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Pr1E11, a novel anti-TROP-2 antibody isolated by adenovirus-based antibody screening, recognizes a unique epitope

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ABSTRACT

TROP-2 is a type I transmembrane glycoprotein that is highly expressed in various epithelial cancer cells, and its increased expression correlates with poor prognosis. Although several anti-TROP-2 antibodies have been described, they were found unsuitable for antitumor therapy use *in vivo* as naked antibodies. In this study, we established a novel anti-TROP-2 antibody, designated Pr1E11, from mice immunized with primary prostate cancer cells. Antibody screening was based on the infection activity of Adv-LacZ-FZ33, which displays an immunoglobulin G binding domain in the adenoviral fiber protein. We found that Pr1E11 specifically binds to TROP-2 with high affinity and recognizes diverse epithelial cancer cell lines and primary pancreatic cancer tissues. Epitope analysis using TROP-2 deletion mutants revealed that binding site of Pr1E11 is a cysteine-rich domain, a unique epitope compared with other available anti-TROP-2 antibodies. In addition, Pr1E11 exhibited low internalization activity, which may make it suitable for naked antibody therapeutics. Our results suggest that Pr1E11 may stimulate different biological activities from other anti-TROP-2 antibodies based on its unique binding epitope, and is a potential candidate for naked antibody therapeutics for various epithelial cancer treatments.

1. Introduction

TROP-2, also known as TACSTD2, EGP-1, and GA733-1, is a type I transmembrane glycoprotein with high homology to TROP-1/EpCAM [1]. Increased TROP-2 expression has been found in diverse epithelial cancers and is associated with poor prognosis. Aberrant TROP-2 expression was found in various epithelial cancer cell lines and clinical tumor samples [2–8]. TROP-2 was also found to contribute to the proliferation and migration of cancer cells and to tumorigenesis [9–11]. For these reasons, TROP-2 is an attractive therapeutic target in cancer.

Several monoclonal antibodies have been used successfully for the treatments of various cancers (e.g. cetuximab and trastuzumab). Recently, antibody-based therapies have become a mainstream strategy for cancer treatment, and many additional antibody-based drugs are undergoing clinical trials [12]. One of them, RS7, is a well-known anti-TROP-2 antibody established by Stein et al. [13]. While RS7 did not show antitumor activity in a mouse xenograft model when used as naked antibody, toxin or radioisotope (RI) RS7 conjugates demonstrated significant antitumor activities, which depended on its high internalization activity [14–16]. By contrast, the anti-TROP-2 antibody AR47A6.4.2 demonstrated antitumor activity *in vivo* as a naked antibody [17]. The use of naked monoclonal antibodies is preferable for clinical uses due to their low toxicity compared with drug-, toxin-, or RI-conjugated antibodies. AR47A6.4.2 was reported to have

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complement-dependent cytotoxicity (CDC) activity *in vitro*; however, the mechanism of *in vivo* antitumor activity has not been well elucidated. Since AR47A6.4.2 did not achieve complete remission in mice xenograft models, we considered that the *in vivo* efficacy of AR47A6.4.2 is not sufficient and there may be room to improve the efficacy and potency of a naked anti-TROP-2 antibody by optimizing its biological activities.

The biological activities of therapeutic antibodies depend mainly on the epitope and binding affinities. Selection of clones that strongly bind a suitable epitope to elicit desirable pharmacological activity is a key step for successful antibody generation. Previously, we established a novel antibody screening system based on the infection activity of modified adenovirus with an IgG binding domain in the adenoviral fiber protein (Adv-LacZ-FZ33) [18]. Using this system, several novel monoclonal antibodies were successfully isolated from hybridomas derived from prostate cancer cell line-immunized mice. Since our system reflects the transfection efficacy of Adv-LacZ-FZ33 mediated by candidate antibodies, clones may be obtained with different characteristics from those obtained by conventional hybridoma screening systems.

In this study, we established a novel anti-TROP-2 antibody using the Adv-LacZ-FZ33 system from primary prostate cancer cell-immunized mice, and characterized its biochemical properties.

