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Plant stanols induce intestinal tumor formation by up-regulating Wnt and EGFR signaling in *Apc*^{Min} mice☆

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Abstract

The rate of *APC* mutations in the intestine increases in middle-age. At the same period of life, plant sterol and stanol enriched functional foods are introduced to diet to lower blood cholesterol. This study examined the effect of plant stanol enriched diet on intestinal adenoma formation in the *Apc*^{Min} mouse. *Apc*^{Min} mice were fed 0.8% plant stanol diet or control diet for nine weeks. Cholesterol, plant sterols and plant stanols were analyzed from the caecum content and the intestinal mucosa. Levels of β-catenin, cyclin D1, epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinase 1/2 (ERK1/2) were measured from the intestinal mucosa by Western blotting. Gene expression was determined from the intestinal mucosa using Affymetrix and the data were analyzed for enriched categories and pathways. Plant stanols induced adenoma formation in the small intestine, however, the adenoma size was not affected. We saw increased levels of nuclear β-catenin, phosphorylated β-catenin (Ser675 and Ser552), nuclear cyclin D1, total and phosphorylated EGFR and phosphorylated ERK1/2 in the intestinal mucosa after plant stanol feeding. The Affymetrix data demonstrate that several enzymes of cholesterol synthesis pathway were up-regulated, although the cholesterol level in the intestinal mucosa was not altered. We show that plant stanols induce adenoma formation by activating Wnt and EGFR signaling. EGFR signaling seems to have promoted β-catenin phosphorylation and its translocation into the nucleus, where the expression of cyclin D1 was increased. Up-regulated cholesterol synthesis may partly explain the increased EGFR signaling in the plant stanol-fed mice.

Keywords: Plant sterols; ApcMin mouse; β-catenin; EGFR; Cholesterol synthesis

1. Introduction

Plant sterol- and stanol-enriched functional foods are designed to lower blood cholesterol, a well-established risk factor of heart diseases. Plant sterols (phytosterols) are naturally existing compounds of plant origin, and they are present in small quantities in an average human diet. Plant stanols are saturated forms of sterols and they are less abundant in nature than plant sterols. Both plant sterols and stanols are poorly absorbed from the intestine and they interfere with cholesterol absorption, leading to increased concentrations of cholesterol and other sterol metabolites in the gut lumen [1]. The elevated level of cholesterol derivatives in the colon may lead to a modified gut environment. High intraluminal cholesterol concentration, from dietary and/or endogenous origin, has been associated with enhanced cell proliferation, aberrant crypt formation and tumor formation in the murine colon [2,3].

In some studies, plant sterols have been demonstrated to inhibit colon tumorigenesis in carcinogen-treated rats [4-6]. However, Quilliot et al. [7] found no protective effect of plant sterol supplement in their study with methylnitrosourea (MNU) rats. They concluded that plant sterols modified gut microflora negatively, which was seen as increased level of feacal coprostanol, a bacterial metabolite of cholesterol associated with colon carcinogenesis [8,9]. The Netherlands Cohort Study on Diet and Cancer did not find association with high dietary intake of plant sterols and lowered risk of colorectal cancer; however, high intake of β-sitostanol was positively associated with cancer of distal colon in men [10].

The risk of developing colorectal cancer increases with age. Mutation in the *adenomatous polyposis coli* (*APC*) tumor suppressor gene is required in developing hereditary colon cancer (familial

Abbreviations: Akt, protein kinase B; Apc, adenomatous polyposis coli; EGFR, epidermal growth factor; ERK1/2, extracellular signal-regulated kinase 1/2; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; LDL, low density lipoprotein; Min, multiple intestinal neoplasia; MNU, methylnitrosourea; PI3K, phosphatidylinostol 3-kinase; SREBP, sterol regulatory element binding protein.

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adenomatous polyposis, FAP) and is found in the majority of sporadic colorectal tumors [11]. The incidence of *APC* mutations in the colon increases in middle age [12] and often at the same time consumption of plant sterol enriched products starts. Therefore, we studied the effect of plant stanol enriched semisynthetic diet on intestinal adenoma formation in the *Apc*^{Min} mouse (*Adenomatous polyposis coli*, Multiple intestinal neoplasia), a well-characterized model of colon cancer. No previous studies have reported the effect of plant sterols or stanols in the tumor prone *Apc*^{Min} mouse. The *Apc*^{Min} mouse carries a heterozygous mutation in the *Apc* gene [13], which leads to spontaneous development of intestinal tumors. Disturbed function of Apc protein results in the activation of the Wnt pathway with concomitant down-regulation of β-catenin degradation and its accumulation in the nucleus [14]. In the nucleus, β-catenin interacts with the Tcf/Lef transcription factor and regulates transcription of genes, e.g., c-*myc* and *cyclin D1* [15,16] that enhance cell proliferation and growth and mediate cellular program for ongogenic transformation in colorectal cancer [17,18]. In addition to Wnt activation, mitogen-activated protein kinase (MAPK) signaling occurs in response to almost any change in the extracellular or intracellular milieu. One subfamily of MAPKs is extracellular signal-regulated kinases (ERK1/2) that are activated through, e.g., growth factor receptor signaling, including the epidermal growth factor receptor (EGFR). The other pathway activated by EGFR phosphorylation is the PI3K/Akt pathway, and through these two pathways EGFR regulates the homeostasis between cell proliferation and maturation in the gut [19,20]. On the membrane EGFRs locate in lipid rafts that are rich in cholesterol and sphingolipids [21-23]. Plant stanols and sterols may be incorporated to cellular membranes, which may result in disturbed membrane properties and receptor function [24-26]. In this study, levels of cholesterol, plant sterols and plant stanols were measured from caecum content and intestinal mucosa in order to detect changes in the extracellular and intracellular milieu of enterocytes after plant stanol feeding. To determine how these changes in the sterol homeostasis affect cell signaling in intestinal tumorigenesis, protein levels of β-catenin and its phosphorylated forms (Ser552 and Ser675), cyclin D1, EGFR, ERK1/2 and Akt were measured from the normal appearing mucosa of small intestine. Finally, microarray analysis was performed to discover the impact of plant stanol feeding on gene expression in the *Apc*^{Min} mouse.

2. Materials and methods

2.1. Animals

The study protocol was approved by the Laboratory Animal Ethics Committee, University of Helsinki. Male and female C57BL/6J *Apc*$^{Min/+}$ mice were bred at the Laboratory Animal Centre in Viikki, Helsinki, from inbred mice originally obtained from the Jackson Laboratory (Bar Harbor, ME, USA). After weaning, the mice were screened for the *Min* genotype using a PCR assay described by Dietrich et al. [27]. The 5-week old *Apc*^{Min} mice were stratified by weight and litter background and assigned into experimental groups of 15 mice each group, eight male and seven female mice into both groups. The mice were housed in plastic cages in humidity- and temperature-controlled facilities with 12-h light–dark cycle. They were fed ad libitum and had free access to tap water. The mice were weighed and monitored weekly and record on their growth was kept throughout the experiment. Rapid loss of weight and a relapse of the rectum were indications of impaired health. One mouse from both experimental groups was sacrificed before the end of experiment due to a considerable weight-loss within 1 week. These mice were excluded from the analysis. The total number of mice included in the data is 28 (14 mice per group).

2.2. Diets

The control diet was a modified high-fat (41% of energy from fat; 13% butter, 5.5% rapeseed oil, 1.1% sunflower oil), very low fiber AIN93-G diet. The plant stanol diet was similar to control diet but contained 0.8% (w/w) plant stanol (sum of campestanol and sitostanol). The protein, carbohydrate, fat, fatty acid, cholesterol, plant sterol, vitamin and mineral contents of both diets were otherwise similar (Table 1). Plant stanol was added to the diet in form of freeze-dried plant stanol ester enriched products found on market in Finland 2003. The control diet was prepared with the same freeze-dried products without plant stanol enrichment. The nutrient content of the products with or without added plant stanol was similar because they were purchased when possible from the same manufacturer in Finland (Supplementary Table 1). The origin of plant sterols in both diets was vegetable oils added to the diets. The approximate daily intake of plant stanol was calculated to be 20 mg per mouse in the plant stanol group.

