Candidate markers for stem and early progenitor cells, Musashi-1 and Hes1, are expressed in crypt base columnar cells of mouse small intestine

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Abstract Musashi-1, a neural RNA-binding protein, is important for maintaining neural stem cells. Both Musashi-1 and Hes1, a transcriptional factor regulated by Musashi-1, are expressed in the small intestine. Here we show that Musashi-1 is present in a few epithelial cells just above the Paneth cells in the small intestinal crypt, the putative position of stem cells, whereas Hes1 is expressed in lower crypt cells just above the Paneth cells, including Musashi-1-positive cells. Musashi-1 and Hes1 were not expressed in Paneth cells. Notably, Musashi-1 and Hes1 were coexpressed in the crypt base columnar cells located between the Paneth cells. These findings suggest that not only the cells just above Paneth cells but also the crypt base columnar cells between the Paneth cells have stem cell characteristics.

Key words: Musashi-1; Hes1; Small intestine; Stem cell; Crypt base columnar cell

1. Introduction

Small intestinal epithelium is composed of four major cell types: enterocytes, goblet cells, enteroendocrine cells, and Paneth cells. All differentiated epithelial cells in the small intestinal mucosa are believed to originate from multipotent stem cells located just above the crypt base [1–3]. Although recent studies have identified and characterized stem cells in several tissues including the central nervous system [4–6], the stem cells in the small intestinal epithelium have not been well defined, due in part to the lack of good molecular markers for stem cells.

Musashi-1 (Msi-1), a neural RNA-binding protein, has been isolated as a mammalian homologue of a *Drosophila* protein that is required for asymmetric division of sensory neural precursor cells [7,8]. It was subsequently demonstrated that Msi-1 is selectively expressed in neural progenitor cells, including stem cells, and has key roles in the maintenance of the stem cell state and its differentiation [9–12]. Thus, Msi-1 is suggested to be a mammalian neural stem cell marker [4]. On the other hand, recent studies demonstrated that Hes1, a transcriptional factor regulated by Notch signaling [13], is essential for the self-renewing activity of neural stem cells and for repression of their commitment to the neuronal lineage [14–16]. More recently, Imai et al. reported that Msi-1 potentiates *Hes1* promoter activity, suggesting a close link between Msi-1 and Hes1 [17]. And others have reported that both Msi-1 and Hes1 are expressed in the small intestine [4,18]. In the present study, therefore, we investigated Msi-1 and Hes1 expression in the small intestinal epithelial cells of the mouse, and attempted to identify putative stem cells.

2. Materials and methods

2.1. Cell lines and culture

IEC6, originated from rat small intestinal epithelium, and Intestine407, originated from human small intestinal epithelium, were cultured in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2. All media and chemicals used in the cell cultures were obtained from Gibco BRL (Grand Island, NY, USA).

2.2. Animals and tissue preparation

ICR mice (Shizuoka Laboratory Animal Center, Shizuoka, Japan) were used in this study. The mice were kept in isolator cages in a barrier facility under a 12 h light cycle and maintained under specific pathogen-free conditions. Postnatal and adult ICR mice were killed at P1, 7, 14, or 21 (day of birth was defined as postnatal day P0). The small intestine was immediately removed and subjected to immunohistochemistry and RNA extraction. All animal procedures followed the guidelines for animal experiments of Kyoto University.

