ABSTRACT The nuclear factor of activated T cell (NFAT) proteins are a family of transcription factors (NFATc1–c4) involved in the regulation of cell differentiation and adaptation. Previously we demonstrated that inhibition of phosphatidylinositol 3-kinase or overexpression of PTEN enhanced intestinal cell differentiation. Here we show that treatment of intestinal-derived cells with the differentiating agent sodium butyrate (NaBT) increased PTEN expression, NFAT binding activity, and NFAT mRNA expression, whereas pretreatment with the NFAT signaling inhibitor cyclosporine A (CsA) blocked NaBT-mediated PTEN induction. Moreover, knockdown of NFATc1 or NFATc4, but not NFATc2 or NFATc3, attenuated NaBT-induced PTEN expression. Knockdown of NFATc1 decreased PTEN expression and increased the phosphorylation levels of Akt and downstream targets Foxo1 and GSK-3α/β. Furthermore, overexpression of NFATc1 or the NFATc4 active mutant increased PTEN and p27kip1 expression and decreased Akt phosphorylation. In addition, pretreatment with CsA blocked NaBT-mediated induction of intestinal alkaline phosphatase (IAP) activity and villin and p27kip1 expression; knockdown of either NFATc1 or NFATc4 attenuated NaBT-induced IAP activity. We provide evidence showing that NFATc1 and NFATc4 are regulators of PTEN expression. Importantly, our results suggest that NFATc1 and NFATc4 regulation of intestinal cell differentiation may be through PTEN regulation.

INTRODUCTION

The mammalian intestinal mucosa undergoes a process of continual renewal, characterized by active proliferation of stem cells localized near the base of the crypts, progression of these cells up the crypt-villus axis with cessation of proliferation, and subsequent differentiation into one of the four primary cell types (i.e., enterocytes, goblet cells, Paneth cells, and enteroendocrine cells) (Cheng and Leblond, 1974). In the process of differentiation, enterocytes acquire structural features of mature cells, such as microvilli, and express specific gene products such as intestinal alkaline phosphatase (IAP), a brush border enzyme (Traber, 1994). The differentiated enterocytes, which make up the majority of the cells of the gut mucosa, then undergo a process of programmed cell death (i.e., apoptosis) and are extruded into the gut lumen. The cellular mechanisms regulating this tightly regimented process have not been clearly defined. Delineating the molecular factors regulating intestinal proliferation and differentiation is crucial to our understanding of not only normal gut development and maturation, but also aberrant gut growth.

The tumor suppressor protein phosphatase and tensin homologue deleted on chromosome ten (PTEN) antagonizes the activity of phosphatidylinositol 3-kinase (PI3K) by dephosphorylating the D3-phosphate group of lipid second messengers, thus serving as a negative regulator of the PI3K pathway (Cantley and Neel, 1999). PTEN inhibits downstream functions mediated by the PI3K pathway,
such as cell growth and survival, cell migration and invasion, and cell cycle progression through the regulation of the expression of the cyclin-dependent kinase inhibitor protein p27\(^{kip1}\) (Sun et al., 1999), which is induced by PTEN in various cells. Previously we showed that inhibition of PI3K or overexpression of PTEN significantly enhanced intestinal cell differentiation either spontaneously or induced by the short-chain fatty acid sodium butyrate (NaBt) (Wang et al., 2001), a histone deacetylase inhibitor produced in the colon by breakdown of dietary fiber. Knockdown of PTEN attenuates NaBt-increased IAP activity (Wang et al., 2007). Moreover, PTEN expression correlates with expression of Cdx-2, a homeodomain protein required for intestinal epithelial cell differentiation, along the length of the murine colon (Kim et al., 2002). PTEN stimulates Cdx-2 protein expression and the transcriptional activity of the Cdx-2 promoter, thus further indicating a role for PTEN in the process of intestinal differentiation. Despite the importance of PTEN in apoptosis and differentiation, little is known about the regulation of PTEN expression in intestinal cells.

