

Regulation of DNA methylation levels in the process of oral mucosal regeneration of oral ulcer model

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Summary. DNA methylation is an important epigenetic mechanism for cellular maintenance. However, the methylation pattern and the key molecule regulated epigenetically in oral mucosal regeneration is unclear. In this study, we generated a rat oral ulcer model and investigated the cell proliferative activities and DNA methylation patterns immunohistochemically. We also performed immunohistochemical analysis of a regulator of epithelial stem/progenitor cell differentiation in the rat model.

We demonstrated immunohistochemistry using antibodies for the molecules as follows: Ki-67, a marker of cellular proliferation; 5-methylcytosine (5-mC), a marker of DNA methylation; 5-hydroxymethylcytosine (5-hmC), a marker of DNA demethylation; Dnmt1, a maintenance DNA methyltransferase; Dnmt3a and Dnmt3b, *de novo* DNA methyltransferases; and Wnt5a, a regulator of stem/progenitor cell differentiation.

In this model, re-epithelialization was completed at Day 4 after ulceration. Regenerating mucosal hypertrophy reached a peak at Day 5 and appeared normal at Day 14. Ki-67-positive cells increased at Day 2 and returned to normal at Day 6 after ulceration. The ratio of the expression level of 5-mC to 5-hmC declined at Day 5 and returned to normal at Day 6. The expression level of Dnmt1 had not changed compared to the normal control at every time point. On the other

hand, the expression levels of Dnmt3a and Dnmt3b had decreased significantly at Day 5 and returned to normal at Day 6. Moreover, Wnt5a-positive cells increased at Day 5.

In conclusion, oral mucosal regeneration was strictly regulated by DNA methylation. Moreover, Wnt5a might play a critical role in oral mucosal regeneration.

Key words: DNA methylation, 5-mC, 5-hmC, Wnt5a, oral ulcer

Introduction

Oral ulcers can be induced by various causes, such as trauma, microbial infections, systemic disorders, as well as iatrogenic factors (Oton-Leite et al., 2012; Vadhan-Raj et al., 2013; Awad et al., 2015). In some cases, severe oral ulcers can lead to a reduction of oral intake, undernutrition and poor quality of life (Oton-Leite et al., 2012; Vadhan-Raj et al., 2013; Awad et al., 2015). Despite the clinical or experimental development of local therapies with topical agents and systemic therapies for oral ulcers (Oton-Leite et al., 2012; Vadhan-Raj et al., 2013; Lim et al., 2016), it is sometimes difficult to complete re-epithelialization efficiently.

Chemical modifications of DNA have been recognized as key epigenetic mechanisms to determine various cell fates in homeostasis and regeneration (Bestor, 2000; Sen et al., 2010; Plongthongkum et al., 2014). Such DNA modifications include 5-methylcytosine (5-mC) and 5-hydroxymethylcytosine

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(5-hmC), and are regulated by DNA methyltransferases (Dnmts) (Plongthongkum et al., 2014; Wu and Ling, 2014). Dnmt1 maintains DNA methylation patterns after cellular replication (Sen et al., 2010), while Dnmt3a and Dnmt3b are responsible for *de novo* methylation (Bestor, 2000). Generally, DNA methylation is required for stem/progenitor cell maintenance and self-renewal (Bestor, 2000; Sen et al., 2010; Plongthongkum et al., 2014). On the other hand, demethylation is required for differentiation (Bestor, 2000; Sen et al., 2010; Plongthongkum et al., 2014). However, few studies have investigated the dynamic changes of DNA methylation level in the process of oral mucosal regeneration after ulceration.

Activation of endogenous somatic stem/progenitor cells is required for the repair of damaged tissues. The tight regulation between decisions for self-renewal and differentiation to produce daughter cells is also important in this process. Recently, it was suggested that *de novo* methylation might affect Wnt5a expression (Petell et al., 2016; Vaidya et al., 2016). Wnt signaling plays an important role in determining the cell fates in stem/progenitor cells for maintenance versus differentiation (Veltri et al., 2018). Generally, canonical Wnt signaling induces stem/progenitor cell maintenance and proliferation, while noncanonical Wnt signaling induces stem/progenitor cell differentiation (Veltri et al., 2018). Thus, it is suggested that Wnt5a, a noncanonical Wnt ligand, plays an important role in terminal differentiation in several organs, such as those related to the cardiovascular system (Mazzotta et al., 2016), the cerebellum (Subashini et al., 2017), liver (Fan et al., 2017), intestines (Bakker et al., 2012), mammary glands (Ford et al., 2009; Kessenbrock et al., 2017), lungs (Rieger et al., 2016), and skin (Sen et al., 2010).

The purpose of this study was to analyze the dynamic changes of the cell proliferative activities, DNA methylation patterns and Dnmts' expression patterns in the process of oral mucosal regeneration using an animal model in which an oral ulcer was created surgically under anesthesia. Furthermore, we focused on the role of Wnt5a to investigate whether it can act as a key molecule for oral epithelial mucosal regeneration.

Materials and methods

Animals

Thirty-six Wistar rats (males, 6 weeks, 192 to 248 g) were used in this study. An ulcer 2.5 mm in diameter was created using forceps in the right buccal mucosa under anesthesia, using a combination anesthetic (0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol). The ulcer was created to the depth of the muscle layer and 0.2% indigo carmine was dropped on the ulcer floor to mark the ulcerative lesion. The left buccal mucosa was left untreated and used as control. Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University with the

approval of the Institute of Animal Care and Use Committee (approval number 1304301058).

