Transcriptional profiling of human mesenchymal stem cells transduced with reporter genes for imaging

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Wang F, Dennis JE, Awadallah A, Solchaga LA, Molter J, Kuang Y, Salem N, Lin Y, Tian H, Kolthammer JA, Kim Y, Love ZB, Gerson SL, Lee Z. Transcriptional profiling of human mesenchymal stem cells transduced with reporter genes for imaging. Physiol Genomics 37: 23–34, 2009. First published December 30, 2008; doi:10.1152/physiolgenomics.00300.2007.—Mesenchymal stem cells (MSCs) can differentiate into osteogenic, adipogenic, chondrogenic, myocardial, or neural lineages when exposed to specific stimuli, making them attractive for tissue repair and regeneration. We have used reporter gene-based imaging technology to track MSC transplantation or implantation in vivo. However, the effects of lentiviral transduction with the fluc-mrfp-0tk triple-fusion vector on the transcriptional profiles of MSCs remain unknown. In this study, gene expression differences between wild-type and transduced hMSCs were evaluated using an oligonucleotide human microarray. Significance Analysis of Microarray identified differential genes with high accuracy; RT-PCR validated the microarray results. Annotation analysis showed that transduced hMSCs upregulated cell differentiation and antiapoptosis genes while downregulating cell cycle, proliferation genes. Despite transcriptional changes associated with bone and cartilage remodeling, their random pattern indicates no systematic change of crucial genes that are associated with osteogenic, adipogenic, or chondrogenic differentiation. This correlates with the experimental results that lentiviral transduction did not cause the transduced MSCs to lose their basic stem cell identity as demonstrated by osteogenic, chondrogenic, and adipogenic differentiation assays with both transduced and wild-type MSCs, although a certain degree of alterations occurred. Histological analysis demonstrated osteogenic differentiation in MSC-loaded ceramic cubs in vivo. In conclusion, transduction of reporter genes into MSCs preserved the basic properties of stem cells while enabling noninvasive imaging in living animals to study the biodistribution and other biological activities of the cells.

DNA microarray; triple fusion reporters; bioluminescence; positron emission tomography

INCREASING EVIDENCE HAS SHOWN that adult human stem or progenitor cells can be useful for therapeutic treatment of neurodegenerative disorders, cancer, ischemic heart diseases, metabolic defects, and other ailments by differentiating into tissue-specific phenotypes, by secreting chemokines, or, in some cases, by cell fusion (37). Exogenous stem cells have shown to recruit or support endogenous stem cells for tissue repair (30).

Human mesenchymal stem cells (hMSCs) are self-renewing, pluripotent adult stem cells that were originally isolated from bone marrow but that are found virtually in all adult organs (29). hMSCs have the potential to differentiate into bone, cartilage, adipose tissue, and cardiomyocytes, as well as neurons under specific conditions (7, 10, 18, 23, 32). Cultured autologous and allogeneic MSCs have been tested in preclinical models for the repair of bone, cartilage, and tendon/ligament by local delivery of MSCs with an appropriate scaffold (4, 15, 20, 45). Additionally, MSCs support hematopoiesis in bone marrow transplant recipients by providing a tissue microenvironment that supports hematopoietic stem cell proliferation and differentiation (24). MSCs have been reported to ameliorate graft versus host disease caused by allogeneic hematopoietic stem cell transplantation (8). Furthermore, hMSCs administered systemically have been shown to home to neoplasms in vivo and thus have the potential to serve as delivery vehicles for anticancer therapies (31). Finally, hMSCs injected in the settings of myocardial infarction and stroke improve posts ischemic regeneration and function (9, 26).

The promise of MSC therapeutics mandates a better understanding of the long-term fate and dynamics of transplanted MSCs in vivo. Traditionally, the majority of such research relied on ex vivo labeling MSCs by various methods, followed by transplantation and euthanasia of the animals at preset serial posttransplant time points to assess histological or biological evidence of MSC fate. It is necessary to develop a non-invasive, longitudinal approach to monitor the fate of hMSC transplants in vivo.

Recently, several noninvasive, imaging-based monitoring modalities have been used to track stem cell transplants via radiolabels, ferromagnetic particles, and reporter genes (1, 3, 5, 27, 28). However, direct labeling of stem cells with radionuclides such as [F-18]-FDG for positron photon emission tomography (PET) imaging (14, 28), or with [In-111]-oxine or [In-111]-trotoplene for single photon emission computer tomography (SPECT) (3, 12, 19), only allows short-term imaging of infused stem cells, due to the short half-life of radioisotopes. Labeling stem cells with superparamagnetic iron oxide particles or perfluoropolyether for magnetic resonance imaging (MRI) imaging suffers from signal decay as cells divide or die (1, 5). More importantly, the engulfment of these nanoparticles by surrounding macrophages after cell death makes it difficult to distinguish viable from nonviable cells.

