Smooth Muscle Progenitor Cells Derived From Human Pluripotent Stem Cells Induce Histologic Changes in Injured Urethral Sphincter

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ABSTRACT

Data suggest that myoblasts from various sources, including bone marrow, skeletal muscle, and adipose tissue, can restore muscle function in patients with urinary incontinence. Animal data have indicated that these progenitor cells exert mostly a paracrine effect on the native tissues rather than cell persistence of the smooth muscle phenotype. Taken together, the data suggest that hPSC-derived pSMCs facilitate restoration of urethral sphincter function by direct smooth muscle cell regeneration and by inducing native tissue elastin/collagen III remodeling.

SIGNIFICANCE

The present study provides evidence that a pure population of human smooth muscle progenitor cells (pSMCs) derived from human pluripotent stem cells (hPSCs) can restore muscle function in patients with urinary incontinence. Animal data have indicated that these progenitor cells exert mostly a paracrine effect on the native tissues rather than cell persistence of the smooth muscle phenotype. Taken together, the data suggest that hPSC-derived pSMCs facilitate restoration of urethral sphincter function by direct smooth muscle cell regeneration and by inducing native tissue elastin/collagen III remodeling.

INTRODUCTION

Stress urinary incontinence (SUI), a condition defined as the uncontrolled leakage of urine on exertion (e.g., coughing, sneezing, laughing) [1], affects 25%–50% of the female population and causes an estimated $12 billion financial burden annually in the United States [2]. Because SUI often occurs during daily activities, it seriously impairs the quality of life of affected women [3, 4]. It is also associated with an increased incidence of mental disorders, such as anxiety, stress, and depression [5, 6].

Intrinsic sphincter deficiency (ISD), which is caused by injury to either the muscle layers of the urethral sphincter (smooth and striated muscles) or to its innervation, is a significant contributor to SUI [7, 8]. SUI patients with ISD often have more severe urinary incontinence and have a higher rate of no response to the current standard treatment [9]. When conservative management fails, invasive surgery may be necessary. Although the primary success rate of surgery is high, recurrence of incontinence can occur after surgery. The primary effect of surgery is to increase urethral...
support and not to reconstruct the damaged or aged urethral sphincter. This limitation and the progressive loss of the circular smooth muscle layer of the urethral sphincter with aging [10] are thought to contribute to long-term recurrence of incontinence after surgery. The 5-year success rates of two common surgeries for treatment of SUI, the retropubic and transobturator midurethral slings, have been reported to be 51.3%–84.7% and 43.4%–86.2%, respectively [11–13]. These data indicate that a reasonable number of the patients will experience the recurrence of urinary incontinence. Hence, a great need exists for minimally invasive alternative treatments that can effectively restore the damaged urethral sphincter, especially in the aged population. Mesenchymal stem cells (MSCs) and myoblasts have been proposed as viable cell candidates that can regenerate a damaged urethral sphincter.

Various types of MSCs, isolated from bone marrow, skeletal muscle, and adipose tissue, have been tested for treatment of SUI, showing positive effects on urethral function in animals [14, 15] and safety and feasibility in humans [16, 17]. However, these adult stem cells are difficult to harvest and expand in vitro, especially in older patients. Furthermore, because the final cell population for transplantation from these cell sources is heterogeneous (including MSCs, myoblasts, satellite cells, fibroblasts, and so forth), efforts to optimize these stem cell-based therapies have been hampered by the lack of understanding of the mechanisms of action of each cell type. Human pluripotent stem cells (hPSCs) are therefore a viable cell source because of their self-renewal and differentiation capabilities. Large numbers of homogeneous muscle progenitor cells can be generated from hPSCs. Recently, we observed that hPSC-derived myoblasts could restore urethral function in an SUI animal model [18].

Current data from animal models using animal autologous mesenchymal stem cells suggest that MSC/myoblasts/progenitor cells (mixed cell populations) exert their effect in vivo mostly through secretion of paracrine factors, rather than by significant cell replacement [15]. A few studies using human adult stem cell sources, amniotic fluid stem cell (SC)-derived myocytes [19] and myoblasts from patient muscle biopsies [20], have suggested integration of muscle-like cells in vivo. Scaut data are available on the paracrine effects of these human SC/progenitors in vivo. No data are available on the in vivo effects of human induced pluripotent SC (iPSC)-derived muscle progenitor cells. In the present study, we sought to examine the histologic effects of a pure population of smooth muscle progenitor cells (pSMCs) derived from hPSCs (human embryonic SCs and iPSCs) on the urethra of SUI rodents.

