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Original Research

Chloride intracellular channel protein 2 is secreted and inhibits MMP14 activity, while preventing tumor cell invasion and metastasis ☆, ☆☆

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Abstract

The abilities to invade surrounding tissues and metastasize to distant organs are the most outstanding features that distinguish malignant from benign tumors. However, the mechanisms preventing the invasion and metastasis of benign tumor cells remain unclear. By using our own rat distant metastasis model, gene expression of cells in primary tumors was compared with that in metastasized tumors. Among many distinct gene expressions, we have focused on chloride intracellular channel protein 2 (CLIC2), an ion channel protein of as-yet unknown function, which was predominantly expressed in the primary tumors. We created CLIC2 overexpressing rat glioma cell line and utilized benign human meningioma cells with naturally high CLIC2 expression. CLIC2 was expressed at higher levels in benign human brain tumors than in their malignant counterparts. Moreover, its high expression was associated with prolonged survival in the rat metastasis and brain tumor models as well as with progression-free survival in patients with brain tumors. CLIC2 was also correlated with the decreased blood vessel permeability likely by increased contents of cell adhesion molecules. We found that CLIC2 was secreted extracellularly, and bound to matrix metalloproteinase (MMP) 14. Furthermore, CLIC2 prevented the localization of MMP14 in the plasma membrane, and inhibited its enzymatic activity. Indeed, overexpressing CLIC2 and recombinant CLIC2 protein effectively suppressed malignant cell invasion, whereas CLIC2 knockdown reversed these effects. Thus, CLIC2 suppress invasion and metastasis of benign tumors at least partly by inhibiting MMP14 activity.

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Keywords: Distant metastasis, Brain tumor, Benign, Malignant, Invasion

Abbreviations: ANOVA, analysis of variance; APMA, 4-aminophenylmercuric acetate; C6 cells, original C6 glioma cells; CC cells, C6 cells transfected with C-terminally FLAG-tagged rat CLIC2 cDNA; CC3, cleaved caspase 3; CLIC, chloride intracellular channel protein; E-cadherin, epithelial cadherin; EGFP, enhanced green fluorescent protein; empty cells, C6 cells transfected with an empty vector; FACS, fluorescence activated cell sorting; FDR, false discovery rate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GBM, glioblastoma multiforme; GSC, glioblastoma stem-like cell; GST, glutathione S-transferase; MMP, matrix metalloproteinase; NNGH, *N*-Isobutyl-*N*-[4-methoxyphenylsulfonyl]glycyl hydroxamic acid; PCA, principal component analysis; PFS, progression-free survival; PM, plasma membrane; qPCR, quantitative real-time RT-PCR; RNA-seq, RNA sequencing; TAM, tumor-associated macrophages; TIMP2, tissue inhibitor of metalloproteinase 2; VAMP7, vesicle-associated membrane protein 7; VE-cadherin, vascular endothelial cadherin.

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Introduction

The mechanisms and molecules responsible for the invasion and metastasis of malignant tumor cells have received extensive focus in cancer research. In particular, studies have revealed that matrix metalloproteinase (MMP) 2 and its activator MMP14 (also known as membrane type 1-MMP) are deeply involved in tumor cell invasion and metastasis [1,2]. However, the reasons why cells from benign tumors do not invade their surrounding tissues or metastasize to distant organs remain unclear.

To identify the molecules that prevent tumor cell metastasis and invasion, we employed a rat model of malignant tumors [3], in which rat C6 glioma cells were transplanted in the back of the neonatal Wistar rats and macroscopic metastasis was formed in the lung within 4 wk after the transplantation. C6 cells create both intracranial brain tumor models and lung metastasis models in the immunocompetent rats, allowing analyses of distant metastasis to the lung and invasion into normal brain tissue. We compared the gene expression of the primary back and metastatic lung tumors by RNA sequencing (RNA-seq), revealing that a number of genes are expressed at higher levels in non-metastatic cells compared to metastatic cells. Among these, we have focused on chloride intracellular channel protein (CLIC) 2 as a potential inhibitor of the invasive and metastatic activities of tumor cells.

Although CLICs have been identified as chloride ion channel proteins, whether they form ion channels of the organelles or the cell surface remains unclear, but considerable amounts are present in the soluble fractions of the cytoplasm [4,5]. CLIC2 is the least investigated protein among the six CLIC family members [6], partly due to the lack of this gene in murine genome. CLIC2 has been implicated in the mental retardation symptoms [7] and has also been postulated to be responsible for the regulation of ryanodine receptors [8]. Although only a few studies have described the relationship between CLIC2 and cancer, its high cellular expression has been implicated in the prolonged survival of patients with breast, lung, gastric, and liver cancers [9–11]. CLIC2 is expressed in normal blood vessel endothelial cells but not in the malignant cells, while involving in the maintenance of the vascular barrier functions [9]. However, no further in vivo detailed characterization including the efficacy of CLIC2 on cancer pathophysiology has been provided.

In this study, we investigated a possibility that CLIC2 suppresses invasive and metastasizing ability of tumor cells. This study firstly demonstrated that extracellularly secreted CLIC2 causes the suppression. Moreover, the direct binding of CLIC2 to MMP14 and its inhibitory effects on the enzyme activity were demonstrated. High CLIC2 expression was correlated with reduced blood vessel permeability. Overall, CLIC2 may be associated with the benign traits of tumor cells.

Materials and methods

Animals

All animal experiments were conducted in accordance with the Guidelines of the Ethics Committee for Animal Experimentation of Ehime University, Japan. Rat metastasis model [3] and rat glioma model [12] were prepared following the procedure as we reported previously. The tumor-bearing animals were considered dead when they could no longer maintain upright postures.

Clinical samples and tracking of patient prognosis

Surgically resected human specimens of meningioma (39 patients; Grade I: 22 cases, Grade II: 15, Grade III: 2) and glioblastoma multiforme (GBM) (24 cases) were obtained under informed consent and the approval of the local ethics committee at Ehime University Hospital, Japan. A part of the tumors was immediately frozen in liquid nitrogen after surgery and stored at -80°C until use. The surgically resected tissues were subjected to histopathological diagnosis per the World Health Organization (WHO) 2007 classification [13]. The progression free survival (PFS) curves were depicted from the start of treatment until the date of progression or death from any cause.

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR (qPCR) was performed as described elsewhere [14]. cDNA was amplified using primers listed in Table S1. All gene-specific mRNA expression values were presented as relative expression levels normalized to the housekeeping (reference) gene glyceraldehyde 3-phosphate dehydrogenase.

Culture of rat C6 glioma cells and human GBM cells

Enhanced green fluorescent protein (EGFP)-expressing rat C6 glioma cells were established and cultured as described elsewhere [15]. A human glioblastoma (GBM) cell line U251 [12] and SFC-2 cells [16] were cultured as described elsewhere. Wound-healing [3] and proliferation [12] assays were done as described elsewhere.

Cloning of CLIC2 cDNA and establishment of C6 cells expressing CLIC2

C-terminally FLAG-tagged rat CLIC2 cDNA was amplified from the total RNA fraction of EGFP-expressing C6 cells via RT-PCR and cloned into a pCX4-puro retroviral vector (gifted by Dr. Tsuyoshi Akagi, Eisai Co., Tokyo, Japan) using the InFusion cloning method (TaKaRa, Tokyo, Japan) [12]. A linearized pCX4-puro vector was prepared by PCR using PrimstarMax (TaKaRa) with primers listed in Table S2. Virus packaging was executed in 293T cells via *in vitro* packaging, in which pCX4-GFP-Clic2-FLAG was transfected together with pGP and pE-ampho packaging plasmids (TaKaRa). A viral vector carrying an empty pCX4-puro vector was also packaged as the negative control (empty). Infected C6 cells (CC cells) were selected according to the resistance against puromycin. Primers used are listed in Table S2.

