

Construction and Functional Analysis of lncRNAs RMST promoter luciferase reporter plasmid

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Abstract. Long noncoding RNAs (lncRNAs) are abundant in the mammalian transcriptome and many are specifically expressed in the brain. Rhabdomyosarcoma 2-associated transcript (RMST), which are indispensable for neurogenesis. RMST expression is specific to the brain, regulated by the transcriptional repressor REST, and increases during neuronal differentiation, indicating a role in neurogenesis. RMST physically interacts with SOX2, a transcription factor known to regulate neural fate. RMST and SOX2 coregulate a large pool of downstream genes implicated in neurogenesis. It is known that the Myocardin can regulate the downstream gene transcription by combining the SRF-forming complex with the CArG box site of the target gene promoter. By analyzing the promoter sequence of RMST, we found that there were one CArG box binding sites within the promoter. In the present study, the gene fragment of human RMST gene promoter containing CArG box was cloned into empty vector pGL3-Basic to construct RMST promoter luciferase reporter plasmid. Myocardin expression plasmids, together with RMST promoter-luciferase plasmids were transfected into COS-7 cells, and then luciferase assay was performed to analyze the effects of Myocardin on regulating the promoter activity of RMST. The results showed that Myocardin can activate the transcription of RMST. Construction of RMST promoter luciferase reporter plasmid will provide the theory basis for investigating the function of RMST in neurogenesis.

1. Introduction

lncRNAs are typically defined as transcripts longer than 200 nucleotides and lack an appreciable open reading frame [1], which do not cause protein production but regulate the expression of other genes. Interestingly, many lncRNAs are highly expressed in the adult and developing brain. lncRNAs RMST is an important regulatory factor in the differentiation of NSCs into neurons [2], mainly in the midbrain dopaminergic neuronal precursor cells. Knockdown of the lncRNAs RMST gene can significantly inhibit the differentiation of NSCs (neural stem cells) and NPCs (neural precursor cells) into neurons; The overexpression of RMST gene causes the proportion of NSCs and NPCs to differentiate into neurons [3]. Myocardin transcription factor can regulate the expression of genes, such as differentiation gene of SMCs [4]. As a transcription factor, myocardin can bind to CArG box and activate the transcription of target gene. By analyzing the promoter sequence of RMST, we found that there were one CArG box binding sites within the promoter. Thus, in this paper, luciferase reporter gene



plasmids containing promoter was constructed and the role of Myocardin in regulating the transcription of RMST was investigated by luciferase assay.

2. Materials and Methods

2.1. Cell Culture and Genomic extraction.

COS-7 cells and Hela cells were obtained from American Type Culture Collection (ATCC) and were cultured in Dulbecco's Modified Eagle Medium or Nutrient Mixture F-12 medium (DMEM/F-12, GIBCO) respectively. All culture media contain 10% FBS, 100 U/mL streptomycin and penicillin (100U/mL). All cultures were maintained in a 37°C incubator supplemented with 5% CO₂. The genomic DNA of Hela cells was extracted in Universal Genomic DNA Kit (CWBIO) according to the instructions.

2.2. Plasmid Construction.

The RMST promoter containing Myocardin binding site (from -629 to -620) was amplified from genomic DNA of Hela cells by PCR and cloned into a pGL3 luciferase reporter vector. The primers used in PCR reactions were as follows: forward primer-TACTCTCGAGCTAAGCAGCAAAGCATTC, reverse primer – CCTGAAGCTTCAAGGCATTGCCTATAAC. Amplification conditions for PCR are as follows: predegeneration for 5 min at 94°C, denaturation for 30s at 94°C, annealing for 30s at 56°C and extension for 1min at 72°C. PCR reaction was carried out for 33 cycles.

The PCR products and pGL3-Basic vector were digested with restriction enzyme XhoI (Thermo) and HindIII (Thermo) at 37°C for 1.5h and then the gene fragment was linked to pGL3-Basic vector using T4 DNA ligase (Thermo) at 16°C for 12h. The recombinant plasmids were transformed into E.coli DH5 α and confirmed by restriction enzyme digestion and DNA sequencing.

