Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 328 (2005) 348–352
BBRC
www.elsevier.com/locate/ybbrc

Hes1-deficient mice show precocious differentiation of Paneth cells in the small intestine☆

Katsumasa Suzuki[a], Hirokazu Fukui[a], Takahisa Kayahara[a], Mitsutaka Sawada[a], Hiroshi Seno[a], Hiroshi Hiai[b], Ryoichiro Kageyama[c], Hideyuki Okano[d], Tsutomu Chiba[a,*]

[a] *Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, Kyoto, Japan*
[b] *Department of Pathology, Kyoto University Graduate School of Medicine, Kyoto, Japan*
[c] *Institute of Virus Research, Kyoto University Graduate School of Medicine, Kyoto, Japan*
[d] *Department of Physiology, Keio University School of Medicine, Tokyo, Japan*

Received 21 December 2004
Available online 7 January 2005

Abstract

We have previously shown that Hes1 is expressed both in putative epithelial stem cells just above Paneth cells and in the crypt base columnar cells between Paneth cells, while Hes1 is completely absent in Paneth cells. This study was undertaken to clarify the role of Hes1 in Paneth cell differentiation, using *Hes1*-knockout (KO) newborn (P0) mice. Electron microscopy revealed premature appearance of distinct cells containing cytoplasmic granules in the intervillous region in *Hes1*-KO P0 mice, whereas those cells were absent in wild-type (WT) P0 mice. In *Hes1*-KO P0 mice, the gene expressions of cryptdins, exclusively present in Paneth cells, were all enhanced compared with WT P0 mice. Immunohistochemistry demonstrated increased number of both lysozyme-positive and cryptdin-4-positive cells in the small intestinal epithelium of *Hes1*-KO P0 mice as compared to WT P0 mice. Thus, Hes1 appears to have an inhibitory role in Paneth cell differentiation in the small intestine.

Keywords: Hes1; Cryptdin; Musashi-1; Small intestine; Stem cell; Paneth cell

The small intestinal epithelium is characterized by continuous replacement of epithelial cells through proliferation and differentiation of a pool of progenitor cells throughout life. However, the exact mechanism of differentiation of small intestinal epithelium remains unclear. The epithelium of the small intestine is composed of four major types of cells: enterocytes, goblet cells, enteroendocrine cells, and Paneth cells. Although the former three types complete their differentiation during upward migration toward the villous tip, Paneth cells achieve their terminal differentiation as they migrate downwards to the crypt base [1–3].

Hes1, a transcriptional factor regulated by Notch signaling [4], is essential for the self-renewal activity of neural stem cells and for repression of their commitment to the neuronal lineage [5,6]. Recently, Jensen et al. [7] have reported that *Hes1*-deficient mice display excessive differentiation of multiple endocrine cell types in the developing small intestine, suggesting that, similar to its effect on neuronal differentiation, Hes1 may be involved in the inhibition of small intestinal cell differentiation toward endocrine cells. Interestingly, we have recently shown that Hes1 is co-localized with Musashi-1, a possible stem cell marker of small intestinal epithelial cells, in a few cells just above Paneth cells (putative stem cells), and

☆ This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan.
☆☆ *Abbreviations:* KO, knockout; WT, wild-type; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
* Corresponding author. Fax: +81 75 751 4303.
E-mail address: cteya@kuhp.kyoto-u.ac.jp (T. Chiba).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.12.174

in crypt base columnar cells located between Paneth cells [8], suggesting involvement of Hes1 in Paneth cell differentiation as well as maintenance of stem cells. In the present study, therefore, by using *Hes1*-knockout (KO) mice, we tried to elucidate the role of Hes1 in Paneth cell differentiation.

Materials and methods

Animals. Pregnant CD1-*Hes1* mice [9] were kept in isolator cages in a barrier facility under a 12 h light cycle and maintained under specific pathogen-free conditions. *Hes1*-double KO mice die immediately after birth. Therefore, for the study, we sacrificed the *Hes1*-KO and wild-type (WT) newborn (P0) mice immediately after birth. *Hes1*-KO mice were genotyped as reported previously [9]. All animal procedures followed the guidelines for animal experiment of Kyoto University.

