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# Establishment of a highly sensitive and specific anti-EphB2 monoclonal antibody ($Eb_2$Mab-12) for flow cytometry

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## Abstract

Ephrin type-B receptor 2 (EphB2) is a member of the Eph family tyrosine kinase receptors. EphB2 binds to ephrin-B1, ephrin-B2, and ephrin-B3, which are critical regulators of vascular and neural development, influencing cell migration and axon guidance. EphB2 is overexpressed in several tumors, including glioma, breast cancer, hepatocellular carcinoma, and malignant mesothelioma, where it functions as a tumor promoter. Therefore, the development of monoclonal antibodies (mAbs) targeting EphB2 is essential for the diagnosis and treatment of EphB2-positive tumors. In this study, we developed novel mAbs for human EphB2 using the Cell-Based Immunization and Screening method. Among the established anti-EphB2 mAbs, $Eb_2$Mab-12 (mouse $IgG_1$, kappa) showed reactivity toward EphB2-overexpressed Chinese hamster ovary-K1 cells (CHO/EphB2) and an endogenously EphB2-expressing cancer cell line (LS174T), as confirmed by flow cytometry. The dissociation constant ($K_D$) values of $Eb_2$Mab-12 for CHO/EphB2 and LS174T were determined to be $1.7 \times 10^{-9}$ M and $4.4 \times 10^{-10}$ M, respectively, using flow cytometry. Furthermore, $Eb_2$Mab-12 exhibited no cross-reactivity with other members of the EphA and EphB receptors. These results indicate that $Eb_2$Mab-12 possesses high affinity and specificity in detecting EphB2, suggesting its potential application in tumor therapy.

***Keywords:*** EphB2; Monoclonal antibody; Cell-Based Immunization and Screening; Flow cytometry

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## 1. Introduction

The mammalian ephrin and Eph system comprises eight cell surface ephrin ligands (five ephrin-As and three ephrin-Bs) and 14 receptor tyrosine kinases (nine EphA and five EphB receptors).[1-6] The interaction between Eph receptors and ephrins occurs through dimerization or oligomerization, which leads to the tyrosine phosphorylation of Eph receptor and ephrin-B.[7] The phosphorylated tyrosine recruits cytoplasmic effectors containing Src-homology 2 domains, phosphotyrosine-binding domains, and PDZ domains.[8] Consequently, the Eph receptor-ephrin complexes activate bidirectional signaling – forward signaling from the Eph receptors and reverse signaling from the ephrins – which is essential for intercellular communication among similar or different

cell types.[2,6,9] Through this bidirectional signaling, the Eph system regulates tissue development, homeostasis, and regeneration; dysregulation of this system is implicated in various diseases, including cancer.[3,4,10-21] Hence, monoclonal antibody (mAb)-based tumor therapies have been developed for certain Eph receptors.[10,22-29]

Dysregulation of the Eph system is observed in both tumor cells and tumor microenvironment.[19] The Eph system plays distinct roles in tumor development, functioning as both tumor promoters and suppressors depending on the cellular context.[19] Ephrin type-B receptor 2 (EphB2) is overexpressed in several tumors, such as glioblastoma,[30] breast cancer,[31] hepatocellular carcinoma,[32] and malignant mesothelioma,[21] correlating with poor clinical outcomes. In these tumors, EphB2 promotes migration and invasion through forward signaling.[33,34]

In contrast, the expression of EphB2 is downregulated in certain tumors, such as colorectal cancer.[35] In the intestinal epithelium, EphB receptors facilitate the proliferation of stem and progenitor cells.[36] Notably, intestinal epithelial cell migration is impaired in mice lacking EphB2 and EphB3. Without EphB signaling, there is approximately a 50% reduction in the number of proliferating cells.[36] Furthermore, EphB receptor expression is elevated in intestinal adenomas,[37] but functions as a tumor suppressor by inhibiting invasive growth. EphB signaling promotes adherens junction formation in colorectal cancer cells, thereby suppressing cancer progression by inhibiting invasive growth.[38] Loss of EphB2 expression occurs during the progression to carcinoma and initiation of invasive growth.[39]

To evaluate the expression of EphB2 and target EphB2-positive cancer cells, the development of mAbs against EphB2 is essential. Previous studies have developed anti-receptor tyrosine kinase mAbs against the human epidermal growth factor receptor (EGFR) (clone EMab-17),[40] HER2 ($H_2$Mab-19),[41] and HER3 ($H_3$Mab-17)[42] using the Cell-Based Immunization and Screening (CBIS) method. The CBIS method involves immunizing antigen-overexpressed cells followed by high-throughput hybridoma screening using flow cytometry. This study reports the development of the novel anti-EphB2 mAbs using the CBIS method.

