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Myogenic Differentiation of Human Bone Marrow Mesenchymal Stem Cells on a 3D Nanofibrous Scaffold for Bladder Tissue Engineering

Hong Tian^{a,c,*}, Shantaram Bharadwaj^{a,*}, Yan Liu^{a,d}, Haiyun Ma^b, Peter X Ma^b, Anthony Atala^a, and Yuanyuan Zhang^a

^a Wake Forest Institute for Regenerative Medicine, Wake Forest University School of Medicine, Winston Salem, NC-27157, USA

^b Department of Biologic and Materials Sciences, Department of Biomedical Engineering, Macromolecular Science & Engineering Center, University of Michigan, Ann Arbor, MI 48109, USA

^c Department of Medical Genetics, Tongji Medical college of Huazhong University of Science & Technology, Wuhan, China

^d National Engineering Laboratory for Clean Technology of Leather Manufacture, Sichuan University, Chengdu, China

Abstract

Current strategies for engineering bladder tissues include a bladder biopsy for *in vitro* cell expansion for use in reconstructive procedures. However, this approach cannot be used in patients with bladder cancer who need a complete bladder replacement. Bone marrow mesenchymal stem cells (BMSC) might be an alternative cell source to better meet this need. We investigated the effects of soluble growth factors, bladder extracellular matrix (ECM), and 3D dynamic culture on cell proliferation and differentiation of human BMSC into smooth muscle cells (SMC). Myogenic growth factors (PDGF-BB and TGF- β 1) alone, or combined either with bladder ECM or dynamic cultures, induced BMSC to express smooth muscle-specific genes and proteins. Either ECM or the dynamic culture alone promoted cell proliferation but did not induce myogenic differentiation of BMSC. A highly porous poly L-lactic acid (PLLA) scaffold provided a 3D structure for maximizing the cell-matrix penetration, maintained myogenic differentiation of the induced BMSC, and promoted tissue remodeling with rich capillary formation *in vivo*. Our results demonstrates that myogenic-differentiated BMSC seeded on a nanofibrous PLLA scaffold can be potentially used for cell-based tissue engineering for bladder cancer patients requiring cystoplasty.

Keywords

Bone marrow; stem cells; smooth muscle cell; bladder; scaffold

Corresponding author: Yuanyuan Zhang MD PhD, Wake Forest Institute for Regenerative Medicine, Wake Forest University School of Medicine, Medical Center Blvd. Winston-Salem, NC 27157, Tel: (336)713-1189; Fax (336)713-7290; yzhang@wfubmc.edu.

* Authors contributed equally to this work

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1. Introduction

Bladder cancer is the 4th and 8th most commonly diagnosed malignancy in US men and women, respectively. It is estimated that 68,810 new cases of bladder cancer occurred in the United States in 2008 [1]. Bladder augmentation is one of main options for people with bladder cancer. This surgical approach increases or restores bladder capacity and compliance and prevents or improves hydronephrosis and renal failure. Currently, augmentation cystoplasty is usually accomplished by placing a stomach patch or detubularized segment of intestine onto the bladder. The gastrointestinal tract has been the mainstay for genitourinary reconstruction in both the adult and pediatric populations. Although functional, several significant complications can arise from using gastric flaps or intestinal segments for urinary reconstruction. Several serious side effects associated with the use of stomach and bowel tissue include electrolyte abnormalities, infection, intestinal obstruction, mucus production, perforation, and carcinogenicity. Cell-based tissue engineering provides alternative for the patients with bladder cancer or other bladder end-stage diseases[2].

Autologous cells provide a cell source for urological tissue engineering because they can safely be used due to their lack of immunogenicity. However, suitable bladder cells might be not available in patients with bladder cancer or conditions; thus, an appropriate alternative to utilizing bladder cells becomes necessary. Stem cells derived from skin[3], adipose[4,5], skeletal muscle[6] and bone marrow[7–9], hair follicle [10] tissues are potential cell sources and have been under investigation for bladder reconstruction with tissue engineering technology.

