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RESEARCH PAPER

Effective Gene Delivery to Mesenchymal Stem Cells Based on the Reverse Transfection and Three-Dimensional Cell Culture System

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ABSTRACT

Purpose To enhance the level and prolong the duration of gene expression for gene-engineered rat mesenchymal stem cells (MSCs) using non-viral vector.

Methods A novel transfection system based on reverse transfection method and three-dimensional (3D) scaffold was developed. The reverse gene transfection system was evaluated for transfection efficiency compared to conventional methods. Collagen sponge and polyethylene terephthalate non-woven fabric were introduced as scaffolds to perform 3D culture with reverse transfection. pDNA coding TGFβ-1 was delivered to MSCs to assess its ability in inducing chondrogenesis with the 3D non-viral reverse transfection system.

Results The reverse transfection method induced higher transgene levels than the conventional transfection in the presence of serum. The electric charge of the anionic gelatin plays an important role in this system by affecting the release pattern of the gene complexes and through the adsorption of serum protein to the substrate. During a long-time *in vitro* culture, MSCs cultured on 3D scaffolds exhibited a higher transgene expression level and more sustained transgene expression than those cultured and transfected on the two-dimensional substrate.

Conclusions The combination of reverse transfection system with 3D cell culture scaffold benefits the cell proliferation and long-time gene transfection of MSCs.

KEY WORDS gene transfection · mesenchymal stem cells · non-viral gene vector · reverse transfection · three dimensional

ABBREVIATIONS

BCA	bicinchonic acid
CDI	N, N'—carbonyldiimidazole
CPZ	chlorpromazine
DMEM	Dulbecco's modified Eagle's medium
MSCs	mesenchymal stem cells
PEI	poly (ethylene-imine)
PET	polyethylene terephthalate
TGFβ-1	transforming growth factor β-1

INTRODUCTION

Bone-marrow-derived mesenchymal stem cells (MSCs), with their self-renewal ability and multi-lineage differentiation potential, as well as ease of isolation, have generated a

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lot of interest for their potential use in regenerative medicine and tissue engineering (1). However, as MSCs are scarcely distributed in the bone marrow and are often not powerful therapeutically, they are always expanded and genetically modified *in vitro* before being introduced to the body (2). Therefore, the methods of transferring target genes to MSCs would greatly affect their therapeutic applications. Although viral vector is widely used for gene delivery, its immunogenicity and oncogenicity have given rise to a number of adverse effects in clinical trials (3). Non-viral vector, which has several advantages such as the ease of synthesis, cell/tissue targeting, low immune response, and unrestricted plasmid size, can be a good alternative for gene transfection (4). So far, a variety of non-viral delivery approaches have been developed, including calcium phosphate, cationized liposomes, noisomes and cationic polymers (5–11). In the present study, pullulan-spermine, a kind of cationic polymer prepared by the conjugation of pullulan and spermine, was used as the non-viral vector, as it can achieve efficient *in vitro* gene expression in various types of cells (2). Pullulan is a water-soluble polysaccharide with a repeated unit of maltotriose condensed through α-1,6 linkage and known to be a safe material for oral healthcare and pharmaceutical coating applications. It has been demonstrated that this polysaccharide-based carrier can be internalized by different cells through a sugar-recognition receptor on cell surface (2). However, there are several bottlenecks in the clinical use of the non-viral vectors, one of which is their instability in the presence of serum and consequential decrease of the transfection efficiency.

Reverse transfection, also named as substrate-mediated transfection, has been reported by our previous studies to be able to attenuate this negative effect caused by the presence of serum (2). The difference between conventional transfection and reverse transfection lies in the addition order of gene complexes and cells. In conventional transfection, cells are seeded on the culture plates for complete attachment prior to the addition of gene complexes. When the gene complexes are fixed to the substrate, 2D culture plates or 3D scaffolds, before the cells are seeded, such transfection is referred to as reverse transfection. In reverse transfection, gene complexes interacted with substrate through non-specific mechanisms, including hydrophobic, electrostatic, and van der Waals force or specific mechanisms, such as in the form of antigen-antibody or biotin-avidin binding (12,13). In this study, cationic pullulan-spermine/DNA complex was coated on the substrate basically through electronic interactions with anionic gelatin. This reverse gene transfection system also includes Pronectin, a commercial name of fibronectin which is an important extracellular protein known for its binding ability to $\alpha_5\beta_1$ integrins, which play a dominant role in the adhesion of most cultured cell lines.

Three-dimensional (3D) cell cultures have been widely used in biomedical research over the past several years (14–19). Signaling pathway activity, and ultimately the cellular response, can differ depending on whether the cells are present in a 2D or 3D environment (20,21). Compared with being cultured on 2D substrates, cells cultured in 3D scaffolds exhibit improved cell adhesion, show a similar morphology as *in vivo*, and adhere to the matrix through different sets of integrins (22). One mechanism by which 3D scaffolds could increase transfection efficiency may relate to the presence of a large surface area from which to deliver DNA to cells (23). By maintaining an available pool of DNA on a surface, without allowing for polyplex aggregation, transfection efficiency may not only be increased, but may also be sustained for longer periods of time (24). Two kinds of gene containing 3D scaffolds were evaluated and compared in this study: one was mesh-like PET non-woven fabric and the other was porous collagen sponge.

The aim of this study was to enhance the level and prolong the duration of gene expression for rat mesenchymal stem cells (MSCs) using non-viral vector through the construction of a novel transfection system, which was based on both reverse transfection method and 3D scaffolds (Fig. 1). For this purpose, we compared the gene expression level of MSCs and several tumor cell lines in the absence and presence of serum using conventional and reverse transfection method. Then, the gelatin with different negative charges was synthesized and used for reverse transfection to investigate whether the electric charge of anionic gelatin can influence the transfection efficiency. Furthermore, this reverse transfection was performed on PET non-woven fabric and collagen sponge, which may provide a better growth environment for MSCs to evaluate the potential application of this transfection system in tissue engineering. Finally, pDNA coding for transforming growth factor β-1 (TGFβ-1) was delivered to MSCs by 3D reverse transfection method to evaluate their capacity to induce chondrogenesis.

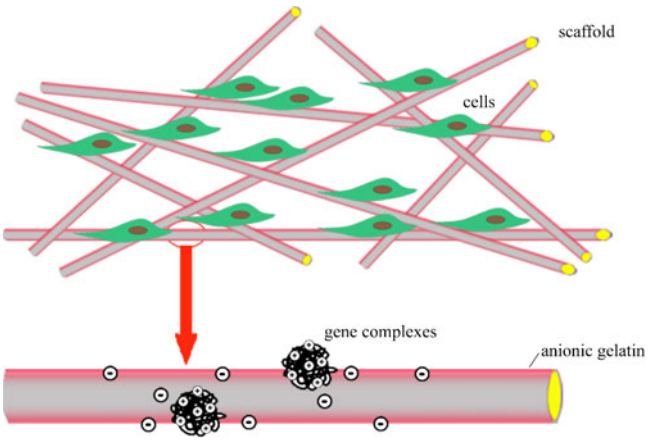

Fig. 1 Representative schematic of reverse transfection combined with three-dimensional system.

