Anti-inflammatory drug resistance selects putative cancer stem cells in a cellular model for genetically predisposed colon cancer

NITIN TELANG

Cancer Prevention Research Program, Palindrome Liaisons Consultants, Montvale, NJ 07645-1559, USA

Received April 12, 2017; Accepted August 23, 2017

DOI: 10.3892/ol.2017.7147

Abstract. Mutations in the adenomatous polyposis coli (Apc) tumor suppressor gene represent the primary genetic defect in colon carcinogenesis. $Apc^{+/-}$ mouse models exhibit pre-invasive small intestinal adenomas. Cell culture models exhibiting Apc defects in the colon and quantifiable cancer risk provide a novel clinically relevant approach. The tumor-derived $Apc^{-/-}$ colonic epithelial cell line 1638N COL-Pr_1 represented the experimental model. The anti-inflammatory drugs sulindac (SUL) and celecoxib (CLX) represented the test compounds. Compared with non-tumorigenic $Apc^{+/+}$ C57COL cells, the $Apc^{+/-}$ 1638N COL cells and $Apc^{-/-}$ 1638N COL-Pr_1 cells exhibited progressive loss of homeostatic growth control. Compared with $Apc^{+/-}$ cells, $Apc^{-/-}$ cells displayed increased expression of biomarkers specific for hyper-proliferation. Treatment of $Apc^{-/-}$ cells with SUL and CLX resulted in inhibition of anchorage-independent colony formation *in vitro*, which is indicative of reduced cancer risk *in vivo*. Mechanistically, SUL and CLX suppressed the expression of the Apc target genes β-catenin, cyclin D1, c-Myc and cyclooxygenase-2. Long-term treatment with high concentrations of SUL and CLX led to the selection of hyper-proliferative drug-resistant phenotypes. The $Apc^{-/-}$ SUL-resistant phenotype displayed spheroid formation and enhanced the expression of the stem cell-specific molecular markers CD44, CD133 and c-Myc. These data demonstrated the growth-inhibitory efficacy of SUL and CLX and indicated that drug resistance leads to the selection of a putative cancer stem cell phenotype. The study outcome validates a stem cell-targeted mechanistic approach to identify testable alternative leads for chemotherapy-resistant colon cancer.

Introduction

According to the American Cancer Society, there will be an estimated 95,270 new colon cancer cases and 49,190 colon cancer-related deaths in 2017 (1). These figures represent overall risk for developing sporadic, familial and hereditary colon cancer in both sexes, which is partly based on common molecular/genetic pathways predisposing to this disease. Germline or somatic mutations in the adenomatous polyposis coli (Apc) tumor suppressor gene represent the primary predisposing genetic defect in the clinical familial adenomatous polyposis (FAP) syndrome, in genetically predisposed early-onset colon cancer, and in the majority of sporadic colon cancers (2).

Genetically engineered mouse models for FAP carry germline mutations in codons 474, 850 or 1638 of the Apc tumor suppressor gene, and exhibit adenoma formation predominantly in the small intestine, rather than in the colon (2-4). Since the colon represents a clinically relevant target organ site for the development of colon cancer, reliable models expressing clinically relevant genetic defects in the target organ site for colon cancer and exhibiting quantifiable cancer risk offer a testable preclinical alternative. Towards this end, Apc-defective colonic epithelial cell culture models derived from $Apc^{1638+/-}$ and $Apc^{850/Min+}$ mice were isolated and characterized. These preclinical cell culture models exhibit spontaneous immortalization as evidenced by telomerase re-expression, loss of homeostatic growth control as evidenced by hyper-proliferation, aberrant cell cycle progression and downregulated cellular apoptosis, and persistent cancer risk as evidenced by anchorage-independent (AI) growth *in vitro* and development of tumors *in vivo*. These colonic epithelial cell culture models for the FAP syndrome have also been utilized as novel experimental approaches to examine the efficacy of selecting mechanistically distinct chemo-preventive agents (5-10).

The preclinical *in vivo* animal models for the FAP syndrome have provided valuable clinically relevant mechanistic evidence for the efficacy of non-steroidal anti-inflammatory drugs (NSAIDs) and selective cyclo-oxygenase-2 inhibitors (COXIBs) in intestinal adenomas (11-13). Subsequently, these agents have also been used as preventive/therapeutic options for clinical FAP and sporadic colon cancer (14-17).

Long-term clinical use of NSAIDs and COXIBs is associated with unacceptable systemic toxicity and significant side effects. Thus, NSAIDs functioning as dual inhibitors of constitutive isoform COX-1 and of inducible isoform COX-2, lead to gastrointestinal toxicity (14,15). COXIBs that function as selective COX-2 inhibitors are associated with cardiovascular

Correspondence to: Dr Nitin Telang, Cancer Prevention Research Program, Palindrome Liaisons Consultants, 10 Rolling Ridge Road, Suite B, Montvale, NJ 07645-1559, USA
E-mail: entitytoo@gmail.com; ntelang3@gmail.com

Key words: anti-inflammatory drugs, colonic epithelium, stem cells

problems and risk of stroke (17-19). Similar to conventional chemotherapeutics, long-term administration of NSAIDs or COXIBs may also lead to acquired tumor resistance due to the emergence of drug-resistant cancer stem cell populations (20-22). Therefore, these limitations emphasize an unmet need for development and characterization of stem cell models exhibiting clinical relevance to genetically predisposed early-onset colon cancer. Furthermore, stem cell models may facilitate identification of novel efficacious preventive/therapeutic agents as testable stem cell-targeted alternatives to existing therapeutic options for colon cancer.

