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Article

Ea8Mab-9: A Novel Monoclonal Antibody Against Erythropoietin-Producing Hepatocellular Receptor A8 for Flow Cytometry

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Abstract: Erythropoietin-producing hepatocellular receptor A8 (EphA8) is a type I transmembrane protein that belongs to the largest Eph family among receptor tyrosine kinases. By binding to its membrane-bound ephrin-A or ephrin-B ligands on adjacent cells, Eph receptors form complexes and mediate bidirectional signaling activities, triggering cell-cell adhesion and repulsion. Increased expression of EphA8 correlates with poor prognosis in some types of cancer. Therefore, developing sensitive monoclonal antibodies (mAbs) for EphA8 has been desired for treatment, diagnosis, and further basic research. In particular, there are no anti-EphA8 mAbs that can be used for flow cytometry. In this study, we established a novel specific and sensitive anti-human EphA8 mAb, which applies to flow cytometry, clone Ea8Mab-9 (mouse IgG1, kappa), by the Cell-Based Immunization and Screening (CBIS) method. Ea8Mab-9 reacted with EphA8-overexpressed Chinese hamster ovary-K1 cells (CHO/EphA8) and EphA8-overexpressed LN229 glioblastoma cells (LN229/EphA8) in flow cytometry. Ea8Mab-9 did not recognize other members of Eph receptors. Furthermore, Ea8Mab-9 demonstrated a high binding affinity for CHO/EphA8 and LN229/EphA8 with dissociation constants of 1.3×10^{-9} M and 1.6×10^{-9} M, respectively. Ea8Mab-9, established by the CBIS method, could be useful for analyzing the EphA8-related biological response using flow cytometry with high affinity and specificity.

Keywords: EphA8; CBIS method; monoclonal antibody; flow cytometry

1. Introduction

Erythropoietin-producing hepatocellular (Eph) receptors are the most prominent family of receptor tyrosine kinases (RTKs) and regulate tissue homeostasis, including cell proliferation and migration, tissue remodeling, angiogenesis, axon guidance, and synaptic plasticity in the nervous system [1–3]. Eph receptors are classified into EphA and EphB subfamilies according to the sequence homology and binding mode to membrane-bound ephrin ligands. The EphA subfamily includes nine molecules, such as EphA1 to EphA8 and EphA10. The EphB subfamily consists of five molecules, such as EphB1 to EphB4 and EphB6. The eight ephrin ligands have been identified, including glycosylphosphatidylinositol (GPI)-anchored ephrin A1 to A5 and transmembrane ephrin B1 to B3. Following the binding to receptors, forward signals are triggered on the receptor side, and reverse signals are generated on the ligand side, controlling various biological homeostasis [3].

EphA8 gene was identified from rat and human DNA encoding as eek (Eph- and elk-related kinase, EEK) in 1991 [4]. Another group has cloned mouse EphA8 molecule in 1997 [5]. EphA8 is one of the members of RTKs; the regulatory mechanism is thought to be based on tyrosine kinase activity. Phosphorylation of Tyr-615 in the EphA8 juxtamemembrane domain mediated a strong association with the SH2 domain of Fyn, a member of Src TKs [6]. Tyr-838 phosphorylation in the EphA8 kinase domain modulates Fyn binding to Tyr-615, resulting in attenuating cell adhesion by cellular cytoskeletal modifications [6]. Interestingly, tyrosine kinase activity-independent functions are also emerging. Ephrin A5-induced EphA8-integrin interaction is promoted by p110γ PI3K in an EphA8

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tyrosine kinase-independent manner [7]. Like representative growth factor receptors such as epidermal growth factor receptor (EGFR), Eph receptors play a role in cell proliferation primarily through forward signaling [8,9]. Not only cell-cell attachment and cell motility, EphA8 controls organ development and axon growth [10,11]. EphA8 induces caspase-dependent apoptotic cell death of ephrin A5-expressing neural epithelial cells during early brain development [10]. Loss of EphA8 disrupts axon guidance during the nervous system in mammals [11]. Furthermore, EphA8 facilitates neurite outgrowth by sustaining MAPK activity in neuronal cells [12].

