Research Article

Optimization of transfection of green fluorescent protein in pursuing mesenchymal stem cells *in vivo*

Mezankimal kök hücrelerin in vivo takibinde yeşil floresan protein aktarılmasının optimizasyonu

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Abstract

Objective: Green Fluorescent Protein (GFP) has been used as a marker of gene expression and a single cell marker in living organisms in cell biology studies. The important areas that GFP is used are expression levels of different genes in different organisms by inserting GFP in these genes and as a marker in living cells. In this study, we tried to optimize transfection of mesenchymal stem cells, (MSCs) used for regeneration of damaged tissues in animals, by GFP containing plasmid vector by which MSCs can be followed *in vivo*.
Material and Methods: To this aim, phM-GFP plasmid vector carrying GFP gene and effectene transfection reagent were used.
Result: The data revealed that twice transfection of MSCs resulted in higher expression of GFP for longer times as compared to once transfected MSCs. On the other hand, leaving the chemical transfection agents in the medium induced apoptosis after a while.
Conclusion: As a conclusion we suggest the transfection of MSCs twice with 48 hours interval and removal of transfection agents after 8 hours which removed toxic and apoptotic effects of the chemicals. *(Turk J Hematol 2008; 25: 172-5)*
Key words: Green fluorescent protein, mesenchymal stem cells, transfection, effectene

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Özet

Amaç: Yeşil floresan proteini (YFP), günümüzde hücre biyolojisi çalışmalarında tanımlayıcı gen ve hücre işaretleyici olarak kullanılmaktadır. YFP'nin oldukça önemli kullanım alanları farklı genlerin içerisine eklenerek bu genlerin farklı organizmalardaki ekspresyonlarının miktar tayininde ve canlı hücreler içerisinde işaretleyici olarak kullanılabilmesidir. Bu çalışmamızda doku tamiri amacıyla ve hayvanlara aktardığımız mezankimal kök hücrelerini (MKH) *in vivo* takip edebilmek amacı ile YFP genini içeren plazmid vektörünün MKH'lara aktarılmasını optimize etmeye çalıştık.
Yöntem ve Gereçler: Bu amaçla YFP geni taşıyan phM-YFP plazmid vektörü ve MKH'lara plazmid vektörün aktarılması amacı ile Effectene Transfeksiyon kiti kullanılmıştır.
Bulgular: Elde edilen sonuçlar, MKH'ların phM-YFP ile iki defa transfekte edilmelerinin tek bir defa transfekte edilmelerine göre daha yüksek oranda ve daha uzun süreli YFP ekspresyonu sağladığını göstermiştir.

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Sonuç: MKH'ların YFP ile işaretlenmesi çalışmalarında transfeksiyon kimyasallarının yeterli bir inkübasyondan sonra uzaklaştırılmasının ve transfeksiyon işleminin 48 saat arayla iki defa yapılmasının MKH'ların aktarıldığı doku veya canlılarda daha uzun süreli ve daha etkin bir şekilde takibine olanak sağlayacağı gösterilmiştir. *(Turk J Hematol 2008; 25: 172-5)*
Anahtar kelimeler: Yeşil floresan protein, mezenkimal kök hücreler, transfeksiyon, effectene

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Introduction

Stem cells have the ability to differentiate into different types of cells and to self-renew [1] and thus can be used for the generation of replacement tissue for damaged tissues and organs. They can be transplanted for the efficient treatment of diabetes [2], Parkinson's disease [3], and heart disease [4]. Adult stem cells can be obtained from the patient's own cells, maintained in cell culture and then given back to the patient [5].

Besides hematopoietic stem cells (HSCs), bone marrow (BM) contains a different cell population that plays important roles in hematopoiesis, known as mesenchymal stem cells (MSCs) or marrow stromal cells. They are composed of endothelial cells, adipocytes, osteoblasts and fibroblasts. MSCs are multipotent precursors present in adult BM, having the ability to differentiate into osteoblasts, adipocytes, chondrocytes, tenocytes, and myoblasts [6,7]. Although MSCs represent a very small fraction of the total population of nucleated cells in the marrow (0.01%-0.001%) [6], they can easily be separated from the HSCs by their ability to adhere to glasses and plastics [8]. They can maintain an undifferentiated and stable phenotype over many generations in vitro. MSCs are found in the fetal BM and liver and adult BM and peripheral blood [9].