2. Materials and methods

2.1. Cells

Primary human prostate cancer cells (Pc1) were isolated from surgically resected tumor tissue. Human peripheral blood mononuclear cells (PBMCs) were obtained from blood donated by healthy volunteers from the Hokkaido Red Cross Blood Center. These experiments were approved by the Institutional Review Board at the Sapporo Medical University. All donors gave written informed consent prior to participation.

Murine myeloma cell line P3U1, human embryonic kidney cell line 293T, human pancreatic cancer cell line BxPC-3, and human ovarian cancer cell line CaOV-3 were purchased from the American Type Culture Collection. Human lung cancer cell line NCI-H322 was purchased from the European Collection of Cell Cultures. Human colon cancer cell line Colo205 was purchased from DS Pharma Biomedical (Osaka, Japan). Human embryonic kidney cell line 293F was purchased from Invitrogen Japan (Tokyo, Japan). Chinese hamster ovary cell line DG44 (CHO/DG44) was kindly provided by Dr. Lawrence Chasin (Columbia University). A FUT8 (α-1,6-fucosyltransferase) knockout CHO cell line was developed at Kyowa Hakko Kirin Co., Ltd [19].

2.2. Immunization, antibody screening, and target antigen identification

Mouse immunization and antibody screening were carried out as previously described [17]. Briefly, Balb/c mice (Japan SLC, Inc., Hamamatsu, Japan) were immunized with Pc1 cells, and harvested splenocytes were fused with P3U1 to generate hybridomas. After selection with HAT medium, hybridoma supernatants and Adv-LacZ-FZ33 were added to a well containing NCI-H322 cells, and a β-galactosidase (β-Gal) reporter gene assay was performed. Antibody reactivity to PBMCs was assessed to exclude monoclonal antibodies that bind leukocyte expressing antigen. The immunoglobulin subclass was determined with the IsoStrip mouse monoclonal antibody isotyping kit (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. To identify the target antigen of established clone, membrane-biotinylated Pc1 cell lysate was immunoprecipitated with the candidate antibody, and antigens were identified by western blotting using horseradish peroxidase (HRP)-labeled streptavidin (SA-HRP) and silver staining. Protein sequence was determined by mass spectrometry using a MALDI-Qq-TOF MS/MS QSTAR Pulsari (Applied Biosystems Japan, Tokyo, Japan). Binding specificity to the candidate antigen was examined by flow cytometry (FCM) analysis of 293T cells transfected with human *TROP-2*.

2.3. Anti-TROP-2 antibodies

The antibody Pr1E11 was purified from hybridoma culture supernatant using Prosep-A (Merck Millipore, Tokyo, Japan) according to the manufacturer's instructions. Recombinant chimeric Pr1E11 (cPr1E11) and chimeric AR47A6.4.2 (cAR47A6.4.2) contain the mouse variable region and human IgG1κ constant region, and were generated as previously described [20]. Briefly, cDNAs of the heavy- and light-chain variable regions were inserted into pKANTEX93 plasmid vector, and transfected into CHO/DG44 or FUT8 knockout CHO cells. Commercially available anti-TROP-2 antibodies were purchased from the following suppliers: 77220 from R&D Systems (MN, USA), MOv16 from Enzo Life Sciences (NY, USA), MM0588-49D6 from ANGIO-PROTEOMIE (MA, USA), YY-01 from Santa Cruz Biotechnology (TX, USA), and 162-46 from BD Biosciences (Tokyo, Japan).

2.4. TROP-2 deletion mutants

The TROP-2 extracellular domain is composed of three domains, a cysteine-rich domain (CRD), a thyroglobulin type-I domain (TY), and a cysteine-poor domain (CPD) [21]. Recombinant full-length and domain-deletion mutants of TROP-2 were generated as follows. cDNAs encoding the full-length TROP-2 extracellular region or its domain-deletion mutants (Fig. 3C) were fused with the cDNA encoding the FLAG epitope (DYKDDDDK) and human IgG1 Fc (TROP-2/FLAG/Fc). All cDNAs were subcloned into the INPEP4 plasmid vector (Biogen-IDEC Inc., MA, USA). 293F cells were transfected with the resultant vectors, and protein was purified from supernatants using Prosep-A. Expression and purity of isolated TROP-2/FLAG/Fc were confirmed by SDS-PAGE analysis. N-terminal amino acid sequences of full-length and mutant TROP-2/FLAG/Fc were confirmed by mass spectrometry.

