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Developmental expression of EphB6 in the thymus: lessons from EphB6 knockout mice☆

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Abstract

A member of the largest family of receptor protein kinases, EphB6, lacks its intrinsic kinase activity, but it is expressed in normal human tissues. To investigate the physiological function of EphB6, we generated EphB6 deficient mice. EphB6$^{-/-}$ mice developed normally, revealed no abnormality in general appearance, and were fertile. Although a developmental increase of EphB6 in the fetal thymus was confirmed, T-cell development in various lymphoid organs of EphB6$^{-/-}$ mice was comparable to those of EphB6$^{+/+}$. Even in fetal thymus organ cultures, any developmental differences of EphB6$^{-/-}$ and EphB6$^{+/+}$ thymocytes were undetectable. The different binding characteristics to ephrin-Fc proteins between EphB6$^{-/-}$ and EphB6$^{+/+}$ thymocytes demonstrated that ephrin-B2 is the unique ligand for EphB6 among eight known ephrins. While EphB6 was a dominant receptor that binds to ephrin-B2 in adult thymocytes, fetal ones also expressed another EphB that binds to ephrin-B2. Overlapping expression of the EphB subfamily in the fetal thymus might compensate for the loss of EphB6 during the thymic development.

Keywords: EphB6; Ephrin-B2; Gene targeted; Thymus

Receptor protein tyrosine kinases play key roles in cellular proliferation and differentiation in a wide variety of cell types. Eph-family receptors are the largest known family of receptor protein tyrosine kinases, consisting of 15 distinct members. The receptors can be classified into two groups, EphA and EphB receptors, based on the sequence homology. Nine ligands for Eph receptors, so called ephrins, are also divided into two classes. Some are membrane-anchored by a glycosylphosphatidylinositol linkage (ephrin-A) and the others through a transmembrane domain (ephrin-B). Generally, ephrin-A and ephrin-B interact with two corresponding groups of receptors, EphA and EphB, with high affinities, respectively. However, the binding affinity varies with each set of Eph and ephrin. Moreover, some Eph receptors promiscuously interact with another group of ephrins [1,2]. In addition, EphB has been shown to act as a ligand and to transduce signals into cells expressing ephrin [3]. These characteristics of the Eph/ephrin family would make an important functional complexity in vivo.

The biological function of the Eph/ephrin family was initially identified as a regulator of axonal guidance and cell migration in the nervous system [2,4]. The Eph/ephrin family also plays an essential role in angiogenesis and embryogenesis [5,6]. To investigate the physiologi-

☆ *Abbreviations:* FTOC, fetal thymus organ culture.

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cal function of the Eph/ephrin family in vivo, six Eph- and four ephrin-gene-disrupted mice have been successfully generated. In addition to the promiscuous binding profiles of Eph/ephrin members, they have extensive and distinct, but overlapping, expression patterns in the nervous system [1,7]. These characteristics predict that there is a high degree of functional redundancy in Eph/ephrin family. However, most of the Eph/ephrin family gene knockout mice revealed distinct phenotypes, indicating that Eph/ephrin family plays an essential role for embryogenesis, especially in tissue morphonegenesis [5,8–17]. EphA4, EphA8, ephrin-A2, and ephrin-A5 genes play important roles in axonal guidance of corticospinal tract, tectal commissure, retinocollicular, and retinal and vomeronasal axons, respectively [9,11,15,18]. EphB2, EphB3, and ephrin-B3 are also involved in axonal path findings of anterior commisure, corpus callosum, and corticospinal tract axons. EphB4 and ephrin-B2 genes are essential for vascular network assembly in the mouse embryos [5,10,12,13,16]. These knockout mice have demonstrated several novel functions of the Eph/ephrin gene family in vivo.

In general, Eph receptors possess intrinsic tyrosine kinase activities, but EphB6 has unique structural characteristics in the kinase domain [19,20]. EphB6 has substitutions in the six invariant amino acids such as lysine in the ATP-binding site and aspartic acid in the phosphotransfer site of the conserved kinase domain. Therefore, EphB6 has no detectable intrinsic tyrosine kinase activity. However, this kinase null receptor is abundantly expressed in normal human tissues, such as the brain and thymus. In the thymus, EphB6 is expressed in $CD4^+CD8^+$ double-positive thymocytes prominently. EphB6 shows T-cell-specific expression in the normal as well as transformed hematopoietic cells [21]. A candidate ligand for EphB6, ephrin-B2, is also reported to be expressed in the thymus [22]. However, it is still unclear whether there are other ligands for EphB6 than ephrin-B2 or not. Although EphB6 has no intrinsic kinase activity, it can transduce signals into the cell in vitro [23]. Here, we generated EphB6 deficient mice to investigate the physiological roles of EphB6 and analyzed the effect of the gene destruction on T-cell development.

