Evaluation of Epithelial Chimerism After Bone Marrow Mesenchymal Stromal Cell Infusion in Intestinal Transplant Patients

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ABSTRACT

Intestinal transplantation is the most effective treatment for patients with short bowel syndrome and small bowel insufficiencies. We evaluated epithelial chimerism after infusion of autologous bone marrow mesenchymal stromal cells (BMSCs) in patients undergoing cadaveric donor isolated intestinal transplantation (I-ITx). BMSCs were isolated from patients' bone marrow via iliac puncture and expanded in vitro prior to infusion. Two out of the 3 patients were infused with autologous BMSCs, and small intestine tissue biopsies collected post-operatively were analyzed for epithelial chimerism using XY fluorescent in situ hybridization and short tandem repeat polymerase chain reaction. We observed epithelial chimeric effect in conditions both with and without BMSC infusion. Although our results suggest a higher epithelial chimerism effect with autologous BMSC infusion in I-ITx, the measurements in multiple biopsies at different time points that demonstrate the reproducibility of this finding and its stability or changes in the level over time would be beneficial. BMSC infusion may have potential implications for improved graft survival, lower immunosuppressant doses, superior engraftment of the transplanted tissue, and higher success rates in I-ITx.

ISOLATED INTESTINAL TRANSPLANTATION (I-ITx) is the most effective treatment for patients with short bowel syndrome and small bowel insufficiencies that require total parenteral nutrition [1–3]. To increase I-ITx graft survival, several approaches have been evaluated including the use of various combinations and dosages of immunosuppressive agents [4]. Recently, a new generation of immunosuppressive agents coupled with accumulating clinical experience improved patient survival rates for I-ITx [3,5]. Even though these advances have helped I-ITx graft survival, success rates lag behind solid organ transplants (e.g., liver, kidney) [2,6,7]. Rejection and sepsis still remain the common causes of graft loss and patient death in I-ITx. In fact, acute cellular rejection is observed more frequently and more severely in I-ITx grafts compared to other organ or tissue transplants [2]. Another limiting factor of this therapeutic surgical modality is the engraftment of the transplanted tissue. Therefore, there is a need for developing new strategies in I-ITx.

A unique aspect of bone marrow mesenchymal stromal cells (BMSCs, also referred to as mesenchymal stem cells [8]) is their ability to modulate immune responses both in vivo [9] and in vitro [10]. BMSCs have the capacity to alter immune responses via direct and indirect interactions with a range of target cell types [11]. BMSCs have been shown to display

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immunosuppressive properties through inhibition of T-cell proliferation, as well as exerting inhibitory effects on B cells, dendritic cells, and natural killer cells [10]. Thus, BMSCs have emerged as a promising new therapeutic approach for managing autoimmune disease [2], improving solid organ transplantation (e.g., kidney transplants) [11–14], and treating graft-versus-host (GVH) disease [15]. A growing body of evidence suggests that multilineage BMSCs enable alternative immunosuppressive and regenerative therapies [1,11,16].

A mechanism that promotes graft tolerance is chimerism, for instance, coexistence of donor and host cells in a tissue. Chimerism has been observed in the bone marrow of transplantation patients following allogeneic hematopoietic cell infusion [17–20]. Transplantation tolerance through donor bone marrow infusion has been shown in primate studies and in pilot clinical trials [20]. Furthermore, donor bone marrow infusion has been performed in murine and porcine models to achieve peripheral chimerism in intestinal transplantation [18,21–23]. In addition, transplantation tolerance through donor BMSC infusion has been successfully shown in primate models and in clinical studies [20]. However, the role of autologous BMSC infusion in epithelial chimerism and methods to study it have not been reported in I-ITx patients. In this study, we evaluated epithelial chimerism in both sex-matched and nonmatched I-ITx patients with and without direct autologous BMSCs. In this study, we treated 2 out of 3 patients with autologous BMSCs and analyzed intestine tissue biopsies collected postoperatively for epithelial chimerism using XY fluorescent in situ hybridization (XY-FISH), and short tandem repeat polymerase chain reaction (STR-PCR).