MATERIALS AND METHODS

Materials

Pullulan with an average molecular weight of 47,300 was purchased from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). Poly (ethylene-imine) (PEI) (MW= 25 kDa), Spermine, gelatin, L-glutamine, and chlorpromazine (CPZ) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Collagen sponges were purchased form Qisheng Co. (Qisheng, China). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin and streptomycin and trypsin were obtained from Gibco BRL (Gaithersberg, MD, U.S.A.). Plasmid DNA coding for luciferase was kindly provided by the Institute of Infectious Diseases, Zhejiang University. Plasmid DNA coding for TGFβ-1 gene was purchased from Origene Co. (Origene, China). The luciferase assay and BCA Protein Assay Kit were purchased from Beyontime Co. (Beyontime, China). All other chemicals were of analytical grade. HeLa cells (human cervical adenocarcinoma cell line), HepG2 cells (human hepatocellular liver carcinoma cell line), and A549 cells (human lung carcinoma cells) were obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

Animals

Three-week-old SD (Sprague–Dawley) male rats (50~60 g) were supplied by Zhejiang University Experimental Animal Center, China. All animals were maintained under constant conditions (temperature 25±1°C) and had free access to a standard diet and drinking water. All of the experimental procedures were in accordance with the Zhejiang University guidelines for the welfare of experimental animals.

Complex Formation and Evaluation

Pullulan-spermine were prepared using an N,N'—carbonyldiimidazole (CDI) activation method as previously reported (25). The molar extent of spermine introduced to the hydroxyl groups of pullulan was 12.3 mole%. Pullulan-spermine/DNA complexes were prepared by mixing the aqueous solution of pullulan-spermine with that of plasmid DNA coding for luciferase gene. Briefly, pullulan-spermine and pDNA dissolved with phosphate-buffered saline solution (PBS, pH 7.4) were mixed together and incubated for 20 min at room temperature and then diluted with PBS with the ultimate concentrations of pDNA of 25 μg/ml. PEI/DNA complexes were prepared at N/P ratio of 10 with the similar approach. The particle size and zeta potential of pullulan-spermine/DNA complexes with the ultimate concentrations of pDNA of 25 μg/ml were determined by laser diffraction spectrometry (Malvern Zetasizer 3000HS, Malvern, UK).

Preparation of MSCs and MSCs Cell Culture

HeLa cells, HepG2 and A549 cells were cultured in DMEM medium containing 10% fetal calf serum (FCS), penicillin (50 U/ml) and streptomycin (50 U/ml) at 37°C in 5% CO_2. Mesenchymal stem cells (MSCs) were isolated from the bone shaft of femurs of three-week-old male SD rats. Briefly, both ends of rat femurs were cut away from the epiphysis, and the bone marrow was flushed out using a syringe (21 gauge needle) with 1 ml of DMEM supplemented with 10% FCS, L-glutamine, penicillin (50 U/ml), and streptomycin (50 U/ml). The cell suspension was placed into two 25 cm^2 flasks and cultured at 37°C in 5% CO_2. The medium was changed on day 4 of culture and every 3 days thereafter. When the cells of the first passage became sub-confluent, usually 7 to 10 days after seeding, the cells were detached from the flask using treatment for 5 min at 37°C with PBS solution containing 0.25 wt% trypsin and 0.02 wt% ethylenediaminetetraacetic acid. Second-passage cells at sub-confluence were used for all experiments.

Conventional Transfection

Two-dimensional transfection was carried out on 24-well culture plates. For conventional transfection, cells were seeded on 24-well culture plates and incubated at 37°C in 5% CO_2 for 24 h. Afterwards, the medium was replaced with DMEM (with 10% FCS or not) containing 40 μl of pullulan-spermine/DNA complexes. After 6 h incubation, the complex was totally removed, and the cells were incubated in DMEM supplemented with 10% FCS, L-glutamine, penicillin (50 U/ml), and streptomycin (50 U/ml). The luciferase assay was carried out according to the manufacturer's instructions. The relative luminescence of each sample was measured by a luminometer (Promega, USA) after adding luciferase assay reagent. Meanwhile, the total protein of each sample was determined by bicinchonic acid (BCA) reagent kit according to the manufacturer's instructions. All the experiments were carried out in triplicate to ascertain reproducibility. Results were expressed as relative light units per mg of cell protein as determined by BCA protein assay.

Reverse Transfection System

Synthesis of Anionic Gelatin

Anionic gelatin was synthesized through conjugation of succinic anhydride to gelatin. Various amounts of succinic

anhydride (37.5 mg, 75 mg and 90.1 mg) were added to 20 ml of 100 mg/ml gelatin solution in dimethyl sulfoxide, followed by agitation at room temperature for 18 h to allow the carboxyl groups to be introduced into the amino groups of gelatin for anionization (2).

Characteristics of Anionic Gelatin

Zeta potentials of 1 mg/ml anionic gelatin (solubilized in 0.01 M PBS) were determined by laser diffraction spectrometry (Malvern Zetasizer 3000HS, Malvern, UK) at 25°C. Substitution degree of amino groups of gelatin was analyzed by the Sörensen formaldehyde titration. Briefly, 0.75 g of anionic gelatin was dissolved in 45 ml of distilled water at 37°C water bath, and then pH was adjusted with sodium hydroxide to reach pH = 9.0. Then, 7.5 ml of 10% formaldehyde was added and allowed to react with anionic gelatin for 20 min, followed by titration with sodium hydroxide to be pH at 9.0. Tg and Ta are the titration volume for gelatin and anionic gelatin, respectively. The substitution degree of amino groups of gelatin is α. $\alpha = (\mathrm{Tg} - \mathrm{Ta})/\mathrm{Tg}$.

In Vitro Gene Release

The release of DNA from the substrates was quantified with fluorescence spectrophotometer (JASCO FP-6500) through binding with Hoechst 33258. Briefly, the surface of each well of the 24-well plate was coated first with 80 μl aqueous solution of 100 μg/ml anionic gelatin with different substitution degree of amino groups, respectively. After 1 h incubation, the well was washed with PBS twice and coated with 40 μl pullulan-spermine/DNA complexes for 0.5 h. Then the well was washed with PBS twice and incubated in 0.5 ml of PBS at 37°C. The supernatant was harvested at various time point (1 h, 15 h, 39 h, 63 h, 97 h), and the fresh medium (PBS with 10% FCS) was added to the plate. At the final time point, the counts remaining on the substrate were also determined. The supernatant was diluted with PBS and mixed with Hoechst 33258 solution. The concentration of DNA was calculated using a calibration curve. The percentage of DNA released was determined as the ratio of the cumulative counts released through a given time divided by the total counts initially delivered to the system (26).