2.5. Flow cytometry

Cells were detached with a 0.02% EDTA solution and suspended in staining buffer (BSA-PBS). Anti-TROP-2 antibodies or isotype controls were added to 10 μg/mL and incubated for 1 h on ice. After washing with staining buffer, cells were incubated with fluorescein isothiocyanate (FITC)- or phycoerythrin-labeled secondary antibody for 30 min on ice. Stained cells were analyzed using EPICS XL-MCL (Beckman Coulter, Tokyo, Japan) or FACS-Calibur (BD Biosciences).

For the competition assay, BxPC3 cells were treated with a mixture containing various concentrations of competitor antibodies and a fixed concentration (0.3 μg/mL) of detector antibodies. Detector antibodies were stained with FITC-labeled secondary antibodies.

To measure internalization, NCI-H322 cells were incubated with an anti-TROP-2 antibody for the indicated times at 37 °C, and antibody remaining on the cell surface was detected by FCM. The percentage of antibody remaining on the cell surface was calculated from mean fluorescence intensity (MFI) according to the formula:

$$\%\ \text{of cell surface remaining antibody} = \mathrm{MFI}_{t=x}/\mathrm{MFI}_{t=0} \times 100$$

$\mathrm{MFI}_{t=0}$ and $\mathrm{MFI}_{t=x}$ are MFI when cells were incubated at 37 °C for 0 min and indicated times, respectively.

