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Original Research

Clostridium butyricum partially regulates the development of colitis-associated cancer through miR-200c

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Abstract: Colitis-associated cancer (CAC), one form of colorectal cancer (CRC),is an increasing concern worldwide. Both diagnosis and current therapy are challenging and bottlenecked. The aim of this study is to investigate novel mechanisms by which the therapeutic *C. butyricum* regulates colitis-induced oncogenesis. Mouse models of CAC were established with 2,4,6-Trinitrobenzenesulfonic acid (TNBS)and azoxymethane (AOM), following by biochemical, clinical and histological analysis. The integrity of epitheliumwas examined by electron microscopy (EM). The epithelial barrier function was evaluated with Ussing chamber. Real time PCR and fluorescent in situ hybridization (FISH) were performed to characterize the effect of *C. butyricum* on miR-200c; cell proliferation assays (MTT) were performed to study the role of*C. butyricum* on epithelial cell proliferation mediated by miR-200c inhibitor; finally, we quantified the proinflammatory cytokines TNF-α and interleukin (IL)-12 by real time PCR. *C. butyricum* ameliorates clinical, histological and biochemical manifestations in colitis-induced CAC models. Further mechanistic studies demonstrated that *C. Butyricum* could lengthen epithelial microvillus and increase TER by decreasing the transepithelial permeability. We also showed that *C. butyricum* facilitates the expression of miR-200c, by which increase the proliferation rate. Finally, we found that *C. butyricum* can regulate the production of proinflammatory cytokines TNF-α and IL-12 through miR-200c. *C. butyricum* may regulate epithelial barrier function through miR-200c, then to be involved in the process of inflammation-associated cancers.

Key words: Colitis-associated cancer; Inflammatory bowel disease; miR-200c; Barrier function; *C. butyricum.*

Introduction

Colitis-associated cancer (CAC), one form of colorectal cancer (CRC),is developed from intestinal inflammation like inflammatory bowel disease (IBD), including mainly ulcerative colitis (UC) and Crohn's disease (CD). The underlying mechanisms by which intestinal inflammation undergoes tumorigenesis are still elusive and may differ in other forms of CRC.Several inflammatory stimulators like dextran sulfate sodium (DSS) (1), oxazolone (2) or enterotoxic bacteria *Bacteroides fragilis* (3)or some inflammatory cytokines pathways, including of NF-κB, PI-3K and Akt pathways (4-6) can drive WNT/β-catenin nuclear accumulation even without any mutations in APC (7).CRCitself is one of the most common causes of cancer deathwith 1.2 million annual new cases and over 600,000 annual deaths worldwide (8, 9). Even though the fact that screening of early-stage CRC allows surgical removal of cancer precursor lesions and potentially reduces mortality of the disease, such as fecal occult-blood testing (FOBT) and colonoscopy, but with obvious limitation and approximately half of CRC patients subject metastases during thecourse of the disease (10, 11), whichrender the long-term survival and prognosis of patients remain quite poor (12). Epithelial-mesenchymal transition (EMT) is a critical step for initiatingtumor metastasis, which is a major cause of failure of cancer treatment (13). The processes of EMT and metastasis are highly regulated by multiple mechanisms, including TGF-β1/ZEB pathways and miR-200 family (14). In short, even promising advances have gradually come to understanding the pathogenesis of inflammation and CRC in recent years, their diagnosis and treatment in clinics are still quite challenging. In order to overcome this clinical challenge, there is a clear need to identify biomarkers that will facilitate the identification of patients with a poor prognosis, and permit personalized treatment for patients with high risk of CRC recurrence.

Numerous miRNAs exhibit abnormal expression in multiple types of inflammation and cancer, and are often associated with diagnosis, staging, progression, prognosis and response to clinical therapies (15-17). The miR-200 family includes five members, miR-200a, miR-200b, miR-200c, miR-429 and miR-141, and plays a critical regulatory role in processes which are associated with inflammation, metastasis and prognosis of malignant tumors: EMT and MET. Particularly, miR-200c is the most representative miRNAin miR-200 family and crucial in regulation of both EMT and MET processes (14) and inflammation (18), which is down regulated in inflammation like IBD (18) and a variety of human cancer typeslike CRC (19, 20). Multiple studies have demonstrated the prognostic value of miR-200c in

different cancers including CRC, for example, the link between overexpression of miR-200c and poor prognosis of CRC patients has been established (20). But, the mechanisms underlie the pathogenesis of miR-200c in IBD and oncogenic feature of miR-200c in CRC are still illusive.

