A comparative study on nonviral genetic modifications in cord blood and bone marrow mesenchymal stem cells

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Abstract The focus of both clinical and basic studies on stem cells is increasing due to their potentials in regenerative medicine and cell-based therapies. Recently stem cells have been genetically modified to enhance an existing character in or to bring a new property to them. However, accomplishment of declared goals requires detailed knowledge about their molecular characteristics which could be achieved by genetic modifications mostly through nonviral transfection strategies. Capable of differentiating into multiple cells, human unrestricted somatic

stem cells (hUSSCs) and human mesenchymal stem cells (hMSCs) seem to be suitable candidates for transfection approaches. Involvement of microRNAs (miRNAs) in many biological processes makes their transfection evaluation valuable. Herein we investigated the efficacy and toxicity of four typically used transfection reagents (Arrest-In, Lipofectamine 2000, Oligofectamine and HiPerfect) systematically to deliver fluorescent labeled-miRNA and Green Fluorescent Protein (GFP) expressing plasmid into hUSSCs and hMSCs. The authenticity of stem cells was verified by differentiation experiments along with flow cytometry of surface markers. Our study revealed that stemness properties of these stem cells were not affected by transient transfection. Moreover the ratios of cell viability and transfection efficiency in both analyzed stem cells were reversed. Considering cell viability, the highest fraction of GFP-expressing cells was obtained using Oligofectamine (~50%) while the highest transfection rate of miRNA was achieved by Lipofectamine 2000 (~90%). Moreover dependency of hMSCs to size of transfected nucleic acid and time-dependency of Oligofectamine and their affection on the yield of transfection were observed. Cytotoxicity assessments also showed that hUSSCs are sensitive to HiPerFect. In addition cells treated by Lipofectamine showed morphological changes. Representing the efficient nucleic acid transfection, our research facilitates comprehensive genetic modification of stem cells and demonstrates powerful approaches to understand stem cell molecular regulation mechanisms,

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which eventually improves nonviral cell-mediated gene therapy.

Keywords Nonviral transfection · USSC · Mesenchymal stromal cells · microRNA · Plasmid

Introduction

Using nucleic acids in gene therapy and cell-based approaches provides a promising therapeutic strategy for a variety of intractable diseases, although little is known about the precise mechanism (Mingozzi and High 2011; Naldini 2011). In bio-market, production of desirable transgenic proteins needs modification of cellular and molecular mechanisms of the hosts (Lico et al. 2008). Recently applied methods to transfect siRNA and microRNA (miRNA) offer ways to inactive genes of interest, thus, provide tools for gene function analyses (Walia et al. 2011; Davidson and McCray 2011). Such applications may be obtained by transfection of investigated nucleic acids into the target cells. Unstable in the presence of nucleases, nucleic acid hardly crosses cell membranes because of its size and charge. Indeed, almost no nucleic acids can permeate cell membranes as naked molecules. For this purpose carriers have been developed with the purpose of delivering genes efficiently into target cells and protecting them from nuclease degradation (Weissig and Torchilin 2001; Wolff and Budker 2005).

Viral vectors have been widely investigated as vehicles for gene delivery. Despite high efficiency, they pose high risks, including insertional mutagenesis, high immunogenicity, DNA size restriction, large-scale constraints and cytotoxicity (Wright 2009). Recently the development of non-integrative viral vectors such as lentiviral and adenoviral vectors provides an important step toward the development of safer vector-based gene transfer technology (Coutant et al. 2008). Lentiviral vectors are not diluted through cell division but support transcription by the cellular machinery, allowing stable transgene expression in non-dividing cells such as postmitotic cells. Non-integrative lentiviral vectors (integrase negative forms) preserve many favorable features of conventional integrative lentiviral vectors, including vector stability, high production yields and infectivity.

Further investigations are still required for their application in clinical purposes.

However, similar to episomal plasmid vectors, adenoviruses remain in epichromosomal form in all known cells except eggs (Okita et al. 2008). Adenoviral vectors have two attractive features: high infection efficiency for both dividing and non-dividing cells and rare integration into the genome (Stadtfeld et al. 2008). The drawbacks for adenoviral-mediated gene delivery are their rapid clearance in dividing cells; in addition their gene expression is not consistently and sufficiently long enough. A recent study about their application in cell reprogramming reported that the adenoviral vector efficiency could be enhanced by combining with various small chemical molecules (Shao and Wu 2010).

Although viral methods have represented more successful gene transfer, from a biosafety point of view, as well as due to the ease of handling and the limited packaging capacity of several viral vectors, non-viral methods are favored. Many different nonviral methods have been developed to deliver nucleic acid to mammalian cells for both stable and transient expression, including DEAE dextran, commercial non-viral vectors (lipids, polymers, dendrimer, poly-ethylenimine, peptides, and nanoparticle-based compounds) and electroporation (Yamano et al. 2010). However, electroporation suffers from many limitations, including the need for large cell numbers, large amounts of nucleic acid, serum-free transfection conditions, and the high level of cell death (Andre and Mir 2004).

Cationic lipids condense nucleic acid through ionic interaction and thus form cationic nano-size complexes which bind to the negatively charged membrane through ionic interaction and enter the cell through spontaneous endocytosis, without significant cytotoxicity (Luo and Saltzman 2000; Dincer et al. 2005). We tested the hypothesis that if under similar conditions, these gene transfer reagents (TRs) are capable of yielding high transfection efficiencies. Four reagents of different formulations: Arrest-In (lipo-polymeric), Lipofectamine 2000 (cationic lipid), Oligofectamine (lipo-polymeric), and HiPerFect (blend of cationic and neutral lipids) were chosen for this study.

In general, transfection efficiency of vectors depends on cell types, kind of nucleic acid, and medium conditions (Yamano et al. 2010). Although

many new systems hold promises to achieve high-transfection efficiencies, a systematic evaluation considering some types of stem cells has not, to our knowledge, been performed yet. Stem cells have been the focus of interest in the field of regenerative medicine not only for their differentiation potential but also for their ability to serve as the live part of target tissue and to give rise to mature and functional cells in vitro and in vivo (Ferguson et al. 2005; Barrilleaux et al. 2006; Feng et al. 2010; Aggarwal and Pittenger 2005). Successful transfection of hMSCs with potentially therapeutic genes, such as coagulation factor VIII and IX for hemophilia, collagen I for osteogenesis imperfecta, interferon- β and interleukin-2 for malignancies and bone morphogenetic protein 2 and 4 for a variety of musculoskeletal defects have been reported (Van Damme et al. 2002; Reiser et al. 2005). hMSCs derived from bone marrow have limited proliferative capacity and their differentiation potential decreases during passaging. Although bone marrow is considered as an accessible and enriched source of hMSC, the isolation procedure is invasive for donors and patients (Kern et al. 2006). Capable of differentiating into multiple cell types, hUSSC is less mature, with more extended life length and longer telomeres. Besides these biological advantages, hUSSC isolated from umbilical cord blood is abundantly available, routinely harvested without risk to the donor. These cells also do not express HLA class II and exhibit less ethical challenges and immunogenicity compared to hMSC and embryonic stem cells. These multipotent stem cells could be expanded up to 20 passages without spontaneous transformation and abnormal karyotype (Bakhshandeh et al. 2011; Kogler et al. 2004). Herein to facilitate dealing with genetically modifications of stem cells, we investigated the efficacy and ability of the four mentioned transfection reagents to deliver fluorescent-miRNA (20 nucleotides) and GFP coding plasmid (7,442 nucleotides) into hUSSCs and hMSCs.

