Gene expression profile of mesenchymal stromal cells after co-culturing with injured liver tissue

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Abstract. Mesenchymal stromal cells (MSCs) are a potential cell source for the development of therapeutic products. Recent studies have shown that the transplantation of MSCs to damaged organs, including the heart, liver and kidneys, results in the restoration of the damaged tissues. However, the mechanisms underlying this regeneration process have yet to be clearly characterized. Consequently, in this study, we focused on the therapeutic potential of MSCs in injured liver tissue by evaluating the gene expression profiles of MSCs in the presence of injured liver and normal liver cells using a microarray chip containing 44,000 genes. In order to mimic the state of liver cell regeneration *in vitro*, we respectively co-cultured MSCs with CCl_4-injured liver cells and normal liver cells from C57BL/6 female mice. After 48 h of co-culturing, MSCs were collected and the RNA was extracted for micro-array analysis. Under conditions of co-culture with normal liver cells, upregulated expression of CXCR6, CCR3, IL-2, IL-11, CD34, CD74, procollagen, FMS-like tyrosine kinase, neuregulin 4, Wnt2 and catenins was noted. Under conditions of co-culture with the CCl_4-injured liver cells, expression of CXCL2, cytoglobin, erythropoietin, v-Erb, hypoxia-inducible factor 3 (α subunit), retinoic acid receptor ß and Vav2 was upregulated. Our research provides information regarding the differential molecular mechanisms that regulate the properties of MSCs in the regeneration of injured liver tissue.

Introduction

Bone marrow (BM) contains heterogenous cells consisting of hematopoietic stem cells (HSCs) and stromal cells, which support the development of HSCs. Among such cells, multipotent stromal cells, or mesenchymal stromal cells (MSCs), perform a supportive role as stromal cells in BM, and also possess the potential to differentiate into a variety of cell types, including osteocytes, chondrocytes, adipocytes and neuronal cells. Recently, the differentiation of MSCs into hepatic cell lineages has been reported. This implies that MSCs might potentially be employed as a source of cell-based therapy for the purpose of tissue regeneration (1,2).

MSCs of this type residing in the BM can differentiate into hepatic cells, which express the liver-specific markers albumin, α-fetoprotein and cytokeratin 18 as a result of stimulation with stem cell factor, epidermal growth factor and hepatocyte growth factor (3). MSCs can also be differentiated into hepatic lineage cells by co-culturing with liver tissues. In previous studies, MSCs were co-cultured with fetal liver cells (1) or liver cells, and were shown to differentiate into functional liver cells secreting albumin and urea after 48 h of co-culture (2).

We previously reported that BM cells ameliorate the pathologic conditions of CCl_4-induced liver injury in mice (4). As MSCs derived from BM are one source of stem cells toward hepatic lineage, they principally differentiate into functional hepatocytes and may contribute to the regeneration of injured liver tissues. Therefore, we analyzed the gene expression profile of MSCs co-cultured with liver cells that mimicked the liver microenvironment *in vitro*. The gene expression profiles of MSCs were assessed after co-culturing with normal liver cells or with liver cells from CCl_4-injected mice via a microarray technique.

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Key words: mesenchymal stromal cells, liver injury, microarray, gene expression

Materials and methods

Mice. Six-week-old C57BL/6 mice were purchased from Koatec (Pyung-Taek, Korea). All mice were bred and housed under specific pathogen-free conditions. All procedures were approved by the Animal Care and Use Committee of the Ewha Womans University School of Medicine.

Cell culture

Isolation and culture of MSCs. Six- to eight-week-old C57BL/6 female mice were sacrificed by cervical dislocation and their limbs were removed. The BM was flushed from the medullary cavities of both the femurs and tibias with serum-free RPMI-1640 medium (Gibco BRL, Carlsbad, CA) using a 25-gauge needle, filtrated through nylon meshes and centri-fugated for 5 min at 1,200 rpm. Isolated BM cells were then incubated in RBC lysis solution (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 10 mM EDTA) and washed twice with phosphate-buffered saline (PBS). The cells were then plated at $1x10^7$ cells/100 culture dishes in Iscove's Modified Dulbecco's Medium (IMDM; Sigma, St. Louis, MO) with 10% heat-inactivated fetal bovine serum (FBS). After 48 h, non-adherent cells were removed via aspiration, and the Mesencult basal medium with 10% mesenchymal stem cell stimulatory supplement (Stem Cell Technologies, Vancouver, Canada; cat. nos. 05501 and 05502) was replenished. Three weeks from the BM isolation, all MSCs used in this study were at 4 passages.

Adipogenic differentiation. MSCs cultured for 3 weeks following primary culture were plated on Mesencult basal medium containing 10% adipogenic stimulatory supplement (Stem Celfigl Technologies; cat. no. 05401). The medium was replenished once every 3 days for 2 weeks.

Liver cell isolation. Six-week-old mice were injected once a day with 10% CCl_4 (10 μl per gram) in mineral oil via the intraperitoneal route for two consecutive days. After 1 week, the mice were sacrificed and their liver tissues were collected aseptically followed by 30 min of collagenase treatment (0.5 mg/ml in RPMI-1640 with 10% FBS; Roche, Indianapolis, IN) at 37˚C. Liver cells were isolated from the collagenase-treated liver tissue following filtration through nylon meshes. For the co-culturing of liver cells and MSCs, the MSCs ($5x10^5$ cells/well) were plated onto the lower chambers of transwell culture plates (Falcon, Bedford, MA), while the liver cells ($3x10^5$/well) were introduced into the inserted upper-chamber (3 μm in pore size) with Dulbecco's modified Eagle's medium (Fig. 1H). After 48 h of co-culture, the inserted upper chambers were removed, and the MSCs in the lower chamber were collected for RNA isolation.

