

immune regulation. They are generally anergic to TCR-mediated proliferative signals in normal mucosal conditions (28, 40, 63). The role of CTLA-4 that is expressed in activated/memory, but not naive T cells, in maintaining peripheral tolerance is well established (64, 65). Nevertheless, to our best knowledge, our observation that CTLA-4 is constitutively expressed in the LP CD4⁺CD25⁻ as well as CD4⁺CD25^{bright} T cells, but not in the PB CD4⁺CD25⁻ T cells is the first report and may be in line with the general view on the acquisition and maintenance of mucosal tolerance. Further study will be needed to determine whether or not blockade of CTLA-4 abolishes the anergic condition of the LP CD4⁺CD25⁻ T cells.

Based on the evidence that the LP CD4⁺CD25⁻ T cells not only expressed CTLA-4, GITR, and *Foxp3*, but also showed anergic cells as compared with the paired PB CD4⁺CD25⁻ T cells, we questioned whether the LP CD4⁺CD25⁻ T cells are also T_R cells as well as the LP CD4⁺CD25^{bright} cells. Unexpectedly, although we could not detect regulatory activity in the LP CD4⁺CD25⁻ subpopulation in our in vitro system at a ratio of 1 T_R:1 responder, it is possible that only a part of the LP CD4⁺CD25⁻ subpopulation expressed CTLA-4, GITR, and *Foxp3* and retains T_R activity, thereby none of the LP CD4⁺CD25⁻ cells showed regulatory function in vitro by the indicated ratio. However, it should be noted that anti-CD3/CD28-stimulated LP CD4⁺CD25⁻ T cells expressed *Foxp3* to an extent similar to the stimulated LP CD4⁺CD25^{bright} T_R cells. Consistent with this, Walker et al. (55) have very recently demonstrated that anti-CD3/CD28-stimulated CD4⁺CD25⁻ peripheral T cells express *Foxp3* and that these activated cells are equally as suppressive as natural-occurring peripheral CD4⁺CD25⁺ T_R cells. In addition, increasing evidence suggests that regulatory CD4⁺ T cells exist in the CD25⁻ compartment in mice as well (26, 30–34), although it is widely accepted that CD4⁺CD25⁺ T cells are the major population that contains T_R cells. In particular, three independent groups, including us, recently demonstrated that several fractions of CD4⁺CD25⁻ T cells, such as α E⁺CD4⁺CD25⁻ (33), LAP⁺CD4⁺CD25⁻ (66), and GITR⁺CD4⁺CD25⁻ (27), could suppress the development of murine CD4⁺CD45RB^{high} T cell-transferred colitis. Further studies will be needed to address the correlation between the anergic memory LP CD4⁺CD25⁻ T cells and T_R function by sorting CD4⁺CD25⁻ T cell fractions, which are enriched T_R cells, such as LP α E⁺CD4⁺CD25⁻, LAP⁺CD4⁺CD25⁻, and GITR⁺CD4⁺CD25⁻ T cells.

In conclusion, the LP CD4⁺CD25^{bright} T cells from both normal individuals and IBD patients were demonstrated to be mucosal T_R cells, indicating that these cells might not only serve as mucosal immune controllers to maintain T cell homeostasis against many stimuli, such as intestinal bacteria, but also as ideal cellular immunosuppressive cells as beneficial therapy for IBD patients in place of chemical immunosuppressants, which retain many side effects.

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Increase of Bone Marrow-Derived Secretory Lineage Epithelial Cells During Regeneration in the Human Intestine

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Background & Aims: We have previously reported that bone marrow (BM)-derived cells contribute to the regeneration of the human intestinal epithelium. To analyze further how these cells arise, proliferate, and differentiate as epithelial cells, histologic analysis was conducted using endoscopic specimens. **Methods:** Thirty biopsy specimens from 14 female, sex-mismatched BM-transplantation recipients were examined. BM-derived cells were identified by fluorescent in situ hybridization (FISH) for the Y chromosome and immunohistochemistry. Multicolor FISH was used to exclude cell fusion. These cells were further analyzed for various differentiation or proliferation markers. **Results:** No evidence of cell fusion was detected. BM-derived cells did not distribute within the crypt as stem cells and rarely expressed Musashi-1. However, BM-derived epithelial cells frequently expressed Ki-67, and some of these cells appeared as pairs of adjacent cells. These cells also expressed markers of all 4 lineages of terminally differentiated cells. During regeneration following graft-vs-host disease, the number of BM-derived cells was substantially increased within Ki-67-positive cells. Interestingly, the number of cells expressing markers for secretory lineage cells was significantly increased within BM-derived cells. This change was unique for BM-derived cells, resulting in a significantly increased proportion of BM-derived cells among secretory lineage cells. **Conclusions:** BM-derived epithelial cells arise via a mechanism other than cell fusion and rarely give rise to stem cells. However, a small proportion of these cells express proliferation markers, and a majority reside as terminally differentiated cells. During regeneration BM-derived cells increase as secretory lineage cells, thereby contributing to restore epithelial functions.

The gastrointestinal (GI) epithelial cells arise from the intestinal stem cells residing in the lower part of the crypt. The intestinal stem cell provides daughter cells, which proliferate and in turn give rise to the 4 main

lineages of terminally differentiated cells, namely, absorptive cells, goblet cells, enteroendocrine cells, and Paneth cells.^{1,2} The terminally differentiated intestinal epithelial cells function not only as mechanical barriers, but they also perform lineage-specific functions, such as nutrition absorption and production of mucin, neuropeptides, or antibiotic peptides. Therefore, a considerable number of functional, terminally differentiated cells in each lineage must be continuously generated to maintain the proper function of the intestinal epithelium.

Much remains unknown, however, about the precise molecular mechanism by which epithelial cells arise, proliferate, and differentiate within the intestinal epithelium.^{3,4} Recent studies have drawn attention to the importance of Wnt signaling in the regulation of the differentiation of immature intestinal epithelial cells.^{5,6} Also, a stepwise differentiation model of intestinal epithelial cells has been proposed, in which differentiation is regulated by a series of transcription factors downstream of Notch signaling.^{7,8} In this model, goblet cells, enteroendocrine cells, and Paneth cells arise from a shared progenitor cell expressing *Math1* and are categorized as secretory lineage cells. In contrast, absorptive cells arise from a distinct progenitor cell expressing *Hes1*.

In the previous studies, we demonstrated that bone marrow (BM)-derived cells contribute to the regeneration of damaged intestinal epithelium as epithelial cells.^{9,10} This suggested that BM-derived cells could be a potential source for epithelial tissue regeneration. However, little is known about how these cells arise or whether they could proliferate and differentiate into

Abbreviations used in this paper: BMT, bone marrow transplantation; GVHD, graft-vs-host disease; FISH, fluorescent in situ hybridization.

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functional, tissue-specific, terminally differentiated cells. Several studies suggest cell fusion between BM cells and tissue-specific cells as one mechanism by which BM-derived nonhematopoietic cells arise,¹¹⁻¹⁵ whereas other studies suggest other mechanisms, including transdifferentiation of BM cells into tissue-specific stem cells.¹⁶⁻²¹ Further studies have reported that an increased proportion of BM-derived cells could improve the function of nonhematopoietic organs,^{17,18,22,23} suggesting that BM-derived cells could possibly express a tissue-specific function within the organ of residence.

In the present study, we demonstrate that BM-derived epithelial cells observed in the human intestine arise via a mechanism other than cell fusion and rarely give rise to intestinal stem cells. Our results show that a small number of these cells express markers of proliferation, but a majority express markers of functional, terminally differentiated epithelial cells within the human intestinal epithelium. During regeneration following epithelial damage, BM-derived epithelial cells increased as secretory lineage cells, thereby supporting both the regeneration and the essential functions of the intestinal epithelium. These results not only provide further support for the use of BM-derived cells to regenerate human intestinal epithelium but also suggest the existence of a unique regulatory system exclusive for BM-derived cells, which changes their differentiation pattern at the site of intestinal inflammation.

Materials and Methods

Bone Marrow Transplant Recipients

We studied total of 14 female recipients who had received sex-mismatched bone marrow transplantation (BMT). All patients were subjected to allogeneic BMT for the treatment of severe hematologic disorders: acute myeloblastic leukemia, aplastic anemia, and acute lymphoblastic leukemia. Three patients developed GI inflammation because of graft-vs-host disease (GVHD). Control of Y-fluorescent in situ hybridization (FISH) staining was provided from 2 males and 2 females (not undergoing transplantation). The details of the patients are summarized in Table 1.

GI Endoscopic Biopsy Specimens

All samples were taken at the Keio University Hospital. We obtained written informed consent from each patient in the formal style after explaining the nature and possible consequences of the studies. The ethics committees of Keio University and Tokyo Medical and Dental University both approved this study. We took endoscopic specimens of the GI tract because patients developed clinical symptoms such as nausea, vomiting, abdominal pain, or diarrhea and were suspected of chronic GVHD, acute GVHD, or other intestinal inflammation. A total of 30 biopsy specimens obtained from

Table 1. Characteristics of Patients

Case	Reason for Transplantation ^a	Time from BMT to sampling (days)	Location of samples ^b	Histologic diagnosis
1	ALL	26	E,S, D	Acute GVHD
		51	E,S, D	Acute GVHD
		77	E,S, D	Acute GVHD
2	MDS	26	E,S, D	n.p.
		161	E,S, D	Chronic GVHD
		381	C	n.p.
3	Aplastic anemia	45	E,S, D	n.p.
		182	E,S, D	n.p.
		288	E,S, D	n.p.
4	AML	32	E,S, D	n.p.
5	AML	21	E,S, D	n.p.
6	ALL	40	E,S, D	Gastric polyp
7	AML	30	E,S, D	n.p.
8	NHL	33	E,S, D	n.p.
9	AML	39	E,S, D	n.p.
10	AML	23	E,S, D	n.p.
11	AML	39	E,S, D	n.p.
12	MDS	27	E,S, D	Acute GVHD
		96	S, D	Acute GVHD
13	MM	27	E,S, D	Esophagitis
		63	E,S, D	Esophageal ulcer
		77	E,S, D	Esophageal granulation
14	MDS	28	E,S, D	n.p.

n.p., no particular finding.

^aALL, acute lymphoblastic leukemia; MDS, myelodysplastic syndromes; AML, acute myelogenous leukemia; NHL, non-Hodgkin lymphoma; MM, multiple myeloma.

^bE, esophagus; S, stomach; D, duodenum; C, colon.

14 female patients were examined and analyzed retrospectively. Details of the specimens examined are also summarized in Table 1.

