

concentrated, and the cDNA of the RNA molecule that had been isolated was finally cloned and its base sequence was determined. The results revealed that Musashi protein binds to sequences that repeat (GUU•••UAG) or (GUU•••UG) 2 or 3 times (consensus sequence: GU<sub>3–5</sub> (G or AG)) [19]. Interestingly, this sequence contributes to the asymmetric division of SOPs, and 15 sites are also present in the 3' untranslated region (UTR) of *tramtrack69* (*ttk69*), which codes a repressor of neuronal differentiation. Although TTK69 protein is expressed in Ila support-cell precursor cells, it had been found not to be expressed in Iib neural precursor cells. Nevertheless, we demonstrated that *ttk69* mRNA is present in Iib neural precursor cells, despite the absence of expression of TTK69 protein [19]. This can be interpreted to mean that two Ila support-cell precursor cells emerge from the initial asymmetric division in *musashi* mutants, but the *ttk69* mRNA in the Iib neural precursor cell is translated into TTK69 protein, and as a result, two Ila support-cell precursor cells emerge. We therefore tested the hypothesis that Musashi protein represses translation of the mRNA by binding to the Musashi-protein-binding sequence of the 3'-UTR of *ttk69* mRNA. Interestingly, in a gain-of-function mutation of the *ttk* gene (*ttk<sup>1</sup>*), a *Drosophila* transposon—P-element vector—was inserted within the exon corresponding to the 3'-UTR of *ttk69* mRNA [20]. As a result, the TTK69 protein was also translated in the Iib neural precursor cells of the *ttk<sup>1</sup>* mutant, as in the phenotype of the *musashi* mutant, and two Ila support-cell precursor cells were produced. This observation can be interpreted as showing that translational repression was eliminated because the Musashi protein was unable to act on the *ttk69* mRNA in the mutant, i.e., the *cis*-element for the translational repression of *ttk69* mRNA in Iib cells resides within the 3'-UTR of *ttk69* mRNA. Actually, in an in vitro experiment utilizing the chimeric Luciferase reporter gene, Musashi protein also repressed the translation of the Luciferase reporter gene through its effect on the 3'-UTR of *ttk69* mRNA. We therefore concluded that Musashi protein specifically binds to a binding sequence that is present on *ttk69* mRNA, and by repressing translation to TTK69 protein, which is a repressor of neuronal differentiation, maintains the potential of Iib neural precursor cells for neural differentiation. *Drosophila* Musashi protein is widely expressed in the compound eye primordium [15] and CNS [14]. It contributes to gene expression at the post-transcription level, i.e., to translation, etc., and it is predicted to possess a variety of context-dependent functions. Investigating whether *ttk69* mRNA is its sole target mRNA would seem to be an important future task.

Another puzzle remains, and that is that before and after SOP division, Musashi protein is almost equally distributed between the Ila cell and Iib cell. However, since Musashi protein only represses the translation of TTK69 protein in Iib neural precursor cells, repression of translation must not occur in Ila support-cell precursor cells despite the presence of Musashi protein [19]. We postulated that the function of

Musashi protein in Ila cells is inactivated by modification at the post-translation level as a result of the actions of upstream signaling pathways. We are currently investigating the underlying mechanism.

The Musashi gene is also expressed in proliferating neural stem/progenitor cells (neuroblasts) [21] in the larval brain of *Drosophila* [14]. The neural stem/progenitor cells (neuroblasts) present in the larval brain differ from the neuroblasts in the embryonic development period, which only divide asymmetrically. After undergoing symmetric self-renewing division in the early stage, they have many characteristics in common with the mammalian NSCs in terms of repeated asymmetric divisions, and are considered a model system for mammalian neural stem/progenitor cells. Our research group is currently investigating the role of the Notch signal in these cells. We believe that the fact that Musashi is expressed in these cells is also important from the standpoint of considering the function of mammalian homologues of Musashi, which will be described next, and we have begun a functional analysis of Musashi in these cells. Overexpression of Musashi in the brain neural stem/progenitor cells of *Drosophila* larvae causes the proliferation of undifferentiated cells (Toriya M, Okano H; unpublished results). Thus, the role of *Drosophila* Musashi in larval neuroblasts may be analogous to that of mammalian Musashi-1, described below. Unlike the asymmetrical division of SOP, the phenomenon of induction of proliferation of neural stem/progenitor cells (neuroblasts) in the larval brain by overexpression of Musashi is difficult to explain by repression of translation of *ttk69* mRNA alone. A different function of Musashi due to a change in context is postulated, and we are currently conducting a genetic analysis in regard to this point. In the future, the Musashi and the Notch signaling systems in the neural stem/progenitor cells (neuroblasts) in larval brain are expected to contribute greatly as model systems to identification and analysis of factors that regulate the proliferation and differentiation of mammalian NSCs.

#### *Musashi-1 as a marker of mammalian neural stem/progenitor cells, and its function*

To elucidate the functions of the MUSASHI gene family in mammals, we first cloned the mouse homologue of Musashi-1 and then analyzed its pattern of expression in detail [5–7]. Based on the results of a series of studies on Musashi-1, we concluded that mammalian Musashi-1 plays important roles in cell fate decision, including the maintenance of the stem-cell state, differentiation, and tumorigenesis [12].

Musashi-1 is a member of the MUSASHI family in vertebrates, and analyses to date have shown that it is strongly expressed in the NSCs of the periventricular area or undifferentiated neural precursor cells of vertebrates as a whole. This has made it possible to map NSCs and progenitor cells in the CNS of a variety of vertebrates by

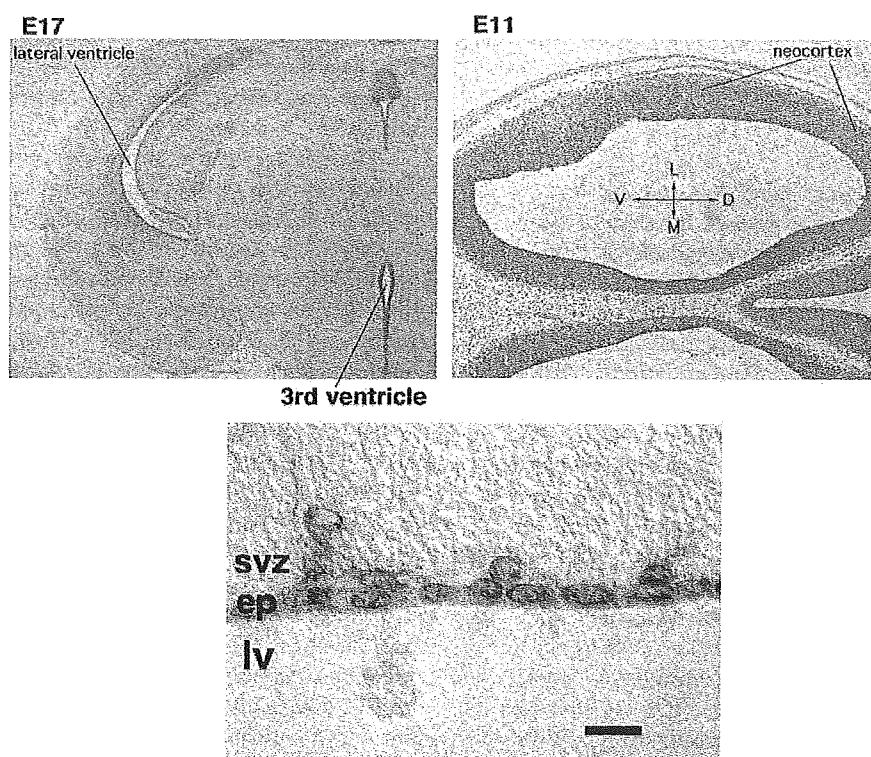


Fig. 1. Expression of Musashi-1 in mouse brain. Upper panel: Fetal forebrain (E17, E11). Lower panel: Adult forebrain (adapted from [7] with permission). In both fetal and adult stages, Musashi-1 protein is localized in the periventricular zone. Complete credit of the lower panel is given to the original source [7] and S. Karger AG, Basel.

using Musashi-1 as a marker. Our group in collaboration with Steven Goldman in the United States used Musashi-1 as a marker and succeeded in identifying NSCs and progenitor cells in the adult human brain [22].

