Chapter 9

An Organ Regeneration Platform for Industrial Production of Hollow Neo-Organs

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/59465

1. Introduction

Organ regeneration technologies aim to restore the original structure and functionality of a diseased organ. In general, healing responses within mammals are characterized by fibrosis and scar tissue formation, not regeneration. Nevertheless, developing mammalian fetuses during the first trimester present wound healing without fibrosis and scar tissue formation (Adzick and Lorenz, 1994). Additionally, compensatory hyperplasia of mammalian kidney or liver secondary to partial nephrectomy or hepatectomy, remodeling of epidermis or bone consequent to injury and regeneration of limb digit tips in humans and mice post-amputation are all examples of regenerative outcomes in adult mammals indicative of an innate regenerative potential within adult mammals (reviewed by Roy and Gatien, 2008).

However, model organisms such as *Hydra*, planaria, zebrafish, *Xenopus* and urodeles (salamanders) present the clearest examples of regenerative outcomes secondary to injury. In these systems, cell-based strategies harnessing pluripotent and tissue specific stem cells as well as dedifferentiation have been leveraged to mediate the regeneration of whole limbs and organs (reviewed by Tanaka and Reddien, 2011). Systematic experimentation with limb regeneration in urodeles has permitted the decipherment of key mechanistic pathways of regeneration at the molecular level. Activation of salient signaling cascades including p53, TGF-β, Delta, ppRB and Wnt/β-catenin have all been associated with limb regeneration (reviewed by Roy and Gatien, 2008). These signaling pathways catalyze a sequence of instructive interactions between mesodermal and ectodermal cell populations that are ultimately responsible for lineage specification (reviewed by Wessels, 1977). In addition, the methodical depletion of macrophages within the first 24 hours subsequent to limb amputation in urodeles has been demonstrated to lead to permanent failure of limb regeneration, extensive fibrosis and disregulation of transcriptional patterns associated with synthesis of extra-cellular matrix (ECM)

components (Godwin et al., 2013). This specific sequence of cellular events associated with regeneration of the urodele limb as defined below recapitulates aspects of embryonic organogenesis and may serve as a model system for establishing the existence of similar pathways in mammals:

Stages in regeneration of urodele limb secondary to amputation:

1. The open wound is enclosed by wound epithelium to form a permissive epithelial structure referred to as the apical ectodermal cap (AEC).
2. Up-regulation of matrix metallo-proteinase (MMP) expression catalyzes structural reorganization of the ECM.
3. Dedifferentiation of cell populations takes place proximal to the plane of amputation.
4. Proliferation and migration of dedifferentiated cells is observed under the AEC.
5. Induction of a *blastema*, a mass of mesenchymal cells that will eventually re-differentiate to create the new muscles, bones, nerves and tendons required to regenerate a functional limb.

An understanding of these stages has already been applied to accelerate regenerative outcomes in mammals. For example, application of MMP1 to digit remnants of adult mice with amputation at the mid-second phalanx significantly improved regeneration of soft tissue and observed rates of wound closure. More multi-potent progenitor cells, capillary vasculature and neuromuscular related tissues were also noted (Mu et al., 2013). Furthermore, recent data on regenerative outcomes in mammals from tissue engineering of bladder, esophagus and intestine provides additional evidence of the existence of a regenerative pathway in adult mammals mimicking aspects of that observed in urodeles, including formation of a neoblastema. This regenerative pathway is characterized by a dependence on adequate vascularization and innervation at the site of regeneration. Importantly, these observations provide insight into a potential mechanism of action for tissue engineered products characterized broadly as instructive signaling between mesenchymal cells of the regenerative construct and host epithelial cell populations. This insight may be harnessed to facilitate development of novel neo-organ products. Here, we review recent progress in regeneration of tubular organs with a particular focus on the gastrointestinal tract that highlights how Tengion's Organ Regeneration Platform™ may harness these fundamental regenerative pathways.

2. Tengion's organ regeneration platform

Tubular organ regeneration involves a specific, temporal sequence of cellular infiltration, vasculogenesis, neurogenesis and the defined differentiation of mucosal, stromal and parenchymal laminar tissue architectures (reviewed by Basu and Ludlow, 2010). Strategies for organ and tissue regeneration must therefore achieve the dual objectives of triggering a true regenerative response while ameliorating any tendency towards fibrotic repair. The methodology first pioneered for regeneration of the bladder may serve as a foundational platform for the

http://dx.doi.org/10.5772/59465

regeneration of any tubular organ including penis, vagina, lung, small intestine, stomach and vessels.

Illustrated by Tengion's Neo-Bladder Augment™ (NBA), this approach initially applied committed cell populations of target organ sourced (bladder)-derived urothelial cells (UC) and smooth muscle cells (SMC) complexed with a biodegradable, synthetic scaffold to trigger *de novo* organogenesis (Oberpenning et al., 1999). The NBA is composed of a biodegradable scaffold seeded with autologous, bladder derived UC and SMC. During implantation, the NBA is wrapped with omentum to facilitate vascularization of the developing neo-organ (Figure 1). This is a critical requirement for successful organ regeneration (Basu & Ludlow, 2010; Basu & Ludlow, 2011; Basu & Ludlow, 2012). Analysis of the matured construct at the cellular level highlights committed SMC interacting with polymer fibers of the biodegradable scaffold (Figure 2). Over time (~3 months), a laminarly organized neo-organ develops. Variations of this approach have recently been described, and the key methodological elements arising from these principal recent reports demonstrating *in vivo* regeneration of neo-bladder or neo-bladder related tissue has been reviewed in detail (Basu & Ludlow, 2010; Basu & Ludlow, 2011; Basu & Ludlow, 2012).

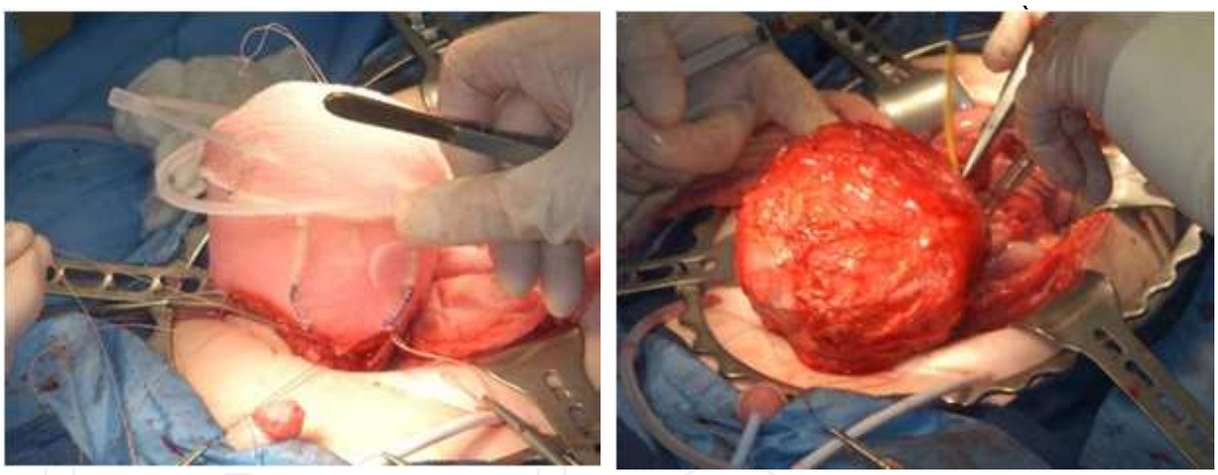

Figure 1. Tengion Neo-Bladder Augment (NBA) during implantation (left) and subsequent to wrapping with omentum (right).

