

## Functions of B cell development-related transcription factors systematically revealed in immature B cells by gene-targeting techniques using chicken DT40 cells

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### ABSTRACT

In vertebrates there are two acquired immune systems, i.e., humoral immune response and cellular immune response. Of mature cells (plasma cells), B cells are differentiated from early progenitor cells and are responsible for humoral immune response, which is an antigen-specific immune system producing antibodies. Normal development and differentiation of B cells require various specific transcription factors. Recently, we systematically analyzed the roles of some transcription factors at immature stage of B cell development using gene-targeting techniques in chicken immature B cell line, DT40 cells, which are very advantageous for analyzing the physiological functions of the B cell-specific transcription factors and others. Many studies using knockout mice have provided important data on the roles of these transcription factors in B cell development. For instance, Aiolos regulates immature B cell apoptosis mediated by B cell receptor signaling. Helios regulates the gene expression of protein kinase Cs (PKCs). E box binding protein 2A (E2A) regulates gene expressions of survivin, IAP2 and caspase-8. Early B cell factor 1 (EBF1) dramatically regulates gene expressions of B lymphocyte-induced maturation protein-1 and PKC $\theta$ . In addition, Paired

box gene 5 (Pax5) regulates gene expressions of p300/CBP-associated factor, histone deacetylase-7 (HDAC7), HDAC9, Aiolos, Origin binding factor-1 (OBF1), Ikaros, E2A, EBF1 and PU.1 dramatically and moderately. Further, Pax5 isoforms A and B differentially regulate other B cell development-related factors. These results, together with enormous previous related data, significantly contribute to the elucidation of roles of these transcription factors in normal development and differentiation of B cells.

**KEYWORDS:** DT40, gene targeting techniques, immature B cells, transcription factors.

### INTRODUCTION

In vertebrates, the body defense against pathogens is mediated by both innate immune system and the acquired immune system. The former is characterized by early non-specific reactions and the latter is characterized by late specific responses as described below in detail. The first line of body defense against pathogens is the innate immune system in every multicellular organism. The innate immune system is characterized by non-specific reactions and consists of physical (skin), chemical (anti-bacterial peptides, lysozyme) and cellular (phagocytes) defenses against various pathogens. The main purpose of the innate immune system is to prevent the

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proliferation, spread and movement of foreign pathogens throughout the human body at the infection sites immediately. On the other hand, the second line of body defense against various pathogens, the acquired immune system, is an antigen-specific immune system and exists only in vertebrates. This system consists of two types of lymphocytes, i.e. B cells and T cells, which are derived from multipotent hematopoietic stem cells in the bone marrow. After differentiation into these two cell types, they would develop into a large number of mature cells. Thus, the distinctive feature of the acquired immune system is clonal expansion of B cells and T cells, which is the rapid proliferation from one or few original cells to enormous number of mature cells. Each of the clone cells derived from the original mature B cell or T cell has the same antigen receptor and thereby eliminates the same pathogen. After all, T cells contribute to cellular immunity. In contrast, B cells differentiate into plasma cells that can produce antibodies and thereby are responsible for humoral immunity [1, 2].

