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Chondrogenic Effect of Precartilaginous Stem Cells Following NLS-TAT Cell Penetrating Peptide-Assisted Transfection of Eukaryotic hTGFβ3

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ABSTRACT

Cell penetrating peptides (CPPs) are a series of promising carriers for delivering exogenous DNA to living cells. Among them, the combination of the human immunodeficiency virus TAT protein (TAT) with the SV40 large T protein nuclear localization signal (NLS) to form NLS-TAT performs well. In the present study, we took advantage of this new carrier to deliver transforming growth factor-beta 3 (TGFβ3) genes. TGFβ3 was expressed by the pEGFP-N1 vector following transfection of rat precartilaginous stem cells (PSCs), which promoted hTGFβ3 protein self-expression. At 24, 48, 72, and 120 h after transfection, the expression levels of hTGFβ3 were found to be elevated as compared with the control. The expression of hTGFβ3 was found to mediate the chondrogenic effect of PSCs. Thus, we determined the expression of the chondrogenesis-related genes type II collagen, Sox 9, and aggrecan in PSCs at 24, 48, 72, and 120 h after transfection. We found that their transcription and translation was augmented, which indicated a trend of active chondrogenesis in the PSCs. Our results demonstrated that NLS-TAT had the ability to deliver exogenous DNA into rat PSCs and could be actively expressed. This process successfully promoted PSC chondrogenesis. Additionally, PSC, may represent a new type of stem cells, and thus show great potential in regenerative repair following cartilage injury. J. Cell. Biochem. 114: 2588–2594, 2013.

KEY WORDS: PEPTIDE-BASED CARRIERS; CELL PENETRATING PEPTIDE; PRECARTILAGINOUS STEM CELLS; CHONDROGENESIS

Transfecting recombined plasmid DNA into cells and promoting expression of specific genes has become a quite common mechanism in gene engineering. However, the traditional transfection method using cationic liposomes or viral vectors as vehicles for gene transfer is often unsatisfactory [Davis and Cooper, 2007]. By contrast, cell-penetrating peptides (CPP) are a series of cationic molecules and have been a subject of recent focus as potential carriers of DNA delivery into cells [Chen et al., 2007; Brasseur and Divita, 2010; Liu et al., 2012]. One of the most typical cell-penetrating peptides is the peptide sequence of the human immunodeficiency virus protein TAT (amino acids 48–60 of the HIV TAT protein, with a sequence of Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Gln). The TAT peptide is thought to promote uptake of macromolecules across plasma membrane and into cells. TAT reportedly binds to cell surface molecules that facilitate its internalization, although the exact process involved remains controversial. Another kind of cell penetrating peptide, the nuclear localization signal (NLS, with the sequence Pro-Lys-Lys-Lys-Arg-Lys-Val) has been identified by the intracellular machinery that translocates macromolecules through nuclear pores. NLS has been suggested as a means to promote nuclear delivery of expression contructs [Eguchi et al., 2005; Wang et al., 2011]. Both CPP and NLS have been reported to facilitate the non-toxic translocation of DNA into cells [Lehto et al., 2011; Suhorutsenko et al., 2011]. Different CPP carriers demonstrate markedly different physicochemical properties when bound to plasmid DNA. This is mainly determined by their three-dimensional structure. When delivering an expression vector, neither TAT nor NLS shows any satisfactory transfection efficiency when used alone. However, the combined peptide delivery system of TAT and NLS, referred to as NLS-TAT (with the sequence Pro-Lys-Lys-Lys-Arg-Lys-Val-Lys-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Gln) serves as a potential tool for the efficient delivery of expression constructs, and shows remarkable effects, especially with the help of Chloroquine which has a multi-functional role in promoting gene expression [Yang et al., 2009].

Conflicts of interest: None.

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Due to the poor proliferation capacity of chondrocytes, several studies have long focused on using articular cartilage regeneration as a method of inducing stem cells to show chondrogenesis [Mehlhorn et al., 2006; Choi et al., 2007]. TGFβ3 is a key requirement for chondrogenesis of stem cells, and studies have shown that TGFβ3 stimulates chondrocyte proliferation and prevents cartilage hypertrophy [Tang et al., 2009]. Precartilaginous stem cells (PSCs) are a type of newly identified stem cells isolated from the La Croix rings of rats [Robinson et al., 1999]. Moreover, we have constructed and verified an immortalized cell strain [Zhang et al., 2008] of PSCs as a model system. In the present study, we took the combined peptide: NLS-TAT as a DNA carrier to deliver the recombined plasmid pEGFP-N1-TGFβ3 into PSCs. Subsequently, we determined the expression of hTGFβ3 and the resulting chondrogenic behavior of PSCs so that we could determine whether NLS-TAT was suitable and effective as a gene delivery system for PSCs. Additionally, we wished to determine whether PSCs thus acquired a chondrogenic trend under the conditions of over-expressed TGFβ3. Chondrogenesis was evaluated by the expression of chondrogenesis-related genes, including collagen type II, aggrecan [Park et al., 2012; Kwon, 2013] and sox9, among which sox9 is predominantly expressed in mesenchymal condensation and cartilage, and function as an early chondrogenic transcription factor, which has also been shown to activate expression of collagen type II and aggrecan [Mochizuki et al., 2006].

