Early Cellular and Stromal Responses in Regeneration Versus Repair of a Mammalian Bladder Using Autologous Cell and Biodegradable Scaffold Technologies

Manuel J. Jayo, Deepak Jain, Belinda J. Wagner and Timothy A. Bertram*

From Tengion, Inc., Winston-Salem, North Carolina

Purpose: Internal organ regeneration holds promise for changing medical technology and decreasing organ shortages. Current medical treatment for internal organ failure is largely limited to organ transplantation. A construct composed of synthetic biopolymer with autologous cells has shown long-term clinical benefit in patients undergoing augmentation cystoplasty. However, to our knowledge early cellular and stromal events during bladder regeneration have not been elucidated.

Materials and Methods: In situ cellular responses to 2 biopolymer implants, including a poly(lactic-co-glycolic acid) (Sigma-Aldrich™) based biodegradable mesh scaffold with autologous urothelial and smooth muscle cells (construct) and a poly(lactic-co-glycolic acid) based biodegradable mesh scaffold alone without cells (scaffold), were compared in a canine model of augmentation cystoplasty. Healing events were correlated with urodynamic assessments.

Results: Construct implants regenerated baseline urodynamics as early as 4 months after implantation. Urodynamics following scaffold implantation failed to return to baseline by study termination at 9 months. Functional differences elicited by construct and scaffold implants correlated with structural differences in the neotissues. Construct stroma had greater vascularization with gently folded, interwoven connective tissue elements. Scaffold stroma was dense, haphazardly organized connective tissue. Urothelium regenerated in response to construct and scaffold implantation. However, only construct had normal stroma, well developed detrusor and abundant α-smooth muscle actin (Vector Laboratories, Burlingame, California) cell staining at early time points, leading to a structurally and functionally complete regenerated bladder wall at 9 months.

Conclusions: Early cellular and stromal events distinguish healing processes that lead to bladder wall regeneration or repair. Construct implants containing cells elicit early healing processes that culminate with the regeneration of complete mucosal and muscular components, whereas the response to scaffold implantation is consistent with reparative healing, that is with mucosal growth but incomplete tissue layer development.

Key Words: bladder; polylactic acid-polyglycolic acid copolymer; regenerative medicine; cystoplasty; implants, experimental

The process of regeneration is associated with the maintenance or restoration of the original structure and function of a tissue or organ.[1] However, injury that exceeds the regenerative capacity of a tissue triggers another mechanism, that is healing by repair and fibrosis, which covers a wound with dense fibrous or scar tissue and structural elements that are different from the original elements.[1] The response of the body to injury is the sum of several factors, including age, injury site and the urgency of restoring homeostasis.[1,2] Many tissues or even organs may be prompted to regenerate if assisted by emerging technologies using some tissue engineering methods to develop regenerative medical products.[1]

The goal of regenerative medicine is to identify optimal products that consistently trigger regenerative healing, and effectively restore function and structure to damaged tissue and whole organs. It is hoped that regenerative medicine will lead to superior patient outcomes. Regenerative medicine approaches have been applied clinically to neurogenic bladder disease that is refractory to medical treatment.[3] Neurogenic bladder affects the bladder wall and causes bladder noncompliance and increased intravesical pressure. When anticholinergic medications and clean intermittent catheterization are ineffective, the patient is at risk for hydronephrosis and/or end stage renal disease. Augmentation enterocystoplasty is used to surgically increase bladder size with a segment of bowel tissue but this procedure can be associated with significant morbidity and metabolic complications.[4]

Tissue engineering approaches to repair large bladder defects began in the early 1900s. Avoiding or overcoming dominant signals that lead to repair by fibrosis has been a challenge.[5–7] A regeneration milestone was achieved for the bladder in dogs[8] and humans[3] using a synthetic, biocompatible scaffold material seeded with autologous UCs and SMCs that demonstrated the clinical usefulness, feasibility and long-term durability of this technology for eliciting regenerative healing without evidence of adverse repair by fibrosis.

Regenerative healing occurs through a temporal sequence of cellular infiltration, blood vessel ingrowth, and site specific in situ differentiation of complete mucosal, stro-

Submitted for publication September 27, 2007.

