Dynamics of Elimination of Plasmids and Expression of *VEGF121* Gene Transfected into Human Mesenchymal Stem Cells by Different Methods

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We compared two methods of transfection (lipofection and electroporation) with plasmid containing *VEGF121* gene in four cultures of mesenchymal stem cells from the human adipose tissue. The efficacy of transfection after 1 day, the dynamics of plasmid elimination after 3, 6, 9 days, and expression of the target gene were evaluated. Transfection by both methods failed in one of 4 cultures. Analysis of the plasmid elimination dynamics showed that the content of plasmids introduced by both methods decreased by 30-69% in all cultures by day 3 and then remained unchanged from day 3 to day 9. The expression of the target gene did not correlate with the content of plasmids in cells and varied by 2-10 times in control cells and cells transfected by both methods. Fluctuation of VEGF121 expression was not related to methylation.

Key Words: *mesenchymal stem cells; DNA methylation; elimination of plasmids; gene expression; transfection*

Mesenchymal human stem cells (MSC) produce a pronounced therapeutic effect (regenerative, anti-inflammatory, and immunomodulatory action), mainly via the paracrine mechanism. This effect can be attained via application of MSC transfected with genetic constructs, which can be an effective treatment for hereditary and acquired diseases.

The persistence and activity of genetic constructs introduced in MSC vary and depend on the construct and method of its introduction into the cells: from months (stable transfection) to weeks or days (transitory transfection) [4,14,15].

From the standpoint of safety of transfected MSC, non-viral transient transfection is more preferable. This approach allows avoiding insertional mutagenesis [11] and immunological reactions in the recipient [5]. Among the basic techniques, electroporation and lipofection are the most safe methods [6,7].

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In stable transfection, transgene expression may last for up to 6 months, in transient transfection it is usually limited by the life-time of the genetic construct in the cell. The latter depends on many factors: method of transfection, type of genetic construct, and source of MSC. For instance, adenoviral transfection of MSC with *VEGF165* (vascular endothelial growth factor) and *GFP* (green fluorescent protein) genes ensures gene expression for 14-24 days [4.13]. The duration of expression after lipofection is approximately the same: about 1 week for *HIF-1*α (hypoxia-induced factor) transfection [15] and about 2 weeks for *eGFP*-carrying plasmid [2].

In stable transfection, the level of expression is considerably modulated by epigenetic reactions (DNA methylation and histone deacetylation) [10,12]. In transient transfection, despite short life-time of the genetic construct in the cell epigenetic processes are involved in reducing the expression [3,8,9].

Here we studied the dynamics of plasmid elimination and expression of *VEGF121* gene introduced by electroporation and lipofection.

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MATERIAL AND METHODS

Experiments were performed on four cultures (MSC-1, MSC-2, MSC-3, and MSC-4). MSC-1 and MSC-2 were isolated from the adipose tissue of female donors during routine surgery (all donors signed informed consent for the use of their cells). Isolation and culturing of cells were performed as described previously [1]. The adipose tissue was a source of MSC-3, and MSC-4 (provided by JSC "ReMeTeks"). All cultures differentiated into osteogenic and adipogenic lineages and were immunophenotyped ($CD44^+$, $CD73^+$, $CD105^+$, $CD14^-$, $CD19^-$).

Transfection was performed with a plasmid carrying *VEGF121* gene (*pS450VEGF121*) under CMV promoter (provided by prof. B. S. Naroditsky, N. F. Gamaleia Institute of Epidemiology and Microbiology, Russian Academy of Medical Sciences). Transfection conditions were optimized using plasmid *pEYFP-C1* (Clontech). The cells (10^5) were resuspended in 100 μl buffer and electroporation was performed (400 V/mm, 55 μsec; Eppendorf Multiporator). Then, the cells were placed to 6-well plates or 25-cm^2 flasks. Lipofection with Unifektin-56 (Unifect Group) was performed after attaining 60-70% confluence (12 U Unifektin and 6 μg plasmid per 10-cm Petri dish or 25-cm^2 culture flask). After both methods of transfection, the medium was changed on the next day and then every 3-4 days.

