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Original Articles

Humanized anti-CD271 monoclonal antibody exerts an anti-tumor effect by depleting cancer stem cells

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ABSTRACT

CD271, known as a neurotrophin receptor, is expressed in various cancers such as hypopharyngeal cancer (HPC) and melanoma. We recently reported that CD271 is a cancer-stem-cell biomarker of HPC, and that its expression is essential for cancer-cell proliferation and is correlated with a poor prognosis in this disease. Here, to develop a therapeutic antibody to CD271, we established a humanized anti-CD271 monoclonal antibody (hCD271 mAb). hCD271 mAb bound to the cysteine-rich domain 1 (CRD1) of human CD271 with high affinity ($K_D = 1.697 \times 10^{-9}$ M). *In vitro*, hCD271 mAb exerted antibody-dependent cell-mediated cytotoxicity (ADCC) activity against SP2/0-CD271 (human CD271-transduced mouse cell line). Treatment with hCD271 mAb also exerted anti-tumor activity in graft models of three cell lines (HPCM2 (patient-derived xenograft cell line of hypopharyngeal cancer), MeWo-Luc (melanoma cell line), and SP2/0-CD271) in mice, resulting in smaller tumors compared to controls and reduced numbers of CD271-positive cells. Collectively, these data suggest that an antibody targeting CD271 is a promising therapeutic strategy.

1. Introduction

Hypopharyngeal cancer (HPC), a malignancy of the hypopharynx, accounts for approximately 10% of all head and neck squamous cell carcinomas (HNSCCs). Unfortunately, approximately 80% of HPC cases are diagnosed at an advanced stage of the disease. Chemoradiotherapy is a treatment option for patients who wish to preserve their larynx, but it often causes life-threatening side effects, and in patients with advanced cancer, the indication for such treatment is limited. Even if the primary lesion is controlled, delayed regional lymph node metastasis, distant metastasis, and additional primary malignancies frequently occur during the course of the disease [1], and the 5-year survival rate of advanced HPC is reported to be less than 30% [1–3]. Likewise, melanoma is an aggressive cutaneous malignancy [4]. Although surgical treatment for early melanoma leads to high cure rates, unresectable advanced and metastatic melanoma present aggressive behaviors, including a tendency to rapidly metastasize and an intrinsic resistance to chemotherapy. Thus, innovative treatment strategies are urgently needed for these cancers.

CD271, known as a low-affinity nerve growth factor (NGF) receptor or p75 neurotrophin receptor, is a member of the tumor necrosis factor receptor (TNFR) superfamily, which binds to several ligands, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and neurotrophin-4 (NT4), with low affinity [5,6]. CD271 is expressed on the basement membranes of normal oral mucosa and normal esophageal mucosa and is regarded as a surface-marker candidate for these tissue stem cells [7,8]. In addition, CD271 is known to play crucial roles in cancers. "Cancer stem cell theory" refers to a mechanism of heterogeneous cancer tissue formation that was proposed decades ago [9]. Cancer stem cells possess several unique

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Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
CDR	complementarity determining region
CRD	cysteine rich domain
CSC	cancer stem cell
DAB	3,3′-diaminobenzidine
DMEM	Dulbecco's Modified Eagle's Medium
ECD	extra cellular domain
ELISA	Enzyme-Linked ImmunoSorbent Assay
FBS	Fetal bovine serum
FITC	fluorescein isothiocyanate
HE	hematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNSCC	Head and neck squamous cell carcinoma
HPC	Hypopharyngeal cancer
HRP	horseradish peroxidase
IgG	Immunoglobulin G
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NOD/scid	Non-obese diabetic/severe combined immunodeficiency
PBMC	Peripheral Blood Mononuclear Cell
PBS	phosphate-buffered saline
PCR	Polymerase Chain Reaction
PDX	patient-derived xenograft
PE	phycoerythrin
RPMI medium	Roswell Park Memorial Institute medium
SDS-PAGE	Sodium dodecyl sulfate - Polyacrylamide gel electrophoresis

properties, such as therapy resistance and tumorigenicity, and are related to a poor prognosis. We previously reported that CD271-positive HPC cells exhibit high tumorigenicity and resistance to cisplatin treatment, and that increased CD271 expression is correlated with a poor prognosis in HPC, suggesting that CD271 is a biomarker for HPC stem cells [10]. We further reported that CD271 plays an essential role in HPC cell proliferation both *in vivo* and *in vitro,* using CD271-knockdown cells [11]. In oral cancer cells, a loss of CD271 also has a negative impact on the initiation of tumor formation *in vivo* [12]. In melanoma cells, CD271 is a crucial determinant of tumorigenicity, stem-like properties, heterogeneity, and plasticity [13]. Moreover, CD271 is reported to be a negative prognostic factor in melanoma [14] and breast cancer [15,16], and to serve as a marker of cancer stem cells in human melanoma [17,18] and esophageal carcinoma [8,19]. CD271 is also reported to accelerate cell proliferation in oral cancer [12], breast cancer [15,16], and brain tumors [20]. Based on these reports, we here sought to develop an anti-CD271 humanized antibody as a therapeutic molecule.

2. Materials and Methods

2.1. Ethics statements

This study was conducted according to the principles expressed in the Declaration of Helsinki and was approved by the Ethics Committees at the Miyagi Cancer Center Research Institute (Natori, Japan). The animal experimental protocols were approved by the Miyagi Cancer Center Animal Care and Use Committee. Peripheral blood mononuclear cells (PBMC) were obtained from healthy volunteers. All volunteers provided signed written informed consent.

