Cancer Cell
Article

ID1 and ID3 Regulate the Self-Renewal Capacity of Human Colon Cancer-Initiating Cells through p21

Catherine A. O'Brien,[1,2,3,7,*] Antonija Kreso,[1,4,7] Paul Ryan,[5] Karin G. Hermans,[1] Lianne Gibson,[1] Yadong Wang,[1] Andrew Tsatsanis,[1] Steven Gallinger,[2,6] and John E. Dick[1,4,*]
[1]Campbell Family Institute, Ontario Cancer Institute, Princess Margaret Hospital
[2]Department of Surgery
University Health Network, Toronto, Ontario M5G 1L7, Canada
[3]Department of Laboratory Medicine and Pathobiology and Department of Surgery, University of Toronto, Toronto, Ontario M5L 1F4, Canada
[4]Department of Molecular Genetics, University of Toronto, Toronto, Ontario M5G 1L7, Canada
[5]Department of Pathology, Bon Secours Hospital, Cork, Ireland
[6]Fred Litwin Centre for Cancer Genetics Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario M7H 2B9, Canada
[7]These authors contributed equally to this work
*Correspondence: cobrien@uhnres.utoronto.ca (C.A.O.), jdick@uhnres.utoronto.ca (J.E.D.)
DOI 10.1016/j.ccr.2012.04.036

SUMMARY

There is increasing evidence that some cancers are hierarchically organized, sustained by a relatively rare population of cancer-initiating cells (C-ICs). Although the capacity to initiate tumors upon serial transplantation is a hallmark of all C-ICs, little is known about the genes that control this process. Here, we establish that ID1 and ID3 function together to govern colon cancer-initiating cell (CC-IC) self-renewal through cell-cycle restriction driven by the cell-cycle inhibitor p21. Regulation of p21 by ID1 and ID3 is a central mechanism preventing the accumulation of excess DNA damage and subsequent functional exhaustion of CC-ICs. Additionally, silencing of ID1 and ID3 increases sensitivity of CC-ICs to the chemotherapeutic agent oxaliplatin, linking tumor initiation function with chemotherapy resistance.

INTRODUCTION

There is increasing experimental evidence from cell fractionation experiments that many, but perhaps not all, tumors are organized as a cellular hierarchy sustained by a so-called cancer-initiating cell (C-IC) or cancer stem cell (CSC) (Al-Hajj and Clarke, 2004; Dick, 2008; O'Brien et al., 2009). Several attributes distinguish C-ICs from the remaining cells of a tumor, including ability to initiate cancer growth in xenotransplantation assays, restoration of the tumor hierarchy by generating non-C-ICs, and capacity for long-term self-renewal (Dick, 2003). It is becoming evident that the acquisition of dysregulated self-renewal mechanisms represents a key step in the generation of C-ICs (Morrison and Kimble, 2006; He et al., 2009). The strongest evidence for C-ICs has come from clonal serial xenotransplantation assays and lentiviral-tracking studies carried out in leukemia by Bonnet and Dick (1997) and Hope et al. (2004). Experimental data are now accumulating that several solid tumors, including breast (Al-Hajj et al., 2003), brain (Singh et al., 2004), and colon (O'Brien et al., 2007; Ricci-Vitiani et al., 2007), adhere to the hierarchical model and contain C-ICs. The focus of our study is on colon C-ICs (CC-ICs), which typically represent a small subset of the total colon cancer cell population and can be prospectively isolated based on the expression of specific cell surface (i.e., CD133 [O'Brien et al., 2007; Ricci-Vitiani et al., 2007], CD44 [Dalerba et al., 2007], CD166 [Dalerba et al., 2007]) or functional (aldehyde dehydrogenase-1; Dylla et al., 2008; Huang et al., 2009) markers. Evidence is also emerging that the C-ICs from several cancers possess properties that make them resistant to chemotherapy and radiation, including expression of drug efflux pumps, altered DNA damage response, or cellular quiescence (Bao et al., 2006; Todaro et al., 2008; Hermann et al., 2007; Schatton et al., 2008; Viale et al., 2009). Collectively, little is known of the molecular regulation of solid tumor C-ICs and whether intrinsic stem cell properties are directly linked to survival mechanisms and therapeutic resistance.

Significance

There is emerging evidence that the capacity for self-renewal is dysregulated in cancer-initiating cells (C-ICs) and that C-ICs also possess properties that make them resistant to chemotherapy and radiation. We found that ID1, ID3, and p21 function together to govern self-renewal of colon cancer-initiating cells (CC-ICs) through cell-cycle restriction and protection from DNA damage accumulation. These components of the tumor-initiating machinery of human CC-ICs are also linked to chemotherapy resistance at the stem cell level, opening the way for stem cell-based therapeutic strategies to manipulate self-renewal as a means to potentiate chemotherapeutic drug combinations.

As a first step to uncover specific factors that govern the maintenance of CC-ICs, we focused on genes that have been implicated in self-renewal properties of somatic or embryonic stem cells, narrowed the list to those that were dysregulated in cancer, and finally to those implicated in human colon cancer specifically. One family of proteins that satisfies these criteria are the inhibitor of DNA binding proteins (IDs), a family of homologous helix-loop-helix (HLH) transcriptional regulatory factors (Gray et al., 2008) (ID1–ID4) with recognized roles in development, senescence, differentiation, angiogenesis, and migration (Fong et al., 2004). The ability of ID proteins to drive self-renewal is well established in embryonic stem cells, where the upregulation of IDs by bone morphogenic protein 4 (BMP4) is required to maintain self-renewal and pluripotency (Hollnagel et al., 1999; Ruzinova and Benezra, 2003). Studies in a murine model of hematopoiesis revealed that *Id1*$^{-/-}$ whole-bone marrow displayed impaired self-renewal capacity relative to wild-type controls (Perry et al., 2007). Similar results were observed in murine cortical neural stem cells where overexpression of Id1 increased self-renewal capacity (Nam and Benezra, 2009). Evidence suggesting that IDs play a role in cancer comes from studies demonstrating increased expression in a variety of solid tumors, including pancreatic (Kleeff et al., 1998), cervical (Schindl et al., 2001), ovarian (Schindl et al., 2003) prostate (Ouyang et al., 2002), breast (Lin et al., 2000; Fong et al., 2003), and colon (Meteoglu et al., 2008; Gray et al., 2008). ID upregulation correlates with both poor prognosis and chemoresistance (Cheung et al., 2004; Hu et al., 2009; Li et al., 2007). Furthermore, studies from Gupta et al. using murine models of breast cancer demonstrated a role for Id1/Id3 in the initiation of metastases, a process that may be closely related to the concept of C-ICs (Gupta et al., 2007). Considering the overall importance of IDs in cancer biology and their role in embryonic and somatic stem cell self-renewal, they represent prime candidates to evaluate in CC-ICs.

In some systems, ID1 functions to maintain self-renewal through repressive effects on expression of the cell-cycle inhibitor, p21/cip1/waf1 (p21) (Ciarrocchi et al., 2007; Jankovic et al., 2007). However, p21 has also been linked to maintenance of self-renewal capacity in leukemic and normal hematopoietic stem cells, indicating that cellular context can impact on functional properties of these regulators (Cheng et al., 2000; Viale et al., 2009). Although, to our knowledge, there are no reports on a role for p21 in CC-IC self-renewal, p21 has a well-established role in protecting colon cancer cells against a variety of stress stimuli, including exposure to radiation and chemotherapy (Mahyar-Roemer and Roemer, 2001; Bene and Chambers, 2009; Gorospe et al., 1996; Sharma et al., 2005; Tian et al., 2000). These studies point to the plausibility that therapy resistance may be linked to tumor initiation and maintenance mechanisms and that pathways driving self-renewal may also function to protect CC-ICs when exposed to environmental stress.

Mechanistic studies on properties governing tumor initiation require large numbers of CC-ICs to carry out functional genomic experiments aimed at identifying the key players in CC-IC function. However, this has proved difficult because CC-ICs are typically rare in primary human cancers, and culture systems that permit genetic studies as well as the production of large numbers of CC-ICs are not well established. Here, we developed a robust culture system that enabled the expansion and genetic manipulation of CC-ICs. Utilizing this system, we interrogated the role of ID1 and ID3 in driving CC-IC self-renewal capacity, as well as their role in determining response to oxaliplatin, a commonly used chemotherapeutic agent in colon cancer (Alberts and Wagman, 2008).

RESULTS

Enrichment of CC-ICs in Primary Human Colon Cancer Cultures

In colon cancer the C-IC fraction is typically small, rendering these cells difficult to identify and making molecular studies aimed at manipulating tumor initiation programs very challenging (O'Brien et al., 2007). We characterized a commercially available cell line, LS174T, and determined that xenografts from this cell line can be initiated with a high frequency (Figure 1A; see Table S2 available online). Furthermore, xenografts derived from LS174T injections were hierarchically organized based on CD44 expression (Figure 1D; Table S3). To determine if the culture conditions could be applied to primary samples, three colon cancers were obtained at the time of surgical resection and established as sphere cultures in serum-free media (Kreso and O'Brien, 2008). There was approximately a 200-fold increase in CC-IC activity in the sphere-cultured cells as compared to in vivo-limiting dilution assay (LDA) results using xenograft cells derived from the same tumor (Figure 1A; Tables S1 and S2).

