Title: Screening for the optimal siRNA targeting a novel gene (HA117) and construction and evaluation of a derivative recombinant adenovirus

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Screening for the optimal siRNA targeting a novel gene (HA117) and construction and evaluation of a derivative recombinant adenovirus

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Abstract

We found a novel gene named as HA117 in our previous research. At this study, we screened for an optimal siRNA targeting the novel gene HA117 using the pSOS-HUS method, verified the results of pSOS-HUS siRNA screening for optimal affinity for the target gene, and constructed and evaluated a recombinant adenovirus carrying the DNA template for transcription of the optimal HA117 siRNA. The pSOS-HUS vector method was successfully utilized as a rapid and effective screen for an optimal siRNA for a target gene. Among five pairs of DNA templates, siRNA transcribed from HAi5 gave the strongest interference with the novel gene HA117; a HAi5-carrying recombinant adenovirus (Ad-HAi5) was successfully

constructed and evaluated, laying a foundation for the further study of HA117 gene function with RNAi technology.

Keywords: HA117 gene; RNA interference; screening; multidrug resistance; recombinant adenovirus

The development of multidrug-resistant tumor cells is a complex process regulated by multiple genes. In a previous study, we established a multidrug-resistant cell line (HL-60/MDR) to facilitate the search for genes involved in the regulation of tumor-cell multidrug resistance [1]. With a combination of suppression, subtractive hybridization and microarray methods, we screened and cloned the novel gene HA117 (Genbank accession number AY230154), which was clearly expressed in the drug-resistant HL60/MDR cell line but not expressed in wild-type HL60 cells. And we found it increased the multidrug resistance of K562 cells and CW-2 cells and could be one of genes regulating tumor cells' drug resistance and a new target of cancer gene therapy[2]. In the present study, we used the plasmid-based pSOS-HUS screening system previously developed in our laboratory to screen for siRNAs targeting the HA117 gene [3]. Double-cloned plasmids were constructed containing both the HA117 gene and DNA templates for complementary siRNA. After transfection of the double-cloned plasmids into 293 cells, we screened for and identified an optimal siRNA for the HA117 gene by measuring green fluorescent protein (GFP) expression levels via microscopic observation and flow cytometry and by quantitating HA117 mRNA expression by everse-transcription qPCR" (RT-qPCR). A recombinant adenovirus for HA117 RNA interference was then constructed and evaluated.

Materials and methods

Plasmids and cell lines

The screening plasmid pSOS-HUS and a red fluorescent protein (RFP)-carrying adenovirus backbone plasmid (pSES-HUS) were developed in collaboration between our laboratory and the Molecular Oncology Laboratory of the University of Chicago. *E. coli* DH5α, *E. coli* BJ5183 and its derivative Adeasy-1 cells, the adenoviral-genome-carrying plasmid pAdeasy-1 and the packaging cell line 293 were generous gifts from Professor Tong-Chuan He of the Molecular Oncology Laboratory of the University of Chicago. The HA117 gene-carrying recombinant plasmid pAdtrack-HA117 and the recombinant adenovirus Ad5-HA117 were propagated in our laboratory [4]. The human colon carcinoma cells CW-2 were bought from the Cell Bank of Chinese Academy of Science, Shanghai, China.

Reagents

Restriction endonucleases, T4 DNA ligase, plasmid DNA isolation, extraction and purification kits and RT-PCR kits were all purchased from TaKaRa Bio Inc. Lipofectamine and an RNA-extraction kit were purchased from Invitrogen Corporation. DNA templates for HA117 gene siRNAs and PCR primers were synthesized by TaKaRa Bio Inc. DNA sequencing was conducted by the Laboratory of Infectious Diseases at Chongqing Medical University. High-quality fetal bovine serum and 1640 medium were Gibco products.

