

Influence of *miRNA-106b* and *miRNA-135a* on butyrate-regulated expression of *p21* and *Cyclin D2* in human colon adenoma cells

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Abstract Epigenetic and posttranslational modifications of the expression of cell cycle-relevant genes or proteins like *p21*, e.g., by miRNAs are crucial mechanisms in the development or prevention of colon cancer. The present study investigated the influence of butyrate and trichostatin A (TSA) as histone deacetylase inhibitors on the expression of colon cancer-relevant miRNA (miR-135a, miR-135b, miR-24, miR-106b, miR-let-7a) in LT97 colon adenoma cells as a model of an early stage of colon carcinogenesis. The impact of distinct miRNAs (miR-106b, miR-135a) on butyrate-mediated regulation of *p21* and *Cyclin D2* gene and protein expression as well as the effect on LT97 cell proliferation (non-transfected, miR-106b and miR-135a mimic transfected) was analyzed. Butyrate and partial TSA reduced the expression of miR-135a, miR-135b, miR-24 and miR-let-7a (~0.5-fold, 24 h) and miR-24, miR-106b and miR-let-7a (~0.5–0.7-fold, 48 h) in LT97 cells. Levels of *p21* mRNA and protein were significantly increased by butyrate and TSA (~threefold and 4.5-fold, respectively, 24 h) in non-transfected but not in miR-106b transfected LT97 cells. Levels of *Cyclin D2* mRNA were significantly reduced by butyrate and TSA (~0.3-fold, 24 h) in non-transfected and miR-135a-transfected LT97 cells, whereas protein levels were predominantly not

influenced. MiR-106b and miR-135a significantly reduced butyrate-/TSA-mediated inhibition of LT97 cell proliferation (72 h). These results indicate that butyrate is able to modify colon cancer-relevant miRNAs like miR-106b and miR-135a which are involved in the regulation of cell cycle-relevant genes like *p21* and might influence inhibition of adenoma cell proliferation.

Keywords Cell cycle · Colon cancer · Histone deacetylase inhibitor · Epigenetic · LT97 cells

Introduction

Colon cancer is the second and third most common form of cancer in woman and men, respectively, representing one of the most frequent causes of all cancer deaths worldwide (Ferlay et al. 2015). Age and genetic predisposition are inevitable risk factors for developing colorectal cancer (CRC). The influence of genetic factors for this disease may be considered as low. It is estimated that 15–30 % of CRC cases exhibit a hereditary influence (Taylor et al. 2010). The familial adenomatous polyposis (FAP), for example, is caused by a mutation in the adenomatous polyposis coli (APC) tumor suppressor gene, whereas a mutation in one of the DNA mismatch repair genes is related to hereditary non-polyposis colorectal cancer (HNPCC, Lynch syndrome) (Rustgi 2007). These hereditary CRC forms as well as closely related variants comprise <5 % of all cancer cases (Fearon 2011; Taylor et al. 2010). In contrast, most cases of CRC are sporadic (75 %) and without identifiable genetic etiology. This form of CRC is mainly influenced by lifestyle factors such as high red meat consumption, high-fat low-fiber diet, alcohol and tobacco

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consumption, obesity as well as lack of physical activity (Durko and Malecka-Panas 2014). Sporadic CRC is also caused by acquired sequential genetic and/or epigenetic alterations in tumor suppressing as well as promoting genes known as the adenoma–carcinoma sequence described by Fearon and Vogelstein (1990). Chromosomal instability, microsatellite instability and CpG island methylator phenotype have been described as three major genetic mechanisms leading to transformed colon epithelial cells (Mundade et al. 2014). These transformed cells are characterized by an enhanced cell proliferation as well as a reduced differentiation and apoptosis (Hanahan and Weinberg 2011). Normally, cell proliferation is controlled by cell cycle regulatory genes and proteins like *p21* and cyclins or cyclin-dependent kinases (CDKs). The expression of these genes can also be regulated on the posttranscriptional level by microRNAs (miRNAs), non-coding small RNA molecules, which function as endogenous repressors of gene expression. Depending on their downstream target genes, miRNAs can exert a function as tumor suppressor or promoter. The expression of miRNAs has also been identified to be dysregulated in colon cancer (Bartley et al. 2011; Schetter et al. 2008; Wu et al. 2011). Another epigenetic mechanism of gene regulation is the modification of histones like (de)acetylation or (de)methylation which also plays a crucial role in colon cancer development (van Engeland et al. 2011).

There is evidence that the consumption of dietary fiber may protect against CRC formation (Aune et al. 2011). Butyrate, a short-chain fatty acid which is formed during bacterial fermentation processes in the colon, may be responsible for the CRC protective effects of dietary fiber. Butyrate is an energy source for normal colon epithelial cells. In contrast, it could be shown that butyrate functions as histone deacetylase inhibitor (HDI), inhibits proliferation and induces apoptosis and differentiation in colon cancer cells mediated, e.g., by induction of *p21* gene expression (Borowicki et al. 2010; Hinnebusch et al. 2002). There is evidence from two studies that these chemopreventive effects of butyrate are mediated also by miRNAs since butyrate as HDI also regulates miRNA expression profiles in colon cancer cells (Hu et al. 2011; Humphreys et al. 2013). Until now, the role of miRNAs in butyrate-mediated chemopreventive effects on colon adenoma cells at an early stage of colon cancer development has not been investigated yet. Therefore, the present study examines the impact of butyrate on the miRNA expression profile and the role of specific miRNAs (miRNA-106b, miRNA-135a) in butyrate-mediated induction of *p21* and *Cyclin D2* gene and protein expression as well as on antiproliferative effects in LT97 colon adenoma cells.

