DOI 10.1002/mnfr.200700448 Mol. Nutr. Food Res. 2008, *52*, S52 – S61

Research Article

Low concentrations of resveratrol inhibit Wnt signal throughput in colon-derived cells: Implications for colon cancer prevention

Christopher Hope[1], Kestutis Planutis[1], Marina Planutiene[1], Mary P. Moyer[2], Karanjodh S. Johal[1], Jaesung Woo[1], Calista Santoso[1], Joseph A. Hanson[3] and Randall F. Holcombe[1]

[1] Division of Hematology/Oncology and Chao Family Comprehensive Cancer Center, University of California, Irvine, CA, USA
[2] Incell Corporation, San Antonio, TX, USA
[3] Johns Hopkins University, Baltimore, MD, USA

Resveratrol is a bioflavonoid which is known to inhibit cell proliferation and induce apoptosis in cancer cell lines at concentrations above 50 μM. It also has colon cancer prevention activity in mouse models and possibly in humans. We have examined the effects of low concentrations of resveratrol on a specific signaling pathway, the Wnt pathway, which is activated in over 85% of sporadic colon cancers. Two colon cancer (HT29 and RKO) and one normal mucosa-derived (NCM460) cell lines were utilized. Cell proliferation was not affected by resveratrol at ≤40 μM for HT29 and NCM460 and <20 μM for RKO though Wnt signal throughput, as measured by a reporter construct, was reduced in RKO and NCM460 at concentrations as low as 10 μM ($p < 0.001$). This effect was most easily appreciated following Wnt pathway stimulation with Wnt3a conditioned medium and LEF1 or LEF1/β-catenin transfection. Resveratrol did not inhibit Wnt throughput in mutationally activated HT29. Low concentrations of resveratrol significantly decreased the amount and proportion of β-catenin in the nucleus in RKO ($p = 0.002$) and reduced the expression of *lgs* and *pygoI*, regulators of β-catenin localization, in all cells lines. Thus, at low concentrations, in the absence of effects on cell proliferation, resveratrol significantly inhibits Wnt signaling in colon-derived cells which do not have a basally activated Wnt pathway. This inhibitory effect may be due in part to regulation of intracellular β-catenin localization.

Keywords: β-Catenin / Colon cancer / Pygopus / Resveratrol / Wnt pathway

Received: October 29, 2007; revised: January 15, 2008; accepted: January 24, 2008

1 Introduction

1.1 Resveratrol

Resveratrol is a bioflavonoid found in the skin of dark grapes, red wine, and peanuts. *In vitro*, this agent has been shown to have antioxidant activity and proapoptotic activity, and to inhibit proliferation of colon cancer cell lines [1]. Resveratrol is also reported to inhibit ribonucleotide reductase, ornithine decarboxylase [2], and IkappaB kinase [3] and is purported to act as a cancer prevention agent for a wide variety of human cancers including colon cancer [4]. The amount of resveratrol in red wine is 5 – 13 μmol/L ("Benton Lane Winery" at http://www.avalonwine.com/Benton-Lane-Winery.php) and it is suggested that even one glass (100 mL, ~1 μmol, 250 μg) of Pinot Noir *per* day provides cancer prevention activity. However, a quantity as small as ¼ mg does not result in serum/plasma concentrations in the range where activity is seen *in vitro*. The suggestion that resveratrol has chemopreventive efficacy in humans, and the evidence that it has chemopreventive efficacy in animal models, at dosages which attain low plasma levels, is perplexing [5]. This could be explained, in part, if activity of resveratrol is present at lower μM concentrations than has previously been appreciated.

Correspondence: Professor Randall F. Holcombe, Division of Hematology/Oncology, Office of Clinical Research and Trials, Chao Family Comprehensive Cancer Center, University of California, Irvine, 101 The City Drive, Building 56, Room 247, Orange, CA 92868, USA
E-mail: rholcomb@uci.edu
Fax: 714-456-2242

Abbreviations: CM, conditioned medium; **FBS,** fetal bovine serum; **SEAP,** secreted alkaline phosphatase

1.2 Colon cancer and Wnt signaling

Colon cancer is the third leading cause of cancer deaths among men and women in the United States ("Cancer facts and figures, 2007" at American Cancer Society, http://www.cancer.org, 2007). The epidemiology of colon cancer suggests that dietary factors play a critical role in tumor initiation and promotion [6], with populations consuming a diet high in fiber, vegetables and fruits exhibiting a lower cancer incidence. Mutations in genes leading to activation of the Wnt signaling pathway are common in colon cancer, seen in over 85% of sporadic cases [7]. Signaling through the Wnt pathway begins with a Wnt ligand which interacts with a cell surface frizzled (Fz) receptor to initiate the signal cascade [8]. Pathway activation prevents degradation of β-catenin which is translocated to the nucleus where it binds to members of the LEF/TCF family of HMG-box transcription factors [9], inducing transcription of growth regulatory genes such as *myc* [10], and *cyclinD1* [11]. Other proteins including legless (lgs), pygopusI (pygoI), and pygopusII (pygoII) complex with LEF/TCFs and β-catenin and facilitate both the translocation of β-catenin into the nucleus and the induction of transcription by the active complex [12, 13]. In colon carcinogenesis, mutations in either APC or β-catenin are early events which constitutively activate the Wnt pathway [14].

Resveratrol can modulate prostaglandin production and inhibit the expression of cyclooxygenase-2 [15, 16] which is overexpressed in colon cancer. Cox2 inhibition may be one mechanism through which resveratrol is acting in relation to cancer chemoprevention [17]. However, the concentrations which display this activity *in vitro* are difficult to attain *in vivo* [18], and thus other mechanisms need to be explored. Since the Wnt pathway is critically important in the development of colon cancer, any inhibitory effects of resveratrol on this pathway are particularly relevant to explore. This study was undertaken to define whether low concentrations of resveratrol, concentrations below those which inhibit proliferation and induce apoptosis, have activity against colon-derived cells *in vitro* and, specifically, whether these low concentrations affected Wnt signaling. We report here that subapoptotic concentrations of resveratrol can inhibit Wnt signal throughput in colon-derived cells and that this effect may be due, in part, to regulation of β-catenin localization. This activity may contribute to resveratrol's cancer prevention activity.

2 Materials and methods

2.1 Cell lines

Three cell lines were utilized for these studies. NCM460 is a cell line derived from normal colonic mucosal cells which were obtained from Incell Corporation (San Antonio, TX) [19]. This cell line was maintained in M3 Base cell culture medium complete (Incell, catalog number M300A-500) with 10% fetal bovine serum (FBS). There are no known *apc* or β-catenin mutations present in this cell line. RKO, a human colon cancer cell line, was kindly provided by Dr. Parker (UCLA). This cell line, originally obtained from a patient with hereditary nonpolyposis colon cancer, displays microsatellite instability (MSI, or RER(+)) and, similar to NCM460, has no known Wnt pathway activation. It was maintained in culture in RPMI1640 media at 5% CO_2 with 10% FBS. HT29, a colon cancer cell line with mutationally activated Wnt signaling due to a truncation mutation in *apc*, was obtained from the American Type Culture Collection (ATCC, Manassas, Virginia). It was maintained in DMEM media at 5% CO_2 with 10% FBS. These three cell lines were selected for study in order to provide both normal-derived and cancer-derived cells, and both Wnt-activated and Wnt-nonactivated cells for analysis.

