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Original Article

Generating tissue-engineered intestinal epithelium from cultured Lgr5 stem cells *in vivo*

Zhassulan Baimakhanov, Yasuhiro Torashima, Akihiko Soyama, Yusuke Inoue, Yusuke Sakai, Mitsuhisa Takatsuki, Fumihiko Fujita, Kengo Kanetaka, Tamotsu Kuroki, Susumu Eguchi*

Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences, Japan

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ABSTRACT

Introduction: Generating tissue-engineered small intestine (TESI) from mature intestinal cells has been established in a mouse model. The purpose of this study was to generate TESI from Lgr5 stem cells *in vivo*.

Methods: We used Lgr5-EGFP mice for intestinal crypt isolation. After seven days, cultured crypts with Lgr5 stem cells were seeded onto a biodegradable polymer and implanted into omentum of NOD/SCID mice.

Results: Engineered intestinal epithelium was generated from Lgr5 stem cells after four weeks of *in vivo* implantation. Intestinal epithelium was immunohistochemically positive for Paneth cells, enteroendocrine cells, goblet cells, microvilli of the absorptive enterocytes and Ki67.

Conclusion: Our observations suggest that transplanted Lgr5 stem cells can differentiate into the intestinal epithelium *in vivo* with further proliferative activity.

The procedure for generating tissue-engineered small intestine (TESI) from donor mature intestinal cells has been established in an animal model [1,2]. Histologically, the engineered small intestine was similar to the native intestine and contained well differentiated epithelium, muscularis, nerves and blood vessels [2]. Previous experiments showed that the TESI could be used as part of an effective treatment strategy in the rat short bowel syndrome model, and could increase the postoperative weight [1]. Additionally, TESI generated from human and mouse small intestines were transplanted into immunodeficient host mouse omentum, and the resulting intestines demonstrated digestive and absorptive functionality, including the ability to absorb sugars [3]. However, despite the progress in research related to the method to generate TESI, there are still many challenges to introducing this type of treatment in clinical practice. One of the main unresolved issues is that current protocols for making TESI require a large number of mature donor intestinal cells, which can cause considerable difficulties due to the critical shortage of donor organs. Therefore, alternative sources of the cells are required. Previously, it has been shown that Lgr5 stem cells constitute multipotent stem cells and generate all cell types of the intestinal epithelium [4]. Furthermore, long term *in vitro* culture conditions have been established, under which organoids were cultured for more than 8 months with continuously expanding, self-organizing crypt-villus like epithelial structures [5].

Considering the previously reported results, in the current study, we aimed to generate the TESI from cultured Lgr5 stem cells *in vivo*. To investigate this possibility, we isolated and cultured small intestinal crypts from heterozygous Lgr5-EGFP-cre/ERT2 mice (both sexes after genotyping, 6–10 weeks; The Jackson Laboratory, Maine, USA) according to the previous reports [4,5]. NOD/SCID mice (female, 7–8 weeks; Charles River Laboratories Japan, Inc., Yokohama) were used as the recipients. Our experiments consisted of at least six different time points, with independent cell preparations every time. All experiments were performed

Abbreviations: PAS, periodic acid-Schiff staining; TESI, tissue-engineered small intestine.

* Corresponding author. Department of Surgery, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan. Fax: +81 95 819 7319.

E-mail address: sueguchi@nagasaki-u.ac.jp (S. Eguchi).

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according to the Guidelines for Animal Experimentation at Nagasaki University. Single isolated crypts cells were cultured in Matrigel (200 crypts per well on 24-well plates). The culture medium containing growth factors (10–50 ng ml^{-1} EGF (Peprotech), 500 ng ml^{-1} R-spondin 1 and 100 ng ml^{-1} Noggin (Peprotech)) was changed every two or three days as previously described [5]. After seven or eight days in a culture without subculturing, the supernatant medium was removed and cultured organoids were mechanically collected with Matrigel by adding the cold culture medium. The supernatant was removed after centrifugation at 70 × g for 5 min and the pellet containing the crypt organoids was resuspended with Matrigel. For the *in vivo* implantation, nonwoven polyglycolic acid (2 mm thickness, 55 mg/cm^3 bulk density; Biofelt, Luxembourg) was used for the scaffold preparation as previously described [2]. The crypt organoids (2000 crypts per scaffold) were loaded on the biodegradable scaffold and directly implanted into the omentum of the NOD/SCID mice and fixed by 6-0 Prolene suture. The recipient mice were sacrificed at four weeks after implantation for an immunohistological analysis. All tissue samples were fixed with 4% paraformaldehyde in phosphate buffered solution and embedded in paraffin with subsequent slicing into 5 μm sections. Hematoxylin and eosin staining was performed according to the standard staining protocol and periodic acid-Schiff staining was according to the manufacturer's protocol (PAS staining kit, Merck KGaa, Darmstadt, Germany). For the immunological staining, deparaffinized tissue sections were treated with heat-induced antigen retrieval (using an autoclave) in a 1 mM EDTA buffer solution containing 10 mM Tris–HCl (pH 9.0). The samples were incubated with the following primary antibodies at 4 °C overnight: anti-mouse lysozyme, Chromogranin A, E-cadherin, villin (all from Santa Cruz Biotechnology Inc., Dallas, USA), a rabbit polyclonal anti-SCG l0 antibody [6], anti-mouse GFP and Ki67 (both from Abcam, MA, USA). The samples were then incubated with secondary antibodies (all from Sigma Aldrich, Missouri, USA) for 1 h at room temperature. The slides were then treated with the DAB substrate-chromogen system (DAKO, CA, USA).