Tissue preparation

All of the rats were euthanized using an intraperitoneal injection of 200 mg/kg pentobarbital at Days 0 to 7 and Day 14 (n=4, in each group) after ulceration and the buccal mucosa was collected for histological and immunohistochemical analysis. Each sample was fixed with 4% paraformaldehyde in a phosphate-buffered saline (PBS) at 4°C overnight (Yamamoto-Fukuda et al., 2010; Akiyama et al., 2014) and embedded in paraffin. Serial sections (5 µm thick) were prepared and then mounted on 3-aminopropyltriethoxysilane-coated slides. For the morphological examination, hematoxylin and eosin (H&E) staining was performed using a standard procedure.

Antibodies

Rabbit polyclonal anti-Ki-67 antibody (product no. NB110-89719) (Novus Biologicals, CO, USA) (1:400) was used as a marker of cellular proliferation. Rabbit polyclonal anti-5-hmC antibody (product no. 39769) (1:500) was from Active Motif (Carlsbad, CA, USA). Mouse monoclonal anti-5-mC antibody (Clone no. 162 33 D3, product no. NA81) (1:400) was from Calbiochem (San Diego, CA, USA). Mouse monoclonal anti-Dnmt1 antibody (Clone no. 60B1220.1, product no. ab92453) (1:250), rabbit polyclonal anti-Dnmt3a antibody (Product no. ab4897) (1:200) and rabbit polyclonal anti-Dnmt3b antibody (Product no. ab119282) (1:200) were from Abcam (Cambridge, MA, USA). A rabbit polyclonal antibody against Wnt5a (Abcam, product no. ab174963) (1:100) was used as a marker for a noncanonical Wnt signaling pathway which regulates epithelial stem or progenitor cell maintenance to differentiation (Ford et al., 2009; Sen et al., 2010; Bakker et al., 2012; Mazzotta et al., 2016; Rieger et al., 2016; Fan et al., 2017; Kessenbrock et al., 2017; Subashini et al., 2017).

Immunohistochemistry

The slides stained with 5-mC, 5-hmC or Wnt5a were visualized by fluorescence conjugate by the indirect immunofluorescent staining and the slides stained with Ki-67 or Dnmts were visualized by 3,3'-diaminobenzidine tetrahydrochloride (DAB) or DAB with Co-Ni/H₂O₂ solution (Adams, 1981) respectively, by the indirect enzyme-labelled antibody method, as described previously (Yamamoto-Fukuda et al., 2010, 2018; Akiyama et al., 2013, 2017). Briefly, the sections were deparaffinized with toluene and rehydrated with serially graded ethanol solutions. Antigen retrieval was performed by autoclave in a 0.01M citrate buffer (pH 6.0) at 120°C for 10 minutes for Ki-67, Dnmts and Wnt5a. They were preincubated with 500 µg/ml normal

goat IgG in 1% bovine serum albumin (Sigma) in PBS for one hour to block a nonspecific reaction. The slides were then incubated overnight with the first antibodies. The slides were washed four times with 0.075% Brij 35 (Sigma) in PBS, and reacted with Alexa Fluor 488 goat anti-rabbit IgG (1:500), Alexa Fluor 546 goat anti-mouse IgG (1:500), HRP goat anti-mouse IgG (1:100) or HRP goat anti-rabbit IgG (1:100) for one hour as the secondary antibody, respectively, and washed with 0.075% Brij 35 in PBS. The slides were then counterstained with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Invitrogen) (1:1000) for 5-mC, 5-hmC and Wnt5a, or counterstained with Hematoxylin for Ki-67. For a negative control, normal mouse or rabbit IgG was used instead of the first antibodies. Fluorescent images were obtained using a Zeiss LSM 780 or 880 confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

Quantitative analysis

For the quantitative analysis of Ki-67 or Dnmts, more than 1,000 cells were counted in random fields at 400 magnification, and the number of positive cells was expressed as a percentage of positive cells per total number of counted cells, respectively, [Ki-67 or Dnmts labeling index (LI); mean $\pm$ standard deviation (SD)] (Yamamoto-Fukuda et al., 2003, 2015).

Image analysis

For the quantitative analysis of 5-mC and 5-hmC, the mean fluorescence intensities were analyzed with

Image J software, which is a public domain, Java-based image processing software package developed at the National Institutes of Health (NIH) (Schneider et al., 2012).

Using an Image J measuring tool, the average value of the fluorescence intensity in each cell nucleus in the basal layer or in the upper layers above the basal layer was measured on a minimum of 1000 cells for each sample from the fluorescence image.

Statistical analysis

All data are presented as mean $\pm$ SD. One-way ANOVA followed by Dunnett's post hoc tests for Ki-67 LI and for Dnmts LI or Tukey's post hoc tests for the ratio of the expression level of 5-mC to 5-hmC was performed using statistical software packages (JMP 13; SAS Institute Inc., Cary, NC). For all tests, 2-tailed p values of <0.05 were considered statistically significant.