MATERIALS AND METHODS

CELL ISOLATION, PURIFICATION, AND CULTURE

Eight Sprague–Dawley rats were provided by the animal center of Tongji Medical College. The tissues located around the La Croix rings [Robinson et al., 1999] were cut down and digested sequentially with Complete™ Typsin Solution (Chemicon International Inc.,CA) and 0.1% collagenase type one (Sigma Chemical Co., MO). When the cells had been dispersed and suspended as single cells in 0.1 M Phosphate Buffer Saline (PBS), they were incubated with rabbit polyclonal FGFR-3 antibody (c-15) (1:100, Santa Cruz Biotechnology Inc., CA) [Robinson et al., 1999] and then purified by an immunomagnetic separation system (Miltenyi Biotech, Bergisch Gladbach, DE). The immuno-selected FGFR-3 positive PSCs were cultured in DMEM/F12 (1:1 ratio) (Thermo Fisher Scienific Inc., CA), which was supplemented with 10% fetal calf serum (Gibco BRL Div. of Invitrogen, Gaithersburg, MD), 100 units/ml penicillin and 100 μg/ml streptomycin sulfate under 5% CO_2 at 37°C. The detailed procedures have been described elsewhere [You et al., 2004; Zhang et al., 2008]. The medium was changed 24 h after seeding the PSCs and then subsequently every 2 days.

IDENTIFICATION OF PSCs BY IMMUNOFLUORESCENT STAINING

The purified PSCs that were obtained at the first passage, and the cells that remained after isolation by immunomagnetic separation (negative control) were seeded into six-well-plates that first had glass coverslips positioned into each well prior to the final addition of complete culture medium. The density of cells was approximately 5×10^5 cells/well. When cells had adhered and were more than 80% confluent, the culture medium was removed and the cells were rinsed three times with PBS and fixed with 4% paraformaldehyde solution for 20 min at room temperature. The cells were then permeabilized with 0.2% Triton X-100 solution for 5 min, following which the cells were incubated with the primary antibody, which was polyclonal rabbit anti-FGFR-3 (1:200 dilution, sc-82, Santa Cruz) at 4°C overnight. Next, detection of the bound primary antibodies was enabled by incubating cells with goat anti-rabbit IgG-Cy3 (Boster Biological Technology, Ltd.,Wuhan,China) for 1 h at 37°C, following which the cells were incubated with 2 μg/ml 4′, 6-diamidino-2-phenylindole (DAPI) staining solution (Boster Biological Technology) for 5 min. After the coverslips with cells were positioned onto new slides, the cells were observed and images recorded under an Olympus fluorescence microscope (CX41, Olympus, Tokyo, Japan).

SYNTHESIS OF PEPTIDE CARRIERS AND PLASMID PREPARATION

The peptide carrier, TAT-NLS was synthesized by Jier Biological Inc., Shanghai, China, with solid phase synthesis using Boc chemistry. Purity of TAT-NLS was 98%. The combined eukaryotic expression plasmid that contained the hTGFβ3 gene was constructed with the pEGFP-N1 vector (Clontech Laboratories, Inc.,Otsu, Shiga, Japan), which was separated and purified from the Hela cell-line, and amplified by polymerase chain reaction (PCR). The endonucleases, *Xho*I and *Bam*HI (TaKaRa Biotech Co., Otsu, Shiga, Japan) were used to digest the endonuclease restriction sites at the Multiple cloning site (MCS). The hTGFβ3 gene was located in the gap and then ligated with DNA ligase to restore the integrity of the loop-like plasmid. The exact method was described in our previous study [You et al., 2011].

PREPARATION OF COMPLEXES

For uptake and expression studies, the DNA/TAT-NLS complex was generated by gradual addition of 5 μg DNA in 50 μl 20 mM Hepes Buffer, pH 7.3, to an equal volume of TAT-NLS in the same buffer. DNA/TAT-NLS solution was incubated at room temperature for 15 min to allow formation of the complex. Prior to transfection, 2.5 ml optiMEM medium (Life technologies, MD) was added to the complexes with gentle mixing. At a charge ratio of 1:10, the final TAT-NLS concentration was 4.10 μM.