Methods

Cell culture

The human colon adenoma cell line LT97 (kind gift from Professor B. Marian, Institute for Cancer Research, University of Vienna, Austria) was used for cell culture experiments. LT97 cells were established from a colon adenoma representing an early stage of development of colon tumors (Richter et al. 2002). The origin, properties and culture conditions of the cell line are described by Klenow et al. (2009). The cell line has recently been authenticated by STR (short tandem repeat) profiling (March 2015) by the Leibniz-Institute DSMZ (German Collection of Microorganisms and Cell Cultures) GmbH. Cells from passages 6–28 were used for cell culture experiments. LT97 cells were seeded into 6-well plates and grown to a confluence of 30–50 % prior to incubation with physiological concentrations of butyrate (2, 4, 10 mM) or 3.3 μ M trichostatin A (TSA), which was used as a HDI-positive control, for 24 and 48 h (Kiefer et al. 2006).

Determination of miRNA expression

For quantification of *miR-135a*, *miR-135b*, *miR-24*, *miR-106b* and *miR-let-7a* expression in butyrate-treated non-transfected as well as transfected cells (*mir-135a*, *mir-106b*), the miScript SYBR Green PCR Kit (Qiagen GmbH, Hilden, Germany) and miScript Primer Assay (Qiagen GmbH, Hilden, Germany) were used according to the manufacturer's instructions. The qPCR experiments were performed using the iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories, Munich, Germany). All reactions were performed using two technical replicates. The expression of miRNAs was normalized to the geometric mean of the two reference genes *RNU6B* and *SnoRD48* based on the equation of Pfaffl et al. (2002). Statistical differences were calculated from three independent experiments.

Transfection of LT97 cells with miRNA mimics

LT97 colon adenoma cells were transfected with miRNA mimics miRNA-106b and miRNA 135a (Syn-hsa-miR-106b-5p miScript miRNA Mimic; Syn-hsa-miR-135a-5p miScript miRNA Mimic, Qiagen GmbH, Germany) using the Saint Red transfection reagent (Synvolux Therapeutics, Netherlands) according to the manufacturer's instructions. Therefore, LT97 cells were seeded into 6-well or 96-well plates and grown to a confluence of 30–50 % prior to transfection. AllStar negative control siRNA and AllStar positive control miRNA-1 (Qiagen GmbH, Germany) were

used as controls in the transfection experiments. Transfection was evaluated in a qualitative manner via fluorescence microscopy (Axiolab, Carl Zeiss AG, Germany) using an AlexaFluor 488-labeled AllStar negative control siRNA (Qiagen GmbH, Germany), and quantification was possible via qPCR using specific primers for miR-135a and miR-106b (Supplementary Figure S2) as well as for the transfection control miRNA-1 (data not shown). Cell culture experiments and incubation with 2, 4 and 10 mM butyrate as well as 3.3 μ M TSA were performed 24 h after transfection.

Isolation of total RNA

RNA was isolated after incubation of non-transfected as well as transfected LT97 cells with 2, 4 and 10 mM butyrate as well as 3.3 μ M TSA for 24 and 48 h. Isolation of total RNA from LT97 cells was performed using the miRNeasy Plus Mini Kit (Qiagen GmbH, Germany) according to the manufacturer's instructions. RNA was eluted in 50 μ l RNase-free water and stored at -80°C . The RNA concentration was measured at 260 nm absorbance with a NanoDrop ND-1000 photometer (NanoDrop Technologies, Wilmington, USA). Furthermore, the RNA integrity was determined using the Agilent RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). Only RNA samples with a RIN (RNA integrity number) >9 were used for further experiments.

cDNA synthesis

Synthesis of complementary DNA was performed using the miScript II RT Kit (Qiagen GmbH, Germany) and the miScript HiFlex Buffer, which allows the detection of miRNA as well as mRNA, according to the manufacturer's instructions. Reverse transcription of 1 μ g total RNA was conducted in a 20 μ l reaction mix for 60 min at 37°C following 5 min at 95°C . The cDNA was diluted 1:50 with RNase-free water for further experiments.

Determination of p21 and Cyclin D2 mRNA expression

Expression of *p21* and *Cyclin D2* mRNA levels was measured using qPCR. The efficiency of qPCR (95–105 %) as well as the optimal annealing temperatures of the specific primers was determined in previous experiments. The qPCR experiments were performed in a 25- μ l reaction mix using the iCycler iQ Real-Time PCR Detection System and iQ SYBR Green Supermix (Bio-Rad Laboratory, Munich, Germany), RNase-free water and 10 pmol of gene-specific primers: *p21* forward 5'-CACTGTCTTGTA

CCCTTGTG-3' and reverse 5'-CTTCCTCTTGGAGAA-GAT-3', *Cyclin D2* forward 5'-CCACCGACTTTAAGTTTGCC-3' and reverse 5'-CTTTGAGACAATCCACGCTG-3', β -actin forward 5'-AGAGCCTCGCCTTTGCCGAT-3' and reverse 5'-CCCACGATGGAGGGGAAGAC-3', *GAPDH* forward 5'-ACCCACTCCTCCACCTTTGAC-3' and reverse 5'-TCCACCACCCTGTTGCTGTAG-3'. The qPCR profile included an initial denaturation step of 2 min at 95°C followed by 40 cycles of denaturation (30 s at 94°C), annealing (30 s at 60°C) and extension (30 s at 72°C) as well as a subsequent melting curve analysis. All reactions were performed using two technical replicates, and results were calculated from three independent experiments. The expression of *p21* and *Cyclin D2* was normalized to the geometric mean of the two reference genes β -actin and *GAPDH* based on the equation of Pfaffl et al. (2002). 106b- and 135a-mimic-transfected cells treated with medium served as treatment control for *p21* and *Cyclin D2* quantification in transfected cells.