## 2. Materials and methods

### 2.1. Antibodies

OptiBuild™ RB545 mouse anti-human EphB2 mAb (clone 2H9; mouse $IgG_1$, kappa) was purchased from BD Bioscience (USA). Alexa Fluor 488-conjugated anti-mouse IgG was purchased from Cell Signaling Technology, Inc. (USA).

### 2.2. Preparation of cell lines

LS174T (human colorectal cancer), LN229 (human glioblastoma), Chinese hamster ovary (CHO)-K1, and P3X63Ag8U.1 (P3U1) cell lines were obtained from the American Type Culture Collection (USA).

pCMV6-myc-DDK vector with EphB2 (Catalog No.: RC223882, Accession No.: NM_004442) was purchased from OriGene Technologies, Inc. (USA). The EphB2 plasmids were transfected into CHO-K1 and LN229 cells using a Neon transfection system (Thermo Fisher Scientific Inc., USA). Stable transfectants were established through cell sorting using the 2H9 conjugated with RB545 and a cell sorter (SH800; Sony Corp., Japan), followed by cultivation in a medium containing 0.5 mg/mL of G418 (Nacalai Tesque, Inc., Japan).

Other Eph receptor cDNAs, including EphA1 (Catalog No.: RC213689, Accession No.: NM_005232), EphA4 (Catalog No.: RC211230, Accession No.: NM_004438), EphA5 (Catalog No.: RC213206, Accession No.: NM_004439), EphA6 (Catalog No.: RC223510, Accession No.: NM_001080448), EphA7 (Catalog No.: RC226293, Accession No.: NM_004440), EphA8 (Catalog No.: RC220352, Accession No.: NM_020526), EphA10 (Catalog No.: RC218374, Accession No.: NM_001099439), EphB1 (Catalog No.: RC214301, Accession No.: NM_004441), and EphB6 (Catalog No.: RC229404, Accession No.: NM_004445), were purchased from OriGene Technologies, Inc. (USA). EphA2 (Catalog No.: HGY095959, Accession No.: NM_004431), EphA3 (Catalog No.: HGY053437, Accession No.: NM_005233), and EphB3 (Catalog No.: HGX039581, Accession No.: NM_004443) cDNAs were purchased from RIKEN DNA Bank (Japan).

EphA2 and EphB3 cDNAs were cloned into a pCAGzeo vector (FUJIFILM Wako Pure Chemical Corporation, Japan), EphB6 cDNA was cloned into a pCMV6 vector, EphA1 cDNA was cloned into a pCAGzeo-ssnPA vector, while EphA3, EphA4, EphA5, EphA6, EphA7, EphA8, EphA10, and EphB1 cDNAs were cloned into a pCAGzeo_ssnPA16 vector.

The plasmids were transfected into CHO-K1 cells and stable transfectants were established through staining with mAbs: an anti-EphA2 mAb (clone SHM16; BioLegend, USA), an anti-EphB3 mAb (clone 647354; R&D Systems Inc., USA), an anti-EphB6 mAb (clone T49-25; BioLegend, USA), and an anti-PA tag mAb (clone NZ-1 for EphA2, EphA3, EphA4, EphA5, EphA6, EphA7, EphA8, EphA10, and EphB1), followed by sorting using SH800. After sorting, the cells were cultivated in a medium containing 0.5 mg/mL of Zeocin (InvivoGen, USA) or 0.5 mg/mL of

G418. This process resulted in the establishment of the Eph receptors-overexpressed CHO-K1 (e.g., CHO/EphB2) clones. In addition, the CHO/PA16-EphB4 cell line had been previously established.[43]

CHO-K1 cells, Eph receptors-overexpressed CHO-K1 cells, and P3U1 cells were cultured in a Roswell Park Memorial Institute (RPMI)-1640 medium (Nacalai Tesque, Inc., Japan), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific Inc., USA), 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B (Nacalai Tesque, Inc., Japan). LS174T, LN229, and EphB2-overexpressed LN229 (LN229/EphB2) cells were cultured in Dulbecco's Modified Eagle Medium (Nacalai Tesque, Inc., Japan), supplemented with 10% FBS, 100 units/mL of penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. All cells were grown in a humidified incubator at 37°C with an atmosphere of 5% $CO_2$ and 95% air.

