Claudin-18 Splice Variant 2 Is a Pan-Cancer Target Suitable for Therapeutic Antibody Development

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Abstract

Purpose: Antibody-based cancer therapies have emerged as the most promising therapeutics in oncology. The purpose of this study was to discover novel targets for therapeutic antibodies in solid cancer.

Experimental Design: We combined data mining and wet-bench experiments to identify strictly gastrocyte lineage – specific cell surface molecules and to validate them as therapeutic antibody targets.

Results: We identified isoform 2 of the tight junction molecule claudin-18 (CLDN18.2) as a highly selective cell lineage marker. Its expression in normal tissues is strictly confined to differentiated epithelial cells of the gastric mucosa, but it is absent from the gastric stem cell zone. CLDN18.2 is retained on malignant transformation and is expressed in a significant proportion of primary gastric cancers and the metastases thereof. In addition to its orthotopic expression, we found frequent ectopic activation of CLDN18.2 in pancreatic, esophageal, ovarian, and lung tumors, correlating with distinct histologic subtypes. The activation of CLDN18.2 depends on the binding of the transcription factor cyclic AMP – responsive element binding protein to its unmethylated consensus site. Most importantly, we were able to raise monoclonal antibodies that bind to CLDN18.2 but not to its lung-specific splice variant and recognize the antigen on the surface of cancer cells.

Conclusions: Its highly restricted expression pattern in normal tissues, its frequent ectopic activation in a diversity of human cancers, and the ability to specifically target this molecule at the cell surface of tumor cells qualify CLDN18.2 as a novel, highly attractive pan-cancer target for the antibody therapy of epithelial tumors.

Gastroesophageal and pancreatic cancers are among the malignancies with the highest unmet medical needs (1). Gastric cancer is the second leading cause of cancer death worldwide. In 2002, ~1.4 million people worldwide developed gastroesophageal cancers and 1.1 million died (2). Esophageal cancer incidence has increased in recent decades, coinciding with a shift in histologic type and primary tumor location. Adenocarcinoma of the esophagus is now more prevalent than squamous cell carcinoma in the United States and Western Europe, with most tumors located in the distal esophagus (3). The overall 5-year survival rates for gastroesophageal cancers are 20% to 25% despite the aggressiveness of established standard treatments associated with substantial side effects (4, 5). For pancreatic cancer, there is an even greater medical need. Due to the advanced state of the disease at the time of diagnosis, the prognosis is extremely poor, with median survival times of less than 6 months and a 5-year survival rate of <5.5%. The lack of a major benefit from the various newer-generation combination chemotherapy regimens for these cancers has stimulated research into the use of targeted agents, such as small molecule – based kinase inhibitors and monoclonal antibodies (mAb; ref. 6). However, mAbs, such as bevacizumab, cetuximab, and trastuzumab, which have emerged as the most promising therapeutic molecules in oncology in the last decade, have thus far failed as single agents to show efficacy in these cancers (7 – 9). This may indicate that growth factor receptors, such as epidermal growth factor receptor and human epidermal growth factor receptor-2/neu, might not be appropriate as targets in these tumors and highlights the fact that novel targets are urgently needed for these clinical indications.

The most successful single agents with antineoplastic activity have been mAbs against highly lineage-specific targets. This type of target ensures that nearly all normal tissues lack the target molecule and are not at risk of toxic effects. This concept has thus far been confined to hematopoietic malignancies because well-characterized lymphocyte differentiation antigens,

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Translational Relevance

This report is of high clinical relevance because it is the first description of the suitability of CLDN18.2 as a target for therapeutic mAbs. In the meantime, a chimerized mAb has been developed and will enter phase Ib trials in patients with gastroesophageal cancer within this year. This antibody is highly cytocidal. Xenografted CLDN18.2-expressing tumor cells in mice are efficiently recognized, with complete remissions observed in a considerable number of mice even in advanced tumor models. The submitted report is the first of a series of articles in preparation featuring the mode of action and pharmacotoxicology of this novel antibody. We would highly appreciate its publication in a journal that is devoted to subjects of clinical relevance, such as *Clinical Cancer Research*.

such as CD20, CD22, CD25, CD33, and CD52 (10–12), could be exploited for this purpose. Anti-CD20 antibodies, such as rituximab, for example, bind exclusively to mature B lymphocytes, resulting in the depletion of B cells. Because CD20 is neither detectable on any other normal cell type nor shared by the stem cell precursor of the B-cell lineage (13, 14), there is no severe dose-limiting toxicity, and normal B-lymphocyte levels are eventually restored by target-negative stem cells. Importantly, even malignant B cells that express CD20 at much lower levels than nonneoplastic B cells can be treated successfully with rituximab.

In the treatment of solid tumors, however, the approach of targeting highly selective tissue differentiation antigens has not been pursued to date due to a lack of suitable candidate molecules.

Recently, through a bioinformatical search strategy for colonocyte lineage genes, we discovered a promising new target candidate for mAb therapy of colorectal cancer (15, 16). Here, we adopted this approach to identify novel targets in gastric cancer and identified the tight junction molecule claudin-18 isoform 2 (CLDN18.2). We show here for the first time that CLDN18.2 is activated in a wide range of human malignancies, including gastric, esophageal, pancreatic, lung, and ovarian cancers, and can be specifically targeted with mAbs.

Materials and Methods

Data mining strategy. A general human expressed sequence tag (EST) file was generated from all available libraries that contained the keyword *Homo sapiens* in the organism field of the dbEST report file at the National Center for Biotechnology Information. From this, a stomach tissue–specific subfile was extracted with ESTs containing the words "stomach" or "gastric" in their description file. In total, we analyzed 6,280,883 ESTs represented in 4,021 cDNA libraries. Stomach tissue ESTs were grouped into clusters assembling overlapping ESTs. The sequence homology search program BLASTN was run for each sequence recorded in the stomach tissue EST subfile against all of the human ESTs in the general list. Thus, for each query EST from the stomach tissue subfile, an "electronic Northern" was generated. Candidates with EST hits derived exclusively from the stomach were subjected to motif and structure analysis by SMART to identify surface molecules topologically resembling CD20 (minimal requirements: at least four hydrophobic regions qualifying as transmembrane domains according to TMHMM and prediction of at least one small extracellular loop).

RNA extraction and reverse transcription-PCR. First strand cDNA preparation and reverse transcription-PCR (RT-PCR) were done as described elsewhere (17, 18). PCR sense primers differentiating between claudin-18 isoforms 1 (5′-TCCACCACCACATGCCAAGTG-3′) and 2 (5′-TGGCTCTGTGTCGACACTGTG-3′) were combined with a shared antisense primer (5′-GTGTACATGTTAGCTGTGGAC-3′). In the real-time RT-PCR assay, the expression levels were normalized to nonexpressing peripheral blood mononuclear cells through a ΔΔCT calculation. Positive expression was defined as an expression level at least 10-fold or higher than the levels in nonexpressing tissues.

DNA bisulfite sequencing. DNA was subjected to bisulfite modification with the use of the CpGenome DNA Modification Kit (Intergen). The reaction products were amplified with primers specific for modified promoter sequences not covering any CpG dinucleotide (sense, 5′-GAGTAGGGTAAGTTTAGGTGGTATTGG-3′; antisense, 5′-CCAATCAATAAAACCACAAACCCCAAACC-3′). Ten individual clones per promoter were sequenced.

