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Yuning Zhou
University of Kentucky, yuning.zhou@uky.edu

Qingding Wang
University of Kentucky, qingding.wang@uky.edu

Heidi L. Weiss
University of Kentucky, heidi.weiss@uky.edu

B. Mark Evers
University of Kentucky, mark.evers@uky.edu

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Nuclear factor of activated T-cells 5 increases intestinal goblet cell differentiation through an mTOR/Notch signaling pathway

Yuning Zhou[a], Qingding Wang[a,b], Heidi L. Weiss[a], and B. Mark Evers[a,b]
[a]Markey Cancer Center and [b]Department of Surgery, University of Kentucky, Lexington, KY 40536

ABSTRACT **The intestinal mucosa undergoes a continual process of proliferation, differentiation, and apoptosis that is regulated by multiple signaling pathways. Previously, we have shown that the nuclear factor of activated T-cells 5 (NFAT5) is involved in the regulation of intestinal enterocyte differentiation. Here we show that treatment with sodium chloride (NaCl), which activates NFAT5 signaling, increased mTORC1 repressor regulated in development and DNA damage response 1 (REDD1) protein expression and inhibited mTOR signaling; these alterations were attenuated by knockdown of NFAT5. Knockdown of NFAT5 activated mammalian target of rapamycin (mTOR) signaling and significantly inhibited REDD1 mRNA expression and protein expression. Consistently, overexpression of NFAT5 increased REDD1 expression. In addition, knockdown of REDD1 activated mTOR and Notch signaling, whereas treatment with mTOR inhibitor rapamycin repressed Notch signaling and increased the expression of the goblet cell differentiation marker mucin 2 (MUC2). Moreover, knockdown of NFAT5 activated Notch signaling and decreased MUC2 expression, while overexpression of NFAT5 inhibited Notch signaling and increased MUC2 expression. Our results demonstrate a role for NFAT5 in the regulation of mTOR signaling in intestinal cells. Importantly, these data suggest that NFAT5 participates in the regulation of intestinal homeostasis via the suppression of mTORC1/Notch signaling pathway.**

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INTRODUCTION

The epithelium of the mammalian intestine 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; Yeung *et al.*, 2011). Over a 3- to 5-d period, the differentiated colonocytes and enterocytes are extruded into the intestinal lumen. Mucin 2 (MUC2), the predominant structural component of the intestinal mucus layer, is exclusively and abundantly expressed by goblet cells in the colon (Tytgat *et al.*, 1995; Garg *et al.*, 2007). MUC2 is secreted into the lumen and forms the mucus layer, acting as a barrier between the luminal contents and the epithelial surface to protect the epithelium from pathogens (Shirazi *et al.*, 2000).

The mechanisms by which committed cells are allocated to the different cell lineages of the intestine are not entirely known.

The nuclear factor of activated T-cell (NFAT) family proteins are a family of transcription factors. Five NFAT family members (NFAT1–5) have been identified and shown to regulate cell differentiation and development in a number of cell types (Santini *et al.*, 2001; Luo *et al.*, 2006; Kao *et al.*, 2009; Zhou *et al.*, 2012). NFAT5 has a large C-terminal region that harbors a hypertonicity-sensitive transactivation domain such that hyperosmotic stress can also activate NFAT5. Unlike NFAT1–4 proteins, the NFAT5 subcellular distribution and its phosphorylation state are not altered by calcineurin (Lopez-Rodriguez *et al.*, 1999, 2001). NFAT5 has been implicated in such diverse processes as embryonic development and cellular

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Address correspondence to: B. Mark Evers (mark.evers@uky.edu).

Abbreviations used: ANOVA, analysis of variance; FCS, fetal calf serum; IBD, inflammatory bowel disease; MUC2, mucin 2; NEC, necrotizing enterocolitis; NFAT, nuclear factor of activated T-cell; NICD, Notch intracellular domain; NTC, nontargeting control; PI3K, phosphatidylinositol 3-kinase; REDD1 (RTP801/Dig2/DDIT4), regulated in development and DNA damage response 1; RT-PCR, reverse transcription PCR; shRNA, short hairpin RNA; siRNA, small interfering RNA; TSC2, tuberous sclerosis complex 2; UC, ulcerative colitis.

Supplemental Material can be found at:
http://www.molbiolcell.org/content/suppl/2014/07/22/mbc.E14-05-0998v1.DC1.html

migration and proliferation (Aramburu *et al.*, 2006). Previously we have shown that 1) NFATc1 and NFATc4 regulate intestinal enterocyte differentiation through induction of phosphatase and tensin homologue deleted on chromosome ten (Wang *et al.*, 2011); 2) NFATc3 inhibits mTOR via induction of regulated in development and DNA damage response 1 (REDD1) and participates in the regulation of goblet cell differentiation (Zhou *et al.*, 2012); and 3) NFAT5 represses canonical Wnt signaling via inhibition of β-catenin acetylation and participates in regulating intestinal enterocyte differentiation (Wang *et al.*, 2013). Together these results suggest differential roles of NFAT in the regulation of intestinal cell differentiation.

mTOR is a member of the phosphatidylinositol 3-kinase (PI3K)-related kinase family and regulates protein translation, cell cycle progression, and cell proliferation (Gingras *et al.*, 2001). The TOR signaling events are essential for epithelial growth, morphogenesis, and differentiation in the vertebrate intestine (Makky *et al.*, 2007). mTOR exists in two complexes: mTORC1 (containing mTOR, Raptor, etc.) and mTORC2 (containing mTOR, Rictor, etc.). The bacterially derived drug rapamycin allosterically inhibits mTOR activity (Guertin and Sabatini, 2007). mTORC1 is partially sensitive to rapamycin treatment, whereas mTORC2 is believed to be rapamycin insensitive (Guertin and Sabatini, 2007). The REDD1 (RTP801/Dig2/DDIT4) protein recently was found to be a negative regulator of mTORC1 signaling (Corradetti *et al.*, 2005). It has been proposed that REDD1 inhibits mTORC1 by displacing tuberous sclerosis complex 2 (tuberin or TSC2) from the 14-3-3 binding protein, allowing TSC2 to inhibit mTORC1 (DeYoung *et al.*, 2008). Consequently loss of REDD1 in this setting induces further mTORC1 activation that drives tumorigenesis (DeYoung *et al.*, 2008).

