Tissue Engineering

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Autologous Bilayered Adipose-Derived Mesenchymal Cell-Gelatin Sheets Reconstruct Ureters in Rabbits

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Abstract

Repair of ureteral defects or strictures due to disease or trauma is usually dependent upon surgery that often requires either reoperation or an alternative treatment. By taking advantage of tissue engineering and regenerative techniques, it may be possible to define new approaches to ureteral repair. In this study, we fabricated autologous bilayered adipose-derived mesenchymal cell (AMC)-gelatin sheets and transplanted them into rabbits to replace surgically excised ureteral segments. AMCs harvested from abdominal adipose tissues of female New Zealand White rabbits were cultured on collagen-coated dishes and labeled with PKH26, a red fluorescent dye, for later identification. Monolayers of the cultured PKH26-labeled AMCs were detached and applied to gelatin hydrogel sheets. Two gelatin sheets were then united with the AMC monolayers apposed together, forming a bilayered AMC-gelatin sheet. Following each partial ureterectomy, a bilayered autologous AMC-gelatin sheet was transplanted, joining the proximal and distal ends of the remaining the ureter (n=9). Control animals underwent the same procedure except that the transplant was achieved with a bilayered acellular-gelatin sheet (n=9). At 4 and 8 weeks after transplantation, the proximal regions of ureters treated with the control bilayered acellular-gelatin sheets exhibited flexures and dilations, which are not characteristic of unoperated ureters. In contrast, the bilayered AMC-gelatin sheet transplanted rabbits did not have ureteral flexures or dilations. About midway between the proximal and distal ends, both the control and experimental reconstructed ureteral walls had smooth muscle layers; however, those in the experimental reconstructed ureteral walls were significantly thicker and better organized than those in the control reconstructed ureteral walls. Some AMCs differentiated into smooth muscle marker-positive cells. The experimental ureteral walls contained smooth muscle cells derived from the PKH26-labeled AMCs and others that were derived through migration and differentiation of cells from the remaining proximal and distal ends of the original ureter. In addition, the lumina of the 8-week reconstructed ureteral tissues in experimental rabbits did not show histological strictures as seen in the control ureters. These results suggest that the bilayered AMC-gelatin sheets have the potential to replace defective tissues and/or reconstruct damaged ureters.

Impact Statement

To reconstruct ureter tissues following partial ureterectomy, we fabricated bilayered adipose-derived mesenchymal cell (AMC)-gelatin sheets based on cell sheet engineering principles. The bilayered AMC-gelatin sheets were transplanted into rabbits to replace a surgically excised ureteral segment. At four and eight weeks after, the ureters that received bilayered AMC-gelatin sheets did not exhibit severe flexures, dilations, or strictures. The experimental ureteral walls had smooth muscle marker-positive cells that were differentiated from the AMCs, and similar cells were present in the adjacent intact ureteral tissues. Therefore, the bilayered AMC-gelatin sheets have the potential to reconstruct ureters damaged through disease or trauma.

Introduction

Treatment of ureteral defects and/or strictures often requires reconstruction by urological surgery using existing and newly developed techniques. Surgical management depends on the location within the ureter, i.e., proximal, middle, or distal portion, of the defect or injury.[1] Proximal and middle ureteral defects and/or strictures are usually reconstructed by endourological management or surgical operations involving primary excision and end-to-end anastomosis either in the form of pyeloureteroplasty or ureteroureterostomy.[2] Distal ureteral injuries are usually managed with ureteral reimplantation to achieve additional length with a downward nephropexy procedure, i.e., a psoas hitch or a Boari flap technique.[3] Recurrence of ureteral strictures requires either reoperation or an alternative treatment.[4] However, these repeated operations are limited by the length of the intact ureteral tissues. Advances in ureteral reconstructive procedures are now creating the possibility of overcoming the surgical limitations associated with ureteral length. Thus, the number of therapeutic surgical choices based upon location of the defect or injury may soon increase.

Tissue engineering has enabled the development of tubular structures, such as blood vessels, bronchial tubes, or gastrointestinal tracts that are produced with biomaterials, cells, or both.[5-12] All engineered tubular structures have two essential requirements, i.e., to replace the structurally and functionally defective tissues and to inhibit post-surgical stenosis. In addition to these requirements, the engineered ureteral tissues must tolerate the urine passing through the lumen and be sufficiently strong to withstand suturing to the existing healthy tissues and simultaneously prevent the leakage of urine.

Based on cell sheet engineering and tissue engineering principles,[13,14] we fabricated novel sheets composed of autologous adipose-derived mesenchymal cell (AMC) monolayers attached to supporting layers of a gelatin hydrogel sheet.[15] To be functionally effective in tissue reconstruction, two of the AMC gelatin sheets were placed together, AMC surface apposed to AMC surface, creating structures that we have designated as “bilayered AMC-gelatin sheets”. After implantation and during the replication and differentiation of AMCs in the bilayered gelatin sheets, it is likely that the implanted

structures provided cell resources and/or paracrine effects that governed further development.[16,17]

In this study, we determined if these developments included reconstruction of ureteral segments in rabbits following partial ureterectomy. Upon finding that to have occurred, we then determined if the reconstructive process included enhancement of smooth muscle layers and reduction in the presence of ureteral flexures, dilations, and strictures compared to control rabbits that received bilayered acellular-gelatin sheets. We also explored the mechanism by which reconstruction of the ureter structures was achieved.

Materials and Methods

Animals

Eighteen female New Zealand White rabbits (3.0-3.5 kg; Japan SLC, Inc., Shizuoka, Japan) at postnatal week 10 were used in this study. The rabbits were treated in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The Animal Ethics Committee of Shinshu University School of Medicine approved the protocol used in this study.

Preparation of AMCs

Rabbits were anesthetized by intramuscular injection of midazolam (1.5 mg/kg; Astellas, Tokyo, Japan) and medetomidine hydrochloride (0.5 mg/kg; Zenoaq, Orion Pharma, Orion Corporation Espoo, Finland), and then further anesthetized by inhalation of 3% sevoflurane (Abbot Japan Co., LTD., Tokyo, Japan). Through an abdominal incision, intraperitoneal adipose tissue (approximately 1.0 g) was harvested, and digested with 0.2% collagenase type I (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in Dulbecco's modified Eagle's medium (DMEM, Gibco, Thermo Fisher Scientific K.K., Kanagawa, Japan) for 90 minutes at 37°C with moderate rotary shaking. After shaking, the solution was centrifuged at 1,000 rpm for 4 minutes, and then the pelleted cells were suspended in culture medium composed of 4.5 g/L glucose-DMEM (Gibco) supplemented with 15% regular fetal bovine serum (Biowest, Nuaille, France) and 0.1% penicillin-streptomycin

(Gibco). After passing through a cell strainer (Life Science, Corning, NY, USA), primary cultures of the suspended cells were established on type I collagen-coated 10-cm culture dishes (AGC Techo Glass, Shizuoka, Japan) and incubated at 37°C in humid air with 5% CO_2 for 10 days. The medium was changed completely every day to wash off unattached cells. We used only the attached and proliferating cells on the type I collagen as AMCs.