Examination of the temporal sequence of neo-bladder regeneration in canine clinical studies illustrates the dichotomy in outcomes between implantation of acellular and cellularized scaffolds (Jayo et al., 2008a,b). This distinction is a principal theme of most studies describing the regeneration of tubular organs. Cell seeded scaffolds mediated a regenerative response within one month post-implantation, characterized by induction of heavily vascularized, smooth muscle-like parenchyma. In contrast, acellular scaffolds triggered a principally fibrotic, reparative outcome characterized by randomly organized collagen fibers with minimal vascularization. Baseline urodynamics were reconstituted within four months of implantation with cell seeded scaffolds, whereas the urodynamic profile of animals implanted with acellular scaffolds remained abnormal throughout the trial period (Jayo et al., 2008a). In a related dog

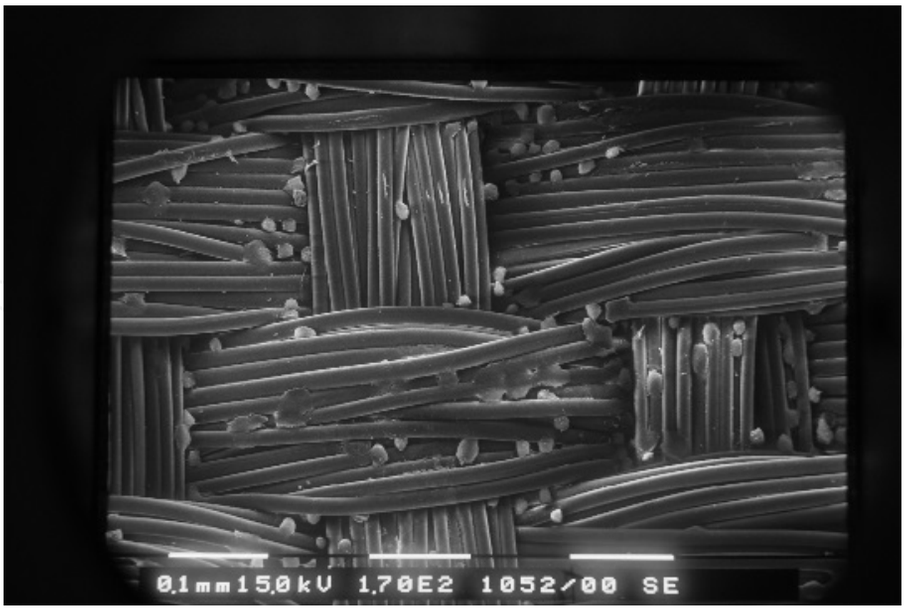

Figure 2. SMC interacting with woven mesh of PGA (poly-glycolic acid), a biodegradable polymer. 170X

study, restoration of tri-laminar bladder wall architecture occurred within three months post-implantation and normal compliance characteristics of a urinary bladder wall developed by 12 months (Jayo et al., 2008b). Regenerated bladders were functionally and structurally stable for up to two years post-implantation (Jayo et al., 2008b). Importantly, although the construct volume was constant at implantation within variably sized dogs, the ratio of the regenerated bladder's volume to host body weight adapted to the recipient animal's size, demonstrating that the neo-organ responds to homeostatic mechanisms regulating organ volume (Jayo et al., 2008b).

Although these canine studies utilized both bladder-derived urothelial and smooth muscle cells, urothelial cells have been shown to *not* be essential for bladder regeneration, thereby greatly facilitating process development and commercialization (Basu & Ludlow, 2010). Neo-urinary constructs seeded only with smooth muscle cells and not urothelial cells nevertheless catalyze complete regeneration of *de novo* urinary-like tissue with patent urothelium, presumably through migration of epithelial cells in *trans* via urine flow or from neighboring tissue (Basu et al., 2012a). However, use of bladder-derived smooth muscle cells is problematic in patients presenting with bladder related malignancies. Therefore, a number of alternate sources of smooth muscle cells have been investigated. Such alternate cell sources may have broad application in the regeneration of additional, laminarly organized tubular organs. Tengion has identified adipose and peripheral blood as alternate sources of smooth muscle cells usable for induction of neo-urinary tissue (Basu et al., 2011a; Basu et al., 2012a). As with other early signaling events during organogenesis or regeneration, instructive interactions between this mesenchymal cell population associated with the regenerative construct and epithelial cell populations within the host are understood to be critical for observed regenerative outcomes.

http://dx.doi.org/10.5772/59465

3. The need for urinary neo-organs

At its most fundamental level, the bladder is responsible for mediating storage and subsequent efflux of urine within a dynamically expandable and contractable container. Though relatively simple in terms of overall histology and structural organization (Figure 3), diseases impacting the bladder have the potential to significantly affect individual quality of life, resulting in continual incontinence or inability to effectively void urine as needed. Several congenital anomalies may result in abnormal bladder development requiring surgical intervention, including posterior urethral valves, bilateral ectopic ureters, bladder extrophy, cloacal extrophy, and spina bifida (myelomeningocele). The resultant clinical outcomes include incontinence and increased risk of renal failure from high pressures in the genito-urinary system.

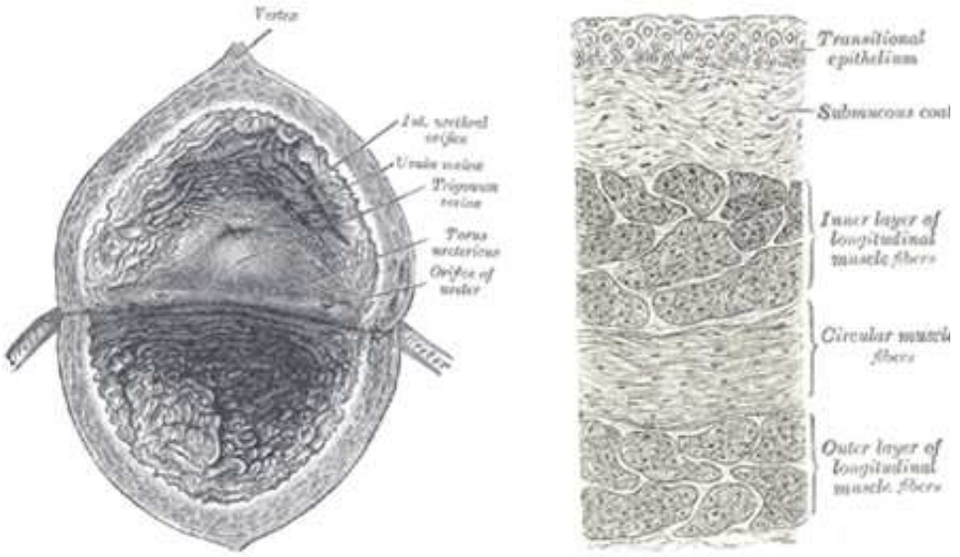

Figure 3. Macroscopic (left) and histological (right) organization of the bladder, illustrating tri-laminar cellular architecture of this hollow, muscular organ. Reproduced from Wikipedia Commons (Open Access) under terms of Open Access agreement.

The current standard of therapy for pediatric patients is bladder augmentation through enterocystoplasty, a procedure which involves the surgical removal of a section of large bowel that is then connected to the existing bladder to increase compliance, decrease pressure, and improve overall urine capacity. These surgeries are relatively complex and costly. Even in patients with good technical outcomes, the procedure is associated with numerous immediate risks and potential chronic complications. A similar surgical procedure is performed in adults requiring bladder replacement, typically secondary to the onset of bladder related malignancies. To this end, cancer of the bladder may be manifested as a broad spectrum of disease presenting across distinct bladder compartments.

The most common types of bladder cancers originate from the internal epithelial lining of the bladder, the urothelium. Less common cancers of the bladder may include squamous cell

carcinoma, adenocarcinoma, sarcoma, and small cell carcinoma. Estimates predict that almost 71,000 new cases of bladder cancer will be diagnosed annually in the USA, with some 14,000 patients succumbing to the disease. Although many patients will have superficial urothelial tumors involving little or no metastasis within the smooth muscle layer, approximately 25% of bladder cancers will invade the detrusor musculature, with the majority of these presenting initially as invasive cancers with metastatic potential.

Invasive cancers often require multi-modality therapy which may involve extirpative surgery, i.e. radical cystectomy and construction of a urinary diversion. Despite the risk of adverse effects, there are approximately 10,000 of these procedures performed per year in the United States, including in 10% of children with congenital bladder abnormalities and 90% of adults with acquired disorders such as bladder cancer. In some cases of severe bladder cancer or other pelvic or abdominal cancers, removal of the entire bladder is indicated. In these circumstances, current standard of care also involves reconstruction of a bladder-like replacement using bowel tissue. Application of bowel tissue for reconstruction of urinary neo-organs is clearly problematic for a number of fundamental reasons, including the fact that bowel is a principally absorptive organ, whereas bladder is designed to store and excrete urine. Exposure of bowel tissue to urine or bladder tissue to bowel-derived micro-organisms has the potential to trigger multiple secondary complications. These may include any of the following:

- **Bowel complications**. Early complications are usually related to the bowel surgery required to harvest tissue for reconstructive use and typically consist of leaks, fistulas and obstructions. Because vitamin B12 is absorbed in the bowel tissue, loss of this tissue can result in anemia and neurologic abnormalities. Additionally, mal-absorption of salts and lipids can lead to diarrhea. Patients with neurogenic bladder are prone to bowel movement problems even before surgery and the removal of bowel tissue may either exacerbate existing conditions or create new motility problems. These difficulties further contribute to substantial physical and psychological morbidity within these patients.

