Nuclear factor of activated T cells (NFAT) signaling regulates PTEN expression and intestinal cell differentiation

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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 $p27^{kip1}$ expression and decreased Akt phosphorylation. In addition, pretreatment with CsA blocked NaBT-mediated induction of intestinal alkaline phosphatase (IAP) activity and villin and $p27^{kip1}$ 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.**

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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,

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Abbreviations used: CSA, cyclosporine A; EMSA, electrophoretic mobility shift assay; Foxo1, forkhead box O1; GSK-3, Glycogen synthase kinase-3; IAP, intestinal alkaline phosphatase; NaBT, sodium butyrate; NFAT, nuclear factor of activated T cell; PTEN, tumor suppressor protein phosphatase and tensin homologue deleted on chromosome ten; RT-PCR, reverse transcription polymerase chain reaction; siRNA, small interfering RNA.

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 of NFAT complexes (Wu *et al.*, 2007). Apart from 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, NFAT also regulates other responsive genes, such as $p21^{waf1}$, $p27^{kip1}$, CD40 ligand, FasL, CDK4, and cyclin A2, indicating that NFAT may be involved in the control of the cell cycle and apoptosis (Viola *et al.*, 2005). NFAT has been shown to regulate cell differentiation and development in a number of cell types. For example, NFAT regulates the development of the cardiovascular system (de la Pompa *et al.*, 1998). Primary keratinocyte cell differentiation is associated with nuclear localization of NFAT; this effect is blocked by CsA (Santini *et al.*, 2001). NFAT also plays a role in adipocyte differentiation (Ho *et al.*, 1998) and stimulation of myogenic differentiation via activation of calcineurin (Delling *et al.*, 2000). Recently Kao *et al.* (2009) have shown that NFAT signaling is required for neuregulin-regulated Schwann cell differentiation. However, the role of NFAT in intestinal cell differentiation is not known.

The purpose of our present study was to determine the role of NFAT signaling in the regulation of PTEN expression and intestinal cell differentiation. Here we show that NFAT activation leads to PTEN expression in intestinal cells. Moreover, overexpression of NFATc1 and NFATc4 results in decreased Akt phosphorylation and increased $p27^{kip1}$ expression. Conversely, silencing NFATc1 results in increased Akt phosphorylation and phosphorylation of Foxo1 and GSK-3α/β, two downstream targets of Akt. Furthermore, NFAT inhibition or silencing of NFATc1 or NFATc4 attenuates NaBT-induced intestinal cell differentiation as shown by decreased IAP activity and decreased villin and $p27^{kip1}$ protein expression. Our results demonstrate that NFATc1 and NFATc4 play an important role in the regulation of PTEN expression. NFATc1 and NFATc4 may regulate intestinal cell differentiation via the induction of PTEN expression.

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 (EMSAs) 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., Me_2SO) (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.

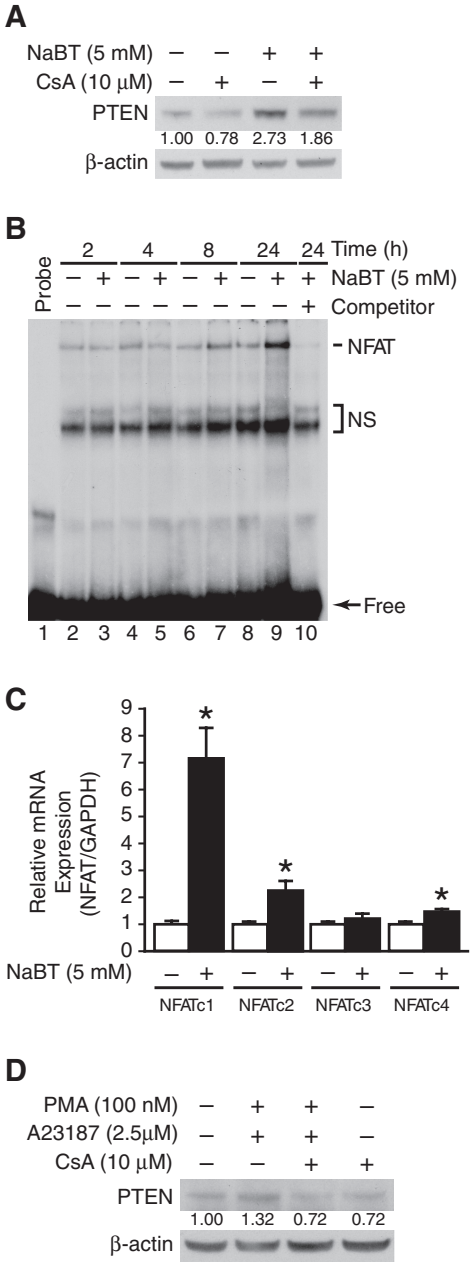

FIGURE 1: NFAT inhibition attenuated NaBT-induced PTEN expression. (A) HT29 cells were treated with NaBT (5 mM), a short-chain fatty acid that increases intestinal cell differentiation, in the presence or absence of CsA for 24 h. Cells were lysed and Western blot analysis was performed using antibodies 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 ^{32}P-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. (Data represent mean ± SD; *, $P < 0.05$ vs. control.) (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.

