Construction of Arel1 Recombinant Plasmid Vector

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Abstract

Arel1 is an E3 ubiquitin ligase with HECT structure which specifically binds to the substrate and degrades it by ubiquitination. In this study, we constructed the recombinant expression vector of Arel1 to explore its function. The total RNA was extracted from placenta and the Arel1 gene was amplified by PCR. Arel1 gene was inserted into pCMV-N-Flag plasmid vector. The gene expression was detected in bacterial colonies by PCR, and the insert was confirmed by restriction enzyme digestion and DNA sequencing. Compared with the original sequence, the matching degree was 100%. We successfully obtained the Arel1 recombinant plasmid expression vector.

Keywords

Arel1; E3 ubiquitin ligase; Recombinant plasmid.

1. Introduction

Arel1 is an ubiquitin ligase and belongs to the HECT family. HECT family shares a conserved catalytic domain at the carboxy-terminus of proteins (HECT domain~ 40-kD) ,with at least 4 aspects of biochemical activity：(1) Binding specific E2 ligase; (2) Accept ubiquitin from E2 to form ubiquitin thioester intermediate at cysteine active site; (3) Transfer ubiquitin to the ε-amino group on the side chain of the substrate lysine by catalyzing the formation of isopeptide bonds; (4) Deliver ubiquitin molecules to the growing end of multi-ubiquitin chains[1]. E2 ligases can transthiol by transferring ubiquitin to the cysteine active site of HECT family. When the ubiquitin molecule is present, the HECT family modifies the substrate in a manner dependent on E2 ligase[1]. The NH2-terminal sequence of the HECT family is not conserved and contains the major sites recognized by specific substrates[2]. The *Arel1* gene is located on chromosome 14q24.3, with a total length of 51853 bp and 20 exons. Northern blot shows that 5.4 kb *Arel1* mRNA can be detected in mouse brain, testis, heart, liver, lung and kidney tissues, but it is hardly expressed in skeletal muscle and spleen[3]. We also found that Arel1 protein expressed in testis. In order to explore the function and role of Arel1 in the testis, we constructed *Arel1* recombinant plasmid expression vector.

2. Material and Methods

2.1 Material

RNA extraction kit (Magen), cDNA first-strand synthesis kit (TaKaRa), PCR Kit (TaKaRa), pCMV-N-Flag (Beyotime Biotechnology), Plasmid extraction kit (OMEGA), BglII/EcoRV enzyme (TaKaRa), T4 DNA Ligase (NEB), rTaq (Takara), Endotoxin-free plasmid extraction Kit (OMEGA).

2.2 Methods

2.2.1 RNA Extraction

(1) Weigh 20 mg of fresh placental tissue into a 1.5 mL centrifuge tube, and then add 600 μL of RL Buffer/β-ME (add 20 μL of β-threoethanol per 1 mL of Buffer RL), centrifuge at 12000×*g* for 5 minutes after sufficient grinding.

(2) Aspirate the supernatant and transfer it to the installed gDNA column, centrifuge at 12000×*g* for 2 min.

(3) Discard the filter column, add 600 μL of 70% ethanol to the filtrate, and mix by pipetting.

(4) Transfer the above mixture to HiPure RNA Mini Column and centrifuge at 8000×*g* for 30 s.

(5) Discard the filtrate and add 600 μL RW1 Buffer to the column, centrifuge at 8000×*g* for 30 s.

(6) Discard the filtrate and add 600 μL RW2 Buffer to the column, centrifuge at 8000×*g* for 30 s.

(7) Repeat (6).

(8) Discard the filtrate, and centrifuge the empty column at 12000×*g* for 2 min.

(9) Place the HiPure RNA Mini Column in a new 1.5 mL centrifuge tube, add 70 μL of RNase free water in the center of the column, Let stand at room temperature for 2 min, and centrifuge at 12000×*g* for 1 min.

(10) Nanodrop detects the RNA concentration and OD260 / OD280 and then stores at -80℃.

2.2.2 cDNA Synthesis

(1) Prepare the following reaction mixture in a 1.5 mL EP tube：

Chemicals	Amount
Oligo dT Primer (50 μM)	1 μL
dNTP Mixture (10 mM each)	1 μL
RNA	Total RNA: ≤5 μg Poly(A)$^+$: ≤1μg
RNase free dH_2O	Up to 10 μL

(2) After the reaction mixture was kept at 65℃ for 5 min, it was quickly cooled on ice to denature the RNA.