Having the ability to migrate towards and engraft at sites of damage, *in vivo*, MSCs elicit a regenerative effect on the injured tissue. This multi-potentiality, their ease of isolation and high capacity for in vitro expansion drew considerable interest in their use in tissue engineering, in vitro and *in vivo* functional studies, and therapeutic trials [10].

This activity underpins the strong interest in the therapeutic use of MSCs in different diseases. Nevertheless, there are several questions related to the applications of MSCs that should be addressed. Very limited data are available related to the final fate of systemically infused MSC. Pereira and his co-workers showed in rodents that MSCs have a broad initial biodistribution followed by a limited capacity for sustained engraftment [11]. Parallel with this result, Liechty and co-workers showed that MSCs have undergone a wide tissue distribution and can differentiate into multiple mesenchymal tissues following peritoneal implantation in fetal sheep [12]. One of the ways to follow the MSCs is to mark them with green fluorescence protein (GFP). GFP is commonly used for monitoring gene expression, protein trafficking within intact cells and pursuing the transfected cells. GFP can easily be visualized by standard fluorescence microscopy to track real-time subcellular localization of a protein of interest.

Transfection is a method commonly used to insert plasmid DNAs, RNAs, siRNAs, and miRNAs into eukaryotic cells. As the cells were transfected with foreign molecules, these molecules could be expressed transiently or permanently. Permanent transfection is generally preferred, since the transferred genetic material is inserted into the genome. A great number of methods and reagents exist, including physical (microinjection, electroporation), chemical (calcium phosphate, DEAE-Dextran), lipophilic (liposomes), and viral (retroviruses) methods [13-16]. In our study, we preferred effectene transfection reagent, since it shows low toxicity and provides transfection of small amounts of DNA, while the transfection procedure could be performed in the complete medium [17].

In this study, we tried to optimize transfection of MSCs with GFP containing phGFP vector and then examined the fate of MSCs both in vitro and *in vivo*.

Materials and Methods

Bone Marrow Harvest: The New Zealand rabbits were sedated with ketamine (35 mg/kg intramuscular) and were anesthetized with sevoflurane (3% to 8%). The iliac crest area was prepared and draped using sterile technique and approximately 5 ml of BM was aspirated into a syringe containing 3000 units of heparin.

Isolation and Culture of MSCs: Between 10 and $15x10^6$ whole marrow cells were placed in a 75 cm^2 tissue culture flask in Dulbecco's modified Eagle's medium (DMEM; Biological Industries, Israel). After 72 hours (h), the nonadherent cells were removed by changing the medium. The medium was completely replaced every 3 days, and the nonadherent cells were discarded. Cultured MSCs were observed under inverted microscope to assess the level of expansion and to verify the morphology at each culture medium change. To prevent the MSCs from differentiating or slowing their rate of division, each primary culture was replaced (first passage) to 3 new plates when the cell density within colonies became 80% to 90% confluent, approximately 2 weeks after seeding. The adherent cells were released from dishes with 0.25% trypsin in 1 mmol/L sodium ethylenediaminetetraacetic acid (EDTA, Sigma, St. Louis, MO, USA), split 1:3, and seeded onto fresh plates. After the twice-passaged cells became nearly confluent, they were harvested and used for the experiments. We demonstrated with these adherence cells after three-passage as excluding hematopoietic markers (CD34- and CD45-) and expressing stromal markers (CD73+ and CD105+) in human MSCs. In addition, we also performed osteogenic differentiation with osteogenic media of rabbit MSCs.

Medium: The cells were routinely cultured in complete medium consisting of DMEM containing selected lots of 10% fetal calf serum (FCS), 2 mmol/L L-glutamine, 100 U/ml of penicillin G and 100 µg/ml of streptomycin (Biological Industries, Israel) at 37°C in a humidified atmosphere of 5% carbon dioxide.