Quantitative methylation-specific PCR. Subsequent to the sodium bisulfite conversion of DNA, a methylation analysis was done by fluorescence-based, real-time PCR assays with the use of methylation-specific CLDN18.2 promoter primers (5′-CGTTTTTATTATATGGTTCGTTTTTGACG-3′, 5′-CAAATAAACAACRCAATTCTAAAACTACCG-3′) as described previously (18). β-Actin amplification (primers 5′-TGGTGATGGAGGAGGTTTAGTAAGT-3′, 5′-AACCAATAAAACCTACTCCTCCCTTAA-3′) allowed the normalization of input DNA. The percentage of methylated alleles was calculated by dividing the CLDN18.2/actin ratio of a sample by the CLDN18.2/actin ratio of bisulfite-modified Universal Methylated DNA (Chemicon) multiplied by 100.

Cyclic AMP–responsive element binding protein transcription factor assay. A commercial assay (Chemicon) was used to analyze the DNA sequences for binding of cyclic AMP–responsive element binding protein (CREB). The following double-strand biotinylated capture probes were synthesized (Operon): bp -66 to -31 of the promoter region containing a predicted consensus sequence for CREB (5′-ATGGTCCGTTCCTGACGTGTACACCAGCCTCTCAGA-3′), a control probe displaying a mutation within the putative CREB response element (5′-ATGGTCCGTTCCTAAAATGTACACCAGCCTCTCAGA-3′), and a control probe methylated at the cytosine (5′-ATGGTCCGTTCCTGAC^{m}GTGTACACCAGCCTCTCAGA-3′). The probes were mixed with nuclear extracts generated from HEK293 cells treated with 100 ng forskolin, transferred to a streptavidin-coated plate, and washed. The phosphorylated CREB transcription factor was detected with a specific mouse anti–phospho-CREB (Ser133) mAb.

Luciferase reporter gene assay. The fragment -1203 to +237 of the *CLDN18.2* promoter was cloned into the pGL4.14 luciferase reporter vector (Promega). The cytosine nucleotide within the CRE site at position -51 to adenine was mutagenized by PCR (mutation of primers in boldface and italics: 5′-CCATTACATGGTCCGTTCCTGA**A**GTGTAACACCAGCCTCTCAGAGAAAAC-3′ and 5′-GTTTTCTCTGAGAGGCTGGTGTACAC***T***TCAGGAACGGACCATGTAATGG-3′ complementary strand). HEK293 cells were transfected with the reporter construct with the use of Lipofectamine 2000 (Invitrogen). After 24 h of culture, Luciferin was added, and the luciferase activity was measured.

Peptide-specific rabbit antisera against CLDN18. The affinity purified anti–claudin-18/n-term antiserum was generated by immunizing rabbits with the synthetic peptide epitope DQWSTQDLYN (amino acids 28-37 in the first extracellular domain), which is specific for this splice variant. The commercial rabbit anti–claudin-18/mid antiserum (Zymed) recognizes both splice variants because it is directed against an undisclosed epitope in the middle part of the human CLDN18 protein.

Immunohistochemistry. The majority of the analyzed tissues (gastric cancers, primary ovarian adenocarcinomas) were archival, formalin-fixed, paraffin-embedded specimens collected by the Department of Pathology of the Community Hospital Aalst. Blocks for tissue microarrays were prepared from these by standard techniques. Many carcinomas were represented twice to correct for potential tumor heterogeneity.

For pancreatic adenocarcinomas, metastases of different tumors in ovaries, metastases of gastric cancers into ovaries (Krukenberg tumors) and normal tissue panels, and commercial tissue microarrays were purchased from Imgenex.[5] After deparaffinization, all slides were subjected to antigen retrieval by boiling in DakoCytomation Target Retrieval solution at 95°C for 30 min (DakoCytomation) and incubated with primary rabbit antiserum for 45 min at room temperature. Bound antibody was visualized with the polymer-based Powervision and a horseradish peroxidase–anti-mouse/rabbit/rat IgG detection kit (ImmunoVision Technologies), and 3,3′-diaminobenzidine as chromogen.

The percentage of neoplastic cells that were labeled with the antibody was scored, as was the relative intensity of labeling (from 0 to 3+). Significant expression was defined as a staining intensity of at least 2+ in at least 60% of cancer cells per individual tumor.

Mouse mAbs against CLDN18.2. Intramuscular DNA immunization of BALB/c mice was conducted on days 1, 16, and 36 with 25 μg of plasmid DNA representing the vector pSecTag2 (Invitrogen), encoding a fusion gene consisting of the IgG κ-leader sequence and the first extracellular domain of CLDN18.2 plus 2.5 μL polyethylenimine-mannose (150 mmol/L) in water. Boosting was done i.p. with 5×10^7 HEK293 cells transfected with a pCR3.1 construct encoding full-length CLDN18.2. Spleen cells from immunized mice were fused with the mouse myeloma cell line P3X63Ag8U.1 (American Type Culture Collection; CRL 1597) with the use of standard hybridoma technology.

Flow cytometry. The cells were washed in PBS with 2% FCS and 0.1% sodium azide, and stained with 10 μg/mL purified hybridoma supernatants, including supernatant 175D10. Allophycocyanin-labeled goat anti-mouse IgG (Dianova) was used as a secondary antibody. Cell counts were plotted against the fluorescence intensity of cells in the allophycocyanin channel with the use of FACSArray equipment (Becton Dickinson).

Results

Transcripts of CLDN18 splice variant 2 are restricted to gastric tissue and detected in several epithelial tumor types. Applying a strategy for the *in silico* discovery of genes highly lineage specific for gastric tissue, we identified claudin-18 (*CLDN18*). CLDN18 belongs to the family of claudins, which are cell surface molecules with four membrane-spanning domains involved in the formation of tight junctions (19). The human *CLDN18* gene has two alternative first exons, giving rise to two protein isoforms (CLDN18.1 and CLDN18.2) differing in the N-terminal 69 amino acids (20), including the first extracellular loop (Fig. 1A). Transcription profiling of a restricted set of tissues had previously shown that these isoforms have different lineage commitments, with CLDN18.1 being predominantly expressed in lung tissue whereas CLDN18.2 displays stomach specificity (20). Moreover, CLDN18 expression had been investigated by other groups in gastric (21, 22) and pancreatic cancers (23), the latter without distinguishing between the two splice variants. We extended the profiling to a comprehensive set of normal and tumor samples by end-point RT-PCR. *CLDN18.1* was confirmed as a lung-specific gene (Fig. 1B) whereas *CLDN18.2* tissue distribution was restricted to normal stomach (Fig. 1B; Table 1). In addition, we detected CLDN18.2 transcripts in the majority of samples derived from gastric cancers, showing that this lineage marker is not lost during the course of malignant transformation (Fig. 1B; Table 2A). Quantification by real-time RT-PCR showed that ~70% of gastric adenocarcinomas display transcript levels comparable with, or even higher than, those in normal stomach tissue (Fig. 1C). In addition to this orthotopic expression, the ectopic activation of CLDN18.2 was detected in neoplasias derived from CLDN18.2-negative tissues, such as pancreatic adenocarcinomas (7 of 11), esophageal tumors (6 of 10), non–small cell lung cancers (30 of 73), and ovarian cancers (Fig. 1B; Table 2A). Notably, in the majority of these specimens, the transcript levels were lower than in gastric tissues (Fig. 1C).

