ESTABLISHING A MODEL OF ORGAN REGENERATION IN THE ADULT MAMMAL: AGE DEPENDENCE AND THE UTILITY OF NON-INVASIVE IMAGING

BY

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A Dissertation Submitted to the Graduate Faculty of
WAKE FOREST UNIVERSITY GRADUATE SCHOOL OF ARTS AND SCIENCES
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
Physiology and Pharmacology
December, 2011
Winston-Salem, NC

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DEDICATION

This work is dedicated to the memory of my late grandfather, George H. Stidworthy, Ph.D.
Thank you for continuing to be an inspiration to me.
ACKNOWLEDGEMENTS

To the members of my committee: Thank you so much for your support, conversations, and criticisms. Your expertise and willingness to discuss this project has made this dissertation possible, and produced a critical graduate student. Thank you.

To the entire Physiology and Pharmacology department, and the Wake Forest Institute for Regenerative Medicine: I am very grateful for the education you have bestowed upon me. This has been a truly exceptional place to learn many different aspects of science, and the knowledge gained from this experience will always travel with me.

To all faculty members, post-docs, technicians, summer students, medical students, residents, fellow graduate students, and other lab members who have played any part in the success of this project: thank you SO much. Whether helping to perform an experiment, discussing the scientific merit of experiments, advising on the research path, or simply forcing me to bring findings into context, your support and assistance have been crucial for my success here. I am extremely grateful for all of your help!

To my network of friends- both those who helped me survive and those who helped me forget the difficult times and long hours- thank you. The former includes many labmates that were inspiring examples of dedication and hard work, and without you I could have given up. The latter includes some of those same friends, and others, who helped me escape when needed for preservation of my own sanity.

To my incredible family, my parents, sisters, nephews, grandparents, and last but certainly not least my loving girlfriend, who have always stood behind me: I would not be here and smiling without you. Even when times get too busy and it seems like decades since our last embrace, you never questioned my purpose or ability. You have always shown me unconditional love, and I will ALWAYS show you the same.
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LIST OF ABBREVIATIONS

STC- Trigone-sparing subtotal cystectomy
AMC- Age-matched Control
CT- Computed Tomography
MRI- Magnetic Resonance Imaging
CMG- Cystometrogram
Bcap- Functional bladder capacity
MV- Micturition Volume
RV- Residual Volume
BP- Basal Pressure
TP- Threshold Pressure
MP- Micturition (Maximum) Pressure
IMP- Inter-micturition Pressure
SA- Spontaneous Activity
Bcom- Bladder Compliance
Emax- Maximum steady state contraction
Cch- Carbachol
PE- Phenylephrine
ATP- Adenosine Tri Phosphate
EFS- Electrical Field Stimulation
IHC- Immunohistochemistry
PCNA- Proliferating Cell Nuclear Antigen
SMA- Smooth Muscle Actin
MHC- Myosin Heavy Chain
VWF- Von Willebrand Factor
ADSC- Adipose Derived Stem Cell
SIS- Small Intestinal Submucosa
BAM- Bladder Acellular Matrix
ABSTRACT

David Mark Burmeister

ESTABLISHING A MODEL OF ORGAN REGENERATION IN THE ADULT MAMMAL: AGE DEPENDENCE AND THE UTILITY OF NON-INVASIVE IMAGING

Dissertation under the direction of

George Christ, Ph.D.

Wake Forest Institute for Regenerative Medicine

Although models of regenerative biology in lower vertebrates (e.g., salamanders, axolotls) have been utilized to study in vivo organ regeneration, there are far fewer opportunities in the adult mammal. One obscure example of mammalian organ regeneration is the urinary bladder, where the regenerative capacities have been known to urologists for some time. Despite anecdotal evidence for de novo growth of the, very few studies have examined the extent of bladder regeneration using animal models. Understanding caveats and strengths of bladder regeneration per se would undoubtedly enhance the field of urological tissue engineering. To this end, the goal of this effort was to characterize the extent of bladder regeneration, with specific emphasis on restoration of function. Furthermore, the impact of age on bladder regeneration, and the prognostic value of non-invasive imaging were evaluated. Removal of a large portion of the urinary bladder (subtotal cystectomy; STC) in adult rats initiated a proliferative response that gave rise to normal bladder wall architecture, including nerves, vessels, urothelium, and smooth muscle. Despite subtle differences in shape and contractility, bladders displayed a normal low pressure, high capacity function, and were able to empty completely 8 weeks after cystectomy. Computed tomography imaging corroborated this increase in bladder volume, and moreover was able to predict a rate of bladder growth that resulted in normal function. Magnetic Resonance Imaging revealed a progressive thickening of the bladder wall, which also conveyed information about bladder wall composition. When STC was performed in moderately aged rats, capacity failed to return to normal values, with further compromised smooth muscle contractility which was accompanied by a delayed proliferative response. Most strikingly, evidence of chronic kidney damage was found. This failure to recover the high-volume function of the bladder was not reversed by local administration of adipose derived stem cells at the time of STC. These studies establish an age-dependent model of mammalian organ regeneration that may be utilized to identify molecular regulators of (un)successful regeneration. While targetable pathways specific to bladder tissue engineering may be leveraged with this model, molecular differences may also lead to an understanding of impaired regenerative capacities due to aging.
CHAPTER 1

Introduction
An estimated 35 million Americans suffer from bladder disease with diverse etiologies, such as inflammation, congenital malformations, infections, or nervous system lesions (BRPRG 2002). These diseases are usually chronic in nature, and lead to a poor quality of life, with an estimated annual cost of 16 billion dollars in the U.S. alone. Although there are many pharmacological treatments that aim to return function, cases that are unresponsive to such treatments require surgical intervention (Flood et al. 1995; Andersson et al. 2009). This is especially true when the low-pressure function of the bladder is compromised, which may lead to destruction of the upper urinary tract, and potentially, end-stage renal disease. Current surgical interventions (usually intestinal segments used for bladder augmentation) have limited efficacy and are associated with substantial side effects such as mucous production, metabolite disturbances, stone formation, and infections (Atala 2011). As such, there has been an increased interest regenerative medicine technology for urological purposes which have also shown great promise (Kanematsu et al. 2007). This may be, in part, due to a natural ability for the bladder to spontaneously regenerate after removal of a large portion of the organ. The purpose of this thesis is to review the current status of the clinical and basic research literature involving bladder tissue engineering (Chapter 2), and to present our recent studies involving de novo regeneration of the urinary bladder in a rat model. As restoration of function is the ultimate aim of tissue engineering/regenerative medicine, particular emphasis will be given to examining bladder function, although mechanisms responsible for successful organ regeneration will also be examined.
Models of organ regeneration, while abundant in lower phylogenetic animals (ie. salamanders, newts, tadpoles) (Adams et al. 2007; Tsonis 2007), are scarce in adult mammals. For the latter, organ regeneration is usually thought to occur only in skin, bone, liver, and muscle, and moreover, these examples are limited in their capacity to fully replace excised/lost tissue (Grefte et al. 2007; Roy et al. 2008). A much more obscure example of organ regeneration is the substantial regenerative capabilities of the urinary bladder. The bladder's regenerative capabilities have been known for over a century, starting as early as 1891 with the work of Schwarz describing a normal sized bladder that had grown after subtotal cystectomy (STC; removal of a majority of the bladder) in dogs (Schwartz 1891). Liang and Goss reported on successful bladder regeneration in rats, where, after STC, bladder regeneration led to bladders about half of the normal size only 2 weeks post-operatively (Liang 1963; Liang et al. 1963). Despite this knowledge, the overwhelming majority of research exploring regeneration of the bladder employs the use of scaffolds for bladder augmentation, and little is known about the regeneration of the bladder following STC alone.

Our overall hypothesis is that there is a regenerative response of the rat bladder elicited by subtotal cystectomy which is compromised in aged animals, and that regenerative response can be monitored non-invasively. We present three sets of experiments, that all examine function of the bladder following subtotal cystectomy. In the first set of experiments (Chapter 3) we perform STC on young adult rodents and characterize time-dependent changes in function of bladder both in vivo and in vitro for up to 8 weeks after STC. In the second set of experiments
(Chapter 4) we examine the effect of moderate aging at the time of STC on bladder function, and report on long-term outcomes (i.e., 6 months) after STC. Additionally we explore mechanisms associated with regeneration via histology, with the overarching hypothesis that age-related differences in bladder regeneration will resemble that seen in other organs (i.e., decreased hyperplasia, angiogenesis, and increased fibrosis, inflammation). Finally, the usefulness of non-invasive imaging (i.e., Magnetic Resonance Imaging, Computed Tomography) as a prognostic indicator of bladder function post-STC will be examined (Chapters 3 and 5). The ultimate goal of the studies presented within this thesis is to establish a model of endogenous bladder regeneration in order to harness this ability for application to the tissue engineering field in urology.
References


CHAPTER 2

Regenerative Capabilities of the Urinary Bladder
REGENERATIVE MEDICINE AND BLADDER DISEASE

The aim of regenerative medicine is, ideally, to restore normal organ function either by replacing a non-functioning organ (end stage disease) or improving function when it is severely impaired. In the case of the bladder, this implies the capacity to store urine at increasing volumes (without increasing intravesical pressure or spontaneous bladder contractions) until complete emptying when socially acceptable. Diverse disease etiologies (e.g. neurogenic, congenital, trauma, infections, etc.) compromise the low-pressure, high-volume function (decreased compliance) of the bladder leading to a number of lower urinary tract symptoms such as urgency, urgency incontinence, frequency, and nocturia.

Antimuscarinic drugs (e.g. oxybutynin, solifenacin, darifenacin) are now the first-line therapy for treatment of detrusor overactivity and the overactive bladder syndrome. Lower urinary tract symptoms can also be treated with alpha-adrenoreceptor (AR) blockers (e.g. doxazosin) alone or in combination with antimuscarinics (Kaplan et al. 2006; Chapple et al. 2009). However, in cases of neurogenic bladder overactivity, where one of the main aims of treatment is to prevent damage to the upper urinary tract, bladder contractility can be reduced with these treatments necessitating the use of clean intermittent catheterization (CIC) for protection of the kidney. With such diverse etiologies for bladder dysfunction, and such a large demand (over 50 million people are estimated to have some type of urinary incontinence), many different classes of drugs have been investigated. These include, for example, beta-3 AR agonists (e.g. mirabegron), and
botulinum toxin-A, but all pharmacological interventions are well beyond the scope of this chapter, and have been discussed in detail elsewhere (Andersson et al. 2009).

In severe cases refractory to pharmacological treatment, high bladder pressures may develop and lead to upper urinary tract deterioration (ie. end stage renal disease, ESRD), particularly if intravesical pressure exceeds 40 cmH$_2$O. Patients that display poorly compliant bladders due to structural or neurogenic reasons are at risk for ESRD and are thus candidates for surgical intervention (Reyblat et al. 2008). Augmentation cystoplasty has been performed in bladder diseases arising from many different etiologies including spinal cord injury, myelomeningocele, interstitial cystitis, idiopathic detrusor overactivity, radiation cystitis, multiple sclerosis, and schistosomiasis.

The purpose of bladder augmentation is to maintain low intravesical pressures, while increasing bladder capacity. Attempts to increase bladder capacity can be traced back to the late 1800’s and throughout the 20$^{th}$ century with many different materials both natural (fascia, dura mater, intestinal segments) and synthetic (teflon, polyvinyl). By the middle of the 20$^{th}$ century, the use of intestinal (usually ileal) segments became commonplace, but was still associated with side effects such as urinary stones, pyelonephritis, metabolic imbalances, infections, and mucous production (Flood et al. 1995). This along with the lack of ability for donor bladder transplantation shaped the need for regenerative medicine/tissue engineering technologies for the bladder.

In one of the first successful neo-organ transplants, bladder constructs were created $in vitro$ by seeding synthetic scaffolds (collagen or collagen/polyglycolic acid
composites) with urothelial cells on the inside and smooth muscle cells on the outside. These constructs were subsequently implanted into patients with myelomeningocele and served as a neo-reservoir to increase bladder capacity (Atala et al. 2006). Despite the importance of this achievement, the implanted neobladders lacked normal innervation required for micturition thus limiting the functionality of the implanted construct. Moreover, this study was performed on otherwise healthy patients (young patients with spinal cord injuries) whereas older patients with compromised health (cancer, diabetes) generally have far less regenerative capacity. (Brooks et al. 1990; Hoenig et al. 2008; El-Ftesi et al. 2009) If this type of technology is to be optimized and applied to other bladder dysfunctions in older patients, an increased understanding of bladder regeneration per se is needed.

The bladder is one organ that has been at the leading edge of tissue engineering perhaps, in part, due to some extent of natural regenerative capacity (Liang 1962; Liang et al. 1963). Despite some controversy on the subject, there are numerous indications that even the human bladder can regenerate after removal of a large portion of the organ (subtotal cystectomy; STC) as outlined in Table 1. Sisk and Neu reported one of the first clinical experiences in 1939, describing a patient who voided through the urethra 8 weeks after STC leaving only a 3 by 3 cm patch of the posterior bladder wall (Sisk et al. 1939). Later studies reported mostly on bladder cancer, with one report claiming that only 6 months after removal of the entire bladder except for the ureterovesical junction and bladder neck, bladder capacity reached 400mL (Tucci et al. 1963).
<table>
<thead>
<tr>
<th>Reference</th>
<th>Major finding</th>
</tr>
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<tbody>
<tr>
<td>(Sisk et al. 1939)</td>
<td>58 year old male underwent extensive STC (leaving 3x3 cm of posterior bladder wall) and voided through his urethra 8 weeks later</td>
</tr>
<tr>
<td>(Folsom et al. 1940)</td>
<td>8 females with interstitial cystitis underwent STC, resulting in bladder capacities up to 600mL (one failure due to pyelonephritis)</td>
</tr>
<tr>
<td>(Richardson 1952)</td>
<td>66 year old male had removal of necrotic bladder tissue above trigone with normal cystogram/urination, and a 350mL capacity one year later</td>
</tr>
<tr>
<td>(Bohne et al. 1957)</td>
<td>7 patients with carcinoma underwent STC, and bladder regeneration did occur, however infections did prevent success in some</td>
</tr>
<tr>
<td>(Portilla Sanchez et al. 1958)</td>
<td>65 year old with bladder cancer underwent STC with a plastic mold and 3 months later a bladder with a transitional epithelium grew larger than the mold</td>
</tr>
<tr>
<td>(Baker et al. 1959)</td>
<td>70 patients with bladder cancer underwent STC, with most resulting in sufficient bladder regeneration (~20% incidence of asymptomatic ureteral reflux)</td>
</tr>
<tr>
<td>(Liang 1962)</td>
<td>11 patients underwent ~75% STC without molds suggests a mechanical stretch stimulus for bladder regeneration</td>
</tr>
<tr>
<td>(Tucci et al. 1963)</td>
<td>A 45 year old male underwent 80-90% STC for bladder cancer leaving only the ureterovesical junction and bladder neck, Normal urination and 400 mL bladder capacity was observed 6 months later</td>
</tr>
<tr>
<td>(Baker et al. 1965)</td>
<td>Several patients presenting recurring multiple transitional cell carcinomas underwent total mucosal excision. Complete epithelial regeneration occurred without the incidence of cancer</td>
</tr>
<tr>
<td>(McCallum 1965)</td>
<td>36 year old male had necrotic tissue (entire bladder except for part of the trigone) removed, and bladder capacity increased from ~45 mL to ~300 mL in 6 weeks, with normal bladder function</td>
</tr>
</tbody>
</table>

Table 1 - A summary of clinical experiences with de novo bladder regeneration.

The studies listed in Table 1 suggest that bladder regeneration can occur in humans without the use of molds or scaffolds. Of note is that the case studies described by these groups most often dealt with patients suffering from invasive bladder cancer, Hunner ulcer, or contracted bladder due to tuberculosis. Needless to say, this process has never been characterized in healthy, young adult humans, and information about the “normal” process of bladder regeneration is not available. Even though unsuccessful accounts of bladder regeneration also exist, citing complications such as infections, these studies certainly show that there are situations in which the bladder can regenerate in situ. It is not clear what situations
permit for successful bladder regeneration in the clinic, and attempts to reveal these circumstances are no longer pursued because of the widespread use of augmentation techniques. However, there is evidence that functional bladder regeneration occurs in both rats and humans and the use of these animal models allows researchers to control these circumstances sufficiently enough for bladder regeneration to occur. Indeed, Daniel Liang in the 1960’s attempted to leverage this idea, and reported similar findings of STC-induced bladder growth both in the human and the rat (Liang 1962; Liang et al. 1963). Despite this knowledge, most studies aiming to optimize urological tissue engineering strategies almost exclusively employ seeded or non-seeded scaffolds for bladder augmentation (Sutherland et al. 1996; Wefer et al. 2001; Drewa et al. 2006; Jayo et al. 2008). Below we briefly describe some techniques used with matrices in bladder regeneration, and reveal the paucity of information on bladder regeneration without the use of scaffolds.

