

Inhibition of Smurf2 translation by miR-322/503 modulates TGF- β /Smad2 signaling and intestinal epithelial homeostasis

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ABSTRACT Smad ubiquitin regulatory factor 2 (Smurf2) is an E3 ubiquitin ligase that regulates transforming growth factor β (TGF- β)/Smad signaling and is implicated in a wide variety of cellular responses, but the exact mechanisms that control Smurf2 abundance are largely unknown. Here we identify microRNA-322 (miR-322) and miR-503 as novel factors that regulate Smurf2 expression posttranscriptionally. Both miR-322 and miR-503 interact with *Smurf2* mRNA via its 3'-untranslated region (UTR) and repress Smurf2 translation but do not affect total *Smurf2* mRNA levels. Studies using heterologous reporter constructs reveal a greater repressive effect of miR-322/503 through a single binding site in the *Smurf2* 3'-UTR, whereas point mutation of this site prevents miR-322/503-induced repression of Smurf2 translation. Increased levels of endogenous Smurf2 via antagonism of miR-322/503 inhibits TGF- β -induced Smad2 activation by increasing degradation of phosphorylated Smad2. Furthermore, the increase in Smurf2 in intestinal epithelial cells (IECs) expressing lower levels of miR-322/503 is associated with increased resistance to apoptosis, which is abolished by Smurf2 silencing. These findings indicate that miR-322/503 represses Smurf2 translation, in turn affecting intestinal epithelial homeostasis by altering TGF- β /Smad2 signaling and IEC apoptosis.

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INTRODUCTION

Ubiquitin modification is implicated in many aspects of cellular physiology by tagging proteins for proteasomal degradation or incorporation into other regulatory complexes (Hershko and Ciechanover, 1998; Hoeller *et al.*, 2006). E3 ubiquitin ligases play a central role in the ubiquitination process, catalyzing the attachment of ubiquitin moieties to target proteins. Smad ubiquitin regulatory factor 2

(Smurf2), a HECT-domain E3 ubiquitin ligase, was initially identified as a negative regulator of transforming growth factor β (TGF- β) signaling by targeting receptor-regulated Smads, including Smad1, Smad2, and Smad3, and the type I receptor (Zhang *et al.*, 2001; Izzi and Attisano, 2004; Lönn *et al.*, 2009). After ligand binding, Smad proteins are phosphorylated by TGF- β receptors and translocate to the nucleus, where they control the transcription of target genes (Ten Dijke and Hill, 2004; Massague *et al.*, 2005; Kamato *et al.*, 2013). The TGF- β -activated Smads must be removed through ubiquitin-dependent degradation in order to exert the biological effects of different ligands in a timely manner. Smurf2 interacts with phosphorylated Smads and induces their rapid ubiquitination and degradation (Lo and Massague, 1999; Gao *et al.*, 2009; David *et al.*, 2013). Subsequent studies extended the repertoire of Smurf2 substrates and show a broader spectrum of Smurf2 biological functions (Li and Seth, 2004; Schwamborn *et al.*, 2007). Induction in Smurf2 expression is linked to telomere attrition, and forced expression of Smurf2 enhances senescence in human fibroblasts (Zhang *et al.*, 2004). Smurf2 also promotes p53 degradation by stabilizing the E3 ligase MDM2 (Nie *et al.*,

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Abbreviations used: Cdk2, cyclin-dependent kinase 2; CHX, cycloheximide; CR, coding region; CUGBP1, CUG-binding protein 1; GFP, green fluorescent protein; IEC, intestinal epithelial cell; miRNAs, microRNAs; Q-PCR, real-time quantitative PCR; Smurf2, smad ubiquitin regulatory factor 2; TGF- β , transforming growth factor- β ; UTRs, untranslated regions.

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2010) and represses tumor growth by controlling the chromatin landscape and genome stability through RNF20 (Blank et al., 2012).

MicroRNAs (miRNAs) are a class of small noncoding RNAs that posttranscriptionally repress the expression of target genes and regulate a variety of cellular processes (Kedde et al., 2007; Omer et al., 2009; Mendell and Olson, 2012). The regulation of mRNA stability and translation by miRNAs is a major mechanism by which mammalian cells control gene expression in response to changes in environmental conditions (Garneau et al., 2007; Mendell and Olson, 2012), but little is known about their roles in the regulation of the ubiquitin system. Generally, miRNAs function by binding to the 3'-untranslated regions (UTRs) of target mRNAs, destabilizing them and/or inhibiting their translation (Eulalio et al., 2008; Leung and Sharp, 2010). Although the exact functions of miRNAs in human development and physiology are not fully elucidated, differential expression of some miRNAs has been linked to several human pathologies. Recently miRNAs have also emerged as major regulators of homeostasis of the intestinal epithelium (Xiao et al., 2011; Ye et al., 2011; Cui et al., 2012; Zhuang et al., 2013), the tissue with the most rapid turnover rate in the body (Sato and Clevers, 2013). For example, miR-503 represses translation of CUG-binding protein 1 (CUGBP1), in turn modulating intestinal epithelial cell (IEC) proliferation and apoptosis (Cui et al., 2012). Other important miRNAs implicated in modulating intestinal epithelial homeostasis are 1) the antiproliferative miRNAs miR-222 (Xiao et al., 2011) and miR-29b (Xiao et al., 2013), 2) the proproliferative miRNAs miR-21 and miR-155 (Bakirtzi et al., 2011), 3) the cell migration-regulatory miR-195 (Zhuang et al., 2013), and 4) the epithelial barrier-regulatory miRNA miR-122b (Ye et al., 2011).

miR-322 is an abundant miRNA that is conserved among many species and is clustered with miR-503 on chromosome X (Griffiths-Jones et al., 2006). miR-322 and miR-503 were studied in myogenesis, where they were shown to promote cell cycle quiescence and differentiation by down-regulating Cdc25 (Sarkar et al., 2010). Two recent studies show that miR-322 regulates the vascular smooth muscle cell phenotype and neointimal formation by inhibiting expression of cyclin D1- and Ca²⁺-regulating proteins calumenin and stromal-interacting molecule 1 (Stim1; Merlet et al., 2013) and that miR-322 and its target, Tob2, control osteogenesis through modulation of *Osx* mRNA degradation (Gamez et al., 2013). Our previous studies show that small intestinal mucosal atrophy after polyamine depletion or fasting is associated with a decrease in the levels of miR-322/503 (Xiao et al., 2011, 2013; Cui et al., 2012). The results reported here further indicate that miR-322/503 regulates *Smurf2* expression and modulates intestinal epithelial homeostasis by altering IEC apoptosis. miR-322/503 directly interacts with *Smurf2* mRNA via its 3'-UTR and represses *Smurf2* translation, thus positively modulating the TGF- β /Smad2 signaling pathway. Moreover, the elevated levels of *Smurf2* achieved by silencing miR-322/503 increase the resistance of IECs to apoptosis.

