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Original Article

RNA-binding protein HuR regulates hsa-let-7c expression by its RNA recognition motif

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Abstract: MicroRNAs (miRNAs) are small noncoding RNAs that control diverse cellular and developmental events through repression of large sets of target mRNAs. miRNAs expressions were mainly regulated at two levels: transcriptional and post-transcriptional. Transcriptional regulation of miRNA-encoding genes produce specific expression patterns of individual miRNA. However, the mechanism of post-transcriptional regulation of miRNAs remains largely unknown. The present study was aimed to clarify whether HuR, an evolutionary conserved AU-rich binding protein, could regulate miRNAs expressions. By means of a computational screen for AUUUA motifs within pri-miRNAs, we found that the downstream of hsa-let-7c but not hsa-miR-21 was enriched of AUUUA motifs. Then we transfected HuR and mutant HuR lacking RNA recognition motif 3 (RRM3) respectively into HEK293T cells. And HuR protein and miRNAs expressions were detected by Western blot and real-time PCR, respectively. The results showed that the overexpression of HuR promoted mature hsa-let-7c expression but not hsa-miR-21 expression. Furthermore, overexpression of HuR deletion mutant lacking RRM3 did not promote hsa-let-7c expression. These results suggest that RRM3 is crucial for HuR mediating mature hsa-let-7c expression. Collectively, these findings proposed a novel role of HuR in biogenesis of miRNAs, possibly by way of post-transcriptional regulation of miRNAs.

Key words: microRNA; HuR; let-7c; post-transcriptional regulation

RNA结合蛋白HuR通过其RNA识别模体调节hsa-let-7c表达

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摘 要：微小RNA (microRNAs, miRNA)是一种短链的非编码RNA，它通过抑制RNA的翻译或促进RNA的降解调控多种细胞活动和机体的发育。机体主要通过转录和转录后两个层面对miRNA的表达进行调控，其中对miRNA转录水平的调控决定了miRNA表达的特异性，而目前我们对其转录后调控的机制还知之甚少。本研究旨在证实RNA结合蛋白HuR是否通过与pri-miRNA中富含AU的序列相结合调节miRNA的表达。利用生物信息学方法对pri-miRNA中的AUUUA模体进行筛查，发现在hsa-let-7c下游富含AUUUA模体，而hsa-miR-21则不具备这一模体。通过转染HuR质粒到HEK293T细胞构建过表达模型，然后用Western blot和real-time PCR分别检测HuR蛋白和miRNAs表达水平。结果显示，过表达HuR能够促进成熟hsa-let-7c而非hsa-miR-21表达。而过表达缺失RNA识别模体3 (RNA recognition motif, RRM3)的突变体HuR则不能促进hsa-let-7c的表达。以上结果提示RRM3是HuR介导成熟hsa-let-7c表达的关键序列，而HuR在调控miRNA生物合成中可能扮演了一种新的角色，即在转录后水平调节miRNA的表达。

关键词：微小RNA；HuR；let-7c；转录后调节
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MicroRNAs (miRNAs) are short (20–23-nucleotide), endogenous, single-stranded RNA molecules that post-transcriptionally regulate gene expression. miRNA genes are transcribed by either RNA polymerase II or III into primary miRNA transcripts (pri-miRNAs), which range in size from hundreds of nucleotides to tens of kilobases. The primary transcripts are next processed in the nucleus by the RNase III enzyme Drosha with co-factors (DGCR8 and RNA helicase) to form miRNA precursors, or pre-miRNAs of ~70–110 nucleotides in length. After nuclear processing, the pre-miRNAs are exported into the cytoplasm by Exportin-5 in complex with Ran-GTP. The RNase III Dicer cleaves off the loop of the pre-miRNAs and generates a roughly 22-nucleotide miRNA duplex. The mature miRNAs are loaded into a functional protein complex containing an argonaute protein at its core to repress target mRNA translation or promote their degradation [1]. Numerous studies have established that miRNAs are important regulators of cell differentiation, proliferation/growth, mobility, and apoptosis. Consequently, dysregulation of miRNA expression has been implicated in various diseases such as cancer, cardiovascular disease, liver disease and immune dysfunction. Recently, growing evidence has demonstrated that the expression of mammalian miRNAs could be regulated at the post-transcriptional level [2, 3]. A particularly well-studied example of post-transcriptionally regulated miRNAs maturation involves repression of let-7 miRNA biogenesis by LIN28 and its homologue LIN28B [4–6].