Results

Morphological changes in the process of oral mucosal regeneration after oral ulcer formation

H&E staining revealed that an oral ulcer was successfully created in the dermis of the buccal mucosa in this study model at Day 0 (Fig. 1A,B). The beginning of re-epithelialization was observed at Day 2 (Fig. 1C) and the completion of re-epithelialization confirmed in all of the rats at Day 4 after ulceration (Fig. 1D). Thickening of the regenerated epithelium continued to increase by Day 5 (Fig. 1E) and invagination of the

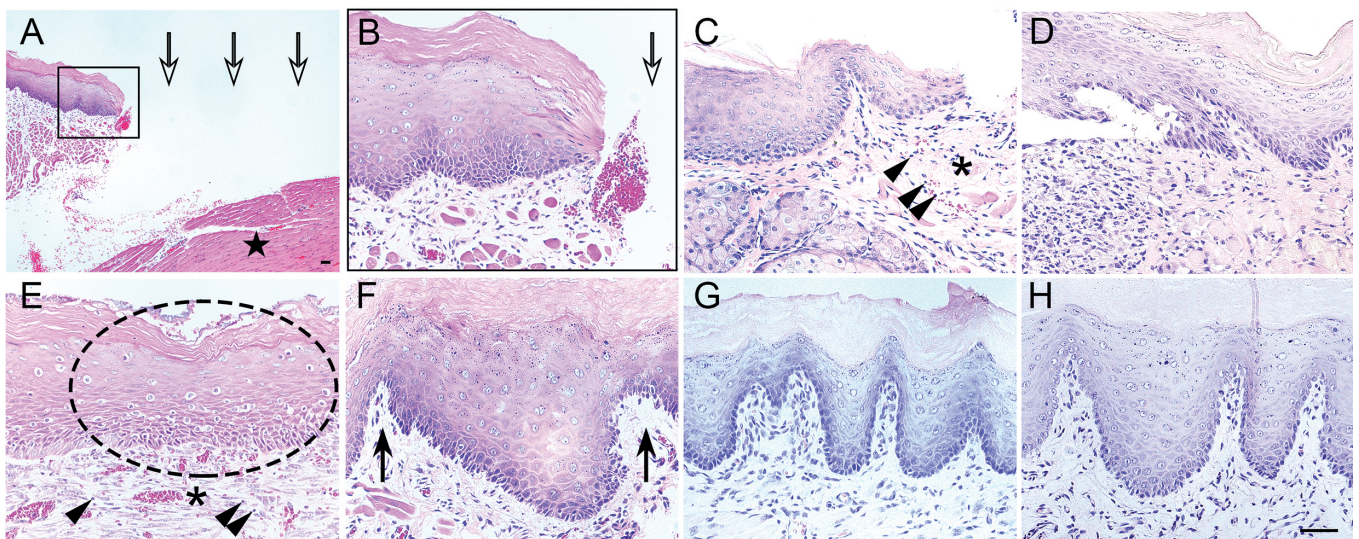
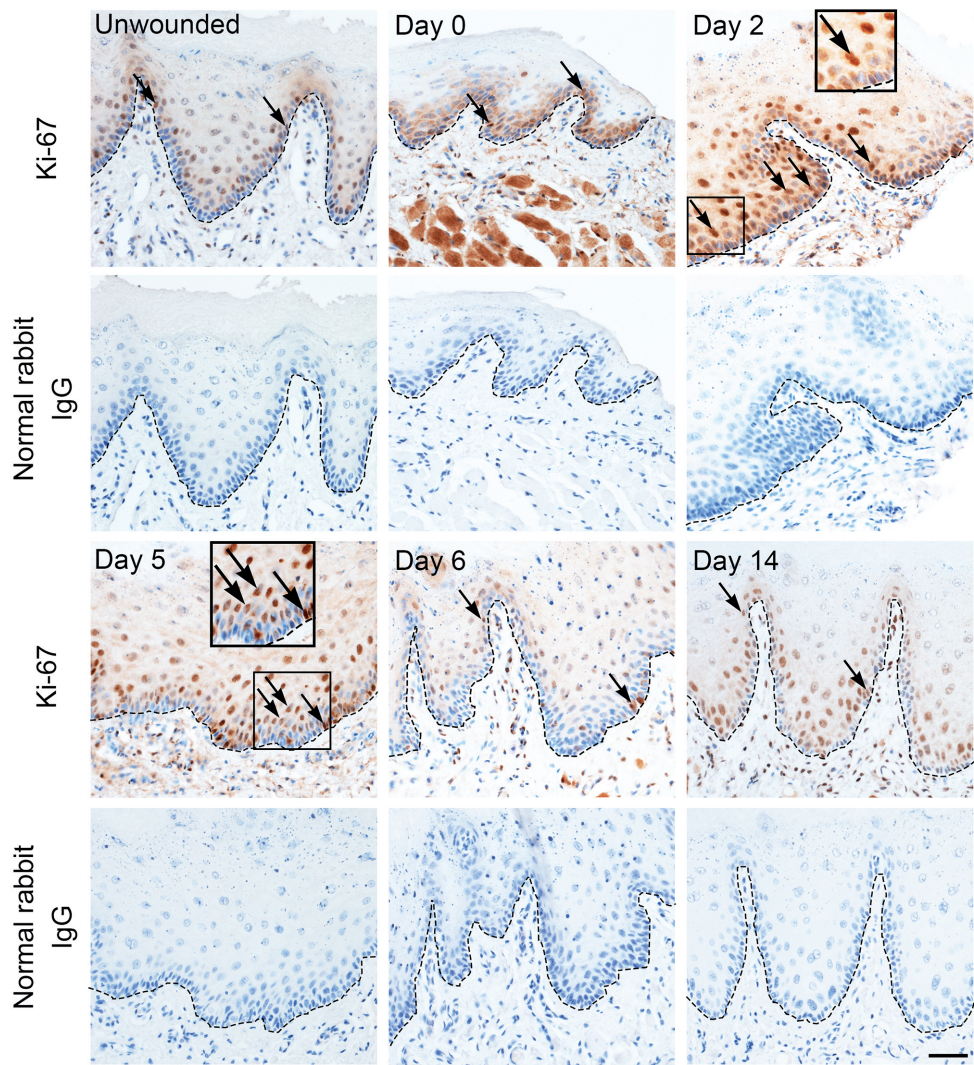