Clostridium butyricum is a spore-forming, gram-positive and obligate anaerobic rod bacterium (21). *C. butyricum*, as a probiotic in humans and animals (22), can increase butyrate production in colon and improve the symptoms of inflammatory bowel diseases (IBD).Further, *C. butyricum* represses the proliferation of CRC cellsby rendering cell cycle arrest and promoting apoptosis, by thus inhibit the development of CRC (23). But, the alternative mechanisms by which CRC metastasis is attenuated by *C. butyricum* need to be explored.

In the present study, by using the experimental model of colon cancer induced by AOM and TNBS, we demonstrated that *C. butyricum* attenuates the colitis associated cancerous responses. Next, we found that *C. butyricum* regulates epithelial barrier function and proliferationthrough regulating mir-200c. Together, this study enables us to better understand the mechanism by which *C. butyricum* improves the outcome of patients suffering inflammation related cancer.

Materials and Methods

Cell culture

Human intestinal cell line Caco2-BBE was cultured according to protocol described previously (24).

C. butyricum

C. butyricum (Shenzhen Kexing Biotech; Shenzhen, China) was cultured in brain heart infusion (BHI) medium (Sigma-Aldrich, St. Louis, MO, USA) for 16 h at 37 °C before the experiments, then centrifuged at 3000 rpm for 5 min, 10^9bacteriawere re-suspended in 500 µl PBS (pH 7.4) and administered by gavage dailyto mice; 10^7 bacteria were re-suspended in 2ml of culture medium for Caco2-BBE cells.

Mice

C57BL/6 mice (8 wk, 18–22 g) were obtained from Chinese Academy of Science and were group housed under a controlled temperature and photoperiod and allowed free access to tap water and standard mouse chow. They were allowed to acclimate to these conditions for at least a week before inclusion in the experiments. All experiments with mice were approved by Zhejiang Academy of Agricultural Sciences, Hangzhou, China.

Induction and assessment of colitis and colitis associated colon cancer (CAC)

Colitis and CAC were induced in mice (n = 6 mice/group) by injection of AOM (10 mg/kg body weight) intraperitoneally and followed by administering 2.5 mg of TNBS (150 µL 50% EtOH) via rectal catheter as described previously (1, 25). The features of CAC were evaluated accordingly: direct visualization of the colon was performed using the coloview system (Karl Storz Veterinary Endoscopy, Goleta, CA). After mice were euthanized by CO_2 method, colon were removed and stored in 4% paraformaldehyde solution, sectioned and stained with haematoxyllin and eosin(H&E) as described previously (26). Myeloperoxidase (MPO) activity was measured as a solid marker for neutrophil infiltration into mucosa based on method described previously (1). To study the role of *C. butyricum* on healing phase of AOM-TNB Sinduced experimental CAC, with control mice (n=20) and C. butyricum treated mice (n=20), we analyzed the survival status of mice for another week after TNBS withdrawal.

Transmission electron microscopy (TEM)

The intestines from 6 mice per group were fixed and analyzed by transmission electron microscopy (TEM) based on protocol described previously (27). Briefly, pieces of ileum were fixed with 2.5% glutaraldehyde, incubated with 1% osmium tetroxide and finally dehydrated with acetone, followed by being embedded in epoxy resin and sectioned. The sections were then stained with uranyl acetate and lead citrate, finally examined under an H-600 Electron Microscope (JEM 1010, Hitachi, Japan) at 80 kV.

miRNAs, plasmid construction, transfection

*mir*Vana® miR-200c mimic (A25576), Anti-miR™ miR-200c Inhibitor (MH12741), miR negative vector (scrambled, AM17110), and anti-miR™ negative control (scrambled, AM17010) were obtained from Thermo Fisher (Carlsbad, CA, USA). Caco2-BBE were transfected with 40 nM of different miRNA construct using Lipofectamine 2000 (Life technology, Carlsbad, CA, USA).

