

Regulation of Caspase Expression and Apoptosis by Adenomatous Polyposis Coli^{1,2}

Tingan Chen, Ivana Yang, Rosalyn Irby, Kenneth H. Shain, Hong Gang Wang, John Quackenbush, Domenico Coppola, Jin Q. Cheng, and Timothy J. Yeatman³

Departments of Interdisciplinary Oncology and Surgery [T. C., R. I., K. H. S., T. J. Y.], Drug Discovery [H. G. W.], Pathology [D. C., J. Q. C.], and H. Lee Moffitt Cancer Center, University of South Florida, Tampa, Florida 33612, and The Institute for Genomic Research, Rockville, Maryland [I. Y., J. Q.]

ABSTRACT

The adenomatous polyposis coli (*APC*) gene, a member of the WNT pathway, has been shown to assign intestinal epithelial cells to a program of proliferation or differentiation through regulation of the β -catenin/TCF-4 complex. Wild-type APC, in certain cellular contexts, appears to induce differentiation and apoptosis, although mutant forms of APC, known to produce polyps and ultimately cancers, may suppress these events. Here, we show that mutant forms of APC can induce repression of select terminal caspases as a potential means of attenuating responses to apoptotic stimuli. Using gene expression profiling to interrogate the intact intestines of *Apc*^{+/^{Min} mice harboring numerous polyps, we identified a reduction in the mRNA expression of both caspases 3 and 7. We additionally identified a reduction in protein levels of caspase-3, caspase-7, and caspase-9 in human colon cancer specimens known to harbor *APC* mutations. A reduction in caspase protein levels resulted in resistance to apoptotic-inducing agents and restoration of caspase levels reinstated apoptotic capacities. Consistent with Wnt pathway involvement, dominant negative TCF/LEF induced caspase protein expression. These data provide support for the hypothesis that one of the functions of APC is the regulation of caspase activity and other apoptotic proteins by controlling their expression levels in the cell.}

INTRODUCTION

The *APC*⁴ gene has been directly implicated in the development of human colon cancer by both germ-line and somatic mutations (1). Loss of APC function is the initiating event in both familial polyposis as well as in the vast majority of sporadic colon cancers. In fact, *APC* mutations have been identified in the earliest histologically identifiable lesions of the adenoma-carcinoma sequence termed aberrant crypt foci (2). APC function has recently been linked to the WNT signal transduction pathway, where it normally functions to target β -catenin for degradation. When mutated, APC causes β -catenin levels to rise and transactivate the TCF/LEF transcription factor with resultant increased expression of cyclin D (3) and c-MYC (4). In addition, APC has been observed to affect cellular adhesion, migration, and apoptosis (1, 5, 6). APC protein levels appear to control development and maturation of normal crypt enterocytes where levels are high in postreplicative cells in the upper portions of crypts and low in the bases of crypts where cells are actively dividing (7). Through the β -catenin/TCF complex, APC now appears to control a critical switch between a pathway of cellular proliferation *versus* one of differentiation (8).

The *Apc*^{+/^{Min} mouse provides a model of colon tumorigenesis where *Apc* germ-line mutation results in the formation of ~100}

adenomatous polyps as well as their precursor lesions, aberrant crypt foci (9). The derived polyps, unlike sporadic cancers and polyps, contain only *Apc* mutations (Ref. 10; a nonsense mutation at codon 850 of *Apc*) in one allele of *Apc* with spontaneous loss of the second wild-type allele. The model demonstrates that a single affected gene can produce the adenoma phenotype. Using both cDNA and oligonucleotide (Affymetrix) microarray technology, we compared the gene expression profile from the colon of the *Apc*^{+/^{Min} mouse to that of its wild-type littermate controls, identifying a number of up-regulated and down-regulated genes. Among these data, we observed significant decreases in the expression levels of caspases 3 and 7. The murine studies were extended to both a human colon cancer cell line stably transfected with an inducible wild-type *APC* gene and sporadic human normal and colon cancer tissues to provide evidence supporting the hypothesis that APC regulates apoptosis by governing the levels of the terminal caspases. We provide additional data demonstrating that caspase levels are not constitutively expressed in human colon cancer and that caspase levels determine the cancer cell's response to proapoptotic stimuli.}

MATERIALS AND METHODS

Microarray Platforms and RNA Preparation

Affymetrix GeneChips. The MGU74a GeneChip was used to assess the *Apc*^{-/-} and *Apc*^{+/^{Min} mouse intestinal samples. These microarray chips interrogate 12,422 unique probe identifier sequences. Data were analyzed using Microarray Suite 4.0 to identify genes exhibiting a 2-fold induction or greater in *Apc*^{+/^{Min} mouse as compared with the *Apc*^{-/-} wild-type animals.}}

cDNA Spotted Arrays. A 27,000 element-cDNA arrays used in this study contain National Institute on Aging and BMAP clone sets, together representing >23,000 unique genes and ESTs.^{5,6} Array fabrication and hybridization assays were performed as described previously (11); detailed protocols are available online.⁷ *APC*^{+/^{Min} and wild-type *APC*^{+/⁺ control RNA were cohybridized to cDNA arrays in triplicate, including an assay reversing Cy3 and Cy5 dyes. Data analysis was performed as outlined previously (12) using TIGR Spotfinder, MIDAS, and MultiExperiment Viewer software packages.⁸ Briefly, individual arrays were normalized using lowess normalization function, followed by replica filtering to remove questionable elements, and finally estimation of local Z score for identification of differentially expressed genes (defined as $Z > 2$). There were ~13,000 genes shared between cDNA arrays, and Affymetrix MGU74A chips were identified using the Resourcerer (13).⁹}}

RNA Preparation and Analysis. Total RNA was extracted from each of 10 intestinal samples derived from the wild-type littermate controls (*Apc*^{-/-}) and the *Apc* mutant mice (*Apc*^{+/^{Min}). RNA was processed as previously described (14) or in accordance with standard Affymetrix protocols. RNA from each of 10 mice was pooled in equimolar amounts before microarray analysis (15).}

Tissue Samples and Cell Lines

Apc^{+/^{Min} ($n = 10$) and *Apc*^{-/-} (littermate controls; $n = 10$) were obtained from Jackson Laboratories (Bangor, ME) and grown until 6 months of age}

Received 12/13/02; accepted 5/28/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ These studies were supported by grants from the National Cancer Institute.

² Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org>).

³ To whom requests for reprints should be addressed, at Department of Surgery and Interdisciplinary Oncology, H. Lee Moffitt Cancer Center, University of South Florida, 12902 Magnolia Drive, Tampa, FL 33612. E-mail: yeatman@moffitt.usf.edu.

