

Genetic networks responsive to sodium butyrate in colonic epithelial cells

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Abstract We performed microarray and computational gene network analyses to identify the detailed mechanisms by which sodium butyrate (SB) induces cell growth arrest and the differentiation of mouse colonic epithelial MCE301 cells. Two thousand six hundred four differentially expressed probe sets were identified in the cells treated with 2 mM SB and were classified into four groups. Of these, the gradually increased group and the gradually and remarkably decreased group contained the genetic networks for cellular development and cell cycles or canonical pathways for fatty acid biosynthesis and pyrimidine metabolism, respectively. The present results provide a basis for understanding the detailed molecular mechanisms of action of SB in colonic epithelial cells.

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1. Introduction

Short-chain fatty acids such as butyrate, acetate and propionate are produced in the colonic lumen as a consequence of the microbial degradation of poorly fermentable dietary fibers [1]. Of these, butyrate in particular is taken up by colonocytes and provides major respiratory fuel for colonic epithelial cells [2], playing an essential role in the maintenance of homeostasis of the colonic epithelium [3]. Previous studies have demonstrated that butyrate suppresses cell proliferation and induces cellular differentiation and apoptosis in a wide variety of cell types [4–7]. An important effect of butyrate is the modulation of gene transcription through the inhibition of histone deacetylase [4,8]; inhibition of this enzyme activity results in the hyperacetylation of histones and consequently facilitates the accessibility of transcription factors to DNA binding sites [4]. In addition, butyrate response elements have been found within the promoters of a number of genes [5,6]. It has been shown that butyrate represses the expression of CCND1 [5] but induces that of CDKN1A [6].

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Abbreviations: AP, alkaline phosphatase; DMEM, Dulbecco's modified Eagle medium; SB, sodium butyrate

DNA microarray technology can provide a view of the expression profiles of many hundreds or many thousands of genes. Using this technology, many genes that are modulated by butyrate have been identified in colonic carcinoma cell lines [9–13]. In our previous study using small-scale cDNA microarrays including 865 genes, we identified 130 genes that were differentially expressed by sodium butyrate (SB) in mouse colonic non-carcinoma epithelial MCE301 cells derived from transgenic mice harboring temperature-sensitive simian virus 40 large T-antigen [14]. More recently, pathway analysis technologies now allow for the mapping of gene expression data into relevant pathway maps based on their functional annotation and known molecular interactions, and have been put to use in biological experiments [15,16]. In the present study, in order to identify the detailed mechanisms by which SB induces cell growth arrest and the differentiation of colonic epithelial cells, we performed global scale time course microarray analysis using the Affymetrix GeneChip[®] oligonucleotide microarray platform with Mouse Expression Array 430A, which were spotted with 22690 probe sets. Additionally, we examined the functional relationships between the candidate genes using the Ingenuity Pathways Analysis tool.

2. Materials and methods

2.1. Cell culture and incubation of the cells with SB

A mouse colonic epithelial cell line, MCE301, was established from transgenic mice harboring the temperature-sensitive simian virus 40 large T-antigen [7]. The cells grow at a permissive temperature (33 °C) and have polarized epithelial cell features. The cells were not transformed, judging by the absence of anchorage-independent growth in soft agar and the lack of tumor formation in nude mice [7]. The cells were cultivated in Dulbecco's modified Eagle medium (DMEM)/F12 medium (Invitrogen Co., Tokyo, Japan) supplemented with 20 ng/ml insulin, 20 ng/ml transferrin, 1.22 ng/ml ethanolamine, 0.0914 ng/ml sodium selenite, 10 ng/ml epidermal growth factor and 2% fetal bovine serum on a collagen type I-precoated culture vessel at 33 °C. SB (Wako Pure Chemical Industries Ltd., Osaka, Japan) at a final concentration of 2 mM was dissolved in the DMEM/F12 medium. The cells were cultured with DMEM/F12 medium containing this compound for 0–24 h at 33 °C.

2.2. Measurement of cell number, apoptosis and alkaline phosphatase activity

The cell number was counted by a hemacytometer. For detection of apoptosis, the formation of DNA ladder was observed according to the method reported previously [17]. Alkaline phosphatase (AP) activity was measured according to our previous method [14].