RESULTS

Smurf2 mRNA is a novel target of miR-322/503

Using standard online software (TargetScan and RNA22), we predicted three binding sites for miR-322/503 within the 3'-UTR of the *Smurf2* mRNA (Figure 1A and Supplemental Table S1), suggesting that the *Smurf2* mRNA is a potential target of miR-322/503. To elucidate the involvement of miR-322/503 in the regulation of *Smurf2* expression, we determined whether miR-322/503 associated with the *Smurf2* mRNA by RNA pull-down assays, using biotin-labeled miR-322 or miR-503 (Figure 1, Ba and Ca). As shown in Figure 1Bb, 24 h after transfection with biotin-labeled miR-322, cells exhibited

elevated miR-322 levels but displayed no changes in the abundance of the housekeeping noncoding RNA *U6* (unpublished data). The levels of *Smurf2* mRNA were highly enriched in the materials from cells transfected with the biotin-labeled miR-322 but not in the pull-down materials from cells transfected with scrambled control miRNA (Figure 1Bc). The enrichment of the *Cugbp1* mRNA product was also examined and served as a positive control, since the *Cugbp1* mRNA is a target of miR-322/503 (Cui et al., 2012). In contrast, increasing the levels of biotin-miR-322 did not increase its interaction with the mRNAs encoding c-Myc and cyclin-dependent kinase 2 (*Cdk2*). In addition, transfection with biotin-labeled miR-322 failed to alter the steady-state levels of total *Smurf2*, *Cugbp1*, c-Myc, and *Cdk2* mRNAs (Figure 1Bd). Consistently, the abundance of *Smurf2* and *Cugbp1* mRNAs was also highly enriched in the materials from cells transfected with the biotin-labeled miR-503, but there were no changes in the levels of c-Myc and *Cdk2* mRNAs between cells transfected with biotin-labeled miR-503 and cells transfected with scrambled miRNA (Figure 1C). These results strongly suggest that the *Smurf2* mRNA is a novel target of miR-322/503 in IECs.

miR-322/503 represses *Smurf2* translation by interacting with *Smurf2* 3'-UTR

To examine the functional consequences of the [miR-322/503-*Smurf2* mRNA] association, we designed the first set of experiments to investigate whether increasing the levels of miR-322/503 through transfection with precursors (pre-miR-322 or pre-miR-503) repressed *Smurf2* expression. As shown, increased levels of either miR-322 by pre-miR-322 transfection or miR-503 by pre-miR-503 transfection decreased *Smurf2* protein levels (Figure 2, A and B), although they did not reduce the levels of total *Smurf2* mRNA (Figure 2C). To determine whether miR-322/503 inhibited *Smurf2* expression by repressing its translation, we examined changes in the levels of new *Smurf2* protein synthesis after ectopic overexpression of miR-322 or miR-503 and demonstrated that newly synthesized *Smurf2* protein decreased significantly in cells transfected with pre-miR-322 or pre-miR-503 compared with cells transfected with scrambled oligomer (Figure 2D). Inhibition of *Smurf2* protein synthesis by miR-322/503 induction was specific, since there was no change in nascent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) synthesis after transfection with pre-miR-322 or pre-miR-503. To further define the roles of miR-322/503 in the regulation of *Smurf2* mRNA translation, we examined the relative distribution of *Smurf2* mRNA in individual fractions from polyribosome gradients after ectopic overexpression of pre-miR-322 or pre-miR-503. Although increasing the levels of miR-322 or miR-503 did not affect global polysomal profiles (unpublished data), the association of *Smurf2* mRNA with actively translating fractions (fractions 8–10) decreased dramatically, shifting to low-translating fractions (fractions 5–7; Figure 2E, top). In contrast, housekeeping *Gapdh* mRNA distributed similarly in both groups (Figure 2E, bottom).

To determine whether this inhibitory effect was mediated through the *Smurf2* CR, we subcloned 3'-UTR or both fractions of the *Smurf2* CR and 3'-UTR into the pmirGLO dual-luciferase miRNA target expression vector to generate pmirGLO-*smurf2*-CR and pmirGLO-*smurf2*-3'UTR reporter constructs (Figure 2F, schematic). Overexpression of miR-322 or miR-503 by transfection with either pre-miR-322 or pre-miR-503 selectively decreased the levels of *Smurf2*-3'UTR luciferase reporter activity (Figure 2F, right) but failed to inhibit the activity of *Smurf2*-CR reporter activity (Figure 2F, left), indicating that miR-322/503 represses *Smurf2* mRNA translation through interaction with its 3'-UTR rather than its CR. We also found that increasing the levels of both miR-322 and miR-503 by cotransfection with pre-miR-322 and pre-miR-503 (Supplemental

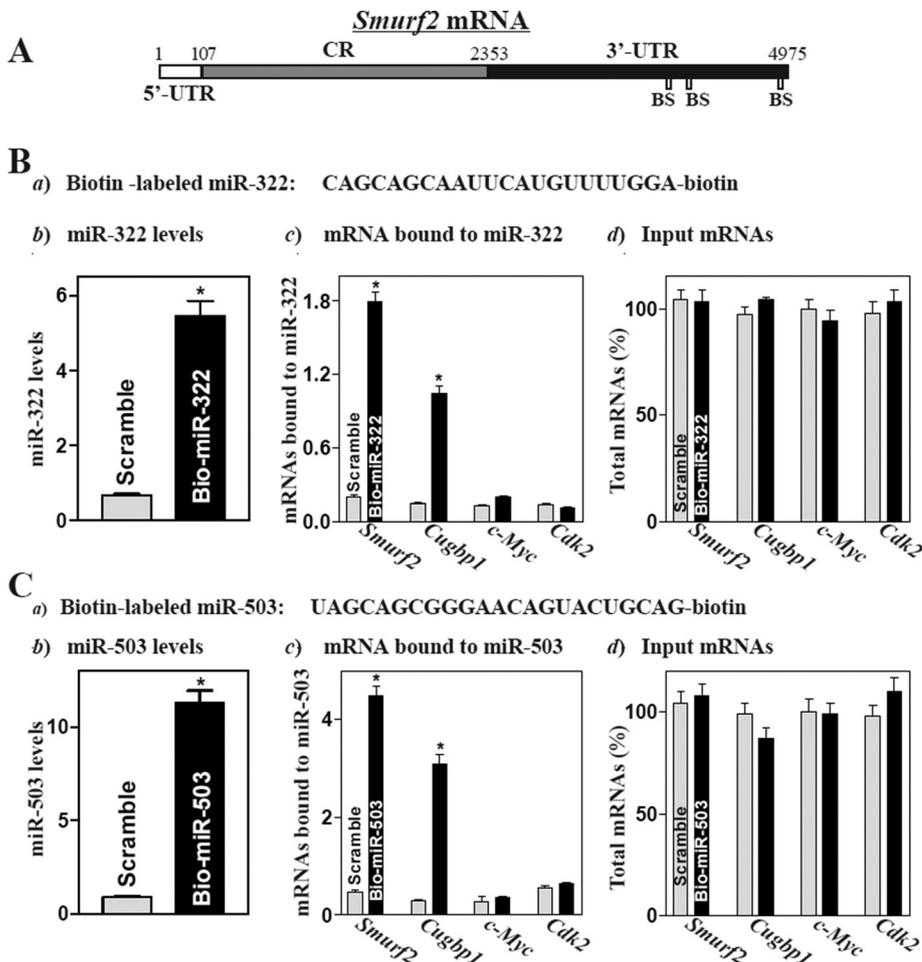


FIGURE 1: miR-322/503 directly interacts with the *Smurf2* mRNA. (A) Schematic representation of *Smurf2* mRNA depicting predicted target sites for miR-322/503 in its 3'-UTR. BS, predicted miR-322/503-binding site. (B) Association of biotinylated miR-322 with mRNAs encoding *Smurf2*, *Cugbp1*, *c-Myc*, and *Cdk2*. (a) Schematic representation of biotinylated miR-322; (b) levels of biotinylated miR-322 as measured by Q-PCR analysis 24 after transfection; (c) levels of *Smurf2*, *Cugbp1*, *c-Myc*, and *Cdk2* mRNAs in the materials pulled down by biotin-miR-322; and (d) levels of total input mRNAs. Values are means \pm SEM from three separate experiments. * $p < 0.05$ compared with cells transfected with control scramble oligomer. (C) Binding of biotinylated miR-503 to *Smurf2*, *Cugbp1*, *c-Myc*, and *Cdk2* mRNAs as measured by biotin pull-down assays used in studies described in B. * $p < 0.05$ compared with cells transfected with scramble oligomer.

