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Original Article

Efficient Modified-mRNA Transfection in Neural Stem Cells

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ABSTRACT

Introduction: Neural stem cells (NSCs) are multipotent stem cells residing in the central nervous system that is capable of self-renewal to support ongoing requirements for neurogenesis in the adult brain. Since NSCs are considered potential candidate cells for neuro-regenerative medicine, applying safe induction methods for them is very important. Synthetic modified-mRNA (mmRNA) as an alternative to traditional DNA- or protein-based methods, is regarded as a powerful tool for inducing short-term gene expression in cells with no genetic manipulation.

Methods: Here, we aimed to develop an optimized condition for mmRNA transfection in primary NSCs. In vitro-transcribed EGFP mmRNA (mmRNAEGFP) was delivered to human embryonic kidney cells (HEK293T) and mouse NSCs by using two commercial agents, Lipofectamine-2000 (LF2000) and TransIT. Also, a plasmid DNA was used to transfect cells considered EGFP-expressing positive control. In addition, the poly(A) tail (poly adenosine tail) elongation and chloroquine (CQ) treatment were performed to improve transfection efficiency. Finally, flow cytometry, fluorescence microscopy, and MTT assays were performed to assess the cells.

Results: In comparison with HEK293T, NSCs were very sensitive to transfection, the efficacy of transfection using DNA/LF2000 was higher in HEK293T cells, but mmRNAEGFP/TransIT showed better transfection efficacy in NSCs. Poly(A) tail elongation; also, treating the cells with CQ before transfection significantly improved its efficacy.

Conclusion: The mmRNA poly(A) tail elongation and the use of specific transfection agents in combination with TLR inhibitors can lead to a more effective transfection in NSCs.

Keywords:
Modified-mRNA
In vitro transcription
Neural stem cell
Chloroquine
Transfection
Cell culture

Introduction

Neural stem cells (NSCs) have been identified as striking therapeutic candidates for neurodegenerative disorders (Ottoboni et al., 2020; Rietze and Reynolds, 2006). However, the development of safe and effective induction methods for their differentiation is an important challenge (Joo et al., 2012; McLenachan et al., 2013; Shih et al., 2002). Over the last few years, various

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differentiation induction techniques based on the application of DNA, RNA, and protein, were examined to provide sources of footprint-free cells for regenerative applications (Badieyan and Evans, 2019). NSCs can be genetically engineered to generate functional cells, such as dopaminergic or cholinergic neurons. A handful of genetic methods are applied for this purpose, amongst which, modified-mRNA (mmRNA) is possibly the only safe method available. Few studies have utilized mmRNA for the production of neuronal cells or inducing neuronal differentiation in candidate cells (e.g. conversion of adult human fibroblasts into neural precursor cells) (Connor et al., 2018).

Typically, adult fibroblasts have been reprogramed into induced pluripotent stem cells (iPSCs) by mmRNAs encoding transcription factors such as Oct4, Sox2, Klf4, c-Myc, Nanog, and Lin28 (Mandal and Rossi, 2013; Rohani et al., 2016; Warren and Lin, 2019; Yakubov et al., 2010). In this context, direct differentiation of somatic cells toward neural precursor cells using SOX2/PAX6 mRNAs was recently done and led to the generation of heterogeneous population of neural precursor cells (Connor et al., 2018). Therefore, governing the fate of produced neurons is a very important concern in cell therapy applications. For this purpose, using mmRNA for the production of defined subtypes of neurons from NSCs seems to be a more effective approach compared to direct trans-differentiation from somatic cells.

NSCs are among the hard-to-transfect cells and even the use of viral vectors yielded a very low transfection efficiency (McLenachan et al., 2013). Also, since they are very sensitive, maintaining their survival during transfection is another important challenge (Lakshmipathy et al., 2004). Consequently, NSC transfection faces numerous technical challenges and very few studies have directly genetically manipulated these cells, and in most of such studies, embryonic stem cells (ESCs), iPSCs, and somatic cells were commonly transfected and then differentiated into the neuronal cells (Ottoboni et al., 2020). Moreover, in the majority of these studies, the transfection method was DNA-based and not intended for differentiation induction purposes, mainly as a way to trace the transplanted cells in the host tissue or manipulate a reporter gene in proof-of-concept experiments (Joshi et al., 2017; Keravala et al., 2008; Pickard et al., 2017).

As described earlier, utilizing mRNA is a safe method but faces three main shortcomings which need to be overcome; the first one is dealing with mRNA immunogenicity and the others are improving its lifespan (Bettinger et al., 2001) and exploring the most compatible transfection technique for it (Bettinger et al., 2001; Ottoboni et al., 2020). After entering the cell, *in vitro*-transcribed mRNA can activate innate immune responses by interacting with different pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and RIG-1, leading to the activation of protein kinase R (PKR) (Kauffman et al., 2016). It has been reported that modification of mRNA through the incorporation of two nucleotide analogs such as pseudouridine (Ψ) and 5-methylcytidine (m5C), hinders ribonuclease (RNase) degradation, reduces the innate immune responses, and improves the stability of synthetic mRNAs (Wang et al., 2013). In addition, using an anti-reverse cap analog (ARCA) and elongation of poly(A) tail (poly adenosine tail) significantly improves the stability and translation efficiency of mRNA (Gómez-Aguado et al., 2020). ARCA cooperates with poly (A) tail synergistically not only in protecting the mRNA from degradation and enhancing its stability but also in the initiation of translation. The eukaryotic initiation factor eIF4G brings cap-binding protein elF4E and poly(A)-binding protein (PABP) together and bridges the two ends of mRNA which protects the mRNA from nuclease degradation and facilitates ribosomes recycling (Michel et al., 2000; Preiss 2002). The longer poly(A) tail, is an indicator of mRNA longevity and it is advantageous for mRNA translation (Gómez-Aguado et al., 2020). Several experiments depicted that an optimized length of poly(A) tail is about 100 adenosines (Gómez-Aguado et al., 2020; Holtkamp et al., 2006; Mockey et al., 2006; Peng and Schoenberg, 2005).