Protein Adsorption

The adsorption of serum protein on the substrate in reverse transfection was determined by BCA assay. Briefly, each well of the 24-well plate was coated first with 80 μl aqueous solution of 100 μg/ml anionic gelatin with different substitution degree of amino groups, respectively, at 37°C for 1 h. In the control group, PBS is used instead of anionic gelatin. After 1 h incubation, the well was washed with PBS twice and coated with 40 μl pullulan-spermine/DNA complexes for 0.5 h. Then the well was washed with PBS twice and incubated in 0.5 ml of DMEM with 10% FCS. After 6 h incubation, the supernatant was harvested. The supernatant was diluted with PBS, and the amount of protein was measured with BCA assay. The adsorbed counts of the serum protein on the substrate were calculated as the initial serum protein content in 10% FCS minus the amount of protein contained in the supernatant. Substrate with no gelatin coating but only gene complexes was set as the control group. The reduced amounts of protein adsorbed on the substrate were determined by comparing the group coated with gelatin with the control group.

Reverse Transfection

For reverse transfection, each well of the 24-well plate was coated first with 80 μl aqueous solutions of 100 μg/ml anionic gelatin and 200 μg/ml Pronectin at 37°C for 1 h. After 1 h incubation, the well was washed with PBS twice and coated with 40 μl pullulan-spermine/DNA complexes for 0.5 h. Then the well was washed with PBS twice and seeded with cells in the medium (with 10% FCS or not). If there was no addition of serum, the medium was replaced with the complete medium containing 10% FCS after incubation with the complex for 6 h.

Treatment with Endocytosis Inhibitors

To evaluate of the effect of endocytosis on the transfection efficiency, cells were pretreated with 5 μg/ml of chlorpromazine (CPZ), a well-known endocytosis inhibitor, for 1 h at 37°C in the culture medium to inhibit the clathrin-mediated endocytosis pathway prior to addition of polyplexes to the cells (27). The cells were incubated at 37°C with the polyplexes for 3 h in the presence of the inhibitors to inhibit the clathrin-mediated endocytosis pathway. Subsequently, the medium was refreshed with DMEM supplemented with 10 % FCS, L-glutamine, penicillin (50U/ml), and streptomycin (50U/ml). The luciferase assay was performed as described above.

Reverse Three-Dimensional Transfection

The collagen sponge and PET non-woven fabric was used as 3D scaffold and cut into discs with diameter of 5 mm and thickness of 3 mm. 3D scaffolds were pre-sterilized with 75% (*v*/*v*) ethanol followed by repeated washing with PBS to remove any residual alcohol. Then, a similar coating procedure with the anionic gelatin, Pronectin and pullulan-spermine/pDNA complex (pDNA coding for

luciferase or TGFβ-1 gene) was performed for the scaffolds. After the coating of the complex, 100 μl of MSCs (2×10^6 cells/ml) were seeded to the scaffold and incubated for 2 h for cell attachment. Then, 2 mL of medium was added slowly to the plate wells, and the seeded scaffolds were transferred to fresh 24-well culture plates containing medium in the next day. The luciferase assay was performed as described above.

Scanning Electron Microscopy Examination

Blank scaffolds and scaffolds with cells were washed with PBS solution and incubated in 10% formalin at 4°C overnight. Samples were then rinsed in PBS and immersed in 1% OsO4 solution for 1 h, then dehydrated in 50%, 70%, 95%, and 100% ethanol for 20 min for each respective ethanol change. Then samples were mounted on specimen holders and dried from CO_2. The samples were finally sputter coated with gold for SEM observation (Hitachi X-650, Tokyo, Japan).

Histology Staining

After 4 weeks co-culture of cells with pullulan-spermine/pDNA encoding for TGFβ-1 gene (as 3D reverse transfection done), cell-seeded scaffolds allocated for histology were fixed in 4% phosphate-buffered (pH 7.4) formalin for at least 12 h, dehydrated with graded ethanol solutions and xylene, embedded in paraffin, and cross-sectioned to a thickness of 20 μm. The sections were mounted on glass slides and stained with hematoxylin and eosin (H&E, to visualize cellular architecture), Alcian blue (glycosaminoglycan, GAG) and Safranin-O (sulfated GAGs) using standard histological techniques. Images were acquired with a light microscope (28–30).

Statistical Analysis

All data were expressed as mean ± standard error (SD). The significance was calculated by Student's *t*-test as indicated by the P value. P values less than 0.05 ($P<0.05$) were considered statistically significant.

RESULTS AND DISCUSSION

Characteristic of Gene Complexes

It was observed that the particle size of pullulan-spermine/DNA complexes increased from 236 nm to 695 nm, while zeta potential of the nanoparticles dropped to be negative with the addition of FCS (Fig. 2a). These alterations could be a function of the adsorption of negatively charged serum proteins to the surface of the nanoparticles, as suggested by many previous studies (31,32). After coating on the substrate, the particle sizes of the nanoparticles were observed by SEM (Fig. 2b), most of which were between 70 nm and 150 nm. It was hypothesized that the physical properties of gene complexes may be changed due to the existence of anionic gelatin. However, the zeta potential of nanoparticles on the reverse transfection system cannot be determined because of the limitation of the current technology.

Influence of Serum on Gene Expression

To investigate the difference of transfection efficiency by conventional transfection method and reverse transfection method, HepG2, HeLa, A549 cells, as well as MSCs were used as model cells. It was observed that the reverse transfection method induced no decrease and even increase

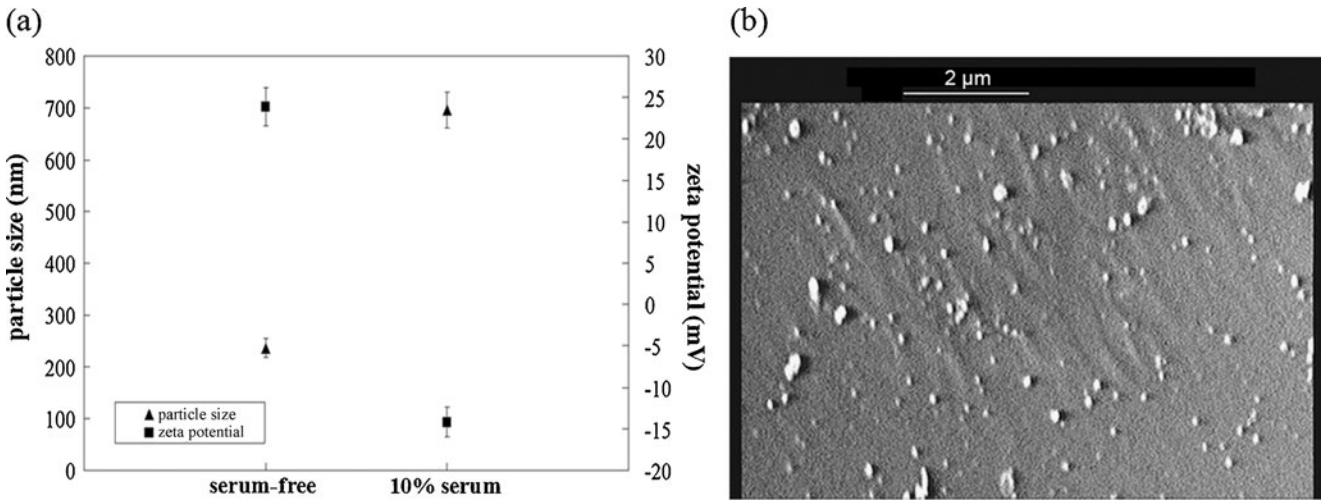

Fig. 2 (**a**) Particle size and zeta potential of pullulan-spermine/DNA complexes in the absence and in the presence of 10% serum; (**b**) the SEM image of pullulan-spermine/DNA complexes on the PET fabric.