Fig. 1. H&E staining of the buccal mucosal tissue after ulceration. Morphological changes in the buccal mucosa at Day 0 (A, B), Day 2 (C), Day 4 (D), Day 5 (E), Day 6 (F) and Day 14 (G) after ulceration. H. Normal buccal mucosa. Open arrow: ulcer formation in the dermis; Star: muscle layer; Arrow heads: infiltration of inflammatory cells in the stroma; Asterisks: increase of collagen fibers in the stroma; Dashed circle: regenerated epithelium; Black arrows: invagination of the epithelium. Scale bar: 50 μ m.

A



B

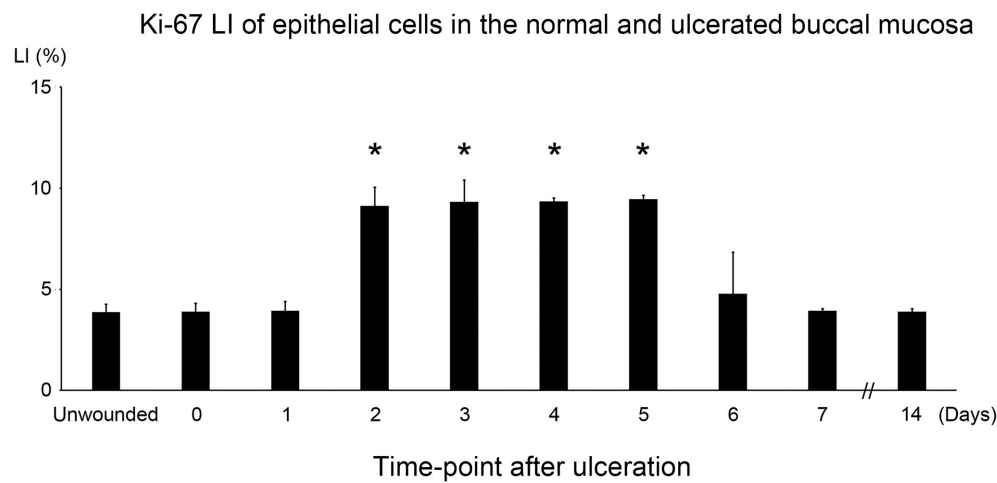
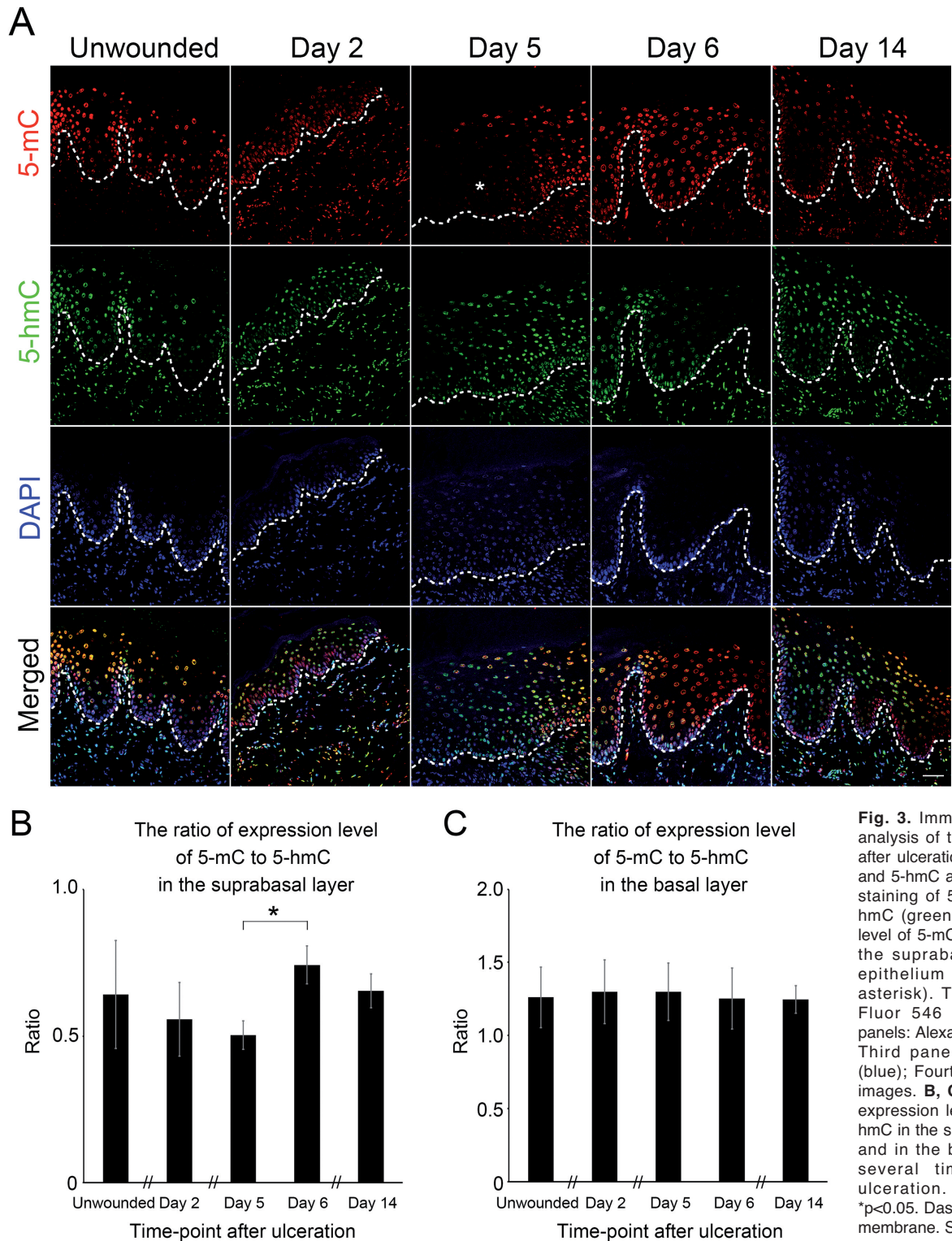