- **Absorption issues**. Use of bowel tissue often leads to electrolyte and metabolic imbalances, which can cause bone loss. Furthermore, certain drugs taken by the patient may be reabsorbed by the implanted bowel tissue, potentially leading to unintended toxic levels of these substances within the bloodstream. The exposure of intestinal surface to urine also results in the inappropriate absorption of ammonium, chloride and hydrogen ions as well as potassium loss, leading to chronic metabolic imbalances or abnormalities.

- **Infection**. Persistent and recurrent infections are typical in patients with bowel tissue reconstruction. Such infections may reach the kidney and become life-threatening. Additionally, bacteria normally found in bowel tissue can serve as a source of infection and septic complications when repositioned into the urinary tract.

- **Stone formation**. One of the consequences of persistent infection is the development of stones. Stones are hard masses which can cause pain, bleeding, obstruction of urine or infections.

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- **Mucus**. Bowel tissue, when repositioned in the urinary tract, continues to secrete mucus. Mucus increases the risk of stone formation and the viscosity of urine, and in the case of bladder augments, may require bladder irrigation and more frequent catheterization.
- **Cancer**. Malignancy, although rare, is a well-recognized complication following bladder augmentation using bowel tissue (enterocystoplasty), as well as from other reconstructive surgeries that incorporate bowel segments into the genitourinary tract.

In addition to cancers and developmental abnormalities, patients may present with neurogenic bladder or dysfunctional bladder due to some form of neurologic disease or condition. Treatment may often require an augmentation of the bladder in order to relieve high pressures and incontinence. Current therapies for neurogenic bladder include medical management through a combination of medication and clean intermittent catheterization and, in advanced cases, surgery. Surgical procedures, such as bladder augmentation, are often considered when other medical and less-invasive treatments fail to adequately lower bladder pressure or reduce the frequency of incontinent episodes.

Ultimately, it is self-evident that the ideal unit of anastamosis for urinary-like tissue is other urinary-like tissue. However, the lack of such material has generally precluded the widespread leveraging of this option. There is therefore clearly a compelling medical need for an improved approach that would eliminate or at least substantially reduce the complications potentially associated with the current standard of care. To this end, identification of bladder-like materials that may be applied towards bladder reconstruction in place of bowel tissue has been attempted. Pilot experiments in 1917 to augment bladder in dogs leveraged fascia (Neuhof, 1917); since then, numerous other biomaterials candidates have been evaluated, including skin, bladder sub-mucosa, small intestinal sub-mucosa, omentum, dura and peritoneum. Synthetic materials candidates have included polyvinyl sponge, Teflon, gelatin, collagen, vicryl and silicone. Failure to achieve successful outcomes with such biomaterials types may be attributed to physical or mechanical failure, lack of biocompatibility and the induction of fibrosis and scarring leading to contraction of the implant and reduction in effective volume over time. Unfortunately, of these candidates the best biomaterial mimicking the physical and mechanical properties of bladder tissue has been bowel. There is therefore a clear and present need for additional, novel technology platforms.

3.1. TE/RM methodologies for bladder replacement and augmentation

Tissue engineering and regenerative medicine approaches offer an alternative, potentially superior methodology to the use of bowel tissue for urinary diversion or replacement. In this methodology, the patient's own cells would be sourced from a bladder biopsy (or other, alternate cell source, see below) and applied to an appropriate, degradable biomaterial scaffold to create a neo-organ or organ-like construct that, upon implantation within the patient and anastamosis to native components of the urinary system, would lead to regeneration of functional, urinary-like neo-tissue capable of storing urine and mediating voiding of urine as needed in response to appropriate neuronal signaling. Such a cell/biomaterial construct would catalyze the regeneration of urinary-like neo-tissue recapitulating native, laminarly organized

bladder wall histo-architecture composed of a luminal urothelial layer and multiple smooth muscle layers, appropriately vascularized and innervated (Figure 3). Regeneration of urinary-like neo-tissue is accompanied by progressive degradation of the biomaterial, such that a seamless transition is achieved between the degrading biomaterial and the regenerating urinary-like neo-tissue.

In preliminary experiments to demonstrate the formation of tissue engineered urothelial-like structures in the rabbit, bladder-derived urothelial cells were used to seed meshes of non-woven polyglycolic acid (PGA), which were subsequently implanted within the peritoneal cavity of athymic mice. Upon recovery, structures composed of degrading biopolymer lined with urothelial cells were observed (Atala et al., 1992). In follow-up studies, combinations of bladder-derived smooth muscle cells and urothelial cells were used to seed tubular-like structures composed of non-woven PGA mesh. Implantation of these constructs subcutaneously within athymic rabbits led to regeneration of urinary-like tubular organoids composed of urothelial cells lining a central lumen and surrounded by layers of smooth muscle cells, as observed within native bladder tissue. Evidence of neo-vascularization was also noted (Atala, et al., 1993). These studies provided preliminary proof of concept to support the potential for *in vivo* regeneration of urinary-like neo-organs through implantation of cell seeded, synthetic bio-polymeric scaffolds.

Although current strategies for creation of bladder-like neo-organs leverage principally non-bladder cell sources, initial work on neo-bladder tissue engineering was dependent on patient-derived bladder biopsies as a source of urothelial and smooth muscle cells. For this approach to be commercially viable, the expansion dynamics of cellular growth for both biopsy-derived urothelial and smooth muscle cell populations must be established. Although smooth muscle cells could be reliably expanded from small bladder biopsies without difficulty, the demonstration that a single biopsy-derived source of bladder urothelial cells could also be expanded to the numbers required for effective seeding of urinary neo-organs was critical for establishing the preliminary bioprocess potential of this methodology (Cilento, et al., 1994). The alternative would involve multiple biopsy sampling to generate sufficient cell numbers for urinary neo-organ seeding, greatly decreasing the attractiveness of this technology for practical application in the clinic.

From a biomaterials perspective, the application of synthetic biopolymers such as PGA for the seeding of urothelial and bladder-derived smooth muscle cells permitted development of modified polymers with continuously tunable physical and mechanical characteristics. To this end, the temporal sequence of polymer hydrolysis may be manipulated by altering the nature and sequence of individual monomer units. In addition, coating by other polymers such as PLGA may be applied to further fine-tune the physical properties of the biomaterial scaffold. Finally, the open, fibrous networked structure of the biomaterial (Figure 4) facilitates angiogenesis and neo-vascularization of the developing neo-organ. Taken together, this binary cell/synthetic biopolymer construct was the foundational technology platform needed for initiating large animal clinical trials and proof-of-concept trials in man.

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Figure 4. Biodegradable PGA felt, with open "spaghetti"-like structure. 170X

3.2. Demonstration of Neo-Bladder formation in large animals

Subsequent to initial proof-of-concept studies showing *de novo* regeneration of urinary-like structures in a small animal model (rabbit), the next step in successful process development is evaluation of the technology in a large animal clinical model such as swine or dog. For the Neo-Bladder Replacement, a key proof-of concept study was provided using a canine total cystectomy (bladder removal) model. 14 beagles were sub-divided into three groups: A (n=2), underwent cystectomy without further intervention. B (n=6) underwent cystectomy followed by implantation with acellular, bladder-shaped biopolymer scaffold and group C (n=6) was subjected to cystectomy and implantation with cell-seeded biopolymeric scaffold. To create the constructs, PGA/PLGA biomaterial scaffolds (Figure 1) were shaped by hand into bladder-shaped structures. Urothelial and smooth muscle cells were sourced from bladder biopsies and expanded separately to numbers sufficiently meaningful to warrant seeding onto the scaffolds (10^6 cells per cm^2 of polymer surface). Urothelial cells were used to seed the interior, luminal surface of the construct, and smooth muscle cells were applied to the exterior, non-luminal surface.