Study was performed in accordance with institutional, state and federal regulations.

* Correspondence: Tengion, Inc., 3929 Westpoint Blvd., Winston-Salem, North Carolina 27103 (telephone: 336-201-0152; FAX: 336-722-2426; e-mail: tim.bertram@tengion.com).

mal and parenchymal tissue layers.[1] Augmentation cystoplasty with an implant composed of biopolymer with autologous UCs and SMCs elicited functional bladder regeneration[3,8] but cell-free implants did not.[7–9] Limited structural and functional characteristics elicited by biopolymer implants alone suggested that healing occurred by repair and not by regeneration. Similarly bladder reduction by cystectomy without augmentation yielded no bladder regeneration.[8]

We compared early cellular and stromal events in regenerative healing with those occurring during reparative healing of a hollow organ. In an established canine model of augmentation cystoplasty[8] we evaluated the healing elicited by a PLGA based biodegradable mesh scaffold with autologous urothelial and smooth muscle cells (construct) or a PLGA based biodegradable mesh scaffold alone (scaffold) as regenerative and reparative healing, respectively. The presence of cells in the implant was the independent variable. Our hypothesis was that early cellular and stromal responses would differ, and may correlate with the longer term functional outcomes elicited by each implant.

MATERIALS AND METHODS

Animals, Study Design and Surgery

Mongrel dogs were used, and all animal procedures were performed in accordance with institutional, state and federal regulations.[10] Male and female mongrel dogs (12 animals per gender) were randomly assigned to the construct group (16) or the scaffold group (8) (table 1). Cystectomy was done to remove greater than 75% of the native bladder tissue, sparing the trigone. Implants were attached with resorbable sutures and wrapped with omentum. The animals were anesthetized with isoflurane inhalant anesthetic during surgery. Before closure implants were tested for leaks using a Foley catheter. After surgery the animals received analgesic therapy with buprenorphine (0.01 mg/kg) subcutaneously in doses given 8 to 12 hours apart for up to 3 days. They voided spontaneously upon Foley/suprapubic catheter removal and regained continence within the first 2 weeks. One, 3, 6 and 9 months after implantation the animals were clinically evaluated and sacrificed for histological/in situ neotissue evaluations.

Three-dimensional bladder-shaped scaffolds were formed from nonwoven PGA felts (Biomedical Structures, Warwick, Rhode Island) and PLGA, packaged, sterilized with ethylene oxide and stored in a desiccator until implant preparation.

TABLE 1. *Study design and postoperative course*

	Construct	Scaffold
No. animals	16	8*
No. planned/actual animals at necropsy:		
1 Mo	4 (4)	2 (2)
3 Mos	4 (4)	2 (2)
6 Mos	4 (4)	2 (1*)
9 Mos	4 (4)	2 (2)
Catheter removal (days):		
Urethral Foley	Within 7	
Suprapubic indwelling	Within 21	
Postop decreased appetite + lethargy resolution (days)	Within 14	
Clinical pathology (urinalysis + hematology)	Hematuria + inflammatory response consistent with surgery + acute phase response to implant, normalized by 30 days	

Animals were younger than 1 year at implantation.
* One animal was sacrificed at 2.5 months due to bladder wall perforation/rupture.

Cell Harvest and Expansion

Bladder tissue was harvested from the 16 animals in the construct group by transmural bladder biopsy. SMCs and UCs were isolated, characterized and expanded separately until cell seeding according to previously published protocols.[11,12] SMCs were cultured in Dulbecco's modified Eagle's medium with fetal calf serum (Invitrogen™). UCs were cultured in serum-free keratinocyte growth medium with epidermal growth factor and bovine pituitary extract (Invitrogen).

Implant Preparation

Sterile scaffolds were hydrated in culture medium and used alone (scaffolds) or seeded with autologous UCs and SMCs (constructs) 7 days or less before implantation.