### 2.3. Development of hybridomas

The animals were housed under specific pathogen-free conditions. All animal experiments were approved by the Animal Care and Use Committee of Tohoku University (Permit number: 2022MdA-001).

Two 5-week-old BALB/cAJcl mice were purchased from CLEA Japan (Japan) to develop mAbs against EphB2. The immunization protocol involved intraperitoneal administration of LN229/EphB2 ($1 \times 10^8$ cells) mixed with 2% Alhydrogel adjuvant (InvivoGen, USA). This initial immunization was followed by 3 additional weekly injections ($1 \times 10^8$ cells/mouse), culminating in a final booster intraperitoneal injection ($1 \times 10^8$ cells/mouse) 2 days before harvesting spleen cells. The harvested spleen cells were subsequently fused with P3U1 cells using PEG1500 (Roche Diagnostics, USA). Hybridomas were cultured in the RPMI-1640 medium with 10% FBS, 5% BriClone (NICB, Ireland), 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B. For hybridoma selection, hypoxanthine, aminopterin, and thymidine (Thermo Fisher Scientific Inc., USA) were added to the medium. The supernatants were subsequently screened using flow cytometry with CHO/EphB2 and CHO-K1 cells.

The cultured supernatant of $Eb_2$Mab hybridomas was then applied to 1 mL of Ab-Capcher (ProteNova, Japan). After washing with phosphate-buffered saline (PBS), the antibodies were eluted with an IgG elution buffer (Thermo Fisher Scientific Inc., USA). Finally, the eluates were concentrated, and the elution buffer was replaced with PBS using Amicon Ultra (Merck KGaA, Germany).

### 2.4. Flow cytometric analysis

Cells were harvested following brief exposure to 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (Nacalai Tesque, Inc., Japan). After trypsinization, the cells were washed with 0.1% bovine serum albumin in PBS (blocking buffer) and treated with 0.01, 0.1, 1, and 10 μg/mL of primary mAbs for 30 min at 4°C. Subsequently, the cells were treated with Alexa Fluor 488-conjugated anti-mouse IgG (1:2,000). In addition, the cells were suspended in 0.01, 0.1, 1, and 10 μg/mL concentrations of the 2H9. Fluorescence data were collected using the SA3800 Cell Analyzer (Sony Corp). Cells were gated on the dot plot based on side scatter and forward scatter, and the fluorescence intensity was determined using FlowJo software (BD Biosciences, USA).

### 2.5. Determination of dissociation constant ($K_D$) by flow cytometry

CHO/EphB2 and LS174T cells were suspended in serially-diluted $Eb_2$Mabs for 30 min at 4°C. The cells were treated with Alexa Fluor 488-conjugated anti-mouse IgG (1:200). The cells were suspended in serially diluted 2H9 conjugated with RB545. Fluorescence data were collected using the BD FACSLyric (BD Biosciences, USA). The $K_D$ was calculated by fitting saturation binding curves to the built-in one-site binding models in GraphPad PRISM 6 (GraphPad Software, USA).

## 3. Results

### 3.1. Development of anti-EphB2 mAbs using the CBIS method

Mice were immunized with LN229/EphB2 cells to develop anti-EphB2 mAb (Figure 1A). The spleen was excised, and splenocytes were fused with myeloma P3U1 cells (Figure 1B). The developed hybridomas were subsequently seeded into ten 96-well plates and cultivated for 6 days. The positive wells were screened by selecting CHO/EphB2-reactive and CHO-K1-non-reactive supernatants using flow cytometry (Figure 1C). A total of 133 positive wells (13.9%) out of 956 wells were obtained. After limiting the dilution of the positive wells and several additional screenings, 12 clones were finally established (Figure 1D).

