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GPR43 mediates microbiota metabolite SCFA regulation of antimicrobial peptide expression in intestinal epithelial cells via activation of mTOR and STAT3

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Abstract

The antimicrobial peptides (AMP) produced by intestinal epithelial cells (IEC) play crucial roles in the regulation of intestinal homeostasis by controlling microbiota. Gut microbiota has been shown to promote IEC expression of RegIIIγ and certain defensins. However, the mechanisms involved are still not completely understood. In this report, we found that IEC expression of RegIIIγ and β-defensins 1, 3, and 4 was lower in G protein-coupled receptor (GPR)43$^{-/-}$mice compared to that of wild-type (WT) mice. Oral feeding with short chain fatty acids (SCFA) promoted IEC production of RegIIIγ and defensins in mice. Furthermore, SCFA induced RegIIIγ and β-defensins in intestinal epithelial enteroids generated from WT but not GPR43$^{-/-}$mice. Mechanistically, SCFA activated mTOR and STAT3 in IEC, and knockdown of mTOR and STAT3 impaired SCFA induction of AMP production. Our studies thus demonstrated that microbiota metabolites SCFA promoted IEC RegIIIγ and β-defensins in a GPR43-dependent manner. The

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data thereby provides a novel pathway by which microbiota regulates IEC expression of AMP and intestinal homeostasis.

Keywords

SCFA; GPR43; IEC; RegIIIγ; Defensins

Introduction

In spite of being home to a diverse community of large numbers of indigenous microorganisms, host intestines live in harmony with microbiota under homeostatic conditions. Among multiple mechanisms involved in the regulation of host responses to microbiota in maintaining intestinal homeostasis, antimicrobial peptides (AMP) that are produced by intestinal epithelial cells (IEC) and Paneth cells serve as the first line of defense against microbiota and occasionally pathogens[1,2]. AMP regulate host responses to microbiota and are involved in the maintenance of intestinal homeostasis. Several distinct families of AMP have been identified including defensins, cathelicidins, and C-type lectins (such as the regenerating islet-derived protein (REG) family). AMP function to kill or inactivate microorganisms rapidly through different mechanisms[1,2]. The spectrum of antimicrobial activity varies for each particular antimicrobial peptide. In general, defensins affect both Gram-positive and Gram-negative bacteria and, in some cases, fungi, viruses, and protozoa[3]. RegIIIγ, on the other hand, is selective for Gram-positive bacteria[4]. Mutually, gut microbiota may also differentially regulate AMP production. For example, although expression of α-defensins is independent of gut microbiota, the expression of RegIIIγ is virtually absent in germ-free (GF) mice and increased upon re-colonization with gut microbiota[4,5].

How microbiota promotes the expression of AMP remains unclear. In mice deficient in MyD88, an adaptor molecule common to most TLRs, IEC expression of RegIIIγ and RegIIIβ is decreased compared to that in wild-type (WT) mice, suggesting that IEC can directly sense bacteria through TLRs and promote the expression of RegIIIγ and RegIIIβ[6,7]. In addition, innate lymphoid cell (ILC) production of IL-22 has also been shown to promote IEC expression of RegIIIγ mRNA. Notably, microbiota drive IL-22 production in ILCs, as demonstrated by the low levels of IL-22 produced by ILCs from GF mice[8]. Thus, gut microbiota can regulate AMP expression, at least partially, through interaction with IEC TLR or stimulating ILC IL-22 production.

Accumulating evidence indicates that the host immune system can sense gut bacterial metabolites in addition to TLR ligands. Recognition of these small molecules greatly impacts the host immune response[9,10]. Among gut microbiota metabolites, short-chain fatty acids (SCFA), which are solely metabolized from indigestible carbohydrates by intestinal bacteria[11], have been shown to have immense impact on the host[12]. Intact production of SCFA is associated with a reduced risk of different diseases, including IBD. As the most abundant SCFA, acetate, propionate, and butyrate, are present in the colonic lumen of humans at high concentrations, ranging from 50 to 150 mM[11,13]. SCFA can function through

the activation of mammalian G protein-coupled receptors (GPCR)[14]. GPR41 and GPR43 are two major host receptors for most SCFA[15]. SCFA can also inhibit histone deacetylase activity (HDAC) to downregulate macrophage pro-inflammatory cytokine production and activate peroxisome proliferator-activated receptor γ to stimulate human colonic epithelial cell expression of angiopoietin-like protein 4[16,17]. In addition, SCFA promote intestinal Treg cell development which is probably also mediated by their HDAC inhibitor activity but independent of GPR43, although it is still debatable as conflicting data have been published[10,18,19].

SCFA butyrate has been demonstrated to induce LL-37/hCAP18, a member of cathelicidin family, in various human colon cell lines[20]. However, the underlying molecular mechanisms by which SCFA regulate IEC AMP production are still not completely understood. It has been reported that MAP kinases respond differently to butyrate stimulation[21]. The MEK-ERK pathway regulates butyrate-induced LL-37 expression, whereas p38/MAP kinase exhibits little effect on AMP production[2122]. C-Jun N-terminal kinase (JNK) also positively regulates cathelicidin production in tissue epithelial cells[23]. The current study was undertaken to investigate the mechanisms of AMP production induced by SCFA. We report here that SCFA promoted RegIIIγ and β-defensin expression in IEC through GPR43, which is mediated by mTOR and STAT3.

Results

1. Expression of RegIIIγ and β-defensins in IEC is impaired in GPR43$^{-/-}$mice

It has been previously reported that microbiota regulate IEC AMP expression differentially[4,5]. As microbiota-derived SCFA can modulate IEC activity through GPR43, we investigated whether GPR43 regulates AMP expression in IEC. We measured the expression of RegIIIγ and β-defensins 1, 3, 4 in C57BL/6 (WT) and GPR43$^{-/-}$mice at both RNA and protein levels. Quantitative real-time PCR (qRT-PCR) revealed that the transcription of RegIIIγ and β-defensin 1, 3, 4 mRNA was compromised in GPR43$^{-/-}$IEC compared to the WT control (Fig. 1A). Consistent with gene expression studies, protein levels of RegIIIγ and β-defensin 1, 3, 4 in GPR43$^{-/-}$mice were significantly lower compared to WT mice (Fig. 1B and 1C). Collectively, these data indicated that SCFA recognition by GPR43 promotes IEC production of RegIIIγ and β-defensins.

