shows impairments in keratinocyte proliferation and the development of the epidermal permeability barrier. These indicate that Bcl11b plays critical roles during skin development. *Bcl11b*$^{KO/KO}$ mice exhibit an interesting feature, i.e., born with eyes open. The hypoplastic epidermis described above may account for this open eyelid.

4-4. Ameloblast in tooth. Bcl11b/Ctip2 is expressed in the ectodermal components of the developing tooth, including enamel epithelial cells and cells of the ameloblast lineage. *Bcl11b*/*Ctip2*$^{-/-}$ mice exhibit multiple defects at the bell stage of embryonic tooth development.[6] The early bell stage (embryonic day 15.5–16.5) is characterized by continued epithelial expansion and differentiation into the inner and outer enamel epithelium, stratum intermedium, and stellate reticulum.[68] Mutant incisors and molars are reduced in size and exhibit hypoplasia of the stellate reticulum that probably harbors stem cells. A well known hallmark of mouse incisors, different from human incisors, is an asymmetric enamel formation, which results from differential distribution of ameloblasts around incisors during development. As a result, the lingual side of mouse incisors lacks ameloblasts and enamel. Interestingly, mutant incisors possess an ameloblast-like cell population at the lingual side. This suggests that Bcl11b functions as a critical regulator of epithelial cell fate and differentiation during tooth morphogenesis. Despite developmental roles delineated in incisors and molars, roles for Bcl11b in maintenance and homeostasis of tooth in adult remain open.

5. *Bcl11b* tumor suppressor gene

Tumor suppressor genes act as inhibitory signals for uncontrolled cell growth and some play a role in DNA repair or cell survival.[69] p53 is one of the most important tumor suppressors, often called the guardian of the genome because of its central role in maintaining the integrity of the cell's DNA by controlling cell cycle inhibition, repair and apoptosis.[70] APC, another important tumor suppressor, is a negative regulator on the Wnt/β-catenin signaling pathway.[71] The signaling in intestines is required for differentiation of enterocytes and secretory cells and also for maintaining stem cells and progenitors within the intestinal crypt.[71)–74] *APC* inactivating mutations or activating β-catenin mutations impair the balance between cell proliferation, differentiation and apoptosis that affects the net number of cells in the tissue. As a consequence, it leads to formation of benign polyps or adenoma cells.

Bcl11b is a tumor suppressor and loss of a *Bcl11b* allele contributes to thymic lymphoma development. On the other hand, Bcl11b is a lineage-specific transcription factor probably responsible for turning on cell type-specific genes. Regulated expression of Bcl11b is important for differentiation of T cells and other types of cells, as described above. There are many other transcription factors affecting both cell differentiation and cancer development, and a well known precedent is β-catenin. Bcl11b and β-catenin have similar properties in cell proliferation, differentiation and apoptosis, and hence deregulation of these properties might be the contribution of loss of a *Bcl11b* allele to lymphomagenesis. In the following sections I will describe haploinsufficiency of Bcl11b for tumor suppression and how Bcl11b is involved in apoptosis, proliferation and differentiation.

Although *Bcl11b* was identified as a tumor suppressor gene in the mouse model, genetic changes were also observed in human malignancy.[32),75),76] Mutations or deletion of *BCL11B* gene were found in approximately 10%–16% of human T-cell acute lymphoblastic leukemia (T-ALL).[32),76),77] This indicates the involvement of Bcl11b in human malignancy as well.

6. Haploinsufficiency of Bcl11b for tumor suppression

As for tumor suppressor genes, loss of two alleles normally contributes to tumorigenesis, and this is known as the Knudson' two-hit theory. However, the Bcl11b tumor suppressor gene is exceptional, belonging to a class of haploinsufficient tumor suppressor genes.[78),79]