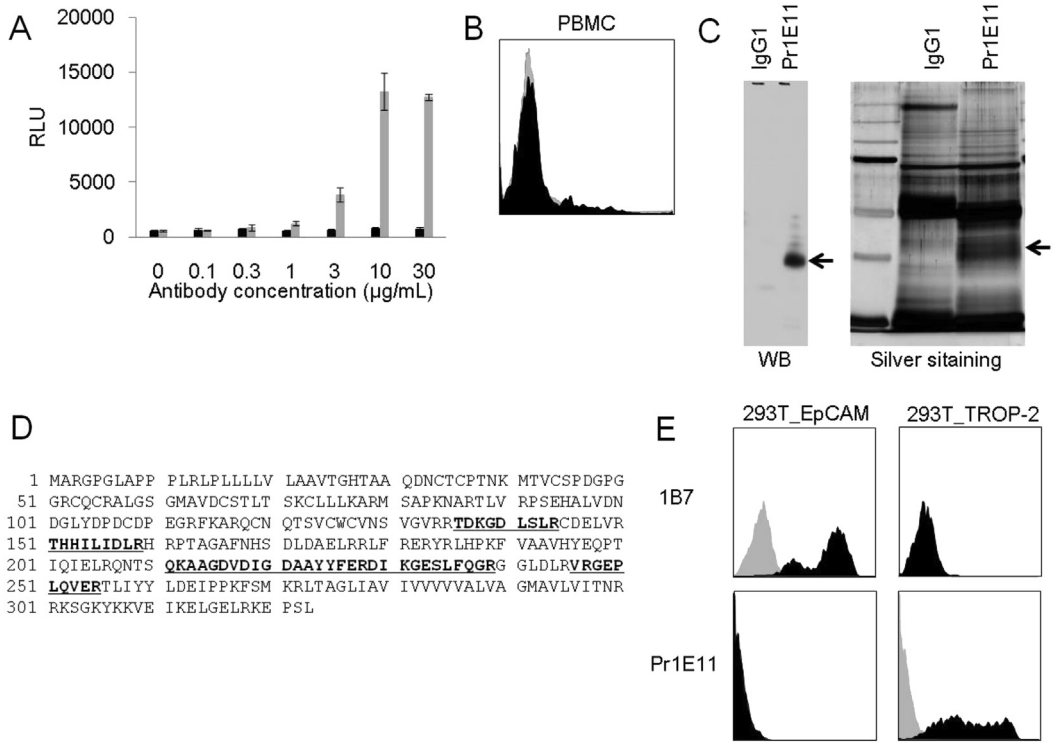

Fig. 1. Isolation and characterization of Pr1E11 as an anti-TROP-2 antibody. (A) β-Gal activity of Adv-LacZ-FZ33-infected cells targeted by Pr1E11. Pc1 cells were infected with Adv-LacZ-FZ33 together with various concentrations of Pr1E11 (gray bar) or control IgG (black bar). Data are presented as the mean ± SD of experiments performed in triplicate. (B) Reactivity of Pr1E11 to PBMCs. PBMCs were incubated with Pr1E11 (black) or control IgG (gray), and detected by FITC-labeled secondary antibody. (C) Isolation of the target of Pr1E11. Lysates of membrane-biotinylated Pc1 cells were immunoprecipitated with the indicated antibodies. The target antigen of Pr1E11 was identified as a band of about 40 kDa (arrow) by WB (left) and silver staining (right). (D) Identification of the target of Pr1E11. The 40 kDa band was excised from the gel and analyzed by mass spectrometry. The underlined sequence was detected, and the protein identified as TROP-2. (E) Reactivity of Pr1E11 with TROP-2 expressing 293T cells. 293T cells were transfected with expression plasmids encoding EpCAM or TROP-2, and treated with anti-EpCAM antibody 1B7, as a positive control for EpCAM staining [18], or Pr1E11, respectively. 1B7 and Pr1E11 are shown in black and the control IgG was shown in gray.

2.6. Immunohistochemistry (IHC)

Frozen human pancreatic cancer tissue (Biochain, CA, USA) was fixed with acetone, and endogenous peroxidase activity was blocked by incubation with 0.3% (v/v) H_2O_2 and 0.1% NaN_3 in PBS. After blocking with BSA-PBS, IHC for TROP-2 was performed using biotinylated anti-TROP-2 antibody, SA-HRP, and 3, 3-diaminobenzidine (DAB). Negative controls were stained with biotinylated isotype-matched antibodies.

2.7. Western blotting (WB)

TROP-2/FLAG/Fc proteins were separated by 5–20% gradient polyacrylamide gels under reducing conditions and transferred to a polyvinylidene difluoride membrane. After blocking with BSA-PBS, the membrane was incubated with the indicated primary antibodies. Membranes were washed with TBST, and incubated with HRP-conjugated secondary antibody. Bands were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Yokohama, Japan) and imaged by LAS4000mini (FUJIFILM, Tokyo, Japan).

2.8. Surface plasmon resonance (SPR)

The antibody binding kinetics was measured using a BIACORE T100 (GE Healthcare, Tokyo, Japan). Anti-Tetra His monoclonal antibody (QIAGEN, Tokyo, Japan) was covalently coupled to CM5 sensor chips (GE Healthcare Biosciences) using an amine coupling kit, and 6 × His-tagged TROP-2/Fc protein (TROP-2/Fc/6 × His, R&D Systems) was captured by immobilized antibodies. Binding kinetics

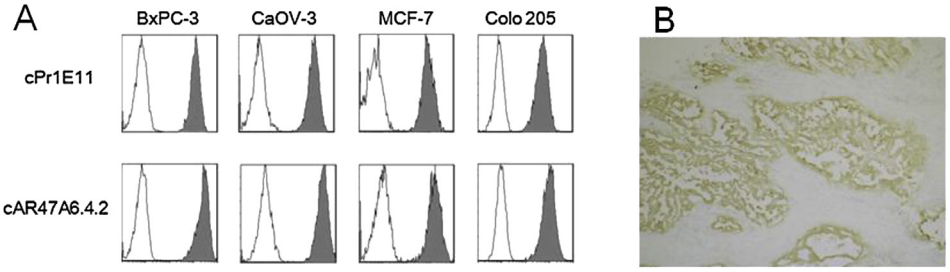

Fig. 2. Reactivity of Pr1E11 to the various cancer cell lines and primary pancreatic cancer tissue. (A) FCM analysis of Pr1E11 binding to epithelial cancer cell lines. The indicated cell lines were stained with Pr1E11 or cAR47A6.4.2 (gray), or isotype control IgG (white), and detected with a FITC-labeled secondary antibody. (B) Frozen pancreatic cancer tissue was stained with biotinylated Pr1E11, and detected by SA-HRP and DAB.