**BLADDER REGENERATION USING CONSTRUCTS**

Many different scaffold and cell combinations have been used to try and restore bladder morphology and function in animal models. While each approach has advantages and disadvantages, they are difficult to compare because of differences in methodology (e.g., outcome measurements). Incorporation of a construct alone does open the door to control for the local administration of pharmacologically active agents within a scaffold to improve bladder regeneration. As a proof of concept study, Kanematsu et al (2003) analyzed the possibility of loading bladder acellular matrix (BAM) with a growth factor (bFGF) for use in an
augmentation model (Kanematsu et al. 2003). They demonstrated sustained release of bFGF from the scaffold both in vivo and in vitro. Moreover, 4 weeks after use for augmentation cystoplasty in rats, bFGF was able to promote angiogenesis and inhibit graft shrinkage in a dose-dependent manner. This study displays the possibility to “load” grafts used for augmentation with growth factors.

As an extension of this Youssif et al. (2005) demonstrated that delivery of VEGF in BAM scaffolds had positive effects early on in regeneration in terms of function and histology in rats. Specifically they showed that exogenous VEGF increased capacity and decreased residual volume 4 weeks after surgery compared to controls, along with enhanced angiogenesis seen at this timepoint, and more smooth muscle content at all timepoints studied. Moreover, there were increased nerve growth factor (NGF) positive cells up to 8 weeks post-surgery, suggesting a synergistic effect between these two growth factors.

This synergistic effect of VEGF and NGF is further supported by a study by Kikuno et al examining augmentation techniques in spinal cord injured rats (Kikuno et al. 2009). Eight weeks after initial spinal cord injury, female Sprague-Dawley rats underwent augmentation cystoplasty using BAM with no growth factor and with NGF and VEGF either alone or in combination. They found 8 weeks after their augmentation surgery, animals that received both growth factors displayed much higher bladder capacity and compliance, and increased smooth muscle and nerve content than in any other group. Taken together these studies indicate that both NGF and VEGF are extremely beneficial in restoring bladder function and architecture.
However, not all growth factors will prove to be indispensable, which is demonstrated in an article by DiSandro et al (DiSandro et al. 1997). They used bladders from normal and epidermal growth factor receptor (EGFR) knockout mice for bladder augmentation in rats. They found that there was no difference in these two groups histologically and both seemed to promote bladder regeneration after injury. However, the authors were quick to point out that while other pathways may atone for the loss of the EGFR function, this pathway may still play a synergistic role in bladder regeneration even though compensatory pathways exist.

Porcine models of augmentation cystoplasty have also been used, and Loai et al. (2010) has incorporated VEGF along with hyaluronic acid into bladder acellular matrix in pigs. They showed that using this glycosaminoglycan and growth factor in combination produced the best epithelialization, neovascularization and smooth muscle regeneration 10 weeks after surgery. It is reasonable to assume that delivering many different growth factors, small molecules or other compounds may aid in regeneration of the urinary bladder, and the incorporation of growth factors into grafts will help explore these possibilities.

In addition to growth factors, the immune response has been studied by Ashley et. al. (2009; 2010) who explored bladder reconstruction with small intestinal submucosa (SIS) grafts. They have found that while most inflammatory cells were present in similar levels in graft and native bladder regions, neutrophils were more abundant in graft sections. Moreover, their finding that regional differences in the intestinal segment that SIS grafts were taken from led to different inflammatory profiles indicates yet one more variable that may play a role in
whether approaches utilizing SIS are successful. This type of approach will be important to characterize the immune response to graft implantation. However this is undoubtedly different from the immune response to STC alone because of the graft itself.

The studies described above only employ the use of scaffolds without cells, however, incorporation of cells into grafts has been shown to greatly enhance bladder regeneration (Oberpenning et al. 1999). As such, there are multiple reports studying the effects of constructs seeded with different cells for bladder regeneration. Table 2 displays several of these studies, and while not meant to be exhaustive, does illustrate the wide variety of cells and graft materials that have been used for augmentation. Most often the control group in these studies is an acellular scaffold which is compared with a scaffold that has been seeded with some type of cells (although a couple of studies using STC alone as a control will be discussed later in this chapter). Because these studies vary on their construct material/ cells, methodologies and assessments, it is not reasonable to compare studies in order to determine which cell type is most promising for promoting bladder regeneration. To date, several studies have generally described both an anti-fibrotic effect, as well as immunomodulatory actions for these cells.
Table 2. Studies illustrating the diversity of cell seeded constructs used for bladder augmentation in animal models. BM-MSCs- Bone-Marrow derived Mesenchymal Stem Cells. SIS- Small Intestinal Submucosa, PGA- Polyglycolic Acid, BAM- Bladder Acellular Matrix, ADSCs- Adipose Derived Stem Cells, PLGA- Poly (lactic-co-glycolic) Acid, SMCs- Smooth Muscle Cells, UCs- Urothelial Cells, PLAC- collagen-poly(lactic acid-co-ε-caprolactone).

<table>
<thead>
<tr>
<th>Reference</th>
<th>Cells used</th>
<th>Scaffold Used</th>
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<tbody>
<tr>
<td>(Chung et al. 2005)</td>
<td>BM-MSCs</td>
<td>SIS</td>
</tr>
<tr>
<td>(Zhang et al. 2005)</td>
<td>BM-Stromal Cells</td>
<td>SIS</td>
</tr>
<tr>
<td>(Drewa et al. 2006)</td>
<td>3T3 Fibroblasts</td>
<td>SIS</td>
</tr>
<tr>
<td>(Drewa et al. 2009)</td>
<td>Hair follicle stem cells</td>
<td>BAM</td>
</tr>
<tr>
<td>(Jack et al. 2009)</td>
<td>ADSCs</td>
<td>PLGA</td>
</tr>
<tr>
<td>(Sharma et al. 2010)</td>
<td>BM-MSCs</td>
<td>poly(1,8-octanediol-co-citrate)</td>
</tr>
<tr>
<td>(Sharma et al. 2010)</td>
<td>SMCs, UCs</td>
<td>SIS</td>
</tr>
<tr>
<td>(Zhu et al. 2010)</td>
<td>ADSCs</td>
<td>BAM</td>
</tr>
<tr>
<td>(Engelhardt et al. 2011)</td>
<td>SMCs, UCs</td>
<td>PLAC</td>
</tr>
</tbody>
</table>

Studies described above have elucidated many growth factors or cells which may be of use in bladder tissue engineering. There has been an extremely large variation in what kind of cells, how many cells, and what kind of material is used. Direct comparison of studies is nearly impossible due to differences in methodology, and a more universal and accepted dogma/methodology would certainly benefit the urological tissue engineering field. Moreover, almost all of these studies have neglected to take into account that some observed regeneration may be due to the natural regenerative capabilities.

Indeed, many of the studies utilize bladder extracellular matrix (BAM), which has been shown to have growth factors preserved in it. BAM was first studied in 1975, and is derived from the lamina propria layer of the bladder (Meezan et al. 1975). This ECM supports growth and differentiation of different cell types, including the urothelium which can rapidly proliferate in response to injury, and involves de- and redifferentiation (Sutherland et al. 1996; Lai et al. 2005; Staack et
Such a diverse and quick response is undoubtedly orchestrated by a variety of growth factors contained within. BAM has been shown to include many different growth factors including VEGF, platelet derived growth factor (PDGF), keratinocyte growth factor (KGF), transforming growth factor β (TGF-β), epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF), among others (Badylak 2002). Additional studies have begun to more rigorously characterize the types and amounts of bioactive molecules. One study quantified these growth factors via ELISA assays, and showed there presence via western blotting, with PDGF, KGF, and VEGF most predominant (Chun et al. 2007).

Different decellularization methods of BAM can produce different, time-dependent results. Many different decellularization techniques have been used, leading to variability in removing cellular debris, which again, makes comparison between studies difficult (Farhat et al. 2008). Yang et al. (2010) compared several different decellularization protocols, using either detergent or distension techniques. They found that there was a positive correlation with the growth factor content and sulfated glycosaminoglycan content, suggesting that preservation of one may be indicative of the other. To date, BAM has been used for many applications such as reconstruction of esophageal, myocardial, skeletal muscle, and penile applications (Badylak et al. 2005; Badylak et al. 2006; Eberli et al. 2007; Moon du et al. 2008). As indicated by animal models of augmentation and the content of the bladder wall (even after decellularization), the properties of the urinary bladder allow it to repair itself following injury (Hodde 2002), although very few studies analyzing this phenomenon in detail exist.
DE NOVO REGENERATON OF THE BLADDER

A select few studies have used animal models to characterize this *de novo* bladder regeneration, with even fewer examining function. Until cystometric methodologies became available in the rat, most all animal studies were based on morphology. Saito et. al. (1996) performed trigone-sparing subtotal cystectomy in male Sprague-Dawley rats, and showed that bladder capacity increased from 13% of sham controls immediately after surgery to ~59% of control values 4 weeks after operation. They also demonstrated that the voiding pressure remained low at this time. While this provides some evidence that the bladder can regain capacity, it says nothing about the timecourse of regeneration, and whether or not normal capacities or pressures are obtained.

In another study, Piechota et al. (1999) used a 50% cystectomy model in male and female rats as a control group for augmentation with BAM scaffolds. 4 months after operation, they found no differences in baseline pressure or bladder opening pressure, but a higher peak pressure, and compliance was found in animals subjected to only cystectomy. Although capacity was lower in these animals (1.52 mL compared to 2.45mL), this study was designed to compare these animals with animals segmented with BAM, and did not mention unoperated controls for comparison. Additionally, the only timepoint examined in this study is four months post-operation, which does not reveal much about the timecourse of bladder regeneration. Similarly, Jack et al. (2009) used STC alone as a control group for cell seeded polyglycolic acid scaffolds. They found that this group regained their baseline bladder capacities by 12 weeks, and concluded that auto-regeneration of
the bladder demonstrates a long-term limitation of the augmentation model. These studies reveal the extent of in vivo functional data on the regenerating bladder, neither examining time-dependent differences in bladder regeneration, or complete examination of all aspects of bladder function (e.g., residual urine).

Function of the detrusor muscle in vitro has been studied in somewhat more detail. A study by Frederiksen et al (2001) demonstrated that active force was decreased 10 weeks after STC despite similar actin concentrations, and actin/myosin ratios to control animals. Another study by Frederiksen et al. (2004) set forth the most complete description of the pharmacology of regenerating bladder to date. Fifteen weeks after STC in female rats, transverse strips were excised from the bladder body and were exposed to contractile stimuli, taking into account the proximity of the strip to the trigone. Antagonists of muscarinic receptors (scopolamine) and α1 adrenoreceptors (AR) (prazosin), as well as a desensitizing agent of P2X1 receptors (α,β-methylene ATP) were used to examine the contribution of each receptor type to contractions evoked by electrical field stimulations (EFS). Additionally, agonists of muscarinic receptors (carbachol), α1 ARs (phenylephrine), and purinergic receptors (α,β-methylene ATP) were used on separate strips. Their findings showed that contractility in response to EFS was not affected by α1-AR blockade, and strips from just above the trigone contracted similarly (in terms of % maximal response) when subjected to scopolamine and α,β-methylene ATP. However in more distal (ie. equatorial) preparations, scopolamine produced a greater inhibition of contractility in control bladders than that from animals that had undergone STC. Moreover, a contractile response remained when
stimulated at 60 Hz, even in the presence of cholinergic, purinergic, and AR blockers, which was similar to that seen in supratrigonal strips. These authors concluded that although the newly formed bladder smooth muscle is well innervated, it has pharmacological properties similar to the supratrigonal tissue from which it had developed.

The idea that bladder tissue formed spontaneously after cystectomy is similar to tissue that remains after STC has been proposed again by this same group (Frederiksen et al. 2008). Whole mount staining of acetylcholinesterase was performed to visualize the pattern of nerves in newly formed detrusor. They found that newly formed tissue contained nerves on the anterior aspect of the bladder that were more slanted against the longitudinally running muscle bundles compared to controls. This pattern of nerves was more similar to what was seen in the trigonal region of the bladder (ie. the tissue that was still present after cystectomy) than what was seen in the body of control bladders.

It might be expected that the process of bladder regeneration may show species variation. For example Lin et al. performed very similar experiments in the rabbit after subtotal cystectomy (Lin et al. 1989). They used responses of the detrusor muscle to bethanechol, ATP, isoproterenol and epinephrine and divided the rabbit detrusor muscle into 5 categories. They showed that, in general, contractile responses to different stimuli were less in the body of bladder that had been subjected to STC. They also showed that the ratio of α:β AR responses were much greater in the cystectomized bladder.
So what can be the criteria for whether or not regeneration is considered a success? Organ regeneration has been broadly defined as the renewal of lost or damaged tissue such that tissue integrity is restored and maintained (Stocum 2010). One inference from this definition, and indeed an important theme in many discussions of organ regeneration is the necessity to regain and/or improve organ function (Lalan et al. 2001; Jenkins et al. 2003; Atala 2006; Gurtner et al. 2007). This implies that measuring success of regeneration for the bladder will include functional studies which, as discussed earlier, are currently lacking. However there is also a lack of data that explores mechanistic aspects of bladder regeneration. It has been suggested that there are three main mechanisms by which regeneration can take place: compensatory hyperplasia, injury-induced activation of stem/progenitor cells, and dedifferentiation of mature cells to form a blastema (Stocum 2010). It is not clear whether the increase in volume seen after STC is simply due to hypertrophy of existing smooth muscle cells, and no clear evidence has been given to support involvement of any of the mechanisms described above.

Additionally, regeneration has been described as a recapitulation of development. Maturation of urothelial and smooth muscle cells would be needed to consider regeneration a success, and this not been proven unequivocally. In addition to the bladder parenchyma, stromal components of the bladder must also be regenerated to demonstrate restoration of tissue morphology and function post-STC. Jayo et al. have discussed regeneration versus repair of the bladder, and described the need for cellular infiltration for blood vessel ingrowth and nerve regeneration, while avoiding the excessive collagen deposition and disordered
tissue commonly seen in simple repair of organs (Jayo et al. 2008). However, this was again completed in a canine model of bladder augmentation. While the study probing motor nerves display reinnervation after STC (Frederiksen et al. 2008), no study has examined angiogenesis in detail. Finally, increased collagen deposition can occur in bladders that still function as in the case with aged bladders and the effect of STC on bladder fibrosis has also not been examined (Zhao et al.). The studies described in this thesis aim to examine these processes in order to determine the extent of bladder regeneration in the rat (Table 1).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Method</th>
<th>Existing Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Function: High Volume</td>
<td><em>In vivo</em> Cystometric</td>
<td>350-400g male rats regained 59% Bcap, but still low MP 4 wks post-STC (Saito), 3 month old rats regained normal Bcap 4 months post-STC (Piechota)</td>
</tr>
<tr>
<td>Function: Low Pressure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Function: Emptying</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Function: Muscle</td>
<td><em>In vitro</em> pharmacology</td>
<td>200 gram female rats tested 15 weeks post-STC displayed normal contractile strength</td>
</tr>
<tr>
<td>contractility</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>IHC for PCNA</td>
<td>None</td>
</tr>
<tr>
<td>Urothelial Maturation</td>
<td>IHC for UP3</td>
<td>None</td>
</tr>
<tr>
<td>Smooth Muscle Maturation</td>
<td>IHC for Smooth Muscle Actin, Desmin, MHC</td>
<td>None</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>IHC for VWF</td>
<td>None</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>Trichrome Staining</td>
<td>None</td>
</tr>
</tbody>
</table>

Table 3. Quantifiable characteristics of bladder regeneration contained within.
REFERENCES


CHAPTER 3

Early stages of *in situ* bladder regeneration in a rodent model

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The following chapter has been published in Tissue Engineering Part A. August 2010; 16(8): 2541-51.

Literature citations and figure formats are adherent to the guidelines set forth in Tissue Engineering Part A.