MATERIALS AND METHODS

Study Population

This case study included 3 patients with short bowel syndrome who have undergone I-ITx and standard immunosuppressive treatment with ($n = 2$) or without ($n = 1$) concomitant administration of BMSC infusion (10^6 BMSCs/kg) at Tepecik Training and Research Hospital (Izmir, Turkey). Pretransplant diagnoses for patients were: (1) superior mesenteric artery (SMA) emboli for patient 1 (no BMSC infusion); (2) SMA trauma and total small bowel resection for patient 2 (BMSC infusion); and (3) Crohn's disease, small bowel fistulas, total small bowel resection, and total colectomy for patient 3 (BMSC infusion). The postoperative periods at the time of biopsy were: (1) 69 months for patient 1; (2) 22 months for patient 2; and (3) 8 months for patient 3 (Table 1). Two of 3 patients (patients 1 and 3) had a sex mismatch with the donor (Table 1). Patients' post-transplant graft biopsies were analyzed using immunohistopathologic methods, STR-PCR for all patients and XY-FISH for patients 1 and 3 who had sex mismatch with the donor. Written informed consents were obtained from each patient for all procedures following a detailed explanation of the objectives and protocols of the study. The study was approved by the institutional review board. Autologous BMSC infusions were performed within the context of therapeutic intervention with permission from the Republic of Turkey, Ministry of Health, Scientific Advisory Committee (Document #19.04.2011-B.10.0.THG.0.14.00.05/18528/48852). Detailed methods on BMSC isolation, culture, and characterization; small bowel transplantation; BMSC infusion; immunohistopathologic analyses; epithelial chimerism analysis with XY FISH and STR-PCR can be found in Materials and Methods. Identification of the SMA and

Table 1. Patient Characteristics, Treatment Details, and Summary of Chimerism Analysis Results

Patient No.	Age/Gender (Weight, kg)	Diagnosis	HLA Mismatch	Donor (Age/Gender)	BMSC Infusion (10^6 BMSCs/kg)	IS	AR Before Discharge	Duration Postsurgery (mo)	CMV Infection	STR-PCR RFU Chimerism Rate (%)	XY-FISH Epithelial Chimerism Rate (%)
1	48/F (54)	SMA emboli	3	Cadaver (25/M)	–	ATG; steroid; FK506; mTOR-inh	None	69	None	34.3	1.7
2	12/F (36)	SMA trauma; total SB resection	2	Cadaver (27/F)	2 doses: d 0; d 15	ATG; steroid; FK506; mTOR-inh	None	22	None	38.9	Not applicable
3	31/F (42)	Crohn's; SB fistulas Total SBR; total colectomy	5	Cadaver (25/M)	2 doses: d 0, d 15	ATG; steroid; FK506; mTOR-inh	None	8	None	7.4	17.5

Abbreviations: ATG, antithymocyte globulin; AR, acute rejection; FISH, fluorescence in situ hybridization; FK506, tacrolimus; HLA, human leukocyte antigen; IS, immunosuppression; BMSC, bone marrow stromal cell; mTOR-inh, mammalian target of rapamycin inhibitor; PCR, polymerase chain reaction; RFU, relative fluorescence unit; SB, small bowel; SBR, small bowel resection; SMA, superior mesenteric artery; STR, short tandem repeat; CMV, cytomegalovirus.

visualization of BMSC infusion is shown in video (http://canarycenter.stanford.edu/bamm-lab/videos/).

BMSC Isolation and Culture

Bone marrow (BM) aspiration was performed at least 4 weeks prior to transplantation to allow sufficient time for expanding BMSCs in vitro. BM (20 mL) was aspirated from the recipients' left and right spina iliaca posterior superior iliac crest under local anesthesia. BM aspirations were sufficient for 2 infusions after in vitro expansion of BMSCs. Isolated BM tissue was transferred at 4°C in an acid-citrate-dextrose containing infusion bag to the Aticell laboratory (Trabzon, Turkey) of the state approved Atigen-Cell Inc (www.atigencell.com, Ankara, Turkey). The clinical-grade autologous BMSCs were cultured, expanded, and biopreserved under Good Manufacturing Practice (GMP) conditions. Briefly, mononuclear cells were isolated from BM using Ficoll density gradient (PAA Laboratories GmbH, Pasching, Austria). Retrieved cells were cultured using GMP-qualified (Advanced Therapy Medicinal Product [ATMP-Ready]) (PAA Laboratories GmbH, Austria), low-glucose Dulbecco's modified Eagle's medium (DMEM; PAA Laboratories GmbH) containing 10% (v/v) autologous human serum (AHS). Monolayer expanded cells were passaged at 70% to 80% confluency using ATMP-Ready trypsin/EDTA solution (PAA Laboratories GmbH) and replated for further expansion. BMSCs used in infusion were serially subcultured up to passage 3 or 4 and cryopreserved in DMEM supplemented with 10% (v/v) dimethyl sulfoxide and AHS. On the day of infusion, BMSCs were thawed, washed 3 times with phosphate-buffered saline (PBS; PAA Laboratories GmbH), and resuspended in PBS containing hydroxyethyl starch (PAA Laboratories GmbH) and AHS at a final concentration of $\sim 2 \times 10^6$ cells/mL.

