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Influence of RNA Interference Mediated CyclinD1 Gene Silencing on The Proliferation and G1 Phase Regulators of Fibroblasts Derived From Keloid

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Abstract In order to investigate the effect of sequence-specific small interfering RNA on suppressing cyclin D1 expression and proliferation and cell cycle and expression of G1 phase regulators of fibroblasts derived from keloid, the plasmid expression vector of siRNA targeted against cyclin D1 was constructed and transfected into fibroblasts with Lipofectamine™ 2000. The changes of cyclin D1 expression were detected by fluorescent quantitative PCR (FQ-PCR), semi-quantitative RT-PCR. The effect of sequence-specific small interfering RNA in suppressing the proliferation of fibroblasts was detected by MTT assay. Flow cytometry were used for evaluation of cell cycle. The expression of cyclin D1, CDK4, pRb and P16 was detected by immunohistochemical method. The results showed that: (1) The sequence- specific siRNA effectively suppressed cyclin D1 expression at both mRNA levels with inhibition rate of 63.68% and 92.83% ($P < 0.01$). (2) Significantly inhibited the proliferation of fibroblasts, and changed cell cycle in percentage of G0/G1 phase cells was increased compared with the controls groups in fibroblasts($P < 0.05$). (3) 72 h after transfection, the expression of cyclin D1, CDK4 and pRb decreased, and the expression of P16 increased. It can be concluded that the plasmid expression vector for the RNAi against cyclin D1 constructed in the study can effectively and specifically suppress cyclin D1 expression, and progression of G1/S is effected by G1 phase related regulatory protein, and suppresses proliferation of fibroblasts derived from keloid.

Key words keloid, RNA interference, cyclin D1, CDK4, pRb, P16, cell cycle

Keloid fibroblasts (KFB) continue to multiply even after the wound, and keloid formation characterized by excessive dermal fibrosis and scarring is a common clinical sequela after wound healing. Understanding the mechanisms of keloid development at a molecular level will benefit disease treatment [1,2]. Keloid formation is concerned with disorder of cell cycle. When the G0 cells are stimulated by external substances, cell would enter G1 phase and begin to proliferate. There are checkpoints in G1/S transition, which are regulated by a series of genes. Key to the control of cell growth at this G1/S junction is cyclinD1 gene of cell cycle regulation with positive effect. After finishing doubling the plasma and structure, cells enter S phase and become more independent than G1 phase [3]. So regulation of KFB cell proliferation is related closely with G1/S checkpoint. The relationship between the abnormal cyclin D1 over-expression and pathogenesis of keloid is one of the hotspots in recent years. Keloid are considered a benign tumor. The high expression of cyclin D1 in keloids, makes it an ideal target for scar of gene therapy.

RNA interference (RNAi) is a process of post transcriptional gene silencing in which siRNA inhibits gene expression in a sequence-dependent manner via degradation of corresponding mRNA, and now has become a powerful tool for studies of gene function, cancer therapy. Compared to antisense oligonucleotide and ribozyme strategies, RNAi strategy seems to have a wider prospect because of powerful ability to suppress gene expression and high stability *in vivo* [4]. So in this study, a siRNA against cyclin D1 plasmid expression vector was constructed and transfected into KFB, the changes of cyclin D1 expression following RNAi was observed and progression of G1/S is effected by G1 phase related regulatory protein, and suppresses of KFB proliferation. They make it possible searching new gene therapy method of the keloid.

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1 Materials and methods

1.1 Materials

The pSUPER plasmid was purchased from OligoEng Corporation. Fetal calf serum (FBS) was purchased from HyClone Company. Reverse transcription system kit, restriction enzyme and T4 DNA ligase were purchased from Promega Company. Trizol reagents, PCR product purification kit and transfection reagent Lipofectamine™ 2000 were purchased from Invitrogen Corporation. MTT, DMSO were purchased from Sigma Company. Monoclonal antibody of cyclin D1, polyclonal antibody of CDK4 were purchased from NeoMarkers Company. Monoclonal antibody of P16 and pRb were purchased from Santa Cruze Company. PCR primers and TaqMan probe were synthesized by Shanghai Biotech Company.

1.2 Tissue sources

The keloids tissue specimens were obtained from The Plastic and Aesthetic Surgery of the Second Affiliated Hospital of Harbin Medical University. Of the six patients (four females, two males, all was Asian) providing tissue for this study, all patients (age range 14 ～38 years) had received no previous treatment for the keloids before surgical excision. The lesions were diagnosed as keloids on the basis of clinical appearance, persistence for several years, extension beyond the original wound margins. Four of the keloids were from the upper thorax or face, one was from the upper back, and one was from the ear lobe. Before excision, a full history was taken and an examination performed, complete with color slide photo documentation and informed consent. A portion of all specimens was sent to the hospital Department of Pathology for histological confirmation of keloid identity.

1.3 Cell culture

Excised keloid specimens were washed in Hanks' balanced salt solution (HBSS) containing 150 mg/L gentamicin and 7.5 mg/L fungizone followed by plain phosphate-buffered saline (PBS) until the washing solution became clear. Epidermis and subdermal fat were removed from sterile biopsies of keloid. The specimens were minced into pieces of 0.5 to 1 cm^3 in sterile tissue culture dishes，and gently overlaid with DMEM (4.5 g of glucose per liter) supplemented with 10% FBS, containing 100 U/ml penicillin, 100 g/L streptomycin. Explants were incubated at 37℃ in a humidified incubator an atmosphere of 5% CO_2 and 95% air. After 12 to 14 days, fibroblasts were harvested from the primary cultures by trypsin treatment and replated. Visual examination and electron microscopy confirmed that cultures contained fibroblasts (KFB).

