

L1-ORF1p and Ago2 are involved in a siRNA-mediated regulation for promoter activity of L1-5'UTR

Liu Shi^{1,2}, Huanhuan Shi¹, Liangliang Lou¹, Xiaoning Yuan¹, Yunfeng Zhu¹

¹College of Life Sciences and Bio-engineering, Beijing Jiao-tong University, Beijing 100044, China

²Department of Psychiatry, University of Oxford, OX3 7JX, UK

Abstract

Introduction. Long interspersed nuclear elements-1 (L1), as the only one self-active retrotransposon of the mobile element, was found to be generally activated in tumor cells. The 5'UTR of L1 (L1-5'UTR) contains both sense and antisense bidirectional promoters, transcription products of which can generate double-strand RNA (dsRNA). In addition, L1-ORF1p, a dsRNA binding protein encoded by L1, is considered to engage in some RNA-protein (RNP) formation. Ago2, one of the RISC components, can bind to dsRNA to form RISC complex, but its role in L1 regulation still remains unclear. Due that the 5'UTR of L1 (L1-5'UTR) contains both sense and antisense bidirectional promoters, so the activities in both string were identified. A dsRNA-mediated regulation of L1-5'UTR, with the feedback regulation of L1-ORF1p as well as other key molecules engaged (Ago1–4) in this process, was also investigated.

Material and methods. Genomic DNA was extracted from HEK293 cells and subjected to L1-5'UTR preparation by PCR. Report gene system pIRESneo with SV40 promoter was employed. The promoter activities of different regions in L1-5'UTR were identified by constructing these regions into pIRESneo, which SV40 region was removed prior, to generate different recombinant plasmids. The promoter activities in recombinant plasmids were detected by the luciferase expression assay. Western blot and co-immunoprecipitation were employed to identify proteins expression and protein-protein interaction respectively.

Results. Ago2 is a member of Agos family, which usually forms a RISC complex with si/miRNA and is involved in post-transcriptional regulation of many genes. Here L1-ORF1p and Ago2 conducts a regulation as a negative feedback for L1-5'UTR sense promoter. L1-ORF1p could form the immune complexes with Ago1, Ago2 and Ago4, respectively.

Conclusions. L1-5'UTR harbors both sense and antisense promoter activity and a dsRNA-mediated regulation is responsible for L1-5'UTR regulation. Agos proteins and L1-ORF1p were engaged in this process. (*Folia Histochemica et Cytobiologica* 2019, Vol. 57, No. 2, 56–63)

Key words: L1-5'UTR; Ago2; L1-ORF1p; antisense promoter; siRNA; HEK293 cells; protein interactions

Introduction

LINE-1 (Long Interspersed Nucleotide Element, L1), an autonomous retrotransposon and a parasitic element, makes up roughly 20% of human genomic DNA. It spreads throughout human genome by a manner of “copy and paste” that propagates its DNA or other DNAs within the genome through RNA intermediate and the mechanism is termed as

target-primed reverse transcription (TPRT) [1, 2]. L1, in human genome, is thought to be activated in germ cell, embryonic cells with early stage of development [3]. Promoter hypermethylation is a common phenomenon for gene deactivation; however, its deactivation is related to promoter hypermethylation in differentiated cells by epigenetic regulation. In addition, L1 as a cis-element can also suppress adjacent gene expression due to unknown mechanism [4]. Moreover, it has been reported that the promoter of L1 provides an alternative promoter site for the expression of other nearby genes. The translocation of L1 containing the 5'UTR promoter sequence to the intron region of the *Met* gene was observed in colorec-

Correspondence address: Yunfeng Zhu
College of Life Sciences and Bio-engineering,
Beijing Jiao-tong University, Beijing, China,
e-mail: zhuyf2004@163.com

tal and liver cancer. This phenomenon has been shown to be associated with an abnormal increase in Met expression and is important for malignant proliferation of cancer cells [5]. Under stress condition, e.g.: heat shock, virus infection, treating by cycloheximide, genotoxic agents and DNA base analogs, L1 can also be activated. The hypermethylation of L1 promoter occurs early in non-small-cell lung cancer (NSCLC), which is independently associated with poor prognosis in stage I NSCLC patients [6]. Physiologically, L1 is considered to be involved in X chromosome inactivation by a Xist mechanism [7]. Activated L1 not only reshapes the genome by arising gene mutations including insertion, deletion and rearrangement, but also contributes to modulation of gene expression by epigenetic mechanism. Given that L1 can lead to deleterious effects, it is important for cell to constrain its activation; however, this gene was identified to be activated ubiquitously in malignant tumor cells [8–10]. It is reported that L1 induces hTERT and ensures telomere maintenance in tumor cell lines [11]. Decreased expression of E-Cadherin and N-Cadherin proteins were observed post L1-ORF2p (L1 encoded ORF2 protein) transfection along with up-regulation of vimentin [12]. Thus, L1 with its feature of widespread distribution in human genome and ubiquitous activation in tumor cells can act as a promising model for us to understand the mechanism of tumorigenesis.