A detailed analysis in the mouse has revealed strong expression of Musashi-1 in the ventricular zone of the neural tube in the embryo stage, when NSC and progenitor-cell division is very active in the CNS [5,7]. Strong expression of Musashi-1 protein has been observed at sites demonstrated in rodents, not only in the ventricular zone of the neural tube in the embryo stage, but also in the neurogenic sites within the postnatal brain including the so-called rostral migratory stream (RMS), which is a migration pathway from the external granule layer of the cerebellum and subventricular zone (SVZ) to the olfactory bulb [6]. In addition, the results of a detailed analysis have shown that Musashi-1 protein is selectively highly expressed in neural precursor cells, including NSCs, in the CNS during the embryonic stage (Fig. 1) [7]. The Musashi-1 protein in the postnatal CNS is expressed in the periventricular ependymal cells and astrocytes (SVZ astrocytes) (Fig. 1), which had been reported to possess stem cell characters in the adult brain [23,24]. Summarizing the above, expression of Musashi-1 protein in the CNS has been observed in NSCs around the ventricular zone of neural tube in the embryonic stage and in periventricular neural stem/progenitor cells (ependymal cells and SVZ

astrocytes) in the adult. Thus, Musashi-1 protein has been confirmed to be continuously expressed in neural stem-cell-like cells. Because of this expression pattern, we have postulated that the RNA-binding protein Musashi-1 is not simply a marker, but is involved in regulating the properties of NSCs through regulation of the translation of specific mRNA. On the other hand, our research group has discovered a second MUSASHI gene in vertebrates and named it Musashi-2 [25]. Musashi-2 protein displays more than 90% homology with Musashi-1 protein at the amino acid level in the RNA-binding domain. Its pattern of expression in the CNS is also very similar to that of Musashi-1 in terms of being strongly expressed in neural stem/progenitor cells, and they have been postulated to play mutually overlapping roles (see below). Nevertheless, Musashi-2 differs in terms of being continuously expressed in only some of the neurons in the CNS and in its level of expression in organs outside the CNS being almost uniform [25]. A thorough analysis of Musashi-2 has only just begun, and we will describe only the results of the analysis centered on Musashi-1 below.

#### *Functions of mammalian Musashi-1 protein*

What sort of roles is mammalian Musashi-1 protein responsible for in NSCs? Musashi-1 protein has been found to function in cooperation with Musashi-2 protein to

activate Notch signaling through repression of translation of the mRNA of the intracellular Notch signal repressor m-Numb, and to maintain the self-renewing ability of NSCs. More specifically, before analyzing the functions of mammalian MUSASHI protein, as described above, we attempted to identify its target RNA sequence by the SELEX method, which we had used when we analyzed *Drosophila* Musashi [19], and we found that it sequence-specifically binds to RNA that possesses a sequence in which (G or A)<sub>n</sub>AGU [ $n = 1-3$ ] is the motif [26]. When we searched for the mRNAs that possess this consensus sequence that are expressed in embryonic CNS, we confirmed that the Musashi-1 protein-binding sequence is present in the 3'-UTR of the *m-Numb* mRNA [27] and that Musashi-1 protein binds to *m-Numb* mRNA *in vivo* as well as *in vitro*. Musashi-1 protein was also shown to be present in the polysomal fraction [26], and was demonstrated to bind directly to a molecule that interacts with both a translation initiation factor and mRNA (Kawahara N, Imai T, Okano H; unpublished data), thereby repressing the target gene(s) at the translational level.

Based on the results of the analysis in *Drosophila*, m-Numb protein binds to the intracellular domain of Notch protein, and by guiding Notch protein endosomes from the cell surface and then to the degradation pathway, it is a modulator that exerts a repressive effect on the Notch signal [28]. Because m-Numb possesses such activity, it is thought that m-Numb protein positively modulates differentiation of neural stem/progenitor cells into neurons in the mammalian CNS, and that down-regulation of m-Numb protein itself from the neuron-production stage onward causes reactivation of the Notch signal and acts as the trigger for astroglia differentiation. Because it represses translation of this intracellular Notch-signal antagonist, Musashi-1 protein was expected to positively regulate Notch signaling, but actually it was confirmed that Musashi-1 protein induces transactivation of the promoter of the Notch signal target *Hes-1* gene [12,26]. In our previous study, we were able to demonstrate that overexpression of Musashi-1 induced the activation of Notch signaling through a pathway dependent on the action of CBF-1(RBP-J), which was measured by the transactivation of *Hes-1* promoter-Luciferase reporter, and the action of Musashi-1 to induce the transactivation of *Hes-1* promoter was found to be dependent on its RNA-binding activity [26].

It is now clear from a great deal of evidence that activation of the Notch signal positively regulates neural stem-cell self-renewal [29–31], and because it exerts an enhancing effect on the Notch signal, Musashi-1 is thought to contribute to retention of the properties of NSCs. We also demonstrated that the product of the second MUSASHI gene, Musashi-2, possesses *m-Numb* mRNA translation repressing activity, the same as Musashi-1 (Imai T and Okano H; unpublished data). Based on the pattern of expression described above as well, Musashi-2 has been postulated to act in the same manner as Musashi-1 in regulating NSCs.

When we combined preparation of knockout mice and functional repression by antisense ablation and conducted a functional double-knockout experiment on the Musashi-1 and Musashi-2 genes in order to elucidate this point, a sharp reduction in the efficiency of neurosphere formation was observed. By contrast, no such phenotype emerged as a result of single knockout (or knockdown) [32]. Thus, it appears that by functioning cooperatively, the Musashi-1 gene and Musashi-2 gene play important roles in maintaining the presence of NSCs or maintaining the undifferentiated state through repression of translation of *m-Numb* mRNA followed by activation of Notch signaling (Fig. 2) [12]. However, whether Musashi-1 and -2 proteins have target mRNA(s) other than *m-Numb* mRNA remains an open question. Future research study will elucidate the precise molecular mechanisms by which these mammalian MUSASHI proteins regulate the translation of their target mRNA(s), and their link to stem-cell maintenance.

Based on the above, although the target mRNA of the MUSASHI family differs in mammals and *Drosophila*, they both contribute to maintaining the character that produces neurons by regulating gene expression at the post-transcription level through repression of translation of target mRNA, and in that sense it seems that the function of the MUSASHI protein family can be said to have been conserved evolutionarily.

#### *Musashi-1 as an epithelial stem-cell marker, and its significance*

While it is true that the results of previous studies have shown that mammalian Musashi-1 increases the self-renewing ability of NSCs by activating Notch signaling through repression of translation of *m-Numb* mRNA, analysis of the upstream signal function involved in expression of the Musashi-1 gene is also important in terms of elucidating the full picture of stem cell regulation. The first step in elucidating it was to compare the Musashi-1 genes (including the base sequence of the 5'-upstream region and the intron region) in humans and mice, in both of which the base sequence of the entire genome has been deciphered, and to identify the sequences that have been conserved in both species. The results suggested that the Tcf/Lef binding sequence (Wnt signal response sequence) and Sox binding sequence are present in the regions that have been conserved outside the protein-coding region. The function of these sequences as transcription control *cis*-elements of the Musashi-1 gene is currently in the process of being analyzed. Wnt signaling and the Sox family transcription factors have each been found to play important roles in the induction and maintenance of intestinal stem cells, hematopoietic stem cells, certain types of neural stem/progenitor cells (Wnt signal: [33–35]), and undifferentiated neural precursor cells (Sox family: [21,22]). Based on what has already been learned, we set up a working hypothesis that expression of Musashi-1 is induced by Wnt signaling and the Sox family

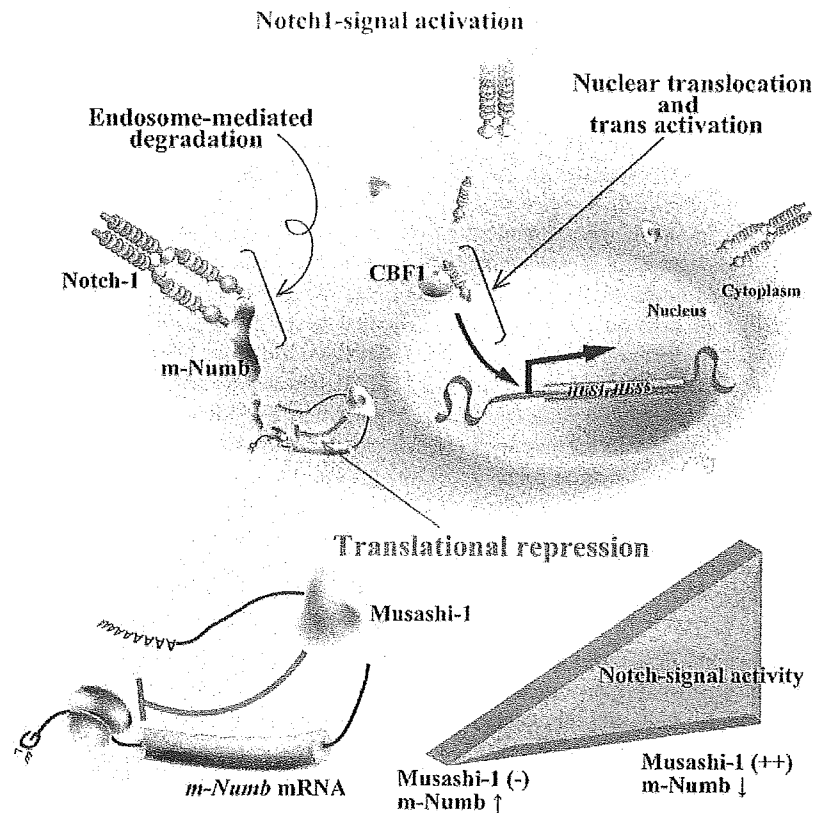


Fig. 2. Mechanism of Notch-1-signal activation by Musashi-1 protein. Musashi-1 protein binds to the 3'-UTR of *m-Numb* mRNA and inhibits its translation [26]. On the other hand, m-Numb protein binds to the intracellular domain of Notch-1 protein and inhibits the activation of the Notch-1 pathway, putatively by guiding Notch-1 protein from the cell surface to the endosome-mediated degradation pathway [28]. Consequently, Musashi-1 protein augments the Notch-1 signaling pathway. Through these mechanisms, Musashi-1 protein acts cooperatively with Musashi-2 protein to maintain the self-renewal of NSCs [12,32].

