Self-renewal as a therapeutic target in human colorectal cancer

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Tumor recurrence following treatment remains a major clinical challenge. Evidence from xenograft models and human trials indicates selective enrichment of cancer-initiating cells (CICs) in tumors that survive therapy. Together with recent reports showing that CIC gene signatures influence patient survival, these studies predict that targeting self-renewal, the key ‘stemness’ property unique to CICs, may represent a new paradigm in cancer therapy. Here we demonstrate that tumor formation and, more specifically, human colorectal CIC function are dependent on the canonical self-renewal regulator BMI-1. Downregulation of BMI-1 inhibits the ability of colorectal CICs to self-renew, resulting in the abrogation of their tumorigenic potential. Treatment of primary colorectal cancer xenografts with a small-molecule BMI-1 inhibitor resulted in colorectal CIC loss with long-term and irreversible impairment of tumor growth. Targeting the BMI-1–related self-renewal machinery provides the basis for a new therapeutic approach in the treatment of colorectal cancer.

Evidence suggests that colorectal cancer usually follows the cancer stem cell model where a subset of cancer cells possess transcriptional and epigenetic programs that endow them with stemness properties, including the capacity for self-renewal1–4. Such cells, functionally defined as CICs, are uniquely capable of initiating and sustaining tumor growth in serial transplantation assays. CICs have a number of other biological properties that distinguish them from the remainder of tumor cells, including resistance to treatment5–7, evasion of cell death8,9 and dormancy10. These properties, together with variation in the ability of CICs to self-renew10, suggest that they may have a central role in tumor recurrence. Although CICs have been characterized in several human cancers2–4,11–13, their clinical relevance remains uncertain. One unattained piece of evidence that would render strong support for the concept that stemness and CICs play a part in human cancer would be to control tumor growth using a clinically relevant method that targets CIC self-renewal.

Bmi-1 represents the first regulator that was strongly linked to self-renewal and has been implicated in the maintenance of stem cells in several tissues14–16. Bmi-1 is part of the polycomb repressive complex 1 (PRC1), where it functions as an epigenetic chromatin modifier targeting many genes17,18. In the intestinal epithelium, Bmi1 expression marks mouse intestinal stem cells and is important for crypt maintenance19,20. Bmi-1 has also been shown to have a role in the propagation of several types of cancer21–25, although only a few studies have directly assessed its impact on CIC function26,27. In the context of colorectal cancer, BMI-1 can be overexpressed in tumors, and its expression has been associated with increased risk of metastasis and high-grade tumors28–31; however, studies on the role of BMI-1 in colorectal CICs and self-renewal have not been reported.

We describe here that BMI-1 is required for CIC function in colorectal tumors and that small-molecule–mediated BMI-1 inhibition reduces tumor burden in primary human colorectal tumor xenograft models. Thus, inhibiting a recognized regulator of self-renewal is an effective approach to control tumor growth, providing strong evidence for the clinical relevance of self-renewal as a biological process for therapeutic targeting.

RESULTS
BM1 knockdown reduces human colorectal cancer growth
We detected BMI-1 expression in most cells of a well-characterized human colon adenocarcinoma cell line, LS174T, and five patient-derived colorectal tumor samples under conditions that permitted expansion in vitro (Fig. 1a,b). When we sorted these samples into phenotypic fractions enriched for colorectal CICs and non-CICs, BMI-1 was expressed in both fractions, similar to other markers used to isolate normal intestinal stem cells and colorectal CICs (Supplementary Figs. 1–3). To determine whether reduction of BMI-1 levels affected the growth of human colorectal cancer cells, we stably

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Received 26 January; accepted 1 November; published online 1 December 2013; doi:10.1038/nm.3418
knocked down BMI-1 transcript levels using a total of four different lentivirus encoded shRNAs. The different shRNAs yielded similar results, and we employed one shRNA for most studies, as it reliably yielded cells with >60% BMI-1 knockdown at the mRNA and protein levels (BMI-1-KD cells) (Fig. 1a,b). In addition to an shRNA to BMI1 (shBMI-1), the lentivirus also encoded eGFP. BMI-1-KD cells were progressively outcompeted and lost their ability to grow in vitro, as evidenced by a decline in the fraction of shBMI-1–expressing eGFP+ cells over time in both cell lines and primary patient-derived cell cultures (Fig. 1c). To evaluate effects on in vivo tumor-forming potential, we injected BMI-1-KD cells subcutaneously into nonobese diabetic–severe combined immunodeficiency (NOD-SCID) and interleukin-2 receptor γc–deficient (NSG) mice. Four weeks following transplantation, BMI-1-KD LS174T cells displayed a 12-fold reduction in tumor growth as compared to control cells expressing an shRNA to luciferase (shLUC) (P < 0.0001, Fig. 1d). Likewise, the mass of tumors from patient-derived colorectal cell cultures was significantly reduced in the BMI-1-KD group of sample 02 (P < 0.0001, Fig. 1d) and sample 04 (P = 0.02, Fig. 1d); no tumors were detected in mice given BMI-1-KD sample 01 cells (Fig. 1d). We obtained similar results using additional shRNAs targeting BMI-1 (Supplementary Fig. 4a). To ensure that the dependence on BMI-1 for tumor cell growth was not due to short-term culturing, we generated single-cell suspensions from five additional colorectal cancer specimens immediately following surgical resection and directly transplanted them without expansion in vitro. We observed considerably reduced tumor growth following injection under the renal capsule in the BMI-1-KD group compared to the control transduced cells expressing shLUC for three of the five tested samples (Fig. 1e). For sample 05 and LS174T cells, BMI-1-KD cells generated smaller tumors upon re-injection into secondary recipients, indicating that BMI-1-KD cells exhibit a sustained reduction in tumor growth (Fig. 1e and Supplementary Fig. 4b). Collectively, these data indicate that human colorectal cancer cells require BMI-1 to generate and maintain tumor growth in vivo.