Materials and methods

Construction of the EphB6 gene targeting vector. To isolate the mouse *EphB6* gene, a mouse 129sv genomic library was screened with a human *EphB6* cDNA probe [20]. Four overlapping clones contained an 18-kb genomic region including the murine EphB6 locus. The *Acc*I–*Xba*I 2.0-kb genomic fragment containing a part of exon 1 and exons 2 and 3 was replaced by a PGK-*neo* cassette (Fig. 1). This replacement deleted the methionine of the translation initiation that would have resulted in the total deficit of EphB6 protein. The targeting vector included a 1.4-kb upstream- and a 7-kb downstream-homologous region. It also included a diphtheria toxin A (DTA) fragment cassette at the 5′ end of the short homologous sequence, as described previously [24].

Generation of the EphB6 knockout mice. J1 and RW4 embryonic stem (ES) cells were electroporated with the linearized targeting vector and selected with geneticin on embryonal fibroblast feeder cells generated from the embryos of the cholecystokinin-B receptor knockout mice [24]. In total, 346 J1 and 208 RW4 ES clones resistant to geneticin were screened by Southern blot analysis using 1.1-kb external and 1.1-kb internal probes. Three and eight clones displayed evidence for homologous recombination of the disrupted *EphB6* gene, respectively. Two J1 ES clones were microinjected into blastocysts of C57BL/6J females and the blastocysts were implanted into pseudopregnant female mice. Eight RW4 ES clones were cocultured with zona-free 8-cells to morula-stage embryos. The morula and blastocyst stage embryos were transferred into the recipients on day 2.5 of pseudopregnancy [25]. Finally, both microinjection and coculture methods successfully generated germ-line chimeras. Each chimera was bred to C57BL/B6 or ICR mice to generate heterozygous mutant F1 mice. All mice were bred and maintained in the Experimental Animal Care Unit at Kobe University School of Medicine.

Immunoblot analysis. Thymocytes were lysed in lysis buffer containing 50 mM HEPES (pH 7.4), 1% Triton X-100, 20 mM sodium chloride, 5 mM EDTA, 2 mM sodium fluoride, 0.02% sodium azide, 1 mM phenylmethylsulfonyl fluoride, 16.5 mg/ml aprotinin, 1.0 mg/ml leupeptin, and 1.25 mg/ml pepstatin. Total cell lysates were clarified by centrifugation at 14,000*g* for 30 min at 4 °C. Proteins were separated on a 7% polyacrylamide gel and transferred onto Immobilon (Millipore, Bedford, MA) filters. Membranes were incubated for 2 h at room temperature in blotto (2% non-fat dried milk, 1% Triton X-100, 50 mM Tris–HCl, pH 7.5, 10 mM EDTA, and 0.01% sodium azide). Membranes were washed and then incubated for 2 h at room temperature with anti-EphB6 monoclonal antibody, followed by 1 h at room temperature with rabbit anti-rat IgG [21]. Signals were visualized after treatment with ^{125}I-protein A (Amersham Biosciences, NJ).

In situ hybridization. In situ hybridization was performed as described [26]. Briefly, embryos were fixed in 4% paraformaldehyde. Serial sections, 5–7 μm thick, were mounted on gelatinized slides. The cRNA probe corresponding to nucleotides 551–1116 of mouse *EphB6* cDNA (GenBank #007680) was synthesized according to manufacturer's conditions (Ambion) and labelled with ^{35}S-UTP (>1000 Ci/mmol; Amersham Biosciences). Sections were hybridized with ^{35}S-labelled cRNA probe. The tissue was subjected to stringent washing at 65 °C in 50% formamide, 2× SSC, and 10 mM DTT and washed in PBS before treatment with 20 μg/ml RNase A at 37 °C for 30 min. Following washes in 2× SSC and 0.1× SSC for 10 min at 37 °C, the slides were dehydrated and dipped in emulsion. And then the slides were exposed for 2–3 weeks in light-tight boxes at 4 °C.

