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# Construction and identification of a model for HJURP gene defect expression in human embryo villus cells

**H.Q. Xue, G. Ting, J.J. Xue, H.Y. Lu, X.Y. Sun, M. Li, Y. Zhou, Z. Lei, Y.P. Zhang**

[1] *Children's Hospital of Shanxi & Women Health Center of Shanxi, Taiyuan (China)*

**Summary**

*Objectives:* To construct a lentiviral vector for RNA interference (RNAi) of the HJURP gene and to identify the silencing efficiency in the human embryo villus cells and to provide a human embryo villus cells multiplication and chromosome segregation. *Materials and Methods:* In accordance with the study, three specific sequences of siRNA targeting HJURP gene were designed, synthesized, then the complementary DNA containing both sense and antisense oligonucleotides of the targeting sequences were annealed and inserted into the lentiviral vector.The correct clonings were confirmed by PCR and sequencing. The most effective recombinant lentivirus vector was screened, and the recombinant plasmids with the lentivirus packaging mixes were co-transfected into 293T cells to obtain packaged lentivirus particles. Then viral titer was determined. The silencing efficiency of target gene in human embryo villus cells was detected by Real-Time PCR. *Results:* DNA sequencing showed that the shRNA sequence was successfully inserted into the lentivirus vector. The recombinant lentiviral vector was successfully transfected into 293T cells. The recombinant lentivirus had a titer of 108 PFU/ml. After silencing HJURP gene in human embryo villus cells, the expression level of HJURP mRNA decreased significantly and the RNAi efficiency was greater than 70%. *Conclusion:* A lentiviral shRNA expression vector targeting the HJURP gene was successfully constructed and may effectively silence the target gene at a cellular level, which provides a experimental model for the influence of HJURP gene expressing inhibition on human embryo villus cells multiplication and chromosome segregation.

*Key words:* Human embryo villus cell; HJURP; RNA interference (RNAi); Lentivirus.

## Introduction

Early spontaneous abortion brings great harm to patients and their families, and 50% of the aborted fetus have chromosome aneuploidies, mainly number abnormality [1]. Previous research considered that nucleosomes are composed of centromere protein CENP-A and histone H4.The newest study shows that HJURP protein is an important component of centromeric nucleosomes, which forms 3D crystal structure with CENP-A and H4 complex [2, 3]. When centromere is in replication, protein CENP-A is recruited into nucleosomes with HJURP protein. HJURP protein can change the conformation of CENP-A protein, which is suitable for nucleosome assembly [4]. HJURP has recently attracted much attention because of the development of research that target this gene. This research adopted the technology of RNA interference which silences HJURP gene, then the authors explored the effect of HJURP gene on the proliferation and chromosome segregation of human embryonic villus cells. RNA interference is small interfering RNA composed of 21-23 nucleotides which induces host cell homologous gene mRNA to degradation, so the expression of target gene is inhibited. The present authors' project was to construct a lentiviral vector for RNA interference (RNAi) of the HJURP gene and to identify the silencing efficiency in the human embryo villus cells. So they can provide a experimental model for the influence of HJURP gene expressing inhibition on human embryo villus cells multiplication and chromosome segregation.

## Material and Methods

Materials used in this study included the following: vector pHBLV-U6-ZsGreen-Puro, vector pSPAX2, vector pMD2G, Lipofiter; Escherichia coli (E.coli) DH5α, fetal bovine serum, trypsogen, Dulbecco's Modified Eagle Medium (DMEM), PBS; the restriction enzymes (BamHI and EcoRI), T4 ligase enzyme, DNA ladder; plasmid DNA mini extraction kit; plasmid DNA maxi extraction kit; DNA gel extraction kit; Sepharose, powdered agar (sagon); 293T cells (ATCC).

*Methods*

*Designing and synthesizing shRNA*

According to shRNA design principles and HJURP (NM AB101211) mRNA sequence, three targeted points were selected and designed, which were shRNA1, shRNA2, and shRNA3. By sequence alignment, these sequences have no sequence homology with human's other genes coding sequences. According to the following structure (Tables 1 and 2): BamH I + STEMP+ Loop + STEMP + termination signal + EcoRI. Nucleotide sequences were synthesized.