TRANSFECTION OF PSCs With pEGFP-N1 Encoding hTGFβ3

Purified second passage PSCs were taken for transfection studies and seeded at a density of 5×10^5 cells/well into six-well-plates with culture medium in the absence of penicillin and streptomycin. When cells achieved a confluence of 80% of the wells, culture medium was removed, the cells were washed once with PBS, and DNA/TAT-NLS in 2.5 ml OptiMEM was added to the cells. After 4 h incubation at 37°C, 2.5 ml complete culture medium was added in the absence of penicillin and streptomycin, supplemented with 100 μM chloroquine. In addition, the culture medium was replaced every other day. The same volumes of OptiMEM culture medium alone were added to PSCs seeded into six-well plates as the blank control.

Fluorescence microscopy. Transfected PSCs were cultured for 24, 48, 72, and 120 h respectively, following which the light-field and EGFP images were recorded using an Olympus inverted fluorescence microscope (CKX41, Olympus,with an excitation filter of 495–525 nm) to visualize the level of expression of foreign proteins of transfected PSCs.

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Before PSCs were seeded into six-well-plates for transfection, coverslips were positioned at the bottom of the wells. When incubated in the culture medium, PSCs adhered to the coverslips. Thus coverslips that had transfected PSCs adhered to their surfaces at 24, 48, 72, and 120 h after were acquired. Next, the coverslip-attached cells were immunofluorescently stained and the images were recorded following the protocols described above using primary antibodies: anti-TGFβ3 rabbit polyclonal antibodies (at 1:200 dilution, sc-82, Santa Cruz), anti-collagen type II rabbit polyclonal antibodies (at 1:100 dilution, ab53047, Abcam Inc., Cambridge), anti-Sox9 rabbit polyclonal antibodies (at 1:200 dilution, sc-20095, Santa Cruz) and anti-Aggrecan rabbit polyclonal antibodies (at 1:100 dilution, sc-25674, Santa Cruz).

SEMI-QUANTITATIVE REAL-TIME PCR

After undergoing the transfection described above, PSCs in each well were washed three times with PBS and treated with Trizol Reagent (Invitrogen, MD) to extract total RNA following culture of the cells for 24, 48, 72, and 120 h following transfection. After the extraction and purification of total RNA, 0.9 μg of total RNA was reverse-transcribed to cDNA with a First Strand cDNA Synthesis Kit Rever Tra Ace-α- (ToYoBo Co., Ltd., Osaka, Japan. Real-time PCR was performed with thunderBird SYBR qPCR Mix (ToYoBo) on the iCycler real time PCR instrument (BIO-RAD, CA) according to the manufacturer's instructions. Following this procedure, cDNA of the hTGFβ3 gene, then Sox9 gene, the Aggrecan gene and the Collagen II gene were amplified using corresponding primers (Table I) and the mRNA expression levels were analyzed using iQ5 (Bio-Rad). Expression levels of the transcripts were normalized to endogenous GAPDH mRNA (as the reference gene) according to the formula $2^{-\triangle\triangle C}$. Control samples were similarly harvested and detected with the same procedures.

WESTERN BLOT ANALYSIS

PSCs cultured for 24, 48, 72, and 120 h after transfection were washed in PBS three times and treated with lysis buffer that consisted of 50 mM Tris–HCl, 0.1% Triton X-100, 0.1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride to obtain whole-cell extracts. Aliquots that contained equal amounts of protein were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The samples were run on 10% gradient cross-linked polyacrylamide gels and transferred to PVDF membranes (0.45 μm Millipore, Billerica, MA). The membranes were then probed with anti-TGFβ3 rabbit polyclonal antibodies (at 1:800 dilution, sc-82, Santa Cruz), anti-collagen type II rabbit polyclonal antibodies (at 1:800 dilution, sc-28887, Santa Cruz), anti-Sox9 rabbit polyclonal antibodies (at 1:800 dilution, sc-20095, Santa Cruz) and anti-Aggrecan rabbit polyclonal antibodies (at 1:700 dilution, sc-25674, Santa Cruz) followed by horseradish peroxidase-conjugated secondary antibodies (at a dilution of 1:7,500). Immunoreactive proteins were visualized by chemiluminescence (Pierce, CA) according to the manufacturer's instructions. The intensity of the blots was quantified by digital image analysis software (Quantity One, version 4.6, Bio-RAD).