Another approach for enhancing protein expression via increasing transfection efficiency is using chloroquine (CQ) (Patel et al., 2019). As a weak base, it rapidly penetrates the cells, accumulates in the acidic compartments such as endosomes, increases their pH and prevents the degradation of nucleic acids by inhibiting hydrolytic enzymes such as nucleases (Bettinger et al., 2001; Liang and Lam, 2012). Moreover, it suppresses the activation of endosomal TLRs by inhibition of endosomal acidification (Kužnik et al., 2011).

In this study, we examined available protocols such as the use of different transfection agents, poly(A) tail

elongation, and CQ treatment, to reach an efficient protocol for mmRNA transfection in NSCs.

Materials and methods

mmRNA Synthesis

Template DNA for *in vitro* transcription (IVT) was constructed in two stages using PCR and molecular cloning. In the first stage, an EGFP DNA fragment was amplified by PCR from plasmid pEGFP-C1 (Clontech) using the following primers: EGFP-fwd: 5′ GCGGCCGCCACCATGGTGAGC 3′; and EGFP-rev: 5′ CTATTACTTGTACAGCTCGTCCATG 3′. After purification using the QIAquick PCR Purification Kit (Qiagen), the PCR product was directly cloned into linearized and blunt-ended pMRNAxp mRNAExpress™ Vector (Cat. number: MR000PA-1, System Biosciences, LLC.). The constructed pMRNA-EGFP vector was then used as a template DNA for IVT after linearizing by *SpeI* (Thermo Fisher Scientific). HiScribe™ T7 ARCA mRNA Kit (Cat. number: 2060S (with tailing), New England Biolabs, Inc.) was utilized for synthesizing capped single-stranded RNA according to the manufacturer's protocol. Also, 1.25 mM of triphosphate derivatives of modified nucleotides Ψ and m5C (TriLink) were used in the IVT reaction. The produced mRNAs contained 40-50 adenosines at the end and were named short poly(A)-tailed mmRNA^{EGFP} (ShP(A)-mmRNA^{EGFP}). Additionally, long poly(A)-tailed mmRNA^{EGFP} (LP(A)-mmRNA^{EGFP}) was produced using poly(A) polymerase (provided by the kit) for further 30-minute incubation at 37°C. Synthesized mmRNAs were then purified using LiCl solution and quantitated by NanoDrop™ 2000c Spectrophotometer. Also, RNA product integrity and length were examined by 1.2% TAE (Tris-acetate-ethylenediamine-tetra-acetic acid) agarose gel electrophoresis.

Cell culture

Human embryonic kidney cells (HEK293T cell line, Pasteur Institute of Iran) were cultured in high-glucose DMEM (Cat. number: 12800, Gibco) supplemented with Glutamax (Cat. number: 35050, Gibco) and 10% FBS (Cat. number: 10270, Gibco). The mNSCs were isolated from the ganglionic eminences of the E13 mouse brain as previously described (Homayouni et al., 2018). Briefly, mNSCs were grown in NSC medium containing neurobasal medium (NB) supplemented with 20 ng/mL EGF, 20 ng/mL bFGF, 1% N2 supplement, 1% B27^{-} supplement, 1 U/mL heparin, 1% L-glutamine, 1% non-essential amino acid (NEAA), 1% penicillin/streptomycin, and 0.1 mM β-mercaptoethanol. Neurospheres appeared after 3 days of culture and their culture medium was changed every other day; when they reached full growth after another four days, they were enzymatically dissociated to form a single cell suspension using 0.05% trypsin-EDTA (Cat. number: 25300, Gibco).

Lipofection

For lipofection experiments, mNSCs and HEK293T cells were transfected with both ShP(A)-mmRNA^{EGFP} and LP(A)-mmRNA^{EGFP} using either Lipofectamine-2000 (LF2000, Life Technologies) or TransIT (Mirus Bio LLC, WI, USA). The transfection procedure as well as mRNA/lipid ratio optimization were accomplished for each agent according to the manufacturer's recommendations. Also, cells transfected with pWPX-LD plasmid using LF2000 were considered EGFP-expressing positive control.

One day prior to transfection, mNSCs were seeded on laminin/Poly-L-ornithine-coated plates in the single cell form at densities of 10^4 and 15×10^4 cells/well in 96- and 24-well plates, respectively. Also, HEK293T cells were seeded on 0.1% gelatin-coated 24 well plates at a density of 75×10^3 cells/well.

CQ treatment and cell survival assay

CQ (Cat. number: C6628 Sigma-Aldrich) was dissolved in DMEM/F12 to produce a 1 mM stock solution. mNSCs were seeded in 96-well plates overnight at a density of 10^4 cells/well. One hour after culturing the cells in the absence or presence of CQ (10, 50, and 100 μM), four groups were transfected with mmRNAs. Cells were monitored for three days and underwent survival assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Briefly, 10 μL MTT (Sigma, St. Louis, MI, USA) solution (5 mg/ml) was added to the culture medium (100 μL) in each well of the 96-well plate. Then, plates were placed in a CO_2 incubator at 37°C to allow mitochondrial uptake. After further 4 hours, the medium was removed and cells were lysed by adding 100 μl of dimethyl sulfoxide (DMSO) to each well. Finally, the absorbance was read at 545 nm with background subtraction at 630 nm using a spectro-

photometric plate reader.