### 3.2. Flow cytometric analysis using anti-EphB2 mAbs

Eight mouse $IgG_1$ clones ($Eb_2$Mab-1, 2, 3, 4, 7, 8, 10, and 12) were selected, and the mAbs were purified from the supernatants (Table 1). The specificity of $Eb_2$Mabs and 2H9 in 14 Eph receptor tyrosine kinases-expressed CHO-K1 was were investigated. 2H9 exhibited reactivity to not only CHO/EphB2 but also to CHO/EphA4, CHO/EphB1, and

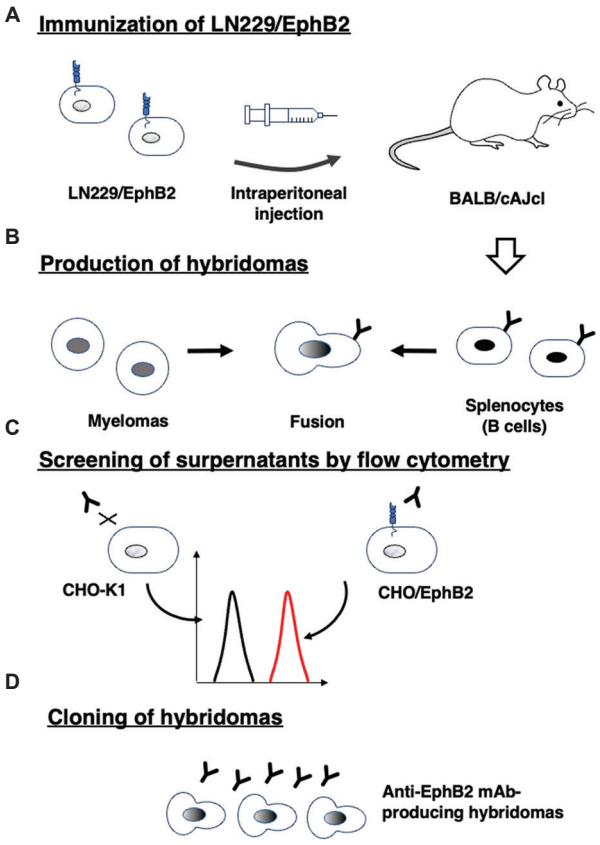


**Figure 1.** The production of anti-EphB2 monoclonal antibodies. (A) LN229/EphB2 cells were immunized into two BALB/cAJcl mice. (B) The spleen cells were fused with P3U1 cells. (C) The supernatant of hybridomas producing anti-EphB2 mAbs was screened through flow cytometry using CHO-K1 and CHO/EphB2 cells. (D) After limiting dilution, the anti-EphB2 mAbs were finally established.
Abbreviations: CHO/EphB2: EphB2-overexpressing Chinese hamster ovary-K1 cells; CHO: Chinese hamster ovary; mAbs: Monoclonal antibodies.

**Table 1. Cross-reactivity and $K_D$ values of $Eb_2$Mabs ($IgG_1$ isotype) in flow cytometry**

| Clones | Isotype | Cross-reactivity | $K_D$ ($\times 10^{-9}$ M) |
|---|---|---|---|
| $Eb_2$Mab-1 | $IgG_1$, kappa | – | 5.9 |
| $Eb_2$Mab-2 | $IgG_1$, kappa | – | 6.4 |
| $Eb_2$Mab-3 | $IgG_1$, kappa | EphA3, EphB1, EphB3 | 1.1 |
| $Eb_2$Mab-4 | $IgG_1$, kappa | – | 3.3 |
| $Eb_2$Mab-7 | $IgG_1$, kappa | – | 5.2 |
| $Eb_2$Mab-8 | $IgG_1$, kappa | EphB3 | 9.5 |
| $Eb_2$Mab-10 | $IgG_1$, kappa | EphB3 | 3.4 |
| $Eb_2$Mab-12 | $IgG_1$, kappa | – | 1.7* |

CHO/EphB2 was used to determine the KD. Cross-reactivity was determined by flow cytometry (Figures S2-S4). $Eb_2$Mab-5 is IgG3. $Eb_2$Mab-6, Eb2Mab-9, and Eb2Mab-11 are IgM. *The data are presented in Figure 4A.
Abbreviations: IgG: Immunoglobin G.

