The miR-223/nuclear factor I-A axis regulates inflammation and cellular functions in intestinal tissues with necrotizing enterocolitis

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We previously demonstrated that microRNA(miR)-223 is overexpressed in intestinal tissue of infants with necrotizing enterocolitis (NEC). The objective of the current study was to identify the target gene of miR-223 and to investigate the role of the miR-223/nuclear factor I-A (NFIA) axis in cellular functions that underpin the pathophysiology of NEC. The target gene of miR-223 was identified by in silico target prediction bioinformatics, luciferase assay, and western blotting. We investigated downstream signals of miR-223 and cellular functions by overexpressing the miRNA in Caco-2 and FHs74 cells stimulated with lipopolysaccharide or lipoteichoic acid (LTA). NFIA was identified as a target gene of miR-223. Overexpression of miR-223 significantly induced MYOM1 and inhibited NFIA and RGN in Caco-2 cells, while costimulation with LTA decreased expression of GNA11, MYLK, and PRKCZ. Expression levels of GNA11, MYLK, IL-6, and IL-8 were increased, and levels of NFIA and RGN were decreased in FHs74 cells. These potential downstream genes were significantly correlated with levels of miR-223 or NFIA in primary NEC tissues. Overexpression of miR-223 significantly increased apoptosis of Caco-2 and FHs74 cells, while proliferation of FHs74 was inhibited. These results suggest that upon binding with NFIA, miR-223 regulates functional effectors in pathways of apoptosis, cell proliferation, G protein signaling, inflammation, and smooth muscle contraction. The miR-223/NFIA axis may play an important role in the pathophysiology of NEC by enhancing inflammation and tissue damage.

Necrotizing enterocolitis (NEC) is a devastating gastrointestinal (GI) disease that is strongly associated with prematurity. It is one of the leading causes of neonatal morbidity and mortality among very low birthweight infants (< 1500 g), with an incidence of 6–10% [1,2] and a mortality of 20–30%. Infants who survive are at risk of long-term complications, including short bowel syndrome, parenteral nutrition-associated cholestasis, poor physical growth, and neurodevelopmental impairment [3,4]. Despite decades of NEC research, its etiology and pathophysiology remain incompletely understood. Multiple mechanisms and contributing factors have been proposed, including immature gut mucosa with impaired host defense immunity, enteral feeding with abnormal

Abbreviations
GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; GLuc, Gaussia luciferase; GNA11, G protein subunit alpha 11; ICAM1, Intercellular adhesion molecule 1; IL-6, Interleukin-6; IL-8, Interleukin-8; LPS, Lipopolysaccharides; LTA, Lipoteichoic acid; MYLK, Myosin light chain kinase; MYOM1, Myomesin 1; NEC, Necrotizing enterocolitis; NFIA, Nuclear factor I-A; PRKCZ, Protein kinase C zeta; RGN, Regucalcin; TLR2, Toll-like receptor-2; TLR4, Toll-like receptor-4; TNF, Tumor necrosis factor.
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Results

Clinical characteristics of recruited infants

As previously reported, there were no statistically significant differences in demographic and clinical characteristics, for example, sex, gestational age, and birthweight, between the infants recruited to the NEC and Surg-CTL groups (Table S1). However, as expected, infants with NEC had significantly longer duration of hospitalization ($P = 0.014$) [8].

Increased expression of miR-223 in NEC tissues and identification of target gene

Expression of miR-223 was upregulated in small intestinal tissues of NEC ($n = 10$) compared with Surg-CTL infants ($n = 10$ in each group; 25.16-fold, $P < 0.001$; Fig. 1A) [8]. In silico target prediction revealed that NFIA was among the top candidate target genes of miR-223 (Table S2). Furthermore, NFIA was decreased by 69.15% in NEC tissues compared with Surg-CTL tissues ($P < 0.001$; Fig. 1B). In NEC tissues, there was an inverse correlation between mRNA levels of miR-223 and NFIA (Fig. 1C).