CLIC2 silencing in CC cells

Stable silencing of CLIC2 in CLIC2-expressing C6 (CC) cells was established as described elsewhere [17]. The target sequence was 5′-ACCCTGAAATTGAGCTCTTTG-3′. A lentiviral vector carrying an irrelevant sequence was also constructed as the negative control by subcloning the sequence 5′-GCGCGCTTTGTAGGATTCG-3′ as described previously. Virus packaging was executed in 293T cells via in vitro packaging, in which CS-Clic2-EG was transfected together with pCMV-VSV-G-RSV-Rev and pCAG-HIVgp packaging plasmids (RIKEN, Wako, Saitama, Japan). Infected cells were selected according to the green fluorescence using FACSAria (Becton Dickinson, CA, USA).

RNA sequencing (RNA-seq)

RNA-seq for C6 cells

Gene expression profiles were compared between EGFP-expressing C6 cells that metastasized to the lungs and those that did not spread from the primary xenografted subcutaneous lesion by RNA-seq. C6 cells were dissociated from dissected tumor masses using a gentleMACS dissociator (Miltenyi Biotec, Tokyo, Japan) and collected by a FACSAria system. Each RNA library was prepared using TruSeq RNA sample prep kit v2-setA (Illumina, CA, USA) according to the manufacturer's instructions and subjected to RNA-seq on a MiSeq NGS sequencer (Illumina). Genes exhibiting prominent differential expression ($P < 0.05$) were selected and analyzed using the "iDEP" (http://bioinformatics.sdstate.edu/idep92/).

RNA-seq of human GBM cells

Isolated DNA samples of 10 GBM stem-like cell (GSC) lines preserved in the Department of Neurosurgery, University of Alabama at Birmingham were used. To demonstrate the difference of biological pathways according to CLIC2 expression, the 10 GSC lines were divided into two groups according to high or low CLIC2 expression (five cell lines each). GSEA software (http://software.broadinstitute.org/gsea/index.jsp) and iDEP were used to examine the different pathways between these two groups.

Primary culture of human meningioma cells and silencing of gene expression

Tumor tissues were surgically obtained from the tumor main body of two patients with meningioma (grade I and grade II), minced, digested with 0.1% trypsin, and then triturated with a Pasteur pipette. Cells were passed through a 70-μm strainer (Falcon; Becton Dickinson Biosciences) and resuspended in high-glucose DMEM supplemented with 10% FBS. Proliferating tumor cells passaged less than six times were subjected to experiments.

Silencing of CLIC2 expression in meningioma cells using siRNA

Primary meningioma cells were transfected by magnetofection using SilenceMag beads (OZ Biosciences, Marseille, France), according to the manufacturer's instructions. The used siRNA duplexes targeting CLIC2 gene and an irrelevant sequence are listed in Table S3.

Synthesis of recombinant proteins using a cell-free system

Glutathione *S*-transferase (GST)-tagged CLIC2, CLIC4 and tissue inhibitor of metalloproteinase 2 (TIMP2) were synthesized using a cell-free protein synthesis system and wheat germ ribosomal RNA [18]. In brief, CLIC2 or CLIC4, TIMP2 cDNA were obtained from CC cells and plasmid (TIMP2 NM_003255 Human Tagged ORF clone: OriGene, Rockville, MD, USA) using cloning primer (Table S4), and each cDNA was inserted into a pEUE01-GST-N2 expression vector containing a GST tag region. The proteins were automatically synthesized by the Robotic Protein Synthesizer Protemist DT II (CellFree Sciences, Matsuyama, Japan). The protein in the mixture was purified using glutathione Sepharose 4B (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions.

Immunoblotting

Tissue samples obtained from human meningioma cases and rat tumors were subjected to electrophoresis and transferred to membranes as described elsewhere [14]. Primary and secondary antibodies are listed in Table S5.

Immunohistochemical staining of animal and clinical cells and tissues

For immunohistochemical experiments, rats were fixed via transcardial perfusion as described elsewhere [19]. The surgically dissected human brain tumor tissues were fixed in 4% paraformaldehyde. Enzyme and immunofluorescence immunohistochemical staining was done as described elsewhere [20]. Primary (Table S6) and secondary antibodies (Table S7) are listed.

Concentration of conditioned medium

Each tumor cells were cultured to confluency in DMEM containing 10% FBS. The medium was then replaced with fresh serum-free medium. The conditioned medium was concentrated by 25 times using an Amicon Ultra-4 (Ultracel-3K) centrifugal filter device (Merck Millipore Ltd., Bedford, MA, USA), which was centrifuged at 7,500 × *g* for 50 min at 4°C.

Fluorescence activated cell sorting (FACS)

Back tumors of the rats dissected 30 d after transplantation were subjected to FACS analysis as described elsewhere [21]. Antibodies are listed in Table S8.

Evaluation of blood vessel permeability with Evans blue

Evans blue dye (0.5% in PBS; Sigma-Aldrich, E2129) was used to evaluate vascular leakage in the rat metastasis model [22]. Rats at 30 d after transplantation were sacrificed 1 h after the injection of Evans blue dye (100 μL/30 g body weight) into the tail vein, then the tumor and lung tissues were dissected, weighed, and incubated in 1 mL of formamide at 50°C for 24 h to extract the dye from the tissues. A microplate reader was used to measure the fluorescence of each supernatant at Ex/Em = 620 nm/680 nm. The Evans blue concentrations were normalized according to the results for the lung tissues in each sample. Part of the tumor masses was fixed in 4% paraformaldehyde and sections were incubated with anti-CD105 antibody, and subsequently treated with DyLight 488-labeled secondary antibody (Table S6). The fluorescence of extravasated Evans blue was evaluated by excitation at 535 nm.

Gelatin zymography

MMP-2 enzyme activity was analyzed in conditioned media of meningioma cells by using a Gelatin Zymography Kit (Cosmo Bio Co., Tokyo, Japan) according to the manufacturer's instructions.

Invasion assay

The invasive activity of U251, SFC-2, primary meningioma, and rat C6 cells was assessed by an in vitro assay method using BioCoat Matrigel Invasion Chambers with 8.0 μm PET Membranes (Corning, NY, USA) as described elsewhere [17]. Recombinant CLIC2 or CLIC4 (0.5 μM) were added into the upper chambers at the same time as seeding each cell.

Immunoprecipitation

To illustrate the binding between recombinant MMP-14 (Anaspec Inc., Fremont, CA, USA; Cat# 72068) and recombinant CLIC2, we performed immunoprecipitation using SureBeads Protein G (Bio-Rad, Hercules, CA, USA). Briefly, SureBeads were washed with PBS containing 0.1% Tween 20 three times. Each primary antibody (Table S9) was added to the beads and incubated for 10 min at room temperature. After washing the beads, antigen-containing lysate (250 ng of recombinant MMP-14 + 250 ng of recombinant CLIC2 or CLIC4 or TIMP2 in PBS + 2 mM $MgCl_2$ +1% BSA) was added to the beads and incubated on a rotator overnight at 4°C. To obtain the immunoprecipitates, beads were magnetized, washed, and further boiled in SDS-PAGE sample buffer followed by magnetization to obtain supernatants.

Assay for MMP14 activity

The inhibitory effects of CLIC proteins on the activity of recombinant MMP-14 were evaluated using a SensoLyte 520 MMP-14 Assay Kit (Anaspec) according to the manufacturer's instructions. Briefly, recombinant MMP-14 was activated by 1 mM 4-aminophenylmercuric acetate (APMA) or recombinant Furin (PEPROTECH, Cranbury, NJ, USA) for 2 h at 37°C. Recombinant CLIC2, CLIC4, TIMP2, and MMP-14 inhibitor (*N*-Isobutyl-*N*-[4-methoxyphenylsulfonyl] glycyl hydroxamic acid; NNGH, BioVision Cat# 2569-5, 25) were added to a microplate together with activated MMP-14. After adding MMP-14 substrate, MMP-14 activity was

determined by measuring the fluorescence of each well at Ex/Em = 490 nm/520 nm in a plate reader (FlexStation 3, Molecular Devices, Tokyo, Japan).

