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Gene Expression Profiling of Human Mesenchymal Stem Cells for Identification of Novel Markers in Early- and Late-Stage Cell Culture

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Human mesenchymal stem cells (hMSCs) are multipotent cells that differentiate into several cell types, and are expected to be a useful tool for cellular therapy. Although the hMSCs differentiate into osteogenic cells during early to middle stages, this differentiation capacity decreases during the late stages of cell culture. To test a hypothesis that there are biomarkers indicating the differentiation potential of hMSCs, we performed microarray analyses and profiled the gene expression in six batches of hMSCs (passages 4–28). At least four genes [necdin homolog (mouse) (*NDN*), EPH receptor A5 (*EPHA5*), nephroblastoma overexpressed gene (*NOV*) and runt-related transcription factor 2 (*RUNX2*)] were identified correlating with the passage numbers in all six batches. The results showed that the osteogenic differentiation capacity of hMSCs is down-regulated in the late stages of cell culture. It seemed that adipogenic differentiation capacity was also down-regulated in late stage of the culture. The cells in late stage are oligopotent and the genes identified in this study have the potential to act as quality-control markers of the osteogenic differentiation capacity of hMSCs.

Key words: cellular therapy, culture stage marker, differentiation, gene expression, stem cell.

Abbreviations: EPHA5, EPH receptor A5; hMSCs, human mesenchymal stem cells; NDN, necdin homolog (mouse); NOV, nephroblastoma overexpressed gene; PBS, phosphate buffered saline; RUNX2, runt-related transcription factor 2.

INTRODUCTION

'Cellular therapy' is a new concept in treating diseases with cells that have regeneration potential. Currently, it is at the clinical research stage; however, the use of cellular therapeutics in regular clinical settings will be implemented in near future. Cellular therapeutics involves the use of cells derived from human tissue, either cultured and/or modified, in regenerating and repairing damaged tissues and consequently improving the functions in the human body. Hence, tissue or embryonic stem cells that have the potential to differentiate into a variety of cell types are one of the prime candidate cells for cellular therapeutics. It is difficult to overview the entire discipline of cellular therapeutics since the cells themselves represent 'life'.

Stem cells, one of the candidates for cellular therapeutics, produce daughter cells identical to themselves that differentiate into other types of cells (*1*). The fate of the stem cells is determined by cellular signaling, although the underlying mechanism is still unknown. It is therefore important to investigate the gene expression patterns that influence the cellular signaling pathways and identify the representative biomarkers that can act as indicators of the differentiation potential of the stem cells. Recently, it has been reported that human somatic cells can be induced to pluripotent stem cells (*2*).

There have been several reports suggesting that cellular therapeutics is a promising treatment for several diseases. C-kit-expressing cells obtained from the bone marrow have been used in cardiac tissue repair in mice experiments (*3*). Previous studies have reported the use of autologous bone marrow cells transplantation for the post-infarction recovery of cardiac function (*4–9*). Cytotoxic T cells have also been used for cellular therapy to protect from infectious diseases in an immunodeficient condition following hematopoietic stem cell transplantation (*10*). Mesenchymal stem cells (MSCs) are also used for therapy expecting immunosuppressive effects (*11*, *12*). Previous studies on MSCs also indicate that these cells possess the ability for chondrogenic (*13*), osteogenic (*14*, *15*) and adipogenic differentiation, and possibly other differentiating capabilities (*16*). In a clinical setting, it is difficult to assess the overall profile of each batch of the cells. We hypothesized the existence of quality-control markers for the differentiation potential of human mesenchymal stem cells (hMSCs) and used gene expression profiling to identify these markers.

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EXPERIMENTAL PROCEDURES

Cell Culture—The hMSCs derived from bone marrow [Lonza (Cambrex), Walkersville, Maryland, USA] were cultured in mesenchymal stem cell growth medium (MSCGM) [Lonza (Cambrex) #PT-3001; mesenchymal stem cell basal medium supplemented with mesenchymal cell growth supplement, L-glutamine and penicillin/streptomycin] at 37°C in CO_2 (5%) incubator. Cells were passaged according to the manufacturer's protocol with slight modification using trypsin–EDTA solution [Lonza (Cambrex) #CC-3232]. Lot numbers of the hMSC batches were as follows: #4F1127, #4F0312, #5F0138, #4F1560, #4F0591 and #4F0760. Informed consent was obtained in Poietics human mesenchymal stem cell systems [Lonza (Cambrex)]. All differentiation procedures were performed according to Lonza (Cambrex) protocol with slight modification.

Osteogenic Differentiation—The hMSCs were plated onto 12-well plates and 24 h later, the medium was changed to MSCGM (as control) or osteogenic induction medium (OIM) [Lonza (Cambrex) #PT-3002; differentiation basal medium containing dexamethasone, ascorbate, mesenchymal cell growth supplement, L-glutamine, penicillin/streptomycin and β-glycerophosphate]. Medium was changed every 3–4 days and cells were differentiated for 21 days.

Calcium Deposition Assay—Calcium deposition was measured using the Stanbio Total Calcium Liquicolor® kit (Stanbio Laboratory, Boerne, Texas, USA; #0150-250) according to the manufacturer's protocol (Cambrex, Stanbio Laboratory). Briefly, the cells cultured on 12-well plates for 22 days (osteogenic-induced for 21 days) were rinsed with phosphate buffered saline (PBS) without calcium and magnesium [Lonza (Cambrex) #17-516Q] and harvested in 0.5 N HCl (600 μl). Calcium was extracted from the cells by shaking the tubes for approximately 20 h at 4°C. Lysates were centrifuged at 500*g* for 2 min at 4°C and 20 μl of the supernatant was used for the assay. Absorption at 560 nm was measured to detect the Ca-ortho-cresolphthalein complexone (OCPC) complex using an EnVision 2103 multilabel reader (PerkinElmer, Waltham, Massachusetts, USA). Calcium deposition was adjusted with the total protein concentration of the samples. Cells harvested in 0.5 N HCl were centrifuged at 15,000 rpm for 10 min at 4°C. The pellet was washed once with PBS without calcium and magnesium, and resuspended in 100 μl of 0.1 N NaOH/0.1% SDS. After overnight incubation at 37°C, the lysate was centrifuged at 15,000 rpm for 10 min at room temperature, and the supernatant was quantitated using the DC protein assay (Bio-Rad Laboratories, Hercules, California, USA) according to the manufacturer's protocol. Absorbance at 620 nm was measured using the EnVision 2103 multilabel reader (PerkinElmer). The standard curve was obtained using bovine serum albumin.

Adipogenic Differentiation—The cells were plated onto a 24 well-plate at 2.1 x 10^4/cm^2, and cultured in MSCGM for 5–6 days. After cells reach confluence, medium was changed to MSCGM (as control) or adipogenic induction medium (AIM) [Lonza (Cambrex) #PT-3004; induction basal medium supplemented with recombinant human insulin, L-glutamine, mesenchymal stem cell growth supplement, penicillin/streptomycin, dexamethasone, indomethacin and IBMX (3-Isobutyl-1-methylxanthine)]. Medium was changed after 3 days into adipogenic maintenance medium (maintenance basal medium supplemented with recombinant human insulin, L-glutamine, penicillin/streptomycin and mesenchymal stem cell growth supplement). After three complete cycles of induction/maintenance, the cells were cultured for 7 more days in adipogenic maintenance medium, replacing the medium every 2–3 days.