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Table 1. — *siRNA information.*

| No. of siRNA | Nucleotide sequences | GC (%) |
|---|---|---|
| siRNA1 | GAGCACAAAGCCATCAAGCAT | 47.62 |
| siRNA2 | GCAAGTATGGAAGTTCGATAT | 38.10 |
| siRNA3 | CCTCGAAGTATTCTTCCTTGA | 42.86 |

*Interference lentiviral vector construction*

The pHBLV-U6-ZsGreen-Puro vector was linearized by BamHI and EcoRI restriction enzyme digestion. Pure linearized vector fragments, then double-stranded DNA fragments (one ul), vector fragments (100-200ng), ligase buffer two ul, T4 DNA ligase one ul, added ddH2O to 20 ul. These substances were combined to-

Table 2. — *Design and synthesis of shRNA targeted sequences.*

| No. of shRNA | 5’ | STEMP | Loop | STEMP | 3’ | |
|---|---|---|---|---|---|---|
| shRNA1(+) | gatccCCGG | GAGCACAAAGCCATCAAGCAT | CTCGAG | | ATGCTTGATGGCTTTGTGCTC | TTTTTTc |
| shRNA1(-) | aattgAAAAAA | GAGCACAAAGCCATCAAGCAT | CTCGAG | | ATGCTTGATGGCTTTGTGCTC | ccggg |
| shRNA2(+) | gatcc | GCAAGTATGGAAGTTCGATAT | TTCAAGAGA | | ATATCGAACTTCCATACTTGC | TTTTTTc |
| shRNA2(-) | aattgAAAAAA | GCAAGTATGGAAGTTCGATAT | TCTCTTGAA | | ATATCGAACTTCCATACTTGC | g |
| shRNA3(+) | gatccgg | CCTCGAAGTATTCTTCCTTGA | TTCAAGAGA | | TCAAGGAAGAATACTTCGAGG | TTTTTTc |
| shRNA3(-) | aattgAAAAAA | CCTCGAAGTATTCTTCCTTGA | TCTCTTGAA | | TCAAGGAAGAATACTTCGAGG | CCg |

