Stem cell models for genetically predisposed colon cancer (Review)

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Abstract. Negative growth regulatory tumor suppressor genes and positive growth regulatory oncogenes serve important roles in initiation/progression of colon cancer. Germline mutation in the adenomatous polyposis coli (APC) tumor suppressor gene represents a primary genetic defect for familial adenomatous polyposis (FAP) syndrome, a predisposing factor for clinical colon cancer. Somatic mutations in the APC gene are common in sporadic colon cancer. Preclinical and clinical efficacy is documented for targeted therapy with non-steroidal anti-inflammatory drugs, selective cyclo-oxygenase-2 inhibitors for prostaglandin biosynthesis and selective inhibitor of ornithine decarboxylase for polyamine biosynthesis. However, these therapeutic options lead to systemic toxicity, acquired tumor resistance and emergence of therapy resistant cancer stem cells. By contrast, non-toxic natural products are unlikely to exhibit drug resistance and may represent testable alternatives for therapy resistant colon cancer. Tumorigenic Apc [-/-] colonic epithelial cell lines derived from preclinical FAP models provide novel cellular models for drug resistant cancer stem cells. Apc [-/-] Sulindac resistant (SUL-R) cells exhibit upregulated expression levels of cancer stem cell markers. Natural products, such as naturally occurring vitamin A derivative all-trans retinoic acid (ATRA) and the anti-cancer agent from Turmeric root curcumin (CUR), represent testable alternatives. Relative to the non-tumorigenic Apc [+/+] C57 COL colonic epithelial cells, the tumorigenic Apc [-/-] 1638N COL and Apc [-/-] 850 ^{MIN} COL cells exhibit aneuploid cell hyper-proliferation and upregulated expression of Apc target genes β-catenin, cyclin D1, c-myc and COX-2. The SUL-R phenotypes exhibit enhanced tumor spheroid formation and upregulated expression levels of stem cell markers CD44, CD133 and c-Myc. Treatment of the SUL-R stem cells with ATRA and CUR inhibits tumor spheroid formation and reduces the expression of stem cell markers. Stem cell models developed for FAP syndrome provide a novel experimental approach to identify mechanistic leads for efficacious natural products as testable alternatives for therapy-resistant, genetically predisposed colon cancer.

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Contents

1. Introduction

Tumor suppressor genes APC, TP53 and SMAD, and oncogenes KRAS and BRAF, operate at distinct phases of multi-step carcinogenesis and thereby, may also represent genetic markers for colon cancer risk (1). Loss of function mutation in the tumor suppressor adenomatous polyposis coli (APC) gene represents a primary genetic defect, leading to clinical Familial adenomatous polyposis (FAP) syndrome, a predisposing factor for colon cancer. At the phenotypic level, truncated protein of mutant APC induces aneuploidy and chromosomal instability, and enhances the risk for tumorigenic transformation in the target tissue (2,3).

Preclinical animal models for genetically predisposed FAP syndrome such as Apc $^{[+/-]}$ 1638N and Apc $^{[+/-]}$ 850^{MIN}/+ mice carry germ line mutations at codons 1638 and 850 of the Apc gene, respectively (4,5). The WNT-β-catenin signaling pathway in general, and the tumor suppressor Apc gene in particular, have been well-documented to play a critical role in colon carcinogenesis. At the mechanistic level, wild type Apc $^{[+/+]}$ regulates the levels of β-catenin via ubiquitination and proteosome mediated degradation. At the structural level, truncated protein of mutant Apc gene exhibits loss of microtubule-binding site, β-catenin-binding sites and axin-binding sites. Loss of microtubule-binding site promotes chromosomal instability and aneuploidy. Loss of β-catenin binding sites favors nuclear translocation of the Apc target gene β-catenin and its expression via the β-catenin-LEF/TCF axis (2,3).

Unlike clinical colonic FAP, mouse models for FAP that carry germline mutations in the tumor suppressor Apc gene, exhibit multiple intestinal neoplasia predominantly in the small intestine. As a viable alternative, experimental models derived from mouse colonic epithelial cells that exhibit

relevant genetic defects and express quantifiable cancer risk promise to provide unique, clinically relevant experimental approaches for genetically predisposed colon cancer.