miRNA expression analysis

Total RNA from Caco2-BBE cells and following cDNA synthesis were prepared as described previously (28). Real time PCRs were performed using iQ SYBR Green Supermix kit (Bio-Rad, Hercules, CA) with the iCycler sequence detection system (Bio-Rad, Hercules, CA) with the universal primer provided in the NCode miRNA first-strand cDNA synthesis kit was used together with following forward primer mir-200c for (5′-CGTCTTACCCAGCAGTGTTTGG-3′).Fold-induction was calculated using the Ct method: $\Delta\Delta Ct = (Ct_{\text{Target gene}}\text{-}Ct_{\text{housekeeping gene}})_{\text{group1}} - (Ct_{\text{Target gene}}\text{-}Ct_{\text{housekeeping gene}})_{\text{group2}}$, and the final data were derived from $2^{-\Delta\Delta Ct}$.18s acts as internal control: sense 5′ ACCACAGTCCATGCCATCAC 3′, antisense 5′ TCCACCACCCTGTTGCTGTA 3′.

Transepithelial resistance (TER) assay

TER, which can mirror epithelial barrier function and migration,was monitored in Caco2-BBE cellby Ussing chamber (Applied BioPhysics, NY, USA) as described previously (29). Since TER is dominated mainly by transepithelial permeability, we then investigated the contribution of permeability *in vitro* using a fluorescein isothiocyanate (FITC)-dextran (4-kDa, Sigma-Aldrich) method in confluent and polarized Caco2-BBE cells. Fluorescence intensity of each sample was measured (485Ex/520Em, Cytofluor 2300; Millipore, Waters Chromatography) and FITC-dextran concentrations were determined from standard curves generated by serial dilution of FITC-dextran.

Fluorescence in situ hybridization (FISH)

FISH was performed with LNA microRNA FISH optimization kit from Exiqon (miRCURY LNA detection; Exiqon, Vedbaek, Denmark) based on kit instruction. Briefly, Caco2-BBE cells grown on slides were fixed, permeabilized, and hybridized with FITC 5′ labeled locked–nuclei-acid incorporated (LNA) miRNAmiR-200c probe, following incubation with mouse anti-FITC antibody (Cell Signaling Technology, Danvers, MA, USA).

Cell proliferation assay

Cell proliferation assay was performed to investigate the contribution of cell proliferation to TER. $2x10^4$ Cells are cultured in flat-bottomed, 96-well tissue culture plates and incubatedwith3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT), following by adding detergent regent provided by commercial TACS MTT Assay Kit (R&D Systems, Minneapolis, MN, USA), the absorbance at 570 nm was read using a spectrophotometer. The data is analyzed by plotting cell number versus absorbance, finally normalized to data from non-treated cells.

Real time PCR analysis

Lipopolysaccharide (LPS), an integral component of the outer cell membranes of Gram-negative bacteria,is used to stimulate inflammatory response in cells(30) and animals (31). Here, the regulation of *C. butyricum* and miR-200c in the production of proinflammatory cytokines was examined in LPS treated Caco2-BBE cells. Real time PCR analysis for proinflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-12 were performed based on study described previously (1)

Statistical analysis

Values were expressed as means ± S.E.M Statistical analysis was performed using unpaired two-tailed Student's *t*-test by InStat v3.06 (GraphPad) software. $P<0.05$ was considered statistically significant.

Results

***C. butyricum* attenuates colitis and CAC induced by TNBS plus AOM by colonoscopy**

To study the regulation of *C. butyricum* on colitis induced CAC, we first established themouse models ofCAC byAOM plus TNBS methods. As shown in Figure 1A, control group showed enormous tumors all over the colon with different sizes, however, *C. butyricum*-treated group only showed mild and much less tumors both in terms of size and quantity. By colonoscopy, we found that there is no evident feature of macroscopic inflammation and carcinomain both control and *C. butyricum* treated mice, all colon fragments demonstrated semi-translucent mucosa characteristic of healthy colon (Figure 1B). However,TNBS induced a rapid and progressiveintestinal inflammation with bloody diarrhea in all the control mice, further, there present plenty of tumors with different sizes covered the whole colon (Figure 1B). *C. butyricum* treated mice exhibited relative mild inflammation, less number of tumors with small sizes, less prominent mucosal edema and less spontaneous bleeding in comparison with control mice.