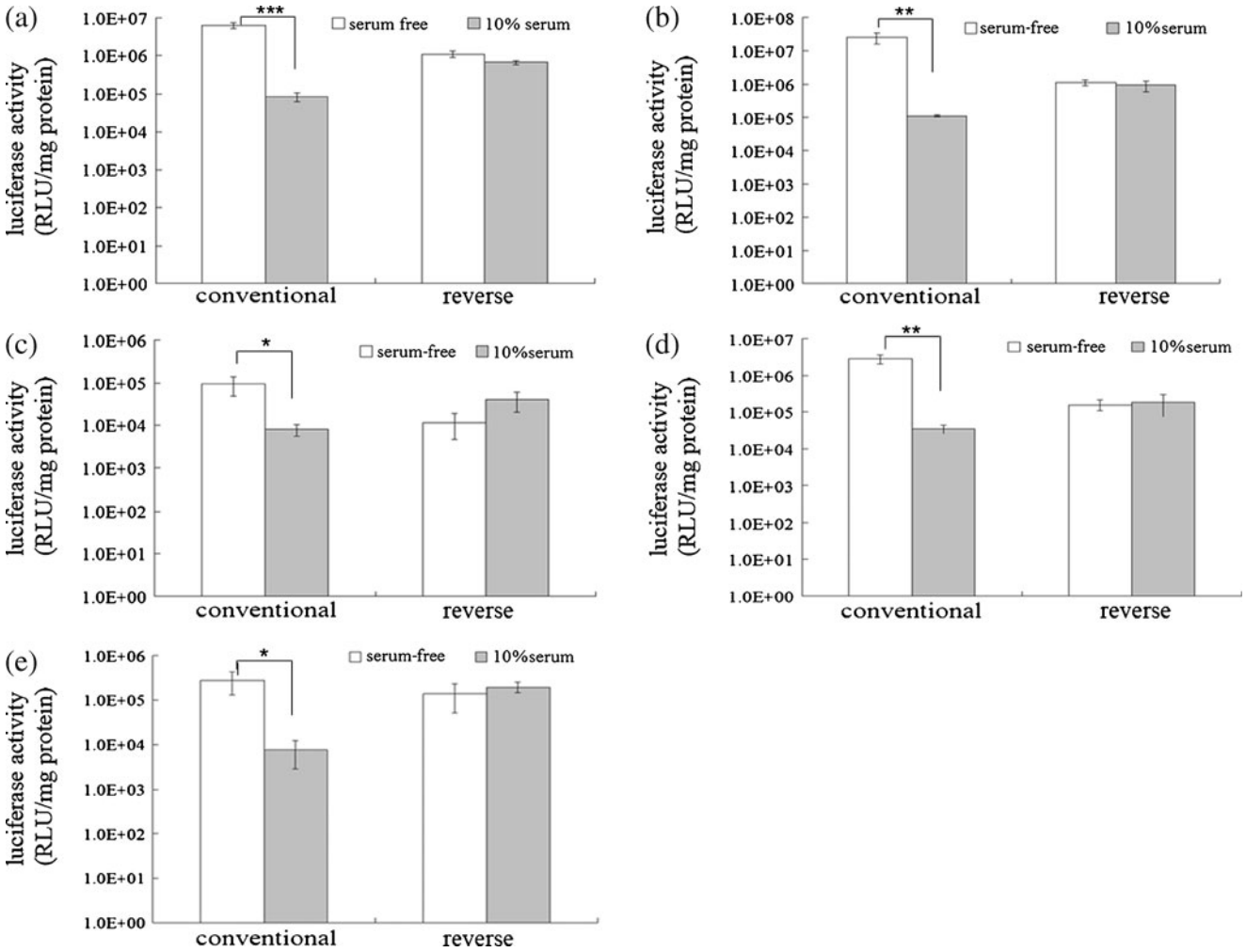

Fig. 3 The influence of the serum with either conventional method or reverse method with pullulan-spermine as the non-viral vector on the gene expression of HeLa (**a**), HepG2 (**b**), A549 (**c**) and MSCs (**d**), or PEI as the gene vector on the gene expression of MSCs (**e**) ($n = 3$, *:$p < 0.05$; **:$p < 0.01$;***:$p < 0.001$).

of the transgene levels in all four cell lines examined with the addition of serum (Fig. 3), while there is a remarkable decrease of gene expression with the addition of serum by conventional method. The improvement of gene transfection efficiency in the presence of serum with reverse transfection method has benefit for the gene transfection of MSCs. As serum is critical for the *in vitro* culture of MSCs, the starvation of MSCs during transfection process may exert an adverse effect on the growth of undifferentiated MSC (33), which will in turn decrease the transfection efficiency. On the other hand, it was reported that non-viral gene complexes enter the nucleus preferentially upon the disassembly of the nuclear envelope during mitotic cell division, so the presence of serum during transfection may facilitate the delivery of plasmid DNA to the nucleus by promoting the proliferation of MSCs (34).

As shown above, the addition of serum can change the particle size and surface charges of the complexes greatly. Here, we hypothesized that the anionic gelatin could act as a shelter for the nanoparticles to avoid adsorption of proteins in the serum; thus, the transfection process can be less affected with the addition of serum. Besides, another possible mechanism is that the gene complexes may be internalized into the cells through different pathways for different transfection methods. Moreover, when the gene vector was changed to be PEI, the gold standard of the polymeric gene transfection agent, the same phenomenon was observed that reverse transfection method could efficiently counteract the serum-incompatibility of PEI.

Characteristics of Reverse Transfection System

Here, we constructed a reverse transfection system using both the anionic gelatin and Pronectin, two important components in this system. Pronectin was introduced here to further improve the affinity of the cells with the substrate

where the gene complexes were located, thus indirectly increasing the interactive opportunities between the cells and the gene complexes. The anionic gelatin has at least two main functions here: one is to increase the adsorption of gene complexes to the substrate through electronic interactions, and the other is to improve the attachment of the cells.