Full-length L1, 6–7kb, is consisted of a 5' untranslated region (5'-UTR), two open reading frames (ORF1 and ORF2) and a 3'-UTR. L1-ORF1p (L1 encoded protein ORF1 protein), a RNA-binding protein facilitating retrotransposition together with L1-ORF2 p [13], was identified to co-localize with RNA-induced silencing complex (RISC) recently [14, 15]. 5'-UTR of L1 containing a sense promoter (SP) and an antisense promoter (ASP) will generate a double-strand RNA (dsRNA) after transcription [16, 17], which implicates an RNA interference mechanism for its regulation. With the constructs of full-length and 3' truncated mutants of L1-5UTR, the promoter activity of L1 was investigated. At the same time, L1-ORF1p as a RNA binding protein was also identified for its function in this process.

The regulation of siRNA trafficking in human cells is executed by RNA-induced silencing complex (RISC). Small interfering RNA molecules are bound to RISC complex followed by inference with corresponding mRNA in cytoplasm. It was previously observed, that L1-5'UTR is negatively regulated by RISC via interaction with Ago2 (ArgonAUT family protein) [18]. However, the exact role of Ago2 (and other proteins of Ago family; Ago1, 3, 4) in regula-

tion negative feedback mechanism of L1 still remains unknown. Therefore, next aim of our study was to check whether the Ago2 protein interacts with L1 particles.

Materials and methods

Cell culture. HEK293 cells were cultured with DMEM medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (Hyclone, Logan, UT, USA). Cells were incubated at 37° with an atmosphere containing 5% CO₂ and saturated humidity. The medium was changed every 2–3 days.

Recombinant plasmids construction. PCR reagents were purchased from Takara Company (Beijing, China) and the approach was followed according to manufacturer's protocol. In brief, the components of PCR reaction were as follows: 2 μl DNA template, 1 μl upstream primer (10 mmol/ml) and downstream primer (10 mmol/ml) respectively, 10 μl of 2 × PCR master mixture (including: Taq, dNTP and buffer), H₂O to a 20 μl final reaction volume. PCR running parameters were as follows: 95°C for 5 min followed by 35 cycles of 94°C for 5 s, annealing at 58°C for 25 s, extension at 70°C for 25 s, and a final extension at 70°C for 10 min.

L1-ORF1p recombinant plasmid: The full-length sequence of L1-ORF1 was amplified by PCR with a template of human genome DNA and cloned into pIRESneo to generate recombinant plasmid pIRES-ORF1-Flag.

L1-5'UTR recombinant plasmids: pCBG99-5UTR-FL: Full-length L1-5'UTR was amplified by PCR with a template of human genome DNA and primers (5'-CCGCTCGAG(XhoII)GAGAGGAGCCAAGATGGC-3' and 5'-CCCAAGCTT(HindIII)CTTTGTGGTTTTATCTACTTT-3'). PCR products were cloned into pCBG99-control vector digested with XhoII/HindIII (removing SV40 promoter) prior to generate pCBG99-5UTR-FL.

pCBG99-5UTR-680 and pCBG99-5UTR-400:

For constructing 3'- truncated mutants of L1-5'UTR, 5'UTR-680 (removing ASP sequence) and 5'UTR-400 (removing ASP and partial SP sequences) of L1-5'UTR were amplified by PCR with a template of pCBG99-5UTR-FL and specific primers

(5'UTR-680: 5'-CCGCTCGAG(XhoII)GAGAGGAGCCAAGATGGC-3' and 5'-CCCAAGCTT(HindIII)GCAGTCTGCCGTTCTCAGA-3; 5'UTR-400: 5'-CCGCTCGAG(XhoII)GAGAGGAGCCAAGATGGC-3', and 5'-CCCAAGCTT(HindIII)TGCAGTTTGATCTCAGACTG-3').