⁴ The abbreviations used are: APC, adenomatous polyposis coli; Zn, zinc; PARP, poly(ADP-ribose) polymerase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 5-FU, 5-fluorouracil; β -gal, β -galactosidase.

⁵ Internet address: <http://lgsun.grc.nia.nih.gov/cDNA/cDNA.html>.

⁶ Internet address: http://www.resgen.com/products/BMAP001_design_specs.php3.

⁷ Internet address: <http://cancer.tigr.org/protocols.shtml>.

⁸ Internet address: <http://www.tigr.org/software/>.

⁹ Internet address: <http://www.tigr.org/tdb/tgi.shtml>.

when stool samples were noted to be hemocult positive. All mice were euthanized, and the small and large intestines were stripped of associated mesenteric fat and blood vessels, opened lengthwise for polyp enumeration, and snap-frozen in liquid nitrogen within 10 min of extirpation. For the *Apc*^{+/^{Min} mice, after evaluation with a dissecting microscope, polyp-bearing tissues were further separated from polyp-free tissues. RNA was then extracted using Trizol (Life Technologies, Inc., Gaithersburg, MD). Before extraction of RNA from *APC*^{+/^{Min} mice, polyp-free intestinal segments were dissected free from polyp-bearing segments. The HT29^{APC} and HT29^{β-gal} cell lines used were a gift from Bert Vogelstein. These cell lines were stably transfected with a Zn-inducible wild-type *APC* or *β-gal* vector. Cells were generally treated for 48 h with 120 μM ZnCl₂ before analysis.}}

Northern Blot Analysis

Ten μg of total RNA (derived from human normal and neoplastic tissues) were fractionated through formaldehyde-containing agarose gels and transferred to nylon membranes. Hybridizations with random primed ³²P-labeled mouse or human caspase-3 and caspase-7 cDNA probes were performed using QuikHyb Hybridization Solution (Stratagene, La Jolla, California). The mouse or human caspase-3 and caspase-7 cDNA probes were synthesized by reverse transcription-PCR using the following primers: mouse caspase-3 forward, AAGATCATAGCAAAGGAGCAG and reverse, GAGTAAGCATACAG-GAAGTCAG; mouse caspase-7 forward, ATTTTAAAGCCGACCTTCCC and reverse, TCCAATCACCATAGTCTCTCC; human caspase-3 forward, ATGCATACTCCACAGCACC and reverse, ACCACCAACCAACCATTTTC; and human caspase-7 forward, TCTGGTGCTGTCTTTTGTCTC and reverse, TTCTTGCTGTTGGCTTCTCT. The PCR fragments were sequenced and confirmed to be the correct sequence for mouse and human caspase-3 and caspase-7.

Western Blot Analysis

Cells were lysed in 50 mM PIPES/KOH (pH 6.5), 2 mM EDTA, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, 5 mM DTT, 20 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, and 2 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged at 4°C for 30 min at 20,000 × g, and the supernatant fraction was recovered. Protein extracts (100 μg) were fractionated through 10% criterion precast gel (Bio-Rad, Hercules, CA) and blotted onto pure nitrocellulose membranes (Bio-Rad). Caspase-3 antibody was purchased from Oncogene Research Products (Boston, MA). Caspase-7 and PARP antibodies were purchased from BD Pharmingen (San Diego, CA). Caspase-8 antibody was purchased from Alexis Biochemicals (San Diego, CA). Caspase-9 antibody was purchased from Calbiochem (San Diego, CA). Final protein detection used a goat antimouse or antirabbit IgG horseradish peroxidase secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

RNase Protection Assays

Total RNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA). The presence of transcripts of the indicated apoptosis-related genes, as well as L32 and glyceraldehyde-3-phosphate dehydrogenase as internal controls, were analyzed with the mAPO-1 and hAPO-1c MultiProbe template sets (BD Biosciences). Probe synthesis, hybridization, and RNase treatment were performed with the RiboQuant MultiProbe RNase Protection Assay System (BD Biosciences) according to the manufacturer's recommendations. Protected transcripts were analyzed by denaturing PAGE on 6% PAGE/urea gel (Ambion, Austin, TX) and quantified on a PhosphorImager with the ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Apoptosis and Terminal Deoxynucleotidyltransferase-mediated Nick End Labeling Assays

Annexin V-phycoerythrin reagents were purchased from BD Biosciences. Caspase-3, caspase-9, and pan-caspase peptide inhibitors (DEVE, LEHD, and ZVAD, respectively) were purchased from Alexis Biochemicals. Cells were washed twice with cold PBS and then resuspended in 1 × binding buffer at a concentration of 1 × 10⁶ cells/ml. One hundred μl of the solution (1 × 10⁵ cells) were transferred to a culture tube and incubated with 5 μl of Annexin

V-phycoerythrin antibody and 5 μl of 7-ADD and incubated 15 min in the dark. Then, cell apoptotic populations were analyzed by flow cytometry. APC induced cell death effect was assessed using tetrazolium salt MTT (Sigma) to measure the viability of the cells. A total of 180 μl of HT29^{APC} or HT29^{β-gal} cell suspension (2.5 × 10⁴ cells/ml) was added to 20 μl of serial 10-fold dilutions of each of 5-FU and anti-Fas antibody (50 ng/ml) in absence or presence of ZnCl₂ (120 μM) in 96-well plates (Corning) and incubated at 37°C for 96 h. Three microtiter wells containing the cells suspended in 200 μl of complete medium (total cell number was equivalent to that in the test wells) were used as controls for cell viability, and three wells containing only complete medium were used as controls for nonspecific dye reduction. After incubation, the cells were treated using MTT dye for 4 h, then DMSO was added to all of the wells. The plates were then read at 540 nm on a microplate reader.

Terminal deoxynucleotidyltransferase-mediated nick end labeling assays were performed using the APO-Direct procedure (BD Pharmingen) according to manufacturer's directions. Briefly, 2 × 10⁶ cells were trypsinized and fixed for 15 min in 1% paraformaldehyde. The cells were washed in cold PBS, resuspended in 70% ethanol, and stored at -20°C overnight. Cells were washed in wash buffer and stained in a solution containing reaction buffer, terminal deoxynucleotidyltransferase enzyme, and FITC-dUTP for 60 min. Cells were washed and analyzed by flow cytometry.