Flow cytometry

Cultured cells were enzymatically dissociated and washed twice with HBSS (Hank's balanced salt solution), centrifuged, and re-suspended in 500 µl of HBSS. EGFP fluorescence in cells was detected by FACSCalibur flow cytometer (Becton-Dickinson Biosciences, San Jose, CA, USA). Flow cytometry data were analyzed using the BD CellQuest Pro software.

Measuring Corrected Total Cell Fluorescence (CTCF) using Image J software

Images were captured from cells using an inverted fluorescence microscope, the cells of interest as well as three regions next to them where no fluorescence signals were selected. Normalized fluorescence intensity was calculated according to the following formula: CTCF= Integrated Density (of the selected cells) - (Area (of the selected cells) × Mean Fluorescence (of the background)) using ImageJ software.

Statistical analysis

Ordinary and repeated measure one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test were used to determine statistically significant differences among groups. GraphPad Prism software (GraphPad.Prism. v8.0.2.263) was used for data analysis. $P<0.05$ was defined statistically significant. All the experiments were performed in triplicate and repeated three times. Data are presented as mean ± standard error of the mean (SEM).

Results

Transfection efficacy of mmRNA^{EGFP} in mNSCs

HEK293T and mNSCs were transfected with ShP(A)-mmRNA^{EGFP} using two different lipofection systems; LF2000 and TransIT. Also, to compare them with EGFP-expressing control groups, both cell lines were transfected with pWPXLD plasmid (pWPXLD/LF2000).

Results of flow cytometry assay 24 hours after ShP(A)-mmRNA^{EGFP} transfection in mNSCs showed that by using TransIT agent, 13.98±0.72 % of cells were EGFP-positive showing 42.46± 2.96 fluorescence intensity of EGFP, while by using LF2000, these parameters were 4.34±0.24% and 18.52±0.65, respectively. On the other hand, in HEK293T cells, ShP(A)-mmRNA^{EGFP} transfection using TransIT, led to 41.84±2.16 % positive cells with 112.78±2.46 fluorescence intensity, while using LF2000, these parameters were 15.56±0.65% and 53.84±1.49, respectively. As a result, in both cell lines, TransIT achieved better outcomes (Figure 1).

Also comparing mmRNA with pWPXLD, in ShP(A)-mmRNA^{EGFP}/TransIT transfected mNSCs, indicated that the percentage of EGFP positive cells and the mean fluorescence intensity of EGFP were respectively 3.4- and 2.97-fold higher than those of pWPXLD/LF2000 transfected cells, while, in ShP(A)-mmRNA^{EGFP}/TransIT transfected HEK293T cells, these parameters were respectively just 0.77- and 0.05-fold higher than those of pWPXLD/LF2000 transfected cells. Subsequently, ShP(A)-mmRNA^{EGFP}/TransIT transfection led to a 4.4-fold increase in the population of EGFP-positive cells and a 59.4-fold increase in the mean fluorescence intensity of EGFP in mNSCs compared to HEK293T cells (Figure 1B&D). Together, mmRNA^{EGFP}/TransIT acted more specifically and efficiently in mNSCs.

Effect of poly(A) tail elongation of mmRNA on transfection efficacy in HEK293T and mNSCs

HEK293T and mNSCs were transfected with ShP(A)- and LP(A)-mmRNA^{EGFP} using TransIT, and their EGFP expression was evaluated by flow cytometry assay up to 96 hours every 24 hours. In HEK293T cells, LP(A)-mmRNA^{EGFP} transfection strongly increased the fluorescence intensity of EGFP which was statistically significant compared to ShP(A)-mmRNA^{EGFP} at all measurement time points ($P<0.0001$). As depicted in figure 2C, the mean fluorescence intensity of EGFP in the LP(A) group 24 hours post-transfection, was 1293.96±5.22 but declined to 110±0.91 after 96 hours, while in the ShP(A) group these values were 64.42±1.58 and 18±1.72, respectively.

Regarding the percentage of EGFP-expressing HEK293T cells at 24-hour post-transfection, there was no significant difference between ShP(A) and LP(A) groups. However, during the following days, a divergence happened between these two groups, and LP(A) group had more EGFP-positive cells ($P<0.01$). Meanwhile, their 96-hour trend showed a steady decline in the number of EGFP-positive cells in both groups while the slope was steeper in the ShP(A) group (Figure 2).