Urodynamics

Bladder capacity and intravesical pressure were measured in dual lumen catheterized animals following the removal of residual urine. One line was connected to a pressure monitoring device and sterile saline at approximately 37C was infused at 20 ml per minute until fluid leakage was observed around the catheter (leak point). The volume in ml of instilled saline (capacity) and intravesical pressure in cm H_2O was recorded at leak point. Compliance values were calculated by dividing the change in bladder capacity by the change in bladder pressure.

Bladder Wall Sample Collection

At necropsy ureters and urethra of retrieved neobladders were clamped, and the neobladders were distended by intravesical instillation of 10% buffered formalin to achieve the same volume and pressure as measured by urodynamics before necropsy. Physiologically distended bladders were immersed in 10% buffered neutral formalin. Neobladders were sampled in standard fashion by dividing the organ into 4 quadrants (dorsal/ventral and right/left). Three full-thickness samples were collected per quadrant, including 2 from the anastomotic interface and 1 from the point most cranial to the surgical interface. A total of 12 samples per neobladder were collected. Native canine bladder was fixed and processed in an identical manner.

Histological Evaluation

Sections from bladder wall samples were stained with hematoxylin and eosin, and Masson's trichrome and VVG stain.[13] PLGA scaffold was visualized with polarized light microscopy.

Blinded morphometric analysis of tissue layer composition and wall thickness was done in all samples from each bladder quadrant using a standard method. Bladder wall samples from animals at 9 months were evaluated microscopically using an ocular reticle calibrated with a stage micrometer. Total bladder wall, mucosa and detrusor muscle

thickness were each measured using a calibrated microscopic reticle.

Two observers performed blinded scoring of all histological samples using hematoxylin and eosin, and VVG and immunohistochemical stains with a 5-point Likert scale, including 0—not present, 1—minimal, 2—mild, 3—moderate and 4—marked. Raw scores were segregated into 2 groups of low (0 to 2) and increased (3 to 4) presence for Pearson's chi-square analyses.

Immunohistochemistry

Antibodies used were αSMA, ED-A fibronectin (Abcam®) and collagen I (Santa Cruz Biotechnology, Santa Cruz, California). Primary antibody incubation and colorimetric detection were performed according to manufacturer protocols.

Statistical Analysis

The mean, SD and regression analysis were calculated with Microsoft® Excel®. The 95% CI and Pearson's chi-square were calculated with JMP®.

RESULTS

We investigated and compared cellular and stromal events 1 month after implantation of a PLGA based biodegradable mesh scaffold with autologous UCs and SMCs (construct) or a cell-free PLGA based biodegradable mesh scaffold. These implant technologies were chosen because they have been shown previously to elicit 2 different long-term functional outcomes of bladder healing. Functional parameter assessment confirmed previous studies suggesting that 2 different processes of healing were occurring in the 2 groups of animals.

Implantation of Constructs and Scaffolds

Table 1 shows the study design, group characteristics, necropsy schedule and postoperative course. In vitro constructs and scaffolds were not watertight. At implantation omentum was used to approximate a vascular source to the implant and form a leak-free barrier. Absent leakage was confirmed in all animals before closing the abdominal incision and skin.

Time Course of Bladder Healing Following Construct and Scaffold Implantation

Urodynamic assessment of construct and scaffold neo-organs. The time course of functional status following bladder augmentation with construct and scaffold implants was assessed with urodynamic studies 1, 3, 6 and 9 months after implantation (fig. 1). Urodynamic functional differences between construct and scaffold implants became evident at 6 and 9 months. Observed changes in capacity and compliance were consistent with the results reported by Oberpenning et al.[8] Regression analysis confirmed that the 2 implant technologies elicited distinct capacity and compliance profiles (fig. 1, *C* and *D*), suggesting that 2 distinct biological processes are involved in healing.