Materials and methods

Cell isolation and culture

With informed consent of the mothers, hUSSC isolation and propagation were carried out as described previously by our laboratory (Bakhshandeh

et al. 2011). Flushing with Hanks balanced salt solution (HBSS, Sigma), hMSCs were isolated from aspirated bone marrow by Ficoll using density centrifuging. Growth or basal medium contained high glucose DMEM supplemented with 10% FBS and a 1% antibiotic (10,000 U penicillin). Cells were cultured under humidified atmosphere of 95% air with 5% CO₂ at 37 °C in 24-well tissue culture polystyrene.

Differentiation tests

For adipogenic differentiation, the cells were cultured for 3 weeks in the presence of 0.5 mM hydrocortisone, 0.5 mM isobutyl methyl xanthine, and 60 mM indomethacin (all from Sigma). For osteogenic induction, the growth medium was supplemented with 250 nM dexamethasone, 50 μ g/mL ascorbic acid-2-phosphate, and 10 mM beta-glycerol phosphate all from Sigma for 21 days. Chondrogenic differentiation was induced in a modified high-density micromass 3D culture for 21 days. Aliquots of $1.5\text{--}2 \times 10^5$ USSCs in 0.5 mL of basal medium were centrifuged at 1,200 rpm for 5 min in 15 mL polypropylene tubes and incubated overnight. Then the medium was supplemented with 250 nM dexamethasone, 50 μ g/mL ascorbic acid-2-phosphate, 1% ITS (6.25 mg/mL insulin, 6.25 mg/mL transferrin, 6.25 ng/mL selenious acid, 1.25 mg/mL bovine serum albumine and 5.35 mg/mL linoleic acid), 200 ng/20 μ l bFGF and 10 ng/mL TGF- β (recombinant human transforming growth factor-beta) all from Sigma to make the chondrogenic induction medium.

To investigate calcium mineralization in osteogenic differentiation, cultured cells were treated with *Alizarin Red*. Briefly, samples were rinsed in PBS, fixed with 4% paraformaldehyde for 1 h, washed with PBS and stained with fresh 1% Alizarin Red S, pH 4.1 for 10 min at room temperature. Wells were then rinsed with 0.1% HCl solution to remove excess stain and visualized under light microscope. *Oil Red* dye was used for detection of accumulated oil droplets in adipogenic differentiated cells. *Alcian Blue* dye was used to identify the presence of glycosaminoglycans (GAG) in the chondrogenic differentiated cells.

Cell viability assessment

Cell viability was assessed by trypan blue exclusion assay. Briefly, 5 μ L of 0.4% trypan blue (Sigma) was added into each sample at the end of transfection time.

After incubation for 30 s at RT, the fraction of blue cells was quantified by microscope (Nikon, Tokyo, Japan) and classified as nonviable or viable if they were positively stained or negatively stained by trypan blue, respectively. The 8×10^4 cells of each types was seeded as the “control”, without any transfection assay.

Propidium iodide (PI) intercalates into double-stranded nucleic acids. It is excluded by viable cells but can penetrate cell membranes of dying or dead cells (Riccardi and Nicoletti 2006). Hence PI as reverse indicator of cell viability was assayed by flow-cytometry to quantify the exact number of viability.

Fluorescent-miRNA (FmiRNA) transfection

FmiRNA transfection was performed by mixing miRCURY LNATM microRNA Power Inhibitor Negative Control B (5 nmol, 3'-fluorescein labeled, Exiqon) with four different TRs. For each transfection reagent, transfection assay was carried out in quadruplicate (experimental replications) simultaneously. Cells were reverse transfected according to the manufacturer's instruction. Briefly, we spotted 37.5 ng or 70 ng fluorescent miRNA in 100 μ L culture medium without serum into a single well of a 24-well plate. Then we added appropriate volume of each TR to each well, mixed thoroughly by pipetting. Incubation for 5–10 min at room temperature to allow formation of transfection complexes was done. After that 8×10^4 cells (USSCs: passage 2 and MSCs: passage 4) in 250 μ L of growth medium (without serum and antibiotics) were seeded into the well, on top of the miRNA/TR complexes and incubated under mild shaking at 37 °C and 5% CO₂ for 6 h. Following incubation, all the transfected cells were visualized by microscope, then washed and harvested for flow-cytometry or viability test. In FACS analyses, the selection criterion was the emission of fluorescence from the positive cells that have absorbed fluorescent-miRNA. The negative cells are the cells that do not absorb the fluorescent-miRNA.

Plasmid DNA (pcDNA) transfection

Plasmid DNA (pEGFP-C1, Invitrogen), encoding the Green Fluorescent Protein (GFP) under the control of the cytomegalovirus promoter/enhancer, was used for investigating large DNA transfection. Vectors were

propagated in competent *Escherichia coli* DH5a cells followed by endotoxin-free plasmid DNA ultrapurification using a QIAGEN Plasmid Maxi kit according to the manufacturer's instructions (Qiagen). Appropriate volume of each TRs was incubated in room temperature with 0.5 μ g or 1 μ g pcDNA for 20 min. Reverse transfection procedure was done as mentioned before. After 6 h, the medium was replaced by DMEM containing 10% FBS and antibiotics and incubated for 72 h. To optimize the incubation time, the transfection of pcDNA by Lipofectamine was investigated at both the 36th and the 72nd h. Then, the transfected cells were visualized by microscope, then washed and harvested for flow-cytometry or viability test. In FACS analyses, the selection criterion was the emission of fluorescence from the positive cells that have absorbed the pcDNA and expressed the GFP. The negative cells are the cells that do not absorb the pcDNA.

Fluorescence microscopy

The miRNA and pcDNA transfected cells were washed once with 1 ml of PBS, followed by addition of 2 ml of PBS. Images were acquired using an inverted fluorescence microscope (TE2000-S; Nikon-Eclipse, Tokyo, Japan).

