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# Mechanical stimulation activates Piezo1 to promote mucin2 expression in goblet cells

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**Key words**
Goblet cells, Mechanical model, mucin2, Piezo1.

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## Abstract

**Background and Aim:** Studies on the regulation of mucin2 expression in intestinal goblet cells by the endocrine system and the immune system have been comprehensive, but the effects of abundant mechanical factors in the intestinal microenvironment on goblet cells are not clear.

**Methods:** We constructed mechanical stimulation models *in vivo* and *in vitro* to explore the effect of mechanical stimulation on intestinal goblet cells. Piezo1 expression and function were regulated through model mouse and drugs to explored whether Piezo1 mediated mechanical stimulation.

**Results:** The results showed that hydrostatic pressure could promote mucus secretion in the mouse colon, and both traction force and shear force could promote the expression of mucin2 in the LS174T cell line. We further found that the Piezo1 protein, which was abundantly expressed in goblet cells, acted as a mechanoreceptor. Knockout of Piezo1 in the intestinal epithelial cells of mice could reduce the promotion of mucus secretion by pressure stimulation, and the specific downregulation of Piezo1 protein in LS174T cells or Piezo1 inhibitor treatment could significantly reduce the promotion of mucin2 expression in goblet cells by mechanical stimulation; however, treatment with a Piezo1 agonist had the opposite effect. Moreover, we found that Piezo1 regulated mucin2 expression through the downstream Erk/$Ca^{2+}$ pathway.

**Conclusion:** In short, our study confirmed for the first time that goblet cells are mechanoreceptive cells that can directly sense mechanical stimulation in the intestinal tract and respond back through the Piezo1-Erk/$Ca^{2+}$-mucin2 pathway.

## Introduction

The intestinal mucus barrier, which comes into contact with the external environment, is the first line of defense for the intestinal epithelium. It selectively allows the penetration of nutrients needed by the human body, while preventing harmful substances from invading the intestinal epithelium.[1–3] Mucin2, the main component of the mucus barrier, is synthesized and secreted by goblet cells. The normal expression of mucin2 by goblet cells is essential to maintain homeostasis.[4–6] Mucin2 is synthesized in the ribosome and the endoplasmic reticulum, glycosylated in the Golgi apparatus, highly polymerized and stored in cells in the form of vesicles waiting for secretory signals.[7] In general, mucin2 is slowly released from goblet cells into the extracellular space, followed by hydration and depolymerization to form a dense gel structure, which acts as a barrier. However, when stimulated by acetylcholine, histamine, prostaglandin, or ischemia–reperfusion, mucin2 will be released in a large amount in a short time, but the exact mechanism is still unclear.[8,9] As a result, researchers have classified its secretion into two forms: regulated vesicle secretion and compound exocytosis.[10] The processes of endocytosis, autophagy, or inflammatory body formation of goblet cells are also necessary for the synthesis and secretion of mucus. When these processes are abnormal, the synthesis and secretion of mucin2 are also blocked.[11] Many classical studies have explained the specific mechanism of goblet cells regulating mucin2 synthesis and secretion, and most of them believe that goblet cells follow the mode of vesicle secretion activated by $Ca^{2+}$.[7] However, the above-mentioned studies mostly focus on the endocrine system and the immune system and do not explore the influence of mechanical factors in the microenvironment of goblet cells.

There are various forms of mechanical stimulation in the intestinal cavity.[12,13] Intestinal peristalsis and contraction constitute a pulling force on the intestinal wall, intestinal contents flow to produce a shear force on the intestinal epithelium, and intestinal epithelial goblet cells are the first to feel intestinal mechanical stimulation. Mechanical stimulation acts on goblet cells constantly, and it is not clear whether mechanical stimulation has any effect on the function of goblet cells. Previous studies showed that the mucus secretion rate of goblet cells at the bottom of the intestinal glandular fossa was slow and that there was mucin accumulation in the cells. The mucus secretion rate of goblet cells on the surface of the intestinal epithelium increased significantly, and no obvious mucus accumulation was observed.[10] This may be related to the stronger mechanical stimulation of intestinal villi. The distribution density of goblet cells in the distal intestine is higher than that in the proximal end, and the mucous layer is


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thicker than that in the proximal end[14,15]; correspondingly, the mechanical stimulation of the distal intestinal wall is also stronger. In addition, previous studies have also found that increased pressure in the respiratory tract can promote the synthesis and secretion of mucus in the trachea,[16] but there is no related study on intestinal goblet cells. In fact, in the process of intestinal peristalsis, the harmful substances enveloped in mucus are also pushed to the distal end.[17] However, mucus can recover quickly at this time and will not cause damage to the intestinal mucosal barrier, which shows that there is a synergistic effect between the intestinal mechanical environment and mucus. Elucidating this mechanism is of great value in understanding the intestinal homeostasis.

In our pre-experiment, we found that the mechanically sensitive protein Piezo1 express abundantly in goblet cells. Piezo1 is the structural basis for goblet cells to receive mechanical stimulation. Piezo1 protein can directly sense mechanical stimulation; open nonselective cation channels; convert perceived mechanical signals into bioelectrical signals; and regulate cell morphology, proliferation, differentiation, secretion, and other behaviors.[18–21] At the same time, Piezo1 acts as a mechanoreceptor in many tissues and organs, such as Piezo1 on the vascular endothelium, which can sense the shear force of blood flow and regulate vascular tension.[18] Renal tubular epithelial cells feel the osmotic pressure of filtrate with the help of Piezo1 and change the reabsorption function of renal tubules.[22,23] Changes in pancreatic intraductal pressure can act on endothelial Piezo1 to induce pancreatitis.[24] Therefore, our goal was to study whether the Piezo1 protein on goblet cells can sense mechanical stimulation and whether it can regulate the synthesis and secretion of mucin2.

In this study, *in vivo* and *in vitro* mechanical stimulation models were established to clarify the phenomenon that mechanical stimulation promoted the expression of mucin2 in intestinal goblet cells. Through the intestinal epithelial Piezo1 knockout mouse model and the specific regulation of Piezo1 expression and related signal molecular activity in LS174T cells, the specific mechanism of mechanical regulation of mucin2 expression mediated by Piezo1 was explored.

## Materials and methods

***Mouse.*** Piezo1$^{flox/flox}$ and PvilliincreT mouse were obtained from Jackson laboratory, and raised in the Animal experimental center of Tongji Medical College. Animals were raised in a SPF environment and healthy male ones were used for experiments when they are 8 weeks. All mice were raised under the specific pathogen-free condition. All experimental procedures were approved by Ethics Committee of Tongji Medical College.

***Hydrostatic pressure model.*** The mice were killed after isoflurane anesthesia, colon was separated and placed in the precooled Krebs liquid. The 3–4 cm colon was cut off and fixed on the hydrostatic device with sutures. The device stimulated the colon with constant hydrostatic pressure and maintain the perfusion state. Intestine was under 20–30 cm hydrostatic pressure stimulation for 30 min. Then the middle part of the intestine was retained and fixed in Carnoy's solution. The sections were stained with Alcian blue and photographed for analysis.

***Alcian blue staining.*** Samples were washed with phosphate-buffered saline (PBS), fixed with Carnoy's fluid at room temperature. After the tissue is embedded and sliced, the staining operation is carried out according to the instructions (Baso, China) and bright field images were captured. Next, a researcher who was not clear about the grouping measured the thickness as previously published[25] (10 measurements per section/two sections per animal/five animals per group) with IMAGEJ.

***Immunohistochemistry.*** Immunofluorescence were carried as previous research,[26] Piezo1 antibody (Proteintech, 15939-1-AP) were diluted at the ratio of 1 to 400 for specific staining. Bright field images were captured and analyzed with IMAGEJ.

***Cell culture.*** Briefly, LS174T cell line was purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology. Cells were cultured by Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% non-essential amino acid and seeded in T25 flask (Corning, NY, USA) with the density of 1–2 × 105/mL. Cells were cultured in 37°C incubator (Panasonic MCO-18AC, Japan) containing 5% $CO_2$. Culture medium was changed every other day. Cells were digested by 0.25% Trypsin–EDTA (Gibco) for 1–2 min and reseeded when cell density reached 80%. All experiments were performed within the passage 8-24.

***Mechanical stimulation model for cells.*** Mechanical traction model; LS174T cells were planted on the matching cell culture plate, cultured to cell fusion, and placed on the mechanical traction instrument (model) produced by Flexcell company. The parameters were 0.2 Hz (15% elongation).