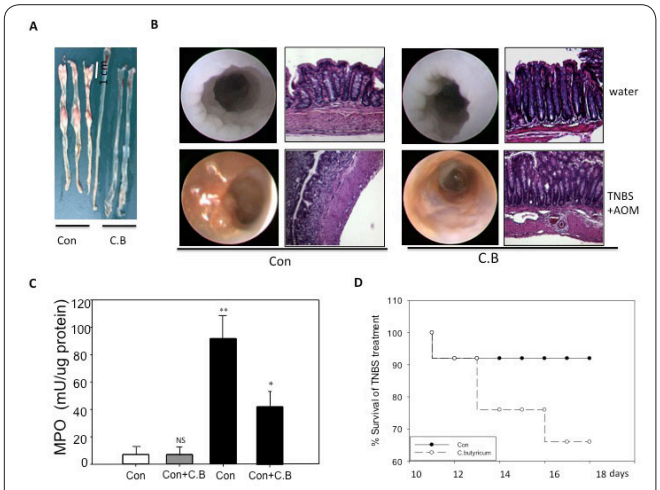

Figure 1. Probiotic *C. butyricum* regulates the process of colitis and colitis-associated cancer (CAC). Experimental colitis/CAC models were established using AOM/TNBS methods, colon was removed from euthanized mice for further analysis. **(A)** Compared to control group with plenty of different sized tumors, *C. butyricum* treated group showed mild and very less tumors spread all around colon. **(B)** Colonoscopic and histological view of CAC mouse: Representative photomicrographs of paraffin-embedded, hematoxylin-stained sections of the distal colon. Original magnification 10 ×; Intestinal inflammation and tumor were evaluated macroscopically *in vivo* using a murine miniature endoscope. Representative images of 6 different mice are shown. **(C)** Colon tissue was collected and subjected for MPO activity measurement. Data are expressed as means ± SEM (n = 9 mice per group). Statistical analysis was performed using an unpaired two-tailed Student's t test. **: $P<0.05$, **: $P<0.01$*. **(D)** After 10 days of 3 % TNBS, mice were given tap water and followed for mortality during recovery phase.

Histological analysis

To study the role of *C. butyricum* on the integrity of intestinal mucosa, we did H&E staining to mice colon samples.Figure 1B showed intact epithelium, a well-defined crypt length, no edema, no neutrophil infiltration into the mucosa or submucosa, and no ulcers or erosions in tap water treated mice. *C. butyricum* treated mice had longer crypt-villi axle, but thinner submucosa, lamina propia or muscle layer. Under the treatment of AOM plusTNBS, control mice display extensive inflammatory, dysplasia or carcinoma lesionsall overentire mucosa (Figure 1B, lower row) with numerous ulcers. Edema of submucosal and muscle layers are obvious. However, in *C.butyricum* group induced by TNBS, the mice colonshowed disperse ulceration and necrosis, and mild edema and less dysplasia, some but much less neutrophil infiltration. Further, mucosa still maintains the overall architecture. Colonoscopy showed that both control and *C. butyricum* mice showed no evidence of macroscopic tumor, displaying a semi-translucent mucosa characteristic of a healthy colon (Figure 1B). However, AOM plus TNBS treated control mice demonstreated a progressive severe, colonic inflammation and numerous tumor-like bodies with bloody diarrhea (Figure 1B). However, *C. butyricum* mice exhibited milder intestinal inflammation, and no tumor noticeable.

MPO activity

MPO activity is regard as an inflammatory indicator of tissue damage and infiltration inflammatory cells. No significant MPO changes were noticed between control

mice (6.473 ± 5.106mUnits/μg protein) and *C. butyricum* mice (6.6994 ± 4.819mUnits/μg protein) (Figure 1C). The values of MPO increased dramatically in TNBS-treated mice compared to control littermates, but *C. butyricum* mice showed significantly lower MPO values (42.5327 ± 18.065 mUnits/μg protein) than TNBS-treated control littermates (91.2167 ± 21.167 mUnits/μg protein), indicating less colon tissue damage and less neutrophil infiltration into lamina propria.