FISH for Human Chromosomes

FISH for chromosomes 1, 18, and Y using formalin-fixed, paraffin-embedded biopsy specimens has already been described.⁹ Paraffin-embedded tissue samples were cut into either 6- μ m- or 3- μ m-thick serial sections and subjected to FISH analysis. The specific DNA probes used were as follows: clone pUC 1.77²⁴ for chromosome 1 (purchased from HSRBB, Osaka, Japan), clone L 1.84²⁵ for chromosome 18, and clone pHY10²⁶ for the Y chromosome. The probes were labeled by nick translation either with biotin-dUTP or DIG-dUTP (Roche Diagnostics, Tokyo, Japan) and detected by incubation with either avidin-FITC or anti-DIG-rhodamine (Roche Diagnostics, Indianapolis, IN). FISH images were captured using a Nikon epifluorescence microscope (Eclipse 800, Tokyo, Japan) coupled to a Sensys CCD camera and analyzed with QUIPS image software (Vysis, Downers Grove, IL). One section per course of endoscopy, containing 1-3 biopsy specimens of the GI tract, was used for the subsequent FISH analysis.

Histology and Immunoperoxidase Staining

Formalin-fixed, paraffin-embedded biopsy specimens were used unless otherwise mentioned. For anti-Musashi-1 antibody (Ab), cryosections prefixed in 4% paraformaldehyde were used. Immunohistochemistry using anti-human cytokeratin Ab (AE1/AE3, DAKO-USA, Carpinteria, CA), anti-human LCA (CD45) Ab (DAKO, Glostrup, Denmark), anti-human chromogranin A Ab (DAKO), anti-human CD10 Ab (clone 56C6, Serotec, United Kingdom), anti-human Ki-67 antigen Ab (ZYMED, San Francisco, CA), and anti-Musashi-1 Ab (14H1) was done as described elsewhere.⁹ Briefly, paraffin-embedded tissue samples were cut into 6- μ m- or 3- μ m-thick serial sections, placed on coated slides, and deparaffinized through a series of xylene and ethanol. Slides were then incubated with primary antibodies at 4°C overnight, followed by biotin-conjugated anti-mouse IgG antibody (E0433, DAKO-USA), biotin-conjugated anti-rabbit IgG antibody (E0435, DAKO-USA), or biotin-conjugated anti-rat IgG antibody (BA4000, Vectastain, Burlingame, CA). The following steps were done using the standard ABC method (Elite ABC kit, Vectastain). Diaminobenzidine tetrahydrochloride (DAB) was used as the substrate for the peroxidase reaction (Vectastain). All slides were counterstained with hematoxylin and observed under a microscope (CH40, Olympus, Tokyo, Japan).

Detection of Y-FISH-Positive Epithelial Cells

Y-FISH-positive epithelial cells were confirmed and quantified as described elsewhere.⁹ Y-FISH-positive, CD45-positive cells did not always show complete lack of cytokeratin staining because of its too intense staining. Therefore, all CD45-positive cells were counted as intraepithelial lymphocytes throughout the study, following exactly the same criteria used in our previous report.⁹ Expression of lineage-specific markers or Ki-67 antigen within the Y-FISH-positive epithelial cells was determined using serial sections. For detection of BM-derived enteroendocrine cells, immunohistochemical staining of chromogranin A was used as a specific marker. For detection of BM-derived goblet cells, alcian blue staining and Y-FISH were performed using a single section. Paneth cells were identified after H&E staining by their eosinophilic granules. Thus, for detection of BM-derived Paneth cells, H&E staining and Y-FISH were performed using a single section. BM-derived absorptive cells were determined as cells with negative staining for lineage markers of the other 3 lineages, negative staining for Musashi-1 or Ki-67 by immunohistochemical analysis and columnar shaped morphology consistent with the differentiated absorptive cells, and positive staining for CD10 in the serial section with Y-FISH. Regenerative epithelium in the sections was identified by the microscopic features of the epithelial cells, such as dense cytoplasm, nuclear swelling, or hyperchromatin.

Statistical Analysis

The results were expressed as the mean \pm standard error of mean (SE). Groups of data were compared by the

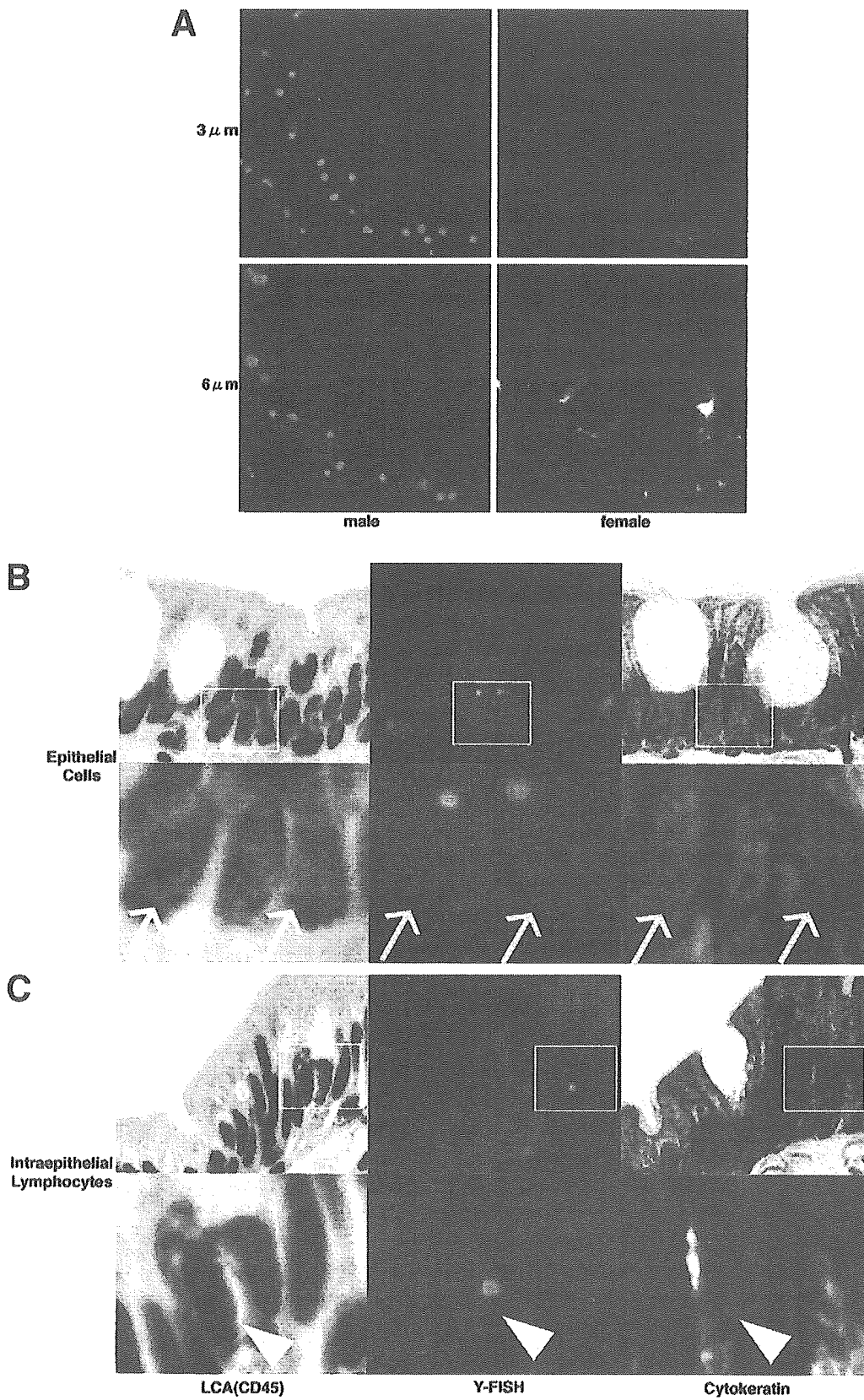
Mann-Whitney *U* test. *P* values less than .05 were considered statistically significant.

Results

BM-Derived Epithelial Cells Are Distributed in the Human Intestine as Euploid Cells With no Evidence of Cell Fusion

We have previously demonstrated the presence of BM-derived epithelial cells in every part of the human GI tract.⁹ In the present study, we attempted to characterize further the BM-derived epithelial cells. For this purpose, we refined our experimental procedure of Y-FISH analysis in this study. In our previous report, we used 6- μ m-thick sections to identify BM-derived epithelial cells. We confirmed that Y chromosomes were also clearly detectable in 3- μ m-thick sections (Figure 1A). We also confirmed that Y chromosome-positive epithelial cells within a female BMT recipient were clearly distinguishable from Y chromosome-positive lymphocytes by the analysis of 3- μ m-thick serial sections (Figure 1B). Therefore, we used 3- μ m-thick sections throughout this study to obtain strict and definite characterization of the BM-derived cells. The results obtained from 3- μ m-thick serial sections were further confirmed by double staining with Y-FISH and specific markers in single sections, as previously described. A total of 330 BM-derived cells were analyzed by this method, and 239 cells (72.4%) were identified as BM-derived epithelial cells and the remainder as BM-derived intraepithelial lymphocytes.

We then examined whether BM-derived epithelial cells detected by the present method arise via the mechanism of cell fusion. Cells generated by cell fusion in vivo are reported to form polyploid cells named *heterokaryons*.^{13,14,27} To reveal the ploidy of BM-derived epithelial cells, we performed multicolor FISH using specific DNA probes for chromosomes 1, 18, and Y. If BM-derived epithelial cells are generated by cell fusion, they will be aneuploid, and, therefore, probes for a single somatic chromosome will be targeted against at least 4 potential target chromosomes within the nucleus of these cells. Thus, we first examined the sensitivity of our FISH method by detecting 4 potential target chromosomes within a single nucleus (Figure 2A). Using probes for chromosomes 1 and 18, we confirmed that our FISH method could detect at least 3 fluorescent signals from 4 potential target chromosomes in a single nucleus at a sensitivity of around 70% (Table 2). Under the same experimental conditions, we examined tissue samples from nor-