The Notch pathway plays a critical role in intestinal cell differentiation (Vooijs *et al.*, 2011). Ligand engagement causes the Notch intracellular domain (NICD) to be cleaved from the membrane and shuttled to the nucleus, where it forms and establishes a transcriptional activator complex leading to the expression of Notch target genes such as Hes1. Inhibition of the Notch/Hes1 pathway leads to an increase in the expression of MUC2, a goblet cell differentiation marker, and the number of goblet cells (Vooijs *et al.*, 2011). We have reported that the REDD1/TSC2 axis plays a role in the regulation of MUC2 expression in intestinal cells (Zhou *et al.*, 2012). However, whether mTOR regulates MUC2 expression and the potential functional interaction of mTORC1 and Notch in intestinal cells is not known.

Previously we showed that NFAT5 represses canonical Wnt signaling and participates in regulating intestinal cell differentiation. In our current study, we found that NFAT5 inhibits mTOR signaling through regulation of REDD1 expression. In addition, we showed that mTORC1 is a positive regulator of Notch signaling in intestinal cells. Moreover, we found that NFAT5 inhibits mTORC1 and contributes to the induction of MUC2 expression. Our study demonstrates the regulation of mTORC1 signaling by NFAT5 and a novel role of mTORC1 signaling in the regulation of intestinal cell differentiation.

RESULTS

NFAT5 activation increases REDD1 expression in HT29 cells

Previously we showed that NFAT5 contributes to intestinal enterocyte differentiation (Wang *et al.*, 2013). In an effort to better delineate the mechanism underlying the effect of NFAT5 in intestinal cells, we performed a whole-genome expression array (Supplemental Table S1). Knockdown of NFAT5 in HT29 cells resulted in a significant decrease of ATF4 target genes, including REDD1 (also known as DDIT4; Jin *et al.*, 2011), cation transport regulator-like protein 1 (CHAC1; Mungrue *et al.*, 2009) and G1 arrest and DNA damage 153 (GADD153, also known as DDIT3; Fawcett *et al.*, 1999; Mungrue *et al.*, 2009). As REDD1 is an mTOR repressor, these results suggest that NFAT5 may inhibit mTOR signaling through the regulation of REDD1 expression in intestinal cells.

To better delineate the role of NFAT5 in REDD1 regulation, we transfected HT29 cells with nontargeting control (NTC) small interfering RNA (siRNA) or siRNA targeting NFAT5. REDD1 mRNA expression was detected by real time reverse transcription PCR (RT-PCR). As shown in Figure 1A, transfection of NFAT5 siRNA decreased REDD1 mRNA expression compared with cells transfected with NTC siRNA. The specificity and efficiency of knockdown of NFAT5 were confirmed by real-time RT-PCR as shown in Figure 1B. To address whether REDD1 mRNA repression paralleled the decrease in REDD1 protein, we performed Western blotting (Figure 1C). Decreased REDD1 protein expression was noted with NFAT5 knockdown and, as a result of mTOR activation, NFAT5 knockdown increased the expression of the phosphorylated Ser-6 (p-Ser-6) compared with cells transfected with NTC siRNA. Consistently, decreased REDD1 protein expression was also found in HT29 cells stably transfected with the short hairpin RNA (shRNA) targeting NFAT5 compared with that in the cells transfected with the control shRNA (Supplemental Figure S1). Therefore NFAT5 inhibition decreases REDD1 expression and activates mTOR signaling.

NFAT5 is an osmoregulator (Aramburu *et al.*, 2006); treatment with sodium chloride (NaCl) increases NFAT5 protein expression in intestinal cells (Chen *et al.*, 2011). For further confirmation that the role of NFAT5 in the regulation of REDD1, HT29 cells transfected with NTC siRNA or siRNA targeting NFAT5 were treated with 100 mM NaCl to stimulate hypertonic shock. Whole-cell lysates were analyzed by Western blotting for detection of REDD1 protein expression (Figure 1D). Treatment with NaCl increased NFAT5 expression. Furthermore, treatment with NaCl increased the expression of REDD1 and, as a result of mTOR inhibition, decreased phosphorylation of Ser-6; the increased REDD1 and decreased p-Ser-6 expression were attenuated by knockdown of NFAT5. These results indicate that NFAT5 activation increases REDD1 expression and inhibits the mTORC1 signal pathway.

NFAT5 regulates REDD1 expression in HCT116, SW480, and Caco-2 cells

To determine whether NFAT5 regulates REDD1 expression in other intestinal cell lines, we transfected HCT116, SW480, and Caco-2 cells with siRNA targeting NFAT5. Knockdown of NFAT5 decreased REDD1 protein expression and increased p-Ser-6 in these cell lines (Figure 2A). For further confirmation of NFAT5-mediated REDD1 induction, HCT116 and Caco-2 cells were transfected with empty vector or plasmid encoding Myc-tagged NFAT5. As shown in Figure 2B, overexpression of NFAT5 increased REDD1 protein expression in HCT116 and Caco-2 cells. These data indicate a role for NFAT5 in REDD1 induction in intestinal cells.