David Burmeister is responsible for almost all data collection and analysis.
Abstract

Surgical removal of \( \approx 70\% \) of the bladder (subtotal cystectomy; STC) was used as a model system to gain insight into the normal regenerative process in adult mammals \textit{in vivo}. Female F344 rats underwent STC and at 2, 4 and 8 weeks post-STC, bladder regeneration was monitored via micro CT scans, urodynamic (bladder function studies) and pharmacologic studies, and immunohistochemistry. CT-imaging revealed a time-dependent increase in bladder size at 2, 4 and 8 wks post STC that positively correlated with restoration of bladder function. Bladders emptied completely at all time points studied. The maximal contractile response to pharmacological activation and electrical field stimulation (EFS) increased over time in isolated tissue strips from regenerating bladders, but was still lower at all time points compared to strips from age-matched control bladders. Immunostaining of the bladder wall of STC rats suggested a role for progenitor cells and cellular proliferation in the regenerative response. Immunostaining and the presence of EFS-induced contractile responses verified innervation of the regenerated bladder. These initial studies establish the utility of the present model system for studying \textit{de novo} tissue regeneration \textit{in vivo} and may provide guidance with respect to optimization of intrinsic regenerative capacity for clinical applications.
**Introduction**

In lower vertebrates such as the axolotl and the newt, regeneration of body parts including limbs, jaws, tail, skin, spinal cord, brain, apex of the heart, etc., are well documented. (1, 2) These amphibian kings of regeneration provide an excellent model for studying the true upper limit of regenerative capacity. However, adult mammals have a much more restricted repertoire for repair and functional replacement of damaged tissues/organs systems that is generally thought to be largely restricted to epidermis, muscle, bone and liver (1). In this regard, a much less well known example is the considerable regenerative capacity of the urinary bladder. The regenerative capacity of the bladder has been known for over a century, with the first report by Schwarz in 1891 describing a normal sized bladder that had grown after subtotal cystectomy (STC; removal of a majority of the bladder) in dogs (3). More recent studies in dogs have confirmed these initials observations (4, 5), and similar findings were made in rats, where STC without augmentation showed that bladder capacity returned to approximately half of the original size two weeks post surgery. (6, 7)

The capacity for the human bladder to regenerate has also been clearly demonstrated, starting with the work of Sisk and Neu in 1939, showing that organ growth ensued after removal of the entire bladder except 3 cm of the posterior bladder wall. (8) Studies by Bohne and Urwiller in the 1950’s and Liang in the 1960’s supported the conclusions by Sisk that bladder regeneration can occur in humans without the use of molds or scaffolds. (6, 7, 9) In this scenario, perhaps it is not surprising that after many years of preclinical work Atala and colleagues have
leveraged the regenerative capacity of the bladder and extended it to the clinic, utilizing a collagen/polyglycolic acid scaffold seeded with autologous cells implanted in patients with myelomeningocele. (10) Despite the unequivocal importance of this seminal clinical study, the implanted neobladders lacked the normal innervation required for micturition, and thus, provided a neo-reservoir, rather than a functional bladder.

In light of the aforementioned considerations, and given the less than optimal alternative methods currently available for bladder repair in humans (e.g., detubularized bowel segments), it is clear that an improved understanding of the naturally occurring regenerative process in the urinary bladder would point toward novel and more effective regenerative therapies for urologic conditions. To this end, we have begun detailed investigations of the morphological and functional characteristics of bladder regeneration in adult rats following STC. The information gained from these studies undoubtedly has important implications for urologic tissue engineering, but likely also has much broader clinical applications as well, as these investigations also provide another important step toward improved understanding of organ regeneration in adult mammals.
Materials and Methods

Animals. A total of 78 female Fisher F344 rats weighing 170-200g underwent subtotal cystectomy (STC; Figure 1), with 9 animals dying post-operatively from urine leakage into the peritoneum (11.5% mortality rate) after STC. Additionally one rat was removed from the study due to the formation of stones which was seen on CT scans and confirmed when opening the bladder.

All protocols were approved by the Animal Care and Use Committee, Wake Forest University.

Figure 1. Subtotal Cystectomy (STC) (A) Cartoon depiction of STC. After dissection of the bladder, the bladder body is excised (~60-70% of the total bladder) leaving the trigone and ureterovesical junction (asterisk) intact. (B) Photographs of STC, where the bladder is held in place with stay sutures before excision.

Trigone-sparing cystectomies. Animals were anesthetized with 2% isoflurane, and the abdominal wall was shaved. Povidone-iodine was used as an antiseptic to
decontaminate the surgical site. A low midline abdominal incision was made, the bladder was identified and the dome was delivered outside of the body. Two stay sutures were made on either side of the bladder, just above the ureteric orifices, using 6-0 polyglycolic acid. The dome portion of the bladder (60-70%) was removed leaving the trigone and ureters intact (trigone sparing cystectomy). The remaining portion of the bladder was then sutured in a continuous fashion using one of the original stay sutures. The abdominal wall and skin were closed in two layers using 3-0 vicryl sutures. Animals were followed for up to 8 weeks post-STC.

CT imaging. CT Scans of the animals were taken with a Siemens MicroCATII @ 70kV, 500μA (BIN Factor of 4, 200° rotation, 500 steps, 73 micron cuts), and the scans were centered on the bladder. Contrast medium (288mg/ml Iothalamate Meglumine, diluted 1:3) was applied via transurethral catheterization and injected until bladder was full. All images were reconstructed using COBRA EXXIM version 4.9.52, and converted to DICOM images with Amira version 3.1. Analysis was done after transfer of images to TeraRecon Aquarius Workstation. Briefly, the entire image except the bladder was removed using the erase function. A bladder template was optimized with the following parameters: Window Width (WW) of 1116, Window Level (WL) of 657, and 11% opacity. Finally, the bladder circumference from the anterior view was selected with the freehand lasso tool, and the volume measurement tool was used for quantification.

Bladder catheter implantation. Catheters were implanted as described previously. (11, 12) Briefly, rats were anesthetized with 2% isoflurane, and the bladder was dissected from ensuing adhesions caused by the previous surgery. The
dome was delivered outside of the body as described above, a small incision was made and a PE-50 intramedic polyethylene catheter (Becton-Dickinson, Sparks, Maryland) with cuff was inserted and anchored with a 5-0 purse string silk suture. The catheter was then tunneled subcutaneously and brought out through the nape of the animals and held in place with cloth tape anchored to the skin via a 3-0 Vicryl suture. The abdominal wall and skin were closed in 2 layers with 3-0 vicryl sutures, and the free end of the catheter was thermally sealed.

_Cystometric analysis._ All cystometric studies were performed 3 days after catheter implantation in conscious, freely moving rats as previously described. (11-13) Briefly, the bladder catheter was connected to a 2-way valve that is, in turn, connected to a pressure transducer and an infusion pump. The pressure transducer is connected to an ETH 400 (CD Sciences, Dover, New Hampshire) transducer amplifier and subsequently connected to a MacLab/8e (Analog Digital Instruments, Castle Hill, New South Wales, Australia) data acquisition board. The pressure transducers and acquisition board were calibrated in cmH\(_2\)O before each experiment. Room temperature saline was infused at a rate of 10mL/hour. Micturition volumes were measured with a silicone coated funnel leading into a collection tube which is connected to a force displacement transducer. All analyses were conducted after a stable voiding pattern of at least 20 minutes was established. The following cystometric parameters were investigated: basal pressure (BP, lowest pressure between voids), micturition pressure (MP, the maximum pressure seen during a micturition cycle), threshold pressure (TP, pressure at which voiding is initiated), intermicturition pressure (IMP, mean bladder pressure between voids),
bladder capacity (B_{cap}, residual volume after last micturition plus amount of saline infused), micturition volume (MV, volume of expelled urine), residual volume (RV, bladder capacity-micturition volume), and bladder compliance (B_{com}, defined as B_{cap}/TP-BP).

**In vitro studies.** After cystometric analysis, animals were sacrificed with CO_{2} inhalation and bilateral thoracotomy, and the bladders were harvested and immediately placed in ice-cold Krebs buffer. The bladders were cut into strips along the longitudinal axis. The strips were denuded of the urothelium, and were attached to tissue holders at one end, and force transducers at the other in an organ bath system (Danish Myo Technology, Aarhus, Denmark) containing 15 ml of Krebs buffer aerated with 95%O_{2}/5% CO_{2} at 37\degree C. Bladder strips were subjected to a resting tension of 2g and allowed to stabilize for at least 60 min. They were then primed using 5 \mu M carbachol and subsequently 60\mu M KCl. Contractions were recorded as changes in tension from baseline in response to both carbachol and electrical field stimulation. Carbachol dose-response curves were generated by adding increasing concentrations of carbachol at \frac{1}{2} log increments starting at 3nM up to 100\mu M. For electrical field stimulation, strips were placed between two platinum electrodes in the organ chamber, and electrical pulses (0.1ms pulse width, 20V in the bath) were delivered, lasting 30 seconds at increasing frequencies (1,2,4,8,16 and 32 Hz) using a S88 stimulator (Grass Instruments, W.Warwick, RI). All tissue responses were normalized to grams of tissue weight.

**Histological analysis.** Bladders not subjected to pharmacological analysis were preserved for histology. After removal of the most superior part of the dome
(~.2 mm) bladders were fixed in 10% Buffered Formalin and processed. Serial 7-
micron cross-sections were sliced along the upper part of the bladder wall (above the uretero-vesical junction). At least 6 sections were examined from the proximal and distal ends of the bladder. Slides were cleared in xylene and rehydrated to water where they were ready for staining. Standard hematoxylin and eosin (H&E), and Gomori's one step trichrome stain (Newcomer Supply Catalog #9176A) were performed. For measurement of bladder wall thickness, at least 5 measurements were taken from 3 different sections of the bladder.

Immunohistochemistry was performed as follows: endogenous peroxidase activity was blocked with Dual Endogenous Enzyme Block (Dako, Ref# S2003) for 10 minutes at room temperature, followed by 10 minute incubation with Serum-Free Protein Block (Dako, Ref# X0909). Then samples were incubated with the following primary antibody dilutions/incubation times: c-kit (Santa Cruz, sc-168, rabbit polyclonal, 1:20 dilution) for 60 minutes, p63 (Lab Vision, #MS-1081-P, mouse monoclonal, 1:200 dilution) for 30 minutes, PGP9.5 (abcam, ab8189, mouse monoclonal, 1:20 dilution) for 60 minutes, UP3(Lifespan biosciences, LS-C40107, mouse monoclonal, 1:10 dilution) for 60 minutes. A heat-mediated antigen retrieval step was also used for PGP9.5 staining. Following primary antibody incubation, slides were treated with 30 minutes each of Biotinylated Universal Link and Streptavidin-HRP (Dako, Ref# K0690). Finally, staining was completed with 5-10 minute incubation with ImmPACT DAB (Diaminobenzidine) from Vector Laboratories (Cat# SK-4105). Immunohistochemistry probing proliferating cell nuclear antigen (PCNA) was performed similarly, without blocking endogenous
peroxidase activity and without using DAB as a chromogen. The primary antibody used was against PCNA (abcam, ab29, mouse monoclonal, 1:3000 dilution) and the secondary antibody was conjugated with Texas Red from Vector Laboratories (Cat# TI-2000).

Statistical Analysis. Statistical evaluations were performed using GraphPad Prism software. (GraphPad software Inc.) For pharmacological analyses, individual response curves were placed in GraphPad software, and a mean curve was fit to the family of curves. The generated values were analyzed via 1-way ANOVAs. 1-way ANOVAs were also performed on cystometric evaluations and CT calculations. P values less that 0.05 were considered significant. All results are expressed as the mean ± SEM.
Results

Rats that successfully recovered from surgery did not show any difference in weight gain compared to non cystectomized animals (data not shown).

CT Imaging. CT imaging of the bladder after STC revealed a progressive increase in bladder size. Figure 2 depicts subsequent images taken in 2 representative animals, while average values for bladder volume 1, 2, 4, 6, and 8 weeks post-STC as determined by TeraRecon analysis are graphed in Figure 2a. The morphology of the regenerated bladder was noted to change after regeneration, becoming more spherical in shape 8 weeks post-STC when compared to the original more pear shaped bladder.

<table>
<thead>
<tr>
<th></th>
<th>Rat 1</th>
<th>Rat 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (pre-STC)</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
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<tr>
<td>1 week post-STC</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>4 weeks post-STC</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>8 weeks post-STC</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure 2. Representative CT Images of the rat urinary bladder in 2 individual animals. Images are shown at 1 week, 4 weeks, and 8 weeks after subtotal cystectomy. Scale bar is 1cm. All images are taken with a Siemens MicroCAT II, with reconstruction on TeraRecon software.
**Cystometric Analysis.** All cystometric parameters from cystectomized and age-matched control animals are shown in Table 1. Age-matched controls were treated as one group since no age-dependent differences were noted in any of the cystometric parameters among the three time points (2, 4, and 8 weeks post-STC). Consistent with CT image analysis, cystometric studies also illustrated the progressive increase in bladder size (0.46± 0.02 mL, 0.72 ± 0.05 mL, and 0.82 ± 0.09 mL at 2, 4, and 8 weeks post STC, respectively). Moreover, bladder capacity at the 8 week time point was not statistically different from controls (0.96 ± 0.05 mL).

<table>
<thead>
<tr>
<th></th>
<th>Bcap (mL)</th>
<th>MV (mL)</th>
<th>RV (mL)</th>
<th>BP (cmH2O)</th>
<th>TP (cmH2O)</th>
<th>MP (cmH2O)</th>
<th>TP/MP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls n=16</td>
<td>0.96±0.05</td>
<td>0.90 ±0.05</td>
<td>0.05±0.01</td>
<td>12.92±0.89</td>
<td>28.14±1.71</td>
<td>49.24±3.72</td>
<td>0.59±0.13</td>
</tr>
<tr>
<td>2 weeks n=10</td>
<td>0.46±0.03</td>
<td>0.42±0.03</td>
<td>0.05±0.02</td>
<td>11.17±2.48</td>
<td>22.36±3.66</td>
<td>30.5±5.87 *</td>
<td>0.75±0.07 *</td>
</tr>
<tr>
<td>4 weeks n=9</td>
<td>0.72±0.05</td>
<td>0.67±0.07</td>
<td>0.06±0.03</td>
<td>14.01±2.64</td>
<td>27.08±3.28</td>
<td>38.15±4.95 *</td>
<td>0.72±0.11 *</td>
</tr>
<tr>
<td>8 weeks n=8</td>
<td>0.85±0.08</td>
<td>0.76±0.06</td>
<td>0.09±0.04</td>
<td>10.83±1.18</td>
<td>25.92±2.6</td>
<td>37.05±3.36</td>
<td>0.7±0.07 *</td>
</tr>
</tbody>
</table>

Table 1. Urodynamic parameters as determined by in vivo cystometry. Age-matched controls revealed no differences and were subsequently grouped together as controls. * Indicates significance as compared to controls. º Indicates significance as compared to 8 weeks animals. # Indicates significance as compared to 4 week animals (P<0.05) Bcap=Bladder capacity, MV=Micturition Volume, RV= Residual Volume, BP=Basal Pressure, TP=Threshold Pressure, MP=Micturition Pressure.

Micturition pressures generated in animals 2 weeks post-STC were reduced (30.5 ± 5.87 cmH2O) compared to controls (49.24 ± 3.72 cmH2O). However, micturition pressures were not significantly different from controls at 4 weeks (38.15 ± 4.95 cmH2O) and 8 weeks (37.31 ± 3.33 cmH2O) post-STC. Importantly, at all time points the bladder was still able to empty completely as evidenced by the similar residual volumes between age-matched control and post-STC animals. (Table 1) There were no significant differences in either the threshold or basal
pressure. A calculated parameter (TP/MP) which reflects the sensory enervation in comparison to the smooth muscle force generation is important in determining the degree of total regeneration of the bladder. However, this ratio was significantly higher than controls at all time points after STC which reflects a faster neurogenic regeneration as compared to a myogenic one.

**CT and cystometric regression.** One of the goals of this initial investigation was to determine if non-invasive imaging could provide guidance regarding bladder regeneration and function. Figure 3a demonstrates that the rate of bladder volume increase is very comparable to that determined by urodynamic studies, which is evidenced by comparable linear regression slopes. Although the actual values of bladder volume may be lower than that obtained via functional methods, the progress of bladder regeneration can be illustrated, and the technique consistent. Moreover, as illustrated in Figure 3b, there was a statistically significant positive correlation observed between micturition pressure determined by cystometric techniques and the bladder circumference determined by CT analysis (r=0.634, P<0.05). These data are consistent with the supposition that the observed increase in bladder size correlates well with the observed increase in bladder function.

**In vitro Studies.** Steady-state concentration response curves were obtained for carbachol-induced contractile responses in bladder strips from both age-matched control and post-STC animals. Mean values for logistic parameter estimates are shown in Table 2, and the data are graphically depicted in Figure 4.
Figure 3. Linear regression involving cystometric and imaging parameters. The top panel shows bladder volume averages for animals studied via cystometry (triangles) and CT imaging (squares). The bottom panel plots micturition pressure determined by cystometry and anterior bladder circumference determined by Tera Recon CT image analysis. Only animals from the 4 and 8 week timepoints, with CT and cystometry data were used (n=9) A positive correlation was found between the two parameters (r=.634, P<0.05).

In short, logistic analysis revealed a significant reduction in the calculated Emax values in isolated detrusor strips from the regenerating bladder at all time points (Figure 4).
However, evidence for functional recovery over time was noted as, for example, the Emax at 8 weeks post-STC was significantly greater than that observed at both 2 and 4 weeks post-STC. As illustrated in Figure 4 and summarized in Table 2, there were no detectable differences in either pEC$_{50}$ or Hill Slope parameters among the groups.