Production of bilayered AMC-gelatin sheets

The adherent proliferating AMCs were dissociated with 0.25% trypsin (Gibco). For later identification, the cell membranes were labeled with the lipophilic red fluorescent dye PKH26 (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. After suspension at $1.0x10^6$ cells/ml in culture medium, the cells were seeded (1 ml) onto temperature-responsive culture dishes ($1.0x10^6$ cells/ml/well, UpCell® 6-cm dish, CellSeed Inc., Tokyo, Japan). The cells were cultured for 7-10 days, reaching over-confluence in which more than 80% of each dish surface was covered by a monolayer of cells.

To harvest the cells after reaching over-confluence, the culture medium was removed, and the temperature of the culture dishes was reduced to 20˚C. This procedure enables detachment of the AMCs from the culture dish while maintaining cell-cell contacts within the monolayer itself.[13,14] The detached AMC monolayers were then applied to a gelatin hydrogel sheet with dry dimensions of 8 mm in diameter and 0.3 mm in thickness (gelatin-layer; Genocel®; Nikke Medical Co., Inc. Osaka, Japan).[15] After wetting, the sheet dimensions were 12-mm diameter and 0.5-mm thickness. Two gelatin sheets, each supporting an AMC monolayer, were then overlaid together with the cell sides juxtaposed with one another to form a single bilayered AMC-gelatin sheet (Fig. 1A). We chose to create the bilayered support for the AMCs out of gelatin rather than collagen. It is generally acknowledged that cells adhere better to collagen than to gelatin; however, gelatin provides a better substratum upon which cell migration can occur. The ability of cells to migrate within the gelatin scaffolding was a valuable property that we sought to preserve.

The bilayered AMC-gelatin sheets were incubated without culture medium at 37°C in humid air with 5% CO_2 for 60 minutes. After the incubation, the bilayered AMC-gelatin

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sheets were cultured with 10 ml R-STEM (Rohto Pharmaceuticals, Osaka, Japan) at 37°C in 5% CO_2 for 3 days (Fig. 1A).

Transplantation of the bilayered AMC-gelatin sheets to replace the excised segments of the ureters

The rabbits from which the adipose tissue was harvested were anesthetized as above, and a catheter (Fr. 8, Terumo, Yokohama, Japan) was inserted through urethra to remove urine. A midline lower abdominal incision was made, and then the left ureter was exposed through the posterior peritoneum and then separated from the abdominal wall (Fig. 1B). After partial ureterectomy of approximately 1 cm, a polyethylene catheter (PE50, Nippon Becton Dickinson, Tokyo, Japan) was inserted into both the proximal remaining ureter end (from the kidney) and the distal remaining ureter end (leading to the bladder, Fig. 1C). The inserted catheter (Fig. 1C) was fixed at that site with a 5-0 silk thread. The purpose of the cathether was two-fold. First, it provided a stable structure upon which to apply and fix the bilayered acellular- and AMC-gelatin sheets. Second, the catheter prevented the exposure of the implanted structures to the urine passing through the ureters.

In each of nine rabbits, a bilayered autologous AMC-gelatin sheet was rolled two times around the inserted catheter (Fig. 1D). The sheet was then covered with an absorbable hemostat (Surgicel, Johnson and Johnson K.K., Tokyo, Japan) to maintain the tightness of the rolled sheet (Fig. 1E). To fix the bilayered AMC-gelatin sheet in place and prevent immediate unravelling of the rolled structure, a biodegradable polyglycolic acid (PGA) nonwoven fabric that was designed to be resorbed after about 2 weeks, was simply wrapped around the implanted bilayered sheet and sutured with a 5-0 silk thread (Fig. 1F). For control rabbits (n=9), the same operation was conducted with bilayered acellular-gelatin sheets. During the operations with each type of sheet (with and without the AMCs), we avoided peeling off each sheet by careful handling. Finally, the abdominal incision was closed, and the urethral catheter removed. The rabbits were maintained for 4 or 8 weeks after surgery.

At 4 weeks after surgery, the ureters of 6 control and 7 experimental rabbits were harvested, and the rabbits were sacrificed with the injection of an overdose of thiopental

sodium solution (Ravonal®, Nippro ES Pharma. Co, Ltd., Osaka, Japan). At 4 weeks after transplantation, two bilayered AMC-gelatin sheet-transplanted rabbits and three control rabbits were anesthetized as above, and the inserted polyethylene catheter was removed from their ureters. These rabbits were kept for another 4 weeks, and then their ureters were harvested, and the rabbits were sacrificed as above.

Histological and immunohistochemical investigations

All of the harvested ureters were fixed in 4% paraformaldehyde for 12 hours, and then immersed in decalcifier solution (OSTEOSOFT®, Sigma-Aldrich) for 12 hours to remove urinary stone-like particles. Then, the samples were embedded in paraffin and cut in 5-µm thick serial cross- and longitudinal-sections. The sections were deparaffinized with xylene, rehydrated with ethanol, and rinsed three times with phosphate-buffered saline. Each section was stained with Masson trichrome (ScyTek Laboratories, Inc., Logan, UT, USA) according to the manufacturer's protocol.

For immunohistochemistry, some of the deparaffinized sections were immersed in 10 mM sodium citrate (pH 6.0) and microwaved at 100°C for 10 minutes for antigen retrieval. The sections were blocked with 1.5% non-fat milk in phosphate-buffered saline for 1 hour at 4°C. Each section was incubated with one of the following antibodies for 12 hours at 4°C to identify smooth muscle cells: smooth muscle actin (SMA, 1:100 mouse monoclonal; Progen Biotechnik GmbH, Heidelberg, Germany), desmin (1:150 rabbit polyclonal; Progen Biotechnik GmbH), or Pax7 (1:1000, rabbit polyclonal; Lifespan Biosciences, Inc., Seattle, WA, USA). After the primary antibody reactions, the sections were incubated with the respective secondary antibodies consisting of donkey anti-mouse or -rabbit IgG, each conjugated with Alexa Fluor 488 (1:250; Molecular Probes, Eugene, OR, USA) for 1 hour at 4°C. After rinsing, they were counterstained with 5 µg/ml 4', 6-diamidino-phenylindole dihydrochloride (DAPI; Molecular Probes).

The bilayered AMC-gelatin sheets that were not used in a transplantation operation were immunohistochemically stained as described above with antibody for the mesenchymal cell marker STRO-1 (1:100, animal poly- or mono-, R&D System, Inc.,

Minneapolis, MN, USA) or the cell junction markers integrin β1 (1:50, rat monoclonal; R&D Systems, Inc.) or N-cadherin (1:100, rabbit polyclonal; Bioss , Woburn, MA, USA).

The Masson trichrome stained samples were observed and photographed with a common optical microscope. To evaluate the thickness of smooth muscle layers, the muscle layers in the Masson trichrome stained samples were randomly viewed and measured in 5-10 locations per tissue sample with a x20 objective lens by using image analysis software (cellSens, Olympus Co., Tokyo, Japan). The immunohistochemically stained samples were observed with a fluorescence microscope (Keyence, Osaka, Japan), and the AMCs were detected by the presence of the PKH26-labled red cells.

Statistical Analysis

Results were expressed as means ± standard deviations. Statistical differences were determined using the Excel® Statistics program (Esumi Co., Ltd. Tokyo, Japan). Comparisons were made by t-tests. P-values less than 0.05 were considered statistically significant.

