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The role of $G\alpha_q/G\alpha_{11}$ signaling in intestinal epithelial cells

Hirosato Mashima[a,*], Noboru Watanabe[b], Masanari Sekine[a], Satohiro Matsumoto[a], Takeharu Asano[a], Kazuhito Yuhashi[a], Noriyoshi Sagihara[a], Shunsuke Urayoshi[a], Takeshi Uehara[a], Junichi Fujiwara[a], Takehiro Ishii[a], Rumiko Tsuboi[a], Hiroyuki Miyatani[a], Hirohide Ohnishi[a,c]

[a] *Department of Gastroenterology, Saitama Medical Center, Jichi Medical University, Saitama 330-8503, Japan*
[b] *Department of Gastroenterology, Akita University Graduate School of Medicine, Akita 010-8543, Japan*
[c] *Japan Organization of Occupational Health and Safety, Kawasaki 211-0021, Japan*

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ABSTRACT

Intestinal homeostasis and the coordinated actions of digestion, absorption and excretion are tightly regulated by a number of gastrointestinal hormones. Most of them exert their actions through G-protein-coupled receptors. Recently, we showed that the absence of $G\alpha_q/G\alpha_{11}$ signaling impaired the maturation of Paneth cells, induced their differentiation toward goblet cells, and affected the regeneration of the colonic mucosa in an experimental model of colitis. Although an immunohistochemical study showed that $G\alpha_q/G\alpha_{11}$ were highly expressed in enterocytes, it seemed that enterocytes were not affected in Int-G_q/G_{11} double knock-out intestine. Thus, we used an intestinal epithelial cell line to examine the role of signaling through $G\alpha_q/G\alpha_{11}$ in enterocytes and manipulated the expression level of $G\alpha_q$ and/or $G\alpha_{11}$. The proliferation was inhibited in IEC-6 cells that overexpressed $G\alpha_q/G\alpha_{11}$ and enhanced in IEC-6 cells in which $G\alpha_q/G\alpha_{11}$ was downregulated. The expression of T-cell factor 1 was increased according to the overexpression of $G\alpha_q/G\alpha_{11}$. The expression of Notch1 intracellular cytoplasmic domain was decreased by the overexpression of $G\alpha_q/G\alpha_{11}$ and increased by the downregulation of $G\alpha_q/G\alpha_{11}$. The relative mRNA expression of *Muc2*, a goblet cell marker, was elevated in a $G\alpha_q/G\alpha_{11}$ knockdown experiment. Our findings suggest that $G\alpha_q/G\alpha_{11}$-mediated signaling inhibits proliferation and may support a physiological function, such as absorption or secretion, in terminally differentiated enterocytes.

1. Introduction

The architectural features of the intestinal epithelium are maintained by a rapid cellular turnover through the continuous replication of multipotential stem cells residing in the niches in the lower part of the crypt [1]. Stem cells give rise to progenitor cells, which are amplified by constant division along the bottom two-thirds of the crypts [2]. These daughter cells migrate up as they proliferate. In the transit amplifying (TA) zone near the top of the crypt, these cells terminally differentiate into the four main cell types: absorptive enterocytes and three secreting cell types: mucus-secreting goblet cells, antimicrobial peptide-secreting Paneth cells and hormone-secreting enteroendocrine cells [3].

Proliferation, differentiation and morphogenesis in the intestinal epithelium are tightly regulated by a number of molecular pathways. Cells adopt an absorptive or secretory cell fate according to the balance between Wnt and Notch signaling, regulating transcription networks that further define the differentiation of intestinal epithelial cells (IECs) [4,5]. The most established effects of Wnt/β-catenin in IECs are those involved in cellular proliferation, in particular the maintenance of the proliferative state of progenitors [4]. The Notch cascade mediates cell-to-cell signaling and has been shown to be essential for the maintenance of the proliferative crypt compartment, as well as for the formation of absorptive enterocytes [4].

The digestive tract consists of a variety of tissues. The coordination of the complex functions of the digestion, absorption, and excretion of a meal is largely achieved by molecules of neuroendocrine origin [6]. Most of the gastrointestinal hormones regulate their target cells through G-protein coupled receptors (GPCRs). Secretin, glucose-dependent insulinotropic polypeptide (GIP), and glucagon-like-peptide-1 (GLP-1)

Abbreviations: Ab, antibody; ACh, acetylcholine; Atoh1, atonal homolog 1; CCK, cholecystokinin; CCK2R, cholecystokinin-2 receptor; DKO, double knock-out; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like-peptide-1; GPCR, G-protein coupled receptor; HE, hematoxylin and eosin; IEC, intestinal epithelial cell; mAChR, muscarinic acetylcholine receptor; NICD, Notch1 intracellular cytoplasmic domain; qPCR, quantitative real-time PCR; siRNA, small interfering RNA; TA, transit amplifying; Tcf1, T-cell factor 1; VIP, vasoactive intestinal peptide

* Corresponding author.
E-mail address: hmashima1-tky@umin.ac.jp (H. Mashima).