(3) Prepare the following reaction solution in the above EP tube：

Chemicals	Amount
The above reaction solution (from step 2)	10 μL
5×PrimerScript II Buffer	4 μL
RNase Inhibitor (40 U/μL)	0.5 μL (20 U)
PrimerScript II RTase (200 U/μL)	1 μL (200 U)
RNase-free Water	Up to 20 μL

(4) Mix slowly.

(5) Reverse transcription under the following conditions: 50℃, 30~60 min.

After 5 min at 95℃ (enzyme inactivation), cool on ice.

2.2.3 PCR Reaction

(1) Primers (Arel1):

ACACGATATCATGTTTTACGTTATTGGTGGAATCACAG (sense);
GGAAGATCTTCAGAGCATGCCAAAGCCCTC (anti-sense).

(2) Reaction system:

Reaction component	Usage amount
PrimerSTAR Max Premix（2×）	25 μL
Primer F	2 μL (10 μM)
Primer R	2 μL (10 μM)
Template cDNA	2 μL
RNase-free Water	19 μL
Total Volume	50 μL

（3） Reaction conditions:

Reaction process	Time	Temperature	
Pre-denaturation	1 min	98℃	
Denaturation	10 s	98℃	×30
Annealing	10s	60℃	
Extend	1min	72℃	

2.2.4 PCR Production Purification

(1) Add Buffer DC to the PCR and mix by pipetting.

(2) Place Spin Column in Collection Tube, transfer the mixed solution in (1) to Spin Colum, centrifuge at 2000 rpm for 1 min at room temperature， discard the filtrate.

(3) Add 700 μL of Buffer WB to the tube, centrifuge at 12,000 rpm for 30 s at room temperature, and discard the filtrate.

(4) Repeat (3)

(5) Place the Spin Column in the Collection Tube， and centrifuge the empty column at 12,000 rpm for 1 min at room temperature.

(6) Place the Spin Column in a new 1.5 mL centrifuge tube, add 25-30 μL of Elution Buffer in the center of the Spin Column, and let stand at room temperature for 1 min.

(7) Centrifuge at 12,000 rpm for 1 min at room temperature, elute the DNA, and store the product at -20℃.

2.2.5 pCMV-N-Flag Transformation and Amplification

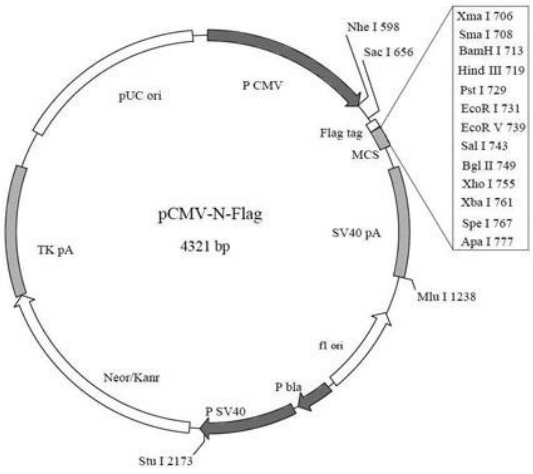

Fig. 1 pCMV-N-Flag Plasmid expression vector

(1) Take *E. coli* strain DH5α from -80℃, thaw on ice.

(2) Add 2 μL of plasmid into 30 μL of competent cells, mix by pipetting, and put it in ice for 30 min.

(3) The above mixture was heat shocked in a 42℃ water bath for 90 s, then quickly transferred it to ice and stay for 2 min.

(4) Add 450 μL of LB liquid medium without antibiotics to the mixture, then put it in a shaking incubator and incubate for 1 h at 37℃.

(5) Centrifuge at 4000×*g* for 5 min at room temperature, discard 400 μL of supernatant, resuspend the cells by gently pipetting the remaining medium. Add the suspension to the plate of LB solid medium containing antibiotics.

(6) Spread the suspension evenly with sterile coating ring, and place it at room temperature for 10 min to make the bacterial solution absorbed.

(7) Seal the plate with parafilm and invert it in a 37℃ incubator for 12-16 h.

(8) Pick a single clone and identify the positive colony by PCR.

(9) The positive colony was cultured in LB liquid medium containing antibiotics in a shaking incubator for 12-16 h (37 ℃, 200 rpm/min).

2.2.6 Extraction of Plasmids

(1) Collect 3-5 mL of bacterial solution and centrifuge at 12000×*g* in a 1.5 mL EP tube for 1 min.

(2) Discard the supernatant and add 250 μL of resuspension buffer to the EP tube.

(3) Add 250 μL of lysate to the bacterial solution, mix upside down, lyse thoroughly until the solution becomes transparent.