2. Feeding SCFA promotes IEC expression of RegIIIγ and β-defensins in WT but not GPR43$^{-/-}$mice

As the most potent agonists for GPR43, SCFA play critical roles in intestinal barrier function[24,25]. Butyrate, in particular, has been demonstrated to induce cathelicidin AMP and β-defensin 1 in human lung epithelial cells[23]. We thus investigated whether SCFA can elicit a similar effect on IEC, and whether GPR43 mediates such effects. Because gut microbiota produces high levels of SCFA in the intestines which may confound the data interpretation, we first treated mice with broad-spectrum antibiotics for 10 days to eliminate gut microbiota as well as their metabolites SCFA, followed by 21 days of butyrate and antibiotics administration in drinking water. IEC were then isolated and assessed for AMP expression. We found decreased expression of RegIIIγ, but no significant difference of β-defensins in

mice receiving antibiotics compared to the control mice (Fig. S1). As expected, in WT mice, feeding butyrate enhanced the expression of RegIIIγ and β-defensin 1, 3, 4 compared to controls in IEC (Fig. 2A). Increased protein levels of RegIIIγ and β-defensins were also detected by Western blot (Fig. 2B and 2C). Feeding butyrate also increased expression of cathelicidin-related antimicrobial peptide (CRAMP) (Fig. 2A). Additionally, mice fed with acetate also slightly increased expression of RegIIIγ and β-defensins in IEC compared to control (Fig. S2). However, feeding butyrate did not affect the expression of RegIIIγ and β-defensin 1, 3, 4 in IEC of GPR43$^{-/-}$mice (Fig. 2D). Together, these data suggested that SCFA serve as potent inducers of AMP production by IEC, which is mediated by GPR43.

We then investigated whether GPR43 and feeding butyrate affect gut microbiota, which could potentially mediate butyrate-induction of AMP production. We fed WT and GPR43$^{-/-}$mice with 300 mM butyrate for 21 days, and gut microbiota assessed by 16s rRNA pyrosequencing of fecal samples. In GPR43$^{-/-}$mice, the abundance of families of Prevotellaceae, Erysipelotrichaceae, and Alcaligenaceae was decreased compared to WT mice (Fig. 3B). At genus level, Alistipes and Lactobacillus were increased while Alloprevotella, Blautia, and Odoribacter were decreased (Fig. 3C), indicating that GPR43 signaling regulated gut microbiota composition. Feeding WT mice butyrate resulted in deviation of the microbial population toward an increased proportion of Bacteroidetes phylum (54.7% to 64.2%, including Prevotellaceae, p=0.06) but decreased proportion of Proteobacteria phylum (7.3% to 4.0%, including Helicobacteraceae and Alcaligenaceae, p=0.05) and Firmicutes phylum (31.4% to 25.5%, including Lachnospiraceae and Erysipelotrichaceae) (Fig. 3A). However, in GPR43$^{-/-}$mice, the increased proportion of Bacteroidetes phylum by feeding butyrate was decreased (from 51% to 55%) compared with WT mice. Furthermore, butyrate did not affect proportion of Proteobacteria phylum in GPR43$^{-/-}$mice. We also found that butyrate feeding increased the abundance of Prevotellaceae family but decreased the abundance of Alcaligenaceae family in WT mice but not in GPR43$^{-/-}$mice (Fig. 3B). At genus level, butyrate feeding increased abundance of Alloprevotella and Odoribacter but decreased abundance of Blautia and Lactobacillus in WT mice but not in GPR43$^{-/-}$mice (Fig. 3C). However, in contrast to the mice treated with antibiotics (Fig. 2), feeding butyrate without depletion of gut bacteria did not increase IEC expression of RegIIIγ and β-defensins in both WT and GPR43$^{-/-}$mice (Fig. S3), possibly due to the high levels of pre-existing SCFA produced by gut microbiota in the intestines.

3. SCFA promote expression of RegIIIγ and β-defensins in both mouse and human IEC *in vitro* in a restricted time window

To determine whether SCFA directly promote the IEC expression of AMP *in vitro*, we utilized intestinal epithelial cell lines and examined RegIIIγ and β-defensins expression after butyrate stimulation. We first used mouse small intestinal epithelial (MSIE) cells, a conditionally immortalized epithelial cell line that retains properties of primary IEC[26]. We treated MSIE cells with butyrate for 48 h and measured RegIIIγ and β-defensins at both gene expression and protein levels. Treatment with butyrate significantly induced the expression of RegIIIγ, β-defensin 1, 3, 4 (Figs. 4A and B). We then determined the kinetics of the butyrate-induced IEC expression of RegIIIγ and β-defensins. We treated the MSIE cells with butyrate and measured RegIIIγ and β-defensins with respect to time over the

course of 72 h. Butyrate treatment did not induce expression of RegIIIγ and β-defensins in early time points of 1 – 12 hrs. Their expression was slightly increased at 24 h, but did not reach significance. Consistently, the expression of RegIIIγ and β-defensins was significantly increased at 48 h, but returned to levels similar to those in controls by 72 h (Fig. S4), indicating that butyrate stimulation occurs within a narrow time window. Treatment with GPR43 agonist elicited similar effect on RegIIIγ and β-defensin 1, 3, 4 expression (Fig. 4C), indicating that GPR43 may mediate the butyrate-induced expression of RegIIIγ and β-defensins in IEC.

To investigate if SCFA also regulate expression of AMP in human IEC, a human colonic epithelial cell line (HT-29) was stimulated with 0.5 mM butyrate for 48 h. Consistently, mRNA expression levels of RegIIIα, the human ortholog of mouse RegIIIγ, β-defensin 1, and LL-37 were enhanced in butyrate-treated HT-29 cells (Fig. 5A). However, butyrate did not affect the expression of α-defensin 5 and α-defensin 6 in HT-29 cells (Fig. 5A). We also demonstrated a similar effect of acetate and propionate on HT-29 cells. While propionate induced mRNA expression of both RegIIIα and β-defensin 1, the expression of α-defensins remained unaffected. Acetate induced β-defensin 1 but not RegIIIα and α-defensins (Fig. S5). Moreover, GPR43 agonist promoted HT-29 cell expression of RegIIIα, β-defensin 1, and LL-37 but not α-defensin 5 and α-defensin 6 (Fig. 5B).

4. SCFA induce expression of RegIIIγ and β-defensins in WT but not GRP43$^{-/-}$small intestinal epithelial enteroids

We next sought to determine if IEC expression of GPR43 directly mediates SCFA-induced AMP production. We established an *ex-vivo* 3-D enteroid culture to recapitulate the comprehensive intestinal microenvironment. Enteroids were generated from WT and GRP43$^{-/-}$mice respectively, and treated with butyrate or PBS control for 48 h. Butyrate treatment did not affect the viability of enteroids as well as the expression of MKI67 (gene for proliferation) and Lgr5 (gene for stemness) (Fig. S6). As shown in Fig 6, butyrate treatment induced the expression of RegIIIγ and β-defensin 1 in WT enteroids, whereas the effect was abrogated in GPR43$^{-/-}$organoids. This data confirmed that GPR43 mediates IEC production of RegIIIγ and β-defensins induced by butyrate.