Construction of recombinant plasmid pSOS-HUS-HA117

The plasmids pAdTrack-HA117 and pSOS-HUS were double-digested with HindIII and KpnI restriction endonucleases. The excised target gene, HA117, and the linearized pSOS-HUS vector were extracted from an agarose gel after digestion, then purified, ligated with T4 DNA

ligase and transformed into competent DH5□ cells. Colonies were picked and amplified for plasmid DNA preparation and restriction digestion. Positive clones were identified by restriction digestion and further confirmed by sequencing.

Design and synthesis of DNA templates for HA117 siRNA

Based on the principles of siRNA design and the full-length cDNA sequence of the HA117 gene[5], we designed specific HA117 gene-insertion-fragment sequences for synthesis. These were designated as follows. HAi1: sense strand, 5'-aCGACAGTATCAACATATCAtttt-3'; antisense strand, 5'-aTTCTCAGCTGGACCATCTGtttt-3; HAi2: sense strand, 5'-aATACGGAGATACGACAGTAtttt-3'; antisense strand, 5'-aTACTG TCGTATCTCCGTATtttt- 3'; HAi3: sense strand, 5'-aCGACAGTATCAACATATCAtttt-3'; antisense strand, 5'-aTGATATGTTGATACTGTCGtttt-3'; HAi4: sense strand, 5'-aTTATGGACCTGAGGCAGATtttt-3'; antisense strand, 5'-aATCTGCCTCAGGTCCATAAttt-3'; HAi5: sense strand, 5'-aGCACCTGAGTACAGACAATtttt-3';antisense strand, 5'-aATTGTCTGTACTCAGGTGCtttt-3'. The lowercase letters indicate sequences complementary to the cohesive ends of the SfiI-digested pSOS-HUS plasmid.

Construction and identification of the pSOS-HUS-HA117 and HAi recombinant plasmids

Plasmid pSOS-HUS-HA117 was digested with SfiI, ligated to the five pairs of DNA templates with T4 DNA ligase and transformed into DH5α competent cells. Colonies were picked and amplified for plasmid DNA preparation and restriction digestion.

Positive clones were identified by restriction digestion and further confirmed by sequencing.

Transfection of the double-cloned plasmids into 293 cells with Lipofectamine

The plasmid pSOS-HUS-HA117 and the five positive double-cloned plasmids were extracted and dissolved in a final volume of 30 μL. The OD values at A260 nm of the samples of HAi1– HAi5 were measured by spectrophotometer at 7.78, 7.32, 9.45, 8.33, 7.96 and 8.57, respectively. The amount of each plasmid solution needed for transfection was calculated to provide 4 μg DNA; therefore the DNA volumes of the double-cloned plasmids HAi1– HAi5 used for transfection were 10.5, 11, 8.5, 9.7, 10 and 9.6 μL, respectively. The packaging 293 cells were cultured in RPM1640 medium containing 10% fetal bovine serum in a 37°C incubator with 5% CO_2. Exponentially growing cells were seeded into six 50-mL cell culture flasks to 50-60% confluence. These 293 cells were incubated for 14 hours before being transfected with the quantified recombinant pSOS-HUS-HA117 plasmid or one of the five double-cloned plasmid DNAs with Lipofectamine. At 24 hours after transfection, relative GFP expression levels were determined by fluorescence microscopy and flow cytometry.

***Measurement of HA117 mRNA expression in the transfected 293 cells by* RT-qPCR**

Six flasks of pSOS-HUS-HA117- or double-cloned plasmid-transfected 293 cells were harvested at 24 hours post-transfection. Total RNA was isolated and amplified by RT-PCR. For the β-actin internal control, the upstream primer was 5′-CTTTGGTATCGTGGAAGGACTC-3′ and the downstream primer was 5′-AGTGGGTGTCGCTGTTGAAGT-3′. For the target gene, the upstream primer was 5′-CAGAGTCAGGGACTTCAGCCTTAT-3′ and the downstream

primer was 5′-CTGTTTCCTTCTCACTCCCAACCA-3′. The amplification conditions were: 94°C for 5 min, 94°C for 30 s, 68°C for 30 s and 72°C for 1 min, for a total of 35 cycles, followed by 72°C for 7 min. The amplification products were separated by agarose-gel electrophoresis and visualized with a UV transilluminator.