Transfection of MSCs by GFP: MSCs grown in 75 cm^2 flask were detached by trypsin-EDTA treatment and replated in 25 cm^2 at a density of $1x10^6$ cells in 5 ml of complete media.

Transfection of MSCs with Monster Green Fluorescent Protein (phMGFP) Vector system (Promega) was conducted using Effectene Transfection Reagent (Qiagen). Transfection was performed as described by the manufacturer.

Briefly, the cells were split the day before transfection to maintain the viability. On the day of transfection, the cells were harvested by centrifugation, the supernatant was removed and the cells were washed once with PBS in a 15 ml Falcon tube. About $1x10^6$ cells were seeded per 60 mm dish in 4 ml growth medium containing serum and antibiotics.

The day before transfection, $5x10^5$ cells were seeded per 60 mm dish in 5 ml appropriate growth medium containing serum and antibiotics. The cells were incubated under their normal growth conditions (37°C and 5% CO_2). The day of transfection, 1 µg DNA was diluted with the DNA-condensation buffer, Buffer EC, to a total volume of 150 µl. 8 µl Enhancer was added and mixed by vortexing for 1s. This mixture was incubated at room temperature (25°C) for 5 min and then was spinned down for a few seconds to remove drops from the top of the tube. 25 µl Effectene Transfection Reagent was added to the DNA-enhancer mixture and was mixed by vortexing for 10s. The samples were incubated for 10 min at room temperature (25°C) to allow transfection-complex formation. While complex formation was in process, the growth medium was gently aspirated from the plate, and the cells were washed once with 4 ml PBS. 4 ml fresh growth medium (can contain serum and antibiotics) was added on to the cells. 1 ml growth medium (can contain serum and antibiotics) was added to the tube containing the transfection complexes. The samples were mixed by pipetting up and down twice, and immediately were added on to the transfection complexes drop-wise onto the cells in the 60 mm dishes. The dish was gently swirled to ensure uniform distribution of the transfection complexes.

Each experiment was conducted three times. The light density was obtained by fluorescent microscope and the light density of the cells was compared between two once- and twice-transfected cells for the different time points.

Results

The cells were successfully grown in DMEM containing selected lots of 10% FCS, 2 mmol/L L-glutamine, 100 U/ml of penicillin G and 100 µg/ml of streptomycin at 37°C in 5% carbon dioxide for the first passage (Figure 1), and attached MSCs (Figure 2) were selected while the suspension hematopoietic stem cells were discarded.

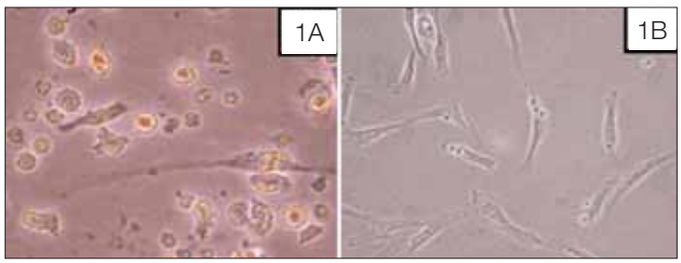

Figure 1. Micrograph of mesenchymal stem cells maintained in DMEM media for (A) 3 and (B) 5 days

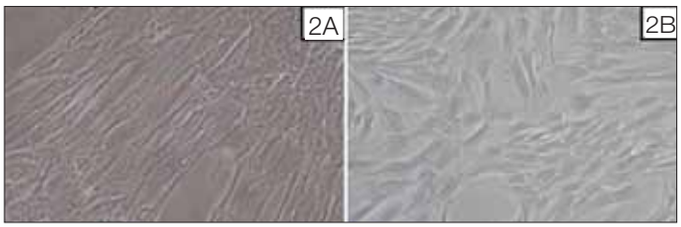

Figure 2. Micrograph of mesenchymal stem cells after (A) 2nd and(B) 3rd passage

At the beginning of the transfection experiments, the transfection chemicals were not removed from the media, which resulted in apoptosis after 72 h on the cells, which were alive and attached to the surface. Later, chemical agents were removed from the media after 8 h, the cells were washed, and the media was refreshed. It was determined that this method significantly prevented apoptosis after 72 h incubation of cells.

