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Involvement of PU.1 in mouse *adar-1* gene transcription induced by high-dose esiRNA☆

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ABSTRACT

Adar-1 gene plays an important role in the negative regulation of RNA interference. We previously showed that increased *adar-1* mRNA level was associated with the rebound of gene expression after RNAi suppression. In this study, we identified a PU.1 binding site upstream from transcription start point of *adar-1* gene and is essential for the promoter activity. Knockdown and over-expression of the PU.1 gene resulted in decreased and increased activity of *adar-1* promoter, respectively. Our results suggest that transcription factor PU.1, could bind to the *adar-1* promoter and play a key role in activating transcription of gene induced by high-dose esiRNAs.

1. Introduction

RNA interference (RNAi), selective degradation of cognate mRNAs by interference RNA (siRNA), is a post-transcriptional gene silencing mechanism conserved among eukaryotic organisms [1]. RNAi is involved in several biological processes, including anti-virus reaction, inhibition of transposition and gene expression regulation. Despite the fact that RNAi is being widely applied in scientific and medical fields as a powerful tool, its mechanisms are not yet well understood. Components participating in this process have been thoroughly studied [2–9]. In contrast, knowledge concerning negative regulation for RNAi is poor.

The first clue of the existence of negative regulation of RNAi came from a plant virus. In tombousvirus, the p19 protein was found to specifically bind duplex siRNAs and block their assembly into RISC (RNA-induced silencing complex) [10,11], thus dampening the silencing of the RNAi. Another example of RNAi suppressor is the Hepatitis C Virus (HCV) core protein, which suppresses the activity of Dicer, which processes dsRNA into siRNA, and thus disturbs the cellular RNA silencing machinery [12].

Negative regulation of RNAi is a newly arising research area. The *Eri-1* gene encoding an exonuclease was discovered in a genetic screen for *C. elegans* mutants with enhanced RNAi efficiency. Mutations of this gene led to increased uptake of siRNAs [13]. Most recently, Fischer et al. reported their findings of trans-splicing in *C. elegans*, which generates the negative RNAi regulator ERI-6/7 [14]. In mammalian cells, ADARs (adenosine deaminases acting on RNA) have been reported to convert adenosine (A) into inosine (I), thus impairing the complementarities between dsRNA and the target mRNA. In addition, ADARs destabilize edited dsRNA, resulting in decreased efficiency of processing this molecule into siRNA by Dicer [15–18]. More interestingly, RNAi negative regulation seems to respond to the amount of RNAs entering the cell. We recently found an inverted correlation between the half-life of RNAi and the dose of siRNA both in cultured cells and in mice [19,20]. RT-PCR analysis further revealed increase of *adar-1* and *meri-1* mRNA induced by injected target esiRNA (endoribonuclease-prepared short interfering RNA) in a dose-dependent manner. More than 4-fold increase was reached upon intravenous injection of 10 μg esiRNA for each mouse. However, injection of 1 μg esiADAR-1 together with 10 μg of esiHBVP (esiRNA corresponding to DNA region encoding P protein of Hepatitis B Virus) did not alter the mRNA levels of *adar-1*.

In this study, we aimed to identify *cis*-elements responsible for the siRNA-induced transcription of *adar-1* in the promoter region and possible *trans*-factors binding to these *cis*-elements.

Abbreviations: RNAi, RNA interference; esiRNA, endoribonuclease-prepared short interfering RNA; esiHBVP, esiRNA corresponding to DNA region encoding P protein of Hepatitis B Virus; adar-1, adenosine deaminase acting on RNA, isoform 1; NP siRNA, non-specific siRNA; NP dsDNA, non-specific double-strand DNA.

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2. Materials and methods

2.1. Construction of plasmids

2.1.1. Construction of reporter plasmids

The 2 kb promoter region of the *adar-1* gene was amplified from genomic DNA of C57 mice. For construction of GFP reporter plasmids, a series of promoter segments were generated in a second round of PCR with various forward primers and a universal reverse primer as listed in Table 1. The forward primers contain an EcoRV and an AseI site, while the reverse primer contains a BamHI site. These amplified DNA fragments were cloned into pBluescriptII KS using restriction sites EcoRV and BamHI. After confirmation of the construction by means of sequencing, each of the promoter fragments was transferred into pEGFP-N1 vector (Clontech, USA) using restriction sites AseI and BamHI.

