Original Article

Autologous adipose-derived stem cell sheets enhance the strength of intestinal anastomosis

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ABSTRACT

Objective: Adipose-derived stem cells (ASCs) are capable of multiple differentiation pathways, imparting immunomodulatory effects, and secreting factors that are important for wound healing. These characteristics can be exploited to decrease the incidence of anastomotic leakage.
Methods: In order to delay local wound healing at the anastomotic site, we induced ischemia in a portion of porcine small intestine by ligating vessels. Then, we injected mitomycin C into the serosa of the small intestine above the ligated vessels. Anastomotic sites were created by 2 cm incisions made in the opposite mesenteric area. ASCs were isolated from the porcine subcutaneous fat tissues and expanded under culture conditions. ASCs were trypsinized and seeded on temperature-responsive dishes and cultured to form confluent sheets. Three ASC sheets were transplanted onto the serous membrane after suturing. The extent of anastomotic wound healing was evaluated by bursting pressure, hydroxyproline content, and mRNA expression of collagen-1 alpha1 and collagen-3 alpha1.
Results: We found that transplantation of ASC sheets increased anastomotic site bursting pressure. Additionally, transplantation of ASC sheets increased the hydroxyproline content of the anastomoses. Furthermore, transplantation of ASC sheets increased mRNA expression of collagen-1 alpha1 and collagen-3 alpha1.
Conclusions: Our findings showed that transplantation of autologous ASC sheets enhanced collagen synthesis and anastomotic strength. Further studies are necessary to identify substances that, in combination with ASC sheets, might enhance collagen synthesis and healing in sites of anastomosis.
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1. Introduction

Anastomotic leakage is a feared complication of colorectal surgery. Wound dehiscence of the anastomotic surgical site leads to not only postoperative morbidity and mortality but also cancer recurrence and poor patient prognosis [1,2]. Although anastomotic devices, surgical techniques, and preoperative care have improved, the rate of anastomotic leakage after colorectal surgery remains high at 3–14% [3,4].

Preoperative chemoradiotherapy for rectal cancer improves local disease control, but can also induce inadequate anastomotic healing and may sometimes lead to severe anastomotic leakage. Previous studies have reported that preoperative chemoradiotherapy influenced the risk of anastomotic leakage postoperatively [5–8].

Generally, intestinal wound healing after anastomosis is divided into three phases: the inflammation phase, proliferation phase, and remodeling phase [9,10]. The strength of the fused intestinal anastomotic site declines in the early postoperative period, and collagen metabolism is necessary in order to provide compensatory strength [10]. In the inflammation phase, collagenase and other matrix metalloproteinases break down the mature collagen at the anastomotic site, resulting in a reduction in the intestinal anastomosis breaking strength because of the decreased concentration of existing collagen [11,12]. In the proliferation phase, fibroblasts are

attracted into the anastomotic site to synthesize a collagen-rich granulation tissue [10,12]. The anastomotic strength depends on the presence of collagenous networks produced by fibroblasts that infiltrate the anastomotic site during the proliferation phase [13–15]. Preoperative chemoradiotherapy can disturb these normal wound healing processes in anastomosis sites, and may sometimes lead to severe anastomotic leakage. Thus, methods to enhance wound healing are needed to reduce the risk of anastomotic leakage in delayed wound healing conditions, such as preoperative chemoradiotherapy.

Regenerative medicine and the tissue engineering field have provided new therapeutic options for preventing anastomotic leakage. Adipose-derived stem cells (ASCs) are capable of multiple differentiation pathways, imparting immunomodulatory effects, and secreting factors that are important for wound healing [16,17]. Because of these characteristics, ASCs have recently become a prime candidate for the repair of damaged tissue and other regenerative medicine applications [18–20]. Several experimental studies have reported that local injections of homogenous ASC suspensions improve the healing of intestinal anastomotic sites [21,22].

In this study, we evaluated the ability of transplanted ASC sheets to improve anastomotic strength and wound healing at intestinal anastomotic sites under delayed wound healing conditions, representative of conditions such as preoperative chemoradiotherapy.

2. Materials and methods

2.1. Animal care

All animals were treated with experimental procedures approved by the Committee for Animal Research of Tokyo Women's Medical University (approval number 15–21 2015). Eleven miniature pigs (6 months of age, 17–20 kg, females, obtained from the Nippon Institute for Biological Science) were used in this study. Four pigs were used to develop delayed wound healing model. Another seven pigs were used to evaluate efficacy of transplanted ASC sheets at intestinal anastomotic sites under delayed wound healing conditions. The pigs were injected intramuscularly with 0.04 mg/kg atropine and 15 mg/kg ketamine as premedication. Then, they received 2.5 mg/kg propofol intravenously. An endotracheal tube was inserted and anesthesia was maintained using sevoflurane and nitrous oxide inhalation with mechanical ventilation.

2.2. Experimental design

The experimental design and procedure for evaluating the transplantation of autologous ASC sheets onto the serous membrane after anastomosis in the pig's small intestine are shown in Fig. 1. Two weeks prior the operation, ASCs were isolated from the subcutaneous fat of pigs and expanded under the culture conditions described later. ASCs were seeded on temperature-responsive dishes and cultured for five days. Eight anastomotic sites and an anastomosis bypass were created under same conditions of the delayed wound healing model by the method described later. The eight sites were divided randomly into ASC sheet transplantation (anastomotic site wrapped with three ASC sheets) and untreated groups (no additional treatment). One pig was euthanized on postoperative day 1 for evaluating mRNA expressions of FGF2, TGF-β1, collagen-1 alpha1, and collagen-3 alpha1 in tissue obtained from the anastomotic site surroundings, and two pigs were euthanized on postoperative day 5. The final four pigs were euthanized on postoperative day 7 for evaluating the strength of and collagen formation in the anastomotic sites.

2.3. Isolation of the ASCs

The lower abdominal subcutaneous adipose tissue was surgically excised from the miniature pigs. The ASCs were isolated from the lower abdominal subcutaneous adipose tissue (20 g). After mincing the tissue into small pieces, the adipose tissue was enzymatically digested with 0.1% Collagenase (Collagenase NB 4G Proved Grade, SERVA Electrophoresis, Heidelberg, Germany) at 37 °C for 1 h. Then, the tissue was placed in a shaker for high-speed shaking at 150 rpm. After centrifugation, the floating adipose tissue was discarded. After filtration and centrifugation at 500 g for 5 min, cells were collected in a pellet. The cells in the pellet were resuspended and cultured in a complete culture medium (Dulbecco's modified Eagle's medium; DMEM, Invitrogen, CA, USA) and supplemented with 10% fetal bovine serum (Japan Bio Serum, Hiroshima, Japan) and with 1% penicillin/streptomycin (Gribco, CA, USA) at 37 °C at 5% CO_2. ASCs were passaged every 3 days at a density of 1.7×10^3 cells/cm^3 until the third passage was achieved.