Flow cytometric analysis. Cell suspensions were prepared by mincing organs and then straining them through fine nylon wool to retain cell clumps. Cells were then washed with PBS by centrifugation at 250*g* for 10 min. Cells were incubated with the optimal concentration of monoclonal antibodies. Fluorescein isothiocyanate (FITC)-conjugated anti-murine CD4 and phycoerythrin (PE)-conjugated anti-murine CD8 were purchased from Caltag Laboratories. For analysis on fusion protein binding, cells were first incubated in 50 μl of 10% normal rabbit serum for 15 min to block non-specific binding of fusion protein, and then incubated with 2.0 μg/ml of eight known mouse ephrin extracellular domains fused to human IgG1Fc (ephrin-A1, A2, A3, A4, A5, B1, B2, and B3-Fc) (R&D Systems, MN) for 20 min at room temperature. As a negative control, human IgG1 was used instead of ephrin-A or B-Fc. The cells were washed and stained with a PE-conjugated $F(ab')_2$ fragment of goat anti-human IgG Fc (Immunotech, Marseilles, France). Analyses were performed on a Becton–Dickinson FACScan flow cytometer.

Fetal thymus organ culture. Fetal thymus organ cultures (FTOC) were done as described previously [27]. Briefly, the thymic lobes of an

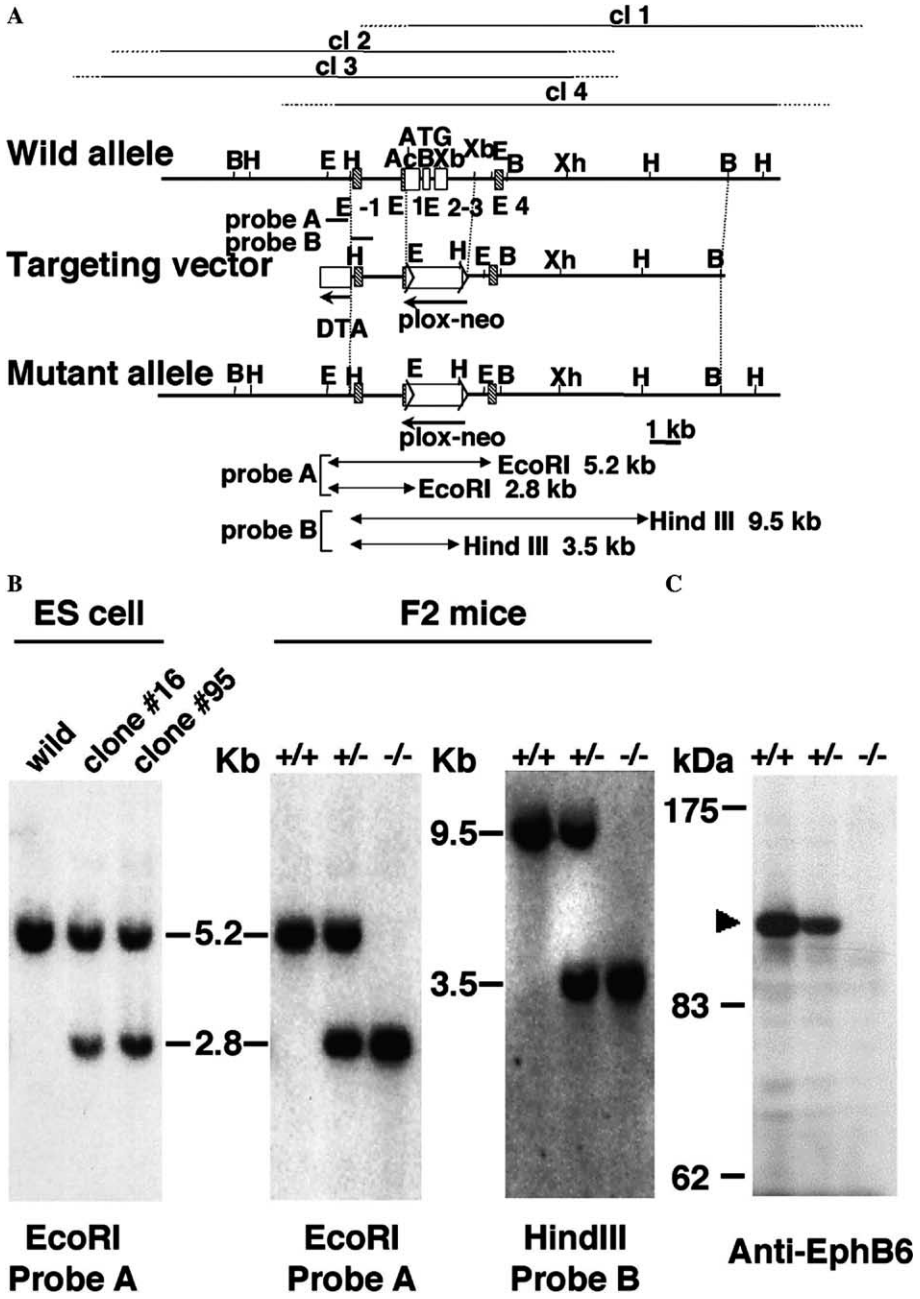

Fig. 1. Disruption of the *EphB6* gene. (A) The targeting vector was designed to replace the *Acc*I-*Xba*I fragment of the mouse *EphB6* gene (wild allele) with a PGK-neo cassette. The predicted mutant allele by homologous recombination is shown as mutant allele. Cross-hatched boxes indicate exons without replacement. Restriction sites indicated are *Acc*I (Ac), *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Xba*I (Xb), and *Xho*I (Xh). (B) Southern blot analysis of homologous recombinant clones and offspring obtained by a heterozygous cross. High molecular weight DNAs of ES cells (wild clone; recombinant clones, clone #16 and clone #95) and F2 wild-type (+/+), heterozygous (+/-), and homozygous (-/-) mice were digested with *Eco*RI and *Hin*dIII Each digest was hybridized with 5′ external probe A or 3′ internal probe B. The positions of the bands corresponding to the wild allele (5.2 kb) and the mutant allele (2.8 kb) are indicated. The probe B confirmed a single integration of the targeting vector in the genomic DNA. (C) The expression of EphB6 receptor protein in the thymus of each genotype. The arrowhead indicates a 135-kDa EphB6 protein.

embryonic day 15.5 (E15.5) were organ cultured on the surface of Nucleopore filters (0.8 μm pore size, Costar, MA) supported on collagen hemostatic sponges (Integra Life Sciences, NJ) in a 24-well plate containing 0.5 ml RPMI1640 medium supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1× non-essential amino acids (Invitrogen, Netherlands) for 5 days at 37 °C under 5% CO_2 in the air.