The direct binding of miR-223 and 3’UTR of NFIA was validated in HEK293 cells by luciferase assay in the presence of mimic miR-223 or mimic-CTL. The luciferase activity of HEK293 cells harboring the NFIA wild-type (WT) 3’UTR reporter plasmid (Fig. 1D) was inhibited by 25.1% in the presence of mimic miR-223 [$n = 4$; $P < 0.001$; Fig. 1E(ii)], compared with that containing the mimic-CTL [Fig. 1E(i)]. Replacing NFIA WT 3’UTR sequence with mutated nucleotides (MT) at the seeding region of miR-223 [Fig. 1E(iii)] or the presence of the irrelevant miR-431 (Fig. S1) did not alter the luciferase activity. Direct binding between miR-223 and 3’UTR of NFIA was further confirmed in the human colorectal cell line Caco-2. The luciferase activity was decreased by 12.4% in the presence of mimic miR-223 and WT NFIA plasmid [$n = 4$, $P < 0.001$; Fig. 1F(ii)], but not in the presence of binding site-mutated plasmid (Fig. 1F(iii)).

Overexpression of miR-223 decreased NFIA expression in Caco-2 cells

Transfection of mimic miR-223 into Caco-2 cells increased miR-223 compared with cells transfected with mimic-CTL ($n = 6$, $P = 0.02$; Fig. 2A) and suppressed NFIA expression by 10.6% ($n = 6$, $P = 0.002$; Fig. 2B). In accordance with mRNA expression, western blot analysis demonstrated that the NFIA protein level was decreased by 27.1% in Caco-2 cells overexpressing miR-223 ($n = 5$, $P = 0.003$; Fig. 2C,D).

Correlation mRNA levels of miR-223 and NFIA with dysregulated gene expressions in NEC tissues

Correlation analysis was performed on levels of miR-223 and NFIA with 52 dysregulated genes in intestinal bacterial colonization, and other conditions that can result in a bacterial/inflammatory response that can lead to subsequent tissue damage [5,6]. In our recent series of studies on expressionally arrays of NEC tissues, we identified aberrant mRNA expression patterns, which suggested a complex mechanism that was initiated at receptor TLR4, mediated via key transcription factors NFKB2, AP-1/FOSL1, FOXA1, and HIF1A, and extended to downstream pathways involving angiogenesis, arginine metabolism, cell adhesion and chemotaxis, extracellular matrix remodeling, hypoxia/oxidative stress, inflammation, and muscular functions [7]. We also reported the first differential microRNA (miRNA) expression array of NEC tissues and provided evidence that miR-431 and its target gene FOXA1 could play a key role in intensifying the inflammatory responses in NEC tissues [8,9]. In a systematic large-scale meta-analysis study, the miRNA-429/200a/b and miRNA-141/200c clusters and their target genes have been suggested as potential biomarkers for NEC [10]. In addition, we reported elevated plasma levels of miR1290 in infants with NEC and suggested that miR1290 could be developed as a biomarker for early diagnosis of NEC [11].

miRNAs are a class of small, single-stranded noncoding RNA molecules (18–24 nucleotides) that can suppress translation and stability of transcripts through partial complementarity to the 3’UTRs of protein-coding mRNAs [12]. Emerging evidence has revealed that miRNAs play important functions in normal cellular and physiologic events, and their dysregulation could be associated with diverse pathologic conditions, including cancer [13], chronic infection, inflammation [14], and acute organ injury [15]. In our previous study, we reported that miR-223 expression was upregulated 25.16-fold in intestinal tissues of infants with NEC compared with noninflammatory surgical control (Surg-CTL) tissues [8]. miR-223 has been reported that its expression was upregulated 25.1% in the presence of mimic miR-223 ($n = 4$; $P < 0.001$; Fig. 1E(ii)], compared with that containing the mimic-CTL [Fig. 1E(i)]. Replacing NFIA WT 3’UTR sequence with mutated nucleotides (MT) at the seeding region of miR-223 [Fig. 1E(iii)] or the presence of the irrelevant miR-431 (Fig. S1) did not alter the luciferase activity. Direct binding between miR-223 and 3’UTR of NFIA was further confirmed in the human colorectal cell line Caco-2. The luciferase activity was decreased by 12.4% in the presence of mimic miR-223 and WT NFIA plasmid [$n = 4$, $P < 0.001$; Fig. 1F(ii)], but not in the presence of binding site-mutated plasmid (Fig. 1F(iii)).

