HDAC6 Inhibition Restores Ciliary Expression and Decreases Tumor Growth

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Abstract

Primary cilia are multisensory organelles recently found to be absent in some tumor cells, but the mechanisms of deciliation and the role of cilia in tumor biology remain unclear. Cholangiocytes, the epithelial cells lining the biliary tree, normally express primary cilia and their interaction with bile components regulates multiple processes, including proliferation and transport. Using cholangiocarcinoma as a model, we found that primary cilia are reduced in cholangiocarcinoma by a mechanism involving histone deacetylase 6 (HDAC6). The experimental deciliation of normal cholangiocyte cells increased the proliferation rate and induced anchorage-independent growth. Furthermore, deciliation induced the activation of mitogen-activated protein kinase and Hedgehog signaling, two important pathways involved in cholangiocarcinoma development. We found that HDAC6 is overexpressed in cholangiocarcinoma and overexpression of HDAC6 in normal cholangiocytes induced deciliation and increased both proliferation and anchorage-independent growth. To evaluate the effect of cilia restoration on tumor cells, we targeted HDAC6 by short hairpin RNA (shRNA) or by the pharmacologic inhibitor, tubastatin-A. Both approaches restored the expression of primary cilia in cholangiocarcinoma cell lines and decreased cell proliferation and anchorage-independent growth. The effects of tubastatin-A were abolished when cholangiocarcinoma cells were rendered unable to regenerate cilia by stable transfection of IFT88-shRNA. Finally, inhibition of HDAC6 by tubastatin-A also induced a significant decrease in tumor growth in a cholangiocarcinoma animal model. Our data support a key role for primary cilia in malignant transformation, provide a plausible mechanism for their involvement, and suggest that restoration of primary cilia in tumor cells by HDAC6 targeting may be a potential therapeutic approach for cholangiocarcinoma. Cancer Res; 73(7); 2259–70. ©2013 AACR.

Introduction

Primary cilia are microtubule-based organelles that function as multisensors of the extracellular environment (1). Interest in primary cilia has increased markedly over the last 15 years, as it was observed that mutations in genes required for the assembly and/or the sensory properties of cilia result in diverse human disorders such as visceral epithelial hyperplasia, polycystic kidneys, pancreas, and liver among other abnormalities (2). Recent observations also suggest a relationship between ciliary structure/function and tumorigenesis. For example, Aurora A kinase mediates ciliary disassembly and is overexpressed in many epithelial cancers (3). Nek8, a kinase expressed in primary cilia that regulates ciliogenesis, is increased in breast cancer (2, 4); and the loss of the von Hippel-Lindau (VHL) tumor suppressor gene inhibits ciliogenesis and is associated with renal cancers (5, 6). Also, mutations in mice of Tg737, the mammalian homolog of Chlamydomonas IFT88, a key component for ciliary formation (7), accelerate the rate at which chemical carcinogens induce liver neoplasms (8). Finally, very recent findings showed reduced expression of cilia in pancreatic ductal adenocarcinoma (2), renal cancer (6), astrocytoma/glioblastoma (9), and breast cancer (10). While these data suggest that ciliary dysfunction may be associated with cancer development, the mechanisms leading to ciliary reduction in tumor cells as well as the consequences of such a loss remain poorly understood and are the subject of the present manuscript.

Cholangiocarcinoma (CCA) is a malignancy thought to be derived from cholangiocytes, the epithelial cells lining the biliary tree. Cholangiocarcinoma is a highly aggressive tumor whose incidence has been increasing worldwide over the past 2 decades, now accounting for 10% to 15% of all hepatobiliary malignancies. Advanced cholangiocarcinoma has a devastating prognosis, with a median survival of less than 24 months (11, 12).
Cholangiocytes normally express primary cilia extending from their apical plasma membrane into the ductal lumen. In cholangiocytes, the primary cilium functions as a multisensor of the extracellular milieu detecting a wide variety of chemical and physical stimuli. Indeed, we reported that cholangiocyte primary cilia are mechano-, chemo-, and osmosensory organelles (13–16).

