Stem Cells, Tissue Engineering and Hematopoietic Elements

Induction of Smooth Muscle Cell-Like Phenotype in Marrow-Derived Cells among Regenerating Urinary Bladder Smooth Muscle Cells

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Tissue regeneration on acellular matrix grafts has great potential for therapeutic organ reconstruction. However, hollow organs such as the bladder require smooth muscle cell regeneration, the mechanisms of which are not well defined. We investigated the mechanisms by which bone marrow cells participate in smooth muscle formation during urinary bladder regeneration, using *in vivo* and *in vitro* model systems. *In vivo* bone marrow cells expressing green fluorescent protein were transplanted into lethally irradiated rats. Eight weeks following transplantation, bladder domes of the rats were replaced with bladder acellular matrix grafts. Two weeks after operation transplanted marrow cells repopulated the graft, as evidenced by detection of fluorescent staining. By 12 weeks they reconstituted the smooth muscle layer, with native smooth muscle cells (SMC) infiltrating the graft. *In vitro*, the differential effects of distinct growth factor environments created by either bladder urothelial cells or bladder SMC on phenotypic changes of marrow cells were examined. First, supernatants of cultured bladder cells were used as conditioned media for marrow cells. Second, these conditions were reconstituted with exogenous growth factors. In each case, a growth factor milieu characteristic of SMC induced an SMC-like phenotype in marrow cells, whereas that of urothelial cells failed. These findings suggest that marrow cells differentiate into smooth muscle on acellular matrix grafts in response to the environment created by SMC. (Am J Pathol 2005, 166:565–573)

Tissue regeneration on acellular extracellular matrix grafts has been studied in various experimental models and organs.^{1–13} Many promising features for these grafts have been reported, including escape from immunological rejection and regeneration of the native tissue on them. Despite these potential advantages, effective tissue renewal on acellular grafts is not demonstrated reproducibly in hollow organs such as the urinary and digestive tracts, or the vasculature. For reconstruction of these organs, smooth muscle regeneration is a crucial requirement, since their functions as reservoirs and/or conduits are dependent on the compliance and contraction of smooth muscle.^{1–5} Although smooth muscle has been shown to regenerate on some acellular grafts without any additional cell transplantation, as in tissue engineering, often this does not extend throughout the whole graft, resulting in a limited contribution of the regenerated component to functional reconstruction of the organs.^{3,8,14} A major drawback of these previous reports was their focus primarily on a phenomenological description of the animal experiments, with less attention given to the potential mechanisms underlying muscle regeneration.

Growth factors are believed to play important roles in muscle regeneration. The presence of multiple growth factors on small intestine submucosa (SIS), the most frequently used acellular matrix graft material, is considered to be the major advantage of the material.^{6,7} However, the specific contribution of each growth factor to tissue regeneration remains unclear. Another current hypothesis proposes that, during regeneration of the urinary bladder on acellular grafts, soluble factors secreted by urothelial cells (UC) within the urinary tract, induce maturation of the smooth muscle layer on the graft via epi-

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thelial-mesenchymal interaction.^{1,10–14} Despite this, no candidate factor from UC capable of inducing smooth muscle formation has been identified. On the other hand, we have reported that exogenous growth factors can be effectively incorporated onto acellular grafts, enabling controlled regeneration of urinary bladder tissue. However, the optimal choice and combination of growth factors to be added could not be determined as long as the functional roles of these growth factors remain unclear.^{8,9} Taken together, elucidation of the mechanisms underlying the regeneration of smooth muscle layer is essential to enable safe and reliable clinical application of these grafts.