Reporter gene imaging modalities rely on genetically engineered cells to express various reporter genes before transplantation. The used reporter genes can usually respond to endog-
enous transcriptional factors and transcription-regulating complexes and can be detected via sensitive imaging devices, such as an optical charge-coupled device (CCD), SPECT, PET, or MRI. In our previous study, MSCs were transduced with a reporter gene fluc-mrfp-ttk (a gift from Sam S. Gambhir, Stanford University) for longitudinal, qualitative, and quantitative imaging of transplants in vivo in small animal models (27). The reporter gene encodes a fusion protein containing functional domains from firefly luciferase (fluc), monomeric red fluorescent protein (mrfp), and a truncated mutant herpes simplex virus 1 thymidine kinase (HSV1-tsr39tk, or ttk), whose products can thus be visualized by bioluminescence imaging (BLI), fluorescence imaging, and PET, respectively (38). BLI of the expression of fluc reporter gene in small animal is highly sensitive but cannot be easily translated into clinical use due to constraints of tissue penetration of visible light photons. However, the reporter gene ttk allows quantitative PET imaging and potential for translation into a clinical setting. Thus, a combined use of these imaging approaches may be more powerful to study the physiology and biology of the transplanted stem cells in vivo.

In recent years, microarrays have been used widely to evaluate the changes in the gene expression profiles associated with the stem cell differentiation (22, 36). Although studies have examined the effects of the transduction of reporter genes on the transcriptional profiles of mouse embryonic stem cells and found the expression of reporter genes has no significant effect on stem cell functions (44), no study has been performed to investigate the effects of transduction on MSCs. Here we report the transduction of hMSCs using lentivirus carrying the fluc-mrfp-ttk triple reporter genes. Though similar reporters have been used previously in the context of stem cell transplants (6, 34, 38, 44), the present study used a second-generation lentiviral vector delivery vehicle and wild-type hMSCs. Furthermore, the MND promoter [a modified myeloproliferative sarcoma virus (MSV) promoter] was used to drive the triple reporter expression as it shows to be more resistant to epigenetic silencing than the EF1α promoter (25). The transduced hMSCs were sorted by fluorescence-activated cell sorting (FACS). BLI and PET imaging were performed to track the fate of MSCs in living animal models. Transcriptional changes associated with the transduction process were evaluated by using a human oligonucleotide microarray. Gene expression results were correlated with in vitro MSC differentiation studies as well as the in vivo bone formation process.

MATERIALS AND METHODS

Lentiviral Vector Construction and Virus Preparation

The detailed construction of the lentiviral vector and preparation of lentivirus have been described previously (27). Briefly, the triple reporter gene fluc-mrfp-ttk, contained in the construct pCDNA3.1-CMV-triplefusion, was inserted into the second generation, self-inactivating lentiviral vector pLVmndLINK14, which drives reporter transcription with a modified MSV promoter to yield the plasmid pH R’Mnd-fluc-mrfp-ttk. Infectious virus was produced by triple transient cotransfection of 293T/17 cells (ATCC, Manassas, VA) with pHR’MND-LRT, pCMVΔR8.91 (packaging vector), and pMD.G (vesicular stomatitis virus protein G pseudotyping, pseudotyping vector) as described by Zielske and Gerson (47). Transduction was performed in serum-free OptiMEM I (Invitrogen) with Lipofectamine2000 (Invitrogen) according to manufacturer’s instructions with the mass ratio of pHR’MND-LRT to pCMVΔR8.91 to pMD.G being 3:3:1. Supernatant containing virus was collected and filter-sterilized through 0.22 μm Steriflip (Millipore) filters prior to storage at –80°C. Virus titer was determined by transduction of K562 cells (ATCC) followed by permeabilization, immunolabeling for firefly luciferase, and flow cytometry as described before (27).

Isolation and Transduction of hMSCs

Bone marrow-derived MSCs were obtained from patients undergoing hip or knee arthroplasty after informed consent under a protocol approved by the Institutional Review Board of the University Hospitals Case Medical Center (27). Primary MSCs were seeded in 175 cm² flasks to 30% confluence in hMSC growth media (DMEM, low glucose, 10% FBS selected for use with hMSCs, 2 mM GlutaMAX, 100 U/ml penicillin, and 100 mg/ml streptomycin). The hMSCs were incubated with virus at a multiplicity of infection (MOI) of 8 or 16 in hMSC growth media containing 8 μg/ml polybrene. For one of the cell preparations, the transduction protocol was repeated, and, in each round, cells were incubated with viral supernatant for 8 h and then in hMSC growth medium for 16 h. Alternatively, cells were incubated with hMSCs at an MOI of 16 for 36 h. After transduction, fresh medium was added to each flask, and cells were incubated for an additional 24 h. Cells were then passaged 1:4 and grown to 80–90% confluence. Three days posttransduction, cells were viewed on an Eclipse TE200 inverted microscope equipped with a fluorescence source and CCD camera. Transduction efficiency was determined by FACS (MoFlo, Dako) at the Cancer Center at the University of Pittsburgh using the excitation wavelength at 575 nm and an emission wavelength at 618 ± 10 nm. Sorted cells were growth for one passage to obtain sufficient number of cells for subsequent experiments.