PCR products were cloned into pCBG99-control vector digested by XhoII/HindIII prior to generate pCBG99-5UTR-680 and pCBG99-5UTR-400, respectively.

pCBG99-actin 680: As a negative control, the sequence of beta-actin with 680bp was prepared by PCR with a template of genomic DNA and primers (5'-CCGCTCGAG (*XhoI*) CTGTGCCCATCTACGAGG-3' and 5'-CCCAAGCTT (*HindIII*) AAAGGGTGTAACGCAACTAA-3'). PCR product was cloned into pCBG99-control digested by *XhoI*/*HindIII* prior to generate pCBG99-actin 680.

pCBG99-5UTR-aFL and pCBG99-5UTR-a680: For investigating ASP activity, antisense sequence of full-length of L1-5'UTR were prepared by PCR with the primers as follows:

5'-CCCAAGCTT (*HindIII*) GAGAGGAGCCAAGATGGC-3' and 5'-CCGCTCGAG (*XhoI*) CTTTGTGGT-TTTATCTACTTT-3'.

Antisense sequence of 3' truncated mutant of 5'UTR-680 was prepared by PCR with the primers as follows:

5'-CCCAAGCTT (*HindIII*) GAGAGGAGCCAA-GATGGC-3' and 5'-CCGCTCGAG (*XhoI*) GCAG TCTGCCCGTTCTCAGA-3.

PCR products were cloned into pCBG99-control digested by *XhoI*/*HindIII* prior to generate pCBG99-5UTR-aFL and pCBG99-5UTR-a680, respectively.

Ago2 recombinant plasmids: pSilencer-Ago2-siRNA1, pSilencer-Ago2-siRNA2, pSilencer-Ago2-Con1, pSilencer-Ago2-Con2: siRNA targeting sequences for Ago2 were designed by RNAi designer (Ambion, USA), two candidates were selected with the sequences as follows:

5'-ACCGAGTTTCGACTTCTACCTGTGTGA-3' and 5'-CAAGACTCTGCGCACCATGTACT-3'

The hairpin structure was designed with the sequences as follows:

siRNA1 gene: sense sequence, 5'-GATCCACCGAGTTCGACTTCTACCTGTGTATTCAAGAG ATACA-CAGGTAGAAGTCGAACTCGGTTTTTTTTGGAAA-3'; anti-sense sequence:

5'-AGCTTTTCCAAAAAACCAGAGTTCGACTTCTACCTGTGTATCTCTTGAATACACAGGTAGAA-GTCGA ACTCGGTG-3';

siRNA2 gene: sense sequence, 5'-GATCCCAAGACACTCTGCGCACCATGTACTTTCAAGAG AAGTACATGGTGCAGAGTGTCTTGTTTTTTTGGAAA-3'; anti-sense sequence:

5'-AGCTTTTCCAAAAACAAGACTCTGCGCACATGTACTTCTCTTGAAGTACATGGTGCAGAGTGTCTTGG-3'.

Double strand sequence was prepared by annealing and ligated into pSilencer2.1-U6/neo (Abcam, Cambridge, MA, USA) to generate recombinant plasmid pSilencer2.1-Ago2-siRNA1 and pSilencer2.1-Ago2-siRNA2, respectively. With same procedure, the control sequences for siRNA1 (con-siRNA1: 5'-ACCTGCTAGTCTATC-

CGTTAGGTA-3') and siRNA2 (con-siRNA2: 5'-CAACACTCTGCGCACTACTGGAACT-3') were designed, synthesized and cloned into pSilencer2.1-U6/neo to generate pSilencer-Ago2-Con1 and pSilencer-Ago2-Con2, respectively.

Western blot analysis. The recombinant plasmids (pSilencer-Ago2-siRNA1, pSilencer-Ago2-siRNA2, pSilencer-Ago2-Con1, pSilencer-Ago2-Con2) were transfected into HEK293 cells using LipofectamineTM2000 reagent (Invitrogen, Carlsbad, CA, USA) for 48 h. Harvested cells were lysed with the lysis buffer (PBS solution including 2 μ g/ml aprotinin, 100 μ g/ml phenylmethyl-sulphonyl fluoride, 2 μ g/ml leupeptin and 1% Nonidet P-40 (Beyotime, Shanghai, China) followed by centrifugation to remove unsolved debris. After fractionating by 12.5% SDS-PAGE, the proteins were transferred into nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were incubated with 5% non-fat milk for 1 h in room temperature and then with the primary antibody (anti-Ago2, anti-FLAG, anti-beta-actin, Abcam) for additional 1 h followed by washing with TBST (Tris Buffered Saline Tween) for three times. The membranes were incubated with horseradish peroxidase-labeled second antibody (Sigma, St Louis, MO, USA) for 1 h and washed with TBST for three times. The signals were visualized by incubating with enhanced chemiluminescence reagent (Amersham, Little Chalfont, UK) for 5 min. Triplicate experiments were conducted independently.

