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이학박사 학위논문

A Study on Application of Mesenchymal Stem Cell Sheet by Endoscopic Transplantation in Inflammatory Bowel Disease Model

염증성장질환 모델에서 내시경이식법을 통한
중간엽줄기세포시트 적용에 관한 연구

울산대학교대학원

의 학 과

박 세 형

A Study on Application of Mesenchymal Stem Cell Sheet by Endoscopic Transplantation in Inflammatory Bowel Disease Model

지도교수 명 승 재

이 논문을 이학박사 학위 논문으로 제출함

2018 년 12 월

울산대학교대학원

의 학 과

박 세 형

박세형의 이학박사학위 논문을 인준함

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2018 년 12 월

Abstract

Background & Aims: Recent progress in regenerative medicine technology has encouraged, clinical trials for harnessing the regeneration and immune modulation potential of stem cells for the treatment of inflammatory bowel diseases (IBD) and has shown some promising results. We investigated the feasibility and utility of intraluminal endoscopic transplantation of rat mesenchymal stem cell (MSC) sheets in murine models of experimental colitis for the condensed delivery of stem cells to the designated lesion.

Methods: Adipose derived-MSCs (AD-MSC) and bone marrow derived-MSCs (BM-MSC) were isolated from enhanced-green fluorescent protein (EGFP)-transgenic rats and were fabricated in sheet forms using temperature-responsive culture dishes. The MSC sheets were endoscopically transplanted to the inflamed areas in electrocoagulation and Dinitrobenzene sulfonic acid (DNBS) colitis models. The effect of transplantation was verified using endoscopic scoring and histological analysis. The fabrication of cell sheets was confirmed by macroscopic and histological analysis and the presence of MSCs in sheets was evaluated with cell surface marker and differentiability tests.

Results: In electrocoagulation model, AD-MSC sheet group showed decreased ulcer size in the transplanted regions. The ulcer size increased by 29.17% in the control group and, 13.10% in sham group, but showed, 17.93% and 35.10% reduction in BM-MSC and AD-MSC sheets groups. In DNBS colitis model, AD-MSC sheet group showed decreased inflammation and

colitis at transplanted regions. The ulcer size decreased by 0.625, 1.15, and 0.5 mm in sham, AD-MSC and BM-MSC groups, respectively. Analysis of murine endoscopic index of colitis severity (MEICS) after 3 days of transplantation, the AD-MSC sheet transplantation group showed a decrease in its score by 3.66 on average, which was significantly different from that of the sham group. The histological analysis showed that the cell sheets had successfully attached to the inflamed mucosa in both the electrocoagulation and DNBS colitis models.

Conclusions: Our results show that endoscopic transplantation of MSC sheets could be a new and effective mode of stem cell therapy for the treatment of IBD.

Key Words: Inflammatory Bowel Diseases, Mesenchymal Stromal Cells, Temperature-responsive Culture Dishes, Cell Sheets, 2,4-dinitrofluorobenzene sulfonic acid-induced Colitis, Electrocoagulation

Abbreviations

IBD: Inflammatory bowel disease

MSC: Mesenchymal stem cell

AD-MSC: Adipose-derived stem cells

BM-MSC: Bone marrow-derived stem cells

EGFP: Enhanced green fluorescent protein

ECM: Extra cellular matrix

Upcell: temperature-responsive culture dish

Electrocoagulation: electrocautery-induced colonic ulcer

DNBS: dinitrobenzene sulfonic acid (2,4-Dinitrobenzene sulfonic acid dihydrate)

IF: immunofluorescence

IHC: immunohistochemistry

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Introduction

1. Inflammatory bowel disease

Inflammatory bowel disease (IBD), which is represented by Crohn's disease and ulcerative colitis, is characterized by uncontrolled inflammation in gut mucosa due to dysfunctions of innate and acquired immune system.[1,2] In IBD, the current treatment approach is focused on inflammation, and steroids, immunomodulators, and biologics are used. However, many patients still suffer from complications, and the rate of intestinal resection is high. Up to now, biological agents such as anti-TNF agents have been widely used for the treatment of IBD; however, the therapeutic effect is weaker than what was initially expected. [3-5] Thus, new therapeutic approaches are strongly needed, and the regenerative medicine could be a good option. **(Fig. 1)**

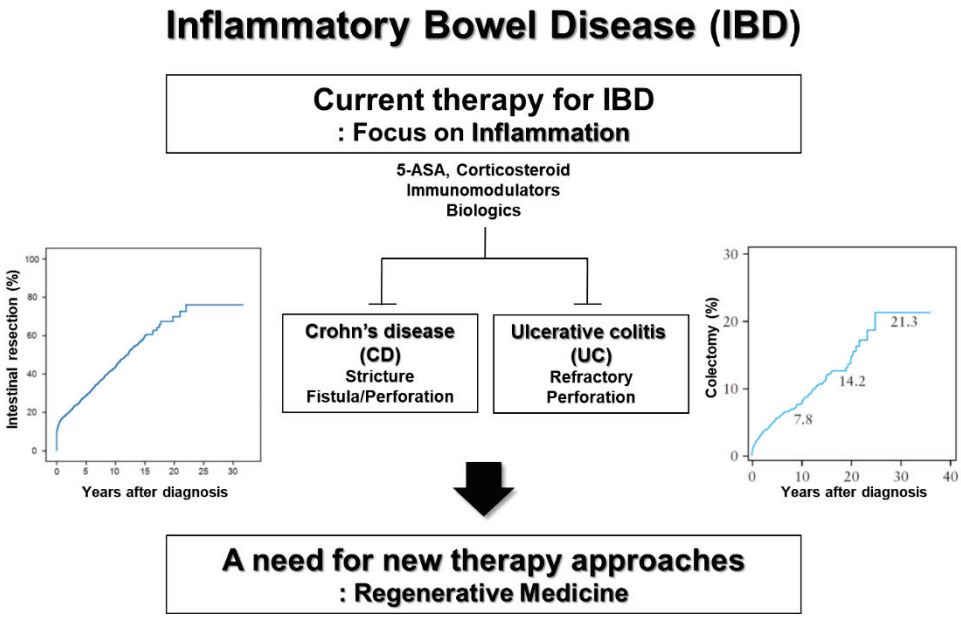

Fig. 1. The emergence of new treatments for IBD using regenerative medicine.

2. Mesenchymal Stem Cell Therapy for IBD

As regenerative medicine, stem cell therapy is widely investigated as a treatment for IBD. Stem cell therapy harnessing the effect of immune regulation ability as well as tissue repairing properties of mesenchymal stem cells (MSC) has been developed for the treatment of intractable diseases **(Table. 1)**.[6] Among the various kinds of stem cells, MSC are particularly promising candidates for the treatment of IBD such as perianal fistula in Crohn's disease, through regulation of inflammatory response and tissue regeneration.[7] Until now, two methods of MSC delivery, have been tried for IBD treatment—intravenous injection for luminal disease, and local injection for perianal fistula.[8-10] However, intravenous injection method has low survival rate of stem cells in the targeted area in luminal diseases, and thus a very high cell number is needed to achieve significant therapeutic results. Also, intravenous injection method is prone to producing side effects because the injected stem cells may migrate to undesired sites.[11,12] Therefore, it is reasonable to consider local delivery of MSCs for luminal disease, but there is no relevant study on this topic to date.

Table 1. Stem Cell Therapy for IBD.

	HSCT		MSC		Intestinal stem cell	
	Allogenic	Autologous	Allogenic	Autologous	Allogenic	Autologous
Immune reconstruction						
Pre-treatment of bone marrow cells	●	●	X	X	○	X
Immunoregulatory action	X	X	○	○	X	X
Modification of genetic predisposition						
Bone marrow cell	●	X	○	X	X	X
Intestinal epithelial cell	X	X	X	X	●	X
Mucosal healing						
Epithelial restitution			○	○	○	○
Anti-inflammatory			○	○	X	X
Proliferation			○	○	○	○
Differentiation/maturation			○	○	○	○

HSCT hematopoietic stem cell, MSC mesenchymal stem cell

3. Cell Delivery for Stem Cell Therapy in IBD

Recently, injection of colon-specific differentiated organoids has been proposed as an alternative for the intravenous injection method.[13,14] Intrarectal infusion of differentiated organoids from LGR5 (colon stem cell marker)-positive stem cells isolated from mouse colon resulted new mucosal formation in the colitis area; however, this method is limited by the need for solidification of materials and the need to temporarily plug the rectum following injection.[13] Although organoids have been reported to show high function stability after differentiation from single stem cells under established conditions,[15] stable maintenance of function after transplantation is yet to be confirmed.

4. Endoscopic transplantation of stem cell sheets for IBD

To overcome such existing technical limitations, we have tried fabricating MSC sheets using a temperature-responsive culture dish, and transplanting them in an endoscopic manner. The cell sheet technology was developed to transfer various types of cells to target organs to be used in various medical fields **(Fig. 2)**.[16-21] Even after being removed from the culture dish, the cell sheets retain their extra cellular matrix in an intact form, and they readily attach to the damaged tissue. With such advantage, endoscopic transplantation of cell sheets derived from buccal mucosa was investigated for the prevention of stricture after submucosal dissection of esophagus.[21-23]

According to a lately published literature, direct cell delivery system using cell sheets was

shown to be useful for optimized stem cell delivery for severe heart failure treatment.[24] Focal transplantation to the lesion may be a more practical approach, considering that intravenous injection has a very low rate of peripheral lesion migration and the possibility of being trapped in the lungs when circulating in the blood vessels. In this aspect, endoscopic transplantation of MSC is an apt candidate method that allows non-invasive and selective transplantation into the lesions, thereby further enhancing the therapeutic effect of MSC and avoiding side effects. Reports on the use of cell sheets method in enteric diseases remain scarce; therefore, we aimed to assess the efficacy of endoscopic transplantation of MSC in two different rat colitis models. We used an electrocoagulation and a DNBS colitis model to transplant MSC sheets by endoscopy, and analyzed the endoscopic phenotype and histology in the transplanted area.

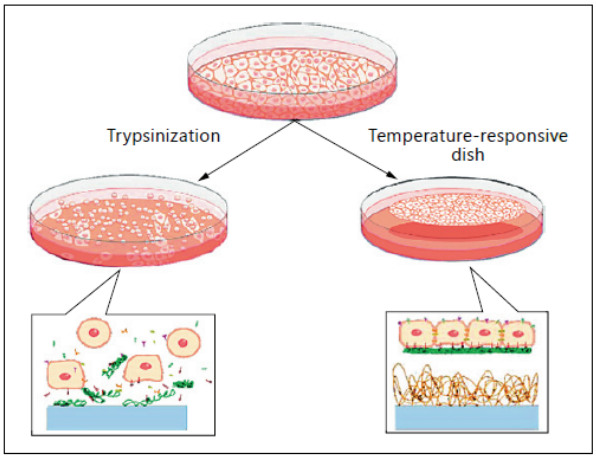

Fig. 2. Temperature-responsive culture dishes.

Materials and Methods

1. Isolation of rat MSC

Adipose-derived stem cells (AD-MSC) were isolated from the inguinal adipose tissue of Sprague Dawley (SD) (CAG-EGFP) transgenic rats, which was processed according to a previously reported method.[25,26] Briefly, the isolated adipose tissue was enzymatically digested with 0.1% type I collagenase (Invitrogen, Carlsbad, CA, USA) at 37°C for 1 h. The stromal vascular fraction was collected after centrifugation at 700 ×g for 5 min. Cells in the stromal vascular fraction were plated on a 10-cm^2 Primaria™ tissue culture dish (BD Biosciences, San Diego, CA, USA) and cultured in complete culture medium consisting of DMEM/F-12 (Invitrogen, Carlsbad, CA, USA) with 10% FBS (Hyclone, Thermo Scientific, Landsmeer, The Netherlands) and 1% Antibiotic-Antimycotic (A-A) (Invitrogen, Carlsbad, CA, USA) at 37°C in a 5% CO_2 incubator. After 24 h, debris was removed by washing with PBS (Life Technologies, Grand Island, NY, USA), and fresh complete culture medium was added. The cells were passaged with 0.25% trypsin-EDTA (Life Technologies, Grand Island, NY, USA) on day 2 and transferred to a new dish. Subcultures were plated at a density of 2.0 × 10^5 cells/10-cm^2 every 3 days.

Bone marrow-derived stem cells (BM-MSC) were isolated from bilateral femurs and tibias of SD(EGFP-CAG) Tg-Rat, which was processed according to a previously reported method.[27,28] A single suspension of bone marrow-derived all nuclear cells was seeded in 10-cm^2 Primaria™

tissue culture dish and incubated at 37°C with 5% CO_2 incubator. After 12 h, nonadherent cells were removed, and adherent cells were cultured in complete culture medium consisting of DMEM, low glucose, GlutaMAX™ Supplement (DMEM+GlutaMAX™, Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS and 1% A-A. The medium was replaced and washed with complete medium every day while growing the cells. The adherent cells were passaged with frequent medium changes to eliminate potential hematopoietic cell contamination.