Recently, novel experimental models have shed new light on this problem. Urothelium implanted ectopically into the omentum or under the renal capsule recruits mesenchymal cells positive for early smooth muscle markers, however these infiltrating cells do not mature into a functional smooth muscle layer.^{14,15} Another model using bone marrow-chimeric animals suggested that the cells repopulating acellular grafts are of bone marrow origin.¹⁶ Since several recent studies have described growth factor-dependent differentiation of stem or precursor cells in different experimental systems,^{17–26} we hypothesized in this study that dedifferentiated mesenchymal cells or circulating bone marrow-derived precursor cells, may undergo differentiation on acellular bladder patches in response to the local growth factor environment elaborated by endogenous bladder cells. To test this hypothesis, we used *in vivo* and *in vitro* models using bone marrow cells. *In vivo*, chimeric rats generated by transplantation of bone marrow cells expressing green fluorescent protein (GFP), were subjected to bladder patch repair and the participation of marrow cells in regeneration of the bladder smooth muscle layer was studied. *In vitro*, marrow-derived stromal cells (MSCs), which can potentially differentiate into various types of mature mesenchymal cells including osteoblast, chondrocyte, adipocyte, and muscle cells,²⁷ were cultured in growth factor environments created by two kinds of bladder cells: smooth muscle cells (SMC) and urothelial cells (UC). The effects of growth factors from either bladder SMC or UC on the phenotype of marrow cells were examined.

Materials and Methods

Bladder Patch Repair with Bladder Acellular Matrix in Bone Marrow-Chimeric Rats

Rats and Bladder Acellular Matrix (BAM)

Wild-type Sprague-Dawley (SD) rat and EGFP (*enhanced GFP*) transgenic SD rat, generated by Dr. Okabe (Osaka University, Osaka, Japan)²⁸ were purchased from Japan Slc. Inc (Shizuoka, Japan). BAM was processed from wild-type SD rat sacrificed for other experiments as described elsewhere.^{8,9} Animals were treated in accordance with NIH animal care guidelines and all animal experiments were approved by Kyoto University Animal Experiment Committee.

Generation of Bone Marrow-Chimeric Rat

Bone marrow transplantation (BMT) was carried out as described elsewhere.²¹ Briefly bone marrow of 10 EGFP-transgenic SD rats was harvested from tibia and femur after euthanization with diethylether overdose. Bone marrow was flushed off from the bones with phosphate-buffered saline (PBS) containing 5% fetal bovine serum (FBS, Hyclone, Logan, UT), homogenized, and injected to 16 lethally irradiated (10 Gray) 6-week-old female SD rats. Two rats died after BMT. Flow cytometry performed 2 months after the BMT determined that both donor and chimera rat peripheral blood contained 25 to 35% of GFP-positive cells after dissolution of red blood cells,²² suggesting the establishment of stable chimerism.

Bladder Patch Repair with BAM

In chimeric animals generated above, the upper half of the bladder was resected, and either simply closed with running 8–0 polyglactin suture (Ethicon, Cornelia, GA) (chimera; partial cystectomy, $n = 7$) or replaced with bladder acellular matrix graft as described previously (chimera; patch repair $n = 7$).⁸ The resected bladder wall was subjected to histological examination to determine the presence of bone marrow-derived cells in the native tissue. The same patch repair was also performed in a group of wild-type SD rats (non-chimera; patch repair, $n = 7$). Two rats in chimera group died after patch repair. At 2, 4, and 12 weeks postoperatively, two rats from each group were evaluated, with the exception of the chimera patch group at 4 weeks ($n = 1$). Briefly, after the rats were sacrificed by overdose of diethylether, the bladder was filled with 0.6 ml of ice-cold PBS via 24-gauge plastic transurethral tube, and the urethra was clamped and ligated for removal of the bladder. The bladder was immediately observed under fluorescence stereomicroscope (Leica, Heerbrugg, Switzerland), under bright field or under ultraviolet lamp with green fluorescent protein filter.²² All images were recorded with a digital camera under fixed exposure to standardize the fluorescence of the image. Bladders were dissected and underwent a serial sucrose gradient, to be frozen and embedded in O.C.T compound (Sakura Finetechnical Co. Tokyo, Japan). Frozen sections of 5 μm thickness were either observed directly under fluorescence microscope, stained by hematoxylin and eosin (H&E) or double-immunostained with rabbit anti-GFP antibody (Clontech, Palo Alto, CA) and mouse anti- α South Muscle Actin antibody. Sections were visualized following incubation with fluorescein-conjugated anti-rabbit, immunoglobulin (Dako Japan, Kyoto, Japan) and Texas-red-conjugated anti-mouse immunoglobulin (Amersham Biosciences, Buckinghamshire, UK). The stained area was analyzed with NIH image software.²⁹