transcription factors, and that it then activates Notch signaling through repression of translation of *m-Numb* mRNA and causes crosstalk between several signal systems involved in the self-renewal of stem cells, and we are currently in the process of testing it. In the future, this Musashi-1 regulatory system may become an important target for screening for low-molecular-mass compounds that cause activation of endogenous NSCs described below. Assuming that it plays the general role of crosstalk with the signal systems involved in maintaining stem cells, Musashi-1 may contribute to maintaining stem-cell systems other than NSCs. When we reviewed the data from the Northern blot analysis we had performed previously, we noticed that the distribution of *musashi-1* mRNA in various organs other than the CNS was high in the small intestine of the adult [5]. Since Musashi-1 is expressed in the crypts, which is where intestinal epithelial stem cells are said to be present, we think that this may represent expression of Musashi-1 in intestinal epithelial stem cells and not a contribution by intestinal ganglion cells. To investigate this, we conducted a joint study with the epithelial stem-cell specialists Christopher Potten and Robert Clarke in the UK and discovered that Musashi-1 is also a marker of intestinal epithelial stem cells [36] and mammary stem cells [37]. The epithelial cells of intestinal

villi of the small intestinal mucosa are replaced within 2–3 days, and this rapid cell turnover, in addition to self-renewal by the intestinal epithelial stem cells present in the crypts of the small intestine, is thought to be maintained by a continuous supply of precursor cells that produce four types of cells that compose the epithelium of the mucosa of the small intestine: enterocytes, goblet cells, entero-endocrine cells, and Paneth cells. In the small intestine, he observed expression of Musashi-1 in the cells up to the 4th–6th position from the bottom of the crypts (especially the 4th), and in the cells in the deepest portion of the large intestine, where the possibility of stem cells is considered to be high [38], suggesting that it can be used as a stem cell marker, especially in the epithelium of the mucosa of the small intestine [36]. The Wnt signal system is known to play an important role in maintaining epithelial stem cells in the intestine [38], and it has been suggested that nuclear localization of stabilized  $\beta$ -catenin reaches its highest level as a result of activation of the canonical pathway of the Wnt signal in the 4th cells from the bottom of the crypts, which is the stem-cell position [38,39]. The fact that expression of Musashi-1 increases in the intestinal epithelium of mutants with functional deficiency of the APC molecule, which constitutively activates the Wnt signal, suggested that the

Wnt signal path performs an important function in expression of Musashi-1 in intestinal epithelial stem cells. Interestingly, a downstream factor of the Notch signal, HES-1 protein, has been found to be expressed in intestinal epithelial stem cells that express Musashi-1 [40]. In other words, the Wnt signal pathway and the Notch signal pathway become activated simultaneously in intestinal epithelial stem cells, and we are currently investigating the biological significance of this phenomenon and whether Musashi-1 is responsible for the simultaneous activation of the two signals by analyzing knockout mice. In addition, as stated above, Musashi-1 is also expressed in mammary epithelial stem cells. The report of Hans Clevers et al. in the past suggested that Tcf-4, a transcription factor that functions in the canonical pathways of the Wnt signal, plays an important role in the stem cells of the mammary epithelium as well as the intestine [41]. This is very interesting in terms of the transcription repression of the Musashi-1 gene, which contains many Tcf binding consensus sequences in its 5'-upstream region, which has been conserved among species, and we are in the process of verifying its role in maintenance of the integrated Wnt > Tcf-4 > Musashi-1 > Notch signal pathway in epithelial stem cells by analyzing stem cell dynamics in knockout mice for associated molecules. Thus, Musashi has begun to attract attention as a regulatory molecule that is also a marker of epithelial stem cells, in addition to NSCs.

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## *Hes1*-deficient mice show precocious differentiation of Paneth cells in the small intestine<sup>☆</sup>

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### 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.

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**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

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<sup>\*\*\*</sup> Abbreviations: KO, knockout; WT, wild-type; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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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'-AAGAGACTAAACTGAGGAGCAGC-3' (sense) and 5'-CGACAGCAGAGCGTGTA-3' (*cryptdin-1*; antisense), 5'-CGCGGGGGCAGCAGTA-3' (*cryptdin-4*; antisense), or 5'-GCA GCAGAATACGAAAGT-3' (*cryptdin-5*; antisense) [11]; mouse *lysozyme* 5'-GGTCTACAATCGTTGTGAGTTGG-3' (sense) and 5'-CTC CGCAGTCCGAATATACTT-3' (antisense); and mouse *GAPDH*, 5'-TTAGCCCCCTGGCCAAAGG-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<sub>2</sub>, 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<sub>2</sub>O<sub>2</sub> 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* ( $\alpha$ -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<sub>2</sub>O<sub>2</sub> 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).

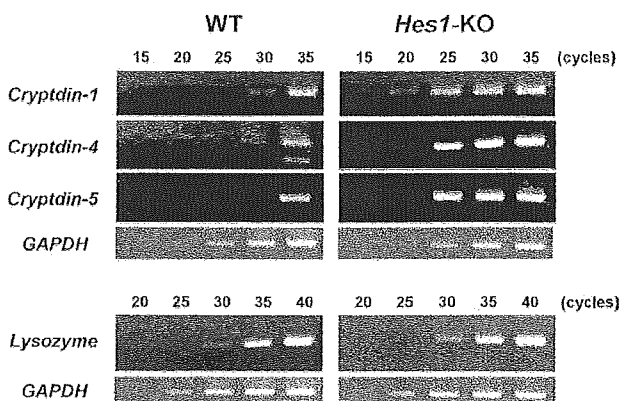


Fig. 1. *Lysozyme* and *cryptdin* mRNA expressions in the small intestine of WT and *Hes1*-newborn mice.



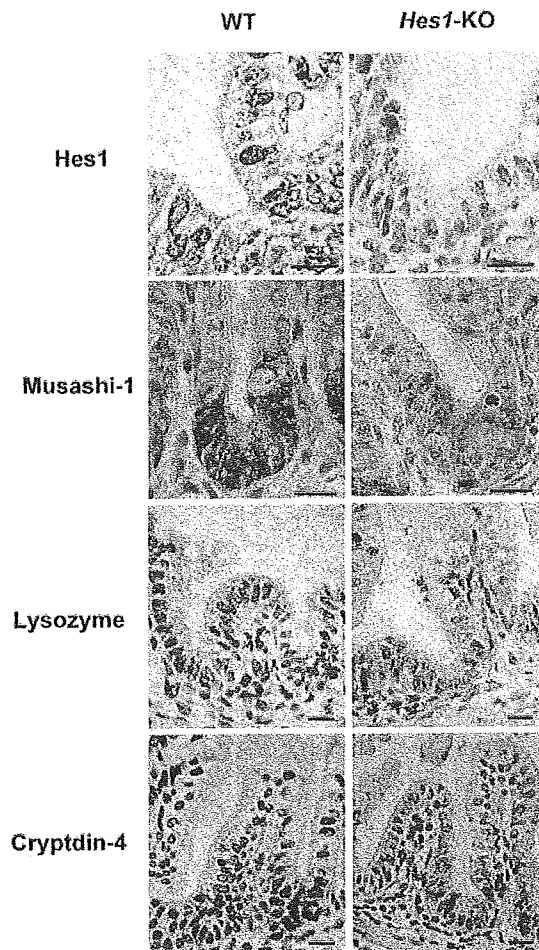


Fig. 2. Immunohistochemistry for Hes1 (bars = 10  $\mu$ m), Musashi-1 (bars = 10  $\mu$ m), lysozyme (bars = 10  $\mu$ m), and cryptdin-4 (bars = 10  $\mu$ m) in the small intestine of WT and *Hes1*-KO newborn mice.

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.

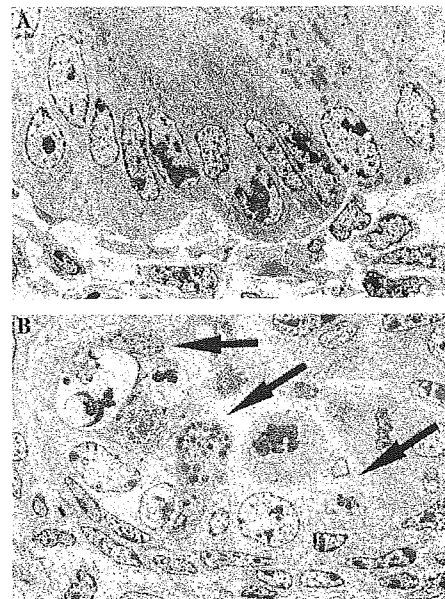


Fig. 3. Electron photomicrograph of the small intestine of a newborn WT mouse (A) and *Hes1*-KO newborn mouse (B). Granule-containing Paneth cells are observed in *Hes1*-KO newborn mouse (arrows in B), while no Paneth cells are seen in WT newborn mouse (bar = 2  $\mu$ m).

