Fecal microbiota transplantation for recurrent *Clostridioides difficile* infection associates with functional alterations in circulating microRNAs

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Author contributions

T.M.M., C.P. and D.K. designed the study, analyzed the data and wrote the paper. AM.S., N.O.M, TO.Y., M.H., N.C., E.B., O.P. and C.P. performed the experiments. T.J. and TO.Y. performed statistical analyses. D.K., T.L., B.R., CL., and P.K. developed the clinical sample cohorts. D.B.L. with N.O.M. provided access to human colonoids and purified whole C. difficile toxins. AM.S., N.O.M, M.H., D.B.L., CL. T.L., T.J., and P.K. provided intellectual input. All authors reviewed the manuscript and approved the manuscript in its final form.

Competing Interests statement

T.M.M. is a consultant advisor for Takeda. The remaining authors declare no competing interest.

Abstract

Background and aims

The molecular mechanisms underlying successful fecal microbiota transplantation (FMT) for recurrent *Clostridioides difficile* infection (rCDI) remain poorly understood. The primary objective of this study was to characterize alterations in microRNAs (miRs) following FMT for rCDI.

Methods

Sera from two prospective multicentre randomized controlled trials were analyzed for miRNA levels using the Nanostring nCounter platform and quantitative RT-PCR. Additionally, rCDI-FMT and toxin-treated animals and *ex vivo* human colonoids were employed to compare intestinal tissue and circulating miRNAs. miRNA inflammatory gene targets in colonic epithelial and peripheral blood mononuclear cells were evaluated by qPCR and 3'UTR reporter assays. Colonic epithelial cells were employed for mechanistic, cytoskeleton, cell growth and apoptosis studies.

Results

miRNA profiling revealed upregulation of 64 circulating miRNAs 4- and 12-weeks following FMT compared to screening, of which the top 6 were validated in the discovery cohort by RT-qPCR. In a murine model of relapsing-CDI, RT-qPCR analyses of sera and cecal RNA extracts demonstrated suppression of these miRNAs, an effect reversed by FMT. In mouse colon and human colonoids, TcdB mediated the suppressive effects of CDI on miRNAs. CDI dysregulated Drosha, an effect reversed by FMT. Correlation analyses, qPCR and 3'UTR reporter assays revealed that miR-23a, miR-150, miR-26b, miR-28 target directly the 3'UTR of IL12B, IL18, FGF21 and TNFRSF9, respectively. miR-23a and miR-150 demonstrated cytoprotective effects against TcdB.

Conclusion

These results provide novel and provocative evidence that modulation of the gut microbiome via FMT induces alterations in circulating and intestinal tissue miRNAs. These

findings contribute to a greater understanding of the molecular mechanisms underlying FMT and identify new potential targets for therapeutic intervention in rCDI.

Keywords

Fecal transplantation, *C. difficile*, microRNA, Drosha

WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Fecal microbiota transplantation (FMT) is highly effective at preventing recurrent *Clostridioides difficile* infection (rCDI). However, the mechanisms of action remain largely unknown. MicroRNAs (miRNAs), short non-coding RNA sequences which bind to complementary sequences of mRNA and can regulate gene expression, may be a potential mechanism by which commensal microbiota communicate with the human host.

NEW FINDINGS

We identified several significant alterations in circulating miRNAs following successful FMT treatment in 2 independent rCDI patient cohorts. miRNA signatures were validated in animal models and human colonoids. We further demonstrate that FMT-regulated miRNAs regulate cell properties and target IL-12B, IL-18, FGF21 and TNFRSF9, integral in pathways linking to inflammation, autoimmunity and cancer.

LIMITATIONS

Deeper characterization of the epitranscriptome in FMT is required.

IMPACT

These results describe a new mechanism of action of FMT against rCDI and provide potential new therapeutic targets for conditions associated with intestinal dysbiosis.

Graphical abstract

(uploaded separately)

Introduction

Fecal microbiota transplantation (FMT) is a well-established treatment for recurrent *Clostridioides difficile* infection (rCDI). Accumulating evidence also supports FMT as a potential treatment for other disorders associated with intestinal dysbiosis, including inflammatory bowel diseases, cancer, metabolic syndrome, and neuropsychiatric disorders.[1]

Despite the effectiveness of FMT in rCDI, its mechanisms of action remain poorly explored. Current evidence suggests the success of FMT may be attributed in part to the reconstitution of intestinal microbiota, restoration of secondary bile acid metabolism, and modulation of immune-mediated inflammatory responses.[2-3] We have previously reported effective FMT for rCDI is associated with activation of the bile acid-farnesoid X receptor (FXR)-fibroblast growth factor (FGF) pathway and decreased serum C-X-C motif chemokine 11 (CXCL11), interleukin-18 (IL-18), tumor necrosis factor-related activation-induced cytokine (TRANCE), IL-12B, CXCL6, and tumor necrosis factor receptor superfamily member 9 (TNFRSF9). [4] The gut microbiota can modify host cell responses to stimuli (e.g., metabolites) through alterations in the host epigenome and, ultimately, gene expression.[5] MicroRNAs (miRNA) are thought to be one way in which the gut microbiota communicates with the human host. These short non-coding RNA molecules (containing about 22 nucleotides) are expressed as individual genes or as parts of longer transcripts and are processed by machinery involving Drosha and Dicer nucleases, which generates the mature miRNAs. Mature miRNAs are loaded on argonaute (Ago)-containing complexes and bind to complementary sequences in the 3-untranslated region (3UTR) of messenger RNAs (mRNAs), resulting in transcript degradation and translational suppression of target genes. [6] Bacterial pathogens clearly alter host microRNA (miRNA) expression,[7] but less is known regarding the effect of commensal bacteria on the host microRNAome. A recent study has demonstrated how host fecal miRNAs, normal components of feces, can enter certain bacteria (e.g., *F. nucleatum* and *E. coli*) and regulate bacterial gene transcription