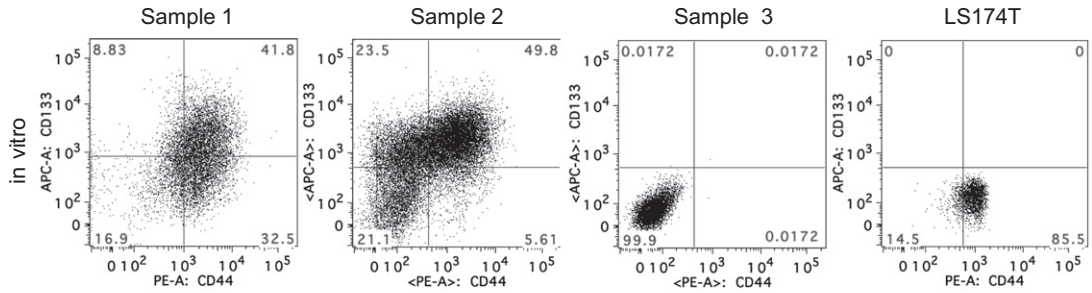
To establish that the primary cultures retained their ability to reestablish a cellular hierarchy, they were transplanted into mice, and expression of known CC-IC surface markers, CD133 and CD44, was assessed. Sample 3 did not express either marker but contained CC-ICs based on in vivo serial passage LDA (Figures 1A–1C). For sample 2, CD44 and CD133 expression during in vitro culture closely resembled the expression from xenograft-derived cells (Figures 1B and 1C). Sample 1 displayed yet a different pattern where CD133 was expressed on a subset of cells in vitro, whereas CD44 was expressed on most cells. However, when sample 1 cells were obtained from xenografts, no CD44 expression was identified, and the CD133 subset remained relatively stable (Figures 1B and 1C). Similar results were observed with LS174T where in vitro culture resulted in expression of CD44 on the majority of cells (Figure 1B), whereas only approximately 50% of xenograft-derived cells expressed CD44 (Figure 1C). To ensure that there was no contamination, each colon cancer sample was checked for nonmalignant cells, including endothelial, hematopoietic, and murine cells (Figure S1).

To determine whether the cell surface phenotype correlated with CC-IC function, CD133^{+} and CD44^{+} subsets were tested using both in vitro and in vivo LDAs and found to be enriched for CC-IC activity (Figure 1D; Table S3), with two exceptions. CD44 expression in LS174T and sample 1 did not enrich from in vitro culture, indicating that CD44 is an unreliable marker of CC-IC activity for these samples under in vitro conditions (Figures 1B and 1D). In contrast, serial transplantation of CD44^{+} and CD44^{-} LS174T xenograft-derived cells demonstrated that CD44^{+} cells possessed increased CC-IC capacity as compared to the CD44^{-} fraction (Figure 1D; Table S3).

A

Sample	CC-IC frequency of xenograft cells in vivo	CC-IC frequency of sphere cells in vivo	SFU frequency in vitro	CFU frequncy in vitro
1	1 in 11498 (18301-7224)	1 in 54 (93-31)	1 in 20 (26-16)	1 in 1.6 (2.1-1.3)
2	1 in 32162 (46614-22191)	1 in 118 (218-64)	1 in 22 (28-17)	1 in 2.4 (2.8-1.9)
3	1 in 57636 (83861-39612)	1 in 580 (1067-317)	1 in 39 (49-31)	1 in 2.7 (3.3-2.1)
LS174T		1 in 111 (156-76)	1 in 40 (72-26)	1 in 2 (2.6-1.6)

B



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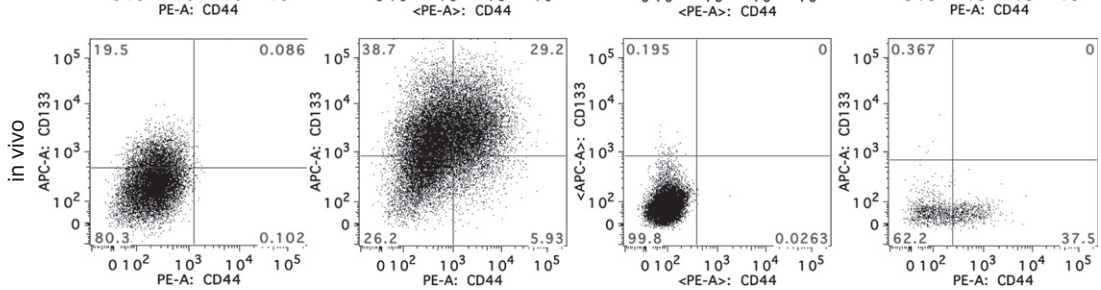


D

Colon cancer sample	Cell subset injected	Sphere LDA			Xenograft LDA		
		CC-IC frequency 1 in x			CC-IC frequency 1 in x		
		Lower	Estimate	Upper	Lower	Estimate	Upper
Sample 2	CD133+	97	62	39	247	121	60
	CD133-	22,439	13,295	7,878	119,974	38,826	12,565
Sample 2	CD44+	173	86	43	674	355	187
	CD44-	7,448	3959	2,105	128,409	40,764	12,941
LS174T	CD44+				358	190	100
	CD44-				14,866	8,748	5,148

Figure 1. In Vitro Expansion and Xenograft Generation of Human Colon Cancer Cells

(A) Comparison of in vivo xenograft formation with in vitro sphere and colony formation at limiting dilution is shown. All data are represented as the frequency of CC-IC, SFU, or CFU; 95% CI is shown in parentheses.

(B) Representative flow cytometric profiles of CD44 and CD133 expression in vitro (n = 5).

(C) Flow cytometric profiles of xenograft-derived cells (n = 5).

(D) Fractionation of CC-IC activity based on CC-IC marker expression. All data are represented as the CC-IC (95% CI). CC-IC frequency 1 in x (x, number of colon cancer cells).

See also Figure S1 and Tables S1, S2, S3, S4, and S5.

Collectively, these findings demonstrate the importance of functionally validating each sample in the context of the system being studied because a change in phenotypic expression does not necessarily correlate to function. Thus, by robust functional criteria we can conclude that the culture conditions we developed resulted in highly enriched CC-ICs that retain their capacity to generate a tumor hierarchy. Recently, a more immune-deficient mouse strain has become available, NOD/SCID γ (NSG), that results in a dramatic enhancement in the detection of C-ICs in some tumors, such as melanoma (Quintana et al., 2008). We observed similar CC-IC frequencies in NOD/SCID versus NSG recipients (Table S4), suggesting that the wide variation observed in melanoma does not necessarily apply to all types of cancer, including the colon cancer samples employed in this study.

Comparison of the Frequency of Cells Capable of In Vitro Sphere and Colony Formation with CC-IC Activity

The gold standard test for enumerating the frequency of C-ICs is in vivo serial transplantation of single C-ICs. However, recently, many groups have commenced utilizing sphere (serum-free media) or colony-forming (10% serum media) assays as attractive surrogates (Todaro et al., 2008; Korkaya et al., 2009). To determine whether these in vitro assays constitute valid surrogates for the measurement of C-IC capacity, each culture was subjected to a detailed quantitative analysis to determine the frequency of colony-forming cells, sphere-forming cells, and in vivo C-ICs. The frequency of sphere and colony-forming cells was 6- and 88-fold greater than in vivo CC-IC frequencies, respectively (Figure 1A). Therefore, it was evident that the number of colon cancer cells capable of forming colonies in vitro was significantly greater than those capable of forming spheres. To determine if colony-forming cells and sphere-forming cells still possessed CC-IC activity, cells from each assay were injected into NOD/SCID mice using LDA. Interestingly, the exact opposite was seen when these cells were injected in vivo: the tumor-initiating capacity was significantly less in the cells cultured in the colony-forming media versus the same cells cultured under sphere conditions. For example when injected in vivo, the LS174T CC-IC frequencies were 1 in 111 in sphere media (Figure 1A) versus 1 in 15,166 in the colony-forming media containing serum (Table S5). This experiment was repeated with a tumor sample taken at the time of surgical resection, and similar results were obtained (Table S5). These results indicate that the sphere assay more closely reflects the in vivo CC-IC frequencies.

Knockdown of ID1/ID3 Expression Reduces Tumor Growth In Vivo

Protein expression of ID1 and ID3 was determined for each of the samples (Figure S2A). To investigate a possible role for ID1 and ID3 in maintaining the CC-IC fraction, the level of expression in CC-IC-enriched and non-enriched fractions was determined by qPCR. There was a trend for *ID1* mRNA expression levels to be higher in the $CD133^+$ and $CD44^+$ fractions, as compared to the negative counterparts (Figure 2A). *ID3* expression levels were similar in both fractions (Figure 2B). To determine the functional significance of ID1 and ID3 expression, we utilized retrovirus-mediated silencing in CC-IC cultures using control (PRS), ID1 knockdown (ID1KD), ID3KD, or combined ID1 and ID3 KD (ID1/ID3KD). To account for potential off-target effects, two independently designed hairpins for ID1 and ID3 were tested (Figure S2B). The two sets of hairpins yielded very similar in vitro results, and the set of hairpins we utilized was previously validated by Gupta et al. (2007). Western blot analysis demonstrated >50% reduction of ID1 and ID3 protein in ID1/ID3KD cells as compared to control cells, indicating efficient KD. However, both ID1 and ID3 are not completely eliminated at the protein level; nevertheless, we will continue to use the nomenclature of ID1/ID3KD to denote this partial KD situation (Figure 2C). Following transduction and selection, colon cancer cells were injected subcutaneously (s.c.) into NOD/SCID mice. The KD of ID1 or ID3 protein expression individually resulted in partial inhibition of tumor growth. However, ID1/ID3KD resulted in a profound decrease in the ability of these cells to form colon cancer xenografts in the LS174T cells (Figures 2D and 2H). There was a complete loss of tumor formation in the ID1/ID3KD group for samples 1, 2, and 3 (Figures 2E–2G).

