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MicroRNA 125 represses nonsense-mediated mRNA decay by regulating SMG1 expression

Gang Wang [1], Bowen Jiang [1], Chenliang Jia, Baofeng Chai *, Aihua Liang

Key Laboratory of Chemical Biology and Molecular Engineering, Ministry of Education, China
Institute of Biotechnology, Shanxi University, Taiyuan 030006, China

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ABSTRACT

Nonsense-mediated mRNA decay (NMD) is a cellular response mechanism that eliminates aberrant mRNA transcripts and thereby prevents the production of potentially deleterious C-terminally truncated proteins. The phosphatidylinositol 3-kinase-related protein kinase SMG1 is considered to be an essential factor in the NMD pathway. We demonstrate that the brain-enriched microRNA, miRNA-125 (miRNA-125a and miRNA-125b) is a bona fide negative regulator of SMG1 in humans. Down-regulation of SMG1 expression is mediated by miRNA-125 binding to a microRNA response element in the 3′ untranslated region of SMG1 mRNA, which leads to degradation of the SMG1 mRNA. In human cells, overexpression of miR-125 represses the endogenous levels of SMG1 protein and suppresses the NMD pathway; however, knockdown of miR-125 up-regulates the NMD pathway. These results suggest the existence of an RNA circuit linking the microRNA and NMD pathways.

1. Introduction

Nonsense-mediated mRNA decay (NMD) is a translation-associated mRNA surveillance mechanism that eliminates mRNAs containing premature translation–termination codons (PTCs). In mammalian cells, the NMD is also involved in pre-mRNA splicing; strong mRNA reduction occurs only when the PTC is located upstream of the last intron [1]. Increasing evidence indicates that the exon junction complex (EJC) mediates the NMD pathway [2,3]. Numerous factors of NMD have been identified: UPF1, UPF2, UPF3A, UPF3B, SMG1, SMG5, SMG6 and SMG7 [2,4]. Of these NMD factors, the RNA helicase UPF1 is functionally the most important factor and its sequential phosphorylation/dephosphorylation cycles are essential for NMD function [4,5]. UPF1 is phosphorylated and regulated by the NMD factor and protein kinase SMG1 (phosphatidylinositol 3-kinase-related protein kinase) [4].

MicroRNAs (miRNAs) are small (~23 nt), regulatory, noncoding RNA molecules that control the expression of their target mRNAs predominantly by binding to the 3′ untranslated region (UTR) [6,7]. The mechanism of how miRNAs silence their target genes remains unclear; nevertheless, most studies overwhelmingly support the two consensus models of translation repression and degradation of the mRNA [6,8–10]. miR-128 was recently reported to repress the NMD function by targeting UPF1 and the EJC core component MLN51 (also known as CASC3 or BTZ) in mammals [11]. Specifically, induction of the brain (and thymus)-restricted miRNA-128 early in brain development reduces the NMD efficiency by targeting the mRNAs encoding UPF1 and MLN51, thereby reducing the functional levels of these proteins [12]. These data suggest the existence of a conserved mRNA circuit that links the microRNA and NMD pathways and results in the induction of cell type-specific transcripts during development.

We report that miRNA-125 (including miRNA-125a and miRNA-125b) represses the expression of SMG1 by degradation of the SMG1 mRNA. This down-regulation of SMG1 by miRNA-125 also inhibits the NMD pathway. These results suggest that an RNA circuit links the microRNA and NMD pathways.

2. Materials and methods

2.1. Mammalian cell culture, transfections and molecular constructs

HeLa cells and HEK 293T cells were cultured in DMEM (HyClone) supplemented with 10% fetal calf serum (HyClone) and grown at 37 °C in a humidified atmosphere of 5% CO_2 according to standard procedures.

Cells were cultured in 12-well or 24-well plates and treated with the transfection agent Lipofectamine™ 2000 (Invitrogen) in conjunction with either plasmids and/or microRNA mimics (or inhibitors), according to the manufacturer's instructions.

SMG1 3′-UTR was amplified by PCR (primers BF336 and BF337; the primer sequences are listed in Table S1) and cloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector

* Corresponding author.
E-mail address: bfchai@sxu.edu.cn (B. Chai).
[1] These authors contributed equally to this work.

