Negative feedback circuitry between MIR143HG and RBM24 in Hirschsprung disease

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A B S T R A C T

Hirschsprung disease (HSCR) is a genetic disorder of neural crest development. It is also believed that epigenetic changes play a role in the progression of this disease. Here we show that the MIR143 host gene (MIR143HG), the precursor of miR-143 and miR-145, decreased cell proliferation and migration and forms a negative feedback loop with RBM24 in HSCR. As RBM24 mRNA is a target of miR-143, upregulation of RBM24 upon an increase in the level of MIR143HG could be attributed to sequestration of miR-143 by MIR143HG (sponge effect). The RBM24 protein was shown to bind to MIR143HG, and subsequently, accelerated its degradation by destabilizing its transcript and facilitating its interaction with Ago2, thus forming a negative feedback between MIR143HG and RBM24. In addition, experiments using siRNA against DROSHA indicated that RBM24 could promote the biogenesis of miR-143. This feedback loop we describe here represents a novel mode of autoregulation, with implications in HSCR pathogenesis.

1. Introduction

Hirschsprung disease (HSCR) is the most common disorder of the enteric nervous system (ENS) at birth. With an incidence of 1:2000–1:5000 live births, this neurodevelopmental birth defect is attributed to a lack of intramural ganglion cells in the myenteric and submucosal enteric nervous system (ENS) at birth. With an incidence of 1:2000 live births, this neurodevelopmental birth defect is attributed to a lack of intramural ganglion cells in the myenteric and submucosal

Abbreviations: HSCR, Hirschsprung disease; miRNA, microRNA; lncRNA, long non-coding RNA; MIR143HG, MIR143 host gene; RBM24, RNA binding motif protein 24; RBP, RNA binding protein; ceRNA, competing endogenous RNA; MIR-143

Mature miRNAs are only generated in defined miRNA biogenesis pathways. MiRNAs are processed from genome-encoded highly structured transcripts that can form local RNA hairpin structures. In general, microRNAs (miRNAs) are classified as “intergenic” or “intronic”, based on their genomic locations. The key difference between intergenic and intronic miRNAs lies in their mechanisms of transcriptional regulation. Intergenic miRNAs are known to have their own promoters, while intronic miRNAs are believed to share common regulatory mechanisms and expression patterns with their host genes [4]. Up to date, mechanisms of the involvement of long non-coding RNAs (lncRNAs) in disease are still poorly understood. Recent studies have revealed that lncRNAs take part in posttranscriptional events by controlling the stability and translation of mRNAs [5] as well as by acting as competing endogenous RNAs (ceRNAs) [6]. The latter are decoy targets that compete for miRNAs and thereby functionally liberate other transcripts targeted by the same miRNAs [6]. So far, only a few lncRNAs, especially those functioning as miRNA host transcripts, are observed to be involved in the ENS development. Thus, it is warranted to investigate the expression, regulation, and function of IncRNA in HSCR, in order to fully explore the underlying molecular mechanisms of this congenital disease.

MIR143 host gene (MIR143HG) is a miRNA precursor of miR-143 and miR-145 (Supplementary Fig. 3). Although miR-145 is known to be involved in neuronal differentiation [7], the role of miR-143, whose miRNA precursor is homologous to miR-145, has not been reported in
the pathological processes of HSCR, and, likewise, the function of MIR143HG in HSCR remains poorly understood.

To understand the regulatory network of MIR143HG and miR-143, we predicted that RBM24 (RNA binding motif protein 24) is a miR-143 potential target gene according to bioinformatics studies. RBM24 is an RNA binding protein (RBP) that is required for myogenic differentiation [8]. Over the past few years, evidence has accumulated regarding pri-miRNAs [9] and IncRNAs as substrates [10] in post-transcriptional regulation of RBPs. However, the molecular effectors of RBM24 are unclear with regard to IncRNAs.

Here we show that MIR143HG, as a pathogenic factor, can positively control the level of RBM24, a protective factor, through miR-143 sponging in HSCR. In turn, RBM24 decays MIR143HG by decreasing its stability and promoting miR-143 synthesis, as shown in vitro. These data indicate the presence of a specific circuitry in which the expression of MIR143HG and RBM24 is reciprocally regulated in order to establish appropriate progression of neuronal migration and proliferation.

