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Negative feedback circuitry between MIR143HG and RBM24 in Hirschsprung disease

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ABSTRACT

Hirschsprung disease (HSCR) is a genetic disorder of neural crest development. It is also believed that epigenetic changes plays 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 plexuses involving varying regions of the gastrointestinal tract [1]. Thus, any aberration of survival, proliferation or migration of enteric neural crest cells (ENCCs) will result in aganglionosis of the distal gut [2]. Although genetic studies have verified that the mutations in *RET* (*RET* proto-oncogene) and *EDNRB* (endothelin receptor type B) are mainly responsible for HSCR [3], the underlying molecular and genetic mechanisms for HSCR still remain unclear.

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 lncRNA 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

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; CCK-8, cell counting kit-8; DMEM, Dubelcco modified Eagle's medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RIP, RNA immunoprecipitation; 3′-UTR, 3′-untranslated region.

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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-mirRNAs [9] and lncRNAs as substrates [10] in post-transcriptional regulation of RBPs. However, the molecular effectors of RBM24 are unclear with regard to lncRNAs.

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. Final diagnosis of HSCR was achieved via pathological analysis for the aganglionosis. Negative controls were randomly selected from patients without HSCR or other congenital malformation received surgical treatment because of intussusceptions or incarcerated and strangulated inguinal hernia without the ischemia or necrosis parts and matched with cases on age and gender. The total population was all Han Chinese.

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% CO_2. All siRNAs listed in Supplementary Table 2, as well as the mimics and inhibitor of miR-143, were purchased from Realgene (Nanjing, China). The siRNAs against MIR143HG were designed by bioinformatics website (http://rnaidesigner.thermofisher.com/rnaiexpress/) 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 $2^{-\Delta\Delta Ct}$. 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 *Kpn*I and *Sac*I 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 2×10^9 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 (1×10^6 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, Mechelen, Belgium) 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 $2^{\wedge(-\Delta\Delta Ct)}$ in this work. Statistical analysis was performed by STATA 9.2, and presented with Graph PAD prism software. Experimental data of tissue