For construction of secreted alkaline phosphatase (SEAP) reporter plasmids, the forward primers for the second round of PCR contain SalI and BglII sites while the universal reverse primer contains EcoRI and NruI sites. The resulting fragments were first cloned into pBluescript II KS using restriction sites SalI and EcoRI. After DNA sequencing, these promoter fragments were transferred into pSEAP2-control (Clontech) with restriction sites BglII and NruI.

2.1.2. Construction of mammalian expression plasmid of PU.1

Full-length mouse *PU.1* cDNA was PCR-amplified with a forward primer (cggaattcagatgttacaggcgtgcaaaatgg, with EcoRI site) and a reverse primer (gaagatctctagtggtggtggtggtggtggtg, with BglII site). The PCR product was cloned into a pBluescript II KS using the EcoRI and BglII sites. After verification of the sequence, the fragment was transferred into pCMV-HA (Clontech, USA) to obtain the plasmid expressing PU.1, named pCMV-HA-PU1.

2.2. Preparation of PU.1 gene-specific esiRNAs and control non-specific esiRNAs

A DNA fragment containing exons 4 and 5 of the mouse *PU.1* gene was PCR-amplified using forward primer (cggaattcggatccgtttcctacatgccccgg, with EcoRI site) and reverse primer (gctctagatcagtggggcgggaggcgccgctc, with XbaI site), and cloned into pBluescript IIKS with corresponding restriction enzymes. After sequencing, the DNA fragment was transferred into a dual promoter plasmid pET-2P, which was derived from pET-22b to contain an extra *tac* promoter in opposite to the T7 promoter [21]. The resulting plasmid pET2P-PU.1 was transformed into the *E. coli* strain BL21 (DE3) to produce double-stranded RNA of the DNA fragment under the induction of IPTG (Isopropyl β-D-1-thiogalactopyranoside). Preparation of esiRNA of *PU.1* was carried out according to the method described by Xuan et al. [21], and the derived product was designated as esiPU.1.

Non-specific esiRNAs were derived from the Hepatitis B Virus P protein and prepared according to the lab protocol previously described [22], designated as esiHBVP.

2.3. Cell culture and transfection

CHO (Chinese Hamster Ovary) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco, USA), 100 μg/ml streptomycin and 100 IU/ml penicillin at 37 °C and 5% CO2. Cells were seeded at a density of 2×10^5/per well in 24-well plates and cultured overnight or to 90% confluence. Each well was then transfected with 0.4 μg of reporter plasmids, together with either 0.4 μg esiHBVP (non-specific esiRNA prepared from the DNA region encoding the P protein of HBV), or 0.4 μg non-specific 21-bp dsDNA, by using Lipofectamine 2000 (Invitrogen, USA). After transfection for 24 h, the expression of reporter genes was measured.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Forty-eight hours after transfection, total cellular RNA was extracted using Trizol (Invitrogen, USA) according to the manufacturer's protocol. Reverse transcription was performed using Reverse Transcriptase XL (AMV) (Takara, Dalian, China). RT-PCR of both β-actin and *PU.1* fragments with different primer concentrations were amplified in the same tube. The primers used for *PU.1* amplification (atgttacaggcgtgcaaaatggaagggttttc and gggcatgtaggaaacctggtgactgaggccggtg) were used at 0.2 μM, whereas the primers for β-actin amplification (gagaccttcaacaccccagc and ccacaggattccatacccaa) were used at 0.02 μM. Amplification was carried out for 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 20 s. The PCR products were analyzed on a 1.5% agarose gel.

Table 1
Primers used for construction of report plasmids.