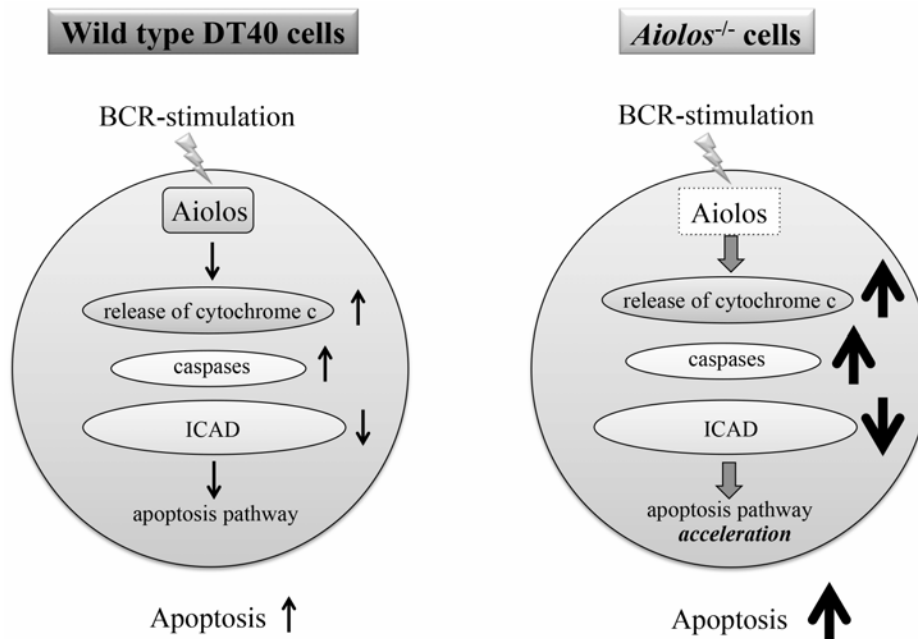
The B cell development requires not only controlled lineage and locus-specific immunoglobulin gene recombination, which establishes the unique antigen specificity of B cell receptors (BCRs), but also developmental stage-specific gene expression, which participates in lymphoid cell proliferation and synthesis of mediators involved in the establishment of immune system. In addition, the normal B cell development requires numerous transcription factors, i.e. E box binding protein 2A (E2A), Early B cell factor 1 (EBF1), Paired box gene 5 (Pax5), PU.1, Aiolos, Ikaros, and so on [3, 4]. However, the detailed understanding of the physiological functions of these transcription factors involved in B cell development has not yet been completely clarified in vertebrate cells. Many experiments using knockout mice have revealed the developmental and physiological roles of disrupted genes coding the corresponding B cell-specific transcription factors [5-10]. But, the roles of these B cell-specific transcription factors remain unknown in cases where the gene disruption causes embryonic lethality or block at the early developmental stage. Therefore, to further investigate the roles of some transcription factors at immature stage of B cell development, we systematically generated chicken

homozygous DT40 mutants, devoid of two alleles using gene-targeting techniques [11-13]. In this review article, we describe the developmental and physiological functions of various B cell-specific transcription factors.

### **Aiolos is involved in the regulation of BCR-mediated signal transduction resulting in apoptosis of immature B cells**

The transcription factor Ikaros family consists of five zinc-finger proteins: Ikaros, Aiolos, Helios, Eos and Pegasus. These proteins except Pegasus are essential for the development and differentiation of lymphocytes [14, 15]. Aiolos, a member of Ikaros family [14], was first detected in precursors of lymphocytes and dramatically enhanced at the pre-B cell stage, and its highest expression was observed in mature B cells [16, 17]. Moreover, the Aiolos gene is widely expressed throughout the normal developmental stage from pro-B cells to mature B cells [18]. In Aiolos-deficient mice, each B cell exhibits an activated cell surface phenotype and possesses an enhanced response against BCR signaling [19]. In addition, Aiolos-deficient mice develop a systemic lupus erythematosus-type autoimmune disease [20]. It was also reported that Aiolos is up-regulated in chronic lymphocytic leukemia [21]. However, its physiological functions remain poorly defined, particularly in each developmental stage of B cells. Interestingly, it was revealed that Aiolos controls gene conversion and cell death in DT40 cells (chicken immature B cells) using gene targeting techniques [22]. However, molecular mechanism of the acceleration of apoptosis in the Aiolos-deficient DT40 mutant, Aiolos<sup>-/-</sup>, remains to be resolved. Moreover, chicken Aiolos is alternatively spliced to form two isoforms, full-length form (Aio-1) and exon 3-lacking shorter form (Aio-2) [23]. But, the difference between Aio-1 and Aio-2 functions is still unknown.

