Chemical conversion of human epidermal stem cells into intestinal goblet cells for modeling mucus-microbe interaction and therapy

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Intestinal goblet cells secrete mucus layers protecting the intestinal epithelia against injuries. It is challenging to study the interaction of goblet cells, mucus layers, and gut microbiota because of difficulty in producing goblet cells and mucus models. We generate intestinal goblet cells from human epidermal stem cells with two small molecular inhibitors Repsox and CHIR99021 in the presence of basic fibroblast growth factor and bone morphogenetic protein 4 at high efficiency (~95%) of conversion for a short time (6 to 8 days). Induced goblet cells are functional to secrete mucus, deliver fluorescent antigen, and form mucus layers modeling the mucus-microbe interaction in vitro. Transplantation of induced goblet cells and oral administration of chemical induction media promote the repair of the intestinal epithelia in a colitis mouse model. Thus, induced goblet cells can be used for investigating mucus-microbe interaction, and chemical cocktails may act as drugs for repairing the intestinal epithelia.

INTRODUCTION

The intestine plays an essential role in the digestion and absorption of digested food and the elimination of undigested food, microbes, and microbial products (1). The functional integrity of the intestinal epithelium involves the coordinated interaction of the mucus layer, intestinal epithelial cells, and immune responses (2). The mucus layer secreted by the goblet cells (GCs) promotes the elimination of gut contents and acts as the first line of defense against various injuries caused by digested food, microbes, and microbial products (3). In addition, GCs can form GC-associated antigen passages (GAPs) that deliver antigens from the intestinal lumen to antigen-presenting dendritic cells in the lamina propria for inducing adaptive immune responses (4, 5). A type of GCs, called sentinel GCs, has been identified in the colonic crypt, which can protect the colonic crypt from bacterial intrusion (6). Accordingly, GCs contribute to the immune regulation in the intestine. The dysfunction of the mucus layer and GCs contributes to several intestinal disorders such as intestinal infections, inflammatory bowel disease, and cystic fibrosis (7). It is, therefore, necessary to study the mechanisms by which intestinal GCs interact with gut microbes and modulate mucosal immunity.

Although intestinal GCs play a critical role in the mucus homeostasis, it is still challenging to isolate and culture GCs from animals and human intestinal tissues. The heterogeneous adenocarcinoma cell line HT-29 can differentiate into a mucus-secreting phenotype and is often used as a cellular model for studying GC differentiation and mucin secretion (7). Besides, intestinal stem cells (ISC) isolated from intestinal tissues or differentiated from pluripotent stem cells such as embryonic stem cells (ES) or induced pluripotent stem cells can differentiate into several intestinal epithelial types including GCs (8–10). However, the processes of obtaining ISC and inducing differentiation are complicated and time consuming. Moreover, the ISC-derived GCs are heterogeneous with a mixture of other intestinal cell types.

In the study, we developed a chemical method to generate GCs rapidly and efficiently (~95%) from human epidermal stem cells (hESC) with two small molecular inhibitors Repsox (R) and CHIR99021 (C) in the presence of basic fibroblast growth factor (bFGF) and bone morphogenetic protein 4 (BMP4). The hESC-derived GCs (hESC-GCs) could express GC-specific marker mucin 2 (MUC2) and form the mucus layer. After exposure to mucin secretagogue signals, hESC-GCs could increase the production of mucins and other bioactive molecules. hESC-GCs were able to deliver the antigen and act as cellular models for investigating antigen-presenting processes. Escherichia coli bacteria could adhere to hESC-GCs. Bacterial infection of hESC-GC cell mass induced the circular, mucus droplet barriers separating bacteria from hESC-GC mass, stimulating the inner mucus layer in the colon. In addition, bacterial infection spontaneously induced hESC-GCs to secrete mucin gels for bacterial colonization in a Transwell culture system. Transplantation of hESC-GCs and oral administration of chemical culture media could promote the recovery of acute colitis in mice models. Thus, hESC-GCs can act as cellular models for investigating mucus-microbe interaction and uncovering the mechanisms of mucus layer formation and mucus barrier dysfunction. hESC-GCs may provide cell sources for transplantation therapy, and chemical cocktails may act as therapeutic drugs for intestinal mucosal diseases.

RESULTS

Direct conversion of hESC into GCs

hESC were cultured and expanded in the EpiLife medium. hESC displayed a cobblestone-like appearance (Fig. 1B, left). Immunostaining assays showed that cultured hESC expressed the epidermal stem cell markers cytokeratin 19 (CK19), CK14, CK5, β1-integrin, and proliferating marker Ki67 (fig. S1A). After differentiation induction with a high concentration of calcium, hESC flattened and expressed the differentiation markers transglutaminase 1 (TGase1) and loricrin (fig. S1B).
Fig. 1. Chemical induction of hESC into GCs. (A) Scheme of induction procedure. (B) Left: Microscope image of hESC. Scale bars, 100 μm. Right: Morphological changes after chemical induction. Scale bars, 100 μm (10×) and 50 μm (20×). Arrow indicates the goblet-like cell. (C) Immunostaining of the MUC2 and E-cad in the induced cells. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). (D) Immunostaining of the MUC2 and β1-integrin in the hESC control without chemical induction. (E) Immunostaining of the UEA-1. Arrow indicates mucus granules. (F to H) Immunostaining of the AGR2, TFF3, and RELMβ. (I) Western blotting of the control hESC and the RCBB-induced cells. (J) Percentage of MUC2-positive (MUC2+) cells in the induced total DAPI-positive cells after 7 days of chemical induction (means ± SEM). (K) Percentages of UEA-1-positive (UEA-1+) cells in the induced total DAPI-positive (DAPI+) cells at the indicated times (means ± SEM). (L) Immunostaining of UEA-1 in the induced cells from day 0 to day 6. (M to P) Immunostaining of the enterocyte marker CYP3A4 and villin, Paneth cell marker LYZ, and enteroendocrine cell marker ChrA. Scale bars, 25 μm (C to H and L to P).
After plating in the collagen-coated plates or glass coverslips, hESC were induced by the chemical induction medium consisting of Dulbecco’s modified Eagle’s medium (DMEM)/F12, 0.5% B27, 0.5% N2, 1% penicillin/streptomycin (P/S), 5 μM Repsox, 3 μM CHIR99021, bFGF (10 ng/ml), and BMP4 (10 ng/ml). The Repsox, CHIR99021, bFGF, and BMP4 were collectively named as RCBB (Fig. 1A). After 6 to 8 days of chemical induction, the cobblestone-like cell morphology was changed into small compact cells, with some cells displaying long, goblet-like morphology (Fig. 1B, right). These induced cells were immunopositive for the epithelial marker E-cadherin (E-cad) and GC marker MUC2 (Fig. 1C and fig. S2A). In contrast, the control hESC did not express the MUC2 (Fig. 1D). Western blotting confirmed the expression of MUC2 in the induced cells (Fig. 1I). The induced cells were also positive for the Ulex europaeus agglutinin-1 (UEA-1, detecting the GC product mucins), showing the presence of mucus granules in the cytoplasm (Fig. 1E, arrow). In addition, the induced cells expressed the GC products, such as anterior gradient protein 2 homolog (AGR2 or named as GOB4), intestinal trefoil factor 3 (TFF3), and resistin-like molecule β (RELMB) (Fig. 1, F to H). These results suggested that the induced cells are GCs, referred to as hESC-GCs. Quantification of MUC2-positive cells showed that 94.98 ± 1.28% of cells were positive for MUC2 at day 7 after induction (Fig. 1J). We further calculated the induction efficiency from day 1 to day 6 by measuring the percentages of the UEA-1-positive cells relative to the total 4′,6-diamidino-2-phenylindole (DAPI)-positive cells after chemical induction. A gradual increase in the number of hESC-GCs was observed during the chemical induction (Fig. 1, K and L). hESC-GCs were negative for markers of other types of intestinal cells, such as the enterocyte markers (CYP3A4 and villin), Paneth cell marker [lysozyme (LYZ)], and enteroendocrine cell marker [chromogranin A (ChrA)] (Fig. 1, M to P). Moreover, the epithelial columnar cell markers CK8/CK18 and CK19 and the epithelial marker, epithelial cell adhesion molecule (EpCAM), were expressed in the hESC-GCs (fig. S2, B to E). Zonula occludens-1 (ZO1) and occludin, the tight junction proteins, were detected around the hESC-GCs (fig. S2, F and G). Western blotting verified that hESC-GCs expressed the epithelial markers and did not express villin, ChrA, and LYZ (fig. S2M). hESC-GCs could proliferate in the induction medium, as evidenced by the Ki67 staining and cell counting kit-8 (CCK-8) assay (fig. S2, H and I).