As we hypothesized that the physiochemical properties of anionic gelatin could affect the transfection efficiency of the developed reverse system, here we characterized the reverse system and evaluated the transfection efficiency of different reverse system. First, we evaluated the effects of succinic anhydride/gelatin mass ratio of anionic gelatin on its physiochemical properties. For this purpose, we synthesized a series of anionic gelatin with various succinic anhydride/gelatin mass ratios. It was found that with the increase of the SA/gel ratio in the synthesis process, the substitution degree of anionic gelatin is higher, and its zeta potential became more negative (Table 1). Then, we investigated the *in vitro* release of the pullulan-spermine/pDNA complex from the substrate coated with anionic gelatin of different SA/gel ratio. It was found that both the initial 15 h release and the cumulative release within 97 h are higher for those substrates modified with the more negative-charged gelatin (Fig. 4). On the other hand, the release rate of the gene complexes after the initial 15 h was almost the same for all kinds of gelatin. At 97 h, all the complexes remained on substrate were measured, so the cumulative release at 97 h implies that the more negative-charged gelatin could immobilize more cationic gene complexes to the substrate; thus, more gene can be released to the supernatant. As the pullulan-spermine/DNA complex was formed by the electronic force between them, we speculated that the presence of the more negatively charged gelatin could facilitate the release of negative charged DNA from the gene complex. Further studies would be carried out to demonstrate this assumption. The release of DNA from the substrate is necessary for the uptake of the cells; therefore, in this case, the more negative-charged gelatin favors the gene transfection. To further explore the underlying mechanisms, we investigated the effect of different-charged gelatin on preventing the serum protein from adsorbing to the substrate. As shown from Fig. 5, all

Table 1 Substitution Degree of Amino Groups of Gelatin and Zeta Potential of the Gelatin and Anionic Gelatin with Different Succinic Anhydride/Gelatin Ratio (SA/gel)

	1	2	3	4
SA/gel ratio(mg/g)	0	1.25	2.5	4.5
substitution degree(%)	0	54	71	75
zeta potential (mV)	−4	−10	−15	−23

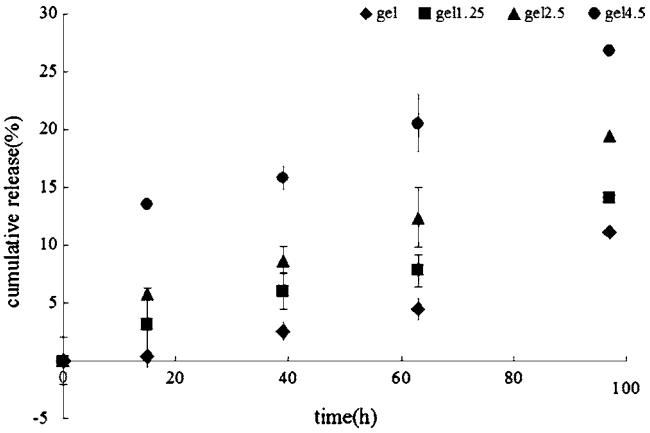

Fig. 4 Cumulative release of the DNA from the reverse transfection system at various time points for different kinds of gelatin.

of the substrate modified with gelatin repelled more serum protein than unmodified substrate, so it was demonstrated that the anionic gelatin can function as a shelter for the nanoparticles to avoid adsorption of proteins in the serum. Moreover, most negative-charged gelatin (gel 4.5) can repel more serum protein than any other kinds of gelatin.

Furthermore, the transfection efficiency of reverse transfection system with different properties was evaluated. When using different-charged gelatin for reverse transfection in the presence of serum, the more negative-charged gelatin showed higher gene expression level (Fig. 6). As mentioned above, this could be explained by the fact that the more negative-charged gelatin can immobilize more gene complexes to the substrate and repel more serum protein than the less negative-charged gelatin. Moreover, it was found that adding the pullulan-spermine/pDNA complex to the substrate either together with or before the anionic gelatin cannot induce as much transgene expression as the adding order used in this study (data not

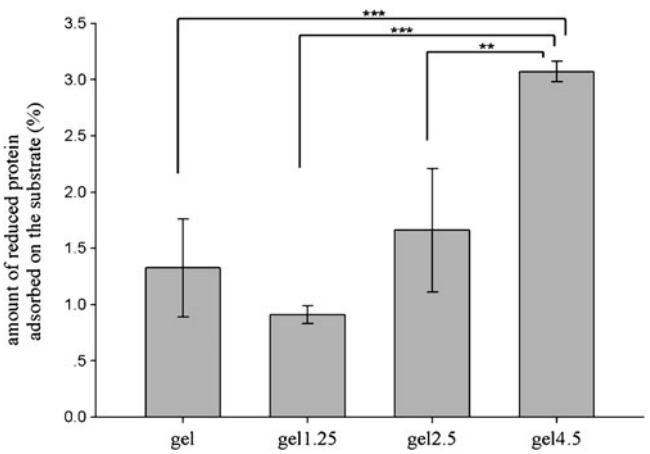

Fig. 5 The reduced amounts of protein adsorbed on the substrate which have been coated with different kinds of gelatin compared with the control group ($n = 3$, **:$p < 0.01$;***:$p < 0.001$).

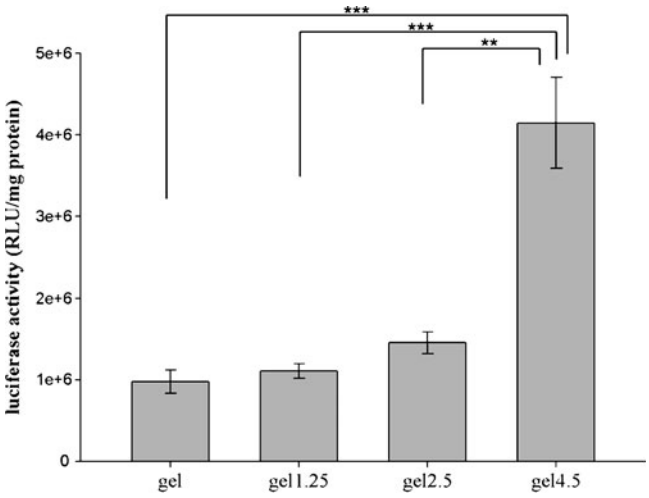

Fig. 6 Gene expression level of reverse transfection system with different kinds of gelatin ($n = 3$, **:$p < 0.01$;***:$p < 0.001$).

shown), where the pullulan-spermine/pDNA complex was added after the adding of anionic gelatin. It was assumed that when the pullulan-spermine/pDNA complex was added after the adding of anionic gelatin, more gene complexes could be exposed to the cells, which were added after the adding of gene complex, so the highest transfection efficiency could be achieved.

Influence of Uptake Inhibitors on the Transfection Efficiency

Since an understanding of cellular uptake pathway and mechanism of the gene complex during transfection procedure could help to facilitate the design of the vector and transfection parameters, we studied the endocytosis of MSCs and HeLa cells by the conventional transfection and the reverse transfection method (35–37). There are two different kinds of endocytosis, which are clathrin-dependent and clathrin-independent pathways (38). The clathrin-dependent pathway is one of the best-characterized uptake mechanisms, which is initiated by the formation of clathrin-coated pits yielding clathrin-coated vesicles and carries the polyplexes into early and late endosomes that finally fuse with lysosomes (39). The clathrin-independent endocytosis pathways include caveolin-mediated endocytosis, caveolin-independent endocytic pathways (also classified as raft-dependent endocytosis), macropinocytosis, and phagocytosis (37). Treatment of cells with the clathrin-dependent

