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Investigation of gene expressions in differentiated cell derived bone marrow stem cells during bone morphogenetic protein-4 treatments with Fourier transform infrared spectroscopy

Jaber Zafari [a], Fatemeh Javani Jouni [b], Ali Ahmadvand [c], Parviz Abdolmaleki [b], Malihe Soodi [d], Rezvan Zendehdel [e,f,*]

[a] *Department of Toxicology and Pharmacology, Faculty of Pharmacy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran*
[b] *Department of Microbiology, Islamic Azad University, Tehran North Branch, Tehran, Iran*
[c] *Computer Science and Informatics Department, EMORY University School of Medicine, Atlanta, GA, USA*
[d] *Department of Toxicology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran*
[e] *Environmental and Occupational Hazards control Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
[f] *Department of Occupational Hygiene, School of Public Health, Shahid Beheshti University, Tehran, Iran.*

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ABSTRACT

A model was set up to predict the differentiation patterns based on the data extracted from FTIR spectroscopy. For this reason, bone marrow stem cells (BMSCs) were differentiated to primordial germ cells (PGCs). Changes in cellular macromolecules in the time of 0, 24, 48, 72, and 96 h of differentiation, as different steps of the differentiation procedure were investigated by using FTIR spectroscopy. Also, the expression of pluripotency (Oct-4, Nanog and c-Myc) and specific genes (Mvh, Stella and Fragilis) were investigated by real-time PCR. However, the expression of genes in five steps of differentiation was predicted by FTIR spectroscopy. FTIR spectra showed changes in the template of band intensities at different differentiation steps. There are increasing changes in the stepwise differentiation procedure for the ratio area of CH2, which is symmetric to CH2 asymmetric stretching. An ensemble of expert methods, including regression tree (RT), boosting algorithm (BA), and generalized regression neural network (GRNN), was the best method to predict the gene expression by FTIR spectroscopy. In conclusion, the model was able to distinguish the pattern of different steps from cell differentiation by using some useful features extracted from FTIR spectra.

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1. Introduction

Stem cells possess the exclusive ability to proliferate by self-renewal or to differentiate into tissues under the effect of suitable molecular prompts. There are various classes of stem cells, all varying in degree of pluripotency. Related to the developmental stage, stem cells are categorized into five main subcategories including those with totipotency, pluripotency, multipotency, unipotency, and oligopotency. Cells with totipotency can be transformed into all cell types, while those with pluripotency can be transformed into most cell types. Cells with multipotency can also be transformed into many cell types, while those with oligopotency can be transformed into a few cell types and those with unipotency into one cell type [1,2]. Mesenchymal stem cells (MSCs) are derived from several organs including the lungs, heart, adipose tissue, bone marrow, and liver [3–8]. MSCs are capable of generating multiple mesenchymal cell lineages under specific culture conditions. Bone marrow mesenchymal stem cells (BMSCs) have potential multilineage and are used in several cell-based treatments.

Male germ cells are derived from a population of primordial germ cells (PGCs) set aside early during embryogenesis [9,10]. Cell proliferation, survival, and differentiation of PGCs are dependent on bone morphogenetic proteins (BMPs) [11]. Bone morphogenetic protein 4 (BMP4) causes the expression of PGCs' specific genes such as Dpp3a (Stella), Fragilis, and mouse vase homologue (Mvh). Western blots, quantitative polymerase chain reaction (qPCR), and immunocytochemistry (ICC) are nowadays used for differentiation assessments. Although such approaches are highly sensitive to specific cell characteristics, they have a destructive nature. This problem does not allow real-time assessments of cell functions [12].

Bio-spectroscopy approaches were recently applied in studying of cells. This method has revealed characteristics of biological systems. Fourier transform infrared (FTIR) spectroscopy is one of the most powerful tools for detecting changes in cellular macromolecules: It provides information about the structures of carbohydrates, lipids, proteins, and nucleic acids, thereby allowing detection, identification, and

* Corresponding author.
E-mail addresses: jaberzafari@yahoo.com (J. Zafari), javani_f@yahoo.com (F.J. Jouni), ali.ahmadvand.66@gmail.com (A. Ahmadvand), parviz@modares.ac.ir (P. Abdolmaleki), soodi@modares.ac.ir (M. Soodi), zendehdel76@yahoo.com (R. Zendehdel).

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quantification of changes in these macromolecules [13–16]. This approach has been widely used to monitor biological processes involved in cancer investigation [17–19], apoptosis detection [20,21], and stem cell research [22–24].

Biostatistics is a mathematical method for describing biological experimental data. This method is based on the probability theory and statistics that can make appropriate models to predict data. The number of biostatistics papers is increasing very fast, while the estimation of an experiment's results requires more sensitive methods [25,26].

The aim of this study is to use a mathematical model to predict real-time PCR results based on the cellular FTIR spectroscopy of the differentiation of BMSCs into PGCs. This differentiation was investigated stepwise five times, while FTIR spectroscopy was employed to detect the changes in cellular macromolecules. Also, the expression of pluripotency (Oct-4, Nanog, and c-Myc) and specific genes (Mvh, Stella, and Fragilis) was investigated by using real-time PCR during differentiation. Four different static models were used to specify gene expressions by using an FTIR spectrum of cells. Models of different well-known regressions, including partial-least squares regression (PLSR), regression tree (RT), boosting algorithm (BA), and generalized regression neural network (GRNN), were initially used to predict the gene expression in selected samples based on the data extracted from FTIR profiles by using a sufficient sampling rate. We also used an ensemble of expert methods, including RT, BA, and GRNN, where the advantages of each model are combined together to get a better performance. The result of this project may provide new visions for the usage of FTIR in gene expression investigations.

