Therapeutic targeting of Id2 reduces growth of human colorectal carcinoma in the murine liver

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Abstract

During development inhibitor of DNA-bind-2 (Id2) regulates proliferation and differentiation. Id2 expression has been detected in cancer cells, yet its cellular function and validity as a therapeutic target remains largely unknown. Immunohistochemical analysis of colorectal cancer (CRC) specimens revealed that Id2 was undetectable in normal colonic mucosa, but occurs in 40% of primary tumors and in most CRC liver metastases ($P < 0.0001$). Additionally, Id2 was expressed in all CRC cell lines assayed. CRC cells with reduced Id2 expression demonstrated reduced proliferation. Analysis of CRC cell cycle regulatory proteins showed that reducing Id2 levels reduces cyclin D1 levels and increased p21 levels. Reduction of Id2 expression also enhanced tumor cell apoptosis, increasing levels of the pro-apoptotic protein Bim/Bod, and cleavage of caspase-7 and poly (ADP-ribose) polymerase. *In vivo* studies show tumors derived from cells with decreased Id2 levels formed smaller tumors with fewer metastases compared with tumors with normal levels ($P < 0.05$). Furthermore, intraperitoneal administration of Id2 small interfering RNA (siRNA) conjugated with the neutral liposome 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine decreased tumor burden in mice compared with control treatment ($P = 0.006$). We conclude that Id2 is upregulated in CRC, and is important in promoting cell survival. *In vivo* targeting of Id2 by siRNA establishes that it is a valid therapeutic target where its expression occurs. *Oncogene* (2008) 27, 7192–7200; doi:10.1038/onc.2008.356; published online 22 September 2008

Keywords

Id2; colorectal carcinoma; Inhibitor of DNA-binding; metastasis; siRNA

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Introduction

Most targeted therapies currently approved by the United States Food and Drug Administration target cell surface receptors or intracellular kinase regulatory proteins, whose expression and/or activation are believed to be critical in tumor growth. Although the clinical results achieved with targeted therapies have been encouraging, overall the benefits are less than anticipated. One potential reason for this limitation observed with targeted therapies is the heterogeneity of cancers themselves; disparate mechanisms may activate similar downstream effectors and lessen the effects of focused targeting strategies. If true, then the ability to identify and effectively target central intercellular regulatory proteins that are essential to these activation pathways, such as transcriptional regulators factors, may prove to be effective therapeutics.

The inhibitor of DNA-bind-2 (Id2) protein belongs to a family of four homologous proteins (Id1-Id4) (Lasorella *et al*., 2001, 2005). The Id proteins are helix–loop–helix-like transcription factors that lack a DNA-binding basic amino acid domain. The Ids are essential for embryogenesis, and they have been functionally implicated in proliferation, differentiation and cell survival (Jen *et al*., 1996; Lasorella *et al*., 2001). The Id proteins are not typically found in most adult tissues. However their reactivated expression, including that of Id2, occurs in some human cancers (pancreatic, neuroblastoma and colon) and may be of prognostic value (Kleeff *et al*., 1998; Rockman *et al*., 2001; Wilson *et al*., 2001; Lasorella *et al*., 2002; Lofstedt *et al*., 2004). The Ids are believed to function primarily as dominant-negative regulators, forming non-DNA-binding heterodimers with other basic helix-loop-helix transcription factors (Benezra *et al*., 1990). Dimerization partners of the Id family encompass members of the Eprotein group (E12, E47 and E2-2), whose downstream targets include the cell cycle inhibitors $p21^{CIP/WAF1}$, $p15^{INK4B}$ and $p16^{INK4B}$ (Pagliuca *et al*., 2000; Rothschild *et al*., 2006). In addition to functioning as a transcriptional repressor, Id2 also binds to retinoblastoma (Rb) tumor suppressor proteins (Iavarone *et al*., 1994; Lasorella *et al*., 2000).

In normal cells, Rb mediates cell cycle arrest through the sequestration of the E2F is preferred nomenclature-transcription factors (Weinberg, 1995). This association is controlled through Rb phosphorylation, which is mediated by the G_1 cyclin–cyclin-dependent kinase complexes, the functions of which are in turn inhibited by the cell cycle inhibitors $p21^{CIP/WAF1}$, $p27^{KIP1}$, and $p57^{KIP2}$ (Weinberg, 1995). Developmental studies in *Rb*-null mice have shown that Rb can suppress Id2-mediated activities, including cell proliferation and survival (Iavarone *et al*., 1994). Although these studies suggest that Rb acts strictly to modulate Id2 activity, additional studies reveal that Id2 can antagonize Rb-mediated repression of E2F target gene transcription. E2F targets constitute the majority of effectors required for cell cycle progression, and loss of E2F sequestration by Rb is generally believed to be a critical step in oncogenesis (Weinberg, 1995; Lasorella *et al*., 1996).

On the basis of the critical aspects of cellular functions mediated by Id2 and its documented reactivated expression in some human cancers, Id2 has been hypothesized to be a potential therapeutic target (Benezra *et al*., 2001; Lasorella *et al*., 2002). The development of new therapeutics, including those that can easily traverse the plasma membrane and target critical intercellular regulators, such as the Id proteins, may lead to greater benefits in terms of patient outcomes.