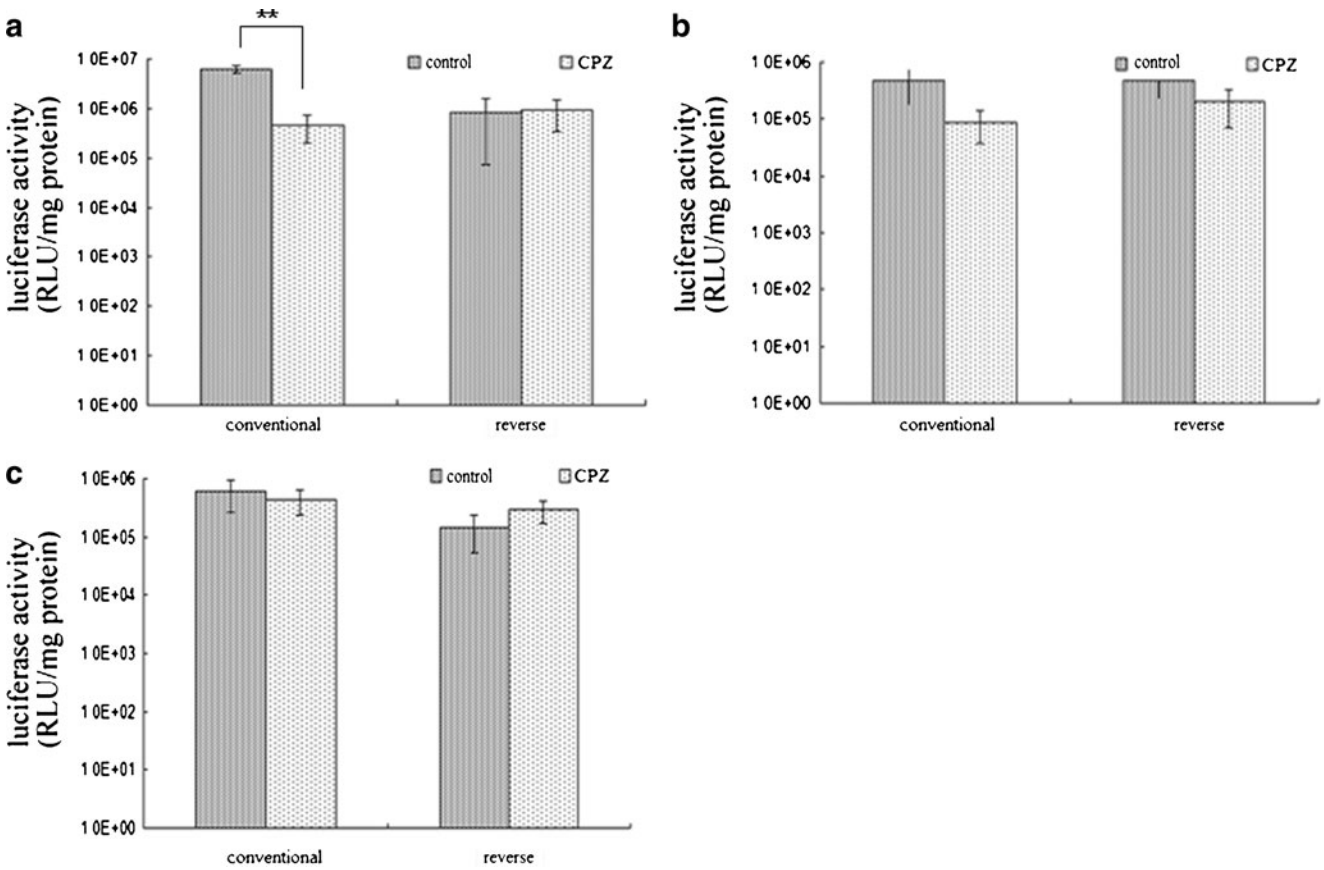

Fig. 7 Transgene expression of HeLa cells (**a**) and MSCs (**b**, **c**) treated with inhibitor (5 μg/ml chlorpromazine(CPZ)) or without inhibitor using the conventional method or reverse method with pullulan-spermine/DNA (**a**, **b**) or PEI (**c**) as the vector in the absence of serum ($n = 3$, **:$p < 0.01$).

pathway inhibitor chlorpromazine (CPZ) may provide a hint of the changes of the cellular uptake with different transfection methods. Chlorpromazine interferes with the endocytosis processes of the cell, so the cytotoxicity of this inhibitor on the cells was evaluated with MTT assay, and a safe concentration window was determined to make sure the alteration of gene expression level is not caused by the decrease of the cell numbers. When MSCs were treated with CPZ during the transfection, changes of the gene expression were vector-dependent. For pullulan-spermine, transfection efficiency decreased both for the conventional method and the reverse method (Fig. 7b). How the reverse transfection influences the gene transfection efficiency may not be a function of changing the clathrin-mediated pathway for pullulan-spermine nanoparticles. On the other hand, when PEI was used to delivery genes, there was only