Shear force model: the cells were inoculated on 24-well and 6-well culture plates (Corning, NY, USA). After growing to fusion, the cells were placed on rocking board in the incubator. The parameters were 0.3 Hz, shear stress = $\eta \times \varphi \times 125 \approx 0.1$ N ($\eta$: viscosity of the medium; $\varphi$: flow rate).

***PCR.*** Quantitative real-time reverse transcription RT-PCR (RT-PCR) was carried out in LightCycler480 using SYBR Green Transcription Master (Roche Diagnostics, IN, USA) as previous research. Transcripts of Piezo1, TRPA1, TRPV1, TRPV4, TRPV6, TLR4, ASIC, Mucin2, and ERK1/2 were amplified using specific primer pairs and conditions while GAPDH was used as internal control. Relative quantification of those genes was performed by the $2-\delta\delta Ct$ method. Primers were listed as follows.

Human primer:

GAPDH: GTCAAGGCTGAGAACGGGAA, AAATGAGCC CCAGCCTTCTC;

Piezo1, CTTCCATGGTATCCGCCTCC, CAGCTTGGCGT TCCTGGATA.

TRPA1; TGTGACGATATGGACACCTTCT, TTGAAGTTT CGGAGATTTGGGTT;

TRPV1; TGCACGACGGACAGAACAC; GCGTTGACAAG CTCCTTCAG.

TRPV4; CCACCCTATATGAGTCCTCGG; TAGGTGCCGTA GTCAAACAGT.


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TRPV6; ACTGACCTCGACTCTCTATGAC; GTGGTGATG ATAAGTTCCAGCAG.

TLR4; AGACCTGTCCCTGAACCCTAT; CGATGGACTTC TAAACCAGCCA.

ASIC; ATGGAAAGTGCTACACGTTCAA; GTTCATCCTGA CTATGGATCTGC.

Mucin2; CTTCCATGGTATCCGCCTCC; CAGCTTGGCGT TCCTGGATA.

Erk: CCCCTTATTTGCACTTCACCTTT; CATCAGCCAAG GTATTTCCACAA.

Mouse primer.

GAPDH: TGAAGCAGGCATCTGAGG; CGAAGGTGGAA GAGTGGGAG.

Piezo1: TCTTACACGGTTGCTGGTTGGT; GCAGGAACA GCGTGAGGAACA.

Mucin2: GCTGACGAGTGGTTGGTGAATG; GATGAGGTG GCAGACAGGAGAC.

***Enzyme-linked immunosorbent assay.*** Samples were obtained according to instructions. (Human Mucin 2 [MUC2] enzyme-linked immunosorbent assay [ELISA] Kit Catalog Number: HM10493, Bio-swamp). Mucin2 levels were determined by the solid-phase sandwich ELISA method. The color change was measured spectrophotometric ally (ChroMate® 4300; Awareness Technology, Inc., Palm City, FL, USA) at a wavelength of 450 nm. The concentration of MUC2 in the samples was then determined by comparing the optical density of the samples to the standard curve.

***Immunofluorescence.*** Immunofluorescence were carried as previous research,[27] Piezo1 antibody (Proteintech, 15939-1-AP) and AGR2 antibody (R&D Systems, AF6068) were diluted at the ratio of 1 to 500 for specific staining.

***Transfection assays.*** For Piezo1 knock-down (Piezo1-KD), LS174T cells were infected by lentivirus packaging Piezo1 siRNA (purchased from Shanghai GeneChem Co., Ltd., China). Cells in control group (CON-KD) were infected by lentivirus containing negative control siRNA. Infected cells were purified by adding 0.5 μg/mL puromycin (Sigma-Aldrich) into culture medium and were passaged at least twice until GFP positive cells reached 99%.

***Semi-wet culture.*** As previous research[28] cell cultures were harvested and 200 ml of LS174T cells suspended in fresh media was added to the apical side of the membrane and 2-mL media to basolateral compartment. The standard condition was maintained by keeping this condition throughout the experiment. Semi-wet interface was produced by 2-mL media in the basolateral compartment and 100-μL media in the apical compartment after confluence. Mechanical stimulation and continuous wetting of the apical surface were achieved by placing the culture plates on a rocking board in the incubator. Butyrate (5-mM, Gibco) were used to induce mucin production, Yoda1 (2.5 μm) and GsMTx4 (0.1 μm) were added in the basolateral compartment to interface Piezo1. Samples were fixed with Carnoy's fluid after 3 weeks' culture, then Alcian blue staining were used to show mucin expression. The results are displayed after analysis by IMAGE J.

***Western blot.*** Cultured cells were directly washed for three times with cold PBS and then lysed in RIPA buffer containing protease inhibitor cocktail (Beyotime Biotechnology, Shanghai, China) and incubated on ice for 30 min. Lysates were centrifuged at 12 000 rpm for 10 min, and the protein content of the supernatant was determined by using the BCA protein assay kit (Beyotime Biotechnology, Shanghai, China). Proteins (50 μg/well) for each group were separated by tris-glycine sodium dodecyl sulfate polyacrylamide gel electrophoresis (Beyotime Biotechnology, Shanghai, China) and were transferred to the polyvinylidene difluoride membranes. Membranes were blocked with 5% (w/v) bovine serum albumin (Sigma-Aldrich) in Tris-buffered saline (once) and Tween-20 (pH 7.4, Sigma-Aldrich) for 1 h and then were incubated with rabbit monoclonal anti-phosphorylated ERK1/2 antibody (1:600, Abcam, Cambridge, UK), rabbit monoclonal ERK1/2 antibody (1:800, Arigo, Taiwan, China), rabbit polyclonal Piezo1 antibody (1: 500 Proteintech,15939-1-AP) and rabbit anti-GAPDH Antibody (1:1000; GAPDH Antibody [Pab], Antgene, Wuhan, China) at 4°C overnight. After washed with in TBST thrice at room temperature for 10 min, membranes were incubated for 1 h at room with HRP-conjugated anti-(rabbit-IgG) antibodies (1:2000; Antgene, Wuhan, China). Blots were developed using Pierce ECL Western Blotting Substrate (Thermo Scientific, Rockford, IL, USA).

***Scanning electron microscopy.*** Cells were processed for transmission electron microscopic observations using modified standard procedures. The tissues were fixed in 2.5% buffered glutaraldehyde for 2 h at 4°C and were subsequently fixed in 1% osmium etroxide for 2 h at room temperature. Then, they were dehydrated in graded alcohol and acetone and embedded in Epon 812 overnight. Next, ultra-thin 60–80 nm sections were cut with a diamond knife using a Leica Ultracut UCT (Leica Microsystems GmbH, Wetzlar, Germany) and stained with uranyl acetate and lead citrate. The structures of the vesicles and endoplasmic reticulum were observed using a HITACHI U8010 transmission electron microscope (Hitachi, Ltd., Tokyo, Japan).

***Calcium imaging.*** LS74T cells were incubated with 1.0-μM Fluo-3 AM solution at 37°C for 30 min, after the fluorescent probe was loaded, then PBS was washed, and the fluorescence signal was collected after washing.

***Ex vivo measurement of mucus thickness.*** According to previous research,[29] we made horizontal perfusion chamber by ourselves. For the ex vivo experiments, mice were anesthetized with isoflurane and killed by cervical dislocation. The distal colon was dissected, flushed, and the longitudinal muscle layer removed. The tissue explant was mounted in a horizontal perfusion chamber. The upper surface of the colonic mucus was visualized by addition of charcoal particles. The mucus thickness was determined by measuring the distance between the epithelial surface and the mucus surface by a micropipette attached to patch clamp viewed through a stereomicroscope. Yoda1 and Ruthenium red were added in the upper room.


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***Statistics.*** The SPSS 21.0 statistical software was used for the data analysis. The data are expressed as the mean ± standard error of at least three independent experiments. Differences between groups were analyzed using one-way analysis of variance (ANOVA), followed by post hoc Bonferroni test. $P < 0.05$ (two-tailed) was considered significant. GRAPHPAD PRISM 6.0 was used to transfer data into graphs.

## Results

***Hydrostatic pressure promotes colonic mucus secretion in mice.*** To explore the effect of mechanical stimulation on mucus expression in intestinal goblet cells, we generated an intestinal perfusion model that simulated the stimulation of hydrostatic pressure. The mice were dislocated and killed after isoflurane anesthesia. The colonic intestines were immediately isolated and placed in the Krebs perfusion device to maintain activity, followed by stimulation by hydrostatic pressure of 20 or 30 cm of $H_2O$. After 30 min, the tissue was fixed with Carnoy's solution, sections were prepared and stained with Alcian, and the thickness of the mucus layer was assessed by photography. The results showed that after hydrostatic pressure stimulation, the thickness of the colonic mucus layer in the control group increased significantly, and the increase in mucus layer thickness under the pressure of 30 cm of $H_2O$ was greater than that of the pressure of 20 cm of $H_2O$ ($P < 0.01$) (Fig. 1a,b).