2.4. Characterization of the ASCs

2.4.1. Flow Cytometry Assay

The isolated ASCs' surface antigen expression was analyzed by flow cytometry according to a previously reported method [23]. One million passage 3 ASCs were suspended in 100 μL PBS containing 10 μg/ml fluorescein isothiocyanate-conjugated primary antibodies specific to mesenchymal stromal cells (MSCs) (CD29, CD44, CD90, and CD105) and hematopoietic cells (CD31 and CD45) (n = 3). The following expression markers reactive with the porcine antigen isoforms were used: Alexa Fluor 647 Mouse Anti-Pig CD29 (BD Bioscience, New Jersey, USA), Anti-CD44 antibody [IM7] (Abcam plc, Cambridge, UK), APC Mouse Anti-Human CD90 (BD-Biosciences), Anti-CD105 antibody [MEM-229] (Abcam plc), PE Mouse Anti-Rat CD31 (BD Biosciences), and Monoclonal Antibody to CD45/LCA (CD45R)-PE (Acris Antibodies, Inc. CA, USA) [24–28]. Cell fluorescence was evaluated with a Gallios flow cytometer (Beckman Coulter, Tokyo, Japan) and the data were analyzed using Karuza for Gallios software (Beckman Coulter).

2.4.2. Differentiation and proliferation of ASCs in vitro

The capacities of passage 3 ASCs to differentiate into adipogenic lineages and osteogenic lineages were evaluated using a previously reported method [23]. For adipogenesis, the medium was switched to an adipogenic medium consisting of a complete medium supplemented with 0.5 μmol/L isobutyl-1-methyl xanthine (Sigma–Aldrich, St. Louis, USA), 0.5 μmol/L dexamethasone (Fuji Pharma, Tokyo, Japan), and 50 μmol/L indomethacin (Wako Pure Chemical Industries, Osaka, Japan). After 14 days, the cells were fixed with 4% PFA and stained with fresh Oil Red O solution (Wako Pure Chemical Industries). For osteogenesis, the medium was switched to a calcification medium consisting of a complete medium supplemented with 50 μmol/L ascorbic acid (Wako Pure Chemical Industries), 10 mmol/L β-glycerophosphate (Sigma–Aldrich), and 100 nmol/L dexamethasone. The cells were incubated for 21 days, and then stained with 1% alizarin red S solution. The proliferation capacities of passage 3 ASCs were evaluated according to the previously reported colony-forming unit assay method [23]. Briefly, 100 cells were cultured in 60-cm^2 dishes for 9 days and stained with crystal violet. Then, proliferation capacity was measured.

2.5. Preparation of ASC sheets

Passage 3 ASCs were seeded on 35-mm-diameter temperature-responsive culture dishes (UpCell; CellSeed, Tokyo, Japan) at a density of 2.4×10^6 cells/dish and cultured in complete culture
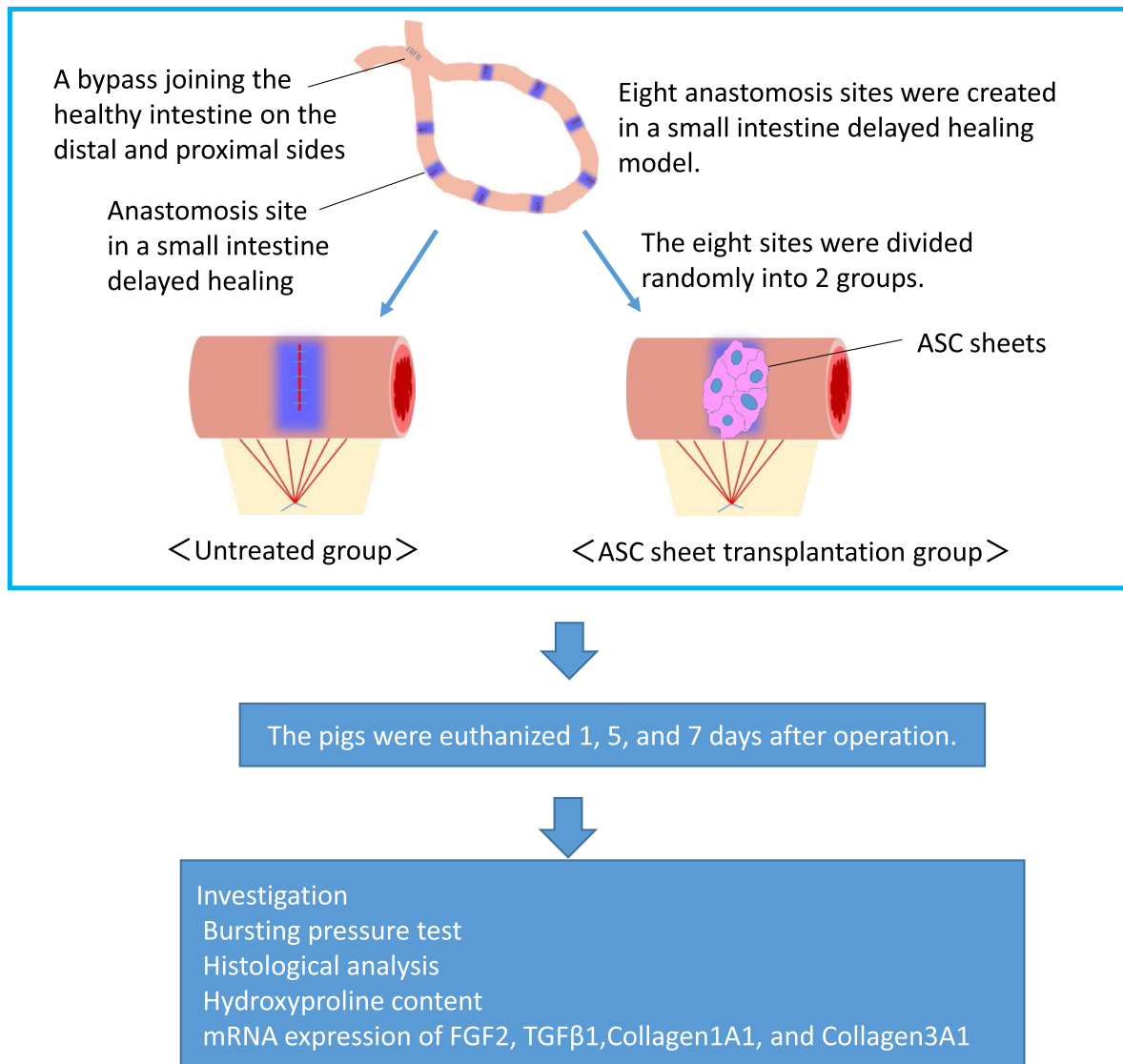
Fig. 1. Experimental design schema outlining the evaluation of autologous ASC sheet transplantation impact on porcine small intestine anastomosis. Two weeks prior the operation, ASCs were isolated from the subcutaneous fat of pigs and expanded under the culture conditions. ASCs were seeded on temperature-responsive dishes and cultured for five days. Eight anastomotic sites were created through blood vessel ligation. Injection of mitomycin C (MMC) induced delayed healing in the small intestine of each pig. The eight sites were divided randomly into adipose-derived stem cell (ASC) sheet transplantation (anastomotic site wrapped with three ASC sheets) and untreated groups (no additional treatment). One pig was euthanized on postoperative day 1 for evaluating mRNA expressions of FGF2, TGF-β1, collagen-1 alpha1, and collagen-3 alpha3 in tissue obtained from the anastomotic site surroundings, and two pigs were euthanized on postoperative day 5. The final four pigs were euthanized on postoperative day 7 for evaluating the strength of and collagen formation in the anastomotic sites.