**Carbachol Response Curve**

![Carbachol Response Curve](image)

**Figure 4.** Carbachol dose response curves from both control animals, and 2, 4, and 8 weeks post-STC. Responses have been normalized to strip weight. Total area under the curve values were 312.8 for controls, 54.65 at 2 weeks, 61.86 at 4 weeks, and 119.7 at 8 weeks post-STC. *Emax values for all STC animals are significantly lower than control tissue. P<0.001. Emax values at 8 weeks post-STC are significantly higher than 2, and 4 week timepoints. P<0.05

<table>
<thead>
<tr>
<th></th>
<th>Emax (g/g tissue)</th>
<th>Log[EC50] (log[carbachol])</th>
<th>Hill Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control n=30 strips, 20 animals</td>
<td>183.2 ± 5.97</td>
<td>-5.85 ± 0.25</td>
<td>1.34 ± 0.30</td>
</tr>
<tr>
<td>2 weeks n=14 strips, 10 animals</td>
<td>30.91 ± 4.49 *</td>
<td>-5.61 ± 0.25</td>
<td>0.91 ± 0.44</td>
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<tr>
<td>4 weeks n=15 strips, 9 animals</td>
<td>35.94 ± 3.37 *</td>
<td>-5.62 ± 0.15</td>
<td>1.33 ± 0.15</td>
</tr>
<tr>
<td>8 weeks n=23 strips, 12 animals</td>
<td>54.03 ± 5.36 @@</td>
<td>-6.05 ± 0.21</td>
<td>1.01 ± 0.46</td>
</tr>
</tbody>
</table>

**Table 2.** Parameters attained from carbachol dose-response curves. *Emax at all time points is significantly lower than control (P<0.001). @@ - Emax values at the 8 week time point is significantly higher than those found at 2, and 4 weeks post STC (P<0.05). There were no differences between groups for either the EC50 or the Hill Slope values.
The maximum contractions of regenerating detrusor smooth muscle strips induced by electrical field stimulation (EFS) was also significantly lower than native tissue at all frequencies tested, and the ratio of native responses to experimental responses was similar across all frequencies (Figure 5). Moreover, there was again functional recovery in that responses generated in bladders 8 weeks post-STC were greater than those 2 weeks post-STC.

![EFS Graph](image)

**Figure 5.** Tissue responses to electrical field stimulation for bladders 2, 4, and 8 weeks post-STC. Responses have been normalized to strip weight and are shown as percent control. Values in parentheses are normalized responses +/-SEM for control tissue at each frequency (n=16 strips, 13 animals). *-All groups different from each other as determined by 1-way ANOVA, P<0.001.

*Histological analysis.* The wall of regenerating bladders apparently retains the architecture of the native bladder. Consistent with this supposition, Figure 6 shows representative examples of H&E staining of both native and 8 week regenerated bladder samples from the same animal. As illustrated, the urothelial,
sub-urothelial, and smooth muscle layers are evident in the regenerating bladders. Similar staining of these layers was also seen with trichrome staining (data not shown). Importantly, bladder wall thickness at the 8 week time point (581.7 ± 19.11µm) was not significantly different from control bladders (558.7 ± 8.68 µm) as shown in figure 6B.

In order to gain some preliminary mechanistic insight into the mechanistic basis for bladder regeneration in this animal model, we compared fluorescent immunostaining with Proliferating Cell Nuclear Antigen (PCNA) in post-STC and age-matched control bladder strips. As shown in Figure 7, there was an increased number of PCNA positive proliferating cells in regenerating bladders 1 week, 2 weeks, and 4 weeks, post-STC.

Figure 6. Histological comparison between native and regenerated bladders 8 weeks post-STC using H&E staining. (a) Average bladder wall thickness of native and 8-week regenerated tissue. Values are mean ± SEM of 10 measurements in 2 different sections of native tissue and 8-week regenerated tissue from 4 animals. Representative images of native tissue (b) and 8-week regenerated tissue (c) from the same animal are shown at 10x magnification.
Figure 7. Fluorescent immunostaining to PCNA shows proliferating cells in the regenerating bladder 2 weeks (b), 4 weeks (c), and 8 weeks (d) post-STC, in greater abundance compared to control tissue (a). PCNA staining is shown with Texas Red, while nuclei are stained with DAPI (blue). All images are at 40x magnification.

In another attempt to begin to characterize the proliferating cell population, immunohistochemical staining was conducted for the presence of CD117 (c-kit) positive cells in the detrusor muscle at all time points after surgery. As shown in Figure 8, c-kit positive staining was much more prominent in regenerating bladder strips at all time points post-STC.
Figure 8. Positive immunostaining for CD117 (c-kit) positive cells in the smooth muscle during bladder regeneration. Staining is demonstrable in the detrusor 2 weeks (b), 4 weeks (c), and 8 weeks (d) post-STC, but not prevalent in control tissue (a). All images on the top row are 200x magnification with scale bars of 50 microns, images in the middle row are 630x magnification with scale bars of 50 microns, and the bottom two images are 1000x magnification with scale bars of 10 microns.

Additionally, in an attempt to evaluate the extent of innervation, immunohistochemistry against protein gene product 9.5 was conducted. As illustrated, the presence of nerves was clearly demonstrable 4-weeks post-STC in the form of both nerve ganglia and bundles (Figure 9). Finally, in order to evaluate the maturity of the bladder urothelial lining, staining was conducted using antibodies to uroplakin III. Once again, as illustrated, these studies revealed a mature urothelium as early as 1 week post-STC. (Figure 9)
Figure 9. Innervation of regenerated bladders shown using immunohistochemistry to PGP9.5 reveals nerves at 2 weeks post-STC (a), 4 weeks post-STC in the form of both nerve ganglia (b) and nerve bundles (c), and 8 weeks post-STC (d). Uroplakin 3 staining reveals a mature urothelium as early as 1 week post-STC (f), maintained 4 weeks post-STC (g), which closely resembles control tissue (e). All images are taken at 40x magnification.
Discussion

Chronic bladder disease can be the result of many pathological conditions (ie. infections, congenital disorders, and inflammation), ultimately reducing bladder function, and adversely affecting the quality of life. As noted in the Introduction, currently available treatment options have both limited efficacy and untoward side effects. (14-19) To circumvent the side effects associated with these treatments, matrix grafts have recently been used. Animal studies with both bladder acellular matrix grafts (BAMG) and small intestinal submucosa (SIS) have shown that urothelium, smooth muscle, nerves, and vasculature have the ability to infiltrate the graft. Atala and colleagues have even extended this to the clinic, utilizing a collagen/polyglycolic acid scaffold seeded with autologous cells implanted in patients with myelomeningocele.(10) Despite the importance of this seminal clinical study, the implanted neobladders lacked normal innervation required for micturition thus limiting the functionality of the implanted construct. Moreover, this study was performed on otherwise healthy patients (young patients with spinal cord injuries) whereas older patients or those with compromised health (cancer, diabetes) generally have a far less regenerative capacity.(20-22) Because of the less than optimal methods currently provided for bladder repair, an improved understanding of the naturally occurring regenerative process would clearly help extend the applicability of this technology.

In this regard, Frederiksen et al. showed that after STC the newly formed detrusor had pharmacological properties similar to the supratrigonal region from which it had developed.(23) A later study by this group demonstrated that the
nerves of the well-innervated regenerated bladder had originated from pre-existing nerves in the detrusor. (24) While these studies do provide some insight into the regeneration of the bladder following STC, the process is still not fully understood. To this end, we report herein our use of a rodent model to examine the early stages of *in situ* bladder regeneration that occurs following STC. The rationale for the use of the rodent model was two-fold: 1) the overall similarity between the lower urinary tracts in rats and humans, and 2) the consistent evidence that bladder regeneration occurs in both.

Consistent with previous studies (25, 26), our current urodynamic investigations confirmed a rapid increase in size of the bladder following STC. Of importance is that, over the time frame of these investigations, the bladder capacity of the regenerating bladder did not exceed the value observed for that same animal prior to STC. The mean values for micturition pressure post-STC were decreased at 2 weeks (≈40%), but similar at 4 and 8 weeks (≈80% of control values in both cases) post-STC (Table 1). To our knowledge, this is the first demonstration of *in vivo* pressure recovery following STC alone. Moreover, this functional recovery in micturition pressure is positively correlated with the increased bladder size detected using non-invasive micro CT imaging techniques (Figs 2, 3). In the future, this latter observation may be of particular importance for monitoring the success of the regenerative process in patients. Interestingly, even in the presence of diminished micturition pressure, the bladder was still able to empty completely (residual volume was the same at all time points). The precise reason for this
observation is not clear, but may be related to, for example, a reduction in urethral outlet resistance. Certainly, this will be the subject of future investigation.

Of note, these studies also documented a greatly reduced contractile response to carbachol (a muscarinic receptor agonist), at all time points examined; albeit with a gradual improvement at 8 weeks post-STC (Table 2, Figure 4). The observed decline in muscarinic contractility is consistent with an earlier study by Saito, et al.(26) An even more marked reduction in detrusor contractility was observed in response to electrical field stimulation (80-90%; Figure 5) As such, the decline in detrusor contractility is clearly much more pronounced than the initial decline in micturition pressure. The reason(s) for this is (are) unclear. Qualitatively, we did not see a difference in smooth muscle bundles (Figure 6), and others have reported no change in smooth muscle actin content or the myosin/actin ratio.(27) Frederiksen et al. (2004) demonstrated that 15 weeks post-STC there was no difference in the contractile response to carbachol between controls and STC animals, and that a small contractile response remained after simultaneous adrenergic, muscarinic, and purinergic receptor blockade.(23) This led to the conclusion that the newly formed detrusor had pharmacological properties similar to the region from which it had developed (supratrigonal rim). It is important to keep in mind that Frederiksen et al. (2004) used supratrigonal and equatorial strips, while we have chosen longitudinally cut preparations. Thus our results involving reduced carbachol contraction following STC could be explained by differing receptor populations, specifically, those more closely related to the trigone and urethra (ie. α-adrenergic).
As illustrated in Figure 6, the regenerating bladder had all layers characteristic of the native bladder. Specifically, a serosal layer was present, along with a mature urothelium, and detrusor smooth muscle bundles. Importantly, proliferating cells were present in all layers of the bladder after STC, which indicates an important role of hyperplasia/cellular proliferation in the regenerative process (Figure 7). Moreover, the abundance of proliferating cells was present in sections taken towards the distal end (dome) of the regenerating bladder, especially in terms of the urothelium. This logical implication is that the regenerative process involves an outgrowth from the distal end of the bladder (where the STC occurred).

Associated with these regenerating smooth muscle bundles is a population of unidentified progenitor cells expressing CD117 (c-kit; Figure 8). This observation is consistent with the supposition that stem/progenitor cells are involved in the proliferation of the detrusor smooth muscle in response to STC. In this regard, c-kit is a tyrosine kinase receptor whose endogenous ligand is stem cell factor (scf).(28) This receptor/ligand combination has been shown to attenuate inflammation, protect from liver damage, and even induce hepatocyte proliferation after 70% hepatectomy.(29, 30) Although most often associated with hematopoietic stem cells, there is evidence that several cell types can respond to and release scf in response to injury.(31) To exactly identify these cells, and where they come from, is a high priority for future studies. In addition, the population of progenitor cells involved in urothelial turnover is also of interest, and several studies have explored the possibility of identifying urothelial stem cells.(32, 33) We found uroplakin 3 staining as early as 1 week post-STC indicating that the barrier function of the urothelium is
maintained during regeneration (Figure 9). That the superficial cells of the urothelium are terminally differentiated at the earliest time point we studied is, although an important finding, not surprising. Normally urothelial turnover is very low, but in response to injury, urothelial cells are known to rapidly proliferate. (34)

The presence of nerves in the newly formed bladder after STC was demonstrated functionally by the response to electrical stimulation of nerves (Figure 5), and morphologically with PGP9.5 staining (Figure 9). Evidence exists for the presence of intramural ganglia in the female rat bladder, similar to what we observed in these studies.(35, 36) The innervation seen post-STC is in agreement with previous studies by Frederiksen et al. (23, 24) in rats describing innervation arising from elongation and branching of existing nerves in the trigone. Taking this into consideration, it is reasonable to assume that nerve growth occurs in parallel to detrusor growth, and not that one precedes the other. What affect this nerve growth has on the development of other aspects of regeneration (ie. angiogenesis) and whether all types of nerves have equal regenerative properties is also of interest, and will be considered in future investigations.

To summarize, in this pilot study, we have described the early functional changes of an in vivo regenerating bladder without the aid of scaffold or cells, and suggested the potential utility of non-invasively monitoring bladder regrowth using micro CT measurements. Further studies are needed to evaluate the functional durability of the observed regenerative response, that is, what happens after 8 weeks post-STC.
Acknowledgements

This work was supported by NIH USPHS (grant no.R21DK081832).

The authors thank Catherine Ward for photographic assistance during surgeries.

Disclosure Statement

No competing financial interests exist.
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CHAPTER 4

Time-dependent functional and morphological changes in the urinary bladder after subtotal cystectomy

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The following chapter was prepared for submission to Aging Cell. The format adheres to WFUSM graduate school requirements.

David Burmeister is responsible for almost all data collection and analysis.
Abstract

Trigone-sparing cystectomy (surgical removal of \( \approx 70\% \) of the bladder, subtotal cystectomy; STC) in 12 week old female rats has previously been shown to induce a robust regenerative response such that normal function is restored within 8 weeks. To evaluate if the age at cystectomy influences bladder regeneration, 12 week and 12 month old female F344 rats underwent STC and were evaluated 1, 2, 4, 8, and 26 weeks post-STC. Bladder regeneration was assessed functionally using urodynamic studies \textit{in vivo} and pharmacologic studies \textit{in vitro}, and complemented with semi-quantitative immunohistochemical studies. Although bladders emptied completely after STC, 12 month old (older) animals displayed abnormally low bladder capacity, and evidence of chronic kidney damage. The maximal contractile response to muscarinic activation was also lower in older animals 26 weeks after STC when compared with 12 week old (younger) rats. However, both these responses and those evoked by adrenergic and purinergic stimuli, showed no differences when normalized to KCl-induced contractions. Immunostaining of the bladder wall of STC rats suggested a delayed proliferation response in older animals, which was mostly absent in younger animals 8 weeks post-STC. Although additional histological studies revealed more blood vessels in younger animals early after STC, ultimately no differences in vascularization or fibrosis were seen in the older animals. The present results suggest that the regenerative capacity of the bladder declines with moderate aging, and that several different mechanisms may be responsible.
Introduction

Animal experiments suggest that the bladder possesses some degree of regenerative potential. Despite some conflicting reports, there are numerous indications that even the human bladder can regenerate after removal of a large portion of the organ (subtotal cystectomy; STC). (Sisk et al. 1939; Liang 1962; Liang et al. 1963). With this in mind, Atala et al. (2006) has engineered autologous neo-bladders and subsequently implanted them into patients with myelomeningocele.

Although the regenerative powers of the bladder have been discussed for decades, the overwhelming majority of animal studies employ the use of scaffolds with or without cells for augmentation, and few studies have examined regenerated bladders after STC alone. Frederiksen et al. (2004; 2008) have examined detailed pharmacological and morphological changes after STC in female rats. Specifically they have shown that newly formed detrusor smooth muscle was similar to the trigone from which it had developed in terms of pharmacological properties, and the pattern of nerves. In terms of function, Saito et al. (1996) found that bladder capacity returned to ~59% of control values 4 weeks after STC in male rats. Additionally, Piechota et al. (1998) demonstrated normal bladder capacities 4 months post-STC performed in 3 month old female rats. We have recently reported on regeneration of the bladder following trigone-sparing cystectomy (STC) in 12 week old female Fisher F344 rats (Burmeister et al. 2010). By 8 weeks post-STC the bladder had regrown to a normal size as determined by both CT imaging and in vivo cystometric analysis, with some recovery in contractile strength of detrusor smooth
muscle *in vitro*. Morphologically, the bladder displayed urothelial, lamina propria, and detrusor muscle layers, and regained normal thickness upon histological evaluation. However, if tissue engineering strategies in urology are to be optimized and applied to bladder dysfunctions in older patients, an increased understanding of natural *de novo* bladder regeneration using animal models is warranted.

The goal of the present study was to distinguish what functional and morphological changes occur when the regenerating bladder ages over time (i.e., the interaction of age and regenerative capacity), with the hypothesis that even moderately-aged animals will display some deficiencies in the regenerative potential of the bladder. We found that several aspects of bladder regeneration are time-dependent, and that the relative age at the time of STC can substantially influence urinary tract function and morphology.
Materials and Methods

Animals

A total of 41-twelve week old (170-200g), and 117-twelve month old (250-300g) female Fisher F344 rats were used in this study. Thirty six younger animals underwent STC and bladders were excised 1,2,4,8, and 26 weeks post-STC for histology, with the remaining animals assigned to urodynamic or pharmacologic studies. Ninety older animals underwent STC a subset of bladders were used for histology 1,2,4,8, and 26 weeks post-STC, with urodynamics and pharmacologic experiments performed at these same timepoints except 1 week. All protocols were approved by the Animal Care and Use Committee, Wake Forest University.