2.3. SDS–polyacrylamide gel electrophoresis and Western blot

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blot were carried out as described elsewhere [18,19]. Anti-acetylated histone H2B rabbit polyclonal and anti-GAPDH mouse monoclonal antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and Chemicon International Inc. (Temecula, CA, USA), respectively. Immunoreactive proteins on the nitrocellulose membrane were visualized by a luminescent image analyzer (LAS-1000 plus, Fuji film Co., Tokyo, Japan) using an enhanced chemiluminescence detection system. Bands of target proteins were quantified by densitometry using Multi-Analyst software (Japan BIO-RAD Laboratories, Tokyo, Japan). Fold induction was normalized by GAPDH [14].

2.4. Separation of total RNA

Total RNA was extracted from the cells using an RNeasy Total RNA Extraction Kit (Qiagen K.K., Tokyo, Japan). Then, RNA samples were treated with RNase-free DNase (Qiagen K.K.) for 30 min at room temperature.

2.5. Microarray and gene network analyses

Gene expression was analyzed using a GeneChip[®] system with Mouse Expression Array 430A which was spotted with 22690 probe sets (Affymetrix, Santa Clara, CA). Sample preparation for array hybridization was carried out following the manufacturer's instructions. In short, 5 µg of total RNA was used to synthesize double-strand cDNA with a GeneChip[®] Expression 3'-Amplification Reagents One-Cycle cDNA Synthesis Kit (Affymetrix). Biotin-labeled cRNA was then synthesized from the cDNA using GeneChip[®] Expression 3'-Amplification Reagents for IVT Labeling (Affymetrix). After fragmentation, the biotinylated cRNA was hybridized to arrays at 45 °C for 16 h. The arrays were washed, stained with streptavidin-phycoerythrin and scanned with a probe array scanner. The scanned chip was analyzed using the GeneChip Analysis Suite software (Affymetrix). Hybridization intensity data were converted into a presence/absence call for each gene, and changes in gene expression between experiments were detected by comparison analysis. The data were further analyzed using GeneSpring software (Silicon Genetics, Redwood City, CA) to extract the significant genes. To examine the gene ontology, including biological processes, cellular components, molecular functions and genetic networks, the data were analyzed using Ingenuity Pathways Analysis tools (Ingenuity Systems, Mountain View, CA), a web-delivered application that enables the discovery, visualization and exploration of molecular interaction networks in gene expression data. The gene lists identified by GeneSpring containing Affymetrix gene ID and natural legalism were uploaded into the Ingenuity Pathways Analysis.

2.6. Real-time quantitative PCR assay

Real-time quantitative PCR was performed on a real-time PCR system (Mx3000P, Stratagene Japan K.K., Tokyo, Japan) using Brilliant SYBR Green qPCR Master Mix (Stratagene Japan K.K.) according to the manufacturer's protocol. Reverse transcriptase reaction was carried out with total RNA by using an oligo d(T)6 primer. Real-time quantitative PCR was performed by using the specific primers listed in Supplementary Material (Table S1). Each mRNA expression level was normalized with respect to the mRNA expression of GAPDH [20].

2.7. Statistical analysis

Data are shown as means ± S.D. Statistical analysis was carried out using Student's *t* test and *P* values less than 0.05 were regarded as significant.

3. Results

3.1. Effects of SB on cell growth and AP activity

When the mouse colonic epithelial MCE301 cells were exposed to 2 mM SB, the cell number remained constant over culture periods from 0 to 24 h (Fig. 1A). Under the same conditions, the cells did not show any DNA laddering (data not shown), indicating that SB induced no apoptotic change [14]. AP is a brush border-associated enzymes which is a good mar-

