Materials and methods

Isolation and cell culture of human bone marrow stromal cells (BMSCs) and dedifferentiated chondrocytes (DECs)

Bone marrow samples and normal human articular chondrocytes were obtained from normal human donors with the approval of the internal Keio Ethics Committee (#13-11). Cells were resuspended in BMSC culture medium [10% fetal bovine serum in Dulbecco's modified Eagle's medium containing 4.5 g/L glucose (DMEM-HG)] with antibiotics/antimycotics supplements (Gibco BRL, Gaithersburg, MD, USA). Chondrocytes were maintained in chondrocyte growth medium (CGM; Bio Whittacker/Clonetics, Walkersville, MD, USA), and cultures were maintained at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. When the cultures reached subconfluence, the cells were harvested with 0.25% trypsin and 1 mM EDTA and were replated with one-half of the harvested cells.

Chondrosphere culture

For chondrogenic differentiation, we established a chondrosphere culture modified from the micromass culture. The cells were harvested with trypsin and EDTA, and the suspension was cytocentrifuged to obtain a concentration of 2.0×10^7 cells/ml. After placing 10-μl drops containing 2.0×10^5 cells each on a 35-mm uncoated culture dish, the cells were allowed to attach to the dish surface for 2 h in a humidified atmosphere at 37°C. Each culture was then flooded with 0.5 ml of each medium for each chondrosphere. The medium for BMSCs consisted of high-glucose DMEM supplemented with 10 ng/ml transforming growth factor (TGF)-β3, 1×10^{-7} M dexamethasone, 50 μg/ml ascorbic acid-2-phosphate, 40 μg/ml proline, 100 μg/ml pyruvate, and 50 mg/ml ITS+ Premix (Becton-Dickinson, Bedford, MA; 6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenious acid, 1.25 mg/ml bovine serum albumin, 5.35 mg/ml linoleic acid). We used CGM and chondrocyte differentiation medium (CDM; Clonetics) for the redifferentiation of dedifferentiated chondrocytes (DECs). On the second day, cells were gently detached from the uncoated dishes, by scraping with a 25-ml pipette, and then passaged on uncoated dishes. The BMSC medium was replaced every day and the DEC medium was replaced every 2 or 3 days. Chondrosphere culture was continued up to 11 months.

Histological and immunocytochemical analysis

Chondrospheres were fixed in 20% formalin, embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin, Alcian blue, and toluidine blue. Immunocytochemical analysis was performed as described [23] by using antibodies against aggrecan and type II collagen.

RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared from chondrosphere cultures with Isogen (Nippon Gene). RNA for RT-PCR was converted to cDNA with a First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotec, Uppsala, Sweden) according to the manufacturer's recommendations. PCR amplification conditions for the cDNAs obtained was performed by 35 cycles of 94°C for 30 s, 58°C for 45 s, and 68°C for 45 s, in which the 68°C step was prolonged by 5 s in every cycle after the first 10 cycles. The reaction products were resolved by electrophoresis on a 2% agarose gel and visualized with ethidium bromide. The PCR primers were as follows: type II collagen (forward): 5′-TTCAGCTATGGAGATGACAATC-3′, type II collagen-(reverse): 5′-AGAGTCCTAGAGTGACTGAG-3′ [24]; aggrecan (forward): 5′-GCCTTGAGCAGTTCACCTTC-3′, aggrecan (reverse): 5′-CTCTTCTACGGGGACAGCAG-3′ [25]; COMP (forward): 5′-CAGGACGACTTTGATGCAGA-3′, COMP (reverse): 5′-AAGCTGGAGCTGTCCTGGTA-3′ [25]; cartilage glycoprotein-39 (forward): 5′-GATAGCCTCCAACACCCAGA-3′, cartilage glycoprotein-39 (reverse): 5′-CTGAGCAGGAGCTGCTTTTT-3′; WISP1 (forward): 5′-GCCCCCAATTCTGCAAGTG-3′, WISP1 (reverse): 5′-TTAGGCTGGAAGGACTGGC-3′; β2-microglobulin (forward): 5′-CTCGCGCTACTCTCTCTTTCTGG-3′, β2-microglobulin (reverse): 5′-GCTTACATGTCTCGATCCCACTTAA-3′ [25].

Flow cytometric analysis

Cells were detached and stained for 30 min at 4°C with primary antibodies and immunofluorescent secondary antibodies. After washing, the cells were analyzed on a FACScan analyzer (Becton Dickinson). Antibodies (anti-human CD14, CD29, CD31, CD34, CD44, CD45, CD105, CD144, CD31, CD50, CD117, CD140α, CD90, CD106, CD166, and isotype control antibodies) were purchased from Beckman Coulter (Miami, FL, USA), Immunotech (Marseilles Cedex, France), Cytotech (Helleback, Denmark), and Pharmingen Pharmaceutical, Inc. (San Diego, CA, USA) (Table 1).

cDNA sequence: oligocapping method sequencing

cDNA libraries were constructed according to the oligocapping method [22,26]. Each plasmid DNA containing a cDNA insert was extracted with MultiScreen 96-well filter plates (Millipore Corp., Billerica, MA, USA). The 5′ end of the cDNA insert was sequenced with a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Briefly, 1 μl of Big Dye Terminator Reaction Mix, 1 μl of 3.2 pmol/μl of primer (5′-TACGGAAGTGTTACTTCTGC-3′), 3 μl of buffer (200 mM Tris-Cl, 10 mM $MgCl_2$, pH 9), and 400 ng of plasmid were mixed in a PCR tube, and the PCR cycle was performed (denaturation, 10 s at 96°C; annealing, 5 s at 50°C; elongation, 4 min at 50°C)

with a GeneAmp PCR System 9600 (Perkin Elmer, Norwalk, CT, USA). Sequencing analysis was performed with ABI3700 (Applied Biosystems, Foster City, CA, USA).

Infection of retroviral constructs

Cloning of the full-length of hTERT cDNA has been described previously [43]. The hTERT cDNA was cloned into pCLXSN with the Gateway System (Invitrogen) to generate pCLXSN-hTERT. Briefly, the cDNA was first cloned into pDONR201 by BP reaction, and then into a destination vector, pDEST-CLXSN, which has a modified cassette containing attR sites and ccdB (Invitrogen) at the multicloning site of pCLXSN (Imgenex Corp., San Diego, CA) [27]. Production of recombinant retroviruses has been described [28]. Briefly, retroviral vector together with a packaging plasmid, pCL-10A1 (Imgenex Corp., San Diego, CA) [27], was transfected into 293T cells by the calcium phosphate method, and the culture fluid was harvested at 60 h post transfection. Production of the LXSN-16E6E7 retrovirus has been described previously [29]. The titers of the recombinant viruses were greater than 1×10^6 drug-resistant colony forming units per milliliter on HeLa cells, and 1 ml of its culture fluid was added to cells in the presence of polybrene (8 μg/ml). Following inoculation with viruses, BMSCs were grown in the presence of G418 (100 μg/ml) or hygromycin B (50 μg/ml), and a polyclonal drug-resistant cell line was established and further analyzed. For combinations of retroviral infections, cells were sequentially transduced with LXSN-E6E7 and LXSN-hTERT, and selected with G418 and hygromycin B, respectively. The stably transduced cells with an expanded life span were named ThMSC1.

Results

Redifferentiation of DECs by chondrosphere culture

Human DECs were isolated from femoral head cartilage and designated as DECs. We used DECs beyond the 5th passage and induced rechondrogenesis by chondrosphere culture. In monolayer culture, the chondrocytes were flat with a fibroblast-like morphology (Fig. 1A and B), but they became round and in close contact with each other after chondrosphere culture at a high density cell condensation of 2×10^7 cells/ml and 2-h incubation (Fig. 1C and D). On day 5, the cells produced an ECM that exhibited metachromasia upon toluidine blue stain (Fig. 1E, F, and G). Expression of type II collagen and aggrecan genes commenced on day 7 (Fig. 1H).

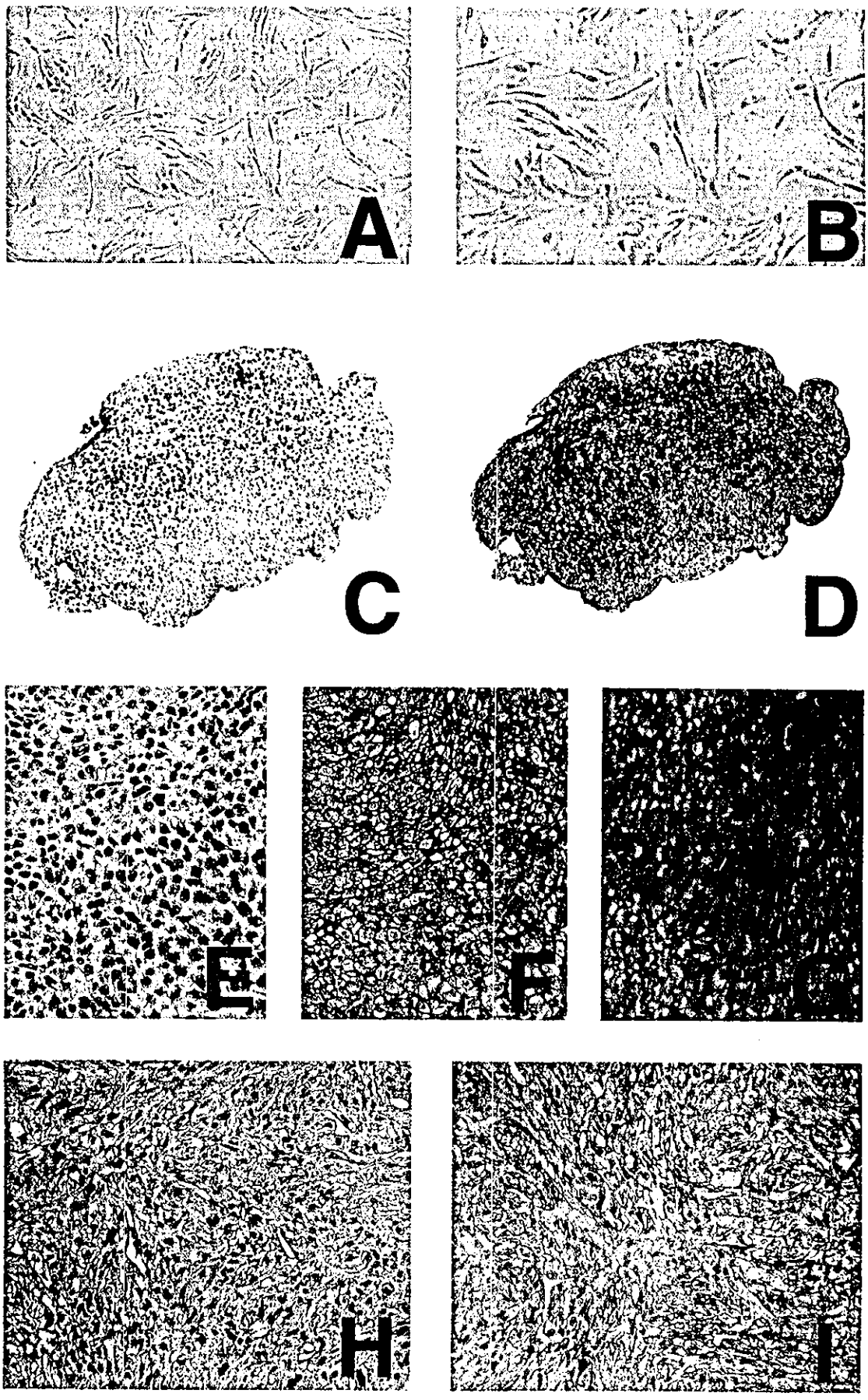
Chondrosphere culture for a long period

We performed micromass culture of monolayered DECs in chondrocyte differentiation medium, formed chondrospheres by detaching the micromasses from the dishes, continued chondrosphere culture, and then compared DEC redifferentiation at 3 weeks, 2 months, and 11 months. The cells were equally embedded in ECM at 3 weeks (Fig. 2A, B, and C) and at 2 months (Fig. 2D, E, and F). At 11 months (Fig. 2G, H, and I), the chondrospheres consisted of three morphologically distinct areas, (1) a high cell density area in the center, (2) an ECM-rich area in the inner layer, and (3) a fibrous area with flattened cells in the outer layer (Fig. 2J). The result of the immunocytochemical analysis showed that aggrecan was undetectable at 3 weeks, and started to be detected in both the cell cytoplasm and also ECM at a later stage (Fig. 2C, F, and I). A slight amount of type II collagen was detected at 2 h of chondrosphere culture, and its expression was enhanced by 3 weeks of culture (Fig. 2K and L).