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Correlation mRNA levels of miR-223 and NFIA with dysregulated gene expressions in NEC tissues

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tissues of infants with NEC reported in our previous study (n = 9–10) [7]. We observed inverse correlations between expression levels of miR-223 and \( GNA11 \), \( MYLK \), or \( MYOM1 \) (P < 0.05), and positive correlations between miR-223 and \( ILS \) or \( TNF \) (P < 0.05) (Fig. 3). A trend of inverse correlation was observed between miR-223 and \( PRKZ \) (P = 0.059) or \( RGN \) (P = 0.088). The expression levels of \( NFIA \)

Fig. 1. Expression of miR-223 and \( NFIA \) in NEC tissues and direct binding of miR-223 and \( NFIA \) by luciferase reporter assay. (A) Relative expression level of miR-223 to U6 and (B) relative expression of \( NFIA \) to GAPDH in human Surg-CTL and NEC tissues were quantified by qPCR (n = 10 for each group). (C) Correlation between miR-223 and \( NFIA \) in NEC tissues. Correlation analysis was performed by the Spearman correlation test. \( r \) represents the Spearman correlation coefficient. n = 10 for each group. (D) Seeding site of hsa-miR-223 and mutation sequence of the \( NFIA \) 3'UTR construct. (E) HEK293 cells were cotransfected with (i) mimic-CTL or (ii) mimic miR-223 and wild-type \( NFIA \) 3'UTR (WT) or (iii) mimic miR-431 with mutated type \( NFIA \) 3'UTR (MT). Luciferase activity was measured at 48 h after transfection. (F) The same experimental procedure was performed on Caco-2 cells. Statistical analysis was performed using paired t-test, two-sided. Data were presented as mean ± SEM, n = 4.
were positively correlated with \textit{GNA11, MYLK, MYOM1, PRKCZ} or \textit{RGN} (\(P < 0.05\)) and inversely correlated with \textit{IL8} or \textit{TNF} (\(P < 0.05\)). There was no significant correlation between \textit{miR-223} and \textit{NFIA} with \textit{IL6} (Fig. S2).

**Effects of overexpressing \textit{miR-223} on gene regulation in Caco-2 and \textit{FHs74} cells**

In Caco-2 cells, overexpression of \textit{miR-223} increased \textit{MYOM1} 1.5-fold (\(P = 0.016\); Fig. 4C1) and decreased \textit{NFIA} by 16.8\% (\(P = 0.045\); Fig. 4D1), or \textit{RGN} by 16.4\% (\(P = 0.038\); Fig. 4H1). The effects of overexpressing \textit{miR-223} on these gene expressions appeared to be unaffected by additional exposure to Lipopolysaccharides (LPS) or lipoteichoic acid (LTA). Levels of \textit{GNA11} (Fig. 4A1), \textit{MYLK} (Fig. 4B1), and \textit{PRKCZ} (Fig. 4E1) were not changed in \textit{miR-223}-overexpressing cells but were increased upon additional exposure to LTA by 1.2- to 1.4-fold (\(P < 0.05\)). \textit{IL-8} was increased by exposure to LPS or LTA by 4.5- to 7.0-fold, but no effect of \textit{miR-223} was observed in all conditions (Fig. 4G1).

In \textit{FHs74} cells, overexpression of \textit{miR-223} significantly increased \textit{GNA11} (1.3-fold; Fig. 4A2), \textit{MYLK} (1.7-fold; Fig. 4B2), \textit{IL-6} (2.8-fold; Fig. 4F2), and \textit{IL-8} (14.1-fold; Fig. 4G2) and decreased \textit{NFIA} by 73.6 \% (\(P < 0.05\)) (Fig. 4D2) or \textit{RGN} by 49.2 \% (Fig. 4H2, \(n = 5\), \(P < 0.05\)). \textit{MYOM1} (Fig. 4C2) and \textit{PRKCZ} (Fig. 4E2) were not affected by \textit{miR-223} overexpression. The presence of LPS or LTA also did not further alter the effects of \textit{miR-223}. Expression of \textit{TNF} was undetectable in \textit{FHs74} cells and in Caco-2 was not affected by overexpression of \textit{miR-223} (Fig. S3). In intestinal tissues of NEC patients, we observed that expression levels of \textit{GNA11, MYLK, MYOM1, NFIA, PRKCZ, and RGN} were significantly downregulated (\(P < 0.02\)), with \textit{IL-6} and \textit{IL-8} significantly increased, compared with those from Surg-CTL subjects (\(n = 10\) for each group; Fig. S4).