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 p27^{kip1} expression

We next determined whether NFATc1 and NFATc4 regulate Akt phosphorylation and p27^{kip1} 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 p27^{kip1} expression in HT29 cells. Previously we have shown that PTEN is crucial for regulation of p27^{kip1} expression and Akt phosphorylation (Kim *et al.*, 2004; Wang *et al.*, 2007). Our results suggested that NFATc1 or NFATc4 decreased Akt phosphorylation and increased p27^{kip1} 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 p27^{kip1} expression and decreased Akt (Ser473) phosphorylation. Collectively, our findings show that NFATc1 and NFATc4 regulate PTEN expression, Akt phosphorylation, and p27^{kip1} expression in intestinal-derived cells.

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

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 (Ser 256), GSK-3α/β (Ser 21/9), 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 (Ser 473) 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

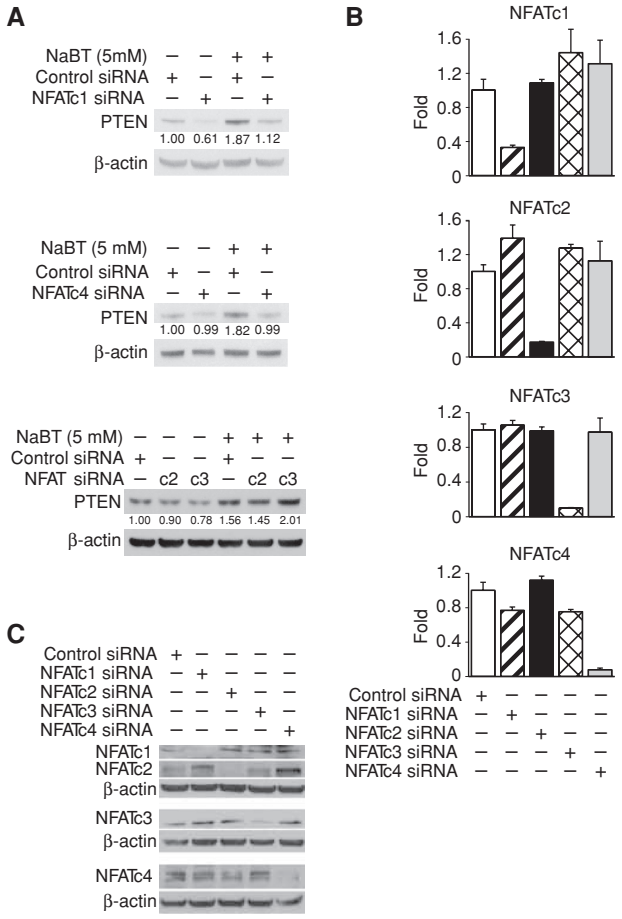

FIGURE 2: Knockdown of NFATc1 and NFATc4, but not NFATc2 and NFATc3, attenuated NaBT induction of PTEN expression in HT29 cells. (A) HT29 cells were transfected with control siRNA or siRNA specific targeting NFATc1 (upper panel), c4 (middle panel), c2 or c3 (lower panel). After a 24-h incubation, transfected 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.

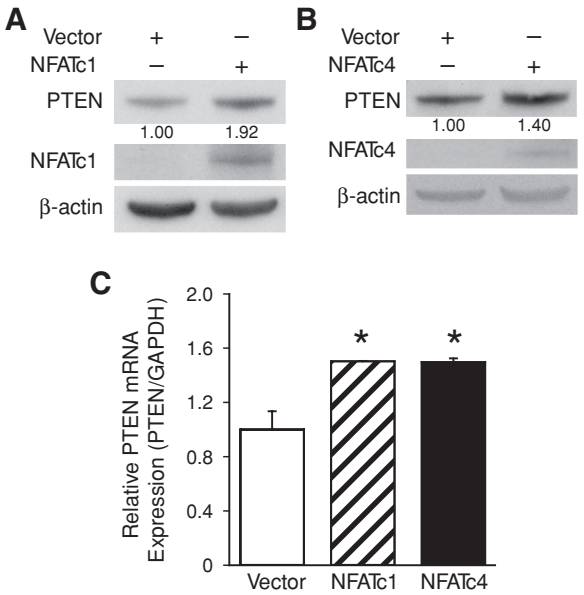

FIGURE 3: NFATc1 and NFATc4 regulation of PTEN expression in HT29. (A, B) HT29 cells were transfected with control vector or active mutants for NFATc1 (A) or NFATc4 (B). After a 48-h incubation, PTEN, NFATc1, and NFATc4 expression was determined by Western blot. β-Actin was blotted to confirm equal loading. 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.)

siRNA was demonstrated by Western blotting (Figure 6D). These results demonstrate that NFATc1 is an upstream regulator of PTEN/Akt signaling.