2. Materials and methods

2.1. Study population and sample recruitment

All experiments with human subjects were approved by the Institutional Ethics Committee of Nanjing Medical University (NJMU Birth Cohort), and all subjects gave written informed consent. The experiments were carried out in accordance with the approved guidelines. Total HSCR colon tissues that were immediately frozen and stored at −80 °C after surgery were recruited from the Department of Pediatric Surgery, Nanjing Children’s Hospital Affiliated to Nanjing Medical University, between 2011 and 2014. Primary diagnosis was confirmed after performing barium enema and anorectal manometry evaluation. The Cell Counting Kit-8 (CCK-8) assay (Beyotime, Nantong, China) was used to detect the cell proliferation. The TECAN infinite M200 Multimode microplate reader (Tecan, Männedorf, Switzerland) was employed in measuring the absorbance at 450 nm. All experiments were performed in triplicate independently.

2.2. Cell culture and transfection

Human 293T and SK-N-BE(2) cells were cultured in complete growth medium DMEM (Hyclone, UT, USA), supplemented with 10% heat-inactivated fetal bovine serum (10% FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 °C, 5% CO2. All siRNAs listed in Supplementary Table 2, as well as the mimics and inhibitors of miR-143, were purchased from Realgene (Nanjing, China). The siRNAs against MIR143HG were designed by bioinformatics website (http://rnaidesigner.thermofisher.com/rnaexpress/) and only the one we listed in the table was highly efficient. Flag-RBM24 was constructed for RBM24 overexpression according to Miyamoto S et al. (2009) (Genscript, Nanjing, China). Lipofectamine 2000 Reagent (Invitrogen, CA, USA) was used in all of the transfection experiments following the manufacturer’s instructions.

2.3. RNA isolation and quantitative real-time RT-PCR (qRT-PCR)

Total RNA, containing miRNA, was extracted from tissue specimens and cell lines using Trizol reagent (Life Technologies, CA, USA) according to the manufacturer’s instructions. qRT-PCR was employed to detect the expression levels of RNA. TaqMan® MicroRNA Assays (Applied Biosystems, CA, USA) were used as the probe for miR-143. Human U6 RNA which was amplified as an internal control. For the detection of mRNA, human GAPDH RNA was used as a control. The miRNA or mRNA levels were calculated according to 2−ΔΔCt. Forward (F) and reverse (R) primer sequences were showed in Supplementary Table 2.

2.4. Protein extraction and western blotting

Total proteins were extracted from tissues or cultured cells using RIPA buffer containing protease inhibitors, while nuclear/cytoplasmic fractionation was carried out using the PARIS Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s specifications. Western blot analysis of whole-cell lysates was performed using standard procedures. Polyclonal anti-RBM24 and monoclonal anti-Ago2 antibodies were purchased from Abcam (ab94567, Abcam Trading (shanghai) Company, China). Monoclonal anti-Flag M2 antibody was purchased from Sigma (F1804, Sigma-Aldrich, St. Louis, MO, US). Anti-GAPDH antibody and the secondary antibodies, including anti-rabbit HRP-linked and anti-mouse HRP-linked were purchased from Beyotime (Nantong, China).

2.5. Dual-luciferase reporter assay

For luciferase reporter experiments, the wild-type and mutated 3′-UTR sequences of RBM24 mRNA were inserted into the KpnI and SacI sites of pGL3 promoter vector (Genscript, Nanjing, China), which were named pGL3-RBM24-wild and pGL3-RBM24-mut, respectively. Cells were plated onto 24-well plates at $10^9$ cells/well and transfected with 100 ng of pGL3-RBM24-wild or pGL3-RBM24-mut, and 50 nM miR-143 mimics/inhibitor and negative control, respectively. Firefly and Renilla luciferase activities were measured consecutively using the Dual Luciferase Assay (Promega, Madison, WI) after 48 h transfection according to the manufacturer’s protocol. Transfection was repeated three times in triplicate. The reporter vectors of MIR143HG were constructed as above.

2.6. RNA immunoprecipitation (RIP)

Human 293T and SK-N-BE(2) cells were transfected with an overexpression vector transiently, then immunoprecipitation was carried out using the RIP Kit (Millipore, CA, USA) following the manufacturer’s specifications. 10% of the cell extract was used for total RNA isolation, with the remaining portion being incubated with antibody-coated beads. Isotype-matched immunoglobulin G (IgG) was used as control. Finally, qRT-PCR analysis was carried out to measure the RNA-protein interaction.

