ABSTRACT SUMMARY
Most malignant tumors are derived from the epithelium; therefore, epithelial cells are ideal targets for cancer therapy. The claudin (CLDN) family, which consists of 27 members, are key components of the epithelial tight junction seal. CLDN3 and CLDN4 are frequently overexpressed in various malignant tumors; however, their low antigenicity has hampered the development of antibody-based CLDN-targeted cancer therapy. In this study, we created a monoclonal antibody to CLDN3/4 by DNA immunization and evaluated its tumor-targeting and anti-tumor activities.

INTRODUCTION
The claudin (CLDN) family is comprised of ~20 tetra-transmembrane proteins that are localized in the lateral membrane between adjacent epithelial cells. An early event in malignant transformation is the loss of cellular polarity, often resulting in translocalization of CLDNs from the lateral membrane to the cell surface. CLDN3 and CLDN4 are particularly promising targets for tumor therapy because they are frequently overexpressed in malignant tumors such as pancreatic, breast, and ovarian cancers.

The C-terminal fragment of Clostridium perfringens enterotoxin (C-CPE) is the most thoroughly characterized CLDN3/4 binder. We previously produced a CLDN-targeting toxin by fusing C-CPE with a protein synthesis inhibitory factor (PSIF) derived from Pseudomonas aeruginosa exotoxin. C-CPE-PSIF showed anti-tumor activity without apparent adverse effects, establishing proof-of-concept for CLDN-targeted cancer therapeutic strategies. However, C-CPE proved unsuitable for clinical use due to its immunogenicity. In this study, we optimized a protocol for immunization with CLDN and developed 8 rat anti-CLDN4 monoclonal antibodies (mAbs), one of which binds to both CLDN3 and CLDN4. We also investigated the tumor-targeting and anti-tumor activities of the anti-CLDN3/4 mAb.

EXPERIMENTAL METHODS
Development of anti-CLDN antibodies: Rats were immunized with plasmids encoding the human CLDN4 sequence. The resulting B cells were fused with myeloma cells. The hybridoma (clone B) that produced the rat anti-CLDN3/4 mAb was screened by fluorescence activated cell sorting (FACS) using CLDN-expressing cells.

Tissue-distribution of anti-CLDN mAb: The mAb was labeled with fluorescent dye (CF750) and injected intraperitoneally into mice bearing human cancer cells (MKN74 human gastric cancer cells or LoVo human colon cancer cells). Tissue distribution was monitored using the Maestro imaging system.

Preparation of rat-human chimeric IgG1: The cDNAs encoding heavy- and light-chain variable region of the anti-CLDN3/4 mAb were cloned into human IgG1-expression vectors. The vectors were then introduced into CHO-S cells, and the transfected cells were cultured for 6 days. The chimeric Ab was purified from the conditioned medium using a Protein G column.

Anti-tumor activity: Antibody-dependent cellular cytotoxicity (ADCC) was evaluated by activation of Fcγ receptor IIIa, as described previously. Mice bearing MKN74 or LoVo cells were injected intraperitoneally with the rat mAb or the human chimeric mAb twice per week for 4 weeks. Tumor volume was monitored during the treatment.

RESULTS AND DISCUSSION
We developed 8 rat anti-CLDN mAbs through DNA immunization. One of these mAbs, clone B, bound to CLDN3 and CLDN4 but not to CLDN1, 2, 5, 6, 7 or 9. Imaging analysis showed that clone B was distributed to tumor tissues in mice bearing MKN74 cells (Fig. 1). A similar distribution pattern was observed in mice bearing LoVo cells (data not shown). These findings indicate that clone B may be suitable for use in cancer-targeted therapeutics.

Anti-tumor activity was detected following administration of clone B (rat IgG) in the mouse models; therefore, to improve druggability of clone B we then developed a human-rat chimeric IgG1 by grafting the heavy- and light-chain variable regions of the rat mAb into human IgG1. The chimeric mAb retained the CLDN-binding specificity of the parental mAb. Although the chimeric mAb itself was not cytotoxic to CLDN-expressing cells in vitro, it did activate Fcγ receptor IIIa-expressing reporter cell in an antigen-binding dependent manner, indicating that the mAb may induce ADCC. Administration of the chimeric mAb attenuated tumor growth in mice bearing MKN74 or LoVo cells, without causing loss of body weight (data not shown, Fig. 2).

CONCLUSION

We created an anti-CLDN3/4 dual-specific mAb by DNA immunization. The anti-CLDN3/4 Ab was distributed to and accumulated in tumor tissues. The human-rat chimeric IgG1 mAb showed anti-tumor activity without apparent adverse effects in mice bearing various human cancer cells. These findings suggest that the anti-CLDN3/4 mAb would be suitable for anti-tumor therapeutic use.

REFERENCES

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Figure 1. In vivo distribution and pharmacokinetics of clone B in tumor-bearing mice. CF750-labeled mAb was administered to mice bearing MKN74 cells. Various tissues were removed at the indicated times, and fluorescence intensity was measured using the Maestro imaging system. The amount of clone B was calculated as the percentage of the injected doses per gram of tissue (%ID/g). Data are expressed as means ± SEM (n = 3).

Figure 2. Anti-tumor activity of rat-human chimeric clone B. Mice bearing LoVo cells were administered either the rat mAb or chimeric mAb twice a week for 4 weeks at 1 mg/kg of body weight. Tumor volume was monitored during treatment and was calculated according to the following equation: tumor volume (mm^3) = 0.5 × (major diameter) × (minor diameter)^2. Data are expressed as means ± SEM (n = 5). *P < 0.05 vs. PBS, **P < 0.01 vs. PBS.