In the present manuscript, we describe that ciliary expression is decreased in cholangiocarcinoma by a mechanism involving overexpression of histone deacetylase 6 (HDAC6). We found that targeting HDAC6 in cholangiocarcinoma cells decreases the tumorigenic phenotype of the cells in a ciliary re-expression–dependent manner in vitro and in an animal model of cholangiocarcinoma. The data not only shed light on the mechanisms by which ciliary disassembly facilitate malignant transformation but also identify a potential molecular target for cholangiocarcinoma.

Materials and Methods

Cell lines and culture

The normal human cholangiocytes (H69 and NHC) and the normal rat (NRC) cell lines were maintained as previously described (15, 17, 18). The human cholangiocarcinoma cell lines [HuCCT-1 (ref. 19) and KMCH (ref. 20)] and the rat cholangiocarcinoma cell line (BDEneu; refs. 21, 22) were cultured in Dulbecco’s Modified Eagles’ Medium (DMEM) supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 100 μg/L insulin.

Real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) and synthesized into cDNA using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative PCR for HDAC6 was carried out using 1 μL of cDNA and the Light Cycler Fast Start DNA MasterPlus SYBR Green I kit (Roche Diagnostics) as previously described (23). The primers used were HDAC6 sense (5’-AGTCTTAGGATGCTATGCGT-3’), HDAC6 antisense (5’-TGGACCCAGTGGCCTTTCAAGG-3’), PTCH1 sense (5’-CGCTGTCCTTCTCTGACCC-3’), and PTCH1 antisense (5’-ATCGACGCTCCAGCAGATG-3’). IFT88 expression was analyzed using the TaqMan Gene Expression Assay (Assay ID Hs00197926_m1) from Applied Biosystems following the manufacturer’s directions. The samples were normalized to 18S rRNA.

Immunofluorescences

Liver sections were incubated with antibodies against acetylated α-tubulin (1:500, Sigma-Aldrich), IFT88 (1:100, Protein-tech), CK19 (1:100, Santa Cruz Biotechnology or Abcam), γ-tubulin (1:500, Sigma-Aldrich), proliferating cell nuclear antigen (PCNA: 1:1,000, Santa Cruz Biotechnology), and/or HDAC6 (1:100, Abcam) overnight at 4°C followed by incubation for 1 hour with fluorescent secondary antibodies (1:100). Nuclei were stained with 4,6-diamino-2-phenylindole (DAPI; Prolong Gold w/DAPI, Invitrogen). For HDAC6-flag expression analysis, cells were transfected with the Addgene plasmid 13823 (Dr. Eric Verdin; ref. 24) using Fugene reagent (Roche). After 3 days of incubation in media without serum, cells were fixed and stained for ciliary markers acetylated α-tubulin and/or IFT88 and ciliated cells were analyzed under the confocal microscopy.

Scanning electron microscopy

Cells were processed as previously described (25).

Chemical and molecular deciliation

Chemical deciliation was carried out by treatment with 4 mmol/L chloral hydrate as previously described (15). Molecular deciliation was obtained by stably transfection with shRNAs against HDAC6, Kif3a, or Cep164 short hairpin RNA (shRNA) plasmids (Supplementary Table S1).

Proliferation assays

Proliferation assays were conducted using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (MTS; Promega) and/or counting cells using the Cellometer Auto4 (Nexcelom Bioscience) cell counter.

Anchorage-independent growth

Anchorage-independent growth was assessed by growing cells in soft agar. About 25,000 cells suspended in 0.4% agar in culture media were layered over a 1% agar layer in a 6-well plate. Media were added twice a week and pictures were taken after 14 to 21 days of incubation. The number and size of colonies were analyzed using the Gel-Pro software.

Invasion assays

Invasion assays were conducted using the CytoSelect 24-Well Cell Invasion Assay Kit (Cell Biolabs, Inc.) following the manufacturer’s directions.

