Claudin-3 gene silencing with siRNA suppresses ovarian tumor growth and metastasis

Yu-Hung Huang[a], Yunhua Bao[a], Weidan Peng[a], Michael Goldberg[b], Kevin Love[b], David A. Bumcrot[c], Geoffrey Cole[c], Robert Langer[b,d,1], Daniel G. Anderson[b,1], and Janet A. Sawicki[a,e,f,1]

[a]Lankenau Institute for Medical Research, 100 E Lancaster Avenue, Wynnewood, PA 19096-3450; [b]David H. Koch Institute for Integrative Cancer Research and [d]Chemical Engineering Department, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Rm E25-342, Cambridge, MA 02139-4307; [c]Alnylam Pharmaceuticals, Inc., 300 Third Street, Cambridge, MA 02142; and [e]Department of Dermatology and Cutaneous Biology and [f]Kimmel Cancer Center, Jefferson Medical School, Thomas Jefferson University, 233 S 10th Street, Philadelphia, PA 19107

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Claudin-3 (CLDN3) is a tight junction protein that is overexpressed in 90% of ovarian tumors. Previous in vitro studies have indicated that CLDN3 overexpression promotes the migration, invasion, and survival of ovarian cancer cells. Here, we investigated the efficacy of lipidoid-formulated CLDN3 siRNA in 3 different ovarian cancer models. Intratumoral injection of lipidoid/CLDN3 siRNA into OVCAR-3 xenografts resulted in dramatic silencing of CLDN3, significant reduction in cell proliferation, reduction in tumor growth, and a significant increase in the number of apoptotic cells. Intraperitoneal injection of lipidoid-formulated CLDN3 siRNA resulted in a substantial reduction in tumor burden in MISIIR/TAg transgenic mice and mice bearing tumors derived from mouse ovarian surface epithelial cells. Ascites development was reduced in CLDN3 siRNA-treated mice, suggesting the treatment effectively suppressed metastasis. Toxicity was not observed after multiple i.p. injections. Importantly, treatment of mice with nonimmunostimulatory 2′-OMe modified CLDN3 siRNA was as effective in suppressing tumor growth as unmodifed siRNA. These results suggest that lipidoid-formulated CLDN3 siRNA has potential as a therapeutic for ovarian cancer.

lipidoid | ovarian cancer | cancer therapy

Ovarian cancer has the highest mortality rate among gynecologic malignancies, and ranks 4th as the most common cancer in women in the United States (1). Treatment of early stage ovarian carcinoma improves the survival rate up to 90%. However, most women have advanced stage metastatic cancer at the time of diagnosis due to the asymptomatic nature of early stages of the disease and the lack of effective screening modalities. The standard treatment for patients with advanced stage epithelial ovarian cancer is surgical debulking followed by chemotherapy with paclitaxel plus a platinum-based therapy (cisplatin or carboplatin). Although ≈80% of patients receiving this therapeutic regimen have an initial favorable response, recurrent disease will occur in a majority of cases. New effective therapies are urgently needed for those patients with advanced-stage ovarian cancer who either do not respond to initial therapy or develop recurrent disease.

Claudins are integral membrane proteins associated with tight junctions. Two members of the claudin protein family, claudin-3 (CLDN3) and CLDN4, are overexpressed in epithelial ovarian tumors relative to normal ovarian tissue (2–5). In fact, they are among the most highly expressed proteins in ovarian tumors. High amounts of CLDN3 and CLDN4 are associated with increased cellular motility and survival of ovarian tumor cells, and an increase in matrix metalloproteinase type 2 (MMP-2) (4). These observations implicate a role for CLDN3 and CLDN4 in ovarian tumorigenesis and metastasis, and suggest their importance as target proteins for development of new diagnostic and therapeutic reagents. CLDN3 and CLDN4 have also been identified as receptors for cytotoxic *Clostridium perfringens* enterotoxin (CPE). Binding of CPE to cells that express CLDN3 and CLDN4 results in cell death, and significantly inhibits ovarian tumor growth in a mouse model (6). Given their high expression in ovarian tumor cells and the possibility that their overexpression may disrupt tight junction barrier function and contribute to tumorigenesis, targeting CLDN3 and CLDN4 using siRNA is an attractive option as a potential therapy for ovarian cancer. Indeed, in vitro siRNA inhibition of CLDN3 and CLDN4 expression reduced the invasive properties of ovarian tumor cells (4).

