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Original Article

Allogeneic transplantation of epidermal cell sheets followed by endoscopic submucosal dissection to prevent severe esophageal stricture in a porcine model

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ABSTRACT

Introduction: Endoscopic submucosal dissection (ESD) is a minimally invasive treatment for early esophageal cancer. However, large mucosal defects after esophageal ESD result in refractory strictures. In the present study, we histologically evaluated the endoscopic transplantation of allogeneic epidermal cell sheets (ECSs) as a feasible therapy for preventing esophageal stricture after circumferential ESD in a porcine model.

Methods: Epidermal cells were isolated from the skin tissue of allogeneic pigs and cultured on temperature-responsive cell culture inserts for 2 weeks. Transplantable ECSs were harvested by reducing the temperature and endoscopically transplanting the sheets to ulcer sites immediately after esophageal ESD. The engraftment of transplanted ECSs was then evaluated in two pigs at 7 days after transplantation. Next, ten pigs were divided into two groups to evaluate the endoscopic transplantation of allogeneic ECSs for the prevention of esophageal strictures after ESD. Allogeneic ECSs were transplanted immediately after esophageal ESD in the transplantation group ($n = 5$), whereas the control group ($n = 5$) did not undergo transplantation.

Results: Most of the transplanted allogeneic ECSs were successfully engrafted at the ulcer sites in the early phase. Fluorescence in situ hybridization analysis revealed that several allogeneic cells were present in the transplanted area at 7 days after ESD. At 14 days after ESD, significant differences in body weight loss, dysphagia scores, and mucosal strictures were observed between the control and transplantation groups. Transplanting allogeneic ECSs after esophageal ESD promotes mucosal healing and angiogenesis and prevents excessive inflammation and granulation tissue formation.

Conclusions: Endoscopic and histological analyses revealed that allogeneic ECSs promoted artificial ulcer healing after ESD, preventing esophageal strictures after ESD.

1. Introduction

Benign esophageal strictures result from chronic gastroesophageal reflux, eosinophilic esophagitis, and the ingestion of corrosive substances [1–3]. Refractory esophageal stricture after endoscopic resection for widespread superficial esophageal carcinoma has been recently recognized as a common issue among patients with benign esophageal strictures [4,5]. Large mucosal defects after esophageal ESD result in long and narrow strictures. Esophageal strictures accompanied by severe dysphagia are treated through frequent sessions of endoscopic balloon dilatation, which is often a painful procedure and is associated with the risk of esophageal perforation [6]. Although several clinical procedures have been reported to prevent post-ESD stricture, no effective preventive procedure for this complication has been established [7–11].

In a previous study, we utilized transplantable cell sheets fabricated on temperature-responsive culture surfaces. These

Abbreviations: ESD, endoscopic submucosal dissection; ECS, epidermal cell sheet; MPO, myeloperoxidase; iNOS, inducible nitric oxide synthase; MP, muscularis propria; PIPAAm, poly-N-isopropylacrylamide; ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition.

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temperature-responsive cell culture inserts, onto which a temperature-responsive polymer poly (*N*-isopropylacrylamide) (PIPAAm) was covalently immobilized, can control its wettability and cell adhesiveness depending on the temperature [12]. Cells attach and proliferate on the surfaces at 37 °C but detach upon a reduction in the temperature below 32 °C. This cellular detachment involves intracellular ATP and cytoskeletal reorganization and does not cause damage to the cell membrane [13,14]. Thus, the cytotoxicity of cell detachment is extremely low, and cell membrane proteins, including receptors, ion channels, cell–cell junction proteins such as cadherin, and extracellular matrix proteins, remain intact after detachment [15].

Oral mucosal epithelial cell sheets (OMCSs) fabricated on temperature-responsive cell culture surfaces have been shown to represent a feasible therapy using cytokines and exosomes [16,17]. Hence, we hypothesized that post-ESD stricture could be prevented by seeding epithelial cells on artificial ulcer sites after ESD. Autologous OMCSs have successfully shown significant efficacy in preventing esophageal stricture after ESD [18–23]. However, autologous cell therapies are generally difficult to prepare, as they require complicated manufacturing processes and strict quality control before transplantation [24,25]. In clinical trials of autologous OMCSs, one-third of patients was excluded because of chronic illness, viral and fungal infections, or epithelial dysplasia in the oral mucosa [26,27]. Furthermore, the occurrence of multiple primary cancers is a well-known phenomenon in patients with esophageal cancer [28,29], and autologous oral mucosa, which is the cell source of OMCSs, naturally presents a risk for the development of premalignant and malignant lesions. Thus, the clinical applicability of autologous OMCSs is currently limited.

Allogeneic epidermal cell-based tissue-engineered products, prepared using the same manufacturing processes, show nearly the same quality and effectiveness in treating skin ulcers, are not required to remain in the whole body for the long term and are applicable to all patients [30–32]. Here, we hypothesized that allogeneic epidermal cell-based tissue-engineered products could prevent severe esophageal strictures after ESD because it is not necessary to maintain the stemness of transplanted cells for long periods. In a preliminary study, we described a porcine model of severe esophageal strictures at 2 weeks after ESD and the preparation of epidermal cell sheets (ECSs) [33]. The underlying mechanism of post-ESD stricture, however, remains elusive. In the present study, we histologically evaluated the endoscopic transplantation of allogeneic epidermal cell sheets (ECSs) as a feasible therapy for preventing esophageal stricture after circumferential ESD in a porcine model.