and growth.[8] However, the alterations in circulating miRNAs of rCDI patients undergoing FMT and the functional effects they may exert on downstream targets remain unknown.

Herein, we characterize the impact of FMT on circulating miRNA signatures to better understand immunological mechanisms relevant to FMT in the treatment of rCDI. Our findings suggest a conserved mechanism involved in regulating host miRNAs by *Clostridioides difficile* and identify new miRNA inflammatory targets in response to FMT.

Materials and Methods

Participants clinical data, sample collection and storage

Randomly selected rCDI subjects participating in 2 clinical trials (capsule vs colonoscopy delivered FMT [9] and fresh vs frozen enema-delivered FMT[10]) comprised the discovery and replication cohorts, respectively. Blood samples were collected between October 2014 and December 2016 (discovery cohort) and July 2012 and September 2014 (replication cohort) and stored at -80°C. Only sera with sufficient volume were selected for miRNA analysis. Healthy controls (n=42; mean [SD] age, 53.3 [20.7]; 30 women [71.4%] were defined as asymptomatic adults undergoing screening colonoscopy recruited from Edmonton, Canada. Clinical and demographic information were collected from medical records. Participant baseline characteristics are shown in Table 1. All participants provided written informed consent under the approvals granted by the research ethics boards of the University of Alberta (Pro 1994 and 49006), St. Joseph's Healthcare (#11-3622), and Hamilton Health Sciences (#12-505) in Canada.

Mouse model of rCDI

A mouse model of rCDI was used to assess miRNA, mRNA and protein levels following infection and treatment with FMT, as described previously.[11] Animal work was approved by the Clemson University Institutional Animal Care and Use Committee (IACUC). Additional details are available in the Supplementary methods. Briefly, 6-8 week old

C57BL/6 mice were given 0.5 mg/mL cefoperazone (MP Biochemicals, cat# 199695) in sterile drinking water (Gibco Laboratories, cat# 15230) ad libitum for five days (-7 days prior infection, n = 42). Two days after cessation of antibiotics, mice received 10^3 *C. difficile* strain 630 spores resuspended in 1mL sterile water, prepped as described previously (day 0, n = 36).[12] A subset of mice was euthanized 4 days post infection (dpi) to assess miRNA during acute infection (n = 9). The remainder of the mice received 0.4 mg/mL vancomycin (Sigma, cat# V2002) on 4 dpi for 5 days (4-9 dpi) ad libitum in sterile drinking water (n = 27). On 11 dpi, FMT prepped from untreated (n = 8), healthy age-matched mice (mFMT) was administered via oral gavage to a group of mice. Each mouse received 100 µL of FMT material diluted in phosphate-buffered saline (PBS); (~0.2 grams fresh fecal material in 1.5 mL pre-reduced PBS, homogenized via mixing and gravity filtering). One group of mice received all antibiotics and mFMT but no *C. difficile* inoculum (handling and experimental control, n = 6). The remainder of infected mice (n = 12) did not receive FMT (noFMT). Fecal sampling was conducted throughout the experiment, and endpoint cecal sampling, to assess *C. difficile* load, was determined by plating 20µL of content from individual samples in 1:10 PBS solution and serially diluted on taurocholate cycloserine cefoxitin fructose agar (TCCFA) under anaerobic conditions (Coy Laboratory Products, Grass Lake, MI, USA). The CFU/mL content was determined after overnight incubation at 37 °C. Mice were euthanized on 21 dpi and cecal contents, tissue and serum were flash frozen in liquid nitrogen and kept at -80°C for downstream analyses.

Mouse toxin challenge model

The animal protocol was approved by the Vanderbilt University Medical Center IACUC. 6-8 week old C57BL/6 mice (Jackson Labs) were observed upon arrival to ensure normal health. Mice were separated into three groups (n = 6 per group) to receive intrarectal instillations of either purified recombinant whole TcdB, TcdA and TcdB, or Hank's Balanced Salt Solution (HBSS) vehicle control, as described elsewhere.[13] Further details on the purification of the toxins are described in the Supplementary methods. Toxins were derived from the VPI 10463 *C. difficile* reference strain and prepared as 15 µg in a total volume of

100 µL per instillation. Mice were anesthetized with isofluorane and confirmed to be sedated by toe pinch. One mL of HBSS was instilled intrarectally to evacuate stools with a flexible plastic gavage applicator (20G x 30MM, gavageneedle.com). Instillation was performed over 30 sec while lightly pinching closed the anus, which was held for an additional 30 sec as previously described.[13] Mice were returned to cages to recover. After two-to-five hours, mice were humanely euthanized by CO_2 inhalation and cervical dislocation. Whole blood was extracted via cardiac puncture and allowed to clot in an RNAse-free microcentrifuge tubes for 15 min at room temperature prior to centrifugation for 15 min at 1500 × g at 4°C. Serum was transferred to a fresh tube and flash frozen in liquid nitrogen. The colon was isolated and dissected from surrounding visceral tissue. The whole colon was washed in chilled, sterile 1x PBS before portions of the middle and distal colon were combined and flash frozen for protein and miRNA analysis.