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exert their signals through the $G\alpha_s$ family of heterotrimeric G proteins [7]. In contrast, cholecystokinin (CCK), gastrin, and acetylcholine (ACh) exert their signals through the $G\alpha_q$ family of G proteins. Mammals express four $G\alpha_q$ class α-subunits, of which two—$G\alpha_q$ and $G\alpha_{11}$—are widely expressed [8].

In order to evaluate the physiological relevance in the regulation of intestinal homeostasis, we generated Int-G_q/G_{11} double knock-out (DKO) (*VilCre*$^{+/-}$*Gnaq*$^{flox/flox}$*Gna11*$^{-/-}$) mice and investigated the role of $G\alpha_q/G\alpha_{11}$-mediated signaling in the intestine [9]. The absence of $G\alpha_q/G\alpha_{11}$-mediated signaling especially impaired Paneth cell maturation and positioning, and regeneration in an experimental model of colitis. In an immunohistochemical study, the expression of $G\alpha_q/G\alpha_{11}$ was abundantly detected near the basolateral membrane of enterocytes, but not in the Paneth cells. Although the Paneth cells in the Int-G_q/G_{11} DKO intestine showed severe alteration, the enterocytes did not show any alteration. Thus, we used a rat intestinal epithelial cell line (IEC-6) to explore the role of $G\alpha_q/G\alpha_{11}$-mediated signaling in enterocytes. We manipulated the expression of $G\alpha_q/G\alpha_{11}$ in IEC-6 cells and evaluated the changes in proliferation, intracellular signaling, and differentiation.

2. Materials and methods

2.1. Cell culture

IEC-6 cells (RBRC-RCB0993), a rat intestinal epithelial cell line, were purchased from RIKEN Cell Bank (Tsukuba, Japan). IEC-6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 25 mM glucose, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified environment of 95% air and 5% CO_2. The medium was replaced every 1–3 days, depending on the harvest time and the degree of confluence. All of the experiments were carried out using IEC-6 cells at the 10–25th passage.

2.2. Construction of retroviruses expressing GNAQ and GNA11

The *GNAQ* and *GNA11* retroviral expression vectors were prepared in a bicistronic vector pMXs-IRES-EGFP and pMXs-IRES-Neo, respectively (Cell Biolabs, Inc., San Diego, CA, USA). The human full-length *GNAQ* gene (GenBank Accession No. NM_002072) was cloned by a PCR using total RNA from CaCO2 cells as a template and the following primers: sense 5′-CTCGAGCCACC**ATG**ACTCTGGAGTCCATCATGG-3′ and antisense 5′-GCGGCCGCTTAGACCAGATTGTACTCCTTCAG-3′. The human full-length *GNA11* gene (GenBank Accession No. NM_002067) was amplified using the following primers: sense 5′- CTCGAGCCACG**ATG**ACTCTGGAGTCCATGATGG-3′ and antisense 5′- GCGGCCGCTCAGACCAGGTTGTACTCCTTG-3′. The PCR products were digested with the XhoI and NotI restriction enzymes and inserted into the XhoI/NotI sites of the pMXs-IRES-GFP and pMXs-IRES-Neo vector, respectively. The whole nucleotide sequences of these constructs were confirmed by sequencing. The XhoI and NotI sites of the above primers are underlined and the start codon is indicated by bold typeface.

2.3. Construction of GNAQ- and/or GNA11-overexpressing IEC-6 cells

The expression vectors (pMXs-GNAQ-IRES-GFP, pMXs-GNA11-IRES-Neo) and the mock vectors (pMXs-IRES-GFP, pMXs-IRES-Neo) that were used as a negative control were transfected with X-tremeGENE 9 DNA transfection reagent (Roche Applied Science, Basel, Switzerland) into PLAT-E cells to obtain the viruses. The IEC-6 cells were infected with the viruses, and the cells expressing GFP were sorted into the medium using a FACS Vantage system (Beckton Dickinson, NJ, USA) at 48–72 h after infection. The IEC-6 cells expressing neomycin-resistant genes were selected with the addition of 400 µg/ml G418 (Invitrogen, Carlsbad, CA, USA) to the culture medium. The cells were named IEC6-cont1 (pMXs-IRES-GFP vector), IEC6-cont2 (pMXs-IRES-Neo vector), IEC6-Gq, IEC6-G11, and IEC6-Gq/11, respectively.

