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Original article

Genetically Engineered Mesenchymal Stem Cells Stably Expressing Green Fluorescent Protein

[1,2] Raheleh Halabian ,[1]Mohamad Hosein Mohammadi,[1]Mohammad Salimi ,[1]Maryam Amani, [3]Amaneh Mohammadi Roushande,[1]Mahnaz Aghaipoor,[1]Nasser Amirizadeh, [1]Majid Ebrahimi, [4]Ali Jahanian Najafabadi, *[1]Mehryar Habibi Roudkenar

Abstract

Objective(s)
Mesenchymal stem cells (MSCs) are nonhematopoietic stromal cells that are capable of differentiating into and contribute to the regeneration of mesenchymal tissues. Human mesenchymal stem cells (hMSCs) are ideal targets in cell transplantation and tissue engineering. Enhanced green fluorescent protein (EGFP) has been an important reporter gene for gene therapy. The aim of this study was establishment of MSCs expressing GFP.

Materials and Methods
MSCs were isolated and characterized by Immunophenotyping. The pEGFP-N1 plasmid was extracted from previously transformed *Escherichia. coli* cells and transfected into MSCs using FuGENE HD transfection reagent. Stable cells were established in the presence of geneticin. Expression of GFP was detected by RT-PCR, western blot analysis and immunoflorecent microscope.

Results
MSCs were successfully isolated and characterized. The MSCs transfected with the pEGFP-N1 plasmid expressed GFP both in mRNA and protein level**s** while cells transfected with empty vector did not.

Conclusion
The results suggested that this engineered cell line will be used in the future studies and can easily be traced *in vivo*.

Keywords: Genetic Engineering, Green Fluorescent Protein, Mesenchymal Stem Cells, Transfection

1- Research Center, Iranian Blood Transfusion Organization, Tehran Iran
*Corresponding authors: Tel: +98 21 88601501-30; Fax: +98-21- 88613410; email: Roudkenar@ibto.ir
2-, Biotechnology Department, Tarbiyat Modares University, Tehran, Iran
3- Department of Anatomy, Faculty of Medicine, Medical University of Tabriz, Tabriz, Iran
4- Molecular Biology, Pasteur Institute of Iran, Tehran, Iran

Introduction

It has been shown that the stem cells derived from bone marrow hold the ability of self renewal and differentiation into hematopoietic and mesenchymal cell lineages (1). The fact that this unique capability is extended to the non-hematopoietic lineages including oval cells, hepatocytes, cholangiocytes (2, 3), neurons (4), skeletal muscle cells (5), and epithelial cells (6) is a relatively new observation (7). Nowadays human mesenchymal stem cells (hMSCs) have been studied for experimental and clinical purposes since the applications of human adult stem cells, such as hMSCs for cell therapy are of great clinical significance. One of the most popular reporter systems in fixed liver tissue is based on GFP, a 27-KDa monomeric protein isolated from jellyfish (8). This protein absorbs blue light and emits green fluorescence without exogenous substrates or cofactors and provides a convenient and efficient way to identify labeled cells. In *in vitro* studies, the hMSCs have been a potential cellular source for clinical applications in regenerative medicine (9). Enhanced green fluorescent protein (EGFP) has been widely used as a new reporter, especially for marking stem cells (10-12). In spite of a few studies dealing with expression of GFP by viral vectors, there is no report on stable expression of GFP by using PEGFP-N1 plasmid as non-viral expression systems in hMSCs (13-15). In this study, we established a mesanchymal stem cell (MSC) stably expressing GFP.

Materials and Methods

Isolation and culture of hMSCs

Heparinized human bone marrow was obtained by aspiration from the posterior iliac of 3 healthy volunteers with 10-20 years old, after informed consent was obtained. Bone mononuclear cells (MNCs) were prepared by density gradient centrifugation with ficoll (below 1.073 g/ml, GE Healthcare, Sweden) then, the cells were washed, counted, and distributed in a 6-well plate at 1×10^6 cells/ml in Dulbecco's modified Eagle's medium-low glucose (MSCs medium, PAA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA). These cells were incubated with 5% CO_2 at 37 °C, and the unattached cells were removed during the period of 24-48 hr. Then the adherent cells were cultured and the medium was replaced every 4 days. After ten days, individual colonies were collected, isolated, cultured and expanded. At an 80% confluence, the cells were detached with a solution of 0.25% trypsin-0.02% ethylenediamine tetraacetic acid (EDTA, Gibco, USA) in PBS and used for passages or transfection.