Fig. 2. Immunohistochemical analysis of the buccal mucosa after ulceration using an anti-Ki-67 antibody. **A.** The slides of normal and ulcerated buccal mucosa stained for Ki-67 or normal rabbit IgG at several time points. **B.** Ki-67 labeling index (LI) of the epithelial cells in the unwounded and ulcerated buccal mucosa. N=4 per group. *p<0.0001. Dashed line: basement membrane; Arrows: positive cells; Inset: high-power view. Scale bar: 50 μ m.

epidermis was observed at Day 6 (Fig. 1F). Infiltration of inflammatory cells, mainly consisting of lymphocytes, and an increase in collagen fibers were observed at Day

2 and increased by Day 5 (Fig. 1C,E). Inflammatory cells and collagen fibers then gradually decreased (Fig. 1F). At Day 14 after ulceration, the regenerated



epithelium and stroma revealed a normal appearance, as on an unwounded mucosa (Fig. 1G,H).

Proliferative activities of epithelial cells in the process of oral mucosal regeneration after oral ulcer formation

The results of the immunohistochemical analysis of proliferative activities of epithelial cells in the oral mucosal epithelium after ulceration using an anti-Ki-67 antibody revealed that Ki-67 LI had significantly increased at Day 2 (Day 2 vs. Unwounded: 9.12 ± 0.92 vs. 3.88 ± 0.37 , $p < 0.0001$, Dunnett) and returned to normal at Day 6 (Day 6 vs. Unwounded: 4.77 ± 2.05 vs. 3.88 ± 0.37 , not significant) after ulceration in the epithelium (Fig. 2A,B). Ki-67-positive cells had increased not only in the basal layer but also in the suprabasal layer of the regenerating buccal epithelium at

Day 5 but had returned to the normal level at Day 6 after ulceration (Fig. 2A).

Analysis of DNA methylation levels and Dnmts' expression in the normal and ulcerated buccal mucosa

As a result, the expression level of 5-mC in the suprabasal layer tended to decrease at Day 5 after ulceration (Fig. 3A). To confirm this tendency, we analyzed the expression ratios of 5-mC to 5-hmC at several time points. As expected, the ratio in the suprabasal layer had decreased at Day 5 and had returned to the normal level at Day 6 after ulceration (Day 5 vs. Day 6: 0.39 ± 0.048 vs. 0.74 ± 0.065 , $p < 0.05$, Tukey) (Fig. 3B). On the other hand, the ratios did not change in the basal layers at every time point (Fig. 3C). We then performed immunohistochemistry using anti-Dnmt1,