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 ( <i>n</i> = 4)	0.00 $\pm$ 0.00	0.03 $\pm$ 0.02	0.00 $\pm$ 0.00	0.21 $\pm$ 0.07
<i>Hes1</i> -KO ( <i>n</i> = 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  $\pm$  SE cells/each villus or intervillous region.

\* Significantly greater than WT *P* < 0.05.

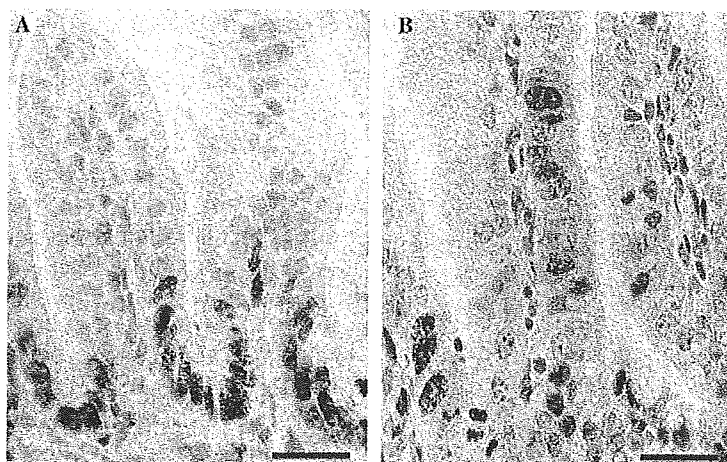


Fig. 4. Ki-67 expression in the small intestine of WT (A) and *Hes1*-KO (B) newborn mice (bars = 25 µm).

#### *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 through-

out 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.

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# Autoamplification of NFATc1 expression determines its essential role in bone homeostasis

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NFATc1 and NFATc2 are functionally redundant in the immune system, but it was suggested that NFATc1 is required exclusively for differentiation of osteoclasts in the skeletal system. Here we provide genetic evidence that *NFATc1* is essential for osteoclast differentiation in vivo by adoptive transfer of *NFATc1*<sup>-/-</sup> hematopoietic stem cells to osteoclast-deficient *Fos*<sup>-/-</sup> mice, and by *Fos*<sup>-/-</sup> blastocyst complementation, thus avoiding the embryonic lethality of *NFATc1*<sup>-/-</sup> mice. However, in vitro osteoclastogenesis in *NFATc1*-deficient cells was rescued by ectopic expression of NFATc2. The discrepancy between the in vivo essential role of *NFATc1* and the in vitro effect of NFATc2 was attributed to selective autoregulation of the *NFATc1* gene by NFAT through its promoter region. This suggested that an epigenetic mechanism contributes to the essential function of *NFATc1* in cell lineage commitment. Thus, this study establishes that *NFATc1* represents a potential therapeutic target for bone disease and reveals a mechanism that underlies the essential role of *NFATc1* in bone homeostasis.

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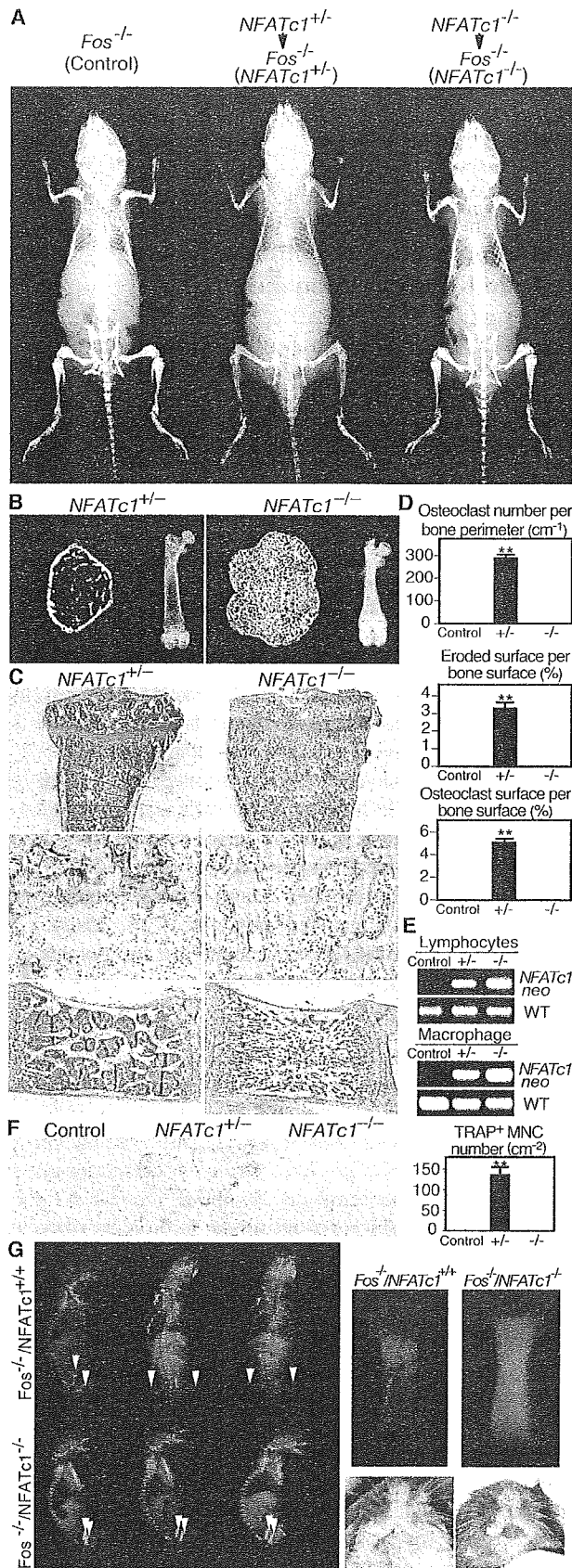
Abbreviations used: AP, activator protein; BMM, bone marrow monocyte/macrophage precursor cell; CBP, CREB-binding protein; ChIP, chromatin immunoprecipitation; ES, embryonic stem; FLC, fetal liver cell; MeCP2, methyl-CpG binding protein 2; RANKL, receptor activator of NF-κB ligand.

The mechanism underlying irreversible cell fate determination is critical for an understanding of biologic systems in vertebrates, where the NFAT family of transcription factors plays important roles (1, 2). Mice deficient in individual NFAT proteins exhibit a relatively mild phenotype in immune cells or neurons (1, 2), which suggests that the different family members play redundant roles in these systems (1–3). Homeostasis of the skeletal system depends on a balance between bone-forming osteoblasts and bone-resorbing osteoclasts (4). The critical role of *NFATc1* in the skeletal system was suggested by selective and strong induction of *NFATc1* in bone marrow monocyte/macrophage precursor cells (BMMs) that were stimulated with a key cytokine for osteoclastogenesis, receptor activator

of NF-κB ligand (RANKL) (5, 6). Although our previous in vitro analyses indicated that NFATc1 is the essential transcription factor for osteoclast differentiation (5), little is known about its in vivo function in osteoclastogenesis because targeted disruption of the *NFATc1* gene in mice results in embryonic lethality. Furthermore, there is a contrasting report that NFATc2 also activates this process and may have a redundant role (7). Considering the close functional similarity of NFATc1 and NFATc2 (3, 7), it is important to determine whether *NFATc1* is essential for osteoclast differentiation in vivo, and, if so, how it exerts an indispensable function in this cell type.

In this report, we provide in vivo genetic evidence for the essential role of *NFATc1* by two novel techniques: adoptive transfer of hematopoietic stem cells to osteoclast-deficient

The online version of this article contains supplemental material.