REDD1/mTOR/Notch pathway is involved in the regulation of goblet cell marker MUC2

Recently we showed that knockdown of mTORC1 repressor REDD1 decreased the expression of the goblet cell differentiation marker MUC2 (Zhou *et al.*, 2012). Notch signaling plays a critical role in the regulation of goblet cell differentiation. Inhibition of the Notch pathway leads to an increase in MUC2 expression and the number of goblet cells (Vooijs *et al.*, 2011; Yeung *et al.*, 2011). To determine whether REDD1 regulates Notch signaling in intestinal cells, we first transfected HT29 cells with NTC siRNA or siRNA targeting REDD1

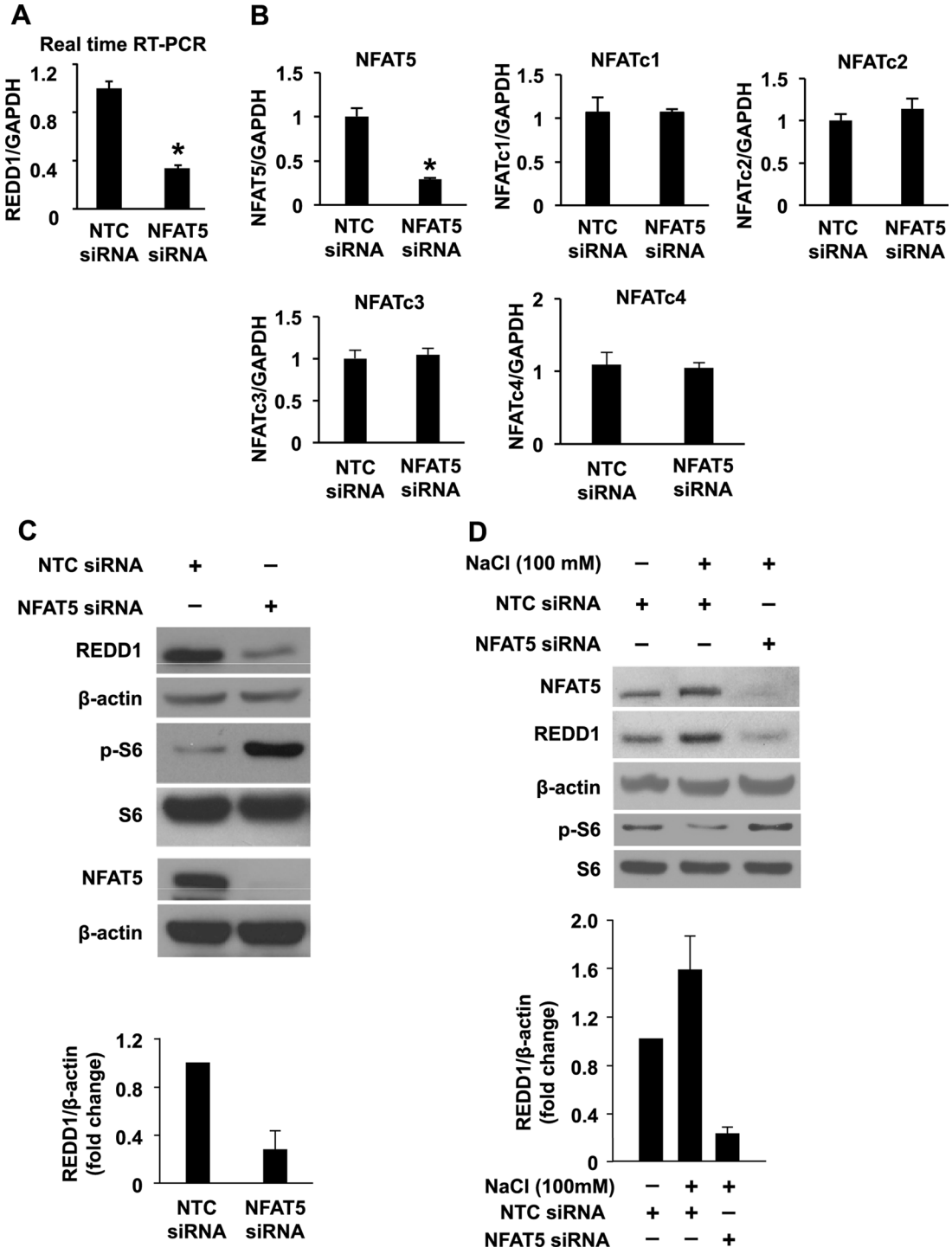

FIGURE 1: NFAT5 regulates REDD1 expression in HT29 cells. (A and B) HT29 cells were transfected with NFAT5 or NTC siRNA. After 48-h incubation, transfected cells were lysed, total RNA was extracted, and real-time RT PCR was performed for analysis of REDD1 (A) and NFAT5 and NFATc1, NFATc2, NFATc3, and NFATc4 mRNA expression (B). (Data represent mean ± SD; *, $p < 0.01$ vs. NTC siRNA as determined by ANOVA.) (C) HT29 cells were transfected with NFAT5 or NTC siRNA. After 48-h incubation, transfected cells were lysed, and Western blot analysis was performed using antibodies against REDD1, p-Ser-6, Ser-6, NFAT5, and β-actin. (D) HT29 cells were transfected with NFAT5 or NTC siRNA. After 24-h incubation, transfected cells were treated with 100 mM NaCl for an additional 24 h and subjected to Western blot analysis using antibodies against NFAT5, REDD1, p-Ser-6, total Ser-6, and β-actin. REDD1 signals from three separate experiments were quantitated densitometrically and expressed as fold change with respect to β-actin.

(Figure 3A). Knockdown of REDD1 increased the Notch transactivator NICD domain and Hes1 expression, suggesting REDD1 negative regulation of Notch signaling in HT29 cells. We next treated HT29 cells with rapamycin. As shown in Figure 3B, treatment with rapamycin decreased the Notch transactivator NCID domain and Hes1 expression, suggesting mTOR-positive regulation of Notch signaling.