Published evidence has strongly supported the concept that inflammation and cancer may be mechanistically linked via a multi-step carcinogenic process consisting of disease initiation, promotion and progression. Inflammation is considered to be a major driver of cancer initiation and progression in the colon, as exemplified by clinical inflammatory bowel disease, ulcerative colitis and colitis-associated colon cancer, and in the preclinical setting by the Apc $^{Min/+}$/DSS mouse model (23,24). The pro-inflammatory inducible nitric oxide synthase-COX-2 (iNOS-COX2) loop that is active in the tumor microenvironment may represent a therapeutic target for cancer. Of note, the anticancer efficacy of selective inhibitors for iNOS and COXIBs have been recently documented in triple-negative breast cancer (25).

In the present study, experiments were designed to i) develop and characterize a cell culture model for tumor-derived Apc$^{-/-}$ colonic epithelial cells, ii) examine the growth-inhibitory efficacy of the prototypic anti-inflammatory agents sulindac (SUL) and celecoxib (CLX) on the developed model, and iii) develop a SUL-resistant (SUL-R) cancer stem cell model that validates a testable cancer stem cell-based alternative approach for the identification of novel stem cell-specific therapeutics for colon cancer.

Materials and methods

Experimental models. The experimental models used in the present study were derived from colonic epithelium of histologically normal descending colon. These colonic epithelial cell lines are spontaneously immortalized, as evidenced by expression telomerase, and exhibit quantifiable risk for tumorigenic transformation (5,6,9).

C57 COL. This cell line is derived from the descending colon of C57BL/6J mice. The spontaneously immortalized cells exhibit the Apc$^{+/+}$ genotype, diploid phenotype and telomerase positivity, but lack AI growth *in vitro* and tumor development *in vivo* (5,6).

1638N COL. This cell line is derived from the descending colon of Apc 1638N$^{+/-}$ mice. The cells carry a mono-allelic mutation in codon 1638 of the Apc gene. The cells exhibit the Apc$^{+/-}$ genotype, ~80-90% aneuploid cell population, telomerase positivity, AI growth *in vitro* and subsequent tumor development *in vivo* (5).

1638N COL-Pr$_1$. This cell line is derived from a primary tumor that developed from a transplanted clone of 1638N COL cells. This tumor-derived cell line exhibits the Apc$^{-/-}$ genotype, a >90% aneuploid cell population, telomerase positivity, loss of homoeostatic growth control and persistence of AI growth *in vitro* (9).

The cell lines were maintained in DME/F12 medium supplemented by 10% heat-inactivated fetal calf serum (Gibco, Grand Island, NY, USA), 0.24 IU (10 μg/ml) insulin and 1 μM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA). The culture medium also contained an antibiotic mixture (100 IU/ml penicillin-100 μg/ml streptomycin mixture, +50 μg/ml fungizone +50 μg/ml gentamycin; all from Gibco). The cell lines were maintained at 37˚C in a humidified atmosphere of 95% air:5% CO_2, and were sub-cultured to ~80% confluency (5,6,9).

Growth assays. The growth assays compared the status of population doubling, saturation density, cell cycle progression and AI colony formation. Population doubling was determined by viable cell counts at 24, 48, 72 and 96 h post-seeding of $1x10^5$ cells. The data were expressed as the mean of the four time points. Saturation density was determined by the viable cell counts at day 7 post-seeding of $1x10^5$ cells. The viable cell counts were determined using a Trypan blue exclusion cell viability assay kit (Sigma-Aldrich). The cell cycle progression was determined by flow cytometry to monitor G_1, S and G_2/M phases of the cell cycle. The data were expressed as G_1: S+G_2/M ratio (7-9).

AI colony formation assay. For this assay, the cell suspension in 0.33% agar (Gibco), with or without the test agent, was overlaid over a basement matrix of 0.6% agar. The cultures were maintained at 37˚C in a humidified atmosphere of 95% air:5% CO_2 for 14 days, and the number of AI colonies formed in 0.33% agar at day 14 post-seeding of 100 cells were determined.

Test compounds. The NSAID pan-COX inhibitor sulindac (SUL) and selective COX-2 inhibitor celecoxib (CLX) (both from Sigma-Aldrich), were used as the test compounds. The stock solutions of these agents (100 mM) were made in 100% ethanol and were serially diluted in the culture medium to obtain the working solutions at concentrations within the pharmacologically achievable dose ranges for SUL and CLX.

Drug-resistant phenotypes. To isolate the sub-population of cells resistant to the cytotoxic effects of SUL and CLX, the Apc$^{-/-}$ 1638N COL-Pr1 cells were maintained in the presence of predetermined cytotoxic concentrations of 20 μM SUL or 20 μM CLX. The surviving drug-resistant cell population was expanded in the presence of 20 μM SUL or 20 μM CLX for at least 5 passages to select the SUL-R and CLX-resistant (CLX-R) phenotypes.