2.3. Tumor scoring and tissue samples

After the 9-week feeding period, the mice were sacrificed by CO_2 asphyxiation. Blood was collected from abdominal aorta and after centrifugation the plasma was stored at −70°C. The small intestine, caecum and colon were removed and opened along the longitudinal axis and rinsed with ice-cold saline. The small intestine was divided into five sections of equal length. The caecum and colon were separated from the small intestine and kept together for analysis. The contents of caecum were collected and stored at −20°C. The intestinal sections were spread flat on microscope slides. Each section of the intestine and colon and caecum was analyzed under inverse light microscope attached to a monitor. The number and diameter of each adenoma was recorded for each section separately. The observers keeping record were blinded to the treatment. The intestinal adenomas were excised from the tissue and the normal-appearing mucosa that was left behind was scraped off from lamina propria. The tissue samples were snap frozen in liquid nitrogen and stored at −70°C. For microarray analysis, a 0.5-cm section was cut from middle of the distal part of small intestine and stored in RNAlater solution at -20 °C (Qiagen).

2.4. Sterol analysis by direct saponification and gas chromatography

Cholesterol, plant sterols and stanols were analyzed as total amount of each sterol instead of separating the esterified and free sterols. The sterol composition was analyzed from the faeces of caecum and duodenal/jejunal mucosa for each mouse by applying a method described by Soupas et al. [28]. A sample of rapeseed oil was used to control interassay variation. Amount of dehydrocholesterol in ethanol was added to each sample as an internal standard. Shortly, ethanol and saturated potassium hydroxide were added to each sample and the esterified sterols were hydrolyzed in a shaking incubator for 30 min in 85°C. After hydrolyzation, water and cyclo-hexane were added and then shaked vigourously to extract sterols into solvent phase. To obtain a detectable concentration of sterols, solvent was evaporated from the samples of the control mice. Silylation was carried out to obtain trimethylsilyl derivatives of sterols. Before silylation, the solvent was evaporated under stream of nitrogen in 50°C. Pyridine and silylation solution (BSTFA: TMCS, 99:1) were added (1:1) and the samples were silylated in an incubator for 30 min in 60°C. The silylation reagent was evaporated under nitrogen in 50°C and remaining sterol sample was dissolved into heptane. The sample was injected into a RTX-5 w/Integra Guard (crossbond 5% diphenyl-95% dimethyl polysiloxane) capillary column (60 m × 0.32 mm i.d., 0,10 μm film) (Restek, Bellefonte, PA, USA) by automated injector of a gas chromatograph equipped with a flame ionization detector (Agilent 6890N Network GC System, Agilent Technologies, USA). Sterols were quantified using an internal standard method. Identification was based on retention times and GC-mass spectrometric analysis.

2.5. Western blotting analysis

Proteins were isolated from normal-appearing mucosa of the distal small intestine which represents approximately 40% of total small intestine. Sample preparation has been described earlier in detail by Misikangas et al. [29]. Samples were fractionated to membranous, cytosolic and nuclear fractions. For proteinase inhibition a solution of 0.4 mM leupeptin, 3.0 μM pepstatin and 1.0 mM PMSF (in DMSO) was used. For whole mucosa lysate 10% Triton-X solution was added to the total homogenate, the sample was efficiently mixed for 20 min with 5 min intervals and centrifuged 15 000×*g* 10 min at 4°C. The supernatant was collected and protein concentration measured as described by Misikangas et al. [29]. All protein samples were stored at −70°C.

Table 1
Analyzed composition of experimental diets

Component	Control diet	Plant stanol diet
	/100 g of diet	
Energy (kJ)*	1959	1965
Protein (g)*	21.3	21.4
Carbohydrate (g)*	51.5	51.1
Crude fat (g)*	19.5	19.8
Water (g)*	4.28	4.37
Ash (g)*	3.41	3.36
Cholesterol (mg)*	50.3	49.0
Plant sterols (mg)**	56.1	68.4
Plant stanols (mg)**	0.8	764.2

* Analysis by Agrifood Research Finland.
** Analysis by Division of Food Chemistry, University of Helsinki.

Proteins were measured and analyzed individually for each mouse in both groups. Fractionated protein sample was used to measure the levels of nuclear β-catenin, cyclin D1 and p53. Total and phosphorylated forms of EGFR, ERK1/2, Akt and phosphorylated β-catenin were measured from whole mucosa lysate. Protein samples were loaded in equal concentrations to 10% SDS-PAGE gels (6.5% gels for EGFR). All samples were analyzed at least in duplicate. An internal standard was used in each gel to control interassay variation. After electrophoresis the proteins were transferred to either nitrocellulose (Hybond ECL membrane, Amersham Pharmacia Biotech.) or PVDF (Hybond P membrane, Amersham Pharmacia Biotech.) membrane. Membranes were incubated overnight in blocking solution containing 3.5% non-fat soya (nuclear β-catenin and lamin B), 5% non-fat milk in Tris-buffered saline with 0.1% Tween. For phospho-β-catenin analysis, Immobilon nitrocellulose membrane (Millipore) and SuperBlock T20 TBS blocking buffer were used (Thermo Scientific).

Membranes were incubated with primary antibody for 2 h or overnight with phospho-specific antibodies. Antibodies towards β-catenin (sc-7199), EGFR (sc-03), ERK1 (sc-94), sterol regulatory element binding protein (SREBP)-2 (H-164,sc-5603) and lamin B (sc-6216) were purchased from Santa Cruz Biotechnology. Other antibodies were anti-cyclin D1 (SP4, NeoMarkers), anti-p53 (#RB-9006-P, NeoMarkers), anti-phospho-EGFR (pY1068, Invitrogen), anti-phospho-p44/42 (#9101, Cell Signaling Technology), anti-phospho-β-catenin (Ser552 #9566 and Ser675 #4176, Cell Signaling Technology), anti-β-catenin (610154, BD Transduction Laboratories) and anti-β-actin (A541, Sigma). For protein detection, secondary antibodies (sc-2030 and sc-2031, Santa Cruz Biotechnology,) and enhanced chemiluminesence reagents, ECL or ECL+ (GE Healthcare), were used. The levels of phosphorylated and total protein were analyzed using the same membrane. After incubating the membrane with phospho-specific antibody, the membrane was stripped in stripping buffer [78 mM Tris–HCl, 2% SDS, 0.68% (v/v) mercaptoethanol] for 20 min in 65°C, incubated in TBS-Tween for 20 min in 65°C and finally in TBS-Tween for 20 min in room temperature. Blots were transferred to X-ray film (Amersham) and scanned and analyzed by GSA-800 Calibrated Imaging Densitometer and Quantity One Program (BioRad Laboratories). β-Actin and lamin B were used to control equal loading of protein samples.

Phosphorylated forms of β-catenin (Ser552 and Ser675) were detected with secondary antibodies (anti-rabbit IRDye 800CW and anti-mouse IRDye 680LT, Odyssey LI-COR) diluted in blocking buffer (SuperBlock T20 TBS, Thermo Scientific) with 0.1% Tween. Ser552 and Ser675 were detected and quantified with Odyssey infrared imager (LI-COR; Lincoln, NE, USA), and the results were controlled to the levels of total-β-catenin and β-actin.

2.6. RNA isolation, microarray and pathway analysis

Total RNA was extracted from ileal mucosa sample by using the RNeasy Mini kit (Qiagen). The tissue sample was stored in stabilization solution (RNAlater, Qiagen) until RNA extraction. Purity and integrity of RNA samples were verified by using spectrophotometry and agrose gel electrophoresis. Equal amounts of total RNA was taken from each mouse to make a pooled sample for control group and plant stanol group. The pooled samples were analyzed by using the Affymetrix microarray platform. The labeling, hybridization and scanning were performed at the Centre for Biotechnology, University of Turku, Finland. The Affymetrix data was normalized by using GC-RMA algorithm, which significantly enhances the quality of detection of low-expressing genes. Genes passing 1.5 fold change limit between the control and the plant stanol treated groups were considered as candidates for being regulated genes, although no statistical comparison was performed. Statistics were applied to detect enriched functional categories or pathways associated with the genes, which diminishes the effect of false positives, as only a large body of genes with similar functions will show as significant enrichment.

2.7. Statistical analysis

The non-parametric Mann–Whitney *U* test was used to compare the data between experimental groups. The associations between variables were analyzed with non-parametric Spearman's correlation test. Statistical analyses were performed using PASW Statistics 18.0 for Windows software (SPSS Inc). *P* values are shown for comparisons.

