

is protective, our data supports the fact that MSCs have the ability to inhibit hypoxia-induced signaling pathways in a HIF-independent fashion.

4.5 Conclusion

This data adds to our work whereby exposure of bladder smooth muscle cells to hypoxia incites a cascade of cellular responses of inflammation and increased extracellular matrix synthesis. However, MSCs have a profound ability to prevent this response by inhibiting pro-inflammatory and pro-fibrotic cytokine production and enhancing anti-fibrotic cytokine secretion. This work has unlocked mechanistic clues that may open avenues for therapeutic intervention.

Chapter 5

Mesenchymal Stem Cell Therapy Inhibited Inflammatory and Pro-Fibrotic Pathways Induced by Partial Bladder Outlet Obstruction and Prevented High Pressure Urine Storage

Bridget Wiafe, Adetola B. Adesida, Thomas Churchill, Peter Metcalfe

5.1 Introduction

Partial bladder outlet obstruction (pBOO) is a urological condition characterized by the increased resistance to urine flow. It affects about 1.1 billion people worldwide, most predominantly, men with benign prostatic hyperplasia. Also, detrusor damage secondary to pBOO affects children born with spina bifida and posterior urethral valves [1, 2] The elevated intravesical pressures result in stretch and hypoxia [3], which trigger a cascade of cellular and molecular responses that culminate in bladder tissue remodeling and fibrosis[2]. Current treatment modalities are associated with high risk of morbidity. Clean intermittent catheterization (CIC) is essential for

the management of urinary retention, however, this is burdensome, associated with urethral trauma, and increases the risk of urinary tract infections [4]. Oral anticholinergic medications are used to relax the detrusor and prevent involuntary bladder contractions, and thereby, decrease the intravesical pressure, but are poorly tolerated [5]. Patients with low capacity, high pressure, poorly compliant bladders may choose to undergo a bladder augmentation using intestinal tissue. However metabolic disturbances, recurrent UTI and long term graft survival are major concerns [6].

Mesenchymal stem cells (MSCs) are undifferentiated adult cells that are immunomodulatory, anti-fibrotic, have multipotent differentiation capacities, and have the ability to repair injured tissue. They represent a potential novel therapy for numerous tissue damage and fibrotic conditions [7]. We have previously demonstrated their incredible ability to inhibit inflammatory and pro-fibrotic response in a hypoxic cell culture model [8]. Also we previously demonstrated the ability of stem cells to mitigate acute inflammatory responses after pBOO in our animal model [9]. However, we now wish to demonstrate their efficacy over a longer term, with analysis across functional, inflammatory and pro-fibrotic pathways.

The TGFβ-SMAD and mTOR signaling pathways are essential mechanisms underlying obstruction-induced fibrosis. The inhibition of these signaling pathways by pharmacological agents led to a significant reduction of pBOO-induced fibrosis [10, 11]. TGFβ1 is a pro-inflammatory cytokine and major mediator of fibrosis in most organs. The canonical pathway involves the activation of smad2/3 by phosphorylation, their trimeric complexing with smad4 and the subsequent translocation of the complex into the nucleus to act as a transcription factor that augments the transcription of target genes. These events lead to increased myofibroblast differentiation and the secretion of concomitant amounts of collagen-rich extracellular matrix.

[12]. MTOR is a serine/threonine kinase that regulates essential biological processes such as cell growth, proliferation, cell cycle progression, survival, and protein and lipid synthesis. mTOR-regulated signaling leads to the activation of the p70 ribosomal S6 protein kinase (p70S6K) and the deactivation of the cellular repressor of translation called, eukaryotic initiation factor 4E binding protein (4EBP) [13]. The mTOR pathway has been used as a marker and fundamental pathway for stretch and smooth muscle hypertrophy [11].

Furthermore, we have previously demonstrated that hypoxic response, defined by HIF 1α, HIF 3α and VEGF is integral to detrusor deterioration [14]. Therefore we hypothesized that MSCs treatment in animal models of pBOO will inhibit the upregulation of these markers and demonstrate a physiologic, clinically relevant improvement after treatment.

5.2 Materials and Methods

5.2.1 Animal and Study Design:

All protocols were reviewed and approved by the University of Alberta Ethics Committee for the Care and Use of Experimental Animals. 20 Healthy adult female Sprague Dawley rats (approximately 250g-300g) were randomly assigned into 5 groups as follows: a) obstructed for 2 weeks (pBOO-2weeks), b) obstructed for 4 weeks (pBOO-4weeks), c) obstructed with MSCs therapy for 2 weeks (pBOO+MSCs-2weeks), d) obstructed with MSCs therapy for 4 weeks (pBOO+MSCs-4weeks), and e) unobstructed controls (n=4 in each group). The animals were housed 2 per cage with free access to food and water.

5.2.2 Induction of pBOO:

Rats in the pBOO-groups underwent urethral ligation under isoflurane anesthesia to create partial bladder outlet obstruction as already described [9, 15]. Briefly, the bladder was exposed and an 18-guage angiocatheter was inserted into the urethra. Then, a 2-0 silk suture was tied around the urethra, and the catheter was removed. All operated rats were provided with cotrimoxazole-water and maintained on meloxicam (1mg/kg) and buprenorphine (0.02mg/kg) to manage pain.

5.2.3 Mesenchymal Stem Cells Treatment:

Immediately after pBOO, the therapy groups were given a tail vein injection of Sprague Dawley bone marrow-derived GFP-expressing MSCs obtained from Cyagen Biosciences Inc, (Sunnyvale, CA). The MSCs were cultured to expand in alpha-minimal essential medium (α-MEM) supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (290μg/ml), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (100mM), sodium pyruvate (1mM), penicillin–streptomycin (100U/ml) (Life Technologies, Burlington, Canada), and 5 ng/ml fibroblast growth factor-2 (FGF-2) (Neuromics, Edina, MN, USA). MSCs between passages 2 and 8 were used for this experiment. One million stem cells in the log phase of growth per 0.5ml PBS was injected per animal. Dosing of MSCs was determined from already published studies [16-18].

At the experimental endpoint, urodynamics was performed and bladder tissue was harvested and part snap frozen for gene expression and protein studies. The other part was fixed in 4% Paraformaldehyde for immunohistochemistry studies.

5.2.4 Urodynamic Studies:

While under mild isoflurane anesthesia, cystometry was performed in rats by firstly exposing the bladder through an abdominal incision. The bladder was emptied and a 27 Gauge needle connected to a pressure transducer (Micro-Med Inc, Louisville, Kentucky) was advanced through the bladder. This infused saline at a constant rate of 100μl/minute. End filling pressure was recorded as the pressure at which urethra leakage was first visualized.

5.2.5 Gene Expression Studies:

Quantitative real-time PCR was used to study gene expression patterns. Bladder tissue was crushed on ice using a mortar and pestle and total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia CA). Sample volumes corresponding to 0.5 microgram were reverse transcribed into cDNA using Quantitect Reverse Transcription kit (Qiagen). Real time PCR reaction was set up according to the instructions of the Kapa Sybr Fast qPCR Kit (Kapa Biosystems, Boston, MA) and ran in a Biorad CFX96 Real time system (Kallang, Singapore). PCR conditions were an enzyme activation at 95^{O}C (3mins) and 40 cycles of denaturing at 95^{O}C (2sec) and annealing-extension at 60^{o}C (30sec). The sequence of primer sets for genes of interest are published in table 5. Relative gene expression were normalized to 2 endogenous controls (β-Actin and GAPDH) and expressed as $2^{-\Delta\Delta ct}$.

Table 5.**1** Primer sequences used to study the expression of target genes

Target gene (NCBI Reference)	**Forward primer sequence (5'-3')**	**Reverse primer Sequence (5'-3')**
collagen type I alpha 1 chain (Col1a1) (NM_053304.1)	GGAGAGTACTGGATCGACCCTAAC	CTGACCTGTCTCCATGTTGCA
collagen type III alpha 1 chain (Col3a1) (NM_032085.1)	GAAAAAACCCTGCTCGGAATT	GGATCAACCCAGTATTCTCCACTCT
mTOR **(**NM_019906.1**)**	TTGAGGTTGCTATGACCAGAGAGAA	TTACCAGAAAGGACACCAGCCAATG
p70 S6K (NM_001010962.2)	GGAGCCTGGGAGCCCTGATGTA	GAAGCCCTCTTTGATGCTGTCC
Smad2/3 (XM_008766216.2)	AGTGTTTGCCGAGTGCCTAAGTG	CCTCAAAACCCTGGTTAACAGACTG
IL-10 (NM_012854.2)	GTTGCCAAGCCTTGTCAGAAA	TTTCTGGGCCATGGTTCTCT
HIF 1α (NM_024359.1)	AAGAAACCGCCTATGACGTG	CCACCTCTTTTTGCAAGCAT
HIF 3α (NM_022528.2)	AGAGAACGGAGTGGTGCTGT	ATCAGCCGGAAGAGGACTTT
TGFβ1 (NM_021578.2)	AAGAAGTCACCCGCGTGCTA	TGTGTGATGTCTTTGGTTTTGTCA
VEGFA (NM_031836.3)	GCACATAGGAGAGATGAGCTTCC	CGCCTTGGCTTGTCACATTT
TNFα (XM_008772775.2)	TCTCAAAACTCGAGTGACAAGC	GGTTGTCTTTGAGATCCATGC
B-Actin (NM_031144.3)	CTAAGGCCAACCGTGAAAAG	TACATGGCTGGGGTGTTGA
GAPDH (XM_017593963)	AGTTCAACGGCACAGTCAAG	TACTCAGCACCAGCATCACC

5.2.6 Mesenchymal Stem Cells Tracking:

To perform MSCs tracking and the immunohistochemistry procedures, formalin-fixed bladder tissue were embedded in paraffin blocks and sectioned into 5µm thickness. Then, the sections were deparaffinised in 3 changes of xylene and brought down to water by passing them through decreasing concentrations of alcohol and water.

