Engineering of BDNF-producing human mesenchymal stem cells with use of polyamidoamine dendrimers

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Abstract

We report the use of polyamidoamine (PAMAM-NH_2) dendrimers alongside with other non-viral vehicles for the in vitro transfection of human bone marrow mesenchymal stem cells (hMSCs) and engineering MSCs for brain-derived neurotrophic factor (BDNF) secretion. Different generations of cationic polyamidoamine dendrimers (generations 3-6) without and with some modifications (neutral PAMAM G4-OH, PAMAM G4 with 25% of hydrophobic $-CH_3$ chains) were first tested on HEK 293T cells. PAMAM-NH_2 G4 dendrimers and Lipofectamine 2000 were next used for hMSCs' transfection and provided GFP reporter expression in around 6% and 20% of MSC, respectively. Both vehicles were then shown to provide BDNF expression in MSCs from bicistronic cassette. Non-viral neurotrophin expression in MSCs can be a safe and easy option to improve autologous stem cells for the therapy of diseases and injuries of neural system.

Keywords: human mesenchymal stem cells, transfection, dendrimers, BDNF

Introduction

Human MSCs represent pluripotent adult stem cell population, which is routinely isolated from bone marrow and under the appropriate conditions can be expanded in vitro while retaining their potential to differentiate into a number of tissue lineages like chondrocytes, osteoblasts, adipocytes, myoblasts, hepatocytes, and presumably even neural tissue. Genetic modification of hMSCs may direct and control their growth and differentiation and provide expression of trophic, anti-tumor or other factors, thus making hMSCs a promising tool in cell-based gene therapy.

Cell-based therapy involving expression of neurotrophic factors can facilitate trophic support to recipient with neurotrauma, neurodegenerative disease and other kinds of pathology. Ronsyn and co-workers [1] have shown that transplantation of multineurotrophin-expressing precursor cells promotes enhanced re-myelination and electrophysiological recovery after traumatic spinal cord injury (SCI) in rats. Grafting of olfactory ensheathing glia transduced with adenovirus vectors

encoding BDNF or neurotrophin-3 (NT-3) reduced lesion volumes in animals with unilateral transection of the dorsolateral funiculus [2]. Other neurotrophic molecules including CNTF and GDNF have also been implicated in neuroprotection and axon outgrowth [3,4]. By now, more than 30 neurotrophic factors have been identified and only a small subset of these tested [5]. We have chosen BDNF, a neurotrophin of the nerve growth factor family, for plasmid-based expression in hMSC since it plays an important role in survival, differentiation, growth and function of human neurons [6]. BDNF can also trigger the differentiation of pluripotent neural crest cells into sensory neurons, is important for myelin formation in peripheral nerve during development [7] and increases the expression of regeneration-associated genes within the cell bodies of the injured axons [8].

In present study, we examined a number of non-viral carriers, including linear polyethylenimine, dendrimers and lipid-based vehicle to produce BDNF-secreting human MSCs, with a focus on polyamidoamine (PAMAM) dendrimers. The advantages of non-viral gene carriers are their relative ease of use and safety, including the avoidance of potential immunogenecity, toxicity and the reversion of the virus to wild type. Non-viral vehicles do not imply transgene insertion into the host genome, thus avoiding the risk of genome destabilization and oncogene activation. Plasmid-based neurotrophin expression in MSCs can be a safe and easy option for MSCs' clinical use in treatment of neuro-degenerative diseases and neural injuries.

Materials and Methods

Vector construction and preparation
All optimization experiments were performed with use of plasmid vector pAAV-IRES-hrGFP (further referred to as pGFP, from Stratagene). Human-recombinant green fluorescent protein (hrGFP) gene was used as a reporter to monitor transfection effectiveness. Multiple cloning site (MCS) and internal ribosome entry site (IRES) sequence from encephalomyocarditis virus (EMCV) upstream of hrGFP allow insertion of gene of interest and its further expression under CMV promoter. We have inserted human *bdnf* sequence (791 bp) into pGFP MCS at the restriction sites *Cla*I and *Eco*RI (recognition sequences were added to 5' termini of amplification primers) by routine molecular cloning procedures. Generated vector, designated as pBDNF-IRES-hrGFP, was confirmed by restriction digest mapping and sequence analysis. Both pGFP and pBDNF-IRES-hrGFP plasmids were propagated in *E. coli* strain DH5α and isolated by Plasmid Maxi kits (Qiagen) according to the manufacturer's instructions. Purified plasmid DNA with the A_{260}/A_{280} ratio of 1.8 was used for transfection.