NFAT signaling is involved in the regulation of intestinal cell differentiation

Because the PI3K/PTEN/Akt pathway and $p27^{kip1}$, 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 $p27^{kip1}$ 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.

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 $p27^{kip1}$ 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)

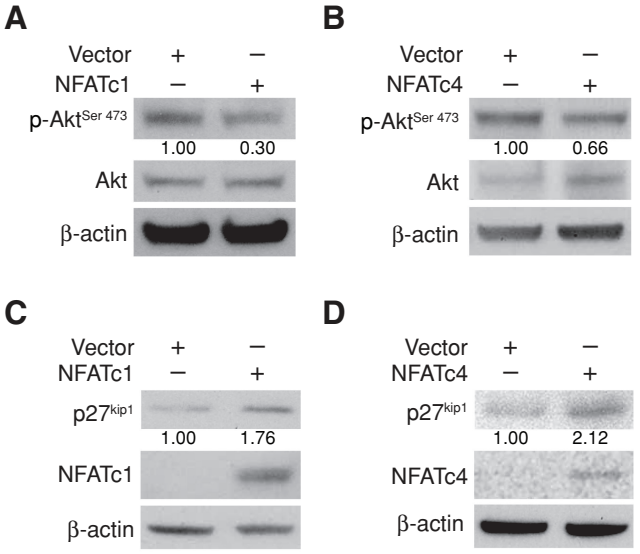

FIGURE 4: Overexpression of NFATc1 or NFATc4 decreased Akt phosphorylation in HT29 cells. HT29 Cells were transfected with pcDNA3.1 control vector or active mutants for NFATc1 (A, C) or NFATc4 (B, D). After a 48-h incubation, phospho-Akt, total Akt, $p27^{kip1}$, and NFATc1 or NFATc4 expression were determined by Western blot. β-Actin was blotted to confirm equal loading. Phospho-Akt and $p27^{kip1}$ signals were quantitated densitometrically and expressed as fold change with respect to total Akt and β-actin, respectively.

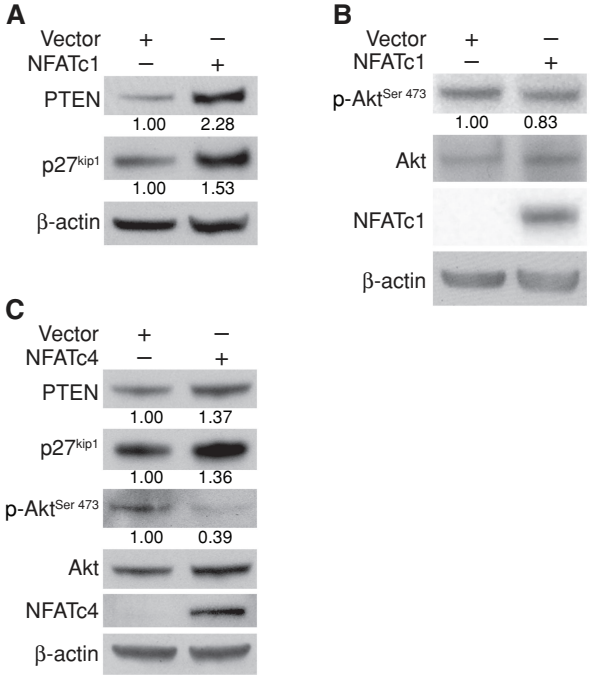

FIGURE 5: NFATc1 and NFATc4 induction of PTEN expression in Caco-2 cells. Caco-2 cells were transfected with pcDNA3.1 control vector or the active mutant for NFATc1 (A, B) or NFATc4 (C). After a 48-h incubation, PTEN and $p27^{kip1}$ (A, C) or phospho-Akt and total Akt (B, C) were determined by Western blot. β-Actin was blotted to confirm equal loading; overexpression was confirmed by expression of NFATc1 and NFATc4 in B and C, respectively. PTEN and $p27^{kip1}$ signals were quantitated densitometrically and expressed as fold change with respect to β-actin. Phospho-Akt signals were quantitated densitometrically and expressed as fold change with respect to total Akt.

portion of the normal colonic mucosa. Taken together, our results suggest that intestinal cell differentiation is regulated, in part, by NFAT/PTEN signaling.