CHO/EphB3 at a concentration of 1 μg/mL (Figure S1). In contrast, $Eb_2$Mab-1, 2, 4, 7, and 12 were recognized as specific to CHO/EphB2, with no cross-reactivity observed even at 10 μg/mL. Conversely, $Eb_2$Mab-3 demonstrated cross-reactivity to EphA3, EphB1, and EphB3. In addition, $Eb_2$Mab-8 and 10 also showed cross-reactivity to EphB3 (Figures S2-4 and Table 1). Flow cytometry was subsequently conducted using the $Eb_2$Mabs and 2H9 against CHO/EphB2, CHO-K1, LN229/EphB2, and LN229 cells. Dose-dependent recognition of CHO/EphB2 cells was observed for the $Eb_2$Mabs at concentrations of 10, 1, 0.1, and 0.01 μg/mL (Figure S5). Among the EphB2-specific $Eb_2$Mabs ($Eb_2$Mab-1, 2, 4, 7, and 12), $Eb_2$Mab-12 showed the high reactivity (Figure 2A). Dose-dependent recognition of CHO/EphB2 cells was also noted for 2H9 at concentrations of 10, 1, 0.1, and 0.01 μg/mL; however, this was less effective compared to $Eb_2$Mab-12 (Figure 2A). Parental CHO-K1 cells were not recognized even at a concentration of 10 μg/mL for either $Eb_2$Mab-12 and 2H9 (Figure 2B). The superior reactivity of $Eb_2$Mab-12 compared to 2H9 was also observed in LN229/EphB2 and LN229 cells (Figure S6). Weak expression of endogenous EphB2 in LN229 had been previously confirmed through quantitative PCR and western blot analyses.[44]

The reactivity of $Eb_2$Mabs and 2H9 against an endogenous EphB2-expressing colorectal cancer cell line, LS174T, was investigated.[45] $Eb_2$Mabs recognized LS174T cells dose-dependently at concentrations of 10, 1, 0.1, and 0.01 μg/mL (Figure S7). Among the $Eb_2$Mabs, $Eb_2$Mab-12 showed the highest reactivity (Figure 3). In contrast, 2H9 was found to react with LS174T cells at concentrations greater than 0.1 μg/mL (Figure 3). These results suggest that $Eb_2$Mab-12 specifically recognizes EphB2 and is effective in detecting endogenous EphB2 through flow cytometry.

### 3.3. Determination of the binding affinity of EphB2 mAbs using flow cytometry

Flow cytometry was conducted, and the geometric mean of the fluorescence intensity was plotted against the concentration of mAbs to determine the $K_D$ values of $Eb_2$Mabs and 2H9. The $K_D$ values of $Eb_2$Mab-12 and 2H9 for CHO/EphB2 were determined as $1.7 \times 10^{-9}$ M and $3.4 \times 10^{-9}$ M, respectively (Figure 4A). While the $K_D$ values for other $Eb_2$Mabs were also determined, $Eb_2$Mab-12 exhibited the highest affinity among the EphB2-specific clones (Table 1). Furthermore, the $K_D$ values of $Eb_2$Mab-12 and 2H9 targeting LS174T were determined to be $4.4 \times 10^{-10}$ M and $1.9 \times 10^{-9}$ M, respectively (Figure 4B). These results indicate that $Eb_2$Mab-12 possesses a superior affinity for both CHO/EphB2 and LS174T compared to 2H9.