The observed decrease in tumor mass upon BMI-1 knockdown suggested a defect in cell proliferation. Consistent with this, BMI-1-KD LS174T cells, when grown as nonadherent spheres in vitro, had a smaller sphere diameter compared to controls (Supplementary Fig. 5a). BMI-1 knockdown did not lead to a statistically significant difference in BrdU uptake (P > 0.05, Supplementary Fig. 5b) in LS174T cells; however, there was a marked increase in the proportion of cells in G0 (Ki67−) (Supplementary Fig. 5c). In accordance with these findings, BMI-1 exerted its effects on sphere initiation through its repressive function of the CDKN2A locus, which encodes p14ARF and p16INK4a (Supplementary Fig. 6a–c and Supplementary Results). We analyzed three primary human colorectal cancer cell cultures and observed a modest 1.7-fold reduction in S phase cells upon BMI-1 knockdown (P = 0.036, Supplementary Fig. 7a,b). There were 1.2-fold more cells in G0 after BMI-1 knockdown (P = 0.050, Fig. 2c,d and Supplementary Fig. 7c,d). In addition, in LS174T cells expressing shBMI-1, there was a marked increase in the proportion of cells expressing cleaved caspase 3 (Supplementary Fig. 5d), indicative of apoptosis induction. In primary cell cultures, we found only a twofold increase (P = 0.04) in cleaved caspase 3 in the BMI-1-KD group (Fig. 2e,f and Supplementary Fig. 7e). Consistent with these small changes in proliferation and apoptosis, inhibiting expression of the CDKN2A locus did not rescue the BMI-1-KD phenotype in primary patient-derived cell cultures (Supplementary Fig. 6d,e and Supplementary Results). Taken together, these data indicate that the reduced tumor mass of BMI-1-KD cells is due in part to the...
BMI-1-KD impairs colorectal CIC self-renewal

In addition to the reduction in tumor mass of BMI-1-KD cells, there was also a decrease in the total number of tumors initiated, suggesting that BMI-1 may regulate colorectal CICs (Fig. 3a). To enumerate the frequency of CICs directly, we employed clonal tumor initiation assays performed with limiting-dilution analyses (LDAs) (Fig. 3b). BMI-1 knockdown decreased the frequency of tumor-initiating cells 12-fold in LS174T cells ($P < 0.0001$, Fig. 3c and Supplementary Table 1). In four primary samples, the frequency was 11-, 65-, 19- and 54-fold lower in the BMI-1-KD groups as compared to controls (Fig. 3c and Supplementary Table 1). These quantitative studies establish that BMI-1 knockdown prevents tumor initiation, thereby functionally lowering the frequency of CICs.

To directly determine whether BMI-1 was affecting CIC self-renewal, we took two additional approaches. We employed the widely used in vitro assay of sphere initiation but used LDA to ensure the accuracy of our measurements. We determined that there was a 283- to 661-fold decrease in the sphere re-initiating cell frequency in the BMI-1-KD group compared to controls (Fig. 3d and Supplementary Table 2). We observed similar results with two additional shRNAs (Supplementary Fig. 8). Therefore, BMI-1 knockdown severely abrogates the self-renewal capacity of colon cancer cells in vitro.

The definitive method to assess the self-renewal potential of CICs is to carry out secondary tumor formation assays in vivo using tumors initiated by a single CIC (see details in Supplementary Methods). We therefore injected shLUC and shBMI-1 cells from three samples at limiting doses into mice; we transplanted tumors generated from a single colorectal CIC into secondary recipients using limiting dilution (Fig. 3e). There was a 60-fold decrease in the frequency of self-renewing CICs in the BMI-1-KD group in LS174T cells ($P < 0.0001$, Fig. 3f).