2.2. Cell lines

The human cell lines used were the three hypopharyngeal patient-derived xenograft (PDX) cell lines (HPCM1 [10,11], HPCM2 [10,11] and HPCM6 (newly established), maintained in RPMI-1640 medium (Wako, Japan) supplemented with 10% fetal bovine serum (FBS) and 100 unit/ml penicillin and 100 μg/ml streptomycin), SK-OV-3 cell line (ovarian adenocarcinoma, maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin, purchased from American Tissue Culture Collection, VA), MeWo-Luc and SK-MEL-2-Luc, (malignant melanoma, maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin, provided from JCRB, Osaka, Japan). MCC148c cell line was established by PDX of cancer tissue derived from a lung squamous cell carcinoma patient [21] and maintained in DMEM (Wako, Japan) supplemented with 10% FBS, 0.4 μg/ml hydrocortisone, 2.5 μM Y-27632 (FocusBiomolecules, PA) and penicillin/streptomycin.

2.3. Animals

Six-week-old female Balb/c mice were purchased from CLEA Japan (Tokyo, Japan). Five-week-old female NOD/scid mice were purchased from Charles River (Kanagawa, Japan).

2.4. Antibodies

The antibodies used in this study are listed inTable S1 .

2.5. Primers

The primers used in this study are listed in Table S2.

2.6. PBMC and NK cells preparation

Blood samples were transferred to BD Vacutainer® CPT™ tubes for isolation of PBMC according to the manufacturer's instructions. NK cells were enriched from PBMCs using the Human NK Cell Isolation Kit (130-092-657, Miltenyi Biotech, Bergisch Gladbach, Germany), followed by activated and expanded by using NK Cell Activation/Expansion Kit (130-094-483, Miltenyi Biotech) following to manufacture's protocol. NK cells were cultured in NK MACS medium (Miltenyi Biotec) containing 5% human AB serum (Biowest, Nuaillé, France) and 500 U/mL IL2. The purity of NK cells was confirmed by FACS Canto II (Becton Dickinson, CA) and $CD3^{-}CD56^{+}$ NK cells occupied over 95% in each sample.

2.7. Plasmid construction

To construct transient expression vectors for full length CD271, cysteine rich domain 1 (CRD1)-deleted CD271 (Δ1), CRD1 and 2-deleted CD271 (Δ1-2), CRD1, 2 and 3-deleted CD271 (Δ1-3) and all CRD-deleted CD271 (Δ1-4), the insert sequence of pBluescript- NGFR (RIKEN, Saitama, Japan) or HPCM2 cell's cDNA was amplified using KOD-FX polymerase (Toyobo, Osaka, Japan) following the manufacture's recommendation and subcloned into the *Eco*RI site (full length CD271) and the *Bam*HI-*Eco*RI site (deletion mutants) of the pcDNA™4/myc-His A (Invitrogen, CA) vector using a ligation kit (Takara Bio, Ohtsu, Japan). To construct constitutive expression vectors for full length CD271 (pBABE-puro-CD271), the insert sequence of pBluescript-NGFR and amplified using PCR method and subcloned into the *Eco*RI site of the pBABE-puro (Addgene, MA) vector using a ligation kit. To construct constitutive expression vectors for purification of CD271 extra cellular domain (ECD) protein, the CD271 ECD coding region gene was amplified by PCR using the pBluescript containing whole CD271 gene as a template. The CD271 ECD segment was cloned through the BamHI and EcoRI sites into the pFastBac 6P1 donor vector (provided from Dr. Kozou Tanaka, Tohoku University) using DNA ligation kit. The insert was sequenced to evaluate the fidelity of cloning.

2.8. Transduction of human or mouse cells

To establish cells transiently expressing CD271 (full length CD271, Δ1, Δ1-2, Δ1-3 and Δ1-4), the plasmids described above were transfected into the 293T cells using FuGENE HD (Promega, WI) following the manufacture's protocol. To establish cells stably expressing full length CD271, pBABE-puro-CD271 or pBABE-puro empty vector was transferred using FuGENE HD (Promega) into PlatA cells (gift from Dr. Kitamura, Tokyo University) following the manufacturer's recommendation. The retrovirus vectors were transduced into SP2/0 and SK-OV-3 cells, and CD271-positive cells (SP2/0-CD271 and SK-OV-3-CD271) and CD271-negative cells (SP2/0-EV and SK-OV-3-EV) were selected by puromycin.

2.9. Purification of CD271 extracellular domain (ECD) recombinant protein

Purification of recombinant protein was performed as described previously [22]. In brief, *E. coli* DH10Bac (Invitrogen) was transformed using the pFastBac-CD271 ECD recombinant donor plasmid. To produce recombinant baculovirus, the pre-cultured Sf21 cells were transfected by recombinant bacmid DNA using a Cellfection II reagent (Invitrogen) according to the manufacturer instruction. Lysis buffer (20 mM Tris-HCl, 10% glycerol, 1% NP-40, 1 mM EGTA, 1 mM DTT, 150 mM NaCl, pH8.0) with Protease Inhibitor Cocktail (Sigma-Aldrich, MO) was added to collected cells and then sonicated and centrifugated. The supernatant was then rotated with Glutathione Sepharose 4B (Sigma-Aldrich, MO) for 60 min at 4 °C. The mixture was added Prescission Protease (GE Healthcare, IL) with Prescission Cleavage buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, pH7.5).

2.10. Establishment of hybridoma cell lines

BALB/c mice were immunized using subcutaneous injections of 40 μg of recombinant CD271-ECD together with Complete Adjuvant (Freund) (BD Biosciences, CA). After several additional immunizations, a booster injection was intravascularly administered 6 and 3 days before harvesting spleen cells. Spleen cells were then fused with SP2/0 cells using HVJ Envelope Cell Fusion Kit (GenomeOne-CF, ISHIHARA SANGYO KAISHA, Osaka, Japan) and grown in GIT medium (Wako, Osaka, Japan) supplemented with 10% FBS, hypoxanthine, aminopterin, and thymidine selection medium supplement (Thermo Fisher Scientific, MA).