Surgical Procedures

Trigone-sparing cystectomies

Animals were anesthetized with isoflurane, and the abdominal wall was shaved. Povidone-iodine was used as an antiseptic to decontaminate the surgical site. A low midline abdominal incision was made, and the bladder dome was dissected down to the level of ureteral insertion. Two stay sutures were made on either side of the bladder, just above the ureteric orifices, using 6-0 polyglycolic acid. The dome portion of the bladder (60-70%) was excised leaving the trigone and ureterovesical junctions intact (trigone-sparing cystectomy). The remaining portion of the bladder was then sutured in a continuous fashion using one of the original stay sutures. The abdominal wall and skin were closed in two layers using 3-0 vicryl sutures. Animals
were allowed to recover and given food and water *ad libitum* for up to 6 months after surgery.

*Bladder catheter implantation*

Rats were again anesthetized with 2% isoflurane, and through a midline incision, the bladder was dissected from ensuing adhesions caused by the previous surgery. The bladder dome was delivered outside of the body as described above, and a small incision was made. A PE-50 intramedic polyethylene catheter (Becton-Dickinson, Sparks, Maryland) with cuff was inserted and anchored with a 5-0 purse string silk suture. The catheter was then tunneled subcutaneously and brought out through the nape of the animals and held in place with cloth tape anchored to the skin via a 3-0 Vicryl suture. The abdominal wall and skin were closed in 2 layers with 3-0 vicryl sutures, and the free end of the catheter was thermally sealed.

*Cystometric analysis*

All cystometric studies were performed 3 days after catheter implantation in conscious, freely-moving rats. Briefly, the previously implanted bladder catheter was connected to a 3-way valve that is, in turn, connected to a pressure transducer and an infusion pump. The pressure transducer is connected to an ETH 400 (CD Sciences, Dover, New Hampshire) transducer amplifier and consequently connected to a MacLab/8e (Analog Digital Instruments, Castle Hill, New South Wales, Australia) data acquisition board. The pressure transducers and acquisition board were calibrated in cmH$_2$O before each experiment. Room temperature saline was infused at a rate of 10mL/hour. Micturition volumes were measured with a silicone-
coated funnel leading into a collection tube which is connected to a force displacement transducer. Analysis begun after a consistent voiding pattern was established. The following cystometric parameters were investigated: basal pressure (BP, lowest pressure between voids), maximum pressure (MP, the highest bladder pressure during micturition), threshold pressure (TP, pressure which initiates a voiding contraction), intermicturition pressure (IMP, mean bladder pressure between voids), bladder capacity (B\text{cap}, residual volume after last micturition plus amount of saline infused), micturition volume (MV, amount of expelled urine), residual volume (RV, bladder capacity-micturition volume), and bladder compliance (B_{com}=\frac{B\text{cap}}{(TP-BP)}).

Renal function

Animals were placed in individual cages on an 18 cage metabolic rack (Allentown, Inc) for separation of urine from feces and food debris. Urine collection began at the start of their diurnal dark cycle, and continued for 20 hours. Before and after urine collection, weights were recorded for all animals, water bottles, and urine tubes. After collection, urine was centrifuged at 3000rpm for 15 minutes to remove any particulate contamination, and the supernatant was separated into 1 mL aliquots and immediately stored at -80°C. Upon euthanasia, at least 2mL of blood was collected from the heart and placed in a serum-separating vacutainer (BD Biosciences, Cat # 367981). Tubes were spun at 2000g, at 4°C, for 15 minutes to separate serum. Supernatant (serum) was removed and placed into 250μL aliquots,
and immediately stored at -80°C. Creatinine (CR) and Blood Urea Nitrogen (BUN) analysis were performed with a Beckman Coulter Synchron CX5 chemistry analyzer (GMI, Ramsey, MN), with output units of serum and urine creatinine as mg/dL. Creatinine clearance was calculated as: 
\[ CC = \left( \frac{[CR]_{\text{urine}} \times \text{Urine volume}}{[CR]_{\text{serum}} \times \text{time}} \right), \]
and normalized per gram body weight.

**Pharmacological studies**

Animals were sacrificed with CO₂ inhalation and bilateral thoracotomy, and the bladders were harvested and immediately placed in ice-cold Krebs buffer. The bladders were cut into approximately equally sized strips along the longitudinal axis. The strips were denuded of the urothelium and suburothelium, and were attached to tissue holders at one end, and force transducers at the other in an organ bath system (Danish Myo Technology, Aarhus, Denmark) containing 15 ml of Krebs buffer aerated with 95%O₂/5% CO₂ at 37°C. Bladder strips were subjected to a resting tension between 1-1.5g and allowed to stabilize for at least 60 min. Strips were then primed with 60mM KCl-induced contractions (recorded as changes in tension from baseline) and repeated until subsequent contractions were consistent. Carbachol (Cch) response curves were generated by stimulating muscarinic receptor with increasing concentrations of Cch at ½ log increments starting at 3nM up to 100μM. Phenylephrine (PE) response curves were generated by stimulating α-1 adrenoreceptors with increasing concentrations of PE in full log increments starting at 10nM up to 100μM. Finally, a single 1mM dose of ATP was given to induce purinergic-mediated contraction. For the electrical field stimulation protocol,
strips were placed between two platinum electrodes in the organ chamber, and electrical pulses (0.1ms pulse width, 20V in the bath) were delivered, lasting 30 seconds at increasing frequencies (1,2,4,8,16 and 32 Hz) using a S88 stimulator (Grass Instruments, W.Warwick, RI). After 30-minute incubation with 10μM atropine this protocol was repeated. Atropine was washed out and 30-minute incubation with 100μM suramin (to antagonize P2 receptors) was followed by a final electrical field stimulation protocol. All tissue responses were normalized to grams of tissue weight.

**Histological analysis**

Bladders not subjected to pharmacological analysis were preserved for histology. Bladders were fixed in 10% buffered formalin overnight, processed, embedded in paraffin and then cut into 7μM axial slices. All stains were performed on at least 2 different areas of the bladder. The first cross section was taken closer to the most distal edge of the bladder (~4mm from the bladder dome), and one was taken from a more proximal location (~4mm distal from the ureterovesical junction). Slides were cleared in xylene and rehydrated to water where they were ready for staining.

Masson’s trichrome stain (Newcomer Supply Catalog #9176A) was performed, along with immunohistochemistry (IHC). IHC was performed as follows: a heat-mediated antigen retrieval step was used with 0.01M citrate buffer at 95°C for 15 minutes Endogenous peroxidase activity was blocked with Dual Endogenous Enzyme Block (DEEB; Dako, Ref# S2003) for 10 minutes at room temperature, followed by 10 minute incubation with Serum-Free Protein Block (PB; Dako, Ref#
X0909). Then samples were incubated with the following primary antibody dilutions/incubation: Von Willebrand Factor (VWF) (Dako, A0082, rabbit polyclonal, 1:200 dilution), α-smooth muscle actin (Sigma, A2547, mouse monoclonal, 5mg/mL concentration), desmin (Santa Cruz, sc-7559, goat polyclonal, 1:50 dilution), and myosin heavy chain (Santa Cruz, sc-12117, goat polyclonal, 1:50 dilution) for 60 minutes each. Following primary antibody incubation, slides were treated with 30 minutes each of Biotinylated Universal Link and Streptavidin-HRP (Dako, Ref# K0690). Finally, staining was completed with 5-10 minute incubation with ImmPACT DAB (Diaminobenzidine) from Vector Laboratories (Cat# SK-4105).

IHC probing proliferating cell nuclear antigen (PCNA) was performed similarly, without blocking endogenous peroxidase activity and without using DAB as a chromogen. The primary antibody used was against PCNA (abcam, ab29, mouse monoclonal, 1:3000 dilution) and the secondary antibody was conjugated with Texas Red from Vector Laboratories (Cat# TI-2000).

Image analysis was performed with ImagePro software 6.3 (Media Cybernetics, Bethesda, MD). One proximal and one distal section were taken for each animal designated for histology, and 4 high magnification images were taken in each section. For trichrome analysis, the color selection tool was used to determine quantity of red (muscle) and blue (collagen) pixels, and the percentage of muscle corresponds to the number of red pixels/ total number of selected pixels. For PCNA analysis, sections were counterstained with 4’,6- diamidino-2-phenylindole (DAPI), and PCNA positive nuclei were counted and divided by the total number of nuclei.
For vessel quantification, images were spatially calibrated, and the inner lumen was traced with the polygon measurement tool.

**Statistical Analysis**

Statistical evaluations were performed using GraphPad Prism software. (GraphPad software Inc.) For pharmacological analyses, individual response curves were placed in GraphPad software, and a mean curve was fit to the family of curves. Two-way ANOVAs with Neumann-Keuls post testing were performed on pharmacological and histological analyses comparing age groups. One-way ANOVAs were performed on cystometric evaluations and pharmacological experiments examining only one age group (i.e., older animals 2, 4, and 8 weeks post-STC). P values less that 0.05 were considered significant. All results are expressed as the mean ± SEM.
Results

Subtotal cystectomy. Of the 36 younger animals (12 weeks old) that underwent STC, 4 died within 3 days because of leakage into the peritoneum (11% mortality rate). When STC was performed in 90 older animals (12 months old), 18 animals died, and 5 were euthanized due to complications such as stones or abcesses (26% mortality/morbidity rate). Older animals that survived STC gained body weight (BW) similarly to age-matched control (AMC) animals in the 8 weeks after operation (264 ± 6g and 267 ± 12g for AMC and STC animals, respectively). However, in these 8 weeks bladder weight (bw) was significantly lower in STC animals (99.6 ± 8.5mg, 87.3 ± 10.3mg, and 122.2 ± 7.8mg for 2, 4, and 8 weeks) compared to controls (160.7 ± 7.2mg, 142.7 ± 10.4mg, 169.7 ± 10.2mg for 2, 4, and 8 weeks).

Six months post-STC there was a difference in BW between younger (AMC; 223.0 ± 5.0g, STC; 230.3 ± 3.3g) and older (AMC; 286.9 ± 7.9g, STC; 289 ± 11.7g) animals, however surgery did not produce a BW different from age-matched controls in either age group. No differences were found in bw due to age or surgery 6 months post STC. Bladders from young animals weighed 121.0 ± 9.2mg and 115.7 ± 6.9mg, while those from older animals weighed 124.6 ± 5.4mg and 141.9 ± 9.8mg for AMC and STC animals respectively.

An observation of increased kidney sizes and ureter dilation was made in older animals subjected to STC, which manifested in a significantly higher average weight of both kidneys from STC animals 6 months after operation (Figure 1A). Of
the 16 older animals that survived 6 months after operation, 8 showed signs of unilateral kidney damage, 3 with bilateral kidney damage, and 5 displayed no detectable kidney damage. None of the younger animals presented with kidney damage 6 months post-STC. This increase in kidney weight reached significance even though there was often a large difference in kidneys from the same animal due to compensation. An example of this is shown with H & E staining (Figure 1C) that depicts two kidneys from the same animal weighing 0.45g and 1.25 g. This compensation is also evident in the variability of creatinine clearance, with only a couple of older STC animals displaying impaired kidney function (Figure 1B).

Figure 1. Effects of STC on kidneys. (A) Average weights of both kidneys reveals a significant increase in kidney weight from older animals 6 months post-STC (P<0.01). (B) Analysis of creatinine clearance as a measure of kidney function 6 months after STC. Means and SEM are shown in black, with individual data points to illustrate variability in the groups. No differences were seen between groups. (C) 5x and 20x images of H & E staining showing two different kidneys from the same older animal post-STC. The left kidney weighed 0.45g, while the right kidney weighed 1.25 g.
**Cystometric Analysis.** All cystometric parameters from cystectomized and age-matched control animals are shown in Table 1. Parameters from urodynamic studies of older age-matched controls 2, 4, and 8 weeks post-STC were similar and thus treated as one group. While there was a trend for bladder capacity to increase in older animals after STC up to 8 weeks, at no time did capacity return to control values. Younger animals 6 months post-STC did have similar bladder capacities compared to age-matched controls.

<table>
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<tr>
<th></th>
<th>Bcap (mL)</th>
<th>MV (mL)</th>
<th>RV (mL)</th>
<th>BP (cmH2O)</th>
<th>TP (cmH2O)</th>
<th>MP (cmH2O)</th>
<th>Bcom (mL/cmH2O)</th>
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<td>0.88 (0.05)</td>
<td>0.88 (0.07) *</td>
<td>0.04 (0.02)</td>
<td>20.36 (3.45)</td>
<td>31.74 (3.84)</td>
<td>55.44 (5.18)</td>
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<td>0.37 (0.04) *</td>
<td>0.35 (0.04) *</td>
<td>0.03 (0.01)</td>
<td>9.18 (1.28) *</td>
<td>16.98 (1.15) *</td>
<td>22.96 (1.86) *</td>
<td>0.05 (0.00) *</td>
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<tr>
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<td>0.47 (0.02) *</td>
<td>0.44 (0.03) *</td>
<td>0.03 (0.008)</td>
<td>10.78 (1.68) *</td>
<td>19.19 (3.01) *</td>
<td>30.71 (3.71) *</td>
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<td>Old-8 weeks, n=9</td>
<td>0.54 (0.06) *</td>
<td>0.51 (0.08) *</td>
<td>0.05 (0.01)</td>
<td>11.75 (1.49)</td>
<td>20.53 (2.71)</td>
<td>33.28 (4.21) *</td>
<td>0.07 (0.01)</td>
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<td>Old-6 month AMC, n=7</td>
<td>0.84 (0.13)</td>
<td>0.74 (0.12)</td>
<td>0.1 (0.02)</td>
<td>16.45 (2.50)</td>
<td>23.69 (2.80)</td>
<td>55.14 (5.32)</td>
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<td>0.08 (0.01)</td>
<td>12.34 (6.29)</td>
<td>21.36 (5.03)</td>
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<td>Young-6 month AMC, n=5</td>
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<td>0.73 (0.07)</td>
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Table 1. Urodynamic parameters as determined by in vivo cystometry. Values show mean (SEM) for 12 week (young) or 12 month old (old) animals. Age Matched controls for old animals at 2, 4, and 8 weeks post-STC revealed no differences and were thus pooled as controls. Bcap- Bladder Capacity, MV- Micturition Volume, RV- Residual Volume, BP- Basal Pressure, TP- Threshold Pressure, MP- Maximal Pressure. Bcom- Bladder Compliance *- Indicates significant difference compared to the corresponding control animal (P<0.05).
Similar differences were seen in micturition volume, while there was no significant residual volume in any STC animals. This indicates that the bladder was able to empty efficiently after STC, regardless of age.

In all STC animals regardless of age or time post-STC, maximum pressures generated during a voiding cycle were significantly lower than their respective age-matched control. While at earlier timepoints (ie. 2, 4, and 8 weeks), older animals also displayed significantly lower basal and threshold pressures, values did normalize by 6 months post-STC. There were no differences in these parameters in younger animals 6 months post-STC. Additionally, bladder compliance significantly decreased in older animals 2 weeks post-STC but did return to normal values by 4 weeks after surgery.

*In vitro Studies.* Maximal steady-state contractions (Emax) of bladder strips in response to carbachol were lower in animals 2 (124.6 ± 12.4 g/g tissue), 4 (68.1 ± 5.6 g/g tissue) and 8 (74.8 ± 7.6 g/g tissue) weeks post-STC compared to controls (392.7 ± 14.4 g/g tissue). Pharmacological data for animals 6 months post-STC are shown in Figure 4. Six months post-STC, carbachol induced Emax values were identical in bladder from younger (356.5 ± 18.1 g/g tissue) and older (357.9 ± 23.1 g/g tissue) age-matched controls (Figure 4A). While bladder strips from younger STC animals 6 months after operation had a lower carbachol induced Emax value (195.5 ± 16.0 g/g tissue) than those from AMC animals, it was significantly higher than Emax values generated by bladder strips from in older STC animals.
(134.2 ± 7.4g/g tissue). There were no detectable differences in either pEC$_{50}$ or Hill Slope parameters among the groups.

KCl-induced contractions of bladder strips showed a similar pattern with those from young STC animals (110.7 ± 17.8g/g tissue) lower than non-cystectomized animals (younger AMC 170.4 ± 16.5g/g tissue; older AMC 148.3 ± 16.2 g/g tissue), but higher than older STC animals (60.5 ± 7.6g/g tissue). When expressed as % KCl contraction, there was no difference in carbachol, phenylephrine, or ATP-induced maximum contractions (Figure 2).