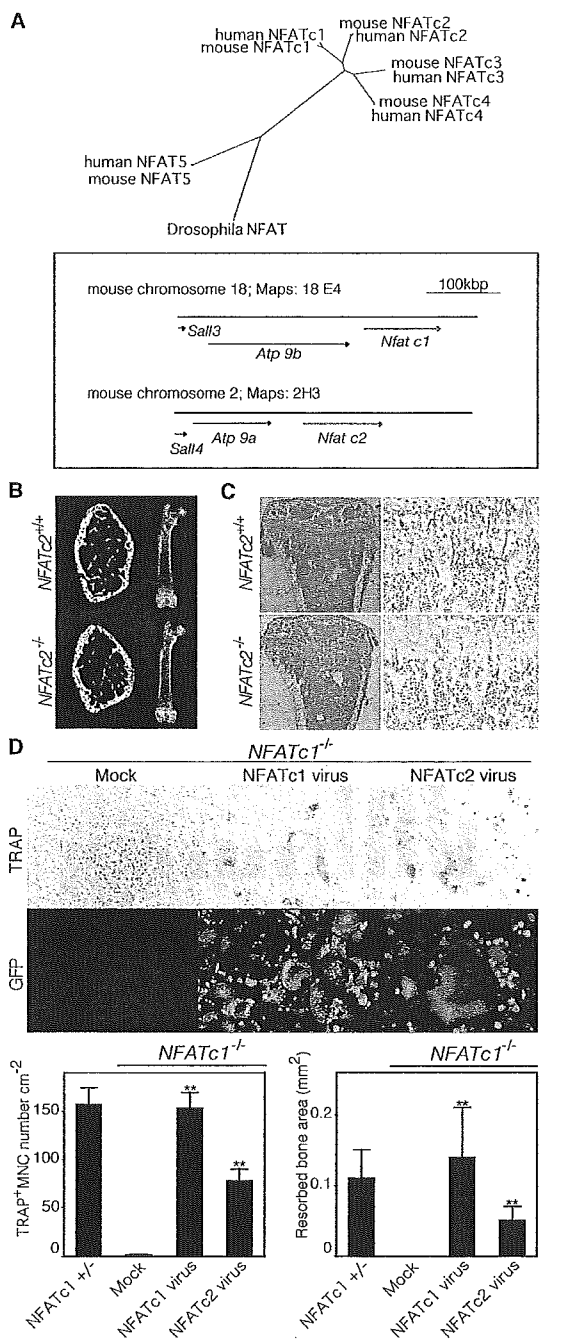
*Fos*<sup>-/-</sup> mice and *Fos*-deficient blastocyst complementation. *NFATc1*-deficient osteoclast precursor cells cannot differentiate into osteoclasts. Conversely, osteoclastogenesis of *NFATc1*-deficient cells is rescued by forced expression of *NFATc2*. The discrepancy between the essential role of *NFATc1* and the in vitro effect of *NFATc2* is attributed to selective autoregulation of the *NFATc1* gene by *NFAT* through its promoter region. Our analysis indicates that the essential role of *NFATc1* in bone homeostasis is not determined by its distinct biochemical properties, but is determined by its unique spatiotemporal activation mechanism during osteoclastogenesis.

**RESULTS**

**In vivo evidence for the essential role of *NFATc1* in osteoclast differentiation shown by the fetal liver cell (FLC) transfer**

Because in vivo analysis of the role of *NFATc1* has been hampered by embryonic lethality (8, 9), one may postulate that a conditional gene targeting strategy should be appropriate to avoid the difficulty. However, there is no desirable promoter to drive *ae* (encoding Cre recombinase) in the very early stage of osteoclast development. Adoptive transfer of hematopoietic stem cells to irradiated lymphocyte-deficient mice (10, 11) is used as an established method to analyze the function of such a gene in the study of lymphocytes (3). Here we applied this method to the skeletal system using mice lacking *c-Fos* (*Fos*<sup>-/-</sup> mice) (12, 13) as osteoclast-deficient recipients. Hematopoietic stem cells that were derived from *NFATc1*<sup>+/-</sup> and *NFATc1*<sup>-/-</sup> FLCs were injected into the liver of busulfan-treated newborn mice of *Fos*<sup>-/-</sup> background, in which no osteoclasts form because of a cell autonomous defect (13). Osteopetrosis is amelio-

**Figure 1. In vivo evidence for the essential role of *NFATc1* in osteoclast differentiation.** (A) Radiographic analysis of *Fos*<sup>-/-</sup> mice transferred with *NFATc1*<sup>+/-</sup> or *NFATc1*<sup>-/-</sup> FLCs. Severe osteopetrosis persists after *NFATc1*<sup>-/-</sup> FLC transfer. (B) Microradiographic analysis of femur (left: microcomputed tomography; right: microradiograph). Bone marrow cavity is filled with unresorbed bone in *NFATc1*<sup>-/-</sup> FLC chimera. (C) Histologic examination of tibia (top: toluidine blue staining; middle: tartrate-resistant acid phosphatase [TRAP] staining for detection of osteoclasts and lumbar vertebra; bottom: toluidine blue staining). No osteoclasts are observed in *NFATc1*<sup>-/-</sup> FLC chimera. (D) Histomorphometric evaluation of osteoclasts in *NFATc1*<sup>+/-</sup> and *NFATc1*<sup>-/-</sup> FLC chimera. (E) Reconstitution of the hematopoietic system of chimeric mice by the donor cells. PCR primers specific for joint region between the *NFATc1* locus and the neomycin-resistant gene (*NFATc1 neo*) and for wild-type (WT) *NFATc1* gene are used. The similar chimeric ratios were confirmed by quantitative PCR. (F) Complete blockade of in vitro osteoclastogenesis in osteoclast precursor cells from *NFATc1*<sup>-/-</sup> FLC chimera. Splenocyte-derived osteoclast precursor cells were cultured in RANKL (50 ng ml<sup>-1</sup>) and M-CSF (10 ng ml<sup>-1</sup>). We counted multinucleated (>3 nuclei) cells (MNCs) positive for TRAP staining. (G) Radiographic analysis of neonates generated by *Fos*<sup>-/-</sup> blastocyst complementation. Note the difference in radio-opacity at the femur (arrowheads). Right panels show the magnified view of the femur of neonates and tooth eruption of 6-wk-old mice. All of the *Fos*<sup>-/-</sup>/*NFATc1*<sup>-/-</sup> chimeric mice exhibited osteopetrosis, but osteoclastogenesis is restored in *Fos*<sup>-/-</sup>/*NFATc1*<sup>+/-</sup> chimeric mice when ES cells are transmitted to the hematopoietic system of recipient mice. \*\*P < 0.001 versus control.



**Figure 2. In vitro compensation of *NFATc1* deficiency by forced expression of *NFATc2*.** (A) Phylogenetic tree analysis for the DNA binding (Rel homology) region of NFAT family proteins (top). A wide-range genomic view of *NFATc1* and *NFATc2* genes suggests that these genes were generated by chromosomal gene duplication (bottom). (B) Loss-of-function analyses of *NFATc2* in osteoclast differentiation. Microradiographic analysis of femur derived from *NFATc2*<sup>-/-</sup> mice at 5 wk of age (see Fig. 1 B). (C) Histologic examination of tibia from *NFATc2*<sup>-/-</sup> mice (left: toluidine blue staining; right: tartrate-resistant acid phosphatase staining [TRAP]). (D) Recovery of osteoclastogenesis in *NFATc1*<sup>-/-</sup> FLCs by retrovirus-mediated expression of *NFATc1* or *NFATc2*. Forced expression of *NFATc2* as well as that of *NFATc1* induces formation of osteoclasts

rated and bone marrow cavities are observed when *NFATc1*<sup>+/-</sup> FLCs are transferred to *Fos*<sup>-/-</sup> mice (Fig. 1, A and B). Tooth eruption, which is not seen in *Fos*<sup>-/-</sup> mice (13), is observed in these mice (unpublished data). In contrast, when *NFATc1*<sup>-/-</sup> FLCs are transferred to *Fos*<sup>-/-</sup> mice, they exhibit severe osteopetrosis and bone marrow cavities remain occupied with unresorbed bone (Fig. 1, A and B). Histologic analyses show that osteoclast number and parameters of bone resorption are normalized only when *NFATc1*<sup>+/-</sup> cells are transferred (Fig. 1, C and D). Repopulation of hematopoietic cells was confirmed by PCR in splenic lymphocytes and monocyte/macrophage precursor cells (Fig. 1 E). Consistently, osteoclast precursor cells that were derived from the *NFATc1*<sup>-/-</sup> FLC chimera could not generate osteoclasts in culture (Fig. 1 F). These results indicate that *NFATc1* is indispensable for osteoclast formation in vivo, and that FLC transfer to osteoclast-deficient mice is a beneficial tool for in vivo analysis of the osteoclast lineage.