Here, we have developed a lipid-like delivery system for i.p. delivery of siRNA to ovarian tumor tissue to test the therapeutic efficacy of CLDN3 siRNA in mouse tumor models. We have tested this approach in 3 mouse models for ovarian cancer, ovarian xenografts in nude mice, MISIIR/TAg transgenic mice (7), and nude mice injected i.p. with mouse ovarian surface epithelial cells (MOSEC) that express firefly luciferase (ID8-Fluc cells) (8). We have used a novel lipid-like molecule, $98N_{12}$-5 (1), to deliver the siRNA intratumorally and i.p. to mice. This molecule belongs to a new class of lipidoid molecules that has recently been shown to deliver siRNA safely and effectively to lung, liver, and peritoneal macrophages in 3 different species, including a nonhuman primate (9).

In all 3 mouse models, tumor growth in CLDN3 siRNA-treated mice was significantly reduced, compared with mice treated with control siRNA. In some mice, tumors even regressed in size. Ascites development in the ID8-Fluc model was suppressed, suggesting that CLDN3 siRNA treatment was effective at inhibiting metastasis. CLDN3 siRNA-treated mice displayed no obvious ill effects from the treatment. The fact that nonimmunostimulatory modified CLDN3 siRNA suppresses tumor growth and stimulatory unmodified siRNA suggests that the therapeutic effects we observe are the result of suppression of CLDN3 protein. These promising results suggest that lipidoid-delivery of CLDN3 siRNA warrants further development as a new therapeutic option for ovarian cancer.

Results

CLDN3 Expression in Human and Mouse Ovarian Tumor Cells. Western blot analysis of membrane proteins prepared from human OVCAR-3 cells, human ovarian ascites cells, ovarian tumors from MISIIR/TAg transgenic mice, and ID8-Fluc cells identified a 22-kDa protein corresponding to CLDN3 (Fig. 1 *A–D*).

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[1]To whom correspondence may be addressed. E-mail: sawickij@mlhs.org, dgander@mit.edu, or rlanger@mit.edu.

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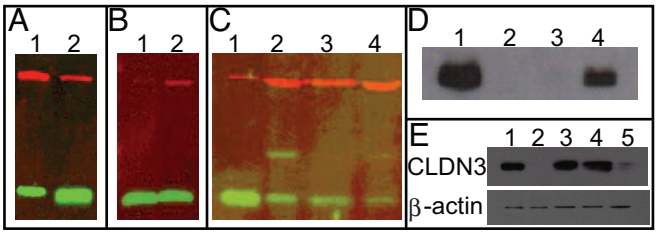

Fig. 1. Western blot analysis of CLDN3 expression in human and mouse tumor cells and in CLDN3 siRNA-treated human OVCAR-3 cells. (*A*) Membrane proteins prepared from LLC PK_1 cells (lane1, positive control) and from OVCAR-3 cells (lane 2). (*A–C*) CLDN3 fluoresces green and β-actin, the loading control, fluoresces red. (*B*) Membrane proteins prepared from LLC PK_1 cells (lane 1, positive control) and from human ovarian ascites cells (lane 2). (*C*) LLC PK_1 cells (lane 1, positive control); membrane proteins from 3 different ovarian tumors from MISIIR/TAg mice (lanes 2–4). (*D*) Membrane proteins from OVCAR-3 cells (lane 1, positive control) and ES2 cells (lane 2, negative contol). Cytoplasmic proteins (lane 3) and membrane proteins (lane 4) from ID8-Fluc cells. (*E*) Small interfering RNA analysis; OVCAR-3 membrane proteins (lane 1, positive control); ES2 membrane proteins (lane 2, negative control); mock transfected OVCAR-3 cells (lane 3); OVCAR-3 cells, control siRNA treated (lane 4); OVCAR-3 cells, CLDN3 siRNA treated (lane 5). CLDN3 (*Upper*); β-actin loading control (*Lower*).