Determination of p21 and Cyclin D2 protein expression

Protein expression of p21 and Cyclin D2 was measured via SDS-PAGE and Western blot analysis. Therefore, butyrate-treated LT97 cells were homogenized in lysis buffer (10 mM Tris/HCl pH 8.0, 75 mM NaCl, 5 % glycerin, 1 mM EDTA, 1 mM DDT, 1 mM sodiumorthovanadate, 1 % Nonidet P40) including a protease inhibitor mix (Protease Inhibitor Roche Complete Mini; Roche Diagnostics Deutschland GmbH, Mannheim, Germany) according to the manufacturer's instructions. Homogenates were incubated on ice for 10 min, and after centrifugation (20 min, $16.000\times g$, 4°C), total protein concentrations were measured using the method of Bradford (1976). For SDS-PAGE, 20 μ g protein was reconstituted in 4 x concentrated loading buffer (0.25 M Tris pH 6.8, 8 % SDS, 40 % glycerol, 20 % 2 β -mercaptoethanol, 0.01 % orange G) for 5 min at 95°C . SDS-PAGE was performed using a 5 % stacking gel (0.125 M Tris/HCl pH 8.8, 6 % acrylamide, 0.1 % SDS, 0.1 % ammonium persulfate, 0.1 % N,N,N',N'-tetramethylethylenediamine, TEMED) and 12 % separation gel (0.375 M Tris/HCl pH 6.8, 12 % acrylamide, 0.1 % SDS, 0.1 % APS und 0.04 % TEMED) for 1.5 h at 100 V in electrophoresis buffer (50 mM Tris/HCl pH 8.5, 0.38 M glycine, 0.1 % SDS). Proteins were blotted on a nitrocellulose membrane (Whatman, Florham Park, NJ) using the Bio-Rad Trans Blot Turbo System (Bio-Rad Laboratories, Munich, Germany). The membrane was treated with Odyssey Blocking Buffer (1:1 in PBS, LICOR Biosciences GmbH, Bad Homburg, Germany) for 1 h and incubated for additional 1 h with the following primary and secondary antibodies, respectively: mouse-anti-p21

(1:500), rabbit-anti-GAPDH (1:2500), mouse-anti-GAPDH (1:1000) (Abcam, Cambridge, UK), rabbit-anti-Cyclin D2 (1:1000) (New England Biolabs, UK), IRDye 800 CW donkey anti-mouse IgG, IRDye 680 LT goat anti-rabbit IgG, IRDye 680 CW donkey anti-mouse IgG, IRDye 800 LT goat anti-rabbit IgG (1:15000) (LI-COR Biosciences GmbH, Bad Homburg). Proteins were visualized and quantified using the Odyssey System (LI-COR Biosciences GmbH, Bad Homburg, Germany). Results are expressed as fold changes in relation to the medium control and the mimic-transfected medium control, respectively.

Determination of cell proliferation

The 4',6-diamidino-2-phenylindol (DAPI) assay was used to examine the time- and dose-dependent effects of butyrate on the proliferation of LT97 adenoma cells. LT97 cells were seeded into 96-well plates and were grown to a confluence of 20–30 % before incubation with 2, 4 and 10 mM butyrate and 3.3 μ M TSA for additional 24, 48 and 72 h. The relative cell number on the basis of a medium control (100 %) was determined using the DAPI assay as described previously (Schlörmann et al. 2012). Determination of cell proliferation of transfected LT97 cells was carried out 24 h after transfection and incubation with the test substances for 24, 48 and 72 h. Results were calculated in comparison with the medium control and the mimic-transfected medium control, respectively.

Statistical analysis

Means and standard deviations (SD) were calculated from at least three independent experiments. Differences in inhibition of cell proliferation were calculated with one-way ANOVA, including Bonferroni posttest with selected pairs, using GraphPad Prism 5.01 (GraphPad Software, Inc. San Diego, CA, USA). Otherwise, comparisons of two groups were done with Student's *t* test (unpaired).

Results

Determination of miRNA expression

The expression levels of *miR-135a*, *miR-135b*, *miR-24*, *miR-106b* and *miR-let-7a* in butyrate and TSA-treated LT97 colon adenoma cells were measured using qPCR. Butyrate and TSA were able to significantly reduce the miRNA expression of *miR-135a* (2, 4, 10 mM butyrate, TSA), *miR-135b* (10 mM butyrate), *miR-24* (4 mM butyrate) and *miR-106b* (4 mM butyrate, TSA) after 24-h incubation. The overall reduction in miR-expression was 0.5-fold (Fig. 1a). After 48-h incubation (Fig. 1b), butyrate

led to a reduction in *miR-24* expression (4 mM, 0.5-fold), *miR-106b* (2, 4, 10 mM butyrate, TSA; \sim 0.6-fold) and *miR-let-7a* (2, 4, 10 mM butyrate; \sim 0.7-fold). Similar results could be obtained for HT29 cells (Supplementary Figure S1). Additionally, the expression of *miR-106b* and *miR-135a* was determined in the respective transfected and butyrate-/TSA-treated LT97 cells. Overall expression of *miR-135a* was 48-fold and 46-fold increased after 24-h and 48-h incubation, respectively, in *miRNA 135a* mimic-transfected cells. Overall levels of *miR-106b* expression in *miRNA-106b* mimic-transfected cells were 50-fold and 55-fold elevated after 24 and 48 h, respectively. An influence of butyrate or TSA on the expression of the distinct miRNAs in transfected cells could not be observed (Supplementary Figure S2).