Microscopic time course of healing. Stromal changes occurring during the healing process with construct and scaf-

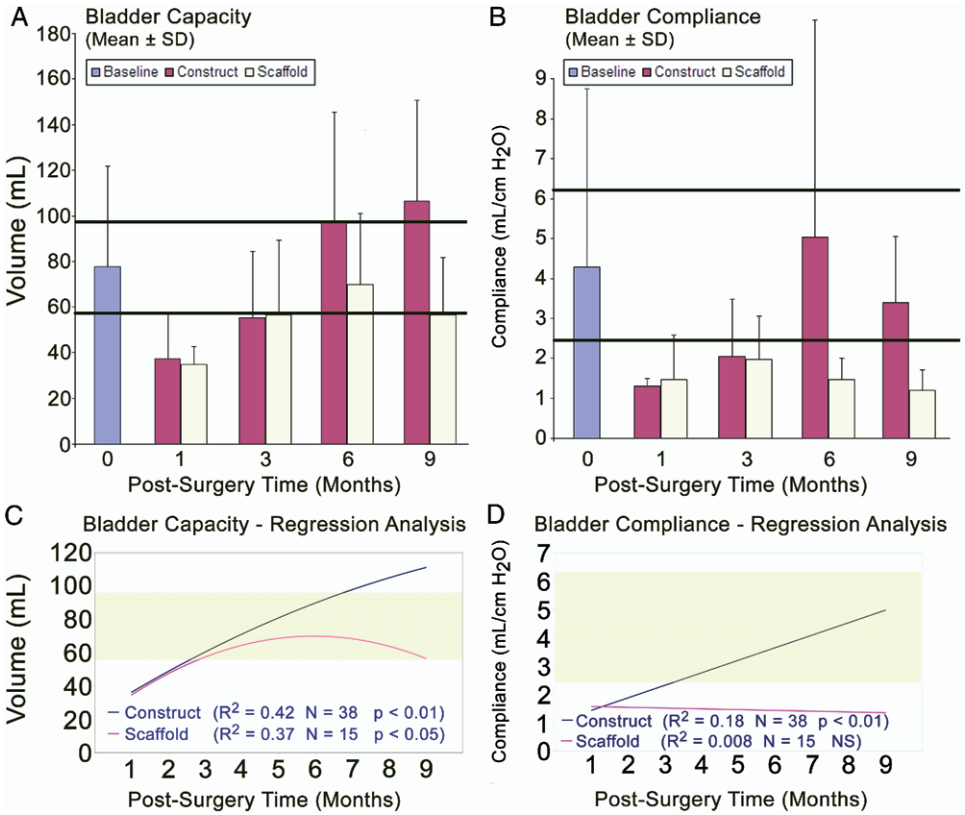

FIG. 1. Mean bladder capacity (*A* and *C*) and compliance (*B* and *D*) at 0 months in all 23 animals, at 1 month in construct in 16 and scaffold in 7, at 3 months in construct in 12 and scaffold in 4, at 6 months in construct in 8 and scaffold in 3, and at 9 months in construct in 4 and scaffold in 2.

fold implants were shown by trichrome staining, which demonstrated muscle and collagenous components as red-pink and blue areas, respectively (fig. 2). At 1 month in each group stromal and cellular responses were distinguishable by blinded evaluation. At 3 months there were differences in the muscle organization and distribution of collagen staining (fig. 2). By 6 and 9 months the muscle organization and overall thickness of bladder walls were distinguishable since construct neobladder muscle layers were organized into bundles of detrusor muscle-like layers that accounted for most bladder wall thickness, whereas scaffold neotissue muscle layers were thin and disorganized (fig. 2).

Neobladder wall morphometry. Thickness of the total bladder wall, mucosal and detrusor muscle layers were measured in tissue sections derived from construct and scaffold neotissues retrieved 9 months after implantation (fig. 3). Mucosal thickness was the same in the 2 groups. However, detrusor muscle layers and the overall wall thickness of construct neobladders were significantly greater (table 2).

Early Cellular and Stromal Responses to Construct and Scaffold Implantation

Elastin, collagen and smooth muscle were visualized in VVG stained sections from neobladder samples taken at 1 month (fig. 4). Marked cellular and tissue activity was observed in each group. However, construct neobladders were organized and composed of a smooth muscle-like parenchyma with delicate fibrovascular tissue radiating around numerous microvessels that extended toward the luminal surface and stromal elements with well developed blood vessels aligned to the mucosal surface (fig. 4, *A*). In contrast, scaffold neotissues had dense collagenous tissue with spindloid cell infiltrate, minimal vascularization and haphazardly organized, thick collagen fibers (fig. 4, *B*).