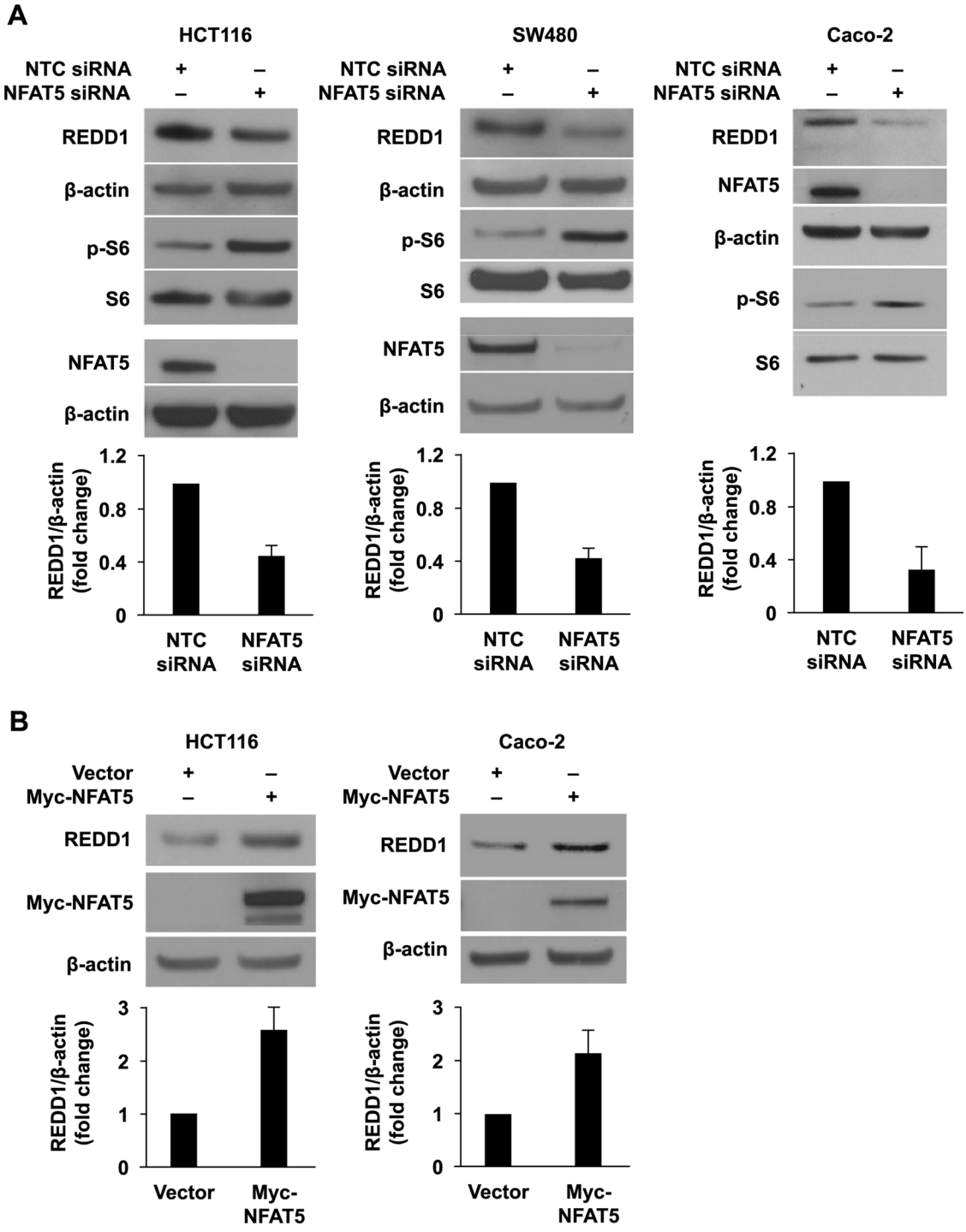

FIGURE 2: NFAT5 regulates REDD1 expression in HCT116, SW480 and Caco-2 cells. (A) HCT116, SW480, and Caco-2 cells were transfected with NFAT5 or NTC siRNA. After 48-h incubation, transfected cells were lysed, and Western blot analysis was performed using antibodies against REDD1, p-Ser-6, Ser-6, NFAT5, and β-actin. (B) HCT116 and Caco-2 cells were transiently transfected with empty vector or a construct expressing Myc-NFAT5. Forty-eight hours after transfection, cells were lysed, and Western blot analysis was performed using antibodies against REDD1, Myc-tag, and β-actin. REDD1 signals from three separate experiments were quantitated densitometrically and expressed as fold change with respect to β-actin.

Previously we showed that knockdown of REDD1 or TSC2 activates mTOR and significantly decreases MUC2 expression (Zhou *et al.*, 2012), suggesting a role for mTOR in the regulation of intestinal goblet cell differentiation. In addition, we have shown that inhibition of PI3K/Akt, an upstream and downstream regulator of mTOR, enhances intestinal enterocyte differentiation (Wang *et al.*, 2001). In this study, we found that decreased mTOR activity was associated with enterocyte differentiation (Figures S2 and S3). For testing whether mTORC1 regulates goblet cell differentiation, HT29 cells were treated with rapamycin (100 nM) for 24 h and MUC2 mRNA