Generation of recombinant adenovirus carrying DNA template of the optimal siRNA against HA117

The adenovirus backbone plasmid pSES-HUS was digested with SfiI, ligated to the selected optimal siRNA template (HAi) and transformed into DH5α competent cells. Colonies were picked and screened by restriction digestion. A positive clone of pSES-HUS-HAi was verified by sequencing. The pSES-HUS-HAi plasmid was linearized with PmeI and cotransformed with the adenoviral homologous shuttle plasmid BJ-Adeasy. The recombinants were identified by PacI digestion. A positive clone was transformed into DH5α competent cells and further confirmed with PacI digestion. The recombinant adenoviral plasmid was linearized and transfected into 293 cells with Lipofectamine for packaging. At 25-30 d post-transfection (when most cells were floating), all cells were harvested and subjected to three freeze-thaw cycles using dry ice. The virus-containing supernatant was collected and used to infect 293 cells for recombinant adenovirus amplification. The infection cycle was repeated three times to generate a high titer of Ad5-HAi5 adenovirus, which was kept at -80°C as a viral stock [6].

Evaluation of HA117 gene silencing by the recombinant-RNA interference adenovirus Ad-HAi5

The CW-2 cells were infected with the recombinant adenovirus carrying the HA117 gene. At 24 hours post-infection, the infected cells were divided into two groups, a control group and an RNAi group. The RNAi group of CW-2 cells was infected with the recombinant-adenovirus-carrying siRNA. The CW-2 cells not infected with recombinant adenovirus were used as a control. After 48 hours of incubation, two groups of cells were harvested, their total RNAs were extracted and the cellular HA117 mRNA expression levels were determined by RT-qPCR.

Statistical analysis

The experimental data were subjected to analysis of variance, with $p < 0.05$ being considered statistically significant.

Results

Restriction digestion of recombinant plasmid pSOS-HUS-HA117

The successfully constructed recombinant plasmid pSOS-HUS-HA117 was double-digested with HindIII and KpnI. The circular plasmid was cleaved into a 4.9-kb pSOS-HUS DNA fragment and a 1.19-kb HA117 gene fragment, as shown in Figure 1.

Restriction digestion of the double-cloned pSOS-HUS-HA117-HAi plasmid

The plasmid pSOS-HUS has a SfiI restriction site between the U6 and H1 promoters. This site is lost after SfiI digestion and all HAi fragments cloned into this site would not have the SfiI restriction site. Therefore, plasmids with positive clones cannot be digested with SfiI [4]. The double-cloned pSOS-HUS-HA117-HAi1 plasmid was digested with SfiI and the uncut, supercoiled-plasmid DNA band could be seen after electrophoresis. HindIII- and KpnI-double

digestion of pSOS-HUS-HA117-HAi1 released a 1.19-kb HA117 band and a 4.9-kb vector band. These results indicated that pSOS-HUS-HA117-HAi1 carried both the HA117 gene and its RNAi fragment, as shown in Figure2.

Sequence verification of the double-cloned plasmids

Each of the five correct double-cloned plasmids was submitted for DNA sequencing. The U6 promoter on the plasmid was used as the starting point to sequence the DNA between the H1 and U6 promoters. The results showed that the sequences between the H1 and U6 promoters corresponded to the HAi sequence, confirming that the five double-cloned plasmids were successfully constructed, as shown in Figure 3.