1.4 Construction of siRNA plasmid expression vector

siRNA-expressing plasmids were constructed by cloning siRNA sequences into pSUPER *via Bgl* Ⅱ and *Hin*d Ⅲ sites. siRNAs targeted against cyclin D1 (NM-053056) were designed by a program available online (http//www.ambion.com/techlib/misc/siRNA-design.htm) and the top-ranked sequence for gene was chosen in this study. The siRNA sequences were further subjected to a BLAST search against human genome and EST databases to ensure that no human gene was targeted. The target sequence of cyclin D1 gene and two DNA chains and one control chains were synthesized as shown in Table 1. The structure of the DNA chains is *Bgl* Ⅱ +sense chain +loop +antisense chain+termination signal+*Hin*d Ⅲ.

Table 1 Oligonucleotides corresponding to the target sequence and the control sequence

siRNA	Sequence
C1	5′ gatcccCAAACAGATCATCCGCAAAttcaagagaTTTGCGGATGATCTGTTTGttttttggaaa 3′
	5′ agcttttccaaaaaaCAA ACAGATCATCCGCAAAtctcttgaaTTTGCGGATGATCTGTTTGggg 3′
C2	5′ gatcccACACGCGCAGACCTTCGAGttcaagagaTAACGAAGGTCTGCGCGTGttttttggaaa 3′
	5′ agcttttccaaaaaaACACGCGCAGACCTTCGTTtctcttgaaTCCGAAGGTCTGCGCGTGTggg 3′
C0	5′ gatcccGCATGTTCGTGGCCTCTAAttcaagagaTTAGAGGCCACGAACATGCtttttggaaa 3′
	5′ agcttttccaaaaaaGCATGTTCGTGGCCTCTAAtctcttgaaGTAGAGGCCACGAACATGCggg 3′

Two equal amount DNA chains were annealed and phosphorylated. To insert the targeting sequence, DNA oligos were designed and cloned into the *Bgl* Ⅱ / *Hin*d Ⅲ sites of the pSUPER vector. The directional cloning was accomplished with the aid of T4 DNA ligase. The ligated plasmid DNA was transfected into competent cells DH5α which was then seeded on solid LB medium containing 0.05 g/L ampicillin and cultured at 37℃ overnight. Three monoclonal colonies were picked out and seeded in 3 ml LB culture fluid and cultured at 37℃ overnight in a rocking bed.

Then pick and miniprep several colonies until a positive clone is located by digesting with *Eco*R Ⅰ and *Hin*d Ⅲ primarily. Finally, sequencing is carried out for further identification. The new plasmid was named as pSUPER-C1 and C2. The negative control plasmid pSUPER-C0 was constructed as the same process. The concentration and purity of the plasmids were detected by an ultraviolet spectrophotometer. The plasmids were stored at –20℃ for subsequent experiments.

1.5 Transfection of plasmids

KFB were seeded on 6-well culture boards and grown to 80% ～ 90% confluence, before the transfection. Only Lipofectamine™ 2000 was used for the transfection in the blank control group. Plasmid pSUPER-C0 was used for the transfection in the negative control group and plasmid pSUPER-C1 and C2 were used for the transfection in the positive experiment group. The common complete medium was replaced by the antibiotics-free medium containing serum. Six hours after the transfection, the medium was replaced by the common complete medium again. After 12, 24, 48 and 72 h the trans fection, expression of EGFP in KFB was observed under an inverse fluorescence microscope.

1.6 MTT assay

KFB grown in 96-well culture boards, grouping and transfection were the same as above. The plate were incubated in 5% CO_2 incubator at 37℃ . The plates were in the presence or absence of the indicated test samples for 12, 24, 48 and 72 h. Stock MTT solution(5 g/L in PBS) 20 μl was added into each well and continued to incubated for 4 h in the same incubator. Supernatant from each well was drawn out carefully and l50 μl DMSO was added. The plate was shaked strongly for 15 min, and the blue crystal was dissolved. Absorbance was measured at 492 nm wavelength using a 96-well multiscanner autoreader. Inhibitory rate= $(1-A1/A0)\times100\%$, $A0$ represents total absorbance of control group, $A1$ represents the absorbance of experimental group.

1.7 Expression of cyclin D1 mRNA detected by FQ-PCR

1.7.1 Total RNA extraction and reverse transcription. Total RNA was extracted from 72 h after the transfected KFB using Trizol reagent. The concentration and quality of total RNA were analyzed by 1% agarose gel electrophoresis and spectrophotometry at 260 nm and 280 nm. Only samples that were not degraded and showed clear 18 S and 28 S bands under ultraviolet light were used for reverse transcription. The cDNA acquired was stored at –20℃ until use.

1.7.2 Primers and probe. The forward primer of cyclin D1 was 5′ AGG TGC TTC TGC CTG TGC 3′ (724～742 bp), and the reverse primer was 5′ CCT TCT TCC TCC CTC ACT TCT C 3′(856～878 bp), and the amplification product was 133 bp. The probe of cyclin D1 was 5′ FAM-TGA GCG CAA GTA CTC CGT GTG GAT CGG CG-TAMRA 3′. The human β-actin served as an internal standard. The forward primer was 5′ ATC TGC TGG AAG GTG GAC AGC GA 3′, and the reverse primer was 5′ CCC AGC ACA ATG AAG ATC AAG ATC AT 3′ , and the amplification product was 118 bp. The probe of β-actin was 5′ FAM-TGA GCG CAA GTA CTC CGT GTG GAT CGG CG-TAMRA 3′.

1.7.3 Construction of recombinant plasmid calibrator. Pure cyclin D1 fragments from classical RT-PCR were joined to pMD18-T vector, resulting in recombinant pMD18-cyclin D1. The recombinant plasmids were confirmed in included cyclin D1 target fragments by sequencing, extracted and purified. The recombinant plasmid DNAs at 10^8 copies/ml were stored at –20℃ untiluse. β-Actin standard template was from the β-actin quantification reagents.

