

ORIGINAL ARTICLE

Transfected human mesenchymal stem cells do not lose their surface markers and differentiation properties

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Abstract

In this study, we evaluated the biological properties of human mesenchymal stem cells transfected (hMSC) with a plasmid vector expressing human cytokine interleukin-12 (IL-12). Surface markers were analysed by immunophenotyping using flow cytometry. Differentiation capability was evaluated towards adipogenesis and osteogenesis. We demonstrated that successfully transfected hMSC retained their surface immunophenotypes and differentiation potential into adipocytes and osteocytes. These results indicate that hMSC may be a suitable vehicle for gene transduction.

Key words: Mesenchymal stem cells, transfection, differentiation, immunophenotyping

INTRODUCTION

Mesenchymal stem cells (MSC) are considered to be a promising platform for cell and gene therapy for a variety of diseases.¹⁻² Work over the past 30 years has resulted in a greater understanding of the biology and therapeutic application of human stem cells in human malignancies.³⁻⁶ The cell population known as MSC is usually isolated from the mononuclear fraction of a bone marrow aspirate, which is then depleted of CD45+ cells and subsequently isolated as the cell population that adheres to plastic tissue culture dishes. MSC can proliferate for many passages in culture and have the ability to give rise to several differentiated cell types, including adipocytes,⁷ chondrocytes,⁸⁻⁹ osteocytes,¹⁰ tenocytes,¹¹ astrocytes and neurons.¹² They are easily obtained from a simple bone marrow aspirate that can be readily expanded to hundreds of millions of cells. MSC are easily transfectable, allowing easy *ex vivo* modification. Because of these properties, MSC have considerable therapeutic potential in several disease processes, including cardiovascular disease, as well as in the treatment of human malignancies.

Transfection is a method used to introduce nucleic acids into mammalian cells. This

technique has been used for a wide variety of applications for protein expression and/or gene analysis. Liposomal and non-liposomal agents are the most common and easy-to-use transfection reagents currently in use. However, all transfection techniques may induce varying levels of cytotoxicity and off-target effects that can mask or alter the cellular response to a gene of interest. Therefore, we would like to study the impact of transfecting a particular gene, interleukin-12 on MSC. This information is important for subsequent application.

In this study, we addressed the biological properties of the transfected MSC in terms of immunophenotypic expression and differentiation potential and made a comparison with that of untransfected normal MSC.

MATERIALS AND METHODS

Bone marrow aspiration

A bone marrow aspirate was taken from the iliac crest of a patient after written informed consent. Sample collection in the study was approved by the institutional research and ethics committee in compliance with good clinical practice guidelines.

MSC isolation and culture

Two ml of bone marrow aspirate was diluted with an equal volume of phosphate buffered saline (PBS) without Ca²⁺ and Mg²⁺ (Gibco, New York, USA) and layered on top of 5 ml Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden). After centrifugation at 2500 rpm for 25 minutes, the mononuclear cells in the interface (density gradient of 1.073 g/cm³) were harvested and washed twice with two parts of PBS by centrifuging at 1000 rpm for 10 minutes. The cell pellet was then suspended in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, New York, USA) and the viable cells were counted by haemocytometer in Trypan Blue exclusion test. 1 X 10⁶ cells were seeded in a 25cm² culture flask containing complete media of DMEM-high glucose and 10% fetal bovine serum (FBS) (Gibco, New York, USA). The cells were incubated in humidified 5% CO₂ incubator until confluency. The culture medium was changed every 3 days.

Transfection

MSC were transfected with pORF-hIL-12 (Invivogen, California, USA) using LipofectamineTM 2000 transfection agent (Invitrogen, USA) according to the manufacturers' instructions and predetermined optimized ratios of DNA (μ g) and LipofectamineTM 2000 transfection reagent (μ l). Genetically engineered MSC were collected 24 hours post transfection and used in the study. To assess human IL-12 production, culture supernatants were collected and measured in duplicate for IL12p70 by using an enzyme-linked immunosorbent assay kit (eBioscience, San Diego, USA) according to the manufacturer's instructions.

