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Original

Characterization and Gene Expression Profiling of Human Mesenchymal Stem Cells

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Abstract **:** Human bone marrow mesenchymal stem cells (hMSCs), one of the tissue stem cells, have the ability to differentiate into adipocytes, osteoblasts or chondrocytes, leading to the attempt to apply these stem cells to regeneration of bone and cartilage. To analyze characteristics of these cells and explain pluripotency of hMSCs, we analyzed gene expression profiles of hMSC clones by using DNA chips that contain over 10,000 genes per chip. We also analyzed their proliferation rates and cell surface markers. As a result, these hMSC clones showed various proliferation rates indicating that hMSCs contains multiple types of cells in their population. Furthermore, analysis of surface markers, especially analysis of CD34 and CD44 showed that these stem cells are not hematopoietic stem cells but mesenchymal origin. Profiling of gene expression by using DNA chip showed that several genes were specifically expressed in hMSCs, and the highest expression was observed in clone no.12 showing that this clone possesses unique characters as a MSC applicable to regeneration research and regenerative medicine.

Keywords: Mesenchymal stem cell, DNA chip, Gene Expression

Introduction

Stem cells are useful for cell and tissue regeneration. Especially, tissue stem cells are widely recognized as highly useful stem cells because there is no bioethical problem and histocompatible difficulty when we use our own tissue stem cells for regeneration or plantation of own tissues. Among them, mesenchymal stem cells (MSCs) isolated from bone marrow have multipotency, that means ability for multilineage differentiation into osteoblasts, chondrocytes, cardiomyocytes as well as adipocytes which make human MSCs to acquire increasing interest as a cell source for therapeutic regeneration of lost tissues [1]. These stem cells are isolated from bone marrow as adherent cells, and separated as CD34 negative non-hematopoietic stem cells by immuno staining [2]. Recently, plantation of bone marrow cells was performed to regenerate osteoblasts [3] or cardiac muscle cells [4, 5], and also purified hMSCs from bone marrow cells were used for regeneration of periodontal tissues [6, 7].

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Despite these clinical approach, effective application of these stem cells are somewhat restricted, because the population of these stem cells in bone marrow is very low, and limited proliferative potential of hMSCs during long-term *in vitro* culture because of progressive telomere shortening [8]. To overcome this disadvantage of hMSCs for basic and clinical research, human telomerase reverse transcriptase (hTERT), a catalytic subunit of telomerase ribonucleoprotein complex with an integral RNA component (hTR), has been genetically transduced into hMSCs, which successfully extended life span of the cells as well as elongated telomere lengths [9].

By using clonal hMSCs established from these improved long-life MSCs, we can overcome the defects of primary MSCs, i.e. small population in bone marrow and short life, easily expand these cells and apply differentiated these cells to clinical trials. Furthermore, we can make detail analysis of these cells in view

of proliferation, surface markers and gene expression because we can perform single cell isolation depending on the long life nature of these cells.

DNA chip technology covering the whole genes has been expanded understanding of our knowledge about many cellular processes including cell cycle, cell differentiation and cell proliferation[10]. Analysis of gene expression in these clonal hMSCs by using DNA chips will help us to understand molecular mechanisms and signaling pathways involved in their characteristics as MSC and in their differentiation pathways.

Here we reporte the analysis of these clonal hMSCs in the aspects of proliferation and cell surface markers. We also analyzed their gene expression profiles by using DNA chips of high density containing over 10,000 human genes on one DNA chip.

Materials and Methods

Cell culture

Human MSC clones were cultured in DMEM supplemented with 10% FBS at 37℃ under 5% CO_2. For osteoblastic differentiation, differentiation medium containing dexamethasone, ascorbate and β-glycerophosphate (Sanko Junyaku Co. Ltd., Tokyo, Japan) was used. For chondrocyte differentiation, differentiation medium containing dexamethasone, ascorbate and TGF-β3 (Sanko Junyaku Co. Ltd., Tokyo, Japan) was used.