2.4. Small interfering RNA (siRNA) transfection

The siRNAs specific for rat *Gnaq* (Stealth siRNAs RSS330736, RSS330737, RSS372821) and *Gna11* (Stealth siRNAs RSS340230, RSS340231, RSS340232) and the matched negative control were purchased from Invitrogen. The *Gnaq*, *Gna11* and negative control siRNAs were transfected twice on two consecutive days using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were named IEC6-sicont (negative control siRNA), IEC6-GΔq (*Gnaq* siRNA), IEC6-GΔ11 (*Gna11* siRNA) and IEC6-GΔq/11 (*Gnaq* + *Gna11* siRNA).

2.5. Measurement of cell growth and DNA synthesis

To measure cell growth, the cells were seeded at a density of 1×10^3 cells/ml in plastic 24-well plates and cultured. After 4, 7 and 10 days, the cells were detached by incubation with 0.05% trypsin/EDTA, and the number of cells was counted using a Cell Counter Plate (Watson, Kobe, Japan). To evaluate DNA synthesis, IEC-6 cells were seeded at a density of 1×10^4 cells/ml in 96-well culture plates. Following serum starvation for 24 h, the cells were cultured for an additional 48 h. BrdU was added for the last two hours of incubation. The DNA synthesis was evaluated using a BrdU incorporation assay kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instruction. CCK-8 was purchased from Peptide Institute (Osaka, Japan), and carbachol was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.6. Western blotting

IEC-6 cells were homogenized in a lysis buffer (100 mM NaCl, 20 mM Tris/HCl (pH7.5), 1% TritonX-100). After centrifugation, the crude extracts were boiled in Laemmli 2× sample buffer. Twenty to eighty micrograms of protein was loaded onto each lane of 7.5% sodium dodecyl sulphate-polyacrylamide gels and run at 200 V. The proteins were then transferred onto nitrocellulose membranes at 60 V for 4 h. The membranes were incubated sequentially with Blocking Ace (Snow Brand Milk Products, Sapporo, Japan), primary antibodies (Abs) and secondary Abs, then were detected using an enhanced chemiluminescence Western blotting detection reagent (Amersham Biosciences, Piscataway, NJ) to visualize the secondary Ab. The experiment was repeated independently at least three times. The densitometry analysis was performed using the ImageJ software program. The primary Abs used in this study were anti-GFP Ab from Thermo Fisher Scientific (Carlsbad, CA, USA); anti-Gq/11 and anti-Notch1 Abs from Abcam (Cambridge, UK); anti-phospho-PKC (pan), anti-PKCα, anti-PKCδ, and anti-Tcf1 Abs from Cell Signaling (Danvers, MA, USA); and anti-actin Ab from Santa Cruz (Dallas, TX, USA). The secondary Abs were horseradish-peroxidase-conjugated donkey anti-rabbit IgG and horseradish-peroxidase-conjugated donkey anti-goat IgG, purchased from Jackson Immuno Research (West Grove, PA, USA).

2.7. Quantitative real-time PCR (qPCR)

The following primers were used for the qPCR: Muc2, sense 5′-CGAAGTGAAGAGTGAGCACG-3′ and antisense 5′-GGATCCGGGTGGTATTCAGC-3′; β-actin, sense 5′- TGAGAGGGAAATCGTGCGTG-3′ and antisense 5′- TCATGGATGCCACAGGATTCC-3′. The reactions were performed using an ABI PRISM 7900HT system (Applied Biosystems), with denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 60 s.

2.8. Statistical analysis

All data are presented as the mean ± standard deviation. The statistical significance of the values obtained was evaluated by Student's *t*-

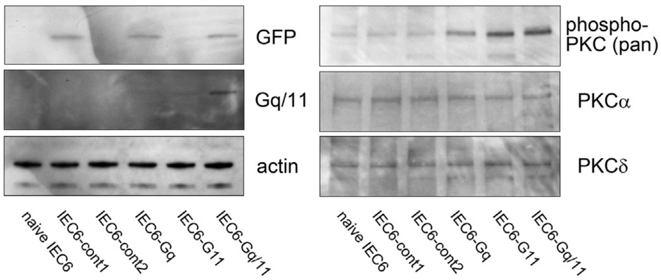

Fig. 1. The generation of $G\alpha_q$ and/or $G\alpha_{11}$-overexpressing IEC-6 cells. Twenty to forty micrograms of protein was loaded onto each lane. Western blotting was performed using anti-GFP, anti-$G\alpha_q/G\alpha_{11}$, and anti-phospho PKC (pan) antibodies. The actin blot was used as an internal control for GFP and $G\alpha_q/G\alpha_{11}$. The PKCα and PKCδ blots were used as internal controls for phospho-PKC (pan). As expected, PKC phosphorylation was elevated in $G\alpha_q/G\alpha_{11}$-overexpressing cells. This antibody detects the endogenous levels of PKC α, βI, βII, δ, ε, η and θ isoforms when it is phosphorylated at a carboxyl terminal residue homologous to serine 660 of PKC βII.

test. A value of $p < 0.05$ was considered to be significant.