Results and discussion

Targeting of the EphB6 gene

The mouse *EphB6* locus was disrupted by homologous recombination in ES cells. According to the strategy

shown in Fig. 1A, a part of exon 1 and exons 2 and 3 were replaced by a PGK-neo cassette. The homologous recombination of ES cells were confirmed by Southern blot analysis (Fig. 1B). Eleven independent ES clones were obtained in which the EphB6 gene was inactivated. Such ES clones were used to generate mice with a germline mutation and two independent ES clones generated several germ-line chimeras.

A genotype analysis of live-born progeny obtained from crosses between C57BL/6J background mice heterozygous for the *EphB6* (EphB6$^{+/-}$) revealed the distribution that follows the Mendelian rule (Table 1). To further confirm that the gene had been completely disrupted, immunoblot analysis was performed. The lysates of adult thymocytes were used, since they showed strong expression of EphB6 [21]. There was a complete absence of 135-kDa full-length as well as any truncated EphB6 proteins in homozygous mutant mice (Fig. 1C).

Mice homozygous for the disrupted *EphB6* gene allele do not show any overt outwardly anatomical phenotype. Although EphB6 was expressed in various tissues including testes [19], EphB6$^{-/-}$ mice were fertile and their crosses generated litter sizes that were comparable to those of wild-type matings (Table 2). The oldest EphB6$^{-/-}$ mice have been alive more than 24 months without any obvious abnormalities in their general appearance compared with their EphB6$^{+/+}$ littermates. The same results were obtained in EphB6$^{-/-}$ ICR mice derived from an independent ES clone. Eph/ephrin family is concerned with tumorigenesis [28]. Recently, EphB6 was reported to suppress malignant phenotypes of unfavorable neuroblastoma [29]. However, we did not macroscopically observe any malignancies in EphB6$^{-/-}$ mice maintained up to 24 months of age.

Characterization of lymphoid organs in EphB6$^{-/-}$ adult mice

Human peripheral CD4-positive lymphocytes as well as human T-cell-derived leukemia/lymphoma cells express EphB6. Thus, we first analyzed lymphoid tissues of the adult (8–15-week-old) C57BL/6 EphB6$^{-/-}$ mice. White blood cell counts in peripheral blood and the percentage of lymphocytes were not altered, compared with those of the wild-type mice. The total numbers of thymocytes were also comparable in both mutant and wild-type mice (Table 3).