**Figure 2.** Responses of bladder strips excised from bladders 6 months after subtotal cystectomy (STC) or age-matched controls (AMC) to various stimuli. (A) Depolarization with potassium chloride (KCl), revealed significantly lower contractile responses in both young and old STC animals compared to their controls (*-P<0.05, **-P<0.01). When expressed as percentage of KCl response, no differences were found amongst the groups when stimulated to carbachol (B), phenylephrine (C), or ATP (D). Young AMC- 7 animals, 14 strips. Young STC- 11 animals, 17 strips. Old AMC- 6 animals, 12 strips, Old STC- 10 animals, 19 strips.
The maximum contractions of regenerating detrusor smooth muscle strips induced by electrical field stimulation (EFS) 6 months post-STC are shown in figure 3. Responses of bladder strips from STC animals were also significantly lower than control tissue at all frequencies tested, and the ratio of native responses to experimental responses was similar across all frequencies (Figure 3). Interestingly, bladder strips from STC animals from both age groups displayed a higher residual response to EFS when subjected to both muscarinic (atropine), and purinergic (suramin) blockade, especially at lower frequencies.

**Figure 3.** Normalized responses of bladder strips to electrical field stimulation. (A) Baseline frequency responses normalized to strip weight reveal lower contractile responses in STC animals compared with their age-matched controls. Electrical field stimulation responses after incubation with (B) 10μM Atropine and (C) 100μM Suramin reveal a significant response remains in STC bladders at low frequencies. Young AMC- 7 animals, 14 strips. Young STC- 8 animals, 13 strips. Old AMC- 6 animals, 12 strips, Old STC- 9 animals, 14 strips.
Histological analysis. Trichrome staining revealed that the bladder wall of regenerating bladders contained urothelial, lamina propria, and smooth muscle layers in both age groups after STC. When the smooth muscle layer was analyzed for % muscle content, older animals after STC had a significantly lower smooth muscle: collagen ratio (Figure 4). Although 2-way ANOVA analysis revealed an overall effect of age, this difference was not evident at all timepoints studied. To examine the maturation of these smooth muscle cells, a continuum of muscle markers were used (Figure 5). These

Figure 4. Quantitation of muscle versus collagen content via analysis of Masson’s Trichrome staining. (A) A 5x trichrome image of native, young bladder. (B) A representative 40x image (1 of 4 per section) depicting muscle layer that was used for quantitative analysis. (C) The same image from (B) that was quantified for % muscle content, with pink representing total muscle pixels, and light blue representing collagen pixels. (D) Quantification of Trichrome analysis utilized 36 images per timepoint (4 images from 3 different sections, from 3 different animals per age and timepoint). 2 way ANOVA revealed an overall effect of age on muscle content (P<0.05).
markers appeared in the same order as seen during bladder development (smooth muscle actin, then desmin, then myosin heavy chain), and usually appeared first in the most proximal sections of regenerating bladder. Moreover, these markers also appeared to be more consistently expressed in younger STC animals at any given timepoint.

**Figure 5.** Analysis of smooth muscle maturation markers in the regenerating bladder. Expression of smooth muscle actin (SMA), desmin (des) and myosin heavy chain (MHC) are shown at timepoints post-STC in younger (top panel) and older (lower panel) animals. Three individual animals (A,B,C) are shown for each age group at each timepoint, and both proximal (BASE) and distal (DOME) sections were stained. Analysis reveals a trend for later expression of these markers in older animals.
The extent of cell proliferation was explored via immunostaining against Proliferating Cell Nuclear Antigen (PCNA) in post-STC and age-matched control bladders (Figure 6). Staining in native bladder always revealed the amount of proliferating cells at < 1%.

![Image of immunostaining results](image)

**Figure 6.** Quantification of Proliferating Cell Nuclear Antigen (PCNA) stain. (A) Top panel shows representative 40x example pictures from younger and older animals up to 8 weeks post-STC. (B) Semi-quantitation of immunohistochemical PCNA staining reveals that there are less proliferating cells present in bladders of older animals at 1,2, and 4 weeks post-STC, but more PCNA positive cells when compared to younger animals at 8 weeks post-STC. *-P<0.05, **-P<0.01, ***-P<0.001.

Staining revealed a higher number of proliferating cells in younger animals 1,2 and 4 weeks post-STC but the opposite was true 8 weeks after operation. Quantification of PCNA staining revealed that in young animals 1,2,4 and 8 weeks
post-STC, the percentage of proliferating cells was 42 ± 4, 42 ± 2, 38 ± 2, and 11 ± 1% respectively. In older animals values were 17 ± 2, 32 ± 2, 29 ± 2, and 31 ± 2% at 1, 2, 4, and 8 weeks. There were no differences in the amount of staining when comparing proximal versus distal sections (data not shown). However, the age-related difference in the amount of PCNA expression appeared to be due to an increased number of proliferating cells in the lamina propria layer.

In an attempt to explore any age-related differences in angiogenesis after STC, the vasculature of bladders was highlighted with von Willebrand factor staining (Figure 7). Quantification of this stain revealed an average baseline vessel circumference of 89.1 ± 7.3 microns and 86.7 ± 2.5 microns for younger and older controls, respectively. Additionally the overall size of blood vessels in the bladder wall after STC did not differ because of age. At early timepoints, however, there were significantly more blood vessels in the bladder wall of younger animals (13.9 ± 2.8, and 31.9 ± 2.2 vessels/20x field of view) compared to older animals (6.6 ± 1.4, and 14.4 ± 2.2 vessels/20x field of view) at 1 and 2 weeks post-STC, respectively.
Figure 7. Quantification of vessel size and number using immunostaining against Von Willebrand Factor (VWF). Representative 20x images show quantification of blood vessels in the bladder wall 2 weeks post-STC in younger (A) and older (B) animals. While no differences were found in terms of blood vessel wall circumference (C), an overall decrease in the total number of vessels per 20x field of view (D) was found in older animals via 2-way ANOVA (B), P<0.001.
Discussion

Numerous chronic bladder conditions adversely affect patient quality of life, and can threaten the upper urinary tract causing end stage renal disease. Current surgical interventions are not always effective and can have unwanted side effects (Rohrmann et al. 1996; Vaught et al. 1996; Kanematsu et al. 2007; Atala 2011). Animal studies using a wide variety of synthetic and natural grafts (both cell seeded and unseeded) have documented a reasonable amount of mesenchymal and stromal incorporation. While one such combination of a collagen/polyglycolic acid scaffold with dual seeded urothelial and smooth muscle cells has had moderate success in young patients with congenital spinal cord disease (Atala et al. 2006), a much larger population of older patients with bladder dysfunction (and a generally reduced regenerative capacity) could benefit from this tissue engineering approach. With rapidly increasing tools for modification of graft materials (e.g. micro/nanoparticles for delivery of growth factors, oxygen, etc.) an understanding of spontaneous bladder regeneration would aid in the identification of targetable pathways to harness natural regenerative mechanisms.

As a first step in this direction, we have utilized a rodent model of subtotal cystectomy to examine time-dependent changes in function and morphology in the regenerating bladder. The lower urinary tracts of the rat and human have many similarities, and both have been shown to be able to regenerate. The difference in age groups chosen for this study is moderate. Well-documented survival curves of Fisher F344 female rats show 12 week ("younger") and 12 month old ("older")
animals display nearly 100% survival. While both age groups represent sexually mature phenotypes (i.e., adults), 12 month old rats are more accurately classified as middle-aged, and mostly do not present issues with baseline bladder function (Elbadawi et al. 1998; Kohan et al. 2000).

Despite this modest difference in age, older animals subjected to STC exhibited an increased mortality/morbidity rate (26%) compared to younger animals (11%). Similar reasons were found to be the cause of death in both age groups (namely urine leakage into the peritoneum), and usually occurred within 3 days of surgery. This indicates an impaired ability of the urothelium and the bladder wall to reseal the bladder immediately after STC in older animals. This is accompanied with decreased PCNA staining 1 and 2 weeks post-STC. The normally quiescent urothelium rapidly proliferates in response to injury (Staack et al. 2005). While this has been suggested to be essential in regeneration of the bladder, the impact of age on urothelial proliferation has not been explored in depth.

The most adverse side effect due to STC in surviving older animals was the finding of kidney damage. Enlarged, edematous kidneys were found and histology revealed chronic kidney damage in the form of hyaline casts. Kidney enlargement was usually unilateral with sufficient compensation by the contralateral kidney, which was further evidenced by normal creatinine clearance in almost all older animals 6 months post-STC. The exact reason for damage to the kidneys is unclear. Hydronephrosis due to high intravesical pressures may be one explanation for urine reflux; however we did not observe increased basal pressures upon urodynamic
investigation. Obstruction of the ureteral orifice into the bladder could be a feasible explanation, but was not obvious upon animal sacrifice. Natural repair mechanisms of the kidney in response to injury (e.g., ischemia/reperfusion) are known to be impaired with aging, and several mechanisms such as reduced epithelial proliferation, decreased stem/progenitor cell function, enhanced susceptibility to apoptosis, and telomere shortening have been reported (Schmitt et al. 2008; Westhoff et al. 2010). While there is no evidence for implicating these processes in our model, any strain on the kidneys after STC may exhaust baseline repair mechanisms in older animals.

Several models of mammalian organ regeneration have shown that the regenerative capacity decreases with age and suggested certain explanations. One recent study examined liver regeneration, in which 70% hepatectomy resulted in age-associated deficiencies in cell proliferation within 48 hours after resection of the liver (Furrer et al. 2011). This was reversed with a serotonin receptor agonist that worked through a VEGF-dependent pathway. Indeed, angiogenesis has been shown to be essential in many models of organ regeneration, and has been reviewed elsewhere (Han et al. 1999; Nomi et al. 2002). In our model, significant age-related changes in morphology were seen, with both cell proliferation (PCNA staining) and the number of newly formed vessels lower in older animals 1 and 2 weeks post-STC. Thus, our current study also illustrates the importance of cell proliferation and angiogenesis in bladder regeneration.
Skeletal muscle is another organ with reported regenerative capacity in the adult mammal and, age dependence of mechanisms involving reduced stem (satellite) cell function and fibrosis have been reported (Barani et al. 2003; Brack et al. 2007; Carlson et al. 2007; Gopinath et al. 2008; Mimeault et al. 2009). We observed a transient increase in collagen content in older bladders subjected to STC but by 6 months STC animals of either age showed no differences in smooth muscle: collagen ratios, suggesting minimal fibrosis. The lack of a definitive bladder stem cell complicates the examination of stem cell number/function (Nguyen et al. 2007; Chan et al. 2010). The label-retaining method has been used to indicate that the basal urothelial cell layer is the stem cell niche for the urothelium (Kurzrock et al. 2008). Consistent with this, PCNA staining in the urothelium seen in this study was localized to basal cells. The majority of other proliferating cells were located in the lamina propria layer, and between (but usually not within) muscle bundles. This coupled with our previous observation of an increase in c-kit positive cells suggests a possible role for the myofibroblast/interstitial cell in regeneration, (Burmeister et al. 2010). While this has been suggested before, the mechanism/origin of de novo smooth muscle formation remains unclear (Faggian et al. 1998). We have found, however, that the continuum of smooth muscle maturation markers are expressed by the smooth muscle in the same order that appears during development (Wu et al. 1999). Moreover, these markers are usually expressed first in the most proximal part of the organ (i.e., closest to the plane of excision) and seem to appear more quickly in younger animals.
Inflammation is another aspect of tissue regeneration that has been shown to negatively influence function/regeneration of, for example, skeletal muscle and the prostate (Begley et al. 2008; Smythe et al. 2008). We have seen substantial immunohistochemical staining for the presence of CD68 (macrophages) and myeloperoxidase (neutrophils) in older animals up to 2 weeks post-STC (data not shown). In a rat bladder augmentation model with porcine small intestinal submucosa, this timepoint did not represent the highest levels of either of these cell populations (Ashley et al. 2009). Although it is not possible to directly compare these models due to the use of a xenograft, there is a need to look at time-dependent changes in inflammatory leukocytes. Additionally, it has been shown that M1 and M2 macrophages elicit differences in repair, and can be pro-fibrotic or pro-regenerative, respectively (Brown et al. 2009). Examining the age-related differences in cells and cytokines mediating inflammation may elucidate targets to alter the process of bladder regeneration.

As organ regeneration is largely measured by a restoration of function, this study aimed to examine bladder function in response to STC (Stocum 2010). The bladder serves as a high capacity reservoir at low intravesical pressures during the storage phase, until micturition which aims to empty the bladder completely. Our urodynamic studies show that bladder capacity in older animals does not return to normal control values in the 8 weeks following STC, a time frame shown previously to be sufficient enough for younger animals (Burmeister et al. 2010). Moreover, by 6 months post-STC, bladder capacity in older animals is still 56% of age-matched
controls, while capacity in younger animals is 96% of controls. This could certainly be due to the delayed proliferative response seen histologically in older animals. Importantly, there was no significant residual volume in any group following STC, and a transient decrease in bladder compliance in older animals 2 weeks post-STC recovered by 4 weeks after operation.

In all STC animals regardless of age, there was a significant reduction in maximum pressures generated during the voiding cycle. While the cause of this is unclear it may involve the urethral sphincter (i.e., reduced outflow resistance), and/or could reflect a reduction in the overall strength of the detrusor smooth muscle. This latter explanation is further evidenced by a reduction in the maximal responses of bladder strips to the depolarizing agent potassium chloride (KCl). While these responses were reduced in younger animals 6 months post-STC, contractility of bladder strips from older animals was even further compromised. Maximal steady state contractile responses to carbachol exhibited a similar pattern between the groups, and significant reductions due to STC have been seen before (Saito et al. 1996). This may differ from previous reports of comparable contractility due to strip preparation (i.e. transverse versus longitudinal) (Frederiksen et al. 2004). Additionally, while other studies have reported a reduction in contractility due to age alone (Zhao et al. 2010), we did not see any effect of age on contractile responses most likely because of the modest age differences discussed earlier.

Interestingly, when expressed as a percent of the total KCl induced response, there were no differences between groups in response to muscarinic (carbachol),
purinergic (ATP) or α-1 adrenergic (phenylephrine) stimulation. This indicates that reductions in contractility seen in bladders from animals subjected to STC is not membrane receptor-mediated, but rather, arises downstream of the receptor. Frederiksen et al. showed that there are no differences in the total amount of smooth muscle actin, and that the ratio of actin/myosin remains unchanged 15 weeks after cystectomy (Frederiksen et al. 2001). However, changes in cross-bridge efficiency/kinetics may be explained by changes in smooth muscle myosin isoforms of either the light or heavy chain. For example, DiSanto et al. has previously shown that the heavy chain isoform SM-A has a decreased maximum velocity and shortening length, and that a change to this isoform may be an explanation for dysfunctional bladders following partial outlet obstruction (DiSanto et al. 2003). Future studies will examine this possibility.

Contractile responses to electrical field stimulation (EFS) were also reduced in animals following STC in both age groups and were abolished with tetrodotoxin (data not shown). Thirty two Hz stimulation produced responses in younger STC animals that were 48% of controls, and those in older animals were 44% of controls. While this is similar to what is seen in older animals this number is in disagreement with the discrepancy seen upon KCl (65%) and carbachol (60%) stimulation in younger animals, which may reflect reinnervation as a limiting factor in bladder regeneration in this age group. It has been shown previously that new nerves formed after STC originate from pre-existing nerves in the detrusor (Frederiksen et al. 2008). This along with tetrodotoxin-resistant responses after electrical field
stimulation clearly displays a capacity for reinnervation; however synaptic potentials and nerve conductance after STC have not been explored. Additionally, there were higher residual EFS-induced contractile responses after both purinergic (suramin) and cholinergic (atropine) blockade, especially at lower frequencies. While adrenergic receptors may contribute to this remaining response, Frederiksen et al. (2004) have previously reported no effect of α-1 adrenergic blockade with prazosin (Frederiksen et al. 2004), so other receptor populations (e.g. tachykinins, TRP channels) may be responsible.

In conclusion, the present study demonstrates that when the bladder is challenged to regenerate following STC, age has some adverse effects on bladder function. Bladder capacity does not normalize causing more frequent voiding, which may be related to the impaired recovery of smooth muscle contractility. While this may cause possible long-term effects on the bladder and urethra, the most concerning finding is that STC in older animals caused significant damage to the kidney. Preliminary insights into the mechanisms responsible for age-associated decreases in regeneration suggest differences in proliferation and angiogenesis in the first 2 weeks after STC. While these differences in regenerative capacity may be exacerbated in truly aged animals, the middle-aged rat may provide a sufficient model for studying deficits in mammalian organ regeneration due to age.
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CHAPTER 5

The utility of non-invasive imaging in vivo to monitor de novo bladder regeneration

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The following chapter was prepared for submission to Radiology. The format adheres to WFUSM graduate school requirements.