2.3. Plasmid transfection.

For transfection experiments, COS-7 cells were plated in 24-well dishes when they were 60-70% confluent. Transfection was carried out using transfection reagent Turbofect (Thermo) following the manual of the manufacturer.

2.4. Luciferase Reporter Assays.

Luciferase activity assay was performed using the Luciferase Assay System (Promega) according to the instructions. Briefly, 24 hours after transfection, the transfected cells were lysed in Cell Culture Lysis Reagent. 20 μ L of cell lysate was added into a 96-well enzyme label plate and reading was initiated by the injection of 100 μ L of Luciferase Assay Reagent into the plate on a SynergyTM 4 (Biotek). Each sample was examined in duplicate and it was repeated in 3 different experiments.

2.5. Statistical analyses.

Data were presented as means $\pm$ SD from at least three separate experiments. The significant difference was examined using the Student's t test. The minimal level of significance was $P < 0.05$.

3. Results

3.1. Construction of RMST Luciferase Reporter Plasmid.

The schematic structure of human RMST promoter containing myocardin binding site was shown in Fig. 1. The RMST promoter sequence from -800 to +100 was cloned from the genomic DNA of the hela cell line.

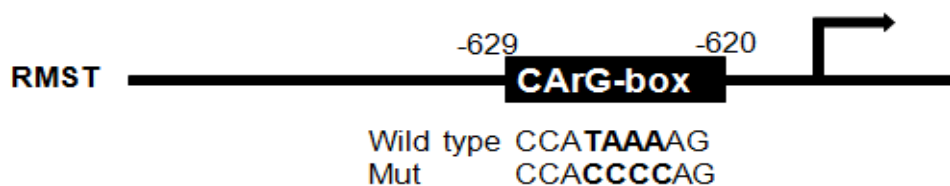


Figure1. A structure schematic of luciferase reporter plasmids containing RMST promoter.

To estimate the PCR amplification of RMST promoter, agarose gel electrophoresis was performed. As shown in Fig. 2, one band emerged nearby at the site of 1000 bp, which represented PCR product of RMST promoter.

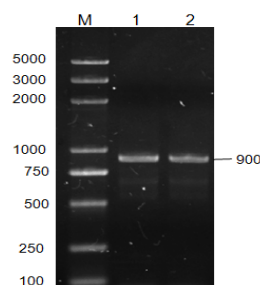


Figure 2. Agarose gel electrophoretic analysis of PCR amplification products (M: DL2000 Plus Marker; 1: PCR products).

As shown in Fig. 3A, the recombinant plasmids were digested with restriction enzyme XhoI (Thermo) and HindIII (Thermo) II at 37°C for 1h. Purified vector fragments and PCR fragments were used in a DNA ligation reaction to generate recombinant plasmids.

As shown in Fig. 3B, the recombinant plasmid was digested with XhoI (Thermo) and HindIII (Thermo) at 37°C for 1h and 1% agarose gel electrophoretic was used to analyze the DNA products, the agarose electrophoresis of DNA showed two major bands: a 900bp band could represent RMST gene promoter; a lower than 5.0-kb band could represent a pGL3-Basic vector. RMST promoter luciferase reporter gene plasmid (p-RMST-Luci) was successfully constructed, confirmed by sequencing.

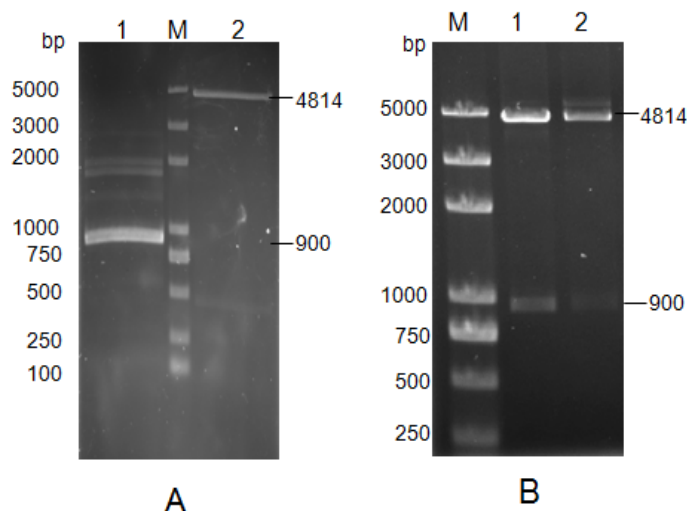


Figure 3. A: Restriction enzyme digestion of pGL3-basic vector and PCR product (M: DL2000 Plus Marker; 1: Double digestion of RMST promoter 2: Double digestion of pGL3-basic vector;).