RESULTS

Gene Expression Profiling of the *Apc*^{+/^{Min} Mouse Identifies Reduced Caspase Levels.} Using both oligonucleotide (Affymetrix) and spotted cDNA arrays, we generated gene expression profiles of the intact large and small intestines of wild-type, polyp negative (*Apc*^{+/⁺) and mutant, polyp-positive (*Apc*^{+/^{Min}) mice. Because of the small size of the individual polyps and the ensuing difficulty in deriving enough RNA for analysis, we elected to analyze pooled RNA from the intact intestines of 10 *Apc*^{+/^{Min} mice harboring a median of 100 polyps/sample. We compared these expression measures with those derived from 10 polyp-free *Apc*^{+/⁺ mice. One observation that emerged immediately from these expression assays was that both caspase-3 and caspase-7 were significantly down-regulated (>2.5 fold) in the *Apc*^{+/^{Min} mice. A Northern analysis of caspase-3 and caspase-7 expression confirmed a significant reduction in expression in colon-bearing polyps and in the adjacent normal mucosa of *Apc*^{+/^{Min} mice relative to the normal mucosa derived from wild-type *Apc*^{+/⁺ mice (Fig. 1A). These data suggest that APC may regulate the expression of at least two terminal apoptotic regulatory molecules, possibly reducing apoptotic events and contributing to the proliferation of polyps and cancers. An evaluation of caspase expression by RNase protection assay additionally confirmed these results by demonstrating a significant reduction (~2.5-fold) in caspase-3 and caspase-7, but not in caspase-8, caspase-6, caspase-11, caspase-12, caspase-2, caspase-1, and caspase-14, in *Apc*^{+/^{Min} mice relative to *Apc*^{+/⁺ mice (Fig. 1B). Furthermore, the data suggest that the degree of caspase repression is dependent on the presence of one or two mutated *Apc* alleles. The caspase expression of colon-bearing polyps presumed to harbor two altered (mutated or deleted) *Apc* alleles was considerably less than that of normal adjacent mucosa with only one mutant *Apc* allele.}}}}}}}}}

The APC Apoptotic Program. Gene expression profiling is a powerful tool that permits the simultaneous evaluation of thousands of genes from multiple families in the same experiment. Here, we present a comprehensive analysis of gene expression in the intact intestines of the *Apc*^{+/⁺ and the *Apc*^{+/^{Min} mouse using two different microarray platforms: the Affymetrix MGU74AGeneChip (Affymetrix) and a high density spotted cDNA array that we constructed. Differentially expressed genes were identified using each platform individually and then genes common to both platforms were identified}}

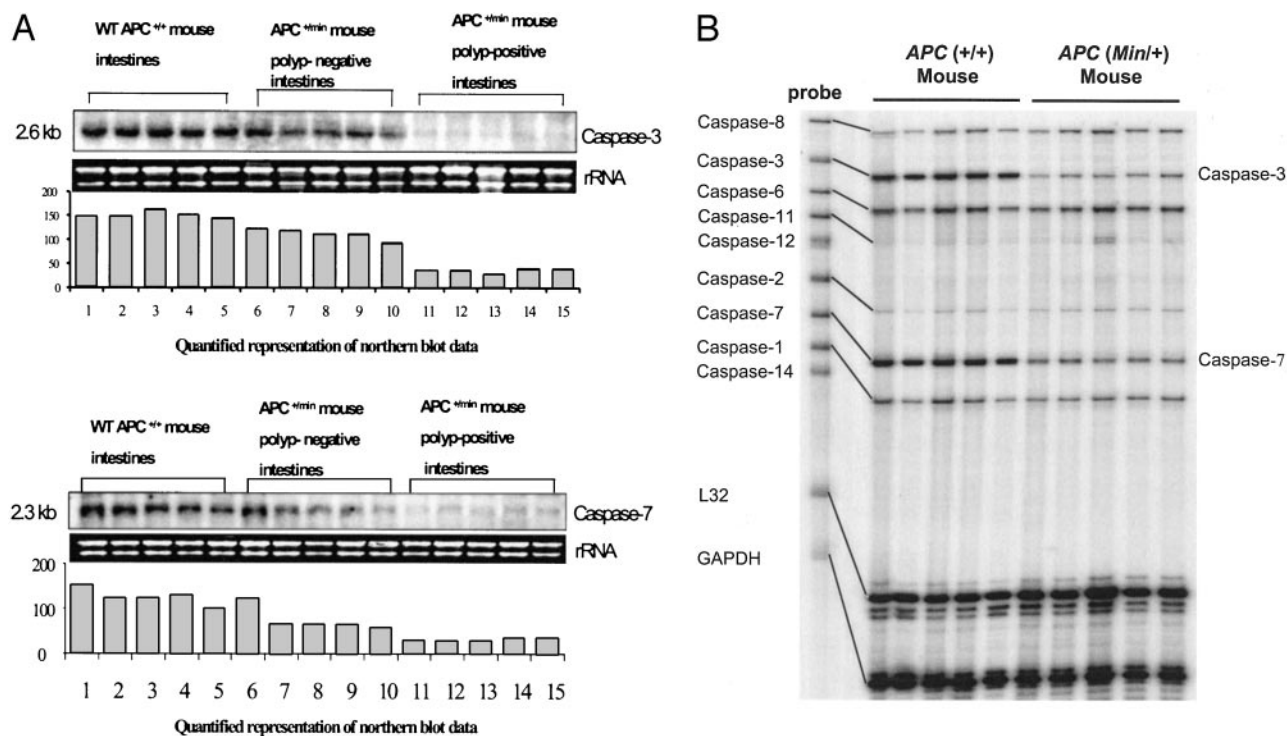


Fig. 1. Caspase-3 and caspase-7 mRNA are down-regulated in the intestines of the *Apc*^{+/Min} mouse. **A**, Northern analysis of RNA derived from the intact intestines of wild-type, polyp-negative *Apc*^{+/+} littermate controls ($n = 5$), polyp-negative portions of intestines from *Apc*^{+/Min} mice ($n = 5$), and polyp-positive (~100 polyps/mouse) *Apc*^{+/Min} mice ($n = 5$) demonstrates a significant reduction in caspase-3 and caspase-7 expression in the polyp-bearing intestines, validating the results of the gene expression analysis. Expression of caspase-3 and caspase-7 was only slightly reduced in the polyp-free portions of intestines from the *Apc*^{+/Min} mice. **B**, RNase protection assay of a panel of caspases confirms down-regulation of caspase-3 and caspase-7 but not caspase-8, caspase-6, caspase-11, caspase-12, caspase-2, caspase-1, and caspase-14. Other caspases such as caspase-9 were not assayed in murine tissues.

using a software program we developed (Resourcerer) to link oligonucleotide data with cDNA array data (13).⁹

A total of 451 genes was identified as differentially regulated using the 2-fold cutoff on oligonucleotide chips (supplemental table),² whereas application of lowess normalization and the local $Z > 2$ criterion (12) resulted in a list of 697 differentially expressed transcripts on cDNA arrays (supplemental table).² A number of differentially regulated genes were also identified as shared between the two microarray platforms (supplemental table).² Among these, we found up-regulation of genes that have been previously implicated in colon

cancer progression, most notably clusterin (16) and osteopontin (15). Of special significance, however, was the observation that both caspase-3 and caspase-7 were down-regulated in *Apc*^{+/Min} mice, suggesting that APC may directly influence the apoptotic pathways. This observation prompted us to separately examine the more extensive lists of differentially regulated genes identified by each of the array platforms. As can be seen in the list of apoptosis-related genes in Table 1, we observed lower levels of proapoptotic molecules such as cytochrome *c* (17) and an apoptosis activator MTD (BCL-2-related ovarian killer protein-like; Ref. 18) in the *Apc*^{+/Min} mouse relative to

Table 1 Differentially expressed genes linked to apoptotic function with the real time RT-PCR validation of a selection of genes.

