



# Development of a specific anti-human EphA3 monoclonal antibody, Ea<sub>3</sub>Mab-20, for flow cytometry

Hiroyuki Satofuka , Hiroyuki Suzuki <sup>\*</sup> , Miu Hirose , Keisuke Shinoda , Takuya Nakamura , Tomohiro Tanaka , Mika K. Kaneko , Yukinari Kato <sup>\*\*</sup>

Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, 2-1 Seiryomachi, Aoba-ku, Sendai, Miyagi, 980-8575, Japan

## ARTICLE INFO

### Keywords:

EphA3  
Monoclonal antibody  
Cell-Based Immunization and Screening  
Flow cytometry  
Immunohistochemistry

## ABSTRACT

Erythropoietin-producing hepatocellular (Eph) receptor A3 (EphA3) is a member of the Eph receptor family, which binds to its respective ligands, ephrins. These interactions are essential for normal development and tissue homeostasis. Dysregulation of EphA3 has been reported to be associated with human hematopoietic malignancies, making it a promising target for therapy and diagnosis. Due to the high similarity of the extracellular domain among Eph receptors (more than 33% amino acid identity), generating highly specific monoclonal antibodies (mAbs) is crucial. We developed anti-human EphA3 mAbs in this study using the Cell-Based Immunization and Screening (CBIS) method. Among them, the clone Ea<sub>3</sub>Mab-20 (IgG<sub>1</sub>, kappa) exhibited high affinity and specificity in flow cytometry. The dissociation constant values of Ea<sub>3</sub>Mab-20 for CHO/EphA3 and Jurkat cells were determined to be  $9.0 \pm 0.3 \times 10^{-9}$  M and  $1.4 \pm 0.1 \times 10^{-9}$  M, respectively. Ea<sub>3</sub>Mab-20 showed no cross-reactivity with other Eph receptors in flow cytometry. Furthermore, Ea<sub>3</sub>Mab-20 demonstrated the suitability for detecting formalin-fixed paraffin-embedded cell samples in immunohistochemistry. Therefore, Ea<sub>3</sub>Mab-20 is valuable mAb for basic research and is expected to contribute to the clinical application of mAb for cancer therapy and diagnosis.

## 1. Introduction

Erythropoietin-producing hepatocellular (Eph) receptors are a family of receptor tyrosine kinases with a single transmembrane domain, classified into A and B categories based on their extracellular domains [1–6]. The extracellular domains of Eph receptors share a highly similar architecture, consisting of a ligand binding domain, a cysteine-rich region with Sushi and epidermal growth factor-like domains, and two fibronectin type III domains in tandem [1–3,7], with more than 33% amino acid identity [8]. Eph receptors interact with their membrane-bound ephrin ligands, with each receptor having preferred ephrin ligands [2]. The mammalian Eph system comprises 14 receptor tyrosine kinases (nine EphA and five EphB receptors such as EphA1 to

EphA8, EphA10, EphB1 to EphB4, and EphB6) and eight cell surface-anchored ephrin ligands (five glycosylphosphatidylinositol-linked ephrin-As such as ephrin A1 to A5 and three transmembrane ephrin-Bs such as ephrin B1 to B3) [4,9]. These interactions are essential for various normal cellular processes during development and serve as key mediators of adult tissue homeostasis [7,10–12].

The expression of Eph receptors and ephrin ligands can be either upregulated or downregulated in cancer cells compared to normal tissues [5,7,13,14]. Aberrant EphA3 regulation has been reported in human hematopoietic malignancies and solid cancers [5,14,15]. High expression and oncogenic functions of EphA3 have been reported in acute lymphoblastic leukemia (ALL) [16], glioblastoma [17], gastric cancer [18], head and neck cancer [19], and prostate cancer [20].

**Abbreviations:** Eph, erythropoietin-producing hepatocellular; mAb, monoclonal antibody; CBIS, Cell-Based Immunization and Screening; ADCC, antibody-dependent cell-mediated cytotoxicity; ALL, acute lymphoblastic leukemia; CAR, chimeric antigen receptor; scFv, single-chain variable fragment; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; FBS, fetal bovine serum; BSA, bovine serum albumin; RPMI, Roswell Park Memorial Institute;  $K_D$ , dissociation constant; FFPE, formalin-fixed paraffin-embedded; GeoMean, geometric mean.

<sup>\*</sup> Corresponding author. Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, 2-1 Seiryomachi, Aoba-ku, Sendai, Miyagi, 980-8575, Japan.

<sup>\*\*</sup> Corresponding author. Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, 2-1 Seiryomachi, Aoba-ku, Sendai, Miyagi, 980-8575, Japan.

E-mail addresses: [hiroyuki.suzuki.b4@tohoku.ac.jp](mailto:hiroyuki.suzuki.b4@tohoku.ac.jp) (H. Suzuki), [yukinari.kato.e6@tohoku.ac.jp](mailto:yukinari.kato.e6@tohoku.ac.jp) (Y. Kato).

<https://doi.org/10.1016/j.bbrep.2025.102130>

Received 1 April 2025; Received in revised form 8 June 2025; Accepted 30 June 2025

2405-5808/© 2025 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

Conversely, low expression and a tumor-suppressive role of EphA3 have been observed in small-cell lung cancer [21]. Additionally, the correlation between EphA3 mutational status and tumorigenesis in lung cancer has been reported [22]. These findings highlight EphA3 as an important therapeutic target for cancer treatment.

In the development of therapeutic monoclonal antibodies (mAbs) targeting EphA3, the mouse mAb (clone IIIA4) and its humanized defucosylated mAb ifabotuzumab (KB004) were developed to target EphA3-overexpressing cells [7,23]. Ifabotuzumab selectively binds to EphA3-positive cancer cells [24] and can stimulate antibody-dependent cell-mediated cytotoxicity (ADCC) [25]. This agent also prevents the proliferation of cancer cells and endothelial cells in the tumor vasculature by inhibiting EphA3 signaling [26]. Ifabotuzumab was evaluated in a Phase I clinical trial for the treatment of patients with hematological malignancies, demonstrating some encouraging clinical responses [24]. Moreover, treatment with IIIA4, conjugated to maytansine or lutetium-177, prevented tumor formation in glioblastoma-bearing mice [17]. Additionally, EphA3-targeted chimeric antigen receptor (CAR)-T cells demonstrated robust antigen-specific killing of human glioblastoma and diffuse midline glioma cell lines in animal models [27,28]. The EphA3 CAR, consisted of single-chain variable fragments (scFv) derived from the anti-EphA3 mAbs IIIA4 [27] and 3C3-1 [28], has been

reported.

Since the development of therapeutic mAbs requires strict specificity to minimize off-target effects caused by cross-reactivity, we aimed to develop anti-EphA3 mAbs with no cross-reactivity to other Eph receptors. We have previously developed several mAbs against various membrane proteins, including Eph receptors, using the Cell-Based Immunization and Screening (CBIS) method [29–33]. The mAbs obtained using this method are prone to recognize conformational epitopes and are suitable for flow cytometry. Furthermore, some of these mAbs also apply to immunohistochemistry, contributing to therapeutic and diagnostic advancements. Therefore, we employed the CBIS method to generate anti-EphA3 mAbs with strict specificity to develop therapeutic and diagnostic agents targeting EphA3.

## 2. Materials and methods

### 2.1. Cell lines

Human glioblastoma LN229, Chinese hamster ovary (CHO)–K1, and mouse myeloma P3X63Ag8U.1 (P3U1) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The human T cell leukemia cell line Jurkat was obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer at Tohoku University (Miyagi, Japan). The human T cell leukemia cell line MOLT-4 was obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka, Japan). These cell lines were cultured as described previously [34].

