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Authors' contributions
YYa, YYo, KM, RT, and FT carried out the experimental work, YYa, KM, TT, RH and YF provided data analysis, YYa, TK, NK and TO designed the study and YYa, NK and TO participated in writing the paper. All authors read and approved the manuscript.

Competing interests
The authors declare that they have no conflict of interest. KM, TT, RH, and YF are Agilent employees.

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REVIEW

Gene-manipulated embryonic stem cells for rat transgenesis

Masaki Kawamata · Takahiro Ochiya

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Abstract Embryonic stem cells (ESCs) are derived from blastocysts and are capable of differentiating into whole tissues and organs. Transplantation of ESCs into recipient blastocysts leads to the generation of germline-competent chimeras in mice. Transgenic, knockin, and knockout gene manipulations are available in mouse ESCs, enabling the production of genetically modified animals. Rats have important advantages over mice as an experimental system for physiological and pharmacological investigations. However, in contrast to mouse ESCs, rat ESCs were not established until 2008 because of the difficulty of maintaining pluripotency. Although the use of signaling inhibitors has allowed the generation of rat ESCs, the production of genetically modified rats has been difficult due to problems in rat ESCs after gene introduction. In this review, we will focus on some well-documented examples of gene manipulation in rat ESCs.

Keywords ES cell · Rat · Transgenic · Pluripotency · Chimera

Introduction

Embryonic stem cells (ESCs) established from the inner cell mass (ICM) of preimplantation blastocysts [1] have been routinely derived from mice since 1981 [2, 3]. These cells have a stable developmental potential to form derivatives of all three embryonic germ layers, the endoderm, mesoderm, and ectoderm, even after prolonged culture [4] and have been used to study the mechanism of cell differentiation. Moreover, they are capable of generating germline chimeras following injection into the blastocyst [5]. Gene manipulation is available, and germline transmission of transgenic ESCs was achieved in 1986 [6]. Soon after this achievement was reported, gene-targeting mice were generated via homologous recombination in ESCs [7]. So far, a huge number of genetically modified mice have been produced via the manipulation of ESCs and used in a range of biomedical researches. However, this technique is unavailable in species other than mice because of a lack of stable ESCs.

The laboratory rat, the first mammalian species domesticated for scientific research, has been used as an animal model for research in physiology, toxicology, nutrition, behavior, immunology, and neoplasia for over 150 years [8–12]. Despite the utility to use rats in experiments, rat ESCs were not established until 2008. The reasons for the failure to develop ESCs in rats are related to the difficulty in maintaining pluripotency in culture despite trials using numerous strategies [13–17]. Our group generated rat ESCs harboring a potential to contribute to chimera but not to develop germ cells [18]. On the other hand, despite the lack of authentic ESCs, several technologies have been developed to alter rats genetically [19–26].

Rat transgenesis from ESCs with 2i+LIF medium

In 2008, germline-competent rat ESCs were first established from blastocysts by using a 2i+LIF medium composed of two signaling inhibitors (MEK inhibitor PD0325901; GSK3 inhibitor CHIR99021), a leukemia inhibitory factor (LIF), and a defined basal culture medium

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containing no fetal bovine serum (FBS) (Fig. 1) [27, 28]. The results of the two studies showed that FBS was a key factor in the induction of differentiation in rat ESCs [29]. This culture medium was also used for the generation of mouse ESCs [30]. Generally, mouse ESCs are cultured on feeder layers of mouse embryonic fibroblasts (MEFs). Further, it was found that the use of DIA-M cells [27] or a mixture of MEFs and L-cells as feeder layers [28] was optimal for isolating rat ESCs. In these conditions, although ESCs maintained pluripotency and contributed to chimeras, only two of nine cell lines achieved germline transmission. Rat-induced pluripotent stem cells (iPSCs) with the potential to contribute to chimeras were also generated by the addition of A-83-01 (Type 1 Tgf β receptor inhibitor) to 2i+LIF in a mouse ESC basal culture medium containing 20% knockout serum replacement (KSR). However, these iPSCs did not achieve germline transmission [31].