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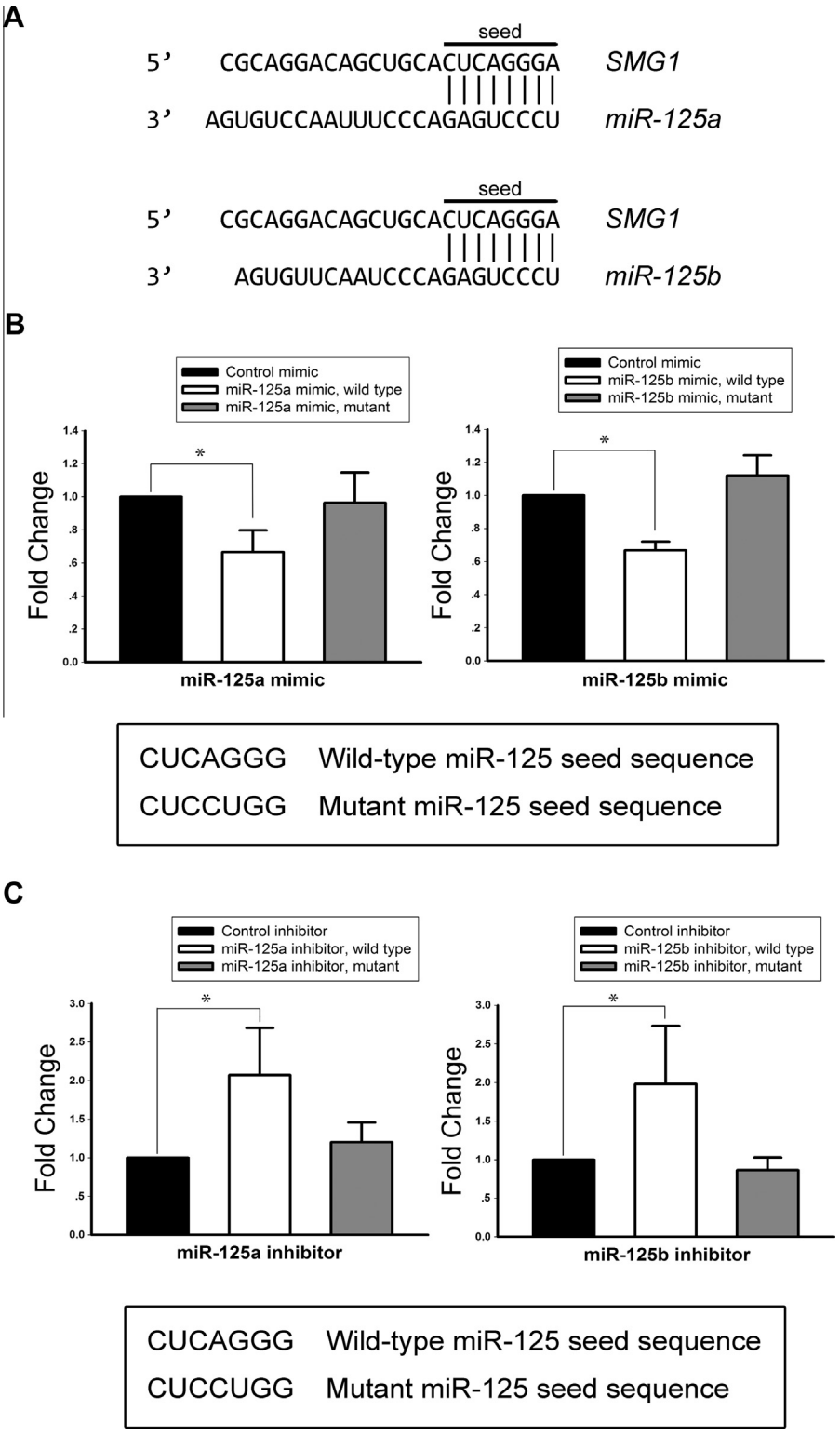

