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**Accurate determination of S-phase fraction in proliferative cells by dual fluorescence and peroxidase immunohistochemistry with 5-bromo-2'-deoxyuridine (BrdU) and Ki67 antibodies**

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**Running headline: DUAL LABELING OF BRDU AND KI67**

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**SUMMARY** To ensure the maintenance of tissues in mammals, cell loss must be balanced with cell production, the proliferative activity being different from tissue to tissue. In the present paper, we propose a new method for the quantification of the proliferative activity, defined as the S-phase fraction of actively cycling cells, by dual labeling with fluorescence and peroxidase immunohistochemistry using BrdU (marker of S-phase) and Ki67 antibodies (marker of G1, S, G2 and M phase) after a one-step antigen retrieval. This method allows reproducible staining of BrdU/Ki67 for the quantification of proliferative activities, demonstrated here in several tissues. In the generative cell zones of fundic and pyloric glandular stomachs, where the majority of cells were cycling, we measured a proliferative activity of 30-31%. In epithelium of the forestomach and the skin, where cycling cells are intermingled with G<sub>0</sub> and differentiated cells, proliferative activities were 21% and 13%, respectively. In the adrenal cortex, in which cycling cells were sparsely distributed, the proliferative activity reached 32%. During the regenerative process in the skin after a lesion, the proliferative activity increased in proximity to the wound. These findings show that the proliferative activity is different between tissues and depends on the physiological or pathological state.. Our results presented here demonstrate the present dual labeling method is useful for estimating the proliferative activity in any tissue containing cells in and out of the cell cycle, under both physiological and pathological conditions.

**Key words:** BrdU, Ki67, stomach, adrenal gland, skin, fluorescence immunohistochemistry, peroxidase immunohistochemistry

## Introduction

To maintain tissue homeostasis, the cell loss by apoptosis should be compensated by a steady supply of cells by proliferation. The balance between cell loss and production is strictly controlled in normal adult tissue, since an excess of cell loss will result in atrophy and progressive tissue failure, while excessive cell production may cause hyperplasia and even tumors. Therefore, the understanding of how such a fine balance is achieved is particularly relevant for human health.

The proportion and the distribution of cells in cell cycle are different from tissue to tissue. For detecting actually proliferating cells in cell cycle, the nuclear protein Ki67 is frequently used, since Ki67 is expressed in all phases of cell cycle including G<sub>1</sub>, S, G<sub>2</sub>, and M, but is absent in resting cells (G<sub>0</sub>) and differentiated cells (Gerdes et al. 1982, 1984, Burger et al. 1986, Lelle et al. 1987). Ki67 immunohistochemistry, however, can only yield information about the proliferative *state* of the cells, but not about the *rate* at which they are produced (Nakajima et al. 1999). To estimate the rate of cell proliferation, the labeling of S-phase by incorporation of [<sup>3</sup>H]thymidine or its nonradioactive pyrimidine analogue 5-bromo-2'-deoxyuridine (BrdU) (Gunduz 1985, Langer et al 1985) is frequently used. These S-phase markers were previously used for analyzing the proliferative activity in tissues having distinct regions composed of proliferating cells; i.e. the generative cell zones of the gastrointestinal tract (Lipkin et al., 1964; Hattori and Fujita 1976; Vries et al. 2010).

In most tissues and organs, however, the proliferating cells are not spatially accumulated like the generative cell zones of the intestinal crypt bottoms, but are dispersed and intermingled with dormant G<sub>0</sub> and/or differentiated cells. For example, in the cortex of adrenal gland, proliferative cells are very sporadically distributed in the zona glomerulosa and ourter half of the zona fasciculata (Ulrich-Lai et al 2006). Previously, [<sup>3</sup>H]thymidine or BrdU were used, but they were only used for localizing S-phase cells in the adrenal gland (Schulte

et al. 2007). In the present study, we developed a new method enabling the quantification of S-phase-fraction (S-phase cells/ actively cycling cells) by the dual labeling fluorescence/oxidase immunohistochemistry using BrdU and Ki67 antibodies.