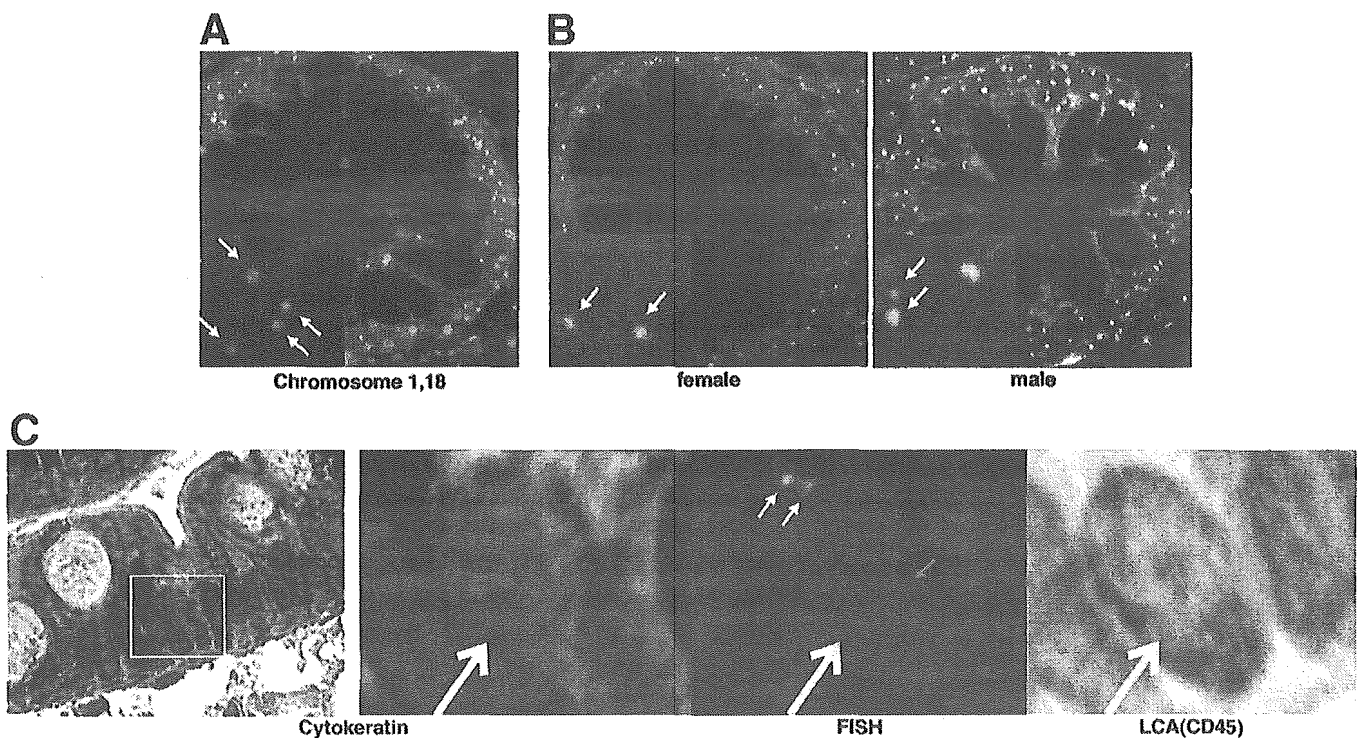


Figure 2. BM-derived epithelial cells show no evidence of heterokaryons. (A) Single-color FISH using centromere probes for both chromosomes 1 and 18 was performed to evaluate the sensitivity of detecting 4 chromosomes within a single nucleus. Analysis of 578 cells using a female colon section revealed that, when 4 potential target chromosomes for FISH are present within a single nucleus, 68.4% of cells show as cells containing at least 3 signals (*inset*) within a single nucleus (original magnification, $\times 800$). (B) Multicolor FISH was performed using male or female colon sections. Probes for chromosome 1 were labeled by FITC (*green dots*), and probes for the Y chromosome were labeled with rhodamine (*red dots*). In the female section, 0–2 green signals from chromosome 1 only were detected within a nucleus (*inset, left*). In the male section, 0–2 green signals from chromosome 1 with a single red signal of the Y chromosome were detected (*inset, right*). Analysis of 220 epithelial cells in male section and 298 epithelial cells in female section revealed that 85.2%–87.6% of epithelial cells showed as cells containing 1 or 2 green signals within a nucleus, both in Y chromosome-negative and Y chromosome-positive cells. No cell showed 3 or more green signals within a nucleus (original magnification, $800\times$). (C) Multicolor FISH was performed using small intestinal section from a post-BMT female recipient. Probes for chromosome 1 were labeled by FITC (*green dots*), and probes for Y chromosome were labeled with rhodamine (*red dots*). Up to 2 green signals from chromosome 1 were detected in the nucleus of a Y chromosome-positive epithelial cell (*yellow arrow*) but never more (*right 3 panels*; original magnification, $12,800\times$). *Right 3 panels* represent the magnified view of the area indicated by the *yellow square* in the *left end panel* showing the lower magnification view of cytokeratin staining (original magnification, $3200\times$). Analysis of 177 Y chromosome-positive and 421 Y chromosome-negative epithelial cells revealed no difference in the number of green signals seen in a nucleus between the 2 groups (1.06 ± 0.713 vs. 1.13 ± 0.703 , respectively, $P = .134$).

mal male, normal female, and female BMT recipients, using specific probes for chromosomes 1 and Y (Figure 2B). Results of multicolor FISH in normal male or female samples showed that up to 2 discrete signals were detected in each nucleus of epithelial cells,

whether or not a signal of the Y chromosome was present (Table 2). Surprisingly, results of multicolor FISH using tissue samples from BMT recipients showed that Y-FISH-positive as well as Y-FISH-negative epithelial cells observed in female BMT recipient

Figure 1. Detection of BM-derived cells by Y-FISH. (A) Y-FISH was performed using paraffin-embedded sections of human male or female colon biopsy specimens. Signals from FITC-labeled DNA probes (*green*) were detected in the DAPI-stained nucleus (*blue*) of both 3- μm -thick and 6- μm -thick sections from a male, but no signal was detected in sections from a female (original magnification, $\times 1600$). (B) Serial section analysis using Y-FISH and immunostaining for CD45 or cytokeratin was performed to detect BM-derived epithelial cells. In 3- μm -thick paraffin-embedded sections of small intestine biopsy specimens obtained from a female post-BMT recipient, male donor BM cell-derived epithelial cells were clearly exhibited as cells positive for both Y-FISH and cytokeratin but negative for CD45 (*arrows*; original magnification, $\times 12,800$). Male donor BM-derived intraepithelial lymphocytes were also clearly observed as cells positive for both Y-FISH and CD45 (*arrowheads*; original magnification, $\times 12,800$). As Y-FISH positive, CD45-positive cells did not always show complete lack of cytokeratin staining, all CD45 positive cells were counted as intraepithelial lymphocytes. A total of 330 BM-derived cells were analyzed, and 239 cells (72.4%) were determined to be BM-derived epithelial cells. *Panels in rows 2 and 4* represent the magnified view of the area indicated by the *yellow square* in the *panels above* (original magnification, $\times 3200$).

Table 2. Results of Multi color FISH

Number of FITC-positive chromosomes in a single nucleus	Normal female epithelial cells		Normal male epithelial cells	Female BMT recipient Y chromosome positive epithelial cells	Female BMT recipient Y Chromosome negative epithelial cells
	Chromosome 1 (FITC)	Chromosome 1 (FITC)	Chromosome 1 (FITC)	Chromosome 1 (FITC)	Chromosome 1 (FITC)
	Chromosome 18 (FITC)	Y (rhodamine)	Y (rhodamine)	Y (rhodamine)	Y (rhodamine)
0	12/578 (2.1%)	44/298 (14.8%)	27/220 (12.3%)	40/177 (22.6%)	79/421 (18.8%)
1	38/578 (6.5%)	129/298 (43.3%)	86/220 (39.0%)	87/177 (49.2%)	206/421 (48.9%)
2	132/578 (22.8%)	125/298 (41.9%)	107/220 (48.6%)	50/177 (28.2%)	136/421 (32.3%)
3	241/578 (41.6%)	0/298 (0.0%)	0/220 (0.0%)	0/177 (0.0%)	0/421 (0.0%)
4 or more	155/578 (26.8%)	0/298 (0.0%)	0/220 (0.0%)	0/177 (0.0%)	0/421 (0.0%)
Average \pm SD	2.84 \pm 0.961	1.27 \pm 0.703	1.36 \pm 0.692	1.06 \pm 0.713	1.13 \pm 0.703

NOTE. Table reflects examined cell population by probe fluorescence. Values indicate number of cells positive for the indicated number of fluorescent signals/total cells examined.

FITC, fluorescein-isothiocyanate.

tissue showed no more than 2 discrete signals within a single nucleus (Figure 2C). Analysis of 177 Y chromosome-positive and 421 Y chromosome-negative epithelial cells revealed no significant difference between the 2 groups in the number of green signals seen in a nucleus (1.06 ± 0.713 vs 1.13 ± 0.703 signals/nucleus, respectively, $P > .1$, Table 2). These results suggest that BM-derived epithelial cells are euploid cells and are not heterokaryons generated by cell fusion. However, because of the limitation of this method, we cannot totally exclude the possibility of cell fusion followed by cytoreductive division.

BM-Derived Epithelial Cells Rarely Give Rise to Intestinal Stem Cells

First, we attempted to determine whether BM-derived epithelial cells reside within the intestinal epithelium as intestinal stem cells. Cells arising from a single intestinal stem cell form continuous, clustered columns of daughter cells along the crypt-villus axis.³ If BM-derived epithelial cells reside in the intestinal epithelium as intestinal stem cells, one would expect to see a single crypt of cells all expressing the Y chromosome marker. By our Y-FISH staining, this is easily demonstrated in a male crypt but never in a female crypt (Figure 3A, left panel). However, crypts of female BMT recipients showed patchy and rarely clustered distributions of Y-FISH-positive cells, even when the population of BM-derived cells was increased because of epithelial regeneration following GVHD (Figure 3A, right panel).

Another distinctive feature of intestinal stem cells is their long lifetime; they remain within the crypt at

least for several years.^{28,29} Therefore, we next attempted to examine whether any BM-derived epithelial cells remain within the intestinal crypt for an extended period. One of our female BMT recipients underwent BMT twice: the first time from a male donor and the second time from a female donor. If any BM-derived epithelial cells do remain within the intestinal crypt for a period of years, Y-FISH-positive epithelial cells should be detected long after the second BMT in this patient. Therefore, we examined GI biopsy specimens taken from this patient at different time points following the first BMT by Y-FISH (Figure 3B). At 45 days after the first BMT, Y-FISH-positive epithelial cells were detected within the small intestinal tissue. However, Y-FISH-positive epithelial cells could not be detected after the second BMT (182 days after first BMT). This suggests that BM-derived epithelial cells do not remain within the crypt for longer than 182 days and therefore lack one of the essential features of intestinal stem cells.