Membrane proteins prepared from LLC PK_1, a renal epithelial cell line known to express CLDN3, served as a positive control (10). Carryover of β-actin in membrane protein preparations allowed for its convenient use as a control for protein loading on gels. CLDN3 expression was not detected in ES2 cells, another human ovarian cancer cell line (Fig. 1*D*, lane 2). The lack of expression in ES2 cells is consistent with previous reports that showed similar results in HOSE-B and A2780) (3). All of the human and mouse tumor cells that we tested expressed CLDN3 in membrane protein preparations. We did not observe CLDN3 expression in cytoplasmic protein preparations of ID8-Fluc cells (Fig. 1*C*, lanes 3 and 4). Based on these results, we chose to use OVCAR-3 cells to generate xenografts for evaluating therapeutic efficacy of siRNA knockdown of CLDN3.

We confirmed by Western blot analysis of membrane proteins prepared from OVCAR-3 cells grown in vitro that the human (h)CLDN3 siRNA sequence we selected as a potential therapeutic agent efficiently suppressed CLDN3 expression (Fig. 1*E*).

Lipidoid-Delivered hCLDN3 siRNA Suppresses OVCAR-3 Xenograft Growth. To test the utility of lipioid formulations to deliver and silence hCLDN3, and the effect of hCLDN3 silencing on the growth of human ovarian tumor cells, xenografts were generated by s.c. injection of OVCAR-3 cells into the hind flank of female nude mice; ≈4 weeks later, when small tumors were visible, baseline tumor volumes were determined (ranging from 50 to 88 mm^3), and mice were distributed into 3 treatment groups composed of tumors with equivalent sizes. Tumors in the test group were injected intratumorally with hCLDN3 siRNA, and tumors in control mice were injected with either Fluc siRNA or with PBS. Mice were treated on day 0 (baseline), and then each tumor was injected again on day 5, 7, and 10. Calipers were used to measure tumor size before each injection and on day 13. At each time point, the fold-increase in tumor volume (tumor volume ÷ baseline tumor volume) for each tumor was calculated. At 5, 7, and 10 days, the mean fold-increase in tumor volume of tumors injected with hCLDN3 siRNA was less than that of both groups of control tumors, although the differences were not statistically significant (Fig. 2*A*). The hCLDN3 siRNA-injected tumors ceased to grow after 2 injections, whereas the control tumors continued to increase in size. The difference in tumor volume between hCLDN3 siRNA-treated and control groups was highly

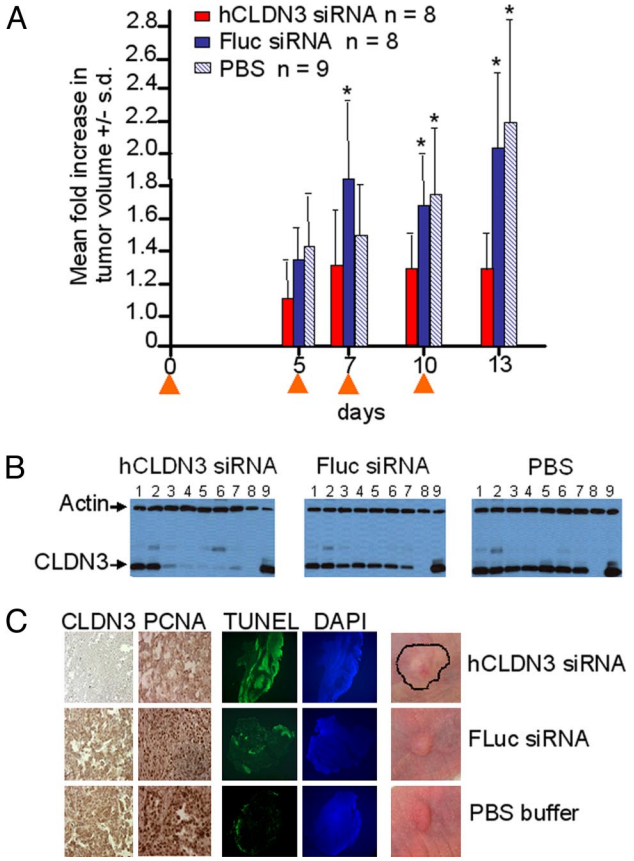

Fig. 2. CLDN3 siRNA treatment of OVCAR-3 xenografts. (*A*) Fold increase (mean ± SD) in tumor volume at various times after intratumoral injection of hCLDN3 siRNA, Fluc siRNA, or PBS (measured against baseline tumor volume at time 0). Orange triangles indicate times of injections. (*B*) Western blot analysis of CLDN3 protein in OVCAR-3 xenografts injected with hCLDN3 siRNA, Fluc siRNA, or PBS. Lanes 1 and 2, tumors before any treatment; lanes 2–7, individual tumors after 4 intratumoral injections of siRNA or PBS; lane 8, membrane proteins from ES2 cells (negative control); lane 9, membrane proteins from OVCAR-3 cells (positive control). (*C*) Sections of xenografts harvested on day 13 and immunostained for CLDN3 and PCNA, or TUNEL stained (with same-section DAPI nuclear stain on the right). Photographs of representative tumors in situ at day 13 are in the column at the utmost right.