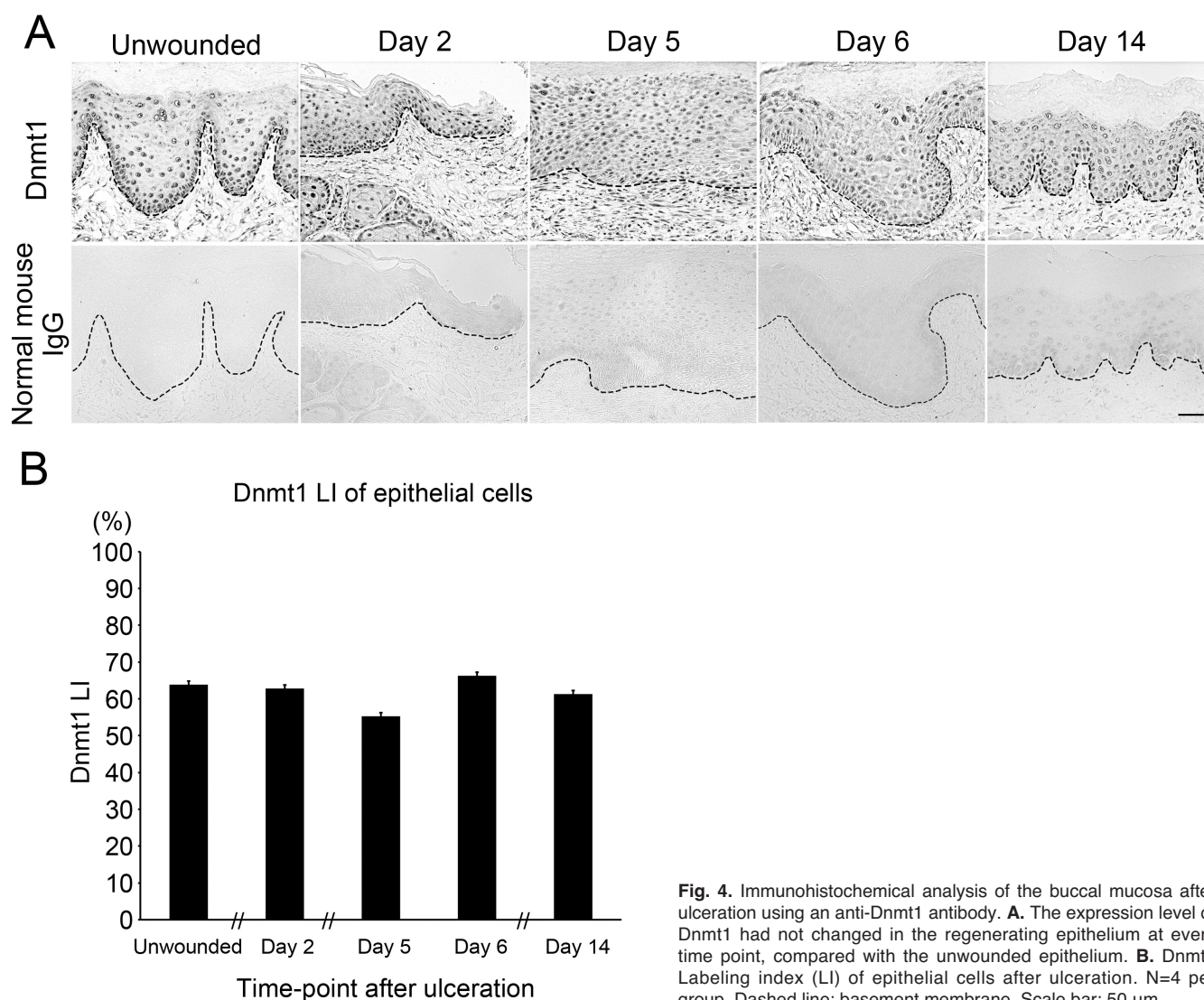


Fig. 4. Immunohistochemical analysis of the buccal mucosa after ulceration using an anti-Dnmt1 antibody. **A.** The expression level of Dnmt1 had not changed in the regenerating epithelium at every time point, compared with the unwounded epithelium. **B.** Dnmt1 Labeling index (LI) of epithelial cells after ulceration. N=4 per group. Dashed line: basement membrane. Scale bar: 50 μ m.

Dnmt3a, and Dnmt3b antibodies. The expression level of Dnmt1 had not changed compared to the normal control at every time point (Fig. 4A,B). On the other hand, the expression level of Dnmt3a and Dnmt3b had significantly decreased at Day 5 (Dnmt3a, Day 5 vs. Unwounded: 18.65 ± 4.94 vs. 31.15 ± 3.56 , $p < 0.001$, Dunnett; Dnmt3b, Day 5 vs. Unwounded: 38.99 ± 18.04 vs. 58.40 ± 1.61 , $p < 0.05$, Dunnett) (Fig. 5A-C).

Analysis of the Wnt5a expression pattern in the regenerating buccal mucosa after ulceration

The results showed that the number of Wnt5a positive cells had increased in both the basal and suprabasal layers of the regenerating epithelium only at Day 5 after ulceration and was the same as the unwounded epithelium at Day 6 after ulceration (Fig. 6).