HuR, a member of the ELAV (embryonic lethal abnormal vision) family of ubiquitously expressed RNA-binding proteins, has numerous functions mostly related to cellular stress response [7]. HuR proteins have three highly conserved RNA recognition motifs (RRMs) and one variable hinge region [8]. HuR affects many post-transcriptional aspects of RNA turnover, from splicing to translation. HuR binds to the AUUUA motifs in the 3' untranslated region (UTR) of the transcripts to stabilize several target mRNAs, such as tumor necrosis factor-α, cyclooxygenase-2 and PAI-2. AU-rich elements (AREs) were first identified as the AUUUA motifs within U-rich regions of the 3' UTR of mRNAs [9]. Like common RNA transcripts, the AREs are also found within the pri-miRNAs, however, whether those AREs play a role in the biogenesis of miRNAs remains unknown.

Using a computation-based approach to search for AUUUA motifs within pri-miRNAs, we found the enrichment of AUUUA motifs in the downstream of hsa-let-7c but not in the hsa-miR-21. In order to clarify whether HuR regulates miRNA expression by AUUUA motifs within the pri-miRNAs, we transfected HuR and mutant HuR lacking RNA recognition motif 3 (RRM3) respectively into HEK293T cells to investigate their influence on miRNAs expression.

1 MATERIALS AND METHODS

1.1 *Plasmids and transfection*

The pcDNA3-HuR and pcDNA3-HuR M1 (mutant HuR lacking RRM3) were kindly provided by Dr. Joan A. Steitz [10]. The plasmid DNA was transfected into cells using the cationic lipid reagent Lipofectamine 2000 (Invitrogen, Carlsbad, CA). In brief, HEK293T cells were cultured in DMEM containing 10% fetal bovine serum. 1×10^5 cells/well were seeded in 6-well plates without antibiotics 1 d before transfection. To each well, 1 μg of DNA was mixed with 3 μL of Lipofectamine 2000 in 0.5 mL of Opti-MEM (Life Technologies, Grand Island, NY), and 6 h later, 0.5 mL of 20% fetal calf serum in DMEM was added to the medium. After 48 h, the cells were assayed for the mRNA or miRNA expression by real-time RT-PCR. All experiments were performed on samples in duplicate and were repeated independently three times.

1.2 *RT-PCR*

Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA was then reverse transcribed into cDNA by use of the RT-PCR Access Kit (Promega, Madison, WI, USA). One microliter of cDNA was used for PCR. The transfected HuR or HuR M1 mRNA was amplified by PCR with the following primers: T7 primer, 5'-TAATACGACTCACTATAGG-3' and SP6 primer 5'-ATTTAGGTGACACTATAG-3'. The PCR mixture was incubated on a DNA Thermal Cycler (Stratagene, La Jolla, CA, USA). After amplification, the products were analyzed by electrophoresis on 1% agarose gels.

1.3 *Quantitation of miRNA expression by real-time PCR*

The expressions of mature hsa-let-7c, hsa-miR-21 and U6 were quantified using a previously published method with modifications [11]. Total RNA was isolated with Trizol (Invitrogen, Carlsbad, CA, USA) according to the instructions of the manufacturer. 100 ng of total

RNA was reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed with Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA). The relative levels of miRNA expressions were quantified using the comparative Ct method with U6 RNA as the endogenous control.

1.4 *AREs predicted in pri-miRNAs*

The whole human genome sequences were downloaded from UCSC (http://genome.ucsc.edu/) and the position information of human miRNAs was from miRBase [12]. Next, the sequence ranging from upstream 1 000 bp to downstream 1 000 bp nucleotide according to the position of pre-miRNAs was extracted by Perl language (http://www.perl.org/). Then the AUUUA motifs in each pri-miRNAs were located automatically. Thus, the frequency of AUUUA motifs in each pri-miRNA was counted.