Flow cytometry. MSCs were analyzed for cell surface marker expression after culturing for 3 weeks. The cells were washed with PBS and stained at 4˚C for 30 min with a combination of the following antibodies: FITC-anti-mouse CD106, FITC-anti-mouse CD34, FITC-anti-mouse CD31, PE-anti-mouse CD73, PE-anti-mouse CD105, PE-anti-mouse CD45. All antibodies were purchased from BD Pharmingen with the exception of PE-anti-mouse CD105, which was obtained from R&D Systems (Minneapolis, MN). Flow cytometric analysis was performed using FACSCalibur and CellQuest software (BD).

Tissue preparation. The liver was perfused via the heart with 4% paraformaldehyde to flush out blood cells, then incubated with 4% paraformaldehyde overnight at room temperature for fixation. After washing twice with water, fixed livers were stored in 70% ethanol at 4˚C and embedded in paraffin. Sections were stained with hematoxylin and eosin.

Cell staining

Albumin staining. MSCs co-cultured with liver cells or not, as described above, were fixed for 10 min in ice-cold methanol. The cells were treated with 1% BSA in PBST (0.05% Tween-20 in PBS) for 30 min to block the unspecific binding of antibodies, then incubated overnight in primary antibody against albumin (Abcam; cat. no. ab19196) at 4˚C (1:800). The next day, the secondary antibody, biotinylated anti-rabbit IgG (DakoCytomation, Denmark) was applied for 30 min. Albumin expression was detected via the streptovidin-HRP/DAB substrate (DakoCytomation) reaction.

Giemsa staining. After 14 days of MSC culture, colony forming units of fibroblasts (CFU-F) were assessed after Giemsa staining. Cells fixed in methanol for 5 min were completely dried, and Giemsa stain solution was added.

Adipocyte staining. Adipocytes differentiated from MSCs were stained with Oil Red O solution. First, the cells were fixed for 5 min in 10% formalin and incubated for 1 h in newly changed 10% formalin. After washing with 60% isopropanol, the cells were dried completely and treated for 1 h with Oil Red O solution.

Isolation of total RNA. Total RNA was extracted from adherent MSCs co-cultured with normal liver tissue or liver tissue from CCl_4-injected mice, as well as from MSCs without co-culture, using TRIzol solution (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. RNA samples were stored at -70˚C until future use.

Microarray analysis. Two-color microarray-based Agilent chips containing 44,000 mouse genes (Digital Genomics, Seoul, Korea) were utilized. Total RNA (50 μg) was prepared from 3 groups of MSCs: the MSC group (untreated MSCs), the liver group (from MSCs co-cultured with normal liver cells) and the CCl_4 liver group (from MSCs co-cultured with the livers of CCl_4-injected mice). Samples from the MSC group were labeled with Cy5 while samples from the liver group were labeled with Cy3, and RNA samples from the MSC group were utilized as RNA references for the comparison of gene expression profiles. These microarray experiments were repeated three times for the different RNA batches. Genes were selected on the basis of differential Cy3/Cy5 expression ratios ≥2 in response. Gene clustering was generated from the gene expression data using Cluster and TreeView software (Eisen Lab; http://rana.lbl.gov/EisenSoftware.htm). Additional filtering was applied with a 2-fold change minimum to analyze the genes expressed in mouse liver tissue using Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA; http://www.ingenuity.com/).

Results

Before the liver cells from CCl_4-injected mice were isolated, liver tissue from the normal (control) and CCl_4-injected mice was fixed and stained with H&E (Fig. 1) so that differences between the two groups might be examined. Fig. 1A and B show characteristic features of the mouse liver, such as anisocytosis (an uneven size of liver cells) and anisokaryosis (an uneven size of liver cell nuclei). In Fig. 1B, a liver section from a CCl_4-injected mouse exhibits vacuolization of the liver cells, indicating that the cells were damaged by the CCl_4 injection.

In order to characterize the MSCs from the liver group, a CFU-F assay was performed (Fig. 2B) and the isolated MSCs were induced to differentiate into adipocytes (Fig. 2C) to confirm their multipotential differentiation. Additionally, CD73, CD105 and CD106 expression, as well as the negative expression of CD31, CD34 and CD45, was observed (Fig. 2D).

To assess the gene expression profiles of the MSCs from the liver and CCl_4 liver groups, we co-cultured liver cells in the upper chamber and MSCs in the lower chamber of transwell plates (Fig. 3). After 48 h of transwell culture, the upper chamber was removed and the cells from the lower chamber, containing MSCs stimulated by liver cells or injured liver cells, were collected. Next, RNAs from the cells of the lower chamber were isolated. Co-culture and RNA isolation was

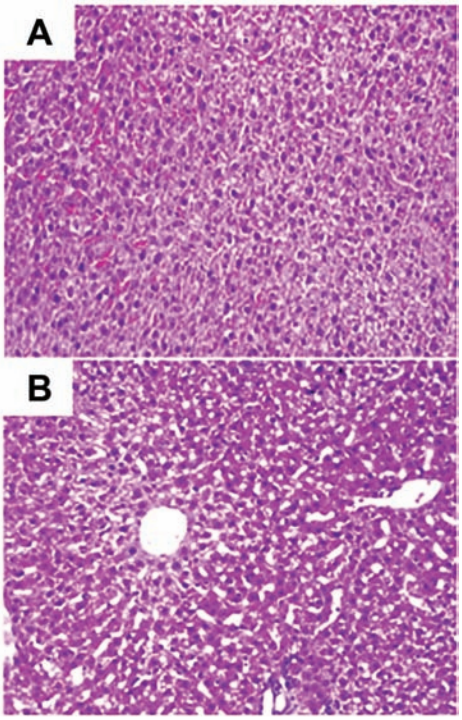

Figure 1. Representative sections of liver tissue stained with hematoxylin and eosin. (A) Normal liver. (B) Injured liver from a mouse 8 days after 2 consecutive injections of CCl_4. White punctuated space among the liver cells from CCl_4-injected mice can be seen. (Original magnification, x200).