Luciferase expression assay. HEK293 cells were cultured with DMEM containing 0.5% calf serum for 24 hours, followed by transfection using 1 μ g pCMV- β -gal (internal reference) combining with 1 μ g different constructs. After culturing for 1 h the transfected cells were lysed and subjected to luciferase and β -Galactosidase assay respectively (Promega, Shanghai, China). The values of luciferase in different groups were normalized by β -Galactosidase to eliminate differences elicited by transfection efficiency. Triplicate experiments were conducted independently.

Co-immunoprecipitation. The cells were harvested and incubated with lysis buffer (20 mM Tris HCl pH 8, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM EDTA) including a cocktail of protease inhibitors (Roche, Basel, Switzerland) on ice for 5 min. The supernatant solution was prepared by centrifuging with 15000 rpm for 10 min at 4°C. 200 μ l cell lysate and 20 μ l 50% bead slurry (protein-A sepharose) were mixed and incubated with gently rocking for 3–4 h at 4°C. Supernatant solution was collected and incubated with 5 μ l anti-FLAG by gently rocking for 4 h at 4°C. After spinning down and washing, the pellet was re-suspended with 20 μ l 2 \times SDS buffer, heated at 95–100°C

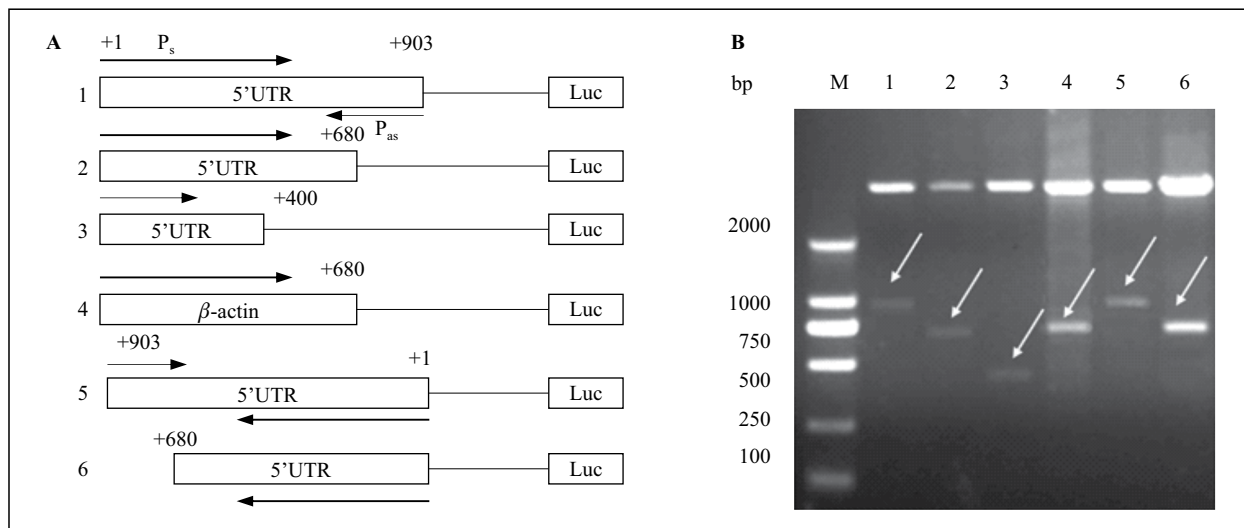


Figure 1. The constructs of full-length and truncated L1-5'UTR. **A.** A schematic map of construction of L1-5'UTR and mutants. 1: pCBG99-5UTR-FL (903bp); 2: pCBG99-5UTR-680 (680bp); 3: pCBG99-5UTR-400 (400bp); 4: pCBG99-actin 680 (680bp); 5: pCBG99-5UTR-aFL (903bp); 6: pCBG99-5UTR-a680 (680bp); **B.** The different constructs were confirmed by electrophoresis. DNA sizes of L1-5'UTR in different constructs were confirmed by digesting with restrictive enzymes as described in materials and methods. M: DNA molecular marker (2000bp, 1000bp, 750bp, 500bp, 250bp, 100bp); 1: pCBG99-5UTR-FL (903bp); 2: pCBG99-5UTR-680 (680bp); 3: pCBG99-5UTR-400 (400bp); 4: pCBG99-actin 680 (680bp); 5: pCBG99-5UTR-aFL (903bp); 6: pCBG99-5UTR-a680 (680bp). Arrows in Fig. 1B mean the different amplicons mentioned above.