The presence of the intravenously injected MSCs in the bladder was detected by incubating the bladder sections in 4′, 6-diamidino-2-phenylindole (DAPI) (Cedarlane, Ontario, Canada) for 10 minutes. Then mounting was carried out with a 1:1 dilution of glycerol and PBS. The

immuno-fluorescence of GFP cells was visualized by an Eclipse Ti-S microscope (Nikon Canada, Mississauga, Canada).

5.2.7 Immunohistochemistry and Histology:

The expression levels and localization of phosphorylated smads 2/3, HIF 1α and HIF 3α were detected by blocking the deparaffinised tissue sections in 5% bovine serum albumin. Overnight incubation in 1:50 dilution of antibodies psmad2/3 (orb99294, biorbyte, San Francisco, California) HIF 1α (ab216842, Abcam, Toronto) and HIF 3α (, PCRP-HIF3A-1B1, IOWA DSHB, Iowa City, IA) was carried out. Following incubation in Horseradish peroxidase-conjugated secondary antibody, immunoreaction was visualized by incubation in a 3, 3'-diaminobenzidine (DAB).

The degree of total collagen deposition in bladder tissue was assessed using Gomori's trichrome staining. Deparaffinised bladder tissue was incubated in Celestin blue solution for 5 minutes followed by incubation in Mayer's haematoxylin for 5 minutes. Slides were then washed under running water for 5 minutes and incubated in Gomori's stain for 15 minutes. Differentiation in 0.5% acetic acid was done for 2 minutes and washed sections were then dehydrated, cleared in xylene and mounted. Microscopic images were captured and 5 randomly selected images per tissue were quantified using imageJ.

5.2.8 Statistical Analysis:

Results are presented as mean (±SEM) of three or more independent experiments run as triplicates. Graphpad prism 6.0 (Graphpad Prism INC, CA, USA) was used to analyze data.

Two-factor analysis of variance (ANOVA) followed by post hoc Bonferroni tests were used to evaluate differences between groups. A p value of < 0.05 was accepted as statistically significant.

5.3 Results

5.3.1 Recruitment of intravenously injected MSCs into the bladder:

GFP-expressing cells were identified throughout the bladder tissue 2 and 4 weeks after therapy. In contrast, no GFP cells were identified in the untreated controls (figure 5.1).

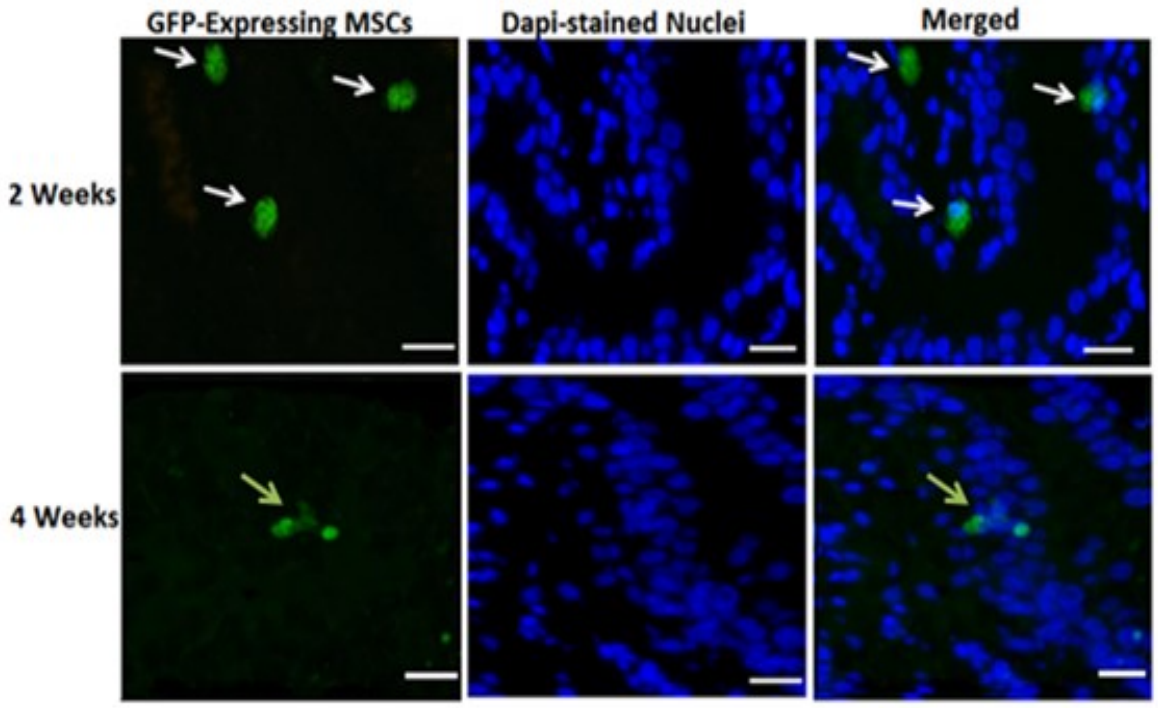

Figure 5.1 the presence of GFP-expressing MSCs in the bladder. Tail vein injected GFP-expressing MSCs (shown by white arrows) were identified in the bladder tissue of rats 2 and 4 weeks after therapy. Scale bar represents 20 µm

5.3.2 Hypoxic Response:

The surgical induction of pBOO resulted in a significant increase in HIF 1- and 3- α transcript levels. HIF 1α expression increased from 1.63 (±0.23) to 4.13 (±0.20) and 8.9 (±0.40) after 2 and 4 weeks of pBOO ($p<0.0001$) respectively. Four weeks after pBOO + MSCs therapy, HIF 1α transcript reduced to normal control levels ($p<0.0001$). HIF 3α transcript levels increased from 0.6 (±0.23) to 5.30 (±1.19) and 8.5 (±1.10) after 2 and 4 weeks of pBOO ($p=0.007$, $p=0.0002$) respectively. However, pBOO + MSCs therapy led to a significant downregulation of transcripts to unobstructed control levels ($p<0.01$).

Immunohistochemistry revealed that HIF 1 and 3α proteins were localized to the lamina propria and detrusor. Two weeks of pBOO resulted in significant increase of HIF 1α in the lamina propria with increase in the smooth muscle levels after 4 weeks of pBOO. Obstruction induced increased levels of HIF 3α in both the lamina propria and detrusor. However, MSCs therapy significantly reduced HIF 1 and 3α levels.

VEGF transcript levels increased from 2.0 (±0.70) to 5.03 (±0.49) and 7.90 (±0.56) after 2 and 4 weeks of PBOO respectively ($p=0.003$). Yet, there was a significant upregulation of VEGF transcripts by 9.5 folds 2 weeks after therapy and a downregulation to control levels 4 weeks after pBOO + MSCs therapy ($p=0.0008$).