Because the role of ID1 in vasculogenesis is well established by Lyden et al. (1999), we investigated whether the drastic difference in tumor size was due to poor vascularization at the site of injection. Control and ID1/ID3KD-transduced LS174T cells were injected into the spleen, a well-vascularized site. A significant reduction in tumor growth and metastatic burden was observed (Figures S2E–2H), suggesting that the effect of ID1/ID3KD was not solely due to effects on vasculogenesis.

ID1/ID3KD Affects Proliferation and Xenograft Microvessel Formation

To elucidate the mechanism of action of ID1/ID3KD, the xenografts generated from each experimental group were examined for microvessel density (MVD), apoptosis, necrosis, and proliferation. MVD was significantly decreased by 50% in the ID1/ID3KD group as compared to control (Figure 3A). This result was anticipated because previous studies have recognized the importance of ID1 expression in vasculogenesis both in endothelial and cancer cells (Lyden et al., 1999; Ling et al., 2005; Swarbrick et al., 2008). There was no significant difference between the experimental and control groups with respect to apoptosis, proliferative index, or percent necrosis (Figures 3B–3E). In contrast to the xenograft results, the in vitro proliferative capacity was 2-fold lower in all three KD groups as compared to control (Figure 3F). Another measure of proliferative capacity is sphere diameter, which showed no significant difference among the three KD groups (Figure S3A). The modest decrease in proliferation observed in vitro could partially explain the significantly smaller xenografts in the ID1KD and ID3KD groups as compared to controls but could not explain the profound decrease in xenograft growth observed in the ID1/ID3KD group. Another mechanism known to be affected by ID proteins is senescence (Swarbrick et al., 2008); however, no differences in the β-galactosidase senescence marker were seen between KD and control groups (data not shown).

The IDs have a well-established role in the inhibition of differentiation in a variety of cell types (Fong et al., 2004). To determine whether ID1/ID3KD was inducing differentiation, the LS174T control and KD xenografts were stained for known colon cancer

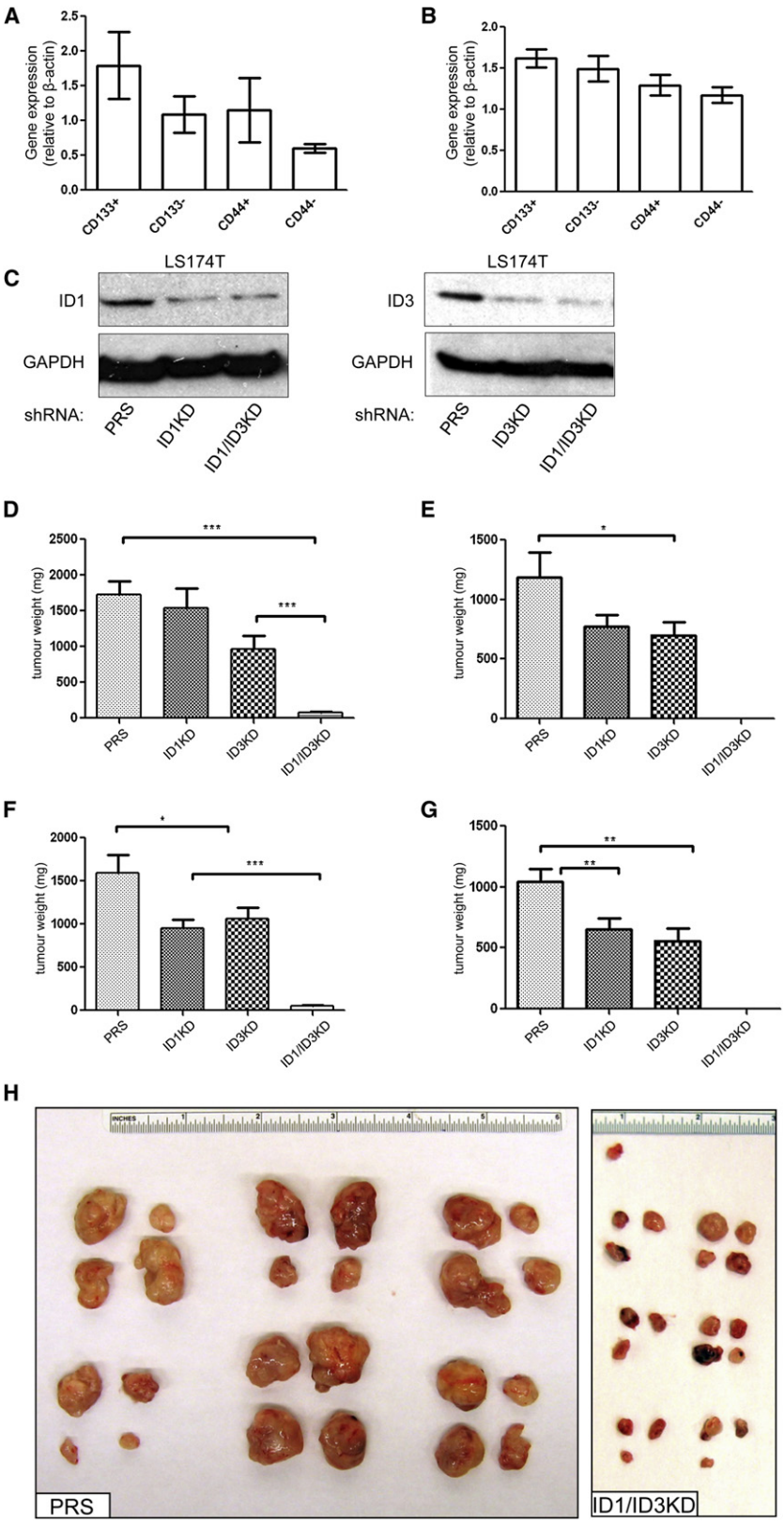


Figure 2. ID1/ID3KD Reduces Tumor Growth In Vivo

(A and B) qPCR for *ID1* (A) and *ID3* (B) expression in the CD133 and CD44 subsets is presented.

(C) Western blot of ID1 and ID3 is illustrated.

(D) Mean tumor weights for LS174T following s.c. injection of PRS, ID1KD, ID3KD, or ID1/ID3KD cells (n = 32 tumors per group) are shown.

(E) Mean tumor weights for sample 1 are presented; no tumors were observed in the ID1/ID3KD group.

(F) Mean tumor weights for sample 2 are shown.

(G) Mean tumor weights for sample 3 are presented; no tumors were observed in the ID1/ID3KD group.

(H) Photographs of excised tumors from mice injected with either ID1/ID3KD or PRS cells are shown.

For (E)–(G) n = 20 tumors/group. Error bars represent ± SD. ***$p < 0.0001$, **$p < 0.001$, *$p < 0.01$.

See also Figure S2.