Immunohistochemistry sections from 1 month construct and scaffold neobladders were blinded and scored for the presence of spindloid/mesenchymal cells, αSMA positive muscle precursor cells, ED-A fibronectin positive myofibroblasts and collagen I positive fibroblasts. Pearson's chi-square analysis confirmed that construct neobladders had more spindloid/mesenchymal cells and αSMA positive muscle precursor cells than scaffold neotissues ($p = 0.0143$ and 0.0057, respectively). However, there were no significant differences in ED-A fibronectin and collagen I positive cells. Similarly no differences were noted in urothelial thickness, the presence of nerve cells or macrophage characteristics.

In construct neobladders αSMA positive spindloid cells were observed in neostromal tissues and around multiple neovessels (arterioles) (fig. 5, *A*). In contrast, αSMA staining was limited to microvessel walls in scaffold neo-neovasculature and it was absent from stroma (fig. 5, *C*). Although collagen I were scored as statistically equivalent on blinded scoring, collagen I staining revealed a greater density of spindloid cells radiating from vessels and foci of individualized cells in scaffold tissues (fig. 5, *D*). Stromal matrix mineralization was commonly observed in scaffold neotissue sections but not in construct neobladders (fig. 5, *B* and *D*).

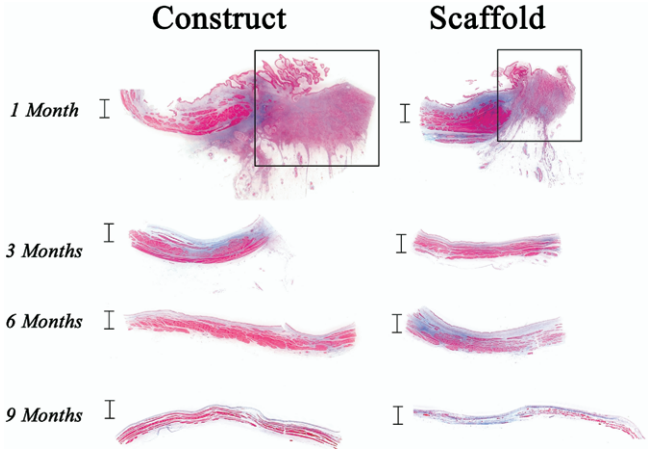

FIG. 2. Digital images reveal histological sections at anastomotic interface between native bladder and implant 1, 3, 6 and 9 months after implantation. PLGA component thickness in construct and scaffold implants was 2.5 mm at definitive surgery. Box indicates implant side of anastomotic interface. Red-pink areas indicate smooth muscle. Blue areas indicate collagenous tissue. Trichrome stain. Bar represents 5 mm.

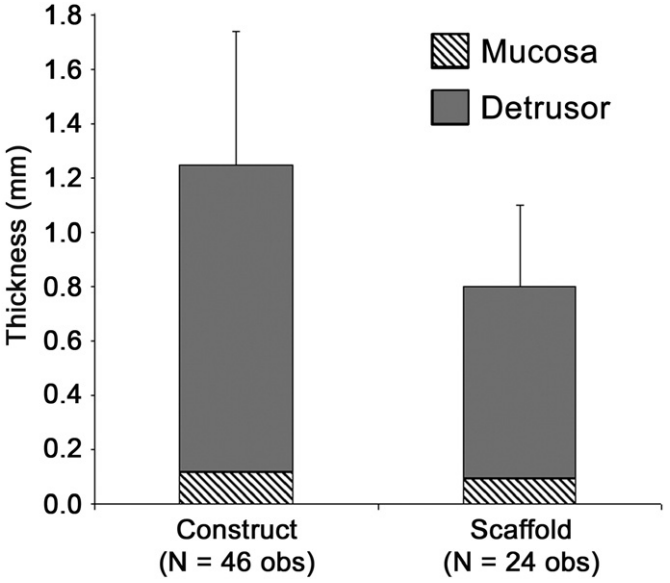

FIG. 3. Bladder wall, mucosa and detrusor muscle thickness data were collected as blinded measurements in tissue samples from construct and scaffold neobladders 9 months after implantation. *obs*, observations.