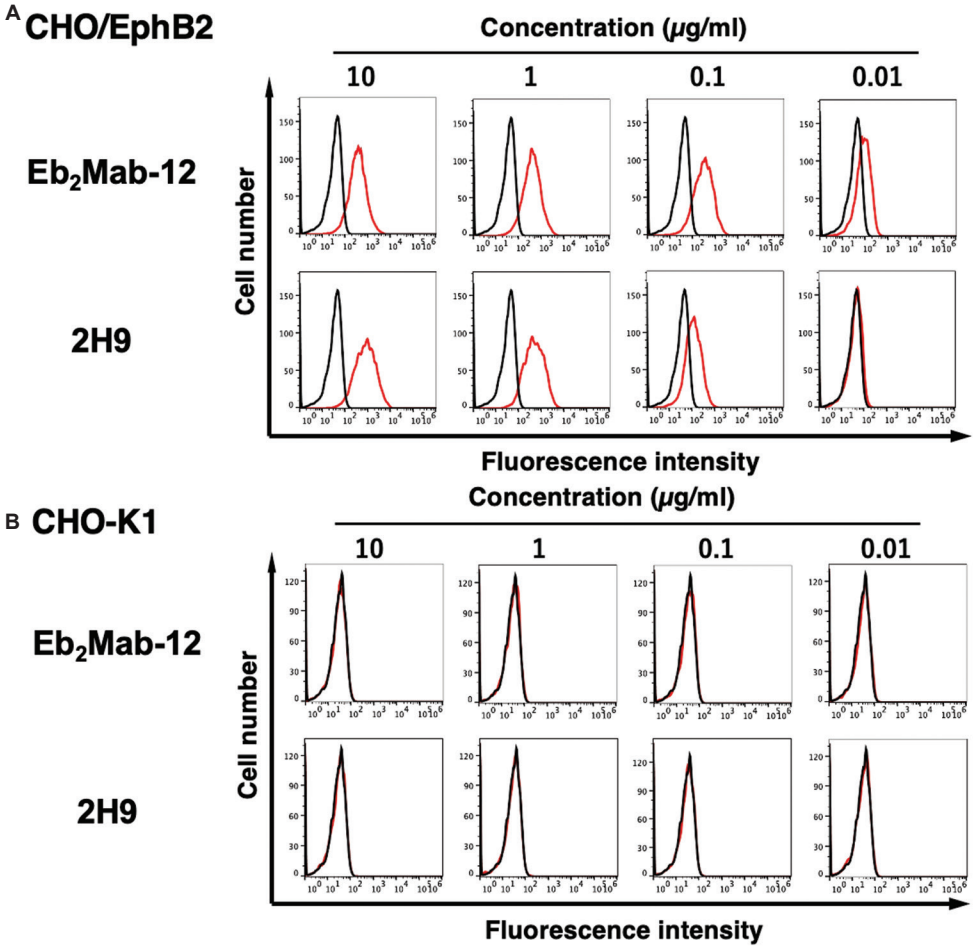


**Figure 2.** Flow cytometry of EphB2-expressed CHO-K1 cells using $Eb_2$Mab-12 and 2H9. CHO/EphB2 (A) and CHO-K1 (B) cells were treated with 0.01–10 μg/mL of $Eb_2$Mab-12 or 2H9 conjugated with RB545 (red line). The $Eb_2$Mab-12 treated cells were further incubated with anti-mouse IgG conjugated with Alexa Fluor 488. Fluorescence data were subsequently collected using the SA3800 Cell Analyzer. The black line represents the negative control (blocking buffer).

Abbreviations: CHO/EphB2: EphB2-overexpressing Chinese hamster ovary-K1 cells; CHO: Chinese hamster ovary; IgG: Immunoglobulin G; mAbs: Monoclonal antibodies.

## 4. Discussion

An anti-EphB2 mAb, clone 2H9, was extensively characterized and developed for tumor therapy as an antibody-drug conjugate.[46] The 2H9 was established through the immunization of mice with the EphB2 ectodomain produced by a baculovirus expression system.[46] However, 2H9 showed cross-reactivity to CHO/EphA4, CHO/EphB1, and CHO/EphB3 (Figure S1). This limitation highlights the need for more specific antibodies that can effectively target EphB2 without such cross-reactivity. In this study, anti-EphB2 mAbs were established using the CBIS method (Figure 1). Among the established mAbs, $Eb_2$Mab-12 exhibited superior reactivity compared to 2H9 in CHO/EphB2 (Figure 2), LN229/EphB2 (Figure S2), and LS174T (Figure 3) cells. Furthermore, $Eb_2$Mab-12 possesses a higher affinity toward CHO/EphB2 and LS174T compared to 2H9 (Figure 4). Importantly, no cross-reactivity was observed for $Eb_2$Mab-12 even at high concentrations (Figures S2-4 and Table 1). Therefore, $Eb_2$Mab-12 is recognized as a highly sensitive and specific anti-EphB2 mAb for flow cytometry.

The interaction of EphB2 with ligands was effectively blocked by 2H9, which also inhibited the autophosphorylation of EphB2.[46] However, 2H9 did not affect the proliferation of EphB2-positive tumor cells.[46] The identification of the epitope is essential to assess the properties of $Eb_2$Mab-12 and 2H9. Hence, the RIEDL insertion for epitope mapping (REMAP) and PA insertion for epitope mapping (PAMAP) methods was developed to determine the conformational epitopes of mAbs. The epitopes of anti-EGFR mAb (EMab-134)[47] and anti-CD44

