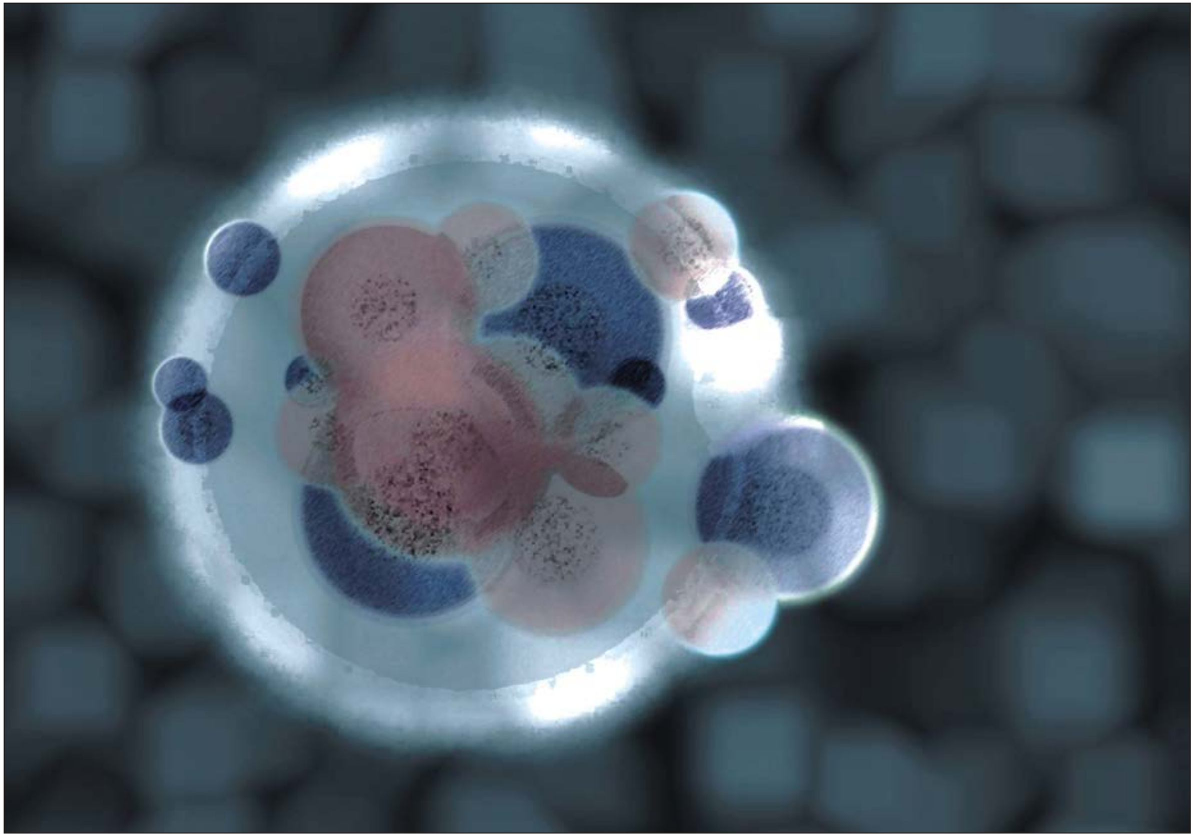

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Gene Expression Changes During the Chondrogenic Differentiation of Human Mesenchymal Stem Cells

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Abstract: To gain more insight into the molecular mechanisms of chondrogenic differentiation induced by transforming growth factor (TGF)-β1 and insulin-like growth factor (IGF)-I and insulin, we performed cDNA microarray experiments during the chondrogenic differentiation of human mesenchymal stem cells (hMSCs), which provide an excellent *in vitro* model system for chondrogenesis. Our repeated cDNA microarray analyses identified the up regulation of 23 transcripts and the down regulation of 25 transcripts after 14 days of chondrogenic induction. We found that many of the up regulated and down regulated genes belonged to overlapping gene categories; specifically, 44 and 40% of the up regulated and down regulated genes were associated with the extracellular matrix and metabolic pathways, respectively. The expression of the identified genes was confirmed by RT-PCR. These analyses suggest that the transcriptional control induced by TGF-β1, IGF-I and insulin signaling during the chondrogenic differentiation of hMSCs is mainly targeted to genes belonging to specialized gene categories.

