

# Sodium butyrate induces differentiation of gastric cancer cells to intestinal cells via the PTEN/phosphoinositide 3-kinase pathway

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## Abstract

NaB (sodium butyrate) inhibits cell proliferation and induces differentiation in a variety of tumour cells. In this study, we aimed to determine whether NaB induced differentiation and regulated the expression of the mucosal factor MUC2 through the PTEN/PI3K (phosphoinositide 3-kinase) pathway. BGC823 cells treated with NaB for 24–72 h showed marked inhibition of cell proliferation and alteration in cellular morphology. NaB treatment markedly increased the expression of PTEN and MUC2, but it decreased the expression of PI3K. These effects were enhanced by intervention with PI3K inhibitors and were reduced by intervention with PTEN siRNA. Hence, we conclude that NaB increased PTEN expression, promoted the expression of MUC2 and induced the differentiation of gastric cancer cells through the PTEN/PI3K signalling pathway.

Keywords: differentiation; gastric cancer; MUC2; PTEN; PI3K; sodium butyrate

## 1. Introduction

Tumour formation can be attributed to abnormal cell differentiation (Markert, 1968). The genes impeded during tumour differentiation can undergo reversible changes under certain conditions (Felsher and Bishop, 1999). Agents that induce differentiation of malignant cells can also induce redifferentiation in these cells and restore their normal phenotype. Redifferentiation is a cell-transformation process and does not produce negative effects in normal cells (Altucci and Gronemeyer, 2001). Therefore, the induction of tumour differentiation can be employed as a useful biological treatment strategy.

Butyrate, a short chain fatty acid, is produced in the colon by the breakdown of dietary fibre (Hague et al., 1993). NaB (sodium butyrate) is a histone deacetylase inhibitor. NaB changes chromatin structure, regulates gene expression and influences cell growth and the expression of proteins and enzymes (Dashwood et al., 2006). It inhibits the growth of a variety of tumour cells and induces cell differentiation, maturation and cell cycle arrest; moreover, it induces tumour-cell apoptosis via mechanisms that remain to be clarified (Lea et al., 2007). Therefore, NaB has the potential for use in cancer therapy because it promotes cell differentiation and exhibits antitumour activity.

PTEN (phosphatase and tensin homologue deleted on chromosome 10) plays an important role in the promotion of intestinal cell differentiation (Li et al., 2004). Thus far, PTEN gene is the first reported tumour-suppressor gene with phosphatase activity. PTEN also induces growth suppression via cell cycle arrest or induction of apoptosis and inhibits cell adhesion and migration. PTEN is not only involved in the regulation of normal cell growth and development, but it also plays an important role in tumorigenesis, progression and metastasis (Tamura et al., 1998).

It was hypothesized that NaB promotes the differentiation of gastric cancer cells via the PTEN/PI3K pathway. The changes in the levels of few related proteins were determined, such as PTEN, PI3K (phosphoinositide 3-kinase), Akt and MUC2 (mucin 2), after NaB-induced differentiation of gastric cancer cells and the effect of PI3K inhibitors and PTEN siRNA (small interfering RNA) intervention on the expression of these proteins was evaluated. The effects of NaB on the proliferation and differentiation of human gastric cancer cells, determination of the underlying mechanisms of these processes and clarification whether NaB induced gastric cancer cell differentiation through PTEN, which regulates the expression of intestinal mucosal factors, were the aims of the study.

## 2. Materials and methods

### 2.1. Cell culture and treatments

BGC823, a poorly differentiated gastric adenocarcinoma cell line, was cultured in complete DMEM (Dulbecco's modified Eagle's medium) containing 10% fetal bovine serum, at 37°C with 5% CO<sub>2</sub> in air. NaB (Sigma), 2 and 5 mM (Augenlicht et al., 2003; Hatayama et al., 2007), and 30 nM Wortmannin (Sigma) were added to the wells. The cells were used for proliferation assay or were lysed directly in each well for total protein extraction, at 48 h after treatment.