We have found that activation of JNK or inhibition of NF-κB increases PTEN expression in human intestinal cells (Kim *et al.*, 2002, 2004; Wang *et al.*, 2007), and these findings have been confirmed by others (Vinciguerra *et al.*, 2008; Park *et al.*, 2009; Ghosh-Choudhury *et al.*, 2010). Two NF-κB recognizing DNA elements have been identified, and inhibition of NF-κB binding to these regions in the PTEN promoter increases PTEN expression (Ghosh-Choudhury *et al.*, 2010). The structure of NFAT and NF-κB DNA-binding domains is strikingly similar; therefore, hypothetically, they could compete for the same promoter regions (Hogan *et al.*, 2003). Indeed, Aurora *et al.* (2010) have shown that NF-κB represses c-FLIP expression through the restriction of NFATc2 promoter binding. Activation of the NFAT signaling pathway induces c-FLIP expression (Ueffing *et al.*, 2008). Induction of NF-κB binding and NFAT dissociation from the same region of the c-FLIP promoter was reversed by IκB inhibition. These results demonstrate the competition between NF-κB and NFAT for DNA binding (Aurora *et al.*, 2010). In our current study, we show that NFATc1 and NFATc4 positively regulate PTEN expression; therefore it is reasonable to postulate that NFAT regulates PTEN expression through the competition with NF-κB for the same promoter regions. Our previous findings have identified a negative regulation between JNK and NF-κB signaling, which plays an important role in the regulation of basal PTEN expression in intestinal cells (Wang *et al.*, 2007). Activation of JNK results in NFAT transactivation, whereas inhibition of the JNK pathway results in the inhibition of NFAT and decreased apoptosis in certain cells (Ortega-Perez *et al.*, 2005; Lin *et al.*, 2010). Taken together, these results suggest that NFAT may play a role in JNK regulation of PTEN expression.

Our results demonstrate that NFATc1 and NFATc4 increased PTEN mRNA and protein 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), upregulate 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). Furthermore, $NFATc2^{-/-}/NFATc4^{-/-}$ mice exhibit a reduced resistin level. Mechanistically, 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

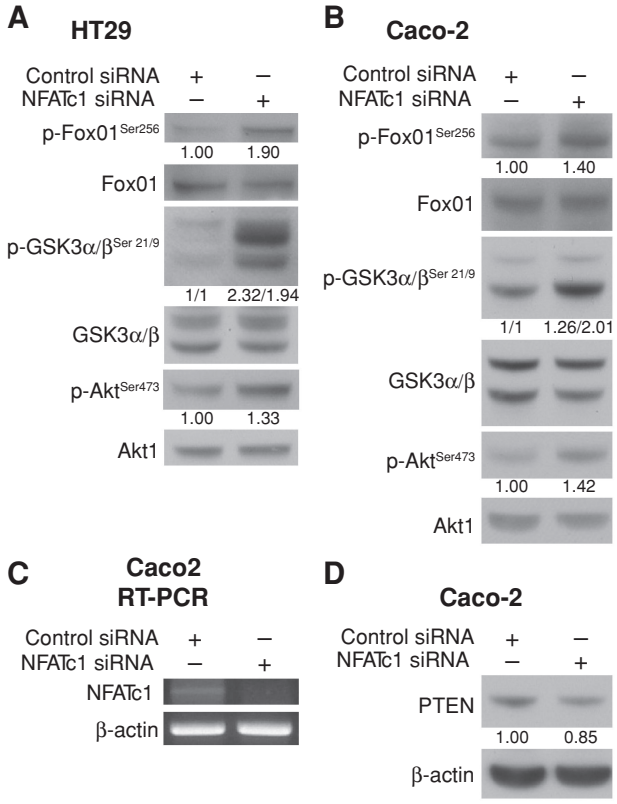

FIGURE 6: Knockdown of NFATc1 increased Akt, Foxo1, and GSK-3α/β phosphorylation. HT29 (A) or Caco-2 cells (B–D) were transfected with control siRNA or siRNA targeting NFATc1. After a 48-h incubation, transfected cells were lysed and Western blot analysis was performed using antibodies against phospho-Foxo1, total Foxo1; phospho-GSK-3α/β, total GSK-3α/β; phospho-Akt, total Akt; and β-actin (A, B). Knockdown of NFATc1 in Caco-2 cells was confirmed by RT-PCR (C). Decreased PTEN expression was noted by Western blotting (D). Phospho-Foxo1, phospho-GSK-3α/β, and phospho-Akt signals were quantitated densitometrically and expressed as fold change with respect to total Foxo1, total GSK-3α/β, and total Akt, respectively. PTEN signals were quantitated densitometrically and expressed as fold change with respect to β-actin.