Flow cytometric analysis was performed to study CD4–CD8 profiles of lymphatic tissues. There were no statistically significant changes in CD4–CD8 profiles of lymphocytes derived from peripheral blood and lymph nodes, and of thymocytes and splenocytes. Moreover, there were no changes in the intensity of CD4 and CD8 staining in the lymphocytes of any lymphoid tissues examined here (Table 4).

Developmental expression of EphB6 in the thymus

We previously reported that EphB6 is abundantly expressed in CD4$^+$CD8$^+$ double-positive thymocytes in 8-week-old mice [21]. The third pharyngeal pouch begins

Table 1
Number of F2 offspring from heterozygous crossing

Genotype	Total no.	Ratio		Females (%)
		Expected	Actual	
+/+	53	1	1.02	47
+/−	103	2	1.98	50
−/−	51	1	0.98	49

Table 2
Number of offspring from matings of EphB6 mutant mice and control animals

Genotype of parents	Average of litter size	No. of litters
+/+	7	11
+/−	7	12
−/−	8	11

Table 3
Parameters of lymphoid tissues of wild-type and EphB6$^{-/-}$ mice

Parameters	Mean value (±SD) for each genotype	
	+/+ (No.[a])	−/− (No.)
Blood		
Leukocytes (10^6/ml)	2.5 ± 1.2 (27)	2.3 ± 1.2 (22)
Granulocytes (%)	15.4 ± 11.5 (27)	20.0 ± 17.7 (22)
Lymphocytes (%)	84.4 ± 11.5 (27)	79.9 ± 17.6 (22)
Erythrocytes	9.9 ± 0.51 (27)	10.0 ± 0.52 (22)
Spleen		
Weight (mg)	82.4 ± 14.8 (22)	76.7 ± 17.6 (21)
Thymus		
Total cell number (10^7)	13.2 ± 4.0 (17)	13.6 ± 4.1 (18)

[a] The number of mice analyzed.

Table 4
CD4-8 profiles of wild-type and and EphB6$^{-/-}$ mice

Parameters	Mean value ± SD for each genotype	
	+/+ (No.[a])	−/− (No.)
Blood		
CD4 (%)	19.5 ± 9.1 (19)	22.0 ± 7.1 (20)
CD8 (%)	8.6 ± 3.2 (19)	11.0 ± 4.0 (20)
Thymus		
DP[b] (%)	85.8 ± 2.5 (23)	84.9 ± 2.1 (25)
CD4 (%)	8.5 ± 2.2 (23)	8.9 ± 1.4 (25)
CD8 (%)	1.64 ± 0.48 (23)	1.8 ± 0.32 (25)
Spleen		
CD4 (%)	23.0 ± 3.1 (17)	23.4 ± 3.0 (18)
CD8 (%)	11.4 ± 1.4 (17)	13.7 ± 2.4 (18)
Lymph node		
CD4 (%)	40.3 ± 4.3 (18)	39.2 ± 4.4 (17)
CD8 (%)	18.3 ± 3.4 (18)	20.7 ± 3.7 (17)

[a] The number of mice analyzed.
[b] DP, CD4$^+$CD8$^+$ double-positive.

to produce the thymus around E12.5. At E14.5, the thymus is easily recognizable. Thus, the developmental expression of EphB6 in the fetal thymus was determined by using a monoclonal antibody specific for EphB6. We could detect a weak expression of EphB6 in the E14.5 thymus. The expression of EphB6 increased during embryonic maturation and reached the highest level at the E17.5 and E18.5 stages (Fig. 2A). The developmental increase of EphB6 expression was consistent with the increase of $CD4^+CD8^+$ double-positive thymocytes in the developing embryonic thymus. The percentage of $CD4^+CD8^+$ double-positive thymocytes was 1% greater at E14.5, 14% at E15.5, 49% at E16.5, 87% at E17.5, and 86% at E18.5.

The expression of the *EphB6* gene in the fetal thymus was also confirmed by in situ hybridization analysis. The *EphB6* mRNA expression in the E15.5 thymus was detected with an anti-sense, but not with a sense probe (Fig. 2B).