Targeting BMI-1 by small-molecule inhibition

We identified a low-molecular-weight compound, PTC-209 (Fig. 4a), by high-throughput screening of compounds using gene expression modulation by small molecules (GEMS) technology, where reduced luciferase BMI-1 reporter activity was measured to identify compounds that lower BMI-1 transcript levels. The GEMS reporter vector contains the luciferase open reading frame flanked by and under post-transcriptional control of the BMI-1 5′ and 3′ untranslated regions (UTRs). Subsequent characterization demonstrated that PTC-209 inhibited not only the UTR-mediated reporter expression (Fig. 4b) but also endogenous BMI-1 expression in human colorectal HCT116 (Fig. 4c) and human fibrosarcoma HT1080 tumor cells (Fig. 4d). The inhibition was dose dependent, with half-maximum inhibitory concentration ($IC_{50}$) at about 0.5 μM in both reporter and ELISA assays (Fig. 4d, b). PTC-209 caused no inhibition of cell growth or viability in HEK293 human embryonic kidney cells after overnight treatment (Fig. 4b) and had limited effects on cell proliferation in HT1080 cells after a 48-h treatment (Fig. 4d), indicating that the activity of PTC-209 is not due to cytotoxicity. BMI-1 facilitates transcriptional silencing through the PRC1 complex by acting as a cofactor for RING1A, a monoubiquitination E3 ligase with activity specific for Lys119 on histone H2A, a mark associated with repressive chromatin structures. PTC-209 selectively reduced global ubiquitination in cells undergoing cell death.
Figure 3 BMI-1 knockdown reduces the frequency of self-renewing colorectal CICs. (a) Representative image of tumors generated in mice that were injected with cells described in Figure 1d. (b) Schematic illustrating the experimental approach taken to assay the frequency of CICs after BMI-1 knockdown (see Online Methods). (c) CIC frequency of shBMI-1– or shLUC-transduced cells as measured by LDA in vivo. Data are expressed as mean and 95% confidence interval (CI). (d) The number of secondary sphere-forming units for the shLUC and shBMI-1 groups. Data are expressed as mean and 95% CI. (e) Schematic representation of the experimental approach taken to quantify the self-renewal ability of a single colorectal CIC in vivo. (f) Frequency of secondary CICs in vivo. Data are expressed as mean and 95% CI. Frequency and probability estimates were computed using the ELDA software (**P < 0.01 and ***P < 0.001).

To more directly establish PTC-209 specificity, we took two genetic approaches using BMI-1-KD and BMI-1 overexpression (BMI-1-OE) lentiviral vectors. In BMI-1-KD colon cancer cells from patient tumor tissue and the LS174T cell line, we found a lower sensitivity to the growth-inhibitory effects of PTC-209 over a range of PTC-209 doses (Fig. 4f–i, Supplementary Figs. 9 and 10 and Supplementary Results), indicating that PTC-209 is specific for BMI-1. Likewise, BMI-1-OE colon cancer cells were relatively less sensitive to the action of PTC-209 than cells expressing endogenous BMI-1 levels (Fig. 4j–l, Supplementary Fig. 11 and Supplementary Results), further supporting the specificity of PTC-209 for BMI-1. In vitro treatment of human colorectal cancer cells with doses between 0.1 and 10 µM reduced BMI-1 protein levels in a dose-dependent manner (Fig. 5a) with a concomitant reduction in cell growth (Fig. 5b). These data indicate that PTC-209 selectively lowers BMI-1 levels and decreases colorectal tumor cell growth. The ability to control the length of BMI-1 inhibition through the addition or removal of the inhibitor provides a critical tool to further investigate the effects of BMI-1 function in colorectal CICs.

**BMI-1 inhibitor irreversibly impairs colorectal CICs**

To determine whether BMI-1 reduction–mediated impairment of colorectal CICs was transitory or irreversible, we exposed three colorectal cancer samples to PTC-209 for 4 d, removed the inhibitor and seeded viable cells at limiting doses in medium lacking inhibitor. For all samples, treatment of cells with the inhibitor at 0.1 µM yielded a statistically significant decrease in the frequency of sphere-initiating cells, ranging from a 2- to 11-fold reduction as compared to vehicle-treated cells (P < 0.01, Fig. 5c and Supplementary Table 4).

We obtained similar results when cells were assayed 5 d following inhibitor removal (Supplementary Fig. 12 and Supplementary Results), indicating that the effects on sphere formation are stable and result from the viability changes induced by drug treatment at the start of the experiment. Therefore, the frequency of sphere-initiating cells was affected after transient downregulation of BMI-1, suggesting that BMI-1 inhibition results in irreversible reduction of CICs.

To examine how PTC-209 affected CICs, we analyzed the response of CIGs and non-CIGs to PTC-209. The proportion of cells with high Wnt activity, as measured by quantifying expression of the T cell factor/lymphoid enhancer factor (TCF/LEF)-eGFP reporter, which was previously used to mark colorectal CIGs, was reduced following PTC-209 treatment (Supplementary Fig. 13a–d and Supplementary Results). PTC-209 treatment also resulted in decreased Lgr5 mRNA levels; Lgr5 is a Wnt target gene and its expression marks mouse
intestine stem cells (Supplementary Fig. 13e). These data support the conclusion that BMI-1 downregulation specifically affects CICs. CD133 and/or CD44 expression was not changed following BMI-1 downregulation (Supplementary Fig. 14), and induction of differentiation markers was variable upon PTC-209 treatment (Supplementary Fig. 15). Analysis of cell cycle and apoptosis markers in CD133+ and CD44+ cells showed an induction of cells in G0 and increased expression of cleaved caspase 3 following PTC-209 compared to shLUC-expressing cells. Data are shown as pools of three, four and two experiments for samples 01 and 03 and LS174T cells, respectively. Data are expressed as mean ± s.d. of 3–9 replicates.

