GPR43 Suppresses Intestinal Tumor Growth by Modification of the Mammalian Target of Rapamycin Complex 1 Activity in Apc(Min/+) Mice

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Highlights of the Study

- G protein-coupled receptor 43 suppresses tumor growth in the intestinal tumor model Apc$^{Min/+}$ mice.
- G protein-coupled receptor 43 does not affect inflammatory cytokines and amino acid transporters expression in Apc$^{Min/+}$ tumors.
- G protein-coupled receptor 43 inhibits mammalian target of rapamycin complex 1 activity independent of the Akt pathway.

Abstract

Objective: G protein-coupled receptor 43 (GPR43), a receptor for short-chain fatty acids, plays a role in suppressing tumor growth; however, the detailed underlying mechanism needs to be comprehensively elucidated. In this study, we investigated the role of GPR43 in inhibiting tumor growth using Apc$^{Min/+}$, a murine model of intestinal tumors. ***Materials and Methods:*** Using GPR43$^{−/−}$ Apc$^{Min/+}$ and GPR43$^{+/−}$ Apc$^{Min/+}$ mice, the number of tumors was analyzed at the end of the experimental period. Immunohistochemistry, quantitative polymerase chain reaction, and Western blotting were performed to analyze cellular proliferation and proliferation-associated signal pathways. ***Results:*** Our results revealed that GPR43 deficiency resulted in increased tumor numbers in Apc$^{Min/+}$ mice. Ki67 was highly expressed in GPR43$^{−/−}$ mice ($p > 0.05$). Increased expression levels of proinflammatory cytokines, including interleukin-6 and tumor necrosis factor-α, and amino acid transporters were not observed in GPR43-deficient mice compared to GPR43-sufficient mice. Furthermore, GPR43-deficient tumor tissues showed enhanced mammalian target of rapamycin-mediated phosphorylated ribosomal protein S6 kinase beta-1 ($p > 0.05$) and phosphorylated eukaryotic translation initiation

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factor 4E-binding protein 1 ($p > 0.05$), but not Akt (protein kinase B) phosphorylation ($p = 0.7088$). ***Conclusion:*** Collectively, GPR43 affords protection against tumor growth at least partly through inhibition of the mammalian target of rapamycin complex 1 pathway.

Introduction

Colon cancer is the third most prevalent cancer, following lung and breast cancer, and is the second leading cause of mortality worldwide [1]. Growing evidence indicates that dietary habits are strongly associated with the development of colorectal cancer, especially decreased intake of dietary fibers and increased intake of refined foods [2]. Dietary fibers are fermented by gut flora to produce short-chain fatty acids (SCFAs) such as butyrate, acetate, and propionate in the colon [2], and it has been reported that butyrate-producing microbiota can inhibit intestinal tumor development by modulating Wnt signaling through G protein-coupled receptor 43 (GPR43) [3].

GPR43 (also called FFAR2) is a receptor for SCFAs and is expressed in intestinal epithelial cells [2]. Previous studies have revealed that GPR43 plays a critical role in SCFA-induced suppression of intestinal inflammation in a mouse model, and GPR43 expression is reportedly lost in several human colon cell lines [4]. It has been suggested that GPR43 suppresses tumor growth by employing several mechanisms; for example, by altering the structure of the gut flora [5]. However, detailed underlying mechanisms, as well as other possibly involved mechanisms, remain elusive.

The Apc$^{Min/+}$ mice model is an animal model of intestinal adenomas that harbors a mutation in the APC gene. Eighty-five percent of human colon cancers develop through an adenoma-carcinoma sequence, and most colon cancers develop APC gene mutations [6]. Thus, Apc$^{Min/+}$ mice have been widely used to investigate human intestinal tumor studies. We investigated the role of GPR43 in this animal model of intestinal tumor development.

Materials and Methods

Animals and Diets

Mice were bred under specific pathogen-free conditions in the Animal Facility at the Kobe University Graduate School of Medicine. Breeding pairs of Apc$^{Min/+}$ mice (C57BL/6 background) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). GPR43 homozygous (GPR43$^{-/-}$) mice (C57BL/6 background) were provided as described previously [7]. GPR43$^{+/-}$ Apc$^{Min/+}$ mice were generated by breeding Apc$^{Min/+}$ mice with GPR43$^{-/-}$ mice. Both male and female littermates were used in this study. All mice were fed a CE-2 diet (CLEA Japan Inc., Meguro-ku, Japan). Mice were allowed free access to food and water ad libitum.