Figure S1) did not synergistically repress *Smurf2* translation, as the levels of *Smurf2* protein and *Smurf2*-3'UTR luciferase reporter activity in cells cotransfected with pre-miR-322 and pre-miR-503 were similar to those observed in cells transfected with pre-miR-322 or pre-miR-503 alone (Supplemental Figure S2).

The second set of experiments examined the influence of decreasing the level of miR-322/503 by transfecting the corresponding antisense oligomer (antagomir) targeting miR-322 (anti-miR-322) or miR-503 (anti-miR-503) on *Smurf2* expression. Transfection with anti-miR-322 or anti-miR-503 decreased the levels of miR-322 and miR-503 (Figure 3A) and increased the level of *Smurf2* protein (Figure 3B), although there were no significant changes in the levels of total *Smurf2* mRNA (Figure 3C). Supporting the notion that the antagomirs were specific, anti-miR322 oligo only decreased miR-322 levels and did not alter miR-503 expression, whereas anti-miR-503 silenced miR-503 expression without affecting miR-322 levels (Supplemental Figure S3). The results presented in Figure 3D

further show that silencing miR-322/503 induced the synthesis of new *Smurf2* protein; this stimulatory effect was mediated via the interaction with the *Smurf2* 3'-UTR, since the levels of luciferase reporter activity were increased in miR-322- or miR-503-silent population of cells only when cells were transfected with the construct of pmirGLO-*Smurf2*-3'UTR (Figure 3E). These results indicate that decreasing the levels of miR-322/503 enhances *Smurf2* translation by reducing formation of the [miR-322/503-*Smurf2* mRNA] complex.

The third set of experiments aimed at further characterizing the specific binding site of miR-322/503 in the *Smurf2* 3'-UTR. To do so, we prepared various reporter constructs that expressed chimeric RNA containing the luciferase and partial transcripts spanning the *Smurf2* 3'-UTR with or without the potential binding site, as indicated in the schematic of Figure 4A. Ectopic overexpression of miR-322 or miR-503 was found to decrease the levels of luciferase reporter gene activity when cells were transfected with plasmid FL-Luc (containing full-length *Smurf2* 3'-UTR) or F3-Luc (containing a predicted binding site located at positions 3974–3998) but not with the F1-Luc (in which the potential binding sites were deleted) or F2-Luc and F4-Luc (which also contains a predicted binding site). Point mutation of the site located at the F3 of the *Smurf2* 3'-UTR was also performed, in which three nucleotides of F3-Luc of the *Smurf2* 3'-UTR were mutated (Figure 4B, schematic). Of interest, *Smurf2* repression by miR-322/503 was completely prevented when this specific binding site was mutated from the *Smurf2* 3'-UTR. Taken together, these results indicate that miR-322/503 interacts with *Smurf2* mRNA predominantly via the specific binding site at the position spanning 3974–3998 and represses *Smurf2* mRNA translation.

miR-322/503-modulated *Smurf2* expression regulates TGF- β /Smad2 signaling

To investigate the implication of miR-322/503-modulated *Smurf2* expression in the regulation of TGF- β /Smad2 signaling pathway, we examined changes in the levels of phosphorylated Smad2 (p-Smad2; the activated form of Smad2 and target for ubiquitin-dependent degradation) in miR-322/503-silenced cells and cells overexpressing green fluorescent protein (GFP)-tagged *Smurf2* after treatment with TGF- β . As expected, exposure of normal IECs to TGF- β induced Smad2 activation rapidly, as indicated by increase in the accumulation of p-Smad2 protein (Figure 5A), although it did not affect total Smad2 (T-Smad2) or *Smurf2* levels. On the other hand, ectopic overexpression of GFP-tagged *Smurf2* decreased the basal level of p-Smad2 in the absence of TGF- β (Figure 5B, left) and prevented TGF- β -induced Smad2 activation. There were no significant differences in the levels of p-Smad2 after treatment with TGF- β in cells overexpressing GFP-tagged *Smurf2* (Figure 5B, right).