Bone marrow mesenchymal stem cells (BMSC) are a good cell source for cell-based therapy because these cells possess plasticity and the ability to self-renew. BMSC are also safe for bladder cancer patients who need autologous tissue-engineered bladder augmentation because bone and/or bone marrow are not targets for bladder carcinoma metastasis. Our previous study demonstrated that BMSC could be induced to differentiate into bladder smooth muscle cells (SMC) and urothelial cells (UC) *in vitro* and *in vivo* through co-culture of bladder cells or conditioned media derived from bladder cell cultures[9]. The co-culture media or conditioned media derived from SMC cultures contain several key growth factors, including transforming growth factor beta (TGF- β), platelet-derived growth factor-BB (PDGF-BB), hepatocyte growth factors (HGF) and vascular endothelial growth factor (VEGF). In continuation of our studies on myogenic differentiation of BMSC, we have sought to optimize other methods for guiding BMSC to SMC *in vitro*. The goal of this study was to explore the effect of myogenic growth factors (PDGF-BB and TGF- β 1) alone or in combination with either bladder extracellular matrix (ECM) or three dimensional (3D) dynamic culture on human BMSC proliferation and myogenic differentiation. We also evaluated the impact of a nanofibrous 3D biodegradable scaffold, Poly-L-Lactic acid (PLLA) polymer, as a potential scaffold for muscle tissue formation of BMSC *in vivo*.

2. Materials and Methods

The overall scheme of research design is represented by a flow chart (Fig. 1).

2.1 Cell culture

Human adult BMSC samples obtained from four different individuals (aged between 19–36 years) were used for this study, provided by a commercial source (Lonza Walkersville Inc). Normal bladder or ureter tissue biopsies were from six patients undergoing ureter reimplantation or kidney transplantation. After separation from urothelial mucosa layer, smooth muscle tissues were digested using 1mg/ml collagenase type IV solution for 30min at 37°C[11]. The tissue was cut into small pieces (approximately 1 mm²) and incubated in high

glucose-Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The culture dish was incubated at standard cell culture conditions (37°C, humidified, 5% CO₂/95% air environment) for 3~4 days. The media was replaced every 2 days and sub-cultured when the cells reached a confluence up to 90%.

2.2 Preparation of bladder extracellular matrix (ECM)

Two types of matrices were used for this study, a bladder tissue-specific ECM and a collagen type IV (col-IV, BD Biosciences) matrix as a control. To obtain tissue-specific ECM, we used the bladder tissue harvested from adult swine as reported previously[12]. The entire organ and tissues were fresh frozen. To prepare the decellularized bladder ECM, the frozen muscle tissue was thinly sliced using a razor blade and decellularized through exposure to a series of solutions with continuous agitation at 4°C as follows. The tissue was washed for two days in deionized water for cells lysis, followed by decellularization with exposure to 1% Triton X-100 for five days with daily changes. The process was completed with two days of rinsing in deionized water followed by one day in phosphate buffered saline (PBS). Completion of decellularization was assessed by sampling the tissue piece (3×5×10 mm) using histology and DNA analysis.

The decellularized ECM was treated with citrate buffer (pH 4.3) for 48 hours at 4°C with constant shaking to cause swelling of the tissue pieces. The buffer was later completely removed and the tissue pieces were frozen for one day at -80°C prior to lyophilization. The lyophilized tissue was powdered using a micro grinder for 15 minutes and stored at -80°C prior to dissolution in 2M urea for three days at 4°C with constant shaking. The undissolved material was removed via centrifugation at 6,000 rpm for 20 min. The solution was then passed through a 40µm filter and the filtrate loaded into dialysis tubing. Dialysis was carried out using distilled water for two to three days. Finally, the dialysate was lyophilized and used as the matrix powder following total protein determination.