Cell culture
Human embryonic kidney 293T cells (HEK 293T) and human bone marrow mesenchymal stem cells (hMSCs) cells were grown in DMEM-Glutamax (Gibco) with 10% heat-inactivated FBS (HyClone). Cells were routinely maintained on plastic tissue culture flasks and plates (Falcon) at 37°C in a humidified atmosphere containing 5% CO_2/95% air. Adult human bone marrow was harvested from routine surgical procedures (pelvic osteotomies) following informed consent, diluted 10-fold in phosphate-buffered saline (PBS) and separated by centrifugation on Ficoll-Paque layer. After centrifugation at 3000g for 30 min, the mononuclear cell layer was recovered from the gradient interface and washed with PBS. The cells were then centrifuged at 1500g for 30 min and resuspended in complete culture medium. The hMSC phenotype was confirmed by FACS analysis with CD90 and CD105 (positive), as well as CD34 and CD45 (negative) using FACS-scan analytical flow cytometer (Becton Dickinson).

Transmission electron microscopy (TEM)
A copper grid of 200 grid mesh precoated with carbon was glow discharged. The solution of a sample was dropped to grids and the grids were touched to filter paper to remove excess liquid, then air-dried under a heat lamp. Then the grids with a sample were stained with 2% uranyl acetate to enhance the contrast between aggregates and background. The observation was made using a Jeol JEM-1010 (Japan) electron microscope.

Ethidium bromide intercalation assay
Ethidium bromide in concentration of 1 molecule of dye per 1 bp was added to plasmid solution its fluorescence was monitored by JASCO-FP 6300 spectrofluorimeter (JASCO GmbH, Germany). The excitation wavelength was 477 nm. Excitation and emission slits were 10 and 5 nm, correspondingly. The emission spectra were recorded between 500 and 800 nm and the position of emission maximum in these spectra was determined. Then 'dye/ plasmid' complex was titrated by a dendrimer and the changes of fluorescent parameters (intensity and λ_{max}^{em}) were recorded.

Transfection experiments
PAMAM-NH$_2$ G3, G4, G5 and G6 dendrimers (EDA core) were obtained from Dendritic NanoTechnologies (Mount Pleasant, Michigan, USA). Generation 4 PAMAM-OH dendrimers and PAMAM dendrimers with 25% hydrophobic chains (further referred to as PAMAM-25%) were obtained from Sigma-Aldrich. Lipofectamine 2000 was obtained from Invitrogen (USA), and linear polyethylenimine (PEI) Exgen-500 was obtained from Fermentas (Lithuania).
HEK 293T (3×10^4 cells/well) cells were seeded in 24-well plates in 1 ml of medium. hMSCs (5×10^4 cells/well) were seeded in 6-well plates in 3 ml of medium. All cells were allowed to grow for 2-3 days before transfection to 65-70% confluence. For HEK 293T transfection, complexes of plasmid DNA (2 ug) and each of dendrimers (G3, G4, G5 and G6) at charge ratio of 1:1 were prepared in 100 ul of 150 mM NaCl solution and the mixtures were incubated for 15 min at room temperature. For hMSC wells, 10 ug of plasmid DNA was diluted in 200 ul of 150 mM NaCl. The medium was replaced by FBS-free medium before transfection. Following 2.5 h treatment of DNA-dendrimer complexes, the medium was replaced by DMEM-Glutamax (Gibco) with 10% heat-inactivated FBS. Transfections of hMSCs with Exgen-500 and Lipofectamine 2000 were made in serum-free medium according to the manufacturer's instructions. hrGFP fluorescence was monitored by microscopy, and percentage of GFP-positive cells was analysed upon fixation with 2% paraformaldehyde by FACS-scan analytical flow cytometer (Becton Dickinson).

Neurotrophin release analysis
Secretion of BDNF protein into the medium was assessed *in vitro* using enzyme-linked immunosorbent assay (ELISA). Supernatant samples were collected 24 hours after transfection and analysed with use of BDNF E$_{max}$® ImmunoAssay System (Promega, USA) according to manufacturer's instructions. Samples from each well were analysed in 3 replicates and pooled for statistical analysis.