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 (Caetano *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 the Th2 response has been shown using peripheral T cells from fetal NFATc1$^{-/-}$ mice (Yoshida *et al.*, 1998). A recent study has shown that NFAT family members may act as tumor suppressors or oncogenes (Robbs *et al.*, 2008). In agreement with our findings showing the induction of PTEN expression, NFATc4 inhibits AP-1-dependent NIH-3T3 cell transformation, and knockdown of NFATc4 induces RasG12V-mediated cell transformation (Yao *et al.*, 2007). The different effects of NFAT isoforms in PTEN regulation imply the possible differences of NFAT isoforms in the regulation of intestinal cell homeostasis.

Our findings that NFATc1 and NFATc4 increase PTEN expression and inhibit Akt phosphorylation suggest that NFAT is an upstream regulator of PTEN/Akt signaling in intestinal cells. However, a previous study has shown that Akt is an important negative regulator of NFAT in early T cell differentiation (Patra *et al.*, 2006). Moreover, Akt inhibits NFAT signaling by reducing NFAT expression levels due to ubiquitination and proteasomal degradation in breast cancer cells (Yoeli-Lerner *et al.*, 2005). In addition to the cross-talk between Akt and NFAT, the forkhead transcription factor, FKHR, is dephosphorylated and activated by constitutively active calcineurin (Shioda *et al.*, 2007) which activates NFAT. Consistently in this study we showed that knockdown of NFATc1 increased the phosphorylation of the forkhead transcriptional factor Foxo1, which further suggests that the interaction between the NFAT and PI3K/Akt signaling pathways represents a contributory mechanism of intestinal cell differentiation.

The role of NFAT in differentiation of a variety of cell types has been shown (Delling *et al.*, 2000; Santini *et al.*, 2001; Kao *et al.*, 2009). In this study, we show for the first time that NFATc1/NFATc4 signaling is associated with intestinal cell differentiation. Results from our laboratory have shown that activation of p38 MAPK is associated with intestinal cell differentiation (Ding *et al.*, 2001). NFAT is a direct target of p38 MAPK, and inhibition of p38 MAPK leads to selective inactivation of NFAT in T cells (Wu *et al.*, 2003). NaBT treatment increased p38 activity in HT29 cells (Ding *et al.*, 2001), and moreover, in our current study, we showed increased NFAT mRNA expression after NaBT treatment. Therefore NFATc1 and NFATc4 might be involved in p38-mediated intestinal cell differentiation.

Treatment of intestinal cells with CsA inhibited the expression of terminal differentiation markers associated with the inhibition of the expression of p27^{kip1}. Our results are consistent with those of Gafter-Gvili *et al.* (2003), who showed the involvement of the calcium/calmodulin-dependent phosphatase calcineurin in keratinocyte terminal 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^{waf1} and p27^{kip1}. The PI3K/Akt pathway plays a critical role in the regulation of p27^{kip1} (Liang and Slingerland, 2003). Akt-mediated regulation of p27^{kip1} occurs at various levels and includes control of p27^{kip1} expression as well as a decrease in protein stability (Liang and Slingerland, 2003). The forkhead transcription factors regulate p27^{kip1} transcription, and Akt has been reported to inhibit the transcription of p27^{kip1} 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 p27^{kip1} 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 p27^{kip1} 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 p27^{kip1} expression; therefore it is possible that calcineurin/NFAT signaling increases intestinal cell differentiation in part through the PTEN/Akt/GSK-3/p27^{kip1} or PTEN/Akt/Foxo/p27^{kip1} 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 p27^{kip1} and Akt phosphorylation. Given the importance of the PTEN/Akt/p27^{kip1} 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