3. Results

3.1. Generation of $G\alpha_q$ and/or $G\alpha_{11}$-overexpressed IEC-6 cells

To determine the functional role of $G\alpha_q/G\alpha_{11}$ signaling, we designed overexpression and knock-down systems using a non-transformed small intestinal epithelial cell line, IEC-6. We generated retroviral vectors to drive the expression of *GNAQ* and/or *GNA11* cDNA. The recombinant retroviral transduction results in the stable integration of the *GNAQ* and/or *GNA11* cDNA transgene into the IEC-6 genome and the stable expression of *GNAQ* and/or *GNA11*. After sorting and/or positive selection with geneticin (G418), equal numbers of IEC6-cont1, IEC6-cont2, IEC6-Gq, IEC6-G11, and IEC6-Gq/11 cells were plated and allowed to grow over a 7-day period. To confirm the overexpression of $G\alpha_q$ and $G\alpha_{11}$, we performed Western blotting using anti-$G\alpha_q/G\alpha_{11}$ and anti-GFP antibodies. As shown in Fig. 1, the band of $G\alpha_q/G\alpha_{11}$ was not detected in naïve IEC6, IEC6-cont1 or IEC6-cont2 cells. It was slightly expressed in IEC6-Gq and IEC6-G11 cells and was clearly overexpressed in IEC6-Gq/11 cells. As expected, the expression of GFP was observed in IEC-cont1, IEC6-Gq, and IEC6-Gq/11 cells, but not in naïve IEC6, IEC6-cont2 or IEC6-G11 cells. The activation of $G\alpha_q$ and $G\alpha_{11}$ results in the stimulation of the phospholipase C (PLC)-β isoform and the consequent inositol 1,4,5-triphosphate-mediated (IP_3-mediated) intracellular calcium mobilization and PKC activation [10]. To confirm the functional expression of $G\alpha_q$ and $G\alpha_{11}$ in IEC-6 cells, the PKC phosphorylation was examined using anti-phospho PKC (pan) Ab and the blots of PKCα and PKCδ were used as internal controls. As shown, the induction of the $G\alpha_q/G\alpha_{11}$ expression resulted in an increase in PKC phosphorylation.

3.2. The effect of $G\alpha_q/G\alpha_{11}$-overexpression in IEC-6 cells

We first analyzed the proliferation of IEC-6 cells by counting the number of cells and measuring the incorporation of BrdU (Fig. 2A, B). We seeded IEC-6 cells at 1×10^4 cell/ml in plastic plates and counted the number of cells on days 4, 7, and 10. As shown, the proliferation was inhibited in IEC6-Gq, and IEC6-G11 cells, and severely inhibited in IEC6-Gq/11 cells. In the BrdU incorporation assay, a similar result was obtained and the proliferation was significantly inhibited in IEC6-Gq/11 cells.

Intestinal homeostasis is preserved by a number of molecular pathways, including Wnt/β-catenin and Notch signaling [4]. Wnt/β-catenin signaling was altered in the Int-G_q/G_{11} DKO intestine [9]. To evaluate Wnt/β-catenin and Notch signaling, we examined the expression of T-cell factor 1 (Tcf1; also known as transcription factor 7) and Notch1 intracellular cytoplasmic domain (NICD) by Western blotting. The expression of Tcf1 increased according to the overexpression of $G\alpha_q/G\alpha_{11}$, while that of NICD decreased (Fig. 2C).

3.3. The effect of the knock-down of the $G\alpha_q/G\alpha_{11}$ expression in IEC-6 cells

We knocked-down the expression of $G\alpha_q$ and/or $G\alpha_{11}$ in IEC-6 cells using siRNA. We investigated the effect of $G\alpha_q/G\alpha_{11}$ knock-down with three types of siRNA. We used cellular homogenate of IEC6-Gq/11 as a positive control and that of the Int-G_q/G_{11} DKO intestine as a negative control. We then selected RSS372821 for *Gnaq* and RSS340230 for *Gna11* and confirmed the knock-down of the $G\alpha_q/G\alpha_{11}$ expression by Western blotting (Fig. 3A, B).

We evaluated the effect on proliferation. IEC-6 cells were seeded at a density of 1×10^4 cells/ml in 96-well culture plates. We incubated the cells with siRNA for two consecutive days and performed the BrdU incorporation assay. As shown in Fig. 3C, the proliferation in IEC6-GΔq, IEC6-GΔ11 and IEC6-GΔq/11 cells was increased in comparison to IEC6-sicont cells.