Cell proliferation assay

Proliferation of hMSC clones was estimated by using cell proliferation assay (CPA) system (GE Healthcare Bio-Science Corp., Piscataway, NJ). Briefly, cells were cultured in DMEM supplemented with 10% FBS containing BrdU for 2hr at 37℃. Then, cells were fixed and blocked (30 min each), and peroxidase conjugated anti-BrdU antibodies were added to the cells. After 30 min at room temp., cells were washed and substrates were added for enzyme reaction. After 5-30 min at room temp., the reaction was ceased and absorbance at 405 nm was measured.

Flow cytometry

Surface markers of hMSC clones were analyzed by flow cytometer EPICS ELITE (Beckman Coulter, Inc. Fullerton, CA). Briefly, hMSC clones were cultured in semi-confluent and pre-incubated with γ-globulin (1mg/ml) at the cell density of $1\sim10^{7}$/ml. Then, diluted FITC-anti-CD34 or FITC-anti-CD44 monoclonal antibodies were added and incubated at 4℃ for 30 min. Cells were washed with PBS and re-suspended in 0.5 ml of PBS at 2-8 ℃ before analysis.

DNA chip analysis

Total RNA was isolated from each hMSC clones with ISOGEN (Nippon Gene, Tokyo, Japan) and treated with RNase-free DNase I. Total RNA of each samples was amplified with T7 polymerase in the presence of aminoallyl-dUTP to produce antisense aminoallyl RNA. The aminoallyl RNA synthesized from hMSC clones was coupled with amino-reactive Cy3. Following fragmentation to 150-300 nucleotides in alkaline solution, the labeled aRNA was hybridized to DNA chips (AceGene, Hitachi Software Engineering Co.,Ltd., Yokohama, Japan) and washed according to manufacture's protocol. Fluorescent signals on the DNA chip were scanned with ScanArray Lite (Hitachi Software Engineering Co.,Ltd.) and analyzed with the QuantArray (Hitachi Software Engineering Co.,Ltd.).

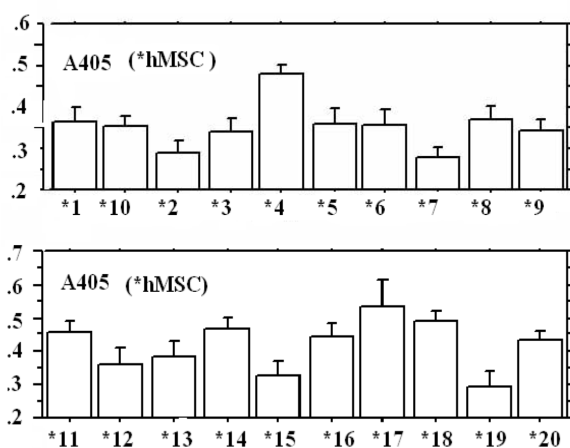

Fig. 1.Proliferation of hMSC clones. Proliferation of hMSC clones were estimated by using cell proliferation assay (CPA) kit. The ordinate indicates absorbance at 405 nm. The horizontal numbers show each clone of hMSC. The absorbance values derived from five wells of each hMSCs were indicated.

Results

Proliferation of hMSC clones

Proliferation of hMSC clones were estimated by using CPA assay. BrdU is incorporated in to cultered cells instead of thymidine and DNA synthesis is detected by anti-BrdU antibody and conjugated enzyme reaction (proliferation ability is indicated by absorbance at 405 nm). Fig. 1 shows the result of DNA synthesis in various hMSC clones. Although these clones were isolated from the pool of human bone marrow cells, these clones showed heterogeneity in its proliferation ability. Among 20 clones shown in Fig. 1, no.17 showed highest proliferation ability and no.4 and no.18 showed the ability next to no.17. Contrary, nos.2, 7 and 19 showed lowest ability. These values were not correlated to the copy number of telomerase gene integrated in the hMSC genome (data not shown). In addition, these ability has no correlation to their differentiation ability, because only clone no.12 which has medium rate of proliferation showed multiple differentiation ability to osteoblasts, chondrocytes and adipocytes.