medium at 37 °C at 5% CO_2 for 2 days. The medium was then changed to complete medium with 16.4 μg/ml ascorbic acid (Wako Pure Chemical Industries) and replaced every 24 h for an additional 2 days. After reaching confluency, the ASC sheets were harvested from the dishes by reducing the temperature to 20 °C for 20 min.

2.6. Creation of delayed wound healing model

Four pigs were used to develop delayed wound healing model. Two pigs were used as delayed wound healing model, another two pigs were used as normal wound healing model. To establish the delayed wound healing model, we induced ischemia by ligation vessels and injected a mitomycin C (MMC) solution into serosa of the small intestine. Surgical procedures were performed through a 9-cm upper abdominal transverse incision under general anesthesia. After the laparotomy, ischemia was induced in eight

portions of porcine small intestine from each pig by ligating six vessels with a 5-0 Vicryl (Ethicon, Tokyo, Japan) at each site. Ligation sites started 20 cm from the terminal ileum and were spaced 15 cm apart toward the proximal side of the small intestine. A MMC solution (composed of 2 ml of 100 μg/ml MMC [Nacalai tesque, Kyoto, Japan], 18 ml saline solution, 2 ml hyaluronate solution (Mucoup®) [Ethicon], and 0.1 ml Indigocarmin [Daiichi Sankyo, Tokyo, Japan]) was injected into the serosa of the small intestine above the ligated vessels in order to inhibit the growth of fibroblast and delay local wound healing at the anastomotic sites (Fig. 2A). A 2-cm incision was made in the opposite mesenteric area, which was closed using layer-to-layer anastomoses with five 5–0 Vicryl sutures (Ethicon, Tokyo, Japan) every 5 mm (Fig. 2B). After the eight anastomosis areas were created, an anastomosis bypass connected the remaining regions of healthy small intestine on either side of the eight anastomotic sites in order to prevent passage of food through the anastomotic sites (Fig. 2A).
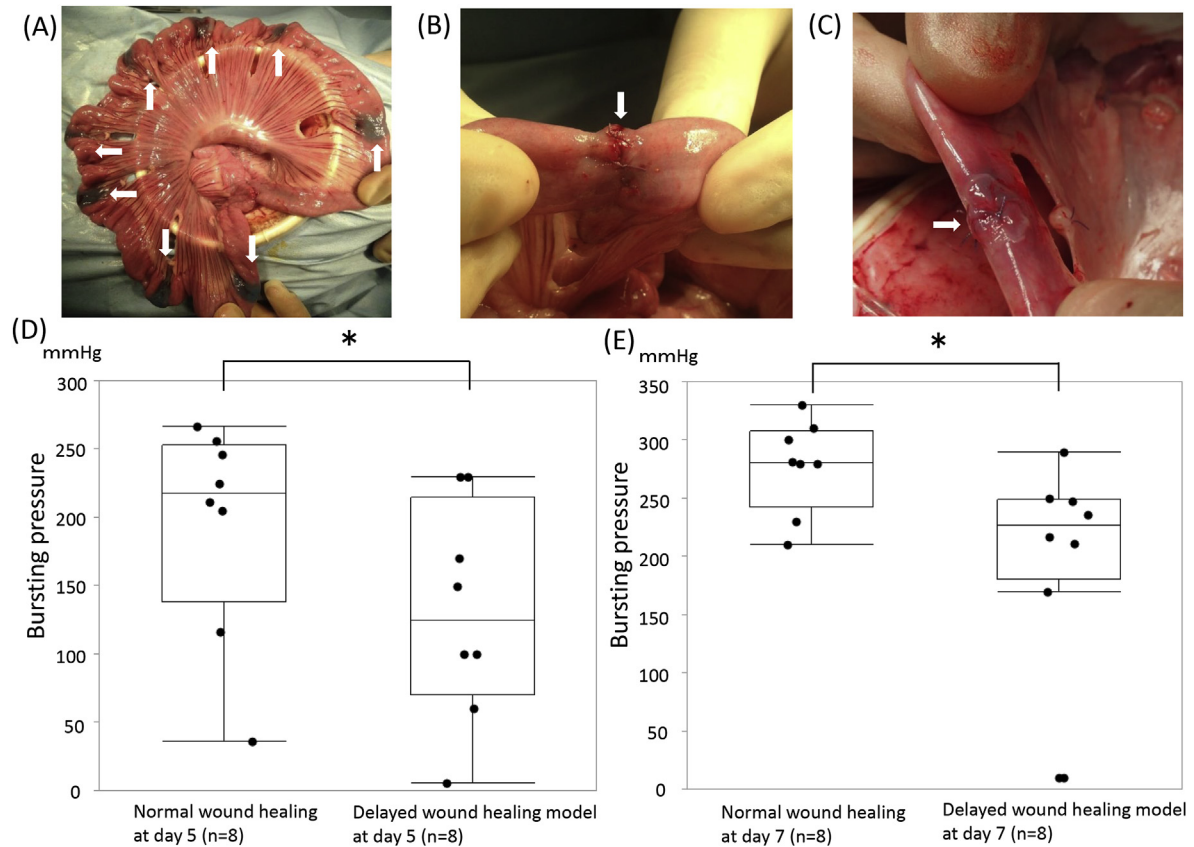
Fig. 2. Surgical procedure for creating the anastomosis delayed wound-healing porcine model and ASC sheet transplantation after anastomosis onto the serous membrane of the small intestine after suturing. A: Ischemia was induced in a portion of porcine small intestine by ligating six blood vessels. MMC solution was injected into the serosa of the small intestine above the ligated vessels. A total of eight anastomotic sites were made in each pig, starting 20 cm from the terminal ileum moving toward the proximal side of the intestine (arrows). An anastomotic site was made every 15 cm. A bypass joining the healthy intestine on the distal and proximal sides of the first and final anastomotic sites, respectively, was created by Braun's anastomosis. B: Thirty minutes after MMC solution injection, a 2-cm incision was made in the opposite mesenteric area and sutured with five stiches using layer-to-layer anastomosis (arrow). After suturing, each anastomotic site was divided randomly into the ACS sheet transplantation or the untreated group (no additional treatment). C: Transplantation of the ASC sheet after anastomosis onto the serous membrane after suturing (arrow). Three ASC sheets were transplanted on each of the four incision sites randomly assigned to ASC sheet treatment. D: The first burst pressures of the normal wound healing model and the delayed wound healing model on postoperative day 5 were 218 ± 79.5 mmHg (n = 8) and 125 ± 79.3 mmHg (n = 8) (P < 0.05), respectively. (P < 0.05). *P < 0.05. E: The first burst pressures of the normal wound healing model and the delayed wound healing model on postoperative day 7 were 280.5 ± 85.6 mmHg (n = 8) and 226.5 ± 85.6 mmHg (n = 8) (P < 0.05), respectively. (P < 0.05). *P < 0.05.