Relative GFP expression levels in transfected 293 cells

The transfected 293 cells were observed and compared at 24 hours post-transfection. The green fluorescence was the weakest in the HAi5-containing 293 cells. All groups of cells were harvested to examine the percentage of GFP-expressing cells by flow cytometry. The data were analyzed with the Chi-square test. The 293 cells transfected with the HAi5-containing double-cloned plasmid had the lowest ratio of GFP expression, which was consistent with the fluorescence-microscopy results. These results suggested that the siRNA transcribed from HAi5 had the strongest ability to interfere with HA117, as shown in Figure 4 and Table 1.

HA117 mRNA expression in the transfected 293 cells

At 24 hours after transfection, the six groups of transfected 293 cells were harvested and the HA117 mRNA expression levels in the different groups were determined by RT-PCR. The PCR products were separated by 2%-agarose-gel electrophoresis and visualized under a UV transilluminator. The HA117 mRNA expression level was the lowest in the group of 293

cells transfected with pSOS-HUS-HA117-HAi5, suggesting that the siRNA transcribed from HAi5 had the strongest ability to interfere with HA117 gene expression, as shown in Figure 5.

Restriction digestion of the recombinant adenoviral plasmid Adeasy-HAi5

After HAi5 was cloned into the adenovirus backbone plasmid pSES-HUS, it was cotransformed with the adenovirus homologous shuttle plasmid Adeasy for recombination. After a successful recombination event, the plasmid digested with PacI should generate 4.5-kb and 23-kb DNA bands. The results shown in Figure 6 indicated a successful recombination.

Infection of 293 packaging cells with Ad-HAi5 recombinant adenovirus to generate high-titer virus

The recombinant adenovirus plasmid Adeasy-HAi5 was transfected into 293 cells for viral packaging. Under fluorescence microscopy, red fluorescence was observed at 24 hours post-transfection in the transfected 293 cells, indicating a successful transfection. Subsequently, the intensity of the red fluorescence gradually increased with time. Simultaneously, the cells gradually transitioned from adhesive growth into a floating condition, suggesting the successful packaging of infectious, intact Ad-HAi5 recombinant adenoviral particles. The process took about four weeks, as shown in Figure 7.

The Ad-HAi5 recombinant adenovirus effectively silences HA117 gene expression

CW-2 cells are highly susceptible to the recombinant adenoviruses Ad-HA117 and Ad-HAi5. At 48 hours postinfection, green fluorescence was observed in CW-2 cells infected with Ad-HA117. At 24 hours after Ad-HA117 infection, the infected CW-2

cells were superinfected with Ad-HAi5, and both green and red fluorescence were observed simultaneously, as shown in Figure 8.

Forty-eight hours later, all groups of cells were harvested and analyzed by RT-qPCR. The Ad-HA117-infected control cells showed a 119-bp DNA band characteristic of the HA117 gene, indicating that exogenous HA117 was effectively expressed in Ad-HA117 infected CW-2 cells. The RNAi group cells infected with Ad-HA117 then superinfected with Ad-HAi5 did not show the DNA band for the HA117 gene, suggesting that the HA117-targeting RNAi successfully silenced the exogenous HA117 gene expression in the CW-2 cells, as shown in Figure 9.

3. Discussion

RNAi silencing is posttranscriptional gene silencing mediated by siRNAs. It features high specificity, high efficiency and inheritability and it is an effective method for studying gene function [7,8]. Currently, siRNAs are usually designed based on software analysis or literature study. Different software-designed siRNAs targeting the same gene may have different interference efficiencies [9-11]. Therefore, if the most efficient siRNA can be quickly and easily screened from among many siRNAs, this would greatly facilitate subsequent successful silencing of target gene expression with RNAi; this capability would be especially helpful in the study of novel gene function.

The convenient and effective siRNA screening plasmid (pSOS-HUS) used in this study is a vector system developed by our group in collaboration with the Cancer Research Institute at the University of Chicago to express small RNA fragments as RNAi [3]. This vector contains two parts: one is a fusion protein of the target-gene product and green fluorescent protein (GFP) and the other is a transcription unit for a 19–21-nt, double-stranded, small RNA fragment (siRNA) under the control of both the U6 and H1 promoters. If this 19–21-nt sequence is completely complementary to a fragment of the target gene, the target gene will

be silenced based on the RNA-interference principle. The silencing effect can be directly monitored under a fluorescence microscope by examining the reduction or loss of GFP expression.