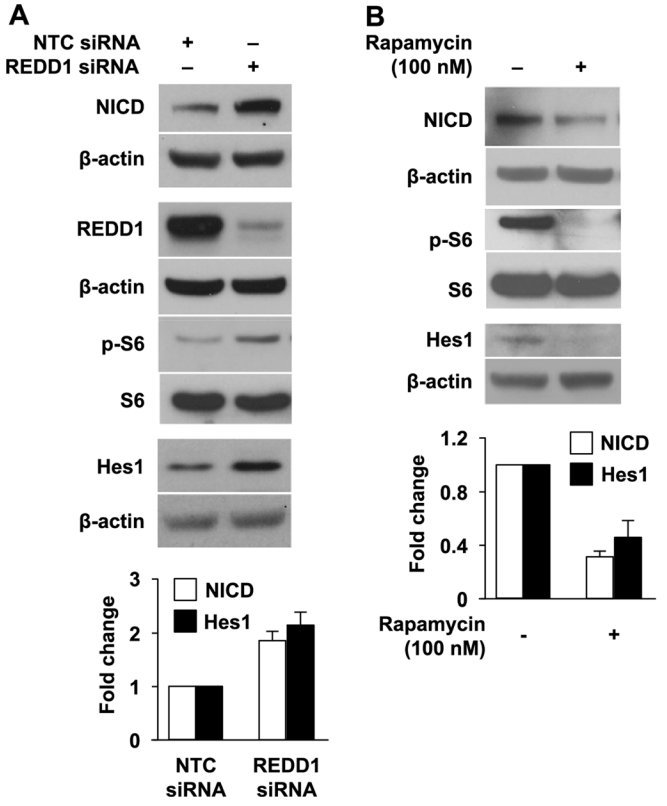

FIGURE 3: Regulation of MUC2 mRNA expression by mTORC1/Notch signaling pathway. (A) HT29 cells were transfected with NTC siRNA or siRNA targeting REDD1. (B) HT29 cells were treated with 100 nM rapamycin for 24 h. Total protein was extracted, and Western blotting was performed using anti-NICD, REDD1, anti–p-Ser-6, anti-Ser-6, Hes1, and anti–β-actin antibodies. NICD and Hes1 signals from three separate experiments were quantitated densitometrically and expressed as fold change with respect to β-actin. (C) HT29 cells were treated with 100 nM rapamycin for 24 h; total RNA was extracted and MUC2 mRNA levels were determined by real-time RT-PCR. (Data represent mean ± SD; *, $p < 0.05$ vs. control as determined by ANOVA.)

levels were analyzed by real time RT-PCR. Consistent with knockdown of REDD1 or TSC2 (Zhou *et al.*, 2012), treatment with rapamycin increased MUC2 mRNA expression in HT29 cells (Figure 3C). Taken together, our results demonstrate the regulation of intestinal goblet cell differentiation by mTORC1/Notch signaling.

NFAT5 inhibits Notch signaling and increases MUC2 expression in intestinal cells

We have shown that NFAT5 inhibits mTORC1 through the regulation of REDD1 expression. In addition, we found that inhibition of mTORC1 inhibits Notch signaling. We next determined whether NFAT5 regulates Notch signaling. HT29 and HCT116 cells were transfected with NTC siRNA or siRNA targeting NFAT5, and the expression of NICD and Hes1 was determined (Figure 4A). Knockdown of NFAT5 increased the protein expression of NICD and Hes1. For further confirmation of the regulation of Notch by NFAT5, Caco-2 cells were transfected with Myc-tagged NFAT5, and the expression of NICD and Hes1 was detected by Western blotting. As shown in Figure 4B, overexpression of NFAT5 decreased NICD and Hes1 protein expression in Caco-2 cells, suggesting the negative regulation of Notch by NFAT5 in intestinal cells.

Our results demonstrated the regulation of mTORC1 by NFAT5. In addition, we showed a role for mTORC1/Notch signaling in the regulation of goblet cell differentiation. MUC2 is expressed in HT29, Caco-2, and HCT116 cells (Barros *et al.*, 2011; Mak *et al.*, 2012; Zhou *et al.*, 2012). HT29 and Caco-2 cell lines have been extensively used as in vitro models for delineating potential pathways leading to differentiation (Wang *et al.*, 2001; Garg *et al.*, 2007; Gobbi *et al.*, 2012; Sodhi *et al.*, 2012). To determine whether NFAT5 regulates goblet cell differentiation, we used RNA from HT29 and Caco-2 cells transfected with NTC siRNA or NFAT5 siRNA for analysis of MUC2 mRNA expression by real time RT-PCR. Knockdown of NFAT5 significantly decreased MUC2 mRNA expression in HT29 and Caco-2 cells (Figure 5A). For further confirmation of the regulation of MUC2 by NFAT5, HCT116 and Caco-2 cells were transfected with empty vector or plasmid encoding Myc-tagged NFAT5. As shown in Figure 5B, overexpression of NFAT5 increased MUC2 mRNA expression in HCT116 and Caco-2 cells. These data indicate a role for NFAT5 in MUC2 induction in intestinal cells. Taken together, our results demonstrate the regulation of intestinal goblet cell differentiation by the NFAT5/mTORC1/Notch axis.

DISCUSSION

We have shown that induction of REDD1 expression enhances, whereas knockdown of REDD1 attenuates, goblet cell differentiation in the HT29 cell line (Zhou *et al.*, 2012). In our present study, we demonstrate by complementary approaches (i.e., siRNA knockdown and overexpression) that NFAT5 regulates REDD1 expression and inhibits mTOR signaling. In addition, mTOR activates but NFAT5 inhibits Notch signaling. Concomitantly the inhibition of mTOR or overexpression of NFAT5 increases the expression of MUC2, a marker of goblet cell differentiation, but knockdown of NFAT5 decreases MUC2 expression. Taken together, our results suggest that intestinal goblet cell differentiation is regulated by NFAT5/REDD1/mTOR signaling.

NFAT5 has been implicated in the signaling pathways regulating cell differentiation of various cell types (O'Connor *et al.*, 2007; Berga-Bolanos *et al.*, 2010). Our previous results showed that NFAT5 inhibits β-catenin signaling and participates in the regulation of intestinal enterocyte differentiation (Wang *et al.*, 2013). In this study, we showed that NFAT5 increases REDD1 expression, inhibits mTOR-dependent Notch signaling, and contributes to MUC2 expression in intestinal cells. Wnt and Notch signaling pathways play important roles in maintaining and regulating intestinal homeostasis (Yeung *et al.*, 2011). As intestinal cells reach the mid-crypt region, Notch and β-catenin/TCF activity is down-regulated, resulting in cell cycle arrest and differentiation (van de Wetering *et al.*, 2002; Yeung *et al.*, 2011). The fact that NFAT5 antagonizes Wnt and Notch signaling is in agreement with the expression patterns of these genes. The expression pattern of NFAT5 in the more differentiated portions of the intestinal mucosa (Wang *et al.*, 2013) suggest that the balance between NFAT5/REDD1 and Wnt or Notch signaling plays an important role in the maintenance of intestinal homeostasis at the crypt–villus axis in intestine. We showed that knockdown of NFAT5 decreased the expression of REDD1. Moreover, REDD1

