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# Urinary bladder smooth muscle engineered from adipose stem cells and a three dimensional synthetic composite

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ABSTRACT

Human adipose stem cells were cultured in smooth muscle inductive media and seeded into synthetic bladder composites to tissue engineer bladder smooth muscle. 85:15 Poly-lactic-glycolic acid bladder dome composites were cast using an electropulled microfiber luminal surface combined with an outer porous sponge. Cell-seeded bladders expressed smooth muscle actin, myosin heavy chain, calponinin, and caldesmon via RT-PCR and immunoflourescence. Nude rats ($n = 45$) underwent removal of half their bladder and repair using: (i) augmentation with the adipose stem cell-seeded composites, (ii) augmentation with a matched acellular composite, or (iii) suture closure. Animals were followed for 12 weeks post-implantation and bladders were explanted serially. Results showed that bladder capacity and compliance were maintained in the cell-seeded group throughout the 12 weeks, but deteriorated in the acellular scaffold group sequentially with time. Control animals repaired with sutures regained their baseline bladder capacities by week 12, demonstrating a long-term limitation of this model. Histological analysis of explanted materials demonstrated viable adipose stem cells and increasing smooth muscle mass in the cell-seeded scaffolds with time. Tissue bath stimulation demonstrated smooth muscle contraction of the seeded implants but not the acellular implants after 12 weeks *in vivo*. Our study demonstrates the feasibility and short term physical properties of bladder tissue engineered from adipose stem cells.



## 1. Introduction

Surgical repair of the urinary bladder using tissue augmentation is often required in the management of bladders damaged by malignancies, trauma, spinal cord injuries, and congenital pediatric disorders such as spina bifida and bladder exstrophy. The surgery is traditionally performed using a pedicle flap of intestine to reconstruct a urinary reservoir. These reconstructive procedures are technically difficult and associated with numerous sequela including secondary malignancies, urinary calculi, intestinal adhesions, and chronic infections. With advancements in the fields of materials science and tissue engineering, interest has grown in using biomaterials synthetically designed for urinary storage to circumvent the problems above. Bladder biomaterials have been used alone, and with cell seeding, for these purposes.

Experiments examining cell-seeded, 'tissue-engineered', bladder materials have been underway since 1992 and traditionally involved surgical harvest of a portion of a patient's bladder to obtain a primary culture of bladder smooth muscle and urothelial cells [1]. These cell cultures were expanded in the laboratory for 6–8 weeks to achieve a cell mass capable of impregnating or seeding biodegradable tissue molds designed to act as scaffolds and infrastructure for the bladder cells. The reconstituted bladder cells seeded into the bladder scaffold formed the basis of an *in vitro* tissue-engineered bladder [2]. Implantation of these and similar cellular engineered constructs into the bladders of rats, dogs, pigs, and humans was performed with promising results over the last several years [3–6]. The clinical utility of extrapolating these models to malignant and pathological bladders has been limited to date, due in part to the lack of healthy and accessible bladder tissue within the patient population in need of bladder replacement. Even within non-malignant bladders, investigators have found transmission of neuropathic cells from neuropathic bladders into the tissue-engineered bladder [7–9]. Tissue engineering critics have also pointed out that the traditional bladder cell harvest procedures are surgically invasive, and the prolonged cell expansion times are

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expensive, subject to contamination, and unrealistic for routine clinical use.

Alternative sources of healthy and abundant bladder tissue are desired for optimal bladder engineering and bladder regeneration. Skeletal muscle cells, bone marrow stromal cells, embryonic, and parthenogenetic stem cells are all currently under investigation as potential future alternatives, however these modalities are in their infancy. For the purpose of this study, we turned to a stem cell source in the form of adipose stem cells (ASCs), and we attempted to tissue engineer bladder smooth muscle using ASCs processed from human lipoaspirate.

Adipose tissue is akin to muscle fibers and bone marrow stroma, in that it is derived from embryonic mesodermal origins and contains a supportive stroma of regenerative pluripotent progenitor cells. Within adipose tissue, we refer to those pluripotent cells as adipose stem cells (ASCs) in accordance to the consensus set forth by the Second Annual International Fat and Applied Technology meeting [10]. Clonal expansions derived from ASCs demonstrate multipotency and self-renewing capacity analogous to mesenchymal bone marrow cells [11,12]. ASCs differentiate into myogenic, adipogenic, osteogenic, chrondrogenic, and neurogenic lineages *in vitro* when cultured in lineage specific conditions [13]. However, unlike bone marrow stem cells, which are difficult to isolate and relatively scarce, ASCs are tremendously abundant and easily accessible. The frequency of ASCs in an aspirate of adipose tissue is approximately 3%, as determined on the basis of adherent colony forming unit cells. In comparison, the frequency of similar cells in bone marrow aspirates is three orders of magnitude less [11,14,15].