2. Characterization of rat mesenchymal stem cells

AD-MSC and BM-MSC at passage 3 were suspended and incubated with an PBS containing 2% FBS (Hyclone, Thermo Scientific, Landsmeer, The Netherlands), followed by primary antibodies: endothelial stem cell marker; CD31 (#555027) (BD Biosciences, San Diego, CA, USA), hematopoietic stem cell marker; CD45 (#554878), CD73 (#551123) (BD Biosciences, San Diego, CA, USA), mesenchymal stem cell marker; CD29 (#562154) (BD Biosciences, San Diego, CA, USA), CD90 (Ab226) (Abcam, Cambridge, UK), CD105 (Ab11414) (Abcam, Cambridge, UK). After incubation for 1 h at 4°C, the cells were washed with PBS and then suspended in 1 ml PBS for analysis. The cells were analyzed with a flow cytometer (Canto™ II; BD biosciences, MD, USA). AD-MSC and BM-MSC were confirmed by measuring their adipogenic and osteogenic abilities, respectively, by using previously reported methods.[29] For each assay, AD-MSC and BM-MSC at passage 3 were plated in a 6-well plate (Nunc, Roskilde,

Denmark) and cultured in complete culture medium (See page 7 for medium condition). For adipogenesis, the medium was switched to the StemPro® Adipogenesis Differentiation medium (Life Technologies, Grand Island, NY, USA). After 14 days, the cells were fixed with 4% paraformaldehyde phosphate buffer solution (PFA) (Wako, Osaka, Japan) for at least 1 h and stained for 2 h with fresh Oil Red-O solution (Wako, Osaka, Japan). For osteogenesis, the medium was switched to the StemPro® Osteogenesis Differentiation Medium (Life Technologies, Grand Island, NY, USA). The cells were incubated for 30 days and then stained with 1% alizarin red-S solution. Each experiment was performed in triplicates.

3. Fabrication of rat Mesenchymal Stem Cell sheets using temperature-responsive culture dish (Upcell)

AD-MSC and BM-MSC at passage 3 – 4 were seeded on 35-mm diameter temperature-responsive culture dishes, UpCell™ (CellSeed, Tokyo, Japan). The UpCell™ dish was pre-coated with 2 ml FBS for 30 min in an incubator before seeding the stem cells. AD-MSC were seeded at 8.0×10^5 cells/dish and cultured in DMEM/F-12 medium with 20% FBS, 5 ng/ml FGF (Invitrogen) and 1% A-A for 48 h **(Fig. 3)**. BM-MSC were seeded at 1.1×10^6 cells/dish and cultured in DMEM+GlutaMAX™ medium supplemented with 20% FBS and 1% A-A for 36 h. After reducing the temperature to 25℃ in a CO_2 incubator, the cells spontaneously detached as contiguous cell sheets and were harvested from the dishes with supplied membrane (Cellshifter™ (CellSeed, Tokyo, Japan)).

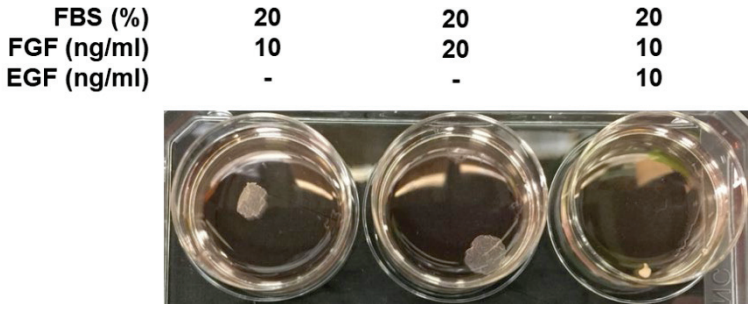

Fig. 3. Establishment of rat mesenchymal stem cell sheets.

The optimal conditions for sheet fabrication of AD-MSC were determined through controlling various culture conditions. For the AD-MSC sheet, the cell sheet was best formed in the culture medium with 20% FBS and 10 ng/ml.

4. Animals

Specific pathogen free six weeks-old (150–200 g) male Sprague Dawley (SD) rats (Orient Bio Inc., Seongnam, Korea) and SD(EGFP-CAG)Tg rats (SLC, Tokyo, Japan) were used. All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the Asan Institute for Life Sciences at Asan Medical Center, consistent with the Institute of Laboratory Animal Resources (ILAR) guidelines. All experiments were performed in accordance with relevant guidelines and regulations. The animals were housed in standard laboratory conditions with a temperature of 21 – 23℃ and 12 h dark/light cycles. Rats were allowed ad libitum access to food and water throughout the study period.

5. Chemicals and devices

2,4-Dinitrobenzene sulfonic acid dihydrate (DNBS) was purchased from MP Biomedicals (Aurora, OH, USA). Endoscopic transplantation was carried out using Karl Storz Coloview^{TM} mini-endoscopic system (KARL STORZ GmbH & Co. KG, Tuttlingen, Germany). For electrocoagulation model, ZATHA Electrosurgical Unit (Jejoong medical, wonju, korea) was used.

6. Electrocautery-induced colonic ulcer (electrocoagulation) model

Electrocoagulation model was generated according to a previously reported method.[30,31] For experimental purposes, animals were fasted for 12 h with ad libitum access to 8% sucrose water in 0.2% saline to prevent dehydration. The 10-cm electrocautery probe (electrode) was made of platinum-coated copper node. The electrode was insulated except for both extremities. For electrocoagulation, the ball-shaped tip of the electrode was introduced through the anus into the 5 cm and 3 cm depths of colon and directly contacted the mucosa; the other tip was connected to the pole of a direct current generator. The position of the tip of electrode directly contacting the mucosa was determined by the direction of the experimenter's finger from the lower abdomen. Bipolar current (18~20 W) was delivered through the electrodes for 2 sec. Electrocoagulation were applied at different depths to prevent overlap formation of ulcer or to prevent perforation **(Fig. 4)**. For the evaluation of the electrocoagulation model, the ulcer sites were directly observed using an endoscopic system immediately after electrocautery. During endoscopic observation, the ulcer diameter was measured using the biopsy forceps (maximum opening width: 4 mm).

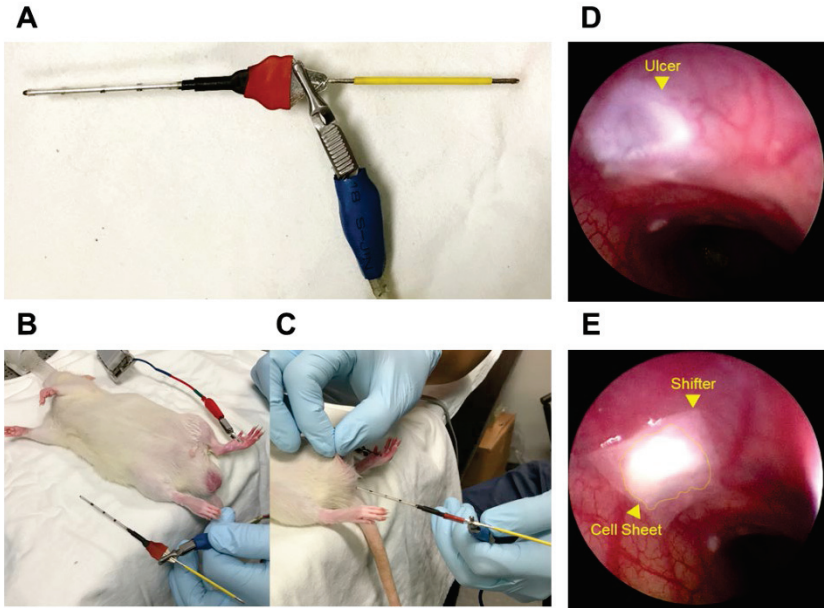

Fig. 4. Electrocautery-induced colonic ulcer modeling.

(A) The specially designed ball-shaped tip monopolar electrode. (B) The other electrode was connected to the hind leg to allow current to flow through the rat. (C) To induce ulceration, the electrode was inserted through the rectum and the current was delivered. (D) Endoscopic image of electrocoagulation. (E) Endoscopic image of a cell sheet attached to an ulcer.

7. Dinitrobenzene sulfonic acid (DNBS) colitis model

Induction of DNBS colitis model was based on the previously reported protocols.[32,33] Briefly, 30 mg /250 μl (in 50% EtOH) DNBS was intrarectally infused via a polyethylene (PE) zonde (Φ 1.8 × 95mm) into 6 weeks old SD rats under isoflurane-induced anesthesia.

8. Endoscopic transplantation of MSC sheets

The MSC sheets were transferred to the Cellshifter™ that was included with the Upcell™ dish using a pair of forceps specifically designed for the endoscope. Then, the MSC sheets laid onto the forceps was attached to the target area **(Fig. 5)**. The experimental groups of the electrocoagulation model were as follows: Control; no procedures performed after electrocoagulation induction, Sham; transplantation with a Cellshifter™ without MSC sheets after electrocoagulation, AD-MSC, BM-MSC; electrocoagulation followed by transplantation of AD-MSC or BM-MSC sheets. Endoscope was used to confirm the location and size of the ulcer and to transplant the MSC sheets onto the electrocautery-induced ulcers. The experimental groups of the DNBS colitis model were as follows: Normal; simulated transplantation only with a Cellshifter™ without MSC sheet in healthy condition, Sham (DNBS control); induced DNBS colitis model, transplantation with a Cellshifter™ without MSC sheets, AD-MSC, BM-MSC; induced DNBS colitis model, followed by transplantation of AD-MSC or BM-MSC sheets.

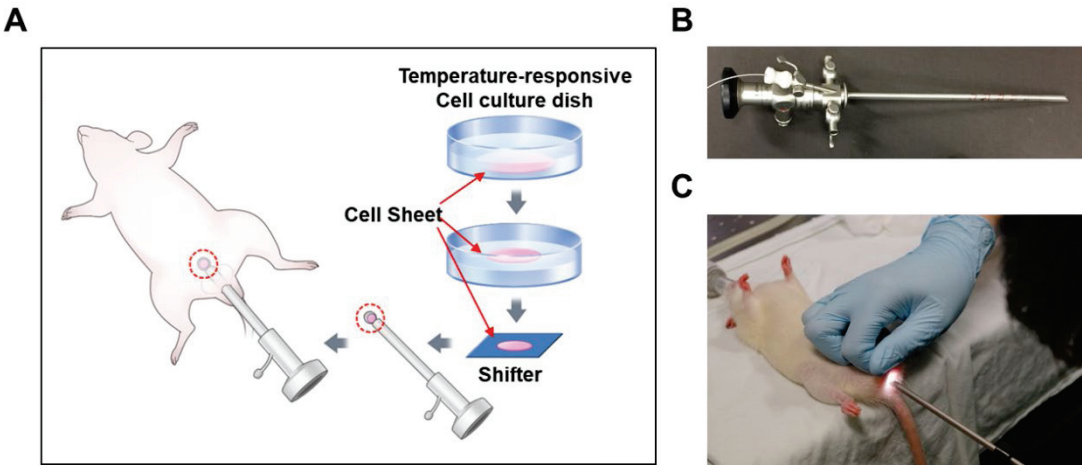

Fig. 5 Endoscopic transplantation of MSC sheets.

(A) Illustration of endoscopic transplantation of MSC sheets using a temperature-responsive cell culture dish. (B) endoscopic equipment for the rat colonoscopy. Channel-inserted forceps was attached to a rat's colonoscopy consisting of a telescope and a sheath. (C) Endoscope procedure through the rectum for endoscopic transplantation of MSC sheet.

9. Assessment of ulcer diameter in electrocoagulation model

The evaluation of colonic ulcer size was performed as follows; the main method was to use endoscopic forceps. The maximum opening width of the forceps used in the transplantation and the basic length of the wire constituting the forceps were measured in advance and used as references **(Fig. 6)**. A complementary approach was to use the Cellshifter^{TM}. During the endoscopic transplantation, the Cellshifter^{TM} was used to transfer the cell sheet. It was used as a scale in the endoscopic evaluation after being applied in uniform size before use in transplantation experiment. The size of Cellshifter^{TM} was 3 × 6 mm and it was used by covering the ulcer or by attaching it to the side.

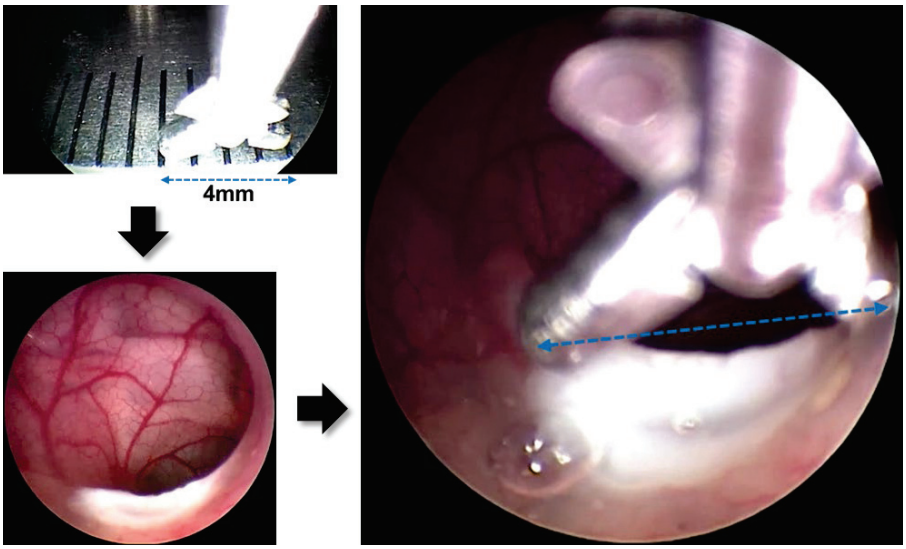

Fig. 6. Measurement of electrocautery-induced colonic ulcer size.

