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J Clin Invest. 1992;**89**(5):1523-1527. <https://doi.org/10.1172/JCI115744>.

Research Article

Colo 320 cells are colonic carcinoma cells known to express abundant c-myc mRNA. Based on the response of several hematopoietic cell lines to chemical inducers of differentiation, we reasoned that such agents might have similar inductive activity in Colo 320 cells. Accordingly, we exposed Colo 320 cells to 5 mM sodium butyrate (NaBT) for 7 d. C-myc expression decreased threefold and self-replicative potential decreased (defined as a greater than 60% decrease in colony-forming capacity in soft agar that did not contain inducer). In an effort to demonstrate a direct cause and effect between myc expression and the colony-forming capacity of Colo 320 cells, we exposed these cells to a 15-base antisense c-myc oligonucleotide (complementary to the translation initiation region of exon II). Cells were also exposed to equimolar (20 microM) amounts of sense and missense oligonucleotides. Subsequently, cells were incubated at 10, 20, 30, and 40 microM antisense DNA for 16 h, then washed and plated in oligonucleotide-free agar medium. We demonstrated that: (a) the oligomers were stable in the extracellular medium and in the cell cytoplasm; (b) the uptake of the oligonucleotides was 0.7%; (c) sense and missense oligonucleotides had no effect on colony-forming capacity; and (d) the antisense c-myc oligonucleotide resulted in a 40-75% concentration-dependent decrease in colony-forming capacity. The specific inhibition of colony-forming capacity by antisense DNA [...]

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c-myc Antisense Oligonucleotides Inhibit the Colony-forming Capacity of Colo 320 Colonic Carcinoma Cells

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Abstract

Colo 320 cells are colonic carcinoma cells known to express abundant *c-myc* mRNA. Based on the response of several hematopoietic cell lines to chemical inducers of differentiation, we reasoned that such agents might have similar inductive activity in *Colo 320* cells. Accordingly, we exposed *Colo 320* cells to 5 mM sodium butyrate (NaBT) for 7 d. *C-myc* expression decreased threefold and self-replicative potential decreased (defined as a > 60% decrease in colony-forming capacity in soft agar that did not contain inducer). In an effort to demonstrate a direct cause and effect between *myc* expression and the colony-forming capacity of *Colo 320* cells, we exposed these cells to a 15-base antisense *c-myc* oligonucleotide (complementary to the translation initiation region of exon II). Cells were also exposed to equimolar (20 μ M) amounts of sense and missense oligonucleotides. Subsequently, cells were incubated at 10, 20, 30, and 40 μ M antisense DNA for 16 h, then washed and plated in oligonucleotide-free agar medium. We demonstrated that: (a) the oligomers were stable in the extracellular medium and in the cell cytoplasm; (b) the uptake of the oligonucleotides was 0.7%; (c) sense and missense oligonucleotides had no effect on colony-forming capacity; and (d) the antisense *c-myc* oligonucleotide resulted in a 40–75% concentration-dependent decrease in colony-forming capacity. The specific inhibition of colony-forming capacity by antisense DNA suggests that the role of *myc* expression in *Colo 320* cells is similar to its role in hematopoiesis, and that the failure to inhibit *myc* expression maintains colony-forming capacity. This system provides a new strategy for inducing differentiation and may provide further insight into the genetic factors that govern the process of colonic carcinogenesis. (*J. Clin. Invest.* 1992. 89:1523–1527.)
Key words: colonic neoplasms • cell differentiation • oncogenes • *myc* • antisense

Introduction

The *c-myc* gene is highly expressed in proliferating, undifferentiated cells, and encodes for a nuclear phosphoprotein that is thought to participate in functions that control cell proliferation and differentiation. The *myc* proteins have been more