2.2 Cell proliferation, cell cycle, and cell size

Cell proliferation was determined utilizing an MTT assay. This assay measures the reduction of a tetrazolium component into an insoluble formazan product by the mitochondria of viable cells. Following 48 h incubation with or without resveratrol, cells were incubated with MTT reagent for 2 h and then lysed with detergent. Solubilized crystals produce a color which is read using an ELISA plate reader at a wavelength of 570 nm. The amount of color produced is directly proportional to the number of viable cells. For cell cycle analysis, cells were plated in a six-well format and treated for 48 h. Cells were recovered by trypsinization, washed with PBS, fixed in 70% ethanol and sorted overnight at -20°C. Fixed cells were washed $\times 3$ in ice cold PBS and incubated with 500 μL of a propidium iodide solution (50 μg/mL) containing 50 μg RNAseA for 30 min in the dark at room temperature. Cell cycle distribution was analyzed by flow cytometry using a Becton Dickinson FACScan system. To characterize cell size following treatment of RKO cells with resveratrol, a trypsinized suspension of spherical cells were placed in a hemocytometer and photographed. The cross-sectional area and distribution of trypan blue-excluding cells was calculated using Image-Pro Plus.

2.3 Wnt throughput assays

In order to evaluate changes in Wnt signaling at low dosages of resveratrol, the colon-derived cell lines were utilized with a Wnt pathway reporter construct designed in our laboratory containing seven LEF/TCF consensus binding sites preceding a secreted alkaline phosphatase (7X-SEAP) reporter (Fig. 1). Activation of Wnt signaling leads to production of SEAP which is secreted into the culture media. SEAP is easily detected and quantified by sampling of the culture media, facilitating sampling without the need for disrupting

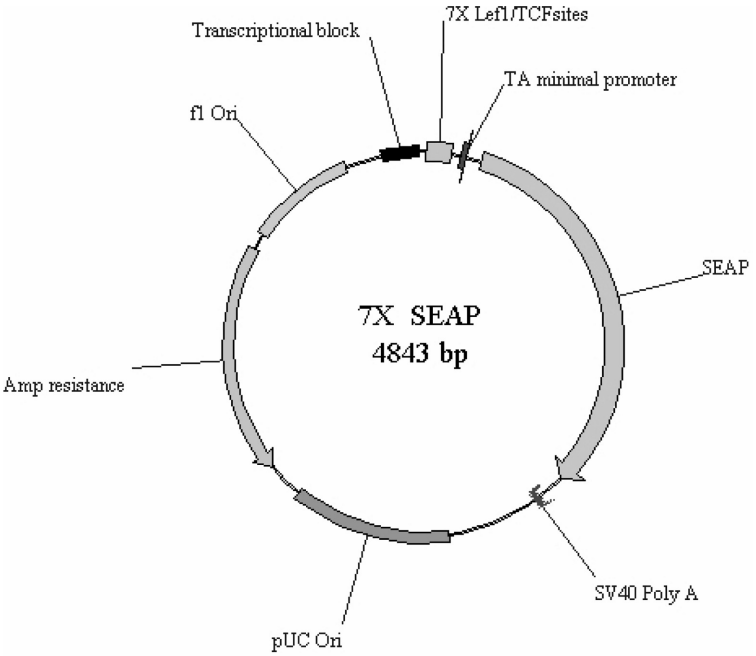

Figure 1. Plasmid map of the 7X-SEAP reporter construct.

transfected cells. Comparative studies of the 7X-SEAP reporter with the "super" TopFlash reporter documented equivalence in sensitivity. For the assays, cells were plated into 24-well plates and allowed to grow for 48 h. Cells were typically between 70 and 90% confluent on the day of transfection. Separate cohorts of cells were treated with Wnt3a conditioned medium (CM; Willert, *et al*., http://www.stanford.edu/~rnusse/assays/W3aPurif.htm) or transfection with LEF1 or LEF1/β-catenin in order to increase Wnt signal throughput, thus facilitating detection of inhibitory activity by resveratrol. Reporter plasmid DNA (1.2 μg) and 0.12 μg of LEF1 expression construct, β-catenin and/or inactive DNA (pBluescript) for a total mass of 1.44 μg DNA *per* well was transfected using Lipofectamine2000 following manufacturers recommendations. Each condition was performed in triplicate. After 48 h in culture with or without resveratrol, media was recovered and 20 μL was assayed using Great EscAPe SEAP detection kit (BD Biosciences) in a 96-well format. Plates were read on a Dynatech Laboratories ML3000 microtiter plate luminometer. Experiments were performed at various concentrations of resveratrol ranging up to 20 μM. All experiments were performed in conjunction with transfection of a control SEAP plasmid lacking LEF/TCF binding sites. Results were additionally normalized to cell counts which were performed to insure that cells were viable and not undergoing apoptosis. Results of the cell counts were consistent with the MTT data. For the very low (5 μM) concentration resveratrol experiments RKO cells were cocultured in 2-D culture with stably transfected Wnt3a producing L-cells for 48 h, in 50:50 RPMI/DMEM media with 10% FBS. Media was assayed for SEAP activity after 48 h of exposure to 5 μM resveratrol or equivalent v/v DMSO only control.

2.4 β-Catenin localization and quantification

β-Catenin localization was evaluated by confocal microscopy and fluorescence immunohistochemistry. Cells were grown in four-well chamber slides (LabTek/Nunc, Rochester, NY) with 10 and 20 μM resveratrol and equivalent v/v DMSO (diluent for resveratrol) as controls. Cells were incubated for 48 – 72 h, fixed, washed, and endogenous peroxidases inhibited by incubating with H_2O_2/methanol for 30 min. Cells were then stained with monoclonal anti-β-catenin antibodies (Transduction Labs/BD Biosciences, San Jose, CA). Following counterstaining with an ABC immunohistochemistry kit (Santa Cruz Biotechnology, Santa Cruz, CA), peroxidase localization with a tyramide-fluorochrome system (Perkin Elmer, Boston, MA) was performed, and visualization was obtained by confocal fluorescence microscopy. Because the cell lines in the resting state, especially NCM460 and RKO, have relatively low levels of β-catenin, the experiments were repeated with the addition of Wnt3a CM to activate the Wnt pathway. Using fluorescence microscopy, 100 cells were visualized and the degree of β-catenin related fluorescence defined with image quantification software. Each cell nucleus was individually mapped as was the cell membrane of each cell. Cells in clusters without distinct cell borders were not evaluated. Nuclear staining across different resveratrol treatment groups was compared as was the calculated nuclear/cytoplasmic β-catenin ratio.