Using this method, we accurately quantified the “proliferative activity”, here defined as the S-phase fraction of a proliferating cell population, in the forestomach, the skin and the adrenal gland, where proliferating cells are sparse in the tissue. In addition, using the same method we showed the effect of a skin lesion on the proliferative activity surrounding the wound.

## **Materials and Methods**

### ***Animals***

Eight weeks-old male C57BL/6 mice (Japan Lab Animals Co., Ltd., Osaka, Japan) were used in this study. BrdU (0.15 mg/g body weight; Sigma; St Louis, MO) was injected intraperitoneally. After 30 minutes, animals were sacrificed by decapitation. Stomach, adrenal gland, and dorsal skin were excised. Sampled tissues were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 48hr. After fixation, the tissues were embedded in paraffin and 3  $\mu\text{m}$ -thick sections were prepared using a microtome. The sections were then mounted on glass slides. For the skin epidermis regeneration study, animals were anesthetized deeply with ether, and then a 3  $\text{mm}^2$  area in the back skin was excised, containing the epidermis, dermis and subcutaneous tissue. After 5 days following the lesion, animals were injected with BrdU and sampled as described above. All studies reported here were approved by the Animal Experimentation Committee of Kyoto University.

### ***Immunohistochemistry***

Paraffin-embedded, glass slide-mounted mouse tissue sections were deparaffinized and hydrated. For antigen retrieval, sections were then boiled under pressure in 10 mM Tris-HCl (pH 9.0) 1 mM EDTA for 2.5 min. After pressurizing was stopped, the cooker was left to cool at room temperature for 1 min, followed by cooling with tap water.

After rinsing in 0.01M phosphate-buffered saline (PBS) for 5 min, the sections were incubated with the mixture of primary rat monoclonal anti-BrdU antibody (code no. BU1/75(ICR1); Abcam: dilution 1:2000 with 0.01M PBS) and rabbit anti-Ki67 antibody

(Abcam; dilution 1:500) overnight at 4°C. After rinsing with 0.01M PBS containing 0.1% Tween20, sections were incubated with the mixture of three species-specific secondary antibodies: donkey anti-rat Alexa 488-conjugated IgG (Molecular Probes; dilution 1:200), donkey anti-rat biotin-conjugated IgG (Jackson ImmunoResearch; dilution 1:1000) and donkey anti-rabbit Alexa 594-conjugated IgG (Molecular Probes; dilution 1:200) for 1 hr at room temperature.

Fluorescent images were acquisitioned with a fluorescence microscope (Zeiss, Oberkochen). After fluorescent pictures acquisition, sections were rinsed then treated with avidin/biotinylated horseradish peroxidase (Vector Lab; dilution 1:200) for 1 hr at room temperature, then visualized with diaminobenzidine (DAB: Wako Pure Chemical, Osaka). Sections were counterstained with hematoxylin, dehydrated, and mounted with Entellan (Merck). Sections were observed and photographed using a light microscope (Zeiss).

### ***Count and statistics***

BrdU-positive cells exhibited both Alexa 488 green fluorophore and peroxidase-DAB brown chromogens. Ki67-positive cells showed Alexa 594 red fluorophore. BrdU/Ki67-double-labeled cells showed the three labels Alexa 488, Alexa 596 and DAB. Cells from the glandular stomach (fundus and pylorus), the forestomach, the skin and the adrenal cortex were counted. Cells with brown-stained granules without Alexa488-fluorescence are melanin-storing cells, and were excluded for BrdU counting. The incidence of Alexa 488-labeled BrdU-positive staining within Alexa 594-labeled Ki67-positive cell population was measured by counting more than 100 Ki67-positive cells each, from tissues collected from four different animals, and was expressed as proliferative activity in %. For statistical analysis, one-way ANOVA followed by Scheffe's multiple

comparisons were applied.