The nuclear factor of activated T cells (NFATc) proteins are a family of transcription factors whose activation is controlled by calcineurin, a calcium-dependent phosphatase. Four distinct genes encoding closely related NFATc proteins (NFATc1–c4) (Yang et al., 2002) have been identified and are involved in multiple biological processes ranging from lymphocyte activation and development to cardiac hypertrophy (Molkentin et al., 1998). NFAT, which exists in a highly phosphorylated form in the cytoplasm, translocates into the nucleus upon dephosphorylation by the phosphatase calcineurin in response to increases in intracellular calcium, where it binds to enhancer elements of specific genes leading to transcriptional activation (Hogan et al., 2003). Cyclosporin A (CsA), a potent and specific inhibitor of calcineurin, is often used to inhibit NFAT transcriptional activity (Lee and Park, 2006). Although additional post-translational events can affect transcriptional activity, the nuclear localization of NFATc1–c4 and the cooperative binding with other transcription factors appears to be a major regulatory mechanism for the transcriptional activity (Lee and Park, 2006). Although additional post-translational events can affect transcriptional activity, the nuclear localization of NFATc1–c4 and the cooperative binding with other transcription factors appears to be a major regulatory mechanism for the transcriptional activity.

The regulation of the expression of a number of cytokine genes, including those for interleukin 2 (IL-2), IL-3, IL-4, IL-5, and gamma interferon, also regulates other responsive genes, such as cell growth and survival, cell migration and invasion, and cell cycle progression through the regulation of the expression of the cyclin-dependent kinase inhibitor protein p27\(^{kip1}\) (Sun et al., 1999), which is induced by PTEN in various cells. Previously we showed that inhibition of PI3K or overexpression of PTEN significantly enhanced intestinal cell differentiation either spontaneously or induced by the short-chain fatty acid sodium butyrate (NaBt) (Wang et al., 2001), a histone deacetylase inhibitor produced in the colon by breakdown of dietary fiber. Knockdown of PTEN attenuates NaBt-increased IAP activity (Wang et al., 2007). Moreover, PTEN expression correlates with expression of Cdx-2, a homeodomain protein required for intestinal epithelial cell differentiation, along the length of the murine colon (Kim et al., 2002). PTEN stimulates Cdx-2 protein expression and the transcriptional activity of the Cdx-2 promoter, thus further indicating a role for PTEN in the process of intestinal differentiation. Despite the importance of PTEN in apoptosis and differentiation, little is known about the regulation of PTEN expression in intestinal cells.

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RESULTS

NFAT activation increased PTEN expression in HT29 cells

Previously we have shown that PTEN plays an important role in the regulation of intestinal cell proliferation and differentiation (Wang et al., 2001). NFAT signaling is involved in regulation of the proliferation and differentiation of various types of cells. To investigate the possible regulatory effect of NFAT on PTEN expression, we used the human colon cancer cell line HT29, which has wild-type (i.e., nonmutated) PTEN (Kim et al., 2004). HT29 is used extensively as a model of intestinal epithelial cell proliferation and differentiation (Wang et al., 2001; Wilson and Browning, 2002). Cells were pretreated with CsA, an inhibitor of calcineurin, followed by treatment with NaBt in the presence or absence of CsA for 24 h. Whole cell lysates were analyzed by Western blot using anti-PTEN antibody (Figure 1A). NaBt treatment resulted in the induction of PTEN expression; this induction was attenuated by pretreatment with CsA. These results suggest that NaBt-induced PTEN expression requires NFAT activation. To determine whether NaBt increased NFAT activity, we performed electrophoretic mobility shift assays (EMSA) with nuclear extracts obtained from HT29 cells treated over a time course with NaBt using an oligonucleotide containing NFAT consensus binding sites. As shown in Figure 1B, treatment with NaBt increased NFAT binding activity; the specificity of the complex was determined using unlabeled cold probe as a competitor. To further determine whether NaBt treatment alters NFAT expression, HT29 cells were treated with NaBt for 24 h, total RNA extracted and NFAT expression determined by real-time RT-PCR. Treatment with NaBt increased NFATc1, NFATc2, and NFATc4 mRNA expression without affecting NFATc3 mRNA levels (Figure 1C), suggesting that NaBt increased NFAT binding activity and PTEN expression through increased NFATc1, NFATc2, and NFATc4 expression.