recently described as interacting with Max, a basic helix-loop-helix zipper protein, and both may belong to a general class of DNA-binding transcription factors (1, 2). The evidence for linkage of *myc* expression and the undifferentiated phenotype has been demonstrated in several cell types, but has most consistently been found in cells of hematopoietic origin (3, 4). Gowda et al. (5) and Emilia et al. (6) demonstrated that the *c-myc* gene was expressed in immature normal myeloid precursor cells, but was repressed in terminally differentiated granulocytes and their immediate precursors. In addition, human acute myeloid leukemic cells that fail to differentiate in vitro also fail to suppress *c-myc* transcription (6). The linkage of *c-myc* expression with hematopoietic cellular differentiation has been formally tested in two ways. First, Dmitrovsky et al. (7) noted that murine erythroleukemia cells transfected with a highly promoted human *c-myc* gene no longer exhibited its normally inducible differentiation program. Secondly, Holt et al. (8) demonstrated that repression of *c-myc* expression using antisense *c-myc* oligodeoxyribonucleotides induced differentiation of HL-60 myeloid leukemic cells. Therefore, in hematopoietic cells the protein *myc* functions to maintain the primitive phenotype and the repression of *c-myc* is a necessary and sufficient step to commit certain primitive cells to differentiate.

Because most nonhematopoietic tumor cells also express *c-myc*, we reasoned that the differentiation function of *c-myc* was likely a broadly applicable principle in multiple lineages (9). Accordingly, we tested the applicability of this model in *Colo 320* cells, a human colonic carcinoma cell line. They were established from a primary human colonic carcinoma and were noted to secrete ACTH, PTH, and serotonin (10). Hence, these were thought to be representative of a neuroendocrine cell type, one of the three cell types found in human colonic mucosa (11). This cell type has been described in other primary colonic tumors and is often referred to as small cell carcinoma of the colon. Clinically, these tumors behave in an unusually malignant manner. Some investigators believe these neuroendocrine cells to be of neural crest origin; however, this is highly controversial, and there is a substantial body of literature that supports the uniform origin of epithelial, mucous, and endocrine cells in colonic mucosa from a common cell of origin (12–15). The *Colo 320* cell line is representative of a small cell neuroendocrine colonic carcinoma. Of particular interest to our laboratory, these cells have multiple copies of *c-myc* and express abundant *c-myc* mRNA (16).

Because markers of differentiation are less well defined in these cells than in cells of the myeloid lineage, we chose to use loss of colony-forming capacity as a marker of differentiation (17, 18). We found that sodium butyrate, a known differentiation-inducing agent (19–21), repressed *c-myc* expression and stimulated differentiation. Based on subsequent experiments using antisense *c-myc* oligonucleotides, we report that the inhibition of *c-myc* expression per se was sufficient to account for a decreased colony-forming capacity.

This work presented in part in May, 1990, at the American Federation for Clinical Research, Washington, D.C.

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Received for publication 6 May 1991 and in revised form 16 December 1991.

Methods

Chemically induced differentiation

Colo 320 cells [CCL 220.1; Amer. Type Culture Collection, Rockville, MD], have been described previously (16). Control cells for chemical differentiation were grown in RPMI 1640 (Gibco Laboratories, Grand Island, NY) with 10% heat-inactivated FCS at 37°C/5% CO₂ for 2–7 d. To induce differentiation, the cells were cultured in RPMI with 10% FCS with 5 mM sodium butyrate for 2–7 d. Serially harvested cells (days 2–7) were evaluated for colony-forming capacity, other markers of differentiation, and *c-myc* expression.

Markers of differentiation

Colony-forming capacity. 10⁴ viable cells were suspended in 1 ml 0.3% agar (Difco Laboratories Inc., Detroit, MI) made with RPMI supplemented with 10% FCS without sodium butyrate, and layered onto 0.6% agar in 35 mm plates with 2 mm² grids. All samples were plated in quadruplicate. The number of colonies (> 40 cells/cluster) was estimated on days 1–16 (22). The number of colonies/plate was calculated as follows: Average number colonies/square × 185 squares/plate (Average number colonies = number colonies in 5 squares/5). The mean values of the quadruplicate determinations and their standard deviations were recorded. A significant decrease in colony-forming capacity was defined as a greater than or equal to 60% decrease in colony-forming ability in the exposed cells when compared with control cells (not exposed to chemical inducers of differentiation).

Phenotypic changes. Cytochrome preparations (10⁵ cells; Shandon cytochrome) were fixed in ethanol: glacial acetic acid (3:1), and stained with hematoxylin and eosin. Immunohistochemical stains for carcinoembryonic antigen (Hybritech, Inc., San Diego, CA), epithelial keratin (23), and neuron specific enolase (Boehringer-Mannheim Biochemicals, Indianapolis, IN) were performed. Alkaline phosphatase activity was measured using *p*-nitrophenyl phosphate as a substrate (20, 24–27).

