A Novel Anti-Cadherin 8 Monoclonal Antibody, Ca_8Mab-4, for Versatile Applications

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Posted Date: 14 January 2026

doi: 10.20944/preprints202601.1012.v1

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Article

A Novel Anti-Cadherin 8 Monoclonal Antibody, Ca8Mab-4, for Versatile Applications

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Abstract

Cadherin-8 (CDH8) is a type II cadherin that plays crucial roles in various aspects of neural development and disease. Although anti-CDH8 monoclonal antibodies (mAbs) are available for Western blotting and immunohistochemistry (IHC), anti-CDH8 mAbs suitable for flow cytometry have not been reported. In this study, we developed novel anti-human CDH8 mAbs (named Ca8Mabs) using a flow cytometry-based high-throughput screening method. Among these, a clone called Ca8Mab-4 (IgG_1, κ) specifically recognized CDH8-overexpressing Chinese hamster ovary-K1 (CHO/CDH8) cells, with no detectable cross-reactivity toward 21 other cadherins, including both type I and type II, by flow cytometry. Additionally, Ca8Mab-4 detected endogenous CDH8 in the human esophageal squamous cell carcinoma cell line TE5. The dissociation constant (K_D) values for Ca8Mab-4 binding to CHO/CDH8 and TE5 were estimated to be 3.8×10^{-9} M and 4.9×10^{-10} M, respectively. Furthermore, Ca8Mab-4 was effective in Western blotting and IHC. Overall, these findings suggest that Ca8Mab-4 is a versatile tool for basic research and holds potential for tumor diagnosis and therapy.

Keywords: cadherin-8; CDH8; monoclonal antibody; Cell-Based Immunization and Screening; flow cytometry; immunohistochemistry

1. Introduction

The cadherin superfamily includes over 100 cell-surface glycoproteins, which are characterized by conserved extracellular cadherin repeats [1,2]. The first identified cadherins (E-Cadherin/CDH1, N-Cadherin/CDH2, P-Cadherin/CDH3) and their closest relatives are classified as classical cadherins. These are divided into type I (CDH1–CDH4 and CDH15) and type II (CDH5–CDH12, CDH18–CDH20, CDH22, and CDH24) cadherins [3]. Classical cadherins are crucial for tissue development and maintenance in vertebrates [4]. In the nervous system, they participate in a wide range of developmental processes, including neurulation, neuronal migration, neurite outgrowth, axonal fasciculation, synaptic differentiation, and synaptic plasticity [5].

Each cadherin in the brain is expressed in specific groups of functionally connected nuclei and laminae [6]. A type II classical cadherin, cadherin-8 (CDH8), plays a crucial role in cold sensation, with its neural circuitry formed by sensory neurons projecting into the spinal cord [7]. The CDH8-expressing sensory neurons were found to connect to CDH8-expressing dorsal horn neurons in the spinal cord, and CDH8 was located near the synaptic junctions formed between these neuronal groups [7].

The neuron-specific transcription factor T-box brain 1 (TBR1) is crucial for brain development [8]. TBR1 haploinsufficiency changed the expression of CDH8, resulting in decreased inter- and intra-amygdalar connectivity and cognitive problems in a mouse model [9]. These developmental abnormalities are likely to impair neuronal activation in response to behavioral stimuli, as evidenced by a reduced number of c-FOS–positive neurons in the TBR1 (+/−) amygdalae [9].

 doi:10.20944/preprints202601.1012.v1

Autism spectrum disorder is characterized by impairments in social communication and learning disability and is implicated to arise from aberrant synaptic connectivity [10]. For instance, rare variants in the neuroligin and neurexin genes, which encode synaptic adhesion molecules that interact across the synaptic cleft, have been linked to increased susceptibility to autism [11,12]. Furthermore, rare familial microdeletions on chromosome 16q21 that disrupt CDH8 were identified in families with autism spectrum disorder and learning disabilities [13]. In a family, three of the four boys with autism and learning disabilities inherited the deletion, but it was not present in their four unaffected siblings or their unaffected mother [13]. Therefore, CDH8 is proposed as a susceptibility factor for autism and learning disabilities.