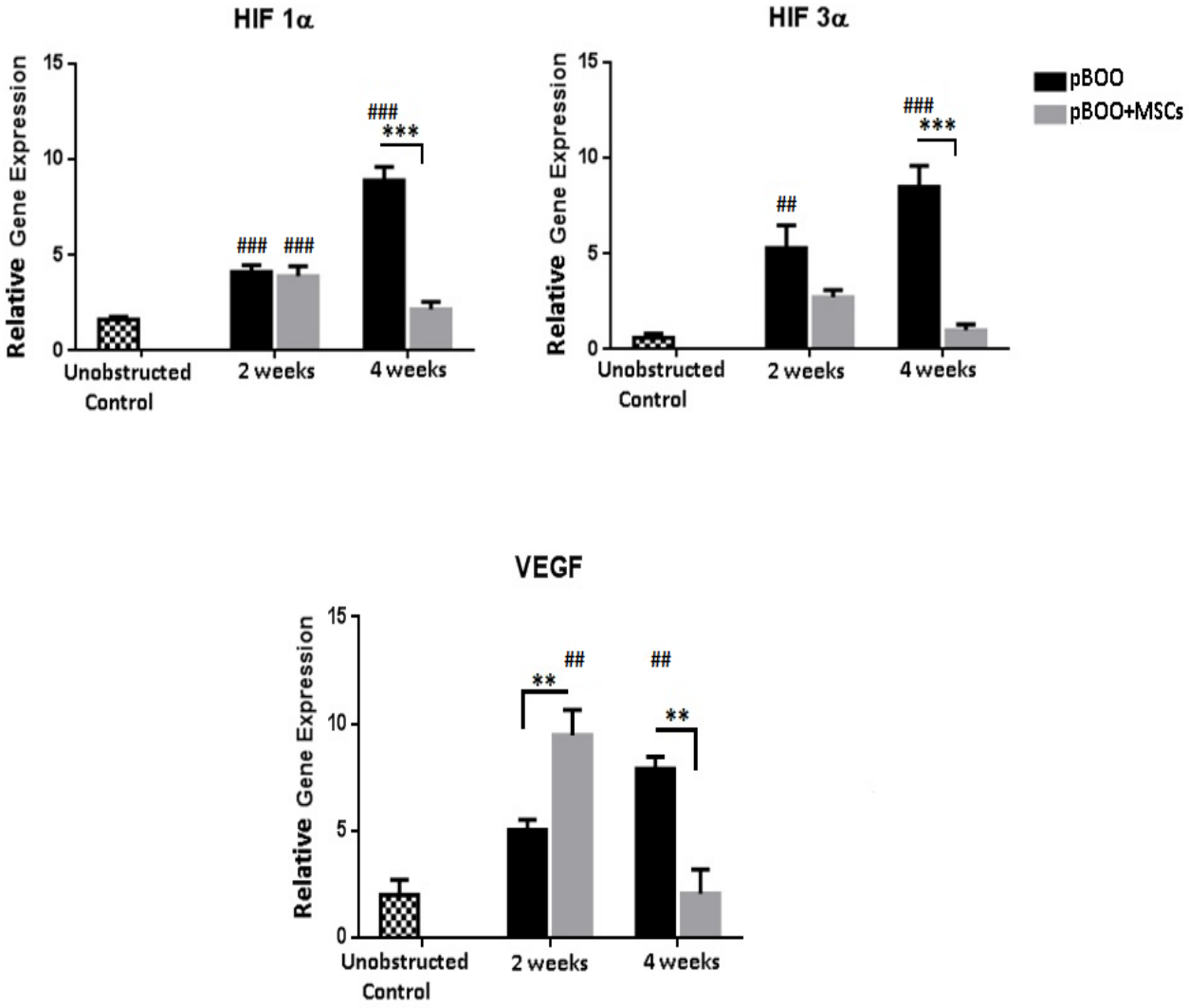

Figure 5.2 pBOO-induced HIF was downregulated by MSCs. PBOO was associated with increasing transcript levels of HIF 1α, HIF 3α and VEGF. MSCs therapy (pBOO+MSCs) led to an increase of VEGF transcripts 2 weeks later and a downregulation of HIF 1α, HIF 3α, and VEGF transcripts 4 weeks later. All gene expression data has been normalized to endogenous controls, β-actin and GAPDH and expressed as $2^{-\Delta\Delta ct}$. **p<005, ***p<0.0005 comparisons between pBOO and PBOO + MSCs for a time point. ## p<0.005 compared to unobstructed controls.

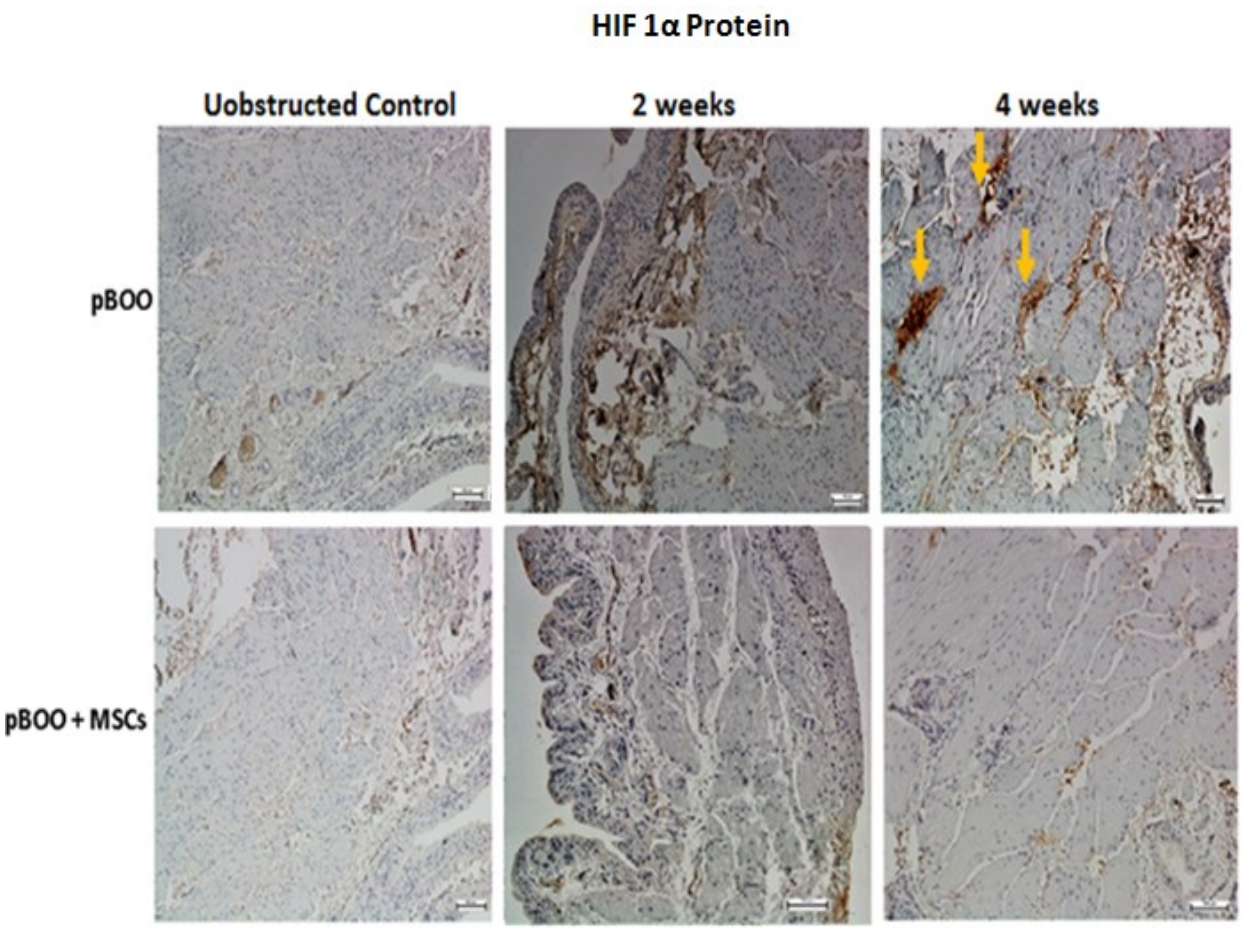

Figure 5.3 immuno-localization of HIF 1α in bladder after pBOO and treatment. HIF 1α levels increased significantly in the lamina propria after 2 weeks of pBOO and in the detrusor (indicated by yellow arrows) after 4 weeks of pBOO. Levels of HIF 1α are significantly reduced in the MSCs treated animals. Representative micrographs were captured at 100x magnification and scale bar represent 100μm