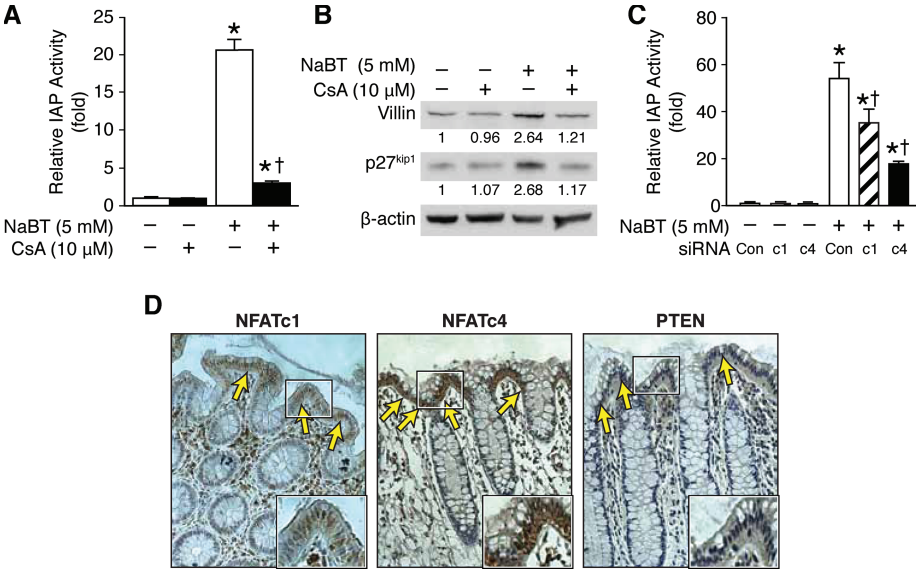

FIGURE 7: Inhibition of NFAT attenuated NaBT-mediated intestinal cell differentiation in HT29 cells. (A, B) HT29 cells were treated with CsA, NaBT, or the combination of NaBT with CsA for 24 h. Cells were lysed and alkaline phosphatase activity was determined (A). (Data represent mean ± SD; *, $P < 0.05$ vs. control; †, $P < 0.05$ vs. NaBT alone.) Villin and $p27^{kip1}$ expression was determined by Western blotting (B). Villin and $p27^{kip1}$ 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. control; †, $P < 0.05$ vs. NaBT alone.) (D) Immunohistochemical analysis of NFATc1, NFATc4, and PTEN protein expression in normal human colon. Human colon tissue sections were fixed and stained with primary anti–human NFATc1, NFATc4, or PTEN antibodies. NFATc1, NFATc4, and PTEN are specifically expressed in the differentiated region (i.e., upper crypts; arrows).

from Calbiochem (San Diego, CA). Mouse monoclonal anti-NFATc1 (clone7A6) antibody, which was used for the immunohistochemical staining, mouse monoclonal anti-NFATc2, mouse monoclonal anti-NFATc3, and goat 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-PTEN antibodies were purchased from Cell Signaling (Beverly, MA). Mouse monoclonal anti-$p27^{kip1}$ (clone 57) antibody was purchased from BD Biosciences (San Diego, CA). Mouse anti-GSK-3α/β and mouse anti-Foxo1 antibodies were from Upstate (Charlottesville, VA). Human NFATc1, NFATc2, NFATc3, NFATc4, and nontargeting control 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, $p27^{kip1}$, 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 (RQ1; Promega, Madison, WI). Synthesis of cDNA was performed with 1 μg of total RNA using the reagents in the Taqman Reverse Transcription Reagents Kit from ABI (#N8080234). 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}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_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_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].

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REFERENCES

Aurora AB, Biyashev D, Mirochnik Y, Zaichuk TA, Sanchez-Martinez C, Renault MA, Losordo D, Volpert OV (2010). NF-κB balances vascular regression and angiogenesis via chromatin remodeling and NFAT displacement. Blood 116, 475–484.

Caetano MS, Vieira-de-Abreu A, Teixeira LK, Werneck MB, Barcinski MA, Viola JP (2002). NFATC2 transcription factor regulates cell cycle progression during lymphocyte activation: evidence of its involvement in the control of cyclin gene expression. FASEB J 16, 1940–1942.

Cantley LC, Neel BG (1999). New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc Natl Acad Sci USA 96, 4240–4245.

Cheng H, Leblond CP (1974). Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian Theory of the origin of the four epithelial cell types. Am J Anat 141, 537–561.

de la Pompa JL *et al*. (1998). Role of the NF-ATc transcription factor in morphogenesis of cardiac valves and septum. Nature 392, 182–186.

Decker EL, Nehmann N, Kampen E, Eibel H, Zipfel PF, Skerka C (2003). Early growth response proteins (EGR) and nuclear factors of activated T cells (NFAT) form heterodimers and regulate proinflammatory cytokine gene expression. Nucleic Acids Res 31, 911–921.

Delling U, Tureckova J, Lim HW, De Windt LJ, Rotwein P, Molkentin JD (2000). A calcineurin-NFATc3-dependent pathway regulates skeletal muscle differentiation and slow myosin heavy-chain expression. Mol Cell Biol 20, 6600–6611.

Deschenes C, Vezina A, Beaulieu JF, Rivard N (2001). Role of p27(Kip1) in human intestinal cell differentiation. Gastroenterology 120, 423–438.

Ding Q, Wang Q, Evers BM (2001). Alterations of MAPK activities associated with intestinal cell differentiation. Biochem Biophys Res Commun 284, 282–288.

Ding QM, Ko TC, Evers BM (1998). Caco-2 intestinal cell differentiation is associated with G1 arrest and suppression of CDK2 and CDK4. Am J Physiol 275, C1193–C1200.