Treatment of human colonoids with *C. difficile* toxins

Deidentified human colon tissue was obtained through the Cooperative Human Tissue Network. On the day of colon resection surgery, normal marginal colon mucosa was resected and placed into Dulbecco's Modified Eagle Medium (DMEM) at 4°C. Within hours, the tissue was prepared into normal human colonoids with Intesticult Organoid Growth Medium (Stem Cell Technologies) using the manufacturer's protocol (https://cdn.stemcell.com/media/files/pis/DX21423-PIS_1_0_0.pdf). Colonoids were suspended in Matrigel matrix (Corning) with standard growth factor concentration for maintenance and passage. Matrigel with reduced growth factors was used for suspending colonoids in the final passage for the experiment. Eight hours prior to toxin exposure, organoids were serum-starved with DMEM and no growth serum. Colonoids were exposed to TcdA (10 pM), TcdB (10 pM), or DMEM vehicle negative control for either 30 min or 6 hours. Colonoid-containing Matrigel was washed once with 1x PBS and flash frozen in liquid nitrogen for protein analysis. For miRNA analysis, colonoids were removed from Matrigel using Gentle Cell Dissociation Reagent (Stem Cell Technologies) and resuspended in

RNAlater (Invitrogen). Colonoids in RNAlater were stored at 4°C overnight before freezing at -80°C.

Serum miRNA isolation and high-content analysis

Human serum was isolated by centrifugation (2200 g for 10 minutes at room temperature) from whole blood and snap-frozen. RNA was isolated from human and animal serum samples (200 µL) using the miRNeasy Serum/Plasma Kit (Qiagen) according to the manufacturer's instructions. Eluted RNA from serum samples was further purified and concentrated by using Amicon Ultra YM-3 columns (3000 kDa MWCO; Millipore).

For high-content miRNA analysis, RNAs following hybridization reactions were processed using the nCounter Prep Station and nCounter Digital Analyser. miRNA levels (n = 800) were analysed using the nSolver software, v3.0 (Nanostring Technologies Inc, Seattle, WA, USA). Normalisation was performed using all miRNAs (n = 110) with coefficient of variation less than 70%.[14]

Additional information is available in the Supplementary Materials and Methods section.

Statistical analysis

All statistical analyses were performed in SPSS v.24 and R 3.5.1. Descriptive statistics for participant characteristics at baseline were reported using mean and standard deviation and percentages. All data are expressed as mean and s.e.m. Systematic within-week changes for each miRNA were examined using a non-parametric longitudinal method (nparLD in R package) followed by Wilcoxon signed-rank test for pairwise comparisons. The association between the metavariables and miRNAs was assessed using Spearman correlation. Heatmaps for capsule and colonoscopy combined and separately for capsule and colonoscopy delivered FMT were generated in R package ComplexHeatmap2.[15] Normality was checked using the Kolmogorov-Smirnov test. Graph generation, fold changes and statistical significance in levels of circulating miRNAs were assessed by qPCR using OriginPro and Wilcoxon matched-pairs signed rank test. Significance differences were considered when *$P < 0.05$. ** $P < 0.01$, *** $P < 0.001$.

Results

FMT modifies circulating miRNAs in rCDI patients

For miRNA profiling, we first analysed serum samples derived from the discovery cohort.[9] Table 1 describes patient baseline characteristics. Sera obtained at screening, 4- and 12-weeks post-FMT from 42 participants who achieved a clinical cure following FMT, were subjected to miRNA analysis using the Nanostring nCounter platform. miRNA profiling of 126 samples revealed the significant upregulation of 64 circulating miRNAs 4- and 12-weeks following FMT, compared to screening (Fig. 1A). The miRNAs with the highest levels detected are depicted in Fig. 1B. Comparable changes in miRNA levels were detected in recipients of either by capsule or colonoscopic FMT (Supplementary Fig. 1). Pathway enrichment analysis identified overlaps between the top three miRNA-regulated pathways, B-cell lymphoma 2 (Bcl-2), Runt-related transcription factor 2 [(RUNX2; linked to NF-kappa-B inhibitor beta (NFKBIB)] and phosphoinositide 3-kinase (PI3K reg class 1A p55-gamma), linking inflammatory signalling to immune cell survival and differentiation (Fig. 1C). Pathway analysis also uncovered commonalities with other diseases (e.g., inflammatory bowel disease and multiple sclerosis) and cell functions such as apoptosis (Supplementary Figures 2 and 3).

We next sought to validate our discovery cohort results. For the replication cohort, we assessed 24 patients at 3 time points: screening, at two and four weeks following FMT.[10] Table 1 describes patient baseline characteristics. The top 6 upregulated miRNAs from the discovery cohort analysis (Fig. 1B) were selected for RT-qPCR validation in our replication cohort. Individual miRNAs were found to be upregulated in 78-94% of samples analysed, with the average changes ranging between 3-and-12 fold (Fig. 1D).