To further demonstrate the role of NFAT in PTEN induction, HT29 cells were treated with phorbol 12-myristate 13-acetate (PMA) plus A23187, pharmacological agents that activate NFAT in intestinal cell types (Duque et al., 2005). Whole cell lysates were analyzed by Western blot using anti-PTEN antibody. Treatment with PMA plus A23187 increased PTEN expression compared with control cells treated with vehicle (i.e., Me₂SO) (Figure 1D); this induction was attenuated by pretreatment with CsA (Figure 1D). Together, our results suggest a role for NFAT activation in PTEN induction in intestinal cells.

Knockdown of NFAT blocked NaBt-induced PTEN expression

Four isoforms of NFAT have been identified. To determine which of the NFAT isoforms are involved in PTEN regulation, individual NFAT isoforms were silenced by transfection of HT29 cells with the relevant siRNA. As shown in Figure 2A, transfection of NFATc1 (Figure 2A, upper panel) or NFATc4 siRNA (Figure 2A, middle panel) blocked NaBt-increased PTEN protein expression compared with cells transfected with nontargeting control siRNA. In contrast, knockdown of NFATc2 or NFATc3 (Figure 2A, lower panel) did not attenuate NaBt-increased PTEN protein expression. The specificity and efficiency of knockdown of individual NFAT isoforms were confirmed by real-time RT-PCR and Western blot as shown in Figure 2, B and C. The results indicate that NFATc1 and NFATc4 are important for NaBt-induced PTEN expression in human intestinal cells.
**NFATc1 and NFATc4 regulation of PTEN expression in HT29 cells**

To better delineate the role of NFATc1 and NFATc4 in PTEN regulation, HT29 cells were transfected with a plasmid encoding active forms of NFATc1 or NFATc4 (Yang et al., 2002; Yang and Chow, 2003). Overexpression of NFATc1 (Figure 3A) or NFATc4 (Figure 3B) increased PTEN protein expression. Overexpression of NFATc1 and NFATc4 was confirmed using anti-NFATc1 and anti-NFATc4 antibodies, respectively. To address whether PTEN mRNA induction paralleled the increase in PTEN protein, real-time RT-PCR (Figure 3C) was performed with total RNA extracted from transfected HT29 cells; PTEN mRNA induction was noted with NFATc1 or NFATc4 overexpression. In addition, cotransfection of an NFATc1 or NFATc4 expression plasmid together with a PTEN promoter construct, which contains 1365 bp of the human PTEN promoter linked to luciferase (Kim et al., 2004), increased PTEN promoter activity in Caco-2 intestinal cells (data not shown). Taken together, these results indicate a role for NFATc1 and NFATc4 in the regulation of PTEN expression in HT29 cells.

**Overexpression of NFATc1 or NFATc4 decreased Akt phosphorylation and increased p27kip1 expression**

We next determined whether NFATc1 and NFATc4 regulate Akt phosphorylation and p27kip1 expression, two PTEN downstream target molecules. Overexpression of NFATc1 (Figure 4, A and C) or NFATc4 (Figure 4, B and D) decreased Akt (Ser473) phosphorylation and increased p27kip1 expression in HT29 cells. Previously we have shown that PTEN is crucial for regulation of p27kip1 expression and Akt phosphorylation (Kim et al., 2004; Wang et al., 2007). Our results suggested that NFATc1 or NFATc4 decreased Akt phosphorylation and increased p27kip1 expression through the induction of PTEN expression in HT29 cells.