DNA was extracted using DNA-Sorb-AM kit (InterLabServis) on days 1, 3, 6, and 9 after transfection. The samples were dissolved in Tris-EDTA buffer and stored at -20°C.

RNA was isolated by standard phenol/chloroform method with additional DNase (Promega) treatment.

DNA and RNA isolated from untransfected samples of all cultures served as negative control.

cDNA synthesis was performed using M-MuLV ("SibEnzyme).

DNA (5 μl) was added to the reaction mixture (25 μl) containing 300 nM primers, 200 μM deoxynucleotide triphosphate, 3 μM Mg^{2+}, 1× working solution of SYBR Green I (Invitrogene), and 0.04 U Taq-polymerase. Real-time PCR was performed on a CFX96 system (Bio-Rad). The reaction protocol was as follows: 3 min at 95°C and 45 cycles (5 sec at 95°C, 10 sec at 60°C, 20 sec at 72°C). The following primers were used: *GAPDH* (F: 5'-AAGGTCGGAGTCAACGGATTT-3', R: 5'-CCAGCATCGCCCCACTTGA-3'), *VEGF121* (F: 5'-CCTTGCTGCTCTACCTCCAC-3', R: 5'- GATGATTCTGCCCTCCTCCTT-3') and *actin β* (F: 5'-CCTGGCACCCAGCACAAT-3', R: 5'-GGGCCGGACTCGTCATAC'3').

DNA samples and control samples for methylation status analysis were treated with restriction endonuclease *Hind*III flanking CMV promoter. After restriction, all samples except positive controls were purified using DNA-Sorb-AM kit (InterLabServis).

The cut plasmid was treated with methylase *M.Sss1* (Sibenzim); DNA was isolated using DNA-Sorb-AM kit (InterLabServis).

Bisulfite conversion of DNA (300 ng) was performed using EZ DNA Methylation Kit (Zymo Research). The efficiency of bisulfite conversion was calculated using Quantification Tool for Methylation Analysis (QUMA) software (http://quma.cdb.riken.jp).

PCR was carried out using CMV promoter-specific primers. Converted DNA (5 μl; in dilutions of 10^{-3} and 10^{-1} for the control and experiment, respectively) was mixed with 200 nM of each primer (F: 5'-TGTTTTTTGTTTGTGTGTTGGA-3', R: 5'-ATACCAAAACAAACTCCCATTA-3') in 25 μl reaction mixture containing 200 μM deoxynucleotide triphosphate and 0.04 U Taq-polymerase. PCR conditions: 2 min at 95°C, 5 cycles (10 sec at 95°C, 30 sec at 62°C, 30 sec at 72°C), 40 cycles (10 sec at 95°C, 30 sec at 58°C, 30 sec at 72°C), 2 min at 72°C; controls: 20 cycles. Electrophoresis was performed in 1.8% agarose gel; amplicons were cut from the gel and isolated using DNA Extraction Kit (Fermentas).

Sequencing was carried out with 3130 Genetic Analyzer (Applied Biosystems). Electrophoregrams were analyzed using Sequence Scanner 1.0 software (Applied Biosystems).

The relative content of *VEGF121* in MSC was calculated as the ratio of relative concentrations of *VEGF121* and *GAPDH* obtained during real-time PCR. The relative amount of mRNA *VEGF121* in MSC was calculated by standardized expression (ΔΔC (t)) for genes *GAPDH* and *Actin β*. The calculations were performed using CFX96 software (Bio-Rad). The data are presented as mean±confidence interval ($p<0.05$). Statistical calculations were performed using Statistica 6 software (StatSoft).