Assays on mNSCs at 24-, 48- and 72-hour time points

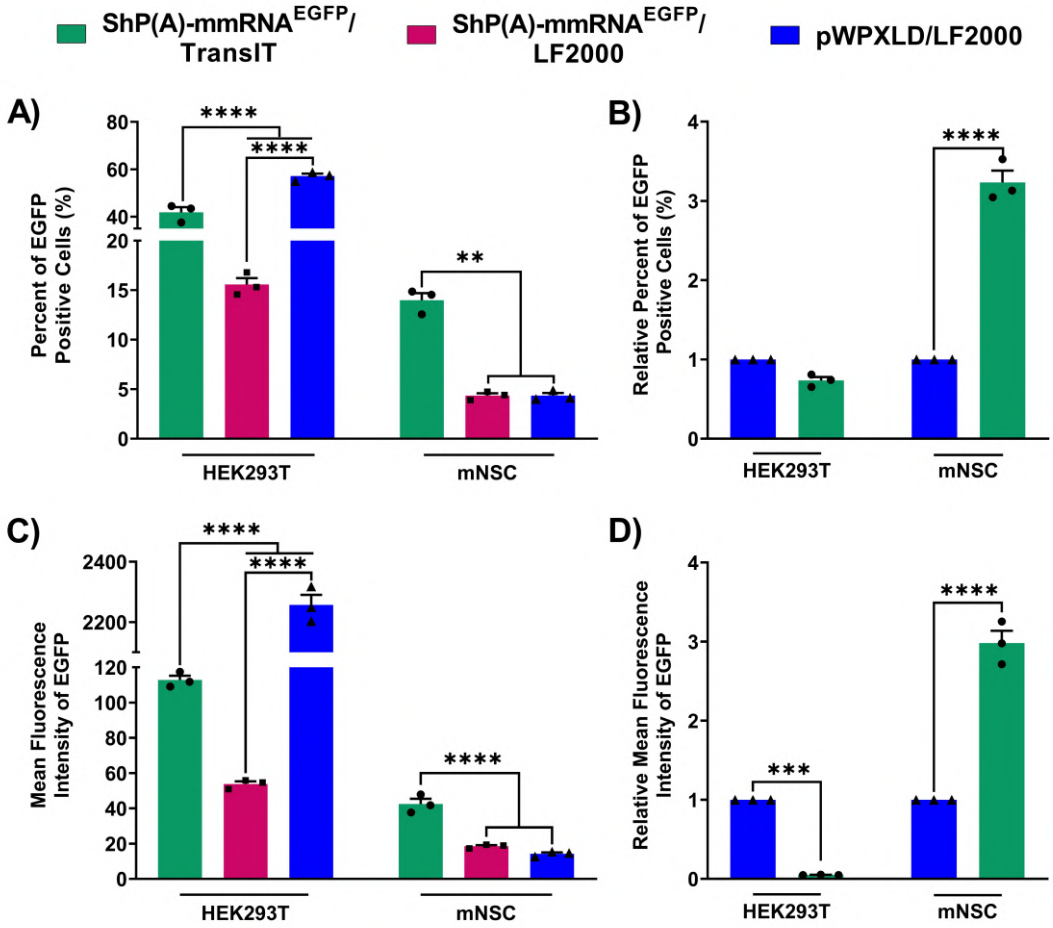

FIGURE 1. Lipofection of HEK293T and mNSCs with mmRNAEGFP and pWPXLD plasmid. HEK293T and mNSCs were transfected with ShP(A)-mmRNAEGFP and pWPXLD plasmid using two dif-ferent lipofection systems. (A) mean percentage of EGFP positive cells, and (C) mean fluores-cence intensity of EGFP in cells were measured by flow cytometry assays 24 hours after trans-fection. The relative differences in the mean percentage of positive cells and the mean fluores-cence intensity of EGFP in cells are presented as ShPA-EGFPIVT/TransIT divided by pWPXLD/LF2000 in B and D, respectively. Overall it can be concluded from this graph that ShPA-EGFPIVT/TransIT worked better for transfection of neural stem cells while for HEK cells pWPXLD/LF2000 is more suitable. One-way (repeated measures) ANOVA followed by Dun-nett's multiple comparisons test were used to assess differences, and P values < 0.05 were con-sidered statistically significant (**: P<0.01; ***: P<0.001 and ****: P<0.0001). All the ex-periments were performed in triplicate and repeated three times. Data are presented as mean ± SEM.

showed that the number of EGFP-positive cells in the LP(A) group was significantly higher (P<0.0001). Also, as shown in figure 3, the number of EGFP-positive cells in both groups decreased over time, with a sharper decline in the ShP(A) group.

Evaluating the mean fluorescence intensity of EGFP in mNSCs (Figure 3C), showed significant differences between the two groups at all evaluated time points (P<0.0001). Accordingly, 24 hours after transfection, the mean fluorescent intensity of EGFP in the LP(A) group was 124.05±5.75 but declined to 43.76±1.55 after 96 hours, while in the ShP(A) group, these values were 36.66±1.68 and 10±0.29, respectively.

CQ enhances the mmRNA transfection efficacy

In order to test the effect of CQ on mmRNA transfection efficacy in mNSCs, cells were treated with CQ then transfected with LP(A)-mmRNA^{EGFP} and monitored for three days after transfection.

Several treatments were tried on mNSCs and as was expected the expression of EGFP protein just happened in LP(A)-mmRNA^{EGFP}/TransIT groups (Figure 4). Survival assay showed that TransIT by itself, and CQ at 50 and 100 µM concentrations, are toxic for the cells. Nevertheless, the viability was not affected in the LP(A)-mmRNA^{EGFP}/TransIT/CQ-10 µM group (Figure 4). These findings indicated that CQ 10 µM enhances the transfection efficiency while exerting a low level of toxicity (Figure 4).