Tumor Counts

At 15 weeks of age, the small intestine and colon were cut open longitudinally, and the intestinal contents were removed with curved forceps. The indigo carmine dye (Daiichi-Sankyo, Chuo-ku, Japan) was used to make the detection of tiny tumors easy. The number of tumors of the entire intestine was counted manually by the same researcher, who was blinded to the experimental groups throughout the study.

Immunohistochemical Staining

All terminal ileum specimens were fixed in formalin and embedded in paraffin. Immunohistochemical staining was performed on the paraffin-embedded sections using the polymer peroxidase method. The sections were boiled in 0.01 M pH 6.0 citrate buffer for 15 min in a microwave oven. The sections were incubated with ki67 antibodies (1:100; Cell Signaling, #12202) overnight at 4°C. The cells positive for ki67 staining were analyzed by 2 researchers, blinded to the experimental protocol, using ImageJ software (NIH) to confirm the consistency of obtained results.

RNA Extraction and Real-Time PCR

Tumor and nontumor tissues were removed from the small intestine, placed in RNAlater™ (Thermo Fisher Scientific, #AM7021), and stored at −80°C until use. Real-time PCR was performed according to a previously described method [8]. Relative expression levels of target genes were standardized to HPRT expression. The primers used are listed in Table 1.

Western Blotting

Tissues were harvested from mice 9–15 weeks of age. Total lysates of tumor and nontumor tissues from the small intestine were extracted. Total lysates were incubated with antibodies against p-p70S6K (1:1,000; Cell Signaling, #9234), p-Akt (1:2,000, Cell Signaling, #4060S), p-4EBP1(1:1,000; Cell Signaling, #9455), and β-actin (1:2,000, Cell Signaling, #8457) overnight. Western blot chemiluminescent signals were captured with an ImageQuant LAS 4000 mini imager (GE Healthcare). The band intensity was measured using ImageJ (NIH).

Statistical Analysis

Data are presented as mean ± standard error of the mean, analyzed by employing Student's *t* test, using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, USA). $p < 0.05$ was considered statistically significant.

Results

Loss of GPR43 Promotes Tumor Development in the Small Intestine and Colon

We first confirmed that GPR43$^{+/+}$ Apc$^{Min/+}$mice and GPR43$^{+/-}$ Apc$^{Min/+}$mice presented a similar number of intestinal tumors (shown in online suppl. Fig. 1; see www.karger.com/doi/10.1159/000518621 for all online suppl.
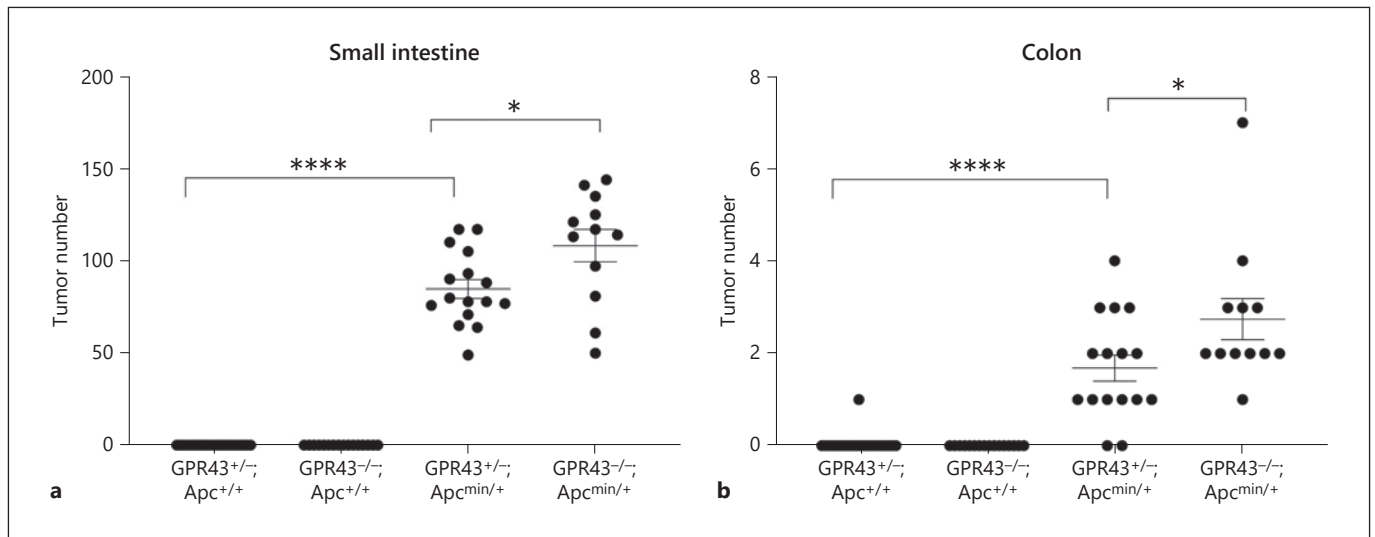
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Fig. 1. GPR43 suppresses tumor growth in the mouse tumor model. Total tumor burden in the small intestine (**a**) and colon (**b**) at 15 weeks of age. GPR43$^{+/-}$ Apc$^{+/+}$, $n = 23$; GPR43$^{-/-}$ Apc$^{+/+}$, $n = 16$; GPR43$^{+/-}$ Apc$^{Min/+}$, $n = 16$; GPR43$^{-/-}$ Apc$^{Min/+}$, $n = 12$. Statistical analysis was performed using Student's t test. $*p < 0.05$, $****p < 0.0001$.