Survival assay

To study the protective effect of *C. butyricum* on severe inflammatory disorder and tumor, experimental mouse model of CAC induced by AOM plus TNBS were employed. Survival curves of *C. butyricum* mice with AOM/TNBS treatment showed 8% death in comparison with the 37% of death in control mice during this period, indicating a higher mortality of control mice during recovery and strong protective effect of *C. butyricum* in AOM/TNBS treated mice (Figure 1D).

Effect of *C. butyricum* on brush border of mice intestine

To study the mechanism by which *C. butyricum* protect mouse from AOM/TNBS treatment, we did the electron microscopy (EM) on mouse mucosa (Figure 2A). Examination of IEC ultrastructure by EM showed similar morphological characteristics in control and *C. butyricum* treated mice, except for the slightly increased size of microvilli at the apical membranes of epithelial cells in *C. butyricum* treated mice compared to control littermates.

***C. butyricum* increases transepithelial barrier function**

We then studied the effect of *C. butyricum* on intestinal barrier function. Caco2-BBE cells grew confluent on

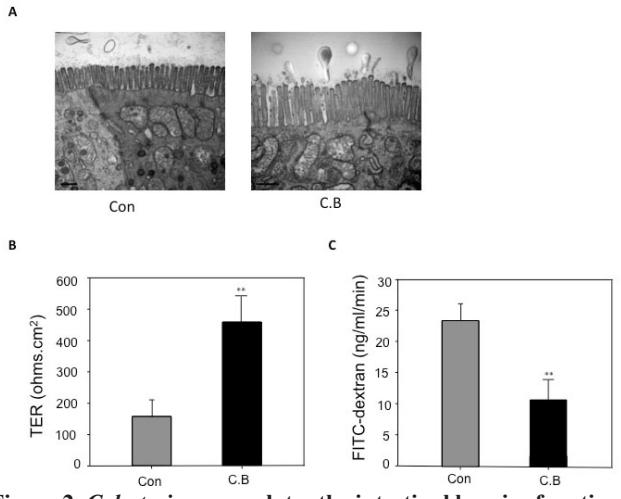

Figure 2. *C. butyricum* regulates the intestinal barrier function. (A) Electro microscope demonstrated that *C. butyricum* increases the length of microvillus of intestinal epithelial cells in comparison with those in control mice. (B) Transepithelial resistance (TER) assay with Ussing chamber in Caco2-BBE monolayer. **(C)** FITC-dextran (4 kDa) was added to the apical side of polarized monolayers of Caco2-BBE cells at 10 mg/ml, and the basolateral reservoir was sampled at 2 h after the addition of FITC-dextran to the apical side. Histograms show mean ± SEM of ng/ml/min FITC-dextran translocation to the basolateral reservoir.

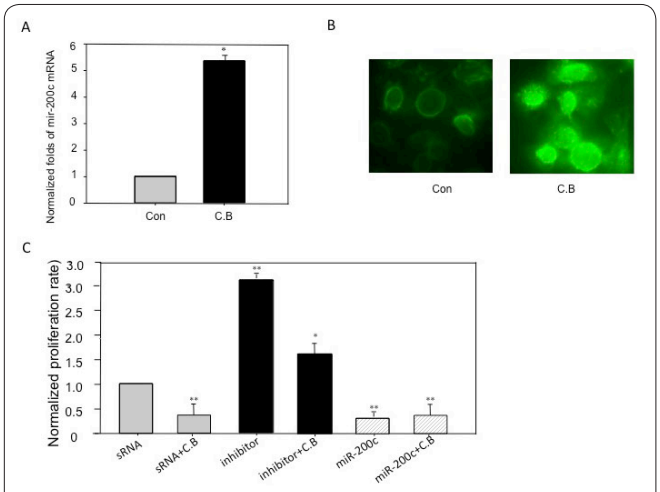

Figure 3. miR-200c mediates the regulation of *C. butyricum* on epithelial cell proliferation. (A) Real time PCR showed that *C. butyricum* increases the transcript level of miR-200c in comparison with non-treated group. **(B)** FISH analysis showed that *C. butyricum* significantly increases the transcript level of miR-200c. **(C)** *C. butyricum* regulates epithelial cell proliferation through miR-200c. Values represent means ± S.E. of three determinations. *p<0.05, **p<0.01, NS, not statistically significant *v.s* control.