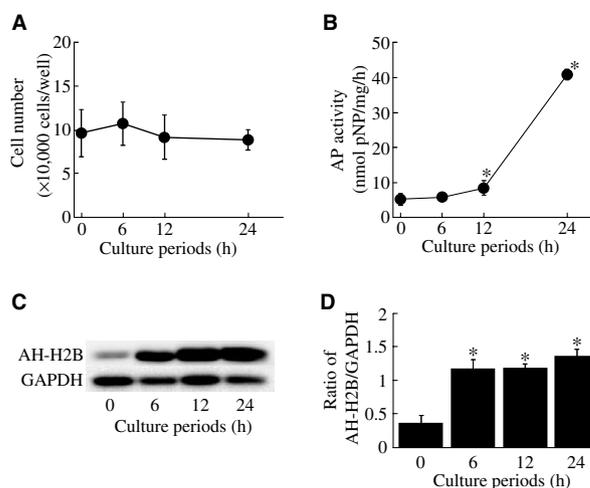


Fig. 1. The effects of SB on cell growth (A), AP activity (B), and the level of histone acetylation (C and D) in MCE301 cells. The cells were cultured in DMEM/F12 medium supplemented with 2 mM SB for 0–24 h. (A) The cell number was counted by a hemacytometer. (B) AP activity was measured using *p*-nitrophenyl phosphate as a substrate, and *p*-nitrophenol (pNP) concentration was determined. (C, D) SDS–PAGE and Western blotting were performed. Signals were visualized by a luminescent image analyzer using an ECL system (C). Bands were quantified by densitometry. Fold induction of acetylated histone H2B (AH-H2B) was normalized by GAPDH (D). Data indicate means ± S.D. for 3–4 different experiments. **P* < 0.05 vs. control (0 h) (Student's *t* test).

ker for the differentiation of colonic cells [6,7]. AP activity was significantly and time-dependently elevated, with levels that were 1.6 times (12 h) and 8.1 times (24 h) higher than those of control cells (0 h) (Fig. 1B). These results show that the treatment of MCE301 cells with SB induces cell growth arrest and a differentiated phenotype at 12 and 24 h [14].

3.2. Effects of SB on the expression level of acetylated histone H2B

As butyrate is known to induce histone acetylation [4,8], the degree of this acetylation was examined. Western blot analysis using an anti-acetylated histone H2B antibody showed that SB at a concentration of 2 mM significantly induced the accumulation of acetylated histone H2B under 6, 12 and 24 h of SB treatment, with levels that were approximately 3.5 times higher than those of control cells (0 h) (Fig. 1C and D).

3.3. The time course global gene expression analysis

Gene expression profiling of MCE301 cells exposed to 2 mM SB for 0, 6, 12 and 24 h were analyzed by GeneChip[®] oligonucleotide expression arrays. Of the 22690 probe sets analyzed, we identified 13216 (percentage present: 58.2%), 13390 (59.0%), 13653 (60.2%) and 13408 (59.1%) probe sets expressed in the cells treated with SB for 0, 6, 12 and 24 h, respectively, indicating that the number of expressed genes was approximately constant among the different samples. Complete lists of genes from all samples are available on the Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE4410>).

3.4. Identification and cluster analysis of differentially expressed genes

Genes that were up- or downregulated by >2.0-fold were examined using the GeneSpring software. When each gene

expression level was compared with that of control samples (0 h), the total number of probe sets that were found to be differentially expressed by SB was 2604; that is, 11.5% of probe sets were affected by SB. Changes in the probe sets of the cells treated with SB for 6, 12 and 24 h were detected in 632 (446 upregulated + 186 downregulated probe sets), 1435 (763 + 672) and 2272 probe sets (946 + 1326), respectively; lists of the genes that were differentially expressed in cells treated with SB are available on our homepage (<http://www.toyamampu.ac.jp/uf/mgrc/html/laboratory.html>). Next, a hierarchical clustering algorithm was utilized within GeneSpring to generate a gene tree representative of the major patterns of gene expression during SB treatment and four major gene clusters were identified: an early increased gene cluster (cluster name: I); a gradually increased gene cluster (II); a gradually decreased gene cluster (III); and a gradually and remarkably decreased gene cluster (IV). These gene clusters of I, II, III and IV contained 329, 691, 585 and 814 probe sets, respectively (Fig. 2). Among differentially expressed genes, there were many whose relationship to SB has been previously reported, such as CCND1 [5,9,12,14], PCNA [13,14], HSP25 [21], FST [12], and SPPI [12,14]. However, a large number of these differentially expressed genes were newly identified genes whose relationship to SB has not been reported previously, such as PPBR, RANBP1, GRN, SAT and TXNIP. Changes in gene