Results

Characterization of bilayered AMC-gelatin sheets

Three days after transplantation of the bilayered AMC-gelatin sheet, the structural organization was examined histologically. The AMCs between the hydrogel sheets of each bilayer were identifiable, but the borders of each hydrogel sheet were indistinct due to cell replication, migration, and integration into the sheets (Fig. 2A). In some cases, the AMCs, or cells derived from the AMCs, had migrated through to the outer surfaces of the hydrogel sheets. Numerous AMCs were positive for the mesenchymal marker STRO-1 (Fig. 2B). The AMCs, or derived cells, expressed the cell junction markers integrin β1 (Fig. 2C) or N-cadherin (Fig. 2D), indicating the formation of cell-cell contacts.

Histological investigation of reconstructed ureters

At 4 weeks after surgery, there was evidence of ureteral reconstruction in both control and experimental groups. Under naked eye inspection, urine leakage from the ureters included in the treated region of either group was not evident. The proximal regions of the ureters

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treated with control bilayered acellular-gelatin sheets exhibited more flexures and dilations (Fig. 3A) that were larger than in normal unoperated ureters (Fig. 3B). Similar flexures and dilations were not present in the distal regions of the control ureters. In contrast, the proximal and distal regions of the ureters treated with experimental bilayered AMC-gelatin sheet transplanted rabbits did not exhibit severe flexures and/or dilations compared to the controls receiving the bilayered acellular-gelatin sheets (Fig. 3B).

At regions treated with the control bilayered acellular-gelatin sheets or experimental AMC-gelatin sheets, the ureteral tissues were composed of the luminal epithelium and underlying smooth muscle layers. In control rabbits, the reconstructed ureteral tissues had fibrotic-like fibroblast layers between the epithelium and the smooth muscle layers (Fig. 3C). In contrast, the ureteral tissues of the bilayered AMC-gelatin sheet-transplanted rabbits had thick smooth muscle layers within the treated regions (Fig. 3D). Additionally, there were no fibroblast layers like those in the controls. The thickness of the formed smooth muscle layers within the reconstructed ureteral walls in the bilayered AMC-gelatin sheet transplanted ureters was significantly greater than in the control ureters (Fig. 3E).

Histological investigation following catheter removal

At 8 weeks after surgery, and at 4 weeks after catheter removal, the reconstructed ureteral structures were intact. Similar to the 4-week findings, the proximal regions of control treated ureters exhibited severe flexures and dilations, while these abnormalities were not present in the distal regions (Fig. 4A). In contrast, there were no severe flexures or dilations in the proximal and distal regions of the bilayered AMC-gelatin sheet-transplanted ureters (Fig. 4B). Four weeks after removal of the catheters, i.e., 8 weeks after the surgery, the reconstructed ureteral tissues of both groups were also composed of a luminal epithelium and underlying smooth muscle layers (Fig. 4C, D). However, the lumina of the reconstructed ureteral tissues in control rabbits were narrowed by increased fibrosis between the epithelium and smooth muscle layers (Fig. 4E). In contrast, histologically, the reconstructed ureteral tissues that received the bilayered AMC-gelatin sheets did not present evidence of luminal strictures (Fig. 4F).

Differentiation of AMCs into smooth muscle cells within the bilayered AMC-gelatin sheets

At 4 weeks after transplantation, about midway between the proximal and distal ends of the reconstructed ureteral walls, the PKH26-labeled AMCs (Fig. 5A) and mature smooth muscle marker SMA-positive cells (Fig. 5B) were present. Some PKH26-labeled AMCs were simultaneously positive for the SMA-antibody (Fig. 5C). At other similar regions of the reconstructed ureteral walls, PKH26-labeled AMCs (Fig. 5D) were present with cells labeled for the immature smooth muscle marker desmin (Fig. 5E). Some of the PKH26-labeled AMCs were simultaneously positive for desmin-antibody (Fig. 5F). In the same or similar regions, the PKH26-labeled AMCs (Fig. 5G) and the myoblast marker Pax7-positive cells (Fig. 5H) were also detected. Similarly, some PKH26-labeled AMCs were simultaneously positive for Pax7-antibody (Fig. 5I). These results showed that in the reconstructed ureteral walls of the bilayered AMCs-gelatin sheet transplanted rabbits, some PKH26-labeled AMCs differentiated into smooth muscle marker-positive cells. Some of these double-positive cells appeared singly, and others were in contact with other double-positive cells.

Reconstruction process of ureter structures in the transplanted regions

At 4 weeks after transplantation, within the reconstructed ureteral tissues of both the control and experimental groups, the gelatin layers that formed either the bilayered acellular-gelatin sheets or the bilayered AMC-gelatin sheets were mostly resorbed, though small fragments of these gelatin layers were still present (see Fig. 6D and H below). In the control rabbits, which received the bilayered acellular-gelatin sheets, the resorbed gelatin layer spaces and the remaining gelatin fragments were populated with PKH26-unlabeled cells (Fig. 6A-D). Thus, it is likely that these cells migrated into the implanted structures from the proximal and distal ends of the original intact ureter that remained following the partial ureterectomy. In the experimental rabbits, which received the bilayered AMC-gelatin sheets, the resorbed gelatin layer spaces and the remaining gelatin fragments were populated with PKH26-labeled cells; however they also contained PKH26-unlabeled cells (Fig. 6E-H). Thus, it is likely that all of the PKH26-labeled cells were derived from the bilayered AMC-gelatin implants, whereas, the PKH26-unlabeled cells probably migrated

from the proximal and distal ends of the original intact ureter that remained following the partial ureterectomy as they did in the control rabbits.

In control rabbits receiving the bilayered acellular-gelatin sheets, about midway between the proximal and distal ends of the reconstructed ureteral walls, some of the PKH26-unlabeled cells were SMA-negative while others were SMA-positive (Fig. 6A). Similarly, at the proximal and distal borders of the control-treated, reconstructed ureteral walls, some PKH26-unlabeled cells were SMA-negative while others were SMA-positive. Other cells in these border regions were positive for either SMA- (Fig. 6B), desmin- (Fig. 6C), or Pax7 (Fig. 6D) proteins.

At both the midway and the border regions of the control-treated reconstructed ureteral walls, the PKH26-unlabeled cells with and without smooth muscle markers such as SMA or desmin proteins, were arranged as layered structures (Fig. 6A-C). Some of these cells, i.e.. Pax7-positive cells were also located within the small gelatin fragments that remained (Fig. 6D).

In experimental rabbits receiving the bilayered AMC-gelatin sheet implants, at regions about midway between the proximal and distal ends of the reconstructed ureteral walls, some PKH26-unlabeled cells were present that probably migrated from the proximal and distal ends of the original remaining intact ureter following the partial ureterectomy (Fig. 6E). Other cells were labeled with PKH26 that were derived from the bilayered AMC-gelatin implant and contained SMA proteins (see Fig. 5A-C). PKH26-unlabeled cells, some of which were SMA-positive and some of which were SMA-negative were located within the micro-spaces that formed between the layers of the PKH26-labeled bilayered AMC sheets (Fig. 6E).