**Effects of overexpressing \textit{miR-223} on proliferation and apoptosis of Caco-2 and \textit{FHs74}**

Overexpression of \textit{miR-223} did not affect proliferation (Fig. 5A) or apoptotic/total cell death of Caco-2 cells.
Fig. 3. Correlation between miR-223 or NFIA and miR-223/NFIA-targeted downstream genes in NEC tissues. (A1-G1) Correlation between miR-223 and miR-223/NFIA-targeted downstream genes in NEC tissues. (A2-G2) Correlation between NFIA and miR-223/NFIA-targeted downstream genes in NEC tissues. Nonparametric correlation analysis was performed by Spearman’s correlation test. $r$ represents the Spearman correlation coefficient. $n = 10$ for each group, except A1, A2 and D1, D2 ($n = 9$).
In the additional presence of LTA, apoptosis (Fig. 5C) and total cell death (Fig. 5E) were increased 1.4- and 1.5-fold \((n = 6, P < 0.01;\) Fig. S5), respectively.

Overexpression of miR-223 inhibited proliferation of FHs74 by 30.4 % after 5 days of culture \((n = 5, P < 0.001;\) Fig. 5B). miR-223 increased apoptotic cell death 1.4-fold \((n = 6, P = 0.046;\) Fig. 5D), and total cell death was not significantly affected \((P = 0.046;\) Fig. 5F). The additional presence of LPS or LTA was not associated with further effects on the cell death data.

Proposed regulatory network of miR-223/NFIA in NEC

MetaCore pathway analysis suggested dysregulation of effector genes in functional categories of cell apoptosis (RGN), proliferation (NFIA, PRKCZ), G protein (GNA11), inflammation (IL-6, IL-8) and smooth muscle contraction (MYOM1, MYLK) signals, with possible links via miR-223/NFIA and C/EBP-\(\alpha\) to multiple downstream transcription factors: HNF4-\(\alpha\), HNF3-\(\alpha\), ATF-2, MEF2C, TCF4, and NF-kB (Fig. 6).

Discussion

Our study presents the first evidence that the miR-223/NFIA axis is aberrantly expressed in intestinal tissues of infants with NEC and suggests that affected downstream signals could reprogram the expression of effector genes in multiple categories of cellular functions, including apoptosis, proliferation, G protein signaling, inflammation, and smooth muscle contraction. We observed increased apoptosis and decreased proliferation of intestinal cells overexpressing miR-223 upon exposure to bacterial toxins in culture. These findings strongly suggest that dysregulation of miR-223 and its target gene NFIA could contribute to the
miR-223/NFIA axis in NEC

Fig. 5. Effects of miR-223 on cell proliferation and apoptosis of Caco-2 cells and FHs74 cells. (A, B) Cell proliferation of Caco-2 cells and FHs74 cells was assessed by MTS assay after transfection with mimic-CTL or mimic miR-223 for 5 days. Statistical analysis was performed by two-way ANOVA with Bonferroni’s correction (n = 5). The data were presented as mean ± SEM. (C–F) Percentage of apoptosis and total cell death were assessed by flow cytometry upon overexpression of mimic miR-223 and stimulation with LPS or LTA. Statistical analysis was performed by paired t-test (n = 6), and the data were presented as mean ± SEM. ***P < 0.001; ****P < 0.0001.

pathophysiology of NEC through escalated inflammation, increased apoptosis, and suppressed cell proliferation, which leads to irreparable tissue damage.