Upon maturation, these constructs were implanted within group C animals. Implanted neo-bladder constructs were wrapped extensively with omentum during surgery, as omentum is a well-established source of pro-angiogenic growth factors (Litbarg, et al., 2007). All animals were monitored over a period of up to 11 months post-implantation and extensive urodynamic and radiographic measurements taken to document urologic functionality. At the completion of the study period, animals were sacrificed and bladder-like structures examined histologically for evidence of tri-laminar bladder wall architecture from regenerated neo-organs. Structural and functional differences in regenerative outcomes were observed between all three groups of canines. Group A dogs regained only minimal urinary reservoir volumes

without approaching pre-cystectomy parameters. Group B dogs presented principally fibrotic neo-bladders with a regenerated luminal urothelial cell layer but minimal regeneration of bladder wall musculature. Urological dynamics were also significantly affected. This ability of acellular constructs to regenerate a normal luminal urothelial cell layer is noteworthy, providing a preliminary indication that prior seeding of constructs with urothelial cells may not in fact be a requirement to achieve full regeneration of native-like urinary neo-tissue from the implanted neo-organ construct. We will re-visit this observation and its implications later in this chapter. However, canines implanted with cell seeded, bio-polymeric neo-bladder constructs were able to regenerate histologically and functionally appropriate neo-bladders with native-like tri-laminar wall histo-architecture and native-like urodynamics (Oberpenning, et al., 1999).

It may also be useful at this point to clearly define functional outcomes that are commonly referred to as "regenerative" versus "reparative" in the context of TE/RM. Organ regeneration involves the replacement and restoration of cellular components and fully developed tissue mass as well as the reconstruction of the organizational, architectural and functional characteristics associated with the particular organ. In this regard, outcomes observed with group C canines above are principally regenerative. In contrast, a reparative outcome is associated with incomplete tissue replacement and may be distinguished by the extensive deposition of collagen and concomitant fibrosis. Outcomes observed with group B canines represent principally reparative pathways. This study and many others demonstrate clearly that regenerative outcomes are mediated by cell seeded constructs only. Acellular constructs are unable to catalyze neo-organ regeneration. This is an important foundational principle for continued development of neo-organ regeneration technology.

Analysis of the dynamics of neo-bladder regeneration in subtotal cystectomized canines serves to further illustrate the dichotomy in outcomes between implantation of acellular and cellularized scaffolds. In another such study, bladder shaped scaffolds composed of woven PGA felt or PLGA (poly-lactic-*co*-glycolic acid) seeded with autologously sourced bladder-derived urothelial cells and smooth muscle cells facilitated a regenerative response within one month post-implantation, as characterized by induction of an extensively vascularized, smooth muscle-like parenchyma. In contrast, acellular PGA/PLGA scaffolds triggered a principally fibrotic, reparative outcome featuring disorganized collagen fibers with minimal vascularization. Baseline urodynamics were reconstituted within four months post-implantation with cell-seeded scaffold, whereas urodynamic profiles of animals implanted with acellular scaffolds remained abnormal throughout the nine-month study (Jayo, et al., 2008).

In a related cystectomized canine study, native-like tri-laminar bladder wall tissue architecture was observed at three months post-implantation with a bladder shaped non-woven PGA felt scaffold seeded with $1.5X10^8$ each of autologously sourced bladder-derived urothelial cells and smooth muscle cells, and normal compliance characteristics of a urinary bladder had developed by 12 months. Regenerated bladders in animals receiving these cell-seeded scaffolds have shown functional and structural stability for up to two years post-implantation. Importantly, although the volume of the cell-seeded scaffold was held constant in this particular study, implantation of the construct within dogs of different sizes that had gained varying amounts

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of weight over the course of the study yielded organs that, as measured by the ratio of bladder capacity to body weight, adapted to the individual recipient animal's size, demonstrating that the regenerated neo-organ was capable of responding to homeostatic mechanisms regulating organ volume (Jayo, et al., 2008).

3.3. Neo-bladder replacement in human pediatric patients-1st clinical trials of a neo-organ

Studies in canines as outlined above established proof-of-concept for the application of cell seeded biodegradable polymeric scaffolds for regeneration of functional, native-like neo-bladders in a large animal cystectomy model (Oberpenning, et al., 1999). Additional data suggested that smooth muscle cells sourced from diseased bladder could potentially be applied successfully to regenerate neo-urinary tissue (Lai, et al., 2002). These data laid the groundwork for initiation of a proof of concept clinical trial in man. In this seminal study, seven pediatric patients presenting with myelomeningocele (a form of spina bifida) were recruited to receive the first ever human neo-organ implants. As previously described in canines, both urothelial and smooth muscle cells were isolated and expanded from autologously sourced bladder biopsies. Up to 5 cell passages over 7-8 weeks was required to generate enough cells to seed the neo-bladder scaffold. Using a sterile pipette, the scaffold exterior was seeded with bladder-derived smooth muscle cells at a seeding density of $5X10^7$ cells/cm^3. After a 48hr recovery period, the luminal surface of the scaffold was seeded with urothelial cells at a density of $5X10^7$ cells/cm^3. The construct was matured in a tissue culture incubator at 37 degrees C for 3-4 days, prior to implantation. Subsequent to implantation, the engineered neo-bladder was cycled (i.e. subjected to serial volume expansion and contraction) as part of regular post-operative care for up to 3 weeks post-implantation; the mechanical forces induced across the neo-bladder during cycling have been found to augment regenerative outcomes. Engineered neo-bladders were found to functionally rescue urologic dynamics and were associated with tri-laminar bladder wall architectures upon histological examination of bladder biopsies recovered at 31 months post-implantation. A number of different scaffold iterations were evaluated, with changes being made over the course of the study to accommodate new data being made available from this and other related studies. Ultimately, an omentum wrapped, collagen/PGA scaffold was found to present best overall regenerative outcomes (Atala, et al., 2006).

3.4. Definition of the cell source used for seeding Neo-Bladders: Adipose

Adipose tissue contains a heterogenous mix of cell types, including adipocytes, endothelial cells, pericytes, SMC, and MSC. Adherent cells that were derived from adipose tissue using different conditions have been referred to as MSC without applying a systematic approach to defining the isolated cell composition; however, distinctly different cell types can share phenotypic characteristics. As more cell-based therapies move from preclinical to clinical evaluation, establishing a cell composition profile that provides a degree of distinction from other cell types will likely become increasingly important for protecting intellectual property rights, gaining regulatory approval, and scaling up for manufacturing. The relevance of cell type characteristics defined from analysis of native tissue or initial cell isolates to expanded

cell populations is unclear because gene and protein expression patterns can be altered by isolation and *in vitro* expansion. Such differences have been documented for MSC and we have observed the same to be true for adipose-sourced smooth muscle-like cells (Ad-SMC).

The more important questions from a product development perspective are what characteristics are associated with the cell population that will be administered and how indicative are those characteristics with regard to cell identity and product potency. Our comparison of Ad-SMC and MSC leveraged established definitions of SMC and MSC and focused on the analysis of smooth muscle phenotype, growth kinetics, *in vitro* tri-lineage differentiation bioactivity and functional responses to small molecules that affect SMC-specific signaling pathways. Unlike methodologies for MSC, isolation and expansion of Ad-SMC is specifically promoted by growth of adipose SVF derived cells at low cell densities in the absence of positive/negative selection through magnetic bead based separation, inductive cytokines or growth factors, high serum (>10%) concentrations or preselected lots of serum. The specific influences of media formulations on isolation and expansion of cells with MSC-like bioactivity has been extensively documented (Basu, et al., 2011). Expansion of Ad-SMC is therefore the default outcome from culture of SVF-derived cell populations in principally basal media formulations at high cell densities not supplemented by inductive cytokines, preselected lots of serum or high concentrations of FBS. This property greatly facilitates process development and manufacturing.