1.5 *Subcellular fractionation and Western blot analysis*

Subcellular fractionation was prepared as previously described [13]. To obtain cytoplasmic fractions, the cells were trypsinized, rinsed with phosphate-buffered saline, incubated in 200 μL of hypotonic buffer A (10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$) supplemented with protease inhibitors on ice, and lysed by addition of 25 μL of buffer A containing 2.5% Nonidet P-40 plus inhibitors. Nuclei were pelleted, and supernatants were saved, freeze-thawed five times, and centrifuged (10 min, 3 500 r/min, 4 °C). Cytosolic fractions were prepared by subjecting cytoplasmic lysates to an additional step of high-speed centrifugation (14 000 r/min for 60 min at 4 °C) and discarding any pelleted material. For preparing nuclear fractions, nuclear pellets were incubated in extraction buffer C (20 mmol/L HEPES, pH 7.9, 0.45 mol/L NaCl, 1 mmol/L EDTA) plus inhibitors, centrifuged and supernatants were saved. The efficiency and quality of nuclei preparation were monitored with a hemacytometer at the end of the nucleus isolation procedure. Western blot was performed as previously described [14]. Cytosolic and nuclear fractions were extracted, and 10 μg of total protein was subjected to electrophoresis. After transferring and blocking, it reacted with primary antibodies at 4 °C overnight. Antibodies against HuR (1:1 000 diluted), lamin A (1:1 000 diluted) and eukaryotic translation initiation factor 5 (eIF5, 1:2 000 diluted) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Target proteins were subsequently detected and quantified using horseradish peroxidase conjugated IgG with enhanced chemiluminescence's system (ECL kit, Pierce Biotechnology).

1.6 *Statistical analysis*

The results are reported as means ± SEM with n representing the number of experiments on cells. Differences between control and experimental groups were analyzed by unpaired Student's t test. All statistical analyses were performed using the software Graphpad Prism 4.0, and $P < 0.01$ was considered significant.

2 RESULTS

2.1 AREs were predicted in pri-miRNAs by bioinformatics

We first analyzed the AUUUA motifs in the pri-miRNAs (for details, see materials and methods). The flowchart was shown as Fig. 1*A*. We found that 371 out of 2 417 *Homo sapiens* pri-miRNAs harbored more than two AREs (data not shown). For example, the hsa-mir-30e had two AUUUA motifs in 1 211–1 215 and 1 558–1 562. The frequency of AUUUA motifs in each pri-miRNA was statistically analyzed, and the frequency chart was shown as Fig. 1*B* indicating that hsa-mir-2054, hsa-mir-1264 and hsa-let-7c possessed the most AUUUA motifs in their pri-miRNAs. However, there was no expression data as to hsa-mir-2054 and hsa-mir-1264, so we chose the hsa-let-7c as an example, which has as much as 15 AUUUA motifs in its pri-miRNA. And miR-21, without any AUUUA motif in its pri-miRNA, was adopted as a reference.

miRNA genes are first transcribed into pri-miRNAs, which can range in size from hundreds of nucleotides to tens of kilobases. However, little is currently known about how the miRNA gene is transcribed, owing to lack of basic information on their gene structure. Referring to some human miRNAs that have been confirmed of their transcription start sites [15–17], we narrowed our search within the sequence of 1 kb upstream and downstream of pre-miRNAs as predicted pri-miRNAs in the present study.

2.2 AREs were enriched in the downstream of hsa-let-7c

The downstream sequence of hsa-let-7c was shown as Fig. 2. This region was highly AU rich (69%), and there were several typical ARE signatures (AUUUA pentamer), which were shown in red in Fig. 2*A*. It has