Figure 3A shows cumulative incidences of thymic lymphomas in *Bcl11b*$^{+/+}$ mice and *Bcl11b*$^{KO/+}$ mice after γ-irradiation.[2] The thymic lymphoma incidence was much higher in *Bcl11b*$^{KO/+}$ mice than wild-type mice. Figure 3B shows cumulative incidences of spontaneously developed thymic lymphomas in *Bcl11b*$^{KO/+}$ mice, *p53*$^{KO/+}$ mice and *Bcl11b*$^{KO/+}$*p53*$^{KO/+}$ doubly heterozygous mice. The incidences in *Bcl11b*$^{KO/+}$ mice and *p53*$^{KO/+}$ mice were low until one year of the age whereas the incidence in *Bcl11b*$^{KO/+}$*p53*$^{KO/+}$ mice was very high. These results suggest that loss of one *Bcl11b* allele does not affect lymphomagenesis in basal conditions but contributes to lymphomagenesis in radiation-induced injury conditions or in the *p53*$^{KO/+}$ heterozygous genetic background. One characteristic of the tumor suppressor gene *Bcl11b* is that its suppressive capacity is haploinsufficient, one wild-type allele being insuffi-

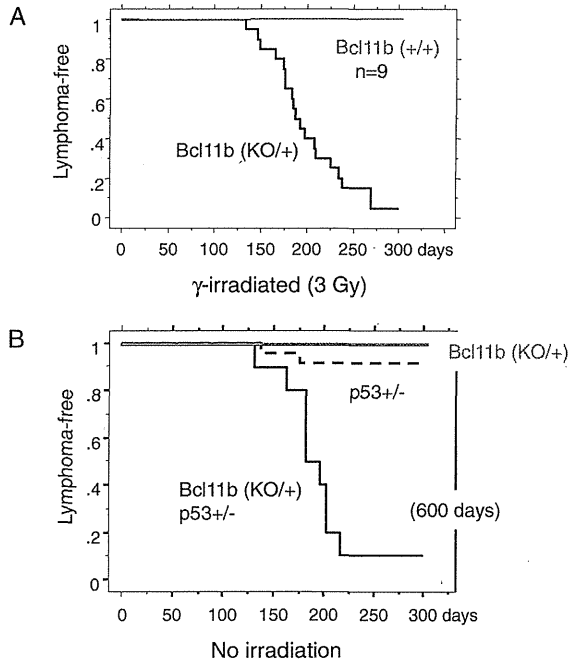

Fig. 3. The cumulative frequency distributions of thymic lymphomas (Kaplan-Mier analysis). (A) Analyzed are the thymic lymphomas that were induced by 3Gy γ-irradiation in *Bcl11b*$^{KO/+}$ mice and *Bcl11b*$^{+/+}$ mice. The curve of *Bcl11b*$^{KO/+}$ mice is shown in black and that of *Bcl11b*$^{+/+}$ mice in gray. (B) Thymic lymphomas spontaneously developed in *Bcl11b*$^{KO/+}$*p53*$^{+/-}$ mice at a high frequency in black, but vary rare in *Bcl11b*$^{+/+}$*p53*$^{+/-}$ mice in black dashed line or *Bcl11b*$^{+/-}$*p53*$^{+/+}$ mice in gray.

cient for tumor suppression. This is based on the finding that most of the thymic lymphomas developed in *Bcl11b*$^{KO/+}$ mice retained the wild-type allele although the thymic lymphomas developed in wild-type mice showed loss of one *Bcl11b* allele at a high frequency.[2),80)] The retention of the wild-type allele was also observed in spontaneously developed thymic lymphomas in *Bcl11b*$^{KO/+}$*p53*$^{KO/+}$ mice.[81)] Of importance, the retention of one *BCL11B* allele was also observed in human T-ALLs having mutations on the *BCL11B* gene.[32),76)] Because loss of one allele is an easier event and much more frequent than loss of two alleles, the haploinsufficiency of Bcl11b/BCL11B tumor suppressor is important from the point of etiological view.

7. Bcl11b and apoptosis

Keeping one *Bcl11b* wild-type allele in most thymic lymphomas may be related to the apoptosis that is often seen in cells with loss of two *Bcl11b* alleles. As described above apoptosis occurs in not all but certain types of thymocytes in *Bcl11b*-knockout mice. An anti-apoptotic property with Bcl11b has been demonstrated using Bcl11b knock-down (KD) Jurkat cells, a T-cell culture line. When Bcl11b expression was heavily reduced using siRNA method, Bcl11b-KD cells underwent apoptosis.[82),83)] On the other hand, we obtained KD cell lines retaining Bcl11b expression at certain levels that were viable and able to proliferate in the serum concentration of 5%.[82)] When the serum concentration was increased from 5% to 10% serum, the Bcl11b-KD cell lines showed growth inhibition due to cell death, accompanying decreased expressions of the CDK inhibitor p27 and the anti-apoptotic protein Bcl-xL. This suggests that the level of apoptosis is related to the cell proliferation rate, which can be controlled by serum concentration in the culture medium.