Culture supernatant of hybridoma cells were screened using ELISA. Each HPCM2-CD271 and HPCM2-EV cells were seeded in plates and fixed in 4% paraformaldehyde. Fixed cells were incubated with supernatant and anti-mouse IgG HRP-conjugated was applied; and the enzyme activity was detected by using the substrate tetramethyl benzidine.

Among these hybridomas, clone #16 produced IgG (mCD271 mA b) with higher reactivity to the ECD, compared to commercially available anti-CD271 antibody ME20.4, in ELISA assay (Fig. S1A). The IgG isotype produced by clone #16 was determined to be IgG2a. The specificity of the antibody was tested by flow cytometry analysis using two human CD271-transfected cell lines, SP2/0-CD271 and SK-OV3-CD271 (Fig. S1B).

For determination of the epitope, the transiently transfected 293T cells (CD271-full length, Δ1, Δ1-2, Δ1-3 and Δ1-4) were seeded in polyethylenimine (PEI) coated plates and incubated for 24 h.

For determination of antibody concentration, plates were coated with anti-mouse IgG2a IgG (ISO2-1 Kit, Sigma-Aldrich, MO), washed and incubated with the culture supernatant of hybridoma cells or anti-mouse IgG2a antibody as a standard (200 μg/ml, Anti-CA IX, H-11, Santa Cruz, TX).

The isotype of the antibody in the hybridoma supernatant was determined using Mouse Monoclonal Antibody Isotyping Reagents (Sigma-Aldrich, St. Louis, MO) following to manufacture's protocol.

2.11. Antibody humanization

Total RNA of the hybridoma cell lines which secrete anti-hCD271 monoclonal antibodies (mAb) (mouse IgG) was isolated using High Pure RNA Isolation Kit (Roche Applied Science, Penzberg, Germany). RT-PCR was performed using SMARTer RACE 5'/3' Kit (Clontech, CA), and the cDNA fragment of the antibody was amplified and then inserted into the cloning vector. *E. coli* JM 109 strain was transformed with the cloning vector and the sequence of the mouse antibody was identified. A human antibody sequence which was highly homologous to the mouse anti-CD271 antibody was selected, and the humanized IgG_1 antibody was established by substituting the amino acids of each CDR region of the mouse anti-CD271 antibody with the amino acids in the CDR regions of the light and heavy chains of the human antibody.

The humanized anti-CD271 antibody (hCD271 mA b) expression vector was prepared by cloning the hCD271 mA b expression unit sequence into the pcDNA 3.4 vector by the Infusion method (pcDNA 3.4 TOPO TA cloning Kit, Thermo Fisher Science, MA). The hCD271 mA b expression vector was transfected into Expi 293 cells and cultured with shaking at 37 °C.

hCD271 mA b was purified from the culture supernatant using Hitrap mabselect SuRe 5 mL column (GE Healthcare, IL) and Bio scale Mini system hydroxyapatite cartridge (BIOLAD, CA).

2.12. Flow cytometry

Flow cytometry was performed as described previously [11]. Fluorescence data were collected using a cell analyzer (FACSCanto II; Becton Dickinson, CA or SONY SA3800).

2.13. Western blotting

Western blot was performed as described previously [11].

2.14. MTT assay

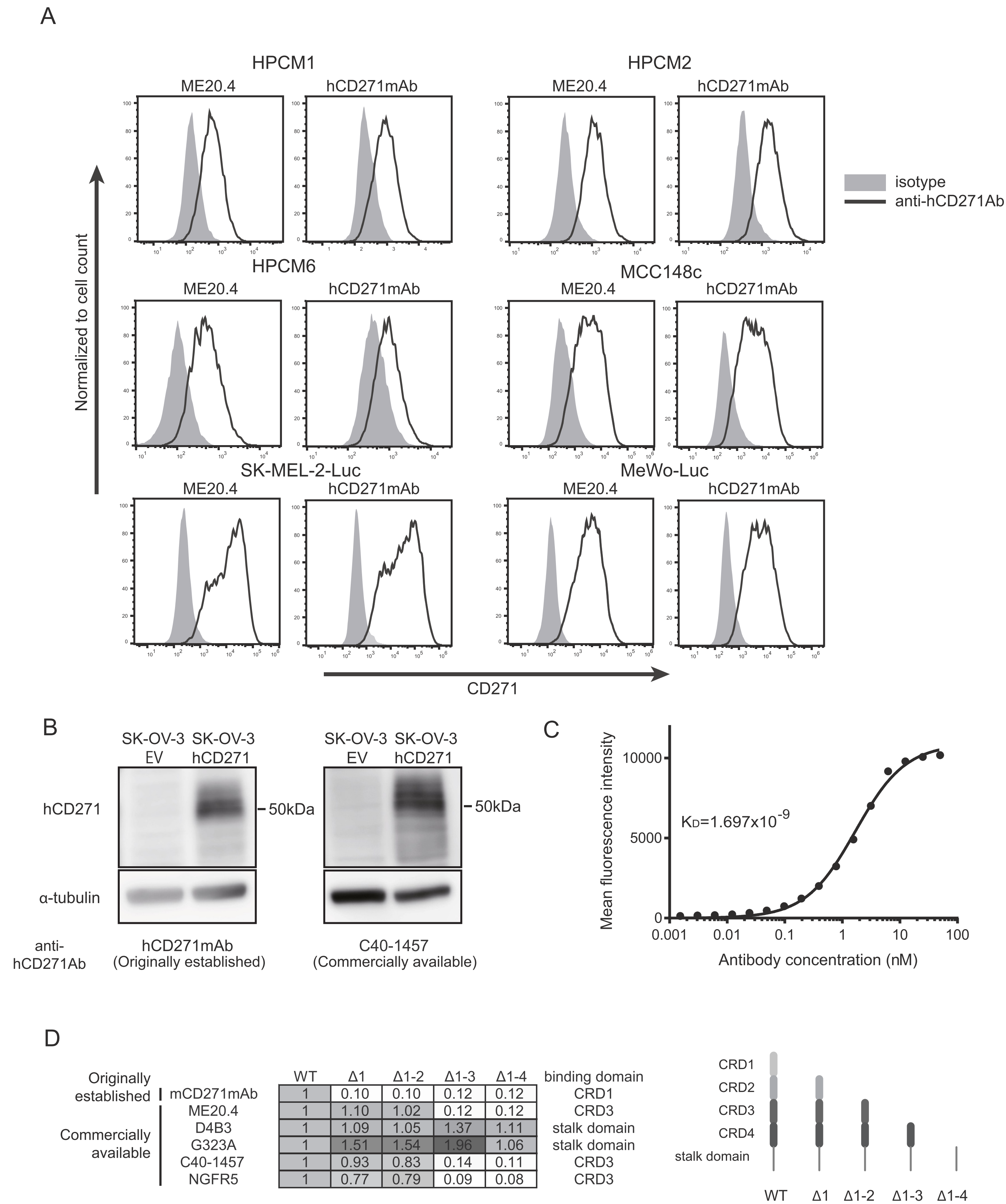
MTT assay was performed as described previously [11].