Tumor formation in mice. Tumor formation was assessed by injecting groups of five male athymic Balb/C *nu/nu* mice with either 1cc of sterile PBS, 10⁷ control *Colo 320* cells, or 10⁷ *Colo 320* cells exposed to 5 mM sodium butyrate for 7 d. Mice were housed under sterile conditions for 4 wk. Mice were weighed and tumors measured weekly. After 4 wk, mice were necropsied and the size of the tumors noted. Tumors were fixed in 10% formalin, and 5- μ m sections were evaluated using hematoxylin and eosin stains (28).

Poly-(A)⁺ RNA isolation–Northern blot analysis

2.5 × 10⁶ control cells or cells cultured in medium containing RPMI with 10% FCS and 5 mM sodium butyrate for 2–7 d were harvested, and poly-(A)⁺ RNA extracted (29). Control and sodium butyrate-treated cells were washed in sterile PBS and lysed (0.2 M NaCl, 0.2 M Tris-Cl, 1.5 mM MgCl₂, sodium dodecyl sulphate, and proteinase K). The lysates were incubated with oligo(dT)cellulose (Collaborative Research Inc., Bedford, MA), suspended in binding buffer (0.5 M NaCl, 10 mM Tris-Cl), and eluted with elution buffer (10 mM Tris-Cl, pH 7.5). 5 μ g of poly-(A)⁺ RNA derived from control cells and from cells exposed to 5 mM sodium butyrate for 2–7 d were subjected to electrophoresis in 1.0% agarose denaturing gels and transferred to nitrocellulose membranes (30). Filters were prehybridized and then hybridized with ³²P-labeled DNA (10⁶ cpm/ml) overnight at 42°C in hybridization buffer (50% formamide/Denhardt's/salmon sperm DNA). Filters were then washed in 2 × standard saline citrate SSC twice, 1 × SSC, 0.1 × SSC, dried, and exposed to x-ray film at –70°C (31).

The filters were probed with a 1.5-kb *Sst* *c-myc* fragment from pHSR-1 (8) containing exon II and reprobed with a 2.1-kb *Pst*I/*Bam*H1 fragment of human γ -actin cDNA subcloned into pGEM3 (32). Probes were radiolabeled using Oligolabeling kit (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) and [³²P]dCTP (sp act 3,000 Ci/mmol; New England Nuclear, Boston, MA) to a sp act of 2 × 10⁸ cpm/ μ g DNA.

Autoradiography was performed and *myc* and actin transcripts evaluated by densitometric scanning. A quantitative estimate of *myc* and actin expression was determined by comparing densitometric values.

Oligonucleotide-induced differentiation

Antisense, sense, and missense *c-myc* oligonucleotides were obtained from the Midland Certified Reagent Co., Midland, TX. These 15-base deoxynucleotides have been described previously (8). The antisense oligonucleotide was complementary to the translation initiation region of the *c-myc* gene located at the beginning of exon II. To insure that the missense oligonucleotide (12 mismatched bp) did not inadvertently anneal to a known sequence, the European Molecular Biology Library (IntelliGenetics, Mountain View, CA) was searched for homologous sequences (allowing up to three mismatched base pairs). The missense oligonucleotide was complementary only to a few reading frames of HTLV-1 and a portion of the neural cell adhesion molecule, NCAM. Each oligomer was labeled using T4 polynucleotide kinase (Boehringer-Mannheim Biochemicals) and α -[³²P]ATP (sp act 6,000 Ci/mM; New England Nuclear). Cell suspensions were evaluated for stability and uptake of the oligonucleotide, as previously described (8, 33), and the resulting effect on colony-forming capacity.

Stability. To determine that the oligonucleotides were stable under experimental conditions, 5' end-labeled pentadecadeoxynucleotides were incubated for 16 h at 37°C in PBS alone, in the presence of 5 × 10⁸ *Colo 320* cells in RPMI with 10% heat-inactivated FCS, and in medium and 10% serum alone. Aliquots from each of these fractions were ethanol precipitated and electrophoresed in a 7 M urea, 8% polyacrylamide gel.