Similar to the controls, experimental rabbits that received the bilayered AMC-gelatin sheet implants had PKH26-unlabeled cells at the proximal and distal borders between the implanted regions and the intact ureteral tissues. Some of these cells were negative for smooth muscle markers while others were positive for them (Fig. 6E-G). Importantly, these cells were arranged in layered structures (Fig. 6E-G). In the same regions, PKH26-labeled cells that were SMA- and desmin-positive (Fig. 6, G respectively) were located alongside

the formed layered structures. In the same regions, PKH26-labeled cells that were SMA- (Fig. 6F), desmin- (Fig. 6G), and Pax7- (Fig. 6H) positive were located within the formed layered structures.

Discussion

Clinical treatment trials for ureteral defects and/or strictures have been conducted with autologous grafts derived from the buccal and lingual oral mucosae,[18,19] ileal and appendix mucosae,[20,21] renal pelvis wall,[22] bladder mucosae,[23] and penile/preputial skin.[24,25] Our study reported here shows that in rabbits, autologously derived bilayered AMC-gelatin sheets have the potential to replace defective ureteral tissue and/or reconstruct the ureters themselves. In our experimental rabbit model, the reconstructed ureters appeared to be structurally adequate to inhibit urine leakage.

Both the control rabbits with bilayered acellular-gelatin sheet implants and the experimental rabbits with the bilayered AMC-gelatin sheet implants developed reconstructed ureteral structures over the 8 weeks of the experiment. Thus, the gelatin-layers alone might have contributed to the ureteral reconstruction, though the control reconstructed ureteral tissues were much less well developed than the experimental reconstructed ones. Additionally, at both 4 and 8 weeks after implantation, the proximal region of the ureters receiving the control bilayered acellular-gelatin sheet implant had flexures, dilations, and strictures; however, none of these defects were present in any region of the ureters receiving the bilayered AMC-gelatin sheet implants.

The presence of flexures, dilations, and strictures in the control rabbits and the absence of them in the experimental rabbits correlates with the subepithelial organization of the reconstructed ureteral tissues in each group. In the control rabbits, the development of smooth muscle layers beneath the epithelium was minimal, but the development of fibrotic tissues between the smooth muscle layers was significant. In contrast, the ureteral smooth muscle layers in the experimental rabbits were thicker and better organized compared to the controls and had smaller fibrotic layers between the epithelium and the smooth muscle layers.

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Some of the PKH26-labeled cells within the bilayered AMC-gelatin sheets differentiated into SMA-positive mature smooth muscle cells, desmin-positive immature smooth muscle cells, or Pax7-positive myoblasts. Similar smooth muscle-development steps were reported by Robbins et al. for fetal rat gubernaculum mesenchymal cells.[26] These findings suggested that the AMCs within the bilayered AMC-gelatin sheets differentiated into smooth muscle cells in stages. Also, some of the SMA-positive smooth muscle cells were present as single cells, while others were in contact with other SMA-positive cells. In addition, PKH26-unlabeled, SMA-positive cells were present in both the control bilayered acellular-gelatin sheet ureter structure walls and the experimental bilayered AMC-gelatin sheet ureter structure walls. This suggests that the PKH26-unlabeled SMA-positive cells were derived from the proximal and distal ends of the original ureteral tissues that were adjacent to the implanted bilayered gelatin sheets. It is probable that PKH26-unlabeled multipotent cells from the proximal and distal ends of the original intact ureter remained following the partial ureterectomy. Thus it is likely that some of these cells migrated through the gelatin sheets that surrounded the proximal and distal ends of the original ureters, whereupon they differentiated into cells destined to become smooth muscle cells.

Historically, cell sheet engineering has been considered to provide mainly paracrine effects for tissue reconstruction.[17, 27, 28] The AMCs, which include mesenchymal stem cells, are also known to have paracrine effects.[29] Our bilayered AMC-gelatin sheets replaced approximately 1 cm of excised ureter, and paracrine effects of mesenchymal stem cells within the bilayered AMC-gelatin sheets are possible, or even likely. Thus, in the experimental rabbits, both differentiation and paracrine stimulation might have induced the thicker smooth muscle layers.[30]

We focused on the reconstruction process that occurred by 4 weeks after implantation of the control or experimental sheets. In the control group, the space of the resorbed gelatin sheet was replaced with PKH26-unlabeled cells, some of which were negative for smooth muscle markers and some were positive for them. Presumably, these PKH26-unlabeled cells migrated from the proximal and distal ends of the remaining original intact ureter. The presence of some PKH26-unlabeled cells that were positive for

smooth muscle protein markers indicates that the cells underwent differentiation following the migration of the parent cells from the remaining ends of the original ureters. Similarly, in the ureters reconstructed with the aid of the experimental bilayered AMC-gelatin sheets, some of the SMA-positive cells did not have PKH26 fluorescence, while others did. These observations for both the control and experimental implants suggest that the gelatin layers themselves could have served as scaffolds that enabled the migration of cells from the ends of the original ureters. Upon arrival, the gelatin scaffold could have facilitated cell replication and differentiation through paracrine effectors to form smooth muscle-like cells and possibly other cell types as well. The presence of AMCs in the experimental bilayer gelatin sheets probably enabled a more rapid and organized start to the ureter-like reconstruction process that was visible in the better organized ureter tissues at 4 and 8 weeks compared to the control bilayered acellular-gelatin sheets.

This study had some limitations that need further investigation. First, in the ureteral tissues derived from the bilayered AMC-gelatin sheets, the development of fibrosis-like fibroblast layers between the epithelium and the smooth muscle layers was inhibited compared to the controls. While some factors secreted from the AMCs might be directly or indirectly related to this effect,[31,32] this study was not designed to explore that aspect of ureteral regeneration. Second, the lumen of the reconstructed ureteral tissues in the bilayered AMC-gelatin sheet-transplanted rabbits did not show histological evidence of strictures at 4 and 8 weeks after implantation. This result might be related to the suppression of fibrosis, but we did not investigate the mechanism. Finally, this study did not explain exactly why the proximal region of the reconstructed ureteral tissues in the control rabbits had flexures and dilations while the bilayered AMC-gelatin sheet-transplanted rabbits did not. This is another topic for future research.

Conclusions

In this study, we fabricated bilayered autologous AMC-gelatin sheets, and then transplanted them into rabbits to replace a surgically excised segment of ureter. At 4 and 8 weeks after transplantation, the proximal region of the reconstructed control ureteral tissues derived from bilayered acellular-gelatin sheets exhibited severe flexures or

dilations. In contrast, neither the proximal nor the distal regions of the experimental reconstructed ureteral tissues that developed from the bilayered autologous AMC-gelatin sheets exhibited these abnormalities. About midway between the proximal and distal ends of the experimental ureteral tissue, the walls had smooth muscle marker-positive cells composed of both the AMC-derived cells and migrating cells that were derived from the adjacent intact ureteral tissue. Together, these cells formed smooth muscle layers that were significantly thicker and better organization than those in the ureteral tissues that developed from the control bilayered acellular cell gelatin-sheets. These results indicate that the bilayered AMC-gelatin sheets have the potential to reconstruct ureters that become damaged through disease or trauma.

Disclosure Statements

No competing financial interests exist.

