Comparative study of BMP-2 gene delivery to Human adipose tissue-derived mesenchymal stem cells with Turbofect and Polyethylenimine

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Abstract. Genetic modification of mesenchymal stem cells (MSCs) with plasmid encoding the bone morphogenetic protein 2 gene (BMP2) is a crucial task in the development of gene therapy and tissue engineering technologies for bone regeneration. The low transfection efficiency of MSC cultures is a limitation for practical applications and makes it necessary to search for optimal protocols that ensure efficient gene delivery while maintaining sufficient cell viability. Comparison of two transfecting reagents (TurboFect and Polyethylenimine) shows that TurboFect is the most effective for MSCs transfection. A higher level of target gene BMP2 expression and osteogenic differentiation can be achieved using the TaqRFP-N-BMP2 plasmid compared with pcDNA3-BMP2.

1. Introduction

Transfection with plasmid is a promising approach of genetic modification of cells. Plasmids are less immunogenic, lower pathogenic and carcinogenic, have a relatively high packing capacity, which makes them more preferable for practical use in comparison with viral vectors. [1]. Transfection with plasmid is used in the development of in vivo and in vitro gene therapy methods and in tissue engineering. MSC cultures are often used in these methods since they have a high proliferative potential and the ability to differentiate into cells of bone, cartilage, adipose and other tissues [2, 3]. Of particular interest are MSCs transfected with BMP2 gene, which is an important inducer of osteogenic differentiation. Such genetically modified cells can be used for bone regeneration [4]. However, MSCs is a cell culture which is difficult to transfect. For better delivery of plasmids into cells transfection reagents which the most well-proven polycationic polymers, such as TurboFect (TF) and Polyethylenimine (PEI) are used [5, 6, 7]. On the other hand, the usage of vectors which sequence is modified in order to enhance transgene expression, increases the functionality of their application [8]. The development of effective MSCs transfection protocols that ensure high cell viability and the level of target genes expression is an important and relevant biotechnological task.
The aim of this work was to compare the efficiency of the most promising transfecting reagents - TF and PEI and vectors pcDNA3-BMP2 and TaqRFP-N-BMP2 for BMP2 gene delivery to human adipose tissue-derived mesenchymal stem cells (hAD-MSCs) for osteogenic differentiation.

2. Materials and methods

2.1. Cell culture
hAD-MSCs were obtained from lipoaspirate of healthy adult donors. The study was approved by the local ethics committee of the “RCMG” (protocol No. 4/2 dated 12.11.2019). AD-MSC were cultured in medium consisting of DMEM (“Paneco”, Russia) with 10% fetal bovine serum (FBS; PAA Laboratories, Canada), 0.584 mg/ml L-glutamine (“Paneco”), 10ng/ml rhFGF2 (Prospec, Israel), 5 000 U/ml heparin sodium (“Paneco”), 5 000 U/ml penicillin (“Paneco”) and 5 000 µg/ml streptomycin (“Paneco”) at 37 °C and 5% CO2.

For osteogenic differentiation hAD-MSCs were incubated after transfection in DMEM with 10% FBS, 0.584 mg/ml L-glutamine, 0.05 mg/ml L-ascorbic acid (Sigma, USA), 2.16 mg/ml β-glycerophosphate (Sigma), 5 000 U/ml penicillin and 5 000 µg/ml streptomycin at 37 °C and 5% CO2 for 14 d.

2.2. Plasmids
Plasmids encoding the reference protein EGFP gene (pEGFP-C1; Clontech, USA) or the target human BMP2 gene, such as pcDNA3-BMP2 and TaqRFP-N-BMP2 (“Eurogen”, Russia) were used. The plasmids were grown in Escherichia coli in a medium with selective antibiotics. Plasmid DNA was purified using the Plasmid Midiprep kit (“Eurogen”) following the manufacturer’s instructions.

2.3. Cell transfection
Cells were seeded into 24-well plate 24 h before transfection. TF (Thermo Fisher Scientific, USA) and PEI (linear 25 kDa, Polysciences, USA) were used for transfection. Cells were transfected according to the manufacturer’s instructions. For the formation of polyplexes TF/PEI and plasmids were incubated for 20 min at 37 °C. hAD-MSCs were cultured with polyplexes in 1 ml of DMEM containing 5% FBS, 0.584 mg/ml L-glutamine, 5 000 U/ml penicillin and 5 000 µg/ml streptomycin at 37 °C for 1 and/or 24 h. Analysis of the transfection efficiency was performed in 24 and 48 h after incubation. Analysis of osteogenic differentiation was performed in 14 d after incubation.

2.4. Cell viability assay
Cell viability after transfection was assessed by the MTT assay. Cells were incubated with 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; “Paneco”) for 2 h at 37 °C. Then the crystals of formazan were eluted using DMSO (“Paneco”) and the optical density was measured on xMark (Bio-Rad, USA) at a wavelength of 570 nm, subtracting the background value at 620 nm.