Monoclonal antibodies (mAbs) that detect CDH8 by Western blotting or immunohistochemistry (IHC) have been developed for various applications; however, suitable mAbs for flow cytometry are not currently available. Using the Cell-Based Immunization and Screening (CBIS) method, our laboratory has previously developed anti-CDH1 [14] and anti-CDH15 [15] mAbs for use in flow cytometry, Western blotting, and IHC. The CBIS method involves high-throughput flow cytometry–based screening, and mAbs produced using this approach usually recognize conformational epitopes, which allows their use in flow cytometry. Notably, some of these mAbs are also compatible with Western blotting and IHC. In this study, we used the CBIS method to develop highly versatile anti-CDH8 mAbs.

2. Materials and Methods

2.1. Cell Lines

Mouse myeloma P3X63Ag8U.1 (P3U1), human glioblastoma (GBM) LN229, and Chinese hamster ovary (CHO)-K1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human esophageal squamous cell carcinoma (SCC) TE5 was obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer at Tohoku University (Miyagi, Japan).

2.2. Stable Transfectants

Genes encoding human *CDH8* (NM_001796.5) were obtained from the RIKEN BioResource Research Center (Ibaraki, Japan). The *CDH8* cDNA was subcloned into the pCAG-Ble vector with an N-terminal MAP16 tag [16]. Additionally, the *CDH8* cDNA with an N-terminal PA16 tag [17] was constructed. These plasmids were transfected into LN229 or CHO-K1cells, and stable transfectants were sorted using an anti-MAP16 tag mAb (clone PMab-1) [16] or an anti-PA16 tag mAb (clone NZ-1) [17] using the Neon transfection system (Thermo Fisher Scientific, Inc.). Finally, MAP16-CDH8-overexpressed LN229 (LN229/CDH8) and PA16-CDH8-overexpressed CHO-K1 (CHO/CDH8) were established.

Type I cadherin-overexpressed CHO-K1 cell lines were previously established in [14]. Type II cadherin-overexpressed CHO-K1 cell lines were established previously [18]. Truncated, seven-domain (7D), and atypical cadherin overexpressed CHO-K1 cell lines were previously established in [18]. Each cadherin expression was confirmed using an anti-CDH1 mAb (clone Ca_1Mab-3 [14],), an anti-CDH3 mAb (clone MM0508-9V11, Abcam, Cambridge, UK), an anti-CDH6 mAb (clone 427909, R&D Systems Inc., Minneapolis, MN, USA), an anti-CDH15 mAb (clone Ca_{15}Mab-1 [15]), an anti-CDH17 mAb (clone 2618, Thermo Fisher Scientific, Inc.), and another anti-PA16-tag mAb (clone NZ-33 [19]) to detect other cadherins.

2.3. Production of Hybridomas

Female BALB/cAJcl mice (CLEA Japan, Tokyo, Japan) were intraperitoneally immunized with LN229/CDH8 cells (1×10^8 cells/injection) mixed with 2% Alhydrogel adjuvant (InvivoGen, San Diego, CA, USA). Following three additional weekly immunizations (1.0×10^8 cells/injection), a

 doi:10.20944/preprints202601.1012.v1

booster dose (1 × 10^8 cells/injection) was administered two days before spleen excision. Hybridomas were produced as previously described [15].

2.4. Flow Cytometry Analysis and Determination of Dissociation Constant Values

CHO/CDH8 and TE5 cells were harvested with 1 mM EDTA were washed with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA; blocking buffer). The cells and incubated with Ca8Mab-4 and flow cytometric data were acquired and the dissociation constant (K_D) values were calculated as described previously [15].

2.5. Western Blotting

Western blotting was performed using 1 μg/mL Ca8Mab-4, 1 μg/mL of NZ-1, or 1 μg/mL of an anti-isocitrate dehydrogenase 1 (IDH1) mAb (clone RcMab-1) as described previously [15].