Table 1. The primers used for real-time PCR in this study

Gene	Forward (5′–3′)	Reverse (5′–3′)
IL-6	GACAAAGCCAGAGTCCTTCAGAGAGATACAG	TTGGATGGTCTTGGTCCTTAGCCAC
TNF-α	AAAATTCGAGTGACAAGCCTGTAG	CCCTTGAAGAGAACCTGGGAGTAG
LTA1	CTGGATCGAGCTGCTCATC	GTTCACAGCTGTGAGGAGC
ASCT2	CGGCACGCCCGGGAGGCTTTC	GAATCTGGGGGCCGGAAGCGG
HPRT	GTTGGATACAGGCCAGACTTTGTTG	CCAGTTTCACTGGTGACACAAACG

material), indicating that heterozygous GPR43 expression does not affect the number of intestinal tumors observed in the Apc$^{Min/+}$ mouse strain. To further determine the GPR43 function, we used GPR43$^{+/-}$ Apc$^{Min/+}$ and GPR43$^{-/-}$ Apc$^{Min/+}$ littermate mice for comparison. Expression of GPR43 was evaluated by real-time PCR, confirming that the genotyping result was reliable (shown in online suppl. Fig. 2). At 15 weeks of age, the GPR43$^{-/-}$ Apc$^{Min/+}$ mice showed a higher number of tumors than GPR43$^{+/-}$ Apc$^{Min/+}$ mice (shown in Fig. 1a, b), suggesting that GPR43 plays an important role in suppressing intestinal tumor development. Ki67, a marker of proliferation in tumor tissues, was enhanced in GPR43$^{-/-}$ Apc$^{Min/+}$ mice. Increased cell proliferation and suppression of apoptosis are important factors that promote tumor growth [9]. To investigate how GPR43 deficiency results in increased numbers of tumor, we performed immunohistochemistry to measure expression levels of ki67 in tumor tissues obtained from both GPR43$^{+/-}$ Apc$^{Min/+}$ and GPR43$^{-/-}$ Apc$^{Min/+}$ mice. The results showed that the GPR43$^{-/-}$ Apc$^{Min/+}$ tumor tissues exhibited higher levels of ki67-positive cells (shown in Fig. 2a, b). Conversely, TUNEL staining, performed to compare the frequency of apoptotic tumor cells, revealed infrequent positive cells in both groups (data not shown). These results suggest that GPR43 deficiency may promote cell proliferation in Apc$^{Min/+}$ mice.