Fluorescence-activated cell sorting (FACS)

Cells were trypsinized, washed twice with PBS, and fixed with 1% paraformaldehyde in PBS. After fixation, FACS analysis was performed on FACS cytometer (Partec Flow Cytometry, Cell Analysis) with fixed cell counts for all experiments. Win MDI 2.8 software was used to create the histograms. Isotype control refers to the 1-day cultured cells without miRNA or pcDNA transfection.

Quantitative real-time RT-PCR

Total RNA was extracted and random hexamer-primed cDNA synthesis was carried out using Revert Aid first strand cDNA synthesis kit (Fermentas). Real-time RT-PCR reactions were performed using MaximaTM SYBR Green/ROX qPCR Master Mix (Fermentas), and monitored in RotorGene 6000 Q real-time analyzer (Corbett) followed by melting curve analysis to confirm PCR specificity. The threshold cycle average was used for

data analysis by Rotor-gene Q software (Corbett). Relative expression was quantified using the comparative $\Delta\Delta C_t$ method. Target genes were normalized against HPRT and calibrated to undifferentiated USSCs and MSCs. The primers used for NANOG were: F' GCTAAGGACAACATTGATAGAAG and R' CTTC ATCACCAATTCGTACTTG, and the primers used for OCT4 were: F' CGCCGTATGAGTTCTGTG and R' GGTGATCCTCTTCTGCTTC.

Statistical analysis

The Student's *t* test was used to determine whether there was a significant difference between the groups and the difference was regarded as significant if *p* value was lower than 0.05. Data are presented as mean $\pm$ standard deviation (SD) of at least three independent experiments.

Results

hUSSC and hMSC characterization

Isolated human stem cells, hUSSCs and hMSCs, were characterized based on their surface markers, reported previously by our laboratory (supplement 1) (Shafiee et al. 2011). Both types of stem cells showed fibroblast-like and spindle-shaped morphology when cultured at low cell density. hUSSCs were rectangle-shaped and hMSCs were square-shaped. After 21 days of osteogenic differentiation using osteogenic medium, the biomineralization and secreted extracellular matrix were observed in monolayer culture, as visualized by Alizarin Red staining (Fig. 1). In addition, hUSSC and hMSCs showed the potential to differentiate to chondrocytes and adipocytes under differentiation inducing conditions. The strong expression and accumulation of acidic proteoglycans was supported by Alcian Blue staining. Cytoplasmic oil vacuoles were stained in red as indicators of adipogenic differentiation (Fig. 1).

Investigation of transfection reagents without constructs

Investigation of cells treated by pure TRs revealed that their toxicities during 6 h incubation were acceptable

(Fig. 2). Remarkably cells exposed to Lipofectamine showed fewer adherences which led to spherical morphology and may result in early apoptosis.

Optimization of transfection conditions

The optimization of incubation time for pcDNA transfection revealed that at the 36th h, despite proper cell viability, the transfection efficiency (indicated by GFP expression) was very low. In contrast the transfection efficiency results at the 72nd h were appropriate (Table 1).

To optimize the appropriate transfection condition, for each stem cell type, seven different combinations of transfection reagents and nucleic acids (either for FmiRNA or pcDNA) were tested. Afterward the results of each stem cell type were compared. In the following, all the increases or decreases are presented in comparison with the standard conditions.

FmiRNA transfection optimization

The results of transfection yield and cell viability for each set of conditions are shown in Table 2. For transfection with Lipofectamine, increase in construct concentration did not improve transfection efficiency significantly. Also increase in Arrest-In volume or construct concentration did not improved transfection yield significantly. In this latter case, decrease in both Arrest-In volume and construct concentration reduced the efficiency dramatically (condition 2–2). Alteration of Arrest-In volume did not affect the cell viability considerably. Therefore the best results were achieved using the standard protocols (conditions 1–1 and 2–3).

pcDNA transfection optimization

The results of transfection yield and cell viability for each set of conditions are shown in Table 3. Decrease in Oligofectamine volume reduced the transfection efficiency while alteration of construct concentration did not change the yield significantly. Noteworthy increase in both Oligofectamine volume and construct concentration (condition 1–3) resulted in unexpected decreased yield although the relevant changes were not significant. Comparing the readings of transfection by Arrest-In revealed that decrease in its volume led to considerable reduced yield. But increase in construct concentration did not alter the transfection efficiency

Fig. 1 The morphology of stem cells in basal medium, and specific staining after a 21-day culture under adipogenic, osteogenic and chondrogenic induction medium. Bars 100 μ m

meaningfully. Overall changes in Oligofectamine or Arrest-In volumes did not affect the cell viability significantly. Altogether the best results were achieved using the standard protocols (conditions 1–1 and 2–1).

FmiRNA transfection

According to FmiRNA transfection procedures (standard conditions), efficiencies of the four different TRs were compared at an early time point (6 h) after transfection for both hUSSCs and hMSCs. The cells were examined by fluorescence microscopy to visualize the green fluorescent cells. In parallel, duplicate transfected cells were washed, harvested and analyzed by flow cytometry to determine the percentage of transfected cells. As expected, all four transfection

reagents were capable of delivering the FmiRNA into both cell types.

In hUSSCs, the highest efficiency, with up to 62% of all cells with fluorescence, was obtained using Lipofectamine (Figs. 3, 7a; Table 4). HiPerfect was inferior in performance in comparison to other commercially available reagents with only 8% efficiency. During this time period, the highest number of viable cells was observed in Lipofectamine assay and the most toxic TR was HiPerfect (Fig. 7c; Table 5).

As expected, Lipofectamine and Arrest-In also showed the highest efficiency in hMSC miRNA-transfection, 86 and 88%, respectively were observed (Figs. 4, 7a; Table 4). Remarkably the highest number of dead cells was observed for these two TRs. On the other hand, Oligofectamine could transfect only 1% of

Fig. 2 The morphology of stem cells before (Control) and 6 h after addition of transfection reagents. The percentage of viable cells (obtained by flow-cytometry of PI indicator) in each assay is shown. Remarkably cells treated by Lipofectamine showed

reduced adherence which led to spherical morphology. Because of low efficiency, HiPerfect reagent was not investigated. Bars 100 μ m

Table 1 Optimization of incubation time for pcDNA transfection by Lipofectamine

Cell type	Viability (36 h) (%)	Viability (72 h) (%)	Efficiency (36 h) (%)	Efficiency (72 h) (%)
USSC	76.5	1	0.54	Near 100
MSC	81	10	0.36	8

the hMSCs with the lowest toxicity during 6 h of assay time (Fig. 7c; Table 5).