**MATERIALS AND METHODS**

**Cell Preparation and Culture**

Human bladder smooth muscle cells (bSMCs) (Lonza, Allendale, NJ, http://www.lonza.com) were cultured in 90% Dulbecco’s modified Eagle’s medium and 10% fetal bovine serum at 37°C in an atmosphere of 95% air and 5% CO2. The cells were transplanted at passage 7. Human pSMCs were differentiated from luciferase (Luc)-tagged/nontagged H9-ESCs and iPSCs. Two iPSC lines reprogrammed from dermal fibroblasts, Luc-tagged retrovirus vector reprogrammed iPSCs (RV-iPSCs) and nonintegrating episomal plasmid reprogrammed iPSCs (Epi-iPSCs; Thermo Fisher Scientific Life Sciences, Waltham, MA, http://www.thermofisher.com), were investigated in the present study. The institutional review board of the Stanford University School of Medicine and the Stanford University Stem Cell Research Oversight Committee approved the present study. Each subject provided written informed consent.

The pSMC differentiation method was adapted from Marchand et al. [21] and Wang et al. [22]. In brief, the stem cells were cultured in chemically defined medium (Roswell Park Memorial Institute 1640 with 1 mM GlutaMAX [Thermo Fisher Scientific Life Sciences], 1% wt/vol nonessential amino acids [Thermo Fisher Scientific Life Sciences], 0.1 mM β-mercaptoethanol, 1% wt/vol insulin-transferrin-selenium [Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com]) supplemented with 50 ng/ml activin A (Peprotech, Rocky Hill, NJ, http://www.peprotech.com) and 50 ng/ml human bone morphogenetic protein 4 (PeproTech), and then 50 ng/ml fibroblast growth factor-2 (PeproTech) and 40 ng/ml vascular endothelial growth factor (Thermo Fisher Scientific Life Sciences). Cells were FACS sorted for CD 31+/34+ markers and then expanded in smooth muscle growth medium (Thermo Fisher Scientific Life Sciences). Luc-tagged pSMCs were used for in vivo tracking. Terminally differentiated SMCs were characterized by SMC markers, proliferation, contractility, and karyotype (unpublished data).

**Animal Care and Generation of SUI Rat Model**

We used female immunodeficient Rowett Nude (RNU) rats (200–250 g) and 8–10-week-old CB17 severe combined immune-deficiency (SCID) female mice (Charles River Laboratories, Hollister, CA, http://www.criver.com/). RNU rats were used to investigate the effects of pSMCs on injured urethra, and SCID mice were used to examine the long-term in vivo survival of pSMCs because they are severely immunocompromised. The animals were maintained at the Stanford University Research Animal Facility in accordance with Stanford University’s Institutional Animal Care and Use Committee guidelines. The durable rat model of SUI was established via transabdominal urethrolysis as described by Rodríguez et al. [23]. Because our previous study showed that the extracellular matrix (ECM) in pelvic connective tissue from women with SUI is modulated by cyclic reproductive hormones [24] and because most SUI patients who might require stem cell therapy will be older and likely estrogen deficient, we performed ovariotomy on the rodents to eliminate the influence of the estrus cycle on the ECM metabolism and to simulate an estrogen-deficiency state [25]. In brief, RNU rats were anesthetized with ketamine (30 mg/kg) and xylazine (3 mg/kg) by intraperitoneal injection. The ovaries were exteriorized through a lower abdominal incision. After the ovarian vessels were ligated, the ovaries were excised between the fallopian tube and the uterine horn. The bladder was identified and the urethra circumferentially detached from the anterior vaginal wall and pubic bone by sharp dissection. The abdominal skin was closed with wound clips. The animal was monitored until recovery.