### 2.2. Construction of plasmids

The CDS (coding sequence) region of human PTEN gene was cloned by PCR. Oligonucleotides with the following sequences were used as primers containing linker segments (underlined)

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**Abbreviations:** CDS, coding sequence; EGFP, enhanced green fluorescent protein; MUC2, mucin 2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NaB, sodium butyrate; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homologue deleted on chromosome 10; siRNA, small interfering RNA.

recognizable by 5'-XhoI and 3'-BamHI. 5'-CCGCTCGAGATGA-CAGCCATCATCAAAGA-3' (forward); 5'-CGGGATCCAGACTT-TTGTAAATTTGTGTATGC-3' (reverse). Subsequently, 1209-bp amplified fragments containing the human PTEN CDS were ligated into the XhoI and BamHI sites of pcDNA3.1.

### 2.3. Transfection for overexpression of PTEN

Transfections were performed with Lipofectamine 2000 (Invitrogen) as directed by the manufacturer. For stable transfection, the cells were transfected with PTEN expression vectors and selected after treatment with 400 µg/ml G418 for 28 days. Transient transfection was performed by a similar method. Phase-contrast microscopy was used to study the size, shape and changes in nuclear morphology of transfected cells.

### 2.4. Gene-silencing experiments

The small interfering RNA (siRNA) target sequence of human PTEN gene was 5'-AACAGTAGAGGAGCCGTCAAA-3' (Kawaguchi et al., 2006). The pSilencer 4.1-CMV neo siRNA expression vector (Ambion) with the inserted RNAi (RNA interference) cassette was transiently transfected into BGC823 cells using Lipofectamine 2000. Subsequently, the cells were incubated for 48 h, and the protein expression was analysed by Western blotting. The pSilencer 4.1-CMV neo negative control was obtained from Ambion.

### 2.5. Cell proliferation assays

The growth rate of the monolayer culture was determined by the previously described methods. In this assay, viable cells converted MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma] to a water-insoluble formazan. The cells were assayed at 0, 1, 2, 3, 4 and 5 days, and the corresponding absorbance values were determined on the Microplate Reader (Bio-Rad) at 570 nm.

### 2.6. Real-time PCR

The total RNA from tissues and cells was obtained using Trizol reagent (Invitrogen). The PCR primers for PTEN (5'-CGGCAGCATCAAATGTTTCAG-3' and 5'-AACTGGCAGGTAGAAGGCAACTC-3') were used as described elsewhere (Meng et al., 2007). Quantitative PCRs were performed using Applied Biosystems Sequence Detection System 7900 with SYBR GREEN PCR MasterMix (ABI). The mRNA copy numbers were determined by melting-curve analysis and by using the cycle threshold values.

### 2.7. Western blot analysis

Equal amounts of protein from different samples subjected to different treatments were electrophoresed on 7.5–12% SDS/polyacrylamide gel and electrotransferred on to PVDF membrane (Millipore). PVDF membranes were blocked for 2 h and subsequently incubated with primary antibodies [PTEN, Santa Cruz; PI3KCA, Abgent; Akt, pAkt, Cell Signaling Technology; MUC2

(Bolt and Mahoney, 1997; Song et al., 2005), Novocastra; GAPDH, KangChen] at 4°C overnight. The HRP (horseradish peroxidase)-conjugated antibodies against mouse or rabbit IgG were used as secondary antibodies. Peroxidase activity was visualized using the enhanced chemiluminescence kit (GE Healthcare). The membrane was stripped and reblotted with GAPDH to determine the result.

### 2.8. Statistical analysis

All data were analysed using SPSS11.0 software. Data are expressed as mean ± S.D. Student's *t* test was used to assess the differences between the controls and test groups. For all comparisons, differences with  $P < 0.05$  were considered statistically significant (two-tailed). Unless indicated, the results have been derived from three independent experiments with similar results. The bars indicate S.D.