Oil Red O staining—The cells were rinsed with 500 μl of PBS and fixed with 10% neutral buffered formalin (500 μl). After washing with sterile water, the cells were washed with 60% 2-propanol (500 μl) for 2–5 min and stained with Oil Red O (500 μl) for 5 min. The cells were rinsed with tap water and stained with Harris' haematoxylin (500 μl) for 1 min and rinsed with the water. Lipid vesicles were observed with microscope Biozero BZ-8000 (KEYENCE, Osaka, Japan).

Chondrogenic Differentiation—The cells (3×10^5) were washed with incomplete chondrogenic induction medium [Lonza (Cambrex) #PT-3003; chondrogenic basal medium containing dexamethasone, ascorbate, ITS (insulin-transferrin-sodium selenite) + supplement, sodium pyruvate, proline, penicillin/streptomycin, L-glutamine] and were resuspended in 0.5 ml of complete chondrogenic induction medium (CCIM; incomplete condrogenic induction medium supplemented with 10 ng/ml of TGF-β3) or MSCGM (as control) and cultured in 15 ml polypropylene culture tubes. The medium was replaced every 3–4 days and the cells were cultured for 24 days.

Safranin-O Stains for in vitro Chondrogenesis—The chondrogenic pellets were fixed in 10% neutral buffered formalin and paraffin embedded. The paraffin sections were stained with Weigert's iron hematoxylin (Wako 298-21741), 0.02% fast green FCF (MP biomedicals 195178) and 0.1% Safranin-O (Sigma HT 90432), followed by observation with microscope Biozero BZ-8000 (KEYENCE).

Total RNA Purification—The hMSCs were cultured on a 10 cm dish, lysed in 600 μl of Buffer RLT (RNeasy® Lysis Buffer) with β-mercaptoethanol and homogenized using a QIA shredder (QIAGEN, Düsseldorf, Germany). Total RNA was purified using RNeasy® mini spin columns according to manufacturer's protocol (QIAGEN). Total RNA was eluted with RNase-free water.

Microarray Analysis—Total RNA (100 ng or 1 μg) was reverse transcribed and amplified using a GeneChip® kit (Affymetrix, Santa Clara, California, USA) and the biotinylated cRNA was hybridized onto the GeneChip® Human Genome U133 Plus 2.0 Array (54,613 probe sets). The data was analysed using GeneChip Operating System software (versions 1.2–1.4), followed by statistical analysis. The data was also analysed using GeneSpring™ (version 7.3) (Agilent, Santa Clara, California, USA). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) (*17*, *18*). They are accessible through GEO Series accession number GSE7637 for the data from 4F1560, and GSE7888 for the data obtained from all six batches. The statistical method for microarray data analysis has been also discussed elsewhere (*19*).

Cluster Analysis—The microarray data of 169 probe sets obtained from six batches of hMSCs was subject to cluster analysis using the Gene Expression Statistical System (NCSS, Kaysville, Utah; Dr Jerry L. Hintze). Fold change of signal intensity to the average signal intensity of early stage was analysed and a double dendrogram was plotted on a log 2 scale.

Gene Ontology Analysis—Gene ontology analysis was conducted using Ingenuity Pathway Analysis (IPA) (Ingenuity® Systems, Redwood City, California, USA), NetAffyx (Affymetrix) and GOTM (Gene Ontology Tree Machine, Vanderbilt University, Nashville, Tennessee, USA) analyses. Probe sets with signal intensity values associated with the passage numbers were subject to analyses. The functional analysis identified the biological function and/or diseases that were most significant to the data set. Genes from the data set that were associated with biological functions and/or diseases in the Ingenuity Pathways Knowledge Base (IPKB) were considered for further analysis.

cDNA Synthesis and Real-time PCR Using Taqman Low-density Array—RT–PCR (reverse transcriptase–PCR) analysis was performed to assess the mRNA levels in six batches of hMSCs using TaqMan® low-density array (TLDA) (Format 48) (Applied Biosystems, Foster City, California, USA). The data was normalized using *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase). Forty-six genes including *GAPDH* as endogenous control are listed in Supplementary Table 1. cDNA was synthesized using a High-capacity cDNA synthesis kit (Applied Biosystems) and Multiscribe reverse transcriptase. cDNA synthesized from 100 ng of total RNA was used for the analysis (2 ng of total RNA per well). Real-time PCR was analysed using 7900 HT real-time PCR system (Applied Biosystems). The conditions for the PCR reaction were as follows: 50°C (2 min) and 94.5°C (10 min), and 40 cycles at 97°C (30 sec) and 59.7°C (1 min). Relative quantification values were calculated by the comparative Ct method using SDS 2.2.2 software (Applied Biosystems).

Pathway Network Analysis—Data were analysed using the IPA (Ingenuity® Systems, www.ingenuity.com). A data set containing gene identifiers and corresponding expression values was uploaded into the application. Each gene identifier was mapped to its corresponding gene object in the IPKB. A fold-change cutoff of 3 for both up- and down-regulation and a p-value cutoff of 0.05 were set to identify the genes to be analysed. These genes, called focus molecules, were overlaid onto a global molecular network developed from information in the IPKB. Networks of these focus molecules were then algorithmically generated based on their connectivity. The functional analysis of a network identified the biological functions and/or diseases that were most significant to the genes in the network. The genes in the networks associated with biological functions and/or diseases in the IPKB were considered for the analysis. Genes and gene products are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least one reference from the literature, textbook or canonical information stored in the IPKB. Human, mouse and rat orthologs of a gene are stored as separate objects in the IPKB, but are represented as a single node in the network. The node colour indicates the degree of up- (red) or down- (green) regulation. Nodes are displayed using various shapes that represent the functional class of the gene product.

Statistical Analyses—Non-parametric analysis was used for microarray data analyses. The Spearman correlation coefficient and two-tailed p-values were calculated. $P < 0.001$ or $P < 0.05$ were considered to be significant. RT–PCR data was analysed with non-parametric analysis. The Spearman correlation coefficient and two-tailed p-values were calculated. To compare the specific passage number and stage, Student's t test was performed. Two-way ANOVA followed by Bonferroni post-test was performed for osteogenesis data. GraphPad Prism® 4 and Microsoft® Office Excel were used for statistical analysis and drawing graphs.

RESULTS

Microarray Analysis of hMSCs—To identify the quality-control markers in different stage of the culture, we performed DNA microarray analyses. Non-parametric analysis and a ratio (max/min of signal intensity) cutoff of 3.071,524 ($1.05^{(28-5)}$; 5% change in each passage from 5th to 28th) showed that the expression level of 341 probe sets out of a total of 54,613 probe sets had a significant association with passage numbers (hMSC lot #4F1560, passage numbers 5, 7, 9, 13, 21 and 28).