1.7.4 Real-time FQ-PCR amplification. The 25 μl PCR mixture for cyclin D1 consisted of 1×PCR buffer, 3 mmol/L $MgCl_2$, 200 μmol/L dNTP, 400 nmol/L primers, 400 nmol/L TaqMan probe, 2U Taq polymerase and 1 μl synthesized cDNA. For cyclin D1 amplification, after one cycle at 93℃ for 5 min, a two-step PCR procedure was processed for 40 cycles at 93℃ for 30 s and at 60℃ for 1 min. Amplification and data acquisition were carried out using the LightCycler system (Roche Molecular Biochemicals, software version 5.1). The inhibition ratio of cyclin D1 expression= (1–the cyclin D1 mRNA copies in the

experiment group/the cyclin D1 mRNA copies in the blank control group)×100%.

1.8 Expression of cyclin D1 mRNA detected by semi-quantitive RT-PCR

After 24, 48 and 72 h the transfection, 1.0×10^6 cells were extracted of total RNA and synthesis of cDNA. Amplification of β-Actin(586 bp) served as an internal standard. The primers used are forward 5′ CCC AGC ACA ATG AAG ATC AAG ATC AT 3′ and reverse 5′ ATC TGC TGG AAG GTG GAC AGC GA 3′. The primers of cyclin D1(478 bp) are 5′ CTT TCT CAA GGA CCA CCG 3′ and reverse 5′ GCA CTT TCT CCG CAG TTT 3′. The PCR products were separated in 1.5% agarose gels visualized by staining with ethidium bromide. Semi-quantitative analysis was performed with the software. The expression intensity of cyclin D1 was denoted with the ratio of the photodensity of the RT-PCR products of cyclin D1 and β-actin.

1.9 Expression of cyclin D1 protein detected by Western blot methods

KFB were transfected with recombinant plasmid for 24, 48 and 72 h. 1×10^7 cells were collected. The sample cells were lysed in protein lysis buffer (50 mmol/L Tris-HCl, 1 mmol/L EDTA, 20 g/L SDS, 5 mmol/L DTT, and 10 mmol/L PMSF) and laid on ice for 30 min lysate were centrifuged at 12 000 r/min for 40 min and the concentration of the protein in each lysate. The total protein separated by 12% SDS-PAGE gels electrophoresis, samples were transferred to PVDF membranes. The membranes were antibody specific for cyclin D1 or the housekeeping protein β-tubulin (1 ∶100 ～1 ∶500 dilution) overnight at 4℃. Each band was analyzed on Image Analysis System. The gene expression level was determined by computerized optical densitometry the ratio of density metric value from the target gene in relation to internal standard.

1.10 DNA content and cell cycle analysis by flow cytometry

After the transfection, 1×10^6 cells were collected. Suspended in 75% ethanol at −20℃ overnight. The cells were incubated in the dark with 50 mg/L of PI and 0.1% of RNase A at 25℃ for 30 min. The percentage of cell cycle phases was analyzed using the cell quest software program, cells were sorted into G0/G1, S and G2/M phases respectively according to their DNA contents on flow cytometry (Becton Dickinson).

1.11 Expression of correlate genes protein detected by immunohistochemical methods

The expression of cyclin D1, CDK4, P16 and pRb protein were detected by the immunohisto chemical method. A petroline section of a breast cancer expressing positively was used as a positive control. PBS was used to replace the anti-antibody to make a negative control. According to the degree of the coloration, the cells were scored as following. Five fields of vision were observed for each coverslip under high power lens randomly, counting arid scoring cells, the sum of the scores was divided by the cell number, the result was considered as the express intensity of gene: (−), 0%～10% cells were positive in each slice, (+), 11%～25% cells were positive, (++), 26%～50% cells were positive, (+++), At least 50 % cells were positive.

1.12 Statistical analysis

Data were expressed as $\bar{x}\pm s$. t Test was used to analyze the results by SPSS10.0 software. P value less than 0.05 was judged statistically significant.

2 Results

2.1 Identification of recombinant plasmid

The recombinant plasmid extracted were double digested with *Eco*RⅠ and *Hin*dⅢ, then checked with 0.5% agarose gel electrophoresis. The recombinant plasmid would produce two bands at 5 kb and 281 bp, while the empty vetor had two bands at 5 kb and 227 bp was also observed (Figure1). Eventually the recombinant plasmid was identiflied to have a correct sequence by DNA sequence analysis, the result of sequencing confirmed that siRNA had been ligated to the vector.

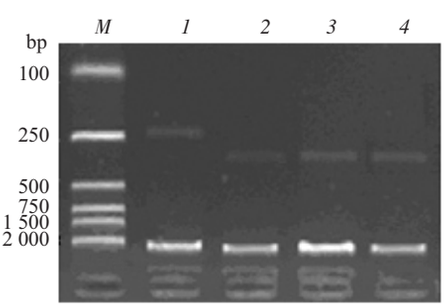

Fig. 1 Identification of recombinant plasmids by restrict endonuclease digestion

M: DL2000 marker; *1*: pSUPER; *2*～*4*: pSUPER-C0, C1 and C2 cut with *Eco*RⅠ/*Hin*dⅢ.

2.2 Results of transfection

After 12 h successfully transfected with recombinant plasmid KFB, expression of EGFP can be seen with an inverse fluorescence microscope, which shift the excitation peak to 488 nm and enhances its fluorescence intensity. After 48 h the transfection, the expression of EGFP was the strongest. After 72 h the transfection, the expression of EGFP attenuated gradually and the rate of transfection was ranged form (6.28±7.30)% (Figure 2).