Key words: TGF-β1, IGF-I, insulin, extracellular matrix, metabolic pathway

INTRODUCTION

Cartilage is essential for skeletal growth and the functional integrity of the skeleton. Cartilage formation is driven by the commitment of undifferentiated mesenchymal precursor cells to the chondrogenic lineage, which triggers chondroprogenitors to form cellular condensations and to differentiate into chondroblasts (Shum and Nuckolls, 2002). Mesenchymal chondroprogenitor cells first proliferate and then differentiate by increasing the cell volume and producing a large amount of cartilage-characteristic extracellular matrix proteins, like type II collagen and aggrecan, that link protein and cartilage oligomeric proteins (Tuckermann *et al*., 2000). The cells then become hypertrophic and start to synthesize type X collagen. The majority of the differentiated chondrocytes appear to undergo apoptosis and are replaced mainly by bone marrow and blood vessels accompanying osteoblasts and osteoclasts that ultimately produce bony tissues. Cartilage cannot repair itself and cartilage degeneration, such as that which occurs in osteoarthritis, leads to serious disability.

Previous results have indicated a role for β-catenin and Wnt signaling in chondrogenesis (Day *et al*., 2005). Moreover, the proliferation and differentiation of chondrocytes is cooperatively regulated by key secreted proteins that include bone morphogenetic proteins (BMPs) 2, 4 and 7-members of the TGF-β superfamily-as well as Fibroblast Growth Factors (FGFs) and Insulin-like Growth Factors (IGFs) (Kronenberg, 2003). Recent comprehensive gene expression analyses have successfully identified the downstream targets of these ligands (Dailey *et al*., 2003). In addition, the sequential differentiation and maturation of chondrocytes from Mesenchymal Stem Cells (MSCs) to hypertrophic chondrocytes in endochondral bone formation is regulated by several transcription factors including

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Sry-type high mobility group box (SOX) 5, 6 and 9 and core binding factor α1 (CBFA1), also known as runt-related gene 2 (RUNX2) family members (Komori *et al*., 1997; Otto *et al*., 1997; Akiyama *et al*., 2002). SOX9 plays an essential role in establishing the condensation of prechondrogenic mesenchymal cells and initiating chondrocyte differentiation (Akiyama *et al*., 2002). The expression of the α1 chain of type II collagen (COL2A1), a major component of the cartilage extracellular matrix, is regulated by SOX9 (Bell *et al*., 1997).

Molecular signaling during chondrogenesis has been studied using expression profiling and microarray technology. Such studies were performed using the mouse embryonal carcinoma cell line ATDC5, immortalized human chondrocyte cell lines like C-28/I2, chondrosarcoma-derived cell lines and murine micromass cultures. BMPs or FGFs were used to enhance cartilage formation in MSCs (Sekiya *et al*., 2005; Solchaga *et al*., 2005). IGFs have been shown to play a central role in chondrogenesis, as indicated by the severe growth failure observed in animals carrying null mutations of Igfs and Igf-I receptor genes. Indeed, IGF-I showed potent chondrogenic effects in MSCs, similar to the effects of TGF-β1 (Longobardi *et al*., 2006). Insulin alone stimulates ATDC5 cells to undergo chondrogenic differentiation (Shukunami *et al*., 1996). Understanding the molecular events through which TGF-β1, IGF-I and insulin cooperatively determine the chondrogenic potential of hMSCs is important for the therapeutic use of these factors in the treatment of cartilage disorders.