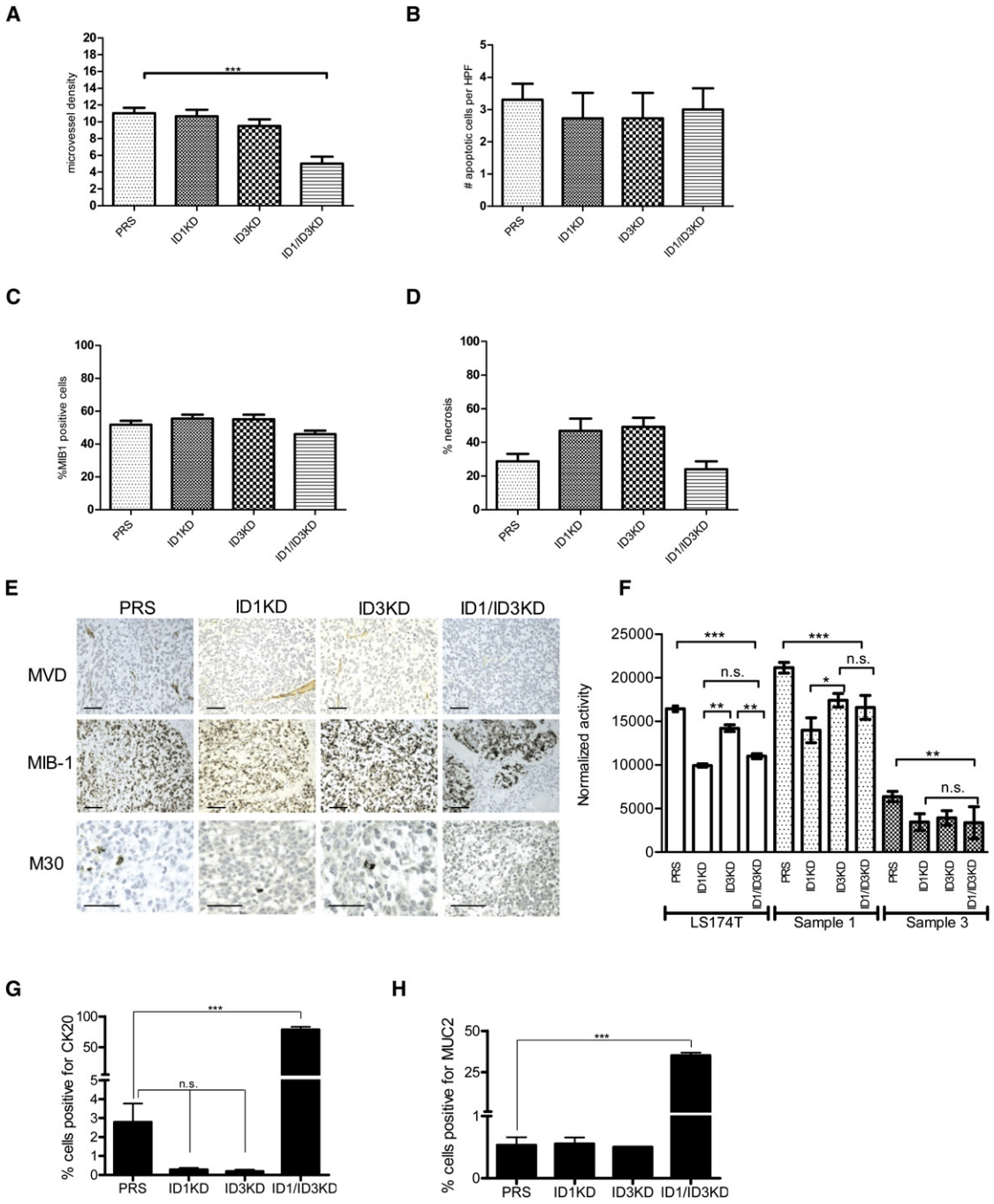


Figure 3. Effect of ID1/ID3KD on Xenograft Histology

(A–D) Quantification of MVD by CD31 staining (A), apoptosis by M30 staining (B), proliferation index by MIB1 staining (C), and percent necrosis (D) are shown.

(E) Histological depiction of the staining (magnification 200×) is illustrated; scale bars represent 50 μm.

(F) Proliferative activity of the PRS and ID1/ID3KD cells for LS174T, sample 1 and 3, is presented.

(G and H) Quantification of CK20 (G) and MUC2 (H) staining is shown.

The histological data are displayed as the mean per high-power field, with ten high-power fields counted per slide, and all error bars represent ± SEM. ***$p < 0.0001$, **$p < 0.001$, *$p < 0.01$. n.s., not significant.

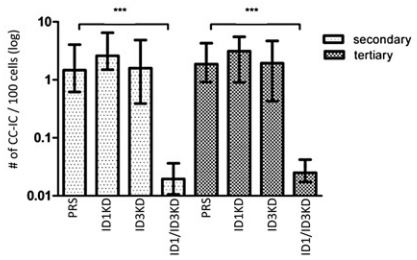
See also Figure S3.

differentiation markers: Muc2 (van de Wetering et al., 2002), and cytokeratin 20 (CK20) (Vermeulen et al., 2008). There was increased expression of differentiation markers in the ID1/ID3KD xenografts, which was not seen in the individual KD or control groups (Figures 3G, 3H, and S3B). This implies that the induction of differentiation is a potential mechanism through

A

CC-IC enriched cultures	Control PRS SRF In Vitro LDA	ID1KD SRF In Vitro LDA	ID3KD SRF In Vitro LDA	ID1/ID3KD SRF In Vitro LDA
Sample 1	1 in 15 (7-33)	1 in 24 (13-45)	1 in 19 (5-58)	1 in 10349 (7923-18209)
Sample 2	1 in 29 (13-51)	1 in 46 (31-75)	1 in 22 (11-43)	1 in 20107 (12497-35280)
Sample 3	1 in 429 (231-657)			1 in 44290 (36518-59173)
LS174T	1 in 39 (24-70)	1 in 62 (34-89)	1 in 25 (11-67)	1 in 3139 (1906-5188)

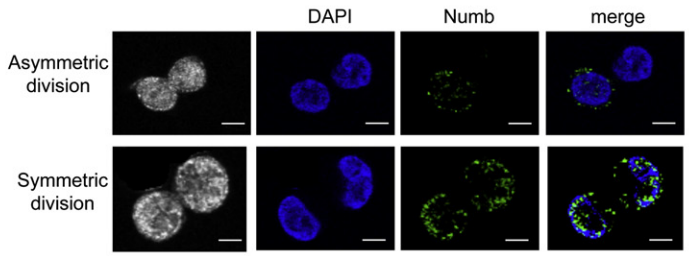
B



C

CC-IC enriched cultures	Control PRS PKH26$^+$ SRF In Vitro LDA	Control PRS PKH26$^-$ SRF In Vitro LDA	ID1/ID3KD PKH26$^+$ SRF In Vitro LDA	ID1/ID3KD PKH26$^-$ SRF In Vitro LDA
Sample 1	1 in 1.2 (1.1-1.4)	1 in 417 (281-619)	1 in 3.9 (2.7-5.6)	0
LS174T	1 in 1.4 (1.2-1.9)	1 in 265 (172-408)	1 in 2.4 (1.6-3.4)	1 in 75876 (34197-168352)

D



E

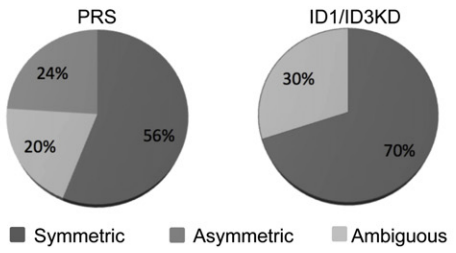


Figure 4. ID1/ID3KD Impairs CC-IC Self-Renewal

(A) Comparison of the sphere-replating frequency (SRF) for PRS, ID1KD, ID3KD, and ID1/ID3KD, as determined by in vitro LDAs, is shown; 95% CI is shown in parentheses.

(B) In vivo serial transplantation assays of LS174T cells derived from PRS, ID1KD, ID3KD, or ID1/ID3KD xenografts are presented.

(C) Comparison of the sphere-replating frequency (SRF) for the PKH26$^+$ and PKH26$^-$ subsets of PRS and ID1/ID3KD cells is shown; in parentheses is the 95% CI.

(D) Confocal images of symmetric and asymmetric division in PKH26$^+$ cells derived from sample 1 are presented: gray scale overlay, DAPI, Numb, and merge (magnification 60×); scale bars represent 10 μm.

(E) Determination of percent asymmetric, symmetric, and ambiguous cell divisions in the PRS and ID1/ID3KD groups (n = 100 cells counted: 50 cells per sample with 2 samples analyzed) is illustrated.

Error bars represent 95% CI. See also Figure S4.

which ID1/ID3KD is exerting its effect (Nam and Benezra, 2009; Anido et al., 2010).

ID1/ID3KD Impairs CC-IC Tumor-Initiating Capacity

To determine whether ID1/ID3KD was affecting the capacity of serial sphere formation, experiments were undertaken where clonally derived primary cells were replated at limiting dilution into secondary sphere-forming assays. The mean sphere-forming capacity decreased on average 300-fold in the ID1/ID3KD versus the other groups (Figures 4A and S4A). These results suggested that ID1 and ID3 together play an essential role in the ability of CC-ICs to sustain propagation through a self-renewal-like mechanism. Despite showing a functional loss of serial sphere formation in the ID1/ID3KD group, we did not identify a significant change in the CD133 or CD44 expression status either in vitro or in vivo (data not shown). Thus, serial sphere formation remains a functional definition, which may or may not be reflected in the phenotypic profile.

To conclusively establish that ID1 and ID3 were affecting the capacity for serial tumor initiation of CC-ICs at the clonal level, in vivo secondary and tertiary transplantation studies were carried out (Figures 4B and S4B). This work could only be carried out using LS174T because the remaining samples did not yield primary tumors in the ID1/ID3KD group (Figures 2E–2G). There was a 200-fold decrease in tumor reinitiation capacity in the ID1/ID3KD group, a difference that was maintained upon multiple passages (Figure 4B). To ensure that KD was maintained, westerns were carried out on the ID1/ID3KD xenograft cells prior to passage (Figure S2C). The decreased capacity for serial tumor initiation was only observed in the ID1/ID3KD group (Figure 4B). Because the in vitro proliferative capacity was equally reduced in all three experimental groups (Figure 3F), our results suggest that the major effect of ID1/ID3KD is not through impaired proliferation but rather through inhibition of the serial tumor initiation capacity of CC-ICs.