Preparation of the plasma membrane fraction

The plasma membrane fraction was prepared according to a method described elsewhere [23].

Preparation of the exosome fraction

Exosome fractions were prepared from the C6 conditioned media using the Total Exosome Isolation Reagent (Invitrogen) according to the manufacturer's instructions.

Statistical analysis

Data are expressed as the mean ± SD or SEM. Group means were compared using the two-tailed unpaired or paired Student's *t*-test, chi-squared test, long-rank test, or one-way analysis of variance (ANOVA) with Tukey's multiple comparison test. All analyses were performed using Prism 8 (GraphPad Software, La Jolla, CA, USA). $P < 0.05$ was considered significant for all tests.

Results

Gene expression in non-metastasized and metastasized cells

The EGFP-tagged rat C6 glioma cells that had been transplanted subcutaneously into the backs within 24 h after birth [3] and allowed to undergo macroscopic metastasis to the lungs ultimately resulted in the death of the animals (Fig. 1A). The primary tumors in the back and the metastasized tumors in the lungs were dissected 5 wk later, whereupon the EGFP-expressing cells were isolated via FACS and then transplanted again into the backs of neonatal Wistar rats or subjected to RNA-seq. The rats transplanted with the primary tumor-derived cells survived longer than those transplanted with the metastatic tumor cells (Fig. 1B). The RNA-Seq revealed the presence of gene expression clusters characteristic either for the lung tumor-derived metastasized or the back tumor-derived non-metastasized cells (Fig. 1C, D). *CLIC2* mRNA was more highly expressed in the primary tumor cells than in the metastasized ones (Fig. 1E, F). By contrast, there were no significant differences in the mRNA expression of *CLIC1, CLIC3, CLIC4, CLIC5*, and *CLIC6* between the primary back and metastatic lung tumors (Fig. S1A). The cells isolated from the metastatic tumors were again transplanted subcutaneously into the back of neonates. Five wk later, qPCR was used to evaluate the *CLIC2* mRNA expression levels in cells isolated from the primary (LB cells) and metastatic (LL cells) tumors (Fig. 1G). The LB cells exhibited higher *CLIC2* mRNA expression levels than the original C6 cells and LL cells.

Establishment of C6 glioma cells with high CLIC2 expression

It was noted that the C6 cells expressed *CLIC2* mRNA at a much lower level than it did *CLIC1* and *CLIC4* mRNAs (Fig. 2A). C6 cells highly expressing CLIC2 were established and designated as CC cells. Additionally, C6 cells transfected with an empty vector were prepared as controls (empty cells). Only the CC cells displayed detectable CLIC2 protein expression (Fig. 2B, C). The high CLIC2 expression level did not affect cell proliferation (Fig. 2D) or cell migration in the wound-healing assay (Fig. 2E). However, the CC cells displayed suppressed invasive activity through the Matrigel-coated chambers compared with the C6 and empty cells (Fig. 2F).

CLIC2 expression was correlated with suppressed metastasis and invasion

The C6, empty, and CC cells were transplanted subcutaneously into the backs of rat neonates. High CLIC2 protein expression in the CC back tumors were confirmed by immunohistochemical staining (Fig. S1B). There were no significant differences in the primary tumor size or lung metastasis frequency between the rats with C6 transplants and those with empty cell transplants (Fig. 3A). However, in the rats with CC cell transplants, smaller primary tumor masses formed (Fig. S1C) and metastasis to the lungs occurred less frequently (Fig. 3A, B). Consequently, the rats with CC cell transplants lived longer than those with C6 or empty cell transplants (Fig. 3C). The CC back tumors contained abundant apoptotic cells bearing cleaved caspase 3 (CC3), but much less $CC3^+$ cells were present in the C6 or empty cell tumors (Fig. 3D). mRNA encoding an anti-apoptotic factor *Bcl-*x_L was less expressed in the CC-back tumors than other two tumors (Fig. 3E). Another anti-apoptotic factor Bcl-2 and pro-apoptotic factor Bax mRNA expression was not changed among the tumor types (Fig. S1D). Each cell type in culture expressed Bcl-x_L, Bcl-2, and Bax to the similar extent (Fig. S1E). The same types of cells were transplanted into the striatum of neonatal rats to generate a model of malignant brain tumor [12]. Consequently, the C6 and empty cells formed large invasive tumors with vague boundaries, whereas the CC cells formed small tumors with clear boundaries (Fig. 3F, G, I). The rats with CC brain tumors lived longer than those with C6 or empty cell tumors (Fig. 3H).

CLIC2 expression in human brain tumors

Next, the expression of *CLIC2* mRNA in tumor tissues of WHO grade I human meningioma, the most benign type of meningiomas, was compared with that in grade II and III meningioma tissues as well as in GBM tissue (Fig. 4A). The mRNA expression level was the highest in the grade I meningioma cells. The expression of CLIC2 protein was diffuse in the grade I lesion, sparse in the grade II lesion, and nearly absent in the grade III tumor (Fig. 4B). Glioma tumor cells of grade I significantly expressed CLIC2 (Fig. 4C). However, only faint CLIC2 expression was detected in grade II, III, and IV glioma except for positive staining in the endothelia of blood vessels. The association of *CLIC1, CLIC2*, and *CLIC4* mRNA expression with progression-free survival (PFS) was investigated in patients with meningioma and GBM (Fig. 4D). The most suitable cut-off value was selected by comparing the area under the curve from the receiver operating characteristic curves for each tumor. PFS was prolonged in the patients with high *CLIC2* expression. By contrast, PFS was not associated with *CLIC1* or *CLIC4* expression, in spite of their high expression levels in the grade I meningioma tumors (Fig. S2).

Ten human glioblastoma stem-like cell (GSC) lines were categorized as having high or low *CLIC2* mRNA expression levels (Fig. 4E) and subsequently subjected to RNA-Seq analysis. CLIC2 expression levels were not significantly correlated with genetic mutation in *isocitrate dehydrogenase* gene, methylation of *O^6-methylguanine DNA-methyltransferase* CpG island, or the subtypes (proneural or mesenchymal) (Table S10). Hierarchical clustering and principal component analysis (PCA) (Fig. 4F) show that the cells with high or low CLIC2 expression display distinct gene expression patterns. Further gene-set enrichment analysis revealed that the cell lines with low *CLIC2* expression were enriched with genes in the hypoxia, inflammation, glycolysis, and mesenchymal pathways, whereas those with high *CLIC2* expression showed gene enrichment in the adhesion-related and proneural pathways (Fig. S3). Thus, the cells with high *CLIC2* expression had benign cell traits, whereas those with low expression featured malignant traits [24,25].