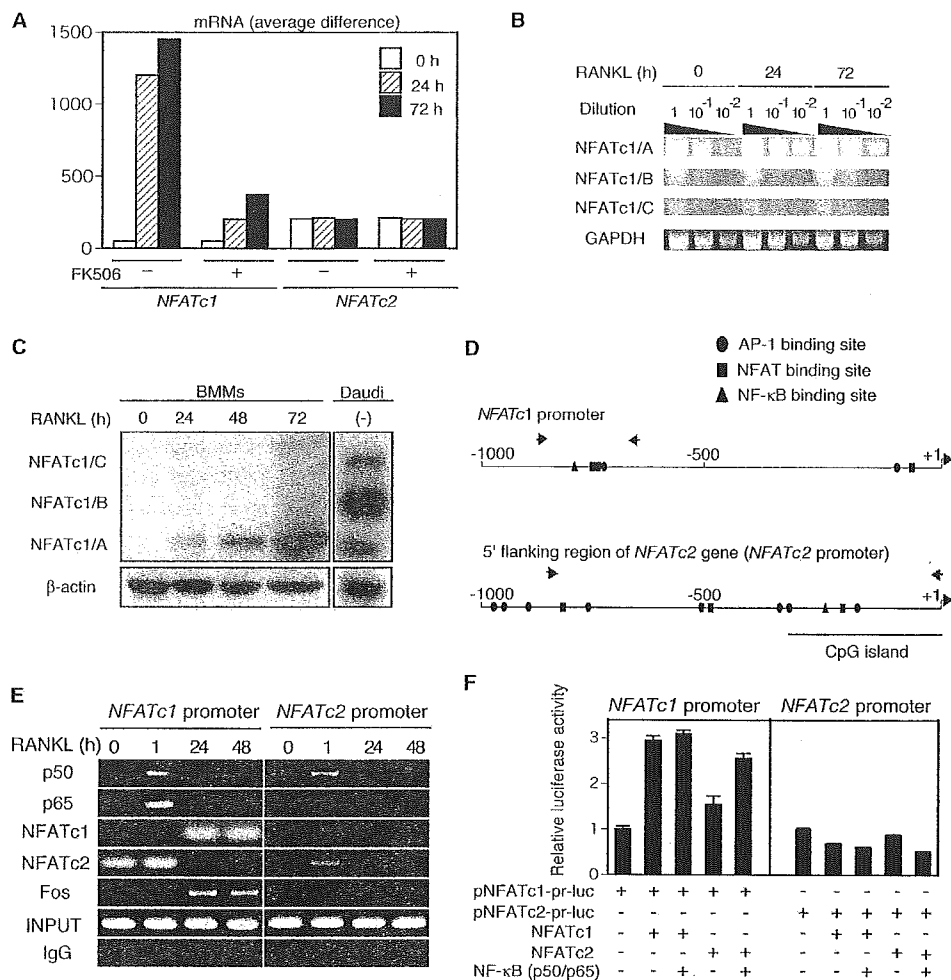
#### *Fos*<sup>-/-</sup> blastocyst complementation

*Rag-2*-deficient blastocyst complementation is an alternative method for analyzing the lymphocyte-specific functions of gene, loss of which results in embryonic lethality (14). To provide additional genetic evidence for the essential role of *NFATc1* in osteoclastogenesis, we applied this method to an analysis of the osteoclast lineage using *Fos*<sup>-/-</sup> blastocysts (referred to as *Fos*-deficient blastocyst complementation). We analyzed chimeric neonates that were generated by injection of wild-type or *NFATc1*<sup>-/-</sup> embryonic stem (ES) cells into *Fos*<sup>-/-</sup> blastocysts. The chimeric mice that result from injection of normal ES cells into *Fos*<sup>-/-</sup> blastocysts (*Fos*<sup>-/-</sup>/*NFATc1*<sup>+/+</sup> chimera) should develop mature osteoclasts, which are derived from the injected ES cells. In fact, all of the chimeric mice recovered from osteopetrosis, and tooth eruption was observed when the injected ES cells were transmitted into the hematopoietic lineage (Fig. 1 G). In contrast, chimeric mice that result from injection of *NFATc1*<sup>-/-</sup> ES cells into *Fos*<sup>-/-</sup> blastocysts (*Fos*<sup>-/-</sup>/*NFATc1*<sup>-/-</sup> chimera) remain severely osteopetrotic, and have a defect in tooth eruption that is due to a lack of osteoclasts (Fig. 1 G and not depicted). Taken together with the results of FLC transfer, these observations provide clear evidence that *NFATc1* is essential for osteoclast differentiation in vivo.

#### *NFATc2* is dispensable for osteoclastogenesis in vivo and in vitro

*NFATc2* is another NFAT protein that is expressed at a low level in osteoclast precursor cells (5, 7). In addition to the similarity of the DNA binding (Rel homology) domain, the genomic view of the *NFATc1* and *NFATc2* loci suggest that *NFATc2* is the evolutionally closest relative of *NFATc1* among the NFAT proteins (1, 2, 15, 16) (Fig. 2 A). In addition

with pit-forming activity on dentin slices (RANKL, 50 ng ml<sup>-1</sup>). Infection efficiency was monitored by GFP, which is expressed bicistronically. \*\*P < 0.001 versus mock.



**Figure 3. Autoamplification of *NFATc1* during osteoclastogenesis.** (A) GeneChip analysis of mRNA expression of *NFATc1* and *NFATc2* in RANKL-stimulated BMMs. Strong induction of *NFATc1* by RANKL is inhibited by FK506 (2.5 μg ml<sup>-1</sup>), which inhibits NFAT activity. *NFATc2* expression is constitutive and is not affected by FK506. (B) Semiquantitative RT-PCR analysis of the mRNA of *NFATc1* isoforms in BMMs. *NFATc1/A* is induced ~10-fold by RANKL. (C) Immunoblot analysis of *NFATc1* isoforms during RANKL-induced osteoclastogenesis in BMMs. A Daudi lymphoma cell line

is shown as a positive control for all three isoforms (A, B, and C) of *NFATc1* (22). (D) A schematic illustration of putative transcription factor binding sites in the 5' flanking region of the *NFATc1* (20) and *NFATc2* genes. Binding sites in the 5' flanking region of the *NFATc2* gene were determined by the TRANSFAC retrieval program. Arrows indicate primers for ChIP experiments. (E) ChIP assay of *NFATc1* and *NFATc2* promoters in RANKL-stimulated BMMs. (F) Luciferase assay of *NFATc1* and *NFATc2* promoters in HEK293T cells.

tion, overexpression of *NFATc2* facilitates the differentiation of osteoclasts (7) (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20051150/DC1>). These results prompted us to investigate osteoclast differentiation in *NFATc2*-deficient (*NFATc2*<sup>-/-</sup>) mice (17). Although *NFATc2* is involved in the regulation of chondrocytes at advanced age, bone development of *NFATc2*<sup>-/-</sup> mice has never been well defined (18). Skeletal development occurs normally, and we observed no increase in bone mass in *NFATc2*<sup>-/-</sup> mice as shown in Fig. 2 B [see also reference 19]. We found no abnormality in the number of osteoclasts, or in the parameters of osteoclastic bone resorption (Fig. 2 C and Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20051150/DC1>) in *NFATc2*<sup>-/-</sup> mice. Consistently, *NFATc2*<sup>-/-</sup> BMMs that are stimulated by

RANKL normally differentiate into osteoclasts with bone-resorbing activity (Fig. S3, available at <http://www.jem.org/cgi/content/full/jem.20051150/DC1> and not depicted). In addition, RANKL induction of *NFATc1* in *NFATc2*<sup>-/-</sup> cells is comparable to that in *NFATc2*<sup>+/+</sup> cells (Fig. S3). Thus, *NFATc2* is dispensable for RANKL-induced *NFATc1* expression and osteoclastogenesis.

#### Osteoclast differentiation of *NFATc1*-deficient cells is rescued by forced expression of *NFATc2*

Because *NFATc1* and *NFATc2* have a similar ability to activate gene expression in T cells (1–3), it has been unexpected that *NFATc1* plays a nonredundant role in the skeletal system. *NFATc1*<sup>+/-</sup> cells differentiate into osteoclasts with bone-

resorbing activity when they are stimulated by RANKL in the presence of M-CSF, whereas *NFATc1*<sup>-/-</sup> cells are unable to generate osteoclasts (Fig. 2 D), despite normal development into the monocyte/macrophage lineage (Fig. S4, available at <http://www.jem.org/cgi/content/full/jem.20051150/DC1>). To investigate the function of NFATc1 and NFATc2 further, we ectopically expressed NFATc1 and NFATc2 under the control of the *LTR* promoter using retrovirus-mediated gene transfer (20) in *NFATc1*<sup>-/-</sup> osteoclast precursor cells. As expected, the formation of bone-resorbing osteoclasts was rescued by retroviral expression of NFATc1. Surprisingly, osteoclast formation recovered, albeit at a reduced efficiency, as the result of forced expression of NFATc2. This demonstrated that forced expression of NFATc2 compensates for the loss of the *NFATc1* gene (Fig. 2 D).

### Selective autoamplification of *NFATc1* during osteoclastogenesis

How can we reconcile the *in vivo* essential role of *NFATc1* in osteoclastogenesis with the observation that *NFATc1* deficiency is compensated for by forced expression of NFATc2? Whereas NFATc2 is expressed constitutively in BMMs at a low level, mRNA of *NFATc1* is induced selectively and potently by RANKL (5) (Fig. 3 A). The induction of *NFATc1*, but not *NFATc2*, is down-regulated by the calcineurin inhibitor, FK506, which suppresses the activity of NFAT (Fig. 3 A). This suggests that *NFATc1* is autoregulated selectively by NFAT when BMMs are stimulated with RANKL, and that the resulting expression pattern may explain the specificity of *NFATc1*. Although *NFATc1* also is autoregulated in T cells (20, 21), the expression pattern during osteoclastogenesis (autoamplification) is characterized by a monotone increase and magnitude of amplification. Three isoforms (A, B, and C) of NFATc1 are well documented in T cells (20, 22), but it remains to be clarified which isoform is involved in osteoclastogenesis. Using specific PCR primers, we found that mRNA expression of the shortest isoform, NFATc1/A, is induced selectively in RANKL-stimulated BMMs (Fig. 3 B); immunoblot analysis yielded consistent results (Fig. 3 C). Therefore, we investigated the regulatory mechanism of the P1 promoter of the *NFATc1* gene, which regulates NFATc1/A induction (20), in comparison with the *NFATc2* promoter. To determine the transcriptional start site of mouse *NFATc2* gene, a 5' rapid amplification of cDNA ends experiment was performed. The putative transcription factor binding sites in the 5' flanking region of the *NFATc1* and *NFATc2* gene are shown in Fig. 3 D. Unexpectedly, these results suggest that not only the *NFATc1* promoter but also the *NFATc2* promoter contain multiple NFAT binding sites.