Determination of p21 and Cyclin D2 mRNA expression

The mRNA expression of *p21* was significantly induced in non-transfected LT97 cells upon treatment with 2, 4 and 10 mM butyrate (up to 2.9-fold) as well as TSA for

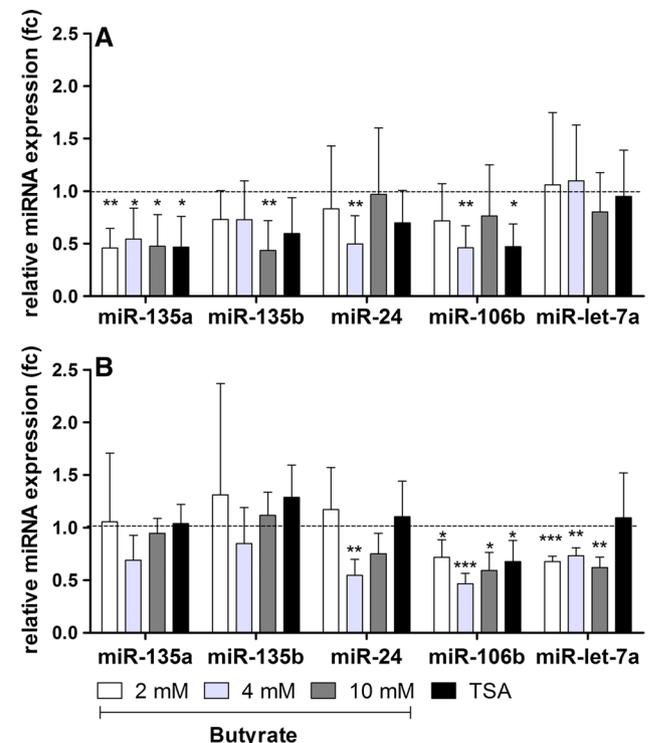


Fig. 1 Expression of miRNAs in LT97 cells after 24-h (a) and 48-h (b) incubation with 2, 4, 10 mM butyrate and 3.3 μ M TSA (trichostatin A). Results represent fold changes (fc) on the basis of a medium control (MC = 1; dashed line). Significant differences *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$ to MC; Student's *t* test (mean + SD, $n = 3$ independent experiments with 2 technical replicates, respectively)

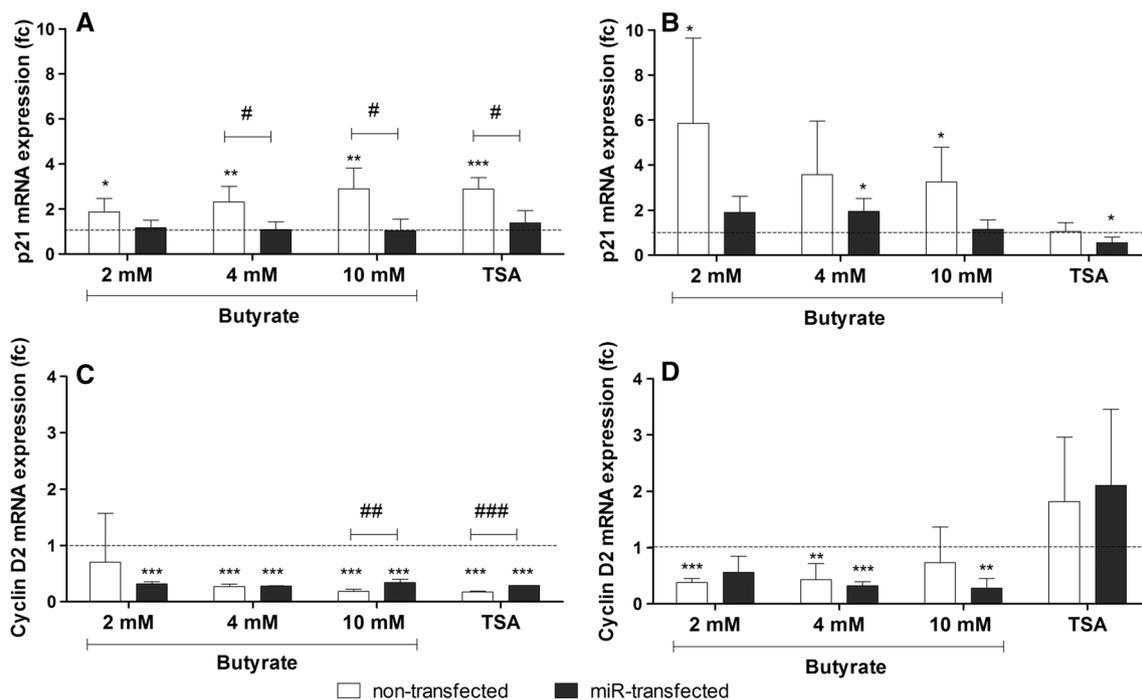


Fig. 2 Expression of *p21* mRNA in non-transfected and miR-106b mimic-transfected LT97 cells after 24 h (a) and 48 h (b) and *Cyclin D2* mRNA expression in non-transfected and miR-135a mimic-transfected LT97 cells after 24-h (c) and 48-h (d) incubation with 2, 4, 10 mM butyrate and 3.3 μ M TSA (trichostatin A). Results represent

fold changes (fc) on the basis of a medium control (MC = 1; dashed line). Significant differences *** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$ to MC; ### $P \leq 0.001$; ## $P \leq 0.01$, # $P \leq 0.05$ between transfected and non-transfected cells; Student's *t* test (mean + SD, $n = 3$ independent experiments with 2 technical replicates, respectively)

24 h (Fig. 2a) compared to the medium control. In miR-106b-transfected cells, *p21* mRNA levels were not increased by butyrate treatment. Furthermore, they were significantly lower compared to *p21* mRNA levels observed in non-transfected LT97 cells. After 48-h incubation (Fig. 2b), 2 mM and 10 mM butyrate were able to significantly increase *p21* mRNA levels in non-transfected cells, and 4 mM butyrate at least tended to induce *p21* mRNA levels ($P = 0.07$). In miR-106b-transfected cells also 4 mM butyrate significantly increased *p21* mRNA levels. TSA led to a significant reduction in *p21* mRNA in transfected cells.