Western blots

Protein fractions were subjected to SDS-PAGE and transferred to nitrocellulose membranes. After blocking, blots were incubated overnight at 4°C with one of the following antibodies: HDAC6 (1:1,000, Santa Cruz Biotechnology), Erk (1:2,000, Abcam), p-Erk (1:1,000, BD Biosciences), Gli1 (1:500, Abcam), IFT88 (1:1,000, Proteintech), Kif3a (1:500, Santa Cruz Biotechnology), Cep164 (1:500, Genetex), IL-6 (1:500, Santa Cruz), bcl-2 (1:1,000, Santa Cruz), actin (1:5,000, Sigma-Aldrich or Abcam), and acetylated-α-tubulin (1:5,000, Sigma-Aldrich); washed and incubated for 1 hour at room temperature with horseradish peroxidase-conjugated (1:5,000, Invitrogen) or IRdye 680 or 800 (1:15,000, Odyssey) corresponding secondary antibody. For protein detection, ECL system or Odyssey Liquor Scanner was used, and the Gel-Pro Analyzer 6.0 software was used for densitometric analysis.

In vivo experiments

All animal experimentation was carried out in accordance with and approved by the Institutional Animal Care and Use Committee. In vivo cell transplantation was carried out in adult fisher 344 male rats (Harlan) with initial mean body weights ranging between 200 and 250 g, as previously
described (21, 26, 27). Five days after tumor implantation, animals were treated daily with tubastatin-A (10 mg/kg body weight intraperitoneally) or vehicle for 7 days. After treatment, animals were euthanized and the livers were removed for analysis.

**Statistical analysis**

Data are expressed as mean ± SE. Statistical analyses were conducted by one-way ANOVA with Bonferroni post hoc test to compare more than 2 groups and by the Student t test to compare 2 groups. Results were considered statistically different at \( P < 0.05 \).

**Results**

**Primary cilia are reduced in cholangiocarcinoma in vivo and in vitro**

To assess the expression of primary cilia in cholangiocarcinoma, we stained liver samples from 21 patients with cholangiocarcinoma and 6 normal controls with the ciliary markers, acetylated \( \alpha \)-tubulin and/or IFT88, and the cholangiocyte marker, CK19. We found that while 100% of bile ducts from normal controls show primary cilia, only 20% are ciliated in cholangiocarcinoma samples (Fig. 1A–E). We found a similar situation when we stained a commercially available cholangiocarcinoma tissue array [AccuMax Array A205(II); Supplementary Fig. S1]. To further explore the differential expression of primary cilia, we assessed their expression on 2 normal human cholangiocyte cell lines (H69, NHC) and the cholangiocarcinoma cell lines HuCCT-1 and KMCH. Ciliary expression was induced by serum starvation (15) and assessed by staining with acetylated \( \alpha \)-tubulin, IFT88, and the centrosome marker, \( \gamma \)-tubulin; our results showed that only 3% (HuCCT-1) and 1.8% (KMCH) of the cholangiocarcinoma cell lines express cilia, whereas in the normal cell lines, cilia were found in 67% (H69) and 61% (NHC) of the cells, respectively (Fig. 1F–H). Finally, to confirm ciliary loss in cholangiocarcinoma cells, we...
conducted scanning electron microscopy of the apical surface of normal and cholangiocarcinoma cells (Fig. 1I–L).