## Results

Ki67 and BrdU stainings were found in all tissues examined with characteristic distribution patterns. In the fundic mucosa of the glandular stomach, Ki67-positive cells were accumulated in the isthmus of the gland (Lipkin et al., 1964). We define the generative cell zone as the innermost to the outermost area delimited by Ki67-positive cells previously reported in [<sup>3</sup>H]-thymidine incorporation studies (Hattori and Fujita, 1976). (**Figure 1A**). A single injection of BrdU labeled many cells in this zone (**Figure 1B, D**). As expected, all BrdU-immunopositive cells were also immunoreactive to Ki67 (**Figure 1C**), but there were some BrdU-negative Ki67-positive cells in the generative cell zone (blue arrows in high magnification photos of **Figure 1A-D**). In the pyloric mucosa of glandular stomach, Ki67-positive generative cell zone was observed near the bottom of the glandular epithelium just above the muscularis mucosae (**Figure 1E**). Similar to fundic mucosa, a number of BrdU-positive cells were observed after the BrdU loading (**Figure 1F-H**).

Double labeling of Ki67 and BrdU were also examined in the forestomach where the epithelium is covered with stratified squamous epithelium (**Figure 2**). In this tissue, Ki67 positive cells represented about 40-60% of total epithelial cells (**Figure 2A**). After BrdU injection, many of Ki67-positive cells were also immunoreactive to BrdU (**Figure 2B-D**).

In the adrenal cortex, Ki67-positive cells were found mainly in the zona glomerulosa (**Figure 3A**), and some were found in the outer half of the zona fasciculata (data not shown). The Ki67-positive cells represent 1-5% of total glomerulosa cells. After BrdU injection, about one third of Ki67-positive cells were also immunoreactive for BrdU (**Figure 3B-D**).

In the skin, Ki67-positive cells constitute about 20-40% of the one to two layered stratified epithelial cells (**Figure 3E**). Ki67 immunoreactivity was also found in sebaceous cells nearby hair follicles. After BrdU injection, double-labeled cells were sporadically found

**(Figure 3F-H).** Cells with brown granules (melanin) but devoid of Alexa 488 signal were also observed. These cells were excluded from the analysis (**dotted blue arrow in Figure 3H**).

To quantify the proliferative activity, we calculated the ratio of BrdU-labeled cells among Ki67-immunopositive cells in each tissue (**Figure 4**). In the generative cell zone of glandular stomach, the proliferative activities of fundic and pyloric gland were  $30.92 \pm 3.43$  % (mean  $\pm$  sem, n=4),  $31.33 \pm 2.12$  %, respectively. In the forestomach, the proliferative activity was  $21.44 \pm 3.95$  %. In the adrenal gland and skin, proliferative activities were  $32.74 \pm 2.47$  % and  $13.30 \pm 1.47$  %, respectively. The proliferative activity in the skin is significantly lower than that in fundic and pyloric stomach and adrenal cortex ( $p < 0.05$ ).

We also performed Ki67 and BrdU stainings in the regenerating skin after 5 days of skin excision. Neighbouring skin near the wound edge became thicker to several layered squamous epithelium, but the Ki67 and/or BrdU stainings were only observed in the stratum basale (**Figure 5**). The incidence of Ki67 immunoreactivity and BrdU incorporation in the epidermis near the wound were markedly increased. However, the skin further from the wound (1 cm distance), did not show any difference in morphology and in immunostainings for ki67 and BrdU (data not shown). The proliferative activity of skin surrounding the lesion and of distal skin was  $52.52 \pm 4.35$  % (mean  $\pm$  sem, n=3) and  $12.88 \pm 2.45$  %, respectively.

## Discussion

By the dual labeling with Ki67 and BrdU, we determined the proliferative activities in various tissues, in which the proportion and spatial distribution of proliferating cells diverged. In the generative zone of the glandular stomach, where the majority of cells are in mitotic cycle, the proliferative activity was 30-35%, for both the fundus and the pylorus. These values of proliferative activity are much higher than those of previous reports (Bykorez and Ivashchenko 1984), because we used Ki67-positive cells as a reference, whereas these authors have used an estimate of the total cells in the generative zone.