To determine whether NFATc1 or NFATc4 regulation of PTEN expression noted in HT29 cells also occurs in other intestinal cell lines, Caco-2 cells were transfected with control vector or a plasmid encoding the active form of NFATc1 or NFATc4. Caco-2 cells, another well-characterized cell model of intestinal differentiation, differentiate to an enterocyte-like phenotype by treatment with NaBT or spontaneously in culture with overconfluence (Ding et al., 1998; Wang et al., 2001). Overexpression of NFATc1 (Figure 5, A and B) or NFATc4 (Figure 5C) increased PTEN and p27kip1 expression and decreased Akt (Ser473) phosphorylation. Collectively, our findings show that NFATc1 and NFATc4 regulate PTEN expression, Akt phosphorylation, and p27kip1 expression in intestinal-derived cells.

** Knockdown of NFATc1 increased phosphorylation of Akt (Ser473) and downstream targets Foxo1 and GSK-3β**

After phosphorylation, Akt is activated and then phosphorylates downstream target molecules such as GSK-3α/β and Foxo1. To determine whether downstream phosphorylation of Foxo1 and GSK-3α/β occurs, HT29 (Figure 6A) and Caco-2 (Figure 6B–D) cells were transfected with nontargeting control siRNA or siRNA targeting NFATc1. Forty-eight hours after transfection, the phosphorylation of Foxo1 (Ser256), GSK-3α/β (Ser21/9), and Akt (Ser473) was analyzed by Western blotting (Figure 6, A and B). In agreement with the decreased Akt (Ser473) phosphorylation by NFATc1 overexpression, knockdown of NFATc1 increased Akt (Ser473) phosphorylation and concomitantly increased Foxo1 and GSK-3α/β phosphorylation in HT29 (Figure 6A) and Caco-2 cells (Figure 6B). The NFATc1 knockdown in Caco-2 cells was confirmed by RT-PCR (Figure 6C). Decreased PTEN expression in Caco-2 cells transfected with NFATc1...

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**TABLE 1:** NFAT inhibition attenuated NaBT-induced PTEN expression.

| Experiment | NaBT (5 mM) | CsA (10 μM) | PTEN | β-actin |
|------------|-------------|-------------|------|---------|
| Free       | –           | +           | –    | +       |
| Competitor | +           | –           | –    | +       |

**FIGURE 1:** NFAT inhibition attenuated NaBT-induced PTEN expression. (A) HT29 cells were treated with NaBT (5 mM), a short-chain fatty acid that increases intestinal cell differentiation, in the presence or absence of CsA for 24 h. Cells were lysed and Western blot analysis was performed using antibodies against PTEN and β-actin. PTEN signals were quantitated densitometrically and expressed as fold change with respect to β-actin. (B) HT29 cells were treated with NaBT (5 mM) over a time course. Nuclear protein was incubated with a 32P-labeled NFAT specific DNA probe alone or in the presence of unlabeled wild-type NFAT oligonucleotide (NS, nonspecific). (C) HT29 cells were treated with NaBT (5 mM) for 24 h. Total RNA was extracted, and NFATc1, NFATc2, NFATc3, and NFATc4 mRNA expression was assessed by real-time RT-PCR. (D) HT29 cells were treated with PMA plus A23187 in the presence or absence of CsA for 2 h. Whole cell lysate was extracted, and Western blot analysis was performed for PTEN protein expression. The membranes were stripped and reprobed with anti-β-actin antibody to control for loading. PTEN signals were quantitated densitometrically and expressed as fold change with respect to β-actin.
Akt signaling. Results demonstrate that NFATc1 is an upstream regulator of PTEN/siRNA was demonstrated by Western blotting (Figure 6D). These results were significant attenuated by the combination treatment with CsA. Furthermore, transfection with NFATc1 or NFATc4 siRNA attenuated NaBT-increased IAP activity (Figure 7C). NaBT induction of PTEN expression in HT29. (A, B) HT29 cells were transfected with control vector or active mutants for NFATc1 (A) or NFATc4 (B). After a 48-h incubation, PTEN, NFATc1, and NFATc4 expression was determined by Western blot. β-Actin was blotted to confirm equal loading. PTEN signals were quantitated densitometrically and expressed as fold change with respect to β-actin. (C) HT29 cells were transfected with control vector or active mutants for NFATc1 or NFATc4. After a 48-h incubation, total RNA was extracted and PTEN mRNA levels were determined by real-time RT-PCR. (Data represent mean ± SD; *, P < 0.05 vs. control.)