5. mTOR mediates SCFA induction of RegIIIγ and β-defensins in IEC

The mammalian target of rapamycin (mTOR), an evolutionarily conserved integrative serine-threonine kinase, is a crucial regulator of physiology of various cells in response to environmental cues and nutrients[27]. mTOR functions through phosphorylating the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and p70 ribosomal S6 kinase 1 (S6K1). SCFA have been shown to activate mTOR in T cells and dendritic cells (DC)[13,28]. We thus investigated whether the mTOR pathway is involved in SCFA-induced RegIIIγ and β-defensins production in IEC. We first treated MSIE cells with butyrate and measured mTOR activation by detecting both phosphorylated-mTOR (p-mTOR) and phosphorylated-4EBP1 (p-4EBP1). As shown in Fig. 7A, butyrate promoted the expression of p-mTOR and p-4EBP1. Consistently, we detected activation of S6K1 by flow cytometry in butyrate-treated MSIE cells (Fig. 7B). Moreover, butyrate activation of mTOR signaling was via GPR43, as evidenced by the activation of mTOR downstream effector S6K1 by a

GPR43 agonist (Fig. S7). To determine that butyrate-induced mTOR activation contributes to IEC production of RegIIIγ and β-defensins, we used mTOR siRNA to specifically knockdown mTOR. MSIE cells were transfected with mTOR siRNA or control non-targeting siRNA, with a transfection efficiency around 50% (Fig. 7C). The cells were then treated with butyrate. Knockdown of mTOR impaired butyrate-induced expression of RegIIIγ and β-defensins at the levels of both mRNA (Fig. 7D) and proteins (Fig. 8E). Collectively, these data demonstrated that butyrate activates the mTOR pathway to positively regulate RegIIIγ and β-defensins production in IEC.

6. STAT3 regulates butyrate promotion of RegIIIγ and β-defensins production in IEC

It has been shown that signal transducers and activator of transcription 3 (STAT3) is required for RegIIIγ production in lung epithelium[29]. Likewise, it is also necessary for IL-22-induced RegIIIγ and β-defensins production in human IEC and alveolar epithelium[30–32]. We postulated that STAT3 regulates SCFA induction of AMP production in IEC. Administration of butyrate led to activation of STAT3 in MSIE cells, evidenced by enhanced phosphorylation of STAT3 (Fig. 8A). Blockade of STAT3 using a specific STAT3 inhibitor HJC0152[33] greatly attenuated butyrate-induced RegIIIγ and β-defensins mRNA expression (Fig. 8B). Secondly, we knocked down STAT3 in MSIE cells with specific siRNA. The obtained transfection efficiency was about 50% (Fig. 8C). In accordance with the data of the STAT3 inhibitor, the knockdown of STAT3 resulted in decreased butyrate-induced IEC expression of RegIIIγ and β-defensins at the levels of both mRNA and proteins (Figs. 8D and E). Together, these data indicated that STAT3 is critical for the butyrate-induced AMP production in IEC.

Discussion

The interplay between gut commensal bacteria and the host provides beneficial effects on host metabolism as well as immune regulation[34–36]. In this report, we have demonstrated that gut microbiota metabolites SCFA promoted IEC expression of RegIIIγ and β-defensins 1, 3, and 4 through GPR43, essential for the maintenance of intestinal homeostasis.

Gut microbiota has been shown to be important for the production of certain AMP in the intestines, in that RegIIIγ and certain β-defensins are virtually absent in GF mice[4,5]. Mice lacking TLR ligands also displayed impaired production of RegIIIγ and RegIIIβ in IEC compared to WT mice, suggesting that microbiota can directly affect IEC AMP production through interaction with TLRs[6,7]. However, substantial amounts of AMP have been detected in MyD88$^{-/-}$mice housed under SPF conditions[4–7]. This indicates the presence of multiple sensors and mechanisms for AMP regulation by microbiota, in addition to direct TLR-TLR ligand interactions. In the current report, we investigated whether SCFA regulated the IEC production of RegIII and the defensin family. Indeed, extraneous butyrate administration promoted IEC production of RegIIIγ, β-defensin 1, 3, and 4, and CRAMP (Fig. 2). This was further supported by *in vitro* studies using human and murine intestinal cell lines (Fig. 4 and 5). Interestingly, a previous report showed that SCFA also promoted β-defensin expression in porcine IEC by using porcine IPEC-J2 intestinal epithelial cells[37], indicating that SCFA can function across different species to promote IEC expression of AMP.

It has been shown that SCFA bind cell-surface receptors such as GPR41, GPR43, and GPR109α[13,38]. SCFA-GPR43 interaction has been reported in regulating intestinal inflammatory responses[39], in that GPR43$^{-/-}$mice demonstrated exacerbated colitis after dextran sodium sulfate (DSS) insult[40]. This further indicates that SCFA affects the intestine via the GPR43 receptor. More recently, it was discovered that low fiber diets induced and perpetuated intestinal inflammation, whereas high fiber diets protected against colitis[41]. The beneficial effect of high fiber intake derives from gut microbiota metabolic products, and subsequent SCFA binding to GPR43 and GPR109a in the intestines. Further studies revealed that SCFA activated the NLRP3 inflammasome through GPCR signaling, which conferred resistance to colitis[41]. Our data demonstrated that production of RegIIIγ and β-defensins in IEC was impaired in GPR43$^{-/-}$mice (Fig. 1). Additionally, administration of a GPR43 agonist enhanced AMP production in both murine and human IEC, indicating that GPR43 is involved in AMP production. Furthermore, butyrate promoted expression of RegIIIγ and β-defensins in WT but not in GPR43$^{-/-}$intestinal epithelial enteroids (Fig. 6). SCFA, especially butyrate, have been shown as HDAC inhibitors, and butyrate-induced HDAC3 inhibition regulates IEC production of retinoic acid, which can potentially contribute to maintenance of intestinal homeostasis[42]. Our data do not rule out the involvement of HDAC-inhibition in SCFA induction of AMP in IEC. It is very likely that SCFA regulate IEC function via multiple mechanisms.

mTOR is a key regulator in various physiological aspects[43]. Among different cell types, SCFA have been shown to activate mTOR in T cells and DCs[27,44]. We showed that SCFA activated the mTOR pathway in IEC (Fig. 7A). Knockdown of mTOR attenuated SCFA-induced AMP production, indicating that activation of mTOR facilitated RegIIIγ and β-defensins production in IEC. Interestingly, butyrate also activated STAT3, which has been implicated in the regulation of immune responses of both innate and adaptive immune cells in the intestines[45]. The blockade of STAT3 signaling compromised RegIIIγ and β-defensins production in SCFA-treated IEC, suggesting that STAT3 is indispensable in SCFA induction of AMP production in IEC (Fig. 8). As activation of STAT3 impacts epithelial cell viability, it is very likely that SCFA activation of STAT3 supports intestinal organoid stemness proliferation, which will affect the AMP secretion by an indirect mechanism as well. Therefore, SCFA activation of mTOR and STAT3 could collaboratively promote AMP expression in IEC.

