〈Research Article〉

Alteration of gene and protein expression of renalase and its transcription factors in Caco-2 cell

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Summary Renalase is an enzyme discovered in 2005 and has been studied in relation to blood pressure regulation. However, its function and regulatory factors in the intestine, where cell proliferation is very active, remain unknown. In this study, we investigated the expression of Renalase and related molecules in the intestine during intestinal epithelial cell differentiation. Caco-2 cells, which are used as a model for intestinal epithelial cell differentiation, were seeded on six-well plates, and cells were collected at 0, 3, 7, 14, and 21 d, with 100% confluence defined as zero days of differentiation. Gene and protein expression dynamics of renalase and related molecules were analyzed by qPCR and immunoblotting. Gene and protein expression of renalase and its receptor, PMCA4b, peaked at 14 d, indicating a positive correlation between renalase and PMCA4b. However, there was no specific correlation between renalase expression and its transcription factor expression. This study provides fundamental insights into the role of renalase in the differentiation of intestinal epithelial cells

Key words: Renalase, Differentiation, Caco-2

1. Introduction

Renalase (RNLS) is a monoamine oxidase discovered in 2005 by Xu et al[1]. RNLS is one of the enzymes secreted by kidneys and has been studied for its role in blood pressure regulation and cardiovascular diseases[2-4]. As research progresses, several functions of RNLS other than blood pressure regulation have been reported. Recombinant RNLS protected renal cells and regulated inflammation in acute kidney injury[5]. RNLS, similar to kidneys,

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attenuates fatty liver ischemia/reperfusion injury by suppressing oxidative stress[6]. However, a study using RP-220 peptides derived from RNLS amino acid sequences reported that it has a cell proliferation effect [7]. A study that investigated the reasons for the cytoprotective and proliferative functions of RNLS, which was discovered as an enzyme, reported that RNLS has a signaling function via the receptor referred to as plasma-membrane calcium ATPase 4b; PMCA4b[8]. From this point on, much attention has been paid to the cytokine-like function of RNLS, and its expression has been reported to be regulated by transcription factors, such as STAT3, Nuclear factorκ-B (NF-κB) p65, HIF-1α, ZBP89, and SP1[7,9,10].

Despite the cytoprotective and proliferative functions of RNLS in various organs, the function of RNLS in the intestinal tract has not been investigated. As a result, our group investigated the function of RNLS in the intestine using a fasting model in which the intestine is exposed to oxidative stress and a model in which RNLS is overexpressed in an intestinal-like cell, Caco-2 cells. Thus, we reported that RNLS was upregulated in the intestine and other organs in response to oxidative stress and that overexpression protects the cells[11]. We discovered that RNLS is predominantly localized near the crypt, where undifferentiated cells reside. During the migration of newly formed cells from the crypt to the apical region of the intestinal villi, intestinal cells differentiate. The fact that RNLS decreases toward the apical region indicates that RNLS is tightly regulated during differentiation. The function of RNLS in the intestine can be clarified in more detail by revealing that RNLS is regulated during differentiation. Elucidation of the expression dynamics of RNLS and related factors during intestinal epithelial cell differentiation may provide new insights into the integrity of the intestine. Although there is a need for a simple model for evaluating the process of intestinal epithelial cell differentiation, Caco-2 cells are frequently used as a model for intestinal epithelial cell differentiation and exploring the gene expression profiling[12-15]. Therefore, we examined how gene and protein expression of RNLS and its transcription factors change in Caco-2 cells on the differentiation time course.

2. Materials and Methods

Cell culture

Caco-2 cells (18H036, European Collection of Authenticated Cell Cultures (ECACC), UK) were incubated at 37°C and 5% CO_2 in Dulbecco's modified Eagle medium (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with a 10% fetal bovine serum and 1% MEM non-essential amino acids solution (Nacalai Tesque, Kyoto, Japan). Cells were seeded in six-well plates at a density of 1.0×10^5 cells. We defined 100% confluence as 0 d of differentiation in this study. Then, the cells were collected at 0, 3, 7, 14, and 21 d of the differentiation time course.

Quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from the cells using Sepasol®-RNA I Super G (Nacalai Tesque). The total RNA concentration was measured using a spectrophotometer (NANO DROP 2000, Thermo Fisher Scientific). Total RNA was adjusted to 100 ng/uL with sterile water. After adding 5× PrimeScript RT Master Mix (Takara Bio, Shiga, Japan) and RNase-free water to the diluted RNA (500 ng/tube), reverse transcription was performed using a thermal cycler (TP 350, Takara Bio, Shiga, Japan). After reverse transcription, we used the KAPA SYBR FAST qPCR kit (Kapa Biosystems, Wilmington, MA, USA) to perform qPCR with QuantStudio 5 (Thermo Fisher Scientific, Waltham, MA, USA). The mRNA levels of TATA box binding protein (Tbp) were used as a housekeeping gene. The cycle threshold (Ct) value of the target gene was normalized by the Ct value of the housekeeping gene ($\Delta\Delta$Ct method). Table 1 lists the sequences of the primers used in this study.

Immunoblotting

Total proteins were extracted from the colon or cells using lysis buffer (1% NP-40, 50 mM Tris, 150 mM NaCl, 1 mM EDTA, including proteinase

Table 1 List of primers

Gene name	Primer Sequence
Alkaline phosphatase intestinal	Forward 5'-CATACCTGGCTCTGTCCAAGA-3'
(Alpi)	Reverse 5'-GTCTGGAAGTTGGCCTTGAC-3'
Renalase	Forward 5'-GAAAAATCATTGCAGCCTCTCA-3'
(Rnls)	Reverse 5'-AAGTTCTGCCTGTGCCTGTGTA-3'
ATPase Plasma Membrane Ca^{2+} Transporting 4	Forward 5'-TGAAGAAACCTGGATCTTTGC-3'
(Atp2b4)	Reverse 5'-AGCAACAACCAGCAACTGTAGTA-3'
Zinc finger protein 148	Forward 5’-GGCTCAAGCCATCCTCCT-3'
(Zfn148)	Reverse 5'-CCTTCCAGTTTGTCGTCAATG-3'
Hypoxia inducible factor	Forward 5’-GAACCTGATGCTTTAACTTTGCT-3'
(Hif1a)	Reverse 5'-TGCTGGTCATCAGTTTCTGTG-3'
Specificity protein 1	Forward 5’-TCACTGTGAATGCTGCTCAA-3'
(Sp1)	Forward 5'-CTGAGCTCCATGATCACCTG-3'
TATA box binding protein	Forward 5'-GCCCATAGTGATCTTTGCAGT-3'
(Tbp)	Reverse 5'-CGCTGGAACTCGTCTCACTA-3'

inhibitor cocktail [Wako, Osaka, Japan] and phosphatase inhibitor [PhosSTOP™, Roche, Basel, Switzerland]). Lysates were centrifuged at 15,000 g for 5 min at 4°C. Total protein concentrations were measured using the BCA protein assay kit (Takara Bio, Shiga, Japan). Then, 2 μg/lane of total protein were used for 8-12 % gradient gel electrophoresis, and proteins were transferred to polyvinylidene fluoride (PVDF) membranes. Primary antibodies listed in Table 2 were incubated with the blotted membranes. The horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling Technology, #7074, US) and anti-mouse IgG (Cell Signaling Technology, #7076, US) were used as secondary antibodies. Signals were detected using the chemiluminescence reagent (EzWestLumi One or EzWestLumi plus,

Table 2 List of antibodies

Antibody name	Company and code	Dilution
Renalase	Proteintech Group, 15003-1-AP	1:1000
PMCA4b	Santacruz Biotechnology, sc-20027	1:1000
HIF1a	Santacruz Biotechnology, sc-13515	1:1000
p-STAT3(Tyr705)	ABclonal Technology, AP0705	1:1000
STAT3	ABclonal Technology, A19566	1:1000
p-p65(Ser536)	ABclonal Technology, AP0475	1:1000
p65	ABclonal Technology, A6667	1:1000
GAPDH	Santacruz Biotechnology, sc-365062	1:1000
Anti-mouse IgG, HRP-linked Antibody	CST, #7076	1:5000
Anti-rabbit IgG, HRP-linked Antibody	CST, #7074	1:5000

ATTO, Tokyo, Japan). Blots were scanned using a chemiluminescence imaging system (FUSION FX7. EDGE, Vilber Lourmat, Marne-la-Vallee, France).

Statistical analysis

Data have been represented as mean ± standard deviation. Comparison between time courses was subjected to one-way analysis of variance followed by Dunnet's post hoc test to evaluate significance. Pearson's correlation method was used to calculate correlation coefficients. Statistical analyses were performed using GraphPad Prism 8.4.3 for Mac (GraphPad Software, San Diego, CA, USA). A value of $p < 0.05$ was considered to indicate a significant difference.