Antibody-dependent cell-mediated cytotoxicity (ADCC) assay.

Lysis of target cells by ADCC was measured by a calcein release method modified. Briefly, target cells from continuous culture were freshly labeled with calcein AM (DOJINDO, Kumamoto, Japan) in assay medium (RPMI-1640 containing 10% FBS, 10 mM HEPES and 2.5 mM probenecid) for 10 min at 37 °C and dispensed to culture plates. Humanized anti-hCD271 mAbs (5 μg/mL at final concentration) were added to the plates containing the target cells and incubated for 1 h at room temperature to allow opsonization. After incubation, effector cells (Effector: Target ratio; 0.25:1–50:1) were added to each well and the plates were incubated for an additional 4 h at 37 °C. Following incubation, the supernatants were collected and fluorescent signals were measured using a Synergy H1 (BioTek Instruments, VT) with excitation/emission at 485/535 nm. Samples and controls were tested at least in duplicate either in the same or separate plates.

2.15. Xenograft assay

Cells (SP2/0-CD271 1×10^4, MeWo-Luc 5×10^5 and HPCM2 4×10^5/50 μL in PBS) were mixed with 50 μL of BD Matrigel Matrix Growth Factor Reduced (BD Biosciences, San Jose, CA). A 100 μL suspension was injected subcutaneously into the bilateral flanks of NOD/scid mice. The tumor diameter was measured using calipers, and the tumor volume was calculated using the following formula:



		WT	Δ1	Δ1-2	Δ1-3	Δ1-4	binding domain
Originally established	mCD271mAb	1	0.10	0.10	0.12	0.12	CRD1
Commercially available	ME20.4	1	1.10	1.02	0.12	0.12	CRD3
	D4B3	1	1.09	1.05	1.37	1.11	stalk domain
	G323A	1	1.51	1.54	1.96	1.06	stalk domain
	C40-1457	1	0.93	0.83	0.14	0.11	CRD3
	NGFR5	1	0.77	0.79	0.09	0.08	CRD3

Fig. 1. Characterization of mouse and humanized anti-hCD271 mAbs. (A) Flow cytometry with hCD271 mA b. Cells were stained with 0.5 μM hCD271 mA b followed by PE-conjugated anti-human IgG. Gray-filled histogram, isotype control. (B) SK-OV-3-CD271 and SK-OV-3-EV cell lysates were subjected to gel electrophoresis, and Western immunoblot analysis was performed with hCD271 mA b. (C) Binding affinity of hCD271 mA b was determined using flow cytometry. SK-OV-3-CD271 cells were suspended in 100 μL of serially diluted hCD271 mA b (0.5 μM–16 pM), and secondary anti-human IgG antibodies were then added. Fluorescence data were collected using flow cytometry. (D) (Left panel) Determination of the binding domain by ELISA. 293T cells transfected with an expression vector of wild-type (WT) or deletion mutants of hCD271 (Δ1, Δ1–2, Δ1–3, and Δ1–4) were incubated with each Ab, and the absorbance was measured. The values were normalized to the absorbance of hCD271 WT-expressing 293T cells. (Right panel) A scheme for the deletion mutant of CD271. mCD271 mA b, anti-CD271 monoclonal mouse IgG.