To establish the normal wound healing model, eight anastomotic sites and an anastomosis bypass were created under same conditions of the delayed wound healing models without blood vessel ligation and MMC injections in the small intestine of the pigs.

Two pigs were euthanized on postoperative day 5, and another two pigs were euthanized on postoperative day 7. To examine the anastomotic strength, anastomotic bursting pressure was measured by the methods described later.

2.7. Transplantation of autologous ASC sheets

Seven pigs were used to evaluate efficacy of transplanted ASC sheets at intestinal anastomotic sites under delayed wound healing conditions by the method described above. After created the delayed wound healing model, the eight portions were divided randomly into two groups: the ASC sheet transplantation group and the untreated group (no additional treatment). Transplantation were performed by wrapping with three ASC sheets without overlapping every sheets on each of the four incision sites. The ASC sheets were adhered without the need for sutures, clips, and any support membrane. After the operation, the animals were allowed free access to water, but were kept in a fasting state for the first 2 days. Then, they were given free access to solid foods.

2.8. Anastomotic pressure test

Anastomotic bursting pressure was measured immediately after the pigs were euthanized with an overdose injection of potassium chloride. We dissected 4-cm segments of each anastomotic site. The anal side of the intestine was clamped 2 cm from the anastomotic site with intestinal forceps to prevent leakage. Then, the tube for the intravenous drip injection was inserted into the other side and tied in place with a 1—0 silk suture to prevent leakage. The tube was then connected to an electronic manometer (PG-100N; Copal Electronics, Tokyo, Japan). The specimen was placed in 0.9% saline solution and air was infused at a rate of 2 ml/s. The bursting pressure was measured at the moment of the first air leakage.

2.9. Histopathological examination

ASC sheets and small intestinal specimens were fixed with 4% paraformaldehyde (Muto Pure Chemical, Tokyo, Japan) and processed into 3-μm-thick paraffin-embedded sections. Hematoxylin and eosin staining and Sirius red staining were performed using conventional methods. To follow the presence of transplanted cells, ASC sheets were labeled with PKH26GL (Sigma—Aldrich) immediately before transplantation. One pig was transplanted with the

ASC sheets labeled PKH26GL and was euthanized after postoperative day 7.

2.10. Hydroxyproline content

Hydroxyproline in tissue hydrolysates are commonly be used as a direct measure of the amount of collagen present [29]. After the bursting pressure tests at the anastomotic sites, two 5-mm segments around the anastomotic sites were excised using a 5-mm punch biopsy, and each sample was weighed. Total hydroxyproline content was quantified using a hydroxyproline assay kit (QuickZyme Biosciences, Leiden, The Netherlands) according to the manufacturer's instructions and a modified version of the method described by Prockop and Udenfriend [29]. Results were represented as micrograms of hydroxyproline per 5 mm of tissue. One pig was used to obtain data for postoperative day 5 data and two pigs were used for postoperative day 7.

2.11. Gene expressions in ASC sheets and the anastomotic site

The mRNA expression of fibroblast growth factor 2 (FGF2) and transforming growth factor (TGF)-β1 in the ASC sheets was compared with that in preconfluent ASCs. Passage 3 ASCs harvested before colony formation were used as preconfluent cells. Furthermore, we evaluated the mRNA expressions of FGF2, TGF-β1, collagen-1 alpha1 (COL1A1), and collagen-3 alpha1 (COL3A1) in 5-mm segments obtained from the surroundings of the anastomotic sites from animals sacrificed on postoperative days 1 and 7. Total RNA extraction was carried out using the RNeasy Mini Kit (QIAGEN INC, Valencia, CA) and RNeasy® Fibrous Tissue Mini Kit (QIAGEN)

according to the manufacturer's instructions. Reverse transcription of total RNA was performed for cDNA synthesis using a reverse transcriptase (SuperScript VILO MasterMix, Life Technologies). Polymerase chain reaction mixes containing target gene primers, the cDNA of each sample, and PrimeScript™ RT Master Mix (Takara, Shiga, Japan) were prepared and real-time polymerase chain reaction was performed using StepOne® (Life Technologies). The following primers were used: Taq Man Gene Expression Assays FGF2, Taq Man Gene Expression Assays TGF-β1, Taq Man Gene Expression collagen type I alpha 1 (COL1A1), and Taq Man Gene Expression Assays collagen type III alpha 1 (COL3A1). Data were represented using the $2^{-\Delta\Delta CT}$ method by using the β-actin gene (*ACTB*: Taq Man Gene Expression Assays β-actin) as a control. All experiments were performed in duplicate.