We then carried out experiments on intestinal epithelium Piezo1 knockout mice in our laboratory (Fig. 1c–e). The results showed that the thickness of the intestinal mucus layer of the model rats was similar to that of the control group before hydrostatic pressure stimulation, but the increase in intestinal mucus layer thickness after hydrostatic pressure stimulation was significantly lower than that of the control group (Fig. 1f,g). Immunohistochemical showed the result of mucus expression is consistent with that of mucus thickness (Fig. 1h,i). This suggests that intestinal epithelial Piezo1 may mediate mechanical stimulation to regulate intestinal mucus secretion.

***Mechanical stimulation affects the synthesis and secretion of mucin2 in cells.*** To further verify that mechanical stimulation promotes the mucus expression of goblet cells, we selected LS174T cells as the object of study *in vitro*. The LS174T cell line strongly expresses mucin2 and mucin5AC and is a common cell line for goblet cell function studies.[30]

Considering that mechanical stimulation in the intestinal tract comes from a variety of sources, it can be divided into two types according to the form of action: traction force and shear force.[31] The force of the former is perpendicular to the stress surface, while the latter is parallel to the stress surface; further, the traction force and shear force cover most of the mechanical forms of the intestine. We constructed two different forms of mechanical stimulation models, for traction force and shear force, *in vitro*.

We first used the Flex-cell device to build a circulatory traction model *in vitro* to simulate the effect of intestinal mechanical traction on the LS174T cells (0.2 Hz, 15% elongation). The RT-PCR and ELISA results showed that the levels of mucin2 mRNA and protein in LS174T cells increased significantly under mechanical traction stimulation for 6, 12, and 24 h (Fig. 2a–c). Under constant-frequency conditions, the ELISA results showed that low-intensity mechanical stimulation did not significantly promote the expression of mucin2 after 24 h of mechanical stretching (0.2 Hz, 10% elongation) (Fig. 2g). It is worth noting that the ELISA results show that the increase in mucin2 in the supernatant was more obvious than that in the cells, suggesting that mechanical stimulation affected not only mucin2 synthesis but also mucin2 secretion.

We then used the warping shaking table to construct a stimulation model to simulate the effect of shear force (rocking board in the incubator, 0.3 Hz, shear stress = η ear s 125 ≈ 0.1 N, where η is the viscosity of the medium μm *in vitro* and φ is the flow rate). The RT-PCR results showed that shear stress could promote the expression of mucin2 in LS174T cells (Fig. 2d). The ELISA results also showed that mucin2 in LS174T cells and supernatant increased significantly after 48 h of shear stress (Fig. 2e,f). There was no significant change in mucin2 in LS174T cells after decreasing the frequency of the shaking table (Fig. 2h) (i.e., weakening the stimulation of shear stress).

***Piezo1 is the mechanoreceptor of goblet cells.*** After confirming that a certain intensity of mechanical stimulation affects the synthesis and secretion of mucin from goblet cells, we then explored the structural basis of the effect of mechanical stimulation on goblet cells. Previous animal experiments suggest that Piezo1 may mediate the effect of mechanical stimulation on intestinal mucus secretion, but whether other ion channels are also involved in this process is a matter of concern. Previous studies have shown that the transient receptor potential (TRP) family ion channels are expressed in intestinal epithelial cells, and their function is similar to that of mechanically sensitive ion channels.[21] The phenomenon of mechanical stimulation of TLR4 and acid-sensing ion channels (ASIC) channels has also been reported in the literature.[32] At the same time, Piezo1 is the first mechanically sensitive ion channel,[18,19,33] which is different from the ion channels mentioned earlier, and its expression in the intestinal epithelium has also attracted the attention of researchers. Therefore, we assessed the expression of many mechanical stimulation-related ion channels in LS174T cells, including TRP. The RT-PCR results showed that the expression of Piezo1 in LS174T cells was significantly higher than that of TRPA1, TRPV1, TRPV4, TRPV6, TLR4, and ASIC (Fig. 3a). TRPA1 and TRPV1 inhibitors could not downregulate the expression of mucin2 in LS174T cells (Fig. 3b). Immunofluorescence showed that Piezo1 protein was expressed on the LS174T cell membrane and in intestinal goblet cells (Fig. 3c,d). Therefore, we believe that the Piezo1 protein is a mechanoreceptor in goblet cells.

***Effect of interfering with Piezo1 on the expression of mucin2 in LS174T cells.*** To study the relationship between Piezo1 and mucin2 expression, we customized shRNA for the Piezo1 gene sequence, introduced the shRNA into LS174T cells with lentivirus as a vector and successfully induced LS174T cell lines with a reduced Piezo1 specificity (Fig. 4a,b). The RT-PCR results showed that the level of Piezo1 mRNA decreased significantly, with a maximum range of more than 50% ($P < 0.05$) (Fig. 4c), and the corresponding mucin2 mRNA level decreased more than 50% synchronously ($P < 0.05$) (Fig. 4d).


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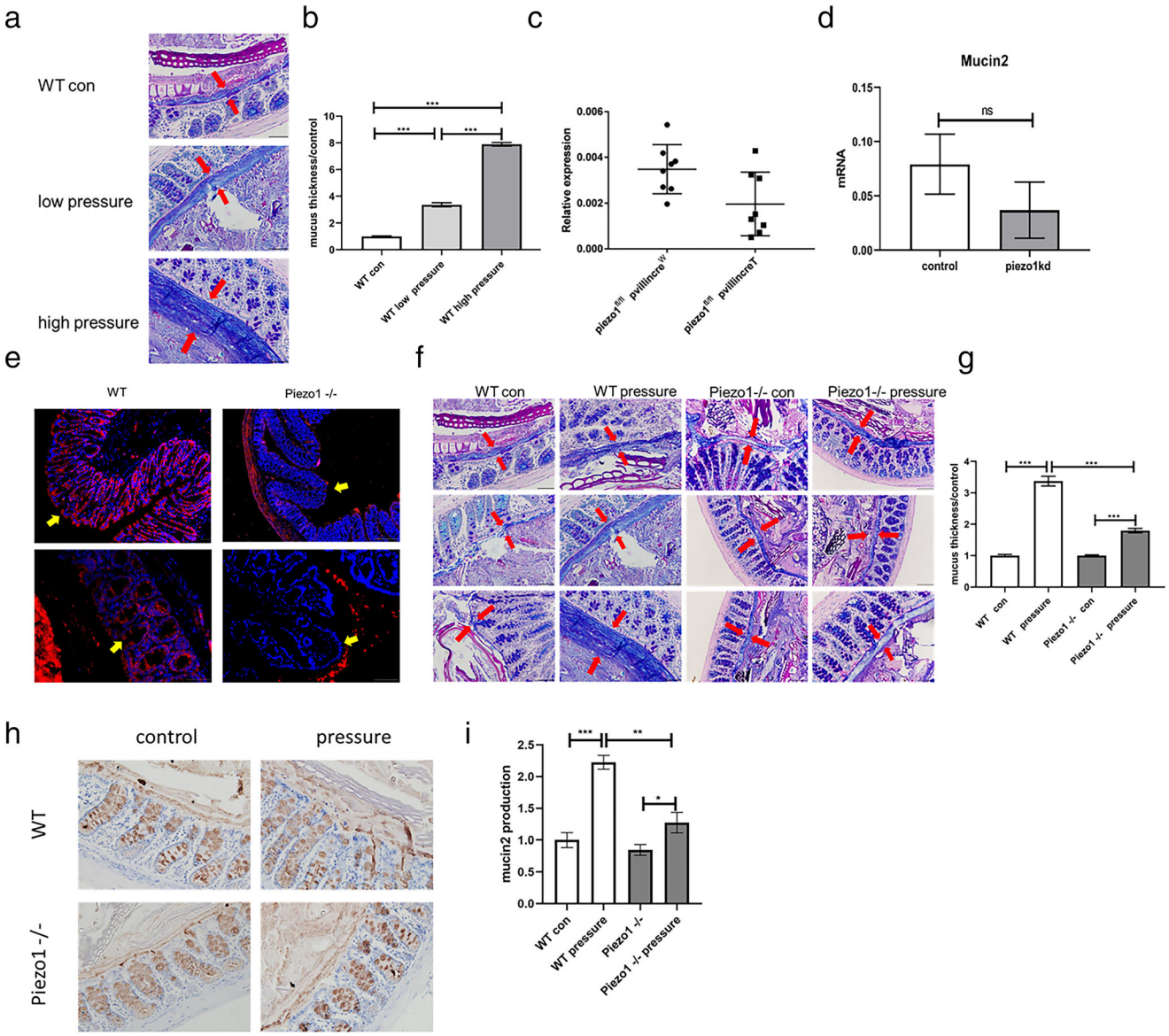