Duque J, Fresno M, Iniguez MA (2005). Expression and function of the nuclear factor of activated T cells in colon carcinoma cells: involvement in the regulation of cyclooxygenase-2. J Biol Chem 280, 8686–8693.

Ewton DZ, Kansra S, Lim S, Friedman E (2002). Insulin-like growth factor-I has a biphasic effect on colon carcinoma cells through transient inactivation of forkhead1, initially mitogenic, then mediating growth arrest and differentiation. Int J Cancer 98, 665–673.

Farrow B, Evers BM (2003). Activation of PPARgamma increases PTEN expression in pancreatic cancer cells. Biochem Biophys Res Commun 301, 50–53.

Gafter-Gvili A, Sredni B, Gal R, Gafter U, Kalechman Y (2003). Cyclosporin A-induced hair growth in mice is associated with inhibition of calcineurin-dependent activation of NFAT in follicular keratinocytes. Am J Physiol Cell Physiol 284, C1593–1603.

Ghosh-Choudhury N, Mandal CC, Ghosh Choudhury G (2010). Simvastatin induces derepression of PTEN expression via NFkappaB to inhibit breast cancer cell growth. Cell Signal 22, 749–758.

Ho IC, Kim JH, Rooney JW, Spiegelman BM, Glimcher LH (1998). A potential role for the nuclear factor of activated T cells family of transcriptional regulatory proteins in adipogenesis. Proc Natl Acad Sci USA 95, 15537–15541.

Hogan PG, Chen L, Nardone J, Rao A (2003). Transcriptional regulation by calcium, calcineurin, and NFAT. Genes Dev 17, 2205–2232.

Kao SC, Wu H, Xie J, Chang CP, Ranish JA, Graef IA, Crabtree GR (2009). Calcineurin/NFAT signaling is required for neuregulin-regulated Schwann cell differentiation. Science 323, 651–654.

Kim S, Domon-Dell C, Kang J, Chung DH, Freund JN, Evers BM (2004). Down-regulation of the tumor suppressor PTEN by the tumor necrosis factor-α/nuclear factor-κB (NF-κB)-inducing kinase/NF-κB pathway is linked to a default IκB-α autoregulatory loop. J Biol Chem 279, 4285–4291.

Kim S, Domon-Dell C, Wang Q, Chung DH, Di Cristofano A, Pandolfi PP, Freund JN, Evers BM (2002). *PTEN* and TNF-α regulation of the intestinal-specific *Cdx*-2 homeobox gene through a PI3K, PKB/Akt, and NF-κB-dependent pathway. Gastroenterology 123, 1163–1178.

Lee M, Park J (2006). Regulation of NFAT activation: a potential therapeutic target for immunosuppression. Mol Cells 22, 1–7.

Liang J, Slingerland JM (2003). Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression. Cell Cycle 2, 339–345.

Lin H, Sue YM, Chou Y, Cheng CF, Chang CC, Li HF, Chen CC, Juan SH (2010). Activation of a nuclear factor of activated T-lymphocyte-3 (NFAT3) by oxidative stress in carboplatin-mediated renal apoptosis. Br J Pharmacol 161, 1661–1676.

Molkentin JD, Lu JR, Antos CL, Markham B, Richardson J, Robbins J, Grant SR, Olson EN (1998). A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell 93, 215–228.

Ortega-Perez I, Cano E, Were F, Villar M, Vazquez J, Redondo JM (2005). c-Jun N-terminal kinase (JNK) positively regulates NFATc2 transactivation through phosphorylation within the N-terminal regulatory domain. J Biol Chem 280, 20867–20878.

Park S *et al*. (2009). RIP1 activates PI3K-Akt via a dual mechanism involving NF-kappaB-mediated inhibition of the mTOR-S6K-IRS1 negative feedback loop and down-regulation of PTEN. Cancer Res 69, 4107–4111.

Patra AK, Drewes T, Engelmann S, Chuvpilo S, Kishi H, Hunig T, Serfling E, Bommhardt UH (2006). PKB rescues calcineurin/NFAT-induced arrest of Rag expression and pre-T cell differentiation. J Immunol 177, 4567–4576.

Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A (2004). Rational siRNA design for RNA interference. Nat Biotechnol 22, 326–330.

Robbs BK, Cruz AL, Werneck MB, Mognol GP, Viola JP (2008). Dual roles for NFAT transcription factor genes as oncogenes and tumor suppressors. Mol Cell Biol 28, 7168–7181.

Santini MP, Talora C, Seki T, Bolgan L, Dotto GP (2001). Cross talk among calcineurin, Sp1/Sp3, and NFAT in control of p21(WAF1/CIP1) expression in keratinocyte differentiation. Proc Natl Acad Sci USA 98, 9575–9580.