Chondrosphere culture of BMSCs

We also generated 7 BMSC strains from two healthy donors, designating them H3-1 to H3-4 and H4-1 to H4-3, and we subjected them to chondrosphere culture, to determine whether they would differentiate into chondrocytes. Expression of chondrocyte-specific genes, such as type II collagen and aggrecan, was induced only when the cells were exposed to TGF-β3. Thus, we supplemented the chondrosphere culture of BMSCs with TGF-β3 in subsequent experiments. However, this is not the case for redifferentiation of DECs. The H4-1 cells were cultured to passage 16 in monolayer culture (Fig. 3A and B) and then placed in chondrosphere culture. On day 5 of chondrosphere culture, the H4-1 cells exhibited round morphology and were surrounded by ECM that stained with alcian blue and exhibited metachromasia with toluidine blue (Fig. 3C–G). To compare the chondrosphere culture with pellet culture, we determined expression of type II collagen and aggrecan in H4-1 cells (Fig. 4A). Aggrecan was induced on day 3 after addition of TGF-β3 in the chondrosphere culture, but not in the pellet culture. On day 5, aggrecan was detected in both cultures. Type II collagen was observed in both cultures on day 3 and day 5. We compared H4-1 with H3-4 at the 7th passage, which is one of other human stromal strains for chondrogenesis. The H3-4 cells were spindle-shaped in morphology (Fig. 3H and I), and aggrecan was detected in them by RT-PCR, but no type II collagen (Fig. 4B).

Chondrogenic differentiation is known to be mediated by mitogen-activated protein (MAP) kinases and blocked by inhibitors [30]. We used two MAPK inhibitors, PD98059, a specific inhibitor of mitogen-or extracellular signal-regulated kinase-1 (MEK1), and SB203580, which inhibits phosphorylation of P38 MAPK. Expression of type II collagen on day 5 was clearly inhibited by SB203580 (Fig. 4C), but not by PD98059 at any time during the chondrogenic differentiation protocol, indicating that chondrocytic differentiation of BMSCs is mediated by the previously reported signal pathway of mesenchymal cells into chondrocytes during embryonic development [9]. Inhibition of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ERK and p38 kinase partially reduced aggrecan expression (Fig. 4C).

Cell surface markers in BMSCs and DECs

The H4-1 chondrogenic progenitors were positive for CD29 (integrin β1), CD44 (Pgp-1/Iy-24), CD105 (endoglin), CDw90 (Thy-1), CD106 (VCAM-1), and CD166 (ALCAM), and negative for CD14 (a marker for macrophage and dendritic cells), CD34, CD45 (leukocyte common antigen), CD144 (VE-cadherin), CD31 (PECAM-1), CD50 (ICAM-3), CD117 (c-kit), and CD140α (PDGFR) (Table 2). H3-4 nonchondrogenic stroma cells were positive for CD29, CD34, CD44, CD105, CD140α, CDw90, CD106, and CD166, and negative for CD14, CD45, CD144, CD31, CD50, and CD117. The DECs were positive for CD14, CD29, CD44, CD105, CD90, and CD106, CD140α, and CD166, and negative for CD34, CD45, CD144, CD31, CD50, and CD117.

Establishment of ThMSC1 cells

To examine the possibility of using the cells as a source of cells for transplantation, they need to have a long life span and retain differentiated capability even after a longer culture period. To produce such cells, we infected H4-1 cells with human telomerase reverse transcriptase subunit (hTERT), E6, and E7. The infected cells, designated as ThMSC1 cells, grew for at least 6 months and proliferated for more than 40 population doublings. The ThMSC1 cells were spindle-shaped, and they elongated more than H4-1 cells in monolayer culture. The ThMSC1 cells did not express type II collagen or aggrecan after 5 days of exposure to TGF-β3 in chondrosphere culture, suggesting that they had lost their capacity of chondrogenesis.

Analysis of the cDNA sequences of cDNA libraries

Profiling of the redifferentiation of DECs (RDEC), H4-1, and ThMSC1 cells was performed; DEC cells at passage 7 were redifferentiated for 7 days of chondrosphere culture and then used for cDNA analysis; H4-1 in monolayer culture at passage 16 and ThMSC1 in monolayer culture at passage 20 were used. The sequenced 5′ ends of 2,628, 2,687, and 671 randomly selected cDNA clones obtained from cDNA libraries of redifferentiation of DECs, H4-1 cells, and ThMSC1 cells, respectively, were sequenced (Table 3), and the cDNA clones were clustered by similarity searches of 5′ end sequences. The threshold of similarity was possession of similarity overlaps with a length ≥50 bp and identity ≥90%. The sequences of representative clones of each group were analyzed with the human RefSeq mRNA (updated September 2001, ftp://ncbi.nlm.nih.gov/refseq/) by a similarity search. The numbers of the groups, each of which could correspond to known genes, are 1,134, 1,150, and 417 for DECs, H4-1 cells, and ThMSC1 cells, respectively. The numbers of genes (i.e., group) having high homology with any of the RefSeq sequences are shown in Table 3. The criterion for high homology was that the representative clone of the group have a similarity overlap with an identity ≥96%. The sequence similarity searches in the above in silico procedures were performed with WU-BLAST 2.0 (http://blast.wustl.edu). The percentages of the novel cDNA clones for redifferentiation of DECs, H4-1 cells, and ThMSC1 cells were 1.0%, 1.0%, and 1.6%, respectively.

The numbers of clones per unigene cluster having high homology (identity over 96%) are shown in Fig. 5A. Approximately 70% of the clusters for redifferentiated DECs and H4-1 cells contained only a single clone, whereas 30% of the clusters contained two or more clones. Fig. 5B shows the distribution of the functional classifications of the genes with identity ≥96% for redifferentiated DECs, H4-1 cells, and ThMSC1 cells. Each slice lists the numbers and percentages of human gene functions assigned to a given category of molecular function, according to the Gene Ontology (available at www.geneontology.org/). Almost all H4-1 and ThMSC1 cells had the same distribution, but redifferentiated DECs and H4-1 cells exhibited a different pattern, especially in "structural protein."

The highly detected genes in redifferentiated DECs, H4-1 cells, and ThMSC1 cells were shown in Table 4. The data showed high expression of two groups, i.e., the ECM-related genes and growth factor-related genes. In redifferentiated DECs, there were 365 clones of ECM-related genes, such as biglycan, chitinase 3-like 1 (cartilage glycoprotein-39), decorin, lamin A/C, and fibromodulin (expression level, 13.9%), and 50 clones of growth factor-related genes, such as insulin-like growth factor binding protein 3 and granulin (expression level, 1.9%). In H4-1 cells, there were 111 clones of ECM related genes, such as EFEMP1, biglycan, lamin A/C, and decorin (expression level, 4.1%), and 155 clones of growth factor-related genes, such as insulin-like growth factor binding protein 3 (IGFBP3) and connective tissue growth factor (CTGF) (expression level, 5.8%).

A marked difference in frequency of ECM-related genes was found between redifferentiated DECs and H4-1 cells

Fig. 3. Chondrogenesis of human marrow stromal cells by chondrosphere culture. H4-1, human marrow stromal cells, in monolayer culture at 16th passage are fibroblast-like in morphology (A). (B) High-power view of A. H4-1 cells at 16th passage for 5 days of chondrosphere culture were round-shaped and surrounded by extracellular matrix (C–G), which showed metachromasie with toluidine blue stain (G). (H and I) Chondrogenesis of H3-4 at 7th passage by a pellet culture. The cells are spindle in morphology. C, E, and H, HE stain; D, F, and I, Alcian blue stain.