The present method allowed us to accurately quantify the proliferative activity, even in tissues in which actively cycling cells are sparsely distributed among many  $G_0$  and/or differentiated cells. In epithelia of the forestomach and the skin, where cycling cells comprise 30-60% of total cells, proliferative activities were 20% and 10–15%, respectively. In the zona glomerulosa of the adrenal cortex, where cycling cells were much less frequent than in the forestomach and skin, the proliferative activity however reached 35%. These findings suggest that the proliferative activity under physiological conditions, reflecting the ability of stem cells to provide the tissue with new cells, is different between tissues. Interestingly, we observed that the proliferative activity does not correlate with the amount of actively cycling cells in the whole tissue examined, or with how localized the cycling cell population is. Indeed, 35% of the 1-5% of cycling cells of the adrenal cortex were in the S-phase, compared with only ~20% of the 40-60% of cycling cells in the forestomach. Moreover, the aggregated cells in the generative cell zones of the stomach and the dispersed cells in adrenal cortex both showed a proliferative rate of 30-35%.

Does the proliferative activity change under pathological conditions? To address this issue, we examined the dynamic changes of Ki67 expression, BrdU incorporation and proliferative activity in the regenerating skin after a skin lesion. In the marginal skin region of

the wound, not only the expression of Ki67, but also the incorporation of BrdU increased in the stratum basale, and the proliferative activity markedly increased in the regenerating epidermis. This suggests that the cell cycle duration of epidermal cells became shorter to allow accelerated regeneration (Clausen et al. 1986). The proliferative activity, as calculated here using the dual labeling method, can be used as a marker to assess the cell regeneration ability under pathological conditions *in vivo*.

For detecting cells in S-phase, [<sup>3</sup>H]thymidine was firstly introduced in 1960s. Although the accuracy of this method has been proven, handling radioactive materials and time-consuming procedures are major drawbacks. BrdU has also been used extensively as an S-phase marker, but pretreatments necessary for visualization of BrdU in formaldehyde-fixed tissues such as hydrolysis by hydrochloride, denaturing by sodium hydroxide or formamide, and digestion by proteinase (Muskhelishvilli et al. 2003), are not compatible with the detection of Ki67 protein (Sasaki et al. 1988). We succeeded to detect both BrdU and Ki67 in paraformaldehyde fixed specimens using a one-step heat activation in pH9.0 buffer. This alkaline-heat activation method induces not only conformational change of covering proteins (Shi et al. 1996; Kajiya et al. 2009), but also denatures DNA permanently. Thus our one-step alkaline-heat activation procedure constitutes a very simple and useful method for the double retrieval of BrdU and Ki67 antigens.

In the present study, we performed dual labeling against Ki67 and BrdU with species-specific secondary antibodies with specific fluorophores or chromogen markers. Using secondary antibodies labeled with specific fluorophores with distinct excitation-emission spectra or labeled with chromogen, we could omit sequential application of the antisera, and could rapidly detect antigens without cross-reactivity. Moreover, the specificity of BrdU labeling was double-checked using Alexa 488 under fluorescent microscopy and DAB chromogen under light microscopy. In this way, we could discriminate brown DAB chromogen

granules from melanin pigments present in the skin and other regions of the body of black-haired C57BL/6 mice. Light microscopic analysis of hematoxylin-counterstained sections improved histological examination on permanent specimens.

In conclusion, we have succeeded in measuring the proliferative activity in various tissues in the body, regardless of the amount of cells actively cycling and their distribution. This dual labeling method could be applied to human tissues such as biopsy specimens and small surgical materials (Sasaki et al. 1988; van Erp et al. 1989; Tsurusawa et al. 1995) Following preincubation with BrdU containing buffer, these tissues can be subject to our dual-labeling immunohistochemistry of Ki67 and BrdU to estimate the proliferative activity of tumors and that under various diseases such as injury and inflammation. By using this method, it would be possible to estimate kinetic parameters in tissue repair and tumor progression.