BMSC Characterization

The phenotypic characterization of the transplanted BMSCs was performed after in vitro monolayer expansion at the GMP facility before infusion. Cells were analyzed for the following mesenchymal stromal cell surface markers: CD73 (BD Pharmingen, San Diego, Calif, United States), CD90 (Beckman Coulter, Miami, FL), CD105 (Beckman Coulter); hematopoietic cell surface markers: CD34 (BD Pharmingen), CD45 (BD Pharmingen); and, human leukocyte antigen DR (HLA-DR, BD Pharmingen) using flow cytometry (Beckman Coulter system equipped with Epics XL-MCL software, USA). Cell viability was determined using trypan blue staining and hemocytometer counting before infusion. Absence of microbial contamination (bacteria, fungus, or mycoplasma) was verified before infusion, using United States Pharmacopoeia XXIV (Chapter 71) sterility test procedure and Hoechst 33258 staining. Endotoxin testing of culture supernatant was performed using a Limulus Amoebocyte Lysate (LAL)-test [24].

Small Bowel Transplantation

For all patients, small bowel grafts were obtained from donor cadavers who met the inclusion criteria for tissue harvesting, such as functional and anatomical sufficiency, absence of systemic infections, and absence of incurable malignancy. On average, the cold ischemia time was 7 hours. The harvested grafts were kept in Viaspan (University of Wisconsin solution, Fresenius Kabi AG, Bad Homburg, Germany) and perfused through the mesenteric artery in the operation theater before transplantation. Next, the abdominal aorta and the vena cava inferior were prepared and anastomosed. Then, the proximal duodenum was anastomosed to the recipient's duodenum and the end of the ileum was ostomized to the abdominal wall. Standard immunosuppressive treatment was employed using antithymocyte globulin (ATG; Fresenius Kabi, Bad Homburg, Germany), steroid (methylprednisolone, Mustafa Nevzat Drug Company Istanbul, Turkey), tacrolimus (FK506, Astellas, Dublin, Ireland), mammalian target of rapamycin (mTOR) inhibitor (Wyeth Laboratories, England; Table 1). The dose of ATG was 4 mg per kg body weight at the preoperative induction, and 3 mg per kg body weight postoperatively with 3-day intervals for a total of 3 doses. Tacrolimus target levels were 15 to 20 mg/dL for the first 4 weeks and 7 to 10 mg/dL after 4 weeks. Oral administration of mTOR inhibitor was started 4 weeks post-transplantation (target blood level was 5 to 8 mg/dL) with tacrolimus. During the induction period, steroid dose was 10 mg per kg body weight with a reduction to half 1 day postoperation, followed by a 10-mg reduction of the total dose per day with a final maintenance dose of 0.3 mg per kg body weight per day. Maintenance immunosuppression doses were kept the same for long-term follow-up.

BMSC Infusion

Two patients (patient 2 and 3) received 2 doses of autologous BMSC infusion at day 0 and day 15 (postsurgery). Based on the patient's body weight, infusion was performed to achieve 1×10^6 cells per kilogram of body weight, which amounted to ~20 mL of cell suspension. The first dose of BMSCs was administered during transplantation surgery (day 0) through the SMA of the graft after releasing the clamps and restoring blood circulation of the transplanted graft. The second dose of BMSCs was administered postoperatively (day 15) to the SMA of the graft via catheterization of the femoral artery under local anesthesia at the coronary angiography unit. To identify the SMA and visualize infusion, we injected nonionic, iodinated, low osmolar radiologic contrast agent, Ultravist 370 mg/mL (Bayer Healthcare, Leverkusen, Germany) and recorded the procedure on video.

Small Bowel Biopsies and Immunohistopathologic Analyses

Tissue biopsies were collected postoperatively (69 months for patient 1, 22 months for patient 2, and 8 months for patient 3) for immunohistopathologic assessment and chimerism analysis. Biopsies were harvested under sedation from the recipient duodenal section (2 biopsies) and transplanted donor intestinal tissue (5 biopsies) starting from distal to proximal sections. The biopsy collection procedure minimized mixing of recipient blood with the biopsy samples due to bleeding. Histologic examination on biopsied tissue was performed using hematoxylin-eosin staining for morphologic assessment and $CD3^+$ T-lymphocyte immunostaining for inflammation response assessment. Three consecutive 5-μm-thick sections were obtained from formalin fixed paraffin embedded blocks for hematoxylin-eosin staining, anti-CD3 (clone UCHT1, Dako Denmark A/S, Glostrup, Denmark) and cytomegalovirus (CMV, clone DDG9+CCH2, Dako Denmark A/S) immunohistochemical stains on Autostainer link 48 (Dako Denmark A/S). Serologic CMV analysis was performed preoperatively for the cadaveric donor as well as pre- and postoperatively for the recipient to verify the absence of active infection.

