**Epigenetic Regulation of Genomes: Nutrient-specific Modulation of Genetic Networks in Bovine Cells**

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**Abstract:** The modern version of epigenetics includes the molecular mechanisms that influence the phenotypic outcome of a gene or genome, in absence of changes to the underlying DNA sequence. A host of genomic interrelationships with the diet evidently exist. The broad topic of nutrigenomics is defined as the interaction between nutrition and an individual's genome. Ruminant species have evolved to metabolize the short-chain volatile fatty acids (VFAs, acetate, propionate, and butyrate) to fulfill up to 70% of their nutrient energy requirements. The potential biological roles of VFAs were investigated using the established Madin-Darby bovine kidney epithelial cell line. Butyrate induces cell cycle arrest and apoptosis in bovine cells. Gene expression profiling indicated that butyrate induces many significant changes in the expression of genes associated with regulatory pathways that are critical to cell growth, immune response and signal transduction. Functional category and pathway analyses of the microarray data revealed that several canonical pathways (the cell cycle G2/M DNA damage checkpoint and G1/S checkpoint regulation; pyrimidine metabolism; and purine metabolism insulin-like growth factor axis components) were significantly affected.

**INTRODUCTION**

The modern version of epigenetics includes the molecular mechanisms that influence the phenotypic outcome of a gene or genome, in the absence of changes to the underlying DNA sequence. As this area continues to unfold, it is becoming increasingly evident that a host of genomic interrelationships with the diet exist which encompasses the broad topic of nutrigenomics, defined as the interaction between nutrition and an individual's genome.

It is now well established that microbial fermentation in the gastrointestinal tract contributes to the energy balance of all mammalian species [1,2]. Short-chain fatty acids (acetate, propionate, and butyrate) are formed during microbial fermentation of dietary fibre in the gastrointestinal tract and then are directly
absorbed at the site of production. Ruminant species have evolved to metabolize
the short-chain volatile fatty acids (VFAs, acetate, propionate, and butyrate) to
fulfill up to 70% of their nutrient energy requirements [2]. While acetate and
propionate hold a prominent position in providing energy to ruminant metabolism,
butyrate, although low in its relative concentrations, appears to be involved in
aspects of metabolism beyond its role as a nutrient. The presented study, using
genomics and molecular techniques, bioinformatics and computation, as well as
knowledge integration, reveals that the short-chain volatile fatty acids (VFA, acetate,
propionate, and butyrate), especially butyrate, are involved in metabolism
not only as nutrient, but also as genomic regulator.

MATERIALS AND METHODS

Madin-Darby bovine kidney epithelial cells (MDBK) were cultured in Eagle’s minimal essential medium
and supplemented with 5% foetal bovine serum At approximately 50% confluence (during the exponential
phase), the cells were treated with 10mM of sodium butyrate for 24h. Cell populations (cell cycle progression
and DNA synthesis) were monitored using flow cytometric analysis. The bovine microarray platform used
was described previously [3]. The data generated from microarray studies was explored using Ingenuity
Pathways Analysis (Ingenuity Systems, www.ingenuity.com), a web-delivered application that enables the
discovery, visualization and exploration of molecular interaction networks in gene expression data. Canonical
pathway analysis, functional analysis, pathways analysis and network generation and functional analysis of
a Network were performed.

RESULTS

Butyrate treatment of MDBK cells arrests cells in G1 phase and induces apoptosis. Average flow cytometric cell cycle distribution from three experiments using butyrate
treatment showed an arrest of cells in the G1 phase with the majority of cells in
G0/G1 (76 ± 2.9%), while 8.9 ± 1% and 15 ± 2.4% of the cells were in S and G2/M
phases, respectively (Figure 1).

To understand the mechanism of cell cycle arrest induced by butyrate, a systematic
search for butyrate-responsive genes in MDBK cells was conducted using a high-
density oligonucleotide microarray, followed by detailed canonical pathway analysis
and regulatory network identification. Using Significance Analysis of Microarray
(SAM), 450 genes significantly regulated by sodium butyrate at a very stringent false
discovery rate (FDR) of 0% were identified. The largest category of genes regulated
by butyrate was cell cycle control related. Fourteen genes related to apoptosis and
extracellular matrix (ECM) were also significantly regulated by butyrate.