Statistics
Results are presented as mean±SE (standard error), n=6. Data were analysed by one-way analysis of variance (ANOVA) with posthoc Newman-Keuls test.

Results

With the aim to investigate plasmid-based solutions for neurotrophin secretion in human MSC, we have tested a number of gene carriers with different transfection mechanisms – PEI, dendrimers and lipid-based vehicle. To select optimal dendrimer generation for gene delivery to human MSCs, we first examined biophysical properties of several middle generation dendrimers and their transfection efficiency on HEK293T cell line.

Biophysical characterization of the dendriplex PAMAM G4-pGFP was made by ethidium bromide intercalation assay (Supportind Information, Figure 4) and by electron microscopy (Supporting information, Figures 5 and 6). The data showed that dendriplex formed has the diameter of 150-200 nm and formed at dendrimer:pGFP charge ratio of 1:1. This ratio was used for further transfection. To compare transfection efficiency of several generations of dendrimers (G3, G4, G5, G6, G4-OH, and G4-25%), we used HEK 293T cells as a common standard for a variety of transfection agents. Transfection results are presented in Figure 1. Representative FACS analysis for transfection using dendrimers PAMAM G4 is presented in Appendix A. Supporting Information (Figure 7).
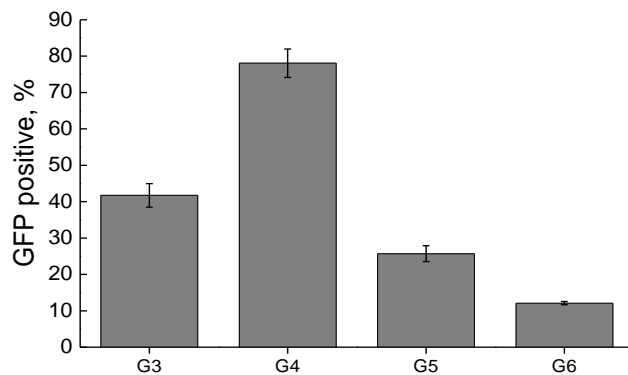

Figure 1. Efficiency of transfection of pGFP by PAMAM G3-G6, PAMAM G4-OH, and PAMAM G4-25% dendrimers in HEK 293T cells. Results are presented as Mean±S.E.M. of 6 experiments. The difference between groups is significant at p<0.05 level by one way ANOVA with posthoc Newman-Keuls test.

As follows from Figure 1, PAMAM G4 dendrimer showed maximal transfection in comparison with PAMAM G3, G5 and G6 dendrimers. Mean fluorescence intensity (MFI) of transfected cells did not differ significantly between dendrimer generations (data not shown). Since G4 dendrimer was the most effective agent, we next tested its alternative modifications, PAMAM-OH and PAMAM-25%. Based on results of analysis we have chosen PAMAM-NH$_2$ G4 for further studies.

In the next set of experiments, we assessed the capacity of PAMAM-NH$_2$ G4 dendrimer for pGFP delivery to human MSCs in comparison with linear PEI and Lipofectamine 2000. All three transfection reagents performed very well in HEK293T cell line (each above 75%, data not shown). However, their effectiveness on hMSC differed significantly (P<0,01), with 6,43±0,85% for PAMAM dendrimer and 20,9±2,1% for Lipofectamine 2000 (Figure 2), whereas linear PEI transfection level was 0,08±0,01%.
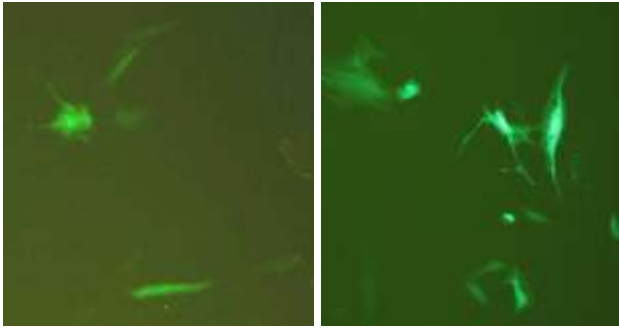
Figure 2. Transfection of hMSCs with G4 dendrimer/DNA complex (left) and Lipofectamine2000 (right).