for 2–5 min and then subjected to SDS-PAGE analysis. The proteins were identified by immune blot using anti-Ago1, anti-Ago2, anti-Ago3, anti-Ago4 antibodies, respectively (Abcam, Shanghai, China).

Statistical analysis. Differences between experiment groups and control were tested using SPSS software v. 11.0 (IBM, New York, NY, USA), in which $p < 0.05$ were considered as significant differences.

Results

Construction of the L1-5'UTR mutants

To elucidate the promoter activity of L1-5'UTR, the full-length and 3' mutants of L1-5'UTR were constructed with SV40-removed luciferase report system and confirmed by sequencing. The schematic map and recombinant plasmids for confirming constructs were shown in Figure 1.

The activity of SP and ASP in L1- 5'UTR were identified

With report system construct of luciferase, the promoter activities of sense and antisense in L1-5'UTR were identified quantitatively. As indicated in Figure 2, both activity of SP and ASP in L1-5'UTR were eval-

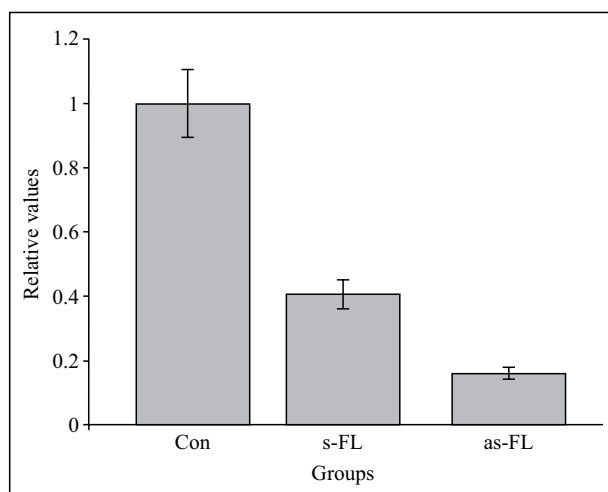


Figure 2. The identification of promoter activities of L1-5'UTR in sense and antisense sequences. The sense and antisense sequences of L1-5'UTR were constructed in immediately upstream of luciferase gene in SV40-removed pCBG99. The relative values of luciferase expressions in different groups were detected as described in Materials and methods. Con: pCBG99-Control (100%); s-FL: pCBG99-5UTR-FL (40%); as-FL: pCBG99-5UTR-aFL (16%). The percentages in parentheses are relative values of luciferase expression compared with control. The value in each group is the mean of triplicates and SEM were labeled by whiskers over the bars

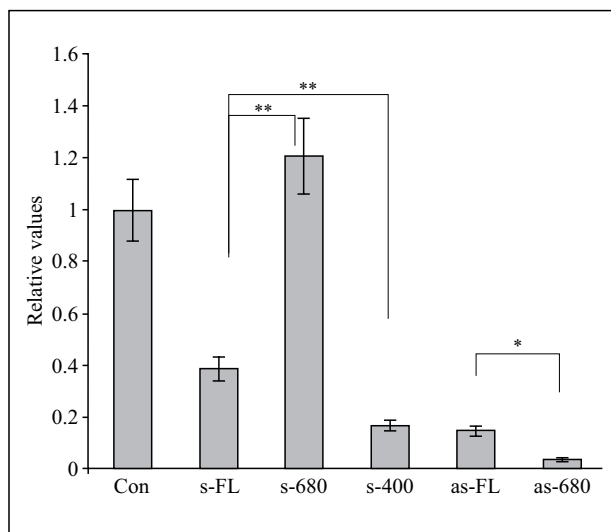


Figure 3. Antisense sequence of L1-5'UTR negatively regulates its promoter activity. Different constructs of L1-5'UTR were transfected into HEK293 cells. The expression of luciferase in different groups was detected and the relative values were calculated by normalizing with control as describing in Materials and methods. Con: pCBG99-Control (100%); s-FL: pCBG99-5'UTR-FL (40%); s-680: pCBG99-5'UTR-680 (120%); s-400: pCBG99-5'UTR-400 (18%); as-FL: pCBG99-5'UTR-aFL (16%); as-680: pCBG99-5'UTR-a680 (5%). The percentages in parentheses are relative values of luciferase expression compared with control. * $p < 0.05$; ** $p < 0.01$

uated by comparing with SV40 and indicated that SP was much stronger than ASP.