Gene ontology analyses showed that the mapped genes corresponded to the probe sets belonging to various categories of molecular and cellular functions such as cell-to-cell signaling and interaction, cellular movement, cell death, cellular assembly, cellular organization and cell cycle, and physiological system development, and biological functions such as hematological system development and function, immune and lymphatic system development and function, tissue development, immune response and embryonic development. The top five disease categories that the genes mapped to, as identified using the IPA software, included cardiovascular, hematological, musculoskeletal, oncogenic and reproductive disorders.

Figure 1 shows the results of cluster analysis obtained from microarray data of six batches of hMSCs in early (passage #4–5), middle (#7–9) and late stages (#22–28). Seventy-nine genes out of the 169 probe sets were categorized by function and disease as per IPA analysis. Networks were analysed for each of the six batches and a representative network is shown in the Supplementary Fig. 1. A list of all top networks in each analysis is shown in Table 1. Many network categories with the top score in each analysis were involved in cancer or regulation of cell cycle. Additionally, specific networks for each sample were generated when the batches were individually analysed.

Calcium Deposition of Osteogenic-induced Cells—In Fig. 2, calcium deposition in hMSC cultures (4F0312, 5F0138, 4F1560, 4F0591, 4F0760) were measured during passages 7, 9, 10 and 19. The results showed that the osteogenic differentiation occurred in early to middle stages and was dramatically suppressed during the late

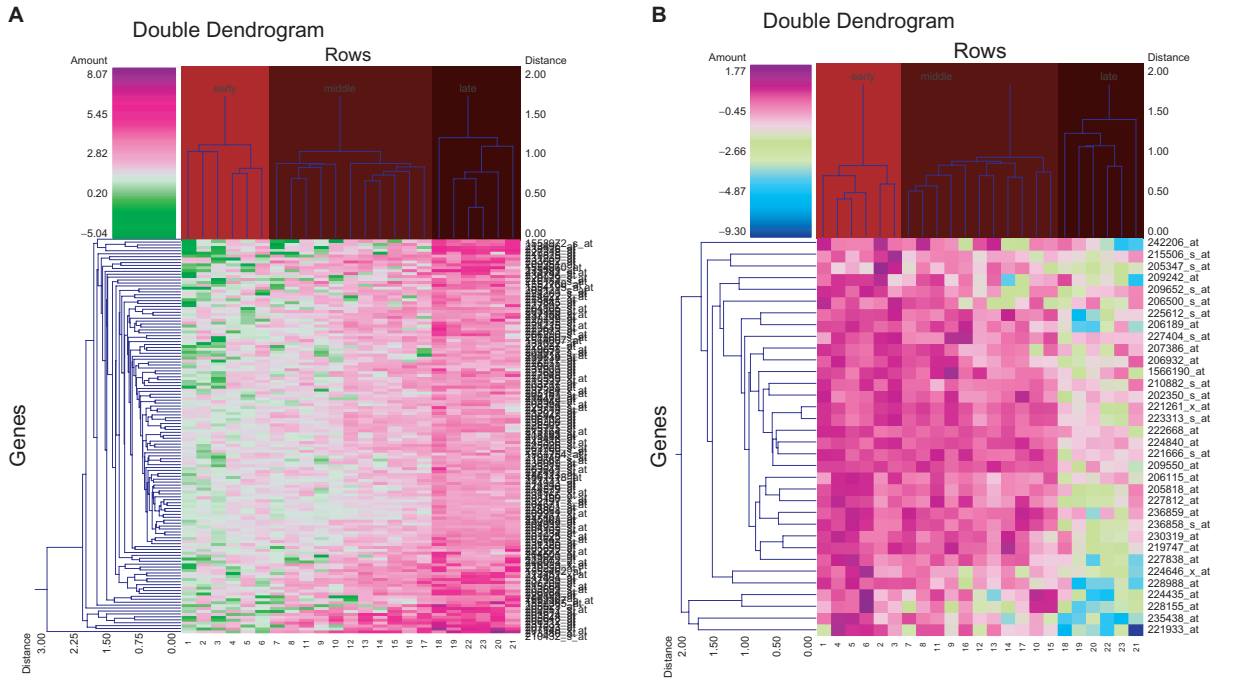

Fig. 1. **Microarray analysis of hMSCs.** One hundred and sixty-nine probe sets extracted from microarray data of six batches of hMSCs. [$n = 6$ in early stage (#4 or 5), passage #9, late stage (#22, 24 or 28), $n = 5$ in passage #7]. Cut off value of signal intensity ratio (max/min of average in each stage) is 2.949145023 [5% change in each passage number from early to late stage; 1.05 $^{(\text{passage number range of average in early and late stage})}$]. Double dendrograms of up-regulated 135 probe sets (A) and down-regulated 34 probe sets (B) are shown.

stages of cell culture. These findings suggest that the expression levels of genes associated with osteogenesis are different at the late stages compared with those at earlier stages of cell culture.

Statistical analysis of microarray and calcium deposition data from three batches (5F0138, 4F1560, 4F0591) of hMSCs in middle (#7–10) and late (#19–28) stages showed that the expression of *NDN* [necdin homolog (mouse)] has a positive correlation with calcium deposition ($P < 0.05$).

Adipogenic Differentiation of hMSCs—Figure 3A shows the results of Oil Red O staining of adipogenic-induced cells. The cells were adipogenic induced for 21 days and lipid was stained with Oil Red O. Adipogenesis of hMSCs seemed to be down-regulated in late culture stage of 5F0138, 4F0591 and 4F0760, while the adipogenic-differentiation capacity seemed to be retained in passage #20 of 4F1560.

Chondrogenic Differentiaion of hMSCs—Figure 3B shows the Safranin-O staining of chondrogeninc-differentiated hMSCs. The cells were differentiated in CCIM for 24 days and stained with Safranin-O. The culture in passages 7, 17 and 22 of 4F0591 showed chondrogenic-differentiated morphology (a, b, c, respectively). The culture in late stage seemed to be chondrogenic differentiated as shown (c). The cells cultured in MSCGM as control did not show any chondrogenic-differentiated morphology (d).

RT–PCR Analysis of hMSCs—The quantitative RT–PCR data showed that some genes had similar expression profiles in all the six batches examined. Up-regulated genes, which were identified as candidates for the stage-specific markers included *EPHA5* (EPH receptor A5), *NOV* (nephroblastoma overexpressed gene), *SERPINE1* [serpin peptidase inhibitor clade E (nexin, plasminogen activator inhibitor type 1), member 1], *ITGA4* [integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)], and down-regulated genes, which are also candidates for the stage-specific markers included *NDN*, *RUNX2* (runt-related transcription factor 2) and *RUNX3* (runt-related transcription factor 3). *NOV* is a growth factor and is involved in the proliferation of bone cancer cell lines (*20*). It is notable that the expression of *NOV* in lot #4F1127 was relatively stable. *SERPINE1* is involved in the protein-binding function and diseases such as heart failure (*21*). *RUNX2* is a member of the runt domain-containing family of transcription factors and suggested to regulate osteogenic differentiation (*22*). *RUNX3* is also a member of the runt domain-containing family of transcription factors and a candidate tumor suppressor (*23*). *EPHA5*, *NOV*, *NDN* and *RUNX2* showed altered expression correlating with passage numbers ($P < 0.01$) (Fig. 4). The results of RT–PCR analysis of 45 genes examined are shown in Supplementary Fig. 2.