2. Materials and methods

2.1. Experimental animals

Fourteen miniature pigs (6 months old, 16–20 kg) were purchased from the Nippon Institute for Biological Science. All of the animals were treated in accordance with experimental procedures approved by the Committee for Animal Research of Tokyo Women's Medical University (11–51 2014).

2.2. Study design

First, the biological and mechanical features of the ECSs were evaluated in vitro and in vivo. The engraftment of 9 transplanted ECSs was evaluated in two pigs at 6 h after transplantation. The engraftment of transplanted ECSs was then evaluated in two pigs at 7 days after transplantation. Next, ten pigs were divided into two groups to evaluate the effectiveness of transplanting allogeneic ECSs for the prevention of esophageal strictures after ESD (Supplemental Fig. S1a). When the esophageal strictures had developed to the point that they compromised more than 80% of the esophageal lumen, the pigs were unable to maintain their weight [24,34]. In a previous study by our group, more than 80% of esophageal strictures had generally developed by 2 weeks after full-circumferential ESD in our porcine models [33]. Therefore, in the porcine model employed for this experiment, we determined that a sample size of ten pigs would provide at least 80% power to detect a 20% decrease in strictures when the α error was set at 0.05. An a priori power analysis based on pilot results was performed using GPOWER 3 [35].

2.3. Preparation and harvesting of allogeneic ECSs

Epidermal cells were isolated from the lower abdominal skin of a pig and suspended in temperature-responsive cell culture inserts (CellSeed Inc., Tokyo, Japan) at a density of 10×10^5 cells/cm^2. The epidermal cells were cultured in modified keratinocyte condition medium for 2 weeks at 37 °C in a humidified atmosphere containing 5% CO_2 [36]. After the epidermal cells had been cultured for 2 weeks at 37 °C, they were transferred to another incubator set at 20 °C for 20 min and then noninvasively harvested as tissue-engineered ECSs.

2.4. ESD and endoscopic transplantation of allogeneic ECSs

ESD was performed in the lower esophagus of each animal. An ECS transplanting test was performed in 4 pigs to analyze the attachment and engraftment of the transplanted allogeneic ECSs (Supplemental Fig. S1b). After full-circumferential ESD, the artificial esophageal ulceration was covered with five to nine sheets in each animal ($n = 5$). The control procedures involved ESD without allogeneic ECS transplantation ($n = 5$). ESD was performed in the lower esophagus of each animal by using an endoscope (XQ260, Olympus, Tokyo, Japan) and the videoscope system (EVIS LUCERA 260, Olympus). Three-quarters of circumferential ESD (40 mm in length) was performed on 4 pigs for the analysis of attachments and engraftments of transplanted allogeneic epidermal cell sheets. Full circumferential ESD (40 mm in length) was performed on 10 pigs to evaluate the safety and efficacy of transplanted allogeneic epidermal cell sheets. To lift the esophageal mucosa, 5–10 mL of 0.04% hyaluronic acid solution (MucoUp, Seikagaku, Tokyo, Japan) and 0.2 mg/mL indigo carmine dye were injected into the submucosal layer of the esophagus. Mucosal incisions of the oral and anal sides were performed by using a hook knife (KD-620LR, Olympus) and a generator (VIO300D, ERBE Elektromedizin, Tübingen, Germany), which was set at end cut mode (effect 2). Submucosal layers were mechanically dissected to avoid thermal damage. To cauterize the blood vessels, hemostatic forceps (Coagrasper, Olympus) were used, and the generator was set at a soft-coagulation mode (40 W, effect 4).

2.5. Postoperative course

Two transplanted pigs were euthanized with an overdose injection of potassium chloride 6 h after transplantation for further analysis after endoscopic observation. The other 12 pigs were fasted for 2 days after the operation and allowed free access to food and water. Two transplanted pigs were euthanized with an overdose injection of potassium chloride 7 days after transplantation for further analysis after endoscopic observation. Ten other pigs were euthanized with an ECS overdose injection of potassium chloride

for further analysis at 14 days after transplantation. A magnification endoscope with a narrow-band imaging system (FQ260Z, Olympus) was used to assess angiogenesis. The increase in the weight of the animals was measured before ESD, at 1 week after ESD, and at 2 weeks after ESD. Swallowing symptoms of dysphagia were scored by the modified Mellow and Pinkas score in a completely blinded manner, as follows: 0 points, no dysphagia; 1 point, ability to eat some solid food; 2 points, ability to eat semisolids only; 3 points, ability to swallow liquids only; and 4 points, complete dysphagia [33,37]. After euthanasia, the esophagus was removed and dissected longitudinally. The rate of mucosal strictures was calculated using the following equation:

$$\text{Rate of mucosal strictures } (\%) = (1 - \text{Lmax})/\text{Lnr} \times 100$$

Where Lmax and Lnr are the lengths of the short axes in the maximal narrow state and the normal state on the oral side, respectively.

2.6. Histological examination

ECSs and esophageal specimens were fixed in 4% paraformaldehyde (Muto Pure Chemical, Tokyo, Japan) and processed into 3 μm-thick paraffin-embedded sections. Hematoxylin and eosin (HE) staining, Azan staining, and Giemsa staining were performed via conventional methods. Sirius red staining was performed using the Picrosirius Red Staining Kit (Polysciences, Inc., Warrington, PA, USA). The rate of mucosal healing was calculated in the center of long-axis sections using the following equation:

$$\text{Mucosal healing rate } (\%) = \text{Lmh}/\text{Lesd} \times 100$$

Where Lmh is the length of the long axes at the oral and anal mucosal healing sites and Lesd is the length of the long axes over the total ESD site.