hESC and hESC-GCs both expressed the transcription factor GATA6 (GATA-binding factor 6) and KLF4 (Kruppel-like factor 4) (fig. S2, J, L, and M), which may facilitate the direct conversion because GATA6 and KLF4 are necessary for GC differentiation (11, 12). GATA 6 expression is restricted to the distal colon epithelial cells (12, 13). However, hESC-GCs did not express GATA4 (fig. S2, K and M), which is generally expressed in the proximal intestine (13, 14). These results implied that hESC-GCs may be distal colon GCs.

As GCs secrete neutral and acidic mucins, we performed periodic acid–Schiff (PAS) staining detecting both neutral and acidic mucins. hESC-GCs were positive for PAS staining (Fig. 2A, left). We further performed Alcian blue (AB) staining to detect acidic mucins specifically. Figure 2A (middle) shows that hESC-GCs were AB positive. AB-PAS staining proved to be positive for both AB and PAS staining (Fig. 2A, right). By contrast, control hESC were negative for PAS and AB staining (Fig. 2A, bottom). Transmission electron microscopy revealed that almost all hESC-GCs have an apical part distended with large rounded mucus globules of moderate electron density and a basal part containing the nucleus (Fig. 2B and fig. S3).

There were few microvilli on the hESC-GCs. hESC-GCs were enriched with mitochondria and endoplasmic reticulum in the cytoplasm (fig. S3). The mucus secretion properties further supported the chemical conversion of hESC into GCs.

**Gene expression analysis of hESC-GCs**

We then performed real-time quantitative polymerase chain reaction (RT-qPCR) analysis of GC-associated genes in hESC-GCs. hESC-GCs increased the mRNA expression of MUC2 and other GC products such as RELMB, Fc-γ binding protein (FCGBP), and zymogen granule protein 16 (ZG16), AGR2, and TFF3, as compared to control hESC group (Fig. 2C). hESC-GCs also up-regulated the gene expression of the transcription factor ATOH1 (protein atonal homolog 1) and SPDEF (SAM pointed domain-containing Ets transcription factor), which are essential for the differentiation of intestinal secretory lineage cells (Fig. 2C). These results suggested that hESC-GCs acquire the key gene expression phenotypes of GCs.

**Effects of small molecules and growth factors on GC induction**

To determine the effects of the small molecules and growth factors, we tested different combinations of them. Treatment with single Repsox (R), bFGF, and BMP4 failed to induce hESC into cells positive for PAS and AB staining (fig. S4, B to D). However, CHIR99021 (C) treatment could induce hESC into PAS- and AB-positive cells (fig. S4E). The addition of Repsox into CHIR99021 could accelerate the morphological changes of hESC into GCs (fig. S4F). bFGF and BMP4 were able to increase the morphological changes and efficiency of conversion (fig. S4, G and H). These data implied that CHIR99021, an inhibitor of glycogen synthase kinase 3 beta (GSK-3β), may play a critical role in GC induction by activation of Wnt signaling. Accordingly, we tested whether another GSK-3β inhibitor could substitute CHIR99021 and found that Kenpaullone (K), another GSK-3β inhibitor, could also induce hESC into GCs (RKBB; fig. S5A).

Wnt activation is associated with the phosphorylation status of GSK-3β because GSK-3β can phosphorylate β-catenin and promote the degradation of β-catenin. Inhibitory phosphorylation of GSK-3β at Ser9 can inhibit the kinase activity of GSK-3β. CHIR99021 is known to inhibit GSK-3β kinase activity by competing the adenosine 5′-triphosphate (ATP)–binding domains and blocking the transfer of the terminal phosphate from ATP to the protein substrate. As a result, CHIR99021 can block phosphorylation of every GSK-3β-targeted substrate including β-catenin, which increases the stability and accumulation of β-catenin in cytosol and nucleus. Nevertheless, we explored whether RCBB activates Wnt signaling via altering the inhibitory phosphorylation of GSK-3β at Ser9 (p-Ser9 GSK-3β). Western blotting revealed that RCBB treatment did not increase the level of p-Ser9 GSK-3β (fig. S6, B and C), indicating that CHIR99021 inhibits activity of GSK-3β not by increasing the inhibitory phosphorylation levels of GSK-3β. A decrease in the ratio of p-Ser9 GSK-3β versus GSK-3β was detected during the initial chemical induction (fig. S6, B and C). Mechanistically, CHIR99021 inhibits GSK-3β activity regardless of its phosphorylation status. Therefore, CHIR99021 can still activate the Wnt signaling. qPCR analysis showed that RCBB treatment led to an increase in the mRNA levels of...
Fig. 2. Characterization of secretory abilities of hESC-GCs. (A) PAS, AB, and AB-PAS staining of hESC-GCs and control hESC. (B) Transmission electron micrograph of hESC-GCs. Almost all the hESC-GCs have large rounded mucus globules (MG) of moderate electron density and a basal part containing the nucleus (N) [top (×1500, 2 μm) and bottom (×3000, 1 μm)]. (C) RT-qPCR analysis of relative mRNA expression levels of mucus-associated genes MUC2, RELMβ, FCGBP, ZG16, TFF3, and AGR2 and GC fate commitment– and differentiation-associated genes ATOH1 and SPDEF in hESC-GCs and control hESC. Values are presented as means ± SEM (n = 3; ***P < 0.001, ****P < 0.0005 versus control group). (D) ALI culture, representative images of mucus secretion after 2 and 7 days of ALI culture in a six-well plate, respectively. Right: Tubes collecting the mucus. Photo credit: Andong Zhao, PLA General Hospital. (E and F) Representative images showing PAS and AB staining of hESC-GCs cultured in the ALI system. (G and H) Representative images of E-cad, MUC2, and CK18 in hESC-GCs cultured in the ALI system. Scale bars, 100 μm (A), 50 μm (E and F), and 25 μm (G and H).
Wnt/β-catenin downstream genes T cell factor 1 (TCF1) and lymphoid enhancer factor (LEF1) and a slight decrease in the mRNA levels of TCF3, TCF4, GSK-3β, and β-catenin as compared to the control hESC (Fig. S6D). A single CHIR99021 also up-regulated the TCF1 and LEF1 and slightly increased GSK-3β in the hESC after 3 days of treatment (Fig. S6E). However, 3-day treatment with RCBB down-regulated the GSK-3β (Fig. S6F), indicating that Repsox, bFGF, and BMP4 also partially affected the GSK-3β. The other two inhibitors of transforming growth factor–β (TGF-β) signaling, A83-01 and SB431542, could substitute Repsox for generating GCs with the increased RELMβ mRNA expression induced by IL-13 treatment (16). Future works should be used to study why hESC-GCs reduced the RELMβ mRNA expression after IL-13 treatment.