For the tumor spheroid formation assay, SUL-R 1638N COL-Pr$_1$ cells were seeded at a density of 100 cells per well in ultralow adherence 6-well plates (Corning/Costar, Corning, NY, USA) in serum-free DME/F12 medium. This culture medium was supplemented with 20 ng/ml epidermal growth factor, 10 ng/ml basal fibroblast growth factor (Sigma-Aldrich), 1% B27, 10 ng/ml leukemia-inhibitory factor (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 5 μg/ml insulin, 1 ng/ml hydrocortisone and 4 μg/ml heparin sodium (Sigma-Aldrich).

The cultures were maintained at 37˚C in a humidified atmosphere of 95% air:5% CO_2, and the spheroids formed on day 14 post-seeding were counted.

Apc target gene and stem cell marker expression assays. The expression of the Apc target gene products β-catenin, cyclin D1, c-Myc and COX-2 was quantified by staining the cells with fluorescein isothiocyanate (FITC)-labeled antibodies for β-catenin (BD Biosciences, San Jose, CA, USA), cyclin D1 and c-Myc (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and COX-2 (Cell Signaling Technology, Beverly, MA, USA), using the optimized protocols recommended by the manufacturers. The antibody-positive cells were sorted by flow cytometry (26). Similarly, the expression of the stem cell markers CD44, CD133 and c-Myc was quantified by sorting of cells positively stained for FITC-labeled antibodies specific for CD44 (Cell Signaling Technology), CD133 (Dako, Carpinteria, CA, USA) and c-Myc (Santa Cruz Biotechnology) following the protocol recommended by the manufacturers. The data were normalized for the fluorescence from cells stained with FITC-labeled IgG (BD Biosciences), and expressed as log mean fluorescence units/10^4 fluorescence events (26).

Statistical analysis. The replicate experiments were performed in triplicate. The data generated were analyzed for statistical significance between the control and the treatment groups by the Student's t-test using the Graph Pad Prism software, version 5.0 (Graph Pad Software, Inc. La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Homeostatic growth control. The data from comparative experiments on the cell culture models are summarized in Table I. Relative to the non-tumorigenic $Apc^{+/+}$ C57 COL cells, Apc-defective tumorigenic $Apc^{+/-}$ 1638 N COL and tumor-derived $Apc^{-/-}$ 1638N COL-Pr_1 cells exhibited a progressive decrease in the population doubling times, increase in saturation density and decrease in the aneuploid G_1:S+G_2/M ratio. In addition, the Apc-defective cell lines exhibited progressive increase in the expression of the Apc target gene products β-catenin, cyclin D1, c-Myc and COX-2.

Growth-inhibitory effects of SUL and CLX. The growth-inhibitory effects of SUL and CLX on the $Apc^{-/-}$ 1638N COL-Pr_1 cells were examined using the AI colony formation assay. In response to treatment with 10 μM SUL and 10 μM CLX, 1638N COL-Pr_1 cells exhibited an 87 and 93.9% reduction (P=0.01) in the number of AI colonies, respectively, compared with the ethanol-treated control (Table II).

Mechanistic targets for the efficacy of SUL and CLX. The experiments conducted to examine the effects of SUL and CLX on the cell cycle progression and on the status of selected Apc target gene product expression are summarized in Table III. In response to treatment with SUL and CLX, the $Apc^{-/-}$ 1638N COL-Pr_1 cells exhibited cell cycle arrest as evidenced by a ~7- and 9-fold increase (P=0.001), respectively, in the aneuploid G_1:S+G_2/M ratio. Additionally, the cell cycle arrest was associated with significant decreases in the Apc target gene products β-catenin (P=0.04), cyclin D1 (P=0.01), c-Myc (P=0.04) and COX-2 (P=0.01).

Drug-resistant phenotypes. The experiments conducted to isolate SUL-R and CLX-R phenotypes are summarized in Table IV. Long-term continuous treatment for at least 5 passages to 1638N COL-Pr_1 cells with high pharmacological doses of SUL and CLX resulted in the emergence of cells that exhibited robust growth and increased number of AI colonies. Relative to the sensitive phenotype, the SUL-R phenotype exhibited a 37.6-fold increase (P=0.001) in saturation density and a 13.4-fold increase (P=0.001) in the number of AI colonies. Similarly, the CLX-R phenotype exhibited a 52.5-fold increase (P=0.001) in saturation density and a ~10-fold increase (P=0.001) in the number of AI colonies.

Table I. Status of homeostatic growth control in $Apc^{-/-}$ 1638N COL-Pr1 cells.