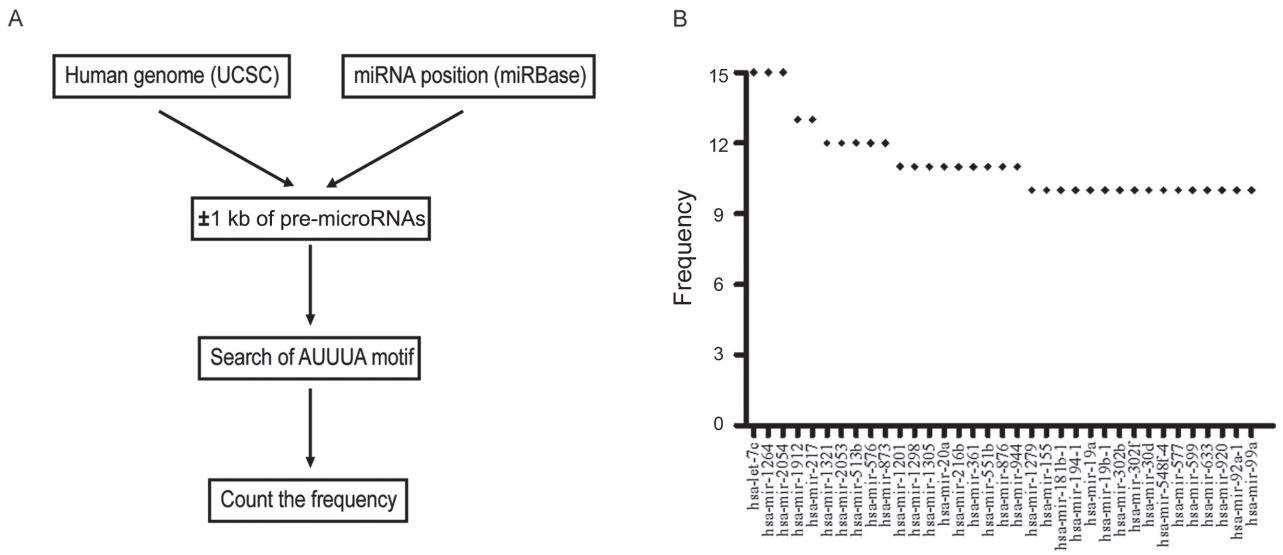

Fig. 1. Bioinformatic prediction of AU-rich elements (AREs) in pri-microRNAs (pri-miRNAs). *A*: The flow chart of prediction of AREs in pri-miRNAs (for details, see methods and materials). *B*: The frequency chart showing the pri-miRNAs harboring more than 10 AREs.