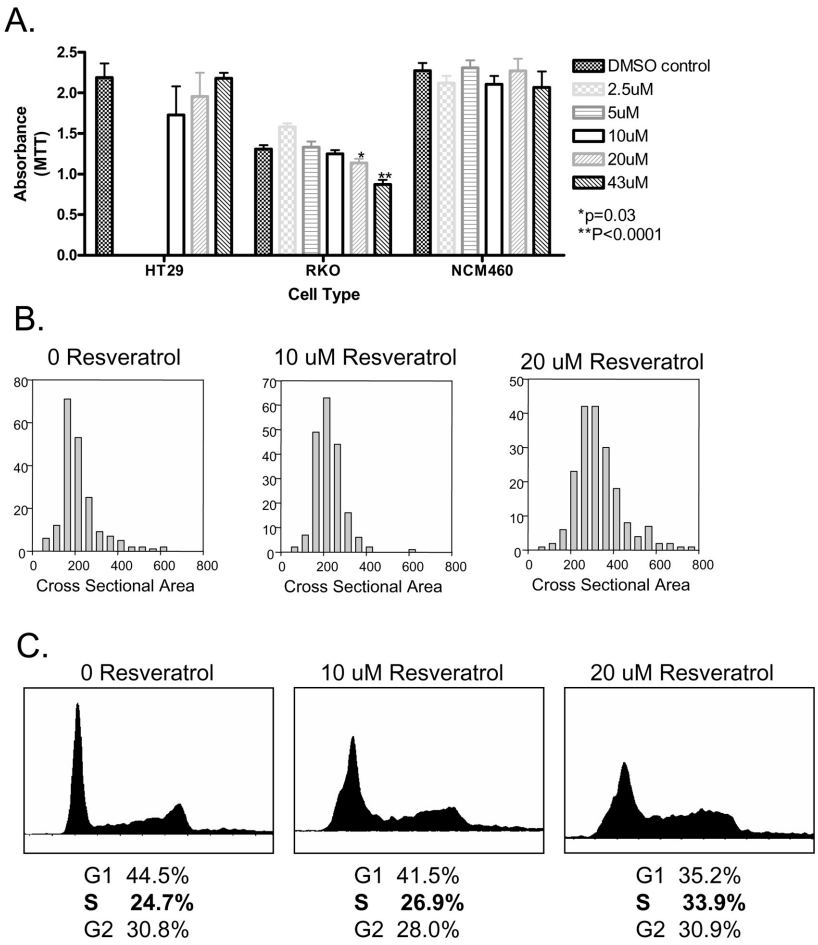

Figure 2. (A) Proliferation of HT29, RKO, and NCM460 cells measured by MTT assay at varying concentrations of resveratrol. Proliferation was not determined for HT29 at 2.5 and 5 μM resveratrol. $*p = 0.03$; $**p < 0.0001$. Error bars represent SEM. (B) RKO cell size as defined by cross-sectional area in microns-squared. *Y*-axis depicts # cells at each size cohort. 200 cells counted under each resveratrol condition. (C) Cell cycle analysis of RKO cells treated with 10 and 20 μM resveratrol and compared to control (0 μM resveratrol). The proportion of cells in S phase increases with increasing resveratrol concentration.

2.5 Expression of *lgs*, *pygol*, and pygoll

Following incubation with or without resveratrol as described above, cells were harvested for mRNA in order to define the expression of *lgs*, *pygoI*, and *pygoII* by quantitative real-time PCR (qRT-PCR). Specific primer sets for each gene were employed and expression was compared to actin controls in all cases. Primer pairs were for *lgs*, *pygoI*, and *pygoII* were obtained from Qiagen (Valencia, CA), catalog numbers QT00051604, QT00032081, and QT00097727, respectively. Temperatures utilized were 48°C for reverse transcription for 30 min and 95°C for DNA polymerase activation for 10 min. Each of 50 cycles were run as follows: 95°C denaturation for 15 s, 55°C annealing for 30 s, 60°C synthesis for 30 s. Actin was utilized as a housekeeping gene for data normalization.

2.6 Statistics

Means, SDs, and standard errors of the mean (SEM) for data from all experimental groups were calculated and comparisons between groups were made with a two-sided unpaired *t*-test with the level of significance defined as $\alpha < 0.05$.

3 Results

3.1 Proliferation and cell cycle analysis

Resveratrol did not affect proliferation of HT29 and NCM460 at concentrations ranging from 2.5 to 40 μM (Fig. 2A). RKO was unaffected at 5 and 10 μM but exhibited a slight decrease in proliferation at 20 μM ($p = 0.03$) and a more significant decrease at 40 μM ($p < 0.001$). For RKO, cell size was noted to be slightly increased at 20 μM (Fig. 2B), possibly indicative of early induction of cell cycle arrest at this concentration for this particular cell line. Mean cell cross-sectional area was $218.8 \pm 92.5\ \mu^2$ with no resveratrol, $226.1 \pm 66.5\ \mu^2$ at 10 μM resveratrol and $331.7 \pm 111.2\ \mu^2$ at 20 μM resveratrol ($p < 0.0001$). The effect of resveratrol on the cell cycle was examined by flow cytometry in RKO cells. Treatment with resveratrol increased the proportion of cells in S phase from 24.7 to

Table 1. Effect of low concentrations of resveratrol on Wnt pathway throughput in colon-derived cell lines

	A			B		
				Plus Wnt3 conditioned media Fold change		
	Control	Lef1	Lef1/β-catenin	Control	Lef1	Lef1/β-catenin
NCM	2.9863 ± 0.8413	8.7168 ± 1.3529 $p = 0.0000$	24.7085 ± 13.1528 $p = 0.00237$	1.0786 ± 0.0719 $p = 0.0963$	1.3807 ± 0.2542 $p = 0.0048$	1.0548 ± 0.1353 p = NS
NCM + 10 μM RES	80.22% ± 1.41 $p = 0.0019$	80.21% ±9.36 $p = 0.0345$	75.21% ± 6.33 $p = 0.0044$	0.7904 ± 0.0299 $p = 0.0055$	0.8897 ± 0.0665 $p = 0.0017$	0.7683 ± 0.0995 p = NS
NCM + 20 μM RES	72.55% ± 9.53 $p = 0.0318$	88.83% ± 9.67 p = NS	71.52% ± 3.27 $p = 0.0171$	0.7133 ± 0.0413 $p = 0.00054$	0.8881 ± 0.0752 $p = 0.01318$	0.7125 ± 0.0207 $p = 0.00139$
RKO	0.0428 ± 0.0163	0.1646 ± 0.1142 $p = 0.0270$	13.3303 ± 13.983 $p = 0.0422$	15.4351 ± 4.0339 $p = 0.00000$	30.7591 ± 9.5413 $p = 0.00002$	3.2482 ± 1.1015 $p = 0.00056$
RKO + 10 μM RES	104.75% ± 5.95 p = NS	81.46% ± 5.59 p = NS	125.43% ± 8.76 $p = 0.0130$	9.8299 ± 1.5027 p = NS	27.7683 ± 0.8956 $p = 0.0036$	1.9354 ± 0.1763 $p = 0.0210$
RKO + 20 μM RES	86.78% ± 1.01 p = 0.NS	53.76% ± 8.06 $p = 0.003$	91.77% ± 14.7 p = 0.NS	10.5443 1.2293 $p = 0.004$	11.7152 0.8649 $p = 0.0117$	2.3713 0.1014 $p = 0.0185$
HT29	0.0512 ± 0.0044	0.0969 ± 0.0127 $p = 0.0041$	0.9086 ± 0.1719 $p = 0.0010$	1.1315 ± 0.1064 p = 0.NS	1.0695 ± 0.0791 p = 0.NS	1.3715 ± 0.1075 $p = 0.0417$
HT29 + 20 μM RES	118.35% ± 5.64 $p = 0.0364$	107.22% ± 2.12 p = 0.NS	107.71% ± 17.99 p = 0.NS	1.3167 ± 0.1419 p = 0.NS	1.2329 ± 0.1240 p = 0.NS	1.5740 ± 0.2187 p = 0.NS

