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RESEARCH PAPER

Optimal Construction and Delivery of Dual-Functioning Lentiviral Vectors for Type I Collagen-Suppressed Chondrogenesis in Synovium-Derived Mesenchymal Stem Cells

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ABSTRACT

Purpose This study aims to deliver both transforming growth factor β3 (TGF-β3) and shRNA targeting type I collagen (Col I) by optimal construction and application of various dual-functioning lentiviral vectors to induce Col I-suppressed chondrogenesis in synovium-derived mesenchymal stem cells (SMSCs).

Methods We constructed four lentiviral vectors (LV-1, LV-2, LV-3 and LV-4) with various arrangements of the two expression cassettes in different positions and orientations. Col I inhibition efficiency and chondrogenic markers were assessed with qPCR, ELISA and staining techniques. Among the four vectors, LV-1 has two distant and reversely oriented cassettes, LV-2 has two distant and same-oriented cassettes, LV-3 has two proximal and reversely oriented cassettes, and LV-4 has two proximal and same-oriented cassettes. Col I and chondrogenic markers, including type II collagen (Col II), aggrecan and glycosaminoglycan (GAG), were examined in SMSCs cultured in 3-D alginate hydrogel.

Results All of the four vectors showed distinct effects in Col I level as well as diverse inductive efficiencies in upregulation of the cartilaginous markers. Based on real-time PCR results, LV-1 was optimal towards Col I-suppressed chondrogenesis.

Conclusion LV-1 vector is competent to promote Col I-suppressed chondrogenesis in SMSCs.

KEY WORDS dual-functioning lentiviral vector · synovial mesenchymal stem cells (SMSCs) · TGF-β3 · type I collagen · chondrogenesis

ABBREVIATIONS

Col I	type I collagen
Col II	type II collagen
LV-dual	recombinant lentiviral vector that expresses both shRNA targeting type I collagen and TGF- β3
LV-T	recombinant lentiviral vector that specifically expresses TGF-β3
shRNA	short hairpin RNA
SMSC	synovium-derived mesenchymal stem cell
TGF-β3	transforming growth factor-β3

INTRODUCTION

Joint cartilage degeneration, typically due to osteoarthritis, remains prevalent among the elderly, while physical trauma-induced cartilage damage may affect all populations (1,2). Great challenges are posed to the treatment of cartilage disorder. So far, a series of therapies has been developed, including biological treatment and surgeries. Alternatively, chondrocyte implantation and stem cell transplantation have been practiced and proved to be

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encouraging for the cure of cartilaginous lesions. In engineered regenerative medicine, autologous cells, no matter committed ones or progenitor cells, in conjunction with a biocompatible scaffold, provide great promise to the therapeutics. Among the stem cells applied for this purpose, synovium-derived mesenchymal stem cells (SMSCs) have been recently recognized as an excellent species for therapeutic chondrogenesis due to their abundance and self-renewal ability of sources as well as the superiority in chondrogenesis (3).

Transforming growth factor-β (TGF-β) superfamilies have long been substantiated to promote various organogenesis, including osteo/chondrogenesis (4–6). Among the families, TGF-β3 is capable of inducing chondrogenesis using SMSCs (6,7). However, an obvious disadvantage of using SMSCs is the inherent expression of type I collagen (Col I) in SMSCs (8,9), which is an undesired component of the cartilage extracellular matrix. Overexpression of Col I would alter the mechanical strength of cartilage and undermine its capability to withstand a certain load. Moreover, in our previous studies, we have found the elevation of Col I expression induced by either exogenous TGF-β3 or endogenously expressed TGF-β3 via a vector (10). Therefore, it is imperative to suppress the expression of Col I in the application of SMSCs for chondrogenesis. RNA interference (RNAi) has provided a solution to meet this end (11,12). With either exogenous small interfering RNA (siRNA) or endogenously expressed shRNA that targets mRNA of Col I, the expression of Col I could be inhibited. In consideration of the above two points, vectors that express TGF-β3 and/or Col I-targeting shRNA would be useful for the delivery of the two in achieving Col I-suppressed chondrogenesis.

Viral delivery systems are known to reach a very high transfection efficiency (13), and the expression duration of delivered genes can be modulated by the selection of suitable vectors. Additionally, the immunogenic problems widely in concern for the application of viral vectors can be undermined to a minimal extent with *ex vivo* cell-based therapy, since the immunogenic viral components, such as envelopes or capsid, have been removed and discarded with the frequent medium change during cell expansion.

Other researchers have designed bipartite vectors to co-produce vascular endothelial growth factor (VEGF) and shRNA targeting inducible NO synthase (14). Previously, we have also successfully constructed a dual-functioning adenoviral vector encoding both TGF-β3 and Col I-targeting shRNA (10,15). The vector was substantiated to be effective in TGF-β3 secretion and Col I inhibition in several cell types. However, the adenoviral vector performs in an episomal manner, which only ensures a transient gene expression. For sustained Col I-suppressed chondrogenesis, a long-term release of TGF-β3, and Col I-targeting shRNA in particular, might be favored. Therefore, lentiviral vectors, which are integrative into the host genome and thus induce a sustained gene expression, are constructed for this purpose. In dual-functioning lentiviral vectors (LV-dual), since two expression cassettes are functioning in the same vector, transcriptional interference might exist to promote or compromise the transcription of each other. As the orientation and location of the two cassettes would affect the expression levels, we constructed four LV-duals by altering the direction and position of the shRNA-expressing cassette. SMSCs were transduced with the lentiviral vectors and encapsulated in three-dimensional alginate hydrogel beads. Expression of Col I and chondrogenic markers was tested. The overall effect of the viral genomic configuration on Col I-suppressed chondrogenesis was assessed to obtain the optimal vector for Col I-suppressed chondrogenesis.

MATERIALS AND METHODS

Construction of Single-Functioning and Dual-Functioning Recombinant Lentiviral Vectors (LV-dual)

To produce recombinant lentiviral vectors that can express TGF-β3 and/or suppress type I collagen expression, we produced recombinant lentiviral vectors with the following configurations. The schematic structures of the recombinant lentiviral vectors and their roles in Col I-suppressed chondrogenesis are shown in Fig. 1a, b.