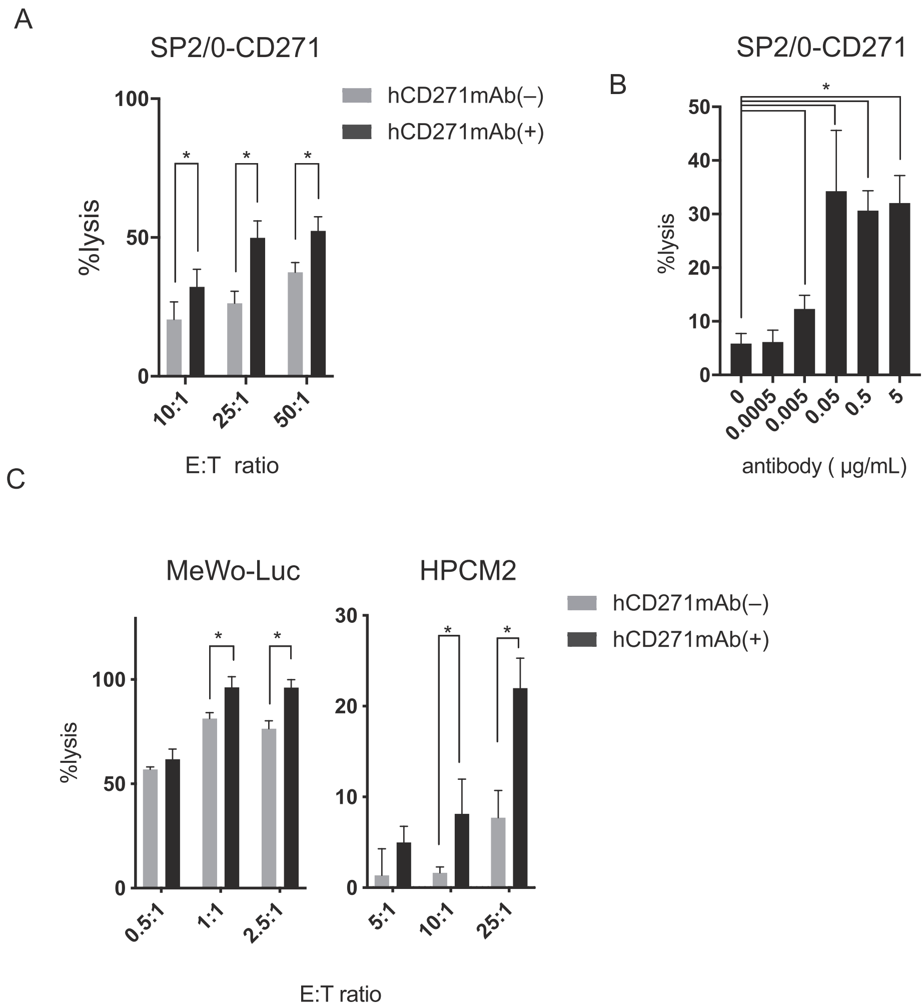


Fig. 2. ADCC assays *in vitro*. (A) The ADCC activity was measured using SP2/0-CD271 (target) and NK (effector) cells. The antibody was used 5 μg/mL at a final concentration of 5 μg/mL. Gray bars indicate the assay using target and effector cells without hCD271 mA b. The values normalized to those of lysed cells by Triton X-100 are indicated. The E:T ratio was the effector cell to target cell ratio. (B) The ADCC activity was measured at various concentrations of the antibody. The E:T ratios was 25:1. (C) The ADCC activity was measured using MeWo-Luc (target) and NK (effector), and HPCM2 (target) and PBMC (effector) cells. The antibody was used at a final concentration of 5 μg/mL. Gray bars indicate the assay using target and effector cells without hCD271 mA b. The values normalized to those of lysed cells by Triton X-100 are indicated. *$P < 0.05$, *t*-test.

volume = $W^2 \times L/2$, where W is the short diameter and L is the long diameter. Statistical analysis was performed using a Tukey-Kramer's test.

2.16. Immunohistochemical (IHC) staining

Immunostaining of human CD271 was performed as previously described [11]. The anti-CD271 antibody was purchased (C40-1457, BD Biosciences, Franklin Lakes, NJ, USA).

2.17. Image analysis

To quantify the IHC staining intensity of CD271-positive area, image data was processed using ImageJ as previously reported [23,24]. We analyzed deconvoluted DAB images, and the high intensity range for the CD271-positive zone was measured.

2.18. Statistical analysis

Statistical analysis was performed using GraphPad Prism vision 7.03 (GraphPad Software, La Jolla, CA). The differences between two groups were analyzed by the unpaired *t*-test. *P*-value < 0.05 was considered to be statistically significant. The dissociation constants (KD) were calculated by fitting the binding isotherms using the built-in one-site binding models in GraphPad PRISM 7.03.

3. Results

3.1. Establishment of a humanized monoclonal antibody specific for human CD271

First, we newly established an anti-CD271 mouse IgG antibody (mCD271 mA b, see Materials and Methods). We then prepared the humanized antibody (hCD271 mA b), which can be applied for cancer therapy. We examined the sensitivity of hCD271 mA b for detecting CD271 in cell lines derived from hypopharyngeal cancer (HPCM1, 2, 6), lung cancer (MCC148c), and melanoma (SK-MEL-2-Luc, MeWo-Luc), and found that all of the staining patterns using hCD271 mA b were similar to those using the commercially available antibody (ME20.4) (Fig. 1A). We further confirmed the specificity of the hCD271 mA b by Western blot analysis. CD271 was clearly detected as a protein of about 50-kDa molecular weight by hCD271 mA b, similar to the detection with a commercially available antibody (clone C40-1457) (Fig. 1B). Next, we analyzed the kinetics of hCD271 mA b with SK-OV-3-CD271 cells using flow cytometry. The dissociation constant (K_D) of hCD271 mA b was estimated to be 1.697×10^{-9} M (Fig. 1C). The ECD of CD271 consists of four cysteine-rich domains (CRDs). We tested the affinity of the mCD271 mA b to four CD271 deletion mutants (Δ1, Δ1–2, Δ1–3, and Δ1–4) and to wild-type CD271 by ELISAs, and found that the antibody bound to wild-type CD271 but not to any of the mutants, suggesting that the antibody recognizes an epitope at the CRD1 domain (Fig. 1D).

