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# Extreme Fluctuations in Wnt/beta-Catenin Signaling as an Approach for Colon Cancer Prevention and Therapy

D. L. Lazarova and M. Bordonaro

Department of Basic Sciences, TCMC, 525 Pine Street, Scranton, PA 18509, USA  
Corresponding author: Michael Bordonaro, Department of Basic Sciences, TCMC,  
525 Pine Street, Scranton, PA 18509, USA  
mbordonaro@tcmedc.org

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**Abstract:** Mutations in the Wnt/beta-catenin (Wnt) signaling pathway initiate most cases of sporadic colon cancers (CC); therefore one approach for CC prevention and therapy is to suppress Wnt activity. However, the prolonged suppression of the signaling pathway is detrimental to the intestinal stem cells that rely on Wnt signaling for survival. Furthermore, CC cells exposed for an extended period of time to a single Wnt signaling - modulating agent may develop resistance to it. A possible solution is suggested by our findings that: (a) butyrate, a colonic metabolite of dietary fiber, and synthetic histone deacetylase inhibitors that mimic the effects of butyrate, hyper-activate Wnt transcriptional activity in CC cells with mutations in the pathway, and (b) high fold changes of Wnt signaling induce high apoptotic levels in the mutated cells. Therefore, diet- and drug-based regimens that result in alternating periods of suppressed and hyper-activated Wnt signalling may become powerful anti-CC preventive and therapeutic approaches.

The hypothesis can be tested by (1) evaluating the effects of drug- and diet-based regimens, which induce maximal fluctuations in Wnt signaling, on the adenoma burden of *Apc/min* mice, an established *in vivo* model of intestinal cancer, (2) epidemiological studies analyzing the dietary habits, such as daily fasting hours and fiber intake, of healthy individuals and patients with positive colonoscopies, and

(3) examining the ability of different diets to produce colonic lumen content with apoptotic and Wnt-modulatory functions.

**Keywords:** Wnt signaling, colon cancer, therapy, prevention, histone deacetylase inhibitors, butyrate, diet

## **Background**

Colorectal cancer (CRC) is the second leading cause of cancer death in the U.S. [23]. About 45% of all CRCs can be prevented by increasing physical activity and fiber intake, and limiting the consumption of energy-dense foods and meat [48]. These recommendations are consistent with the diet on which modern humans have evolved. Paleolithic humans subsisted on a wide variety of foraged plants, seeds, nuts, and lean meats from wild animals; this diet is estimated to have included an intake of more than 100 g of fiber per day [13,32,33]. Only within the last 10,000 years, after the introduction of farming in the Neolithic period, human society has started to rely on a limited number of crops, domesticated animals, and their byproducts [27]. Of all modern human populations, Australian aborigines come closest to the diet of the ancestral hunter-gatherers, with a daily dietary fiber intake of 80 to 130 g [10]. A similar level of dietary fiber intake has been reported for rural Uganda, where Burkitt and Trowell first developed the hypothesis for a protective role of fiber against colon cancer (CC) [11]. Despite the knowledge of what constitutes a high fiber diet [47], epidemiological studies on the effects of fiber in western societies have typically considered an intake of 25-30 g of fiber a day as “a high fiber diet” [4,17,35,36]. Whereas this categorization is justified since the average fiber intake in western countries is only 15-16 g a day [47], it is misleading with respect to the levels of fiber intake that might protect against CC.

Another striking difference between the dietary habits of the past and of contemporary westernized societies is the frequency of meals. Our ancestors mostly subsisted on one meal per day and frequently went without food for several days at a time [31]; whereas, today most individuals consume three regular meals a day with additional snacks. In the past 25 years, the percentage of the population reporting 3 or 4 snacks a day has increased 4-fold [39]. Approximately 60% of Americans eat snacks regularly, consuming up to 35% of their calories from snacks [24,39].

At present, a dietary regimen of frequent small meals (“grazing” behavior) is promoted as healthy; however, epidemiological studies have reported that a smaller number of eating episodes is associated with a reduced risk of CC [15,41,42,46]. Consistent with these results, rodent studies have demonstrated that longer intervals between meals inhibit cancer development [31,43]. Finally, calorie restriction, under which humans have evolved, also seems to protect against various diseases, including

intestinal cancer [30,45]. Interestingly, periods of strictly vegetarian diet (source of fiber) and intermittent fasting are incorporated in many religious traditions around the world [24]. For example, throughout the year, the Eastern Orthodox calendar includes 180-200 days of fasting, during which vegetarian days alternate with periods of abstinence from food [37,38].