RegIIIγ and β-defensins secreted by IEC are known for limiting bacteria and manipulating species composition, especially Gram-positive species. Our findings that SCFA induce IEC RegIIIγ and β-defensins production in IEC suggest a possible autoregulation of microbiota. In light of this, drugs targeting the intrinsic pathways of SCFA-mediated AMP production might serve as promising candidates for clinical applications by promoting host barrier defense and correcting dysbiosis, and restoring homeostatic balance. Nevertheless, the modification of AMP requires carefully monitoring, and dysregulated AMP production can have negative impacts on intestinal homeostasis. Indeed, it was discovered that excessive lipocalin-2 and calprotectin production induced by IL-22 can, in turn, suppress commensal bacteria, resulting in pathogen colonization[46]. In order to preserve intestinal homeostasis, critical elements of host immunity and microbial colonization need to counterbalance each

other. To that end, we present evidence that metabolites from commensal microbiota are capable of inducing host innate immunity to regulate the intestinal environment.

Moreover, we demonstrated that GPR43 signaling regulated gut microbiota and feeding butyrate resulted in alterations in the composition of the microbiota (Fig. 3). Among the bacteria affected by GPR43 signaling, the abundance of Prevotellaceae was decreased in GPR43$^{-/-}$mice compared to WT mice. Feeding WT mice but not GPR43$^{-/-}$mice butyrate increased the abundance of Prevotellaceae. A recent report by Jiminez et al demonstrated more potent effects of butyrate on gut microbiota by rectal administration of 140 mM butyrate, especially when mice developed colitis upon infection with *Citrobacter rodentium*[47]. The difference of the data between Jiminez et al and our current study is likely due to the different routes of butyrate administration. While butyrate by rectal administration would mostly go to the colon, due to potential absorption, only a small portion of oral administered butyrate would reach the intestinal lumen to affect gut microbiota. Even so, in consistent with our results, their study also demonstrated an increased proportion of Bacteroidetes phylum but decreased Firmicutes phylum after rectal administration of butyrate[47]. While our data demonstrated that butyrate promotes IEC AMP production, which can in turn affects microbiota composition, direct antimicrobial effects of butyrate have also been reported[48], both of which can contribute to butyrate-induced microbiota alterations. Whether these changes directly or indirectly affect butyrate regulation of IEC expression of AMP cannot be stated at this point; however, we showed that SCFA-producing phyla were altered by feeding butyrate. While the Bacteroidetes phylum, which produces mainly acetate and propionate, was increased, members of the Firmicutes phylum, which preferentially produce butyrate[49], were decreased. Thus, we are still not clear, at this point, whether and at what levels the butyrate-mediated alternations of gut microbiota contribute to butyrate induction of IEC AMP expression.

Materials and Methods

Mice

C57BL/6 mice were purchased from the Jackson Laboratory and housed in the Animal Resource Center at UTMB. GPR43$^{-/-}$(Ffar2^{tm1Lex}) mice were obtained from Bristol-Myers Squibb and maintained in the Animal Resource Center at UTMB. All experiments were reviewed and approved by the Institutional Animal Care and Use Committees of UTMB.

Reagents

OPTI-MEM were purchased from Life Technologies. GPR43 agonist (phenylacetamide agonist #44, Amgen Inc)[50] was kindly provided by Dr. Warren N. D'Souza, Amgen Inc. STAT3 inhibitor HJC0152 was kindly provided by Dr. Jia Zhou, University of Texas Medical Branch. McCoy's 5A and Lipofectamine RNAiMAX Transfection Reagent were purchased from Thermo Fisher Scientific. Sodium butyrate (MKBZ0227V), sodium propionate (SLBL9015V), and sodium acetate (SLBH4840V) were purchased from Sigma-Aldrich. Predesigned siRNA directed against mouse mTOR (SI01005683), STAT3 (SI01435287), and negative control siRNA (1027310) were purchased from Qiagen. Antibodies against phosphor-STAT3 (Tyr705), phosphor-mTOR (S2448), phosphor-4E-BP1

(T70), phosphor-p44/42 MAPK (Thr202/Tyr204), phosphor-NF-κB p65 (Ser536), phosphor-S6k1 Ribosomal protein (S235/236) (D57.2.2E), β-actin, and HRP-conjugated anti-rabbit secondary Ab were purchased from Cell Signaling Technology.

SCFA treatment

Mice were given drinking water containing 1g/l metronidazole (Sigma-Aldrich), 0.5g/l vancomycin (Hospira), 1g/l ampicillin (Sigma-Aldrich), and 1g/l kanamycin (Fisher Scientific) for 10 days, followed by SCFA feeding. Butyrate or acetate were added to drinking water with a final concentration of 300 mM. Mice were sacrificed 21 days later for subsequent studies.

Isolation of IEC

After cleaning, small intestines were sliced into small pieces (<0.5 cm), and incubated with 5mM EDTA in HBSS buffer containing 5% FBS at 37°C for 40 min. Cells were collected by passaging supernatant through a 100-μM cell strainer (BD Falcon). After washing with PBS, IEC were separated from a 20%/40% Percoll interface (Amersham Pharmacia Biotech).

Epithelial cell culture

MSIE cells[26] were maintained in RPMI 1640 medium with 5 U/ml murine IFNγ, ITS (5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenous acid), 100 U/ml penicillin/streptomycin, and 5% FBS at 33°C and 5% CO_2. After reaching 80% confluence, cells were starved at 37°C for 16 h before subsequent experiments. HT-29 cells were cultured in complete McCoy's 5A Medium with 10% FBS at 37°C and 5% CO_2.

siRNA transfection

siRNA transfection in MSIE cells was performed by using Lipofectamine RNAiMAX Transfection Reagent according to the manufacturer's instructions. Briefly, 3×10^5 MSIE cells were incubated with 50 nM siRNA and 10 μl LipofectamineRNAiMAX Transfection reagent in 1ml OPTI-MEM medium for 6 h at 33°C, followed by normal growth medium for 40 h at 33°C. MSIE cells were then starved for 6 h at 33°C before treatment. Transfection efficiency was determined at 40 h post transfection. The expression of RegIIIγ and β-defensins was determined at 48 h after administration with SCFA.