The multipotentiality of ASCs, combined with their vast availability, ease of procurement, and ability to differentiate into functional and contractile smooth muscle makes them an attractive source of smooth muscle for bladder tissue engineering. To investigate this hypothesis, we attempted to use ASCs as a cell source to tissue engineer urinary bladder muscle. We created a novel synthetic three dimensional bladder mold, impregnated it with smooth muscle cells derived from ASCs, and evaluated the tissue-engineered bladder material *in vitro*, *in vivo* in a rat surgical model, and *ex vivo* in isometric tissue baths.

## 2. Materials and methods

### 2.1. Isolation and culture of adipose stem cells (ASCs)

Human adipose stem cells (ASCs) were obtained from liposuction procedures under local anesthesia (HSPC#98-08 011-02) as previously described [15]. Raw lipoaspirate was washed with phosphate-buffered saline (PBS), digested with 0.075% collagenase (Sigma, St Louis, MO) and centrifuged. The cell pellet was suspended in 160 mM $NH_4Cl$ for 10 min, filtered to 100 μm, and incubated at 37 °C/5% $CO_2$ in non-inductive media consisting of DMEM (Mediatech, Herndon, VA), 10% Fetal Bovine Serum (FBS; HyClone, Logan, UT), and 1% antimicrobial solution (Penicillin G/Amphoteracin B/Streptomycin; Mediatech, Herndon, VA). Cell populations that demonstrated clonal differentiation into osteogenic, chondrogenic, and adipogenic lineages were considered adipose stem cells [13].

### 2.2. Scaffold construction

85:15 Poly(D,L-lactic-co-glycolic acid) was purchased (PLGA; latide:glycolide 85:15; viscosity 0.65 dL/g; Birmingham Polymers, Birmingham, AL) for preparation of the bladder scaffolds. The poly-lactic-glycolic acid (PLGA) microfiber mats that comprised the scaffold lumen were prepared using electropulling as previously described [16]. Briefly, PLGA was dissolved in chloroform (15% w/w; viscosity ~150 cp) and pulled through a 25 gauge needle (BD, Franklin Lakes, NJ) using 28 kV DC. An additional electrode, located below the needle electrode and 15 cm away from the ground collector electrode, had a floating potential and functioned as second collector, and the PLGA solution was electropulled from the floating electrode toward the ground collector. The PLGA microfibers were collected on the distal electrode to form a fiber mat 1 mm thick. The fiber mat was freeze dried at 100 mTorr and −110 °C (VirTis, Warminster, PA) for 24 h, then vacuum sealed until use. The outer PLGA sponge portion of the scaffold was fabricated from 85:15 PLGA by solvent casting and particulate leaching. PLGA (20% w/w) was dissolved in a 67:33 (w:w) mixture of chloroform/methanol. Sucrose particles (diameter range = 100–200 μm) were added to create a 95% v/v sucrose:PLGA paste (95% porosity). The paste was cast and shaped in Teflon rings (dia = 10 mm; height = 1 mm) and lypholized for 12 h. Sucrose was leached from the casts in sterile distilled water. The luminal microfiber mats were fused to the outer porous sponges and shaped into a dome to create the final scaffold. To do so, the PLGA sponge was set on top of an equally shaped piece of microfiber mat and both were placed inside of a 5 L chloroform vapor chamber (8 mL 100% chloroform; 5 mTorr) for 30 min under their own weight. After removal from the vapor chamber, they were shaped by gently placing them into a dome-shaped Teflon mold (inner dia = 10 mm; concavity = 3 mm) such that the PLGA mat formed the concave (luminal) surface of the final structure (Fig. 1) and freeze dried for 24 h at 100 mTorr and −110 °C. The final construct was disinfected in 3% hydrogen peroxide solution × 3 min, washed in sterile water, and stored in sterile PBS for up to 14 days until ready for clinical or analytical use. Scanning electron micrography (SEM) was used to assess the structural features of the scaffolds.

### 2.3. Smooth muscle tissue engineering

Undifferentiated ASCs at passages 3 through 5 were differentiated into smooth muscle cells (SM-ASCs) by incubation in smooth muscle inductive media (SMIM) consisting of Medium MCDB 131, 1% FBS, 100 U/mL heparin for 6 weeks at 37 °C/5% $CO_2$ as previously described [17]. The media was changed every 5 days. Cell splitting was not required. ASCs in non-inductive media were used as controls. SM-ASCs were labeled with 1:200 dialkylcarbocyanine (*Vybrant DiI*; Molecular Probes, Eugene, OR) for 20 min the day prior to scaffold seeding. For scaffold seeding, SM-ASCs were trypsinized, suspended in SMIM ($1\times10^6$ cells/50 uL), and pipette directly onto PLGA bladder scaffold ($1\times10^6$ cells/scaffold). Tissue-engineered constructs were incubated at 37 °C/5% $CO_2$ in SMIM for 14 days. Unseeded PLGA scaffolds and scaffolds seeded with undifferentiated ASC were prepared identically as controls.