When viable cells from sample 11 were injected following ex vivo treatment with 10 µM PTC-209, no tumors formed, whereas equal numbers of viable cells generated tumors in the control group (Fig. 5f). Therefore, transient depletion of BMI-1 levels irreversibly reduces the potential of colorectal cancer cells to initiate tumors.

**Therapeutic targeting of colorectal CICs**

Having established that BMI-1 inhibition has an irreversible effect on colorectal CIC function, we evaluated the ability of PTC-209 to inhibit the growth of an established primary human colon cancer xenograft in nude mice. Pharmacokinetic analysis demonstrated that PTC-209 effectively inhibited BMI-1 production in tumor tissue in vivo (Supplementary Table 5). The general health, blood indices and numbers of hematopoietic stem cells and peripheral blood leukocytes of control and BMI-1 inhibitor treatment groups were not altered (Supplementary Table 6, Supplementary Fig. 18 and Supplementary Results). For mice transplanted with cells from sample 01, 9 d following the initial drug administration, at which point control animals...
reached endpoint and were killed, the tumor volume of the PTC-209–treated group was significantly reduced \((P = 0.0005, \text{Fig. 6a})\). Continued treatment of animals with PTC-209 for an additional week did not affect the overall health of the mice, and tumors did not increase in size. Likewise, for mice given cells from sample 03, 9 d after PTC-209 initiation, the drug-treated tumors displayed a significantly smaller tumor volume as compared to the control tumors \((P = 0.002, \text{Fig. 6b})\). LS174T cell–derived tumors displayed significantly

Figure 6 Therapeutic targeting of the BMI-1–related self-renewal machinery. (a–e) Tumor growth curves of colorectal tumors that were treated with PTC-209, where each point represents the mean tumor volume ± s.e.m. of at least eight tumor measurements. mg kg\(^{-1}\), mg per kg body weight. (f,g) Tumor growth curve of recipients bearing LIM1215 cell–derived (f) and HCT1116 cell–derived (g) tumors that were previously treated with control or PTC-209 (data shown in d for LIM1215 and e for HCT1116); treatment was terminated and tumor size monitored for an additional 30 d without any additional treatment. (h) Frequency of colorectal CICs following PTC-209 treatment in vivo. At the end of in vivo dosing experiments (as shown in b and c), tumor cells were isolated and viable cells were injected into secondary animals in a limiting-dilution series; the recipients did not receive any further PTC-209 treatment during these secondary assays. Frequency and probability estimates were computed using the ELDA software. The mean and 95% CI are shown. \((i,j)\) Tumor growth curves of previously PTC-209–treated tumors. Colorectal tumors, which were treated in the experiments in d and retransplanted for the experiments in i or treated in the experiments in e and retransplanted for the experiments in j, were transplanted to assess possible development of resistance. Once transplanted cells formed tumors, recipients received daily PTC-209 for the indicated number of days. For a–g and i–j, \(P\) values were derived by comparing values from the control- or PTC-209–treated groups that were normalized to the tumor size on day 0 of each experiment \((* P < 0.05, ** P < 0.01 \text{ and} *** P < 0.001)\). Squares represent mean tumor volume, and data are expressed as mean ± s.e.m.
reduced tumor volumes in the BMI-1 inhibitor group ($P < 0.0001$, Fig. 6c). We saw similar results using tumors derived from human colon cancer cell lines LIM1215 and HCT116 cells (Fig. 6d,e). These data indicate that inhibition of BMI-1 with PTC-209 halts growth of preestablished tumors in vivo.

To test whether BMI-1 inhibitor–treated recipients should receive continuous BMI-1 inhibitor to control tumor size, we discontinued PTC-209 treatment and observed xenografts derived from two independent samples for the following 30 d. In recipients bearing LIM1215 cell–derived tumors, the previously PTC-209–treated and the control–treated recipients displayed similar tumor volumes when examined 30 d following PTC-209 termination (Fig. 6f). In recipients bearing HCT116 cell–derived tumors (from Fig. 6e), significantly smaller tumors were present 30 d following PTC-209 termination ($P = 0.008$; Fig. 6g). Although there is heterogeneity between these two models, for the HCT116 group, tumors from inhibitor–treated animals did not grow back when inhibitor treatment was stopped.

To confirm whether the tumors harvested at the end of BMI-1 inhibitor treatment exhibited an irreversible decrease in CICs, we transplanted tumor cells of two samples at the end of the in vivo dosing regimen into secondary recipients at limiting doses. PTC-209 treatment in primary tumors reduced the frequency of functional tumor-initiating cells by 16-fold for LS174T cell–derived tumors ($P < 0.0001$) and fourfold for sample 03 tumors ($P = 0.01$, Fig. 6h and Supplementary Table 7). These data demonstrate that in vivo treatment with a BMI-1 inhibitor reduces the frequency of functional colorectal CICs.