2.5. Flow Cytometry for EGFP analysis
To determine transfection efficiency cells were removed from the plates, centrifuged at 1200 rpm for 5 min and the number of cells synthesizing EGFP was counted on CyFlow® Space flow cytometer (Partec, USA). Analysis was performed using the FloMax software.

2.6. Real-time PCR
Expression levels of osteogenic differentiation markers such as BMP2, OCN, and OPN were analyzed by RT-PCR assay using “SYBR Green I” intercalating dye (“Eurogen”). Total RNA was isolated from cells using the RNeasy Plus Mini Kit (Qiagen, Germany). Synthesis of the first strand cDNA on the RNA matrix was performed using the RevertAid kit (Thermo Fisher Scientific). The analyzed genes expression levels were normalized by expression values of the reference genes: GAPDH и ACTB.
2.7. Alizarin red staining
Extracellular matrix mineralization (ECM) was detected by staining cells with Alizarin Red (Sigma). Cells were washed from the medium with phosphate-buffered saline (pH 7.4), fixed in 70% ethanol and stained with 40 mM alizarin red S solution pH = 4.1 for 5 minutes.

2.8. Statistical analysis
Statistical processing and graphing of the results were performed in SigmaPlot 12.0 (Germany). All data are expressed as the mean ± standard error (mean ± SD). Groups were compared using Student's t-test or Mann-Whitney U-test depending on the Gaussian distribution. Differences were considered statistically significant at the level below 5 % (p < 0.05).

3. Results and discussion
For the hAD-MSCs transfection two transfecting reagents from the class of cationic polymers, TF and PEI, which had previously demonstrated their effectiveness for the transfection of HEK293 [5, 6], HeLa [5] and BM-MSC [7], were chosen. A comparison of the cell transfection efficiency was made for 1 and 24 h of incubation using plasmid encoding EGFP. Previously, we had adjusted the conditions for MSCs transfection with polyplexes in a ratio of 4 µl/ml TF + 2 µg/ml pEGFP-C1 [9]. Polyplexes with PEI were obtained at two concentrations: 3 µg/ml PEI + 1 µg/ml pEGFP-C1 as recommended by the manufacturer and 6 µg/ml PEI + 2 µg/ml pEGFP-C1 which corresponds to the concentration of the plasmid during transfection with TF. It was shown that the most efficient transfection was observed when hAD-MSCs were incubated with polyplexes for 1 h. The number of transfected cells was 15.33±1.45% for 4 µl/ml TF + 2 µg/ml pEGFP-C1, 2.96±0.28% for 3 µg/ml PEI + 1 µg/ml pEGFP-C1 and 3.22±0.29% for 6 µg/ml PEI + 2 µg/ml pEGFP-C1 (figure 1a, b). Thus, TF is significantly more efficient in promoting the delivery of plasmids into hAD-MSC cultures than PEI.

![Figure 1](image_url)

Figure 1. hAD-MSCs transfection with TF/PEI with pEGFP-C1 plasmid during 1 or 24 h incubation. Transfection efficiency analysis was performed 24 h after incubation. a – Evaluation of transfection efficiency by fluorescence microscopy through the accumulation of EGFP in hAD-MSCs after 1 h. b – Evaluation of transfection efficiency by flow cytometry. *p <0.05 (vs the control).
The viability analysis of hAD-MSCs in 24 and 48 h after transfection showed that incubation of cells with complexes for 1 h had the least cytotoxic effect on cells than incubation for 24 h. The living cells count after 24 h was 76.98±5.31% for 4 µl/ml TF + 2 µg/ml pEGFP-C1, 71.55±7.11% for 3 µg/ml PEI + 1 µg/ml pEGFP-C1 and 43.01±7.42% for 6 µg/ml PEI + 2 µg/ml pEGFP-C1 (figure 2). Thus, the usage of PEI resulted in significantly higher cell death compared with TF. Therefore, hAD-MSCs were incubated with 4 µl/ml TF + 2 µg/ml plasmid and 3 µg/ml PEI + 1 µg/ml plasmid for 1 h for further analysis.

To assess the efficiency of the target gene expression two plasmids encoding the BMP2 gene, pcDNA3-BMP2 and TaqRFP-N-BMP2, were chosen. The sequence of the TaqRFP-N-BMP2 plasmid is optimized for expression in mammalian cells and the mRNA translation enhancement [10]. It was shown that after 14 d of hAD-MSCs transfection with polyplexes, BMP2 expression is significantly higher when using the TaqRFP-N-BMP2 plasmid compared with pcDNA3-BMP2 (figure 3). At the same time, TF also provides better transfection in comparison with PEI.