2.7. Immunofluorescent cytology and histology

The strictures of ECS were evaluated by immunofluorescent cytology. After fixation using 4% paraformaldehyde (PFA) (Wako Pure Chemicals, Osaka, Japan), harvested ECSs were incubated for 5 min with PBS containing 0.5% Triton X at room temperature. After blocking for 1 h with 5% bovine serum albumin, ECSs were treated with anti-Ki67 (Dako, Glostrup, Denmark) at 4 °C overnight. Finally, treated ECSs were placed on grass slides, and coverslips were mounted in Prolong Gold with DAPI (Thermo Fisher Scientific, Waltham, MA, USA).

The protein expression of ECSs and resected esophageal specimens was evaluated by immunofluorescent histology. Deparaffinized sections were washed with PBS and subjected to proteinase K treatment (Dako) or heat treatments with Target Retrieval Solution, Citrate pH 6 (Dako) or Target Retrieval Solution, EDTA pH 9 (Dako). Each section was treated with the following primary antibodies: anti-pancytokeratin (Enzo Life Sciences, Farmingdale, NY, USA), anti-cytokeratin 5 (Abcam, Cambridge, UK), anti-integrin beta 4 (Santa Cruz Biotechnology, Dallas, TX, USA), anti-p63 (Santa Cruz Biotechnology) and anti-CD31 (Thermo Fisher Scientific) anti-Vimentin (Dako) at 4 °C overnight. All sections were treated with fluorescent conjugated secondary antibodies. Coverslips were mounted in Prolong Gold with DAPI. The sections were observed using confocal laser microscopy (FV1200, Olympus). The clone, code, dilution, and antigen retrievals of immunohistochemistry are summarized in Supplemental Table 1.

2.8. Immunohistochemistry

The protein expression of resected esophageal specimens was evaluated by immunohistochemistry. After pretreatment, each section was treated with the following primary antibodies: anti-pancytokeratin, anti-Ki67, anti-myeloperoxidase (MPO) (Dako), anti-inducible nitric oxide synthase (iNOS) (Abcam), and anti-CD3 (Abcam). All sections were treated with a secondary antibody (EnVision™/HRP, Dako), and hematoxylin was used for counterstaining. The clone, code, dilution, and antigen retrievals of immunohistochemistry are summarized in Supplemental Table 1.

2.9. FISH analysis of X and Y chromosomes

To confirm the presence of transplanted cells, allogeneic ECSs, which were fabricated from male pigs, were transplanted into female pigs. Paraffin-embedded sections of specimens at 7 days after transplantation were used to detect the transplanted cells. Fluorescence in situ hybridization (FISH) analysis was performed in association with Chromosome Science Lab Inc. After deparaffinization, the sections were heated and digested with pepsin (0.1% pepsin and 0.1 After washing and drying the sections, probes for pig chromosomes X and Y (Chromosome Science Lab, Sapporo, Japan) were applied to the washed and dried sections and incubated on a 90 °C hot plate for 10 min. The sections were then hybridized at 37 °C overnight. After washing the sections, the sections were stained with DAPI, and coverslips were mounted. The sections were observed by fluorescence microscopy (CW-4000 systems, Leica, Cambridge, UK).

2.10. Evaluation of the wound environment

The rate of mucosal healing was calculated in the center of the long-axis sections by the following equation:

$$\text{Mucosal healing rate } (\%) = \text{Lmh}/\text{Lesd} \times 100$$

Where Lmh is the length of the long axes at the oral and anal mucosal healing sites and Lesd is the length of the long axes at the total site of ESD.

Fibrosis and atrophy of the muscularis propria (MP) were numerically graded using a modified version of Honda's scoring system, as follows: 0 points, no atrophic or fibrotic change in the MP evident in any of the examined sections; 1 point, atrophy or fibrosis present but confined to only part of the MP; 2 points, atrophy or fibrosis present but confined to the full-thickness MP; and 3 points, transmural fibrosis of the MP [38]. Hypertrophy of the submucosal layer in the ulcer sites after esophageal ESD was evaluated by Sirius red staining. The thickest areas of submucosal layers were measured and averaged in an area of 5 mm at intervals of 1 mm.

Angiogenesis was histologically evaluated by fluorescent immunohistochemistry. The area surrounded by CD31-positive cells in the field of the ulcer site was measured at 5 random locations (× 600 magnification of each sample) in each pig using ImageJ (U.S. National Institutes of Health, Bethesda, MD).

Inflammation of ulcer sites after ESD in the control and transplantation groups was evaluated by hematoxylin and eosin (HE) staining and immunohistochemistry. Inflammatory cells, MPO-positive cells, iNOS-positive cells, and CD3-positive cells in the field of the ulcer site were counted and averaged at 5 random high-power fields (HPF) in each pig (× 400 magnification).

2.11. Statistical analysis

The data are expressed as the mean ± standard deviation. All statistical analyses were performed using the SAS-JMP program for

Windows (SAS Institute, Inc., Cary, NC, USA). Continuous values were evaluated with Student's *t test*. Probability values (P) of <0.05 were considered statistically significant.

3. Results

3.1. Characteristics of ECSs

All cultured ECSs were noninvasively harvested as a transplantable contiguous cell sheet after incubation at 20 °C for 20 min. The harvested ECSs were 20 mm in diameter and were composed of proliferating epidermal cells arranged in a cobblestone-like pattern (Fig. 1a–c). Cell structures and protein expression in both apical and basal progenitor cells were similar to those in the epidermis (Fig. 1d and e).