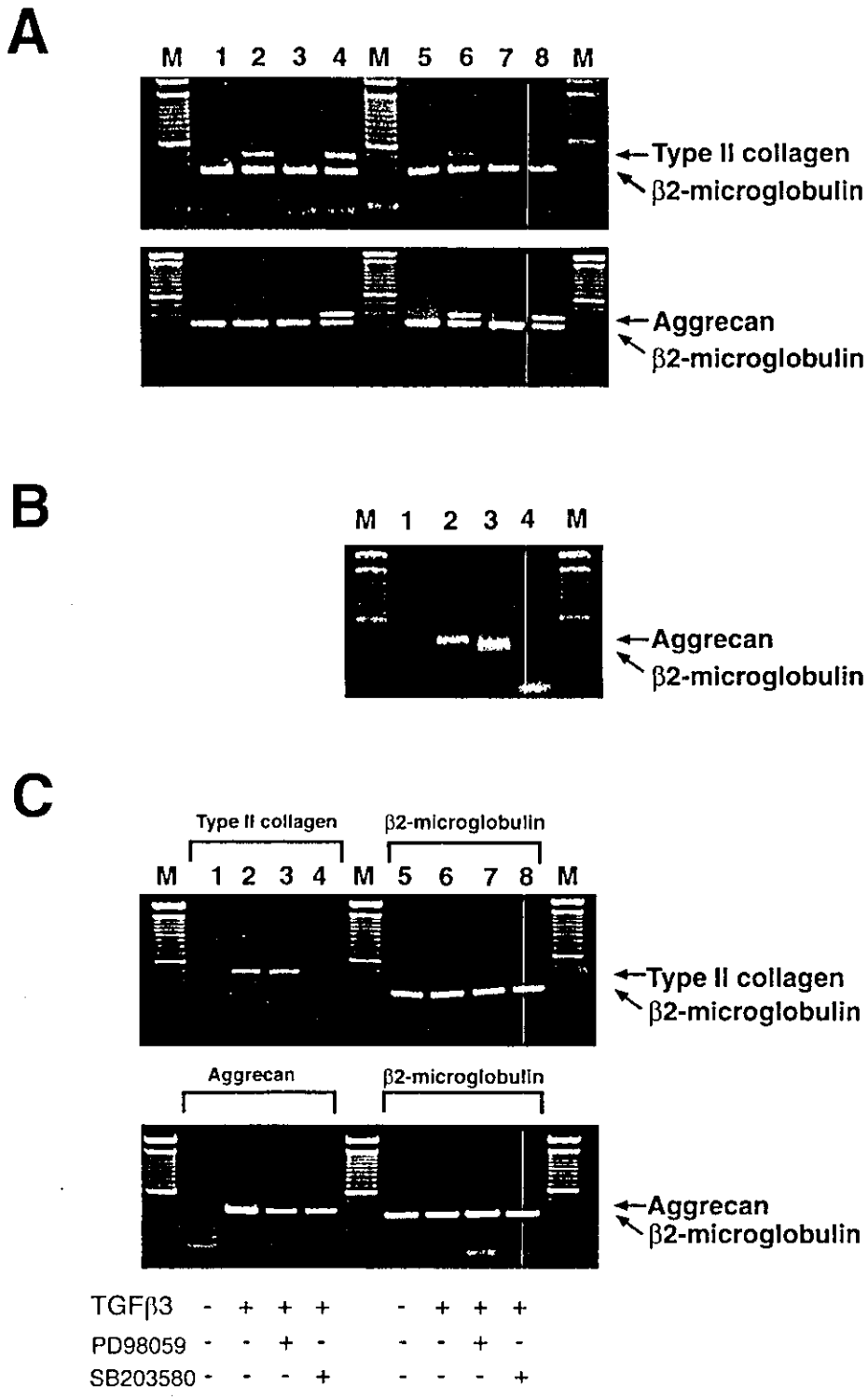


Fig. 4. Regulation of type II collagen and aggrecan by the pellet culture and chondrosphere culture with transforming growth factor (TGF)-β3. (A) Reverse transcription-polymerase chain reaction (RT-PCR) analysis of type II collagen (upper column) and aggrecan (lower column) expression in H4-I cells. Expression of β2-microglobulin is also shown as a loading control for each lane. Lanes 1 and 5, 3rd and 5th day of pellet culture without TGF-β3, respectively; lanes 2 and 6, 3rd and 5th day of pellet culture with TGF-β3, respectively; lanes 3 and 7, 3rd and 5th day of chondrosphere culture without TGF-β3, respectively; lanes 4 and 8, 3rd and 5th day of chondrosphere culture with TGF-β3, respectively. (B) RT-PCR analysis of aggrecan expression in H3-4 (70th passage) by the pellet culture. RNA was isolated from cells on 7th day of pellet culture. Lane 1, type II collagen; lane 2, aggrecan; lane 3, β2-microglobulin; lane 4 served as a negative control (H20) for β2-microglobulin primers. (C) RT-PCR analysis of type II collagen (lanes 1–4 of upper column) and aggrecan (lanes 1–4 of lower column) expression in H4-I cells on 5th day of chondrosphere culture. H4-I cells were differentiated in the chondrosphere culture protocol without TGF-β3 (lanes 1 and 5) or with TGF-β3 (lanes 2 and 6). The cells were exposed to MAPK inhibitors, 20 μM PD98059 (lanes 3 and 7), or 10 μM SB203580 (lanes 4 and 8) throughout the differentiation protocol. Expression of β2-microglobulin is also shown as a loading control (lanes 5–8). M represents 100-bp ladder of molecular markers.

Table 2
Flow cytometric analysis of cell surface markers in H4-1, H3-4, and dedifferentiated chondrocytes (DEC)[a]

Cells	CD14	CD29	CD34	CD44	CD45	CD105	CD144
H4-1	−	+	−	++	−	+	−
H3-4	−	++	++	++	−	++	−
DEC	+	++	−	++	−	++	−
	CD31	CD50	CD117	CD140a	CD90	CD106	CD166
H4-1	−	−	−	−	+	+	++
H3-4	−	−	−	+	++	+	++
DEC	−	−	−	+	++	+	++

[a] Peak intensity was estimated in comparison with isotype controls. (++), strongly positive (more than 10 times of the isotype control); (+), weakly positive; (−), negative (less than twice of the isotype).

(Table 5). The number of clones for ECM-related genes, which accounted for most of the structural proteins in Gene Ontology, was 3.4-fold higher than H4-1 cells (Fig. 5C). The percentage of the ECM-related genes was 15.0% in redifferentiated DECs, as follows: proteoglycans, 230 clones; cartilage oligomeric matrix protein (COMP), 85 clones; cartilage glycoprotein-39, 58 clones; EFEMP1, 13 clones; and collagens 5 clones. The proteoglycans in the redifferentiated DECs were mostly leucine-rich small proteoglycans, such as biglycan, 145 clones; decorin, 31 clones; fibromodulin, 23 clones; lumican, 12 clones, and other proteoglycans were osteoglycin, 7 clones; aggrecan, 6 clones; and tenascin C, syndecan 4, testican, fibrillin 1, osteonectin, and osteoglycin, 1 clone each.

The frequency of ECM-related genes in H4-1 cells was much lower (4.3%) than in redifferentiated DECs. The proteoglycans in H4-1 cells were biglycan, 26 clones; decorin, 12 clones; lumican, 5 clones; osteonectin, 5 clones; fibromodulin, 3 clones; and testican and glypican, 1 clone each.

Growth factors, such as TGF-β, fibroblast growth factor 7 (FGF7), vascular endothelial growth factor C (VEGFC), growth differentiation factor 10 (GDF10), and platelet-derived growth factor C (PDGFC) (1 clone each) were observed in redifferentiated DECs. Granulin, a putative growth factor, was detected at 20 clones in redifferentiated DECs. Connective tissue growth factor (CTGF), 24 clones; FGF7, 7 clones; granulin, 7 clones; and PDGFC, 1 clone; inhibin βA, 1 clone were detected in H4-1 cells. Among the growth factor-related genes, insulin-like growth factor binding protein 3 (IGFBP3) was highly observed in redifferentiated DECs (30 clones) and in H4-1 (131 clones). Gremlin or bone morphogenetic protein (BMP) antagonist 1 (CKTSF1B1), a member of cysteine knot superfamily, was detected at 6 clones in H4-1 cells. In the CCN family, WNT1-inducible signaling pathway protein 1 (WISP1) and WISP2 were observed in H4-1 cells (1 clone each). Among the Wnt family and signal pathway, frizzled-related protein (FRZB) was seen at 5 clones; dickkopf homolog 3, 3 clones; Wnt5a, 1 clone, in redifferentiated DECs.

Among the cell surface markers, CD29, CD44, CD46, CD54, CD55, CD68, CD90 (Thy-1), and CD105 were detected in redifferentiated DECs and CD10, CD24, CD29, CD44, CD55, CD59, CD73, CD90, CD97, CD105, CD119, and CD164 in H4-1 cells, while CD29, CD44, CD90, and CD105 were highly expressed in both redifferentiated DECs and H4-1 cells.

In the integrin family, integrin α11, β1 (CD29) and β5 were seen in redifferentiated DECs, while integrin α5 and β1 were detected in H4-1 cells. In the cadherin family, cadherin 11 was expressed in redifferentiated DECs, and cadherin 11 and 13 were observed in H4-1 cells. Among the cellular adhesion molecules cerebral cell adhesion molecule (C-CAM), junctional adhesion molecules, and intercellular adhesion molecule 1 (ICAM-1 or CD54) were expressed in redifferentiated DECs, while vascular cell adhesion molecule 1 (VCAM1) and melanoma adhesion molecule were expressed in H4-1 cells. Among the matrix metalloproteinase-related genes, matrix metalloproteinase 1 (MMP 1), MMP 2, MMP 3, tissue inhibitor of metalloproteinase 1 (TIMP 1), TIMP 3, a disintegrin, and metalloproteinase domain 15 (ADAM 15) were detected in redifferentiated DECs, while MMP 2, TIMP 3, and ADAM 9 were observed in H4-1 cells. Among the cytokine receptors, oncostatin M receptor was detected at 3 clones and the tumor necrosis factor receptor superfamily was observed at 6 clones. Among transcriptional factors, v-ets avian erythroblastosis virus E26 oncogene-related gene (ERG) was detected at 1 clone, v-ets avian erythroblastosis virus E26 oncogene homolog 2 (ETS2), 2 clones, retinoic acid receptor gamma (RARG), 2 clones, SRY-box 9 gene (Sox 9) gene, 1 clone, in redifferentiated DECs. The sequences of the cDNAs were deposited in Genbank (AU279383–AU280837; total 1,455 clones).

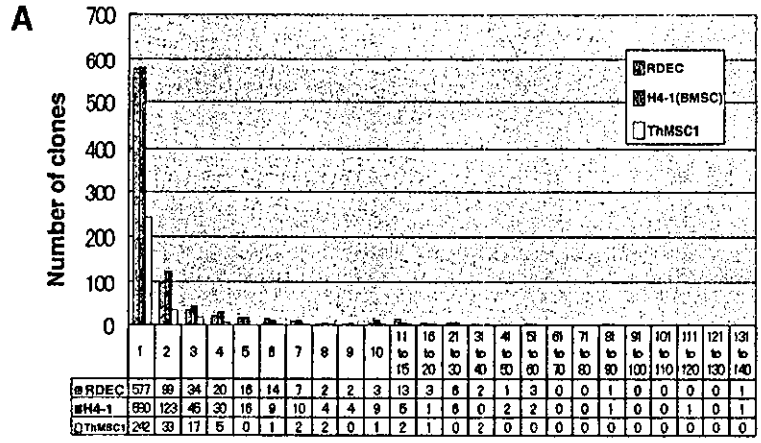
Table 3
General analysis of RDEC, H4-1 (BMSC), and ThMSC1 cDNA libraries[a]

	RDEC	H4-1 (BMSC)	ThMSC1
Total number of cDNA clones successfully sequenced at the 5′ end	2,628	2,687	671
Number of clusters	1,134	1,150	417
Number of clusters with high similarity to RefSeq mRNA[b]	804	849	308
Number of clusters with low similarity to RefSeq mRNA	334	301	109
Percentage of clones with low similarity to RefSeq mRNA in the cDNA library[c]	13%	11%	12%

[a] "High" and "low" similarity means ≥96% and <96%, respectively. RDEC, redifferentiated dedifferentiated chondrocytes; BMSC, bone marrow stromal cells.

[b] Each cluster represents a RefSeq mRNA.

[c] Each percentage represents the number of clones with low similarity to RefSeq mRNA divided by the total number of cDNA clones sequenced.



