ACE2 contributes to the maintenance of mouse epithelial barrier function

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ABSTRACT

Background: The whole world was hit hard by the coronavirus disease-19 (COVID-19). Given that angiotensin I converting enzyme 2 (ACE2) is the viral entry molecule, understanding ACE2 has become a major focus of current COVID-19 research. ACE2 is highly expressed in the gut, but its role has not been fully understood and thus COVID-19 treatments intending to downregulate ACE2 level may cause untoward side effects. Gaining insight into the functions of ACE2 in gut homeostasis therefore merits closer examination, and is beneficial to find potential therapeutic alternatives for COVID-19. Methods: We took advantage of *Ace2* knockout mice and isolated intestinal organoids to examine the role of ACE2 in intestinal stemness. Inflammatory bowel disease (IBD) mouse model was established by 4% dextran sodium sulfate. LGR5 and KI67 levels were quantitated to reflect the virtue of intestinal stem cells (ISCs). FITC-dextran 4 (FD-4) assay was used to assess intestinal barrier function. Results: Western blotting identified the expression of ACE2 in colon, which was consistent with the results of immunofluorescence and RT-PCR. Moreover, $Ace2^{-/-}$ organoids showed decreased LRG5 and KI67 levels, and elevated calcium concentration. Furthermore, the permeability of $ace2^{-/-}$ organoids was markedly increased compared with $ace2^{+/+}$ organoids. Collectively, $ace2^{-/-}$ mice were more susceptible than $ace2^{+/+}$ mice to IBD, including earlier bloody stool, undermined intestinal architecture and more pronounced weight loss. Conclusions: Our data reveal that ACE2 contributes to the proliferation of intestinal stem cells and hence orchestrates the mucosal homeostasis.

1. Introduction

COVID-19 rages worldwide and causes more than 592,495 as of July 17, 2020. Given the absent treatment of proven efficiency and limited understanding of 2019-nCoV, gearing up for a protracted war against the pandemic needs detailed research. Angiotensin I converting enzyme 2 (ACE2), a zinc metalloprotease which shares homology with ACE, is the entry molecule of 2019-nCoV. 2019-nCoV binds to ACE2 through its surface spike protein and hence infects the target cell, causing severe injuries [1]. Gaining insight into the regulation of ACE2 is therefore pivotal in a bid to develop potential alternatives for COVID-19 [2,3].

ACE2 is widely known as a negative regulator of the Renin-Angiotensin System (RAS). Besides, it is highly expressed throughout the gut system (https://www.proteinatlas.org/ENSG00000130234-ACE2/tissue). Kotfis et al. reported the appeared gastrointestinal diseases in COVID-19 patients [4]. Moreover, COVID-19 patients have significant alterations in fecal microbiomes compared with healthy volunteers, and during hospitalization microbiomes associated with reduction of ACE2 correlate inversely with 2019-nCoV load [5], suggesting the involvement of ACE2 in 2019-nCoV replication. Sander et al. further confirmed that ACE2 upregulation exacerbates the outcomes of COVID-19 by facilitating 2019-nCoV entry into the host cell [6]. Logically, the implication of ACE2 blockers (ACEIs) would constitute a novel strategy to treat COVID-19. However, some unpredicted side effects induced by ACEIs may occur due to the pivotal role of the ACE2/RAS axis in maintaining a variety of cellular processes, including autonomous regulation in gut [7,8]. Therefore, the safe administration of ACEIs has been intensely discussed. Zhang

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et al. reported a lower risk of mortality of COVID-19 patients with ACEIs administration than those with non-ACEIs/ARBs antihypertensive drugs [9]. Emerging studies also supported the continuation of ACEIs or ARBs in COVID-19 patients due to a lower risk of mortality [10—13]. Nevertheless, impaired ACE2 expression results in intestinal dysbiosis [14] and exacerbates inflammatory phenotypes [15—17]. Exploring the role of ACE2 in gut homeostasis and elucidating the pertinent mechanism are therefore urgent for developing adequate treatment options.

