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A BTB-ZF protein, ZNF131, is required for early B cell development

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ABSTRACT

Members of the BTB-ZF transcription factor family play important roles in lymphocyte development. During T cell development, ZNF131, a BTB-ZF protein, is critical for the double-negative (DN) to double-positive (DP) transition and is also involved in cell proliferation. Here, we report that knockout of *Znf131* at the *pre*-pro-B cell stage in mb1-Cre knock-in mouse resulted in defect of pro-B to pre-B cell transition. ZNF131 was shown to be required for efficient pro-B cell proliferation as well as for immunoglobulin heavy chain gene rearrangement that occurs in the proliferating pro-B cells. We speculate that inefficient gene rearrangement may be due to loss of cell proliferation, since cell cycle progression and immunoglobulin gene rearrangement, which would occur in a mutually exclusive manner, may be interconnected or coupled to avoid occurrence of genomic instability. ZNF131 suppresses expression of Cdk inhibitor, p21[cip1], and that of pro-apoptotic factors, *Bax* and *Puma*, targets of p53, to facilitate cell cycle progression and suppress unnecessary apoptosis, respectively, of pro-B cells. There results demonstrate the essential roles of ZNF131 in coordinating the B cell differentiation and proliferation.

1. Introduction

Members of transcription factor families play critical roles in various aspects of lymphocyte development and their functions. The BTB-ZF family transcription factors (e.g. Bcl-6 [1], Th-POK [2], LRF [3], PLZF [4] and Miz1 [5,6]) carry broad complex, tramtrack, bric a brac (BTB) domain and various numbers of Zinc-finger (ZF) domains [7]. We previously reported that ZNF131, a member of BTB-ZF, is required for T cell development and mature T cell activation [8]. Knockout of the *Znf131* gene at double-negative (DN) 2 stage of T cell differentiation resulted in severe suppression of DN to double-positive (DP) transition. Vigorous cell proliferation during this transition step generates a large numbers of thymocytes subjected to the selection of the DP population, a key step for central tolerance establishment. Knockout of *Znf131* in DP, a later stage of thymocytes development, does not affect the cell numbers of DP population. However, the induction of various genes (e.g. cytokines) required for the effector functions of T cells and cell proliferation upon T cell receptor (TCR) activation is significantly suppressed [8]. One of the possible mechanisms for suppression of proliferation is the elevated expression of p21[cip1] encoded by *cdkn1a*, an inhibitor of the Cyclin-Cdk complex [8]. ZNF131 may suppress the transcription of *cdkn1a*. Another link between cell cycle regulation and ZNF131 has recently been reported. HAUS5, one of the building blocks of the augmin complex [9], was shown to be a target of ZNF131. The augmin complex is required for the proper formation of the spindle between kinetochores and centrosomes. Thus, ZNF131 may be required for the normal progression of mitosis as well.

During B cell development, cell expansion takes place in pro-B and early pre-B cells [10]. DNA replication and non-homologous DNA end joining (NHEJ) required for the completion of immunoglobulin gene rearrangement proceed in a mutually exclusive manner [11]. If these two processes occur concomitantly, significant genome instability could be induced, resulting in various types of mutations including translocations of chromosomes. This would lead to eventual development of malignant tumors. Therefore, at the pro-B cell stage, immunoglobulin heavy chain gene rearrangement has to proceed in the G1 phase of the cell cycle. In

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addition, p53 actively arrests cell cycle progression or induces apoptosis during the pro-B cell stage if genome instability arises. In fact, in the absence of p53, pro-B cell lymphoma frequently develops in mouse [12]. The pre-B cell stage is divided into early and late; extensive cell proliferation is induced by the IL-7 and pre-B cell receptor (preBCR) signal in the early stage, while the loss of IL-7 signal suppresses cell cycle progression and stimulates immunoglobulin light chain gene rearrangement in the late stage [13,14] [15]. DNA rearrangement of TCRβ locus proceeds during the DN2 through DN3 stages when cell cycle is arrested. Once TCRβ gene is successfully rearranged, the TCRβ chain and the surrogate TCRα chain (pTα) are expressed on the cell surface, which triggers cell proliferation [16]. Thus, in both pre-B cell stage of B cell development and "DN to DP" stage of T cell development, cell proliferation and DNA rearrangement are temporally separated to avoid the occurrence of genome instability.

To elucidate the roles of ZNF131 in B cell development, mb1-Cre mouse was generated to knockout *Znf131* at the *pre*-pro-B cell stage. In mb1-Cre mouse, the *mb1*, the expression of which is induced during the *pre*-pro-B cell stage, is replaced with the Cre recombinase gene [17]. Upon induction of knockout of *Znf131* at this stage, B cell development was arrested at the transition from pro-B to pre-B cell stage. Vigorous proliferation of the pro-B cells was significantly suppressed at this stage, and immunoglobulin heavy chain rearrangement was partially suppressed. Our data reported here demonstrate that ZNF131 is required for the proliferation of B cells as well as for immunoglobulin gene rearrangement.

