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 öncelikli kullanılan alanları farklı genlerin içerisinde eklenebilecek bu genlerin farklı organizmalarındaki ekspresyonlarının miktarını tanımlayınca ve canlı hücreler içerisinde işaretleyici olarak kullanılabilmesidir. Bu çalışmadında doku tanımlayıcı amaca ve hayvanlara aktardığımız mezankimal kök hücrelerini (MKH) in vivo takip edebilmek amacıyla YFP genini içeren plazmid vektörünün MKH’lara aktarılması optimize etmeye çalıştık.

Yöntem ve Gereçler: Bu amaçla YFP geni taşıyan phM-YFP plazmid vektörü ve MKH’lara plazmid vektörünün aktarılması amacı ile Effectene Transfeksiyon kiti kullanılmıştır.

Bulgular: Elde edilen sonuçlar, MKH’ların phM-YFP ile iki defa transekte edilmişlerinin tek bir defa transakte edilmelerine göre daha yüksek oranda ve daha uzun süre YFP ekspresyonu sağladığı göstermiştir.
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 tissue. This multi-potentiality, their ease of isolation and high capacity for in vitro expansion drew considerable interest in their 

Liver and adult BM and peripheral blood [9].

Sonuç: MKH’ların YFP ile işaretlenmesi çalışmamıda transfeksiyon kimyasallarının yeteri bir inkübasyondan sonra uzaklaştırılması ve transfeksiyon işlemi 48 saat arayla iki defa yapıldığını MKH’ların aktarıldığı doku veya canlarda daha uzun sürele ve daha etkin bir şekilde takibe olanak sağlayacağı göstermiştir. (Turk J Hematol 2008; 25: 172-9)

Anahtar kelimeler: Yaşlı floresan protein, mezenkimal kok hücreler, transfeksiyon, effectene


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 1x10^5 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 1x10⁶ cells were seeded per 60 mm dish in 4 ml growth medium containing serum and antibiotics.

The day before transfection, 5x10⁵ 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% CO₂). 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 spun 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.

**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 transected 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.
References


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.