To better understand the function of Id2 in colorectal cancer (CRC), we used small hairpin RNA (shRNA) to establish stable cell lines with reduced Id2 levels, and found that these cell lines had increased doubling times (that is, reduced proliferation). Examination of cycle

regulatory proteins demonstrated knockdown of Id2 levels were accompanied by decreased levels of cyclin D1 and increased levels of the cell cycle inhibitory protein p21. Repression of Id2 protein levels also decreased cell survival. Western analysis showed that increased levels of the pro-apoptotic Bcl-2 family member *Bim/Bod* accompanied the knockdown of Id2, in addition to enhanced cleavage of the anti-apoptotic proteins caspase- 7 and poly (ADP-ribose) polymerase. *In vivo* studies in mice further substantiated the critical function of Id2 in CRC progression, as tumors derived from cells with suppressed Id2 levels formed smaller tumors and fewer metastases than those expressing normal levels of Id2. Finally, we established the validity of Id2 as a therapeutic target by showing that intraperitoneal administration of anti-Id2 small interfering RNA (siRNA) incorporated into the neutral liposome 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) in mice significantly decreased CRC tumor burden in the murine liver compared with treatment with a control siRNA–DOPC complex.

Results

Id2 expression in human colon mucosa, primary colon tumors, liver metastases specimens and human CRC cell lines

Given the paucity of published data regarding Id2 expression in human CRC and CRC metastasis, we first evaluated levels of Id2 in paraffin-embedded samples of human primary colon tumors, adjacent normal colonic mucosa and CRC liver metastases by immunoperoxidase staining (Figure 1a). No Id2 was detected in nonmalignant mucosa (0 of 40), but Id2 was expressed in 81 of 194 (42%, $P < 0.0001$) primary adenocarcinomas and in 47 of 56 liver metastases (84%, $P < 0.0001$ vs normal mucosa, $P < 0.0001$ vs primary adenocarcinomas). Id2 protein expression was also detected in all six CRC cell lines assayed (Figure 1b).

Id2 expression and proliferation of CRC cells

Id2 has been reported to regulate the proliferation of human tumor cells (Lasorella *et al*., 2002). To examine the effects of Id2 on proliferation *in vitro*, we reduced Id2 expression in HCT-116 cells by shRNA targeting. Western analysis of lysates from the siRNA-transfected clones sh-Id2C14 and sh-Id2C15 showed reductions in Id2 of more than 85% compared with controls (parental cells and cells transfected with scrambled sequence (shRNA-control (sh-Con), Figure 1b). This targeting was specific for Id2, as levels of Id1 and Id3 did not change (Id4 protein was not detected) (Figure 2a). Proliferation rates, as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were similar in the two controls (doubling time for parental cells, 26.0 ± 1.2 h vs 25.4 ± 1.0 h for sh-Con) but longer for clones with reduced Id2 expression (sh-Id2C14, 36.0 ± 2.7 h; sh-Id2C15, 33 ± 1.3 h) (Figure 2b). Similar results showing an increase in cell doubling time occurring with a reduction of Id2 levels were also obtained with Celltiter-Glo assays (Promega, WI, USA) (data not shown). Next, to see if knockdown of Id2 affected cell cycle regulatory proteins in CRC cells, we used western blotting of cyclin molecules. Only cyclin D1 level (~75% reduction compared to control), and not levels of cyclin D2, D3, A (data not shown) or E (data not shown), was affected by reduction of Id2 expression (Figure 2c). To see if the repression of Id2 levels affected the expression of cell cycle inhibitors in CRC cells, we assessed p15, p16, p21, p27 and p53 by western analysis. Levels of p21 increased twofold (as determined by densitometry) in the Id2-suppressed clones, whereas levels of the other cell cycle regulators were not altered (Figure 2c).

Id2 expression and apoptosis of CRC cells

To determine if the impeded growth of clones with reduced Id2 expression resulted from changes in spontaneous apoptosis, we used fluorescence-activated cell sorting to examine

control and shRNA-targeted cells to assess the percentage of sub-G_0 cells. Knockdown of Id2 expression in CRC cells resulted in four- to sixfold increases in the apoptotic (sub-G_0) populations (mean 12% in sh-Id2C14 cells and 8% in sh-Id2C15 cells) compared with those of the controls (1.5% in parental cells and 2% in sh-Con cells, Figure 3a). Western analysis was utilized to determine if the observed increase in apoptosis resulted from changes in expression of apoptotic regulatory proteins. Examination of the Bcl-2 family members (Bcl-2, Bcl-xl, Bad, Bax and Bim/Bod) showed that only levels of the proapoptotic Bcl-2 family member Bim/Bod increased by twofold in response to reduction of Id2 expression in HCT-116 cells (Figure 3b). Further examination of the apoptotic regulatory proteins caspase-7 and caspase-9 reveled that although the caspase-9 protein remained uncleaved, caspase-7 underwent a threefold increase in cleavage owing to knockdown of Id2 as did the downstream caspase-7 target poly (ADP-ribose) polymerase (Figure 3c).

Knockdown of Id2 expression and tumor growth *in vivo*

To understand the effect of Id2 on the growth of CRC cells *in vivo*, we injected nude mice subcutaneously with sh-Con or shRNA-targeted clones with reduced Id2 levels and assessed the resulting tumors. Tumors from cells deficient in Id2 expression were significantly smaller than those from cells with normal Id2 levels ($451 \pm 97 mm^3$ for sh-Con vs $161 \pm 59 mm^3$ for sh-Id2C14 ($P = 0.03$) and $192 \pm 62 mm^3$ for sh-Id2C15 ($P = 0.017$)) (Figure 4a). To ascertain the effect of Id2 expression on hepatic metastasis of CRC cells, cells of each clone were injected into the spleen of nude mice and the resulting liver metastases were assayed. Cells with reduced Id2 expression produced fewer detectable metastases than did cells with normal Id2 levels (mean 7 ± 2 hepatic metastases for sh-Con vs 2 ± 1 for sh-Id2C14 ($P = 0.022$) or 3 ± 2 for sh-Id2C15 ($P = 0.045$)) (Figure 4b). The mean volumes of the metastases were smaller for the reduced-Id2 clones than for the siRNA control cells (mean $286 \pm 43 mm^3$ for sh-Con vs $55 \pm 15 mm^3$ for sh-Id2C14 ($P = 0.015$) or $57 \pm 24 mm^3$ for sh-Id2C15 ($P = 0.02$) (Figure 4c).