**Figure 1** Hydrostatic pressure promotes colonic mucus secretion in mice. (a) Alcian staining of mucus in wild type mouse colon after hydrostatic pressure stimulation, 400×. (b) Statistical analysis of colon mucus thickness in panel (a). (c,d) Reverse transcription-PCR analysis of Piezo1 and mucin2 mRNA in control and Piezo1−/− mouse colon. (e) Immunofluorescence for Piezo1 (red) in control and Piezo1−/− mouse colon, 200× and 400×. (f) Alcian staining of mucus in wild type and Piezo1−/− mouse colon after hydrostatic pressure stimulation, 400×. (g) Statistical analysis of colon mucus thickness in panel (f). (h) Immunohistochemistry of mucin2 wild type and Piezo1−/− mouse colon with and without pressure, 400×. (i) Statistical analysis of mucin2 production in panel (h). [Color figure can be viewed at wileyonlinelibrary.com]

We further assessed the expression of mucin2 in LS174T cells after downregulation of Piezo1 by ELISA, and the results showed that mucin2 also decreased by more than 50% ($P < 0.05$) (Fig. 4e).

After obtaining the results that the expression of Piezo1 was related to mucin2 in LS174T cells, we further studied the effect of the open state of the Piezo1 protein channel on mucin2. In the absence of mechanical stimulation, the Piezo1 channel was blocked by GsMTx4 (MCE Co., Ltd., initial concentration of 2 mm mol/L, with experimental concentrations of 0.005 μmol/L and 0.01 μmol/L), a specific inhibitor of the Piezo1 channel,[34] and the treatment times were 12 hours and 24 hours. The RT-PCR results showed that the expression of mucin2 mRNA was significantly inhibited by treatment with 0.005 μmol/L and 0.01 μmol/L GsMTx4 (Fig. 4f,g). The ELISA results for mucin2 in cells were consistent with the trend of the RT-PCR results (Fig. 4h). Electron microscope also suggested that GsMTx4 significantly inhibited the synthesis of mucin2 (supporting information Fig. S1).

We also studied the effect of the Piezo1 protein channel activation on mucin2. In the absence of mechanical stimulation, LS174T cells


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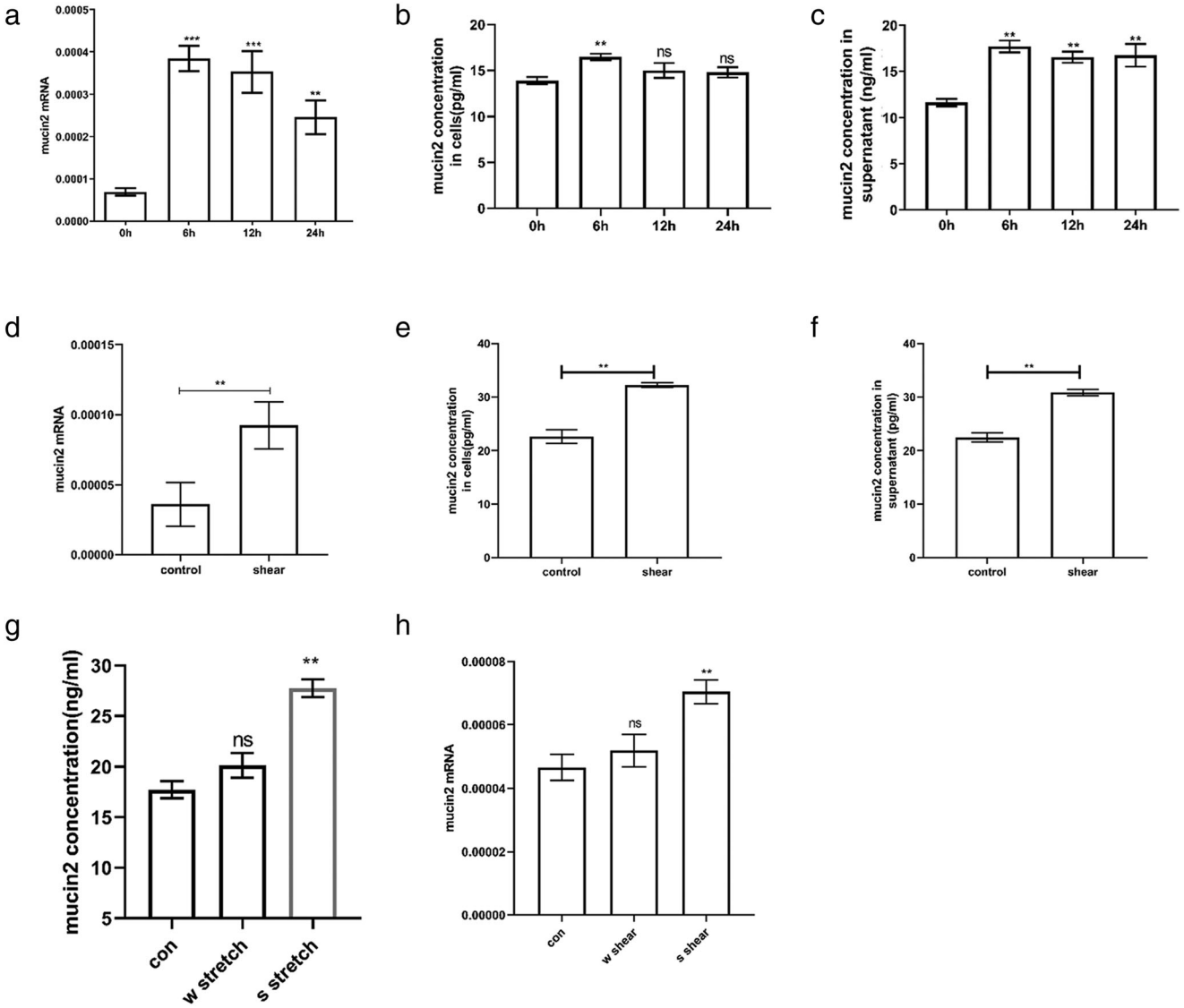



**Figure 2** Mechanical stimulation affects the synthesis and secretion of mucin2 in cells. (a) Reverse transcription PCR analysis of mucin2 in LS174T cells after 6, 12, and 24 h traction force. (b,c) ELISA analysis of mucin2 in LS174T cells and supernatant after 6, 12, and 24 h traction force. (d) Reverse transcription PCR analysis of mucin2 in LS174T cells after 24 h shear force. (e,f) ELISA analysis of mucin2 in LS174T cells and supernatant after 24 h shear force. (g,h) ELISA analysis of mucin2 in LS174T Cells after low intensity and strong intensity mechanical force (compared with control $*P < 0.015$, $**P < 0.01$, and $***P < 0.001$).

were treated with the Piezo1 agonist Yoda1[35,36] (MCE company, initial concentration of 50 mmol/L, experimental concentrations of 2.5 μmol/L, 25 μmol/L, and 50 μmol/L) at 6 hours, 12 hours and 24 hours, respectively. The RT-PCR results showed that the level of mucin2 mRNA increased with the extension of the treatment time (Fig. 4i–k). However, interestingly, the peak mucin2 mRNA appeared at different time points after treatment with different concentrations of Yoda1. The increasing trend of mucin2 was the most significant when treated with 2.5 μmol/L Yoda1 for 6 hours, 25 μmol/L Yoda1 for 12 hours and 50 μmol/L Yoda1 for 24 hours. The ELISA results showed that the expression of mucin2 increased significantly after treatment with 2.5 μmol/L, 25 μmol/L and 50 μmol/L Yoda1 for 24 hours (Fig. 4l).