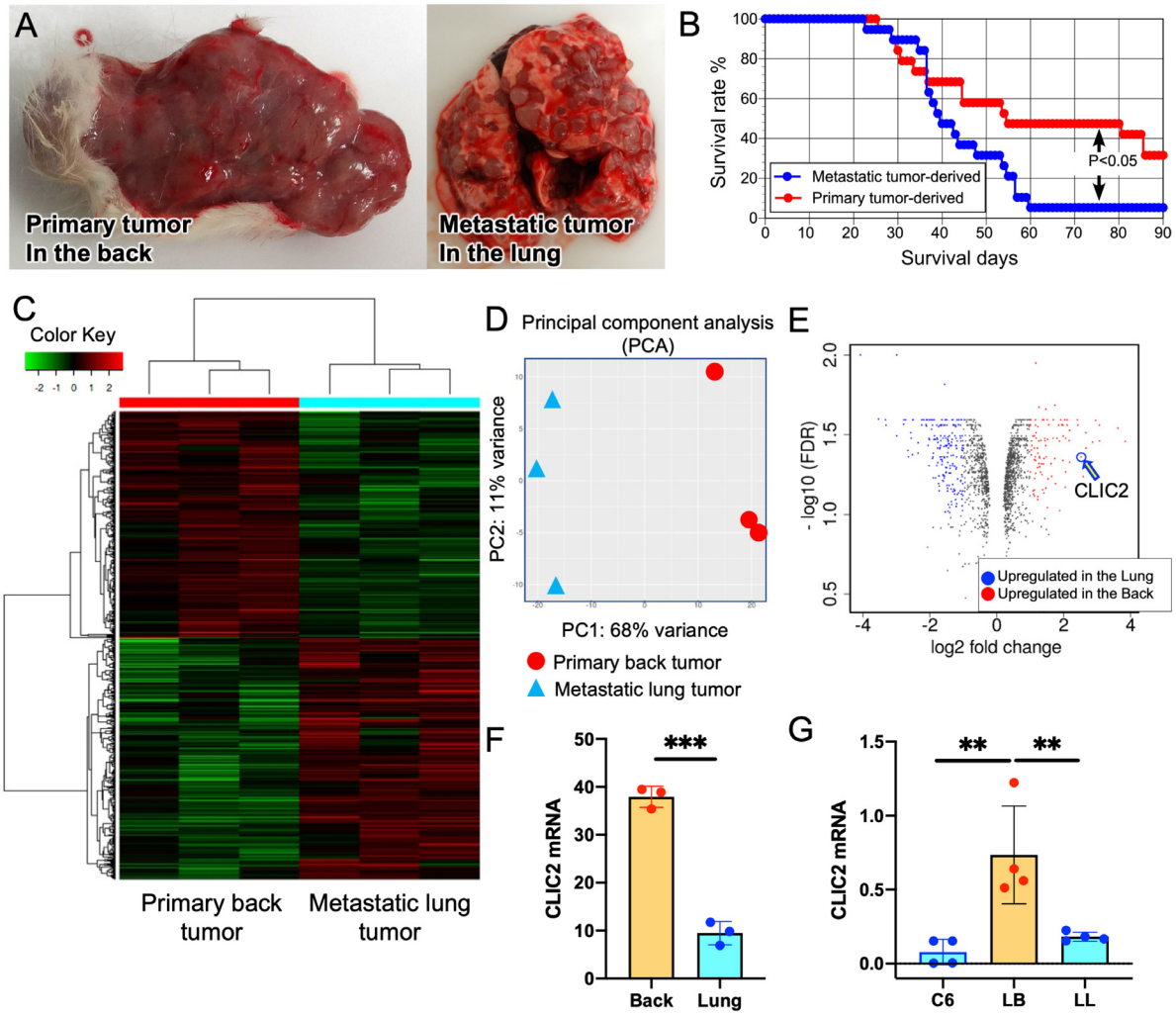

Fig. 1. CLIC2 was predominantly expressed in non-metastatic cells. (A) Primary back tumors and metastatic lung tumors in the rat at 35 d after the transplantation of C6 glioma cells. (B) Survival of the rats after the transplantation of primary and metastatic tumor cells. Log-rank test. n = 19 for each group. (C, D) Hierarchical clustering (C) and principal component analysis (PCA) (D) indicating the substantial difference in thousands of genes between cells in the primary back and metastatic lung tumors. (E) Volcano plot analysis on the gene expression profile shows the predominant expression of CLIC2 in the back tumor-derived non-metastatic cells. FDR; false discovery rate. (F) Comparative RNA-Seq analysis of the *CLIC2* mRNA expression levels in primary (Back) and metastatic (Lung) tumor cells. Unpaired two-tailed t test. n = 3. (G) *CLIC2* mRNA expression in C6 cells, primary back tumor-derived cells (LB), and metastatic lung tumor-derived cells (LL). Ordinary ANOVA with Tukey's multiple comparison test. n = 4. Data are expressed as mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$.

CLIC2 suppressed permeability of tumor blood vessels

Evans blue dye was injected into the rats with C6 and CC tumors via the tail veins [26] to investigate the relationship between CLIC2 and vascular barrier functions. The C6 tumor mass-stained blue or displayed red fluorescence, suggesting its increased vessel permeability (Fig. 5A, B). FACS analyses revealed that the total $CD45^+$ leukocyte and $CD45^+/CD11b^+$ macrophage counts were lower in the CC than in the C6 tumors (Fig. 5C). Thus, the CC tumors contained less numbers of tumor-associated macrophages (TAMs) than the C6 tumors; TAM plays a significant role in aggravation of malignant tumors partly by producing angiogenic factors [27]. Moreover, the $CD45^-/CD31^+$ cell fraction, which contained endothelial cells [28], was also lower in the CC tumors (Fig. 5D), suggesting suppressed angiogenesis in the CC tumors.

An increased level of epithelial cadherin (E-cadherin) and vascular endothelial cadherin (VE-cadherin) expression may prevent tumor cell invasion and metastasis [29–31]. The marked localization of E-cadherin along the endothelium was observed in the CC tumors, whereas its expression was nearly absent in the vasculature in the C6 tumors (Fig. 5E). E-cadherin is expressed by endothelial cells [32], and C6 glioma cells have been reported to stimulate or maintain E-cadherin expression by endothelial cells [33]. However, the *E-cadherin* mRNA expression levels did not differ significantly between these two types of tumors (Fig. 5F). Moreover, a significantly high level of E-cadherin protein expression was obvious in the CC tumors but not in the C6 tumors (Fig. 5G). CLIC2 protein expression was lower in the grade II than the grade I meningioma tumors (Fig. 5H). Additionally, the levels of the active form of MMP2 were increased and those of VE-cadherin were decreased in the grade II tumors. These results suggest that the effects of CLIC2 may be correlated with MMP2 activity, because the latter is responsible for the degradation of cadherins [34,35]. However, the mRNA expression levels of the genes encoding *MMP2* and *VE-cadherin* were not