2.3. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using the single-step guanidinium thiocyanate phenol–chloroform method (Trizol; Gibco BRL). To generate cDNA, 5 μg of total RNA was reverse-transcribed using 200 U of SuperScript II RT (Gibco BRL) in a total reaction volume of 20 μl. For the following PCR, pairs of oligonucleotide primers for mouse *Msi-1*, mouse *Hes1*, and mouse *Glyceraldehyde-3 phosphate dehydrogenase* (*GAPDH*) were prepared: mouse *Msi-1*, 5′-CGAGCTCGACTCCAAAACAAT-3′ (sense) and 5′-GGCTTTCTTGCATTCCACCA-3′ (antisense); mouse *Hes1*, 5′-CTACCCCAGCCAGTGTCAAC-3′ (sense) and 5′-AAGCGGGTCACCTCGTTCAT-3′ (antisense); mouse *GAPDH*, 5′-TTAGCCCCCCTGGCCAAGG-3′ (sense) and

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Abbreviations: Msi-1, Musashi-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; (RT-)PCR, (reverse transcription-)polymerase chain reaction

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5′-CTTACTCCTTGGAGGCCATG-3′ (antisense). One microliter of reverse-transcription product was amplified by PCR in a 50 μl reaction volume containing 10 pmol of the above primer sets, 1.25 U Ampli-Taq DNA polymerase (Applied Biosystems, Branchburg, NJ, USA), PCR buffer [final concentration: 20 mM Tris–HCl (pH 8.4), 50 mM KCl], 2.5 mM $MgCl_2$, 10 mM dithiothreitol, and 1 mM dNTP. The PCR amplification was performed as follows: 95°C for 10 min, 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final extension step of 72°C for 5 min.

2.4. Immunohistochemistry

The small intestine removed from ICR mice was fixed with 4% paraformaldehyde overnight in 0.1 M phosphate-buffered saline (PBS; pH 7.4) at 4°C, embedded in paraffin and OCT compound (Tissue-Tek; Sakura Finetechnical, Tokyo, Japan), and cut at a thickness of 6 μm. Immunostaining for Msi-1, Hes1, and Ki-67, a proliferation marker, was performed as previously described [10,19]. In brief, sections were treated with 3% H_2O_2 in methanol for 20 min to quench endogenous peroxidase activity. The sections were then placed in 0.01 M citrate buffer (pH 6.0) and treated with microwave heating for 10 min to facilitate antigen retrieval. The sections were immunostained using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. Sections were incubated with 3% bovine serum albumin in PBS for 30 min and then incubated with anti-mouse Msi-1 antibody (final dilution 1:1000) [10], anti-mouse Hes1 antibody (kindly supplied by Dr. T. Sudo, Toray Industries, Tokyo, Japan, final dilution 1:1000) [19], or anti-mouse Ki-67 antibody (Dako Cytomation, Copenhagen, Denmark; final dilution 1:200, according to the manufacturer's instructions) at 37°C for 30 min. The sections were incubated with biotinylated secondary antibody for 40 min. After washing with PBS, avidin–biotin complex was applied for 30 min. The sections were then incubated in 3,3′-diaminobenzidine tetrahydrochloride with 0.05% H_2O_2 for 3 min and counterstained with Mayer's hematoxylin.

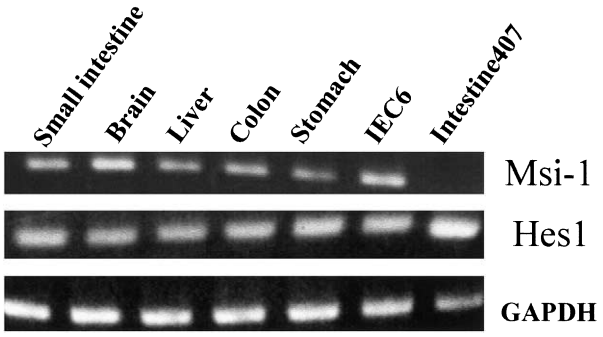

Fig. 1. RT-PCR analysis of *Msi-1* and *Hes1* mRNA expression in mouse normal tissues, IEC6, and Intestine407 cell lines. *Msi-1* and *Hes1* expression was detected in all tissues examined and IEC6. *Hes1* was expressed in Intestine407. *GAPDH* expression is shown as internal control.