2.7. Cell transwell assay

About 100 μl of cell suspension with serum-free medium were seeded in the upper chamber ($10^5$ cells/ml) and the lower chamber was filled with medium containing 10% fetal bovine serum. The cells were incubated for 48 h at 37 °C. Those that did not traverse the membrane were removed by a cotton swab and those on the lower surface of the membrane were stained with crystal violet staining solution (Beyotime, Nantong, China) and photographed under 40× magnification (five views per well). All experiments were performed in triplicate independently.

2.8. Cell proliferation assay

The Cell Counting Kit-8 (CCK-8) assay (Beyotime, Nantong, China) was used to detect the cell proliferation. The TECAN infinite M200 Multimode microplate reader (Tecan, Männedorf, Switzerland) was employed in measuring the absorbance at 450 nm. All experiments were performed in triplicate independently.

2.9. Statistical analysis

The results of qRT-PCR were analyzed with the method of $2^{ΔΔCt}$ in this work. Statistical analysis was performed by STATA 9.2, and presented with Graph PAD prism software. Experimental data of tissue...
samples were presented as box plot of the median and range of log-transformed relative expression level using Wilcoxon rank-sum (Mann–Whitney) test. The top and bottom of the box represent the seventy-fifth and twenty-fifth percentile, respectively. The whiskers indicate the 10th and 90th points. The results obtained from experiments with in vitro assays are presented as mean ± SEM from three separate experiments in triplicates per experiment by double-sided Student’s t-test. Pearson correlation analysis was used to analyze the relationship of expression level of tissues between case and control groups. Results were considered statistically significant at p < 0.05.

3. Results

3.1. Clinical information analysis

The clinical information, including age, gender and body weight, was obtained from participants among 96 HSCR patients and 96 normal controls. The ages of HSCR patients and matched controls were 124.21 ± 6.66 and 115.19 ± 5.49 days, respectively, while the body weight were 5.48 ± 0.13 and 5.25 ± 0.11 kg, respectively. All the clinical information showed no significant difference between HSCR cases and normal controls (Table 1).

3.2. The host gene MIR143HG was inversely regulated by miR-143 in HSCR

Initially, we examined the expression of MIR143HG in 96 paired HSCR and control samples. MIR143HG was shown to be overexpressed in colon tissues from HSCR as shown in Fig. 1a left panel, suggesting that it may be involved in the pathological development of HSCR. However, surprisingly, the expression of miR-143 was significantly lower in the same HSCR tissues than in matched controls as shown in Fig. 1a right panel. Such observations prompted us to explore the possible role of MIR143HG and miR-143 in HSCR by examining their functions in cell migration and proliferation. The Cell Counting Kit-8 (CCK8) assay (Fig. 1b) and cell transwell assay (Fig. 1c) showed, respectively, that the proliferation and migration rates of human 293T and SK-N-BE(2) cells were significantly increased after down-regulated expression of MIR143HG or up-regulated expression of miR-143.

The previous studies identified that the local negative feedback regulation of host genes by miRNAs is essential for animal development [11]. MIR143HG is a long non-coding RNA residing at 5q32. A potential regulation of host genes by miRNAs is essential for animal development. MIR143HG or up-regulated expression of miR-143.

3.3. LncRNA MIR143HG functions as a ceRNA in HSCR

In order to explore the potential mRNA targets of miR-143 as well as the role of MIR143HG in HSCR, the miRBase, PicTar and TargetScan were used to predict potential target gene(s) of miR-143. RBM24, IGFBP5 (insulin-like growth factor binding protein 5) and MAPK7 (mitogen-activated protein kinase 7) were obtained (Supplementary Table 1). To measure the expression levels of these genes, quantitative real-time PCR (qRT-PCR) was employed to examine their mRNA levels. RBM24 was the only candidate gene that showed significant up-regulation in HSCR versus normal colon tissues (Fig. 3a). Western blot analysis also showed that the level of RBM24 was higher in HSCR (Fig. 3b). Its expression is correlated with MIR143HG expression in both HSCR (Supplementary Fig. 1c) and control tissues (Supplementary Fig. 1d). Cell proliferation and migration were investigated after 48 h transfection, and it was demonstrated that knock-down of RBM24 suppressed both processes (Fig. 3c,d).