snap well filters (Corning Costar, Corning, NY, USA), and were treated with $2x10^7$ CFU ml^{-1}*C. butyricum* for 2h; relative transepithelial resistance (TER) was measured with Ussing chambers (Physiologic Instruments, San Diego, CA, USA).As shown in Figure 2B, *C. butyricum* increased TER to 420.65 ± 94.5 ohms.cm^2 from 150.9 ± 71.1 ohms.cm^2 in non-treated cells. Since TER is dominant by the transepithelial permeability, to further investigate the contribution transepithelial permeability to TER, we examined transepithelial permeability using (Figure2C) FITC-dextran (molecular weight: 4-kDa) method. Non-treated cells showed a FITC-dextran flux (ng/ml/min) of 23.6 ± 3.96. In comparison, a∼2-fold decrease in FITC-dextran flux was observed in C. butyricum treated cells (11.8 ± 4.03).

***C. butyricum* increases the transcripts of mir-200c in Caco2-BBE cells**

To investigate the effect of *C.butyricum* on expression of mir-200c, we treated the Caco2-BBE cells with 2×10^7 of *C. butyricum*. We found that *C. butyricum* can stimulate the transcription of mir-200c significantly when compared to control cells (Figure 3A).

FISH assay confirmed this result that miR-200c is increased at transcript level after *C. butyricum* treatment, in comparison with non-treated Caco2-BBE cells (Figure 3B); further, miR-200c is mainly located at cytosolic pool, after induced by C. butyricum, miR-200c in both cytosolic pool and nucleus are positively regulated.

***C. butyricum* regulated the proliferation of Caco2-BBE cells through miR-200c**

In MTT assay, *C. butyricum* alone did not change the proliferation rate of Caco2-BBE cells significantly in comparison with non-treated cells.Over-expression of miR-200c dramatically inhibited Caco2-BBE cell-sproliferation, and*C. Butyricum* treatment of miR-200c-transfected cells could not restore its proliferation rate. miR-200c inhibitor accelerated the proliferation of Ca-

co2-BBE cells to a very high level, but, co-treatment of cells by inhibitor and *C. butyricum* slowed down significantly its proliferation, but not resumed to its original level (Figure 3C).

mir-200c mediates the regulation of *C. Butyricum* on transepithelial barrier function

To further study the effect of interaction between *C. butyricum* and miR-200c on intestinal barrier function, we performed the TER analysis. Single treatment by either *C. butyricumn* or miR-200cincreased TER significantly, further, co-treatment by both of *C. butyricumn* and miR-200c synergistically strengthen the transepithelial TER. On the contrary, miR-200c inhibitor compromised the TER when compared to scramble RNA treated cells. Further, *C. butyricumn* rescued the compromised TER to a significant high level (Figure 4A). In transepithelial permeability experiment by FITC-dextran method, we found that *C. butyricum* decreased the passages of FITC-dextran in comparison with negative control. Similarly, miR-200c decreased the concentration of FITC-dextran through Caco2-BBE cells; co-treatment with miR-200c and *C. butyricum* synergistically inhibited the concentration of dextran the most. Further, miR-200c inhibitor could accelerate the passages of FITC-dextran, but co-treatment by miR-200c and *C. butyricum* tightened monolayer of Caco2-BBE and resumed the FITC-dextran concentration to its original level (Figure 4B).

C. butyricum regulates the production of pro-inflammatory cytokines through mir-200c in LPS treated Caco2-BBE cells

Pro-inflammatory cytokines play central roles in pathogenesis of both IBD and CAC(1). Enhanced intestinal permeability and consequent immune cell infiltration is thought to stimulate the production of pro-inflammatory cytokines. We found that either miR-200c, its specific inhibitor, or *C. butyricum* did not change the expression of both TNF-**α** and IL-12, remaining at basal level. Under the treatment of LPS, for TNF-α (Figure 5A), LPS increased its production of TNF-αsignificantly in comparison with non-treated cells,*C. butyricum* decreased TNF-α expression in comparison with non-treated cells but still higher than non-LPS treated cells.Overexpression of miR-200c attenuated the increased expression of TNF-α by LPS, however, co-treatment of miR-200c and *C. butyricum* could not resume the TNF-α to its original level. Interestingly, miR-200c specific inhibitor treated Caco2-BBE cell secreted dramatically high level of TNF-α under the stimulation of LPS, and co-treatment of this inhibitor and *C. butyricum* rescued the high TNF-α to its lower level. Similar results were seen for IL-12 as shown in Figure 5B.