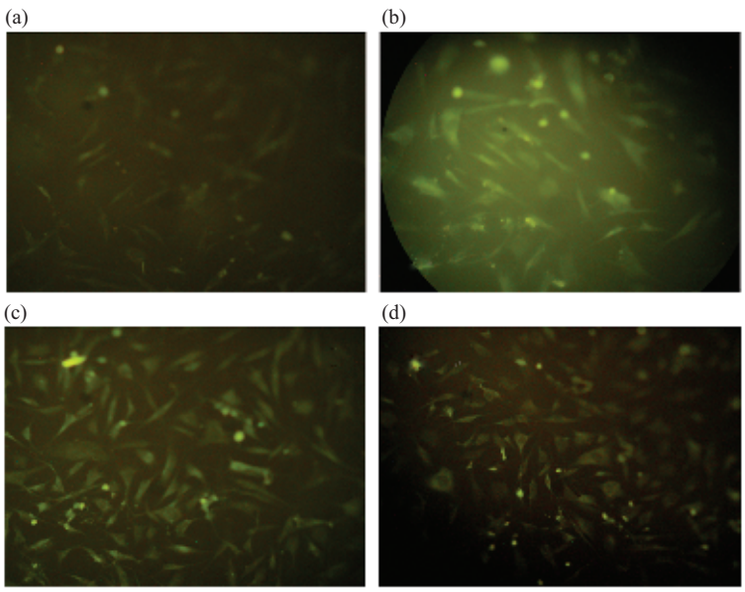

Fig. 2　Expression of EGFP in transfection after with pSUPER-C KFB at different stage post transfection

(a)～(d) Transfected 12, 24, 48 and 72 h after with pSUPER-C KFB, respectively. Magnification: 200×.

2.3 Cell proliferation analysis

Transfected of KFB were cultured for 24, 48 and 72 h and cell proliferation was evaluated by MTT assay. Transfected pSUPER-C1 and C2 caused a significant decrease in proliferation as early as 24 h after transfection of KFB (26.51% and 31.95% , $P < 0.05$), respectively, and continued to 72 h (52.74% and 81.37%, $P < 0.05$), in a time-dependent manner inhibit proliferation of experiments group KFB(Figure 3), as the transfected pSUPER and pSUPER-C0 weakly inhibit cell proliferation ($P > 0.05$).

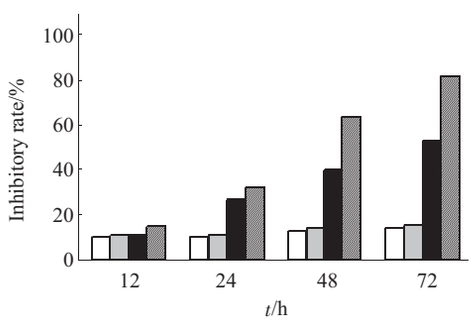

Fig. 3　Detection on proliferation of transfected with pSUPER-C KFB by MTT colorimetry

The results were expressed as ($\bar{x} \pm s$) from independent experiments, $P < 0.05$ *vs* lipofectamine control group. □: pSUPER; ▨: pSUPER-C0; ■: pSUPER-C1; ▩: pSUPER-C2.

2.4 Inhibition of cyclin D1 mRNA expression

We examined three siRNA-expressing vector pSUPER-C which target human cyclin D1 as shown in Figure 4. The PCR products showed a band of 478 bp, and the β-actin product located at 586 bp. 48 h after the pSUPER-C1 and C2 KFB were yielded obviously weaker cyclin D1 band compared with the control cells (43.28% and 68.17 %), while the transfected pSUPER-C0 KFB showed no difference with the contro1 ($P < 0.05$). Of the three siRNA-expressing vectors，pSUPER-C2 potently suppressed the synthesis of cyclin D1 mRNA, in a time-dependent manner. PCR products quantification showed that 72 h after transfected pSUPER-C2 suppressed cyclinD1 mRNA production to 86.27% of that in the control cells ($P < 0.05$).

2.5 Standard curve construction

The increase in the fluorescence signal of the reporter was proportional to the amount of specific PCR products, providing highly accurate and reproducible quantification. The number of PCR cycles to reach the fluorescence threshold was the cycle threshold (*C*t). The *C*t value for each sample was proportional to the l g of the initial amount of input

cDNA. By plotting the *C*t value of an unknown sample on the standard curve, the amount of target sequences in the sample could be calculated. We used different concentrations (c) of β-actin and cyclin D1 standard templates including 10^3, 10^4, 10^5, 10^6, 10^7 and 10^8 copies/ml to perform quantitative PCR and calculate the standard curves, respectively. The standard curves were *C*t (β-actin) =–3.64 lg$c$ and *C*t (cyclin D1) =–3.96 lg$c$. The correlation coefficients were both 0.999(Figure 5).

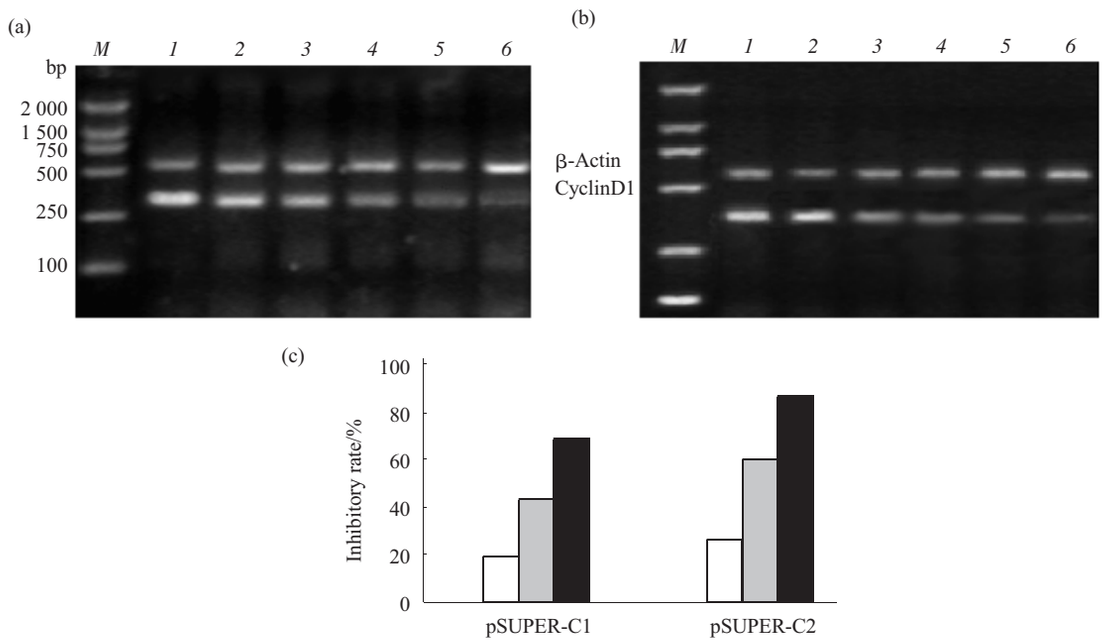

Fig. 4 The mRNA level of cyclin D1 gene in KFB after transfection of pSUPER-C detected by RT-PCR