B: Agarose gel electrophoretic analysis of recombinant plasmids. M: DL2000 Plus Marker; 1,2: Double digestion of RMST recombinant plasmid.

3.2. Luciferase Assay.

To analyze whether the transcription activity of RMST promoter is regulated by myocardin, the RMST promoter plasmids or mutant RMST promoter plasmids (CArG box binding sites mutation, Figure 1) were transfected into COS7 cells together with pcDNA3.1 empty vector or pcDNA3.1- myocardin expressive plasmids for 24h. As shown in Fig. 4A, compared to the control group, the overexpression of myocardin significantly upregulated the activity of RMST promoter approximately 3-fold, furthermore, myocardin has no significant influence on mutant RMST promoter plasmids (Fig. 4B), suggesting that myocardin could activate the transcription of RMST and RMST might be one target gene of myocardin.

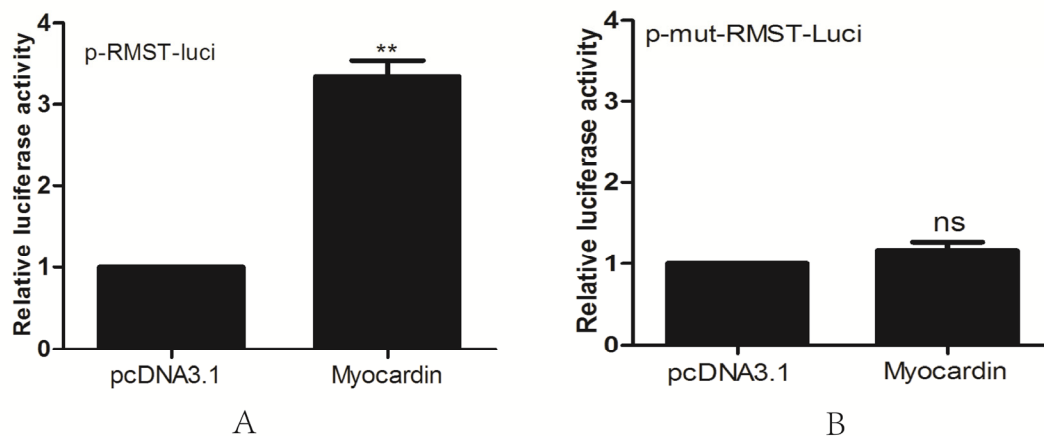


Figure 4. (A) myocardin increased the transcriptional activity of RMST promoter. (B) myocardin has no significant influence on the transcriptional activity of mutation RMST promoter (**, $P < 0.01$, $n = 4$; Data are presented as means $\pm$ SD)

4. Discussion

RMST plays an important role in neuronal differentiation [5]. To explore the function of RMST, as an indispensable gene for neurogenesis, it will provide more knowledge about the role and regulation of neuronal differentiation in physiological and pathology. In this study, RMST promoter luciferase reporter plasmid was successfully constructed. Luciferase assay confirmed that myocardin can transactivate the RMST promoter. some transcription factors such as REST and SOX2 have been shown to interact with RMST and regulate of neural stem cell fate [2].

Christopher W. Uhde et al. report that Lmx1a (LIM homeobox transcription factor 1) can co-express with RMST, which can promote mice brain development together [6]. Maxime Bouchard [7] also identified RMST (Ncrms), which is expressed in the developing mid-hindbrain region under the control of Pax2.

From literatures, we know that RMST might play an important role in the regulation of neuronal differentiation. But, it is not clear whether myocardin can regulate neural differentiation, and the mechanism is unclear. So further study is needed to illuminate the role of RMST and myocardin in neurogenesis.

References

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