The CLDN18.2 protein is a highly selective gastric lineage marker expressed in short-lived differentiated cells but not the stem cell zone of stomach mucosa. To assess the tissue distribution of the CLDN18.2 protein, a set of normal human tissues (one to nine donors per tissue type) was investigated by immunohistochemistry with the rabbit anti–claudin-18/n-term and anti–claudin-18/mid antibodies. In the large majority of tissues, CLDN18.2 was not detected (Table 1; Fig. 2A). As implied by transcript analysis, stomach mucosa was the only tissue in which we detected the CLDN18.2 protein (Fig. 2B, *top left*). Surprisingly, the CLDN18.2 protein was predominantly observed in the epithelial cells of the pit region and the base of gastric glands, including exocrine and endocrine cells (Fig. 2B, *top* and *bottom right*). The staining was strongly membrane associated, with an additional cytoplasmic component in the endocrine cells. However, in the neck region of the gastric glands, no significant CLDN18.2 staining was detectable (Fig. 2B, *bottom left*). The upper isthmus of the neck zone is considered to harbor gastric stem cells. However, due to the lack of definitive markers of lineage commitment, gastric stem cells cannot be defined at the single-cell level. It is generally agreed and well supported by extensive cell positional experiments that they are located in the vicinity of the highly proliferative, post-progenitor cell population (24, 25), which we identified by anti-Ki67 staining. Negligible CLDN18.2 expression was observed in both the highly Ki67+ mitotic, post-progenitor population as well as the adjacent cell zone harboring the stem cell pool (Fig. 2C). Our data establish CLDN18.2 as a highly selective marker for gastric cells activated at an advanced stage of differentiation.

The CLDN18.2 protein is a pan-cancer target expressed in primary lesions and metastases of several human cancer types. To determine the prevalence of human cancers that are positive for the CLDN18.2 protein, tumor tissue sections were examined by labeling with a rabbit anti–claudin-18/mid antibody (Table 2B). A staining intensity ≥2+ in at least 60% of tumor cells was defined as a significant level of labeling to estimate the number of patients that may benefit from an approach that targets CLDN18.2. CLDN18.2 was detected in 77% of primary gastric adenocarcinomas. Of these, 56% displayed significant expression of CLDN18.2 (Table 2B; Fig. 3A). Labeling was localized to the membrane and partially accompanied by cytoplasmic staining. Down-regulation (expression scores not within the range defined as significant) of CLDN18.2 expression occurred more frequently in intestinal than in diffuse gastric cancers (significant expression in 46% versus 75%; Table 2B). The CLDN18.2 protein was also frequently detected (51%) in a set of lymph node metastases of gastric cancer (Table 2B). In 21 of 24 patients, matched nodal metastases displayed the same CLDN18.2 expression status as the primary tumors from which they were derived (exemplified in Fig. 3A). Interestingly, in distant metastases of gastric cancer into ovaries, significant

[5] http://www.imgenex.com/tissue_array.php

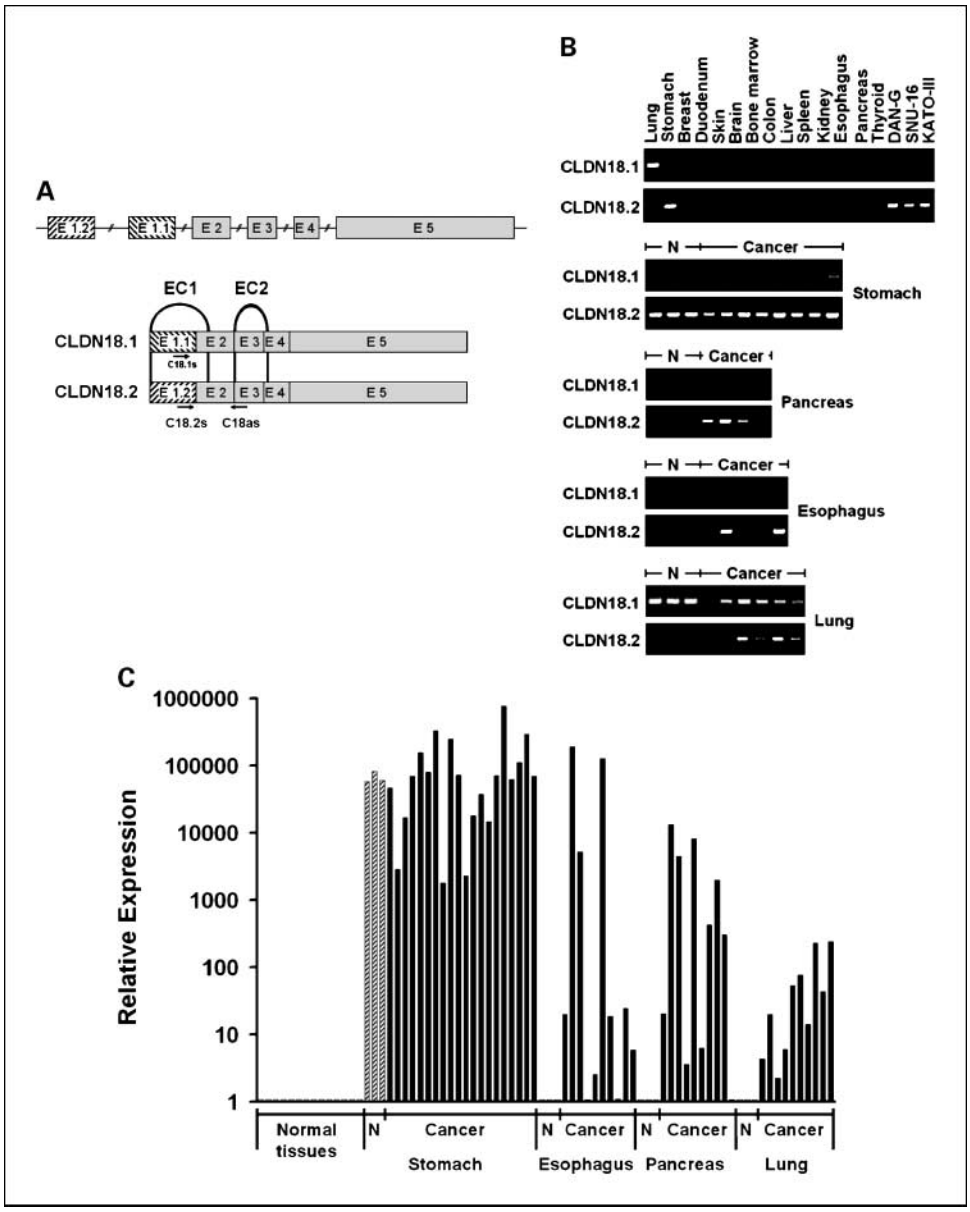

Fig. 1. Profiling of CLDN18.1 and CLDN18.2 transcripts in a panel of human tissues. *A,* genomic structure of the *CLDN18* locus (*top*). Hatched boxes, exons unique for CLDN18.1 (E1.1) or CLDN 18.2 (E1.2), respectively; bottom, exon composition of CLDN18 variants; arches, the two extracellular domains; arrows, the primers used for RT-PCR. *B,* comparative analysis of the CLDN18 isoforms in normal human tissues (*N*), primary tumor specimen, and tumor cell lines by end-point RT-PCR. *C,* quantification in normal human tissues (*N*), primary tumor specimen, and tumor cell lines by real-time PCR.