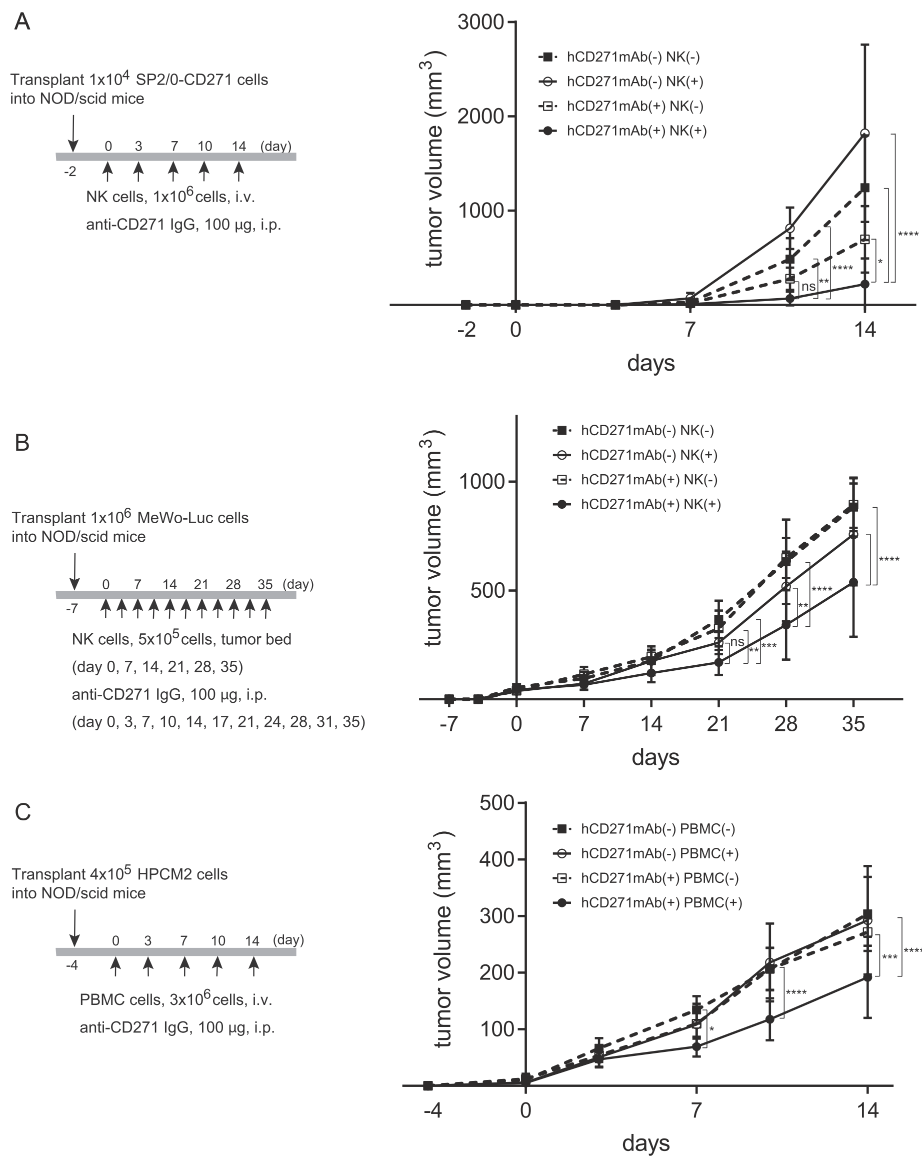


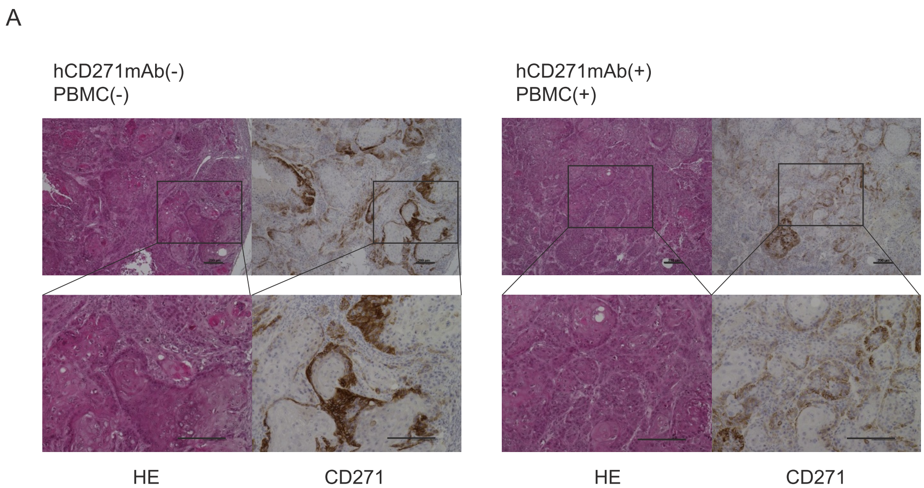
Fig. 3. Anti-tumor activity of the hCD271 mA b *in vivo*. The indicated tumor cells SP2/0-CD271 (A), MeWo-Luc (B), and HPCM2 (C) were xenografted into NOD/scid mice, and humanized anti-CD271 mAb (hCD271 mA b) and effector cells (NK or PBMC) were injected. The tumor size was measured weekly. i. p., intraperitoneal. PBMC, peripheral blood monocytes. Values are means ± SEM. Asterisks indicate statistical significance ($^*P < 0.05$, Tukey-Kramer's test).

3.2. hCD271 mA b-mediated ADCC in vitro

We next investigated whether hCD271 mA b could induce ADCC against CD271-positive cancer cells. For *in vitro* ADCC assay, we used hCD271-introduced mouse myeloma cell line, SP2/0-CD271, because NK cells possessed lower killing activity without the antibody against SP2/0-CD271 than other CD271-positive cell lines such as HPCM2, MeWo and SK-OV3. The SP2/0-CD271 cells were killed by NK cells more efficiently in the presence of hCD271 mA b than its absence (Fig. 2A), indicating that hCD271 mA b-mediated ADCC is effective for killing cells. We next examined the ADCC activity at various antibody concentrations, and found that it increased in an antibody dose-dependent manner (Fig. 2B). We examined the ADCC activity using other cell lines MeWo-Luc and HPCM2, and got similar results (Fig. 2C). We also checked whether hCD271 mA b elicited complement-dependent cytotoxicity, and found no such activity against SP2/0-CD271 cells (data not shown). We further examined the effect of hCD271 mA b on the *in vitro* proliferation of HPCM2 cells. There was no difference in the HPCM2 cell growth in the presence versus the absence of hCD271 mA b in the culture medium (Fig. S2), indicating that hCD271 mA b does not have a direct effect on cell proliferation *in vitro*.