**Deciliation of normal cholangiocytes induces proliferation, anchorage-independent growth, and invasion**

To explore the potential relationship between ciliary loss and cholangiocyte phenotype, we assessed the effect of deciliation on normal human (i.e., H69) and rat (NRC) cholangiocyte cell lines. First, we induced chemical deciliation by ClHy treatment (15); deciliation by this manipulation increased normal cholangiocyte proliferation by 2-fold (Fig. 2A). We complemented this approach of chemical deciliation by molecular deciliation using specific shRNAs against IFT88, Kif3a, or Cep164 (Supplementary Fig. S2); this approach caused an increase in cell proliferation by 1.89-, 1.77-, and 1.63-fold, respectively (Fig. 2B). The increased proliferation was confirmed by nucleotide incorporation assays (Supplementary Fig. S3). Furthermore, cell-cycle analysis showed an increase in G2–M and a decrease in G1–G0 phases in IFT88-shRNA deciliated cells compared with normal NT-shRNA controls (Supplementary Fig. S4), further supporting increased proliferation. To further characterize the effect of ciliary loss, we assessed anchorage-independent growth and invasion and found that deciliation by IFT88 shRNAs induced both parameters by 6- and 3-fold, respectively, and by 3-fold and 2-fold, respectively, when deciliation was induced by Kif3a shRNAs (Fig. 2C and D). Finally, we analyzed the status of the hedgehog signaling pathway by real-time PCR (RT-PCR) of patched mRNA and Western blots for Gli1, interleukin (IL)-6, and Bcl2; and the mitogen-activated protein kinase (MAPK) signaling pathway by Western blotting and found that deciliation induced activation of both hedgehog and MAPK signaling pathways.
Taken together, these data suggest that experimentally induced deciliation stimulates a phenotypic transformation of normal cholangiocytes in culture to a malignant phenotype.

HDAC6 is overexpressed in cholangiocarcinoma and decreases ciliary expression

To explore the potential mechanisms involved in the cholangiocarcinoma decreased ciliary expression, we analyzed HDAC6, a tubulin deacetylase reported to induce ciliary resorption in the immortalized retinal pigment epithelial cell line hTERT-RPE1 (3); moreover, evidence in the literature suggests an important role for HDAC6 overexpression in tumorigenesis (28–31). We assessed the expression of HDAC6 by Western blot analysis on 2 different cholangiocarcinoma cell lines (Hucct-1 and KMCH) and found increased expression in both (on average, 100%) of HDAC6 compared with normal cultured cholangiocytes (H69). Overexpression of HDAC6 correlated with the decreased amount of its target, acetylated α-tubulin (Fig. 3A), supporting the validity of our analyses. The mRNA level was analyzed by qRT-PCR and no significant differences were found (Fig. 3B), suggesting a posttranscriptional regulatory pathway. Using confocal immunofluorescence microscopy, we also observed that HDAC6 was overexpressed in liver specimens from 10 patients with cholangiocarcinoma compared with 11 normals (Fig. 3C). We found a similar situation when we stained the commercially available cholangiocarcinoma tissue array [AccuMax Array A205(II); Supplementary Fig. S5].

To assess the role of HDAC6 overexpression in cholangiocyte ciliary loss, we transfected NHC cells with a HDAC6-flag expression vector. Confocal immunofluorescences using anti-flag and HDAC6 antibodies showed the expression of the HDAC6-flag construct (Fig. 4A). As predicted, when the cells were co-stained with the ciliary markers, acetylated-α-tubulin and IFT88, the cells that were positive for the flag epitope showed reduced cilia expression compared with the surrounding nontransfected cells (Fig. 4B). After stable transfected cells were obtained by antibiotic selection, Western blot analysis showed the overexpression of HDAC6 in the stably transfected cells using anti-flag antibodies (Fig. 4C). In addition to the decreased ciliary expression (Fig. 4D), these cells showed an increased proliferation rate (1.7-fold; Fig. 4E), increased anchorage-independent growth (1.5-fold; Fig. 4F), and decreased protein levels of acetylated-α-tubulin compared with empty vector-transfected cells (Fig. 4G). Taken together, these results suggest that the overexpression of HDAC6 in normal cholangiocytes correlates with the reduction of primary cilia expression and the acquisition of a malignant-like phenotype.