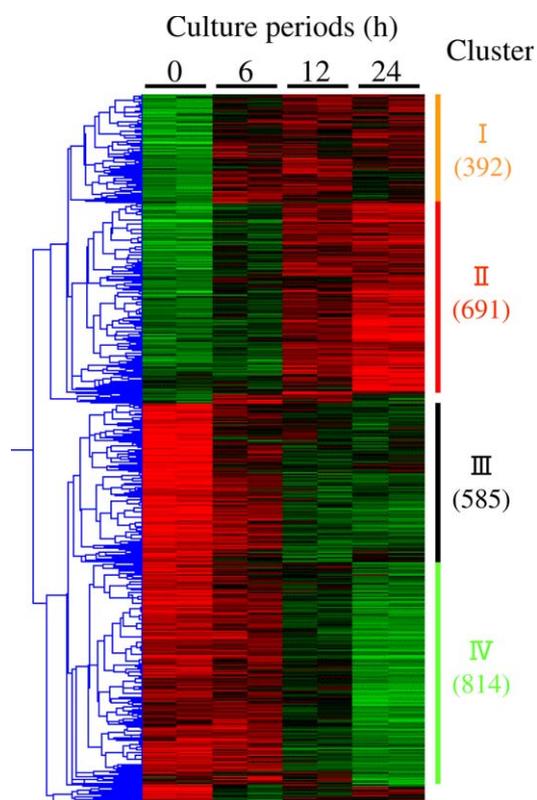


Fig. 2. Hierarchical clustering of 2604 probe sets differentially expressed by >2.0 in the cells treated with 2 mM SB after 0, 6, 12 and 24 h. Clustering was performed by GeneSpring software. Each probe set is represented by a single row of colored bars. Intensity in the green and red color range indicates downregulated and upregulated probe sets, respectively. Intensity in the black color indicates no change (relative expression: 1). The number in parentheses indicates the number of probe sets.

expression of PPBR, RANBP1, GRN, SAT and TXNIP were confirmed by the real-time quantitative PCR (Supplementary Material Fig. 1S).

3.5. Functional category and pathway analyses

The functional category and pathway analyses of the probe sets of the four clusters obtained by the hierarchical clustering of differentially expressed genes in cells treated with SB were explored using the Ingenuity Pathways Knowledge Base. When the functional category analysis of these four clusters was performed, clusters I, II, III and IV contained 165, 305, 29 and 433 focus genes, respectively. Moreover, genes that were significantly and functionally categorized as having molecular and cellular functions were observed in clusters II and IV, but not in clusters I and III (Supplementary Material Fig. S2). There were 14 categories, including amino acid metabolism, cell cycle, cell death, cell morphology, etc. Based on the significance and number of genes, the top 3 categories affected by SB treatment in cluster II were cell death, cellular movement or lipid metabolism, and in cluster IV, cell cycle, cellular assembly and organization or DNA replication, recombination and repair. The genes associated with these categories are listed in Supplementary Material (Tables S2 and S3).

Next, to determine the biologically relevant networks and pathways of the genes identified here, pathway analysis was carried out on the up- and downregulated genes using the Ingenuity Pathways Knowledge Base. The networks describe functional relationships between gene products based on known interactions reported in the literature. Several significant pathways were recognized in up- and downregulated genes that belonged primarily to clusters II and IV. Of upregulated networks, the most significant network (score: 51; 33 genes) including IGF2, CDKN1C and RB1 was associated with cell death (P -value: $4.79E-11$ to $3.86E-3$; 25 genes) and cellular development (P -value: $4.94E-9$ to $3.86E-3$; 23 genes). In this network, differentiation-related genes whose nodes and edges were highlighted in blue were observed (Fig. 3). Moreover, canonical pathway analysis of upregulated genes that primarily belonged to cluster II showed metabolic pathways including valine, leucine and isoleucine degradation (ratio: 11/66; P -value: $1.44E-5$), fatty acid biosynthesis (ratio: 5/18; P -value: $4.53E-4$) or fatty acid metabolism (ratio: 13/119; P -value: $5.27E-4$) and a signaling pathway of antigen presentation (ratio: 6/42; P -value: $4.92E-3$). The genes of these canonical pathways are listed in Supplementary Material (Table S4).