miR-223 is a highly conserved miRNA known to be involved in the regulation of hematopoiesis, immune response, and inflammation [20–22]. It exerts its influence through binding to a variety of target genes in specific tissues and pathologic conditions. We report upregulation of miR-223 in intestinal tissue of infants with NEC and identified the transcription factor NFIA as a binding target of miR-223. Our observation is in line with reports on hematopoietic cells and the central nervous system in which the miR-223/NFIA axis is suggested to play important roles in regulating development, cell proliferation, and malignant progression [16,17,23]. While not previously elaborated in NEC tissues, upregulation of miR-223 and potential target genes involving TLR and NF-κB signaling has been reported in the inflamed colonic mucosa of patients with ulcerative colitis (UC) [18]. Schönaeuf et al. demonstrated that levels of circulatory and fecal miR-223 correlated with disease activity and could be potential biomarkers for inflammatory bowel diseases (IBD) [24]. In the mouse model of induced colitis, treatment with miR-223 antagonir significantly improved acute intestinal conditions. The effect was suggested to be mediated by reactivating claudin-8 and preserving the integrity of the gut epithelial barrier [22]. miR-223 has been reported to serve both pro-inflammatory [22,25,26] and anti-inflammatory [27–29] functions, possibly dependent on specific target genes and disease conditions. In the FHs74 model, we observed upregulated expression of two pro-inflammatory cytokines, IL6 and IL8, upon overexpression of miR-223, suggesting potent pro-inflammatory roles of miR-223 in NEC. Our results are consistent with those reported about CMT93 cells where overexpression of miR-223 induced expression of inflammatory cytokines TNF-α, IL-6, and COX-2, possibly mediated by FOXO3a and NF-κB activities [25]. NFIA has been
acknowledged as a master molecular transcriptome regulator that modulates myeloid differentiation and maturation and acts as an immune defense mechanism during sepsis [30]. Nevertheless, its implications in NEC or other inflammatory bowel conditions have not been reported.

The aberrant colonization of Gram-positive (e.g., Clostridium spp.) or Gram-negative bacteria (e.g., E. Coli and Klebsiella spp.) in the gut is an important risk factor of NEC. 30% of infants with NEC also suffer from neonatal sepsis [31]. In this context, our study has further elucidated the effects of miR-223 overexpression on downstream gene expression signals and functions upon exposure of gut epithelial cells to LPS and LTA in culture. Our results showed upregulation of GNA11, MYLK, MYOM1, and IL-8, and downregulation of NFIA and RGN in Caco-2 and/or FHs74 cells upon overexpression of miR-223. GNA11,
MYLK, and PRKCB were increased in Caco-2 cells with the additional presence of LTA. Importantly, these affected genes are known to play important roles in the maintenance of intestinal homeostasis, including regulation of cellular differentiation and proliferation, apoptosis, smooth muscle contraction, epithelial barrier permeability, and tight junction integrity, as well as immune and inflammatory responses. A brief summary of their relevant functions is described in Table S3. Furthermore, expression levels of these regulated genes were correlated with levels of miR-223 or NFIA in tissues of patients with NEC. We also observed significant dysregulation of these signals in NEC tissues compared with the Surg-CTL counterparts. In line with the regulatory pattern of the cell models, IL8 and IL-6 were upregulated in NEC tissues, while NFIA and RGN were decreased. Network analysis demonstrated that miR-223 could downregulate target gene NFIA and mediate through multiple transcription factors, of which some are reported targets of miR-223 (e.g., FOXA1, MEF2C, HNF4-α, C/EBP-α, and NF-kB) [16,32,33]. Furthermore, the dysregulation of downstream transcription factors, FOXA1, C/EBP-α, HNF4-α, ATF2, HNF3-α, TCF4, and NF-kB, has been implicated in inflammatory GI conditions such as NEC and IBD [7,34–38].

While both Caco-2 and FHs74 have been applied as cell models for studying NEC biology [39–41], we did observe some differences in the response of these two cell lines to miR-223 overexpression and bacterial toxin exposure, possibly due to intrinsic variations of tissue origin, disease condition, and expression level of regulatory genes and receptors. For example, we observed significantly lower or undetectable expression levels of TNF, Toll-like receptor 2 (TLR2), and TLR4 in FHs74 cells (Fig. S6), which could lead to their lack of response to LPS and LTA. Nevertheless, dysregulation of the miR-223/NFIA axis in these intestinal cells has consistently directed to multiple detrimental activities, including increased pro-inflammatory cytokines, disturbance of homeostasis, induction of apoptosis, and inhibition of the cellular replenishing mechanism.

In summary, our study has provided evidence on the importance of the miR-223/NFIA network in intestinal tissues affected by NEC and its probable contribution to disease pathophysiology. Despite advances in neonatal care over the past decades, morbidity and mortality outcomes of NEC have remained disappointing [2,42]. Development of novel therapeutic tactics, such as targeting miRNAs using specific antagonists or mimics and genetically engineered extracellular vesicles, has been proposed and under preclinical and clinical trials for various diseases [43,44]. Our findings provide new insights into the molecular mechanisms of NEC relating to regulatory pathways involving miRNA. The pathways suggested by our results offer new targets for diagnostic and therapeutic options not only for NEC but also for other conditions with underlying intestinal inflammation.