The dietary regimen of the Orthodox Christian church may explain the health benefits of the Greek-Mediterranean diet [37,38], since high fiber intake (resulting in high concentrations of colonic butyrate) and intermittent fasting differently affect Wnt/beta-catenin (Wnt) signaling, a pathway deregulated in most CCs [3]. Thus, we have observed that butyrate hyper-activates Wnt transcriptional activity in CC cells with mutations in the Wnt pathway, but not in cells without such mutations [5,6,26]. The fold change of Wnt signaling is linearly correlated to the levels of apoptosis in butyrate-treated CC cells [26], and the causal character of this correlation has been demonstrated by the fact that CC cells, in which the induction of Wnt signaling by butyrate is suppressed via genetic means, exhibit lower apoptotic levels [26]. The effects of butyrate, a histone deacetylase inhibitor (HDACi), on Wnt and apoptosis are mimicked by other structurally different HDACis, such as Trichostatin A, SAHA, MSH275, and LBH-589 [5 and unpublished data].

Fasting, on the other hand, may suppress the Wnt pathway. The possibility that Wnt signaling is silenced in colonic cells in the absence of nutrients, and induced in the presence of metabolites, is supported by our findings that butyrate induces autocrine Wnt activity in CC cells [5], and the report that glucose moderately stimulates autocrine Wnt signaling in a different cell type [2]. The possible physiological meaning of these observations is that Wnt signaling, a pathway that at moderate levels maintains cell proliferation, should be “on” in the presence of nutrients and allow for colonic cell proliferation; however, in the absence of nutrients, the pathway should be “off” and thus, suppress cell proliferation. Based upon the findings that the pathway responds to physiological concentrations of nutrients, Sethi *et al.* have proposed that Wnt signaling is one of the cell sensors for nutritional cues, and thus, “the pathway is at the crossroads of nutritional regulation” [40].

The correlation between the fold change in Wnt activity and the physiological response of the cells (apoptosis) that we have observed in CC cells is consistent with other reports. Thus, it has been shown that cells respond to fold changes in signaling inputs (e.g., Wnt ligands), rather than to absolute levels of these inputs [14,18,19]. This suggests that Weber's law of physiology, that perception of a sensation is based not upon the level of a stimulus, but rather upon the magnitude of the stimulus relative to background levels, is valid at the cellular level.

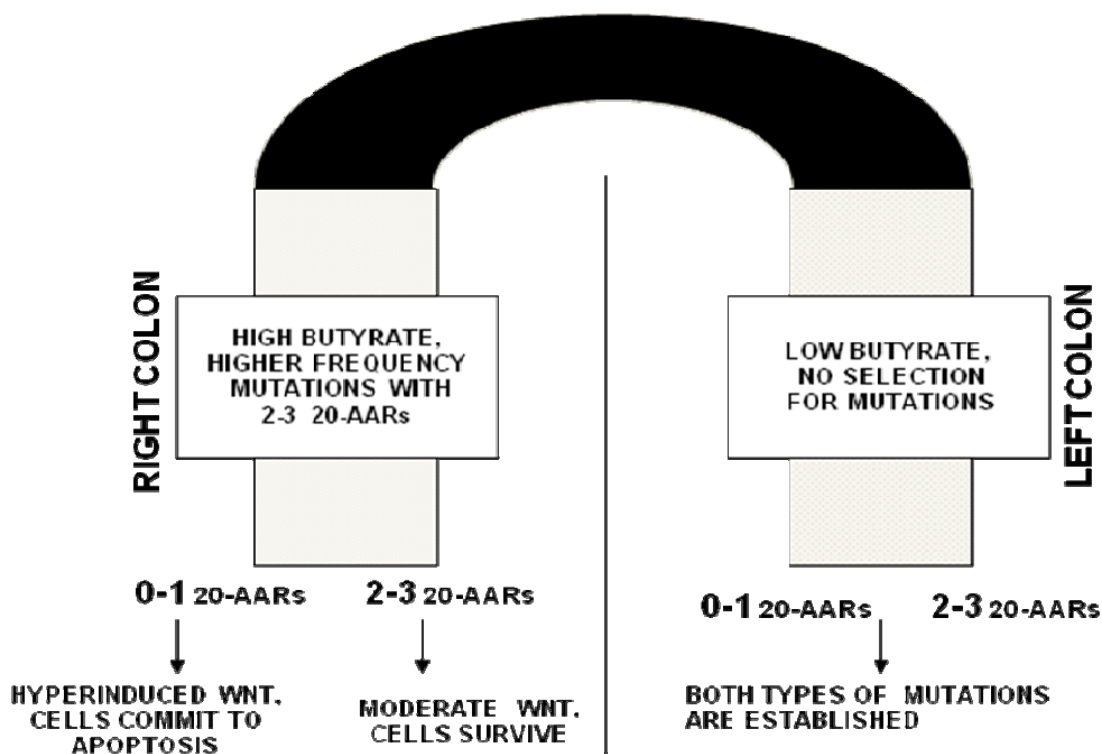
Based upon these reports, we hypothesize that inducing fluctuations in Wnt signaling is a powerful approach to eliminate neoplastic colonic cells. Additional support for the hypothesis comes from a report that distinct mutations in the Wnt pathway are selected along the segments of the colon [1]. Most sporadic CCs exhibit biallelic protein-truncating mutations in *APC* [16], and as a result some of the seven