In downregulated networks, a significant network (score: 47; 33 genes) including CCND1, PCNA and BRCA1 was found to be associated with cell cycle (P -value: $1.10E-7$ to $1.91E-2$; 16 genes) and DNA replication, recombination and repair (P -value: $3.87E-7$ to $3.86E-2$; 19 genes). In this network, we observed cell cycle progression-related genes whose nodes and edges were highlighted blue (Fig. 4). Canonical pathway analysis of downregulated genes that belonged primarily to cluster IV showed metabolic pathways including pyrimidine metabolism (ratio: 18/146; P -value: $9.58E-6$) or carbon pool by folate (ratio: 5/19; P -value: $6.5E-4$) and signaling pathways including cell cycle G1/S checkpoint regulation (ratio: 10/43; P -value: $3.87E-6$) or cell cycle G2/M DNA damage checkpoint regulation (ratio: 8/34; P -value: $3.49E-5$). The genes of these canonical pathways are listed in Supplementary Material (Table S5).

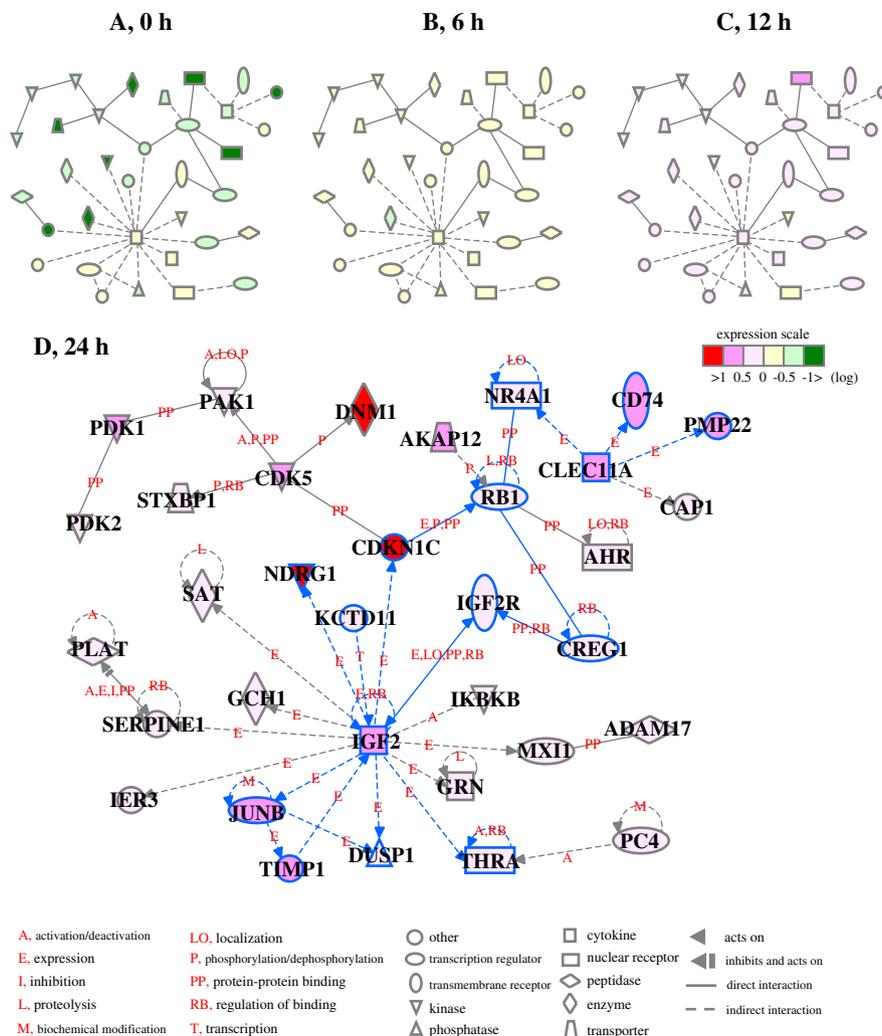


Fig. 3. A network of genes that were upregulated in cells treated with SB after 24 h. Probe sets including cluster II were analyzed by the Ingenuity Pathways Analysis software. The network is displayed graphically as nodes (genes) and edges (the biological relationships between the nodes). The node color indicates the expression level of the genes. Nodes and edges are displayed with various shapes and labels that present the functional class of genes and the nature of the relationship between the nodes, respectively. The nodes and edges of genes associated with cell differentiation are highlighted in blue.

4. Discussion