ID1/ID3KD Influences Asymmetric Cell Division

The profound loss of CC-ICs and the direct effects we observed on tumor initiation prompted us to determine whether ID1 and ID3 were influencing the asymmetric versus symmetric fates of

the daughter cells derived when CC-ICs divided. However, the ability to address this question was dependent on finding a method to purify the CC-IC fraction. The CC-IC-enriched cultures were labeled with PKH26, a lipophilic fluorescent dye, which labels relatively quiescent cells within a bulk population (Cicalese et al., 2009; Pece et al., 2010). We found that human colon cancer contains a small population of slowly dividing cells that retain the PKH26 dye and are enriched for CC-ICs, whereas the majority of the cells that progressively lose the dye through proliferation are depleted of CC-IC function. The $PKH26^+$ population was significantly higher in the control (4.5% ± 1.7%) versus ID1/ID3KD (0.5% ± 0.3%) group, suggesting that there were fewer label-retaining cells in the ID1/ID3KD group (Figure S4C). To determine if this correlated with the frequency of sphere-forming cells, in vitro serial-replating LDAs were carried out using flow-sorted $PKH26^+$ and $PKH26^-$ cell subsets for both control and ID1/ID3KD cells. For the control group the sphere-initiating frequency was 1 in 1.2–1.4 in the $PKH26^+$ cells, representing a significant enrichment over bulk cultures, much better than established cell surface markers (CD133 and CD44). The frequency of sphere-initiating cells in the $PKH26^-$ fraction was significantly lower (1 in 265–417) than the $PKH26^+$ fraction. In the ID1/ID3KD group, sphere-initiating cells were only detected in the $PKH26^+$ subset with a frequency of approximately 1 in 2.4–3.9 (Figure 4C). There were no sphere-initiating cells detected in the ID1/ID3KD $PKH26^-$ subset for sample 1; however, in LS174T there were very rare (1 in 75,876) sphere-initiating cells at (Figure 4C).

We hypothesized that the difference in the initiating capacity and PKH26 labeling in the ID1/ID3KD versus control group was related to a shift in the proportion of symmetric versus asymmetric cell divisions. To further investigate this possibility, Numb was utilized as a marker of asymmetric cell division (Cicalese et al., 2009; Pece et al., 2010; Kharas et al., 2010). $PKH26^+$ cells from each group were stained for Numb expression to determine the dominant mode of cell division (Figure 4D). The control cells underwent both asymmetric and symmetric divisions at a frequency of 24% and 56%, respectively. In contrast in the ID1/ID3KD group approximately 70% of dividing cells displayed equal distribution of Numb, with 30% of the cell divisions being ambiguous (Figure 4E). In the cells undergoing symmetric division, approximately 90% of the daughter cells expressed CK20 (Figure S4D), a marker of colon cancer differentiation; in the remaining 10% approximately half was weakly positive, and the remainder was negative. This result suggests that the majority of cell divisions that the ID1/ID3KD cells undergo are symmetric giving rise to two differentiated progeny. Collectively, this work along with our functional serial tumor initiation experiments provide strong support that ID1/ID3 is governing CC-IC stem cell functions.

ID1/ID3KD Increases Sensitivity to Oxaliplatin

The chemoresistance of C-ICs has emerged as an important cellular property that enables tumors to regrow following initial cytoreductive therapy. Prior literature has identified a potential role for ID1 in maintaining the chemoresistance of cancer cells in a variety of solid tumors; however, to our knowledge, no such data exist for colon cancer (Cheung et al., 2004). Oxaliplatin is a chemotherapeutic agent commonly used in colon cancer therapy. In LS174T the IC_{50} for the control cells was approximately 200 μM oxaliplatin versus 50 μM in the ID1/ID3KD group; similar results were obtained with sample 2 (Figures 5A and 5B). The enhanced chemosensitivity was also reflected in the approximate 2-fold increase in apoptotic cells following oxaliplatin exposure (IC_{50}), as determined by both annexinV and cleaved caspase-3 staining (Figures 5C–5E). To determine whether oxaliplatin had any effect on in vitro propagation of CC-ICs, sphere-replating assays were carried out in the presence of oxaliplatin. In vitro treatment of the control transduced cells with oxaliplatin (IC_{50}) did not significantly change sphere-replating capacity. In contrast the ID1/ID3KD cells displayed a further 5-fold decrease in sphere-replating capacity following treatment (Table 1; Figure 5F). To determine if this result could be translated to an in vivo model, xenograft studies were carried out with ongoing oxaliplatin treatment to transplanted mice (LS174T). Despite a highly significant decrease in tumor growth in the ID1/ID3KD versus control groups, the addition of oxaliplatin led to a further significant decrease in mean tumor weight (Figure 5G). There was no significant difference in the tumor weights in the oxaliplatin versus vehicle-treated control groups. This was likely related to the dose of oxaliplatin; however, due to toxicity in immune-deficient mice. we were unable to increase the dose. Nevertheless, even in the absence of an objective tumor response in the control group, our results demonstrated a further decrease in xenograft formation in the ID1/ID3KD oxaliplatin-treated group as compared to the vehicle-treated ID1/ID3KD group, indicating that ID1/ID3KD was potentiating tumor cell killing.

ID1/ID3KD Decreases Tumor-Initiating Capacity through Downregulation of p21

To further delineate the molecular machinery by which ID1/ID3 affects tumor-initiating capacity, we investigated a known target, p21. ID1-mediated repression of p21 represents a mechanism to preserve self-renewal capacity in endothelial progenitor cells (Ciarrocchi et al., 2007). Our interest was to determine whether p21, a cell-cycle inhibitor, was playing a similar role in CC-ICs. Interestingly, western blot analysis in the CC-IC lines revealed that p21 expression levels in vitro were high in the parental and control transduced cells but undetectable in the ID1/ID3KD cells (Figure 6A); the exact opposite of the pattern reported in endothelial progenitor cells (Ciarrocchi et al., 2007). Examination of *p21* expression by qPCR revealed a trend toward increased expression in the $CD133^+$ versus $CD133^-$ subsets; however, this did not reach statistical significance. No difference was detected in the level of *p21* mRNA expression in the $CD44^+$ and $CD44^-$ fractions (Figure 6B). Evaluation of p21 protein expression using immunohistochemistry revealed clearly higher levels in both the $CD133^+$ and $CD44^+$ fractions, as compared to their negative counterparts (Figure 6C). From these results we can infer that the effect of ID1/ID3 on p21 is likely through stabilization of the protein as opposed to regulation at a transcriptional level.

In contrast to endothelial progenitor cells, silencing of p21 in leukemic and normal hematopoietic cells impairs stem cell self-renewal (Cheng et al., 2000; Viale et al., 2009), although, to our knowledge, no studies have explored lowered p21 expression in the context of ID1/ID3KD. To determine whether the

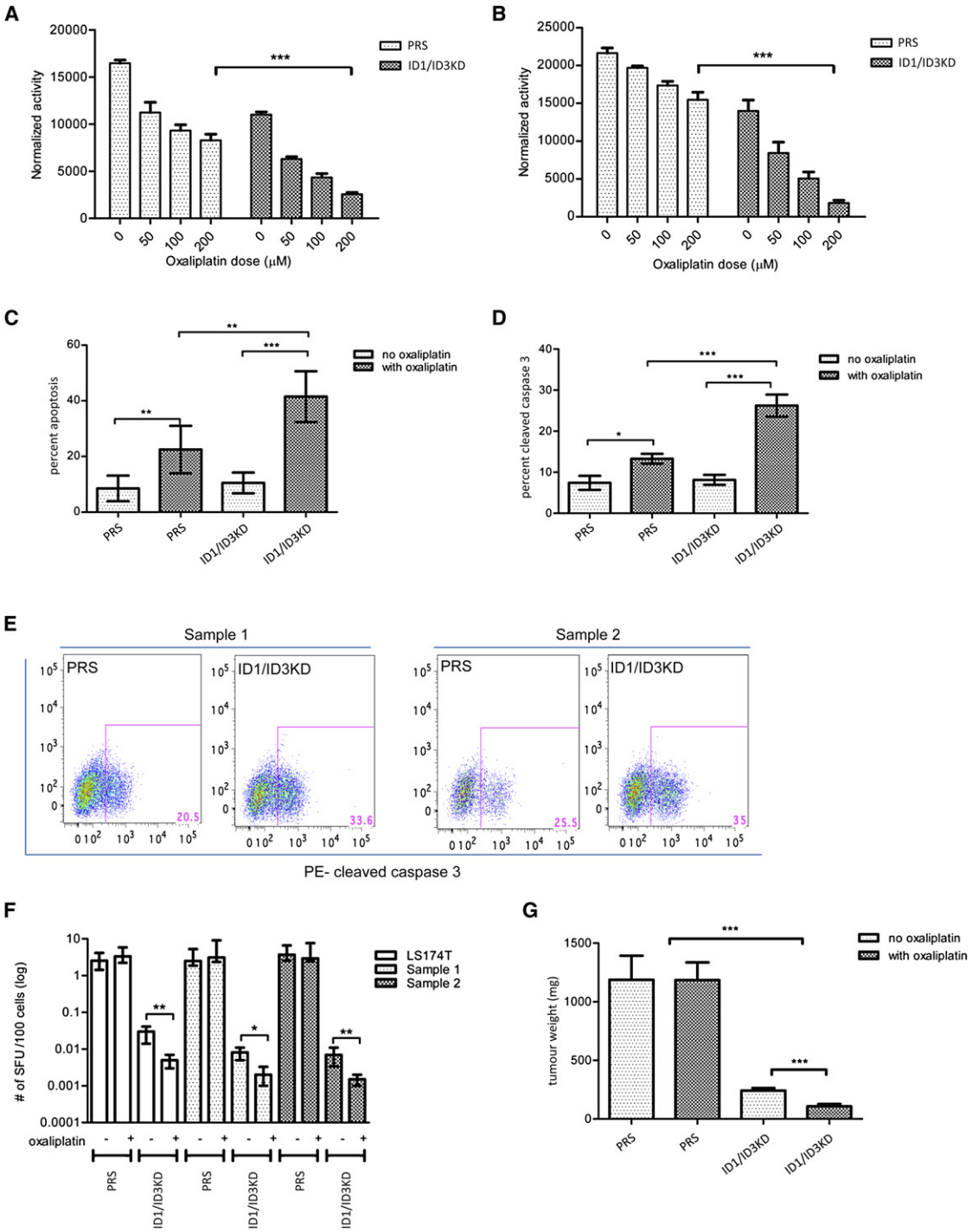


Figure 5. ID1/ID3KD Increases the Sensitivity of CC-ICs to Oxaliplatin

(A and B) Proliferation analysis following a 48 hr oxaliplatin exposure for LS174T and sample 2 (mean ± SEM) is illustrated.