Gene introduction was available in the rat ESCs cultured in 2i+LIF medium. However, they were sensitive to electro-physical stimulation induced by the conventional electroporation method, which led to cell death. A nucleofection method was found to be more efficient and convenient for gene introduction in rat ESCs [28]. Furthermore, FBS was temporally added into an electroporation medium as well as a 2i+LIF cell-culture medium to aid viability [27]. Each group obtained stable transfectant clones in which the CAG-eGFP-IRES-pac plasmid was randomly integrated in their genome after selection with puromycin [27, 28]. Although five overt coat color chimeras were born after injection of the clone, they either died perinatally or were euthanized due to jaw abnormalities. The reasons for their abnormalities might have been chromosomal instability in the transfectant ESC line [27]. On the other hand, Hirabayashi et al. [32] succeeded in the germline transmission of a transfectant rat ESC line harboring a humanized Kusabira-Orange (huKO) gene using the 2i+LIF culture medium. A CAG/huKO-neo plasmid was introduced into ESCs by electroporation, and then stable clones were obtained by neomycin selection. In the 2i+LIF medium, 1,000 U/ml of rat LIF [33] was substituted for the human LIF used in the previous works (100 U/ml [27]; 10 U/ml [28]). It is possible that the rat LIF is better for the maintenance of rat ESCs [34]. Kobayashi et al. [35] overcame the difficulty to generate interspecific chimeras between rats and mice using rat ES or iPS cells cultured in a 2i+rat LIF medium. Thus, using rat LIF might be an option to keep rat ESCs stable.

Rat transgenesis from ESCs with YPAC medium

Our group developed a new culture medium (YPAC medium) including the additional signaling inhibitors of Rho-associated kinase (Y-27632) and A-83-01 to the 2i [36]. The four inhibitors, Y-27632, PD0325901, A-83-01, and CHIR99021, are collectively referred to as YPAC. A mouse ESC basal culture medium containing FBS (20% vol/vol) and MEFs was used, but LIF was not necessary in our study (Fig. 1). In the culture condition, the majority of cell lines (six out of six) demonstrated chimerism and germline transmission and could be stably transfected with a reporter transgene to produce genetically modified rats. These three cell lines were derived from each of the following strains: Wistar, LEA (Long Evans Agouti), and hybrid Wistar/LEA [36].

Since the medium contained 20% serum, the ESCs were tolerant to the damage induced by electric stimuli during gene introduction. In our procedure, a transgene in which the Venus gene was transcribed by the *Oct4*-promoter (*Oct4*-Venus) was introduced in the ESCs by the nucleofection method. When the manipulated cells were plated,

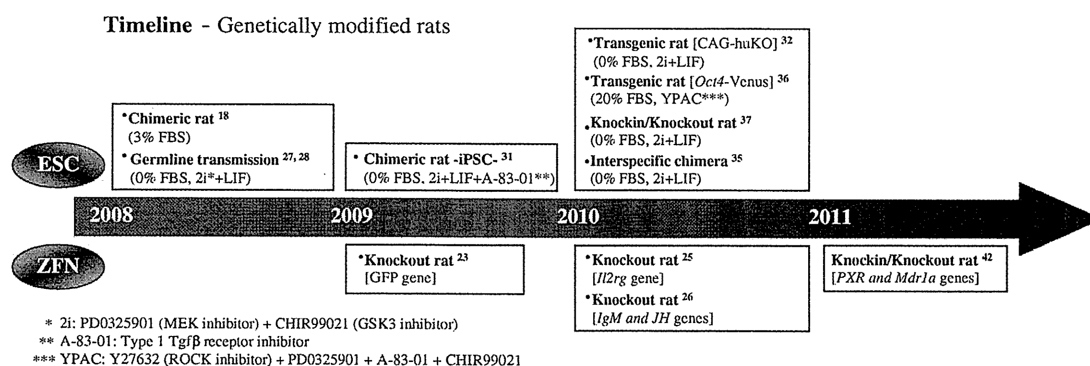

Fig. 1 Timeline of rat transgenesis using ESC or ZFN technology since 2008. The *parentheses* indicate the culture conditions. The *brackets* indicate transgenes or targeted genes

the use of matrigel (2% at final concentration) was effective for selecting stable clones because they are retained to adhere on MEFs [36]. It is generally known that rat ESC colonies tend to detach from MEFs [27, 28, 36]. This phenomenon enhances the ESCs' attachment to each other, leading to clone contamination. As the transgene did not include a selection cassette, Venus-positive clones were picked and expanded without drugs. In this cloning process, we found an advantage of using ESCs for the generation of transgenic rats because we were able to choose high-quality clones mimicking an endogenous *Oct4* expression pattern. While the majority of the clones exhibited a heterogeneous expression pattern in undifferentiated cells, only a few clones maintained homogenous expression after long-term culture (Fig. 2a). This homogeneity corresponds to the expression pattern of endogenous OCT4 protein. *Oct4*-Venus transgenic rats were generated through germline transmission of the selected clones without any adverse effects of gene introduction on chimera contribution (Fig. 2b). The Venus fluorescence was also detected in germ cells of the transgenic fetal gonads (Fig. 2c). Moreover, we could trace the fluorescence only in undifferentiated ESCs from their blastocysts during the establishment process and long-term culture (Fig. 2d) [36].