EphA8 expression has also been reported to be associated with cancer. EphA8 upregulation is observed in various cancers, including oral tongue squamous cell carcinoma (OTSCC) [13], ovarian cancer [14], gastric cancer [15], and breast cancer [16]. EphA8 and ephrin A5 contribute to the invasiveness of stem cells isolated from MDA-MB-231, a triple-negative invasive breast cancer cell line [17]. In contrast, tumor suppressor functions of EphA8 have also been proposed. Reducing the expression of miR-10a, a promoter of cancer invasion, increases EphA8 expression and suppresses cancer progression, such as colorectal cancer and glioma [18,19]. Further research is necessary to clarify the role of EphA8 in promoting or suppressing cancer functions.

Previously, we have developed various monoclonal antibodies (mAbs) against human Eph receptors, including EphA2 [20], EphB2 [21], and EphB4 [22], by the Cell-Based Immunization and Screening (CBIS) method. This method can efficiently obtain a wide variety of antibodies that recognize modifications and three-dimensional structures of extracellular domains of membrane protein. Since flow cytometry is used for high-throughput screening in the CBIS method, mAbs that can be used for that application are the first choice. However, anti-EphA8 mAbs suitable for flow cytometry are not yet commercially available.

In this study, we have successfully established an anti-human EphA8 mAb (clone Ea$_8$Mab-9) for flow cytometry using the CBIS method.

2. Materials and Methods

2.1. Cell Lines and Stable Transfectants

LN229, Chinese hamster ovary (CHO)-K1, and P3X63Ag8U.1 (P3U1) cells were obtained from the American Type Culture Collection (Manassas, VA). The cDNA encoding human EphA8 (Accession No. NM_020526; Catalog No.: RC220352) was purchased from OriGene Technologies, Inc. (Rockville, MD, USA). The open reading frame of EphA8, excluding the signal sequence, interleukin 2-signal sequence, and PA16 tag [23] or MAP16 tag [24] of the N-terminus of EphA8, were subcloned into the pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) using the In-Fusion HD Cloning Kit (Takara Bio, Inc., Shiga, Japan). The plasmid was transfected into the cell lines using a Neon transfection system (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Subsequently, LN229 and CHO-K1, stably overexpressed EphA8, which deleted amino acids 1st to 27th with N-terminal MAP16 tags (from now on described as LN229/EphA8 and CHO/EphA8, respectively) and LN229 which stably overexpressed EphA8 which deleted amino acids 1st to 27th with N-terminal PA16 tag (from now on described as LN229/PA16-EphA8) were established using a cell sorter (SH800; Sony Corp., Tokyo, Japan) by staining tag systems.

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), EphA10 (Catalog No.: RC218374, Accession No.: NM_001099439) EphB1 (Catalog No.: RC214301, Accession No.: NM_004441), EphB2 (Catalog No.: RC223882, Accession No.: NM_004442), EphB6 (Catalog No.: RC229404, Accession No.: NM_004445), were purchased from OriGene Technologies, Inc. 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 (Ibaraki, Japan).

EphA2 and EphB3 cDNAs were cloned into a pCAGzeo vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). EphB6 cDNA was cloned into a pCMV6 vector. EphA1 cDNA was cloned

into a pCAGzeo-ssnPA vector. EphA3, EphA4, EphA5, EphA6, EphA7, EphA8, EphA10, and EphB1 cDNA were cloned into a pCAGzeo_ssnPA16 vector.