Reporter plasmid	Primer sequence	Promoter segment
GFP series		
pEGFPN1-2k	5′-cggatatcattaattcctgaaagcctcccagatta	(−2000)–(−1)
pEGFPN1-600	5′-cggatatcattaattacccagggatatggaagt	(−600)–(−1)
pEGFPN1-200	5′-cggatatcattaatgagtggtgaggaaggcggtg	(−200)–(−1)
pEGFPN1-180	5′-cggatatcattaatggcgtggactacggcg	(−180)–(−1)
pEGFPN1-160	5′-cggatatcattaatagcccttatggtgggc	(−160)–(−1)
pEGFPN1-140	5′-cggatatcattaattgctgctgctcggctacc	(−140)–(−1)
pEGFPN1-100	5′-cggatatcattaatttggcccaacagttggg	(−100)–(−1)
pEGFPN1-200ΔPU1	5′-cggatatcattaatttggcccaacagttggg	(−200)–(−1), ΔPU1
pEGFPN1-180 + PU1	5′-cggatatcattaattgaggaaggggcgtggac 5′-cgggatccagtgccggcaaggcccgcac[a]	(−180)–(−1), +PU.1
SAP series		
pSEAP2-2k	5′-cggtcgacagatcttcctgaaagcctcccaga	(−2000)–(−1)
pSEAP2- 600	5′-cggtcgacagatcttacccagggatatggaag	(−600)–(−1)
pSEAP2-200	5′- cggtcgacagatctgagtggtgaggaaggc	(−200)–(−1)
pSEAP2-180	5′- cggtcgacagatctggcgtggactacggcg	(−180)–(−1)
pSEAP2-160	5′- cggtcgacagatctagcccttatggtgggc	(−160)–(−1)
pSEAP2-140	5′- cggtcgacagatcttgctgctgctcggctac	(−140)–(−1)
pSEAP2-100	5′- cggtcgacagatctgagtggcggtgggcgtg	(−100)–(−1)
pSEAP2-200ΔPU1	5′- cggtcgacagatctgagtggcggtgggcgtg	(−200)–(−1), ΔPU1
pSEAP2-180 + PU1	5′- cggtcgacagatcttgaggaaggggcgtgg 5′-cggaattcgcgaagtgccggcaaggcccgcac[a]	(−180)–(−1), +PU1

[a] The reverse primers are bolded and indicated.

2.5. Alkaline phosphatase activity assay

The SEAP (secreted alkaline phosphatase) activity of the culture media was measured 24 h after transfection by performing a colorimetric assay according to recommendation [23]. Briefly, 50 μl of 4-fold diluted culture medium was heat-treated at 65 °C for 30 min, and then was added to 150 μl of SEAP assay solution containing 20 mM pNPP (para-nitrophenylphosphate), 1 mM $MgCl_2$, 10 mM l-homoarginine and 1 M diethanolamine, pH 9.8. The reaction mixture was transferred to a 96-well plate and further incubated at 37 °C for 15 min before reading at 405 nm for absorbance.