We further sought to examine whether any of the BM-derived cells express a specific molecular marker for intestinal stem cells, although no definitive markers have been confirmed. One candidate for such a marker is Musashi-1, an RNA-binding protein initially identified in neural stem cells.³⁰ Recent studies have demonstrated that Musashi-1 is expressed exclusively in the stem cell region of the murine small intestine.^{31,32} However, little is known about the expression of Musashi-1 in the human intestine.³³ Therefore, we first sought to establish whether Musashi-1 could also be used as a molecular marker for

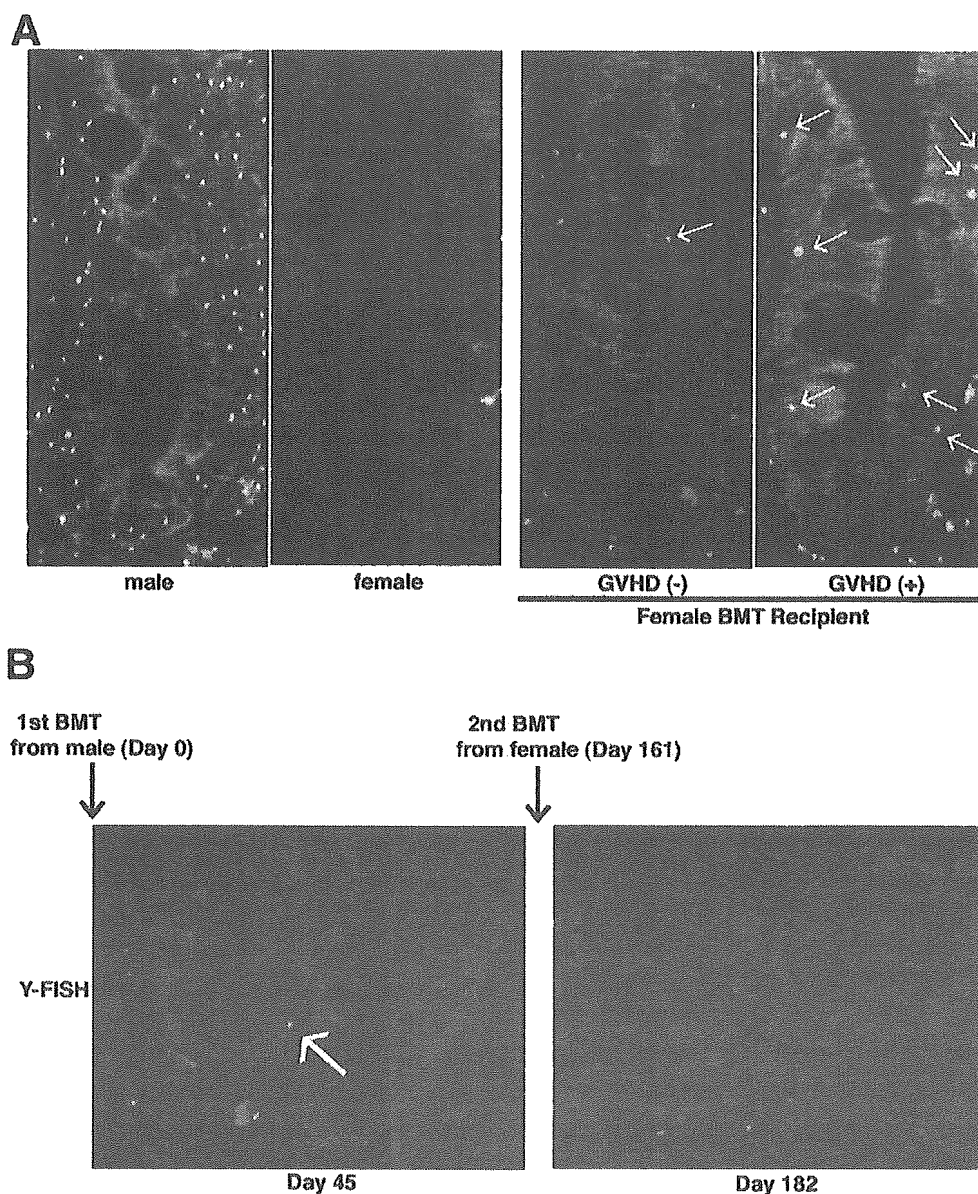


Figure 3. BM-derived epithelial cells rarely give rise to intestinal stem cells. (A) The distribution of Y-FISH-positive cells within the longitudinal section of a single crypt was examined in the human small intestine. Y-FISH-positive cells (green dots) dominated the crypt of a male, but no such cells were detected in the crypt of a female (original magnification, 1200 \times). The distribution of Y-FISH-positive epithelial cells (green dots) within the crypt of a female BMT recipient was patchy, both in sections with GVHD (+) (original magnification, 1200 \times) or without inflammation because of GVHD (GVHD (-) (original magnification, 1200 \times). (B) The presence of BM-derived cells within the intestinal epithelium during 2 courses of BMT in a female patient was examined by Y-FISH. After the first BMT, which was from a male donor, Y-FISH-positive cells (arrow) were observed within the small intestinal epithelium at 45 days post-BMT (left; original magnification, 1600 \times). However, after the second BMT, which was from a female donor and was performed at 161 days after the unsuccessful first BMT, Y-FISH-positive cells could no longer be found in the small intestinal section taken at 182 days after first BMT (right; original magnification, 1600 \times).

intestinal stem cells in humans. The results from immunohistochemical analysis of normal human small intestinal tissues showed that Musashi-1 protein is expressed in the human intestinal epithelial cells residing in the lowest part of the crypt (Figure 4A). Musashi-1-positive cells distributed up to several epithelial cells from the lowest part of the crypt, but Paneth cells were completely negative for the stain (Figure 4B). Immunostaining of the adjacent section

for Ki-67 revealed that the majority of Musashi-1-positive cells within a single crypt distributed beneath the lowest border of Ki-67-positive cells but left the possibility that few double-positive cells may exist (Figure 4A). These findings suggest that Musashi-1-positive epithelial cells distribute within the lowest part of the human crypt but are distinct from Paneth cells or the Ki-67-positive cells. This distribution coincides with the predicted stem cell region of the

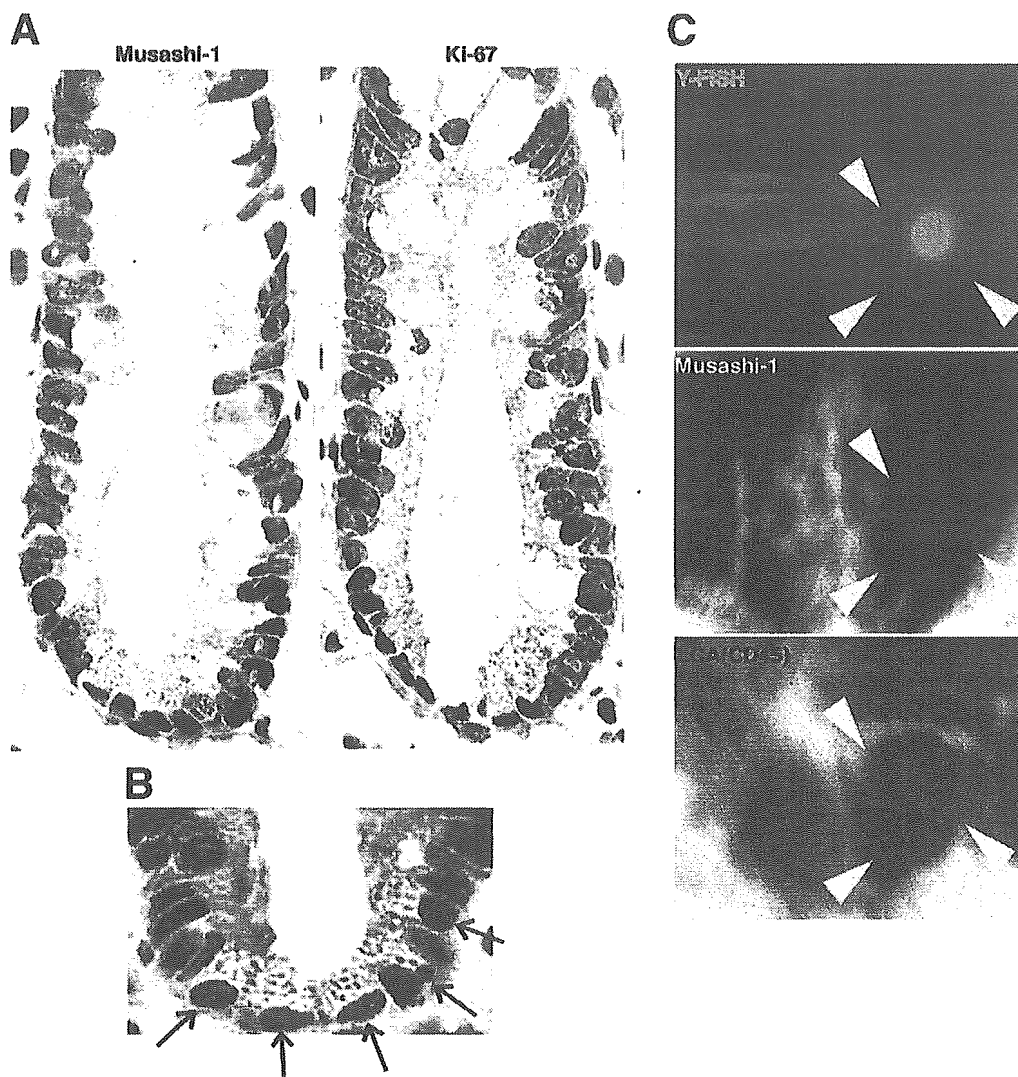


Figure 4. BM-derived epithelial cells rarely coexpress Musashi-1. (A) Distribution of the expression of Musashi-1 protein within a human small intestinal crypt was examined by an immunohistochemistry using a polyclonal antibody specific for human Musashi-1. Intense staining of epithelial cells residing in the lower part of the crypt was clearly demonstrated. Musashi-1-positive cells distributed over the predicted stem cell region but also appeared to include a few more cells other than just stem cells. Immunostaining of the adjacent section for Ki-67 further confirmed that Musashi-1-positive epithelial cells within a single crypt distribute beneath the lowest border of Ki-67-positive cells (original magnification, 1000 \times). (B) A magnified view of a human small intestinal crypt immunostained for Musashi-1. Paneth cells were clearly negative for Musashi-1 (arrow; original magnification, 1200 \times). (C) Serial section analysis of small intestinal sections taken from a female BMT recipient was performed to detect Musashi-1-positive BM-derived epithelial cells. Sections were examined by immunostaining for Musashi-1, CD45, and Y-FISH. A single cell was found to be double positive for Y-FISH and Musashi-1 (arrowhead) but negative for CD45 (original magnification, 12,800 \times). Of a total of 30,973 epithelial cells, 2346 cells were Musashi-1 positive, 239 cells were Y-FISH positive, but only 2 epithelial cells were double positive.

intestinal crypt, but, at the same time, it seems to indicate that Musashi-1-positive cells include a lot of cells other than the stem cells in the human intestine. The specificity of the staining was confirmed by the finding that preincubation of the antibodies with specific blocking peptides completely abrogated the observed immunoreactivities (data not shown). Under these experimental conditions, we examined whether BM-derived epithelial cells are distributed as Musashi-1-positive epithelial cells in the human in-

testine. Within the intestinal epithelium of BMT recipients, Musashi-1-positive BM-derived epithelial cells were detected at extremely low frequency (Figure 4C). Of 30,973 epithelial cells examined, 2346 cells were found to be Musashi-1 positive, and 239 cells were Y-FISH positive, but only 2 cells coexpressed both Musashi-1 and Y-FISH (0.08% of Musashi-1-positive epithelial cells).