Surface markers on hMSC clones

Surface markers of hMSC clones were analyzed by using anti-CD monoclonal antibodies and flow cytometer (Fig. 2). In this

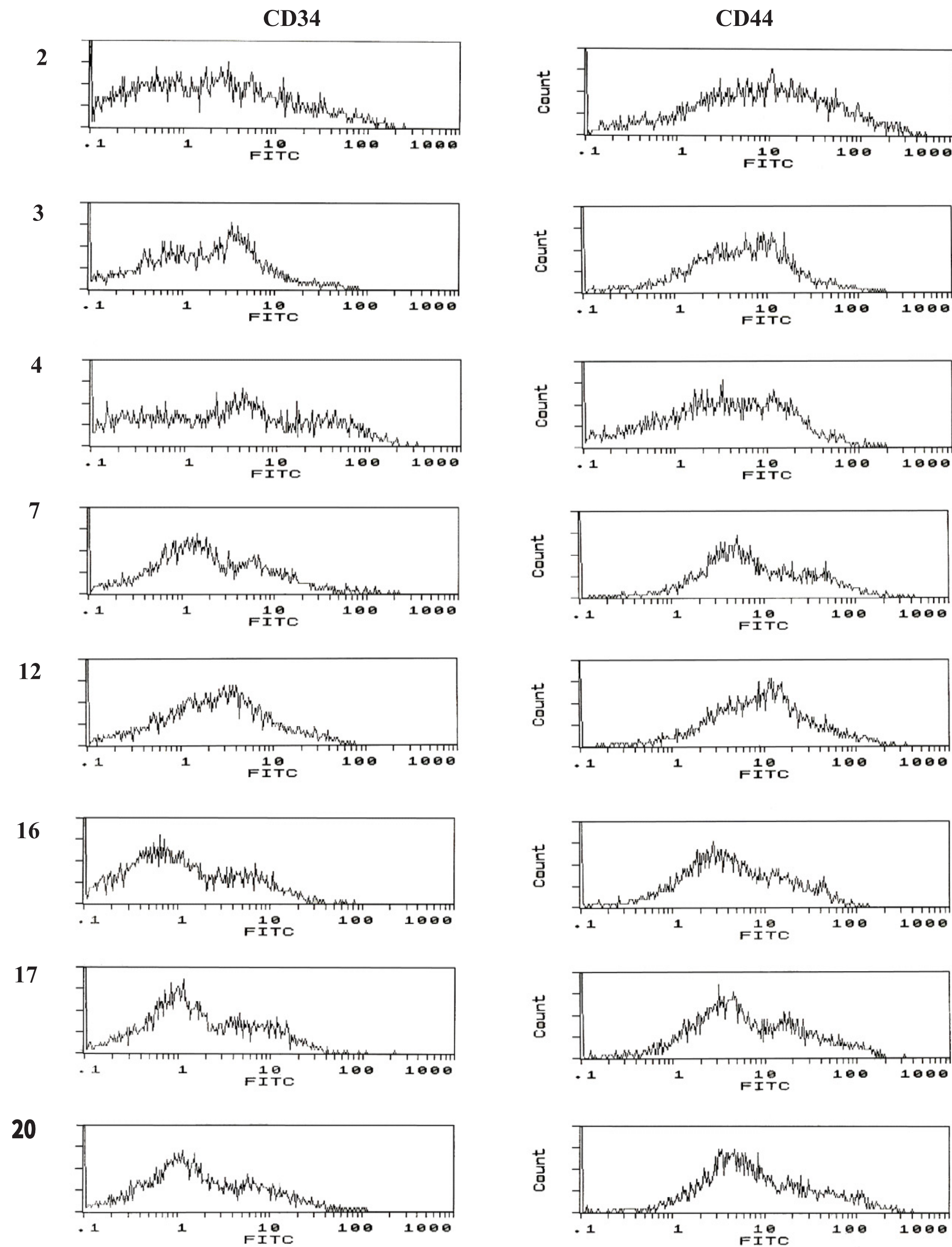

Fig. 2 Analysis of surface markers on hMSC clones. Clone numbers 2, 3, 4, 7, 12, 16, 17 and 20 were cultured and the expression of CD34 or CD44 on their surface was analyzed by the reaction to anti-CD34 or anti-CD44 monoclonal antibodies. Binding of these antibodies on cell surface was estimated by using flow cytometer. The ordinate indicates relative counts of positive cells. The horizontal bar shows FITC intensity (log scale). The small bars in the figure shows threshold values

experiment, we used anti-CD34 and anti-CD44 monoclonal antibodies: the former antibody selectively recognizes hemotopoietic stem cells and the latter recognized mesenchymal stem cells in the population of bone marrow cells. We selected clone no.17 and no.4 as typical clones with high proliferation ability. Also we selected no. 2 and no.7 as typical clones with low proliferation ability, and other four clones, 3, 12, 16 and 20 with medium ability.

As a result, almost all clones showed low reactivity for anti-CD34. Among them, clone no.4 with high differentiation ability contained small population of CD34-positive group (about 30% positive of total cells). For CD44, almost all clones showed

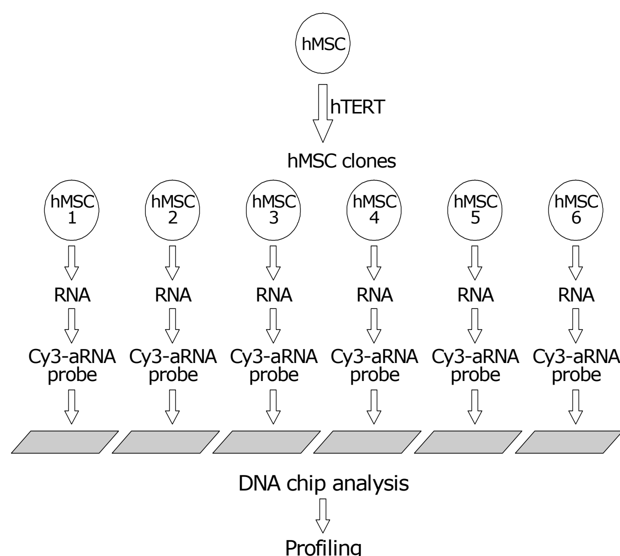