David Burmeister is responsible for all imaging analysis and almost all histology data collection/analysis.
Abstract

Subtotal cystectomy (STC; removal of ~70% of the bladder) in young adult rats has been shown to induce a regenerative response such that the bladder will regenerate to a normal volume, while maintaining overall function. We investigated the potential for in vivo Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) scans to noninvasively track this regenerative process. Twelve week old female Fisher F344 rats underwent STC and were scanned via CT and MRI 2, 4, 8, and 12 weeks afterwards, at which times urodynamic testing was also performed. After euthanasia, bladders were excised for histological processing or for vascular perfusion of the bladder with a silicone-based contrast agent (Microfil ®; FlowTech, Inc). Bladder volumes determined 2 weeks post-STC via CT scanning were able to predict in vivo pressure generation in the bladder 8 weeks post-STC. MRI Scans demonstrated an initial decline followed by a time-dependent increase in the thickness of the bladder wall, which was indistinguishable from pre-operative values by 8 weeks post-STC. Moreover, bladder wall thickness was negatively correlated with cystometric bladder capacity early on (2 and 4 weeks post-STC), and maximum pressures at the 8 and 12 week timepoints. Trichrome analysis did not reveal any significant fibrosis after STC; however the collagen: smooth muscle ratio positively correlated with MRI-determined bladder wall thickness at 8 and 12 weeks. Taken together, the results of this study demonstrate a potential for non invasive imaging as a prognostic indicator for the functional success or failure of bladder regeneration.
Introduction

Tissue engineering, aiming to restore function in damaged or diseased organs, is a promising approach for the field of urology. In the case of the bladder, engineered constructs may be more effective than traditional surgical interventions and may prevent unwanted side effects (Atala et al. 2006; Atala 2011). Many different animal models have been used extensively to examine the effectiveness of different cell/scaffold combinations in augmentation of the bladder (Yoo et al. 1998; Oberpenning et al. 1999; Obara et al. 2006; Urakami et al. 2007; Roth et al. 2011; Sharma et al. 2011). In addition to bladder augmentation models, several studies have suggested that removal of a large part of the bladder without replacement also results in some degree of bladder regeneration, with functional restoration (Liang et al. 1963; Saito et al. 1996; Piechota et al. 1999; Frederiksen et al. 2004; Burmeister et al. 2010).

In either of these bladder regeneration models, experiments examining the functional restoration of the bladder have their caveats/limitations (Andersson et al. 2011). One such disadvantage is that with current urodynamic approaches only one timepoint may be studied in a given rat (i.e. the experiment is terminal). Non-invasively monitoring the bladder would not only give information on the regeneration process over time but may also lead to insights on bladder function. This would greatly reduce the number of animals needed for a given study, alleviating some of the costs and ethical concerns associated with the use of animals. Implantation of an indwelling catheter also has its technical difficulties, and even if
the surgical procedure is completed successfully, there may be catheter problems (e.g., blockage/kinking or displacement) sometimes caused by the animals gnawing on the exposed end. Moreover, it is not known what effect the invasive surgical procedure per se has on the function of the regenerating bladder. Certainly, alternative methods are needed to study the timecourse of regeneration and the functional changes that occur.

Non-invasive imaging has great prognostic value in the clinic, and imaging techniques have also been applied to tissue engineering in, for example, cardiac, cartilage and bone regeneration (Young et al. 2008; Lau et al. 2010; Saldanha et al. 2011). Although a few studies have examined Magnetic Resonance Imaging (MRI) of grafts in the bladder, little is known about how changes in the morphology of the bladder reflect on bladder function (Cheng et al. 2007). The current study explores the usefulness of non-invasive imaging as an alternative method to study bladder function during regeneration induced by subtotal cystectomy (STC).
Materials and Methods

Animals

A total of 30-12 week old (170-200g) female Fisher F344 rats were used in this study; 28 animals underwent STC (see below), with 2 control animals used for Microfil® studies. Additionally, retrospective analysis was performed on previously published data from animals of the same strain, age, and gender (Burmeister et al., 2010). All methods were approved by the Animal Care and Use Committee, Wake Forest University.

Trigone-sparing cystectomies

Animals were anesthetized with 2% isoflurane, and the abdominal wall shaved. After disinfecting the surgical site with Povidone-iodine, a low midline abdominal incision was made. The bladder was identified and dissected down to the level of the ureterovesical junction. Two stay sutures were made on either side of the bladder, just above the ureteric orifices, using 6-0 polyglycolic acid. The dome portion of the bladder (~60-70%) was excised leaving the trigone and ureterovesical junctions intact (i.e., trigone-sparing cystectomy; STC). The remaining portion of the bladder was then sutured in a continuous fashion using one of the original stay sutures. The abdominal wall and skin were closed in two layers using 3-0 vicryl sutures. Animals were allowed to recover and given food and water ad libitum for up to 12 weeks after surgery.

Magnetic Resonance Imaging (MRI)
All MRI experiments were performed in a 7T horizontal bore magnet (Bruker Biospin, Billerica, MA) equipped with an actively shielded gradient insert. RF signal transmission and reception was performed with a 50mm I.D. quadrature Litzcage RF coil (Doty Scientific, Columbia, SC) tuned and matched with each rat to 300.2 MHz. Rats were anesthetized with 2% isoflurane and the bladder was manually expressed. The animal was then placed in the RF coil in the prone position with the bladder centered in the RF coil. Anesthesia was maintained during the scan via 1.5% isoflurane. Body temperature was kept constant by thermostatically controlled warm air (SA Instruments, Stoney Brook, NY), and the bladder was centered in the RF coil via a Rapid Acquisition with Relaxation Enhancement (RARE) spin echo pulse sequence with an echo train of 8 echos with parameters TR=1500ms, TE=40ms, FOV = 6 cm, matrix = 128x128, slice thickness = 2 mm, NEX=1.

A 3D FLASH pulse sequence allowed for the acquisition of 8 slices, each 250 microns thick. This reduced partial volume effects, thus allowing for better detection of the inner and outer surfaces of the bladder wall. The coronal 3D FLASH slab was positioned using the tri plane localizers as scout images so that the slices in the center of the slab were perpendicular to the surface of the bladder wall. Scanning parameters were as follows: TR=50ms, TE=6ms, flip angle = 15 degrees, FOV = 3 cm, matrix = 256x256, giving an in-plane resolution of 117um, NEX=8. Acquisition was also respiratory gated to avoid breathing motion artifacts. Analysis of bladder wall thickness was performed using TeraRecon 3D visualization and image analysis software using the linear measurement tool. This was performed in triplicate, on five different locations of the bladder wall, denoted “base” to “dome”.

**Bladder catheter implantation**

Rats were again anesthetized with 2% isoflurane, and through a midline incision, the dome of the bladder was dissected from ensuing adhesions caused by the previous surgery. A small incision was made in the bladder dome, and a PE-50 intramedic polyethylene catheter (Becton-Dickinson, Sparks, Maryland) with cuff was inserted and anchored with a 5-0 purse string silk suture. The catheter was then tunneled subcutaneously and brought out through the back of the neck, and subsequently held in place with cloth tape anchored to the skin via a 3-0 Vicryl suture. The abdominal wall and skin were closed in 2 layers with 3-0 vicryl sutures, and the free end of the catheter was thermally sealed.

**Cystometric analysis**

All cystometric studies were performed 3 days after catheter implantation in conscious, freely-moving rats. The indwelling catheter was connected to a 3-way valve that is, in turn, connected to a pressure transducer and an infusion pump. The pressure transducer was connected to an ETH 400 (CD Sciences, Dover, New Hampshire) transducer amplifier and consequently connected to a MacLab/8e (Analog Digital Instruments, Castle Hill, New South Wales, Australia) data acquisition board. The pressure transducers and acquisition board were calibrated in cmH$_2$O before each experiment. Room temperature saline was infused at a rate of 10mL/hour. Micturition volumes were measured with a silicone-coated funnel leading into a collection tube which is connected to a force displacement transducer, which was also calibrated before each experiment. Analysis begun after a consistent
voiding pattern was established. The following cystometric parameters were investigated: basal pressure (BP, lowest pressure between voids), maximum pressure (MP, the highest bladder pressure during micturition), threshold pressure (TP, pressure which initiates a voiding contraction), intermicturition pressure (IMP, mean bladder pressure between voids), bladder capacity ($B_{\text{cap}}$, residual volume after last micturition plus amount of saline infused), micturition volume (MV, amount of expelled urine), residual volume ($R_V$, bladder capacity-micturition volume), and bladder compliance ($B_{\text{com}} = B_{\text{cap}} / (\text{TP-BP})$).

*Microfil® Infusion*

After euthanasia, the thoracic cavity was opened, and a blunt 16 gauge needle was inserted into the left ventricle. This needle was connected to silicon tubing (Cole Parmer ID SKU 957021), which was thread through a perfusion pump. The free end of the tubing was inserted into the solutions to be perfused. A small incision was made in the right atria, and immediately afterward 50mL of heparinized saline was perfused through the vasculature using the perfusion pump at a speed of 15-20mL/min. After perfusion of saline, 100mL of 10% neutral buffered formalin (NBF) was perfused through the blood vessels at the same speed. During this perfusion, Microfil MV-122 (Flow Tech Inc. Carver, MA) was prepared according to the manufacturer’s instructions (22.5 mL solution/2.5 mL curing agent). After NBF perfusion, this 25mL of working Microfil solution was perfused at an initial rate of 10mL/min for 1 min, and 5mL/min for 3 min. Bladders were removed by ligating both ureters and proximal urethra and excising distal to the sutures. CT Scans of the
animals were taken with a Siemens MicroCATII @ 70kV, 500μA (BIN Factor of 1, 360° rotation, 360 steps).

**Histology**

Bladders not assigned to Microfil infusion were preserved for histological analysis of smooth muscle content. Bladders were fixed in 10% buffered formalin overnight, processed, embedded in parrafin and then cut into 7μM axial slices. Slides were cleared in xylene and rehydrated to water. Massons trichrome stain (Newcomer Supply Catalog #9176A) was performed on at least 2 different areas of the bladder (a distal and a proximal location). Four high magnification images were taken in each section, and image analysis was performed with ImagePro software 6.3 (Media Cybernetics, Bethesda, MD). The color selection tool was used to determine quantity of red (muscle) and blue (collagen) pixels, and the percentage of muscle corresponds to the number of red pixels/ total number of selected pixels.

**Statistical Analysis**

Statistical evaluations and regression analysis were performed using GraphPad Prism software. (GraphPad software Inc.) One-way ANOVAs with Neumann-Keuls post testing were performed on bladder wall thickness, cystometric parameters, and Trichrome analysis. A two-way ANOVA was used to determine any regional variations in bladder wall thickness. P values less that 0.05 were considered significant. All results are expressed as the mean ± SEM.
Results

Subtotal cystectomy

Of the 28 rats that underwent STC, three animals died within three days after surgery due to urine leakage into the peritoneum (11% mortality rate). All animals were followed via MRI scanning, with 5 animals undergoing cystometric studies at 2, 4, and 12 weeks, and the remaining rats were sacrificed at 8 weeks after urodynamic testing.

CT Scanning

We have previously shown that CT scanning reflects a rate of bladder growth consistent with cystometric bladder capacity, and that anterior bladder circumferences measured via CT scans positively correlate with maximum pressures generated by the bladder (Burmeister et al, 2010). In addition to the animals listed above, a retrospective analysis was performed on these previously published results. Regression analysis (Figure 1) revealed that total bladder volume measured by CT scans 2 weeks post-STC is negatively correlated with maximum pressures generated in vivo 8 weeks post-STC. Surprisingly, these 2 week volumes were not predictive of any other cystometric parameter, including bladder capacity (data not shown).

Microfil® perfusion techniques reveal a unique pattern of vessels 2 weeks post-STC, with newly formed vessels in the regenerating portion of the bladder (Figure 2). Dilation/widening of blood vessels is apparent in the bladder base as compared
with control bladders. Additionally, a dense network of small blood vessels forming at the distal part of the bladder dome appeared with more twists and convolutions. There were not enough observations to justify a quantitative analysis of this observation (n=2).

Figure 1. Retrospective regression analysis of animals subjected to STC reveals a predictive value for CT Scanning (Burmeister et al., 2010). X-axis represents bladder volume 2 weeks post-STC as determined by CT Scanning, and Y-axis represents maximum pressure (MP) generated by bladders 8 weeks post STC as determined by in vivo urodynamic studies in the SAME ANIMAL. While it has been previously shown that scans done at the time of urodynamic analysis can be revealing for function, this analysis displays that animals with minimal bladder growth seen upon CT scanning 2 weeks post-STC display bladder overactivity, while the animal with a large bladder volume 2 weeks after surgery generates maximum pressures ~13 cmH2O. Normal bladder function was seen in animals with intermediate bladder volumes determined by CT 2 weeks post-STC. $y = -46.65x + 52.12$, $r=0.82$, $P=0.02$. 
**Figure 2.** Images obtained via Microfil vascular perfusion. Bladder vasculature demonstrates a distinct pattern, especially at distal locations. Representative images of highlighted bladder vasculature via perfusion with Microfil (FlowTech, Inc) in a control bladder (A) and one 2 weeks post-STC (B).

**MRI scanning**

Analysis of sagittal slices of MRI scans revealed an initial decrease in bladder wall thickness after STC, which normalized to control values by 8 weeks post-STC (Figure 3). Values for bladder wall thickness were 402.1 ± 18.82 microns, 269.6 ± 12.21 microns, 315.3 ± 17.55 microns, 384.7 ± 19.11 microns, and 399.8 ± 25.12 microns for 0, 2, 4, 8, and 12 weeks, respectively. Analysis of bladder wall thickness variations from the distal (dome) to proximal (base) bladder demonstrated no differences between any timepoints studied (8 weeks shown in Figure 3D).
Figure 3. MRI analysis of the bladder wall via MRI scanning. The bladder wall normalizes thickness 8 weeks after STC. (A) Example of a sagittal view of a control (pre-STC) bladder visualized by magnetic resonance imaging (MRI), magnified in (B). (C) 1 Way ANOVA analysis of quantified bladder wall thickness using MRI scans reveals that the bladder wall is thinner than pre-STC values 2 and 4 weeks post-STC (P<0.01). (D) Analysis of regional wall thickness shows no differences from control (n=24) and 8 weeks post-STC (n=14).

Trichrome analysis and regression

A representative trichrome image is shown in Figure 4, and analysis revealed no changes in smooth muscle: collagen ratio at any time after STC. Percent smooth muscle values were 66.16 ± 1.41, 64.14 ± 3.35, 65.46 ± 2.09, 59.10 ± 1.67, and 63.48 ± 1.35% at 0, 2, 4, 8, and 12 weeks post-STC, respectively. However, the ratio of
smooth muscle to collagen was positively correlated with MRI-determined bladder wall thickness at the 8 and 12 week timepoints (P=0.031).

![Image](image_url)

Figure 4. Quantification of smooth muscle to collagen ratio using semi-quantitation of Masson’s trichrome staining. (A) Representative image of excised STC bladder used for quantification by choosing pixel intensity (B). (C) Analysis shows no differences amongst the groups, however linear regression reveals that the amount of smooth muscle is correlated with bladder wall thickness determined via MRI (P<0.05).

Cystometric analysis and regression

All cystometric parameters are displayed in Table 1. Bladder capacity was higher at 8 weeks compared to every other timepoint. There were no other differences seen in any other cystometric parameter over time. Linear regression analysis between
cystometric parameters and bladder wall thickness at the terminal timepoint revealed different correlations when examining early and late timepoints (Figure 5).

In the early time period after STC (i.e. 2, and 4 weeks) bladder wall thickness was negatively correlated with bladder capacity (P=0.018). In later timepoints (i.e., 8 and 12 weeks) bladder wall thickness was negatively correlated with maximum pressures obtained during the micturition cycle (P=0.015).

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<th>Bcap (mL)</th>
<th>MV (mL)</th>
<th>RV (mL)</th>
<th>BP (cmH2O)</th>
<th>TP (cmH2O)</th>
<th>MP (cmH2O)</th>
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<tr>
<td>2 weeks post-STC, n=4</td>
<td>0.39 (0.03)</td>
<td>0.36 (0.09)</td>
<td>0.03 (0.01)</td>
<td>11.82 (2.51)</td>
<td>19.36 (3.67)</td>
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<td>4 weeks post-STC, n=5</td>
<td>0.42 (0.06)</td>
<td>0.50 (0.08)</td>
<td>0.05 (0.02)</td>
<td>12.40 (2.74)</td>
<td>21.96 (3.81)</td>
<td>37.56 (6.51)</td>
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<td>8 weeks post-STC, n=8</td>
<td>0.78 (0.12)*</td>
<td>0.76 (0.15)</td>
<td>0.06 (0.02)</td>
<td>16.42 (6.02)</td>
<td>27.67 (10.20)</td>
<td>45.78 (13.99)</td>
<td>0.15 (0.06)</td>
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<tr>
<td>12 weeks post-STC, n=5</td>
<td>0.50 (0.04)</td>
<td>0.46 (0.04)</td>
<td>0.04 (0.01)</td>
<td>8.45 (1.22)</td>
<td>16.59 (1.64)</td>
<td>29.99 (4.22)</td>
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Table 1. Urodynamic parameters as determined by in vivo cystometry. Values show mean (SEM) for animals 2, 4, 8 and 12 weeks post-STC. Bcap- Bladder Capacity, MV- Micturition Volume, RV- Residual Volume, BP- Basal Pressure, TP- Threshold Pressure, MP- Maximal Pressure. Bcom- Bladder Compliance. *- Bladder capacity was higher 8 weeks post-STC compared with all other timepoints (P<0.05).