DISCUSSION

hMSCs will be used for cellular therapeutics in clinical settings in the near future. The importance of quality control of the cells will be significant as the use of cellular therapeutics becomes more common. In this report, we report on profiling the gene expression of

Table 1. **List of the networks in hMSCs.**

Analysis	Molecules in network	Score	Focus molecules	Top functions
4F0591-#9	BUB1B, CCNB1, CDC2, CDKN3, CENPF, CGREF1, Cyclin E, DLG7, E2f, ERCC6L, FOXM1, KRT8, LRP1, MAD2L1, MEOX2, NDC80, NFkB, NUF2, NUSAP1, OLR1, PBK, PCSK1, PLK1, PTTG1, RAD51AP1, Rb, RNA polymerase II, RRM2, SERPINB2, SPC25, TFPI2, TNFSF9, TRADD, TYMS, UBE2C	65	30	Cell Cycle, Cancer, Reproductive System Disease
4F1560-#28	ADH1B, ALDH1A3, BEX1 (includes EG:55859), BMP15, CD80, CHI3L1, DBP, DLX1, ENPP1, FGF5, GBP2, IGF2, Igfbp, IGFBP5, KRT19, LBP, MEOX2, Mmp, NFkB, NOV, PCSK1, PCSK5, PEG10, PYCARD, RAGE, SEPP1, SERPINB2, SERPING1, ST8SIA1, Tgf beta, TLR1, TNFAIP6, TNFSF15, TRAF4, TSLP	57	31	Cancer, Cellular Growth and Proliferation, Neurological Disease
4F0760-#9	ANKRD1, Ap1, BIRC3, CCNB1, CCR2, CDC2, CENPE, COL15A1, FBLN5, Jnk, LATS1, Mmp, MSR1, NDC80, NFkB, NRL, NUF2, OMD, PBK, Pdgf, PDGF BB, PDGFD, PRDM1, S100A4, SERPINB2, SLC37A4, SORBS3, SPC24, SPC25, TFPI2, THBD, TNFRSF8, VANGL2, VAV3, VSNL1	56	29	Cancer, Cell Cycle, Reproductive System Disease
5F0138-#24	ARL4C, BUB1 (includes EG:699), BUB1B, CCNB1, CCNB2, CCNF, CDC2, CDC7, CDC20, CDC25C, CDKN3, CENPE, CENPH, Cyclin B, Cyclin E, FBXO5, FOXM1, GINS1, GPNMB, IL6, KIAA0101, KIF11, KIF22, KIF2C, MRVI1, NDC80, NUF2, PBK, PLK4, PTTG1, SLC7A7, SPC25, UBE2C, VTCN1, ZWINT (includes EG:11130)	52	33	Cell Cycle, Cancer, DNA Replication, Recombination, and Repair
4F1560-#9	14-3-3, AURKA, BIRC5, CCNB1, CDC20, CDC25C, CDCA8, Cyclin B, Cyclin E, E2f, IGF2, MAD2L1, NDC80, NFkB, NUF2, OLR1, PBK, PRR11, RAD51AP1, Rb, RGS7, RNA polymerase II, RRM2, Scf, SERPINB2, SFN, SFRP4, SPC24, SPC25, TNFRSF8, TOP2A, TYMS, UBE2C, UHRF1, ZNF74	52	27	Cancer, Cell Cycle, Reproductive System Disease
4F0591-#28	AEBP1, ALDH1A3, ANGPT1, ANKRD1, BEX1 (includes EG:55859), C1R, CGREF1, CXCL16, DIRAS3, GAD1, HDAC9, ID4, IL1, IL1R1, KRT18, KRT19, MEOX2, Mmp, MYBL1, MYPN, NFkB, OLR1, PAK1IP1, Pdgf Ab, PLAT, PYCARD, RIPK4, SERPINB2, SERPINF1, SERPING1, TFPI2, Tgf beta, TNFRSF19, TNFRSF11B, TSLP	51	30	Cancer, Cardiovascular Disease, Cell Death
4F0312-#7	A2M, ACAN, ASPN, BRCA1, C1R, C1S, CEBPD, CYP27A1, DDIT3, DDIT4, ESR1, FGF7, GDF15, IL1, Jnk, KSR2, MAD2L1, Mek, Mek1/2, Mmp, NFkB, NOTCH3, NOX4, NR4A1, OSMR, P38 MAPK, PCK2, Pdgf, PDGF BB, SERPINB2, STAT, TNFAIP6, TNFSF9, TNFSF15, TRIB3	50	25	Cell Cycle, Inflammatory Disease, Cellular Development
4F0312-#28	ANKRD1, BEX1 (includes EG:55859), BLK, CD36, CDKN2B, CTSL2, ENPP1, F2RL1, FABP5, FKBP5, FUS, G0S2, GAD1, GDF15, IGFBP5, IGHG1, IL1, N-cor, NFkB, OLR1, PAPPA2, PLAT, PNRC1, Rxr, RXRA, SERPINB2, STMN2, Tgf beta, THRB, Thyroid hormone receptor, TNFRSF19, TNFRSF11B, TNFSF13B, TRIP13, VSNL1	50	29	Cancer, Cellular Growth and Proliferation, Immunological Disease
4F1560-#7	14-3-3, AURKA, BIRC5, CCNE2 (includes EG:9134), CDC25A, CDCA8, CEBPA, CENPF, CSPG4, Cyclin A, Cyclin E, E2f, ESPL1, FEN1, FMOD, Histone h3, Mapk, MCM8, MCM10, MDM4, NUSAP1, OIP5, PP2A, PRR11, PTTG1, Rb, RGS7, RRM2, SFRP4, SMPDL3A, SOS2, TOP2A, TTK, TYMS, UBE2C	50	27	Cell Cycle, Cancer, Reproductive System Disease
4F0312-#9	ABCA1, ACAN, AEBP1, Akt, ANXA11, ASPN, C1R, C1S, CD36, DKK, F2RL1, FBLN1, FGF7, FOXE1, GDA, GDF15, HSD11B1, IGF2, IGFBP2, Insulin, LDL, LEPR, Mapk, NFkB, NTF3, P38 MAPK, PDGF BB, PTX3, SLC7A7, THBS2, TNFAIP6, TNFSF9, Wnt, WNT2, WNT16	49	23	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry

(continued)