2.6. IHC Using Cell Blocks

The formalin-fixed paraffin-embedded (FFPE) cell sections were stained with Ca8Mab-4 (0.1 or 10 μg/mL), MpMab-2 (10 μg/mL, IgG_1 isotype control, http://www.med-tohoku-antibody.com/topics/001_paper_antibody_PDIS.htm), or NZ-33 (0.01 μg/mL) using the *BenchMark* ULTRA *PLUS with* OptiView DAB IHC Detection Kit *or* ultraView Universal DAB Detection Kit *(Roche Diagnostics,* Indianapolis, IN, USA*).*

3. Results

3.1. Development of Anti-CDH8 mAbs by the CBIS Method

An immunogen, LN229/CDH8, was prepared as described in the Materials and Methods. LN229/CDH8 (1 × 10^8 cells/mouse) was intraperitoneally injected five times into two BALB/cAJcl mice (Figure 1A). Hybridomas were produced by fusing splenocytes with myeloma P3U1 (Figure 1B). The supernatants of the hybridoma were screened to identify those positive for CHO/CDH8 and negative for CHO-K1 (Figure 1C). As a result, 54 positive wells out of 956 (5.6%) were found. Limiting dilution was then performed to clone hybridomas producing anti-CDH8 mAb (Figure 1D). Finally, 4 clones were established, and the purified mAbs were prepared.

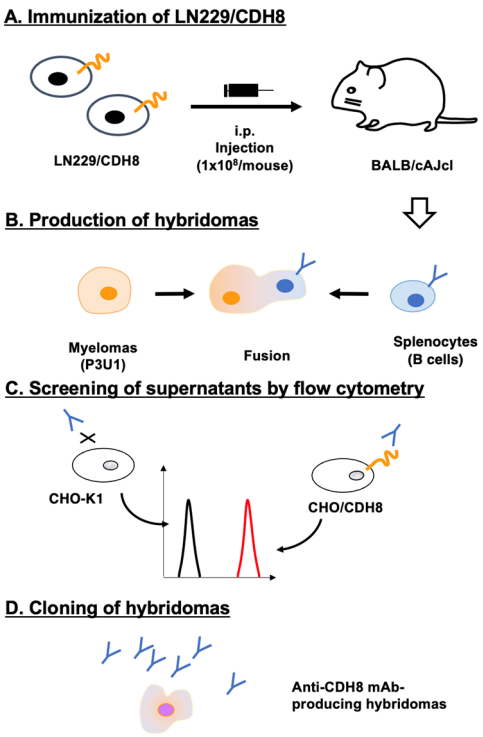

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Figure 1. Schematic representation of anti-CDH8 mAbs production. (A) BALB/cAJcl mice were intraperitoneally injected with LN229/CDH8. (B) After five immunizations, splenocytes were fused with P3U1. (C) The supernatants from hybridomas were screened using CHO-K1 and CHO/CDH8 by flow cytometry. (D) Ca8Mabs, anti-CDH8 mAb-producing hybridoma clones, were established through limiting dilution.

3.2. Flow Cytometry Analyses of Ca8Mab-4 Against CHO-K1, CHO/CDH8, and TE5

Among four clones, we selected Ca8Mab-4 (IgG_1, κ) based on its reactivity in flow cytometry and suitability for Western blotting (http://www.med-tohoku-antibody.com/topics/001_paper_antibody_PDIS.htm). Figure 2 shows the flow cytometry analysis using Ca8Mab-4 against CHO/CDH8 and CHO-K1. Ca8Mab-4 reacted in a dose-dependent manner with CHO/CDH8 from 10 to 0.01 μg/mL (Figure 2A). In contrast, Ca8Mab-4 did not recognize CHO-K1 even at 10 μg/mL (Figure 2A). Additionally, Ca8Mab-4 reacted with human esophageal SCC TE5 in a dose-dependent way (Figure 2B), indicating that TE5 expresses endogenous CDH8. The binding affinity of Ca8Mab-4 was assessed through flow cytometry. The fitted binding isotherms of Ca8Mab-4 binding to CHO/CDH8 and TE5 are shown in Figure 2C. The K_D values were 3.8×10^{-9} M for CHO/CDH8 and 4.9×10^{-10} M for TE5. These findings demonstrate that Ca8Mab-4 has a high binding affinity for CDH8-positive cell lines.