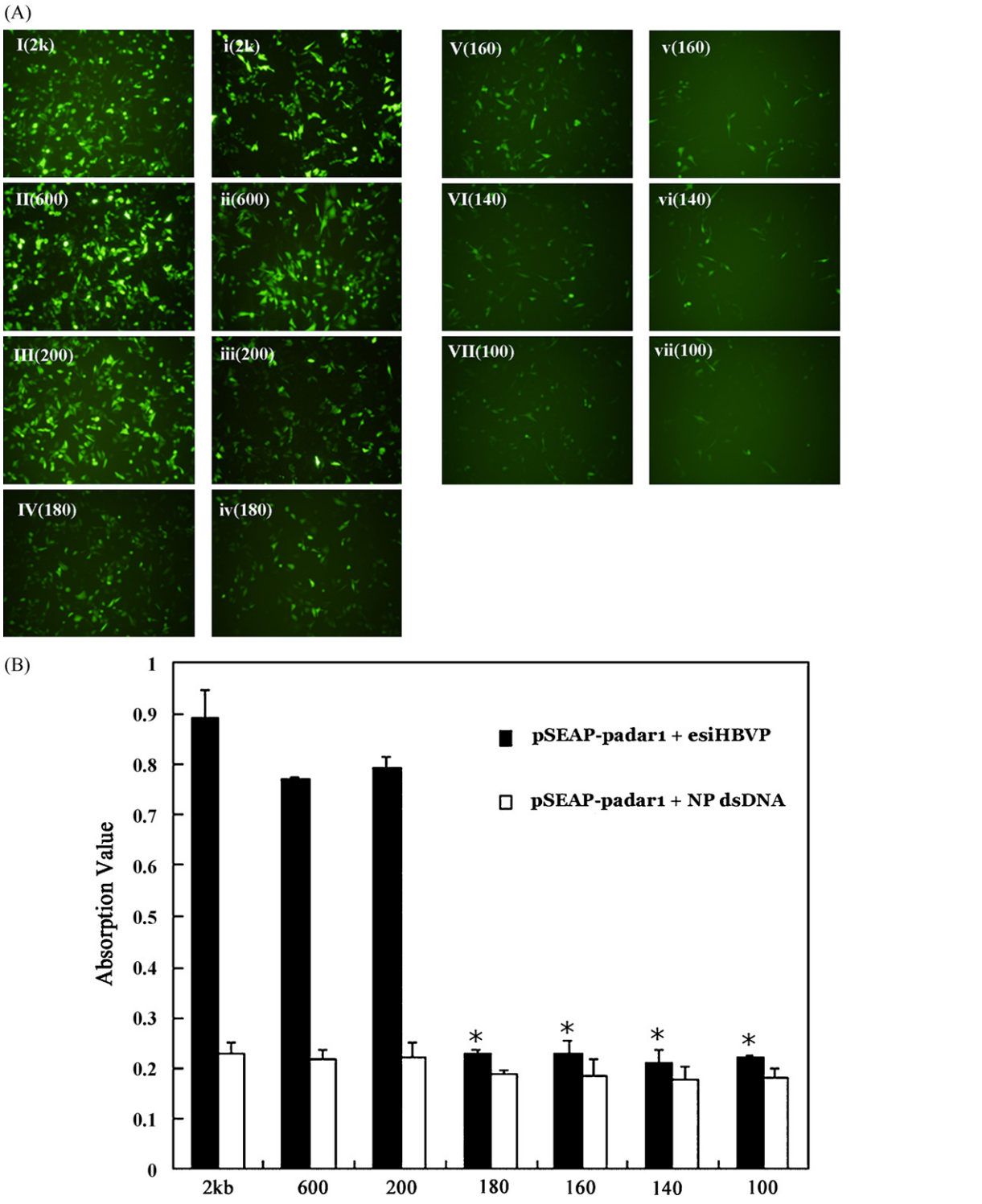

Fig. 1. Reporter gene assay of truncated *adar-1* promoters. The transcription activities of different truncated *adar-1* promoter fragments responding to esiRNA induction was determined by GFP reporter gene assay and secreted alkaline phosphatase (SEAP) reporter assay separately. (A) GFP assay of truncated *adar-1* promoters. CHO cells were co-transfected with pEGFP reporter plasmids containing truncated *adar-1* promoters and esiHBVP (I–IX), or 21-bp non-specific dsDNAs with 2-bp overhang at the 3′ end (i–ix). (B) SEAP activity assay of truncated *adar-1* promoters. CHO cells were co-transfected with pSEAP2 reporter plasmids containing truncated *adar-1* promoters and NPsiRNA or 21-bp non-specific dsDNA. The data represent the mean of three independent experiments, with error bars indicate s.e.m. of triplicate samples. Statistical analysis was performed by on way ANOVA, followed by the Tukey–Kramer's post hoc test for multiple comparisons. *$P < 0.005$.

2.6. Detection of GFP expression

CHO cells were transfected with pEGFP-N1-padar1 series reporter plasmids together with NPesiRNAs or control dsDNAs, and the expression of GFP was investigated 24 h post-transfection under the LEICA DM RA2 microscope (objective 20×) at 488 nm.

2.7. Western blot

HA-tag antibody was purchased from Beyotime Institute of Biotech (Jiangsu, China). Transfected cells were lysed in lysis buffer containing 50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100 mM dithiothreitol and 0.1% bromophenol blue. Proteins were resolved on 10% SDS-polyacrylamide gels and subjected to Western blot according to standard protocols. Antibodies were used at 1:500 dilutions, and HRP-conjugated goat anti-mouse secondary antibody was used at 1:2000 dilutions.

2.8. Prediction of cis-transcription element in adar-1 gene promoter

The cloned adar-1 gene promoter sequence was input into the transcription factor binding site database—MAPPER (multi-genome analysis of positions and patterns of elements of regulation) at: http://mapper.chip.org/mapper/mapper-main, to search for candidate promoter element(s). The sequence was selected according to scores and ranks.

2.9. Statistical analyses

Statistical analysis of the data from Alkaline phosphatase activity assays were performed by using SPSS-15 software. Error bars indicate standard error of the means (s.e.m.) of triplicate samples and presented in figures. Statistical analysis was performed by one way ANOVA, followed by the Tukey–Kramer's post hoc test for multiple comparisons. *P* values were also calculated and presented.