RESULTS

Transfection efficiency. The results of experiments on transfection efficiency (day 1) and plasmid dynamics (days 3-9) are presented in Table. 1. Transfection of culture MSC-4 by all the used methods failed (Table 1), despite the fact that transfection was carried out in parallel with the culture MSC-3. For this reason, the data obtained on this culture were excluded from further analysis. After standardization by *GAPDH* gene DNA, the lipofection efficiency in cultures was similar and ranged from 1.27 to 1.92, the efficiency of electroporation varied in a greater range (from 0.39 to 2.51). The efficiency of transfection by both methods was minimum for MSC-2 culture and maximum for MSC-3 culture. The efficiency of transfection by both

TABLE 1. Content of Plasmids in MSC Cultures at Different Terms after Transfection (*M*±*m*)

MSC culture	Method of transfection	Relative amount of *VEGF121*							
		day 1		day 3		day 6		day 9	
		abs.	%	abs.	%	abs.	%	abs.	%
MSC-1	Lipofection	1.56±062	100	0.46±0.34	29.5	0.36±0.11	23.1	0.22±0.10	14
	Electroporation	1.59±1.02	100	0.52±0.38	32.7	0.34±0.17	21.4	0.20±0.145	12.6
MSC-2	Lipofection	1.27±0.97	100	0.67±0.24	52.8	0.33±0.32	26	0.08±0.07	6.3
	Electroporation	0.39±0.79	100	0.27±0.31	69.2	0.27±0.31	69.2	0	0
MSC-3	Lipofection	1.92±0.49	100	0.98±0.16	51	1.00±0.20	52	1.18±0.25	61.5
	Electroporation	2.51±0.15	100	0.70±0.50	27.9	0.78±0.20	31.1	0.69±0.29	27.5
MSC-4	Lipofection	0	0	0	0	0	0	0	0
	Electroporation	0	0	0	0	0	0	0	0

methods in different cultures and for different methods was similar ($p>0.05$).

Dynamics of plasmid elimination from MSC. Analysis of the dynamics of plasmid elimination from different cultures revealed substantial individual differences (Table 1). Significant differences in the content of plasmids on days 1 and 3 after transfection by both methods were revealed for MSC-1 and MSC-3 ($p<0.05$). After lipofection, the number of plasmids in MSC-3 was significantly higher than in other cultures on day 9 and than in MSC-1 on day 6. After electroporation, significant differences between this culture and MSC-2 were revealed on day 9.

Individual differences, in our opinion, do not allow using the same standard protocol for transfection of all cultures. In each case, optimization is required, which considerably complicates cell therapy with the use of MSC transfected by nonviral methods.

All cultures for each transfection type were presented by homogeneous groups, which allowed us to pool them (Fig. 1). The number of plasmids in cells transfected by electroporation and lipofection decreased by day 3 and then remained practically unchanged.

At all stages of observation, the content of plasmids in transfected cells was significantly higher than in the control and did not depend on the method of transfection. The number of plasmids in cells decreased 3-fold over the first 3 days after transfection (significant difference from day 3) and then decreased by 5% from baseline every 3 days (insignificant differences).

Evaluation of the expression of transfected VEGF121 gene. After electroporation, expression on days 1 and 3 significantly differ from the control. On days 6 and 9, the differences were not detected (Fig. 2). After lipofection, no differences in the expression from the control were found at all terms. At the same time at all stages analyzed, the mean level of *VEGF121* expression after lipofection was 1.5-2 times higher than in the control. Analysis of the correlation for each culture separately and integrally for both methods of transfection revealed no relationships between plasmid content in cells and expression levels (r=-0.2, $p=0.8$ for electroporation and r=-0.6, $p=0.4$ for lipofection). It was also noted that the expression level of the target gene in the control samples on days 6 and 9 differed from the corresponding value on day 3. Enhanced expression of the target gene on days 1 and 3 after electroporation could be caused by increased content of plasmids in MSC. Further studies are required for verification of this assumption.