Flow cytometry

MSIE cells were treated with SCFA for 48 h then stained for Live/Dead dye and phospho-S6k1 (S235/236). Cells were sampled on a LSRII Fortessaflow cytometer (BD Biosciences), and data analyzed by using FlowJo7.6 software (Tree Star).

Western blot

Total protein of cells was extracted and the concentration determined by a BCA Protein Assay kit. Four μg of protein was separated electrophoretically by NuPAGEBis-Tris mini gels (Life Technologies) and probed with phospho-mTOR, phospho-4EBP1, phospho-STAT3, phosphor-p44/42 MAPK, or phosphor-NF-κB p65 Abs overnight, followed by 1h HRP-conjugated anti-rabbit secondary Ab. Membranes were stripped and re-probed for actin

as loading control. Protein/β-actin relative expression was calculated using ImageJ (10.2) software.

Quantitative Real-time PCR

Total RNA was extracted with TRIzol (Life Technologies, Carlsbad, CA) and quantified for cDNA synthesis. Quantitative real-time PCR reactions were performed by using SYBR Green Gene Expression Assays (Bio-Rad, Hercules, CA, USA). The primers were listed in supplementary Table 1. All data were normalized to GAPDH mRNA expression.

Enteroid culture

The intestines were cut longitudinally, minced, and treated with 2 mM EDTA. The tissues were then treated with PBS containing 43.3 mM sucrose and 54.9 mM sorbitol, and filtered through a 70-μm cell strainer. Supernatant was collected and centrifuged. The resulting pellet containing detached crypts was re-suspended in Matrigel (BD Bioscience) with 0.5μg/ml rEGF (R&D Systems2028-EG), 1μg/ml rNoggin (R&D Systems1967-NG/CF), 5μg/ml rR-spondin (R&D Systems 3474-RS), and 1μg/ml rWnt3a (R&D Systems 35036-WN/CF). 50μl Matrigel with 500 crypts was plated, and advanced DMEM/F12 media (Invitrogen 12634-010) with 1x N2 supplement (R&D Systems AR009), and 1x B27 (Invitrogen 12587-010). Five days later, the enteroids were treated with 0.5 mM butyrate.

16S rRNA pyrosequencing analysis

Fecal bacterial DNA was isolated using a MoBio PowerFecal kit (MoBio, USA). The isolated DNA was amplified using universal V3-V4 16S rRNA V3-V4 region primers. Sequencing was performed with an Illumina MiSeq instrument. The raw sequencing reads were trimmed and filtered based on initial quality assessment using FASTQC. The reads were trimmed to 350 bases and filtered to exclude reads with low quality, two or more unknown characters, sequencing adapters. To identify the presence of known bacteria, the subsequences were analyzed using CLC Genomics Workbench 8.5 Microbial Genomics Module. Reference based OTU picking was performed using the SILVA SSU v119 97% database. Sequences present in more than one copy but not clustered to the database were then placed into de-novo OTUs (97% similarity) and aligned against the database with 80% similarity threshold using MUSCLE alignment.

Statistical analysis

Student’s t test in Prism 5.0 (Graphpad) was used to determine levels of significance for comparisons between samples. Where appropriate, mean ± SEM is represented on graphs. *p< 0.05; **p < 0.01.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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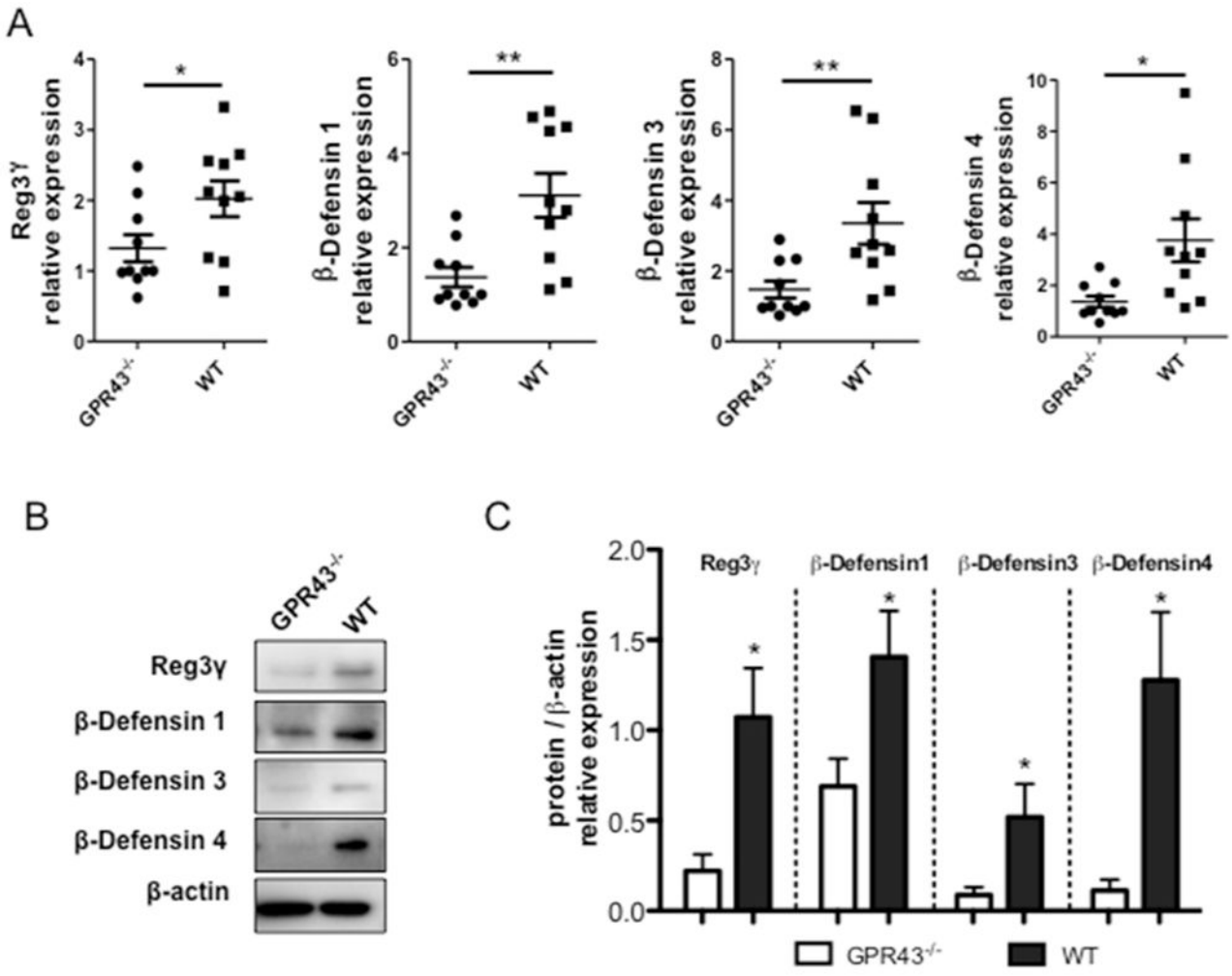