To improve our understanding of the global molecular basis of cellular signaling in chondrogenic differentiation, we searched for genes that were up regulated or down regulated after TGF-β1-dependent, IGF-I-dependent and insulin-dependent chondrogenic differentiation in hMSCs, which were initially isolated from bone marrow and characterized by the expression of various cell surface markers. MSCs are progenitor cells that have the potential to differentiate into several mesenchymal tissue lineages, including cartilage, bone, muscle and fat and thus represent a potential source of pluripotent cells for autologous bone tissue engineering. The results from our transcriptome analysis suggest that at the onset of chondrogenesis, a number of genes involved in the extracellular matrix and metabolic pathways are induced by the combination of TGF-β1, IGF-I and insulin in hMSCs.

MATERIALS AND METHODS

Cell cultures: hMSCs were purchased from Cambrex (New Jersey). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) and antibiotic/antimycotic solution (100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 250 ng mL^{-1} amphotericin B). The chondrogenic differentiation of hMSCs was induced by adding Chondrocyte Differentiation Medium (CDM) SingleQuots Kit CC-4408 (Cambrex, New Jersey) -including TGF-β1, IGF-I analog, long R3 IGF-I (R3-IGF-I), insulin, transferrin and gentamicin/amphotericin B-to DMEM containing 5% FBS, 100 μM ascorbic acid-2-phosphate, 1 mM sodium pyruvate and 350 μM proline.

Cells were stained to reveal chondrogenic differentiation using Alcian blue (Wako Pure Chemical, Osaka), which binds sulfated proteoglycans. Briefly, the cells were washed twice with phosphate-buffered saline and fixed for 2 h in a 10% formalin solution. Alcian blue (pH 2.5) was added to the cells and the samples were incubated at room temperature for 2 h. Excess stain was removed and the cells were rinsed twice with 70% ethanol to remove residues before visualization.

RT-PCR method: Total cellular RNA was extracted using TRIzol reagent according to the manufacturer's instructions (Invitrogen). RT-PCR was performed using the SuperScript one-step RT-PCR system and gene-specific primers according to the conditions recommended by the manufacturer (Invitrogen). Reaction mixtures containing total RNA (500 ng of each), 0.2 mM dNTPs, 0.8 μM each primer, 2 units of enzyme mixture including SuperScript II RT, platinum Taq DNA polymerase and 1x buffer with 1.2 mM $MgSO_4$ were kept at 50°C for 30 min and then at 94°C for 2 min; PCR was then performed. The PCR profile was 22-27 cycles at 94°C for 15 sec, 55°C for 30 sec and 70°C for 1 min. The RT-PCR primers were designed based on human sequences in GenBank. These sequences are listed in Table 1.

cDNA microarray: Total RNA was verified using an RNA 6000 Nano assay and an Agilent Bioanalyzer (Agilent Technologies). Ten micrograms of total RNA was reverse transcribed into cDNA using cyanine 3-dCTP (Cy3) or cyanine 5-dCTP (Cy5) (PerkinElmer). Labeled probes were hybridized using an Agilent human 1 cDNA microarray kit (Agilent Technologies), which included 12,814 unique clones from Incyte UniGene 1 and human drug target clone sets. The microarray was scanned on both Cy3 and Cy5 channels using an Agilent DNA microarray scanner (Agilent Technologies). The raw data from the microarray assay were normalized according to the manufacturer's guidelines using Feature Extraction software from Agilent Technologies. A ratio of normalized intensity >2.5 or <0.4 was used to generate a list of genes with significant changes in expression during chondrogenic differentiation. The expression profile data were evaluated using duplicate experiments.