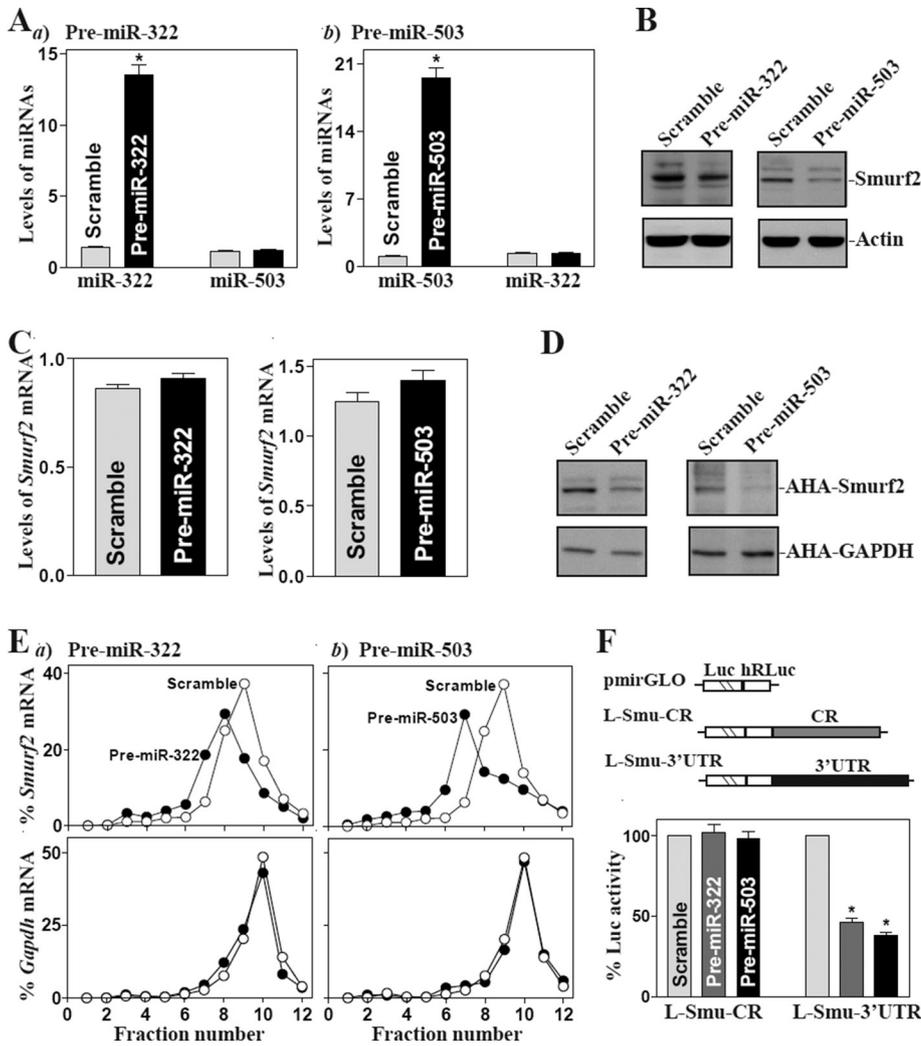


FIGURE 2: Ectopic overexpression of miR-322/503 inhibits Smurf2 translation. (A) Levels of miR-322 (a) and miR-503 (b) 48 h after transfection of pre-miR-322 or pre-miR-503. Values are means \pm SEM from three separate experiments. * $p < 0.05$ compared with scramble. (B) Levels of Smurf2 protein after ectopic miR-322/503 overexpression. Whole-cell lysates were prepared for Western blotting; equal loading was monitored by assessing β -actin levels. (C) The levels of *Smurf2* mRNA as examined by Q-PCR analysis. Left, cells transfected with pre-miR-322; right, cells transfected with pre-miR-503. (D) New synthesis of Smurf2 protein. After cells were exposed to L-azidohomoalanine (AHA), cell lysates were incubated with the reaction buffer containing biotin/alkyne reagent; the biotin-alkyne/azide-modified protein complex was pulled down by paramagnetic streptavidin-conjugated Dynabeads. (E) Distributions of *Smurf2* (top) and *Gapdh* (bottom) mRNAs in each gradient fraction of polysomal profile after ectopic miR-322/503 overexpression. After fractionation through sucrose gradients, total RNA was isolated from different fractions; the levels of *Smurf2* and *Gapdh* mRNAs were measured by Q-PCR analysis and plotted as percentage of total *Smurf2* or *Gapdh* mRNA level in the samples. (F) Levels of reporter activities after ectopic overexpression of miR-322/503. Results expressed as means \pm SEM data from three separate experiments. * $p < 0.05$ compared with cells transfected with scrambled RNA.

Moreover, the increased levels of endogenous Smurf2 after antagonizing miR-322 through transfection with anti-miR-322 were also associated with a decrease in the levels of p-Smad2; this effect was prevented by silencing Smurf2 (Figure 5C). In miR-322-silenced cells, increased Smurf2 suppressed TGF- β -induced Smad2 activation by enhancing p-Smad2 degradation (Figure 5D). In fact, the levels of p-Smad2 protein in miR-322-silenced cells treated with TGF- β were much lower than those observed in miR-silenced cells without TGF- β treatment. Similarly, the increase in levels of endog-

enous Smurf2 by antagonism of miR-503 had an identical stimulatory effect on p-Smad2 degradation after treatment with TGF- β (Figure 5, E and F). In addition, increased levels of Smurf2, by either ectopic gene expression or silencing miR-322/503, failed to alter the cellular abundance of T-Smad2. These results indicate that increased Smurf2 by miR-322/503 silencing inhibits TGF- β /Smad2 activation by enhancing p-Smad2 degradation.

Induced Smurf2 by miR-322/503 silencing protects IECs against apoptosis

To investigate the biological significance of miR-322/503-modulated Smurf2 expression, we examined their possible involvement in the control of intestinal epithelial homeostasis by measuring changes in apoptosis in miR-322/503-silenced cells. Increased levels of endogenous Smurf2 resulting from anti-miR-322 transfection (Figure 3) did not directly induce apoptosis (Figure 6A, a and b, left). There were no apparent differences in cell viability between miR-322-antagonized populations and control cells, including no morphological features of apoptosis and no detectable levels of active caspase-3 regardless of treatment with or without the miR-322 antagonist. To determine whether increased Smurf2 by antagonizing miR-322 altered the susceptibility of IECs to apoptosis, we exposed cells to treatment with tumor necrosis factor- α (TNF- α) plus cycloheximide (CHX). When control cells were exposed to TNF- α /CHX for 4 h, we observed morphological features characteristic of programmed cell death, elevated annexin V staining, a classic indicator of apoptotic cells (Figure 6, Aa, right, and B, left), and increased levels of active (cleaved) caspase-3 (Figure 6C). Increasing Smurf2 by miR-322 silencing protected IECs against TNF- α /CHX-induced apoptosis, as indicated by a decrease in the percentages of apoptotic cells (Figure 6, Ab and B). This protective effect remained intact when cells were transfected with control siRNA (Figure 6Ac), but it was lost when Smurf2 expression was silenced by siRNA targeting Smurf2 (siSmurf2). The percentage of apoptotic cells (Figure 6, Ad and B, right) and level of active caspase-3 protein (Figure 6C) in miR-322-silenced cells transfected with siSmurf2 were higher than those in miR-322-antagonized cells transfected with C-siRNA after exposure to TNF- α /CHX. Consistently, the increase in Smurf2 levels by miR-503 silencing also elicited antiapoptotic effects after exposure to TNF- α /CHX, and this protective effect was prevented by Smurf2 silencing (Supplemental Figure S4). Furthermore, ectopic overexpression of GFP-tagged Smurf2 protected IECs against TNF- α /CHX-induced apoptosis, because the percentage of apoptotic cells and the level of active

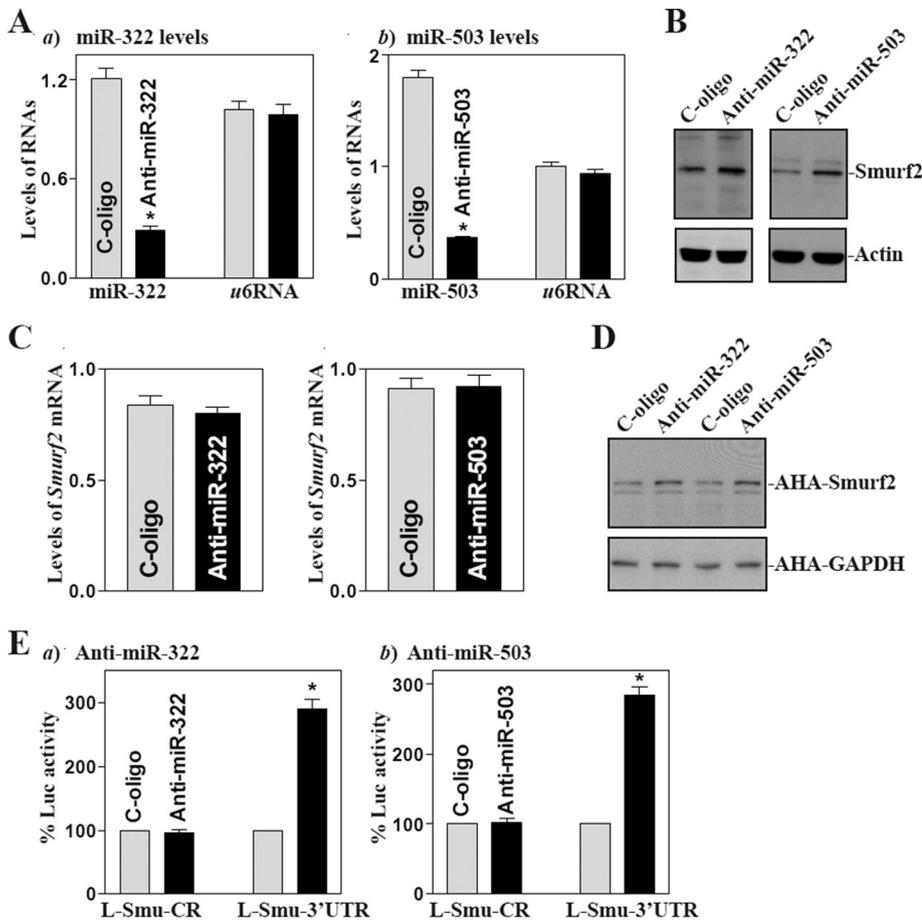


FIGURE 3: miR-322/503 silencing enhances Smurf2 translation. (A) At 48 h after transfecting IECs with oligomers targeting miR-322 (anti-miR-322) or miR-503 (anti-miR-503) or with a control oligo (C-oligo), the levels of miR-322 (a) and miR-503 (b) were measured by Q-PCR. Values are means \pm SEM from three separate experiments. * $p < 0.05$ compared with cells transfected with C-oligo. (B) Levels of Smurf2 protein as measured by Western immunoblotting analysis in cells described in A. (C) Levels of *Smurf2* mRNA as examined by Q-PCR. (D) New synthesis of Smurf2 protein as measured by Click-iT protein analysis using L-azidohomoalane (AHA). (E) Reporter activities after miR-322/503 silencing. Results expressed as means \pm SEM data from three separate experiments. * $p < 0.05$ compared with cells transfected with C-oligo.

caspase-3 in cells overexpressing GFP/Smurf2 decreased compared with those observed in control cells after treatment with TNF- α /CHX (Figure 7). These results indicate that Smurf2 is an antiapoptotic factor in IECs, whereas miR-322/503 silencing protects cells against apoptosis by increasing Smurf2.