Gene targeting rats from ESCs

Tong et al. [37] achieved for the first time the production of knockout rats via homologous recombination in rat ESCs. A targeting vector was constructed to disrupt the tumor suppressor gene *p53* (also known as *Tp53*). Targeting efficiencies in two ESC lines derived from the DA (Dark Agouti) strain were 1.12–3.70%. Many properly targeted cell lines cultured in the 2i+LIF medium developed chromosome abnormalities. Over 65% of the cells were polyploid. This phenomenon is similar to that reported in previous works [27, 28]. However, after subcloning round and compact colonies, two out of 20 clones had euploid chromosome numbers leading to the production of a viable knockout [34]. This achievement is historic because the p53 knockout rat validates the culminated effort of many to enable targeted genetic engineering in rat ESCs. Another group also succeeded in gene targeting in a hypoxanthine phosphoribosyltransferase (*hprt*) locus by homologous recombination in rat ESCs [38]. Although these *hprt* heterozygous clones cultured in the 2i+LIF medium maintained pluripotency, aneuploid cells did emerge in the cultures. However, approximately 2% of geneticine-resistant colonies achieved recombination correctly. The efficiency was similar to that originally reported for mouse

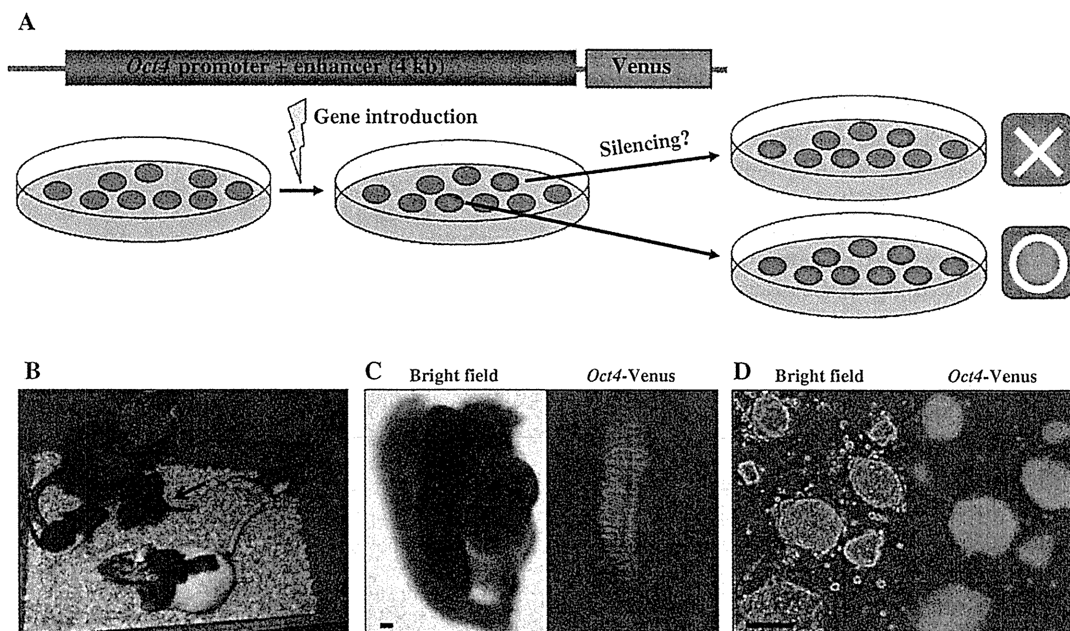

Fig. 2 Transgenesis in rat ESCs. **a** *Oct4*-Venus transgene is introduced in rat ESCs by a nucleofection method. Some clones receive random integration of the transgene, with subsequent green fluorescence. After subcloning and passaging, Venus fluorescence was decreased in a majority of the clones (*upper*), while a minority of the clones expressed the fluorescence homogeneously. **b** The *Oct4*-Venus transgenic rat (*arrow*) was produced through germline transmission of the recombinant ESCs from a chimeric rat. **c** *Oct4*-Venus positive-germ cells in E16.0 gonad of the transgenic rats. **d** An ESC line derived from the *Oct4*-Venus transgenic rat. Venus fluorescence was kept in undifferentiated cells after 18 passages. All *scale bars*, 100 μm

and human ESCs [39–41]. Thus, these reports suggest that rat ESCs are readily amenable to gene targeting by homologous recombination using the basic methodology that has proved so effective in mouse ESCs.