CLDN18.2 labeling was prevalent in 69% of patients (exemplified in Fig. 3A). Metastatic lesions of primaries of unknown origin into the ovaries displayed significant CLDN18.2 labeling in 50% of patients. In contrast, ovarian metastases derived from colorectal cancer were not positive for CLDN18.2 at any significant level (Table 2B).

In addition to this orthotopic expression, the CLDN18.2 protein was detected in malignancies of the pancreas, esophagus, and ovaries (Fig. 3B; Table 2B). Most interestingly, correlation with histologic subtypes was observed—adenocarcinoma, but not squamous cell cancers, of the esophagus; mucinous, but not serous, ovarian cancers; and ductal pancreatic adenocarcinomas, but not pancreatic islet cancers, were CLDN18.2 positive.

In summary, our data qualify CLDN18.2 as a pan-cancer target that is expressed in a diverse variety of epithelial tumor types and is maintained in disseminated cancer cells.

Transcriptional regulation of CLDN18.2 correlates with genomic hypomethylation of promoter CpG islands and involves the transcription factor CREB. The notion that a tightly regulated gastric differentiation marker escapes silencing in a number of epithelial tumor entities originating from unrelated tissue types raised our interest in the transcriptional regulation of CLDN18.2. The methylation of CpGs in promoter regions has been reported as a mechanism for both the lineage-specific expression of differentiation genes (26, 27) as well as the ectopic activation of genes in cancer (28). The treatment of peripheral blood mononuclear cells as well as the CLDN18-negative cell line SNU-I with the methylation inhibitor 5-aza-2′-deoxycytidine resulted in the robust induction of CLDN18.2 but not CLDN18.1 (Fig. 4A).

The methylation status of individual CpG dinucleotides (positions -78, -66, -51, -10, +8, +22) in the promoter region of *CLDN18.2* (Fig. 4B) was assessed by sequencing bisulfite-treated DNA (Fig. 4C) whereas the relative methylation level of the sequence region of interest was determined by methylation-specific real-time PCR (Fig. 4D). In tissues and cells without

detectable expression of CLDN18.2, such as peripheral blood mononuclear cells, normal human lung tissue, and SNU-I cells, CpGs in the promoter region were completely methylated. In contrast, the constitutive expression of CLDN18.2 in normal gastric tissue, stomach, and lung cancers as well as the chemically induced activation of this molecule in primary cells and tumor cell lines were associated with hypomethylation of the promoter region (Fig. 4C and D).

Sequence analysis of the *CLDN18.2* promoter region led to the identification of binding sites for several transcription factors, including CREB, at positions -47 to -54 (Fig. 4B). CREB occupancy in the promoter regions of several genes has been reported to be blocked by the methylation of the central CpG dinucleotide in the prototypical CRE palindrome (5′-TGACGTCA-3′; refs. 29, 30). Interestingly, the respective CpG dinucleotide (position -60) in the *CLDN18.2* promoter is

Table 1. Expression of CLDN18.2 transcript and protein in normal human tissues as measured by end-point RT-PCR and immunohistochemistry

	RT-PCR	Immunohistochemistry	
		a-CLDN18/mid	a-CLDN18/n-term
Abdominal organs			
Esophagus	0/6	0/2	0/3
Stomach	10/11	7/7	9/9
Small intestine	—	0/1	0/2
Colon	0/3	0/5	0/8
Liver	0/4	0/1	0/3
Pancreas	0/5	0/1	0/3
Gall bladder	—	0/1	0/2
Urogenital organs			
Breast	0/5	0/1	0/6
Ovary	0/5	0/1	0/3
Kidney	0/4	0/5	0/8
Testis	0/4	0/1	0/2
Cervix	0/2	0/1	0/1
Placenta	0/3	0/1	0/2
Uterus	0/4	—	0/1
Bladder/urothelium	0/5	0/1	0/2
Prostate	0/5	0/1	0/5
Endometrium	—	0/1	0/2
Cardiopulmonary system			
Lung	0/10	0/1	0/4
Heart	0/5	0/1	0/2
Endothelia	—	0/1	0/1
Nervous system			
Total brain	0/5		
Cerebral cortex	0/1	0/1	0/3
Cerebellum	0/1	0/1	0/2
Nucleus caudatus	0/1	—	—
Hippocampus	0/1	—	—
Corpus callosum	0/1	—	—
Pons	0/1	—	—
Pituitary gland	0/1	0/1	0/2
Retina	0/1	0/1	0/2
Others			
Skin	0/3	0/1	0/2
Skeletal muscle	0/4	0/1	0/2
Thyroid	0/3	0/1	0/2
Adrenal gland	—	0/1	0/2
Salivary gland	—	0/1	0/2
Hematopoietic system			
Bone marrow	0/1	0/2	0/3
Spleen	0/3	0/1	0/2
Lymph node	0/5	0/1	0/2
Tonsil	0/2	—	—
Thymus	1/2	—	—
PBMC	0/5	—	—
PBMC, activated	0/5	—	—
B lymphocytes	0/2	—	—
CD4+ T lymphocytes	0/2	—	—
CD8+ T lymphocytes	0/2	—	—
Granulocytes	0/2	—	—
Dendritic cells	0/2	—	—

Abbreviation: PBMC, peripheral blood mononuclear cells.

Table 2. Expression of CLDN18.2 transcript in human cancer tissues as determined by end-point RT-PCR (*A*) and immunohistochemistry (*B*)

(A)

Tumor type	RT-PCR
Gastric AC	46/48 (96%)
Pancreatic AC	7/11 (63%)
Esophageal cancer	6/10 (60%)
Non–small cell lung cancer	30/73 (41%)
Colorectal carcinoma	2/16
Hepatocelluar carcinoma	2/12
Breast cancer	1/16
Ovarian cancer	2/13
Prostate cancer	0/11
Renal cell cancer	0/12
Malignant melanoma	0/8
Head and neck cancers	1/5

(B)