These studies thus demonstrate that BM-derived epithelial cells show a patchy distribution within the

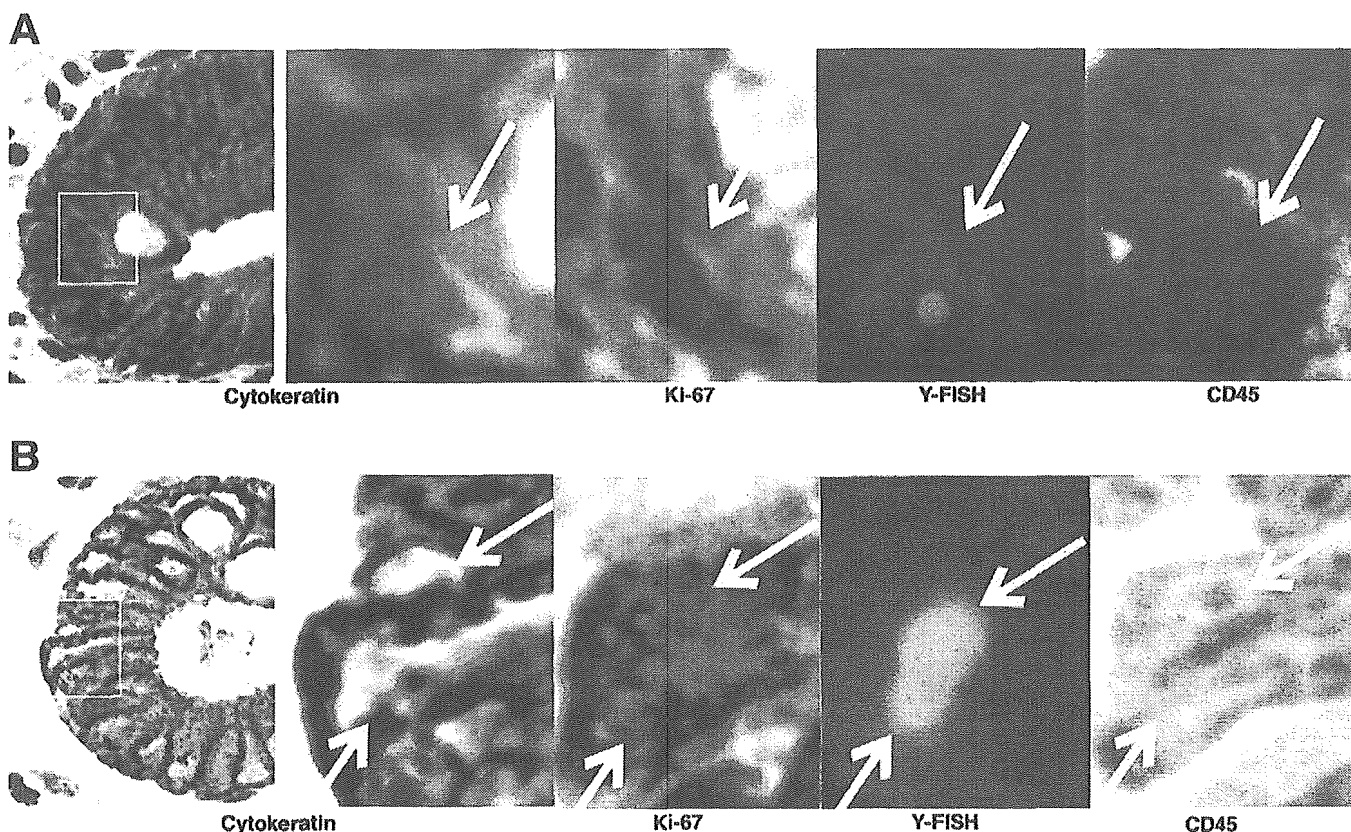


Figure 5. BM-derived epithelial cells distribute as Ki-67–positive cells and divide within the human intestinal epithelium. (A) Serial section analysis of small intestinal sections taken from a female BMT recipient was performed to detect Ki-67–positive BM-derived epithelial cells. Sections were examined by immunostaining for Ki-67, cytokeratin, CD45, and Y-FISH (yellow arrow). Of a total of 30,973 epithelial cells, 2098 cells were Ki-67 positive, and 26 cells were positive for both Ki-67 and Y-FISH (right 4 panels; original magnification, 12,800 \times). Right four panels represent the magnified view of the area indicated by the yellow square in the left end panel showing the lower magnification view of cytokeratin staining (original magnification, 3200 \times). (B) Serial sections analyzed for Ki-67–positive BM-derived epithelial cells were further examined for clustering of BM-derived epithelial cells, which would suggest cell division of these cells within the intestinal epithelium. A maximum of 2 adjacent cells were found to be positive for Y-FISH, Ki-67, and cytokeratin (yellow arrow) but negative for CD45 (right 4 panels; original magnification, 12,800 \times). Right 4 panels represent the magnified view of the area indicated by the yellow square in the left end panel showing the lower magnification view of cytokeratin staining (original magnification, 3200 \times).

crypt but do not continue to reside within the epithelium and rarely express Musashi-1 protein. Therefore, we conclude that BM-derived epithelial cells rarely distribute as intestinal stem cells.

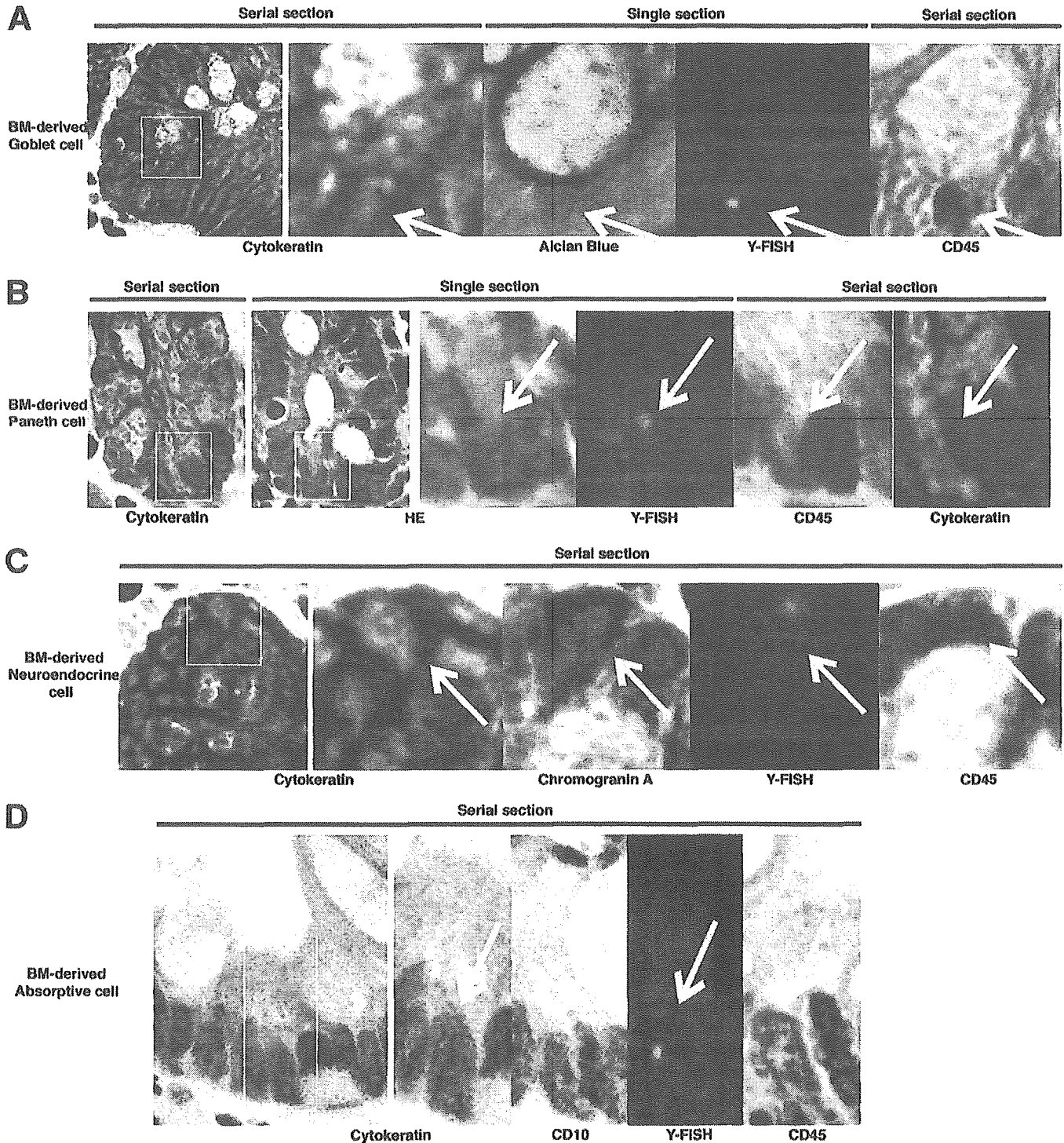
BM-Derived Epithelial Cells Distribute as Ki-67-Positive Cells and Express Markers of Terminally Differentiated Functional Epithelial Cells Within the Human Intestine

The intestinal epithelium is composed of 4 main lineages of terminally differentiated cells and proliferative cells expressing Ki-67 antigen, along with stem cells.⁴ We examined whether BM-derived epithelial cells distribute as Ki-67–positive cells that proliferate within the epithelium. Serial section analysis using CD45, cytokeratin, and Ki-67 immunostaining along with Y-FISH revealed the presence of Ki-67–positive BM-

derived cells within the intestinal epithelium of BMT recipients (Figure 5A). Of 30,973 epithelial cells examined, 26 cells were determined to be Ki-67–positive BM-derived cells (10.9% of BM-derived epithelial cells, 1.2% of Ki-67–positive epithelial cells). However, Ki-67 also stains cells in G1,³⁴ and, therefore, the results show merely the proliferative potential of the BM-derived epithelial cells. If actively proliferating BM-derived epithelial cells exist, pairs of Ki-67–positive BM-derived epithelial cells should be found adjacent to one another. Within the intestinal epithelium of BMT recipients, such pairs of adjacent Ki-67–positive, Y-FISH–positive cells were indeed observed (Figure 5B). These results suggested that the BM-derived epithelial cells could express Ki-67 antigen and proliferate within the crypt. However, clusters of 3 or more BM-derived epithelial cells were not observed, and only 3 pairs of adjacent cells were found among the 30,973 epithelial cells examined.

We then attempted to determine whether BM-derived cells distribute as terminally differentiated epithelial cells that express lineage markers related to specific functions. When a single section was stained sequentially with alcian blue and Y-FISH, BM-derived goblet cells producing acidic mucin were observed within the intestinal epithelium of the BMT recipients (Figure 6A). When a single section was

stained sequentially with H&E and Y-FISH, BM-derived Paneth cells containing the characteristic eosinophilic granules were observed within the intestinal epithelium of the BMT recipients (Figure 6B). Serial section analysis using CD45, chromogranin A, and cytokeratin immunostaining with Y-FISH revealed the presence of BM-derived neuroendocrine cells expressing chromogranin A within the intestinal



epithelium of the BMT recipients (Figure 6C). Serial section analysis using CD45, CD10,³⁵ and cytokeratin immunostaining with Y-FISH revealed the presence of BM-derived absorptive cells expressing CD10 within the intestinal epithelium of the BMT recipients (Figure 6D). These findings suggested that BM-derived cells distribute as all 4 lineages of terminally differentiated epithelial cells associated with lineage-specific functions. However, the dividing potential of the BM-derived epithelial cells is extremely limited, suggesting that new BM cells are constantly being recruited, which in turn mature into terminally differentiated cells after a short residence within the epithelium.