2.3 BMSC proliferation on bladder ECM

Culture dishes were coated with bladder ECM solution at a final concentration of 0.1mg/ml by overnight incubation on a rocker at 4°C. The next day, excess matrix solution was aspirated and the plates washed with PBS solution. The coated dishes were sterilized by γ -irradiation (10⁶ rad) and used immediately or stored at 4°C. BMSC were cultured on 12 well plates coated with bladder ECM (1000 cells/well). Cell counts were manually performed using a standard hemocytomete for 6 wells at each specific time point (Day 1, 3, 5 and 7).

For collagen-IV (Col IV) coating, the stock solution was diluted in 10 mM acetic acid to obtain a final concentration of 5 µg/ml. The solution was filtered through a 0.20 µm cellulose acetate filter and tissue culture dishes were coated by incubation at room temperature for 1 hour. Following this, excess solution was aspirated and the plates washed gently with PBS (2 times). The coated plate was allowed to dry in the culture hood before use. All coated plates were rinsed with PBS before culturing cells.

2.4 Differentiation of human BMSC in vitro

To induce SMC differentiation, human BMSC were treated with myogenic growth factors [5 ng/ml platelet-derived growth factor (PDGF-BB) and 2.5 ng/ml transforming growth factor (TGF- β 1)], to which we added bladder ECM or col-IV, either with 3D dynamic culture or static culture, or with both. Differentiated BMSC were assessed with RT-PCR, Western blots and immunocytochemistry.

Human BMSC were cultured on matrices (bladder ECM and col-IV) for 7 and 14 days either using a static culture or 3D dynamic culture system as described before[12] for differentiation. For proliferation study, BMSC were cultured on bladder ECM or col-IV coating for day(s) 1,

3, 5 and 7. BMSC (p5) were cultured in 12-well dishes at 2,500 cells/well, either in the presence or absence of bladder ECM coating. To study the effects of 3D dynamic culture on human BMSC growth, BMSC were seeded on the culture plates for 6 h and then loaded on an orbital shaker (Belly Dancer, Stovall, Greensboro, NC) at 40 revolution per minute (rpm) for up to 6 days. A combination of matrix and growth factors (5.0 ng/ml PDGF-BB and 2.5 ng/ml TGF- β 1) for 14 days was also employed to study myogenic differentiation.

2.5 RNA extraction and RT-PCR

Total cellular RNA was isolated from cell cultures using TRIzol. Complementary DNA (cDNA) was synthesized from RNA using SuperScriptTM II Reverse Transcriptase reagents (Invitrogen) according to the manufacturer's instructions. A touch down PCR was used for the detection of SMC-specific transcripts. The PCR primer sequences are listed in Table 1. The amplified products were analyzed by electrophoresis (15 μ l/well) using a 1.5% agarose gel incorporating SYBR-safe DNA gel stain (Invitrogen) for visualization. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene.

2.6 Immunoblotting

Immunoblotting was performed to identify expression of SMC-specific proteins. Briefly, cells were homogenized in lysis buffer [50 mM Tris HCl (pH 7.5), 150 mM NaCl, 0.5% TX-100 and protease inhibitors (Complete Mini, Roche) added freshly before use]. The total protein content was determined by the method of Lowry. Protein samples (25–50 μ g/well) were loaded on to a 10% SDS-polyacrylamide gel after denaturation by boiling for 5 minutes. The separated proteins were transferred on to a nitrocellulose membrane following gel electrophoresis. The membrane was blocked by incubation in TBST [10mM Tris (pH 7.5), 150mM NaCl, 0.1% Tween 20] containing 5% nonfat dry milk at room temperature with constant shaking for 1 hour. The membrane was probed with α -smooth muscle actin [α -SMA] (1:5000), calponin (1:1000) and smooth muscle myosin heavy chain (MHC, 1:1000) monoclonal antibodies (Sigma). The housekeeping protein, β -actin, was used as a load control. The primary antibodies were left overnight on the membrane at 4°C. The membrane was later washed three times with TBST and incubated with the secondary antibody (anti-mouse-HRP, 1:2000) at room temperature for one hour. Protein hybridization was detected by using the chemiluminescent reagent, SuperSignal West Femto reagent (Pierce) and images captured using a Fujifilm imaging system (LAS 3000). For quantification of band intensities, the Multi Gauge V 3.0 software from Fujifilm was used and the values normalized to β -actin in that particular lane.