3. Results

3.1. Identification of a cis-element in the adar-1 promoter region

Since the transcription start point of the *adar-1* gene has not been characterized yet, we prepared a series of fragments covering various regions of the *adar-1* promoter counting from the base before AUG start cordon as −1, and examined their activity using two reporter-systems. Similar transcription (<10% difference) of the reporter genes upon esiRNA-induction was observed for the 2 kb full length promoter, the 600 bp, and the 200 bp fragments (Fig. 1A: I–III; Fig. 1B: panels 1–3). In contrast, removal of the 20 base pairs from −200 bp to −180 bp led to a 65% decrease in the reporter gene transcription (Fig. 1A: IV–VII; Fig. 1B: panels iv–vii). This 20 bp segment thus likely contains a *cis*-element which is essential for regulating transcription of the *adar-1* gene in response to esiRNA-induction.

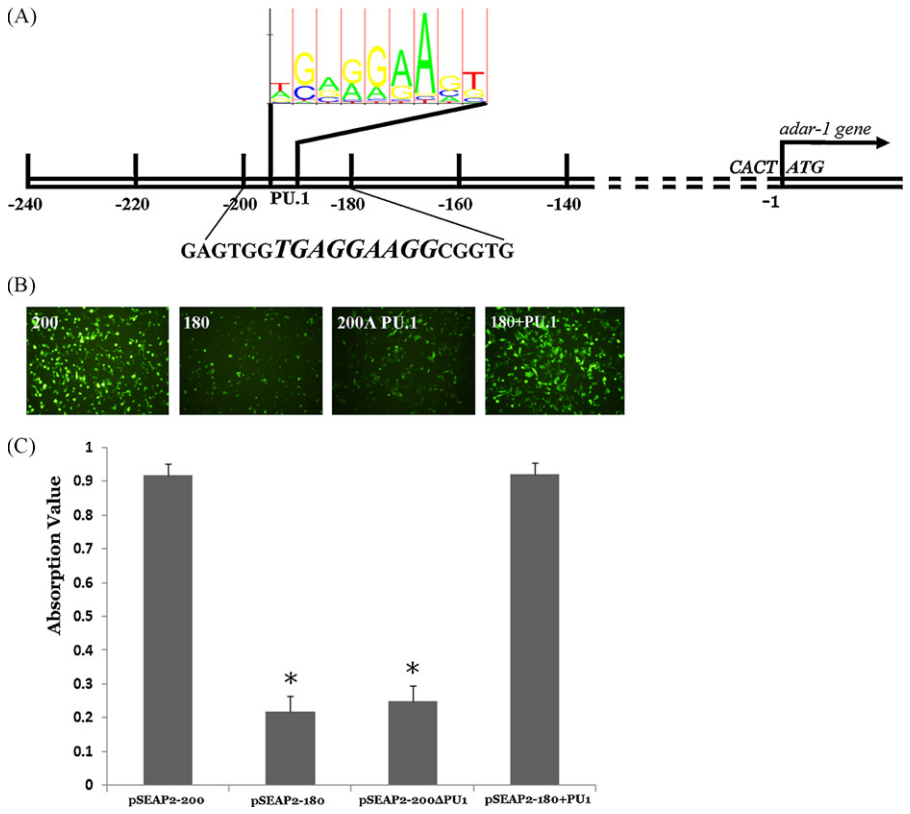

Fig. 2. Identification of the 9-bp PU.1 binding sequence as the key *cis*-element for esiRNA-induced *adar-1* gene transcription activation. (A) Schematic presentation of PU.1 binding site predicted by database and the actual PU.1 binding sequence in the 200-bp promoter region upstream of the *adar-1* gene. Size of the colour letters represents the preference of a nucleotide at the positions. The sequence of in bold and italic indicates the 9-bp *cis*-element in the *adar-1* promoter. (B) Green fluorescence in CHO cells transfected with 0.4 μg pEGFP reporter plasmids containing the 200-bp *adar-1* promoter (labelled as 200), the same fragment but with the 9-bp PU.1 site deleted (200ΔPU.1), the 180-bp long promoter fragment (180), or the same fragment with the 9-bp PU.1 binding sequence added at the 5′ end (180 + PU.1), together with 0.4 μg esiHBVP. (C) SEAP activities in CHO cells transfected with pSEAP2 reporter plasmids containing the same promoter sequences described in (B). The data represent the mean of three independent experiments, with error bars indicate s.e.m. of triplicate samples. Statistical analysis was performed by on way ANOVA, followed by the Tukey–Kramer's post hoc test for multiple comparisons. *$P < 0.005$.