significant on day 13 [1.3-fold (hCLDN siRNA) vs. 2.1-fold (Fluc siRNA) and 2.2-fold PBS; $P = 0.0010$].

Western blot analysis of membrane proteins prepared from tumors harvested on day 13 showed a very significant reduction in the amount of CLDN3 protein in tumors injected with hCLDN3 siRNA (Fig. 2*B*). Injection of tumors with control Fluc siRNA had very little effect, if any, on the amount of CLDN3 protein with no significant difference from those in uninjected tumors and tumors injected with PBS (Fig. 2*B*). Immunostaining of tumor sections for CLDN3 protein agreed well with the Western blot analysis results. Very little CLDN3 protein was detected in tumors injected with hCLDN3 siRNA compared with sections from control tumors (Fig. 2*C*). To evaluate the amount of cell division in tumors after treatment, tumor sections were immunostained for proliferating cell nuclear antigen (PCNA). The nuclear PCNA staining index for sections from tumors injected with hCLDN3 siRNA was very low compared with control tumors (Fig. 2*C*). TUNEL staining of tumor sections revealed that a majority of cells in the hCLDN3 siRNA-injected tumors were apoptotic, whereas the number of cells undergoing apoptosis in control tumors was very low (Fig. 2*C*). The gross appearance and texture of tumors injected with

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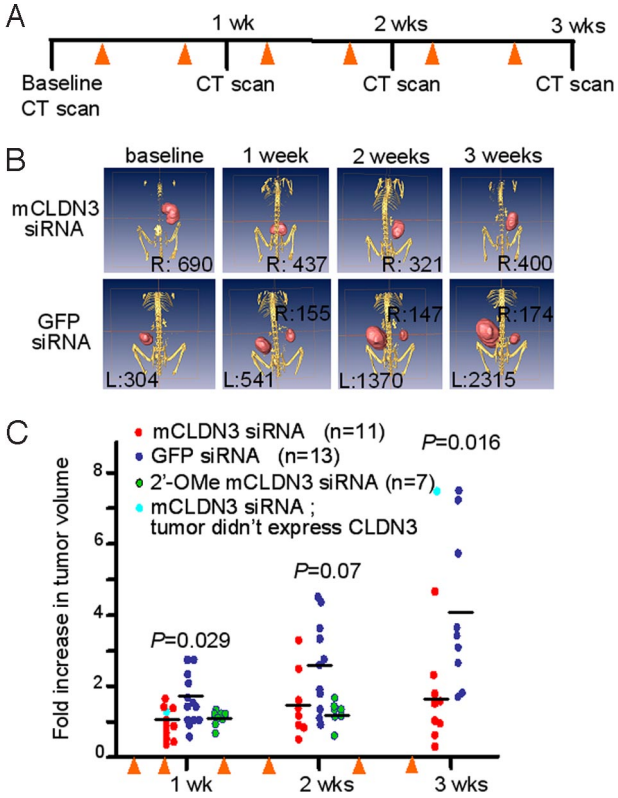

Fig. 3. Testing therapeutic efficacy in ovarian tumor-bearing MISIIR/TAg mice. (*A*) Treatment schedule. Orange triangles indicate times of i.p. injections. (*B*) Representative reconstructed CT scans of ovarian tumors of MISIIR/TAg mice treated with mCLDN3 siRNA or with GFP siRNA. Numbers in each panel are the tumor volumes (mm^3). L, left-side tumor; R, right-side tumor. (*C*) Fold increase in tumor volume at 1, 2, and 3 weeks compared with baseline tumor volume. Each point represents a single tumor. The mean tumor volume at each time point is designated by a horizontal line. Orange triangles indicate times of i.p. injections.