ASP can suppress SP activity

Owing to transcripts from SP and ASP in L1-5'UTR will generate a dsRNA, the effect of dsRNA on SP activity was investigated by constructing different ASP mutants. pCBG99-5UTR-680, a construct with 223bp removing in 3 terminus of 5'UTR, and pCBG99-5UTR-400, a construct with 503bp removing in 3 terminus of 5'UTR displays the strongest and weakest SP activity respectively by comparing with pCBG99-5UTR-FL, a construct with full-length of L1-5'UTR. Meanwhile, the remaining ASP activity in these truncated mutants was also determined by constructing pCBG99-5UTR-a680 and pCBG99-5UTR-a400 (data not shown) (Fig. 3). Apparently, ASP activity negatively regulates SP activity and a dsRNA-mediated regulation was responsible for this mechanism.

Argonaut 2 is involved in siRNA-mediated regulation for L1-5'UTR

To elucidate a dsRNA-mediated regulation further, Argonaut 2 (Ago2), a key component of RISC (RNA-induced Silencing Complex) playing an important role in siRNA processing was investigated. Co-transfection of constructs of Ago2-siRNA with either pCBG99-5UTR-680 or pCBG99-5UTR-FL was conducted. As indicated in Figure 4, luciferase expression in construct of pCBG99-5UTR-FL was increased significantly, whereas moderate and null increases were shown in constructs of pCBG99-5UTR-680 and pCBG99-

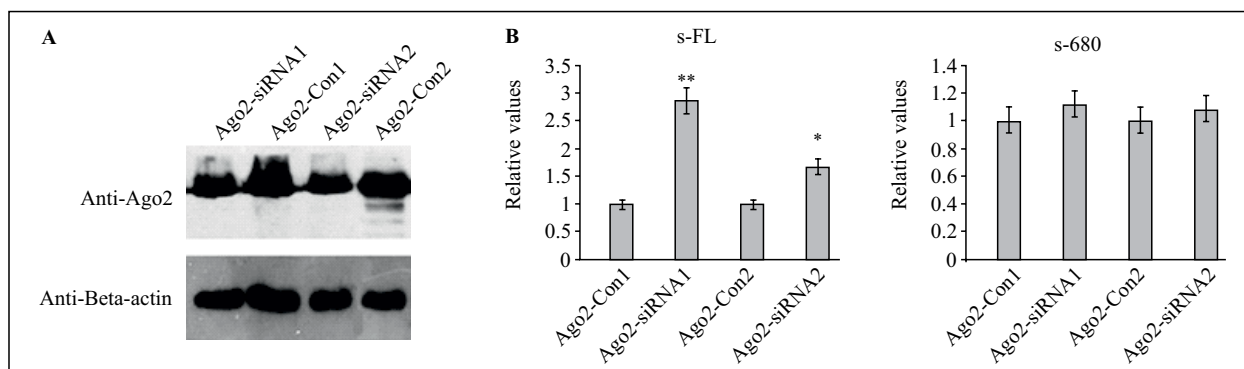


Figure 4. Ago2 is involved in the regulation of L1-5'UTR activity. HEK293 cells were transfected by either pCBG99-5UTR-FL or pCBG99-5UTR-680 combined with Ago2-siRNA constructs. The expressions of luciferase in different groups were detected by measuring the OD value at 415 nm absorbance the relative values were calculated by normalizing with respective control (transfected by either pCBG99-5UTR-FL or pCBG99-5UTR-680 only). Ago2-siRNA1: pSilencer-Ago2-siRNA1; Ago2-con1: pSilencer-Ago2-Con1; Ago2-siRNA2: pSilencer-Ago2-siRNA2; Ago2-con2: pSilencer-Ago2-Con2 s-FL: pCBG99-5'UTR-FL; s-680: pCBG99-5'UTR-680. **A.** The expressions of Ago2 in transfected HEK293 cells with different siRNA constructs where assessed by Western blot. **B.** The relative values of luciferase expressions. S-FL: Ago2-con1 (100%); Ago2-siRNA1 (280%); Ago2-con2 (100%); Ago2-siRNA2 (170%); S-680: Ago2-con1 (100%); Ago2-siRNA1 (110%); Ago2-con2 (100%); Ago2-siRNA2 (105%). The percentages in parentheses are relative values of luciferase expression compared with control. The value in each group is the mean of triplicates and SEM were labeled by whiskers over the bars * $p < 0.05$; ** $p < 0.01$