2. Materials and Methods

2.1. Animal Use

Male adult Vistar rats (aged = 4–6 weeks old), derived from the original stocks obtained from Razi Laboratory (Tehran, Iran), were maintained under the standard conditions with free access to food and water at the animal facility of Tarbiat Modares University. To satisfy the moral concerns, all experiments were conducted in accordance with the guidelines set by the National Research Council (affiliated to Tarbiat Modares University).

2.2. Isolation, Culture, and Treatment of MSCs

The primary source for bone marrow stem cells (BMSCs) is bone marrow, although recent reports indicate BMSCs can be isolated from other sources such as peripheral blood [27], fat [28], skin [29], vasculature [30], and muscle [31]. BMSCs are isolated, as described by Woodbury [32]. In our study, eight to 10 week-old rats were killed, and their tibias and femurs were dissected and placed in phosphate buffered saline (PBS) on ice. Marrow was flushed out under sterile conditions by cutting two ends and using a syringe filled by 10 ml of αMEM (Invitrogen LT, Merelbeke, Belgium). Cells were seeded in αMEM supplemented with 20% of fetal bovine serum (FBS, Invitrogen LT, Merelbeke, Belgium), 100 U/ml of penicillin (Gibco, Germany), and 100 U/ml of streptomycin (Gibco, Germany) in a 75 cm^2 flask and incubated at 37 °C with 5% humidity. After 24 h, non-adherent cells were discarded and adherent cells were thoroughly washed twice with phosphate buffered saline (PBS). BMSCs were allowed to grow until 70%–80% confluence of the surface area of the flask. Confluent BMSCs were passaged and plated out at 1:2 or 1:3 dilutions every five to six days by using 0.25% trypsin and 1 mM of EDTA (Invitrogen LT, Merelbeke, Belgium), and the media was changed every other day. MSCs were purified from other cells based on their adherent properties. Spindle-shaped cells were used from 5th passages for treatment (Fig. 1). These cells were frozen in αMEM with 93% FBS and 7% DMSO (MERCK, Darmstadt, Germany) in liquid nitrogen [33]. After four or five passages, when the density of the cultured BMSCs was approximately 5×10^4 cells cm^{-2}, the cells were ready to be treated with BMP4 [32]. BMP4 (25 ng ml^{-1}; Chemicon, USA) was added to the 4th passage BMSCs at different times (0, 24, 48, 72, and 96 h), according to the method described by Mazaheri et al. [34]. To study the change of macromolecules in the differentiation procedure, each time was considered as the first step (0, 24, 48, 72, and 96 h).

2.3. Quantitative Analysis of Gene Expression

According to the manufacturer's recommendations, the total amount of RNA was extracted from the BMSCs treated with BMP4 in each step (0, 24, 48, 72, and 96 h) by using RNeasy Mini Kit (Qiagen, USA). Concentrations of RNA were determined by UV spectrophotometry (Eppendorff, Germany). The cDNAs were synthesized from 500 ng of DNAase-treated RNA samples with a Quantitect Reverse Transcription Kit by using oligo (dT) primers. The specific primers used for PCR reactions are listed in Table 1, and they were synthesized by Pishgam (Tehran, Iran). PCRs were performed by using Master Mix and Eva Green in an Applied Biosystems Step One™ thermal cycler (Applied Biosystems, USA). The PCR program started with an initial melting cycle to activate the polymerase for 5 min at 95 °C, followed by 40 cycles of melting (30 s at 95 °C), annealing (30 s at 58 °C), and extension (30 s at 72 °C). The quality of PCR reactions was confirmed by melting curve analysis. Efficiency was determined by using a standard curve for each gene (logarithmic dilution series of cDNA from the tests). For each sample, the reference gene (Gapdh) and target gene were amplified in the same run. The reference genes were approximately equal. The target genes were normalized to a reference gene and expressed relative to a reference gene as calibrator.

2.4. FTIR Analysis

Cells were suspended with the trypsin (0.5 g L^{-1})/EDTA (0.2 g L^{-1}) buffer and then washed three times in the normal saline solution (NaCl,

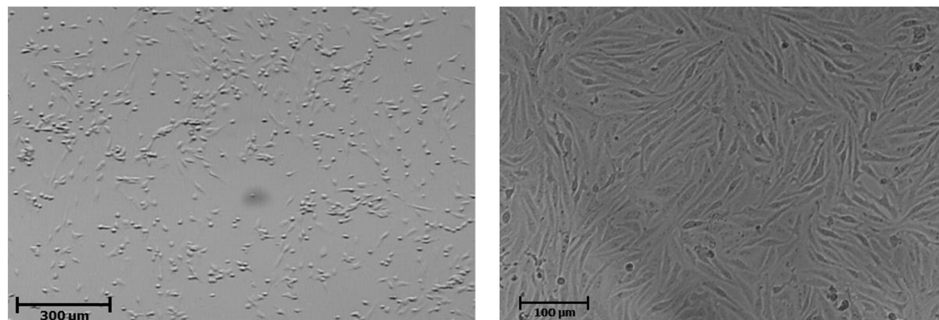

Fig. 1. Primary cell culture of bone marrow mesenchymal stem cells: (A), the population of heterogeneity of cells after 4 h culture; (B), the population of homogeneity of cells after five-passage culture.

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