In the present study, we examined the expression of ACE2 in gut, monitored the influence of ACE2 on the regenerative capacity of ISCs, explored the consequences of *ace2* deficiency in IBD mouse models.

2. Methods

2.1. Ethics statement

All procedures and assays were approved by the Institutional Animal Care and Use Committee of Jining Medical University.

2.2. Animal

Ace2 knockout mice on the C57BL/6 background and wild type littermates were purchased (Cyagen, China). All animals were housed at sperm free animal facility at Jining medical university. All procedures involving animals were conducted within IACUC guidelines under approved protocols.

2.3. Acute inflammatory bowel disease mouse model

4% dextran sodium sulfate (DSS, MP Biomedicals) was added to drinking water for 5 days. IBD was scored by the following standards: (i) weight loss (no loss = 0; <5% = 1; 5—10% = 2; 10—20% = 3; >20% = 4); (ii) stool (normal = 0; soft, watery = 1; very soft, semi-formed = 2; liquid, sticky, or unable to defecate = 3); (iii) bloody stool test (no blood test positive within 2 min = 0; purple positive after 10 s = 1; light purple positive within 10 s = 2; heavy purple positive within 10 s = 3) (Leagene); (iv) Histological injury and inflammation were scored by parameters including edema, destruction of the epithelial monolayer, crypt loss, infiltration of immune cells into the mucosa.

2.4. Histology

Tissues were fixed and then embedded in paraffin. 3 μm slice was sections stained with hematoxylin and eosin (H&E).

2.5. Immunofluorescence

For immunofluorescence, gut tissue slides or intestine organoids were blocked with 3% BSA, 0.2% TWEEN 20 in PBS, and incubated with primary antibodies (1:100 dilution) (MUC2 Monoclonal Antibody, KI-67 Antibody, LGR5 Monoclonal Antibody, Alexa Fluor™ 488 Phalloidin, MitoSOX™ Red Mitochondrial Superoxide Indicator). After PBS washing, Goat anti-Mouse IgG (H + L) Alexa Fluor 488 and Dapi was loaded for 60 min and 15 min, respectively.

2.6. Intestinal barrier function assays

Mice were fasted for 4 h and orally gavaged with fluorescein isothiocyanate (FITC)-dextran (average molecular weight: 4,000, 0.6 mg/g) (MedChemExpress). Fluorescence intensity of plasma was measured in 4 h (excitation nm/emission 520 nm). Meanwhile, the albumin level in fecal was measured by ELISA (Bethyl Laboratories) according to the manufacturer's protocol.

2.7. Intestinal organoids culture

Intestine organoids were isolated and cultured according to the protocol (Stemcell technology). Small intestine was washed with cold PBS for 15 times until supernatant was clear. Resuspended the intestine in cell dissociation reagent (Stemcell technology) for 15 min at room temperature. Removed the liquids and washed the digested intestine with PBS. The supernatant was filtered through 70 μm cell strainer. Centrifuged the fractions at 1300 rpm for 5 min and resuspend the pellet in the complete intestiCult organoid growth medium (Stemcell technology). The medium was exchanged every two days.

2.8. Measurement of intracellular Ca^{2+}

Intestinal organoids were loaded with Fura-2 (2 μM, MedChemExpress) for 15 min and mounted with an inverted phase-contrast microscope (Zeiss). Calcium imaging was performed with the calcium imaging set-up (Molecular Devices) and data was analyzed by Metafluor.

2.9. Western blotting

Tissue or organoids were lysed with RIPA cell lysis buffer (Roche). The extracts were centrifuged at 13,000 rpm for 20 min at 4 °C and the protein concentration of the supernatant was determined. Total protein (60 μg) was subjected to 8% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane (VWR) and the membranes were then blocked overnight at 4 °C with 10% non-fat dried milk in tris-buffered saline (TBS) containing 0.1% Tween-20. The membranes were incubated overnight at 4 °C with the antibody directed against (MUC2 Antibody, KI-67 Antibody, LGR5 Antibody) (1:1000) (ThermoFisher). A GAPDH antibody (1:1000) (ThermoFisher) was used for a loading control. Specific protein bands were visualized after subsequent incubation with the anti-rabbit IgG conjugated to horseradish peroxidase and a super signal chemiluminescence detection procedure.