**DISCUSSION**

Previously we have shown that the overexpression of PTEN enhances, whereas knockdown of PTEN attenuates, enterocyte-like differentiation of the HT29 and Caco-2 human colon cancer cells demonstrating a role for PTEN in intestinal cell differentiation (Wang et al., 2001, 2007). In our present study, we demonstrate by complementary approaches (i.e., chemical inhibition, siRNA knockdown, and overexpression) that NFATc1 and NFATc4 regulate PTEN expression, Akt phosphorylation, and p27kip1 expression. Consistent with these results, inhibition or knockdown of NFATc1 and NFATc4 attenuates brush-border enzyme activity and villin expression, which are markers of enterocyte differentiation. In addition, NFATc1, NFATc4, and PTEN are expressed in the upper (i.e., differentiated) intestinal mucosa, thus further suggesting the association of NFATc1 or NFATc4 with intestinal differentiation. Our findings demonstrate the role of NFATc1 and NFATc4 in the regulation of the PTEN/Akt signaling pathway and intestinal cell differentiation. Finally, to determine the location of NFATc1, NFATc4, and PTEN expression in vivo, sections of normal human colon were obtained from adult patients and analyzed (Figure 7D). Interestingly, a majority of NFATc1, NFATc4, and PTEN expression was specifically localized to the intestinal cells in the differentiated fractions of the colonic mucosa. That is, intense staining for NFATc1, NFATc4, and PTEN was located in the upper crypt portion of the colon. Minor staining was noted in the lower crypt region of the colon. Therefore these findings demonstrate the NFATc1, NFATc4, and PTEN are co-localized specifically in the more differentiated portions of the intestinal mucosa, thus further suggesting the association of NFATc1 or NFATc4 with intestinal differentiation.

**NFAT signaling is involved in the regulation of intestinal cell differentiation**

Because the P38/PTE[N/Akt pathway and p27kip1, a downstream target protein of Akt, play important roles in the regulation of intestinal cell differentiation (Deschenes et al., 2001; Wang et al., 2001), we next determined whether inhibition of NFAT signaling inhibits differentiation. Treatment with NaBT increased IAP activity (Figure 7A) and villin and p27kip1 protein expression (Figure 7B); these increases were significantly attenuated by the combination treatment with CsA. Furthermore, transfection with NFATc1 or NFATc4 siRNA attenuated NaBT-increased IAP activity (Figure 7C). These results demonstrate the regulation of intestinal cell differentiation by NFATc1 and NFATc4.

Our findings demonstrate the role of NFATc1 and NFATc4 in the regulation of the PTEN/Akt signaling pathway and intestinal cell differentiation. Finally, to determine the location of NFATc1, NFATc4, and PTEN expression in vivo, sections of normal human colon were obtained from adult patients and analyzed (Figure 7D). Interestingly, a majority of NFATc1, NFATc4, and PTEN expression was specifically localized to the intestinal cells in the differentiated fractions of the colonic mucosa. That is, intense staining for NFATc1, NFATc4, and PTEN was located in the upper crypt portion of the colon. Minor staining was noted in the lower crypt region of the colon. Therefore these findings demonstrate the NFATc1, NFATc4, and PTEN are co-localized specifically in the more differentiated portions of the intestinal mucosa, thus further suggesting the association of NFATc1 or NFATc4 with intestinal differentiation.