3. Results

3.1. Plant stanol feeding increases adenoma number but not size

The mice grew well and there was no difference in weekly body weight gain between the groups (Supplementary Fig. S1). The number of adenomas in the entire small intestine was higher in the plant stanol group (median 63,5; min 34; max 103) than in the control group (median 36; min 24; max 77; $P=.002$; Fig. 1A), and the effect was seen in both sexes (male $P=.01$, female $P=.054$). Only 4 out of 14 control mice and 6 of 14 plant stanol mice had adenomas in the colon or caecum, and there was no statistical difference between the groups in the multiplicity of colonic or caecal adenomas. The size of intestinal adenomas did not differ between the groups ($P=.383$; Fig. 1B). The median size of the adenomas in plant stanol fed mice was 1.17 mm (min 0.93; max 1.32) and in control mice 1.21 (min 0.99; max 1.32).

3.2. Plant stanol increases cholesterol level in the caecum content

Plant stanol feeding increased the cholesterol level by 6-fold in the caecum content compared to the control diet ($P<.001$; Table 2). Levels of plant stanols (sitostanol and campestanol) and plant sterols (sitosterol, campesterol, brassicasterol) were increased in the caecum content after plant stanol feeding (all $P<.001$; Table 2). There was a positive correlation between number of adenomas in the small intestine and the cholesterol level in the caecum content (Spearman correlation coefficient $r^s=0.570$; $P<.01$; Fig. 2).

3.3. Plant stanol does not affect cholesterol level in the intestinal mucosa

Plant stanol feeding resulted in higher levels of plant stanols (campestanol, sitostanol) in the mucosal tissue compared with the control diet ($P<.001$ for both; Table 2). Levels of plant sterols (campesterol, sitosterol) were, however, lower in the intestinal mucosa of plant stanol fed mice ($P<.001$ for both; Table 2). Mucosal cholesterol level was unchanged after plant stanol feeding (median 200.9 μg/100 mg in both diet groups, $P=.408$; Table 2.).

3.4. Plant stanol increases level of nuclear β-catenin and nuclear cyclin D1

Levels of nuclear β-catenin and cyclin D1 were increased by plant stanol feeding ($P=.043$ and $P=.065$, respectively; Fig. 3A and B). Levels of nuclear β-catenin and cyclin D1 correlated positively (Spearman's correlation coefficient $r^s=0.816$, $P<.001$). No change in nuclear p53 was detected ($P=.382$).

3.5. Plant stanol increases the levels of EGFR and phosphorylated ERK1/2

Levels of EGFR, ERK1/2 and Akt were analyzed from whole mucosa lysate. The level of total EGFR was higher in the plant stanol group compared to the control group ($P=.031$; Fig. 3C). A higher level of phosphorylated EGFR indicated that plant stanol feeding activated EGFR pathway ($P=.089$; Fig. 3D). Total and phosphorylated EGFR correlated positively with nuclear β-catenin ($r^s=0.692$, $P<.001$ and $r^s=0.554$, $P=.002$, respectively) and with nuclear cyclin D1 ($r^s=0.743$, $P<.001$ and $r^s=0.631$, $P<.001$, respectively).

Plant stanol feeding resulted in higher level of phosphorylated ERK1/2 (p44/42; $P=.039$; Fig. 3E). No difference was seen in the total amount of ERK1/2 ($P=.435$). The level of total Akt was somewhat higher in the mucosa of plant stanol fed mice ($P=.077$, data not shown). There was no difference in the level of phosphorylated Akt between the groups ($P=.491$). Phosphorylated EGFR did not correlate with phospho-ERK1/2 or phospho-Akt ($r^s=0.049$, $P=.806$ and $r^s=0.267$, $P=.170$, respectively).

3.6. Plant stanol increases β-catenin phosphorylation at Ser675

Phosphorylated EGFR correlated positively with nuclear β-catenin, therefore we measured levels of Ser675 and Ser552 phosphorylated β-catenin, which are both related to EGFR signaling and are known to have transcriptional activity [30,31]. Phospho-Ser675 was significantly higher in plant stanol mice than in the control mice ($P=.027$; Fig. 4A). The level of phospho-Ser552 was increased after plant stanol feeding ($P=.077$; Fig. 4D). Phosphorylated EGFR correlated positively with phospho-Ser675 and phospho-Ser552 ($r^s=0.639$, $P<.001$ and $r^s=0.544$, $P=.003$, respectively; Fig. 4B and 4E). Both phosphorylated

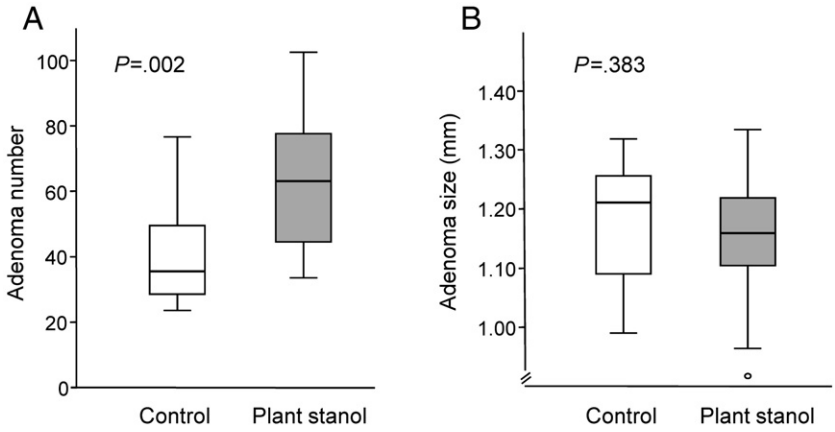

Fig. 1. The number and size of adenomas in the small intestine. Plant stanol feeding increased adenoma number in the small intestine of Apc^{Min} mice (A). No effect was detected in the adenoma size (diameter, mm) between the groups (B). Results are presented as box-plots, where the box represents the interquartile range and contains 50% of values. The whiskers extend to the maximum and minimum values. The median is indicated by a line across the box. n=14 in both groups.

forms of β-catenin correlated positively with nuclear cyclin D1 (Ser675 r^s=0.904, P<.001 and Ser552 r^s=0.837, P<.001; Fig. 4C and F).

3.7. Cholesterol synthesis, p53 pathway and extracellular matrix remodeling genes are up-regulated by plant stanol feeding

An Affymetrix microarray assay was carried out to detect previously uncharacterized changes in the intestinal mucosa at the gene expression level. After enrichment analysis, we observed that genes encoding enzymes of the cholesterol synthesis pathway were up-regulated in the intestinal mucosa of plant stanol fed mice and the pathway was significantly enriched (P<.001; Fig. 5, Supplementary Table S2). Many of these up-regulated enzymes take part in consecutive reactions in sterol biosynthesis e.g. farnesyl diphosphate synthetase (FDPS), farnesyl diphosphate farnesyl transferase 1 (FDFT1), and squalene epoxidase to yield 2,3-oxidosqualene from geranyl pyrophosphate. In addition, the expression of 7-dehydrocholesterol reductase, the enzyme converting 7-dehydrocholesterol to cholesterol, was up-regulated in the intestinal mucosa of plant stanol fed Apc^{Min} mice when compared to control mice.

According to enrichment analysis, expression of several genes of the p53 pathway was regulated in the intestinal mucosa of plant stanol fed mice (Supplementary Table S3, Fig. S2). The data show, that expression of p53 and its downstream target p21 were up-regulated after plant stanol feeding implying of enhanced regulation of cell cycle arrest. The gene expression of cyclin D1 was up-regulated, which supports our Western blotting data of the protein level of cyclin D1.