2.7 Immunocytochemistry

Cells were seeded in four-well chamber slides and allowed to adhere overnight. Cells were washed twice with PBS, fixed with 4% paraformaldehyde and permeabilized with 100% cold acetone. The slides were then blocked using serum-free protein block solution (Dako) and incubated overnight at 4°C with monoclonal primary antibodies α SMA (Sigma, 1:100), calponin (Sigma, 1:10,000), desmin (Santa Cruz, 1:50) and myosin heavy chain (MHC) (Santa Cruz, Myosin, 1:50) diluted in antibody dilution solution (Dako). Excess primary antibody was removed by washing twice in PBS and followed by fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary antibody (1:200) incubation at room temperature for 45 minutes. Following incubation, unbound secondary antibody was removed by PBS washes and the slides were mounted in propidium iodide containing mounting media for visualization by fluorescence microscopy. Cells cultured using the 3-D dynamic culture system was also analyzed for smooth muscle-specific protein expression after 14 days.

2.8 In vivo differentiation studies

Growth factor-induced BMSC was seeded onto a nanoporous matrix, PLLA scaffold, created in the lab of Professor Peter Ma [13]. The PLLA matrix was processed and seeded with cells as described earlier [13]. Briefly, the PLLA matrix was pre wet with absolute alcohol for 30 min, washed three times with PBS (30 min each) and incubated twice in serum-containing media (2 h each). The BMSC (1×10^6) were re-suspended in 50–100 μ l of culture media and slowly loaded onto the matrix. The matrix was left undisturbed for 6–12 h before culture medium was added. The matrix was then implanted subcutaneously into nude mice and analyzed 1 month after implantation. The implants were processed for immunocytochemistry with smooth muscle-specific antibodies, desmin and myosin. For quantitation of immunohistochemical images, cells in four random fields (400x) were counted. A total number of 300–400 cells were counted for each of the experimental condition. The overall positively stained cells were expressed as a percentage of the total cell number in that particular field per cm^2 .

2.9 Statistical analysis

Triplicate values were averaged and expressed as mean $\pm$ standard deviation (SD). Each experiment was repeated three times and statistical differences assessed by Student's two sample *t* test. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1 Characterization of proliferation of human BMSC

Cell counts taken on day 1, 3, 5 and 7 showed a constant increase in cell numbers on both bladder ECM coated and non-coated culture plates with time (Fig. 2). However, the numbers of BMSC significantly increased a greater amount on the coated plates than that on non-coated plates on days 3, 5 and 7, indicating that the tissue-specific bladder ECM coating enhanced BMSC proliferation. Although cell numbers were lower on the non-coated plates on day 1, this decrease was not significant when compared to the coated-plate cell numbers.

Proliferation of BMSC was also analyzed in the presence and absence of myogenic growth factors using static and 3D dynamic culture systems (Fig. 3). Cell proliferation increased when the 3D dynamic culture was used, irrespective of the presence of myogenic growth factors. In experiments with a combination of growth factors and 3D dynamic culture conditions cell numbers nearly doubled on day 6 (1.7-fold) compared to the static cultures under similar conditions. However, in experiments without growth factors, 3D dynamic cultures were marginally better than static cultures in promoting cell growth (1.2-fold on day 6) (Fig. 3). These data indicate that 3D dynamic culture stimulates both non-induced and induced BMSC proliferation, but did not induce myogenic differentiation of BMSC. Myogenic growth factors retarded cell growth in both static and dynamic culture conditions *in vitro*.