**Cell Transplantation and Tissue Collection**

The rats were divided into six groups: group 1, urethrolysis plus saline injection (sham-saline group, n = 28); group 2, urethrolysis plus bSMC injection (bSMC group, n = 24); group 3, urethrolysis...
plus H9-ESC-derived pSMC injection (H9-pSMC group, n = 22); group 4, urethrolysis plus RV-iPSC-derived pSMC injection (RV-iPSC-pSMC group, n = 18); group 5, urethrolysis plus Epi-iPSC-derived pSMC injection (Epi-iPSC-pSMC group, n = 9); and group 6, intact RNU rats served as controls (pure control group, n = 26). Cell transplantation was performed 3 weeks after urethrolysis. After the rats were anesthetized with 1%–3% isoflurane, 2 × 10⁶ cells suspended in 50 μl of smooth muscle growth medium (Thermo Fisher Scientific Life Sciences) were injected periurethrally using a 28.5-gauge insulin syringe. The SUI rats in the sham-saline group underwent injections with 50 μl of saline only. Rat urethras and bladders were collected 5 weeks after injection. The proximal part of the urethra was embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Tokyo, Japan, http://www.sakura-finetek.com) for histologic study. The middle part of the urethra was used for RNA extraction, and the distal part of the urethra and bladder were used for protein extraction. For SCID mice (n = 10), 1 × 10⁶ pSMCs in 50 μl of Matrigel (50% wt/vol; BD Biosciences, San Jose, CA, http://www.bdbiosciences.com) were injected directly into the adductor longus muscles. After 6 months of monitoring, the mice were sacrificed and the hind leg skeletal muscles harvested and embedded in OCT (Sakura Finetek) and stored at −80°C for further studies.

In Vivo Bioluminescence Imaging of Transplanted Smooth Muscle Progenitor Cells

For in vivo cell tracking, the Luc-tagged pSMCs were injected into the urethral sphincter region of the RNU rats (n = 38) or the hind legs of SCID mice (n = 20). Transplanted cell survival was monitored via bioluminescence imaging (BLI) using the Xenogen in vivo Imaging System (Caliper Life Sciences, Waltham, MA, http://www.perkinelmer.com). In brief, D-luciferin (Biosynth, Itasca, IL, https://http://www.biosynth.com) was administered intraperitoneally at a dose of 375 mg/kg body weight 15 minutes before image acquisition. The animals were placed in a light-tight chamber, and photons emitted from luciferase-expressing cells were collected with integration times of 2 minutes. BLI signal was quantified in maximum photons per second per cm² per steradian and presented as Log₁₀ (photons per second). Images were obtained every 2 days until the disappearance of signal and then once per week for 5 weeks or 6 months, respectively.

Immunofluorescence Staining

The cryostat sections of rodent tissues were prepared and fixed, as described previously [26]. The slides were treated with 0.25% vol/vol Triton-100 in phosphate-buffered saline (PBS) for 10 minutes at room temperature. After washing with PBS-Tween (PBS-T; 0.01% vol/vol Tween in PBS) and blocking with 1% wt/vol bovine serum albumin in PBS-T, the slides were incubated with primary antibodies overnight at 4°C followed by appropriate secondary antibodies in a moisture chamber. Primary antibodies against the following molecules were used: smoothelin (1:50; rabbit polyclonal antibody; Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com), human nuclei (1:50; mouse polyclonal antibody; EMD Millipore Corporation, Temecula, CA, http://www.emdmillipore.com), skeletal muscle-α (1:100; mouse monoclonal antibody; Thermo Fisher Scientific Life Sciences). Secondary antibodies were mouse anti-rabbit-λG- fluorescein isothiocyanate (1:100; Sigma-Aldrich), goat anti-mouse-λG-tetramethylrhodamine (1:100; Sigma-Aldrich), and goat anti-mouse-IgM-Alexa Fluor 594 (1:300; Thermo Fisher Scientific Life Sciences). The slides of noncell-injected hind leg muscle (contralateral hind leg) from the same mouse were used as the negative controls. 4´,6-Diamidino-2-phenylindole (DAPI) was used to stain the nuclei. After washing and mounting, the slides were examined with a fluorescence microscope. RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction

The expression of genes for rat elastin, rat collagen I/III, human elastin and human endogenous retroviruses (ERV-3) were analyzed using real-time quantitative PCR (RT-qPCR). Because ERV-3 is present in the human genome at a single genomic locus but is absent from the rodent genome, it can be used as a marker to qualify human cells in rodent tissue [27]. RNA extraction from the tissue sample was performed with the RNA-STAT-60 reagent (Tel-Test, Inc., Friendswood, TX, http://www.tel-test.com). The cDNA was generated from total RNA, as described previously [28]. The PCR primers used to amplify rat elastin, human elastin, and ERV-3 are listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous reference. RT-qPCR was performed using the Mx3005P Multiplex Quantitative PCR System with MxPro QPCR software (Stratagene, La Jolla, CA, http://www.genomics.agilent.com). Brilliant SYBR Green QPCR Master Mix (Stratagene) was used to perform PCR. RT-qPCR was performed, as described previously [24]. The amplifications were performed after a 10-minute hot start at 95°C in a three-step protocol with 30 seconds of denaturation (94°C), 1 minute of annealing (55°C), and extension at 72°C for 30 seconds. Forty cycles were performed. All PCR reactions were performed in duplicate. For comparatively quantitative PCR, GAPDH was used as an endogenous reference against which the different template values were normalized. Relative quantification of the targeted gene corrected for the quantity of the normalizer gene (GAPDH) was divided by one normalized control sample value (calibrator sample) to generate the relative quantification to calibrator. For qPCR, standard samples were generated from defined numbers (0.5 × 10³, 2 × 10³, 4 × 10³, 8 × 10³, 10 × 10³) of human bSMCs and used to prepare a standard curve. Each human cell contains one copy of ERV-3 transcript. Hence, the number of copies will be equal to the number of cells. The number of human cells in rodent tissue was calculated using this standard curve. All the RT-qPCR analyses were performed using MxPro QPCR software (Agilent Genomics, Santa Clara, CA, http://www.agilent.com).