Epithelial Chimerism Analysis With XY-FISH

Comparative chimerism analysis of donor and recipient biopsies after BMSC infusion was performed via XY-FISH analysis of sex chromosomes after transplantation from a sex-mismatched donor in patients 1 and 3. X and Y chromosomes of cells in tissue biopsies were evaluated using chromosome enumeration probe (CEP) X spectrum Green (locus Xp11.1-q11.1) and Y spectrum Orange (locus Yq12) direct label fluorescent DNA probes (Abbott Molecular, USA) according to manufacturer's instructions. Hybridization of the CEP X/Y DNA probe in the XY-FISH stained tissue samples were imaged using a fluorescence microscope (Olympus BX51, Japan) with a 100× oil immersion objective at the Department of Medical Biology and

Genetics in School of Medicine at Dokuz Eylul University (Izmir, Turkey). Villus epithelial cell nuclei with X or Y sex chromosome signal were included in this analysis. 4′,6-diamidino-2-phenylindole (DAPI) staining was used to identify cell nuclei and to distinguish individual chromosome pairs for each cell in 2 tissue sections per biopsy sample. XY-FISH analysis was performed based on inclusion of epithelial cell nuclei with exclusion of potential cell overlay and single villus epithelial cells. To quantify epithelial chimerism, the number of cells with XX chromosomes (ie, recipient's cells) and cells with XY chromosomes (ie, donor cells) were determined and the fraction of the recipient's cells was calculated (ie, the number of XX cells divided by the total number of counted cells).

Chimerism Analysis With STR-PCR

Chimerism analysis on tissue biopsies was performed using STR-PCR (AmpFlSTR Identifiler PCR Amplification Kit and Applied Biosystems Gene Mapper ID v3.2 software, Applied Biosystems, Carlsbad, Calif, United States) at the Institute of Forensic Medicine (Izmir, Turkey). PCR relative fluorescence unit (RFU) ratio comparison between recipient and donor biopsies was performed by evaluating 16 standard STR loci (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, FGA, and Amelogenin) in tissue biopsies (3 biopsies for patients 1 and 3, 2 biopsies for patient 2). RFU ratios were determined using 9 informative loci (D21S11, D7S820, CSF1PO, D13S317, D16S539, D2S1338, D19S433, vWA, and D18S51) for patient 1, 5 informative regions (D8S1179, THO1, D16S539, D2S1338, and 18S51) for patient 2, and 7 informative regions (D8S1179, D21S11, D13S317, D16S539, D2S1338, vWA, and D5S818) for patient 3. The locus that has 3 or 4 alleles were plotted to show the genes coming from both the donor and the recipient separately at the transplant. From the transplanted graft biopsies (3 biopsies per patient), RFU rates of patient specific peaks and donor specific peaks were used as a measure of chimerism as described earlier [25,26]. Oral mucosal swaps and peripheral blood samples were also collected from patients at the time of biopsy to be included in STR-PCR analysis for the confirmation of the absence of microchimerism. RFU ratios were statistically compared using one way analysis of variance with Tukey post hoc test for multiple comparisons with statistical significance threshold set at $P < .05$.

RESULTS

Characterization of Infused BMSCs

The phenotypic characterization of the BMSCs showed greater than 90% positivity for mesenchymal stromal cell surface markers before infusion: $CD73^+$: 95.8% (±2.3%), $CD90^+$: 95.7% (±4.8%), $CD105^+$: 90.7% (±5.2%). Hematopoietic cell surface markers were low and detected to be $CD34^+$: 1.9% (±1.2%), $CD45^+$: 2.2% (±2.2%), and human leukocyte antigen (HLA) DR was 0.8% (±1.0%). The cell viability of the BMSCs was 94.0% (±1.4%) before infusion.

Histologic, Immunohistochemical, and Pathologic Evaluation of Tissue Biopsies

Evaluation of biopsy samples taken concomitantly from recipient's duodenum and jejunum of the transplanted graft revealed normal tissue organization and cellular morphology (Fig 1A–C). All patients were negative for CMV as determined by serologically and immunohistochemically (data not shown). $CD3^+$ T lymphocyte immunostaining of tissue biopsies showed normal levels of lymphocyte infiltration in the epithelium and superficial lamina propria in all 3 patients (Fig 1D–F). Histologic evaluation on biopsy sections did not show immune rejection signs, such as crypt destruction and inflammatory response [7]. Furthermore, there was no sign of rejection in routine clinical assessments in any of the patients throughout the 6-month follow-up period after the tissue biopsies were collected.