3. Results

Rnls and its receptor Atp2b4 expression levels were significantly altered

Alpi is a marker of intestinal cell maturation, which was significantly upregulated at 3 d compared to 0 d. It attained its peak expression level at 14 d (Fig. 1B). *Rnls* was significantly upregulated at 3 d compared to 0 d and peaked at 14 d. Then, on day 21, it decreased to the same level as 0 d (Fig. 1C). Similarly, *Atp2b4*, the *Rnls* receptor gene, was significantly upregulated at 3 d compared to 0 d, peaking at 14 d (Fig. 1D).

Transcription factors, Zfn148, and Sp1, were upregulated at 21 d and positively correlated to the Rnls

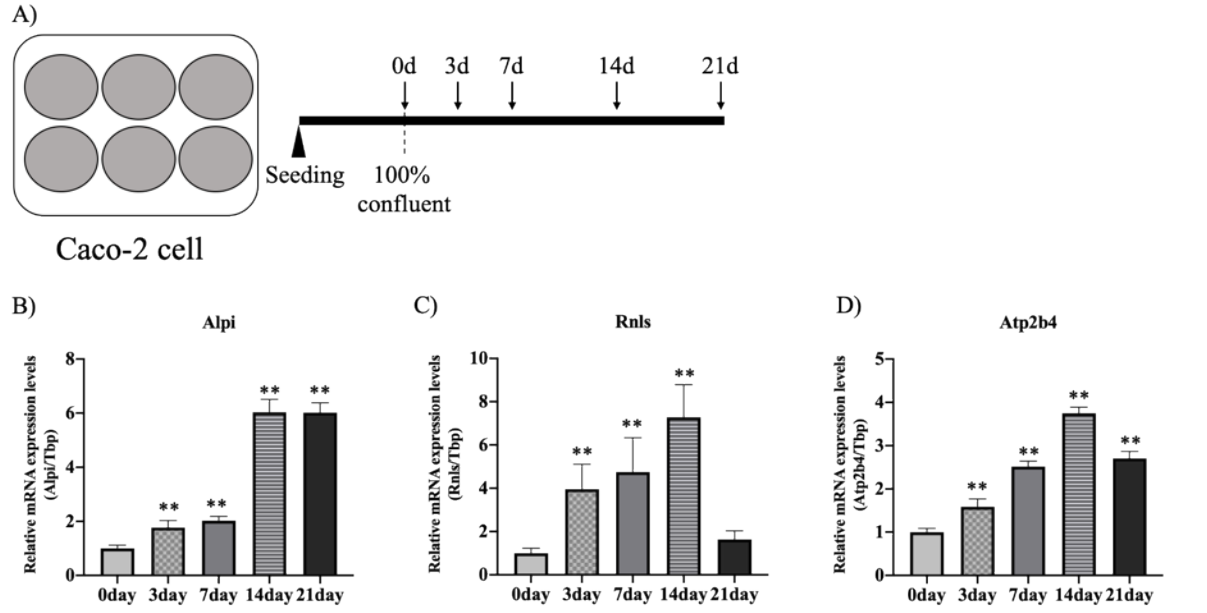

Fig. 1. Alteration of gene expression in *Alpi, Rnls* and *Atp2b4*. A) Sample collection time course in this study. B) Gene expression level of alkaline phosphatase intestinal (*Alpi*). C) Gene expression level of renalase (*Rnls*). D) Gene expression level of ATPase Plasma Membrane Ca^{2+} Transporting 4 (*Atp2b4*). Data are mean ± SD (n=6). Asterisks indicate the significant differences (**p < 0.01 vs 0day).

