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Observing better transfection efficiency in primary cultured VSMCs: Comparison and development of two different protocols

[Primer VDKH kültüründe daha etkili bir transfeksiyon gözlenmesi: İki farklı protokolün karşılaştırılması ve geliştirilmesi]

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ABSTRACT

Aim: In this study, we have evaluated the procedure of transient transfection efficiency of vascular smooth muscle cells (VSMCs) with Lipofectamine (Life Technologies) and FuGENE (ROCHE, FuGENE HD Reagent) transfection reagents using the pCH110 eukaryotic assay vector containing the *lac*Z reporter gene. We also did try these attainments to transfect VSMCs with Ras^{N17} DNA and affirmed our findings.

Material Methods: Plasmid pcH110, which has been purified by cesium chloride gradient centrifugation, was used in all transfections as the assay vector. And c-H-Ras was observed via Western-blot technique.

Results: Briefly, the transfection with FuGENE has been given the best results, comparing with Lipofectamin. Under our culture conditions for VSMCs, FuGENE transfection efficiency could be augmented by simply increasing the amount of plasmid DNA 1.5–3 times above the recommended concentration without any visible cytotoxicity. With the FuGENE reagent, optimal transfection efficiency was obtained for primary culture of VSMCs within the recommended concentrations, but at the top of the range. The results indicate that optimization of the transfection process should include plasmid DNA concentrations above the levels suggested by the manufacturers, in order to accomplish the highest transfection efficiency. And, these finding was also supported with our Western-blot results when VSMCs have been transiently transfected with Ras^{N17} DNA.

Conclusion: According to the difficulty of transfections of primary cell culture, we used FuGENE reagent with different DNA and plasmid ratio describes by the manufacturer and obtained better transfection efficiency in primary cultured vascular smooth muscle cells. Our findings, precisely implicates to use FuGENE reagent for to get a better transfection efficiency in primary cultured cells, especially in primary cultured VSMCs.

Keywords: Transfection, FuGENE, Lipofectamine, pCH110, VSMCs.

Conflict of interest: Authors have no conflict of interest.

ÖZET

Amaç: Bu çalışmada, lacZ taşıyıcı genini içeren pCH110 ökaryotik vektörünü kullanarak, Lipofektamin (Life Technologies) transfeksiyon kiti ve FuGENE (ROCHE, FuGENE HD) deney kitini kullanarak vasküler düz kas hücrelerinin (VDKH) geçici transfeksiyon verimliliğini eş zamanlı olarak test etmek istedik. Ayrıca aynı teknikler üzerinden VDKH'lerini Ras^{N17} DNA'sı ile transfekte ederek, bulgularımızı destekledik.

Materyal Metot: Sezyum klorid gradient santrifügasyonu ile pürifiye edilmiş pCH110 plasmidi, deney vektörü olarak tüm transfeksiyonlarda kullanılmıştır. Ras proteininin olası artışı, transfeksiyon sonrasında Western-blot tekniği ile gösterilmiştir.

Sonuçlar: Özetle, Lipofektaminle kıyaslandığında, FuGENE ile transfeksiyon daha iyi sonuçlar elde etmemizi sağlamıştır. VDKH'leri için oluşturduğumuz deney kültür ortamı altında, FuGENE ile etkili bir transfeksiyon için basit olarak kullanılan plazmid DNA'nın miktarını 1,5-3 kat oranında, tavsiye edilen konsantrasyonların üzerinde ve görülür sitotoksisiteden uzak olarak, arttırmak yeterli görünmektedir. Primer VDKH kültürü için FuGENE reaktifi kullanılarak, optimal transfeksiyon etkisi belirlenen konsantrasyonlar içerisinde gözlenmesine karşın, bu değerlerin kit için belirlenmiş maksimum veriler olması dikkat çekicidir. Sonuçlar, yüksek oranda transfeksiyon etkinliğine ulaşmak için, transfeksiyon işleminin optimizasyonunda kullanılan plazmid DNA konsantrasyonlarının firmanın önermiş olduğu düzeylerden daha fazla kullanılma gerekliliğini işaret etmektedir. Ayrıca, bu bulgular, VDKH'lerinin geçici Ras^{N17} DNA'sı ile transfeksiyonu sonucu uygulanan Westren-blot sonuçları ile de desteklenmiştir.