(a) Transfection of pSUPER-C1. (b) Transfection of pSUPER-C2. (c) Quantitation of inhibition of cyclin D1 mRNA. *M*: DL 2000 marker; *1*: Control KFB; *2*: Transfected pSUPER and pSUPER-C0; *2*～*6*: 24, 48 and 72 h after transfected pSUPER-C1 and C2 of KFB. Each level of PCR product of cyclin D1 gene was quantitated and normalized to the level of β-Actin. Inhibitory rate was calculated by comparing to the control cells. The results were expressed as $\bar{x}\pm s$ from independent experiments. □: 24 h; ▨: 48 h; ■: 72 h.

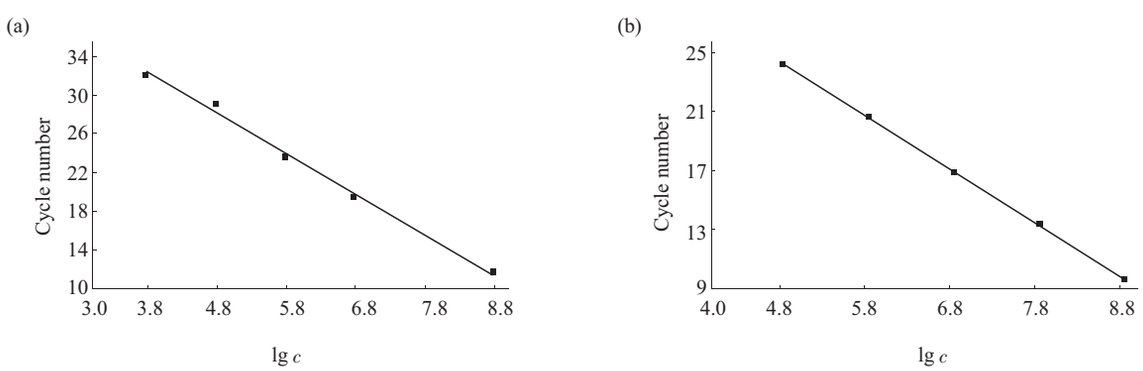

Fig. 5 Fluorescent quantitative PCR curve of β-actin and cyclin D1 standard sample

(a) β-Actin. (b) Cyclin D1.

2.6 FQ-PCR analysis

The expression of cyclin D1 mRNA in after 72 h transfected KFB detected by a highly sensitive FQ-PCR assay. For each experimental sample, the amount of cyclin D1 and β-actin was determined from the appropriate standard curve. Then, cyclin D1 amount was divided by the β-actin amount to obtain. the recombinant plasmid pSUPER-C1 and C2 were potently suppressed the expression of cyclin D1 mRNA compared with the control cells (63.68% and

92.83%, $P < 0.05$). On the basis of these results, we selected pSUPER-C2 as the most highly functional siRNA expressing vector in further studies (Figure 6).

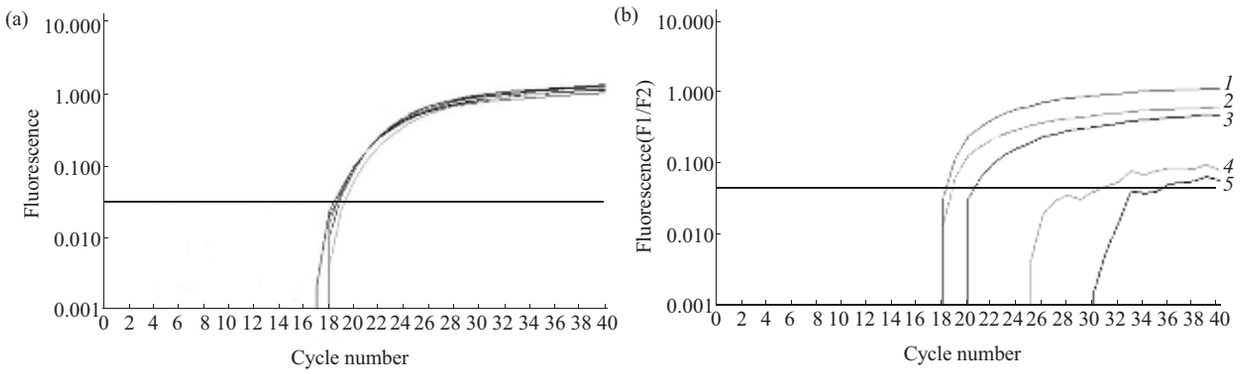

Fig. 6　The mRNA level of β-actin and cyclin D1 gene in KFB after transfection of pSUPER-C detected by real-time fluorescent quantitative PCR

(a) β-Actin. (b) Cyclin D1. *1*: Control KFB; *2*: Transfection of pSUPER-C0; *3*～*5*: 72 h after transfected pSUPER-C0, C1 and C2 of KFB.

2.7　Inhibition of cyclin D1 protein expression

The expression of cyclin D1 protein in each group KFB was detected by Western blot methods. As shown in Figure 7, the bands representing cyclin D1 and β-tubulin were specifically reacted. The each group KFB showed alike expression level of β-tubulin at 51 ku. The control KFB showed significantly higher level of cyclin D1 at 34 ku compared with 48 h after transfected experiment KFB. The expression of cyclin D1 protein in 72 h after transfected pSUPER-C1 and C2 is obviously droped (48.76% and 72.53%), and pSUPER- C2 restrains expression of cyclin D1 protein higher than pSUPER-C1($P < 0.05$).