***Regulation of mucin expression in LS174T cells by mechanical stimulation mediated by Piezo1.*** To determine whether the Piezo1 protein plays a role in mediating mechanical stimulation in LS174T cells, we constructed a Piezo1-specific downregulated LS174 cell line and used it in the traction model. The results showed that the promoting effect of traction on mucin2 expression in LS174T cells was significantly inhibited (Fig. 5a–c). We also studied the effect of the functional state of the Piezo1 protein on mucin2. The results showed that GsMTx4, an inhibitor of the Piezo1 channel (Fig. 5d), inhibited the synthesis of mucin2 in a dose-dependent manner under traction, while the expression of mucin2 could not be further enhanced after treatment with the Piezo1 protein agonist Yoda1 (2.5 μmol/L


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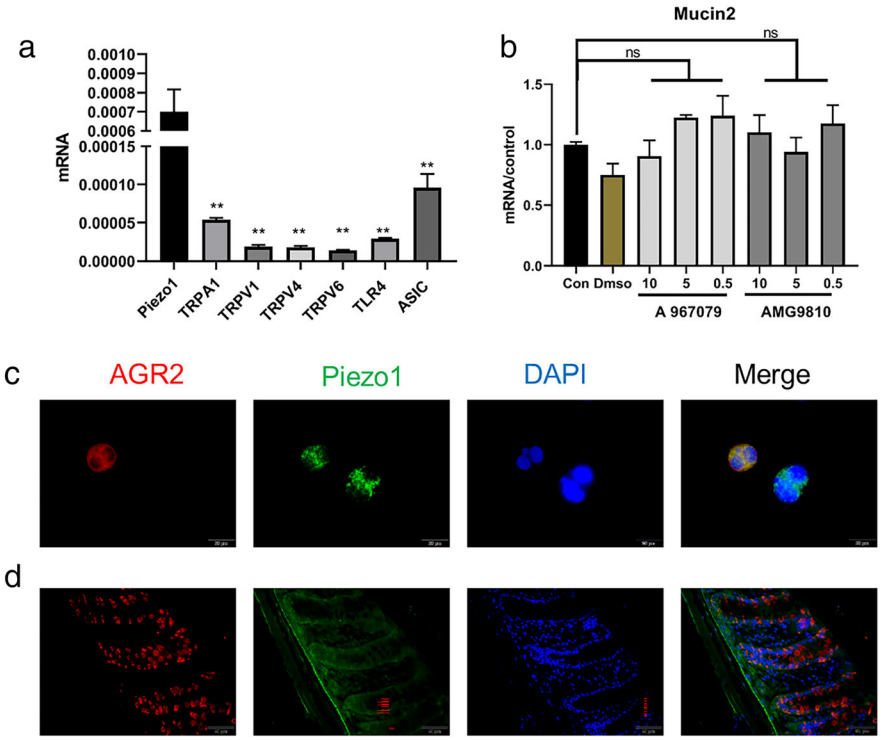



**Figure 3** Piezo1 is the mechanoreceptor of goblet cells. (a) Reverse transcription-PCR analysis of Piezo1, TRPA1, TRPV1, TRPV4, TRPV6, TLR4, and ASIC in LS174T cells. (b) Reverse transcription-PCR analysis of mucin2 mRNA after A 967079 and AMG 9810 intervention for 24 h. (c,d) Immunofluorescence for co-localization of AGR2 and Piezo1 in LS174T cells and murine intestine, 400× (compared wih control *P < 0.015,**P < 0.01, and ***P < 0.001). [Color figure can be viewed at wileyonlinelibrary.com]

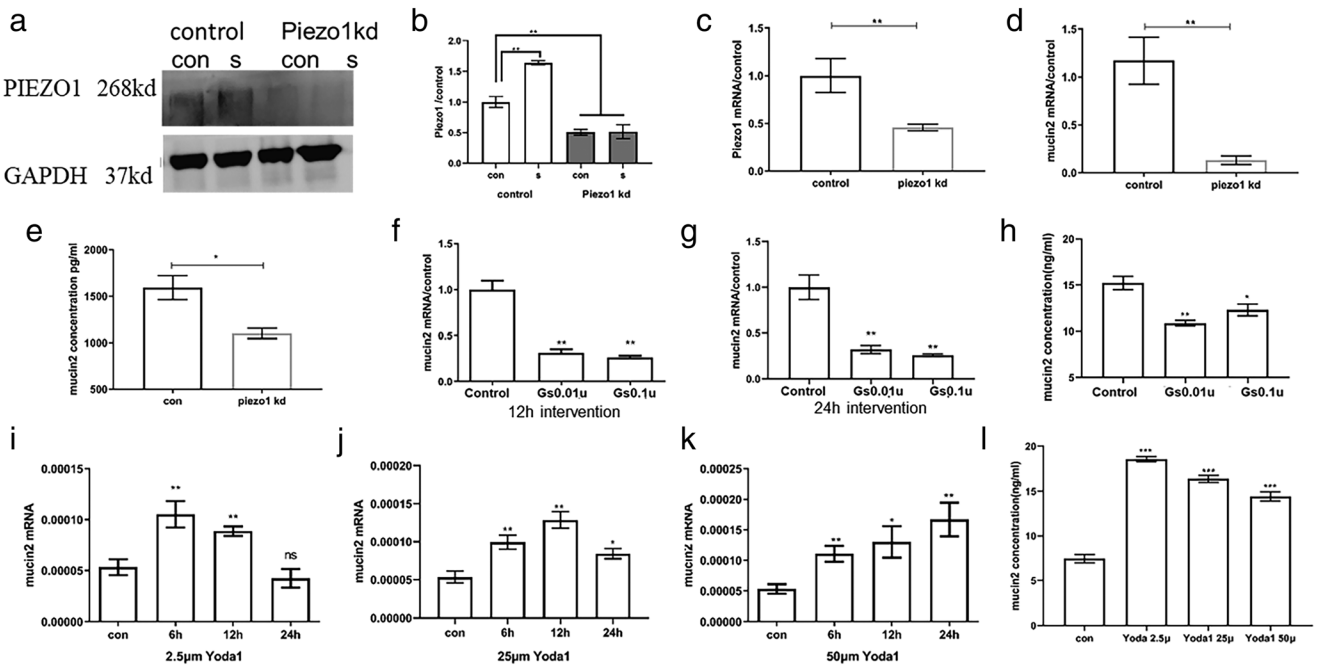



**Figure 4** Effect of interfering with Piezo1 on the expression of mucin2 in LS174T cells. (a) Western blot of Piezo1 in control and Piezo1 knock-down LS174T cells before and after traction force. (b). Statistical analysis of Piezo1 in panel (a). (c,d) Reverse transcription (RT)-PCR analysis of Piezo1 and mucin2 mRNA in control and Piezo1 knock-down LS174T cells. (e) Enzyme-linked immunosorbent assay ELISA analysis of mucin2 in control and Piezo1 knock-down LS174T cells. (f,g) RT-PCR analysis of mucin2 mRNA in LS174T cells after 0.01 and 0.1 μm GsMTx4 intervention for 12 and 24 h, respectively. (h) ELISA analysis of mucin2 in LS174T cells after 0.01 and 0.1 μm GsMTx4 intervention 24 h. (i–k) RT-PCR analysis of mucin2 mRNA in LS174T cells after 2.5, 25, and 50 μm Yoda1 intervention for 6, 12, and 24 h, respectively. (l) ELISA analysis of mucin2 in LS174T cells after 2.5, 25, and 50 μm Yoda1 intervention for 24 h.