The diameter of the ulcer was measured based on the maximum opening width of the endoscopic channel-inserted forceps.

10. Post-transplantation procedure

The endoscope was reinserted after excising the lower abdomen, and the colon was sampled after pinning the target area **(Fig. 7)**. During sacrifice, the transplanted area was examined with endoscope before dissecting the colon, and the area was probed with tiny pin that was inserted from the outer colon towards the inner colon. After transplantation, we utilized an endoscope to accurately locate and analyze the transplanted area. In the EC model, on the day of transplantation (D0) and 1 day after transplantation (D1), the rats were sacrificed to perform macroscopic observation and fixation for histologic analysis. In the DNBS colitis model, the rats were sacrificed at either 1 day (D1) or 3 days (D3) post-transplantation for histologic analysis (EGFP positive signal percentage index: negative - 0, weak positive - 50, positive – 100. Success rate score index: negative - 0, weak positive - 0.5, positive – 1) (MSC cell sheet transplanted animals, n = 9)

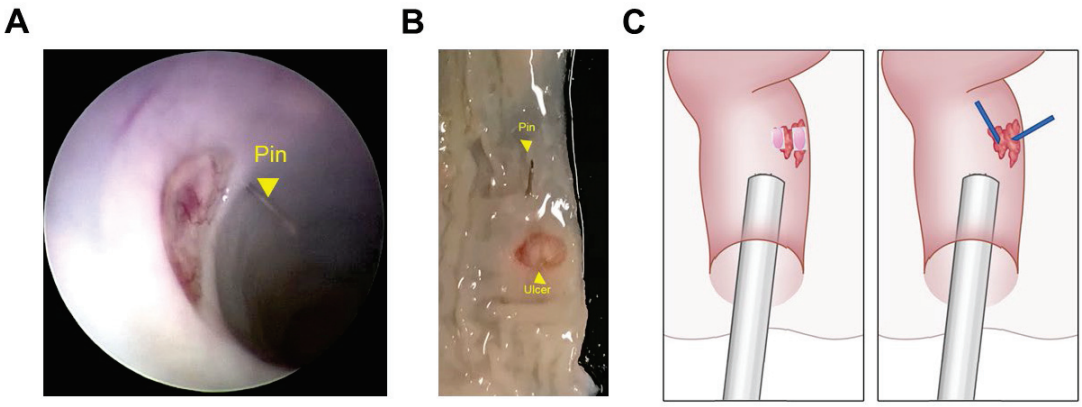

Fig. 7. MSC sheet transplantation site-marking method for histological analysis after endoscopic transplantation.

(A) To mark the MSC sheet transplanted sites, the pin was inserted into the colon during endoscopy with the lower abdomen open. (B) Longitudinal section of the colon that was pinned. (C) An illustration showing the process of extracting the portion of the MSC sheet.

11. Assessment of disease activity in DNBS colitis model

The disease activity of the DNBS models was evaluated by the sum of the scores in each category—a percentage of weight loss from body weight, characteristics of the stool and the presence of blood—from the day of DNBS administration to the day of sacrifice following cell sheet transplantation **(Table 2)**.[34,35] The endoscopic score DNBS colitis severity was evaluated by the sum of the scores in murine endoscopic index of colitis severity (MEICS)—thickening of the colon, changes of the vascular pattern, fibrin visible, granularity of the mucosal surface and stool consistency—from the day of DNBS administration to the day of sacrifice following cell sheet transplantation **(Table 3)**.[34,35]

Table 2. Scoring system for calculating disease activity index (DAI)

score	weight loss	Stool consistency	Occult/gross bleeding
0	None	Normal	None
1	1%-5%	-	-
2	5%-10%	Loose	Slightly bleeding
3	10%-15%	-	-
4	>15%	Diarrhea	Gross bleeding

Table 3. Endoscopic colitis score (murine endoscopic index of colitis severity; MEICS)

	Murine endoscopic index of colitis severity (MEICS)				
	0	1	2	3	Total
Thickening of the colon	Transparent	Moderate	Marked	Non-transparent	0–3
Changes of the vascular pattern	Normal	Moderate	Marked	Bleeding	0–3
Fibrin visible	None	Little	Marked	Extreme	0–3
Granularity of the mucosal surface	None	Moderate	Marked	Extreme	0–3
Stool consistency	None + Solid	Still shaped	Unshaped	Spread	0–3
				overall:	0–15

12. Histological analysis of the colon tissue

At 1 h after transplantation (D0) and the day after (D1 and D3), the lower abdomen of the model was excised and the colon was removed. The colon was longitudinally sectioned to confirm the attachment of the MSC sheets on the target area. The subsequent process was performed as mentioned previously.[36] The colon tissue in which the MSC sheets was transplanted and the surrounding tissue were fixated with 4% PFA (Wako), and paraffin tissue sections were constructed. Serial sections of tissue blocks were subjected to immunofluorescence analysis. Heat-induced antigen retrieval was performed with Target Retrieval Solution (pH 6.0) (DAKO, Agilent Technologies, Santa Clara, CA, USA) for 1 h in a steamer followed by cooling for 30 min. Nonspecific binding was blocked with Protein Block Serum-Free solution (DAKO) for 20 min. Tissue sections were then incubated consecutively overnight at 4°C with primary antibodies, rabbit anti-GFP (MBL, #598; 1:200, Nagoya, Japan) and FITC mouse anti-E-Cadherin (BD, #612131; 1:300), diluted in Antibody Diluent, Background Reducing solution (DAKO). Then the sections were washed with PBS and then incubated for 1 h at room temperature with Alexa Fluor 594-conjugated goat anti-rabbit secondary antibodies (Cell signaling, #8889, 1:500; MA, USA). The slides were then washed and mounted using Antifade Mounting Medium with DAPI (VECTASHIELD, Vector). Serial sections of tissue blocks were subjected to hematoxylin-eosin staining and immunohistochemical staining. Heat-induced antigen retrieval was performed with Target Retrieval Solution (pH 6.0) (DAKO) for 1 h in a steamer followed by cooling for 30 min.

Nonspecific binding was blocked by applying Protein Block Serum-Free solution (DAKO) for 20 min. Endogenous peroxidase activity was blocked for 30 min by using Peroxidase-Blocking Solution (DAKO). Tissue sections were then incubated overnight at 4°C with primary antibodies, rabbit anti-GFP (MBL, #598; 1:2000) diluted in Antibody Diluent, Background Reducing solution (DAKO). Sections were then incubated for 20 min at room temperature with horseradish peroxidase–conjugated secondary antibodies (EnVision, DAKO), and then for 1 min at room temperature with diaminobenzidine (DAB+, DAKO). Sections were washed three times with Wash Buffer (DAKO) after each incubation. The sections were counterstained with Harris hematoxylin, rehydrated with a graded series of ethanol solutions, and then mounted. The extent of the MSC sheet attachment and changes in the surroundings of the transplanted area were analyzed according to a previously published protocol[36]. Immunofluorescence stained tissues were visualized by confocal scanning microscopes (LSM 780; Carl Zeiss Microscopy GmbH, Germany). Bright-field images (HE and immunohistochemical stain) were obtained by auto imaging system (EVOS FL Auto; Life Technologies).

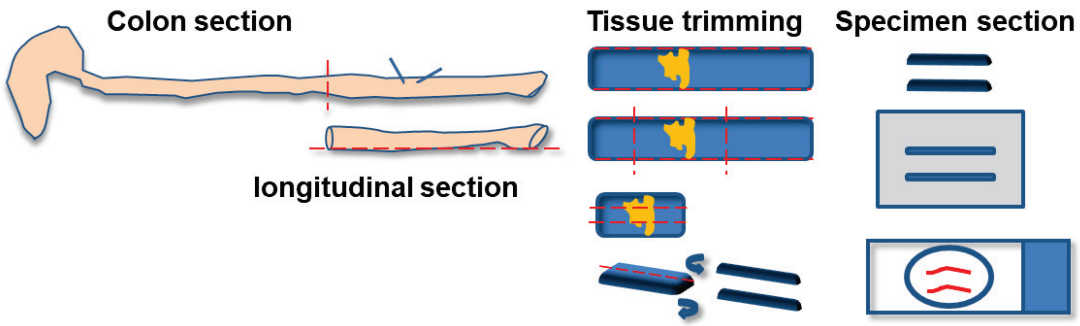

Fig. 8. Colon tissue sampling scheme of the MSC sheet transplant site for histological analysis.

13. Statistical analysis

Quantitative data are presented as means ± standard error of the mean (SEM) and were analyzed with the Student's t-test and a two-way analysis of variance. Statistical significance was determined using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). P-values less than 0.05 were considered statistically significant. All experiments were conducted in groups of three animals per group and at least three experiments were performed. Tissue staining results are representative of at least three independent determinations. The numbers of samples are indicated in the figure legends.

Results

1. Rat mesenchymal stem cell sheet fabrication

For the characterization of mesenchymal stem cells, AD-MSC and BM-MSC were isolated from SD-rats (normal rats). Isolated AD-MSC and BM-MSC were stained with cell surface markers and analyzed with flow cytometry using the following markers: hematopoietic stem cell marker, CD45 (negative); endothelial stem cell marker, CD31 (negative) and; mesenchymal stem cell markers, CD29, CD73, and CD90 (positive) **(Fig. 9).**

In the differentiation analysis, the formation of oil and accumulation of calcium in the AD-MSC and BM-MSC was found, showing the differentiation capacity of AD-MSC and BM-MSC **(Fig. 10)**.

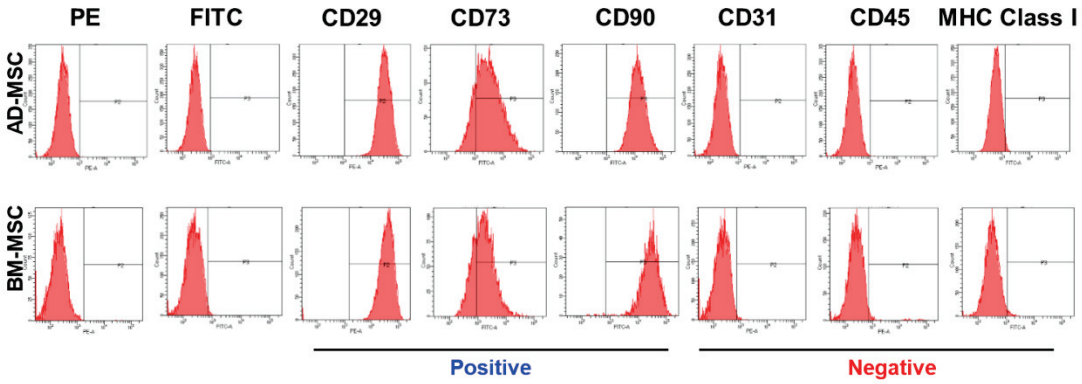

Fig. 9. Characterization of rat mesenchymal stem cell markers.

Histogram of cell surface markers showing the AD-MSC (upper) and BM-MSC (lower).

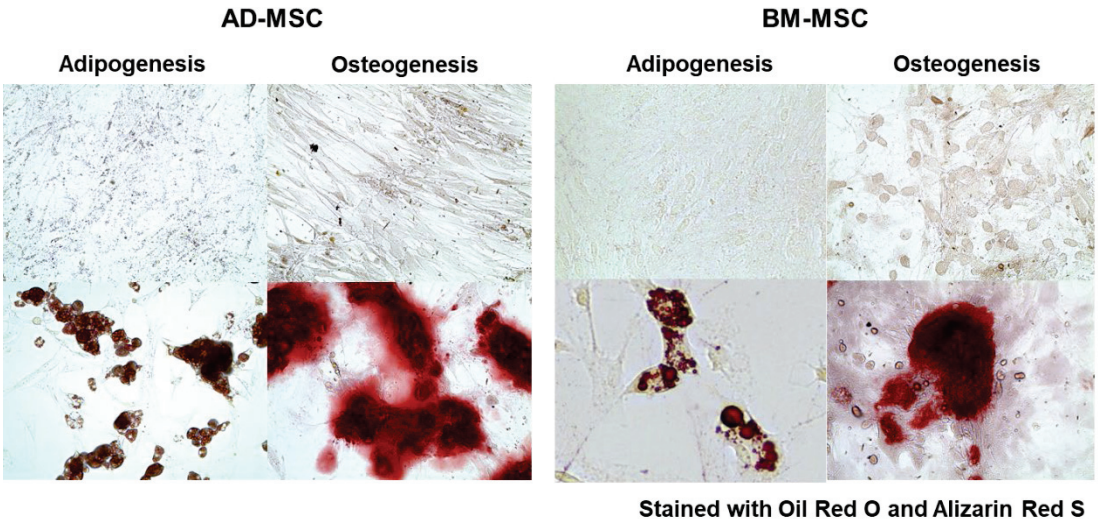

Fig. 10. Verification of differentiation ability of rat mesenchymal stem cells.

Differentiation capacity of AD-MSC and BM-MSC for differentiating into adipocytes and osteoblasts with alizarin red. (upper panel; undifferentiated condition, lower panel; differentiated condition). Extracellular calcium deposition in the osteoblasts is shown in red, the accumulation of lipid vacuoles in the adipocytes are shown in red.

All the experiments were performed three times.