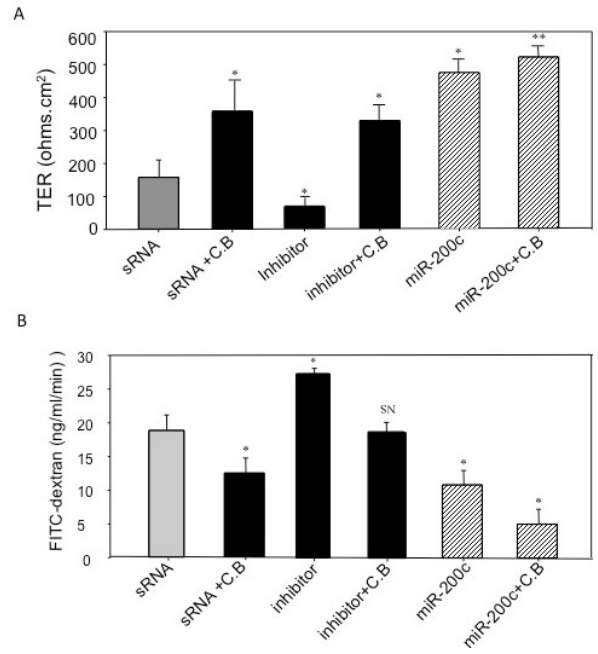

Figure 4. miR-200c mediates the regulation of *C. butyricum* on epithelial barrier function. (A) *C. butyricum* regulates transepitheial TER through miR-200c, ussing chamber was used to examine the TER in our treated groups; **(B)** miR-200c mediates the regulation of *C. butyricum* on transepithelial permeability by FITC-dextran method. Values represent means ± S.E. of three determinations. *p< 0.05, **p< 0.01; NS, not statistically significant versus control.

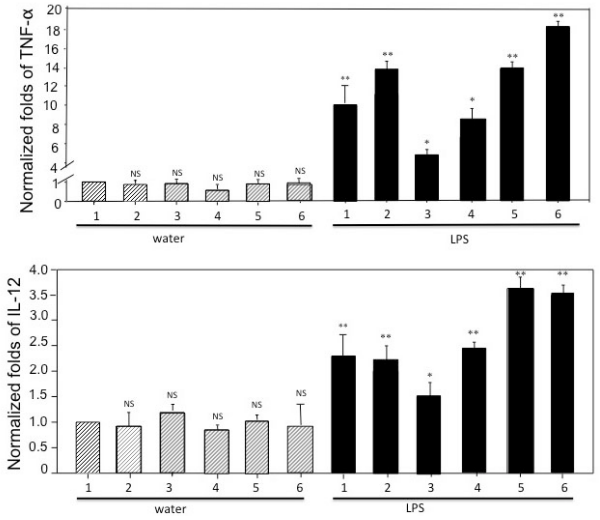

Figure 5. Balances between miR-200c and *C. butyricum* determined the production of proinflammatory cytokines TNF-a and IL-12. Caco2-BBE cells were transfected with different constructs of miR-200c, then subjected to inflammation stimulator LPS, real time PCR was used to quantify proinflammatory cytokines TNF-a (A) and IL-12 (B). Here, 1: scramble RNA (sRNA), 2: sRNA+C.B, 3: miR-200c, 4: miR-200c+C.B, 5: miR-200c inhibitor, 6: inhibitor+C.B. Values represent means ± S.E. of three determinations. * $p < 0.05$, **$p < 0.01$; NS, not statistically significant versus control.