Therefore, to investigate the physiological functions of Aiolos, we analyzed Aiolos<sup>-/-</sup> established by us (Fig. 1) [24]. As a result, the lack of Aiolos certainly accelerates apoptosis of DT40 cells mediated by BCR signaling mimicked by phorbol 12-myristate 13-acetate (PMA)/ionomycin treatment. Moreover, the Aiolos-deficiency and BCR signaling cooperatively control apoptotic cell death through dramatically



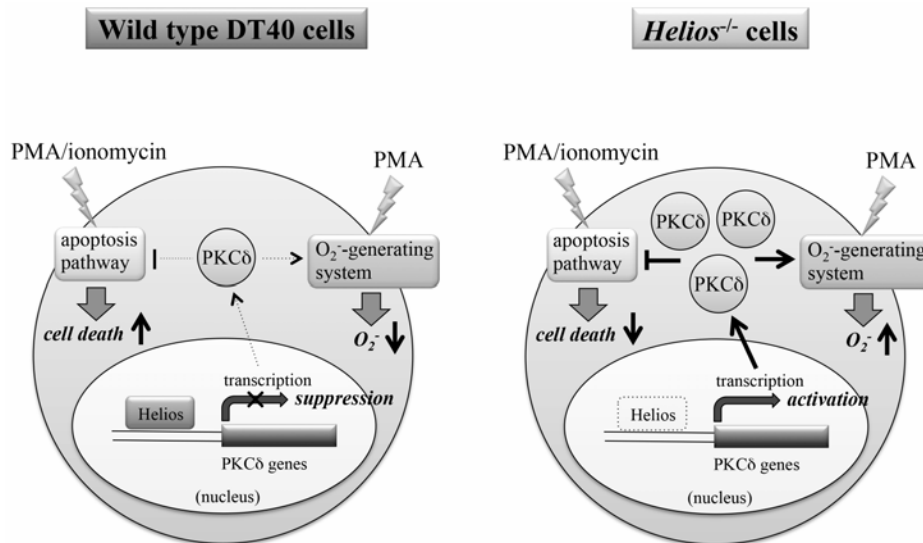
**Fig. 1. Aiolos is involved in the regulation of BCR-mediated signal transduction resulting in apoptosis of immature B cells.** Concerning BCR-stimulation, the Aiolos-deficiency causes elevation of cytochrome *c* release from mitochondria to cytosol and caspase (caspase-3, -8 and -9) activities, resulting in drastically diminished amounts of ICAD followed by increased DNA fragmentation. As a result, apoptosis should be dramatically accelerated in *Aiolos*<sup>-/-</sup> cells compared with wild-type DT40 cells.

elevated cytochrome *c* release from mitochondria to cytosol, and elevated activities of caspases (caspase-3, -8 and -9), resulting in drastically diminished amounts of inhibitor of CAD (ICAD) followed by increased DNA fragmentation. Very interestingly, the re-expression study revealed that Aio-2, but not Aio-1, controls PMA/ionomycin-mediated apoptosis *via* up-regulation and down-regulation of the protein kinase C $\delta$  (PKC $\delta$ ) and bak genes, respectively. These findings may contribute to resolving the molecular mechanisms of negative selection of B cells and also auto-immune diseases.

#### **Helios is involved in the regulation of some immature B cell functions *via* transcriptional regulation of PKCs**

As mentioned above, Helios is also a member of Ikaros family [14]. Though Helios is constitutively expressed in hematopoietic cells [14], it is mainly detected in T cells after differentiation and involved in both T cell development and function [14, 25]. Helios is also expressed in B cells [14, 26, 27], and silencing of Helios is critical for normal function of B cells [26]. However, in B cells, the

physiological role of Helios remains to be elucidated yet, because its expression level is very low. To know the physiological role of Helios in B cells, we generated and analyzed the Helios-deficient DT40 cells, *Helios*<sup>-/-</sup> (Fig. 2) [28]. The Helios-deficiency brings about significant increases in transcriptions of four protein kinase C (PKC) isoforms; PKC $\delta$ , PKC $\epsilon$ , PKC $\eta$  and PKC $\zeta$  [28], whereas their expressions are drastically down-regulated in *Aiolos*<sup>-/-</sup> [24]. In addition, *Helios*<sup>-/-</sup> is remarkably resistant against PMA/ionomycin treatment, which mimics the BCR-mediated stimulation. In the presence of PMA/ionomycin, their viability is remarkably higher than that of DT40, and their DNA fragmentation is less severe than that of DT40 and is in the opposite manner for the Aiolos-deficiency. The resistance against the PMA/ionomycin-induced apoptosis of *Helios*<sup>-/-</sup> is sensitive to Rottlerin (selective inhibitor for PKC $\delta$  and PKC $\theta$ ) but not to Go6976 (selective inhibitor for PKC $\alpha$  and PKC $\beta$ ). In addition, the Helios-deficiency causes remarkable up-regulation of the Rottlerin-sensitive superoxide (O<sub>2</sub><sup>-</sup>)-generating activity, which is induced by BCR-mediated