CTCF measurements 24 hours after transfec-

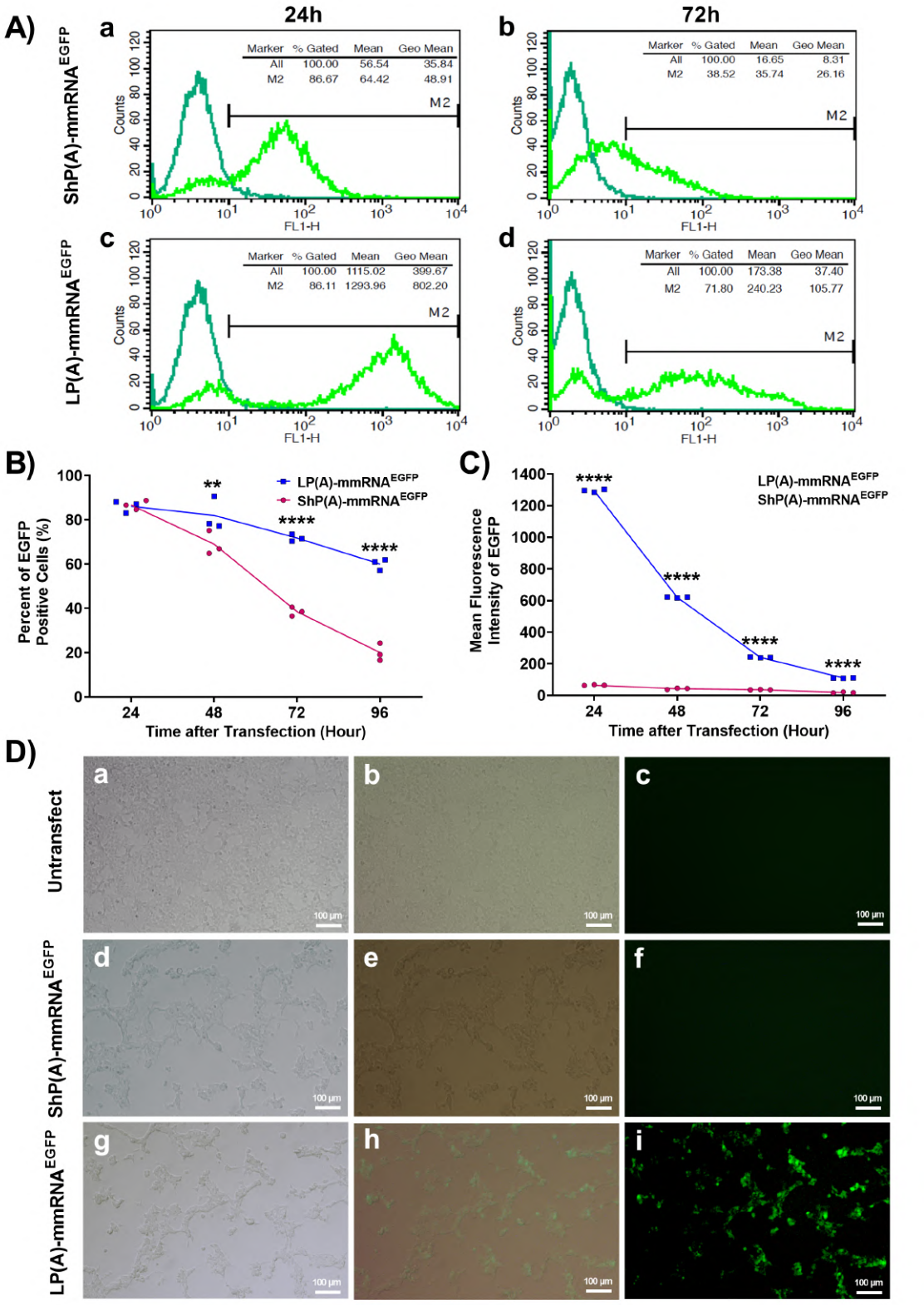

FIGURE 2. The efficacy of transfection in HEK293T cells using ShP(A)- and LP(A)-mmRNAEGFP. ShP(A)- and LP(A)-mmRNAEGFP transfection was performed in HEK293T cells using TransIT. (A) The histogram plots of flow cytometry assays represent the percentage of EGFP-positive cells and their fluorescence intensity 24 and 72 hours post-transfection in the ShP(A)- and LP(A)-mmRNAEGFP transfected groups. (B) Line graphs represent the percentage of EGFP-positive cells, and (C) mean fluorescence intensity of EGFP expression for each group for up to 96 hours post-transfection. (D) Inverted phase-contrast microscopic images in bright (a, d, and g), semi bright/semi fluorescence (b, e, and h), and fluorescence light (c, f, and i) fields from dif-ferent groups. LP(A) group showed better results in the mean number of transfected HEK cells and their fluorescent intensity during different periods (****: $P < 0.0001$). One-way ANOVA (repeated measures) followed by Dunnett's multiple comparisons test was used to assess differ-ences among groups and P values < 0.05 were considered statistically significant (**: $P < 0.01$, and ****: $P < 0.0001$). All the experiments were performed in triplicate and repeated three times. Data are presented as mean ± SEM.