Table 1. **Continued.**

Analysis	Molecules in network	Score	Focus molecules	Top functions
5F0138-#9	ABCA1, AEBP1, ARG2, BGN, C1q, C1R, C1S, CYP2B6 (includes EG:1555), DDIT4, ENPP1, FABP5, FADS1, GDF15, HABP2, N-cor, NCOR-LXR-Oxysterol-RXR-9 cis RA, NFkB, Nr1h, OLR1, PCK2, PDGF BB, PTGDR, Rxr, SCD, SERPING1, SFTPD, SORBS3, SREBF1, SYNE1, THBS2, Thyroid hormone receptor, TNFAIP6, TNFSF9, TRIB3, VDR	46	27	Respiratory Disease, Inflammatory Disease, Lipid Metabolism
4F1127-#9	ACAN, Alkaline Phosphatase, Ap1, ASPN, C3, CCL2, CCNO, COL13A1, CP, FABP5, GEM, HMOX1, HOMER2, IGFBP5, IL1, JAG1, LDB3, LDL, Mmp, MMP28, NFkB, P38 MAPK, Pdgf, PDGF BB, RGS4, SERPINB2, SPINT2, SPP1, TAC1, Tgf beta, TNFAIP6, TNFRSF11B, TXNIP, VitaminD3-VDR-RXR, ZNF335	45	24	Cellular Development, Cellular Growth and Proliferation, Skeletal and Muscular System Development and Function
4F1127-#22	AEBP1, ANKRD1, C1q, C1R, CBR3, CD36, CFH, ENPP1, FLNC, FOXF1, G0S2, HAMP, HDL, HIST2H2AA3, HIST2H2BE, HIVEP1, IGKC, KCNAB1, KCND2, KRT17, LDB3, LY6E (includes EG:4061), MYOZ1, NFkB, OLR1, PLK3, POU2F2, REG3A, RIPK4, SLC40A1, TNFRSF19, TNFRSF10D, TNFSF9, TSLP, VSNL1	44	32	Genetic Disorder, Metabolic Disease, Molecular Transport
4F0760-#28	Alpha Actinin, CDH1, CTSH, Cyclin A, Cyclin E, E2f, EDN1, GAST, ICAM2, Integrin, ITGA2, ITGA6, KRT7, KRT18, LAMC2, MARCKSL1, Mek1/2, Mmp, MYOZ2, OCLN, PCOLCE, PCOLCE2, Plc beta, PLCB4, PRPS1, Rb, S100A4, SCG5, SDPR, SERPINB2, SMURF2, TFPI2, TGFB1, TNFRSF11B, TSPAN8	44	26	Cardiovascular System Development and Function, Cell Morphology, Skeletal and Muscular System Development and Function
4F0591-#7	AMELX, AQP4, ARNT2, BAT3, beta-estradiol, BIRC5, CATSPERB, CDCA8, CEBPA, CGREF1, DLGAP1, GLIPR1, GPR37, GRIN1, GTSE1, HSPA2, HSPA5, INS1, LITAF, NCAPG (includes EG:64151), NFkB, NPAS1, PLGLB2, RAB31, RAGE, retinoic acid, RPS14, RPS4X, RRM2, SCG2, STXBP4, TF, TGFBI, TP53, TRHDE	38	16	Cell Death, Cancer, Respiratory Disease
4F1127-#7	Actin, ADIPOQ, Akt, Ap1, BCL9, BIRC5, CCL2, CPE, EGR2, ERCC6L, HIST1H4C, Histone h3, HOMER2, IL1, IL8, Jnk, KRT18, LDL, NFkB, OSBP, P38 MAPK, PBK, PDGF BB, PDGFC, PLK1, POSTN, PRDX4, SERPINA3, SFRP4, SLC2A3, Tgf beta, TIFA, TNFRSF11B, TNFRSF1B, TPT1	35	17	Cellular Growth and Proliferation, Cellular Development, Hematological System Development and Function
5F0138-#7	Akt, Ap1, ASNS, CALM2, DAD1, DDIT4, FSTL1, G0S2, GARS, GDF15, HTRA1, JAK3, LDHA, LDL, LOX, MMP1 (includes EG:4312), NFkB, P38 MAPK, PCK2, PCOLCE, PDGF BB, PDGFC, PDPN, RND3, RPN2, SFRP1, SLC7A1, TCR, TGFB1, TIMP4, TNFRSF8, TNFSF9, TRIB3, UGDH, WNT2	30	15	Cancer, Cellular Movement, Cellular Development

The top networks in each analysis data analyzed by IPA are listed.

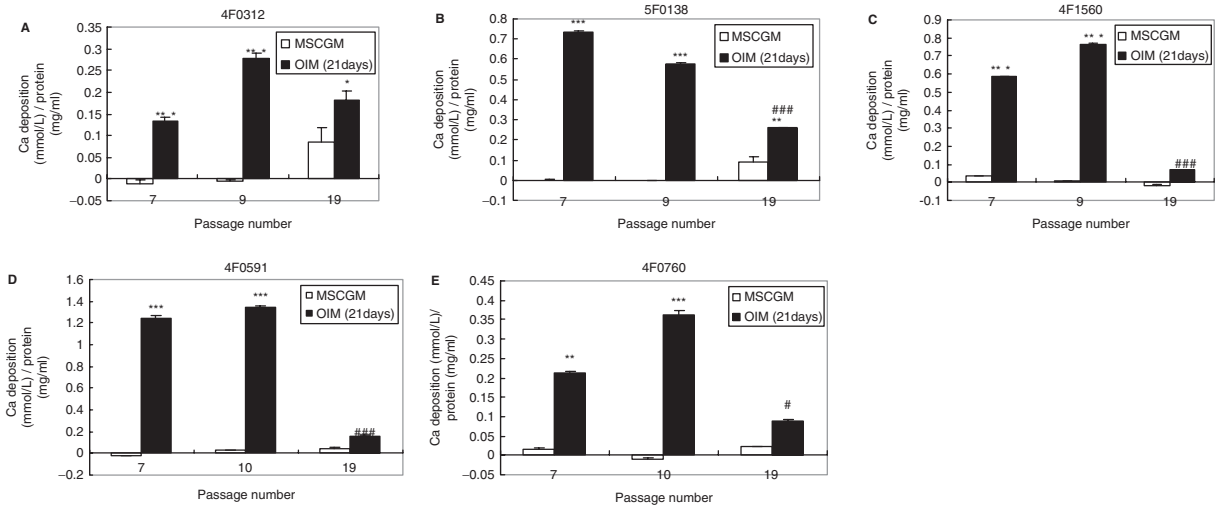

Fig. 2. **Calcium deposition of hMSCs.** The cells in each passage numbers indicated were plated on 12-well plates and cultured in MSCGM (control; clear column) or OIM (osteogenic differentiated; filled column) for 21 days. The amounts of calcium deposition in 4F0312, 5F0138, 4F1560, 4F0591 and 4F0760 are indicated in (A, B, C, D and E), respectively. Calcium deposition divided by protein concentration is showed as mean + SEM in triplicate. $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$ when osteogenesis in MSCGM was compared to that in OIM in each passage number. ### $P<0.001$, ## $P<0.01$, # $P<0.05$ when osteogenesis in passage #19 was compared to that in passage number 7 ($n=3$).

hMSCs through early and late stages of cell culture. Replication was performed by testing six different batches of cells. All six batches examined showed a marked decrease in culture growth rate with increasing passages.

The hMSC potential for osteogenic differentiation was down-regulated in all the batches of hMSCs examined during the late culture stage. The osteogenic differentiation was observed in all the batches of hMSCs examined for passages 7, 9 and 10. Also, every batch examined showed a down-regulation of the osteogeneic process during the 19th passage. As previously stated, four genes, *NDN*, *EPHA5*, *NOV* and *RUNX2* showed altered expression depending on the culture stage. *EPHA5* and *NOV* were up-regulated as the cells were further passaged, while *NDN* and *RUNX2* were down-regulated.