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## Figure Legends

**Figure 1 Dual fluorescence and peroxidase immunohistochemistry with BrdU and Ki67 antibodies in glandular stomach.** Fundic gland mucosa (A-D) and pyloric gland mucosa (E-H) stained with Ki67-Alexa 594 (A, E), BrdU-Alexa 488 (B, F), merged images of Ki67-Alexa 594 and BrdU-Alexa 488 to see the colocalization (C, G), and BrdU-peroxidase-DAB counterstained with hematoxylin (D, H). Squared areas of A-D are magnified in the bottom of each photograph. Red arrows indicate S-phase cells (BrdU+/Ki67+ cells), and blue arrows indicate non-S-phase cells (BrdU-/Ki67+) in mitotic cycle. gcz, generative cell zone; mm, muscularis mucosae. Bars=20  $\mu$ m.

**Figure 2 Dual fluorescence and peroxidase immunohistochemistry in forestomach covered with stratified squamous epithelium.** Sections are stained with Ki67-Alexa 594 (A), BrdU-Alexa 488 (B), merge of photo-A and photo-B (C), and BrdU- peroxidase-DAB counterstained with hematoxylin (D). Squared areas of A-D are magnified in the bottom of each photograph. Note three types of cells: red arrows indicate S-phase cells (BrdU+/Ki67+ cells), blue arrows non-S-phase cells (BrdU-/Ki67+), and black arrows non-cycling cells. Bars=20  $\mu$ m.

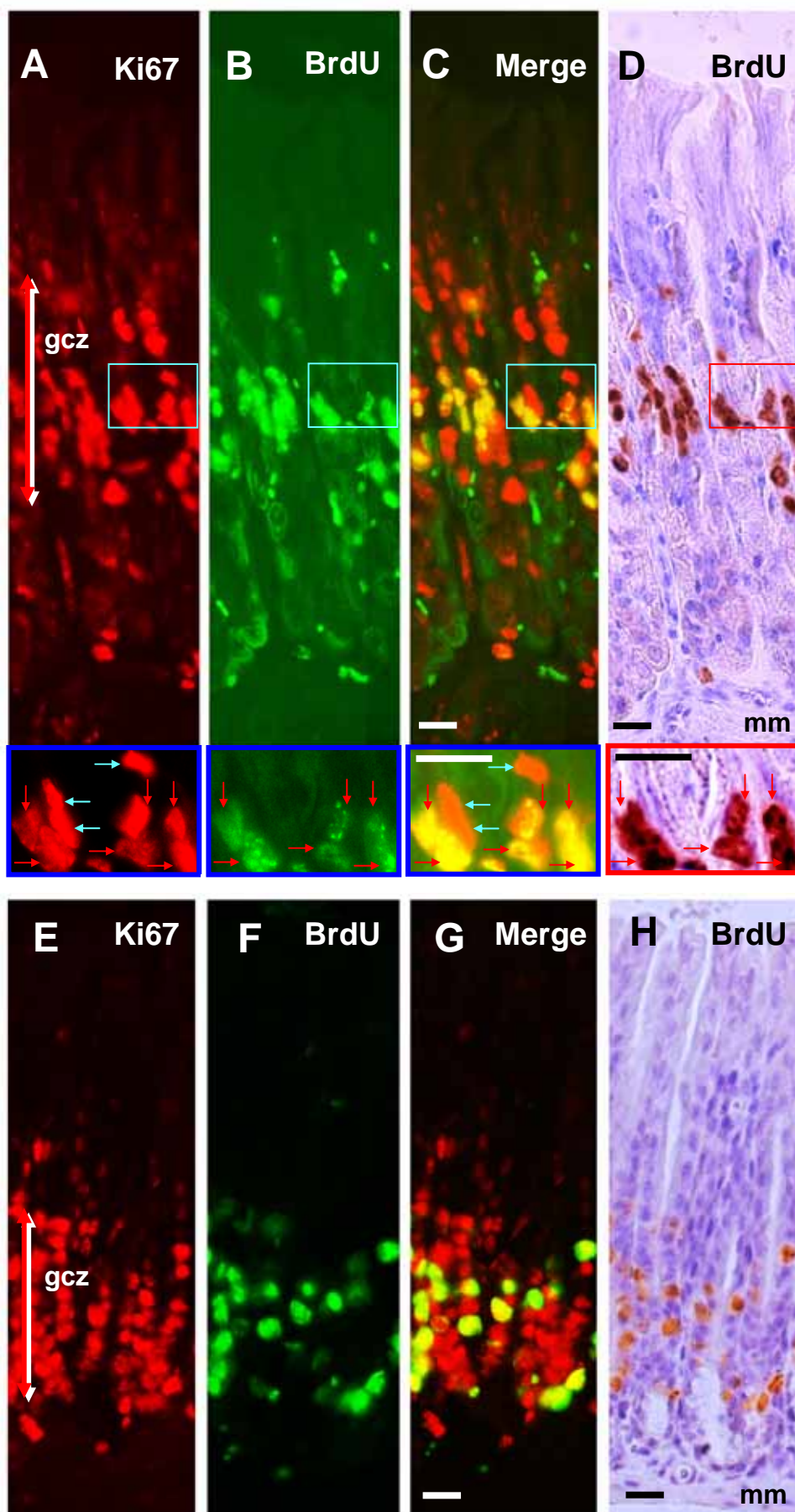
**Figure 3 Dual fluorescence and peroxidase immunohistochemistry in adrenal cortex (A-D) and back skin (E-H).** Sections are stained with Ki67-Alexa 594 (A, E), BrdU-Alexa 488 (B, F), merged images of Ki67-Alexa 594 and BrdU-Alexa 488 to see the colocalization (C, G), and BrdU- peroxidase-DAB counterstained with hematoxylin (D, H). Red arrows indicate S-phase cells (BrdU+/Ki67+ cells) and blue arrows non-S-phase cells (BrdU-/Ki67+). A dark-blue dotted arrow indicates melanin containing cells in the skin. ep, epithelial cells; hf,