3.2. Engraftment of ECSs in a short period after transplantation

Almost all transplanted ESCs had stably attached at the ulcer sites of esophageal ESD within 6 h after transplantation (8/9; 83%) (Fig. 2a). Several attached basal cells were observed to proliferate, as shown in Fig. 2b.

Under endoscopic observation, transplanted allogeneic ECSs were barely detectable at the ulcer sites of esophageal ESD at 7 days after transplantation. Healthy granulation tissue without a white coating was observed at the transplanted ulcer site (Fig. 2c–e). Interestingly, fluorescence in situ hybridization (FISH) analysis revealed that several Y chromosome-positive allogeneic cells were present in the same region (Fig. 2f). Double immunofluorescence analysis demonstrated that several epithelial cells with an epithelial-to-mesenchymal transition (EMT)-phenotype, which showed strong positive CK5 signals and weak positive vimentin signals, also existed around the submucosal layer (Fig. 2g).

3.3. Esophageal strictures, dysphagia, and mucosal healing after ESD

Full-circumferential ESD with or without allogeneic ECS transplantation was successfully performed in all pigs. From postoperative day 7 to day 14, the increase in body weight in the transplantation group (1.01 ± 1.31%) was greater than that in the control group (−1.46 ± 0.90%) (P = 0.010) (Fig. 3a). The dysphagia score of the control group (3.4 ± 0.5%) was significantly higher than that of the transplantation group (1.0 ± 0.70%) (P < 0.001) (Fig. 3b). On postoperative day 14, pinhole-sized strictures were observed only in the control group (Fig. 3c and d). In contrast, in the transplantation group, the esophageal lumen was slightly narrowed, and the elasticity of the lumen was lower than that in the control group (Fig. 3c and d). The rate of mucosal stricture in the control group (85.7 ± 4.5%) was significantly higher than that in the transplantation group (62.0 ± 1.7%) (P < 0.001) (Fig. 3e). The rate of esophageal mucosal healing in the transplantation group (51.0 ± 5.6%) was significantly higher than that in the control group (26.6 ± 11.0%) (P = 0.027) (Fig. 3f).

3.4. Wound healing environment at the ulcer site after ESD

Neovascularization containing red blood cells was observed in the transplantation group (Fig. 4a, Supplemental Figs. S2a and b). In contrast, small vessels and red blood cells were rarely observed