20-amino acid repeats (20-AARs), which contribute to the ability of APC to control the levels of beta-catenin, are removed. Albuquerque *et al.* [1] reported that mutations in APC resulting in retention of two 20-AARs are more frequent in right than left (descending/sigmoid) colon tumors (70% versus 32%). Neoplasms with different number of total retained AARs are expected to exhibit various Wnt signaling levels [1]; thus, preservation of two to three 20-AARs allows for residual ability of APC to control beta-catenin levels, and results in moderate Wnt activity levels; whereas, APC mutants with zero or one 20-AARs allow higher accumulation of beta-catenin, and higher Wnt activity levels. A possible interpretation of this study is that the conditions in the right colon select against APC mutations resulting in high Wnt signaling levels, but favor APC mutations resulting in moderate Wnt signaling levels. Based upon the causal correlation between fold induction of Wnt signaling and apoptotic levels in butyrate-treated CC cells [7,8], and the fact that dietary butyrate is present at its highest levels in the right colon [20,28,29], we posit that in the right colon fiber-derived butyrate induces apoptosis of neoplastic cells that hyper-induce the Wnt pathway (e.g., cells with APC protein mutants of zero or one 20-AARs). However, cells with APC mutants of 2 or 3 20-AARs do not respond to butyrate with high fold induction of Wnt activity, and therefore, they do not undergo apoptosis and may develop into neoplasms in both the right and left colon (Fig.1).

### Hypothesis

Since extreme fluctuations in Wnt signaling lead to efficient apoptosis of neoplastic colon cells with mutations in the pathway, we posit that diet- and drug-based regimens that elicit Wnt signaling fluctuations *in vivo* are a promising approach for CC prevention and therapy.