At four weeks after transplantation, implanted Lgr5 stem cells in the biodegradable scaffold had grown larger and into a spherical form (Fig. 1A). The histological analysis revealed that the generated TESI from Lgr5 stem cells had proliferated. Additionally, a large quantity of differentiated mucosal epithelium was observed (Fig. 1B) with surrounding multiple blood vessels (Fig. 1C) and formed cyst-like structures with the cavity. Furthermore, neuronal cells around the generated intestinal epithelium (Fig. 1D) were observed using SCG 10 antibody, which has been shown to be a strong neuronal growth-associated protein marker [6]. The cell origin of the generated TESI was immunohistologically confirmed by detection of the specific Lgr5-GFP^+ labeled cell marker at the bottom of the engineered intestinal crypt (Fig. 1E). The small intestine of a Lgr5-EGFP-cre/ERT2 donor mouse (Fig. 1F) was used as a positive control. Further, we confirmed that the epithelial differentiation was comparable to that of the native small intestine. The intestinal epithelium in generated TESI was immunohistochemically positive for goblet cells (Fig. 2A; PAS staining), Paneth cells (Fig. 2B; lysozyme), microvilli of the absorptive enterocytes (Fig. 2C; villin), enteroendocrine cells (Fig. 2D; Chromogranin A), epithelial adherens junction protein – E-cadherin (Fig. 2E) and were still strongly positive for the proliferation marker Ki67 (Fig. 2F).

The herein described technique was successful in transplanting Lgr5 stem cell that differentiated into the intestinal epithelium *in vivo*. Furthermore, at four weeks after implantation, there remained a large number of Ki67-positive epithelial cells. Therefore, the proliferative activity of the intestinal epithelium and the maintenance of the intestinal morphology containing all four differentiated epithelial cells were confirmed. There are limitations to this protocol because of a lack of muscularis in the generated TESI. Future research into co-culture methods with mesenchymal stromal cells to generate the muscular layer in the TESI is being