Similar to its regulation of MIR143HG, miR-143 is also able to suppress RBM24 expression (Fig. 4a). The dual luciferase reporter system was also employed to further confirm RBM24 is a direct target of miR-143. The binding region of the 3’UTR of RBM24 mRNA with wild-type or mutant seed sequence recognizing sites was cloned into a dual-luciferase reporter (Supplementary Fig. 1b). As shown in Fig. 4b, the luciferase signal of the wild-type reporter was suppressed by miR-143 mimics, similar to MIR143HG. These results indicate that RBM24 is also directly regulated by miR-143. Endogenous RBM24 levels were also measured upon RNAi of MIR143HG in order to validate the crosstalk between RBM24 and MIR143HG, and data showed a decrease in RBM24 when MIR143HG was down-regulated (Fig. 4c). These results indicate that RBM24 expression was regulated by MIR143HG.

Taken together, our results demonstrate that both MIR143HG and RBM24 are the direct targets of miR-143, and their expression levels are inversely correlated with that of miR-143. MIR143HG expression can relieve the suppression of RBM24 expression by miR-143, indicating that MIR143HG is a competing endogenous RNA for the binding of miR-143 with RBM24 (Fig. 4d).

3.4. RBM24 specifically regulates MIR143HG expression and stability

To further determine whether RBM24 regulates MIR143HG expression, human 293T and SK-N-BE(2) cells were transfected with an RBM24-expressing plasmid. We found that upon transient expression of RBM24, the level of MIR143HG transcript was decreased (Fig. 5a). The dual luciferase reporter system was employed to determine whether MIR143HG is a direct target of miR-143. Reporter plasmids containing either the wild-type or mutant binding site of MIR143HG downstream a luciferase gene were constructed. These constructs were referred to as pGL3-MIR143HG-wild and pGL3-MIR143HG-mut. Co-transfection of the pGL3-MIR143HG-wild and miR-143 mimics in SK-N-BE(2) cells resulted in a decrease of the luciferase signal when compared with the control vector only with the wild-type. In contrast, the effects of miR143 mimics or miR-143 inhibitor were abrogated when pGL3-MIR143HG-mut was applied (Fig. 2b), confirming that MIR143HG could directly bind to miR-143.

Table 1

<table>
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<th>Variable</th>
<th>Control(n = 96)</th>
<th>HSCR(n = 96)</th>
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<td>Age(days, mean, SE)</td>
<td>124.21(6.66)</td>
<td>115.19(5.49)</td>
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<tr>
<td>Weight (kg, mean, SE)</td>
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<td>5.25(0.11)</td>
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<td>Sex(%)</td>
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<td>Male</td>
<td>60(62.50)</td>
<td>72(75.00)</td>
<td>0.062*</td>
</tr>
<tr>
<td>Female</td>
<td>36(37.50)</td>
<td>24(25.00)</td>
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* Student’s t-test.

** Two-sided chi-squared test.
Fig. 1. MIR143HG and miR-143 are involved in HSCR. (a) The expressions of MIR143HG and miR-143 in 96 HSCR tissues and 96 control tissues. The expression of MIR143HG was significantly higher in patient tissues than in control tissues (left panel), and the expression of miR-143 was significantly lower in HSCR (right panel). Data were presented as box plot of the median and range of log-transformed relative expression levels. The top and bottom of the box represent the seventy-fifth and twenty-fifth percentile. The whiskers indicate the 10th and 90th points. (b) Human 293T and SK-N-BE(2) cell lines were transfected with MIR143HG siRNA or miR-143 mimics to regulate their expression levels. Knockdown of MIR143HG and transfection with a miR-143 mimics promoted cells proliferation. Cell viability is shown as the mean value of absorption at 450 nm (±SE). (c) Knockdown of MIR143HG and miR-143 mimics promoted cells migration in the cell transwell assay. The representative images show the invasive cells at the bottom of the membrane stained with crystal violet (left panel). Scale bars, 180 μm. Quantifications of cell migration is given as number of counted cells (in %) which were observed to migrate (right panel). *p < 0.05, **p < 0.01, ***p < 0.001.