	Relative to $Apc^{+/+}$ C57 COL	
Biomarker	$Apc^{+/-}$ 1638N COL	$Apc^{-/-}$ 1638N COL-Pr_1
Population doubling[a]	-50.0%	-64.7%
Saturation density[b]	+2.5X	+7.6X
Aneuploid cell population[c]	+25%	+75%
Aneuploid G_1:S+G_2/M ratio[c]	-54.8%	-77.4%
Apc target genes[d]		
β-catenin	+4.8%	+85.7%
Cyclin D1	+1.9X	+3.9X
c-Myc	+1.1X	+2.9X
COX-2	+65.9%	+2.1X

[a]Determined from the exponential growth phase. [b]Determined at day 7 post-seeding. [c]Determined from flow cytometry-based cell cycle analysis at day 3 post-seeding. [d]Determined at day 3 post-seeding, log mean fluorescence units. Apc, adenomatous polyposis coli; COX, cyclo-oxygenase; X, fold-change.

Table II. Effect of SUL and CLX on AI growth of $Apc^{-/-}$ 1638N COL-Pr1 cells.

Treatment	Concentration	AI colony no.[a]	Inhibition (% control)
EtOH	0.01%	37.8±5.0	-
SUL	10 μM	4.9±1.2[b]	87.0
CLX	10 μM	2.3±0.5[c]	93.9

[a]Determined at day 14 post-seeding of 100 cells; values are presented as mean ± standard deviation (n=18 per treatment group). [b,c]P=0.01 compared with the EtOH-treated control. Data were analyzed using the Student's t-test. AI, anchorage-independent; Apc, adenomatous polyposis coli; EtOH, ethanol; SUL, sulindac; CLX, celecoxib.

Table III. Effect of SUL and CLX on cell cycle progression and status of Apc target gene expression in $Apc^{-/-}$ 1638N COL-Pr1 cells.

Treatment	Concentration	Aneuploid G_1:S+G_2/M ratio	Log Mean FU[a]			
			β-catenin	Cyclin D1	c-Myc	COX-2
EtOH	0.01%	0.9±0.4	7.9±0.2	14.8±0.9	6.8±0.7	14.3±0.9
SUL	10 μM	9.7±1.2[b]	4.2±0.2[d]	4.2±0.4[f]	3.4±0.2[h]	7.1±0.8[j]
CLX	10 μM	7.6±0.9[c]	3.5±0.2[e]	4.0±0.4[g]	3.3±0.2[i]	5.8±0.6[k]

[a]Mean ± standard deviation (n=3 per treatment group). [b,c]P=0.001, [d,e,h,i]P=0.04 and [f,g,j,k]P=0.01 compared with the EtOH-treated control. Data were analyzed by the Student's t-test. EtOH, ethanol; SUL, sulindac; CLX, celecoxib; Apc, adenomatous polyposis coli; FU, fluorescence units.

Table IV. Drug-resistant phenotypes from $Apc^{-/-}$ 1638N COL-Pr1 cells.

Cell type	Treatment	Biomarker	
		Saturation density[a]	AI colony no.[b]
SUL-S	20 μM SUL	1.7±0.4	1.3±1.2
SUL-R	20 μM SUL	65.6±4.4[c]	18.7±3.3[d]
Δ SUL-S		+37.6X	+13.4X
CLX-S	20 μM CLX	1.2±0.5	1.8±1.7
CLX-R	20 μM CLX	64.2±4.3[e]	20.4±3.8[f]
Δ CLX-S		+52.5X	+10X

[a]Viable cell number ($x10^5$) at day 7 post-seeding; values are presented as mean ± standard deviation (n=3 per treatment group). [b]AI colony number at day 14 post-seeding of 100 cells; values are presented as mean ± standard deviation (n=18 per treatment group). [c,d]P=0.001 compared with SUL-S. [e,f]P=0.001 compared with CLX-S. Data were analyzed by the Student's t-test. Apc, adenomatous polyposis coli; AI, anchorage-independent; SUL-S, sulindac-sensitive; SUL-R, sulindac-resistant; CLX-S, celecoxib-sensitive; CLX-R, celecoxib-resistant; X, fold change.

The data generated from the experiments designed to examine the stem cell characteristics of the SUL-R phenotype are summarized in Table V. Compared with SUL-sensitive (SUL-S) cells, the SUL-R cells exhibited a ~7-fold increase (P=0.01) in the number of tumor spheroids. As regards stem cell-specific molecular markers, SUL-R cells exhibited a 5.7-fold increase (P=0.01) in the expression of CD44, a 3.6-fold increase (P=0.02) in the expression of CD133, and a 1.7-fold increase (P=0.02) in the expression of c-Myc, compared with the SUL-S cells.

Discussion

Loss-of-function mutations in the APC and p53 tumor suppressor genes and gain-of-function mutations in the RAS and RAF oncogenes represent major genetic defects associated with the initiation/progression of colon cancer, promoting the emergence of immortalized aberrantly hyper-proliferative cancer phenotypes (2,17). Conventional chemotherapy involving combinations of mechanistically distinct cytotoxic pharmacological agents is an established treatment option for colon cancer. However, long-term chemotherapy is associated with systemic toxicity, acquired tumor resistance and emergence of chemo-resistant cancer stem cells, compromising the therapeutic efficacy and promoting disease progression (17,20-22). These limitations emphasize an unmet need to identify testable alternatives that are effective for the prevention/therapy of genetically predisposed as well as sporadic colon cancer. The experiments in the present study utilized a cell culture model for Apc-defective colon cancer to evaluate the growth-inhibitory efficacy of the prototypic anti-inflammatory agents SUL and CLX, and to isolate and characterize drug-resistant cancer stem cells.