FMT modifies intestinal tissue miRNAs in a mouse model of rCDI

The concerted increase of circulating miRNAs by FMT suggests that *C. difficile*-associated dysbiosis may regulate miRNAs through a conserved mechanism. We tested this hypothesis by employing a mouse model of rCDI.[8] Animals pre-treated with cefoperazone

received 10^3 *C. difficile* spores, and 4 days post-infection (dpi) were exposed to vancomycin. At 11 dpi, a group of mice received fresh FMT derived from healthy mice. Sera collected 21 dpi were subjected to RT-qPCR. Parallel to what we found in rCDI patients, the same top 5 miRNAs (miR-4454 has not been characterised in mice) and in addition two potentially functional miRNAs were also upregulated after FMT. *C. difficile* recurrence (Supplementary Figure 4) resulted in downregulation of the tested circulating miRNAs in mice (Fig. 2A) concomitant with a decrease in animal body weight (Supplementary Figure 5). To examine the time-dependence of miRNA regulation by CDI, we assessed miRNA levels during the acute phase of infection (4 dpi). RT-qPCR analysis revealed that the suppression of circulating host miRNAs may be an early sign of CDI (Supplementary Figure 6). Importantly, the inhibitory effects of rCDI on miRNA levels was reversed 10 days post-FMT (Fig. 2A).

We next tested whether the circulating miRNA changes reflect the effects of rCDI on colonic tissue. RNA extracts from the ceca were analysed by RT-qPCR. Our results showed that tissue miRNA levels altered similarly to circulating miRNAs (Fig. 2B). Comparisons of the changes of individual microRNA levels in matched tissue and serum samples, from all animals, showed significant and positive correlation between circulating and ceca-expressed miRNAs (Supplementary Figure 7). This supports the notion that alterations in circulating miRNAs may be originating from colonic tissues. Furthermore, we found even stronger positive correlation between matched colonic and circulating miRNAs derived from an 84-year-old male patient with fulminant CDI treated with FMT by colonoscopy (Supplementary Figure 8). In mice, tissue miRNA levels downregulated by rCDI reached statistical significance in the early phase of infection (Supplementary Figure 9), while FMT upregulated the tissue miRNAs, coinciding with the reduction of *C. difficile* load (Supplementary Figure 4) and the recovery of animal body weight (Supplementary Figure 5).

Toxin B suppresses miRNAs in the intestinal mucosa

C. difficile pathogenicity is primarily mediated by exotoxins that induce cell death. We investigated the effects of purified toxin A (TcdA) and toxin B (TcdB) on host miRNA regulation. Mice were treated by intrarectal instillation with a combination of TcdB and TcdA, TcdB alone (15μg), or vehicle (HBSS), and colonic tissues and sera were collected 2-5 hours post instillation. RT-qPCR analysis of serum RNA extracts showed the toxins had no effect on circulating miRNAs (Supplementary Figure 10). However, the expression of the same miRNAs was suppressed in colonic tissues of *C. difficile* infected mice. Notably, TcdB suppressed miRNA levels, an effect enhanced by its combination with TcdA (Fig. 3A). The discrepancy between circulating and tissue miRNAs in this model suggests the impact of the toxins is luminally confined and may not lead to changes in circulating miRNA levels due to local and time-restricted exposure of animals to toxins in early time points (2-5 hours).

The above findings suggest the effect of CDI on miRNA regulation is mediated by the direct effect of TcdA and/or TcdB on colonic epithelial cells and the observed alterations in circulating miRNAs in FMT-treated patients may result from changes at the tissue level. To test this hypothesis, we exposed normal human colon organoids (colonoids) to TcdA or TcdB and analysed miRNA expression. Colonoids from freshly isolated human colon mucosa were treated with TcdA (10 pM), TcdB (10 pM) or vehicle (DMEM), and cell extracts were obtained 30 min and 6 hours later. RT-qPCR analysis showed that 30 min after exposure to TcdB, all miRNAs studied were reduced (Fig. 3B), an effect sustained for 6 hours (Fig. 3C). These results show that miRNA suppression in CDI is attributed to the activity of TcdB and further support the hypothesis that FMT-mediated upregulation of circulating miRNAs is driven, at least partly, at the epithelial level.

FMT counteracts *C. difficile* effects on miRNA biogenesis

The evidence collectively show that the miRNAs studied adhere to a common mode of regulation, albeit they are encoded by different genes and controlled by different

mechanisms at the level of transcription. In fact, the miRNAs studied are located in different chromosomes, are intragenic (exonic or intronic) or intergenic, and their expression is under the control of distinct regulatory elements. Hence, their regulation may be a result of a universal mechanism. Following transcription, miRNA maturation is a process shared by most miRNAs, and their concerted upregulation by FMT suggests that miRNA processing may be affected by *C. difficile*-associated dysbiosis. Therefore, we analysed the effects of *C. difficile* and FMT on the expression of enzymes playing a central role in miRNA biogenesis (Drosha, Dicer1, Ago2). RT-qPCR analysis of cecal RNA extracts from rCDI mice suggested that CDI suppresses Drosha expression by 50% (Fig. 4A, left panel), with minor changes on Dicer1 and Ago2 mRNA levels (Supplementary Figure 11). A more pronounced effect (> 80% decrease) on Drosha expression was evidenced at the protein level (Fig. 4A, right panel). Importantly, the effects on both Drosha mRNA and protein were reversed by FMT (Fig. 4A). Similarly, in colonic tissues of mice treated with exotoxins, Drosha mRNA levels dropped by 40% (Fig. 4B). In human colonoids, TcdB showed small but significant effects on Drosha mRNA (Fig. 4C, left panel), while western blot analysis revealed a robust decrease (>60%) in protein levels (Fig. 4C, right panel).