	1	2	3	4	5	6	7	8	9	10	11 to 15	16 to 20	21 to 30	31 to 40	41 to 50	51 to 60	61 to 70	71 to 80	81 to 90	91 to 100	101 to 110	111 to 120	121 to 130	131 to 140
RDEC	577	99	34	20	16	14	7	2	2	3	13	3	6	2	1	3	0	0	1	0	0	0	0	1
H4-1	660	123	46	30	16	9	10	4	4	9	5	1	6	0	2	2	0	0	1	0	0	1	0	1
ThMSC1	242	33	17	5	0	1	2	2	0	1	2	1	0	2	0	0	0	0	0	0	0	0	0	0

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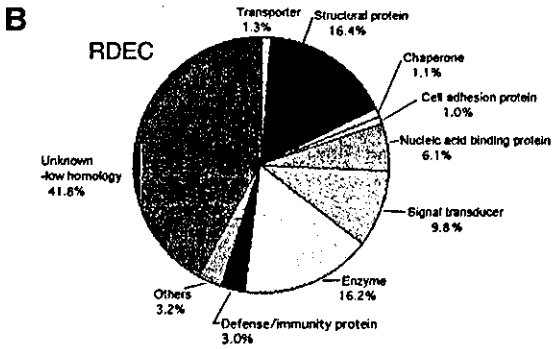
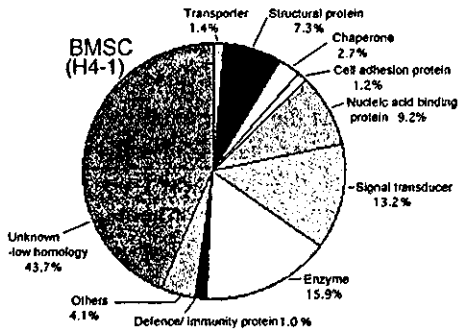
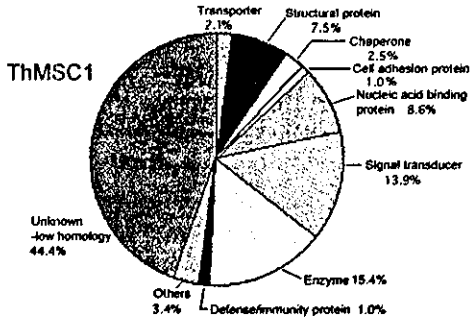




category of molecular function	RDEC	BMSC	ThMSC1
transporter	1.3%	1.4%	2.1%
structural protein	16.4%	7.3%	7.5%
chaperone	1.1%	2.7%	2.5%
cell adhesion protein	1.0%	1.2%	1.0%
nucleic acid binding protein	6.1%	9.2%	8.6%
signal transducer	9.8%	13.2%	13.9%
enzyme	16.2%	15.9%	15.4%
apoptosis	0.1%	0.3%	0.2%
defense/immunity protein	3.0%	1.0%	1.0%
others	3.2%	4.1%	3.4%
unknown low homology	41.8%	43.7%	44.4%



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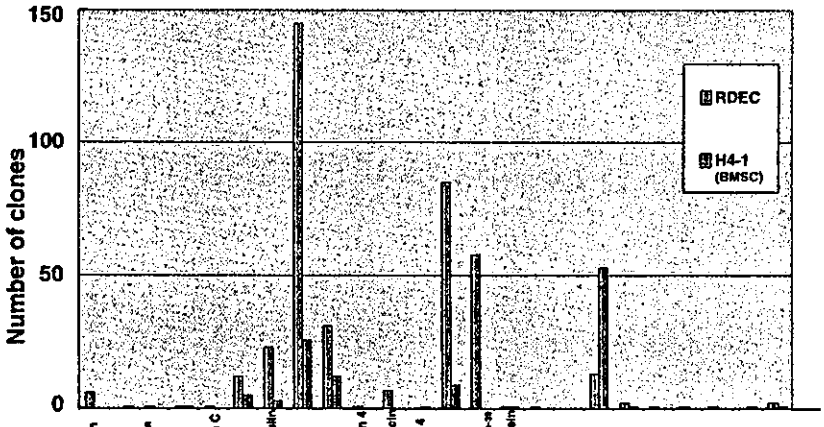

	Aggrecan	Versican	Chondroitin sulfate 4	Tesican	Tenascin C	Lumican	Fibromodulin	Biglycan	Decorin	Syndecan 4	Osteoglycin	Glypican 4	COMP	Cartilage glycoprotein-39	Link protein	Thrombo spondin 1	Thrombo spondin 2	EFEMP1	EFEMP2	Col 1 A1	Col 3 A1	Col 5 A2	Col 6 A1	Col 6 A3	Total
RDEC	6	0	1	1	1	12	23	145	36	1	7	0	85	58	1	1	0	13	2	1	1	1	0	2	393
H4-1	0	1	0	1	0	5	9	26	12	0	0	1	9	0	1	0	0	53	1	0	0	0	1	1	115

Table 4
The 15 most highly expressed genes in the cDNA library for RDEC, H4-1 (BMSC), and ThMSC1 cells[a]

Ranking	Gene title	Gene symbol	Gene ID	Identity	Clones	Expression level
RDEC						
1	Biglycan	BGN	NM_001711.1	100%	145	5.50%
2	Transforming growth factor, beta-induced	TGFBI	NM_000358.1	100%	133	5.06%
3	Cartilage oligomeric matrix protein	COMP	NM_000095.1	98%	85	3.24%
4	Chitinase 3-like 1	CHI3L1	NM_001276.1	99%	58	2.21%
5	Complement component 1, r subcomponent	C1R	NM_001733.1	99%	51	1.94%
6	Decidual protein induced by progesterone	DEPP	NM_007021.1	100%	41	1.56%
7	Procollagen-proline, 2-oxoglutarate 4-dioxygenase	P4HB	NM_000918.1	97%	32	1.22%
8	Decorin	DCN	NM_001920.1	99%	31	1.18%
9	Insulin-like growth factor binding protein 3	IGFBP3	NM_000598.1	97%	30	1.14%
10	Vimentin	VIM	NM_003380.1	100%	28	1.07%
11	Heat shock 70-kDa protein 8	HSPA8	NM_006597.1	99%	27	1.03%
12	Lamin A/C	LMNA	NM_005572.1	99%	23	0.88%
12	Fibromodulin	FMOD	NM_002023.2	98%	23	0.88%
14	Pyruvate kinase, muscle	PKM2	NM_002654.1	99%	21	0.80%
15	Granulin	GRN	NM_002087.1	100%	20	0.76%
H4-1 (BMSC)						
1	Insulin-like growth factor binding protein-3	IGFBP3	NM_000598.1	97%	131	4.88%
2	Transforming growth factor, beta-induced	TGFBI	NM_000358.1	100%	119	4.43%
3	Eukaryotic translation elongation factor-1 alpha 1	EEF1A1	NM_001402.1	99%	88	3.28%
4	Proteinase inhibitor, clade H (heat shock protein 47)	SERPINH2	NM_001235.1	99%	56	2.08%
5	EGF-containing fibulin-like extracellular matrix protein 1	EFEMP1	NM_004105.2	99%	53	1.97%
6	Vimentin	VIM	NM_003380.1	100%	43	1.60%
7	Heat shock 70-kDa protein 8	HSPA8	NM_006597.1	99%	42	1.56%
8	Actin, beta	ACTB	NM_001101.2	99%	27	1.00%
9	Biglycan	BGN	NM_001711.1	100%	26	0.97%
9	Procollagen-proline, 2-oxoglutarate 4-dioxygenase	P4HB	NM_000918.1	97%	26	0.97%
11	Connective tissue growth factor	CTGF	NM_001901.1	98%	24	0.89%
12	Amyloid beta (A4) precursor-like protein 2	APLP2	NM_001642.1	99%	21	0.78%
13	Lamin A/C	LMNA	NM_005572.1	99%	20	0.74%
14	Pyruvate kinase, muscle	PKM2	NM_002654.1	99%	15	0.56%
14	Complement component 1, r subcomponent	C1R	NM_001733.1	99%	12	0.45%
15	Decorin	DCN	NM_001920.1	99%	12	0.45%
ThMSC1						
1	Insulin-like growth factor binding protein 3	IGFBP3	NM_000598.1	97%	37	5.51%
2	Transforming growth factor, beta-induced	IGFBI	NM_000358.1	100%	36	5.37%
3	Vimentin	VIM	NM_003380.1	100%	19	2.83%
4	Eukaryotic translation elongation factor-1 alpha 1	EEF1A1	NM_001402.1	99%	12	1.79%
4	Heat shock 70-kDa protein 8	HSPA8	NM_006597.1	99%	12	1.79%
6	EGF-containing fibulin-like extracellular matrix protein 1	EFEMP1	NM_004105.2	99%	10	1.49%
7	Lactate dehydrogenase A	LDHA	NM_005566.1	99%	8	1.19%
7	Lamin A/C	LMNA	NM_005572.1	99%	8	1.19%
9	Biglycan	BGN	NM_001711.1	100%	7	1.04%
10	Pyruvate kinase, muscle	PKM2	NM_002654.1	99%	6	1.04%
11	Upregulated by 1.25-dihydroxyvitamin D-3	VDUP1	NM_006472.1	99%	4	0.60%
11	Immunoglobulin superfamily containing leucine-rich repeat	ISLR	NM_005545.1	100%	4	0.60%
11	P311 protein	P311	NM_004772.1	99%	4	0.60%
11	RAP1B, member of RAS oncogene family	RAP1B	NM_015646.1	98%	4	0.60%
11	Karyopherin alpha 2	KPNA2	NM_002266.1	99%	4	0.60%

[a] RDEC, redifferentiation of dedifferentiated chondrocytes; BMSC, bone marrow stromal cells; EGF, epidermal growth factor.

Fig. 5. General analysis of cDNA library from redifferentiated dedifferentiated chondrocytes (DEC), bone marrow stromal cells H-1 (BMSC), and ThMSC1. (A) The number of clones per unigene cluster that belongs to high similarity (identity over 96%). Approximately 70% of redifferentiated DEC (RDEC) and H4-1 clones (BMSC) was a single clone. For ThMSC1, 242 clones (78%) were nonrepetitive clones. (B) Distribution of the molecular functions of clones for redifferentiated DEC (RDEC), H4-1 (BMSC), and ThMSC1. Each slice lists the numbers and percentages of human gene functions assigned to a given category of molecular function according to the Gene Ontology (GO). (C) The number of clones on extracellular matrix genes for redifferentiated DEC (RDEC) and H4-1 (BMSC).

Table 5
The genes (identity over 96% of similarity) with the classification of molecular function[a]