Further analysis showed that apoptosis occurred in S phase of the cell cycle and impaired activation of the cell-cycle checkpoint kinase Chk1. Activated Chk1 through phosphorylation, which is induced by DNA replication stress and subsequent formation of single-stranded DNA, leads to the arrest of cell cycle progression to allow DNA repair. Failure of the cell cycle arrest may be a cause of apoptosis in Bcl11b-KD cells by inducing proapoptotic signals. Consistently, radiation with UV, an agent producing single-stranded DNA, enhanced apoptosis more in those Bcl11b-KD Jurkat cell lines than in control Jurkat cells. Therefore, the apoptosis may be a reflection of deregulated cell cycle progression leading to DNA replication stress and of an accumulation of DNA damages during the S phase. These suggest that Bcl11b plays a role in the recovery for DNA replication stress. The anti-apoptotic property of Bcl11b may contradict the tumor suppressor function because apoptosis has been considered as a mechanism to eliminate deleterious cells with damaged DNA. However, a different interpretation is also possible. Hyperplastic or dysplastic cells in premalignant lesions often exhibit apoptotic phenotype together with high mitotic index.[84),85)] Therefore, the apoptosis may be a phenotype of precancerous cells, and the cells susceptible to apoptosis can be progressed to a rapidly progressive tumor when they acquire the ability to escape apoptosis.

8. Premalignancy and cell proliferation

Premalignant conditions are recognizable lesions that are strongly associated with the development of malignant neoplasia. They differ from normal conditions and hence may possess properties unique to

them. Normal cells possess checkpoint function that can perceive and arrest aberrant cell cycle triggered by cancer-promoting stimuli. The checkpoint functions as an inducible barrier against clonal cell expansion and genomic instability leading to tumor development. Accordingly, premalignancy might be related to impairment or inability of the checkpoint barrier function. Indeed, premalignant legions in human tissues exhibit signatures of persistent functioning of checkpoint, for instance, elevated protein expressions relevant for DNA damage responses.[84),85)]

γ-irradiation to *Bcl11b*$^{KO/+}$ mice confers the thymus atrophic, the cell number being reduced approximately one tenth, and the atrophic thymus will develop thymic lymphomas. This implies that the atrophic thymus is in a premalignant condition and comprises a lesion harboring premalignant cells. Supporting evidence is that the mice that had received thymocytes from the atrophic thymus developed thymic lymphomas at a high frequency.[86),87)] Other studies also show the presence of premalignant cells in atrophic thymus.[88),89)] In general, a small thymus with increased apoptosis and an expanded proportion of immature DN thymocytes characterizes the premalignant cells. For instance, transgenic mice expressing the oncogene *Lmo2* develop T-cell leukemia after a long latency period, keeping atrophic thymus before the development.[90),91)] Of note, the induction of atrophic thymus is caused by γ-irradiation but not a direct consequence of irradiation, because the thymus is recovered from damages within one week after γ-irradiation and atrophy of the thymus begins approximately three weeks after.[92)] To characterize γ-ray induced atrophic thymus is important to elucidate how loss of Bcl11b contributes to premalignancy and tumor development at initial stages.

Cell proliferation of clonal origin is a hallmark of premalignant cells. Clonal proliferation of certain thymocytes was observed in about a half of atrophic thymuses in *Bcl11b*$^{KO/+}$ mice at as early as 60 days after irradiation.[92)] Clonality was determined by assaying specific V(D)J rearrangements with three primer sets designed for the *TCRβ* locus. Recombination leading to the V(D)J rearrangements occurs in thymocytes at DN3 stage before β-selection. Figure 4A shows positions of primers and band patterns of PCR products in gel electrophoresis. Normal thymocytes within a thymus exhibit a DJ rearrangement pattern of all six distinct bands reflecting polyclonal origin of thymocytes. However, atrophic thymuses showed a few prominent band patterns of rearrangement (Fig. 4A), indicating clonal expansion of a few parental thymocytes having passed β-selection. The thymus is here designated as C-type thymus (C stands for clonal expansion) and the other thymuses are called T-type thymus (T stands for normal thymus).