### 2.2. Plasmid construction and establishment of stable transfectants

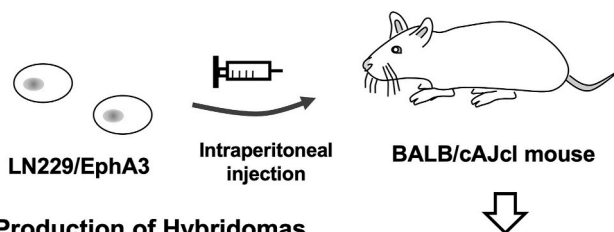
The gene encoding human *EPHA3* (NM\_005233) was obtained from the RIKEN BioResource Research Center (Ibaraki, Japan). The open reading frames, excluding the signal sequences, were subcloned into the pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) with an N-terminal PA16 tag (GLEGGVAMPGAEDDVV) [35] or a MAP16 tag (PGTGDGMVPPGIEDKI) [36], using the In-Fusion HD Cloning Kit (Takara Bio, Inc., Shiga, Japan). The constructed vectors were named pCAG-PA16-EphA3 or pCAG-MAP16-EphA3. The plasmids were transfected into CHO–K1 and LN229 cells, and stable transfectants were established using SH800S cell sorter (Sony Corporation, Tokyo, Japan) to select the highest expression of EphA3-expressing transfectants as described previously [29]. Other Eph receptor-expressed CHO–K1 cells (e.g., CHO/EphA2) were established and the cell surface expression was confirmed as reported previously [29].

The EphA3-knockout Jurkat cells were generated using the clustered regularly interspaced palindromic repeats (CRISPR)/Cas9 system with EphA3-specific guide RNAs (CCTGGCTTACCTTCATTGGA). Knockout cell lines were isolated using the SH800S cell sorter based on the loss of reactivity to an anti-EphA3 mAb IIIA4.

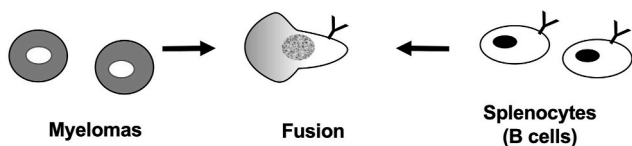
### 2.3. Hybridoma production

All animal experiments were approved by the Animal Care and Use Committee of Tohoku University (Permit number: 2022MdA-001) and carried out per the NIH (National Research Council) Guide for the Care and Use of Laboratory Animals. Two six-week-old female BALB/cAJcl mice (CLEA Japan, Tokyo, Japan) were intraperitoneally immunized with LN229/EphA3 cells ( $1 \times 10^8$  cells) with Alhydrogel adjuvant (2 %, InvivoGen). After three weekly immunizations ( $1 \times 10^8$  cells), a final booster injection ( $1 \times 10^8$  cells) was administered two days before splenocyte harvesting. Hybridomas were generated as described previously [31]. The hybridoma supernatants that were negative for CHO–K1 cells but positive for CHO/EphA3 cells were identified using flow cytometry (SA3800 Cell Analyzer, Sony Corporation). To produce purified mAbs, hybridomas were cultured in serum-free Hybridoma-SFM medium (Thermo Fisher Scientific, Inc.). The culture supernatant was

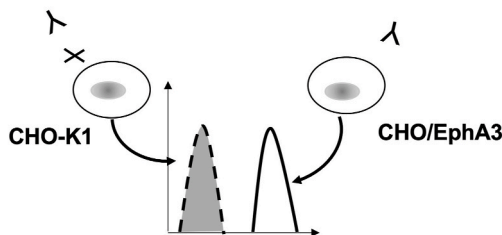
### A. Immunization of LN229/EphA3



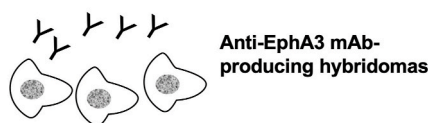
### B. Production of Hybridomas



### C. Screening of supernatants by flow cytometry

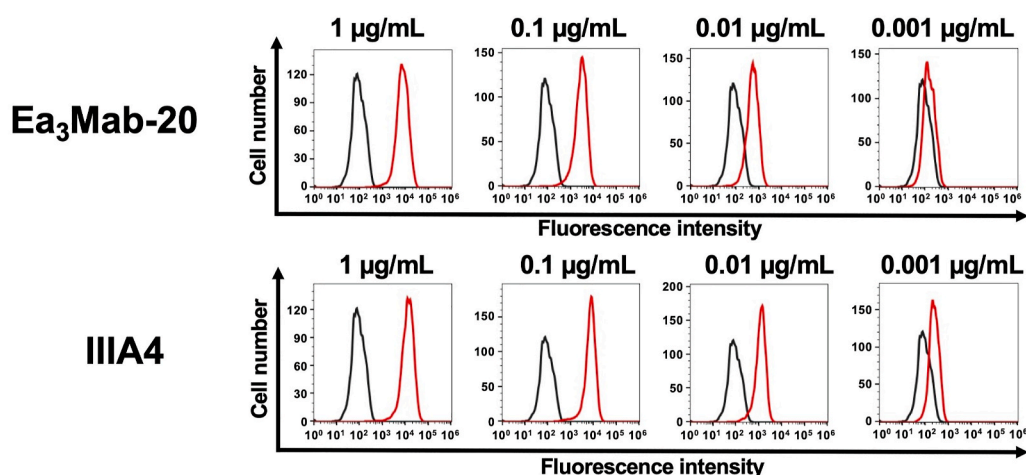


### D. Cloning of Hybridomas by limiting dilution

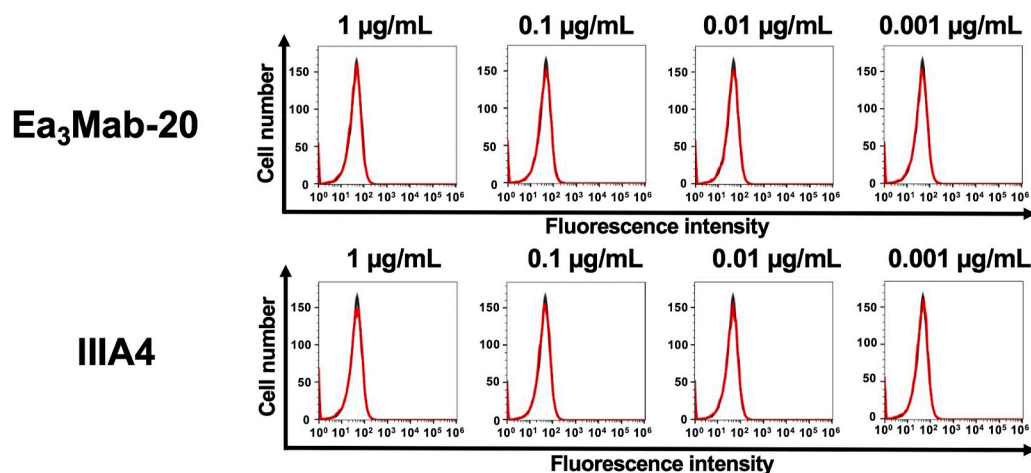


**Fig. 1.** Schematic illustration of anti-EphA3 mAb production using the CBIS method. (A) Two female BALB/cAJcl mice were intraperitoneally injected with LN229/EphA3 cells. (B) Splenocytes were harvested and fused with P3U1 myeloma cells using PEG1500. (C) Hybridoma supernatants were screened by flow cytometry using CHO/EphA3 and parental CHO–K1 cells to identify anti-EphA3-specific mAbs. (D) Hybridoma clones producing antigen-specific mAbs were isolated through the limiting dilution method.