Plasmid transfection

The pcDNA used in this study had identical expression cassette, encoding for the GFP gene under control of

Table 2 Evaluation of FmiRNA transfection with different ratios of reactants and constructs after 6 h; Lipofectamine and Arrest-In were selected because of their appropriate primary results (Efficiency and viability were assessed by flow cytometry)

Cell type	Condition	Transfection reagent	FmiRNA (ng)	Transfection efficiency (%)	Viability (%)
USSC	1–1 ^a	1 µL Lipofectamine	37.5	65	84
	1–2	1 µL Lipofectamine	75	67	84
	2–1	1 µL Arrest-In	75	50	81
	2–2	1 µL Arrest-In	37.5	15	82
	2–3 ^a	2 µL Arrest-In	37.5	50	82
	2–4	3 µL Arrest-In	37.5	60	83
	2–5	3 µL Arrest-In	75	62	80
MSC	1–1 ^a	1 µL Lipofectamine	37.5	71	63
	1–2	1 µL Lipofectamine	75	75	60
	2–1	1 µL Arrest-In	75	72	65
	2–2	1 µL Arrest-In	37.5	61	71
	2–3 ^a	2 µL Arrest-In	37.5	75	63
	2–4	3 µL Arrest-In	37.5	65	63
	2–5	3 µL Arrest-In	75	78	60

Efficiency and viability were assessed by flow cytometry

^a Standard condition according to manufacturer's instruction

Table 3 Evaluation of pcDNA transfection with different ratios of reactants and constructs after 72 h; Oligofectamine and Arrest-In were selected because of their appropriate primary results (Efficiency and viability were assessed by flow cytometry)

Cell type	Condition	Transfection reagent	pcDNA (µg)	Transfection efficiency (%)	Viability (%)
USSC	1–1 ^a	2 µL Oligofectamine	0.5	48.5	64
	1–2	1 µL Oligofectamine	0.5	35	68.2
	1–3	2 µL Oligofectamine	1	43.1	66.6
	1–4	1 µL Oligofectamine	1	45	65
	2–1 ^a	2 µL Arrest-In	0.5	38	64.4
	2–2	1 µL Arrest-In	0.5	21	72.6
	2–3	2 µL Arrest-In	1	43	66.5
	2–4	1 µL Arrest-In	1	23	62.7
MSC	1–1 ^a	2 µL Oligofectamine	0.5	3.5	84.3
	1–2	1 µL Oligofectamine	0.5	2.4	87.4
	1–3	2 µL Oligofectamine	1	2	85
	1–4	1 µL Oligofectamine	1	2.3	81.6
	2–1 ^a	2 µL Arrest-In	0.5	2	70.8
	2–2	1 µL Arrest-In	0.5	1.1	84.2
	2–3	2 µL Arrest-In	1	2	72
	2–4	1 µL Arrest-In	1	1	84.5

Efficiency and viability were assessed by flow cytometry

^a Standard condition according to manufacturer's instruction

the CMV promoter. 72 h post-transfection the cells were examined by fluorescence microscopy to visualize the GFP expressing cells. In parallel, duplicate transfected cells were washed, harvested and analyzed by flow cytometry to determine the percentage of GFP expressing cells compared to untransfected samples.

In hUSSCs, the Lipofectamine was the most efficient reagent in transfecting the pcDNA with the

efficiency of approximately 100% (Fig. 5). In contrast, quantitative analysis by flow cytometry demonstrated that nearly 0% of cells transfected with Lipofectamine expressing GFP (Fig. 7b). A logical explanation could be the fact that dead cells were washed out after microscopic visualization and before harvest for flow-cytometry. Cell viability assessments supported this explanation, too. Other TRs transfected pcDNA into hUSSCs with efficiencies below 50%

Fig. 3 Phase-contrast, fluorescent microscopy and flow-cytometry analysis of USSCs transfected by FmiRNA. White arrows and red arrows show viable and apoptotic cells, respectively. FACS diagrams: color areas— isotype controls, white areas—test conditions, vertical axis—cell number and horizontal

axis—fluorescent emission. Numbers correspond to the percentage of transfection for each transfection reagent. Standard protocols were used for transfections, 3 μ L were used in the case of HiPerFect. Bars 100 μ m. (Color figure online)

Table 4 Transfection efficiencies represented as averages ($n = 4$) $\pm$ SD

Condition	Yield (%)			
	Lipofectamine 2000	Arrest-In	Oligofectamine	HiPerFect
FmiRNA-USSC	62 $\pm$ 7	52 $\pm$ 5	11 $\pm$ 4	8 $\pm$ 1.7
FmiRNA-MSC	86.22 $\pm$ 6	88 $\pm$ 3	1 $\pm$ 0.5	9.47 $\pm$ 2.3
pcDNA-USSC	0 $\pm$ 0.5 ^a	39 $\pm$ 3	47.38 $\pm$ 2	26.25 $\pm$ 4
pcDNA-MSC	8.05 $\pm$ 1.1	2.11 $\pm$ 0.4	3.67 $\pm$ 0.8	4.36 $\pm$ 0.5

^a According to fluorescent images, this efficiency is near to 100%

Table 5 Cell viabilities represented as averages ($n = 4$) $\pm$ SD

Condition	Yield (%)			
	Lipofectamine 2000	Arrest-In	Oligofectamine	HiPerFect
FmiRNA-USSC	35 $\pm$ 4	30 $\pm$ 6	18 $\pm$ 2	14 $\pm$ 1
FmiRNA-MSC	55 $\pm$ 12	35 $\pm$ 10	90 $\pm$ 1	85 $\pm$ 1
pcDNA-USSC	1 $\pm$ 1	40 $\pm$ 8	55 $\pm$ 7	50 $\pm$ 12
pcDNA-MSC	10 $\pm$ 1	70 $\pm$ 10	98 $\pm$ 2	97 $\pm$ 2

(Fig. 7b; Table 4). The lowest toxicity was seen for HiPerFect (Fig. 7d; Table 5).

As illustrated, the highest GFP expressing hMSCs had been transfected by Lipofectamine yielding $\sim$ 8% transfection efficiency (Fig. 6). Transfection with the other commercial transfection reagents resulted in less than 5% efficiency (Fig. 7b; Table 4). Transfection of hUSSCs resulted in significantly higher ratio in comparison to hMSCs. In this assay, the proportion of viable cells and transfected cells of each TR showed an inverse trend for both cell types. Predictably, the lowest viable cell numbers were detected in the Lipofectamine transfection assay (Fig. 7d; Table 5).

The influence of transient transfection on stemness properties

Quantitative transcriptional evaluation of OCT4 (Papamichos et al. 2009) and Nanog (Matsuoka et al. 2010; Brandner 2010) (two stemness indicators) on FmiRNA transfected USSCs and MSCs (by lipofectamine and after 6 h of incubation) revealed no significant changes (Fig. 8). Thus transient transfection does not alter the stemness property of these stem cells.