DISCUSSION

Smurf2 is an important E3 ubiquitin ligase and plays a critical role in defining substrate specificity and subsequent protein degradation by the 26S proteasomes (Lin *et al.*, 2000; Ten Dijke and Hill, 2004; Xu *et al.*, 2012), but the exact mechanisms that control Smurf2 abundance have not been identified. The present study demonstrates a novel function of miR-322/503 in the regulation of Smurf2 translation and provides insight into the control of Smurf2 expression at the posttranscriptional level. Both miR-322 and miR-503 interact with the *Smurf2* mRNA via its 3'-UTR and repress Smurf2 translation, but they do not appear to affect *Smurf2* mRNA stability, since miR-322/503 overexpression specifically reduced Smurf2 nascent translation and anti-miR-322 and anti-miR-503 antagonists elevated it, but neither intervention affected total *Smurf2* mRNA levels. Our study further shows direct evidence of the site of miR-322/503 in the

Smurf2 3'-UTR, as point mutation of this site within the *Smurf2* 3'-UTR prevented miR-322/503-induced repression of Smurf2 translation. These findings advance our understanding of the molecular mechanisms underlying the regulation of *Smurf2* gene expression and indicate that association of miR-322/503 with the *Smurf2* mRNA controls its translation and therefore modulates the Smurf2-dependent ubiquitin system. Furthermore, our findings strongly suggest that control of *Smurf2* mRNA translation by miR-322/503 is relevant for maintaining intestinal epithelial homeostasis by altering IEC apoptosis.

Our results also indicate that miR-322/503 interacted with the *Smurf2* 3'-UTR but not its CR in IECs. Studies using various ectopic reporters bearing partial transcripts spanning the *Smurf2* 3'-UTR with or without the miR-322/503 binding site further demonstrated that the 3974-3998 site of the *Smurf2* 3'-UTR was the predominant and functional site through which miR-322/503 interacted with and repressed Smurf2 translation, whereas two other predicted sites spanning positions 3721-3774 and 4919-4921 in the *Smurf2* 3'-UTR exhibited a lesser effect. Consistent with our results, several previous studies showed that miR-322/503 directly interact with the 3'-UTRs of *Cdc25*, *Tob2*, *Ccnd1* (encoding cyclin D1), and *Cal* (encoding calumenin) mRNAs (Sarkar *et al.*, 2010; Gamez *et al.*, 2013; Merlet *et al.*, 2013) and thus destabilizes them and/or represses their translation. In some instances, miR-322/503 was also found to associate with the CRs of target mRNAs for their regulatory actions; for example, we recently reported that miR-503 represses CUGBP1 translation by directly interacting with the *Cugbp1* CR rather than the 3'-UTR (Cui *et al.*, 2012).

Although the specific molecular mechanism by which miR-322/503 association with *Smurf2* mRNA represses Smurf2 translation is unknown, our recent studies show that miR-503 inhibits CUGBP1 translation in normal IECs by enhancing the recruitment of *Cugbp1* mRNA to processing bodies (P-bodies; Cui *et al.*, 2012), where mRNAs are sorted for translation repression and/or degradation (Buchan and Parker, 2009; Kulkarni *et al.*, 2010; Yu *et al.*, 2012). Silencing P-body-resident proteins such as Ago2 or RCK prevents miR-503-induced repression of CUGBP1 translation, whereas Ago2 overexpression enhances the inhibitory effect of miR-503. In support of this notion, our previous studies also demonstrated that miR-222-induced repression of CDK4 translation and miR-195-induced *Stim1* mRNA destabilization are directly linked to increased translocation of *Cdk4* and *Stim1* mRNAs to P-bodies (Xiao *et al.*, 2011; Zhuang *et al.*, 2013). It is unclear, however, whether miR-322/503 alters the subcellular localization of the *Smurf2* mRNA to P-bodies in normal IECs and whether an increase in the recruitment of *Smurf2* mRNA to P-bodies affects its translation rate. These possibilities are being investigated in our ongoing studies.