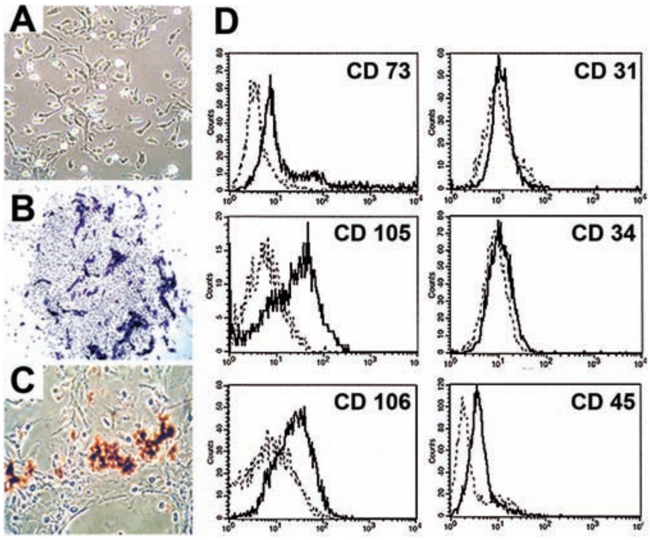

Figure 2. Preparation and characterization of isolated MSCs. The newly planted bone marrow cells were divided into adherent and non-adherent cells after 48 h of culture. (A) Adherent cells, or MSCs, proliferated and formed CFU-F. (B) This was confirmed by Giemsa staining on day 14. (C) MSCs cultured for 3 weeks were differentiated into adipocytes and stained red with Oil Red O (original magnification A-C, x100). (D) For the phenotypic markers, the MSC markers (CD73, CD105 and CD106) were positive. In contrast, the hematopoietic (CD34 and CD45) and endothelial (CD31) cell markers were not detectable in the cells after 21 days of culture.

repeated three times for each of the experimental groups, and the expression profile was presented as the mean values from these three microchip assay analyses.

Genes upregulated in the liver group as compared with the untreated MSC group are listed in Table I. Genes upregulated in the CCl_4 liver group as compared with the MSC group are shown in Table II. In addition, Tables III and IV show IPA-filtered genes that evidenced a >2-fold change in the gene expression of MSCs from the liver and CCl_4 liver groups, respectively. The results of microarray analysis of MSCs from the liver group demonstrate that inhibitor of DNA binding 1, Forkhead box G1, Wnt2, CD34, CXCR6 (receptor for CXCL16), tissue inhibitor of metalloproteinase 3, periostin, procollagen type 1, IL-2, neuregulin 4, CCR3 (receptor for RANTES, MCP-2, -3, -4), IL-11, CD74 (Ii chain of class II MHC molecules) and catenins were upregulated. When co-cultured with the CCl_4-injured liver cells, the expression of RAR-related orphan receptor ß, retinoic acid receptor ß, forkhead box G1, neural cell adhesion molecule 1, matrix metallopeptidase 12, insulin-like growth factor binding protein 6, nerve growth factor receptor, chordin, CXCL2, cytoglobin, erythropoietin, v-Erb, hypoxia inducible factor 3 (α subunit), Vav2 and hepatic nuclear factor 4α was upregulated.

Immunostaining analysis of albumin was conducted on MSCs co-cultured with liver cells from mice treated or untreated with CCl_4. After 48 h of co-culture with CCl_4-treated liver cells, the MSCs expressed albumin (Fig. 4). Therefore, MSCs derived from BM cells seemed to differentiate into functional hepatocytes under the specific conditions provided by a damaged liver.

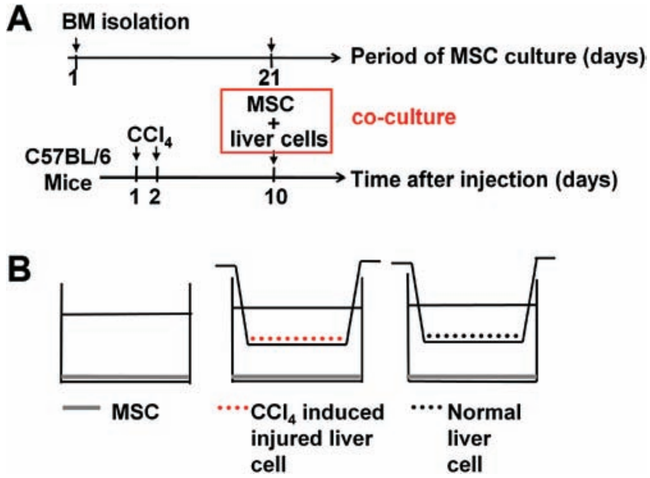

Figure 3. Co-culturing of MSCs and liver cells in transwell plates. (A) MSCs and injured livers were established according to the indicated co-culture schedule. (B) MSCs co-cultured with normal or CCl_4-injured liver cells on the transwell insert co-culture system.

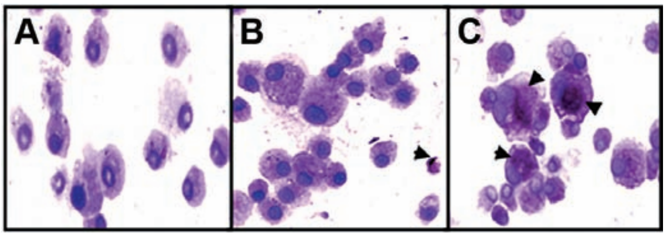

Figure 4. Albumin detection in co-cultured MSCs by immunocytochemistry. MSCs cultured alone (A) or co-cultured with normal liver cells (B) did not express albumin. However, MSCs co-cultured with CCl_4-injected liver cells (C) evidenced albumin positivity.

Table I. Genes upregulated in MSCs co-cultured with normal liver cells.