Materials and methods

Clinical specimen

Intestinal specimens were collected during surgery from infants with stage III NEC (n = 10) in the Prince of Wales Hospital, a tertiary hospital affiliated with the Chinese University of Hong Kong [8]. These samples were histologically examined to confirm NEC diagnosis. In addition, Surg-CTL tissues were obtained from infants who underwent intestinal surgery because of noninflammatory conditions, including congenital small bowel atresia (n = 7), intestinal obstruction (n = 2), and elective closure of ileostomy (n = 1).

Ethics statement

Written informed consents were obtained from the parents for all cases. This study has been approved by the Joint Chinese University of Hong Kong and New Territories East Cluster Clinical Research Ethics Committee (2014.159). The study methodologies conformed to the standards set by the Declaration of Helsinki.

Cell culture

Human epithelial cell lines Caco-2 (colon carcinoma) and FHs74 (fetal small intestine), and HEK293 (embryonic kidney) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Caco-2 was cultured in Eagle’s Minimum Essential Medium (EMEM; ATCC) supplemented with 20% FBS (Gibco, Carlsbad, CA, USA). FHs74 was cultured in Hybri-Care Medium containing 10% FBS (Gibco) and 10% epidermal growth factor (EGF; PeproTech, Rocky Hill, NJ, USA). HEK293 was grown in EMEM (ATCC) containing 10% FBS (Gibco).

Identification of target gene and luciferase reporter assay

Potential target genes of miR-223 were predicted by six databases: miRTarBase 4.0, DIANA TOOLS TarBase v7.0, DIANA-microT web server v5.0, TargetScan Human Release 6.2, microRNA.org, and miRDB as previously described [9]. The characteristics of these databases are summarized in Table S4. The reporter plasmid was
constructed with the full-length 3'UTR of NFIA (NM_005595.1) cloned into the pEZX-MT05 plasmid (GeneCopoeia, Rockville, MD, USA). Site-directed mutation [16] of the binding site was generated using the Q5-site-directed mutagenesis kit [New England Biolabs (NEB), Ipswich, MA, USA] and confirmed by DNA sequencing (BigDye Terminator v3.1 Cycle Sequencing Kit; Applied Biosystems, Forster City, CA, USA). All primers used are listed in Table S5. Mimic miR-223 (mirVanaTM miRNA Mimics; Ambion, Austin, TX, USA) or scrambled mimic control (mimic-CTL; mirVanaTM miRNA Mimics; Ambion) and reporter plasmids were cotransfected into the Caco-2 or HEK293 cells using Lipofectamine® 3000 (Invitrogen, Carlsbad, CA, USA). Luciferase activities of reporter proteins Gaussia luciferase (Gluc) and secreted alkaline phosphatase (SEAP) were measured (Secrete-Pair Dual Luminescence Assay Kit; GeneCopoeia). The luciferase activity of Gluc was normalized with SEAP. The transfection of each miRNA mimics was performed in triplicate in each set of experiment. HEK293 cells or Caco-2 cells were transfected with NFIA 3'UTR reporter plasmid (pEZX-MT05) using Lipofectamine® 3000 in 6-well plates. After 48 h, the cells were split into a new 6-well plate in selection medium containing G418 (0.8 mg·mL−1; Sigma-Aldrich, Saint Louis, MO, USA). After 21 days, selected cells stably harboring the exogenous plasmids were expanded in a 75-cm² flask, and seeded in a 24-well plate for luciferase reporter assay. Mimic miR-223, mimic miR-431 (an irrelevant miRNA), and mimic-CTL (all mimics from mirVana™ miRNA Mimics; Ambion) were transfected into these cells with Lipofectamine® 3000. After incubation for 48 h, the medium was collected for measurement of luciferase activity.