To investigate the role of Drosha suppression in mediating the CDI effects on the regulation of miRNA levels we knocked down Drosha in colonic epithelial cells (NCM356) by means of siRNA. We verified that the knockdown of Drosha mimics the effects of TcdB on Drosha protein levels in these cells (Fig. 4D). RNA extracts from these cells were subjected to RT-qPCR for the levels of the primary transcripts (pri-miRNAs) and the mature forms of three different miRNAs, known to be transcriptionally regulated by distinct mechanisms.[16-18] The results showed that upon Drosha inhibition the levels of the pri-miRNAs (Fig. 4E, upper panel) are increased with the concurrent and significant decrease in mature miRNA levels (Supplementary Figure 12). We then measured the levels of these primary transcripts in colonoids treated with TcdB. In the same line, we found that though the mature miRNAs are suppressed by TcdB (Fig. 3), the levels of the respective pri-miRNAs are increased (Fig. 4E, lower panel).

Combined, these data suggest that Drosha expression is decreased in response to CDI, a phenomenon regulated at both the transcriptional and post-transcriptional level. Furthermore, they attribute the concerted changes in miRNA levels to the dysregulation of miRNA biogenesis machinery (proposed model, Fig. 4E) by rCDI and its recovery by FMT treatment.

FMT-regulated miRNAs possess functional properties

We next investigated the downstream effects of FMT-regulated circulating miRNAs in our rCDI patient cohorts compared to healthy controls. We assessed miRNAs predicted to target specific chemokines and cytokines, which we previously found to be downregulated by FMT.[4] Based on TargetScan prediction analyses we found that the levels of 6 miRNAs in FMT-treated rCDI patients inversely correlate with the serum levels of FGF21, IL-12B, IL-18 and TNFRSF9 proteins (Supplementary Figure 13). miR-23a, miR-26b and miR-130a are predicted to target the 3'UTR of FGF21 mRNA, miR-23a is predicted to target the 3'UTR of IL-12B, miR-150 the 3'UTR of IL-18, while miR-20a and miR-28 are the 3'UTR of TNFRSF9. Overexpression of these miRNAs in colonic epithelial cells showed that miR-26b, miR-23a, miR-150 and miR-130a suppresses the mRNA levels of FGF21, IL-12B, IL-18 and TNFRSF9 respectively (Fig. 5A). These findings were further validated by 3'UTR reporter assays, where mutations in their target sequences within the 3'UTRs reversed their suppressive effects (Fig. 5B), suggesting that the four miRNAs directly target these mRNAs.

In addition to the changes observed for these miRNAs in the mouse model of rCDI (Fig. 2) human colonoids and mouse colonic tissues treated with TcdB (Fig. 3) and FMT-treated rCDI patients (Fig. 1A), we found rCDI decreases the levels of these four miRNAs in sera when compared to healthy controls (Fig. 5C). The use of miRNA inhibitors against miR-26b and miR-150, in PBMCs derived 4 weeks after FMT treatment, partly (FGF21) or completely (IL-18) reversed the effect of FMT on their expression, suggesting a functional role for these two miRNAs in FMT therapy (Supplementary Figure 14). Pre-treatment of colonic epithelial cells with these cytokines revealed that though FGF21, IL-12B and

TNFRSF9 had no effects (Supplementary Figure 15), IL-18 sensitises cells to TcdB but not TcdA (Fig. 5D), an effect validated in a second mucosal cell line (Fig. 5E). The identified miRNA-target interactions provide novel links between inflammation and metabolism (Supplementary Figure 16).

FMT-regulated miRNAs modulate susceptibility to *C.difficile* toxin effects

We next examined whether miRNAs upregulated by FMT can affect the colonic epithelial response to *C.difficile* toxins. Firstly, we tested the effect of miRNAs up-regulation, alone or in combination with TcdB, on cell survival. The results showed that individual miRNAs had minor effects on TcdB cytotoxicity. However, combination of miR-23a-3p and miR-150-5p significantly increased cell survival (Fig. 6A). A live-cell analysis assay verified the above findings (Supplementary Figure 17) and suggested that miR-23a-3p and miR-150-5p alone (Supplementary Figure 18) or in combination confer a growth advantage to cells treated with TcdB (Fig. 6B). In accordance, the viability of a second mucosal cell line exposed to TcdB was significantly increased by the combined overexpression of miR-23a-3p and miR-150-5p (Fig.6C).