GPR43 Deficiency Enhances the mTORC1 Pathway

It is well known that inflammation is associated with tumor growth. Reportedly, the overexpression of interleukin (IL)-6 promotes cachexia and increases intestinal tumors in Apc$^{Min/+}$ mice [10], and blocking tumor necrosis factor (TNF)-αin mice decreases colorectal carcinogenesis [11]. Therefore, we assessed the expression of TNF-α and IL-6 in GPR43$^{+/-}$Apc$^{Min/+}$ and GPR43$^{-/-}$ Apc$^{Min/+}$ mice by real-
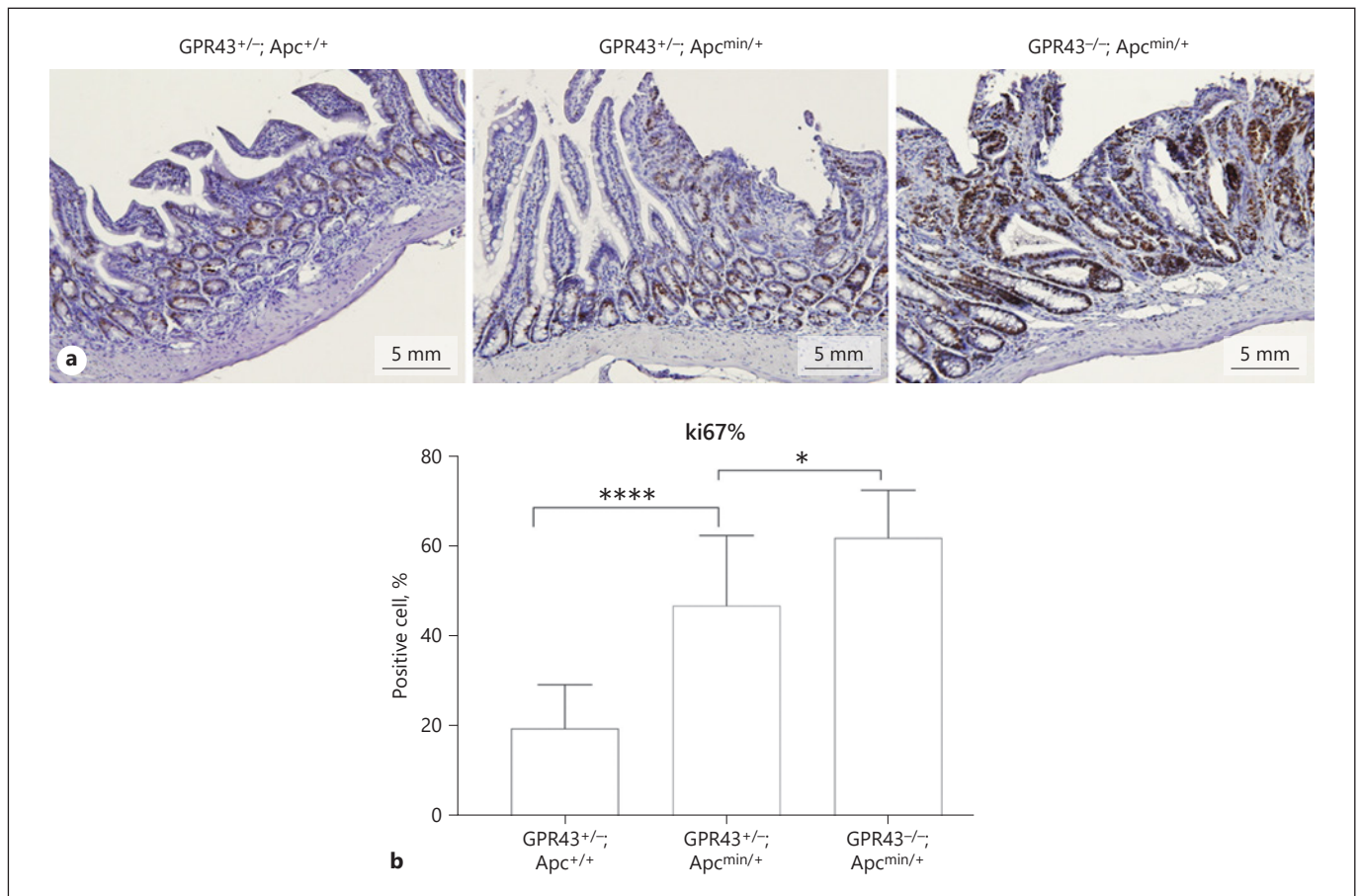
Fig. 2. GPR43 deficiency promotes cell proliferation in Apc$^{Min/+}$ mice. **a** Representative picture of staining analysis. **b** Staining of proliferative marker (ki67) was enhanced in GPR43$^{-/-}$ Apc$^{Min/+}$mice. The ki67-positive cells in each group were assessed using ImageJ. GPR43$^{+/-}$ Apc$^{+/+}$, $n = 10$; GPR43$^{+/-}$ Apc$^{Min/+}$, $n = 14$, GPR43$^{-/-}$ Apc$^{Min/+}$, $n = 7$. Scale bar, 5.0 mm. Magnification, ×200. Statistical analysis was performed using Student's t test. *$p < 0.05$, ****$p < 0.0001$.