Novel transcript profiling technologies allow the simultaneous measurement of changes in gene expression of many hundreds or many thousands of genes. With these technologies, it has been demonstrated that many genes that were modulated by butyrate were identified in colonic carcinoma cell lines [9–13] and in the colonic non-carcinoma cell line MCE301 in the previous [14] and present studies. Although it is important to identify the individual genes that are differentially expressed, there is an increasing need to move beyond this level of analysis. To this end, in the present study, we carried out pathway analysis on the SB-induced genes in MCE301 cells using the Ingenuity Pathways Analysis tool. The present study successfully identified gradually increased and gradually and remarkably decreased gene clusters including cell death- or cellular development-related and cell cycle- or DNA replication-related genetic networks in colonic non-carcinoma epithelial cells treated with SB. To the best of our knowledge, this is

the first report of the identification of the influence of butyrate on genetic networks in colonic epithelial cells using pathway analysis software.

In the present study, 2604 differentially expressed probe sets were identified in the cells treated with 2 mM SB, and were classified into four gene clusters, two of which, the gradually increased group (cluster II) and the gradually and remarkably decreased (cluster IV) group, contained the molecular and cellular functions for cell death or cellular development and cell cycles or DNA replication, recombination and repair, respectively. Based on the present results together with those of previous studies [5,6,9–14], we suggest that a decrease in cell cycle-related genes in cluster IV (Table S2) may be closely associated with the cell growth arrest induced by SB. Of particular interest is our identification of the cell cycle-associated genetic network whose core contains CCND1, PCNA and BRCA1 (Fig. 4). In this network, a high expression level of CCND1 was maintained during the cell cycle progression stage. On the other hand, the addition of