Although miR-23a-3p and miR-150-5p had minor effect on the early TcdB-mediated cytotoxicity they promoted cell survival (Supplementary Figure 19). Using a real-time Caspase3/7 detection assay, we found that TcdB induces cell apoptosis in a dose-dependent manner (Supplementary Figure 20). miR-23a-3p and miR-150-5p had additive effects in reducing susceptibility to TcdB-induced apoptosis over time (Fig. 6D). A major cytopathic effect attributed to TcdB is the induction of cell morphological and cytoskeleton changes, which can be visualized by staining colonic epithelial cells with fluorescence conjugated phalloidin. Using this method, we observed major morphological changes to epithelial cells such as loss of actin stress fibres, spindle-like formation and cell rounding when exposed to both TcdA and TcdB. Quantification of the ratio of rounded and spindle-like cells, revealed that TcdB-induced cytopathic effects were significantly counteracted by the overexpression of miR-23a-3p and miR-150-5p (Fig. 6E, Supplementary Figure 21).

Discussion

We report the first study to examine the effects of FMT for rCDI on miRNA signatures. Our findings in a clinical rCDI cohort demonstrated the concerted regulation of miRNA expression by FMT. Validating these observations in a mouse model of rCDI and organoids, we provide evidence that miRNA processing in colonic epithelial cells is directly altered by *C. difficile* toxins and may be affected by *C. difficile*-associated dysbiosis. Conditional knockout of the miRNA-processing enzyme *Dicer* in murine intestinal epithelial cells, which secrete fecal miRNAs, has been shown to modulate the gut microbiota and exacerbate colitis. This phenotype can be rescued via wild-type fecal transplantation.[8] From this observation, we hypothesized that colitis due to CDI can suppress circulating miRNAs, which can be restored by FMT. In support of our hypothesis, predominant suppression of miRNAs was observed in two independent cohorts of rCDI patients but was differentiated from observed miRNA suppression in other colitis patient cohorts, such as in patients with chronic inflammatory bowel diseases (IBD; ulcerative colitis[14] and Crohn's disease[19]) and associated colorectal cancer.[20] For example, miR-30 family members are upregulated in both ulcerative colitis and Crohn's disease, but suppressed in rCDI, while miR-23a-3p is upregulated only in ulcerative colitis and downregulated in rCDI. These differences may be leveraged for future diagnostic purposes and could help identify IBD patients who can benefit from biotherapeutic products such as FMT, which currently demonstrate variable efficacy compared to CDI patients.

The miRNA signature in the mouse models suggest that changes in circulating miRNAs reflect alterations in the intestinal epithelial cells. Importantly, the effects of *C. difficile* infection on miRNA levels are reversed in both sera and tissues after FMT, parallelling the post-FMT serum miRNA changes observed in patients with CDI following FMT. TcdA and TcdB have been causally linked to the pathogenetic mechanisms of CDI.[21] At the cellular level, toxins induce cytoskeleton reorganisation and tight junction disruption resulting in cell rounding and cell death.[22] Here, we show that TcdB suppresses key inflammation-related miRNAs in murine intestinal tissues and human colonoids. Moreover, we identify

two miRNAs upregulated by FMT, miR-23a and miR-150, which exert cytoprotective effects against TcdB. Interestingly, we found that IL18, previously shown to contribute to mucosal damage[23], sensitizes colonic epithelial cells to TcdB. In addition, we show that IL18 is a direct target of miR-150, suggesting a new mechanism by which FMT may counteract *C. difficile*-induced epithelial disruption. Additional miRNA-regulated cytokines may be involved in the regulation of anti-inflammatory effects of FMT. We found that miR-23a targets IL12B, an essential activator of Th1 cell development, associated with CDI recurrence.[24] Collectively, these data support a role for miRNAs suppressed by toxins as a new pathogenic mechanism for CDI. We propose that the restoration of these miRNAs by FMT contributes to the protection of epithelial barrier integrity. Changes in circulating miRNAs may also contribute to extra-colonic manifestations of CDI including cardiac, renal, and neurologic impairment.[22]

Our analyses of the miRNA biogenesis machinery illustrate that downregulation of miRNAs is likely through the suppression of Drosha by CDI/TcdB and is restored following FMT. Inhibition of Drosha results in defective microRNA processing with accumulation of primary (unprocessed) miRNA transcripts. Our findings suggest the biphasic regulation of the microprocessor by CDI. The temporal effect of TcdB suggests that early miRNA regulation is attributed to a non-transcriptional mechanism. In fact, Drosha protein is reduced in response to TcdB before its mRNA levels are suppressed. Different types of stress have been associated with the stability of Drosha protein. Under oxidative stress, Drosha is phosphorylated by p38 MAPK at the N-terminus. This results in disruption of its interaction with DGSCR8, relocation to the cytoplasm and protein degradation.[25] Indeed, the cytotoxic effect of TcdB has been shown to depend on assembly of the host epithelial cell NADPH oxidase (NOX) complex and the production of reactive oxygen species.[26] Other metabolic inputs may be involved. The mTOR nutrient/amino acid sensor activates MDM2 which catalyses Drosha ubiquitination marking it for degradation.[27] The long-term suppression of both Drosha mRNA and protein may involve a gene transcription regulatory mechanism. The transcription factor c-Myc activates *Drosha* gene and upregulates Drosha protein,[28]

and is under the control of the Wnt/beta catenin pathway, which is suppressed by both toxins.[29-30]