Targeting HDAC6 induces ciliary restoration and reverses the malignant phenotype of cholangiocarcinoma cells

As HDAC6 overexpression seems to contribute importantly to cholangiocarcinoma ciliary loss, we inhibited HDAC6 expression by specific shRNAs (Fig. 5A) or with the HDAC6 inhibitor, tubastatin-A (32). These approaches both induced an increase in acetylated-α-tubulin levels and the restoration of primary cilia expression in the cholangiocarcinoma cell lines (3.3- and 18-fold, respectively; Fig. 5A, D, and E); and the restoration of primary cilia correlated with downregulated Hedgehog (Hh) and MAPK signaling pathways (Fig. 5C), as well as decreased cell proliferation rates (decreased in...
average by 50%; Fig. 5B and F) and invasion (decreased by 40%; Fig. 5G). To analyze whether the restoration of cilia is a major reason for these phenotypic changes, we repeated the experiments in KMCH cells stably transfected with IFT88-shRNA to prevent ciliogenesis. In the experiments in which cholangiocarcinoma cells were prevented from developing HDAC6

Figure 4. Effect of HDAC6 overexpression in normal cholangiocytes. A, NHC cells were transfected with HDAC6-flag expression vector, and protein expression was analyzed by confocal immunofluorescence using anti-flag (green) and anti-HDAC6 antibodies (red); nuclei were stained in blue with DAPI. B, confocal immunofluorescence using anti-flag (purple), anti-acetylated-α-tubulin (red), and anti-IFT88 (green) showed that cells overexpressing HDAC6 do not grow cilia compared with nontransfected surrounding cells. C, NHC cells were stably transfected with empty vector or with HDAC6-flag expression vector. Western blot analysis showed the expression of HDAC6-flag. D, confocal immunofluorescence on cells cultured 2 days in cilia-promoting media showed ciliary expression by acetylated-α-tubulin staining (red) and centrioles (purple). Note that cilia are easily detected by the basal bodies in empty vector–transfected cholangiocytes (EV), whereas they are mainly absent on the HDAC6-flag–transfected cells. Nuclei are stained in blue with DAPI. E, MTS proliferation assay comparing empty vector (EV) and HDAC6-flag stably transfected cells (P < 0.05). F, colony number after 14 days of growth in soft agar (P < 0.05). G, Western blot analysis of acetylated-α-tubulin on NHC cells stable transfected with EV and HDAC6-flag.
Figure 5. Targeting of HDAC6 induces cilia restoration in tumor cells. A, CCA cells were stably transfected with HDAC6-specific or with nontarget (NT) control shRNAs. HDAC6 and acetylated-α-tubulin protein expression was analyzed by Western blotting. Ciliary expression was analyzed by confocal immunofluorescence. The ciliary marker, acetylated-α-tubulin in red; the centrioles marker γ-tubulin in green; and nuclei were stained with DAPI in blue. B, proliferation analysis by MTS (P < 0.001). C, MAPK and Hh signaling pathways were analyzed by Western blotting of pERK and Gli1, respectively. D, CCA cells were treated for 2 days in ciliary-promoting media in the absence or presence of the HDAC6 inhibitor tubastatin-A. Acetylated-α-tubulin protein expression was analyzed by Western blotting (D), and the expression of cilia was analyzed by confocal microscopy (E). Cilia are stained in red with the ciliary marker IFT88 and nuclei in blue with DAPI. F, tubastatin-A treatment reduced proliferation rates in 2 different CCA cell lines (*, P < 0.05). G, invasion assays comparing vehicle treated (control) and tubastatin-A treated KMCH cells (*, P < 0.05).
HADC6 inhibition by tubastatin-A treatment reduces tumor growth and induces ciliogenesis in vivo

On the basis of the in vitro activity of tubastatin-A on cholangiocarcinoma cell lines, we tested the effect of this drug using a recently developed syngeneic rat orthotopic model of cholangiocarcinoma (21, 26, 27). Tumors were removed after treatment with tubastatin-A or vehicle for 7 days. The mean tumor weights in animals treated with tubastatin-A was 6-fold lower than vehicle-treated controls (0.33 ± 0.09 vs. 1.81 ± 0.51 g, P < 0.05). Similarly, the ratios of tumor weight to liver weight and body weight were also significantly reduced (5- and 5.6-fold, respectively) by tubastatin-A treatment (Fig. 7A–D). Furthermore, confocal immuno-fluorescence microscopy showed a greater frequency of ciliated cholangiocytes in the treated animals compared with controls (29% vs. 1.4% ciliated cells per high-power field; Fig. 7E, G). Finally, the amount of PCNA-positive cells was significantly reduced in the treated tumors compared with vehicle controls (34% vs. 65% PCNA-positive cells per high-power field), indicating decreased proliferation (Fig. 7F, H). These data indicate that a drug that inhibits HDAC6 can significantly reduce the growth of cholangiocarcinoma in vivo.