Bulgular: Elde ettiğimiz veriler, özellikle primer hücre kültürlerinin transfeksiyonunun zorluğu karşısında gerçekleştirdiğimiz VDKH kültüründe transfeksiyonu FuGENE reaktifi ile daha başarılı olabileceğini ve özellikle etkinliğinin yadsınmayacak ölçüde daha effektif olabileceği gösterilmiştir.

Anahtar kelimeler: Transfeksiyon, FuGENE, Lipofektamin, pCH110, VDKH.

Çıkar çatışması: Yazarların hiçbirinin arasında herhangi bir çıkar çatışması yoktur.

Introduction

The transfer of recombinant genes into a variety of eukaryotic cultured cells, commonly known as transfection, is an extensively used approach in gene expression studies. A large number of plasmid DNA delivery methods have been developed for mammalian cells. Calcium phosphate precipitation and DEAE-dextran transfection were two of the early methods developed for DNA delivery [1]. These methods appear to facilitate DNA binding to cell membranes and entry of DNA into the cell via endocytosis. Both methods, as well as electroporation, tend to have harmful effects on the cells [2].

Transfection by cationic liposomes is better tolerated by the cells and has the additional advantage of simplicity. Cationic liposomes interact efficiently with negatively charged nucleic acid molecules and DNA-bound lipids associate with the cell membrane, leading to DNA internalization. Since these synthetic molecules were first introduced by [3], and the number and variety of commercially available forms have greatly increased.

As demand for rapid, high efficiency transfections becomes more intense, a number of other products, including non-liposomal lipids, synthetic polymers, etc., have been developed that mediate transport of genes into cells. However, a problem associated with the majority of non-viral gene-delivery agents is their relatively low transfection efficiency [4].

A wide variety of factors can influence transfection efficiency. Essential parameters which should be taken into consideration when optimizing transfection efficiency include cell type or cell line to be used, culture conditions, and transfection vector. For instance, certain cell types or lines are intrinsically easier to transfect than others, although the exact reason for these differences is currently unknown. Even clonal variability in DNA uptake has been reported in mouse L cells [5]. Another important factor influencing the success or failure of transfection is the quality of the transferred DNA, as well as its size, configuration, and quantity.

In order to study the effects of Ras transfection involved in Phosphoinositede-3 kinase-Akt protein kinase B (PI3-K-Akt/PKB) signaling, it was essential for us to transiently introduce recombinant plasmid DNA into the primary cultured VSMCs [6] with high efficiency. We evaluated two transfection systems: FuGENE (ROCHE, FuGENE HD Reagent) and Lipofectamine (Life Technologies). In view of the fact that the majority of cell lines can survive in a serum-free environment for a limited time, we decided to try transfections with the Lipofectamine reagent, which is recommended in the absence of serum (with Opti-MEM) for maximal activity. On the other hand, it is well known that transfections in the presence of serum (%1) may accomplish better cell growth, function and viability, and may reduce the cytotoxic effects of transfection reactions [7]. For these reasons, we also tested the FuGENE reagent. Thus, we describe here the optimized transfection parameters for both Lipofectamine and FuGENE with primary cultured VSMCs.

Materials and Methods

Plasmid DNA

Plasmid pCH110 (gifted from Uğur Yavuzer, PhD., Akdeniz Univ. Sch. of Med. Dept. of Physiology) was used in all transfections as the assay vector. It contains a functional *lac*Z gene and is routinely used for screening and normalizing expression in eukaryotic cells. The vector was purified by cesium chloride gradient centrifugation and its concentration determined spectrophotometrically.