Table 1: RT-PCR primer sequences. F and R indicate forward and reverse primer sequences, respectively. The number of PCR cycles is shown

Target	Primer sequences	PCR cycles
CDR1	F: 5'-TGGAAGACCTGGATTTTTCG-3', R: 5'-ACATCTTCCAATGGCCTCAG-3'	22
FAD2	F: 5'-ACCTGCCCTACAATCACCAG-3', R: 5'-TGTGACCCACACAAACCAGT-3'	27
SC4MOL	F: 5'-ACATGGGAAAACCAATGGAA-3', R: 5'-TTCCAAATGGAGCCTGAAAC-3'	25
COL11A1	F: 5'-TGGAATGAGGGGAGAAGATG-3', R: 5'-TCCTGGTTTTCCTTGTGGTC-3'	25
SOX9	F: 5'-GGAGGCAGAGGAGGCCACGGAG-3', R: 5'-ACTCGTTGACATCGAAGGTCTC-3'	27
COL2A1	F: 5'-GAGCTGCTGGCCGCGTTGGACC-3', R: 5'-CCTGCTTGCCGGGCTCACCCGA-3'	27
GAPDH	F: 5'-AGAACATCATCCCTGCCTCTACTGG-3', R: 5'-AAAGGTGGAGGAGTGGGTGTCGCTG-3'	22

RESULTS AND DISCUSSION

Chondrogenic differentiation of hMSCs: When TGF-β1, IGF-I and insulin were added to the culture medium, the cells rapidly lost their fibroblastic morphology. As shown in Fig. 1A, hMSCs showed a dramatic alteration in cell morphology after 7 days of treatment in chondrogenic differentiation medium. This alteration was accompanied by the rapid biosynthesis of glycosaminoglycan and shown by Alcian blue staining (Fig. 1B). Chondrogenic differentiation occurs when MSCs are grown under conditions that include a three-dimensional culture, a serum-free medium and the addition of a member of the TGF-β superfamily (Heng *et al*., 2004). To induce chondrogenic differentiation in hMSCs, different factors such as FGFs and BMPs have been used (Sekiya *et al*., 2005; Solchaga *et al*., 2005). In particular, addition of IGF-I and BMP2 to hMSC has been shown to induce markers of osteogenesis (Koch *et al*., 2005). IGF-I chondroinductive actions were equally potent to those induced by TGF-β1 and the two growth factors had additive effects (Longobardi *et al*., 2006). Besides TGF-β1 and IGF-I, insulin was added to chondrogenic differentiation medium. By microarray analysis, IGF-I and insulin were shown to induce different patterns of gene expression in mouse fibroblast NIH-3T3 cells. Interestingly, more than half of the genes up regulated by IGF-I are associated with mitogenesis and differentiation, whereas none of the genes specifically up regulated by insulin are associated with these processes (Dupont *et al*., 2001). On the other hand, the set of genes revealed to be differentially regulated in another system did not overlap at all (Mulligan *et al*., 2002). It is still not understood how the specificity of IGF-I versus insulin signaling is controlled in chondrogenesis. The divergent effects of IGF-I and insulin may be explained by specificity in the intracellular signals generated by IGF-I and insulin. We chose to study the additive action of IGF-I and insulin on an accepted model of chondrocyte differentiation. Transferrin, which is known as a major angiogenic molecule produced by hypertrophic chondrocytes during endochondral bone formation, was also included in the chondrocyte differentiation medium.

In the presence of TGF-β signaling during chondrogenic differentiation, MSCs synthesize aggrecan, link protein, fibromodulin, cartilage oligomeric matrix protein, decorin, type II collagen and chondroadherin, all components of the normal articular cartilage matrix (Barry *et al*., 2001). We checked the expression of well-known cartilage-specific genes. An expression analysis for the known chondrogenic markers SOX9 and COL2A1 using semi-quantitative RT-PCR in differentiated hMSCs was completed prior to the microarray analysis to validate the RNA quality and to verify the chondrogenic differentiation in these trials. In this manner, we showed that SOX9 and COL2A1 were induced during the course of hMSC differentiation into a chondrogenic lineage (Fig. 1C). The gene expression of SOX9 increased until it plateaud on day 14 of the culture period.