2.12. Statistical analyses

All numerical data are presented as mean ± standard deviation (SD). Comparisons between the two groups were performed using the Student *t* test with JMP® 11 (SAS Institute Inc., Cary, NC, USA). Probability values (P) less than 0.05 were considered significant.
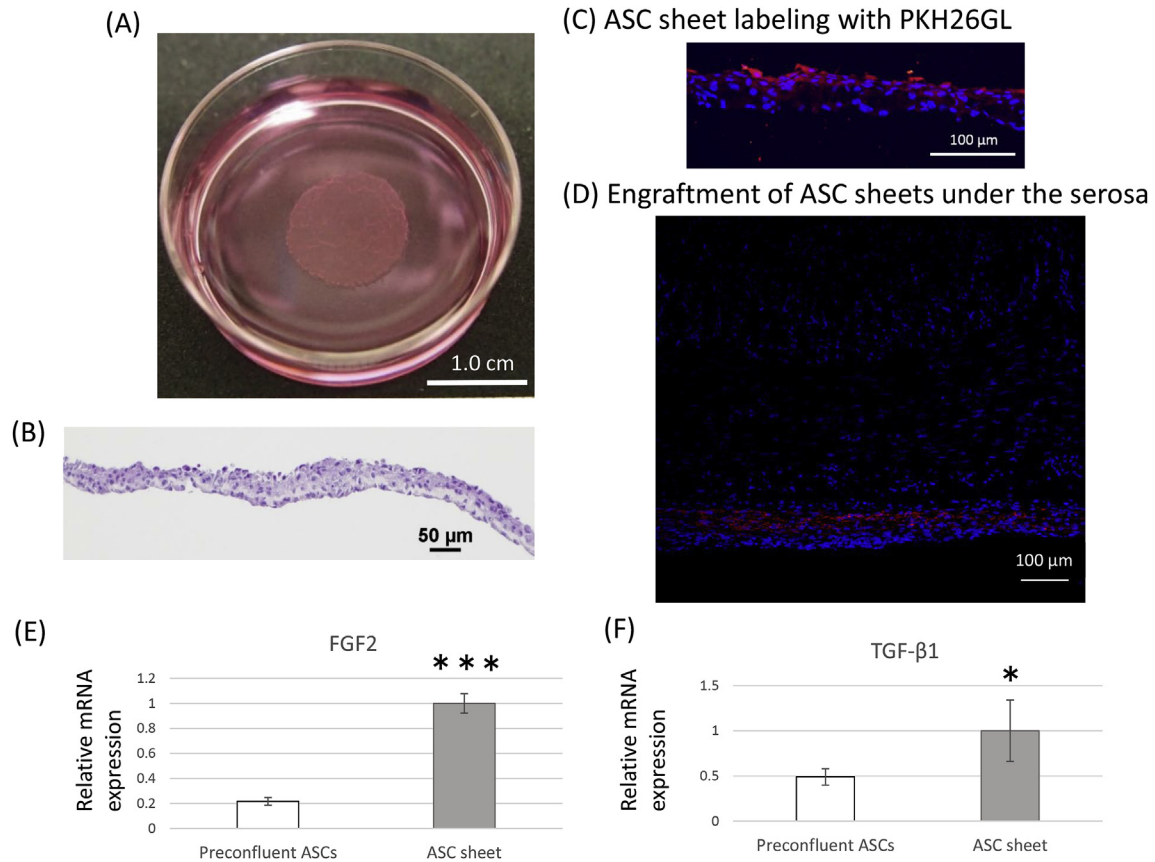
3. Results

3.1. Characterization of the ASCs

The expression profiles of known surface markers specific to MSCs in the primary cultured ASCs were as follows: 88.0 ± 6.8% CD29+, 86.7 ± 5.4% CD44+, 85.5 ± 3.5% CD90+, and 81.8 ± 3.1% CD105+ in Flow Cytometry Assay. The expression profiles of
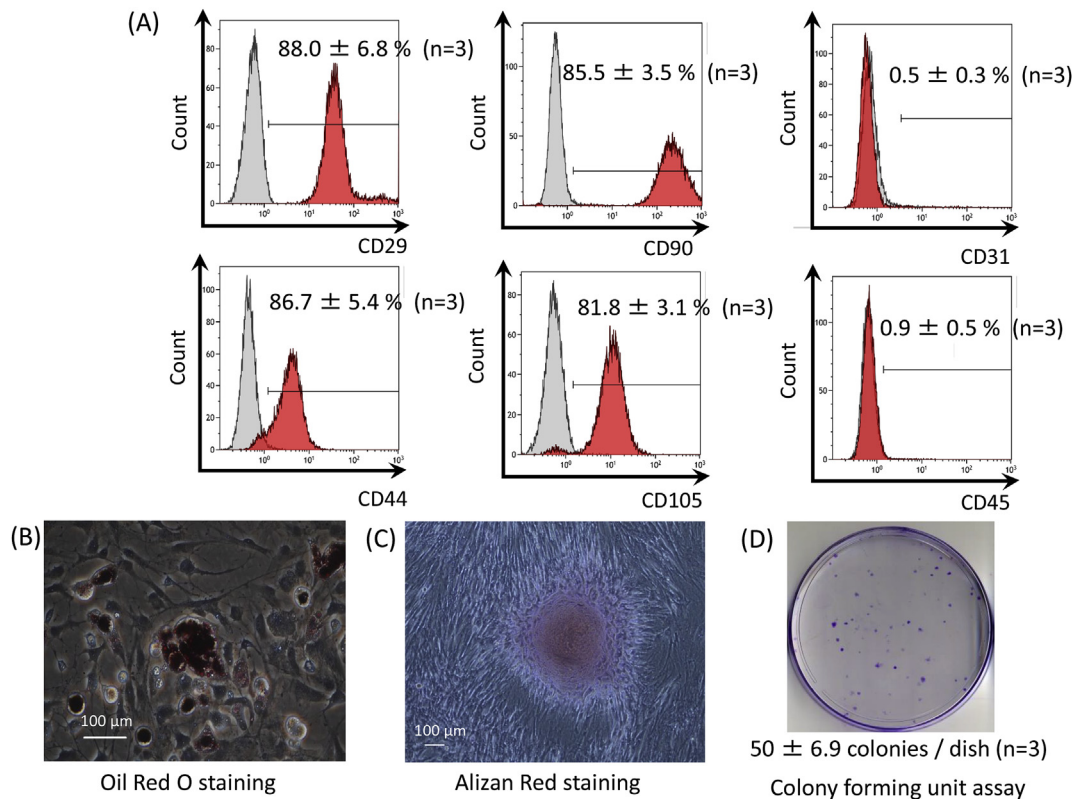
Fig. 3. Characteristics and biopotency of primarily cultured ASCs from miniature pigs. A: Flow cytometric analysis of ASC primary cultures showed the expression of known surface markers specific to mesenchymal stromal cells (MSCs) (CD29, CD44, CD 90, and CD105) and was negative for hematopoietic markers such as CD31 and CD45. B: After 2 weeks of culture in an adipogenic medium, ASCs stained positively for Oil Red O. The scale bar is 100 μm. C: After 4 weeks of culture in an osteogenic induction medium, ASCs stained positively for Alizan Red. The scale bar is 100 μm. D: One hundred cells cultured for 7 days in 60-cm² dishes were stained with crystal violet to assess the number of cell colonies and confirm the proliferation ability. The average number of colonies was 50 ± 6.9 (n = 3).

(A)

1.0 cm

(B)

50 μm

(C) ASC sheet labeling with PKH26GL

100 μm

(D) Engraftment of ASC sheets under the serosa

100 μm

(E)

(F)

Fig. 4. Preparation of autologous ASC sheets and their engraftment and analysis of gene expression of the ASC sheet. A: After temperature reduction to 20 °C for 20 min, ASCs were successfully harvested as cell sheets without enzymatic treatment. B: Hematoxylin and eosin stain showed that ASC sheets were composed of five overlaying layers of cell. C: To follow the presence of transplanted cells, ASC sheets were labeled with PKH26GL immediately before transplantation. D: Retention of PKH26GL-labeled ASC sheets under the serous membrane of the anastomotic site after 7 days. E: The mRNA expression levels of FGF2 in the ASC sheets were significantly higher than those in preconfluent ASCs (n = 4) (P < 0.05). F: The mRNA expression levels of TGF-β1 in the ASC sheets were significantly higher than those in preconfluent ASCs (n = 4) (P < 0.05). *P < 0.05, ***P < 0.001.

hematopoietic markers were as follows: $0.5 \pm 0.3\%$ CD31+ and $0.9 \pm 0.5\%$ CD45+ in Flow Cytometry Assay (Fig. 3A). These results indicate that the ASCs used in this study possessed MSC-like properties.