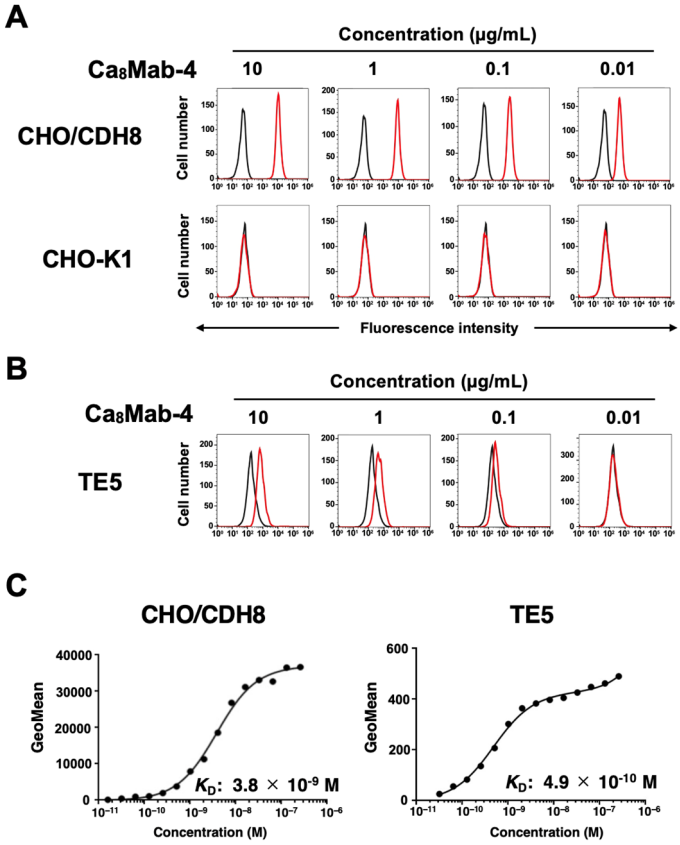

Figure 2. Flow cytometric analysis of Ca8Mab-4. (A) CHO-K1 and CHO/CDH8 were treated with Ca8Mab-4 at the indicated concentrations (red) or with blocking buffer (black, negative control). (B) Human esophageal SCC TE5 was treated with Ca8Mab-4 at the indicated concentrations (red) or with blocking buffer (black, negative control). The mAbs-treated cells were incubated with Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were collected using the SA3800 Cell Analyzer. (C) The determination of the dissociation constant of Ca8Mab-4. CHO/CDH8 and TE5 were suspended in serially diluted Ca8Mab-4. Then, cells were treated with Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were subsequently collected using the SA3800 Cell Analyzer. The K_D values were calculated by GraphPad PRISM 6.

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3.3. Determination of the Specificity of Ca8Mab-4 Using CDHs-Overexpressed CHO-K1

We previously established CHO-K1 cells, which overexpressed type I cadherins (CDH1–CDH4 and CDH15) [14,15], type II cadherins (CDH5–CDH12, CDH18–CDH20, CDH22, and CDH24), a truncated cadherin (CDH13), 7D cadherins (CDH16 and CDH17), and an atypical cadherin (CDH26) [18]. Therefore, the specificity of Ca8Mab-4 to those cadherins was determined. As shown in Figure 3A, Ca8Mab-4 recognized CHO/CDH8 but did not react with other cadherins-overexpressed CHO-K1 cells. The cell surface expression of each cadherin was confirmed in Figure 3B. These results indicate that Ca8Mab-4 is a specific mAb to CDH8 among those CDHs.