In Vitro Differentiation Assay of Transduced hMSCs

Transduced hMSCs were assayed for transgene expression and differentiation potential into osteogenic, chondrogenic, and adipogenic lineages in vitro as described below (27).

Osteogenic potential assay. We seeded 5.0 × 10⁴ hMSCs per well in 12-well tissue culture plates and allowed them to attach overnight. The next day (day 0) culture medium was completely replaced with either complete medium (DMEM + 10% FBS) or osteogenic medium (OS medium) composed of complete medium with 0.1 μM dexamethasone (Dex), 2 mM β-glycerophosphate, and 0.05 mM ascorbate 2-phosphate. Cultures were harvested on days 10, 20, and 28. Calcium deposition into the cell layer was extracted with 0.6 N HCl and then quantified using a Calcium Assay kit from BioAssay Systems according to the manufacturer’s instructions (HAYWARD, CA). Absorbance was read at 575 nm on a Tecan microplate reader and compared with that of standard solutions prepared in parallel.

Chondrogenic potential assay. We centrifuged 2.5 × 10⁶ hMSCs in 0.5 ml of defined medium at 500 g for 5 min. The formed pellets were then incubated at 37°C in 95% humidified air and 5% CO₂. The defined medium consists of DMEM-high glucose supplemented with ITS + Premix (Collaborative Bio-medical Products, Bedford, MA) with pyruvate (1 mM, GIBCO-BRL), ascorbate 2-phosphate (100 μM; Wako, Osaka, Japan), Dex (10⁻⁷ M), and 10 ng/ml recombinant human transforming growth factor (TGF)-β1 (Peprotech, Rocky Hill,
For histology, aggregates were fixed for 15 min in 10% neutral-buffered formalin, dehydrated in a series of graded alcohols, paraffin embedded, and sectioned at a thickness of 5 μm. The slides were finally stained with toluidine blue (0.2%).

Adipogenic potential assay. We plated 1.0 × 10⁴ hMSCs in adipogenic medium (growth medium plus 5 mg/ml insulin, 10⁻⁶ M Dex, 0.5 mM isobutylmethylxanthine, and 60 mM indomethacin). At 8 and 14 days in culture, the detached cells were fixed by the addition of 4% paraformaldehyde to a final concentration of 0.5% and stained for triglycerides in a 1 mg/ml solution of Nile red in PBS. The number of adipogenic cells was quantified at the Flow Cytometry Core Facility at Case Western Reserve University (CWRU) National Cancer Institute Core Facility. Cells cultured in growth medium served as the baseline for measuring Nile red staining.

Ceramic Cube Implantation in Mice, BLI, and PET Imaging

The studies were approved and conducted in accordance with the institutional guidelines for care of laboratory animals at the CWRU. Fibronectin-coated ceramic cubes loaded with hMSCs expressing the triple reporter were implanted subcutaneously into NOD-SCID mice (~8 wk old) under anesthesia. Cubes loaded either with cells that were transduced with the virus backbone without reporter genes or with cells that were untreated or with either with cells that were transduced with the virus backbone expressing the triple reporter were implanted subcutaneously into CWRU. Fibronectin-coated ceramic cubes loaded with hMSCs without reporter genes or with cells that were untreated or with cells that were transduced with the virus backbone expressing the triple reporter were implanted subcutaneously into Case Western Reserve University (CWRU) National Cancer Institute Core Facility. Cells cultured in growth medium served as the baseline for measuring Nile red staining.

Histological Analysis for In Vivo Osteogenesis

Selected mice were euthanized at 2, 4, 6, 8, 10 wk after BLI or PET imaging by 0.5 ml Fatal-Plus anesthesia. Ceramic cubes were removed from the animals and fixed in 10% formalin in phosphate-buffered saline for at least 24 h, washed in water, and decalcified in RDO (Apex Engineering, Plainfield, IL) for standard histology. Serial sections, cut at 5 μm, were stained with Mallory-Heidenhain and examined for bone formation.

RNA Isolation, Preparation of cRNA, and Microarray Hybridization

Cells were detached from the cell culture plate with trypsin-EDTA (0.25%) from Invitrogen, and immediately the same volume of DMEM was added to inactivate the trypsin. Cells were centrifuged for 5 min at 300 g. RNA was extracted from the cell pellet with RNeasy Midi Kit from Qiagen (Valencia, CA) according to the recommended protocol. Total RNA was given to the Gene Expression Array Core Facility at CWRU. cRNA was prepared and hybridized to Affymetrix Human U133 plus 2.0 GeneChips (Santa Clara, CA) according to the manufacturer’s instructions. Briefly, 3 μg of RNA was used in a reverse transcription reaction (SuperScript II; Life Technologies, Rockville, MD) to generate first-strand cDNA. After second-strand synthesis, double-stranded cDNA was used in an in vitro transcription reaction to generate biotinylated cRNA. After purification and fragmentation, 15 μg of biotin-labeled cRNA was used in a 300 μl hybridization cocktail containing spiked control. We loaded 200 μl of cocktail onto Affymetrix microarrays (Santa Clara, CA) and hybridized it for 16 h at 45°C with agitation. Standard posthybridization washes and double-stain protocols used an Affymetrix GeneChip Fluidics Station 450. Arrays were scanned with the high-resolution Gene Array scanner 3000. All microarray data were submitted to the National Center for Biotechnology Information Gene Expression Omnibus (GEO series GSE9941; http://www.ncbi.nlm.nih.gov/geo/).