hair follicle; zg, zona glomerulosa; zf, zona fasciculata. Bars=20  $\mu$ m.

**Figure 4 Proliferative activity in forestomach, fundic and pyrolic glandular stomach, adrenal cortex, and skin.** Proliferative activity is shown as a ratio of BrdU-labeling among Ki67-labeled cells. The values are expressed as means  $\pm$  SEM (n=4).

**Figure 5 Dual fluorescence and peroxidase immunohistochemistry in the regenerating skin marginal to the wound after 5 days of injury.** Sections are stained with Ki67-Alexa 594 (A), BrdU-Alexa 488 (B), merged images of Ki67-Alexa 594 and BrdU-Alexa 488 to see the colocalization (C), and BrdU- peroxidase-DAB counterstained with hematoxylin (D). Red arrows indicate S-phase cells (BrdU+/Ki67+ cells) and blue arrows non-S-phase cells (BrdU-/Ki67+). Ki67 and/or BrdU stainings were only observed in the stratum basale, but not in the thickened stratum spinosum (\*: asterisk). Note the high incidence of S-phase cells (BrdU+/Ki67+ cells) in the stratum basale marginal to the wound. Margin of wound is shown in broken line. Squared area of D shows the area of photographed region of fluorescent data of A-C. Bars=20  $\mu$ m.