Immunophenotyping analysis

For immunophenotypic analysis, medium was removed from the flask and confluent MSC cell layers were washed twice with PBS (Gibco, New York, USA) after detachment with 0.25% trypsin-EDTA (Gibco, New York, USA). The cell pellet was suspended in FACSFlowTM (Beckton-Dickinson, Ontario, Canada) at concentration of 1 X 10⁷ cells/ml and incubated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated monoclonal antibodies (Beckton-Dickinson, Ontario, Canada) in dark, room temperature for 30 minutes. A panel of monoclonal antibodies was used in the study, namely CD3, CD14, CD29, CD34, CD44, CD45, CD54, CD56, CD 73, CD90, CD105 and

HLA-DR. After incubation, the cell suspension was washed with PBS to remove any unlabelled antibodies. The cells were resuspended in FACSFlowTM and analysed using FACScan flow cytometer (Becton Dickinson, San Diego, CA, USA). A total of 10000-gated events were collected and analysed using CellQuest software and FACS Calibur (Beckton-Dickinson, Ontario, Canada). The results were checked with its isotype controls.

Differentiation study

MSC were differentiated towards adipogenic and osteogenic lineages. Untransfected normal MSC were used as positive control. As negative control, both transfected and untransfected MSC were cultured identically in standard medium (DMEM high glucose supplemented with 10% FBS) without differentiation stimulants.

Adipogenic differentiation was performed using 1 μ M dexamethasone (Sigma-Aldrich, USA), 0.2mM indomethacin (Sigma-Aldrich, USA), 0.1 mg/ml insulin (Sigma-Aldrich, USA) and 0.5mM 3- isobutyl-1-methylxanthine (Sigma- Aldrich, USA) according to approved procedure by Jaiswal *et al.*¹³

Osteogenic differentiation of MSC was carried out according to Jandeora *et al.*¹⁴ using 100nM dexamethasone (Sigma-Aldrich, USA), 10mM β -glycerophosphate (Sigma-Aldrich, USA) and 0.5mM L-ascorbic acid-2-phosphate (Sigma-Aldrich, USA).

After 2-3 weeks of culture in induction medium, differentiated MSC were subjected to cytochemical staining. Adipogenic induced MSC were stained with Oil Red O using a standard protocol while osteogenic induced MSC were evaluated by Alizarin Red S.

Statistical analysis

The experiment was carried out at least twice with independent samples. Data was expressed as mean and standard deviations. The results were analyzed with the Paired Student's t test. Two-sided p values <0.05 were considered statistically significant.

RESULTS

Human IL-12 protein expression

Lipofectamine transfection reagent induced a high amount of 412 pg/ml IL-12p70 protein 24h post transfection in IL-12 transfected MSC (10-fold increase over the untransfected MSC controls) (P=0.017) (Figure 1). These IL-12 transfected MSC constitutively expressed and