Elastin Staining

Rodent tissues were embedded in OCT compound on dry ice and stored at −80°C until cryosectioned. The cryosections were cut at 10 μm and mounted on superfrost slides (Thermo Fisher Scientific Life Sciences). The sections were warmed up for 15 minutes to room temperature before fixing in 4% wt/vol cold paraformaldehyde (Thermo Fisher Scientific Life Sciences) in PBS (pH 7.4) at room temperature for 15 minutes. The sections were washed three times in fresh PBS (5 minutes per wash) to remove OCT and then rinsed in water once. The elastin fibers (black) were stained in Weigert’s Resorcin-Fuchsin solution for 2–4 hours according to the manufacturer’s instruction (Electron Microscope Sciences, Hatfield, PA, http://www.electronmicroscopy-sciences.com).
The excess solution was removed with 95% vol/vol ethanol. The slides were differentiated with 1% vol/vol acid alcohol and then wash in water. Cell nuclei (dark blue) were stained with Weigert’s iron hematoxylin working solution (Poly Scientific R&D Corporation, Bay Shore, NY, http://www.polyrnd.com) for approximately 30 seconds. The slides were washed well in running water and then counterstained with van Gieson’s solution for 3–5 minutes for collagen fibers (red pink). The excess stain was rinsed from the slides with distilled water.

Quantitative Macro-Based Image Analysis to Compare Elastin Content

Quantification of elastin from tissue slides was performed using macro-based image analysis. The macro was trained to recognize the range of color intensities represented by the elastin stain in ×40 magnified images. Bright-field histologic images (×40) were imported into Image-Pro Plus, version 7.0 (Media Cybernetics, Silver Spring, MD, http://www.mediacy.com). The area occupied by purple/black-stained elastic matrix components in the histological tissue sections were analyzed using customized visual basic Image-Pro macros based on segmentation algorithms, developed by Image-IQ, Inc. (Cleveland, Oh, http://www.image-iq.com). The stained tissues were segmented using a customized color palette to identify the range of color hues represented by the stained elastic matrix. The segmentation algorithm was iteratively trained over the multiple images and the parameters optimized. For each tissue section, manual and arbitrary regions of interest (ROIs) were drawn to analyze the matrix-containing regions. This procedure was repeated three times per image, and, although in effect, the same image was repeat analyzed, this procedure incorporated randomization in the selection of ROIs, thereby rendering the data set more representative. This strategy was repeated for six different images taken for each of three replicate tissue sections per animal in each test or control group, leading to a large enough sample size. Images of elastic-stained histological sections were analyzed quantitatively for the amount of elastin. The area for the ROIs in each tissue section was measured and exported to MS Excel (Microsoft, Redmond, WA, http://www.microsoft.com), and a pseudocolored overlay image was saved, showing the outline of the identified tissue regions. Each of the measurements was expressed as a percentage of the total area of the ROI, thereby yielding the percentage content for each ECM component and cells in the ROI and then normalized to similarly acquired data from healthy tissue controls. The relative normalized distribution of elastic matrix was estimated as a function of tissue treatment.