3. Results

3.1. Expression of Msi-1 and Hes1 mRNAs in adult mouse tissue and intestinal cell lines

The PCR-amplified products obtained using Msi-1-specific and Hes1-specific primers had clear bands of the predicted sizes, 304 bp and 322 bp, respectively. The PCR product sequence was confirmed using the dideoxy chain termination procedure. *Msi-1* mRNA expression was detected in small intestine, colon, stomach, liver, and IEC6. *Msi-1* mRNA expression was not detected in Intestine407. On the other hand,

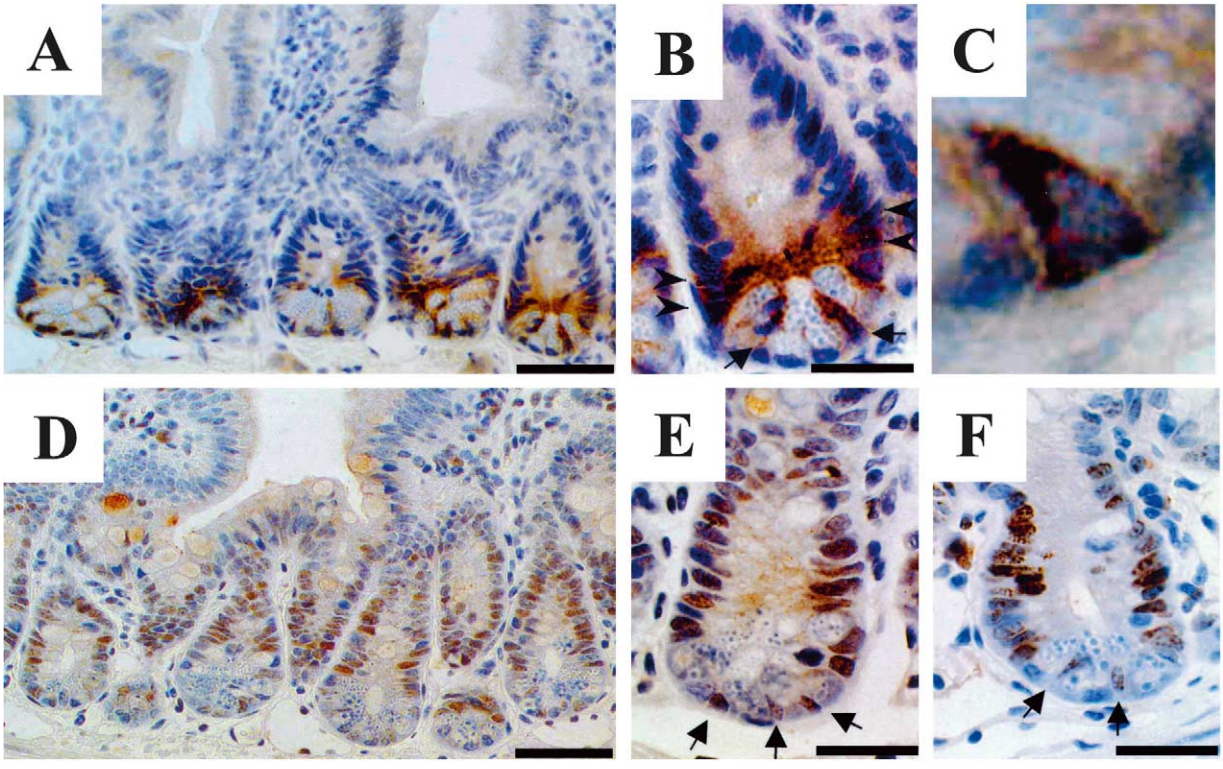

Fig. 2. Immunohistochemical analysis of Msi-1 (A–C), Hes1 (D,E), and Ki-67 (F) in adult mouse small intestine. A few crypt cells just above the Paneth cells (arrowhead) and the crypt base columnar cells between the Paneth cells (arrow) were stained with Msi-1 antibody. Msi-1 was absent in the Paneth cells. No Msi-1-positive cells were detected at the villous epithelium (A,B). Higher magnification of a crypt base columnar cell. Msi-1 reactivity was located in the cytoplasm, and the cell nucleus was not stained (C). Hes1 was predominantly expressed in mid to lower crypt cell nuclei and immunoreactivity gradually increased toward the crypt base. Goblet and Paneth cell nuclei were not stained. Crypt base columnar cells were also stained with Hes1 antibody (arrow) (D,E). Mid to basal part of crypt epithelial cells were stained with Ki-67 antibody. Moreover, crypt base columnar cells were also positive for Ki-67 (arrow) (F). Bars indicate 50 μm (B,E,F), and 100 μm (A,D).