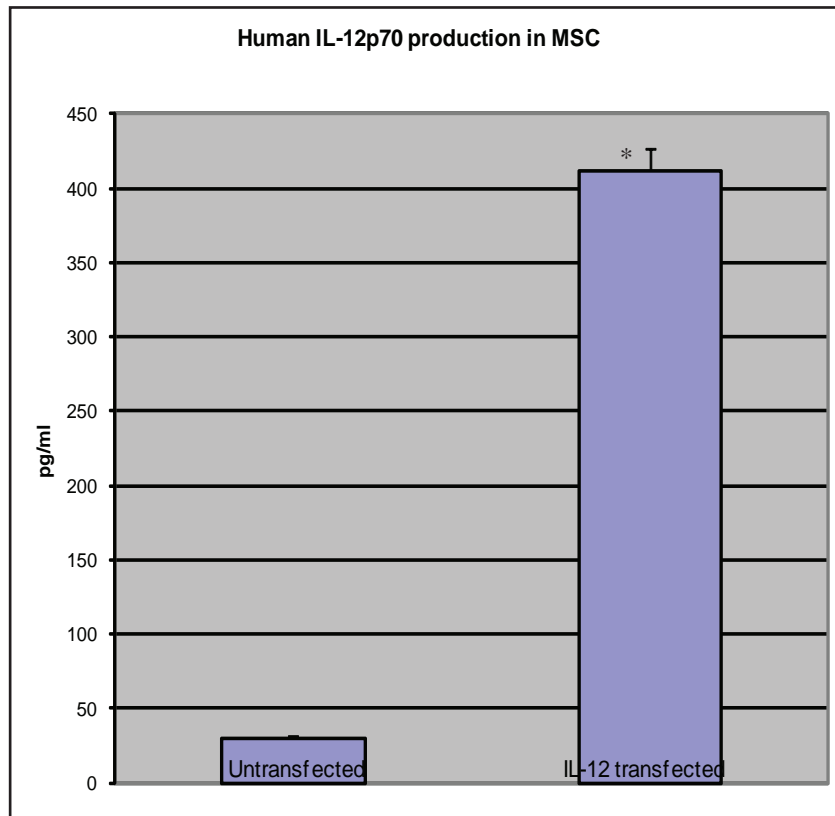


FIG. 1: Human IL-12 production in transfected and untransfected MSC. The average is represented as a bar graph and standard deviation is represented as error bars. Asterisk indicates significance compared to untransfected MSC ($p < 0.05$)

secreted IL-12 as more than 150 pg/ml of IL-12 was still detectable after 3 weeks (data not shown).

Immunophenotypes of MSC

The quality of MSC transfected with human interleukin-12 was monitored by assessing

their morphology after *in vitro* culturing and comparing with that of normal MSC. Analysis by light microscopy revealed that transfected MSC retained typical morphology of MSC as fibroblast-like cells (Figure 2).

Flow cytometry indicated that all the culture expanded MSC represented a homologous cell

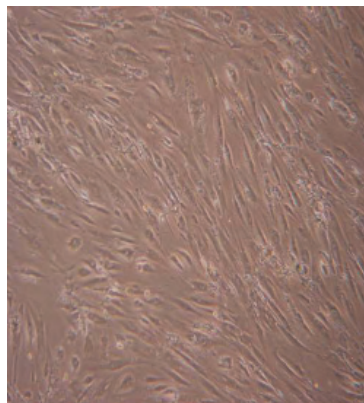


FIG. 2: Morphology of IL-12 transfected MSC (Magnification: 40X)

population. A single peak of fluorescence was shown for a variety of cell markers. Figures 3(a) and 3(b) represent immunophenotypic analysis of MSC from flow cytometry prior and after transfection, respectively. Both untransfected MSC and transfected MSC showed their immunophenotype in a similar manner. In our study, expression of more than 1% is considered as positive. These cells constitutively expressed CD29, CD44, CD54, CD73 and CD105. Cells of the haematopoietic lineages were not detected in the human MSC cultures as demonstrated by the absence of cells expressing those markers such as CD3, CD14, CD34, CD45, CD56 and HLA-DR.

Statistical analysis on percentage of CD markers expressed in untransfected and transfected MSC revealed no significance change for all the CD markers tested (Table 1).

Differentiation and cytochemical staining

To prove that MSC still maintain their stem cell character after liposomal mediated transfection, we differentiated IL-12 transfected MSC into adipocytes and osteocytes (Figure 4). Their ability to differentiate was compared to that of untransfected normal MSC.