Fig. 2. MIR143HG was regulated directly by miR-143 in HSCR. (a) Human 293T and SK-N-BE(2) cells were transfected with miR-143 mimics or inhibitor for 48 h, qRT-PCR was then performed to evaluate the expression level of MIR143HG. (b) SK-N-BE(2) cells were co-transfected with miR-143 mimics or miR-control, renilla luciferase vector pRL-SV40 and MIR143HG luciferase reporters for 48 h. Both firefly and renilla luciferase activities were measured in the same sample. Firefly luciferase signals were normalized with renilla luciferase signals (left panel). A miR-143 inhibitor was co-transfected with the reporter in the cells (right panel). *Significantly different compared with control (p < 0.05), **p < 0.01, ***p < 0.001.
cells to ~2.84 h in RBM24-siRNA cells (Fig. 6a right panel), indicating that RBM24 destabilizes MIR143HG.

Furthermore, given earlier evidence that the RNA binding protein HuR suppresses target mRNA expression by facilitating its interaction with let-7/RISC [12], we examined whether there was a similar suppression mechanism in the case of RBM24. With bioinformatics analysis, MIR143HG was predicted to be associated with several miRNAs including let-7 and miR-143. MicroRNAs can direct the RNA-induced silencing complex (RISC) to down-regulate gene expression. Ago2 is a necessary component of RISC [13]. Ago2 RNA immunoprecipitation (RIP) analysis in human 293T cells (Fig. 6b left panel) showed robust enrichment of MIR143HG and silencing RBM24 lowered the interaction of Ago2 with MIR143HG (Fig. 6b right panel). U1 was used as positive control (Supplementary Fig. 2a). These results indicate that RBM24 promotes the association of RISC with the MIR143HG transcript.

Together, the above data suggest that RBM24 is able to accelerate MIR143HG decay and destabilize the MIR143HG transcript in vitro.

3.5. MIR143HG interacts with RBM24 physically

After transcription, a non-coding RNA (ncRNA) likely associates with proteins to form a ribonucleoprotein complex that governs ncRNA stability, degradation and function [14]. Since the RNA binding protein HuR was described to have a nuclear/cytoplasmic distribution during muscle differentiation [15], we were interested in the subcellular localization of RBM24 in HSCR. Western blot analysis demonstrated that RBM24 accumulated in the nuclear compartment when RBM24 is overexpressed. We also silenced MIR143HG with siRNA in the 293T cell line. As indicated by previous results, down-regulated MIR143HG inhibited the expression of RBM24. We observed that RBM24 mainly localizes to the cytoplasm when expression levels of RBM24 are low, as represented by conditions of MIR143HG inhibition (Supplementary Fig. 2b). Next, a RIP assay was carried out to analyze the interaction between MIR143HG and RBM24 (Fig. 7a). MIR143HG was extracted from the IP material and detected in association with RBM24 in RBM24 IP samples but not in IgG IP samples from both human 293T and SK-N-BE(2) cells (Fig. 7b). These results indicate that RBM24 binds to MIR143HG transcript directly.

3.6. RBM24 controls miR-143 biogenesis

During the maturation of the mammalian miRNAs, the first step is the nuclear cleavage of the pri-miRNA, which liberates the pre-miRNA. This process is performed by the DROSHA RNase III endonuclease. The nuclear cut by DROSHA defines one end of the mature miRNA [16]. We hypothesized that RBM24 has a direct effect on miR-143 processing by cooperating with DROSHA cleavage. As expected, Flag-RBM24 promoted miR-143 biogenesis, while siRNA against RBM24 produced a consistent decrease in miR-143 accumulation (Fig. 8a,b). To further support these data, we performed qRT-PCR to test miR-145, which is also generated from MIR143HG. We observed an inverse correlation relative to miR-143, i.e. expression levels of miR-145, as those for MIR143HG, were elevated upon RBM24 knockdown and decreased upon RBM24 overexpression (Supplementary Figs. 2c
In addition, we depleted cellular DROSHA by RNAi-mediated knockdown to verify our hypothesis. Results showed that the expression level of MIR143HG and miR-145 were up-regulated (Fig. 8c and Supplementary Fig. 2d) with DROSHA knockdown, while the level of miR-143 decreased (Fig. 8d). These results show that RBM24 could take control of the biogenesis of miR-143 by cooperating with DROSHA.