Immunophenotyping of hMSCs

Presence of the cell surface antigens CD105, CD166, CD45, CD90, CD34, CD73 and CD14 ((DAKO, Denmark) were detected by flow cytometry. To do this, single cell suspensions derived from individual MSC donors (third passage) were washed twice in PBS supplemented with 0.5% (v/v) bovine serum albumin (BSA). Cell suspensions were incubated with titrated concentrations of the corresponding fluorescent conjugated antibodies for 30 min at 4 ºC (10^6 cells/ml in PBS/0.5% BSA). Then the samples were analyzed in a Partec pas III Flow cytometer (Partec, Germany).

Plasmid propagation and extraction

pEGFP-N1 plasmid was kindly provided by Yoshikazu Kuwahara (Department of pathology, IDAC, Tohoku University, Japan). Bacterial strain *Escherichia coli* DH5α (Cinagen, Tehran, Iran) was used for propagation and preparation of the recombinant plasmid. The recombinant plasmid was extracted with High Pure Plasmid Extraction Kit™ according to the manufacturer's instruction (Roche, Germany). The schematic representation of the recombinant plasmid is depicted in Figure 1.

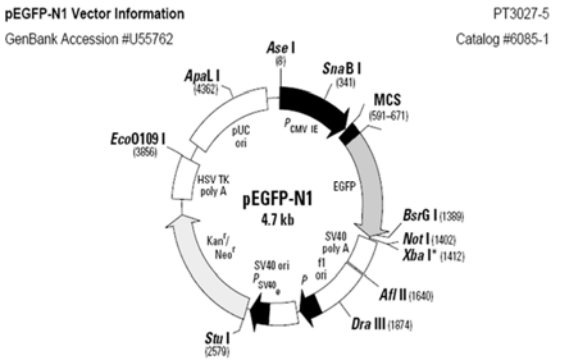

Figure 1. pEGFP vector. This is a shuttle vector that makes it possible to clone in a prokaryotic system and express in a eukaryotic system.

Establishment of GFP-expressing cells

Third passage of the hMSC cells were transfected with the pEGFP-N1 vector using FuGENE HD transfection reagent according to manufacturer's (Roche, Germany) protocol. One day before transfection, 7×10^5 cells/well were cultured in a six-well plate. For transfection, 2 μg plasmid DNA was diluted in 100 μl of transfection reagent (mixed with 6 μl of FuGENE HD) in a sterile tube and incubated at room temperature for 15 min. Following the incubation, the transfection complex was added to the cells. After 48 hr, the medium was replaced with a selection medium containing an antibiotic. In fact, the MSCs capable of stably expressing GFP were selected in a medium containing 350 μg/ml geneticin (Roche, Germany) for at least 14 days, and this led to isolation of several stable clones (Stable expression of GFP approves the integration of its gene into the hMSCs genome).

Reverse transcriptase (RT)-PCR

Total RNA was extracted from the *hMSC*-GFP by using Trizol (Invitrogen, USA), and 500 ng of this RNA was reverse transcribed by using random hexamers and the superscript pre-amplification system for first strand cDNA synthesis as instructed by the manufacturer (Invitrogen, USA).

PCR was performed by Taq DNA polymerase (Roche, Germany) and the PCR condition included an initial denaturation at 94 °C for 5 min, followed by 30 cycles of 30 sec at 94 °C, 30 sec at 59 °C and a final extension of 1 min at 72 °C. β-actin expression was evaluated for normalization.

Primer set for GFP consisted of a forward (5′-GACGACGGCAACTACAAGA-3′) and a reverse (5′-GATGCCGTTCTTCTGCTT-3′) primer, which amplify 717 base pairs (bp) of GFP. For normalization, expression of b-actin was examined by using a primer pair including a forward (5′-TTC TAC AAT GAG CTG CGT GTG G-3′) and a reverse (5′-GTG TTG AAG GTC TCA AAC ATG AT-3′) primer.