For the fabrication of cell sheets, AD-MSC and BM-MSC were isolated from SD(EGFP-CAG)Tg-rats (Tg-Rats). EGFP fluorescence of AD-MSC and BM-MSC isolated from Tg-Rat was used to track the cell sheets after transplantation. EGFP fluorescence through antibody labeling showed an amplified signal compared with that of the EGFP originating from Tg-Rats **(Fig. 11)**. Various conditions were screened to obtain the optimal cell sheet fabrication conditions for AD-MSC and BM-MSC. 1) Cell numbers: Considering the characteristics of the Upcell™ dish, in order to prepare a cell sheet, culture needed to done in such a way that the cells would not be substantially confluent (8.0×10^5 cells/dish of AD-MSC and 1.1×10^6 cells/dish of BM-MSC). 2) Growth factors: In the UpCell™ dish, various growth factors were added to the medium to obtain the best culture conditions for the stem cell sheets. The AD-MSC sheets required 20% FBS and 5 ng/ml FGF in culture medium. The BM-MSC sheets required 20% FBS in culture medium. 3) Incubation times: After seeding in the Upcell™ dish, the optimal time to detach into cell sheets was measured. As a result, AD-MSC sheets were formed 48 h after cell seeding, and BM-MSC sheets were formed 36 h after seeding, at a temperature condition changed from 37℃ to 25℃, after incubation. After adjusting for the aforementioned conditions, we were able to produce viable cell sheets using AD-MSC and BM-MSC. Histological analysis of the AD-MSC and BM-MSC sheets was carried out and it was verified that the sheets were well formed **(Fig. 12)**. Immunoblotting was performed for the EGFP fluorescent tag isolated from Tg-Rats to confirm EGFP expression **(Fig. 13)**.

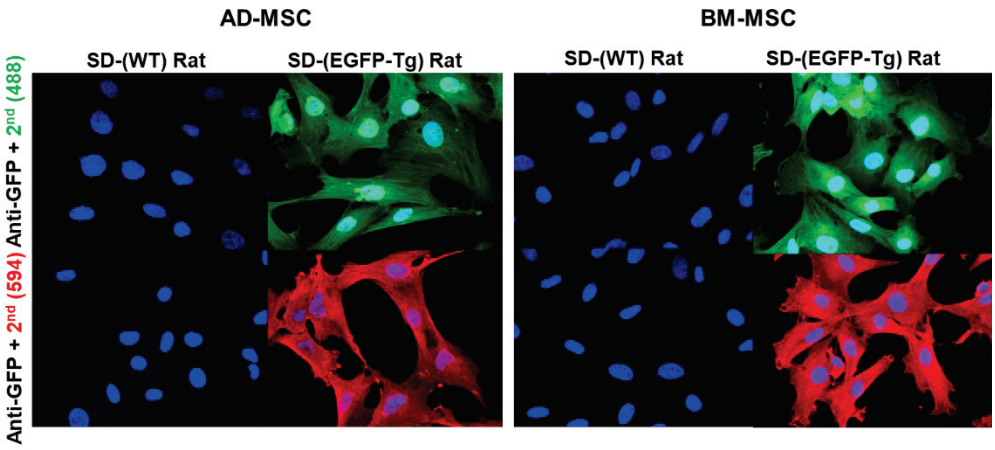

Fig. 11. Immunocytochemistry with anti-EGFP Ab.

EGFP signal of SD-Tg AD-MSC and BM-MSC 1 day after seeding on a culture dish. AD-MSC and BM-MSC were used at passages 3.

All the experiments were performed three times. Green: AD-MSC or BM-MSC from EGFP transgenic rats, Red: EGFP antibody labeled by Alexa Flour 594 Blue: nucleus.

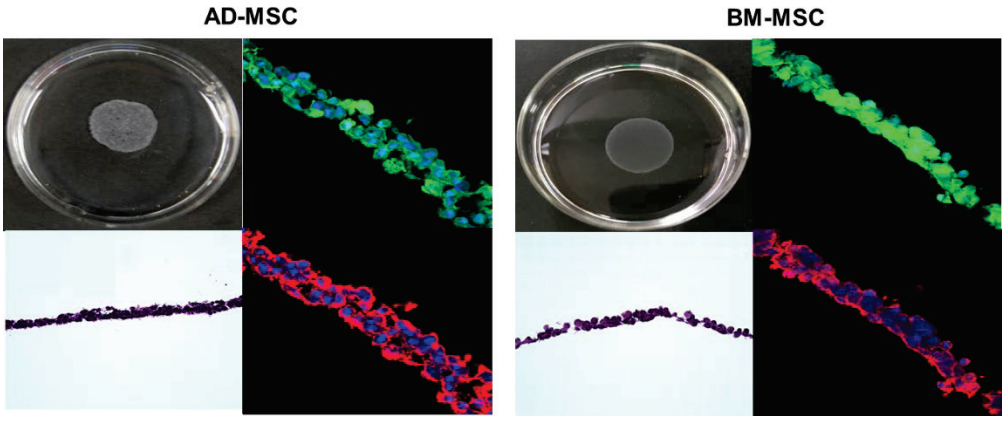

Fig. 12. Histological analysis of rat MSC sheets.

Fabricated AD-MSC and BM-MSC sheets. AD-MSC and BM-MSC sheets by hematoxylin-eosin staining. EGFP-expressing monolayered AD-MSC and BM-MSC sheets. AD-MSC and BM-MSC were used at passages 3 or 4.

All the experiments were performed three times. Green: AD-MSC or BM-MSC from EGFP transgenic rats, Red: EGFP antibody labeled by Alexa Flour 594 Blue: nucleus.

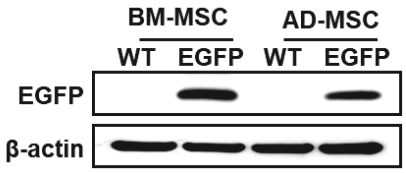

Fig. 13. Expression of EGFP protein in MSCs isolated from Tg-rats.

EGFP protein expression levels in Tg-rat isolated AD-MSC and BM-MSC.

All the experiments were performed three times.

2. Verification of adhesion of the MSC sheets in animal model of colitis.

Before confirming the therapeutic effect on colonic lesions through endoscopic transplantation, we confirmed that the MSC sheets were able to adhere to the colon mucosa. The MSC sheets were attached to the extracted colon as observed by longitudinal sectioning; the sheets were washed in PBS solution and analyzed by molecular imaging for adhesion maintenance according to lesion severity. The MSC sheet was found to be attached to the colon and was incubated at 37℃ for 10 min. After the incubation, the colon was sprayed with micropipette, and then immersed in the solution, followed by washing with shaking for 10 times. After each washing, molecular imaging analysis was performed.

In the normal colon, no fluorescence signal was detectable from the attached MSC sheet after light washing **(Fig. 14A)**. In the colitis-induced colon, it was confirmed that the attached MSC sheet retains its ability to adhere and maintain fluorescence signal even after strong washing **(Fig. 14B)**. MSC sheets were attached to the colon lesion and it was confirmed that they remained adhered to each other over time. Molecular imaging analysis of the colon one day after endoscopic transplantation of MSC sheets showed that the fluorescence signals could be detected in two of the transplantation sites **(Fig. 15A)**. In addition, the fluorescence signal of the MSC sheet was confirmed in the analysis of the colon 3 days after the transplantation **(Fig. 15B)**.

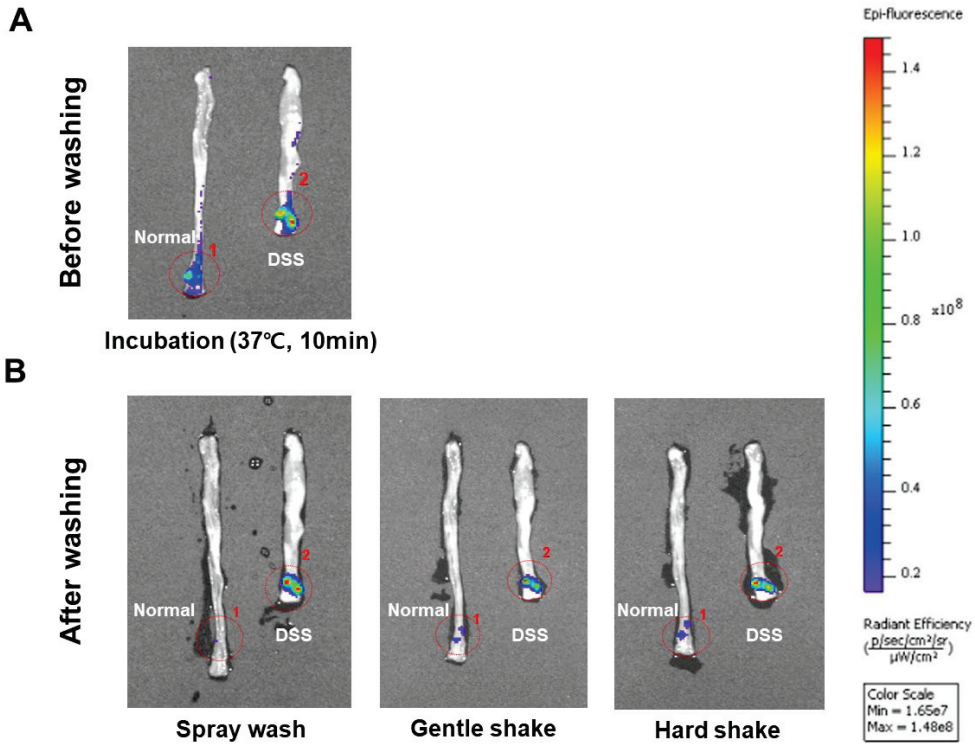

Fig. 14. Maintenance of MSC sheets attached to the colon lesions.

(A) Molecular image analysis after longitudinal sectioning of the incubated (37℃, 10 min) MSC sheets attached to the colon (B) Molecular imaging of MSC sheets for detection of fluorescence signals in normal colon and in lesion areas, according to the washing method (spray wash or shake wash). All experiments were performed three times.

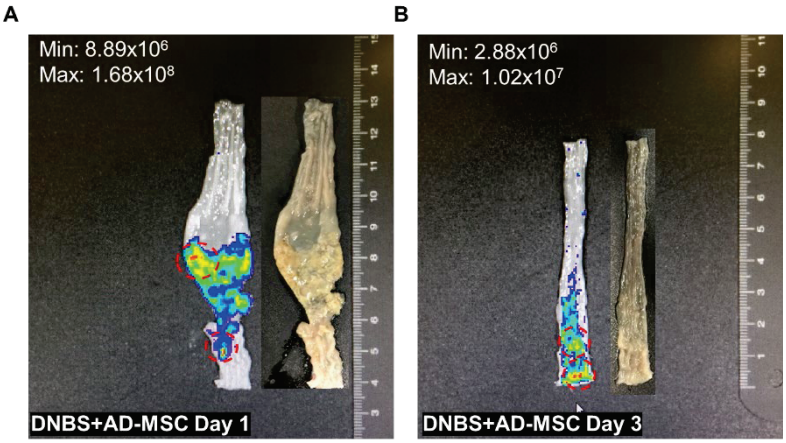

Fig. 15. MSC sheets are retained implantation in the colon lesions.

(A) Molecular imaging of the colon 1 day after transplantation of AD-MSC sheets in the colon of DNBS-induced colitis rats. (B) Molecular imaging of the colon 3 days after transplantation of AD-MSC sheets in DNBS-induced colitis rats.

3. Endoscopic transplantation of MSC sheets in animal models

AD-MSC and BM-MSC were isolated from Tg-Rats and were cultured in temperature-responsive culture dishes to fabricate MSC sheets **(Fig. 16A)**. The methodology for the endoscopic transplantation in the electrocoagulation model **(Fig. 16B)** and DNBS colitis model **(Fig. 16C)** is schematically represented. After electrocoagulation, MSC sheets were transplanted on Day 0 and analyzed 1 day after transplantation in the electrocoagulation model. In addition, 4 days after induction of DNBS colitis, MSC sheets were transplanted and analyzed on days 1 and 3 after transplantation in the DNBS colitis model.

The fabricated MSC sheets were transferred to the Cellshifter^{TM} for endoscopic transplantation. The Cellshifter^{TM} was held with endoscopic forceps and the MSC sheets were housed in the endoscope sheath **(Fig. 17A)**. During the endoscopic transplantation, the MSC sheets held by forceps were deployed and attached to the transplantation site. The attached area was pressed gently by forceps to reinforce the transplantation of MSC Sheets to the inflamed region **(Fig. 17B)**.