## 3. Results

### 3.1. Effect of NaB on BGC823 cell differentiation

NaB-treated BGC823 cells showed morphological changes characteristic of differentiating cells. Further, NaB induced marked changes in cell morphology, such as elongation/stretching of the cells; while untreated cells appeared hexagonal, NaB-treated cells appeared elongated (Figure 1A). NaB increased MUC2 levels in a time-dependent manner (Figures 1B, 1C), which was indicative of differentiation. In addition, the growth of NaB-treated BGC823 cells was inhibited in a time-dependent manner (Figure 1D). These results suggested that BGC823 cells underwent differentiation after NaB treatment.

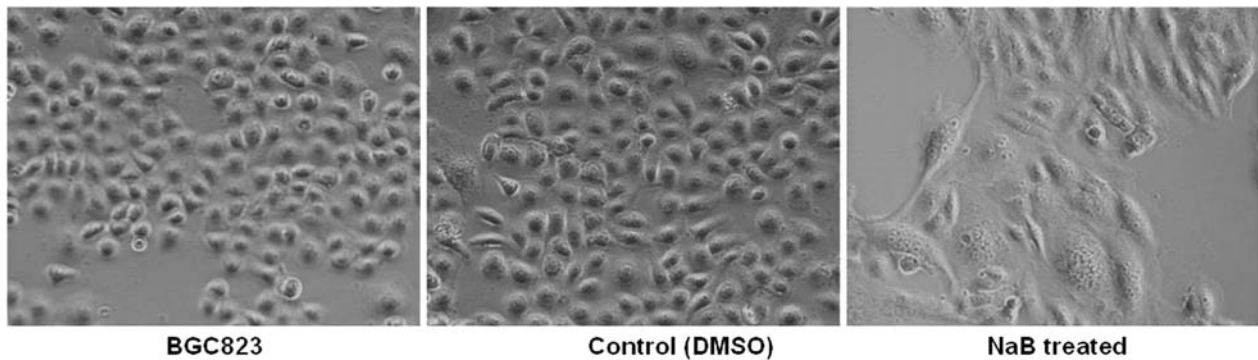
### 3.2. Inhibition of cell proliferation in gastric cancer due to overexpression of PTEN

The BGC823 cells showed exogenous PTEN expression after treatment with pEGFP (enhanced green fluorescent protein)-PTEN (Figures 2A, 2B). However, low levels of endogenous PTEN protein were detected by Western blotting in tumour cells, suggesting that NaB might compromise the expression of PTEN, which was similar to the tumour-suppressor effect of PTEN. Furthermore, MTT assay for cell viability, which was performed every day from day 1 to day 5, revealed that the proliferation of cancer cells treated with pEGFP-PTEN was significantly lower ( $P < 0.01$ ) than that of control cells treated with pEGFP or PBS (Figure 2C).

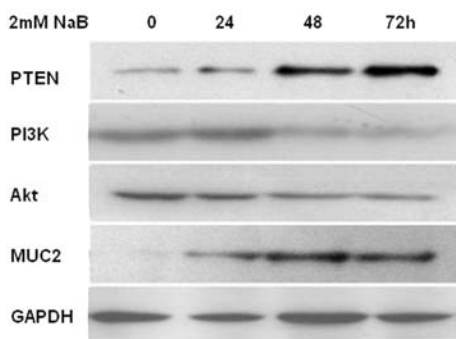
### 3.3. NaB increases PTEN expression in BGC823 cells

Western blotting revealed an increase in PTEN expression after NaB treatment (Figure 1B). MUC2 expression was increased, and PI3K level was decreased after NaB treatment in comparison with their levels in the internal control. These results suggest a general regulation of PTEN expression by NaB.