Electron microscopy. After P0 mice had been sacrificed, their small intestines were immediately fixed in 1% glutaraldehyde and 1.4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2), postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, dehydrated in a graded ethanol series, and embedded in Epon resin. After staining with uranyl acetate and lead citrate, the ultrathin sections were examined under an electron microscope (Hitachi H-700, Tokyo) [10].

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 SuperScript II RT (Gibco-BRL) in a total reaction volume of 20 μl. For the following PCR, pairs of oligonucleotide primers for mouse *cryptdin-1*, *cryptdin-4*, and *cryptdin-5*, mouse *lysozyme*, and mouse *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) were prepared: mouse *cryptdin-1*, *-4*, and *-5*, 5′-AAGAGACTAAAACTGAGGAGCAGC-3′ (sense) and 5′-CGACAGCAGAGCGTGTA-3′ (*cryptdin-1*; antisense), 5′-CGGCGGGGGCAGCAGTA-3′ (*cryptdin-4*; antisense), or 5′-GCA GCAGAATACGAAAGT-3′ (*cryptdin-5*; antisense) [11]; mouse *lysozyme* 5′-GGTCTACAATCGTTGTGAGTTGG-3′ (sense) and 5′-CTC CGCAGTTCCGAATATACTT-3′ (antisense); and mouse *GAPDH*, 5′-TTAGCCCCCCTGGCCAAGG-3′ (sense) and 5′-CTTACTCCTT GGAGGCCATG-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, 35, 30, 25, 20, or 15 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.

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 perpendicularly at a thickness of 6 μm. Immunostaining for Musashi-1, Hes1, Lysozyme, Cryptdin-4, and Ki-67, a proliferation marker, was performed as described previously [8,12,13]. 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-Musashi-1 antibody (final dilution 1:1000) [12], anti-Hes1 antibody (kindly supplied by Dr. T. Sudo, Toray Industries, Tokyo, Japan, final dilution 1:1000) [13], anti-lysozyme antibody (DAKO, Carpinteria, CA, USA, final dilution 1:200), anti-cryptdin-4 (α-defensin-4) antibody (Santa Cruz Biotechnology, CA, USA, final dilution 1:100) 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. To count the number of lysozyme-positive or cryptdin-4-positive cells, well-oriented areas from the villus to the intervillous region were selected. These positive cells were counted in at least 15 different areas from the villus to the intervillous region for each section, and the results were averaged.

Results

Lysozyme and cryptdin mRNA expressions in the small intestine of WT and Hes1-KO P0 mice

Lysozyme mRNA expression in the small intestine of *Hes1*-KO P0 mice was slightly increased compared with that of WT P0 mice. Moreover, in the small intestine of WT mice, only faint expression of *cryptdin-1*, *-4*, and *-5* mRNAs was observed at 35 cycles of PCR, whereas expression was greatly enhanced in *Hes1*-KO mice (Fig. 1).

Hes1, Musashi-1, lysozyme, and cryptdin-4 protein expression in the small intestine of WT and Hes1-KO P0 mice

A few Hes1-positive cells were present in the intervillous region of the small intestine of WT P0 mice, but no such cells could be detected in *Hes1*-KO mice (Fig. 2). On the other hand, Musashi-1 expression was observed in the cells located in the intervillous region of both *Hes1*-KO and WT P0 mice. However, the intensity of Musashi-1 immunoreactivity in *Hes1*-KO P0 mice was less than that in WT P0 mice (Fig. 2).

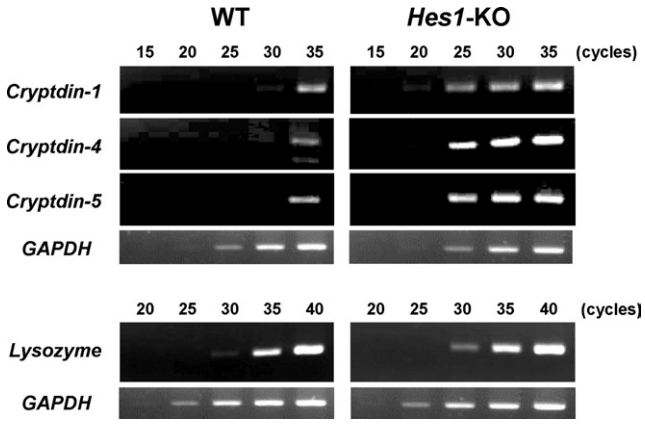

Fig. 1. *Lysozyme* and *cryptdin* mRNA expressions in the small intestine of WT and *Hes1*-newborn mice.