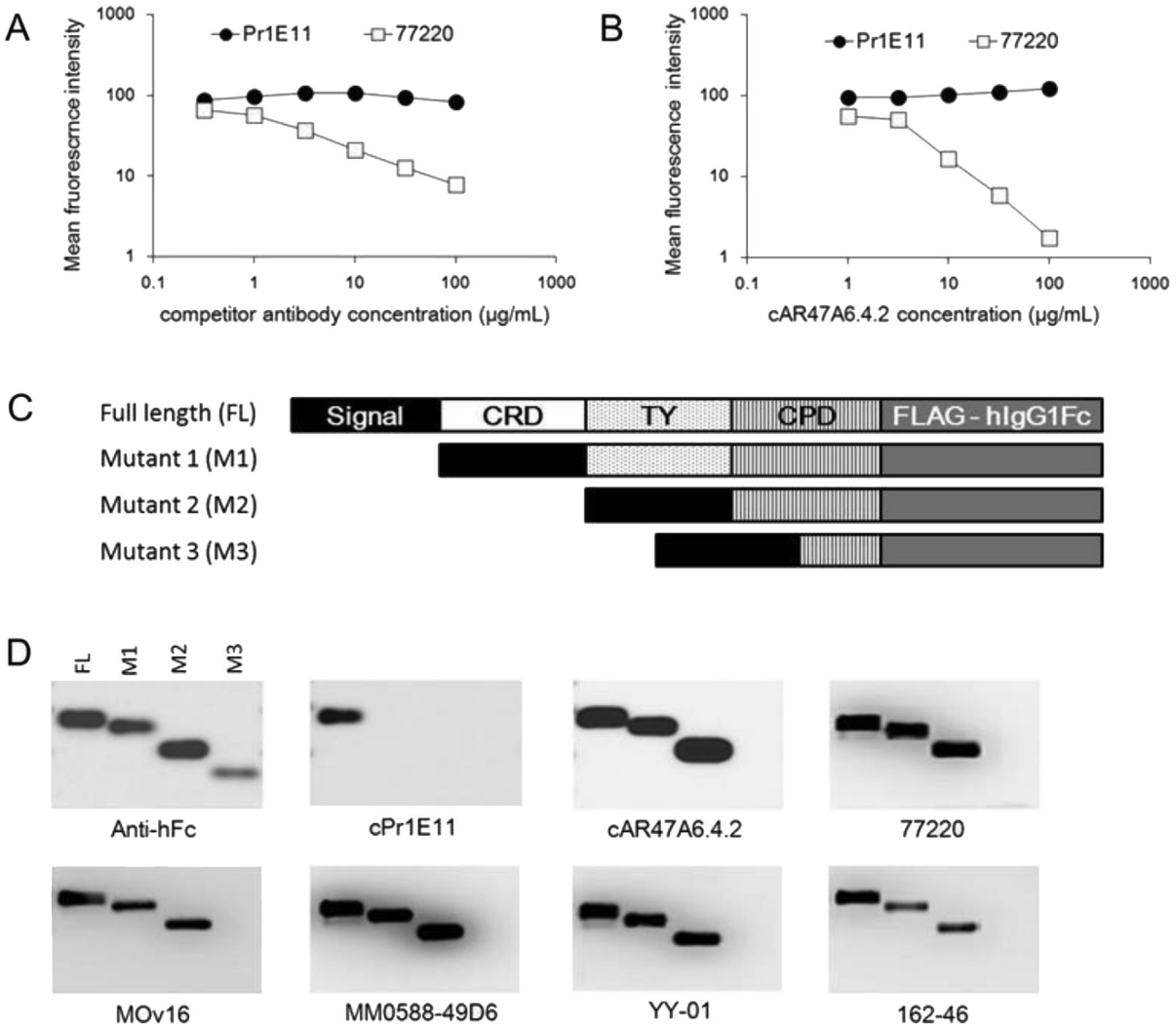

Fig. 3. Analysis of binding domain of anti-TROP-2 antibodies. (A) Competitive FCM assay with cAR47A6.4.2. cAR47A6.4.2 binding to BxPC3 cells in the presence of Pr1E11 (closed circles) or 77220 (open squares) as competitor antibodies was measured by FCM using FITC-labeled goat anti-human κ antibody. (B) Competitive FCM assay with Pr1E11 or 77220. Specific binding of Pr1E11 (closed circles) and 77220 (open squares) in the presence of cAR47A6.4.2 as a competitor antibody was measured by FCM using FITC-labeled goat anti-mouse IgG. (C) Schematic presentation of domain-deletion constructs. Signal, signal sequence (black area); CRD, cysteine-rich domain (C34 to K72); TY, thyroglobulin type-I domain (C73 to C145); CPD, cysteine-poor domain (D146 to T274); FLAG-hIgG1Fc, human IgG1 Fc tagged with FLAG epitope. Mutant 3 is missing the N-terminus of the CPD (L179 to T274). (D) Recognition of TROP-2/FLAG/Fc mutants by the indicated TROP-2 antibodies was determined by WB. FL, full length; Mut 1, mutant 1; Mut 2, mutant 2; Mut 3, mutant 3.

was monitored with the single-cycle kinetics method. N-terminal amino acid sequence of the TROP-2/Fc/6 × His protein used in this study was confirmed by mass spectrometry before use.

3. Results

3.1. Isolation of Pr1E11 and identification of target antigen

Mice were immunized with Pc1 cells, and hybridomas were generated and screened using Adv-LacZ-FZ33 (see Methods for details). Colonies that exhibited high β-Gal activity were cloned and the reactivity of each clone to PBMCs was evaluated. We selected a clone (Pr1E11) that demonstrated strong β-Gal activity and no reactivity to PBMCs (Fig. 1A and B). Pr1E11 was purified from hybridoma supernatants and the isotype was determined to be IgG1κ (data not shown). To identify the target antigen, biotinylated Pc1 cell lysate was immunoprecipitated