Cyclin D2 mRNA levels were significantly reduced upon butyrate and TSA treatment for 24 h both in non-transfected (0.2-fold; except 2 mM butyrate) and also in miR-135a-transfected LT97 cells (0.3-fold; Fig. 2c). Furthermore, 10 mM butyrate and TSA led to significantly lower *Cyclin D2* mRNA levels in non-transfected compared to transfected cells. After 48 h (Fig. 2d), the incubation with 2 and 4 mM butyrate led to a significant reduction (0.4-fold) in *Cyclin D2* mRNA levels in non-transfected cells, whereas 4 and 10 mM butyrate significantly reduced *Cyclin D2* mRNA expression in transfected cells (0.3-fold).

Determination of p21 and Cyclin D2 protein expression

Protein levels of p21 were significantly increased in non-transfected cells after incubation with 2 and 4 butyrate (4.1-fold) as well as TSA (5.0-fold), but were reduced by 10 mM butyrate (0.4-fold) after 24 h (Fig. 3a). The expression of p21 was not affected in miR 106b-mimic-transfected LT97 cells after butyrate incubation, but was significantly increased by TSA (1.7-fold). The protein expression of p21 was significantly lower in transfected cells compared to non-transfected cells (2 and 4 mM butyrate, TSA). In general, levels of p21 protein were lower after 48 h (Fig. 3b) compared to the incubation for 24 h. A significant increase in p21 protein levels could be observed after 48-h incubation with 2 mM butyrate in non-transfected LT97 cells compared to the medium control and also to the transfected cells. In contrast, 10 mM butyrate and TSA significantly decreased p21 protein levels on non-transfected cells.

Cyclin D2 protein expression in non-transfected as well as miR-135a-transfected LT97 cells was unaffected by incubation with butyrate and TSA (24 h; Fig. 3c). After 48 h of incubation, 10 mM butyrate significantly decreased

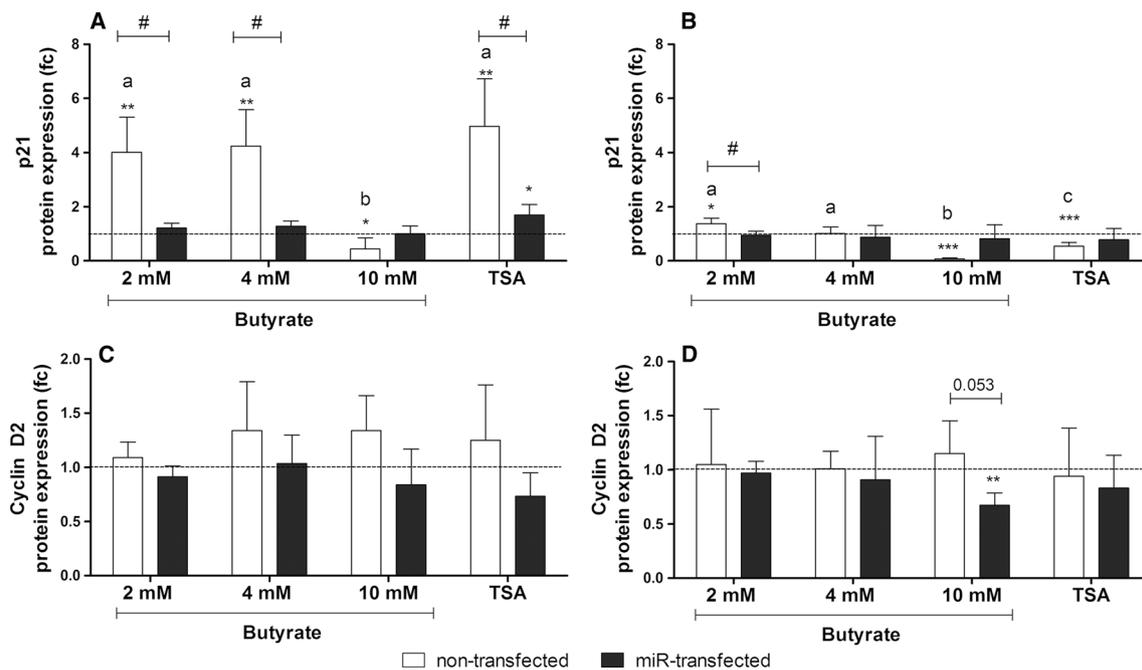


Fig. 3 Expression of p21 protein in non-transfected and miR-106b mimic-transfected LT97 cells after 24 h (a) and 48 h (b) and Cyclin D2 protein expression in non-transfected and miR-135a mimic-transfected LT97 cells after 24-h (c) and 48-h (d) incubation with 2, 4, 10 mM butyrate and 3.3 μ M TSA (trichostatin A). Results represent

fold changes (fc) on the basis of a medium control (MC = 1; dashed line). Significant differences $***P \leq 0.001$, $**P \leq 0.01$; $*P \leq 0.05$ to MC; $^{\#}P \leq 0.05$ between transfected and non-transfected cells; $^{a,b,c}P \leq 0.05$ between concentrations (represented by different letters); Student's *t* test (mean + SD, $n = 3$ independent experiments)

Cyclin D2 protein levels in transfected cells (Fig. 3d). Compared to non-transfected cells, the decrease was not significant, but a trend could be observed ($P = 0.053$). The results for p21 and Cyclin D2 protein expression are also

reflected by representative Western blots shown in Fig. 4a, b.

Determination of cell proliferation

Cell proliferation analysis revealed that all butyrate concentrations as well as TSA were able to modulate the adenoma cell proliferation in a time- and dose-dependent manner (Fig. 5). Transfection of cells also resulted in a reduction in cell proliferation (data not shown). Therefore, the results obtained for transfected cells were calculated in comparison with the respective medium control. Treatment of cells for 24 h (Fig. 5a) with butyrate and TSA already resulted in reduced cell numbers of non-transfected as well as miR-106b-/miR-135a-transfected cells (down to ~59–46 %) compared to the medium control.