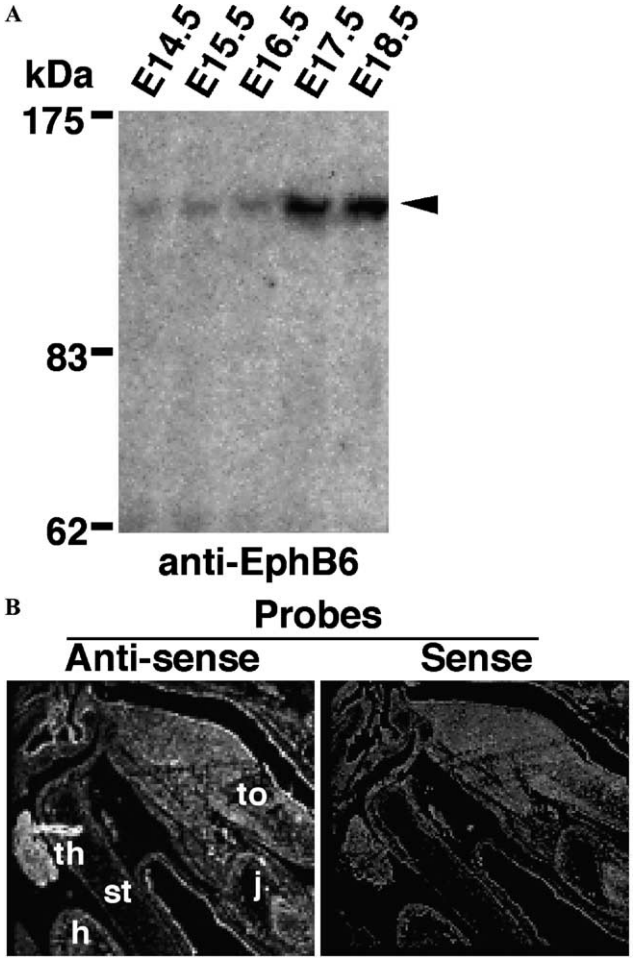

Fig. 2. Developmental increase of EphB6 expression in the thymus. (A) The cell lysates of whole thymus at E14.5, 15.5, 16.5, 17.5, and 18.5 embryos were subjected to an immunoblot analysis using anti-EphB6 specific monoclonal antibody. The arrowhead indicates the 135-kDa EphB6 protein. (B) Specific expression of *EphB6* transcripts in the thymus. A sagittal cryosection through the upper thorax regions at E15.5 mouse embryo was hybridized with an anti-sense *EphB6* probe (left panel) or a control sense probe (right panel). h, heart; j, jaw; st, sternum; th, thymus; to, tongue.

Redundant expressions of EphB subfamily in fetal thymocytes

The expression of ephrin-B2, which is a candidate ligand for EphB6, has been reported in the thymus [30]. However, it remains to be clarified whether other ephrins including ephrin-A could bind to EphB6 or not. Ephrin-B2 binds to all EphB except EphB5 with various affinities [2]. Thus, we first investigated the binding profiles of eight known ephrin-A and ephrin-B to the thymocytes derived from 8-week-old $EphB6^{-/-}$ and $EphB6^{+/+}$ mice. Among eight ephrin-Fc, ephrin-A1-Fc, -A2-Fc, -A3-Fc, -B1-Fc, -B2-Fc, and -B3-Fc could bind to the wild-type thymocytes. The binding of ephrin-A4-Fc or -A5-Fc to both $EphB6^{-/-}$ and $EphB6^{+/+}$ thymocytes was observed as scarcely as that of control human IgG. While ephrin-A1-Fc, -A2-Fc, -A3-Fc, -B1-Fc, and -B3-Fc bound to both $EphB6^{-/-}$ and $EphB6^{+/+}$ thymocytes constantly, the specific binding of ephrin-B2-Fc to wild-type thymocytes was almost abolished in 8-week-old $EphB6^{-/-}$ thymocytes (Figs. 3A and B). The binding profiles of ephrin-B2-