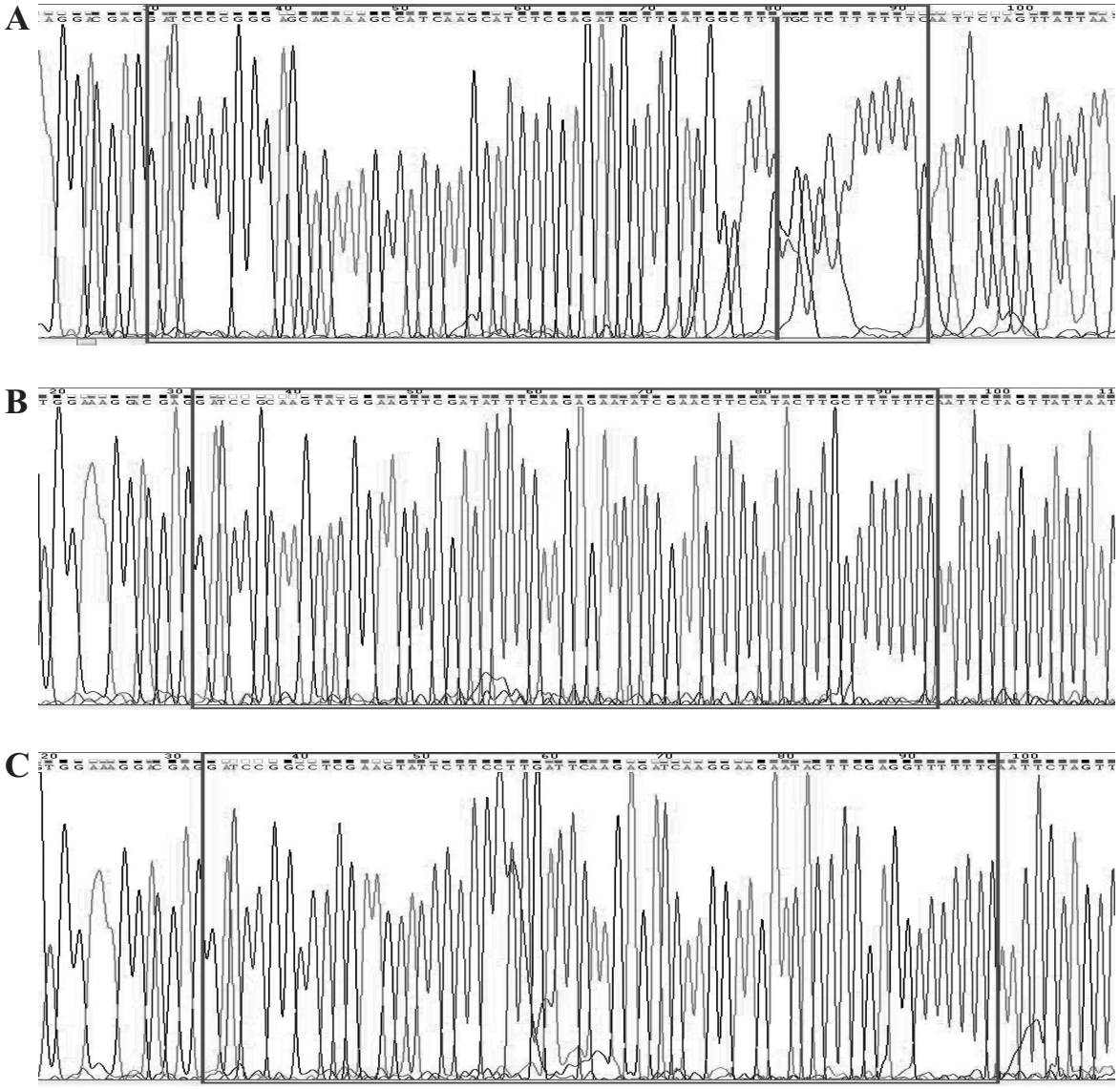


Figure 1. — Identification of recombinant lentivirus vectors. A: shRNA1 sequencing result of the interference vectors; B: shRNA2 sequencing result of the interference vectors; C: shRNA3 sequencing result of the interference vectors.