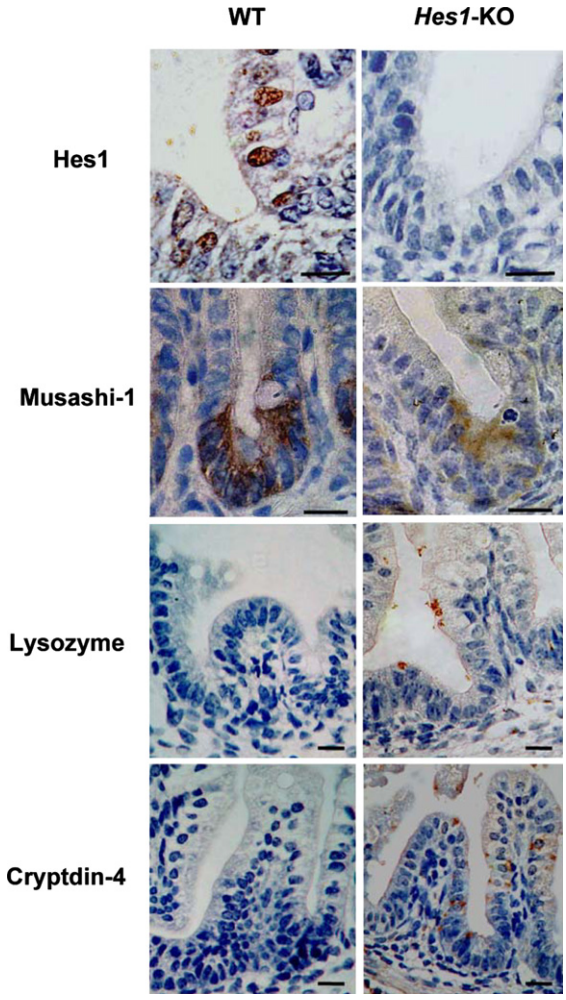

Fig. 2. Immunohistochemistry for Hes1 (bars = 10 μm), Musashi-1 (bars = 10 μm), lysozyme (bars = 10 μm), and cryptdin-4 (bars = 10 μm) in the small intestine of WT and *Hes1*-KO newborn mice.

Although lysozyme-positive and cryptdin-4-positive cells were hardly detected throughout the small intestinal epithelium of WT P0 mice, a few lysozyme-positive and cryptdin-4-positive cells were observed not only in the intervillous regions but also in the villi of *Hes1*-KO P0 mice (Fig. 2 and Table 1).

Electron microscopy

As in WT mice [8], there were no crypt structures in *Hes1*-KO mouse small intestine immediately after birth (P0). Normally, mature Paneth cells contain a large number of cytoplasmic granules. In WT P0 mice, we could hardly observe granule-containing cells throughout the small intestinal epithelium (Fig. 3A). In contrast, a few granule-containing cells could be detected in the villi or in the intervillous regions of *Hes1*-KO P0 mice (Fig. 3B). These granules contain high-density core matrix with low-density peripheral halo, resembling that of Paneth cells [14]. However, we usually observed immature Paneth cells with small granules, but hardly detected mature Paneth cells with large granules in *Hes1*-KO P0 mice. There were no obvious differences in the other cells in the intervillous region between *Hes1*-KO and WT mice.