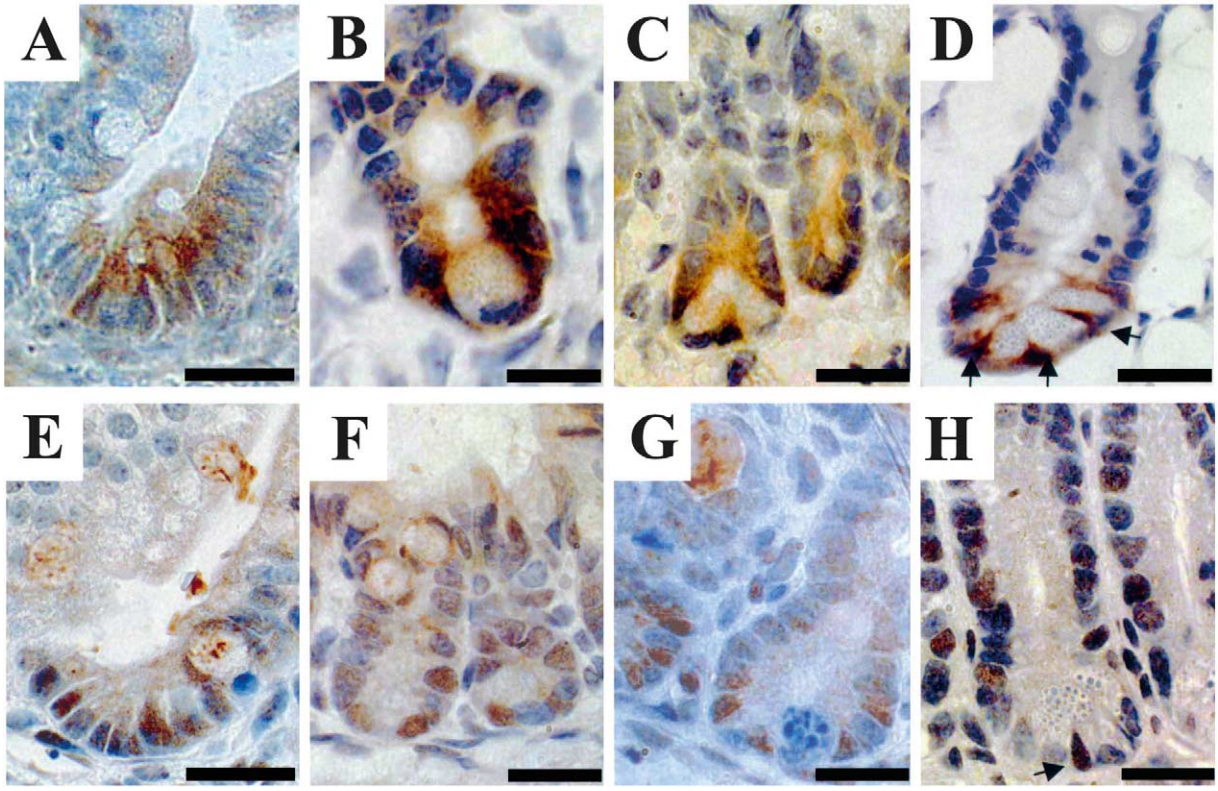

Fig. 3. Expressions of Msi-1 (A–D) and Hes1 (E–H) protein during the development of mouse small intestine. In P1 mice, there were no crypt structures. Both Msi-1 and Hes1 were detected in the intervillus epithelial cells (A,E). In P7 mice, small crypt structures were observed in the intervillus space and single Paneth cell began to appear in the crypt base. Almost all crypt cells were stained by both Msi-1 and Hes1 antibodies, but not the Paneth cells (B,F). In P14 mice, Msi-1 was expressed at the lower part of the single crypt. Msi-1 was not expressed in the Paneth cells (C). On the other hand, Hes1 was also expressed in the crypt cells, and the cells adjacent to the Paneth cells had strong reactivity to the Hes1 antibody (G). Two or three Paneth cells were observed in the intestinal crypt of P21 mice (D,H). In addition to the cells just above the Paneth cells, the crypt base columnar cells between Paneth cells were stained with both Msi-1 (D) and Hes1 (H) antibodies (arrow). Bars indicate 50 μm.

Hes1 mRNA expression was detected in all samples examined (Fig. 1).

3.2. *Msi-1 and Hes1 protein expression in adult mouse small intestine*

In adult mouse small intestine, Msi-1 expression was observed in a few cells just above the Paneth cells. The Msi-1-positive cells were located where the stem cells are thought to be located [1–3,20–22]. In contrast, Paneth cells were completely devoid of Msi-1 immunoreactivity. In addition to those Msi-1-positive cells just above the Paneth cells, crypt base columnar cells located between the Paneth cells were stained with the anti-mouse Msi-1 antibody. On the other hand, Msi-1 immunoreactivity was not detected in villus structures or the upper part of the crypt (Fig. 2A–C).