3.3. Anti-tumor effect of hCD271 mA b in vivo

We next investigated the anti-tumor effect of hCD271 mA b in cancer graft models in mice using the SP2/0-CD271, MeWo-Luc, and HPCM2 cell lines. Cells from the three cell lines were subcutaneously implanted into the bilateral flanks of NOD/scid mice, hCD271 mA b was then administered together with effector NK cells or PBMCs, and the resulting tumors were observed. hCD271 mA b was intraperitoneally administered, and the effector cells were administered into the tail vein or tumor bed. In the mice with SP2/0-hCD271-transplanted cells, the tumor development with hCD271 mA b compared with the control IgG administration was significantly reduced on day 14 (Fig. 3A). The body weight was slightly decreased in those receiving hCD271 mA b and NK cells as compared with the other groups, probably because the tumor



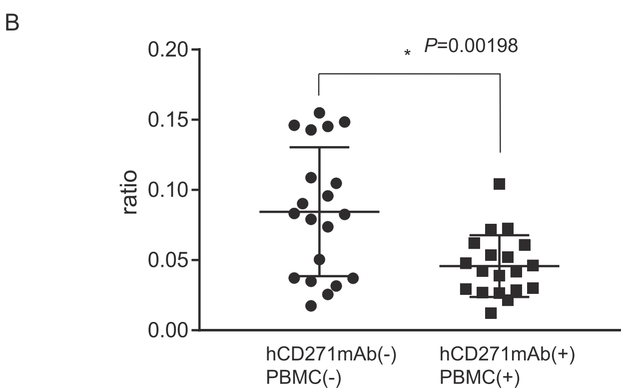


Fig. 4. CD271-positive cells were decreased by humanized anti-hCD271 mA b treatment. (A) Representative images of Hematoxylin & eosin (HE) staining and immunohistochemical staining for CD271 in tumors obtained from mice with HPCM2 xenografts. (B) Ratio of the CD271-positive area/ total area. HE, Hematoxylin & eosin staining.

growth was smaller than that in the other groups (Fig. S3A). Similar results were obtained in the graft models using MeWo-Luc and HPCM2 cells, which both express endogenous CD271 (Fig. 3B and C). In the mice with MeWo-Luc xenografts, the body weight was not significantly different among the treatment groups (Fig. S3B). In the mice with HPMC2-xenografts, the body weight was slightly decreased except for the hCD271 mA b and PBMC-treated group, in which tumors were significantly suppressed (Fig. S3C). These data indicated that the HPCM2 cells secrete some toxic substance(s) in mice.

The tumors that developed in the graft models using HPCM2 cells were dissected, and tumor sections were stained with hematoxylin and eosin or immunohistochemically for CD271. Although there was little difference in the hematoxylin and eosin staining between tumors treated with or without hCD271 mA b and PBMCs, the immunohistochemical staining clearly showed that CD271-positive cells were decreased in the tumors treated with hCD271 mA b and PBMCs (Fig. 4A and B). These data suggested that hCD271 mA b is effective for suppressing CD271-positive tumor cell growth, through ADCC.

4. Discussion

In this study, we sought to establish a humanized mouse mAb against human CD271 for application as a clinical therapy for tumors. Various mAbs are known to exert anti-tumor activity through ADCC [25–27]. Since the efficacy of ADCC is determined by the antibody's affinity, we selected mouse mCD271 mA b, which was specific for the human CD271 extracellular domain, because it had the highest affinity among our mAb clones screened, and we humanized it to obtain hCD271 mA b. The K_D of hCD271 mA b was calculated to be 1.697×10^{-9} M. Most antibodies used for clinical trials have K_D values between 10^{-9} M and 10^{-10} M; for example, those of Herceptin, Erbitux, Opdivo, and Rituxan are 5.0×10^{-9} M, 3.8×10^{-10}, 2.6×10^{-9} M, and 8.0×10^{-9} M, respectively [28], indicating that the affinity of therapeutic antibodies for tumors is in the range of K_D values of 10^{-9} M or less. Accordingly, the hCD271 mA b has sufficient affinity to be applied for tumor treatment.

Tumors exhibit heterogeneous target antigen expressions, and low antigen expression causes insufficient ADCC [29]. We previously reported that CD271 is expressed heterogeneously in primary hypopharyngeal cancers and in xenograft tumors of the hypopharyngeal PDX cell line, HPCM2, that CD271-positive HPCM2 cells are more tumorigenic than CD271-negative cells, and that CD271-positive cells can generate CD271-negative cells [10]. In this study, we demonstrated that the tumor growth in mice with transplanted HPCM2 cells was significantly inhibited by treatment with hCD271 mA b together with PBMCs. Although the CD271 staining was heterogeneous in HPCM2 tumor tissues even without treatment, the CD271-positive cells were significantly decreased after treatment with the antibody and PBMCs, suggesting that ADCC caused the death of the CD271-positive cells in HPCM2 tumors. We previously reported that CD271-positive cells exhibit cancer stem cell-like characteristics in human HPC, such as high tumorigenicity and chemotherapy resistance [10]. In colon cancer, LGR5-positive cells serve as cancer stem cells, and the selective ablation of LGR5-positive cells leads to tumor regression, followed by tumor regrowth driven by the re-emergence of LGR5-positive cells [30]. Another report suggested that the targeting of $CD133^+$ cells, known as a cancer stem cell marker, leads to a tumor response in breast, ovarian, and gastrointestinal cancer [31,32]. These data suggest that the targeting of cancer stem cells is a promising therapeutic strategy, and that a combination therapy of anti-CD271 antibody and chemotherapy could result in improved outcomes.