DISCUSSION

We compared early cellular and stromal events in regenerative healing with those occurring during reparative healing of a hollow organ. We used an established canine model of augmentation cystoplasty with 2 technologies, including an autologous dual cell seeded PGA scaffold (construct) and a cell-free PGA scaffold (scaffold), to elicit a regenerative or a reparative healing response.[8] The significant differences in urodynamic parameters 6 and 9 months after implantation confirmed the results of Oberpenning et al[8] and suggested that 2 distinct healing processes had occurred. To our knowledge this study provides new structural data to support and confirm that the 2 implant technologies (construct and scaffold) elicit 2 distinct healing outcomes. The histological time course and morphometric analyses of bladder wall revealed visible and statistically significant differences between construct and scaffold neotissues at 9 months (figs. 3 and 4).

TABLE 2. *Bladder wall morphometric analysis*

	No. Animals	Mean ± SD mm Implant Thickness (95% CI)		
		Mucosa	Detrusor	Bladder Wall
Construct	46	0.115 ± 0.061 (0.097–0.132)	1.133 ± 0.491 (0.991–1.275)	1.248 ± 0.543 (1.091–1.405)
Scaffold	24	0.093 ± 0.020 (0.085–0.102)	0.709 ± 0.299 (0.589–0.828)	0.802 ± 0.308 (0.679–0.925)
p Value (2-tailed t-test)		0.1017	0.0002	0.0004

Regeneration and repair are 2 broad categories of healing responses that are distinguished by structural and functional characteristics. Regeneration restores cellular components, tissue organization and architecture, and function. In repaired tissue restoration is incomplete or disordered with excessive collagen deposition and in extreme cases scar tissue formation.[14] At the cellular level regenerating and repairing tissues have different proportions of stromal (fibroblasts, vessels and nerves) and parenchymal (epithelial and smooth muscle) components. Myocyte precursors are also seen in regenerating but not in repairing tissue. None of these differences has been shown to correlate with long-term healing outcome, although these studies largely focused on a skin healing model.[15–17] Our results demonstrate that there are significant cellular and stromal changes evident in early stages of the healing process that result in divergent healing outcomes. The presence of myogenic spindloid αSMA positive precursor cells observed 1 month following construct implantation was associated with the structural and functional regeneration of a complex internal organ, that is the bladder.

Native urothelium has a low turnover rate. However, mucosal defects or denudation rapidly activates adjacent UCs to replicate, migrate and re-epithelialize the defect.[18,19] The detrusor muscle comprises greater than 75% of the bladder wall. Unlike UCs, SMCs do not respond as rapidly to injury. Our study would suggest that the myogenic spindloid precursor cellular response facilitated regeneration of the parenchyma in the construct group (fig. 5, *A*).

The cellular composition of pre-detrusor muscle (myofibroblastic) tissue differed between the 2 groups. In construct neobladders αSMA positive cells predominated with minimal fibroblastic collagen type I positive cells and fewer myofibroblastic ED-A fibronectin positive cells. These proportions were reversed in scaffold neotissues with ED-A fibronectin positive ≥ collagen type I positive > αSMA positive cells. These findings suggest that scaffold neotissue healed via reparative processes, in contrast to the construct, which healed by a regenerative process. Histology at later time points showed that scaffold neotissues were richer in stromal connective tissue and devoid of key cellular elements (fig. 2), as reflected in decreased bladder functionality at 9 months. In contrast, the early presence of myogenic spindloid α-SMA positive precursor cells in construct neobladders resulted in a functionally regenerated bladder with greater detrusor muscle, less fibrous connective tissue and native viscoelastic stromal composition.