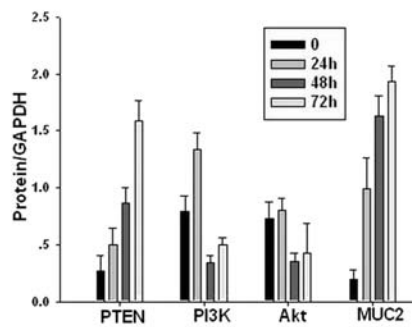
A



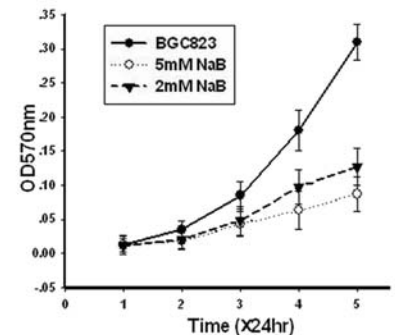
B



C



D



**Figure 1** Effects of NaB on gastric cancer cell differentiation

(A) Morphological change. (B) Increase in PTEN expression of BGC823 cells after treatment with NaB. After treatment of BGC823 cells with NaB for various time periods, total protein was extracted, and Western blotting was performed for analysing the levels of PTEN/PI3K and MUC2 proteins. (C) Densitometry results of the blots. (D) MTT array for changes in cell proliferation.

### 3.4. Inhibition of PTEN attenuates NaB-mediated MUC2 induction

First, the efficacy of PTEN siRNA inhibition was analysed. PTEN expression was decreased by 80% at 48 h after transfection with PTEN siRNA (Figure 3A). On the basis of these results, we then treated cells with NaB at 24 h after transfection with siRNA. In comparison with cells transfected with non-targeting scrambled control siRNA, BGC823 cells transfected with PTEN siRNA showed attenuated NaB-mediated MUC2 induction (Figure 3B). Immunoblotting analysis confirmed that the expression of PTEN was significantly inhibited for 48 h after transfection with PTEN siRNA. Increased MUC2 expression was noted in NaB-treated cells. Transfection of cells with non-targeting control siRNA had no effect on MUC2 expression. The data indicate that PTEN is an important regulator of MUC2 expression.

### 3.5. Inhibition of PI3K enhances NaB-mediated MUC2 induction

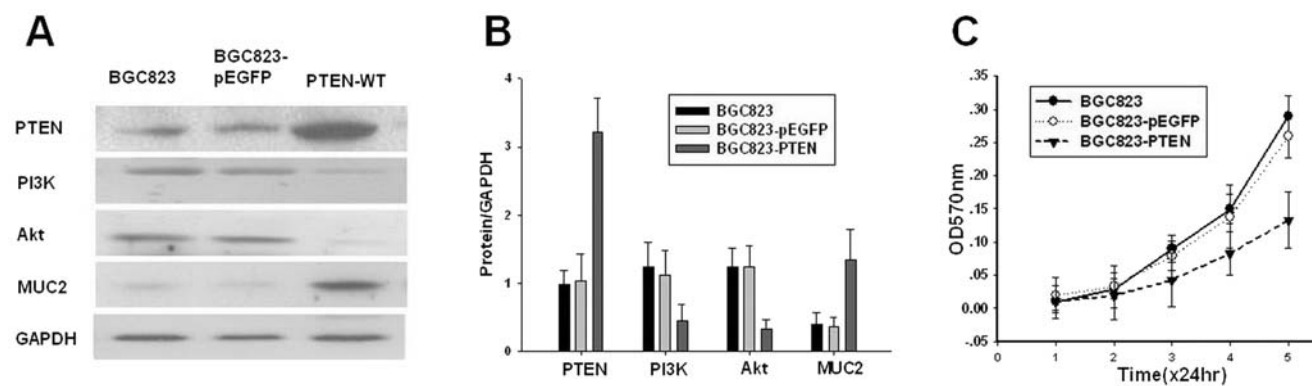
To investigate the possible regulatory effect of NaB on MUC2 expression, BGC823 cells were pretreated with a selective PI3K

inhibitor, Wortmannin, and subsequently treated with NaB for 48 h. As expected, NaB increased MUC2 protein expression, and this induction was remarkably enhanced by pretreatment with Wortmannin (Figure 3C). These results suggest that NaB-induced MUC2 expression requires PTEN activation.