**FIGURE 2:** Knockdown of NFATc1 and NFATc4, but not NFATc2 and NFATc3, attenuated NaBT induction of PTEN expression in HT29 cells. (A) HT29 cells were transfected with control siRNA or siRNA specific targeting NFATc1 (upper panel), c2 (middle panel), c2 or c3 (lower panel). After a 24-h incubation, transfected cells were treated with NaBT (5 mM) for an additional 24 h. Cells were lysed and Western blot analysis was performed using antibodies against PTEN and β-actin. PTEN signals were quantitated densitometrically and expressed as fold change with respect to β-actin. (B, C) HT29 cells were transfected with control siRNA or siRNA targeting NFATc1, c2, c3, or c4. After a 48-h incubation, total RNA (B) and total protein (C) were extracted and real-time RT-PCR and Western blot performed for analysis of NFATc1, NFATc2, NFATc3, and NFATc4 mRNA and protein expression, respectively.
NFAT may play a role in JNK regulation of PTEN expression. In addition, we found that overexpression of NFATc1 or NFATc4 increased PTEN promoter activity, suggesting that the increased PTEN expression may be mediated, at least in part, through regulation of transcription. However, after computer analysis of PTEN promoter sequences (AF406618), there are no typical NFAT binding sites located in the PTEN promoter region, implying an indirect regulation of PTEN expression by NFATc1 and NFATc4. Results from our laboratory and others have shown that several transcription factors, such as EGR1 and peroxisome proliferator-activated receptor gamma (PPARγ), up-regulate PTEN expression (Farrow and Evers, 2003; Tamguney and Stokoe, 2007). A cooperative interaction between NFAT and EGR1 regulating expression of several genes has been reported (Decker et al., 2003). Molecular analysis indicates that PPARγ is a target of NFAT (Yang et al., 2002). Two distinct NFAT binding elements are located in the PPARγ2 gene promoter, and stable expression of the active form of NFATc4 increases the expression of PPARγ2 (Yang et al., 2002). Resistin, a cytokine involved in inflammation and insulin resistance, increases PTEN expression (Shen et al., 2006). Further- more, NFATc2−/−/NFATc4−/− mice exhibit a reduced resistin level. Mechanically, NFAT is recruited to the transcription loci and regulates resistin gene expression upon insulin stimulation (Yang et al., 2006). It is possible that EGR1, PPARγ, or resistin might be involved in NFATc1- and NFATc4-mediated PTEN induction. We are currently investigating this possibility.

The importance of NFATc1 and NFATc4, but not NFATc2 and NFATc3, in NaBt-mediated PTEN induction is shown in our current study. Analysis of mice deficient for NFAT suggests different roles for

\[ \begin{align*}
\text{NFATc1} & \quad \text{NFATc4} \\
\beta\text{-actin} & \quad \beta\text{-actin} \\
\text{Akt} & \quad \text{Akt} \\
p\text{Akt}^{\text{Ser } 473} & \quad p\text{Akt}^{\text{Ser } 473} \\
\text{PTEN} & \quad \text{PTEN} \\
p\text{p27\text{kip1}} & \quad p\text{p27\text{kip1}} \\
\end{align*} \]
the NFAT family of transcription factors in the regulation of cell proliferation and apoptosis. Although NFATc1 and NFATc2 share high sequence similarity, mice deficient in either of these two genes have markedly divergent phenotypes. NFATc2−/− mice consistently showed a marked increase in lymphocyte proliferation and develop neoplastic transformation of cartilage cells (Cao et al., 2002). NFATc1−/− is lethal due to a defect in the development of cardiac valves and septa (de la Pompa et al., 1998). Moreover, an important role of NFATc1 in the development of T lymphocytes and in the differentiation of Th2 response has been shown (Delling et al., 2000). NFATc1−/− is associated with intestinal cell differentiation. Their results suggest that CsA prevents or retards premature and spontaneous terminal differentiation of hair keratinocytes in vivo via inhibition of calcineurin-dependent expression of p21<sup>WAF1</sup> and p27<sup>KIP1</sup>. The PI3K/Akt pathway plays a critical role in the regulation of p<sup>27</sup><sup>KIP1</sup> (Li et al., 2003). Akt-mediated regulation of p<sup>27</sup><sup>KIP1</sup> occurs at various levels and includes control of p<sup>27</sup><sup>KIP1</sup> expression as well as a decrease in protein stability (Li et al., 2003). The forkhead transcription factors regulate p<sup>27</sup><sup>KIP1</sup> transcription, and Akt has been reported to inhibit the transcription of p<sup>27</sup><sup>KIP1</sup> by inactivating forkhead. In addition, our previous study has shown that GSK-3β, another downstream target of Akt, plays a crucial role in the regulation of nuclear p<sup>27</sup><sup>KIP1</sup> protein expression (Wang et al., 2008). Genetic studies have shown that the forkhead transcription factors are essential for the formation and differentiation of gut endoderm (Ewton et al., 2002). Moreover, the importance of p<sup>27</sup><sup>KIP1</sup> in intestinal cell differentiation has been demonstrated (Deschenes et al., 2001). We showed that NFAT inhibits phosphorylation of Akt, GSK-3, and Foxo1 in combination with the increase of p<sup>27</sup><sup>KIP1</sup> expression; therefore it is possible that calcineurin/NFAT signaling increases intestinal cell differentiation in part through the PTEN/Akt/GSK-3/p<sup>27</sup><sup>KIP1</sup> or PTEN/Akt/Foxo/p<sup>27</sup><sup>KIP1</sup> pathway.