3.2. Identification of a PU.1 binding sequence

To identify possible *cis*-element in the 20-bp segment, we searched for matching promoter elements using the database at http://mapper.chip.org. Three candidates with sequence similarity were found, among which the PU.1 gene binding sequence (TGAGGAAGG) showed the highest score. This sequence was thus selected for further analysis (Fig. 2A).

We then prepared the 200-bp *adar-1* promoter segment with and without the PU.1 binding site (TGAGGAAGG) and examined their activity in regulating transcription of the reporter genes. In both assay systems, deletion of PU.1 binding sequence drastically affected reporter gene transcription induced by esiRNA, while adding back this 9-bp element restored promoter activity (Fig. 2B and C). These results clearly suggest that the PU.1 binding sequence is the key *cis*-element for esiRNA-induced activation of the *adar-1* gene transcription.

3.3. Knock-down of PU.1 gene impaired reporter gene transcription

Specific esiRNAs were prepared to knock down the *PU.1* gene, and used to transfect CHO cells together with high dose non-specific esiRNAs and the reporter plasmids. Degradation of *PU.1* mRNA in the transfected cells was confirmed (Fig. 3). Both GFP and SEAP assays revealed that knock-down of the *PU.1* gene significantly decreased transcription of the reporter genes. These data imply that the transcription factor PU.1 responds to the signal of high-dose esiRNA at high doses and enhances the transcription of the *adar-1* gene.

3.4. Over-expression of PU.1 gene enhances gene transcription by adar-1 promoter

We constructed pCMV-HA-PU.1, a plasmid expressing the *PU.1* gene under the CMV promoter. Since there are abundant researches

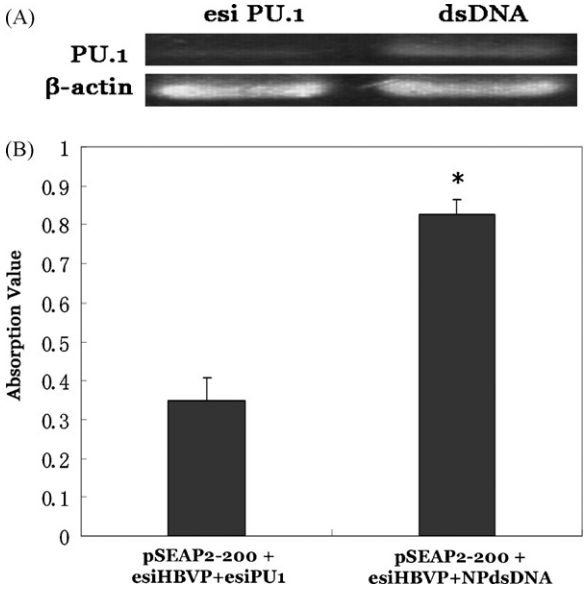

Fig. 3. Effect of PU.1 gene knock-down on reporter gene transcription of pSEAP2-200 induced by esiRNA in CHO cells. (A) RT-PCR detection of endogenous mRNA level of *PU.1* gene in CHO cells transfected with either 0.4 μg esiPU1 (left) or 0.4 μg 21-bp dsDNA (right). Messenger RNA level of β-actin was used as an internal control. (B) SEAP activities in culture media of CHO cells co-transfected with 0.2 μg pSEAP2-200 and either 0.4 μg esiHBVP (left) or 0.4 μg 21-bp dsDNA (right). The data represent the mean of three independent experiments with error bars indicate s.e.m. of triplicate samples. Statistical analysis was performed by on way ANOVA, followed by the Tukey–Kramer's post hoc test for multiple comparisons. $^{*}P < 0.005$.