STATISTICAL ANALYSIS

Data were expressed as mean ± standard deviation (SD). Differences between sample means were analyzed by two-factor ANOVA and two-tailed student–Newman–Keuls multiple comparisons test using SPSS version 13.0 software (SPSS Inc., Chicago, IL). An alpha value of $P < 0.05$ was considered statistically significant. For all statistical analyses, experiments were independently repeated three times with cells from different donor SD rats (if not indicated otherwise) and three samples per trial for each experiment.

RESULTS

PURIFICATION AND IDENTIFICATION OF PSCs

Immunomagnetically purified PSCs were observed to be uniformly short fusiform or triangular cells with two or three short axons when cultured in DMEM/F12. PSCs stained positive for FGFR-3 when assayed by immunofluorescence (Fig. 1A), while the cells that remained after immunomagnetic separation were negative for FGFR-3 expression (Fig. 1B).

IDENTIFICATION OF THE RECOMBINED PLASMID

To demonstrate the location of the hTGFβ3 gene in the recombined plasmid, 1.5% agarose gel electrophoresis was carried out, which showed DNA fragments after the recombinant vector was treated with *Xho*I and *Bam*HI (Fig. 2). In addition, DNA sequencing demonstrated that the shorter DNA fragment obtained from the former procedure corresponded to hTGFβ3.

TRANSFECTION OF PSCS WITH PEPTIDE-DNA COMPLEXES AND EXPRESSION OF EGFP

To show that the recombinant plasmid had been delivered into PSCs by NLS-TAT and that the cargo DNA could be actively expressed in

TABLE I. PCR Primers and the Corresponding Gene Products

Gene	Primer sequence	Product length (bp)
Sox9 (XM_343981.2)	F:5′-GTGGGAGCGACAACTTTACC-3′ R:5′-GAGAACGAAACCAGGGCTACT-3′	85
Collagen type II (NM_012929.1)	F:5′-aCAAGAGCAAGGGAAGAAGCA-3′ R:5′-TGGACAGTAGACGGAGGAAAG-3′	131
Aggrecan (NM_022190.1)	F:5′-AGAATCCATAACTGCCCCAAC-3′ R:5′-GTCACGCCCTCCACTAACTCT-3′	90
hTGFβ3 (NM_003239.2)	F:5′-CACCCAGGAAAACACCGAGTC-3′ R:5′-GCGGAAAACCTTGGAGGTAAT-3′	86
GAPDH	F:5′-CCACTTTGTGAGCTCATTTCCT-3′ R:5′-TCGTCCTCCTCTGGTGCTCT-3′	140

F, forward sequence; R, reverse sequence.

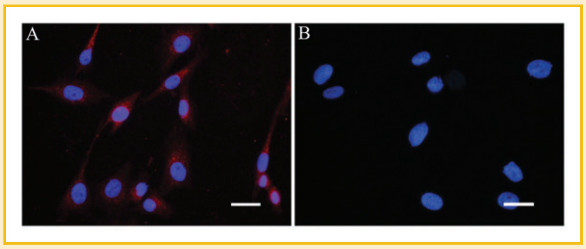

Fig. 1. Fluorescence microscopy shows the specific expression of FGFR-3 by PSCs (A), while the cells that remained after immunomagnetic separation were negative for FGFR-3 expression (B). Excitation filter was 555–585 nm. Scale bar = 25 μm.

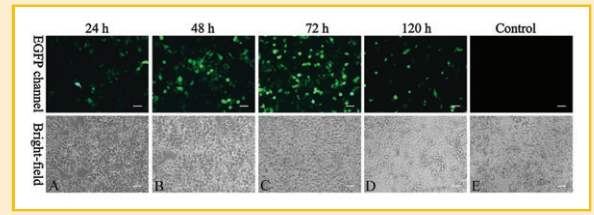

Fig. 3. Fluorescent micrograph of EGFP and light field of PSCs, which were taken at different time points after DNA/NLS-TAT complex-assisted transfection. Images in the upper row show green fluorescence that was emitted by PSCs that were incubated for 24, 48, 72, and 120 h successively following transfection. Bright-field images in the lower row correspond to those images in the upper row. The last lane (E) shows cells that had been incubated in the absence of the DNA/NLS-TAT complex as blank control. Excitation filter was 495–525 nm. Scale bar = 50 μm.