## A CHO/EphA3



## B CHO-K1



**Fig. 2.** Flow cytometry analysis of anti-EphA3 mAbs against CHO/EphA3 and CHO-K1 cells.

CHO/EphA3 (A) and CHO-K1 (B) cells were treated with Ea<sub>3</sub>Mab-20 or the commercially available anti-EphA3 mAb IIIA4 at the indicated concentrations. Cells were stained with (red lines) or without (black lines) anti-EphA3 mAbs, followed by staining with Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were subsequently acquired using the SA3800 Cell Analyzer.

collected, and the mAbs were purified using the Ab-Capture Kit (ProteNova Inc., Kagawa, Japan) according to the manufacturer's instructions.

### 2.4. Flow cytometry

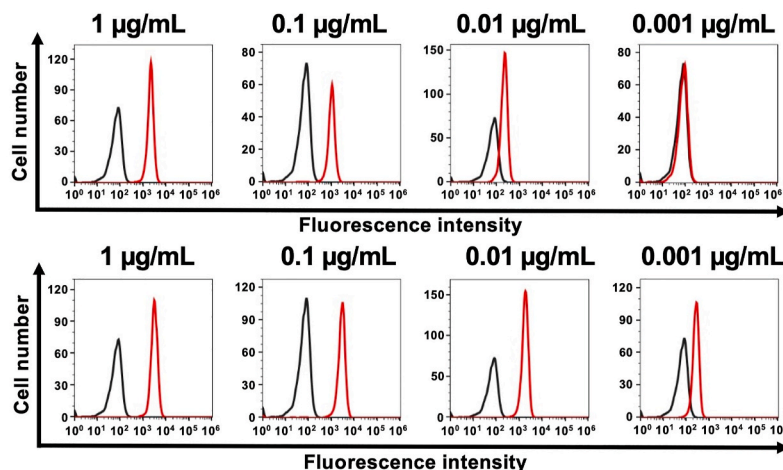
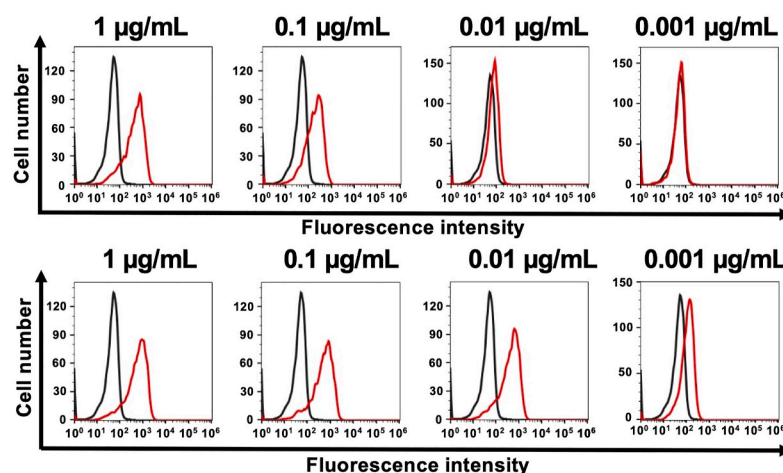
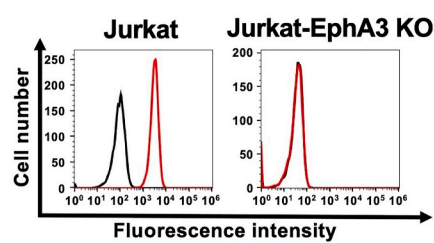
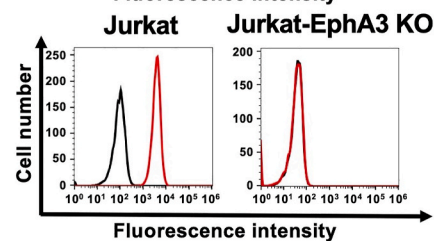
CHO/EphA3 cells were detached using 1 mM ethylenediaminetetraacetic acid (EDTA; Nacalai Tesque, Inc., Kyoto, Japan). Jurkat and MOLT-4 cells were harvested from cultured cell suspensions by centrifugation. The cells were washed with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) as a blocking buffer and incubated with primary mAbs for 30 min at 4 °C. After washing, the cells were stained with Alexa Fluor 488-conjugated anti-mouse IgG (1:2000 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA) for 30 min at 4 °C. Data were acquired using the SA3800 Cell Analyzer and analyzed using FlowJo software (BD Biosciences, Franklin Lakes, NJ, USA).

### 2.5. Determination of dissociation constant values using flow cytometry

CHO/EphA3 and Jurkat cells were treated with serially diluted Ea<sub>3</sub>Mab-20 and IIIA4 (10–0.0006 µg/mL). Subsequently, the cells were stained with Alexa Fluor 488-conjugated anti-mouse IgG (1:200 dilution) for 30 min at 4 °C. The experiment was repeated three times, and the data were collected using the SA3800 Cell Analyzer. The dissociation constant ( $K_D$ ) values were determined as described previously [29].

### 2.6. Immunohistochemical analysis

Formalin-fixed paraffin-embedded (FFPE) CHO/EphA3 and CHO-K1 cell blocks were prepared using iPGell (Genostaff Co., Ltd., Tokyo, Japan). Staining was performed using the VENTANA BenchMark ULTRA PLUS (Roche Diagnostics) with the recommended protocol and the ultraView Universal DAB Detection Kit.

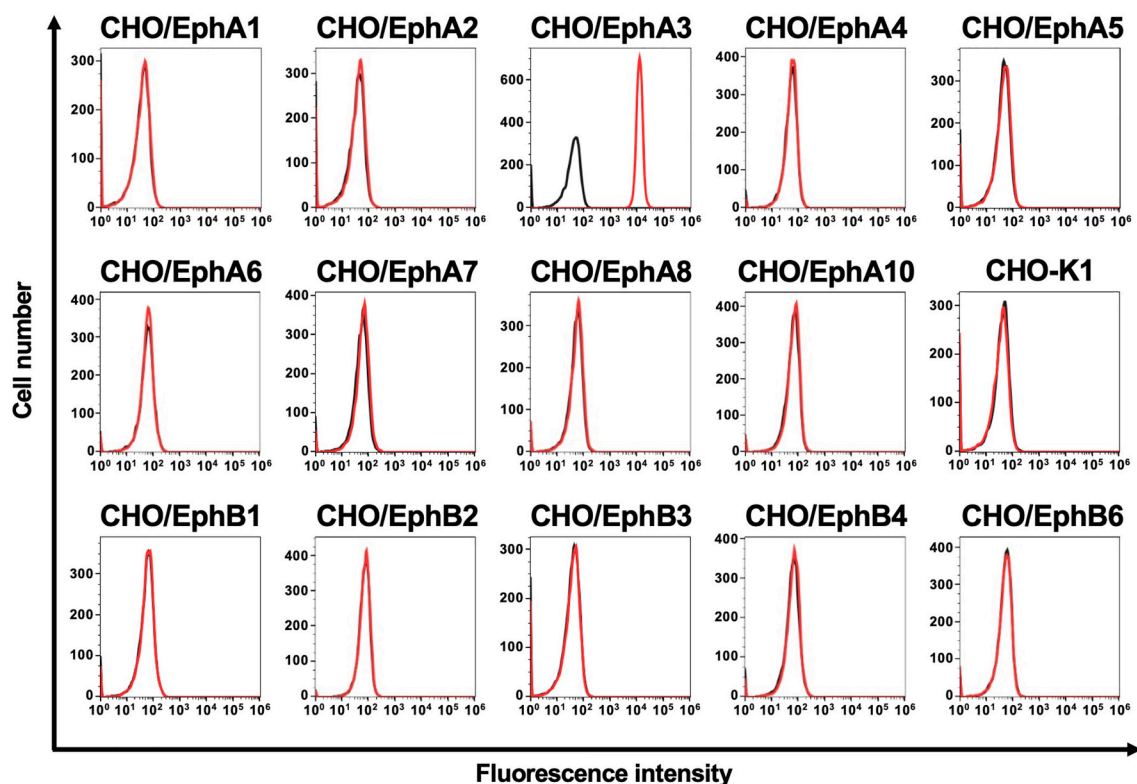
**A Jurkat****Ea<sub>3</sub>Mab-20****IIIA4****B MOLT-4****Ea<sub>3</sub>Mab-20****IIIA4****C****Ea<sub>3</sub>Mab-20****IIIA4**