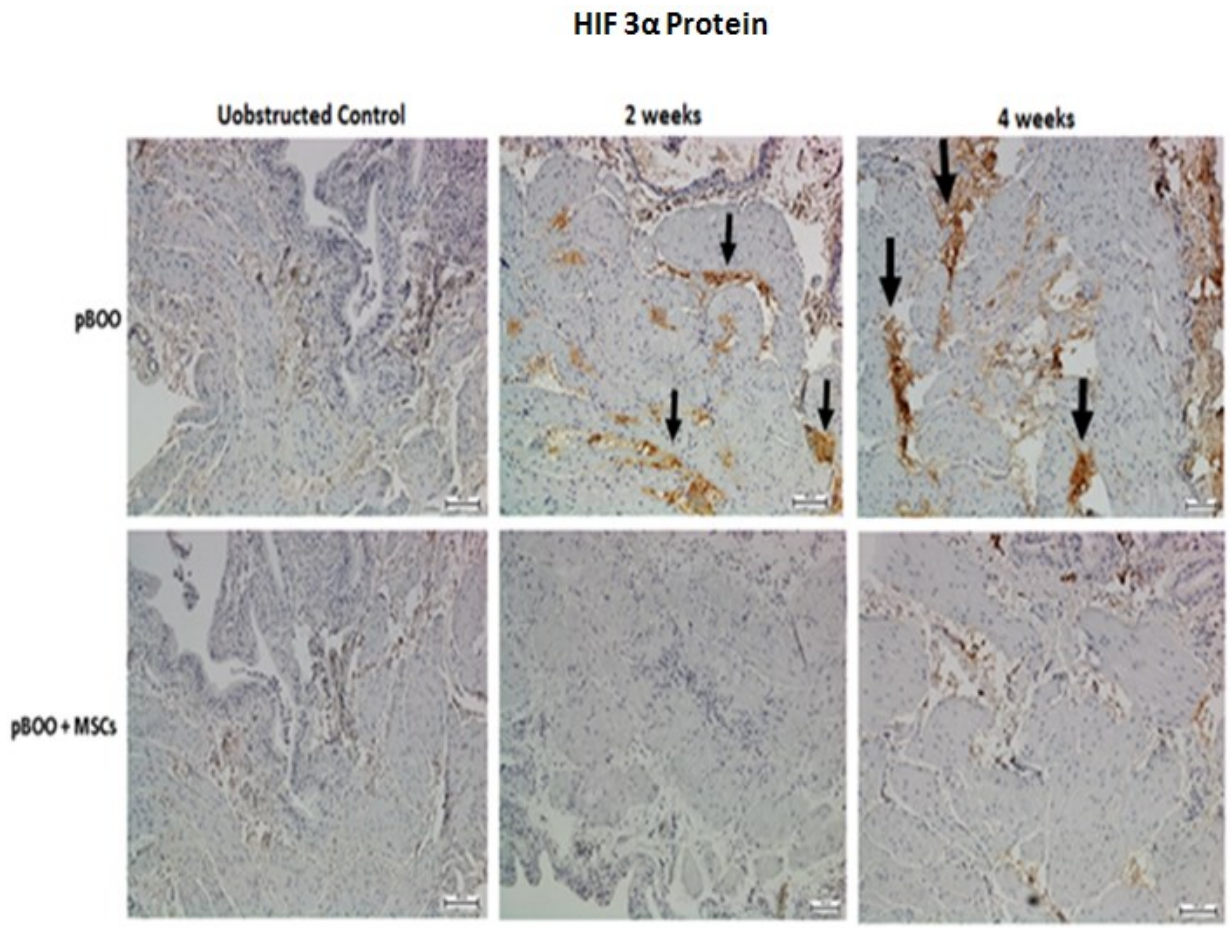

Figure 5.4 HIF 3α was upregulated after pBOO but was downregulated after MSCs therapy. HIF 3α is identified as brown staining in the lamina propria and detrusor (identified by black arrows). Images shown were captured at 100x magnification.

5.3.3 Pro- and Anti-inflammatory response

TNFα transcript levels rose from 2.50 (±0.33) in unobstructed controls to 8.03 (±0.60) and 10.23 (±0.79) after 2 and 4 weeks of obstruction respectively ($p<0.01$). Notwithstanding, 2 and 4 weeks after pBOO + the single injection of MSCs, there was a significant downregulation to control levels ($p<0.0001$ for all). pBOO resulted in a significant decline in interleukin 10 expression in a time dependant manner ($p=0.03$). However, IL-10 expression in the pBOO + MSCs therapy

groups was significantly higher than the pBOO controls ($p<0.05$). IL-10 expression in the 2-week pBOO + MSCs therapy group was not significantly different from the unobstructed control levels ($p=0.222$). Yet, the 4- week pBOO + MSCs therapy group showed a significantly lower transcript levels compared to the normal controls ($p=0.035$).

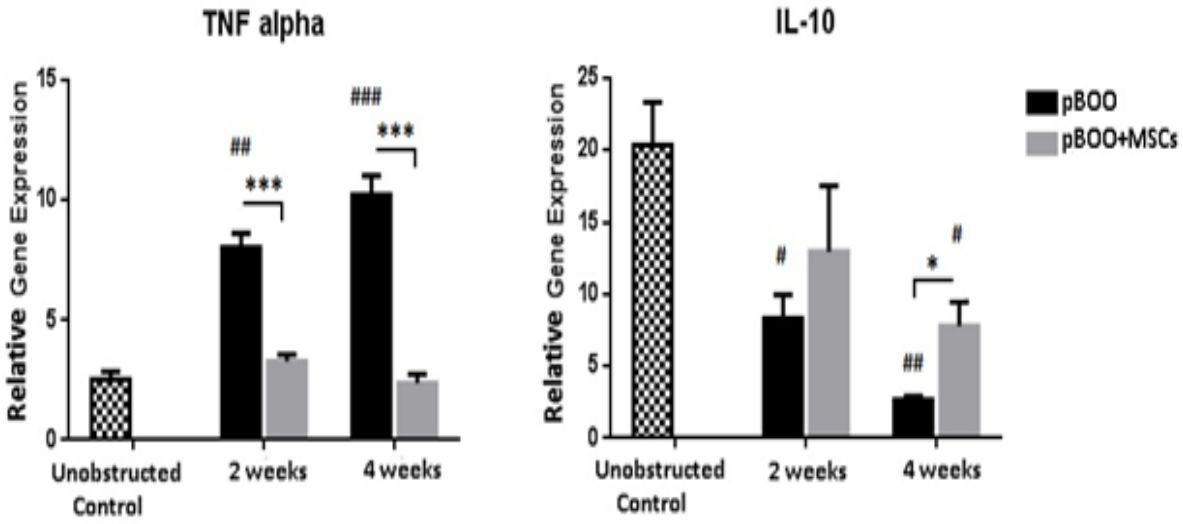

Figure 5.4 MSCs therapy inhibited pro-inflammatory cytokine and increased anti-inflammatory cytokine. TNF: IL-10 ratio increased in response to pBOO and this trend was reversed by MSCs therapy. * $P<0.05$, *** $p<0.0005$ represent comparisons between untreated pBOO and MSCs-treated pBOO groups. # $P<0.05$ compared to unobstructed controls

5.3.4 Pro-fibrotic and hypertrophy response:

Increase in TGFβ1 expression was directly related to the duration of bladder obstruction: Unobstructed control transcript level which was 1.37 (±0.23), increased to 5.50 (±0.42) and

10.73 (±1.19) after 2 and 4 weeks of obstruction respectively (p<001). Nonetheless, transcript reverted to normal control levels 4 weeks after MSCs therapy. Similarly, Smad 2/3 transcript levels rose from 1.87 (±0.35) to 6.43 (±0.55) and 8.53 (±1.00) after 2 and 4 weeks of pBOO (p<0.001). MSCs therapy led to a significant downregulation of transcript levels to 3.5 (±0.55) and 2.67 (±0.52) after 2 and 4 weeks of therapy respectively (p<0.05). In the same manner, immunohistochemistry showed increased phosphorylated smad 2/3 protein levels that was directly related to the duration of bladder obstruction. Phosphorylated smad 2/3 protein was localized to the urothelium and the detrusor muscle. Interestingly, bladder obstruction induced a significant increase in mainly the urothelial levels of psmad2/3. However, MSCs treatment led to a significant decrease in psmad2/3 levels (figure 5.5).

The mechanistic target of rapamycin (mTOR) transcript levels which was 1.47 (±0.24) in normal controls was 11.07 (±1.49) and 16.93 (±1.16) after 2 and 4 weeks of pBOO (p<0.0001). Four weeks after MSCs therapy, mTOR transcript levels were significantly reduced to 4.63 (±0.62) (p<0.001). Similarly, p70 S6K transcript levels rose from 3.43 (±0.18) to 8.03 (±2.19) and 12.13 (±0.70) (p=0.006) in the 2- and 4- weeks obstructed groups respectively. Transcript levels were significantly reduced to 3.90 (±0.35) 4 weeks after MSCs therapy (p=0.0019).

Collagen 1 transcript levels also increased in direct relation to the duration of pBOO. In contrast, Collagen 3 transcript levels peaked after 2 weeks of obstruction. Collagen 1 expression which was 1.9 (±0.85) increased to 6.5 (±0.95) and 8.57 (±0.52) after 2 and 4 weeks of pBOO. Transcript levels were comparable to normal control levels in obstructed rats 4 weeks after MSCs therapy (p=0.97). Collagen 3 expression which was 0.73 (±0.17) increased to 10.30

(±1.26) and 6.90 (±0.60) in 2 and 4 weeks of PBOO. However, MSCs therapy resulted in a significant reduction of collagen 3 transcript to normal control levels after 4 weeks (p=0.004).