Figure 1. Decreased production of RegIIIγ and β-defensins in IEC of GPR43$^{-/-}$mice

IEC from small intestine were isolated from WT and GPR43$^{-/-}$mice respectively. **(A)** IEC expression of RegIIIγ and β-defensins 1, 3, 4 in GPR43$^{-/-}$and WT mice determined by qRT-PCR and normalized against *gapdh*. Pooled data from 2 independent experiments. n=5 mice/group. **(B)** Protein levels of RegIIIγ and β-defensins were determined by Western blot. Data are reflective of 2 independent experiments. n=4 mice/group. **(C)** Protein/β-actin relative expression of RegIIIγ and β-defensins were compared between GPR43$^{-/-}$and WT mice. Data were combined from 2 independent experiments. *p<0.05; **p<0.01.

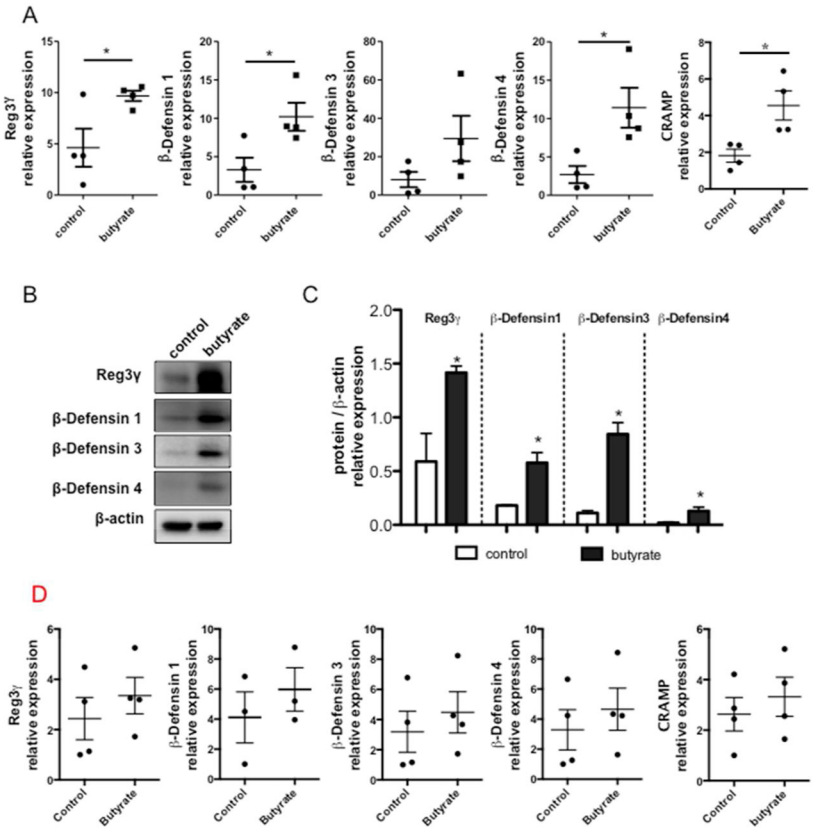

Figure 2. Butyrate feeding promotes IEC expression of RegIIIγ and β-defensins *in vivo*

WT and GPR43$^{-/-}$C57BL/6 mice were treated with antibiotics in drinking water for 10 days, and then fed with or without 300 mM butyrate in drinking water for 21 days. **(A)** IEC expression of RegIIIγ and β-defensins 1, 3, 4, and CRAMP in WT mice were determined by qRT-PCR and normalized against *gapdh*. Data are reflective of 2 independent experiments. n=4 mice/group. **(B)** Protein levels of RegIIIγ and β-defensins determined by Western blot in WT mice. Data are reflective of 2 independent experiments. **(C)** Protein/β-actin relative expression of RegIIIγ and β-defensins were compared between control and butyrate-treated WT mice. Data were combined from 2 independent experiments. *p<0.05. **(D)** IEC expression of RegIIIγ and β-defensins 1, 3, 4 in GPR43$^{-/-}$mice were determined by qRT-PCR and normalized against *gapdh*. Data are reflective of 2 independent experiments. n=4 mice/group.

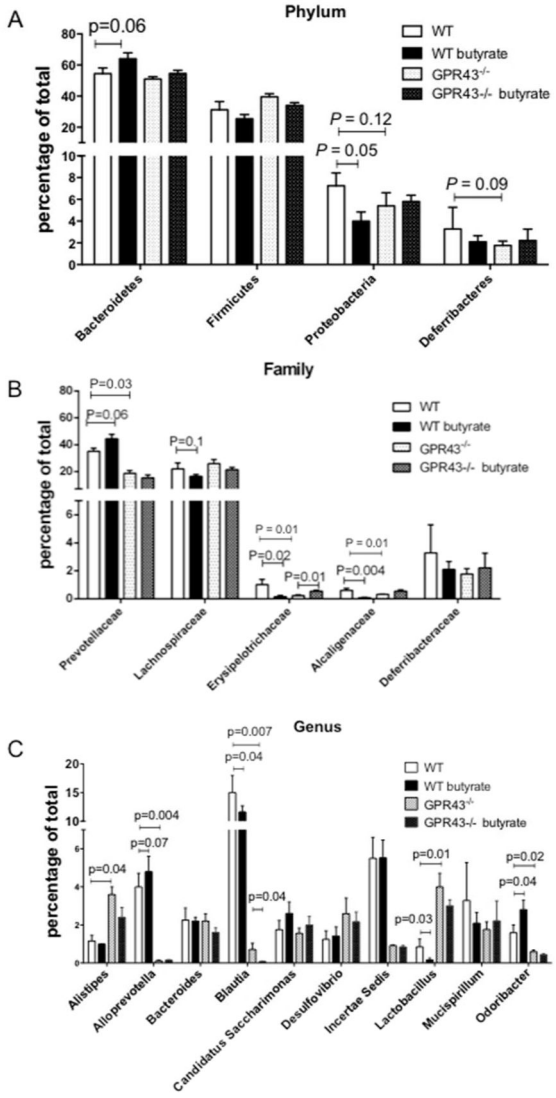

Figure 3. Butyrate feeding changes gut microbiota

WT and GPR43$^{-/-}$C57BL/6 mice were fed with 300 mM butyrate for 21 days. Gut microbiota prior and after feeding butyrate was determined by 16s rRNA sequencing. Bar charts for fecal bacterial composition of phylum level (**A**), family level (**B**), and genus level **(C)**. N=4–5 mice/group.