The strongest inhibitory effects on LT97 cell proliferation were observed after 48- and 72-h (Fig. 5b, c) treatment with butyrate and TSA (down to 27–9 % and 18–7 %, respectively). No significant differences between non-transfected and transfected cells regarding proliferation inhibitory effects of butyrate and TSA could be measured after 24 and 48 h. In contrast, after 72 h, inhibition of proliferation of miR-135a-transfected cells was significantly lower compared to non-transfected cells after incubation with 4 and 10 mM butyrate as well as TSA. Inhibition of proliferation of miR-106b-transfected LT97

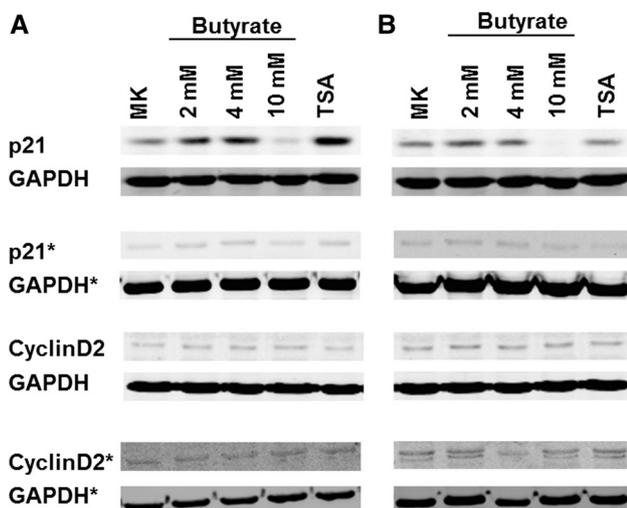


Fig. 4 Representative Western blots of p21 protein levels in non-transfected and miR-106b mimic-transfected LT97 cells (p21*, GAPDH*) and Cyclin D2 protein levels in non-transfected and miR-135a mimic-transfected LT97 cells (CyclinD2*, GAPDH*) after 24-h (a) and 48-h (b) incubation with 2, 4, 10 mM butyrate and 3.3 μ M TSA (trichostatin A) in relation to GAPDH protein levels, respectively

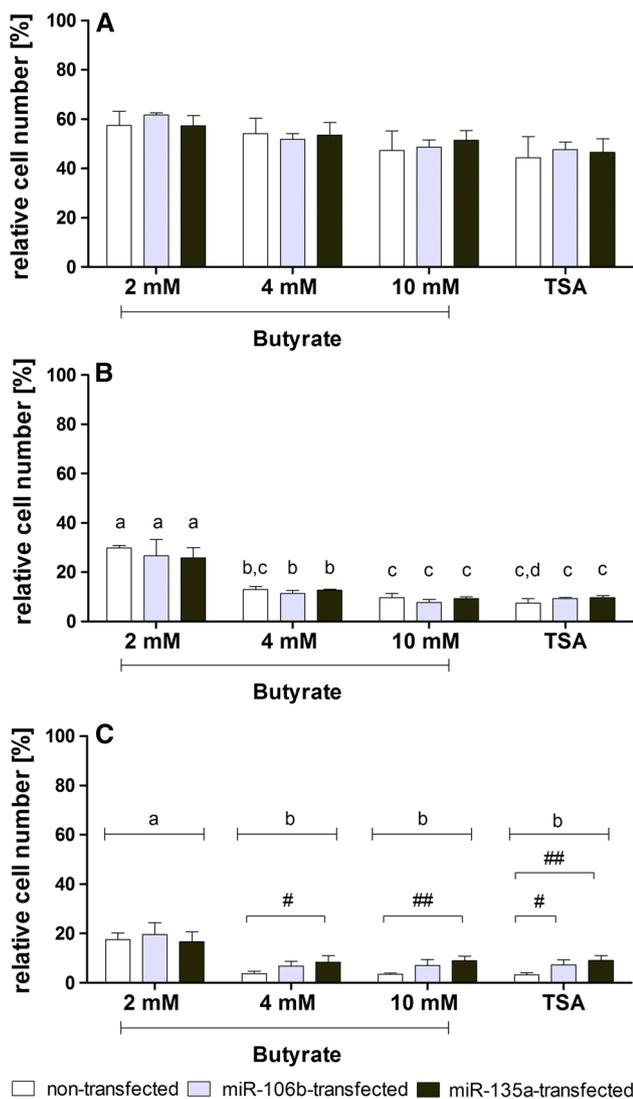


Fig. 5 Cell proliferation (relative cell number) of non-transfected and miR-106b mimic or miR-135a mimic-transfected LT97 cells after 24-h (a), 48-h (b) and 72-h (c) incubation with 2, 4, 10 mM butyrate and 3.3 μ M TSA (trichostatin A). Results represent fold changes (fc) on the basis of a medium control (MC = 100 %). All results were significantly different compared to MC ($P \leq 0.001$). Significant differences: # $P \leq 0.05$, ## $P \leq 0.01$ between transfected and non-transfected and ^{a,b,c,d} $P \leq 0.05$ between different concentrations of butyrate and TSA (represented by *different letters*); Student's *t* test (mean + SD, $n = 3$ independent experiments with 6 technical replicates, respectively)

cells by TSA was also significantly lower compared to non-transfected cells.