3.2 In vitro differentiation studies

Immunocytochemical data showed that BMSC strongly expressed α SMA and calponin, irrespective of the induction conditions (Fig. 4). However, desmin and myosin, which are more specific markers for smooth muscle cells, were expressed at higher intensity on the BMSC seeded on bladder ECM coating dishes compared to BMSC seeded on col-IV with the addition of myogenic growth factors (Fig. 4). Neither the bladder nor the collagen coatings alone enhanced desmin or myosin expression. However, a significant and specific expression of desmin and myosin was observed when the cells were cultured using the 3D dynamic system and myogenic growth factors, compared to static culture (Fig. 5).

Next, we performed reverse-transcriptase PCR analysis for smooth muscle-specific transcripts. The α SMA transcript was expressed at high levels irrespective of the experimental conditions (Fig. 6). In general, it was observed that addition of growth factors increased levels of transcript, especially with calponin and to a lesser extent, myosin. In Western blot analysis, when BMSC was treated with growth factors, the cells expressed the smooth muscle-specific proteins, myosin, irrespective of the presence or absence of coatings (Fig. 7). Calponin and α SMA were expressed at higher levels throughout the different experimental conditions. This suggests that myogenic growth factors, but not ECMs, play an important role in expressing muscle-specific markers in BMSC.

3.3 In vivo studies

Overall, the scaffolds harvested after 30 days of implantation in nude mice, contained cells uniformly infiltrated throughout the porous PLLA scaffold in all of the cell-seeded experimental grafts (data not shown). The growth factor-induced BMSC expressed myogenic proteins, i.e. desmin and myosin, compared to little or no expression of myogenic protein in cell-free or BMSC-alone grafts (Fig. 8A). The percentage of cells expressing the immunohistochemical markers per cm² is shown in fig 8B. In cell-free PLLA grafts, host cell penetration was observed but with limited cell numbers. The overall percentage of positively stained cells for desmin: PLLA alone-7.8%, BMSC-23.3% and GF-induced BMSC-80%; myosin: PLLA alone-5%, BMSC-23.4% and GF-induced BMSc-57.9%; myosin (Fig 8B). SMC-seeded PLLA were used as positive controls and showed predictable staining (desmin-85% and myosin-62%).

4. Discussion

The urinary bladder is a hollow organ with flexible muscular walls which primarily functions in storing and voiding urine. SMC are the dominating cell type involved in the maintenance of the bladder contractile function and structural integrity. Hence, it is critical to generate sufficient amount of SMC from stem cells for use in *de novo* bladder reconstruction with tissue engineering technology. Myogenic differentiation of embryonic or adult stem cells is commonly induced by addition of cytokines/growth factors, extracellular matrix (ECM) and physical/mechanical environment (i.e. dynamic culture conditions). In this study, we optimized the differentiation capability and efficiency of human BMSC in giving rise to SMC for bladder tissue engineering and regeneration. Our data demonstrate that a myogenic growth factor cocktail (PDGF-BB plus TGF- β 1) alone or when combined either with bladder ECM coatings or 3D dynamic culture systems, can induces myogenic expression in human BMSC. Although ECM coatings and dynamic culture individually failed to induce BMSC to differentiate into SMC, both factors significantly promoted cell proliferation. Moreover, the use of a highly porous poly L-lactic acid (PLLA) scaffold provided a 3D structure to maximize the cell-matrix penetration, maintain myogenic differentiation of growth factor-induced BMSC and promote tissue remodeling with revascularization *in vivo*.