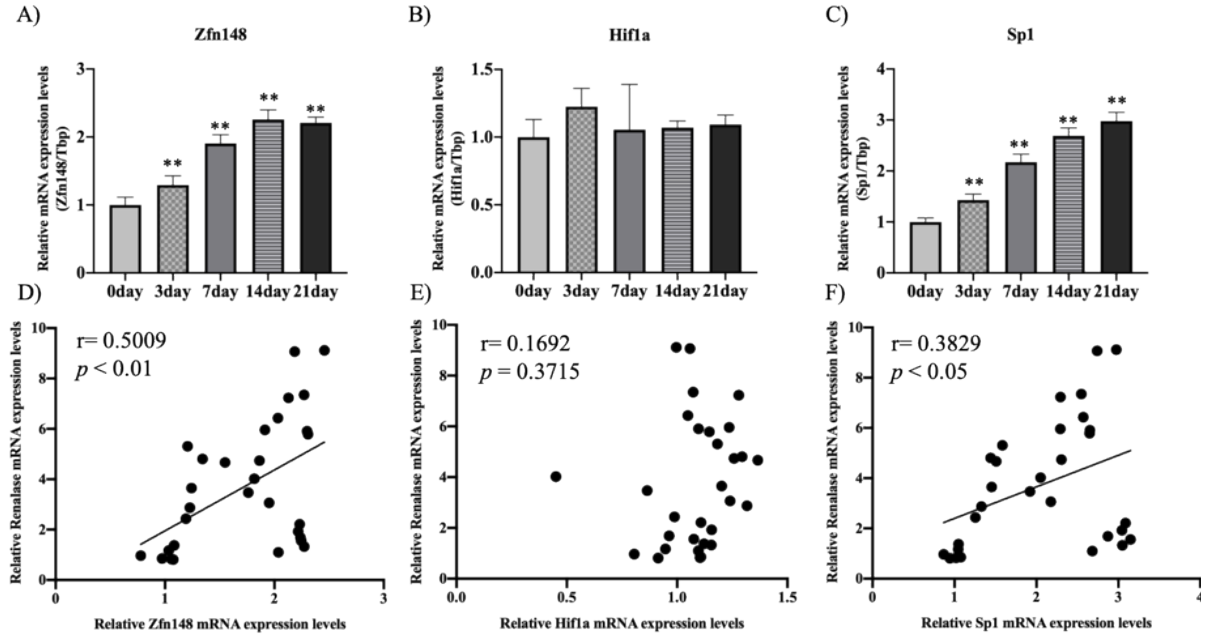

Fig. 2. Alteration of gene expression in *Zfn148, Hif1*a and *Sp1* and correlation of *Rnls* with each gene. A) Gene expression level of zinc finger protein 148 (*Zfn148*). B) Gene expression level of hypoxia inducible factor (*Hif1a*). C) Gene expression level of specificity protein 1 (*Sp1*). D) Correlation of *Rnls* with *Zfn148*. E) Correlation of *Rnls* with *Hif1a*. F) Correlation of *Rnls* with *Sp1*. Data are mean ± SD (n=6). Asterisks indicate the significant differences (**p < 0.01 vs 0day).

expression level

Zfn148, Hif-1α, and *Sp1* are transcription factors of *Rnls*. *Zfn148* and *Sp1* were significantly upregulated at 3 d compared to 0 d and peaked at 14 d (*Zfn148*) and 21 d (*Sp1*) respectively (Fig. 2A, C). Then, we discovered that these genes positively correlated with Rnls expression level (Fig. 2D, F). *Hif-1α* was not changed and did not correlate with

Rnls (Fig. 2B, E).

Protein levels of RNLS and PMCA4b were similarly changed and positively correlated with gene expression

RNLS was significantly upregulated at 14 d compared to 0 d and decreased to the same level on day 21 as 0 d (Fig. 3A). PMCA4b, the protein name of *Atp2b4*, was significantly upregulated at 7 d compared to 0 d and peaked at 14 d (Fig. 3B). PMCA4b expression levels positively correlated with the expression level of RNLS (Fig. 3C).

The expression levels of transcription factors for RNLS were irregular and did not correlate with RNLS expression

RNLS transcription factors include p65, HIF-1α, and STAT3. The phosphorylation level of p65 was significantly downregulated at 14 and 21 d and negatively correlated with RNLS expression level (Fig. 4A, D). HIF-1α expression level was significantly upregulated at 3 and 7 d but did not correlate with RNLS expression level (Fig. 4B, E). The phosphorylation level of STAT3 was significantly upregulated at three days but did not correlate with RNLS expression level (Fig. 4C, F).

4. Discussion

In this study, Caco-2 cells were used as a model for intestinal epithelial cell differentiation, and samples were collected at 0, 3, 7, 14, and 21 d to examine the gene and protein expression dynamics of RNLS and its related factors.