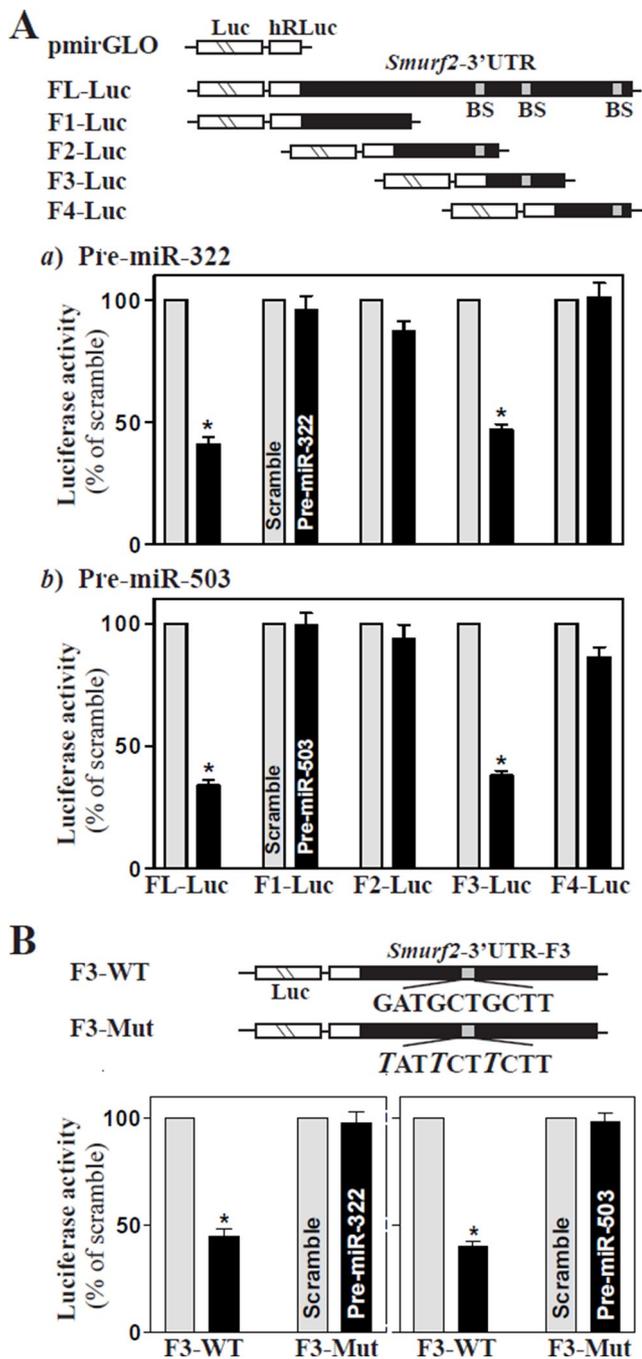


FIGURE 4: Changes in activities of *Smurf2* 3'-UTR luciferase reporters after deletion of miR-322/503-binding site. (A) Effect of 5' deletion of *Smurf2* 3'-UTR on its luciferase reporter activity in cells transfected with pre-miR-322 (a) or pre-miR-503 (b). Top, schematic of plasmids of different chimeric firefly luciferase *Smurf2* 3'-UTR reporters. BS, predicted miR-322/503-binding site. Bottom, *Smurf2* 3'-UTR luciferase reporter activity. At 24 h after transfection with pre-miR-322 or pre-miR-503, cells were cotransfected with *Smurf2* F-Luc constructs and a *Renilla* luciferase reporter. Firefly and *Renilla* luciferase activities were assayed 24 h later. Results were normalized to the *Renilla* luciferase activity and expressed as means \pm SEM of data from three separate experiments. * $p < 0.05$ compared with cells transfected with control scrambled oligomer. (B) Effect of point mutation of specific miR-322/503-binding site (schematic) in *Smurf2* 3'-UTR on luciferase reporter activities after ectopic overexpression of miR-322 (left) or miR-503 (right). * $p < 0.05$ compared with cells transfected with control scrambled oligomer.

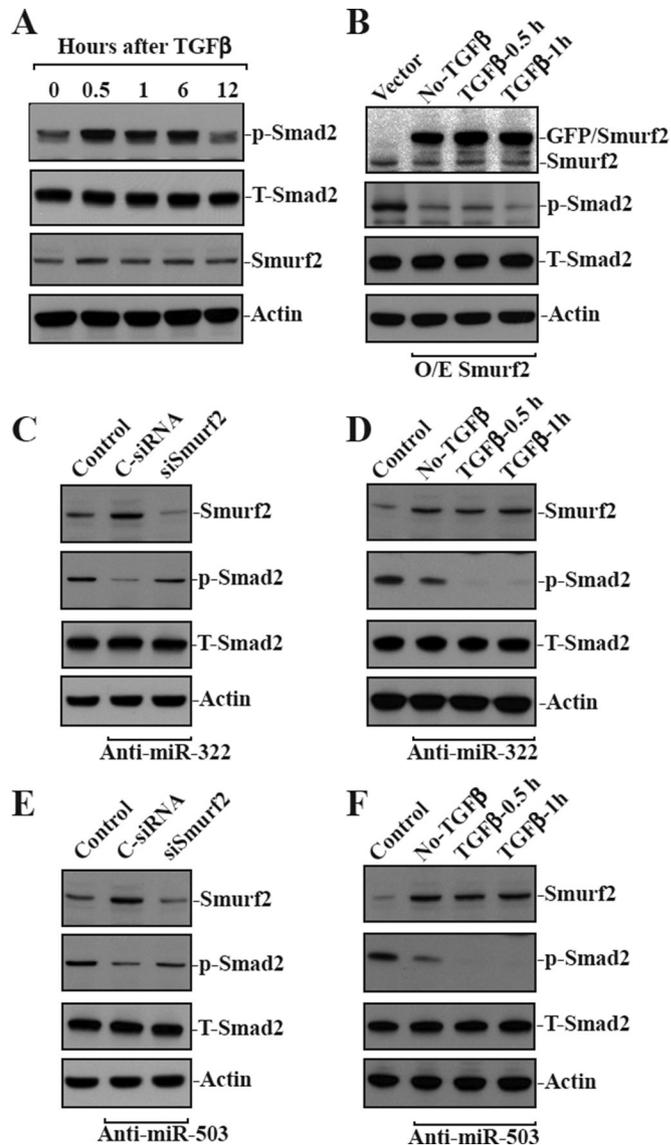


FIGURE 5: miR-322/503-modulated expression of *Smurf2* regulates the degradation of phosphorylated Smad2. (A) Levels of phosphorylated Smad2 (p-Smad2) and total Smad2 (T-Smad2) in cells treated with TGF- β (1 ng/ml) for different times. The levels of p-Smad2 and T-Smad2 were measured by Western blotting analysis using anti-p-Smad2 or anti-Smad2 antibody. (B) Changes in the levels of p-Smad2 and T-Smad2 in cells overexpressing GFP-tagged *Smurf2* after treatment with TGF- β . After cells were transfected with the *Smurf2* expression vector or control vector for 24 h, they were exposed to TGF- β for 0.5 or 1 h. (C) Levels of p-Smad2 and T-Smad2 48 h after cotransfecting cells with anti-miR-322 and control siRNA (C-siRNA) or siRNA targeting *Smurf2* mRNA (siSmurf2). (D) Changes in the levels of p-Smad2 and T-Smad2 in miR-322-silenced cells exposed to TGF- β . Cells were initially transfected with anti-miR-322 for 48 h and then exposed to TGF- β for 0.5 or 1 h. (E) Levels of p-Smad2 and T-Smad2 48 h after cotransfecting cells with anti-miR-503 and siSmurf2 for 48 h. (F) Changes in the levels of p-Smad2 and T-Smad2 in miR-503-silenced cells exposed to TGF- β for different times.

Our results presented here also indicate that the miR-322/503-modulated expression of *Smurf2* regulates TGF- β /Smad2 signaling pathway. Increased levels of endogenous *Smurf2* by miR-322/503 silencing not only decreased basal levels of p-Smad2 (activated Smad2 protein) in no-TGF- β -stimulated cells but also prevented