Shen YH, Zhang L, Gan Y, Wang X, Wang J, LeMaire SA, Coselli JS, Wang XL (2006). Up-regulation of PTEN (phosphatase and tensin homolog deleted on chromosome ten) mediates p38 MAPK stress signal-induced inhibition of insulin signaling. A cross-talk between stress signaling and insulin signaling in resistin-treated human endothelial cells. J Biol Chem 281, 7727–7736.

Shioda N, Han F, Moriguchi S, Fukunaga K (2007). Constitutively active calcineurin mediates delayed neuronal death through Fas-ligand expression via activation of NFAT and FKHR transcriptional activities in mouse brain ischemia. J Neurochem 102, 1506–1517.

Sun H, Lesche R, Li DM, Liliental J, Zhang H, Gao J, Gavrilova N, Mueller B, Liu X, Wu H (1999). PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway. Proc Natl Acad Sci USA 96, 6199–6204.

Tamguney T, Stokoe D (2007). New insights into PTEN. J Cell Sci 120, 4071–4079.

Traber PG (1994). Differentiation of intestinal epithelial cells: lessons from the study of intestine-specific gene expression. J Lab Clin Med 123, 467–477.

Ueffing N, Schuster M, Keil E, Schulze-Osthoff K, Schmitz I (2008). Up-regulation of c-FLIP short by NFAT contributes to apoptosis resistance of short-term activated T cells. Blood 112, 690–698.

Vinciguerra M, Veyrat-Durebex C, Moukil MA, Rubbia-Brandt L, Rohner-Jeanrenaud F, Foti M (2008). PTEN down-regulation by unsaturated fatty acids triggers hepatic steatosis via an NF-κBp65/mTOR-dependent mechanism. Gastroenterology 134, 268–280.

Viola JP, Carvalho LD, Fonseca BP, Teixeira LK (2005). NFAT transcription factors: from cell cycle to tumor development. Braz J Med Biol Res 38, 335–344.
Wang Q, Wang X, Evers BM (2003). Induction of cIAP-2 in human colon cancer cells through PKCδ/NF-κB. J Biol Chem 278, 51091–51099.
Wang Q, Wang X, Hernandez A, Kim S, Evers BM (2001). Inhibition of the phosphatidylinositol 3-kinase pathway contributes to HT29 and Caco-2 intestinal cell differentiation. Gastroenterology 120, 1381–1392.
Wang Q, Zhou Y, Wang X, Chung DH, Evers BM (2007). Regulation of PTEN expression in intestinal epithelial cells by c-Jun NH2-terminal kinase activation and nuclear factor-κB inhibition. Cancer Res 67, 7773–7781.
Wang Q, Zhou Y, Wang X, Evers BM (2006). Glycogen synthase kinase-3 is a negative regulator of extracellular signal-regulated kinase. Oncogene 25, 43–50.
Wang Q, Zhou Y, Wang X, Evers BM (2008). p27 Kip1 nuclear localization and cyclin-dependent kinase inhibitory activity are regulated by glycogen synthase kinase-3 in human colon cancer cells. Cell Death Differ 15, 908–919.
Wilson CA, Browning JL (2002). Death of HT29 adenocarcinoma cells induced by TNF family receptor activation is caspase-independent and displays features of both apoptosis and necrosis. Cell Death Differ 9, 1321–1333.
Wu CC, Hsu SC, Shih HM, Lai MZ (2003). Nuclear factor of activated T cells c is a target of p38 mitogen-activated protein kinase in T cells. Mol Cell Biol 23, 6442–6454.
Wu H, Peisley A, Graef IA, Crabtree GR (2007). NFAT signaling and the invention of vertebrates. Trends Cell Biol 17, 251–260.
Yang TT, Chow CW (2003). Transcription cooperation by NFAT.C/EBP composite enhancer complex. J Biol Chem 278, 15874–15885.
Yang TT *et al*. (2006). Role of transcription factor NFAT in glucose and insulin homeostasis. Mol Cell Biol 26, 7372–7387.
Yang TT, Xiong Q, Enslen H, Davis RJ, Chow CW (2002). Phosphorylation of NFATc4 by p38 mitogen-activated protein kinases. Mol Cell Biol 22, 3892–3904.
Yao K, Cho YY, Bergen HR 3rd, Madden BJ, Choi BY, Ma WY, Bode AM, Dong Z (2007). Nuclear factor of activated T3 is a negative regulator of Ras-JNK1/2-AP-1 induced cell transformation. Cancer Res 67, 8725–8735.
Yoeli-Lerner M, Yiu GK, Rabinovitz I, Erhardt P, Jauliac S, Toker A (2005). Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT. Mol Cell 20, 539–550.
Yoshida H *et al*. (1998). The transcription factor NF-ATc1 regulates lymphocyte proliferation and Th2 cytokine production. Immunity 8, 115–124.