Cellular uptake of oligonucleotide. 5 × 10⁵ *Colo 320* cells in 100 μ l RPMI with heat-inactivated FCS were exposed to 10 μ M of 5' end-labeled oligonucleotide for 16 h at 37°C. FCS was heated to 65°C for 30 min to inactivate DNases. The cells were washed twice and the supernatants saved (extracellular fraction). Nonidet-P40 lysis buffer was then added, and nuclei were prepared by centrifugation. This phase represented the cytoplasmic fraction. The remaining material was the nucleoplasmic fraction. All uptake experiments were performed in duplicate. The percent uptake by the cells was calculated by the number of counts in the cytoplasmic fractions/total number of counts in the cell pellet, cell wash, and culture-medium supernatant (8, 33). To insure that the radioactive counts in the cytoplasmic fraction was due to labeled probe rather than a labeled phosphate, ethanol-precipitated samples of the cytoplasmic fraction were electrophoresed in 7 M urea/8% polyacrylamide gels and autoradiographed.

Oligonucleotide exposure. 20 μ M of *c-myc* antisense, sense, or missense oligonucleotides were added to 10⁵ *Colo 320* cells in 100 μ l of RPMI with heat-inactivated serum, and incubated for 16 h at 37°C. Cells were washed and colony-forming capacity determined. In subsequent experiments, cells were exposed to 10, 20, 30, or 40 μ M antisense oligonucleotide for 16 h at 37°C to demonstrate a dose-dependent decrease in colony-forming capacity. Viability of cells was demonstrated by trypan blue exclusion.

Results

Sodium butyrate inhibited *c-myc* mRNA accumulation and colony-forming capacity in *Colo 320* cells. *Myc* expression began to decrease after 2 d exposure to sodium butyrate (see Fig. 1), and after a 5-d exposure the cells showed a significant decrease (\geq 60%) in colony-forming capacity (see Fig. 2). Other markers of differentiation such as morphological changes, alkaline phosphatase synthesis, and immunohistochemical stains were not helpful. Tumors formed in 9 of the 10 athymic mice injected with *Colo 320* cells. Tumor size and histology were similar in mice-receiving control cells and cells exposed to sodium butyrate for 7 d. Because *c-myc* repression preceded the significant decline in colony-forming capacity, we tested a

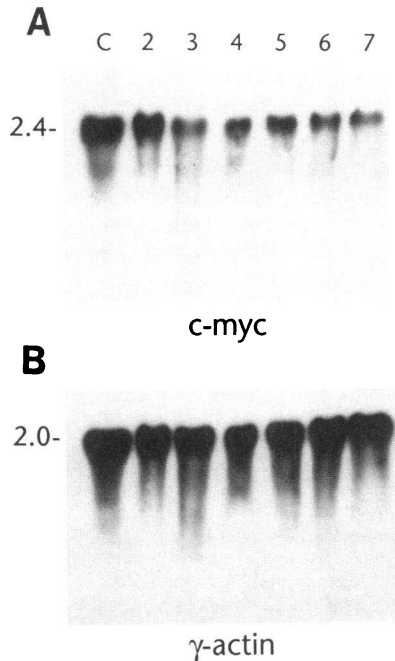


Figure 1. *Myc* expression in *Colo 320* cells exposed to 5 mM sodium butyrate for 0–7 d. 5 μ g of poly-(A)+RNA were loaded into each lane and electrophoresed in a 1.0% agarose denaturing gel. The 2.4-kb *myc* transcript is shown in *A* and the 2.0-kb actin transcript in *B*. A significant decrease in *myc* mRNA is noted in *Colo 320* cells after 2 d exposure to sodium butyrate.

possible cause and effect relationship between *c-myc* expression and colony-forming capacity by using antisense methodology.

The uptake of 5' end-labeled oligonucleotides (antisense, sense, and missense) by *Colo 320* cells was 0.6–0.8% after 16 h. Denaturing gel electrophoresis of the labeled oligonucleotide in the extracellular medium and in the cytoplasm of the cultured cells demonstrated that the oligonucleotide was stable for up to 16 h in the presence of cells (Fig. 3).