A: Wnt pathway throughput (SEAP activity). Absolute value ± SD. For resveratrol conditions% of nonresveratrol activity ± SD.
B: Fold change due to Wnt3 conditioned media.
Rows with cell line only listed: p value is for Wnt3a, Wnt3a/LEF1, or Wnt3a/LEF1/β-catenin *vs.* control.
Rows which list resveratrol concentration: p value is for comparison to similar condition without resveratrol.
NS = not significantly different ($p > 0.05$).

26.9 to 33.9% upon exposure to 10 and 20 μM, respectively (Fig. 2C), indicating cell cycle arrest at S phase.

3.2 Evaluation of Wnt pathway activity

Wnt signal throughput was evaluated with the 7X-SEAP reporter construct for each of the cell lines (Table 1). Wnt signal throughput was induced in NCM460 cells following transfection of LEF1 or LEF1/β-catenin. Treatment with Wnt3a CM did not augment Wnt signal throughput further by itself, or in the setting of LEF1/β-catenin transfection, but was synergistic following LEF1 transcription inducing a small (1.38-fold) but significant ($p = 0.0048$) increase. RKO cells responded to LEF1 and LEF1/β-catenin transfection with an increase in Wnt signal throughput and were extremely responsive to the addition of Wnt3a CM under all conditions, displaying up to a 30-fold increase. Despite intrinsic Wnt pathway activation, HT29 cells could be induced to further increase Wnt signal throughput following transfection with LEF1 and LEF1/β-catenin. HT29 cells were minimally responsive to Wnt3a CM either by itself or in combination with LEF1 or LEF1/β-catenin.

Low dose resveratrol, at 10 and 20 μM (concentrations at which no significant effect on cell proliferation was seen), significantly reduced Wnt signal throughput in NCM460 under every condition: without additional Wnt stimulation, following transfection of LEF1 and LEF1/β-catenin, and with the addition of Wnt3a CM. Wnt signal throughput was reduced by 20–30% (Table 1). Ten and twenty micromolar resveratrol did not affect Wnt throughput in unstimulated RKO cells but did significantly reduce it in nearly all conditions following augmentation of the Wnt signal through either transfection or treatment with Wnt3a CM. Resveratrol was ineffective in these cells only following LEF1/β-catenin transfection in the absence of Wnt3a CM (Table 1). The effect of 10 μM resveratrol in reducing Wnt signal throughput in RKO cells in the presence of Wnt3a CM is depicted in Fig. 3. Resveratrol trended to reduce Wnt throughput under control conditions ($p = 0.056$) and significantly reduced Wnt throughput following LEF1 transfection ($p = 0.002$) and following LEF1/β-catenin transfection ($p < 0.001$). The lowest concentration of resveratrol tested in RKO cells was 5 μM but no effect on Wnt throughput was seen at this concentration (Table 2). These latter experiments were conducted following exposure to Wnt3a producing cells in side-by-side coculture rather than the addition of Wnt3a CM. HT29 cells were generally unresponsive to resveratrol and, while not significant, the trend at 20 μM was for an increase, rather than a decrease in Wnt signal throughput (Table 1).

Table 2. Effect of a very low concentration of resveratrol on Wnt throughput in RKO cells

	A			B		
					Plus Wnt3 coculture Fold change	
	Control	Lef1	Lef1/β-catenin	Control	Lef1	Lef1/β-catenin
RKO	0.0288 ± 0.0125	0.1826 ± 0.0205 $p = 0.0004$	11.4874 ± 1.8236 $p = 0.0004$	30.8831 ± 6.2975 $p = 0.0012$	64.7230 ± 6.8884 $p = 0.0001$	2.8199 ± 0.5251 $p = 0.0045$
RKO + 5 μM RES	ND	ND	ND	27.1597 ± 5.7014 $p = 0.49$	61.5837 ± 3.0671 $p = 0.51$	2.9715 ± 0.1805 $p = 0.47$

A: Wnt pathway throughput (SEAP activity). Absolute value ± SD.
B: Fold change due to Wnt3a coculture.
Top row: *p* value is for Wnt3a, Wnt3a/LEF1, or Wnt3a/LEF1/β-catenin *vs.* control.
Bottom row: *p* value is for comparison to similar condition without resveratrol.

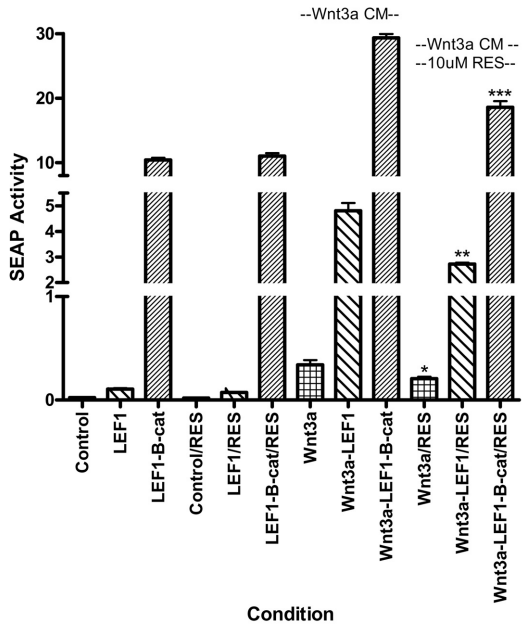

Figure 3. Wnt throughput as measured by SEAP activity following transfection of the 7X-SEAP reporter in RKO cells under various conditions. The three left bars represent Wnt throughput (control, transfected LEF1, and transfected LEF1-β-catenin) in the absence of Wnt3a CM and resveratrol. The next three bars represent Wnt throughput (control, transfected LEF1, and transfected LEF1-β-catenin) in the absence of Wnt3a CM but in the presence of 10 μM resveratrol. The next three bars represent Wnt throughput under the various conditions following the addition of Wnt3a CM. The three right bars represent Wnt throughput following Wnt3a CM in the presence of 10 μM resveratrol. LEF1 transfection, LEF1/β-catenin transfection, and the addition of Wnt3a CM each significantly increase Wnt throughput. Resveratrol decreases Wnt throughput under each transfection condition in the presence of Wnt3a (*$p = 0.056$; **$p = 0.002$; ***$p < 0.001$). All data represents Wnt throughput as measured by SEAP activity rather than fold increase to facilitate comparisons between different subgroups. Error bars represent SEM.