CLDN3 siRNA before their resection on day 13 was very different from that of control tumors, and appeared to reflect the difference in the degree of apoptosis (Fig. 2*C Right*). The CLDN3 siRNA-injected tumors were very flat and soft, and had a whitish margin of cells surrounding a slightly raised nub of tumor cells. In contrast, control-treated tumors were very hard and had a distinct raised profile (Fig. 2*C*).

Lipidoid-Delivered mCLDN3 siRNA Suppresses Ovarian Tumor Growth in MISIIR/TAg Mice. To further test the utility of CLDN3 siRNA as a therapeutic for ovarian cancer, we administered $98ND_{12}$-5(1) formulations of mCLDN3 siRNA by i.p. injection to ovarian tumor-bearing MISIIR/TAg mice. Control mice received i.p. injections of GFP siRNA. Mice bearing tumors were initially identified by abdominal palpation. These mice were then microCT scanned, and baseline tumor volumes were determined from reconstructed images. Mice were distributed into 2 groups having tumors of equivalent sizes (ranging from 75 to 976 mm^3). Mice received 2 injections of siRNA formulation per week for 3 weeks, and tumor volumes were assessed by CT scanning once a week (Fig. 3*A*). Representative reconstructed micro CT scans from which tumor volumes were determined are shown in Fig. 3*B*. After each week treatment, the mean fold increase in tumor volume relative to the baseline volume of mice injected with mCLDN3 siRNA was significantly less that that of control mice injected with GFP siRNA (at 3 weeks, $P < 0.02$) (Fig. 3*C*). After 3 weeks of treatment with CLDN3 siRNA, the average increase in tumor size relative to baseline measurements was 1.7-fold. Tumor growth was completely suppressed or even regressed in ≈40% of mice injected with mCLDN3 siRNA. In contrast, the volume of all tumors in control mice increased 2-fold or more (mean = 4.1-fold). Also, only 22% (2/9) of mice treated with CLDN3 siRNA developed ascites over the 3-week treatment period, compared with 75% (6/8) of control mice. Clinical chemistry analyses of metabolic enzymes in serum of siRNA-treated mice showed that all of the enzymes were present at normal levels (Table 1).

Western blot analysis of membrane proteins prepared from several tumors harvested after the last CT scan at 3 weeks showed reduced amounts of CLDN3 expression in tumors from mice injected with mCLDN3 siRNA compared with amounts in mice injected with GFP siRNA (Fig. S1). Interestingly, there was no trace of CLDN3 expression in the large tumor from a mCLDN3 siRNA-treated mouse (Fig. 3*C*), probably accounting for its nonresponsiveness to the siRNA treatment.

Lipidoid-Delivered 2′-OMe Modified mCLDN3 siRNA Suppresses Ovarian Tumor Growth in MISIIR/TAg Mice. Concern that the therapeutic effects we observe might be due to induction of cytokines by unmodified CLDN3 siRNA led us to test its immunostimulatory activity and to test the immunostimulatory activity and therapeutic effect of 2′-OMe-modified CLDN3 siRNA. We found unmodified CLDN3 siRNA to be slightly immunostimulatory. However, human and mouse modified CLDN3 siRNA was nonstimulatory as determined by ELISA assays on peripheral blood mononuclear cells (PBMC) and serum from mice treated with modified mCLDN3 (Table S1 and Fig. S2). We also determined that 2′-OMe modified human and mouse CLDN3 siRNAs suppress CLDN3 protein in OVCAR-3 and ID8-Fluc ovarian tumor cells, respectively (Fig. S3).

To test therapeutic efficacy, tumor-bearing MISIIR/TAg mice were injected i.p. twice a week for 2 weeks with $98ND_{12}$-5(1)-formulated 2′-OMe modified mCLDN3. Tumor volumes were determined from reconstructed CT images (Fig. S4), and the fold increase in tumor volume over a 1- and 2-week period was determined (Fig. 3*C*). After 1 week, the mean tumor volume increased by 1.1-fold, nearly identical to the fold increase observed in mice treated with unmodified mCLDN3 siRNA. After 2 weeks, the mean tumor volume increased by 1.2-fold compared with 1.6-fold for mice treated with unmodified mCLDN3 siRNA. We conclude that 2′-OMe modified mCLDN3