The C-type thymus or clonal expansion was also detected in atrophic thymuses that were γ-ray induced in *Bcl11b*$^{+/+}$ wild-type mice.[93)] In this case, irradiation is required 4 times of 2.5 Gy at one-week interval for efficient lymphoma development, because *Bcl11b*$^{+/+}$ mice are much less susceptible to thymic lymphomas than *Bcl11b*$^{KO/+}$ mice. Approximately a half of those C type thymuses showed allelic loss at *Bcl11b* locus, suggesting that the allelic loss contributes to lymphomagenesis and possibly to clonal expansion of thymocytes. However, those atrophic thymuses did not exhibit the activation of DNA damage checkpoints such as γH2AX, Chk1, Chk2 or p53,[93)] which is a hallmark of human precancerous cells.[84),85)] This was an unexpected result to us. Further study is necessary to elucidate relationship among the atrophic thymus, premalignancy and the activation of checkpoint function. Collectively, these results suggest that loss of one *Bcl11b* allele in *Bcl11b*$^{KO/+}$ mice contributes to clonal cell proliferation at an early stage of thymic lymphoma development.

9. Bcl11b and cell differentiation

Figure 4B shows flowcytometry of T-type and C-type thymuses. Despite clonal cell expansion, a half of the C-type *Bcl11b*$^{KO/+}$ thymuses comprised thymocyte subtypes in the same proportion as normal thymus, mostly consisting of CD4$^+$CD8$^+$ DP cells. This indicates their retention of the capability to differentiate from DN3 cells to DP cells and further to SP cells. Accordingly, the C-type thymocytes have properties to undergo many rounds of cell division cycle within the thymus and to capable to differentiate. These capabilities must have been acquired at a developmental stage after β-selection. It is because, if not, the clonally proliferating thymocytes would have shown normal, but not skewed, distribution of the V(D)J recombination patterns.

The other half of the C-type *Bcl11b*$^{KO/+}$ thymuses consisted mostly of immature thymocytes with differentiation arrest at DN or/and ISP stages (the third low in Fig. 4B). This suggests that this class of C-type thymocytes lacks the capability to differentiate to DP cells. The differentiation arrest, a