In our previous study, BMSC gained a SMC-like phenotype, i.e. expressed SMC marker and exhibited remarkable contractile function when co-cultured with either bladder cells or conditioned medium derived from bladder SMC [9]. Expression of several important growth factors (TGF- β 1, PDGF-BB, HGF and VEGF) has been detected in SMC co-culture and the conditioned media. The co-culture system is the closest possible *in vitro* setup that mimics *in vivo* surrounding and allows for soluble regulatory proteins/factors, of which growth factors seem to be a major player. However, it is very difficult to characterize these interactions *in vitro* for all the soluble factor or proteins secreted by SMC. Numerous studies have been reported on growth factor cocktails inducing stem cells to differentiate into SMC for vessel tissue engineering and several other studies have reported on myogenic differentiation of stem

cells into SMC for visceral tissue engineering in animal models. Several cytokine growth factors cocktail have been successfully used in inducing embryonic or adult stem cell differentiation into SMC, such as TGF- β 1 alone [14–16], TGF- β 1 plus PDGF[16], TGF- β 1 plus AA[14], sphingosylphosphorylcholine (SPC) or SPC plus TGF- β 1 [15], and TGF- β 1 plus HGF [17] are commonly used for myogenic differentiation of stem cells into vessel SMC. In this study, we used commercially-available growth factors (TGF- β 1 plus PDGF-BB) [16,18] to induce differentiation of human BMSC into SMC. Our data demonstrated that TGF- β 1 plus PDGF-BB clearly enhanced the expression of SMC-specific transcripts and proteins, particularly myosin expression. We observed that human BMSC not only expressed α -SMA and calponin, but also desmin before myogenic induction. This is different from BMSC derived from dogs and rodents, which do generally do not express desmin [8,17].

In addition to cytokines/growth factors, ECM coatings and dynamic culture systems can also promote cell proliferation and differentiation of stem cells. ECM plays an important role in enhancing cell adhesion and proliferation, and particularly in determining the developmental fate of embryonic stem (ES) cells. Collagen IV (Col-IV) has been reported to direct ES cell differentiation to mesodermal lineages in both mouse and human ES cells [16]. In this study, both bladder ECM and col-IV failed to induce human BMSC differentiation into cells expressing myosin, the most definitive marker of differentiated SMC (Fig 5). However, both ECM coatings significantly enhanced cell proliferation of the GF-induced BMSC and promoted expressing α -SMA, calponin and desmin (Figs 4 and 5). ECM alone did not appear to completely induce adult mysenchymal stem cells to myogenic differentiation, but did stimulate the cell growth *in vitro*. Mechanical strength is often used in myogenic, osteogenic and chondrogenic differentiation of stem cells for use in the skeletal tissue system [19]. The bladder wall is continuously exposed to mechanical stresses due to the dynamic nature of filling and voiding of urine. In this study, we used a 3D dynamic culture to mimic this mechanical stress on BMSC for cell proliferation and differentiation. The dynamic culture could stimulate cell proliferation by generating force and allowing uniform distribution of nutrients. Induced BMSC proliferated more rapidly in a 3D dynamic culture than a static culture, but failed to induce myogenic differentiation. Overall, our study showed that use of bladder ECM or a 3D dynamic culture could significantly promote BMSC proliferation, but not myogenic differentiation *in vitro*. We have observed that growth factors added to a dynamic culture system or ECM coating improve the efficiency of myogenic-differentiated BMSC in expressing SMC-specific transcripts and protein markers *in vitro*.

The selection of a biodegradable scaffold is another critical factor for bladder tissue engineering. An ideal scaffold for bladder or other hollow organ tissue engineering must possess the proper biomechanical/physical assets along with a highly porous structure. Biomechanical properties provide the scaffold with a hollow structure that can maintain the correct shape of the bladder during tissue regeneration. The porosity of the biomaterial aids cell infiltration into the matrix *in vitro* and promotes vascularization of cell-scaffold constructs at early stages of implantation.