Induction of adipogenic differentiation was apparent by the accumulation of lipid-rich vacuoles within the cells and stained red with

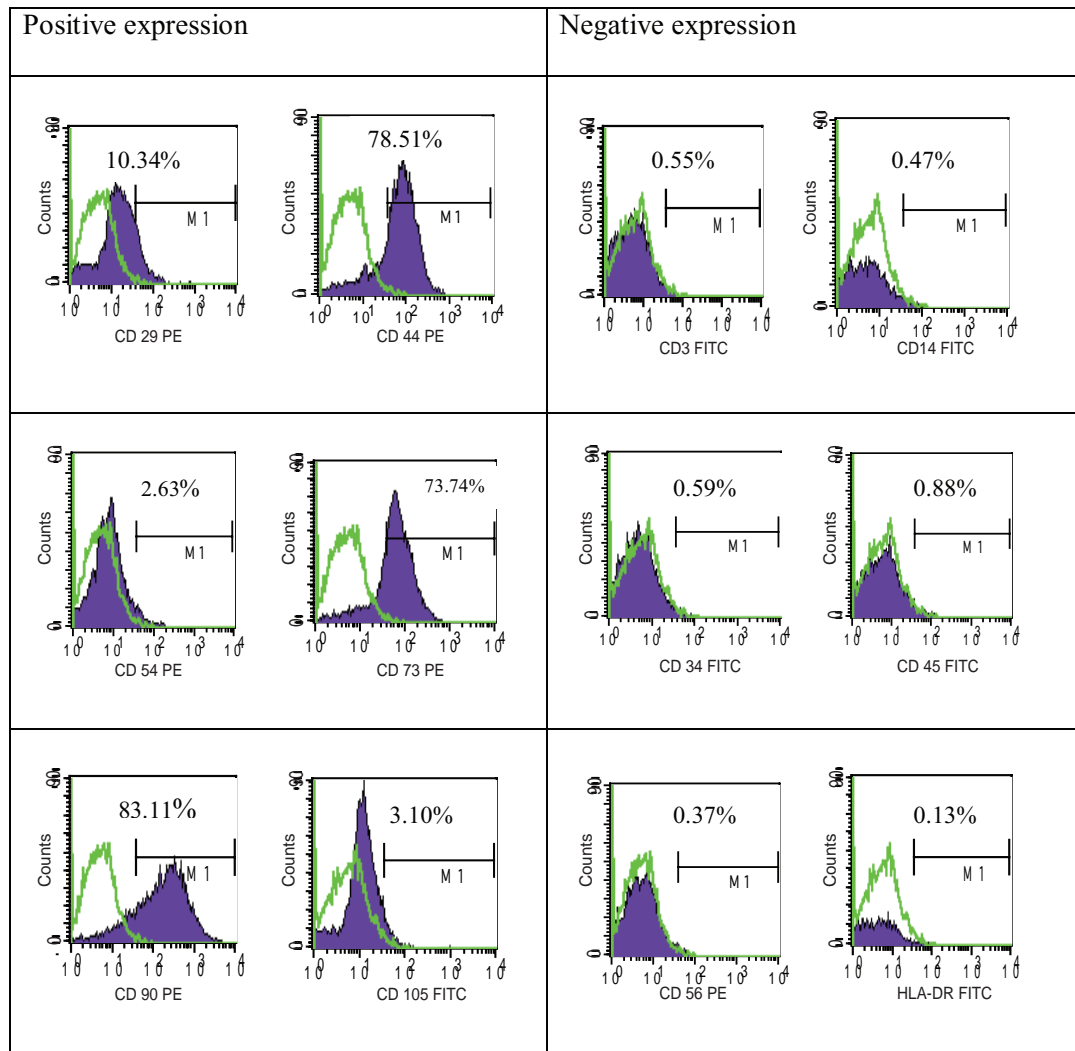


FIG. 3: (a) Representative immunophenotypic analysis showed untransfected MSC express CD29, CD 44, CD54, CD73, CD90 and CD105 but negative for CD3, CD14, CD34, CD45, CD56 and HLA-DR. The filled overlays represent MSC specific monoclonal antibody fluorescence, whereas the unfilled overlays represent fluorescence from non-specific isotype control antibodies.