with Pr1E11, and an approximately 40 kDa band was detected by WB and silver staining (Fig. 1C). The protein sequence of the putative Pr1E11 target was identified by mass spectrometry as TROP-2 (Fig. 1D). Specificity of the antibody was evaluated using 293T cells transfected with plasmid vectors encoding *TROP-2* or *EpCAM* cDNA, as positive or negative controls, respectively. Pr1E11 recognized TROP-2 expressing 293T cells, while it did not bind to EpCAM-expressing 293T cells (Fig. 1E).

3.2. Reactivity of Pr1E11 to cancer cell lines and tissue

FCM analysis was performed to examine the expression of TROP-2 in various tumor cell lines. Pr1E11 bound strongly to epithelial cancer cell lines BxPC-3, CaOV-3, and Colo205 cells, displaying binding intensities comparable to cAR47A6.4.2 (Fig. 2A).

IHC staining of pancreatic cancer tissues with Pr1E11 revealed that Pr1E11 recognized ductal pancreatic cancers (Fig. 2B).

3.3. Binding affinity

The equilibrium dissociations constants (K_D) of each antibody were determined by SPR analysis. Antibody-antigen interactions were investigated with the single-cycle kinetics method using

Table 1
Kinetic rate constants and equilibrium constants for antibody binding to TROP-2/Fc/6 × His. TROP-2/Fc/6 × His was captured on a CM5 sensor chip with an anti-Tetra His monoclonal antibody. The indicated anti-TROP-2 antibodies were analyzed. Data are presented as the mean values from two independent experiments.

	k_a (1/Ms)	k_d (1/s)	K_D (nM)
Pr1E11	3.89×10^5	1.01×10^{-4}	0.261
cPr1E11	4.16×10^5	5.80×10^{-5}	0.143
cAR47A6.4.2	2.21×10^5	2.18×10^{-4}	1.01
77220	5.55×10^5	6.10×10^{-4}	1.10
MOv16	1.83×10^5	1.84×10^{-4}	0.929

BIACORE technology. Pr1E11 and cPr1E11 exhibited a K_D value around 5-fold lower than cAR47A6.4.2, 77220, and MOv16, with a high association constant (k_a) and a low dissociation constant (k_d; Table 1).