The functional category and pathway analysis of differentially expressed genes in
cells treated with butyrate were explored using the Ingenuity Pathway Knowledge base.
Four canonical pathways (the cell cycle G2/M DNA damage checkpoint and G1/S
checkpoint regulation; pyrimidine metabolism; and purine metabolism) were significantly
(P<0.05) regulated. Cell cycle checkpoint pathways (the G2/M DNA damage checkpoint
and G1/S checkpoint regulation) were impaired due to treatment with butyrate; for the
first time, two pathways critical for the regulation of purine and pyrimidine metabolism
were linked to the induced biological effects of butyrate (Table 1). These findings
underlie mechanisms of butyrate induced cell arrest in the progression of the cell cycle.
Global functional analysis also indicated that the levels of many genes involved in the
cell cycle, cellular movement, DNA replication, recombination and repair, cellular
growth and proliferation are significantly and consistently dysregulated. The top 10
dysregulated functions induced by butyrate are listed in Table 2.
Fig. 1: Butyrate treatment arrested cells in G1/G0 phases.

For flow cytometric analysis, exponential growing cells (normal cells) and butyrate treated cells were first pulse labeled with BrdU for 30 min. Collected cells were stained with diluted fluorescent (Fluorescent isothiocyanate, FITC) anti-BrdU antibody and then stained with DNA marker (7-ADD). The fluorescent signal generated by FITC was acquired in a logarithmic mode (DNA synthesis), and fluorescent signal from the DNA-content marker 7-ADD was normally acquired in the linear signal amplification mode. Two colour staining separated cells into three populations, G1/G0, S and G2/M phases. Butyrate treatment arrested cells in G1/G0 and almost eliminated the S cell population.

Table 1: Canonical pathways regulated by butyrate in MDBK cells.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>-log(p-value)</th>
<th>Differential expressed genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell cycle: G2/M DNA damage checkpoint regulation</td>
<td>3.60E+00</td>
<td>TP53, CDC25B, CDC25C, CCNB1, CCNB2, CDC2, CHEK1</td>
</tr>
<tr>
<td>Pyrimidine metabolism</td>
<td>3.32E+00</td>
<td>NME5, ITPA, ENTPD2, DKC1, RRM2, POLA2, REV3L, DTYMK, NXN, TK1, RFC3, NME6</td>
</tr>
<tr>
<td>Cell cycle: G1/S checkpoint regulation</td>
<td>2.88E+00</td>
<td>TP53, E2F4, PA2G4, TGFBI, CDK4, CDC25A, HDAC, E2F, EBPI</td>
</tr>
<tr>
<td>Purine metabolism</td>
<td>2.83E+00</td>
<td>NME5, PKLR, RRM2, REV3L, NME6, ADSS, IMPDH2, ITPA, ENTPD2, IMPDH1, MPHOSPH1, ADA, POLA2, PDE5A, GMPR, RFC3</td>
</tr>
</tbody>
</table>
Table 2: Major cellular functions targeted by butyrate.

<table>
<thead>
<tr>
<th>Function</th>
<th>-log(p-value)</th>
<th>Number of genes targeted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell cycle</td>
<td>1.9E-11-3.56E-02</td>
<td>82</td>
</tr>
<tr>
<td>Cellular movement</td>
<td>2.84E-07-3.64E-02</td>
<td>44</td>
</tr>
<tr>
<td>DNA replication, recombination, and repair</td>
<td>4.57E-06-3.52E-02</td>
<td>75</td>
</tr>
<tr>
<td>Cellular assembly and organization</td>
<td>1.75E-05-3.1E-02</td>
<td>48</td>
</tr>
<tr>
<td>Cellular growth and proliferation</td>
<td>8.51E-05-3.35E-02</td>
<td>97</td>
</tr>
<tr>
<td>Cellular function and maintenance</td>
<td>3.2E-04-3.27E-02</td>
<td>9</td>
</tr>
<tr>
<td>Cell death</td>
<td>6.15E-04-3.27E-02</td>
<td>94</td>
</tr>
<tr>
<td>Cell-to-cell signaling and interaction</td>
<td>9.14E-04-3.61E-02</td>
<td>27</td>
</tr>
<tr>
<td>Nucleic acid metabolism</td>
<td>9.14E-04-1.36E-03</td>
<td>5</td>
</tr>
<tr>
<td>Cellular development</td>
<td>1.04E-03-3.56E-02</td>
<td>23</td>
</tr>
</tbody>
</table>