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Figure Legends

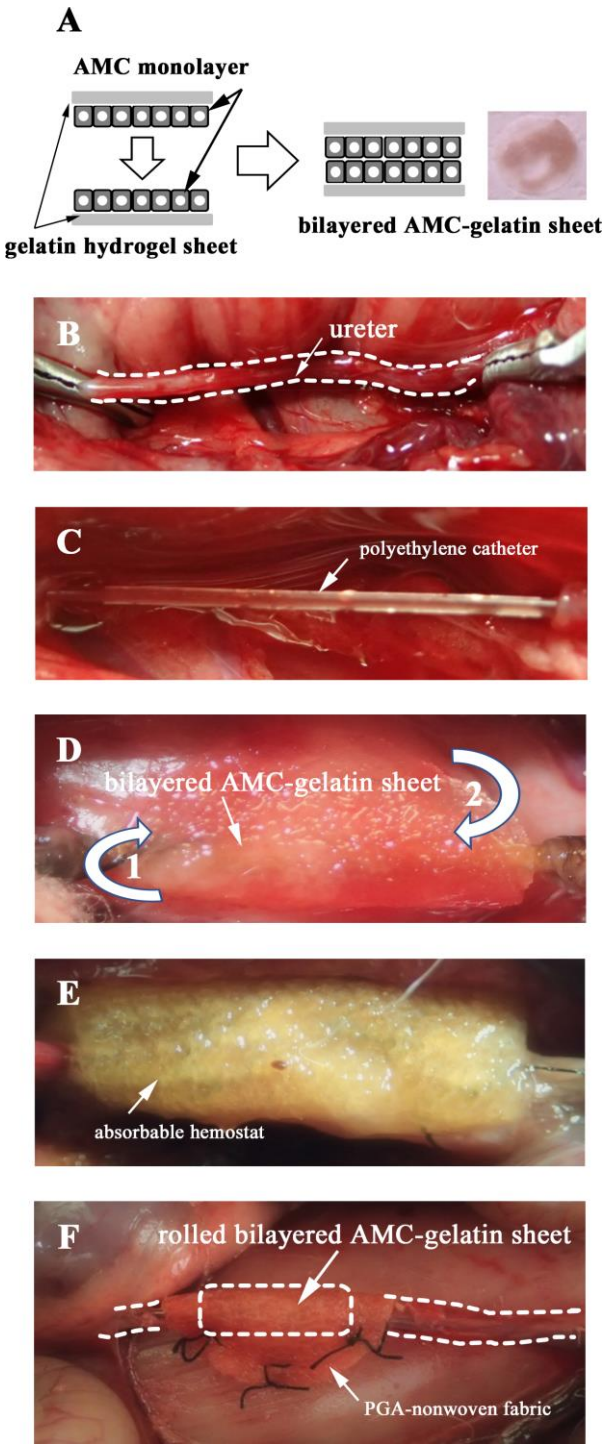

Figure 1.

Production and transplantation of bilayered adipose-derived mesenchymal cell-gelatin sheets. (**A**) Monolayers of adipose-derived mesenchymal cells (AMCs) detached from thermally responsive culture dishes were harvested and then applied to a gelatin hydrogel

sheet. Bilayered AMC-gelatin sheets were assembled by placing two AMC monolayer sheets together, with the cells facing one another. (**B**) Just prior to excision, the left ureter (dashed white lines) was exposed and separated from posterior abdominal wall. (**C**) Partial ureterectomy of approximately 1 cm was conducted, and then a polyethylene catheter was inserted to connect the proximal remaining ureteral portion (leading to the kidney) with the distal remaining ureteral portion (leading to the urinary bladder). (**D**) The bilayered autologous AMC-gelatin sheet was rolled twice around the inserted catheter. (**E**) The rolled bilayered AMC-gelatin sheet was covered with an absorbable hemostat to maintain the tightness of the rolled AMC-gelatin bilayer. (**F**) A polyglycolic acid (PGA) nonwoven fabric was simply wrapped around the implanted bilayered AMC-gelatin sheet. The PGA nonwoven fabric was then sutured to fix the rolled bilayered sheet in place.

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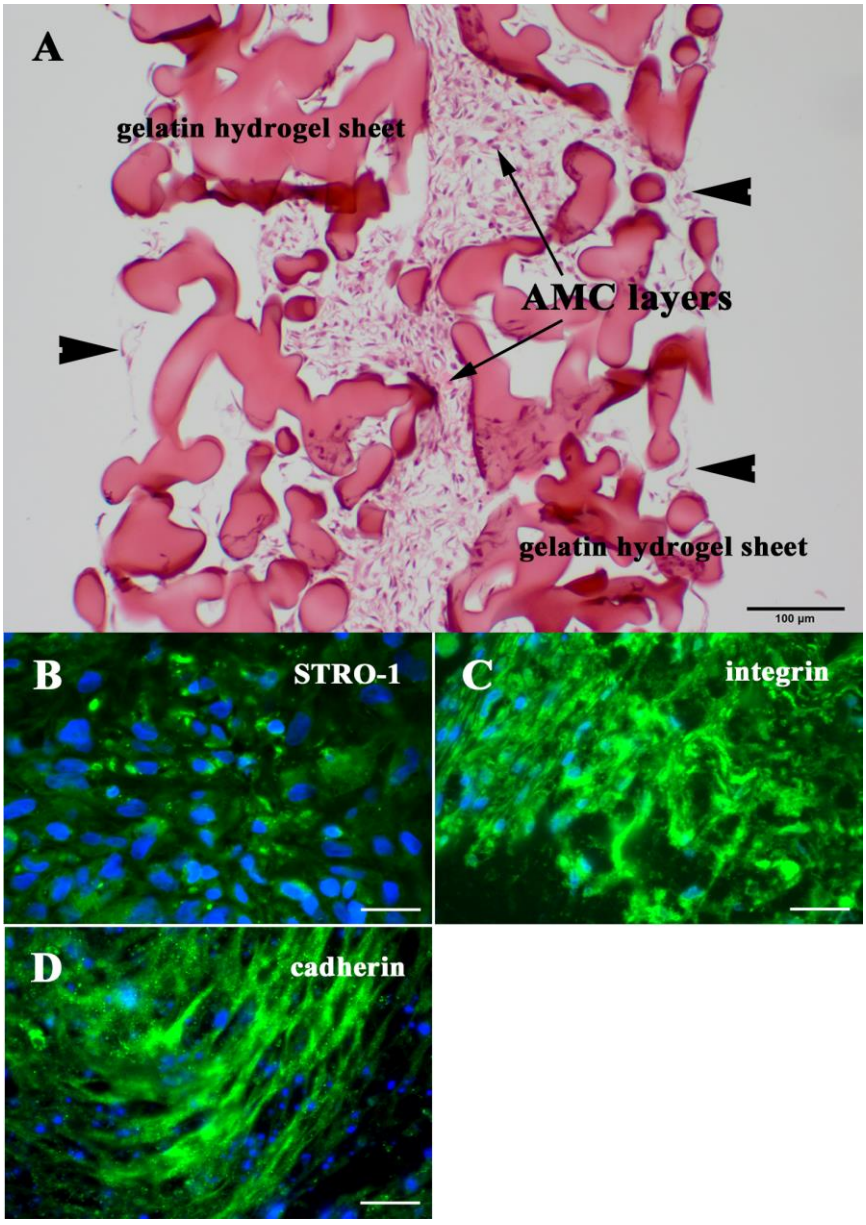

Figure 2.