Tumor type	Any labeling	≥2+ in ≥60% of cells significant labeling	No labeling
Primaries			
Gastric AC	51/66 (77%)	37/66 (56%)	15/66 (23%)
Diffuse		15/20 (75%)	
Intestinal		21/45 (46%)	
Esophagus AC	17/22 (78%)	11/22 (50%)	5/22 (22%)
Esophagus SCC	0/7	0/7	7/7
Pancreatic AC, ductal	8/10 (80%)	6/10 (60%)	2/10 (20%)
Pancreatic islet cell carcinoma	0/5	0/5	5/5
Ovarian AC	4/42 (10%)	4/42 (10%)	38/42 (90%)
Serous	0/25	0/25	25/25
Mucinous	4/17 (24%)	4/17 (24%)	13/17 (76%)
Metastases			
Gastric AC, LN metastasis	19/29 (66%)	15/29 (51%)	10/29 (34%)
Gastric AC, ovarian metastasis	32/33 (96%)	23/33 (69%)	1/33 (4%)
Colorectal cancer, ovarian metastasis	6/28 (21%)	0/28	22/28 (79%)
Unknown primary, ovarian metastasis	8/12 (66%)	6/12 (50%)	4/12 (33%)

Abbreviations: AC, adenocarcinoma; SCC, squamous cell carcinoma; LN, lymph node.

methylated in tissues that do not express this gene (Fig. 4C and D). To determine whether the *CLDN18.2* promoter is responsive to activation by CREB, protein-DNA binding assays were done (Fig. 4E). The incubation of the nuclear cell extract from forskolin-treated HEK293 cells as a source of CREB protein with a biotinylated oligonucleotide capture probe representing the wild-type CRE site of *CLDN18.2* resulted in the formation of a specific protein-DNA complex. This was inhibited by excess amounts of the unlabeled competitor and also in the presence of oligonucleotide probes, in which the cytosine at position -60 bp was either mutated or altered by methylation.

We then linked the promoter region to a luciferase reporter gene. The transfection of this plasmid construct into forskolin-treated HEK293 cells resulted in significant increases in luciferase activity (Fig. 4F). Site-directed mutagenesis of the CpG dinucleotide within this *cis*-element disrupted the ability of this basal promoter to significantly activate gene expression and completely abolished promoter activity. In summary, these results suggest that CREB is directly involved in the transcriptional control of the human *CLDN18.2* gene.

Splice variant 2 of CLDN18 can be specifically targeted with the use of mAbs. The tissue distribution of CLDN18.2 makes it an attractive candidate for therapeutic antibodies. As a tetraspannin, CLDN18.2 has two extracellular loops. However, because mAbs binding to extracellular epitopes of claudins have not been described thus far, it is not known whether members of this family are accessible drug targets on the surface of tumor cells. A further challenge was to avoid the cross-reactivity of such antibody drugs with the closely related splice variant 1 of CLDN18 because this protein is expressed in normal lung tissue. The two variants only differ by 8 of the 51 amino acids in the first extracellular domain, and the sequences of their second extracellular loops are identical. To generate splice variant–specific antibodies, we immunized with DNA encoding a secretion domain attached to the first extracellular loop of CLDN18.2 and boosted with CLDN18.2-transfected tumor cells. Classic hybridoma technology was used to obtain a panel of antibodies directed against CLDN18.2. To identify antibodies with the desired binding characteristics, screening of supernatants was done with the use of flow cytometry and HEK293 cells transfected with CLDN18.1, CLDN18.2, or the vector backbone, and human gastric cancer cell lines with constitutive CLDN18.2 expression. As exemplified by mAb 175D10, antibodies were identified that bind exclusively to CLDN18.2 (Fig. 5A) and detect physiologic densities of the epitope (Fig. 5B) on the surfaces of living cells.

Discussion

Our primary objective was to identify a differentiation antigen of the gastric lineage suitable in an analogous manner to the concept of targeting CD20 in lymphoma treatment as target for antibody therapy in gastric cancer. Our findings not only support CLDN18.2 as a candidate target for the development of therapeutic antibodies but also provide new insights into the tissue expression and regulation of this tissue-specific tight junction protein.

First, by profiling a comprehensive set of human tissues, we showed that CLDN18.2 is a lineage-specific marker that is highly selective for short-lived gastric epithelial cells and is absent from the vast majority of healthy tissues. These results refine and extend previous reports, which have been done in a smaller panel of normal tissues and were restricted to transcriptional profiling (20, 22, 31). Specifically, we did not find expression of CLDN18.2 in skin or duodenal Paneth cells, as reported by others for the human or mouse orthologue of this gene (22, 32, 33). Unexpectedly, however, we unraveled the absence of CLDN18.2 from the stem cell zone of gastric glands. Stem cells give rise to all gastrointestinal epithelial cell lineages and thereby regulate their normal homeostasis and turnover, which occurs every two to 7 days (25, 34, 35). The exclusive expression of CLDN18.2 in differentiated gastric cells, in combination with the fact that transient GI toxicity is a frequent and manageable adverse event, makes this molecule highly attractive as a target for the development of safe and potent drugs.

Second, we found sustained membranous CLDN18.2 protein expression in a considerable number of cancers arising from gastric epithelial lineages. Previously, Sanada et al. (22) showed that, overall, half of the tested stomach cancer samples retained CLDN18.2, which correlated with a survival benefit. The down-regulation of CLDN18.2 was preferentially observed in intestinal gastric cancers, which is in line with our findings. Matsuda et al. (21), however, reported robust CLDN18.2 expression in only one third of tested gastric cancer samples and described a tendency toward CLDN18.2 down-regulation at the invasive front, which is in contrast to our study and those of others (22). Compared with these previous reports, CLDN18.2 expression was more frequently sustained in our study (any membrane labeling in 77% and staining intensity of at least 2+ in at least 60% of cells in 56% of cases). This may be attributed to the different origins of tested specimens (Caucasian versus Japanese), the different proportions of intestinal and diffuse subtypes within the test samples, and the different scoring systems used to categorize the state of CLDN18.2 protein expression. Thus far, our study is the only one in which CLDN18.2 membrane labeling has been analyzed in metastases of stomach cancer, in which frequencies are