From where do Ad-SMCs originate and what is their relationship to MSC? Adipose is a heavily vascularized tissue and a number of studies have implicated the perivascular niche as a potential source of both MSC as well as smooth muscle and endothelial cells. Pericytes with MSC differentiation potential have been isolated directly from blood vessels as well as from multiple organ systems throughout the body. However, although ACTA2/SMAA+cells have been localized to all capillaries, arterioles and venules of the adipose-derived vascular bed, expression of STRO-1, a key MSC-specific marker, is tightly associated with endothelium and additionally found only within a subset of blood vessels. Furthermore, expression of the stem cell-specific markers Oct4 and telomerase was observed only rarely, suggesting that truly pluripotent progenitors are uncommon within adipose. In their entirety, these observations point to MSC, endothelium, and smooth muscle occupying distinct spaces within the broader peri-vascular niche. Nevertheless, there remains the potential for considerable ebb-and-flow across developmental lineages. For example, endothelial cells appear capable of lineage switching towards a smooth muscle cell like phenotype in response to TGF-β or the depletion of pro-angiogenic factors and loss of endothelial cell-cell contact. In addition, adherent cell types with endothelial and smooth muscle phenotypes as well as limited mesenchymal differentiation potential have been identified to circulate in adult peripheral blood. Such circulating smooth muscle cells may contribute to the population of adipose-derived smooth muscle cells, although we have been unable to purify them directly from human adult peripheral blood in meaningful numbers (our unpublished observations).

Given that MSC in long term culture also follow a smooth muscle cell-like differentiation pathway, we believe that taken together, the published data as well as our observations are consistent with the peri-vascular niche of adipose SVF as a source for a broad continuum of smooth muscle cells, smooth muscle progenitors, MSC and partially lineage committed MSC-

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like cell types with variable and overlapping degrees of proliferative and differentiation potential. Modulation of SVF derived cell phenotypes and functionality away from MSC and towards SMC is possible through selection of media formulations during cell isolation and expansion. Our studies provide methodological validation for the utility of non-bladder sources of smooth muscle-like cells for applications in urologic regenerative medicine, thereby bypassing the potential for isolation and expansion of transformed SMC associated with bladder biopsies derived from patients presenting with bladder-related malignancies.

3.5. Other approaches to tissue engineering Neo-Bladders

The sequential process development of the smooth muscle cell seeded/biodegradable synthetic biopolymer scaffold neo-bladder regenerative platform as outlined above represents by far and away the most clinically advanced such neo-organ product candidate developed to date. No other methodology has even come close to clinical trials in humans or to commercial manufacture. However, it is still worthwhile at this point to critically examine some alternative approaches that have been presented in the literature and discuss why these have proven to be ultimately unsuitable for commercialization.

From a biomaterials perspective, we have already discussed why the application of synthetic, biodegradable polymers is preferable for industry. Briefly, such biomaterials may be sourced or manufactured with defined chemical composition and offer reliable and reproducible physical and mechanical properties that are continuously tunable by modification of basic polymer chemistries. In contrast, naturally sourced biomaterials such as bladder-derived sub-mucosa present variable chemical compositions that are donor specific, and may not be readily sourced, as is in fact the case with human bladder-derived sub-mucosa.

This has led to the use of xenogeneically sourced biomaterials as substitute. Notwithstanding the fact that porcine derived intestinal sub-mucosa is an established product for multiple surgical applications, scaffolds made of decellularized bladder sub-mucosa were actually evaluated in the first human neo-bladder clinical trials head to head against scaffolds composed of principally synthetic polymers (Atala, et al., 2006). The most favorable regenerative outcomes were generated from implanted neo-bladders composed principally of PGA, whereas the least favorable regenerative outcomes were observed within patients implanted with neo-bladders constructs composed of decellularized bladder sub-mucosa. The course of the clinical trial was appropriately modified to incorporate these findings. Taken together, these observations reinforce the application of synthetic polymers over natural sourced biomaterials for commercial viability.

From a cell sourcing perspective, a number of investigators are actively exploring the utility of smooth muscle cells derived by the directed differentiation of MSC isolated from bone marrow or adipose. A key characteristic of such stem and progenitor cell populations is a requirement for exposure to combinations of exogenous growth factors, extracellular matrix components, or other defined factors to drive differentiation along defined developmental lineages. For example, in one recent report, adipose-derived MSC was differentiated into smooth muscle-like cells using inductive media containing 100U/ml heparin for up to 6 weeks prior to seeding polymeric bladder dome-like scaffold structures that demonstrated some

evidence of functionality in a rat cystectomy model (Jack, et al., 2009). In additional, related studies, TGF-β or small molecule agonists targeting the TGF-β signaling pathway (e.g., sphingosyl-phosphorylcholine, bradykinin and angiotensin II) have also been used to induce a smooth muscle-like phenotype from adipose or bone marrow derived MSC (Gong, et al., 2008; Kim, et al., 2008a; Jeon, et al., 2006; Kim, et al., 2008b). In a less targeted approach, epigenomic reprogramming with the DNA demethylating agent 5-azaC, has been used to direct bone marrow-derived MSC towards a cardiomyocyte-like phenotype (Xu,, et al., 2004). Dedifferentiated adipocytes may also be driven along a smooth muscle lineage using TGF-β and have been reported to contribute towards bladder tissue regeneration in a mouse bladder injury model (Sakuma, et al., 2009). Finally, methods for TGF-β induced differentiation of smooth muscle cells from bone-marrow derived cells have been described (Kanematsu, et al., 2005; Becker, et al., 2008). These studies invariably involve demonstrations of *in vitro* directed differentiation of SMC-like cells derived from MSC, using various combinations of growth factors and cytokines over an extended time period. Any *in vivo* data is typically based in small animals, is often subcutaneous and therefore of little relevance to product development and manufacturing.

Finally, the use of the peritoneal cavity as a living bioreactor for maturation of tubular organs has been proposed. It is well established that implantation of foreign material into the peritoneal cavity may frequently trigger a fibrotic response, with encapsulation of the foreign matter by multiple layers of fibroblasts. We have observed similar, non-regenerative outcomes associated with implantation of certain synthetic biopolymers such as poly-caprolactate within the renal parenchyma (Basu et al., 2012). However, this normally unwelcome outcome might be leveraged for induction of tubular neo-tissue formation.

In the first such demonstration of this approach, silastic tubing was implanted into the peritoneal cavity of rats/rabbits. At 2 weeks post-implantation, the tube was observed to be coated with several layers of myofibroblasts, collagen and a layer of mesothelium. Removal of the silastic tube followed by eversion of the cellular construct resulted in formation of a vessel-like structure, with laminar wall architecture mimicking that of native vasculature. Anastamosis of this neo-vessel with the vasculature of the original host animal demonstrated evidence of function and patency for up to 4 months post-implantation (Campbell, et al., 1999).

Similarly, neo-organs resembling bladder, uterus and vas deferens could be engineered by grafting biomaterials templates of appropriate shape within the peritoneal cavity of rabbits. After 2-3 weeks of implantation within the environment of this "living bio-reactor", neo-organ like frameworks composed of myo-fibroblastic layers could be harvested and grafted onto the appropriate organs of the original host to demonstrate functionality and eventual regeneration of urinary-like tissue (Campbell, et al., 2008). Although interesting, it is difficult to envision how such a strategy can ever be feasible in practice. Clearly, undergoing major surgery and all associated post-operative care and monitoring simply to create the neo-organ implant will not be an attractive option, given the alternatives currently under development. Therefore, we do not anticipate that tissue engineering of bladder using the peritoneal cavity as a living bioreactor will become a commercially viable strategy.

3.6. Key learnings from development of Neo-Bladder: Factors facilitating commercial viability of organ regeneration platform

The Neo-Bladder experience at Tengion highlights certain key factors that may impact the commercial success of an organ regeneration platform. These same principles are pertinent to discussion of non-bladder tubular organs as well as to regenerative technologies targeting solid organs such as kidney, liver and pancreas. We outline these principles below:

1. A biodegradable, synthetic scaffold based on well-characterized biomaterials (e.g., PGA, PLGA) that can be manufactured reproducibly with defined characteristics is desirable.
2. A population of committed cell types (e.g., smooth muscle cells) that is easily isolatable and expandable is required for scaffold seeding to trigger regeneration *in vivo*.
3. A purified population of defined stem cells in neither needed nor desirable. Cost of goods increases significantly with requirements to monitor stem cell potential and to control directed differentiation.
4. Engineering of an entire organ *in vitro* or within the peritoneal cavity is neither needed nor desirable. Instead, an *in vitro* cell seeded scaffold is adequate to trigger the innate regenerative response in the mammalian body, resulting in *de novo* organogenesis *in vivo*, including in humans.