**hESC-GCs deliver fluorescent antigen**

GCs have been demonstrated to deliver antigen from the intestinal lumen to the antigen-presenting cells, the lamina propria dendritic cells, via GAPs (4, 5). We treated the cultured enhanced green fluorescent protein (EGFP)–positive hESC-GCs with cell membrane–impermeable model antigen rhodamine B labeled dextran (RB-dextran) (10 kDa) for 2 hours. Fluorescent images showed that RB-dextran granules were present in the cytoplasm of EGFP–positive GCs and were close to the Hoechst 33342–positive nucleus, indicating that hESC-GCs could take up the fluorescent antigen (Fig. 4A and fig. S8A). GCs in the small intestine and colon could form GAPs (fig. S8B) (4, 5). We transplanted EGFP–positive hESC-GCs into the intestine and colon of mice and found that EGFP–positive hESC-GCs could attach the small intestinal epithelia and take up the RB-dextran in vivo, evidenced by EGFP–positive epithelial cells containing a DAPI nucleus and dextran, which indicated the GAP formation (Fig. 4B and fig. S8C, white squares and red arrowheads). We also found that hESC-GCs could attach the colon epithelia and take up RB-dextran (fig. S8D). We further cocultured EGFP–positive hESC-GCs with dendritic cells and found that EGFP–positive hESC-GCs could take up more RB-dextran than dendritic cells (Fig. 4C). These results indicated that hESC-GCs may take up fluorescent antigens and possibly form GAPs.

Knoop et al. (5) has also demonstrated that acetylcholine can induce GAP formation by acting on muscarinic Ach receptor 4 (mACHR4) expressed by GCs and that activation of epidermal growth factor receptor (EGFR) can inhibit GAP formation. We found that both hESC and hESC-GCs expressed messenger RNAs for the mACHR4 and EGFR (Fig. 4, D and E). Immunocytochemistry analyses demonstrated the expression of mACHR4 on hESC and hESC-GCs, but many mACHR4 were in the cytoplasm, not on the cell membrane (Fig. 4F). Although bacterial infection did not significantly change the expression level of mACHR4 in hESC-GCs (Fig. 4, H, I, and K), more mACHR4 were located on the cell membrane (Fig. 4J). Activation of EGFR [phosphorylated EGFR (p-EGFR)] was detected in hESC-GCs, and bacterial infection led to an increased activation of EGFR (Fig. 4, G and I).

Because GC-intrinsic myeloid differentiation primary response (MYD88)–dependent microbial sensing can activate EGFR and inhibit GAP formation (5), we evaluated the MYD88 signaling components in hESC-GCs and hESC. RT-qPCR showed that hESC-GCs and hESC expressed TLR1, TLR2, TLR3, TLR4, and TLR5, with hESC-GCs expressing slightly higher levels of TLR1, TLR4, and TLR5 (Fig. 4F). In addition, hESC-GC expressed the mRNAs of MYD88 and other MYD88 signaling components trn receptor associated factor 6 (TRAP6), interleukin 1 receptor associated kinase 4 (IRAK4), nuclear factor κB (NF-κB), and interferon alpha 1 (IFNA1) (Fig. 4G). Immunostaining assays verified the presence of MYD88 in the cytoplasm of hESC-GCs and hESC (Fig. 4F and fig. S8E). Bacterial infection increased MYD88 in the hESC-GCs (Fig. 4G), which is consistent with the increased activation of EGFR. Moreover, bacterial infections increased the expression of RELMβ (Fig. 4, right). Together, these above results indicated that hESC-GCs could deliver fluorescent antigens.

**E. coli adherence to hESC-GCs**

We compared the adherence of *E. coli* strains to HeLa cells (a conventional immortalized cell line used currently for this assay) and...
hESC-GCs. We performed the adherence assays using wild-type strains with enteropathogenic (EPEC), enteroinvasive (EIEC), enterohemorrhagic (EHEC), and enterotoxigenic (ETEC) *E. coli*. These strains adhered robustly to hESC-GCs cultured in monolayers, like HeLa cells (Fig. 5A). These visual observations suggested the adherence of *E. coli* to hESC-GCs.

When we cultured the hESC-GCs in cell mass and infected the cell mass with *E. coli* strains for 1 to 2 days, we observed an interesting phenomenon. The clustered hESC-GCs could secrete mucus and form many small, circular, bright mucus droplets surrounding the edge of the cell mass (Fig. 5B). The mucus droplets could retain their shapes for several days, and bacteria could not destroy the droplet barriers. We could only observe few bacteria in the droplets (Fig. 5C and movie S2). However, circular mucus droplets were not easily observed when hESC-GCs were cultured in monolayers with...
Developing mucus-microbe interaction models

GCs can secrete mucus to form a mucus layer that provides the frontline host defense against endogenous and exogenous irritants and a good habitat for microbial colonization because oligosaccharides of MUC2 offer numerous microbial attachment sites and energy source (1). However, it is hard to study the microbial community residing in the mucus layer across the length of the intestinal tract, given the difficulty to sample this region in vivo, especially in humans. We developed cellular models to study the spatial organization of the gut microbial ecosystem. We cultured hESC-GCs in the upper chamber in the Transwell system. In the lower chamber, bacteria were added into the culture medium, and the upper chamber was filled with bacteria-free medium. After 3 to 4 days, a viscoelastic, gel-like mucus layer was formed in the lower chamber (Fig. 5D, Figs. S9A and S3). However, the mucus layer could not form without bacteria in the lower chamber (Fig. S9B). Apparent microbial colonization was observed in the mucus layer, with much higher bacteria in the mucus layer than in the culture medium (Fig. S9C). In particular, bacteria were trapped and fixed in the mucus layer right under the upper chamber (Fig. S9C). After fixation with Carnoy’s solution, the mucin gel formed in the Transwell system was positive for AB and PAS staining, and the bacteria were positive for crystal violet staining (Fig. 5D). The mucus layer could be stained by the lectin UEA-1 (Fig. 5D). We developed another indirect culture system by culturing hESC-GCs on the 24-well plates and covering the cells with glass coveslips on the top. Bacteria were then added to the culture. Three to 4 days after coculture, a big, complex gel-like mucus layer was formed in the lower chamber (Fig. 5D). Likewise, the gel-like layers were colonized by bacteria and positive for PAS, AB, crystal violet staining, and UEA-1 immunofluorescent staining (Fig. S9D). This phenomenon indicated that microbes could spontaneously stimulate the hESC-GCs to secrete mucins and form a gel-like mucus layer for bacterial colonization.

We then investigate whether hESC-GCs could secrete mucin to form gel-like mucus without the bacterial infection. We replaced the DMEM/F12-based medium with HCM (hepatocyte culture medium) and cultured the hESC-GCs in a Transwell system or 24-well or 6-well plates and found that hESC-GCs could secrete mucins to form viscoelastic, gel-like mucus for 2 to 3 days of culture. When the gel-like mucus was fixed with Carnoy’s solution, thin, transparent, gel-like networks were observed (Fig. 5E, left). The gel-like networks were colonized by bacteria and positive for crystal violet staining (Fig. 5E). When hESC-GCs were cultured in HCM and in the presence of E. coli, the formed gel-like mucus was thicker after fixation (Fig. 5F, left). The thick gel-like layers were positive for AB and PAS staining and UEA-1 staining (Fig. 5F). The gel-like networks were colonized by many bacteria, observed by crystal violet staining (Fig. 5F). These results suggest that hESC-GCs can spontaneously form gel-like mucus colonized by bacteria after bacterial infection, recapitulating the intestinal outer mucus layer.