Classification	Subclassification	Gene title	Gene symbol	Gene ID	Identity	RDEC	H4-1	ThMSC1
Extracellular matrix	Proteoglycan	Aggrecan	PG1	NM_001135.1	99%	6	0	0
		Versican	PG2	NM_004385.1	99%	0	1	0
		Chondroitin sulfate 4	CSPG4	NM_001897.1	100%	1	0	0
		Testican	SPOCK	NM_004598.2	99%	1	1	0
		Tenascin C	HXB	NM_002160.1	99%	1	0	0
		Lumican	LUM	NM_002345.1	99%	12	5	1
		Fibromudulin	FMOD	NM_002023.2	98%	23	3	0
		Biglycan	BGN	NM_001711.1	100%	145	26	7
		Decorin	DCN	NM_001920.1	99%	31	12	3
		Syndecan4	SDC4	NM_002999.1	100%	1	0	0
		Osteoglycin	OGN	NM_033014.1	99%	7	0	0
		Glypican 4	GPN4	NM_001448.1	99%	0	1	0
	Matrix protein	Cartilage oligomeric matrix protein	COMP	NM_000095.1	98%	85	9	1
		Cartilage glycoprotein-39	CHI3L1	NM_001276.1	99%	58	0	0
		Cartilage linking protein 1	CRTL1	NM_001884.1	99%	1	1	1
		Thrombospondin 1	THBS1	NM_006988.2	100%	1	0	0
		Thrombospondin 2	THBS2	NM_003247.1	100%	0	0	0
		EGF-containing fibulin-like Extracellular matrix protein 1	EFEMP1	NM_004105.2	99%	13	53	0
		EGF-containing fibulin-like Extracellular matrix protein 2	EFEMP2	NM_016938.1	99%	2	1	0
	Collagen	Collagen, type I, α1	COL1A1	NM_000088.2	99%	1	0	0
		Collagen, type III, α1	COL3A1	NM_000090.2	100%	1	0	0
		Collagen, type V, α2	COL5A2	NM_000393.1	99%	1	0	0
		Collagen, type VI, α1	COL6A1	NM_001848.1	99%	0	1	0
		Collagen, type VI, α3	COL6A3	NM_004369.1	99%	2	1	0
Growth factor	Growth factor	Transforming growth factor, β1	TGFb1	NM_000660.1	99%	1	0	0
		Fibroblast growth factor 7	FGF7	NM_002009.2	100%	1	7	1
		Vascular endothelial growth factor	VEGF	NM_003376.1	100%	1	0	0
		Vascular endothelial growth factor C	VEGFC	NM_005429.1	100%	1	0	0
		Growth differentiation factor 10	GDF10	NM_004962.2	99%	1	0	0
		Connective tissue growth factor	CTGF	NM_001901.1	98%	0	24	3
		Platelet-derived growth factor C	PDGFC	NM_016205.1	100%	1	1	0
		Heparin-binding epidermal growth factor-like growth factor	DTR	NM_001945.1	99%	0	0	1
		Granulin	GRN	NM_002087.1	100%	20	7	1
Growth factor-related protein		Transforming growth factor, β-induced	TGFB11	NM_000358.1	100%	133	119	36
		Insulin-like growth factor binding protein 3	IGFBP3	NM_000598.1	97%	30	131	37
		Gremlin	CKTSF1B1	NM_013372.1	99%	0	5	0
CCN family		WNT1 inducible signaling pathway protein 1	WISP1	NM_003882.1	99%	0	1	0
		WNT1 inducible signaling pathway protein 2	WISP2	NM_003881.1	99%	0	1	0
Wnt	Wnt family	Wingless-type MMTV integration site family, member 5A	WNT5A	NM_003392.1	98%	1	0	0
	Wnt signal pathway	Frizzled-related protein	FRZB	NM_001463.1	98%	5	0	0
		Dickkopf homolog 3	DKK3	NM_013253.1	100%	2	0	0
Cell surface markers		10	CALLA	NM_007288.1	99%	0	1	0
		24		NM_013230.1	99%	0	1	0
		29		NM_002211.1	100%	2	3	0
		44	H-CAM, Pgp-1	NM_000610.1	99%	11	7	2
		46	MCP	NM_002389.1	99%	1	0	0
		54	ICAM11	NM_000201.1	100%	1	0	0
		55	DAF	NM_000574.1	100%	1	1	1
		59		NM_000611.1	99%	1	5	2
		68		NM_001251.1	100%	1	0	0
		73		NM_002526.1	99%	0	1	0
		90	Thy-1	NM_002526.1	99%	4	2	1
		97	GR1	NM_001784.1	100%	0	1	01
		105	ENG	NM_000118.1	100%	4	5	1
		164	MGC-24	NM_006016.1	100%	0	2	

Table 5 (*continued*)

Classification	Subclassification	Gene title	Gene symbol	Gene ID	Identity	RDEC	H4-1	ThMSC1
Cell adhesions	Integrin	Integrin, α5	ITGA5	NM_002205.1	100%	0	3	0
		Integrin, α11	ITGa11	NM_012211.1	100%	1	0	0
		Integrin, β1	ITGB1	NM_002211.1	98%	2	3	0
		Integrin, β-like 1	ITGBL1	NM_004791.1	99%	0	0	0
		Integrin, β2	ITGB2	NM_000211.1	99%	0	0	1
		Integrin, β5	ITGB5	NM_002213.1	100%	1	0	0
	Cadherin	Cadherin 11	CDH11	NM_001797.1	100%	1	1	0
		Cadherin 13	CDH13	NM_001257.1	98%	0	2	1
	Cell adhesion molecules	Cerebral cell adhesion molecule	C-CAM	NM_016174.1	99%	1	0	0
		Vascular cell adhesion molecule 1	VCAM1	NM_001078.1	99%	0	1	2
		Junctional adhesion molecule 3	JAM3	NM_031470.1	100%	1	0	0
		Melanoma adhesion molecule	MCAM	NM_006500.1	99%	0	1	0
		Intercellular adhesion molecule 1	ICAM1	NM_000201.1	100%	1	0	0
		Intercellular adhesion molecule 3	ICAM3	NM_002162.2	98%	0	0	0
MMP	MMP	Matrix metalloproteinase 1	MMP1	NM_002421.2	99%	4	0	0
		Matrix metalloproteinase 2	MMP2	NM_004530.1	99%	7	2	0
		Matrix metalloproteinase 3	MMP3	NM_002422.2	100%	3	0	0
		Matrix metalloproteinase 14	MMP14	NM_004995.2	99%	0	0	0
TIMP	TIMP	Tissue inhibitor of metalloproteinase 1	TIMP1	NM_003254.1	99%	2	0	0
		Tissue inhibitor of metalloproteinase 3	TIMP3	NM_000362.2	98%	2	2	0
ADAM	ADAM	A disintegrin-like and metalloprotease with thrombospondin type 1 motif, 1	ADAMTS1	NM_006988.2	99%	0	0	0
		A disintegrin and metalloproteinase domain 9	ADAM9	NM_003816.1	99%	0	1	0
		A disintegrin and metalloproteinase domain 10	ADAM10	NM_001110.1	99%	0	0	0
		A disintegrin and metalloproteinase domain 15	ADAM15	NM_003815.2	99%	2	0	0
Receptor	Type 1	Interleukin 6 signal transducer	IL6ST	NM_002184.1	99%	1	0	0
		Oncostatin M receptor	OSM	NM_003999.1	99%	3	0	0
		Interleukin 13 receptor, α1	IL13RA1	NM_001560.1	100%	1	0	0
	Type II	Interferon-γ receptor 1	IFNrR1	NM_000416.1	100%	0	2	0
	Type III	Tumor necrosis factor receptor superfamily, member 1A	TNFRSF1A	NM_001065.1	99%	4	5	2
		Tumor necrosis factor receptor superfamily, member 10d	TNFRSF10D	NM_003840.1	100%	1	0	0
		Tumor necrosis factor receptor superfamily, member 10b	TNFRSF10B	NM_003842.1	100%	1	1	0
		Lymphotoxin β receptor (TNFR superfamily, member 3)	LTBR	NM_002342.1	100%	0	0	0
	Other (inner cellular)	Retinoic acid receptor, γ	RARG	NM_000966.1	100%	2	0	0
Transcription	ETS	V-ets avian erythroblastosis virus E26 oncogene homolog 2	ETS2	NM_005239.1	100%	2	0	0
		Ets variant gene 5	ETV5	NM_004454.1	98%	0	0	0
		V-ets avian erythroblastosis virus E26 oncogene related	ERG	NM_004449.2	100%	1	0	0
SOX	SOX	SRY (sex determining region)-box 9	SOX9	NM_000346.1	99%	1	0	0

[a] EGF, epidermal growth factor.

Expression of cartilage-related genes in DECs and BMSCs

The results of RT-PCR assays of parallel samples were consistent with the cDNA profiling data (Fig. 6). The expression of the COMP genes was detected at a higher level in the redifferentiated DECs than in H4-1 cells (Fig. 6A), and expression of cartilage glycoprotein-39 was found in both DECs and redifferentiated DECs (Fig. 6B). Expression of WISP1 was observed in H4-1 cells but not in DECs or redifferentiated DECs (Fig. 6C).

Discussion

BMSCs were successfully cultured for more than 25 passages. H4-1 cells proliferated and retained differentiation capability even after 48 population doublings

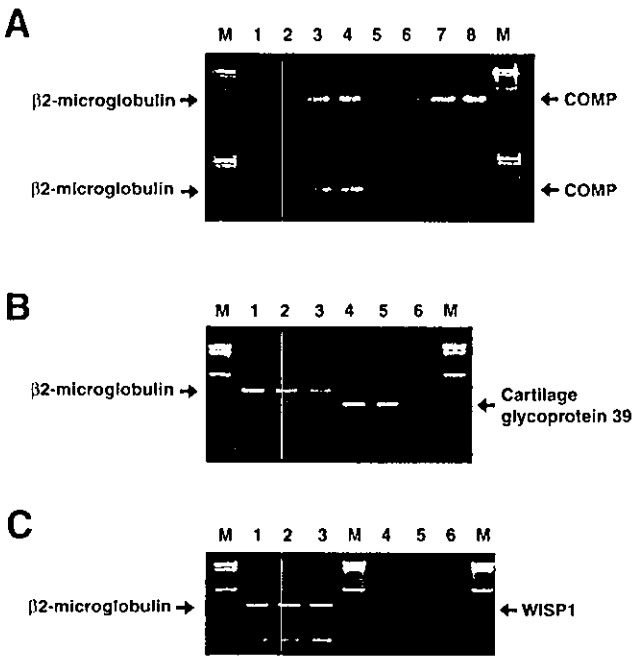

Fig. 6. The expression pattern of cartilage-related gene was consistent with the results of random sequencing analysis. (A) Reverse transcription-polymerase chain reaction (RT-PCR) analysis of cartilage oligomeric matrix protein (COMP) expression (lanes 5–8) in redifferentiation of dedifferentiated chondrocytes (DEC) at 1 week (upper column) and in the monolayer culture of H4-1 (lower column). Expression of β2-microglobulin is used for an internal control of each RNA (lanes 1–4). Lanes 1 and 5, 20 cycles of PCR; lanes 2 and 6, 25 cycles; lanes 3 and 7, 30 cycles; lanes 4 and 8, 35 cycles. (B and C) RT-PCR analysis of cartilage glycoprotein-39 (B) and WISP 1 (C). M, molecular markers; lanes 1 and 4, monolayer culture of DEC; lanes 2 and 5, redifferentiation of DEC at 1 week, lanes 3 and 6, monolayer culture of H4-1.

(PDs), while most marrow-derived mesenchymal cells reached senescence before 30 PDs. Human marrow progenitor cells have been successfully cultured for more than 60 cell doublings with multipotency under specific culture conditions [31]. H4-1 cells had a surface marker pattern similar to that of mesodermal progenitor cells, and thus it is of interest to investigate whether it undergoes not only chondrogenic differentiation but differentiation into other mesenchymal phenotypes under specific inducing conditions.

The method of chondrosphere formation, which has been modified in this study, promoted redifferentiation of dedifferentiated chondrocytes. Pellet culture is the method most commonly used for chondrogenesis of marrow mesenchymal stem cells [15], and comparison of the chondrosphere culture method and pellet method revealed that they have similar effects in inducting chondrogenesis in vitro in terms of increasing cell density to a high level. Based on the evidence that chondrocytes markers such as aggrecan and type II collagen were rapidly induced, 2-h cell aggregation by the chondrosphere method sufficiently promoted differentiation. Furthermore, since extremely abundant ECM was produced by the cultured cells, the chondrosphere culture did not require a scaffold for chondrogenesis. We conclude that chondrosphere formation is sufficient for chondrogenesis of dedifferentiated chondrocytes and H4-1 cells.