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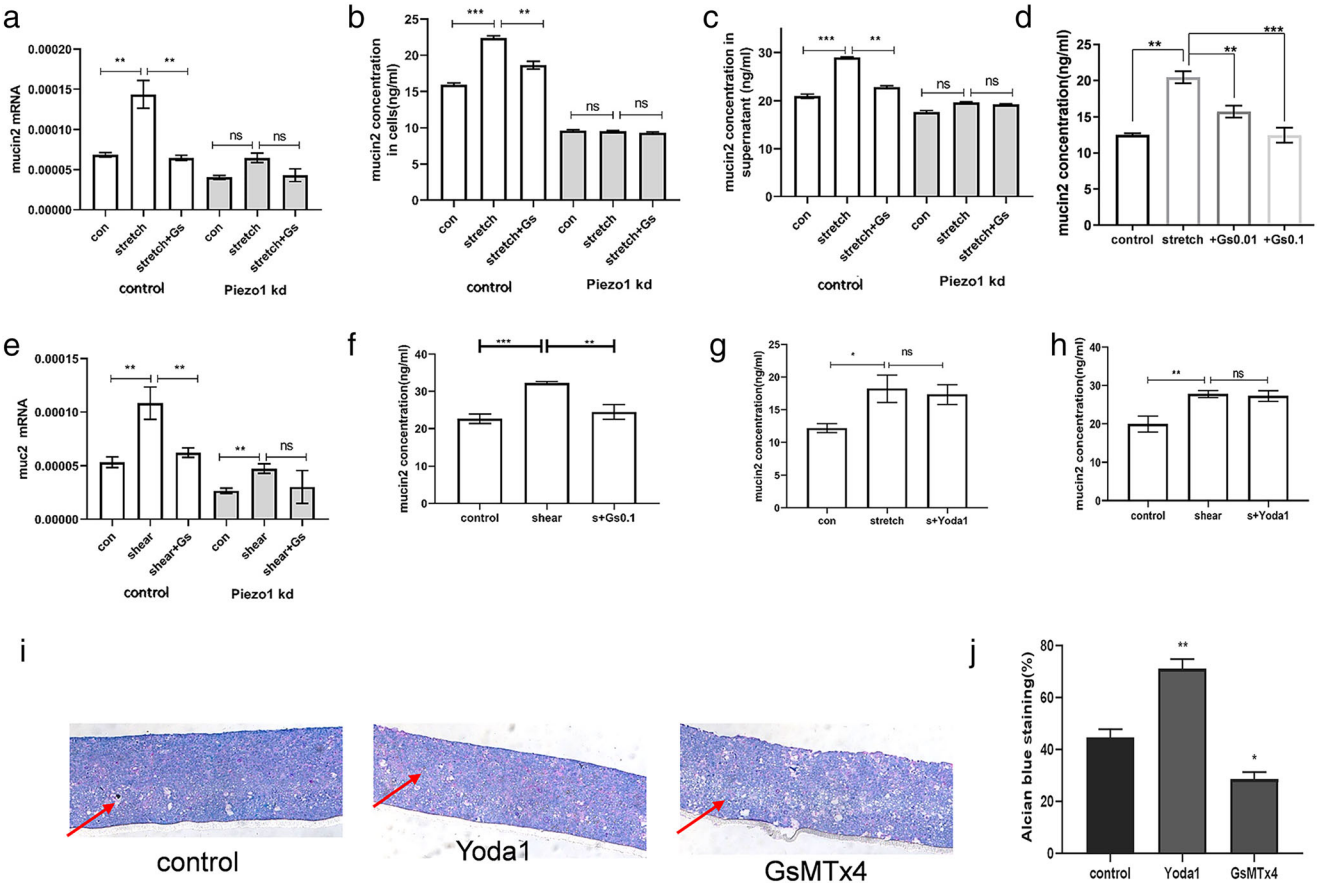



**Figure 5** Regulation of mechanical stimulation on LS174T cells mucin2 expression is mediated by Piezo1. (a–c) Reverse transcription (RT)-PCR and enzyme-linked immunosorbent assay (ELISA) analysis of mucin2 in control and Piezo1 knock-down LS174T cells before and after stretch stress. (d) RT-PCR analysis of mucin2 in LS174T cells after 0.01 and 0.1 μm GsMTx4 intervention with stretch. (e,f) RT-PCR and ELISA analysis of mucin2 in control and Piezo1 knock-down LS174T cells before and after shear stress. (g,h) ELISA analysis of mucin2 in LS174T cells under stretch and shear stress with 2.5-μm Yoda1. (i) Alcian staining of LS174T cells after semi-wet culture, 400×. (j) Statistical analysis of mucin expression in panel (i). [Color figure can be viewed at wileyonlinelibrary.com]

for 24 hours) (Fig. 5g). Thus, mechanical stimulation is mediated by the Piezo1 protein in LS174T cells.

The results of the treatment under shear stress were basically consistent with those under traction stimulation. The experimental results showed that the promoting effect of shear stress on mucin2 was weakened after downregulation of Piezo1 expression in LS174T cells (Fig. 5e). GsMTx4 could inhibit the promoting effect of shear stress on mucin2 synthesis (Fig. 5f). The two effects were shown to be basically the same. Effect of Yoda1 was similar (Fig. 5h).

After obtaining the above-mentioned results, we further explored the effect of the Piezo1 channel on the morphology of the intestinal mucous layer. We used a semi-wet model[28] to culture LS174T cells. After 3 weeks of culture, a mucus layer was formed on the Transwell chamber. After the cells grew to complete confluency and the model entered the mechanical stimulation environment, we added Yoda1 (2.5 μmol/L) and GsMTx4 (0.01 μmol/L) into the lower chamber of the Transwell chamber and assessed the mucin of the formed mucus layer by Alcian staining. The results showed that the mucin staining in the Yoda1 treatment group was denser than that in the control group, while the result was the opposite after GsMTx4 treatment (Fig. 5i,j).

***Piezo1 mediates the regulation of mucin expression by mechanical stimulation in LS174T cells through the Erk pathway.*** We explored the possible downstream pathway of mechanical stimulation that regulates mucin2 synthesis in goblet cells through Piezo1. It has been noted that air pressure can affect the mucus synthesis of airway epithelial cells through the Erk pathway.[16] We confirmed by RT-PCR that the expression of Erk was significantly downregulated in LS174T cells after downregulation of Piezo1 and treatment with ruthenium red (Fig. 6a,b), while the expression of Erk increased under mechanical stimulation (Fig. 6c,d), suggesting that Erk may be the downstream pathway of Piezo1. The Results of the Western blot assessment of mechanically stimulated LS174T cells


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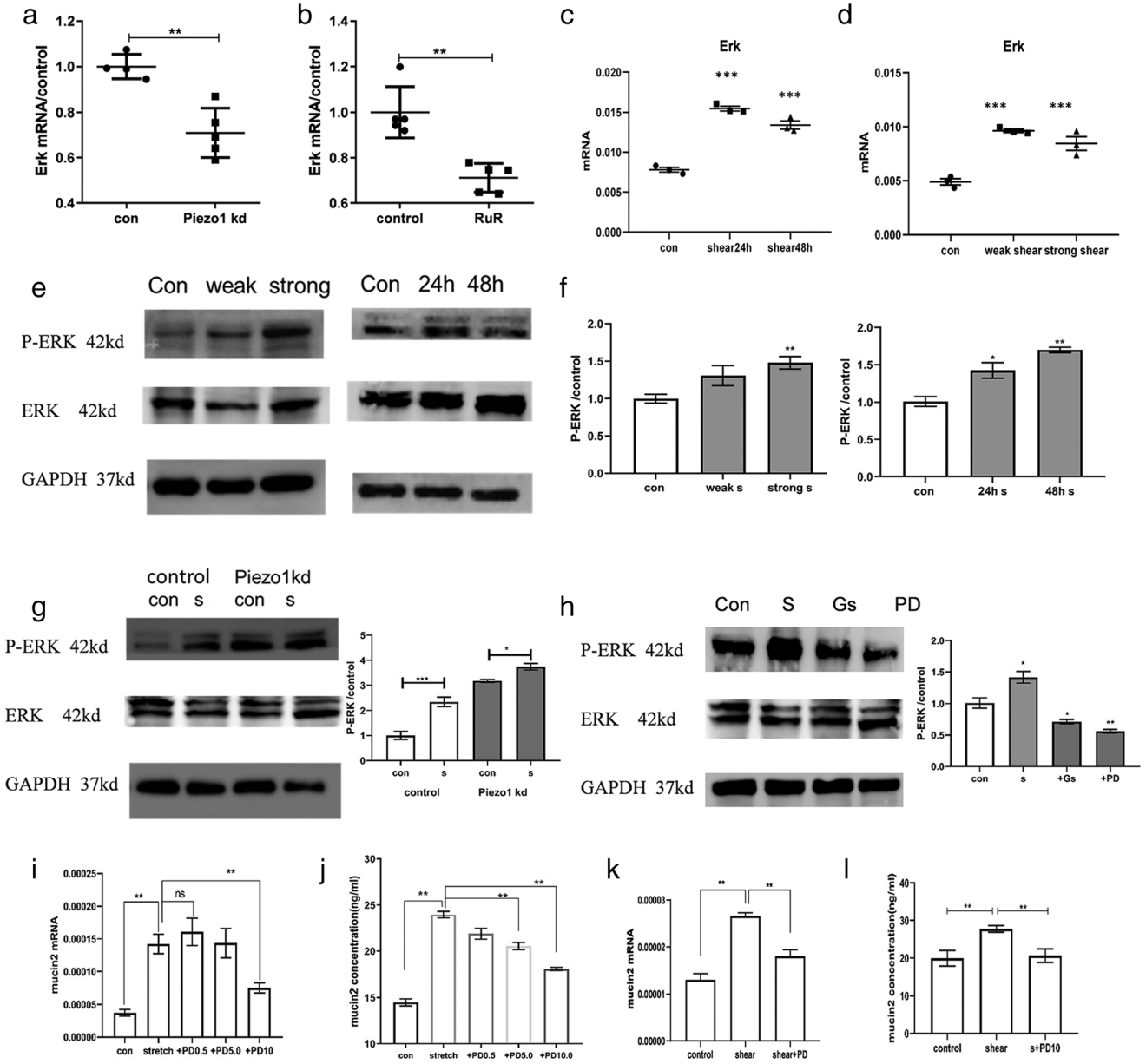