![Fig. 4. Delivering fluorescent antigen by hESC-GCs.](http://advances.sciencemag.org/)

(A) Fluorescent images of RB-dextran and EGFP in the hESC-GCs after incubation with RB-dextran. (B) Fluorescent images of RB-dextran and EGFP in the small intestines of mice receiving transplantation of EGFP-positive hESC-GCs and injection of RB-dextran. White squares and arrows indicating the GAP formation. (C) Fluorescent images of RB-dextran and EGFP in cocultured EGFP-positive hESC-GCs and EGFP-negative human dendritic cells. (D to F) RT-qPCR for the mRNA expression of mAChR4, EGFR, and MYD88 signaling components in the hESC-GCs and hESC. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (G and H) Western blotting of hESC-GCs and hESC-GCs infected with enteroinvasive (EIEC) E. coli (hESC-GCs EIEC). p-EGFR, phosphorylated EGFR. (I) Quantification of Western blotting analysis of hESC-GCs and hESC-GCs EIEC. The expression level of p-EGFR and mAChR4 in hESC-GCs EIEC was normalized to that of the control hESC-GCs.

*P < 0.05. (J) Immunostaining of p-EGFR, mAChR4, and MYD88 in hESC-GCs and hESC-GCs EIEC. (K) RT-qPCR analysis of the mRNA expression of EGFR and mAChR4 in hESC-GCs EIEC and hESC-GCs (mean ± SEM, n = 3). (L) RT-qPCR analysis of the EIEC mRNA expression of TLR2, TLR4, and TLR5 and MYD88 in hESC-GCs EIEC (mean ± SEM, *P < 0.05 versus control hESC-GCs).
Fig. 5. **Bacterial adherence assay and mucus-microbe interaction model.** (A) hESC-GCs and HeLa cultured in monolayers were incubated with the indicated *E. coli* strains, assessed by crystal violet staining. (B and C) Representative images of hESC-GCs cultured in cell mass and infected with the indicated *E. coli* strains. Clustered hESC-GCs formed circular mucus droplets [top (10×) and bottom (20×)]. High-power images (C). (D) hESC-GCs were cultured in the upper chamber of the Transwell system, and bacteria were added in the lower chamber. After 3 to 4 days, a viscoelastic, gel-like mucus layer was formed. Photo credit: Andong Zhao, PLA General Hospital. PAS, AB, crystal violet, and UEA-1 staining of the gel. (E) hESC-GCs cultured in HCM formed viscoelastic mucus. Left: Appearance of the thin, transparent gel after fixation of the mucus. Right: PAS, AB, AB-PAS, and UEA-1 staining of the gel. (F) hESC-GCs cultured in HCM and infected with EIEC formed viscoelastic mucus. Left: Appearance of the thick, opaque gel after fixation of the mucus. Right: PAS, AB, AB-PAS, and UEA-1 staining of the gel. The lower panels showed the costaining of AB, PAS, and crystal violet in the gel. Scale bars, 100 μm (A to F).
Transplantation of hESC-GCs and chemical culture media promote the recovery of acute colitis

We next tested the transplantation of the cultured hESC-GCs. We induced acute colonic mucosal damage in immunodeficient NOD-SCID IL2rg⁻/⁻ (NSG) mice with colitis-inducing dextran sulfate sodium (DSS) for 5 days (Fig. 6A, right). Most of the mice developed acute colitis characterized by weight loss, bloody stool, diarrhea, and epithelial injury in the distal colon. At 7 and 10 days after DSS administration, we dissociated EGFP-positive hESC-GCs into small fragments (Fig. 6B), suspended them in a Matrigel-containing phosphate-buffered saline (PBS), and instilled them in recipient mice. After 3 hours of transplantation, EGFP-positive cells attached to the luminal surface (Fig. 6C). After 7 days of cell transplantation, recipient mice were euthanized, and the recipient colons showed varying degrees of recovery (Fig. 6A, right). Multiple EGFP-positive areas appeared as well-demarcated patches in the treated distal colons (Fig. 6D). The transplanted hESC-GCs were adapted to the colonic tissue, and mucin gel was observed around the hESC-GCs (Fig. 6D). The transplanted EGFP-positive cells were immunopositive for GC marker MUC2 (Fig. 6E). Hematoxylin and eosin (H&E) staining showed that transplantation of hESC-GCs promotes the repair of the mucus layer and intestinal epithelia in the acute colitis model (Fig. 6F). Notably, many transplanted EGFP-positive hESC-GCs adhered to the outer mucus layer and died or were removed out with feces soon. Only a small number of transplanted hESC-GCs could attach the ulcerated sites of colons. Therefore, increasing the survival and decreasing the removal of hESC-GCs through feces are important for transplantation therapy.

We further investigated whether oral administration of the chemical culture medium containing RCBB could promote the colon epithelial regeneration after acute damage. The administration of DSS for 7 days led acute damage to colon epithelia in the adult C57BL/6 mice, and then the drinking water was replaced with the chemical culture medium in the experimental group, and the control group was administered with the basal culture medium without the chemical compounds. After 14 days of oral administration, mice were euthanized, and histological analysis revealed that chemical culture medium enhanced the regeneration of colon epithelia in the experimental group, with thicker epithelial layers as compared to the control group (Fig. 6G). AB-PAS staining showed more GCs in the injured colon epithelial sites after oral administration of chemical culture media as compared to control media (Fig. 6H). Immunostaining analysis revealed that more UEA-1-positive mucus granules were present in the injured epithelia of chemical culture medium group than those observed in the control group (Fig. 6I). These results suggest that chemical culture medium promotes the recovery of the injured intestinal epithelia.

Effects of chemical treatment on the differentiation of mouse intestinal organoids

We further investigated the effects of the chemical treatment on the differentiation of adult mouse intestinal organoids (or ISC) in vitro. Mouse small intestinal crypts were embedded in Matrigel and cultured in the presence of growth factors including EGF, Noggin, and R-spondin 1 (ENR). Under the ENR condition, intestinal organoids developed many crypt-villus structures (fig. S10A). After 4 days of culture in the ENR condition, passaged intestinal organoids were then cultured in the ENR condition plus the RCBB cocktail for another 4 days. We found that RCBB decreased the proliferation of crypt cells, as evidenced by decreased numbers of crypt buds and Ki67-positive cells and reduced size of organoids compared to those observed with ENR cultures (fig. S10, A and H). We then compared the differentiation of intestinal organoids cultured in different conditions. Intestinal organoids could spontaneously differentiate into alkaline phosphatase–positive and CYP3A4-positive enterocytes (fig. S10, B and D), PAS-positive and MUC2-positive GCs (fig. S10, C and E), LYZ-positive Paneth cells (fig. S10F), and ChR-A-positive enteroendocrine cells (fig. S10G), which were consistent with previous reports (8, 19, 20). Intestinal organoids cultured in the ENR + RCBB condition also could differentiate into the four types of intestinal epithelial cells (fig. S10, B to G). However, RCBB increased the GC differentiation as compared to the ENR condition (fig. S10, C and E). These results suggest that RCBB may promote the differentiation of mouse intestinal organoids into intestinal epithelial cells, especially GCs.

DISCUSSION

The intestinal mucus layer secreted by GCs provides the first line of defense against injury caused by digested food, microbes, and microbial products. Moreover, GCs contribute to mucosal immune responses by secreting antimicrobial proteins, chemokines, and cytokines and forming GAPS (4–6). The dysfunction of GCs has been associated with multiple diseases, including inflammatory bowel disease and cystic fibrosis, indicating that GCs are not always innocent bystanders and can be active participants in disease pathogenesis (1, 21). However, it is challenging to independently investigate the functions of GCs because of the difficulty in isolating GCs from intestinal epithelial cells. Recent studies have reported to differentiate ISC or pluripotent stem cells into intestinal epithelial cells, including enterocytes, GCs, Paneth cells, and enteroendocrine cells (8, 9, 22). However, those differentiation procedures involve complex, long-time procedures, a combination of various growth factors, and a three-dimensional culture system. Moreover, only a small subset of GCs was obtained within the induced intestinal epithelial cell types. In our study, we have induced a homogeneous population of GCs from hESC with a simple, rapid method. The induction process requires 6 to 8 days of chemical induction, without three-dimensional culture systems. The two small molecules used are cost effective and easy to be synthesized. Compared to ISC or PSC, hESC is relatively easier to obtain from the skins.