Discussion

Colon cancer is mainly a lifestyle-associated disease, which is predominantly influenced by nutrition (Durko and Malecka-Panas 2014). The consumption of dietary fiber is

associated with a lower risk of CRC development (Aune et al. 2011; Bingham et al. 2003). In particular, the short-chain fatty acid butyrate, which is formed during bacterial fermentation of dietary fiber in the colon, has chemopreventive effects on colon cancer cells. Several studies showed that butyrate functions as a HDI, reduces proliferation and induces apoptosis as well as differentiation. Butyrate regulates the expression of genes and proteins involved in cell cycle control like p21 and Cyclin D1, leading to a cell cycle arrest in human colon cancer cells (Hamer et al. 2008; Hinnebusch et al. 2002; Siavoshian et al. 2000). Lately, Hu et al. (2011) as well as Humphreys et al. (2013) showed that butyrate also influences miRNA expression in HCT116 and HT29 colon cancer cells, respectively. Therefore, it can act both via its function as HDI and by regulation of miRNA which are dysregulated in CRC (Bartley et al. 2011; Schetter et al. 2008; Slattery 2000; Wu et al. 2011). In colon tumors for example, miR-let-7, miR-24, miR-34, miR-125 and miR-143 are down-regulated, whereas miR-21, miR-135 and miR-106b are up-regulated (Faber et al. 2009; Wu et al. 2011). The deregulation of these miRNAs may influence the development of colon cancer. Therefore, in the present study we investigated for the first time the influence of butyrate on the expression of proliferation and cell cycle-relevant miRNAs (miR-135a, miR-135b, miR-24, miR-106b, miR-let-7a) in LT97 colon adenoma cells as an early stage of transformed cells. The HDI TSA was used as a positive control (Hu et al. 2011; Kiefer et al. 2006). All analyzed miRNAs were down-regulated to a certain degree by butyrate or TSA after 24- or 48-h incubation, respectively. Studies revealed that miR-135a and miR-135b exhibit an oncogenic potential, by inhibiting expression of APCs (Nagel et al. 2008). Recently, Valeri et al. (2014) demonstrated that miR-135b promotes cancer progression by acting as a downstream effector of oncogenic pathways in colon cancer. Therefore, a down-regulation may be associated with a reduced risk of CRC. In the present study, especially miR-135a was significantly reduced by butyrate and TSA after 24 h (~0.5-fold) in LT97 cells and partially also in HT29 cells (~0.5-fold, Supplementary Figure S1). But, LT97 colon adenoma cells lack the two alleles of APC (Richter et al. 2002). Therefore, APC cannot be considered as target gene of miR-135a in these cells.

In contrast, miR-24 is associated with a tumor-suppressing activity by reducing the expression of DHFR (dihydrofolate reductase) and inhibiting cell proliferation (Mishra et al. 2009). Mishra et al. (2009) showed that miR-24 is down-regulated in 45 % of the investigated colorectal cancer tumors. On the other hand, Bartley et al. (2011) demonstrated that miR-24 is up-regulated in neoplasm, dysplasia and adenocarcinoma tissue, indicating a differentiated role for this miRNA in the development of CRC.

In the present study, only 4 mM butyrate significantly down-regulated miR-24 expression in LT97 cells, whereas TSA or the other butyrate concentrations had no effect. Therefore, we conclude that histone deacetylation has no or only minor effects on miR-24 expression. Xie et al. (2013) also demonstrated that miR-24 expression is not altered by treatment with TSA or DAC (5-aza-20-deoxycytidine).

The expression of miR-106b was significantly reduced upon treatment with both HDIs butyrate and TSA, especially after 48 h. Several studies have shown that miR-106b is overexpressed in colon cancer cells and that it has oncogenic potential (Hu et al. 2011; Schetter et al. 2008; Wu et al. 2011). A validated target gene of miR-106b is the cell cycle regulator p21. MiR-106b binds to the 3'UTR and inhibits the translation of p21 (Hu et al. 2011). In the present study, butyrate and TSA were able to reduce miR-106b expression in LT97 colon adenoma cells, especially after 48-h treatment and in HT29 cells (Supplementary Figure S1). These results are in line with that of Hu et al. (2011) and Humphreys et al. (2013) who analyzed the impact of butyrate on miR-106b expression in HCT116 cells and also in HT29 cells, respectively, representing progressed stages of colon cancer development. In contrast, the present study indicates that HDIs like butyrate and TSA are also capable of altering miRNA expression in adenoma cells representing an early stage of colon cancer development. The miR-let-7a binds to kras-mRNA, resulting in its degradation and inhibition of the MAP kinase signaling cascade. Therefore, miR-let-7a could exhibit tumor growth-suppressing potential, as shown by Akao et al. (2006) for DLD-1 human colon cancer cells. In the present study miR-let-7a was significantly down-regulated after 48-h treatment with butyrate, whereas TSA showed no effect. A reduction in miR-let-7a might have negative effects on development of CRC. Caspase 3 (*CASP3*) is a validated target gene of miR-let-7a (Vergoulis et al. 2012) as an effector of apoptosis, too. Tsang and Kwok (2008) could demonstrate an inverse interaction between miR-let-7a and *CASP3* in HepG2 and A431 cells, and Medina et al. (1997) showed an induction of *CASP3* activity by butyrate and TSA in Jurkat cells and LIM 1215 colon cancer cells. In a former study, we could also confirm that butyrate induces apoptosis in LT97 cells by induction of *CASP3* activity (Schlörmann et al. 2012). Therefore, butyrate could induce apoptosis in LT97 colon adenoma cells via *CASP3* induction mediated by miR-let-7a. This could possibly compensate the detrimental effects described above.