At the third step of our studies we analyzed the delivery and expression of plasmid pBDNF-IRES-hrGFP in human MSCs and measured the release of BDNF into culture medium. PAMAM G4 dendrimer and Lipofectamine 2000 were used for transfection, whereas linear polyethylenimine was excluded from further studies due to its low efficiency in MSCs. The results of these transfections are presented at two-scale diagram in Figure 3 together with BDNF release level, measured by ELISA.
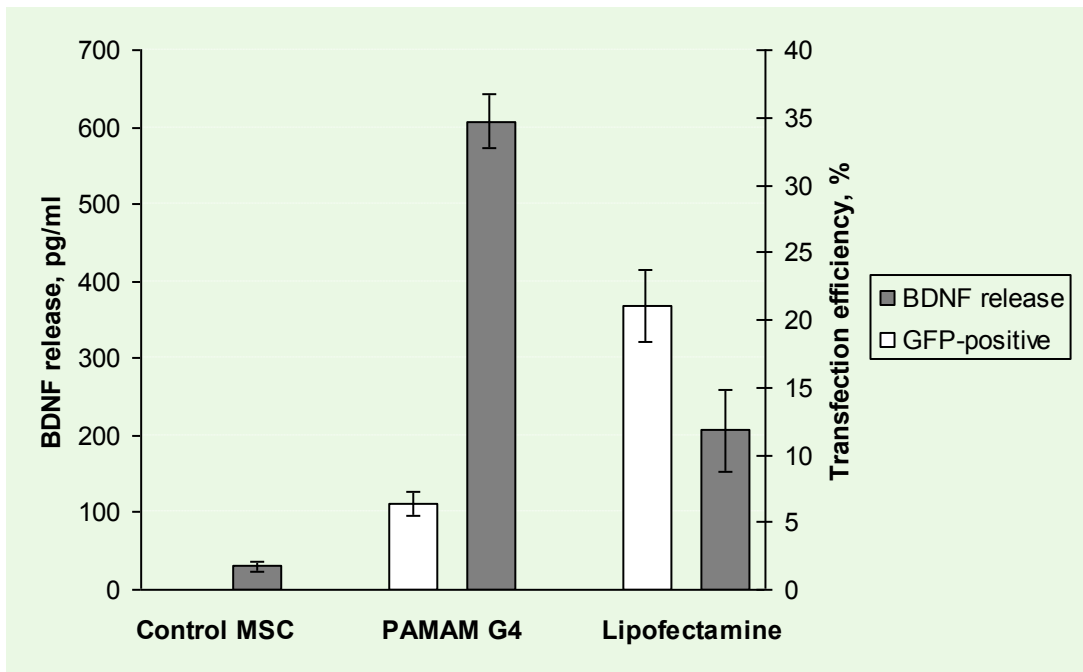

Figure 3. Transgene delivery and BDNF secretion in hMSCs, transfected with pBDNF-IRES-hrGFP plasmid vector.

As presented on Figure 3, mean BDNF secretion levels for G4 dendrimer and Lipofectamine 2000-transfected hMSCs were 607 and 206 pg/ml, respectively. The values differed significantly ($p < 0.01$, one way ANOVA with posthoc Newman-Keuls test). Such difference was not the case in HEK293T, where secretion levels were 8,4 and 8,57 ng/ml for G4 dendrimer and Lipofectamine-mediated delivery, with mean transfection efficiencies of 72% and 78%, respectively.

Discussion

A number of transfection vehicles was proposed for primary and stem cell transfection, each having unique properties facilitating gene delivery. Particularly, polyethylenimine is a polymer with high cationic-charge density, which effectively condenses DNA. PEI/DNA complexes were shown to interact with cell surface proteoglycans (syndecans) resulting in internalization by endosomes. Lipid-based vehicles, known to deliver DNA in membrane-merging liposomes, were presented in our study by Lipofectamine, proven effective in many studies [9,10].