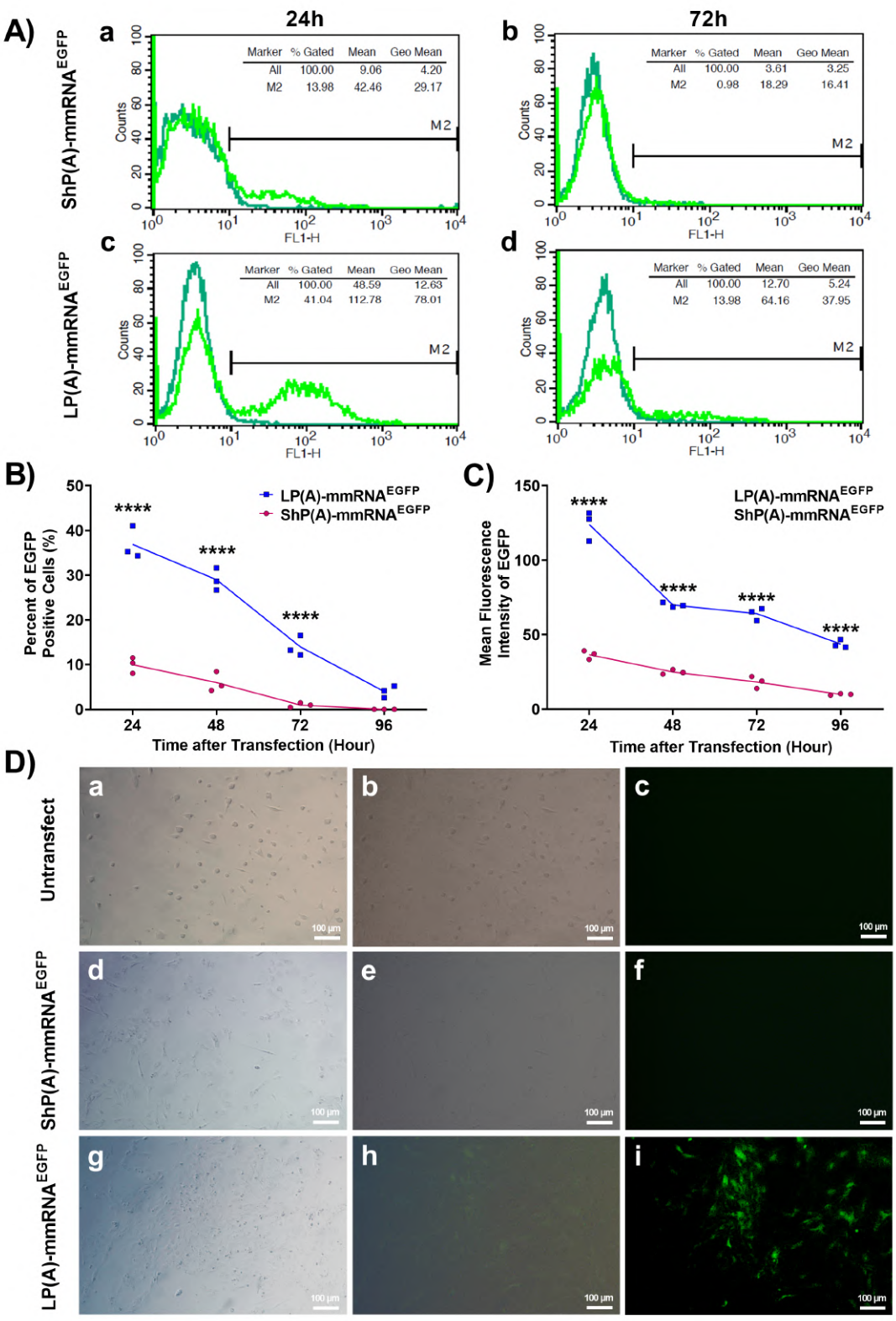

FIGURE 3. The efficacy of transfection in mNSCs using ShP(A)- and LP(A)-mmRNAEGFP. ShP(A)- and LP(A)-mmRNAEGFP transfection was performed in mNSCs using TransIT. (A) The histogram plots of flow cytometry assays represent the percentage of EGFP-positive cells and their fluores-cence intensity 24 and 72 hours post-transfection in the ShP(A)- and LP(A)-mmRNAEGFP trans-fected groups. (B) Line graphs represent the percentage of EGFP-positive mNSCs, and (C) mean fluorescence intensity of EGFP expression for each group for up to 96 hours post-transfection. LP(A) group showed better results in the mean number of transfected cells and their fluorescent intensity during different periods (****: $P < 0.0001$). (D) Inverted phase-contrast microscopic images in bright (a, d, and g), semi bright/semi fluorescence (b, e, and h), and fluorescence light (c, f, and i) fields from different groups. One-way (repeated measures) ANOVA followed by Dunnett's multiple comparisons test was used to examine differences among groups. All the ex-periments were performed in triplicate and repeated three times. Data are presented as mean ± SEM.