Fig. 1. miRNA-125 targets the 3′-UTR of SMG1. (A) Predicted base pairing of mature miR-125a and miR-125b seed sequences in the 3′-UTR of SMG1 (http://www.targetscan.org/). (B) Luciferase expression in HEK 293T cells transfected for 24 h with miR-125a mimic, miR-125b mimic or a negative-control miRNA mimic (Biomics). The cells were co-transfected with the pmirGLO dual-luciferase miRNA Target Expression Vector (Promega) reporter containing SMG1 3′-UTR sequence, including the putative miR-125a or miR-125b target site shown in (A). The mutant has the indicated 2 nt mutation in the target sequence. The Renilla luciferase activity was normalized to Firefly luciferase activity levels. (C) Luciferase expression in HeLa cells transfected for 48 h with miR-125a inhibitor, miR-125b inhibitor or a negative-control inhibitor (Biomics). The cells were co-transfected with the pmirGLO dual-luciferase miRNA Target Expression Vector (Promega) reporter introduced as in (B). The Renilla luciferase activity was normalized to Firefly luciferase activity.

(Promega). The resulting construct was named pmirGLO-SMG1-WT. The miR-125a mimic (or inhibitor), miR-125b mimic (or inhibitor), and miR-control (miR-67) mimic (or inhibitor) were synthesized by Biomics.

2.2. Dual-luciferase reporter assays

Luciferase reporter assays were performed using the pmirGLO Dual-Luciferase miRNA Target Expression Vector reporter system. HeLa cells or HEK 293T cells were co-transfected with either pmirGLO-SMG1-WT (or pmirGLO-SMG1-MU) plasmids and the miR-125 mimic (or inhibitor) or pmirGLO-SMG1-WT (or pmirGLO-SMG1-MU) plasmids and the control mimic (or inhibitor). Firefly and Renilla luciferase activities were quantified using the Dual-Luciferase Reporter Assay System (Promega), and Renilla luciferase activity was normalized to the Firefly luciferase activity.

2.3. Protein extractions and Western blotting analysis

HeLa cells were cultured and transfected with miR-125a mimics or miR-125b mimics. Forty-eight hours post-transfection, the cells were washed in cold PBS, and protein was extracted using the cell lysis buffer for Western and IP protocols (20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100; Beyotime), according to the manufacturer's instructions.

The protein extracts were boiled and resolved by 6% SDS-PAGE and electroblotted onto 0.45-μm pore-size nitrocellulose membranes (Millipore) for 2 h at 90 V using a Tran-Blot Electrophoretic Transfer Cell (Bio-Rad). The membranes were soaked in a blocking buffer (5% skim milk, 140 mM NaCl, 27 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, and 0.5% Tween-20) for 1 h at room temperature and then incubated at 4 °C overnight with the mouse monoclonal anti-SMG1 antibody (catalog No. Sc-135563; Santa Cruz) diluted 1:200 in blocking buffer. The membranes were washed 3 times with wash buffer (140 mM NaCl, 27 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, and 0.5% Tween-20) and incubated with HRP-goat anti-mouse IgG (catalog No. 81-6520; Invitrogen), diluted 1:10,000 in blocking buffer, at room temperature for 1 h. Proteins were detected using a highly sensitive enhanced chemiluminescent substrate (SuperSignal West Dura Extended Duration Substrate; Pierce Chemical).

2.4. RNA extractions and quantitative RT-PCR (qRT-PCR) assays

HeLa cells or HEK 293T cells were cultured and transfected with miR-125 mimics (or inhibitors) as described above. Forty-eight hours post-transfection, the cells were washed with phosphate-buffered saline (PBS), and total RNA was isolated using RNAiso Plus (TAKARA) according to the manufacturer's protocol. A total of 500 ng of RNA was used for reverse transcription using PrimerScript® RT Master Mix (TAKARA). Quantitative RT-PCR (qRT-PCR) assays included 0.4 μM of each primer and 12.5 ng of cDNA in SYBR *Premix Ex Taq*™ II Tli RnaseH Plus (TAKARA). PCR reactions were performed in an ABI StepOne Plus (Applied Biosystems) thermocycler. Quantification of gene expression was based on the ΔΔCT method and normalized to the β-actin gene levels. Melting curve and electrophoresis analyses were performed to confirm PCR product specificities and exclude nonspecific amplification. The PCR primers for SMG1 are BF328 and BF329; the PCR primers for TBL2 are BF332 and BF333; PCR primers for β-actin are BF330 and BF331 (primer sequences are listed in Table S1).