RT–PCR data indicated that the expression of *NDN* in all batches examined decreased during the late stages of culture. The expression of *NDN* in lot #4F1127, #4F0312 and #5F0138 was relatively stable until the 14th passage, which was then followed by a decrease in expression during the late stages. Microarray data also showed that the expression of *NDN* in passages 22–28 were decreased compared to that in passages 4–8. Furthermore, our results showed a positive correlation between the expression of *NDN* in hMSCs and the potential to differentiate into osteogenic cells as measured by the calcium deposition rates. Previous reports suggested that necdin, an *NDN* homolog, interacts with IL-1α precursor (*24*). The expression of *NDN* in hMSCs decreases with increasing passages. It is possible that *NDN* down-regulation is involved in activation of IL1-Myd88 pathway by dying cells (*25*).

Every batch showed a passage-dependent increase in the expression level of *EPHA5*. EPHA5 is transmembrane receptor protein tyrosine kinase, known as Ephrin A5 receptor, and belongs to the ephrin receptor subfamily. Recently, it has been shown that EPHA5 is involved in cellular growth and tumor malignancies (*26*, *27*). Also, it is known that the expression level of human *EPHA5* mRNA is high in primary human breast carcinoma cells (*28*).

NOV/CCN3 is a growth factor that plays several roles in cellular migration, growth, proliferation and chemotaxis. The previous finding that *NOV* inhibits the proliferation of a cancer cell line is consistent with the observation that *NOV* expression level is increased in the senescing phase, which coincides with the low proliferative stage of hMSCs. Furthermore, in primary skin fibroblasts, NOV/CCN3 protein increases the expression of human *SERPINE1* mRNA level (*29*). This is consistent with our observation that the expressions of *SERPINE1* as well as *NOV* are up-regulated during the late stages of cell culture. Mutant human SERPINE1 (T333R; A355R), which lacks the protease-inhibitory activity, decreases the quantity of rat laminin and inhibits matrix accumulation (*30*). On the other hand, previous finding indicated that the expression of mouse *Myod1* (myogenic differentiation 1) mRNA level and Myog (myogenin) protein decreased in C2/4 cells (subclone of C2C12 mesenchymal cells) stably expressing NOV, which suggests that NOV suppresses the myogenic differentiation of C2/4 cells (*31*).

The expression of *RUNX2* was also decreased in late stage of the culture. *RUNX2* is a member of the runt family of transcription factors and suggested to be involved in osteogenesis (*22*). It is possible that down-regulated osteogenic differentiation of hMSCs is caused by the decreased expression of *RUNX2*. Recent reports have shown that 3D cultures of human adipose tissue-derived endothelial and osteoblastic progenitors generate osteogenic–vasculogenic constructs (*32*). It might be

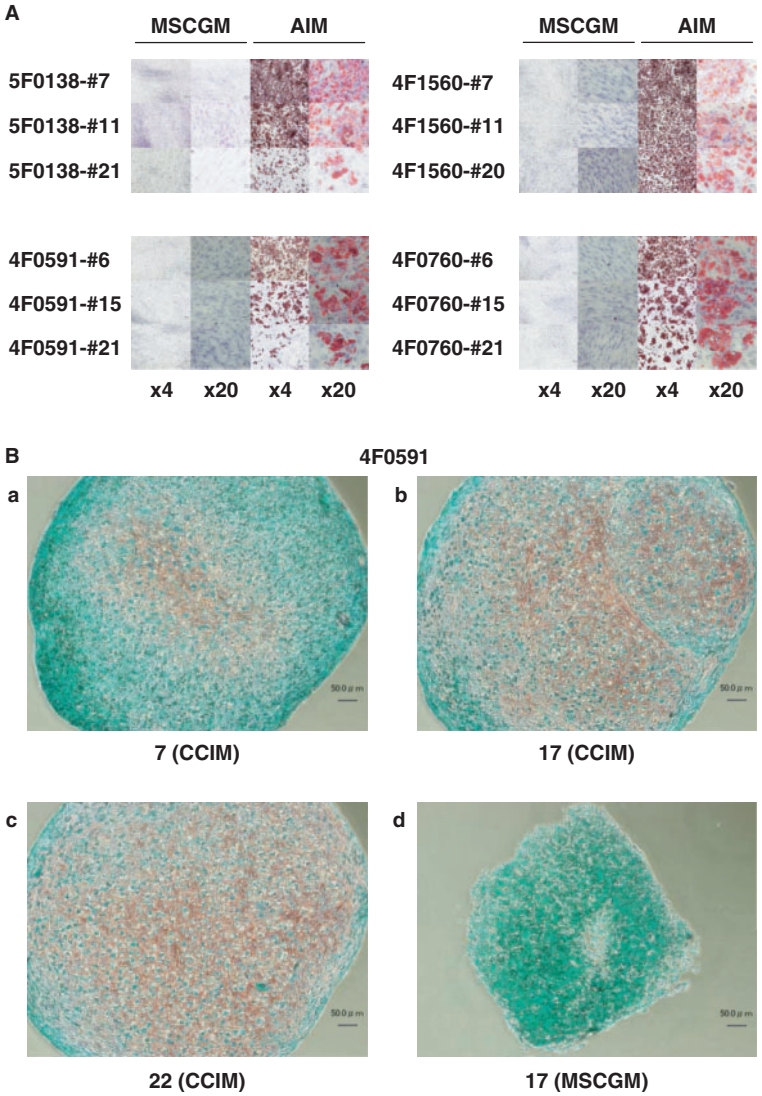

Fig. 3. **Adipogenic differentiation and chondrogenic differentiation of hMSCs.** (A) The cells in each passage numbers indicated were plated on 24-well plates and cultured in MSCGM (as control) or AIM (adipogenic differentiation medium) for 21 days. The cells were stained with Oil Red O. (B) The cells in passage. #7, #17 or #22 of 4F0591 were cultured in CCIM for 24 days and stained with Safranin O (a, b, c, respectively). Proteoglicans stained red. The cells in passage #17 were also cultured in MSCGM (as control) for 24 days (d).

interesting to investigate the gene expression profile of the 3D culture of hMSCs.

In conclusion, microarray and RT–PCR data of the six batches of hMSCs suggested that four genes, *EPHA5*, *NOV*, *NDN* and *RUNX2* have the potential to act as stage-specific markers during hMSC culture. These genes can be used as candidates for quality control markers of the culture status with regard to the differentiation potential for future clinical application of hMSCs for cellular therapeutics. We reported that the capacity of hMSCs for osteogenic differentiation was highly suppressed during the late culture stages. *NDN* or *RUNX2* may be a quality control marker of hMSC capacity for osteogenic differentiation. The observations of adipogenic differentiation of hMSCs suggested that each batch shows different transition in differentiation potential. It seemed that the capacity tends to be suppressed in late stage of the culture. The observations of chondrogenic differentiation suggested that the differentiation potential of hMSCs is retained in late stage of the culture. It seems that the cells in the late stage have limited differentiation potential (oligopotent). Furthermore, network analysis and gene expression analysis revealed that the expression profiles are distinct for each passage number. These findings imply the importance of quality control for safe application of hMSCs for cellular therapy and usefulness of expression analysis for finding marker genes. Phenotype profiling and profiling at the genome level, including chromosomal analysis, might need more research in the future. The profiling of the cells, in both differentiated and 3D states, will also need to be investigated for future clinical applications.