Discussion

Rat transgenesis via gene manipulation in ESCs was demonstrated in 2010, marking the beginning of a new era in rat genetics. Although some problems remain in the rat ESC handling, a combination of the methods described in this manuscript as well as newly devised techniques will lead to the discovery of a gold standard method to routinely generate genetically modified rats from ESCs. Recently, not only knockout but also knockin rats have been generated using ZFN-mediated homologous recombination [42]. This knockin strategy will make it possible to introduce temporal control and tissue-specific changes in genes in rat models by combining *Cre/loxP* and an inducible gene expression system. ZFN technology also possesses several advantages, such that the time frame to obtain mutant animals is short, ZFN-mediated homologous recombination in embryos does not require a selection marker, and time-consuming backcrossing is avoided [42]. However, this technology remains expensive to purchase, which is an obstacle for most researchers. In contrast, researchers can apply gene targeting by using ESCs, as is routinely done in mouse research. Therefore, ESC is also required to expand knockout rat lines. There is another advantage of using ESCs when generating transgenic rats. Useless transgenic animals are frequently generated with the conventional method. However, as described in this manuscript, we can choose ESC clones in which a transgene is correctly expressed, leading to the generation of high-quality transgenic rats [36]. Moreover, we can analyze gene function in chimeric animals by using ESCs. Recent reports have shown that this chimeric strategy is effective in identifying gene functions in vivo in terms of developing a more clinically relevant stochastic model [43]. Thus, we speculate that using both the ESC and ZFN strategies will be necessary for routine rat transgenesis.

We now have an opportunity to find new gene functions that have been concealed or questioned in mutant mice. We have accumulated genetic information and a vast amount of research data on physiology and pharmacology in rats. Thus, a combination of these studies will lead to the discovery of new and profound mechanisms of human diseases and the manufacture of medicines to cure patients. Furthermore, rats with their larger sizes make it possible to extract sufficient quantities of samples, such as blood, without killing the animals and to perform difficult surgeries, such as those in brain tissue; all of this emphasizes the advantages of gene-modified rats. We hope that researchers will create many genetically modified rats and open up a powerful new platform for the study of human diseases.

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Impairment in differentiation and cell cycle of thymocytes by loss of a *Bcl11b* tumor suppressor allele that contributes to leukemogenesis

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Key words: T-ALL, BCL11B, type B abnormalities, haploinsufficiency, cell cycle of thymocytes

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4 Figures, no Table,

4 Supplementary Figures

ABSTRACT

Genetic changes in T-ALL are classified into type A abnormalities leading to arrest at a specific stage of T-cell differentiation and type B abnormalities that target cellular processes including cell cycle regulation. Mutations and deletion of a *BCL11B* haploinsuffiecient tumor suppressor allele have been found in 10-16% of T-ALL subgroups. Analysis of *Bcl11b*$^{KO/+}$ mice revealed impaired T-cell differentiation at two different stages and attenuation of γ-ray induced cell-cycle arrest at S/G2/M phase in immature CD8 single positive cells. Hence, those phenotypes provided by loss of a *Bcl11b* allele favor that *Bcl11b* mutation belongs to type B abnormalities.

Key words: T-ALL, BCL11B, type B abnormalities, haploinsufficiency, cell cycle of thymocytes

1. Introduction

T-cell acute lymphoblastic leukemia (T-ALL) is a common pediatric leukemia and an aggressive malignancy of thymocytes that accounts for about 15% of ALL cases [1, 2]. Leukemic transformation of immature thymocytes is caused by a multistep pathogenesis involving numerous genetic abnormalities that drive normal T-cells into uncontrolled cell growth and clonal expansion. Despite the diversity in genetic alterations, the biological processes that are targeted seem conserved throughout all T-ALL cases and affect T-cell differentiation, T-cell receptor signaling that affects cell survival, and cell cycle. The current knowledge of oncogenic and tumor suppressive mutations in T-ALL suggests a classification of these genetic defects into type A and type B abnormalities [3]. Type A abnormalities such as *TAL/LMO* alterations are a class that delineates distinct molecular-cytogenetic T-ALL subgroups and are thought to cause arrest at a specific stage of normal T-cell differentiation. The other class is type B abnormalities including *CDKN2A/2B* and *NOTCH1* mutations that are shared by several different T-ALL subgroups, and they target cellular processes including cell cycle regulation and synergize with the type A mutations during T-cell pathogenesis.

Bcl11b (B-cell CLL/lymphoma 11b) belongs to Kruppel-like C_2H_2 type zinc finger transcription proteins, the largest family of transcription factors in eukaryotes [4-7]. Although *Bcl11b* knockout (KO) mice die shortly after birth, loss of Bcl11b function is known to lead to arrest of thymocyte differentiation at different stages, indicating many roles in T-cell development [8-13]. The *Bcl11b* gene was first identified as a tumor suppressor gene by analysis of γ-ray induced mouse thymic lymphomas [4, 14], a model of T-ALL [15], and genetic changes were found in more than a half of the lymphomas [4, 16, 17]. Recently, mutations and deletions of the human orthologue *BCL11B* have been found

in 10%-16% of T-ALL [18-20], and of interest, these mutations are detected irrespective of T-ALL subgroups. Accordingly, *Bcl11b* mutation may be classified into a type B abnormality, though consequences of the mutation were not investigated.