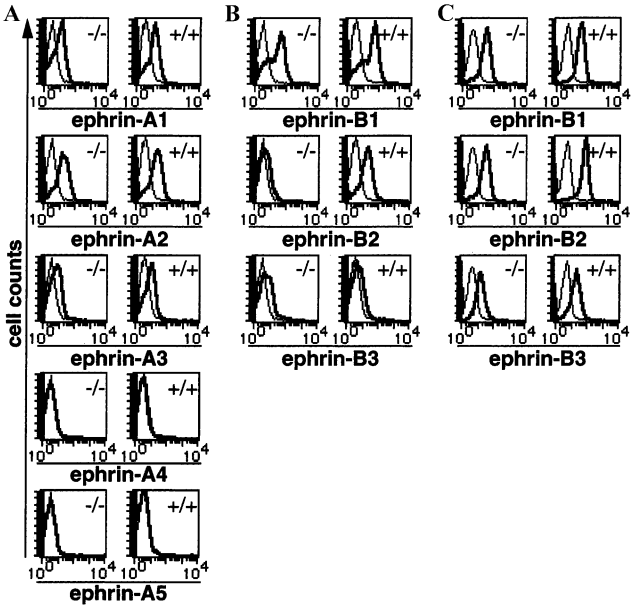

Fig. 3. Binding profiles of ephrin-Fc fusion proteins to $EphB6^{-/-}$ thymocytes. Thymocytes of week-old $EphB6^{-/-}$ mice (left panel) and $EphB6^{+/+}$ mice (right panel) were incubated with ephrin-A-Fc (A) or ephrin-B-Fc (B), followed by staining with PE-conjugated anti-human IgG antibody (solid line). As a negative control (thin line), thymocytes were incubated with human IgG instead of ephrin-Fc. (C) Fetal thymocytes at E15.5 were incubated with ephrin-B2-Fc, followed by staining with PE-conjugated anti-human IgG antibody (solid line). As a negative control (thin line), thymocytes were incubated with human IgG instead of ephrin-Fc. Comparable binding of ephrin-B2-Fc to both $EphB6^{-/-}$ (left panel) and $EphB6^{+/+}$ (right panel) thymocytes at E15.5 are shown. Results are representative of three or more independent experiments.

Fc to EphB6$^{-/-}$ and EphB6$^{+/+}$ thymocytes in neonate (2 days after birth) were the same as those of adult mice (data not shown).

Next, we compared the ephrin-B2-Fc binding abilities between EphB6$^{-/-}$ and EphB6$^{+/+}$ fetal thymocytes. At E15.5, ephrin-B2-Fc showed significant binding to both EphB6$^{-/-}$ and EphB6$^{+/+}$ thymocytes. Fetal thymocytes derived from both genotypes could also bind to ephrin-B1. However, the binding capacity for ephrin-B1 was lower than that for ephrin-B2 in the fetal ones. The binding of ephrin-B3 to both EphB6$^{-/}$ and EphB6$^{+/+}$ fetal thymocytes was also detected. The binding of ephrin-B3 to fetal thymocytes is the weakest among three ephrin-Bs. These results clearly demonstrated that only ephrin-B2 is the high affinity ligand for EphB6 among eight known ephrins. They further indicate that EphB6 is the dominant EphB receptor that binds to ephrin-B2 in the adult thymocytes.

Present studies have shown that not only EphB6 but also another EphB receptor that binds to ephrin-B2 with a higher affinity than to ephrin-B1 or ephrin-B3 is expressed in the embryonic thymus at the protein level. Ephrin-B2 has been shown to interact at a high affinity with EphB1, B2, B3, and B4, besides EphB6 [31]. Ephrin-B1 also binds to EphB1, B2, and B3, but the binding affinity of ephrin-B1 to EphB4 was much lower than that of ephrin-B2 to EphB4 [2,32]. Ephrin-B3 is able to bind to EphB2 and B3 at least, but not to EphB4 [6,31]. It is still controversial whether ephrin-B3 binds to EphB1 or not. Among the *EphB* genes, *EphB3* and *EphB4* mRNAs have been reported to express in the mouse embryonic thymus [33]. Considering the differences in their binding affinities of each set of EphB and ephrin-B, the most likely EphB protein expressed abundantly in the developing mouse thymus would be EphB4. However, all the EphB subfamily appeared to be expressed in the embryonic thymus. The identification of EphB isoforms expressed in the fetal thymus would be essential to explore the functional role of EphB6 in the thymic development.

Among the *EphA* genes, the *EphA1* and *EphA2* mRNAs have been shown to be expressed in the adult mouse thymus, [28,34]. A high expression level of *EphA2* mRNA was also shown by in situ hybridization in the fetal mouse thymus [35]. However, there is no report about expression of EphA in the mouse thymus at protein level. Ephrin-A1 interacts with ephA1 to A7 with different affinities [7,36]. Ephrin-A2 and -A3 interact at high affinity with EphA3, A5, A6, A7, and A8 [7,37–39]. Ephrin-A5 binds to EphA3, A4, A5, and A8 with high affinity [37,38]. There is no report about the expression of EphA6 or EphA7 in the thymus. Taken together with these reports, our finding that ephrin-A1, -A2, and -A3, but not -A4 and -A5 bound to adult thymocytes suggests that EphA6 and A7 might also be expressed on the adult mouse thymocytes.