GenBank Accession	Common_Name/ Role_Guess	Assay	Log ₂ (APC/WT)	QT-PCR (APC/WT)
U54803 AU016275	Caspase 3	Affymetrix cDNA	-1.55 -1.06	
U67321	Caspase 7	Affymetrix	-1.17	
AA409687 AI849373	Cytochrome <i>c</i>	cDNA cDNA	-0.931 -0.860	-2.105 -1.971
AI838402 AI847702	Mad protein (Max dimerizer) apoptosis activator Mtd; Bcl-2-related ovarian killer protein-like	cDNA cDNA	-1.08 -0.632	-2.352
AI847285 AU016757	apoptosis signal-regulating kinase 2 Myc proto-oncogene protein (C-MYC)	cDNA cDNA	0.590 1.05	1.50
AW553554	E1B 19K/Bcl-2-binding protein homolog	cDNA	1.12	
AI854349	Serine/threonine protein kinase SGK	cDNA	1.14	
AF007769	Neuronal apoptosis inhibitory protein 1	Affymetrix	1.42	1.778
X16009	mitogen regulated protein/proliferin (MRP/PLF)	Affymetrix	1.98	49.428
D38117 AU018274	Calpain 2 large (catalytic) subunit	Affymetrix cDNA	2.90 1.18	5.359

¹Down-regulated genes shown in green; up-regulated genes shown in red.

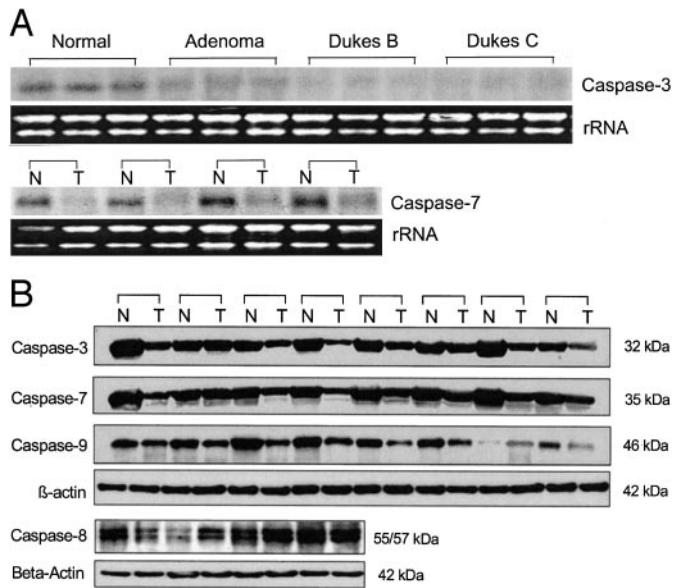


Fig. 2. Caspases are down-regulated in the majority of representative human colon cancer specimens. *A*, Northern analysis demonstrates down-regulation of caspase-3 and caspase-7 to occur both in adenomas and cancers of Dukes stage B and C. *B*, Western blot analysis demonstrates protein levels of caspase-3, caspase-7, and caspase-9 but not caspase-8 were down-regulated in human colon cancer specimens when compared with paired, adjacent, normal mucosal controls.

the wild-type *Apc*^{+/+} mouse. The *MAD* (19) gene controlling cell growth was also down-regulated. Conversely, antiapoptotic genes were increased in expression such as *c-MYC* (17), *NAIP* (20), and calpain (21). We have validated the expression of a number of these genes using real-time reverse transcription-PCR. These analyses have identified a set of genes regulated by APC, some of which have been previously linked to the function of apoptosis. We propose these candidate genes as members of an APC apoptotic program. The full list of genes identified by both platforms is available as supplementary data on line.¹⁰

Caspase Expression in Human Colon Cancer Tissue Specimens.

Our initial evaluation of gene expression profiles of the *Apc*^{+/*Min*} mouse model suggested that APC may control apoptosis by regulating the expression of specific terminal caspases. To additionally investigate this hypothesis, the expression of numerous caspases in human colon neoplastic specimens relative to normal mucosal controls was examined. Caspase-3 mRNA levels were observed to be reduced incrementally in adenomatous polyps and cancers (Fig. 2*A*). Similarly, caspase-7 mRNA levels were substantially reduced relative to adjacent, paired normal mucosa in four of four specimen pairs tested (Fig. 2*A*). Using Western analysis, protein levels for caspase-3, caspase-7, and caspase-9 but not caspase-8 were reduced in human colon cancer specimens relative to adjacent normal mucosal controls (Fig. 2*B*). In the majority of invasive tumor samples examined, caspase protein expression was reduced by >50%.

Effect of APC Expression on Caspase Levels. Because the caspases have been regarded as redundant and constitutively expressed, it was necessary to determine whether we could demonstrate that caspase expression levels were regulated by APC expression levels. Moreover, we sought to demonstrate that the selective repression of terminal caspases could, in fact, significantly alter the degree of apoptosis achieved with proapoptotic stimuli. Using a well-described *in vitro* model, a null *APC*^{-/-} human colon cancer cell line (HT-29) stably transfected with a Zn-inducible wild-type APC vector

or a β -gal control [the mRNA levels of caspase-3, caspase-7, and caspase-9 were demonstrated by RNase protection to rise in cells after induction of wild-type APC expression (Fig. 3*A*)]. Similarly, protein levels of caspase-3, caspase-7, and caspase-9 but not caspase-8 were shown to be up-regulated with induction of wild-type APC expression (Fig. 3*B*). This increased expression of caspases also resulted in increased levels of caspase cleavage products for caspase-3, caspase-7, and caspase-9 as well as an increase in the basal rate of PARP cleavage (Fig. 3*B*).

Dominant Negative TCF/LEF Enhances Caspase Expression.

To investigate the mechanism underlying APC regulation of caspase expression, we evaluated the potential for TCF/LEF to effect caspase protein expression. Transient transfection with the dominant negative TCF/LEF construct resulted in significant increases in caspase protein expression (caspase-3, caspase-7, and caspase-9), whereas *c-Myc* and cyclin-D1 were suppressed (Fig. 4).