Microarray analyses: To determine the molecular events during the chondrogenic differentiation of hMSCs, especially those induced by TGF-β1, IGF-I and insulin, we employed cDNA microarray technology. We identified 23 genes that were up regulated and 25 genes that were down regulated after 14 days of treatment in chondrogenic differentiation medium. The experiments were performed at least twice and the mean and Standard Deviation (SD) values were determined for each gene. Under our assay conditions, a more than 2.5-fold or less than 0.4-fold difference was considered to indicate a candidate novel marker of chondrogenic differentiation (Table 2 and 3).

We classified the 48 genes according to their functions as follows: extracellular matrix, metabolism, protein modification, transcription, cell growth, cytoskeleton, ion transport and unknown (Table 2 and 3). The frequencies of these functionally classified genes are shown in Fig. 2A and B. Remarkably, the categories to which the up regulated and down regulated genes belonged overlapped. We found that genes involved in the extracellular matrix and metabolic pathways significantly accounted for 44 and 40%, respectively, of the up regulated and down regulated genes.

Cartilage matrix proteins have important roles in the maintenance and organization of chondrocyte phenotypes. The presence of cartilage-specific matrix

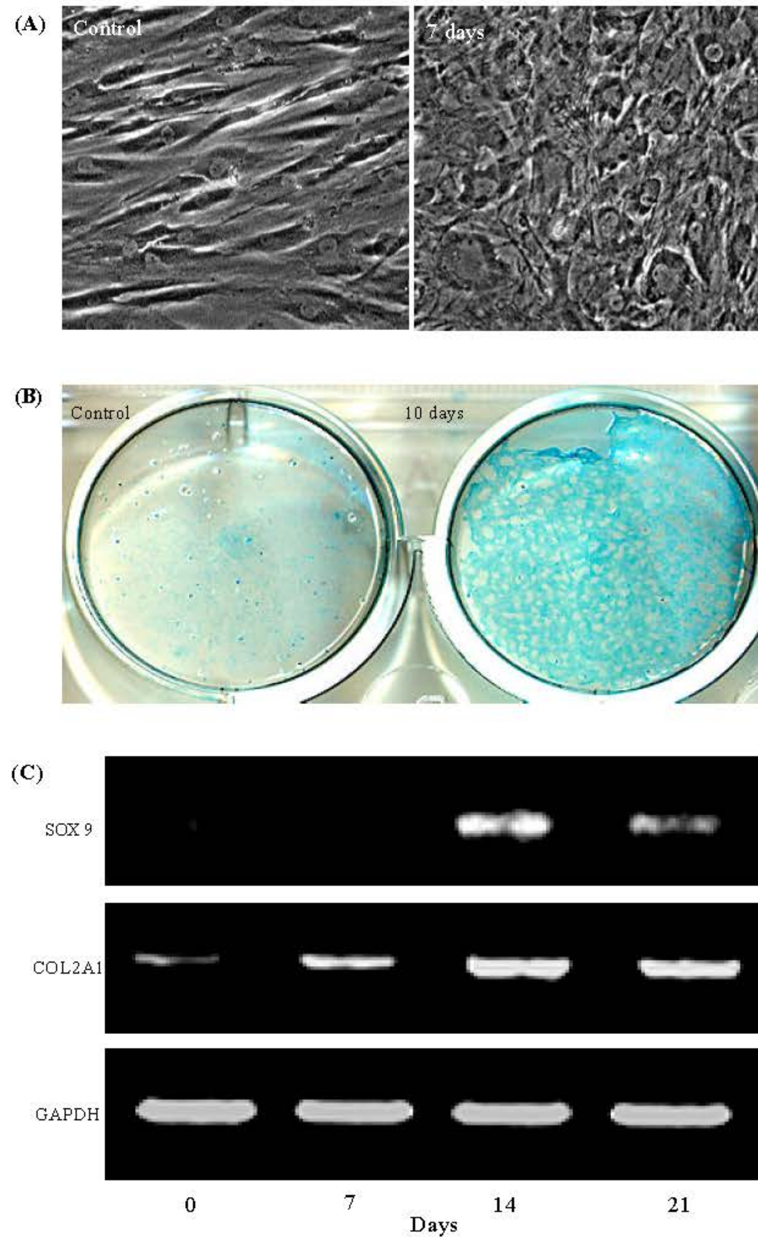