Comparative data on the Apc-defective colonic epithelial cells provided evidence for loss of homeostatic growth control. These data are consistent with the previously published data on the cell culture models for the FAP syndrome (5-10). It is also noteworthy that comparison between $Apc^{+/-}$ 1638N COL and $Apc^{-/-}$ 1638N COL-Pr_1 cells revealed a progressive biomarker modulation relevant to hyper-proliferation, aberrant cell cycle progression and Apc target gene expression in favor of 1638N COL-Pr_1 cells. Collectively, these data suggest that an Apc defect correlating with mutation and allelic deletion of the Apc tumor suppressor gene, emergence of aneuploid cell populations and upregulation of Apc target genes, such as β-catenin, cyclin D1, c-Myc and COX-2, may have facilitated a progressive loss of homeostatic growth control and advent of tumorigenic transformation. This interpretation of the present data is consistent with emergence of genetic instability, aneuploidy and chromosomal instability, corresponding to lack of tumor-suppressive function of the Apc gene (2,5,6).

AI growth represents an *in vitro* surrogate marker for cancer risk of the tumorigenically transformed phenotype (7,9,10). Thus, inhibition of AI colony formation in the $Apc^{-/-}$ 1638N COL-Pr_1 cells by the prototypic anti-inflammatory drugs SUL and CLX suggests their ability to reduce cancer risk. These data are consistent with similar effects by several mechanistically distinct chemo-preventive agents in the cell culture models for the FAP syndrome (5,9,10), and complement their *in vivo* efficacy to inhibit adenoma formation in the animal models for the FAP syndrome (11-13,17).

Mechanistic experiments on $Apc^{-/-}$ 1638N COL-Pr_1 cells demonstrated that these cells exhibited cytostatic growth arrest in response to treatment with SUL and CLX, as evidenced by increased G_1:S+G_2/M ratio. This inhibitory effect was associated with decreased expression of β-catenin, cyclin D1, c-Myc and COX-2. Collectively, these data are consistent with the

Table V. Drug-resistant stem cells in SUL-R Apc$^{-/-}$ 1638N COL-Pr1 cells.

Stem cell marker[a]	SUL-S	SUL-R	δ-SUL-S
Tumor spheroids	2.3±1.7	18.7±3.3	+7.1X[b]
CD44 (FU)	2.1±0.6	14.4±1.5	+5.7X[c]
CD133 (FU)	3.1±1.2	14.2±1.3	+3.6X[d]
c-Myc (FU)	2.6±0.7	7.0±0.5	+1.7X[e]

[a]Mean ± standard deviation (n=3 per treatment group). [b,c]P=0.01 compared with the SUL-S phenotype. [d,e]P=0.02 compared with the SUL-S phenotype. Data were analyzed by Student's t-test. SUL-S, sulindac-sensitive; SUL-R, sulindac-resistant; FU, log mean fluorescence units; Apc, adenomatous polyposis coli; X, fold-change.

evidence that c-Myc and COX-2 are established early response genes that drive the process of cell proliferation in response to oncogene/hormone/growth factor-mediated stimulus, and that β-catenin, cyclin D1, c-Myc and COX-2 represent established Apc target genes (2,17). It is also noteworthy that these Apc target genes are documented to be downregulated by several NSAIDs and COXIBs in the preclinical *in vivo* models for the FAP syndrome (11-13,17,27-29).

Metabolic and pharmacokinetic profiles of the prototypic NSAID SUL have provided evidence that hepatic metabolism of this pro-drug generates sulfide and sulfone derivatives. The sulfide derivative functions as a potent inhibitor of prostaglandin E2 synthesis, while the sulfone derivative exerts potent anti-proliferative and pro-apoptotic effects, independent of the COX status, within the pharmacologically achievable concentration ranges (28,30). Thus, the sulfide and sulfone derivatives of SUL represent bio-active metabolites functioning via distinct COX-dependent and -independent pathways in the animal model for the FAP syndrome, colon carcinoma-derived cell culture models *in vitro*, as well as tumor xenograft models *in vivo* (28,30). Although SUL, as well as its metabolites, are individually effective in cell culture models (5,10,30), little evidence is available as to whether SUL may be directly metabolized to generate these metabolic derivatives in the cell culture models. In this regard, reliable colon-derived cell culture models offer relevant experimental approaches to examine the preventive/therapeutic efficacy of anti-inflammatory agents directly on the target cells (5,7,9,10). The growth-inhibitory efficacy of SUL in the present experimental system raises an intriguing possibility, that either the prodrug exerts direct inhibitory effects, or that target cells are able to metabolize SUL and generate growth-inhibiting bio-active agents.