To evaluate the abilities of isolated ASCs to differentiate into adipogenic and osteogenic lineages, cells were stained with Oil Red O (Fig. 3B) and Alizan Red (Fig. 3C), respectively. Proliferation capacity was confirmed by colony-forming assay (Fig. 3D). The number of colonies in each sample was 50 ± 6.9 (n = 3). These results indicate that the ASCs used in this study possessed MSC-like properties.

The mRNA expression levels of FGF2 in the ASC sheets were significantly higher than those in the preconfluent ASCs (n = 4) (P < 0.001) (Fig. 4E). The mRNA expression levels of TGF-β1 in the ASC sheets were significantly higher than those in preconfluent ASCs (n = 4) (P < 0.05) (Fig. 4F). These results indicate that the ASCs sheet release these growth factors which regulate the proliferation of fibroblasts and collagen synthesis.

### 3.2. Preparation of autologous ASC sheets and their engraftment

By reducing the temperature of confluent ASCs cultured on temperature-sensitive dishes to 20 °C for 20 min, ASCs were harvested as cell sheets without enzymatic treatment (Fig. 4A). Hematoxylin and eosin stains showed that ASC sheets were composed of five laminated cell layers (Fig. 4B). To confirm the presence of transplanted ASCs after postoperative day 7, cell sheets were labeled with the fluorescent dye PKH26GL immediately before transplantation at the anastomotic site, but after suturing (Fig. 4C). Fluorescence microscopy of frozen sections of transplanted anastomotic sites on postoperative day 7 revealed PKH26GL-positive ASCs retained under the serosa layer (Fig. 4D).

3.3. Anastomotic burst pressure test

The first burst pressures of the normal wound healing model and the delayed wound healing model on postoperative day 5 were 218 ± 79.5 mmHg (n = 8) and 125 ± 79.3 mmHg (n = 8) (P < 0.05), respectively (Fig. 2D). The first burst pressures of the normal wound healing model and the delayed wound healing model on postoperative day 7 were 280.5 ± 85.6 mmHg (n = 8) and 226.5 ± 85.6 mmHg (n = 8) (P < 0.05), respectively (Fig. 2E). Using this model, we evaluated the ability of transplanted ASC sheets to improve anastomotic strength and wound healing at the intestinal anastomotic sites under delayed wound-healing conditions.

The first burst pressures of untreated and ASC sheet transplantation groups on postoperative day 5 were 118.5 ± 85.9 mmHg (n = 8) and 146.5 ± 58.8 mmHg (n = 8), respectively. The first burst pressures of untreated and ASC sheet transplantation samples on postoperative day 7 were 226 ± 87.7 mmHg (n = 16) and 267 ± 49.1 mmHg (n = 16), respectively (Fig. 5A and B). The anastomosis burst pressure exhibited by the ASC sheet transplantation group was higher compared to the burst pressure of the untreated group, and this difference reached statistical significance

(A)

(B)
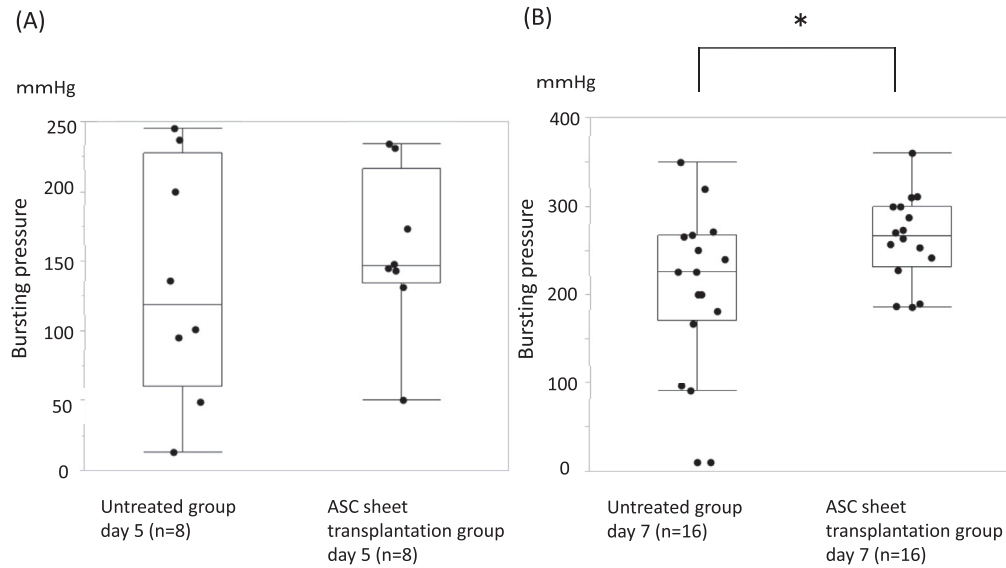
Fig. 5. Anastomotic burst pressure test. A: The first burst pressures of untreated and ASC sheet transplantation samples on postoperative day 5 were 118.5 ± 85.9 mmHg (n = 8) and 146.5 ± 58.8 mmHg (n = 8), respectively. B: On postoperative day 7, untreated and ASC sheet transplantation sample first burst pressures were 226 ± 87.7 mmHg (n = 16) and 270 ± 49.1 mmHg (n = 16), respectively. The ASC sheet transplantation group showed a higher anastomosis burst pressure compared to the burst pressure of the untreated group, which reached statistical significance on postoperative day 7. *P < 0.05.

(A) Untreated group at day 7

(B) ASC sheet transplantation group at day 7

(C)