Table 2
Composition of sterols in caecum content and intestinal mucosa

Sterol (μg/100mg)	Control group	Plant stanol group
Caecum content:		
Cholesterol	41.1 (10.1; 79.4)	252.4 (189.7; 363.1) *
Sitosterol	37.0 (16.9; 91.2)	90.3 (52.8; 106.0) *
Campesterol	23.6 (11.4; 61.23)	76.3 (48.7; 107.7) *
Brassicasterol	6.1 (3.2; 13.7)	13.0 (8.3; 16.4) *
Sitostanol	1.2 (0.63; 2.6)	1571.3 (986.3; 2619.2) *
Campestanol	3.3 (2.6; 4.2)	581.5 (371.8; 1016.4) *
Intestinal mucosa:		
Cholesterol	200.9 (180.0; 233.9)	200.9 (184.0; 245.2)
Sitosterol	2.9 (2.1; 4.8)	1.7 (1.0; 3.2) *
Campesterol	9.0 (6.3; 11.9)	3.9 (3.2; 5.7) *
Sitostanol	0.2 (0.2; 0.3)	12.9 (4.4; 38.1) *
Campestanol	0.8 (0.6; 1.0)	6.5 (3.5; 15.7) *

* P<.001; values represent median (min; max).

3.8. Plant stanol feeding does not affect nuclear SREBP-2, but slightly increases SREBP-2 precursor content

While plant stanol feeding up-regulated genes of the cholesterol synthesis, we analyzed SREBP-2 from nuclear extracts (active form 68 kDa) and total homogenates (precursor 125 kDa and active 68 kDa). The SREBP-2 content in the nucleus increases in cholesterol depleted cells [32]. We did not detect any difference in the level of active SREBP-2, however, the level of SREBP-2 precursor was somewhat increased in plant stanol mice (P=.206; Fig. 6A). The ratio of the precursor to the mature SREBP-2 was elevated in plant stanol mice (P=.141; Fig. 6B). There was a strong positive association between SREBP-2 precursor and p-EGFR (r^s=0.553, P=.002; Fig. 6D), tot-EGFR (r^s=0.719 P<.001), phospho-Ser675-β-catenin (r^s=0.867, P<.001; Fig. 6E) and cyclin D1 (r^s=0.836, P<.001).

4. Discussion

The present study shows that feeding 0.8% plant stanol enriched diet induces intestinal tumor formation in Apc^{Min} mice by over 70% compared to control diet. To our knowledge, pure plant sterols and stanols have not been studied earlier in the Apc^{Min} mouse. Plant

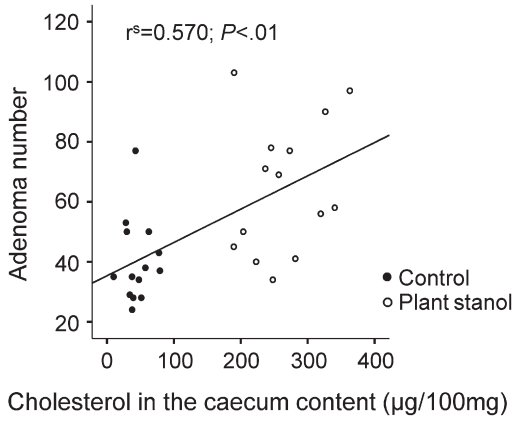

Fig. 2. The number of adenomas in the small intestine correlated positively with the cholesterol level in the caecum content (r^s=0.570; P<.01).

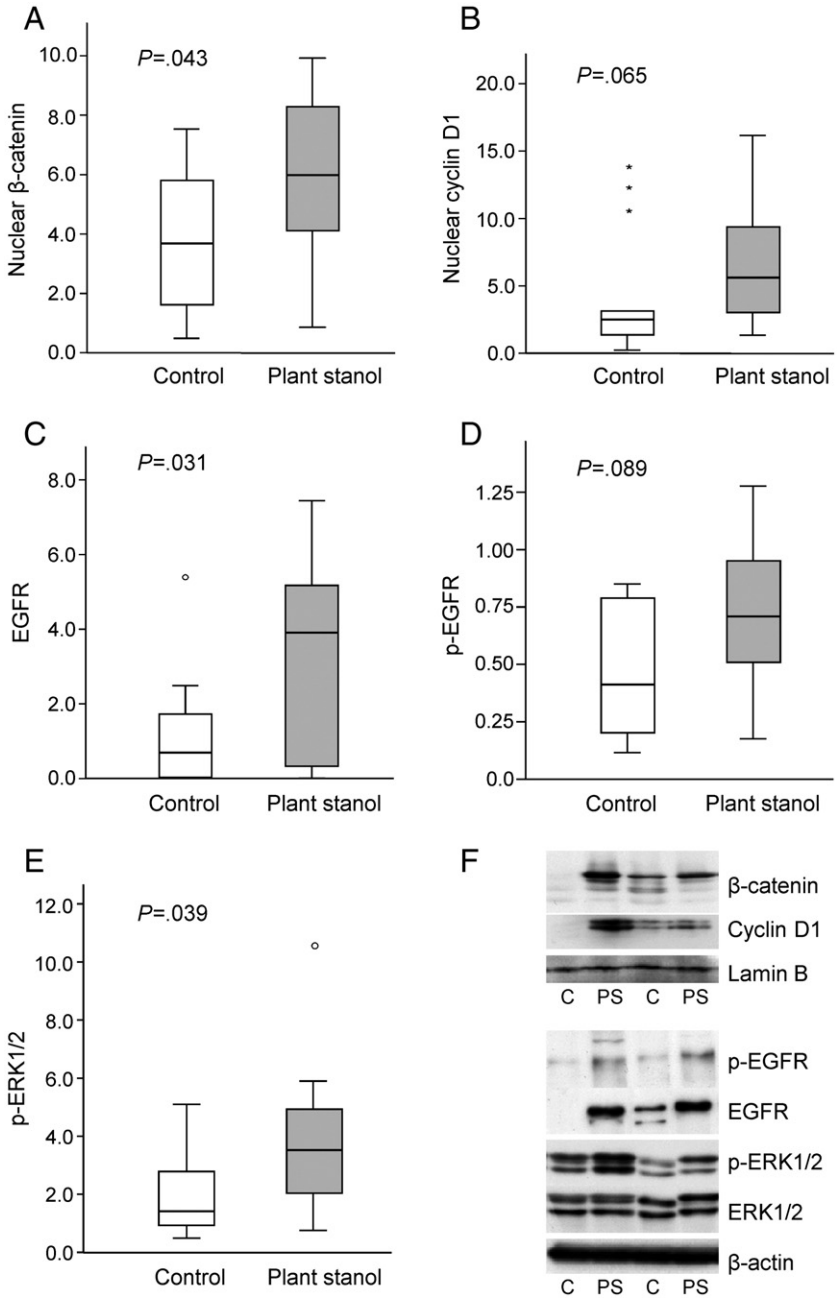

Fig. 3. β-Catenin, cyclin D1, EGFR and ERK1/2 were analyzed from the intestinal mucosa by Western blotting. Plant stanol feeding increased the level of nuclear β-catenin (P=.043)(A), nuclear cyclin D1 (P=.065) (B), total EGFR (P=.031) (C), phosphorylated EGFR (P=.089) (D) and phosphorylated ERK1/2 (P=.039) (E). Box-plots represent the result from all mice from both groups (n=14 mice both groups). Representative bands from Western blotting are shown in F. C=control, PS=plant stanol.

stanol feeding resulted in sixfold higher cholesterol level in the caecum content and our data show that there was a positive correlation between feacal cholesterol level and adenoma number. The elevated level of intraluminal cholesterol is associated with increased carcinogenesis in vivo [2,3].

In our study, enhanced adenoma formation was accompanied by the activation of the Wnt/β-catenin and EGFR signaling, two major pathways in colon carcinogenesis, and these changes were detectable already in the normal appearing mucosa. Activation of the Wnt pathway by plant stanol feeding was seen as higher level of nuclear β-catenin and elevated level of nuclear cyclin D1, the target gene of β-catenin. In humans, most sporadic colon cancer cases exhibit loss of the tumor suppressor APC [33], which leads to the accumulation of β-catenin in the nucleus and activation of the Wnt pathway. This is considered an initiating step in the colon carcinogenesis [14,18]. Similarly, the role of EGFR signaling has been related with early stages of colon carcinogenesis such as microadenoma formation [34], and inhibition of EGFR signaling is shown to inhibit polyp formation [35,36]. Previously, increased levels of both total and phosphorylated EGFR were seen in *Apc*-null tumors of Apc^{Min} mice as well as in the intestinal mucosa, where Apc function is reduced [37]. In the present study, plant stanol feeding accelerated formation of intestinal adenomas in Apc^{Min} mice and up-regulated the levels of total and phosphorylated EGFR already in the non-transformed, normal-appearing intestinal mucosa. This suggests that EGFR has a constitutive role in the initiation of intestinal tumorigenesis.