**Figure 2.** hAD-MSCs viability after 1 or 24 h incubation with TF/PEI with pEGFP-C1 plasmid. The results of MTT assay after 24 and 48 h after transfection are presented. *p < 0.05 (vs the control).

**Figure 3.** BMP2 relative expression after transfection of hAD-MSCs with pcDNA3-BMP2 and TaqRFP-N-BMP2 plasmids. The results of RT-PCR assay after 14 d after transfection are presented. *p < 0.05 (vs the control).

**Figure 4.** Expression levels of osteogenic differentiation markers were analyzed by RT-PCR assay after 14 d after transfection hAD-MSCs with pcDNA3-BMP2 and TaqRFP-N-BMP2 plasmids. *p < 0.05 (vs the control).
Analysis of the osteogenic effect of polyplexes with plasmids encoding the BMP2 gene revealed that the best osteogenic differentiation of hAD-MSCs was facilitated by polyplexes based on the TaqRFP-N-BMP2 plasmid in combination with TF. The usage of 4 µl/ml TF + 2 µg/ml TaqRFP-N-BMP2 led to a significant increase in the gene expression of osteogenic markers OCN (8.87±2.07) and OPN (2.85±0.82) (figure 4) and ECM mineralization after 14 d (figure 5) compared with the control, which indicated the development of osteogenic properties in hAD-MSC culture.

![Figure 5. ECM mineralization in hAD-MSCs detected by Alizarin Red staining. The assay was carried out 14 d after transfection of hAD-MSCs with pcDNA3-BMP2 and TaqRFP-N-BMP2 plasmids.](image)

4. Conclusions
The study has shown that TF is more preferable to use for transfection of hMSCs from adipose tissue. It provides a more efficient delivery of plasmids into cells while maintaining a sufficiently high level of viability of the transfected cells in contrast to the linear PEI. In addition, a higher level of BMP2 gene expression and osteogenic differentiation can be achieved using the TaqRFP-N-BMP2 plasmid compared with pcDNA3-BMP2. The selected transfection conditions (4 µl/ml TF + 2 µg/ml TaqRFP-N-BMP2 for 1 h) can be exploited for transfection of MSCs in tissue engineering and in gene therapy technologies to facilitate bone regeneration.

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References
[1] Park J, Ries J, Gelse K, Kloss F, von der Mark K, Wiltfang J, Neukam F W and Schneider H 2003 Bone regeneration in critical size defects by cell-mediated BMP-2 gene transfer: a comparison of adenoviral vectors and liposomes Gene Ther 10 1089-98
[2] Logovskaya L V, Bukharova T B, Volkov A V, Vikhrova E B, Makhnach O V and Goldshtein D V 2013 Induction of osteogenic differentiation of multipotent mesenchymal stromal cells from human adipose tissue Bull. Exp. Biol. Med. 155(1) 145-50
[3] Augello A and De Bari C 2010 The regulation of differentiation in Mesenchymal Stem Cells Hum. Gene Ther. 21(10) 1226-38
[4] Perez J R, Kouroupis D, Li D J, Best T M, Kaplan L and Correa D 2018 Tissue engineering and cell-based therapies for fractures and bone defects Front Bioeng. Biotechnol. 6 105
[5] Reed S E, Staley E M, Mayginnes J P, Pintel D J and Tullis G E 2006 Transfection of mammalian
cells using linear polyethylenimine is a simple and effective means of producing recombinant
adeno-associated virus vectors J Virol Methods 138(1-2) 85-98
[6] Rahimi P, Mobarakheh V I, Kamalzare S, SajadianFard F, Vahapour R and Zabihollahi R 2018
Comparison of transfection efficiency of polymer-based and lipid-based transfection reagents
Bratisl Lek Listy. 119(11) 701-5
[7] Gonzalez-Villarreal C, Fernandez S S, Dominguez A S, Rivas G P, Treviño E G, Rocha H R and
Rodriguez H M 2018 Bone marrow mesenchymal stem cells: improving transgene expression
level, transfection efficiency and cell viability J BUON 23(6) 1893-903
[8] Šimčíková M, Prather K L J, Prazeres D M F, and Monteiro G A 2015 Towards effective non-
viral gene delivery vector Biotechnol. Genet. En.g Rev. 31(1-2) 82-107
[9] Nedorubova I A, Bukharova T B, Zagoskin Y D, Vasilyev A V, Grigoriev T E, Mokroussova V
O, Chvalun S N, Goldshtein D V and Kulakov A A 2020 Development of osteoplastic material
impregnated with plasmid encoding bone morphogenetic protein-2 Biotekhnologiya 36(4) 59-
64
[10] pTagRFP-N vector https://evrogen.ru/products/vectors/pTagRFP-N/pTagRFP-N.pdf