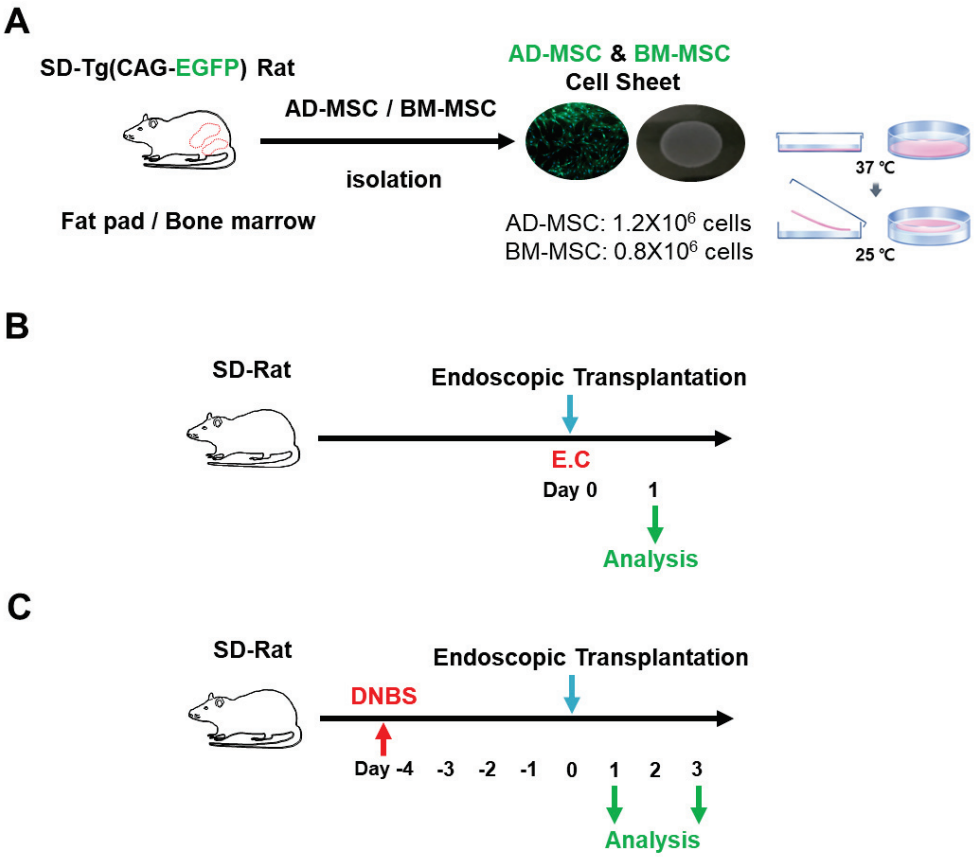

Fig. 16. Rat MSC sheets fabrication scheme and animal modeling.

(A) The scheme of MSC sheets fabrication. (B) Study design for electrocoagulation ulcer model. (C) Study design for DNBS-colitis model.

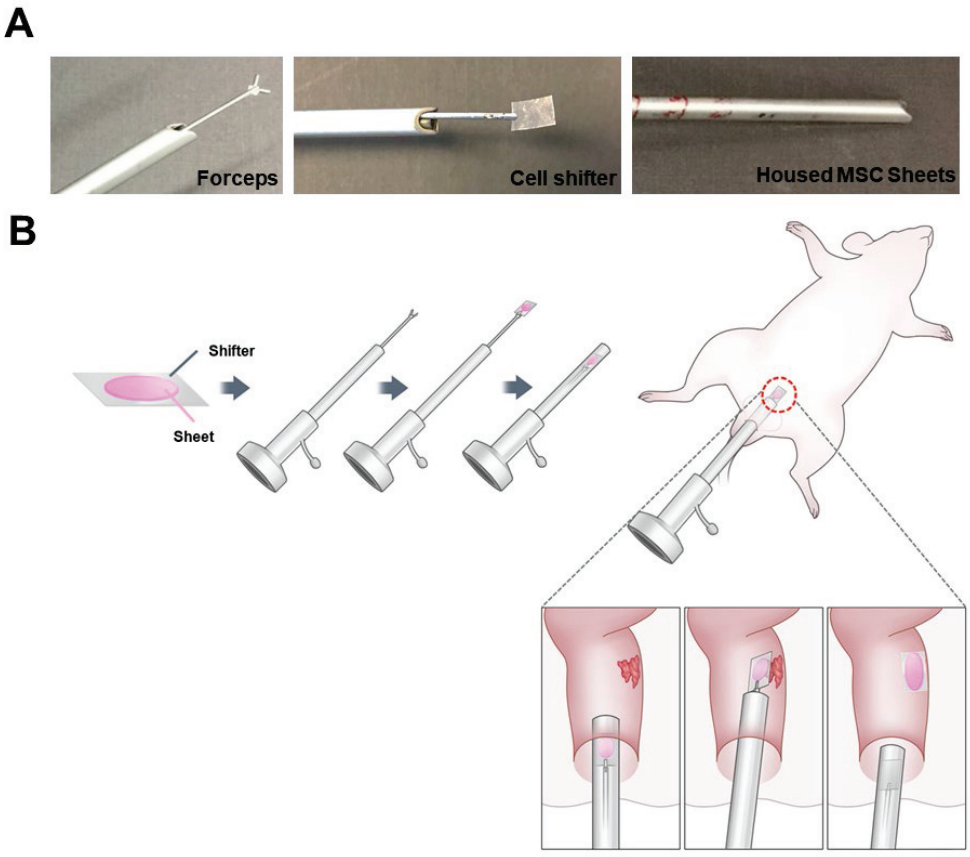

Fig. 17. Endoscopic cell sheet transplantation scheme.

(A) Endoscopic forceps for MSC sheets transplantation (left). The forceps holding a Cellshifter™ (middle). MSC sheets placed on a Cellshifter™ was housed in colonoscope (right). (B) Endoscopic transplantation process.

4. Analysis of the electrocoagulation ulcer model after endoscopic transplantation

The transplanted site was traced with an endoscope on the day of (D0) **(Fig. 18A upper panel)** and 1 day (D1) after transplantation **(Fig. 18A, lower panel)**. Macroscopic analysis was performed 1 day after MSC sheets transplantation. **(Fig. 18B).**

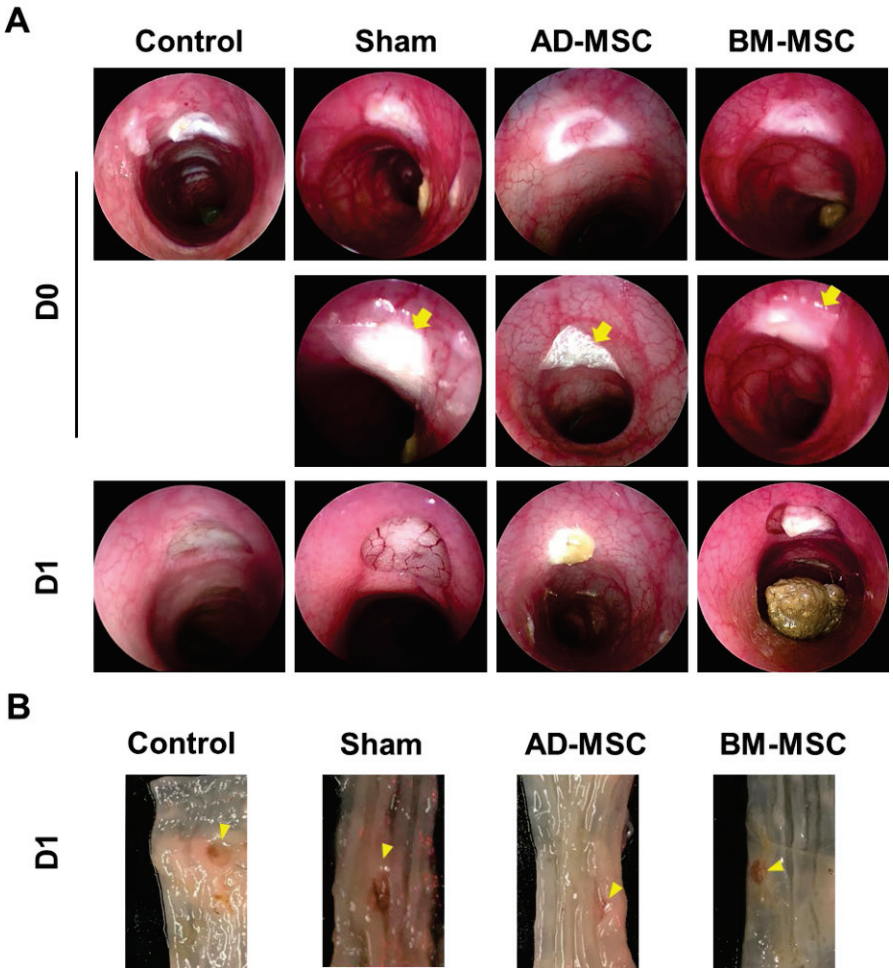

Fig. 18. Reduced ulcer size of lesion in the ulcer after MSC sheet transplantation in the electrocoagulation ulcer model.

(A) Endoscopic imaging of the colon induced by electrocoagulation ulcer (upper). and MSC sheets transplanted at the site of induction of electrocoagulation ulcer (middle). 1 day after MSC sheets endoscopic transplantation (lower). (B) Macroscopic representation of the ulcer formation on colon in 1 day after MSC sheets endoscopic transplantation. The yellow arrow points to the transplanted ulcer site. Control; only electrocoagulation, Sham; transplanted without MSC sheets, AD-MSC; AD-MSC sheets transplanted, BM-MSC; BM-MSC sheets transplanted.

Before transplantation, the inflamed colon in each animal was endoscopically observed to select the appropriate sites for MSC sheets transplantation. The following procedure was performed to identify changes in ulcer size before and after transplantation. Cell sheets for transplantation was transplanted into the ulcer by the Cellshifter^{TM} **(Fig. 19A left, D0)**.

The evaluation of colonic ulcer size was performed as follows: The method used endoscopic forceps. The maximum opening width of the forceps used in the transplantation and the basic length of the wire constituting the forceps were measured in advance and used as references **(Figure. 19A right, D1)**.

An endoscopy movie was used to measure the size change in the ulcer that occurred between the day of (D0) and the day after transplantation (D1). The control and sham groups showed an average of 1.00 mm and 0.50 mm increase in diameter, respectively. Conversely, the AD-MSC sheets group showed an average of 1.13 mm ($p = 0.007829$) decrease in diameter, and the BM-MSC sheets group showed an average of 0.57 mm decrease in ulcer size in a successfully transplanted site ($p = 0.045675$) **(Fig. 19B)**. These apparent size changes in the ulcer represent 29.17% increase in the control group, 13.10% increase in the sham group, 17.93% reduction in the BM-MSC sheets group, and 35.10% reduction in the AD-MSC sheets group. Consequently, AD-MSC and BM-MSC sheets transplantation effectively reduced the ulcer size compared to the sham group (sham vs. AD-MSC, $p = 0.00002$ and sham vs. BM-MSC, $p = 0.0007$) **(Fig. 19C)**. When comparing between AD-MSCs and BM-MSCs, AD-MSCs reduced ulcer size more effectively than BM-MSCs ($p = 0.0364$).

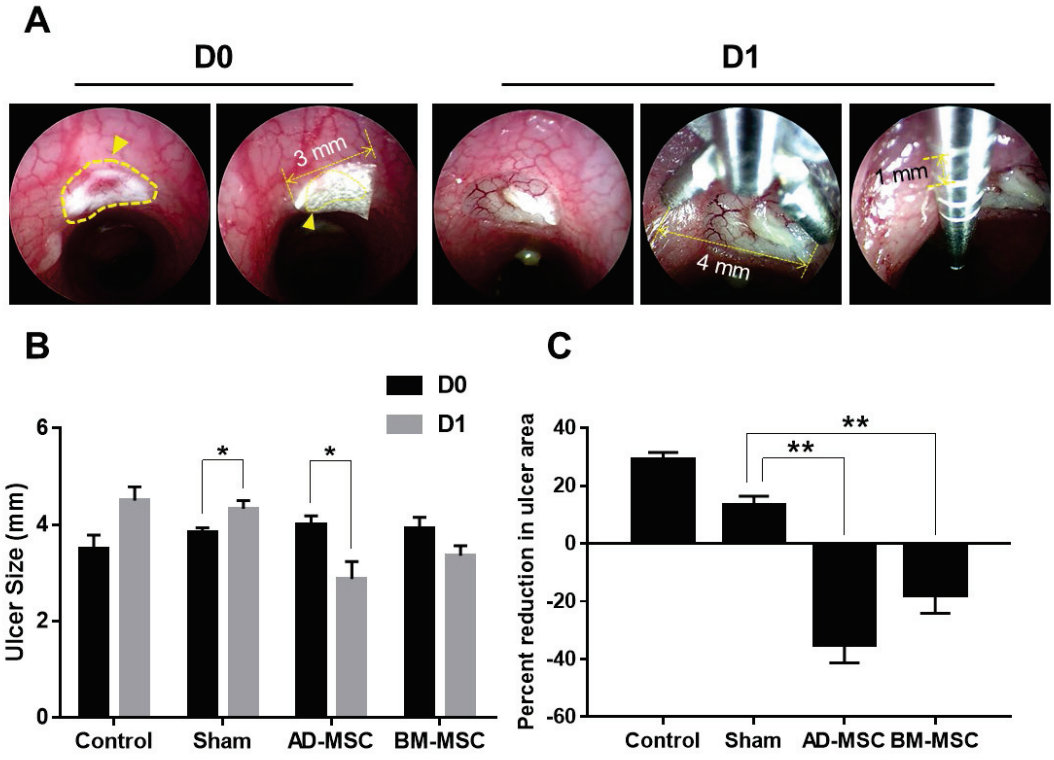

Fig. 19. Evaluation of MSC sheet endoscopic transplantation in electrocoagulation ulcer model.

(A) Cell sheets were transplanted the ulcer by the Cellshifter^{TM}. The yellow line points to the ulcer site. The yellow arrow indicates the covered MSC sheets by Cellshifter^{TM}. (left panel D0). The evaluation of colonic ulcer size. The maximum opening width of the endoscopic forceps used in the transplantation and the basic length of the forceps wire node (right panel D1).