Fig. 1: Effect of chondrogenic differentiation medium on hMSCs. (A) Morphology of hMSCs cultured in chondrogenic differentiation medium or control medium for 7 days. (B) Chondrogenesis and chondrocyte differentiation after 10 days of culture. Cells were stained with Alcian blue to show the synthesis of sulfated glycosaminoglycans. (C) Effect of chondrogenic differentiation medium on the expression of chondrocyte-specific marker genes in hMSCs. RT-PCR analysis of SOX9, COL2A1 and GAPDH genes was performed at the indicated time points

proteins in the cell signify the developmental stages of chondrogenesis. Most notably, COL10A1, COL11A1, COL1A1 and COL3A1 were up regulated, whereas, COL6A3 and COL1A2 were down regulated (Table 2 and 3). COL10A1 is known as a direct transcriptional target of RUNX2 during chondrogenesis (Zheng *et al*., 2003). Type XI collagen is a trimer consisting of 3 different polypeptides (α1 [A1], α2 [A2] and α3 [A3]) and COL11A1 is essential for the normal formation of cartilage collagen fibrils (Li *et al*., 1995). Many of these proteins are

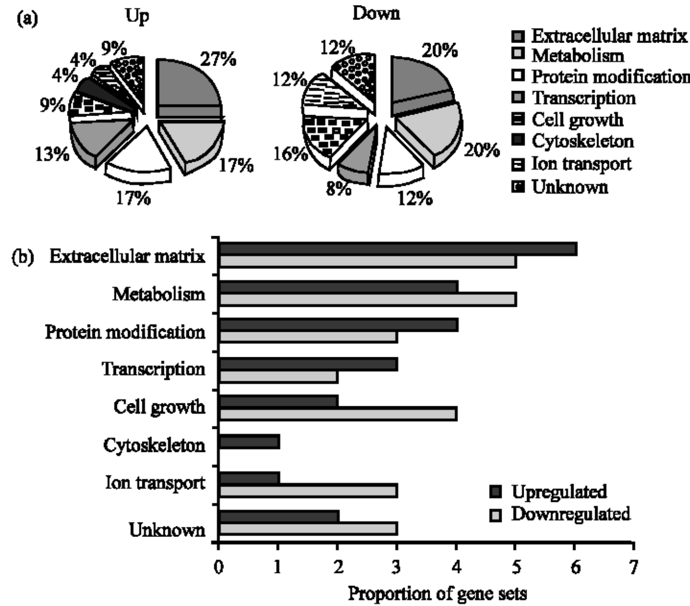

Fig. 2: Distribution of differentially expressed gene sets and corresponding molecular classifications. (A) Genes exhibiting more than a 2.5-fold change in gene expression were categorized according to molecular function; the results are shown according to the population (%). (B) The proportion of genes that are either up regulated or down regulated by more than 2.5-fold after 14 days of treatment in chondrogenic differentiation medium

Table 2: cDNA microarray results showing up regulation during chondrocyte differentiation