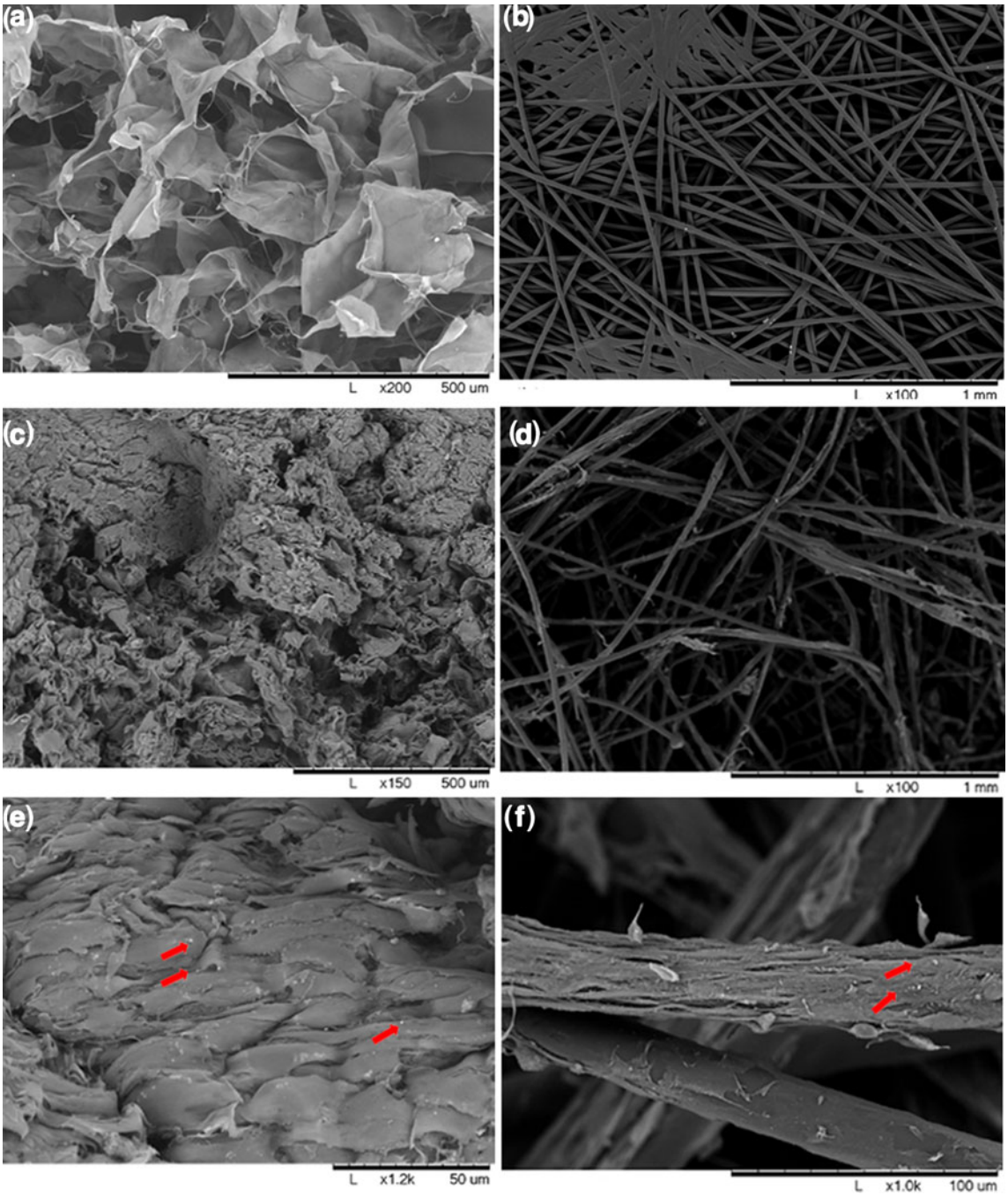

Fig. 8 SEM photograghs of 3D scaffolds: surface morphology of the blank collagen sponges (**a**) and the blank PET fabric (**b**); MSCs grown on the collagen sponge (**c**, **e**) and PET fabric (**d**, **f**) (*arrows* remaining gene complexes).

a slight decrease of gene expression with conventional method and two-fold increase with reverse method (Fig. 7c). Hence, it can be proposed that change of the transfection method might alter the numbers of the PEI/pDNA nanoparticles that could enter the MSCs through the clathrin-mediated endocytosis. As nanoparticles can enter the HeLa cells through both clathrin-mediated endocytosis and caveolae-mediated uptake, HeLa cells were always used to investigate the uptake mechanism of specific nanoparticles (39, 40). For HeLa cells with pullulan-spermine as the vector, there is almost no change of the gene expression with the addition of CPZ by reverse transfection (Fig. 7a), whereas as much as 10-fold decrease of the transgene level was observed with conventional transfection method. Therefore, for HeLa cells, the internalization pathway is different using different transfection method. In conclusion, different transfection methods might change the cellular uptake pathway of gene complexes in a cell-type-and vector-dependent way.

3D Transfection

As the efficiency of non-viral gene delivery is related to the proliferation of the cells, which could improve the gene transfection, a better culture environment is required. To create a better culture environment, the surface area of culture substrate should be increased, and the biomaterials are preferred to be biocompatible. Until now, several 3D scaffolds have been designed to demonstrate their feasibility in proliferation enhancement and gene transfection improvement, and both collagen sponges and PET non-woven fabric are widely used due to their excellent biocompatibility (41–45). In the present study, based on the reverse transfection system, non-woven PET fabric and the collagen were used, respectively, to construct a 3D scaffold. Non-woven PET fabric is a nondegradable polymer scaffold with a fibrous structure, while the porous collagen scaffold is biodegradable but has a poorer mechanical property when compared with PET fabric.

To compare them with 2D transfection system based on the reverse transfection method, the 3D transfection systems were constructed using these two materials. As seen from the SEM images (Fig. 8), both scaffolds used in this study possess a large surface area, and after 9 days co-culture of MSCs with the gene complexes, cells grew well, and some complexes still remained in this system. It was shown that the transfection efficiency of both 2D and 3D systems reached the peak at around the fifth day when incubated with the gene complex (Fig. 9). At the fifth and ninth days, gene expression on the 2D substrate was higher than on the collagen sponge. However, there was a remarkable change of gene expression level on the fourteenth day. Gene expression level on 2D substrate dropped rapidly, but cells cultured on the collagen sponge still kept a sustained gene expression on the fourteenth day and twenty-first day. As for the PET fabric, cells kept a high and sustained gene expression at all the time point within three weeks. Therefore, PET fabric might be superior to collagen sponge as a scaffold for *in vitro* 3D reverse transfection.

Although it was observed that there were significant differences among 2D and 3D culture system only at the fourteenth day, the average gene expression level of 3D culture was higher than that of 2D system except for on the ninth day. As shown in Fig. 9, there was remarkable decrease in the gene expression level for 2D system since the fourteenth day, while 3D system allows a sustained and high expression in all the 21 days. On the tenth day, the MSC cultured on 2D system became fully confluent, and a lot of cells no longer adhered to the plate, but were suspended in the medium. Therefore, the remarkable decrease of gene expression for 2D system since day 14 may be caused by the bad cell status. Thus, the 3D system will be of great help when MSCs need to express an exogenous gene for a long period of time.

The matrix mechanics and fluid transport can influence gene transfer in many aspects. Signaling pathway activity, and ultimately the cellular response, can differ depending on whether the cells are present in a 3D or 2D environment and how stiff the scaffold is (46–48). Therefore, it was presumed that the difference of the mechanical properties of the 2D substrate, collagen sponge and PET fabric scaffold may account for the distinct gene expression of cells cultured on them. The contraction of collagen sponge with cells grown on it can lead to the collapse of the pore and thus hinder the nutrition and waste transport. Therefore, a new biodegradable scaffold with

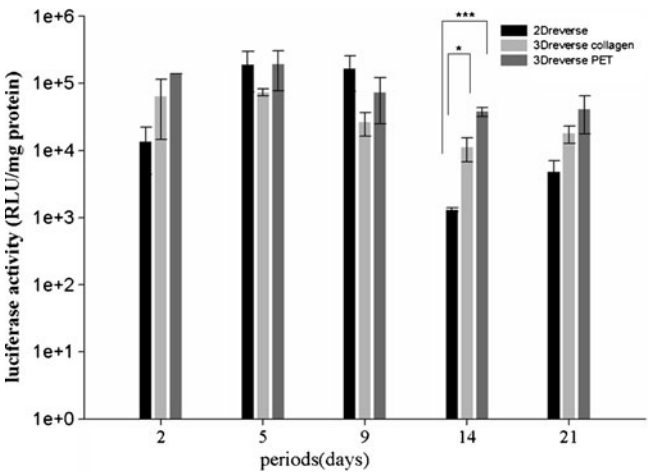

Fig. 9 Comparison of gene expression of MSC on 2D culture plates and 3D scaffolds with reverse transfection method for 21 days ($n = 3$, *:$p < 0.05$;***:$p < 0.001$).

improved mechanical property should be designed in the future for *in vivo* use.