time PCR; however, the expression of both inflammatory genes did not demonstrate any difference (Fig. 3a, b). This suggests that in our current setting, GPR43 affords protection against tumor development not through the modulation of the inflammatory pathway. Furthermore, tumor cells require a large number of nutrients and amino acids for rapid cell proliferation [12]. Both L-type amino acid transporter (LAT1) and ASCT2 are important amino acid transporters, and knockdown of LAT1 and ASCT2 with RNA interference exhibits reduced cell proliferation [13]. We speculated whether GPR43 deficiency can modulate the expression of amino acid transporters. Apc$^{Min/+}$ tumors demonstrated higher expression levels of these transporters than Apc$^{+/+}$ intestinal tissue; however, no difference was observed between GPR43$^{+/-}$ Apc$^{Min/+}$ and GPR43$^{-/-}$ Apc$^{Min/+}$ tissues (shown in Fig. 3c, d). The mammalian target of rapamycin complex 1 (mTORC1) pathway plays an extremely important role in cell proliferation. It promotes cell growth by promoting protein translation, as well as ribosome biogenesis, and the prototypic mTOR pathway is activated by the PI3K/Akt pathway; however, it can receive multiple signal inputs in an Akt-independent manner [14]. Protein kinase S6k1 and 4EBP1 are downstream of the mTORC1 pathway and are activated by phosphorylation. We hypothesized that GPR43 deficiency enhances the mTORC1 pathway. As expected, Western blotting revealed enhanced phosphorylation of p70S6K (p-p70S6K) and 4EBP1 (p-4EBP1) in GPR43$^{-/-}$ Apc$^{Min/+}$ mice, but interestingly, phospho-Akt (p-Akt) enhancement was not observed (shown in Fig. 4a, b).
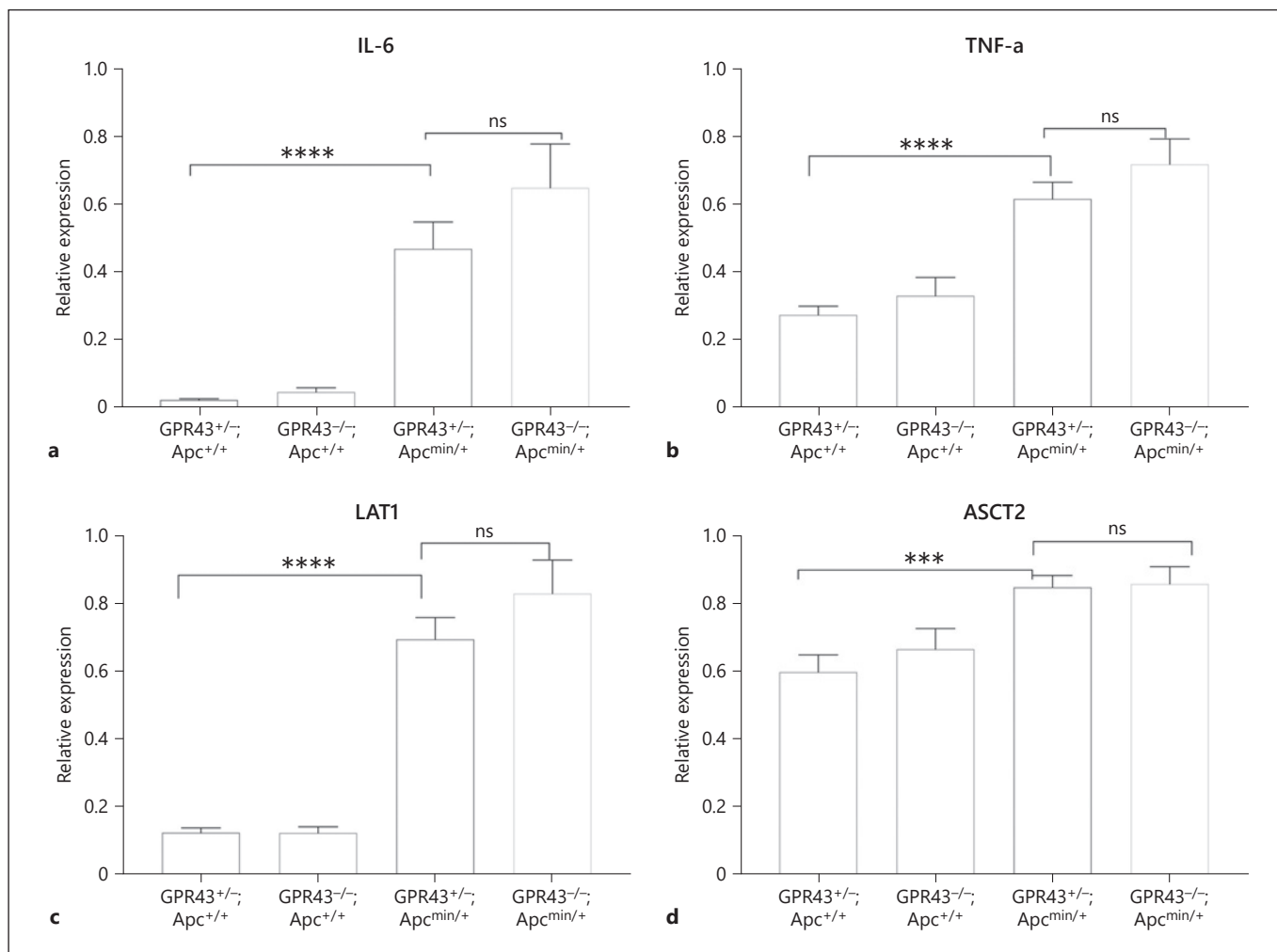
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Fig. 3. Real-time PCR analysis does not reveal any difference in the small intestine. Normal tissues were harvested from GPR43$^{+/-}$ Apc$^{+/+}$ ($n = 20$) and GPR43$^{-/-}$ Apc$^{+/+}$ mice ($n = 14$); tumor tissues were harvested from GPR43$^{+/-}$ Apc$^{Min/+}$ ($n = 16$) and GPR43$^{-/-}$ Apc$^{Min/+}$ mice ($n = 12$). All tissues were harvested from the small intestine. **a–d** Gene expression levels were analyzed. Data are presented as mean ± standard error of the mean. ***$p < 0.0005$, ****$p < 0.0001$, ns, no significance, $p > 0.05$. Statistical analysis was performed using Student's t test.