3.3 Intracellular β-catenin localization

In order to determine whether resveratrol was modulating Wnt signaling by influencing β-catenin translocation to the nucleus, nuclear and cytoplasmic β-catenin was quantified for each cell line following 48 h of treatment with different concentrations of resveratrol with and without Wnt3a CM. As anticipated based on the lack of effect on Wnt signal throughput, no change was seen in HT29. Similarly, no significant effect on β-catenin localization was seen in NCM460. In RKO cells, however, a significant reduction in the amount of nuclear β-catenin was seen at 10 μM ($p < 0.001$). Exposure to Wnt3a CM increased nuclear β-catenin ($p < 0.001$) but this increase was abrogated by 20 μM resveratrol ($p < 0.01$ *vs*. Wnt3a CM with 0 μM resveratrol, Fig. 4A). To insure that these results represented changes in the distribution of β-catenin, rather than the total cellular content, the nuclear/cytoplasmic ratio was examined following Wnt3a stimulation and in the presence of 20 μM resveratrol. A significant reduction in the nuclear/cytoplasmic ratio was seen in RKO cells (Figs. 4B and C, $p < 0.001$).

3.4 Expression of intracellular modulators of β-catenin localization

Quantitative real time PCR was utilized to define the effect of resveratrol at concentrations ranging from 5 to 20 μM on the expression of *lgs*, *pygoI*, and *pygoII* in NCM460, RKO, and HT29 (Fig. 5). *Lgs* expression was significantly reduced in HT29 at all concentrations and at 10 μM in NCM460. Other concentrations showed a nonsignificant trend toward reduction, especially in NCM460. *PygoI* was reduced in all cell lines except at 20 μM in RKO and 5 μM in NCM460, though the only statistically significant decrease was seen at 10 μM in HT29 ($p = 0.0155$). *PygoII* overall was minimally changed, with a slight reduction at 10 μM in RKO ($p = 0.0068$) and an increase in mRNA levels in HT29 at 5 μM ($p < 0.001$).

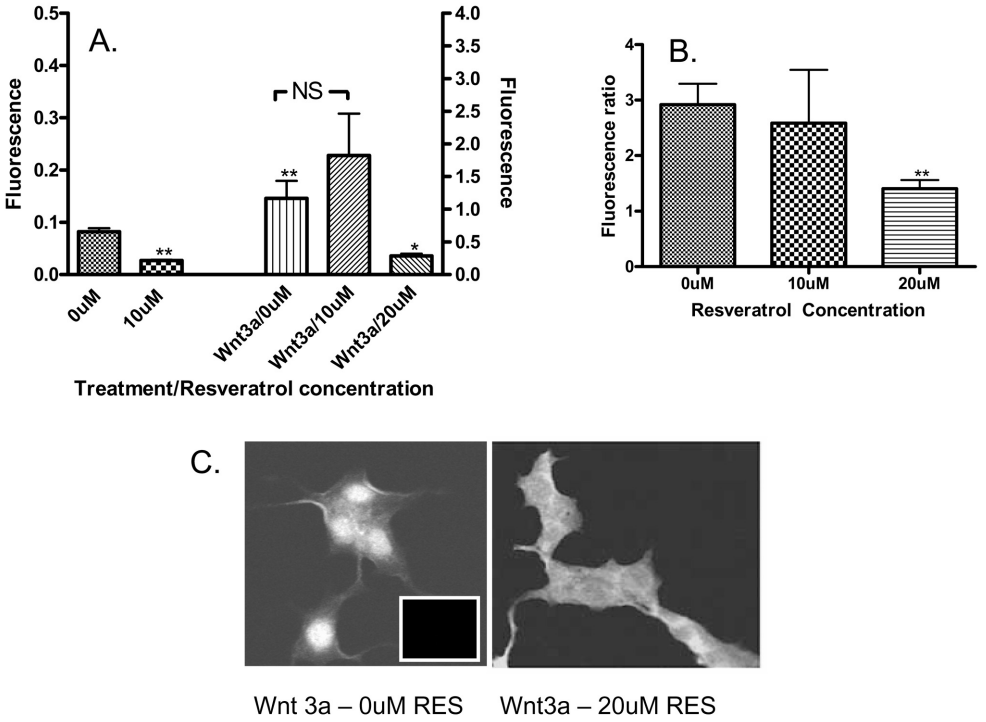

Figure 4. (A) Quantity of nuclear β-catenin as measured by nuclear fluorescence intensity in RKO cells at 0 and 10 μM resveratrol and following the addition of Wnt3a CM at 0, 10, and 20 μM resveratrol. 10 μM resveratrol reduces nuclear β-catenin ($p < 0.01$) in the absence of Wnt3a stimulation. 20 μM resveratrol reduces nuclear β-catenin following Wnt3a stimulation ($p < 0.05$). Wnt3a alone increases the amount of nuclear β-catenin compared to nonstimulated baseline ($p < 0.01$). Error bars represent SEM. (B) Nuclear/cytoplasmic ratio of β-catenin in RKO cells following stimulation with Wnt3a CM as a ratio of fluorescence intensity. Twenty micromolar resveratrol significantly reduces the nuclear/cytoplasmic ratio ($p < 0.01$). Error bars represent SEM. (C) Visual representation of β-catenin fluorescence intensity and localization in RKO cells following stimulation with Wnt3a and exposure to 0 μM (control) and 20 μM resveratrol. Inset (left panel) depicts fluorescence intensity of secondary IgG antibody only control.

4 Discussion

We describe here that resveratrol inhibits Wnt signal throughput in RKO cells and NCM460 cells, both colon-derived cell lines lacking intrinsic activation of the Wnt pathway. These effects were noted at concentrations of resveratrol below those that inhibited cell proliferation. Additionally, pathway inhibition was seen under various conditions which increase Wnt throughput, such as treatment with Wnt3a CM or transfection with LEF1 and/or β-catenin. However, no effect on Wnt throughput was seen in HT29 which harbors a Wnt-activating mutation in *apc*.