We next examined the changes in Wnt/β-catenin and Notch signaling. The expression of Tcf1 did not change but the expression of NICD was markedly increased according to the downregulation of $G\alpha_q/G\alpha_{11}$ (Fig. 3D). This was in sharp contrast to the results of the overexpression experiment (Fig. 2C).

3.4. The effect on the differentiation of IEC-6 cells

In the Int-G_q/G_{11} DKO mice study, the maturation of Paneth cells was impaired and differentiation toward goblet cells was induced [9]. We then evaluated the relative mRNA expression of a goblet cell marker (*Muc2*) using a qPCR. As shown in Fig. 4, the relative expression of *Muc2* was increased in IEC6-GΔ11 and IEC6-GΔq/11 cells, but it was not changed in the overexpression experiment.

4. Discussion

The gastrointestinal system is a rich source of neuroendocrine hormones that interact with at least 10 families of GPCRs containing more than 30 known receptor subtypes [6]. Although the physiological relevance of the regulation of intestinal homeostasis is unclear, the sheer number of potential $G\alpha_q/G\alpha_{11}$-coupled receptors suggests the importance of this G protein family members in the regulation of the intestinal functions [6]. In our previous *in vivo* study, the enterocytes in the Int-G_q/G_{11} DKO intestine showed no alterations; however, an immunohistochemical study clearly showed that $G\alpha_q/G\alpha_{11}$ were abundantly expressed near the basolateral membrane of enterocytes [9]. In the present study, we used a rat intestinal epithelial cell line (IEC-6) and investigated the role of $G\alpha_q/G\alpha_{11}$ in enterocytes, in which $G\alpha_q/G\alpha_{11}$ was overexpressed or downregulated with a retroviral system or siRNA.

When $G\alpha_q/G\alpha_{11}$ was overexpressed in IEC-6 cells, the number of cells and the incorporation of BrdU were clearly inhibited (Fig. 2A, B). In contrast, when the expression of $G\alpha_q/G\alpha_{11}$ was knocked-down, the incorporation of BrdU was increased (Fig. 3C). These results suggested that signaling through $G\alpha_q/G\alpha_{11}$ inhibited the proliferation of IEC-6 cells. Potential ligands interacting with GPCRs accompanied by $G\alpha_q/G\alpha_{11}$ in intestine are CCK, gastrin, and ACh. We therefore evaluated the BrdU incorporation in naïve IEC-6 cells with the addition of CCK or carbachol, a stable muscarinic acetylcholine receptor (mAChR) agonist, to the culture medium. Cholecystokinin-2 receptor (CCK2R, originally known as the CCK-B receptor) is a member of the G-protein-coupled seven transmembrane domains receptor superfamily that binds both amidated gastrin and CCK [11]. As shown in Supplementary Fig. 1, CCK did not affect the proliferation, but carbachol dose-dependently inhibited the cell growth with a maximum effect at 1 mM in naïve IEC-6 cells. The peptide hormone gastrin is a well-recognized growth factor for the colonic epithelium [12,13]. The deletion of the functional gastrin gene resulted in decreased colonic proliferation in mice [14,15].