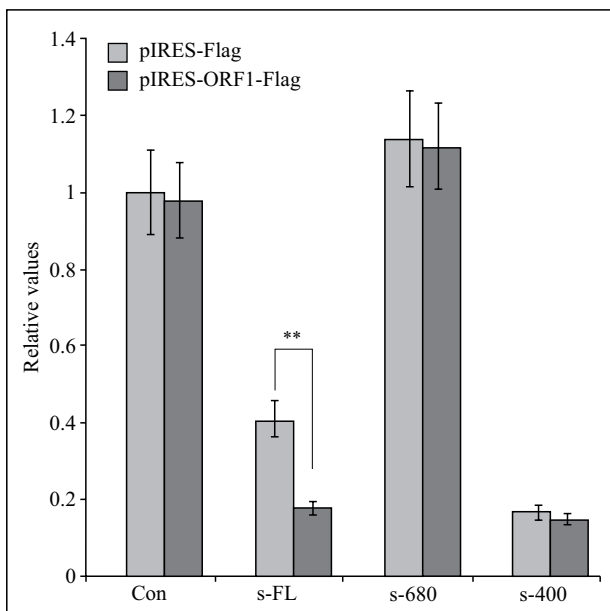


Figure 5. L1-ORF1p modulated L1-5'UTR activity. pIRES-ORF1-Flag was co-transfected with one of constructs of L1-5'UTR in HEK293 cells and the expressions of luciferase in different groups were detected by measuring the OD value at 415 nm absorbance. After normalizing with control (co-transfection of pIRES-Flag with pCBG99-Control), the relative values of expression in different groups were plotted. Con: pCBG99-Control (pIRS-FLAG: 100%, pIRS-ORF1-FLAG: 97%); s-FL: pCBG99-5UTR-FL (pIRS-FLAG: 40%, pIRS-ORF1-FLAG: 19%); s-680: pCBG99-5UTR-680 (pIRS-FLAG: 112%, pIRS-ORF1-FLAG: 110%); s-400: pCBG99-5UTR-400 (pIRS-FLAG: 19%, pIRS-ORF1-FLAG: 18%). The percentages in parentheses are relative values of luciferase expression compared with control. The value in each group is the mean of triplicates and SEM were labeled by whiskers over the bars **p < 0.01

-5UTR-400, respectively. Suggesting that Ago2 can suppress L1-5'UTR promoter activity and the mechanism by which a siRNA-mediated regulation may account for this fact.

L1-ORF1p is involved in siRNA-mediated regulation for L1-5'UTR

Given the RNA binding activity of L1-ORF1p, the effect of L1-ORF1p on siRNA-mediated regulation for L1-5'UTR was investigated. With different constructs of L1-5'UTR, the promoter activity was investigated in L1-ORF1p over-expressed HEK293 cells. As indicated in Figure 5, over-expression of L1-ORF1p resulted in decrease of luciferase expression significantly in full-length 5'UTR construct, whereas less effect on other truncated mutants. It indicates that L1-ORF1p is involved in siRNA-mediated L1-5'UTR regulation.

L1-ORF1p can form immune complex with the members of Argonaut family

Owing that both L1-ORF1p and Ago2 are RNA-binding proteins, the interaction between them was investigated with which may account for the mechanism of L1-ORF1p. As indicated in Figure 6A, a RNA-independent interaction between Ago2 and L1-ORF1p was identified by co-immunoprecipitation. Furthermore, the other members of Argonaut family in human were also investigated and indicated that Ago1, Ago2, Ago4 but not Ago3 can form immune complex with L1ORF1p, respectively (Fig. 6B). Thus, the interaction between Ago2 (or other members of Argonaut family) and L1-ORF1p may account for the mechanism of L1-ORF1p for its feedback regulation to L1-5'UTR.