2.10. Real-time PCR

Total RNA was extracted according to the manufacturer's instructions (TAKARA). After DNAse digestion reverse transcription of total RNA was performed using Transcriptor High Fidelity cDNA Synthesis kit (TAKARA) according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification of the respective genes were set up in a total volume of 20 μl using 40 ng of cDNA, 500 nM forward and reverse primer and 2x GoTaq® qPCR Master Mix SYBR Green (TAKARA). Cycling conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s and 72 °C for 30 s. For the amplification the following primers were used (5`->3′orientation):

2.11. Ace2

fw CACTCCTGCCACACCACGTT;
rev TGGTCTTTAGGTCAAGTTTACAGCC.
Tbp(TATA box-binding protein).
fw CACTCCTGCCACACCACGTT;
rev TGGTCTTTAGGTCAAGTTTACAGCC.
Specificity of PCR products was confirmed by analysis of a melting curve. Real-time PCR amplifications were performed on a CFX96 Real-Time System (Bio-Rad). All experiments were done in duplicate. Amplification of the house-keeping gene Tbp was performed to standardize the amount of sample RNA. Relative quantification of gene expression was achieved using the Δct method.

2.12. Statistics

Data are provided as means $\pm$ SEM, n represents the number of independent experiments. All data were tested for significance using Student's unpaired two-tailed *t*-test or ANOVA and only results with $p < 0.05$ were considered statistically significant.

3. Results

3.1. ACE2 is expressed in mouse intestinal organoids

Ace2 knockout mice were included and colon tissue was isolated. As illustrated in Fig. 1A–B, western blotting and RT-PCR confirmed the expression of ACE2 in the colon and validated *ace2* knockout mouse strain. Immunofluorescent assay further showed the ubiquitous abundance of ACE2 in the colon (Fig. 1C). By organotypic cultures and *ace2* genetic mice, we ascertained the high expression of ACE2 in intestinal organoids (Fig. 1D).

3.2. Ace2 deficiency hampers the permeability and stemness of ISCs

To determine the function of ACE2 on intestinal barrier function, we isolated intestinal organoids from *ace2*$^{+/+}$ and *ace2*$^{-/-}$ mice. *Ace2* deficiency did not cause obvious structure disruption, as reflected by phallodin staining (Fig. 2A). Of note, LGR5 (marker of ISCs) and KI67 (marker of proliferation) were markedly lower in *ace2*$^{-/-}$ organoids than that in *ace2*$^{+/+}$ organoids (Fig. 2A). Organotypic analysis further showed that *ace2* deficiency led to a slower regeneration of intestinal organoids, as evidenced by a smaller size and slowed differentiation (Fig. 2B). In full accordance with the findings above, western blotting showed that the expressions of LGR5 and KI67 in *ace2*$^{-/-}$ organoids were significantly lower than

that in *ace2*$^{+/+}$ organoids (Fig. 2C). *Ace2* deficiency led to an increased FITC fluorescence in organoids, suggesting that ACE2 contributes to intestinal barrier function (Fig. 2E). Much less expected was the unchanged plasma level of FITC in *ace2* KO mice after oral gavage of FD-4, which may be explained by the various mechanistic pathways involved in the maintenance of mucosal complexity in vivo.

3.3. Ace2 knockout mice are susceptible to IBD

The lack of obvious barrier dysfunction would suggest ACE2 likely become more relevant under pathophysiological settings, such as IBD. As illustrated in Fig. 3A–C, *ace2* deficiency exaggerated the progression of IBD, as evidenced by more pronounced weight loss, earlier bloody feces and higher feces album level. Morphological assays showed that *ace2*$^{-/-}$ mice were more susceptible than *ace2*$^{+/+}$ mice to intestinal architecture destroy and crypts loss as well as infiltration of inflammatory cells, obvious edema and reduction in colon length (Fig. 3D). Additionally, marked loss of LGR5 and MUC2 was observed in *ace2*$^{-/-}$ IBD mice (Fig. 3E).