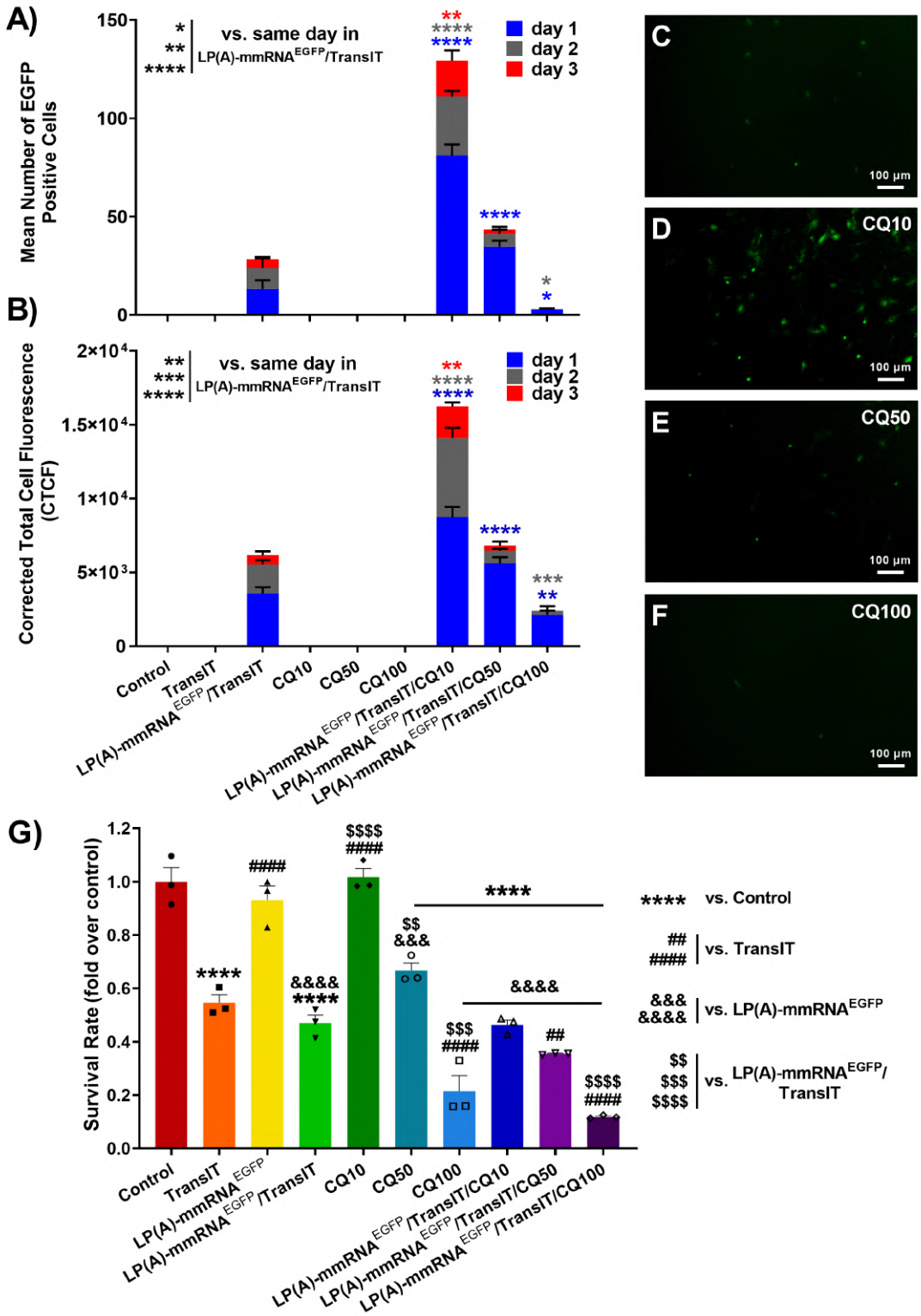

FIGURE 4. CQ treatment and its impact on mmRNA transfection efficacy and cell survival. The mNSCs were transfected with LP(A)-mmRNAEGFP in the presence and absence of different concentra-tions of CQ. (A) The number of EGFP-positive cells and (B) corrected total cell fluorescence (CTCF) was measured for up to 3 days after transfection. (C-F) Fluorescent microscopy images of LP(A)-mmRNAEGFP/TransIT-transfected mNSCs in the presence and absence of different concentrations (10, 50 and and 100 µM) of CQ. (G) MTT cell survival assay of mNSCs in differ-ent treatment groups. Altogether this graph shows that LP(A)-mmRNAEGFP/TransIT-transfection in presence of CQ 10 µM yields better results and CQ is necessary for getting an adequate amount of sustained transfection at least for 3 days. Repeated measures one-way ANOVA (A&B) and ordinary one-way ANOVA (G), followed by Dunnett's multiple comparisons test were used to assess differences among groups. Representations of symbols are as follow: *, &, $, and # for $P < 0.05$; **, &&, $$, and ## for $P < 0.01$; ***, &&&, $$$, and ### for $P < 0.001$; and ****, &&&&, $$$$, and #### for $P < 0.0001$. All the experiments were done in three replicates and repeats. Data are presented as mean ± SEM.

tion showed that CQ at 10 and 50 μM concentrations effectively improved the transfection efficacy of LP(A)-mmRNA^{EGFP}/TransIT regimen (P<0.0001). Likewise, this regimen could significantly maintain the detectable amount of EGFP expression that long lasted for two days (P<0.01) (Figure 4).

Discussion

The present study investigated the challenges of mmRNA transfection in NSCs. Our findings showed that the mmRNA transfection efficacy was 14% in NSCs and 40% in HEK293T cells while using plasmid transfection these rates became 5% and 55% in each respective cell line, reflecting that mmRNA transfection was more efficacious method in NSCs.

Furthermore, comparing the intensity of EGFP expression in mmRNA transfected cells, it was found that the fluorescence intensity of EGFP in NSCs was only half of that of HEK293T cells, indicating that the mmRNA entering NSCs was properly able to increase the protein expression.

This was also remarkable that transfection with mmRNA was almost three times more successful than the plasmid in terms of the percentage of transfected NSCs and their EGFP fluorescence intensity. Also, the use of the specific carriers for mmRNA improved the transfection efficacy more than the conventional agents such as Lipofectamine.

There are few similar reports in which mmRNA-transfection method was applied to NSCs (McLenachan et al., 2013). Nevertheless, in most of them, DNA constructs were used to transfect neural cells, and various efficacies were reported (e.g. a remarkable success rate of 80% using PEI/DNA polyplexes for *in vivo* transfection of neurons) (Chang et al., 2016).

Results of the present study showed that mmRNA-transfection works more properly than DNA for NSCs. Only in one similar study, McLenachan et al. (2013) reported that in NSCs, the efficacy of mmRNA-transfection was much higher than DNA-transfection (50% versus 10%), while the intensity of protein expression was three times higher in DNA-transfected NSCs.