We have constructed a dual-functioning adenovirus previously (10). To construct lentiviral plasmid that had the same arrangement of TGF-β3 and shRNA-expressing cassettes with adenoviral vector (pLVX-3), the recombined adenoviral shuttle vector was digested with the restriction endonuclease PspOM I. The linearized recombinant adenoviral vector was further digested with the restriction endonuclease Sal I and subjected to gel electrophoresis for extraction of the fragment containing TGF-beta3 cDNA sequence, U6 promoter and Col I-targeting shRNA, which was about 1,600 bp long. Meanwhile, the pLVX-IRES-ZsGreen vector (Clontech Laboratories Inc., Mountain View, CA) was double digested with restriction endonucleases Xho I and Not I. The fragment from the recombined adenoviral shuttle vector and the linearized pLVX-IRES-ZsGreen vector were ligated. Recombinant plasmid was amplified, purified and verified through enzymatic digestion and sequencing.

LV-4 differed from the existing LV-3 in that the shRNA-expressing cassette was in the same direction with TGF-β3-expressing cassette in LV-4, whereas in LV-3 the directions for the two were reverse. In LV-1 and LV-2, we moved the shRNA-expressing cassette elsewhere out of the multiple

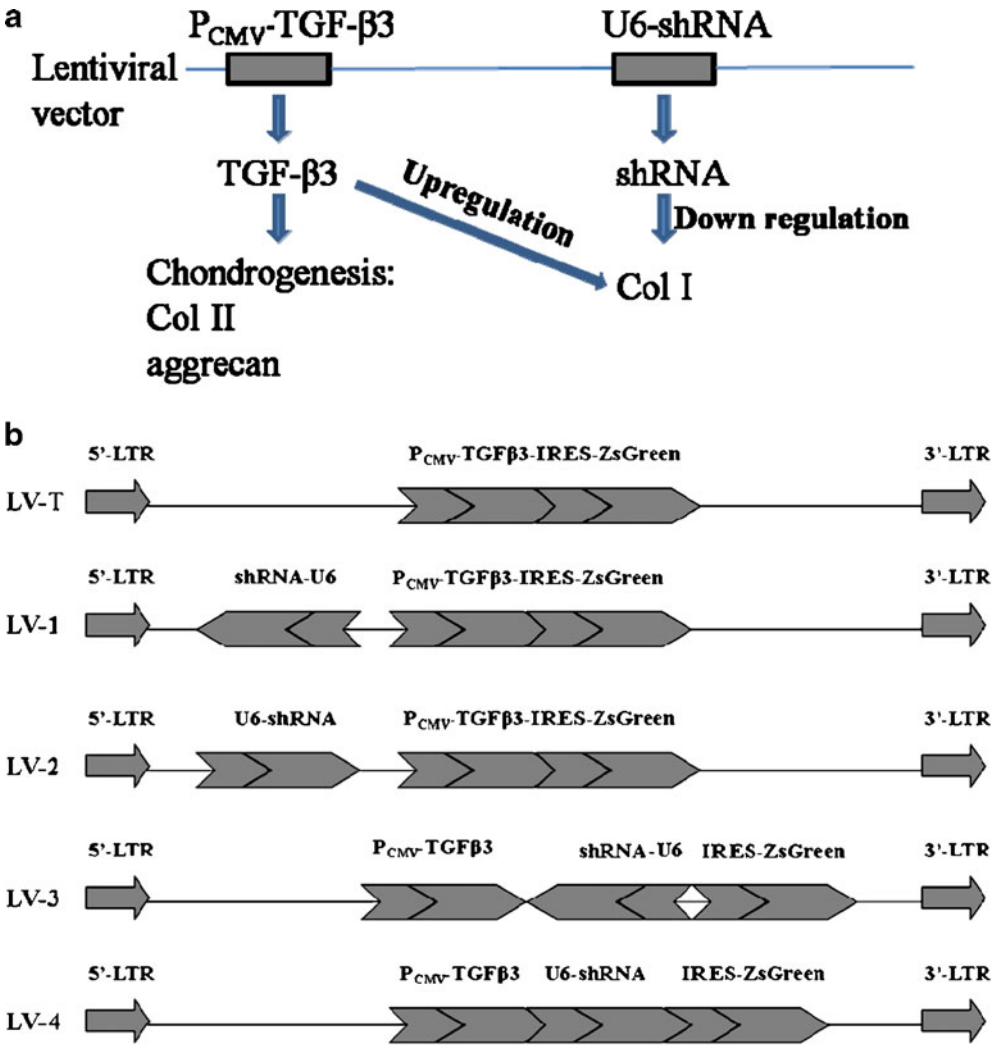

Fig. 1 Schematic roles and structures of recombinant lentiviral vectors. (**a**) The relationship of the molecules in our research. (**b**) The structures of the recombinant lentiviral vectors.

cloning sites (MfeI and FseI restriction sites), to avoid the potential interference between the two expressing cassettes. In LV-1, the two cassettes were in reverse directions, while in LV-2 they were in the same direction.

For detailed procedures, we firstly constructed lentiviral plasmid vector carrying only TGF-β3-expressing cassette (pLVX-T) as the backbone for the others and also as a control in subsequent experiments. TGF-β3 sequence was amplified through PCR using primers as shown in Table I. Both the TGF-β3 sequence and pLVX vector were digested with XhoI and NotI, and the two linear segments were ligated.

shRNA-expressing cassette was amplified from recombinant dual-functioning adenoviral donor vector (pDNR) with primers carrying various restriction sites at the ends. The segment was digested with corresponding restriction endonucleases and ligated with pLVX-T which had been

Table I Primers Designed for Vector Construction. (Ta: Annealing Temperature)

Amplicon	Restriction sites	Primer sequence 5'-3'	Length (bp)	Ta (°C)
TGF-β3	XhoI/NotI	F:CCGCTCGAGATGAAGATGCACTT R:ATAAGAATGCGGCCGCTCAGCTACATTTAC	1,259	50
shRNA cassette-1	FseI/MfeI	F:AGACTACAATTGTGCAGGAAGAGGGCCTAT R:CATCAAGGCCGGCCACGCGTAAAAAACAATCACCTG	~350	55
shRNA cassette-2	MfeI/FseI	F:AGACTAGGCCGGCCGACCATGTTCACTTACCTAC R:CATCAACAATTGACGCGTAAAAAACAATCACCTG	~400	58
shRNA cassette-4	NotI/BamHI	F:AAGGAAAAAAGCGGCCGCATATAGCAGGAAGAGGGCCTAT R:CGGGATCCACGCGTAAAAAACAATCACCTG	~350	55

digested with the same restriction endonucleases. The primers for the amplification of TGF-β3 and shRNA-expressing cassettes for pLVX-1, pLVX-2 and pLVX-4, respectively, were designed and are listed in Table I.