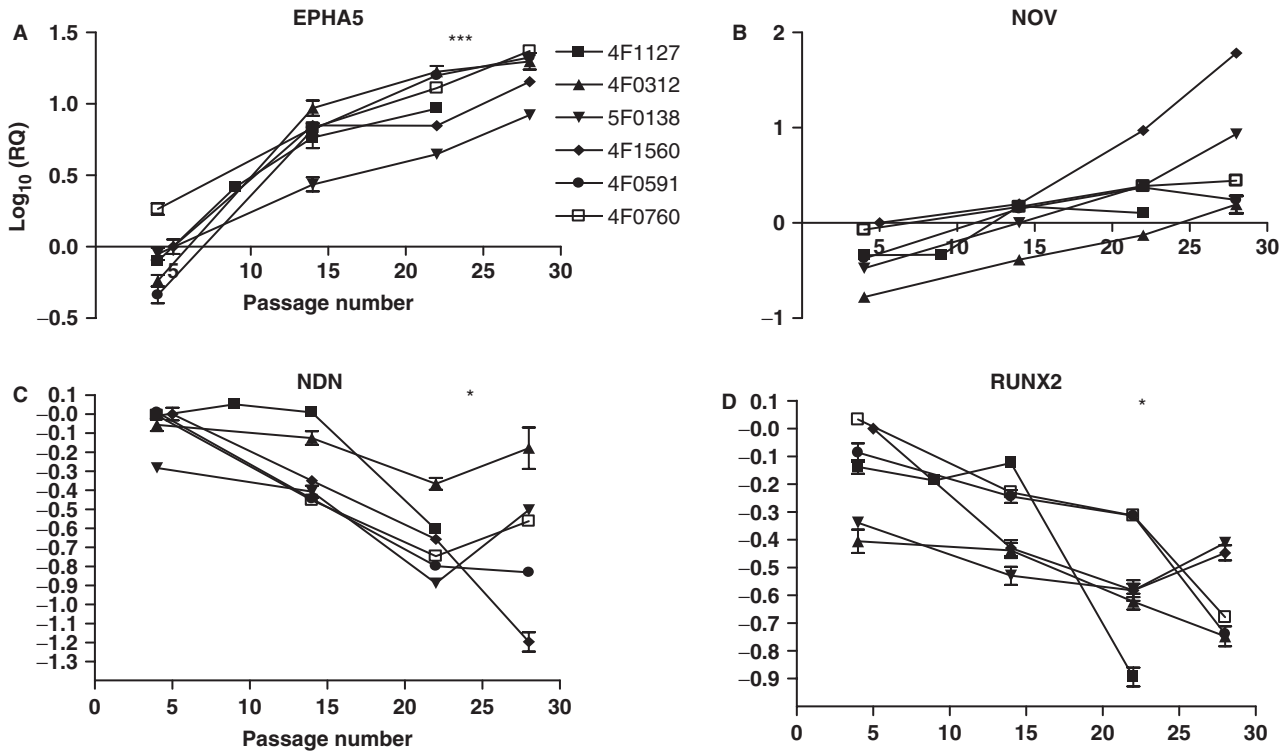

Fig. 4. **Gene expression profiles of culture stage markers suggested for hMSCs.** Individual plots for six batches of hMSCs obtained from RT–PCR data. The expression of *EPHA5* (A), and *NOV* (B) increased, while that of *NDN* (C) and *RUNX2* (D) decreased as the cells were further passaged in each batch. Relative quantity value was plotted on a log 10 scale. The expression of four genes (A–D) was correlated with passage numbers ($P < 0.01$). $^{***}P < 0.001$, $^{*}P < 0.05$ when the expression in passage #14 was compared to that in late stage (passage #22 and #28) ($n = 6$ in passage #14, $n = 10$ in late stage).

Supplementary data are available at *JB* online.

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REFERENCES

1. Clevers, H. (2005) Stem cells, asymmetric division and cancer. *Nat. Genet.* **37**, 1027–1028
2. Takahashi, K., Tanabe, T., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861–872
3. Fazel, S., Cimini, M., Chen, L., Li, S., Angoulvant, D., Fedak, P., Verma, S., Weisel, R.D., Keating, A., and Li, R.K. (2006) Cardioprotective c-kit+ cells are from the bone marrow and regulate the myocardial balance of angiogenic cytokines. *J. Clin. Invest.* **116**, 1885–1877
4. Rosenzweig, A. (2006) Cardiac cell therapy-mixed results from mixed cells. *N. Engl. J. Med.* **355**, 1274–1277
5. Wollert, K.C., Meyer, G.P., Lotz, J., Lichtenberg, S.R., Lippolt, P., Breidenbach, C., Fichtner, S., Korte, T., Hornig, B., Messinger, D., Arseniev, L., Hertenstein, B., Ganser, A., and Drexler, H. (2004) Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. *Lancet* **364**, 141–148
6. Schächinger, V., Erbs, S., Elsässer, A., Haberbosch, W., Hambrecht, R., Hölschermann, H., Yu, J., Corti, R., Mathey, D.G., Hamm, C.W., Suselbeck, T., Assmus, B., Tonn, T., Dimmeler, S., and Zeiher, A.M. (2006) Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. *N. Engl. J. Med.* **355**, 1210–1221
7. Assmus, B., Honold, J., Schächinger, V., Britten, M.B., Fischer-Rasokat, U., Lehmann, R., Teupe, C., Pistorius, K., Martin, H., Abolmaali, N.D., Tonn, T., Dimmeler, S., and Zeiher, A.M. (2006) Transcoronary transplantation of progenitor cells after myocardial infarction. *N. Engl. J. Med.* **355**, 1222–1232
8. Lunde, K., Solheim, S., Aakhus, S., Arnesen, H., Abdelnoor, M., Egeland, T., Endresen, K., Ilebekk, A., Mangschau, A., Fjeld, J.G., Smith, H.J., Taraldsrud, E., Grøgaard, H.K., Bjørnerheim, R., Brekke, M., Müller, C., Hopp, E., Ragnarsson, A., Brinchmann, J.E., and Forfang, K. (2006) Intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction. *N. Engl. J. Med.* **355**, 1199–1209
9. Janssens, S., Dubois, C., Bogaert, J., Theunissen, K., Deroose, C., Desmet, W., Kalantzi, M., Herbots, L., Sinnaeve, P., Dens, J., Maertens, J., Rademakers, F., Dymarkowske, S., Gheysens, O., Cleemput, J.V., Bormans, G., Nuyts, J., Belmans, A., Mortelmans, L., Boogaerts, M., and Van de Werf, F. (2006) Autologous bone marrow-derived stem-cell transfer in patients with ST-segment elevation myocardial infarction: double-blind, randomised controlled trial. *Lancet* **367**, 113–121