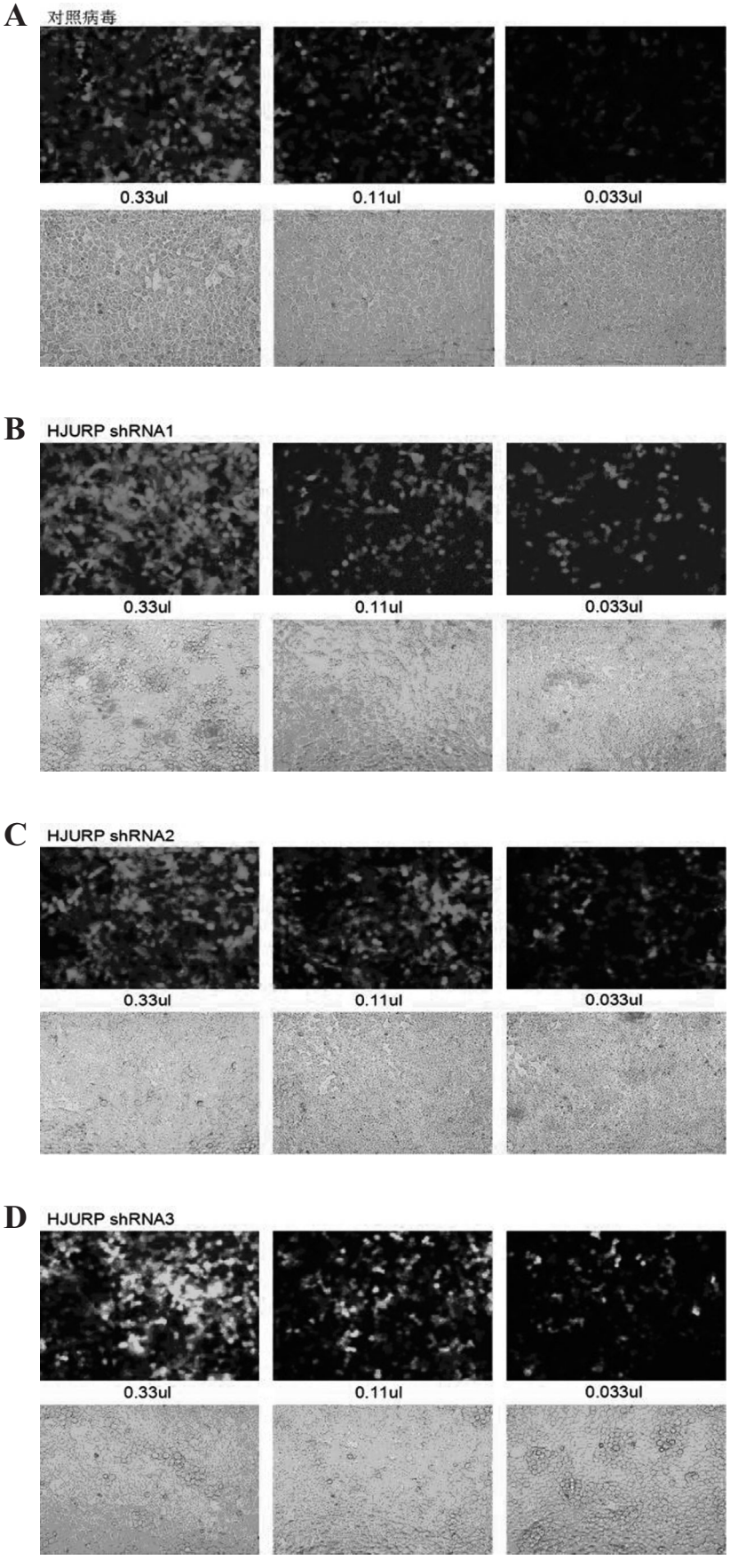


Figure 2. — Fluorescent microscopy of 293T cells after infection with lentivirus and the detection of lentivirus. 0.033 ul were taken per hole measuring virus titer.
A: control group viral titer = 4*10^4*30%*MOI(1)* Virus diluted multiples (30)*10^3=3*10^8 PFU/ml.
B: HJURP shRNA1 viral titer = 4*10^4*10%*MOI(1)* Virus diluted multiples (30)*10^3=1*10^8 PFU/ml.
C: shRNA2 HJURP shRNA2 viral titer = 4*10^4*50%*MOI(1)* Virus diluted multiples (30)*10^3=5*10^8 PFU/ml.
D: shRNA3 HJURP shRNA3 viral titer = 4*10^4*50%*MOI(1)* Virus diluted multiples (30)*10^3=5*10^8 PFU/ml.

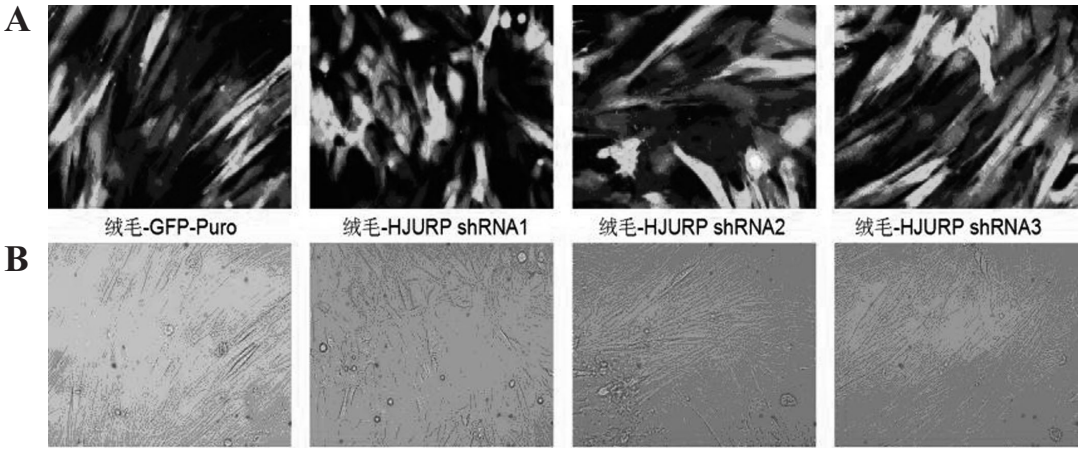


Figure 3. — Fluorescent microscopy of human embryo villus cells after infection with lentivirus and the detection of lentivirus. A: GFP expression (original magnification: ×40); B: Optical microscopy (original magnification: ×40).

gether overnight at 4°C. The recombinant vector was then transformed into the freshly prepared E. coli competent cells. Verification of the positive clones was conducted using sequencing.