Finally, to assess whether tumors growing in secondary recipients were generated by cells with acquired resistance to PTC-209, we isolated and re-injected previously treated LIM1215 cell– and HCT116 cell–derived tumor cells into new recipients. Following tumor establishment, we treated recipients with PTC-209. Compared to the control, there was a reduction in tumor size following PTC-209 treatment for both samples (Fig. 6i), indicating that tumors remained sensitive to the BMI-1 inhibitor. Taken together, our data indicate that colorectal CICs are irreversibly impaired upon BMI-1 inhibition and that a small molecule targeting BMI-1 can control tumor growth in a preclinical model of primary human colorectal cancer.

**DISCUSSION**

Our results establish that BMI-1 regulates primary human colorectal cancer cells, including functionally defined CICs. The identification of a molecular regulator of CICs enabled us to successfully test the relevance of the cancer stem cell model in colorectal cancer; if CICs are clinically relevant, then therapeutically targeting them should result in effective and durable therapies.

Self-renewal is largely measured in functional assays that require proliferation, making it difficult to investigate molecules that affect both processes. BMI-1 does not function solely as a self-renewal regulator, as it is also expressed in tumor cells that do not possess CIC activity. Although BMI-1 downregulation caused cell cycle exit and increased apoptosis in both phenotypically defined CIC and non-CIC fractions of primary colorectal cancer (Supplementary Figs. 16 and 17), these effects were much smaller than the impact on CIC self-renewal. In addition, temporary downregulation of BMI-1 by transient exposure to the BMI-1 inhibitor or siRNAs (Supplementary Results) against BMI-1 resulted in permanent impairment of functional colorectal CICs. Moreover, effects on self-renewal were not obtained by transiently inhibiting *PCNA*, the gene encoding proliferating cell nuclear antigen, which is involved in proliferation (Supplementary Fig. 19 and Supplementary Results). Collectively, our data indicate that colorectal cancer cells are highly dependent on BMI-1, which functions as a pleiotropic regulator that maintains the viability and proliferative capacity of colorectal cancer cells in general but also governs CIC self-renewal.

The finding that BMI-1 plays a central part in colorectal CIC self-renewal opened an avenue to target the stemness function of CICs with small-molecule inhibition. PTC-209 reduced tumor volume and reduced the number of functional colorectal CICs, even following short-term treatment, a result that contrasts with the enrichment of CICs observed following conventional chemotherapy. The effectiveness of transient inhibition suggests that colorectal cancer cells may be reliant on BMI-1 to sustain growth and clonal maintenance. An important consideration is how PTC-209 will affect normal human intestinal stem cells. In mice expressing Cre-inducible diphtheria toxin receptor, targeted ablation of *Bmi1*-positive cells using diphtheria toxin leads to crypt loss. However, with PTC-209, no changes in digestive function in the treated mice were identified (data not shown), indicating that the dose we used to lower tumor burden does not noticeably affect the intestinal system. Of note, in our studies, we lowered BMI-1 levels in xenografts and in cell culture, whereas mouse studies analyzed the effect in knockout animals where Bmi-1 is absent. Interestingly, transient depletion of Bmi-1 in cells leads to crypt loss, but the remaining crypts can repopulate the damaged area through crypt fission.

Targeting CIC stemness determinants including self-renewal has been proposed as a therapeutic goal and our study provides strong evidence for this concept. A number of molecules and gene signatures that underlie stemness have been identified, and future studies should be undertaken to identify and target other key pathways driving self-renewal in the CIC subset. Our study provides a template for preclinical evaluation of other CIC targets that focuses on primary samples and clonal *in vitro* and *in vivo* CIC assays.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Note:** Any Supplementary Information and Source Data files are available in the online version of the paper.

**ACKNOWLEDGMENTS**

We thank R. Lopez and P. Lo from the Animal Research Centre, University Health Network and A. Khandani, P. Penttila, and S. Zhao from the Sickkids Flow Cytometry facility for their skillful assistance. We are grateful to all members of the Dick lab, especially M. Anders, E. Laurenti, N. Mbong, S. Doulatov, M. Milyavsky and F. Notta, as well as C. Gedy and J. Wang for their advice and critical comments. We thank E. Lechman (University Health Network, Toronto), J. Moffat (University of Toronto) and T. Chiba (Chiba University) for lentiviral vectors expressing shRNAs to BMI1. We also thank members from the Department of Pathology (Toronto General Hospital), including F. Meng, M. Sukhram, V. Son, H. Begley and P. Shaw, for providing colon cancer samples. This work was supported by funds to PTC Therapeutics from the Wellcome Trust and to J.E.D. from 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, a Canada Research Chair, the Princess Margaret Hospital Foundation and funds to E.L.-F. by Fonds National de la Recherche, Luxembourg and the Marie Curie Actions of the European Commission (FP7-COFUND, PDR 2012-2 4735314). This research was funded in part by the Ontario Ministry of Health and Long Term Care (OMOHLT). The views expressed do not necessarily reflect those of the OMOHLT.