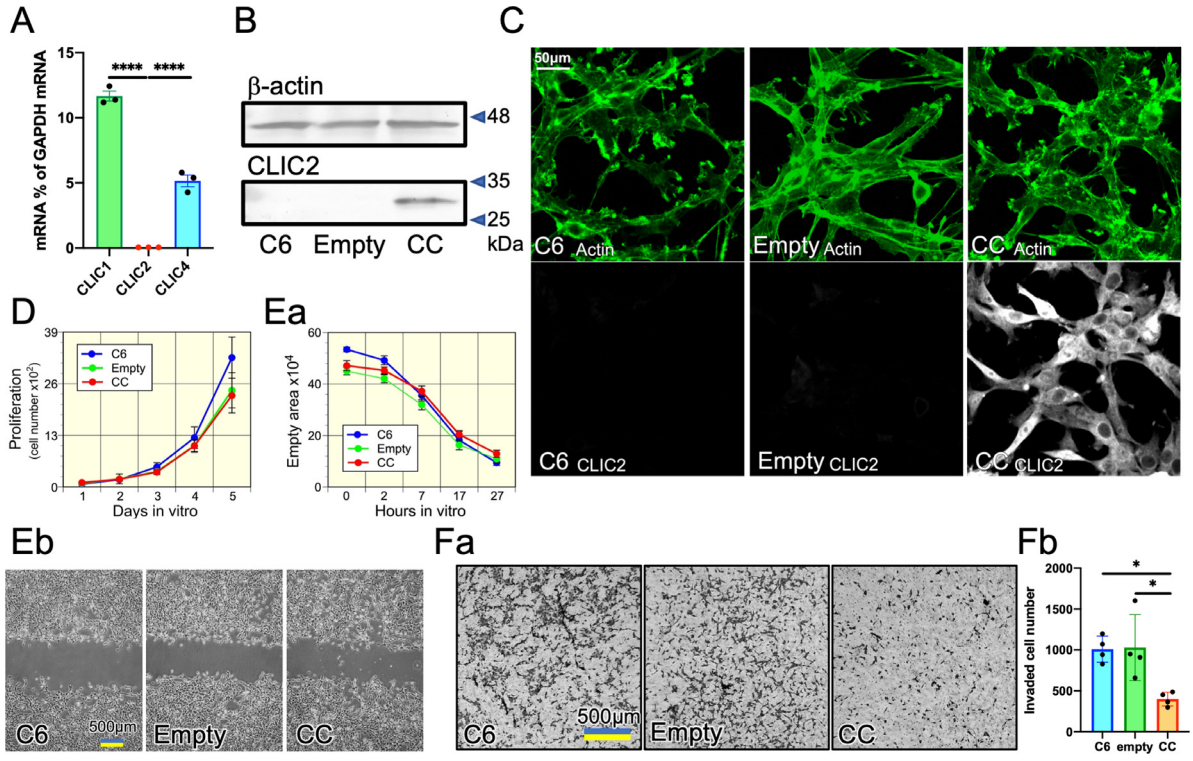

Fig. 2. Establishment of C6 cells with high CLIC2 expression (CC). (A) Original C6 glioma cells significantly expressed mRNA encoding CLIC1 and CLIC4, but not CLIC2 mRNA. (B, C) Establishment of C6 cells with high CLIC2 expression (CC). The original C6 cells (C6) and C6 cells transfected with an empty vector (empty) did not express CLIC2 as revealed by immunoblotting (B) and immunocytochemical staining (C). (D, E) High CLIC2 expression did not affect cell proliferation (D; n = 5), and cell migration (E; n = 3). F) CC cells showed weaker invasive activity, as revealed by the invasion assay. n = 4. Ordinary ANOVA with Tukey's multiple comparison test. $^{*}P < 0.05$, $^{****}P < 0.0001$.

significantly different between the grade I and II meningioma tumors (Fig. S2).

Secretion of CLIC2 and inhibitory effects on MMP 14 activity

Furthermore, *CLIC2* silencing did not affect the expression of the inactive proMMP2 protein (Fig. 5I) and *MMP2* mRNA (Fig. S4A) in cultured meningioma cells, but it did increase gelatin degradation (Fig. 5I). Surprisingly, the CLIC2 protein was released extracellularly as a soluble protein, which challenges the theory that CLICs are constituents of the ion channels, as revealed by immunoblotting of the concentrated conditioned media (Fig. 5J). The grade I meningioma cells released more CLIC2 and less of the active MMP2 than the grade II cells (Figs. 5J, S4B). CLIC2 was not present in the $CD9^{+}/CD81^{+}$ exosome fractions released by the meningioma cells (Fig. S4C), suggesting that it was present as a colloidal dispersion in extracellular space, allowing CLIC2 to act on the surface of the other cells. The grade I cells also released only a trace amount of CLIC4 and no CLIC1, whereas the meningioma cells (Fig. S4D), and meningioma and GBM tumors expressed mRNAs of both genes significantly (Fig. S4E).

CLIC2 was localized in granules, with immunoreactivity for vesicle-associated membrane protein 7 (VAMP7), a marker of secretory granules in grade I meningioma cells (Fig. 6A) [36]. MMP2 and MMP14 were colocalized with VAMP7. CLIC2 was also localized to the Golgi apparatus (Figs. 6A and S4F). *CLIC2* silencing increased the release of active MMP2 (Fig. 6B). Although the CC cells (but not the C6 and empty cells) secreted CLIC2, they secreted lower amounts of MMP2 than the C6 and empty cells did (Fig. 6C). The MMP14 protein was expressed at similar levels by all three types of cells (Fig. 6D). However, its presence in the plasma membrane (PM) fraction was the lowest in the CC cells, which also released it in the highest amounts among the various cell types [37]. *MMP14* mRNA was expressed at similar levels by the meningioma grade I and II tumors (Fig. S2) and was not affected by *CLIC2* silencing (Fig. S4G).

GST-tagged recombinant CLIC2 was synthesized using the wheat germ protein synthesis system [18]. The recombinant CLIC2 protein was highly soluble in aqueous buffers. Mutual binding between the GST-tagged recombinant CLIC2 and His-tagged recombinant MMP14 was examined using the immunoprecipitation assay, whereupon they were confirmed to bind to each other (Fig. 6E). The presence of the recombinant TIMP2 did not affect the binding of CLIC2 to MMP14 (Fig. S5A). Recombinant CLIC2 inhibited the activity of MMP14 that had been activated with APMA (Figs. 6F and S5B) [38], whereas recombinant CLIC4 did not (Fig. S5C). The inhibitory effect of CLIC2 was not much different from that of a broad spectrum and water-soluble MMP inhibitor NNGH [39,40] and was more significant than TIMP2 (Fig. 6G) [41,42] against furin-activated recombinant MMP14 (Fig. S5B) [42,43]. A mixture of TIMP2 and CLIC2 did not show any synergistic activity. Homogenates of CC cells had weaker MMP14 activity than those of C6 and empty cells, suggesting that CLIC2 inhibits MMP14 activity inside the cells (Fig. 6H).

Recombinant CLIC2 added to the cultured media suppressed invasion of human GBM cell lines U251 (Fig. 6I), SFC-2 cells (Fig. S5D) as well as C6 (Fig. S5E) cells in Matrigel-coated invasion chambers. However, CLIC4 did not suppress C6 cell invasion (Fig. S5F). *CLIC2* silencing accelerated the invasion of the meningioma cells (Fig. 6J) and CC cells (Fig. S5G).

Discussion

In this study, CLIC2 was found out to be a secretable and soluble protein despite that it has been said to consist an ion channel [4,6,44].