PSCs following transfection, the mammalian EGFP reporter gene was used in the functional gene assay: When transfected PSCs were incubated for 24 h, dim green fluorescence was displayed in a small population of PSCs (Fig. 3A). More cells that emitted more intense green fluorescence, were seen at 48 and 72 h following transfection (Fig. 3B,C). This indicated that the peptide-DNA complexes entered PSCs and the hTGFβ3 gene could be efficiently expressed when delivered by NLS-TAT. Following 120 h after transfection (Fig. 3D), green fluorescent cells decreased sharply.

hTGFβ3 Expression in PSCs

Human TGFβ3 mRNA expression (Fig. 4A) and protein expression (Fig. 4B) was detected by semi-quantitative real time PCR and Western blot analysis, respectively. As expected, hTGFβ3 gene expression (Fig. 4A) in the transfection group was relatively intense and increased progressively from 24 to 72 h as compared with the control-group ($P < 0.01$). However, at 120 h after transfection, mRNA expression of hTGFβ3 declined gradually as compared with the previous time-points, yet remained considerably higher as compared with the control group ($P < 0.01$). Similarly, TGFβ3 protein expression (Fig. 4B) increased as compared with the control group and showed significant differences ($P < 0.05$). However, unlike the observations made following mRNA expression by real time PCR, protein expression of TGFβ3 increased noticeably between 48 and 72 h and sustained high expression levels up to and including 120 h post-transfection ($P < 0.01$). At the same time, hTGFβ3 protein expression was also detected by immunofluorescent staining (Fig. 6) at all four detection time-points. As compared with the blank control, transfected PSCs showed more intense red fluorescence and retained staining intensity up to 120 h post-transfection.

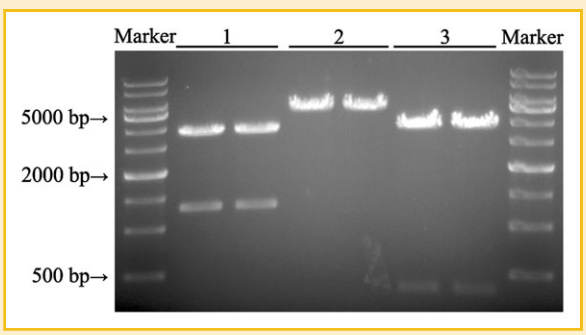

Fig. 2. The recombined plasmid pEGFP-N1-TGFβ3 was identified by restriction enzyme digestion (*Xho*I and *Bam*HI). The first two lanes show the recombined plasmid digested by *Xho*I and *Bam*HI. The lower band was found to locate between 2,000 and 500 bp, and indicates the hTGFβ3 gene fragments. The second two lanes show the recombined plasmid that was digested only by *Xho*I. The third two lanes show the empty plasmid pEGFP-N1 that was digested by *Xho*I and *Bam*HI.

CHONDROGENESIS-RELATED GENE EXPRESSION

Expression of the chondrogenesis-related genes: sox9, collagen II and aggrecan were assayed by semi-quantitative real-time PCR (Fig. 5A) and Western blot analysis (Fig. 5B). After data was normalized by GAPDH, it was found that consistent with the expression of hTGFβ3 the expression of sox9, collagen II and aggrecan increased as compared with the control at the same time points ($P < 0.05$). Among the three chondrogenesis-related genes, expression of collagen type II increased more prominently, while it was not that noticeable for that of aggrecan. The result also showed that transcription of sox9, collagen type II and aggrecan decreased by 120 h after transfection of hTGFβ3 whose mRNA expression decreased notably at 120 h post-transfection as compared with the expression levels seen at 24, 48, and 72 h. By contrast, protein expression of the chondrogenesis-related genes did not alter and did not decrease at 120 h post-transfection as compared with 72 h ($P < 0.05$), which also corresponded to the trend in expression of hTGFβ3. Images of immunofluorescently stained PSCs also showed collagen type II, sox9, and aggrecan expression following transfection (Fig. 6). Though it cannot be quantified, the red fluorescent intensity retained a trend of escalating expression over the time-points of 24–120 h.

DISCUSSION

Delivering exogenous DNA into living cells and promoting expression of the functional gene have represented a quite common and effective method in gene engineering. Many kinds of carriers have been applied including cationic liposomes and viral vectors, but each of them have disadvantages. Therefore, confirmation or validation of