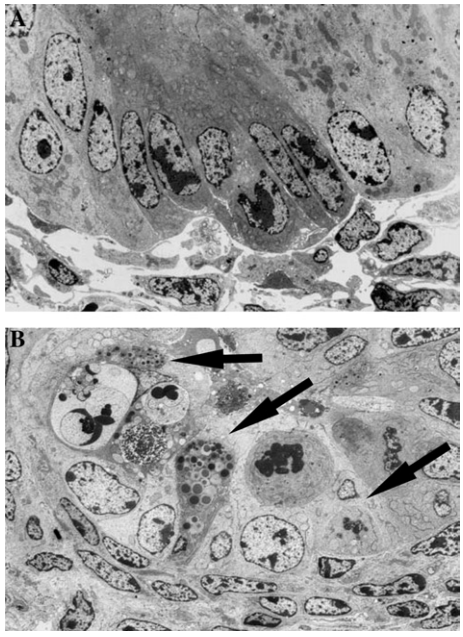

Fig. 3. Electron photomicrograph of the small intestine of a newborn WT mouse (A) and *Hes1*-KO newborn mouse (B). Granule-containing Paneth cells are observed in *Hes1*-KO newborn mouse (arrows in B), while no Paneth cells are seen in WT newborn mouse (bar = 2 μm).

Table 1
Number of lysozyme-positive and cryptdin-4-positive cells in the small intestine of WT and *Hes1*-KO newborn mice

	Lysozyme-positive cells		Cryptdin-4-positive cells	
	Villus	Intervillous region	Villus	Intervillous region
WT ($n = 4$)	0.00 ± 0.00	0.03 ± 0.02	0.00 ± 0.00	0.21 ± 0.07
Hes1-KO ($n = 8$)	$0.81 \pm 0.07^{*}$	$0.49 \pm 0.05^{*}$	$2.68 \pm 0.29^{*}$	$0.82 \pm 0.19^{*}$

The number of lysozyme-positive and cryptdin-4-positive cells was evaluated as described in Materials and methods. Results are expressed as means ± SE cells/each villus or intervillous region.

* Significantly greater than WT $P < 0.05$.

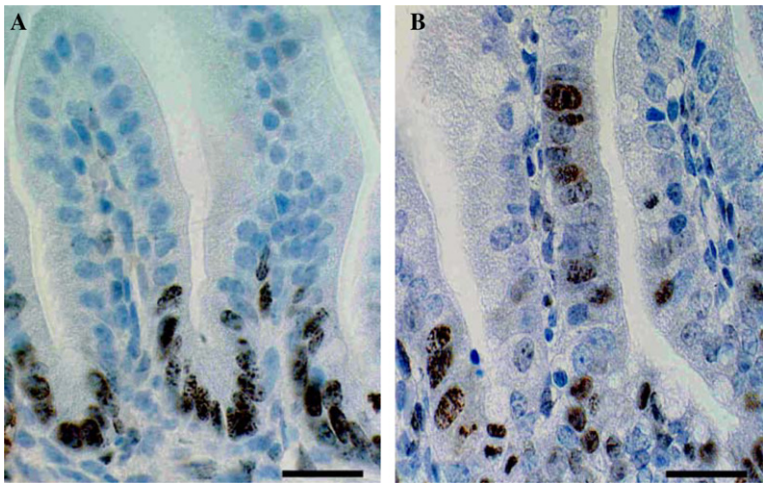

Fig. 4. Ki-67 expression in the small intestine of WT (A) and *Hes1*-KO (B) newborn mice (bars = 25 μm).

Ki-67 expression in the small intestine of WT and Hes1-KO P0 mice

In WT P0 mice, Ki-67-positive cells were present exclusively in the intervillous region of the small intestine, at a position corresponding to that of Hes1-positive cells. In contrast, in *Hes1*-KO P0 mice, Ki-67-positive cells were not localized to the intervillous region, but showed an irregular distribution throughout the epithelium (Fig. 4). This change was mirrored by a decrease in the number of Ki-67-positive cells in the intervillous region of *Hes1*-KO mice compared with WT P0 mice.

Discussion

We have shown previously that, in mouse small intestine, Hes1 is present in the nuclei of the cells just above Paneth cells (putative stem cells), and in crypt base columnar cells between Paneth cells, while Paneth cells are completely devoid of Hes1 staining, suggesting an inhibitory role for Hes1 in Paneth cell differentiation [8]. To further elucidate the role of Hes1 in Paneth cell differentiation, we used *Hes1*-KO newborn mice in this study. Previous studies have reported that no Paneth cells are present immediately after birth in normal mouse small intestine [8].