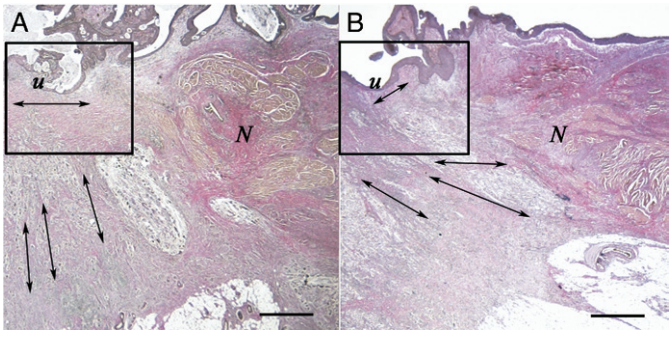

FIG. 4. Sections demonstrate cellular and stromal neobladder tissue elements 1 month after implantation, including anastomotic interface between native bladder (*N*), and implant from construct (*A*) and scaffold (*B*) neobladders. Urothelium (*u*) is also shown. Box indicates anastomotic site. Arrow in box indicates well developed blood vessels aligned to mucosal surface (*A*) and fibrovascular tissue (*B*). Arrows indicate VVG stain. Bar represents 1,000 μ.

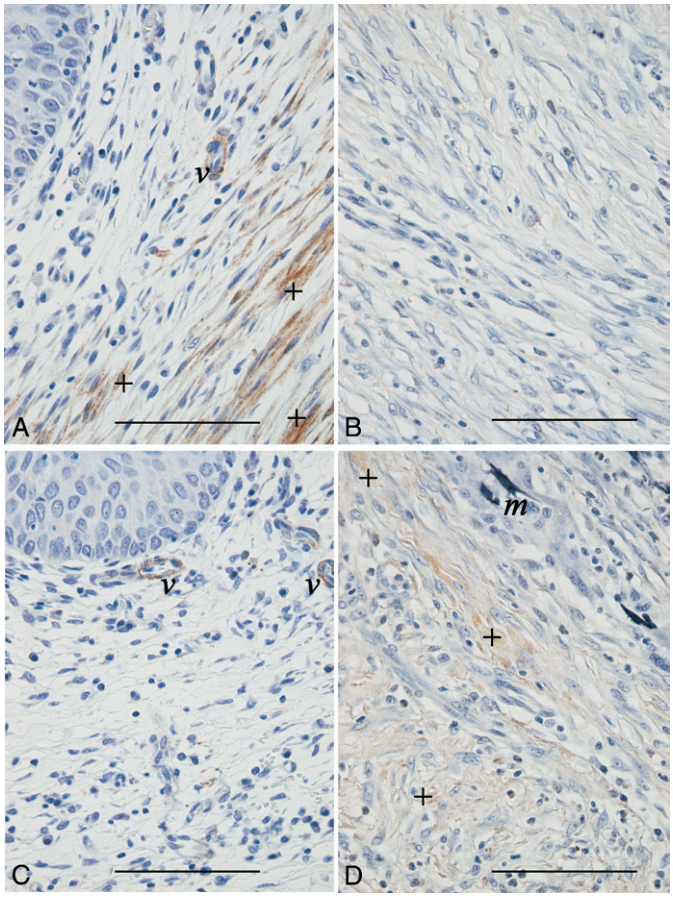

FIG. 5. αSMA (*A* and *C*) and collagen I (*B* and *D*) in tissue sections from construct (*A* and *B*) and scaffold (*C* and *D*) neobladders 1 month after implantation. Note spindloid cells (+) and vessels (*v*) with perivascular cells positive for αSMA (*A* and *C*), and collagen I deposition (+) and stromal matrix mineralization (*m*) (*B* and *D*). Immunohistochemical staining. Bar represents 100 μ.

CONCLUSIONS

Using an established in vivo model of tissue healing we detected early cellular and stromal events that lead to bladder regeneration. Regenerative healing occurred when autologous UCs and SMCs were seeded onto the implant (construct) and reparative healing occurred when the implant remained cell free (scaffold). This study elucidates important tissue compositional differences between regenerative and reparative healing processes. Specifically the early presence of myogenic spindloid αSMA positive precursor cells and appropriately permissive stromal elements was elicited by construct implants and appeared to correlate with functional organ regeneration. Future experiments will address the origin of progenitor cells and the local or systemic factors that influence their appearance, proliferation and differentiation of these cells in the microenvironment of regenerating tissue.