The SUL-R phenotype exhibits enhanced expression of cancer stem cell markers, such as spheroid formation, and positive expression of CD44, CD133 and c-Myc. In this context, it is noteworthy that CD44^{+} and CD133^{+} stem cells have been documented in several human colon carcinoma-derived cell lines (31-33), and positive expression of these markers in colon carcinoma-derived HCT-116 cells have been correlated with the metastatic phenotype (31). In addition, as regards the upregulated expression of CD44 and c-Myc, supportive lines of evidence suggest that these Apc target genes are upregulated in colon cancer stem cells (20-22,31-33). Furthermore, the SUL-R phenotype also raises the possibility that these resistant cells may display altered expression of non-COX targets, such as β-catenin, E-cadherin, mitochondrial apoptotic proteins, cyclin-dependent kinase inhibitor p21$^{WAF1/CIP1}$ and peroxisome proliferator-activated receptor-γ (PPAR-γ). Of note, abrogation of the expression of p21 and PPAR-γ has been documented in SUL-R colon tumors in the Apc$^{Min/+}$ mouse model, suggesting that loss of these proteins characterizes resistance to SUL (34,35).

Wnt/β-catenin signaling regulates telomerase activity in cancer and cancer-initiating stem cells. At the mechanistic level, β-catenin transcriptionally regulates the gene encoding Tert, which represents the enzymatic subunit of the telomerase enzyme (36). Furthermore, several natural compounds and their derivatives, as well as synthetic small molecules, achieve a potent inhibition of telomerase in cell culture models for epithelial cancers (37-40). In this regard, the present colonic epithelial cell culture model differing in the expression of the tumor suppressor Apc gene, together with a model for Apc$^{-/-}$ drug-resistant telomerase-expressing colonic cancer stem cells, may provide novel mechanism-based experimental approaches for the identification of efficacious stem cell-targeted therapeutic agents.

Anti-inflammatory agent-based monotherapy for genetically predisposed, early-onset colon cancer and/or early sporadic colon cancer, similar to long-term sequential chemotherapy with fluoropyrimidines, oxaliplatin and irinotecan for metastatic colon cancer, may enhance the risk of acquired tumor resistance and resultant emergence of drug-resistant cancer stem cells (41,42). It is noteworthy that drug-resistant cancer stem cells have been isolated and characterized from colon carcinoma-derived cell culture models (31-33) and from luminal A and triple-negative breast carcinoma-derived cell culture models (43,44).

With regard to the future research directions, it is noteworthy that our previously published data on the Apc$^{+/-}$ 850^{Min} COL model for the FAP syndrome have documented the growth-inhibitory efficacy of several mechanistically distinct pharmacological agents, such as CLX and difluoromethyl ornithine, and naturally occurring agents, such as epigallocatechin gallate, curcumin and eicosapentaenoic acid (7,8). Additionally, selected Chinese nutritional herbs have provided promising leads for their growth-inhibitory mechanisms in the 850^{Min} COL model (Telang *et al*, unpublished results). Based on the abovementioned observations, future experiments on the Apc$^{-/-}$ SUL-R cancer stem cell model will examine whether relatively non-toxic natural products or nutritional herbs are able to directly target the cancer stem cell phenotype. In these experiments, optimized assays for stem cell markers will represent the quantitative mechanistic endpoints for evaluating the extent of stem cell targeting by the test compounds.

However, it must be mentioned that the present cell culture-based approaches provide only a limited validation for their clinical relevance and translatability. Previous evidence for therapeutic targeting of cancer stem cells from patient-derived xenografts of gastric cancer (45), pancreatic ductal adenocarcinoma (46) and colonic organoids derived from induced pluripotent stem cells from APC mutant familial

adenomatous polyposis patients (47) support the validity of future experimental approaches.

Acknowledgements

The author acknowledges productive collaborations with former colleague Dr Meena Katdare, and former research fellow Dr Guo Li for their extensive participation in the research program 'Preclinical models for genetically predisposed early onset colon cancer'. This program has been funded in the past through NCI Contract Research Master Agreement CN 75029-63, NIH Grant CA 29502-S1 and philanthropic support to Strang Cancer Prevention Center.