Colony-forming capacity was decreased by 50% in the antisense-exposed cells, but not in the cells exposed to sense or missense oligonucleotides (Fig. 4). Moreover, exposure to increasing doses of antisense oligonucleotide resulted in a dose-dependent decrease in colony-forming capacity (Fig. 5). Cell viability was 95% in both control and antisense exposed cells when assessed by trypan blue exclusion.

Discussion

Colo 320 cells are small cell neuroendocrine colonic carcinoma cells known to actively express the *c-myc* protooncogene. Based on prior observations in hematopoietic cells, we pro-

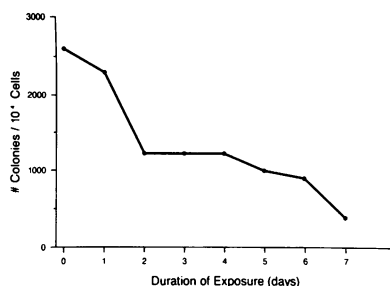


Figure 2. The colony-forming capacity of *Colo 320* cells after exposure to 5 mM sodium butyrate for 1–7 d. The number of colonies/plate were determined after the cells had been cultured on double-agar plates for 15 d. Note that a 60% decrease in colony-forming capacity occurs after 5 d exposure. Each value is the mean of quadruplicate determinations.

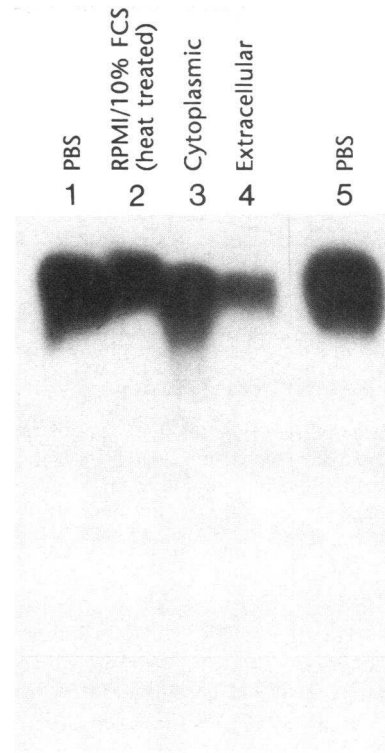


Figure 3. Stability of 5' α [32 P]ATP-labeled-antisense *c-myc* oligonucleotide. The 15-base nucleotide was incubated in PBS (lanes 1 and 5), in medium with heat-inactivated serum (lane 2), and in the presence of *Colo 320* cells for 16 h at 37°C. The extracellular medium was removed and the cells lysed. The cell lysates (cytoplasmic fraction) and extracellular fractions were ethanol precipitated, and the oligonucleotide loaded into lanes 3 and 4, respectively. Oligonucleotide samples were electrophoresed in a 7-M urea/8% polyacrylamide gel (see Methods).

posed that *myc* expression was necessary to maintain the undifferentiated phenotype in these cells. Using sodium butyrate as an inducing agent, we first aimed at defining a reliable and reproducible indicator of this phenotype and the induction of a more differentiated phenotype. Light microscopic morphology, immunohistochemical stains, quantitative analyses of alkaline phosphatase synthesis, and tumor-forming ability in athymic mice and colony-forming capacity were independently assessed. Decreased colony-forming capacity was a reproducible indicator of commitment. When evaluating the cytocentrifuge preparations of induced cells, we could not reproducibly identify a change in cell morphology. Immunohistochemical stains (carcinoembryonic antigen, keratin markers [AE1, AE3], and neuron-specific enolase) were negative in both induced and uninduced cells; this is not totally unex-

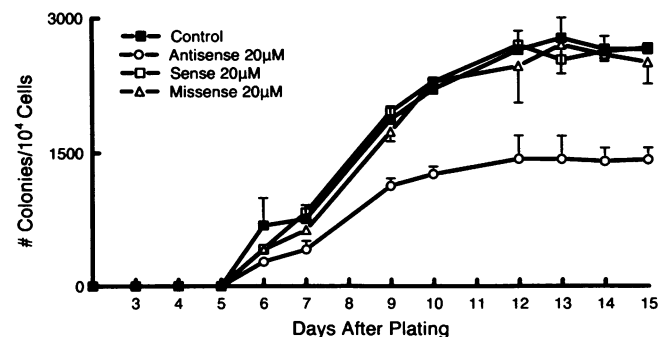


Figure 4. Colony-forming capacity of *Colo 320* cells exposed to 20 μ M antisense, sense, or missense oligonucleotide for 16 h at 37°C. Cells were washed and 10^4 viable cells cultured on double-agar plates. Colonies were counted on days 1–15. Each value is the mean \pm SD of quadruplicate determinations.