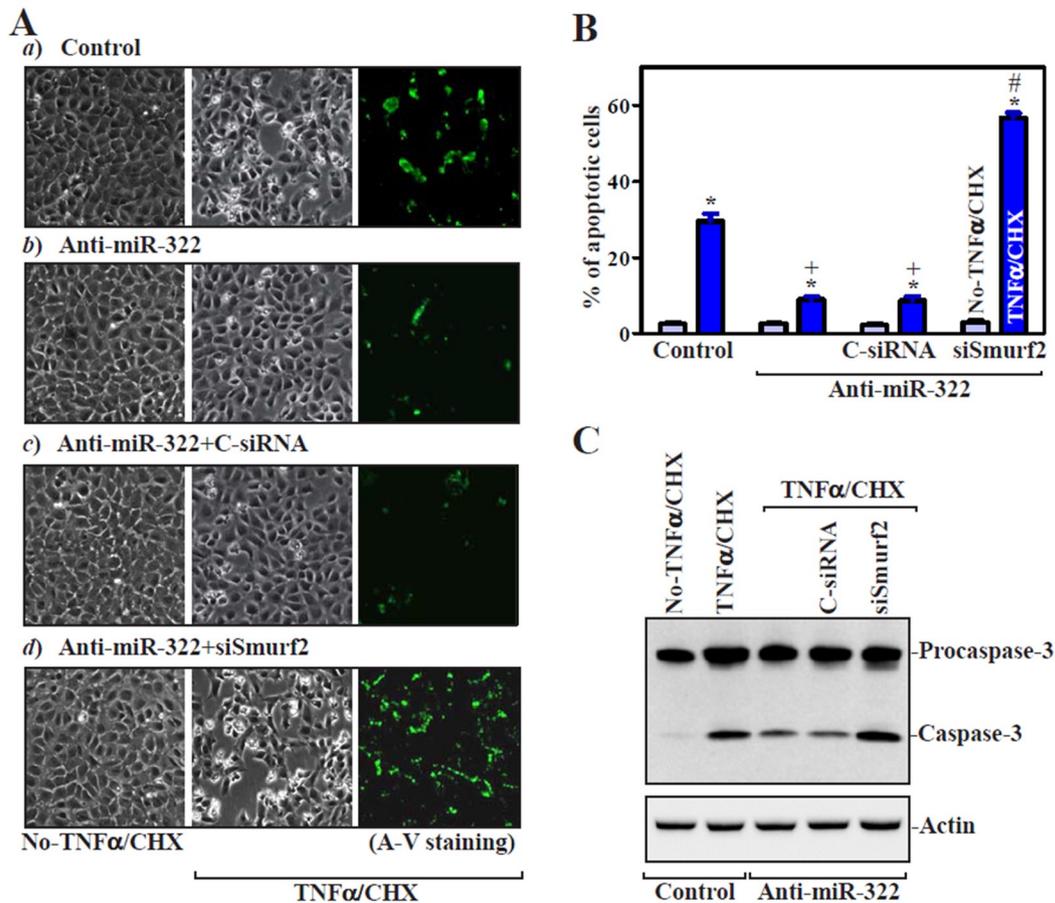


FIGURE 6: miR-322 silencing protects IECs against apoptosis by induction in the levels of endogenous Smurf2. (A) TNF- α /CHX-induced apoptosis after various treatments. Cells were transfected with the anti-miR-322 or control oligo (control); 48 h after transfection, apoptosis was measured after 4 h of treatment with TNF- α /CHX. (a) Cells transfected with Con-oligo; (b) cells transfected with anti-miR-322; (c) cells transfected with anti-miR-322 and control siRNA (C-siRNA); (d) cells transfected with anti-miR-322 and siRNA targeting Smurf2 (siSmurf2). Original magnification, $\times 150$. (B) Percentages of apoptotic cells after different treatments as described in A. Values are means \pm SEM of data from three experiments. * $p < 0.05$ compared with cells untreated with TNF- α /CHX; + $p < 0.05$ compared with cells exposed to TNF- α /CHX; # $p < 0.05$ compared with cells cotransfected with anti-miR-322 and C-siRNA and then exposed to TNF- α /CHX. (C) Changes in levels of caspase-3 in cells described in A. Whole-cell lysates were harvested, and the levels of procaspase-3 and caspase-3 were assessed by Western blot analysis. β -Actin immunoblotting was performed as an internal control for equal loading.

TGF- β -induced Smad2 activation by increasing p-Smad2 degradation. Smad proteins consist of two conserved globular domains, MH1 and MH2, connected by a linker region (Shi and Massague, 2003). The MH1 domain binds DNA, whereas the MH2 domain binds membrane receptors for activation, nucleoporins for nuclear translocation, and other members of the Smad family to form transcriptional complexes. On the other hand, the linker region of Smads functions as a critical regulatory domain, and its phosphorylation activates Smad proteins and then induces their degradation. In Smad2 the linker region contains four SP phosphorylation sites for proline-directed kinases and also has a PPAY motif that binds Smurf2 ubiquitin ligase (Zhu *et al.*, 1999; Sapkota *et al.*, 2007). Exposure to TGF- β induces phosphorylation of Smad2 at the C-terminus, as well as at an interdomain linker region. TGF- β -induced linker phosphorylation marks the activated Smad proteins for proteasome-mediated destruction, and this process is tightly regulated by numerous factors, including Nup214 and Nedd4L (Sapkota *et al.*, 2007; Gao *et al.*, 2009). To the best of our knowledge, the present study provides the

first evidence that miR-322/503 regulates TGF- β /Smad2 signaling by altering Smurf2 expression. Because the lowering of miR-322/503 by transfection with their respective antagonists was similar to that observed in IECs exposed to cell stimuli such as polyamine depletion (Cui *et al.*, 2012), the induced expression of Smurf2 by miR-322/503 silencing likely reflects biologically relevant events.

Finally, our findings show that miR-322/503-modulated Smurf2 expression has cellular function, playing a role in maintaining homeostasis of the intestinal epithelium by regulating IEC apoptosis. The epithelium of the intestinal mucosa is subject to rapid and dynamic self-renewal, and its homeostasis is preserved through strict regulation of cell proliferation, migration, differentiation, and apoptosis (Wang *et al.*, 1991; Sato and Clevers, 2013). IECs continuously replicate within the crypts of intestine, and this process is counterbalanced by apoptosis (Liu *et al.*, 2003; Rao and Wang, 2011). Apoptosis occurs in the crypt area, where it maintains a critical balance in cell number between newly divided and surviving cells, and at the luminal surface of the intestinal mucosa, where differentiated

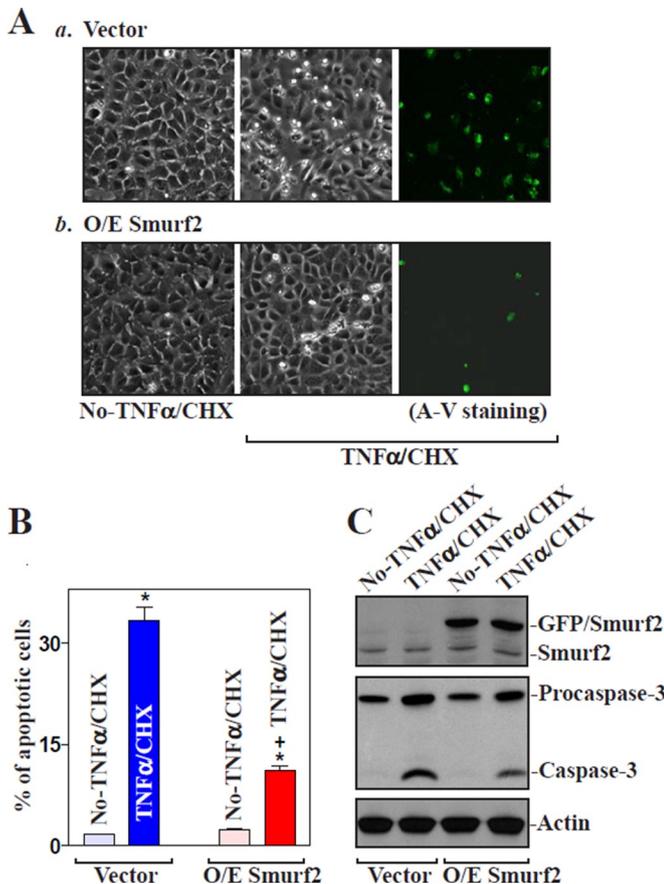


FIGURE 7: Ectopic overexpression of Smurf2 protects IECs against apoptosis. (A) Images of apoptotic cell death after treatment without (left) or with (middle and right) TNF α /CHX for 4 h. (a) Cells transfected with control vector (vector) and (b) cells transfected with the vector expressing GFP-tagged Smurf2 protein (Smurf2) for 24 h. Original magnification, $\times 150$. (B) Percentage of apoptotic cells in study as described in A. Values are means \pm SEM of data from three experiments. * $p < 0.05$ compared with No-TNF α /CHX. ** $p < 0.05$ compared with cells that were transfected with control vector and then treated with TNF α /CHX for 4 h. (C) Representative immunoblots for GFP/Smurf2, procaspase-3, and caspase-3 in cells described in A, as measured by Western immunoblotting analysis.

cells are lost. Our previous studies and studies from other laboratories (Seiler and Raul, 2007) demonstrated that NF- κ B (Li *et al.*, 2001), Akt kinase (Zhang *et al.*, 2004), ATF-2 (Xiao *et al.*, 2007), XIAP (Zhang *et al.*, 2009), MEK1 (Wang *et al.*, 2010), and CUGBP1 (Cui *et al.*, 2012) are involved in the control of IEC apoptosis, and these signals are highly regulated by cellular polyamines. Of interest, polyamines also regulate expression of miR-322/503 in IECs, and depletion of cellular polyamines decreases the level of miR-322/503 (Xiao *et al.*, 2011; Cui *et al.*, 2012). Our present studies provide additional evidence that the miR-322/503-modulated protein Smurf2 is a new member of this family of regulators of the control intestinal epithelial homeostasis, since increasing endogenous Smurf2 by silencing miR-322/503 protected cells against apoptosis, and this effect was abolished when Smurf2 expression was silenced. Taken together, our findings suggest that miR-322/503-mediated Smurf2 repression increases the susceptibility of IECs to apoptosis under pathological conditions, thus representing a novel therapeutic target for patients with intestinal mucosal atrophy and/or impaired epithelial integrity.