Microarray Data Analysis

The quality of the microarray hybridization results for each sample was evaluated first before any analysis was made. Housekeeping control and spiked control genes were qualitatively examined to ensure that microarray hybridization experiments were valid according to the Data Analysis Fundamentals Manual from Affymetrix, GeneSpring software (Agilent Technologies, Santa Clara, CA) was also used to display the plot results for the hybridized control as well as internal controls. Correlation coefficients of the microarray results were also calculated by GeneSpring for quality control. Microarray data were analyzed as described previously (43). In brief, the hybridization results were first scaled to a target value of 15 using GCOS v1.2 from Affymetrix, and the differential gene expression of the transduced cells vs. the control cells was analyzed for each sample pair. The “presence” status of each gene in each sample, and the differential change status of each gene in each sample pair were computed statistically by GCOS. A gene was considered upregulated in the transduced cells if it was labeled as “present” or “marginally” in the transduced cells and if the fold change was “increased” or “marginally increased” according to GCOS. In contrast, a gene was considered downregulated if it was labeled as “present” or “marginal” in the transduced cells and if the fold change was “decreased” or “marginally decreased” in the transduced cells. The analysis was performed with Microsoft Excel and Access. The genes that were differentially expressed in all the sample pairs were considered and were further analyzed by SAM (developed by Stanford University). A fold change of 2.0 and a delta value ≥1.0 were used as the criteria for SAM analysis. The filtered genes were then further analyzed by SAM. The subset genes obtained from SAM were used either for hierarchical cluster analysis with Cluster 3.0 and Java Treeview or for function and biological process analysis with Ingenuity Pathway Analysis (IPA, Redwood City, CA).

Gominer (http://discover.nci.nih.gov/gominer/) was used for annotation analysis, and results were tabulated in Table 1. To identify putative interrelationships between subsets of dif-
ferentially regulated transcripts, we generated biological associated networks using Pathway Studio 4 (Ariadne Genomics) and IPA. These software packages utilize information derived from literatures to identify regulatory relationships between genes and various biological processes and molecular functions.

Quantitative RT-PCR

Four optimal Taqman primers and probe sequences for our genes of interest were designed and produced by Applied Biosystems (Foster City, CA). The primers, probes, and the total RNA for microarray studies were given to the Gene Expression Array Core Facility. A 384-well plate set-up was used on a PRISM 7900HT Sequence Detection System from Applied Biosystems to perform RT-PCR.

Data Analysis for Imaging and Functional Cell Culture Studies

Image data as well as function analysis data are given as means ± SD. For statistic analysis, the two-tailed Student’s t-test was used. Differences between control and positive results were considered significant at \( P < 0.05 \).

RESULTS

MSC Transduction

The mean titer of lentivirus produced by transient cotransfection was \( 8 \times 10^8 \) infectious particles/ml. Transduction of hMSCs at an MOI of 16 yielded a transduction efficiency of 87.4% as measured by FACS (Fig. 1B). This batch of cells was used for subsequent studies. Using stem cells from a different source and a MOI of 8 achieved a transduction efficiency of 83.2% when cells were transduced two rounds with 8 h in each round (27). Moreover, the same cells with a transduction MOI of 16 and an incubation time of 36 h led to 99.4% transduction efficiency (data not shown). Also, the signal intensity from the mrfp shifted to a significantly higher value.

In Vitro Characterization of Pluripotential Capacity

In vitro functional assays have been performed previously and shown that transduced hMSCs displayed in vitro luciferase activity 4,000-fold greater and \( tk \) activity 9-fold greater than untransduced hMSCs. These hMSCs were visible by fluorescence microscopy 3 days posttransduction (27).

Stem cells were induced to differentiate into adipocytes and assessed for the proportion of cells positively stained with Nile red, a measure of cellular lipid content. We found that 21.6 ± 1.5% of hMSCs transduced with the triple reporter genes had an adipocytic phenotype vs. 13.6 ± 1.2% for cells transduced with empty vector and 15.3 ± 1.9% for untransduced hMSCs after 2 wk (Fig. 2A). In contrast, 1 wk incubation with the induction medium led to a lower differentiation rate of MSCs into adipocytes (4.75 ± 1.51, 8.0 ± 0.93, 7.0 ± 0.49 for hMSCs transduced with the triple reporter genes, hMSCs transduced with the empty vector, and hMSC controls). However, the MSCs in the control medium showed a relatively low rate of positive phenotype (≤3.0%).