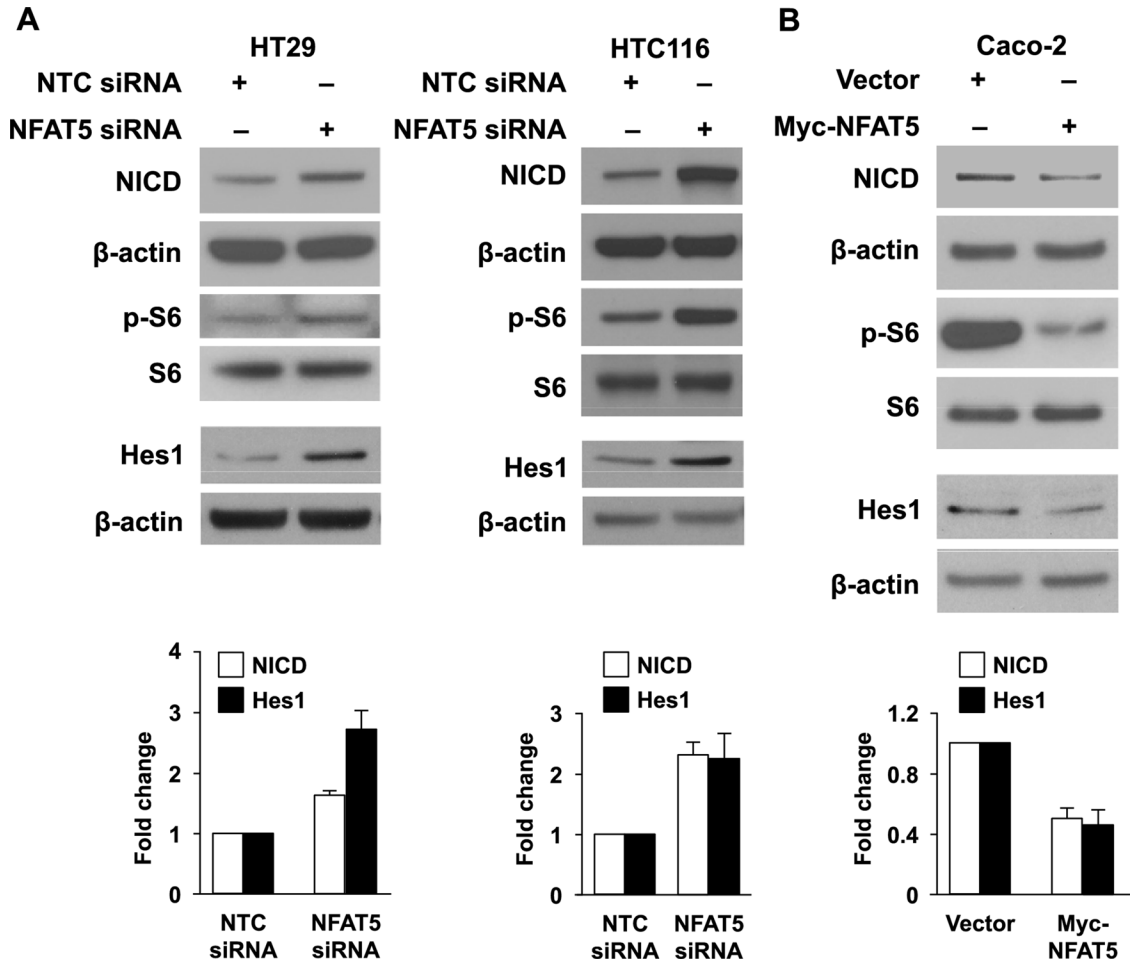

FIGURE 4: NFAT5 inhibits Notch signaling. (A) HT29 or HCT116 cells were transfected with NTC siRNA or siRNA targeting NFAT5. (B) Caco-2 cells were transfected with control vector or NFAT5 plasmid. After 48-h incubation, total protein was extracted and subjected to Western blotting using anti-NICD, anti–p-Ser-6, anti-Ser-6, Hes1, and anti–β-actin antibodies. NICD and Hes1 signals from three separate experiments were quantitated densitometrically and expressed as fold change with respect to β-actin.

inhibits Wnt/TCF activity (Feng *et al.*, 2012). Therefore these findings suggest that NFAT5 positively regulates the expression of REDD1 and thus contributes to its inhibitory role not only on mTOR but also on Wnt signaling in intestinal cells.

Although NFAT5 inhibits mTOR and Notch signaling and also contributes to MUC2 expression, NFAT5 may also regulate goblet cell differentiation through Wnt signaling. We showed that NFAT5 negatively regulates Wnt signaling in intestinal cells (Wang *et al.*, 2013). NFAT proteins repress canonical Wnt signaling via their interaction with dishevelled protein and participate in regulating neural progenitor cell proliferation and differentiation (Huang *et al.*, 2011). In the intestine, the Wnt signaling pathway has been implicated in regulating the balance between proliferation and differentiation (van de Wetering *et al.*, 2002). Wnt signaling controls the differentiation of the secretory cell lineage of the epithelium (Yeung *et al.*, 2011). High Wnt activity interferes with goblet cell differentiation (Sansom *et al.*, 2004), and inhibition of Wnt signaling favors the goblet cell phenotype (Yeung *et al.*, 2011; Heuberger *et al.*, 2014). Moreover, Wnt stimulates cell growth by activating the TSC-mTOR pathway (Inoki *et al.*, 2006). Taken together, these results have shown that multisignaling pathways are integrated and contribute to the maintenance of intestinal cell homeostasis.