**Fig. 2. Helios is involved in the regulation of some immature B cell functions via transcriptional regulation of PKC $\delta$ .** The Helios-deficiency causes remarkable up-regulation of PKC $\delta$  gene expression (to ~300%) in comparison with wild-type DT40 cells. In *Helios*<sup>-/-</sup> cells, increased PKC $\delta$  inhibits the BCR-mediated apoptosis pathway, but it promotes the O<sub>2</sub><sup>-</sup>-generating system induced by PMA stimulation.

signaling. These data suggest that Helios may contribute to the regulation of the BCR-mediated apoptosis and the O<sub>2</sub><sup>-</sup>-generating activity via transcriptional regulation of the four PKCs (especially PKC $\delta$ ) in immature B cells. Our findings, together with previous data, may significantly help in the understanding of the B cell-specific expressions of PKC genes and molecular mechanisms of both the BCR-mediated apoptosis involved in negative selection and the O<sub>2</sub><sup>-</sup>-generating system in immature B cells.

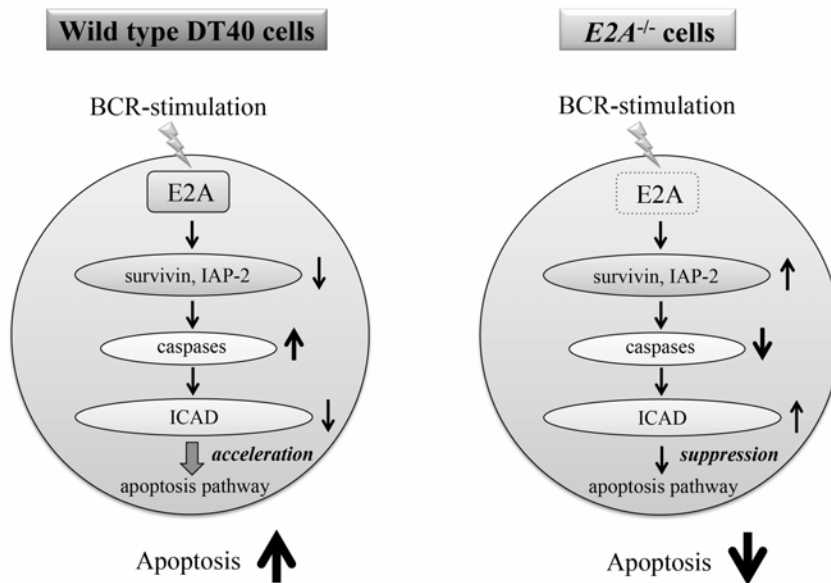
#### **E2A is involved in the regulation of BCR-mediated apoptosis via promoting caspase activation caused by down-regulation of survivin and IAP2 gene expressions in immature B cells**

E2A has been directly implicated in the transcription regulation of several B lineage-specific genes and shown to be essential for immunoglobulin (Ig) H and L-chain recombination [29]. Moreover, E2A is required to initiate expressions of some B lineage-specific genes such as EBF1, mb-1 and B29, but not to maintain expressions of these genes [29]. In general, E2A has also been shown to promote proliferation and survival of various cell types [29, 30]. E2A-deficient mice develop T cell-derived lymphoma, enforcing expression of inhibitor of

differentiation proteins, which indirectly inhibit E2A function [31]. It is also known that E2A plays important roles in apoptosis of B cells [32, 33]. In addition, Id3 protein, an E2A antagonist, induces growth arrest and apoptosis in B cell progenitor [34]. However, the role of E2A in controlling the BCR-mediated apoptosis of immature B cells still remains unclear. To know the role of E2A in BCR-mediated apoptosis of immature B cells, we generated and analyzed the E2A-deficient DT40 cell line, E2A<sup>-/-</sup>. The E2A-deficiency prevents in part immature B cells from apoptosis mediated by BCR signaling (Fig. 3) [35]. In addition, the E2A-deficiency suppresses the BCR-mediated apoptosis, through relatively elevated survivin plus IAP-2 amounts, and reduces caspase-3, -8 and -9 activities, resulting in relatively increased amounts of ICAD, compared with those in the presence of E2A, followed by the diminution of DNA fragmentation. These findings may contribute to resolving the molecular mechanisms of negative selection of B cells and also auto-immune diseases.