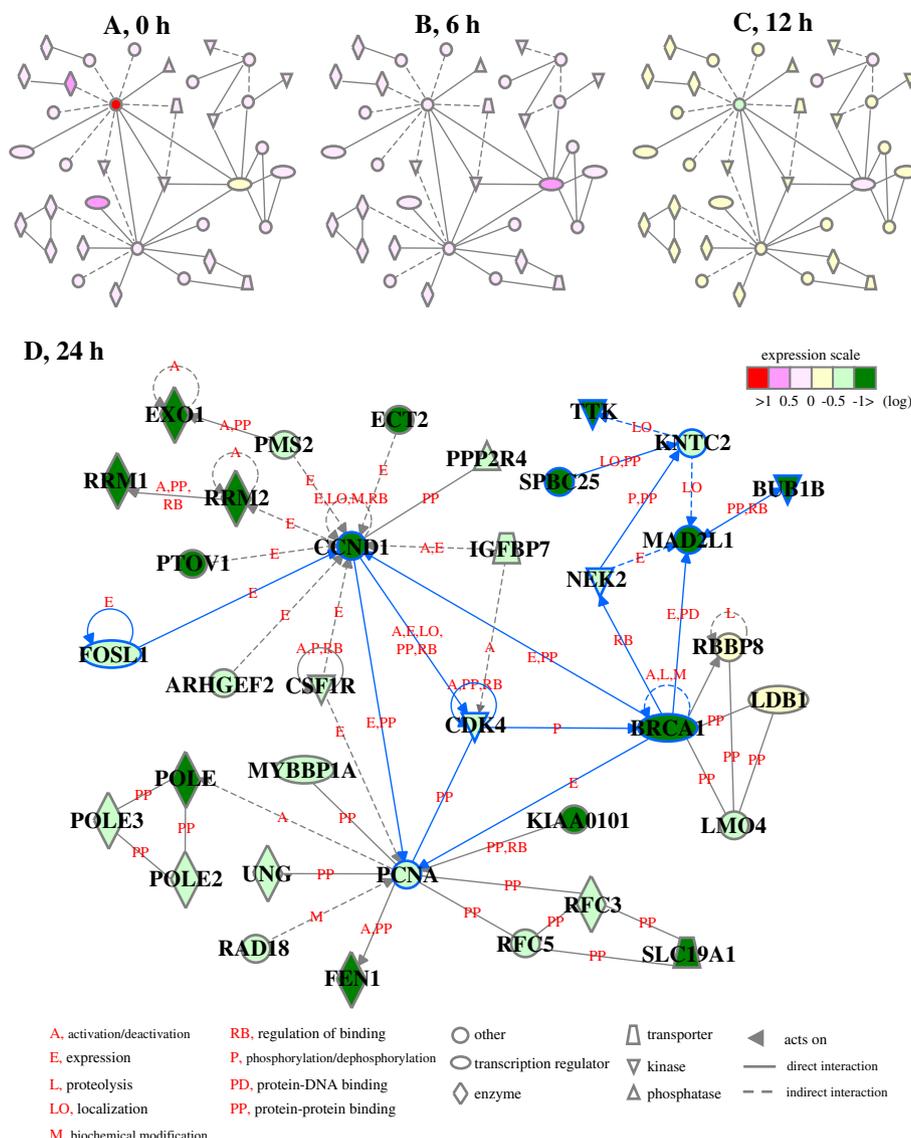


Fig. 4. A network of genes that were downregulated in cells treated with SB after 24 h. Probe sets including cluster IV were analyzed by the Ingenuity Pathways Analysis software. The network is displayed graphically as nodes (genes) and edges (the biological relationships between the nodes). The node color indicates the expression level of the genes. Nodes and edges are displayed with various shapes and labels that present the functional class of genes and the nature of the relationship between the nodes, respectively. The nodes and edges of genes associated with cell cycle progression are highlighted in blue.

SB to the cells induced a dramatic decrease in CCND1 in a time-dependent manner. We believe that this decrease in the expression of CCND1 may be due to the action of SB via butyrate-response elements within the promoter region of the gene [5] and to be suppression of the gene products, FOSL1 [22] and ECT2 [23], that indicate the positively regulated expression of CCND1. Almost all genes in this network declined gradually, and only BRCA1 was transiently elevated at 6 h and then gradually declined. Previous research has shown that BRCA1 protein increases arrest in the cell cycle progression of cells [24]. In addition, BRCA1 protein is reported to indicate the positively regulated expression of CCND1 [25], PCNA [26], NEK2 [27] and MAD2L1 [28]. In the present cell system, cell cycle-related genes, especially those participating in this genetic network (Fig. 4), may be closely associated with the cell growth arrest caused by SB. Interestingly, the cellular development-associated genetic net-

work was found to be contain many upregulated genes related to cell differentiation (Fig. 3), suggesting that this network may be correlated with the colonic cell differentiation induced by SB. Indeed, PMP22 [29], RB1 [30], NDRG1 [31], IGF2R [32], IGF2 [33], JUNB [34], TIMP1 [35] and THRA [36] have been reported to be expressed in the small intestine or colon. This network includes IGF2 at its center. IGF2 is reported to be involved in the expression of NDRG1, THRA, JUNB and DUSP1 in mutant mouse fibroblasts with a homozygous knockout of IGF1R [37]. The differentially expressed genes or functionally categorized genetic clusters and genetic networks identified here are likely to be involved in cell growth arrest and differentiation induced by SB in colonic epithelial cells.

In conclusion, a detailed knowledge of changes in gene expression using global scale microarray and computational pathway analyses will provide a basis for understanding the

detailed molecular mechanisms of the action of SB in colonic epithelial cells.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.febslet.2006.04.048](https://doi.org/10.1016/j.febslet.2006.04.048).

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