The plasmids were also transfected into CHO-K1 cells and stable transfectants were established by staining with an anti-EphA2 mAb (clone SHM16; BioLegend, San Diego, CA, USA), an anti-EphB3 mAb (clone 647354; R&D Systems Inc., Minneapolis, MN, USA), an anti-EphB6 mAb (clone T49-25; BioLegend), and an anti-PA tag [25] mAb (clone NZ-1 for EphA2, EphA3, EphA4, EphA5, EphA6, EphA7, EphA10, and EphB1), and sorted using SH800. After sorting, cultivation in a medium containing 0.5 mg/mL of Zeocin (InvivoGen, San Diego, CA, USA) or 0.5 mg/mL of G418 was progressed. These Eph receptors-overexpressed CHO-K1 (e.g., CHO/EphA1) clones were finally established. CHO/PA16-EphB4 was previously described [22].

CHO-K1, P3U1, and each Eph receptor-overexpressed CHO-K1 cells were also cultured in a Roswell Park Memorial Institute (RPMI)-1640 medium (Nacalai Tesque, Inc.) that was supplemented with 10% heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific Inc.), 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Nacalai Tesque, Inc.). LN229 and LN229/EphA8 were cultured in a Dulbecco's Modified Eagle Medium (DMEM) (Nacalai Tesque, Inc.) that was supplemented with 10% heat-inactivated FBS (Thermo Fisher Scientific Inc.), 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Nacalai Tesque, Inc.). Then, cells were cultured in a humidified incubator, supplied with 5% CO_2 and 95% air at 37°C.

2.2. Antibodies

The secondary Alexa Fluor 488-conjugated anti-mouse IgG was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

2.3. Development of Hybridomas

For developing anti-EphA8 mAbs, two 6-week-old female BALB/cAJcl mice purchased from CLEA Japan (Tokyo, Japan) were immunized intraperitoneally with 1×10^8 cells of LN229/PA16-EphA8. The immunogen was harvested after brief exposure to 1 mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Inc.). Alhydrogel adjuvant 2% (InvivoGen) was added as an adjuvant in the first immunization. Three additional injections of 1×10^8 cells of LN229/PA16-EphA8 were performed without an adjuvant addition every week. A final booster immunization was performed with 1×10^8 cells of LN229/PA16-EphA8 intraperitoneally two days before harvesting splenocytes from mice. We conducted cell-fusion of the harvested splenocytes from immunized mice with P3U1 cells using polyethylene glycol 1500 (PEG1500; Roche Diagnostics, Indianapolis, IN) under warming conditions.

Hybridomas were cultured in the RPMI-1640 medium, supplemented as shown above. We further added hypoxanthine, aminopterin, and thymidine (HAT; Thermo Fisher Scientific, Inc.), 5% BriClone (NICB, Dublin, Ireland), and 5 μg/mL of Plasmocin (InvivoGen) into the medium. The hybridoma supernatants were screened by flow cytometry using CHO/EphA8 and parental CHO-K1 cells. The cultured supernatant of Ea8Mab-9-producing hybridomas was filtrated and purified using Ab-Capcher Extra (ProteNova, Kagawa, Japan).

2.4. Flow Cytometric Analysis

CHO-K1, CHO/EphA1, CHO/EphA2, CHO/EphA4, CHO/EphA6, CHO/EphA7, CHO/EphA8, CHO/EphB1, CHO/EphB6, LN229, and LN229/EphA8 cells were harvested after brief exposure to 1 mM EDTA. CHO/EphA3, CHO/EphA5, CHO/EphA10, CHO/EphB2, CHO/EphB3, CHO/EphB4 were harvested after brief exposure to 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Inc.). Cells were washed with 0.1% bovine serum albumin in phosphate-buffered saline (PBS) and treated with primary mAb for 30 min at 4°C. Afterward, cells were treated with Alexa Fluor 488-conjugated anti-mouse IgG (1:1000) following the collection of fluorescence data using the SA3800 Cell Analyzer (Sony Corp.).