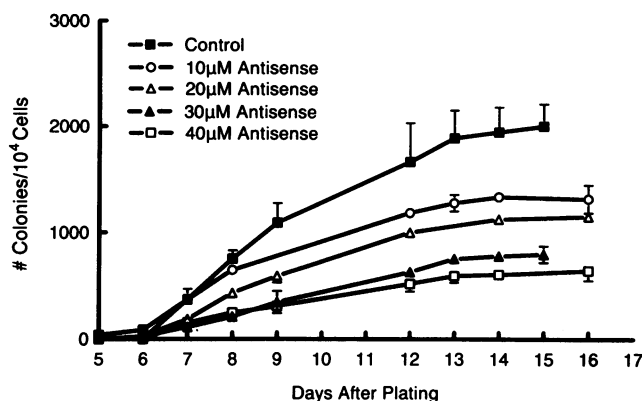


Figure 5. Colony-forming capacity of *Colo 320* cells after exposure to increasing concentrations of antisense *c-myc* oligonucleotide for 16 h at 37°C. Cells were washed and 10^4 viable cells plated on double-agar plates. Colonies were counted on days 1–16. Each value is the mean \pm SD of quadruplicate determinations.

pected in these poorly differentiated cells (20, 24). Alkaline phosphatase activity, a biochemical indicator used by other investigators to correlate to a particular state of differentiation in colonic carcinoma cells, actually increased after exposure to sodium butyrate (data not shown). These changes are similar to the change in activity described in other colonic carcinoma cells (20, 21, 24, 25), and were a consistent finding in our cells. However, because alkaline phosphatase activity may actually decrease in *Colo 320* cells after exposure to other chemical inducers (2% dimethylsulfoxide; unpublished data) and in other carcinoma cells (19, 22), we argued that alkaline phosphatase activity was not a reliable indicator of induced differentiation.

Conceptually, an ideal biological marker of malignant cellular behavior would have been tumor-forming ability in athymic mice. The pitfall of this technique was that it required a minimum of 4 wk before quantifiable tumor growth could be assessed. During this period of time the cells were not in the presence of differentiating agents, and the fraction of cells that had not undergone commitment had time to reestablish their full colony-forming capacity. In vitro, cells exposed to and then removed from inducing agents regain their baseline colony-forming capacity within 14 d after removal of the agent (20). Hence, we were unable to detect differences in tumor histology, size, or metastases between animals receiving control cells and those receiving cells exposed to sodium butyrate for 7 d. We concluded that the loss of colony-forming capacity was the most sensitive and quantitative measure of an induced commitment towards differentiation. The fact that *c-myc* repression preceded the decline in colony-forming capacity was compatible with the notion that the decrease in *myc* transcription was the cause, and not the result, of the decreased colony-forming capacity.

To formally demonstrate a direct cause and effect relationship between *c-myc* repression and inhibition of colony-forming capacity, we utilized an antisense oligodeoxynucleotide method. We demonstrated that the *c-myc* antisense and control oligonucleotides were stable in the presence of *Colo 320* cells and took up the oligonucleotide intact. We noted a dose-dependent decrease in colony-forming capacity after cells were exposed to antisense oligonucleotide. The lack of effect by

equimolar amounts of sense or missense 15-base nucleotides on colony-forming capacity implies a specific inhibition by *myc* antisense. This supports the conclusion that *myc* expression is required to maintain colony-forming capacity and its repression is sufficient to inhibit it.

These data strongly suggest that the role of *c-myc* in *Colo 320* cell differentiation is similar to its role in hematopoiesis, and that unrepressed *myc* expression maintains this colony-forming capacity and represents a primitive cell phenotype. Future studies designed to clarify the molecular determinants of *c-myc* repression in normal colonic epithelial cells may yield important information about one of the fundamental molecular defects in colorectal carcinoma cells.

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