Western Blot Analysis

Protein extraction from rodent tissue was performed, as described previously [29]. The total protein concentrations were determined using the Bradford method (Bio-Rad, Hercules, CA, http://www.bio-rad.com). The samples were not reduced for analysis of collagen proteins and were reduced for analysis of elastin protein with a sodium dodecyl sulfate (SDS) sample buffer containing 5% wt/vol of 2-mercaptoethanol and boiled for 10 minutes. Samples (70 μg per lane) were subjected to 8% polyacrylamide gels (SDS-polyacrylamide gel electrophoresis; Bio-Rad). The gels were blotted onto nitrocellulose membranes (Bio-Rad) in an electrophoretic transfer cell. After blocking with 5% vol/vol nonfat milk, the blots were probed with goat anti-rat α-elastin (1:500; Abcam Inc., Cambridge, MA, http://www.abcam.com) at room temperature for 1 hour or mouse anti-rat collagen III (1:500; Abcam Inc.) at 4°C overnight. After washing three times with phosphate-buffered saline with 0.1% vol/vol Triton (pH 7.4), the membrane was then incubated with horseradish

Table 1. Primer pairs used for real-time qPCR analysis

| Target gene   | Forward primer | Reverse primer              |
|---------------|----------------|------------------------------|
| Rat-elastin   | ATCGTGGCTTAGGAGTCTCAACA | TGGGAACCGACACCAAGGAACTTT    |
| Rat-GAPDH     | GCCAGCTCTGTCCTATAAGACA | TGTTAACCGCGCTCCGATA         |
| Rat-collagen 1| GAAGGCAACAGTCGATTCCACC | GACGTCTTCGCCAAGGCTCC       |
| Hu-collagen 1 | TGCTTATGGCTATAGGAG   | ATCCAAACCAGCAGAAA          |
| Hu-ERV-3      | CAGCCCGGAGCTTGCTGTGA | TGGGAATCTCCCAATACCTGG       |
| Hu-elastin    | GTCCAGGTTGCTCTTCTAGGT | GGGCCCAACTGTTCCTTTG         |
| Hu-GAPDH      | CTCAGGCAACACTTTGTCGAAGCTCA | GGTCTTACTCGTAGGAAGGCCATGG   |

Abbreviations: Hu, human; qPCR, quantitative polymerase chain reaction.

Figure 1. In vivo tracking of transplanted pSMCs with BLI. To assess longitudinal cell survival in vivo, rats were monitored for 5 weeks. (A): Representative images show that the BLI signal persisted in rats treated with Luc-tagged RV-iPSC-pSMCs. (B): The disappearance rate of BLI signal in RV-iPSC-treated rats at different times after cell transplantation. Abbreviations: BLI, bioluminescent imaging; iPSC, induced pluripotent stem cell; Luc, luciferase; pSMCs, smooth muscle progenitor cells; RV, retrovirus vector.
peroxidase (HRP)-conjugated mouse anti-goat IgG (1:50,000; GE Healthcare, Pittsburgh, PA, http://www.gelifesciences.com) and sheep anti-mouse IgG (1:2,000; GE Healthcare) for 1 hour at room temperature. The blots were washed with PBS-T three times, developed by chemiluminescence, and reprobed with rabbit anti-GAPDH polyclonal antibody (1:2,000; Abcam, Inc.) and then a 1:10,000 dilution of donkey anti-rabbit IgG conjugated to HRP (GE Healthcare). The relative densities of the bands were assessed using ImageJ, version 1.48 (NIH, Bethesda, MD, http://www.imagej.nih.gov). The optical density (OD) values of the bands were normalized to the OD values of the GAPDH bands and then graphed.

Statistical Analysis
Statistical analyses were performed using SPSS, version 19 (SPSS Inc., Chicago, IL, http://www.ibm.com). The results are expressed as the mean ± SEM. Kruskal-Wallis one-way analysis of variance on ranks, followed by the Wilcoxon method, was used to compare the variables. Comparisons between two groups were performed using the nonparametric Mann-Whitney U test. A value of p < .05 was considered significant.

RESULTS

In Vivo Identification and BLI of Transplanted pSMCs
After in vivo injection of luciferase-tagged H9-pSMCs and RV-iPSC pSMCs, the BLI showed that the transplanted cells were mainly clustered in the periurethral region. The BLI signal in the H9-pSMC-injected rats had disappeared at day 9 after injection. In the RV-iPSC-pSMC group, two rats continued to exhibit weak BLI signal 35 days after cell transplantation (Fig. 1A, 1B).

In Vivo Integration of Transplanted pSMCs
To investigate long-term in vivo differentiation and integration of pSMCs, we performed immunofluorescence (IF) staining of the rat urethral sphincter and mouse leg muscle to detect human nuclei and human smoothelin (a smooth muscle cell protein). IF staining confirmed the presence of human nuclei-positive/smoothelin-positive cells at the injection site at day 12 after pSMC injection (Fig. 2A, 2B). BLI of SCID mice injected with pSMCs in the hind leg also confirmed that BLI could identify these cell clusters at the injection site. The BLI signal had disappeared at day 5 in one mouse treated with H9-pSMCs but had re-emerged approximately 7 weeks after cell injection (Fig. 3A). Furthermore, IF staining showed integration of human nuclei-positive/smoothelin-positive cells in the skeletal muscle of mouse hind leg at day 66 after H9-pSMC injection (Fig. 3B).