Fig. 3 DNA chip analysis of hMSC clones. Schematic representation of DNA chip analysis in this experiment.

medium response in which no.4 showed relatively low response to CD44 (about 33% positive of total cells).

DNA chip analysis

To understand detail molecular mechanism involved in the maintenance of undifferentiated state and pluripotency of hMSC clones, we analyzed gene expression profiles in 10 hMSC clones (nos. 2, 3, 4, 7, 12, 15, 16, 17, 19 and 20) with different ability of differentiation by high-density DNA chip. Cy-3 labeled probes made from each mRNA isolated from above hMSC clones were hybridized to one oligonucleotide-type DNA chip (Fig.3). Totally, we used 10 DNA chips for analysis of all above MSC clones. Among 10,000 gene probes on one DNA chips, about 4,800 genes were confirmed as valid spots. Comparing with gene expression profiles of 10 clones, 40 genes, including transcription-related genes, growth factor genes, signal regulating genes and some enzymes were differentially up- or down-regulated. These genes are the candidates related to multipotency of hMSCs. Typical 14 genes were shown in Fig.4 and their expression patterns especially expression of four genes (p53-regulating protein, ubiquitin-conjugated enzyme E2, CCAAT/enhancer-binding protein and AT-rich sequence-binding protein 1) in five hMSC clones (nos. 2, 3, 4, 7 and 12) were indicated in Fig. 5. Particularly, clone no.12 showed high level of gene expression for these genes comparing with other hMSC clones.