### 3.4. Ace2 deficiency induces calcium overload in intestine organoids

Proper intracellular calcium concentration is a necessity to maintain gut homeostasis. Calcium overload and ROS production are equally involved in cellular dysfunctions, such as regeneration of stem cells. To explore the nature and mechanism whereby *ace2* deficiency undermines the virtue of ISCs and hence the epithelial renewal, intracellular calcium concentration was examined by calcium imaging. As demonstrated in Fig. 4A and B, ATP-induced $[Ca^{2+}]_i$ was significantly higher in *ace2*$^{-/-}$ organoids than that in
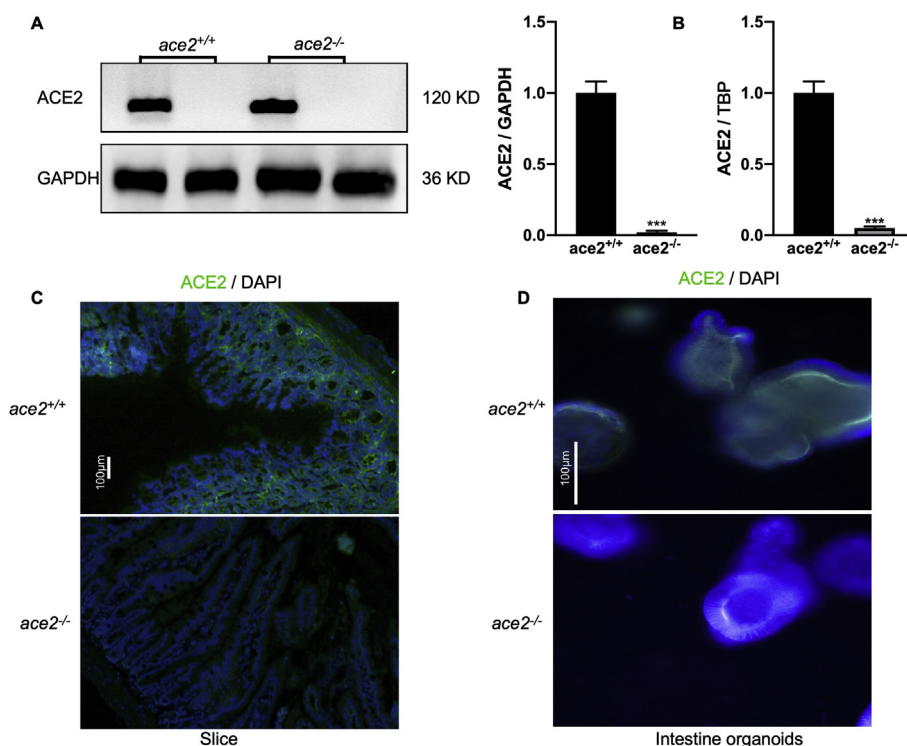
Fig. 1. ACE2 is highly expressed in gutA. Original western blot pictures and bar charts (= 4) illustrating the protein abundance of ACE2 in the colon tissue and organoids isolated from *ace2*$^{+/+}$ and *ace2*$^{-/-}$ mice. B. RT-PCR results illustrating the mRNA level of *ace2* in