Characterization of AMCs within bilayered gelatin sheets at 3 days after implantation. (**A**) AMCs were distributed between the two gelatin hydrogel layer sheets. Some of the AMCs, or cells derived from them, had migrated through the gelatin hydrogel sheets to reach the exterior surfaces of each sheet (arrowheads). (**B**) Numerous AMCs (or derived cells) were positive for the mesenchymal marker STRO-1 (green). Blue: DAPI-stained nuclei. Scale bar: 20 µm. (**C and D**) Cell junction markers integrin β1 (**C**, green) or N-cadherin (**D**, green) indicated the presence of cell-cell junctions. Blue: DAPI-stained nuclei. Scale bar: 20 µm.

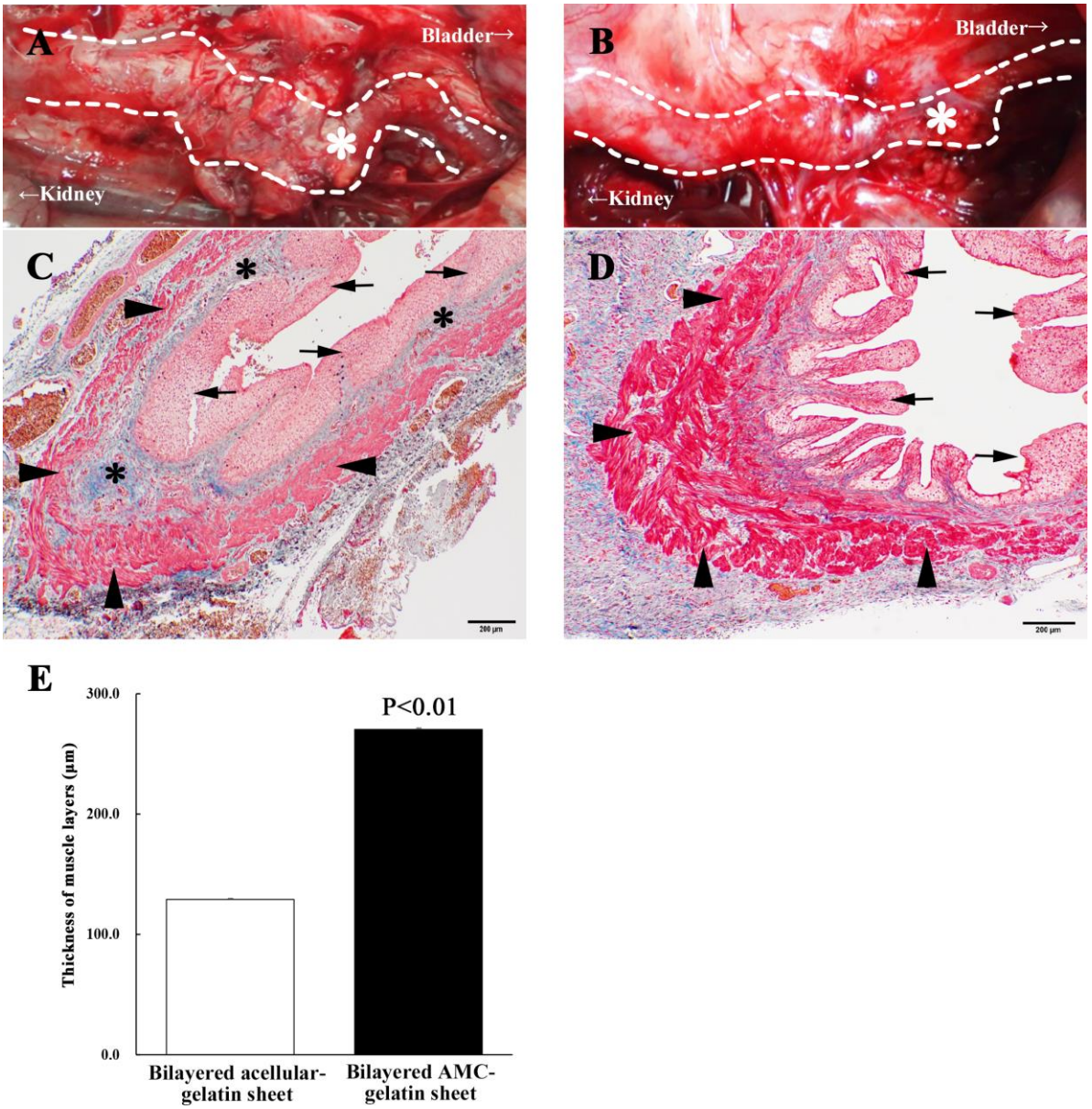

Figure 3.

Histology of the reconstructed ureter structures at 4 weeks after transplantation. (**A**) In control rabbits, the proximal regions (from the kidney) of the ureters treated with control bilayered acellular-gelatin sheets exhibited flexures and dilations compared to normal ureters (Fig. 1B). Dashed white lines: ureter including treated regions (asterisk). The distal regions did not exhibit flexures and dilations. (**B**) In experimental rabbits, the proximal regions of the ureters treated with the bilayered AMC-gelatin sheets did not show severe flexure and/or dilations like that of the controls (A). Dashed white lines: ureter including treated regions (asterisk). Similar findings were present in the distal regions. (**C and D**) At

regions treated with the bilayered acellular- or AMC-gelatin sheets, the ureteral tissues were composed of epithelium (arrows) and smooth muscle layers (arrowheads). (**C**) The reconstructed ureteral tissues of control rabbits had fibrosis-like fibroblast layers (asterisks) between the epithelium and smooth muscle layers. **(D)** In contrast, the bilayered AMC-gelatin sheet transplanted ureteral tissues did not have the fibroblast layers like those in the controls. (**E**) Thickness of smooth muscle layers within the reconstructed ureter walls in the bilayered AMC-gelatin sheet transplantation group was significantly greater than in the controls.