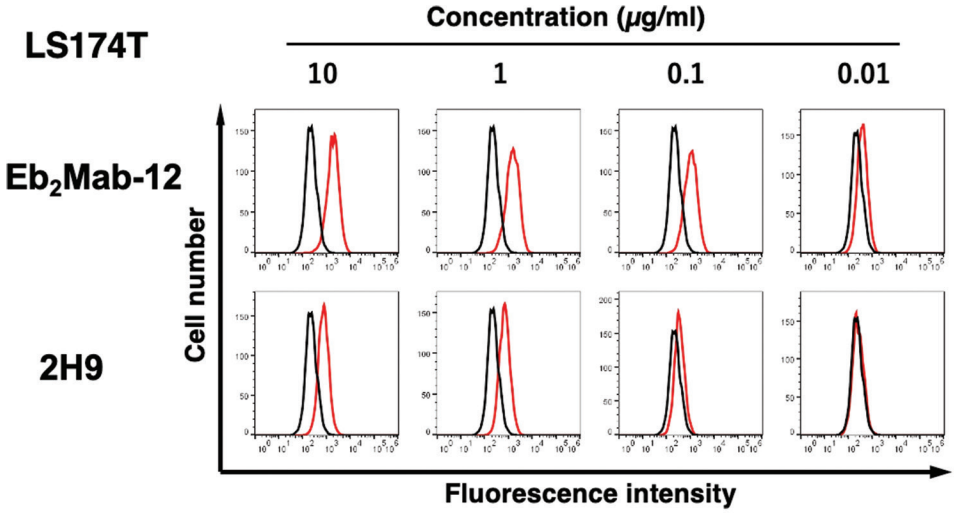


**Figure 3.** Flow cytometry of endogenous EphB2-expressing cells using $Eb_2$Mab-12 and 2H9. LS174T cells were treated with 0.01 – 10 μg/mL of $Eb_2$Mab-12 or 2H9 conjugated with RB545 (red line). The $Eb_2$Mab-12 treated cells were further incubated with anti-mouse IgG conjugated with Alexa Fluor 488. The fluorescence data were subsequently collected using the SA3800 Cell Analyzer. The black line represents the negative control (blocking buffer).
Abbreviation: IgG: Immunoglobulin G.