Meanwhile, recombinant lentiviral vector that encodes shRNA only (pLVX-sh) was also constructed for comparison, using Lenti-X expression vectors (Clontech Laboratories Inc., Mountain View, CA). Briefly, two oligonucleotides are annealed to form a short stretch of double-stranded DNA carrying shRNA-encoding sequence with NotI and BamHI overhangs on each end and inserted into pLVX. The sequence of one strand is given as 5'-GATCCG-CAATCACCTGCGTACAGAA-TTCAA-GAGA-TTCTGTACGCAGGTGATTG-TTTTTT-ACGCGT-G-3'.

For verification via enzymatic digestion, the restriction endonucleases Sal I and Hind III were used separately with 1 μg of the recombinant plasmid prior to agarose gel electrophoresis.

Recombinant lentiviral plasmids (pLVX-T, pLVX-sh, pLVX-1, pLVX-2, pLVX-3, pLVX-4) were then used to generate lentiviral vectors (LV-T, LV-sh, LV-1, LV-2, LV-3, LV-4) according to the manual of Lenti-X^{TM} Lentiviral Expression Systems (Clontech, Mountain View, CA). Among them, LV-T referred to the recombinant lentivirus that expresses TGF-β3 only, while LV-sh was the one that expresses shRNA against type 1 collagen. LV-1, LV-2, LV-3 and LV-4 all belonged to the LV-dual family, which express both TGF-β3 and shRNA.

Porcine Synovial Mesenchymal Stem Cell (pSMSC) Isolation and Culture

Porcine SMSCs were isolated from porcine synovium as described by De Bari *et al.* (16). Briefly, after being harvested aseptically from knee joint of mature swine, the synovial tissues were washed triplicately with phosphate-buffered saline (PBS) solution with penicillin/streptomycin and minced finely. Following digestion with 0.1% collagenase II, cells were released and then propagated in complete growth medium containing high-glucose DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin. The medium was changed every 3–4 days. Subculture with 1:3 dilution ratio was carried out when cultures reached 90% confluence. The above-mentioned cell culture-related reagents were purchased from Gibco (Invitrogen, Carlsbad, CA).

Lentiviral Transduction and Selection of ZsGreen-Positive Cells

Aliquots of filtered lentiviral stocks were thawed. Culture medium and polybrene stock solution were supplemented to adjust the volume to accommodate the transduction. An MOI of 10 was applied, and the final polybrene concentration was adjusted to 2 μg/mL. Viral supernatant was added to the cells and allowed for transduction for 8 h before it was replaced with fresh growth medium. The cells were incubated for 48 h before they were trypsinized and washed with PBS. FACS was used to select the ZsGreen-positive cells, which had been successfully transduced with recombinant lentiviral vectors. The excitation wavelength was 470 nm, while the emission wavelength was 520 nm. The sorted cells were then plated in dishes for amplification, marked as P0. Subsequent passages were labeled as P1, P2, etc.

Amplification and Three-Dimensional Culture of pSMSC

ZsGreen-positive cells were amplified in 2-D culture flasks for four passages according to the standard culture protocols. Then the cells were trypsinized and rinsed with washing buffer (0.15 M NaCl (Sigma-Aldrich)+25 mMN, 2-hydroxyethylpiperazine-N0-ethanesulfonic acid (HEPES) (Hyclone) in distilled water). After that, the cells were resuspended in sterile 1.2% alginate (Sigma) at 7×10^6 cells/mL. Forty μL of cell suspension was slowly dropped into a beaker containing 102 mM $CaCl_2$ (Sigma-Aldrich) solution for polymerization for 10 min. Finally, four beads were placed into each well of the 24-well plate with 1 mL of chondrogenic medium which contained high-glucose DMEM (Gibco), 100 nM dexamethasone (Sigma), ascorbic acid 2-phosphate (50 μg/mL; Sigma), sodium pyruvate (100 μg/mL; Gibco), proline (40 μg/mL; Sigma), penicillin (100 U/mL), streptomycin (100 μg/mL) (Gibco), and 5 mL of ITS premix in 500 mL of medium (6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 μg/mL selenous acid, 5.35 μg/mL linoleic acid, and 1.25 μg/mL bovine serum albumin (BD Biosciences, Bedford, MA). Medium was replaced and collected for ELISA assay every three days. In parallel, SMSCs without transduction were embedded into alginate hydrogel as negative control. pSMSCs without transduction are also cultured with manually added TGF-β3 protein every three days at a concentration of 10 ng/mL as a positive control. In total, there were seven groups: Neg (negative control), Neg/TGFβ3 (positive control), LV-T, LV-1, LV-2, LV-3 and LV-4. One day after gelation was defined as Day 0. At day 21 and day 42, the beads were collected for RNA analysis, CGC quatification as well as staining.