Accession	Symbol	Name	Mean
NM_207624	*ACE*	Mus musculus angiotensin converting enzyme (Ace), transcript variant 1, mRNA [NM_207624]	2.21
NM_007392	*ACTA2*	Actin, α2, smooth muscle, aorta	2.64
NM_007395	*ACVR1B*	Activin A receptor, type 1B	2.09
NM_009633	*ADRA2B*	Adrenergic receptor, α 2b	2.78
NM_011784	*AGTRL1*	Angiotensin receptor-like 1	2.59
NM_011784	*AGTRL1*	Angiotensin receptor-like 1	2.34
NM_009914	*CCR3*	Chemokine (C-C motif) receptor 3	2.08
NM_133654	*CD34*	CD34 antigen	4.22
NM_010545	*CD74*	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	2.19
NM_007664	*CDH2*	Cadherin 2	2.01
NM_007664	*CDH2*	Cadherin 2	2.01
NM_007693	*CHGA*	Chromogranin A	2.31
NM_016673	*CNTFR*	Ciliary neurotrophic factor receptor	2.84
NM_007742	*COL1A1*	Procollagen, type I, α1	3.22
NM_007743	*COL1A2*	Procollagen, type I, α2	2.14
AK076297	*COL27A1*	Procollagen, type XXVII, α1	2.27
AK008121	*CTNNA1*	Catenin (cadherin associated protein), α1	2.41
AK077879	*CTNNAL1*	Catenin (cadherin associated protein), ß1	2.92
NM_030712	*CXCR6*	Chemokine (C-X-C motif) receptor 6	3.40
NM_030206	*CYGB*	Mus musculus cytoglobin	2.49
NM_009998	*CYP2B10*	Cytochrome P450, family 2, subfamily b, polypeptide 10	2.03
NM_007822	*CYP4A14*	Cytochrome P450, family 4, subfamily a, polypeptide 14	2.32
NM_007824	*CYP7A1*	Cytochrome P450, family 7, subfamily a, polypeptide 1	2.04
NM_010045	*DARC*	Duffy blood group, chemokine receptor	2.52
NM_010106	*EEF1A1*	Eukaryotic translation elongation factor 1 α1	2.12
NM_010109	*EFNA5*	Ephrin A5	2.13
NM_207667	*FGF14*	Fibroblast growth factor 15	2.04
NM_008005	*FGF18*	Fibroblast growth factor 2	2.44
AK005502	*FLT1*	FMS-like tyrosine kinase 1	2.06
AK034946	*FMO1*	Flavin containing monooxygenase 1	2.13
NM_008241	*FOXG1*	Forkhead box G1	4.55
NM_008126	*GJB3*	Gap junction membrane channel protein ß3	2.80
NM_008127	*GJB4*	Gap junction membrane channel protein ß4	2.44
NM_013920	*HNF4G*	Hepatocyte nuclear factor 4, γ	2.55
NM_008296	*HSF1*	Heat shock factor 1	2.53
NM_010495	*ID1*	Inhibitor of DNA binding 1	4.69
NM_008344	*IGFBP6*	Insulin-like growth factor binding protein 6	3.19
NM_008350	*IL-11*	Interleukin 11	3.13
NM_019451	*IL-1F5*	Interleukin 1 family, member 5 (δ)	2.45
NM_008366	*IL-2*	Interleukin 2	3.20
NM_008368	*IL-2RB*	Interleukin 2 receptor, ß chain	3.03
NM_013565	*ITGA3*	Integrin α3	2.00
NM_008501	*LIF*	Leukemia inhibitory factor	2.32
NM_009158	*MAPK10*	Mitogen activated protein kinase 10	2.08
NM_008598	*MGMT*	O-6-methylguanine-DNA methyltransferase	2.03
NM_011846	*MMP17*	Matrix metallopeptidase 17	2.14
NM_010808	*MMP24*	Matrix metallopeptidase 24	2.01
AK038264	*MSH3*	MutS homolog 3 (*E. coli*)	2.36
NM_008634	*MTAP1B*	Microtubule-associated protein 1 B	2.37
NM_013607	*MYH11*	Myosin, heavy polypeptide 11, smooth muscle	2.21
NM_013607	*MYH11*	Myosin, heavy polypeptide 11, smooth muscle	2.21

Table I. Continued.

Accession	Symbol	Name	Mean
X15052	*NCAM1*	Neural cell adhesion molecule 1	2.25
NM_010875	*NCAM1*	Neural cell adhesion molecule 1	2.14
NM_010900	*NFATC2IP*	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2 interacting protein	2.10
NM_008700	*NKX2-5*	NK2 transcription factor related, locus 5 (*Drosophila*)	2.60
AK012322	*NR2F2*	Nuclear receptor subfamily 2, group F, member 2	2.91
X76653	*NR2F2*	Nuclear receptor subfamily 2, group F, member 2	2.40
NM_032002	*NRG4*	Neuregulin 4	3.19
NM_008814	*PDX1*	Pancreatic and duodenal homeobox 1	2.30
NM_015784	*POSTN*	Periostin, osteoblast specific factor	3.28
NM_008969	*PTGS1*	Prostaglandin-endoperoxide synthase 1	2.31
NM_008973	*PTN*	Pleiotrophin	2.76
NM_011243	*RARB*	Retinoic acid receptor,ß	3.06
NM_009084	*RPL37A*	Ribosomal protein L37a	2.10
NM_009115	*S100B*	S100 protein, ß polypeptide, neural	2.55
NM_011347	*SELP*	Selectin, platelet	2.07
AK038807	*SLIT2*	slit homolog 2 (*Drosophila*)	2.20
AK020817	*SMUG1*	Single-strand selective monofunctional uracil DNA glycosylase	2.14
NM_009235	*SOX15*	SRY-box containing gene 15	2.00
NM_011443	*SOX2*	SRY-box containing gene 2	2.56
NM_025285	*STMN2*	Stathmin-like 2	3.03
NM_019507	*TBX21*	T-box 21	2.20
NM_011595	*TIMP3*	Tissue inhibitor of metalloproteinase 3	3.31
NM_021406	*TREM1*	Triggering receptor expressed on myeloid cells 1	2.22
AK048623	*TRP63*	Transformation related protein 63	2.19
AK006986	*TYK2*	Tyrosine kinase 2	2.70
NM_011707	*VTN*	Vitronectin	2.07
NM_023653	*WNT2*	Wingless-related MMTV integration site 2	4.22
NM_009523	*WNT4*	Wingless-related MMTV integration site 4	2.56

Table II. Genes upregulated in MSCs co-cultured with CCl_4-injured liver cells.