Discussion

The key findings reported here relate to the potential role of primary cilia in the pathogenesis of cholangiocarcinoma. Our data show that (i) the expression of primary cilia is decreased in cholangiocarcinoma in vivo and in vitro; (ii) chemical or molecular deciliation of normal cholangiocytes induces a malignant phenotype characterized by increased proliferation, anchorage-independent growth, invasion and activation of Hh and MAPK signaling pathways; (iii) HDAC6 is overexpressed in cholangiocarcinoma in vivo and in vitro; (iv) molecular overexpression of HDAC6 causes decreased ciliogenesis and induces proliferation and anchorage-independent growth in normal cholangiocytes (i.e., a malignant phenotype); (v) molecular downregulation of HDAC6 or its pharmacological inhibition by tubastatin-A induces restoration of primary cilia in cholangiocarcinoma cells and reduces cell proliferation, anchorage-independent growth and invasion in a ciliary-dependent manner, and (vi) the HDAC6 inhibitor, tubastatin-A, reduces tumor growth in a cholangiocarcinoma animal model. The data are consistent with an important role for HDAC6 and primary cilia in the pathogenesis of cholangiocarcinoma.

Recently, four different tumors were described to be devoid of primary cilia. In pancreatic cancer cells, the absence of cilia is independent of ongoing proliferation and ciliogenesis can be reversed by inhibiting Kras pathways (2). These findings are consistent with our results, as HDAC6 is activated by Kras and the incidence of Kras mutations in cholangiocarcinoma is estimated to be 54% to 67% (3, 33). The absence of primary cilia has also been reported in sporadic clear cell renal carcinoma; in this case, ciliary loss is mediated by dysfunction of the protein of the vHL tumor suppressor gene, pVHL (6). pVHL binds and stabilizes microtubules by protecting them from depolymerization, which is a prerequisite for ciliogenesis (6). Interestingly, pVHL inactivation induces HEF1 and AuroraA (34), and these events lead to HDAC6 activation and ciliary resorption (3). Aberrant ciliogenesis is also found in cells derived from astrocytomas/glioblastomas; it has been proposed that this deficiency likely contributes to the phenotype of these malignant cells (9). Finally, primary cilia are decreased in breast cancer as well, by unknown mechanisms (10). Taken together, our results and these previous studies suggest that the loss of primary cilia is a common feature in many epithelial tumors. However, our studies are the first to show the reduction of cilia and the overexpression of HDAC6 in cholangiocarcinoma. Moreover, our data are first to show...
that restoration of primary cilia by targeting HDAC6 is a potential therapeutic approach for cholangiocarcinoma and perhaps other tumors characterized by defective ciliogenesis.

On the other hand, the situation is different in medulloblastoma and basal cell carcinoma; these tumors are mainly ciliated and cilia are required for the growth of tumors bearing an activation mutation at the level of smoothened (ciliary-dependent activator of the Hh signaling pathway). In contrast, if the tumors have an activation mutation of the downstream effector of the pathway, the transcription factor Gli2, primary cilia play a similar role as described in the present work, that is, inhibition of tumor development (35, 36). In cholangiocarcinoma, consistent with our results, the aberrant activation of Gli transcription factors has been described (37, 38).