RNA Isolation and qPCR Analysis

RNA from monolayer was extracted using TRIZOL® Reagent (Invitrogen, Singapore) according to the procedure described in the manual. For RNA extraction from alginate

hydrogel constructs, the cells were first released by dissolving the alginate beads in 55 mM sodium citrate (Sigma, Singapore) for 5 min, and then RNA was obtained with TRIZOL® Reagent. Then, 500 ng of RNA from each group was converted to cDNA for the subsequent real-time PCR experiments using M-MLV reverse transcriptase (Promega, USA). Then qPCR was performed using iQTM qPCR system (Bio-Rad, USA) under the following conditions: 40 cycles in total, each cycle including 30 s for denaturation at 95°C, 30 s for annealing at 58°C and 30 s for extension at 72°C. The threshold cycle values of each sample were normalized against corresponding housekeeping gene RPL4. Then quantitative data were normalized against that of the negative control sample to yield the relative fold change. Triplicates were conducted for each sample. Sequences of all the primers for real-time PCR are as follows (5'-3'): Col I: CCTGCGTGTACCCCACTCA, ACCAGACATGCCTCTTGTCCTT; type II collagen (Col II): GCTATGGAGATGACAACCTGGCTC, ACAACGATGGCTGTCCCTCA; aggrecan: CGAGGAGCAGGAGTTTGTCAAC, ATCATCACCACGCAGTCCTCT; RPL4: CAAGAGTAACTACAACCTTC, GAACTCTACGATGAATCTTC.

Quantitative Analysis of hTGF β3 Expression by Specific ELISA

Conditioned medium collected from SMSCs-alginate constructs from the six groups (Neg/TGF-β3, LV-T, LV-1, LV-2, LV-3 and LV-4) was stored at −20°C for further analysis by specific ELISA kit (R&D Systems, Minneapolis, MN) following the manufacturer's instructions. Duplicates were performed for each time point. The concentration of TGF-β3 from each group was calculated using SkanIt Software 2.2.

Cell Viability Test

Cell viability was tested using WST-1 assay (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2 H-5-tetrazolio]-1,3-benzene disulfonate assay, Roche Diagnostics, Germany). Briefly, at day 42 of the culture period, 10 μL of WST solution was added into each well containing one sample bead in 100 μL of medium. After 2 h incubation, the absorbance at 450 nm of the medium was determined by a microplate reader (Multiskan Spectrum, Thermo). Each group was conducted in triplicate.

Quantitative Immunofluorescent Ataining of Col II Protein

At day 42, SMSCs-alginate constructs from each group were dissolved in sodium citrate. The suspension was homogeneously aliquoted into the wells of a 96-well plate and frozen. Then, the solid phase of the mixture was freeze-dried overnight. The product was fixed with glutaraldehyde for 1 h and blocked with 10% (v/v) goat serum for 30 min. After washing with PBS, the residual proteins on the surface of the wells were incubated with mouse anti-pig Col II primary antibody (2 ng/mL in PBS, Chemicon) at room temperature for 2 h. After three washes with PBS, the samples were further incubated with AlexaFluor 546 rabbit anti-mouse IgG (1:200, Invitrogen, Carlsbad, CA, USA) for 1.5 h. After three washes with PBS, Infinite 200 fluorescent microplate reader (TECAN Systems, San Jose, CA, USA) was used to determine the fluorescent density in each well, with the excitation wave length of 560 nm and emission wave length of 590 nm. Each sample was performed in triplicate.

Biochemical Analysis

After 42 days of culture, constructs were taken out, washed three times with deionized water to remove the salt in the medium followed by freeze drying (36 h), and digested with 1 mL of papain per sample for biochemical analysis (17,18). Dimethylmethylene blue dye was added into the digested solution, and absorption at 525 nm was measured using a UV-VIS spectrophotometer (Multiskan Spectrum, Thermo) to quantify sulfated glycosaminoglycan (GAG) content (19,20).

Histology and Immunohistochemistry

For 3D constructs, samples from each group at Day 42 were fixed in 4% (w/v) neutral buffered paraformaldehyde, embedded in paraffin and sectioned (5 μm thick). Then, sections from all groups were deparaffined and stained with Safranin-O for glycosaminoglycans (GAGs) and Masson's Trichrome for total collagen (Invitrogen, Carlsbad, CA). For immunohistochemistry, the specimens were fixed in glutaraldehyde (2.5%, 30 min) and blocked with 1% goat serum (w/v, in PBS) for 1 h. Afterwards, primary antibodies for Col I (2 ng/mL in PBS, Santa Cruz Biotechnology) and Col II (2 ng/mL in PBS, Chemicon) were applied for 2 h (4°C), respectively. Following three PBS washes, the sections for Col II or Col I were, respectively, incubated with HRP-conjugated secondary antibodies (1 μg/mL in PBS, Invitrogen, Carlsbad, CA) at room temperature for 1 h. The presence of Col II was observed using the DAB Substrate kit (Clontech, Mountain View, CA).

Statistical Analysis

The results are indicated as mean±SD. Where appropriate, ANOVA was used to analyze results, and difference was considered to be statistically significant at $P<0.05$.

RESULTS

Construction of Single-Functioning and Dual-Functioning Recombinant Lentiviral Vectors

The configurations of the recombinant vectors are given in Fig. 1b. Among them, LV-1 is characterized with two distant and reversely oriented cassettes, while LV-2 has two distant and same-oriented cassettes. On the other hand, LV-3 has two proximal and reversely oriented cassettes, while LV-4 has two proximal and same-oriented cassettes.

The bands which appeared after enzymatic digestion with MluI or EcoRI and gel electrophoresis are shown in Supplementary Material Fig. S1a, b. Following MluI digestion, pLVX and pLVX-T were expected to give rise to linearized bands at 8,204 bp and 9,450 bp, respectively, while 5,241 bp/4,514 bp, 5,591 bp/4,120 bp, 8,144 bp/1,656 bp and 8,481 bp/1,237 bp were expected after the digestion of pLVX-1, pLVX-2, pLVX-3 and pLVX-4, respectively. For EcoRI digestion, bands at approximately 8,204 bp, 9,000 bp/450 bp, 7,384 bp/1,882 bp/450 bp, 7,574 bp/1,692 bp/450 bp, 8,504 bp/862 bp/450 bp and 8,284 bp/1,060 bp/450 bp were anticipated to appear for pLVX, pLVX-T, pLVX-1, pLVX-2, pLVX-3 and pLVX-4, respectively. The resultant bands as appeared in Supplementary Material Fig. S1a, b were consistent with expectations. DNA sequencing further verified the successful recombination of the plasmids, and no single mutation was noted through sequencing.