Human mesenchymal stem cells have been successfully isolated and can differentiate into osteocytes, chondrocytes, and adipocytes [15]. The surface antigen phenotype of H4-1 cells, c-kit negative and CD140α low or negative cells, showed differences from the surface antigens of human mesenchymal stem cells [15,31]. When FACS sorting is set for this population, $CD34^-$, c-kit^-, and $CD140\alpha^{-\text{ or low}}$, the fraction of human bone marrow cells will be highly enriched in chondrogenic progenitor cells. CD29, CD44, CD90, CD105, CD106, and CD166 were detected in both H4-1 cells and DECs in this study.

The consistency between the result of flow cytometric analysis and the frequency of the clusters (Table 6) indicates that the expression profiling by large-scale sequence analysis of the cDNA libraries obtained by oligocapping method [32] is valid, the same as previously reported large-scale sequencing analyses [33,34] and expression tag analyses [35]. Expression of "structural protein" on Gene Ontology, including the ECM, was much higher by redifferentiated DECs than by H4-1 and ThMSC1 cells, implying that the cells are mainly engaged in synthesizing ECM. Leucine-rich small proteoglycans having a molecular weight of 30 to 50 kDa include decorin, biglycan, fibromodulin, and lumican. Redifferentiated chondrocytes rapidly produced these small proteoglycans to embed themselves in the ECM, and

Table 6
Consistency between frequency of the clones and flow cytometric analysis[a]

Surface marker	H4-1 (BMSC)		Dedifferentiated chondrocytes	
	Flow cytometric analysis (folds)	cDNA analysis (clones)	Flow cytometric analysis (folds)	cDNA analysis (clones)
CD14	1	0	3	0
CD29	10	5	90	2
CD34	1	0	1	0
CD44	33	7	300	12
CD45	1	0	1	0
CD105	3	5	7	4
CD144	1	0	1	0
CD31	1	0	1	0
CD50	1	0	1	0
CD117	1	0	1	0
CD140a	1	1	2	1
CD90	5	2	43	4
CD106	5	1	6	0

[a] H4-1 (flow cytometric analysis), H4-1 (cDNA analysis), and dedifferentiated chondrocytes (flow cytometric analysis) were in the monolayer culture. Dedifferentiated chondrocytes (cDNA analysis) were in the condition of chondrosphere culture. BMSC, bone marrow stromal cells.

produced larger molecular weight proteoglycans, such as aggrecan at a later stage of differentiation. This pattern of expression for proteoglycans in redifferentiated DECs resembles the higher expression of biglycan, decorin, and fibromodulin by human mesenchymal stem cells by the pellet culture protocol [25].

Elongation factor 1 αI (EF-1 α) is one of the highly expressed genes among "nucleic acid binding proteins" (88 clones in H4-1 cells and 15 clones in redifferentiated DECs). The marked difference between these H4-1 cells and DECs may be attributable to a stage of cellular aging, since the level of EF-1 α expression during aging, e.g., an irreversible decrease in EF-1 α, is found in aged mesenchymal cells [36,37]. Among growth factors, genes of the CCN family, including CTGF, WISP1 (CCN4), and WISP2 (CCN5), were highly detected in H4-1. WISP1 and WISP2 are expressed at high levels in fibroblasts, and overexpressed in colon tumors [38]. The proteins they encode bind to decorin and biglycan, and may prevent the inhibitory activity of decorin and biglycan. The next most highly expressed genes of growth factors in H4-1 cells were FGF7/keratinocyte growth factor. This growth factor has a mitogen potentiality for epithelial cells, but for neither fibroblasts nor endothelial cells [39]. One of the growth factor-related genes is gremlin, whose protein acts as an antagonist of BMP signaling by preventing BMPs from interacting with their receptors [40,41]. Gremlin was highly detected during the growth phase of H4-1 cells, and this protein may inhibit differentiation of these cells through BMP inhibition at this stage.

The expression pattern of chondrocyte-specific genes in dedifferentiated chondrocytes is different from that of ATDC5 cells, which are a mouse embryonal carcinoma-derived chondrogenic cell line. ATDC5 cells exhibit a multistep differentiation process encompassing the stages from chondrogenesis to enchondral ossification [42]. Early-phase differentiation is characterized by expression of type II collagen, followed by induction of the aggrecan gene [42]. Late-stage differentiation is characterized by the start of expression of short-chain collagen type X genes. By contrast, marrow-derived mesenchymal stem cells express the aggrecan genes at an early stage and then type II collagen during chondrogenic differentiation [15]. The time course of ECM expression in redifferentiated DECs in this study resembles that of mesenchymal stem cells during chondrogenic differentiation rather than that of differentiating embryonal cell carcinoma cells.

These established human mesenchymal cells with expression profiling provide a powerful model for a study of chondrogenic differentiation and our further understanding of cartilage regeneration using redifferentiated DECs and BMSCs. BMSCs with a chondrogenic potential and dedifferentiated chondrocytes are useful candidate cell sources for transplantation in osteoarthritis and rheumatoid arthritis.

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In vivo cardiovasculogenesis by direct injection of isolated adult mesenchymal stem cells

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Abstract

The characterization of mesenchymal stem cells (MSCs) is of biological and clinical interest. We demonstrate that isolated MSCs, defined by $CD34^{low}$ $c\text{-}kit^{+}$ $CD140a^{+}$ $Sca\text{-}1^{high}$, are able to differentiate into cardiomyocytes, endothelial cells, and pericytes or smooth muscle cells by direct injection into adult heart. In skeletal muscle and lung, they also contributed to formation of the vasculature. MSCs did not transform into malignant cells or form excess extracellular matrix. This study suggests that MSCs may supply an ideal donor source of cardiovascular cells in patients with cardiopulmonary diseases.

Keywords: Regeneration; Transplantation; Cell therapy; Cardiomyocyte; Marrow stroma

Introduction

The existence of stem cells for nonhematopoietic cells in bone marrow was first suggested by Cohnheim [1] about 130 years ago. Recently, it was reported that marrow-derived mesenchymal stem cells (MSCs) differentiate into most somatic cells including osteoblasts, chondrocytes, myoblasts, and adipocytes such as adult neural stem cells [2] when placed in appropriate in vitro [3,4] and in vivo environments [5] or injected into blastocysts [6]. We have shown that stromal cells are able to generate cardiomyocytes in vitro [7,8]. However, most of these experiments were performed with heterogeneous stromal cells that had been obtained by adherence to plastic culture dishes [9]. Since it was demonstrated that bone marrow-derived stromal cells were purified to a homogeneous population that met the criteria for nonhematopoietic stem cells [10], these cells have been termed mesenchymal stem cells because they generate an array of cells, defined as mesenchymal cells.

Major advances have been made in the prevention, diagnosis, and treatment of cardiovascular diseases. However, morbidity and mortality from cardiovascular diseases continue to be an enormous burden experienced by many individuals with substantial economic cost. Recently, it was reported that adult bone marrow-derived cells can be reprogrammed to differentiate into various cell types, including liver cells [11], and neuronal-type cells [12] in vivo. In this study, our results present two novel findings in marrow-derived MSCs, i.e., the presence of a set of surface marker proteins and site-specific differentiation into cardiovascular cells in adult.

Materials and methods

Cell culture

Bone marrow-derived stromal cells were cultured by using methods previously described [4]. Bone marrow cells

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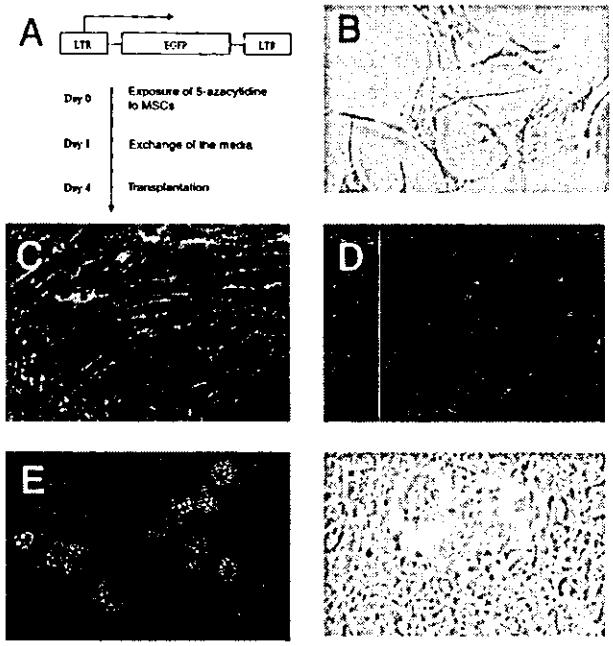

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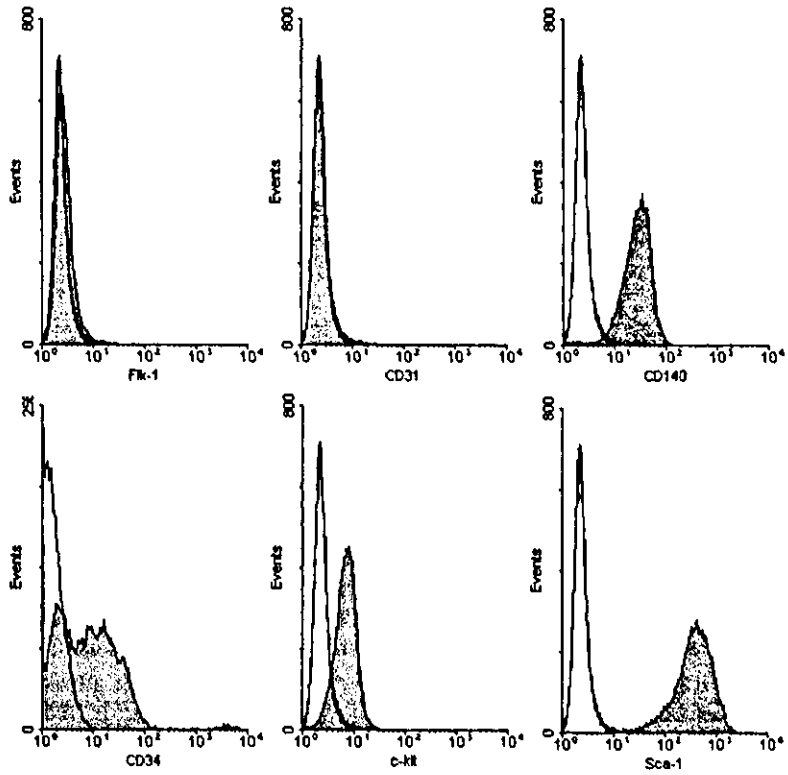

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Surface Marker	Expression	Surface Marker	Expression	Surface Marker	Expression
Flk-1	-	CD14	-	CD54	-
CD31	-	CD29	++	CD90	-
CD144	+	CD41	+	CD102	-
CD34	+	CD44	+++	CD105	-
c-kit	+	CD45	-	CD106	-
Sca-1	+++	CD49b	-	Ly-6c	++
CD140	++	CD49d	-	Ly-6g	-

were obtained from female C3H/HeJ mice. Cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% fetal bovine serum and penicillin (100 μg/ml)/streptomycin (250 ng/ml)/amphotericin B (85 μg/ml) at 33°C in humid air with 5% CO_2. After a series of passages, attached marrow stromal cells became homogeneous and were devoid of hematopoietic cells. Immortalized cells were obtained by frequent subculture for more than 4 months. To induce demethylation of genomic DNA, cells were treated with 3 μmol/l of 5-azacytidine (Sigma Chemical Co., St. Louis, MO, USA) for 24 h.