the colon and organoids isolated from *ace2*$^{+/+}$ and *ace2*$^{-/-}$ mice. C. Immunofluorescent staining of ACE2 in the colon from *ace2*$^{+/+}$ and *ace2*$^{-/-}$ mice. D. Immunofluorescent staining of ACE2 in the organoids isolated from *ace2*$^{+/+}$ and *ace2*$^{-/-}$ mice. *($p < 0.05$),** ($p < 0.05$), *** ($p < 0.001$) indicate significant difference (two-tailed unpaired *t*-test).
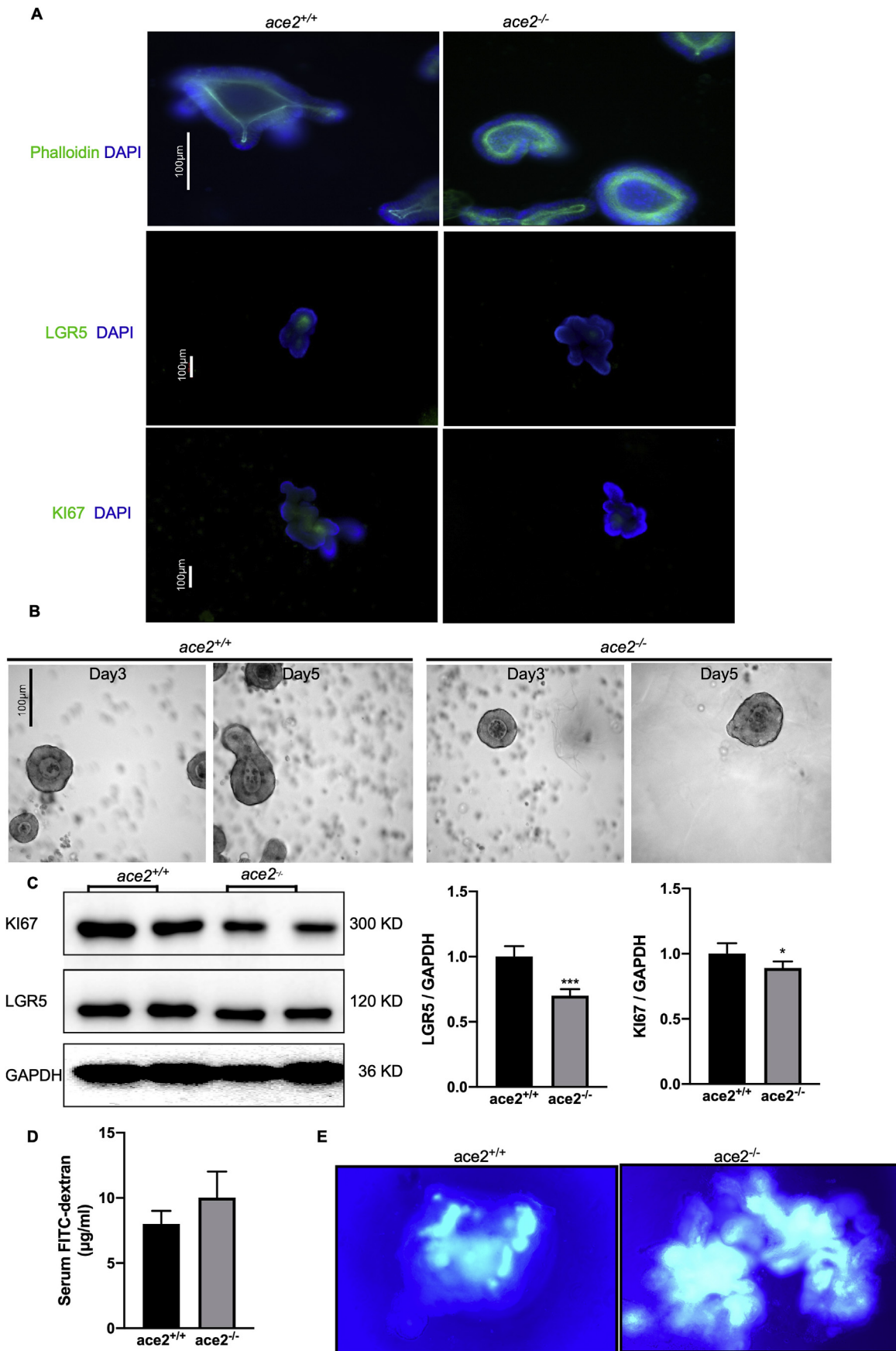
Fig. 2. Ace2 deficiency influences the permeability and regeneration capacity of intestinal organoids. A. Immunofluorescent staining of F-ACTIN, LGR5 and KI67 in the organoids isolated from ace2$^{+/+}$ and ace2$^{-/-}$ mice. B. Original pictures illustrating the morphological changes of organoids isolated from *ace2*$^{+/+}$ and *ace2*$^{-/-}$ mice. C. Original western blot pictures and bar charts ($n = 4$) illustrating the protein abundance of LGR5 and KI67 in the organoids