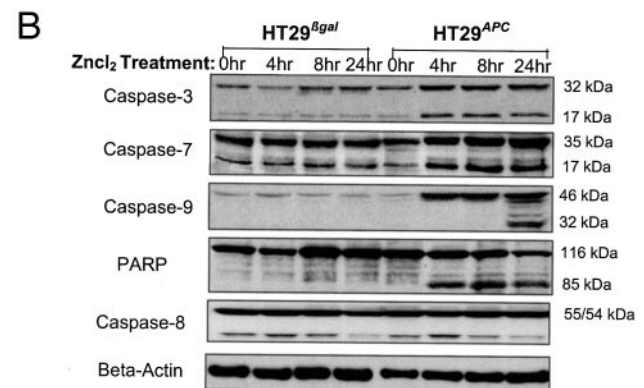
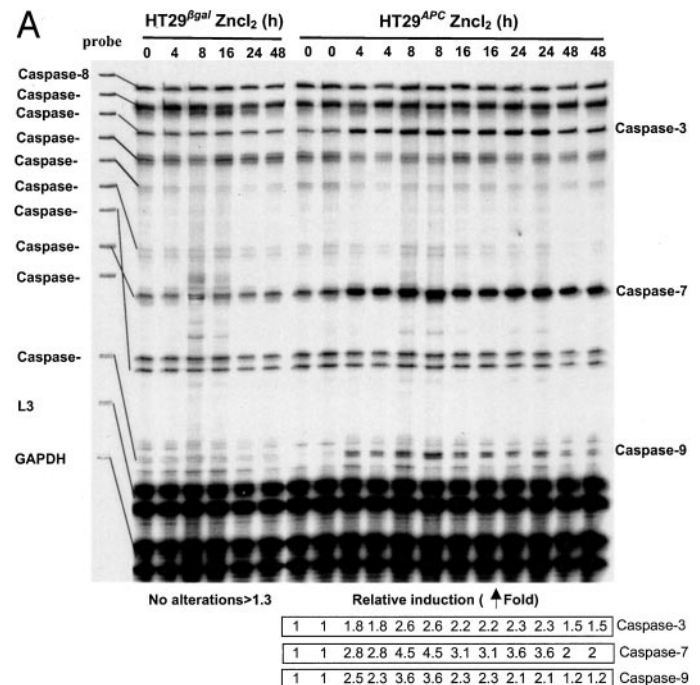


Fig. 3. Caspase regulation by Zn-induced wild-type APC expression in APC-deficient HT29 colon cancer cells. *A*, RNase protection assay demonstrates reduced expression of caspase-3, caspase-7, and caspase-9 but not other tested caspases over a 48-h time course in HT29^{APC} cells after induction of APC expression by ZnCl₂ (120 μ M) exposure. No alterations in gene expression were observed in HT29^{β-gal} control cells similarly exposed to ZnCl₂. *B*, Western analysis demonstrates an increase in protein levels of caspase-3, caspase-7, and caspase-9 but not caspase-8 with wild-type APC induction by ZnCl₂ in HT29^{APC} over a 24-h time course. This increased expression of caspases also resulted in increased levels of caspase cleavage products for caspase-3, caspase-7, and caspase-9 as well as increased basal PARP cleavage as a measure of apoptosis secondary to induction of APC expression.

¹⁰ Internet address: <http://cancer.tigr.org/data/APC>.

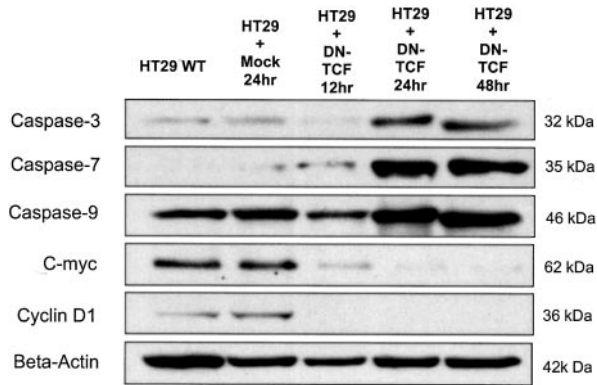


Fig. 4. Effect of dominant negative TCF/LEF on caspase expression. Protein expression of caspase-3, caspase-7, and caspase-9 was significantly enhanced when TCF/LEF function was inhibited for >24 h. Inhibition of the expression of known TCF/LEF targets, c-Myc and cyclin-D1, is also noted.

Effect of APC and Caspase Expression on Induced Apoptotic Events. To assess the baseline effect of wild-type APC protein expression in human colon cancer cells containing endogenous inactive APC alleles, transfected HT29^{APC} cells were incubated with or without ZnCl₂ for 48 h, and apoptosis was then assessed by annexin-V staining analyzed by flow cytometry (Fig. 5A). These data indicated that the fraction of apoptotic cells was increased ~3-fold because of induction of APC alone. When we exposed these cells to apoptotic stimuli (5-FU, anti-Fas antibody, and their combination), the fraction of apoptotic cells increased ~6-fold in those expressing APC relative to controls absent of APC expression. Furthermore, this level of apoptotic induction translated into significant effect on cell survival in APC expressing cells (Fig. 5B) but not controls as anticipated (Fig. 5C). To determine whether an alteration in caspase levels could affect the degree of apoptosis achieved with exposure to apoptotic stimuli in APC-expressing cells, peptide inhibitors of selective caspases were evaluated (Fig. 5D). We demonstrated that the caspase-3 inhibitor (DEVD), the caspase-9 inhibitor (LEHD), and the pan-caspase inhibitor (ZVAD) were all able to significantly reduce (by ~50% or more) the fractional apoptosis produced by proapoptotic stimuli. Interestingly, the peptide inhibition of caspase-8, a protein demonstrated to not be influenced by APC, did not affect apoptosis. Taken together, these data suggest that the specific reduction of caspase-3, caspase-7, and caspase-9 associated with mutant forms of APC might have a significant effect on apoptotic events.

To additionally demonstrate a role for caspase levels in the regulation of apoptosis in human colon cancer, caspase 3 was transiently transfected into wild-type APC^{-/-} HT29 cells, and apoptosis was monitored after activation by 5-FU + Fas ligand (Fig. 5E). These experiments demonstrated that overexpression of caspase-3 in the absence of apoptotic stimuli resulted in an approximate 3-fold basal increase in fractional apoptosis relative to controls (Fig. 5E). Subsequent stimulation with 5-FU and anti-Fas antibody significantly increased basal levels of apoptosis from ~30 to ~75% (Fig. 5E). Collectively, these data provide evidence that caspase levels are regulated by APC and directly affect the efficiency of apoptosis when cells are exposed to apoptotic stimuli.