BM-Derived Epithelial Cells Distributed as Secretory Lineage Epithelial Cells Within the Regenerative Epithelium Following GVHD

We have previously shown that BM-derived epithelial cells increase in number and contribute to tissue regeneration in the recovery phase from GVHD.⁹ However, whether this increase is correlated with a change in the proliferation and/or differentiation of the BM-derived epithelial cells remains unclear. If the substantial increase of BM-derived cells is due to the increased proliferation of BM-derived epithelial cells within the epithelium, the frequency of Ki-67-positive BM-derived cells should also be increased in BMT recipients who developed GVHD. Thus, we first compared the expression of Ki-67 within BM-derived epithelial cells in BMT recipients who did (GVHD [+]) or did not develop GVHD (GVHD [-]). We also compared the expression in

GVHD (+) BMT recipients with or without regenerative epithelium. The total number of BM-derived epithelial cells was substantially increased in GVHD (+) recipients, as compared with GVHD (-) recipients: GVHD (-), 0.76%; GVHD (+) regenerative epithelium (-), 0.97%; GVHD (+)/regenerative epithelium (+), 1.31% of total epithelial cells. The low frequency observed in GVHD (-) recipients was compatible with previous reports.³⁶ Analysis of the expression of Ki-67 showed that the proportion of Ki-67-positive BM-derived epithelial cells among all Ki-67-positive epithelial cells increased substantially in GVHD (+) recipients with regenerative epithelium, as compared with GVHD (-) recipients or GVHD (+) recipients without regeneration, along with the total number of BM-derived epithelial cells. However, the increase observed in GVHD (+) recipients with regenerative epithelium was not statistically significant compared with GVHD (-) recipients: GVHD (-), 1.0 ± 0.23 cells; GVHD (+)/regenerative epithelium (-), 1.2 ± 1.7 cells/100 Ki-67-positive cells, $P > .1$; GVHD (+)/regenerative epithelium (+), 1.9 ± 1.17 cells/100 Ki-67-positive cells, $P = .076$. These results suggest that BM-derived epithelial cells increase in number via increase of Ki-67-expressing epithelial cells in case of severe intestinal inflammation and the following regeneration of the damaged epithelium but rapidly become resident as terminally differentiated intestinal cells. We next examined whether the increase of BM-derived epithelial cells during regeneration from GVHD is correlated with a change in lineage distribution of the BM-derived epithelial cells. For this purpose, we compared the expression of lineage markers

Figure 6. BM-derived epithelial cells express markers of functional, terminally differentiated epithelial cells within the human intestine. (A) A single section of a small intestinal specimen taken from a female BMT recipient was subjected to double-staining analysis to detect BM-derived goblet cells. The section was first stained by Y-FISH and subsequently with alcian blue. Expression of cytokeratin and the absence of CD45 were confirmed by the immunostaining. Of a total of 30,973 epithelial cells examined, 4151 cells were positive for alcian blue, and 26 cells (arrow) were double positive (right 4 panels; original magnification 19,200 \times). Right 4 panels represent the magnified view of the area indicated by the yellow square in the left end panel showing the lower magnification view of cytokeratin staining (original magnification, 3200 \times). (B) A single section of a small intestinal specimen taken from a female BMT recipient was subjected to double-staining analysis to detect BM-derived Paneth cells. The section was first stained with H&E to demonstrate the specific eosinophilic granules and subsequently stained by Y-FISH. Expression of cytokeratin and the absence of CD45 were confirmed by the immunostaining. Of a total of 30,973 epithelial cells examined, 428 cells were positive for Paneth cell-specific granules, and a single cell (yellow arrow) was double positive (right 4 panels; original magnification, 19,200 \times). Right 4 panels represent the magnified view of the area indicated by the yellow squares in the left 2 panels showing the lower magnification view of H&E or cytokeratin staining (original magnification, 3200 \times). (C) Serial section analysis of small intestinal sections taken from a female BMT recipient was performed to detect BM-derived neuroendocrine cells. Sections were examined by immunostaining for chromogranin A, cytokeratin, CD45, and Y-FISH (arrow). Of a total of 30,973 epithelial cells, 1074 cells were chromogranin A positive, and 14 cells were double positive for chromogranin A and Y-FISH (right 4 panels; original magnification, 12,800 \times). Right 4 panels represent the magnified view of the area indicated by the yellow square in the left end panel showing the lower magnification view of cytokeratin staining (original magnification, 3200 \times). (D) Serial section analysis of small intestinal sections taken from a female BMT recipient was performed to detect BM-derived absorptive cells. Sections were examined by immunostaining for CD10, cytokeratin, CD45, and Y-FISH (arrow). Of a total of 30,973 epithelial cells, 15,968 cells were CD10 positive, and 159 cells were double positive for CD10 and Y-FISH (right 4 panels; original magnification, 12,800 \times). Right 4 panels represent the magnified view of the area indicated by the yellow square in the left end panel showing the lower magnification view of cytokeratin staining (original magnification, 4800 \times).

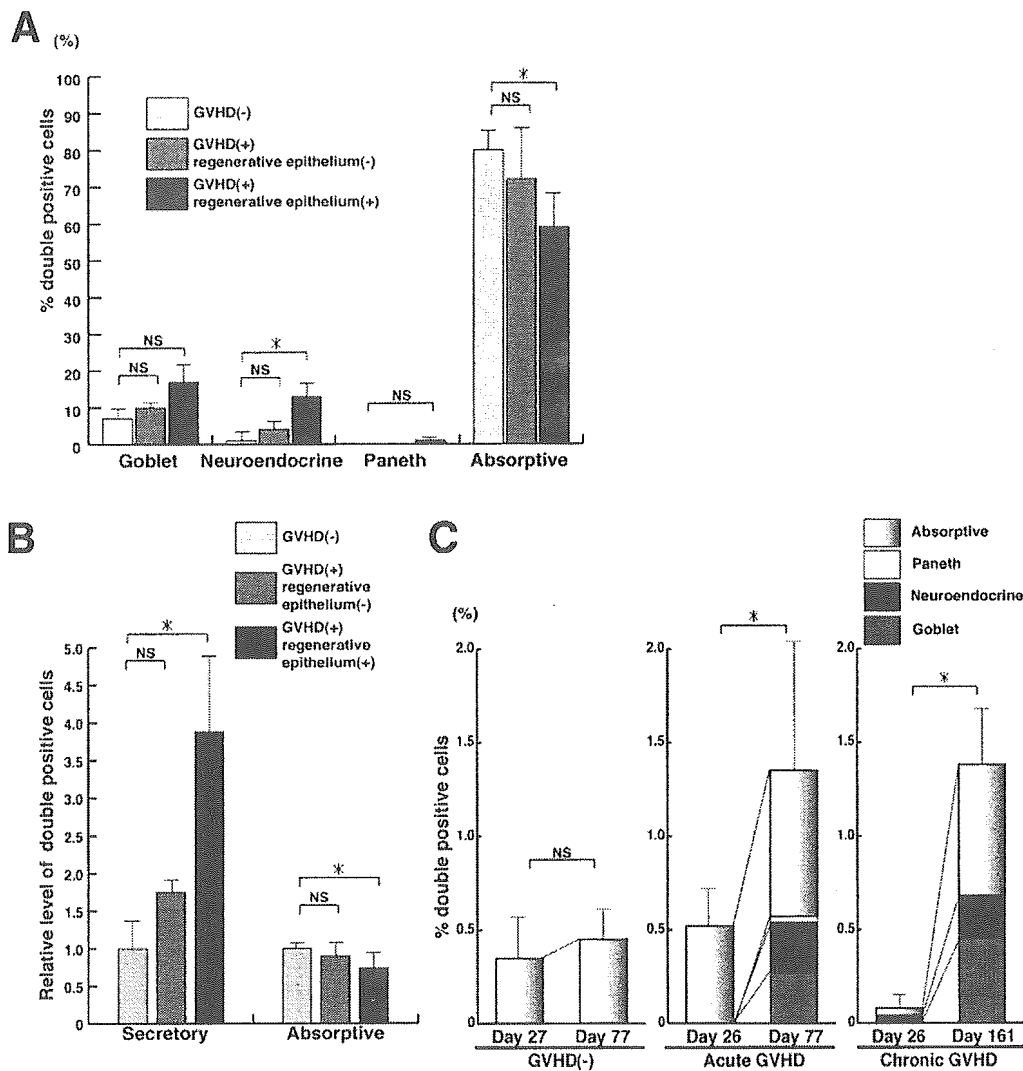


Figure 7. BM-derived epithelial cells proliferate and increase as secretory lineage cells in the regenerative epithelium following GVHD. (A) Coexpression of lineage-specific differentiation markers within BM-derived epithelial cells was compared in the same 3 groups of patients: patients who did not develop GVHD (GVHD(-), 13,629 epithelial cells); patients who developed GVHD but without regenerative epithelium (GVHD(+)/regenerative epithelium(-), 2976 epithelial cells); and patients who developed GVHD accompanied with regenerative epithelium (GVHD(+)/regenerative epithelium(+), 7114 epithelial cells). Coexpression of the lineage-specific markers was examined in a total of 239 Y-FISH-positive epithelial cells by analysis of serial sections or double staining of a single section, as formerly described. BM-derived neuroendocrine cells showed a significant increase, whereas BM-derived goblet cells and BM-derived Paneth cells also showed substantial increase in GVHD(+)/regenerative epithelium(+) compared with GVHD(-). In sharp contrast, BM-derived absorptive cells showed a significant decrease in GVHD(+)/regenerative epithelium(+) compared with GVHD(-). GVHD(+)/regenerative epithelium(-) showed no significant difference compared with GVHD(-). Values are shown as mean \pm SE. *Indicates $P < .05$. NS indicates $P \geq .05$. (B) Coexpression of secretory cell lineage or absorptive lineage markers within BM-derived epithelial cells was compared in the same 3 groups of patients. The results in Figure 7B were reanalyzed in terms of secretory or absorptive lineage, and data were presented as the relative level of each lineage within BM-derived cells compared with GVHD(-). Secretory lineage cells were found to be significantly increased, whereas absorptive lineage cells were significantly decreased in GVHD(+)/regenerative epithelium(+). GVHD(+)/regenerative epithelium(-) showed no significant difference compared with GVHD(-). Values are shown as mean \pm SE. *Indicates $P < .05$. NS indicates $P \geq .05$. (C) Series of biopsy specimens taken at different time points post-BMT from 3 cases were examined for coexpression of lineage-specific differentiation markers in BM-derived epithelial cells. A case that did not develop GVHD (GVHD[-]) showed no increase of BM-derived epithelial cells between day 27 (3235 total epithelial cells) and day 77 (1790 total epithelial cells), and the differentiated BM-derived epithelial cells were dominated by absorptive lineage cells. In sharp contrast, a case that developed acute GVHD around day 77 post-BMT showed a significant increase in the frequency of BM-derived epithelial cells between day 26 (968 total epithelial cells) and day 77 (3870 total epithelial cells). This increase was associated with a substantial increase of BM-derived secretory lineage cells within epithelial cells after the development of GVHD. Another case that developed chronic GVHD also showed a significant increase (0.13 ± 0.075 vs 1.46 ± 0.278 cells/100 epithelial cells, $P = .002$) in the frequency of BM-derived epithelial cells between day 26 (1454 total epithelial cells) and day 161 (1257 total epithelial cells). This was also associated with a substantial increase of BM-derived secretory lineage cells within epithelial cells (0.04 vs 0.68 cells/100 epithelial cells after the development of GVHD). Values are shown as mean \pm SE. *Indicates $P < .05$. NS indicates $P \geq .05$.

