

Lab Report THG

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1 Introduction

Cell therapies in general enable the treatment of a patient by repairing, substituting or supporting cells or tissue by transplantation of (modified) cells, either from patients themselves (autologous), or from a donor (allogenic) [1]. A promising approach in the field of regenerative medicine is the application of mesenchymal stem cells [2].

Nucleic acid therapies, especially in form of messenger RNA (mRNA), are seen as novel therapeutic modalities providing an auspicious approach regarding various treatments against e.g. cancer, infectious, and genetic diseases, in forms of immunotherapies, vaccines or protein replacement, [3, 4] showing “long lasting or even curative effects” [5]. Other therapeutics that can be used instead of mRNA are, among others, DNA plasmids, small interfering RNA, microRNA and Aptamers [6].

Advantages of using mRNA are its fast and easy degradation by nucleases [7], as well as the fact that it is not entering the nucleolus [8], thus, being unable to rewrite the human genome [8]. Moreover, mRNA proved to be cost effective, relatively simple in production and seems to be able to target undruggable pathways [6]. However, the short degradation time can be seen as a disadvantage, since there is only a small period of time available to express genes into the human body. Another disadvantage is that mRNA is a large, charged molecule [3] that additionally activates the immune system [7]. By modifying sequences of the mRNA it is possible to enhance its stability, as well as circumventing potential problems regarding its charge or immunogenicity [3].

2 Methods

2.1 mRNA Transfection of Cells

In the first part of the practical, the aim was to detach neonatal human foreskin fibroblasts (NuFF) from a cell culture flask, and seed them into a 6-well plate afterwards. Therefore, in the beginning, the medium in the cell culture flask was aspirated, and the cells were washed with 10 ml of phosphate buffered saline (PBS) to remove any contaminations as e.g. dead cells. Next, the fibroblasts, adhered on the ground of the cell culture flask, were detached by adding 5 ml of a Trypsin/EDTA (T/E) solution, and incubated at 37 °C for 3 min. Successful detachment of the fibroblasts was verified under the microscope. After that, 5 ml of medium were added to the T/E solution to inactivate the trypsin, and thus, preventing the fibroblasts from damage. Then, the cell suspension was centrifugated at 300 x g for 5 min. The supernatant was aspirated and 10 ml of

medium were added for resuspension of the pelleted cells. Next, the fibroblasts were counted using a Neubauer hemocytometer. Therefore, 15 μl of a solution, consisting of cell suspension and Trypan blue, using a dilution factor of 1:2, were given into the device. The viable cells, appearing white, of 4 big squares were counted. Out of the counted cells, the amount of $\frac{\text{cells}}{\text{ml}}$ in the solution was calculated by the following formula:

$$C (\text{cell conc.}) \left[\frac{\text{cells}}{\text{ml}} \right] = \frac{\text{Counted cells (alive)}}{\text{Number of big squares}} \cdot \text{dilution factor (Trypan blue)} \cdot 10^4 \quad (1)$$

Last, 3 wells of a 6-well plate were filled with 10^4 cells and 1.5 ml medium each, and incubated overnight. The volume x of cell suspension to fill in $10^4 \frac{\text{cells}}{\text{well}}$ was determined as:

$$x = \frac{\text{wanted cells} \left[\frac{\text{cells}}{\text{well}} \right]}{\text{determined cell concentration} \left[\frac{\text{cells}}{\text{ml}} \right]} \quad (2)$$

In the second part of the practical, the cells were transfected with GFP mRNA. Therefore, mRNA transfection complexes were prepared in 1.5 ml low bind tubes. For further determination of a successful transfection, two negative controls were prepared additionally. The three tubes were vortexed and incubated at RT for 15 min. The complexes/controls were then added to the cells in the well plate and incubated overnight. The exact composition of each well is listed in Table 1. With the two practical groups, one used a higher concentration of mRNA and transfection reagent, and the other one used a lower concentration, to be later able to observe the influence of the concentration.