(C) Percent apoptotic cells as measured by annexin V analysis (mean ± SEM) is shown.

(D) Cleaved caspase-3 staining (mean ± SEM) is presented.

(E) Cleaved caspase-3 FACS plots of oxaliplatin-treated ID1/ID3KD and PRS cells from sample 1 and 2 (C–E n = 10 per group) is illustrated.

(F) LDA analysis of SFUs after exposing ID1/ID3KD cells to oxaliplatin (IC_{50}) is shown; error bars represent 95% CI.

(G) Tumor weight following oxaliplatin treatment is presented. PRS or ID1/ID3KD LS174T cells were injected s.c. and the tumors allowed to grow until 0.5 cm^3; mice were then treated with oxaliplatin (mean ± SD).

***p < 0.0001, **p < 0.001, *p < 0.01.

Table 1. Comparison of the SRF for the Control versus ID1/ID3KD Cells in the Presence and Absence of Oxaliplatin

CC-IC-Enriched Cultures	PRS SRF In Vitro LDA	PRS with Oxaliplatin SRF In Vitro LDA	ID1/ID3KD SRF In Vitro LDA	ID1/ID3KD with Oxaliplatin SRF In Vitro LDA
Sample 1	1 in 40 (19–53)	1 in 32 (11–43)	1 in 12,392 (9,048–20,810)	1 in 52,379 (30,007–75,228)
Sample 2	1 in 27 (15–39)	1 in 34 (13–41)	1 in 14,740 (8,660–29,105)	1 in 64,399 (49,583–79,332)
LS174T	1 in 39 (24–70)	1 in 30 (17–45)	1 in 3,493 (2,400–6,930)	1 in 19,215 (13,901–28,985)

All data are represented as the SRF for each group; the 95% CI is shown in parentheses.

decreased expression of p21 observed in the ID1/ID3KD group was playing a functional role in maintaining tumor-initiating capacity, a genetic rescue experiment was designed to reintroduce p21 into the ID1/ID3KD cells. Sample 2 and LS174T were tested by in vivo xenograft formation; ID1/ID3KD/p21 overexpressing (OE) xenografts were significantly larger than those generated by injection of ID1/ID3KD cells alone, although they remained significantly smaller than controls (Figures 6D and 6E). This partial rescue of tumor formation indicated that the decreased expression of p21 in the ID1/ID3KD cells was functionally important for tumor initiation and maintenance. Secondary LDA experiments revealed a sustained increase in CC-IC frequency in the ID1/ID3KD/p21OE cells as compared to ID1/ID3KD alone (Figure 6F), establishing that CC-IC sustainability is affected by p21. Our preliminary data suggest that one possible mechanism by which ID1/ID3 may influence p21 protein stability is through inhibitory effects on phosphatase and tensin homolog (PTEN). Genetic KD of ID1/ID3 in our model system results in the re-expression of PTEN at the protein level (Figure S2D). Our results lend additional support to the notion that the ID/p21 regulatory axis is important in tumor initiation and warrants further investigation, including confirmation of the role of PTEN.

Effect of ID1/ID3KD on Cell Cycle and Accumulation of DNA Damage-Induced Foci

It is well established that p21 inhibits cell-cycle progression by binding to G1 cyclin/CDK complexes (Abbas and Dutta, 2009). Because p21 protein expression was decreased following ID1/ID3KD, it was important to determine whether this reflected a change in cell-cycle distribution. The mean proportion of cells in G0/G1 was significantly higher in the control versus ID1/ID3KD cells, whereas the opposite was observed for cells in S phase. There was no significant difference between the two groups in the proportion of cells in G2/M; however, there was a trend toward ID1/ID3KD having more cells in G2/M (Figure 7A). In support of the cell-cycle results, BrdU incorporation experiments demonstrated that the ID1/ID3KD group exhibited a 2- to 3-fold increase in BrdU incorporation, as compared to the control cells (Figures 7B and 7C). p21 has previously been shown to play a critical role in leukemia stem cell (LSC) maintenance, where it imposes a cell-cycle restriction that in turn acts to limit the accumulation of DNA damage in LSCs (Viale et al., 2009). In our samples there was an approximate 2-fold increase in γH2AX foci in the ID1/ID3KD versus control cells (Figures 7D and 7E). These results suggest that the reduced levels of p21 and the subsequent lack of cell-cycle restriction resulted in an accumulation of DNA damage leading to CC-IC exhaustion.

DISCUSSION

Our findings establish that ID1 and ID3 govern self-renewal of CC-ICs derived from primary colon cancer samples. Silencing both genes together resulted in a dramatic loss of tumor-initiating potential. Second, we found that ID1/ID3 orchestrate their regulation of CC-ICs via p21, providing a linkage of the ID1/ID3-p21 regulatory axis with maintenance of tumor initiation capacity in any solid tumor C-IC. Finally, we show that in the presence of oxaliplatin, a commonly used chemotherapeutic agent, ID1/ID3 functions to protect the tumor-initiating capacity of CC-ICs. Following ID1/ID3KD, exposure to oxaliplatin resulted in a further decrease in tumor-initiating capacity. Thus, our study connects the capacity for serial propagation of tumor-initiating cells with CC-IC chemoresistance, knowledge that could be exploited in future therapeutic strategies.

ID1/ID3 Govern Self-Renewal

As expected from studies of ID family proteins in other model systems, we observed effects on proliferation and angiogenesis upon silencing of ID1 and ID3 individually or together. However, these effects alone could not explain the drastic decrease in xenograft growth observed in the ID1/ID3KD group. The ID proteins have a central role in maintaining cells in an immature state; in keeping with this the ID1/ID3KD group displayed increased expression of markers of intestinal cell differentiation, a finding that correlates with a decrease in self-renewal potential. Emerging data indicate that the *ID* genes play an important role in maintaining C-ICs in a variety of solid tumors (Hollnagel et al., 1999; Nam and Benezra, 2009; Perry et al., 2007). Most recently, Andio et al. reported that TGF-β-induced inhibition of $CD44^+$ glioblastoma-initiating cells was due to repression of ID1 and ID3, pointing to a potential link between ID genes and C-IC self-renewal (Anido et al., 2010). Our data support this prior work by providing direct evidence that ID1/ID3 govern CC-IC maintenance.

Moreover, we demonstrate that ID1/ID3KD dramatically alters the ratio of asymmetric and symmetric cell divisions in the CC-IC fraction. This provides another line of evidence linking ID1/ID3 with canonical stem cell properties. Numb staining of $PKH26^+$ control cells suggested that these cells normally undergo both asymmetric and symmetric cell divisions. In contrast following ID1/ID3KD the majority of divisions became symmetric, giving rise to two daughter cells both displaying evidence of differentiation, as demonstrated by increased CK20 staining. These results are in keeping with prior studies in normal neural stem cells where high ID1 expression drove self-renewal by promoting asymmetric cell division (Nam and Benezra, 2009). They found that ID1 was expressed as a gradient with the highest levels in