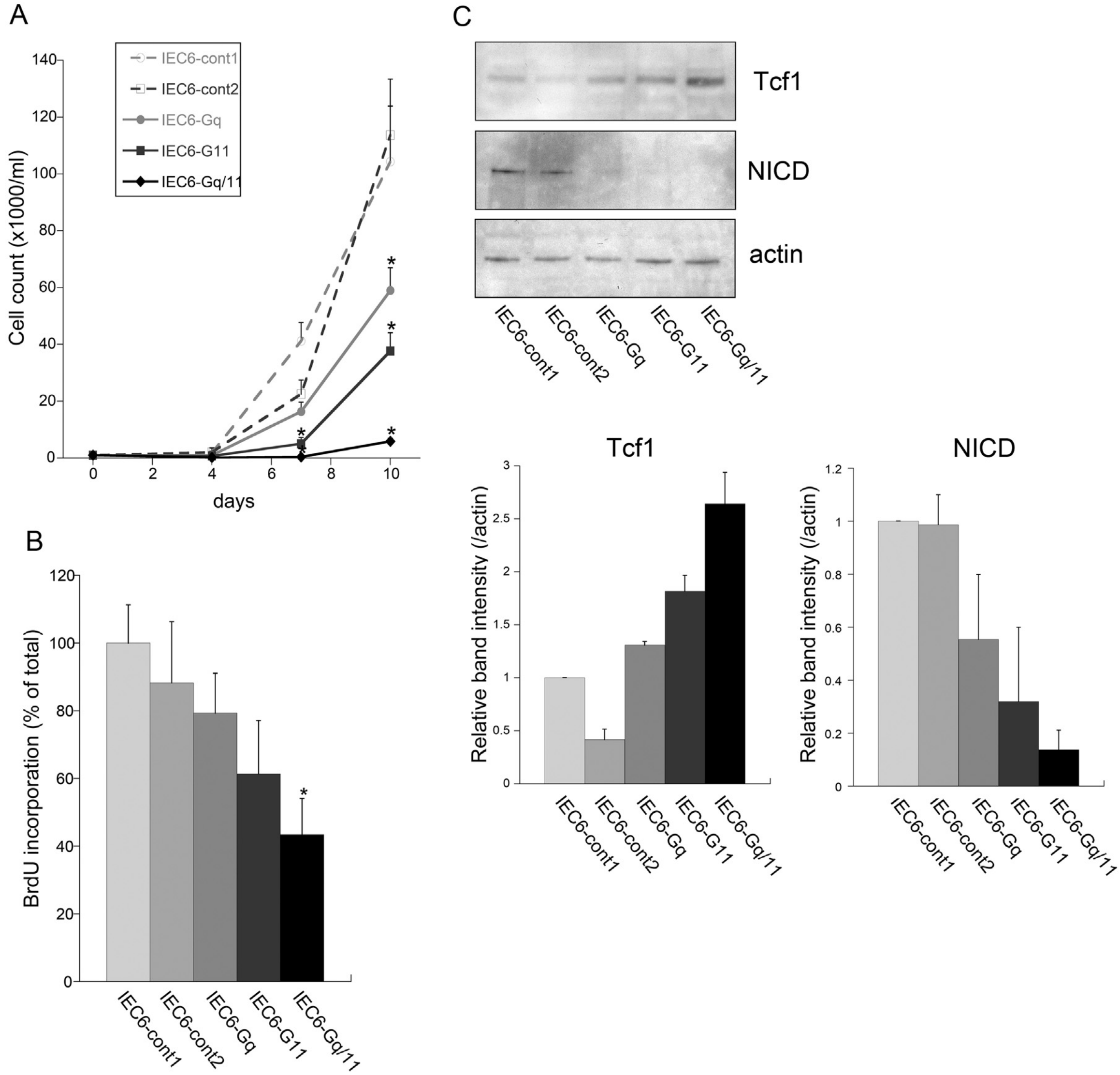

Fig. 2. The effect of $G\alpha_q/G\alpha_{11}$-overexpression in IEC-6 cells. A. IEC-6 cells were seeded at a density of 1×10^3 cells/ml in plastic 24-well plates and cultured. After 4, 7 and 10 days, the cells were detached and the number of cells was counted. B. IEC-6 cells were seeded at a density of 1×10^4 cells/ml in 96-well culture plates. Following serum starvation for 24 h, the cells were cultured for an additional 48 h. BrdU was added for the last two hours of incubation. The DNA synthesis was evaluated according to the incorporation of BrdU. The data are expressed as the mean ± standard deviation (n = 4 per cell type, $^*P < 0.05$). C. Western blotting using anti-T-cell factor 1 (Tcf1) and anti-Notch1 antibodies. Twenty (Tcf1) and 80 µg (Notch1 intracellular cytoplasmic domain (NICD)) of protein was loaded onto each lane. The actin blot was used as an internal control. Three independent experiments were performed with similar results. Representative figures are shown. The ImageJ densitometry analysis of the three experiments is shown in the lower panels.

The overexpression of CCK2R has been shown to mediate the rapid progression of colorectal cancer [16], and gastrinomas have been linked in case reports to increased colon polyposis [17]. CCK2R is expressed in murine basal colonic crypts [18], where the stem cells and progenitor cells are located. Gastrin and CCK may therefore play a proliferative role in immature cells but not in mature enterocytes.

In rat and human intestinal epithelial cells, the main subtype of mAChR is the m3 receptor with perhaps a minor contribution by the m1 receptor [19]. The m3 mAChR mainly mediates secretory responses, such as ions and enzymes, but it has been reported to be capable of stimulating colon cancer cell growth [20]. ACh is reported to stimulate the proliferation of neural stem cells and stem cell-derived progenitor cells expressing m2, m3, m4 mRNA, and m2 protein during neural cell lineage progression *in vitro* [21]. The m3 mRNA was reported to be localized to the lower two-thirds of the villi and not to the crypt in rat jejunum by *in situ* hybridization [22], overlapping with the localization of enterocytes. Reynolds et al. reported that the m3 receptor was localized to the basal membrane of human colonic epithelium in immunohistochemistry [23]. Carbachol dose-dependently inhibited the cell growth of IEC-6 cells. Therefore, ACh may inhibit the proliferation of enterocytes and play a role in the secretory or absorptive functions.