The regulation of miRNA levels by *C. difficile* may not rely solely on the effects of toxins on mucosal cells. miRNA suppression, mediated by surface layer proteins of specific *C. difficile* ribotypes, has been proposed to attenuate the host's immune response resulting in a more persistent infection in mice.[31] Interestingly, the suppression of circulating miRNAs upon depletion of regulatory T cells (Tregs), reported in mouse models of autoimmune diseases,[32] suggest potential links between Treg function, CDI, miRNAs and FMT. Recent experimental evidence has linked the effectiveness of FMT for colitis with the induction of IL-10 and TGB-β, cytokines critical for Treg accumulation in the intestine.[33] Whether FMT-directed immunosuppression aids in the recovery from *C. difficile* requires further investigation.[3]

Intriguingly, in *C. difficile* naïve animals treated with FMT we observed a trend towards higher Drosha and Dicer mRNA levels and alterations in the miRNA profiles. These control animals were conditioned with cefoperazone (an antibiotic against both Gram-positive cocci and Gram-negative bacteria) to facilitate *C. difficile* colonization. This observation proposes that host-microbe interactions regulate the host miRNA biogenesis machinery, which in turn may affect directly or indirectly, through the immune response, gut microbiota composition.

Here, we reported changes in circulating and colonic miRNAs in the context of FMT for rCDI, validating our observations across two independent randomized trials. Using two different animal models, human colonoids and colonic epithelial cells, we substantiated that *C. difficile* highjacks miRNA biogenesis and show how miRNA restoration contributes to the therapeutic effects of FMT. Our findings strongly support the need for further mapping of the epitranscriptomic changes associated with FMT. While our data in rCDI mice suggests that TcdB impacts Drosha rather than Dicer1 or Ago2 expression, studies with conditional knockout mice of the miRNA-processing machinery, such as *Dicer* or

Drosha, may reveal additional molecular mechanisms or metabolic pathways affected by CDI-induced colitis.

Together, these results provide novel and provocative evidence that modulation of the gut microbiome via FMT induces changes in circulating miRNAs, and a subset of these miRNAs downregulates inflammatory protein expression and protects epithelial cells. These findings add new insight into the molecular mechanisms underlying *C. difficile* pathogenesis and FMT and identify new potential targets for therapeutic intervention.

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Figure Legends

Table 1. Participant baseline characteristics for discovery and replication cohorts.

Figure 1. Fecal Microbiota Transplantation (FMT) in patients with *C. difficile* infection regulates the levels of circulating miRNAs. *(A)* Heatmap representation of the significantly upregulated circulating miRNAs 4- and 12-weeks after FMT treatment compared to the screening time point (n = 42), as assessed by the nCounter Nanostring platform. *(B)* Representative box plots depicting the miRNAs with highest levels of detection. Box plots denote mean % change ± s.e.m., inner boxes represent mean, and error bars represent 95% confidence interval. Statistical significance of FMT effect on circulating miRNAs was determined by non-parametric longitudinal method followed by Wilcoxon signed-rank test for pairwise comparisons; $^{**}P < 0.01$, $^{***}P < 0.001$. *(C)* The overlapping top three miRNA-regulated pathways, as assessed by the Metacore network analysis software. *(D)* Validation of top 6 upregulated miRNAs in the replication cohort 2- and 4-weeks after FMT as assessed by RT-qPCR. Fold changes and statistical significance in circulating levels of miRNAs was determined by OriginPro and Wilcoxon matched-pairs signed rank test; $^{***}P < 0.001$.

Figure 2. FMT reverses the effects of *C. difficile* on circulating and tissue miRNAs in a mouse model of recurrent CDI. Box plots depicting the changes in miRNA levels in *(A)* sera and *(B)* ceca from animals treated with FMT, infected with *C. difficile* (CDI), and infected with *C. difficile* and treated with FMT (CDI+FMT) compared to FMT donors. Box plots denote mean % change ± s.e.m., inner boxes represent mean, and error bars represent 95% confidence interval. miRNA levels were assessed by RT-qPCR and were normalised against *(A)* RNU1A1 and cel-miR-39 (spike-in) or *(B)* RNU5G and 5S rRNA, and compared to control (donor) samples, set as 100%. Statistical significance was determined by Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (compared to donor) and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ (compared to CDI).