In conclusion, our results clearly showed that the adult mouse thymus expressed not only EphB6 but also EphA interacting to ephrin-A2 with a high affinity at the protein level. Mouse thymocytes could bind to both ephrin-A2 and ephrin-B2. However, there would be no functional redundancy between EphA and EphB6, because the latter binds only to ephrin-B2 with a high affinity among all known ephrins.

Normal CD4$^+$/CD8$^+$ differentiation in FTOC

To study the role of EphB6 for T-cell development during embryogenesis, we also performed fetal thymus organ cultures (FTOC). After 5 days of culture, the percentages of CD4$^-$CD8$^-$ double-negative, CD4$^+$CD8$^+$ double-positive, CD4$^+$ single-positive, and CD8$^+$ single-positive thymocytes in the cultured thymic lobes derived from EphB6$^{-/-}$ mice were comparable with those from EphB6$^{+/+}$ mice (Fig. 4). Conclusively, we could not detect any abnormalities in EphB6$^{-/-}$ thymocyte differentiation in vitro. In addition to no remarkable phenotypes in the lymphoid tissues of EphB$^{-/-}$ mice, the in vitro results also raise a possibility that other EphB expressed in the fetal thymus might functionally compensate for the loss of *EphB6* in vivo.

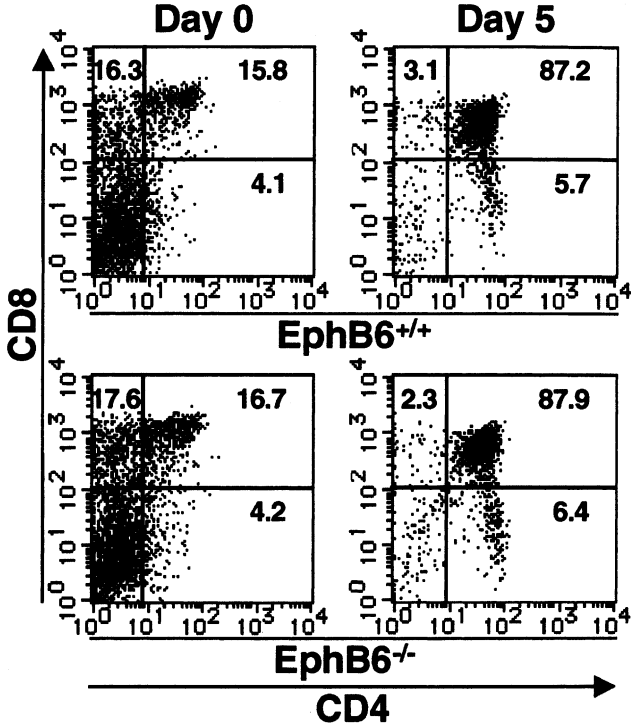

Fig. 4. Development of CD4$^+$/CD8$^+$ thymocytes in FTOC. Thymus lobes from EphB6$^{+/+}$ and EphB6$^{-/-}$ littermate embryos at E15.5 were cultured for 5 days. Thymocytes obtained from fresh or cultured thymus lobes were stained with FITC-conjugated anti-CD4 antibody and PE-conjugated anti-CD8 antibody. The results of the two-color flow cytometric analyses are shown. The percentage of each cell population is indicated in each panel. Results are representative of three or more independent experiments.

The thymus had been reported to express 10 of the 15 *Eph* genes [19,28,33,34,40,41]. In *EphA2* knockout mice, any discernable phenotype was not exhibited in histological sections of the thymus [8]. Moreover, any phenotypic changes in the thymus have not been referred in other *Eph*/*ephrin*-gene knockout mice. Generations of double knockout mice lacking EphB6 and other Eph or ephrin would be necessary to avoid the functional redundancy of Eph/ephrin family in T-cell development.

Further analysis of EphB6 targeted mice will be helpful to investigate the function of *Eph*-family receptors not only in the regulation of T-cell differentiation, but also in the central nervous system where EphB6 is expressed as strongly as in the thymus. The availability of EphB6$^{-/-}$ mice could facilitate exploration of novel functions of this kinase-defective receptor.

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