4. Discussion

Studies on molecular genetics of HSCR patients and using animal models of HSCR have, in parallel, revealed a great deal of the main pathways involved in this multigenetic disease [17]. With the discovery of epigenetic modifications and functional ncRNAs [18], the RNA-mediated regulation can now be explored for the understanding of the
pathogenesis of HSCR. In this study, we investigated certain RNAs by focusing on their possible interactions and identified a feedback loop of their combined regulations in HSCR, as schematically illustrated in Fig. 9.

![Image](image1)

**Fig. 6.** RBM24 destabilizes MIR143HG transcript and accelerates the degradation of MIR143HG. (a) RBM24 shortens the half-life of MIR143HG transcript. The level of MIR143HG transcript was determined by qRT-PCR analysis and the relative half-life of MIR143HG transcript was calculated. Human 293T cells were treated with siRNA against RBM24 and then treated with actinomycin-D (14 μg/ml) over a 5 h period at 1 h interval (left panel). Knockdown of RBM24 in SK-N-BE(2) cells with actinomycin-D (20 μg/ml) over a 2 h period at 0.5 h interval (right panel). Total RNAs were purified, and the levels of MIR143HG and GAPDH transcripts were analyzed using qRT-PCR. The relative levels of MIR143HG transcript were normalized with the levels of GAPDH transcript and plotted along with time to calculate the relative half-life of MIR143HG in the presence or absence of RBM24. The x axis represents the time after addition of actinomycin-D, and the y axis represents the relative levels of remaining MIR143HG transcript in the cells. (b) RBM24 is necessary for the degradation of MIR143HG. The association of MIR143HG with Ago2 was assessed by RIP analysis in the 293T cell line (left panel). Ago2 IP was conducted in cells transfected with RNAi against RBM24 (right panel).

![Image](image2)

**Fig. 7.** RBM24 binds to MIR143HG transcript in vitro. (a) An RNA immunoprecipitation assay was carried out using extracts from human 293T or SK-N-BE(2) cells transiently expressing Flag-tagged RBM24 or mock. The anti-Flag antibody was used to immunoprecipitate potential RBM24-RNA complexes, whereas the anti-IgG antibody was used as the control. (b) MIR143HG transcript in potential RBM24-RNA complexes was detected by qRT-PCR in human 293T cells (left panel) and SK-N-BE(2) cells (right panel).

Our study revealed that MIR143HG may contribute to the development of HSCR based on our findings from CCK8 and cell transwell assays following the confirmation of the high expression of MIR143HG in HSCR.
clinical samples. Our data from human 293T and SK-N-BE(2) cell lines suggest that MIR143HG serves as a competing endogenous RNA in the regulation of RBM24 by miR-143. These results for the first time highlight the significance of ceRNAs in HSCR development. So far, many of the genes with known ceRNA interactors have been implicated in human disease [19]. We also propose that additional ceRNA regulation may also contribute to congenital disease initiation and progression. In addition, this observation in HSCR provides another evidence to support that ceRNA crosstalk is crucially relevant in various pathophysiological conditions, as has been suggested by previous reports [20,21].

A previous study found that the expression of RBM24 is muscle-specific [22]. We discovered that RBM24 is also expressed in colon tissues and regulates cell proliferation and migration in human 293T and SK-N-BE(2) lines. Our data show that when RBM24 is over-expressed, it gets more abundant in the nucleus than in the cytoplasm. The subcellular localization after silencing MIR143HG suggests that there might exist a threshold value for RBM24 recruitment to the nucleus. To identify such threshold value would be helpful toward understanding the negative RBM24/MIR143HG feedback loop in HSCR. Another question is what could prompt this recruitment of RBM24 to the nucleus? We propose that MIR143HG is responsible for such recruitment. The up-regulated expression of MIR143HG might trigger the negative RBM24/MIR143HG loop. Furthermore, the expression of MIR143HG may serve as a predictive marker for clinical outcomes in HSCR in addition to biopsy that is commonly used for preoperative diagnosis. Nevertheless, this is one possibility that has to be explored in future studies.