References

1. American Cancer Society: Facts & Figures-2016. American Cancer Society, Atlanta, GA, 2016.
2. Fodde R, Smith R and Clevers H: APC, signal transduction and genetic instability in colorectal cancer. Nat Rev Cancer 1: 55-67, 2001.
3. Fodde R, Edelman W, Yang K, Van Leeuwen C, Carlson C, Renault B, Breukel C, Alt E, Lipkin M, Khan PM, *et al*: A targeted chain termination mutation in the mouse Apc gene results in multiple intestinal tumors. Proc Natl Acad Sci USA 91: 8969-8973, 1994.
4. Oshima M, Oshima H, Kitagama K, Kobayashi M, Itakura C and Takata M: Loss of Apc heterozygosity and abnormal tissue building in nascent intestinal polyps in mice carrying a truncated Apc gene. Proc Natl Acad Sci USA 92: 4482-4486, 1995.
5. Katdare M, Kopelovich L and Telang N: Efficacy of chemopreventive agents for growth inhibition of Apc [+/-] 1638NCOL colonic epithelial cells. Int J Mol Med 10: 427-432, 2002.
6. Telang NT, Li G and Katdare M: Prevention of early onset familial/hereditary colon cancer: New models and mechanistic biomarkers (review). Int J Oncol 28: 1523-1529, 2006.
7. Telang N and Katdare M: Combinatorial prevention of carcinogenic risk in a model for familial colon cancer. Oncol Rep 17: 909-914, 2007.
8. Telang N and Katdare M: Novel cell culture model for prevention of carcinogenic risk in familial polyposis syndrome. Oncol Rep 21: 1017-1021, 2009.
9. Telang N and Katdare M: Putative cancer initiating stem cells in a model for familial adenomatous polyposis (FAP) syndrome. Int J Mol Med 30 (Suppl 1): S10, 2012.
10. Telang N: Preventive efficacy of the non-steroidal anti-inflammatory drug Sulindac on cell culture models for genetically predisposed early onset colon cancer. Int J Mol Med 34 (Suppl 1): S15, 2014.
11. Beazer-Barkley Y, Levy DB, Moser AR, Dove WF, Hamilton SR, Vogelstein B and Kinzler KW: Sulindac suppresses tumorigenesis in the Min mouse. Carcinogenesis 17: 1757-1760, 1996.
12. Jacoby RF, Siebert K, Cole CE, Kelloff G and Lubet RA: The cyclooxygenase-2 inhibitor celecoxib is a potent preventive and therapeutic agent in the Min mouse model of adenomatous polyposis. Cancer Res 60: 5040-5044, 2000.
13. Jacoby RF, Cole CE, Tutsch F, Newton MA, Kelloff G, Hawk ET and Lubet RA: Chemopreventive efficacy of combined piroxicam and difluoromethylornithine treatment on Apc mutant Min mouse adenoma, and selective toxicity against Apc mutant embryos. Cancer Res 60: 1864-1870, 2000.
14. Baron JA, Cole BF, Sandler RS, Haile RW, Ahnen D, Bresalier R, McKeown-Eyssen G, Summers RW, Rothstein R, Burke CA, *et al*: A randomized trial for aspirin to prevent colorectal adenomas. N Engl J Med 348: 891-899, 2003.
15. Girdiello FM, Hamilton SR, Krush AJ, Piantadosis S, Hylind LM, Cellano P, Brooker SV, Robinson CR and Offerhans GL: Treatment of colonic and rectal adenomas with Sulindac in familial adenomatous polyposis. N Engl J Med 328: 1313-1316, 1993.
16. Stienbeck G, Lynch PM, Phillips RK, Wallace MH, Hawk E, Gordon GB, Wakabayashi N, Saunders B, Shen Y, Fujimura T, *et al*: The effect of Celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. N Engl J Med 342: 1946-1952, 2000.
17. Gupta RA and Dubois RN: Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. Nat Rev Cancer 1: 11-21, 2001.
18. Solomon SD, McMurray JJ, Pfeffer MA, Wilters J, Fowler R, Finn P, Anderson WF, Zauber A, Hawk E and Bertagnolli M; Adenoma Prevention with Celecoxib (APC) Study Investigators: Cardiovascular risk associated with Celecoxib in a clinical trial for colorectal adenoma prevention. N Engl J Med 352: 1071-1080, 2005.
19. Bresalier RS, Sandler RS, Quan H, Bolognese JA, Oxenius B, Horgam K, Lines C, Ridell R, Morton D, Lanas A, *et al*: Cardiovascular events associated with rofecocixb in a colorectal adenoma chemoprevention trial. N Engl J Med 352: 1092-1102, 2005.
20. Dalerba P, Dylla SJ, Park IK, Liu R, Wang X, Cho RW, Hoey T, Gurney A, Huang EH, Simeone DM, *et al*: Phenotypic characterization of human colorectal cancer stem cells. Proc Natl Acad Sci USA 104: 10158-10163, 2007.
21. O'Brien CA, Pollett A, Gallinger S and Dick JE: A human colon cancer cell capable of initiating tumor growth in immunedeficient mice. Nature 445: 106-110, 2007.
22. Ricci-Vitiani L, Lombardi DG, Pillozi E, Biffoni M, Todaro M, Peschele C and De Maria R: Identification and expansion of human colon cancer initiating cells. Nature 445: 111-115, 2007.
23. Balkwill F and Mantovani A: Inflammation and cancer: Back to virchow? Lancet 357: 539-545, 2001.
24. Tanaka T, Kohno H, Suzuki R, Hata K, Sugie S, Niho N, Sakano K, Takahashi M and Wakabayashi K: Dextran sodium sulfate strongly promotes colorectal carcinogenesis in Apc(Min/+) mice: Inflammatory stimuli by Dextran sodium sulfate results in development of multiple colonic neoplasms. Int J Cancer 118: 25-34, 2006.
25. Basudhar D, Somsudaram V, Glyn S, Ridnour LA, Cheng RY and Wink DA: Regulatory effects of NOS2-COX2 in breast progression in tumor microenvironment. Int J Mol Med 38 (Suppl 1): S81, 2016.
26. Katdare M, Osborne M and Telang NT: Soy isoflavone genistein modulates cell cycle progression and induces apoptosis in HER-2/neu oncogene expressing human breast epithelial cells. Int J Oncol 21: 809-815, 2002.
27. Boolbol SK, Dannenberg AJ, Chadburn A, Martucci C, Guo XJ, Ramonetti JT, Abreu-Goris M, Newmark HL, Lipkin ML, DeCosse JJ and Bertagnolli MM: Cyclooxygenase-2 over-expression and tumor formation are blocked by Sulindac in a murine model for familial adenomatous polyposis. Cancer Res 56: 2556-2560, 1996.
28. Mahmood NN, Boolbol SK, Dannenberg AJ, Mestre JR, Bilinski RT, Martucci C, Newmark HL, Chadburn A and Bertagnolli MM: The sulfide metabolite of Sulindac prevents tumors and restores enterocyte apoptosis in a murine model for familial adenomatous polyposis. Carcinogenesis 19: 87-91, 1998.
29. Mahmood NN, Bilinski RT, Churchill MR, Edelmann W, Kucherlapati R and Bertagnolli MM: Genotype phenotype correlation in murine Apc mutation: Differences in enterocyte migration and response to sulindac. Cancer Res 59: 353-359, 1999.
30. Williams CS, Goldman AP, Sheng H, Morrow JD and Dubois RN: Sulindac sulfide, but not Sulindac sulfone, inhibits colorectal cancer growth. Neoplasia 1: 170-176, 1999.
31. Chen KL, Pan F, Jiang H, Chen JF, Pei L, Wie FW and Liang HJ: Highly enriched CD133(+)CD44(+) stem-like cells with CD133(+) CD44(high) metastatic subset in HCT116 colon cancer cells. Clin Exp Metastasis 28: 751-763, 2011.
32. Wang C, Xie J, Guo J, Manning HC, Gore JC and Gou N: Evaluation of CD44 and CD133 as cancer stem cell markers for colorectal cancer. Oncol Rep 28: 1301-1308, 2012.
33. Zhou JY, Chen M, Ma L, Wang X, Chen YG and Lui SL: Role of CD44(high)/CD133(high) HCT-116 cells in the tumorigenesis of colon cancer. Oncotarget 7: 7657-7666, 2016.
34. Greenspan EJ, Nichols FC and Rosenberg DW: Molecular alterations associated with Sulindac-resistant colon tumors in ApcMin/+ mice. Cancer Prev Res (Phila) 3: 1187-1197, 2010.
35. Zeilstra J, Joosten SP, Dokter M, Verwiel E, Spaargaren M and Pals ST: Deletion of the WNT target and cancer stem cell marker CD44 in Apc(Min/+) mice attenuates intestinal tumorigenesis. Cancer Res 68: 3655-3661, 2008.
36. Hoffmeyer K, Raggioli A, Rudolf S, Anton R, Hierholzer A, Del Valle I, Hein K, Vogt R and Kemlar R: Wnt/β-catenin signaling regulates telomerase in stem cells and cancer cells. Science 336: 1549-1554, 2012.