Figure 7. Effect of tubastatin-A on cholangiocarcinoma growth in vivo. The effect of tubastatin-A was tested on an orthotopic, syngeneic CCA model in rats. A, livers were removed after 7 days of treatment and tumors were dissected. Tumor weights, tumor/liver weight, and tumor/body weight ratios were calculated and compared (B–D). Tumor sections were stained with the ciliary marker acetylated-α-tubulin in red and the cholangiocyte marker CK-7 in green (E) or with the proliferation marker PCNA in green (F); nuclei were stained in blue with DAPI. The amount of ciliated and PCNA-positive cells per field were quantified (G, H); *, P < 0.05, n = 5 for controls and n = 6 for tubastatin-A.
interact with histones (i.e., inhibition of HDAC6 inhibits deacetylation of α-tubulin without affecting histone acetylation; ref. 39). HDAC6 not only deacetylates α-tubulin but also cortactin, Hsp90, and the redox regulatory proteins, Prx and II (40). Moreover, HDAC6 has been identified as a key regulator of many processes that are linked to cancer (e.g., cell survival, motility, and metastasis), making it an attractive therapeutic target (39). On the basis of our results, it appears that the antitu- morigenic effect of HDAC6 inhibition in cholangiocarcinoma mainly depends on ciliary restoration, as KMCH cells stably transfected with ift88 shRNA did not respond to tubastatin-A treatment. We acknowledge that the involvement of the other targets of HDAC6 cannot be confidently excluded.

Previous work with embryonic kidney cells, mammary epithelial cells, mouse embryonic fibroblasts, and ovarian and cancer cell lines showed that HDAC6 is not only important for Ras- or ErbB2-dependent oncogenic transformation of primary cells but also is required for maintaining the anchorage-inde-
dependent growth of established cancer cell lines (28). The exact molecular mechanisms mediating such a tumor-promoting effect remains unknown. Our results in cholangiocarcinoma cells suggest that HDAC6 mediates the oncogenic-induced loss of primary cilia and the subsequent derepression of tumorigenic signaling pathways such as Hh and MAPK. The mechanisms by which HDAC6 is overexpressed in cholangiocarcinoma remain to be elucidated. As we found alterations in protein but not in mRNA levels for HDAC6, posttranslational regulations (e.g., HCAC6 turnover rates, the downregulation of microRNAs potentially targeting HDAC6 mRNA, etc.), are possibilities.

Hedgehog and extracellular signal–regulated kinase (ERK)1/2 pathways are both activated in cholangiocarcinoma (37, 38, 41), and the dual targeting of these pathways coordinate
dy proliferation and survival of cholangiocarcinoma cells (41). IL6 and bcl-2, both targets of hedgehog signaling (42–44), are also activated in cholangiocarcinoma (45, 46). Our results show that the experimental deciliation of normal cholangiocytes induced the activation of both hedgehog and MAPK pathways, consistent with the concept that cilia normally act as a negative regulator of these pathways in cholangiocytes. Importantly, our results also show that the restoration of primary cilia, by targeting HDAC6 in cholangiocarcinoma cells, decreases Hh and MAPK signaling pathways.

Michaud and Yoder speculated that genes and proteins involved in the structure or function of primary cilia may represent new targets for small-molecule inhibitors, siRNAs, or antibody therapeutics (47). On the basis of our data, we would extend this concept by suggesting that restoration of primary cilia and their complex multisensory signals by HDAC6 targeting could act as a tumor suppressor mecha-
nism. Indeed, the rapidly evolving field of HDAC inhibitors promises to generate very potent and specific HDAC6 inhibitors, like tubastatin-A (32) and the more recently developed ACY-1215 (48). The fact that mice lacking HDAC6 are viable and develop normally (49) suggests that HDAC6-specific targeting may have minimal adverse effects. Furthermore, our in vivo experiments on a rat cholangiocarcinoma model using tubastatin-A showed a significant decrease in tumor growth associated with an increased ciliary expression, sug-

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S.A. Gradilone, P.S. Bogert, G.B. Gajdos, N.F. LaRusso Development of methodology: P.S. Bogert, N.F. LaRusso Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.A. Gradilone, B.N. Radtke, P.S. Bogert, B.Q. Huang, N. F. LaRusso Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.A. Gradilone, G.B. Gajdos, N.F. LaRusso Writing, review, and/or revision of the manuscript: S.A. Gradilone, B.N. Radtke, N.F. LaRusso Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B.N. Radtke

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