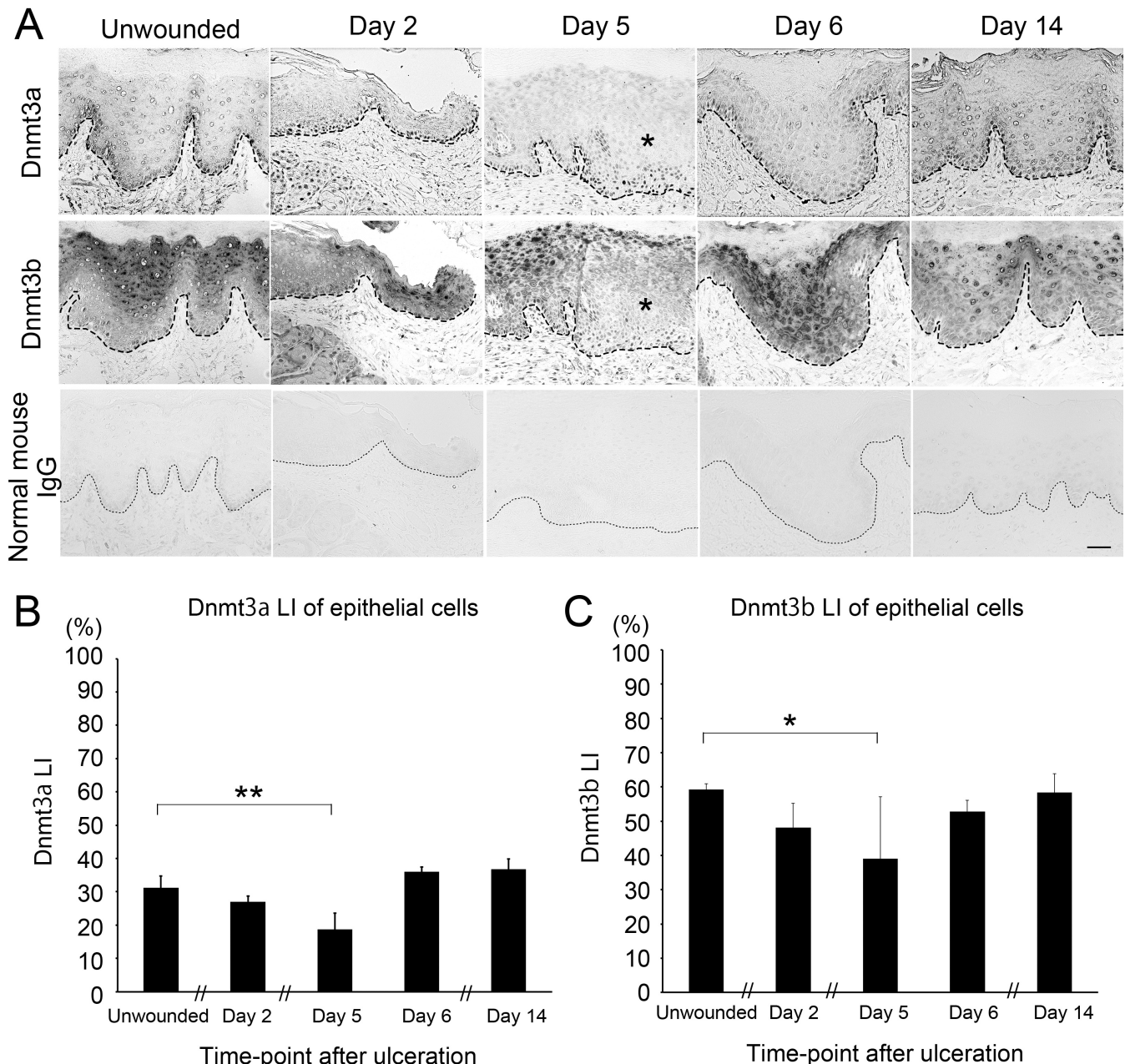


Fig. 5. Immunohistochemical analysis of the buccal mucosa after ulceration using anti-Dnmt3a and anti-Dnmt3b antibodies. **A.** The expression level of Dnmt3a and Dnmt3b had decreased in the basal and in the suprabasal layers of the regenerating epithelium only at Day 5 after ulceration (asterisk) but had not changed at the other time points. Dnmt3a (**B**) and Dnmt3b (**C**) Labeling index (LI) of epithelial cells after ulceration. N=4 per group. * $p < 0.05$, ** $p < 0.001$. Dashed line: basement membrane. Scale bar: 50 μ m.

Discussion

In this model, oral mucosal re-epithelialization was observed at Day 2 and was complete at Day 4 after ulceration. Regenerating mucosal hypertrophy reached a peak at Day 5 after ulceration. An immunohistochemical analysis for Ki-67 revealed that the proliferative activities were upregulated from Day 2 to Day 5 and returned to normal at Day 6 after ulceration. Ki-67-positive cells had increased not only in the basal layer but also in the suprabasal layer of the regenerating buccal epithelium. Previously, epithelial stem cells were to be localized only in the basal layer of epidermis and these cells become activated and contribute substantially to repair in wound healing spanning from the basal layer

to the suprabasal layer (Blanpain and Simons, 2013). Recently, it has been suggested that stem/progenitor cells in the epidermis undergo asymmetrical cell division temporally and spatially in wound healing (Ouspenskaia et al., 2016). Based on this, it has been suggested that an increase in Ki-67-positive cells in the suprabasal layer might divide asymmetrically into stem or proliferating progenitor cells, and that this stem/progenitor cell proliferation might play an important role during the proliferative stage in oral epithelial regeneration.

DNA methylation due to Dnmts is thought to be tightly regulated in stem/progenitor cells and necessary for maintaining stemness or progenitor function (Bestor, 2000; Sen et al., 2010). In the previous study, it was reported that differentiation-related genes of the