4. Neo-urinary conduit: Introduction

The possibility of creating a TE/RM product facilitating urinary diversion without leveraging native GI tissue was the rationale underlying the development of the Neo-Urinary Conduit (NUC). In its simplest configuration, the NUC is a cell/scaffold construct that, upon implantation within the body and attachment to native ureters, mediates efflux of urine from the kidneys directly to the external surface of the body. As with the Neo-Bladder Replacement and related TE/RM products, the NUC construct serves as a template to catalyze the regeneration of native-like urinary tissue concomitant to degradation of the biomaterial scaffold following implantation.

4.1. GLP preclinical analysis of de novo neo-urinary conduit formation in a porcine cystectomy model

32 Gottingen swine with total cystectomy and incontinent ureterostomy (8 animals per data point composed of 4 males and 4 females each) were used in a GLP preclinical analysis to determine the safety and functionality of tissue-engineered NUC constructs seeded with autologous smooth muscle cells derived from the bladder, blood or adipose tissue. Of the 32 animals, the first group (4 males, 4 females) was implanted with NUC seeded with bladder-derived smooth muscle cells. A second group was implanted with NUC scaffold seeded with adipose-derived SMCs, a third group was implanted with a NUC scaffold seeded with blood-derived SMCs, and the 4th group was implanted with unseeded NUC scaffold only. Device

effect and performance was monitored through ultrasound imaging, pyelogram, as well as urine and blood analysis at different time-points of the study. At the completion of the recovery period (Day 84+/-5), all animals were euthanized and a necropsy performed for harvesting the kidneys, conduits, and associated organs and tissues for histological preparation and pathological examination.

4.2. Alternate cell sourcing of SMC for seeding of NUC

Tissue engineering principles have been successfully applied to provide implantable cell-seeded matrices for use in the reconstruction, repair, augmentation or replacement of laminarily organized luminal organs and tissue structures, such as a bladder or a bladder segment or component, typically composed of urothelial and smooth muscle layers. Smooth muscle cells may be derived from the patient's own tissue, including the bladder, urethra, ureter and other urogenital tissue. However, there are challenges associated with dependence upon the development and maintenance of cell culture systems from the primary organ site as the basic unit for developing new and healthy engineered tissues, as for example during treatment of cancerous bladder tissue. Clearly, such cancerous cells are inappropriate for populating an implantable neo-bladder scaffold or matrix. We have therefore attempted to identify and characterize alternative sources of smooth muscle cells capable of reconstituting urologic characteristics de novo upon implantation on appropriate synthetic, biopolymer scaffold constructs, and to demonstrate functionality *in vivo*.

Numerous studies have indicated that adherent, fibroblast-like cells with typical smooth muscle cell characteristics may be recovered from the mononuclear fraction of peripheral blood, cord blood or bone marrow. These smooth muscle-like cells may be pericytes, which are recruited to developing arterioles, capillaries and venuoles. Additionally, it is well established that adipose tissue is a readily available source of adherent cell types and may therefore also represent an alternative source of smooth muscle cells useful for urologic application. Indeed, adipose isolated during abdominoplasty precedures is rich with capillaries, providing a potential source of isolatable smooth muscle-like pericytes. Although smooth muscle cells have also been isolated from other tissue sources such as skeletal muscle and omentum, we chose to focus on recovery of smooth muscle cells from peripheral blood and adipose, as these represent the source tissue with maximum potential clinical utility. A porcine cystectomy model was selected to evaluate the performance of peripheral blood and adipose-derived smooth muscle cells relative to bladder-derived smooth muscle cells upon application in a cell/scaffold composite.

Direct plating of the peripheral blood-derived mononuclear fraction from swine resulted in outgrowth of colonies with typical smooth muscle cell morphology. 100% of animals screened (n=24) generated smooth muscle cell colonies, with 2.44 $X10^3$-2.37 $X10^6$ smooth muscle cells recovered at passage zero from 50mls of peripheral blood. Recovery of smooth muscle cells was unaffected by changes in media formulation, cell density or surface coatings (data not shown). A similar approach was used to investigate the potential application of subcutaneous or lipoaspirate-derived adipose as a source of smooth muscle cells. The stromal-vascular fraction (SVF) of adipose represents a heterogenous population of cells including endothelial

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cells, smooth muscle cells as well as progenitor cells with limited mesenchymal potential. We were able to generate colonies (expandable into monolayers) of smooth muscle cells from porcine adipose with 100% efficiency (*n*=24), with a cell recovery rate of 1.37 $X10^5$-4.36 $X10^5$ cells/g adipose tissue. In comparison, smooth muscle cells could be isolated from bladder tissue with a recovery rate of 1.29 $X10^6$-9.3 $X10^6$ cells/g bladder tissue. Expansion of smooth muscle cell colonies from peripheral blood or adipose resulted in the formation of a cell monolayer with a typical whirled, "hill-and-valley" organization characteristic of cultured bladder-derived smooth muscle cells (Figure 5). Enrichment of smooth muscle cells was facilitated by use of high cell densities and high glucose media, which has been shown to specifically select against the growth and expansion of mesenchymal stem cells (Basu, et al., 2011).

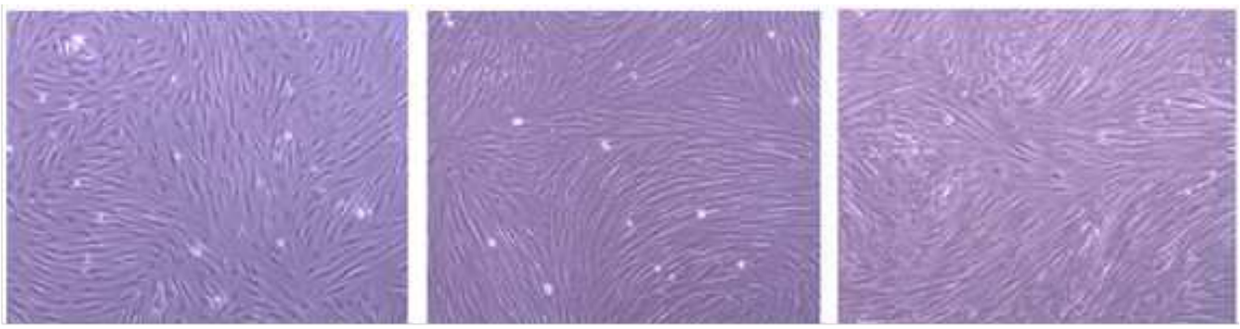

Figure 5. Healthy SMC in culture present a well-established appearance of flattened, spindle-like, fibroblastic morphology, with characteristic "hill-and-valley" organization.

Analysis of the functional properties of peripheral blood or adipose-derived smooth muscle cells *in vitro* demonstrates that they are indistinguishable from bladder-derived smooth muscle cells. Increased expression of proteins associated with smooth muscle contractility is a characteristic feature of smooth muscle cell differentiation and maturation. Myocardin is a key transcription factor required for smooth muscle cell differentiation and acts to mediate the expression of smooth muscle markers essential for contractility including SM22, α-smooth muscle actin (SMαA), smooth muscle myosin heavy chain (SMMHC) and calponin (CNN). Expression of the smooth muscle markers SMMHC and CNN is generally regarded as diagnostic of mature smooth muscle cells.

Application of adipose and peripheral blood-derived smooth muscle cells for urologic regenerative medicine is contingent on being able to secure adequate cell numbers within an acceptable time frame. Towards this end, we have observed that smooth muscle cell colonies (from a 50ml sample of porcine peripheral blood or 7-25g porcine adipose) are identifiable within 7 days post seeding, and may be passaged within 14 days. In fact, tens of millions of smooth muscle cells may be recovered from bladder, peripheral blood or adipose within 2-4 weeks (*n*=24). Bladder and adipose-derived smooth muscle cells were expanded for 2 passages prior to harvesting of cells for seeding a synthetic, neo-urinary conduit scaffold. Peripheral blood-derived smooth muscle cells were expanded for 3-4 passages to generate equivalent cell numbers. On average, 30-40 $X10^6$ smooth muscle cells were used to seed a neo-urinary conduit scaffold.