Our results demonstrate the regulation of intestinal goblet cell differentiation by NFAT5/REDD1/mTOR/Notch signaling pathway. Notch signaling is required for formation and self-renewal of tumor-initiating cells and for repression of secretory cell differentiation in colon cancer (Sikandar *et al.*, 2010). Inhibition of Notch activation using pharmacological approaches or genetic manipulation results in the predominance of the goblet cell lineage in the intestine and colon (van Es *et al.*, 2005). mTOR promotes Notch activation in certain types of cells (Ma *et al.*, 2010; Pear, 2010; Francipane and Lagasse, 2013; Li *et al.*, 2014), although rigorous proof was lacking that mTOR regulates Notch-1 activation in intestinal cells. In the present study, we show that the decreased mTORC1 activity is associated with increased intestinal cell differentiation. Signal transducer and activator of transcription 3 (STST3) has been identified as a downstream molecule of mTORC1 (Kim *et al.*, 2009) and acts as a mediator between mTORC1 and Notch signaling pathways (Ma *et al.*, 2010; Li *et al.*, 2014). In addition, our previous results showed that the progressive decrease of STAT3 protein level and binding activity occurs at a time associated with increased Caco-2 cell differentiation (Wang and Evers, 1999). However, loss of STAT3 does not affect intestinal cell differentiation (Mair *et al.*, 2010). STAT3 signaling may not be involved in mTOR-mediated regulation of

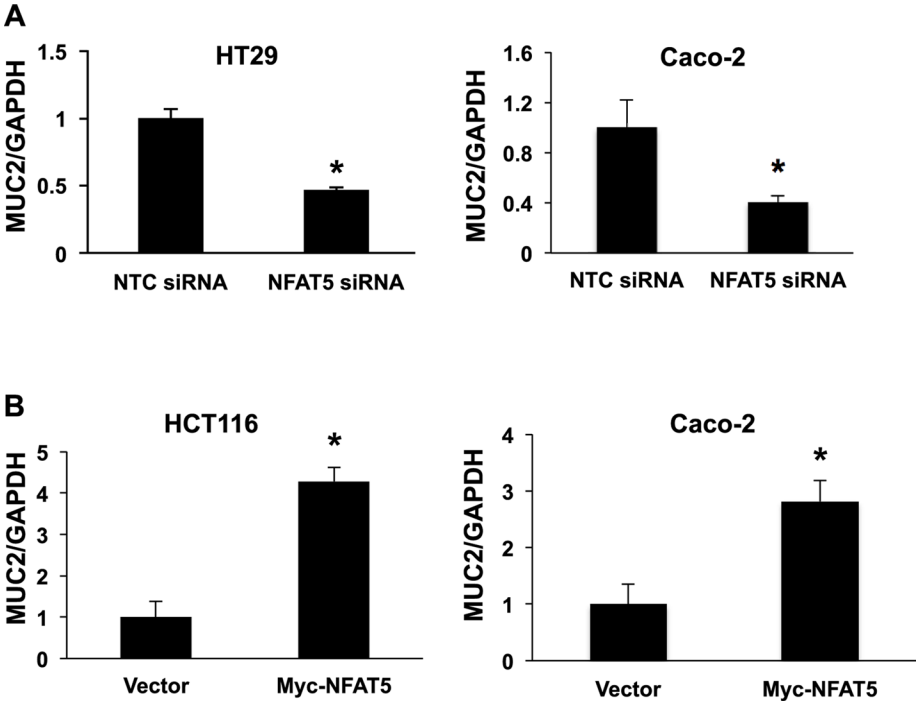

FIGURE 5: NFAT5 regulation of MUC2 mRNA expression. (A) HT29 and Caco-2 cells were transfected with NTC siRNA or siRNA targeting NFAT5. (B) HCT116 and Caco-2 cells were transfected with control vector or NFAT5 plasmid. After 48-h incubation, total RNA was extracted, and MUC2 mRNA levels were determined by real-time RT-PCR. (Data represent mean ± SD; *, $p < 0.01$ vs. control siRNA as determined by ANOVA.)

goblet cell differentiation. How mTORC1 regulates Notch signaling and goblet cell differentiation remains to be defined.

Mucins, secreted by goblet cells, form a semipermeable mucous layer between the lumen and the intestinal epithelium, thus protecting the epithelial surface of the gastrointestinal tract (Shirazi *et al.*, 2000; Einerhand *et al.*, 2002). Abnormal regulation of goblet cell differentiation has been shown to be associated with the pathogenetic mechanisms leading to ulcerative colitis (UC), one type of inflammatory bowel disease (IBD), and necrotizing enterocolitis (NEC). In UC, the mucus layer, which protects the host from the enormous amount of luminal microbes, is defective (Gersemann *et al.*, 2012). This is accompanied by an insufficient differentiation of goblet cells arising from the intestinal stem cells. Defects in this intestinal shield enable the luminal microorganisms to attack the epithelium. The number of MUC2-positive goblet cells is significantly decreased in neonatal rats with NEC (Clark *et al.*, 2006). Consistently, Notch activity is increased in intestinal tissues of patients with NEC, and Notch activation is required for the induction of NEC in mice (Sodhi *et al.*, 2012; Lu *et al.*, 2014). Inhibition of Notch signaling leads to increased goblet cells and reduced severity of experimental NEC (Sodhi *et al.*, 2012).

Abnormal activation of mTOR signaling is also consistently observed in the colonic epithelial cells of human IBD patients with active disease (Deng *et al.*, 2010). Inhibitors of TORC1 (rapamycin and rapalogs) have proven to be effective in IBD (Garcia-Maurino *et al.*, 2012). Moreover, gut ischemia has been implicated in the pathogenesis of NEC. Treatment with rapamycin significantly attenuated ischemia/reperfusion injury in the gut (Puglisi *et al.*, 1996). Our results show that rapamycin inhibits Notch, suggesting that the potential role of rapamycin in the treatment of UC and NEC may be through the inhibition of Notch and the increase of goblet cell differentiation. Although the direct link between the intestinal inflammatory process and NFAT5 activation is not known, patients with IBD present with increased luminal hyperosmolality, which may result in NFAT5 activation in intestinal epithelial cells (Neuhofer, 2010). In addition, we have shown that NFAT5 is abundantly expressed in intestinal epithelial cells (Wang *et al.*, 2013). Therefore, since results in our current study show that NFAT5 contributes to goblet cell differentiation, and NFAT5 has previously been shown to protect cells from osmotic stress (Lopez-Rodriguez *et al.*, 2004; Aramburu *et al.*, 2006), it is possible that a feedback activation of NFAT5 may play a defensive role in the process of IBD. The use of conditional NFAT5 knockout mice will be helpful to better delineate this process. Nevertheless, we now identify a novel role for NFAT5 in intestinal epithelial differentiation, suggesting that further exploration of the NFAT5/mTOR/Notch signaling pathway may offer novel therapeutic approaches to UC and NEC.