2. Materials and methods

2.1. Mice

The *Znf131* floxed mice were described previously [8]. $mb1^{Cre/+}$ mice were provided by Dr. Michael Reth [17]. Mice were bred and housed in specific pathogen-free conditions. All the mice including littermates and age-matched controls were examined at the age of 6–12 weeks. Genotyping was performed by PCR, using genomic DNA extracted from mouse tails. The experimental protocols were approved by the Animal Use and Care Committee of the Tokyo Metropolitan Institute of Medical Science.

2.2. Flow cytometry and cell sorting

Single cell suspensions from spleen and bone marrow were incubated with ice-cold red blood cell (RBC) lysis buffer and stained with specific combinations of fluorophore or biotin-conjugated antibodies (all from BioLegend), such as IgM, CD19, B220, CD43, Ter119, CD11b, Gr-1 and CD3ε. Surface markers were detected and analyzed with FACS CantoII (BD). Gating and analysis were performed using FlowJo software (Tree Star). For sorting, lineage$^+$ cells were first depleted by magnetic cell separation with streptavidin MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions. *Pre*-pro-B cells were sorted as Lin$^-$ IgM$^-$ B220$^+$ CD43$^+$ CD19$^-$ and pro-B cells were sorted as Lin$^-$ IgM$^-$ B220$^+$ CD43^{high} CD19$^+$ using FACS AriaIII (BD).

2.3. Real-time quantitative reverse transcriptional PCR (RT-qPCR)

Total RNA was extracted with RNAiso Plus regent (TaKaRa) according to the manufacturer's instructions. Five hundred ng of total RNA was converted to cDNA using the random primer and PrimeScript RT reagent Kit (TaKaRa). Expression of the indicated genes was measured by RT-qPCR with SYBR Premix Ex TaqII (TaKaRa) and LightCycler480 system (Roche). The relative quantification of target genes was given by the CT values, and the CT value of *Hprt* or *L32* was subtracted to obtain ΔCT. The relative mRNA expression level of targeted genes was determined as $2^{-\Delta CT}$. The experiment was performed in triplicate.

2.4. Detection of rearrangement

PCR using genomic DNA form the pro-B cells was conducted. Serial dilutions of genomic DNA were analyzed by PCR with primers specific for V_HJ558 (5′-CGAGCTCTCCARCACAGCCTWCATGCARCTCARC-3′) or V_H7183 (5′-CGGTACCAAGAASAMCCTGTWCCTGCAAATGASC-3′) and J_H3 (GTCTAGATTCTCACAAGAGTCCGATAGACCCTGG-3′). PCR products were separated by 1% agarose gel electrophoresis, were transferred to Hybond-N membranes and were quantitatively analyzed by Southern blot with J_H3 probe (5′-AGCCTTCAGGACCAAGATTCTCTGCAAACG-3′).

2.5. Bromodeoxyuridine (BrdU) incorporation and flow cytometry

For determination of BrdU incorporation *in vivo*, mice were injected i.p. with 1 mg BrdU (Sigma-Aldrich) at 2 and 4 h before the analysis. Single cell suspensions from bone marrow were stained for cell surface markers, treated with IC Fixation Buffer and Permeabilization Buffer (eBioscience), followed by digestion with recombinant DNaseI (Roche) and staining with anti-BrdU Ab (BioLegend). Cells were analyzed with FACS CantoII (BD).

2.6. Statistical analysis

Unpaired two-tailed Student *t*-test were carried out using GraphPad Prism software (GraphPad Software) to determine the statistical relationship of different groups. In all experiments, the results were representatives of at least two independent experiments.

3. Results

3.1. ZNF131 is required for the transition from pro-B to pre-B cell stage

ZNF131 floxed mouse line ($ZNF131^{fl/f}$) was crossed to mb1-Cre knockin mouse line ($mb\text{-}1^{Cre/+}$) in which Cre recombinase replaced the mb1 gene that is specifically induced at the *pre*-pro-B cell stage. By the pro-B cell stage, *Znf131* was knocked out and the amount of the *Znf131* mRNA was significantly reduced (data not shown). The numbers of immature and mature B cells in spleen of $ZNF131^{fl/fl}$ $mb1^{Cre/+}$ mouse was less than 1% to those of wild type mice, resulting in about 85% reduction of the B220$^+$ B cell number (Fig. 1A). In bone marrow, the transition from the pro-B to pre-B cell stage was blocked (Fig. 1B). The numbers of the pro-B cell was about a half of those of the wild type (Fig. 1C), suggesting the reduced proliferation of the proB cells.

3.2. Expression of transcription factors essential for the lineage decision and for early development of B cells was not affected by the absence of ZNF131

Similar to T cell development, the commitment and determination of B cells lineage are established by the network of several transcription factors [18]. To elucidate the role of ZNF131 in the transcriptional network for the B cell lineage commitment and the following differentiation, mRNA expression of several key transcription factors was analyzed by qRT-PCR. Three essential genes