Fig.1



**Fig.2**

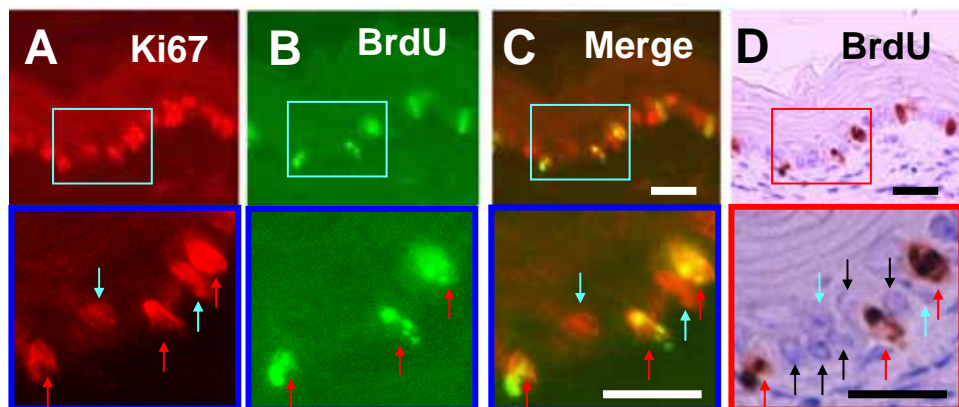
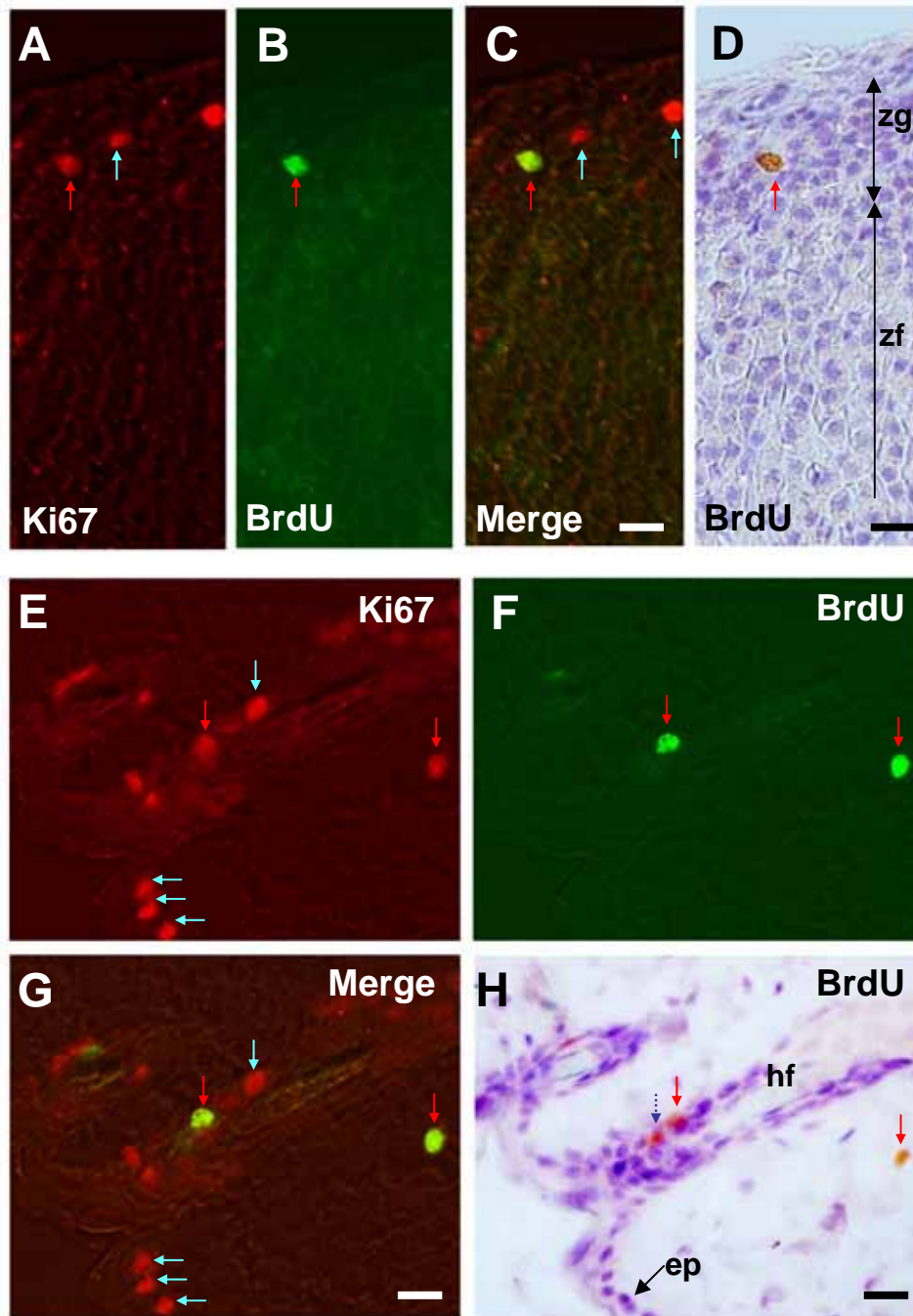