The 850^{MIN}/+ preclinical animal model for FAP syndrome has been extensively utilized to investigate chemo-preventive efficacy of mechanistically distinct pharmacological agents (6-8), and natural products (9,10). Targeted therapy with non-steroidal anti-inflammatory drugs, selective cyclo-oxygenase-2 (COX-2) inhibitors and selective ornithine decarboxylase (ODC) inhibitors have shown efficacy in clinical FAP and sporadic colon cancer (11,12), as well as in preclinical FAP model as monotherapy as well as combinatorial therapy (13-16). Long-term therapy with pharmacological agents is associated with systemic toxicity and acquired tumor resistance (17).

In contrast to drug resistance by the pharmacological agents (17), non-toxic natural products are less likely to induce treatment related tumor resistance, and therefore, may represent testable alternatives for therapy resistant colon cancer (10,18,19).

Combinatorial approaches utilizing *in vitro* tissue culture and *in vivo* transplantation assays have been optimized for isolation and characterization of putative cancer stem cells. The cell culture assays include i) Drug efflux positive side population, ii) Aldehyde dehydrogenase-1 (ALDH-1) positive cells, iii) cells forming non-adherent tumor spheroids, iv) Phenotypes positive for cluster of differentiation (CD44, CD133), v) Phenotypes positive for nuclear transcription factors Octamer binding transcription factor-4 (Oct-4), Kruppel-like factor-4 (Klf-4), sex determining region-box-2 (SOX-2), cellular Myc (c-Myc) and DNA-binding homeobox nuclear transcription factor (NANOG), and vi) cells positive for resistance to conventional chemo-endocrine therapy and/or to targeted therapy (20-22). *In vivo* transplantation assays have documented cancer initiating properties of colon cancer stem cells (23-26). Additionally, maintenance of the stem cell phenotype is dependent on the expression of transcription factors Oct-4, Klf-4, Sox-2 and c-Myc (27-29). Collectively, the expression of these proteins represents stem cell specific cellular and molecular markers, and cancer stem cell models expressing these markers facilitate identification of stem cell targeted testable alternatives for therapy resistant colon cancer (30).

Based on the importance of cancer initiating stem cells in colon cancer progression (23-25), and of published evidence on the parental colonic epithelial cell culture models for the FAP syndrome (31,32), current research has been extended to develop colon cancer stem cell models, and has been summarized in the review.

The present review summarizes the experimental evidence for i) Colonic epithelial cell derived models for the FAP syndrome, ii) Isolation and characterization of drug resistant stem cell phenotypes, and iii) Stem cell targeted efficacy of natural products as testable alternatives for chemotherapy resistant colon cancer.

2. Cellular models

Combination of *in vitro* organ culture/cell culture assays have been effectively used to investigate the aspect of cancer initiation in multi-step colon carcinogenesis. For example, organ cultures from histo-pathologically normal colonic crypts treated with the carcinogen dimethyl hydrazine produce hyper-cellular aberrant crypt foci upon transplantation (33). Apc mutant colonic epithelial cells exhibit spontaneous immortalization *in vitro* and tumorigenic transformation upon *in vivo* transplantation (18,19,30-32).

1638N COL and 850^{MIN} COL models. Colonic epithelial cell culture model developed from the descending colon of wild type Apc $^{[+/+]}$ mice retains the original Apc $^{[+/+]}$ genotype. In contrast, cells derived from anchorage independent colonies from descending colons of Apc $^{[+/-]}$ mice exhibit Apc $^{[-/-]}$ genotype. Lack of expression of the tumor suppressor gene Apc leads to chromosomal instability, aneuploidy and upregulated expression of Apc target genes (1-3). Therefore, Apc $^{[+/+]}$ C57 COL cells represent an appropriate control for Apc$^{[-/-]}$ 1638N COL and Apc$^{[-/-]}$ 850 ^{MIN} COL cells. These models described in Table I are utilized to monitor the status of aneuploidy, cell proliferation, and expression of Apc target genes. The data for biomarker modulation are summarized from prior publications on the Apc $^{[+/-]}$ 1638N COL model (18,31) and on the Apc $^{[+/-]}$ 850 ^{MIN} COL model (19,32).