We have previously shown that bladder-derived smooth muscle cells may be used to seed a synthetic, biopolymer scaffold which upon implantation into an *in vivo* clinical model of bladder cystectomy resulted in the regeneration of a fully functional *de novo* bladder augment (Jayo, et al., 2008a,b). However, because use of bladder-derived smooth muscle cells may not be clinically ideal, we proceeded to evaluate the *in vivo* clinical efficacy of peripheral blood and adipose-derived smooth muscle cells in a 3 month porcine clinical model of urinary incontinence. Bladder, adipose and peripheral blood-derived smooth muscle cells were used to seed PGA/PLGA-based scaffolds to create a regenerative, neo-urinary conduit permitting efflux of urine from the ureters directly to the external body surface.

We observed that constructs composed of smooth muscle cells obtained from blood or bladder sources regenerated a patent conduit composed of an urothelial cell lining and smooth muscle layer that did not result in alterations to the upper urinary tract. No evidence was found for elevated creatinine, metabolic abnormalities or altered hematological parameters. Histological characteristics of the regenerated urological tissue forming the neo-urinary conduit were generally similar regardless of the origin of the smooth muscle cell population. In contrast, scaffold-only implanted animals developed patent urothelial-lined conduits composed primarily of fibrous connective tissue and limited smooth muscle development. This group also had a high frequency of hydroureter and hydronephrosis. In both groups, early post-operative management of the conduit lumen and stoma was required to maintain patency for the study duration (Basu, et al., 2012).

This study demonstrates that a synthetic, biopolymeric scaffold composite seeded with autologous smooth muscle cells derived from multiple potential cell sources (blood, fat or bladder) is capable of being used to re-create a patent neo-urinary conduit. The regenerated conduit was not associated with sequela commonly found with urinary diversions generated from intestinal tissue or with non-cell seeded synthetic scaffolds. This ability to create urologic structures *de novo* from synthetic scaffolds seeded by peripheral blood or adipose-derived smooth muscle cells will greatly facilitate the translation of urologic tissue engineering technologies into clinical practice.

4.3. Clinical trials of NUC

Based on successful outcomes in GLP porcine cystectomy models as outlined above, Tengion has initiated Phase I clinical trials of NUC constructs in human patients requiring urinary by-pass. This Phase I study "Incontinent Urinary Diversion Using an Autologous Neo-Urinary Conduit" is currently recruiting patients (as of time of writing), with the objective of implanting up to 10 patients by 2015. The objective of the study is to evaluate if NUC constructs, made in the laboratory by a combination of the patient's own adipose derived smooth muscle cells in combination with defined, degradable biomaterial scaffolds, may be used to form a functional conduit to safely facilitate passage of urine from kidneys to outside the body subsequent to radical cystectomy in patients presenting with bladder cancer.

Primary outcome indices over a 12 month post-implantation timeframe include structural integrity and conduit patency. CT scans will be used to demonstrate that urine may flow safely through the NUC construct. Additional measures of primary outcomes up to 12 months post-

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implantation include an evaluation of any product or procedure related adverse events. Similarly, secondary outcome indices will include analysis of NUC structural integrity and patency over a 12-60 month post-implantation timeframe. CT scan and renal ultrasound will be applied to demonstrate that urine is able to flow safely through the NUC construct up to 60 months post-implantation. Procedural and product related adverse events will also be monitored to 60 months post-implantation. Finally, the overall safety of the NUC construct will be assessed through evaluation of non-product/procedural related adverse events and patient vital signs. Eligibility for this clinical trial has been adjusted to be as inclusive as possible while ruling out any patients that present with secondary conditions likely to negatively impact regenerative outcomes:

4.4. Regeneration of muco-cutaneous region at skin-conduit junction

Successful implantation of NUC requires the regeneration of a native-like muco-cutaneous region at the skin/conduit junction following implantation. A muco-cutaneous junction is a region where the *luminal mucosa* transitions to skin. Such muco-cutaneous junctions occur throughout the body at orifices including mouth, nostrils, anus, eyelids and components of the genito-urinary system. Typically, such junctions involve transitioning of epithelium to epidermis, *lamina propia* to dermis and smooth muscle to skeletal muscle. An *in vitro* tissue engineered human lip-like element reproducing the transitional morphology of human lip including epidermal skin, vermillion and oral mucosa has recently been reported (Peramo et al., 2012). This observation notwithstanding, it should be appreciated that the regeneration of a muco-cutaneous junction at the skin/conduit interface of a synthetic neo-organ such as the NUC has never before existed in nature. This indicates that context-appropriate signaling between construct and host is nevertheless possible leading to regeneration of cell types relevant to the neo-organ.

In the case of the urinary system, urine exits the body via the *urethral meatus*, a distinct structure incorporating features that defend the opening against local and/or ascending infections, emptying into the vaginal vestibule in females and the *fossa navicularis* in males. Specifically, this muco-cutaneous junction is a non-keratinized, stratified squamous epithelium composed of glycogen rich cells that provide substrate for a protective endogenous lacto-bacterial flora. Also, as the epithelium nears the skin, it is associated with acid phosphatase activity and lysozyme-like immuno-reactivity indicative of the presence of macrophages that secrete bacterial compounds. In the pre-clinical study in cystectomized pigs discussed previously, implanted NUC was observed to catalyze formation of a native-like transition between the urinary mucosa and skin epithelium with structural features resembling that of muco-cutaneous junctions observed in native urethras (Basu et al., 2012a). These observations demonstrate that the *de novo* regeneration of transitional junctions including muco-cutaneous junctions may be catalyzed by regenerative constructs such as Tengion's Organ Regeneration Platform™, opening the door to regeneration of components of the gastro-intestinal tract, where many such transitional zones are located.

5. The gastro-intestinal tract: Introduction

Individual components of the GI represent locally specialized, iterative variations of essentially the same laminarly organized tubular histologic architecture as the bladder, and therefore should be amenable to regenerative therapies based on the same platform technologies successfully applied to the bladder. However, from a commercial perspective, the small intestine represents by far the most pressing current clinical need, with small bowel transplantation representing an unsatisfactory current standard of care for pediatric small bowel syndrome (SBS) (http://digestive.niddk.nih.gov/ddiseases/pubs/shortbowel/). Additionally, esophageal replacement or augmentation for esophageal cancer may also be commercially viable.

Signaling interactions between endodermal epithelium and mesenchymal cells derived from splanchic mesoderm mediate axial differentiation of the primitive gut tube during embryogenesis into distinctive functional domains. These individual components of the GI including the oral cavity, esophagus, stomach, small intestine and colon represent locally specialized iterative variations of essentially the same laminarly organized tubular histologic architecture as the bladder. The molecular genetics underlying these differentiative signaling cascades is complex, but is broadly understood to involve members of the *hedgehog* family of ligands, including *sonic hedgehog*, *desert hedgehog* and *indian hedgehog*. *Hedgehog* proteins bind the multi-pass transmembrane receptor *patched-1*, thereby blocking *patched-1*-mediated inhibition of the seven-pass membrane protein *smoothened*. *Smoothened*-mediated signal transduction regulates the transcriptional response to *hedgehog* activation within the target tissue. In mouse models and human patients, mutations in *hedgehog* signaling proteins and downstream targets lead to a variety of severe malformations of the GI tract. Genetic analysis of GI formation has been extensively reviewed in detail elsewhere (Van den Brink, 2007). Conservation of signaling between mesenchymal and epithelial cell populations during organogenesis of bladder and GI suggests that the organ regeneration platform appropriate for bladder regeneration may also function in an independent positional context to catalyze regeneration of the GI, including but not limited to small intestine and esophagus.