Discussion

Recent studies demonstrate strong efforts to develop and apply efficient nonviral gene delivery systems that

could be competitive with viral vectors (Falk et al. 2002; Lam and Dean 2010; Luo and Saltzman 2000). Few approaches to optimize the efficiency of gene transfer and establishment of proficient transfection protocols have been described only for some types of cells (Dinser et al. 2001; Yamano et al. 2010; Gonzalez et al. 2007; Yamauchi et al. 2010). Because of variation in transfection yield and cytotoxicity of each method, selection of an appropriate method for transfection of a given cell type is crucial. In most reports, cell lines have been routinely used for systematic evaluation of TR performance. Contrary to cell lines, stem cells can promote tissue healing through extensive proliferation, differentiation and growth factor secretion at the site of injury. Moreover genetic modification of stem cells provides a powerful tool to understand complex molecular regulation of development (Kern et al. 2006). Hence stem cell transfection strategy seems to be an ideal approach for cell-mediated gene therapy and developmental biology. However, the transfection efficiency is lower in stem cells compared to cell lines (Hoelters et al. 2005). To the best of our knowledge, this is the first study for comparative assessment of different TRs in two types of human stem cells.

hUSSCs can be considered as a preferable allogenic alternative source to hMSCs for stem cell regeneration. This adherent stem cell has unique biological characteristics and is capable of broad replication in culture as well as differentiation under controlled

Fig. 4 Phase-contrast, fluorescent microscopy and flow-cytometry analysis of MSCs transfected by FmiRNA. *White arrows* and *red arrows* show viable and apoptotic cells, respectively. FACS diagrams: *color areas*—isotype controls, *white areas*—test conditions, *vertical axis*—cell number and *horizontal*

axis—fluorescent emission. *Numbers* correspond to the percentage of transfection for each transfection reagent. Standard protocols were used for transfections, 3 μ L were used in the case of HiPerfect. Bars 100 μ m. (Color figure online)

Fig. 5 Phase-contrast, fluorescent microscopy and flow-cytometry analysis of USSCs transfected by GFPpcDNA. *White arrows* and *red arrows* show viable and apoptotic cells, respectively. FACS diagrams: *color areas*—isotype controls, *white areas*—test conditions, *vertical axis*—cell number and *horizontal axis*—

fluorescent emission. *Numbers* correspond to the percentage of transfection for each transfection reagent. Standard protocols were used for transfections, 3 μ L were used in the case of HiPerFect. *Bars* 100 μ m. (Color figure online)

Fig. 6 Phase-contrast, fluorescent microscopy and flow-cytometry analysis of MSCs transfected by GFPpcDNA. *White arrows and red arrows* show viable and apoptotic cells, respectively. FACS diagrams: *color areas*—isotype controls, *white areas*—test conditions, *vertical axis*—cell number and *horizontal*

axis—fluorescent emission. *Numbers* correspond to the percentage of transfection for each transfection reagent. Standard protocols were used for transfections, 3 μ L were used in the case of HiPerfect. *Bars* 100 μ m. (Color figure online)

Fig. 7 **a** Percentage of FmiRNA transfection into stem cells, analyzed by flow-cytometry, Vertical axis 0–100%, **b** percentage of pcDNA transfection into stem cells, analyzed by flow-cytometry, vertical axis 0–50%, **c**, **d** percentages of viable cells after transfection compared to untransfected cells as “control”.

Significant differences with $p < 0.05$ between controls and transfected cells or between transfected USSCs and MSCs are indicated by (*) or (#), respectively.?: More explanations are provided in the main text

Fig. 8 Investigation of stemness property of stem cells after transient transfection by relative transcriptional evaluation of Nanog and OCT4 (stemness indicators)

conditions into multiple stromal lineages (Fig. 1, supplementary figure 1) (Bakhshandeh et al. 2011; Kogler et al. 2004). Because of promising therapeutic utility of hMSCs and remarkable potentials of hUSSCs, we have chosen these two types of stem cells for transfection evaluation.

To establish gene transfer systems capable of yielding high-transfection efficiencies for stem cell modifications, four typically used and biochemically

different transfection reagents (Arrest-In: lipo-polymeric, Lipofectamine 2000: cationic lipid, Oligofectamine: lipo-polymeric, and HiPerFect: blend of cationic and neutral lipids) were comparatively studied. These reagents were reported to be efficient in some previous studies (Gheisari et al. 2008; Yamano et al. 2010; Akimov et al. 2009; Nabzdyk et al. 2011).

Typically β -galactosidase, luciferase, chloramphenicol transferase or GFP are being used as reporter genes to test transfection reagents, mostly cationic liposomes and polymers in combination with plasmids (Maurer et al. 2001). Transfection efficiencies of two nucleic acids; (1) fluorescent labeled-miRNA (FmiRNA, 20 nucleotides) and (2) GFP encoding-plasmid (pcDNA, 7,442 nucleotides) with different sizes under the same conditions were investigated as another aim of this research. RNA interference and miRNA provide great opportunities for desired genetic modifications. In addition, substitution of supplementary stimulating growth factors with direct transfection of small-RNAs to induce intentional differentiations or gene-mediated therapy (Djuranovic et al. 2011; Chitwood and Timmermans 2010; Poliseno et al. 2008; Tarantino et al. 2010) encouraged us to evaluate

miRNA transfection systematically in different conditions. As explained, in this study transfection efficiency and cytotoxicity have been analyzed in relation to cells of interest, transfection reagent and kind of nucleic acid. For optimization we investigated the effects of different ratios of reactants and constructs on transfection yield. Regarding the low yield of transfection achieved by HiPerFect, this reagent would not be a good option despite its low toxicity; therefore it was not considered for optimization. In addition, the increase and decrease in the volume of Lipofectamine was not investigated because of high toxicity and low experimental precision.

Considering that the best results as well as the need for smaller amounts of costly reagents and constructs were achieved by standard protocols, we used these transfection conditions for further investigations (Tables 2, 3). Since the analyzed cells were adherent, reverse transfection method (see “[Materials and methods](#)”) was used for both types of nucleic acids. By applying this strategy, FmiRNA transfection efficiency increased notably compared to other reports on nonviral transfection of these stem cells (Hoelters et al. 2005; Trompeter et al. 2011; Liu et al. 2009). Mild shaking during transfection period might have helped in view of elevating transfection efficiency.

The rate of plasmid transfection in hMSCs was in line with the most previous reports (Peister et al. 2004; Falk et al. 2002; Lakshmipathy et al. 2004; Weissinger et al. 2003) while Hoelters et al. (2005) reported higher transfection efficiency. Our lower yield could be because of the difference in methods for determination of transfection efficiency, which was fluorescence microscopy and Neubauer chamber in their study and flow cytometry in ours.