Cell culture

VSMCs were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies), supplemented with 10% fetal bovine serum, allowed to grow near confluency and harvested with trypsin. Cells were then pelleted by centrifugation and resuspended in fresh growth medium. Viable cell counts were determined by trypan blue staining and $2.5x10^5$ or $1x10^6$ VSMCs were seeded per well in either six well or 35 mm tissue culture plates in 2 ml of complete growth medium. Cells were incubated overnight at 37 ºC in a 5%CO_2 atmosphere to give 70–80% confluence before the transfection. For VSMCs, we tried lower and higher cell plating densities, but the values mentioned above resulted in optimal culture confluence recommended for transfections. Therefore, this seeding protocol was maintained throughout all the experiments presented.

In situ β-galactosidase staining

Transfection efficiency was determined by in situ staining of cells expressing β-galactosidase (β-gal) [8]. Twenty-four hours after transfection, cells were washed with Phosphate buffer saline (PBS), fixed for 5 min in 2% formaldehyde, 0.2% glutaraldehyde in PBS at room temperature, rinsed three times with PBS and stained overnight at 37 ºC with 0.1% X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$ in PBS. Stained cells were photographed using Olympus E-330 digital camera on an Olympus CKX41 inverted microscope. Results are presented as the average of three independent transfection experiments for each plasmid DNA concentration. The percent of stained cells was determined from manual counts of photographed wells (from three separate fields per transfection with at least 100 cells counted per field).

Ras Western-Blot Analysis

VSMCs were directly lysed in Laemmle buffer containing 10 mM dithiothreitol. c-H-Ras was separated on 15% polyacrylamide gel and electroblotted to nitrocellulose membranes. Ras was detected using anti-c-H-Ras

antibody (1:500, Calbiochem, US). Primary antibody was detected with horseradish peroxidase-coupled secondary antibody (1:4000, Sigma, US) and a chemiluminescent substrate (Bio-Rad, US).

Statistical analysis

SPSS statistical software Version 11.0.1 was used for statistical analysis. All data were expressed as mean ±S.E.M. Normally distributed data were analyzed by one-way ANOVA and were Bonferroni-corrected for repeated measures over time. All experiments were performed at least 3 times. Representative results of Western blot analysis are shown. A probability value $P < 0.05$ was regarded as significant.

Results and Discussion

In our initial experiments, we tried the method as described in the study done by Weber and et al. [6] with VSMCs and obtained an average of 1% transfection with low viability, which low efficiency is considering the total amount of plasmid DNA used (40 µg). This is time consuming method giving low transfection efficiency, so we evaluated two further systems: Lipofectamine (Life Technologies) and FuGENE (ROCHE).

In order to achieve optimal transfection efficiency we varied all parameters as per the manufacturers' guidelines and directions. For Lipofectamine we tested four different lipid volumes (4, 6, 8 and 10 µl) within the suggested range of 2–5 µg pCH110 and 6 h exposure of cells to the complexes. Even, the viability was high and the observed toxicity of cells was low, efficiency of transfection was not satisfied (data not shown). Each transfection was carried out in the absence of serum; we used Opti-MEM I Reduced Serum Medium (Life Technologies) during exposure of cells to DNA-liposome complexes.

We also tried three different exposures of cells to the complexes: 3, 5 h and overnight with 3 and 4 µg pCH110 and 6-8 µl FuGENE reagent, and found out that 6 h, followed by addition of complete growth medium for 24 h, was optimal under our conditions (data not shown).

Finally, we tested concentrations of plasmid DNA within the 1–2 µg suggested by the manufacturer for 6-well dishes. Plasmid DNA concentrations below and above the recommended levels were also tested (Table 1 and Figure 1). Using Lipofectamine, the highest transfection efficiency averaging 12,3 % transfected VSMCs was obtained with 4 µg pCH110 DNA, which is not within the recommended range, even an average of 9,4 % was seen using 3 µg Plasmid DNA with VSMCs, which is also above the suggested amount. Using DNA below 2 µg for VSMCs resulted in a drastic decrease in transfection efficiency (Table 1 and Figure 1B).