To determine the biologically relevant networks and pathways of the differentially expressed genes, pathway analysis was done on the integrated total data sets using the Ingenuity Pathways Knowledge Base. A total of 26 regulatory networks were linked to butyrate. These networks had an Ingenuity Pathway Analysis (IPA) score $\geq 10$, indicating a less than $10^{-10}$ chance that the genes in the network are associated together solely due to random events. The networks describe functional relationships between gene products based on known interactions reported in the literature. The top five networks that are targeted by butyrate are listed in Table 3. The first network was centred on upregulated TGFβ1. In addition, the most significant characterization of this network was the downregulated expression of the genes such as IGFBP6, E2F4, CDC2, CKS, CCNA2, CCNB1 and CHEK1, which are all related to cell cycle progression. The significant changes to this network may indicate the important role of TGFβ1 in the cell cycle regulation induced by butyrate. The second network associated with up- and downregulated genes is also related to the cell cycle as well as cellular movement. Genes associated with this network were mostly downregulated genes such as TP53, CDC48, CCNG1, CHD3, CDC20, and MAD2L1. Only three upregulated genes (BIRC5, THBS2 and CTSF) were involved in this network. The third network identified contains upregulated IGF2, IGFBP3, INHBA, MMP1, MMP13, etc., as well as downregulated genes such as IGFBP4, MCM2, MCM3, MCM4, MCM5, MCM6, and ORC1L. Major cellular functions or diseases associated with this network are cancer, cellular movement and cell death.