We recently reported that CD271 is essential for cancer-cell proliferation, and that knocking down CD271 causes cell-cycle arrest in HPCM2 cells [11]. CD271 is known as a nerve growth factor receptor, and its downstream signaling molecules, including MEK/ERK, AKT, and NFκB have been identified. Here, using an MTT assay, we found that hCD271 mA b did not inhibit the proliferation of HPCM2 cells. Furthermore, we performed the cell proliferation assay using other newly established anti-CD271 mouse IgGs, but none of the IgGs tested showed an inhibitory effect (data not shown). We checked the proliferation ability under the stimulation of ligands of CD271 (NGF, BDNF, NT3, and NT4); however, alteration was seen in HPCM2 cells (data not shown). We speculated that CD271 could work with co-receptor(s) and stimulate the proliferation activity, and the ligand-receptor interaction of CD271 is not important for cancer cell proliferation. Further study is required to elucidate the precise mechanisms of cell proliferation involving the CD271 pathway.

ADCC activity generally depends on the affinity of the antibody and the amount of antigen. We found a positive correlation between the *in vitro* ADCC activity and the CD271 expression in hypopharyngeal cell lines (HPCM1, 2, and 6, data not shown.) By flow cytometry, CD271-positive cells represented 23.4%, 82.2%, and 73.8% of the HPCM2, SP2/0-CD271, and MeWo-Luc cells, respectively (Fig. S4). Thus, it is reasonable that the ADCC activity was higher in the SP2/0-CD271 than in the HPCM2 cells. In MeWo-Luc cells, the ADCC activity was lower than in the SP2/0-CD271 cells, despite having almost the same CD271 expression. Since protein glycosylation varies between cell types, and the antigenic modulation by glycosylation affects antibody affinity [33–35], further study is needed to elucidate the mechanism of antigen recognition by the anti-CD271 antibody in the MeWo-Luc cells.

CD271 is expressed in normal tissues, such as in the basement membrane of the oral cavity and esophagus. CD271 is also expressed in neural cells and plays an important role in axonal growth. Thus, it is possible that an anti-CD271 antibody administered into patients could cause adverse effects such as neuronal damage, mucositis, and dermatitis. An effective treatment should include a drug delivery system that transports the antibody specifically to cancer tissues. Recently, a system for delivering nano-sized therapeutic agents to cancers that relies largely on enhanced permeability and retention (EPR) effects caused by the leaky nature of the cancer vasculature was reported [36]. In CAR-T (T cells expressing chimeric antigen receptors) therapy, a recent report demonstrated a new CAR system in which two different antigen stimulations are required simultaneously to activate T cells [37]. Further study will be needed to develop effective treatments using anti-CD271 antibodies.

Taken together, a newly established humanized anti-CD271 antibody, hCD271 mA b, could be a useful immunotherapeutic molecule for CD271-positive cancers such as hypopharyngeal cancer and melanoma. Our data also suggest that cancer stem cell-targeted therapy could be advantageous for HPC.

Conflicts of interest

The authors have no competing ethical or financial interests to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.07.011.

Table S1
A list of antibodies used in this study.

Application	Antibody	clone, production number	
Flow cytometory	anti-human CD271	ME20.4, sc-13577	Santa Cruz
	anti-mouse IgG FITC-conjugated	poly 4053	Biolegend
	anti-human IgG antibody PE-conjugated	409303	Biolegend
	human IgG1k isotype control	GTX16193	GENETEX
ELISA	anti-human CD271	ME20.4, sc-13577	Santa Cruz
		D4B3, 8238	Cell Signaling Technology
		G323A	Promega
		C40-1457 (RUO), 560927	BD Biosciences
		NGFR5, ab3125	Abcam
	anti-mouse IgG (H + L chain) HRP-conjugated	330	MBL
	Anti-CA IX mouse IgG2a	H-11, sc-393581	Santa Cruz
Western Blotting	anti-human CD271	C40-1457 (RUO), 560927	BD Biosciences
	anti-α-tubulin	B-5-1-2, sc-23948	Santa Cruz
	anti-mouse IgG (H + L chain) HRP-conjugated	330	MBL
	anti-human IgG (H + L chain) HRP-conjugated	206	MBL
MTT assay	human IgG1k isotype control	GTX16193	GENETEX
Immunohistochemistry	anti-human CD271	C40-1457 (RUO), 560927	BD Biosciences

Table S2
A list of primers used in this study.

Primer name	Purpose	F/R	Sequence (5′ to 3′)
Primer#1	pcDNA™4/myc-His A-full length CD271	Forward	cgaattcgccaccatgggggcaggtgccac
		Reverse	cgaattcacaccggggatgtggcagtg
Primer#2	pcDNA™4/myc-His A-CRD1-deleted CD271 (Δ1)	Forward	aaGGATCCGCCACCATGccctgcctggacagcgt
		Reverse	ttgaattcTCAcaccggggatgtggc
Primer#3	pcDNA™4/myc-His A-CRD1 and 2-deleted CD271 (Δ1–2)	Forward	aaGGATCCGCCACCATGcgctgcgcctacggcta
		Reverse	ttgaattcTCAcaccggggatgtggc

(*continued on next page*)

Table S2 (*continued*)

Primer name	Purpose	F/R	Sequence (5′ to 3′)
Primer#4	pcDNA™4/myc-His A-CRD1,2 and 3-deleted CD271 (Δ1–3)	Forward	aGGATCCGCCACCATGgagtgccccgacggcac
		Reverse	ttgaattcTCAcaccggggatgtggc
Primer#5	pcDNA™4/myc-His A-all CRD-deleted CD271 (Δ1–4)	Forward	aaGGATCCGCCACCATGgaggagatccctggccg
		Reverse	ttgaattcTCAcaccggggatgtggc
Primer#6	pBABE-puro-CD271	Forward	cgaattcgccaccatgggggcaggtgccac
		Reverse	cgaattcacaccggggatgtggcagtg
Primer#7	pFastBac-CD271 ECD	Forward	AAAGGATCCggaggtgccaaggaggca
		Reverse	TTTGAATTCTCAgtcggtggtgcctcgggt
Primer#8	Sequence primer for pFastBac	Forward	ATACCGTCCCACCATCG
		Reverse	GATCCTCTAGTACTTCTCG

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