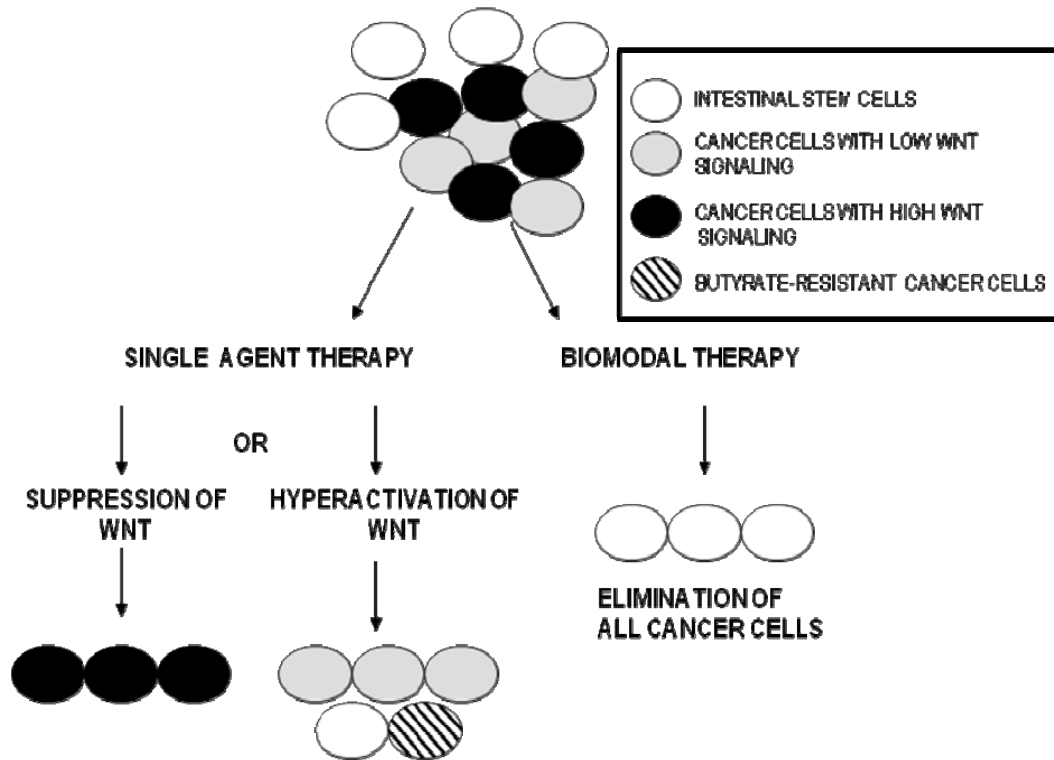
We have established that CC cell populations *in vitro* are heterogeneous in terms of Wnt activity levels [26], and this finding concurs with the *in vivo* observations that cells within a single colonic neoplasm exhibit different Wnt signaling levels [9,21,22,44]. *In vitro*, exposure of CC cells to butyrate increases Wnt activity levels per cell, and increases the number of Wnt signaling-positive cells; however, a fraction of cells in each cell population exhibit lower levels of Wnt signaling [26]. The cell heterogeneity in terms of Wnt signaling levels may explain why extreme fluctuations in the pathway are more effective in eliminating neoplastic cells than are approaches that either suppress or hyper-induce Wnt signaling. Both extremes of the pathway (suppression and hyper-induction) elicit apoptosis [49, and reviewed in 7,8]; however, exposure to only one therapeutic mode (e.g., hyper-activation or suppression of Wnt activity) would eliminate only one of the two (high or low Wnt signaling) cell populations, and prolonged application of only one therapeutic approach may result in drug-resistance and toxicity (e.g., killing of the



**Figure 1. High butyrate concentrations in the right colon may select against APC mutants with zero or one 20-AARs.** Albuquerque *et al.* [1] reported that mutations in APC resulting in retention of two 20-AARs are more frequent in right than left (descending/sigmoid) colon tumors (70% versus 32%). Based upon the causal correlation between fold induction of Wnt signaling and apoptotic levels in butyrate-treated CC cells [7,8], and the fact that dietary butyrate is present at its highest levels in the right colon [20,28,29], we posit that in the right colon, fiber-derived butyrate induces apoptosis of neoplastic cells that hyper-induce Wnt activity (e.g., cells with APC mutants of zero or one 20-AARs).

adult stem cells that rely on Wnt signaling, and selection for gene expression to survive the apoptotic stimulus). In contrast, in a bimodal approach to CC prevention or therapy, the short-term Wnt signaling-suppressive mode will eliminate cells with

lower Wnt activity levels, and the following short-term Wnt signaling-inducing mode will eliminate cells with hyper-induced Wnt activity. The alternating administration of the two modes will generate extreme fluctuations in the Wnt pathway, maximizing cell death. This hypothesis is presented in Figure 2.



**Figure 2. Comparison between single-mode and bimodal approaches to eliminate colon cancer cells with deregulated Wnt signaling.** Colonic neoplasms are heterogeneous in terms of Wnt signaling levels; therefore, the exposure to only one therapeutic mode (e.g., hyper-activation or suppression of Wnt signaling) will eliminate only some of the cancer cells. In addition, the prolonged application of only one therapeutic agent may result in drug-resistance and toxicity (e.g., killing of the adult stem cells that rely on Wnt activity). In contrast, in a bimodal approach to CC prevention or therapy, the short-term Wnt signaling - suppressive mode will eliminate cells with lower Wnt activity levels, and the following short-term Wnt signaling - inducing mode will eliminate cells with hyper-induced Wnt activity. The alternating administration of the two modes will generate extreme fluctuations in the Wnt pathway, maximizing cell death.

### Testing the hypothesis

How can we test the hypothesis that extreme fluctuations in Wnt signaling result in effective CC-preventive and therapeutic approaches?