Discussion

GPR43 is mainly expressed in intestinal epithelial cells, and GPR43 is also expressed in the small intestine of Apc$^{Min/+}$ mice. It has been reported that GPR43 deficiency enhances the downstream signals of the cAMP-PKA-CREB pathway, leading to histone deacetylase overexpression [15]. It has also been shown that the butyrate-producing probiotic called *Clostridium butyricum* could inhibit high-fat diet induced intestinal tumor development in Apc$^{Min/+}$ mice through GPR43 [3]. These observations indicate the importance of the interactions between gut flora, SCFAs, and GPR43 in the intestinal tumor de-

velopment. However, to the best of our knowledge, the possible modification of mTORC1 activation by GPR43 in the intestinal tumor has not been previously reported.

Chronic inflammation is linked to tumorigenesis, including cell survival and proliferation [16]. GPR43$^{-/-}$ mice reportedly exhibit severe inflammation in a mouse model of dextran sulfate sodium colitis. Nuclear factor-κB regulates numerous genes involved in inflammatory responses in activated M1 macrophages, including IL-6 and TNF-α [17], both of which are highly expressed in tumors. However, our results failed to demonstrate any increased expression of IL-6 and TNF-α in the GPR43-deficient tissue, indicating that the increased tumor phe-
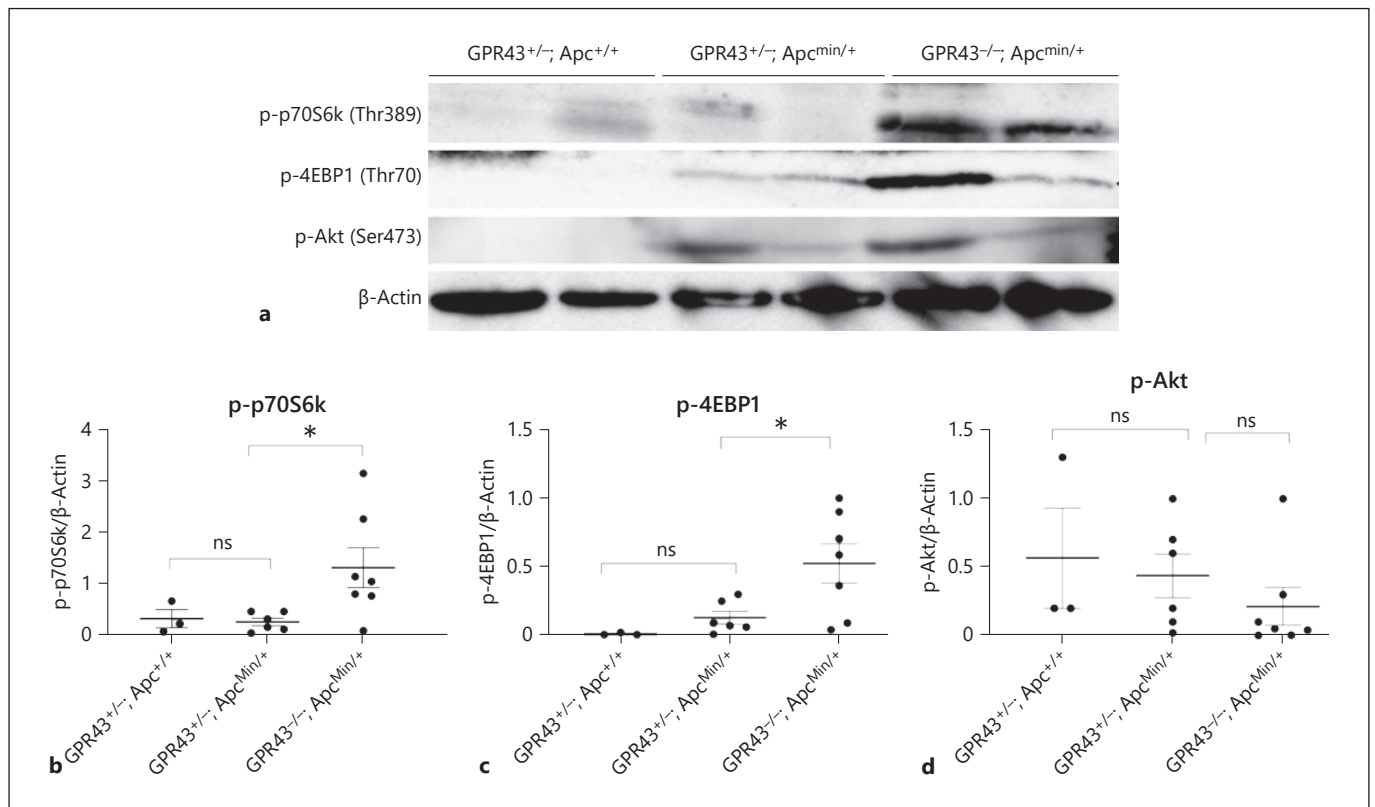
Fig. 4. GPR43 deficiency enhanced the mTORC1 pathway independent of the Akt signaling pathway. Total lysates of tumor or nontumor tissues from the small intestine were extracted. Representative picture of Western blotting analysis (**a**) and the band intensity of p-p70S6K (**b**), p-4EBP1 (**c**), and p-Akt (**d**) in each group are shown. GPR43$^{+/-}$ Apc$^{+/+}$, $n = 3$; GPR43$^{+/-}$ Apc$^{Min/+}$, $n = 6$; GPR43$^{-/-}$ Apc$^{Min/+}$, $n = 7$. Statistical analysis was performed using Student's t test. ns, no significance, $*p < 0.05$.