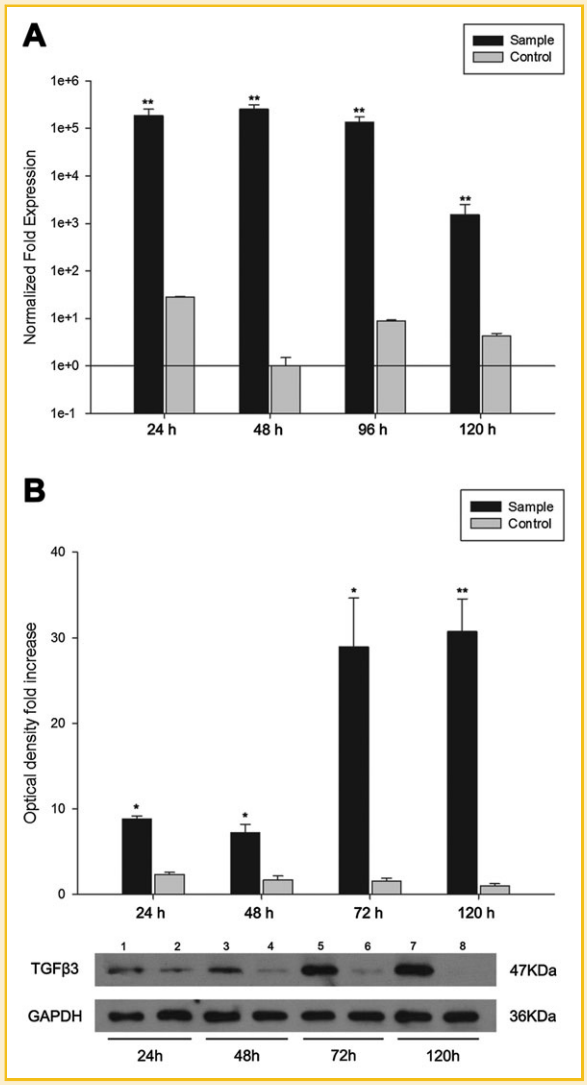

Fig. 4. Expression of hTGFβ3 was analyzed by semi-quantitative real-time PCR (A). The relative mRNA expression of hTGFβ3 was normalized to GAPDH. The error bar represents standard deviations ($P < 0.05$). Protein expression of hTGFβ3 was determined by Western blot (B). The histogram represents normalized band densitometry readings averaged from three independent samples. Corresponding to the former test, GAPDH was also used as an internal control and served to normalize the data. Error bars represent standard deviations ($P < 0.05$). The P values are as follows: $^{*}P < 0.05$; $^{**}P < 0.01$. Sample: the transfected PSCs, Control: the blank control.

the most effective carrier for gene delivery remains controversial. Recently, with the advent of CPPs, they have been shown to be very attractive candidates for the delivery of therapeutic macromolecules because of their low toxicity and robust operation [Suhorutsenko et al., 2011; Wang et al., 2011]. However, not all of the CPPs have a satisfactory transfection efficiency and acceptable gene expression for a corresponding biological effect [Margus et al., 2012]. Furthermore, most investigators in the field are more concerned in determining whether exogenous DNA or oligonucleotides can penetrate the plasma membrane with the assistance of CPPs, and in determining the mechanism of this delivery. Studies regarding CPPs as a practical method of gene therapy and applying them to preclinical and clinical applications are rare. However, one type of CPPs, namely TAT, has been used in patients [van den Berg and Dowdy, 2011]. Thus it is of great importance to test the feasibility of CPPs applied to some additional specific fields of application.

As for PSCs, they are FGFR-3 positive cells located at the La Croix ring of rats and serve as cartilaginous precursor stem cells. It was Robinson et al. [1999] who separated PSCs in 1999 and suggested PSC is a new type of stem cell, and provided a new method for repair of cartilage injury. A strong foundation for further research of the differentiation of chondrocytes from purified PSCs was described by You et al. [2004].

In previous studies done by our group, PSCs were induced to differentiate into chondrocytes with TGFβ3. Furthermore, a trial of promoting self-secretion of TGFβ3 by PSCs has also been studied, although shortcomings of traditional transfection methods could not be overcome [You et al., 2011]. All the experiments noted above indicated that PSC represents a new kind of previously under-appreciated cartilaginous precursor stem cell, which has great potential in the repair of cartilage injury.

In the present study, we selected TAT as our vector for DNA delivery to PSCs. To enhance the ability of TAT to penetrate through the plasma membrane and show exogenous gene expression, NLS was added to the N-terminal of TAT (NLS-TAT) [Yang et al., 2009]. By using this vector, green fluorescence was found emitting from PSC, which indicated not only the expression of the reporter gene EGFP, but also the expression of hTGFβ3. Results of real-time PCR and Western blot confirmed the conclusion made above.

Nevertheless, it is worthwhile to note that the protein expression of TGFβ3 remained elevated as compared with the control 120 h after transfection, and that the expression of EGFP could also be detected 120 h after transfection, which is quite different from the condition of transient transfection using traditional transfection reagents [Davis and Cooper, 2007]. For traditional transfection methods using cationic liposomes, expression of exogenous DNA can only be retained for approximately 72 h and decreases sharply thereafter with gradually decreasing EGFP-mediated green fluorescence, under conditions where this reporter gene is used.