Table 1. Results of enzyme assays

	T.BIL.	LDH	ALT	GGT	ALK	CREAT	CPK
siRNA	mg/dL	U/L	U/L	U/L	U/L	mg/dL	U/L
mCLDN3	0.1	849 ± 206	41 ± 10	3	70 ± 13	0.2	5,634 ± 3176
GFP	0.1	1,448 ± 247	38 ± 13	3	33 ± 3	0.2	8,028 ± 3987

Enzyme assays (mean ± SD) performed on serum from MISIIR/TAg mice that had been injected i.p. with either mCLDN3-siRNA ($n = 3$) or GFP-siRNA ($n = 3$) (175 μg per injection; 2 injections per week, for 3 weeks). CREAT, creatine; ALK, alkaline phosphate; LDH, lactate dehydrogenase; CPK, creatine kinase; T.BIL., total bilirubin; ALT, alanine transaminase; GGT, gamma glutamyltransferase.

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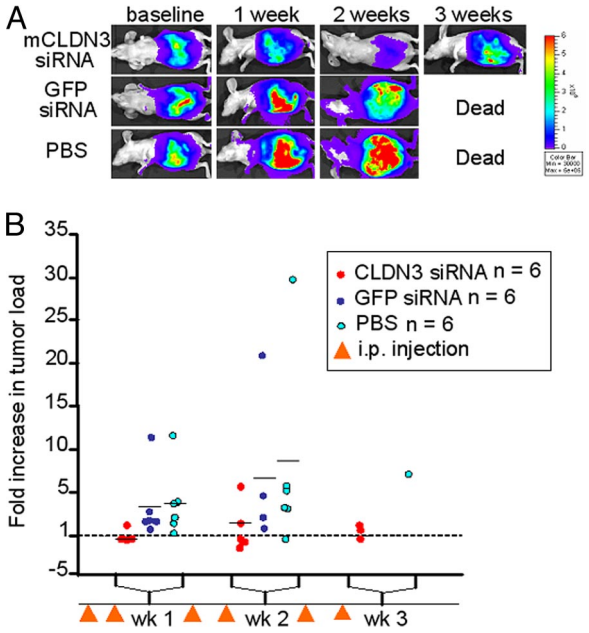

Fig. 4. Testing therapeutic efficacy in ID8-Fluc model. (*A*) Representative optical images of mice before treatment (baseline) and 1, 2, and 3 weeks after i.p. injections of mCLDN3 siRNA, GFP siRNA, and PBS. In each panel, a pseudocolor image representing emitted light is superimposed over grayscale reference image of mouse. Photons/sec are indicated in the color scale bar. (*B*) Fold increase in tumor load at 1, 2, and 3 weeks compared with baseline tumor load. Each point represents a single tumor. The mean tumor volume at each point is designated by a horizontal line.

siRNA is as effective as unmodified mCLDN3 siRNA in suppressing ovarian tumor growth in mice.

Lipidoid-Delivered mCLDN3 siRNA Suppresses Ovarian Tumor Growth and Metastasis in ID8-Fluc Model. We performed a third test of therapeutic efficacy of CLDN3 siRNA by using *nu/nu*.BALB/c female mice that developed tumors in organs throughout the peritoneal space after i.p. injection of ID8-Fluc cells (Fig. S5). Tumor load in these mice can be assessed by using optical imaging to detect bioluminescence from luciferase expressed in the tumors and, then, quantifying relative light units (RLU) (Fig. S6). Baseline optical images were obtained 6–7 weeks after mice were injected with ID8-Fluc cells. The total RLU per mouse for baseline images ranged from 3×10^7 to 4×10^8 RLU. Mice were distributed into 3 groups having equivalent tumor loads ($n = 6$ per group). The treatment schedule for these mice was the same as that used for the MISIIR/TAg mice, i.e., 2 i.p. injections of $98N_{12}$-5(1)-formulated siRNA per week for 3 weeks. Mice were optically imaged once a week. Representative optical images of mice are shown in Fig. 4*A*. The fold increase in tumor load (RLU at time point X ÷ baseline RLU) was determined at different times after treatment (Fig. 4*B*). Total suppression of tumor growth was achieved in a high percentage of tumors only in mice treated with mCLDN3 siRNA. Also, except for one PBS-injected mouse, all control mice died during the third week of treatment, whereas 50% of the mCLDN3 siRNA-treated mice survived. All mice that died succumbed to cancer after rapidly developing ascites. This difference in survival between mCLDN3 siRNA-treated mice and control mice suggested that mCLDN3 siRNA suppressed metastasis of solid tumors. Ascites fluid that accumulates in the abdomen attenuates the bioluminescence emitted by tumor cells. Thus, the use of bioluminescence may have resulted in low assessments of tumor load in control mice and an underestimate in the effectiveness of the siRNA treatment on the suppression of solid tumor growth.