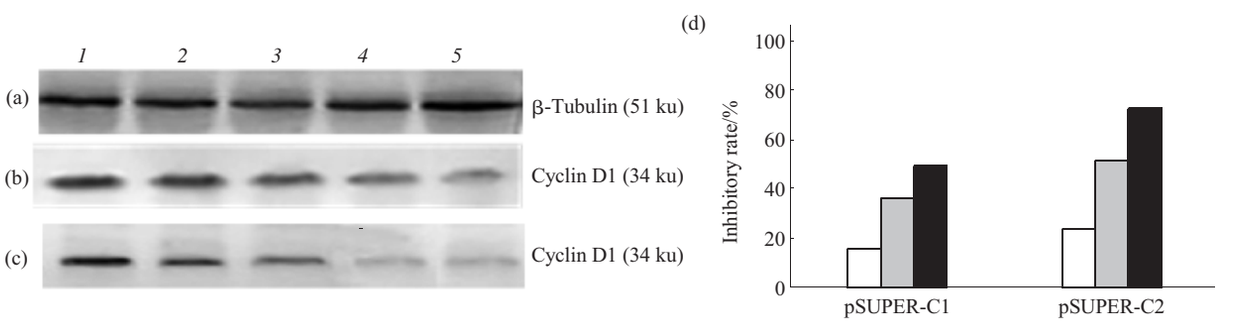

Fig. 7　The protein level of cyclin D1 gene in KFB after transfection of pSUPER-C detected by Western blot

(a) β-Tubulin. (b) Transfection of pSUPER-C1. (c) Transfection of pSUPER-C2. (d)Quantitation of inhibition percentage of cyclin D1 protein. □: 24 h; ▨: 48 h; ■: 72 h. *1*: Control KFB; *2*: Transfection of pSUPER-C0; *3*～*5*: 24, 48 and 72 h after transfected pSUPER-C1 and C2 of KFB. Each level of Western blot reacted of cyclin D1 gene was quantitated and normalized to the level of β- tubulin. Inhibitory rate was calculated by comparing to the control cells. The results were expressed as $\bar{x}\pm s$ from independent experiments.

2.8　Cell cycle analysis

Figure 8 illustrates changes in DNA content distribution, the transfected with pSUPER-C2 from 48 to 72 h, the percentage of cells in G0/G1 phase increased and S phase correspondingly decreased, and G2/M phase decreased obviously. When different transfected pSUPER-C1 and C2 for 72 h, the percents of G0/G1 phase cells were increased to 59.62% and 69.54%, S phase cells were decreased to 26.76% and 18.61% and G2/M phase cells were decreased to 12.36% and 7.75 % in a time-dependent manner ($P < 0.05$).

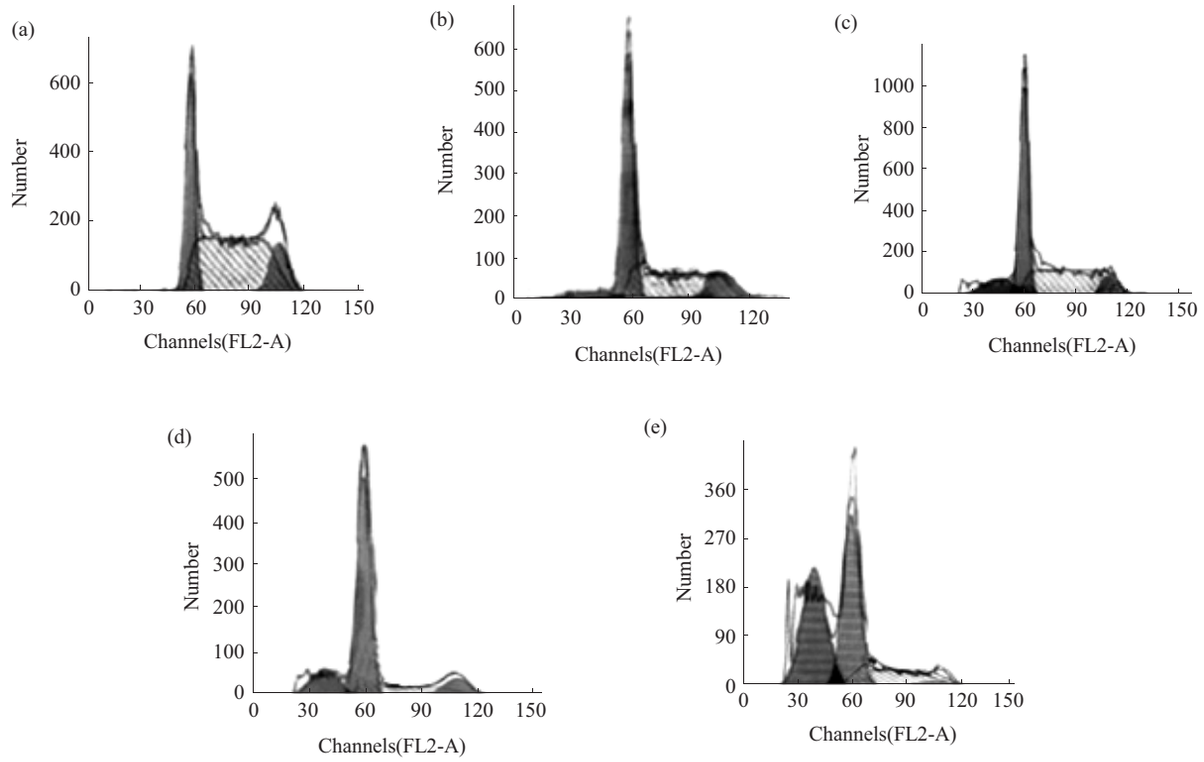

Fig. 8　The cell cycle in KFB after transfection of pSUPER-C detected by flow cytometry

(a) Control KFB. (b), (c) 48 and 72 h after transfected pSUPER-C1. (d), (e) 48 and 72 h after transfected pSUPER-C2 of KFB.