Targeting Id2 *in vivo* and colon cancer progression

On the basis of reduced tumor growth with Id2 silencing, we next asked whether a relevant therapeutic treatment approach could be utilized. We used Id2 siRNA sequences incorporated into the neutral lipo-some DOPC, shown earlier to efficiently reduce *in vivo* gene expression (Landen *et al.*, 2005). Bioluminescence imaging of tumor cell growth in nude mice showed that siRNA to Id2 impaired the growth kinetics of HCT-116 CRC cells compared with those of control cells at 30 days after hepatic inoculation (mean activity 1.8×10^6 photons for control vs 2.8×10^5 photons for Id2 siRNA–DOPC) (Figure 4a). At the termination of the experiment, the hepatic tumor burden in the control group (control Sh-DOPC) was much greater than in the siRNA-Id2 mice (mean $932 \pm 112 mm^3$ for control siRNA–DOPC vs $377 \pm 100 mm^3$ for Id2 siRNA–DOPC ($P = 0.006$, Wilcoxon rank-sum test)) (Figures 5b and c). Cellular proliferation in the excised tumor samples (assessed by immunohistochemical staining for proliferating cell nuclear antigen) was reduced in the Id2-targeted group compared with those in the control group ($P = 0.02$) (Figure 6a). TdT-mediated UTP nickend labeling staining showed that CRC cells growing in the liver of mice treated with siRNA to Id2 had four times more apoptotic cells ($P = 0.002$) than did tumors treated with control siRNA (control Sh-DOPC) (Figure 6b). Finally, Id2 expression in hepatic tumors was shown by immunohistochemistry (data not shown) and western analysis to be substantially reduced in the Id2-siRNA-treated group compared with controls (Figure 6c).

Discussion

Earlier studies have documented that members of the Id family of proteins are expressed in solid tumors, yet their function and therapeutic value, especially in human CRC, remains largely unknown (Kleeff *et al*., 1998; Rockman *et al*., 2001; Wilson *et al*., 2001; Lasorella *et al*., 2002; Lofstedt *et al*., 2004). The experiments described in this study demonstrate that Id2 represents a potential target in cancers where its expression occurs, and is critical for the growth of CRC cells. Id2 was not expressed in normal human colonic tissue. However, Id2 expression was present in greater than 40% of primary colon tumors and in almost all liver metastases studied, suggesting that Id2 may be a contributing factor for CRC metastasis. This observation is of particular clinical interest as the most common site of metastasis is the liver, and hepatic tumor burden is the most common cause of death in patients with metastatic CRC. These results, contrary to *Id2* germline analysis where *Id2* suppressed tumor formation (Russell *et al*., 2004), suggesting that Id2 may have opposing functions in developmental and oncogenic tissues. Similar functions have also been described for other Rb regulatory proteins including E2F (Weinberg, 1996). This dichotomy may be dependent upon the availability of additional co-regulators that direct Id2 function. Id2 was also expressed in all six of the human CRC cell lines assayed, and reduction of Id2 levels in CRC cells by siRNA decreased proliferation rates and increased spontaneous apoptosis. Further, our *in vivo* models showed that knockdown of Id2 expression, through either the creation of stable Id2-deficient clones or the systemic administration of siRNA to Id2, severely impaired the ability of CRC cells to grow in the liver, the major site of CRC metastasis.

Consistent with findings in other oncogenic tissues, reduction of Id2 levels in CRC cells with siRNA decreased proliferation rates (Lasorella *et al*., 1996, 2002). Earlier reports indicated that Id2 interferes with E2 transcription (Pagliuca *et al*., 2000; Rothschild *et al*., 2006) and Rb sequestration of E2F (Iavarone *et al*., 1994; Lasorella *et al*., 2000), both critical cell cycle regulators. Our examination of cell regulatory proteins revealed that reduction of Id2 levels in CRC cells downregulated cyclin D1 levels. These findings are contrary to those of an earlier study in which ectopic overexpression of Id2 in an Rb mutant osteosarcoma cell line decreased cyclin D1 levels (Lasorella *et al*., 1996). In cells with functional Rb, such as HCT-116 cells (Broude *et al*., 2007), cyclin D1 is required for cell cycle progression (Lukas *et al*., 1995). The ability of Id2 expression to modulate cyclin D1 may depend on the presence of functional Rb and its coordination of cell cycle progression. We also showed that knockdown of Id2 increased levels of the cell cycle inhibitor p21 in the absence of changes in p53 levels (Lasorella *et al*., 1996). Expression of p21 can be upregulated by members of the E2 family (Lasorella *et al*., 1996; Prabhu *et al*., 1997), which are antagonized by Id heterodimerization. Thus, decreasing Id2 levels could enhance E2 homodimerization, leading to upregulation of *p21* in CRC cells. To further examine the relationship between Id2 and E2 gene regulation, we assessed levels of the E2 target gene *Axin2* (Hughes and Brady, 2005). Levels of Axin2 remained unchanged in HCT-116 cells regardless of Id2 levels (data not shown). This may suggest that other members of the Id family, whose levels remain unchanged in our Id2-siRNA-targeted cells, can regulate other aspects of E2 gene control.