RBM24 was particularly interesting to us due to its contribution to the stability of mRNA. RBM24 shares high similarity with RBM38 in the RRM region [23] and is reported to have an effect on several important RNA substrates via mRNA stability, such as p21, myogenin and p63 [23,24]. Besides identifying RBM24 as a ceRNA effector, we show that RBM24 binds to MIR143HG and reduces its level, thus yielding a

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Fig. 8. RBM24 controls the biogenesis of miR-143. (a, b) RBM24 promotes the biogenesis of miR-143 in human 293T and SK-N-BE(2) cells. The expression of miR-143 was detected by qRT-PCR. The level of miR-143 is increased by ectopic expression of RBM24. Knockdown of RBM24 led to a decrease of miR-143 levels. (c) RBM24 cooperates with DROSHA in human 253T cells. After knock-down of cellular DROSHA by siRNA, the mRNA expression levels of DROSHA was detected by qRT-PCR. (d) The expression levels of miR-143 were evaluated by qRT-PCR. *p < 0.05, **p < 0.01, ***p < 0.001.

Fig. 9. Proposed model of the feedback loop between MIR143HG and RBM24 in HSCR. MIR143HG is over-expressed in HSCR and triggers the development of disease by decreasing cell proliferation and migration. RBM24 is the direct target of miR-143. Up-regulated MIR143HG favors RBM24 accumulation in the nucleus through its function as a sponge for miR-143. Elevated RBM24 protein in the nucleus interacts with MIR143HG directly to accelerate its degradation by lowering the stability of the MIR143HG transcript and promoting RISC binding to MIR143HG, thus forming a negative feedback loop.
negative feedback control. A negative feedback loop often maintains cell homeostasis [25]. A previous report show that negative feedback signal network underlies oncogene-induced senescence [26]. In this mechanism, MIR143HG acts as a disease trigger, the negative feedback mechanism could be used to balance the pathogenic effect of MIR143HG, thus preventing human colon from aganglionosis. This negative feedback circuitry between lncRNA and RBPs is novel in our understanding of gene regulation.

Very importantly, our data for the first time uncover a role for RBM24 as a post-transcriptional inhibitor of lncRNA. These findings also indicate that RBPs and miRNAs can function jointly in the suppression of a shared target lncRNA.

Interestingly, our results showed that the expression of miR-143 was contrary to that of MIR143HG, and, in contrast, the expression of miR-145 was consistent with that of MIR143HG. These results are totally different from those in cancer [27,28]. Our initial thinking for such a phenomenon relied on the cooperation with DROSHA cleavage. As expected, depletion of cellular DROSHA inhibited processing, causing the down-regulation of miR-143. These results suggest that RBM24 may be associated with the biogenesis of miR-143 at the posttranscriptional level by acting in the same pathway as DROSHA. Moreover, we speculated that there may exist some mechanism(s) that can attract RBM24 to the location of miR-143 specifically but not to the location of miR-145, in a way similar to RBM24 that mediates exon inclusion rather than intron [29]. It should be noted that miRNA levels are tightly controlled, and the biogenesis of miRNAs is regulated at various steps by a variety of factors. Certainly more studies are needed to clarify the function of RBM24 in the process, such as whether it works as a splicing enhancer.

Disease is a complex interplay of pathogenic and protective factors. In this work, MIR143HG and miR-143 were identified as pathogenicity factors, while, the negative feedback between MIR143HG and RBM24 was considered as protective factor. However, the deregulation of MIR143HG and miR-143 seems to outweigh the counteracting negative feedback circuit, resulting in disease manifestation. Possibly, amplification of the protective role of the feedback circuit may be a strategy to antagonize pathogenesis. In summary, this study is the first to discover an abnormal InC RNA MIR143HG-regulated network in HSCR, both transcriptionally and post-transcriptionally. As a complete understanding of the interplay among RBPs, miRNAs, and lncRNAs is needed, we just began to get into the complexity of post-transcriptional gene control. We believe that to fully understand this negative feedback which may play a protective role in the development of HSCR will not only help the elucidation of an autoregulatory mechanism but also be valuable in developing strategies for the treatment of HSCR.

Author contributions

Conceived and designed the experiments: YX, WT, CXD; Performed the experiments: ZYS, RJZ, HX; Analyzed the data: PFC, HXJ; Wrote the paper: CXD, ZYS, RJZ, BH. All authors discussed the results and commented on the manuscript.

Conflict of interest

The authors have declared no conflict of interest.

Transparency Document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jbbdis.2016.08.017.

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