Chimerism Analysis

In XY-FISH analysis, we focused on epithelial chimerism by only including the epithelial cells in quantification that can be accurately identified morphologically and by molecular DNA probing using fluorescent microscopy. We analyzed epithelial chimerism in patients 1 and 3, who had cadaveric donor sex-mismatch (Table 1). XY-FISH analysis on patient 1 tissue biopsies displayed a total of 232 countable villus epithelial cells in 2 histologic sections per biopsy, 4 of which (1.7%) were identified to have an XX chromosome-positive signal. On the other hand, XY-FISH analysis on patient 3 tissue biopsies displayed a total of 349 countable villus epithelial cells in 2 histologic sections per biopsy, 61 of which (17.5%) displayed an XX chromosome-positive signal. Representative XY-FISH images taken at a single focal plane displayed XX-positive host epithelial cells among XY-positive donor epithelial cells in intestinal tissue biopsies of patient 1 (Fig 2A–C) and patient 3 (Fig 2D–F). However, this method is not suitable to evaluate sex-matched patients, where we utilized STR-PCR.

Representative STR-PCR results for TH01 informative region for patient 2 were presented for recipient's blood sample, recipient's small bowel biopsy, recipient's oral swab sample, donor small bowel biopsy, and transplanted small bowel biopsy (Fig 3A–E). STR-PCR analysis on peripheral blood samples, small bowel biopsies, and oral mucosal swabs confirmed absence of microchimerism, where patient specific peaks (e.g., 6 and 8) appeared in the same informative regions of the spectrum (Fig 3A–C). On the other hand, biopsy samples from transplanted tissues revealed chimeric effect associated with cellular integration of donor (Fig 3D) and recipient tissues (Fig 3E), where patient specific peaks (e.g., 6 and 8) and donor specific peaks (e.g., 7 and 9.3) appeared in the same spectrum. Mean STR-PCR RFU rates indicative of chimerism were 34.3%, 38.9% and 7.4% in patients 1, 2 and 3, respectively (Table 1 and Fig 3F). Even though patient 2 (with BMSC infusion) had a shorter postoperative period (22 months) compared to patient 1 (without BMSC infusion, 69 months), patient 2 RFU rate was significantly greater than patient 1 ($P < .05$, Fig 3F). Patient 3 (postoperative period of 8 months) displayed lower RFU rate compared to patients 1 and 2 ($P < .05$, Fig 3F).

DISCUSSION

Epithelial chimerism was previously shown to be 0.18% to 0.26% in graft biopsies collected up to 770 days postsurgery in intestinal transplant patients without any BMSC infusion