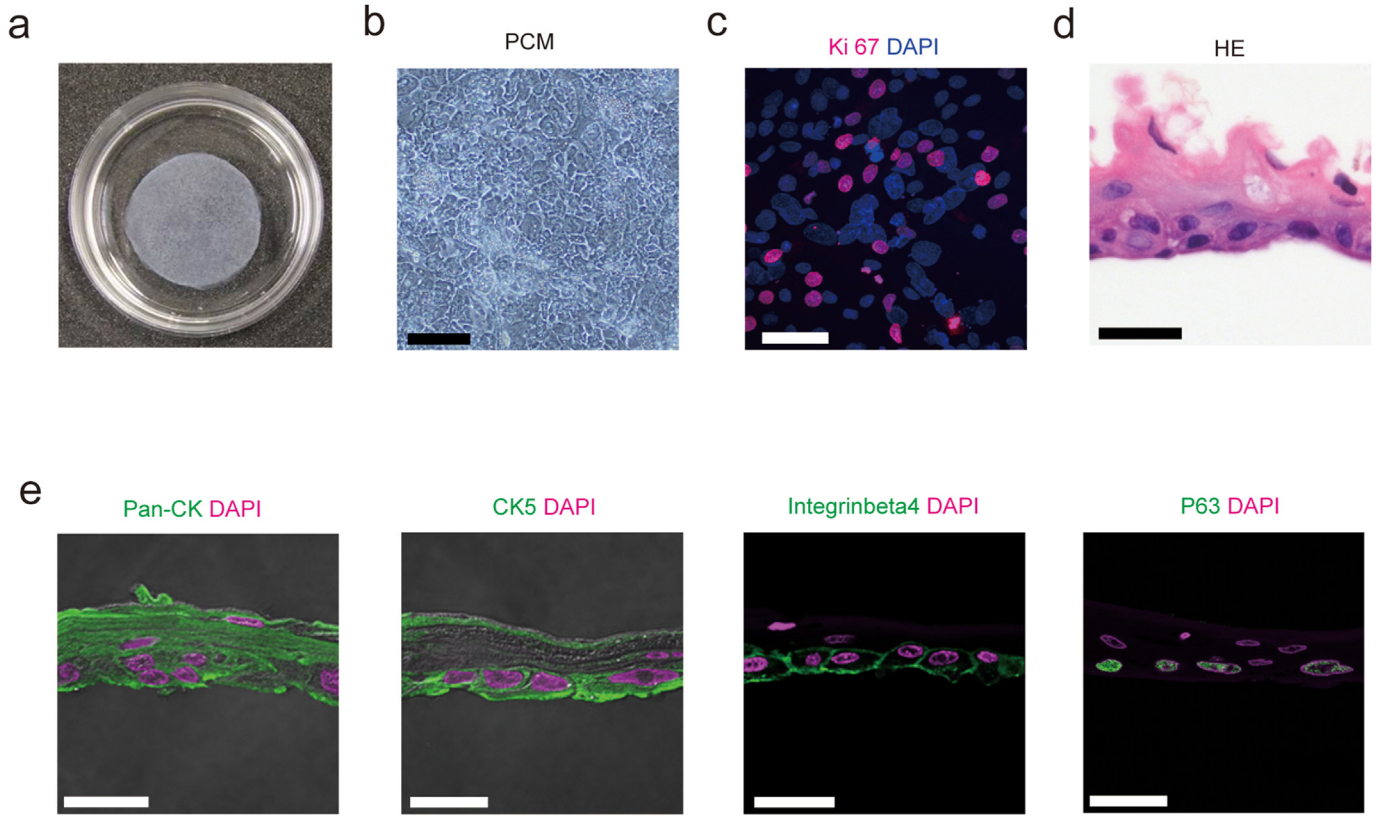

Fig. 1. Characterization of tissue-engineered ECSs. (**a**) The ECS was harvested noninvasively from a temperature-responsive cell culture insert. The diameter of the fabricated ECS is approximately 20 mm. (**b**) Epidermal cells arranged in a cobblestone-like pattern in tissue-engineered ECSs were observed via phase contract microscopy (PCM). The scale bar indicates 50 μm (**C**) Several proliferated cells were present in tissue-engineered ECSs. The scale bar indicates 20 μm. (**d**) HE staining showing that the ECS was composed of layers of basal cells and keratinized cells. The scale bar indicates 50 μm. (**e**) According to histological analysis, cells in the basal layer were similar to basal cells in the epidermis. The scale bars indicate 20 μm.

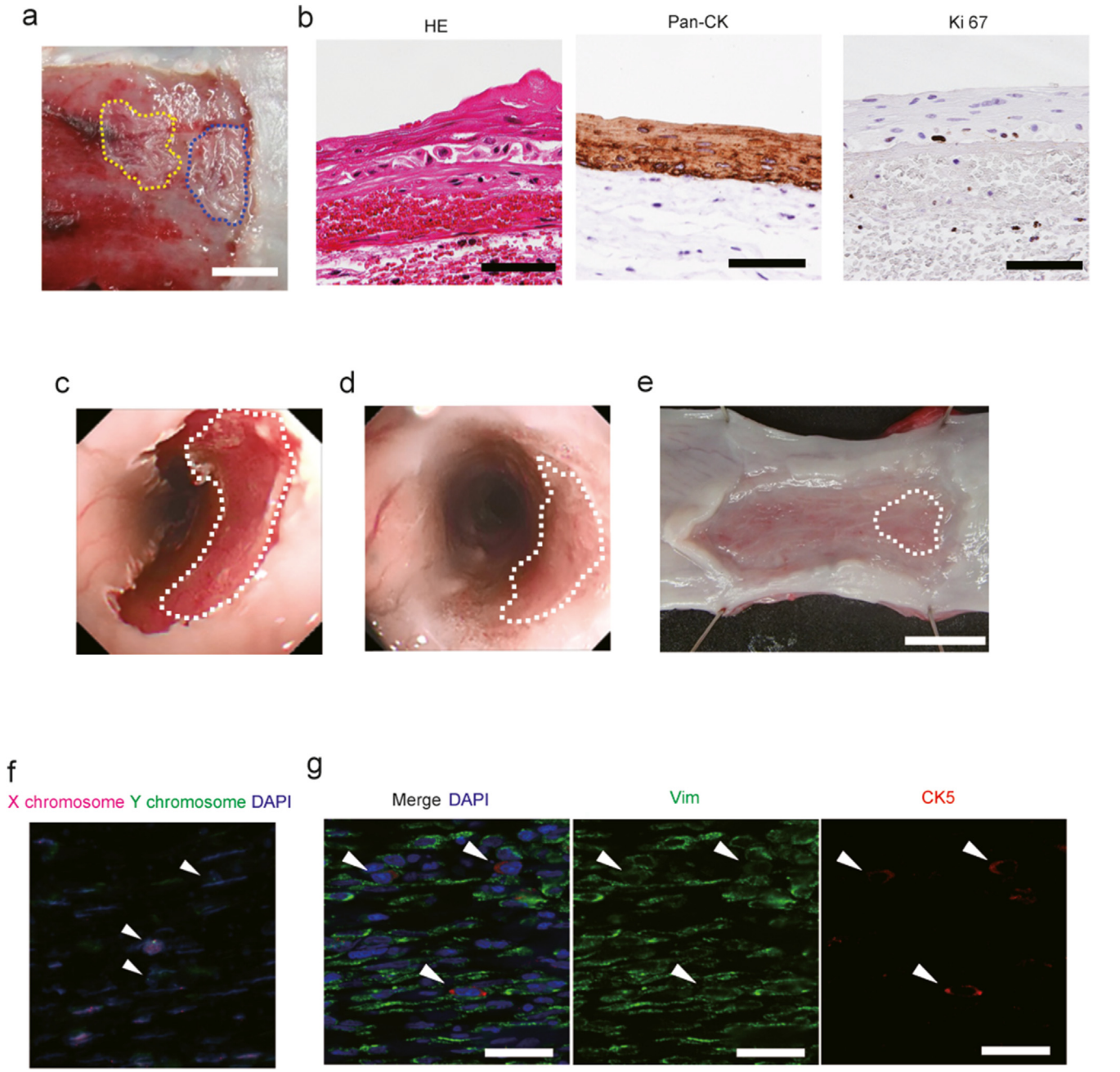

Fig. 2. Engraftment of allogeneic ECSs to artificial esophageal ulcer sites after circumferential ESD at 6 h and 7 days posttransplantation. (**a-b**) Transplanted allogeneic ECSs attached immediately to the artificial ulcer surface after transplantation. The transplanted ECSs were attached histologically, and several basal cells proliferated at the ulcer site at 6 h after transplantation. The white and black scale bars indicate 10 mm and 50 μm, respectively (**c-e**) Endoscopic examination of the ulcer sites immediately after ECS transplantation (c) and 7 days after ECS transplantation (d) and macroscopic findings at the ulcer sites 7 days after ECS transplantation (e). The ulcer sites where allogeneic ECSs were transplanted are surrounded by white-dotted lines. The scale bar indicates 10 mm. (**f**) Some of the transplanted ECSs produced from male pigs were placed at ulcer sites in female pigs. Red dots indicate an X chromosome, and green dots indicate a Y chromosome. (**g**) Several epithelial cells with an EMT-like phenotype were found at the ulcer sites with transplanted ECSs. The scale bars indicate 20 μm.