In order to optimize higher expression levels of GFP in the long-term, we conducted two parallel experiments. In the first group, the transfection was performed once (Figure 3), while for the second group the transfection procedure was performed twice (Figure 4), with a 48-h interval. After transfections, both groups of cells were incubated for 48-, 96-, 144- and 216 h in CO_2 incubator, and the fluorescent light density was examined under fluorescent microscope. The results revealed that, after 48 h of incubation (Figure 3A), the first group of cells reflected fluorescent green light. However, the light density was low as compared to light density of incubated cells reflected after 96- (Figure 3B), 144- (Figure 3C), and 216 h (Figure 3D) ($p<0.05$, $p<0.05$ and $p<0.01$). Twice-transfected cells also reflected higher fluorescent light density after 48 h (Figure 4A) as compared to once-transfected cells. The light density of the second group was also increased in parallel to increasing time from 48 h to 96 (Figure 4B), 168 (Figure 4C) and 216 h (Figure 4D) ($p<0.05$).

Discussion

Marking of stem cells is a widely used strategy to follow stem cells in living animals and cells. There are a number of ways to follow cells in living organisms, including microinjection, electroporation, calcium phosphate precipitation, DEAE-Dextran, liposomes and adenoviruses, lentiviruses, and retroviruses [13-16]. It was clearly shown by Meier and coworkers that effectene transfection method has low toxicity and better transfection efficiency in neuronal cells [17]. One other important property of effectene transfection reagent is its ability to work in the complete medium [17].

Tomiyama and coworkers in 2007 used the same strategy to characterize BM cells in adipose tissue in the rat [18]. Our data revealed that MSCs transfected twice with GFP vector with 48 h time interval exhibited higher expression of GFP and reflected much more fluorescent light density for a longer time as compared to once-transfected MSCs. Removing the chemical agents used for the transfection of MSCs inhibited apoptosis resulting from the cytotoxic effects of the transfection agents. These results give vital data regarding how to conduct transfection to follow MSCs transferred to tissues and living organisms.

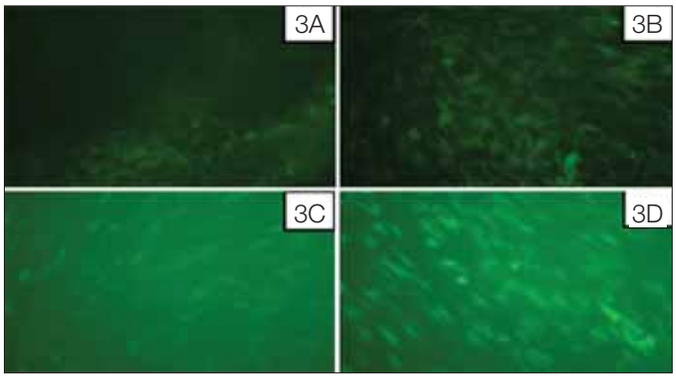

Figure 3. Micrographs of once-transfected mesenchymal stem cells incubated for (A) 48-, (B) 96-, (C) 144-, and (D) 216 hours p<0,05, p<0,05 and p<0,01

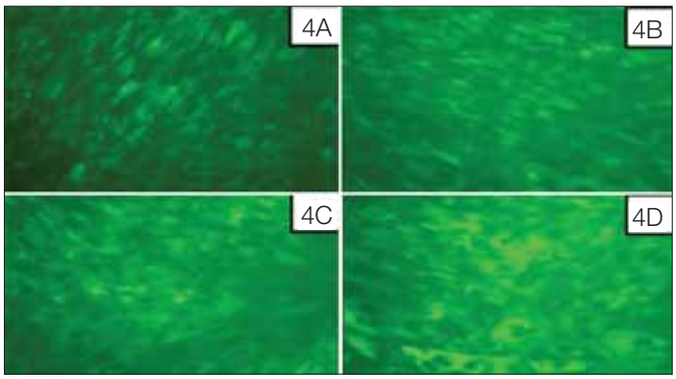

Figure 4. Micrographs of twice-transfected mesenchymal stem cells incubated for (A) 48-, (B) 96-, (C) 144- and (D) 216 hours p<0,05

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