3. Results and discussion

3.1. miRNA-125 targets the 3′-UTR of SMG1

We used the miRNA algorithm TargetScan (http://www.targetscan.org/) to predict and identify miRNAs that have the potential to regulate the NMD by targeting the NMD factor SMG1. This algorithm identified miR-125a and miR-125b as potential regulators of SMG1 (Fig. 1A). We found that the 3′-UTR of SMG1 contains a sequence motif that is identical to the seed sequence of miR-125 (Fig. 1A). Both of the miR-125 isoforms (125a and 125b) share perfect sequence homology in this seed sequence but vary towards the 3′-end of the mature miR molecule (Fig. 1A).

To determine whether miR-125 directly targets the 3′-UTR of SMG1, we amplified the 500-nt-long 3′-UTR of SMG1 from human genomic DNA and cloned it into a dual-luciferase miRNA Target Expression Vector. Co-transfection of both the dual-luciferase vectors and miR-125a or miR-125b mimic (a double-stranded form of miR-125 that is processed into a functional single-stranded miRNA in cells) into HEK 293T cells led to a 40% reduction in normalized luciferase values compared with relevant controls, including a control miRNA mimic (Fig. 1B). Introduction of a two-nucleotide mutation in the miR-125 seed sequence in the 3′-UTR of SMG1 abrogated this regulation (Fig. 1B).

We also detected luciferase activity in HeLa cells co-transfected with the dual-luciferase vectors and miR-125a or miR-125b inhibitor. Following transfection, luciferase activity increased from SMG1 3′-UTR reporter vectors (Fig. 1C). Introduction of a two-nucleotide mutation in the miR-125 seed sequence in the 3′-UTR of SMG1 abrogated this regulation (Fig. 1C). These data indicate that the predicted binding sites (miRNA response elements, MREs) in the seed sequence are critical for the direct and specific binding of miR-125 to the SMG1 mRNA.

3.2. miRNA-125 represses SMG1 expression

We transfected miR-125a mimic or miR-125b mimic into HeLa cells to detect the ability of miR-125 to repress the expression of SMG1. The miR-125a mimic decreased SMG1 protein levels by ~40%, and the miR-125b mimic decreased SMG1 protein levels by ~60% (Fig. 2).

Many studies indicate that to silence gene expression, miRNA either represses the translation of its target mRNA or promotes its degradation [6]. A more recent analysis of reduced protein production in mammals demonstrated that mammalian miRNA predominantly acts to decrease target mRNA levels through miRNA-mediated destabilization of the target mRNA [6,13].

To determine whether miRNA-125 promoted SMG1 mRNA degradation, we quantified the SMG1 mRNA levels in cells transfected with miRNA-125 mimic or inhibitor. The miR-125a and miR-125b mimic decreased SMG1 mRNA levels by ~60% (Fig. 3A), whereas the miR-125a or miR-125b inhibitor increased SMG1 mRNA levels

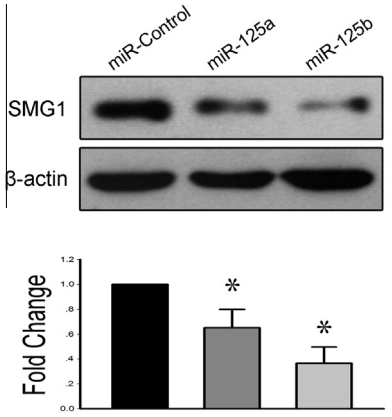

Fig. 2. miR-125 reduces SMG1 protein levels. Western Blot analysis of endogenous SMG1 protein levels in HeLa cells 48 h after transfection with mature miR-125a mimic, miR-125b mimic or a random negative-control miRNA mimic (Biomics). β-Actin was used as the internal control. Bottom panels show the mean of three experiments; error bars represent standard deviation (SD).