Accession	Symbol	Name	Mean
AK051467	*AGTPBP1*	ATP/GTP binding protein 1	2.07
NM_011784	*AGTRL1*	Angiotensin receptor-like 1	2.33
NM_019577	*CCL24*	Chemokine (C-C motif) ligand 24	2.37
NM_009139	*CCL6*	Chemokine (C-C motif) ligand 6	2.26
NM_133654	*CD34*	CD34 antigen	2.56
NM_007650	*CD5*	CD5 antigen	2.33
NM_009873	*CDK6*	Cyclin-dependent kinase 6	2.80
NM_009893	*CHRD*	Chordin	3.08
AK003879	*COL27A1*	Procollagen, type XXVII, α1	2.57
NM_007758	*CR2*	Complement receptor 2	2.39
NM_008176	*CXCL2*	Chemokine (C-X-C motif) ligand 2	2.60
NM_030206	*CYGB*	Cytoglobin	4.90
NM_201640	*CYP4A10*	Cytochrome P450, family 4, subfamily a, polypeptide 10	2.09
NM_007824	*CYP7A1*	Cytochrome P450, family 7, subfamily a, polypeptide 1	2.29
AK009701	*DAPK1*	Death associated protein kinase 1	2.08
NM_010109	*EFNA5*	Ephrin A5	2.04
NM_207655	*EGFR*	Epidermal growth factor receptor	2.23

Table II. Continued.

Accession	Symbol	Name	Mean
NM_007942	*EPO*	Erythropoietin	2.73
XM_136682	*ERBB4*	v-Erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)	2.46
NM_021272	*FABP7*	Fibroblast growth factor 1	2.22
NM_207667	*FGF14*	Fibroblast growth factor 15	2.42
NM_008005	*FGF18*	Fibroblast growth factor 2	2.90
AK008922	*FGF22*	Fibroblast growth factor 23	2.32
NM_008011	*FGFR4*	Fibroblast growth factor receptor 4	2.17
NM_008241	*FOXG1*	Forkhead box G1	3.46
NM_008055	*FZD4*	Frizzled homolog 4 (*Drosophila*)	2.52
NM_008107	*GDF1*	Growth differentiation factor 1	2.06
NM_008160	*GPX1*	Glutathione peroxidase	2.06
AK002213	*GSTM1*	Glutathione S-transferase, mu 1	2.63
NM_016868	*HIF3A*	Hypoxia inducible factor 3, α subunit	2.22
NM_008261	*HNF4A*	Hepatic nuclear factor 4, α	3.03
NM_010495	*ID1*	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	2.11
NM_010496	*ID2*	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	2.10
NM_008344	*IGFBP6*	Insulin-like growth factor binding protein 6	3.18
NM_019451	*IL1F5*	Interleukin 1 family, member 5 (δ)	2.37
NM_001005608	*ITGB4*	Integrin ß4	2.00
XM_140451	*LAMA3*	Laminin, α3	2.27
BC070467	*MAP2K7*	Mitogen activated protein kinase kinase 7	2.02
AK053819	*MAPK8IP1*	Mitogen activated protein kinase 8 interacting protein 1	2.30
NM_008605	*MMP12*	Matrix metallopeptidase 12	3.34
NM_008611	*MMP8*	Matrix metallopeptidase 8	2.22
NM_133250	*MUTYH*	MutY homolog (*E. coli*)	2.06
AK048336	*MYST4*	MYST histone acetyltransferase monocytic leukemia 4	2.08
X15052	*NCAM1*	Neural cell adhesion molecule 1	3.36
NM_010896	*NEUROG1*	Neurogenin 1	2.14
NM_033217	*NGFR*	Nerve growth factor receptor (TNFR superfamily, member 16)	3.11
NM_008725	*NPPA*	Natriuretic peptide precursor type A	2.49
AK041047	*NR1D1*	Nuclear receptor subfamily 1, group D, member 1	2.22
X76653	*NR2F2*	Nuclear receptor subfamily 2, group F, member 2	2.61
AK012322	*NR2F2*	Nuclear receptor subfamily 2, group F, member 2	2.58
NM_013630	*PKD1*	Polycystic kidney disease 1 homolog	2.09
AK040305	*POLI*	Polymerase (DNA directed), ι	2.00
BC042707	*PROK1*	Prokineticin 1	2.37
NM_008973	*PTN*	Pleiotrophin	2.54
NM_011243	*RARB*	Retinoic acid receptor, ß	4.88
NM_011244	*RARG*	Retinoic acid receptor, γ	2.05
NM_134257	*RGS3*	Regulator of G-protein signaling 3	2.09
BC058269	*RORB*	RAR-related orphan receptor ß	5.32
NM_009115	*S100B*	S100 protein, ß polypeptide, neural	2.31
AK038807	*SLIT2*	slit homolog 2 (*Drosophila*)	3.00
AK090367	*SMUG1*	Single-strand selective monofunctional uracil DNA glycosylase	2.56
NM_011445	*SOX6*	SRY-box containing gene 6	2.93
NM_011448	*SOX9*	SRY-box containing gene 9	2.73
NM_009291	*STRA6*	Stimulated by retinoic acid gene 6	2.21
NM_011578	*TGFBR3*	Transforming growth factor, ß receptor III	2.00
NM_009380	*THRB*	Thyroid hormone receptor ß	2.95
AK006986	*TYK2*	Tyrosine kinase 2	2.46
NM_009500	*VAV2*	Vav 2 oncogene	2.14
M89800	*WNT6*	Wingless-related MMTV integration site 6	2.34

Table III. Gene expression profiles in MSCs co-cultured with normal liver cells.