**Figure 6** Piezo1 mediates the regulation of mucin expression by mechanical stimulation in LS174T cells through Erk pathway. (a–d) Reverse transcription-PCR analysis of mucin2 in LS174T cells after Piezo1 knock-down, RuR intervention and different shear stress. (e–g) Western blot of phosphorylated ERK and total Erk in control and Piezo1 knock-down LS174T cells before and after mechanical stimulation. (h) Western blot of phosphorylated ERK and total Erk in LS174T cells after PD98059 intervention. (i–l) Reverse transcription-PCR and ELISA analysis of mucin2 in LS174T cells after mechanical stimulation with PD98059.

showed that the expression of phosphorylated Erk was significantly increased under mechanical stimulation (Fig. 6e,f). After the downregulation of Piezo1, the expression of phosphorylated Erk in LS174T cells also decreased, and there was no obvious response to mechanical stimulation (Fig. 6g). Besides, the expression of phosphorylated Erk was downregulated by GsMTx4 and the Erk pathway inhibitor PD98059 (Fig. 6h). Therefore, we believe that the Erk pathway is likely to be the key signaling molecule downstream of Piezo1.

To confirm that the Erk pathway is involved in the regulation of mucin2 expression by mechanical stimulation, we used treatment with PD98059 (0.5 μmol/L, 5.0 μmol/L, and 10.0 μmol/L), an inhibitor of the Erk pathway, for 24 hours in conjunction with the mechanical traction model. The RT-PCR and ELISA results showed that PD98059 inhibited the promoting effect of mechanical stimulation on mucin expression in LS174T cells in a dose-dependent manner (Fig. 6i,j). The same experimental results are obtained by the shear force model (Fig. 6k,l).


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***Piezo1 mediates the secretion of mucin by mechanical stimulation in LS174T cells through $Ca^{2+}$.*** In the experimental results outlined earlier, the increase in mucin2 in the supernatant was more obvious under mechanical traction stimulation; thus, we speculated that mechanical stimulation may have a certain effect on mucin secretion. Previous studies have shown that Piezo1 channels non-selectively allow cation influx, of which $Ca^{2+}$ is the main part, and that $Ca^{2+}$ is the key signaling molecule regulating vesicle release.[37] It is not known whether the Piezo1-mediated influx of $Ca^{2+}$ regulates the release of mucin2 in goblet cells. To verify this conjecture, we first verified Piezo1-mediated $Ca^{2+}$ influx. The results showed that the $Ca^{2+}$ influx decreased significantly after blocking the Piezo1 channel (Fig. 7a). Then, we switched to a $Ca^{2+}$- and $Mg^{2+}$-free medium when mechanical stretch stimulation was performed on LS174T cells. After stimulation, ELISA was used to assess changes in mucin2 in the culture medium. The results showed that the promoting effect of mechanical traction stimulation on mucin2 expression was significantly inhibited and the effect was basically the same as that of GsMTx4 treatment (Fig. 7b,c).

After verifying the change in mucin2 expression in the open state of the Piezo1 channel at the cellular level, we further studied the effect of the open state of the Piezo1 channel on mucus secretion in mouse intestinal mucosa isolated *in vitro*. Based on a previous study,[29] we constructed and improved the device for measuring the thickness of the intestinal mucus layer *in vitro* (Fig. 7d) and successfully assess the thickness of the mucus layer of the mouse colon *in vitro* under constant temperature and oxygenation conditions. The thickness of the mucus layer of the proximal colon was 222.3 ± 65.6 μm and that of the distal colon was 435.5 ± 54.4 μm, which was generally consistent with the reference. It was also observed that the mucus layer thickness of isolated small intestinal mucosa increased significantly with time under perfusion conditions (Fig. 7e). On this basis, the Piezo1 protein agonist Yoda1 (400 μmol/L) and inhibitor ruthenium red (200 μmol/L) were added to the apical chamber of the intestinal mucosa, and the mucus layer thickness was measured again after treatment for 30 min. The results showed that compared with the control group, the increase in mucus layer thickness was significantly increased after Yoda1 treatment ($P < 0.01$), while the increase in mucus layer thickness was significantly decreased after ruthenium red treatment ($P < 0.01$) (Fig. 7f). The functional changes of Piezo1 significantly affected the mucus secretion of intestinal mucosa in mice.

## Discussion

In these experiments, through the construction of a mechanical model, it was confirmed that goblet cells are mechanically sensitive cells and that mechanical stimulation can act on the Piezo1-Erk/$Ca^{2+}$ pathway in goblet cells to regulate the synthesis and secretion of mucin2 (Fig. 8). The goblet cells in the intestine can respond directly to mechanical stimulation in the intestine both *in vivo* and *in vitro*, which may be one of the mechanisms of the quick mucin-secretion ability in the goblet cells at the tip of the

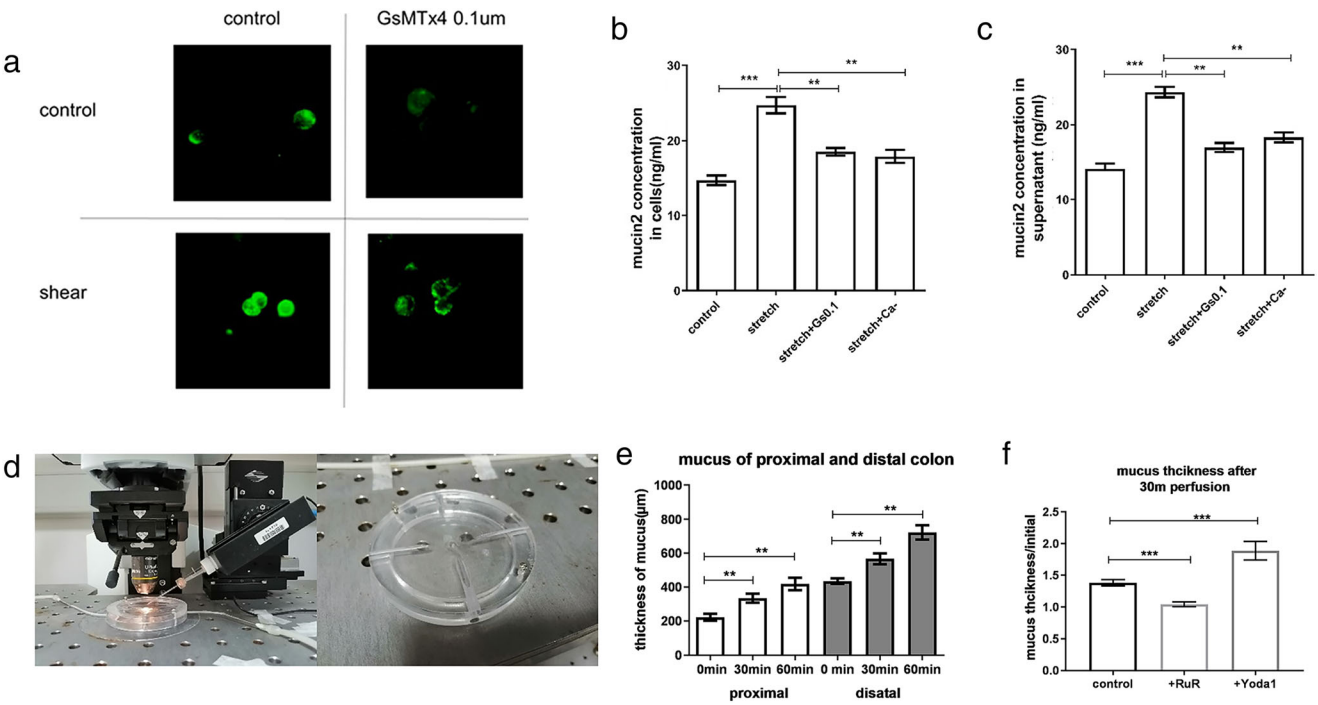



**Figure 7** Piezo1 mediates the secretion of mucin by mechanical stimulation in LS174T cells through Ca2+. (a) Calcium imaging showed Ca2+ influx in LS174T cells under shear stress. (b,c) Enzyme-linked immunosorbent assay analysis of mucin2 in LS174T cells after mechanical stimulation with Ca2+ free medium. (d) Image of self-made horizontal Ussing chamber. (e) Results of measurement of mucus thickness in proximal and distal colon on horizontal Ussing chamber. (f) Measurement of colon mucus thickness after RuR or Yoda1 intervention. [Color figure can be viewed at wileyonlinelibrary.com]