**AUTHOR CONTRIBUTIONS**

ARTICLES


COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Colorectal cancer cell isolation and xenograft establishment. Human colorectal cancer tissue was obtained with patient consent, as approved by the Research Ethics Board at the University Health Network in Toronto. Tumor specimens were washed in PBS, minced with a razor blade and incubated with collagenase A (3 mg mL⁻¹, Roche) for 60 min at 37 °C. After this enzymatic digestion, the sample was filtered through a 40-µm cell strainer, and the cells were mixed with ammonium chloride solution (Stemcell Technologies) for 5 min to lyse red blood cells. Cells were then used for downstream applications.

Eleven patient-derived tumors were used (ranging from stage II to IV, including both tumors from the colon and liver metastases) to capture the diversity of primary colorectal tumor tissue. For the generation of xenografts, cells were injected with Matrigel under the capsule of NSG and/or NOD-SCID mice (male or female, 8-10 weeks of age) as previously described. For LDA analysis and inhibitor studies, cells were injected subcutaneously. Animal work was carried out in compliance with the ethical regulations approved by the Animal Care Committee, University Health Network, Toronto, Ontario, Canada.

Colorectal cancer in vitro culture. Isolated colorectal cancer cells were plated in DMEM/F-12 containing epidermal growth factor (EGF) and fibroblast growth factor (FGF). The in vitro culture medium contained DMEM/F-12:1 (1:1 ratio) supplemented with penicillin-streptomycin (1%), fungizone (1 µg mL⁻¹), l-glutamine (2 mM), nonessential amino acids, sodium pyruvate (1 mM), HEPES, heparin (4 µg mL⁻¹), B27 supplement ( Gibco), N2 supplement ( Gibco), heparin (Sigma), EGF (20 ng mL⁻¹) and basic FGF (10 ng mL⁻¹). Cells were cultured in suspension culture flasks at 37 °C in a 5% CO₂–humidified incubator. Under these conditions, cells grew as nonadherent aggregates, or spheres, and were passaged weekly by dissociating the cells with trypsin and replating in aforementioned medium. The patient-derived cell lines, as well as the cell lines LS174T, LIM1215 and HCT116, were authenticated using short tandem repeat profiling and tested for mycoplasma.

Viral transduction of colorectal cancer cells. Cells were dissociated and filtered through a cell strainer to eliminate clumps of cells. Cells were diluted to 5E5 cells per mL and plated in 500-µL volumes in a 24-well dish. Viral particles were added at a multiplicity of infection of 1 to 20 using virus that was concentrated by ultracentrifugation. After a 48-h transduction, cells were washed and resuspended in culture medium for in vitro culture or injected into recipient mice as described above. The shRNA vectors expressing shBMI-1 or shLUC also express eGFP, which allowed tracking of the percentage of transduced cells for each sample. The transduction efficiency varied with the amount of viral particles added. For LS174T cells, >90% transduction efficiency was observed as assessed by flow cytometric analysis of eGFP. For the patient-derived samples that had been cultured in vitro under conditions that enrich for CICs, usually >60% of the cells were transduced with the shRNA vectors, and commonly >90% transduction efficiency was observed. For patient-derived colon cancer cells that had not been previously cultured in vitro, the transduction efficiency varied across samples but was generally <50% (sample 05, 24%; sample 06, 43%; sample 07, 13%; sample 08, 21% and sample 09, 18% eGFP+ cells).

Limiting dilution analysis. For in vitro LDAs, transduced colorectal cancer cells were dissociated into single cells, and eGFP-expressing cells were sorted using fluorescence-activated cell sorting into 96-well plates at the indicated cell doses. SytoxBlue was used to exclude dead cells. For each cell dose, at least 12 wells were seeded with cells, and for the lower cell doses, generally 96 wells were plated at a single-cell dose. Six to eight weeks later, wells containing spheres were scored, and the number of positive wells was used to calculate the frequency of sphere-forming units using the ELDA software (http://bioinf.wehi.edu.au/software/elda/index.html) provided by the Walter and Eliza Hall Institute. For in vivo LDAs, transduced colorectal cancer cells either from primary tumors or from cultured cells were dissociated into single cells or diluted serially to the desired cell doses. Cells were injected subcutaneously into the flanks of NOD-SCID or NSG mice, and the number of tumors formed out of the number of sites injected was scored to determine the frequency of colorectal CICs calculated using the ELDA software. In cases where the transduced cell population was not eGFP+ before re-injection, the percentage of eGFP-expressing cells was used to calculate the eGFP+ tumor mass, which was further used to calculate the frequency of initiating cells.

Experimental approach to quantify the self-renewal ability of a single colorectal CIC in vivo. This protocol is in reference to Figure 3e. Following shRNA transduction, a limiting dilution series of cells is injected subcutaneously into primary mice to calculate the cell dose at which tumors are derived by a single CIC. Tumor cells seeded at this cell dose and thus derived from a tumor that was generated by one CIC, are isolated and viable cells are re-injected into secondary animals in a limiting dilution series to enumerate the secondary CIC frequency, which reads out the number of colorectal CICs present in the primary clonal tumor. By determining the number of CICs in the primary tumors (through the use of the serial LDAs), one can compute how many colorectal CICs were generated by the original CIC that seeded the tumor.