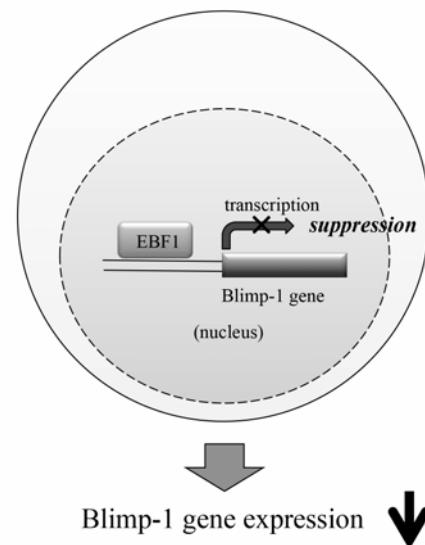
#### **EBF1 is involved in transcriptional regulation of Blimp-1 and PKC $\theta$**

EBF1 is a transcription factor having an atypical zinc-finger and helix-loop-helix motif and is essential



**Fig. 3. E2A is involved in the regulation of BCR-mediated apoptosis via promoting caspase activation caused by down-regulation of survivin and IAP2 gene expressions in immature B cells.** Concerning BCR-stimulation, The E2A-deficiency causes elevation of survivin and IAP-2 proteins, resulting in the down-regulation of caspase (caspase-3, -8 and -9) activities. Inhibition of caspases should drastically cause the increase in amounts of ICAD followed by decreased DNA fragmentation. As a result, apoptosis should be suppressed in  $E2A^{-/-}$  cells compared with wild-type DT40 cells.

for the development and differentiation of lymphocytes [36, 37]. In mice, EBF1 participates in the generation of pre-pro B cells (the first specified progenitors of B cells) from common lymphoid progenitors (CLPs) and also in transcriptional regulations of various genes (e.g., mb-1 and Pax5) involved in B cell development [6, 38, 39]. During B cell development, EBF1 is detected throughout from CLPs to mature B cells [40]. However, in immature B cells, the physiological role of EBF1 remains to be elucidated. To know the physiological role of EBF1 in immature B cells, we analyzed the phenotype of EBF1-deficient DT40 cells,  $EBF1^{-/-}$ , generated by us (Fig. 4) [41]. The EBF1-deficiency brings about significant increases (to ~800%) in both mRNA and protein levels of B lymphocyte-induced maturation protein-1 (Blimp-1), the gene of which is the master gene for plasma cell differentiation [42]. In addition, both transcription and protein synthesis of Blimp-1 are remarkably down-regulated (to ~20%) by the re-expression of EBF1. Chromatin immunoprecipitation (ChIP) assay revealed that EBF1 binds to proximal 5'-upstream regions around two putative EBF1 binding motifs of the gene *in vivo*. These results suggest that EBF1 participates



**Fig. 4. EBF1 inhibits transcription of Blimp-1.** The EBF1-deficiency causes dramatic up-regulation of transcription of Blimp-1, resulting in the accumulation of Blimp-1 protein (to ~800%) in comparison with wild-type DT40 cells.

in the transcriptional regulation of the Blimp-1 gene in immature B cells and thereby plays a key role in B cell differentiation.