A number of clones were isolated by limiting dilution. Subcloned cells were maintained and again exposed to 5-azacytidine for 24 h. We selected a unique population of immortalized cells that expressed the hematopoietic stem markers $CD34^{low/-}$ and $c\text{-}kit^{+}$ and the mesenchymal marker $CD140a^{+}$ for subsequent experiments, because this cell population is believed to be one of the most upstream progenitors among our isolated cell population. The retroviral vector used in this study contains enhanced green fluorescent protein (EGFP) driven by the long terminal repeat enhancer/promoter of the Moloney murine leukemia virus. The ecotropic virus-producing GP + E86 cells were created as a packaging cell. Supernatants from the vector-producing cells, with a titer of 5×10^5 particles per milliliter, were used to transduce MSCs. Gene transduction was performed by three exchanges of the culture media of MSCs plated at density of 1×10^5 cells per 10 cm of tissue culture dish with the supernatant of the retroviral supernatant over 72 h with polybrene. Following transduction of the EGFP gene, the cells were maintained and used as donor cells in subsequent experiments. The transduction efficiency was determined by fluorescence-activating cell sorting (FACS) analysis.

FACS analysis

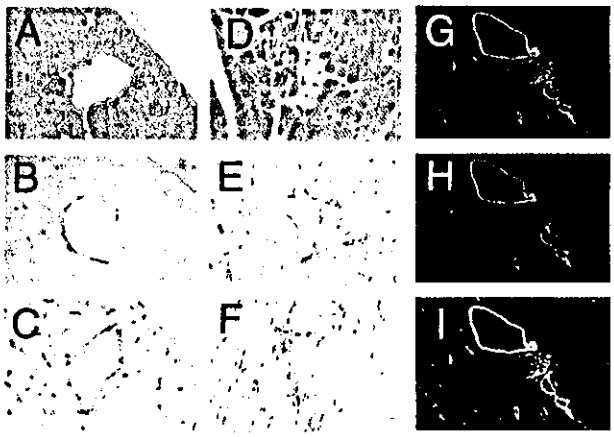
All samples were treated by water lysis to delete red blood cells. Cells were incubated with 0.01 μg/μl monoclonal antibody in Hanks' balanced salt solution (containing 0.1% albumin and 0.1% sodium azide) per 1×10^6 cells. When the first antibody was conjugated with biotin, cells were then washed twice and incubated with 1 μg of streptoavidin-phycoerythrin (GibcoBRL, Gaithersburg, MD, USA) for 30 min on ice. Purified antibodies in the first step were stained with 1 μg of fluorescein isothiocyanate-conjugated goat anti-mouse antibody. Controls included cells stained with individual isotypes (mouse IgG_1 or rat IgG_{2a}). Incubation was performed in the presence of 10 μg of mouse immunoglobulin to prevent nonspecific antibody binding. Anti-mouse Flk-1 (KDR or VEGF-receptor 2), CD31 (PECAM-1), CD34, c-kit, Sca-1 (Ly-6A/E), CD140a, CD144 (VE-Cadherin; endothelial cell-specific marker), CD14 (a marker for macrophages and dendritic cells), CD29 (integrin β1), CD41 (integrin αIIβ chain), CD44 (Pgp-1/Ly-24), CD45 (leukocyte common antigen), CD49b (integrin α2 chain), CD49d (integrin α4 chain), CD54 (ICAM-1), CD90 (Thy-1), CD102 (ICAM-2), CD105 (Endoglin), CD106 (VCAM-1), Ly-6C, Ly-6G (Gr-1), and isotype control antibodies were purchased from Pharmingen Pharmaceutical, Inc. (San Diego, CA, USA). Dead cells were removed by washing twice with Hanks' balanced salt solution, then propidium iodide was added to each test tube at a concentration of 1 μg/ml just before acquisition by FACScan flow cytometry (Becton Dickinson, Bedford, MA, USA) with the argon laser at 488 nm. List mode data for 30,000 to 50,000 cells were collected in the propidium iodide gate.

Cellular transplantation

Following priming by 5-azacytidine, the cells were cultured for an additional 3 days (Fig. 1A). Then these cells were harvested with 0.05% trypsin and 0.25 mM EDTA and suspended as single cells at a concentration of 1×10^5 cells/μl with phosphate-buffered saline (PBS). Cell viability in suspension, determined by 0.05% erythrosine dye exclusion, was 90% to 95%. After general anesthesia of the recipient mice by an intraperitoneal injection of pentobarbiturate 0.05 mg/g body weight, a transverse incision was made under the arch of a rib. The xiphoid process was inverted to the head, and the liver was pushed inferiorly so that the heart became visible through the diaphragm. Immediately before implantation, the cell suspension was drawn up into a 50-μl Hamilton syringe with a 31-gauge needle. A 10-μl portion of the cell suspension was injected into the ventricular myocardium or the inferior vena cava of syngeneic adult recipient mouse (C3H/HeJ, purchased from Clea Japan, Inc., Tokyo, Japan), aged 8 to 10 weeks [13]. Cell transplantations were also performed into the quadrant

Fig. 1. In vitro characteristics of the mesenchymal stem cells (MSCs) as a pluripotent stem cell model. (A) Construction of the recombinant retrovirus carrying enhanced green fluorescent protein (EGFP) cDNA and experimental procedure of cell transplantation. (B) Phase-contrast micrograph of isolated MSCs at the semiconfluent stage. (C–F) In vitro differentiation of the isolated MSCs. (C) Cell clusters spontaneously beat with the characteristic findings specific to cardiomyocytes such as branched structure. (D) Myotubes with multinuclei do not spontaneously beat. (E) Clusters of adipocytes. (F) Bone matrix produced by the cells.

Fig. 2. Flow cytometric analysis of cell surface markers in murine mesenchymal stem cells (MSCs). Endothelial cell markers Flk-1 and CD31 are negative. Mesenchymal marker CD140a and hematopoietic cell markers c-kits and Sca-1 are positive. Although CD34 expression has two peaks, negative and low positive at the periods of the transplantation, the more $CD34^{low}$ cells are generated the longer cultivation after the exposure to 5-azacytidine. Further phenotypic analysis was shown in the table (details in the supplements). The stainings of MSCs were performed according to Pharmingen protocols. All the antibodies used were from Pharmingen.



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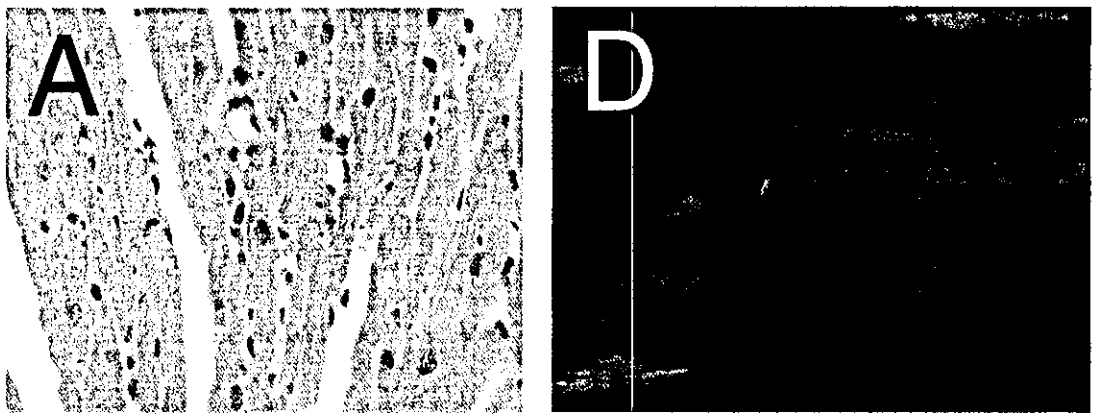

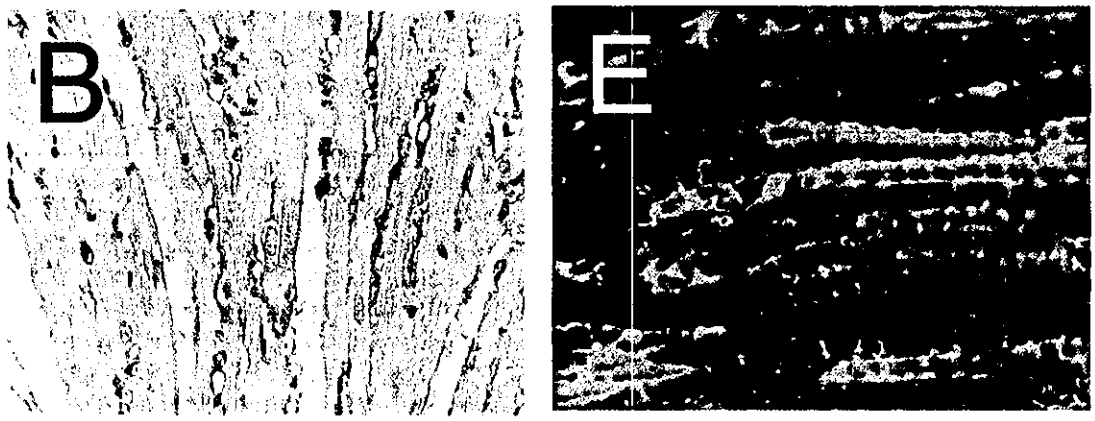

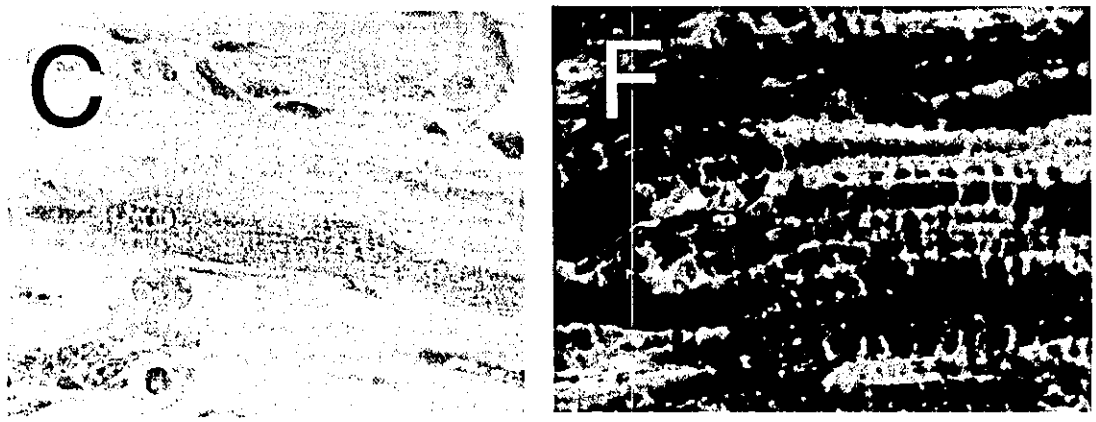

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muscles at a dose of 1×10^6 and 1×10^8 cells per each mouse. At 1, 4, 8, and 12 weeks after the transplantation, three to five mice were killed, and the lung, heart, thymus, liver, spleen, kidney, stomach, small intestine, colon, and brain were harvested from each animal. All animals received humane care in compliance with the *Principles of Laboratory Animal Care,* formulated by the National Society for Medical Research, and the *Guide for the Care and Use of Laboratory Animals,* prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985). All mice were maintained in a laminar-flow rack in our animal facility.