Because the direct conversion of hESC into GCs was rapid, we investigated the molecular mechanisms by which hESC are induced into GCs. Wnt signaling is an important signaling pathway for regulating the GC differentiation in the intestine (23, 24). Inhibition of Wnt signaling in the intestine resulted in a remarkable reduction in the secretory cells including GCs, enteroendocrine cells, and Paneth cells (23). Activation of Wnt signaling could promote GC differentiation in the intestinal organoid development (25). In addition, activation of Wnt signaling with GSK-3β inhibitor 6-bromoindirubin-3-oxime (BIO) could induce differentiation of human ES into all four intestinal differentiated cell types including GCs (10). The transcription factor SPDEF, a critical regulator for GC differentiation, maturation, and function, was found a downstream target of Wnt signaling in the intestine (26). Consistent with these results, activation of Wnt signaling by CHIR99021 used in the study played a critical role in the GC induction from hESC (fig. S4). CHIR99021 is a GSK-3β inhibitor by competing the ATP-binding domain of GSK-3β, which can inhibit the GSK-3β–mediated phosphorylation of every substrate (27). Activation of Wnt after chemical induction was observed,
Fig. 6. Improving the acute colitis by transplantation of hESC-GCs and chemical culture media. (A) The right panel displayed the recipient colon at 7 days after the initiation of DSS administration, and the left panel displayed the recipient colon at 7 days after cell transplantation (n = 10). (B) Representative fluorescent images of EGFP-positive hESC-GCs. (C) Representative fluorescent image of EGFP-positive hESC-GCs areas attaching the mucosal layer in the distal colon at 3 hours after transplantation. (D) Representative fluorescent images of EGFP-positive hESC-GCs areas overlapping the damaged region after 7 days of transplantation. (E) Representative images showed that EGFP-positive transplanted area was immunopositive for MUC2. (F) H&E staining of distal colon sections in acute colitis mice and in colitis-induced mice transplanted with hESC-GCs after 7 days of cell transplantation. (G to I) H&E, AB-PAS, and UEA-1 staining of colon sections in acute colitis–induced mice treated with chemical culture media containing RCBB (Chemical, n = 8) and control basal media without RCBB (Control, n = 8) after 14 days of oral administration. Scale bars, 25 μm (E), 100 μm (G to I).
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Cytokines, IL-4, and IL-13, induced by nematode infection, could increase the mucus secretion and cause expulsion of parasites (17). Although IL-13 treatment induced the mRNA expression of MUC2, FGBP, and SPDEF, IL-13 resulted in decrease in the mRNA of RELMβ, which was up-regulated in intestinal GCs after parasite infection and LS174T cells after IL-13 treatment (16, 17, 36). Why hESC-GCs down-regulate RELMβ in response to IL-13 remains to be studied in the future.

GCs can form GAPs and deliver luminal substances to antigen-presenting cells in the lamina propria, inducing adaptive immune responses (4, 5, 37-39). The GAPs can be identified by using the ability of GCs to take up fluorescently labeled dextran (fig. S8B). After exposure to fluorescent RB-dextran in vitro, hESC-GCs actively took up the dextran into their cytoplasm (Fig. 4A). However, the in vitro uptake experiment did not demonstrate the GAP formation in vivo because of the in vitro culture environment. After transplantation of EGFβ-positive hESC-GCs into the small intestine and colon, EGFP-positive hESC-GCs attached the small intestine and colon and transcellularly took up the fluorescent dextran, manifested by EGFβ-positive epithelial cells containing a DAPI nucleus and dextran, indicating the GAP formation (Fig. 4B and fig. S8C, white squares and arrows). Because transplanted EGFP-positive hESC-GCs did not attach the underlying lamina propria dendritic cells and immunostaining for labeling the dendritic cells was not used, the direct delivery of dextran antigen to dendritic cells was not easily observed in the present study. Nonetheless, the results indicated the GAP formation by hESC-GCs to some extent. After coculturing EGFβ-positive hESC-GCs and human dendritic cells, we observed that EGFβ-positive hESC-GCs took up more dextran granules than human dendritic cells (Fig. 4C). Because of lack of the intestinal epithelial barrier, the cultured dendritic cells were directly incubated in dextran-containing medium, which resulted in difficulty in observing the delivery of dextran from hESC-GCs into dendritic cells. Newberry and colleagues (5, 37-43) have demonstrated that acetylcholine induces GAP formation via mACHr4 expressed by intestinal GCs and that activation of EGFβ in GCs can inhibit the GAP formation. hESC-GCs and hESC were found to express mACHr4 and EGFβ, evidence by RT-qPCR, immunocytochemistry, and Western blotting (Fig. 4 and fig. S8). Cultured hESC-GCs had much lower levels of EGFβ activation than hESC (Fig. 4, G and I, and fig. S8, E and F), which may possibly explain why hESC-GCs could take up many dextran antigens in the culture setting. Colon GC-intrinsic MYD88-dependent microbial sensing can activate EGFβ and inhibit GAP formation (5). We found the mRNA expressions of the MYD88 signaling components in hESC-GCs and hESC (Fig. 4F). Bacterial infection can activate the MYD88 signaling and subsequent activation of EGFβ (5). Bacterial infection increased the activation of EGFβ in hESC-GCs and hESC (Fig. 4, G, I, and G, and fig. S8, E, F, and H), consistent with the increased expression of MYD88 signaling components (Fig. 4, J and L). Although bacterial infection did not induce significant changes in the mACHr4 (Fig. 4, H, I, and K, and fig. S8, G and H), bacterial infection induced the translocation of mACHr4 onto the cell membrane (Fig. 4I). Therefore, these data supported that hESC-GCs may form GAPs. On the other hand, hESC-GCs have distinct functions in differentiating into the intestinal epithelium including GCs (1, 21). The TLR2 ligand PCSK increased mucus secretion from hESC-GCs, in accordance with previous study (35). Bacterial infection is the major factor inducing GC secretion (1). Treatment with LPS resulted in increased mucus secretion from hESC-GCs. TLR4-mediated immune responses also regulate the GC secretion. TLR4 cytokines, IL-4, and IL-13, induced by nematode infection, could increase the mucus secretion and cause expulsion of parasites (17). Although IL-13 treatment induced the mRNA expression of MUC2, FGBP, and SPDEF, IL-13 resulted in decrease in the mRNA of RELMβ, which was up-regulated in intestinal GCs after parasite infection and LS174T cells after IL-13 treatment (16, 17, 36). Why hESC-GCs down-regulate RELMβ in response to IL-13 remains to be studied in the future.

EGFβ signaling pathway is another important pathway involved in GC differentiation. TGFβ signaling was found to restrict the GC differentiation in the conjunctiva by inhibiting the expression of SPDEF in a Smad3-dependent manner (30). In the intestine, depletion of either Smad3 or Smad4, the downstream mediators of TGFβ signaling pathway, resulted in gastric and duodenal mucinous adenocarcinoma that were characterized by an abundance of GCs (31, 32). In the airway epithelia, activation of Smad signaling also restricted the GC differentiation (33). These results collectively suggested that TGFβ signaling play a global role in restricting the GC differentiation (30). Therefore, the inhibitors of TGFβ-signaling, such as Repsox, SB431542, and A83-01, promoted the GC induction from hESC in the present study (fig. S5). bFGF has been used to induce definitive endoderm into the gut lineage cells (34). bFGF and BMP4 were shown to act synergistically with BIO and DAPT to enhance the differentiation of human ES into the intestinal epithelium including GCs (10). Notch signaling pathway suppresses the GC differentiation from ISC (15). The addition of the Notch pathway inhibitor DAPT could increase the maturation of hESC-GCs (fig. S7), but was dispensable for GC induction from hESC.