### Recruitment of NFATc1 and other transcription factors to the *NFAT* promoters

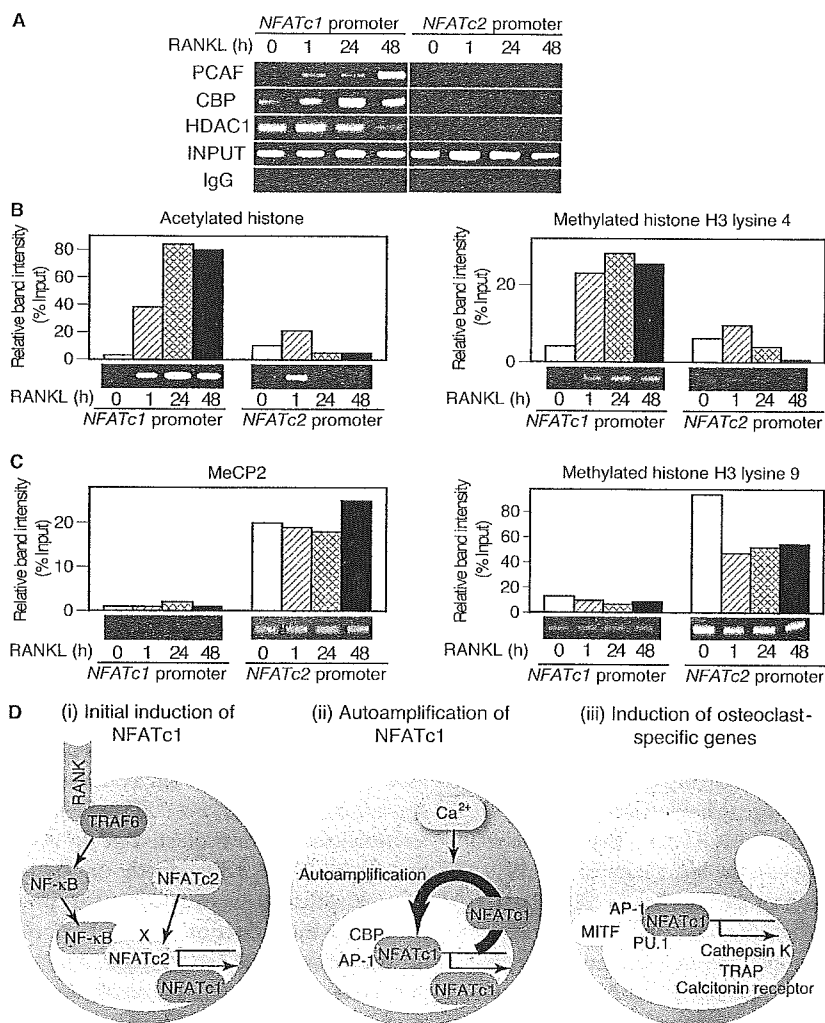
Chromatin immunoprecipitation (ChIP) analysis shows that NFATc2 is recruited to the *NFATc1* promoter at the earliest phase of osteoclast differentiation (Fig. 3 E). 24 h after RANKL stimulation, NFATc1 mainly occupies the promoter instead of NFATc2, and c-Fos is concomitantly recruited to

the promoter (Fig. 3 E); this is consistent with the strong induction of *NFATc1* at this time point. This occupancy persists during terminal differentiation of osteoclasts, and indicates that autoamplification of *NFATc1* occurs during this process and that c-Fos may contribute to it. This is congruent with the critical role of c-Fos in RANKL-mediated induction of *NFATc1*, which was reported previously (5, 23). NF- $\kappa$ B is activated rapidly by RANKL (24), and a recent study suggests that NF- $\kappa$ B activity is important for RANKL-mediated induction of *NFATc1* at the early phase of osteoclastogenesis (25). Consistent with this, NF- $\kappa$ B components p50 and p65 are recruited to the *NFATc1* promoter 1 h after RANKL stimulation. However, this recruitment is not observed after 24 h (Fig. 3 E), which suggests that NF- $\kappa$ B is important for the initial induction of *NFATc1* in cooperation with NFATc2. Although NFATc2 is detected on the *NFATc1* promoter without RANKL stimulation, *NFATc1* is not induced under this condition, which suggests that additional stimulation, such as NF- $\kappa$ B activation, is important for the efficient triggering of this promoter. This is consistent with the observation in the luciferase assay that the *NFATc1* promoter is not activated fully by NFATc2 alone, but is activated markedly by the coexpression of NFATc2 and NF- $\kappa$ B (Fig. 3 F). The basal calcineurin activity in osteoclast precursor cells may contribute to the nuclear localization of NFATc2 without RANKL addition (Fig. S5, available at <http://www.jem.org/cgi/content/full/jem.20051150/DC1>). Because osteoclast differentiation was not affected in *NFATc2*<sup>-/-</sup> mice, the function of NFATc2 evidently can be compensated by other factors, although it may contribute to physiologic regulation of the initial induction of *NFATc1*. A reporter plasmid that is driven by the *NFATc1*-P1 promoter, but not by the *NFATc2* promoter, is activated markedly by NFAT in cooperation with NF- $\kappa$ B (Fig. 3 F). These results indicate that the *NFATc1* promoter is distinct from the *NFATc2* promoter in that the former is exclusively under autoregulation by NFAT; this further suggests that an autoregulatory mechanism contributes to the essential role of *NFATc1*.

### Epigenetic regulation of *NFATc1* gene determines its unique spatiotemporal induction pattern during osteoclastogenesis

To gain insight into the selective recruitment of NFATc1 to the *NFATc1* promoter, we examined the association of transcriptional cofactors with these promoters. *NFATc1* promoter is associated increasingly with transcriptional coactivators with histone acetylase activity, such as CREB-binding protein (CBP) and p300/CBP-associated factor (PCAF), but is dissociated with histone deacetylase 1 (HDAC1; Fig. 4 A). It is notable that histone acetylation in the *NFATc1* promoter increased gradually after RANKL stimulation and that the high acetylation status was sustained, but this was not observed in the *NFATc2* promoter (Fig. 4 B). Methylation of histone H3 lysine 4, which is characteristic of the transcriptionally active locus, is up-regulated exclusively in the *NFATc1* promoter (Fig. 4 B). Conversely, the *NFATc2* promoter is associated con-





**Figure 4. Epigenetic regulation of *NFATc1* promoter underlies the selective autoamplification of *NFATc1*.** (A) Analysis of chromatin modification–related factors in *NFATc1* and *NFATc2* promoters by ChIP in RANKL-stimulated BMMs during osteoclastogenesis. (B) ChIP assay for acetylated histone and methylated histone H3 lysine 4. (C) ChIP assay for MeCP2 and methylated histone H3 lysine 9. (D) A schematic diagram of three stages of osteoclast differentiation that are governed by *NFATc1*. (i) *NFATc2* is recruited to the *NFATc1* promoter at the very early phase, but this is not enough to activate the *NFATc1* promoter. The binding of RANKL to receptor activator of NF-κB (RANK) results in the recruitment of TNF receptor–associated factor (TRAF) 6, and leads to the activation of downstream molecules, such as NF-κB (25, 34). Cooperation of *NFATc2* and NF-κB activates the initial induction of

*NFATc1*, but because *NFATc2*-deficient mice have no obvious defect in osteoclast differentiation, unknown factor(s) (shown as X) may compensate for the loss of *NFATc2* in these mice. (ii) RANKL–RANK interaction cooperates with immunoreceptors to activate the calcium signals (28), which stimulate the *NFATc1* activation by way of calcineurin (5). *NFATc1* binds to its own promoter, which leads to the robust induction of *NFATc1*; AP-1 (containing c-Fos) is critical for this autoamplification. Selective recruitment of *NFATc1* to the promoter of *NFATc1*, but not *NFATc2*, is explained in part, by epigenetic regulation. (iii) Several osteoclast-specific genes, such as *cathepsin K*, *TRAP*, and *calcitonin receptor*, are activated by a transcriptional complex that contains *NFATc1* and other cooperators, such as AP-1, PU.1, and microphthalmia-associated transcription factor (MITF).

stantly with methylated DNA-binding proteins, such as the methyl-CpG binding protein 2 (MeCP2) (Fig. 4 C), which suggests that the *NFATc2* promoter specifically is silenced during osteoclast differentiation. Consistent with this notion, we detected high levels of methylated histone H3 lysine 9 only in the *NFATc2* promoter (Fig. 4 C). These results suggest that epigenetic regulation underlies the specific autoamplification of *NFATc1*.