In some cells (RKO), our data suggest that the effect of low concentrations of resveratrol on Wnt signaling may be at least partially due to inhibition of β-catenin nuclear translocation. A different plant flavonoid, apigenin has been shown to affect Wnt signaling by reducing nuclear β-catenin and the expression of *c-myc* and *cyclinD1* in the prostates of TRAMP mice [20]. However, other mechanisms must be operative, as Wnt throughput inhibition was seen in NCM460 in the absence of demonstrable effects on β-catenin quantity or intracellular localization. Low concentrations of resveratrol also affect the expression of *lgs, pygoI* and, to a lesser extent, *pygoII*, the proteins for which are involved in β-catenin nuclear transport and localization. It is possible that resveratrol's activity in eliciting an S-phase arrest [21], in conjunction with reductions in *lgs* and *pygoI*, may affect the cellular distribution of β-catenin in a way that reduces Wnt signal throughput. The S-phase arrest mediated by resveratrol, confirmed here in RKO cells, occurs at lower concentrations than its cytotoxic and apoptotic effects [22–26]. Recently, it has been suggested that β-catenin transcriptional activity is regulated not only through the levels of protein degradation, but also *via* regulation of its nuclear localization [27]. During S-phase pause or arrest, APC protein is increased [28]. This occurs even in cells harboring truncating mutations since the N-terminal 20 amino acid segment of APC, which is essential for cell proliferation [29], binds to β-catenin serving as a cytoplasmic "sink" and preventing its translocation to the nucleus [28]. Thus, an increase in cytoplasmic APC would retain β-catenin in this compartment, reducing its transcription activating functions. *Lgs* and *pygoI*, alternatively, serve as nuclear "sinks" for β-catenin so a reduction in their abundance, as we demonstrate here following exposure to resveratrol, would additionally contribute to a reduction in

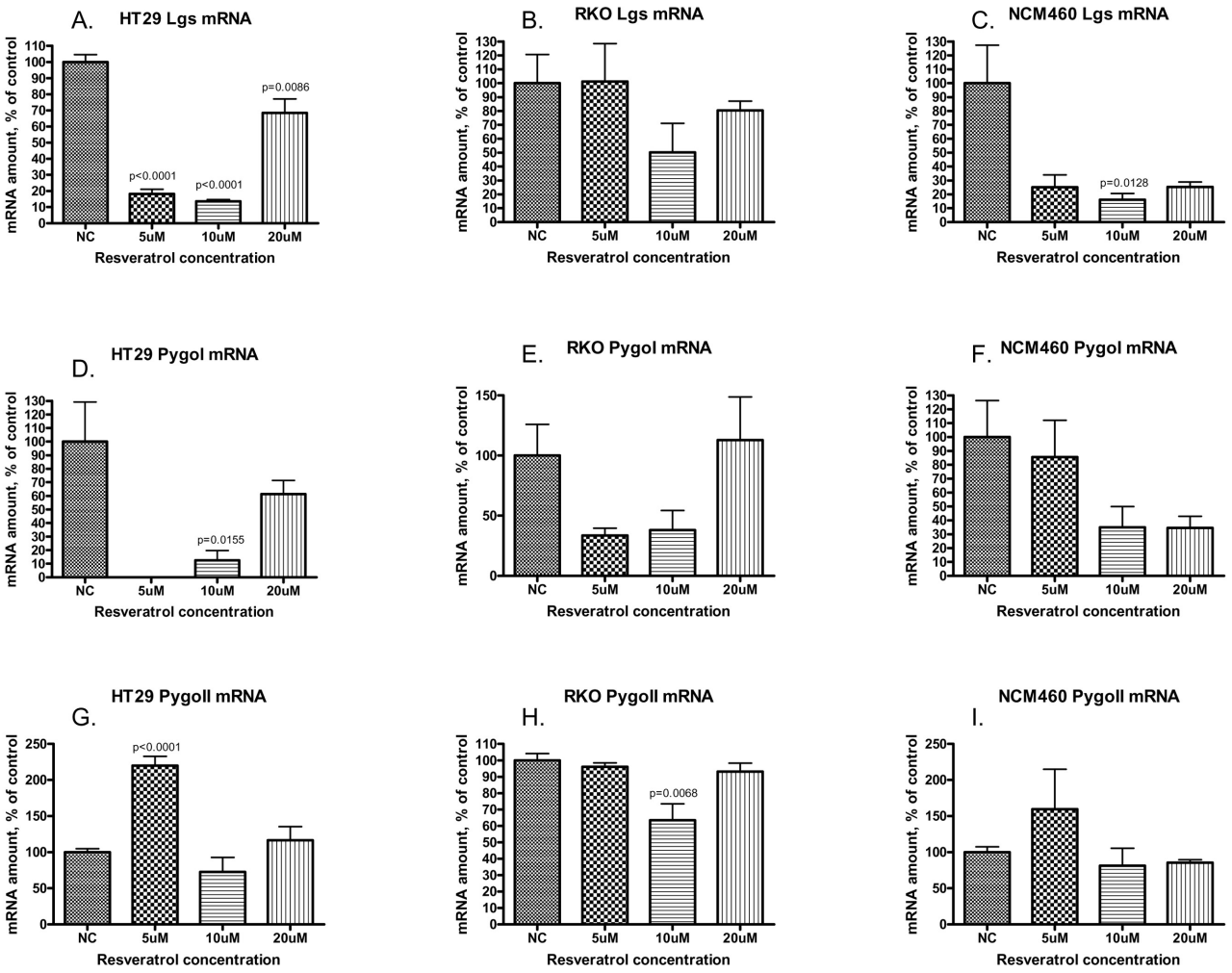

Figure 5. Expression of *lgs* (A – C), *pygol* (D – F) and *pygoII* (G – I) measured by quantitative real-time PCR and represented as amount of mRNA, normalized to actin, and graphed as percent of control (*y*-axis). Cell lines tested included HT29 (A, D, G), RKO (B, E, H), and NCM460 (C, F, I). Resveratrol concentrations included 5, 10, and 20 μM (*x*-axis). Error bars represent SEM. Levels of significance noted when $p < 0.05$.

β-catenin dependent Wnt throughput [13, 30]. However, the fact that resveratrol had a measurable effect on β-catenin localization by immunohistochemistry only in RKO cells, while inhibiting Wnt signal throughput in both RKO and NCM460, and that the effects on *lgs*, *pygoI*, and *pygoII* do not directly correlate with Wnt throughput inhibition, suggest that resveratrol's inhibition of Wnt signaling likely involves multiple mechanisms that may be partially cell type dependent.

The effect on Wnt signaling has implications for cancer treatment or cancer prevention, though resveratrol has various other effects in humans including beneficial activity related to cardiovascular disease [31], protective effects following brain damage and ischemia [32] and antiaging properties (reviewed in ref. [33]). In relation to the cancer chemopreventive effects, the concentrations attained *in vivo* are significantly lower than the concentrations required for inhibition of cell growth *in vitro*, raising the question as to how resveratrol exhibits this activity in animal models and perhaps in humans [34]. A recent pharmacokinetic study of single dose resveratrol confirmed that peak plasma concentrations of the parent compound following a single large 5 g ingestion reached only 539 ng/mL (2.4 μM) [35]. Our finding that low concentrations of resveratrol can inhibit Wnt signaling may provide a partial explanation to this conundrum. Cooperativity with other biologically active flavanoids and polyphenols in wine, grapes, and other nutritional sources and increased concentrations of resveratrol locally in the colon may also contribute to resveratrol's *in vivo* activity.