## 4. Discussion

It has been established that increased histone acetylation plays an important role in the tumour cell differentiation (Mompalmer, 2003). Cao et al. (2001) have suggested that NaB, which is a histone deacetylase inhibitor, inhibited histone deacetylase activity and markedly increased histone acetylation. Consequently, it affected DNA replication and transcription and triggered a series of changes, including the regulation of the expression of some genes that mainly regulate the cell cycle and apoptosis-related genes, such as PTEN (Yi et al., 2005).

It was found that NaB significantly inhibits the proliferation of BGC823 cells in a time-dependant manner. During treatment with NaB, the shape of BGC823 cells was changing into multinuclear, and the cell morphology became irregular. After treatment, the cells



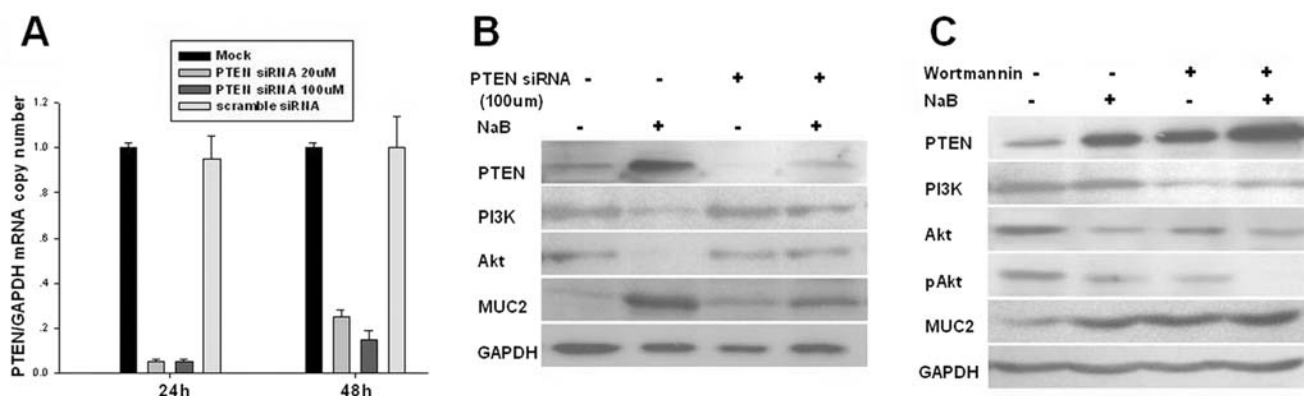
**Figure 2** Inhibition of cell proliferation in gastric cancer due to overexpression of PTEN (A) Western blot analysis of PTEN/PI3K and MUC2 proteins from gastric cancer cells overexpressing PTEN; (B) Densitometry analysis of the blots. (C) MTT array for proliferation change due to overexpression of PTEN.

exhibited high expression level of PTEN and MUC2 and low expression level of PI3K. It was shown that NaB increases MUC2 expression in gastric cancer cells. Further, NaB stimulated MUC2 production in the human colon cancer cell line LS174T (Hatayama et al., 2007). Whether elevated MUC2 expression contributed to the increase of PTEN expression was determined. The expression of MUC2 in BGC823 cells overexpressing PTEN was assessed. Overexpression of PTEN increased MUC2 expression. To confirm the role of PTEN in MUC2 regulation, BGC823 cells were transfected with non-targeting control siRNA or PTEN siRNA. Similar to the induction of MUC2 by overexpression of PTEN, transfection with PTEN siRNA almost blocked PTEN expression and attenuated NaB-mediated MUC2 induction, thereby suggesting that PTEN plays a role in MUC2 regulation in gastric cancer cells.