Table 1: Composition of different samples

Well	Samples	Transfection medium (Opti-MEM) [ml]	Transfection reagent (Lipofectamin 2000) [μl]	mRNA (Stock: 1000 ng/ μl) [μl]
1	Negative control 1	1	-	-
2	Negative control 2	1	2/4	-
3	mRNA GFP	1	2/4	1/2

2.2 Viability of Transfected Cells, Detection of Exogenous Protein Expression

The cell viability after mRNA transfection was determined by colorimetric measurement. Therefore, a Presto Blue solution was produced by diluting 400 μl of Presto Blue with 3.6 ml of medium. Then, the medium was aspirated from the cells and 1 ml of the Presto Blue solution was added per well. Additionally, one blank well with only Presto Blue

solution was prepared, which was subsequently used as measurement reference value. The well plate was incubated for 45 min, so that the color could be absorbed and metabolized by the cells. Afterwards, 100 µl were transferred three times per well (triplicate) into a 96-well plate. By using a Mithras spectrometer with an excitation wavelength of 535 nm, and an emission wavelength of 600 nm the cells were observed. With fluorescence microscopy it was investigated whether green fluorescence light was emitted by GFP (max. wavelength 509 nm, filter 530 nm) after excitation with blue light (max. wavelength 488 nm, filter 485), thus, if the mRNA transfection was successful. Last, flow cytometry was applied with a FACS-Scan for quantifying the transfection efficiency. As preparation, the medium was aspirated from the wells, and the cells were washed with 1 ml of PBS each. 1 ml of T/E was added per well, and the well plate was incubated. 1 ml of medium was added, the suspensions were transferred into FACS tubes and centrifuged at 600 x g for 3 min. Then, the supernatant was aspirated, and the cell pellet was resuspended with 1 ml of PBS.

3 Results

3.1 Number of Cells

By applying Formula 1, the number of $\frac{\text{cells}}{\text{ml}}$ was calculated as:

$$C (\text{cell conc.}) = \frac{27}{4} \cdot 2 \cdot 10^4 = 135\,000 \frac{\text{cells}}{\text{ml}}$$

The volume required to prepare wells with $10^4 \frac{\text{cells}}{\text{well}}$ was determined via Formula 2 as:

$$x = \frac{30\,000 \frac{\text{cells}}{\text{well}}}{135\,000 \frac{\text{cells}}{\text{ml}}} = 0.22 \frac{\text{ml}}{\text{well}}$$

3.2 Presto Blue Assay

The

diagrams

in

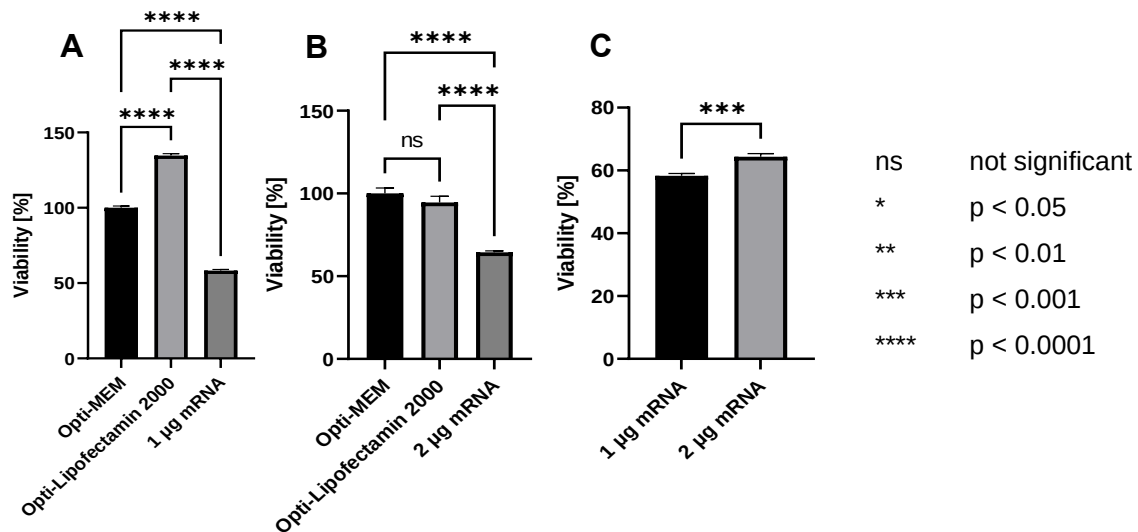


Figure 1 show the cell viability of cells transfected with 1 µg (A) and 2 µg (B) GFP mRNA in relation to the Opti-MEM negative control, that was taken as reference value. The Opti-Lipofectamin 2000 (OL) negative control was also set in relation to the Opti-MEM negative control. One-way ANOVA revealed a significantly lower cell viability for cells transfected with 1/2 µg of GFP mRNA compared to the Opti-MEM control. Diagram A additionally shows a significantly higher viability for the OL control. A t-test revealed a significantly differing cell viability for cells transfected with 1 µg and 2 µg GFP mRNA (58.3 % vs. 64.4 %).