Symbol	Name	Location	Accession	Fold change
ACE	Angiotensin I converting enzyme (peptidyl-dipeptidase A) 1	Plasma membrane	NM_207624	2.206
ACTA2 (includes EG:59)	Actin, α2, smooth muscle, aorta	Cytoplasm	NM_007392	2.642
ACVR1B	Activin A receptor, type IB	Plasma membrane	NM_007395	2.092
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	Extracellular space	NM_009621	-3.448
ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif, 5 (aggrecanase-2)	Extracellular space	AK046558	-3.19
APAF1	Apoptotic peptidase activating factor 1	Cytoplasm	NM_009684	-2.001
BCL10	B-cell CLL/lymphoma 10	Cytoplasm	AK080820	-2.157
BCL2L11	BCL2-like 11 (apoptosis facilitator)	Cytoplasm	NM_207680	-2.062
C3	Complement component 3	Extracellular space	NM_009778	-6.849
CASP3	Caspase 3, apoptosis-related cysteine peptidase	Cytoplasm	NM_009810	-2.423
CAV1	Caveolin 1, caveolae protein, 22 kDa	Plasma membrane	NM_007616	-2.479
CD34	CD34 molecule	Plasma membrane	NM_133654	4.217
CD36	CD36 molecule (thrombospondin receptor)	Plasma membrane	NM_007643	-2.174
CD74	CD74 molecule, major histocompatibility complex, class II invariant chain	Plasma membrane	NM_010545	2.192
CD86	CD86 molecule	Plasma membrane	NM_019388	-2.618
CDC42	Cell division cycle 42 (GTP binding protein, 25 kDa)	Cytoplasm	NM_009861	-2.217
CDH2	Cadherin 2, type 1, N-cadherin (neuronal)	Plasma membrane	NM_007664	2.012
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	Plasma membrane	NM_011926	-2.506
CFLAR	CASP8 and FADD-like apoptosis regulator	Cytoplasm	NM_009805	-2.389
CNTFR	Ciliary neurotrophic factor receptor	Plasma membrane	NM_016673	2.836
COL18A1	Collagen, type XVIII, α1	Extracellular space	NM_009929	-2.326
COL1A1	Collagen, type I, α1	Extracellular space	NM_007742	3.218
COL1A2	Collagen, type I, α2	Extracellular space	NM_007743	2.143
COL27A1	Collagen, type XXVII, α1	Extracellular space	AK076297	2.270
CUL1	Cullin 1	Nucleus	NM_012042	-3.055
CXCL3	Chemokine (C-X-C motif) ligand 3	Extracellular space	NM_009140	-2.439
CXCL12	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	Extracellular space	NM_013655	-2.329
CXCR6 (includes EG:10663)	Chemokine (C-X-C motif) receptor 6	Plasma membrane	NM_030712	3.400
CYP2B6 (includes EG:1555)	Cytochrome P450, family 2, subfamily B, polypeptide 6	Cytoplasm	NM_009998	2.027
EEF1A1	Eukaryotic translation elongation factor 1 α1	Cytoplasm	NM_010106	2.120
EFNA5	Ephrin-A5	Plasma membrane	NM_010109	2.130
ERBB2IP	erbb2 interacting protein	Extracellular space	NM_001005868	-2.107
FN1	Fibronectin 1	Plasma membrane	NM_010233	-2.439
GNA13	Guanine nucleotide binding protein (G protein), α13	Plasma membrane	NM_010303	-2.128
HGF	Hepatocyte growth factor (hepapoietin A; scatter factor)	Extracellular space	NM_010427	-2.439
ID1	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	Nucleus	NM_010495	4.690
IGF2	Insulin-like growth factor 2 (somatomedin A)	Extracellular space	NM_010514	-2.528
IGFBP6	Insulin-like growth factor binding protein 6	Extracellular space	NM_008344	3.192
IL1B	Interleukin 1, ß	Extracellular space	NM_008361	-3.906
IL1RN	Interleukin 1 receptor antagonist	Extracellular space	NM_031167	-2.924

Table III. Continued.

Symbol	Name	Location	Accession	Fold change
ITGA3	Integrin, α3 (antigen CD49C, α3 subunit of VLA-3 receptor)	Plasma membrane	NM_013565	2.002
ITGA6	Integrin, α6	Plasma membrane	AK045391	-2.551
KITLG (includes EG:4254)	KIT ligand	Extracellular space	NM_013598	-2.171
MAF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	Nucleus	NM_001025577	-2.309
MAPK9	Mitogen-activated protein kinase 9	Cytoplasm	NM_207692	-2.003
MCL1	Myeloid cell leukemia sequence 1 (BCL2-related)	Cytoplasm	NM_008562	-2.547
MMP17	Matrix metallopeptidase 17 (membrane-inserted)	Extracellular space	NM_011846	2.142
MMP24	Matrix metallopeptidase 24 (membrane-inserted)	Extracellular space	NM_010808	2.012
NFATC2	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	Nucleus	AK081853	-2.237
NR3C1	Nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	Nucleus	NM_008173	-2.134
PTGS1	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	Cytoplasm	NM_008969	2.310
PTPRC	Protein tyrosine phosphatase, receptor type, C	Plasma membrane	NM_011210	-3.067
PTX3	Pentraxin-related gene, rapidly induced by IL-1 ß	Extracellular space	NM_008987	-2.182
RARB	Retinoic acid receptor, ß	Nucleus	NM_011243	3.058
SELP	Selectin P (granule membrane protein 140 kDa, antigen CD62)	Plasma membrane	NM_011347	2.070
SLIT2	Slit homolog 2 (*Drosophila*)	Extracellular space	AK038807	2.200
SOCS3	Suppressor of cytokine signaling 3	Cytoplasm	NM_007707	-2.268
SPP1	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	Extracellular space	NM_009263	-2.060
TNFRSF9	Tumor necrosis factor receptor superfamily, member 9	Plasma membrane	AK019885	-2.000
TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	Plasma membrane	NM_008764	-2.538
UBA1	Ubiquitin-like modifier activating enzyme 1	Cytoplasm	NM_009457	-2.660
VTN	Vitronectin	Extracellular space	NM_011707	2.070