(D)
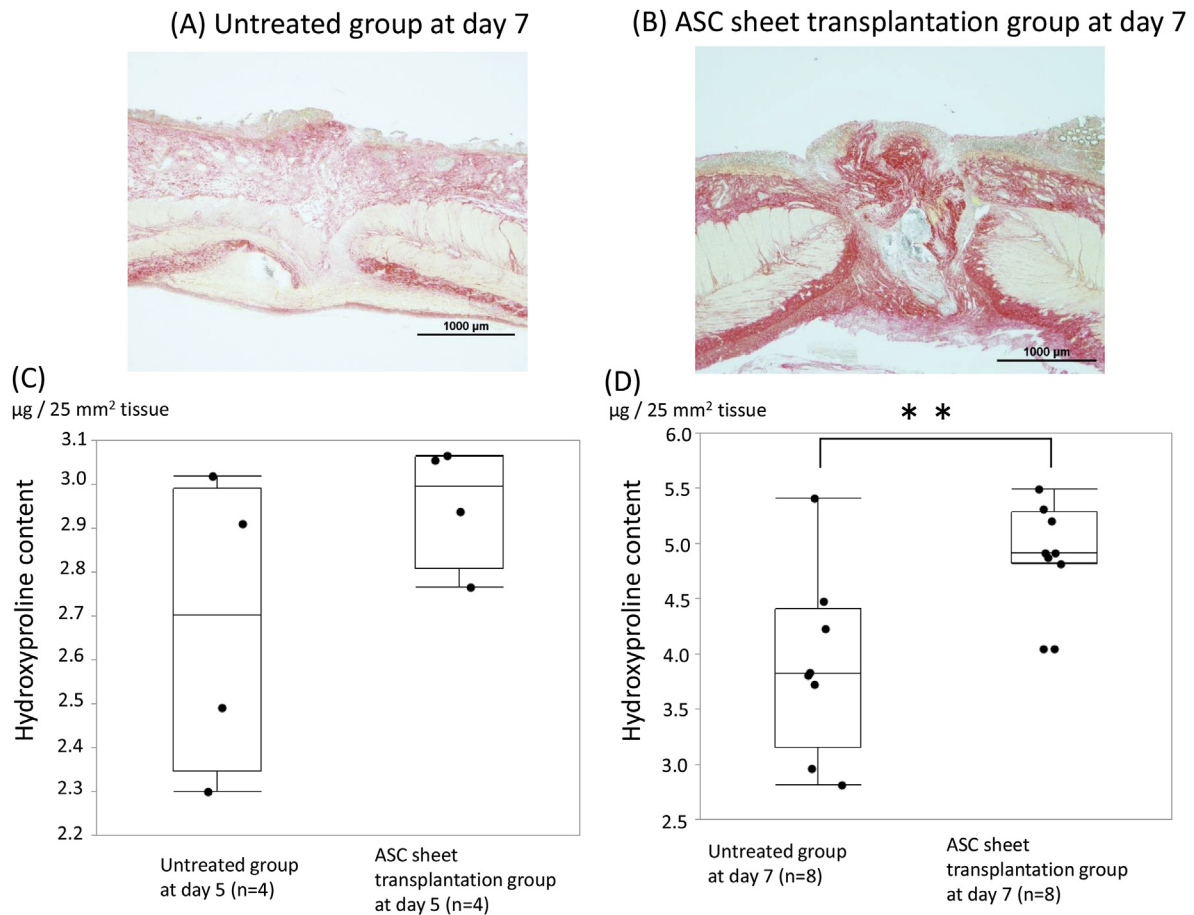
Fig. 6. Evaluation of collagen synthesis at the anastomotic sites and retention of ASC sheets on the serous membrane of the anastomotic site after 7 days postoperatively. Sirius red staining of anastomotic sites on postoperative day 7. A: Untreated group; B: ASC sheet transplantation group. The submucosal layer of intestinal tissue in the ASC sheet transplantation group stained more deeply with Sirius red staining than the untreated group. 5-mm biopsy punch tissue samples around the suture knots were measured for hydroxyproline content. At every anastomotic site, two tissue samples were collected and measured for hydroxyproline content. The hydroxyproline content in ASC sheet transplantation and untreated samples was compared on postoperative days 5 (n = 4) and 7 (n = 8) after anastomosis. C: On postoperative day 5, the hydroxyproline content at the anastomotic sites in the ASC sheet transplantation group was not significantly higher than that in the untreated group. D: The ASC transplantation sample hydroxyproline content was significantly higher than that in the untreated group on postoperative day 7. **P < 0.01.

on postoperative day 7 (P < 0.05). The transplantation of ASC sheets increased anastomotic site bursting pressure, which was closed to the value of the bursting pressure of normal wound healing conditions.

3.4. Histological examination of anastomoses region

Anastomotic sites in untreated and the ASC sheet transplantation groups on postoperative day 7 showed differences in Sirius red staining. The submucosal layer of intestinal tissue in the ASC sheet transplantation group stained more deeply with Sirius red staining than the untreated group (Fig. 6A and B). These results indicated that ASC sheet transplantation enhanced collagen synthesis around anastomosis sites.

3.5. Hydroxyproline content

The hydroxyproline content of untreated and ASC sheet transplantation groups on postoperative day 5 were 2.70 ± 0.34 µg/mg (n = 4) and 2.99 ± 0.14 µg/mg (n = 4), respectively. The hydroxyproline content untreated and ASC sheet transplantation groups of on postoperative day 7 were 3.82 ± 0.83 µg/mg (n = 8) and

4.91 ± 0.43 µg/mg (n = 8), respectively (Fig. 6D). By postoperative day 7, the higher hydroxyproline content of the ASC sheet transplantation group compared to the untreated group reached statistical significance (P < 0.01) (Fig. 6C).

3.6. Analysis of gene expression of anastomotic sites

On postoperative day 1, the mRNA expression of FGF2 at the anastomotic sites was significantly higher in the ASC sheet transplantation group than that in the untreated group (n = 4) (P < 0.01) (Fig. 7A). On postoperative day 1, COL1A1 and COL3A1 expressions were significantly higher in the ASC sheet transplantation group than those in the untreated group (n = 4) (P < 0.05) (Fig. 7C and D). On postoperative day 7, the mRNA expressions of COL1A1 and COL3A1 were significantly higher in the ASC sheet transplantation group than those in the untreated group (n = 4) (P < 0.05) (Fig. 7E and F).