The data for prevention of colon cancer by resveratrol in animal models are somewhat contradictory, both in its efficacy as well as information regarding the effective dose range, though activity at low dosages, and therefore in circumstances where low serum concentrations are attained,

has been reported. Tessotore *et al*. [36] demonstrated activity of very low dose resveratrol of 0.2 mg/kg/day in reducing aberrant crypt foci (ACF) in the colon in an azoymethane-induced tumor model. In another carcinogen-based model, utilizing 1,2-dimethylhydrazine, resveratrol at 8 mg/kg/day reduced both ACF and colonic tumors [37, 38]. In genetic models utilizing the $APC^{min/+}$ mouse, which harbors a single allele mutation in *apc*, and therefore has intrinsically activated Wnt signaling, Schneider [39] demonstrated profound activity at dosages as low as 0.3 mg/mouse/day in reducing intestinal tumors. In this study, expression of the Wnt target gene, *cyclinD1*, as well as other markers of cell cycling, were reduced. We have confirmed the effects of resveratrol on cell cycling in colon-derived cells *in vitro* in our study. Other reports suggest that resveratrol dosages as high as 90 mg/kg [40] are ineffective or that only extremely high dosages up to 500 mg/kg show activity [41]. Sale [42] utilized dosages of 60 and 240 mg/kg, and found the former ineffective but the latter effective in inducing a more modest reduction in intestinal tumorigenesis. Overall, these studies indicate that resveratrol may have activity in both carcinogen-induced tumor models as well as in the Wnt activated $APC^{min/+}$ mouse, but that the effective dose is unclear, with activity reported utilizing dosages ranging from < 1 to 500 mg/kg *per* day. The effect of resveratrol on Wnt signaling in animal models has not been explored.

This is the first report describing a Wnt inhibitory activity of resveratrol. While the precise mechanisms require further study, resveratrol does affect β-catenin nuclear localization in some cells and the expression of genes encoding proteins involved in this process. The effects on Wnt signaling are seen at concentrations of resveratrol below those that induce apoptosis and inhibit cellular proliferation. Wnt inhibition appears to be restricted to cell lines without a mutationally activated Wnt pathway. While most colon cancers have a mutationally activated Wnt pathway, Wnt signaling is also of paramount importance in regulating proliferation of normal colonic mucosa and in the regulation of the colonic stem cell compartment [43, 44]. Because of this, the effects of resveratrol in nonmutationally activated cell lines may be most relevant in relation to colon cancer prevention rather than treatment. Further studies in animal models and humans on the influence of resveratrol on the Wnt pathway are warranted.

Supported by CA82450 (R. F. H.).

The authors have declared no conflict of interest.

5 References

[1] Szende, B., Tyihak, E., Kiraly-Veghely, Z., Dose-dependent effects of resveratrol on proliferation and apoptosis in endothelial and tumor cell cultures, *Exp. Mol. Med.* 2000, *32*, 88 – 92.

[2] Khanduja, K. L., Bhardwaj, A., Kaushik, G., Resveratrol inhibits N-nitrosodiethylamine-induced ornathine decarboxylase and cyclooxygenase in mice, *J. Nutr. Sci. Vitaminol*. 2004, *50*, 61 – 65.

[3] Holmes-McNary, M., Baldwin, A. S., Jr., Chemopreventive properties of trans-resveratrol are associated with inhibition of activation of the I-k-B kinase, *Cancer Res*. 2000, *60*, 3477 – 3483.

[4] Bauer, J. A., Sinclair, D. A., Therapeutic potential of resveratrol: The *in vivo* evidence, *Nat. Rev. Drug Discov.* 2006, *5*, 493 – 506.

[5] Gescher, A. J., Steward, W. P., Relationship between mechanisms, bioavailability and preclinical chemopreventive efficacy of resveratrol: A conundrum, *Cancer Epidemiol. Biomarkers Prev.* 2003, *12*, 953 – 957.

[6] Mason, J. B., Nutritional chemoprevention of colon cancer, *Semin. Gastroint. Dis*. 2002, *13*, 143 – 153.

[7] Robbins, D. H., Itzkowitz, S. H., The molecular basis of colon cancer, *Med. Clin. North Am.* 2002, *86*, 1467 – 1495.

[8] Cadigan, K. M., Nusse, R., Wnt signaling: A common theme in animal development, *Genes Dev.* 1997, *11*, 3286 – 3305.

[9] Van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M. *et al*., Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF, *Cell* 1997, *88*, 789 – 799.

[10] He, T. C., Sparks, A. B., Rago, C., Hermeking, H., *et al*., Identification of c-MYC as a target of the APC pathway, *Science* 1998, *281*, 1509 – 1512.

[11] Testu, O., McCormick, F., Beta-catenin regulates expression of cyclinD1 in colon carcinoma cells, *Nature* 1999, *398*, 422 – 426.

[12] Thompson, B. J., A complex of Armadillo, Legless, and Pygopus coactivates dTCF to activate wingless target genes, *Curr. Biol*. 2004, *14*, 458 – 466.

[13] Kramps, T., Peter, O., Brunner, E., Nellen, D., *et al*., Wnt/wingless signaling requires BCL9/legless-mediated recruitment of pygopus to the nuclear beta-catenin-TCF complex. *Cell* 2002, *109*, 47 – 60.

[14] Polakis, P., The many ways of Wnt in cancer, *Curr. Opin. Genet. Dev.* 2007, *17*, 45 – 51.

[15] Patel, B., Patel, S., Hoffman, R., Inhibition of cyclo-oxygenase-2 expression in mouse macrophages by 4-(3-methyl-but-1-enyl)-3,5,3′,4′-tetrahydroxystilbene, a resveratrol derivative from peanuts, *Phytother. Res*. 2005, *19*, 552 – 555.

[16] Kundu, J. K., Shin, Y. K., Kim, S. H., Surh, Y. J., Resveratrol inhibits phorbol ester-induced expression of COX-2 and activation of NF-kappaB in mouse skin by blocking IkappaB kinase activity, *Carcinogenesis* 2006, *27*, 1465 – 1474.

[17] Shankar, S., Singh, G., Srivastava, R. K., Chemoprevention by resveratrol: Molecular mechanisms and therapeutic potential, *Front. Biosci*. 2007, *12*, 4839 – 4854.

[18] Boocock, D. J., Faust, G. E., Patel, K. R., Schinas, A. M., *et al*., Phase I dose escalation pharmacokinetic study in healthy volunteers of resveratrol, a potential cancer chemopreventive agent, *Cancer Epidemiol. Biomarkers Prev.* 2007, *16*, 1246 – 1252.