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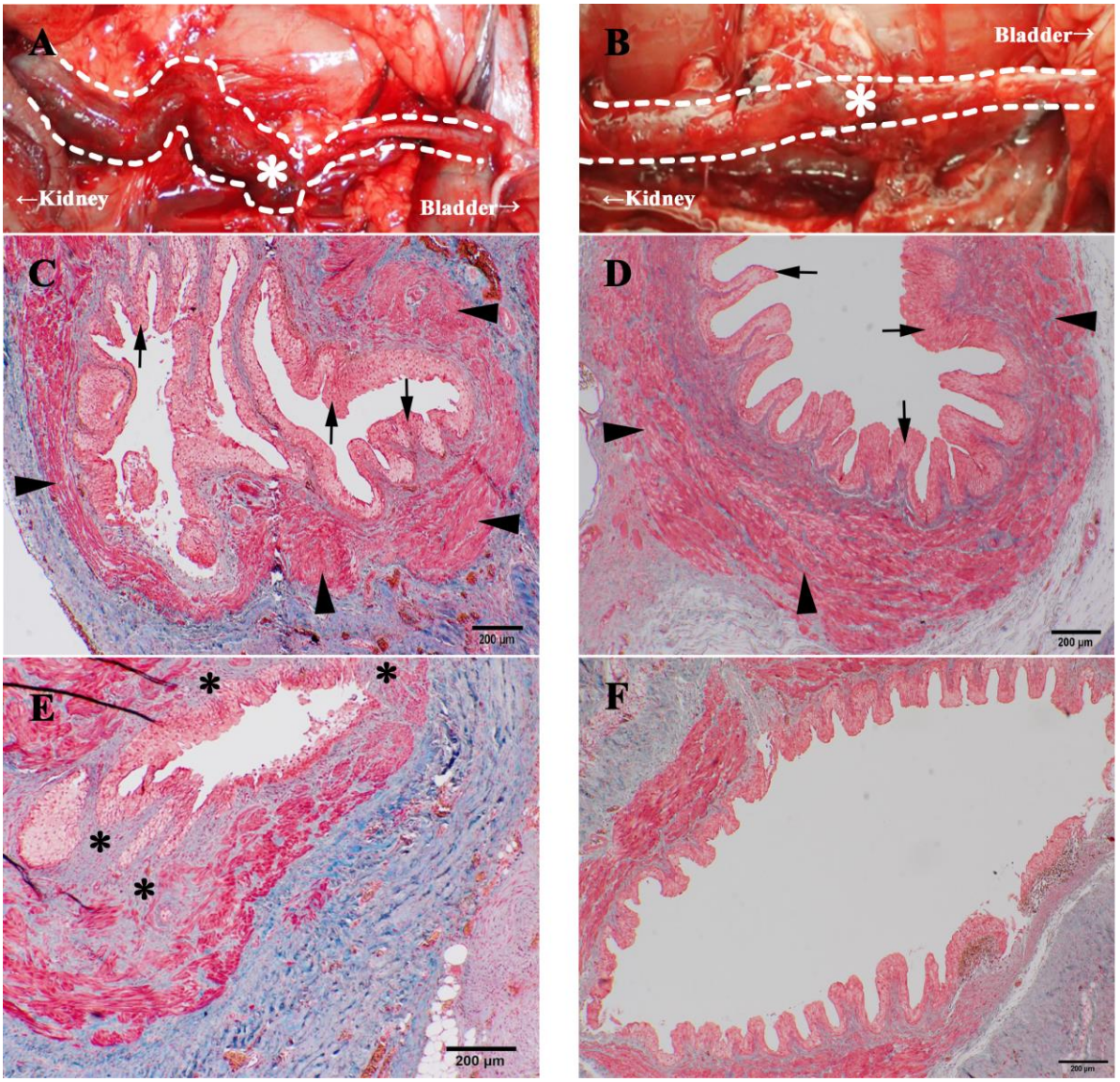

Figure 4.

Histology of the reconstructed ureter structures at 8 weeks after transplantation (4 weeks after removal of the catheter). (**A and B**) The proximal regions of the ureters treated with control bilayered acellular-gelatin sheets (A) exhibited severe flexures and dilations; however, the bilayered AMC-gelatin sheet-transplanted ureters did not (B). The distal regions did not exhibit flexures and dilations in either group. Dashed white lines: ureter including control- (A, asterisk) or experimental- (B, asterisks) treated regions. (**C and D**) The reconstructed ureteral tissues of both control (C) and experimental (D) groups were composed of epithelium (arrows) and smooth muscle layers (arrowheads). (**E and F**) While lumina of the reconstructed ureteral tissues in control rabbits (E) were narrow with

increased fibrosis (asterisks) between the epithelium and smooth muscle layers, the bilayered AMC-gelatin sheet-transplanted ureteral tissues did not exhibit the histological stricture-like lumina (F). Scale bars: 200 μm.

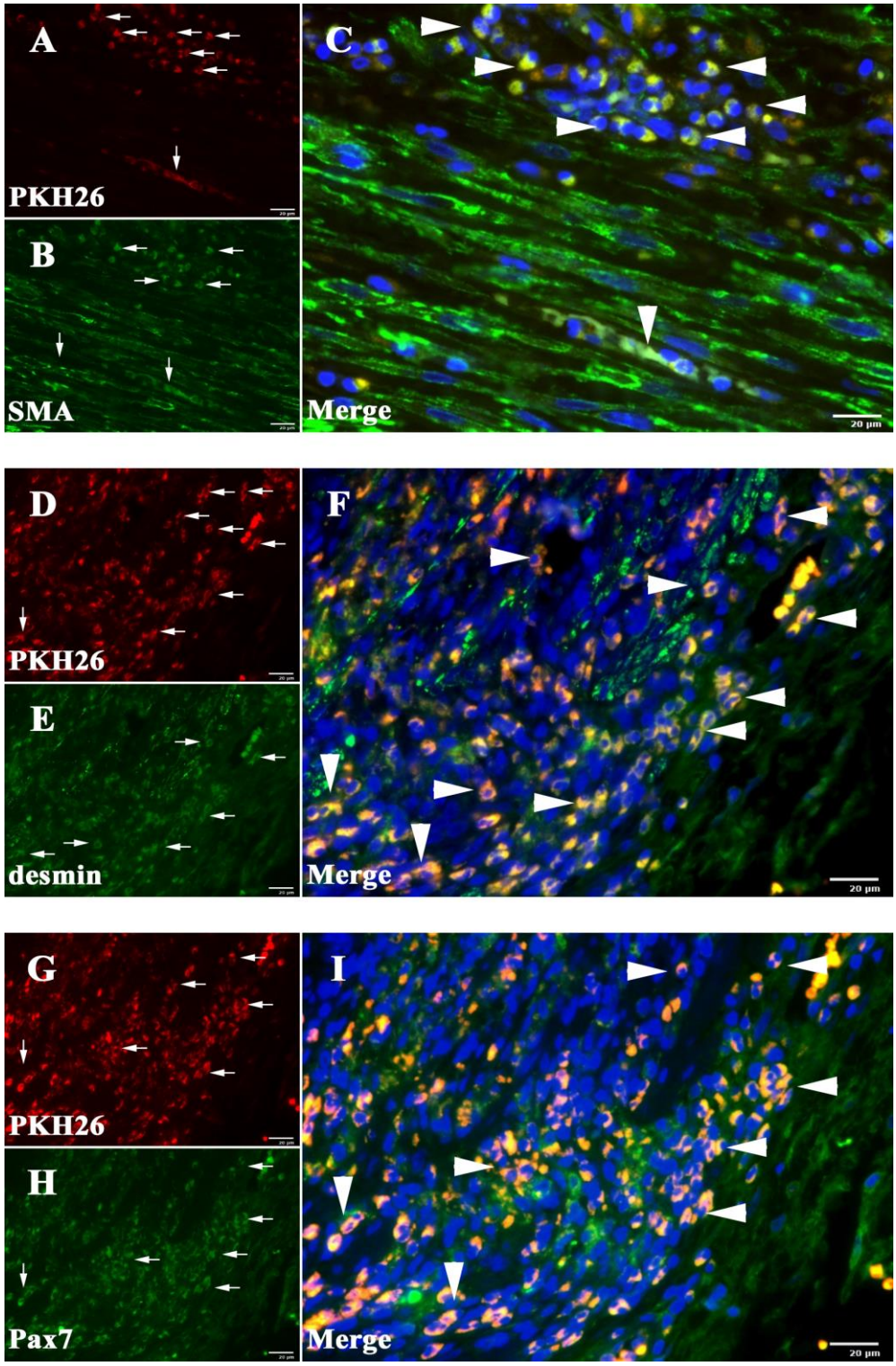

Figure 5.