(4) Add 400 μL of neutralization buffer to the lysis solution, invert and mix to generate a white flocculent precipitate, centrifuge at 12000×*g* for 13 min at room temperature.

(5) Pipette about 750 μL of supernatant into the filter column and centrifuge at 12000×*g* for 1min.

(6) Discard the filtrate, add 500 μL of protein removal buffer, and centrifuge at 12000×*g* for 30 s.

(7) Discard the filtrate, add 600 μL of washing solution, centrifuge at 12000×*g* for 30 s, and discard the filtrate.

(8) Repeat (7).

(9) Centrifuge with empty column at 12000×*g* for 2 min.

(10) Put the filter column in a new 1.5 mL EP tube, add 30 μL of eluent to the center of the column, let it stand for 2 min, and centrifuge at 12000×*g* for 1 min.

(11) Repeat (10).

(12) Measure the concentration of plasmid and store at -20 ℃.

2.2.7 Construction of Recombinant Plasmid pCMV-N-Flag-Arel

（1） Restriction enzyme digestion:

Reaction components	Amount
Restriction enzyme	1 μL
10× buffer	2 μL
DNA	≤1 μg
ddH_2O	Up to 20 μL
Total Volume	20 μL

（2）Connect the vector and target gene. The reaction system and conditions are as follows:

Reaction components	Usage amount
Target gene	3.5 μL
Vector	1.5 μL
T4DNA ligase	0.5 μL
10×buffer	1 μL
ddH_2O	Up to 10 μL
Total Volume	10 μL

(3) Transformation of recombinant plasmid into E. coli strain DH5α and amplification.

(4) Three colonics were randomly picked and their inserts were amplified by PCR.

2.2.8 The Insert was Confirmed by Restriction Enzyme Digestion and DNA Sequencing

(1) The reaction system is as follows：

Reaction components	Amount
Restriction enzyme Ⅰ	0.5 μL
Restriction enzyme Ⅱ	0.5 μL
10× buffer	2 μL
DNA (plasmid)	≤1 μg
ddH_2O	Up to 20 μL
Total Volume	20 μL

(2) Put the mixture into 37℃ water bath for 1 h.

(3) The insert was confirmed by DNA sequencing.

3. Result

3.1 RNA Extraction from Placental Tissue

RNA was extracted from placental tissue and detected by agarose gel electrophoresis. There were three bands shown in Fig. 2: 28sRNA, 18sRAN, and 5sRNA. It indicated that the total RNA was successfully extracted without degradation.

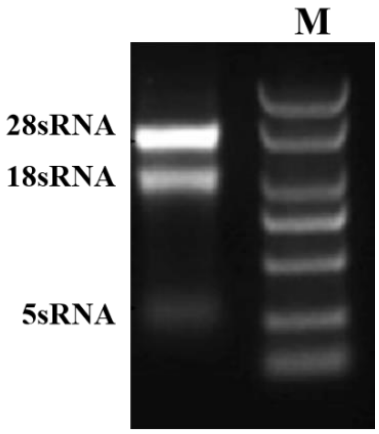

Fig. 2 Agarose gel electrophoresis for RNA

3.2 PCR Amplification of Arel1

The nucleic acid sequence of *Arel1* is displayed on NCBI genebank, and it is 2472bp. The cDNA obtained by reverse transcription was used as a template for PCR amplification. The PCR product was detected by agarose gel electrophoresis. The bands of PCR product are located in the sites of 2000bp-3000bp in Fig. 3, which is consistent with the molecular weight of *Arel1*.

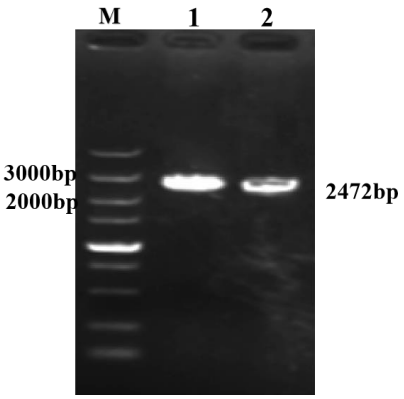

Fig.3 PCR amplification of *Arel1*. 1 & 2 are different samples of PCR product.

3.3 Target Gene in Colony was Amplified by PCR

The obtained target gene and the plasmid expression vector pCMV-N-Flag were treated by restriction digestion respectively to construct the recombinant plasmid pCMV-N-Flag-Arel1 which was transformed into DH5α cells. The bacterial solution was spread on a plate containing KaNa antibiotics, incubating at 37℃ overnight, and then colony PCR was used to identify positive single colony. The results are shown in the figure below, the bands of colony PCR is consistent with the bands of target gene amplified by PCR.