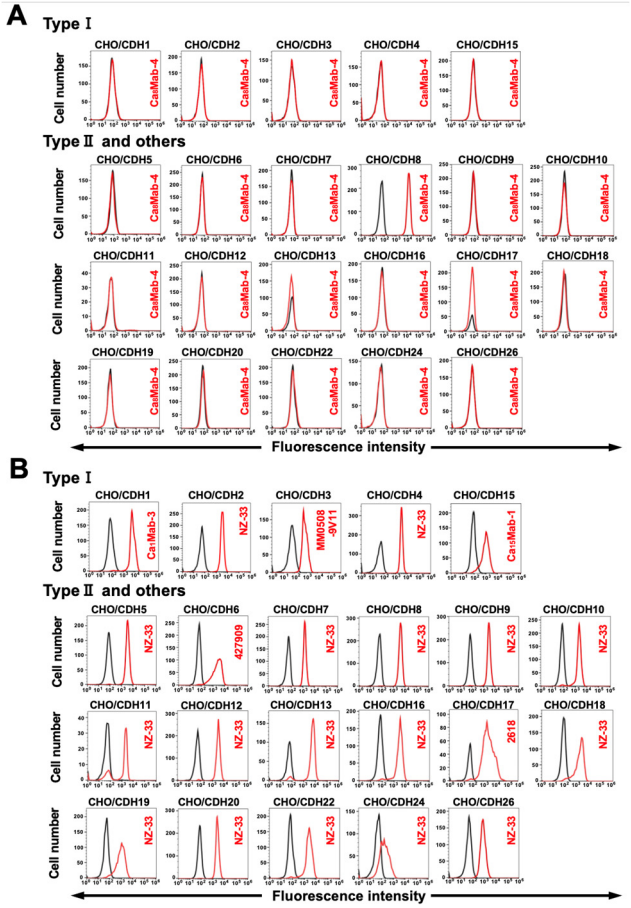

Figure 3. Specificity of Ca8Mab-4. (A) The type I cadherins (CDH1, CDH2, CDH3, CDH4, and CDH15), type II cadherins (CDH5, CDH6, CDH7, CDH8, CDH9, CDH10, CDH11, CDH12, CDH18, CDH8, CDH20, CDH22, and CDH24), a truncated cadherin (CDH13), 7D cadherins (CDH16 and CDH17), and an atypical cadherin (CDH26)-overexpressed CHO-K1 were treated with 10 μg/mL of Ca8Mab-4 (red) or with control blocking buffer (black, negative control), followed by treatment with anti-mouse IgG conjugated with Alexa Fluor 488. (B) Each cadherin expression was confirmed by 1 μg/mL of an anti-CDH1 mAb (clone Ca1Mab-3), 1 μg/mL of an anti-CDH3 mAb (clone MM0508-9V11), 1 μg/mL of an anti-CDH6 mAb (clone 427909), 1 μg/mL of an anti-CDH15 mAb (clone Ca15Mab-1), 1 μg/mL of an anti-CDH17 mAb (clone 2618), and 1 μg/mL of an anti-PA16-tag mAb (clone NZ-33) to detect other CDHs, followed by the treatment with Alexa Fluor 488-conjugated secondary mAbs. The fluorescence data were collected using the SA3800 Cell Analyzer.

3.4. Western Blotting Using Ca8Mab-4

We next tested whether Ca8Mab-4 is suitable for Western blotting. Whole-cell lysates from CHO-K1 and CHO/CDH8 were analyzed. Ca8Mab-4 detected bands around 63–100 kDa in CHO/CDH8, but not in CHO-K1 (Figure 4A). An anti-PA16 mAb (NZ-1) primarily detected 100 kDa in CHO/CDH8 (Figure 4B). An internal control, IDH1, was detected by RcMab-1 (Figure 4C). These results demonstrate that Ca8Mab-4 can detect CDH8 in Western blotting.

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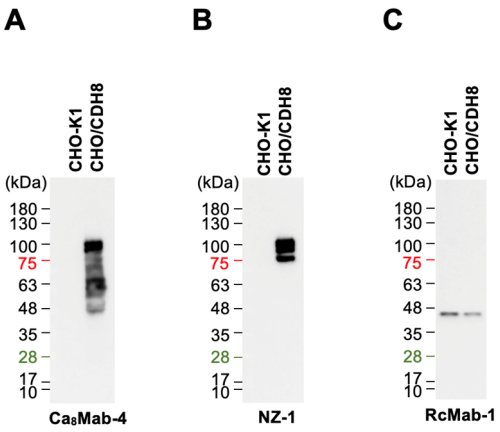

Figure 4. Western blotting using Ca8Mab-4. Cell lysates (10 μg/lane) from CHO-K1 and CHO/CDH8 were electrophoresed and transferred to polyvinylidene difluoride membranes. The membranes were incubated with 1 μg/mL of Ca8Mab-4 (A), 1 μg/mL of NZ-1 (B), or 1 μg/mL of RcMab-1 (an anti-IDH1 mAb) (C), followed by the treatment with anti-mouse (Ca8Mab-4) or anti-rat IgG (NZ-1 and RcMab-1)-conjugated with horseradish peroxidase.