However, our findings showed that both the intensity (expression) and the percentage of transfected cells were higher in the mmRNA group. Given that the percentage of transfection is a more important indicator of the transfection efficacy, our study confirmed McLenachan et al. (2013) study where the mRNA method was more desirable for NSCs.

Regarding the effect of mRNA concentration, 1 μg of mRNA was used in our study for 15×10^4 cells, and in McLenachan et al. (2013) study, it was 0.5-1 μg for 10^5 cells, which were almost identical. However, in the case of cells, McLenachan et al. (2013) transfected neurospheres as well as monolayer cultured NSCs, and based on their results, NSCs showed a better transfection ratio in the monolayer culture while better survival rate in the neurosphere form. They found that transfecting floating neurospheres with higher concentrations of mmRNA in the presence of Lipofectamine, had a better outcome due to less cell damage. They also found that when mmRNA concentration exceeds 0.8 μg, cell death occurs in the monolayer form, while neurospheres still stay alive (McLenachan et al., 2013).

Our findings showed that the 200-adenosine tail had a significant increasing effect on the rate of mmRNA translation in NSCs, as it tripled the percentage of transfected cells and their EGFP fluorescence intensity in the early 24 hours after transfection. However, during the same period in HEK293T cells, the percentage of transfection did not change, while the fluorescence intensity showed up to 20-fold increases.

However, in McLenachan et al. (2013) study, no sharp decline happened in the percentage of transfected NSCs even until 100 hours post-transfection, while a decrease in protein expression occurred within 48 hours after transfection. It should be noted that the poly(A) tail length in their study was around 130 adenosines.

In studies where non-neuronal cells such as endothelial cells, fibroblasts, and HEK293T cells were transfected with mmRNA, no change in the percentage of transfected cells over time was reported, while there was a decrease in protein expression (Avci-Adali et al., 2014). Another report on mmRNA transfection in dendritic cells showed that increasing the length of poly(A) tail from 64 to 100 adenosines, enhances the rate of protein expression by up to 35-fold (Mockey et al., 2006). Also, similar findings by Holtkamp et al. showed that the 120-adenosine tail exhibits the highest mmRNA stability and translation efficiency (Holtkamp et al., 2006). This incremental effect stems from the synergistic cooperation of the 5′ cap with poly(A) tail and the resulting pseudo-circular structure of the mmRNA (Michel et

al., 2000; Preiss, 2002), which can facilitate ribosome recycling and protect mmRNA against exonucleases (López-Lastra et al., 2005).

We have shown that the use of TransIT carrier for transfection of NSCs with mmRNA is far more effective than LF2000 and yielded a higher percentage of transfection and EGFP expression. However, McLenachan et al. (2013) concluded that Lipofectamine was less toxic and almost more effective. Studies on other cell lines revealed different degrees of success for each pair of particular cell and its lipofection agent (McLenachan et al., 2013). In the study by Adali et al., HEK293T, fibroblasts and endothelial cells were evaluated for mRNA transfection using Lipofectamine, Mirus and Stemfact carriers and their results showed that for HEK293T as well as endothelial cells both TransIT and LF2000 and for fibroblasts LF2000 work better (Avci-Adali et al., 2014). Therefore, cells respond differently to distinct carriers and future innovations in the production of mRNA carriers can lead to better results.

A part of our study focused on improving the quality of mmRNA transfection using CQ, and we found that CQ at 10 μM concentration can be effective in improving the percentage of transfected cells as well as the intensity of protein expression without causing severe toxicity in cells. CQ has already been used by numerous studies to optimize DNA transfection (Erbacher et al., 1996; Hasan et al., 1991; Zhang and Mallapragada, 2011); however, there are few reports on its efficacy on mRNA transfection. For instance, Bell et al. used Xentry-protamine-mediated transfection of EGFP mRNA in A549, HepG2, and AGS cells, and reported that the use of CQ at concentrations of 50 to 100 μM could increase the expression of EGFP from 0 to 2-4% (Bell et al., 2018).

CQ improves the mRNA-transfection rate by reducing mRNA degradation, preventing the activation of immune reactions, and increasing endosomal escape (Kužnik et al., 2011; Liang and Lam 2012). As a weak alkali, it accumulates in acidic vesicles and subsequently, reduces the activity of hydrolytic enzymes, including nucleases, which in turn, prevents mRNA degradation (Liang and Lam, 2012); it also suppresses the activation of endosomal TLRs by nucleic acids as their activation requires an acidic environment (Kužnik et al., 2011). In addition, by binding directly to nucleic acids, CQ affects their structure and obscures their TLR-binding epitope, thus, TLR cannot activate the IFN and NF-κB signaling pathways and therefore the translation inhibition mechanism remains inactive (Kužnik et al., 2011). Furthermore, CQ-containing copolymers effectively improve endosomal escape by increasing the pH of the endosomes and preventing the endosomes from attaching to the lysosomes (Durymanov and Reineke, 2018).

Conclusion

By using mRNA-specific transfection agents and improving the quality of synthesized mmRNA alongside using TLR inhibitors like CQ, it is possible to achieve higher transfection efficacy in NSCs.

Acknowledgment

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethics approval

All of the laboratory experiments on cells were performed in accordance with standard procedures and approved by the Royan Institute Cell Culture and Utilization Committee (Approval ID: IR.ACECR.ROYAN.REC.1397.083).

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