Cell Culture

Isolation of Marrow-Derived Stromal Cells (MSC)

Bone marrow was harvested from tibia and femur dissected from 3-weeks-old male SD rats, euthanized by

diethylether overdose.²⁷ Bone marrow was flushed off the bones with α minimum essential medium (α MEM) with 15% FBS, homogenized, and incubated in 25-cm² cell culture flasks (Corning, Corning, NY).²⁷ Seventy-two hours later, and on alternate days thereafter, the culture medium was replaced. Adherent cells were cultured until confluent ($\sim$ 1 week, on average) and were subjected to subsequent experiments. Typically, MSC were harvested from 3 to 4 rats at a time, and expanded to 2 to 3 $\times$ 10⁷ cells in total at 1 week.

Isolation of Bladder Cells

Bladder smooth muscle and urothelial cells were isolated from eight 10-week-old female SD rats at each experiment by a combination of the procedures described elsewhere.^{30–33} Briefly, dissected rat bladders were incubated on a silicone dish in 1000 U/ml dispase (Godo Shusei, Tokyo, Japan) for 12 hours at 4°C. The mucosa was peeled off, minced, and digested in 0.05% trypsin and 0.02% EDTA for 10 minutes at 37°C. The cell suspension was sieved through a cell strainer, re-suspended in keratinocyte serum-free medium (Gibco, Long Island, NY) at a density of 1 $\times$ 10⁵ cells/ml, and was seeded in 12-well cell culture dishes coated with collagen type IV (Nitta Gelatin Co., Osaka, Japan). The medium was changed 48 hours later and every other day thereafter. The remaining bladder tissue was digested with collagenase type IV (Sigma, St. Louis, MO, 250 U/ml) for 30 minutes at 37°C. Smooth muscle cells were suspended in Medium 199 (Sigma) with 10% FBS and plated in 25 cm² cell culture flasks. The medium was changed 24 hours later, and every other day thereafter.

Culture of Bone Marrow Cells in Conditioned Media from Bladder Cells

Retrieval of Conditioned Media

When each population of bladder cells (UC or SMC) reached 50 to 70% confluency, primary culture media were changed to α MEM with 15% FBS. Each medium was retrieved after 48 hours, passed through 0.22- μ m nitrocellulose filter, and used as conditioned medium (CM).³⁴ SMC and UC after retrieval of CM were either lysed in lysis buffer (100 mmol/L Tris-HCl (pH 7.4), 75 mmol/L NaCl, 1% Triton-X 100, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride), or trypsinized and seeded on 8-well chamber slides for immunostaining, as controls of each cellular phenotype.

Incubation of MSC in Conditioned Media

Confluent MSC were passaged and seeded at cell density of 1 $\times$ 10⁵/ml on 4-cm culture dish (Primaria, Becton Dickinson, NJ), either in SMC-conditioned medium (SMC-CM), UC-conditioned medium (UC-CM), or α MEM containing 15% FBS as a control. The media were changed every 3 days thereafter and the culture was maintained for up to 8 days. Cells were lysed on day 0 (before

first passage), day 3, 6, or 8 as described³⁵ or passaged again onto 8-well chamber slides for immunostaining.

Cell Growth Assay

MSC were plated in 96-well culture plates at the same density and conditions as described above. At 1, 3, 4, and 6 days, the cells were washed twice with PBS and frozen at -20°C . DNA content was determined by fluorescence intensity of the DNA-binding dye, Hoechst 33258 (Nacalai tesque, Kyoto, Japan) bound to cell lysates as described elsewhere.²⁷

Immunofluorescence Microscopy

MSC before and after incubation in control medium or bladder cell-conditioned media were seeded in chamber slides in standard culture medium, and allowed to adhere overnight. Cells were washed twice with PBS, fixed with 4% paraformaldehyde, and incubated with antibodies for α SMA (Sigma 1:100), calponin (Sigma, 1:100), and cytokeratin 18 (CK 18, Sigma 1:100). Cells were visualized by fluorescence microscopy following incubation with fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin (Dako Japan).