**Linear regression with pre-operative MRI Scans**

In addition to the correlations mentioned above, regression analysis revealed a relationship of pre-operative bladder wall thickness values with several important parameters (Figure 6). Pre-operative (baseline) wall thickness values were positively correlated with the eventual bladder compliance and smooth muscle: collagen ratio seen at 2 and 4 weeks post-STC (P=0.049, and 0.016, respectively). Additionally, baseline wall thickness was negatively correlated with residual volume seen at 8 and 12 weeks post-STC (P=0.033).
Figure 5. Linear regression analysis of cystometric parameters with bladder wall thickness at respective terminal timepoints. (A) At early timepoints (i.e., 2, 4 weeks) after STC, the bladder capacity is negatively correlated with bladder wall thickness. (B) At later timepoints (i.e., 8, and 12 weeks) Wall thickness is negatively correlated with maximum pressure during voiding. $P<0.05$ in all cases.
Figure 6. Linear regression analysis with bladder characteristics with pre-operative bladder wall thickness. The pre-operative thickness of the bladder wall is positively correlated with bladder compliance (A) and smooth muscle content (B) seen at early timepoints post-STC. (C) Residual volume seen at later timepoints is negatively correlated with the initial bladder wall thickness before surgery. P<0.05.
Discussion

Animal models of bladder regeneration (both with and without the use of augmentation constructs) have given insight into the usefulness of bladder tissue engineering approaches. However, for regeneration to be considered a success, it must be shown that bladder function is maintained/ restored. Current methods for examining bladder function in animal models carry several drawbacks. The terminal nature of the experiments dictates that any given animal can only be studied once throughout the regeneration process. Additionally, the challenging surgery carries with it technical difficulties, and may itself adversely effect the function of the bladder. In the studies described above, we investigated the potential of Magnetic Resonance Imaging (MRI) and Computed Tomography (CT) imaging techniques to serve as a functional correlate in order to circumvent invasive/terminal methods currently used to study the timecourse of bladder regeneration.

Previously we have shown that non-invasive CT imaging accurately reflects the rate of bladder growth, and that the circumference of the bladder measured with these scans is positively correlated with maximum pressures (MP) generated during urodynamic studies. Here we report a new analysis of the results obtained from those same animals. This showed that the volume calculated via CT scanning at 2 weeks negatively correlated with MP seen at 8 weeks in the same animal. This indication that CT scanning can be predictive of bladder function is not trivial, and could be used for assessment of interventions to enhance smooth muscle function (e.g. delivery of stem cells, growth factors, or gene therapy). In this case, it seems
that there is an optimum rate of bladder growth, such that an extremely small increase in capacity by 2 weeks post-STC results in overactivity (i.e. high pressure and non voiding contractions). On the other hand, an extremely large amount of bladder growth within 2 weeks post-STC results in a substantial deficiency for the smooth muscle to generate pressure.

We have also shown via vascular contrast and CT scanning that there is a significant amount of angiogenesis, leading to a distinct pattern of blood vessels 2 weeks after STC. Although the extent of vessel formation/branching could be quantified, we did not do so in this study because the Microfil® contrast agent used is permanent, and the experiment is therefore terminal. We chose instead to focus on non-invasive means of imaging, which excludes this procedure. In this regard, one group has used contrast enhanced MRI to quantify angiogenesis after engraftment of an acellular matrix onto the rabbit bladder (Cheng et al. 2007). Specifically they have shown that the contrast agent Gadomer correlated with the microvascular density seen when analyzed histologically. While this procedure may be technically difficult in a rat, it should be considered when considering non-invasive means to monitor bladder regeneration.

Although the size of the rat bladder is rather small, we were able to overcome this by using a 7 Tesla magnet along with respiratory gating. While a few instances of motion artifact prevented the acquirement of analyzable images (8/90 possible scans), most scans were successful. In addition, both long scan times and a large number of observations prevented scans to be repeated, but this could
certainly be done if fewer animals were needed. We found that bladder wall thickness drastically decreased after STC, but had returned to normal values by 8 weeks post-STC. This is consistent with our previous finding in which we found no difference in histologically determined bladder wall thickness 8 weeks post-STC (Burmeister et al. 2010). Although thinner in the 2-4 weeks after STC, the bladder wall did not display any significant fibrosis when subjected to trichrome analysis of smooth muscle: collagen ratio at any timepoint studied.

Cystometric analysis revealed higher bladder capacities at 8 weeks post-STC, but this was not maintained 12 weeks post-STC. The reason for this is unclear, however there was a high variability in cystometric performance at the 8 week timepoint, despite the increased number of observations. At this timepoint, some animals were also subjected to CT scanning as well as MRI scanning. It is possible that the manual bladder emptying during MRI, and the transurethral filling of the bladder with contrast during CT scanning has some consequence on the regeneration process. The effect of mechanical stimuli on bladder regeneration has been postulated dating back to the 1960’s (Liang 1966). In fact, the importance of mechanical forces on bladder regeneration has recently been illustrated in a canine model of bladder augmentation (Boruch et al. 2010). After augmentation cystoplasty with both bladder acellular matrix and small intestinal submucosa, long term catheterization (thus preventing the physiological filling of the bladder) produced massive deficits in regeneration in terms of urothelial and smooth muscle infiltration and fibrosis. Clearly, mechanical forces within the bladder may affect bladder regeneration in our model as well.
MRI derived wall thickness did, however, reveal several relationships with urodynamic parameters. We chose to separate analyses into early (2 and 4 weeks) and late (8 and 12 weeks) timepoints to reflect two different phases of bladder regeneration, as 8 weeks has been previously shown to be sufficient time for regeneration in this model. At the earlier timepoints, wall thickness was negatively correlated with bladder capacity, possibly due to non-optimal proliferation/organization of the smooth muscle in the regenerating bladder wall. This same relationship was not seen at the later timepoints. Instead, wall thickness at 8 and 12 weeks was negatively correlated with the maximum pressures generated in the bladder, despite the fact that thicker bladder walls were also associated with an increased smooth muscle: collagen ratio. The clinical impact of this finding is unclear, as it has been shown that bladder wall thickness is positively correlated with intravesical pressure during urine flow in women with lower urinary tract symptoms (Kuhn et al. 2011).

Baseline bladder wall thickness (before STC) may provide useful information as to the extent of bladder regeneration following STC. Baseline bladder wall thickness was predictive of both bladder compliance and the percent of smooth muscle found in the bladder at 2 and 4 weeks post-STC. Although collagen content of the bladder contributes to its increasing volume accommodation at low pressures (i.e., compliance) detrusor smooth muscle cells also play a significant role (Andersson et al. 2004). Additionally, at later timepoints, increased pre-operative wall thickness was associated with a decreased residual volume after regeneration. All of these correlations point to an overall improved outcome of bladder regeneration in
animals that have a thicker bladder wall before surgery. This observation suggests that it may be possible to predict whether or not a given individual is a good candidate for surgical bladder repair before the operation takes place.

To summarize, we have demonstrated that non-invasive imaging may be a powerful prognostic tool for bladder regeneration strategies. In animal models these methods may reveal functional insights while avoiding disadvantages associated with functional analyses. Because tissue engineering strategies are often measured by the amount of functional restoration after regeneration, invasive means to monitor bladder function will always have a place in research. However, CT and MRI may circumvent invasive surgery to provide insight into bladder function, thus allowing researchers to follow the regeneration process longitudinally in the same animal, and reducing the number of animals needed.
References


CHAPTER 6

Discussion and Perspectives for the Future
The studies presented within this thesis have established the rodent bladder as a model of organ regeneration in adult mammals. The methods used this far for characterizing this model have demonstrated that the plasticity/accomodation of the bladder is sufficient enough to maintain function after removal of a large portion of the organ. Our results indicate that bladder regeneration following STC is associated with a robust proliferative response leading to a mature smooth muscle and urothelial layer. No apparent fibrosis was seen, newly formed nerves and vessels were apparent. In addition to the studies mentioned here, recommendations for future areas of interest cover multiple topics including contributing cell populations, contractility deficits, mechanical stimuli, regeneration incompetent scenarios, and targetable pathways.

Identifying which cell population is responsible for the formation of new tissue presents a demanding, albeit worthwhile challenge. While hyperplasia was demonstrated within, the actual stem/progenitor cell population associated with the regenerative response has been elusive. An upregulation of c-kit positive cells were seen in detrusor smooth muscle after STC. Although this membrane receptor has been shown to be associated with progenitor cells responsible for liver and cardiac regeneration, it is also associated with myofibroblasts in the lamina propria layer of the bladder. This layer was also identified as containing an abundance of proliferating cells outside of the urothelium. Given that these cells are also involved with signaling between urothelial and smooth muscle cells, this seems to be an attractive candidate for the cell population involved in regeneration. Examining the
immediate response to STC should reveal large populations of proliferating cells that may also express this receptor.

The observed deficit in contractility seen after STC, in the face of relatively normal in vivo function is not fully understood. A similar percent of control contractions were seen to a number of different stimuli. This suggests that the differences in contractility observed are due to changes in the second messaging cascade, or in the contractile machinery itself. It should be determined whether or not there are differences in myosin isoforms in the regenerating bladder which account for reduced contractility. Additionally it would be revealing to conduct a number of in vitro experiments (i.e., electrophysiology) to examine if membrane potentials of smooth muscle (or both sensory and motor nerves) from regenerating bladders are normal. Differences in the status of ion channels (e.g. a prolonged opening of BK channels) could inhibit activation of excitable cells in the bladder, leading to a reduction in maximum contractility.

The importance of mechanical stimuli (e.g., physiological filling of the bladder) has been suggested before, and may account for some differences in function seen when animals also undergo non-invasive imaging. Filling and emptying of the bladder leads to mechanical forces on the bladder wall, and this cycle would certainly occur more rapidly following STC. In this regard it would be interesting to look at mechanoreceptors in the bladder wall that are involved with afferent signaling. Namely transient receptor potential channels (TRPV1, TRPV4) may be up or down regulated in response to STC surgery. Antagonists (e.g.,
capsazepine) are available to determine the extent of involvement of these receptors.

In organ regeneration models studies in lower vertebrates, loss of function studies have been used to gain key molecular insights into organ regeneration per se. While we have seen a reduction in regenerative capacity because of age, an even older age group may enhance this age-related decline. While it is to be expected that mortality rates would increase in an older age group, differences in regeneration per se would be exacerbated. Alternatively, taking advantage of genetic manipulations possible in mice may also bring about regeneration-incompetent stages in order to help determine key molecular players in the regenerative process. While the STC surgery may be difficult in these animals due to the smaller size, it may be fruitful to embark on these experiments.

Much of the possibilities raised above are ultimately aimed at identifying targetable pathways in order to alter bladder regeneration. Although not reported herein, we have attempted to deliver Adipose Derived Stem Cells (ADSCs) locally to the site of injury at the time of STC in older animals. The current prevailing thought with stem cell treatments are not that cells provided incorporate into regenerating tissue themselves, but rather act as a paracrine mediator of several processes (e.g. angiogenesis, inflammation). When delivered in our model, ADSCs did not seem to aid in recovering function in older animals after STC. This could be due to the release of chemotactic agents from the ADSCs that create a milieu for pro-inflammatory or anti-regenerative cytokines/cells. Perhaps the ADSCs paracrine effect results in signals that both inhibit and stimulate processes associated with
regeneration. Regardless, the general effect of stem cell therapy seems to be convoluted enough to necessitate other means altering the process. Exploration of other pathways discussed herein may be a good beginning, but a top-down approach to the molecular biology of regeneration (i.e. 2-D DIGE or microarray experiments) would give major clues as to what time-dependent events drive the process of bladder regeneration. In either case, identifying specific targetable pathways for the alteration of bladder regeneration would definitely allow for a more meaningful translational value.

In summary, in order to gain insight on what specific growth factors, cytokines or other molecules are needed to facilitate urological tissue engineering strategies into regeneration compromised patients, more studies are needed. With the studies described in this thesis, the regeneration of the urinary bladder has been well documented from a functional standpoint. Although age-dependence and non-invasive imaging were suggested as an approach to alter and monitor the process of regeneration, identifying specific molecular targets to harness this regenerative potential will be of utmost importance for the clinic.
David Mark Burmeister, B.S.
Ph.D. Candidate
Wake Forest University School of Medicine

EDUCATION
Ph.D.  Wake Forest University Health Sciences, Winston-Salem, NC
   Physiology and Pharmacology, Present
B.S.   College of Charleston Honors Program, Charleston, SC
   Biology / Pre-Pharmacy, 2005

PROFESSIONAL EXPERIENCE
May 2007 to Present  Wake Forest University School of Medicine, Winston Salem, NC
   Ph.D. Student, Department of Physiology/Pharmacology, Wake Forest Institute for Regenerative Medicine
   -Utilizing multi-disciplinary studies to examine endogenous bladder regeneration in the rodent
   -Evaluating the delivery of Adipose Stem for enhancement of endogenous bladder regeneration

Jan 2007 to May 2007  Wake Forest University School of Medicine, Winston Salem, NC
   Research Rotation, Department of Physiology and Pharmacology
   -Utilized proteomics to study receptor populations from brain regions implicated in schizophrenia.
   -Performed 2D-DIGE experiments to examine protein changes in serum from cocaine-exposed monkeys.

Jan 2004 to August 2005  Medical University of South Carolina (MUSC), Charleston, SC
   Research Assistant, Department of Biochemistry and Molecular Biology
   -Investigated regulation of the expression of the protooncogene Bcl-2 in leukemia cells.
   -Utilized site-directed mutagenesis of bcl-2 to examine the mRNA stabilizing protein nucleolin.

SKILLS
- Small animal surgery: Performed a number of rodent survival surgeries including subtotal cystectomy of the bladder, bladder catheterization, and iliac artery endothelial balloon injury.
- Non-invasive imaging: Performed micro CT and micro MRI scanning of rodents to visualize the urinary bladder including transurethral administration of contrast agents.
- In vivo physiology: Utilized data acquisition software (LabChart, AD Instruments) to examine bladder pressure generation and voided volume after bladder catheterization in a rodent.
- In vitro pharmacology: Utilized data acquisition software (LabChart, AD Instruments) to measure smooth muscle contractility from the urinary bladder, urethra, and aorta from rats and monkeys.
- Cell Culture: Utilized aseptic technique to perform primary culture of bladder and vascular smooth muscle cells, and adipose derived stem cells from rats and sheep.
- Histology: Perfomed basic histological techniques including both frozen and paraffin-embedded sections. Completed both brightfield microscopy (ie. H&E, Trichrome) and fluorescent (immunohistochemistry) techniques for semi-quantitation.
- Molecular Biology: Isolated and homogenized proteins for use in Western blotting analysis, and HPLC, MS identification.
- Computer Software: Demonstrated proficiency in Microsoft Office Suite (Microsoft Word, Excel, Powerpoint), Image J (NIH), GraphPad, Chart, ImagePro

PUBLICATIONS
PODIUM PRESENTATIONS


HONORS AND AWARDS

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<td>2010</td>
<td>Graduate Student Travel Award- American Society for Pharmacology and Experimental Therapeutics-Experimental Biology Meeting</td>
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<td>Runner Up, Graduate Student and Post Doctorate poster competition- Society for Pharmacology and Experimental Therapeutics, Integrative and Systems Biology division</td>
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<td>2008</td>
<td>Drug Discovery, Development, and Regulatory Affairs Travel Award- Southeastern Pharmacological Society meeting</td>
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<td>2008</td>
<td>Invited Talk for Wake Forest Graduate Student Research Day Tissue Engineering Panel with Dr. Anthony Atala- “If a rat can do it, why not a human?”</td>
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TEACHING AND MENTORING

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<td>2010-2011</td>
<td>Lectured Physical therapy students on the cellular basis of muscle physiology/dysfunction</td>
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<td>2010</td>
<td>Guest lecturer for pre-nursing students at Winston Salem State University on the urinary system</td>
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<td>Trained and mentored undergraduate and medical students as part of the Wake forest Institute for Regenerative Medicine Summer Scholars Program</td>
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<td>Served on Curriculum committee Wake Forest University Department of Physiology and Pharmacology</td>
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PROFESSIONAL AFFILIATIONS

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<tr>
<td>2008-Present</td>
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<td>Member- National Society of Collegiate Scholars</td>
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EXTRACURRICULAR ACTIVITIES

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<td>2009</td>
<td>Participated in developing ASPET’s Integrative, Systems, Translational and Clinical Pharmacology website.</td>
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<td>2008-2011</td>
<td>Presented over 20 research Poster Presentations at various conferences/meetings</td>
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<tr>
<td>2006</td>
<td>Organized and completed a cross-country cycling trip from Hilton Head, SC to San Francisco, CA.</td>
</tr>
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<td>2000-2001</td>
<td>Volunteered at Volunteers in Medicine Clinic (VIM) in Hilton Head, SC</td>
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