37. Nasuni I, Seimiya H and Tsuruo T: Telomerase inhibition, telomere shortening, and senescence of cancer cells by tea catechins. Biochem Biophys Res Commun 249: 391-396, 1998.
38. Ramchandran C, Fonseca HB, Jhabvala P, Escalon EA and Melnick SJ: Curcumin inhibits telomerase activity through human telomerase reverse transcritpase in MCF-7 breast cancer cell line. Cancer Lett 184: 1-6, 2002.
39. Jagadeesh S, Kyo S and Banerjee PP: Genestein represses telomerase activity via both transcriptional and posttranslational mechanisms in human prostate cancer cells. Cancer Res 66: 2107-2115, 2006.
40. Arndt GM and MacKenzie KL: New prospects for targeting telomerase beyond the telomere. Nat Rev Cancer 16: 508-524, 2016.
41. Dean M, Fojo T and Bates S: Tumor stem cells and drug resistance. Nat Rev Cancer 5: 275-284, 2005.
42. Lobo NA, Shimono Y, Qian D and Clarke MF: The biology of cancer stem cells. Annu Rev Cell Dev Biol 23: 675-699, 2007.
43. Telang N: Putative cancer initiating stem cells in cell culture models for molecular subtypes of clinical breast cancer. Oncol Lett 10: 3840-3846, 2015.
44. Telang N: Growth inhibitory efficacy of natural products in a model for triple negative molecular subtype of clinical breast cancer. Biomed Rep 7: 199-204, 2017.
45. Nguyen PH, Giraud J, Staedel C, Chambonnier L, Dubus P, Cheveret E, Boeuf H, Gauthereau X, Rousseau B, Fevre M, *et al*: All-trans retinoic acid targets gastric cancer stem cells and inhibits patient-derived gastric carcinoma tumor growth. Oncogene 35: 5619-5628, 2016.
46. Roife D, Dai B, Kang Y, Perez MVR, Pratt M, Li X and Fleming JB: Ex Vivo testing of patient-derived xenografts mirrors the clinical outcome of patients with pancreatic ductal adenocarcinoma. Clin Cancer Res 22: 6021-6030, 2016.
47. Crespo M, Vilar E, Tsai SY, Chang K, Amin S, Srinivasan T, Zhang T, Pipalia NH, Chen HJ, Witherspoon M, *et al*: Colonic organoids derived from human induced pluripotent stem cells for modeling colorectal cancer and drug testing. Nat Med 23: 878-884, 2017.