in the control group. The vessel density in the transplantation group was significantly higher than that in the control group ($P = 0.001$) (Fig. 4b). When the infiltration of inflammatory cells at the ulcer sites after ESD was compared between the control and transplantation groups (Fig. 4c–f, Supplemental Fig. S2c), the number of inflammatory cells, anti-myeloperoxidase (MPO)–positive leukocytes, and anti-inducible nitric oxide synthase (iNOS)–positive leukocytes in the transplantation group was found to be significantly lower than that in the control group ($P < 0.001$, $P = 0.003$, and $P = 0.010$, respectively). The number of CD3-positive leukocytes in the transplantation group was similar to that in the control group ($P =$ n.s.). Based on observations in Azan-stained histological sections, the mean atrophy score of the MP layer in the control group was significantly higher than that in the transplantation group ($P = 0.009$) (Fig. 4g and h, Supplemental Fig. S2d). According to the results from Sirius red-stained histological sections, the hypertrophic submucosal layers were significantly thinner after esophageal ESD in the transplantation group than in the control group ($P = 0.012$) (Fig. 4i, Supplemental Fig. S2e).

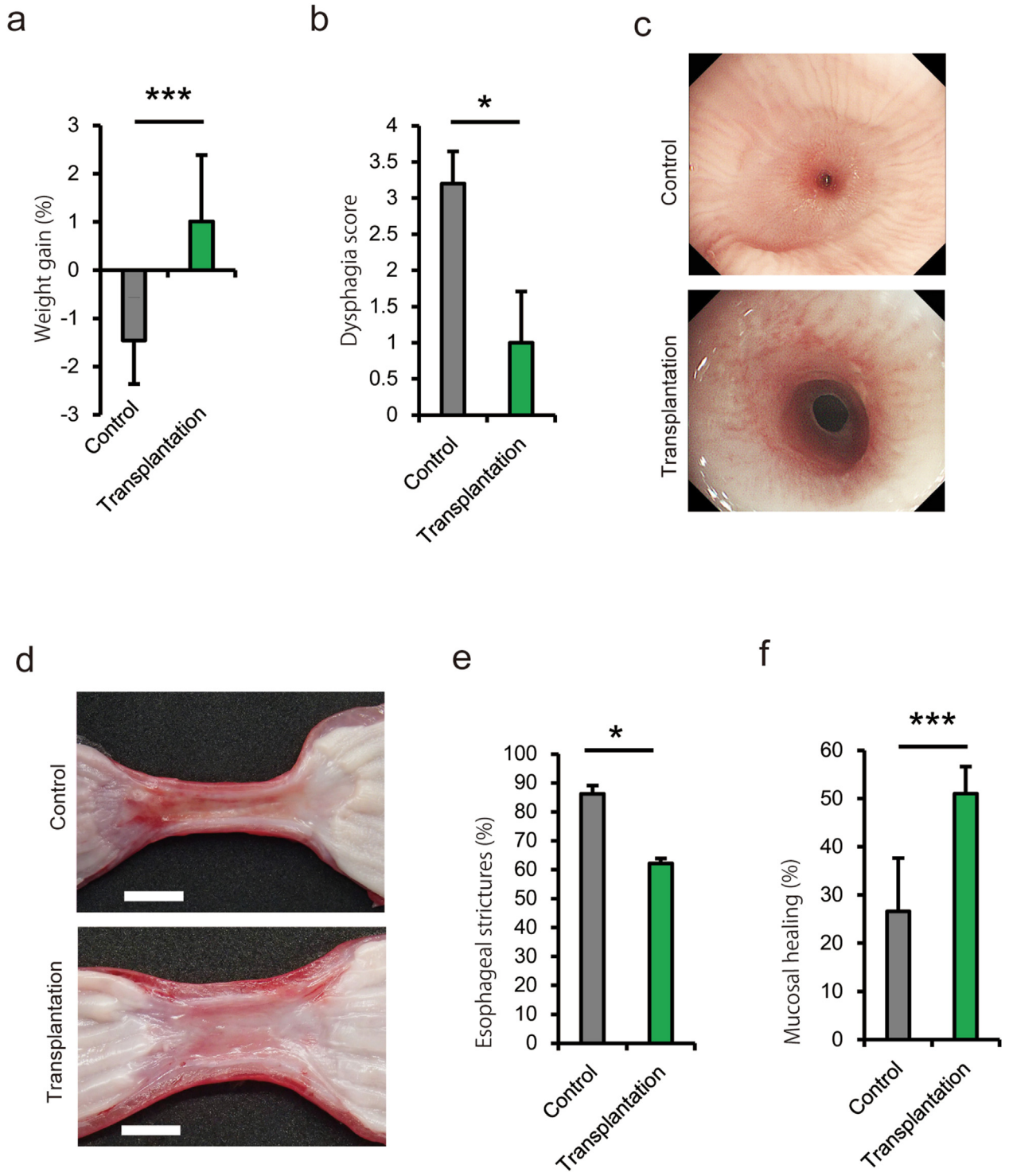

Fig. 3. Clinical and histological evaluations of the control and transplanted groups at 14 days after esophageal ESD. (**a, b**) The graphs show the rate of the increase in body weight and dysphagia scores (**c, d**) Endoscopic and macroscopic findings in the esophageal lumen in the control and transplanted groups. Scale bars indicate 10 mm (**e, f**) The graphs show the esophageal stricture rate and mucosal healing rate. The asterisk indicates $P < 0.001$. The triple asterisk indicates $P < 0.05$.