In conclusion, our results demonstrate that NFAT5 contributes to the regulation of the mTOR repressor REDD1 and thus inhibits mTORC1. Furthermore, our study demonstrates a positive regulation of Notch by mTORC1 and a novel role for the NFAT5/mTORC1/Notch axis in the regulation of goblet cell differentiation (summarized in Figure 6). In the normal intestine, Notch signaling regulates cell proliferation in the base of crypts and inhibits goblet differentiation in the upper portion of crypts. NFAT5 is expressed mainly in differentiated epithelial cells. Our findings suggest that NFAT5 plays a critical role in the regulation of intestinal homeostasis through negative regulation of Wnt and mTORC1-dependent Notch signaling.

MATERIALS AND METHODS

Materials

Sodium butyrate and antibody against β-actin were purchased from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal anti-NFAT5 antibody was from Thermo Fisher Scientific (Rockford, IL). Rabbit polyclonal anti-REDD1 antibody was purchased from Proteintech Group (Chicago, IL). Rabbit monoclonal anti-Notch1 (NICD) antibody was from Epitomics (Burlingame, CA). Rabbit anti-Hes1 antibody was from Abcam (Cambridge, MA). Rapamycin and antibodies against phospho-mTOR (Ser-2448), mTOR, p-Ser-6 (pS235/236), and Ser-6 antibodies were purchased from Cell Signaling (Beverly, MA). The plasmid encoding human Myc-tagged NFAT5 was from Alex Toker (Boston, MA). Human NFAT5, REDD1, and NTC siRNA SMARTpool were purchased from Dharmacon (Lafayette, CO). siRNA SMARTpool, consisting of four siRNA duplexes, was designed using an algorithm made up of 33 criteria and parameters that effectively eliminate nonfunctional siRNA (Reynolds *et al.*, 2004). Non-targeting Control Pool (D-001810-10-50) is designed and microarray tested for minimal targeting of human, mouse, or rat genes, according to the manufacturer's description.

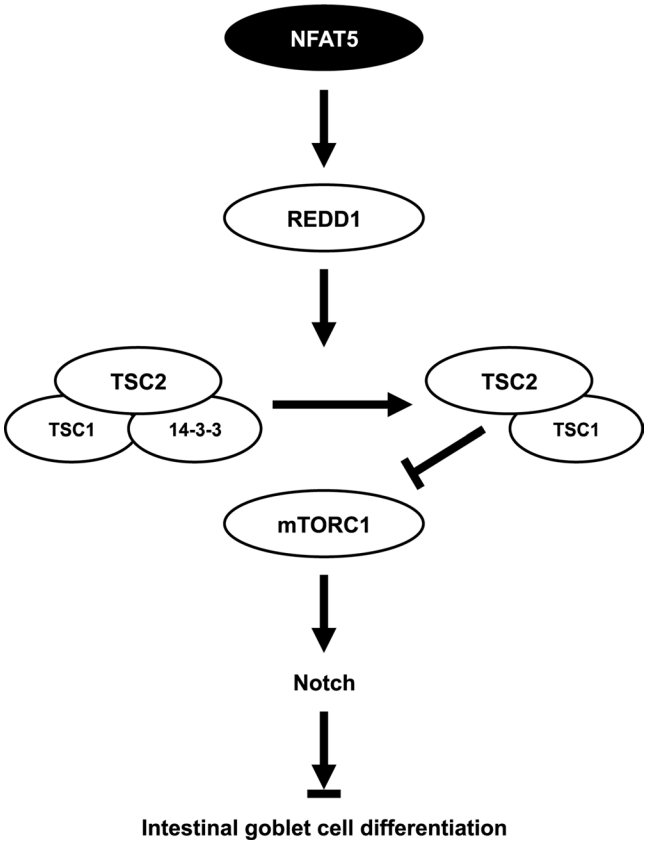

FIGURE 6: NFAT5/REDD1/mTOR/Notch pathway model. REDD1 is proposed to inhibit mTORC1 by displacing TSC2 from the 14-3-3 binding protein, allowing TSC2 to inhibit mTORC1. Activation of NFAT5 increases REDD1 expression resulting in inhibition of mTORC1 signaling, leading to decreased Notch signaling and an increase of goblet cell differentiation.

Cell culture, transfection, and treatment

The human colon cancer cell lines HT29 and HCT116 were maintained in McCoy's 5A supplemented with 10% fetal calf serum (FCS). SW480 and Caco-2 were maintained in DMEM supplemented with 10% FCS and MEM supplemented with 15% FCS, respectively. Cells were transfected with the siRNA duplexes and plasmids by electroporation (Gene Pulser; Bio-Rad, Hercules, CA) and lipofectamine 2000 (Invitrogen, CA), respectively, as we have described previously (Wang *et al.*, 2003, 2006).

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. REDD1, NFAT5, NICD, Hes1, phospho-mTOR, mTOR, p-Ser-6, Ser-6, 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 reagents in the Taqman Reverse Transcription Reagents Kit (ABI N8080234). TaqMan probe and primers for human NFATc1, NFATc2, NFATc3, NFATc4, NFAT5, REDD1, MUC2, 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).

Statistical analysis

Comparisons of real time quantitative reverse transcription PCR (qRT-PCR) were performed between control siRNA versus NFAT5 siRNA and control vector versus Myc-NFAT5, or across rapamycin treatment groups using a two-sample *t* test or analysis of variance (ANOVA) with pairwise comparisons using a contrast statement. Bar graphs represent mean ± SD levels in each group. p values < 0.05 were considered statistically significant.

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