MATERIALS AND METHODS

Chemicals and cell culture

Tissue culture medium and dialyzed fetal bovine serum were from Invitrogen (Carlsbad, CA), and biochemicals were from Sigma-Aldrich (St. Louis, MO). The antibodies recognizing Smurf2, Smad2, and β -actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling (Danvers, MA), and the secondary antibody conjugated to horseradish peroxidase was from Sigma-Aldrich. Pre-miR miRNA precursors and anti-miR miRNA inhibitors of miR-322 and miR-503 were purchased from Ambion (Austin, TX). Biotin-labeled miR-322 and miRNA-503 were custom made by Dharmacon (Lafayette, CO).

The IEC-6 cell line, derived from normal rat intestinal crypt cells (Quaroni *et al.*, 1979), was purchased from the American Type Culture Collection (Manassas, VA) at passage 13 and was maintained in T-150 flasks in DMEM supplemented with 5% heat-inactivated fetal bovine serum. IEC-6 cells at passages 15–20 exhibited no significant changes in biological function or characterization (Wang *et al.*, 1995; Liu *et al.*, 2009) and were used in all experiments.

Plasmid construction

The chimeric firefly luciferase reporter construct of the Smurf2 CR or 3'-UTR was generated as described previously (Zou *et al.*, 2010). The full-length Smurf2 CR or its 3'-UTR and different 3'-UTR fragments with or without predicted miR-322/503-binding site were amplified and subcloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI) to generate the pmirGLO-Luc-Smurf2-CR and pmirGLO-Smurf2-3'UTR. The sequence and orientation of the fragment in the luciferase reporter were confirmed by DNA sequencing and enzyme digestion. Transient transfections were performed using the Lipofectamine Reagent as recommended by the manufacturer (Invitrogen). The luciferase reporter constructs were transfected into cells along with pRL-null, a Renilla luciferase control reporter vector from Promega, to monitor transfection efficiencies as described previously (Liu *et al.*, 2009). Luciferase activity was measured using the Dual Luciferase Assay System, and the levels of pmirGLO-Luc-Smurf2-CR or Smurf2-3'UTR luciferase activity were normalized to Renilla luciferase activity and further compared with the levels of luciferase mRNA in every experiment. All of the primer sequences for generating these constructs are list in Supplemental Table S2. The vector expressing GFP-tagged Smurf2 protein was obtained from OriGene (Rockville, MD).

Reverse transcription and quantitative real-time PCR analyses

Total RNA was isolated by using the RNeasy mini kit (Qiagen, Valencia, CA) and used in reverse transcription (RT) and PCR amplification reactions as described (Yu *et al.*, 2011). The levels of *Gapdh* PCR product were assessed to monitor the evenness in RNA input in RT-PCR samples. Real-time quantitative PCR (Q-PCR) analysis was performed using 7500-Fast Real-Time PCR Systems with specific primers, probes, and software (Applied Biosystems, Foster City, CA). For miRNA studies, levels of miR-322 and miR-503 were also quantified by Q-PCR by Taqman MicroRNA assay; levels of small nuclear RNA *U6* were measured as endogenous control.

Western blotting analysis

Whole-cell lysates were prepared using 2% SDS, sonicated, and centrifuged (12,000 rpm) at 4°C for 15 min. The supernatants were boiled for 5 min and size fractionated by SDS-PAGE (7.5% acrylamide). After transfer of proteins onto nitrocellulose

filters, the blots were incubated with primary antibodies recognizing Smurf2, Smad2, or phosphorylated Smad2; after incubations with secondary antibodies, immunocomplexes were developed by using chemiluminescence.

Analysis of newly translated protein

New synthesis of nascent Smurf2 protein was detected by Click-iT protein analysis detection kit (Life Technologies, Grand Island, NY) and performed following the manual with minor modification (Xiao *et al.*, 2013). Briefly, cells were incubated in methionine-free medium and then exposed to L-azidohomoalanine. After mixing of cell lysates with the reaction buffer containing biotin/alkyne reagent and CuSO₄ for 20 min, the biotin-alkyne/azide-modified protein complex was pulled down using paramagnetic streptavidin-conjugated Dynabeads. The pull-down material was resolved by 10% SDS-PAGE and analyzed by Western immunoblotting analysis using the antibody against Smurf2 or GAPDH.

Polysome analysis was performed as described (Yu *et al.*, 2012). Briefly, cells at ~70% confluence were incubated for 15 min in 0.1 mg/ml cycloheximide, lifted by scraping in 1 ml of polysome extraction lysis buffer, and lysed on ice for 10 min. Nuclei were pelleted, and the resulting supernatant was fractionated through a 10–50% linear sucrose gradient to fractionate cytoplasmic components according to their molecular weights. The eluted fractions were prepared with a fraction collector (Brandel, Gaithersburg, MD), and their quality was monitored at 254 nm using a UV-6 detector (ISCO, Louisville, KY). After RNA in each fraction was extracted, the levels of each individual mRNA were quantified by Q-PCR in each of the fractions.

Biotin-labeled miR-322/503 pull-down assays

Binding of miR-322 or miR-503 to target mRNAs was examined by biotin-labeled miR-322 or miR-503 as described previously (Orom and Lund, 2007; Nonne *et al.*, 2010). Briefly, biotin-labeled miR-322 or miR-503 was transfected into cells for 24 h, and then whole-cell lysate was collected. Cell lysates were mixed with streptavidin-Dynal beads (Invitrogen) and incubated at 4°C with rotation overnight. After the beads were washed thoroughly, the bead-bound RNA was isolated and subjected to RT followed by Q-PCR analysis. Input RNA was extracted and served as control.

Determination of apoptosis

Apoptosis was induced by treatment with TNF- α in combination with CHX as described previously (Li *et al.*, 2001). After various experimental treatments, cells were photographed with a Nikon (Melville, NY) inverted microscope before fixation. Annexin V staining of apoptosis was carried out by using a commercial apoptosis kit (Clontech Laboratories, Palo Alto, CA) and performed according to the protocol recommended by the manufacturer. Briefly, cells were rinsed with 1 \times binding buffer and resuspended in 200 μ l of 1 \times binding buffer. A 5- μ l amount of annexin V was added on slides and incubated at room temperature for 10 min in the dark. Annexin-stained cells were visualized and photographed under fluorescence microscope using a dual filter set for fluorescein isothiocyanate and rhodamine, and the percentage of apoptotic cells was determined.

Statistics

Values are means \pm SEM from three to six samples. Immunoblotting results were repeated three times. The significance of the difference between means was determined by analysis of variance. The level of significance was determined by using Duncan's multiple-range test (Harter, 1960).

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