First, diet- and drug-based regimens that elicit fluctuations in Wnt activity can be tested in the *Apc<sup>Min</sup>/+* mice, a murine model of intestinal cancer. These mice develop intestinal adenomas due to a truncating mutation in *Apc*. Similar mutations in the human *APC* gene are responsible for most cases of sporadic CC and underlie the inherited syndrome familial adenomatous polyposis [3,16]. The drug-based regimens can consist of the stop-and-go application of any of the synthetic HDACis that are either FDA-approved, or are in clinical trials. For example, we have found that both SAHA and LBH589 hyper-induce Wnt transcriptional activity in neoplastic colon cells [5, and data not shown]. In order to generate the beneficial Wnt activity fluctuations, the drug regimen should utilize an intermittent application of the maximal non-toxic doses of the HDACis, and should allow for periods of complete withdrawal from the drugs. The diet-based regimen should also aim at creating maximal fluctuations of Wnt signaling in the intestinal tract; therefore, diet rich in fermentable fiber should not be delivered *ad libitum*, but rather in one to two servings a day, allowing for adequately long periods of fasting that can suppress Wnt activity. The control regimens should support a consistent delivery of the active agents throughout the testing period. We expect that regimens resulting in intermittent high concentrations of intestinal butyrate or another HDACi will be most effective in preventing and/or slowing down the progression of intestinal neoplasms. Since there is no sensitive technical method that measures fluctuations of Wnt signaling in individual cells *in vivo*, the proposed experiments will only demonstrate a possible correlation between Wnt signaling fluctuations and adenoma burden. However, the ability of different regimens to modulate the Wnt pathway in colonic cells can be measured by the third experimental approach proposed below.

Second, if dietary habits such as high fiber intake and fewer eating episodes a day result in extreme fluctuations of Wnt signaling, epidemiological studies can ascertain whether subjects with this dietary pattern are relatively protected against CC. Therefore, data on dietary intake of fiber and frequency of eating episodes need to be gathered from patients with positive colonoscopies and healthy individuals. Epidemiological and molecular biology approaches can also address a corollary of the main hypothesis. Thus, if the high levels of butyrate in the right colon eliminate, through apoptosis, neoplastic cells that hyper-induce Wnt signaling, then any right-sided colonic neoplasms developed in patients with higher fiber intake are likely to be more resistant to butyrate/HDACis, and this pattern will not hold for individuals with low fiber intake. Interestingly, right-sided neoplasms develop more frequently at older age [12,34], and this age-related increase in incidence may reflect the fact that the lesions in the proximal colon take longer time to establish, since only mutant cells relatively resistant to butyrate give rise to right-sided cancers. To test the hypothesis, primary neoplastic cells from resected CC can be assayed for Wnt activity levels in

the absence and presence of butyrate. The fold induction of Wnt signaling by butyrate should be compared between left and right CCs separately for patients with relatively low fiber intake and patients with high fiber intake. If the hypothesis is true, for patients with higher fiber intake, the cells from right-sided CCs should exhibit low fold induction of Wnt signaling in the presence of butyrate.

Third, the Wnt signaling - modulating ability of diets that are associated with different risk for CC (e.g., Western, Okinawan, Greek-Mediterranean) can be tested with the *in vitro* gastrointestinal model TIM1/2 (operated by TNO Netherlands). It is possible that the CC-protective diets, such as the Okinawan and Greek-Mediterranean, are able to induce strong hyper-induction of Wnt signaling; whereas, the Western type diet may not have the potential to elicit high levels of Wnt activity. The *in vitro* gastrointestinal model consists of TIM1 that simulates the stomach and the small intestine, and TIM2, the compartments of which contain a high density metabolic-active microflora of human origin, simulates the conditions of the large intestine. The input for the *in vitro* digestive system can be a complete meal representative of each diet. The colonic content from each meal and from a simulated fasting, can be collected and tested for their ability to modulate Wnt activity and induce apoptosis in a panel of normal and neoplastic colonic cells. This approach will allow comparative analyses between the three diets independent of variations in intestinal microbiota.

### **Implications of the hypothesis**

(1) Establishing whether the effect of fiber on CC incidence is modified by the frequency of eating episodes will facilitate the formulation of dietary recommendations for CC prevention. (2) Knowledge of whether dietary habits, such as fiber intake, correlate with the sensitivity of colon neoplasms to HDACis can result in targeted therapeutics for a group of CC patients. (3) Ascertaining whether dietary regimens, which result in extreme fluctuations of Wnt signaling, are preventive and/or therapeutic may lead to novel strategies applicable to CCs and other cancers with deregulated Wnt pathway. (4) Comparative proteomics between normal and neoplastic cells that are exposed to colonic content with Wnt signaling - modulating ability may reveal molecular targets for anti-CC therapy and prevention, and suggest diagnostic markers for colonic neoplasms.

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