Discussion

In this study, we show suppression of ovarian tumor growth in 3 different mouse models after lipidoid-mediated delivery of a siRNA targeting CLDN3 expression. A primary component of these formulations was the delivery molecule, $98N_{12}$-5(1), which belongs to a class of previously undescribed lipid-like molecules. It has previously been used to deliver siRNA and miRNA to lung, liver, and peritoneal macrophages, and to silence target genes effectively in 3 different species, including nonhuman primates (9).

We selected CLDN3 as the target for siRNA therapy, because it is one of the most highly up-regulated genes in ovarian cancer (2). Also, its expression has been shown to promote migration, invasion, and survival of ovarian cancer cells (4, 11). It is an important clinical target, because it is overexpressed in 90% of ovarian tumors, including all 4 major subtypes (serous, mucinous, clear cell, and endometrioid), and expressed at very low levels in normal tissues.

Studies using OVCAR-3 xenografts in nude mice allowed evaluation of CLDN3 siRNA therapeutic efficacy directly in human ovarian tumor cells, whereas the use of tumor-bearing MISIIR/TAg transgenic mice allowed for evaluation of the therapy in immunocompetent animals. Importantly, the i.p. injection of siRNA into these mice allowed us to validate the therapeutic efficacy associated with this administration route. Based on a recent report indicating that i.p. chemotherapy for advanced ovarian cancer improves overall and disease-free survival (12), the National Cancer Institute posted a rare Clinical Announcement recommending that physicians use this mode of drug administration in treating advanced ovarian cancer patients. The significant reduction in ovarian tumor loads and in ascites production that we observed in MISIIR/TAg mice and mice injected with ID8-Fluc cells after i.p. injections of CLDN3 siRNA, and the high expression of CLDN3 protein we observed in human ascites cells, suggest that this therapy holds promise for the treatment of patients with metastatic ovarian cancer. To our knowledge, this is the first time that siRNA therapy has been shown to suppress ascites tumor development.

Use of tumor-bearing MISIIR/TAg transgenic mice and in C57BL/6J mice injected with ID8-Fluc MOSEC cells also allows for evaluation of the therapy in immunocompetent animals, an important consideration, given the ability of certain siRNA sequences to induce inflammatory cytokines and type I interferons (13). Relative to known immunostimulatory sequences, the unmodified CLDN3 siRNA resulted in measurable, but low, immunostimulation (Table S1 and Fig. S2). However, modified CLDN3 siRNA was nonstimulatory and suppresses ovarian tumor growth as effectively as the unmodified CLDN3 siRNA after i.p. injection of formulated material. This result suggests that the therapeutic effects we observe are the direct result of suppression of CLDN3 protein.

Also to efficacy, the safety of lipidoid-siRNA formulations is an important factor in considering their utility for clinical application. It has been reported that CLDN3 is expressed at very low levels in several normal tissues in humans including the lungs, kidneys, breast, uterus, pancreas, and thyroid (2, 14). Colon, small bowel, and prostate are the only normal tissues that show appreciable expression (3, 15); i.p. administration of CLDN3 siRNA formulations may reduce the concern of adverse effects of silencing CLDN3 in healthy tissues that reside outside the peritoneum. We have not observed any formulation-dependent toxicity in noncancerous tissues or in metabolic parameters after multiple i.p. injections of lipidoid formulated CLDN3 siRNA over a period of several weeks.

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Last, as recently reviewed, different members of the claudin gene family are abnormally regulated in several human cancers (11). Also to its frequent overexpression in ovarian tumors, CLDN3 is overexpressed in breast (16), prostate (15), and endometrial cancer of serous papillary or clear-cell histology (17). There is evidence that it is overexpressed in other tumor types as well, including lung and kidney (14). The CLDN3 siRNA therapy that we have shown here to suppress ovarian tumor growth and metastasis in preclinical studies may also be effective in the treatment of other cancers. More broadly, lipidoids have the potential to delivery siRNAs to target other claudin proteins that are specifically overexpressed in other tumor types.