The intestinal epithelium is the most highly regenerative tissue within adult mammals and may therefore be expected to be most amenable towards tissue engineering or regenerative medicine methodologies. Perhaps the best described strategy for regeneration of intestine and other components of the gastrointestinal tract involves the use of *in vivo* derived organoid units, formed from incompletely disassembled clusters of epithelial and mesenchymal cells generated through partial digestion of intestinal epithelium and therefore likely incorporating resident intestinal stem cells. In one such study, autologous organoids were derived from the intestine of 6 week old mini-pigs and used to seed PLLA scaffold tubes that were subsequently matured within the peritoneal cavity of the original donor. Seven weeks post-implantation, this tissue-engineered small intestine recapitulated the gross overall laminar organization of native small intestine (SI) (Sala, et al., 2009). Significantly, acellular scaffolds did not result in the regeneration of tissue engineered gastrointestinal structures. These data notwithstanding, anastamosis of these tissue engineered small intestines to host SI within a large animal model

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remains to be demonstrated. Additionally, up to 10cm of host SI was harvested to derive donor organoids that are not readily expandable *in vitro*. Whether organoid units capable of seeding a scaffold structure may be isolated from diseased human intestine, and how much diseased donor material may be needed, remain factors yet to be elucidated. The requirement to leverage the peritoneal cavity as a bioreactor for tissue engineering may also impede widespread application of this approach. The bladder-derived organ regeneration platform of biopolymeric scaffold seeded with smooth muscle cells may be applicable for regeneration of SI. To this end, stomach derived smooth muscle cells were used to seed a collagen-based scaffold prior to implantation within surgically isolated ileal loops of dogs for eight weeks, prior to reanastamosis to the native intestine. Acellular collagen scaffold was used as a control. By 12 weeks post-surgery, macroscopic analysis of the cell seeded scaffold implantation site demonstrated regeneration of neo-mucosa resembling native mucosa. However, in animals containing an acellular scaffold, the implant site remained ulcerated up to 12 weeks post-implantation. Additional histological data showed significantly enhanced vascularization, epithelialization and organization of the circular muscle layer at the cell seeded scaffold defect site relative to acellular control (Nakase, et al., 2006).

Increasing the number of smooth muscle cells seeded onto the scaffold increased the area of regenerated SI tissue, although no concomitant increase in the thickness of the smooth muscle layer was observed (Nakase, et al., 2007). Nevertheless, these data suggest that a simple regenerative platform composed of biodegradable scaffold nucleated with smooth muscle cells may be adequate to facilitate SI regeneration. Although this approach must be reproduced using a directly anastamosed tubular scaffold and alternate sources of smooth muscle cells, if successful, this methodology represents the most straightforward, clinically relevant and commercially viable strategy for regeneration of the SI. This organ regeneration platform technology may also be leveraged for regeneration of the esophagus. In one such example, patch defects were created in the abdominal esophagus of 27 female rats, subsequently implanted with gastric acellular matrix (GAM). Of the 24 survivors, none showed evidence of regeneration of the lamina muscularis mucosa even 18 months post-implantation (Urita, et al., 2007). In contrast, a study of a canine model of esophageal resection and replacement demonstrated that PGA tubes seeded with a mixture of keratinocytes and fibroblasts triggered regeneration of smooth muscle laminar organization similar to native esophagus within 3 weeks post-implantation, whereas acellular PGA tubes formed esophageal strictures were associated with near complete obstruction within two to three weeks (Nakase, et al., 2008). In another dog study, cervical esophageal defects were patched with either small intestinal submucosa (SIS) alone, or SIS seeded with autologous oral mucosal epithelial cells. After four weeks, dogs implanted with cell seeded SIS showed almost complete re-epithelialization and minimal evidence of inflammation and, by eight weeks post-surgery, regeneration of the underlying smooth muscle layer. Acellular SIS grafted animals presented only partial re-epithelialization and a more extensive inflammatory response by four weeks, and no muscular regeneration by eight weeks. Attempts to introduce an acellular SIS tubular construct into the cervical esophagus of piglets were also unsuccessful, demonstrating scarification and a minimal regenerative response (Doede, et al., 2009).Progress has also been made in efforts to tissue engineer the stomach. Stomach derived organoid units, (analogous to the SI organoids

used to tissue engineer the SI) upon seeding of a biopolymeric scaffold, triggered reconstitution of the gastric and muscularis mucosa in stomach tissue engineered within the peritoneal cavities of swine (Sala, et al., 2009). In another study, circular defects were created in the stomach of seven dogs and a composite biodegradable scaffold ("New-sheet"), soaked with either autologous peripheral blood or bone marrow aspirate, was sutured over the defect. By 16 weeks post implantation, the defect site had formed regenerated stomach with evidence of re-epithelialization, formation of villi, vascularization and fibrosis within the sub-mucosal layer. However, minimal regeneration of the smooth muscle layer was observed, as shown by expression of smooth muscle α-actin, though not calponin, a marker of mature smooth muscle cells (Araki, et al., 2009).Though strictly not a tubular organ, the anal sphincter is a component of the gastrointestinal tract and is critical in regulating patency of the large intestine. Recent efforts to engineer the anal sphincter leverage the same general platform used to catalyze bladder regeneration. To this end, smooth muscle cells isolated from human internal anal sphincter were seeded onto fibrin gels poured around a central mold. Cell mediated contraction of the gel around the mold resulted in the formation of a 3D cylindrical tube of sphincteric smooth muscle tissue. Although *in vivo* anastamosis remains to be demonstrated, this bioengineered anal sphincter demonstrated contractile properties and response to defined neurotransmitters consistent with the functionality of native anal sphincter (Somara, et al., 2009; Hashish, et al., 2010). Use of alternatively sourced smooth muscle cells may facilitate the transition of engineered sphincter towards commercial production.

Preclinical rodent studies were initiated in our laboratory to evaluate the ability of constructs composed of smooth muscle cell seeded PLGA coated PGA patches to support regeneration of esophagus. In these studies, small (3-5 mm^2) defects were introduced within the esophagus of rodents, such that the entire esophageal wall was removed within the defect. Injuries were subsequently repaired with cell seeded patch constructs and allowed to regenerate *in vivo* for up to 10 weeks post-implantation. Evidence of early regenerative outcomes was observed at 8 days post-implantation in the form of developing bundles of smooth muscle. Complete regeneration of longitudinal and circularly oriented musculatures and luminal epithelia were observed at 10 weeks post-implantation of construct. Importantly, as with previous studies, vascularization of the regenerative construct by wrapping with omentum during implantation was crucial for a successful regenerative outcome. These small animal data establish the utility of SMC coated PGA felt-based biomaterials for tissue engineering and regeneration of the esophagus (Basu et al., 2012b; Basu et al., 2013).

For the small intestine (SI), patch based constructs composed of adipose derived smooth muscle cell seeded PGA felts were observed to support full regenerative outcomes upon implantation within rodent models of SI injury. Regeneration of intestinal epithelia with microvilli as well as regenerated bundles of smooth muscle cells were observed within 3 months post-implantation. Studies were also initiated to evaluate the regenerative potential of cell seeded biomaterials constructs within the context of a tube. In addition to supporting the proliferation and migration of SI derived cells, such tubular constructs must have appropriate physical and mechanical properties that complement those of native tissue. In particular, within the GI tract, tubular constructs must be capable of supporting peristaltic movement of

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food or fecal matter. Commercially sourced PGA braided tubes were observed to have physical properties reminiscent of SI, and were therefore applied in the context of tubular cell seeded constructs for evaluation of regenerative outcomes within rodent SI. As with the patch, complete regeneration of intestinal epithelia and partial regeneration of bundles of smooth muscle cells was observed at 3 months post-implantation. Implanted animals were capable of eating normally, gained weight and passed fecal material in a manner comparable to healthy controls. A detailed analysis of regenerative outcomes in rodents implanted with SMC seeded tubular constructed has been previously presented (Basu et al., 2011b). As with all other tubular organs, presence of a cellular component on the regenerative construct is essential to a successful regenerative outcome. This data notwithstanding, observed difficulties in regeneration of SI musculature from simple constructs derived from biomaterials used in bladder regeneration suggest that there are elements unique to SI currently missing from the current generation of construct prototypes. One such potential factor is the role of the enteric nervous system in facilitating regenerative outcomes within the GI tract.

6. Summary

1. Tengion's foundational Organ Regeneration Platform™ composed of adipose-sourced smooth muscle cells complexed with a synthetic, biodegradable scaffold, is broadly applicable to regeneration of multiple tubular organs.
2. Constructs function by manipulating fundamental signaling pathways between mesenchymal and epithelial cell populations to trigger regenerative outcomes mimicking organogenesis during development and regeneration in organisms such as urodeles, including induction of a neo-blastema.
3. Tubular organ regeneration is influenced by adequate vascularization and cellular constituents present at the local site.
4. Mucosal regeneration is influenced by blood supply with organ specific events controlling biological events.

This ability to apply the same fundamental signaling elements that mediate the regenerative process through application of the same combination of cells and biomaterials towards regeneration of multiple tubular neo-organs will greatly facilitate development of a manufacturing platform with commonalities throughout bio-processing.

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