3.4. Epitope analysis

To examine whether Pr1E11 binds a different epitope than cAR47A6.4.2, we performed a competitive FCM assay. Antibody 77220 inhibited cAR47A6.4.2 binding to BxPC-3 cells in a dose-dependent manner, whereas Pr1E11 did not (Fig. 3A). In the reciprocal assay, competition with cAR47A6.4.2 resulted in a decreased binding intensity of 77220, whereas Pr1E11 binding was unchanged (Fig. 3B).

To determine the antibody binding site, the reactivity of each antibody to TROP-2 deletion mutants (Fig. 3C) was investigated by WB. While cPr1E11 only reacted with full-length TROP-2, cAR47A6.4.2 and 77220 recognized full-length, and also mutants 1 and 2 (Fig. 3D). We also evaluated the binding properties of four additional commercially available antibodies, MOv16, MM0588-49D6, YY-01, and 162-46. All of these antibodies bound to full-length and mutants 1 and 2, identical to cAR47A6.4.2.

3.5. Internalization activity

Antibody internalization activity was evaluated by FCM. NCI-H322 cells were incubated with cPr1E11 or cAR47A6.4.2 for up to 3 h at 37 °C. cPr1E11 exhibited lower internalization activity than cAR47A6.4.2 (Fig. 4A). After a 180 min incubation, the fluorescence intensity of cPr1E11 was about 50% of the initial intensity, while cAR47A6.4.2 retained only 30% (Fig. 4B).

4. Discussion

We have established a novel anti-TROP-2 antibody, Pr1E11, from a primary prostate cancer cell-immunized mouse-derived hybridoma using the Adv-LacZ-FZ33 system. Pr1E11 specifically bound to TROP-2 with high affinity. This is significant, as aberrant TROP-2 expression has been observed in diverse epithelial cancer cells. Pr1E11 also strongly recognized several epithelial cancer cell lines, which is consistent with previous reports [3,4,7]. In addition, Pr1E11 clearly stained primary pancreatic cancer tissue sections. Our results suggest that Pr1E11 can recognize TROP-2 when expressed in clinical samples, even if unknown post-translational modifications to TROP-2 are present in primary cancer cells.

Because, to our knowledge, only cAR47A6.4.2 has shown significant antitumor activity in mouse xenograft models when used as a naked antibody [17], we performed a side-by-side analysis of epitope recognition between this antibody and Pr1E11. Pr1E11 did not inhibit cAR47A6.4.2 binding in a competitive FCM experiment, suggesting that the binding site of PrE11 is distinct from cAR47A6.4.2. The extracellular domain of TROP-2 consists of three parts, CRD, TY, and CPD. To determine the epitopes of our antibodies, we generated three domain-deletion mutants of TROP-2/FLAG/Fc. Using WB, we determined that Pr1E11 recognized full-length, but demonstrated no binding to any deletion mutants. By contrast, cAR47A6.4.2 reacted to full-length, and also mutants 1 and 2, but not mutant 3, which is an N-terminal deletion of CPD (L179 to T274). Surprisingly, five additional antibodies tested displayed binding properties similar to cAR47A6.4.2. These results clearly demonstrate that the binding site of Pr1E11 is CRD, while the other antibodies bind to the N-terminal region of CPD.