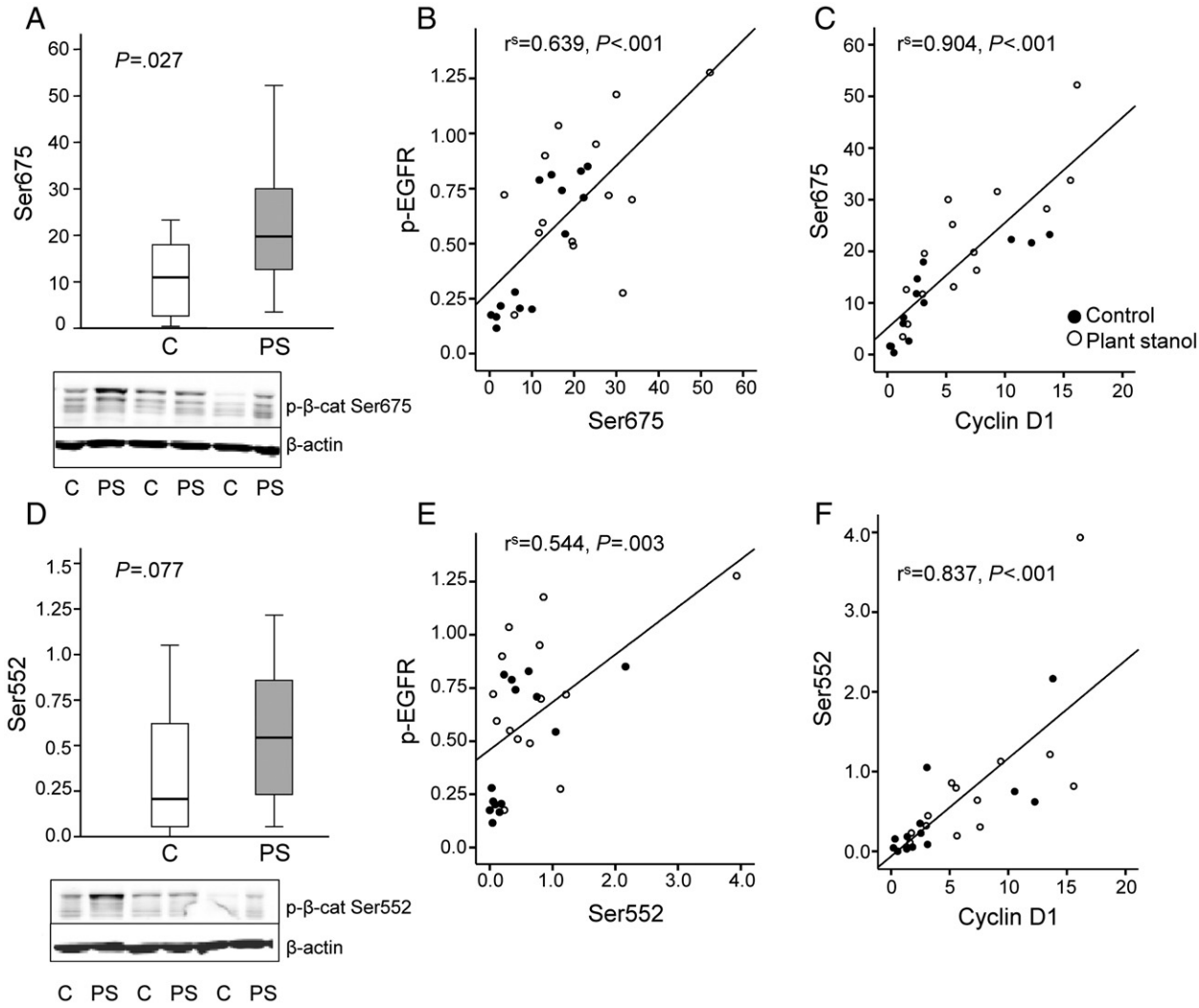

Fig. 4. Phosphorylated β-catenin at Ser675 and Ser552 were analyzed from the intestinal mucosa by Western blotting. Plant stanol feeding increased the level of Ser675-phosphorylated β-catenin (A). The correlation between phosphorylated EGFR and β-catenin phosphorylated at Ser675 (B). The correlation between β-catenin phosphorylated at Ser675 and nuclear cyclin D1 (C). Plant stanol feeding resulted in increased level of Ser552-phosphorylated β-catenin (D). The correlation between phosphorylated EGFR and β-catenin phosphorylated at Ser552 (E). The correlation between β-catenin phosphorylated at Ser552 and nuclear cyclin D1 (C). Representative bands of phosphorylated β-catenin from Western blotting are shown in A (Ser675) and 4D (Ser552) below box-plots. C=control, PS=plant stanol.

The positive correlation between phosphorylated EGFR and nuclear β-catenin suggests a causal relationship between them. In fact, evidence of convergence between Wnt and EGFR signaling exists [38], and it has been shown that upon EGF stimulation β-catenin translocates into the nucleus without any alterations in its stability or phosphorylation state by APC-dependent glycogen synthase kinase-3-β (GSK-3-β) [39]. We show that levels of β-catenin phosphorylated at Ser675 and Ser552 were increased in plant stanol fed mice, and both these forms of phospho-β-catenin are known to relate to EGFR signaling. Ser675 residue of β-catenin is phosphorylated by a serine-threonine kinase Pak1, which is activated by EGFR signaling [31] and K-Ras/Rac1 cascade [40,41]. Phosphorylation of β-catenin at Ser552 residue is induced by Akt, which is activated downstream of EGFR [30]. In this study, plant stanol feeding resulted in stronger increase in phospho-Ser675-β-catenin than in phospho-Ser552. Moreover, there was no difference between experimental groups in the level of phosphorylated Akt, which implies that EGFR may have signaled through other downstream targets than Akt. Finally, we saw a strong positive correlation between the levels of phosphorylated-β-catenin and nuclear cyclin D1. It has been demonstrated that phosphorylation of β-catenin at Ser552 and Ser675 promote transcriptional activity of β-catenin [30,42].

Plant stanols may have disturbed cholesterol balance at the cell membrane and consequently activated membrane receptors such as EGFR and its downstream targets. Although plant stanol feeding had no effect on total cholesterol level in the intestinal mucosa, the total level of plant sterols and stanols was higher in plant stanol fed mice than in control mice. Plant sterols can be incorporated to cellular membranes displacing cholesterol, which results in perturbation of membrane properties and receptor function [24-26]. Evidence provided by numerous studies show that EGFRs are located in lipid rafts rich in cholesterol and sphingolipids [21-23]. Cholesterol depletion from plasma membrane with methyl-β-cyclodextrin increases the number of EGFR in the plasma membrane [43] and enhances EGF binding and EGFR phosphorylation [43,44]. Moreover, ligand-independent activation of EGFR, tyrosine phosphorylation of EGFR downstream targets (e.g., ERK) [45,46], and activation of Ras [46] have all been found in cholesterol depleted membranes. In contrast, addition of cholesterol to cell membrane reduces EGFR activation [43]. Cellular cholesterol