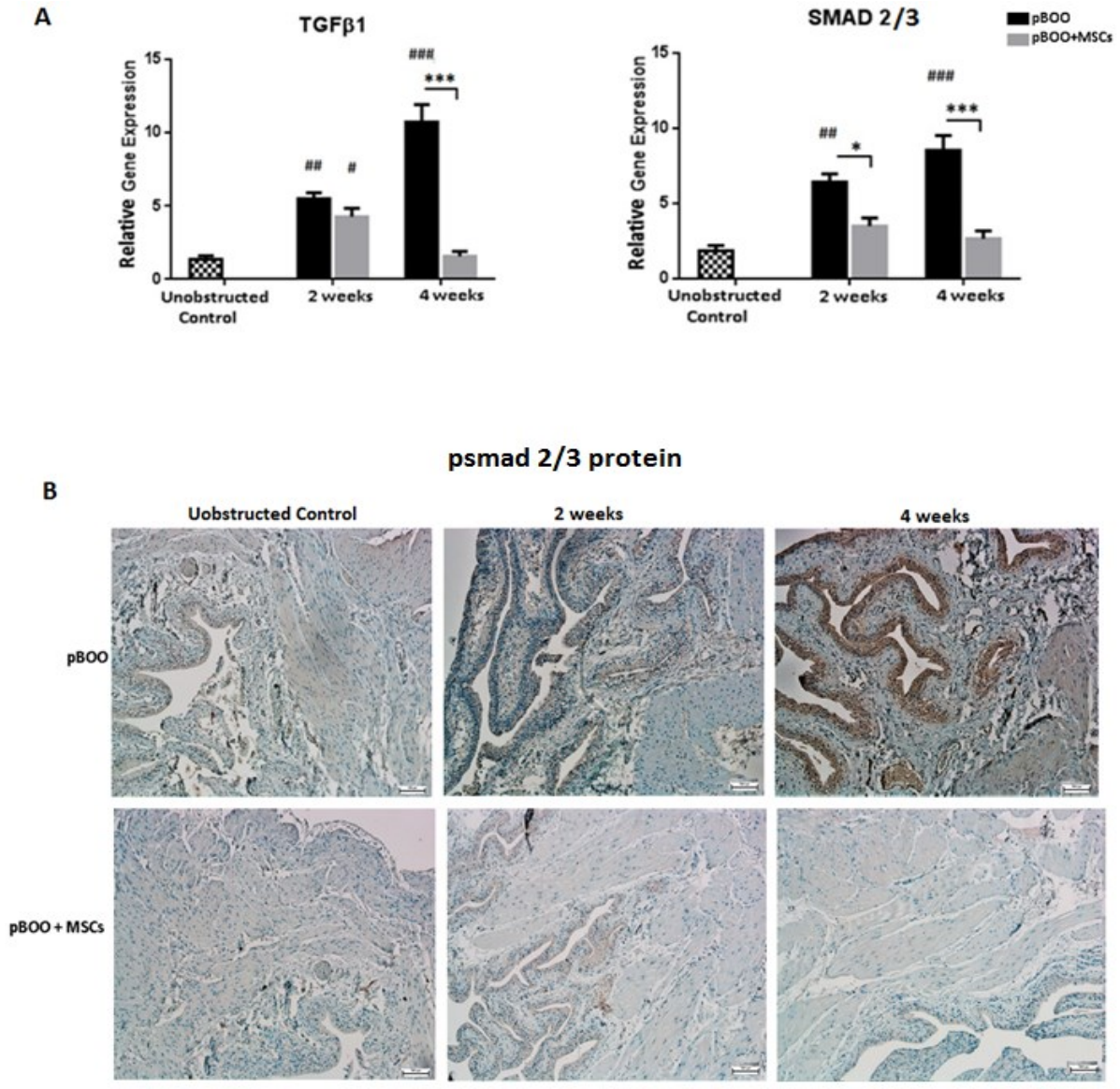

Figure 5.5 increased TGFβ1 expression and activity in pBOO was inhibited by MSCs. A. TGFβ1 and smad 2/3 transcripts were upregulated after pBOO but inhibited by MSCs therapy. B. phosphorylated smad 2/3 protein levels increased according to the duration of pBOO and decreased significantly after MSCs therapy. Note the increased urothelial levels of psmad2/3 in the 2 and 4 week obstructed animals. Scale bar is 100µm

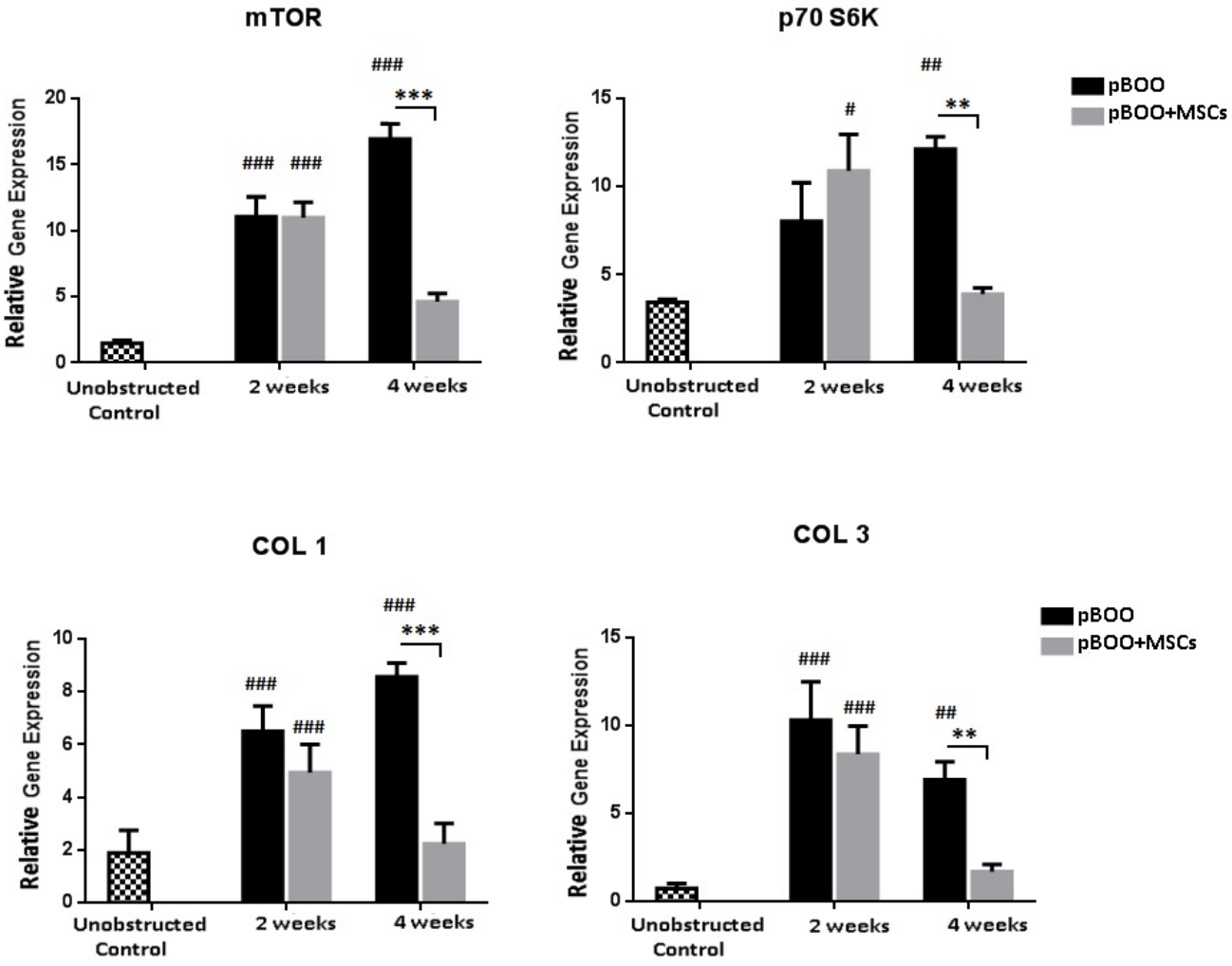

Figure 5.6. **Pro-fibrotic genes upregulated by pBOO were inhibited by MSCs therapy**. MTOR, p70 S6K, COL 1 and 3 transcript levels were significantly upregulated in the pBOO group but these were significantly reduced 4 weeks after pBOO plus MSCs therapy. Total RNA from 4 animals per group were reversed transcribed and used for gene expression analysis. ***P<0.0005, **p<0.005 represent comparisons between untreated pBOO and MSCs-treated pBOO groups. ## p<0.005 compared to unobstructed controls.

5.3.4 Histology:

pBOO was associated with detrusor muscle hypertrophy which was seen as increased muscular mass. Trichrome staining revealed an increased collagen deposition in the lamina propria, and interfascicular spaces of the smooth muscle layer of the pBOO groups. Furthermore, the 4-week obstructed animals had the highest collagen deposition and a distorted histology of the smooth muscle layer due to intrafascicular collagen infiltration. In agreement with the gene expression data, the connective tissue infiltration of detrusor smooth muscle bundles was inhibited by the single injection of MSCs. The amount of collagen in the bladder wall of the 4-week pBOO + therapy group was significantly lower and they had normal bladder histology.