This is one of the key reasons why it is a real challenge to induce differentiation of stem cells using cationic liposome as vehicles for gene transfer and promoting exogenous gene self-expression.

TGFβ3 is a key requirement for chondrogenesis of mesenchymal stem cells (MSCs) and PSCs [You et al., 2011]. In the presence of TGFβ3, PSCs differentiate into a chondrogenic cell-line [Tang et al., 2009] that produces cartilage-specific matrix proteins such as sox9, collagen type II, and aggrecan. We tested three indexes of chondrogenesis to confirm chondrogenesis of PSCs after transfection. The results of real-time PCR and Western blot analysis showed that sox9, collagen type II and aggrecan have different degrees of enhanced expression, but the trend was in parallel to that of TGFβ3 protein, which was also indicated, albeit indirectly, by immunofluorescence staining. Thus biologically active TGFβ3 was expressed after exogenous TGFβ3 gene delivery into PSCs, which was assisted by NLS-TAT. Expression was sustained at levels and over a time span that promoted chondrogenesis.

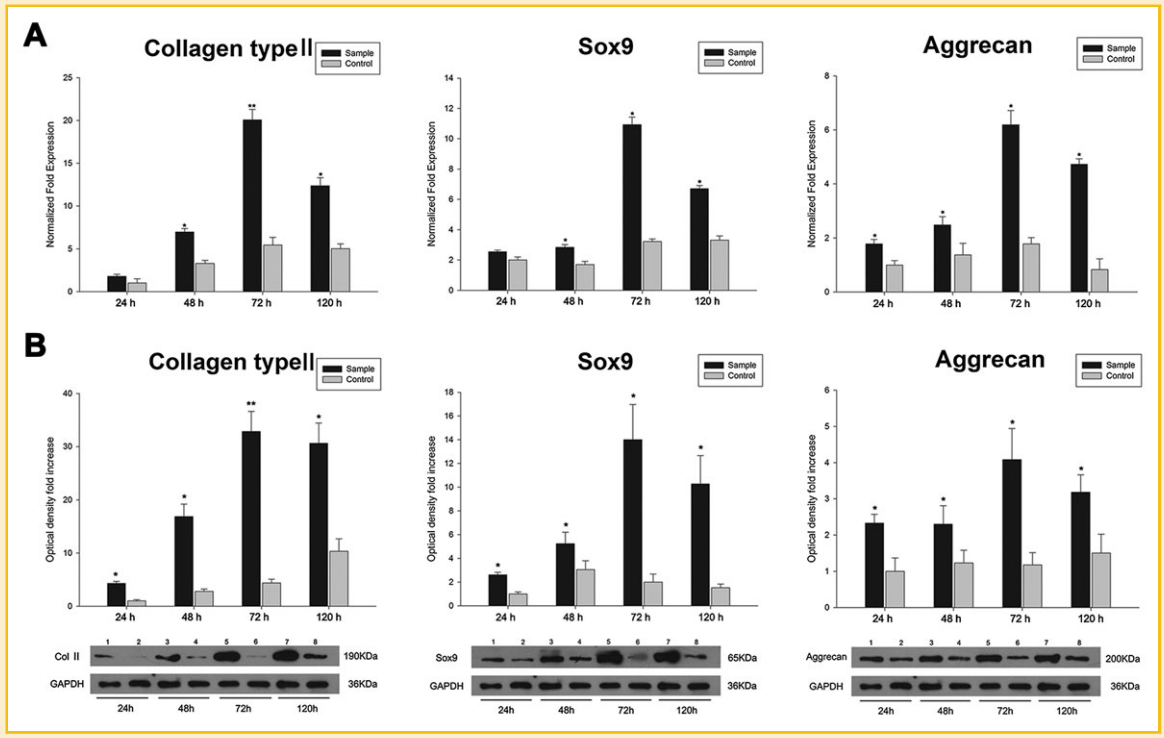

Fig. 5. Results of chondrogenesis-related gene expression as determined by semi-quantitative real-time PCR (A) and Western blot (B). The relative mRNA and protein expression of collagen type II, sox9 and aggrecan were normalized to GAPDH. From 24 to 72 h, mRNA and protein expression corresponding to the three chondrogenesis-related genes was found to increase ($P < 0.05$). Though the expression of those genes did not increase continually (at 120 h) after transfection, their expression still remained elevated. Furthermore, protein expression of chondrogenesis-related genes at 120 h after transfection did not show any appreciable difference ($P < 0.05$) as compared with their corresponding protein expression levels at 72 h. The error bar represents standard deviations ($P < 0.05$). The *P* values are described as follows: $^{*}P < 0.05$; $^{**}P < 0.01$. Sample: the transfected PSCs, Control: the blank control.