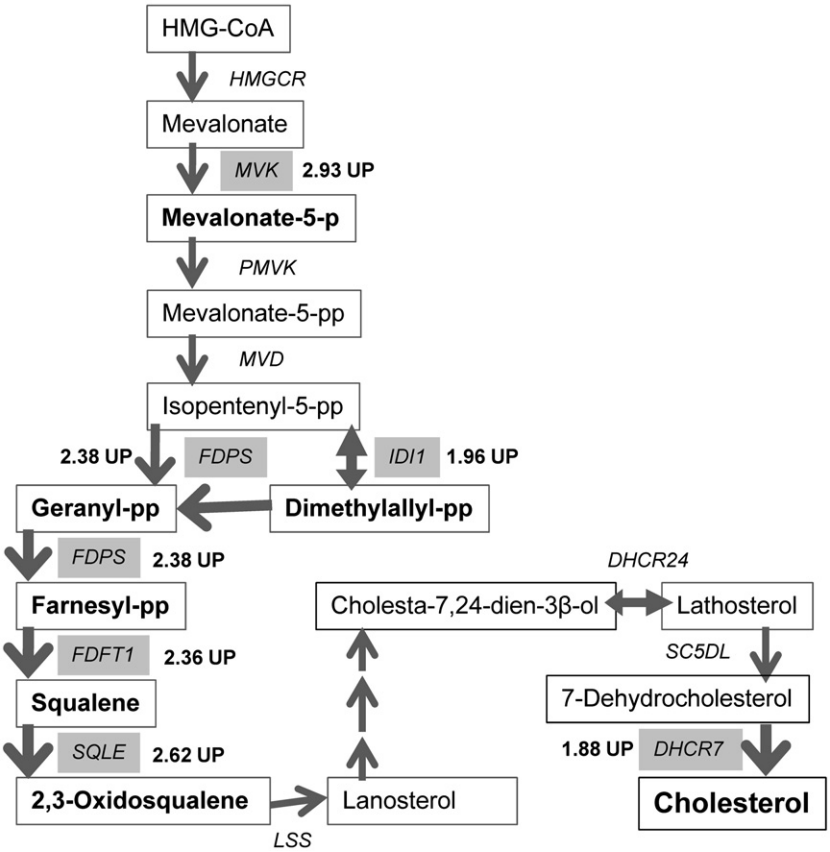

Fig. 5. Cholesterol synthesis was up-regulated in the plant stanol fed mice. Total RNA was extracted from mucosa of small intestine. Equal amounts of total RNA was taken from each mouse to make a pooled sample for control group and plant stanol group. The pooled samples were analyzed by using the Affymetrix microarray platform. Biosynthesis of cholesterol was significantly enriched among putatively regulated genes in plant stanol fed mice. The gene expression of enzymes synthesizing, e.g., GPP, FPP, squalene and cholesterol were up-regulated by plant stanol feeding. Fold-change values represent the change in gene expression in the plant stanol group compared to the control group. *HMGCR*, 3-hydroxy-3-methylglutaryl-CoA reductase, HMG-CoA reductase; *MVK*, mevalonate kinase; *PMVK*, phosphomevalonate kinase; *MVD*, mevalonate decarboxylase; *IDI1*, isopentenyl-diphosphate delta-isomerase; *SQLE*, squalene epoxidase; *LSS*, lanosterol synthetase; *DHCR24*, 24-dehydrocholesterol reductase; *SC5DL*, sterol-C5-desaturase; *DHCR7*, 7-dehydrocholesterol reductase.

depletion has been shown to activate ERK1/2 directly [47], and this may be the case in our study, since phosphorylated EGFR did not correlate with its downstream target ERK1/2. Presumably, plant stanol feeding increased the level of phosphorylated EGFR and ERK1/2 independent of each other as a consequence of disturbed cholesterol balance in enterocytes.

The pathway analysis on our microarray data shows that plant stanol feeding up-regulated the transcription of several consecutive enzymes of cholesterol synthesis in the intestinal mucosa. This indicates that reduced cholesterol absorption probably stimulated a compensatory cholesterol synthesis in enterocytes. When intracellular free cholesterol level is low, SREBP-controlled transcription of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase), a key enzyme regulating cholesterol biosynthesis, and LDL-receptor, is increased. As a result, cholesterol synthesis and cholesterol uptake are increased. Although we saw no change in HMG-CoA reductase at the gene expression level, a 2% sitosterol diet has been demonstrated to up-regulate HMG-CoA-reductase activity and receptor-mediated LDL binding without any change in total or esterified cholesterol concentrations in rat jejunal mucosal cells [48]. Patients with hypercholesterolemia are often treated with statins that inhibit the HMG-CoA-reductase activity. A meta-analysis of 20 case–control studies found a significant association between statin usage and reduced risk of colon cancer [49]. In addition, atorvastatin [50] and pitavastatin [51] were shown to reduce intestinal polyp formation in the *Apc*^{Min} mouse. Plant stanols and statins lower blood cholesterol in different mechanisms which may partly explain their different effects on colon cancer development. Statins inhibit the conversion of HMG-CoA to mevalonate and reduce the synthesis of the downstream products, such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GPP). Our results show, that the expression of enzymes producing FPP and GPP was up-regulated in the intestinal mucosa of plant stanol fed mice. FPP and GPP are post-translational modifiers of several proteins including Ras, Rho, Rab and lamin B [52,53]. These intermediates of the sterol biosynthesis could modify and activate Ras, which in turn activates EGFR and its downstream effectors [53]. A higher farnesyl pyrophosphate synthase activity is found in human colorectal cancer samples when compared to normal surrounding mucosa and it is shown to regulate cell proliferation [54]. Taken together, enhanced EGFR activity in plant stanol fed *Apc*^{Min} mice may be a result of increased synthesis of modifiers that activate Ras. Furthermore, EGFR signaling resulted in increased phosphorylation of β-catenin, increased level of nuclear β-catenin and increased level of its transcriptional target cyclin D1.

Although genes of the cholesterol synthesis were up-regulated after plant stanol feeding, the nuclear SREBP-2 content was not changed. There was no difference in the level of mucosal cholesterol between groups and possibly therefore no difference in the nuclear SREBP-2 was seen. The synthesis of SREBP-2 precursor may be under regulation, too [55,56]. The regulatory pool of cholesterol synthesis consists of newly synthesized cholesterol, plasma membrane

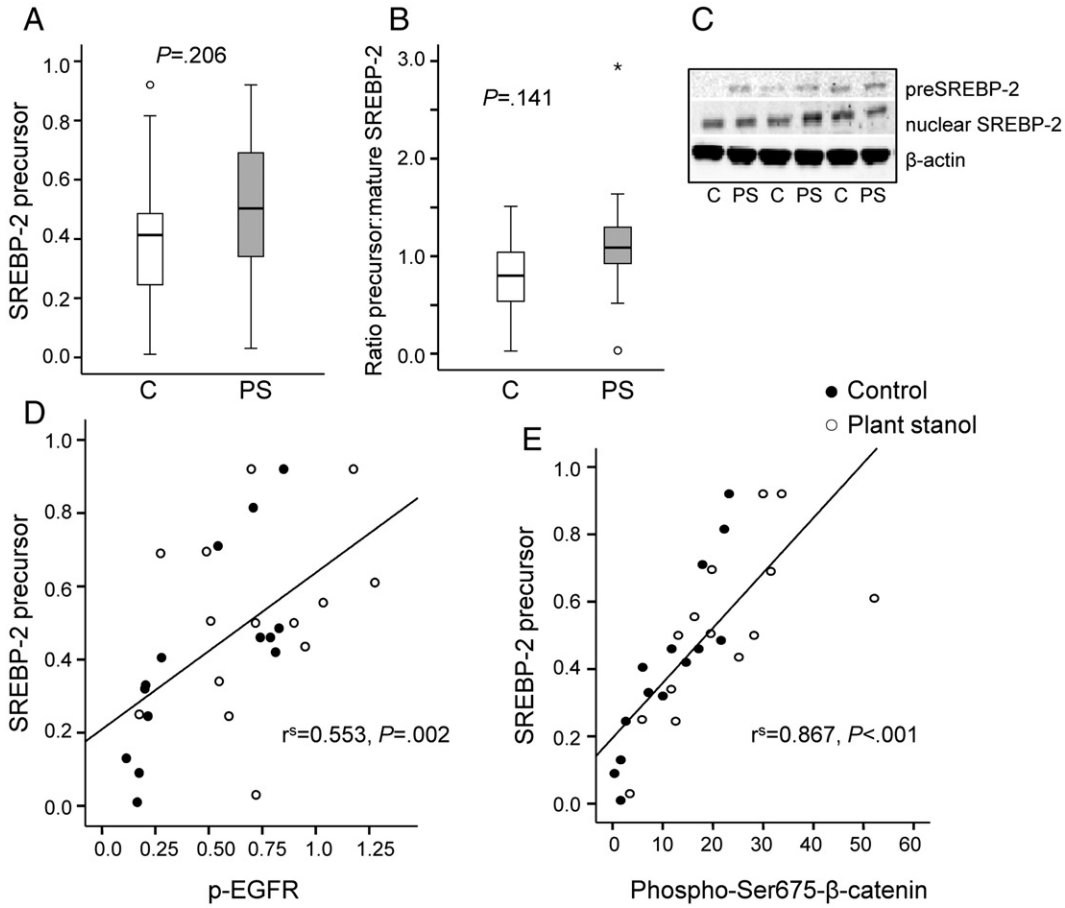

Fig. 6. Precursor and mature forms of SREBP-2 were analyzed from nuclear extracts and total homogenates of the intestinal mucosa. Plant stanol feeding elevated the level of precursor SREBP-2 (A) and the ratio of precursor to mature SREBP-2 in the total homogenate (B). No difference was seen in the nuclear SREBP-2 content. Representative bands from Western blotting are shown in C (C=control, PS=plant stanol). The precursor form of SREBP-2 correlated strongly with levels of phosphorylated EGFR (D) and phospho-Ser675-β-catenin (E).