Proliferation, differentiation and morphogenesis in the intestinal epithelium are tightly regulated by a number of molecular pathways, including Wnt/β-catenin and Notch signaling [4]. Wnt/β-catenin signaling and the Tcf family transcription factors play a central role in proliferation during intestinal development [24]. Western blotting using homogenates of the mucosa of the Int-G_q/G_{11} DKO intestine revealed that the expression of Tcf1 was decreased, while that of NICD was unchanged [9]. We then examined the expression of Tcf1 and NICD in IEC-6 cells. In Wnt/β-catenin signaling, the expression of Tcf1 was increased according to the overexpression of $G\alpha_q/G\alpha_{11}$ (Fig. 2C), which was a sharp contrast to the result of Int-G_q/G_{11} DKO intestine [9], and was unchanged in the knock-down experiment (Fig. 3D). Wnt/β-catenin signaling plays a pivotal role in the proliferation and differentiation of intestinal stem and progenitor cells [4], but may act differently in terminally differentiated cells. The Wnt pathways include canonical Wnt signaling, which is referred to as the Wnt/β-catenin pathway, and noncanonical Wnt signaling, which is β-catenin-independent and

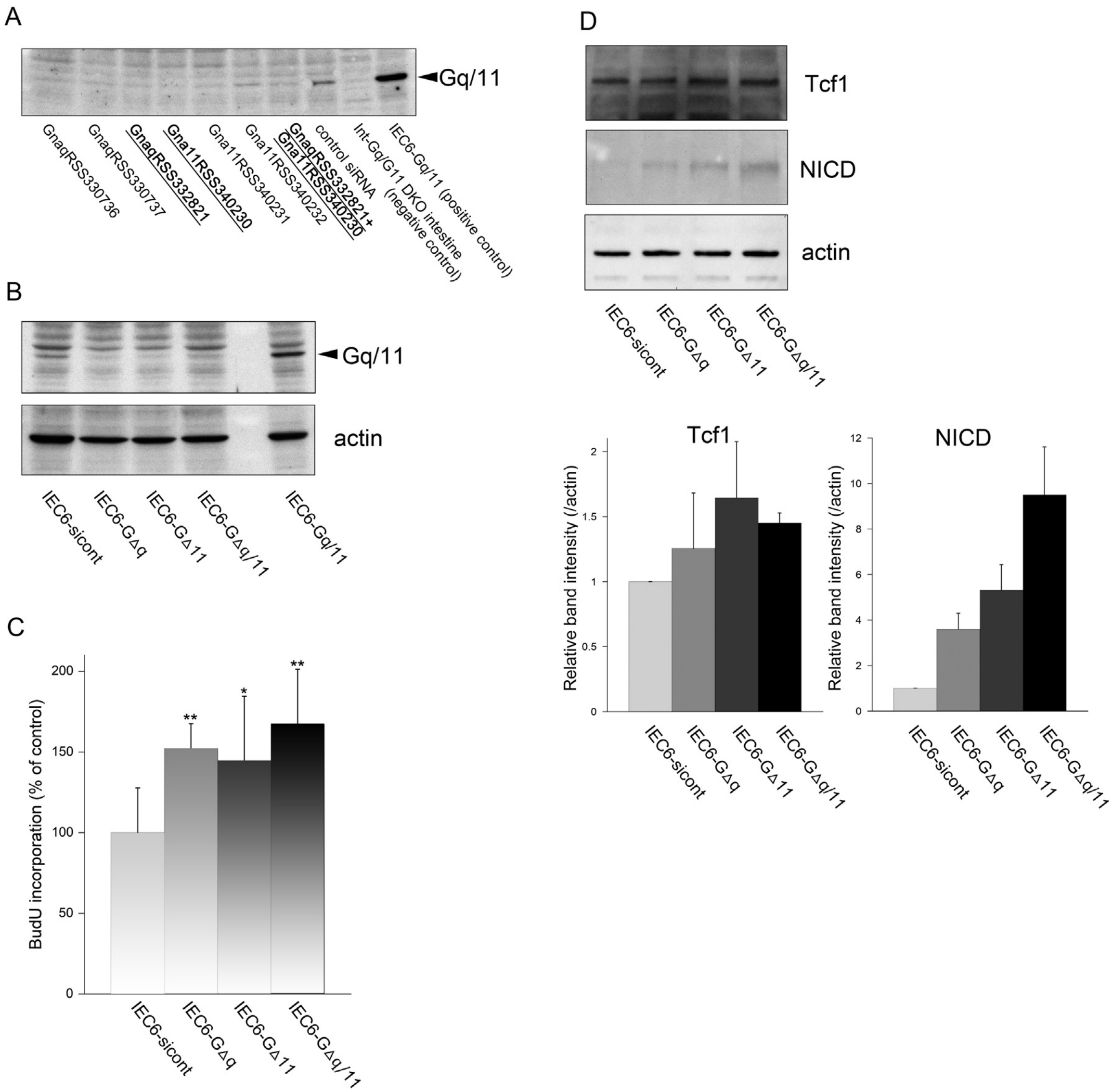

Fig. 3. The effect of the knock-down of the $G\alpha_q/G\alpha_{11}$ expression in IEC-6 cells. A. The selection of siRNA for the knock-down of *Gnaq* and *Gna11*. Eighty micrograms of protein was loaded onto each lane. Cellular homogenate from IEC6-Gq/11 was used as a positive control and that of the Int-G_q/G_{11} DKO intestine was used as a negative control. B. Confirmation of the knock-down of $G\alpha_q/G\alpha_{11}$. C. IEC-6 cells were seeded at a density of 1×10^4 cells/ml in 96-well culture plates. The DNA synthesis was evaluated according to the incorporation of BrdU. The data are expressed as the mean ± standard deviation (n = 4 per cell type, $^*P < 0.05$, $^{**}P < 0.01$). D. Western blotting of Tcf1 and NICD. The blot of actin was used as an internal control. Three independent experiments were performed with similar results. Representative figures are shown. The ImageJ densitometry analysis of the three experiments is shown in the lower panels.

subdivided into two general categories: the Wnt/Ca^{2+} and Wnt/JNK pathways. Fzd is a seven-pass transmembrane receptor and is associated with $G\alpha_q/G\alpha_{11}$ in the Wnt/Ca^{2+} pathway. This signaling pathway is known to antagonize the canonical Wnt/β-catenin signaling [25]. If this pathway plays a role in $G\alpha_q/G\alpha_{11}$-overexpressing cells, the expression of Tcf1 is expected to be decreased. Thus, signaling through $G\alpha_{q/11}$ affects the Wnt/β-catenin pathway in a different fashion from Wnt/Ca^{2+} pathway in this cell system.