Western blot analysis

Total proteins were extracted by using lysis M buffer (Roche, Germany) according to manufacturer's instruction. The proteins were separated on a 12% SDS-PAGE and trans blotted onto PVDF membranes in a buffer containing 25 mM Tris HCl pH 8.3, 192 mM glycine and 20% methanol. Then, the PVDF membranes were incubated in 10 mM PBST (0.1% Tween 20 in PBS 10 mM) containing 3% skimmed milk for 12 hr. Afterwards the membranes were incubated with anti-GFP polyclonal antibodies (rabbit IgG, 1:10,000 dilution in PBST) for 1 hr at room temperature followed by 5 times washing with PBST. Then the membranes were incubated with HRP conjugated-anti-rabbit secondary antibodies (1:10,000 dilutions in PBST) at room temperature for 1 hr with gentle rocking. Finally, the membranes were washed 5 times with PBST and the immunoreactive bands were developed by ECL kit (Amersham, USA).

Immunofluorescence microscopy

MSCs which were supposed to be capable of stable expression of GFP were further evaluated for two months after transfection by fluorescence microscopy on a Nikon Eclipse TE300 inverted microscope (Nikon instruments; Melville, NY) with filter sets designed for EGFP. Digital images were taken using a nikon E8400 color camera (Nikon, Japan). After transfection, MSCs were trypsinized and the transfection efficiency was determined by counting GFP expressing cells versus non-expressing cells under the fluorescent microscope.

MTT assay

Cell viability following transfection was determined by MTT assay. To do this, 1×10^4 transfected cells/well were seeded in a 96-well plate and on seventh day the cells were incubated with 10 μl of 3-(4,5-dimethlthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT, Sigma) with a concentration of 5 mg/ml at 37 °C in 5% CO_2 atmosphere for 4 hr. Then the reaction was stopped by addition of 10% SDS in 0.01 M HCl, and cell viability was measured using a microplate reader.

Results

Isolation and culture of hMSCs

MNCs were isolated from human (10-20 years old) bone marrow by density gradient fractionation and adherence to plastic flasks.

After 10 days, individual colonies were selected, cultured and expanded in tissue dishes. Following reaching 80% confluence, cells were detached by trypsinization and passaged every 4-5 days for 12 passages and showed no morphologic changes. Fibroblast-like morphological features could be seen in typical primary or passaged hMSCs (Figure 2).

Immunophnotyping of hMSCs

Human bone marrow-derived MSCs were analyzed routinely for the presence of typical MSC-related cell surface antigens by flow cytometric analysis. These cells showed the typical antigenic profile of MSC and were positive for CD166, CD105, CD90 and CD73 antigens. They were also negative for the antigens CD45, CD34 and CD14 (Figure 3).

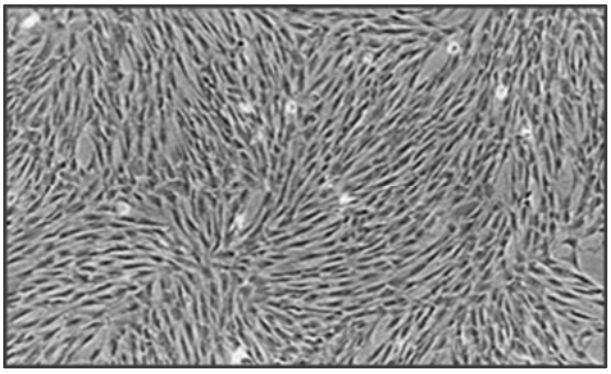

Figure 2. MSCs which are approximately 90% confluent. Isolated mesanchymal Stem Cells present fibroblast like cells.

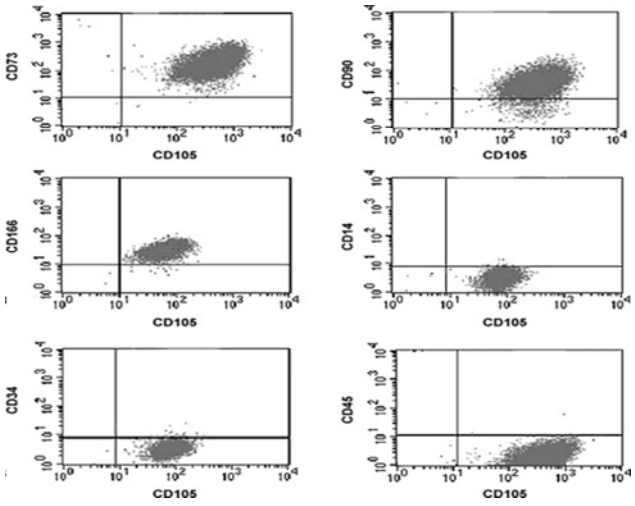

Figure 3. Cell surface antigen profile of adult human bone marrow-derived MSC. Flow cytometric analysis demonstrates that propagated MSC (passage 3) are positive for the antigens CD166, CD105, CD90 and CD73 but negative for CD45, CD34 and CD14.