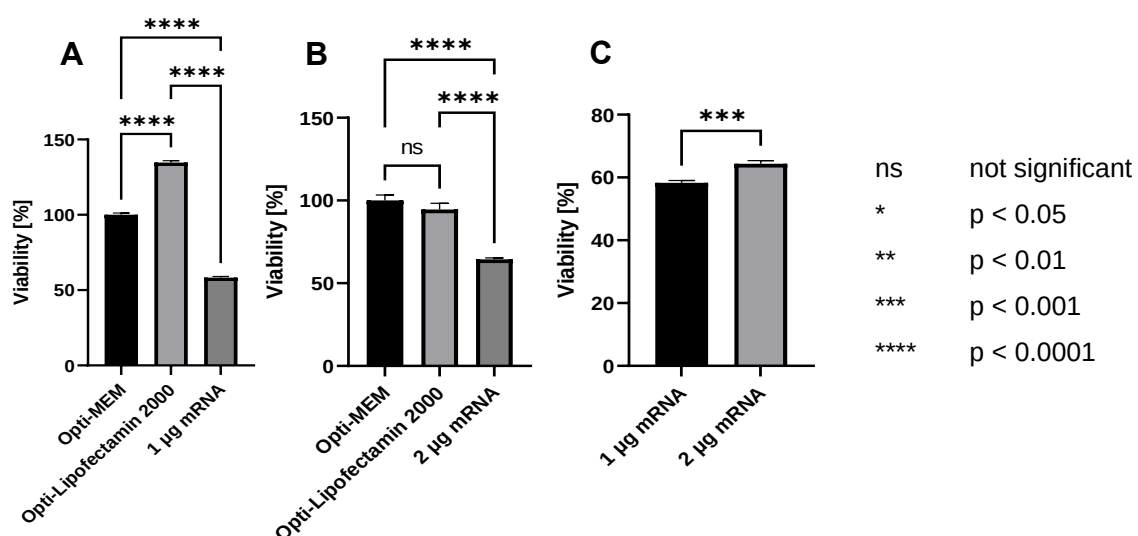


Figure 1: Relative cell viability after transfection of NuFF with 1 µg (A)/ 2 µg (B) GFP mRNA, and of OL control in relation to Opti-MEM negative control. C: comparison of cell viability for cells transfected with 1/2 µg GFP mRNA. Statistical analysis was done with ANOVA (A, B) and t-test (C)

3.3 Fluorescence Microscopy (FM)

The FM images depicted in Figure 2 are showing a brightfield image of the fibroblasts (left), an image taken in green channel to visualize fluorescence (middle), and an overlay image of the two previous ones (right) for verifying that the fluorescence is emitted by the fibroblasts. It can be observed that the transfection of NuFF cells with GFP mRNA was successful. However, the images do not show a difference in fluorescence intensity between cells transfected with 1 μ g GFP mRNA (top) and 2 μ g GFP mRNA (bottom).