Initially, we measured the *Alpi* gene to confirm whether Caco-2 cells were differentiating appropriately. *Alpi* expression started to increase significantly after 3 d and reached its peak expression at 14 d. Therefore, the peak of differentiation was attained at 14 d, indicating that Caco-2 cells are differentiating appropriately. Next, we examined the gene expression dynamics of *Rnls* and its receptor, *Atp2b4*. Similar to *Alpi*, the expression of *Rnls* and *Atp2b4* genes peaked at 14 d and declined at 21 d. The expression of each protein exhibited the same dynamics as gene expression, and there was a positive correlation between RNLS and PMCA4b expression. Atp2b4 encodes the protein PMCA4b, which is the only RNLS receptor currently identified[8]. Therefore, its expression level may have increased in sync with the increase in RNLS expression. When RNLS acts as a signaling factor, it increases the phosphorylation of ERK and Akt, which are cell growth factors, via PMCA4b[16]. RNLS may also have PMCA4b-mediated effects in this differentiation process. Interestingly, RNLS expression in both gene and protein increased up to 14 d, which is the peak of differentiation, but decreased

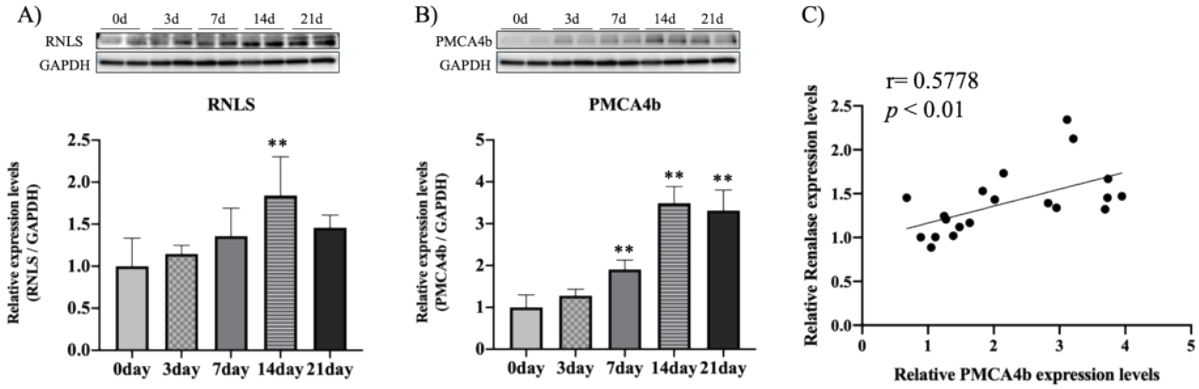

Fig. 3. Alteration of protein expression in RNLS and PMCA4b and correlation of RNLS with PMCA4b. A) Protein expression level of RNLS. B) Protein expression level of PMCA4b. C) Correlation of RNLS with PMCA4b. Data are mean ± SD (n=4). Asterisks indicate the significant differences (**p < 0.01 vs 0day).