Western Blotting

The protein content of cell lysates was measured using the BioRad Protein Assay Kit (BioRad Laboratories, Hercules, CA). Cell lysates were resolved by sodium dodecylsulfate polyacrylamide electrophoresis (SDS-PAGE) under reducing conditions, and immunoblotted with antibodies against CK 18 (1:4000), α SMA (1:5000), smooth muscle myosin heavy chain (SM-MHC, Santa Cruz, 1:100), and calponin (1:1000) using methods described elsewhere.³⁵ Expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also evaluated with anti-GAPDH antibody (1:200; Chemicon, Temecula, CA) as an internal control.

Quantification of Growth Factors in Conditioned Media

The content of growth factors in the conditioned media was assessed by enzyme-linked immunosorbent assay (ELISA). The kits used were: rat hepatocyte growth factor (HGF) assay (Tokushu Men-eki Kenkyujo, Tokyo, Japan), mouse vascular endothelial growth factor (VEGF) (R&D Systems, Inc., Minneapolis, MN), mouse SCF (R&D), human bFGF (R&D), and human transforming growth factor β (TGF β) (R & D). For mouse kits, rat VEGF (R&D) and rat stem cell factor (SCF) (Peprotech, London, UK) were used as standards. The detection of platelet-derived growth factor-BB (PDGF-BB) was carried out with antibodies kindly given from Mochida Pharmaceutical Co. (Tokyo, Japan)³⁶ with rat PDGF-BB protein (R&D) as standard. For human kits, data are presented as percentage of control level (α MEM + 15%FBS).

Simulation of Conditioned Media by Exogenous Growth Factors

The conditions determined above were simulated by adding exogenous growth factors into α MEM with 0.5% FBS. SMC-CM was simulated by adding 40 ng/ml human recombinant HGF (kindly provided from Snow Brand, Tokyo, Japan) and 5 ng/ml recombinant TGF β (R&D). UC-CM was simulated by adding 50 ng/ml bFGF (kindly provided from Kaken Pharmaceuticals Co., Tokyo, Japan) and 100 ng/ml rat VEGF (R&D). Other cells were cultured in α MEM with 0.5% FBS containing no or each single growth factor at the dosage as described above. MSC were incubated for 6 days, and cell growth and phenotypic changes were evaluated as described above.

Blockade of Growth Factor Activity in Conditioned Media by Neutralizing Antibodies

Growth factor activity in conditioned and control media was neutralized with blocking antibodies, and the phenotypes of MSC cultured for 6 days under these conditions, were examined by the methods described above. TGF β and/or HGF in SMC-CM were blocked by 35 μ g/ml pan-specific TGF β antibody (R&D), 1 μ g/ml anti-c-met antibody (Sigma), or both. bFGF in UC-CM and control medium (α MEM + 15% FBS) was blocked by 20 μ g/ml anti-bFGF antibody (Upstate Cell Signaling Solutions, Lake Placid, NY).

Results

Bladder Patch Repair by Bladder Acellular Matrix, in Bone Marrow-Chimeric Rats

GFP-positive cells were present in the bladder wall excised at surgery 2 months after bone marrow transplantation. They were mainly distributed in the submucosal area (Figure 1A). In the smooth muscle layer, fewer cells were located at the periphery of smooth muscle fascicles (Figure 1B). No cells that were positive for both α SMA and GFP were detected.

Two weeks after the bladder patch repair with acellular matrix, dense infiltration of GFP-positive cells was observed into the graft (Figure 2, A and B). Most of the cells were white blood cells, although a band of α SMA-positive cells was observed beneath the regenerating urothelium. Few cells stained positively for both GFP and α SMA, suggesting that the majority of α SMA-positive cells were of native bladder origin (Figure 2B). There were also few double-positive cells in the graft at 4 weeks.