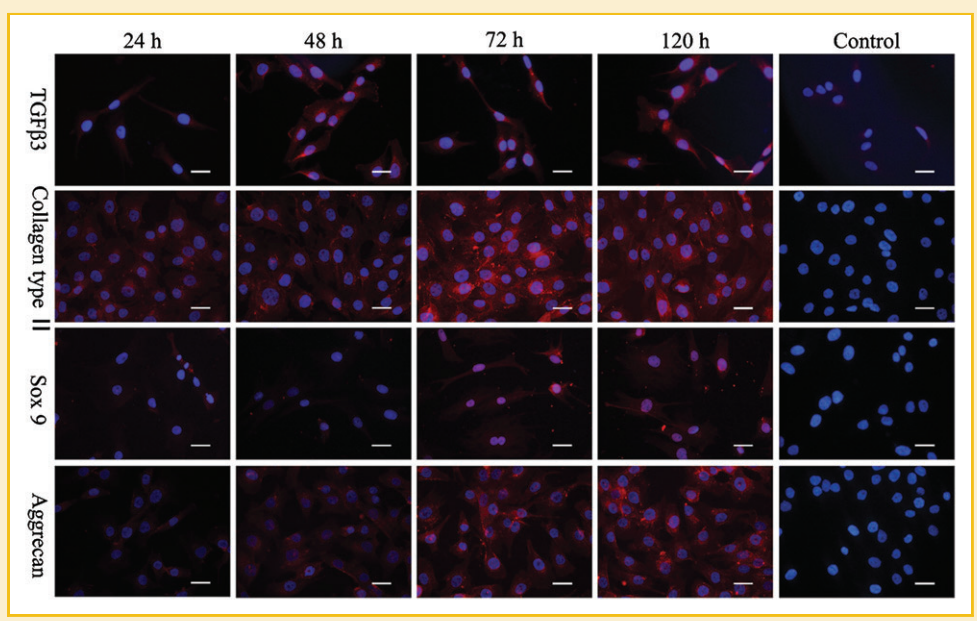

Fig. 6. Images of PSCs were tested with immunofluorescent stains at different time-points after transfection. hTGFβ3 protein and the chondrogenesis-related proteins: Collagen type II, sox9 and aggrecan were labeled with red fluorescence. In each part of the figure, the rows from top to bottom indicate the PSCs, which were tested by different primary antibodies, while the lanes show PSCs that were incubated at different detection times after transfection. The last lane indicates the blank control group. Compared with the blank control, images that indicated the detection of hTGFβ3 (the first row) showed more intense fluorescence at time points of 72 and 120 h post-transfection, while fluorescence of the cells at 24 h was not particularly apparent. This trend was also notable in the images for collagen type II, sox9 and aggrecan, although to different extents. The excitation filter was 555–585 nm. Scale bar = 25 μm.

However, there were drawbacks from our study, which are challenging to explain. First, TGFβ3 mRNA increased up to 1×10^5 times when compared with the control at 24, 48, and 72 h after transfection. When comparing this observation with the increased range of TGFβ3 protein which remained at approximately 10×, the sharp increase of mRNA was controversial. Plasmid pEGFP-N1-TGFβ3 added to the culture medium was focused on and regarded as the probable factor if the bias in expression differences really existed. Thus, we attempted many ways to prevent contamination of the recombined plasmid such as rinsing the cells and re-acquiring them by centrifuge before extraction of total RNA. However, we still did not find significant difference between the two results. Though the mechanisms of CPP-mediated cellular transfection are still under investigation, it is believed that endocytosis is one of the major uptake mechanisms [Madani et al., 2011]. Therefore, it is reasonable to predict that exogenous TGFβ3 folded in macropinosomes, and could not be subsequently removed by washing. This may help explain the inappropriate increase in mRNA expression of TGFβ3.

Secondly, mRNA of sox9, collagen type II and aggrecan began to decrease at 120 h after transfection. However, the corresponding decline in protein expression was not detected at the same time points. Whether this phenomenon suggests the decline of chondrogenesis-related proteins as a function of time has not been determined. However, NLS-TAT has shown many advantages including durable time ranges of continued exogenous gene expression as compared with other nonviral vehicles for gene transfer applications.

In conclusion, we have demonstrated that the recombinant plasmid expressing biologically active hTGFβ3 could be successfully delivered into PSCs using NLS-TAT as vehicles for gene transfer. With increasing expression of TGFβ3, we confirmed that chondrogenesis-related genes were showing signs of initial gene expression and that PSCs showed chondrogenesis. This study represents an important exploration for the application of CPPs and the development of regenerative stem cell repair approaches in chondrocyte injury.

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