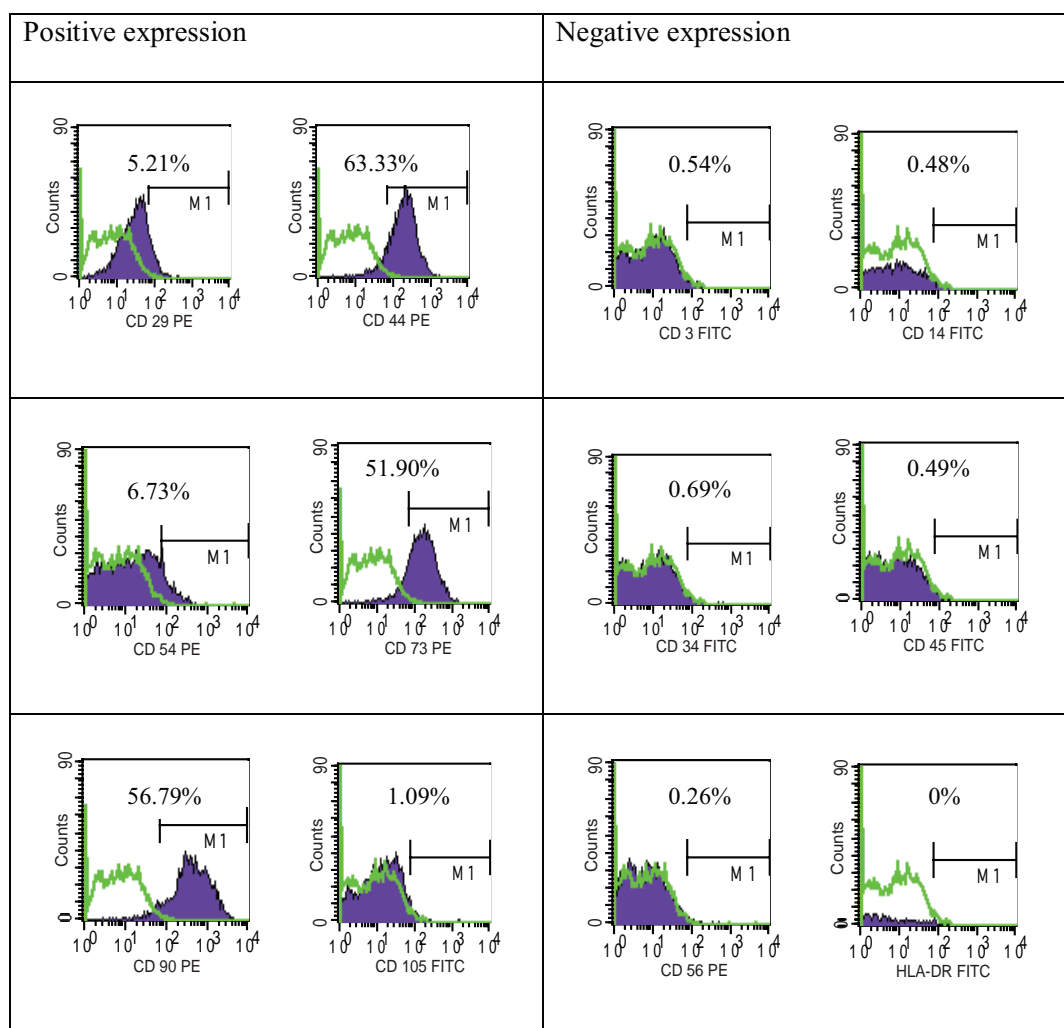


FIG. 3: (b) Representative immunophenotypic analysis showed IL-12 transfected MSC maintain expression of the panel of CD markers tested which is positive for CD29, CD 44, CD54, CD73, CD90 and CD 105 but negative for CD3, CD14, CD34, CD45, CD56 and HLA-DR. The filled overlays represent MSC specific monoclonal antibody fluorescence, whereas the unfilled overlays represent fluorescence from non-specific isotype control antibodies.

Oil Red O staining. Evidence of osteogenic differentiation was sought with Alizarin Red S, where detectable calcium deposition in differentiated MSC was stained orange red. The ability of these transfected MSC to differentiate into adipocytes and osteocytes in adipogenic induction medium and osteogenic induction medium, respectively, indicates that the stem cell character of transfected MSC is unperturbed despite liposomal transfection.