3.5. IHC Using Ca8Mab-4 in FFPE Cell Blocks

We next tested whether Ca8Mab-4 is suitable for IHC in FFPE sections from CHO-K1 and CHO/CDH8. Ca8Mab-4 showed intense membranous and cytoplasmic staining in CHO/CDH8 but not in CHO-K1 (Figure 5A). Additionally, an anti-PA16 tag mAb (NZ-33) exhibited a similar staining (Figure 5B). Ca8Mab-4 also showed a membranous staining in TE5, but the isotype control mAb (MpMab-2) did not. These results indicate that Ca8Mab-4 can detect exogenous and endogenous CDH8 in IHC of FFPE sections of cultured cells.

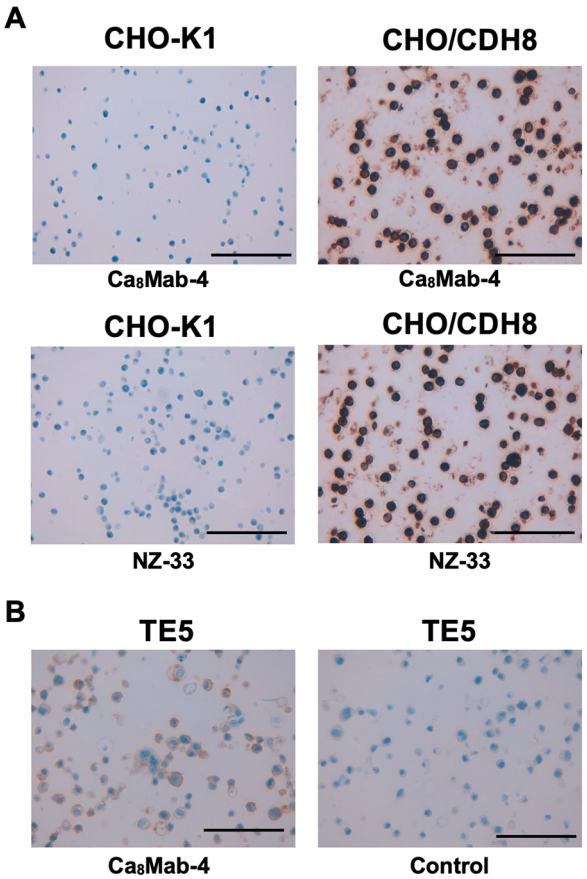

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Figure 5. Immunohistochemistry using Ca8Mab-4 in formalin-fixed paraffin-embedded cell blocks. (A) CHO-K1 and CHO/CDH8 sections were treated with 0.1 μg/mL of Ca8Mab-4 or 0.01 μg/mL of NZ-33. The staining was performed using *BenchMark* ULTRA *PLUS with* the ultraView Universal DAB Detection Kit. (B) TE5 sections were treated with 10 μg/mL of Ca8Mab-4 or 10 μg/mL of MpMab-2 (IgG_1 isotype control). The staining was performed using *BenchMark* ULTRA *PLUS with* the OptiView DAB IHC Detection Kit. Scale bar = 100 μm.

4. Discussion

CDH8 has five extracellular cadherin repeats, one of which mediates calcium-dependent homophilic and heterophilic interactions [20]. In this study, we developed a novel anti-CDH8 mAb using the CBIS method (Figure 1). A clone Ca8Mab-4 showed strong recognition of both exogenous and endogenous CDH8 in flow cytometry and IHC (Figures 3 and 5). Importantly, Ca8Mab-4 exhibited high affinity (Figure 2C) and specificity for CDH8 without detectable cross-reactivity to other 21 cadherins, including type II, type I, 7D, truncated, and atypical CDH (Figure 3). Therefore, Ca8Mab-4 could be helpful for isolating CDH8-positive cells using fluorescence-activated cell sorting. Since cross-reactivity data are unavailable for commercially supplied mAbs, careful validation is necessary, and caution should be exercised when using these mAbs. Additionally, identifying the epitope recognized by Ca8Mab-4 will be crucial for developing highly specific anti-CDH8 mAbs. Furthermore, Ca8Mab-4 is suitable for IHC of cell block specimens (Figure 5). Notably, IHC was performed on an automated slide-staining system, ensuring standardized and reproducible staining conditions. Overall, Ca8Mab-4 is a versatile antibody with broad applications in basic research and potential clinical use.