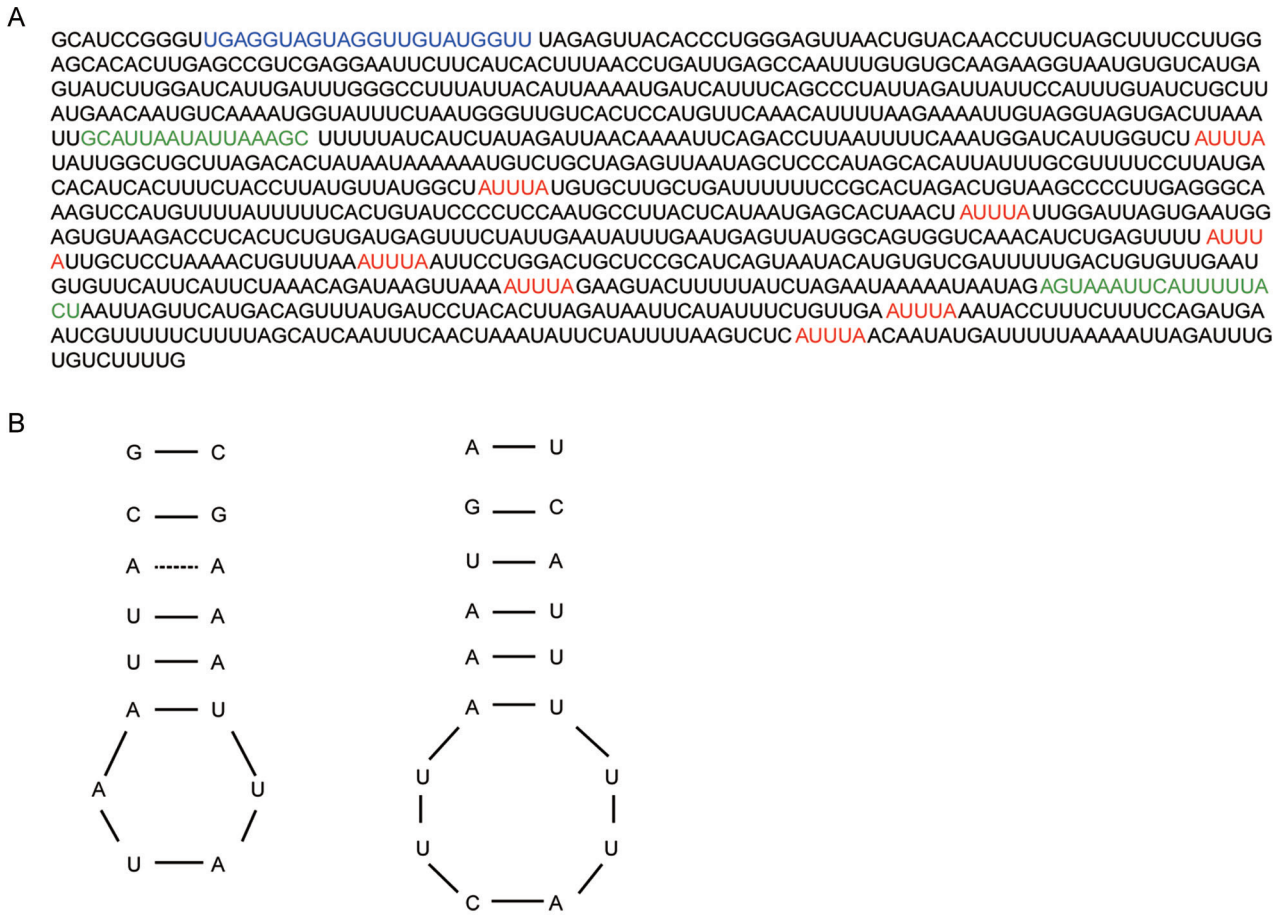

Fig. 2. The sequence downstream of hsa-let-7c is enriched of AU-rich elements. *A*: The mature hsa-let-7c sequence was in blue. The AU-rich elements were in red. The HuR binding motifs were in green. All the sequence was downloaded from miRBase. *B*: Secondary structures of the two HuR binding motifs predicted by mfold web server.

been reported that AREs in the 3' UTR of mRNAs were the *cis*-acting elements regulating the mRNA decay by interaction with sequence-specific RNA-binding proteins [18], and HuR, an RNA binding protein characteristic of a 17- to 20-base-long RNA motif rich in uracils, has been identified to bind to the AREs and stabilize its targets [19]. So we analyzed the secondary structure of downstream sequence of hsa-let-7c by mfold web server (http://www.bioinfo.rpi.edu/applications/mfold)[20], and found two HuR RNA binding motifs as shown in green in Fig. 2*A*. In addition, the secondary structures of the two putative HuR motifs are shown in Fig. 2*B*. Therefore, the enrichment of AREs in the downstream of hsa-let-7c and the existence of HuR binding motif suggested that this region might also play a role in the expression of hsa-let-7c.

2.3 HuR promoted hsa-let-7c expression

In the HEK293T cells, HuR was constitutively expressed and localized predominantly in the nucleus as previously reported [10]. While transiently transfected, the HuR content in the cytosol significantly increased in consistence with HuR in the nucleus extract (Fig. 3*A*, *B*). After transfection of HuR for 48 h, the cytoplasmic HuR content upregulated to 3.13 ± 0.10 folds (Fig. 3*C*), while HuR in nuclear fractions increased to 1.99 ± 0.16 folds (Fig. 3*D*). However, the antibody from Santa Cruz Biotechnology only recognized the native HuR but could not detect the HuR mutant proteins possibly as a result of lacking RRM3, though we confirmed our successful transfection by detecting RNA transcribed from the plasmid (Fig. 4). Next, we measured the expression of mature hsa-let-7c after transient transfection of HuR or HuR M1, with the level of hsa-miR-21 treated as control. As shown in Fig. 3*E*, overexpression of HuR increased hsa-let-7c to 1.88 ± 0.16 folds, whereas overexpression of HuR M1 had no influence on hsa-let-7c level. In contrast, either HuR or HuR mutants had no effect on hsa-miR-21 expression (Fig. 3*F*). Taken together, these results suggested that HuR increased the expression of hsa-let-7c, which was carried out through its RRM3.

3 DISCUSSION

The present study showed that overexpression of HuR in HEK293T cells up-regulated mature hsa-let-7c expression, but not mature hsa-miR-21 expression. However, overexpression of HuR mutant lacking RRM3 did not promote hsa-let-7c expression, indicating that RRM3 was important for HuR's regulation on let-7c. Thus, our study suggests that HuR regulates miRNA biogenesis in a pattern of post-transcriptional regulation.

Hsa-let-7c is the member of an intronic miR-99a/let-7c/miR-125b miRNA cluster located in C21orf34 on chromosome 21 [21]. Evidence has shown that the expression of let-7c is frequently reduced in lung cancers both *in vitro* and *in vivo* [22]. However, HuR showed a nuclear overexpression in non-small cell lung cancer tissue specimens [23]. These contradictory results seemed to be explained by the fact that let-7c was located at fragile sites which frequently deleted in lung cancers [24].