Twelve weeks after the bladder patch repair, fluorescence on the graft was still elevated compared to native bladder, but was less intense than observed at 2 weeks (Figure 3A). In the native bladder, the localization of GFP-positive cells was similar to that observed pre-operatively, but some GFP-positive cells in the submucosal area were also positive for α SMA (Figure 3B). The regenerating smooth muscle layer on the graft, which stained

Figure 1. Bladder of bone marrow-chimeric rat and the distribution of transplanted marrow-derived cells in the native bladder. Two months after bone marrow transplantation, the bladder wall was excised at the surgery and was stained for α SMA (red) and GFP (green). **A:** Submucosal area: relatively higher numbers of transplanted GFP-positive cells, which are negative for α SMA, are distributed. **B:** Smooth muscle layer: fewer cells are located at the periphery of smooth muscle fascicles. **Bar,** 100 μ m (magnification, $\times 400$). After fixation in 4% paraformaldehyde for 3 hours at 4°C, the specimen was processed in a serial sucrose gradient, frozen and embedded in cryocompound. Frozen sections (5- μ m thick) were stained with hematoxylin and eosin (H&E) or double-immunostained with rabbit anti-GFP antibody (1:200) and mouse anti- α SMA antibody (1:100), and then visualized following incubation with fluorescein-conjugated anti-rabbit immunoglobulin and Texas-red-conjugated anti-mouse immunoglobulin.

positive for α SMA, consisted of a mixture of GFP-positive and -negative cells (Figure 3C). GFP cells were scattered in the graft as patchy foci, where GFP signal was 100- to 200-fold more intense compared with the native bladder tissue. Double-positive cells were mainly located among the regenerating smooth muscle. The double-positive rate among GFP-positive cells in the graft area ranged from 1.2% to 4.6% per cross-section.

In partial cystectomy group, clustering of GFP-positive cells was initially observed around two marking nylon sutures, but not along the anastomosing bioabsorbable sutures at any time point. In non-chimera patch repair group, little autofluorescence was observed and no cell was stained positive for anti-GFP antibody throughout the study.

Phenotypic Change of Bone Marrow-Derived Cells in Conditioned Media from Bladder Cells

In SMC-CM and UC-CM, MSC proliferated at approximately the same rates, reaching a plateau around day 3 (Figure 4A). Before passage, MSC expressed little or no α SMA, and no other SMC markers (calponin and myosin). After 8 days of culture, MSC cultured in SMC-CM expressed elevated levels of α SMA, myosin, and calponin, which were expressed at lower levels or not at all in MSC cultured in control medium (α MEM + 15% FBS) or in UC-CM (Figure 4, B and C). These phenotypes were expressed homogeneously in the majority of MSC cultured under the same condition (Figure 4B). In contrast,

2 weeks post-op

Figure 2. Two weeks after the bladder patch repair with acellular matrix. **A:** Fluorescence stereomicroscopy: in chimeric rats, fluorescence was increased in the graft in patch repair rat, or around the marking nylon suture in partial cystectomy rat. The fluorescence along the 8-0 polyglactin suture was insignificant, suggesting that inflammatory response to the anastomosing suture material may not be the chief cause for the infiltration of marrow-derived cells. No increase in fluorescence was detected in non-chimeric rats, which excludes the possibility of autofluorescence in GFP chimera. White arrows indicate the location of marking sutures. **B:** Histology: The specimen was processed as in Figure 1. GFP-positive marrow-derived cells infiltrated the graft. A band of α SMA-positive cells is observed beneath the regenerating urothelium. Few cells are double-stained for GFP and α SMA. **Bar,** 100 μ m (magnification, $\times 400$).

the UC marker (CK 18) was not expressed in MSC cultured under any condition.