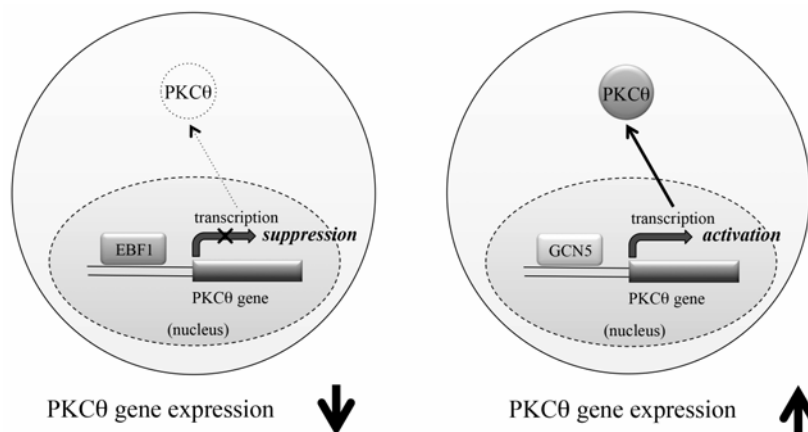
In addition, we revealed that general control non-repressible 5 (GCN5) [43, 44] and EBF1 are involved in the regulation of PKC $\theta$  transcription in the opposite manner in immature B cells (Fig. 5) [45]. The GCN5-deficiency in DT40 causes drastic down-regulation of transcription of PKC $\theta$ . In contrast, The EBF1-deficiency causes remarkable up-regulation of that of PKC $\theta$ , and the re-expression of EBF1 dramatically suppresses transcription of PKC $\theta$ . ChIP assay revealed that GCN5 binds to the 5'-flanking region of the PKC $\theta$  gene and acetylates histone H3, and EBF1 binds to the 5'-flanking region of the gene surrounding putative EBF1 binding motifs.

**Pax5 is involved in the transcriptional regulation of IgM H- and L-chains, chromatin-modifying enzymes (PCAF, HDAC7, HDAC9) and transcription factors (Aiolos, Ikaros, EBF1, E2A, PU.1 and OBF1) in different manners in immature B cells**

Pax5 belongs to the Pax transcription factor family (Pax1 ~ Pax9) and is essential for the development of B cells [46, 47]. Gene knockout studies in mice revealed that B cell development is arrested at the early pro-B cell stage in bone marrow [48]. Moreover, Pax5 plays important roles throughout B cell development from the pro-B to mature B cell stages, and its down-regulation in stimulated B cells promotes the terminal differentiation into plasma cells [46, 47, 49]. And then the Pax5 gene consists of two promoters, two first exons 1A and

1B and nine other exons, resulting in the generation of two types of transcripts by alternative splicing followed by production of two isoform proteins: Pax5A and Pax5B in humans, mice and chicken [50, 51]. Transcription of the former is B cell specific phenomenon, while that of the latter is performed in not only B cells but also other cell types [50]. However, individual roles of Pax5 and the two Pax5 isoforms remain to be elucidated.

To know the physiological functions of Pax5 and its isoforms, we generated Pax5-deficient DT40 mutant cells, Pax5<sup>-</sup>, devoid of the Pax5 gene existing on Z sex chromosome that is monosomy in chickens (USCS Genome Browser data base) [52, 53]. The Pax5<sup>-</sup> cells are obviously distinct from the homozygous Pax5-deficient DT40 mutant cells previously reported by Nera *et al.* [54]. The discrepancy must be due to the fact that the Pax5 homologues exist on other autosomes, but the real reason for this discrepancy is still unsolved. We first analyzed several characteristics of Pax5<sup>-</sup> using some experimental methods including immunoblotting, reverse transcription-polymerase chain reaction (RT-PCR) and so on [52]. The Pax5-deficiency dramatically increases gene expressions of IgM H- and L-chains. Moreover, in Pax5<sup>-</sup>, whole and secreted forms of IgM H-chain mRNA are dramatically elevated, and membrane-bound form of IgM H-chain mRNA and IgM L-chain mRNA are considerably increased. In addition, p300/CBP-associated factor (PCAF) and histone deacetylase-9 (HDAC9) mRNA levels are