Table IV. Gene expression profiles in MSCs co-cultured with CCl_4-injured liver cells.

Symbol	Name	Location	Accession	Fold Change
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	Extracellular space	NM_009621	-3.724
ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif, 5 (aggrecanase-2)	Extracellular space	AK046558	-4.255
AHR	Aryl hydrocarbon receptor	Nucleus	NM_013464	-2.930
AKT1	v-akt murine thymoma viral oncogene homolog 1	Cytoplasm	NM_009652	-2.083
APAF1	Apoptotic peptidase activating factor 1	Cytoplasm	NM_009684	-2.209
BCL10	B-cell CLL/lymphoma 10	Cytoplasm	AK080820	-2.532
C3	Complement component 3	Extracellular space	NM_009778	-4.415
CASP3	Caspase 3, apoptosis-related cysteine peptidase	Cytoplasm	NM_009810	-2.597
CASP4	Caspase 4, apoptosis-related cysteine peptidase	Cytoplasm	NM_007609	-2.360
CAV1	Caveolin 1, caveolae protein, 22 kDa	Plasma membrane	NM_007616	-2.297
CCL24	Chemokine (C-C motif) ligand 24	Extracellular space	NM_019577	2.368
CCRL1	Chemokine (C-C motif) receptor-like 1	Plasma membrane	NM_145700	-2.060
CD5	CD5 molecule	Plasma membrane	NM_007650	2.327
CD34	CD34 molecule	Plasma membrane	NM_133654	2.561
CD86	CD86 molecule	Plasma membrane	NM_019388	-2.558

Table IV. Continued.

Symbol	Name	Location	Accession	Fold Change
CDK2	Cyclin-dependent kinase 2	Nucleus	NM_183417	-2.091
CFLAR	CASP8 and FADD-like apoptosis regulator	Cytoplasm	NM_207653	-2.498
COL18A1	Collagen, type XVIII, α1	Extracellular space	NM_009929	-2.239
COL27A1	Collagen, type XXVII, α1	Extracellular space	AK003879	2.572
CUL1	Cullin 1	Nucleus	NM_012042	-2.727
CXCL2	Chemokine (C-X-C motif) ligand 2	Extracellular space	NM_008176	2.600
CXCL10	Chemokine (C-X-C motif) ligand 10	Extracellular space	NM_021274	-3.448
EFNA5	Ephrin-A5	Plasma membrane	NM_010109	2.040
EGFR	Epidermal growth factor receptor [erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian]	Plasma membrane	NM_207655	2.230
ERBB2IP	erbb2 interacting protein	Extracellular space	NM_001005868	-2.089
GNA13	Guanine nucleotide binding protein (G protein), α13	Plasma membrane	NM_010303	-2.083
GPX1	Glutathione peroxidase 1	Cytoplasm	NM_008160	2.063
HGF	Hepatocyte growth factor (hepapoietin A; scatter factor)	Extracellular space	NM_010427	-2.222
HNF4A	Hepatocyte nuclear factor 4, α	Nucleus	NM_008261	2.144
ID1	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	Nucleus	NM_010495	2.113
ID2	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	Nucleus	NM_010496	2.095
IGF2	Insulin-like growth factor 2 (somatomedin A)	Extracellular space	NM_010514	-2.547
IGFBP6	Insulin-like growth factor binding protein 6	Extracellular space	NM_008344	3.185
IL1B	Interleukin 1, ß	Extracellular space	NM_008361	-2.457
ITGA6	Integrin, α6	Plasma membrane	AK045391	-2.360
KITLG (includes EG:4254)	KIT ligand	Extracellular space	NM_013598	-2.876
LAMA3	Laminin, α3	Extracellular space	XM_140451	2.272
MAF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	Nucleus	NM_001025577	-2.254
MAP2K7	Mitogen-activated protein kinase kinase 7	Cytoplasm	BC070467	2.019
MAP3K1	Mitogen-activated protein kinase kinase kinase 1	Cytoplasm	NM_011945	-2.091
MCL1	Myeloid cell leukemia sequence 1 (BCL2-related)	Cytoplasm	NM_008562	-2.155
NCOR1	Nuclear receptor co-repressor 1	Nucleus	AK035813	-3.040
NFATC2	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	Nucleus	AK081853	-3.096
NGFR	Nerve growth factor receptor (TNFR superfamily, member 16)	Plasma membrane	NM_033217	3.110
NOTCH1	Notch homolog 1, translocation-associated (*Drosophila*)	Plasma membrane	NM_008714	-2.865
NR1D1	Nuclear receptor subfamily 1, group D, member 1	Nucleus	NM_145434	-2.275
PKD1	Polycystic kidney disease 1 (autosomal dominant)	Plasma membrane	NM_013630	2.086
PTEN	Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	Cytoplasm	AK030750	-2.000
PTPRC	Pprotein tyrosine phosphatase, receptor type, C	Plasma membrane	NM_011210	-3.293
RARB	Retinoic acid receptor, ß	Nucleus	NM_011243	4.876
RARG	Retinoic acid receptor, γ	Nucleus	NM_011244	2.050
RGS3	Regulator of G-protein signaling 3	Nucleus	NM_134257	2.091
SLIT2	Slit homolog 2 (*Drosophila*)	Extracellular space	AK038807	3.000
STAT1	Signal transducer and activator of transcription 1, 91 kDa	Nucleus	AK041814	-3.25
TGFBR3	Transforming growth factor, ß receptor III	Plasma membrane	NM_011578	2.000
THRB	Thyroid hormone receptor, ß [erythroblastic leukemiaviral (v-erb-a) oncogene homolog 2, avian]	Nucleus	NM_009380	2.947
TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	Plasma membrane	NM_008764	-3.978