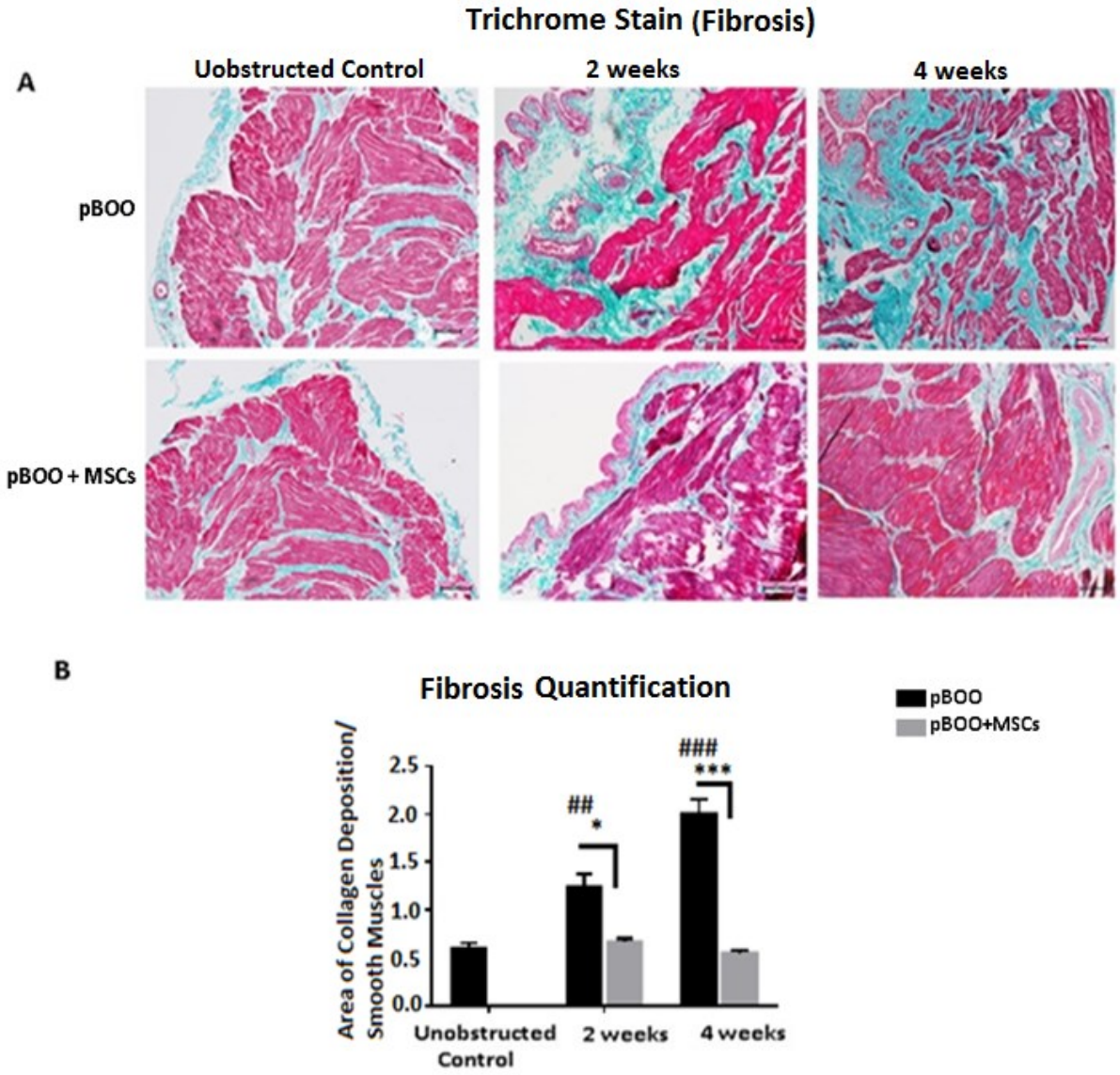

Figure 5.7. Surgical induction of bladder outlet obstruction led to a significant deposition of total collagen and was prevented by MSCs therapy. A. Gomori's trichrome staining results show high infiltration of the lamina propria and detrusor muscle bundles with collagen (green stain) in pBOO groups. Smooth muscle fibres stained red. Representative images from controls and treatment groups have been shown. Images were captured with 100X magnification and scale bars represents 100µm. Note the distortion of the detrusor smooth muscle histology of the 4 week pBOO group due to concomitant collagen deposition. The treatment groups show normal bladder tissue histology and normal collagen levels. B. quantification of the collagen stained areas in the bladder tissue per area of smooth muscle staining. Image J was used to measure the

collagen density of 5 randomly selected images of each bladder tissue. The value was divided by the corresponding smooth muscle density of each sample.

5.3.5 Urodynamics:

End filling bladder volumes increased significantly after pBOO and MSCs therapy did not alter the increased bladder capacities. Remarkably, the induction of pBOO was associated with high pressure urine storage. End filling detrusor pressure was 5.17 (±0.12) cm H_2O in normal controls but an increased pressure of 10.63 (±0.23) cm H_2O ($p=0.006$) and 12.00 (±1.86) cm H_2O ($p=0.002$) were recorded at the time urethral leakage was visualized in the 2- and 4- week obstructed rats respectively. Mesenchymal stem cell therapy on the other hand resulted in a significant reduction in urine storage pressures to 6.15 (±0.64) cm H_2O ($p=0.01$) and 3.53 (±0.78) cm H_2O ($p<0.0001$) respectively.

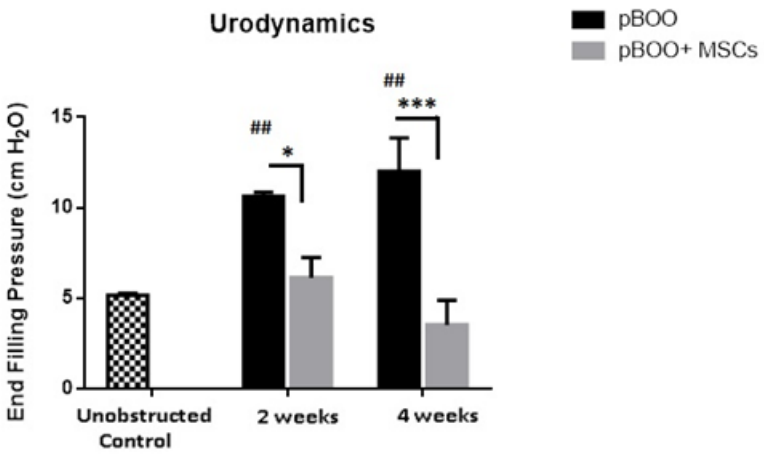

Figure 5.8 pBOO induced high pressure urine storage was reduced by MSCs. Functional assessment of the bladder showed increasing urine storage pressure induced by pBOO. The high pressure urine storage is inhibited by MSCs therapy. * $p<0.05$, *** $p<0.0005$ represent comparisons between untreated pBOO and MSCs-treated pBOO groups. ## $p<0.005$, ### $p<0.0005$ compared to normal controls.

5.4 Discussion

pBOO is a serious urological condition that exposes the bladder to high pressure urine storage which poses a high risk of renal damage. Molecular studies have delineated the detrimental effects of increased inflammation, mechanical stretch, hydrodynamic pressure and hypoxia which are believed to be the causative factors in the pathophysiology of pBOO. Common among these findings were increased collagen synthesis and extracellular matrix remodeling [19-21]. In humans and whole animal models of pBOO, the resultant infiltration of bladder smooth muscle fibers with connective tissue is destructive to the bladder's contractile component. Current therapies have failed to effectively target the phases of inflammation and fibrosis in pBOO. However, mesenchymal stem cell therapy has shown promising experimental outcomes in the management of a myriad of inflammatory-fibrotic conditions including pBOO. Nonetheless, the mechanisms of action of this therapy are not well understood. We recently showed that both a direct and indirect co-culture of bladder smooth muscle cells with mesenchymal stem cells attenuates hypoxia-induced inflammatory and fibrotic pathways [22].

Findings from this study reaffirms that pBOO is associated with several molecular changes which are progressive with time. We recorded an early rise in end filling detrusor pressure and this was associated with substantial gene expression changes after 2 weeks of bladder obstruction. The upregulation of pro-inflammatory markers including HIF 1α, HIF 3α, TGFβ1, and TNFα was further exacerbated by the marked decline in IL-10 levels. IL-10 is a well-studied anti-inflammatory and anti-fibrotic cytokine that is known to inhibit inflammatory markers such as TNFα and IL-6. From the current study, the inverse relationship between the duration of pBOO and IL-10 expression is novel. Previous studies have shown that hypoxia is one of the factors that can downregulate IL-10 levels [14, 22]. Therefore, one likely explanation for the

reduced IL-10 expression in the pBOO groups may be the presence of pBOO-induced hypoxia. Moreover, a number of studies found lower serum levels of IL-10 in patients with type 2 diabetes compared to normal controls and in subjects with unstable angina compared to those with stable angina [23-25]. Some studies have also found a correlation between coronary disease, leptospirosis, or malaria severity, and IL-10 to TNFα ratio. A low ratio was associated with disease progression or severity [26-28]. In the current study, since IL-10 levels declined over time as TNFα levels increased, the IL-10 to TNFα ratio seemed to correlate with pBOO progression.

With the profound systematic improvements seen with MSCs treatment in this study, it becomes apparent that the single injection of MSCs has both short and long-term benefits. The identification of GFP-MSCs in bladder tissue 2 and 4 weeks after therapy confirms the ability of stem cells to migrate to the tissue of injury. Even though we could not show their relative expression in non-target organs, the therapeutic effects we found were paramount. Noticeably, the immunomodulatory effects of the MSCs therapy were detected early, with a significant downregulation of TNFα, and upregulation of IL-10 and VEGF within 2 weeks after MSCs therapy. This is in contrast to the modulation of pro-fibrotic genes that occurred rather later (4 weeks after therapy).

We believe that the initial immunomodulatory responses are mediated by the paracrine mechanisms of the MSCs whereas the pro-fibrotic changes occur once the injected MSCs engraft and repopulate the bladder. With regards to paracrine immuno-regulation, MSCs are known to secrete factors such as IL-10 and VEGF which inhibits pro-inflammatory cytokines like TNFα and stimulate angiogenesis and tissue injury repair [29, 30]. In the current study, changes in gene expression of VEGF correlated with the expression of HIF 1 and 3α. However, we believe that

the initial rise in VEGF after 2 weeks of therapy which did not correspond to rises in HIF 1 and 3 α, may have been induced by the MSCs therapy in order to stimulate angiogenic growth and healing of the bladder. Eventually, both IL-10 and VEGF expressions were significantly reduced 4 weeks after therapy. This outcome may imply that the immunomodulatory effects of MSCs is potent, but may either be short-lived or cools off once long term pro-fibrotic pathway modulation is initiated. From immunohistochemistry, the HIF 1 and 3α immuno-localization to the lamina propria and detrusor correlates with the pattern of pBOO-induced collagen deposition. This observation supports the causative relationship between hypoxia and fibrosis. Even though HIF 1α has been a good marker for hypoxia, from our results, HIF 3α may be an emulous marker too.