In a graphical representation of the molecular relationships between genes/gene products (Network Pathway), genes or gene products are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least one reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge Base. Figure 2 shows the third network listed in Table 3 as an example of graphical representations. This network contains upregulated IGF2, IGFBP3, INHBA, MMP1, MMP13, etc., as well as downregulated genes such as IGFBP4, MCM2, MCM3, MCM4, MCM5, MCM6, and ORC1L.
<table>
<thead>
<tr>
<th>Network</th>
<th>Molecules in network</th>
<th>Score</th>
<th>Focus genes</th>
<th>Top functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ACTN1, ASGR2, CCNA2, CCNB1, CDC2, CDC25A, CDC25B, CHEKI, CKS1B, CLDN4, CYC1, E2F4, EEF1A1, ELAVL1, FEN1, HNRPK, HTRA1, IGFBP6, ITPA, LOXL1, LTC4S, MXII, PPIA, PPIG, PRODH, RAD51, RAD51AP1, RAD54L, RBM3, SERPINH1, SFRS3, TGFBI1, TNFAIP2 (includes EG: 7127), TXNIP, UBE2C</td>
<td>54</td>
<td>35</td>
<td>Cell cycle, cancer, reproductive system disease</td>
</tr>
<tr>
<td>2</td>
<td>ANLN, AURKA, AURKAIP1, AURKB, BIHC5, BUB1, BUB1B, CCNB2, CCNG1, CDC20, CDC48, CDK4, CENPA, CHD3, CTSF, DNL7, DNMT1, FUBP1, GNL3, GTSE1, HSP90AA1, HSP49B (includes EG: 3313), INCENP, KIF14, KIF23, MAD2L1, NASP, NUSAP1, PRC1, REV3L, THBS2, TK1, TP53, TUBB4, VRK1</td>
<td>54</td>
<td>35</td>
<td>Cell cycle, cellular movement, cancer</td>
</tr>
<tr>
<td>3</td>
<td>ANKRDL1, CIQA, CDC45L, CENPF, CTF5, CTSD, DUSP1, FST, G1P2, HELLS, HGF, HSPB2, IGF2, IGFBP3, IGFBP4, INHBA, KIF22, MCM2, MCM3, MCM4, MCM5, MCM6, MMP1, MMP7, MMP13, ORCIL, PFDN6, PGM2, PLAT, PLAUR, PSAP, RRM2, TFP12, TIMP3, UHRF1</td>
<td>54</td>
<td>35</td>
<td>Cancer, cellular movement, cell death</td>
</tr>
<tr>
<td>4</td>
<td>ANXA2, ARG1, BCAT1, CCNA2, CDC2L1, CEBP4, CKS2, CREG1, CTSB, DDX21, E2F4, ESIPL1, FBL, GADD45A, HNRPH1, HNRPK, LGMN, MTHFD1, MYC, NCL, NFYA, NFYC, NOL5A, PABPC4, PCBP2, PLAT, PTG1, RPL14, RPS12, RPS4X, SERPINI1, SNRP70, TNC, TUBB2A, UBE2C</td>
<td>25</td>
<td>22</td>
<td>Gene expression, cell cycle, cardiovascular disease</td>
</tr>
<tr>
<td>5</td>
<td>ANXA3, ARID4A, BDPI, BRF1, BRM1, CBFA2T3, CBX1, CBX5, CCNA1, CDC25A, CDC25C, CDK4, CDK8, CHAF1A, CHEKI, DNMT1, GJA1, GJA7, GTF3C2, HDAC8, HIST1H1B, HIST2H3C, HRAS, PHF5A, PLOD1, RAD51, RB1, RBPP4, RUNX1T1, SAP30, SDCCAG10, SHFM1, SUV39H1, TLR1, TOP2A</td>
<td>18</td>
<td>18</td>
<td>Cancer, cell cycle, reproductive system disease</td>
</tr>
</tbody>
</table>

Differentially expressed genes are in bold (↑ up or ↓ down regulated)
Fig. 2: An integrated network with both up- and downregulated genes in cells treated with butyrate for 24 h. Data set was analyzed by the Ingenuity Pathways Analysis software (Ingenuity® Systems, www.ingenuity.com). The graphical pathway network showed is the third network from Table 3. This network contains upregulated IGF2, IGFBP3, INHBA, MMP1, MMP13, etc., as well as downregulated genes such as IGFBP4, MCM2, MCM3, MCM4, MCM5, MCM6, and ORC1L. Notes and edges are displayed with various shapes and labels that present the functional class of genes and the nature of the relationship between the notes, respectively.

**DISCUSSION**

During the last two decades, we have come to realize that lipids function as both energy stores and signaling molecules. They play pivotal roles in regulating a wide variety of cellular processes. As a particular group of lipids, short-chain fatty acids have been studied intensively [1,4]. Short-chain fatty acids are a major energy source in ruminants and contribute up to 70% of their energy requirements [2,5,6]. Of these short-chain fatty acids, butyrate is the major source of energy for colonocytes [7,8]. Short-chain fatty acids are organic acids produced by anaerobic fermentation of dietary fibre in the gastrointestinal tract of mammalian species and then directly absorbed at the site of production. Short-chain fatty acids, especially butyrate, are important nutrients and function as signaling molecules. The molecular mechanisms involved in biochemical and molecular actions of these nutrients remain largely unknown. Few definitive studies in cattle, if any, have addressed the capabilities of short-chain fatty acids as signaling molecules to modulate gene expression and the proteometric outcomes at cellular and molecular levels.