In addition to its effect on cellular proliferation, we also showed that reduction of endogenous Id2 levels increased spontaneous apoptosis in CRC cells. This increase seems to be independent of p53, which remained unchanged in our Id2-deficient CRC cells (data not shown). Similar findings attributing anti-apoptotic functions to Id2 have been reported for T cells (Cannarile *et al*., 2006) and prostate cancer, in which this effect was also independent of p53 (Asirvatham *et al*., 2007). Examination of apoptotic regulatory proteins showed that reduction of Id2 resulted in increased levels of Bim/Bod, which antagonizes the survival functions promoted by Bcl-2 and its homolog Bcl-xl (Hsu *et al*., 1998; O'Connor *et al*.,

1998). Our data suggests that Id2 represses Bim/Bod expression. The ability of Id2 to downregulate Bim/Bod levels may have a function in promoting colon cancer cell survival (Hsu *et al*., 1998). Additionally, in our study the knockdown of Id2 expression increased cleavage of caspase-7. Although activation of this apoptotic pathway has not previously been linked to Id2 expression, loss of Id1 expression has been shown to increase poly (ADP-ribose) polymerase cleavage (Zhang *et al*., 2007), itself a marker of apoptosis (Oliver *et al*., 1998), suggesting that the Id proteins may function in promoting cell survival.

We also showed the importance of Id2 in CRC growth through the use of stable transfectants with reduced Id2 expression and by targeting Id2 *in vivo* using liposomal-conjugated siRNA. Tumors arising from the stable cell lines lacking Id2 demonstrated reduced growth and tumorgenicity in both subcutaneous and in orthotopic (liver metastases) models. Tumors that did grow at either site were smaller than tumors derived from cells with normal Id2 levels. Similar results were achieved by targeting human CRC cells implanted in the livers of mice *in vivo* with liposome-conjugated siRNA to Id2. Although the siRNA–DOPC complex is not specific for the human cancer cells implanted in the murine liver, we sought to determine if siRNA to Id2 had any effect on the mouse homolog of Id2. A Basic Local Alignment Search Tool (BLAST) homology search was performed and found that the regions targeted by the human siRNA utilized in our studies demonstrated no homology with the concomitant murine sequence; in addition, transient transfection with the human specific siRNAs did not reduce endogenous Id2 expression in a murine hepatic endothelial cell line (Langley *et al*., 2003; data not shown). These findings suggest that Id2 liposomal siRNA designed to inhibit human Id2 *in vivo* had no effect on murine Id2; thus we believe that the observed changes in tumor growth were mediated exclusively by blocking the Id2 expressed by the tumor cells.

In summary, our study here demonstrates that Id2 is an important mediator in CRC growth and metastasis, and a promising therapeutic target in tumors where its expression occurs. Our results indicate that Id2 enhanced cell proliferation, survival and *in vivo* tumor growth, all critical aspects of CRC progression. The use of anti-Id2 therapeutic targeting may improve existing targeted treatments and provides a unique opportunity to circumvent the ability of Id2-expressing tumors, such as CRC, to grow and metastasize.

Materials and methods

Human tissue specimens

Formalin-fixed, paraffin-embedded specimens of colon adenocarcinoma, adjacent non-malignant colonic mucosa and colon cancer liver metastases were obtained from an established tissue bank through a protocol approved by the institutional review board of The University of Texas MD Anderson Cancer Center, Houston, TX, USA. Histopathologic confirmation was provided by the MD Anderson Cancer Center's Department of Pathology.

Histopathologic preparation and immunohistochemical staining of tissue specimens

Patient tissue specimens were analyzed with an anti-Id2 antibody (C-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) as described earlier (Parikh *et al*., 2004). Tumor xenografts from mice (generated as described below) were fixed in 10% neutral formalin (for paraffin embedding) or optimum cutting temperature solution (Miles, Elkhart, IN, USA). Immunostaining was done using standard procedures as described elsewhere (Camp *et al*., 2006; Yang *et al*., 2006). Staining for the endothelial cell-specific marker CD31 was performed with anti-mouse CD31 (PECAM-1) antibody (BD Biosciences, Franklin Lakes, NJ, USA) and goat anti-mouse Alexa-594 conjugated antibody (Molecular Probes, Eugene, OR, USA). Apoptotic cells were detected with a TdT-mediated UTP nick-end labeling Dead-End labeling kit (Promega, Madison, WI, USA) according to the manufacturer's

protocol. Image analysis of the immunohistochemical stains was done with NIH Image J 1.34 software. All analyses represent an average of a minimum of five randomly selected fields per specimen.

Cell lines and culture conditions

The human CRC cell lines Geo, HCT-116, HT-29, RKO and SW-480 were obtained from the American Type Culture Collection (Manassas, VA, USA); KM12 cells were obtained from IJ Fidler (MD Anderson Cancer Center). All cells were maintained in complete minimum essential medium supplemented with 10% fetal bovine serum, 2mm l-glutamine, penicillin and streptomycin (Life Technologies, Grand Island, NY, USA).

Preparation of shRNA expression plasmids and siRNA cell lines

Small interfering RNA plasmids were created with pSilencer 4.0. (Ambion, Austin, TX, USA). The target sequences for Id2, 5′-gcggtgttcatgatttctt-3′ and 5′-caaagcactgtgtgtgggctga-3′ (Invitrogen/Life Technologies), were ligated into the expression plasmid. HCT-116 cells were transfected with 0.5 ng of each siRNA plasmid and grown in selective medium containing 700 μg/ml hygromycin B (Roche Diagnostics, Mannheim, Germany). Negative controls were scrambled Id2 target sequences with no significant homology to other vertebrate genes.