Transduced or untransduced hMSCs were incubated in OS medium or control medium for osteogenic assay. Calcium was extracted and measured as a marker for osteoblastic differentiation (Fig. 2B). Within 20 days, the transduced and sorted MSCs had a production of 53.8 ± 7.9 \( \mu \)g of calcium, while a further incubation with the OS medium up to 4 wk produced only a mild increase of calcium amount to 57.2 ± 1.8 \( \mu \)g. The untransduced cells or cells that were transduced

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Table 1. GO annotation analysis

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GO, gene ontology.
with the empty vectors only have a slight increase of calcium production with time; 8.7 ± 1.8 μg and 11.1 ± 3.3 μg calcium were produced at week 4, which is significantly lower than that from cells with reporter gene transduction (P < 0.05). Also the calcium amount in the control medium had no significant difference compared with that in the OS medium until after week 4.

For chondrogenic assay, chondrogenic aggregates were fixed and stained with toluidine blue for visual assessment of chondrogenic potential. As shown in Fig. 2C, transduced cells have slightly higher staining density than the control cell, and the size of its aggregates was slightly smaller.

Images of Cube Implants

The ceramic cubes loaded with transduced cells were visible with BLI 30 min after implantation. The BLI signal from some of the cubes was visible beyond 2 mo after implantation (Fig. 3). Compared with the wild-type hMSC or empty cubes in the same animal, only the cubes loaded with reporter transduced hMSC produced strong signal. The microPET imaging performed on the mice showed signals from the implanted ceramic cubes with reporter transduced hMSCs 9 wk after implantation.

Histology

The osteogenic potential of transduced and untransduced hMSCs was further assessed by histological examination as shown in Fig. 4. Six weeks after implantation, empty ceramics were uniformly negative for bone formation as shown previously(27), while hMSCs transduced with the reporter genes showed typical regions of bone formation on the surfaces of the ceramic implants (Fig. 4A). The hMSC population transduced with the empty vector and untransduced cells also produced bone (Fig. 4, B and C).

Microarray Study

A human gene chip was used to assess the transcriptional changes in the transduced cells vs. the untransduced control cells. Overall correlation of gene expression between different arrays was high, with a coefficient >0.9 for all the arrays as shown in Fig. 5. The signal intensity of internal controls shows similar patterns, and their normalized values are very close. The spiked control genes for all the arrays have a low variability in the signal intensity. All these data indicate the high reliability and reproducibility of the microarray data. To accurately and reliably determine gene expression profiles, GCOS was first used to statistically evaluate gene expression changes in each sample pair by combining 1) the “presence” or “absence” status of each gene and 2) the statistical “decrease” or “increase” trend for differential change. Only those genes that were differentially changed in all the pairs were further exported to SAM for further analysis. Using very stringent criteria of expression fold change ≥2.0 and a delta value of 1.0, SAM analysis generated a 0.02% false discovery rate (percentage of genes that may be falsely identified to be positive), which is considerably low. Finally, 91 upregulated and 81 downregulated probes were identified, corresponding to 87 and 69 upregulated and downregulated genes, respectively. A further hierarchical cluster was performed using the obtained genes. The transduced samples can be clearly distinguished from the untransduced control samples. Furthermore, for most of the genes identified, the differential change for the low signal population and high signal population had similar trend (Fig. 5).

In this study, four genes were used for RT-PCR analysis to validate the microarray results. SOX4 [SRY (sex determining region Y)-box 4] and CDKN1C [cyclin-dependent kinase inhibitor 1C (p57, Kip2)] had a downregulation of 6.6- and 6.5-fold, respectively, while IL-8 (interleukin 8), and PMAIP1
(phorbol-12-myristate-13-acetate-induced protein 1) were upregulated 4.1- and 3.7-fold, respectively. This correlates well with their respective upregulation and downregulation in the microarray (2.5-fold for SOX4, 3.1 for CDKN1C, 3.0 for PMAIP1). More importantly, these genes have the same expression patterns in both the low signal and high signal cell populations.