Overexpression of miR-223 upon LPS or LTA treatment
Caco-2 or FHs74 cells were seeded in 24-well plates and incubated for 16 h before transfection. Mimic miR-223 or mimic-CTL was introduced into the cells by Lipofectamine RNAiMAX (Invitrogen). After 24 h, the cells were treated with lipopolysaccharide from Escherichia coli 0127: B8 (LPS; Sigma-Aldrich) or lipoteichoic acid (LTA) from Staphylococcus aureus (LTA; InvivoGen, San Diego, CA, USA) at 50 μg·mL−1 for 24 h [45–47].

Western blot analysis
Caco-2 cells were lysed with RIPA buffer (Sigma-Aldrich) supplemented with 1× protease inhibitor (Roche, Basel, Switzerland) and 1X phosphatase inhibitor (Roche) after transfection with mimic miR-223 or mimic-CTL. Twenty microgram of proteins was separated by electrophoresis using 10% polyacrylamide gel (Bio-Rad, Hercules, CA, USA). The proteins were transferred onto nitrocellulose membrane (GE Healthcare, Chicago, IL, USA) and incubated with rabbit anti-NFIA (1 : 2000; Thermo Fisher, Waltham, MA, USA, Cat. No. PA5-52252) or rabbit anti-GAPDH (1 : 5000; Cell Signaling, Danvers, MA, Cat. No. CST5174) in 4 °C overnight. The membranes were then incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (Cell Signaling, Cat. No. CST7074) for 1 h in room temperature. Chemiluminescent signals were detected by the ECL Prime Western Blotting Detection Reagent (GE Healthcare) and analyzed by the ImageJ software (National institutes of Health, Bethesda, MD, USA). GAPDH was used as the loading control.

Real-time polymerase chain reaction of miRNA and mRNA
miRNA was extracted from intestinal tissues, Caco-2 cells, or FHs74 cells using the mirRNAeasy Mini Kit (Qiagen, Hilden, Germany), and reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and microRNA RT primers (Applied Biosystems). PCRs were performed using the 7300 Real-Time PCR System (Applied Biosystems) with TaqMan MicroRNA Assays (Applied Biosystems, hsa-miR-223, assay ID: 002295, UGUCAGUUUGUCAAAUACCCCA; U6 snRNA, assay ID: 001973), cDNA templates, and Universal Master Mix (Applied Biosystems). The reaction was performed in duplicate at: one cycle of 95 °C for 10 min, 45 cycles of 95 °C for 15 s, and 60 °C for 1 min. U6 was amplified as an internal control. All reactions were performed in parallel with no-template control reactions. The level of specific miRNAs was expressed as the relative expression to U6/ GAPDH.

Total RNA was extracted from intestinal tissues, Caco-2, or FHs74 using the RNeasy Mini Kit (Qiagen). cDNA was generated by the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PCRs were performed using the 7300 Real-Time PCR System (Applied Biosystems) with TaqMan probes (Applied Biosystems; Table S6), cDNA templates, and TaqMan Gene Expression Master Mix (Applied Biosystems). The reaction was performed in duplicate at: one cycle of 50 °C for 2 min, one cycle of 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. GAPDH was amplified as the internal control.

Cell proliferation by MTS assay
Caco-2 or FHs74 was seeded in 96-well plates at the density of 2.5 or 4.0 × 10³ cells and transfected with mimic miR-223 or mimic-CTL. Cell proliferation was measured daily for 5 days using the CellTiter 96® AQueous One Solution Cell Proliferation Assay Kit containing 3-(4,5-dimethyl-2-y1)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
Apoptosis assay by flow cytometry

Caco-2 cells were transfected with mimic miR-223 or mimic-CTL for 24 h before treatment with LPS or LTA for 24 h, whereas transfected FHs74 cells were incubated for 48 h and treated with LPS or LTA for 48 h. The percentage of apoptotic and total dead cells were measured by the Apoptosis Detection Kit I (BD Biosciences, Franklin Lakes, NJ, USA) containing phycoerythrin (PE) annexin V and 7-amino-actinomycin (7-AAD) reagents, and analyzed by the FACSCalibur/LSRFortessa Cell Analyzer and CellQuest/FACSDiva software packages (BD Biosciences).

Network analysis

The MetaCore Analysis Suite (Clarivate Analytics, Philadelphia, PA, USA) was used to map regulatory networks of validated target genes downstream of the miR-223/NFIA axis. Multiple algorithms were applied to show direct gene–gene interaction and the shortest path for interactions.