In similar conditions, the efficiency of pcDNA transfection in hUSSCs was much higher than hMSCs (Fig. 7; Tables 4, 5). Actually hUSSCs illustrated higher susceptibility for transfection of large nucleic acids than hMSCs (Figs. 5, 6). However, we couldn't conclude such judgment for FmiRNA transfection since both cell types represented acceptable efficiency for transfection of FmiRNA (Figs. 3, 4).

Unlike for hUSSCs, significant difference between efficiencies of pcDNA and FmiRNA transfections was observed in hMSCs. Such difference might be the result of difference in GFP expression in hUSSCs and hMSCs or size-dependency of nucleic acid transfection in both stem cell types. Altogether it revealed that

the nature of the cell itself plays a critical role in effectiveness of transfection as suggested by others (Yamano et al. 2010; Lee and Kim 2005).

Toxicity of cationic lipids is another important aspect when using liposomes. Toxicity is mainly caused by free liposomes, which do not complex nucleic acid. For optimal transfer efficiencies, higher nucleic acid concentrations require higher amounts of TR, which inevitably increase toxicity. In addition to reagent dose, other factors that could influence cytotoxicity are the presence or absence of serum and the charge ratios of TR/nucleic acid complexes and incubation time (Yamano et al. 2010). For pcDNA transfection (assessed by GFP expression), our study showed that the optimum incubation time to achieve the appropriate transfection efficiency along with acceptable cell viability was 72 h (Table 1).

Even though it has been shown that cell cytotoxicity is an inevitable side-effect of any TRs (Pouton and Seymour 2001), for in vivo transplantation dead cells are harmful. Therefore, having acceptable toxicity is an essential factor for choosing a TR. Thus, lowering the toxicity rate is a crucial part of any transfection optimization. Our investigation on cells treated with pure TRs revealed that their toxicity rates during 6 h incubation were acceptable (Fig. 2). Remarkably cells exposed to Lipofectamine showed reduced adherences which led to spherical morphology and may result in early apoptosis. A recent study has reported similar changes in morphology and consequent apoptosis of cultured human lung epithelial cells which were treated with Lipofectamine for 6 h (Kongkaneramt et al. 2008). The dependency of the production of reactive oxygen species on Lipofectamine-induced apoptosis has also been described in the mentioned paper.

Under determined conditions, we observed that the ratio of cell viability and transfection in both analyzed stem cell types were inverse, as has been reported by other groups (Madeira et al. 2010; Gheisari et al. 2008). The largest number of viable hMSCs was found after transfection with HiPerFect or Oligofectamine with nearly the lowest transfection efficiencies. In transfections with Oligofectamine and HiPerFect, significant differences were observed between viability of hUSSCs and that of hMSCs but not between transfection efficiencies. Overall it seems that in comparison to hMSCs, hUSSCs are more sensitive to Oligofectamine and HiPerFect and thus, these two TRs are not recommended for hUSSC transfection.

In consistency with previous reports (Zhang et al. 2004; Hoelters et al. 2005), most efficient TR in FmiRNA transfection, with respect to the number of viable cells, was Lipofectamine followed by Arrest-In. The efficiency of small-RNA transfer is even higher than in a study, in which hematopoietic stem cells were transfected with siRNAs by electroporation (Oliveira and Goodell 2003). The two other reagents (Oligofectamine and HiPerFect) showed much lower efficiencies. The transfection rates of these two latter reagents might increase if higher concentrations of reagent and/or nucleic acid are used, but it would be too costly for practical use.

In plasmid transfection, Oligofectamine showed the highest transfection efficiency considering cell viability again followed by Arrest-In. Worth mentioning is, that the performance of Oligofectamine seems to be time-dependent. Its efficiency after 6 h incubation was very low for FmiRNA transfection while the best performance in pcDNA transfection was observed for Oligofectamine after 72 h evaluation.

As the last assay, the possible influence of transient transfection on stemness of these stem cells was investigated by transcriptional evaluation of OCT-4 and Nanog as two stemness markers (Fig. 8). The results suggest that stemness properties of these stem cells were not affected by transient transfection.

Altogether studying the biological action of each reagent in delivering the genetic material into stem cells can be considered as an interesting approach for future studies.

Conclusion

Stem cells have been mainly investigated for their regenerative potential; however, in many cases it may be necessary to enhance or change some features of the cells by genetic modifications. Although non-viral methods commonly have the disadvantage of a low nucleic acid transfer yield, biosafety is an advantage, especially when they are considered for clinical trials. Herein we investigated the potency of four transfection reagents with different biochemical structures to transfect miRNA or plasmid DNA into hMSC and hUSSC. Our findings indicated that the mentioned transfection reagents can offer a feasible approach for non-viral genetic modification of stem cells satisfactorily. Such studies are the first steps in eliminating

supplementary inducing factors in order to direct desirable differentiation or functional gene analyses and eventually develop an in vivo gene transfer system.

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Conflict of interest None.

References

- Aggarwal S, Pittenger MF (2005) Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 105:1815–1822. doi:10.1182/blood-2004-04-1559
- Akimov IA, Kabilova TO, Vlassov VV, Chernolovskaya EL (2009) Inhibition of human cancer-cell proliferation by long double-stranded RNAs. *Oligonucleotides* 19:31–40. doi:10.1089/oli.2008.0151
- Andre F, Mir LM (2004) DNA electrotransfer: its principles and an updated review of its therapeutic applications. *Gene Ther* 11(Suppl 1):S33–S42. doi:10.1038/sj.gt.3302367
- Bakhshandeh B, Soleimani M, Ghaemi N, Shabani I (2011) Effective combination of aligned nanocomposite nanofibers and human unrestricted somatic stem cells for bone tissue engineering. *Acta Pharmacol Sin* 32:626–636. doi:10.1038/aps.2011.8
- Barrilleaux B, Phinney DG, Prockop DJ, O'Connor KC (2006) Review: ex vivo engineering of living tissues with adult stem cells. *Tissue Eng* 12:3007–3019. doi:10.1089/ten.2006.12.3007
- Brandner S (2010) Nanog, Gli, and p53: a new network of stemness in development and cancer. *EMBO J* 29:2475–2476. doi:10.1038/emboj.2010.162
- Chitwood DH, Timmermans MC (2010) Small RNAs are on the move. *Nature* 467:415–419. doi:10.1038/nature09351
- Coutant F, Frenkiel MP, Despres P, Charneau P (2008) Protective antiviral immunity conferred by a nonintegrative lentiviral vector-based vaccine. *PLoS One* 3:e3973. doi:10.1371/journal.pone.0003973
- Davidson BL, McCray PB Jr (2011) Current prospects for RNA interference-based therapies. *Nat Rev Genet* 12:329–340. doi:10.1038/nrg2968
- Dincer S, Turk M, Piskin E (2005) Intelligent polymers as nonviral vectors. *Gene Ther* 12(Suppl 1):S139–S145. doi:10.1038/sj.gt.3302628
- Dinser R, Kreppel F, Zaucke F, Blank C, Paulsson M, Kochanek S, Maurer P (2001) Comparison of longterm transgene expression after non-viral and adenoviral gene transfer into primary articular chondrocytes. *Histochem Cell Biol* 116:69–77
- Djuranovic S, Nahvi A, Green R (2011) A parsimonious model for gene regulation by miRNAs. *Science* 331:550–553. doi:10.1126/science.1191138