As shown in Table 1, the differentially expressed genes belong to several different classes, including the genes associated with apoptosis, development, cell proliferation, cell cycle, immune response, transport, translation, RNA processing, or metabolism. Immune response-related genes include IL-8 (4.03-fold), PSME2 (3.19-fold), CRLF1 (cytokine receptor-like factor 1, 3.9-fold), etc. Upregulation of some ubiquitin cycle genes such as ISG15 (2.96-fold) and UBE2D3 (2.06-fold) was also detected. Clusterin, a gene associated with immune response, apoptosis, and lipid metabolism (22), was downregulated in the transduced cells. Many genes that are associated with cytoskeleton organization and biogenesis, cell adhesion were also altered in the transduced cells, including KRTAP1–3 (keratin-associated protein 1–3, 5.4-fold) and NEFM (neurofilament, medium polypeptide 150 kDa, 3.0-fold), and the downregulation of many collagen type genes such as COL14A1, COL15A1, COL8A2, and COL11A1. FGF5 (fibroblast growth factor 5, 2.8-fold), whose family members possess broad mitogenic and cell survival activities, are involved in embryonic development, cell growth, morphogenesis, tissue repair, and tumor growth and invasion. FGF7 (positive regulation of cell growth), a potent epithelial cell-specific growth factor, has found roles in morphogenesis of epithelium, reepithelialization of wounds, hair development, and early lung organogenesis. It is interesting to note that COX6A1 (cytochrome c oxidase subunit VIa polypeptide 1) has the highest upregulation, up to several thousands, and may function for electron transport and generation of precursor metabolites and energy. Cell adhesion downregulation was mostly involved with protocadherin genes such as PCDHB10 (−3-fold), PCDHB5 (−3.1-fold), and PCDHB6 (−2.1-fold), which may play roles in specific cell-cell signaling and communications.

IPA software was utilized for pathway analysis of the differentially expressed genes. The differentially expressed transcripts were found to be involved in the eicosanoid signaling pathway, interferon signaling pathway, axonal guidance signaling pathway, and wnt/β-catenin signaling pathway (Table 2). Some genes were observed to participate in the pathways that may damage the cells and cause cytotoxicity. For example, CDKN2A, PMAIP1, UNC5B, and E2F7 were...
involved with the p53 signaling pathway, while insulin growth factor 1 (IGF1), FGF7, and IL8 may be involved with the hepatic fibrosis. Figure 6 shows some of the genes that are involved in cell proliferation (Fig. 6A) and cell differentiation (Fig. 6B). It is obvious that some genes can have either positive or negative effects on cell proliferation and differentiation. The interactions of some of the genes that were differentially expressed are shown in Fig. 6C. It seems that several genes such as IL8, EGR1, and IGF1 interact with many other genes and have multiple roles in affecting cell proliferation, growth, death, or differentiation. Interestingly, cell differentiation genes could appear either upregulated, including LIF, COL13A1, and EGR1, or downregulated, such as LEPR (a gene related to adipocytes), MGP (matrix Gla protein), PTGER4 (regulation of ossification), and COMP (a cartilage-related gene), which are also related to stem cell bone formation and cartilage matrix regulation.

DISCUSSION

Transduction of Triple Reporter Into hMSCs

The hMSCs used in this study was transduced with the lentivirus carrying the triple reporter genes at an efficiency of 87.4%. Our study showed that the increased incubation time and MOI can cause increased transduction efficiency (41, 42). As shown previously, a transduction efficiency of 83.2% was achieved when MSCs of a different source were transduced at a MOI of 8 for 16 h. In contrast, an MOI of 16 achieved nearly 99.4% of transduction efficiency with an incubation time of 36 h. In this study, two populations of cells have been sorted by FACS and further analyzed accordingly. The high signal population of sorted hMSCs may be caused by the enhanced virus transduction and high gene copy numbers that are integrated into the genome of the hMSCs (13). These results indicate that there may exist an appropriate incubation time, after which transduction efficiency is not significantly affected but more copies of genes are integrated into each cell.

Our previous enzymatic assay and fluorescent examination results have shown all three components of the triple fusion reporter gene were working properly (27). Other assay results showed that reporter-transduced hMSCs have a normal growth pattern compared with untransduced hMSCs. More importantly, there appeared to be no severe adverse changes in cell phenotype and pluripotentiality as demonstrated by adipogenic, osteogenic, and chondrogenic assays performed on both transduced and untransduced cells. From this batch of hMSCs used in experiments, the adipogenic and osteogenic assays showed a certain extent of enhancement in transduced cells compared with either the untransduced MSCs or MSCs transduced with empty vectors. This may be associated with, in addition to the potential difference(s) in donor MSCs, the differential changes of some genes that are related to matrix organization, skeleton formation or lipid metabolism, and bone formation, which can be found in the microarray results.

Long-term Monitoring of hMSCs

BLI and PET are sensitive methods to monitor stem cell fate in vivo. BLI can enable real-time noninvasive visualization of gene expression in living animals with respect to localization, level, and duration of the transplanted genes in the same animal along the time scale. PET was able to detect both cubes and correlated well with the BLI signal from the same mice. BLI data indicate that cells were well engrafted into the ceramic cubes, and signal intensity was increased. After 4 wk, the signal began to decline. The signal increase may be due to the cell proliferation as the proliferation process is always initiated before cells differentiate (33). The mechanisms for the cell signal decline are not fully understood. This may be caused by
the death of some donor cells (25) due to cell differentiation itself or due to the decreased oxygen transport caused by the secreted calcium matrix. Further experiments will be needed to elucidate the mechanism of this phenomenon. Our histological analysis correlates well with the bone formation process. At week 6, bone was found in all the ceramics except those without cells (Fig. 4). The enhancement in in vitro osteogenic assays (Fig. 2B), however, indicated that reporter-transduced MSCs not only augmented the potential for osteogenic differentiation, but also shortened the timing for such differentiation for this batch of cells. Since the cells used in this study were sorted, these results demonstrate that the basic properties of the hMSCs were not affected by the transduction or expression of the reporter genes.