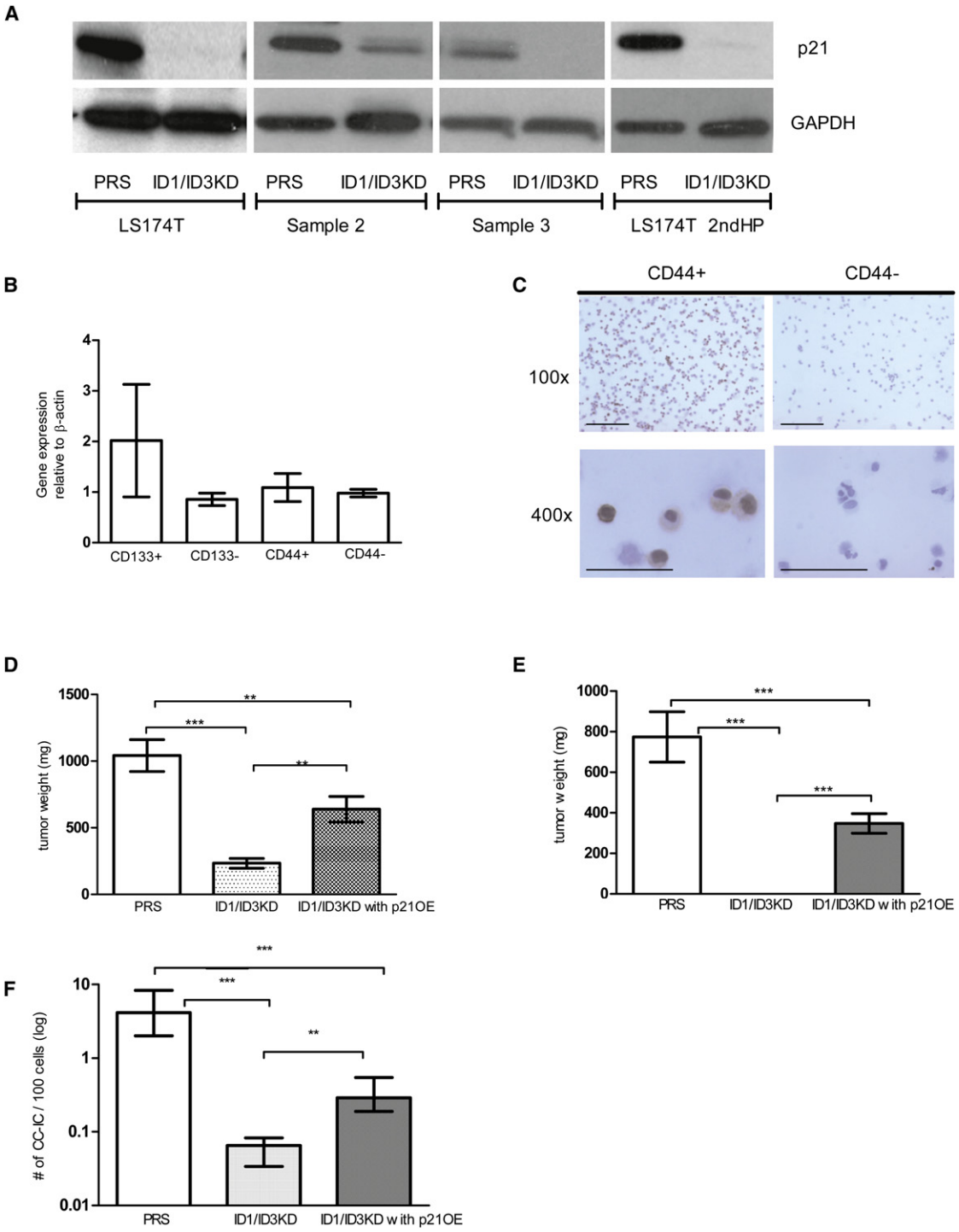


Figure 6. ID1/ID3KD Decreased the Self-Renewal Capacity of CC-IC through p21 Downregulation

(A) Western blot analysis of p21 protein levels in PRS versus ID1/ID3KD cells is presented. Displayed are LS174T, samples 1 and 2, and LS174T transduced with second set of ID1/ID3KD hairpins. GAPDH was utilized as the housekeeping gene.

(B) p21 mRNA expression in CC-IC-enriched and nonenriched fractions (n = 3 replicates) is shown; error bars indicate ± SEM.

(C) Immunohistochemical staining for p21 on $CD44^{+}$ and $CD44^{-}$ cell subsets is illustrated. Top scale bars (100×) represent 100 μm; bottom scale bars (400×) represent 50 μm.

(D and E) Tumor weights for LS174T and sample 2, and three groups, PRS, ID1/ID3KD, and ID1/ID3KD with p21 overexpression (ID1/ID3KDp21OE), are presented. In both samples expression of p21 partially rescued the effect of ID1/ID3KD on xenograft growth; mean ± SD is shown (n = 20 injections per group).

(F) In vivo secondary transplantation LDA with LS174T.

Error bars represent 95% CI. ***$p < 0.0001$, **$p < 0.001$.

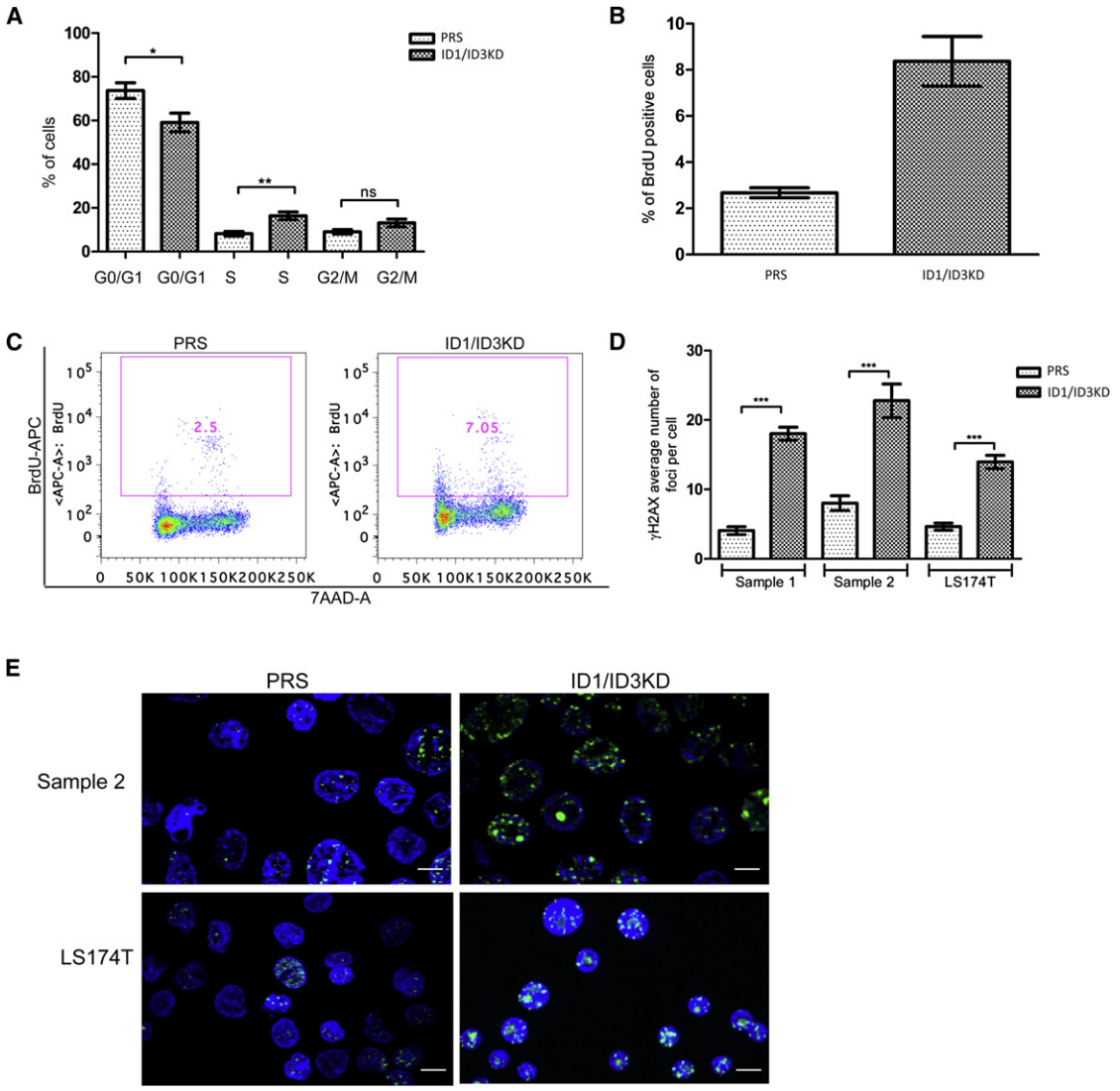


Figure 7. Effect of ID1/ID3KD on Cell Cycle and DNA Damage

(A) Cell-cycle distribution was determined by flow cytometry (n = 3 replicates).

(B and C) BrdU incorporation analysis (n = 3 replicates) is illustrated.

(D) Quantification of γH2AX staining (n = 100 cells counted) is shown.

(E) Confocal images of γH2AX staining (×60) are presented; scale bars represent 10 μm.

For bar graphs, mean ± SEM is shown; ***p < 0.0001, **p < 0.001, *p < 0.01. ns, not significant.

self-renewing neural stem cells and the lower levels in non-stem cells correlating with increased expression of markers of neural cell differentiation (Nam and Benezra, 2009). Similarly, CC-IC confocal imaging of ID1 and ID3 in our PKH26$^+$ and PKH26$^-$ colon cancer cells demonstrated ID1/ID3 staining in both subsets; however, the expression level appeared higher in the PKH26$^+$ cells, as compared to the PKH26$^-$ cells (data not shown). It is possible that, similar to neural stem cells (Nam and Benezra, 2009), ID expression may be on a continuum where colon cancer cells expressing the highest combined levels have the capacity to initiate tumors. Our work, as well as that of others, clearly indicates that ID1/ID3 play a central role in both normal stem cell and C-IC maintenance, making it essential that the molecular mechanisms driving this process are better understood.