One characteristic of *Bcl11b* is haploinsufficient in its tumor suppressive capability, one wild-type allele being insufficient for tumor suppression. This is based on that most of the thymic lymphomas developed in *Bcl11b*$^{KO/+}$ mice retained the wild-type allele although thymic lymphomas developed in wild-type mice showed loss of one *Bcl11b* allele at a high frequency [16, 17]. The retention of the wild-type allele was observed in spontaneously developed thymic lymphomas in *Bcl11b*$^{KO/+}$*p53*$^{KO/+}$ mice [16], and importantly, also in T-ALLs having mutations on the *BCL11B* gene [19, 20]. These indicate that only loss of one *Bcl11b* allele can affect lymphomagenesis in mice and humans. Haploinsufficient capability of *Bcl11b* is also reported in tissue development [21, 22]. Despite the importance of *Bcl11b* heterozygosity in lymphomagenesis, its effect on T-cell development was not studied in detail. Hence, we have examined differentiation, apoptosis, and cell cycle of thymocytes at different developmental stages in *Bcl11b*$^{KO/+}$ mice. In this paper we show that loss of a *Bcl11b* allele leads to differentiation arrest at certain immature stages and deregulation of the response to γ-radiation in cell cycle, a phenotype that may be explained by the type B alteration.

2. Materials and methods

2.1. Mice

Bcl11b$^{KO/+}$ mice were generated as described [5]. *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice of the BALB/c background were subjected to γ-irradiation of 1 Gy or 3 Gy at 8 weeks of age as described [23]. Thymus or left and right thymic lobes were isolated at indicated times

after irradiation and subjected to analysis. Mice used in this study were maintained under specific pathogen-free conditions in the animal colony of Niigata University. All animal experiments comply with the guidelines by the animal ethics committee for animal experimentation of the University.

2.2. Flow cytometry

Flow cytometric analysis was performed as previously described [5]. In brief, single cell suspensions of thymocytes were prepared from thymus and, 1-2 x 10^6 cells were incubated with antibodies in phosphate-buffered saline containing 2% fetal calf serum and 0.2% NaN_3 for 15 min at 4 ^{0}C. The monoclonal antibodies (mAbs) used were anti-CD4-APC or -PerCP-Cy5.5 (RM4-5 BioLegend), anti-CD8-APC or -PE (53-6.7 eBioscience), anti-CD25-FITC (PC61 BioLegend), anti-CD44-PE (IM7 Biolegend), and anti-TCRβ-FITC or -APC (H57-597 BioLegend). To prevent nonspecific binding of mAbs, we added CD16/32 (93 eBioscience) before staining with labeled mAbs. Dead cells and debris were excluded from the analysis by appropriate gating of FSC and SSC. Cells were analyzed by a FACScan or a FACSCalibur (Becton-Dickinson) flow cytometer, and data were analyzed using the Flow-Jo software (Tree-Star, Inc).

Apoptosis was measured by Annexin V assay following the manufacturer's instructions (BD Bioscience). Briefly, thymocytes were washed in cold PBS and resuspended in binding buffer (HEPES buffer supplemented with 2.5 mM $CaCl_2$). These were incubated with FITC-labeled Annexin V (BD Bioscience) for 15 min at room temperature. Flow cytometry was performed on thymocytes gated on the basis of their forward and side light scatter with any cell debris excluded from analysis. Apoptotic cells were defined as $FITC^+$ cells.

For BrdU incorporation experiments, we injected mice intra-peritoneally with

100 μl of BrdU solution (10 mg/ml). In indicated cases, irradiation was performed at 1 h after BrdU injection. Thymuses were isolated 1 h or 5 h after BrdU administration and thymocytes were fixed with cytofix/Cytoperm (BD Bioscience) and analyzed with the use of the BD Bioscience BrdU Flow Kit according to Manufacturer's instruction. In brief, cells were suspended at a concentration of 1-2 x 10^6 cells/ml, fixed, permeabilized and incubated with DNaseI (300μg/ml) for 60 min at 37 °C. After washing, cells were incubated with FITC conjugated anti-BrdU antibodies for 20 min at room temperature. Cells were resuspended in staining buffer and analyzed by FACSCalibur flow cytometer.

Statistical analysis was done using t-test.