Currently, two types of scaffolds are commonly used in experimental and clinical applications for bladder tissue engineering: natural collagen acellular matrices such as small intestinal submucosa (SIS) [8,20–23], bladder submucosa (BSM)[24], and bladder acellular matrix graft (BAMG)[25], and synthetic polymers, such as polyglycolic acid (PGA) and poly (lactic-co-glycolic acid) (PLGA) [2,26]. Natural collagen acellular matrixes are produced by removing all cellular components from a piece of tissue to obtain a biological scaffold. This material contains numerous growth factors that promote tissue regeneration[21,27]. In animal studies of partial cystoplasty, biologic materials alone (without seeded cells) facilitated tissue regeneration with SMC infiltration, vascularization and innervation [20,21]. However, the use of natural collagen matrices for bladder regeneration is limited by the size of the bladder defect

to be replaced. A recent study showed that extensive bladder augmentation with either cell-seeded or cell-free natural collagen matrix did not improve bladder regeneration [22]. One reason for this finding may be that the collagen matrix is too soft to maintain the shape of the organ during tissue remodeling, and as a result the scaffold collapses after implantation. For replacement of large amounts of tissue, a PGA-PLGA cell-seeded scaffold is preferred. PGA-PLGA can provide a 3D structure to allow loading of more cells during the *in vitro* phase, and this can lead to the formation of complete tissues *in vivo*.

A biodegradable PLLA polymeric scaffold is a new generation of synthetic polymer scaffold that possesses better biomechanical/physical features with highly enhanced porosity. PLLA as a nanofibrous scaffold has been successfully used in the tissue engineering of several organs, e.g. bone[28], cartilage[13] and liver[29] both *in vitro* and *in vivo*[30]. This nanostructured PLLA scaffold mimics natural ECM and provides macro and micro pores and fibers with diameters that facilitate gas and nutrition exchange along with a better cell-matrix infiltration, thereby promoting cell metabolism. The PLLA scaffold also maintains the physiological strength of the replaced tissue *in vivo*. This biodegradable scaffold can be maintained in the body for 3 to 6 months without any cytotoxic effects or immunological reactions [28–30]. In this study, the growth factor-induced BMSC displayed abundant cell-matrix infiltration and SMC differentiation *in vitro*. The cell-seeded PLLA scaffold induced smooth muscle tissue regeneration *in vivo* with profuse capillary formation, which was superior to those induced by cell-seeded natural collagen matrix. The benefit of using PLLA as a substrate is that its porous structure provides cellular interactions with the polymer, prompting cell infiltration, proliferation and tissue formation in 3D construction. Additionally, PLLA provides adequate physical properties to hold the shape of the implant and prevent collapse during healing processes of the tissue.

5. Conclusion

Human BMSC, as an alternative cell source, can be converted into smooth muscle -like cells by induction with myogenic growth factors, i.e. PDGF-BB and TGF- β 1. Growth factors alone or when combined with either bladder ECM coatings or a dynamic culture system, induced BMSC to express smooth muscle-specific genes and proteins in BMSC *in vitro*, but not with just ECM coatings or just the dynamic culture system. Thus, we conclude that ECM coatings or a dynamic culture system can significantly enhance cell proliferation of myogenic-differentiated BMSC. Furthermore, a nanofibrous 3D PLLA polymer scaffold with porous structure provides an optimal microenvironment for facilitating cell-matrix penetration and retention of myogenic-differentiated BMSCs, thereby promoting tissue remodeling with rich capillary formation *in vivo*. Therefore, myogenic-differentiated BMSC seeded on this nanofibrous PLLA scaffold can be potentially used in cell-based tissue engineering for patients with bladder cancer who need cystoplasty.

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Fig. 1. Schematic representation of research design
myogenic differentiation of human BMSC by growth factors, bladder ECM and dynamic culture conditions.