Expression of GFP in MSCs

Stable engineered MSCs were generated in the presence of geneticin. RT-PCR was performed to investigate the expression of GFP by the transfected cells. GFP mRNA was expressed by the isolated stable clones, but there was no expression in the MSCs transfected with empty vector (Figure 4).

Transfection efficiency was about 60 percent as determined by observation of the cells under the fluorescent microscope. Next, western blot analysis was performed for detection of GFP protein. Cells transfected with the recombinant vector expressed GFP protein, whereas the cells transfected with empty vector do not (Figure 5).

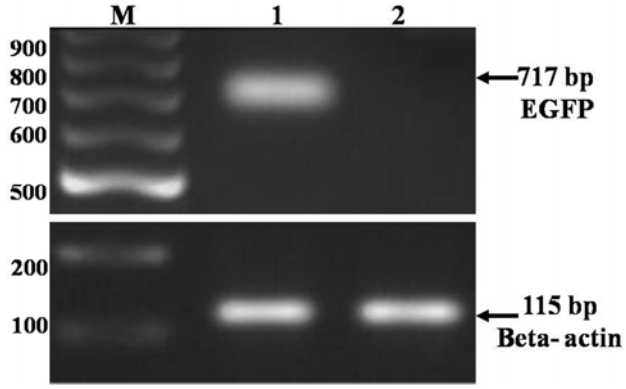

Figure 4. (A) Expression of GFP by stable clones of MSCs. RNA was extracted from cells transfected with the recombinant vector and also cells transfected with empty vector. cDNA was synthesized and RT-PCR was performed using a pair of primers specific for GFP. MSCs transfected with the recombinant plasmid expressed GFP mRNA and revealed a 717-bp fragment of GFP mRNA following PCR (Lane 1), whereas MSCs transfected with empty vector revealed no expression (Lane 2). Lower figure indicates the expression of b-actin in both stable clones of MSC, i.e., transfected with the recombinant or the empty vector. M; 100-bp ladder marker.

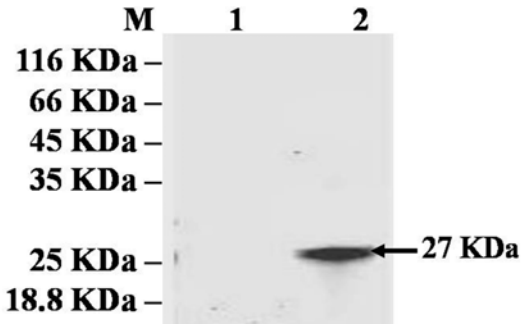

Figure 5. Western blot analysis of GFP expression by the stables clones. MSCs transfected with the recombinant vector expressed GFP protein (Lane 2), whereas in MSCs transfected with the empty vector expression was not observed (Lane 1).

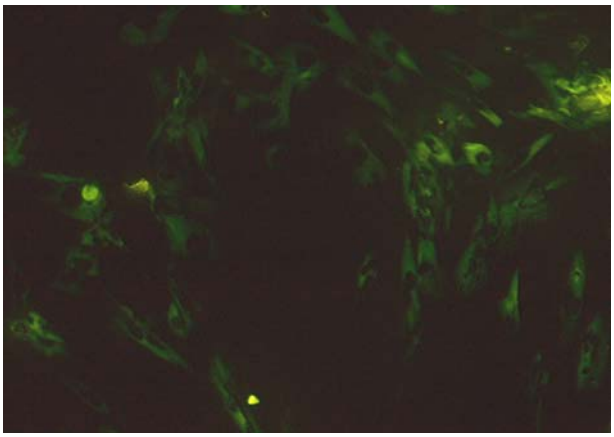

Figure 6. Fluorescence microscopy showed GFP expression in transfected mesenchymal stem cells.