Three-Dimensional Gene Expression of Col I, Col II and Aggrecan

Twenty-one and 42 days after 3-D culture of non-transduced and transduced pSMSCs in chondrogenic medium, gene expression profile of Col I, Col II and aggrecan was assessed with qPCR, as shown in Fig. 2. It was observed that Col I was elevated either in Neg/TGF-β3 or LV-T group at both day 21 and day 42. Col I expression was lower in LV-1 and LV-3 than that in Neg/TGF-β3 at day 21 and day 42. On the contrary, LV-2 transduction resulted in a comparable Col I level at day 21 with Neg/TGF-β3, while it decreased Col I expression significantly at day 42. In comparison, LV-4 elicited the expression of Col I to a higher extent at day 42 compared to Neg/TGF-β3.

We also performed quantitative analysis of Col II and aggrecan mRNA levels, since the two are the primary components of cartilage extracellular matrix and therefore can be trusted as markers for chondrogenesis. Groups of Neg/TGF-β3, LV-T, LV-1 and LV-4 presented much higher expression of Col II relative to that in Neg at both day 21 and 42. Results obtained for LV-2 and LV-3 showed some differences between day 21 and day 42. Col II expression was much higher in LV-2 at day 21 compared to Neg, while the level decreased significantly at day 42. Contrarily, LV-3 remarkably promoted Col II expression at day 42. The trends for aggrecan expression were similar to those for Col II, except that the level in LV-1 was very low at day 21.

Quantitative Analysis of TGF-β3 Expression by Specific ELISA

The curves of TGF-β3 expression at the protein level in LV-T, LV-1, LV-2, LV-3 and LV-4 groups were obtained throughout the culture period of 42 days, as shown in Fig. 3. In all five groups, there was a high expression level initially, reaching up to 120 ng/mL for LV-4 and 8 ng/mL for LV-T, followed by a drastic decline within the first 15 to 18 days. The expression levels then remained at a relatively stable platform afterwards, although there were minor fluctuations or rebounding particularly for LV-4. By comparing the expression levels among these five groups, LV-4 always had the highest expression of TGF-β3, whereas LV-T seemed to have the lowest expression level of all. The platform expression levels of LV-1, LV-2 and LV-3 were close to one another, around 2 ng/mL.

Cell Viability Test at Day 42 by WST Assay

Comparison of absorbance levels obtained from WST assay is demonstrated in Fig. 4a. All six groups (Neg/TGF-β3, LV-T, LV-1, LV-2, LV-3 and LV-4) displayed more or less higher absorbance values in relation with the value in Neg group, indicating higher cell viability in these groups. Among them, LV-4 group had the highest cell viability, significantly higher than either the Neg group or any other group.

Quantitative Analysis of Col II Protein Expression

The intensity following fluorescent staining of Col II was illustrated in Fig. 4b. In the vertical axis for percentage of induction, the level of blank was set as 0, while the fluorescent level in Neg was labeled as 100%, with all other groups being demonstrated as the ratio relative to that in Neg group. All of the six groups with either manually supplemented TGF-β3 or TGF-β3 expressed endogenously via the lentiviral vectors (Neg/TGF-β3, LV-T, LV-1, LV-2, LV-3 and LV-4) showed higher amount than the Neg group. Among them, LV-2 and LV-4 showed the highest Col II protein levels.

Quantitative Analysis of Glycosaminoglycan (GAG)

Figure 4c shows, that there was a basal expression of GAG even in Neg group. In comparison, all other groups showed higher production of GAG. Among them, cells transduced with LV-3 had the highest GAG levels at day 42.