We found that Ca8Mab-4 recognized the human esophageal SCC TE5 cell line in flow cytometry and IHC (Figure 3 and Figure 5). Although we examined the reactivity of Ca8Mab-4 in other esophageal SCC and glioblastoma cell lines, TE5 is the only cell line recognized by Ca8Mab-4. No studies have examined the role of CDH8 in tumors. Further studies will be essential to clarify the roles of CDH8 in tumor proliferation and metastasis, as well as its expression in various human tumors. Although the extracellular domain of cadherins mediates calcium-dependent homophilic binding [2], CDH8 was reported to make not only homophilic binding, but also heterophilic one with another type II cadherin, CDH11 [21]. Since CDH11/OB-cadherin is predominantly expressed in mesenchymal cells and involved in fibrosis [22], the interaction between CDH8-positive tumor cells and CDH11-positive mesenchymal cells in the tumor microenvironment should be investigated in future studies [23].

We previously cloned cDNAs from hybridomas and produced recombinant mouse IgG_{2a}-type mAbs to enhance antibody-dependent cellular cytotoxicity (ADCC). Using human tumor xenograft models, antitumor activities have been evaluated [24,25]. We have cloned the cDNA of Ca8Mab-4, and the IgG_{2a}-type Ca8Mab-4 will be produced and evaluated for *in vitro* ADCC and antitumor efficacies in mouse tumor xenograft models.

Using *in situ* hybridization, *CDH8* was detected in the developing cortex of a 9-week-old human embryo [13]. As shown in Figure 5, Ca8Mab-4 is suitable for IHC. Therefore, Ca8Mab-4 will contribute to the analysis of the distribution and subcellular localization of CDH8 in the human central nervous system. CDH8 is a TBR1 target in the cortex [26], and these have been implicated as risk factors in behavioral disorders such as autism [27,28]. This pathway is consistent with the hypothesis that dendritic defects contribute to the pathogenesis of disorders arising from aberrant neuronal wiring. Ca8Mab-4 will also help clarify the hypothesis.

The CDH8-mediated adhesive code that determines neuronal connectivity has been clarified in mouse models [7,29,30]. In the mouse retina, the dendrites of over 40 different retinal ganglion cells (RGCs) arborize within the inner plexiform layer [31]. The dendrites are limited to one or several distinct sublaminae. Within these sublaminae, RGC dendrites receive synaptic inputs from at least 70 types of interneurons, including amacrine and bipolar cells [32,33]. In TBR1-expressing RGCs, the TBR1-CDH8 axis is required for their laminar specification [34]. Therefore, Ca8Mab-4 would be an

 doi:10.20944/preprints202601.1012.v1

essential tool to distinguish or isolate CDH8-positive RGCs in human retina or *in vitro* differentiated RGCs from induced pluripotent stem cells [35].

Credit authorship contribution statement: Takuya Nakamura: Investigation; **Keisuke Shinoda:** Investigation; **Hiroyuki Suzuki:** Investigation, Writing – original draft; **Mika K. Kaneko:** Conceptualization; **Yukinari Kato:** Conceptualization, Funding acquisition, Project administration, Writing – review and editing. All authors have read and agreed to the published version of the manuscript

Funding Information: This research was supported in part by Japan Agency for Medical Research and Development (AMED) under Grant Numbers: JP25am0521010 (to Y.K.), JP25ama121008 (to Y.K.), JP25ama221153 (to Y.K.), JP25ama221339 (to Y.K.), and JP25bm1123027 (to Y.K.), and by the Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research (KAKENHI) grant no. 25K10553 (to Y.K.).

Institutional Review Board Statement: The animal study protocol was approved by the Animal Care and Use Committee of Tohoku University (Permit number: 2022MdA-001) for studies involving animals.

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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