3. Results

3.1. Differentiation of thymocytes

Development of αβ T cells in the thymus proceeds through three major stages defined according to their expression pattern of CD4 and CD8 molecules on cell surface, i.e. in order of maturity, $CD4^-CD8^-$ double negative (DN), $CD4^+CD8^+$ double positive (DP), and $CD4^+CD8^-$ or $CD4^-CD8^+$ single positive cells (CD4SP or CD8SP cells, respectively) [7, 24]. DP thymocytes undergo rearrangement at the TCRα locus and hence a small fraction ($TCR\beta^{high}$-DP cells) expresses the αβ T-cell receptor (TCRαβ) complex on cell surface before progressing to $TCR\beta^{high}$-SP stage. Immature $CD8^+$ single positive (ISP) cells exist between DN and DP stages, and they express CD8 but lack αβ TCR. DN thymocytes can be further divided into four subpopulations based on the surface expression of CD44 and CD25, with the developmental progression being $CD44^+CD25^-$ (DN1) to $CD44^+CD25^+$ (DN2) to $CD44^-CD25^+$ (DN3) and then to $CD44^-CD25^-$ (DN4) cells.

Fig. 1A shows flow cytometric analysis of thymocytes using differentiation

markers that were obtained from *Bcl11b*$^{+/+}$ wild-type and *Bcl11b*$^{KO/+}$ heterozygous mice. Figs. 1B, 1D and 1E show the percentage of DP, TCRβ^{high} and ISP cells, respectively, and Supplementary Fig. 1 (A-D) shows the absolute cell number of the thymocyte subsets. Analysis with CD4 and CD8 markers showed a higher percentage of DP cells (Fig. 1B) and a lower ratio of SP cells /DP cells (Fig. 1C) in *Bcl11b*$^{KO/+}$ thymus than in *Bcl11b*$^{+/+}$ thymus. The absolute number of DP cells was increased in *Bcl11b*$^{KO/+}$ thymus (Supplementary Fig. 1B). These results suggest developmental arrest of a certain fraction of thymocytes at DP stage in *Bcl11b*$^{KO/+}$ mice. Expression of TCRβ marker in total thymocytes showed a significantly decreased percentage of TCRβ^{high} cells in *Bcl11b*$^{KO/+}$ mice (Fig. 1D). This suggests arrest at TCRβ^{low} DP stage possibly before rearrangement at the TCRα locus.

Cells in the CD8^{+} fraction consist of mature CD8SP cells highly expressing TCRβ and immature ISP cells with much lower expression. The percentage and the absolute number of ISP cells were higher in *Bcl11b*$^{KO/+}$ thymus than *Bcl11b*$^{+/+}$ thymus (Fig. 1E and Supplementary Fig. 1A), suggesting arrest also at the ISP stage. The CD8^{+} fraction may include γδ T cells [10]. However, their percentage was very low in *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ CD8^{+} fractions and no marked difference was observed between them (Supplementary Fig. 2), indicating that the higher percentage of ISP cells is due to an accumulation of precursors to DP cells. On the other hand, analysis of DN cell subtypes using CD44 and CD25 markers did not show significant differences in the percentage or the cell number between *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice (Supplementary Fig. 3). This suggests no impairment during development of immature DN thymocytes. Together, these results suggest developmental arrest at the two different ISP and DP stages in *Bcl11b*$^{KO/+}$ heterozygous mice.

3.2. Apoptosis and proliferation of thymocytes

T-cell development is tightly related to apoptosis. Accordingly, we examined the

percentage of Annexin V-positive cells, an indicator of apoptosis (Fig. 2A). Annexin V^+ cells were observed in CD8SP cells at a significant level but very low in ISP, DP and CD4SP cells. Comparison between *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ CD8SP thymocytes revealed a 3-fold increase in *Bcl11b*$^{KO/+}$ mice (Fig. 2B). This suggests that loss of a *Bcl11b* allele provides increased susceptibility to apoptosis in CD8SP cells.

Deregulation of cell cycle is a hallmark of leukemic transformation. We examined BrdU incorporation of thymocytes, an indicator of cell cycle progression, in mice 1 h after BrdU administration (Supplementary Fig. 4). About 40% of ISP cells and 8% of DP cells showed BrdU incorporation whereas very low percentages of CD4SP and CD8SP cells showed BrdU incorporation, consistent with a previous report [24]. Comparison between *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice did not show significant differences in ISP and DP cells.