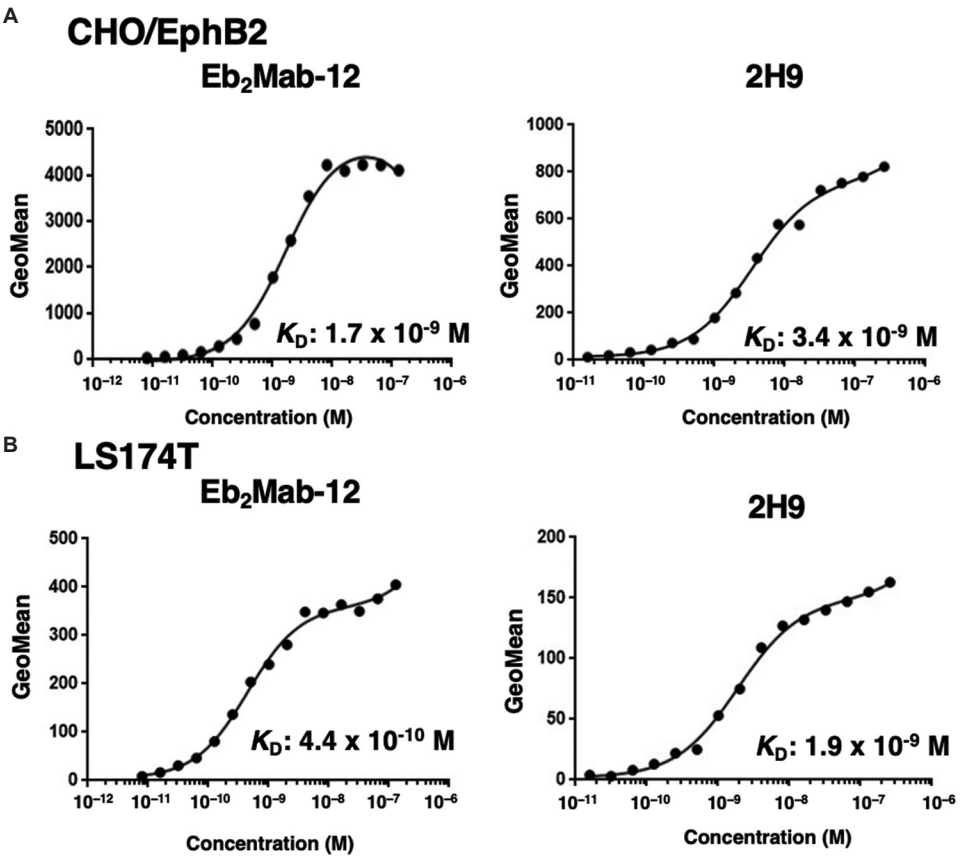


**Figure 4.** The binding affinity of $Eb_2$Mab-12 and 2H9. CHO/EphB2 (A) and LS174T (B) cells were suspended in serially diluted $Eb_2$Mab-12. The cells were treated with anti-mouse IgG conjugated with Alexa Fluor 488. The cells were also suspended in serially diluted 2H9 conjugated with RB545. The fluorescence data were subsequently collected using the BD FACSLyric, followed by the calculation of the $K_D$ using GraphPad PRISM 6.
Abbreviation: IgG: Immunoglobulin G.

mAb ($C_{44}$Mab-46)[48] were successfully determined using the REMAP method. In addition, the epitopes of anti-mouse CD39 mAb ($C_{39}$Mab-1) were identified using both REMAP and PAMAP methods.[49] Therefore, further studies are required to elucidate the epitope and biological activities of $Eb_2$Mab-12.

The expression of EphB2 in more than 100 cell lines was investigated using flow cytometry, with LS174T exhibiting the highest expression (Figure 3). Since LS174T is a transplantable cancer cell line in BALB/c nude mice,[50] the *in vivo* anti-tumor effects of $Eb_2$Mab-12 can be evaluated. To achieve this, conversion of $Eb_2$Mab-12 (mouse $IgG_1$) to mouse $IgG_{2a}$ subclass is essential for enhancing effector activation ability. Previously, recombinant mAbs were produced, converting them into the mouse $IgG_{2a}$ subclass from mouse $IgG_1$. In addition, defucosylated $IgG_{2a}$ mAbs were produced using fucosyltransferase 8-deficient CHO-K1 cells to enhance the antibody-dependent cellular cytotoxicity and *in vivo* anti-tumor effect in mouse xenograft models.[51] Therefore, a class-switched and defucosylated version of $Eb_2$Mab-12 could significantly contribute to the treatment of EphB2-positive tumors in pre-clinical studies. The properties of $Eb_2$Mabs ($Eb_2$Mab-3, 8, and 10), which showed cross-reactivity, were also determined (Table 1). Such mAbs may be useful when targeting multiple EphBs for mAb-based therapies.

Cancer-specific mAbs (CasMabs) have been developed against HER2 ($H_2$Mab-250),[52] podocalyxin (PcMab-6),[53] and podoplanin (LpMab-2),[54] demonstrating reactivity with cancer cells, but not normal cells in flow cytometry. The strategy used in this study is also applicable for selecting anti-EphB2 CasMabs from $Eb_2$Mabs (Table 1) or from clones derived from remaining positive wells. It has been confirmed that EphB2 is detectable in certain normal epithelial cells, prompting the screening of anti-EphB2 CasMabs. The unique properties of $H_2$Mab-250 could facilitate the development of HER2-targeting chimeric antigen receptor (CAR)-T-cells, which are currently undergoing a clinical phase I study in the US.[55] Therefore, the development of $Eb_2$Mabs for CAR-T applications is deemed necessary for treating EphB2-positive tumors.

## 5. Conclusion

An anti-EphB2 mAb, $Eb_2$Mab-12 exhibits high affinity and specificity to EphB2, suggesting its potential application in tumor diagnosis and therapy.

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## Conflict of interest

The authors have no conflicts of interest.

## Authors contributions

*Conceptualization:* Mika K. Kaneko, Yukinari Kato
*Formal analysis:* Rena Ubukata
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*Methodology:* Mika K. Kaneko
*Writing – original draft:* Rena Ubukata, Hiroyuki Suzuki
*Writing – review & editing:* Hiroyuki Suzuki, Yukinari Kato

## Ethics approval and consent to participate

All animal experiments were approved by the Animal Care and Use Committee of Tohoku University (Permit number: 2022MdA-001).

## Consent for publication

Not applicable.

## Availability of data

The data presented in this study are available in the article.

## Further disclosure

The paper has been uploaded to a preprint server (DOI: 10.20944/preprints202406.0704.v2).

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