ID1/ID3 Function through Effects on p21

We initially hypothesized that the mechanism by which ID1/ID3KD was decreasing tumor-initiating capacity may be similar to the effect observed in endothelial progenitor cells where ID1KD resulted in increased p21 levels and a subsequent decrease in self-renewal capacity (Ciarrocchi et al., 2007). Unexpectedly, we observed the exact opposite: p21 was highly expressed in the parental and control transduced cells, whereas the ID1/ID3KD cells displayed an almost complete loss of p21 expression in all samples tested. Moreover, CD133$^+$ and

$CD44^+$ CC-IC-enriched fractions expressed significantly higher p21 protein levels as compared to their negative counterparts. This result suggests that in the context of CC-ICs, p21 may function to maintain ID1/ID3-dependent tumor-initiating potential.

Interestingly, an association between ID1 overexpression and high levels of p21 was initially identified in a mouse mammary carcinoma model that showed ID1 overexpression resulted in tumors that continued to proliferate despite high levels of p21 (Swarbrick et al., 2008). These authors hypothesized that ID1 must act downstream of p21, rendering cells refractory to p21-dependent cell-cycle arrest. However, this was not the case in our colon cancer samples because the reintroduction of p21 resulted in a partial rescue of the ID1/ID3KD-induced tumor-initiating defect, providing strong genetic evidence that p21 plays a functional role in maintaining CC-ICs. One possible explanation for the rescue only being partial is that the effect of ID1/ID3 on proliferation is independent of their combined effects on p21 and self-renewal. Each of the ID1KD, ID3KD, and ID1/ID3KD groups exhibited an approximate 2-fold decrease in proliferative capacity, yet the effect on self-renewal was only seen in the ID1/ID3KD cells. Alternatively, ID1/ID3KD may have influenced other downstream pathways in addition to p21 that are involved in the maintenance of CC-ICs. Studies of a variety of normal and neoplastic stem cell systems are pointing to the integrated functioning of multiple genetic and epigenetic components working together to maintain self-renewal potential (Morrison and Spradling, 2008; Shackleton et al., 2009; He et al., 2009).

One possible mechanism by which ID1/ID3 may regulate p21 is through inhibition of PTEN. Lee et al. have previously shown that ID1 can negatively regulate PTEN at a transcriptional level in MCF7 human breast cancer cells (Lee et al., 2009). Furthermore, attenuation of PTEN has been shown to increase p21 levels through stabilization of the protein (Lin et al., 2007). Our preliminary data also show that ID1/ID3KD results in the re-expression of PTEN. In renal cell carcinoma, p21 stabilization is one of the key mechanisms by which PTEN-deficient tumors escape chemotherapy-induced cell death (Lin et al., 2007). The notion that p21, a cell-cycle inhibitor, is maintaining self-renewal seems counterintuitive. However, there is strong evidence from murine models of normal hematopoietic and leukemic stem cells that p21 is an important regulator of self-renewal. In the absence of p21, hematopoietic and leukemic stem cells underwent functional exhaustion and were unable to maintain the clone (Cheng et al., 2000; Viale et al., 2009).

Our results support a role for p21 in the prevention of CC-IC exhaustion through cell-cycle restriction and the resulting accumulation of DNA damage as shown by increased γH2AX foci. These results are consistent with a number of publications over the past 20 years that have recognized a role for p21 in the protection of cancer cells from stress and DNA damage (Mahyar-Roemer and Roemer, 2001; Bene and Chambers, 2009; Gorospe et al., 1996; Sharma et al., 2005; Tian et al., 2000). Bunz et al. (1998) were the first to demonstrate that $p21^{-/-}$ colon cancer cells treated with a DNA-damaging agent undergo aberrant progression through S and M phases of the cell-cycle-triggering apoptosis. Also in support of our findings, there are numerous reports linking p21 expression with protection of colon cancer cells from apoptosis induced by a wide range of insults, including exposure to radiation (Tian et al., 2000), chemotherapeutic agents (Mahyar-Roemer and Roemer, 2001; Bene and Chambers, 2009), and cryotherapy (Sharma et al., 2005). Finally, clinical trial data from patients with rectal tumors undergoing neoadjuvant chemoradiation show association between increased p21 expression and the development of resistance resulting in decreased disease-specific survival (Kuremsky et al., 2009; Rau et al., 2003). Taken together, these observations support the clinical relevance of our findings and extend the functional roles of p21 to include preservation of CC-IC self-renewal.

Role of ID1/ID3 in Chemoresistance

Our studies also provide a direct link between the capacity of CC-ICs for serial tumor initiation and chemoresistance. Although existing literature supports a role for ID1 and p21 in maintaining chemoresistance in some solid tumors (Cheung et al., 2004; Hu et al., 2009; Li et al., 2007), to our knowledge, the effect of ID1/ID3KD on colon cancer cells and their response to treatment with oxaliplatin has not been investigated. We found that the dose of oxaliplatin that reduced overall cell proliferation did not inhibit the sphere-replating capacity of control transduced cells. This finding may in part explain clinical observations related to oxaliplatin treatment. When oxaliplatin is used in the adjuvant setting with 5-fluorouracil (5-FU), the tumor response rates are in the range of 40%–50%, whereas the actual survival advantage conferred is on average less than 10% (Alberts and Wagman, 2008; Chau and Cunningham, 2009). This suggests that the response rates may actually be monitoring decreased proliferative capacity of the bulk cancer cells; however, if there are CC-ICs surviving despite oxaliplatin treatment, the drug may not affect their function. In contrast, oxaliplatin treatment of ID1/ID3KD cells decreased tumor initiation capacity, which warrants further investigation of the linkage between CC-ICs and chemoresistance. If this linkage is universally important, then understanding the mechanisms that drive C-IC self-renewal will lead to the development of therapeutic agents that target this essential aspect of tumor maintenance and may potentiate the efficacy of chemotherapies.

In conclusion our study demonstrates the feasibility of utilizing primary human cancer cells to enrich for CC-IC activity thereby providing a powerful tool to carry out genetic approaches to unravel the molecular pathways sustaining tumor growth. Our findings point to the central role that ID1/ID3 and p21 play in regulating the tumor-initiating program of CC-ICs and in governing their response to chemotherapy. Collectively, our findings put forth self-renewal pathways as potential targets for the development of effective therapies to eradicate CC-ICs.

EXPERIMENTAL PROCEDURES

Culture and Xenografting of Colon Cancer Cells

Human colon cancer specimens were obtained with informed patient consent as approved by the Research Ethics Board (University Health Network, Toronto). Cells were isolated and cultured in DMEM/F-12 with EGF and bFGF as previously described by Kreso and O'Brien (2008). Unless otherwise noted, tumor cells were injected s.c. into NOD/SCID mice using procedures that conform to the standards approved by the Animal Care Committee (Ontario Cancer Institute, Toronto).

Viral Vectors

Short hairpins to ID1 and ID3 were obtained from Dr. J. Massague (Memorial Sloan-Kettering Cancer Center) (Gupta et al., 2007). Dr. J. Moffat (University of Toronto) provided a second set of hairpins to ensure that the effects were not off target. For combined KD, cells were sequentially infected and puromycin selected. For p21 overexpression a pBabe construct (Dr. G. Peters) was utilized for both overexpression and control viruses.

LDAs

Viable cells were diluted, and defined cell doses were (i) injected into mice to assess CC-IC frequency in vivo and (ii) plated in 96-well plates to assess the number of sphere-forming units (SFUs) in vitro. The ELDA website was used to determine the estimated cell frequency (http://bioinf.wehi.edu.au/software/elda/index.html). For in vitro LDAs a number of cell doses were tested, and the lowest cell dose was one cell per well, all plated in a fixed volume of 200 μl per well. Any well with one or more spheres was considered positive for ELDA. To assess the number of colony-forming units (CFUs), similar methods were used, but cells were plated in medium containing 10% serum.

Statistical Analysis

PRISM software was used to analyze results; values are reported as mean ± SD unless otherwise indicated. The limiting dilution function (http://bioinf.wehi.edu.au/software/elda/index.html) was used to calculate the estimated cell frequencies for LDAs; 95% confidence intervals (CIs) are reported for each frequency.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, five tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.ccr.2012.04.036.

ACKNOWLEDGMENTS

We would like to thank Eric Lechman, Faiyaz Notta, Elisa Laurenti, and Michael Milyavsky for their critical review of this manuscript; Fanong Meng, Mayleen Sukhram, and Viktor Son for providing colon cancer samples; PRP for assistance with immunohistochemistry; and Shane Harding, Peter Dirks, and Ian Clarke for their assistance with planning experiments. This work was supported by funds from the Krandel Fund at Princess Margaret Hospital, Genome Canada through the Ontario Genomics Institute, Ontario Institute for Cancer Research and a Summit Award with funds from the province of Ontario, the Canadian Institutes for Health Research, and a Canada Research Chair. This research was funded in part by the Ontario Ministry of Health and Long Term Care (OMOHLTC). The views expressed do not necessarily reflect those of the OMOHLTC.

Received: March 4, 2010
Revised: September 14, 2011
Accepted: April 23, 2012
Published: June 11, 2012

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