The siRNA for RNAi can be obtained by in vitro preparation (such as chemical synthesis), in vitro transcription or by digesting a large dsRNA fragment using RNase III. It can also be obtained by in vivo synthesis through siRNA expression using plasmid or viral vectors, or through a siRNA expression cassette prepared by PCR [12]. Among these cassettes, the adenoviral vector for intracellular siRNA expression is convenient and inexpensive; making it one of the most widely used biological viral vectors. It has many advantages including a wide host range, low pathogenicity for humans and effective proliferation [13-16]. Here, we used the adenoviral shuttle plasmid pSES-HUS to design a double promoter containing a SfiI restriction site. Upon insertion of the siRNA transcription template of the target gene into this site, the siRNA can be made. Based on this shuttle plasmid and the adenoviral backbone plasmid, a recombinant RNA-interference adenovirus was constructed for the target gene. After infecting the cells, siRNA for the target gene was stably expressed for a long period of time as it executed its RNAi function.

Using the screening plasmid pSOS-HUS, we successfully identified the strongest-interfering siRNA (HAi5) from among five siRNA transcription templates for the target gene HA117, and we constructed an HAi5-carrying recombinant adenovirus (Ad-HAi5) to target HA117 using the modified adenovirus shuttle plasmid pSES-HUS. Subsequent functional analysis showed that Ad-HAi5 effectively silenced HA117 gene expression through RNA interference. This study thus established a framework for further functional studies of the novel gene HA117 using RNA interference. Additionally, our results showed that pSOS-HUS is a quick and convenient screening system for selecting the best siRNA transcription template for a target gene.

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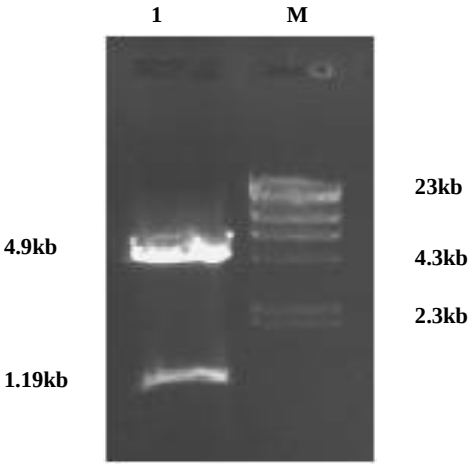
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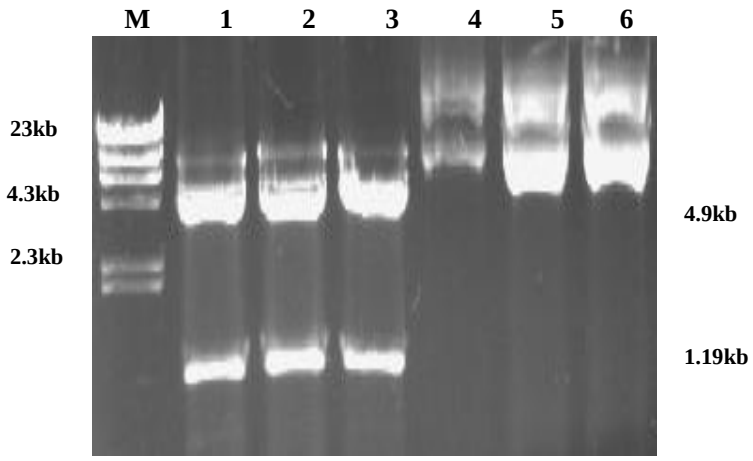
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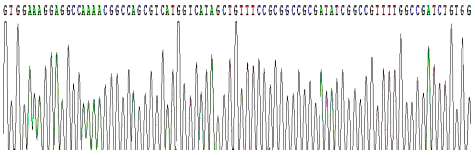