Aneuploid cell proliferation. Apc $^{[+/+]}$ C57 COL cells are diploid, while the two Apc $^{[-/-]}$ cell lines exhibit detectable aneuploid cell population. Status of aneuploid cell hyper-proliferation demonstrates that the two Apc $^{[-/-]}$ cells lines exhibit about a 50 to 59% reduction in population doubling times and about a 3 to 8 fold increase in the saturation density. These biomarker modulations result in emergence of aneuploid cell population exhibiting altered aneuploid quiescent (G_1) and proliferative ($S+G_2/M$) ratio (Table II). These data suggest that hyper-proliferative aneuploid cell population may represent relevant target cells for carcinogenic transformation in Apc mutant colonic epithelial cell models. Consistent with this aspect, the two cell lines exhibit anchorage independent colony formation *in vitro* that represents a surrogate end point for *in vivo* tumorigenic transformation (19,32). Furthermore, *in vivo* transplantation of Apc $^{[-/-]}$ 1638N COL cells produce rapidly growing tumors at the transplant site (30).

Apc target gene expression. Expression of downstream target genes of Apc (1-3,34) in the two Apc $^{[-/-]}$ 1638N COL and 850^{MIN} COL cell lines demonstrates that Apc gene is essentially undetectable, while that of its target genes β-catenin, cyclin D1, c-Myc and COX-2 is significantly increased relative that in Apc $^{[+/+]}$ C57 COL cell line (Table III).

The data on the immuno-fluorescence of cyclin D1, β-catenin, c-Myc, COX-2, CD44 and CD133 are generated using a quantitative immunofluorescence assay adapted for the Apc 1638N COL model (30). The assay monitors fluorescent events in cells stained with appropriate FITC labeled antibodies and sorted using a flow cytometer. The data are normalized for fluorescence in FITC-IgG stained cells, and presented as log mean fluorescent units (FU) per 10^4 fluorescent events.

Statistical analysis. The data on population doubling times, saturation density and Apc target genes are analyzed using one-way ANOVA with Dunnett's multiple comparison

Table I. Cellular models.

Cell line	Apc genotype	Origin and characteristics
C57 COL	+/+	Epithelial cell line derived from normal descending colon of Apc $^{[+/+]}$ C57BL J6 mouse. Spontaneously immortalized. Non-tumorigenic.
1638N COL	-/-	Colonic epithelial cell line derived from AI colony from Apc $^{[+/-]}$ 1638N COL cells. Spontaneously immortalized. Tumorigenic.
850 ^{MIN} COL	-/-	Colonic epithelial cell line derived from AI colony from Apc $^{[+/-]}$ 850 ^{MIN} COL cells. Spontaneously immortalized. Tumorigenic.

Apc, adenomatous polyposis coli.

Table II. Aneuploid cell hyper-proliferation in 1638N COL and 850 ^{MIN} COL cell lines.

	Colonic epithelial cell line[a]				
End point marker	Apc$^{[+/+]}$ C57 COL	Apc $^{[-/-]}$ 1638N COL	P-value	Apc $^{[-/-]}$ 850 ^{MIN} COL	P-value
Population doubling time, h	34.0±3.8	17.0±1.9	0.037	14.0±1.6	0.037
Saturation density, x10^5	7.7±0.5	32.5±2.1	0.014	67.9±7.5	0.014
Aneuploid phenotype, %	Undetectable	22.8±2.5		81.7±9.1	
Aneuploid G_1:S+G_2/M	Undetectable	3.3±0.2		0.6±0.2	

[a]Mean ± SD, n=4 per treatment group. Population doubling time C57 COL > 1638N COL. C57 COL > 850 ^{MIN} COL. Saturation density C57 COL < 1638N COL. C57 COL < 850 ^{MIN} COL. Data were analyzed by ANOVA and Dunnett's multiple comparison test (α=0.05). Apc, adenomatous polyposis coli.

post-hoc test with a threshold of α=0.05. The data on tumor spheroids are analyzed using the chi square (χ^2) test. The χ^2 values for the differences between the control and treatment groups greater than 3.84 (P<0.05) are considered significant. The data on CD44, CD133 and c-Myc are analyzed using the two-sample Student's t test. P<0.05 was considered to indicate a statistically significant difference. The statistical analyses were performed using the Microsoft Excel 2013 XLSTAT-Base software.