Electron microscopic observation in the present study revealed premature appearance of granule-containing cells, which resembled Paneth cells, in the small intestinal epithelium of *Hes1*-KO mice. In addition, we found that, in *Hes1*-KO mice, the gene expressions of cryptdins-1, -4, and -5, which are exclusively present in Paneth cells, were all enhanced compared with those in WT P0 mice. Furthermore, immunohistochemical studies demonstrated that, although lysozyme-positive and cryptdin-4-positive cells were hardly detected throughout the small intestinal epithelium of WT P0 mice, significant numbers of lysozyme-positive and cryptdin-4-positive cells were detected in the small intestinal epithelium of *Hes1*-KO P0 mice. These data confirmed precocious development of Paneth cells immediately after birth in *Hes1*-KO mice, suggesting that Hes1 has an inhibitory role in Paneth cell differentiation. Interestingly, we found in this study that lysozyme-positive and cryptdin-4-positive cells were detected not only in the intervillous regions but also in the villi of *Hes1*-KO P0 mice. These results may suggest that Hes1 also plays a role in the distribution of Paneth cells in the small intestinal epithelium.

Jensen et al. [7] have previously reported that *Hes1*-KO mice show precocious and excessive differentiation of multiple endocrine cell types in the small intestinal mucosa. Taken together, these findings indicate that Hes1 appears to act as a general negative regulator in differentiation of the small intestinal epithelium. Yang et al. [15] have shown that, in addition to disturbance of enteroendocrine and goblet cell development, development of Paneth cells is disturbed in *Math1*-deficient mice. Because Hes1 antagonizes the transcriptional activity of *Math1* [16], Hes1 may exert a general inhibitory action on differentiation of small intestinal cells, including Paneth cells, through antagonization of *Math1* transcriptional activity.

In this study, we observed that, in contrast to WT mice, Ki-67-positive cells in *Hes1*-KO newborn mice were not localized to the intervillous region, but were irregularly distributed throughout the epithelium. As strong Ki-67 staining is generally observed in proliferating progenitor cells in the intervillous region of normal newborn mice as well as in the crypt base of adult mice [8], our data may indicate that Hes1 has a role in localizing progenitor cells in the intervillous region or in the crypt base. Alternatively, precociously developed cells in

Hes1-KO mice, that have lost their proliferation activity, may have intermingled with Ki-67-positive progenitor cells.

We have previously shown that Musashi-1 is co-localized with Hes1 in the cells of the intervillous region of newborn mice [8]. Imai et al. [17] have reported that Musashi-1 enhances promoter activity of the *Hes1* gene. Our present study revealed that expression of Musashi-1 protein was decreased in *Hes1*-KO newborn mice. This suggests a positive feedback mechanism for Musashi-1 expression by Hes1, decelerating the differentiation process. Alternatively, as Musashi-1 is considered to be a stem cell marker in the small intestine [18], the decrease in Musashi-1-positive cells in *Hes1*-KO newborn mice may merely reflect a decrease of the stem cell pool by enhancement of precocious differentiation of the stem or progenitor cells in the intervillous region.

In summary, we have clearly demonstrated precocious development of Paneth cells in the small intestine of *Hes1*-deficient mice, indicating that Hes1 plays an inhibitory role in Paneth cell differentiation. It was noteworthy that, although crypt base columnar cells are strongly positive for Hes1, adjacent Paneth cells are completely devoid of Hes1. Thus, the mechanism of such sudden loss of Hes1 expression in crypt base columnar cells with resulting terminal differentiation toward Paneth cells needs to be elucidated in future studies.

Acknowledgments

We thank Dr. T. Sudo in Toray Industries, Tokyo, Japan, for kindly providing anti-Hes1 antibody. We also thank M. Fujioka (Central Laboratory for Electron Microscopy, Kyoto University Graduate School of Medicine) for technical assistance with the electron microscopy.

References

[1] H. Cheng, C.P. Leblond, Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian Theory of the origin of the four epithelial cell types, Am. J. Anat. 141 (1974) 537–561.

[2] M. Bjerknes, H. Cheng, The stem-cell zone of the small intestinal epithelium. I. Evidence from Paneth cells in the adult mouse, Am. J. Anat. 160 (1981) 51–63.

[3] C. Booth, C.S. Potten, Gut instincts: thoughts on intestinal epithelial stem cells, J. Clin. Invest. 105 (2000) 1493–1499.