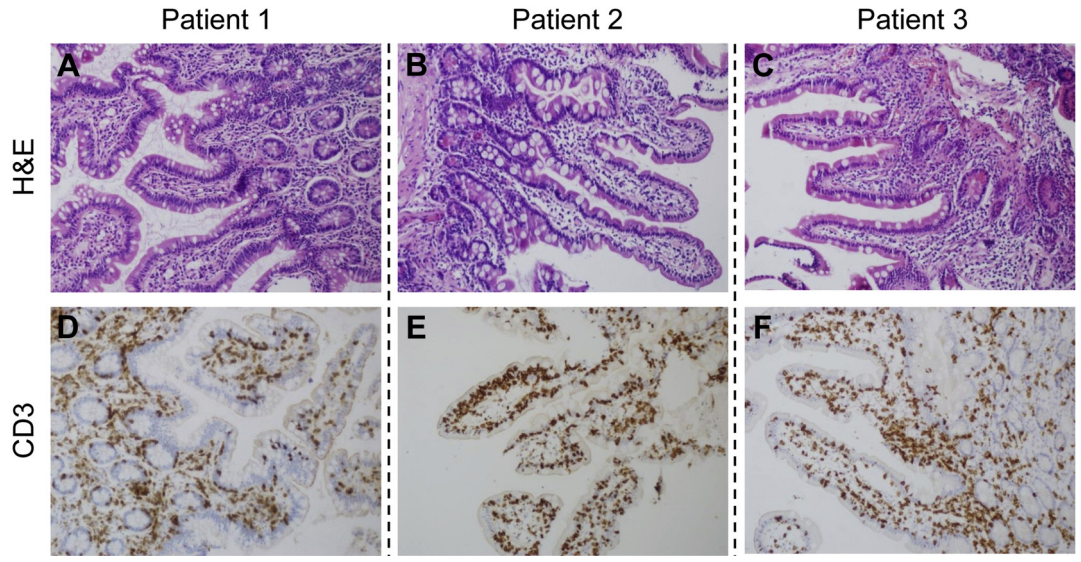

Fig 1. Histologic and immunohistologic analysis results on tissue biopsies obtained from 3 patients postsurgery (69 months for patient 1, 22 months for patient 2, and 8 months for patient 3). **(A–C)** Hematoxylin and eosin (H&E) staining of small bowel graft biopsies (patients 1–3) showed presence and normal morphology of crypts, regular villus morphology, and no sign of inflammation in all patients. **(D–F)** CD3 T-lymphocyte immunohistologic staining of tissue biopsies (patients 1–3) indicated normal lymphocyte presence, especially in the epithelium and superficial lamina propria in all patients. Images were taken using a 20× objective.

[27]. Likewise, in this study, we show epithelial chimerism of 1.7% in graft biopsies collected 69 months (2070 days) postsurgery in patient 1 without BMSC infusion (Fig 2). In the case of autologous BMSC infusion, epithelial chimerism level was observed to be as high as 17% in graft biopsies collected 8 months (240 days) postsurgery from patient 3 (Fig 2). Intestinal mucosal crypts have stem cells, which allow regeneration, repair and repopulation of the small intestinal mucosa [28]. A putative regenerative role of infused autologous BMSCs is to mediate immune tolerance by a chimeric effect and enhance engraftment in transplantation [9]. We observed 10-fold higher number of XX type epithelial cells (Fig 2) in patient 3 with BMSC therapy 8 months after surgery compared to patient 1 without BMSC therapy 69 months after surgery. Enhanced epithelial chimeric effect in the transplanted tissue may have a critical role in long-term graft survival in I-ITx. However, these studies need to be compounded with repeat confirmatory data in multiple biopsies at different time points to demonstrate the reproducibility of enhanced chimeric effect and its stability or changes in level over time. Furthermore, it was also reported in the literature that even after considerable mucosal regeneration after acute graft rejection and in some cases up to 8 years of survival, there was no crypt replacement by recipient cells [29].

Lymphoid chimerism has been demonstrated earlier with no infusion of recipient BMSCs in small bowel transplants [30]. Even though the replacement of infiltrating lymphocytes of the graft with recipient cells is well characterized [30,31], there is a scarcity of studies in the literature investigating the replacement of donor epithelial cells with recipient cells upon BMSC infusion. It is known that donor lymphocyte-derived transient chimerism may trigger acute immune rejection as reported earlier in the peripheral circulation of the recipient [30]. This chimeric effect was not observed in the peripheral circulation of the BMSC-infused patients, whose blood, small bowel biopsy, and oral swab samples were analyzed postoperatively at reported biopsy times using STR-PCR (Fig 3A–C). On the other hand, post-transplant chimeric effects observed in STR-PCR analysis in graft biopsies may be attributed to the presence of recipient's cells in the lamina propria (e.g., T lymphocytes, dendritic cells, and monocyte-derived macrophages) [32]. Indeed, it is possible to anticipate greater numbers of monocyte derived macrophage like cells in the lamina propria proportional to graft survival [32]. Immunohistologic staining for anti-CD3 revealed similar T-lymphocyte presence in graft biopsies in all patients (Fig 1D–F). Histologic and immunohistologic staining of graft biopsies confirmed absence of immune rejection.

Quantification of chimerism has been considered as a method to evaluate engraftment and potential graft rejection following stem cell transplantation [9,25,33]. Several approaches have been used to analyze chimerism, STR-PCR being the most widespread technique [25] with high sensitivity based on the utilized markers [34]. In addition, chimerism in sex-mismatched transplantations have been assessed earlier using XY-FISH with specific probes for the sex chromosomes [35]. This method has been shown to yield high sensitivity in tissue samples [36]. Therefore, to enhance quantification accuracy and reliability, we performed STR-PCR analysis in combination with XY-FISH for sex-mismatched transplant patients.