The dendrimers were also found to self-assemble electrostatically with plasmid DNA, forming nanometer-scale complexes. Specifically, PAMAM dendrimers used in our study are based on an ethylenediamine core and branched units are built from methyl acrylate and ethylenediamine. The first attempts to analyze DNA delivery using PAMAM dendrimers with EDA core were made in the work of J. Haensler and F.C. Szoka [11], where PAMAM G2-G10 with EDA core showed themselves as effective delivery vehicles for transfection of pCLUC4 plasmid encoding firefly luciferase into adherent cell lines CV-1 (monkey fibroblast). Moreover, dendrimers of higher generations were more effective than smaller ones. In contrast, in paper [12] PAMAM G5 dendrimers showed higher efficiency than PAMAM G10 dendrimer for the transfection of COS-1 cells (monkey kidney fibroblast) and PAMAM G5, G7 and G10 dendrimers were more or in the same degree effective than Lipofectamine for a wide range of cells. Later in paper [13] authors showed the higher transfection efficiency of PAMAM G5 dendrimer in comparison with PAMAM G2 dendrimer (EDA core) for transfection of lacZ gene in cell lines U937 (human macrophage-like) and NIH/3T3 (mouse fibroblast).

To address the question on dependence of efficiency of transfection on dendrimer generation, we analyzed the transfection of reporter plasmid pGFP by PAMAM G3-G6, PAMAM G4-OH, PAMAM G4-25% dendrimers with EDA core into model HEK293T line. The third, fourth, fifth and sixth generations of PAMAM-NH_2 dendrimers (PAMAM G3, G4, G5 and G6) possess 32, 64, 128 and 256 surface amino groups, respectively; their molecular weights are respectively 6.9, 14.2, 28.8 and 60 Da, with corresponding molecular diameters 3.1, 4, 5.3 and 6.7 nm. PAMAM G4-OH differs from PAMAM G4 by surface groups – it has 64 surface -OH groups which are neutral at pH 7.4. PAMAM G4-25% has 48 NH_3^+ groups (75%) and 16 CH_3 groups (25%) [11–13]. As follows from Figure 1, the maximal transfection rate (up to 80%) was shown by PAMAM G4 dendrimer. G3 showed ~41%, G5 - ~22%, G6 - ~13%, G4-OH - 0%, G4-25%~0.2%. Our results are in contradiction with above mentioned results of J. Haensler and F.C. Szoka [11], but in a good agreement with other authors [12,13]. For explanation of container properties of PAMAM dendrimers Svenson and Tomalia advanced an opinion on the balance between shape of dendrimers, flexibility of their surface groups and surface charges [14]. At this, maximal container properties had the dendrimers of 'middle' generations – 4th and 5th. Seemingly, we observe a similar law: 'middle' dendrimers are more effective than lower and higher generations of dendrimers.

Optimization on HEK cells allowed us to select from middle-generation dendrimers the one with highest transfection efficiency - PAMAM-NH_2 G4 dendrimer. Alternative modifications of G4 dendrimer - PAMAM-OH and PAMAM-25% - differed from PAMAM-NH_2 both in the kinetics of complex formation and transfection efficiency, which may evidence the predictive value of ethidium bromide intercalation assay for vehicles with essential biochemical differences. However, kinetic disrepancies between generations of PAMAM with NH_2 active groups – G3, G4, G5 and G6 – were not sufficient to predict their transfection rates.

We next compared transfection efficiencies of PAMAM-NH_2 G4 dendrimer and widely used commercial reagents – linear PEI-based Exgen500 and lipid-based Lipofectamine 2000 on MSCs

from human bone marrow. Transfection of hMSCs with the G4/pGFP dendriplex resulted in 6,43±0,85% GFP-positive cells *versus* 20,9±2,1% for Lipofectamine 2000 and less than 0,1% for linear PEI. G4/pGFP dendriplex and linear PEI had some advantage in terms of cell viability, possibly due to lower genotoxic effects. Thus, PAMAM G4 dendrimer and Lipofectamine 2000 as relatively effective transfection agents were selected for generation of BDNF-secreting MSCs, whereas linear polyethylenimine was excluded from further studies due to its low efficiency in hMSCs. Noteworthy, the efficiency of PAMAM G4 dendrimer in human MSCs in our research is in contradiction with the results of Santos *et al.* [15], reporting very low levels of hMSC transfection efficiency for G5, G6 and G7 dendrimers and no effect of lower generations. Despite reported low efficiency, dendrimer-mediated transient expression of BMP-2 was sufficient for osteogenic differentiation of MSCs [15].