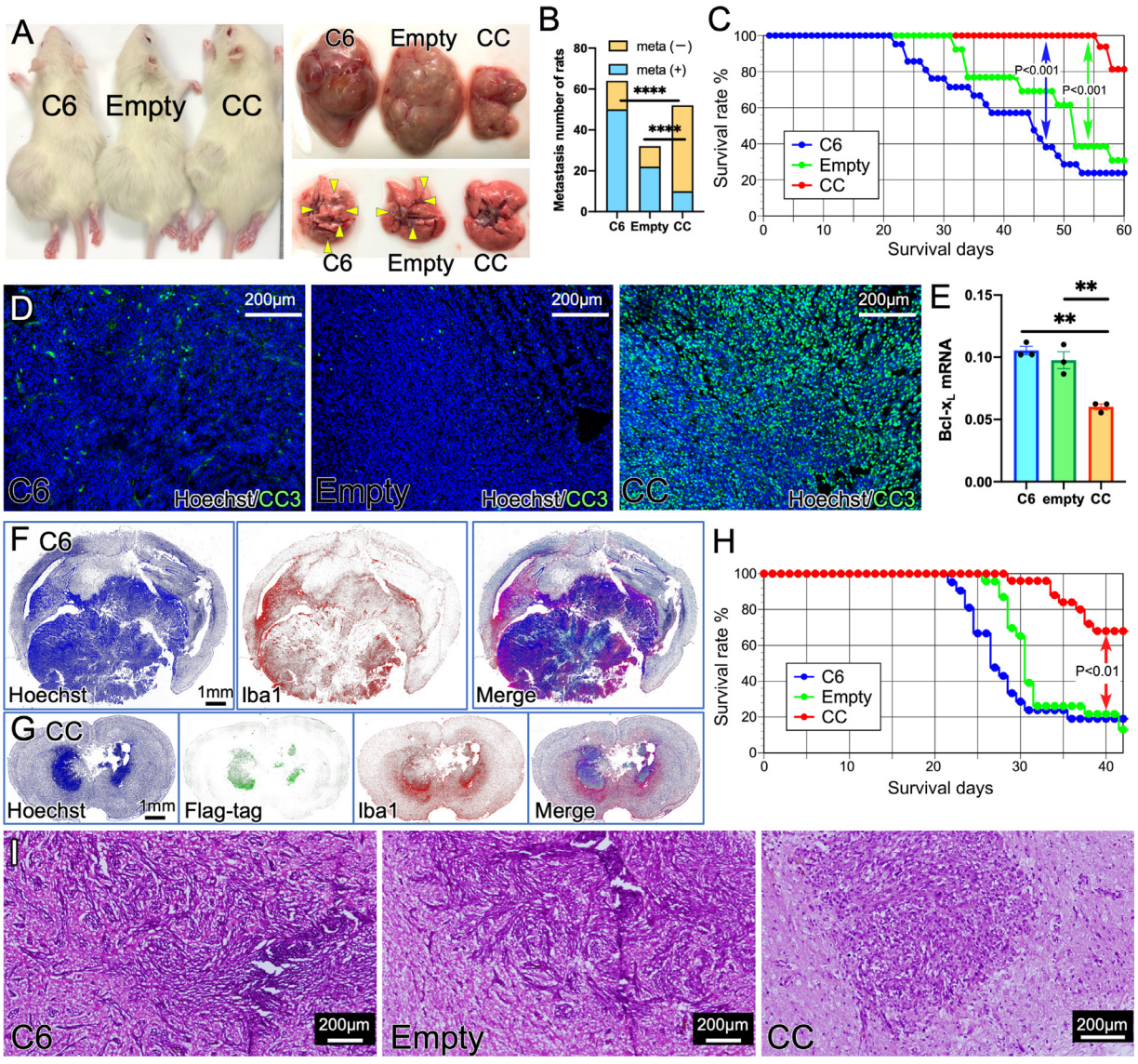

Fig. 3. CLIC2 suppressed metastasis and invasion in the rat tumor models. (A) Comparison of primary and metastatic tumors of C6, empty, and CC cells at 35 d after glioma cell transplantation. (B) Rates of lung metastasis of the three types of cells. Chi-square test. n = 64 (C6), 32 (empty), and 52 (CC). $^{****}P <$ 0.0001. (C) Survival of the rats up to 60 d after glioma cell transplantation. Log-rank test. n = 21 (C6), 13 (empty), and 16 (CC). (D) Expression of CC3 in the back tumors. E) Bcl-x_L-mRNA expression in the back tumors. n = 3. Ordinary ANOVA with Tukey's multiple comparison test. $^{**}P < 0.01$. (F–G) Brain tumor models prepared by transplanting C6 cells (F) or CC cells (G) into the striatum of neonatal rats. H) Survival of the rats up to 42 d after brain tumor cell transplantation. n = 21 (C6), 23 (empty), and 25 (CC). Log-rank test. I) Brain tumor tissues stained with hematoxylin and eosin.

Furthermore, CLIC2 suppressed invasive and metastatic activities of tumor cells, which were at least in part attributable to its inhibitory effects of MMP14 activity through the direct binding to the enzyme. The CLIC2 action was also correlated with the reduced blood vessel permeability [9]. CLIC2 expression was associated with less invasive and less metastatic traits, resulting in prolonged overall survival or PFS in the present animal and clinical studies.

In benign tumor cells, CLIC2 was localized in the Golgi apparatus and secretory granules together with MMP14. It is presumable that binding of CLIC2 to MMP14 in the secretory granules may prevent the insertion of the enzyme into the membrane, leading to its secretion into the extracellular milieu. This is because reduced MMP14 activity in the CC cell homogenates was observed compared to those of C6 and empty cells and also because recombinant CLIC2 bound to recombinant MMP14 while inhibiting its activity. In addition to the intracellular actions of CLIC2, CLIC2 may play a critical role in the extracellular milieu as a secreted soluble protein; recombinant CLIC2 added to the culture medium inhibited the invasive activities of malignant cells. Furthermore, overexpressed CLIC2 suppressed malignant cell invasion, and CLIC2 silencing abolished the effects. Thus, there should be two mechanisms underlying the actions of CLIC2: intracellular and extracellular mechanisms.

In malignant cells, MMP14 on the surface of malignant cells activates MMP2, degrades extracellular matrix proteins and cell adhesion molecules with activated MMP2 [45], leading to the invasion and metastasis of tumor cells [46]. MMP14 is also reported to promote angiogenesis [46] and generate abnormal vascularization [31]. In this study, we found that high CLIC2 expression was correlated with decreased blood vessel permeability, TAM