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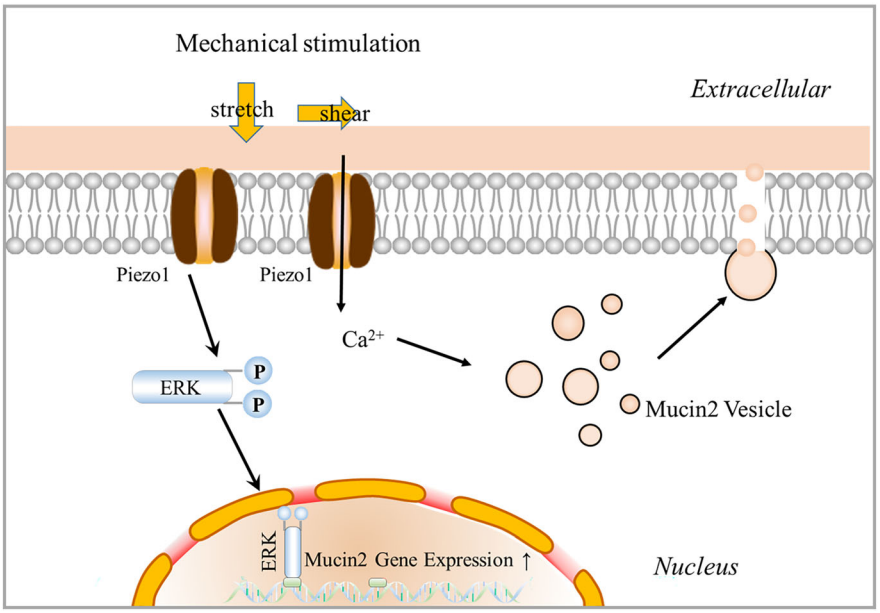



**Figure 8** Mechanical stimulation activates Piezo1 to promote mucin2 expression in goblet cells. Stretch and shear force signals via Piezo1 in goblet cells, resulting in phosphorylated Erk and Ca2+ influx. Phosphorylated Erk drives mucin2 expression and Ca2+ promotes mucin2 secretion. [Color figure can be viewed at wileyonlinelibrary.com]

midgut villi,[10] as the goblet cells at the tip of the villi are the first to feel changes in mechanical stimulation in the intestinal cavity. In the past, it was thought that local intestinal immune and endocrine regulation played a dominant role in intestinal mucus synthesis and secretion.[10] Our study proposed for the first time that intestinal mechanical stimulation can directly affect mucin synthesis and secretion without endocrine feedback regulation and also revealed the corresponding regulatory mechanism.

Previous studies have shown that mucin secretion in goblet cells is mainly induced by $Ca^{2+}$ mobilization agents such as acetylcholine and histamine[38–40] and that PGE2 and IL-13,[41] produced by the immune system, can also regulate mucin synthesis. However, our study found that goblet cells are mechanically sensitive and the mechanosensitive channel Piezo1 protein on the surface of goblet cells is the structural basis of their perception of external mechanical stimuli. Although there was no difference about mucus thickness between WT con and Piezo1−/− con group at rest, the mucus secretion of the experimental group was significantly less than that of the control group during mechanical stimulation, which indicated that regulatory effect of Piezo1 was more significant during stress. This new pathway may be a supplement to the immune function of goblet cells and is beneficial for goblet cells to provide rapid feedback when they receive external stimuli.

The contraction of intestinal smooth muscle, the change in intestinal pressure, and the transmission of intestinal contents can all produce mechanical stimulation of the intestinal epithelium. In this experiment, two forms of mechanical stimulation were simulated, and the effects of the form and time of mechanical stimulation on mucin synthesis and secretion of LS174T cells were discussed. We found that under physiological conditions, the traction force and shear force in the intestine can promote the synthesis and secretion of mucin. Although the forms of traction force and shear force are different, the Piezo1 protein, as a mechanically sensitive ion channel on the cell membrane, can sense a change in membrane surface tension.[36,42] The ultimate effect of traction and shear stimulation may be to change the cell membrane surface tension, and thus the effect on goblet cell function is similar. Traction and shear stimulation forces can be produced in the process of intestinal peristalsis, which are beneficial to the digestion and absorption of food. Goblet cells correspondingly increase mucus secretion in this process, which plays an important synergistic effect and is conducive to the transport and transport of intestinal substances. These cells also block the interference of substances that may be harmful to the internal environment.[43]

With the increase in mechanical stimulation intensity, mucus synthesis and secretion increase *in vivo* and *in vitro*, but low-intensity mechanical stretch stimulation cannot significantly promote the expression of mucin2, which may be related to the lack of stimulation intensity, leading to no obvious change in the membrane surface tension. We demonstrated that the Piezo1 protein cannot be fully activated. The experiment also showed that under the condition of mechanical stretch stimulation, the activation of Piezo1 protein by Yoda1 stimulation cannot further promote the expression of mucin2 induced by mechanical traction stimulation, which may be because the intensity of mechanical traction stimulation is strong enough to activate all Piezo1 proteins and the use of Yoda1 cannot further increase the opening of Piezo1 protein. In short, the activation of Piezo1 protein is closely related to the intensity of mechanical stimulation, with threshold effects and saturation phenomena.

The mechanical environment in the intestinal tract is more diverse than that in the respiratory tract, and the intestinal barrier structure is also more complex. Although our study cannot completely simulate the mechanical microenvironment in the intestinal tract, the traction force and shear force in the simulation study included the main forms of mechanical forces in the intestinal tract, which is of certain significance for exploring the mechanical microenvironment for the study of intestinal homeostasis.

Previous studies have shown that gas pressure can promote mucus synthesis in bronchial epithelial cells through the Erk pathway.[16] The Erk pathway is closely related to mucin synthesis.[44] Our study also confirmed that mechanical stimulation


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of the intestinal goblet cell affects the downstream Erk pathway. Besides, mechanical stimulation with Erk pathway inhibitor not only inhibited pErk but also significantly downregulated the expression of mucin2, which was close to that of the control group. Therefore, we considered that Erk pathway played a predominant role in mediating the regulation of mucin expression by Piezo1. However, the downstream pathway of Piezo1 may not be unique. Some studies have suggested that Piezo1 may affect nuclear gene transcription and translation[19] through cytoskeletal proteins, which may be a feasible research direction. In addition, the opening of Piezo1 can promote $Ca^{2+}$-based cation influx,[45] and we also confirmed that Piezo1 can regulate goblet cell mucin secretion through $Ca^{2+}$. However, there are many downstream pathways of $Ca^{2+}$, and whether there are other regulatory pathways is also worthy of study.

The physiological functions of different organs and tissues are different, but they are all under the regulation of the mechanical environment. This study confirmed that mechanical stimulation-activated Piezo1 promotes goblet cell mucin expression and participates in the regulation of gastrointestinal physiological function. Previous studies have also confirmed that mechanical stimulation plays an important physiological role in the respiratory tract, urinary tract, vasculature, and skeletal system through the Piezo1 protein.[19,46–48] However, the complexity of human structure determines the different mechanical environments of different organs, so Piezo1, as a mechanically sensitive protein, adapts to the mechanical forms in different environments and plays a corresponding effect, which may be the reason why our research is different from others. As a widely distributed mechanically sensitive ion channel, the role of the Piezo1 protein in various organs and tissues is worthy of further exploration. Mechanical stimulation has a direct regulatory effect on mucin synthesis and secretion with the help of the Piezo1 protein, which can provide a new target for follow-up research on the pathogenesis of intestinal diseases such as IBS and IBD and help to guide the treatment of the disease.

In short, our study explored the effect of mechanical stimulation on intestinal mucus for the first time and proposed that goblet cells are mechanically sensitive cells. The Piezo1 protein on the cell membrane is a mechanical sensor, and mechanical stimulation regulates goblet cell mucus synthesis and secretion through the Piezo1-Erk/$Ca^{2+}$ pathway. This study investigated the effect of the intestinal mechanical microenvironment on goblet cell function from two aspects of phenomenon and mechanism, which is expected to provide a new research direction for the pathogenesis and treatment of intestinal diseases.

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## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1.** Electron microscope of LS174T cells after GsMTx4 treatment, and the red arrow points to the mucin2 vesicle.


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