Overall, these data suggest that lack of expression of the tumor suppressor Apc gene favors upregulated expression of its target genes. Thus, Wnt signaling pathway in general, and Apc target genes in particular, may represent important therapeutic targets for colon cancer (34).

3. Drug resistant stem cells

Chronic exposure of Apc $^{[-/-]}$ colonic epithelial cells to cytostatic concentrations of sulindac (SUL) favors the emergence of actively proliferating sulindac resistant (SUL-R) cells. Status of expression of select stem cell markers in the SUL-R phenotype isolated from Apc $^{[-/-]}$ 1638N COL cells and from Apc $^{[-/-]}$ 850 ^{MIN} COL models demonstrate a significant increase in tumor spheroid formation in SUL-R cells relative to that in sulindac sensitive (SUL-S) cells. In addition, the expression of other stem cell markers CD44, CD133 and c-Myc are significantly increased in SUL-R cells as quantified by the cellular immunofluorescence assay and presented as log mean fluorescent units (Table IV).

In this context, it is notable that in addition to functioning as stem cell markers, CD44 and c-Myc represent well-established Apc target genes (2,3), while CD133 represents a well-documented stem cell marker in various organ site cancers including colon cancer (35-37). Collectively, these data demonstrate that the drug resistant phenotypes from the two Apc $^{[-/-]}$ cell lines are enriched for cancer stem cells.

4. Chemo-preventive natural products

The Apc 850^{MIN}/+ mouse model for FAP syndrome (38) has exhibited growth inhibitory efficacy for adenoma formation in response to treatment with several mechanistically distinct pharmacological agents such as the non-steroid anti-inflammatory drug SUL and selective COX-2 inhibitor celecoxib (CLX), and selective polyamine synthesis inhibitor difluoro methyl ornithine (DFMO) (6-8,13,14). Natural products such as tea polyphenol epigallo catechin gallate (EGCG) and active agent from Turmeric Curcumin (CUR) have also demonstrated preventive efficacy in the Apc 850 ^{MIN}/+ model (9,10). In addition. Low dose combinations of select pharmacological agents have documented enhanced efficacy in the Apc 850 ^{MIN}/+ mouse model (7,14,15) as well as in patients (16,39). Consistent with these *in vivo* data, published evidence has documented similar efficacy of pharmacological agents in the 1638N COL model (18,31), and of pharmacological and natural agents in the 850 ^{MIN} COL model (19,32). Unlike pharmacological agents, natural products rarely exhibit systemic toxicity and are less likely to induce acquired drug resistance. These aspects favor

Table III. Status of Apc and Apc target genes in Apc $^{[-/-]}$ colonic epithelial cells.

	Colonic epithelial cell line[a]				
Target gene	Apc$^{[+/+]}$ C57 COL	Apc $^{[-/-]}$ 1638N COL	P-value	Apc $^{[-/-]}$ 850 ^{MIN} COL	P-value
Apc	17.6±1.7	Undetectable		Undetectable	
β-catenin	4.7±0.3	8.2±0.5	0.020	9.0±0.8	0.014
Cyclin D1	3.0±0.1	15.1±1.4	0.014	16.1±1.5	0.020
c-Myc	2.1±0.2	6.8±0.6	0.031	6.6±0.4	0.031
COX-2	3.9±0.4	14.3±1.6	0.020	12.8±1.8	0.031

[a]Mean ± SD, N=4 per treatment group. Data are presented as the log mean FU. β-catenin, cyclin D1, c-Myc and COX-2. C57 COL < 1638N COL. C57 COL < 850 ^{MIN} COL. Data were analyzed by ANOVA and Dunnet's test (α=0.05). Apc, adenomatous polyposis coli; COX-2, cyclooxygenase; FU, fluorescence units.

Table IV. Stem cell marker expression in drug resistant Apc $^{[-/-]}$ cells.