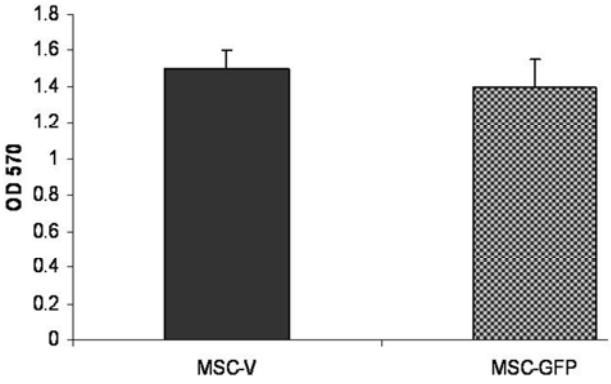

Figure 7. Cell viability of stable MSCs following transfection with pEGFP vector was determined by MTT assay on day 7 post transfection. There was no difference between the viability of the cells transfected with the empty vector (MSC-V) or the recombinant vector (MSC-GFP) (Mean ± SD: $P > 0.05$).

Finally the expression of GFP by the stable clones of MSCs was confirmed by florescence microscopy (Figure 6). Eventually, no toxicity effect by GFP was observed as determined via MTT assay (Figure 7).

Discussion

In this study, MSCs were isolated and transfected with a recombinant plasmid containing GFP gene. BrdU, fluorescent dye, green fluorescent protein, magnetic, and isotope labeling techniques are some of the most widely used techniques for stem cell labeling in stem cell transplants fields. The selection of each of these tracing techniques depends on the aims of the experiment and also the stem cells characteristics. The BrdU and fluorescent dye techniques lie among the firstly used tracing methods because of their convenience. But the problem with these techniques is the gradual decreasing in their labeling intensity as the time passes, and also their inability for *in vivo* tracing of labeled stem cells (16-21). Although MRI and isotope labeling techniques are used for *in vivo* tracing of stem cells because of their non-invasive properties, the former technique can result in false positives and the latter is limited by the specific stem cell markers (22-25).

GFP is widely applied for stem cell tracing because of the possibility of its stable expression, high specificity, and its easy *in vivo* tracing, however, its application is limited by certain cytoxicity of high GFP levels (10, 26). Lee *et al* transduced hMSCs by a GFP-retroviral vector (13). Viral vectors have been also used in other similar studies (14-15). But in the present study the non-viral pEGFP-N1 vector was used for transfection of hMSCs and stable expression of GFP in these cells. It should be noted that the safety of viral vectors is a major concern in application of MSCs in transplantation. Therefore, the fact that the vector used in the present study is a plasmid-based vector but not a viral derived vector would be considered as an advantage over the other studies. Recently, improvement of a bacterial fluorescence has been reported by Yoo *et al*. In their study the fluorescence of bacterial cells expressing a variant of GFP (GFPm) was reduced to background levels by entire replacement of the leucine residues of GFPm by 5, 5, 5- trifluoroleucine. The median fluorescence of cells expressing this fluorinated protein was improved about 650-fold when compared to that of expressing non-fluorinated GFPm (26). The possibility of repairing the liver injuries in wild-type mice by transplantation of both wild type and green-GFP bone marrow has been reported by Beaudry *et al*. and they detected the GFP expression in the transplanted cells (27). The therapeutic effects of tyrosine hydroxylase (TH) transfected NdSCs-D-BMSCs on Parkinson's disease through different transplantation protocols were investigated, which resulted in GFP expression by 62.1% of the transfected cells 5 day post transfection

and the rate of co-expression with TH was 83.5% (28). Also, GFP or GFPm has been widely used in cardiomyocytes regeneration (29, 30), retinal pigment epithelium cell repair (31), and skin repair (32). In the present study, FuGENE HD transfection reagent was used for transfection of the pEGFP-N1 into the MSCs. Simple methodology, minimal optimization, low cytotoxicity and ability to provide high transfection efficiency even in the presence of serum are advantages of FuGENE HD transfection reagent. Taking together, in this study we reported the stable expression of GFP in bone marrow derived mesanchymal stem cells and our results suggested that this technique would be applicable for labeling the MSCs in their potential future applications. Despite the advantages of GFP labeling of stem cells and transgenic mice, there is some limitations including (i) it is difficult to prepare a continuous, high performance and stably GFP expressing clone; (ii) high levels of GFP expression could affect cell growth by decreasing the growth rate which suggests that GFP may be cytotoxic; (iii) it is impossible to amplify GFP signals, therefore when its expression by stem cells is low it could not be detected and can't be used for quantitative analysis.

Conclusion

In this study the MSCs were successfully isolated and a cell line stably expressing GFP was established. In future studies, this cell line will be used and can easily be traced *in vivo*.

Acknowledgment

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