Microarray Analysis and Correlations

A very important question associated with the reporter gene transduction of stem cells is whether the transduction will lead to any abnormal changes of MSCs themselves. To answer this question, a human Affymetrix microarray chip was used to detect the transcriptional changes of hMSCs after reporter gene transduction. Many classes of genes were changed, including a few number of pregnancy genes, which are associated with embryo or fetus development. The differential change of immune response-related genes may be caused by the viral infection process, while the upregulation of some ubiquitin cycle genes may be associated with the increased protein degradation. The differential change of many genes that are associated with matrix organization and skeleton formation, which may explain the difference in osteogenic assay between transduced and wild-type MSCs or empty-vector transduced MSCs.

As shown in Fig. 6, some genes that are associated with cell proliferation are upregulated, and some are downregulated. Some genes such as CDC42 (2.2-fold) have roles in either cell growth, proliferation (positive), cell death, or differentiation (negative). Similarly, IL-8 has positive roles in cell proliferation, differentiation, and cell death. In contrast, some genes have either positive or negative impact on the same biological process. For example, EGR1, CDKN2A, and CDKN2B have positive and negative roles on cell death, along with positive roles in cell differentiation, as shown in Fig. 6. PMAIP1, involved with cellular apoptotic responses to interferon, double-stranded RNA, and virus infection (40), and antiapoptosis genes SERPINB2 and TNFAIP3 were all upregulated in this study. Our in vitro cell culture with respect to the cells with low signal intensity shows rapid cell growth beyond a seeding density, possibly indicating the insignificant impact on the cell growth caused by the transduction. The combined effects of these genes may have offset the overall effects as demonstrated in other studies (44). However, hMSCs with high mrfp signal intensity seem to have delayed cell growth rate, which needs to be confirmed with further experiments. A proper gene transduction is necessary to maximally maintain cell properties.

A number of genes that are associated with cell differentiation were found to be differentially expressed in the transduced cells, as shown in Fig. 6. Among them, a few genes are associated with lipid metabolisms or regulation of ossification and cartilage formation. For example, PTGS2 (5.4-fold), PTGIS (3.1-fold), and PTGDS (2.2-fold) regulates lipid synthesis. PTGIS, prostaglandin I2 (prostacyclin) synthase, encodes a member of the cytochrome P450 superfamily of enzymes that are monooxygenases and catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids, and other lipids. On the other hand, MGP, which regulates the ossification process, is an important inhibitor of calcification in vivo, whose downregulation may correlate with the increased capability of osteogenic differentiation (35, 46). PTGER4, another gene that mediates PGE2 induced expression of EGR1, has shown roles in osteoporosis in knockout mice (39); its down-regulation may lead to changes in osteogenesis. It is found that cartilage oligomer protein was also downregulated after trans-
duction. An identification of genes responsible for osteoblast
differentiation from human mesodermal progenitor cells shows
this gene is constantly upregulated in TGF-β/H9252-treated cell
cultures (36). SOX4, a gene that was downregulated, as
demonstrated by microarray and RT-PCR, may mediate
downstream effects of parathyroid hormone (PTH) and
PTH-related protein in bone development. COL13A1 is also
involved with the endochondral ossification as revealed by
the annotation analysis; its upregulation may contribute to
the increased osteogenesis. IGF1 (−4.0-fold) and IGF2
(−3.85-fold) may be associated with the bone differentia-
tion process (22). It is also reported that the Wnt signaling
pathway may be involved in the bone formation and main-
tenance process; in this study, several genes related to the
Wnt signaling pathway were found to be differentially
expressed, including PRICKLE1, SFRP4, TCF7, and NLK.
Overall, the upregulation and downregulation of some of
these positive and negative genes may, in combination,
contribute to a certain degree of changes in osteogenesis and
adipogenesis after viral transduction of the reporter genes,
as observed in the in vitro assays. Similarly, a previous work
of ours also showed a slightly enhanced adipogenic and
osteogenic differentiation potential, although the difference
was not as large (27). There is a possibility that the trans-

<table>
<thead>
<tr>
<th>Pathway</th>
<th>P Value</th>
<th>Upregulated Genes</th>
<th>Downregulated Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eicosanoid signaling</td>
<td>0.0012</td>
<td>PTGS2</td>
<td>PTGER4, PTGDS, PTGIS</td>
</tr>
<tr>
<td>Interferon signaling</td>
<td>0.0200</td>
<td>OAS1, IFIT1</td>
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<td>Axonal guidance signaling</td>
<td>0.0257</td>
<td>H Hipp, CDC42, GNAS</td>
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<tr>
<td>Wnt/β-catenin signaling</td>
<td>0.0316</td>
<td>NLK</td>
<td>IGF1, NRP2, UNC5B NTRK2</td>
</tr>
<tr>
<td>p53 signaling</td>
<td>0.0026</td>
<td>PMAIP1, E2F7</td>
<td>CDKN2A, SFRP4, SOX4</td>
</tr>
<tr>
<td>Hepatic fibrosis</td>
<td>0.0257</td>
<td>IL8</td>
<td>FGF7, IGF1</td>
</tr>
<tr>
<td>Hepatic stellate cell activation</td>
<td>0.0282</td>
<td>IL8</td>
<td>IGF1</td>
</tr>
</tbody>
</table>