isolated from *ace2*$^{+/+}$ and *ace2*$^{-/-}$ mice. D. Intestinal barrier functional assays measuring FITC-dextran in the serum of *ace2*$^{+/+}$ and *ace2*$^{-/-}$ mice. E. Intestinal barrier functional assays measuring FITC-dextran in the organoids isolated from *ace2*$^{+/+}$ and *ace2*$^{-/-}$ mice.
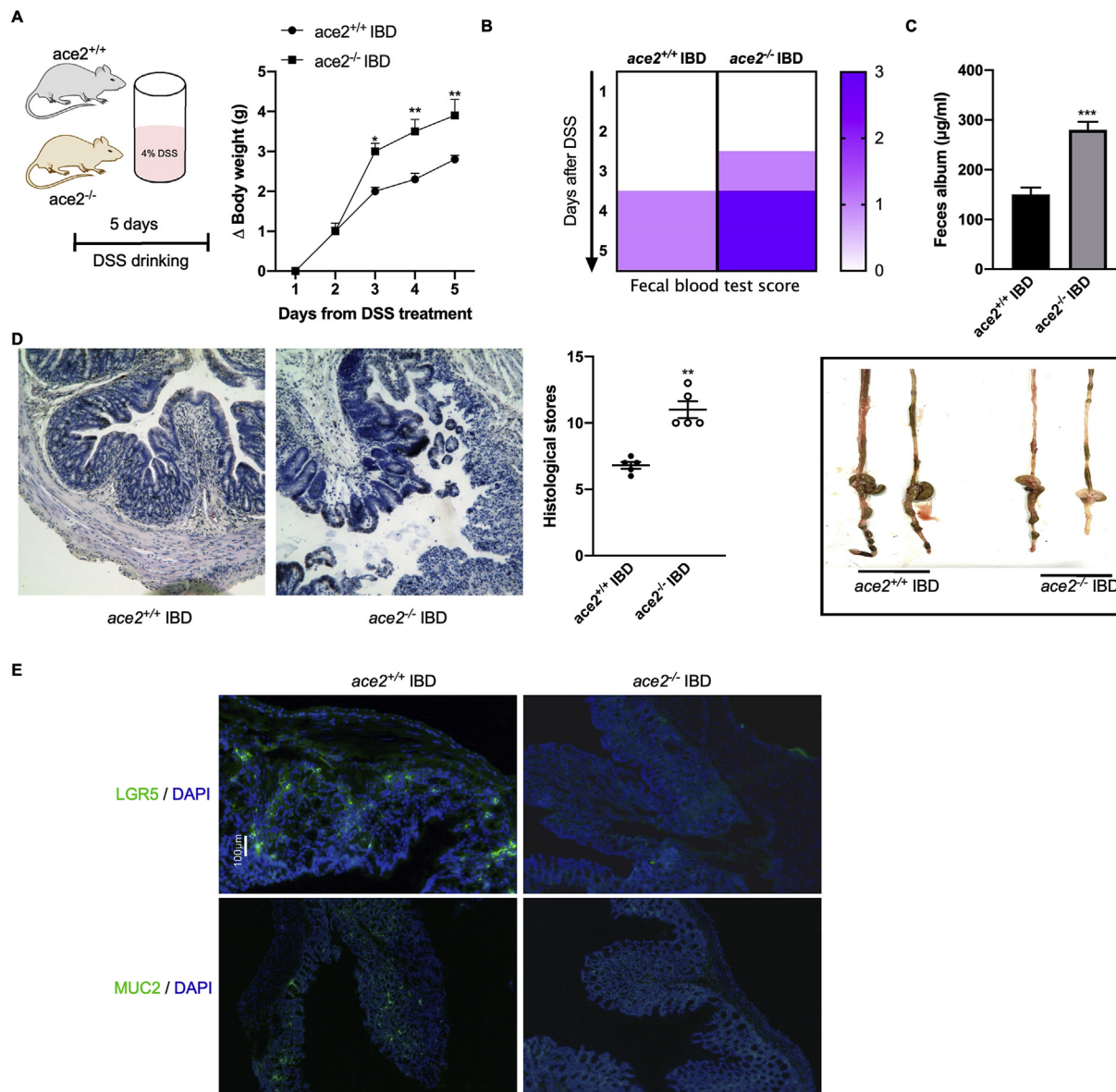
Fig. 3. Ace2 knockout mice are susceptible to IBD. A. Body weight of male *ace2*⁺/⁺ and *ace2*⁻/⁻ IBD mice at 6 weeks of age. B. Intestinal barrier functional assays measuring serum FITC-dextran of male *ace2*⁺/⁺ and *ace2*⁻/⁻ IBD mice. C. Intestinal barrier functional assays measuring albumin from fecal of male *ace2*⁺/⁺ and *ace2*⁻/⁻ IBD mice. D. Combined scores of the mucosal injury in *ace2*⁺/⁺ and *ace2*⁻/⁻ IBD mice. E. Immunofluorescent original pictures (*n* = 4) showing the expression of LGR5 and MUC2 in the colon isolated from *ace2*⁺/⁺ and *ace2*⁻/⁻ IBD mice.