DISCUSSION

The penultimate control of apoptosis has generally been linked to activation of the terminal caspases, rather than to regulation of their intracellular protein levels. Through proteolytic cleavage, caspases are converted to active caspases, which can then exert their direct effects. To date, it has been generally assumed that caspases are constitutively

expressed and that regulation of apoptosis is directly controlled through modification of their activity by upstream events (22, 23), a number of which have been attributed to the action of tumor suppressor genes (24, 25). For example, regulation of apoptotic activity has been linked to prevention of AKT activation by the tumor suppressor PTEN, and the p19ARF tumor suppressor gene induces apoptosis by binding to MDM2 and preventing P53 degradation. The RB tumor suppressor inhibits apoptosis through the induction of BCL-X_L, and P53 induces apoptosis through the induction of the proapoptotic BAX gene. Similarly, apoptosis has been observed to be regulated by a number of oncogenes, including c-MYC, E1A, and RAS (24). Despite the majority of reports describing upstream regulation of apoptosis, there is some evidence that caspase levels are subject to transcriptional regulation with resultant effects on apoptotic potential. For example, cells lacking STAT1 demonstrate decreased levels of caspase-1, caspase-2, and caspase-3 with subsequent attenuated responses to apoptotic stimuli (26). Experimental knockout models confirm the potential for profound defects in apoptosis in animals lacking certain caspases (27, 28). Conversely, caspase-3 mRNA and protein levels have been shown to increase after chemotherapy treatment (29). We report the first example of a tumor suppressor gene, APC, regulating caspase expression. These data provide direct evidence that the regulation of apoptosis may be finely controlled at multiple levels in the cascade of events that leads to cell death.

Using two different microarray platforms (oligonucleotide and spotted cDNA), gene expression profiling has led to the observation that expression of select terminal caspases (caspase-3 and caspase-7) were significantly reduced in the *Apc*^{+/^{Min}} mouse where a single mutation is responsible for the inactivation of *Apc* through protein truncation. These reductions in mRNA levels were subsequently linked to reduced protein levels that had a significant effect on the level of apoptosis achieved by the affected cell population. We have also demonstrated a reduction of caspase-3, caspase-7, and caspase-9 in human colorectal tumor specimens suggesting that APC may exert control over both initiator (upstream) caspases (-9) and effector (downstream) caspases (-3 and -7). It is interesting to note that not all members of the caspase family are affected but rather that only a few appear to be tightly regulated by APC. Moreover, specific peptide inhibition of caspase-3, caspase-7, and caspase-9, but not caspase-8, was effective in reducing apoptotic events.

The observation that caspase regulation occurs in the *Apc*^{+/^{Min}} mouse where genetic alterations common to somatic human colon cancers (e.g., RAS, P53) do not occur suggests that APC itself is ultimately responsible for the caspase regulation. Because dominant negative TCF/LEF was shown to significantly induce caspase expression, we believe the Wnt pathway can now be implicated in the control of the apoptotic program. Furthermore, because alterations in apoptosis were demonstrated in the HT29 colon cancer cell line harboring and inducible APC-producing vector but functionally null for P53 expression, the observed apoptosis is likely independent of other tumor suppressor genes such as P53. These findings suggest that whereas APC has been shown to regulate a master switch determining a program of proliferation or alternatively differentiation, in certain cellular contexts, APC might also regulate a program of apoptosis.

With the caveat that gene expression profiles faithfully represent mRNA expression but may not reflect protein expression, they can still provide important clues regarding the regulation of apoptotic events by APC. Apoptosis is a process likely determined by a balance of competing pro- and antiapoptotic molecules. APC appears to tip this balance in favor of apoptosis though downstream alterations in gene expression. In this regard, mutant APC, expressed by the *Apc*^{+/^{Min}} mouse, appears to repress genes with reported proapoptotic function such as cytochrome *c* (17), MAD (19), and MTD (18).