4. Discussion

Collagen synthesis plays an important role in the healing process, as it impacts anastomotic strength, and can reduce the risk of
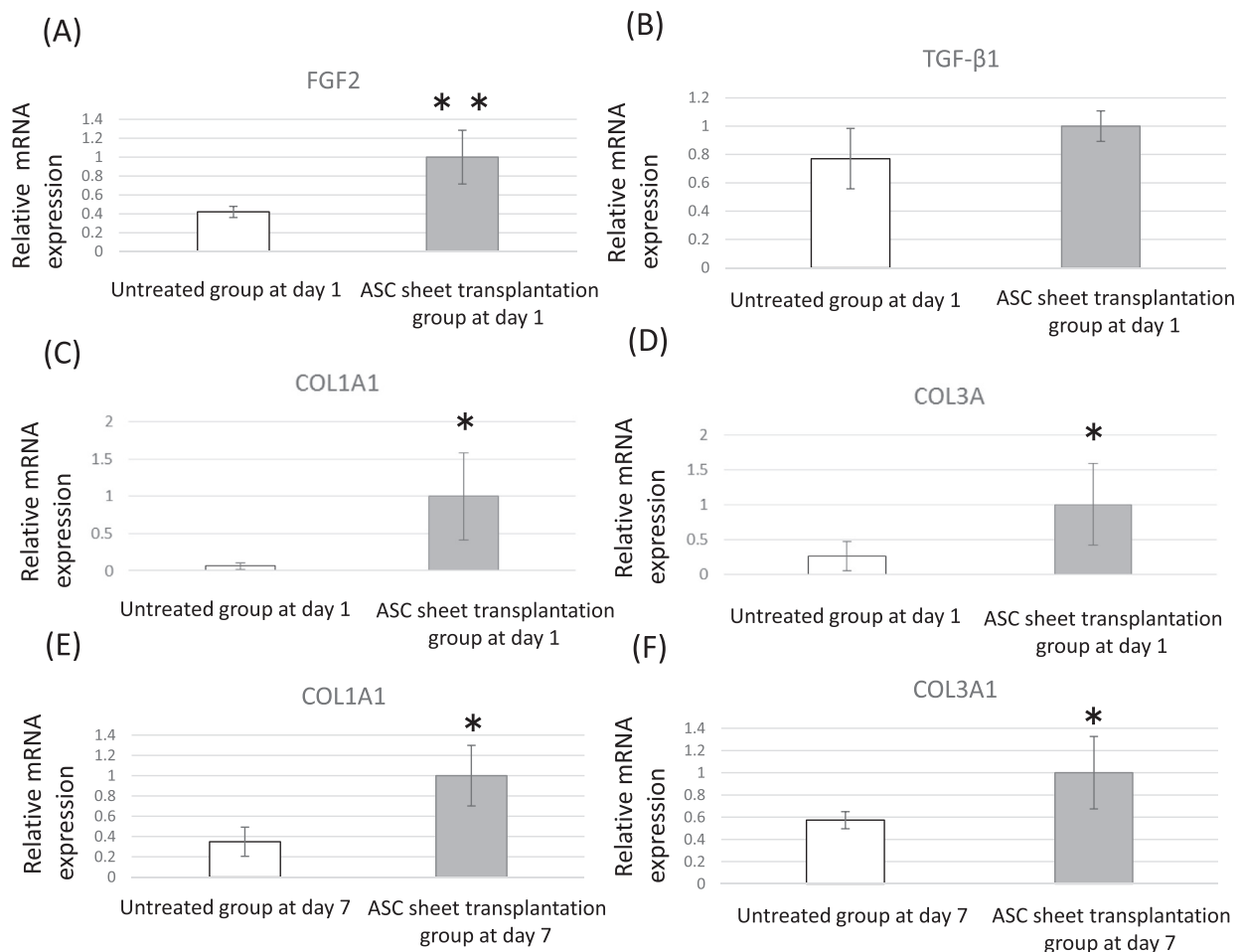
Fig. 7. Analysis of gene expression of the anastomotic sites. A: On postoperative day 1, the mRNA expression of FGF2 at the anastomotic site in the ASC sheet transplantation group was significantly higher than that in the untreated group (n = 4) (P < 0.001). B: On postoperative day 1, the mRNA expression of TGF-β1 at the anastomotic sites in the ASC sheet transplantation group was not significantly higher than that in the untreated group (n = 4). C: On postoperative day 1, collagen-1 alpha1 in the ASC sheet transplantation group was significantly higher than that in the untreated group (n = 4) (P < 0.05). D: On postoperative day 1, the mRNA expression of collagen-3 alpha1 in the ASC sheet transplantation group was significantly higher than that in the untreated group (n = 4) (P < 0.05). E: On postoperative day 7, the mRNA expression of collagen-1 alpha1 in the ASC sheet transplantation group was significantly higher than that in the untreated group (n = 4) (P < 0.05). F: On postoperative day 7, the mRNA expression of collagen-3 alpha1 in the ASC sheet transplantation group was significantly higher than that in the untreated group (n = 4) (P < 0.05). *P < 0.05, **P < 0.01.

dehiscence [10−14,30]. MMC is an antibiotic produced by fungus *Streptomyces caespitosus* [31]. MMC was originally introduced as an antibiotic and has found a place as antitumor agent [31,32]. MMC forms a covalent linkage with deoxyribonucleic acid (DNA) and inhibits DNA synthesis, functional as a powerful alkylating agent [31]. It is the best known that MMC inhibit fibroblast proliferation [32−35]. In this study, we created the delayed wound healing model using with MMC, because it was difficult to create delayed wound healing model with chemoradiotherapy in pigs in our laboratory. In this study, MMC was introduced to inhibit fibroblast activity, and therefore, reduce collagen synthesis at the anastomotic sites to induce lower than normal wound healing conditions. Using this model, we evaluated the ability of transplanted ASC sheets to improve anastomotic strength and wound healing at the intestinal anastomotic sites under delayed wound healing conditions. We found that the transplantation of ASC sheets increased anastomotic site bursting pressure, which was closed to the value of the bursting pressure of normal wound healing conditions. Additionally, the collagen synthesis of the anastomoses significantly increased in the ASC sheets transplantation group.

Further studies are necessary to gain insight into the mechanisms behind this process. In the proliferation phase, the migration and proliferation of fibroblasts into the wound are largely regulated by potent tissue growth factors such as TGF-β1, platelet-derived growth factor, and FGF2. This leads to collagen synthesis and a rapid increase in anastomotic strength [10,12,13]. In our study, the mRNA expression levels of FGF2 and TGF-β1 in the ASC sheets were significantly higher than those in the preconfluent ASCs. The mRNA expression of FGF2 in the tissue obtained from the anastomotic sites was significantly higher in the ASC sheet transplantation group than in the untreated group. Thus these growth factors released by the ASC sheets might impact the healing of the anastomotic site and stimulate the fibroblasts and collagen synthesis around the anastomotic site.

Although several experimental studies have reported that local injection of cell suspensions of ASCs into intestinal anastomotic sites has a beneficial effect on the healing process at the anastomotic sites under normal wound-healing conditions [21,22], it is difficult to compare these results with those of the present study as the experimental animals, injury models, and suturing pitches are different. Okano et al. developed cell culture dishes coated with a temperature-responsive polymer, *N*-isopropylacrylamide, to create cell sheets [36]. Using this temperature-responsive dish, various cells can be harvested as cell sheets with intact cell−cell junctions and their underlying extracellular matrix. The cell sheets can maintain their functional capability and structure after transplantation, and with the presence of deposited extracellular matrix on the basal surface, the cell sheets can therefore be transplanted without the need for sutures or clips [36,37]. As we used large animals and created delayed wound-healing conditions, we consider that our study results are more relevant clinically and that the delayed wound-healing conditions created were more efficient to test our hypothesis than those used in previous studies [21,22].

In conclusion, we demonstrated that the transplantation of autologous ASC sheets enhanced collagen synthesis and the anastomotic site strength even under delayed wound-healing conditions. This new approach might be a feasible and promising strategy to prevent anastomotic leakage in patients with delayed wound-healing conditions.

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Conflict of interest

Teruo Okano, Ph.D is a founder and member of the board of CellSeed Inc., which has licences for certain cell sheet-related technologies and patents from Tokyo Women's Medical University. Masayuki Yamato, Ph.D is Ownership or partnership: Share holder of CellSeed Inc., Tokyo, Japan. Another authors have no financial relationships to disclose.

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