Differentiation of smooth muscle cells from PHK26-labeled cells in the transplanted bilayered AMC-gelatin sheets. (**A-C**) At 4 weeks after transplantation, at about midway between the proximal and distal ends of the reconstructed ureter structures, PKH26-

labeled AMCs (A, red, arrows) and mature smooth muscle SMA-positive cells (B, green, arrows) were present. Some of the PKH26-labeled AMCs were simultaneously positive for the SMA-antibody (C, yellow, arrowheads). (**D-F**) At regions similar to those in Fig. 4A-C, other PKH26-labeled AMCs (D, red, arrows) and immature smooth muscle marker desmin-positive cells (E, green, arrows) were also present. Some of the PKH26-labeled AMCs were simultaneously positive for desmin antibody (F, yellow, arrowheads). (**G-I**) At regions nearby Fig. 5D-F, PKH26-labeled AMCs (G, red, arrows) and myoblast marker Pax7-positive cells (H, green, arrows) were also detected. Some of the PKH26-labeled AMCs were simultaneously positive for Pax7-antibody (I, yellow, arrowheads). Blue: DAPI-stained nuclei. Scale bars: 20 μm.

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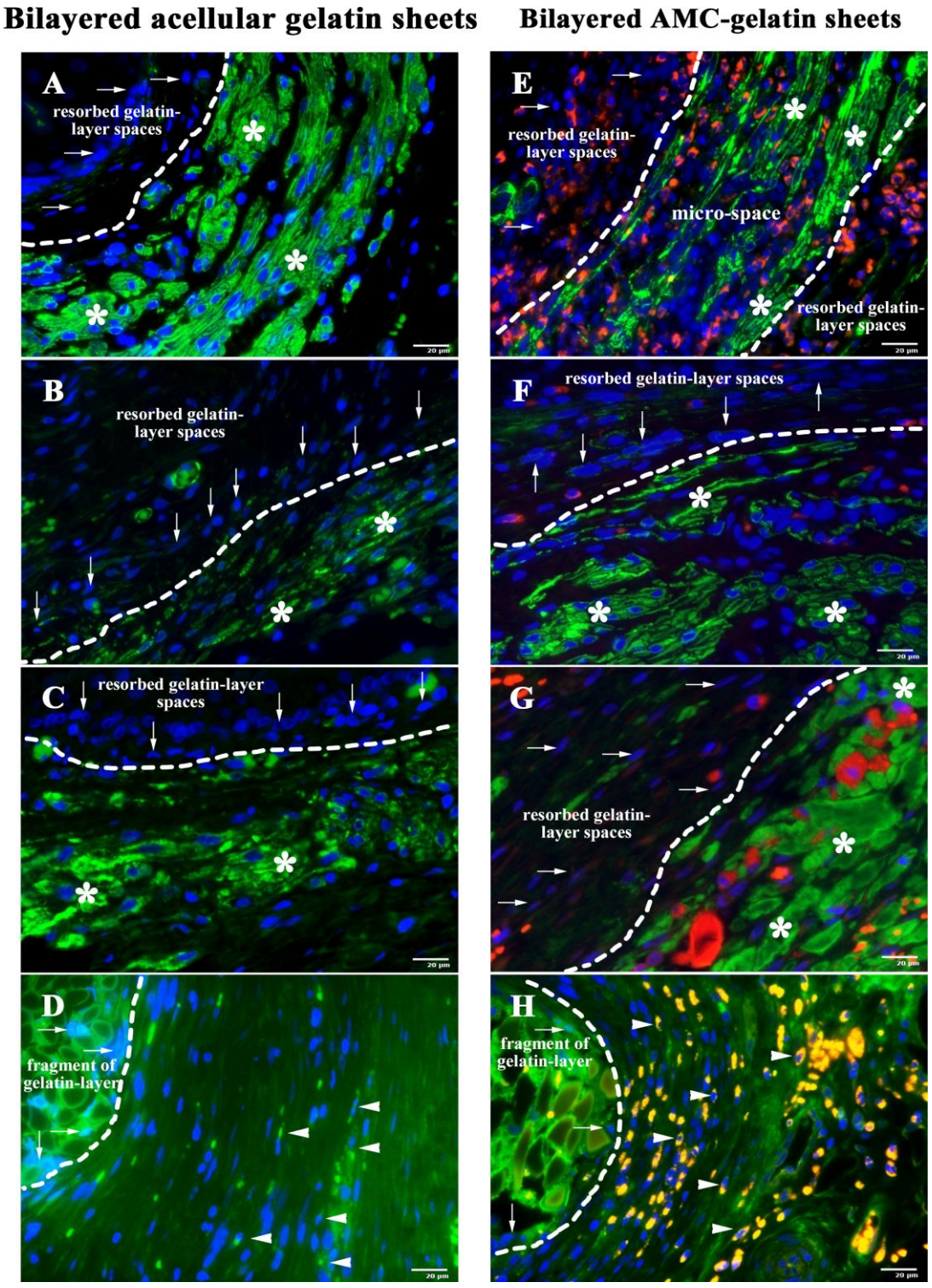

Figure 6.

Reconstruction process of ureter structures. (**A**) At about midway between the proximal and distal ends of the reconstructed ureteral walls in the control rabbits, there were numerous PKH26-unlabeled cells (white arrows) located in the resorbed gelatin layer

spaces. These cells probably migrated from the proximal and distal ends of the intact ureter that remained after the partial ureterectomy. Some of the cells were SMA-positive smooth muscle cells (green, asterisks). (**B-D**) At the borders of the control treated regions, the PKH26-unlabeled cells that migrated from the original intact ureter to the resorbed gelatin layer spaces were arranged as layered structures (white arrows). SMA- (B, green, asterisks), desmin- (C, green, asterisks), and Pax7- (D green, arrowheads) positive cells, which also migrated from the original intact ureter were located alongside the formed layered structures or the remaining small fragments of the gelatin layer. (**E**) At about midway between the proximal and distal ends of the reconstructed ureteral walls in the bilayered AMC-gelatin sheet-transplanted rabbits, PKH26-unlabeled SMA-positive muscle cells (asterisks) that migrated from the adjacent intact tissue after ureteral excision were present within micro-spaces formed between the PKH26-labeled AMC (red) monolayers (between dashed white lines). White arrows: PKH26-unlabeled cells that were negative for SMA antibody. (**F and G**) At the proximal and distal borders of the experimental bilayered AMC-gelatin sheet-transplanted regions, SMA- (F, green, asterisks) and desmin- (G, green, asterisks) positive muscle cells were present along with adjacent intact ureter-derived PKH26-unlabeled cells (white arrows). Red: PKH26-labeled AMCs. (**H**) At regions similar to (F and G), the PKH26-labeled AMCs that migrated from the bilayered AMC-gelatin sheets were also present along with intact ureter-derived cells (white arrows). Some of the PKH26-labeled cells differentiated into Pax7-positive cells (yellow, arrowheads). Blue: DAPI-stained nuclei. Scale bars: 20 μm.

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