[19] Moyer, M. P., Stauffer, J. S., Manzano, L. A., Tanzer, L. R., Merriman, R. L., NCM460, A normal human colon mucosal epithelial cell Line, *In Vitro Cell. Dev. Biol. Anim.* 1996, *32*, 315 – 317.

[20] Shukla, S., MacLennan, G. T., Flask, C. A., Fu, P., *et al*., Blockade of beta-catenin signaling by plant flavonoid apigenin suppresses prostate carcinogenesis in TRAMP mice, *Cancer Res*. 2007, *67*, 6925 – 6935.

[21] Wolter, F., Akoglu, B., Clausnitzer, A., Stein, J., Downregulation of the cyclin D1/Cdk4 complex occurs during resveratrol-induced cell cycle arrest in colon cancer cell lines, *J. Nutr.* 2001, *131*, 2197 – 2203.

[22] Joe, A. K., Liu, H., Zuzui, M., Vural, M. E., *et al*., Resveratrol induces growth inhibition, S-phase arrest, apoptosis and changes in biomarker expression in human cancer cell lines, *Clin. Cancer Res*. 2002, *8*, 893 – 903.

[23] Delmas, D., Passilly-Degrace, P., Jannin, B., Cherkaoui, M., Latruffe, N., Resveratrol, a chemopreventive agent, disrupts the cell cycle control of human SW480 colorectal tumor cells, *Int. J. Mol. Med*. 2002, *10*, 193 – 199.

[24] Park, J. W., Choi, Y. J., Jang, M. A., Lee, Y. S., *et al*., Chemopreventive agent resveratrol, a natural product derived from grapes, reversibly inhibits progression through S and G2 phases of the cell cycle in U937 cells. *Cancer Lett.* 2001, *61*, 179 – 189.

[25] Bernhard, D., Tinhofer, I., Tonko, M., Hubl, H., *et al*., Resveratrol causes arrest in the S-phase prior to Fas-independent apoptosis in CEM-C7H2 acture leukemia cells, *Cell Death Differ*. 2000, *7*, 834 – 842.

[26] Haider, U. G., Sorescu, D., Griendling, K. K., Volmar, A. M., Dirsch, V. M., Resveratrol increases serine 15-phosphorylated by transcriptionally impaired p53 and induces a reversible DNA replication block in serum-activated vascular smooth muscle cells, *Mol. Pharmacol*. 2003, *63*, 925 – 932.

[27] Krieghoff, E., Behrens, J., Mayr, B., Nucleo-cytoplasmic distribution of beta-catenin is regulated by retention, *J. Cell. Sci*. 2006, *119*, 1453 – 1463.

[28] Schneikert, J., Grohmann, A., Behrens, J., Truncated APC regulates the transcriptional activity of beta-catenin in a cell cycle dependent manner, *Hum. Mol. Genet*. 2007, *16*, 199 – 209.

[29] Schneikert, J., Behrens, J., Truncated APC is required for cell proliferation and DNA replication, *Int. J. Cancer* 2006, *119*, 74 – 79.

[30] Townsley, F. M., Cliffe, A., Bienz, M., Pygopus and legless target armadillo/beta-catenin to the nucleus to enable it transcriptional co-activator function, *Nat. Cell Biol*. 2004, *6*, 626 – 633.

[31] Hung, L. M., Chen, J. K., Huang, S. S., Lee, R. S., Su, M. J., Cardioprotective effect of resveratrol, a natural antioxidant derived from grapes, *Cardiovasc. Res*. 2000, *47*, 549 – 555.

[32] Virgili, M., Contestabile, A., Partial neuroprotection of *in vivo* excitotoxic brain damage by chronic administration of the red wine anti-oxidant agent, trans-resveratrol in rats, *Neurosci. Lett*. 2000, *281*, 123 – 126.

[33] Baur, J. A., Sinclair, D. A., Therapeutic potential of resveratrol: The *in vivo* evidence, *Nat. Rev. Drug Discov.* 2006, *5*, 493 – 506.

[34] Gescher, A. J., Steward, W. P., Relationship between mechanisms, bioavailability and preclinical chemopreventive efficacy of resveratrol: A conundrum, *Cancer Epidemiol. Biomarkers Prev.* 2003, *12*, 953 – 957.

[35] Boocook, D. J., Faust, G. E. S., Patel, K. R., Schinas, A. M., *et al*., Phase I dose escalation pharmacokinetic study in healthy volunteers of resveratrol, a potential cancer chemopreventive agent, *Cancer Epidemiol. Biomarkers Prev.* 2007, *16*, 1246 – 1252.

[36] Tessitore, L., Davit, A., Sarotto, I., Caderni, G., Resveratrol depresses the growth of colorectal aberrant crypt foci by affecting bax and $p21^{CIP}$ expression, *Carcinogenesis* 2000, *21*, 1619 – 1622.

[37] Sengottuvelan, M., Viswanathan, P., Nalini, N., Chemopreventive effect of trans-resveratrol – a phytoalexin against colonic aberrant crypt foci and cell proliferation in 1,2-dimethylhydrazine induced colon carcinogenesis, *Carcinogenesis* 2006, *27*, 1038 – 1046.

[38] Sengottuvelan, M., Nalini, N., Dietary supplementation of resveratrol suppresses colonic tumor incidence in 1,2 dimethylhydrazine-treated rats by modulating biotransforming enzymes and aberrant crypt foci development, *Br. J. Nurtition.* 2006, *96*, 145 – 153.

[39] Schneider, Y., Duranton, B., Gosse, F., Schleiffer, R., *et al*., Resveratrol inhibits intestinal tumorigenesis and modulates host-defense-related gene expression in an animal model of human familial adenomatous polyposis, *Nutr. Cancer* 2001, *39*, 102 – 107.

[40] Ziegler, C., Rainwater, L., Whelan, J., McEntee, M. F., Dietary resveratrol does not affect intestinal tumorigenesis in $Apc^{Min/+}$ mice, *J. Nutr.* 2004, *134*, 5 – 10.

[41] Gignac, E. A., Bourquin, L. D., Influence of resveratrol and sulindac on intestinal tumor numbers in Min mice, *FASEB J.* 2001, *15*, A630.

[42] Sale, S., Tunstall, R. G., Ruparelia, K. C., Potter, G. A., *et al*., Comparison of the effects of the chemopreventive agent resveratrol and its synthetic analog trans 3,4,5,4-tetramethoxystilbene (DMU-212) on adenoma development in the $Apc^{Min/+}$ mouse and cyclooxygenase-2 in human derived colon cancer cells, *Int. J. Cancer* 2005, *115*, 194 – 201.

[43] de Lau, W., Barker, N., Clevers, H., WNT signaling in the normal intestine and colorectal cancer, *Front. Biosci*. 2007, *12*, 471 – 491.

[44] Pinto, D., Clevers, H., Wnt control of stem cells and differentiation in the intestinal epithelium, *Exp. Cell Res*. 2005, *306*, 357 – 363.