Since PTEN is a potential tumour suppressor of gastric cancer, and the inhibition of PI3K enhances NaB-mediated intestinal cell differentiation (Wang et al., 2001), we analysed the role of PTEN in NaB-induced differentiation of BGC823 cells. Treatment with NaB

increased MUC2 expression level (Figure 1B), which is a differentiation marker of intestinal cells (Gendler and Spicer, 1995), and this increase was attenuated by the transfection of PTEN siRNA, thereby suggesting that PTEN plays a role in NaB-mediated differentiation.

Enhanced gastric cancer cell differentiation by NaB was observed. The signalling pathways involved in this regulation process were delineated further. We observed that NaB, which induces gastric cancer cell differentiation, increased MUC2 expression. PTEN activation and PI3K inhibition resulted in NaB-mediated MUC2 induction. Treatment of the BGC823 gastric cancer cells with NaB activated PTEN and inhibited PI3K activation. Consistent with these findings, the inhibition of PTEN activity attenuated NaB-mediated MUC2 induction, whereas the inhibition of PI3K and activation of PTEN enhanced this induction. In addition, we observed negative regulation of PTEN and PI3K signalling on MUC2 expression. Inhibition of PI3K increased PTEN activity, while activation of PI3K inhibited PTEN activity. In contrast, the inhibition of PTEN increased PI3K activation. Our



**Figure 3** PTEN/PI3K changes NaB-mediated MUC2 induction (A) siRNA-mediated reduction of PTEN mRNA expression in BGC823 cells. Normalized PTEN/GAPDH mRNA levels were measured at 24 and 48 h after transfection and compared with the corresponding levels in control cells treated with Lipofectamine 2000 only and those treated with scrambled siRNA; (B) Knockdown of PTEN attenuates MUC2 induction by NaB. BGC823 cells were transfected with PTEN siRNA or control siRNA. Cells were harvested 48 h after transfection and treated with NaB for an additional 24 h. Whole-cell protein was extracted, and Western blotting was performed for the analysis of protein expression; (C) Inhibition of PI3K enhances NaB-mediated MUC2 induction. BGC823 cells were pretreated with a specific PI3K inhibitor, wortmannin, for 2 h and subsequently treated with NaB for 48 h. Total protein was extracted, and Western blotting was performed for the analysis of protein expression.

findings indicate that PTEN and PI3K regulate the MUC2 induction in gastric cancer cells. Butyrate-stimulated MUC2 production in LS174T cells was inhibited by MEK inhibitor U0126, implicating the involvement of ERK (extracellular-signal-regulated kinase) cascades in this process, which indicates the complexity of the process regulating cell differentiation (Hatayama et al., 2007).

Wang et al. (2001) showed that NaB-induced intestinal cell differentiation was associated with the activation of PTEN. Conversely, inhibition of PTEN attenuated NaB-induced intestinal cell differentiation. PTEN activity has been associated with intestinal cell death. Overexpression of PTEN in colorectal cancer cells resulted in cell cycle arrest and enhanced cell death through inhibition of PI3K (Sun et al., 1999). We found that the blockade of PTEN/PI3K pathway by pharmacological (i.e. wortmannin) or genetic mechanisms (i.e. transfection with PTEN siRNA) attenuated the induction of MUC2 by NaB.

In summary, in investigating the signalling mechanisms regulating MUC2 expression and function in gastric cancer cells, we found that MUC2 is a downstream target of the PTEN/PI3K pathway. The PTEN/PI3K signalling pathway may alter gastric cancer cell differentiation through the regulation of MUC2 expression. MUC2 expression was regulated through the PTEN/PI3K pathway in NaB-treated BGC823 cells. The regulation mechanism of MUC2 and PTEN warrants further clarification.

#### Author contribution

Zhigang Bai carried out the experiments and wrote the paper. Yingjiang Ye constructed plasmids, transfected and performed Western blot analysis and real-time RT-PCR. Shan Wang was responsible for the gene-silencing experiments. Zhongtao Zhang designed the experiments, analysed the data and wrote the paper.

#### Funding

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

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Received 19 December 2009/17 July 2010; accepted 19 August 2010

Published as Immediate Publication 19 August 2010, doi 10.1042/CBI20090481