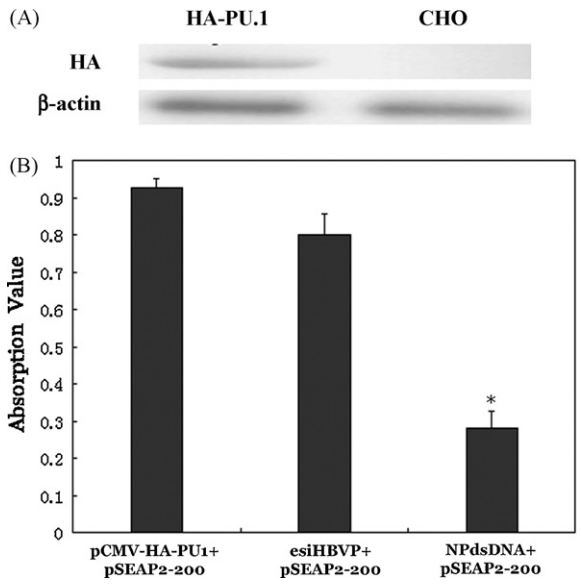

Fig. 4. Effect of over-expression of *PU.1* gene on reporter gene transcription of pSEAP2-200 induced by esiRNA in CHO cells. (A) Western blotting of HA-tagged PU.1 in CHO cells transfected with 0.4 μg pCMV-HA-PU1 (left), or with 0.4 μg pCMV-HA (right). Beta-actin was taken as internal control. (B) SEAP activity of culture media of CHO cells co-transfected with pCMV-HA-PU1 plus 0.4 μg pSEAP2-200 (left column), with 0.4 μg esiHBVP plus 0.4 μg pSEAP2-200 (middle right column), or with 0.4 μg NPdsDNA plus 0.4 μg pSEAP2-200 (right column). The data represent the mean of three independent experiments, with error bars indicate s.e.m. of triplicate samples. Statistical analysis was performed by on way ANOVA, followed by the Tukey–Kramer's post hoc test for multiple comparisons. $^{*}P < 0.005$.

reported that PU.1 is a versatile transcription regulator which controls lymphoid and myeloid cell proliferation and development, so we the effect of PU.1 over-expression on CHO cell proliferation, and phase-contrast pictures showed that there was little difference in cell numbers in control and PU.1 transient transfected cells (data not shown). Then, we transfected CHO cells together with reporter plasmid pSEAP2-200 and high-dose of esiRNA (0.8 μg/ml). Expression of *PU.1* was confirmed by Western blot (Fig. 4A, left panel). About a 3-fold higher alkaline phosphatase activity was found in culture medium of CHO cells transfected with pCMV-HA-PU.1, compared to those transfected with control dsDNA (Fig. 4B, compare left and right columns). This increase in alkaline phosphatase activity is comparable to high-dose esiHBVP induced one (Fig. 4B, compare left and middle columns). Therefore, over-expression of *PU.1* enhanced the activity of the *adar-1* promoter, providing additional evidence that *PU.1* plays a key role in esiRNA-induced *adar-1* gene transcription.

4. Discussion

PU.1, also known as Spi1 (spleen focus forming virus proviral integration oncogene spi1), is an ETS-family transcription factor, once reported as an important, versatile regulator of B lymphoid and myeloid genes [24,25]. Now, PU.1 has also been shown to be involved in other differentiation and developmental processes [26,27].

The core sequence of PU.1 binding site in downstream gene promoter is a $^{C}/_{A}GGA_{A}/^{T}$ centered within a longer recognition sequence, with these flanking sequences defining binding specificity [28]. However, PU.1 does not perform its transcriptional regulation function independently, but usually collaborates with other transcription factors such as IRF4/8 [29,30] and the homeodomain protein HOXC13 [31].

In this study, we identified a 9-bp PU.1 binding site in the *adar-1* gene promoter region which is essential for the esiRNA-induced

promoter activity and demonstrated that the transcription factor PU.1 indeed plays a key role in responding to high-dose esiRNA and activating transcription of the *adar-1* gene. Our data suggest that RNAi machinery may be negatively regulated via induction of expression of a number of genes coding for enzymes in a cascade leading to destabilization of siRNAs. Transcription factor PU.1 may be located upstream of this pathway and stimulate transcription of the *adar-1* gene. Collectively, our data demonstrate that PU.1 has the ability to sense high-dose siRNAs and activate gene transcription of *adar-1* expands our understanding of RNA interference and reveals a distinct pathway may allow the immune system to tailor it response to exogenous small dsRNAs and maintain the balance of internal homeostasis. However, the detailed knowledge of negative regulation of siRNA is still in mystery and there still remains to be thoroughly investigated.

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