**Fig. 3.** Flow cytometry analysis of anti-EphA3 mAbs against endogenous EphA3 expressing cancer cells.

Jurkat (A) and MOLT-4 (B) cells were treated with Ea<sub>3</sub>Mab-20 and IIIA4 at the indicated concentrations (red lines). The black line represents the negative control, which was stained without anti-EphA3 mAbs. (C) Jurkat and EphA3-knockout Jurkat cells were treated with 1 µg/mL of Ea<sub>3</sub>Mab-20, 1 µg/mL of IIIA4, or blocking buffer. The mAb-treated cells were incubated with Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were subsequently collected using the SA3800 Cell Analyzer.



## Ea<sub>3</sub>Mab-20



**Fig. 4.** Cross-reactivity of Ea<sub>3</sub>Mab-20 in Eph receptor-expressed CHO-K1 cells.

The 14 Eph receptors (EphA1 to A8, A10, B1 to B4, and B6)-expressed CHO-K1 cells and parental CHO-K1 cells were treated with 10 µg/mL of Ea<sub>3</sub>Mab-20 (red lines). The black line represents the negative control, which was stained without anti-EphA3 mAbs (blocking buffer). The mAb-treated cells were subsequently incubated with Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were collected using the SA3800 Cell Analyzer.

### 3. Results

#### 3.1. Development of anti-human EphA3 mAbs

Two BALB/cAJcl mice were immunized with LN229/EphA3 cells (Fig. 1A). After harvesting splenocytes from these mice, cell fusion with P3U1 cells was performed (Fig. 1B). The resulting hybridomas were seeded into 96-well plates. After colony formation, supernatants were collected and analyzed by flow cytometry-based high-throughput screening to identify those that were negative for CHO-K1 cells and positive for CHO/EphA3 cells (Fig. 1C). Subsequently, 20 hybridomas producing anti-EphA3 mAbs were cloned by limiting dilution. Finally, anti-EphA3 mAb clones, including Ea<sub>3</sub>Mab-20 (IgG<sub>1</sub>, kappa), were established (Fig. 1D).

#### 3.2. Flow cytometry using Ea<sub>3</sub>Mab-20 and IIIA4

We assessed the reactivity of Ea<sub>3</sub>Mab-20 against CHO/EphA3 and CHO-K1 cells. Ea<sub>3</sub>Mab-20 recognized CHO/EphA3 cells in a dose-dependent manner at concentrations ranging from 1 to 0.001 µg/mL (Fig. 2A). However, Ea<sub>3</sub>Mab-20 did not bind to CHO-K1 cells at any concentrations (Fig. 2B). This result indicates that Ea<sub>3</sub>Mab-20 recognizes EphA3 on the cell surface. A commercially available anti-human EphA3 mAb (IIIA4) exhibited a similar pattern of reactivity with CHO/EphA3 and CHO-K1 cells (Fig. 2). Next, we analyzed the reactivity of Ea<sub>3</sub>Mab-20 against endogenous EphA3-expressing cells, Jurkat (Fig. 3A) and MOLT-4 (Fig. 3B) cells [16], using IIIA4 as a positive control (Fig. 3). Furthermore, to confirm the reactivity of Ea<sub>3</sub>Mab-20 to

EphA3, we generated EphA3-knockout Jurkat cells by disrupting the EphA3 gene using CRISPR/Cas9 system (Fig. 3C). The specific reactivity of Ea<sub>3</sub>Mab-20 to EphA3 on Jurkat cells was clearly demonstrated by flow cytometry.

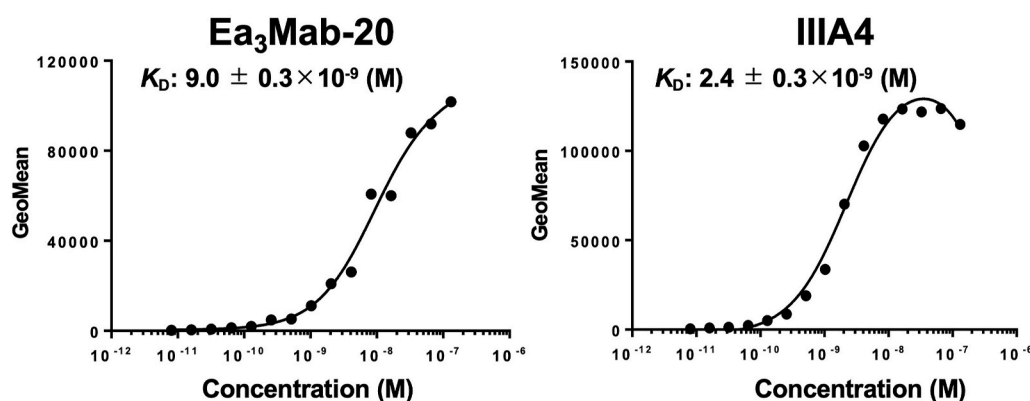
#### 3.3. Specificity of Ea<sub>3</sub>Mab-20 using CHO-K1 cells overexpressed various Eph receptors

We previously established CHO-K1 cells that overexpressed each human Eph receptor (EphA1 to A8, A10, B1 to B4, and B6) [29]. Using these cell lines, we analyzed the specificity of anti-EphA3 mAbs. Among the 20 clones producing anti-EphA3 mAbs, 13 highly reactive clones were selected for analysis. Staining data with 10 µg/mL of Ea<sub>3</sub>Mab-3, 4, 7, 9, 15, and 20 showed no cross-reactivity among the Eph receptors (Fig. 4 and Supplementary Table 1). However, IIIA4 exhibited slight but significant reactivity with CHO/EphA6 cells (Supplementary Fig. 1), indicating that IIIA4 does not exhibit complete specificity for EphA3. Ea<sub>3</sub>Mab-20 exhibited the highest binding affinity and specificity among these mAbs.