MTOR is a principal pathway mainly implicated in cancers and allograft rejections of organ transplants. As a result, rapamycin, a potent mTOR inhibitor is used as a primary immunosuppressant in solid organ transplantation. Also, several ongoing research are targeting the mTOR pathway for the design of inhibitors as potential therapeutic agents. However, so far there have been only moderate successes with the use of such drug candidates due to the challenges of drug resistance and bioavailability [31]. Given the fibrogenic potency of TGFβ, research has also focused on the discovery of respective inhibitors for the prevention of fibrosis. However, not much success has been achieved so far [32]. Therefore, from this study, the demonstrated ability of stem cell therapy to target these principal pathways is remarkable. Since the biological activity of TGFβ1 is executed by phosphorylating its downstream mediators Smad2 and Smad3, thus, phosphorylated smad2/3 is an important indicator of enhanced TGFβ1 activity. In this study, pBOO-induced increase of Psmad2/3 was exclusively observed in the urothelial layer. This observation may indicate that the urothelium is a constant target for pBOO-induced urodynamic pressure and stretch. Thus, it not surprising that the inhibition of this pro-

fibrotic pathway by MSCs therapy was associated with improved functional outcomes. Therefore, this work has unraveled mechanistic cues underlying the anti-fibrotic effects derived from stem cells.

From this study, we have demonstrated that the significant benefits of MSCs therapy can be divided into 2 main phases: early anti-inflammatory stage and a secondary anti-fibrotic mechanism. Ultimately, both phases were associated with significant reduction in total collagen deposition and in end filling pressure. Most importantly, this effect was seen with a normalization of bladder histology and the prevention of high pressure urine storage, bladder decompensation, smooth muscle hypertrophy and fibrosis induced by pBOO.

5.5 Conclusion

Partial bladder outlet obstruction results in significant upregulation of inflammatory and pro-fibrotic genes and subsequent bladder decompensation. However, the intravenous injection of bone marrow-derived mesenchymal stem cells prevents these changes and preserves the normal function of the bladder. This work has elucidated the ability of MSCs to target inflammatory and pro-fibrotic pathways to prevent bladder deterioration due to pBOO. It lays an important foundation for the prevention and potential treatment of end stage fibrotic bladder with mesenchymal stem cells.

Chapter 6

General Discussion, Conclusions and Future Recommendations

Bridget Wiafe

6.1 General Discussions

For many years our understanding of the pBOO pathology centred around the causative factors: mechanical stretch, hydrodynamic pressure, and inflammation. Notwithstanding, previous research had identified hypoxia in obstructed bladder tissue. However, its ramification was not yet understood. At the cellular level, the ability of MSCs to inhibit hypoxia-induced inflammatory and fibrotic pathways was also not known. These created a big gap in our understanding of the pBOO pathology and consequently limited possibilities of therapeutic interventions. Thus, the characterization of the isolated effects of hypoxia on the bladder is an essential contribution to this field.

The study recorded in chapter 3 of this thesis clearly illustrated that exposure of bladder smooth muscle cells to hypoxia induced an intense hypoxic, pro-inflammatory and fibrotic gene expression that closely resembled the pBOO pathology. This work was exhaustive, exploring multiple fibrotic pathways induced by hypoxia. Notably, the hypoxia signaling pathways disrupted the equilibrium between extracellular matrix anabolism and catabolism. Consequently, ECM production increased with a decreased rate of breakdown leading to a net increase of

collagen and other ECM components. Thus, these results reaffirm that hypoxia plays a key role in bladder tissue pathology.

While the different organs of the body are exposed to different oxygen tensions for physiological reasons, the level of hypoxia cells are exposed to, determine to a large extent their level of adaptive response. In order to create an in vitro model that closely replicates the pBOO, we chose to expose bladder cells to hypoxia (i.e. 3% O_2). This is because from literature, the best estimate of the oxygen tension in the normal bladder is about 80mmHg which corresponds to about 10% oxygen tension [1]. A previous study found that 2 weeks of obstruction resulted in 70% reduction in blood flow [2]. Therefore, we believe that an oxygen tension of 3% is physiologically relevant to the pBOO condition. Thus we are confident that the findings of the in vitro study illustrate the isolated effects of hypoxia-induced bladder damage that can be attributable to pBOO. The ability of hypoxia to initiate and sustain pro-fibrosis in bladder cells may explain why pBOO results in progressive bladder damage.

Hypoxia plays a central role in the pathophysiology of many conditions and in cancer, decades of research has focussed on the discovery of molecular targets for HIF synthesis, stabilization, dimerization as well as signaling. However, despite advancements in high-throughput screening and computational molecular modeling technology, no direct HIF inhibitor candidate has been approved for cancer therapy. Most clinical trials were halted for lack of safety and/or efficacy [3]. In animal models of pBOO, the inhibition of HIFα using 17-DMAG, prevented bladder fibrosis over a short term. 17-DMAG is a soluble analogue of geldanamycin and it targets HSP70 which is responsible for HIFα folding and stabilization [4].

Essentially, a deeper understanding of the differential expression and regulation of the HIFα subtypes is very important. This is because most HIF inhibitors target only HIF 1α. The challenge here is that findings from some studies have revealed that inhibiting one HIF α subtype may stimulate the other subtypes in compensatory mechanisms leading to a poorer disease prognosis and drug resistance [5, 6]. This therefore underscores the importance of our work in characterizing the expressivity of all HIF α subtypes in the bladder. From this study, the involvement of HIF 3α in the bladder was identified for the first time. Its intense expression in prolong hypoxia was conspicuous when the HIF 1 and 2 α levels had subsided after an initial increase. This finding appear to be supportive of an inhibitory or compensatory relationship between the HIF 3α and HIF 1 and 2α subtypes. Importantly, this outcome has serious implications on the potential choice of anti-HIF treatment for pBOO.

Further investigations to confirm the HIFα inhibitory mechanisms may design experiments that inhibit HIF 3α expression in bladder smooth muscle cells exposed to prolong hypoxia. siRNA or specific antibodies could be used to inhibit HIF 3α expression. Increase of the other HIFα subtypes in the absence of HIF 3α and a subsequent downregulation of HIF 1 or 2α when HIF 3α is overexpressed may confirm an inhibitory association between these subtypes. Essentially, the upregulation of HIF 3α in response to prolong hypoxia provides the bases for future investigation into whether HIF 3α levels of the bladder is a potential marker of chronic hypoxia. To investigate this hypothesis, levels of bladder expressivity of all the HIF α subtypes could be measured in animal models of pBOO at different disease stages in order to establish possible correlations between expressivity patterns and disease stage and progression.

In chapter 4, through co-culture with MSCs, we were able to elucidate the anti-fibrotic nature of MSCs and its ability to inhibit HIF-induced fibro-inflammatory pathways. Both the paracrine and cell to cell mechanisms had equal anti-fibrotic potencies measured by increased IL-10 synthesis and a corresponding reduction in hypoxia induced extracellular matrix. This result provides basis to support the potential use of MSCs-conditioned media in the treatment of pBOO. As a follow up to this co-culture study, mass spectrometry can be carried out on culture media to investigate the types of MSC-derived trophic factors that are mediating paracrine roles and on cells to determine the receptors and proteins playing roles in cell-cell contact.

In order to determine the translatability of these molecular mechanisms, animal models of pBOO were used. Chapter 5 describes the ability of MSCs to inhibit hypoxic, inflammatory and fibrotic pathways which was determined by the injection of MSCs in rats with pBOO. MSCs transfection with the green fluorescent protein (GFP) made tracking of injected MSCs easier. The identification of GFP-MSCs in the bladder reaffirmed the ability of MSCs to home in to sites of injury. Even though we could not determine relative localization of the systemically injected MSCs to non-target organs like the lungs, spleen and liver, the MSCs inhibitory effects on hypoxic, inflammatory and fibrotic pathways was profound. Thus benefits of MSCs to inhibit inflammatory and fibrotic pathways was demonstrated in vitro and confirmed in vivo. However, as a follow up to the MSCs localization experiments, the proliferative capacity of the bladder-recruited MSCs can be investigated by immuno-staining for markers such as Ki67 and 5-bromo-2'-deoxyuridine. Furthermore, the expression of markers such p53, p21 senescence-associated β galactosidase will also be important in determining the in vivo viability of the transplanted cells. Ultimately, the fate of the bladder-localized MSCs may be tracked by staining for some known

bladder specific markers such myosin, desmin, smoothelin and caldesmon. Thus, the identification of the GFP-MSCs co-expressing these bladder specific markers may be indicative that transplanted viable and replicating MSCs are engrafted and repopulating the bladder.

Experimentally, the time point for the in vivo model was chosen to coincide with the phase where the pro-fibrotic stage is emerging in a more established inflammatory milieu; Outcomes of MSCs treatment was measured after 2 and 4 weeks. Initial work by our lab has shown that MSCs intervention in the first week of pBOO mainly borders on inhibition of inflammatory factors [7]. From the results recorded in chapter 5, it does not appear that we lost any information by not including a 3-week after treatment time point. Rather, it might be much more beneficial to measure outcomes beyond 4 weeks to know how long the effects of therapy will last.