Immunoprecipitation and western analysis

Cells were solubilized in 20mm Tris-Cl (pH 8.0), 137mm NaCl, 1% Triton X-100, 1mm Na_3VO_4 and 2mm ethylene-diaminetetraacetic acid with protease inhibitor (Roche Diagnostics). Cell lysates were separated on 8% sodium dodecyl sulfate polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Amersham, Arlington Heights, IL, USA). Membranes were probed with antibodies against Id2, Id1, Id3 (Santa Cruz Biotechnology), vinculin (Serotec, Raleigh, NC, USA), cyclin D1, cyclin D2, cyclin D3, cyclin A, cyclin E, p21, p27, p16, p53, Bcl-2, Bcl-xl, Bad, Bax, Bim/Bod, poly (ADP-ribose) polymerase, caspase-9 and caspase-7 (all from Cell Signaling Technology, Danvers, MA, USA). After incubation with the appropriate antibodies, immunostained proteins were detected by chemiluminescence (New England Nuclear, Boston, MA, USA). For western analysis of *in vivo* tumor sections, tumors were emulsified in protein lysis buffer as described above.

MTT analysis of cell proliferation

In vitro proliferation was assessed with tetrazolium salt MTT as described earlier (Gray *et al.*, 2005). Briefly, 2000 cells of each clone were plated per well onto 96-well microtiter plates in minimum essential medium with 10% fetal bovine serum. One plate was developed immediately after cells had adhered (at approximately 4 h), and other plates were developed every 24 h for 4 days. Assays were done by incubating each plate with 20 μl of MTT substrate for 2 h followed by removal of medium and addition of 200 μl of dimethylsulfoxide. Plates were read at a wavelength of 570 nm.

Flow cytometry and cell cycle analysis

Each clone was grown to 85–90% confluence in Eagle's minimum essential medium supplemented with 2mm l-glutamine, 12.5% fetal bovine serum, 100 U/ml penicillin and 100 ng/ml streptomycin). Cells were trypsinized, washed in phosphate-buffered saline and fixed in 70% ethanol at 4 °C for 2 h. DNA was stained with propidium iodide (10 mg/ml of phosphate-buffered saline) and DNase-free RNase (Roche Diagnostics) (2.5 μg/ml of phosphate-buffered saline) for at least 30 min before cells were subjected to flow cytometry

in a Coulter EPICS XL (Beckman Coulter, Fullerton, CA, USA). Cell cycle profiles were generated by MultiCycle software (Phoenix Flow Systems, San Diego, CA, USA).

In vivo tumor growth and siRNA targeting

Six- to eight-week-old male athymic nude mice were purchased from the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD, USA) and maintained under specific pathogen-free conditions. All animal experiments met the requirements of MD Anderson's Animal Care Facility and the National Institutes of Health guidelines on animal care and use. Each HCT-116 clone was prepared and inoculated into 10 mice as described elsewhere (Gray *et al*., 2005). For studies involving stable cell lines, 5.0×10^5 viable sh-Con, sh-Id2C14 or sh-Id2C15 cells were injected in 0.1 ml of Hanks balanced salt solution either subcutaneously into the right rear flank, liver or spleen (for metastasis studies) of each nude mouse. For the *in vivo* siRNA delivery experiments, 1.0×10^6 HCT-116 cells expressing the lenti-Luc reporter gene (Arumugam *et al*., 2006) were suspended in 0.1 ml of Hanks balanced salt solution and inoculated into the liver of each mouse. After 4 days, controlor Id2-liposomal–siRNA (3 μg) complexes were given as a 200 μl intraperitoneal bolus with identical treatment given every 3 days thereafter. After 32 days, mice were sacrificed (CO_2 inhalation) and total body weight, liver weight and tumor volume were calculated. Tumor volume was calculated as ((length/2) $\times$ (width^2)) $\pm$ s.e.m. For metastasis studies, tumor volume was determined as the sum of the individual metastatic foci in each liver. Tissue specimens from each treatment group were snap-frozen, fixed in formalin or frozen in optimum cutting temperature solution.

Bioluminescence imaging

Bioluminescence imaging was done as described earlier (Arumugam *et al*., 2006) by using a cryogenically cooled IVIS 100 imaging system coupled to a data-acquisition personal computer equipped with Living Image software (Xenogen, Hopkinton, MA, USA). For *in vivo* studies, before each imaging session, mice were anesthetized with isoflurane and injected intraperitoneally with 15 mg/ml of luciferase potassium salt (Sigma-Aldrich, St Louis, MO, USA) in phosphate-buffered saline at a dose of 150 mg/kg body weight. Initial images were obtained to establish baseline tumor volume/proton activity at 3 days after the hepatic inoculation of tumor cells (to allow tumor establishment), and additional images were obtained at 14 and 28 days after inoculation.

Preparation of liposomal siRNA

All Id2 and Id2 control sequences used for the *in vivo* experiments were purchased from Ambion (Landen *et al*., 2005). Briefly, DOPC and siRNA (a control Id2-scrambled sequence or an Id2-specific sequence as described above) were mixed and lyophilized. The siRNA–liposome complexes were purified by separating the free siRNA from the liposomes by using 30 000 nominal molecular weight limit filter units (Millipore, Billerica, MA, USA). The lyophilized preparations were hydrated in 0.9% saline at a concentration of 5 μg/200 μl before being administered *in vivo*.

Quantitation of autoradiograms

Autoradiographs were quantified in the linear range of the film by scanning the image using a Hewlett Packard Scanjet scanner and quantitated with Scion Image software program. Each sample measured was calculated as the ratio of the average area over the average area of actin or vinculin for immunoblots or as the ratio above the average area of basal/control levels of the protein of interest in immunoprecipitations.

Statistical analyses

Statistical analyses were performed with InStat 2.01. software (GraphPad Software, San Diego, CA, USA), with Student's t-tests or Fisher's exact tests as appropriate. Significance ($P < 0.05$) was determined with 95% confidence intervals.