Since the short-chain volatile fatty acids (VFAs, acetate, propionate, and butyrate) fulfill up to 70% of their nutrient energy requirements, cells derived from ruminant
species are presumed to be equipped to respond to VFA with increased sensitivity compared to other species [2]. Our studies revealed, however, that butyrate induced cell cycle arrest and apoptosis in an established bovine MDBK cell line [9]. In this study, the detailed mechanisms by which butyrate induces cell growth arrest and apoptosis were analyzed using global gene expression profiles with high-density oligonucleotide microarrays and the Ingenuity Pathways Knowledge Base. Gene expression profiling indicated that butyrate induces many significant changes in gene expression involving many regulatory pathways that are critical to cell growth, immune response and signal transduction. The functional category and pathway analyses of the microarray data revealed that four canonical pathways (the cell cycle G2/M DNA damage checkpoint and G1/S checkpoint regulation; pyrimidine metabolism; and purine metabolism) were significantly perturbed.

The significance calculated for each canonical pathway is a measurement of the likelihood that the pathway is associated with the dysregulated genes by random chance. Interestingly, four pathways (the cell cycle G2/M DNA damage checkpoint and G1/S checkpoint regulation; pyrimidine metabolism; and purine metabolism) showed significant regulation by butyrate. Cell cycle checkpoint pathways (G2/M DNA damage checkpoint and G1/S checkpoint regulation) are impaired due to the treatment with butyrate. For the first time, two pathways critical for the regulation of purine and pyrimidine metabolism were linked to the induced biological effects of butyrate. These findings underline mechanisms of butyrate induced cell arrest in the progression of the cell cycle.

Insulin-like growth factor binding proteins (IGFBPs) modulate IGF action and regulate cell growth and apoptosis by preventing IGF from interacting with their own receptors. In this study, insulin-like growth factor (IGF2) was upregulated by butyrate, which is consistent with other published data [10]. Microarray and real-time RT-PCR results confirmed that IGFBP6 was downregulated by butyrate. IGFBP-6 has a 100-fold higher affinity for IGF2 than IGF1 [11]. It seems paradoxical that while IGF2 is upregulated significantly by butyrate, its highest affinity binding protein is downregulated. These results may suggest different functions for various IGFBP members in regulating apoptosis and cell cycle progression. It would be intriguing to see how IGFBPs exert their actions in cell growth and apoptosis via an IGF-independent fashion. In addition, extensive repression of cyclin-dependent kinases as well as cell cycle related genes, such as CDC2/CDK1, CDC20, CDC25A, CCNG1, CCNB1, CCNB2, CCNA2, CCNG1, and PCNA, may be closely associated with the cell growth arrest induced by butyrate. They are consistent with the growing body of evidence suggesting that disruption of the coordinate between regulation of DNA synthesis and cyclin-dependent kinase activity is an important feature of apoptosis.

The results also showed that TGFBI was significantly upregulated. Studies have shown that TGFBI regulates anterior pituitary cell proliferation and hormone secretion. There is also a report that indicates that butyrate and TGFBI inhibit pituitary cell proliferation and regulate the expression of 7B2, PC1, and PC2 in a cell culture model of pituitary tumours [12]. However, to our best knowledge, there is no previously reported relation between butyrate and the expression of TGFBI. TGFBI is a multifunctional peptide that controls proliferation, differentiation, and other functions in many cell types. TGFBI acts synergistically with TGFA in inducing transformation. It also acts as a negative autocrine growth factor. We speculate that the upregulated expression of both IGF2 and TGFBI may have a synergistic effect in inducing cell apoptosis.
CONCLUSION

The present findings provide an example of the epigenetic regulation of the genome at work and a basis for understanding the full range of the biological roles and molecular mechanisms that butyrate may play in human and animal cell growth, proliferation, and energy metabolism. The results illustrate the potential for the nutritional manipulation of gene activity for the purpose of enhancing human health, such as in cancer prevention. This study also generated comprehensive information on the experimental system, which can be used in many functional genomic studies in bovines and provided a basis for understanding the full range of the biological roles and molecular mechanisms of butyrate in animal cell growth, proliferation, and energy metabolism. The results illustrate the potential for exploiting the nutritional manipulation of gene activity to enhance animal production efficiency in a drug residue-free format.

REFERENCES


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