- Falk A, Holmstrom N, Carlen M, Cassidy R, Lundberg C, Frisen J (2002) Gene delivery to adult neural stem cells. *Exp Cell Res* 279:34–39
- Feng Y, Sun Y, Jia W, Zhang C (2010) Platelet-rich plasma and 1, 25(OH)₂ vitamin D₃ synergistically stimulate osteogenic differentiation of adult human mesenchymal stem cells. *Biotechnol Lett* 32:635–642. doi:[10.1007/s10529-009-0198-8](https://doi.org/10.1007/s10529-009-0198-8)
- Ferguson C, Larochelle A, Dunbar CE (2005) Hematopoietic stem cell gene therapy: dead or alive? *Trends Biotechnol* 23:589–597. doi:[10.1016/j.tibtech.2005.09.005](https://doi.org/10.1016/j.tibtech.2005.09.005)
- Gheisari Y, Soleimani M, Azadmanesh K, Zeinali S (2008) Multipotent mesenchymal stromal cells: optimization and comparison of five cationic polymer-based gene delivery methods. *Cytotherapy* 10:815–823. doi:[10.1080/14653240802474307](https://doi.org/10.1080/14653240802474307)
- Gonzalez G, Pfannes L, Brazas R, Striker R (2007) Selection of an optimal RNA transfection reagent and comparison to electroporation for the delivery of viral RNA. *J Virol Methods* 145:14–21. doi:[10.1016/j.jviromet.2007.04.013](https://doi.org/10.1016/j.jviromet.2007.04.013)
- Hoelters J, Ciccarella M, Drechsel M, Geissler C, Gulkan H, Bocker W, Schieker M, Jochum M, Neth P (2005) Nonviral genetic modification mediates effective transgene expression and functional RNA interference in human mesenchymal stem cells. *J Gene Med* 7:718–728. doi:[10.1002/jgm.731](https://doi.org/10.1002/jgm.731)
- Kern S, Eichler H, Stoeve J, Kluter H, Bieback K (2006) Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* 24:1294–1301. doi:[10.1634/stemcells.2005-0342](https://doi.org/10.1634/stemcells.2005-0342)
- Kogler G, Sensken S, Airey JA, Trapp T, Muschen M, Feldhahn N, Liedtke S, Sorg RV, Fischer J, Rosenbaum C, Greschat S, Knipper A, Bender J, Degistrici O, Gao J, Caplan AI, Colletti EJ, Almeida-Porada G, Muller HW, Zanjani E, Wernet P (2004) A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. *J Exp Med* 200:123–135. doi:[10.1084/jem.20040440](https://doi.org/10.1084/jem.20040440)
- Kongkanermit L, Sarisuta N, Azad N, Lu Y, Iyer AK, Wang L, Rojanasakul Y (2008) Dependence of reactive oxygen species and FLICE inhibitory protein on lipofectamine-induced apoptosis in human lung epithelial cells. *J Pharmacol Exp Ther* 325:969–977. doi:[10.1124/jpet.107.136077](https://doi.org/10.1124/jpet.107.136077)
- Lakshmipathy U, Pelacho B, Sudo K, Linehan JL, Coucouvanis E, Kaufman DS, Verfaillie CM (2004) Efficient transfection of embryonic and adult stem cells. *Stem Cells* 22:531–543. doi:[10.1634/stemcells.22-4-531](https://doi.org/10.1634/stemcells.22-4-531)
- Lam AP, Dean DA (2010) Progress and prospects: nuclear import of nonviral vectors. *Gene Ther* 7:439–447. doi:[10.1038/gt.2010.31](https://doi.org/10.1038/gt.2010.31)
- Lee M, Kim SW (2005) Polyethylene glycol-conjugated copolymers for plasmid DNA delivery. *Pharm Res* 22:1–10
- Lico C, Chen Q, Santi L (2008) Viral vectors for production of recombinant proteins in plants. *J Cell Physiol* 216:366–377. doi:[10.1002/jcp.21423](https://doi.org/10.1002/jcp.21423)
- Liu YP, Hu YD, Wang F, Ling Y, Kong YZ, Li P (2009) Effect of human telomerase reverse transcriptase gene antisense oligonucleotide on sensitivity of gemcitabine in pancreatic cancer cell. *Zhonghua Yi Xue Za Zhi* 89:2391–2394
- Luo D, Saltzman WM (2000) Synthetic DNA delivery systems. *Nat Biotechnol* 18:33–37. doi:[10.1038/71889](https://doi.org/10.1038/71889)
- Madeira C, Mendes RD, Ribeiro SC, Boura JS, Aires-Barros MR, da Silva CL, Cabral JM (2010) Nonviral gene delivery to mesenchymal stem cells using cationic liposomes for gene and cell therapy. *J Biomed Biotechnol* 2010:735–349. doi:[10.1155/2010/735349](https://doi.org/10.1155/2010/735349)
- Matsuoka J, Yashiro M, Sakurai K, Kubo N, Tanaka H, Muguruma K, Sawada T, Ohira M, Hirakawa K (2010) Role of the stemness factors Sox2, Oct3/4, and Nanog in gastric carcinoma. *J Surg Res*. doi:[10.1016/j.jss.2010.11.903](https://doi.org/10.1016/j.jss.2010.11.903)
- Maurer N, Fenske DB, Cullis PR (2001) Developments in liposomal drug delivery systems. *Expert Opin Biol Ther* 1:923–947. doi:[10.1517/14712598.1.6.923](https://doi.org/10.1517/14712598.1.6.923)
- Mingozzi F, High KA (2011) Therapeutic in vivo gene transfer for genetic disease using AAV: progress and challenges. *Nat Rev Genet* 12:341–355. doi:[10.1038/nrg2988](https://doi.org/10.1038/nrg2988)
- Nabzdyk CS, Chun M, Pradhan L, Leger FW (2011) High throughput RNAi assay optimization using adherent cell cytometry. *J Transl Med* 9:48. doi:[10.1186/1479-5876-9-48](https://doi.org/10.1186/1479-5876-9-48)
- Naldini L (2011) Ex vivo gene transfer and correction for cell-based therapies. *Nat Rev Genet* 12:301–315. doi:[10.1038/nrg2985](https://doi.org/10.1038/nrg2985)
- Okita K, Nakagawa M, Hyunjong H, Ichisaka T, Yamanaka S (2008) Generation of mouse induced pluripotent stem cells without viral vectors. *Science* 322:949–953. doi:[10.1126/science.1164270](https://doi.org/10.1126/science.1164270)
- Oliveira DM, Goodell MA (2003) Transient RNA interference in hematopoietic progenitors with functional consequences. *Genesis* 36:203–208. doi:[10.1002/gene.10212](https://doi.org/10.1002/gene.10212)
- Papamichos SI, Kotoula V, Tarlatzis BC, Agorastos T, Papazisis K, Lambropoulos AF (2009) OCT4B1 isoform: the novel OCT4 alternative spliced variant as a putative marker of stemness. *Mol Hum Reprod* 15:269–270. doi:[10.1093/molehr/gap018](https://doi.org/10.1093/molehr/gap018)
- Peister A, Mellad JA, Wang M, Tucker HA, Prockop DJ (2004) Stable transfection of MSCs by electroporation. *Gene Ther* 11:224–228. doi:[10.1038/sj.gt.3302163](https://doi.org/10.1038/sj.gt.3302163)
- Poliseno L, Pitto L, Simili M, Mariani L, Riccardi L, Ciucci A, Rizzo M, Evangelista M, Mercatanti A, Pandolfi PP, Rainaldi G (2008) The proto-oncogene LRF is under post-transcriptional control of MiR-20a: implications for senescence. *PLoS One* 3:e2542. doi:[10.1371/journal.pone.0002542](https://doi.org/10.1371/journal.pone.0002542)
- Pouton CW, Seymour LW (2001) Key issues in non-viral gene delivery. *Adv Drug Deliv Rev* 46:187–203
- Reiser J, Zhang XY, Hemenway CS, Mondal D, Pradhan L, La Russa VF (2005) Potential of mesenchymal stem cells in gene therapy approaches for inherited and acquired diseases. *Expert Opin Biol Ther* 5:1571–1584. doi:[10.1517/14712598.5.12.1571](https://doi.org/10.1517/14712598.5.12.1571)
- Riccardi C, Nicoletti I (2006) Analysis of apoptosis by propidium iodide staining and flow cytometry. *Nat Protoc* 1:1458–1461. doi:[10.1038/nprot.2006.238](https://doi.org/10.1038/nprot.2006.238)
- Shafiee A, Kabiri M, Ahmadbeigi N, Yazdani SO, Mojtahed M, Amanpour S, Soleimani M (2011) Nasal septum-derived multipotent progenitors: a potent source for stem cell-based regenerative medicine. *Stem Cells Dev* 20:2077–2091. doi:[10.1089/scd.2010.0420](https://doi.org/10.1089/scd.2010.0420)
- Shao L, Wu WS (2010) Gene-delivery systems for iPS cell generation. *Expert Opin Biol Ther* 10:231–242. doi:[10.1517/14712590903455989](https://doi.org/10.1517/14712590903455989)
- Stadteld M, Nagaya M, Utikal J, Weir G, Hochedlinger K (2008) Induced pluripotent stem cells generated without viral integration. *Science* 322:945–949. doi:[10.1126/science.1162494](https://doi.org/10.1126/science.1162494)
- Tarantino C, Paoletta G, Cozzuto L, Minopoli G, Pastore L, Parisi S, Russo T (2010) miRNA 34a, 100, and 137