ace2⁺/⁺ organoids, indicating a calcium overload caused by ace2 deficiency. Moreover, elevated ROS production was observed in *ace2*⁻/⁻ organoids.

4. Discussion

The coronavirus pandemic rages the world and causes various challenges for gastroenterology. Given that IBD patients may be particularly susceptible to 2019-nCoV, efforts are underway to investigate the role of ACE2 in the gut system. Accumulating evidence indicate that modulation of ACE2 expression influences the severity of colitis [18,19]. However, there has been a debate with respect to the role of ACE2 in the gut. Elevated ACE2 level is observed in the colon isolated from the IBD mouse model [20], and ACEI could markedly rescue the progression of DSS-induced

experimental colitis [21]. Nevertheless, Hashimoto et al. reported that *ace2* deficiency causes a high risk of colitis due to hampered immune cell trafficking as well as alterations of gut microbiota [19]. In full accordance with the findings above, our data showed that lack of ACE2 reduced the expressions of LGR5 and MUC2 in the colon, indicating a necessity of ACE2 in maintaining the stemness of ISCs.

The barrier function requires the complexity of epithelial, which relies on the differentiation of ISCs. ISCs renew the epithelial lining and hence counteract epithelial damage, restoring the barrier function in response to various insults such as infections [22]. Intestinal organoids, deemed ideal for investigating gastrointestinal pathology, incorporate a diverse array of the physiological features of the in vivo intestinal tissue, including a polarized epithelial layer surrounding a functional lumen and all the cell types of the
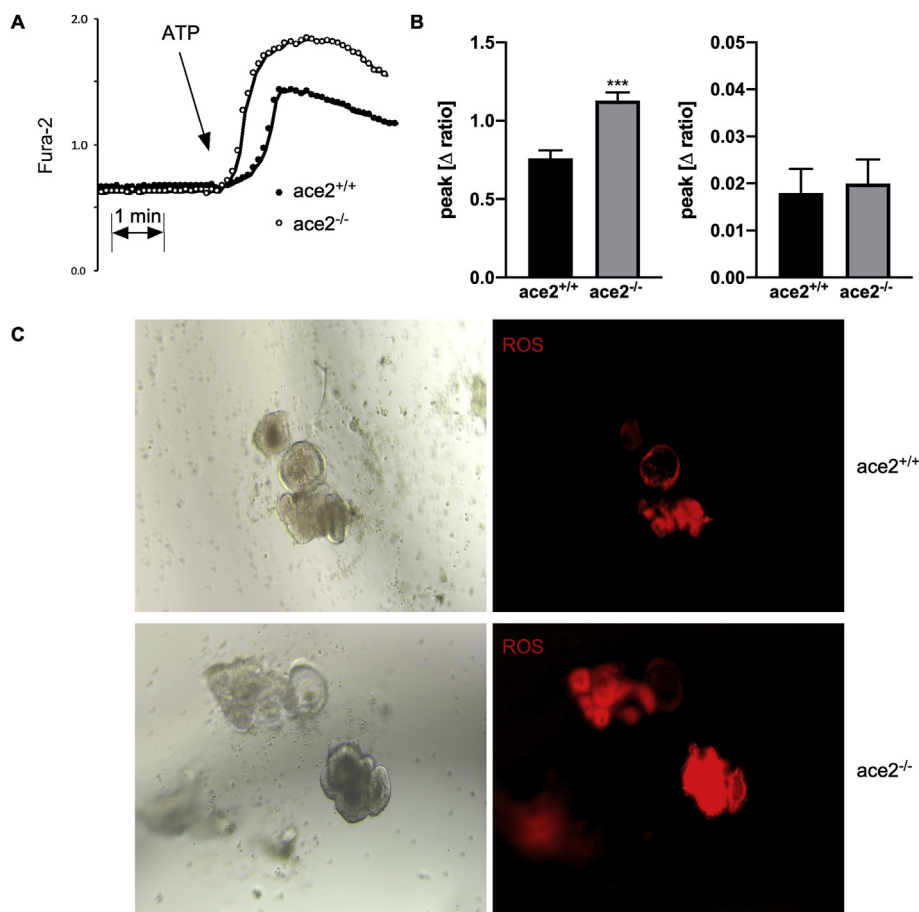
Fig. 4. Ace2 deficiency induces calcium overload in intestine organoidsA. Representative tracings showing the Fura-2 fluorescence ratio in the organoids isolated from ace2$^{+/+}$ and ace2$^{-/-}$ mice. Arithmetic means ($\pm$ SEM, $n = 5$) of the slope and the peak of the change in Fura-2 fluorescence following readdition of Ca^{2+} reflecting Ca^{2+} entry in the ace2$^{+/+}$ and ace2$^{-/-}$ organoids. B. Arithmetic means ($\pm$ SEM, n = 5) of the slope and the peak of the change in Fura-2 fluorescence following readdition of Ca^{2+} reflecting Ca^{2+} entry in the ace2$^{+/+}$ and ace2$^{-/-}$ organoids. C. Immunofluorescence image of ROS in the ace2$^{+/+}$ and ace2$^{-/-}$ organoids.