DISCUSSION

Mesenchymal stem cells can be harvested from human bone marrow and expanded *in vitro*.

Although only 0.001-0.1% of nucleated cells in the bone marrow are MSC, millions of cells can be generated after a few passages in culture, with no spontaneous differentiation.¹⁵ We were able to generate a large number of MSC from a single bone marrow aspirate for experimental purposes.

Unlike haematopoietic stem cells in which differentiation occurs during *in vitro* culture, resulting in less repopulation capacity than in freshly explanted marrow,¹⁶⁻¹⁷ MSC do not differentiate during *in vitro* cell expansion as MSC differentiation requires specific signals. Undifferentiated MSC as well as differentiated adipocytes and osteocytes from MSC in our study

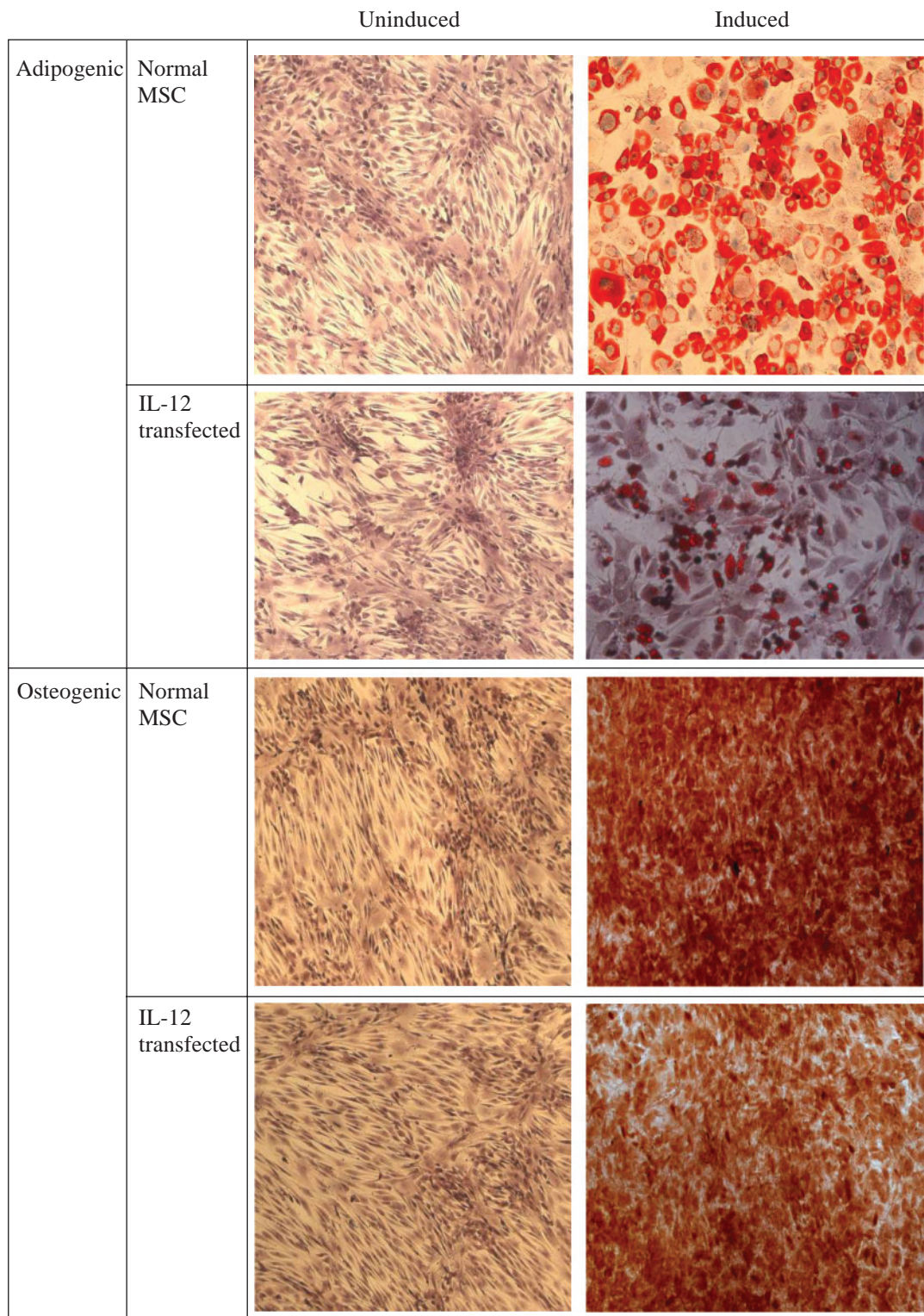


FIG. 4: Differentiation abilities of IL-12 transfected MSC compared with normal untransfected MSC: Cytochemical staining of adipogenic and osteogenic differentiated MSC and uninduced control cells, stained with Oil Red O and Alizarin Red, respectively. Magnification: 40X

TABLE 1: Average percentage of CD markers expression in untransfected and transfected MSC as measured by FACS analysis. Values represent mean \pm standard deviation

CD Markers	Human MSC (%)		P Value
	Untransfected	Transfected	
CD 29	7.87 \pm 3.493107	5.12 \pm 0.127279	0.477
CD 44	79.935 \pm 2.015254	66.805 \pm 4.956819	0.229
CD 54	3.015 \pm 0.544472	6.48 \pm 0.353553	0.115
CD 73	79.315 \pm 7.884241	53.225 \pm 1.873833	0.165
CD 90	87.525 \pm 6.243753	63.04 \pm 8.838835	0.262
CD 105	2.44 \pm 0.933381	1.06 \pm 0.042426	0.273
CD 3	0.365 \pm 0.26163	0.375 \pm 0.233345	0.705
CD 14	0.26 \pm 0.296985	0.425 \pm 0.077782	0.480
CD 34	0.38 \pm 0.296985	0.515 \pm 0.247487	0.161
CD 45	0.475 \pm 0.572756	0.425 \pm 0.091924	0.907
CD 56	0.23 \pm 0.19799	0.28 \pm 0.028284	0.807
HLA-DR	0.07 \pm 0.084853	0.44 \pm 0.622254	0.594