The main difference in terms of outcome between the in vitro and in vivo systems is the HIF regulation by MSCs. In the in vitro experiments, HIF expression was unaffected by MSCs intervention whereas in vivo, HIF expression was modulated by MSCs. In an attempt to explain these outcomes; consideration must be given to the differences in time points between the in vitro and in vivo experiments. In the in vitro experiment, probably, the duration of hypoxia (72 hours) was still acute so that MSCs could potently inhibit the inflammatory and fibrotic pathways without affecting HIF levels. However, MSCs may suppress HIF levels in longer hypoxia durations. This view is supported by the fact that in our in vivo findings, MSCs did not significantly affect HIF transcript levels 2 weeks after treatment. However, MSCs significantly down-regulated HIF transcripts 4 weeks after treatment. Thus, in the first two weeks after therapy, the MSCs-induced downregulation of high pressure urine storage and inflammatory factors occurred while HIF was still upregulated. Therefore, the upregulation of VEGF 2 weeks

after therapy may be a synergistic response of MSCs secretion and HIF upregulation. Thus, the reversion of VEGF to normal unobstructed levels 4 weeks after therapy coincides with the MSCs-induced suppression of HIF levels.

Central to our results is the activation of the TGFβ-Smad pathway in pBOO which has pro-fibrotic sequela. This coupled with the upregulation of the mTOR-p70 S6K pathway and the loss of IL-10, it was not surprising that these events culminated in fibrosis of the bladder tissue. Therefore, against such background, the demonstrated ability of mesenchymal stem cells to inhibit these multiple fibrotic pathways in the presence of pBOO was exhaustive. An analysis of our results revealed that, the anti-fibrotic effects of MSCs were executed at the molecular and physiological levels. The molecular effects of MSCs was profound inhibiting both inflammatory and fibrotic pathways in both HIF independent and dependent manners. The anti-fibrotic effects of MSCs at the physiological level potently inhibited the high urine storage pressure associated with pBOO. This outcome had positive effects on blood flow to the bladder. Thus hypoxia and the hypoxia-induced pro-fibrotic signaling in the obstructed bladder tissue was significantly reduced. The angiogenic stimulation capacity of MSCs may have also contributed to the reduction of obstructed bladder hypoxia. Figure 6.1 summarizes these findings.

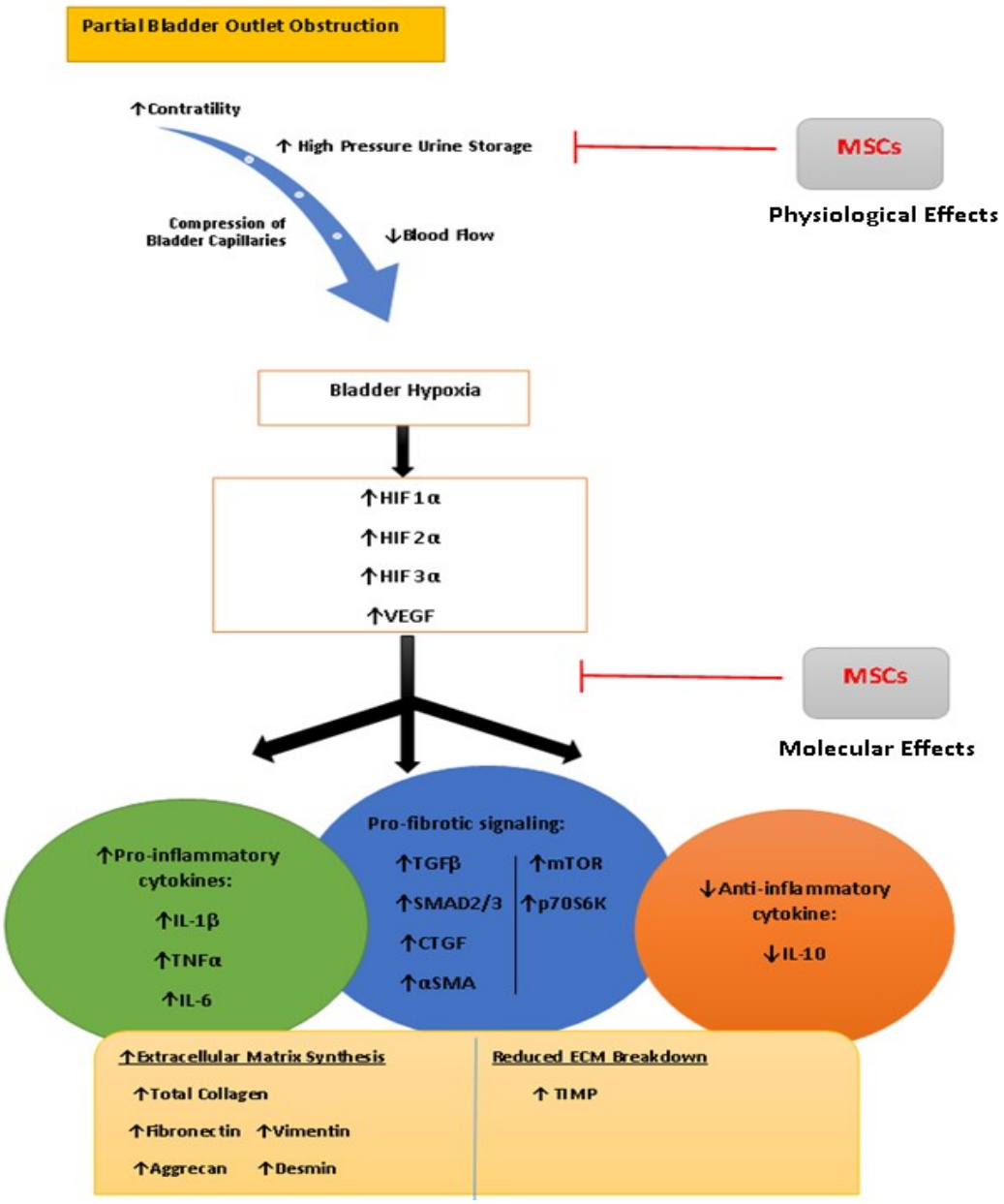

Figure 6.1. Molecular and physiological benefits of MSCs anti-fibrotic effects on pBOO pathology. A schematic drawing summarizing the in vitro and in vivo findings of the pBOO pathology and the MSCs anti-fibrotic effects. The induction of pBOO, increased bladder contractility and high pressure urine storage which compresses blood vessels reducing blood flow to the bladder. The resulting tissue hypoxia induces significant inflammatory and fibrotic changes to the bladder. MSCs potently inhibit these disease mechanisms at the molecular and physiological levels preventing bladder deterioration.

6.2 Potential Clinical Benefits

The therapeutic potential of mesenchymal stem cell therapy can not be over-emphasized. The outcome of our study is exciting in the wake of the high morbidity associated with the current pBOO treatment regimes. The results of this thesis supports the future use of mesenchymal stem cell therapy for the treatment of pBOO from different aetiologies. The clinical relevance of our work is shown by the fact that the anti-fibrotic effects of MSCs were eminent in the presence of continuous hypoxia (in vitro) or pBOO (in vivo). In fact, the anti-fibrotic effects of MSCs prevailed for 4 weeks in the presence of bladder obstruction. Clinically, even though pBOO of many anatomical causes may be relieved by surgery, some causes of functional bladder obstruction such as spina bifida and diabetes require management in the presence of nerve damage or other causes of the obstruction. Thus, the results described in this thesis gives hope that MSCs therapy may be effective in preventing bladder deterioration and fibrosis.

Given the huge potential of our findings for clinical applications, some work is still required to ensure increased efficacy and effectiveness. First of all, beyond the anti-fibrotic effects shown as well as the recommendations given, there must be investigation into the ability of MSCs to regenerate damaged bladder nerves which is associated with the pBOO pathology. This will ensure complete recovery of bladder function and maximize benefits of MSCs to children with spina bifida and other sufferers of pBOO. MSCs are known to secret factors such as nerve growth factor, insulin-like growth factor, Brain-derived neurotrophic factor, VEGF and other neuroprotective factors which promotes nerve repair and regeneration [8]. After MSCs transplantation in animal models of pBOO, immunostaining for axonal markers such as neurofilaments, myelin, GAP-43, and βIII-tubulin could be performed in order to assess the degree of nerve regeneration. Secondly, the safety of transplanted cells for clinical application is

an important concern. Consequently, there is the need to use improved in vivo tracking systems such as MRI to trace the fate of transplanted cells for longer periods. Bioluminescence imaging of luciferase-transfected MSCs has also been effective in the non-invasive real time ex vivo tracking of transplanted whole animals [9, 10]. Moving forward, it will be important to confirm these findings in larger animal models such as the pig, sheep, and cow. This is because these large animals are more similar to humans in terms of body weight, anatomy, physiology and pathophysiology [11]. Thus, findings from such results will be much more translatable to humans.

6.3 Conclusions

Hypoxia induced significant inflammatory and fibrotic effects in bladder smooth muscle cells similar to pBOO. Furthermore, MSCs have the ability to mitigate multiple inflammatory and pro-fibrotic pathways to prevent bladder deterioration due to pBOO. The outcomes of this study are promising and lay an important foundation for the prevention and potential treatment of end stage fibrotic bladder with mesenchymal stem cells.

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Chapter 1

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Chapter 2

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