The yellow arrow points to the transplanted ulcer site. Control; only electrocoagulation, Sham; transplanted without MSC sheets, AD-MSC; AD-MSC sheets transplanted, BM-MSC; BM-MSC sheets transplanted. Cellshifter^{TM} size: 3 × 6 mm, Forceps width: 4 mm, Forceps wire node length: 1 mm.

Further, we analyzed the success rate score for the electrocoagulation ulcer model and performed a histological analysis of the EGFP signal to decide the success of attachment of the engrafted MSC sheets to the damaged lesion site **(Fig. 20A)**. MSC sheets were transplanted into two sites per animal. The success of transplantation was determined based on the site of transplantation. According to the histological analysis of MSC sheet- endoscopic transplanted sites, the success rate was 37.6% for the AD-MSC group and 35.2% for the BM-MSC group **(Fig. 20B)**. The success rate score was 0.39 for the AD-MSC group and 0.25 for the BM-MSC group **(Fig. 20C)**.

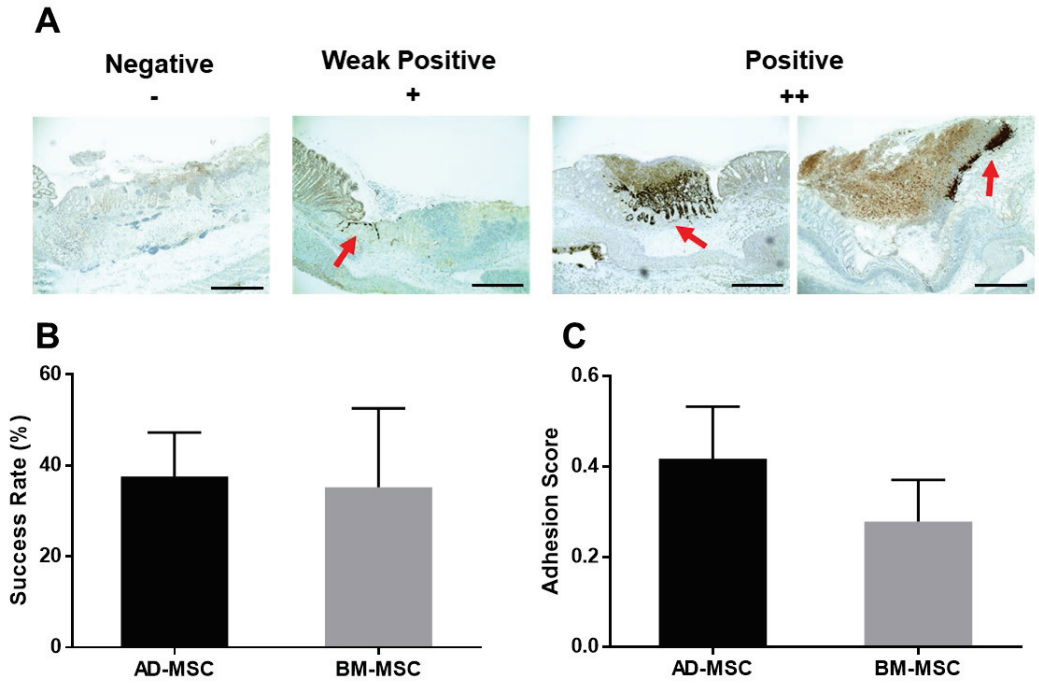

Fig. 20. Evaluation of MSC sheet endoscopic transplantation in electrocoagulation ulcer model.

(A) Representative histological analysis based on the transplantation success. The red arrow indicates the positive signal of EGFP by immunohistochemistry. (C and D) Success rate and adhesion score of the electrocoagulation ulcer model.

Con; only electrocoagulation, Sham; transplanted without MSC sheets, AD-MSC; AD-MSC sheets transplanted, BM-MSC; BM-MSC sheets transplanted. EGFP positive signal percentage index: negative - 0, week positive - 50, positive – 100. Success rate score index: negative - 0, week positive - 0.5, positive – 1. MSC sheets transplanted animal, n = 9 (transplanted site, n = 18).

Colons with and without the transplanted MSC sheets were stained with hematoxylin and eosin or anti-EGFP antibody and the transplanted MSC sheets in EGFP-Tg rats were confirmed by immunohistochemistry and immunofluorescence analysis. On the day of the transplant tissue, a very strong EGFP fluorescent signal was observed **(Fig. 21)**. Analysis of the tissue at 1 day after transplantation showed that both AD-MSC and BM-MSC cell sheets were retained in the ulcer **(Fig. 22)**. In addition, each MSC sheet migrated to and infiltrated into the ulcer area due to electrocoagulation **(Fig. 22, magnified)**. The patterns of engrafted MSC sheets were like crypts, because they filled the damaged and empty areas.

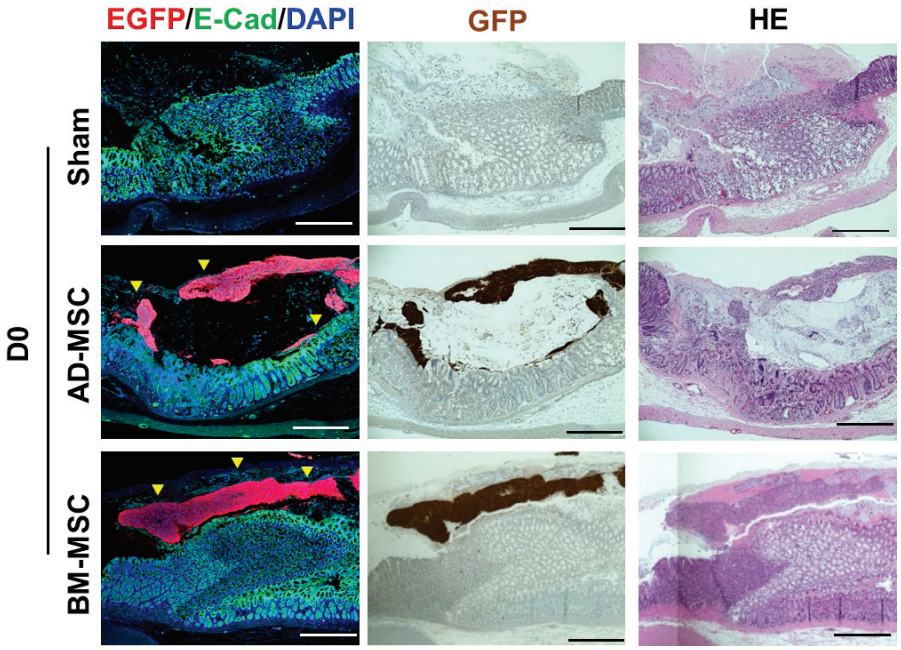

Fig. 21. Histological analysis of MSC sheet endoscopic transplantation in electrocoagulation ulcer model.

Histological analysis of colon tissue 0 day after MSC sheets. (middle; AD-MSC, lower; BM-MSC) attachment group. Con; only electrocoagulation, Sham; transplanted without MSC sheets, AD-MSC; AD-MSC sheets transplanted, BM-MSC; BM-MSC sheets transplanted. The red fluorescent signal is an EGFP signal in immunofluorescence, Dark brown stain was EGFP signal in DAB stain. GFP/E-Cad/DAPI; immunofluorescence stain, Brown GFP; immunohistochemistry, HE; Hematoxylin and eosin stain. Bar represents 200 μm.

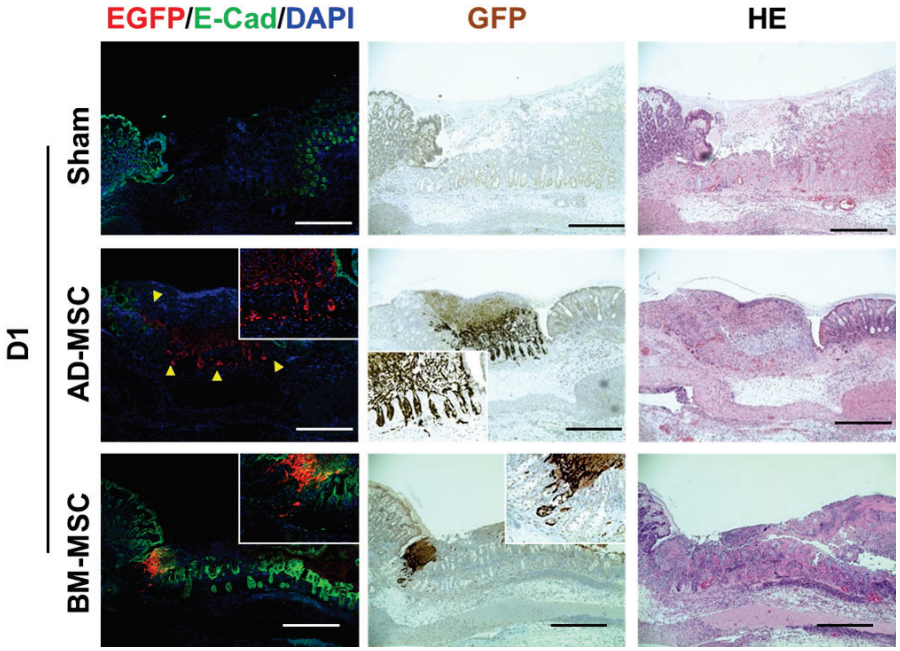

Fig. 22. Histological analysis of MSC sheet endoscopic transplantation in electrocoagulation ulcer model.

Histological analysis of colon tissue 1 day after MSC sheets. (middle; AD-MSC, lower; BM-MSC) attachment group. Con; only electrocoagulation, Sham; transplanted without MSC sheets, AD-MSC; AD-MSC sheets transplanted, BM-MSC; BM-MSC sheets transplanted. The red fluorescent signal is an EGFP signal in immunofluorescence, Dark brown stain was EGFP signal in DAB stain. GFP/E-Cad/DAPI; immunofluorescence stain, Brown GFP; immunohistochemistry, HE; Hematoxylin and eosin stain. Bar represents 200 μm.

5. Analysis of DNBS colitis model after endoscopic transplantation

As carried out for the electrocoagulation ulcer model, the transplanted site was traced with an endoscope on the day of (D0) **(Fig. 23A upper panel)** and 3 days (D3) after transplantation **(Fig. 23A, lower panel)**. The transplantation of AD-MSC and BM-MSC cell sheets, compared to sham, showed a decrease in colitis as seen by endoscopic observation. The sham, AD-MSC, and BM-MSC sheet groups showed reductions in ulcer size of 0.625 mm, 1.15 mm, and 0.5 mm, respectively. Although the AD-MSC group showed more reduction in ulcer size than the other groups, it was not statistically significant **(Fig. 23B)**. According to the MEICS analysis, at 3 days after transplantation, the AD-MSC sheet transplantation group showed a decrease in its average score by 3.66, which was significantly different from that of the sham group. The BM-MSC sheets transplantation group showed a decrease of 4 in the average score on D3 compared to D0, but did not show any statistically significant difference compared with the sham group that showed an average score decrease of 2.25 on D3 **(Fig. 23C)**.

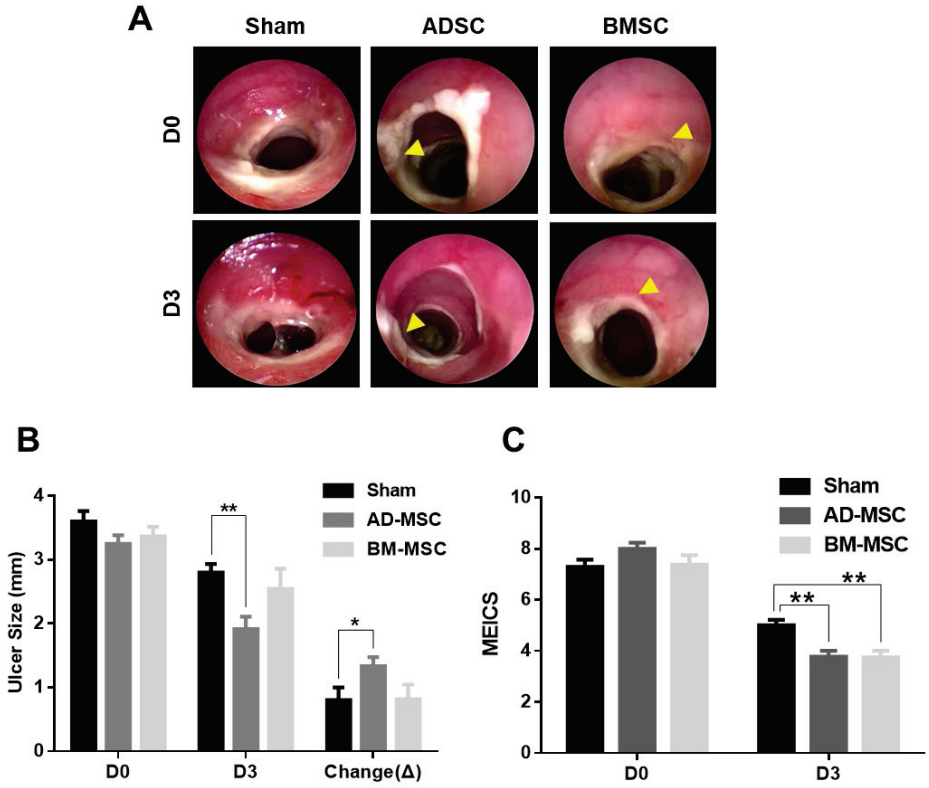

Fig. 23. Evaluation of MSC sheet endoscopic transplantation in DNBS colitis model.