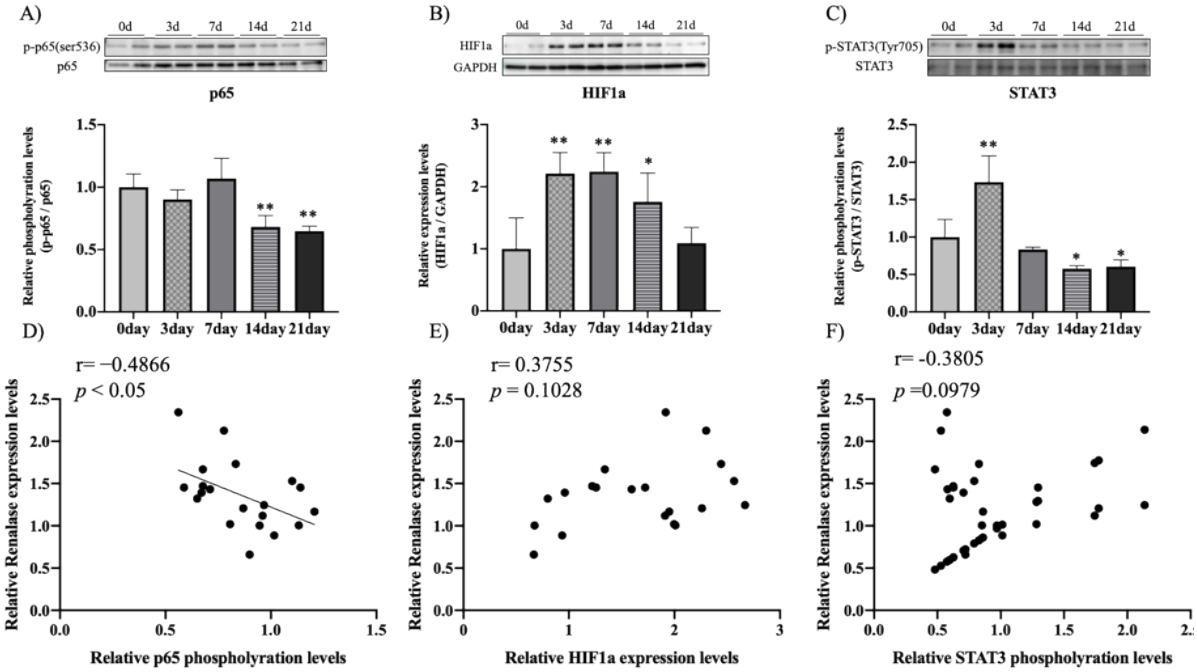

Fig. 4. Alteration of protein expression in p65, HIF1a and STAT3 and correlation of RNLS with each protein. A) Phosphorylation level of p65. B) Protein expression level of HIF1a. C) Phosphorylation level of STAT3. D) Correlation of RNLS with p65. E) Correlation of RNLS with HIF1a. F) Correlation of RNLS with STAT3. Data are mean ± SD (n=4). Asterisks indicate the significant differences (*p < 0.05, **p < 0.01 vs 0day

by 21 d. This indicates that RNLS may be involved in differentiation signals for up to 14 d. However, whether the ligand-receptor relationship between RNLS and PMCA4b is also present and involved in the intestinal tract needs to be tested in the future. It is possible that RNLS may be involved in differentiation other than through PMCA4b-mediated effects. TGF-β1 is involved in the differentiation signal[17]. In fact, we discovered that TGF-β1 expression was significantly upregulated when RNLS was overexpressed in Caco-2 cells (data are not shown). Therefore, RNLS may be involved in differentiation by inducing TGF-β1. Although this study revealed an increase in RNLS, it did not clarify whether the RNLS gene is directly involved in differentiation. Therefore, the role of RNLS in the differentiation mechanism of intestinal epithelial cells can be clarified in the future by establishing KO cells and conducting similar experimental procedures.

When we focused on the expression dynamics of RNLS transcription factors during the differentiation process, we discovered that *Zfn148* and *Sp1* genes exhibited similar expression dynamics to those of *Rnls*, with a positive correlation. However, there was no consistency in the protein expression of the transcription factor HIF-1α or the phosphorylation rates of p65 and STAT3. Furthermore, p65 is a transcription factor whose phosphorylation is enhanced in response to cellular stresses, such as oxidative stress and inflammatory response. It exhibited a negative correlation, which differed from our previous study[11]. Then, it activated the transcription of anti-stress genes, such as superoxide dismutase 2[18]. However, in this case, p65 may not be a regulator of RNLS because the differentiation process is associated with cell maturation. STAT3 is a molecule that forms a positive feedback loop with RNLS in other organs, enhancing each other's expression and phosphorylation[9,19]. Previous studies have focused on cell proliferation in cancer and may not involve the cell differentiation process in intestinal epithelial cells. *Zfn148* and *Sp1* genes, which encode the transcription factors ZBP89 and SP1, respectively, were discovered to be significantly associated with RNLS. However, both genes are ubiquitous genes and transcription factors that are involved in

various processes in the cell. In fact, their expression remains high even after 21 d, and we cannot be certain that they are specific RNLS regulators. We were unable to identify RNLS-specific transcription factors in intestinal epithelial cell differentiation. Whether the transcription factors examined in this study jointly regulate RNLS expression in a time-dependent manner or whether other novel transcription factors are involved in intestinal epithelial cell differentiation needs to be clarified in the future.

In this study, we investigated the expression of RNLS genes and proteins, as well as the expression dynamics of the related molecules, during intestinal epithelial cell differentiation, using a Caco-2 cell differentiation model. Previous studies have also indicated the importance of RNLS in the intestinal tract[20], and RNLS may become a target factor for various diseases in the intestinal tract in the future. This study may provide the basis for the function and regulation of the RNLS gene in intestinal epithelial cells.

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Conflicts of Interest

All authors declare that there is no conflict of interest regarding the publication of this article.

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