KLF4 is required for the terminal differentiation of GCs in the intestine (11). The expression of KLF4 is present both in the hESC and hESC-GCs (fig. S2M), which may, in part, explain why hESC can be easily converted into GCs. GCs have distinct functions in different segments of the intestine. GATA6 is required for normal maturation of GCs in the colon, whereas, in the small intestine, GATA6 does not play a role in commitment, differentiation, or maturation of GCs (12-14). However, GATA4 is expressed in the proximal small intestines and absent in the distal colons (14). Consistent with these results, we found that hESC-GCs expressed GATA6 but not GATA4, indicating that hESC-GCs might be restricted to colons. However, more study will be needed to determine whether the induced hESC-GCs belong to small intestines or colons, such as comparing the global gene expression profiles of hESC-GCs with those of human GCs in the small intestines and colons.

hESC-GCs were functional because hESC-GCs could secrete mucins and respond to several mucin secretagogue signals. Cholinergic agent (CCh) has been widely used to induce secretion of GCs (4, 5), which was consistent with increased secretion in hESC-GCs after CCh treatment. Activation of TLR2 can stimulate mucus secretion in GCs (1, 21). The TLR2 ligand PCSK increased mucus secretion from hESC-GCs, in accordance with previous study (35). Bacterial infection is the major factor inducing GC secretion (1). Treatment with LPS resulted in increased mucus secretion from hESC-GCs.
can express EGFR and mAChR4 and MYD88 signaling components, indicating that hESC in the skin surface may also form GAPs involved in the adaptive immune responses.

The relationship between gut microbiota and the host maintains the intestinal homeostasis. A disturbance of this relationship can result in intestinal disorders such as inflammatory bowel diseases and metabolic syndromes. The mucosal layer plays a critical role in maintaining a beneficial relationship between microbes and the host. The advance in research of the interaction between host and microbes has been hampered by the lack of a suitable model system recapitulating the interactions at the mucosal layer. We found that EIEC, EPEC, EHEC, and ETEC E. coli strains could adhere to hESC-GCs in monolayers and damaged the cells (Fig. 5A). When cultured in cell mass, hESC-GCs could form circular mucus droplets surrounding the edge of cells, which largely separated bacteria from cell mass (Fig. 5, B and C, and movie S2). The inner mucus layer in the colon is immunoprotective to bacteria and protects intestinal epithelia against the lumen microbiota (18). Accordingly, the mucus droplet barrier function is like that of the inner mucus layer. It is thus hypothesized that intestinal GCs may form the inner mucus layer in the manner of mucus droplet barrier, in which many layers of droplet barriers constitute the inner mucus layer. Without the bacterial infection, hESC-GCs cultured in cell mass did not form the circular, mucus droplet barriers, supporting the idea that luminal microbiota are involved in the formation of mucus layers in the intestine (44, 45). Although the mechanism underlies the formation of circular droplets and the components in the droplets remain to be investigated, this cell-mucus droplet barrier–bacteria model may be used to study how the mucus barrier can be destroyed, reconstructed, or enhanced in vitro by disturbing the bacteria and GCs.

In addition, we developed indirect coculture models of hESC-GCs and bacteria, in which mucus layers colonized by bacteria were spontaneously formed (Fig. 5D and fig. S9A). This in vitro coculture model had a membrane barrier to separate bacteria from hESC-GCs, which stimulates the separation between intestinal GCs and luminal microbiota. It will be a valuable alternative to study the fine-scale spatial organization of the gut microbial ecosystem. Moreover, the cellular model enables the study of severe gut microbiome perturbations such as antibiotic therapy or pathogen invasion, which cannot be performed in humans for obvious ethical reasons. Without infection, it was hard for hESC-GCs cultured in DMEM/F12-based chemical induction media to form viscoelastic, gel-like mucus. However, hESC-GCs cultured in HCM could easily form viscoelastic, transparent, gel-like mucus in the absence of bacterial infection (Fig. 5E). This phenomenon implied that the culture setting had some effects on the secretory properties of hESC-GCs. HCM may possibly recapitulate the gut microenvironment better. Bacterial infection induced thick, more viscoelastic, gel-like mucus (Fig. 5F), indicating that bacterial infection could enhance the mucus secretion by hESC-GCs.

The transplantation experiments showed that some of transplanted hESC-GCs attached the denuded regions of recipient colonic epithelia, secreted mucus, and improved the repair of damaged mucus barrier to some extent. However, most of the transplanted hESC-GCs adhered to the outer mucus layer and died or were removed with feces soon. Only small number of transplanted hESC-GCs could attach the ulcerated sites of colons. Therefore, increasing the survival and decreasing the removal of hESC-GCs through feces are important for transplantation therapy. Moreover, the transplantation method used in our study could not precisely deliver hESC-GCs into the targeted lesion site. Injection of hESC-GCs under the view of the endoscope may facilitate the precise delivery of hESC-GCs to the lesion site, which will enhance the beneficial effects of hESC-GCs on recovery of colon epithelia.

We further conducted preliminary experiments to explore whether small molecules and growth factors directly could improve the recovery of colon mucus layer in the colitis model. Oral administration of chemical induction media improved the repair of the lesioned colon epithelia as compared to the control (Fig. 6G). In addition, more GCs were regenerated in the repaired colon epithelial sites. It is possible that these chemical factors may modulate the proliferation and differentiation of resident ISC into intestinal epithelial cells including GCs and subsequently contribute to the improvement of GC regeneration. Given that colon epithelia can self-heal, it is necessary to determine the relative contribution of the chemical factors to the GC regeneration in the future. Thus, an alternative colitis model (IL-10−/− knockout mice) that has abnormal functions of GCs or reduced numbers of GCs may be used, instead of the DSS-induced colitis model (4). Despite IL-10−/− mice used, much attention should be paid to precise evaluation of the effects of chemical recipes on GC regeneration when considering the genetic deficits on the GC differentiation, maturation, and function. In addition, further works are required to investigate which concentrations of the chemical compounds and how to deliver them provide the best effects on the regeneration of colon epithelia.

Because the chemical cocktails could promote the colon epithelial regeneration, we further examined the effects of RCBB on the intestinal ISC. We cultured adult small intestinal organoids in the ENR condition (8) and added RCBB into the ENR condition. RCBB was found to slightly inhibit proliferation of intestinal organoids and promote GC differentiation into four types of intestinal cells, especially GCs. Notably, the efficiency of RCBB-induced GC differentiation from intestinal organoids was lower than that observed during the reprogramming of hESC by RCBB (fig. S10, C and E). The differences in the effects of RCBB on hESC and ISC may be attributed to their different tissue origins. ISC have the intrinsic potential to differentiate into enterocytes, Paneth cells, and enteroidendocrine cells in vivo and in vitro. By contrast, hESC derived from skin do not have the inherent potential to undergo the intestinal differentiation. Therefore, specific differentiation of ISC into GCs requires the inhibition of other cell fate commitments simultaneously, which is hard to achieve. For example, Notch signaling suppresses the secretory cell differentiation of ISC by directing enterocyte differentiation (46, 47). Thus, it is necessary to inhibit Notch signaling for efficiently inducing secretory cell differentiation from ISC (19, 46, 47). Without inhibition of Notch signaling, therefore, RCBB may not promote GC differentiation from ISC with high efficiency. However, DAPT-mediated inhibition of Notch signaling is dispensable for reprogramming of hESC into GCs by RCBB, although DAPT could slightly promote the reprogramming into GCs (fig. S7). This difference may partly contribute to the differences in the reprogramming efficiency.

In addition to the gastrointestinal tract, many organs throughout the body maintain epithelial homeostasis by forming a mucosal barrier that acts as a lubricant and helps to preserve a near-sterile epithelium. Upper and lower respiratory tract, genitourinary system, and ocular surface all have mucosal barriers secreted by GCs. Our methods of generating gut GCs provide proof of principle that a purified population of intestinal GCs can be chemically
induced, and it is possible to produce other organ-specific GCs with similar methods.