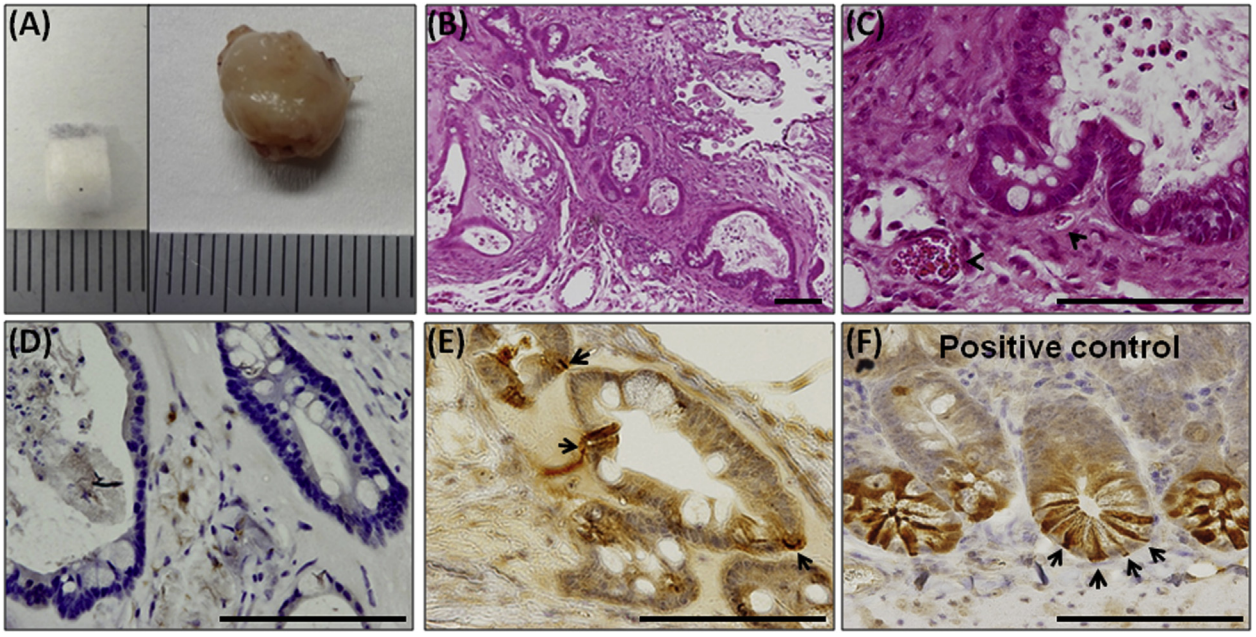

Fig. 1. Generated small intestinal epithelium from cultured Lgr5 stem cells four weeks after transplantation. (A) A macroscopic observation showed that the TESI had grown larger and into a spherical form relative to the initial scaffold size. (B, C) Hematoxylin and eosin staining showed a large quantity of differentiated mucosal epithelium surrounded with multiple blood vessels (C; arrowheads), which formed cyst-like structures with the cavity. Immunohistochemistry confirmed the existence of neuronal cells around the intestinal epithelium (D), the Lgr5-GFP^+ labeled cell marker at the bottom of the engineered intestinal crypt (E; arrows). The small intestine of a Lgr5-EGFP-cre/ERT2 donor mouse (F) was used as the positive. Scale bars: 100 μm.

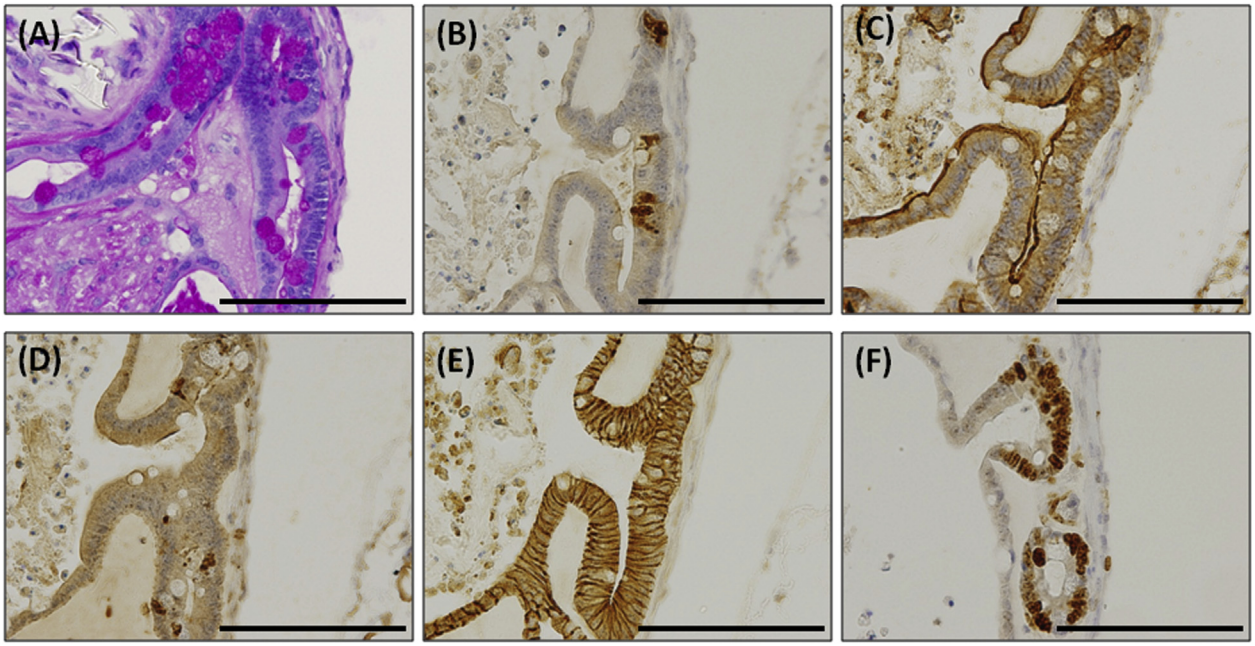

Fig. 2. Morphology of the differentiated intestinal epithelium in generated TESI from cultured Lgr5 stem cells four weeks after transplantation. The PAS staining showed goblet cells (A). Immunohistochemical staining showed the existence of Paneth cells (B), microvilli of the absorptive enterocytes (C), enteroendocrine cells (D), epithelial adherens junction protein – E-cadherin (E) and the expression of the proliferation marker Ki67 (F). Scale bars: 100 μm.

considered. Nevertheless, under pathological conditions like inflammatory bowel disease, rapid regenerative response is probably complicated from the endogenous cell source alone. Therefore, we believe that the current technique of generating small intestinal epithelium from Lgr5 stem cells offers a viable therapeutic opportunity for the treatment of inflammatory bowel disease by encouraging regeneration to damaged epithelia. Further investigations of this protocol should be conducted to determine the effectiveness of ex-vivo expanded epithelial stem cells transplantation technology, which may generate novel clinical applications for improving the intestinal function and have implications for tissue replacement.

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