- modulate differentiation of mouse embryonic stem cells. *Faseb J* 24:3255–3263. doi:[10.1096/fj.09-152207](https://doi.org/10.1096/fj.09-152207)
- Trompeter HI, Abbad H, Iwaniuk KM, Hafner M, Renwick N, Tuschl T, Schira J, Muller HW, Wernet P (2011) MicroRNAs MiR-17, MiR-20a, and MiR-106b act in concert to modulate E2F activity on cell cycle arrest during neuronal lineage differentiation of USSC. *PLoS One* 6:e16138. doi:[10.1371/journal.pone.0016138](https://doi.org/10.1371/journal.pone.0016138)
- Van Damme A, Vanden Driessche T, Collen D, Chuah MK (2002) Bone marrow stromal cells as targets for gene therapy. *Curr Gene Ther* 2:195–209
- Walia B, Satija N, Tripathi RP, Gangenahalli GU (2011) Induced pluripotent stem cells: fundamentals and applications of the reprogramming process and its ramifications on regenerative medicine. *Stem Cell Rev*. doi:[10.1007/s12015-011-9279-x](https://doi.org/10.1007/s12015-011-9279-x)
- Weissig V, Torchilin VP (2001) Cationic bolosomes with delocalized charge centers as mitochondriaspecific DNA delivery systems. *Adv Drug Deliv Rev* 49:127–149
- Weissinger F, Reimer P, Waessa T, Buchhofer S, Schertlin T, Kunzmann V, Wilhelm M (2003) Gene transfer in purified human hematopoietic peripheral-blood stem cells by means of electroporation without prestimulation. *J Lab Clin Med* 141:138–149. doi:[10.1067/mlc.2003.14](https://doi.org/10.1067/mlc.2003.14)
- Wolff JA, Budker V (2005) The mechanism of naked DNA uptake and expression. *Adv Genet* 54:3–20. doi:[10.1016/S0065-2660\(05\)54001-X](https://doi.org/10.1016/S0065-2660(05)54001-X)
- Wright JF (2009) Transient transfection methods for clinical adeno-associated viral vector production. *Hum Gene Ther* 20:698–706. doi:[10.1089/hum.2009.064](https://doi.org/10.1089/hum.2009.064)
- Yamano S, Dai J, Moursi AM (2010) Comparison of transfection efficiency of nonviral gene transfer reagents. *Mol Biotechnol* 46:287–300. doi:[10.1007/s12033-010-9302-5](https://doi.org/10.1007/s12033-010-9302-5)
- Yamauchi J, Hayashi Y, Kajimoto K, Akita H, Harashima H (2010) Comparison between a multifunctional envelope-type nano device and lipoplex for delivery to the liver. *Biol Pharm Bull* 33:926–929
- Zhang M, Bai CX, Zhang X, Chen J, Mao L, Gao L (2004) Downregulation enhanced green fluorescence protein gene expression by RNA interference in mammalian cells. *RNA Biol* 1:74–77