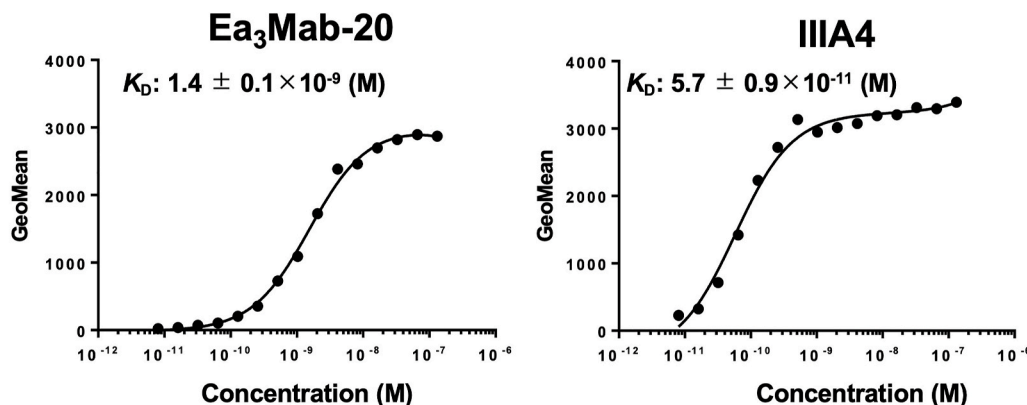
#### 3.4. Determination of binding affinity of Ea<sub>3</sub>Mab-20 and IIIA4 using flow cytometry

The binding affinity of Ea<sub>3</sub>Mab-20 and IIIA4 was determined using CHO/EphA3 and Jurkat cells. The average  $K_D$  values obtained from three independent measurements for Ea<sub>3</sub>Mab-20 were  $9.0 \pm 0.3 \times 10^{-9}$  M for CHO/EphA3 cells (Fig. 5A and Supplementary Fig. 2) and  $1.4 \pm 0.1 \times 10^{-9}$  M for Jurkat cells (Fig. 5B and Supplementary Fig. 2). The

## A CHO/EphA3



## B Jurkat



**Fig. 5.** Measurement of the binding affinity of Ea<sub>3</sub>Mab-20 and IIIA4.

CHO/EphA3 (A) and Jurkat (B) cells were treated with 100  $\mu$ L of serial dilutions of Ea<sub>3</sub>Mab-20 and IIIA4 (10–0.0006  $\mu$ g/mL). Subsequently, fluorescence data were collected using the SA3800 Cell Analyzer. The fluorescence data's geometric mean (GeoMean) values were plotted, and the  $K_D$  values were calculated using GraphPad PRISM 6 software.

average  $K_D$  values for IIIA4 were  $2.4 \pm 0.3 \times 10^{-9}$  M for CHO/EphA3 cells and  $5.7 \pm 0.9 \times 10^{-11}$  M for Jurkat cells (Fig. 5 and Supplementary Fig. 2).

### 3.5. Immunohistochemistry using anti-EphA3 mAbs

Ea<sub>3</sub>Mab-20 was assessed for its application in immunohistochemistry using FFPE CHO–K1 and CHO/EphA3 cell sections. We used the VENTANA BenchMark ULTRA PLUS system for the detection. Apparent membranous staining by Ea<sub>3</sub>Mab-20 was observed in CHO/EphA3 cells (Fig. 6A) but not in CHO–K1 cells (Fig. 6B). In contrast, IIIA4 is unavailable for staining FFPE cell sections (Fig. 6A). In addition, we could not detect endogenous EphA3 in Jurkat cells (Supplementary Fig. 4). These results indicate that Ea<sub>3</sub>Mab-20 is suitable for detecting EphA3-positive cells not only in flow cytometry but also in FFPE samples.

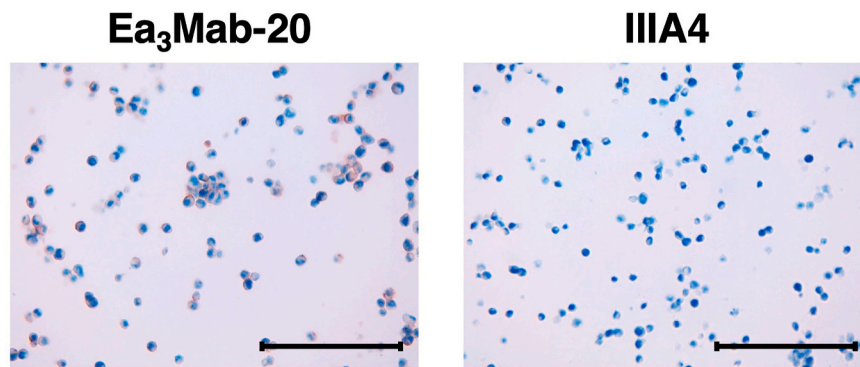
## 4. Discussion

Using the CBIS method, we generated novel anti-EphA3 mAbs, including Ea<sub>3</sub>Mab-20, with confirmed specificity for EphA3. Ea<sub>3</sub>Mab-20 is suitable for flow cytometry (Figs. 2 and 3) and immunohistochemistry

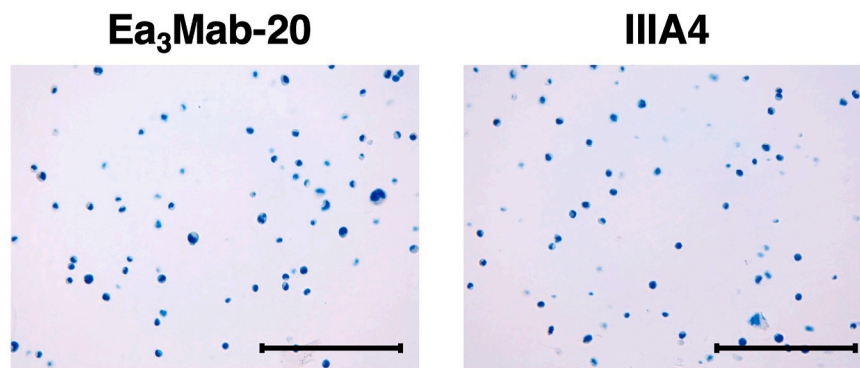
(Fig. 6). Furthermore, the specificity of Ea<sub>3</sub>Mab-20 was confirmed using EphA3-knockout Jurkat cells (Fig. 3C). Since Ea<sub>3</sub>Mab-20 does not cross-react with other Eph receptors expressed in CHO–K1 cells (Fig. 4), it is versatile for basic research and is expected to contribute to the development of clinical applications of mAbs for cancer therapy and diagnosis.

The cross-reactivity of IIIA4 with EphA6 may be due to the similarity of the extracellular domain among Eph receptors. The amino acid identity and similarity of the extracellular domain among human Eph receptors range from 33 to 65% and 72–91%, respectively (Supplementary Fig. 3A). The extracellular domain of EphA6 is the most similar in the phylogenetic tree among Eph receptors (Supplementary Fig. 3B), exhibiting a high amino acid identity (64%) and similarity (91%) with that of EphA3. Furthermore, the IIIA4 has been reported to recognize a conformation-specific epitope within the ephrin-binding domain (amino acids 29–202) of EphA3 [37]. The structural similarity of this epitope between EphA3 and EphA6 may explain why an anti-EphA3 mAb IIIA4 cross-reacts with EphA6. Additionally, several clones of the obtained anti-EphA3 mAbs showed cross-reactivity with various Eph receptors, including EphA6 (Supplementary Table 1). Since the development of therapeutic mAbs requires strict specificity to avoid

## A CHO/EphA3



## B CHO-K1



**Fig. 6.** Immunohistochemical staining of paraffin-embedded cell sections of CHO/EphA3 and CHO-K1 cells. Sections of CHO/EphA3 (A) and CHO-K1 (B) cells were stained with 10  $\mu\text{g}/\text{mL}$  of Ea<sub>3</sub>Mab-20 and IIIA4 using the VENTANA BenchMark ULTRA PLUS. Scale bar = 100  $\mu\text{m}$ .

off-target effects caused by cross-reactivity, Ea<sub>3</sub>Mab-20 is a promising candidate for further development as a therapeutic and diagnostic agent.