cholesterol, lipoprotein-derived cholesterol and absorbed cholesterol. What in the cholesterol pool specifically regulates SREBP-2 conversion is not well-known. Harding et al. [57] demonstrated that plant sterol feeding to hamsters increased levels of hepatic plant sterols and down-regulated processing of SREBP-2. There was no change in the hepatic free cholesterol level, however, cholesterol synthesis was increased and cholesterol absorption decreased. They speculated that, in addition to cholesterol, free plant sterols may bind to the SCAP sterol sensing domain and down-regulate conversion of SREBP-2 into its active form. Similarly, we suggest, that conversion of the SREBP-2 precursor was reduced by increased mucosal plant stanol content. Interestingly, we found strong positive associations between SREBP-2 precursor and activated EGFR, phospho-Ser675-β-catenin and cyclin D1. This supports our hypothesis that plant stanol feeding induced alterations in the regulation of cholesterol metabolism, which was associated with activated EGFR signaling.

The gene expression of p53 tumor suppressor and its downstream target p21 were up-regulated in the intestinal mucosa of plant stanol fed mice. P53 responds to intracellular (e.g., DNA damage) and extracellular stress signals, and it controls the fate of damaged and stressed cells through initiation of apoptosis, cell cycle arrest and DNA repair [58]. Evidently, plant stanols induced cellular stress (e.g., increased β-catenin and EGFR signaling, disturbed cholesterol homeostasis), which up-regulated the gene expression of p53 to regain normal cell function. The protein level of p53 was, however, not different between the groups and the response of p53 pathway was not attained.

Plant stanols were supplemented to the diet as plant stanol esters, in the form that these compounds are present in the human diet. As a matter of fact, plant stanol esters were added to the experimental diet as freeze-dried plant stanol ester enriched food products that were on market in Finland 2003. The estimated daily intake of plant stanol was 20 mg for a mouse consuming 12 kcal of energy, which is equivalent to 5 g intake for a man consuming 3000 kcal per day. A potential plant sterol intake in humans was assessed in simulation studies, where the intake of plant sterols was accounted for virtual consumption of enriched products [59,60]. Simulation studies suggest that the daily intake of plant sterols could exceed 8 g [59] or 13 g [60] depending on the enrichment scenario. A meta-analysis by Katan et al. [61] stated that daily intake of 2 g plant sterols or stanols lower serum LDL cholesterol approximately by 10%, and that there is little additional effect at higher intake than 2.5 g.

In conclusion, plant stanols induce tumor formation in the small intestine of the Apc^{Min} mouse by activating Wnt and EGFR signaling pathways that are both known to play a key role in early colon tumorigenesis. Plant stanols themselves, by an unidentified mechanism, or changes in the cholesterol metabolism induced by plant stanol feeding may have enhanced EGFR signaling within enterocytes. Activation of EGFR seems to have promoted β-catenin phosphorylation and its translocation into the nucleus, where the expression of cyclin D1 was increased. The increased level of EGFR alone may have increased tumorigenesis in plant stanol fed Apc^{Min} mice, however, the presence of *Apc* mutation in the intestinal mucosa may predispose to stronger effects of plant stanols. Mutations of the *Apc* become more

prevalent in the colon of middle-aged people, who are more likely to consume cholesterol lowering, plant sterol or stanol enriched foods. Therefore, further studies are needed to evaluate the relationship between high plant stanol intake and colon cancer in wild-type, "healthy" mice and humans.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2012.07.002.