The expression of ERV-3 was examined by RT-qPCR in 60 RNU rats and 10 SCID mice to quantify the in vivo survival of hPSC-derived pSMCs. Gene expression of human ERV-3 was extremely low or undetectable in the urethras of the pSMC-treated rats 5 weeks after injection (Fig. 2C) and in the hind leg muscles of
pSMC-treated mice 3 months after transplantation. Given that cell survival was documented by the BLI signal and IF tissue staining, the qPCR data suggest that the surviving population of transplanted pSMCs might be less than the detection range of this assay.

Effect of Human pSMCs on the Muscle Layers of the Urethral Sphincter

IF was performed on the rat urethral sphincter tissue for smoothelin and skeletal α-smooth muscle actin expression to determine whether the injected human pSMCs induced a myogenic response. Rats in the sham-saline group had disjointed and thinner smooth and striated urethral muscle layers compared with the control group. The urethral sphincters of the H9-pSMC-, RV-iPSC-pSMC-, and Epi-iPSC-pSMC-treated group showed smooth muscle regeneration with a thicker layer of smooth and striated muscle and intense smoothelin expression, similar to that of the intact control rats. In contrast, the SUI rats transplanted with human terminally differentiated bSMCs showed sphincter atrophy with a thin smooth muscle layer and weak smoothelin expression, similar to the SUI rats in the sham-saline group (Fig. 4).

Effects of hPSC-pSMCs on Urethral Elastic Fibers

To evaluate the effect of hPSC-derived pSMCs on elastogenesis in the rat urethra, urethral tissues were stained and analyzed using a quantitative macro-based image system. In the sham-saline group, the elastic fibers were significantly shorter and disorganized compared with those of the control rats. Treatment with H9-pSMCs and iPSC-pSMCs appeared to have restored elastin organization and content. The elastin fibers in these groups were much longer and thicker than those in the sham-saline group. The elastic fibers in the SUI rats transplanted with bSMCs were fragmented and not as well organized as those in the intact controls or the pSMC-treated group (Fig. 5A).

The elastic fiber density in the urethra of the SUI rats was significantly lower than that in the pure control rats \((p < .001)\). In contrast, the elastic fiber density in the SUI rats transplanted with H9-pSMCs was significantly increased \((p = .0022)\) compared with the sham-saline group (Fig. 5B).

We performed qPCR analysis and immunohistochemistry for gene expression of human elastin in the rat urethra to determine whether transplanted cells were the source of the increased elastin fibers. Expression of the human elastin gene was undetectable in the hPSC-pSMC-treated rats, suggesting that the increases in elastic fibers are not attributable to the transplanted hPSC-derived pSMCs.

hPSC-pSMCs Induced ECM Remodeling in the Lower Urinary Tract of SUI Rats

To further confirm and quantify the effects of hPSC-derived pSMCs on ECM remodeling, we examined the protein level of elastin in the distal rat urethra by Western blot. Because the distal section of the rat urethra was too small to extract enough protein...
for Western blot and the lower portion of the rat bladder was injured and ischemic during transabdominal urethrolysis, we also included the bladder in the analysis. We examined the protein levels of elastin and collagen III in rat bladders with or without pSMC transplantation. As shown in Figure 6, the protein level of collagen III in the sham-saline group was significantly lower than that in the pure control group (p = .02). SUI rats in sham-saline group also showed a decreasing trend in elastin protein compared with the pure control group. The SUI rats treated with H9-pSMCs had significantly higher levels of elastin and collagen III proteins in the rat bladder compared with the sham-saline group (p, .05 for both). RV-iPSC-pSMC-treated and Epi-pSMC-treated rats also displayed increasing trends in elastin and collagen III content in the rat urethra and bladder compared with the sham-saline group, although the difference was not significant.

**DISCUSSION**

The present study provides evidence that periurethral injection of a purified population of human ESC/iPSC-derived pSMCs can facilitate the restoration of sphincter structure in a SUI rat model with chronic urinary sphincter injury. The present results also suggest a novel and promising type of hPSC-derived pSMCs for the regeneration of damaged smooth muscles for clinical applications.