3.3. Effect of γ-irradiation on cellularity of thymocytes

Bcl11b$^{KO/+}$ mice developed thymic lymphomas when γ-irradiated, but not spontaneously [17]. Accordingly, of importance may be the effect of *Bcl11b*$^{KO/+}$ heterozygosity on radiation-induced cell damages. Thus, we examined cell number of thymocytes from mice 4 h after 1 Gy or 3 Gy of γ-irradiation. Fig. 3A shows expression of CD4/CD8 and TCRβ in thymocytes. Fig. 3B summarizes the cell number of ISP and DP cells in *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice. One Gy irradiation led to a two-fold decrease in ISP cells and a minimal decrease in DP cells. The decreases did not differ between *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice. On the other hand, 3 Gy irradiation reduced the cell number to approximately one tenth in ISP cells and to about one half in DP cells probably by inducing apoptosis differently. Comparison between *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice also did not show marked difference in the decreases.

3.4. Effect of γ-irradiation on cell cycle

We next examined radiation effect on cell cycle of thymocytes. To monitor cell cycle, we administrated BrdU at 1 h before γ-irradiation. Accordingly, analysis of thymocytes was performed in mice 5 h after BrdU administration and 4 h after irradiation, and for comparison in mice 1 h and 5 h after BrdU administration without irradiation. Fig. 4A show the percentage of cells in four different areas in DN, ISP and $TCR\beta^{low}$-DP cells in unirradiated mice. Control panels without BrdU administration are included. The four areas were determined by BrdU incorporation (vertical axis) and FSC value (horizontal axis), an indicative of cell size. $BrdU^{+}$ FSC-large (FSC^{L}) represents cells in S or G2/M phases of cell cycle whereas $BrdU^{+}$ FSC-small (FSC^{S}) represents G1 cells that have passed S phase after BrdU administration. $BrdU^{-}FSC^{L}$ and $BrdU^{-}FSC^{S}$ cells are thymocytes present in S/G2/M and G1 phase, respectively, that have not passed S phase for 5 h after BrdU administration.

In unirradiated mice 1 h after BrdU administration, the percentage of $BrdU^{+}FSC^{S}$ cells was much less than that of $BrdU^{+}FSC^{L}$ cells in DN, ISP, and $TCR\beta^{low}$-DP cells irrespective of *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ genotypes, indicating that BrdU-incorporated S-phase cells did not yet progress into G1 phase. However, in mice 5 h after, both $BrdU^{+}FSC^{L}$ cells and $BrdU^{+}FSC^{S}$ cells were observed at significant levels in DN, ISP, and $TCR\beta^{low}$-DP cells. The percentage of $BrdU^{+}FSC^{S}$ cells was more than that of $BrdU^{+}FSC^{L}$ cells in each subset. No differences were observed between *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice. These results indicated that certain fractions of thymocytes in S phase progressed to G1 phase for 4 h in those thymocyte subsets.

γ-irradiation affected the cell cycle progression and the consequence was different depending on subsets. Fig. 4B shows the percentage of cells in four different areas in DN, ISP and $TCR\beta^{low}$-DP cells in mice 5 h after BrdU administration and 4 h after 1 Gy or 3 Gy irradiation. $TCR\beta^{low}$-DP cells in both irradiated mice little contained $BrdU^{+}FSC^{S}$ cells

irrespective of *Bcl11b*$^{+/+}$ or *Bcl11b*$^{KO/+}$ genotypes, indicating radiation-induced arrest at S or G2/M phase. In contrast, ISP cells comprised BrdU^{+}FSC^{S} cells at significant percentages even after irradiation, indicating the progression from S to G1 phase. One Gy irradiated mice contained more BrdU^{+}FSC^{L} cells than BrdU^{+}FSC^{S} cells whereas 3 Gy irradiated mice exhibited less BrdU^{+}FSC^{L} cells than BrdU^{+}FSC^{S} cells. Comparison between *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice revealed a significant increase in the percentage of BrdU^{+}FSC^{S} cells in *Bcl11b*$^{KO/+}$ ISP cells in both 1 Gy and 3 Gy irradiated mice (Fig. 4C). Differences in radiation effect between *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice were also observed in DN cells, though the DN cell fraction comprised a mixture of immature thymocytes consisting of different subsets. Together, those findings indicated that BrdU^{+}FSC^{L} ISP cells, but not BrdU^{+}FSC^{L} DP cells, progressed to BrdU^{+}FSC^{S} cells more in *Bcl11b*$^{KO/+}$ mice than *Bcl11b*$^{+/+}$ mice. This suggests that *Bcl11b*$^{KO/+}$ heterozygosity attenuates radiation-induced cell-cycle arrest at S or G2/M phase in ISP cells.