show similar morphology and cytochemical characteristics as reported by Mok PL *et al.*¹⁸⁻¹⁹

More importantly, as shown in this study, transfected MSC retained their stem cell character. After transfection and culture expansion, the transfected MSC maintained their phenotypic profiles and their ability to undergo adipogenic and osteogenic differentiation when exposed to the appropriate stimuli. This is consistent with the research findings by Lee K *et al.* and Böcker W *et al.*²⁰⁻²¹ although their studies utilized viral transfection.

In the study by Lakshmipathy and Aluigi *et al.*²²⁻²³, nucleofection (Amaxa Biosystem) was used as a method of transfection and shown to be superior for transfection of embryonic stem cells and other hard-to-transfect adult stem cells. Comparison was made in Lakshmipathy's study with electroporation as well as lipid-based transfection reagents, FuGENE6 and DOTAP (Roche Applied Science). However, transfection of MSC using Lipofectamine (Invitrogen) also yielded high transfection efficiency as shown in our study. One should consider that optimal conditions are required for efficient liposomal transfection. These include reduced serum conditions, pre-optimized DNA to liposome ratios and sufficient cell density for transfection. Usage of Lipofectamine has been reported in

several studies in mouse embryonic stem (mES) cells. It generally achieves 20%-25% transfection efficiency in mES cells.²⁴ A modified method of Lipofectamine transfection even increased transfection efficiency to 50% under optimal condition.²⁵

To our knowledge, this is the first report demonstrating gene transfer into human bone marrow-derived MSC using Lipofectamine™ 2000 transfection agent (Invitrogen) with maintenance of transgene expression, without affecting the immunophenotypic characteristics and differentiation ability into adipocytes and osteocytes of MSC.

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