Viral vectors. The H1-driven shRNA–elongation factor-1 (EF-1x)–eGFP vector had shRNA sequences to BMI-1 and luciferase (as the control) and was a gift from T. Chiba. The second shRNA, contained in the H1–shRNA–human phosphoglycerate kinase (hPGK)–eGFP vector, had shRNAs designed to target BMI-1 and RFP as a control. Viral vectors (pLKO1) were obtained from The RNAi Consortium shRNA Library (shGFP, shBMI-1 A2 (shRNA 3 in this study) and shBMI-1 A5 (shRNA 4 in this study). Viral transduction of colorectal cancer cells using the pLKO1 shRNAs was carried out by seeding cells in 7T5s with 6E6 cells per flask, and 2 mL of appropriate virus was added to reach a total volume of 25 mL. The bidirectional overexpression MA1 vector was used to overexpress humanized Revilla luciferase as a control or BMI-1 cDNA; transgene expression was driven by the spleen focus-forming virus promoter.

T cell factor/lymphoid enhancer factor reporter assay. Cells were stably transduced with lentiviral TCF/E-β-deficient reporter vectors (Signal Reporter, Qiagen), and clones were selected using puromycin and eGFP cell sorting. For the reporter assay, cells were treated for the indicated times and concentrations. Cells were washed, stained with SytoxBlue for viability and processed for flow cytometry using the BD LSR II HTS. The previously characterized Wnt inhibitor iCRT14 was used as a control (Sigma). Cells were treated with iCRT14 for 24 h before processed for flow cytometry.

CDKN2A studies. Retroviral RNAi vectors were used to target mouse p63 (as a control, shCtrl) and human p14, p16 and p14/p16 (combined knockdown using an shRNA that targets the common exon 2 of the CDKN2A locus) as previously described. Cells were transduced and selected with hygromycin. Transduced cells in each condition were subsequently transduced with either shBMI-1– or shLUC-expressing lentiviruses. After primary spheres formed, cells were dissociated, and eGFP+ cells were plated in a limiting dilution series in order to measure sphere re-initiation potential.

Western blotting. Cells were lysed with RIPA lysis buffer containing protease inhibitors (Roche). Lysates (10–100 µg per lane) were separated by SDS-PAGE, and protein was transferred onto a polyvinylidene fluoride membrane (Immobilon-P membrane, Millipore). Specific antibodies to BMI-1 (1:1,000; ab14389, Abcam), p15 (1:750; 4822, Cell Signaling), p16 (1:100; JC8, Santa Cruz), p18 (1:1,000; 118.2, Santa Cruz), β-catenin (1:1,000; 5F1, Calbiochem), actin (1:5,000; 8226, Abcam) and GAPDH (1:10,000; 71.1, Sigma) were detected using the appropriate secondary horseradish peroxidase–conjugated antibodies (Amersham) and visualized by an enhanced chemiluminescence detection system (Pierce, GE Healthcare).

Flow cytometry and FACs. Cells were stained with antibodies to human CD44 (1:100; 550989, BD Biosciences) and/or CD133/1 (1:50; 130-090-826, Milteny), as well as SytoxBlue to mark viable cells. Antibodies to CD45 (1:100; 340934, BD) and CD31 (1:100; 340297, BD) were used to exclude hematopoietic and endothelial cells, respectively. For K67 (1:50; 51-36535X, BD) and cleaved caspase 3 (1:50; 550821, BD) analyses, cells were fixed and permeabilized before the intracellular staining was carried out. Additional antibodies used in this manuscript were specific for CD166 (1:100; 559263, BD Pharmingen), CD29 (1:100; 559883, BD Pharmingen), CD24 (1:100; 555427, BD Pharmingen) and
and injected into mice or plated in limiting doses affected tumor cell growth, cells were plated with the inhibitor for 4 d of viable cells was recorded after 6 and 9 d in triplicate, and dose-response curves were used, consisting of four mixed siRNAs targeting each gene (BMI-1 or PCNA). As a control, a nontargeting siRNA was used. DMEM/HAM-F12 1:1 (antibiotic-free) medium containing either 8% HiPerFect transfection reagent (Qiagen) or 200 nmol siRNA was prepared. 50-µl aliquots of transfection reagent were first transferred into appropriate wells of a 24-well plate before adding 50 µl of siRNA solution and gently mixing the two solutions together by pipetting. The plate was incubated at room temperature for 20 min with occasional swirling. Dissociated colon cancer cells were plated at 7.5 × 10^5 cells per mL, and 0.4-ml aliquots were added to the transfection mixture. The final concentration of the transfection mixture was 0.8% HiPerFect and 20 nmol siRNA. Cells were incubated for 24 h before the transfection mixture was removed and replaced with fresh suspension medium (full with antibiotics). Cells were returned to the incubator for 6 d to allow the target protein to be knocked down. Single-cell suspensions were generated, and cells were seeded with a fixed number of viable cells per well of a 96-well plate for limiting-dilution analysis (1,000, 100, 10 and 1 cell per well).