In summary, we developed an efficient and rapid method to chemically induce hESC into GCs. hESC-GCs could secrete mucus, respond to mucin secretagogues, and deliver fluorescence antigen. In addition, several E. coli strains can adhere to hESC-GCs, and hESC-GC cell mass can form a mucus droplet barrier against bacterial infection. We established indirect coculture models of bacteria and hESC-GCs for recapitulating and researching the mucus-microbe interaction. Moreover, our study revealed that transplantation of hESC-GCs and chemical compounds could provide beneficial effects on improving intestinal mucus layer, which will be helpful in the therapy of intestinal diseases.

**MATERIALS AND METHODS**

**Cell culture**

hESC were obtained from the Otwo Biotech Inc., which isolated hESC from the newborn foreskin. hESC were grown on plates coated with collagen IV (ColV, Sigma-Aldrich) in EpiLife medium (Invitrogen, #MEPI500CA) supplemented with 0.06 mM Ca²⁺, 1% Human Keratinocyte Growth Supplement (Invitrogen, #S0015), and 1% P/S (Invitrogen). Cultures were routinely maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

**Chemical induction of hESC into goblet cells**

hESC were plated on collagen I (Corning)—coated 6-well plates (Corning), 24-well plates (Corning), or glass coverslips (15 mm). Cells were cultured in EpiLife medium until 70 to 80% confluence. The culture medium was replaced by the chemical induction medium consisting of DMEM/F12 (Invitrogen, #10565018), 0.5% N2 (Gibco, #17502048), 0.5% B27 (Gibco, #17504044), and 1% P/S, supplemented with 5 μM Repsox (MedChem Express, #HY-13012), 3 μM CHIR99021 (MedChem Express, #HY-10182), bFGF (10 ng/ml; PeproTech, #100-18B-50), and BMP4 (10 ng/ml; PeproTech, #120-05ET-10). The control group was treated with the basal medium consisting of DMEM/F12 (Invitrogen) supplemented with 0.06 mM Ca²⁺, 1% Human Keratinocyte Growth Supplement (Invitrogen, #S0015), and 1% P/S (Invitrogen). Cultures were routinely maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

**Chemical induction efficiency**

The chemical induction efficiency was calculated. Briefly, 10 randomly selected visual fields were used to count cell numbers. The total number of MUC2-positive and UEA-1-positive cells was determined, and the chemical induction efficiency was calculated as the percentages of MUC2-positive cells and UEA-1-positive cells among the total DAPI-positive cells. The data are presented as the means ± SEM from triplicate samples.

**Quantitative real-time PCR**

Total RNA was extracted from indicated cell samples by using TRIzol (Invitrogen) as instructed. RNA was reverse-transcribed by using the PrimeScript RT reagent kit with a genomic DNA eraser (Takara, #RR047A). Quantitative real-time PCR involved the use of TB Green Premix Ex Taq II (Takara) in a QuantStudio Real-Time PCR system (Applied Biosystems). The relative expression levels were normalized to that of the internal control (glyceraldehyde-3-phosphate dehydrogenase). All primer sequences are in table S1.

**Western blotting**

Cells in culture were washed with PBS and lysed in radioimmunoprecipitation assay buffer, and protein concentration in the sample lysate was determined with the bicinchoninic acid (BCA) protein assay. Total lysates were separated on a 10% SDS–polyacrylamide gel electrophoresis gel electrophoresis. After staining with polyvinylidene difluoride membrane (Millipore), nonspecific binding was blocked with 5% milk in tris-buffered saline–Tween for 1 hour at room temperature. The epitope of interest was probed with the appropriate primary antibody at 4°C overnight. Membranes were then incubated with appropriate secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology). Electrochemiluminescence was performed on the ChemiImager 5500 imaging system according to the manufacturer’s instructions (Alpha Innotech Co.). The following primary antibodies were used: rabbit anti-MUC2, rabbit anti-p–EGFR, rabbit anti-mAchR4, mouse anti-MYD88, rabbit anti-CK19, mouse anti-CK8, rabbit anti-CK18, mouse anti-CYP3A4, mouse anti-villin, mouse anti-ChrA, mouse anti-LYZ, rabbit anti-HNF4α, rabbit anti-GATA6, mouse anti-GATA4, mouse anti-KLF4, rabbit anti–GSK-3β (Cell Signaling Technology), rabbit anti–phospho–GSK-3β (Ser), and rabbit...
anti-β-actin. β-Actin expression was used as the internal control. The relative intensity was determined by the ratio of the specific marker to β-actin, as measured by densitometry.

**PAS, AB, and AB-PAS staining**

Cells were fixed in 4% paraformaldehyde or Carnoy’s solution for 10 min and stained with a PAS staining kit (Sigma-Aldrich), AB (Sigma-Aldrich), and AB-PAS staining kit (Solarbio) according to the manufacturer’s instructions.

**Transmission electron microscope**

Cell samples were fixed with 2.5% glutaraldehyde in PBS and post-fixed with 1.0% osmium tetroxide in the same buffer, followed by dehydration with a graded series of ethanol. Next, cells were treated with propyleneoxide and then embedded in epoxy resin and sectioned. The ultrathin sections were contrasted with ethanolic uranyl acetate and lead citrate and observed under a transmission electron microscope (JEOL JEM-1210, Japan).

**Mucin secretion**

hESC-GCs were treated with 1 mM cholinergic agent CCh (Sigma-Aldrich) or PCSK (20 µg/ml) (Santa Cruz Biotechnology) for 2 hours, followed by analysis of PAS assay of mucin contents in cell lysates and culture media and RT-qPCR analysis of MUC2 mRNA expression levels. hESC-GCs were treated with LPS (50 µg/ml) (Sigma-Aldrich) for 24 hours or IL-13 (50 ng/ml) (PeproTech) for 1 and 3 days before RT-qPCR analysis of gene expression changes.

**PAS assay**

hESC-GCs treated with CCh or PCSK were disrupted in PBS using sonication (Sonics VCX105, USA) to obtain soluble proteins. Protein concentration was measured with a BCA protein assay kit. All proteins from different groups were diluted to the same concentration. The method to measure the mucus glycoprotein was previously described (48, 49). Briefly, cellular soluble fractions and culture media from different groups were first incubated with 0.1% periodic acid (Sigma-Aldrich) for 2 hours at room temperature, followed by the addition of the Schiff reagent (Sigma-Aldrich) and further incubation for 30 min at room temperature. The optical density value of the resulting solutions was taken at 550-nm wavelength as a measure of the amount of PAS-positive contents. Data were expressed as the fold change relative to the mean value of the control group.

**ALI culture of hESC-GCs**

hESC-GCs were seeded onto the Transwell system coated with collagen I and grown to confluence in the chemical induction medium. After 1 week of ALI culture, the mucus secreted in the upper chamber moved, establishing the transition to an ALI culture condition.

**Antigen-delivering model in hESC-GCs**

hESC before chemical induction and hESC-GCs were transduced with pLenti-CMV-EGFP-3FLAG vectors to express EGFP. As a result, hESC-GCs were labeled with fluorescent EGFP. For modeling antigen deliver in vitro, EGFP-positive hESC-GCs were cultured in the upper chamber of the Transwell. RB-dextran (10 kDa; Sigma-Aldrich) as a model antigen was added onto the upper chamber at 0.1 mg/ml. After incubation of 2 hours before imaging, Hoechst 33342 was added for nuclear staining. Confocal z-stacks were taken to analyze the distribution of the RB-dextran using the Leica TCS SP-8 microscope. In addition, EGFP-positive hESC-GCs were cocultured with human dendritic cells (obtained from ScienCell) on the collagen I-coated glass coverslips. After incubation with RB-dextran and Hoechst 33342, cells were imaged by the Leica TCS SP-8 microscope.