Quantification of Growth Factors in Conditioned Media

UC-CM and SMC-CM contained distinctly different levels of growth factors from each other (Figure 5). SMC-CM contained relatively higher levels of TGF β and HGF than UC-CM, while UC-CM contained higher levels of VEGF and bFGF than SMC-CM. PDGF-BB and SCF remained below physiologically relevant levels in both media.

Simulation of the Effect of Conditioned Media by Exogenous Growth Factors

Based on these results, the conditioned media were simulated by adding exogenous growth factors into α MEM + 0.5%FBS. MSC cultured in simulated UC-CM acquired a condensed configuration, resulting in higher cell counts at confluency, compared with MSC cultured in simulated SMC-CM (Figure 6, A and B). After 6 days incubation,

MSC incubated in simulated SMC-CM (TGF β + HGF) expressed all SMC markers, while MSC incubated in simulated UC-CM (bFGF + VEGF) expressed little or no SMC markers (Figure 6, B and C). MSC incubated in α MEM + 0.5%FBS also expressed certain level of SMC markers (Figure 6C).

Blockade of Growth Factors in Conditioned Media by Neutralizing Antibodies

Neutralizing antibodies against TGF β and/or HGF did not abolish the expression of α SMA in MSC cultured in SMC-CM. On the contrary, neutralization of bFGF in UC-CM and control medium (α MEM + 15% FBS) induced increased expression of α SMA in MSC (Figure 7).

Discussion

This study demonstrates the involvement of bone marrow cells in the formation of smooth muscle tissue on acellular grafts, and the differential effect of growth factors from

12 weeks post-op

Figure 3. Twelve weeks after the bladder patch repair with acellular matrix. **A:** Fluorescence stereomicroscopy: fluorescence on the graft was observed as patchy foci, and was still prominent at this time point, though less intense. **B:** Native bladder: Immunostaining for α SMA (red) and GFP (green) shows, scattered overlapping cells in the submucosal region (arrow), and few cells in the muscle layer (asterisk). **C:** Histology: The picture was taken at the mid-point between the border and the center of the graft. The four corners of the three right panels (immunofluorescence) are indicated by asterisks in the left panel (H&E). Regenerating smooth muscle structures were observed. The layer consisted of substantial amount of cells positive for both GFP and α SMA. The specimen was processed as in Figure 1. Bar, 100 μ m (magnification, $\times 400$).

bladder SMC and UC on phenotypic changes in marrow cells. Marrow cells acquired an SMC-like phenotype in the growth factor environment created by SMC in culture, while the growth factor milieu of UC failed to induce such a phenotype. These findings suggest that differentiation of infiltrating immature bone marrow cells to an SMC-like phenotype may contribute to regeneration of bladder muscle on acellular matrix grafts under influence from local SMC, but not from UC.

Although the present results suggest minimal involvement of the urothelium in maturation of smooth muscle tissue on acellular grafts, as postulated by other authors, our findings may not necessarily be in conflict with those reports.^{1,10–14} The growth factors secreted predominantly from urothelium (bFGF and VEGF) are potentially angiogenic, and neovasculature created by them may generate an environment suitable for survival of infiltrating marrow-derived cells, SMC, and UC themselves (Figure 2). In addition, mature smooth muscle tissue does not regenerate beneath ectopically isolated urothelium, but only in the presence of adjacent native bladder smooth muscle tissue.^{14,15} This is consistent with our *in vitro* observations, where MSC failed to express SMC markers under growth factor conditions characteristic of the UC

environment, specifically under bFGF-enriched conditions (Figures 4 and 6). Since inhibition of bFGF activity led to SMC marker expression under conditions in which it was not otherwise attained, loss of the inhibitory effects of bFGF appear necessary for smooth muscle maturation (Figure 7).