References

[1] Rao AV, Janezic SA. The role of dietary phytosterols in colon carcinogenesis. Nutr Cancer 1992;18:43-52.
[2] Kendall CW, Janezic SA, Friday D, Rao AV. Dietary cholesterol enhances preneoplastic aberrant crypt formation and alters cell proliferation in the murine colon treated with azoxymethane. Nutr Cancer 1992;17:107-14.
[3] Rao AV, Janezic SA, Friday D, Kendall CW. Dietary cholesterol enhances the induction and development of colonic preneoplastic lesions in C57BL/6J and BALB/cJ mice treated with azoxymethane. Cancer Lett 1992;63:249-57.
[4] Raicht RF, Cohen BI, Fazzini EP, Sarwal AN, Takahashi M. Protective effect of plant sterols against chemically induced colon tumors in rats. Cancer Res 1980;40:403-5.
[5] Deschner EE, Cohen BI, Raicht RF. The kinetics of the protective effect of beta-sitosterol against MNU-induced colonic neoplasia. J Cancer Res Clin Oncol 1982;103:49-54.
[6] Janezic SA, Rao AV. Dose-dependent effects of dietary phytosterol on epithelial cell proliferation of the murine colon. Food Chem Toxicol 1992;30:611-6.
[7] Quilliot D, Boman F, Creton C, Pelletier X, Floquet J, Debry G. Phytosterols have an unfavourable effect on bacterial activity and no evident protective effect on colon carcinogenesis. Eur J Cancer Prev 2001;10:237-43.
[8] Panda SK, Chattoraj SC, Broitman SA. Correlation of neomycin, faecal neutral and acid sterols with colon carcinogenesis in rats. Br J Cancer 1999;80:1132-6.
[9] Peuchant E, Salles C, Jensen R. Relationship between fecal neutral steroid concentrations and malignancy in colon cells. Cancer 1987;60:994-9.
[10] Normén AL, Brants HA, Voorrips LE, Andersson HA, van den Brandt PA, Goldbohm RA. Plant sterol intakes and colorectal cancer risk in the Netherlands Cohort Study on Diet and Cancer. Am J Clin Nutr 2001;74:141-8.
[11] Powell SM, Zilz N, Beazer-Barclay Y, Bryan TM, Hamilton SR, Thibodeau SN, et al. APC mutations occur early during colorectal tumorigenesis. Nature 1992;359:235-7.
[12] Luebeck EG, Moolgavkar SH. Multistage carcinogenesis and the incidence of colorectal cancer. Proc Natl Acad Sci USA 2002;99:15095-100.
[13] Su LK, Kinzler KW, Vogelstein B, Preisinger AC, Moser AR, Luongo C, et al. Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene. Science 1992;256:668-70.
[14] Näthke IS. The adenomatous polyposis coli protein: the Achilles heel of the gut epithelium. Annu Rev Cell Dev Biol 2004;20:337-66.
[15] He TC, Sparks AB, Rago C, Hermeking H, Zawel L, da Costa LT, et al. Identification of c-MYC as a target of the APC pathway. Science 1998;281:1509-12.
[16] Shtutman M, Zhurinsky J, Simcha I, Albanese C, D'Amico M, Pestell R, et al. The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc Natl Acad Sci USA 1999;96:5522-7.
[17] van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, et al. The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell 2002;111:241-50.
[18] Bienz M, Clevers H. Linking colorectal cancer to Wnt signaling. Cell 2000;103:311-20.
[19] Prenzel N, Fischer OM, Streit S, Hart S, Ullrich A. The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. Endocr Relat Cancer 2001;8:11-31.
[20] Krasinskas AM. EGFR signaling in colorectal carcinoma. Patholog Res Int 2011:932932.
[21] Balbis A, Posner BI. Compartmentalization of EGFR in cellular membranes: role of membrane rafts. J Cell Biochem 2010;109:1103-8.
[22] Patra SK. Dissecting lipid raft facilitated cell signaling pathways in cancer. Biochim Biophys Acta 2008;1785:182-206.
[23] Pike LJ. Growth factor receptors, lipid rafts and caveolae: an evolving story. Biochim Biophys Acta 2005;1746:260-73.
[24] Awad AB, Chinnam M, Fink CS, Bradford PG. beta-Sitosterol activates Fas signaling in human breast cancer cells. Phytomedicine 2007;14:747-54.
[25] Ratnayake WM, L'Abbé MR, Mueller R, Hayward S, Plouffe L, Hollywood R, et al. Vegetable oils high in phytosterols make erythrocytes less deformable and shorten the life span of stroke-prone spontaneously hypertensive rats. J Nutr 2000;130:1166-78.
[26] Mora MP, Tourne-Peteilh C, Charveron M, Fabre B, Milon A, Muller I. Optimisation of plant sterols incorporation in human keratinocyte plasma membrane and modulation of membrane fluidity. Chem Phys Lipids 1999;101:255-65.
[27] Dietrich WF, Lander ES, Smith JS, Moser AR, Gould KA, Luongo C, et al. Genetic identification of Mom-1, a major modifier locus affecting Min-induced intestinal neoplasia in the mouse. Cell 1993;75:631-9.
[28] Soupas L, Huikko L, Lampi A-M, Piironen V. Oxidative stability of phytosterols in some food applications. Eur Food Res Technol 2005;222:266-73.
[29] Misikangas M, Pajari A-M, Päivärinta E, Mutanen M. Promotion of adenoma growth by dietary insulin is associated with increase in cyclin D1 and decrease in adhesion proteins in Min/+ mice mucosa. J Nutr Biochem 2005;16:402-9.
[30] Fang D, Hawke D, Zheng Y, Xia Y, Meisenhelder J, Nika H, et al. Phosphorylation of beta-catenin by AKT promotes beta-catenin transcriptional activity. J Biol Chem 2007;282:11221-9.
[31] Galisteo ML, Chernoff J, Su YC, Skolnik EY, Schlessinger J. The adaptor protein Nck links receptor tyrosine kinases with the serine-threonine kinase Pak1. J Biol Chem 1996;271:20997-1000.
[32] Brown MS, Goldstein JL. The SREBP pathway: Regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell 1997;89:331-40.
[33] Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. Cell 1996;87:159-70.
[34] Fichera A, Little N, Jagadeeswaran S, Dougherty U, Sehdev A, Mustafi R, et al. Epidermal growth factor receptor signaling is required for microadenoma formation in the mouse azoxymethane model of colonic carcinogenesis. Cancer Res 2007;67:827-35.
[35] Roberts RB, Min L, Washington MK, Olsen SJ, Settle SH, Coffey RJ, et al. Importance of epidermal growth factor receptor signaling in establishment of adenomas and maintenance of carcinomas during intestinal tumorigenesis. Proc Natl Acad Sci USA 2002;99:1521-6.
[36] Buchanan FG, Holla V, Katkuri S, Matta P, DuBois RN. Targeting cyclooxygenase-2 and the epidermal growth factor receptor for the prevention and treatment of intestinal cancer. Cancer Res 2007;67:9380-8.
[37] Moran AE, Hunt DH, Javid SH, Redston M, Carothers AM, Bertagnolli MM. Apc deficiency is associated with increased Egfr activity in the intestinal enterocytes and adenomas of C57BL/6J-Min/+ mice. J Biol Chem 2004;279:43261-72.
[38] Hu T, Li C. Convergence between Wnt-β-catenin and EGFR signaling in cancer. Mol Cancer 2010;9:236.
[39] Lu Z, Ghosh S, Wang Z, Hunter T. Downregulation of caveolin-1 function by EGF leads to the loss of E-cadherin, increased transcriptional activity of beta-catenin, and enhanced tumor cell invasion. Cancer Cell 2003;4:499-515.
[40] Lee S-H, Kunz J, Lin S-H, Yu-Lee L. 16-kDa prolactin inhibits endothelial cell migration by down-regulating the Ras-Tiam1-Rac1-Pak1 signaling pathway. Cancer Res 2007;67:11045-53.
[41] Wang Z, Pedersen E, Basse A, Lefever T, Peyrollier K, Kapoor S, et al. Rac1 is crucial for Ras-dependent skin tumor formation by controlling Pak1-Mek-Erk hyperactivation and hyperproliferation in vivo. Oncogene 2010;29:3362-73.
[42] Zhu G, Wang Y, Huang B, Liang J, Ding Y, Xu A, et al. A Rac1/PAK1 cascade controls β-catenin activation in colon cancer cells. Oncogene 2012;31:1001-12.
[43] Ringerike T, Blystad FD, Levy FO, Madshus IH, Stang E. Cholesterol is important in control of EGF receptor kinase activity but EGF receptors are not concentrated in caveolae. J Cell Sci 2002;115:1331-40.
[44] Pike LJ, Casey L. Cholesterol levels modulate EGF receptor-mediated signaling by altering receptor function and trafficking. Biochemistry 2002;41:10315-22.
[45] Lambert S, Vind-Kezunovic D, Karvinen S, Gniadecki R. Ligand-independent activation of the EGFR by lipid raft disruption. J Invest Dermatol 2006;126:954-62.
[46] Chen X, Resh MD. Cholesterol depletion from the plasma membrane triggers ligand-independent activation of the epidermal growth factor receptor. J Biol Chem 2002;277:49631-7.
[47] Wang P-Y, Weng J, Anderson RGW. OSBP is a cholesterol-regulated scaffolding protein in control of ERK 1/2 activation. Science 2005;307:1472-6.
[48] Nguyen LB, Shefer S, Salen G, Tint GS, Ruiz F, Bullock J. Mechanisms for cholesterol homeostasis in rat jejunal mucosa: effects of cholesterol, sitosterol, and lovastatin. J Lipid Res 2001;42:195-200.
[49] Taylor ML, Wells BJ, Smolak MJ. Statins and cancer: a meta-analysis of case–control studies. Eur J Cancer Prev 2008;17:259-68.
[50] Swamy MV, Patlolla JMR, Steele VE, Kopelovich L, Reddy BS, Rao CV. Chemoprevention of familial adenomatous polyposis by low doses of atorvastatin and celecoxib given individually and in combination to APCMin mice. Cancer Res 2006;66:7370-7.
[51] Teraoka N, Mutoh M, Takasu S, Ueno T, Yamamoto M, Sugimura T, et al. Inhibition of intestinal polyp formation by pitavastatin, a HMG-CoA reductase inhibitor. Cancer Prev Res (Phila) 2011;4:445-53.
[52] Guruswamy S, Rao CV. Multi-target approaches in colon cancer chemoprevention based on systems biology of tumor cell-signaling. Gene Regul Syst Biol 2008;2:163-76.
[53] Dimitroulakos J, Lorimer IA, Goss G. Strategies to enhance epidermal growth factor inhibition: targeting the mevalonate pathway. Clin Cancer Res 2006;12:4426s-31s.
[54] Notarnicola M, Messa C, Cavallini A, Bifulco M, Tecce MF, Eletto D, et al. Higher farnesyl diphosphate synthase activity in human colorectal cancer inhibition of cellular apoptosis. Oncology 2004;67:351-8.
[55] Kim H-J, Takahashi M, Ezaki O. Fish oil feeding decreases mature sterol regulatory element-binding protein 1 (SREBP-1) by down-regulation of SREBP-1c mRNA in mouse liver. A possible mechanism for down-regulation of lipogenic enzyme mRNAs. J Biol Chem 1999;274:25892-8.

[56] Field FJ, Born E, Murthy S, Mathur SN. Gene expression of sterol regulatory element-binding proteins in hamster small intestine. J Lipid Res 2001;42: 1-8.
[57] Harding SV, Rideout TC, Jones PJH. Hepatic nuclear sterol regulatory binding element protein 2 abundance is decreased and that of ABCG5 increased in male hamsters fed plant sterols. J Nutr 2010;140:1249-54.
[58] Levine AJ, Hu W, Feng Z. The P53 pathway: what questions remain to be explored? Cell Death Differ 2006;13:1027-36.
[59] De Jong N, Pijpers L, Bleeker JK, Ocké MC. Potential intake of phytosterols/-stanols: results of a simulation study. Eur J Clin Nutr 2004;58:907-19.
[60] Kuhlmann K, Lindtner O, Bauch A, Ritter G, Woerner B, Niemann B. Simulation of prospective phytosterol intake in Germany by novel functional foods. Br J Nutr 2005;93:377-85.
[61] Katan MB, Grundy SM, Jones P, Law M, Miettinen T, Paoletti R. Efficacy and safety of plant stanols and sterols in the management of blood cholesterol levels. Mayo Clin Proc 2003;78:965-78.