for terminally differentiated cells within BM-derived epithelial cells. We observed significant differences in the differentiation pattern of BM-derived epithelial cells between GVHD (-) recipients and GVHD (+) recipients with regeneration (Figure 7A). BM-derived neuroendocrine cells showed a significant increase (1.0 ± 1.99 vs 12.9 ± 3.78 cells/100 Y-FISH-positive epithelial cells, $P = .002$), whereas BM-derived goblet cells (6.7 ± 2.77 vs 17.2 ± 5.0 cells/100 Y-FISH-positive epithelial cells, $P = .053$) or BM-derived Paneth cells (0.0 ± 0.0 vs 1.1 ± 0.87 cells/100 Y-FISH-positive epithelial cells, $P = .056$) showed a substantial increase in GVHD (+) recipients with regeneration compared with GVHD (-). In sharp contrast, BM-derived absorptive cells showed a significant decrease (79.8 ± 4.61 vs 59.1 ± 9.06 cells/100 Y-FISH-positive epithelial cells, $P = .039$) in GVHD (+) recipients with regeneration compared with GVHD (-). It has recently been proposed that goblet cells, enteroendocrine cells, and Paneth cells arise from a shared progenitor cell and are categorized as secretory lineage cells, which are distinct from absorptive lineage cells.^{7,8} Reanalysis of the data in terms of BM-derived secretory lineage cells and BM-derived absorptive lineage cells showed that secretory lineage cells were significantly increased (3.88 ± 1.02 , $P = .013$), whereas absorptive lineage cells were significantly decreased (0.74 ± 0.11 , $P = .039$) in GVHD (+) recipients with regeneration (Figure 7B). Such changes in the lineages of terminally differentiated BM-derived cells were not observed in GVHD (+) recipients without regeneration (secretory lineage cells, 1.75 ± 0.16 , $P > .1$; absorptive lineage cells, 0.9 ± 0.19 , $P > .1$).

To confirm that BM-derived secretory lineage cells increased during regeneration from GVHD, we further examined series of biopsy specimens taken at different time points from 3 different cases (Figure 7C). The first case, which did not develop GVHD (GVHD [-]), showed no increase of BM-derived epithelial cells (0.47 ± 0.154 vs 0.5 ± 0.139 cells/100 epithelial cells, $P > .1$), and the differentiated BM-derived epithelial cells were dominated by absorptive lineage cells. In sharp contrast, the second case, which developed acute GVHD around day 77 post-BMT, showed a significant increase in the total number of BM-derived epithelial cells (0.52 ± 0.184 vs 1.56 ± 0.574 cells/100 epithelial cells, $P = .037$) associated with an increase of the secretory lineage cells (0.0 vs 0.571 cells/100 epithelial cells) during regeneration from GVHD. The third case, which developed chronic GVHD, showed the same change as the second case. These results show that BM-derived epithelial cells increase in number via increase of Ki-67-positive cells and, at the same time, generate more

BM-derived cells of secretory lineage function in cases of severe intestinal inflammation and the following regeneration of the damaged epithelium. However, in any case, no less than 50% of terminally differentiated BM-derived epithelial cells were absorptive cells. This may be due to the surrounding environment of the small intestine, in which many absorptive cells are required and generated, compared with cells of other lineages.

To examine whether this change in differentiation pattern is unique to BM-derived cells, we examined the differentiation pattern of non-BM-derived cells. Interestingly, the differentiation patterns of both the total epithelial cells (Figure 8A) and the Y-FISH-negative epithelial cells (Figure 8B) were virtually the same and showed no difference among recipients with GVHD (-), GVHD (+) without regeneration, and GVHD (+) with regeneration. To examine further the contribution of BM-derived cells to the composition of the entire epithelium, we compared the proportions of BM-derived cells in epithelial cells of each lineage. Surprisingly, the proportions of BM-derived goblet cells and neuroendocrine cells were significantly increased in GVHD (+) recipients with regeneration as compared to GVHD (-) recipients (goblet cells, 0.29 ± 0.09 vs 1.39 ± 0.38 cells/100 goblet cells, $P = .006$; neuroendocrine cells, 0.16 ± 0.53 vs 3.95 ± 1.34 cells/100 neuroendocrine cells, $P = .001$), whereas the proportion of BM-derived absorptive cells remained at the same level (Figure 8C). The proportion of BM-derived secretory lineage cells was significantly increased (0.24 ± 0.07 vs 1.92 ± 0.51 cells/100 secretory lineage cells, $P = .003$) up to 8-fold in GVHD (+) recipients with regeneration as compared with GVHD (-) recipients. No changes of the proportions of terminally differentiated BM-derived cells were observed in GVHD (+) recipients without regeneration. Collectively, these results suggested that the change in differentiation pattern at the site of intestinal inflammation is unique to BM-derived cells. They also suggested that the proportion of BM-derived secretory lineage cells increases at the site of intestinal inflammation, thereby supporting the specific functions of the epithelium during regeneration from epithelial damage.

Discussion

The present study provides evidence that human BM-derived intestinal epithelial cells are euploid, with only rare cells expressing the stem cell marker Musashi-1 expression and no detectable cells showing the long-term survival thought to be characteristic of stem cells. A few BM-derived cells showed evidence of proliferation,

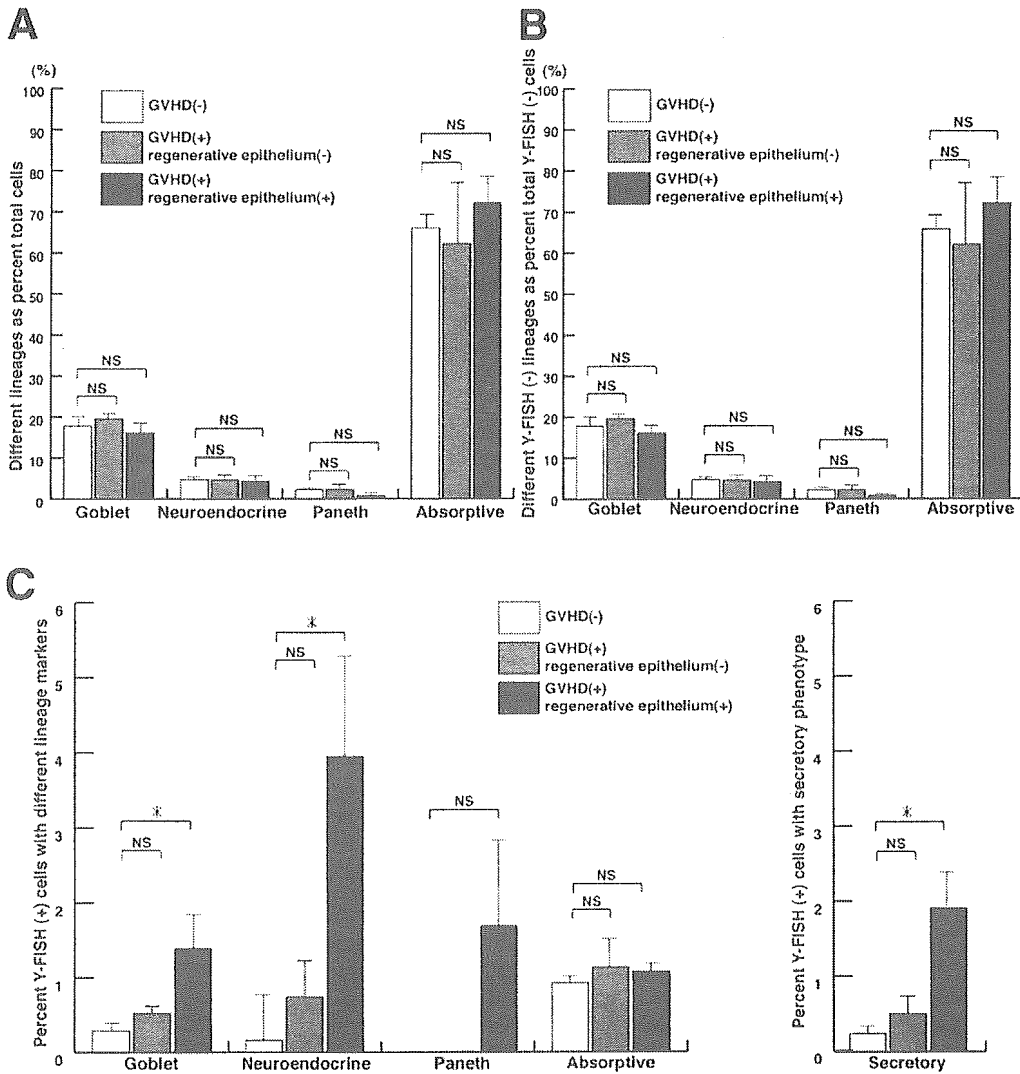


Figure 8. The proportion of BM-derived epithelial cells among total secretory lineage cells increases in the regenerative epithelium following GVHD. (A) The differentiation pattern in a total of 30,973 epithelial cells was compared in the 3 groups of patients described in Figure 7A. No difference was observed among the groups. Values are shown as mean \pm SE. NS indicates $P \geq .05$. (B) The differentiation pattern in a total of 30,734 Y-FISH-negative epithelial cells was compared in the 3 groups. No difference was observed among the groups. Values are shown as mean \pm SE. NS indicates $P \geq .05$. (C) The proportion of BM-derived cells within each lineage was compared in the 3 groups. The proportions of BM-derived goblet cells within total goblet cells and of BM-derived neuroendocrine cells within total neuroendocrine cells were significantly increased in GVHD (+)/regenerative epithelium (+) compared to GVHD (-). Consequently, the proportion of BM-derived secretory lineage cells within total secretory lineage cells was significantly increased up to 8-fold in GVHD (+)/regenerative epithelium (+) compared with GVHD (-), whereas no difference was seen in the proportion of BM-derived absorptive cells. Values are shown as mean \pm SE. *Indicates $P < .05$. NS indicates $P \geq .01$.

whereas a majority express markers of terminal differentiation.