4. Discussion

This study investigated histological analysis of the allogeneic transplantation of ECSs to prevent esophageal stricture after large ESD in a porcine model. FISH analysis revealed that transplanted allogeneic cells were present at 7 days after transplantation. The transplanted epidermal cells with an EMT-like phenotype migrated into the submucosal layer. Moreover, we obtained evidence that allogeneic ECSs promote mucosal healing and angiogenesis in artificial ulcers after esophageal ESD and prevent excessive inflammation, granulation tissue formation, and MP damage after esophageal ESD (Supplemental Fig. S3).

The regenerative cellular events that occur in wound healing after the transplantation of ECSs remain unclear. We previously demonstrated that partial EMT occurs within the basal layer cells of ECSs after grafting in an in vitro model [39]. In the current study,

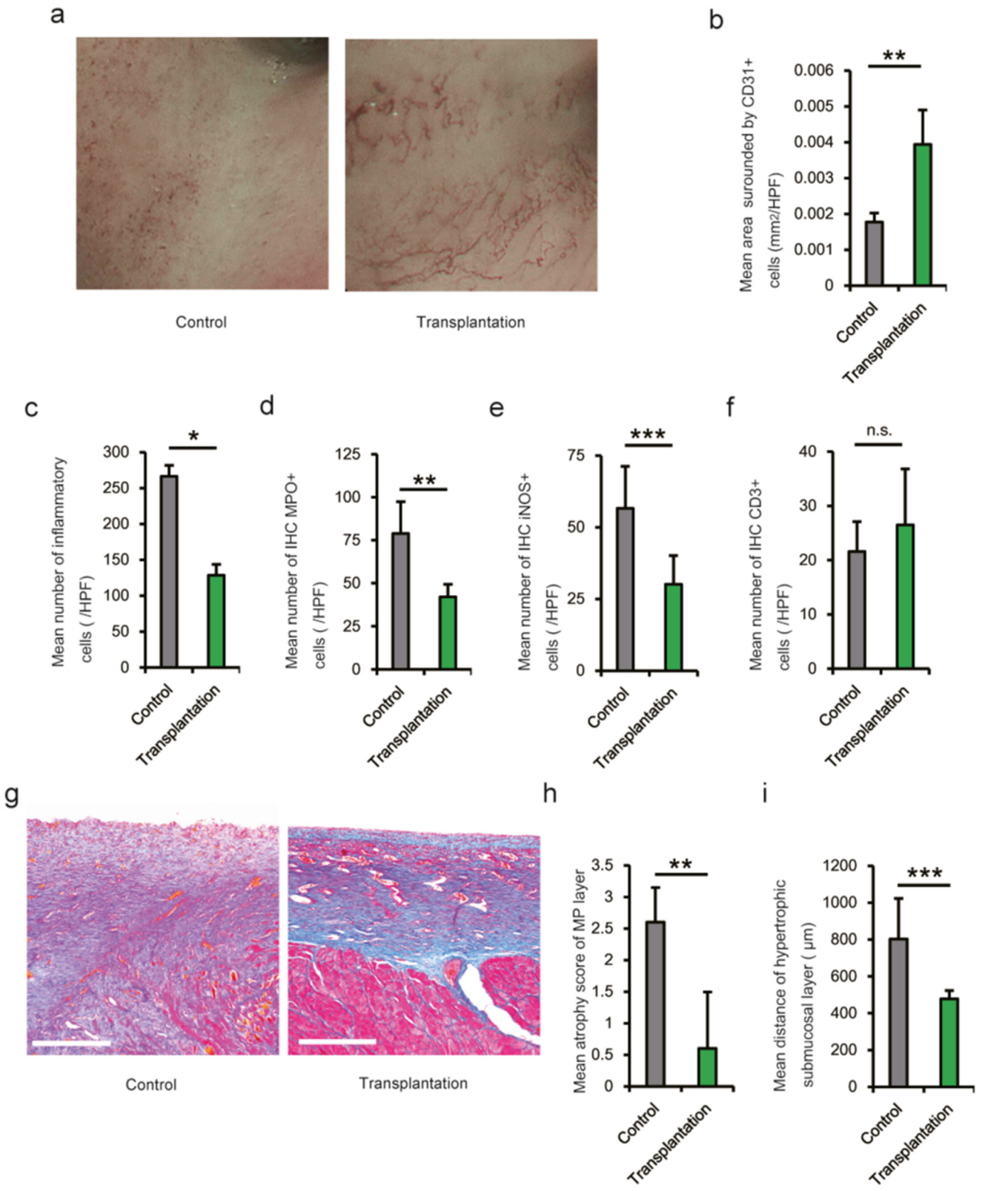

Fig. 4. Characteristics of ulcer sites after ESD with or without allogeneic ECS transplantation at 14 days postoperation. (a) Magnified endoscopic observation of artificial esophageal ulceration through narrow band imaging (NBI) in the control and transplantation groups. (**b**) The graphs show the numbers of CD31-positive cells in a high-power field (HPF) at the ulcer sites (**c-f**) The graphs show the numbers of inflammatory cells, myeloperoxidase (MPO)-positive cells, inducible nitric oxide synthase (iNOS)-positive cells, and CD3-positive cells in an HPF at the ulcer sites. (**g**) Azan-stained sections from the control and transplantation groups showing that allogeneic ECS transplantation protects against atrophy and fibrosis of the muscularis propria (MP). Scale bars indicate 500 μm (**h, i**) The graphs show the atrophy score for the MP layer and the distance of the hypertrophic submucosal layer. The asterisk indicates $P < 0.001$. The double asterisk indicates $P < 0.01$. The triple asterisk indicates $P < 0.05$.

the engraftment of ECS basal layer cells in the ulcer site after ESD was also demonstrated in an animal model. Thus, partial EMT of transplanted ECSs occurs in the ulcer site after ESD.

The porcine model of esophageal stricture employed in this work was previously established [33,34]. In almost all cases, long esophageal strictures (>2 cm), which represent a risk for the development of refractory strictures, develop at 2 weeks after ESD [40]. More than three-fourths of the esophageal circumference and a longitudinal diameter of more than 40 mm have been reported as risk factors for esophageal stricture after ESD [41]. These features are included in the porcine model of esophageal stricture.

To prevent esophageal stricture after ESD, several basic science studies have suggested the importance of protecting the artificial ulcer site from mechanical and chemical damage, suppressing inflammation, and preventing adhesion of the wound bed during the acute phase [38,42–44]. However, the biological mechanisms underlying mucosal healing and stricture at the ulcer site after esophageal ESD remain elusive. In this study, the biological mechanisms of mucosal healing and stricture at the ulcer site after ESD were determined by comparing the control group with the transplantation group.

Autologous OMCS transplantation after esophageal ESD suppresses major proinflammatory cytokines that induce esophageal stricture in serum [17]. Likewise, allogeneic ECS transplantation after ESD suppressed excessive inflammation to improve the wound environment. For instance, angiogenesis induced by allogeneic ECSs supplies nutrition and oxygen to granulation sites. At 14 days, the ulcer sites in the transplantation group animals remained in the proliferative phase. In contrast, the ulcer sites of the control group animals remained in the active inflammatory phase. Thus, a prolonged inflammatory phase may have caused excessive fibrosis and stricture after esophageal ESD in the control group.