The binding affinity of IIIA4 is approximately 3.8-fold higher than that of Ea<sub>3</sub>Mab-20 when analyzed using CHO/EphA3 cells (Fig. 5). However, this ratio increases to approximately 24.6-fold when using Jurkat cells endogenously express EphA3 (Fig. 5). This discrepancy may be due to the endogenous expression of EphA6 in Jurkat cells [35], which could have influenced the affinity analysis through the cross-reactivity of IIIA4 with EphA6. The mAbs with high specificity for Eph receptors, such as Ea<sub>3</sub>Mab-20, are crucial for clinical applications, even though the binding affinity of Ea<sub>3</sub>Mab-20 ( $K_D$ :  $9.0 \pm 0.3 \times 10^{-9}$  M) is lower than that of IIIA4 ( $K_D$ :  $2.4 \pm 0.3 \times 10^{-9}$  M) in CHO/EphA3 cells.

Several preclinical studies reported that anti-EphA3 CAR-T cells are effective against glioblastoma [27,28]. Regarding the binding affinity of CARs consisting of scFv, it has been reported that a low-affinity CAR ( $K_D$ :  $1.4 \times 10^{-8}$  M), which exhibits more than 40-fold lower affinity for CD19 compared to existing scFvs derived from FMC63, enhances CAR-T cell expansion and prolongs persistence in pediatric patients with ALL [38]. Additionally, it has been noted that a faster off-rate, which reflects the rate at which the antibody dissociates from the antigen, is preferred, particularly in CAR-T therapies. The on-rate and off-rate of Ea<sub>3</sub>Mab-20 should be determined. However, its affinity for CHO/EphA3 and Jurkat cells is on the order of  $10^{-9}$  M, which may provide sufficient potential for CAR development.

We have previously developed two methods for epitope mapping: PA insertion for epitope mapping (PAMAP) and RIEDL insertion for epitope

mapping (REMAP) [39–43]. These approaches have successfully identified the epitopes of various mAbs, including anti-mouse CD39 mAb (C<sub>39</sub>Mab-1) [39], anti-CD44 mAbs (C<sub>44</sub>Mab-5 and C<sub>44</sub>Mab-46) [40,41], and anti-EGFR mAbs (EMab-51 and EMab-134) [42,43]. Further investigation is necessary to determine the epitope of Ea<sub>3</sub>Mab-20. If Ea<sub>3</sub>Mab-20 targets a linear and non-glycosylated epitope, this finding could facilitate the development of broadly applicable and highly specific mAbs against other Eph family members through peptide-based immunization strategies.

To effectively target EphA3-positive cancer cells using Ea<sub>3</sub>Mab-20 (IgG<sub>1</sub>), generating a class-switched variant with a mouse IgG<sub>2a</sub> backbone would be beneficial. Additionally, our previous studies demonstrated that defucosylated IgG<sub>2a</sub> mAbs enhance ADCC activity and exhibit more potent antitumor effects in mouse xenograft models [44,45]. Developing a class-switched and defucosylated version of Ea<sub>3</sub>Mab-20 could improve its therapeutic efficacy against EphA3-positive cancers in preclinical research. Furthermore, it is also essential to evaluate whether Ea<sub>3</sub>Mab-20 demonstrates ADCC activity or drug-induced cytotoxicity when conjugated with cytotoxic agents.

Altogether, Ea<sub>3</sub>Mab-20 reacts with EphA3 without cross-reactivity with Eph family members and is suitable for flow cytometry and immunohistochemistry. EphA3 is a potential therapeutic target, especially in hematopoietic malignancies and brain cancers. Therefore, Ea<sub>3</sub>Mab-20 is a highly sensitive and versatile mAb for basic research and is expected to contribute to clinical applications such as antibody therapy and tumor diagnosis.



## CRediT authorship contribution statement

**Hiroyuki Satofuka:** Writing – original draft, Investigation, Funding acquisition. **Hiroyuki Suzuki:** Writing – review & editing, Investigation, Funding acquisition. **Miu Hirose:** Investigation. **Keisuke Shinoda:** Investigation. **Takuya Nakamura:** Investigation. **Tomohiro Tanaka:** Investigation, Funding acquisition. **Mika K. Kaneko:** Conceptualization. **Yukinari Kato:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

## Author disclosure statement

The authors have no conflict of interest in this article.

## Funding information

This research was supported in part by Japan Agency for Medical Research and Development (AMED) under Grant Numbers: JP24am0521010 (to Y.K.), JP24ama121008 (to Y.K.), JP24ama221339 (to Y.K.), JP24bm1123027 (to Y.K.), and JP24ck0106730 (to Y.K.), and by the Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research (KAKENHI) grant nos. 24K11652 (to H. Satofuka), 22K06995 (to H. Suzuki), 24K18268 (to T.T.), and 25K10553 (to Y.K.).

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrep.2025.102130>.