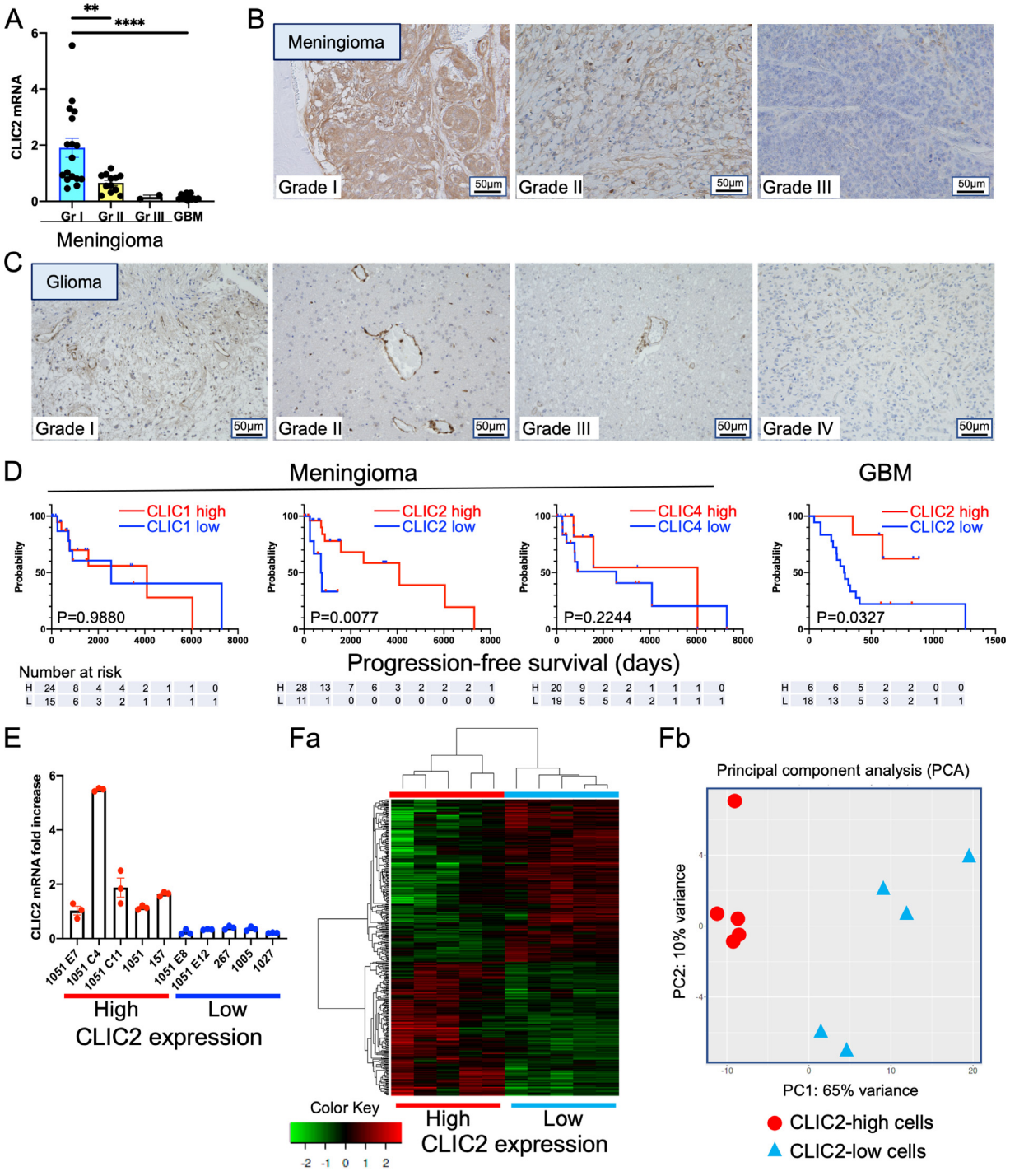

Fig. 4. CLIC2 was highly expressed in benign human tumors. (A) *CLIC2* mRNA expression in human meningioma and GBM tissues. Ordinary ANOVA with Tukey's multiple comparison test. n = 22 (grade I), 15 (grade II), 2 (grade III), and 24 (GBM). (B) Representative immunohistochemical staining for CLIC2 in grade I, II, and III meningioma tissue sections. (C) Representative staining for CLIC2 in grade I, II, III, and IV glioma tissue sections. (D) High CLIC2 expression was correlated with longer PFS in patients with meningiomas and GBM. Log-rank test. E) Ten human GSC lines could be divided into high and low CLIC2 expression groups. n = 3. F) Hierarchical clustering (Fa) and PCA (Fb) revealed the distinct gene expression pattern between high and low *CLIC2* gene expression. $^{**}P < 0.01$, $^{****}P < 0.0001$. Data are expressed as mean ± SEM.

infiltration, angiogenesis in the rat metastasis model, and increased apoptosis in the tumor cells with decreased expression of anti-apoptotic factor Bcl-x_L. These may be the causes for the smaller tumor formation in the back and the brain of the rat models. CLIC2 is reported of its involvement in the maintenance of the vascular barrier functions in normal blood vessel [9]. Therefore, CLIC2 may maintain the vascular barrier function through inhibition of MMP14. Secreted CLIC2 likely binds to MMP14 on the surface of other types of cells (e.g., stromal or vascular cells) in the benign tumor mass. Therefore, the degradation of the extracellular matrix and adhesion molecules and activation of MMP2 were suppressed in the CLIC2-

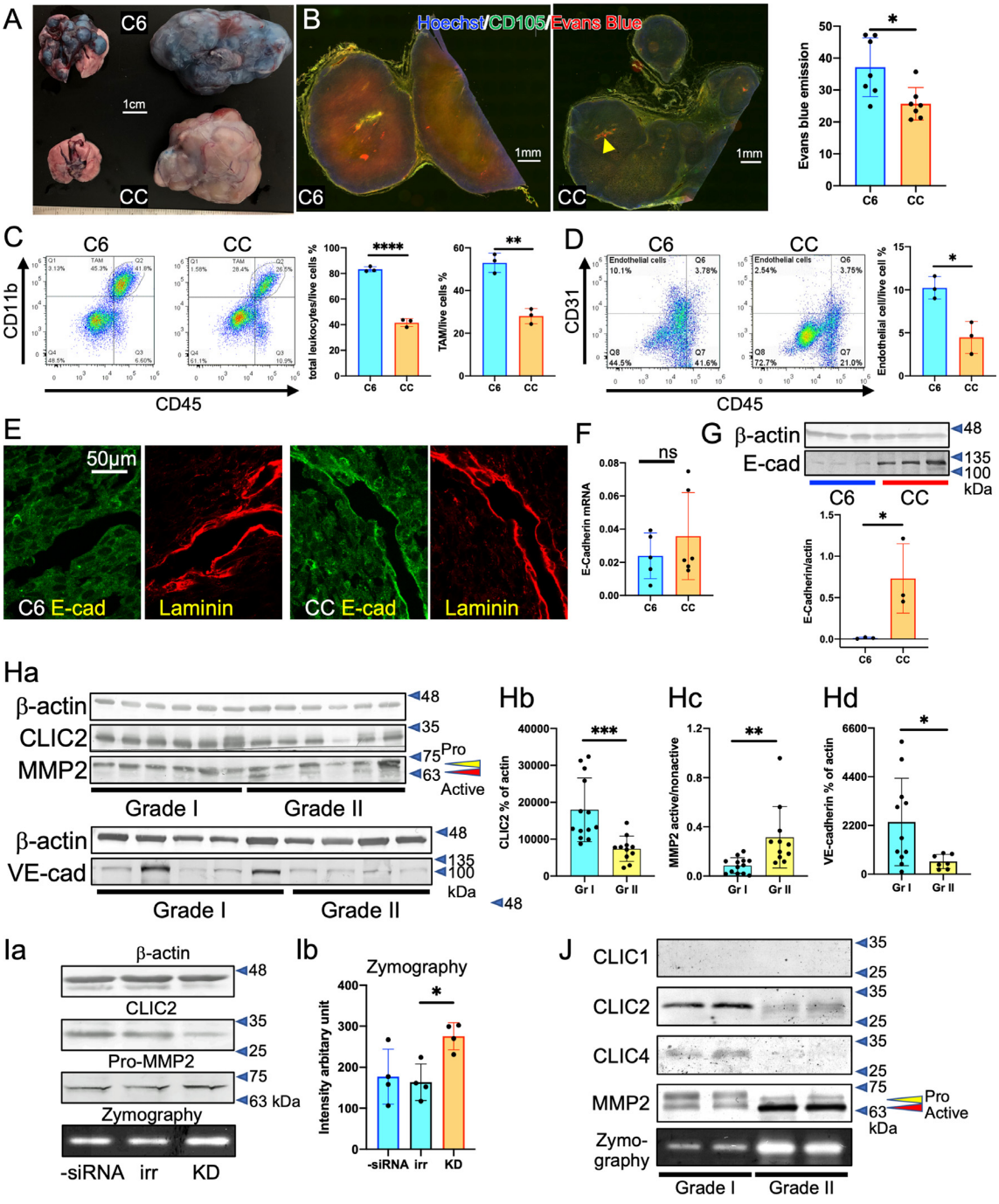

Fig. 5. Effects of CLIC2 on tumor blood vessels and MMP2; relationship with extracellular secretion of CLIC2. (A) Increased vascular permeability of C6 tumors compared with that of CC tumors. (B) Evans blue dye-infiltration in the back tumor sections were observed under a fluorescence microscope. The dye accumulated in the vessels (arrowhead) without leakage in the CC tumors. The extravasation of the dye was indicated by the fluorescence intensity. n = 7. C) The total $CD45^+$ leukocyte and $CD45^+/CD11b^+$ macrophage or TAMs counts were lower in the CC tumors than in the C6 tumors. n = 3. D) The CC tumors had fewer $CD31^+/CD45^-$ cells, presumable endothelial cells. n = 3. E) Epithelial (E)-cadherin expression in the vascular endothelium of the CC tumors was higher than that in the C6 tumors. F) E-cadherin mRNA expression did not differ between the different tumors. n = 5 (C6) and 6 (CC). G) The E-cadherin protein levels were higher in the CC tumors. n = 3. H) The meningioma grade I tumors (n = 11~13) exhibited higher CLIC2 protein expression, a smaller active-matrix metalloproteinase 2 (MMP2)/inactive MMP2 (proMMP2) protein ratio, and higher vascular endothelial (VE)-cadherin protein expression, compared to grade II tumors (n = 7~11). The red and yellow arrowheads denote active MMP2 and proMMP2, respectively. Data are expressed as mean ± SD. Unpaired two-tailed *t* test. I) CLIC2 and proMMP2 expression in human meningioma cells that were either untreated (−siRNA) or treated with irrelevant (irr) or CLIC2-targeting (KD) siRNA sequences. *CLIC2* silencing increased gelatinolysis in the zymographic assay. Representative data (Ia) and statistical analysis by ANOVA and Tukey's multiple comparison test (Ib). n = 3. J) Immunoblotting of the concentrated conditioned media and zymography. CLIC2 was released by grade I but not grade II meningioma cells. Active MMP2 (red arrowhead) was more highly released by the grade II cells than proMMP2 (yellow arrowhead). Slight CLIC4 release was detected, whereas CLIC1 release was absent. Grade II meningioma cells had strong gelatinolytic activity. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ "(Color version of figure is available online.)"