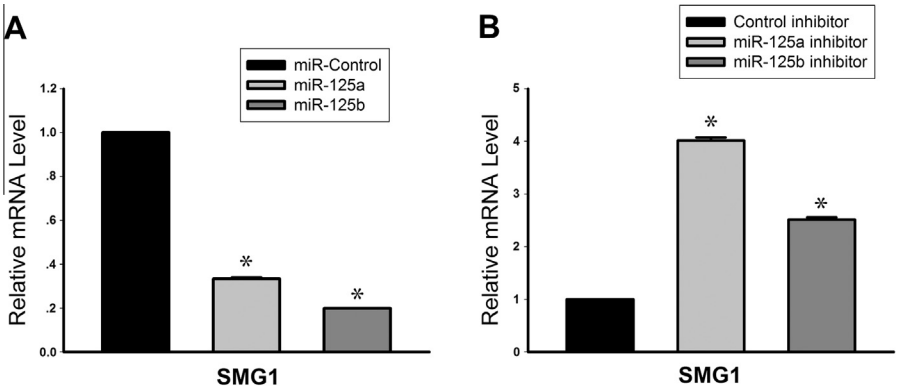

Fig. 3. miR-125 reduces SMG1 mRNA levels. Quantitative polymerase chain reaction (qPCR) analysis of HeLa cells transfected for 48 h with the indicated miRNA mimics (A) or inhibitors (B). Shown are the results from three independent experiments, normalized to β-actin mRNA levels.

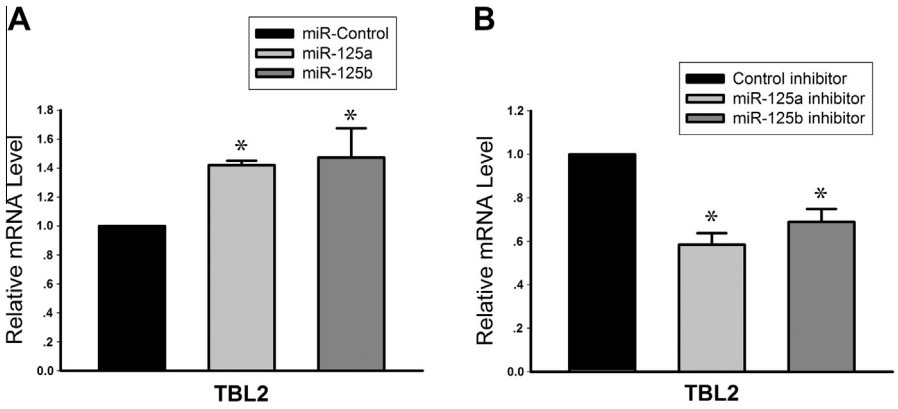

Fig. 4. miR-125 regulates the NMD. Quantitative polymerase chain reaction (qPCR) analysis of the NMD substrate TBL2 mRNA in HeLa cells transfected for 48 h with the indicated miRNA mimics (A) or inhibitors (B). Shown are the results from three independent experiments, normalized to β-actin mRNA levels.

(Fig. 3B). Our results suggest that miR-125a and miR-125b decreased SMG1 levels by degradation of the target SMG1 mRNA.

3.3. miRNA-125 represses NMD by down-regulating SMG1 expression

The discovery that SMG1 is down-regulated by miR-125 suggested the possibility that miR-125 regulates the NMD. To study this mechanism, we first examined whether miR-125 up-regulates transcription of the known NMD substrate TBL2 [11,14]. TBL2 mRNA has a long 3′-UTR, a feature recently shown to trigger mammalian NMD [11,15]. We found that TBL2 mRNA was up-regulated in HeLa cells transfected with the miR-125a mimic or the miR-125b mimic (Fig. 4A), whereas the miR-125a inhibitor or the miR-125b inhibitor decreased TBL2 mRNA levels in HeLa cells (Fig. 4B). These data indicate that miR-125 regulates the NMD. In addition, we detected the levels of other NMD substrates in cells transfected with miR-125 mimics and obtained results similar to those from our studies on the regulation of TBL2 transcription (data not shown).

In the past decade, miRNA regulation of gene expression has attracted widespread attention, resulting in rapid advances. miRNAs regulate the expression of their target mRNAs predominantly by binding to the 3′ UTR. A single 3′ UTR may have binding sites for many miRNAs or multiple sites for a single miRNA, suggesting that the post-transcriptional control of gene expression exerted by these regulatory RNAs is complex [6]. Recently, miRNA-125a and miRNA-125b were shown to inhibit the p53 tumor suppressor gene and tumor necrosis factor alpha-induced protein 3 (TNFAIP3, A20) [8,16,17]. Our findings help to elucidate the mechanism of miRNA-125 regulation of gene expression and indicate that miRNA-125 is a credible regulator in the NMD pathway.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.129.

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