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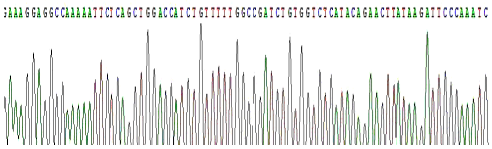

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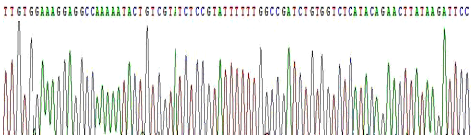

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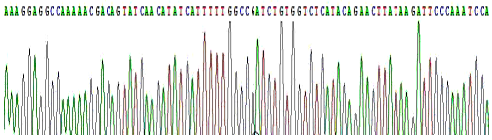

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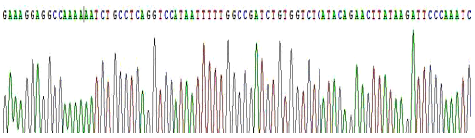

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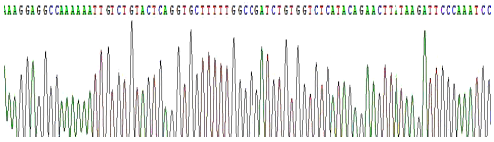

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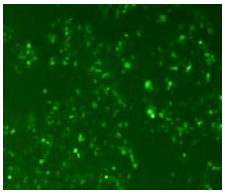	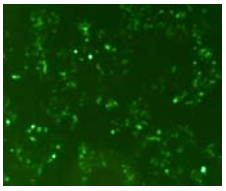	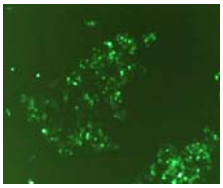
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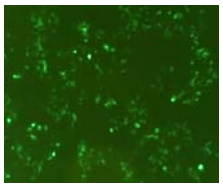	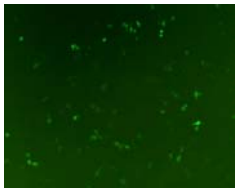	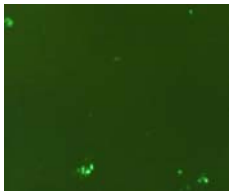
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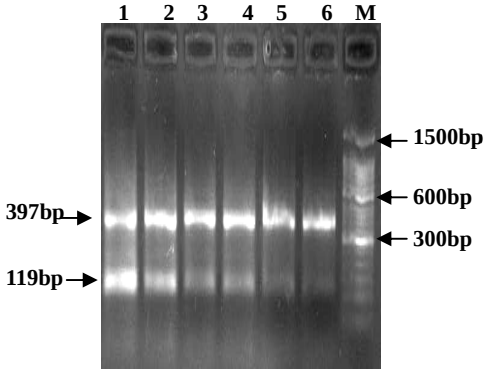
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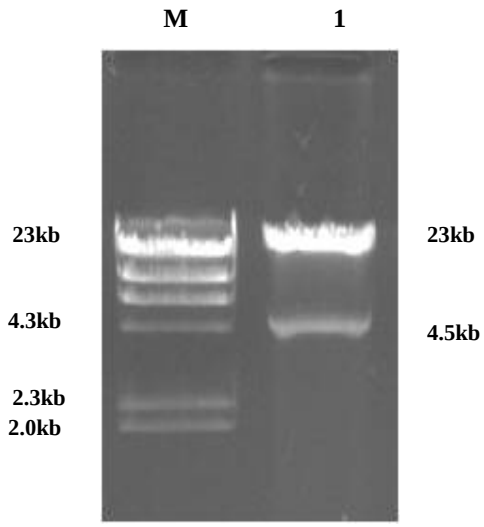
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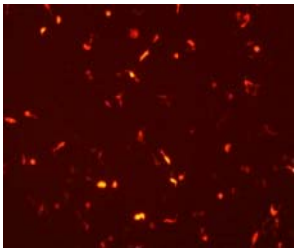
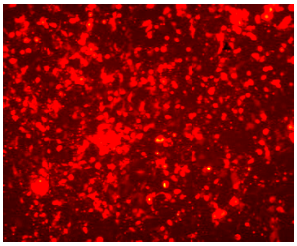
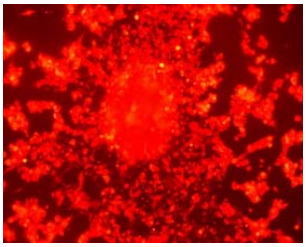
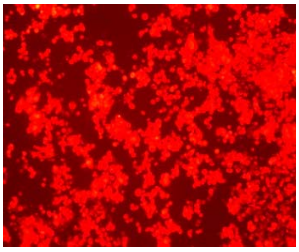
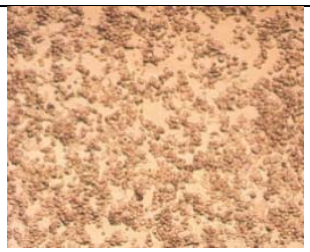
 **24h**	 **14d**
 **20d**	 **28d**