### 2.4. Assessment of SM-ASC viability and differentiation on PLGA scaffolds

SM-ASC density within the cell-seeded scaffolds was assessed using an epiflourescence inverted microscope filtered for DiI. Scaffolds were stained for live and dead cells with 4 μM calcein acetoxymethyl and 2 μM ethidium homodimer-1 (Invitrogen, Carlsbad, California). Total RNA was isolated from cell-seeded constructs at 14 days *in vitro*. RNA was lysed by rinsing the scaffolds directly in RNA lysis buffer (β-mercaptoethanol/RLT; RNeasy Kit, Qiagen, CA). Two-step RT-PCR was performed using 1 μg of total RNA with reverse transcription × 1 h at 42 °C and cDNA amplification over 35 cycles (94 °C × 30 s, 53 °C × 1 min, and 72 °C × 1 min) with 1 μM of 5′ and 3′ primer. The following primers were used: αSMA: (5′)ACCCACAATGTCCCCATCTA, (3′)TGATCCACA-TCTGCTGGAAG, 595 bp; MHC: (5′)GGACGACCTGGTTGTTGATT, (3′)GTAGCTGCTTGATGGCTTCC, 656 bp; Calponin: (5′) ATGTCCTCTGC-TCACTTCA, (3′)TTTCCGCTCCTGCTTCTCT, 453 bp; Caldesmon: (5′)AGATTGA-AAGGCGAAGAGCA, (3′)TTCAAGCCAGCAGTTTCCTT, 397 bp; SM22: (5′)ATGGCCAACAAGGGTCC, (3′)CTTCAAAGAGGTCAACAG, 349 bp; Smoothelin: (5′)ATGGCGGACGAGGCCTTAG, (3′)CCTCAATCTCCTGAGCCC, 358 bp. Reaction products were analyzed by electrophoresis of 10 μL aliquots in 1.5% agarose with ethidium bromide staining.

Real-time quantitative PCR analysis was performed for human αSMA, calponin and MHC message RNA using an ABI 7000 Prism Sequence detection system with 900 nM of upper and lower primer, 250 nM Taqman probe, 50 ng cDNA and 1 × PCR master mix (Promega, Wisconsin), amplified × 40 cycles with degradation at 95 °C × 15 s and annealing and elongation at 60 °C for 60 s. Probes were synthesized by BioSource (Camarillo, CA) with a 5′FAM and a 3′TAMRA quencher. Reactions were run in triplicate. Standard curves were prepared from 1:5 standard dilutions of purified GAPDH cDNA. Relative copy numbers of mRNA were calculated from using automated software analysis (ABI Prism 7000) based on exponential amplification cycles. Smooth muscle mRNA copy numbers were divided by GAPDH mRNA copy numbers and reported as the ratio of marker to GAPDH. The following real-time PCR primers and probes were used: αSMA: (5′)GACAGCTACGTGGGTGACGAA, (3′)GATGCCATGTTCTATCGGGTACT, (probe)FAM-CACAGAGCAAAAGAGG-AATCCTGACCCTG-TAMRA; Calponin: (5′)CCTGCCTACGGGCTGTCA, (3′)CTCCCGCTGGTGGTCA-TACTT, (probe)FAM-CCGAGGTTAAGAACAAGC-TGGCCCA-TAMRA; MHC (5′)CAACCT-GAGGGAGCGGTACTT, (3′)GAGTAGATGGGCAGGTGTTTATAGG, (probe) CAGGGCTAATATATACGTACTCTGGCCTCTTCTGC-TAMRA.

### 2.5. Bladder augmentation model

Animal studies were performed in strict accordance to Animal Research Committee guidelines at UCLA. Adult female *Rnu* athymic rats (NIH-*Foxn1rnu*, Charles River, Wilmington, MA) weighing 200–250 g were used. For partial cystectomy and bladder augmentation, rats were anesthetized with 2% isoflurane continuous flow. A 1 cm supra-pubic incision exposed the urinary bladder. Partial cystectomy was performed by removing the dome of the bladder to create a 50% total bladder defect. Transected bladder was augmented with the tissue-engineered SM-ASC seeded scaffolds ($n = 15$) or acellular unseeded scaffolds ($n = 15$) using interrupted 6-0 prolene sutures at the 12-, 3-, 6-, and 9-O'clock positions.