As for Notch signaling, the expression of NICD was decreased with the overexpression of $G\alpha_q/G\alpha_{11}$ where the proliferation was inhibited and increased in the knock-down experiment where the proliferation was enhanced (Figs. 2C and 3D). During the regeneration of the intestinal epithelia, the activation of Notch promoted proliferation by suppressing goblet cell differentiation [26]. Thus, the effect on the proliferation of IEC-6 cells through $G\alpha_q/G\alpha_{11}$ signaling relies more on Notch signaling than on Wnt/β-catenin signaling. However, when we evaluated the expression of *Muc2*, a goblet cell marker, the expression of *Muc2* was elevated to 2–3 fold in the knock-down experiment (Fig. 4), which suggested that goblet cell differentiation was not suppressed. The elevated *Muc2* expression in the knock-down experiment was somewhat consistent with the results in the Int-G_q/G_{11} DKO intestine, in which the differentiation of Paneth cells toward goblet cells was induced [9].

In IEC-6 cells, the proliferation of $G\alpha_q/G\alpha_{11}$-overexpressing cells was inhibited, while that of $G\alpha_q/G\alpha_{11}$-knock-down cells was increased. Wnt/β-catenin signaling and Notch signaling were changed according to the level of $G\alpha_q/G\alpha_{11}$ expression, which was not consistent with the results obtained with Int-G_q/G_{11} DKO mice. Signaling through $G\alpha_q/G\alpha_{11}$ probably works in a context-dependent fashion, based on whether the cells are stem cells, progenitor cells, proliferating cells, or terminally differentiated cells. In differentiated enterocytes, signaling through $G\alpha_q/G\alpha_{11}$ inhibits the proliferation and may have an effect on physiological functions, such as absorption or secretion.

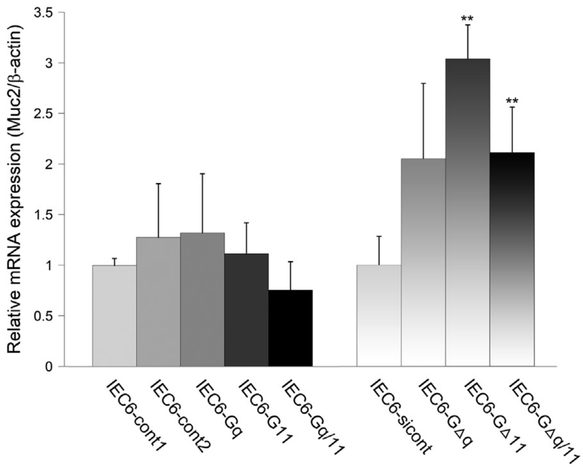

Fig. 4. The relative mRNA expression of goblet cell marker, *Muc2*. A quantitative real-time PCR was performed. The expression of β-actin was used as an internal control. The data are expressed as the mean ± standard deviation from $n = 3$ per cell type. Two independent experiments showed similar results. Representative figures are shown. $^{**}P < 0.01$.

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2018.01.003.

Appendix B. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2018.01.003.

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