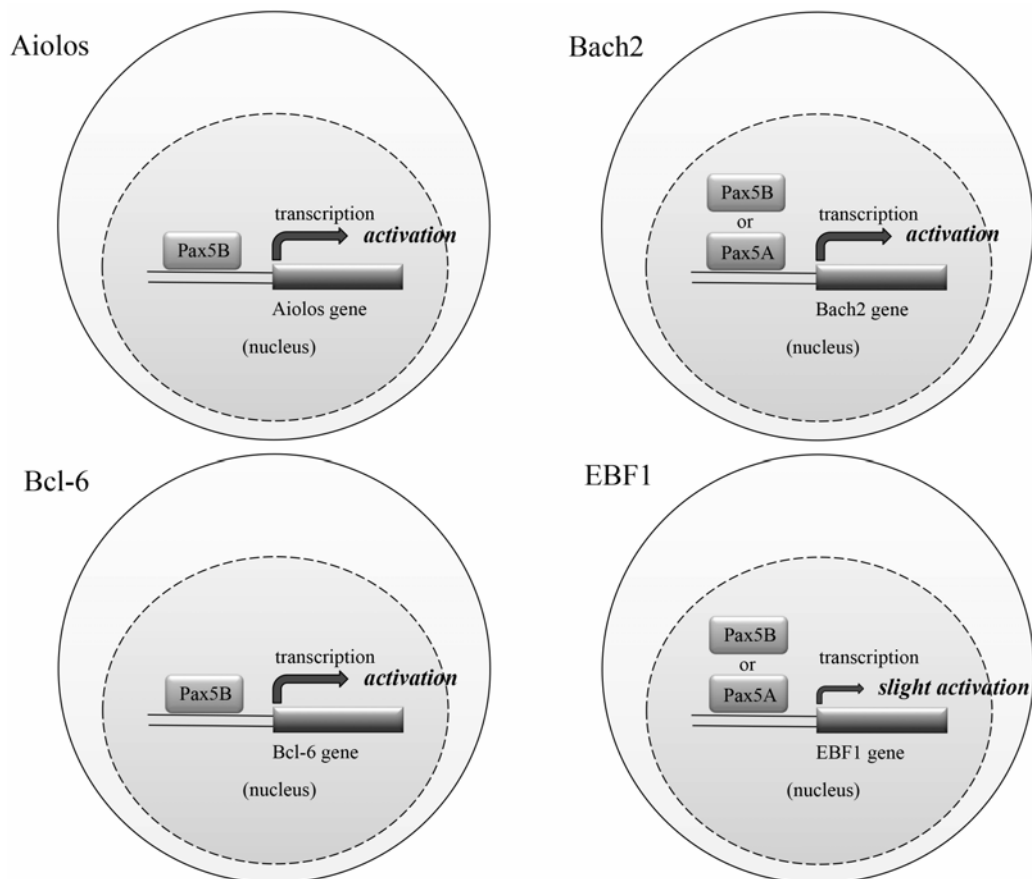


**Fig. 5. EBF1 and GCN5 oppositely regulate transcription of the PKC $\theta$  gene in immature B cells. EBF1 suppresses transcription of PKC $\theta$ , but GCN5 activates it.**

dramatically elevated, and the HDAC7 mRNA level is slightly decreased. Aiolos and OBF1 mRNA levels are obviously decreased, and Ikaros and E2A mRNA levels are slightly elevated. The EBF1 mRNA level is completely decreased, and the PU.1 mRNA level is remarkably reduced.

With regard to these changed genes, there are very interesting and important phenomena on alterations in their gene expression patterns in Pax5<sup>-</sup> during continuous cultivation, although the phenomena are not entirely related to the function of Pax5 [52]. Increased protein levels of IgM H- and L-chains in Pax5<sup>-</sup> are dramatically decreased during cultivation and finally at the later cultivation stage reach comparable levels in DT40 cells. In addition,

PCAF and HDAC9 mRNA levels are gradually elevated during cultivation, and the HDAC7 mRNA level is moderately changed. Aiolos and OBF1 mRNA levels are gradually reduced during cultivation, whereas dramatically elevated Ikaros and E2A mRNA levels are gradually decreased until the later stage. The completely decreased EBF1 mRNA level remains unchanged during cultivation, and the remarkably reduced PU.1 mRNA level is gradually elevated until the later stage. These results, together with our previous findings, revealed that gene expressions of the two immunoglobulin proteins are indirectly regulated by HDAC2 through opposite regulations of gene expressions of Pax5, Aiolos, EBF1, Ikaros and E2A in DT40 cells.