(A) Endoscopic images of MSC sheets transplantation in DNBS colitis model. The yellow arrow points to the transplantation site. (B) The changes in ulcer size before and after D0 between D3 (±SEM n = 12, each). **P < 0.01, *P < 0.05. (C) Endoscopic score changes of D0 and D3, as detailed in Table 3.. D0; transplantation day, D3; 3 days after transplantation (±SEM n = 12, each). **P < 0.01. Sham: transplantation of Cellshifter^{TM} without MSC sheets, AD-MSC; AD-MSC sheets transplanted, BM-MSC; BM-MSC sheets transplanted.

DNBS colitis was induced 4 days prior to MSC sheets transplantation, and body weight changes **(Fig. 24A),** as well as disease activity index (DAI) **(Fig. 24B)** were analyzed following the MSC sheet transplantation. There was no significant difference in body weight and disease activity index among the experimental groups.

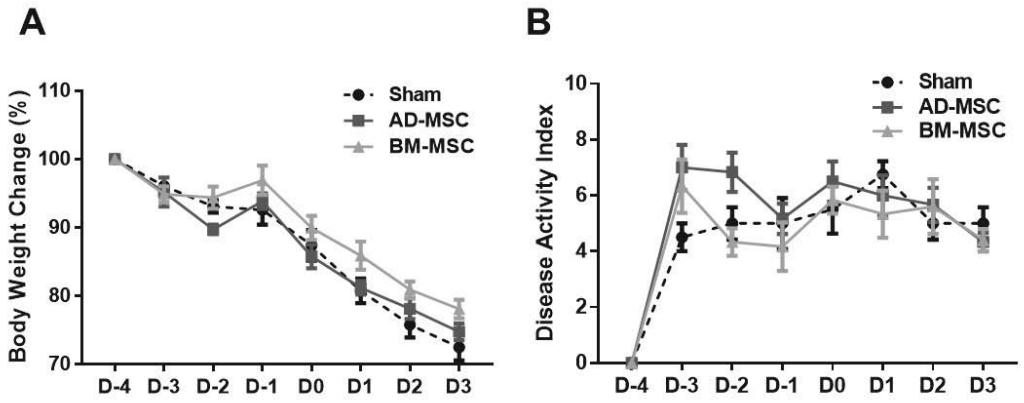

Fig. 24. Evaluation of MSC sheet endoscopic transplantation in DNBS colitis model.

(A) The time-course body weight change on transplanted and non-transplanted MSC sheets. (B) The evaluation of animal model clinical severity assessed by DAI scores. DAI calculated by the combined score of weight loss, stool consistency, and bleeding, as detailed in Table 2. D0; transplantation day, D3; 3 days after transplantation (±SEM n = 12, each). **P < 0.01. Sham: transplantation of Cellshifter^{TM} without MSC sheets, AD-MSC; AD-MSC sheets transplanted, BM-MSC; BM-MSC sheets transplanted.

Histological analysis of the colon with an endoscope transplanted to the DNBS colitis lesion showed that the sheets had fluorescence in the colon colonized with the MSC sheets **(Fig. 25)**. Hematoxylin and eosin stain, immunohistochemical stain, and immunofluorescence staining with anti-EGFP antibody at 1 day after transplantation of MSC sheets confirmed that the AD-MSC and BM-MSC sheets were attached to the inflammatory site **(Fig. 26)**. Analysis of the tissues transplanted with AD-MSC sheets revealed that the EGFP cells were intact. In the BM-MSC group, EGFP positive signal was detectable, but predominantly the signals from immune cells were observed at the transplanted site. However, in some cases, a crypt pattern of fluorescence signals, which were similar to those found in the electrocoagulation ulcer model, were obtained at the colitis site. In the histological analysis of the colon 3 days after MSC sheet transplantation, EGFP positive signals were detected in the AD-MSC group, but the fluorescence intensity was lower than that of the day 1 tissue after transplantation. The BM-MSC group remained difficult to distinguish from preexisting tissues **(Fig. 27)**.

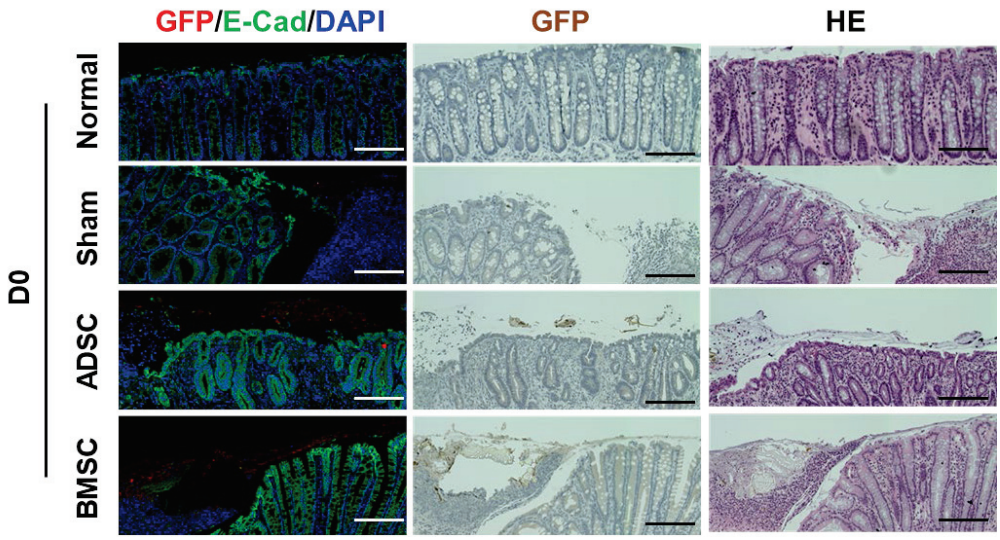

Fig. 25. Histological analysis of MSC sheet endoscopic transplantation in DNBS colitis model.

Histological analysis of colon tissue the day (D0) after MSC sheets. Normal; healthy condition, Sham; transplanted CellshifterTM without MSC sheets, AD-MSC; AD-MSC sheet transplanted, BM-MSC; BM-MSC sheet transplanted. The red fluorescent signal is an EGFP signal in immunofluorescence, Dark brown stain was EGFP signal in DAB stain; EGFP/E-Cad/DAPI; immunofluorescence stain, Brown GFP; immunohistochemistry, HE; Hematoxylin and eosin stain. Bar represents 200 μm.

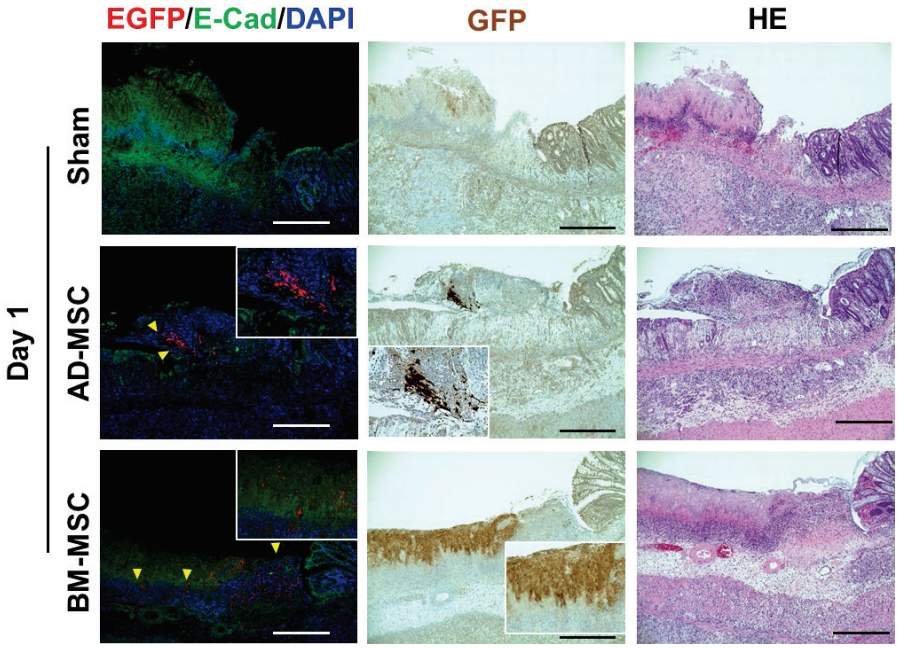

Fig. 26. Histological analysis of MSC sheet endoscopic transplantation in DNBS colitis model.

Histological analysis of colon tissue 1 day after MSC sheets. (middle; AD-MSC, lower; BM-MSC) attachment group. Normal; healthy condition, Sham; transplanted Cellshifter^{TM} without MSC sheets, AD-MSC; AD-MSC sheets transplanted, BM-MSC; BM-MSC sheets transplanted. The red fluorescent signal is an EGFP signal in immunofluorescence, Dark brown stain was EGFP signal in DAB stain. GFP/E-Cad/DAPI; immunofluorescence stain, Brown GFP; immunohistochemistry, HE; Hematoxylin and eosin stain. Bar represents 200 μm

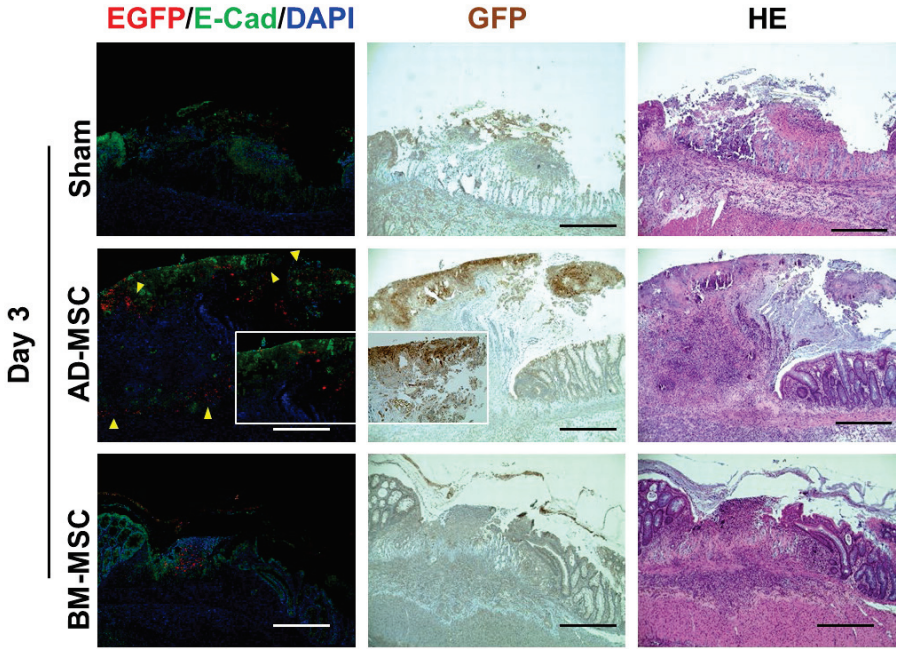

Fig. 27. Histological analysis of MSC sheet endoscopic transplantation in DNBS colitis model.

Histological analysis of colon tissue 3 day after MSC sheets. (middle; AD-MSC, lower; BM-MSC) attachment group. Normal; healthy condition, Sham; transplanted Cellshifter^{TM} without MSC sheets, AD-MSC; AD-MSC sheets transplanted, BM-MSC; BM-MSC sheets transplanted. The red fluorescent signal is an EGFP signal in immunofluorescence, Dark brown stain was EGFP signal in DAB stain. GFP/E-Cad/DAPI; immunofluorescence stain, Brown GFP; immunohistochemistry, HE; Hematoxylin and eosin stain. Bar represents 200 μm

Discussion

To propose a new therapeutic approach in the field of stem cell therapy for IBD, we endoscopically transplanted MSC sheets in rat model colitis and demonstrated the utility of endoscopic transplantation of the MSC sheets. After isolating rat AD-MSCs and BM-MSCs, we successfully produced AD-MSC and BM-MSC sheets using a temperature-responsive dish. The electrocoagulation model for colonic ulcer was generated with an electrode, and the transplanted site was analyzed to confirm whether the MSC sheets were able to help recovery of coagulated ulcers. In the DNBS colitis model, we confirmed the anti-inflammatory effect of MSC sheets transplanted endoscopically onto inflammatory ulcer sites. Our results show that the endoscopic transplantation of MSC sheets onto the inflamed lesions may potentially act as a novel approach for treating IBD.

Until now, cell therapy in IBD treatment has been mainly experimented with hematopoietic stem cell transplantation and MSC transplantation for perianal fistula of Crohn's disease.[37,38] Hematopoietic stem cell transplantation has been studied for a long period, but it can only be applied to a small subset of patients because myeloablation needs to precede the transplantation.[37] In a study of the efficacy and safety of autologous BM transplantation in patients with refractory Crohn's disease, autologous BM transplantation achieved a medication-free response in 80% of patients at 6 months. Since then, more than 50% of patients have experienced recurrence, but 80% have achieved remission with medication. In general,

side effects related to infection are frequent, so caution is needed. In contrast, MSC transplantation has been recognized as a promising therapeutic agent for regenerative medicine in IBD[6].