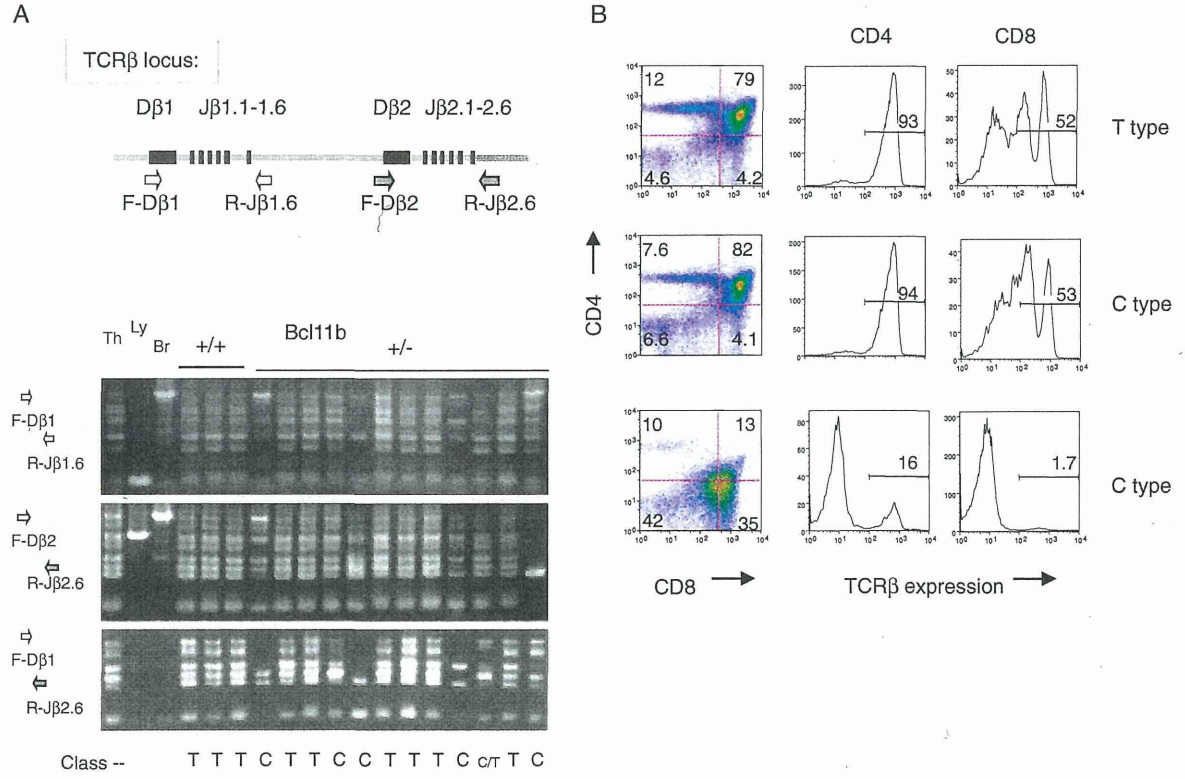

Fig. 4. Clonal proliferation of thymocytes in atrophic thymuses after γ-irradiation of *Bcl11b*$^{KO/+}$ mice. (A) The upper diagram shows part of the *TCRβ* locus and the relative location of PCR primers. The lower panel shows gel electrophoresis of PCR products with three different sets of primers, F-Dβ1 and R-Jβ1.6 (top), F-Dβ2 and R-Jβ2.6 (middle), and F-Dβ1 and R-Jβ2.6 (bottom). Thymuses are classified into two group, T type thymus (similar to normal thymus) and C type thymus (showing clonal expansion) depending on band patterns of PCR products. The T type thymus shows all six different bands as normal thymus does, whereas the C type thymus shows skewed band patterns, a few bands being more prominent than the other bands. The latter pattern indicates the presence of clonally proliferating thymocytes. (B) Flowcytometry of CD4, CD8 and TCRβ expression on thymocytes, showing differences in differentiation level. The vertical axis shows CD4 expression and the horizontal axis displays CD8 expression (left); the vertical axis shows cell number and the horizontal axis displays TCRβ expression of thymocytes in the CD4 quadrant (middle) and in the CD8 quadrant (right). Some C type thymuses showed differentiation arrest and lower expression of TCRβ on surface.

hallmark of cancer, was found only in this subgroup of the *Bcl11b*$^{KO/+}$ C-type thymocytes, but not seen in the *Bcl11b*$^{+/+}$ C-type thymocytes.[91] Of note, nevertheless approximately a half of the *Bcl11b*$^{+/+}$ C-type thymocytes lost one allele of *Bcl11b*. This suggests that acquired loss of a *Bcl11b* allele did not affect the differentiation arrest in those thymocytes. Though it is not clear at which developmental stage the acquisition of loss occurred, it is likely that the *Bcl11b* allelic loss took place at the stage after V(D)J recombination because of limited V(D)J recombination patterns detected. Li *et al.*[46] demonstrated that the expression of Bcl11b begins at the early DN1 cell stage in thymus. The DN1 cell population is multipotent and has the potential to differentiate into macrophage, NK cells, T cells and others. Actual T cell precursors within the DN1 population are CD117^{high}, whereas CD117^{low} DN1 cells are precursors for NK and γδT cells or others.[94] Bcl11b expression was observed in CD117^{low} DN1 progenitor cells but not in CD117^{high} DN1 cells.[46] Hence, *Bcl11b* heterozygosity can affect lymphomagenesis at CD117^{high} DN2 stage after CD117^{high} DN1 stage. Though impaired differentiation is a hallmark of cancer, it would not alone be sufficient for DN or ISP thymocytes to acquire malignancy. The number of thymocytes in C-type thymuses is low (one tenth in average relative to normal thymus) and hence the C-type thymocytes are obviously not fully malignant. To establish full malignancy, some of the C-type

thymocytes must acquire an additional genetic or epigenetic change(s). Together, these findings suggest that the differentiation arrest is related to *Bcl11b*$^{+/-}$ genotype and may be ascribed to Bcl11b loss in immature thymocytes after early CD117^{low} DN1 stage and possibly before the ISP or DP stage.