In conclusion, our results demonstrate that NFATc1 and NFATc4 play an important role in the regulation of PTEN and expression of its downstream target p<sup>27</sup><sup>KIP1</sup> and Akt phosphorylation. Given the importance of the PTEN/Akt/p<sup>27</sup><sup>KIP1</sup> pathway in intestinal cell differentiation, NFATc1 and NFATc4 may represent key modulator proteins for intestinal cell proliferation and differentiation through the regulation of PTEN expression.

**MATERIALS AND METHODS**

**Materials**

NaBT, PMA, and antibody against β-actin were purchased from Sigma Chemical (St. Louis, MO). CsA and A23187 were purchased...
expression was determined by Western blotting (B). Villin and p27 \( \text{kip1} \) signals were quantitated densitometrically and expressed as fold change with respect to determined. (Data represent mean ± SD; †, \( P < 0.05 \) vs. NaBT alone.) Villin and p27 \( \text{kip1} \) expression was determined by Western blotting (B). Villin and p27 \( \text{kip1} \) signals were quantitated densitometrically and expressed as fold change with respect to β-actin. (C) HT29 cells were transfected with NFATc1, NFATc4, or control siRNA. After a 24-h incubation, transfected cells were treated with NaBT (5 mM) for 24 h. Cells were lysed and alkaline phosphatase activity was determined. (Data represent mean ± SD; †, \( P < 0.05 \) vs. NaBT alone.) NFATc1, NFATc4, and PTEN antibodies were purchased from Cell Signaling (Beverly, MA). Mouse monoclonal anti-NFATc1 antibody, which was used for the immunohistochemical staining and Western blot, and rabbit anti-villin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-NFATc1 antibody, which was used for the Western blot, and rabbit anti-NFATc4 antibody, which was used for the immunohistochemical staining and Western blot, were from Affinity BioReagents (Golden, CO). Rabbit anti-Akt, anti-phospho-Akt (Ser473), anti-phospho-GSK-3α/β (Ser 21/9), anti-phospho-Foxo1 (Ser256), and rabbit anti-β-actin antibodies were purchased from BD Biosciences (San Diego, CA). Mouse monoclonal anti-NFATc1, NFATc2, NFATc3, NFATc4, and nontargeting siRNA SMARTpool were purchased from Dharmacon (Lafayette, CO). siRNA SMARTpool, consisting of four siRNA duplexes, was designed using an algorithm composed of 33 criteria and parameters that effectively eliminate nonfunctional siRNA (Reynolds et al., 2004).