	Colonic epithelial cell line							
	Apc $^{[-/-]}$ 1638N COL				Apc $^{[-/-]}$ 850^{MIN} COL			
Drug resistance	TS[a]	CD44[b]	CD133[b]	c-Myc[b]	TS[a]	CD44[b]	CD133[b]	c-Myc[b]
SUL-S	2.3±0.6	2.5±0.5	3.1±0.4	2.6±0.5	3.8±0.4	2.1±0.5	2.4±0.6	3.4±0.4
SUL-R	18.7±3.5	15.8±2.9	14.2±2.8	7.0±0.3	19.8±2.9	14.8±1.8	15.3±1.5	7.8±1.4
P-value	0.005	0.010	0.010	0.020	0.010	0.010	0.010	0.020
χ^2	7.88				6.63			
δSUL-S	+7.1X	+5.3X	+3.6X	+1.7X	+4.2X	+6.0X	+5.4X	+1.3X

[a]TS counts performed on day 14 post-seeding of 100 cells. Mean ± SD, n=3 per treatment group. Data were analyzed by χ^2 test. [b]Immunofluorescence assay performed on day 3 post-seeding and presented as log mean FU. Mean ± SD, n=3 per treatment group. Data were analyzed by the two-sample Student's t test. Apc, adenomatous polyposis coli; TS, tumor spheroids; CD, cluster of differentiation; c-Myc, cellular Myc; SUL-S, sulindac sensitive; SUL-R, sulindac resistant; SD, standard deviation; X, fold.

the concept that natural products may function as potential testable alternatives for therapy resistant colon cancer.

The evidence for stem cell targeted modulation by the naturally occurring vitamin A derivative ATRA demonstrates that this endogenous metabolite at its maximum cytostatic concentration induces a significant decrease in the number of tumor spheroids, and significantly reduces the expression of CD44, CD133 and c-Myc in the Apc$^{[-/-]}$ 1638N COL model (Table V). Similarly, the natural agent CUR induces a significant inhibition in tumor spheroid formation, and a significant reduction in CD44, CD133 and c-Myc expression in the Apc $^{[-/-]}$ 850 ^{MIN} COL model (Table VI). Collectively, these data offer a proof of concept for stem cell targeted inhibitory efficacy of natural products ATRA and CUR.

This evidence taken together, validates an approach to identify other natural products as stem cell targeted testable alternatives. In this context, it is noteworthy that several mechanistically distinct natural products including sulforaphane, benzyl isothiocynate, quercetin, all trans-retinoic acid, and carnosol have documented inhibitory efficacy in stem cell models established from breast, pancreas, gastric and prostate cancer (40-47).

5. Study conclusions

The data discussed in the present review support a speculative validation for following mechanistic leads that are relevant to novel cellular models for FAP syndrome, isolation and characterization of drug resistant colonic epithelial stem cells and identification of efficacious natural products as testable alternatives for stem cell targeted therapeutic potential.

a) Apc $^{[-/-]}$ 1638N COL and Apc $^{[-/-]}$ 850^{MIN} COL cells exhibit hyper-proliferation of aneuploid phenotype, and upregulated expression of Apc target genes β-catenin, cyclin D1, c-Myc and COX-2.

b) Long-term treatment with SUL facilitates emergence of SUL-R phenotype. This drug resistant phenotype exhibits increased tumor spheroid formation and increased expression of stem cell markers CD44, CD133, and c-Myc, relative to the SUL-S phenotype.

c) Treatment with vitamin A derivative ATRA and Turmeric component CUR results in inhibition of tumor spheroid formation and in downregulated expression of CD44, CD133 and c-Myc.

Table V. Inhibition of stem cell marker expression in sulindac resistant Apc $^{[-/-]}$ 1638N COL cells.