The expression of mammalian miRNAs can be regulated at post-transcriptional level through the modulation of nuclear and cytoplasmic miRNA processing events. Increasing evidence has demonstrated that RNA-binding proteins modulate miRNA biogenesis through their RNA-binding domain. The KH-type splicing regulatory protein (KSRP) interacts with the terminal loop of pre-let-7a-1 and promotes its maturation by KH domain 3 and 4 [25]. hnRNP A1, a protein implicated in RNA processing, binds to the loop of pri-miR-18a and promotes its cleavage by Drosha [26]. These findings have thrown a new light upon the regulation on miRNAs. Transcriptional regulation on miRNA gives a miRNA its specificity, and affects the miRNA expression through promoting or repressing the transcription of its gene, which in a sense is a *de novo* regulatory pattern for miRNA biogenesis and usually takes a longer time. However, RNA-binding proteins regulate miRNA expression through binding to pri-miRNAs or even pre-miRNAs and affecting their cleavage and stability as well as miRNA maturation. Owing to acting on post-transcriptional level, they can yield a faster result in regulating miRNA expression. Thus RNA-binding proteins may play a crucial role in some acute stresses and reactions. HuR is an RNA-binding protein widely expressed in a variety of tissues, and let-7c has been demonstrated to be involved in tumorigenesis, ovarian insufficiency and cardiac hypertrophy, so our findings are helpful to explore the pathogenesis of these diseases. However, it remains to be investigated how HuR promotes let-7c expression after binding to this miRNA.