2.9　Correlate protein expression analysis

The positive staining KFB with brown-yellow granulations in nuclei and cytoplasm, but mainly, cyclin D1, pRB, P16 protein expression were observed in the nuclei, CDK4 protein expression were mainly in cytoplasm. We found that the expression of cyclin D1, CDK4 and pRb protein was down regulated to 23.72%, 15.17% and 20.53% in transfected pSUPER-C2 of KFB with a knock down of cyclin D1 gene (Figure 9), respectively. On the contrary, the expression of P16 protein was upregulated to 74.15% (Figure 9), compared with that in contro1 KFB. These results indicated that the expression of some proliferation and apoptosis related target genes was altered subsequently after knockdown of cyclin D1 by a siRNA-expressing vector.

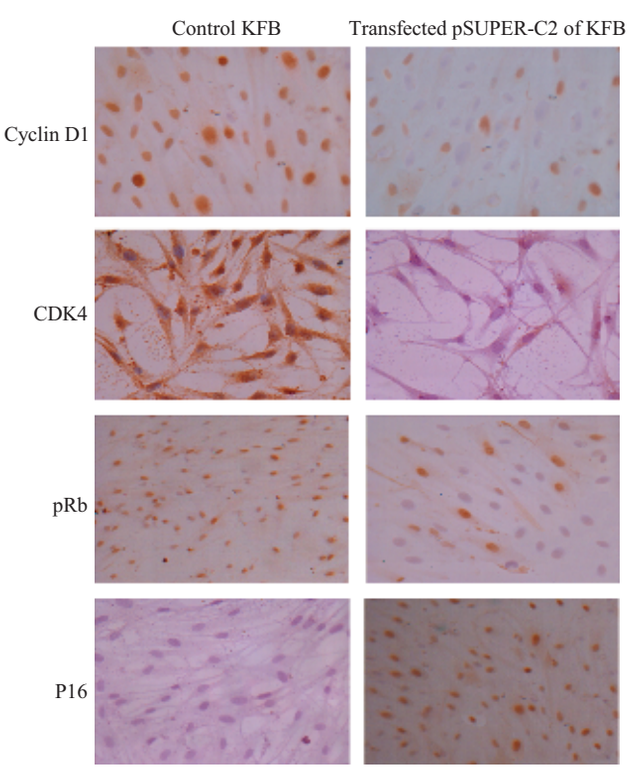

Fig. 9　The expression of correlate genes protein in KFB after transfection of pSUPER-C2 detected by immunohistochemical method (×400)

3 Discussion

Keloid is one of the difficulties and warm spots in the clinic work of plastic surgery. Keloids are considered a benign tumor, the clinical manifestation of keloid is that cicatricial tissue extensively grows to surpasses the original surface to grow invasively to the surroundings, and operating on a keloid usually stimulates high risk of relapse. Currently, there is no effective method to prevent such scarring during wound healing. Understanding the mechanisms of keloid development at a molecular level will benefit disease treatment. KFB continue to multiply even and accumulation of extracellular matrix (ECM) proteins is a manifestation of increased collagen synthesis and/or reduced matrix degradation, and keloid formation characterized by deformed appearance and dysfunction is a common clinical sequela after wound healing. Researchers find out that the keloid formation is concerned with disorder of cell cycle, then loss of cell cycle control. Thus, mechanisms may lead to deregulated expression of cyclin D1 in keloid. Cyclin D1 is a key regulation protein in cell cycle progression, which can accelerate cell cycle from G0 to G1 and increase cell proliferation. Consequently, inhibition of cyclin D1 function may be an important target of keloid prevention and therapy[5,6].

RNA interference (RNAi) is a conserved cellular mechanism by which double-stranded RNA (dsRNA) silences the corresponding homologous gene. These 21～23 nucleotides with long，double-stranded small interfering RNA (siRNA) molecules can direct degradation of eukaryotic mRNAs in a sequence-specific manner. Two major discoveries have highlighted the potential of RNAi application in gene therapy. The introduction of synthtic siRNA of 21 nucleotides into cells can result in efficient gene silencing, and siRNAs expressed from RNA promoters within expression vectors also trigger RNAi [7]. This study demonstrated the step is helpful to clone the ectogenesis fragment (siRNA) into the plasmid vector pSUPER. siRNAs generated by a pSUPER in mammalian cells brings about sustained gene inactivation without cytotoxicity and loss of function phenotypes to be assayed over longer periods of time. pSUPER has several key features that equip it for the job. It contains an RNA polymerase Ⅲ promoter, a well-defined transcription start site and a termination signal that consists of five Ts [8]. The pSUPER vector was constructed by inserting the report gene enhanced green fluorescent protein (EGFP) into pSUPER vector. This EGFP carries a mutation in its chromophore, which shifts the excitation peak to 488 nm and enhances its fluorescence intensity. EGFP provides an opportunity to visualize only cells expressing this protein under wavelength of 488 nm. Therefore, the pSUPER vector is a new and powerful system to analyze gene function in a variety of mammalian cell types. To target a specific mRNA for degradation, a portion of the mRNA target sequence must be known and a segment of the target mRNA that will be used for targeting by the cognate siRNA duplex must be chosen . Synthesize several siRNA duplexes for the specificity of the knockdown experiments. Furthermore, a nonspecific siRNA duplex may be needed as a control [9]. So, we selected two oligonucleotides of cyclin D1 siRNA. Structure of single target sequence itself is apt to forming hairpin by self-folding when performing the annealing reaction which need step-cooling or ideal degree gradient. This study demonstrated the step is helpful to clone the ectogenesis fragment into the plasmid vector pSUPER.