Gene name	Gene description	RefSeq Accession No.	Gene function	Mean (N = 2, >2.5)	SD
CDR1	Cerebellar degeneration-related protein 1, 34 kDa	NM_004065	Unknown	7.00	1.66
COL10A1	Collagen, type X, alpha 1	NM_000493	Extracellular matrix	6.08	0.66
CREB3L1	cAMP responsive element binding protein 3-like 1	NM_052854	Transcription	3.51	0.21
NR1H2	Nuclear receptor subfamily 1, group H, member 2	NM_007121	Transcription	3.42	0.78
FADS2	Fatty acid desaturase 2	NM_004265	Fatty acid biosynthesis	3.33	0.25
SC4MOL	Sterol-C4-methyl oxidase-like	NM_006745	Sterol biosynthesis	3.07	0.07
SORL1	Sortilin-related receptor, L (DLR class) A repeats-containing	NM_003105	Protein transport	3.02	0.42
ACTA2	Actin, alpha 2, smooth muscle, aorta	NM_001613	Cytoskeleton	2.99	0.10
CLU	Clusterin	NM_001831	Extracellular matrix	2.90	0.54
SAA1	Serum amyloid A1	NM_000331	Extracellular matrix	2.86	0.62
COL11A1	Collagen, type XI, alpha 1	NM_001854	Extracellular matrix	2.84	0.88
TMEM168	Transmembrane protein 168	NM_022484	Unknown	2.83	0.35
TEAD1	TEA domain family member 1	NM_021961	Transcription	2.82	0.63
SCD	Stearoyl-CoA desaturase (delta-9-desaturase)	NM_005063	Fatty acid biosynthesis	2.76	0.34
KDELR1	KDEL endoplasmic reticulum protein retention receptor 1	NM_006801	Protein transport	2.70	0.65
COL1A1	Collagen, type I, alpha 1	NM_000088	Extracellular matrix	2.69	0.10
IDI1	Isopentenyl-diphosphate delta isomerase 1	NM_004508	Cholesterol biosynthesis	2.69	0.37
FKBP9	FK506 binding protein 9, 63 kDa	NM_007270	Protein folding	2.64	0.40
INSIG1	Insulin-induced gene 1	NM_005542	Cell growth	2.61	0.13
COL3A1	Collagen, type III, alpha 1	NM_000090	Extracellular matrix	2.58	0.21
KCTD12	Potassium channel tetramerisation domain containing 12	NM_138444	Potassium ion transport	2.58	0.43
IGFBP3	Insulin-like growth factor binding protein 3, transcript variant 2	NM_000598	Cell growth	2.57	0.24
PRSS23	Protease, serine, 23	NM_007173	Proteolysis	2.53	0.04

regulated by the sequence-specific transcription factor SOX9 (Bell *et al*., 1997; Zhang *et al*., 2003). Further investigation of SOX9-dependent and SOX9-independent transcriptional regulation may provide a novel therapeutic target for cartilaginous diseases, including osteoarthritis. Among the extracellular matrix genes, matrix G1a protein (MGP) was down regulated. Mgp-deficient mice exhibited the inappropriate calcification of various cartilages, including the growth plate, which eventually led to a short stature, osteopenia and fractures (Luo *et al*., 1997).

The IGF signaling system is one of the major regulators of endochondral ossification. The chondrogenic-promoting activity of IGF-I is thought to be primarily transduced through a phosphatidyl inositol

Table 3: cDNA microarray results showing down regulation during chondrocyte differentiation