		Stem cell marker			
Treatment	Concentration	TS[a]	CD44[b]	CD133[b]	c-Myc[b]
EtOH	0.01%	15.5±1.5	16.6±1.0	17.0±1.2	8.2±1.2
ATRA	2 μM	4.8±0.5	4.2±0.2	5.2±0.3	3.5±0.4
P-value		<0.01	0.01	0.01	0.03
χ^2		7.74			
δEtOH, %		-69.0	-74.7	-69.4	-57.3

[a]TS count performed on day 14 post-seeding of 100 cells. Mean ± SD, n=3 per treatment group. Data were analyzed using a χ^2 test. [b]Immunofluorescence assay performed at day 3 post-seeding and presented as log mean FU. Mean ± SD, n=3 per treatment group. Data were analyzed using the two-sample Student's t-test. TS, tumor spheroid; CD, cluster of differentiation; c-Myc, cellular Myc; EtOH, ethanol; ATRA, all trans-retinoic acid; SD, standard deviation, FU, fluorescent unit; X2, chi square. TS, tumor spheroids; CD, cluster of differentiation; c-Myc, cellular Myc; EtOH, ethanol; ATRA, all trans-retinoic acid.

Table VI. Inhibition of stem cell marker expression in sulindac resistant Apc $^{[-/-]}$ 850^{MIN} COL cells.

		Stem cell marker			
Treatment	Concentration	TS[a]	CD44[b]	CD133[b]	c-Myc[b]
EtOH	0.01%	24.8±1.2	15.3±1.9	16.4±2.2	8.3±1.2
CUR	20 μM	4.0±0.2	3.5±1.5	5.3±1.8	3.8±0.8
P-value		<0.01	0.01	0.01	0.03
χ^2		7.74			
δEtOH, %		-83.9	-77.1	-67.7	-54.2

[a]TS count performed on day 14 post-seeding of 100 cells. Mean ± SD, n=3 per treatment group. Data were analyzed using a χ^2 test. [b]Immunofluorescence assay performed at day 3 post-seeding and presented as log mean FU. Mean ± SD, n=3 per treatment group. Data were analyzed using the two-sample Student's t-test. TS, tumor spheroids; FU, fluorescent units; CD, cluster of differentiation; c-Myc, cellular Myc; EtOH, ethanol; CUR, curcumin; SD, standard deviation..

d) Cancer stem cell models developed from drug resistant Apc $^{[-/-]}$ colonic epithelial cells provide a novel approach for FAP-mediated colon carcinogenesis. Additionally, this approach identifies potential leads for Apc target genes as actionable molecules in sporadic clinical colon cancer that frequently exhibits somatic mutation in the APC gene.
e) Present experimental approach facilitates identification of testable alternatives for therapy resistant colon cancer.

6. Future prospects

Current preclinical experimental approach using mouse derived colonic epithelial cell lines and drug resistant stem cell models taken together, provides conceptual mechanistic leads for potential clinically translatable promise of natural products as testable alternatives for therapy resistant colon cancer. To confirm this clinical potential, future experimental directions need to be focused on development of colonic organoids enriched in chemotherapy resistant stem cells from patient derived tumor xenografts. Supporting such an approach is the documented evidence that CRISPR-Cas9 mediated gene editing relevant to colon specific truncal mutations in human intestinal organoids provides a model for multi-step carcinogenic process of colon cancer (48). Additionally, in colon carcinoma derived HCT-116 cells CRISPR-Cas9 based correction of mutant β-catenin restores its normal function, inhibits cell proliferation *in vitro* and tumor growth *in vivo* (49). Collectively, the CRISPR-Cas9 mediated gene editing may provide a valuable approach for gene therapy of colon cancer. It is also notable that targeted gene modifications in the tumor suppressor genes APC, TP53 and SMAD4, and in the oncogene KRAS have been documented to promote cellular aneuploidy and invasive tumorigenic transformation in cultured human intestinal stem cells (50).

Future investigations on the development of human tissue derived colon cancer stem cell models, colonic organoids from chemotherapy resistant patient derived xenografts and CRISPR-Cas9 mediated gene editing together may provide potentially valuable mechanistic leads to identify efficacious stem cell targeted pharmacological agents and natural products. These research directions may validate gene therapy based experimental approaches for secondary prevention/therapy of clinical colon cancer.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

NT reviewed the published data, prepared the manuscript and read and approved the final manuscript.

Ethics approval and consent to participate

The data presented in the manuscript are generated from non-clinical samples. The cell line models developed from mouse colonic epithelial cells lines are summarized in Table I. Therefore, ethics approval and consent to participate are not required.

Patient consent for publication

Not applicable.

Competing interests

The author declares that they have no competing interests.

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