In order to maximize transfection efficiency using FuGENE, the following parameters were optimized: ratio of DNA to FuGENE reagent, length of exposure of cells to complexes, and quantity of plasmid DNA. We tested 1-6 µg pCH110 and observed that 3:6 and 4:8 DNA to FuGENE ratios resulted in high transfection efficiencies, while some cytotoxicity was detected at 6:12 (Table 1 and Figure 1A). Regarding length of exposure of cells to complexes, we tested 5, 16 and 24 h with 3 µg and 4 µg pCH110 and a 4:8 ratio of DNA to FuGENE (data not shown). The highest transfection efficiency without visible toxic effects was detected after 24 h. Therefore, in all subsequent experiments a ratio of 4 µg DNA to 8 µl FuGENE was used, and complexes were incubated with cells for 24 h.

Lastly, different concentrations of plasmid DNA were tested within the recommended range of 1–2 µg for 35 mm dishes, as well as above this range (Table 1 and Figure 1). Unexpectedly, we obtained the highest transfection efficiency for VSMCs using plasmid concentrations 2–3 times above the suggested values: for Lipofectamine transfection, this was an average of 12 % transfected cells with 4 µg pCH110 and for FuGENE transfection, an average of 66 % transfected cells with again 4 µg pCH110 (Figure 2). Attempts were made to further increase the quantity of DNA (with 5 and 6 µg of pCH110), but excessive cell death was observed in both transfection method due to the cytotoxic effects of Lipofectamine-DNA and FuGENE-DNA complexes (Figure 1).

After having the best transfection ratios and the efficiency, we transfected our VSMCs with a Ras^{N17} DNA by using FuGENE reagent. Afterwards, the Western-blot results were promising (Figure 3).

We are aware that transfection techniques may modify cellular activity; particularly those of the cell membrane, so functional assays should be taken into account in addition to transfection efficiency [9, 10]. For this reason, it is important to check morphologically transformed cells under high magnification during transfection for signs of cytotoxicity. However, as we have observed decreased cell survival percentages after being used the DNA:FuGENE and/or Lipofectamine ratios of 5:10 and 6:12, it can easily be told that using higher FuGENE and/or Lipofectamine concentrations could become toxic for cultured cells (Figure 1).

In conclusion, under our culture conditions for VSMCs, transfection efficiency can be augmented simply by increasing the amount of plasmid DNA. Therefore, optimization of the transfection process should include plasmid DNA 2–3 times above the recommended concentration (FuGENE) in order to accomplish the highest efficiency of transfection. And by the means of our assays, the transfection done by FuGENE reagent has become more effective in our primary cultured VSMCs as compared with Lipofectamine; especially comparing the results, as observed in the experiments of which having been done with using the ratio of 4:8 (DNA:FuGENE and /or Lipofectamine), we obtained better transfection efficiency and higher cell survival percentage by using FuGENE reagent (Figure 1).

Table 1. Transfection efficiency of Lipofectamine and FuGENE transfection reagents with various plasmid DNA (pCH110) concentrations in VSMCs.

DNA (mg)	FuGENE (ml)	Lipofectamine (ml)	%Transfected cells			
			With Lipofectamine	Cell survival %after transfection with Lipofectamin	With FuGENE	Cell survival %after transfection with FuGENE
1	2	2	3.1 ±0.4	16.3	4.6 ±0.3	20.19
2	4	4	5.2 ±1.3	22.6	10.7 ±0.7	24.7
3	6	6	9.4 ±1.7	43.9	33.2 ±2.4	57.9
4	8	8	20.2 ±1.2	81.3	66.3 ±4.3	92.4
5	10	10	7.1 ±2.1	29.2	21.2 ±3.1	44.3
6	12	12	5.8 ±0.6	23.7	14.4 ±1.9	28.6

Note: data are presented as the average of three independent transfections. % Transfected cells are defined as the percentage of cells exhibiting β-gal expression 24 h after transfection. Counts of three separate fields for each transfection, with at least 100 cells counted per field, were made for each independent experiment. Cell survival percentages are defined as the un-stained cells, which were counted at the same separate fields chosen for each transfection and independent experiment.