To date, several SCs or progenitor cells have been tested for SUI treatment. Most have been harvested from adult patients and further expanded. The resulting cell population for transplantation has been heterogeneous, making it difficult to understand their mechanisms of action and to optimize efficacy. Hence, no consensus on the most clinically applicable SC type has emerged. Although the quest for the “ideal” cell in SUI treatment is still ongoing, comparison data have suggested that the best therapeutic outcomes are achieved by cells that are phenotypically as close as possible to those in the targeted organs [30]. An example is muscle progenitor cells that show therapeutic potential in SUI associated with urethral sphincter deficiency [31, 32]. Human ESCs and iPSCs have been successfully differentiated into vascular muscle progenitor cells in vitro using chemically defined media in various laboratories [21]. These progenitor cells have been tested in human diseases and have shown promising results [33]. The advantages of ESC/iPSC-derived muscle progenitor cell therapy include unlimited cell source, uniform cell population, in vitro myogenic commitment, off-the-shelf immediate availability, and potentially improved therapeutic efficacy. We tested a pure population of smooth muscle progenitor cells derived from human PSCs (H9-ESCs and iPSCs) in a urethral injury animal model and confirmed restoration of muscle function [18]. In the present study, we investigated the mechanism of action of the human pSMCs on the injured urethra.
The molecular etiology of SUI is complex and incompletely defined. Possible etiologies include abnormal ECM metabolism modulated by genetic predisposition, trauma, mechanical stress load, and aging [34, 35]. The female urethra, composed of smooth and striated muscle cells, is surrounded by a fibrous-muscular system consisting of elastic fibers, collagen fibers, and SMCs. In addition to the cellular components of the sphincter, these fibers maintain a delicate balance between elastic extensibility and mechanical resistance to achieve continence; thus, changes to any component can break this balance, leading to the occurrence of SUI. Previous studies have observed fragmented and disorganized elastic fibers and significant changes in collagen content in the periurethral tissues of women with SUI [36]. We, and other investigators, have also shown that the genes of proteins involved in elastin and collagen metabolism are differentially expressed in the vaginal tissues of SUI women and continent women [37–39]. These findings suggest an essential role of abnormal ECM homeostasis in the pathogenesis of SUI. Consistent with these published data on human tissues, in the present study, we observed short and disorganized elastic fibers and a thin muscle layer in the urethral sphincter of SUI rats 8 weeks after urethrolysis. Therefore, the SUI rats simulate the structural and functional alterations in SUI patients associated with urethral intrinsic sphincter deficiency and aged urethral muscle [40].

The present study also demonstrated that human pSMC-treated SUI rat tissues recovered markedly from surgical injury compared with the control rats. The elastic fibers were reoriented in the proximal urethra, and the tissue content of rat elastin and collagen III increased in the urethra and bladder of the pSMC-treated rats. Such recovery did not occur in the saline-treated or terminally differentiated bSMC-treated rats, suggesting that the hPSC-derived pSMCs can promote ECM remodeling in the lower urinary tract. These findings correlate with our functional studies using urethral pressure measurements, such as the leak point pressure (LPP), to evaluate the effect of pSMCs on the SUI rat model [18]. The animal studies demonstrated that SUI rats treated with pSMCs (derived from both iPSCs and ESCs) improved LPP values compared with the rats in the sham-saline group. Therefore, remodeling of the ECM proteins might contribute to recovery of urethral sphincter function in hPSC-pSMC-treated rats. Smooth muscle progenitor cells have been documented to secrete a number of autocrine/paracrine factors that could have therapeutic effects, including pro-proliferative,

**Figure 5.** Assessment of elastin fibers in the proximal urethra of the rat. (A): Representative images of cross-section of proximal urethra with Weigert’s Resorcin–Fuchsin’s elastin and van Gieson’s collagen staining. Elastic fiber shown as dark blue, collagen as red pink, and other tissue elements as yellow. Scale bars = 100 μm. (B): Quantification of elastin fibers was assessed using Image-Pro Plus software and expressed as a percentage of the arbitrary ROIs. Each bar represents the mean value ± SEM. Abbreviations: bSMC, human bladder smooth muscle cells; H9-pSMC, surgery plus H9-pSMC injection; Hu-bSMC, surgery plus injection of human bladder smooth muscle cells; pSMCs, smooth muscle progenitor cells; Pure control, no surgery and no treatment; ROIs, regions of interest; Sham saline, surgery plus saline injection.
angiogenic, and cytoprotective factors, such as vascular endothelial growth factor, hepatocyte growth factor, and neuregulin 1 [41–43]. Future studies will investigate which cytokines secreted from pSMCs induce tissue recovery in SUI.