For modeling antigen delivery in vivo, adult C57BL/6 mice were administered 3% DSS for 5 days to induce colitis. Before 2 days of transplantation, mice were intraperitoneally injected with cyclosporin A (50 mg/kg) to suppress immune rejection. After 2 days of transplantation of EGFP-positive hESC-GCs into the small intestine and colon, lysine-fixable RB-dextran (2 mg/ml; Invitrogen, D1817) was injected into the desired small intestine and colon [100 µl for each intestinal area with a 28G insulin syringe, which was described previously (50)]. The normal mice without DSS treatment and cell transplantation were also injected with RB-dextran into the desired segments of the small intestine and colon. After incubation of 30 min, mice were euthanized, and the regions where dextran was injected were collected. The following tissue processing method was according to the previous method with some modifications (50). The collected segments were fixed in 10% formalin solution at room temperature for 30 min and further fixed by Carnoy’s solution for 4 hours. The segments were followed by incubation in methanol for 2 × 30 min, in ethanol for 2 × 20 min, in xylene for 2 × 25 min, and in liquid paraffin for 2 × 30 min before paraffin embedding. After paraffin embedding, 6-µm intestinal sections were incubated with DAPI to stain the nuclei. The section slides were imaged by the Leica TCS SP-8 microscope.

**Gene expression analysis, Western blotting, and immunostaining of hESC-GCs and hESC after bacterial infection**

hESC-GCs and hESC were cultured in a 60-mm petri dish and coverslips. The experimental hESC-GCs and hESC were infected with EIEC E. coli, and the control hESC-GCs and hESC were not infected, respectively. Followed 1 day of injection, all the cell groups were performed for RT-qPCR, Western blotting, and immunostaining analyses of the EGFR, mAChR4, and MYD88 signaling components.

**Bacterial adherence assays**

Bacterial adherence assays were performed according to the previous studies (51). Briefly, HeLa cells and hESC-GCs were cultured on collagen-coated coverslips in 24-well plates in monolayers. After washes with antibiotic-free medium, cells were incubated with 0.5 ml of the indicated E. coli strain-containing media (~10⁷ colony-forming units/ml) for 1 hour in a 37°C, 5% CO₂ incubator. Then, the bacteria-containing media were removed and washed three times with fresh media to remove nonadherent bacteria and cultured in bacteria-free media for 3 hours. The cells were then washed five times with ice-cold PBS to remove nonadherent bacteria further and fixed in cold methanol and stained in 2.5% crystal violet.

hESC-GCs were cultured on the collagen-coated coverslips in cell mass and infected with the indicated E. coli strains, respectively. After 1 to 2 days of culture, circular, bright mucus droplets were imaged and videoed.

**Indirect coculture of hESC-GCs and bacteria**

hESC-GCs were cultured in the upper chamber of the 12-well Transwell plates or 6-well Transwell plates (Corning). In the lower chamber of Transwell, E. coli or Staphylococcus was added. After 3 to 4 days, the
mucus layers were formed and collected for PAS, AB, and crystal violet staining. In another indirect coculture system, hESC-GCs were seeded on the collagen-coated 24-well plates and covered with glass coverslips. Bacteria were added to the culture medium. After 3 to 4 days, the mucus layers were formed and collected for analysis.

**hESC-GCs cultured in HCM and bacterial infection**

hESC-GCs were cultured in HCM (Lonza, CC-3198) supplemented with RCBB. Within 2 to 3 days, the culture medium was changed into viscoelastic, gel-like mucus. The gel-like mucus was collected and fixed by Carnoy’s solution. After fixation, the thin gel was performed for PAS, AB, AB-PAS, and UEA-1 staining. In addition, hESC-GCs cultured in HCM were infected with the indicated E. coli strain. After 1 to 2 days of infection, the culture medium was changed into more viscoelastic, gel-like mucus containing many bacteria. After fixation with Carnoy’s solution, the thick gel was analyzed by the PAS, AB, AB-PAS, UEA-1, and crystal violet staining.

**Transplantation and chemical treatment experiment in animals**

All animal experimental procedures in this study were performed in accordance with the recommendations of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Animal Experimentation Ethics Committee of the Fourth Medical Center of PLA General Hospital. Female NSG mice (the immunodeficient strain) aged 8 weeks old and female C57BL/6j mice aged 8 weeks old were purchased from the SPF (Beijing) Biotechnology Co., Ltd. Transplantation of hESC-GCs was performed as described on days 7 and 10 following the initiation of DSS-induced colonic injury (52, 53). Acute colitis was induced by feeding adult female NSG mice and C57Bl/6j mice with 3.0% DSS (molecular weight, 40,000) dissolved in drinking water for 5 days.

For transplantation of EGFP-positive hESC-GCs into the distal colon of the NSG mice, EGFP-positive hESC-GCs were released from the collagen-coated plates and mechanically dissociated into small sheets of epithelial tissue. Cell fragments of 500 to 1000 EGFP-positive hESC-GCs were resuspended in 200 μl of diluted Matrigel (1:20) in PBS, which were anally instilled into the colonic lumen of each recipient by using a syringe and a thin, flexible catheter 4 cm in length and 2 mm in diameter (n = 10 per group). After infusio, the anal verge was glued for 12 hours to prevent luminal contents from being excreted immediately. After the procedure, mice were maintained as usual. Mice were euthanized and analyzed after 7 days of transplantation.

For chemical treatment, the above chemical induction medium containing RCBB was orally administered into the C57BL/6j mice with colitis (n = 8 per group). The control group was administered with the basal medium without RCBB (n = 8 per group). After 14 days of administration, both groups were euthanized, and colon samples were collected for histological and immunohistochemical analyses.

**Stereomicroscopy, tissue histology, and immunohistochemistry**

For transplantation experiments, whole distal colons of recipients and their fluorescence were imaged using a fluorescence microscope equipped with the phase-contrast setting. In some experiments, engrafted cells were imaged with a fluorescent stereomicroscope system. For experiments of chemical treatment, whole colons were collected for analysis. For histology and immunohistochemistry, colon tissues were fixed with Carnoy’s solution for 4 hours, and then samples were incubated in 2 × 30 min in methanol, 2 × 20 min in ethanol, 2 × 25 min in xylene, and 2 × 30 min in liquid paraffin before paraffin embedding. Colon sections (4 to 6 μm) were subjected to conventional H&E stain, PAS, AB, AB-PAS staining, and immunohistochemistry for MUC2 and UEA-1.

**Small molecules induce the differentiation of mouse ISC**

We then investigated the effects of the RCBB chemical cocktail on the proliferation and differentiation of mouse ISC. Proximal small intestinal crypts were isolated from adult C57BL/6j mice as previously described (8). Isolated crypts were embedded in Matrigel (growth factor reduced, Corning). The basal DMEM/F12 medium supplemented with 1% N2, 1% B27, 1 mM N-acetylcysteine (MedChem Express), and 1% P/S was added, containing the ENR growth factors including EGF (50 ng/ml; PeproTech), Noggin (100 ng/ml; PeproTech), and R-spondin 1 (500 ng/ml; PeproTech). To investigate the effects of the RCBB cocktail on the crypts, passages intestinal crypts were first cultured under ENR condition for 4 days and then replaced with ENR + RCBB. After 4 days of chemical induction, crypts were fixed, and fixed cells were used for alkaline phosphatase assay and PAS staining and immunostained for the intestinal cell markers CYP3A4, LYZ, and ChrA and the proliferation marker Ki67. The images were taken by a light microscope and a confocal microscope.

**Statistical analysis**

All quantified data were statistically analyzed and are presented as means ± SEM. Statistical significance of differences between groups was determined by Student’s t test. *P < 0.05 was considered significant.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/7/16/eabb2213/DC1

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**REFERENCES AND NOTES**

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