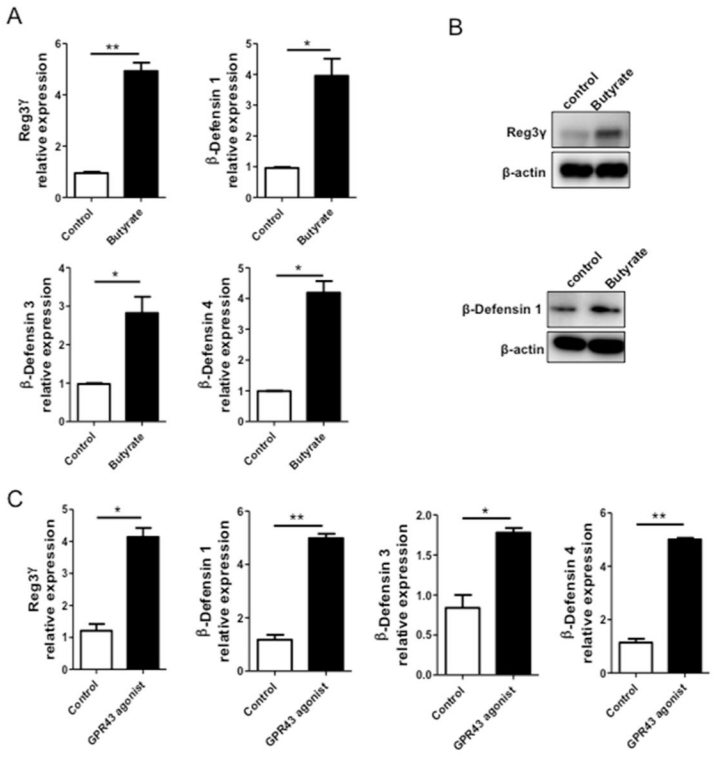

Figure 4. Butyrate and GPR43 agonist induce expression of RegIIIγ and β-defensins in mouse IEC

MSIE cells were treated with 0.5 mM butyrate for 48 h. **(A)** The expression of RegIIIγ and β-defensins mRNA determined by qRT-PCR and normalized against *gapdh*. **(B)** Protein levels of RegIIIγ and β-defensin 1 determined by Western blot. **(C)** MSIE cells were treated with 5 μM GPR43 agonist. The expression of RegIIIγ and β-defensins were determined by qRT-PCR at 48 h. *p<0.05; **p<0.01. Data are reflective of 3 independent experiments.

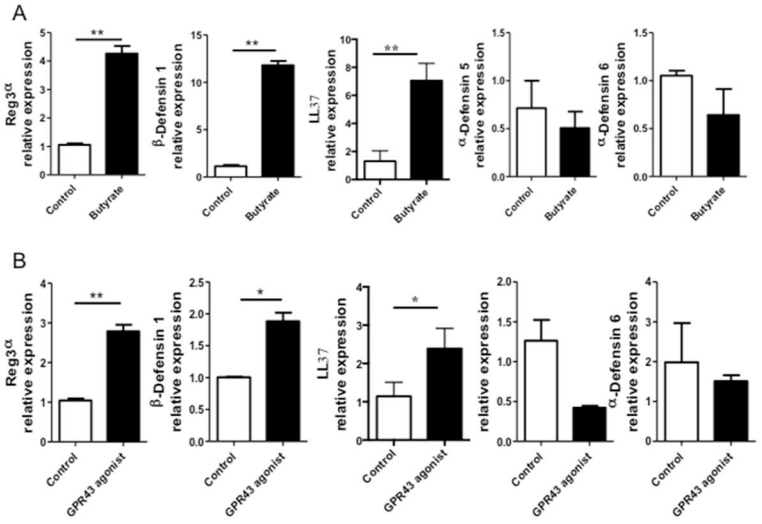

Figure 5. Butyrate and GPR43 agonist induce expression of RegIIIγ and β-defensins in human IEC

HT-29 cells were treated with 0.5 mM butyrate for 48 h. (**A**) The expression of α-defensins, β-defensins, RegIIIα, and LL37 were determined by qRT-PCR and normalized against *gapdh*. **(B)** HT-29 cells were treated with 5 μM GPR43 agonist 48 h, and the expression of α-defensin 6, β-defensin 1, RegIIIα, and LL37 was determined by qRT-PCR. *p<0.05; **p<0.01. Data are reflective of 3 independent experiments.

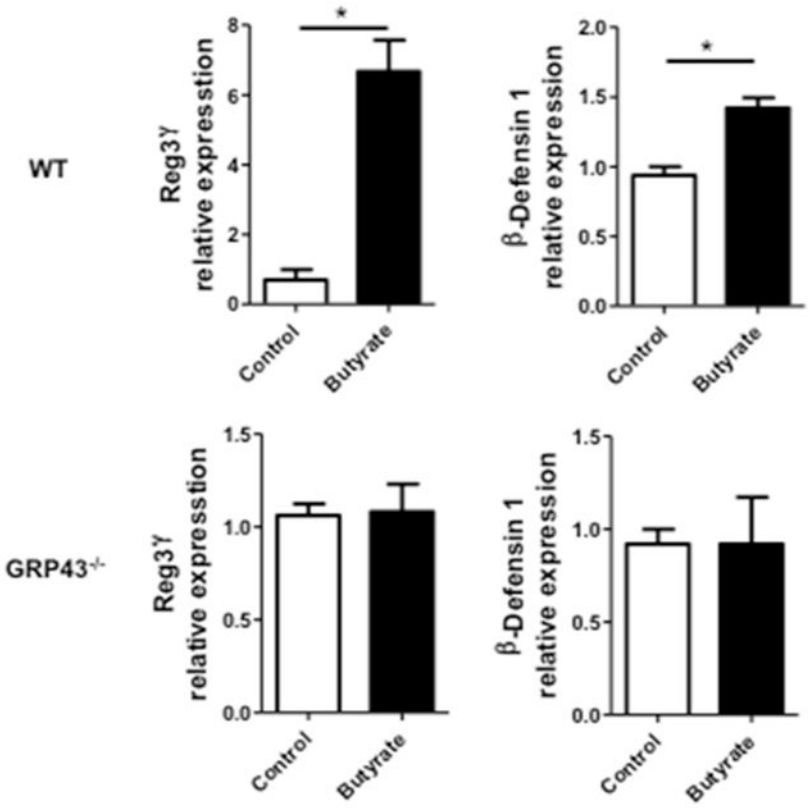

Figure 6. Butyrate induces expression of RegIIIγ and β-defensins in WT but not GPR43$^{-/-}$intestinal epithelial enteroids