MSC transplantation is an ideal therapeutic approach, because MSCs are easier to extract than other stem cells and less are immunogenic,[39]; thus, they can be used for treatment after extraction from the patient. In addition, MSCs are known to not only regulate the immune response, but also promote tissue repair, suggesting further benefits in treating IBD. The remaining challenges are the scientific assessment of MSC treatment for conventional IBD treatments in randomized controlled trials. The likelihood of using allogenic MSCs and the immunomodulatory properties suggests promising and safer prospects for testing IBD therapies. Remarkable obstacles remain with regard to the design and interpretation of studies on MSC, including patient selection, disease stages, disease activity, and long-term safety.

Recently, the injection of colon-specific differentiated organoids was reported.[13,14] Since the organoid originates from the stem cells of the intestinal mucosa and forms, the basic unit of the intestinal membrane, it can sufficiently amplify the organoid in vitro from a single cell of normal mucosa. In addition, it can be used as a useful method for future regenerative medicine in intractable intestinal diseases by transplanting amplified organoids into injured mucosa, such as intractable ulcers in the intestinal mucosa, to promote the recovery of intestinal mucosa. However, this method utilizes solidification materials that are yet to be clinically approved. In addition, allogenic transplantation of organoids is likely to induce

immunogenicity, which may prevent stable delivery and sufficient therapeutic efficacy.

In this study, we made continuous efforts to overcome the various obstacles that were presented while trying to optimize the endoscopic transplantation procedure. We first decided to use rats as the model for analysis in this pre-clinical study. However, the experiment with rat model was not easy because of several points. Cell sheet fabrication using rat MSCs was more complicated than using human or mouse-derived MSCs. Direct cell transplantation into the colon was not well tolerated in experimental murine models due to the presence of mucus and stools. In addition, it was not easy to deliver fabricated cell sheets into the colon. Therefore, we experimented with various disease models; the electrocoagulation model was employed to reveal the therapeutic possibility of MSC transplantation through endoscopic transplantation. We focused on the ulcer size and appropriately evaluated the attached cell sheet. The DNBS colitis model, one of the representative experimental colitis models, was employed to perform endoscopic transplantation of MSC sheets in animal models that mimic IBD. This model has been used to suggest that this study is clinically applicable, effective method in the field of cell therapy of IBD.

One of the most difficult aspects of this study was the process of finding a specific lesion in an animal model and transplanting with the cell sheet stored in the endoscope. A sufficient fasting time (~12 hours) was provided before transplantation for clean intestine conditions. An enema was performed to remove the stools and mucosa from the ulcer as much as possible. Procedures for transplanting cell sheets were performed after pre-endoscopy. The pre-

endoscopy was able to designate lesions suitable for transplantation and establish the depth and clockwise direction of the lesion from the anus. The cell sheets were transplanted according to the determined depth and clockwise direction and the information was used for the follow-up after transplantation. Histopathological analysis of the post-transplantation was performed by marking the location of the ulcer by physically positioning the needle on it.

Electrocoagulation ulcer model helped wound healing and confirmed ulcer size reduction. In the DNBS colitis model used in this study, which differs in that broad and overall colitis occurs in the middle-lower distal part of the colon, ulcer size and MEICS of the lesions were changed; however, there was no significant difference in body weight and DAI expressing overall inflammation.

In the transplantation experiment, two type of cell sheets were transplanted. Based on the results of the currently obtained DNBS colitis model, the further study is to cover as many colitis lesions as possible via multiple grafting at one- or two-day intervals. These transplantations do not restore the overall inflammation through the cell sheet, but can be effectively applied for local delivery to localized severe pathologies. It may be a useful transplantation experiment for fistula model in further studies.

The limitations of this study include the lack of long-term observation following MSC sheet transplantation. It is also necessary to perform molecular biological analysis and immune response analysis at the transplanted area. Both the electrocoagulation model and the DNBS colitis model rats recovered within 4 to 5 days of the experiment, making it difficult to observe

and compare the long-term effects of the disease and therapeutic strategies.

Despite these limitations, we have clearly shown that MSC sheets reduce the ulcer induced from physical mucosal damage (electrocoagulation model), and mitigates the severity of inflammation due to colitis condition (DNBS model); therefore, the therapeutic effect on IBD through modulation of immune response may be expected. In addition, no beneficial effects were observed in body weight and disease activity index in the DNBS colitis model, because the endoscopic transplantation of MSC sheets was not enough to cover the entire colon ulcer in the colitis model. Therefore, we suggest that our method could be applied as an adjunctive method to the existing IBD treatment of ulcers associated with colitis.[40,41)] From the results presented, it is worthwhile to note that there was significant reduction in ulcer size and colitis score under conditions of AD-MSC and BM-MSC sheet transplantation, in both the electrocoagulation and DNBS model.

Conclusion

The benefits of our endoscopic transplantation of MSC sheets seem to be largely due the direct application of cells onto the lesions. The novel method described herein has no need for scaffolds or solidification of cells to enhance cell attachment, and the MSCs themselves preserve the characteristics of extra cellular matrix (ECM). Our results suggest that the transplantation of MSC sheet is a highly practical technology for clinical application, especially for enteric diseases, because cell sheets can be directly applied to colonic lesions via endoscope and can exert immediate protective effects. In addition, endoscopic transplantation is safer than conventional single cell injection techniques and has high potential for stable transplantation into the lesion sites. The MSC sheets can be directly transplanted with endoscopes, making them suitable for treating the various symptoms of colon disease and restoring tissue integrity at the surgical site.

In conclusion, the results of the present study suggested that endoscopic transplantation of MSC sheets could be a new strategy in stem cell therapy for the effective treatment of IBD. This new method will provide new possibilities in the field of regenerative medicine for IBD treatment.

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국문초록

배경 및 목적: 최근 염증성 장 질환 (IBD)의 치료를 위해 줄기세포를 이용하여 재생의학 기술이 발전하고 있다. 중간엽줄기세포 (MSC)의 재생 및 면역조절 잠재력을 활용하기 위한 임상 시험들이 진행되고 있으며 몇 가지 유망한 결과를 보여주고 있다. 이에 본 연구에서는 특정된 병변에 줄기세포의 효과적이고 밀집된 전달을 위해서 대장염 동물 모델에서 랫드 중간엽줄기세포 시트 (MSC sheet)의 내시경적 이식법의 타당성 및 유용성을 조사했다.

방법: EGFP (enhanced green fluorescent protein) 형질전환 랫드로부터 지방 유래 MSC (AD-MSC)와 골수 유래 MSC (BM-MSC)를 분리하고 온도반응성 배양 접시를 사용하여 시트 형태로 수립하였다. 수립된 MSC 시트는 전기응집궤양 모델 (electrocoagulation ulcer)과 Dinitrobenzene sulfonic acid (DNBS) 대장염 모델에서 염증 부위에 직접적으로 내시경을 이용하여 이식되었다. MSC 시트의 내시경 이식의 효과는 내시경 스코어링 및 조직학 분석을 사용하여 확인하였다. 세포 시트의 제작은 육안 확인 및 조직학적 분석에 의해 확인되었으며, 시트를 구성하는 MSC 의 검증은 세포 표면 마커 및 분화능 테스트로 평가되었다.

결과: Electrocoagulation 모델에서 AD-MSC 시트 이식 그룹은 이식 부위의 궤양 크기가 감소함을 보여주었다. 궤양 크기는 대조군에서 29.17%, 모의시술군에서 13.10% 증가했지만 BM-MSC 및 AD-MSC 시트 군에서 17.93% 및 35.10% 감소했다. DNBS 대장염 모델에서 AD-MSC 시트 그룹은 이식 부위에서 염증과 대장염이 감소함을 보여주었다. 궤양의 크기는 각각 모의시술, AD-MSC 및 BM-MSC 군에서 0.625, 1.15 및 0.5 mm 감소했

다. AD-MSC 시트 이식 군은 이식 3 일 후 설치류 대장염 중증도 내시경 수치 (MEICS)를 분석한 결과 평균 3.66 의 호전이 있었고 모의시술군과 통계학적으로 유의한 차이가 있었다. 조직학적 분석 결과, 세포 시트가 electrocoagulation 과 DNBS colitis model 에서 염증이 있는 점막에 성공적으로 부착되었음을 알 수 있었다.

결론: MSC 시트의 내시경적 이식은 IBD 치료를 위한 줄기세포치료의 새롭고 효과적인 방법이 될 수 있다는 가능성을 제안한다.

중심단어: 염증성 장 질환, 중간엽줄기세포, 온도 감응형 세포배양접시, 세포 시트, 2,4-dinitrofluorobenzene sulfonic acid-induced Colitis, 전기응집 궤양

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지난 만 4 년동안의 박사학위 과정동안 지도해주신 명승재 교수님께 먼저 감사의 말씀을 올립니다. 이 풍납동 캠퍼스에 발을 들인지 햇수로 6 년이라는 시간동안 첫 교수님과의 면담과 다시 진학을 하기로 하였던 그 순간, 연구의 큰 난관에 봉착하였을 때, 다시 좋은 결과로 이어지고 마무리가 되어가던 그 매순간에 명 교수님의 끊임없는 지원과 세심한 배려가 없었다면, 본 논문은 빛을 보지 못했을 것입니다. 또한, 세부적인 연구내용을 진행하는 과정에서 황성욱 교수님의 번뜩이는 아이디어와 꼼꼼하고 냉철한 분석에 따른 가르침이 없었다면, 본 연구의 진행은 현재까지도 절반 이상 나아가지 못했을 것입니다. 황 교수님, 그동안 주셨던 가르침 잊지 않겠습니다. 그리고 소속과 전공이 다름에도 불구하고 누구보다도 연구과정에서 어머니와 같은 마음으로 엄격하고 따뜻한 지도를 아끼지 않았던 심인경 교수님께 깊은 존경과 감사의 말씀을 올립니다. 이 논문의 시작점이자 끝인 줄기세포들에 대한 내용들과 세포 시트를 제작을 완성까지 하는 지난한 과정들은 함께 부딪혀가며 나아가던 기억들은 잊혀 지기 쉽지 않을 것 같습니다. 돌이켜보면 이 연구의 시작점이 되었고, 형태를 갖추어 나아가는데 있어 물심양면으로 지원해주신 김상엽 교수님께도 감사의 말씀을 올립니다. 그간 교수님들의 지도와 조언들이 있었기에 비로소 다른 연구자들에게 연구결과를 보이고 학위 논문으로도 완성이 되었습니다. 항상 부족하고 미련했던 저를 이끌어 주신 점 잊지 않고 배우는 자세와 겸손하고 지치지 않는 연구자로 나아가겠습니다.

여기 서울아산병원에 와서 연구원으로 그리고 박사 학위 과정 대학원생으로 있던 시간동안 Myung's Lab 식구들과 연구소 내 많은 선생님들의 도움이 있었습니다. 처음 이 연구실에 들어올 때 가족처럼 지냈던 도은주 박사님과 류연미 박사님, 배상문 박사님께 감사합니다. 박사님들이 계셔서 많은 실험들을 조율해주시고 연구실 생활에 도움을 주시어 원활하게 본 연구가 진행되고 마무리될 수 있었습니다. 항상 살뜰히 실험실의 살림과 궂은일을 묵묵히 맡아 오셨던 박선하 선생님 감사합니다. 그동안의 배려와 지원으로 걱정없이 연구에 매진할 수 있었습니다. 학위 과정 초반에 같이 시작하여 어느덧 석사를 졸업 후 각자의 분야에서 연구원으로 활약하는 태훈이와 진학이 에게도 고맙단 인사를 하고 싶습니다. 너희들 덕분에 대학원 생활이 마냥 힘들지만은 않았어. 그리고 이 실험에 집중하느라 많은 도움이 되지 못해 감사함 보다 먼저 미안한 마음이 드는 자영쌤, 동희쌤 앞으로도 좋은 연구자가 되는 길에 제가 미약하게나마 도움이 될 수 있기를 바랍니다. 그리고 독립적인 연구자를 목표로 나아갈 형일쌤, 어떠한 역경과 고난이 있더라도 반드시 그 끝에는 훌륭한 성과가 있을 거라 믿어 의심치 않습니다. 그리고 옆 실험실에서 같이 울고 웃으며 많은 실험들을 본인 일처럼 도와주었던 혜진쌤과 유나쌤, 서로 많은 도움을 주고받으며 같이 성장했던 소중한 나날들이었습니다.

끝으로, 본 연구 결과를 완성하면서 느낀 것이 있다면 그것이 비록 미약한 연구일지라도 반드시 혼자가 아닌 많은 사람들의 노력과 도움이 있어야 완성될 수 있다는 점일 것입니다. 연구는 끊임없이 소통하고 부딪혀가며 한 발씩 내딛어가며 진행되고 그것이 쌓여 한번에 도약할 수 있는 순간이 연구자로 살아가는데 가장 큰 보람이자 행복이 아닐까 생각합니다. 비록 중간에 크나큰 좌절이 있었지만 좋은 교수님과 선생님들이 있어서 저에겐 크나큰 영광이자 행운이었습니다. 앞으로도 늘 부족함을 알고 그것을 채워 나가는 연구자로 매진하겠습니다. 미처 언급하지 못했지만, 저를 걱정하고 격려해주셨던 모든 분들께도 진심으로 감사하다는 말씀을 전합니다. 부디 이 글을 읽는 모든 분들께 앞으로의 건승과 좋은 결과들이 함께 하길 기원합니다. 감사합니다.

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박 세 형 올림