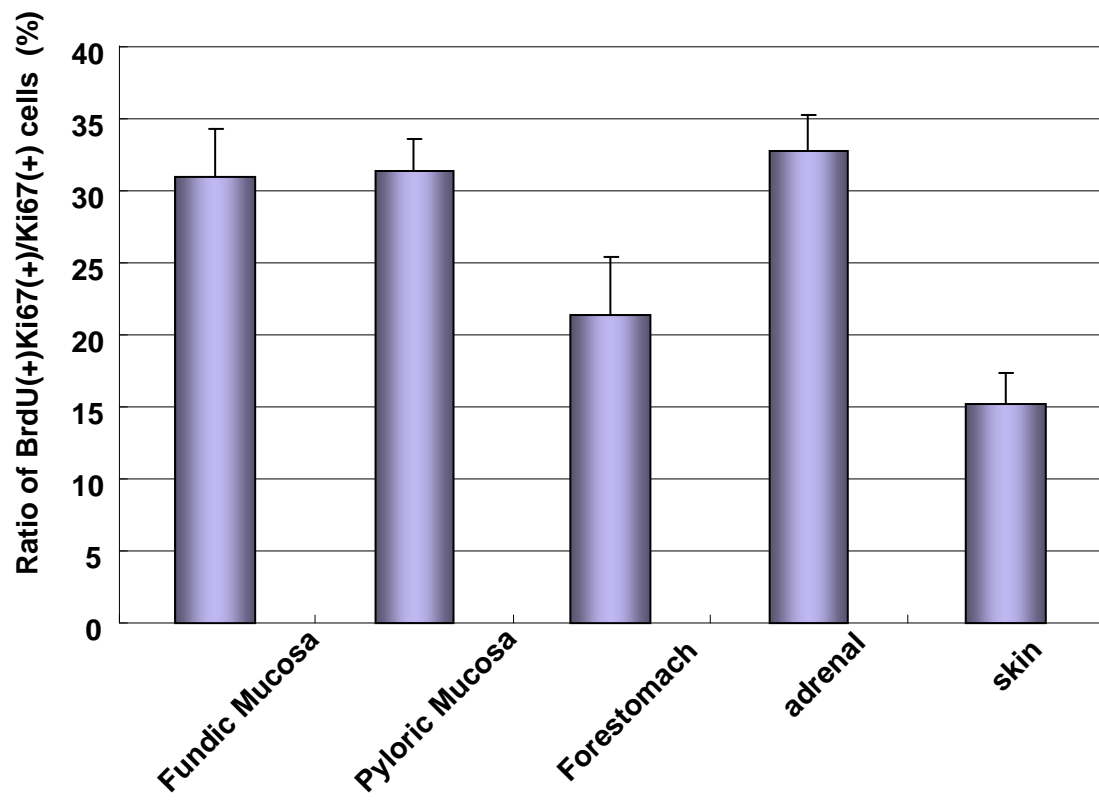


Fig.3



**Fig.4**



**Fig. 5**