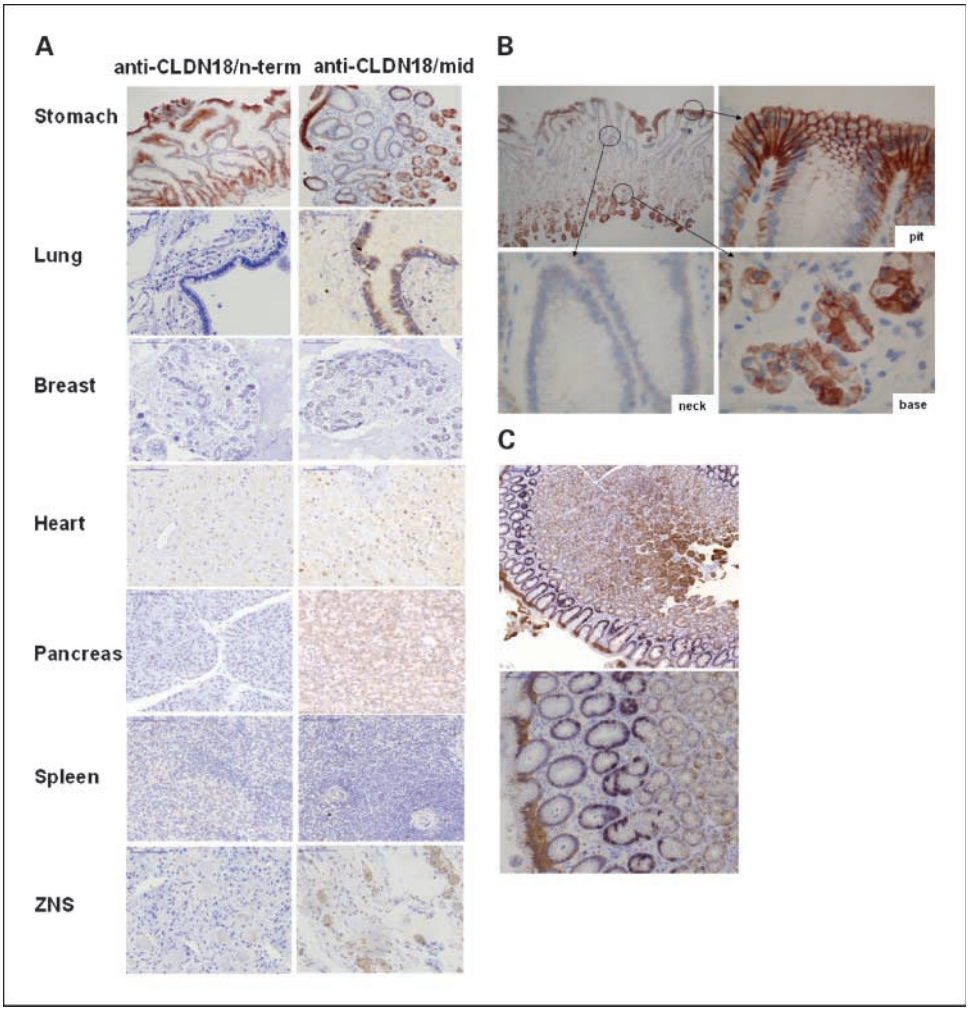

Fig. 2. Immunohistochemistry of normal human tissues with CLDN18.2 antibodies. *A,* sections prepared from normal human tissues were stained with anti – claudin-18/n-term (1:300) and anti – claudin-18/mid (1:50) antibodies. The anti – claudin-18/mid antibody labels lung due to cross-reactivity with variant CLDN18.1. *B,* epithelia from the antral gastric region were stained with the anti – claudin-18/n-term antibody (*top left*). Close-ups show CLDN18.2 protein expression status at the tip and the base of glands but not in the neck region. *C,* double staining of gastric tissue sections with Ki67 (*dark purple*) and anti – claudin-18/mid antibodies (*brown*).

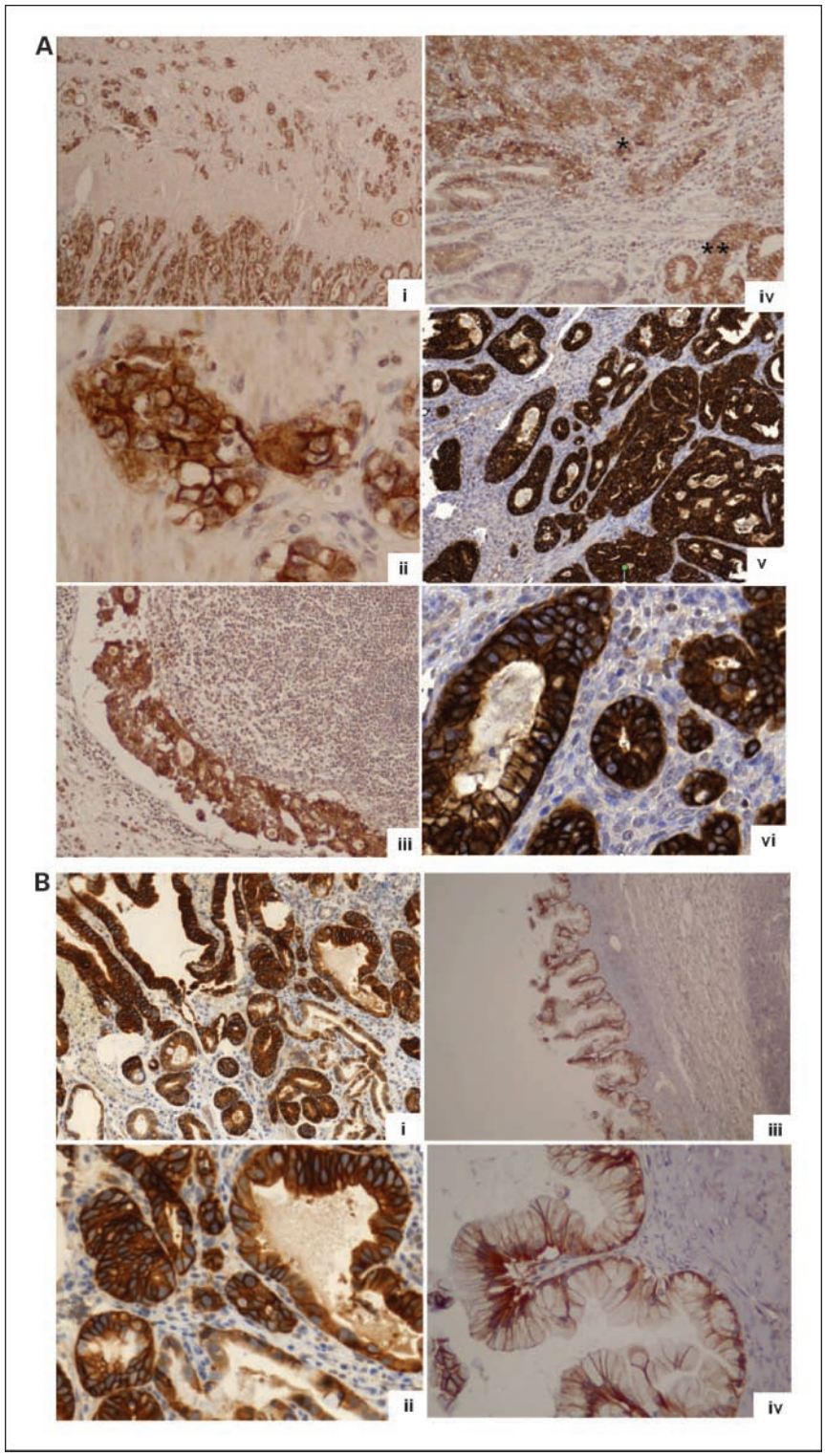

Fig. 3. Immunohistochemistry of tumor tissues with CLDN18.2 antibodies. *A,* tissue sections obtained from gastric tumors expressing CLDN18.2. Overview (*i*) and close-up (*ii*) of a primary lesion and a lymph node metastasis (*iii*) obtained from the same patient. Gastric cancer specimen from a second patient (*iv*). Overview (*v*) and close-up (*vi*) from a gastric cancer metastasis into ovary obtained from a third patient. *B,* overviews and close-ups of an adenocarcinoma of the esophagus (*i, ii*) and of a mucinous ovarian cancer (*iii, iv*), respectively. All cases shown in (*A*) and (*B*) had been scored as 3+ staining in 90% to 95% of cells. *, tumor tissue; **, normal tissue.

comparable with those in primary gastric cancer specimens, supporting the potential value of this candidate therapeutic target in advanced disease.