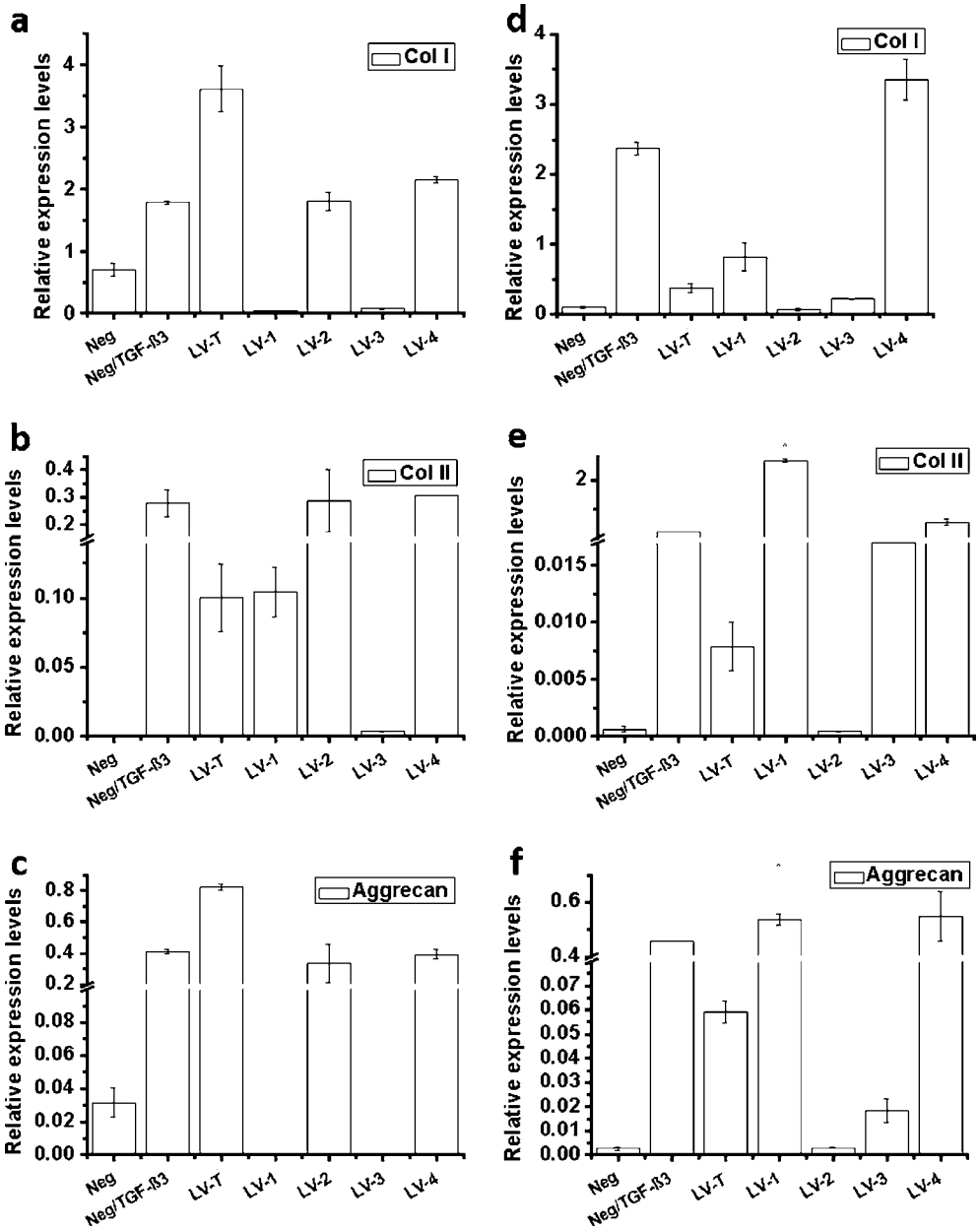

Fig. 2 Relative gene expression determined by qPCR at day 21 (**a**, **b**, **c**) and day 42 (**d**, **e**, **f**). (**a**, **d**) Col I; (**b**, **e**)Col II; (**c**, **f**) aggrecan.

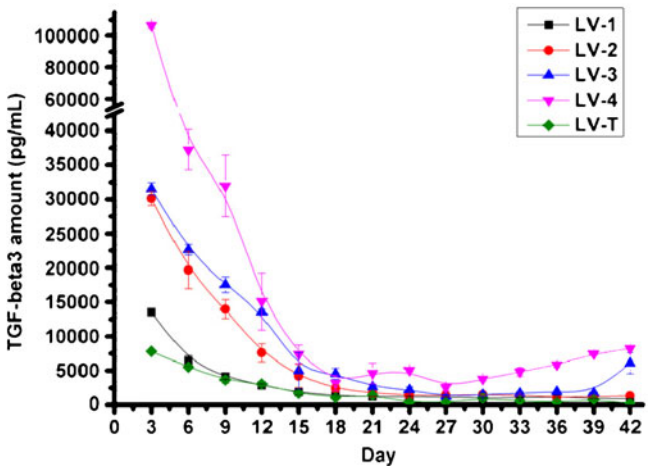

Fig. 3 Quantity of TGF-β3 determined by specific ELISA.

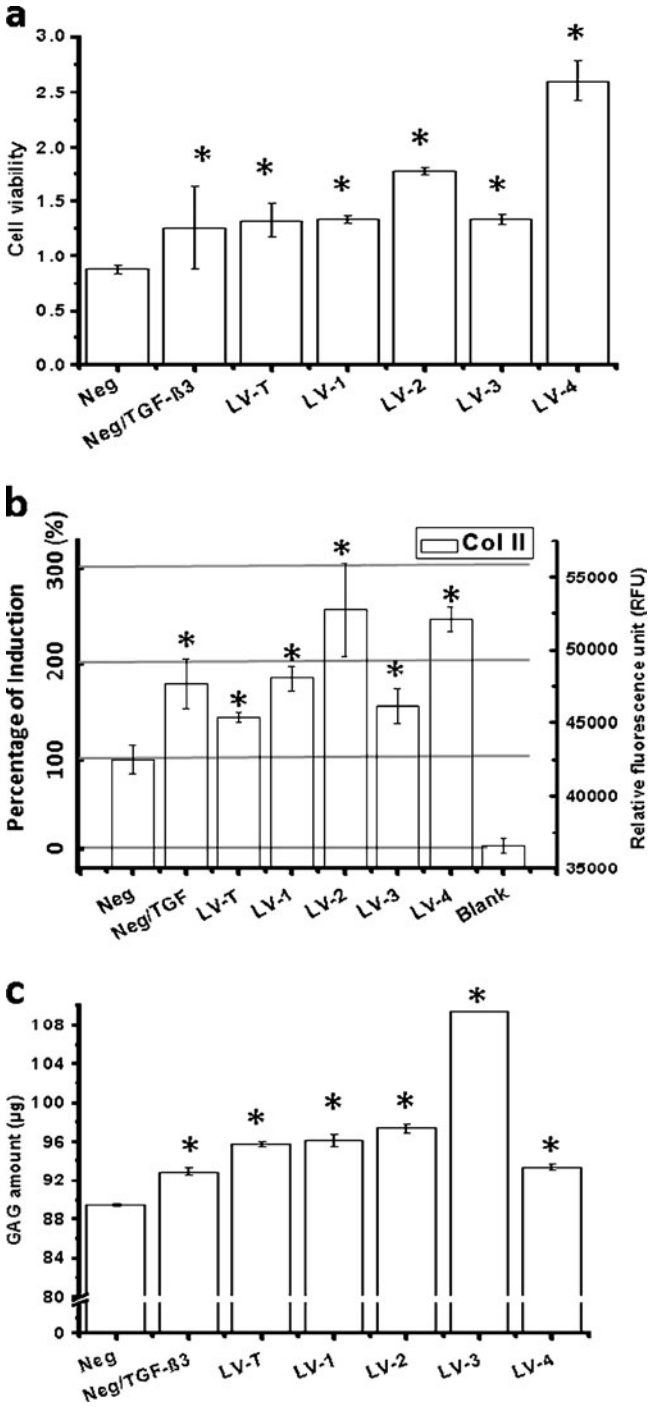

Fig. 4 Cell viability and quantity of ECM components at day 42. (**a**) Cell viability was determined by WST assay. Type II collagen (**b**) was determined by immunofluorescent staining. (**c**) The amount of GAG was measured by biochemical assay. $n = 3$; error bars represent SEM. $^*P < 0.05$.

Immunohistochemical Analysis

Safranin O staining was performed in order to visualize GAG in the cross-sections of the beads cultured for 42 days. GAG was stained in red surrounding the cells, as shown in Fig. 5a. In the Neg group, there was a minimal amount of GAG observed in between the cells, if not zero amount. With Neg group being the ground mark, the rest of the groups were above the ground in terms of GAG assessment, with LV-1 group showing the most obvious GAG staining. LV-3 also demonstrated a large production and homogeneous distribution of GAG within the cross-section.