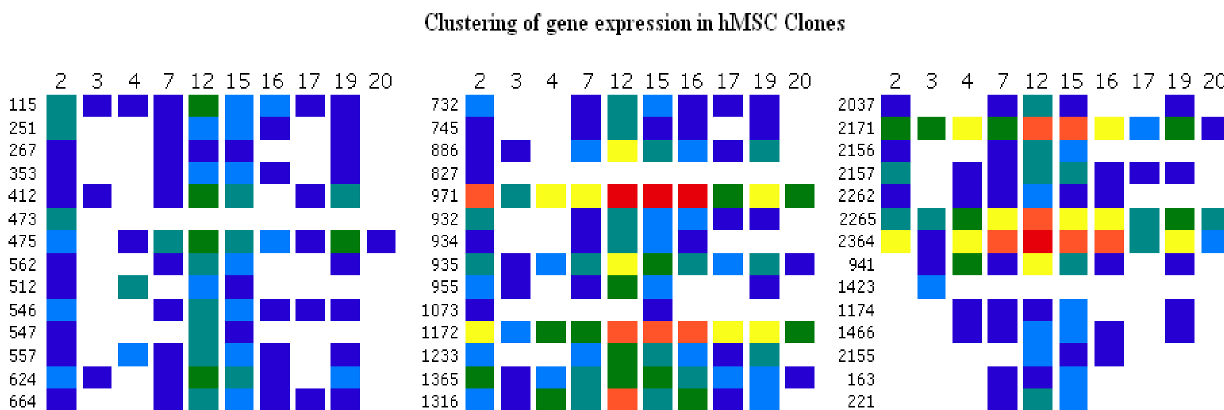

Fig. 4 Profiling of gene expression in hMSC clones. The ordinate indicates analyzed genes on DNA chips (indicated by gene number on DNA chips). The horizontal numbers show each clone of hMSC. Profiles of gene expression in clones 2, 3, 4, 7, 12, 15, 16, 17, 19 and 20 were analyzed. Each color shows relative intensity of gene expression. The order of color representation is red>orange>yellow>green>glaucous>blue>dark blue.

Discussion

In this paper, we characterized newly established human mesenchymal stem cell (hMSC) clones in their proliferation ability, expression of surface makers and levels of gene expression.

Human MSCs derived from small population of bone marrow cells originally posses low ability of cell proliferation. In this point of view, immortalized hMSCs are valuable in its high ability of proliferation and useful for detail analysis of these cells. This time, we used several hMSC clones established from immortalized hMSCs for analysis of single character of each MSC contained in whole MSC pool, because hMSC showed some heterogeneity: seemed to contain various types of stem cells with low or high ability of differentiation. It seems to be important to isolate single stem cell which has multipotency of differentiation for efficient regenerative therapy. In this study, we characterized proliferation ability, surface makers and gene expression of these various MSC clones and showed their heterogeneity correlating to their differentiation ability.

In the aspect of proliferation of these clones, several clones (nos.4, 17 and 18) showed high ability of proliferation and clones nos. 2, 7 and 19 showed low ability indicating that introduction