[4] S. Jarriault, C. Brou, F. Logeat, E.H. Schroeter, R. Kopan, A. Israel, Signalling downstream of activated mammalian Notch, Nature 377 (1995) 355–358.

[5] C. Akazawa, Y. Sasai, S. Nakanishi, R. Kageyama, Molecular characterization of a rat negative regulator with a basic helix–loop–helix structure predominantly expressed in the developing nervous system, J. Biol. Chem. 267 (1992) 21879–21885.

[6] Y. Sasai, R. Kageyama, Y. Tagawa, R. Shigemoto, S. Nakanishi, Two mammalian helix–loop–helix factors structurally related to *Drosophila* hairy and enhancer of split, Genes Dev. 6 (1992) 2620–2634.

[7] J. Jensen, E.E. Pedersen, P. Galante, J. Hald, R.S. Heller, M. Ishibashi, R. Kageyama, F. Guillemot, P. Serup, O.D. Madsen, Control of endodermal endocrine development by Hes-1, Nat. Genet. 24 (2000) 36–44.

[8] T. Kayahara, M. Sawada, S. Takaishi, H. Fukui, H. Seno, H. Fukuzawa, K. Suzuki, H. Hiai, R. Kageyama, H. Okano, T. Chiba, Candidate markers for stem and early progenitor cells, Musashi-1 and Hes1, are expressed in crypt base columnar cells of mouse small intestine, FEBS Lett. 535 (2003) 131–135.

[9] M. Ishibashi, S.L. Ang, K. Shiota, S. Nakanishi, R. Kageyama, F. Guillemot, Targeted disruption of mammalian hairy and enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix–loop–helix factors, premature neurogenesis, and severe neural tube defects, Genes Dev. 9 (1995) 3136–3148.

[10] M. Sawada, K. Takahashi, S. Sawada, O. Midorikawa, Selective killing of Paneth cells by intravenous administration of dithizone in rats, Int. J. Exp. Path. 72 (1991) 407–421.

[11] D. Darmoul, D. Brown, M. Selsted, A.J. Ouellette, Cryptdin gene expression in developing mouse small intestine, Am. J. Physiol. 272 (1997) G197–G206.

[12] Y. Kaneko, S. Sakakibara, T. Imai, A. Suzuki, Y. Nakamura, K. Sawamoto, Y. Ogawa, T. Miyata, H. Okano, Musashi1: an evolutionally conserved marker for CNS progenitor cells including neural stem cells, Dev. Neurosci. 22 (2000) 139–153.

[13] T. Ito, N. Udaka, T. Yazawa, K. Okudera, H. Hayashi, T. Sudo, F. Guillemot, R. Kageyama, H. Kitamura, Basic helix–loop–helix transcription factors regulate the neuroendocrine differentiation of fetal mouse pulmonary epithelium, Development 127 (2000) 3913–3921.

[14] A. Ahonen, Histochemical and electron microscopic observations on the development, neural control and function of the Paneth cells of the mouse, Acta Physiol. Scand. Suppl. 398 (1973) 1–71.

[15] Q. Yang, N.A. Bermingham, M.J. Finegold, H.Y. Zoghbl, Requirement of Math1 for secretory cell lineage commitment in the mouse intestine, Science 294 (2001) 2155–2158.

[16] C. Akazawa, M. Ishibashi, C. Shimizu, S. Nakanishi, R. Kageyama, A mammalian helix–loop–helix factor structurally related to the product of *Drosophila* proneural gene atonal is a positive transcriptional regulator expressed in the developing nervous system, J. Biol. Chem. 270 (1995) 8730–8738.

[17] T. Imai, A. Tokunaga, T. Yoshida, M. Hashimoto, K. Mikoshiba, G. Weinmaster, M. Nakafuku, H. Okano, The neural RNA-binding protein Musashi1 translationally regulates mammalian numb gene expression by interacting with its mRNA, Mol. Cell. Biol. 21 (2001) 3888–3900.

[18] C.S. Potten, C. Booth, G.L. Tudor, D. Booth, G. Brady, P. Hurley, G. Ashton, R. Clarke, S. Sakakibara, H. Okano, Identification of a putative intestinal stem cell and early lineage marker; musashi-1, Differentiation 71 (2003) 28–41.