For therapeutic antibodies, the epitope recognized is an important factor that affects both Fc-dependent and independent activities. A clear example of this is the mechanistic difference between therapeutic antibodies rituximab and tocitumomab, which target different epitopes on CD20. Rituximab is a type I anti-CD20 antibody that redistributes CD20 into lipid rafts and shows strong CDC activity but low direct killing activity. By contrast, tocitumomab is a type II anti-CD20 antibody that does not change CD20 redistribution and has no CDC activity, but strongly induces direct cell death [22]. As TROP-2 contributes to various cell behaviors, including proliferation and migration, our results suggest that there is the potential for Pr1E11 to demonstrate distinctive biological activities compared with other antibodies currently in use.

Anti-TROP-2 antibody RS7 rapidly internalizes following antigen binding [15], and had no antitumor activity in mouse xenograft models when used as a naked antibody. To maximize ADCC and/or CDC activity, it is desirable that antibodies are not internalized, as internalization leads to reduced cell surface antigen, decreasing the availability of the Fc region [12]. For these reasons, we considered

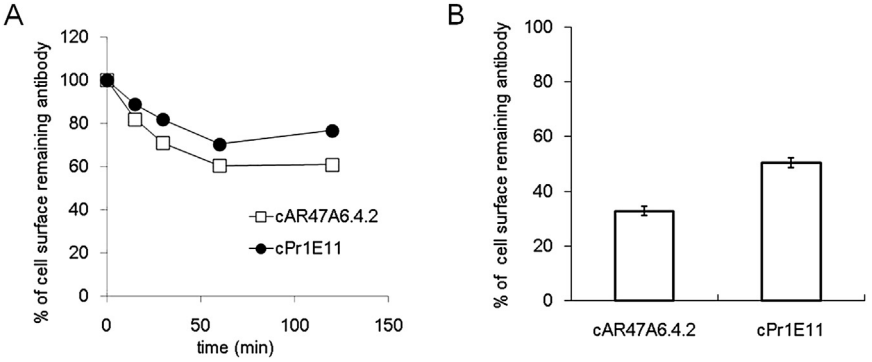

Fig. 4. Internalization of anti-TROP-2 antibodies. (A) Time course of antibody internalization. NCI-H322 cells were incubated with anti-TROP-2 antibodies at 37 °C to allow internalization. Remaining cell surface antibodies were detected by FITC-labeled anti-human IgG. (B) Remaining antibody bound on the cell surface after a 180 min incubation. Assays were performed in triplicate, data are presented as mean ± SE.

that the low *in vivo* efficacy of naked RS7 might be due to its very high internalization activity; therefore, low internalization activity is a desirable quality for an anti-TROP-2 antibody that can be used *in vivo* as a naked antibody. We conducted a side-by-side analysis of internalization activities of cPr1E11 and cAR47A6.4.2 to compare the activity of cPr1E11 with that of AR47A6.4.2, which showed antitumor activity *in vivo*. It was an unexpected finding that cPr1E11 strongly induced Adv-LacZ-FZ33 infection, but exhibited lower internalization activity than cAR47A6.4.2. These results suggest that cPr1E11 may exhibit superior *in vivo* efficacy compared with cAR47A6.4.2, as a higher number of antigens for ADCC and/or CDC activities would be available.

We measured the binding affinities of the various antibodies using SPR and TROP-2/Fc/6 × His. We found that Pr1E11 had greater affinity than cAR47A6.4.2. High affinity binding is a desirable property, as antibodies can occupy target antigens, even at low concentrations. These results suggest that the potency of Pr1E11 might be superior to those of other naked anti-TROP-2 antibodies.

In conclusion, we established a novel anti-TROP-2 antibody, designated Pr1E11, from human primary prostate cancer cell-immunized mice using the Adv-LacZ-FZ33 infection system. TROP-2 is widely expressed on a variety of epithelial cancers; therefore, Pr1E11 has the potential to be an antibody-based therapy for various epithelial cancers. Pr1E11 had lower internalization activity than cAR47A6.4.2, which has demonstrated antitumor activity as a naked antibody. It is possible that the unique binding epitope of Pr1E11 mediates not only different internalization activity, but also additional biological functions. Together, our results suggest that Pr1E11 may exhibit antitumor activity superior to AR47A6.4.2 as a naked antibody. Further evaluation of *in vitro* and *in vivo* efficacy is needed for its future application.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.051.

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