Abbreviations and Acronyms

αSMA	=	α-smooth muscle actin
PGA	=	polyglycolic acid
PLGA	=	poly(lactic-co-glycolic acid)
SMC	=	smooth muscle cell
UC	=	urothelial cell
VVG	=	Verhoeff-van Gieson

REFERENCES

1. Stocum DL: Regenerative Biology and Medicine. Burlington, Massachusetts: Academic Press 2002.
2. Stanger BZ, Tanaka AJ and Melton DA: Organ size is limited by the number of embryonic progenitor cells in the pancreas but not the liver. Nature 2007; **445:** 886.
3. Atala A, Bauer SB, Soker S, Yoo JJ and Retik AB: Tissue-engineered autologous bladders for patients needing cystoplasty. Lancet 2006; **367:** 1241.
4. Metcalf PD, Cain MP and Gilley DA: What is the need for additional bladder surgery after bladder augmentation in childhood? Presented at annual meeting of American Association of Pediatrics Section on Urology. Washington, D. C., October 8–10, 2005.
5. Hansen OH, Rasmussen F and Hansen JP: Alloplastic replacement of the partially resected urinary bladder in dogs. Scand J Urol Nephrol 1974; **8:** 7.
6. Kelami A, Dustmann HO, Ludtke-Handjery A, Carcamo V and Herlld G: Experimental investigations of bladder regeneration using Teflon-felt as a bladder wall substitute. J Urol 1970; **104:** 693.
7. Yoo JJ, Meng J, Oberpenning F and Atala A: Bladder augmentation using allogenic bladder submucosa seeded with cells. Urology 1998; **51:** 221.
8. Oberpenning F, Meng J, Yoo JJ and Atala A: De novo reconstitution of a functional mammalian urinary bladder by tissue engineering. Nat Biotechnol 1999; **17:** 149.
9. Kropp BP, Rippy MK, Badylak SF, Adams MC, Keating MA, Rink RC et al: Regenerative urinary bladder augmentation using small intestinal submucosa: urodynamic and histopathologic assessment in long-term canine bladder augmentations. J Urol 1996; **155:** 2098.
10. Guide for the Care and Use of Laboratory Animals. National Research Council Institute of Laboratory Animal Resources. Washington, D. C.: National Academy Press 1996.
11. Kim BS and Atala A: Mesenchymal cell culture: smooth muscle. In: Methods of Tissue Engineering. Edited by A Atala and RP Lanza. San Diego: Academic Press 2002; pp 287–292.
12. Lai JY and Atala A: Epithelial cell culture: urothelium. In: Methods of Tissue Engineering. Edited by A Atala and RP Lanza. San Diego: Academic Press 2002; pp 243–246.
13. Dellman HD and Brown EM: Textbook of Veterinary Histology. Philadelphia: Lea & Febiger 1976.
14. Clark LD, Clark RK and Heber-Katz E: A new murine model for mammalian wound repair and regeneration. Clin Immunol Immunopathol 1998; **88:** 35.
15. Beare AH, Metcalfe AD and Ferguson MW: Location of injury influences the mechanisms of both regeneration and repair within the MRL/MpJ mouse. J Anat 2006; **209:** 547.
16. Hinz B: Formation and function of the myofibroblast during tissue repair. J Invest Dermatol 2007; **127:** 526.
17. Martin P: Wound healing—aiming for perfect skin regeneration. Science 1997; **276:** 75.
18. Baskin LS, Hayward SW, Sutherland RA, DiSandro MS, Thomson AA and Cunha GR: Cellular signaling in the bladder. Front Biosci 1997; **2:** d592.
19. Varley C, Hill G, Pellegrin S, Shaw NJ, Selby PJ, Trejdosiewicz LK et al: Autocrine regulation of human urothelial cell proliferation and migration during regenerative responses in vitro. Exp Cell Res 2005; **306:** 216.