Recent studies both in vitro and in vivo have demonstrated that fusion between BM cells and tissue-specific cells could be one mechanism by which BM-derived nonhematopoietic cells arise.¹¹⁻¹⁵ Cells generated in vivo through this mechanism form polyploid cells called *heterokaryons*, which may subsequently give rise to 2 euploid cells by cytoreductive division.^{13,14,27} In the present study using multicolor FISH, no BM-derived epithelial cell examined was found to be a heterokaryon. We have previously reported that no epithelial cell expressing 2 discrete signals of Y-FISH within a single nucleus could be found in the intestinal epithelium of a male BMT recipient whose donor was male.⁹ These findings together suggest that BM-derived epithelial cells are eu-

ploid cells and not heterokaryons generated by cell fusion. Several reports support the absence or the rare contribution of cell fusion in vivo. A recent report has evaluated cell fusion events in vivo using the Cre/lox system and suggested that epithelial cells of lung, liver, and skin can develop from BM-derived cells without cell fusion.²⁰ However, our present method cannot exclude the possibility of cell fusion followed by cytoreductive division.

Although several reports have demonstrated the presence of BM-derived epithelial cells within the intestine, it has never been clarified how these cells arise, proliferate, or function within the intestinal epithelium.^{9,37-40} In other nonhematopoietic tissues, BM-derived cells are reported to distribute as tissue-specific cells via tissue-specific stem cells²² or intermediate progenitor cells.^{18,41}

Our present results show that BM-derived epithelial cells virtually never express functional or molecular features of intestinal stem cells. On the other hand, we showed that Ki-67–positive, BM-derived epithelial cells occasionally appear to divide at least once within the intestinal epithelium. Moreover, coexpression of various lineage-specific differentiation markers has been frequently observed within BM-derived epithelial cells. Coexpression of these lineage-specific differentiation markers directly reflect the lineage-specific functions of the BM-derived cells, and, thus, we have demonstrated for the first time that BM-derived epithelial cells adopt the specific phenotype of differentiated epithelial cells. However, we did not observe clusters of 3 or more BM-derived epithelial cells, of over 3 adjacent cells, and pairs of BM-derived epithelial cells were rare (3 pairs observed within 30,973 epithelial cells). From these results, we suggest that BM-derived epithelial cells reside as late transit cells with limited dividing potential,² which in turn give rise to differentiated, functional epithelial cells within an extremely short period. This is indeed advantageous for the repair of the damaged epithelium because BM-derived cells can immediately function within the epithelium in response to severe tissue injury and thereby support the essential functions of the intestinal epithelium.

A novel finding to emerge from our study is that the proportion of BM-derived epithelial cells expressing markers of secretory lineages increases during epithelial regeneration in response to GVHD. In contrast, the proportion of non-BM-derived, resident epithelial cells expressing markers of secretory lineage did not change during GVHD-associated regeneration. This suggests that, during inflammation and epithelial damage because of GVHD, differentiation of BM-derived epithelial cells toward secretory lineage may be regulated differently compared with resident epithelial cells. This mechanism could have advantages for epithelial repair if the BM-derived secretory lineages express factors such as Trefoil peptides,⁴² which promote restitution or mediators of defense such as mucin and antimicrobial peptides. Because Notch signaling^{43,44} regulates the decision between secretory and absorptive lineage,^{7,45,46} future examination of the expression of this pathway in BM-derived epithelial cells during GVHD and regeneration could be of interest. However, another possibility that BM-absorptive cells have shorter survival time and therefore appear to decrease in relative proportion during inflammation and epithelial damage because of GVHD must also be considered. Effects of inflammatory mediators or mesenchymal cell-derived factors on differentiation of

BM-derived epithelial cells to secretory lineage could also be of interest.

In the human intestine, mesenchymal cells such as myofibroblasts are also reported to arise from BM-derived cells and increase their population during regeneration from intestinal inflammation.^{47,48} This suggests that both BM-derived epithelial cells and BM-derived mesenchymal cells may share the same origin within the BM cells, such as mesenchymal stem cells. A recent report using a mouse BM transplantation model has suggested that BM-mesenchymal stem cells may be the origin of BM-derived gastrointestinal epithelial cells.⁴⁹ However, there are series of studies suggesting hematopoietic stem cells as the origin of BM-derived intestinal epithelial cells.^{37,50} Thus, further studies are needed to determine the exact population within BM cells that give rise to BM-derived intestinal epithelial cells or BM-derived mesenchymal cells in the human intestine.^{51,52}

In conclusion, BM-derived epithelial cells arise via a mechanism other than cell fusion and virtually never give rise to intestinal stem cells. However, these cells reside as late transit cells, which in turn give rise to differentiated, functional epithelial cells within an extremely short period. During regeneration following epithelial damage, BM-derived cells increase as functional secretory lineage cells, thereby supporting the regeneration and the essential functions of the intestinal epithelium. These results not only provide further evidence for the use of BM-derived cells to regenerate human intestinal epithelium but also suggest the existence of a unique regulatory system targeting BM-derived cells, which can change the differentiation pattern at the site of intestinal inflammation.

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A Specific Genetic Alteration on Chromosome 6 in Ulcerative Colitis-associated Colorectal Cancers¹

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ABSTRACT

Various genetic alterations in ulcerative colitis (UC)-associated colorectal cancers (CRCs) have been reported. However, almost all studies have shown abnormalities of the known genes that have been demonstrated in sporadic CRCs. To identify novel genetic alterations, we selected unintentionally 35 microsatellite markers, and performed allelotype study in CRCs or dysplastic lesions from UC. High frequency of loss of heterozygosity (LOH; 62.5%) was detected on chromosome 6 (*D6S468*) but not on other chromosomes. With four additional microsatellite markers around the *D6S468* locus, we determined the commonly deleted region between two loci, *D6S1543* and *D6S1580*, in UC-associated CRCs and dysplasia. Interestingly, there was no LOH in this region in sporadic CRCs and severely inflamed lesions of longstanding and extensive UC without cancers. These results indicated the presence of novel tumor suppressor genes on chromosome 6 related to the carcinogenesis of UC but not to sporadic CRCs.

INTRODUCTION

UC³ is a chronic inflammatory bowel disease with unknown etiology. It is well known that longstanding and extensive UC is high-risk for CRCs (1). Although the adenoma-carcinoma sequence is known as the molecular mechanism for carcinogenesis of sporadic CRCs (2), it is not solely applied to UC-associated CRCs. In sporadic CRCs, mutation of the *APC* gene is an early event, and mutation of *p53* is a late event in their carcinogenesis. In contrast, mutation of *APC* gene is rare and mutation of *p53* is an early event in the carcinogenesis in UC-associated CRCs (2–5). MSI is another important mechanism of carcinogenesis (6–8). Two phenotypes, MSI-H and low frequency of MSI (MSI-L), have been described in sporadic CRCs (9). In CRCs of hereditary nonpolyposis colon cancer with MSI-H phenotype, the mutation of *hMLH1* or *hMSH2* has been detected (10–12). In UC-associated CRCs, it has been reported that the frequency of MSI is low, and those cases are related to *hMLH1* promoter hypermethylation (13). These facts suggest that there are different mechanisms of carcinogenesis between sporadic CRCs and UC-associated CRCs, and novel tumor suppressor genes may exist in UC-associated CRCs.

MATERIALS AND METHODS

Samples. A total of 12 paired samples of CRCs or dysplastic lesions and corresponding noncancerous lesions derived from UC were obtained at Keio University Hospital (Shinjuku-ku, Tokyo, Japan), Kitasato Institute Hospital

(Minato-ku, Tokyo, Japan), and Hyogo College of Medicine Hospital (Nishinomiya-shi, Hyogo, Japan). Twenty paired samples of sporadic CRCs and corresponding noncancerous lesions and 20 samples of severe inflamed lesions in longstanding and extensive UC were obtained at Keio University Hospital. All of the samples were fixed in formalin, embedded in paraffin, and attached individually to glass slides. Genomic DNA was extracted according to the methods described elsewhere (14).

Microsatellite Markers. Thirty-nine microsatellite markers were used in this study (Figs. 1 and 2). We referred to the database of National Center for Biotechnology Information (NCBI) for sequences of primers to make PCR products of <200 bp.

LOH Analysis. We performed LOH analysis according to the methods described elsewhere (15).

RESULTS

Allelotype Study. The results of allelotype study using paired samples of CRCs or dysplastic lesions and corresponding noncancerous lesions from UC are summarized in Fig. 1. LOH was found at 16 of 35 loci and was distributed in 12 chromosomes. At the *D6S468* locus on chromosome 6, 8 of 12 cases were informative, and 5 (62.5%) of 8 cases showed LOH. In contrast, the frequency of LOH was extremely low and was <25% on other chromosomes. LOH on chromosome 6 was detected in four of six CRCs. Four cases with LOH on chromosome 6 consisted of one case with well-differentiated adenocarcinoma and three with moderately or poorly differentiated adenocarcinomas. Interestingly, one case of dysplastic lesions also showed LOH. It means there is no significant relationship with pathological diagnosis of neoplasia. Besides there was no association with clinical courses such as duration of disease, location of tumor, or resistant properties for medication.

Deletion Mapping on Chromosome 6. We then performed LOH analysis on chromosome 6 using the same samples from UC according to the results of the allelotype study. An additional four microsatellite markers were selected around the *D6S468* locus (see the deletion map of chromosome 6 in Fig. 2A). We determined the commonly deleted region between two loci, *D6S1543* and *D6S1580*. The distance of these two loci is ~6 cM apart. Typical patterns of LOH analysis were also shown in Fig. 2B. LOH (arrowhead) was detected in the tumor at the loci of *D6S1543*, *D6S468*, and *D6S283*, but not at the loci of *D6S1606* and *D6S1580*.

LOH Analysis at the *D6S283* Locus in Sporadic CRCs. We then assessed whether the genetic alteration on chromosome 6 was specific to UC-associated CRCs or not. We investigated LOH analysis in paired samples of cancer and corresponding noncancerous lesions from 20 sporadic CRCs at the *D6S283* locus. The reason why we used this locus was that the *D6S283* locus is the commonly deleted region and showed clear bands of alleles in UC-associated CRCs. In any cancerous lesions of the 20 sporadic CRCs, no LOH was detected at the *D6S283* locus (Table 1). Even in three patients with moderately or poorly differentiated adenocarcinoma, no LOH was detected.

LOH Analysis at the *D6S283* Locus in Longstanding and Extensive UC without CRC. To investigate whether the genetic alteration occurred in the chronic inflammation process, we carried out

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³ The abbreviations used are: UC, ulcerative colitis; CRC, colorectal cancer; LOH, loss of heterozygosity; MSI, microsatellite instability; MSI-H, high frequency of MSI