## References

- [1] A. Barquilla, E.B. Pasquale, Eph receptors and ephrins: therapeutic opportunities, *Annu. Rev. Pharmacol. Toxicol.* 55 (2015) 465–487, <https://doi.org/10.1146/annurev-pharmtox-011112-140226>.
- [2] T.K. Darling, T.J. Lamb, Emerging roles for eph receptors and ephrin ligands in immunity, *Front. Immunol.* 10 (2019) 1473, <https://doi.org/10.3389/fimmu.2019.01473>.
- [3] L.Y. Liang, O. Patel, P.W. Janes, J.M. Murphy, I.S. Lucet, Eph receptor signalling: from catalytic to non-catalytic functions, *Oncogene* 38 (2019) 6567–6584, <https://doi.org/10.1038/s41388-019-0931-2>.
- [4] E.B. Pasquale, Eph receptors and ephrins in cancer progression, *Nat. Rev. Cancer* 24 (2024) 5–27, <https://doi.org/10.1038/s41568-023-00634-x>.
- [5] O.J. Buckens, B. El Hassouni, E. Giovannetti, G.J. Peters, The role of Eph receptors in cancer and how to target them: novel approaches in cancer treatment, *Expert Opin. Invest. Drugs* 29 (2020) 567–582, <https://doi.org/10.1080/13543784.2020.1762566>.
- [6] A. Poliakov, M. Cotrina, D.G. Wilkinson, Diverse roles of eph receptors and ephrins in the regulation of cell migration and tissue assembly, *Dev. Cell* 7 (2004) 465–480, <https://doi.org/10.1016/j.devcel.2004.09.006>.
- [7] E.B. Pasquale, Eph receptor signalling casts a wide net on cell behaviour, *Nat. Rev. Mol. Cell Biol.* 6 (2005) 462–475, <https://doi.org/10.1038/nrm1662>.
- [8] M.P. Beckmann, D.P. Cerretti, P. Baum, T. Vanden Bos, L. James, T. Farrah, C. Kozlosky, T. Hollingsworth, H. Shilling, E. Maraskovsky, et al., Molecular characterization of a family of ligands for eph-related tyrosine kinase receptors, *EMBO J.* 13 (1994) 3757–3762, <https://doi.org/10.1002/j.1460-2075.1994.tb06685.x>.
- [9] H. Hirai, Y. Maru, K. Hagiwara, J. Nishida, F. Takaku, A novel putative tyrosine kinase receptor encoded by the eph gene, *Science* 238 (1987) 1717–1720, <https://doi.org/10.1126/science.2825356>.
- [10] E.M. Lisabeth, G. Falivelli, E.B. Pasquale, Eph receptor signaling and ephrins, *Cold Spring Harbor Perspect. Biol.* 5 (2013), <https://doi.org/10.1101/cshperspect.a009159>.
- [11] E. Battle, D.G. Wilkinson, Molecular mechanisms of cell segregation and boundary formation in development and tumorigenesis, *Cold Spring Harbor Perspect. Biol.* 4 (2012) a008227, <https://doi.org/10.1101/cshperspect.a008227>.
- [12] E.B. Pasquale, Eph-ephrin bidirectional signaling in physiology and disease, *Cell* 133 (2008) 38–52, <https://doi.org/10.1016/j.cell.2008.03.011>.
- [13] G. Hanover, F.S. Vizeacoumar, S.L. Banerjee, R. Nair, R. Dahiya, A.I. Osornio-Hernandez, A.M. Morales, T. Freywald, J.P. Himanen, B.M. Toosi, N. Bisson, F. J. Vizeacoumar, A. Freywald, Integration of cancer-related genetic landscape of Eph receptors and ephrins with proteomics identifies a crosstalk between EPHB6 and EGFR, *Cell Rep.* 42 (2023) 112670, <https://doi.org/10.1016/j.celrep.2023.112670>.
- [14] H.Q. Xi, X.S. Wu, B. Wei, L. Chen, Eph receptors and ephrins as targets for cancer therapy, *J. Cell Mol. Med.* 16 (2012) 2894–2909, <https://doi.org/10.1111/j.1582-4934.2012.01612.x>.
- [15] M. London, E. Gallo, Critical role of EphA3 in cancer and current state of EphA3 drug therapeutics, *Mol. Biol. Rep.* 47 (2020) 5523–5533, <https://doi.org/10.1007/s11033-020-05571-8>.
- [16] S. Charmsaz, F. Al-Ejeh, T.M. Yeadon, K.J. Miller, F.M. Smith, B.W. Stringer, A. S. Moore, F.T. Lee, L.T. Cooper, C. Stylianou, G.T. Yarranton, J. Woronicz, A. M. Scott, M. Lackmann, A.W. Boyd, EphA3 as a target for antibody immunotherapy in acute lymphoblastic leukemia, *Leukemia* 31 (2017) 1779–1787, <https://doi.org/10.1038/leu.2016.371>.
- [17] B.W. Day, B.W. Stringer, F. Al-Ejeh, M.J. Ting, J. Wilson, K.S. Ensby, P. R. Jamieson, Z.C. Bruce, Y.C. Lim, C. Offenhausser, S. Charmsaz, L.T. Cooper, J. K. Ellacott, A. Harding, L. Leveque, P. Inglis, S. Allan, D.G. Walker, M. Lackmann, G. Osborne, K.K. Khanna, B.A. Reynolds, J.D. Lickliter, A.W. Boyd, EphA3 maintains tumorigenicity and is a therapeutic target in glioblastoma multiforme, *Cancer Cell* 23 (2013) 238–248, <https://doi.org/10.1016/j.ccr.2013.01.007>.
- [18] B. Nasri, M. Inokuchi, T. Ishikawa, H. Uetake, Y. Takagi, S. Otsuki, K. Kojima, T. Kawano, High expression of EphA3 (erythropoietin-producing hepatocellular A3) in gastric cancer is associated with metastasis and poor survival, *BMC Clin. Pathol.* 17 (2017) 8, <https://doi.org/10.1186/s12907-017-0047-y>.
- [19] S.H. Kim, W.H. Lee, S.W. Kim, H.U. Je, J.C. Lee, H.W. Chang, Y.M. Kim, K. Kim, S. Y. Kim, M.W. Han, EphA3 maintains radioresistance in head and neck cancers through epithelial mesenchymal transition, *Cell. Signal.* 47 (2018) 122–130, <https://doi.org/10.1016/j.cellsig.2018.04.001>.
- [20] R. Wu, H. Wang, J. Wang, P. Wang, F. Huang, B. Xie, Y. Zhao, S. Li, J. Zhou, EphA3, induced by PC-1/PrLZ, contributes to the malignant progression of prostate cancer, *Oncol. Rep.* 32 (2014) 2657–2665, <https://doi.org/10.3892/or.2014.3482>.
- [21] J. Peng, Q. Wang, H. Liu, M. Ye, X. Wu, L. Guo, EPHA3 regulates the multidrug resistance of small cell lung cancer via the PI3K/BMX/STAT3 signaling pathway, *Tumour Biol* 37 (2016) 11959–11971, <https://doi.org/10.1007/s13277-016-5048-4>.
- [22] E.M. Lisabeth, C. Fernandez, E.B. Pasquale, Cancer somatic mutations disrupt functions of the EphA3 receptor tyrosine kinase through multiple mechanisms, *Biochemistry* 51 (2012) 1464–1475, <https://doi.org/10.1021/bi2014079>.
- [23] M.E. Vail, C. Murone, A. Tan, L. Hii, D. Abebe, P.W. Janes, F.T. Lee, M. Baer, V. Palath, K. Bebbington, G. Yarranton, C. Llerena, S. Garic, D. Abramson, G. Cartwright, A.M. Scott, M. Lackmann, Targeting EphA3 inhibits cancer growth by disrupting the tumor stromal microenvironment, *Cancer Res.* 74 (2014) 4470–4481, <https://doi.org/10.1158/0008-5472.CAN.14-0218>.
- [24] R.T. Swords, P.L. Greenberg, A.H. Wei, S. Durrant, A.S. Advani, M.S. Hertzberg, B. A. Jonas, I.D. Lewis, G. Rivera, D. Gratzinger, A.C. Fan, D.W. Felsher, J.E. Cortes, J. M. Watts, G.T. Yarranton, J.M. Walling, J.E. Lancet, KB004, a first in class monoclonal antibody targeting the receptor tyrosine kinase EphA3, in patients with advanced hematologic malignancies: results from a phase 1 study, *Leuk. Res.* 50 (2016) 123–131, <https://doi.org/10.1016/j.leukres.2016.09.012>.
- [25] N. Tomasevic, K. Luehrs, M. Baer, V. Palath, D. Martinez, J. Williams, C. Yi, S. Sujatha-Bhaskar, R. Lanke, J. Leung, W. Ching, A. Lee, L. Bai, G. Yarranton, C. Bebbington, A high affinity recombinant antibody to the human EphA3 receptor with enhanced ADCC activity, *Growth Factors* 32 (2014) 223–235, <https://doi.org/10.3109/08977194.2014.984808>.
- [26] X.Y. Lv, J. Wang, F. Huang, P. Wang, J.G. Zhou, B. Wei, S.H. Li, EphA3 contributes to tumor growth and angiogenesis in human gastric cancer cells, *Oncol. Rep.* 40 (2018) 2408–2416, <https://doi.org/10.3892/or.2018.6586>.
- [27] L. Lertsumitkul, M. Iliopoulos, S.S. Wang, S.J. McArthur, L.M. Ebert, A. J. Davenport, R. Endersby, J.R. Hansford, K.J. Drummond, R. Cross, M.R. Jenkins, EphA3-targeted chimeric antigen receptor T cells are effective in glioma and generate curative memory T cell responses, *J. Immunother. Cancer* 12 (2024), <https://doi.org/10.1136/jitc-2024-009486>.
- [28] P. Martins, R.C.J. D'Souza, N. Skarne, L. Lekieff, S. Horsefield, M. Ranjankumar, X. Li, T.T. Le, F. Smith, C. Smith, J. Burrows, B.W. Day, R. Khanna, EphA3 CAR T cells are effective against glioblastoma in preclinical models, *J. Immunother. Cancer* 12 (2024), <https://doi.org/10.1136/jitc-2024-009403>.
- [29] R. Ubukata, H. Suzuki, M. Hirose, H. Satofuka, T. Tanaka, M.K. Kaneko, Y. Kato, Establishment of a highly sensitive and specific anti-EphB2 monoclonal antibody. Eb2Mab-12) for Flow Cytometry, 2025, <https://doi.org/10.36922/mi.5728>. MI.
- [30] T. Tanaka, H. Suzuki, G. Li, S. Fujisawa, M.K. Kaneko, Y. Kato, Ea 1 Mab-30: a Novel Monoclonal Antibody Against Erythropoietin-Producing Hepatocellular Receptor A1 for Versatile Applications Preprint, 2025.
- [31] T. Tanaka, Y. Kaneko, H. Yamamoto, G. Li, S. Fujisawa, H. Satofuka, K. Shinoda, T. Nakamura, M.K. Kaneko, H. Suzuki, Y. Kato, Development of a novel anti-erythropoietin-producing hepatocellular receptor B6 monoclonal antibody Eb(6) Mab-3 for flow cytometry, *Biochem. Biophys. Rep.* 41 (2025) 101960, <https://doi.org/10.1016/j.bbrep.2025.101960>.
- [32] H. Satofuka, H. Suzuki, T. Tanaka, G. Li, M.K. Kaneko, Y. Kato, Development of an anti-human EphA2 monoclonal antibody Ea2Mab-7 for multiple applications, *Biochem. Biophys. Rep.* 42 (2025) 101998, <https://doi.org/10.1016/j.bbrep.2025.101998>.
- [33] R. Nanamiya, H. Suzuki, M.K. Kaneko, Y. Kato, Development of an Anti-EphB4 monoclonal antibody for multiple applications against breast cancers, *Monoclon.*