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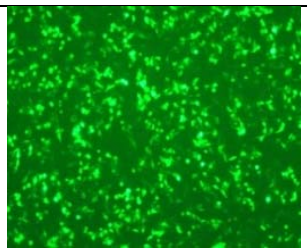

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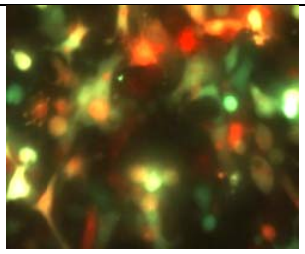

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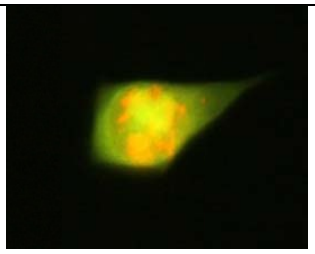

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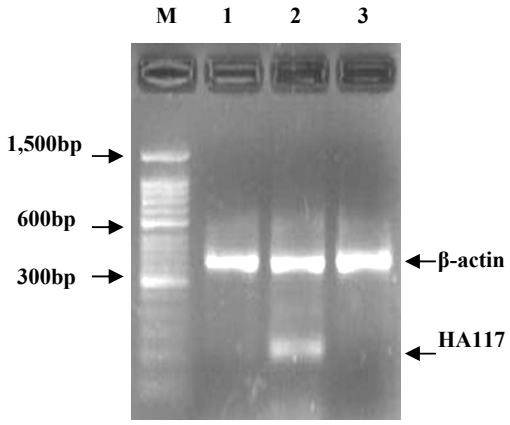
M
1
2
3
1,500bp
600bp
300bp
β-actin
HA117

Table1 GFP expression in transfected 293 cells

Groups	Number of cells expressing GFP	Number of cells not expressing GFP	Total number of cells	Percentage of cells expressing GFP (%)
Control	**1.72×10^7**	**0.88×10**	**2.60×10^7**	**66.23**
HAi1	**1.65×10^7**	**1.69×10^7**	**3.34×10^7**	**49.57**
HAi2	**0.79×10^7**	**1.26×10^7**	**2.05×10^7**	**38.73**
HAi3	**0.23×10^7**	**2.52×10^7**	**2.75×10^7**	**8.49**
HAi4	**0.60×10^7**	**3.30×10^7**	**3.90×10^7**	**15.42**
HAi5	**0.03×10^7**	**2.35×10^7**	**2.38×10^7**	**1.36**

Notice : vs other groups, the GFP proportion of cells with pSOS-HUS-HA117-HAi5 transfected was lowest, $P<0.05$.