Purification of recombinant plasmid and packaging plasmid was carried out with DNeasyV RBlood and Tissue Kit as per the manufacturer's recommend -dations. Purified DNA was evaluated for quality and yield by spectrophotometry. A260/A280 were between 1.7 and 1.8, and DNA concentration was larger than one ug/ul.

*Recombinant lentivirus packaging and titering*

Using recombinant lentiviral vectors, packaging plasmids pSPAX2 and pMD2G were tranfected together in 293T cells. After six hours, the culture medium was changed to complete medium.The supernatant was concentrated and collected twice after culturing for 48 and 72 hours. The virus titer of the 293T cells was determined using the dilution gradient method and calculated as follows: virus titer (PFU/ml) = (counted fluorescent cells %×MOI(1)×corresponding dilution times).

*Examination of cell transfection and the transfection rate*

Packaged recombinant lentivirons were classified into three groups based on multiplicity of infection (MOI) values, and were transfected into the human embryo villus cells separately. The culture medium was changed to complete medium at 48 hours following transfection. Cells were cultured for three days, following which the expression level of GFP in the cells was observed using an inverted fluorescence microscope. Quantitative PCR (qPCR) was also performed. Total RNA was extracted at day 4 following transfection using TRIzol reagent.Reverse transcription of the RNA to cDNA was conducted using the RNA as a template, and then PCR amplification of the HJURP gene was performed using the cDNA as a template. GAPDH was selected to be as endogenous control.

HJURP-F: 5'TGCAGCGGCTGATAGAGAAG3';
HJURP-R: 5'CACTGCAGGCTGGATCTCTC3';
GAPDH-F: 5'CGCTCTCTGCTCCTCCTGTT3';
GAPDH-R: 5'CCATGGTGTCTGAGCGATGT3'.

## Results

*Vector construction and verification of the positive clone using sequencing*

Three recombinant lentiviral vectors were amplified, then sequenced. DNA sequencing results demonstrated that the sequence was correct and showed no mutations (Figure 1).

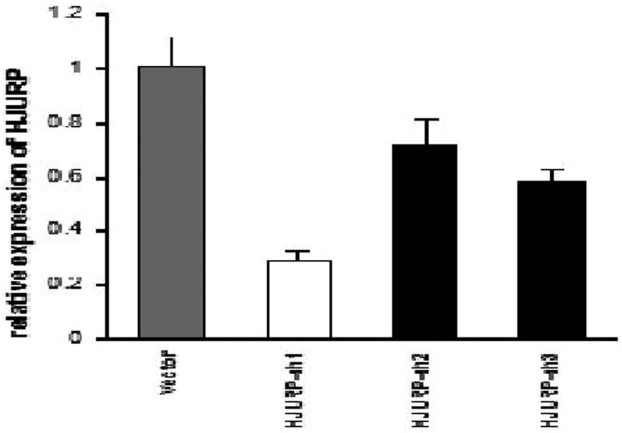


Figure 4. — Effect of HJURP interference on HJURP expression in human embryo villus cells. Real-time PCR for HJURP mRNA expression. *$P < 0.05$ *vs.* control group and experimental group.

*Lentivirus packaging and viral titer*

293T cells were transfected using the successfully constructed HJURP RNAi lentiviral vectors and pSPAX2, pMD2G coated plasmids. Significant expression of GFP was observed under the fluorescence microscope after 48 hours, and the biological titer for the collected and concentrated virus was 1x108 PFU/ml, as determined by the hole dilution method (Figure 2).