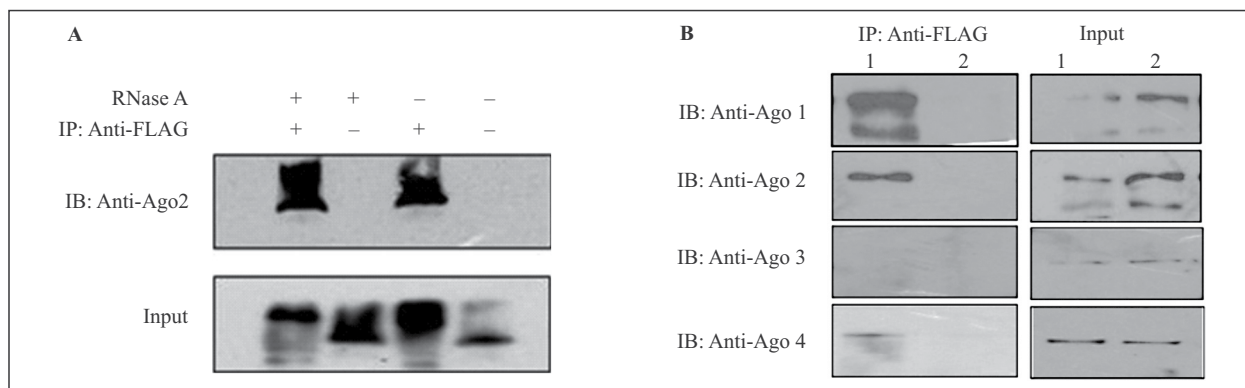


Figure 6. L1ORF1p formed a complex with the members of Argonaut family, Ago1, Ago2 and Ago4. Transfected HEK293 cells were lysed and subjected to immunoprecipitation with anti-L1ORF1p. The complexes were fractionated by SDS-PAGE and immune-blotted with anti-Ago1, anti-Ago2, anti-Ago3 and anti-Ago4, respectively. 1: pIRES-L1ORF1-Flag; 2: pIRES (control). **A.** RNA-independent interaction of L1-ORF1p and Ago2. **B.** The interactions of L1-ORF1p with other members of Argonaut family, Ago1 and Ago4.

Discussion

SP and ASP in L1-5'UTR were identified for their activities by different constructs with luciferase report system. To elucidate a dsRNA (siRNA precursor) mediated regulation for L1-5'UTR, we constructed differently truncated mutants of 3' terminus of L1-5'UTR (removing ASP sequence) and found that the activity of SP has a negative relationship with ASP. It is implicated that a siRNA mediated regulation was employed for L1-5'UTR. Furthermore, Ago2, a critical component in RISC (RNA- Induced Silencing Complex) [18] was investigated and, resultantly, a negative regulation for L1-5'UTR was identified. Our result is consistent with previous report, in which L1-5'UTR transcripts was identified as the first endo-siRNA in germ cells and L1 retrotransposition was suppressed by endogenously encoded small interfering RNAs [19] and Ago2 [14, 20]. Thus, Ago2 plays a crucial role in siRNA-mediated regulation for L1-5'UTR. Consistently, the depletion of endo-siRNA was reported in human breast cancer cells. In addition, L1-ORF1p, a RNA binding protein and co-localizing with RISC [14], is also involved in a negative feedback regulation of L1-5'UTR by siRNA-mediated mechanism. Given that both Ago2 and L1-ORF1p are RNA binding proteins, an interaction was hypothesized and as a result that L1-ORF1p can form immune complex with Ago2 with RNA independent manner even that the domains responsible for direct interaction need to be identified further. In addition, more members, but not Ago3, of Argonaut family (Ago1, Ago2, Ago4) were identified to interact with L1-ORF1p, suggesting that L1-ORF1p is involved in both siRNA and miRNA regulation based on the fact that Ago2 usually forms a complex with siRNA and Ago1/Ago4 usually forms a complex with miRNA [21]. Usually, Argonaut proteins and miRNAs/siRNAs are localized in processing bodies and, under stress conditions, re-localized to stress granules where it results in mRNA cleavage with miRNA/siRNA-dependent manner [21]. So, the interaction of L1-ORF1p with Argonauts may also account for that L1-ORF1p is involved in the process of miRNA/siRNA maturing and processing.

Taken together, both SP and ASP are identified in L1-5'UTR and a siRNA mediated regulation is identified for L1-5'UTR. L1-ORF1p, a RNA binding protein, is involved in siRNA mediated feedback regulation for L1-5'UTR by forming a complex with Ago1, Ago2 and Ago4, respectively.

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Conflict of interest

The authors declare no conflict of interest.

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