Hes1 was expressed in the nuclei of the cells at the lower part of the crypt including Msi-1-positive cells just above Paneth cells, but not in the goblet or Paneth cells. In the crypt–villus axis, Hes1 immunoreactivity was most prominent in the cells just above the Paneth cells and gradually decreased toward the villus tip. Of note, the nuclei of Msi-1-positive crypt base columnar cells between Paneth cells were also strongly positive for Hes1 (Fig. 2D,E). In addition to a few cells just above the Paneth cells, crypt base columnar cells between the Paneth cells were also positive for Ki-67 staining (Fig. 2F).

3.3. *Expression of Msi-1 and Hes1 protein during the development of mouse small intestine*

There was no crypt formation in mouse small intestine at P1. At P1, both Msi-1 and Hes1 expression were detected in the cells located at the intervillus region (Fig. 3A,E). Incomplete crypts were observed at P7, and a single Paneth cell appeared in some of those crypts. Expression of both Msi-1 and Hes1 was detected in a few cells adjacent to the Paneth cells in the crypt base, whereas Msi-1 and Hes1 immunoreactivity was completely absent in the Paneth cells (Fig. 3B,F). From P7 to P14, crypts became longer and some crypts became bifurcated. Msi-1 and Hes1 expression were still observed in the cells adjacent to the Paneth cells (Fig. 3C,G). Morphologically, crypt formation was almost complete at P21 and more than two Paneth cells were observed in each crypt base. Hes1-positive cells localized at the lower to middle portion of the crypts and further increased in number from P14. A few Msi-1-positive cells were localized just above the Paneth cells and thus their distribution was more restricted than that of Hes1. In addition, the crypt base columnar cells began to appear between the Paneth cells, and were immunoreactive for both Msi-1 and Hes1 (Fig. 3D,H) on P21. Villus enterocytes, goblet cells, and Paneth cells were not immunoreactive for Msi-1 or Hes1 throughout the developmental period.