In this study, based on STR-PCR analysis, chimerism was observed to be significantly greater ($P < .05$) in patient 2 graft biopsy samples compared to patient 1, who did not receive

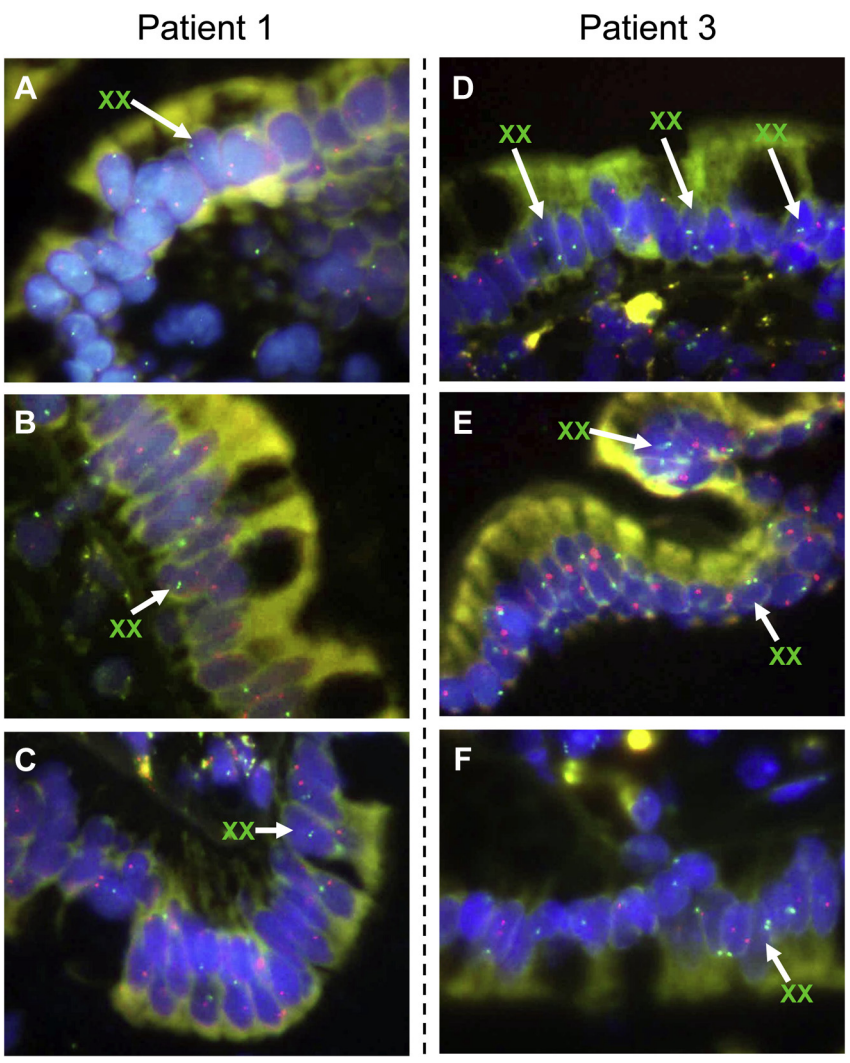

Fig 2. Representative images of XY fluorescence in situ hybridization (FISH) analysis on tissue biopsies obtained from sex-mismatched patients (1 and 3). **(A–C)** XY-FISH analysis for patient 1 revealed that XX-stained host cells were present among the XY-stained donor cells, displaying epithelial chimerism of 1.7%. **(D–F)** XY-FISH analysis on patient 3 tissue biopsies displayed a higher level of epithelial chimerism at 17.5%. XY-FISH images were taken at a single focal plane using a 100× oil-immersion objective with a fluorescent microscope to demonstrate typical XX-stained host epithelial cells among XY stained donor epithelial cells.

BMSC infusion, but did possess a longer postoperative duration (69 months) compared to patient 2 (22 months). On the other hand, STR-PCR analysis on patient 1's graft biopsies indicated significantly greater chimerism compared to patient 3, who received BMSC infusion with a significantly shorter postoperative duration (8 months). XY-FISH analysis displayed 10 times greater epithelial chimerism in patient 3 compared to patient 1. The difference between the chimerism analysis performed for patients 1 (1.7% by XY-FISH and 34.3% by STR-PCR) and patient 3 (17.5% by XY-FISH and 7.4% by STR-PCR) with these methods can be attributed to the differences in the sensitivities of these techniques. Differences in the quantitative results obtained from these 2 methods have been reported in specific cases where STR-PCR was considered to be the method of choice for chimerism quantification after stem cell transplantation [25]. It should be noted that we performed XY-FISH by scoring up to 349 cell nuclei in the limited number of tissue biopsies obtained from consenting patients post-transplantation.

Achievement of effective doses of BMSCs at the target tissue can be hampered by sequestration of these cells in peripheral tissues, such as lung, brain, liver and myocardium, when BMSCs are administered via the peripheral venous route [37–40]. While it is known that BMSCs can be attracted to inflammatory regions via chemotactic effects following peripheral administration [37,41–43], extensive inflammatory reaction would be necessary to induce such attraction [44]. Therefore, autologous BMSCs were administrated via the SMA of the graft to enable effective and direct access to the tissue graft. This surgical protocol is in parallel with catheter-based cell therapies for myocardial diseases [45–47].