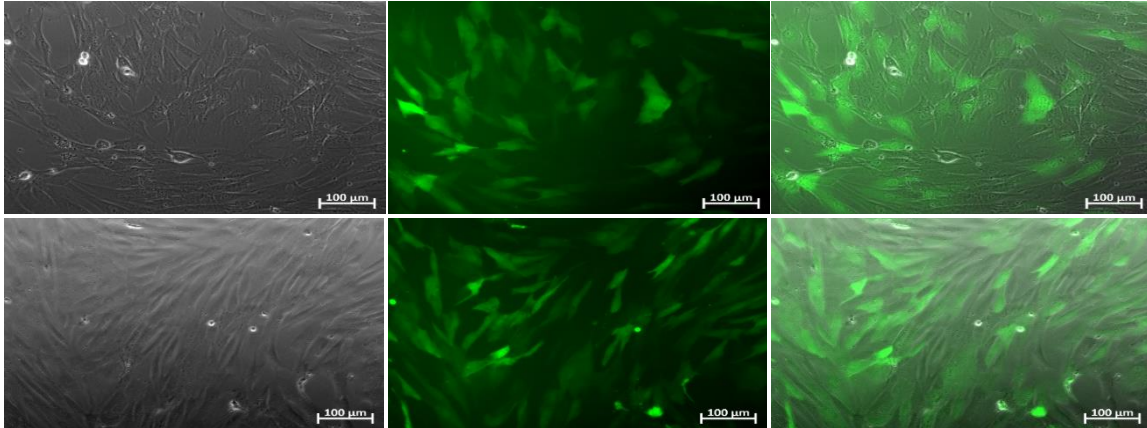


Figure 2: FM images showing brightfield (left), green channel (middle) and overlay images of both (right) when using 1 μ g GFP mRNA (top) and 2 μ g GFP mRNA (bottom)

3.4 Flow Cytometry

The histograms in Figure 3 show the amount of cells measured with flow cytometry on the y-axis and their fluorescence on the x-axis when using 1 μ g of GFP mRNA (left) and 2 μ g of GFP mRNA (right). The black curve is representing the OL control, whereas the red curve shows the fibroblasts. The total amount of cells transfected with mRNA, and thus, expressing GFP is higher when using 2 μ g mRNA (81.4 %) than if using 1 μ g mRNA (69.3 %). The histogram for 1 μ g mRNA shows a higher overlap between the OL control and fibroblasts not expressing GFP than the histogram for 2 μ g mRNA.

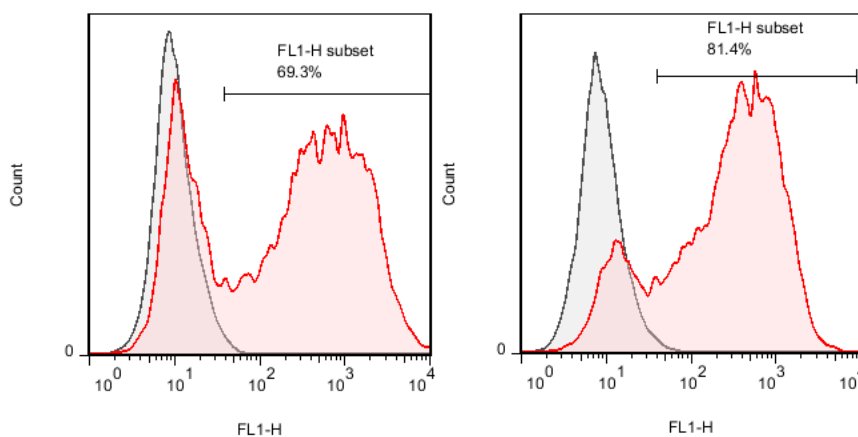


Figure 3: Histograms showing flow cytometry results for cells transfected with 1 μ g (left) and 2 μ g (right) GFP mRNA

4 Discussion

The determined cell viability, tested with the Presto Blue assay, for samples containing GFP mRNA was significantly lower than in both control samples indicating mRNA and/or GFP having a cytotoxic effect on NuFF. A potential cell toxicity of GFP was also discussed by other scientists [9]. As there was a significantly higher viability/no significant difference in viability for the OL controls in comparison to the Opti-MEM controls it can be concluded that a decreasing cell viability is mainly influenced by the transfection of GFP mRNA. Since it was shown that GFP mRNA is cytotoxic it might be expected that a higher concentration of GFP mRNA leads to a lower viability. However, in this experiment the viability was significantly higher for a concentration of 2 µg GFP mRNA than for 1 µg GFP mRNA. This might be due to statistical uncertainties, as the mean values were calculated of only three measurement values. Since the difference in GFP mRNA concentrations used in this experiment is minor, it might not have an impact on the viability at all. Furthermore, the different concentrations were prepared by two groups on different well plates. These influences might also result in the huge difference between the OL viability values as they should theoretically be the same.

Fluorescence microscopy is a fast method to determine whether cell transfection with GFP mRNA was successful or not, thus if the GFP is metabolized by the cells. As fluorescence microscopy is only a qualitative method, flow cytometry was conducted for quantitative measurement of the transfected GFP mRNA. The data indicates that using a higher concentration of GFP mRNA leads to an increased transfection of cells. It has to be considered that this effect has an upper limit due to the cytotoxicity of GFP mRNA.

5 Literature

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