4. Discussion

This study has investigated changes in differentiation, cell survival and cell cycle during T-cell development in mice of the *Bcl11b*$^{KO/+}$ heterozygous genotype, which provides susceptibility to thymic lymphomas [4, 23], a mouse model of T-ALL [15, 26]. In *Bcl11b*$^{KO/+}$ mice, increases were observed in the percentage and cell number of ISP and DP cells, indicating differentiation arrest at ISP and DP stages. No impairment was observed in immature thymocytes at DN stages. Previous studies using *Bcl11b*$^{KO/KO}$ mice revealed the requirement of Bcl11b function for thymocyte differentiation at DN2 and DN3 stages [5, 11-13]. These data indicate difference in the effect on differentiation between loss of one *Bcl11b* allele and loss of both alleles. The difference may be important in light of the

contribution of loss of one *Bcl11b* allele alone to lymphomagenesis.

Difference in cell survival was observed between *Bcl11b*$^{KO/+}$ and *Bcl11b*$^{+/+}$ mice. The percentage of apoptotic cells in CD8SP cells was increased in *Bcl11b*$^{KO/+}$ mice. The increased apoptosis may be related to impairment in TCR (T-cell receptor) signaling that provides signaling for cell survival. This is because CD4-Cre; *Bcl11b*$^{flox/flox}$ mice lacking Bcl11b activity after DP stage fail to express a TCR complex on cell surface [8, 10]. The result suggests that impairment in the TCR signaling is also present in *Bcl11b*$^{KO/+}$ mice.

TCR or preTCR signaling leads to the activation of a cascade of signaling molecules and eventually to the downstream activation of RAS-MAPK pathway, PI3K-AKT pathway and others [3, 27]. Multiple components in the signaling pathway are targeted by either mutations or chromosomal translocations in T-ALL, and the genetic changes result in the activation of TCR or preTCR signaling. Accordingly the activating mutation of TCR or preTCR signaling is classified as a type B abnormality [3]. However, loss of Bcl11b results in an adverse effect, inactivation of the signaling, and hence it may not be a factor contributing to lymphomagenesis.

Difference in cell cycle was observed in radiation-induced injury conditions though not in basal conditions. In both *Bcl11b*$^{+/+}$ and *Bcl11b*$^{KO/+}$ mice, irradiation led to arrest at S and G2/M phase in DP cells but not much in ISP cells. Most ISP cells progressed from S to G1 phase after irradiation, indicating a reduced capability in radiation-induced arrest at ISP stage. Of importance is that the reduced capability of arrest was more marked in *Bcl11b*$^{KO/+}$ mice. This suggests that loss of a *Bcl11b* allele attenuates the cellular response to irradiation that results in arrest at S or G2/M phase. The attenuated cellular response leads to rapid cell cycle progression at S phase, which may decrease the time to repair and hence raise the chance for accumulation of mutations. It was reported that thymocytes at a stage after pre-TCR signaling and before completion of TCRα

rearrangement, which includes ISP cells, undergo the malignant transformation in Notch1-activated conditions [28]. Therefore, ISP cells might be cells of origin in thymic lymphomas in *Bcl11b*$^{KO/+}$ mice.

Regulatory role for Bcl11b in cell cycle was studied using Bcl11b knock-down (KD) Jurkat cells, a T-cell culture line [29, 30]. Bcl11b-KD cell lines showed cell death with decreased expression of the anti-apoptotic protein Bcl-xL and also a decrease in the CDK inhibitor p27. The decrease of p27 may promote cell cycle progression during S phase. Furthermore, activation of the cell-cycle checkpoint kinase Chk1 was deregulated in Bcl11b-KD cells. The activated Chk1 through phosphorylation leads to arrest of cell cycle at S phase [29]. Therefore, this deregulation may abrogate S phase checkpoint, consistent with the attenuated arrest of ISP cells in irradiated *Bcl11b*$^{KO/+}$ mice.

T-ALL is a heterogeneous disease comprising different genetic abnormalities of oncogenic and tumor suppressive genes that are associated with specific patterns of gene expressions, as determined by microarray analysis [31-33]. Type A abnormalities in T-ALL may delineate distinct molecular-cytogenetic T-ALL subgroups and are thought to cause arrest at a specific stage of normal T-cell differentiation. Results in this study demonstrated that loss of a *Bcl11b* allele in thymocytes affects T-cell differentiation leading to developmental arrest. The arrest may contribute to thymic lymphoma development. However, the consequence was not at a specific stage but at different ISP and DP stages, which may not support that *Bcl11b* mutation belongs to a group of type A abnormalities. Type B abnormalities target cellular processes such as cell cycle regulation. Our results showed that loss of a *Bcl11b* allele impairs cell cycle regulation of ISP cells in radiation-induced injury conditions. This favors that the loss of a *Bcl11b* allele belongs to the group of type B abnormalities, consistent with that *BCL11B* mutations were detected across the major molecular subtypes of T-ALL [22].

Appendix A. Supplementary date

Supplementary data associated with this article can be found, in the online version, at doi:

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