Third, we reported that, in the context of malignant transformation, CLDN18.2 does not remain restricted to the gastric cell lineage but does occur in tumors derived from organs that normally do not contain a CLDN18.2-positive cell population. This was already implied by previous data (23), showing significant levels of antibody labeling in 50% of pancreatic cancer specimens, including early lesions, such as pancreatic intraepithelial neoplasia. We showed for the first time that CLDN18.2 is a pan-cancer target and is aberrantly activated in neoplasias of the lung, ovary, and esophagus. In all tumor types, we observed associations with distinct histologic subtypes, suggesting that CLDN18.2 is linked to a specialized genetic program. Jovov et al. (36) have recently described that

CLDN18.2 is activated during the course of metaplastic transition of stratified squamous cell epithelium of the esophagus to specialized columnar epithelium. This alteration arises in the setting of gastroesophageal reflux and predisposes for the development of distal esophageal adenocarcinoma. This suggests that ectopic CLDN18.2 activation may be an early event in esophageal adenocarcinoma.

Our data imply that a considerable number of patients suffering from cancers of desperate prognosis may be eligible for CLDN18.2 targeting therapies under the presumption that significant labeling is defined as a staining intensity ≥2+ in at least 60% of tumor cells. This cutoff is arbitrary but more stringent than the immunohistochemical criteria that are internationally required for the administration of approved antibodies to solid tumors, such as cetuximab (any membranous epidermal growth factor receptor protein expression in a minimum of 1% colon cancer cells) or trastuzumab (3+ membranous staining in at least 10% of breast cancer cells or 1+ to 2+ membranous staining in at least 10% of breast cancer cells combined with *Her2/neu* gene amplification). It is not known thus far which fraction of cells within a tumor have to express the target antigen to ensure the clinical benefit of mAb treatment. Moreover, the secondary mechanisms occurring consecutive to Fc-mediated immune effects on binding to the antigen-specific target cell and their potential effect on surrounding target-negative cells are still not understood. To investigate these questions in future studies, antibody targets such as CLDN18.2 are required, which are less heterogeneously expressed than those addressed by currently approved antibodies.

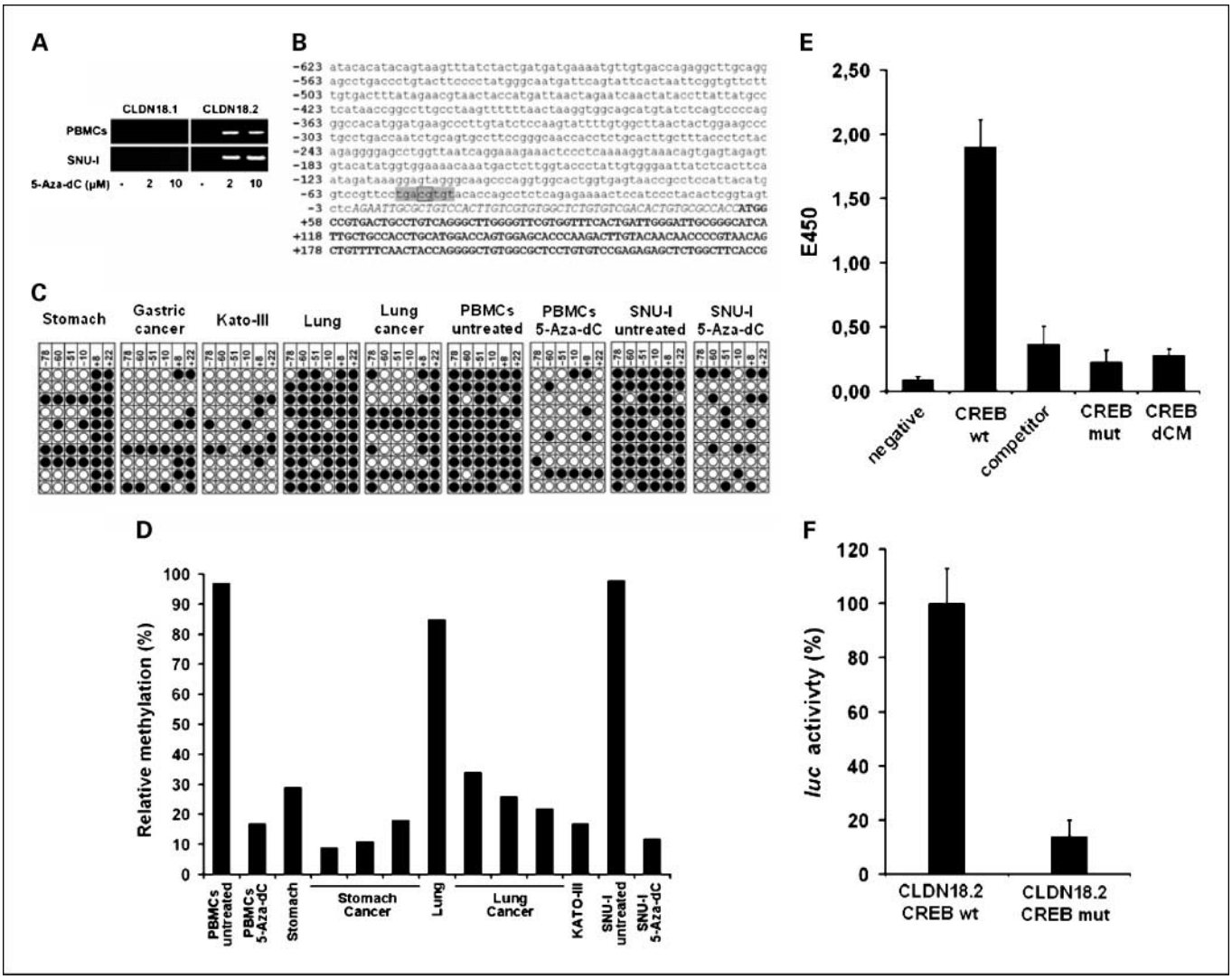

Fig. 4. The effect of promoter methylation and the role of CREB on expression of CLDN18.2. *A,* freshly isolated peripheral blood mononuclear cells (*PBMCs*) and the gastric cancer cell line SNU-I were cultured in medium supplemented with different concentrations of the methylation inhibitor 5-aza-2′-deoxycytidine (5-Aza-dC) for 72 h. *B,* the 5′ untranslated region of the CLDN18.2 transcript (*uppercase italics*) and the translated region (*bold*). Gray, the minimal CREB binding consensus sequence; boxed, the central CpG considered as a critical site for methylation. *C,* analysis of promoter methylation in different human tissues and cell types. Filled circles, methylated CpG dinucleotides; open circles, unmethylated CpG dinucleotides. *D,* relative methylation of promoters as analyzed by quantitative methylation-specific real-time PCR. All cancer specimens subjected to this analysis were previously confirmed as CLDN18.2 positive by RT-PCR. *E,* formation of DNA-protein complexes was measured using biotinylated oligonucleotide duplexes without the CREB binding site (neg), the wild-type CREB binding site of the CLDN18.2 promoter (CREB wt) alone, or in combination with an unlabeled competitor probe in excess, variants in which the central cytosine is either mutated (mut) or methylated (dCM). *F,* the relative luciferase activities in HEK293 cells transiently transfected with constructs representing the CLDN18.2 promoter region and a variant with mutated CREB binding site. Values, means ± SD from three different experiments.