**Cell culture, transfection, and treatment**

The human colon cancer cell lines HT29 and Caco-2 were maintained in McCoy’s 5A supplemented with 10% of FCS and MEM supplemented with 15% of fetal calf serum (FCS), respectively. HT29 and Caco-2 cells were transfected with the siRNA duplexes and plasmids by electroporation (Gene Pulser; Bio-Rad, Hercules, CA) and lipofectamine 2000 (Invitrogen, Carlsbad, CA), respectively, as we have described previously (Wang et al., 2003, 2006). Caco-2 cells were transiently transfected with a human PTEN promoter construct and Renilla reporter pRL-null, an internal control to normalize for variation in transfection efficiency, using Lipofectamine 2000, and the luciferase activity was determined and normalized to the Renilla activity as we have described previously (Kim et al., 2004).

**Western blot analysis**

Total protein was resolved on a 10% polyacrylamide gel and transferred to polyvinylidene fluoride membranes. Membranes were incubated for 1 h at room temperature in blotting solution. PTEN, phospho-Akt (Ser473), Akt, p27\(^{\text{kip1}}\), villin, NFATc1, NFATc2, NFATc3, NFATc4, phospho-Foxo1, Foxo1, phospho-GSK-3α/β, GSK-3α/β, and β-actin were detected with specific antibodies following blotting with a horseradish peroxidase–conjugated secondary antibody and visualized using an enhanced chemiluminescence detection system.

**Quantitative real-time RT-PCR analysis**

Total RNA was extracted and DNase-treated (Qiagen, Valencia, CA). Synthesis of cDNA was performed with 1 μg of total RNA using the reagents in the TaqMan Reverse Transcription Reagents Kit from ABI (Foster City, CA). The TaqMan probe and primers for human PTEN, NFATc1, NFATc2, NFATc3, NFATc4, and GAPDH were purchased from Applied Biosystems (Foster City, CA). Quantitative real-time RT-PCR analysis was performed with an Applied Biosystems Prism 7000HT Sequence Detection System using TaqMan universal PCR master mix as we have described previously (Kim et al., 2004).

**Preparation of nuclear extracts and EMSAs**

The nuclear extracts were prepared from HT29 cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce, Rockford, IL) according to the manufacturer’s protocol. Nuclear extracts (10 μg) were incubated with 40,000 cpm of \(^{32}\text{P}\)-labeled NFAT consensus oligonucleotide (5′-CGC CCA AAG AGG AAA ATT TGT TTC ATA-3′) (Santa Cruz Biotechnology) for NFAT binding activity as we have described previously (Wang et al., 2007).

**Alkaline phosphatase enzyme activity assay**

Protein was extracted from cells with lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM Na\(_2\)EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na\(_2\)VO\(_4\), 1 μg/ml leupeptin), and the concentrations of the supernatant cell lysates were determined. The supernatant cell lysates (20 μl) were used to determine alkaline phosphatase activity by a commercially available kit (Sigma), and the data were normalized by the protein amounts used, as we have previously described (Wang et al., 2008).

**Immunohistochemical analysis**

Formalin-fixed, paraffin-embedded tissue samples of normal human colon were used; the samples were taken from the adjacent histologically normal colon removed at the time of resection for colon...
cancer. Tissue was processed for routine immunohistochemical staining using antibodies against human PTEN, NFATC1, or NFATc4. Negative controls (including no primary antibody or isotype-matched mouse immunoglobulin G) were used in each assessment.

Statistical analysis

Data were analyzed using analysis of variance for a two-factor experiment. Main effects and interaction were assessed at the 0.05 level of significance. Fisher’s least significant difference procedure was used for multiple comparisons with Bonferroni adjustment for the number of comparisons. All statistical computations were carried out using SAS, Release9.1 [R1].

ACKNOWLEDGMENTS

We thank Karen Martin, Donna Gilbreath, and Nathan L. Vanderford for manuscript preparation and Tatsuo Uchida for statistical analysis. This work was supported by Grants P20 CA150343 (UK GI SPORE) and R01 DK48498 from the National Institutes of Health.

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