In Vitro Differentiation of MSCs

MSCs are multipotent cells that can be induced to differentiate along a variety of tissue-specific pathways, including bone cells (osteoblasts), cartilage cells (chondrocytes), and fat cells (adipocytes) (49). For chondrogenic induction of adult MSCs, cells were collected into high-density pellets or in appropriate 3D biomaterials and treated with specific growth factors. Growth factors most commonly used for chondrogenic differentiation of MSCs belong to the TGF-β superfamily (50,51). Under these conditions, MSCs synthesize a cartilage-specific extracellular matrix (ECM) rich in glycosaminoglycan (GAG) and type II collagen and express cartilage markers (51).

To evaluate the efficiency of gene delivery based on the constructed reverse and 3D systems and the possibility of gene-engineered MSCs for the clinical therapy of cartilage defect, pDNA coding for TGFβ-1 was delivered to MSCs to assess its ability in inducing chondrogenesis. As seen from

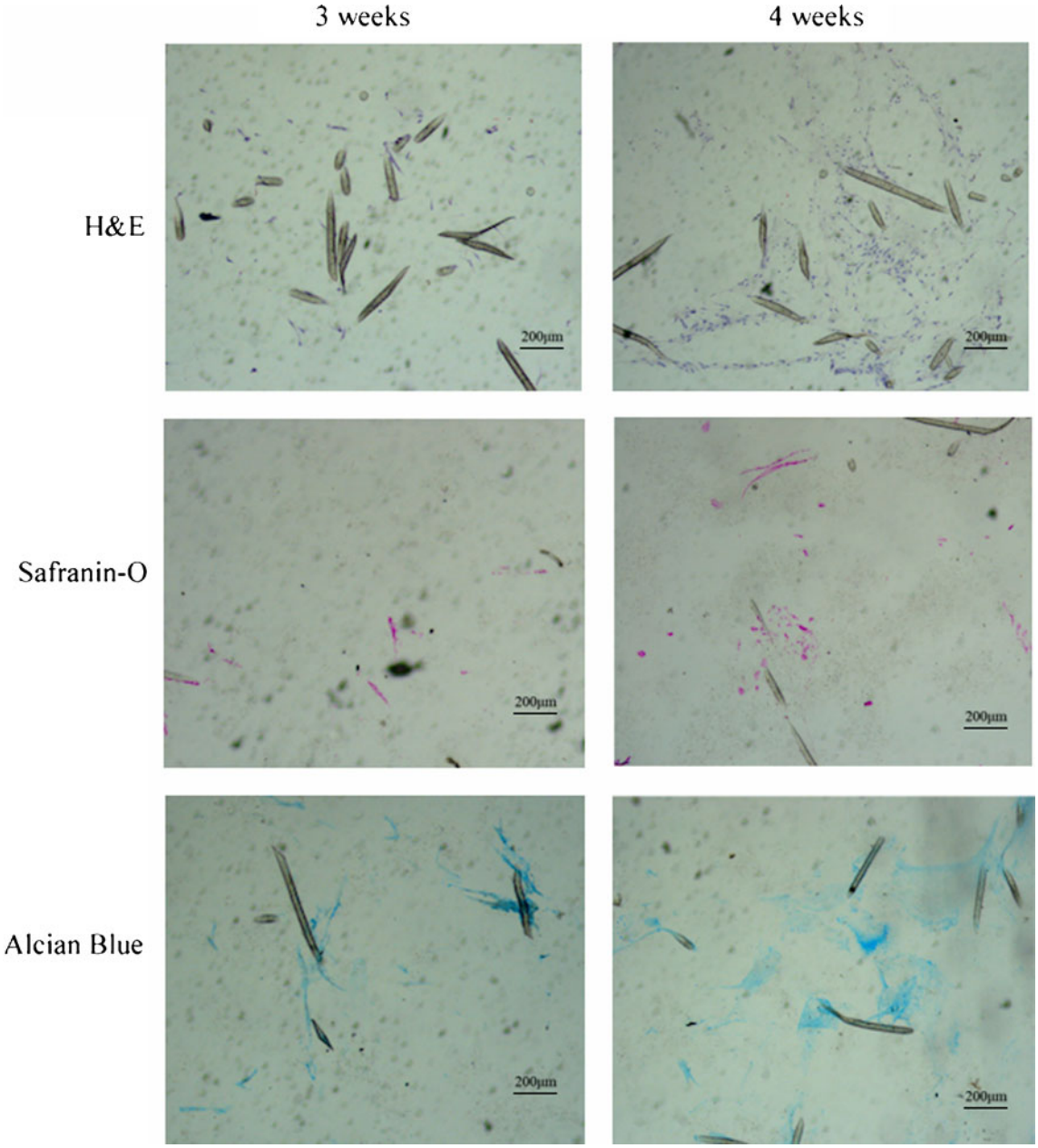

Fig. 10 Histological observation of the gene-engineered MSCs grown on PET scaffolds for 3 weeks and 4 weeks with H&E staining, Safranin-O and Alcian Blue staining.

Fig. 10, H&E staining showed that more cells grew in the scaffold after 4 weeks culture than 3 weeks. Safranin-O staining of the samples showed that accumulation of sulfated GAGs could be observed in both groups. Alcian blue staining displayed that the distribution of the cartilaginous matrix GAG improved with time. Our data indicate that MSCs transfected with non-viral vector and pDNA coding for TGFβ-1 by reverse transfection method show their capacity to differentiate into the cartilage, and a differentiation period of 4 weeks is preferable over 3 weeks. Future studies utilizing an *in vivo* animal model are necessary to validate the feasibility of the MSCs transfected with non-viral vector and pDNA coding for TGFβ-1 for cartilage regeneration. Therefore, TGFβ-1 gene-engineered MSCs using the non-viral vector are promising in the treatment of cartilage-related disease.

The reverse transfection has received increasing attention, and the understanding of mechanism of the reverse system is crucial for its application and further improvement (52–54). However, the mechanism studies of the reverse system were not reported previously. We hypothesized that the gene transfer levels in the reverse transfection system are dependent on the interactions among the cells, substrate and gene complexes and made a preliminary mechanism research in this area and found some interesting phenomenon. For instance, the more negative-charged gelatin could immobilize more cationic gene complexes to the substrate; thus, more genes could be released to the supernatant, which can favor the gene transfection. The anionic gelatin can function as a shelter for the nanoparticles to avoid adsorption of proteins in the serum.

CONCLUSIONS

We have successfully developed a novel transfection system based on the reverse transfection and 3D transfection system and compared the underlying mechanism for the cellular uptake of gene complex by the conventional and reverse transfection method. As compared with conventional transfection method, which is serum-resistant for non-viral gene delivery to various cell types, reverse transfection induced no decrease of, and even increased, the transgene levels with the addition of serum in all the cells tested. Such a distinction for different transfection methods might be caused by the alteration of the physical properties of the nanoparticles after the addition of serum and the corresponding change of the uptake mechanism. Also, three-dimensional scaffold provides a good environment for cell proliferation and showed enhanced gene expression as compared with the 2D system in the long run. Therefore, TGFβ-1 gene-engineered MSCs using non-viral vector and 3D reverse transfection system are promising in the treatment of cartilage-related disease. Further studies will be conducted to implant the gene-engineered MSCs for the *in vivo* treatment of the cartilage related disease

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