Intestinal epithelial enteroids were generated from either WT or GPR43$^{-/-}$mice, and treated with 0.5 mM butyrate. The expression of RegIIIγ and β-defensins were determined by qRT-PCR at 48 h and normalized against *gapdh*. *p<0.05. Data are reflective of 2 independent experiments.

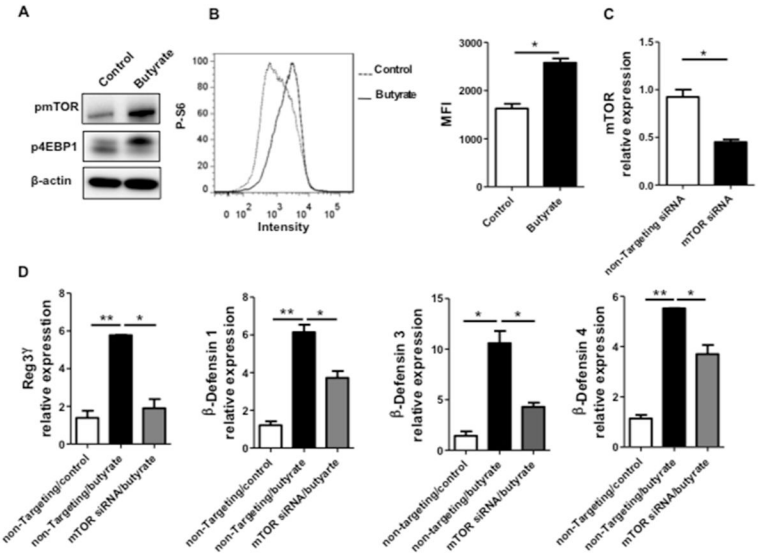

Figure 7. mTOR regulates butyrate induction of RegIIIγ and β-defensins in IEC

MSIE cells were treated with 0.5 mM butyrate for 1 h. **(A)** Phosphorylation of mTOR and 4E-BP1 were determined by Western blot, with β-actin as a loading control. **(B)** The expression of phosphorylated S6K1 was determined by flow cytometry at 48 h post butyrate treatment. Combined median fluorescence intensity (MFI) was presented. **(C and D)** MSIE cells were transfected with mTOR siRNA or control non-targeting siRNA, followed by 0.5 mM butyrate for 48 h. **(C)** siRNA knockdown efficiency was confirmed by RT-PCR at 40 h post-transfection. **(D)** The expression of RegIIIγ and β-defensins were determined by qRT-PCR and normalized against *gapdh* 48 h post-treatment. *p<0.05; **p<0.01. Data are reflective of 3 independent experiments.

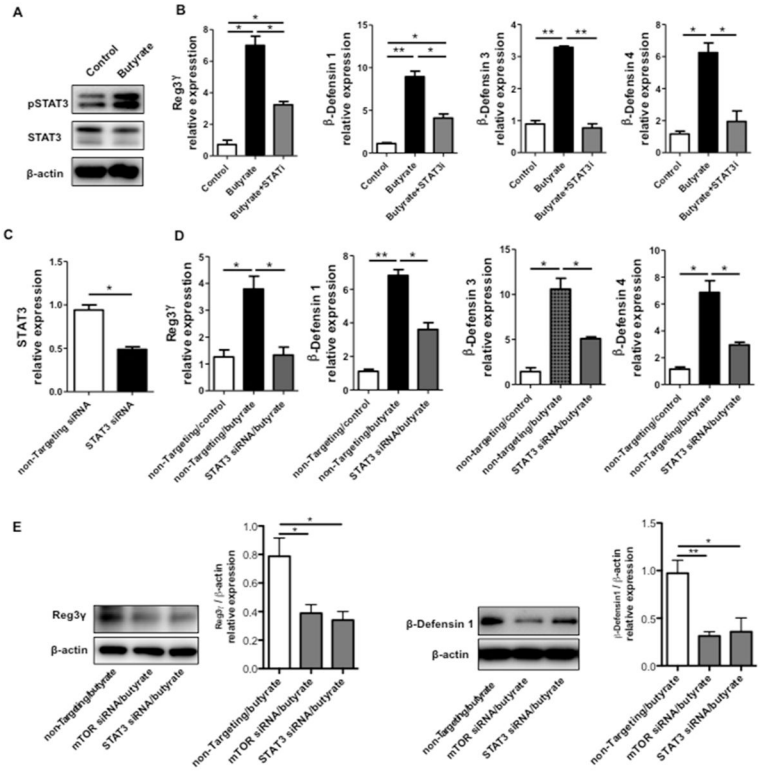

Figure 8. STAT3 regulates butyrate induction of RegIIIγ and β-defensin in IEC

MSIE cells were treated with 0.5 mM butyrate for 1 h. **(A)** Phosphorylation of STAT3 was determined by Western blot, with total STAT3 and β-actin as loading controls. **(B)** MSIE cells were treated with 0.5 mM butyrate in the presence or absence of 5 μM STAT3 inhibitor HJC0152. The expression of RegIIIγ and β-defensins was determined by qRT-PCR at 48 h and normalized against *gapdh*. **(C and D)** MSIE cells were transfected with STAT3 siRNA or control non-targeting siRNA and then treated with 0.5 mM butyrate. **(C)** siRNA knockdown efficiency was confirmed by RT-PCR at 40 h post-transfection. **(D)** The expression of RegIIIγ and β-defensins were determined by qRT-PCR 48 h post-transfection and normalized against *gapdh*. **(E)** Protein levels of RegIIIγ and β-defensin were determined by Western blot and combined protein/β-actin relative expression was presented. *p<0.05; **p<0.01. Data are reflective of 3 independent experiments.