Animal studies using animal MSCs support the hypothesis that MSCs exert their effect of restoring urethral sphincter function mainly through these paracrine mechanisms, rather than via cell regeneration or terminal differentiation of transplanted cells [15, 44, 45]. However, recent studies using human stem/progenitor cells derived from human adult tissues have shown that the transplanted human cells can survive in the host environment [19, 20], suggesting that cell regeneration might also be a mechanism of action. However, the intervals between cell transplantation and tissue collection in these studies were too short (<2 weeks); thus, it is uncertain how long these human stem/progenitor cells can survive and proliferate in vivo. In the present study, we confirmed that transplanted hPSC-pSMCs can be detected by IF staining up to at least 10 weeks in the cell transplantation region of the SCID mice and that the pSMCs were integrated into the surgically injured urethra in RNU rats. Additional evidence of long-term in vivo cell survival is the persistence of the BLI signal in pSMC-treated rats and signal re-emergence in some of the pSMC-treated mice. Furthermore, these pSMCs preserved their SMC phenotype in vivo, confirmed by the presence of smoothelin in the human cells.

ERV-3 is a marker that can be used to qualify human cells in rodent tissues [46]. RT-qPCR assay for the gene expression of ERV-3 to examine the amount of the surviving cells in rat urethra showed that the expression level of ERV-3 was extremely low or undetectable in the urethra of pSMC-treated rats at day 35 after transplantation. The expression levels in tissues were many-fold lower than those in the pure bladder SMCs we used to develop the calibration curve (Fig. 2). We believe this is because the number of integrated cells was small, which increased the error introduced by RNA extraction and PCR. Therefore, the ERV-3 assay is not reliable when the cell numbers in tissue samples are low. Given that a large number of rodent muscle cells died during urethrolysis, the recovery of urethral sphincter function cannot be solely attributed to the small number of engrafted cells. Consequently, the regenerative effects of hPSC-derived pSMCs likely result from a combination of cell integration and paracrine effect on native tissue ECM remodeling.

The present study has shown that transplantation of human ESC-derived and iPSC-derived pSMCs facilitate the restoration of elastin fiber and damaged urethral sphincter in RNU rats. Because of evolving advances in genetic reprogramming of adult somatic cells into cells with pluripotent stem cell properties, we examined different human PSC lines to determine whether the in vivo effect of human pSMCs is similar, regardless of the origin of the PSC line. The RV-iPSCs tested in the present study were genetically reprogrammed with the use of a retrovirus. With this method, viral particles can integrate into the host genome and cause mutations in the genome. Therefore, these cells are unlikely to be used in clinical treatment. However, the Epi-iPSCs might be a promising source of cells for SUI treatment, because they are reprogrammed using a nonintegrative method. In addition, this autologous cell strategy could reduce the host immune responses [47] and avoid the ethical issues associated with hESCs. Our data suggest that the in vivo effects of smooth muscle progenitor cells are likely to be similar regardless of the source of the hPSCs.

Study Limitations
One limitation of the present study was the use of rodents to test the in vivo effect of human cells, because rodent regenerative properties differ from those of humans. Future clinical trials might
provide additional human data. Another possible source of error was inclusion of the rat bladder in the Western blot assay for elastin and collagen III. We did so because we did not have sufficient RNU rat urethra for the assay. It has been reported that stem/progenitor cells can migrate to the injury site following gradients of chemokines [48]. Given that the innervation and blood supply of the rat bladder were likely damaged during urethroplasty and that BLI demonstrated that the transplanted cells were localized around the lower urinary tract, the transplanted cells were likely to have exerted the same effect on the rat bladder and the urethra.

**Conclusion**

Our findings indicate that periurethral transplantation of hPSC-derived pSMCs can promote the restoration of injured rat urethra. The mechanism of action of these cells involves modulation of ECM metabolism and pSMC proliferation and integration to regenerate the muscle layer in the lower tract. Further investigations are needed to identify the detailed paracrine pathways by which hPSC-derived pSMCs modulate ECM metabolism and to determine whether these findings apply to human tissues.

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**Author Contributions**

Y.L. and Y. Wen: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; Z.W. and Y. Wei: collection and/or assembly of data, data analysis and interpretation; P.W., M.G., and G.S.: collection and/or assembly of data; A.R.: conception and design; R.R.P.: conception and design, provision of study material; B.C.: conception and design, data analysis and interpretation, manuscript writing, final approval of the manuscript.

**Disclosure of Potential Conflicts of Interest**

The authors indicated no potential conflicts of interest.

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