In this study，to explore a stable suppression of cyclin D1 expression in KFB *via* RNAi, two tatget sites(586～604 bp and 869～887 bp) were expressed in KFB *via* designed using an online software and recombinants pSUPER-C1 and pSUPER-C2 were constructed to direct the siRNAs transcription in transfected cells. The RT-PCR primers of cyclin D1 were designed sites 627～1105 bp. Our preliminary results demonstrated that the expression of cyclin D1 mRNA level was significantly suppressed to 59.86% and 82.57% in 72 h after transfected KFB with pSUPER-C1 and C2 as compared to vacant vector transfected and control KFB, respectively. We quantified cyclin D1 mRNA expression using TaqMan real-time fluorescent quantitative PCR (FQ-PCR) assay. The principle of TaqMan method is based on the cleavage of an internal dual-labelled probe which annealed to the amplification target sequence, and cleaved by Taq polymerase during the extension phase. At the beginning, no fluorescence is emitted due to the proximity of the quencher and reporter dyes attached to the 3′ and 5′ ends of the probe. However, during each PCR cycle one molecule of reporter dye is cleaved for each target molecule amplified. The released fluorescence is measured in real time. The threshold cycle value (*C*t) is the number of cycles

before the emitted fluorescence passed the threshold. The lg of the number of targets initially present is proportional to *C*t and can be measured using a standard curve. Compared with the traditional competitive RT-PCR, this method is more sensitive, accurate, specific and quicker, especially suitable for cyclin D1 detection. We amplified the housekeeping gene β-actin. According to each standard curve, we got the copy numbers of β-actin and cyclin D1, respectively. The ratio between copy numbers of cyclin D1 and β-actin represented the normalized cyclin D1 for each sample and could be compared with that of other samples [10]. The inhibitory effect was shown 92.83% be specific because no non-specific down-regulation of gene expression was observed, as demonstrated by the β-actin control. We reported here the optimizing method in constructing these recombinant plasmids pSUPER-C2 serves other plasmid-based RNAi research. The expression of cyclin D1 protein level was significantly suppressed to 82.57% in transfected KFB with pSUPER-C2, as demonstrated by the β-tubulin control.

The normal operation of cell cycle is determined by the regulatory system composed of cyclin, cyclin-dependent kinases (CDKs) and cyclin-dependent kinases inhibitor (CDKI). Cyclin D1/CDK4/P16-pRb pathway is one of the key signal transduction pathways for G1/S checkpoint in cell cycle [11]. Cyclin D1 is an oncogene of cell cycle regulation with positive effect. When combined with CDK4 to form a compound, it will cause changes in CDK4′ s structure and activate it. Then the activated CDK4 will lead to the phosphorylation of Rb (pRb) protein, unblock Rb′ s inhibition to E2F accordingly and motivate the synthesis of DNA, making the cells transit from G1 to S, resulting in accelerating proliferation [12]. P16 as a tumor suppressor gene, is one of the CDKI proteins. It binds to and inhibits CDK4, thus preventing CDK4 -dependent inhibits phosphorylation of Rb and resulting in a block during the G1-S phase transition of the cell cycle [13]. Thus repressing cyclin D1 gene or its protein expression will not produce much influence to normal cells. This provides a precondition for interference therapy based on cyclin D1, in this experiment, a cyclin D1 siRNA expression vector was transfected into KFB. Our finding cyclin D1 gene expression could be blocked, and have decrescendo effects on activing capacity of pro CDK4 and pRb. Significant up-regulation of P16 were also observed in experiment KFB with knockdown of cyclin D1. So, change by G1 phase related regulatory protein to stoppage cell cycle progression. 72 h after transfected pSUPER-C1 and C2 of KFB caused a significant repressed in cell proliferation (52.74% and 81.37%), respectively. Flow cytometry analysis showed that 72 h after transfected pSUPER-C1 and C2 into KFB the percentage of cells in G0/G1 phase increased and that S phase correspondingly decreased, and the percentage of cells G2/M phase decreased obviously. The cell cycle arrest lead to suppresses proliferation. We reported here the optimizing method in constructing these recombinant plasmids pSUPER-C2 serves other plasmid-based RNAi research.

This study indicated that the inhibition of cyclin D1 expression *via* a siRNA expression vector could cause the alteration of G1 phase related regulatory protein and cell cycle arrest, and suppresses cell proliferation. Although the biological features of cells with downregulated expression of cyclin D1 await further investigation. Nevertheless, the issue of delivery and KFB-targeting is still a major challenge before RNAi coming into a practical reality.

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RNA 干扰 Cyclin D1 基因表达对瘢痕疙瘩成纤维细胞增殖和 G1 期调控的影响

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摘要　为研究 siRNA 干扰瘢痕疙瘩成纤维细胞 cyclin D1 基因表达，对瘢痕疙瘩成纤维细胞的增殖、细胞周期和 G1 期调控的影响，构建了靶向 cyclin D1 的 siRNA 表达质粒．利用 Lipofectamine™ 2000 转染体外培养的瘢痕疙瘩成纤维细胞，应用荧光定量 PCR、RT-PCR 检测 cyclin D1 mRNA 的干扰效果, 应用 MTT 法、流式细胞仪检测细胞增殖和细胞周期的变化,应用免疫组织化学染色检测成纤维细胞中 cyclin D1、CDK4、P16、pRb 蛋白表达的影响．主要结果如下：a．靶向 cyclin D1 的特异性 siRNA 序列可以高效地抑制成纤维细胞 cyclin D1 基因表达，对照组与实验组在 mRNA 水平其表达抑制率分别为 63.68% 和 92.83% ($P < 0.01$)；b．可以显著抑制瘢痕疙瘩成纤维细胞的增殖，改变细胞周期分布，G0/G1 期细胞比例显著高于各对照组 ($P < 0.05$)，细胞分裂被阻滞；c．免疫组化染色发现，转染 72 h 后，过表达的 cyclin D1、CDK4 和 pRb 蛋白，在瘢痕疙瘩成纤维细胞中均出现了不同程度的表达下调，而低表达的 P16 则呈上调表现．由上述结果可见，构建的靶向 cyclin D1 的 RNAi 表达质粒，可有效地抑制瘢痕疙瘩成纤维细胞 cyclin D1 基因表达，通过改变 G1 期相关周期蛋白的水平，影响 G1/S 期的进程，显著地抑制成纤维细胞的增殖．

关键词　瘢痕疙瘩，RNA 干扰，cyclin D1，CDK4，pRb，P16，细胞周期

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