notype was not due to aberrant immunological activation. SCFAs, such as butyrate, are known to fuel intestinal epithelial cell growth [18], and hence we speculated whether GPR43 deficiency might upregulate some genes to take-up alternative nutrients such as amino acids. Cancer progression is linked to increased nutrient uptake to support cellular proliferation [19]. Among over 60 amino acid transporters, LAT1 and ASCT2 are major pro-tumoral transporters with high-level expression in several cancer types, including colon cancer, and the knockdown of both amino acid transporters can suppress cell growth [12]. However, unfortunately, similar levels of LAT1 and ASCT2 expression can be detected in both GPR43$^{+/-}$ Apc$^{Min/+}$ and GPR43$^{-/-}$ Apc$^{Min/+}$ mice, indicating that the increased tumor proliferation is possibly not associated with increased amino acid transporter expression.

mTORC1 is critical for the proliferation of Apc-deficient mice [13]; however, the interconnection between GPR43 and the mTORC1 pathway has not yet been investigated. Signaling associated with the mTORC1 pathway has been intensively studied, and the classical mechanism of mTORC1 regulation through the Akt pathway activation is widely known. Conversely, mTORC1 can also be activated in an Akt-independent pathway. In the current study, we observed that the expression of p-p70S6K and p-4EBP1 was stronger in GPR43$^{-/-}$ Apc$^{Min/+}$ mice, suggesting that GPR43 deficiency induces mTORC1 pathway activation to promote cell proliferation in Apc$^{Min/+}$ mice.

Interestingly, our results indicate that GPR43 deficiency does not affect Akt phosphorylation. Akt-independent mechanisms of mTOR regulation have been reported in several studies, and researchers have revealed that the Ras/MEK/ERK pathway may also activate mTOR via phosphorylation of tuberous sclerosis complex 2 (TSC2). TSC is a genetic disorder characterized by numerous benign tumors, and patients with TSC frequently show extracellular signal-regulated kinase activation [20]. Furthermore, the mTOR pathway is regulated by energy-sensing pathways,

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with intracellular energy depletion suppressing mTOR activation through regulation of AMP-activated protein kinase, which is activated by calmodulin-dependent protein kinase β, associated with increased intracellular calcium. Additionally, the binding of hormones and cytokines to Gq-couple receptors activate AMP-activated protein kinase [21]. Moreover, a previous study has reported that the mTORC1 pathway remains active even following complete inhibition of the PI3K/Akt pathway in a breast cancer cell line [22]. In addition to the above findings, mTORC1 couples with amino acids to regulate cell proliferation [23]. However, the involvement of amino acids, especially that of transporters, appeared contradictory in our study.

Although our results showed that GPR43 deficiency led to the enhancement of the mTORC1 pathway without affecting phosphorylation of Akt, the direct evidence of the interaction between those is missing in this study. We only tested expression levels of very limited number of major amino acid transports with negative findings in the current study; however, a lot more transporters exist. The interactions between GPR43 and mTORC1 pathway might be far more complicated, and GPR43 might be inhibiting the WNT pathway and indirectly influencing mTORC1 activation. Comprehensive analysis such as RNA sequencing data using tumor tissues from GPR43-deficient and -sufficient mice might be one good way to investigate it further as a future study.

Conclusion

We observed that the expression of p-p70S6K and p-4EBP1 was enhanced in GPR43$^{-/-}$ Apc$^{Min/+}$ mice, but not p-Akt. This may suggest that GPR43 inhibits tumor development partly by suppressing the mTORC1 pathway independent of the Akt pathway. The exact mechanism of GPR43 in regulating the mTORC1 pathway in Apc$^{Min/+}$ mice warrants further investigation.

Statement of Ethics

All animal experiments were approved by the Institutional Animal Care and Use Committee of Kobe University (Permission No. P160505).

Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

Funding Sources

Author Contributions

L.K. and N.H. conceived and designed the experiments. L.K., N.H., Y.S., Y.Y., R.Y., M.O., Z.T., I.K., and Y.K. analyzed the data and drafted the manuscript.

Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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