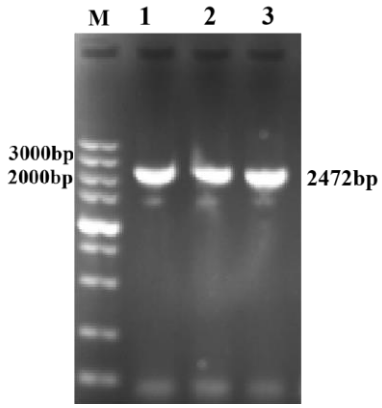

Fig. 4 PCR amplification of the target gene in pCMV-N-Flag-Arel1.
1, 2, 3 are different positive single colonies.

3.4 The Insert Confirmed by Restriction Enzyme Digestion

The positive colonies were cultured overnight in LB liquid medium containing antibiotics, and the bacterial liquid was collected to extract the plasmid. The insert was confirmed by restriction enzyme digestion and enzymatic product was verified by agarose gel electrophoresis. The result is shown in the figure below, which is consistent with the PCR amplified product.

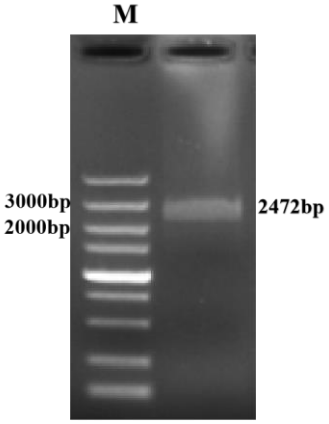

Fig. 5 The insert was confirmed by restriction enzyme digestion

3.5 DNA Sequencing Result

The DNA sequencing result of the recombinant plasmid was compared with the original DNA sequence. The result is shown in the figure below, and the matching rate is 100%. This shows the successful construction of *Arel1* recombinant plasmid vector.

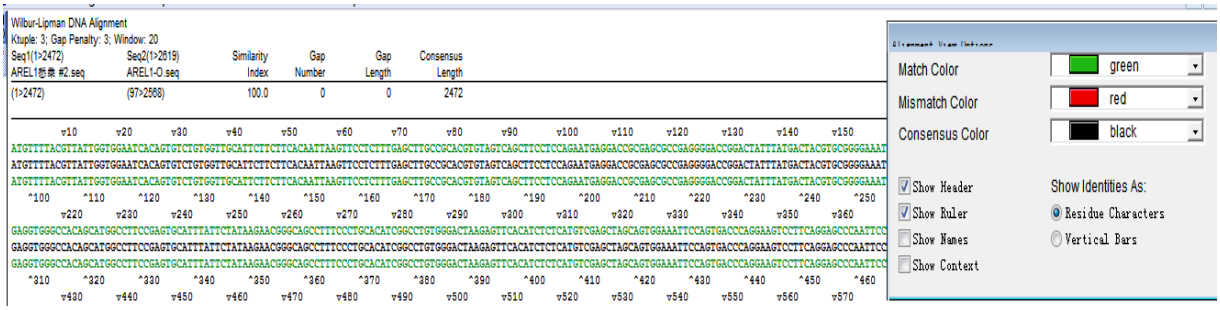

Fig. 6 DNA sequence result

4. Discussion

The characteristic of HECT E3 ligase catalysis is that it first produces an E3-Ub intermediate form with the ubiquitin molecule through a thioester bond, then transfer the Ub to the substrate molecule. This particular reaction may provide an effective site for regulating the process of ubiquitination[4]. The abnormal HECT E3 ligase-mediated ubiquitination can cause cancer[5], nervous system diseases, autoimmune diseases[6], and hypertension[7]etc. Therefore, it is of great significance to explore the mechanism of E3 ubiquitin ligase of HECT family. Arel1 contains a HECT domain at the C-terminus and is located in the cytoplasm. When cells are stimulated by apoptosis signals, IAP antagonists such as SMAC, HtrA2 and ARTS localized in the mitochondrial inner and outer interstitial stroma will be released into the cytoplasm. Finally, Arel1 interacts with and ubiquitination degrades IAP antagonists to inhibit the occurrence of apoptosis[3]. At present, Arel1 has no reports besides being an E3 ubiquitin ligase to inhibit apoptosis. In this study, we have found that Arel1 protein locates in testis. In order to explore the function and regulatory mechanism of Arel1 in the testiswe constructed the recombinant plasmid expression vector of Arel1.

References

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