Taken together, our findings demonstrated a novel

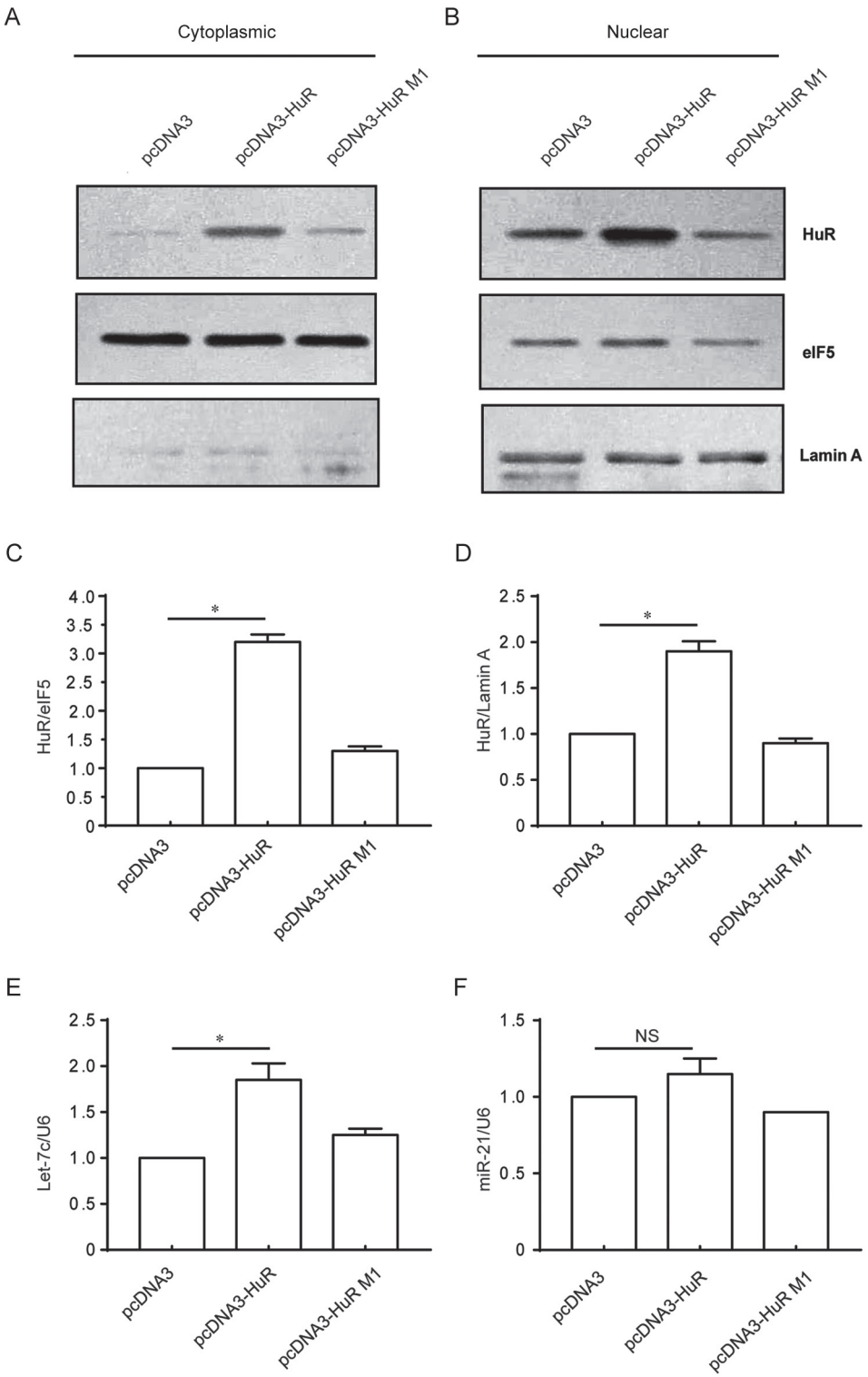

Fig. 3. Overexpression of HuR in HEK293T cells upregulated hsa-let-7c expression but not miR-21 expression. The cells were plated and cultured with 10% FBS. After transfection of pcDNA3, pcDNA3-HuR or pcDNA3-HuR M1 plasmid for 48 h, the expressions of HuR and HuR M1 in either cytoplasmic (*A*) or nuclear extracts (*B*) from 293T cells were detected using antibodies directed to the indicated proteins by Western blotting. The levels of eukaryotic translation initiation factor 5 (eIF5, a cytoplasmic protein) and lamin A (a nuclear protein) in the same samples were assessed by Western blotting in order to ascertain the quality of the fractionation procedure and to detect loading differences. The ratios of HuR/eIF5 and HuR/lamin A were shown in *C* and *D*, respectively. Meanwhile, the relative expressions of hsa-let-7c (*E*) or hsa-miR-21 (*F*) to U6 RNA were analyzed respectively by real-time RT-PCR. All the experiments were done in duplicate, $n = 3$. The data are presented as means ± SEM. $^{*}P < 0.01$. NS: not significant.

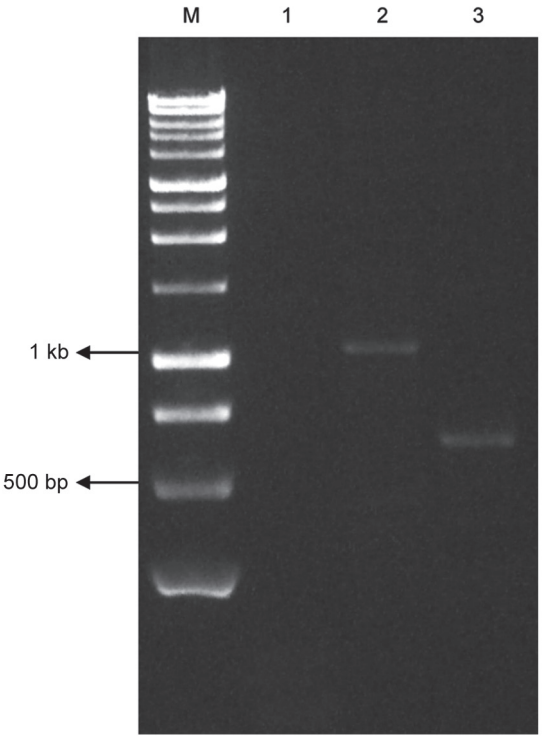

Fig. 4. Transfected HEK293T cells expressed HuR or HuR M1 mRNAs. RT-PCR using T7 primer and SP6 primer was performed without (lane 1; negative control) or with mRNA isolated from the cells transfected with pcDNA3-HuR or pcDNA3-HuR M1 (lane 2–3). The PCR products of HuR or HuR M1 were accordingly 1 145 and 714 base pairs. Products were visualized by ethidium bromide staining and compared to a 1 kb-base pair ladder (lane M).

role of HuR in biogenesis of miRNAs through the possible way of post-transcriptional regulation. It is noteworthy that the direct interaction of HuR and the exact ARE(s) of pri-miRNAs still needs confirming by RNA-EMSA and RNA-IP assay.

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