Fig. 2. Effect of bladder ECM on cell proliferation of human BMSC *in vitro*

Human BMSC (p4) were cultured on tissue culture dishes coated with decellularized bladder muscle tissue matrix (ECM) or non-coated dishes for 1, 3, 5 and 7 days. Cell counts were recorded manually on indicated days. Error bars indicate standard deviation. * $P < 0.05$

Fig. 3. Effect of dynamic culture on cell growth of induced and non-induced BMSC
Human BMSC (p5) were cultured under static or 3-D dynamic culture conditions for 2 and 6 days in the presence and absence of growth factors. Manual cell counts were performed on triplicate 6 wells on indicated days. Cell numbers were significantly higher when BMSC were cultured under dynamic conditions with or without the myogenic growth factors on day 6. * $P \leq 0.05$

Fig. 4. Impact of growth factors and ECM on myogenic differentiation of human BMSC by immunofluorescence analysis

Human BMSC (p4) were cultured on matrices in the presence and absence of myogenic growth factors (GF) for 14 days prior to staining with α -smooth muscle actin (α SMA, top row), desmin (second row), myosin (third row) and calponin (last row). Magnification 200x

Fig. 5. Effect of 3D dynamic culture on myogenic differentiation of human BMSC using immunocyto-chemistry analysis

BMSC (p5) were either cultured under static or constant 3D dynamic movement at 40 rpm for 14 days prior to staining for desmin (left panels) and myosin (right panels). Cells were stained with FITC-conjugated secondary antibody (green) and PI (red) for visualizing the nucleus. Cells were treated with growth factors, TGF β 1 and PDGF-BB (bottom row) or cultured with regular medium (middle row). Smooth muscle cells (p3) served as positive control for immunostaining (top row). Magnification 200x

Fig. 6. Impact of growth factors and ECM on myogenic differentiation of BMSC by RT-PCR analysis

Human BMSC (p4) were cultured on different matrices (bladder ECM and col-IV) in the presence and absence of growth factors (TGF- β 1 and PDGF-BB) for 14 days prior to smooth muscle-specific transcript analysis by RT-PCR. GF: Growth factor, SMC: Bladder SMC as positive control, UC: Urothelial cells as negative control, Col-IV: collagen type IV, H₂O: water control.

A

B

Fig. 7. Effect of growth factors and ECM on myogenic differentiation of BMSC with immunoblot analysis

A) Human BMSC (p4) were cultured on different matrices (bladder ECM and col-IV) in the presence and absence of growth factors (TGF- β 1 and PDGF-BB) for 14 days prior to Western blot analysis. GF: Growth factor, SMC: Bladder SMC as positive control, UC: Urothelial cells as negative control, Col-IV: collagen-IV. **B) Quantification of immunoblot.** Band intensities were measured using the Multi Gauge V 3.0 software (Fujifilm) and the individual band intensities plotted after normalizing to the load control, β -actin.

Fig. 8.

A) Myogenic differentiation of human BMSC grown on 3D nano-fibrous PLLA scaffold *in vivo*. Histoimmunochemical (desmin and myosin) analysis performed on tissue cross-sections of implanted PLLA seeded with SMC, PLLA alone, PLLA seeded with non-treated BMSC and PLLA seeded with BMSC cultured with growth factors for 14 d. The implanted scaffold was retrieved after 1 month in nude mice. Black arrows indicate specific immunohistochemical staining whereas the white arrow indicates background staining of the PLLA matrix. Images are shown at 400x. **B) Quantification of cells expressing smooth muscle-specific markers.** For quantization of the immunohistochemical images, positively stained cells were counted in four random fields as mentioned in Materials and Methods. The overall positively stained cells were expressed as a percentage of the total cell number per cm².

Table 1

Sequence information of primers used for RT-PCR and their expected product size.

Target gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	size (bp)
MHC	GGACGACCTGGTTGTTGATT	GTAGCTGCTTGATGGCTTCC	656
ASMA	ACCCACAATGTCCCATCTA	TGATCCACATCTGCTGGAAG	595
Calponin	ATGTCCTCTGCTCACTTCA	TTCCGCTCCTGCTTCTCT	453
GAPDH	CGGATTGGTCGTATTGG	TCAAAGGTGGAGGAGTGG	861