4. Discussion

Stem cells of the small intestinal epithelium are believed to be located just above the Paneth cells in the crypt base, and migrate both upward toward the lumen of the gut and downward toward the crypt base as they mature [1–3,23,24]. In the present study, cells at the intervillus region were stained for both Msi-1 and Hes1 in the developmental period. But, in adult mice, Msi-1 was expressed in only a few cells just above the Paneth cells of the small intestinal crypt, and Hes1 was present in the cells at the lower part of the crypt, including Msi-1-positive cells. Msi-1 is present specifically in neural stem cells, and has an important role in the maintenance of the neural stem cell state and its differentiation [4,9,10]. Moreover, recent studies demonstrated that Hes1 is positively regulated by Msi-1, and is involved in neural stem cell self-renewal [17]. Thus, co-localization of Msi-1 and Hes1 in the cells just above the Paneth cells in adult mice strongly suggests that these cells represent progenitor cells, or stem cells of the small intestinal mucosa, supporting the concepts reported previously [1–3,21]. Furthermore, an attractive hypothesis derived from the present study is that Notch signaling plays an important role in the maintenance of the intestinal stem cells. However, it may also be noted that Hes1-positive cells were more broadly distributed than Msi-1-positive cells. Thus, it may be considered that although Hes1 and Msi-1 double positive cells represent stem cells, most Hes1-positive cells are transit-amplifying cells of the crypt and villous epithelial cell lineage.

Another interesting finding of the present study is that in addition to the cells just above the Paneth cells, crypt base columnar cells located between Paneth cells were also strongly positive for both Msi-1 and Hes1, while Paneth cells themselves were completely devoid of Msi-1 and Hes1 immunoreactivity. Furthermore, the Msi-1- and Hes1-positive cells just above and between the Paneth cells were both positive for Ki-67, indicating their high proliferative activity. These data suggest that crypt base columnar cells also have stem cell-like characteristics. Previously, Cheng et al. reported electron microscopic observations that the labeling of crypt base columnar cells after injection of [^{3}H]thymidine was followed by the labeling of Paneth cells, and suggested that Paneth cells originate from crypt base columnar cells [20,25,26]. Our present data might support this hypothesis. Furthermore, a recent report showed that crypt base columnar cells have a similar expression profile of EphB/EprinB to that of stem cells, which is distinct from Paneth cells [27].

In this study, we found that in addition to Hes1 single positive cells, Msi-1 and Hes1 double positive cells just above and between Paneth cells were both positive for Ki-67, indicating their high proliferating activity. Furthermore, we found that those Msi-1 and Hes1 double positive cells were also positive for bromodeoxyuridine (data not shown). It appears that most Ki-67-positive cells that have only Hes1 but not Msi-1 expression are transit-amplifying cells. However, since stem cells are believed to be in a dormant state generally, the reason why Msi-1 and Hes1 double positive putative stem cells expressed Ki-67 in our study needs to be clarified in a future study.

It has been believed that all the differentiated epithelial cells in the small intestinal mucosa, including Paneth cells, originate from common pluripotent stem cells located just above the Paneth cells [1–3], and that crypt base columnar cells might be transit cells in the Paneth cell lineage rather than stem cells [28]. However, the present study clearly demonstrated that not only the cells just above the Paneth cells but also the crypt base columnar cells were positive for both Msi-1 and Hes1. Thus, although it is not clear at present whether the Msi-1 and Hes1 double positive cells just above the Paneth cells and those located between the Paneth cells represent distinct progenitor cells of different cell lineage, it is possible that the cells just above the Paneth cells are the progenitor cells for crypt cells and villous epithelial cells that migrate toward the villus tip, while crypt base columnar cells between Paneth cells are the progenitor cells for only Paneth cells. In this regard, it may be noted that although both Msi-1 and Hes1 are absent in Paneth cells, Msi-1 disappears but Hes1 remains in the epithelial cells of the lower crypt just above the Msi-1 and Hes1 double positive putative stem cells. Thus, the mechanism of differentiation involving Msi-1 and Hes1 might be different between the putative stem cells just above the Paneth cells and those located between the Paneth cells.

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