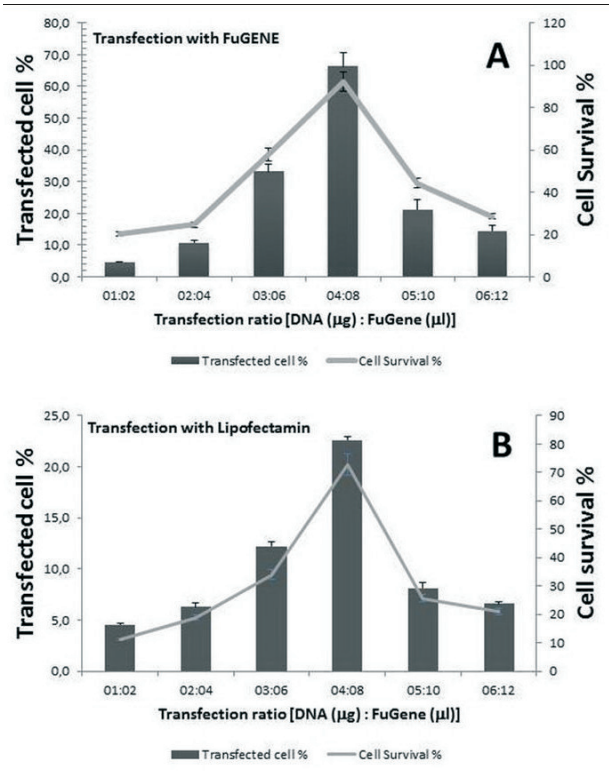

Figure 1. Transfection efficiency and cell survival of VSMCs at the indicated DNA:FuGENE (A) and DNA:Lipofectamine (B) ratios, exhibiting β-gal expression 24 h after transfection. Counts of three separate fields for each transfection, with at least 100 cells counted per field, were made for each independent experiment. Cell survival percentages are defined as the un-stained cells, which were counted at the same separate fields chosen for each transfection and independent experiment.

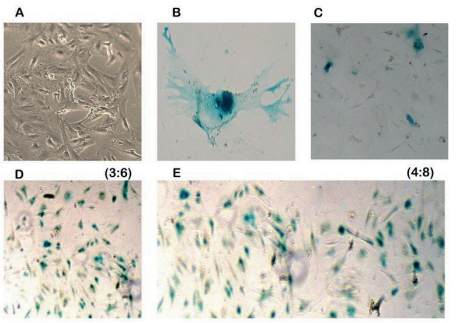

Figure 2. Non-transfected, control VSMCs (**A**). Typical view of a VSMC stained with β-Gal after being transfected with pCH110 DNA (**B**). VSMCs transfected with a ratio 1:2 (1 µg DNA/2 µl Transfection reagent) by using Lipofectamine reagent (Red arrows, marking the transfected cells while the black arrows showing the non-transfected VSMCs) (**C**). Best transfection efficiencies observed by using FuGENE HD reagent (**D**; a ratio of 3:6, **E**; a ratio of 4:8)

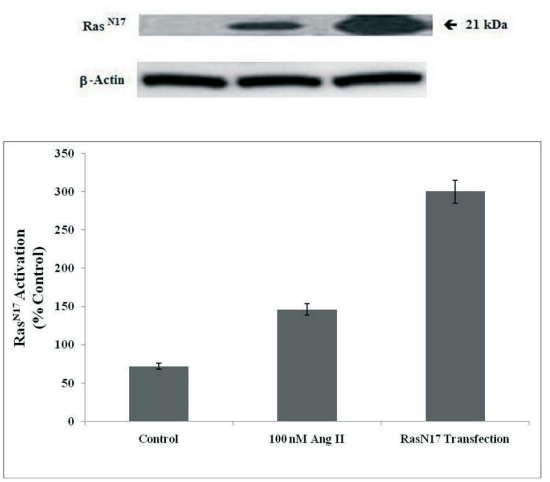

Figure 3. Ras^{N17} transfected VSMCs, has observed giving nearly 3 fold more Ras activity comparing the cells only stimulated with Angiotensin II (Ang II). The transfection has done with FuGENE reagent by the means of ratio 4:8.

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Conflict of interest: Authors have no conflict of interest

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