Other means of staining techniques were applied as well. Masson's Trichrome staining was performed to visualize total collagen, including Col I, Col II and other types. In Fig. 5b, the LV-dual groups (LV-1, LV-2, LV-3 and LV-4) all showed aggregation of collagens around the cells. Amongst them, LV-2 and LV-4 had the most obvious visualization of collagens.

In immunohistochemistry staining for type I and Col II, Col I and Col II were stained as brown against the background. In Fig. 5c, Col II was visually apparent in LV-1, LV-2 and LV-4, while LV-3, LV-T and Neg/TGF-β3 also showed visualization of Col II to some extent. All these groups demonstrated higher expression of Col II in comparison with Neg.

In Fig. 5d, more Col I was observable in Neg and Neg/TGF-β3. Comparatively, Col I was rarely detected in LV-dual groups.

DISCUSSION

RNA polymerase binds to the promoter of an expressing cassette and initiates the elongation of the transcriptional process. In the theory of position effect, an enhancer of a promoter may function to affect the expression of an adjacent gene, leading to the variation of the expression levels (21). On the other hand, negative transcriptional interference occurs due to any of the following mechanisms: promoter competition (22), sitting duck mechanism (23), occlusion (24), collisions (25) and roadblock (26). When two expression cassettes are located in proximity, whether they are in convergence or tandem, transcriptional interference might occur. Even if the two cassettes are a distance away from each other, there might also be transcriptional interference, whereby one transcriptional activity would impact a second transcriptional activity.

In our design, we constructed four lentiviral vectors by altering the location and orientation of the shRNA-expressing cassette. Col I-suppressed chondrogenesis was examined to assess the overall effect of these various lentiviral vectors. Among these four LV-duals, TGF-β3 cassette and shRNA cassette are closely proximal in LV-3 and LV-4, while the two cassettes are far apart in LV-1 and LV-2. Therefore, transcriptional interference is expected to be lower in LV-1 and LV-2. Similarly, as the shRNA cassette is in the reverse orientation of TGF-β3 cassette in LV-1 and LV-3, the interference would be lower than that

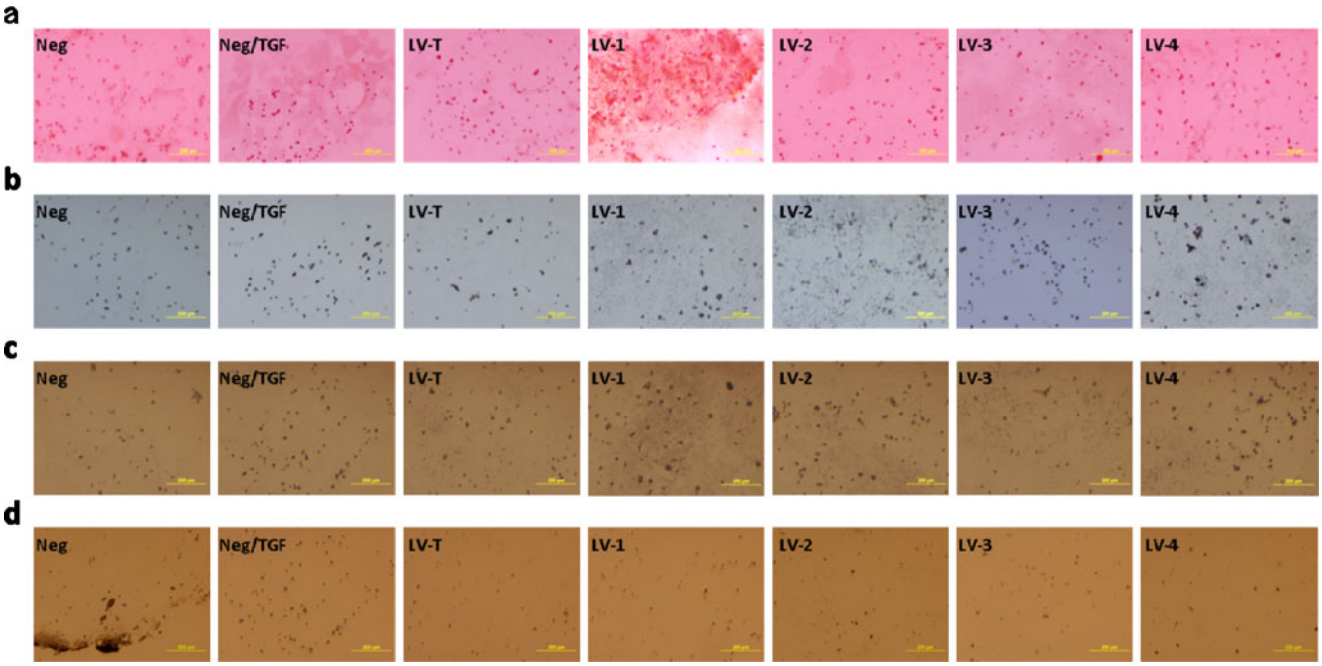

Fig. 5 Chemical and immunohistochemical staining on the cross-sections of the constructs cultured for 42 days. (**a**) Safrainin O staining for glycosaminoglycan; (**b**) Masson's Trichrome staining for total collagen; (**c**) Immunohistochemical staining for Col II; (**z**) Immunohistochemical staining for Col I.

in LV-2 and LV-4, where the two cassettes are in the same direction and therefore transcription activities on the two cassettes proceed on the same strand of the double-stranded DNA. By altering the relative positions and orientations of TGF-β3 and Col I-targeting shRNA cassettes, we could expect various degrees of transcriptional interference between the two cassettes, which would result in different efficiencies in Col I-suppressed chondrogenesis (27,28). We aim at finding the optimal construct that performs best in both Col I suppression and chondrogenic induction in engineered cartilage tissues.