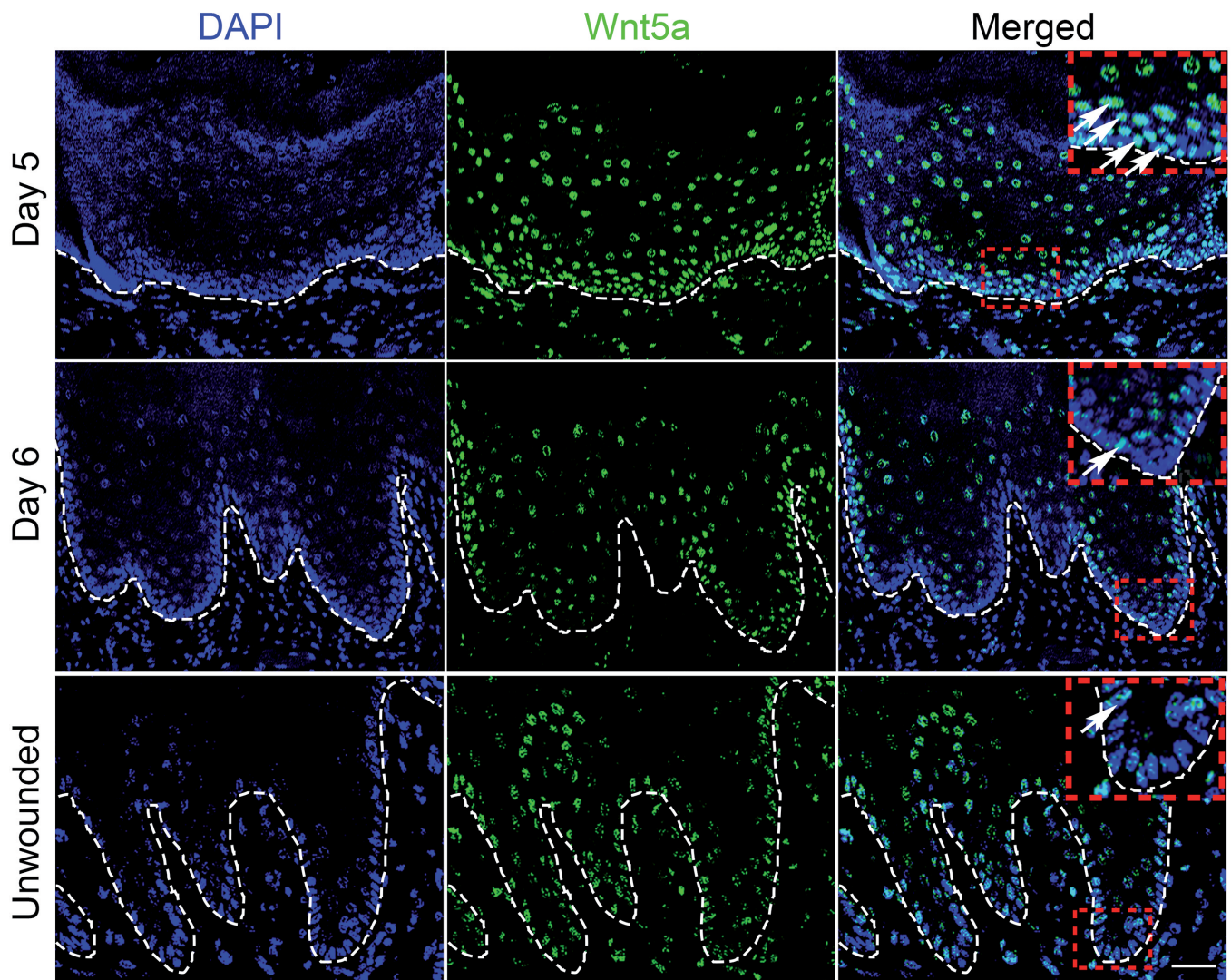


Fig. 6. Immunohistochemical analysis of the buccal mucosa after ulceration using anti-Wnt5a antibody. The number of Wnt5a-positive cells had increased at Day 5 in both the basal and suprabasal layers of the regenerating epithelium after ulceration. By contrast, it was the same as the unwounded epithelium at Day 6 after ulceration. Dashed line: basement membrane; Arrows: positive cells; Inset: high-power view. Scale bar: 50 μ m.

epidermis were enriched for promotor methylation in undifferentiated keratinocytes (Sen et al., 2010; Plongthongkum et al., 2014). Once undifferentiated keratinocytes were induced to differentiation, most of the methylated promotor region of the genes was demethylated (Sen et al., 2010). Moreover, it was also demonstrated that the knockdown of Dnmt in undifferentiated keratinocytes induced terminal differentiation efficiently (Sen et al., 2010). In this study, it was revealed that the expression ratio of 5-mC to 5-hmC in the epithelial cells in oral mucosa was significantly reduced and the expression level of Dnmt3a and Dnmt3b was also reduced in the regenerating epithelium at Day 5 after ulceration. In other words, a decrease of DNA methylation levels was observed in the suprabasal layers of the regenerating hypertrophic epithelium at Day 5. These results indicated that the stimulation of stem/progenitor proliferative activities following demethylation played a crucial role for epithelial cell differentiation in the thickened regenerating epithelium after ulceration. Interestingly, the expression level of Dnmt1 had not changed compared to the normal control at every time point. And thus, this process might not depend on maintenance DNA methyltransferase activity but on *de novo* DNA methyltransferase activities. Recently, it has been revealed that *de novo* methylation due to Dnmt3a and Dnmt3b may regulate stem/progenitor cell differentiation (Rajasingh et al., 2011; Avgustinova and Benitah, 2016; Ouspenskaia et al., 2016). To investigate whether Wnt5a, a noncanonical Wnt ligand, could play an important role for the epithelial cell differentiation controlled by DNA methylation and demethylation for oral mucosal re-epithelialization, we analyzed the Wnt5a expression pattern in the regenerating epithelium at Day 5, when the expression levels of Dnmt3a and Dnmt3b were lower and the proliferating cells in the regenerating epithelium were expected to begin differentiation. We also analyzed the Wnt5a expression pattern in the regenerating epithelium at Day 6, when the expression levels of Dnmt3a and Dnmt3b had returned to normal. According to the results, the number of Wnt5a positive cells had increased at Day 5 not only in the basal layers but also in the suprabasal layers, and was the same as normal epithelium at Day 6 after ulceration. These results indicated that the temporal reduction of the expression level of Dnmt3a and Dnmt3b might dominantly induce the reduction of the DNA methylation level in the suprabasal layers of the regenerating epithelium and could induce downregulation of stem/progenitor cell proliferation. Furthermore, it was suggested that a decrease in Dnmt3a and Dnmt3b expression might accelerate stem/progenitor cell differentiation through the upregulation of Wnt5a expression.

In this model, it was indicated that the DNA methylation level was critically controlled between proliferation and differentiation in the process of oral mucosal regeneration after ulceration. Moreover, Wnt5a,

representing a noncanonical Wnt ligand, might possibly play an important role in the regulation of stem/progenitor cell maintenance to differentiation. In conclusion, it was suggested that the temporal and spatial regulation of the DNA methylation level and Wnt5a expression pattern may possibly play an important role in oral mucosal regeneration, although further investigation is required.

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