intestinal epithelium. We found that *ace2* deficiency decreased LGR5 and KI67 expressions and slowed the development of intestinal organoids, indicating the necessity of ACE2 in supporting epithelial turnover and ensuring the healing after insults. Additionally, it is well-documented that intracellular calcium concentration plays a vital role in the differentiation of ISCs [23]. Calcium overload results in mitochondrial dysfunctions and elevated reactive oxygen species (ROS) in mitochondria production, which dictates cell fate [24–27]. We herein observed a markedly higher intracellular calcium concentration and ROS level in *ace2*$^{-/-}$ intestinal organoids. We postulate that ACE2 may dictate the stemness of ISCs by orchestrating calcium perturbation.

Lamers et al. reported the high expression of ACE2 in enterocytes and the readily infected enterocytes by 2019-nCoV [28]. However, Lines of evidence suggest the increased risk for 2019-nCoV infection of pregnant and older patients with IBD [29,30], pointing out the putative hazard of ACEIs or ARBs administration. In conclusion, neither ACE2 downregulation nor ACEI is the ideal treatment for COVID-19 patients with gut diseases, and combination therapy of ACEI and calcium blocker merits further investigation.

Authors' contributions

Study concept and design: Jing Yan. Data acquisition: Wei Yu, Xianhong Ou. Data analysis and interpretation: Xiaofan Liu, Shuaixu Zhang, Xinxin Gao. Drafting of the manuscript; Jing Yan. Critical revision of the manuscript for important intellectual content: Jing Yan. Statistical analysis: Hongju Cheng, Baoliang Zhu. Obtained funding: Jing Yan, Wei Yu. Administrative, technical, or material support: Wei Yu; study Supervision: Jing Yan.

Ethics approval and consent to participate.

Not applicable.

Declaration of competing interest

All authors disclose that they have not any potential conflicts of interest.

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