2.5. Determination of Dissociation Constant (K_D) by Flow Cytometry

CHO/EphA8 and LN229/EphA8 were suspended in 100 μL serially diluted Ea$_8$Mab-9 (10 μg/mL to 0.0006 μg/mL), after which Alexa Fluor 488-conjugated anti-mouse IgG (1:200) was added. Fluorescence data were subsequently collected using the BD FACSLyric (BD Biosciences, Franklin Lakes, NJ, USA), following the calculation of the dissociation constant (K_D) by fitting the binding isotherms into the built-in one-site binding model in GraphPad PRISM 6 (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Development of Anti-EphA8 mAbs Using the CBIS Method

To develop anti-EphA8 mAbs, we employed the CBIS method using EphA8-overexpressed cells. Anti-EphA8 mAbs-producing hybridoma were screened by using flow cytometry (Figure 1). Two female BALB/cAJcl mice were intraperitoneally immunized with LN229/PA16-EphA8 every week for a total of 5 times. Subsequently, hybridomas were seeded into 96-well plates, after which flow cytometric analysis was used to select CHO/EphA8-reactive and parental CHO-K1-nonreactive supernatants of hybridomas. Afterward, we obtained some highly CHO/EphA8-reactive supernatants of hybridomas. We finally established the highest sensitivity clone, Ea$_8$Mab-9 (mouse IgG$_1$, kappa), by limiting dilution and additional analysis.
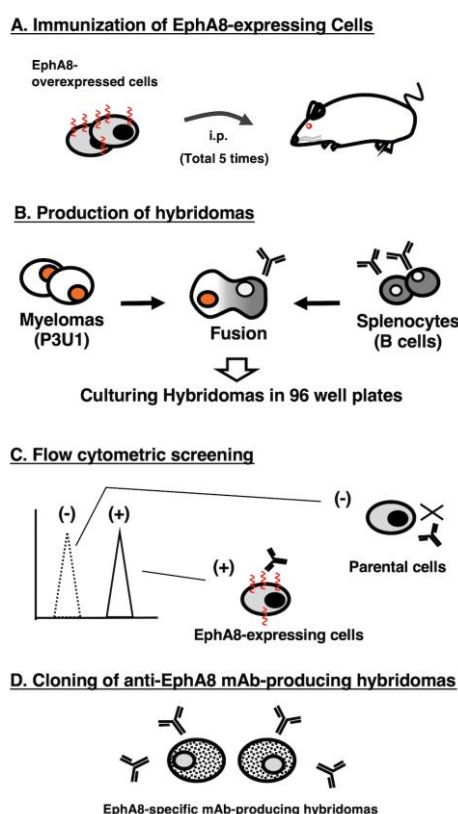

Figure 1. A schematic diagram of anti-EphA8 mAbs development. The procedure of antibody development using the Cell-Based Immunization and Screening (CBIS) method. **(A)** LN229/PA16-EphA8 cells were immunized into two mice by intraperitoneal injection. **(B)** The spleen cells from antigen-immunized mice were fused with myeloma P3U1 cells by PEG1500. **(C)** The culture supernatants of hybridoma were screened by flow cytometry using CHO-K1 and CHO/EphA8 to select EphA8-specific mAb-producing hybridomas. **(D)** After limiting the dilution of hybridomas to obtain the single clone and additional screening, Ea$_8$Mab-9 (mouse IgG$_1$, kappa) was finally established.

3.2. Flow Cytometric Analysis

Flow cytometric analysis was conducted using Ea$_8$Mab-9 against CHO-K1, CHO/EphA8, LN229, and LN229/EphA8 cells. Results indicated that Ea$_8$Mab-9 recognized CHO/EphA8 (Figure 2A, upper)