Donor bone marrow infusion has been demonstrated in murine and porcine models earlier in small bowel transplantation [18,21–23]. Transplantation tolerance through donor bone marrow infusion has been successfully shown in primate models and in clinical studies [20]. Moreover, single and multiple infusions of BMSCs have been tested in

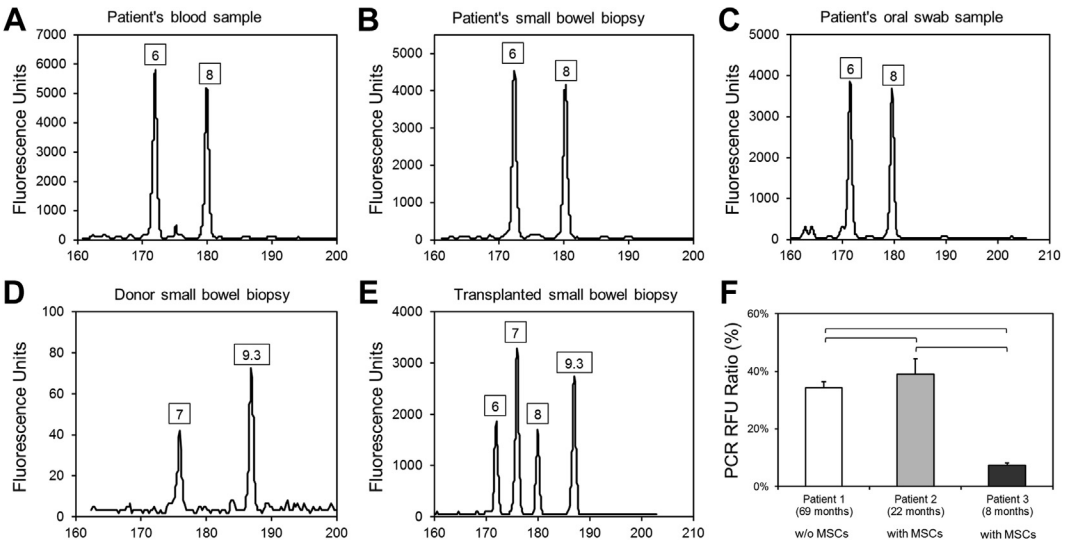

Fig 3. Chimeric effect was compared between donor and recipient biopsies by short tandem repeat (STR) analysis used for genetic fingerprinting. (**A**) TH01 STR locus for patient 2 blood sample. (**B**) TH01 STR locus for patient 2 small bowel biopsy. (**C**) TH01 STR locus for patient 2 oral swab sample. (**D**) TH01 STR locus for donor (cadaver) small bowel biopsy. (**E**) Transplanted intestine biopsy (22 months after surgery) STR results for patient 2 showing the TH01 informative locus. (**F**) PCR RFU ratio comparison between recipient and donor biopsies was performed by evaluating 16 standard STR regions (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818, FGA, and Amelogenin) in tissue biopsies (three biopsies for patients 1 and 3, two biopsies for patient 2). PCR relative fluorescence unit (RFU) ratio comparison between recipient and donor is presented for patient graft survival with and without autologous BMSC infusion. BMSC infusion was performed via femoral artery catheterization for direct infusion to the superior mesenteric artery of the graft during surgery and post-operation (at 15th day). Even though patient 2 (with BMSCs) had a shorter graft service compared to patient 1 (without BMSCs), PCR RFU rates indicative of chimerism were significantly greater ($P < .05$) in patient 2 biopsies compared to patient 1. These results indicate regeneration capacity of autologous BMSC infusion in small bowel villus epithelial tissue. PCR RFU ratios presented here for each patient is the average of informative STR loci. In plots **A**–**E**, the x-axis represents length of PCR products in base pairs and the y-axis represents the fluorescence intensity in arbitrary units. Brackets connecting individual samples indicate statistically significant difference ($P < .05$, n=8, ANOVA with Tukey's posthoc test for multiple comparisons).

earlier clinical studies for acute graft-versus-host disease [48]. Since this is a pilot clinical study performed with a limited number of cases, the selection of postoperative sampling and the use of BMSC infusion doses were limited. Similar postoperative follow-up studies have been reported in literature for clinical tests with a limited number of cases [31]. Future randomized and case–control studies comparing outcomes with or without BMSC infusion and at different BMSC infusion doses in larger patient populations are needed to provide further insight into such therapeutic approaches in I-ITx.

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