**DISCUSSION**

Our results illustrate that the essential importance of a gene is not only attributable to the specific biochemical function of the coded protein, but also is determined epigenetically by the distinct regulatory mechanism of gene expression. The role of *GATA-1* and *GATA-3* in erythropoiesis and Th2 cell differentiation are examples in which the essential importance of one member of a group of structurally related pro-

teins of interchangeable function is based on an autoregulatory mechanism (26, 27). These studies suggest that autoregulation of transcription factors is one of the critical mechanisms for cell fate determination (2). In a future study, generation of mice with an *NFATc2* knock-in into the *NFATc1* locus will be an intriguing strategy to provide the conclusive *in vivo* evidence.

As depicted in Fig. 4 D, in the differentiation of osteoclasts, preexisting NFATc2 cooperates with other RANKL-stimulated transcription factors, such as NF- $\kappa$ B, to activate the initial induction of *NFATc1*, followed by an autoamplification phase of *NFATc1*. It was reported that the activator protein (AP)-1 component, c-Fos, is critical for RANKL-mediated induction of *NFATc1* (5, 23). Consistent with this, c-Fos is recruited selectively to the *NFATc1* promoter at the autoamplification phase (Fig. 3 E). The composite NFAT/AP-1 site is observed exclusively in the *NFATc1* promoter (Fig. 3 D) (20). This lends further support to the notion that the cooperation of NFAT and AP-1 is responsible for the specific activation of the *NFATc1* promoter and its autoamplification.

Calcium signal-mediated activation of NFATc1 also triggers the autoamplification loop of *NFATc1* and ensures a sustained *NFATc1*-dependent transcriptional program (5, 28) in which osteoclast-specific genes are activated by a transcriptional complex that involves NFATc1, AP-1, and other cooperators (5, 29). Accumulating evidence indicates that NFATc1 regulates many osteoclast-specific genes, such as *cathepsin K* (29), *TRAP* (5, 30), *calcitonin receptor* (5, 30), and *osteoclast-associated receptor* (30), in cooperation with other transcription factors (e.g., PU.1 and microphthalmia-associated transcription factor), although the components of the transcriptional complex are not always the same (30) (Fig. 4 D, iii). A distinct pattern of calcium oscillation, which efficiently keeps NFATc1 transcriptionally active in the nucleus (31), may explain the specific spatiotemporal expression of NFATc1 in the osteoclast lineage (5), in contrast to T cells. FK506 inhibited osteoclast formation, even when it was added at the late phase of osteoclastogenesis (48–72 h after RANKL stimulation, unpublished data); this suggests that calcium signaling is also critical for the autoamplification of *NFATc1*.

Successful application of FLC transfer and blastocyst complementation, first established in the immune system, provided genetic evidence that *NFATc1* is essential for osteoclast differentiation and maintenance of the skeletal system. Modulation of the *NFATc1* autoamplification pathway has promise as a strategy for suppressing the excessive osteoclast formation that is characteristic of a variety of bone diseases.

## MATERIALS AND METHODS

**Mice and analysis of bone phenotype.** Generation of *Fos*<sup>-/-</sup> and *NFATc1*<sup>-/-</sup> mice has been described previously (8, 13). *NFATc2* mice (17) were provided by L.H. Glimcher (Harvard Medical School, Boston, MA). Histologic, histomorphometric, and microradiographic examinations were performed using essentially the same methods as described (32). Statistical analysis was performed using Student's *t* test. All mice were kept in a specific pathogen-

free environment, and all animal experiments were performed with the approval of the institutional committee of Tokyo Medical and Dental University.

**In vitro osteoclastogenesis and retroviral gene transfer.** Bone marrow cells or FLCs were cultured in  $\alpha$ -MEM (GIBCO BRL) containing 10% FBS (Sigma-Aldrich) and 10 ng ml<sup>-1</sup> M-CSF (R&D Systems). After 2 d, adherent cells were used as osteoclast precursor cells. In osteopetrotic mice, osteoclast precursor cells were obtained similarly from splenocytes. We described the method of RANKL-induced *in vitro* osteoclastogenesis, retroviral gene transfer, characterization of osteoclasts, and immunostaining (5, 24, 32). All data are expressed as mean  $\pm$  SEM ( $n = 6$ ). Retroviral vectors, pEGZ-NFATc1/A (NFATc1 virus) and pEGZ-NFATc2 (NFATc2 virus), were described previously (20), and infection efficiencies of both retrovirus vectors into osteoclast precursor cells were  $\sim$ 40%, as described previously (24).

**Fetal liver cell transfer.** *NFATc1*<sup>-/-</sup> and *NFATc1*<sup>+/-</sup> embryos were obtained by crossing *NFATc1*<sup>+/-</sup> parental mice. Total liver cells (10<sup>7</sup> cells) from embryonic day (E) 13.5 embryos were injected into the liver of recipient *Fos*<sup>-/-</sup> newborn (2 d) mice, which were analyzed at 6 wk of age. The recipient mice were treated with busulfan (Sigma-Aldrich) in utero by i.p. administration to pregnant mice (12.5 mg kg<sup>-1</sup>) twice at 17.5 and 18.5 d postcoitum.

***Fos*<sup>-/-</sup> blastocyst complementation.** Establishment of *NFATc1*<sup>-/-</sup> ES cell lines has been described (33). *NFATc1*<sup>-/-</sup> ES cells or wild-type ES cells (E14K) were injected into E3.5 *Fos*<sup>-/-</sup> blastocysts that were obtained by fertilizing *Fos*<sup>-/-</sup> gametes from *Fos*<sup>-/-</sup> paternal and maternal mice using *in vitro* fertilization technique. Resultant blastocysts were transferred to pseudopregnant foster mothers to generate chimeric mice.

**RT-PCR and GeneChip analysis.** RNA extraction, semiquantitative RT-PCR, and GeneChip analysis were performed as described previously (5, 32). The following PCR primers were used to detect NFAT isoforms. NFATc1/A: 5'-GGTAACCTCTGCTTTCTAACCTTAAGCTC-3' (sense) and 5'-GTGATGACCCAGCATGCACCAGTCACAG-3' (antisense); NFATc1/B: 5'-CCCATCCGCCAGGCTACAGCCGCAGTAA-3' (sense) and 5'-TTCGGTAAGTTGGGATTTCTGAGTGGTACC-3' (antisense); NFATc1/C: 5'-CCCATCCGCCAGGCTACAGCCGCAGTAA-3' (sense) and 5'-TGAGTGGTACCAGATGTGGGTCCAGTTTAT-3' (antisense).

**Chromatin immunoprecipitation.** ChIP was performed with the ChIP Assay Kit (Upstate Biotechnology) according to the manufacturer's instructions, using antibodies against NFATc1, NFATc2, p300/CBP-associated factor, histone deacetylase 1, p50, CBP, MeCP2 (Santa Cruz Biotechnology, Inc.), p65, acetylated histone, mono/di/trimethyl-histone H3 lysine 4, dimethyl-histone H3 lysine 9 (Upstate Biotechnology), c-Fos (Calbiochem), and normal IgG (Santa Cruz Biotechnology, Inc.). The purified DNA was analyzed by PCR using primers that detect sequences containing the *NFATc1*-P1 promoter: 5'-CCGGGACGCCCATGCAATCTGT-TAGTAATT-3' (sense) and 5'-GCGGGTGCCCTGAGAAAGC-TACTCTCCCTT-3' (antisense) and the *NFATc2* promoter: 5'-TTAT-CAGGGAGCACTGCCCATCTCCGCTTT-3' (sense) and 5'-CGGTCTGGCCTGAGCGACAGGCCAGACAA-3' (antisense).

**Luciferase reporter gene assay.** The reporter plasmid pNFATc1P1-0.8 kb-luc (pNFATc1-pr-luc) was described previously (20). pNFATc2-0.8 kb-luc (pNFATc2-pr-luc) was constructed by inserting a 0.8-kb NheI-Xho fragment of the mouse *NFATc2* promoter into the same sites of the pGL3 basic promoter vector (Promega). The mouse *NFATc2* promoter region was amplified by PCR using following primers: 5'-GCTAGCTGTTTGTGACTGTTATCATGCTGGG-3' (sense) and 5'-CTCGAGCTTCCTGCTCAAGGCACCTGTTGCAG-3' (antisense). Transfection into HEK293T cells and measurement of luciferase activity were performed as described (5) using the Dual-luciferase reporter assay system (Promega).

**Online supplemental material.** Fig. S1 shows the gain-of-function analyses of *NFATc1* and *NFATc2* in osteoclasts. Fig. S2 shows the histomorphometric evaluation of osteoclasts in *NFATc2*<sup>-/-</sup> mice. Fig. S3 shows the normal osteoclast differentiation and expression of NFATc1 in *NFATc2*<sup>-/-</sup> monocyte/macrophage precursor cells that were stimulated with RANKL/M-CSF. Fig. S4 shows the development into the monocyte/macrophage lineage from *NFATc1*<sup>-/-</sup> FLCs. Fig. S5 shows the effect of FK506 on the recruitment of NFATc2 to the *NFATc1* promoter without RANKL stimulation. Also, details of the methods for phylogenetic tree analysis and 5' rapid amplification of cDNA ends analysis are available. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20051150/DC1>.

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