10. Peggs, K.S., Verfuerth, S., Pizzey, A., Khan, N., Guiver, M., Moss, P.A., and Mackinnon, S. Adoptive cellular therapy for early cytomegalovirus infection after allogeneic stem-cell transplantation with virus-specific T-cell lines. *Lancet* **362**, 1375–1377
11. Dazzi, F., van Laar, J.M., Cope, A., and Tyndall, A. (2007) Cell therapy for autoimmune diseases. *Arthritis Res. Ther.* **9**, 206–214
12. Ringdén, Ó., Uzunel, M., Rasmusson, I., Remberger, M., Sundberg, B., Lönnies, H., Marschall, H.-U., Dlugosz, A., Szakos, A., Hassan, Z., Omazic, B., Aschan, J., Barkholt, L., and Le Blanc, K. (2006) Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. *Transplantation* **81**, 1390–1397
13. Derfoul, A., Perkins, G.L., Hall, D.J., and Tuan, R.S. (2006) Glucocorticoids promote chondrogenic differentiation of adult human mesenchymal stem cells by enhancing expression of cartilage extracellular matrix genes. *Stem Cells* **24**, 1487–1495
14. Nuttelman, C.R., Tripodi, M.C., and Anseth, K.S. (2004) *In vitro* osteogenic differentiation of human mesenchymal stem cells photoencapsulated in PEG hydrogels. *J. Biomed. Mater. Res. A* **68**, 773–782
15. Kulterer, B., Friedl, G., Jandrositz, A., Sanchez-Cabo, F., Prokesch, A., Paar, C., Scheideler, M., Windhager, R., Preisegger, K.-H., and Trajanoski, Z. (2007) Gene expression profiling of human mesenchymal stem cells derived from bone marrow during expansion and osteoblast differentiation. *BMC Genomics* **8**, 70–84
16. Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143–147
17. Barrett, T., Troup, D.B., Wilhite, S.E., Ledoux, P., Rudnev, D., Evangelista, C., Kim, I.F., Soboleva, A., Tomashevsky, M., and Edgar, R. (2007) NCBI GEO: mining tens of millions of expression profiles-database and tools update. *Nucleic Acids Res.* **35**, D760–D765
18. Edgar, R., Domrachev, M., and Lash, A.E. (2002) Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res.* **30**, 207–210
19. Toda, K., Ishida, S., Nakata, K., Matsuda, R., Shigemoto-Mogami, Y., Fujishita, K., Ozawa, S., Sawada, J., Inoue, K., Shudo, K., and Hayashi, Y. (2003) Test of significant differences with a priori probability in microarray experiments. *Anal. Sci.* **19**, 1529–1535
20. Benini, S., Perbal, B., Zambelli, D., Colombo, M.P., Manara, M.C., Serra, M., Parenza, M., Martinez, V., Picci, P., and Scotlandi, K. (2005) In Ewing's sarcoma CCN3(NOV) inhibits proliferation while promoting migration and invasion of the same cell type. *Oncogene* **24**, 4349–4361
21. Heymans, S., Lupu, F., Terclavers, S., Vanwetswinkel, B., Herbert, J.-M., Baker, A., Collen, D., Carmeliet, P., and Moons, L. (2005) Loss or inhibition of uPA or MMP-9 attenuates LV remodeling and dysfunction after acute pressure overload in mice. *Am. J. Pathol.* **166**, 15–25
22. Mundlos, S., Otto, F., Mundlos, C., Mulliken, J.B., Aylsworth, A.S., Albright, S., Lindhout, D., Cole, W.G., Henn, W., Knoll, J.H.M., Owen, M.J., Mertelsmann, R., Zabel, B.U., and Olsen, B.R. (1997) Mutaions involving the transcription factor CBFA1 cause cleidocranial dysplasia. *Cell* **89**, 773–779
23. Yamamura, Y., Lee, W.L., Inoue, K., Ida, H., and Ito, Y. (2006) RUNX3 cooperates with FoxO3a to induce apoptosis in gastric cancer cells. *J. Biol. Chem.* **281**, 5267–5276
24. Hu, B., Wang, S., Zhang, Y., Feghali, C.A., Dingman, J.R., and Wright, T.M. (2003) A nuclear target for interleukin-1α: Interaction with the growth suppressor necdin modulates proliferation and collagen expression. *Proc. Natl. Acad. Sci. USA* **100**, 10008–10013
25. Chen, C.-J., Kono, H., Golenbock, D., Reed, G., Akira, S., and Rock, K.L. (2007) Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. *Nat. Med.* **13**, 851–856
26. Wan, D., Gong, Y., Qin, W., Zhang, P., Li, J., Wei, L., Zhou, X., Li, H., Qiu, X., Zhong, F., He, L., Yu, J., Yao, G., Jiang, H., Qian, L., Yu, Y., Shu, H., Chen, X., Xu, H., Guo, M., Pan, Z., Chen, Y., Ge, C., Yang, S., and Gu, J. (2004) Large-scale cDNA transfection screening for genes related to cancer development and progression. *Proc. Natl. Acad. Sci. USA* **101**, 15724–15729
27. Herath, N.I., Spanevello, M.D., Sabesan, S., Newton, T., Cummings, M., Duffy, S., Lincoln, D., Boyle, G., Parsons, P.G., and Boyd, A.W. (2006) Over-expression of Eph and ephrin genes in advanced ovarian cancer: ephrin gene expression correlates with shortened survival. *BMC Cancer* **6**, 144–150
28. Woelfle, U., Cloos, J., Sauter, G., Riethdorf, L., Jänicke, F., van Diest, P., Brakenhoff, R., and Pantel, K. (2003) Molecular signature associated with bone marrow micrometastasis in human breast cancer. *Cancer Res.* **63**, 5679–5684
29. Lin, C.G., Chen, C.-C., Leu, S.-J., Grzeszkiewicz, T.M., and Lau, L.F. (2005) Integrin-dependent functions of the angiogenic inducer NOV (CCN3). *J. Biol. Chem.* **280**, 8229–8237
30. Huang, Y., Haraguchi, M., Lawrence, D.A., Border, W.A., Yu, L., and Noble, N.A. (2003) A mutant, noninhibitory plasminogen activator inhibitor type 1 decreases matrix accumulation in experimental glomerulonephritis. *J. Clin. Invest.* **112**, 379–388
31. Sakamoto, K., Yamaguchi, S., Ando, R., Miyawaki, A., Kabasawa, Y., Takagi, M., Li, C.L., Perbal, B., and Katsube, K. (2002) The nephroblastoma overexpressed gene (NOV/ccn3) protein associates with Notch1 extracellular domain and inhibits myoblast differentiation via Notch signalling pathway. *J. Biol. Chem.* **277**, 29399–29405
32. Scherberich, A., Galli, R., Jaquiery, C., Farhadi, J., and Martin, I. (2007) 3D perfusion culture of human adipose tissue-derived endothelial and osteoblastic progenitors generates osteogenic constructs with intrinsic vascularization capacity. *Stem Cells* **25**, 1823–1829