**Fig. 6. Pax5 isoforms are involved in the transcriptional regulation of B cell development-related genes in different manners.** Pax5A and Pax5B remarkably rescue transcription of Bach2. Pax5B certainly recovers transcription of Aiolos, but Pax5A has no effect on it. In addition, Pax5B causes dramatic up-regulation of Bcl-6, and Pax5A shows a slight positive effect on its expression. On the other hand, both Pax5A and Pax5B relatively increase the transcription of EBF1.

### **Pax5 isoforms are involved in transcriptional regulation of B cell development-related genes in different manners**

To understand individual roles of isoforms Pax5A and Pax5B, we carried out re-expression study using cloned cDNAs of Pax5A and Pax5B in Pax5<sup>-</sup> [55]. The resultant transfectants were used for the complementation assay of six B cell development-related genes: activation-induced cytidine deaminase (AID), Aiolos, BTB and CNC homology 2 (Bach2), B cell lymphoma-6 (Bcl-6), EBF1 and OBF1, whose transcriptions are remarkably down-regulated in Pax5<sup>-</sup>. Among them, the transcription of Bach2 is remarkably rescued by Pax5A and Pax5B (to ~130% and ~150%, respectively). Pax5A has no effect on the transcription of Aiolos, while Pax5B certainly recovers it (to ~60%). Pax5B causes dramatic up-regulation of Bcl-6 (to ~120%), whereas Pax5A shows slight positive effects on its expression (to ~35%). Both Pax5A and Pax5B weakly increase the transcription of EBF1 (to ~15%). These results of the four genes (Aiolos, Bach2, Bcl-6 and EBF1) are shown in Fig. 6. Our data obtained in this study may contribute to understanding the role of each Pax5 isoform during B cell development.

### **CONCLUSION**

The acquired immune system of vertebrates is the antigen-specific immune system, which consists of B cells (responsible for humoral immunity) and T cells (responsible for cell immunity). B cells differentiate into plasma cells that produce antibodies. The B cell development requires numerous transcription factors, i.e. EBF1, Pax5, PU.1, E2A, GATA-3, Aiolos, Ikaros, Helios and so on [3, 4]. In addition, the Ig gene expression also requires numerous transcription factors, i.e. USF, TFEB, Ig/EBP, NF-IL6, OCA-b, YY-1, E2A, PU.1 and so on [56-63].

As a first attempt to know respective roles of these transcription factors, using gene-targeting techniques, we have systematically generated five chicken DT40 mutants, devoid of Aiolos, E2A, Helios, EBF1 and Pax5, respectively. Analyses of these resultant mutants revealed physiological roles of the five transcription factors in immature B cells as follows. 1) The Aiolos-deficiency accelerates immature B cell apoptosis mediated by BCR signaling *via* elevation in cytochrome *c* release

from mitochondria to cytosol [24]. 2) Helios is involved in the control of immature B cell functions *via* regulation of protein kinase Cs gene expression [28]. 3) E2A is involved in the regulation of survivin, IAP2 and caspase-8 gene expressions [35]. 4) EBF1 functions as a strong repressor of Blimp-1 gene expression, and EBF1 and GCN5 oppositely regulate PKC $\theta$  gene expression [41, 45]. 5) Protein and mRNA levels of IgM H- and L-chains artificially and excessively accumulated in Pax5-deficient DT40 mutants are rapidly and dramatically reduced through various generations during continuous cultivation [52, 53]. 6) Pax5 isoforms A and B possess distinct functions in the regulation of B cell development-related gene expressions [55].

These results, together with those of DT40 mutants, devoid of remaining transcription factors (will be obtained in the near future), may significantly help in the understanding of the overall picture of the acquired immune system of vertebrates, including development and differentiation of lymphocytes, negative selection of B cells, BCR-mediated apoptosis, O<sub>2</sub><sup>-</sup> generating system and so on. In addition, these results could contribute to resolving the molecular mechanism of auto-immune diseases in the near future.

### **ACKNOWLEDGMENTS**

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### **CONFLICT OF INTEREST STATEMENT**

There are no conflicts of interest.

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