Acknowledgments

Abbreviations

CRC	colorectal carcinoma
Id2	inhibitor of DNA-bind-2
Rb	Retinoblastoma protein
shRNA	short hairpin RNA
siRNA	small interfering RNA

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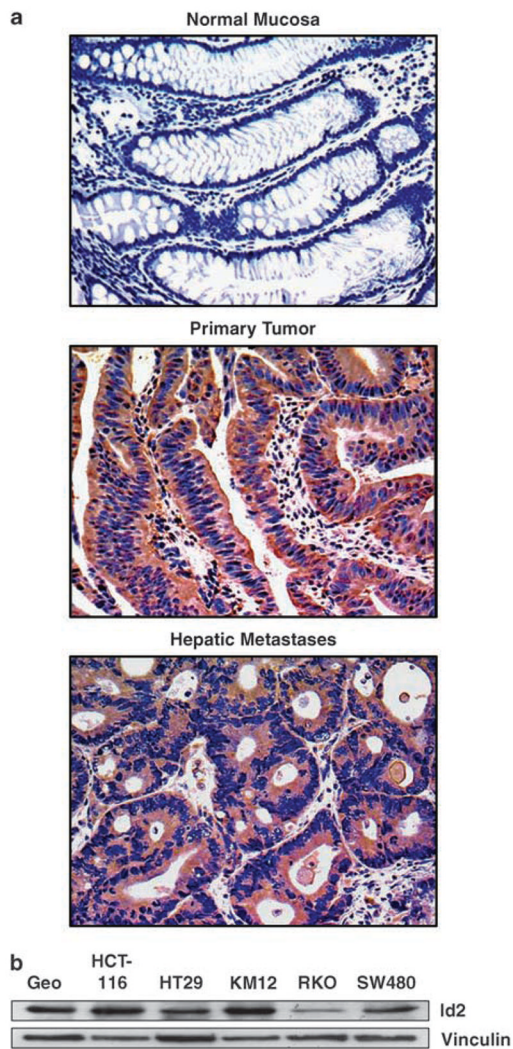
Figure 1.
Assessment of Id2 expression in human colon tissues and cell lines. (**a**) Representative tissue sections of nonmalignant human colonic mucosa, primary colon adenocarcinoma, colon adenocarcinoma and liver metastasis were subjected to immunoperoxidase staining to detect expression of Id2 protein. (**b**) Western blot analysis of Id2 expression in six colon adenocarcinoma cell lines. Vinculin was used as an internal loading control. Id2, inhibitor of DNA-bind-2.
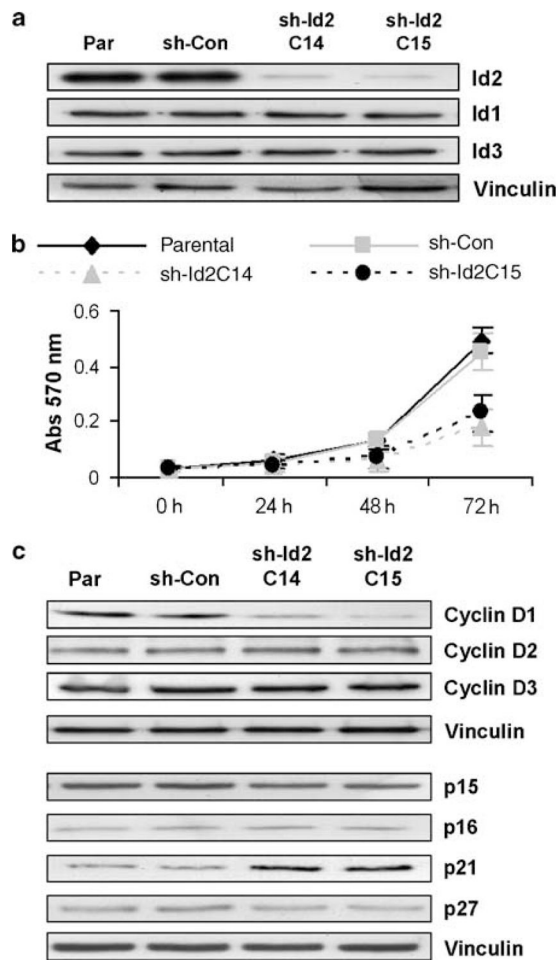
Figure 2.
Effect of Id2 expression on proliferation and cell cycle protein expression in HCT-116 cells. (**a**) Western blot analysis of Id2 levels in parental (Par) and shRNA-control (sh-Con) cells and two shRNA clones (sh-Id2C14 and sh-Id2C15). (**b**) MTT analysis of growth rates of control cells (Par and sh-Con) or clones with reduced Id2 expression (sh-Id2C14 and sh-Id2C15). Bars indicate standard error of the mean. (**c**) Western blot analysis of cell cycle regulatory proteins from control cells (Par and sh-Con) and stable cell lines with reduced Id2 expression (sh-Id2C14 and sh-Id2C15). Vinculin was used as an internal loading control. Id2, inhibitor of DNA-bind-2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
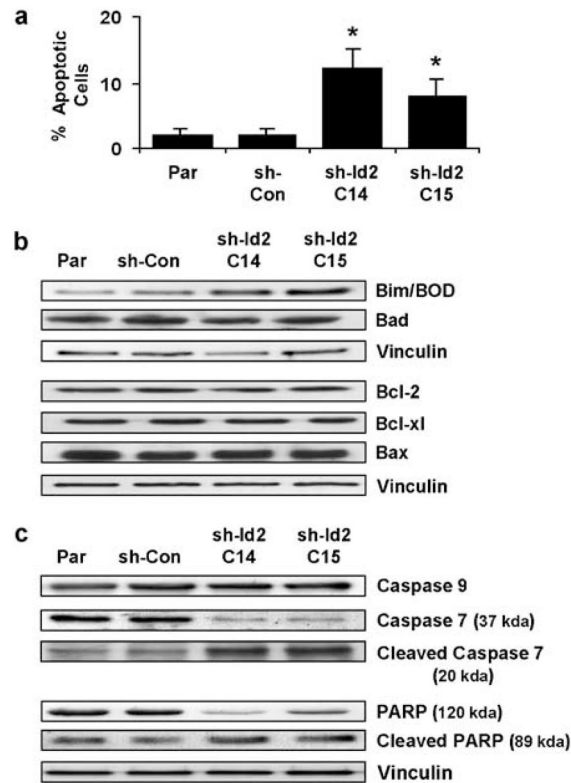
Figure 3.