For TGF-β3 secretion, according to ELISA results, LV-T had the lowest expression profile, while all LV-duals express higher amount of TGF-β3. This phenomenon suggests that the expression of TGF-β3 could be elevated by the introduction of shRNA cassette, and this elevation is more obvious when the two cassettes are located close to each other and when they are in the same direction, as the expression level in LV-4 is much higher than that in LV-3, and the expression in LV-3 is higher than that in LV-2 and LV-1. The results have indicated a positive effect of proximity and same direction on the transcription of TGF-β3 cassette. A possible hypothesis to explain this phenomenon might be the attraction or recruit of type II RNA polymerase, which binds to CMV promoter, by type III RNA polymerase, which binds to U6 promoter. An alternative hypothesis to explain this phenomenon might be the higher local concentration of nucleotides and other regulation factors at the site of the two cassettes attracted by type III RNA polymerase. A higher concentration of type II RNA polymerase, nucletotides and regulation factors by this effect would therefore result in higher expression of TGF-β3.

For the mRNA level of Col I, it was found that TGF-β3, whether manually supplemented or endogenously expressed through the recombinant lentiviral vectors, was able to elicit the transcription of Col I. This has complicated the situation where Col I, an undesired protein of cartilage extracellular matrix, is inherently produced in SMSCs, which adds on more justification to the necessity for Col I suppression. For LV-duals, some were found to be effective in reducing the Col I mRNA level. The expression levels of Col I in these groups may not be significantly lower than that in the Neg group. However, by comparing the expression levels of Col I in these groups with Neg/TGF-β3 or LV-T group, they did serve to down-regulate the expression of Col I and compromise the elevating effect imposed by TGF-β3. Of the four LV-duals, LV-4 had the least effect, or the expression level of Col I in LV-4 was even elevated at day 42 of 3-D culture compared to either Neg/TGF-β3 or LV-T group. LV-1 was more efficient in Col I suppression in the shorter term (day 21), whereas LV-2 had a high Col I inhibition efficiency in the longer term (day 42). LV-3 was supposed to be the most effective among all the vector configurations, by down-regulating Col I expression compared to either Neg/TGF-β3 or LV-T at both time points. Comparatively, LV-1 and LV-2 are suboptimal.

Higher cell viability was observed in all other groups than the Neg group. As TGF-β3 was either manually supplemented to the cells or endogenously expressed with the transduction of the lentiviral vectors in the cells in these groups, the higher cell viability can be attributed to the presence of TGF-β3, which is a growth factor that controls such cellular activities as cell proliferation, differentiation and apoptosis. The highest cell viability occurred in the cells transduced with LV-4, which is corresponding to the ELISA result where LV-4 group had the highest expression of TGF-β3 protein. This leads to the assumption that TGF-β3 promotes cell viability on a concentration-dependent basis. Higher amount of TGF-β3 within a certain limit may better promote cell viability and proliferation.

With endogenously expressed TGF-β3, chondrogenesis induced by these lentiviral vectors was explored. Based on qPCR results, for Col II expression, LV-1 and LV-4 had high levels at both day 21 and 42. In comparison, LV-2 promoted Col II expression in the shorter term, as opposed to LV-3, which resulted in a high Col II expression in the long run. Aggrecan expression had similar patterns, except that LV-1 only promoted aggrecan expression in the longer term.

Since qPCR only represents the real-time status of transient expression of the markers at the time of testing, we further assessed the overall Col II accumulation during the 42 days of culture by quantitative immunofluorescent staining, which takes account of the overall 42-day time period for accumulative protein levels. Therefore, despite some differences between the day 21 and day 42 qPCR results, e.g., Col II mRNA level in LV-2 was quite low at day 42, while it was much higher at day 21, all the groups had higher levels of Col II protein compared to Neg group in quantitative immunofluorescent staining. Immunohistochemical staining further substantiates the above observations, with LV-1 and LV-4 showing highest demonstration of Col II. Since Col II is a major component of the extracellular matrix of cartilage, the expression of Col II at both mRNA and protein levels corroborates the assumption that the cells are differentiating towards the chondrocyte phenotype.

GAG, as the brick for the organization of cartilage ECM, was also quantitated with biochemical assays. The amount of GAG with lentiviral transduction all showed higher levels than that in the Neg group. This was also observed in Safrainin O staining. These results provide the evidence that the lentiviral vectors were capable of inducing the cells into the chondrocyte lineage, by producing more constituents to construct cartilage ECM. By comparing the four LV-duals, LV-1 and LV-3 seemed to have a better expression profile of GAG and also a better distribution as observed by Safranin O staining on the cross-sections. It is noteworthy that in our hydrogel system, both the biochemical assay and immunofluorescent staining for Col II are adopted as qualitative and semi-quantitative references with lower resolution compared to qPCR assay. Therefore, hereby, we are making comparisons relative to the Neg group, with the result being that all groups showed higher Col II levels than Neg. The quantitative relations between the experimental groups and Neg, as well as among all the experimental groups, are analyzed based on qPCR results for examination at transcriptional level.

The result obtained from Masson's Trichrome staining was, to some degree, consistent with the result from immunohistochemical staining of Col II. This is primarily because Col II constitutes the principal collagen matrix in cartilage, about 90-95% of the macrofibrilar framework, while Col I, in spite of its existence, makes up only a small portion of total collagen, as observed in the Neg group in Col I immunohistochemical staining (29). Other collagen subtypes such as type IX and type X collagen are also in small amount (29). Although we are expecting an increase in Col II and decrease in Col I, the two effects would not be offset due to the imbalance between the quantities of the various collagen subtypes.

CONCLUSION

Comprehensively in consideration of all the above factors, LV-4 was able to express the highest level of TGF-β3, which led to the best induction of chondrogenesis and Col I expression simultaneously. However, as the increase of Col I level was unable to be compensated by the insertion of shRNA cassette, LV-4 goes out of our choice to meet our objective of Col I-suppressed chondrogenesis. LV-3 had the best Col I suppression efficiency, while it tended to induce chondrogenesis only in the longer term. LV-1 and LV-2 had suboptimal Col I down-regulation, whereas LV-1 had a better profile for chondrogenic induction. Therefore, LV-1 was determined as the optimal choice. However, more work has to be done in order to improve the inhibitive efficiency and coordinate between Col I suppression and chondrogenic induction.

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