and LN229/EphA8 (Figure 2B, upper) dose-dependently. Ea₈Mab-9 did not react with parental CHO-K1 (Figure 2A, lower) and LN229 (Figure 2B, lower) cells even at 10 μg/mL of Ea₈Mab-9. Ea₈Mab-9 could react to EphA8 specifically in flow cytometry.
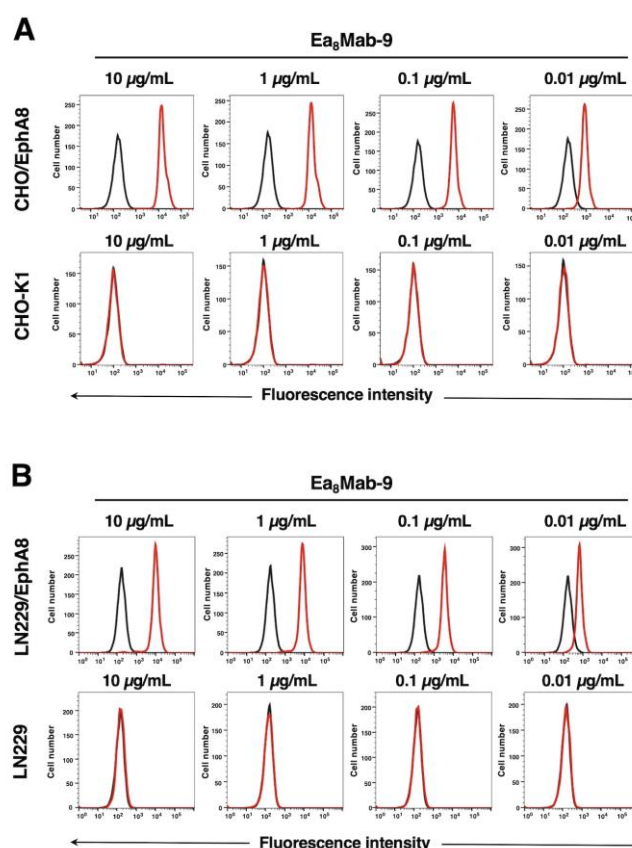

Figure 2. Flow cytometric analysis of Ea₈Mab-9. (A) CHO/EphA8 (upper panels) and CHO-K1 (lower panels) cells were treated with 0.01–10 μg/mL of Ea₈Mab-9 (red line), followed by treatment with Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were collected using the SA3800 Cell Analyzer. Black line, control (no primary antibody treatment). **(B)** LN229/EphA8 (upper panels) and LN229 (lower panels) cells were treated with 0.01–10 μg/mL of Ea₈Mab-9 (red line), followed by treatment with Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were collected using the SA3800 Cell Analyzer. Black line, control (no primary antibody treatment).

3.3. Specificity of Ea₈Mab-9 to Eph Receptor-Expressed CHO-K1 Cells

We have established the all Eph receptor-overexpressed CHO-K1 cells, EphA1 to A8, A10, B1 to B4, and B6 respectively. Using the fourteen cell lines, the specificity of Ea₈Mab-9 was evaluated. As shown in Figure 3, Ea₈Mab-9 could recognize only CHO/EphA8, but not other Eph receptors-expressed cell lines. We confirmed the specificity of Ea₈Mab-9 among Eph receptors.
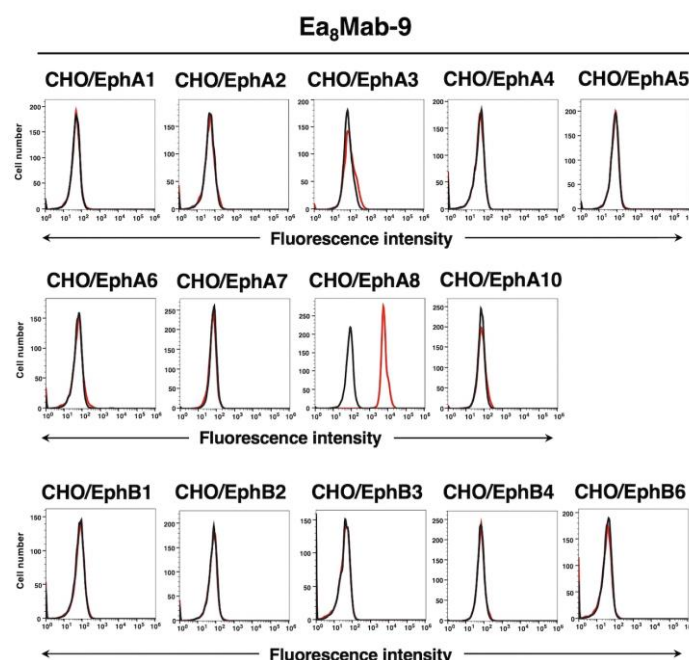
Figure 3. Flow cytometry of Ea₈Mab-9 in Eph receptor-expressed CHO-K1 cells. CHO-K1 cells which expressed each of the fourteen Eph receptors were treated with 10 µg/mL of Ea₈Mab-9 (red line) or control blocking buffer (black line) followed by the treatment with anti-mouse IgG conjugated with Alexa Fluor 488.