*Examination of the interference rate of HJURP-siRNA.*

Relative qPCR was performed with GADPH as the internal reference and the results were analyzed using the standard curve method. The results indicated that the mRNA expression level of HJURP decreased significantly following transfection of three recombinant lentiviral vectors into the human embryo villus cells (Figure 3). In ad-

dition, the corresponding rates for the empty vector increased, further demonstrating that these three plasmids exhibited a significant inhibiting influence on HJURP 3. Furthermore,the knockout effectiveness for HJURP-shRNA1 plasmid was better compared with the other two plasmids ($p < 0.05$) (Figure 4). Therefore, the HJURP-shRNA1 plasmid may have the most efficient inhibiting effect on HJURP.

## Discussion

HJURP (Holliday junction recognition protein) is called Holliday cross recognition protein.The gene is located in 2q37.1, approximately 17.73kb, including nine exons, and the cDNA has 2529 nucleotides, encoding a protein of 748 amino acids. HJURP gene is expressed in human and mammals, and Scm3 is homologous to HJURP in budding yeast. HJURP proteins are involved in the assembly of centromere and kinetochore, and the movement of spindle microtubule. In order to understand the important role of HJURP protein in chromosome segregation, the HJURP gene was inhibited in embryonic villus cells in this study and the effect of HJURP gene on cell proliferation and chromosome segregation will be verified by subsequent experiments. The authors constructed RNA interference lentiviral vector, so the expression of HJURP gene was inhibited.

The phenomenon of RNA interference was first found in the plant's natural antiviral mechanism. The DICER enzyme cleaves the double-stranded RNA that is produced in the process of replication of the virus into some fragments of 21-23 bp, namely siRNA [5-6]. In this process, siRNA and a variety of proteins are combined to form RNA induced silencing complexes (RISC). The sense strand of siRNA is cleaved by protein Argonaute 2 (Ago2). The antisense strand of siRNA is complementary to homologous mRNA sequence, thus leading to specific gene silencing [7]. The technology of RNA interference has been a tool for gene function research [8, 9], basic research [10-14], and drug development research [15, 16].

At present, there are two ways to achieve RNA interference: synthesis of siRNA and vector mediated generation of siRNA in cells. The latter method is that oligonucleotide chains composed of siRNA and the complementary sequence are cloned into the vector. Then the recombinant vector is transfected into the target cells to express shRNA which is processed into siRNA in cytoplasm, playing the role of RNA interference.

Vectors used in RNA interference include non-viral vectors and viral vectors. Non-viral vectors mainly include liposomes, but the transfection efficiency is low and they have cytotoxicity, furthermore the effect of inhibition is weak and duration is shorter [17]. So we usually choose the virus vector. In virus vectors, retrovirus only infect the cells in the stage of division, and the capacity is limited. The foreign genes carried by adenovirus are generally not integrated into the host cell genome, namely instantaneous infection. The lentivirus vector has high infectivity and high expression, which can infect dividing and non-dividing cells, etc. Then the foreign gene can be integrated into the host cell genome [18]. So the lentivirus vector is most suitable for long term expression of shRNA and gene silencing.

By RNA interference, downregulation of HJURP gene can lead to a decrease in the number of CENP-A, defects of cells in mitosis, abnormal chromosome separation, and aneuploid [19]. Inhibition of HJURP gene expression by siRNA can cause abnormal chromosome fusion, leading to chromosome instability and cell aging [20]. HJURP proteins are involved in many links in chromosome segregation. Not only do they participate in the structure and function of centromere, but they have a relation to the spindle check point in monitoring mechanism. The present authors assume that silencing HJURP gene by RNA interference results in the chromosome non-separation in human embryonic chorionic villi cells leads to the formation of chromosome aneuploid and spontaneous abortion. This study will provide a new breakthrough in the further exploration of the molecular mechanism of spontaneous abortion and lay the theoretical and experimental foundation for selective prevention and treatment.

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Corresponding Author:
H.Q. XUE, M.D.
Children's Hospital of Shanxi &
Women Health Center of Shanxi
No. 13, Xin-min Bei Jie Street
Taiyuan 030013 (China)
e-mail: pyxhq2013@hotmail.com