BMI-1 inhibitor studies. To determine the IC50 values, 100,000 cells were plated, and four different concentrations (tenfold dilutions starting at 10 µM) of BMI-1 inhibitor PTC-209 were added; DMSO was used as a control. The total number of viable cells was recorded after 6 d and 9 d in triplicate, and dose-response curves were fitted to the data. To determine whether pretreatment with the inhibitor affected tumor cell growth, cells were plated with the inhibitor for 4 d in vitro and injected into mice or plated in limiting doses in vitro without adding further inhibitor. Trypan blue exclusion was used to count viable cells. The tumor weights were recorded, and the in vitro sphere-initiating cell frequency was calculated after inhibitor treatment by evaluating the number of wells containing spheres. For the experiments where LDAs were set up following recovery of PTC-209-treated cells, 6-well plates were seeded with 1E6 cells per well (4 mL medium per well) and incubated overnight. Cells were subsequently treated for 4 d in triplicate with either DMSO vehicle or PTC-209 (0.01, 0.1, 1 and 10 µM). Drug treatments were washed off and 4 mL fresh suspension medium added to all wells. To assess cell viability following the 4 d treatment window, cells were trypsinized and counted at 0, 24, 72 and 120 h after removal of the drug. Long-lasting effects of the drug treatment on sphere-forming ability were assessed by plating LDAs (50,000, 10,000, 1,000,100, 10 and 1 cell per well) using the cells obtained 120 h after the 4-d drug treatment.

Untranslated region–mediated luciferase reporter expression and cytotoxicity measurements. HEK293 cells were transfected with a GEMS reporter vector that contains the luciferase open-reading frame flanked by and under post-transcriptional control of the BMI-1 5’ and 3’ UTRs. The resulting stable cells (FS) were treated with PTC-209 or vehicle control overnight, and then luciferase reporter activity and cytotoxicity were determined using Bright-Glo and CellTiter-Glo assays (Promega), respectively. The assays were run in triplicate for each point, and the percentage of inhibition was calculated against vehicle control.

Rescue experiments with BMI-1-KD or BMI-1-OE and PTC-209. Following transduction of colon cancer cells with the shBMI-1 or shLUC virus, PTC-209 was added. The number of surviving transduced (eGFP+) cells was determined using SytoxBlue dead stain exclusion by flow cytometry. For both the control and BMI-1-KD group, the number of viable eGFP+ cells in the PTC-209 treatments was normalized to the mean number of cells in the 0-nM PTC-209–treated group to allow for comparison between different experiments. Data are shown as a pool of three, four and two experiments for samples 01 and 03 and LS174T cells, respectively, in Figure 4f. Individual data are shown in Supplementary Figure 9. Likewise, colon cancer cells from samples 03 and 11 and LS174T cells were transduced with lentiviral vectors overexpressing luciferase (LUC-OE) or BMI-1 (BMI-1-OE); three to sixfold BMI-1–OE was observed (Supplementary Fig. 11a). 5 × 10^3 to 1 × 10^4 transduced cells were plated in the presence of the indicated concentrations of PTC-209. Four days following PTC-209 addition, the number of surviving transduced cells was recorded using SytoxBlue dead stain exclusion and eGFP expression by flow cytometry. For both the LUC-OE and BMI-1-OE groups, the number of viable eGFP+ cells in the PTC-209 treatments was normalized to the average number of cells in the 0-nM PTC-209–treated group.

In vivo PTC-209 dosing. For the experiments where mice were dosed with the drug in vivo, tumor cells were injected subcutaneously into nude mice (male, aged 8–10 weeks), and when the tumors reached an approximate 0.2 cm^3 volume, PTC-209 was administered subcutaneously once a day at a dose of 60 mg per kg body weight (control animals received equal volumes of vehicle, 14% DMSO, 36% polyethylene glycol 400 and 50% polypropylene glycol). Tumor volume measurements were recorded every 3–7 d until the endpoint was reached.

Statistical analyses. 11 different patient tumor samples (ranging from stage II to IV, including both tumors from the colon and liver metastases) were used in this study to capture the diversity of colon cancer specimens. Sample sizes were dependent on the number of viable cells obtained following single-cell isolation from tumor tissue. Generally, at least five animals were used per group. For limiting-dilution analyses, at least ten tumors were analyzed to ensure a large enough sample for statistical comparison. No animals or samples were excluded from any analysis. Animals were randomly assigned groups for in vivo inhibitor studies; no formal randomization method was applied when assigning animals for treatment. Group allocation and outcome assessment was not done in a blinded manner, including for animal studies. P values were derived using two-tailed Student’s t-tests, unless otherwise indicated in the figure legend. LDAs for frequency determinations, as well as the corresponding Pvalues, were generated using the ELDA software, which took into account whether the assumptions for LDA were met (http://bioinf.wehi.edu.au/software/elda/index.html, provided by the Walter and Eliza Hall Institute). In each group of data, estimate variation was taken into account and is indicated in each figure as s.d. or s.e.m. For all graphs, *P = 0.01–0.05, **P = 0.001–0.01 and ***P < 0.001.