Effect of Id2 expression on apoptosis in HCT-116 cells. (**a**) Graphical representation of proportions of apoptotic control cells (Parental (Par) and scrambled-siRNA control (sh-Con)) and stable cell lines with reduced Id2 expression (sh-Id2C14 and sh-Id2C15) as determined by fluorescence-activated cell sorting analysis. (**b**) Western analysis of Bcl-2 family members Bim/Bod, Bcl-2, Bcl-xl, Bad and Bax from control cells (Par and sh-Con) and stable cell lines with reduced Id2 expression (sh-Id2C14 and sh-Id2C15). Vinculin levels were probed to ensure equal loading of all samples. (**c**) Western analysis of caspase-7/caspase-9 and PARP from control cells (Par and sh-Con) and stable cell lines with reduced Id2 expression (sh-Id2C14 and sh-Id2C15). Vinculin levels were probed to ensure equal loading of all samples. Id2, inhibitor of DNA-bind-2; PARP, poly (ADP-ribose) polymerase.
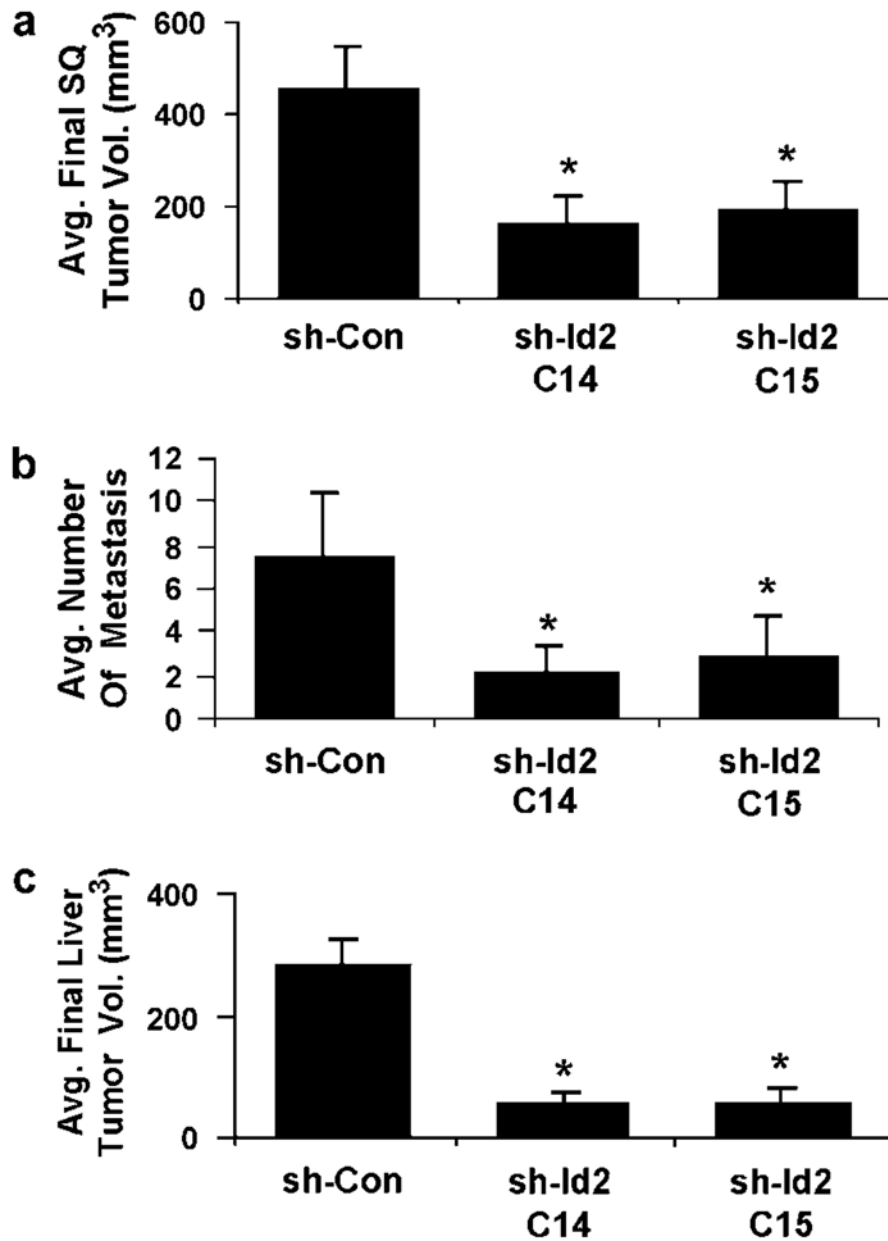
Figure 4.
Effect of Id2 expression on the growth and metastasis of HCT-116 colon cancer cells *in vivo*. (**a**) Mean tumor volumes after subcutaneous injection of control (shRNA-control (sh-Con)) cells or clones with reduced Id2 expression. Bars indicate standard error of the mean for 10 mice. Volumes of both siRNA clones were different from the sh-Con control ($P = 0.03$ for sh-IdC14 and $P = 0.017$ for sh-Id2C15). (**b**) Average numbers of liver metastases after splenic injection of control (sh-Con) cells or clones with reduced Id2 expression. Bars indicate standard error of the mean for 10 mice. Both clones produced fewer metastases than did control cells ($P = 0.022$ for sh-Id2C14 and $P = 0.045$ for sh- Id2C15). (**c**) Mean volumes of metastases from control cells (sh-Con) and clones with reduced Id2 expression. Total volume is the sum of individual metastases in each liver. Data are means ± s.e.m. from 10