Discussion

In this study, we focused on the therapeutic potential of MSCs in injured liver tissues. Using a microarray containing 44,000 genes, we assessed the gene expression profiles of MSCs in the presence of injured liver cells and normal liver cells. The results demonstrate that MSC gene responses to co-culturing with liver cells occurred in a condition-specific manner.

The results of the microarray analysis of MSCs co-cultured with normal liver cells demonstrate that genes associated with the inflammatory process were upregulated; e.g., CXCR6 (receptor for CXCL16), CCR3 (receptor for RANTES, MCP-2, -3, -4), IL-2, IL-11, CD34 and CD74 (Ii chain of class II MHC molecules). Although CXCR6 (5), IL-11 (6) and CD34 (7) were reportedly expressed in human MSCs, these genes were also upregulated in mice MSCs co-cultured with normal liver cells. It is also worth noting that the expression of CD74, an invariant chain of class II MHC molecules, was upregulated. CD74 is required for the macrophage migration inhibitory factor-induced activation of the extracellular signal-regulated kinase-1/2 MAP kinase cascade, cell proliferation and PGE_2 production (8). The procollagen (type 1 and $\alpha 1$) gene was also upregulated and is believed to be associated with the tissue repair process. Neuregulin 4 is one of the neuregulins, a diverse family of EGF-like ligands that are sensitive to ADAM (a disintegrin and metalloproteinase) for the cleavage of the extracellular domain (9). Wnt signaling affects the developmental process of stem cells, including MSCs. In MSCs, signaling via the Wnt/ß-catenin pathway stimulates osteoblastogenesis and inhibits adipogenesis by regulating the relative levels of tissue-specific transcription factors (10). It has also been reported that the Wnt/ß-catenin pathway contributes to the activation of liver progenitor cells (11). Therefore, in MSCs co-cultured with normal liver cells, Wnt2 and catenin were also upregulated and may be associated with differentiation into hepatocytes.

The results of the microarray analysis of MSCs co-cultured with liver cells from CCl_4-injected mice demonstrate that genes associated with hypoxia response were upregulated; e.g., cytoglobin, hypoxia inducible factor 3 (α subunit) and erythropoietin. Cytoglobin, the gene involved in cell proliferation – possibly via collagen synthesis – and expressed predominantly in fibroblasts and associated cell types, is significantly elevated under hypoxic conditions (12). In this study, cytoglobin was upregulated in both the liver and CCl_4 liver groups. Hypoxia also induces the upregulation of erythropoietin, Sox6, and particularly Sox9, which is a key regulator of the differentiation of MSCs into chondrocytes. Another upregulated gene in the CCl_4 liver group was Vav2, the guanine nucleotide exchange factor. The primary function of this gene is the regulation of collagen phagocytosis, which is an $\alpha 2\beta 1$ integrin-dependent extracellular remodeling process (13). CXCL2 (Groß) is a ligand of CXCR2 and contributes to the rapid mobilization of HSCs with enhanced engraftment properties (14). Upregulation of the v-Erb gene can be considered in the context of epidermal growth factor receptors. In particular, it is noticeable that the expression of hepatic nuclear factor 4α, a critical transcription factor in hepatocyte differentiation (15), was upregulated.

MSCs co-cultured with liver cells from CCl_4-injected mice evidenced elevated levels of several growth factors, including epidermal growth factors. The observed up-regulation of frizzled homolog 4 reflects the possibility that the Wnt/ß-catenin pathway is relevant to the co-culturing of MSCs with liver cells.

Several genes were upregulated in common in the liver and CCl_4 liver groups; e.g., angiotensin receptor-like 1, CD34, procollagen type XXVII, cytochrome P450, fibroblast growth factor, forkhead box G1, hepatocyte nuclear factor 4, inhibitor of DNA binding 1, IL-1, matrix metallopeptidases, retinoic acid receptor ß, S100 protein ß, slit homologue 2 and tyrosine kinase 2. As well, several genes were downregulated in both groups; e.g., C3, CCR2, ADAM metallopepidase with thrombospondin type 1 and 5, CD86, cullin 1, Erbb2 interacting protein, GNA13, hepatocyte growth factor, integrin $\alpha 6$, NFATc2 and protein tyrosine phosphatase receptor type C. In the liver group, Socs3 was downregulated. Socs3 is a key inhibitor of cytokines that utilize gp130 (e.g., IL-23R and IL-6R), whereas Socs1 is believed to inhibit any cytokines that utilize γc (16). In the CCl_4 liver group, PTEN was downregulated compared to the untreated MSCs. PTEN is a tumor suppressor gene and functions as a lipid phosphatase that decreases the PI3K signaling pathway. In the absence of PTEN, HSCs are driven into the cell cycle; the loss of PTEN frequently promotes the formation of a variety of tumors (17).

In summary, MSCs co-cultured with normal liver cells exhibited the potential for differentiation into functional liver cells via upregulation of the genes associated with inflammatory response. MSCs co-cultured with CCl_4 liver cells differentiated into functional liver cells and upregulated genes related to hypoxic stress. In the CCl_4-injected mice, hypoxic-induced responses were involved in the regeneration process of liver cells via MSCs. We suggest that a diverse repairing pathway contributes to the regeneration process of liver cells by MSCs.

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