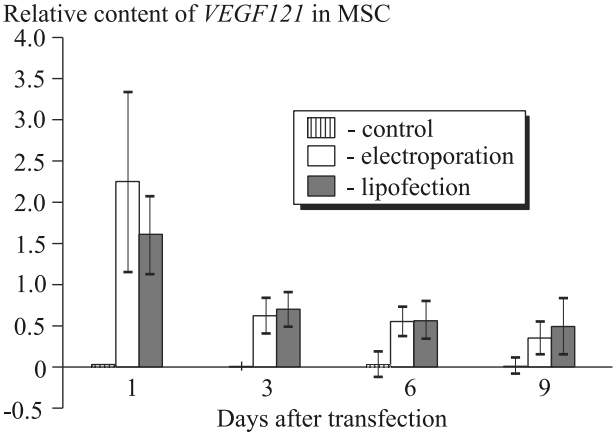

Fig. 1. Dynamics of elimination of plasmid with *VEGF121* gene after lipofection and electroporation (summary data for all cell cultures).

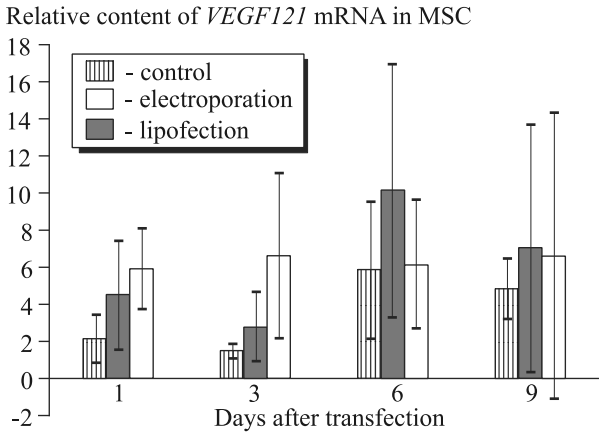

Fig. 2. Dynamics of expression of the *VEGF121*gene after lipofection and electroporation in all cell cultures (normalized by *GAPDH* and *Actin* β genes).

VEGF121 expression levels in the control and after lipofection varied at different terms, while after electroporation they remained practically the same.

We also noted a 2-4-fold increase in *VEGF121* gene expression on day 6 in both transfected by lipofection and in untransfected cells (in the latter case $p<0.05$). This can be explained by the fact that culture medium was changed on the same or next day, *i.e.* cells were cultured in depleted medium. Increased expression of *VEGF121* appears to be a normal reaction to changed culturing conditions.

The data on plasmid elimination from MSC suggest that the content of plasmids in cells was significantly higher than in controls even on day 9, but the level of expression of days 6 and 9 did not differ from the control. The question arises as to whether methylation of CMV promoter affects the expression level.

Methylation of four CpG-pairs (2, 5, 6 and 7) in CpG site of CMV promoter was evaluated on days 1, 3, and 6 after transfection. The degree of bisulfite conversion for samples selected on days 1 and 3 was 100% and for a sample selected on day 6 it was 92.3%.

CpG-dinucleotides of CMV promoter were only slightly susceptible to methylation (Table 2). The second CpG-dinucleotide always contained two peaks (thymine and cytosine), which attested to partial methylation of this base pair. This methylation level should not affect the function of the genetic construct.

Epigenetic effects of the cells on plasmids were not confirmed. Methylation was negligible and, in most cases, partial, and hardly could influence the expression of the inserted gene.

Lipofection and electroporation are not considered to be the most effective methods of MSC transfection, but they seem to be the safest method for subsequent cells application in therapy. Developers of commercial kits for both methods offer optimized transfection protocols for various types of cells, but in our experiments these proposals have not been confirmed. Further search for effective methods of non-viral transfection of MSC is required.

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TABLE 2. Methylation of CpG-Pairs

No. of CpG-pair	Time after transfection, days			Negative control	Positive control
	1	3	6		
22	C	C	C	T	C
55	T	T	T	T	C
66	T	T	T	T	C
67	T	T	C	T	C

Note. C: cytosine, T: thymine.

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