Na et al. reported the insufficient efficacy of allogeneic OMCS transplantation to prevent post-ESD esophageal stricture in a porcine model [45]. There could have been problems with the quality control of OMCSs, such as cell proliferation ability, density, and bacterial contamination. These issues make it impossible to judge the efficacy of allogeneic OMCS transplantation to prevent esophageal strictures after ESD. Interestingly, pig oral mucosal epithelial cells proliferated poorly and were unable to form cell sheets without feeder layers [46]. Thus, we have established a standardized culture system and protocol for the efficient and suitable transplantation of ECSs [33,36]. The cell structures and proliferation of ECSs were maintained in the present study.

Another possible drawback of allogeneic ECS transplantation is the immune rejection of transplanted cells, potentially detracting from the justification for applying this model in humans. In this study, variations in the major histocompatibility complex were minimized by selecting a closed swine colony [47], thus allowing for the omission of immunosuppressive drugs. The immunogenicity of ECSs may be clinically solved using low-pathogenicity keratinocytes, which are prepared from a low-pathogenicity cell source, such as neonatal skin [48]. In the humanized SCID mouse, tissue-engineered allogeneic skin contained only purified populations of keratinocytes engrafted for more than 28 days [49]. Thus, the transplanted epidermal cells are finally rejected after the transplantation of allogeneic ECSs. However, The rejection of transplanted allogeneic ECSs may not be observed within a week after the transplantation of allogeneic ECSs. In clinical settings, allogeneic tissue-engineered skin therapies provide biological dressings, matrix materials, cytokines, and regulatory molecules to maintain a suitable environment for wound healing without any immunological problems [50,51]. Moreover, the risk of immunological rejection can be minimized if the cell source for transplantation can be selected from a cell bank based on the human leukocyte antigen matching score, as in haplotype-based banking of human pluripotent stem cells [52]. To prevent allograft rejection and esophageal strictures, the combination therapy with steroids and transplanting allogeneic epidermal cell sheets to large mucosal defect after esophageal ESD will be expected in the future [14].

This study had several limitations. First, the number of pigs was small, corresponding to the minimum number calculated to be sufficient. Second, this study did not compare the efficacy of allogeneic and autologous ESCs in preventing esophageal strictures after ESD.

In conclusion, we developed a new minimally invasive surgical procedure using a tissue engineering approach, and our results demonstrate the effectiveness of allogeneic ECSs in preventing esophageal stricture after ESD with a focus on endoscopic and histological findings. Allogeneic ECSs and extensive ESD provide an optimal treatment strategy for widespread superficial esophageal carcinoma.

Author contributions

Shinichiro Kobayashi primarily designed and wrote this article. Nobuo Kanai helped with ESD and endoscopic observations, contributed to the discussion, and reviewed/edited the manuscript. Masayuki Yamato confirmed the research projects and edited the entire contents of the main body of the text. Susumu Eguchi supervised the research projects and approved the final submission of this article.

Declaration of competing interest

Prof. Masayuki Yamato are shareholders of Cell Seed Inc.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2022.06.008.

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