Statistical analysis

Expression levels of regulatory genes in NEC and Surg-CTL tissues were compared using the unpaired t-test (two-sided). Correlation analyses between miR-223 or NFIA and the regulatory genes were performed using Spearman’s correlation test. Data of luciferase reporter assay, western blot and cell apoptosis assays were analyzed by the paired t-test (two-sided). The proliferation of mimic miR-223- or mimic-CTL-transfected cell lines was compared by two-way ANOVA with Bonferroni’s correction. P values ≤ 0.05 were considered statistically significant. All data are presented as mean ± SEM. Statistical analyses were performed with the GRAPHPAD PRISM 5.0 software (La Jolla, CA, USA).

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Conflict of interest

The authors declare no conflict of interest.

Data Accessibility

The data that support the findings of this study are openly available in Gene Expression Omnibus at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE68054, reference number GEO:GSE68054.

Author contributions

KYYC, KL, and PCN designed the study; YZW and KTL collated the data and carried out data analyses; HSL, KHL and YHT carried out patient recruitment and collection of clinical data; HSL and KL contributed to the data analysis; YZW, KYYC and KL wrote the manuscript. All authors have read and approved the final submitted manuscript.

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**Supporting information**

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

**Table S1.** Clinical characteristics of NEC and Surg-CTL infants.

**Table S2.** Top candidate target genes of miR-223.

**Table S3.** A brief summary of functions of potential regulatory genes.

**Table S4.** Bioinformatics tools for miRNA prediction.

**Table S5.** Primers used for site-directed mutagenesis and DNA sequencing.

**Table S6.** Taqman assays used for target gene validation and miR-223 functional study.

**Fig. S1.** Specific binding of mimic miR-223 to 3'UTR of NFIA in stably transfected HEK293. HEK293 cells were constructed to stably express wild type 3'UTR NFIA (WT) reporter gene and were co-transfected with (i) mimic-CTL or (ii) mimic miR-223, or (iii) the irrelevant mimic miR-431 (negative control). Luciferase activity was measured after incubation for 48 hours. Statistical analysis was performed by paired-t test. The data were presented as mean ± SEM, n = 4.

**Fig. S2.** Correlation between miR-223 or NFIA and IL6 in NEC tissues. There were no significant correlations between expression levels of IL-6 and miR-223 or NFIA. Correlation analysis was performed by Spearman correlation test. r represents Spearman correlation coefficient. n = 10 for each group.

**Fig. S3.** Expression of TNF in Caco-2 cells transfected with mimic-CTL or mimic miR-223, and stimulated with LPS or LTA. The level of TNF was not affected by overexpression of miR-223, with or without exposure to LPS or LTA. Gene expression was normalized with GAPDH. Statistical analysis was performed by paired-t test. The data were presented as mean ± SEM, n = 5.

**Fig. S4.** Expressions of NFIA and targeted downstream genes were quantified by qPCR in EC tissues. Results were presented as fold change of respective expressions in NEC versus Surg-CTL. Comparisons of all individual regulatory genes between NEC and...
Surg-CTL were statistically significant ($P < 0.05, n = 10$, Unpaired-$t$ test).

**Fig. S5.** (A) Representative flow cytometric dot-plot of mimics- transfected Caco-2 cells upon LPS or LTA stimulation. Apoptosis and total cells death of mimic-CTL and mimic miR-223-transfected Caco-2 cells upon stimulation with LPS or LTA were measured by flow cytometry. The lower right (LR) quadrant represented apoptotic cells; the upper right quadrant (UR) plus lower right quadrant (LR) were total cell death. (B) Representative flow cytometric dot plot of mimics-transfected FHs74 cells upon LPS or LTA stimulation. Apoptosis and total cells death of mimic-CTL and mimic miR-223-transfected FHs74 cells upon stimulation with LPS or LTA were measured by flow cytometry. The lower right (LR) quadrant represented apoptotic cells; the upper right quadrant (UR) plus lower right quadrant (LR) were total cell death.

**Fig. S6.** Expression of *TLR2* and *TLR4* in Caco-2 cells and FHs74 cells. Expression of *TLR2* and *TLR4* were measured by qPCR. Gene expressions were normalized with *GAPDH*. $n = 1$ for each group.