10. Cancer stem cells and C-type thymocytes

Recently, McCormack *et al.* have shown an interesting finding of premalignant thymocytes in atrophic thymus, using the *Lmo2*-transgenic mouse model.[90] The *LMO2* oncogene causes a subset of human T cell lymphoblastic leukemias. They used a combination of *in vivo* cell fate mapping, in which the *Lmo2* gene was constitutively expressed in the thymus but not in the bone marrow (BM), and transplantation of thymocytes from young *Lmo2*-transgenic mice. As a result, they found self-renewing thymocytes in atrophic thymus of *Lmo2*-transgenic mice that were committed T cells at the DN3 stage. Of interest, these self-renewing DN3 cells possessed many features of cancer stem cells, including the ability to serially transplant, the ability to generate mature T cells, and the expression of several genes typical of hematopoietic stem cells including stem cell marker. Thymic atrophy in *Lmo2*-transgenic mice is probably caused by loss of the entry of progenitor cells from BM due to the development of self-renewing cells within the thymus. They also showed that the *Lmo2*-induced premalignant thymocytes can survive after a high-dose irradiation, consistent with cancer stem cell hypothesis. The importance of cancer stem cells in leukemia therapy has been pointed out in relapsed acute lymphoblastic leukemia in humans.[91),95]

As described above, some of the C-type thymocytes in atrophic thymus have two properties, to undergo many rounds of cell cycle within the thymus and to capable to differentiate into DP and SP cells. In the respect of self-renewal and differentiation, they are similar to the *Lmo2*-induced premalignant thymocytes. Analyses of *Bcl11b*$^{KO/KO}$ mice[22),46] showed that the arrested Bcl11b-KO DN2 cells started to self-renew. This may implicate Bcl11b deficiency in the generation of cancer stem cells or premalignancy. Interestingly, a subtype of human leukemias, CML (chronic myeloid leukemia), possesses self-renewal and cell lineage capabilities, and thereby the cells are assumed to be leukemia-initiating or cancer stem cells.[95),96] Some of the C-type thymocytes have the phenotypes of CML. Therefore, those C-type thymocytes might be lymphoma stem cells. If so, they will be a model for therapy.

Acknowledgments

The author thanks Dr. Hiroshi Hiai for critical reading this manuscript and helpful comments. The author is also grateful for people in his laboratory who have contributed to works described here.

Competing financial interests

The authors declare no completing interests.

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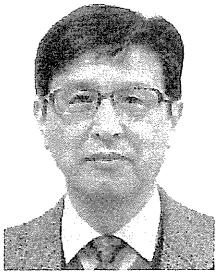
(Received Oct. 7, 2011; accepted Jan. 11, 2012)

Profile



Ryo Kominami was born in 1947 and graduated from Okayama University School of Medicine at 1971. He started his research career at the Department of Biochemistry under the direction of Professor Masami Muramatsu at Tokushima University School of Medicine in 1972 and he received his PhD in 1976. After two years of study as a postdoctoral fellow at Frederic Cancer Research Institute near Washington DC in U.S.A., and then returned to Cancer Institute in Tokyo in 1979 and served as Researcher. He became Assistant Professor at the University of Tokyo in 1982 and was promoted to Associate Professor in 1983. He studied repetitive sequences, including minisatellites that were useful for individual identification. He is Professor of Biochemistry at Niigata University School of Medicine since 1987 and serving as a Vice Dean of the Graduate School of Medical and Dental Sciences at Niigata University. He isolated the *Bcl11b* tumor suppressor gene by analyzing radiation-induced mouse thymic lymphomas and found a regulatory role for Bcl11b in T-cell development. He was awarded the Japan Radiation Research Society Award in 2007.