In marked contrast, medium conditioned by SMC promoted expression of multiple SMC markers in MSC (Figures 4 and 6). Despite this, the effect of principal growth factors in SMC-CM (TGF β and HGF) on differentiation of MSC to an SMC-like phenotype is modest compared to the significant negative effect of bFGF on marker expression, as neutralization of TGF β and/or HGF did not abolish SMC marker expression in MSC. These findings may indicate that these growth factors may not specifically promote differentiation, but that their effects may comply with the spontaneous differentiation that occurs under reduced serum conditions (Figures 6 and 7). Proliferation appears to be inversely associated with expression of the SMC-like phenotype, since MSC proliferated at a lower rate in the presence of TGF β and HGF (SMC-CM associated factors) compared to bFGF and VEGF (Figure 6A). bFGF and VEGF are known to promote proliferation of marrow cells,^{24,25} while the effect of HGF on mesenchy-

Figure 4. Marrow cells take distinct phenotype in conditioned media of bladder cells. **A:** Growth curve of MSC cultured in SMC-CM, UC-CM, and control medium. MSC proliferated at equivalent rate in SMC-CM and UC-CM. **B:** Immunofluorescence for α SMA in cultured SMC and MSC cultured in SMC-CM, UC-CM, and control medium (magnification, $\times 400$). The cells were fixed with 4% paraformaldehyde, incubated with α SMA antibody (1:100), and visualized with fluorescein-isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin. The nucleus was counterstained with Hoechst 33258 dye. **C: Left:** Cultured SMC expressed α SMA, SM-MHC and calponin. UC expressed cytokeratin. MSC expressed little or no α SMA and no other SMC markers. **Right:** MSC cultured in SMC-CM expressed stronger expression of all three SMC markers than MSC cultured in control medium or in UC-CM.

mal cells is primarily mitogenic as opposed to mitogenic, and TGF β is a known growth inhibitor for mesenchymal cells, including urinary tract SMC.^{37,38} However, it should also be noted that cell proliferation is not the only determinant of the cellular phenotype, since MSC proliferated at approximately the same rate in SMC-CM and UC-CM and expressed totally different phenotypes (Figure 4A). Since growth factors elicit context-dependent effects on both proliferation and differentiation, the ultimate effect on cells reflects a combination of positive and negative activities of several distinct factors. Interestingly, PDGF-BB, which is frequently associated with proliferation and differentiation of SMC,^{20,21,23,39} inhibited the expression of SMC markers in MSC under the same conditions as described above (data not shown), possibly suggesting negative effects of this factor on differentiation of MSC to smooth muscle, similar to the effects of bFGF.

These *in vitro* findings are consistent with our *in vivo* findings using the bladder patch repair model with acellular grafts, in which marrow cells participated in smooth muscle regeneration. Though marrow cells prevailed the graft initially, the infiltrating α SMA-positive cells at 2 weeks were mainly of native bladder origin, and not from

Figure 5. Bladder cells create distinct growth factor environments in conditioned media. Immunoassay results for HGF, TGF β , VEGF, and bFGF are presented. **A and B:** Since levels of HGF and VEGF in control medium were nearly undetectable, these data are presented as concentration of rat growth factor level. **C and D:** The levels of TGF β and bFGF are presented as percentage of control level (ie, 15% FBS), because considerable levels of these factors were also detected in control medium. These growth factors could be classified in three categories. **A and C:** 1) Growth factors that were predominantly present in SMC-CM (HGF and TGF β . **B and D:** 2) Growth factors that were predominantly present in UC-CM (VEGF and bFGF). 3) Growth factors that remained below physiological levels (<20 pg/ml, PDGF-BB and SCF).

bone marrow (Figure 2). Marrow cells acquire an SMC-like phenotype intermingled with infiltrating SMC during the time course of the experiment. Our observations with SMC-CM and medium supplemented with growth factors, suggest that the growth factor environment that promotes the observed phenotypic changes, derives from native SMC proliferating into the graft from adjacent bladder tissue, but not from UC (Figure 3).