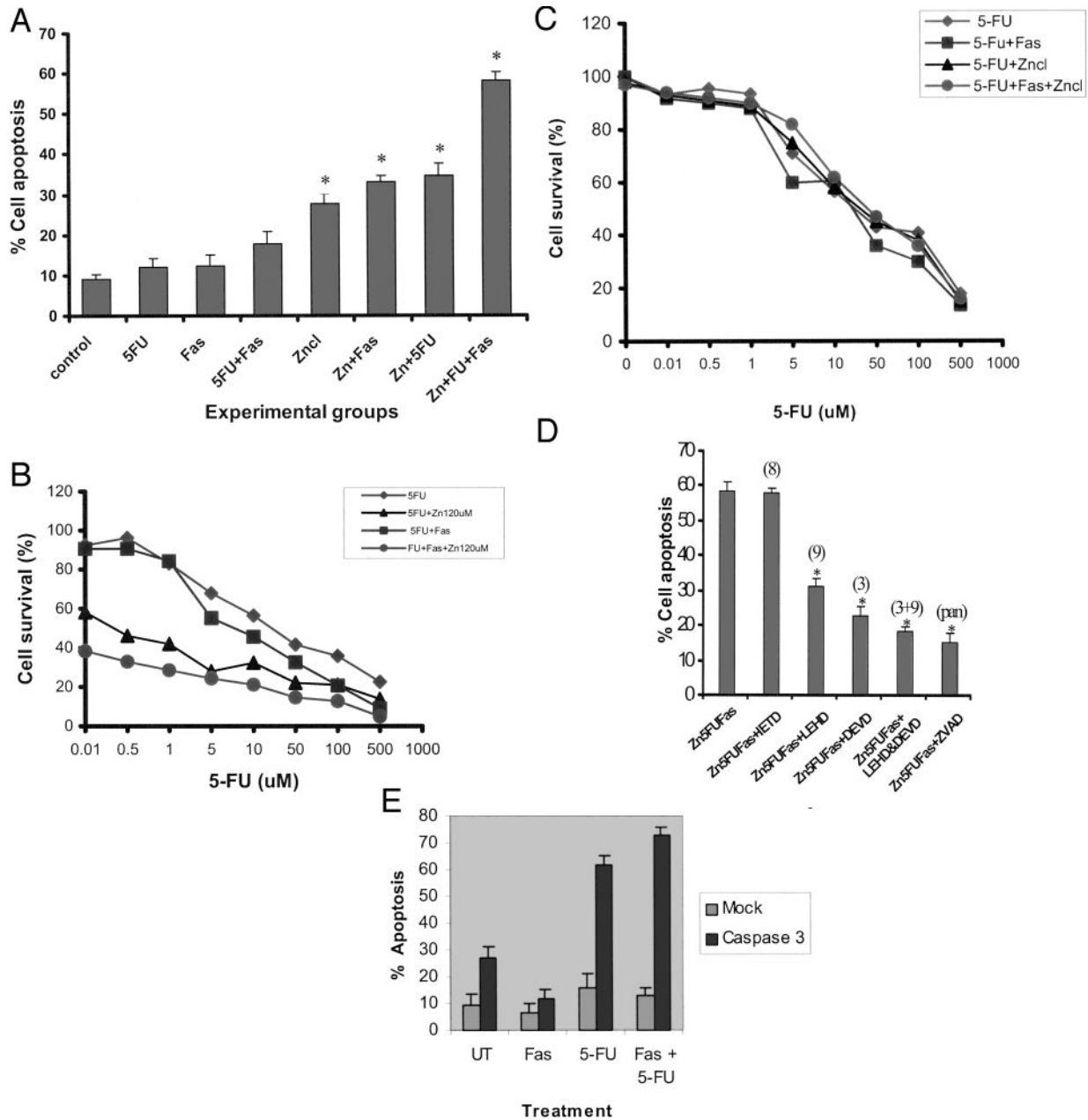


Fig. 5. Expression of wild-type APC and associated caspases regulates apoptosis and cell survival. *A*, apoptosis secondary to 48-h exposure to anti-Fas antibody (50 ng/ml), 5-FU (5 μ M), or their combination is enhanced by the induction of wild-type APC by ZnCl₂ exposure (120 μ M). *B*, wild-type APC induced by ZnCl₂ exposure significantly reduces the percentage cell survival as measured by a MTT assay after exposure to apoptotic stimuli (5-FU and anti-Fas antibody). The effect is more obvious at lower 5-FU doses. *C*, there is no differential effect of proapoptotic stimuli (5-FU and anti-Fas antibody) on HT29 ^{β -gal} control cells deficient in wild-type APC production. *D*, peptide inhibitors of caspase-9 (LEHD, 20 μ M), caspase-3 (DEVD, 50 μ M), their combination (LEHD + DEVD), and pan-caspase inhibitors (ZVAD, 50 μ M) all demonstrated a significant reduction in apoptosis induced by wild-type APC achieved through ZnCl₂ induction of HT29^{APC} cells. On the other hand, peptide inhibitors of caspase-8 (IETD, 50 μ M) were unable to suppress apoptosis induced by APC in conjunction with proapoptotic stimuli. *E*, caspase-3 transient transfection is capable of restoring apoptotic responses to proapoptotic stimuli (5-FU and anti-Fas antibody) in wild-type HT29 cells deficient in APC and caspase-3 expression. Statistically significant differences are marked by an asterisk ($P < 0.05$, two-sided *t* test).

Similarly, genes with antiapoptotic function appear to be up-regulated. These include *c-MYC* (17), *SGK* (30), calpain (21), and *NIAP*. These data provide evidence that two pathways, the WNT pathway and the phosphatidylinositol 3'-kinase pathway, may control apoptotic events through the intersection of glycogen synthase kinase-3, which can alter *c-MYC* levels directly via phosphorylation or indirectly via TCF/LEF (31). Of particular interest is the finding that the *NIAP* gene, which has recently been shown to directly and selectively inhibit caspase-3 and caspase-7 (20) activity, is up-regulated in the context of mutant APC. This would suggest that even when APC induced repression of caspase expression is incomplete, the expression of *NIAP*

may ensure effective caspase-3 and caspase-7 inhibition. Similarly, osteopontin, a protein we have previously demonstrated to be up-regulated in human colon cancer (15), is known to promote antiapoptotic activity (32). Somewhat puzzling is the observation that a number of proapoptotic insulin-like growth factor binding proteins are up-regulated in HT29^{APC} cells. We interpret this result as a compensatory mechanism in colon cancer cells known to highly overexpress insulin-like growth factor receptor (and depend on it for growth); however, compensation at the extracellular level may ultimately be ineffective in subverting the effect of APC on terminal caspases. Collectively, these profiles provide a number of genes that may be

part of a larger APC-controlled apoptotic program that represents an altered balance between pro- and antiapoptotic molecules.

We have provided evidence for a novel mechanism by which the APC tumor suppressor gene, when mutated, may alter the balance of antiapoptotic and proapoptotic molecules and support a proliferative cellular program. The observed down-regulation of the terminal caspases, in concert with APC mutation, may result in a significant reduction in apoptotic activity, which may contribute to tumor growth or resistance to apoptotic stimuli such as chemotherapeutic drugs. The expression of wild-type APC appears to be related to the expression of caspase-3, caspase-7, and caspase-9 and may also represent a normal control mechanism in crypt maturation and differentiation. These data imply that expression of full-length APC regulates the expression and activation of terminal caspases and provides a mechanism for the control of apoptotic activity.

ACKNOWLEDGMENTS

We thank Shrikant Mane and Steve Enkemann for expert help in the hybridization of oligonucleotide arrays, and Merek Willoch for tumor bank assistance. We also thank Alexander I. Saeed, Vasily Sahrov, Joseph White, Jerry Li, Nirmal Bhagabati, Wei Liang, John Braisted, Tracey Currier, Maria Klapa, and Mathangi Thiagarajan for software support.