P < 0.05. Shown are genes that are involved in the pathways and gene expression changes.
duction conditions as well as cell source variation can affect the degree of this enhancement.

Although several genes are related to mesenchymal tissues or cell differentiation, fewer of them are marker genes for cell differentiation or play a crucial role in regulating cell differentiation. MSCs’ chondrogenic differentiation was associated with the early expression of fibromodulin and cartilage oligomeric matrix protein deposition, followed by an increased aggregan and versican core protein synthesis at the intermediate stage, which also involved the small leucine-rich proteoglycans decorin and biglycan. The appearance of type II collagen and chondroadherin (2) indicates the maturation of the chondrocytes. However, none of these key genes was differentially expressed in the transduced cells. For adipogenic differentiation, a cascade of steps involved with several key regulatory genes is initiated after a cycle of postconfluent proliferation, including the CCAAT/enhancer binding proteins, as well as the peroxisome proliferator-activated receptor-γ, which is early expressed in the early adipogenesis and responsible for initiation and maintenance of the adipocytes phenotype in vivo (16, 17). These genes are found necessary and/or sufficient for the transition frompreadipocytes to adipocytes. Interestingly, some antiproliferative or responsive genes for growth arrest such as GAS1, BTG1, WEE1, PPP2R5A, and PTPRM (16) were not reported in this study, correlating with our cell growth rate. Leptin, a late-stage mature adipocyte-specific marker gene or hormone that regulates adipose tissue mass through hypothalamic effects on satiety and energy expenditure and acts through the leptin receptor (LEPR), a single-transmembrane-domain receptor of the cytokine receptor family, plays a role in skeletal growth and development (11) and affects cartilage generation directly. However, these genes were not found differentially expressed in the transduced cells. CBFA1/OSF2 has been reported to be necessary for
osteogenesis. CBFA1 activates osteocalcin, an osteoblast-specific gene expressed in fully differentiated osteoblasts. CBFA1-deficient mice lack bone formation because of the maturation arrest of osteoblast (36). Not surprisingly, these gene and osteogenic differentiation markers such as osteocalcin and osteonectin (21) were not differentially expressed in the transduced MSCs. This confirmed that reporter gene transduction did not cause the loss of the cell differentiation properties. Transduced hMSCs remain to be stem cells and maintain the basic properties of stem cells.

We also conducted microarray studies using the hMSCs that were transduced with the backbones of the virus without any reporter genes. SAM identified 35 differentially expressed genes, such as ARIH1 [Ariadne homolog, ubiquitin-conjugating enzyme E2 binding protein, 1 (Drosophila)], CCT5 [chaperon containing TCP1, subunit 5 (epsilon)], CSNK1A1 (casein kinase 1, alpha 1), EGFR [epidermal growth factor receptor [erythroleukemia viral (v-erb-b) oncogene homolog, avian]], IRAK1BP1 (interleukin-1 receptor-associated kinase 1 binding protein 1), PTGER3 [prostaglandin E receptor 3 (subtype EP3)], and PTPN22 [protein tyrosine phosphatase, nonreceptor type 22 (lymphoid)]. Interestingly, few genes overlap with those that were transduced with the reporter genes. The viability, proliferation, and differentiation studies have shown that the hMSCs transduced with empty vector are quite similar to the control cells. This may indicate that the incorporation or the expression of the triple reporter genes in the hMSCs may have effects on the overall gene expression profiles from the genetic point of view. In the future, interesting genes can be selected for more defined and clear functional studies. Furthermore, hMSCs from more individuals can be used for more universal or general conclusions (36).

**Conclusion**

Microarray studies of hMSCs after transduction with the triple reporter genes using lentivirus were performed to investigate the effects of transduction on stem cell properties. A number of genes were found to be upregulated or downregulated when comparing reporter transduced MSCs with wild type. The expression of an essential set of genes crucial to the basic characteristics of MSCs was not significantly changed, and the properties of stemness was observed. The in vitro functional studies did show certain degree of alterations in differentiation, but the differentiation potentials were preserved. The specific triple reporter gene approach that we adopted resulted in a viable method for labeling and imaging stem cells. Noninvasive imaging in real time and in repeated fashion as proposed here is an effective method for tracking stem cell transplantation. Imaging small animal models by using micro-PET imaging can be easily translated into human studies by using clinical PET imaging in the future.

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