3.4. Determination of the Binding Affinity of Ea₈Mab-9 by Flow Cytometry

The binding affinity of Ea₈Mab-9 was assessed with exogenously EphA8-expressed CHO/EphA8 and LN229/EphA8 using flow cytometry. Results showed that the K_D values of Ea₈Mab-9 were 1.3×10^{-9} M (CHO/EphA8) and 1.6×10^{-9} M (LN229/EphA8) (Figure 4). These results demonstrate that Ea₈Mab-9 possesses a high affinity for EphA8.
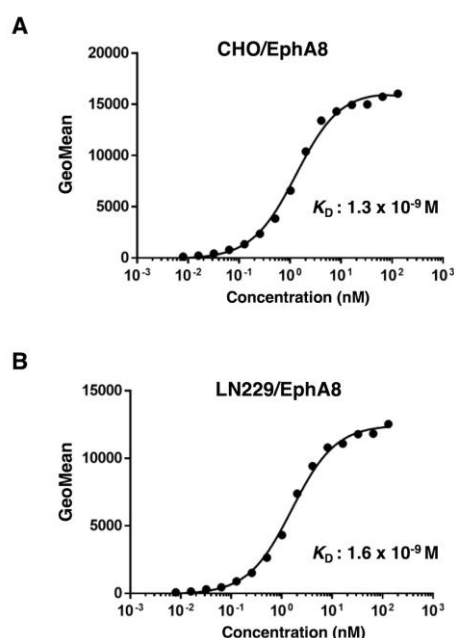

Figure 4. Determination of the binding affinity of Ea₈Mab-9 against EphA8-expressed cells. CHO/EphA8 **(A)** and LN229/EphA8 **(B)** cells were suspended in 100 µL of serially diluted Ea₈Mab-9 (10 µg/mL to 0.0006 µg/mL). Then, cells were treated with Alexa Fluor 488-conjugated anti-mouse IgG. Subsequently, fluorescence data were collected using the BD FACSLyric, following the calculation of the dissociation constant (K_D) by GraphPad PRISM 6 software.

4. Discussion

In this study, we developed a novel anti-EphA8 mAb, Ea$_8$Mab-9 using the the CBIS method and showed the high affinity and specificity in flow cytometry (Figures 2–4). We also established other anti-EphA8 mAb clones for flow cytometry (http://www.med-tohoku-antibody.com/topics/001_paper_antibody_PDIS.htm#EphA8). Ea$_8$Mab-9 and other clones would contribute to the elucidation of EphA8 functions in various researches.