Figure 3. Downregulation of miRNAs in mouse colonic tissues and human colonoids treated with *C. difficile* toxins. *(A)* Box plots depicting the changes in miRNA levels in colonic tissues from animals treated with TcdB and combination of TcdA with TcdB, compared to HBSS-treated controls (Vehicle). Box plots denote mean % change ± s.e.m., inner boxes represent mean, and error bars represent 95% confidence interval. miRNA levels were assessed by RT-qPCR, were normalized against RNU5G and 5S rRNA, and compared to control samples, set as 100%. Changes in miRNA levels *(B)* 30 min and *(C)* 6h in colonoids treated with TcdA or TcdB, compared to DMEM-treated controls (Vehicle). miRNA levels assessed by RT-qPCR, were normalized against RNU1A1 and 5S rRNA and are expressed as mean ± s.e.m. compared to control samples, set as 1. Statistical significance was determined by Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Figure 4. *C. difficile* infection suppresses Drosha expression and miRNA processing. *(A)* Drosha mRNA and protein levels in ceca from animals treated with FMT, infected with *C. difficile* (CDI), infected with *C. difficile* and treated with FMT (CDI+FMT), compared to FMT donors. *(B)* Drosha mRNA levels in colonic tissues from animals treated with TcdB or combination of TcdA with TcdB, compared to controls (Vehicle). *(C)* Drosha mRNA and protein levels in colonoids treated with toxin A or toxin B, compared to DMEM-treated controls (Vehicle). *(D)* Drosha protein levels in NCM356 colonic epithelial cells upon knockdown of Drosha by means of siRNA (left) and treated with toxin B (right), compared to non-targeting siRNA (siControl) and DMEM-treated controls (Vehicle), respectively. *(E)* Pri-miRNA levels in NCM356 cells upon knockdown of Drosha (upper panel), and in colonoids treated with TcdA or TcdB (lower panel), compared to non-targeting siRNA (siControl) and DMEM-treated controls (Vehicle), respectively. mRNA and pri-miRNA levels assessed by RT-qPCR were normalized against beta-Actin and GAPDH and are expressed as mean ± s.e.m. compared to control samples, set as 1. Drosha protein levels were assessed by immunoblot analysis. alpha-Tubulin was used as the loading control. Statistical significance was determined by Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{\#\#}P$

< *0.01* (compared to CDI). *(E)* Lower right panel, schematic representation of the proposed model on microRNA regulation by *C. difficile* infection.

Figure 5. Effects of Fecal Microbiota Transplantation (FMT)-regulated miRNAs in patients with *C. difficile* infection, on circulating proteins. *(A)* Effects of miR-26b-5p, miR-23a-3p, miR-150-5p and miR-28-5p overexpression on the levels of FGF21, IL12B, IL18 and TNFRSF9 mRNAs, respectively, in colonic epithelial cells. Gene expression data normalized against beta-Actin and GAPDH are expressed as mean ± s.e.m. compared to miR-C transfected cells, set as 1. *(B)* Effects of miR-26b-5p, miR-23a-3p, miR-150-5p and miR-28-5p on the activity of IL12B, IL18 and FGF21 mRNA 3'UTRs, as assessed by luciferase reporter assays. 3'UTR sequences were cloned in a reporter vector downstream of the *Renilla* Luciferase gene. The reporter vector was transfected in colonic epithelial cells and luciferase activity was measured 24 hours after the overexpression of the respective miRNAs in the same cells. Direct targeting of the 3'UTR by the miRNA was validated by assays employing deletion mutants (DM) of the respective miRNA target sequences. *Renilla* Luciferase activity was normalized against the activity of the *Firefly* Luciferase gene, expressed by the same vector. miR-C (cel-miR-39-3p), a non-targeting miRNA was used as negative control. *(C) C. difficile* infection effect on the levels of circulating miR-26b-5p, miR-23a-3p, miR-150-5p and miR-28-5p in patients. The levels of serum miRNAs in *C. difficile* patients compared to healthy controls (n = 42), as assessed by the nCounter Nanostring platform. Box plots denote mean % change ± s.e.m., inner boxes represent mean, and error bars represent 95% confidence interval. *(D)* Effects of IL-18 on toxin-mediated cell growth inhibition. NCM356 cell growth was monitored in real-time as % of confluence (IncuCyte). *(E)* Effects of IL-18 on TcdB-mediated cell growth inhibition. NCM460 cell survival was assessed by measuring metabolically active cells (Cell-Titer Glo) and is expressed as mean ± s.e.m. compared to untreated cells, set as 100%. Statistical significance was determined by Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to miR-C *(A* and *B),* healthy controls *(C)* and TcdB alone *(D* and *E)*, and $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, compared to wild type 3'UTRs).

Figure 6. Functional effects of Fecal Microbiota Transplantation (FMT)-regulated miRNAs on colonic epithelial cells. *(A)* Effects of FMT-regulated miRNAs overexpression on TcdB-mediated cell growth inhibition. NCM356 cell survival was assessed by measuring metabolically active cells (Cell-Titer Glo) and is expressed as mean ± s.e.m. compared to miR-C transfected cells, set as 100%. *(B)* Effects of miR-23a-3p and miR-150-5p overexpression on TcdB-mediated NCM356 cell growth inhibition. Cells were monitored in real-time as % of confluence (IncuCyte). *(C)* Effects of miR-23a-3p and miR-150-5p overexpression on TcdB-mediated NCM460 cell growth inhibition. Cell survival was assessed by measuring metabolically active cells (Cell-Titer Glo) and is expressed as mean ± s.e.m. compared to miR-C transfected cells, set as 100%. *(D)* Effects of miR-23a-3p and miR-150-5p overexpression on TcdB-induced cell apoptosis. NCM356 cell apoptosis was monitored in real-time as activated caspase3/7 fluorescence (IncuCyte). *(E)* Effects of miR-23a-3p and miR-150-5p overexpression on TcdB-mediated cytoskeleton rearrangements. NCM356 cytoskeleton organisation was studied by fluorescence microscopy after phalloidin staining. miR-C (cel-miR-39-3p), a non-targeting miRNA, was used as negative control. Statistical significance was determined by Student's *t*-test, $*P < 0.05$, $**P < 0.01$.