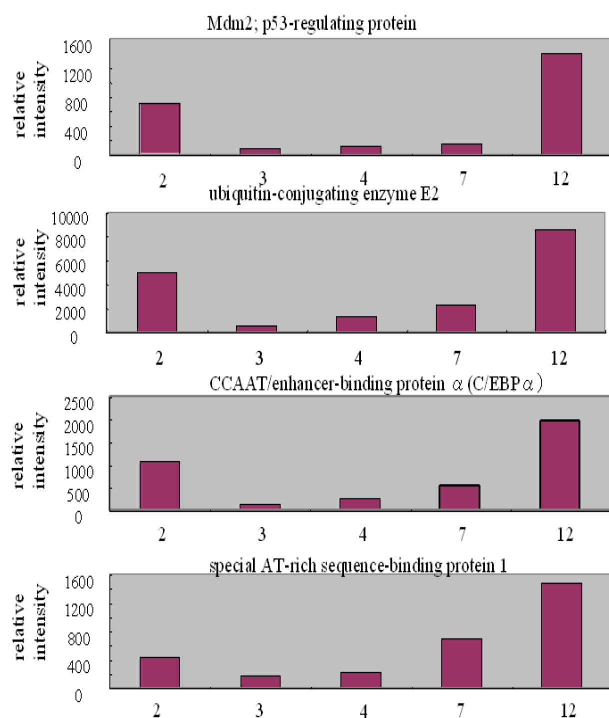

Fig. 5 Gene expression in hMSC clones. Gene expression levels of four genes in five hMSC clones were shown. The ordinate indicates relative intensity of expression analyzed by DNA chips. The horizontal numbers show each clone of hMSC.

of telomerase gene into these MSCs promotes their long-term proliferation depending on the expression level of functional telomerase. The values of DNA synthesis shown in Fig. 1 were not correlated to the copy number of telomerase gene integrated in the hMSC genome (data not shown), so transcription efficiency of telomerase gene seemes to be different in each MSC clone which leads the difference of proliferation ability. Generally, cells with high proliferation rate show low ability of differentiation. In the case of this study, only clone no.12 which has medium level of proliferation ability showed multiple differentiation ability to osteoblasts, chondrocytes and adipocytes indicating that this differentiation ability depends on other factors than the expression level of telomerase mRNA or the ability of proliferation.

Next, analysis of cell surface markers was performed by using flow cytometer. In this experiment, we analyzed the expression of CD34 and CD44. CD34 is expressed on hematopoietic stem cells and endothelial progenitor cell (EPC)- like cells [11] but recent study showed that CD34 positive cells had wide potency of differentiation including osteoblastic differentiation. CD44 is a transmembrane adhesion molecule with hyalluronic acid binding domain [12]. It is also expressed on some tumor cells and its variants are associated to metastasis of these tumors. Because this molecule is expressed on chondrocytes, it is thought that this molecule is a kind of surface marker of some stem cells including MSCs. The expression patterns of these molecules on hMSC clones shown in Fig. 2 indicated that almost all these clones were not hematopoietic stem cells but relatively has hyalluronic acid binding domain and seems to belong to mesenchyme origin. Only clone no.2 and 4, especially no.4 showed CD34 positive population. Clone no.4 also showed relatively weak signals in the reaction to CD44, suggesting that clone no.4 possesses more broad differentiation ability, in other word, more primitive stem cell than other clones.

Finally, we performed DNA chip analysis of these hMSC clones to make their gene expression profiles and identify MSC-specific and multipotency-related genes. Fig. 3 shows the strategy of DNA chip analysis and Fig.4 shows a part of profiling results. Fig. 4 shows that there are several genes specifically expressed in these MSCs. Among them, cysteine-rich protein 61 (Cyr61) [13] and connective tissue growth factor (CTGF) [14] are the most prominent genes. These two genes may play some important roles in the maintenance or differentiation of MSCs. Furthermore, there were several MSC clones that highly expressed above genes. These clones (nos. 12, 15 and 16), especially no.12 showed highest expression patterns of above genes (Fig. 5) indicating that no.12 has some unique character between hMSC clones. In fact, only no.12 showed multiple differentiation ability (differentiated to osteoblasts, chondrocytes and adipocytes) among these clones (data not shown). We also suppose there is some regulation of gene expression in no.12 associated to its DNA demethylation, because regulation and reorganization of DNA methylation are the most common epigenetic events in mammalian cell development [15].

We are planning to perform further analysis of these hMSC clones especially in their differentiation ability *in vitro* and *in vivo*, and another detail profiling of gene expression using DNA chips of higher density.

Acknowledgments

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