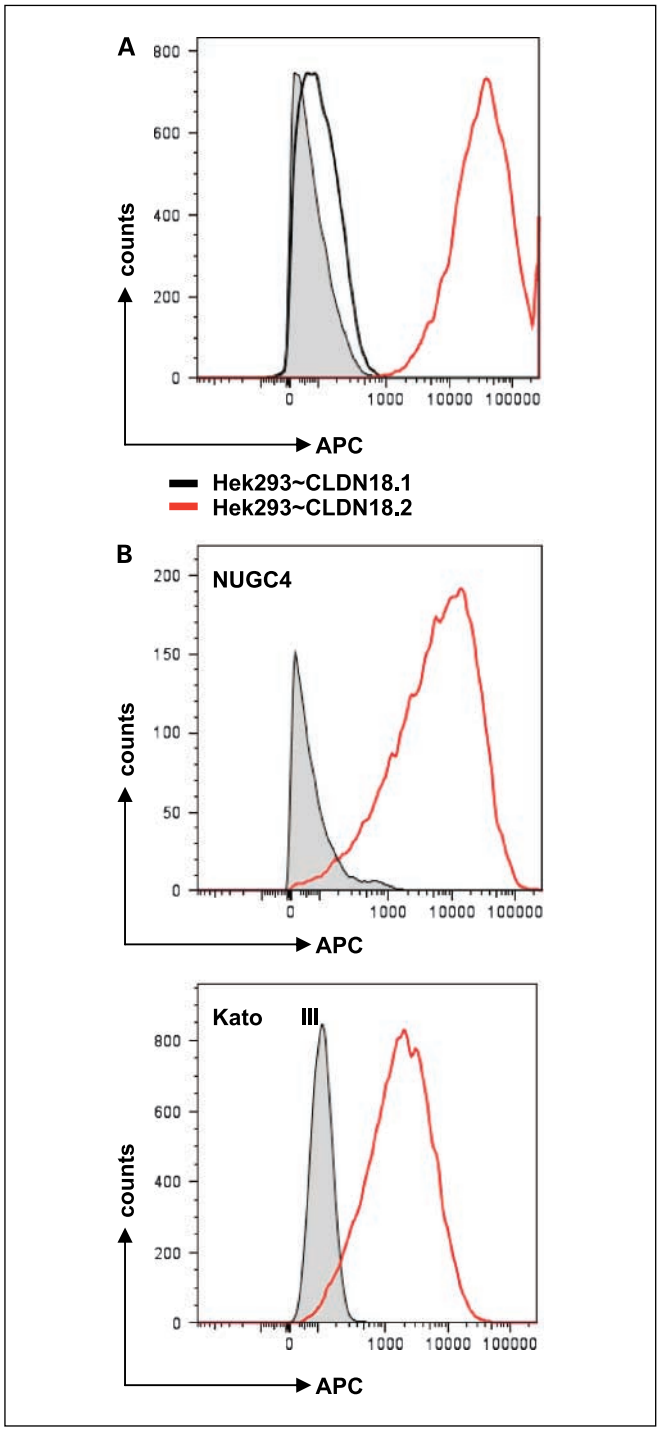

Fig. 5. Targeting CLDN18.2 with therapeutic mAbs. Flow cytometric analysis of the binding of the anti-CLDN18.2 mouse mAb 175D10 (*unfilled histograms*) to cell surface epitopes as compared with an irrelevant mouse IgG1 isotype control (*gray histograms*). *A,* labeling of HEK293 cells stably transfected with CLDN18.1 (*black line*) and CLDN18.2 (*red line*) by mAb 175D10. *B,* labeling of the human stomach cancer cell lines NUGC4 (*top*) and KATO III (*bottom*) by mAb 175D10. Both cell lines have been shown to express CLDN18.2 but not CLDN18.1 by RT-PCR and immunofluorescence microscopy. Dead cells were excluded from the analysis.

Fourth, we identified *cis*-regulatory elements of CLDN18.2 expression. This is particularly interesting given that CLDN18.2 expression is tightly repressed in the vast majority of normal tissues, and it frequently escapes this stringent silencing in the course of malignant transformation. Our results indicate that the transcription factor CREB is required to switch on CLDN18.2 transcription, which is completely blocked by the methylation of a central CpG within its binding site. This was further confirmed by analysis in a panel of normal and neoplastic human tissues either lacking or expressing CLDN18.2. The CREB binding domain is conserved in *CLDN18.2* orthologues from other species, supporting its role as a dominant regulator of basic CLDN18.2 transcription in constitutively expressing primary tissues. This does not exclude the involvement of other factors in regulating CLDN18.2 transcription. It has been reported that treatment of the gastric cancer cell line MKN45 with phorbol-12-myristate 13-acetate enhances prevalent levels of CLDN18.2 expression via activator protein-1 motifs (37).

Fifth, we showed that CLDN18.2 can be targeted by antibodies. mAbs labeling members of the claudin family at the surfaces of tumor cells have not been previously described. We not only provided proof that claudins per se can be targeted by mAbs. We also presented the first mAb that binds exclusively to CLDN18.2 and not to CLDN18.1. Given that the CLDN18.1 splice variant is expressed in lung, which is a tissue that is highly relevant to toxicity, such splice variant specificity is a necessary requirement to allow further consideration of cytotoxic mAb therapies based on this tight junction molecule.

In principle, mAbs against CLDN18.2 may be designed to induce tumor cell death via several independent mechanisms. One is attack by complement and cytotoxic immune effector cells, which may result in potent cytocidal effects independent of the cellular functions of CLND18.2. Additionally, mAbs may interfere with the biological function of CLDN18.2 in cancer cells, which is, thus far, not precisely known. As a typical representative of the growing claudin family (38), CLDN18 is most likely to be involved in the formation of tight junctions (19). The key functions of tight junctions are the maintenance of a luminal barrier, paracellular transport, and signal transduction. Involvement in tumorigenesis has been reported for other members of the claudin family (reviewed in ref. 39). In ovarian cancer, claudin-3 and claudin-4 proteins are highly overexpressed (40), and the overexpression of these claudins increases cell invasion and motility (41). In colon cancer, increased expression of claudin-1 has been associated with increased growth and metastasis of xenograft tumors in athymic mice (42). These claudins, in contrast to CLDN18.2, are widely expressed in healthy tissues, including those relevant to toxicity (31).

The aim of ongoing studies is to assess which modes of action are required to generate anti-CLDN18.2 mAbs, which combine both high safety and antitumoral activity, and whether this can be translated into a clinical benefit.

Disclosure of Potential Conflicts of Interest

Several of the authors (U. Sahin, M. Koslowski, D. Usener, G. Brandenburg, Ö. Türeci) are named as inventors on patent applications describing CLDN18.2 as therapeutic target. Several authors are associated with the University spin-off company Ganymed Pharmaceuticals either as employees (U. Sahin, M. Koslowski, D. Usener, G. Brandenburg, Ö. Türeci) or advisory board member (C. Huber).

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