Materials and Methods

The materials and methods used for membrane protein preparations, immunoblotting, immunohistochemistry, PBMC isolation and culture, and siRNA transfection and cytokine assays can be found in *SI Results*.

Cell Lines and Cultures. OVCAR-3 cells (ATTC) were maintained at 37 °C in 5% CO_2 in RPMI-1640 medium (ATCC) supplemented with 20% FBS (ATLANTA) and 0.01 mg/mL bovine insulin. ES-2 cells (ATCC) were grown in McCoy's 5-a medium (Cellgro), 10% FBS. ID8, a cell line derived from spontaneous in vitro malignant transformation of C57BL6 MOSEC, was a generous gift from Paul F. Terranova and Katherine F. Roby (University of Kansas, Lawrence, KS). These cells were maintained in DMEM (Cellgro), supplemented with 4% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL sodium selenite (Cellgro). To establish stable transfectants containing the firefly luciferase gene (Fluc), LipofectAMINE 2000 (Invitrogen) was used to transfect the cells with the plasmid pCAG/Fluc (18). Cells were maintained in medium containing G418 (1 mg/mL) (Cellgro), and clones were picked and expanded to generate cell lines. ID8-Fluc cells were maintained in medium containing G418 (600 μg/mL).

Small Interfering RNA Formulations. All siRNAs were synthesized by Alnylam. Sequences are in *SI Materials and Methods*. For in vitro studies, 1.2×10^6 cells were seeded per 100-mm culture dish. When cells had grown to ≈70% confluency, a mixture of 30 μL of siRNA (20 pmol/μL) and 30 μL of LipofectAMINE 2000 (Invitrogen) was added to the medium in each dish. After 48 h, cells were harvested and membrane protein preparations were made. For in vivo studies, siRNA sequences were formulated with $98N_{12}$-5(1), PEG ceremide, and cholesterol, as previously described (9); siRNA formulations were injected intratumorally into xenografts (25 μg per injection) or i.p. (for MISIIR/TAg and ID8-Fluc-injected mouse models) (175 μg per injection).

Mouse Models. To generate xenografts, 2×10^6 OVCAR-3 cells in 100 μL of PBS containing 20% cold Matrigel (BD Biosciences) were injected s.c. into the flank of 8-week-old female athymic nude-*Foxn1*^{nu}/*Foxn1*$^{+}$ (nude) mice (Harlan). Caliper measurements of tumor length and width were taken at various times over a 2 week period, and used to calculate tumor volume (length × width^2 × 0.52).

To generate tumors in a syngeneic mouse model, 6–8-week-old *nu*/*nu*.BALB/c female mice (Harlan) were injected i.p. with 5×10^6 ID8-Fluc cells suspended in 200 μL of DMEM containing no additives.

MISIIR/TAg transgenic mice were initially obtained from Denise Connolly, Fox Chase Cancer Center. Male MISIIR/TAg mice were bred to C57BL/6J females to generate hemizygous females for experiments. Transgenic mice were identified by PCR amplification of transgene sequences from genomic DNA by using primers and conditions as described in ref. 7. Assay of metabolic serum enzymes of siRNA-treated MISIIR/TAg mice was performed by LabCorp.

All experiments with mice were approved by the Lankenau Institutional Animal Care and Use Committee (IACUC).

Imaging. Optical imaging to detect luciferase activity in mice was performed by using an IVIS 100 series Bioluminescence Imaging System (Caliper Life Sciences) as described previously with a 5-min integration time for image acquisition (19).

For microCT scans, mice were injected i.p. with 250 μL of Optiray Pharmacy Ioversol Injection 74% contrast medium (741 mg of ioversol/mL); 10 min later, mice were anesthetized and underwent CT scanning by using a MicroCAT 1A scanner (ImTek) (40 kV, 600 μA, 196 rotation steps, total angle of rotation = 196). Images were reconstructed by using RVA3 software program (ImTek). Tumor volumes were determined by using Amira 3.1 software (Mercury Computer Systems).

Statistical Analysis. Statistical significance was determined by using a 2-tailed Student's *t* test. We considered a value of $P < 0.05$ to be statistically significant.

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