EphA8 regulates brain development and neural outgrowth in normal tissues [10,12], and its overexpression in cancer has been reported [13,14,16]. EphA8 TK-dependent activation appears to be an essential mechanism for its function. Indeed, Tyr-phosphorylated EphA8 regulates cell-cell adhesion by interacting with Fyn [6]. On the other hand, TK-independent roles of EphA8 have also been noted [7]. Recently, it has become evident that the RTKs' modification mediated by Ser/Thr kinases is closely related to the regulation and function of receptors. EGFR internalization is mediated by Ser/Thr phosphorylation by p38 MAPK [26,27]. EphA2, the most extensively studied Eph receptors, is phosphorylated Ser-897 by p90 ribosomal S6 kinase and correlates tumor malignant progression [28]. Cellular stress triggers these modifications to RTKs [29,30]. The role of EphA8 has also been addressed. In breast cancer, EphA8 suppresses cell apoptosis via Akt activation and correlates with poor prognosis [16]. Simultaneously, EphA8 expression may mediate the resistance to paclitaxel [16]. Also, in normal tissue, EphA8 induces apoptosis of ephrin A5-expressing neural epithelial cells during the brain development of mice [10]. EphA8 has been reported to govern apoptosis in both tumor and normal tissues. Furthermore, EphA8 is involved in gastric cancer malignancy through Akt signaling and interaction with ADAM10 [15]. The expression of EphA8 significantly correlates with only TNM stage, but not other risk factors such as age, gender, drinking, and smoking history in OTSCC patients.[13] Although the TK dependency was not clarified in these reports, the TK-independent regulation of EphA8 by Ser/Thr kinases may function through a non-canonical TK-independent mechanism. Since EphA8 has been reported to cooperate with stem cells [17], the relationship between EphA8-expressing cells and other membrane protein markers such as CD44 and CD133 might be evaluated [31,32]. To analyze the population of EphA8-expressing cancer, it is necessary to detect naïve EphA8 in cancer cells. Because Ea$_8$Mab-9 recognizes EphA8 with high affinity, it will be helpful for diagnosis and experiments using flow cytometry.

Eph receptors have been widely studied about cancer and are attracting attention as therapeutic targets [2,33]. Clinical trials of various modalities, including compounds, antibody drugs, and chimeric antigen receptor (CAR)-T cells, have been conducted targeting Eph receptors and ephrin ligands such as EphA2, EphA3, EphA5, EphB4, ephrin A4, and ephrin B2 [2,34]. However, there are currently no drugs approved specifically for Eph receptors or ephrin ligands. The clinical tests targeting EphA8 have not been confirmed yet. Although the specific antibody is also desired to clarify the character of EphA8 molecule in basic research, commercially available antibodies for EphA8 is not abundant. In particular, no commercially available anti-EphA8 antibodies are suitable for flow cytometry. Ea$_8$Mab-9 has high affinity and is amenable to flow cytometry (Figures 2–4). Ea$_8$Mab-9 is expected to lead to a more detailed elucidation of the role of EphA8 in cancer, including the tumor microenvironment and in neuronal research. Furthermore, we have previously enhanced antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) activities by switching isotypes and eliminating fucosylation in mAbs [35–38]. Since Ea$_8$Mab-9 is mouse IgG$_1$, which lacks ADCC activity, its mouse IgG$_{2a}$ version will be generated to examine the efficacy of antitumor activities in tumor xenograft models in the future study.

Author Contributions: Tomohiro Tanaka: Investigation, Writing – original draft, **Haruto Yamamoto**: Investigation, **Yu Kaneko**: Investigation, **Keisuke Shinoda**: Investigation, **Takuya Nakamura**: Investigation, **Guanjie Li**: Investigation, **Shiori Fujisawa**: Investigation, **Hiroyuki Satofuka**: Investigation, **Mika K. Kaneko**: Conceptualization, Funding acquisition, **Hiroyuki Suzuki**: Funding acquisition, Writing – review and editing, **Yukinari Kato**: Conceptualization, Funding acquisition, Project administration, Writing – review and editing, All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Not applicable.

Data Availability Statement: All related data and methods are presented in this paper. Additional inquiries should be addressed to the corresponding authors.

Conflicts of Interest: The authors declare no conflict of interest involving this article.

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