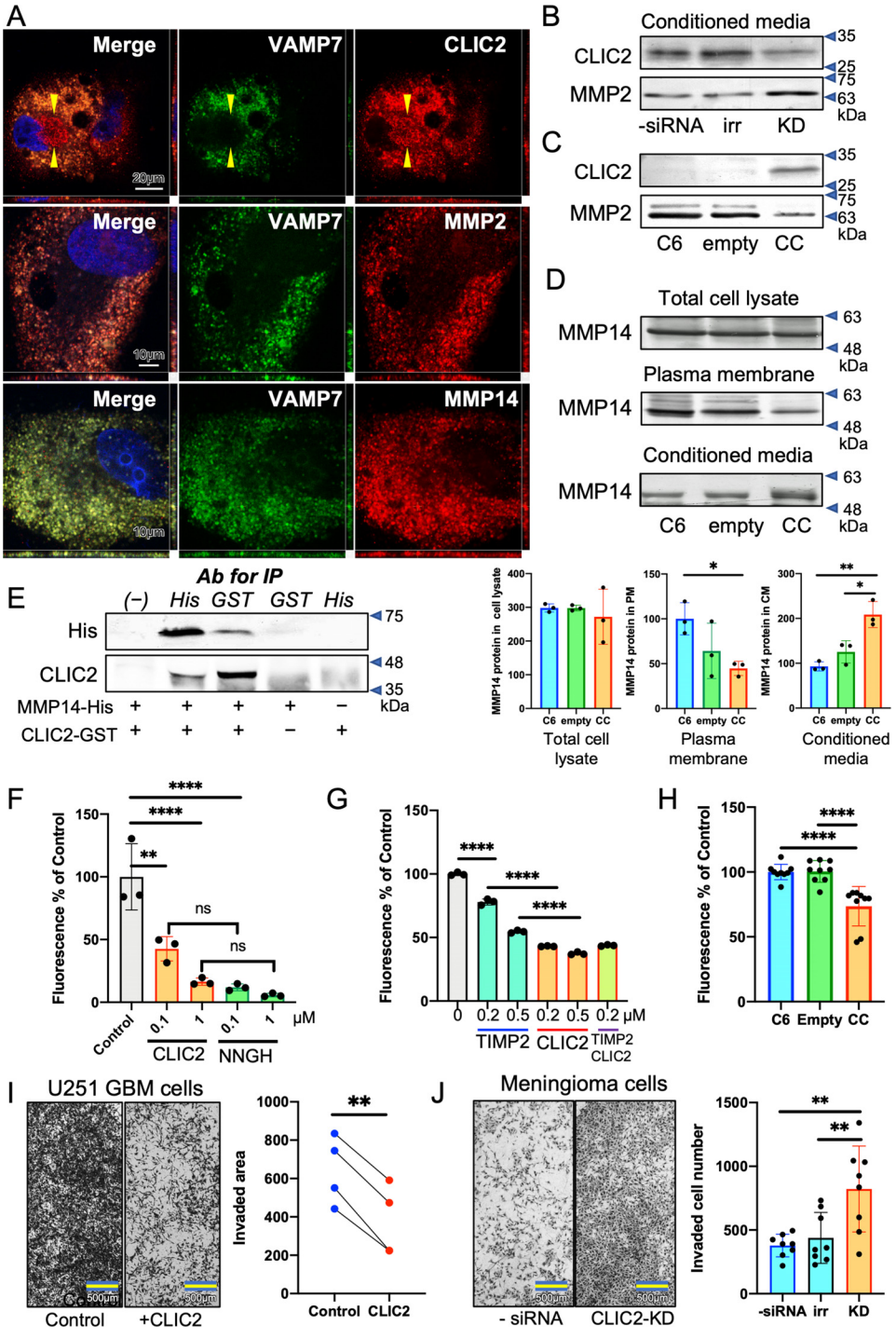

Fig. 6. Secretion of CLIC2 and its inhibitory effects on tumor cell invasion. (A) CLIC2, MMP2, and MMP14 immunoreactivity was detected in vesicle-associated membrane protein 7-positive (VAMP7^{+}) secretory granules. CLIC2 was localized to the Golgi apparatus (arrowheads). (B) The media conditioned by grade I meningioma cells contained CLIC2 and active MMP2. *CLIC2* silencing increased the amount of MMP2 released. (C) The CC (but not C6 and empty) cells secreted CLIC2 significantly. The C6 and empty cells released greater amounts of MMP2 than the CC cells. (D) Expression and secretion of MMP14 by C6, empty, and CC cells, and plasma membrane (PM) localization of the protein. The MMP14 expression levels in the whole-cell lysates were almost the same among the three types of cells, but its localization in the PM fraction was reduced in the CC cells. The CC cells secreted more MMP14. n = 3. (E) Immunoprecipitation assays revealed the binding of His-tagged MMP14 with glutathione *S*-transferase (GST)-tagged CLIC2. F) Recombinant CLIC2 inhibited the activity of 4-aminophenylmercuric acetate (APMA)-treated MMP14 to a similar extent as the synthetic MMP inhibitor *N*-Isobutyl-*N*-[4-methoxyphenylsulfonyl] glycyl hydroxamic acid (NNGH). n = 3. G) Recombinant CLIC2 inhibited MMP14 activity more significantly than did recombinant tissue inhibitor of metalloproteinase 2 (TIMP2). No synergistic activity was found when TIMP2 and CLIC2 were added simultaneously. n = 3. H) Activities of MMP14 in the homogenates of C6, empty, and CC cells. n = 9. I) Recombinant CLIC2 prevented the invasion of U251 cells. n = 4. J) *CLIC2* silencing increased the invasive activity of meningioma cells (n = 8). Data are expressed as mean ± SD. Ordinary ANOVA with Tukey's multiple comparison test (A, F, H, I, J, and L). Paired (I) two-tailed t test. $^{**}P < 0.01$, $^{****}P < 0.0001$.

secreting cells and their neighboring cells, leading to the inhibition of invasion and metastasis of tumor cells with benign traits.

MMPs have long been implicated in the invasion and metastasis of malignant tumors [1,31], and many inhibitors of MMPs have been developed and tested in clinical trials [47,48]. However, none of them have been approved for clinical use to date. Inhibition of specific MMP is desired as a potential treatment for cancer therapy. This study provided insights into why benign tumors and normal cells do not spread to distant organs. We showed that CLIC2 expression levels varied greatly depending on whether the cells metastasized or not, even when they were derived from the same metastasized C6 cells. Whether this variation in CLIC2 expression is a result of or a cause for the invasive or metastatic abilities of tumor cells and how CLIC2 expression is regulated require further studies for clarification. Nevertheless, our present findings on the inhibitory effects of CLIC2 on MMP14 provide a novel avenue for the development of interventions based on MMP inhibition for treating malignant tumors. The inhibitory action on MMP14 activity of the recombinant CLIC2 is more significant than the recombinant TIMP2. Moreover, when considering gene therapy for highly invasive malignant tumors such as GBM using the CLIC2 gene, it does not have to be selective for malignant tumor cells, which would reduce technical difficulties, since the CLIC2 gene is expected to be effective if it is introduced not only into malignant tumor cells but also into surrounding normal cells.

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Author contribution

SO: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, AU: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, HY: Methodology, Writing - original draft, Supervision, SO: Investigation, YS, EH: Investigation, EU: Investigation, AI: Methodology, Investigation, MEC: Methodology, Investigation, YN: Investigation, Resources, DY: Investigation, Resources, YO: Resources, MN, AI: Resources, SS: Resources, JK: Investigation, Resources, HW: Resources, YT: Investigation, Resources, YW: Resources, IN: Methodology, Resources, TK: Conceptualization, Resources, Supervision, Project administration, JT: Conceptualization, Methodology, Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neo.2021.06.001.

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