Gene name	Gene description	RefSeq Accession No.	Gene function	Mean (N = 2, <0.4)	SD
PTX3	Pentaxin-related gene, rapidly induced by IL-1 beta	NM_002852	Extracellular matrix	0.10	0.01
IGFBP2	Insulin-like growth factor binding protein 2, 36kDa	NM_000597	Cell growth	0.11	0.02
PDGFB	Platelet-derived growth factor beta polypeptide	NM_002608	Cell growth	0.15	0.05
EMP1	Epithelial membrane protein 1	NM_001423	Cell growth	0.19	0.02
COL6A3	Collagen, type VI, alpha 3	NM_004369	Extracellular matrix	0.21	0.08
IER3	Immediate early response 3	NM_052815	Unknown	0.25	0.01
MGP	Matrix Gla protein	NM_000900	Extracellular matrix	0.28	0.02
AIM1	Absent in melanoma 1	NM_001624	Unknown	0.30	0.02
SELENBP1	Selenium binding protein 1	NM_003944	Unknown	0.31	0.02
CLIC1	Chloride intracellular channel 1	NM_001288	Ion transport	0.31	0.06
EEF1D	Eukaryotic translation elongation factor 1 delta	NM_032378	Protein biosynthesis	0.32	0.07
AADAC	Arylacetamide deacetylase (esterase)	NM_001086	Metabolism	0.32	0.10
COL1A2	Collagen, type I, alpha 2	NM_000089	Extracellular matrix	0.33	0.08
AKR1C1	Aldo-keto reductase family 1, member C1	NM_001353	Bile acid metabolism	0.34	0.02
NDUFB7	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7, 18kDa	NM_004146	Electron transport	0.35	0.02
ISLR	Immunoglobulin superfamily containing leucine-rich repeat	NM_005545	Cell adhesion	0.35	0.03
HEXA	Hexosaminidase A (alpha polypeptide)	NM_000520	Carbohydrate metabolism	0.36	0.09
ENO2	Enolase 2, (gamma, neuronal)	NM_001975	Glycolysis	0.36	0.08
TTN	Titin, transcript variant N2-A	NM_133378	Protein modification	0.37	0.10
ARNT	Aryl hydrocarbon receptor nuclear translocator	NM_001668	Transcription	0.37	0.02
PDGFRA	Platelet-derived growth factor receptor, alpha polypeptide	NM_006206	Cell growth	0.37	0.03
C1R	Complement component 1, r subcomponent	NM_001733	Proteolysis	0.38	0.01
PABPN1	Poly(A)-binding protein, nuclear 1	NM_004643	mRNA processing	0.38	0.03
SLC4A7	Solute carrier family 4, sodium bicarbonate cotransporter, member 7	NM_003615	Ion transport	0.38	0.05
CD63	CD63 antigen	NM_001780	Protein transport	0.38	0.10

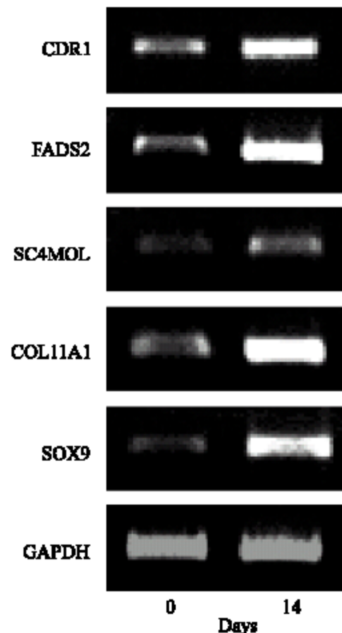

Fig. 3: Effect of chondrogenic differentiation medium on the expression of candidate genes in hMSCs. RT-PCR analysis of the indicated genes after 14 days of treatment in chondrogenic differentiation medium

3 (PI3)-kinase signaling pathway (Oh and Chun, 2003). PI3-kinase may be directly or indirectly responsible for chondrogenesis through currently defined metabolic genes. In our data sets, mRNA encoding insulin-induced gene 1 (INSIG1) was markedly up regulated, along with transcripts for IGF binding protein (IGFBP) 3, whereas IGFBP2 displayed the opposite behavior (Table 2 and 3).

Validation of microarray expression data: To validate the microarray data using independent methods, novel markers were selected for the analysis of gene expression using RT-PCR. We were particularly interested in Cerebellar Degeneration-Related protein 1 (CDR1), whose function is unknown and two metabolic enzyme genes, Fatty Acid Desaturase 2 (FAD2) and sterol-C4-methyl oxidase-like (SC4MOL). Gene expression patterns for CDR1, FAD2, SC4MOL, COL11A1 and the known cartilage marker SOX9 were selected; the expression patterns for these genes matched those obtained by microarray analyses (Fig. 3). The microarray expression patterns of these genes were therefore validated through alternative experimental means, suggesting that the microarray data correspond to the actual gene expression patterns.

CONCLUSIONS

The present transcriptome analysis of TGF-β1, IGF-I and insulin-induced chondrogenesis provided several lines of new, unexpected findings. Further study will clarify how these concomitant changes in gene expression are brought about and are organized into the concerted cellular event of chondrogenic differentiation in hMSCs.

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