REFERENCES

- Morin, P. J., Vogelstein, B., and Kinzler, K. W. Apoptosis and APC in colorectal tumorigenesis. *Proc. Natl. Acad. Sci. USA*, *93*: 7950–7954, 1996.
- Yuan, P., Sun, M. H., Zhang, J. S., Zhu, X. Z., and Shi, D. R. APC and K-ras gene mutation in aberrant crypt foci of human colon. *World J. Gastroenterol.*, *7*: 352–356, 2001.
- Tetsu, O., and McCormick, F. β -Catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature (Lond.)*, *398*: 422–426, 1999.
- He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. Identification of c-MYC as a target of the APC pathway. *Science (Wash. DC)*, *281*: 1509–1512, 1998.
- Goss, K. H., and Groden, J. Biology of the adenomatous polyposis coli tumor suppressor. *J. Clin. Oncol.*, *18*: 1967–1979, 2000.
- Strater, J., Koretz, K., Gunthert, A. R., and Moller, P. *In situ* detection of enterocytic apoptosis in normal colonic mucosa and in familial adenomatous polyposis. *Gut*, *37*: 819–825, 1995.
- Shih, I. M., Wang, T. L., Traverso, G., Romans, K., Hamilton, S. R., Ben-Sasson, S., Kinzler, K. W., and Vogelstein, B. Top-down morphogenesis of colorectal tumors. *Proc. Natl. Acad. Sci. USA*, *98*: 2640–2645, 2001.
- van de Wetering, M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der Horn, K., Battle, E., Coudreuse, D., Haramis, A. P., Tjon-Pon-Fong, M., Moerer, P., van den Born, M., Soete, G., Pals, S., Eilers, M., Medema, R., and Clevers, H. The β -catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell*, *111*: 241–250, 2002.
- Moser, A. R., Luongo, C., Gould, K. A., McNeley, M. K., Shoemaker, A. R., and Dove, W. F. ApcMin: a mouse model for intestinal and mammary tumorigenesis. *Eur. J. Cancer*, *31A*: 1061–1064, 1995.
- Shoemaker, A. R., Luongo, C., Moser, A. R., Marton, L. J., and Dove, W. F. Somatic mutational mechanisms involved in intestinal tumor formation in Min mice. *Cancer Res*, *57*: 1999–2006, 1997.
- Tsai, J., Sultana, R., Lee, Y., Perte, G., Karamycheva, K., Antonescu, V., Cho, J., Parvizi, B., Cheung, F., and Quackenbush, J. Resource: a database for annotating and linking microarray resources within and across species. *Genome Biol.*, *2*: software 0002.0001–0002.0004, 2001.
- Yang, I., Chen, E., Hasseman, J., Liang, W., Wang, S., Sharov, V., Saeed, A., White, J., Li, J., Lee, N., Yeatman, T., and Quackenbush, J. Within the fold: assessing differential expression measures and reproducibility in microarray assays. *Genome Biol.*, *3*: research0062, 2002.
- Yamori, T., Kimura, H., Stewart, K., Ota, D. M., Cleary, K. R., and Irimura, T. Differential production of high molecular weight sulfated glycoproteins in normal colonic mucosa, primary colon carcinoma, and metastases. *Cancer Res.*, *47*: 2741–2747, 1987.
- Agrawal, D., Chen, T., Irby, R., Quackenbush, J., Chambers, A. F., Szabo, M., Cantor, A., Coppola, D., and Yeatman, T. J. Osteopontin identified as lead marker of colon cancer progression, using pooled sample expression profiling. *J. Natl. Cancer Inst. (Bethesda)*, *94*: 513–521, 2002.
- Klefsjöm, J., Verschuren, E. W., and Evan, G. c-Myc augments the apoptotic activity of cytosolic death receptor signaling proteins by engaging the mitochondrial apoptotic pathway. *J. Biol. Chem.*, *277*: 43224–43237, 2002.
- Inohara, N., Ekhterae, D., Garcia, I., Carrio, R., Merino, J., Merry, A., Chen, S., and Nunez, G. Mtd, a novel Bcl-2 family member activates apoptosis in the absence of heterodimerization with Bcl-2 and Bcl-XL. *J. Biol. Chem.*, *273*: 8705–8710, 1998.
- James, L., and Eisenman, R. N. Myc and Mad bHLHZ domains possess identical DNA-binding specificities but only partially overlapping functions *in vivo*. *Proc. Natl. Acad. Sci. USA*, *99*: 10429–10434, 2002.
- Maier, J. K., Lahoua, Z., Gendron, N. H., Fetni, R., Johnston, A., Davoodi, J., Rasper, D., Roy, S., Slack, R. S., Nicholson, D. W., and MacKenzie, A. E. The neuronal apoptosis inhibitory protein is a direct inhibitor of caspases 3 and 7. *J. Neurosci.*, *22*: 2035–2043, 2002.
- Perrin, B. J., and Huttenlocher, A. Calpain. *Int. J. Biochem. Cell Biol.*, *34*: 722–725, 2002.
- Elbaz, M., Avni, A., and Weil, M. Constitutive caspase-like machinery executes programmed cell death in plant cells. *Cell Death Differ.*, *9*: 726–733, 2002.
- Weil, M., Jacobson, M. D., Coles, H. S., Davies, T. J., Gardner, R. L., Raff, K. D., and Raff, M. C. Constitutive expression of the machinery for programmed cell death. *J. Cell Biol.*, *133*: 1053–1059, 1996.
- Macleod, K. Tumor suppressor genes. *Curr. Opin. Genet. Dev.*, *10*: 81–93, 2000.
- Macleod, K. F., Hu, Y., and Jacks, T. Loss of Rb activates both p53-dependent and independent cell death pathways in the developing mouse nervous system. *EMBO J.*, *15*: 6178–6188, 1996.
- Kumar, A., Commane, M., Flickinger, T. W., Horvath, C. M., and Stark, G. R. Defective TNF- α -induced apoptosis in STAT1-null cells due to low constitutive levels of caspases. *Science (Wash. DC)*, *278*: 1630–1632, 1997.
- Zheng, T. S., and Flavell, R. A. Divinations and surprises: genetic analysis of caspase function in mice. *Exp. Cell Res.*, *256*: 67–73, 2000.
- Matikainen, T., Perez, G. I., Zheng, T. S., Kluzak, T. R., Rueda, B. R., Flavell, R. A., and Tilly, J. L. Caspase-3 gene knockout defines cell lineage specificity for programmed cell death signaling in the ovary. *Endocrinology*, *142*: 2468–2480, 2001.
- Droin, N., Dubrez, L., Eymen, B., Renvoize, C., Breard, J., Dimanche-Boitrel, M. T., and Solary, E. Up-regulation of CASP genes in human tumor cells undergoing etoposide-induced apoptosis. *Oncogene*, *16*: 2885–2894, 1998.
- Brunet, A., Park, J., Tran, H., Hu, L. S., Hemmings, B. A., and Greenberg, M. E. Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a). *Mol. Cell Biol.*, *21*: 952–965, 2001.
- Wang, Q., Wang, X., Hernandez, A., Hellmich, M. R., Gatalica, Z., and Evers, B. M. Regulation of trail expression by the PI3-kinase/Akt/GSK-3 pathway in human colon cancer cells. *J. Biol. Chem.*, *24*: 24, 2002.
- Geissinger, E., Weisser, C., Fischer, P., Scharl, M., and Wellbrock, C. Autocrine stimulation by osteopontin contributes to antiapoptotic signalling of melanocytes in dermal collagen. *Cancer Res.*, *62*: 4820–4828, 2002.
- Hegde, P., Qi, R., Abernathy, K., Gay, C., Dharap, S., Gaspard, R., Hughes, J., Snesrud, E., Lee, N., and Quackenbush, J. A concise guide to cDNA microarray analysis. *Biotechniques*, *29*: 552–562, 2000.
- Hegde, P., Qi, R., Gaspard, R., Abernathy, K., Dharap, S., Earle-Hughes, J., Gay, C., Nwokekeh, N. U., Chen, T., Saeed, A. I., Sharov, V., Lee, N. H., Yeatman, T. J., and Quackenbush, J. Identification of tumor markers in models of human colorectal cancer using a 19,200-element complementary DNA microarray. *Cancer Res.*, *61*: 7792–7797, 2001.