To provide efficient BDNF production in hMSCs, we have constructed pBDNF-IRES-hrGFP plasmid, allowing the expression of human *bdnf* gene under potent CMV promoter with chicken β-globin intron as an enhancer. Simultaneous expression of humanized recombinant green fluorescent protein (hrGFP) from the same transcript via the IRES-driven second open reading frame serves as a marker for both transfection efficiency and *bdnf* transgene expression. BDNF secretion into culture medium was assessed using routine ELISA protocol. hMSC transfection rates for pBDNF-IRES-hrGFP plasmid vector did not differ significantly from those for pGFP for either PAMAM G4 and Lipofectamine 2000, respectively.

We found that both vehicles delivered pBDNF-IRES-hrGFP plasmid into hMSCs and provided BDNF secretion in the medium. Interestingly, despite significantly higher transfection efficiency and MFI (5323±234 a.u. for Lipofectamine *vs* 1981±189 a.u. for G4), Lipofectamine-transfected cells showed markedly lower BDNF release (206±53,5 pg/ml) compared to PAMAM G4 dendrimer (607±34,9 pg/ml). Standard errors given also suggest that dendrimer-driven BDNF expression was more consistent between the experiments. We found this effect to be specific for MSCs, whereas pBDNF-IRES-hrGFP-transfected HEK293T cells showed almost equally high transfection level and BDNF expression for both delivery vehicles.

Reduced BDNF release by Lipofectamine 2000-transfected MSCs can be possibly explained by higher sensitivity of stem cells to the influence of lipid-based transfection on membane properties and BDNF release mechanism, or vector readout inhibition due to higher GFP synthesis. Unlike other neurotrophins from the same family NT-3 and NGF, BDNF is normally expressed within the regulated secretory pathway from dense core vesicles [16]. Possible distruption of binding with sorting receptor carboxypeptidase E or endosomal protein sortilin may cause reduction of BDNF sorting into the vesicles, as well as the interference of lipid-based reagent with granule maturation and release.

Despite the unexpected difference between the two delivery agents, neurotrophin release level, provided by either vehicle, was in general in a good agreement with the data on plasmid-based NT-3 expression in human MSCs by Ronsyn and co-workers [1]. Lipofectamine-mediated BDNF expression in neuroendocrine cells AtT-20 (~0,2 ng/ml), reported by Heymach and colleagues [9], was even closer to our results in MSCs. Adenoviral transduction of MSC at MOI of 300 and 1000 have also resulted in comparable BDNF production levels (434 and 931 pg/ml, respectively) [17].

Transient expression of neurotrophins is required by neurons in the vicinity of their final targets to sustain their survival and complete differentiation in development [18] and may be useful in SCI therapy [6]. Importance and beneficial effects of BDNF, delivered by injections, hydrogels, viral vectors or cell-based gene therapy, were reported in many studies on neurologic injuries and diseases [19–21]. Grafting of BDNF-transduced MSCs to cystic sites of SCI resulted in a significant increase in the extent and diversity of host axonal growth, including

serotonergic, coerulospinal, and dorsal column sensory axons [22]. Intracerebral injection of BDNF-transfected MSC resulted in improved function and reduced ischemic damage in a rat model of middle cerebral artery occlusion, indicating a possible usefulness of this approach for the treatment of stroke [23].

Some authors also suggested that over-expression of neurotrophic factors may direct the differentiation of human MSCs to neural lineage [24,25]. Particularly, BDNF-transduced hMSCs could convert MSCs into neuronal cell types, demonstrating the expression of neural markers and appropriate electrochemical properties [25]. However, the possibility of terminal neuronal differentiation of MSCs is still considered as controversial [26].

In conclusion, transplantation of BDNF-transfected MSCs may benefit the outcome of neurologic injury by neuroprotection, stimulation of axonal regeneration, and possibly cell replacement due to trans-differentiation. We have proposed plasmid-based solution for BDNF expression in human MSCs using various non-viral delivery agents. Development of a rapid, easy and safe protocol for neurotrophin expression in autologous MSC may facilitate trophic support and/or neural differentiation of MSC, paving a way to cell-based therapy of acute nerotraumas and other neurologic diseases.

Appendix A. Supporting Information
Supporting Information associated with this article can be found, in the online version, at

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