The marrow cells may be recruited onto the graft through the circulation, or from adjacent native bladder tissues, as they are already present in native bladder after bone marrow transplantation (Figure 1). The regenerated smooth muscle layer comprised both marrow cells and non-marrow cells, in sharp contrast to the native bladder muscle layer, in which marrow-derived cells were barely detected (Figure 3). In this model, marrow cells participated in remodeling on the graft, but did not significantly remodel the native bladder tissue. This observation may reflect the need for a specific biological context in which marrow-derived cells participate in remodeling of smooth muscle tissue.¹⁹ Although such findings have also been explained as cell fusion in several models,³⁶ our *in vitro* findings and other studies suggest that differentiation from marrow cells to SMC could take place without fusion.^{20,41} The present *in vitro* model has clearly shown that MSC acquire a SMC-like phenotype under the influence of soluble factors elaborated by SMC. This occurs without direct contact of the cells, although

Figure 6. Simulation of conditioned media by exogenous growth factors reproduced the phenotype change in marrow cells. Growth factors are added to α MEM + 0.5%FBS based on the ELISA data. **A:** Growth curve of MSC. MSC cultured in simulated UC-CM showed higher proliferation, which may be attributed to condensed cellular configuration. **B:** Immunofluorescence for SMC markers (magnification, $\times 400$). The cells were fixed with 4% paraformaldehyde, incubated with α SMA and calponin antibodies (1:100 for each), and visualized with FITC-conjugated anti-mouse immunoglobulin. The nucleus was counterstained with Hoechst 33258 dye. **C:** Immunoblotting; simulated SMC-CM and UC-CM induced the similar differential phenotypic change in MSC.

presumably cell-cell interactions could also occur *in vivo*, further promoting phenotypic changes.

The clinical implications of our findings remain indeterminate. The extent of α SMA expression among GFP-positive cells was modest, and the precise percentage of marrow-derived cells within regenerated smooth muscle could not be assessed because not all of the donor marrow cells expressed GFP in our model. Our observation may point to a novel method of tissue regeneration, provided that recruitment and differentiation of precursor

cells that repopulate the graft can be appropriately controlled.¹⁶ However, elucidation of the mechanisms underlying recruitment of cells to the graft, that have the potential to differentiate into SMC will be crucial to achieve this goal. In addition, the function of the newly formed tissue in contributing to low-pressure storage of urine and appropriate voiding behavior remains to be determined. Bone marrow cells are known to participate in cardiac regeneration and formation of vascular neo-intimal SMC, but their contribution to organ function has been found to be either subtle (in cardiac muscle), or negative (for vessels).^{18,19,24,40,41} In order for acellular matrix grafts to be used successfully in bladder reconstruction, it is imperative that the newly formed smooth muscle functions equivalently to healthy native smooth muscle. Whether marrow cell-derived smooth muscle cell could attain such function should be the crucial point to be clarified in the further study.

A major finding of our study is the demonstration that specific growth factor conditions elaborated by native bladder cells are able to promote differentiation of mesenchymal cells that infiltrate the acellular graft. We should note that the matrix graft itself is another potential source of growth factors. As SIS contains growth factors such as TGFβ, VEGF, and bFGF,^{6,7} growth factors would remain on other animal-derived acellular grafts. Given these issues, decellularization method is critical for interpreting the results of acellular graft studies. As growth factors bind to extracellular matrix by electrostatic interactions with heparan sulfate and other charged molecules, they would be lost from matrix preparations under conditions of high ionic strength or in the presence of strong detergents.^{3,9} Thus, different results between previous studies may reflect different prevailing levels of growth factors within the matrix grafts. Although many promising results have been reported for bladder reconstruction using acellular matrices, it is necessary to evaluate them in the context of the underlying tissue remodeling processes. Conversely, if there exists optimal growth factor conditions for tissue regeneration, reproduction of this environment within the graft matrix would offer a reproducible and effective method for organ reconstruction.

In summary, the present findings elucidate some of unknown mechanisms in bladder regeneration process, and provide new perspectives for organ reconstruction with novel biomaterials.

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Figure 7. The effect of blocking antibody for growth factors in conditioned media. Blockade of TGFβ and/or HGF in SMC-CM did not block the expression of SMC marker. Inhibition of bFGF in UC-CM and in the control medium induced higher expression of α SMA.

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