The specific role of miR-106b and miR-135a regarding proliferation and cell cycle-relevant genes was further investigated in LT97 colon adenoma cells. Therefore, the expression of p21 and *Cyclin D2* as target gene of miR-106b (Hu et al. 2011) and predicted target of miRNA-135a (Schultz et al. 2008), respectively, was studied in mimic-

transfected as well as non-transfected LT97 cells. The mRNA as well as the protein expression of p21 was significantly enhanced upon 24-h treatment with 2, 4 and 10 mM butyrate and TSA, while miR-106b levels were significantly reduced upon treatment with 4 mM butyrate and TSA. Enhanced mRNA and protein levels due to butyrate treatment were also shown in former studies with HT29 or HCT116 (Hu et al. 2011; Humphreys et al. 2013; Siavoshian et al. 2000) as well as LT97 cells (Borowicki et al. 2010). After 48-h treatment of LT97 cells, the induction of p21 mRNA levels was less pronounced and not dose dependent compared to 24 h, mainly due to the relative high standard deviations. Also p21 protein expression was reduced after 48-h treatment of LT97 cells with 2, 4 and 10 mM butyrate compared to 24 h, though miR-106b levels were significantly reduced upon butyrate treatment at the respective concentration. These effects could be explained by a saturation of p21 induction and also by enhanced apoptotic processes as shown for example in Jurkat or LIM 1215 cells (Medina et al. 1997) as well as LT97 cells (Schlörmann et al. 2012). It could also be shown that p21 protein is cleaved by DEVD effector caspase resulting in a p15 fragment due to butyrate treatment in LIM 1215 cells (Chai et al. 2000). These effects could also be responsible for the significantly reduced p21 protein expression in LT97 cells treated with 10 mM butyrate. In contrast, p21 mRNA and protein levels were not influenced in miR-106b mimic-transfected cells, especially after 24-h treatment with butyrate or TSA. These results are in line with results obtained by Hu et al. (2011) who analyzed p21 mRNA and protein expression in butyrate-treated miR-106b-transfected HCT116 cells, representing a late stage of tumor development. In contrast to their results, we also found significantly reduced p21 mRNA levels in miR-106b-transfected compared to non-transfected LT97 adenoma cells even after treatment with butyrate and TSA. These results indicate that miR-106b is involved in the inhibition of butyrate-mediated induction of p21 protein levels in LT97 cells mainly due to degradation of p21 mRNA levels rather than inhibition of protein translation. Also these results might demonstrate a differential role for miR-106b in different stages of cancer progression or different types of cancer cells. Down-regulation of p21 mRNA levels due to miR-106b could also be confirmed by several studies using different cell types (Ivanovska et al. 2008; Kan et al. 2009; Zhao et al. 2012). The down-regulation of mRNA and protein levels of the CDK inhibitor p21 which normally induces cell cycle arrest could lead to an induction of downstream targets like Cyclins (A, D and E) (Hinnebusch et al. 2002; Ivanovska et al. 2008). This induction results in enhanced cell proliferation due to an increased transition of the G1 to the S phase and could promote the transformation of preneoplastic cells.

In the present study, we investigated for the first time the role of miR-135a as a predicted target of Cyclin D2. It could be shown that Cyclin D2 along with other Cyclins (D1, D3) is overexpressed in colon tumors and colon cancer cells like HT29 cells (Mermelshtein et al. 2005). Cole et al. (2010) could demonstrate that Cyclin D2 expression is up-regulated immediately after APC loss. LT97 cells are characterized by a loss of the two APC alleles (Richter et al. 2002), indicating an oncogenic potential for Cyclin D2 in LT97 cells. In the present study, the expression of Cyclin D2 mRNA in non-transfected and miR-135a-transfected LT97 cells was significantly reduced by butyrate and TSA, especially after 24-h treatment. This reduction was significantly lower in miR-135a-transfected cells compared to non-transfected cells. Cyclin D2 protein expression was marginally influenced by butyrate and TSA treatment or transfection with miR-135a. Only treatment with 10 mM butyrate (48 h) led to significantly reduced Cyclin D2 expression levels in miR-135a-transfected LT97 cells. Similar results could be obtained by Siavoshian et al. (2000) who showed that *Cyclin D1* mRNA was reduced upon butyrate treatment without effects on protein expression and Cyclin D3 protein expression increased without modified mRNA levels in HT29 cells. The authors explained this effect by a butyrate-mediated stabilization of these cyclins or induction of differentiation processes. Taken together, the results of the present study indicate that Cyclin D2 probably does not function as a target of miR-135a in LT97 colon adenoma cells. Further studies have to elucidate the influence of butyrate on the expression of cell cycle regulatory D Cyclins as potential targets of chemoprevention.

The inhibitory effects of butyrate on colon cancer cell proliferation have been demonstrated in several studies (Hinnebusch et al. 2002; Hu et al. 2011; Humphreys et al. 2013; Kautenburger et al. 2005). Hu et al. (2011) showed that the transfection of HCT116 colon cancer cells with miR-106b mimics resulted in reduced butyrate-mediated inhibition of cell proliferation after 24-h treatment. In contrast, in the present study, a significant reduction in inhibition of proliferation in miR-106b-transfected LT97 adenoma cells could be detected only after 72-h treatment with TSA. Additionally, transfection with miR-135a led to a significant reduction in butyrate-mediated inhibition of cell proliferation also after 72-h treatment. The differences between our study and that by Hu et al. might be explained by the sensitivity of LT97 cells toward butyrate due to the different stages of colon cancer development. Kautenburger et al. (2005), for example, could show that LT97 adenoma cells are more sensitive to butyrate treatment in comparison with HT29 colon cancer cells due to a higher butyrate uptake. The reduced butyrate uptake in HT29 cells is due to a down-regulation of the MCT-1 transporter (Cuff

et al. 2005). Therefore, the inhibitory effects of butyrate on LT97 cell proliferation might exceed the miR-mimic effects until 72-h treatment.

These results indicate that both miR-106b and miR-135a might be involved in regulation of butyrate-mediated inhibition of LT97 colon adenoma cell proliferation. According to our knowledge, this is the first study that demonstrates the ability of the gut fermentation product butyrate to modify miRNA expression profiles in colon adenoma cells at an early stage of colon carcinogenesis. The modification of miR-106b expression might contribute to the induction of cell cycle regulatory proteins like p21 as a mechanism of chemoprevention. The role of miR-135a in butyrate-mediated inhibition of LT97 cell proliferation and its impact on potential targets like Cyclin D1 have to be further investigated.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Compliance with ethics guidelines This article does not contain any studies with human or animal subjects performed by any of the authors.

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