Discussion

In current study, by *in vivo* mouse model and *in vitro* cell model, we established the concept that miR-200c plays important role in mediating the protective effect of *C. butyricum* against colitis-associated cancer, which shed some new insight of *C. butyricum's* application in attenuating inflammation related disorders.

In the mouse model of CAC induced by AOM/TNBS, we noticed that, by following histologic studies, there are still lots of features of colitis, such as tremendous neutrophil filtration into lamina propria, necrosis and edema, or the bloody colon lumenby colonoscopy. Nevertheless, dysplasia and carcinoma are the predominant phenomena in AOM/TNBS induced cancer model.

To date, the treatments of intestinal inflammation and CRC are limited of drug effectiveness,resistance and or-

gan toxicities (32), or surgery timeliness,such that, other alternative therapeutic options are urgent. *C. butyricum,* thriving even at low pH and high temperature, is one probiotic and gram-positive anaerobe present in intestines of healthy animals and humans, rendering it potential applications against various conditions, such as inflammatory disorders and tumorigenesis (22), for example, treating patients with specific immunotherapy (SIT) and probiotic *C. butyricum* together significantly improved the clinical symptoms of UC (33). Basically, *C. butyricum* produces high levels of butyrate and acetate in colon, which are part of energy sources (34); further, *C. butyricum* decrease the intestinal permeability and strengthens TER, by reinforcing various components of the colonic barrier such as balancing intestinal microflora, re-shaping immune response, promoting epithelial migration, proliferation (as shown in our research), inducing production of mucins, intestinal trefoil factor, transglutaminase activity, antimicrobial peptides, heat shock proteins, Toll like receptors (35, 36),tight junction proteins (37, 38), adherent junction proteins (39) and other epithelial cell structural proteins (40).*C. butyricum* can also interact with miRNAs to exert different bio-functions as mentioned in our study. In addition, *C. butyricum* inhibits *E.coli* viability and causes E.coli-induced apoptosis, by which prevents *E.coli*-induced intestinal disorders through (41). Further, *C. butyricum* promotes the growth of *Lactobacillus* and *Bifidobacterium* and inhibits antibiotic-associated diarrhea (42).

Similar to our study in which *C. butyricum* facilitates the expression of proinflammatory cytokines TNF-α and IL-12, some other studies also found that *C. butyricum* could regulate inflammatory disorder by regulating the balance between proinflammatory cytokines TNF-α, IFN-γ, IL-1βand anti-inflammatory cytokines IL-10 (35, 43-45). Besides, *C. butyricum* produces butyrate and acetate in colon as energy sources and has been used as a probiotic in humans and animals (22, 33).

As a known tumor suppressor, miR-200cis involved in prognosis and oncogenesis of almost all kinds of cancer types, including bladder cancer (46), breast cancer (47), CRC(48), endometrial cancer (49), esophageal cancer (50), gastric cancer (51), head and neck cancer (52), liver cancer (53), lung cancer (54), renal cancer (55) and many others. The proposed mechanismsare variable, for example,miR-200c targets at transcription factors ZEB (39), p53 (56) to regulate EMT; miR-200c can modify metastasis by targeting HMGB1(57), VEGFR (58), ZNF217 (59); miR-200c optimizes the sensitivity to many anti-cancer drugs like doxorubicin, paclitaxel, trastuzumab, cetuximabor cisplatin etc through molecules like TGF-β, RhoE, TrkB and other molecules (60-62); miR-200c also targets some stem cell markersBMI1,NCAM1, CD133 (62-64).

The interaction of endothelial/epithelial barrier function and cancer metastasis was intensively studied lately, basically,the interaction and penetration of epithelial/endothelial cells by metastasizing tumor cells is a checkpoint for metastasis (65), increase of barrier function reduced the penetration of tumor cells through mesothelial cells (66). The epithelium in CRC is more leaky than those in healthy controls (66).Study demonstrated that hepatocyte growth factor (HGF) decreased TER and increased paracellular permeability in human vascular endothelial cells, by which stimulating invasion of breast cancer cells (67, 68).

The current study, for the first time, established the linkage betweenprobiotic *C. butyricum* and tumor suppressor miR-200c, laying the foundation for the clinic use of *C. butyricum* or miR-200c on the inflammatory disorders or cancers.

Acknowledgments

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