- Antib. Immunodiagn. Immunother. 42 (2023) 166–177, <https://doi.org/10.1089/mab.2023.0015>.
- [34] T. Tanaka, H. Suzuki, Y. Isoda, T. Asano, T. Nakamura, M. Yanaka, S. Handa, N. Takahashi, S. Okuno, T. Yoshikawa, G. Li, R. Nanamiya, N. Goto, N. Tateyama, Y. Okada, H. Kobayashi, M.K. Kaneko, Y. Kato, Development of a sensitive anti-human CCR9 monoclonal antibody (C(9)Mab-11) by N-Terminal peptide immunization, *Monoclon. Antib. Immunodiagn. Immunother.* 41 (2022) 303–310, <https://doi.org/10.1089/mab.2022.0027>.
- [35] Y. Fujii, M. Kaneko, M. Neyazaki, T. Nogi, Y. Kato, J. Takagi, PA tag: a versatile protein tagging system using a super high affinity antibody against a dodecapeptide derived from human podoplanin, *Protein Expr. Purif.* 95 (2014) 240–247, <https://doi.org/10.1016/j.pep.2014.01.009>.
- [36] Y. Fujii, M.K. Kaneko, Y. Kato, MAP tag: a novel tagging system for protein purification and detection, *Monoclon. Antib. Immunodiagn. Immunother.* 35 (2016) 293–299, <https://doi.org/10.1089/mab.2016.0039>.
- [37] C. Vearing, F.T. Lee, S. Wimmer-Kleikamp, V. Spirkoska, C. To, C. Stylianou, M. Spanevello, M. Brechbiel, A.W. Boyd, A.M. Scott, M. Lackmann, Concurrent binding of anti-EphA3 antibody and ephrin-A5 amplifies EphA3 signaling and downstream responses: potential as EphA3-specific tumor-targeting reagents, *Cancer Res.* 65 (2005) 6745–6754, <https://doi.org/10.1158/0008-5472.CAN-05-0758>.
- [38] S. Ghorashian, A.M. Kramer, S. Onuoha, G. Wright, J. Bartram, R. Richardson, S. J. Albon, J. Casanovas-Company, F. Castro, B. Popova, K. Villanueva, J. Yeung, W. Vetharoy, A. Guvenel, P.A. Wawrzyniecka, L. Mekkaoui, G.W. Cheung, D. Pinner, J. Chu, G. Lucchini, J. Silva, O. Ciocarlie, A. Lazareva, S. Inglott, K. C. Gilmour, G. Ahsan, M. Ferrari, S. Manzoor, K. Champion, T. Brooks, A. Lopes, A. Hackshaw, F. Farzaneh, R. Chiesa, K. Rao, D. Bonney, S. Samarasinghe, N. Goulden, A. Vora, P. Veys, R. Hough, R. Wynn, M.A. Pule, P.J. Amrolia, Enhanced CAR T cell expansion and prolonged persistence in pediatric patients with ALL treated with a low-affinity CD19 CAR, *Nat. Med.* 25 (2019) 1408–1414, <https://doi.org/10.1038/s41591-019-0549-5>.
- [39] Y. Okada, H. Suzuki, T. Tanaka, M.K. Kaneko, Y. Kato, Epitope mapping of an anti-mouse CD39 monoclonal antibody using PA scanning and RIEDL scanning, *Monoclon. Antib. Immunodiagn. Immunother.* 43 (2024) 44–52, <https://doi.org/10.1089/mab.2023.0029>.
- [40] T. Asano, M.K. Kaneko, J. Takei, N. Tateyama, Y. Kato, Epitope mapping of the Anti-CD44 monoclonal antibody (C(44)Mab-46) using the REMAP method, *Monoclon. Antib. Immunodiagn. Immunother.* 40 (2021) 156–161, <https://doi.org/10.1089/mab.2021.0012>.
- [41] T. Asano, M.K. Kaneko, Y. Kato, Development of a novel epitope mapping system: RIEDL insertion for epitope mapping method, *Monoclon. Antib. Immunodiagn. Immunother.* 40 (2021) 162–167, <https://doi.org/10.1089/mab.2021.0023>.
- [42] M. Sano, M.K. Kaneko, T. Asano, Y. Kato, Epitope mapping of an antihuman EGFR monoclonal antibody (EMab-134) using the REMAP method, *Monoclon. Antib. Immunodiagn. Immunother.* 40 (2021) 191–195, <https://doi.org/10.1089/mab.2021.0014>.
- [43] R. Nanamiya, M. Sano, T. Asano, M. Yanaka, T. Nakamura, M. Saito, T. Tanaka, H. Hosono, N. Tateyama, M.K. Kaneko, Y. Kato, Epitope mapping of an anti-human epidermal growth factor receptor monoclonal antibody (EMab-51) using the RIEDL insertion for epitope mapping method, *Monoclon. Antib. Immunodiagn. Immunother.* 40 (2021) 149–155, <https://doi.org/10.1089/mab.2021.0010>.
- [44] K. Ishikawa, H. Suzuki, T. Ohishi, T. Nakamura, M. Yanaka, G. Li, T. Tanaka, A. Ohkoshi, M. Kawada, M.K. Kaneko, Y. Katori, Y. Kato, Antitumor activities of anti-CD44 monoclonal antibodies in mouse xenograft models of esophageal cancer, *Oncol. Rep.* 52 (2024), <https://doi.org/10.3892/or.2024.8806>.
- [45] K. Ishikawa, H. Suzuki, T. Ohishi, G. Li, T. Tanaka, M. Kawada, A. Ohkoshi, M. K. Kaneko, Y. Katori, Y. Kato, Anti-CD44 variant 10 monoclonal antibody exerts antitumor activity in mouse xenograft models of oral squamous cell carcinomas, *Int. J. Mol. Sci.* 25 (2024), <https://doi.org/10.3390/ijms25179190>.