mice. Both clones produced lower volume metastases than did control cells ($P \leq 0.02$). Id2, inhibitor of DNA-bind-2; siRNA, small interfering RNA.
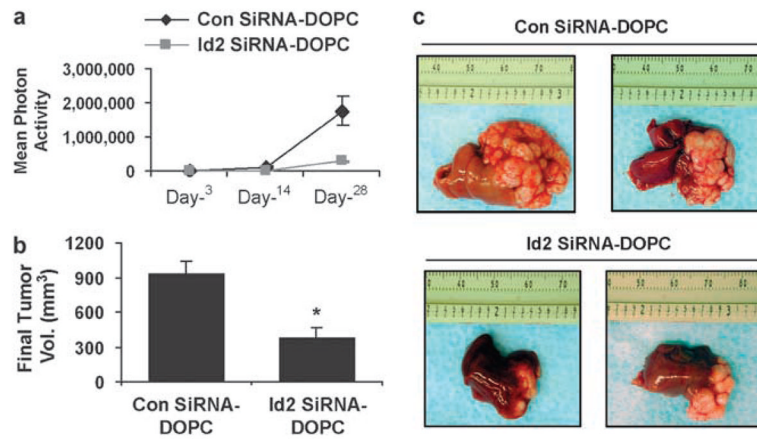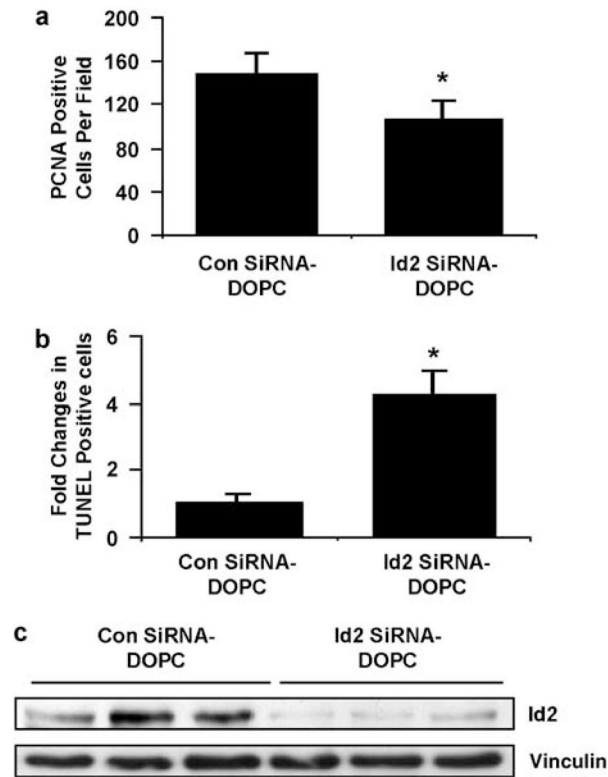
Figure 5.
Effect of *in vivo* administration of liposomal-conjugated siRNA to Id2 on CRC tumor growth in the liver. (**a**) Graphical plot of proton activity from bioluminescence imaging of mice inoculated with 1.0×10^6 HCT-116 cells and imaged at 3, 14 and 28 days after hepatic injection while being treated at 3-day intervals with either control (control siRNA–DOPC) or Id2-specific siRNA (Id2 siRNA– DOPC). (**b**) Mean tumor volumes at 32 days after inoculation, with continuous intraperitoneal injections every 3 days of control (control siRNA–DOPC) or Id2–specific siRNA (Id2 siRNA–DOPC) (10 mice per group). *P = 0.0045 vs control siRNA. (**c**) Photographs of excised livers from mice treated with either nonspecific or Id2-targeted siRNA. Id2, inhibitor of DNA-bind-2; siRNA, small interfering RNA; CRC, colorectal cancer; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine.

Figure 6.
Effect of *in vivo* administration of liposomal-conjugated siRNA to Id2 on CRC tumor growth in the liver. (**a**) Graphical plot of immunohistochemistry (IHC) staining for proliferating cell nuclear antigen (PCNA) in tumor sections from samples in Figure 5c. Treatment groups; control liposome (control siRNA–DOPC) and Id2-targeted liposome (Id2 siRNA–DOPC). *P = 0.02 between treatment groups. (**b**) Graphical representation of 'fold' change in TUNEL-positive cells (apoptotic cells) in tumor sections from control-siRNA- and Id2-siRNA-treated mice observed in five fields at ×20. Average *P = 0.002 between treatment groups. (**c**) Western analysis of tumor homogenates for Id2 expression in liposomal treatment groups from Figure 5c; control liposome (control siRNA–DOPC) and Id2-targeted liposome (Id2 siRNA–DOPC). Id2, inhibitor of DNA-bind-2; siRNA, small interfering RNA; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine; TUNEL, TdT-mediated UTP nick-end labeling.