Histological analyses

Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections (6 μm) were mounted on poly-L-lysine-coated slides. After deparaffinization with xylene, tissues were rinsed in acetone or ethanol. Slides were incubated in 0.3% H_2O_2 in methanol for 30 min. After washing in PBS, tissues were preblocked for 30 min with 5% normal swine serum. They were incubated overnight at 4°C with mouse monoclonal antibody against recombinant green fluorescent protein (Clontech Laboratories, Inc., Palo Alto, CA, USA, catalogue no. 8362-1) at 1:500. After rinsing in PBS, slides were incubated with horseradish peroxidase-conjugated swine anti-mouse immunoglobulin diluted at 1:100 with 1% bovine serum albumin (BSA) in PBS, and washed in cold PBS. Staining was developed by using a solution containing diaminobenzidine and 0.01% H_2O_2 in 0.05 M Tris-HCl buffer, pH 6.7. Slides were counterstained with methylgreen or hematoxylin. Harvested organs were fixed in 10% formaldehyde and stained with hematoxylin and eosin, or anti-EGFP. Slices with positive signals for EGFP were further stained with anti-CD31 (PECAM-1) (M-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The total numbers of EGFP-positive cells per section were counted.

Frozen sections (6 μm) of the samples were used to detect the donor cells and the differentiation status by using alkaline phosphatase and fluorescence. After fixation with acetone and the blocking with PBS containing 5% rabbit serum, anti-GFP antibody was overlaid at 4°C overnight. Following three washes with PBS, the slides were incubated with anti-mouse IgG antibody conjugated with alkaline phosphatase for 1 h at room temperature. Visualization was achieved through the alkaline phosphatase detection system. In the case of fluorescence, anti-CD31 or anti-desmin (Bio-Science Products AG, Emmenbrücke, Switzerland) was used for first antibody, and rat anti-mouse IgG antibody conjugated with tetramethylrhodamine isothiocyanate (T4280, Sigma) or goat anti-mouse IgG antibody conjugated with rhodamine (M116, Leinco Technology, Inc., St. Louis, MO, USA) was used for second antibody, respectively.

Results

Gene transduction

Following retrovirus-mediated gene transduction of isolated MSCs derived from marrow stroma with the EGFP gene and subsequent culture (Fig. 1A and B), the cells were evaluated by FACS analysis and confocal laser microscopy. After three 24-h exposures of the cells to the retroviral vector, 70% to 80% of the cells expressed the reporter EGFP gene. The isolated MSCs retained the ability to generate cardiomyocytes, skeletal muscle cells, adipocytes, and osteoblasts in vitro (Fig. 1C–F).

Surface analysis of mesenchymal stem cell line

A true mesenchymal stem marker has yet to be identified. The putative MSCs ($CD34^{low/-}$ $c\text{-}kit^+$ $CD140a^+$), used in this experiment, expressed CD34 on the fourth day after the 5-azacytidine treatment (Fig. 2). Sca-1, CD29, and CD44 were expressed in all mesenchymal cell lines examined at moderate to high levels. Further surface analysis showed that these cells also expressed CD41, CD144, and Ly-6C. The cells were negative for Flk-1, CD31, CD14, CD45, CD49b, CD49d, CD54, CD90, CD102, CD105, CD106, and Ly-6G. It was previously reported that human mesenchymal stem cells were successfully isolated and could differentiate into osteocytes, chondrocytes, and adipocytes [14]. Our surface antigens analyses showed the differences from those on human MSCs, which express CD105 but not CD34.

Fig. 3. Generation of endothelial cells by the isolated mesenchymal stem cells (MSCs). The MSCs expressing enhanced green fluorescent protein (EGFP) were directly injected into the heart, and were histologically analyzed in the heart 1 week (A–C) or 3 months (D–F) after the grafting ($n = 41$). Sections for donor-derived cells were stained with hematoxylin and eosin (A and D), anti-EGFP antibody (B and E), or anti-CD31 antibody (C and F) by peroxidase method. CD31 staining demonstrates that the donor MSCs differentiated into the endothelial cells. The vessels contained red blood cells (D), suggesting that the vessels connected to the host vascular system and supplied blood to the tissues. The merge (I) of green fluorescence of injected MSCs (G) and rhodamine of CD31 (H) clearly demonstrated that the MSCs differentiated into the endothelium.

Fig. 4. Differentiation of the isolated mesenchymal stem cells (MSCs) into cardiomyocytes. Enhanced green fluorescent protein (EGFP)-labeled MSCs could be recognized morphologically as the cardiomyocytes in the heart 3 months after transplantation (A, hemotoxglin and eosin staining; B and C, peroxidase staining). The EGFP-positive donor cells exhibit cardiomyocyte-specific features such as a single nucleus in the middle of the cells, branching, striation, and intercalated disks. The injected donor cells labeled with EGFP were also detected by green fluorescence. (D) Green fluorescence of EGFP-labeled donor cells. (E) Immunohistochemistry against desmin (red). (F) Merge.

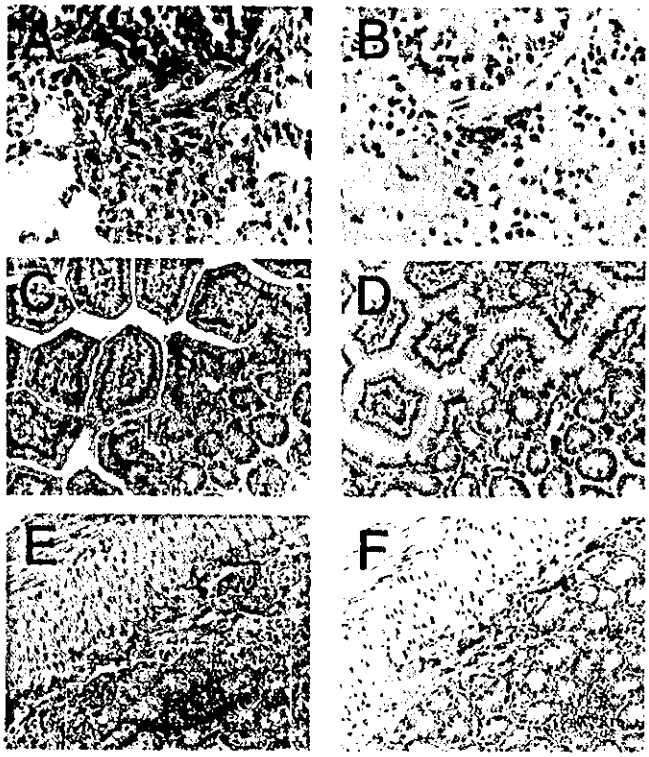

Fig. 5. Donor cell distribution after intravenous injection of the mesenchymal stem cells (MSCs) expressing enhanced green fluorescent protein (EGFP). EGFP immunoreactivities were detected in the lung (A and B), the small intestine (C and D), and the stomach (E and F). Recipient tissues (n = 28) were examined at 3 months after the transplantation of MSCs.

Engraftment of mesenchymal stem cells

Cells, which were grafted into the heart, generated neoangiogenesis near the injection site within 1 week after transplantation. The number of vessels increased significantly in the injection region and EGFP-positive donor cells could be identified in these vessels (Fig. 3A and B). Immunohistochemistry with anti-CD31, a marker for endothelium, confirmed that the donor cells of the newly formed vessels had differentiated into endothelium (Fig. 3C). At 3 months after transplantation into the heart, the donor-derived endothelium that formed vessels were observed in in vivo environments (Fig. 3D–F).

Furthermore, we assessed the fluorescence conjugated to the anti-CD31 antibody. Double-positive cells for GFP and CD31 were clearly demonstrated in the injection area (Fig. 3G–I). Donor cells engrafted in the heart appeared to maintain the characteristics of stem cells, which continue to provide the progenies, i.e., differentiated endothelial cells, in this case. At this time point, cardiomyocytes expressing EGFP protein were present in juxtapositions of both donor to donor and donor to host cells in the heart around the injection area (Fig. 4A–C). These cells formed striated muscles with a branched structure, which distinguished them from skeletal muscle cells. Another cardiac muscle-specific finding, an intercalated disk, was found at the longitudinal end of donor cells. Moreover, the alignment of the implanted cells observed in this study was parallel to the host cardiomyocytes, suggesting that the donor cells possibly cooperate with cardiac contraction. We reconfirm the presence of donor cells by visualization with alkaline phosphatase as well as with EGFP. Positive signals for alkaline phosphatase (data not shown) and the green fluorescence were clearly recognized in the injected donor cells (Fig. 4D–F).

Quantification of the EGFP-positive MSCs from serial sections showed that an estimated 2,500 donor cells were viable in the ventricle 1 week after grafting 1×10^6 cells (0.25%) into the heart. The number of endothelial cells and cardiomyocytes originating from the injected cells were 1,625 and 75 cells, respectively. The remaining donor cells could not be characterized using immunohistochemistry. The number of donor-derived cells in the heart 3 months after transplantation decreased to 1,100 cells, including 275 endothelial cells and 25 cardiomyocytes.

We injected mesenchymal stem cells intravenously to clarify whether vasculogenesis could occur elsewhere, especially in the lung. At 1 week after intravenous injection,

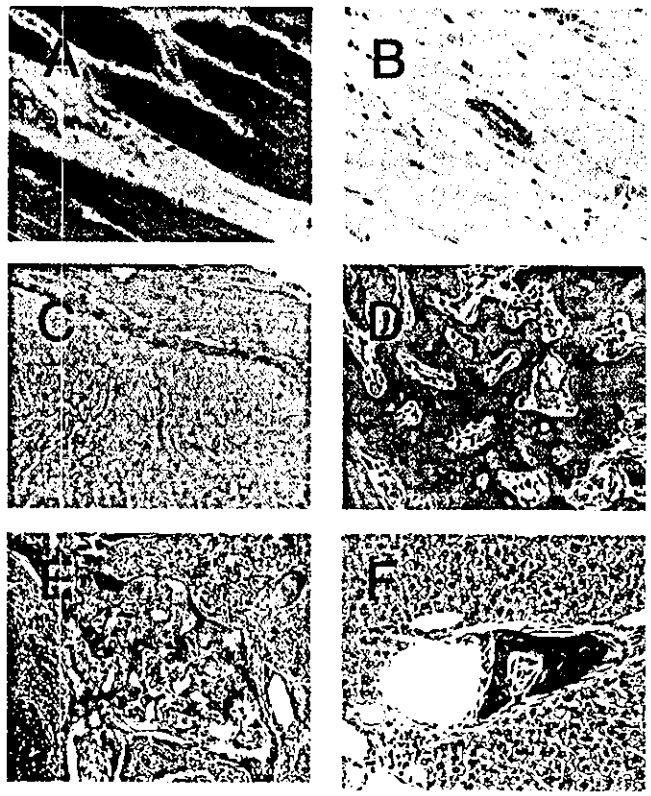

Fig. 6. Generation of endothelium and skeletal muscle by the mesenchymal stem cells (MSCs). The MSCs also differentiated into the skeletal muscle cells and fused to the host skeletal muscle 3 months after the grafting (A and B). Bone formation was detected in the skeletal muscle after a mega-dose injection of the cells (C and D). Similarly, osteogenesis was seen in the spleen (E) and the liver (F) 2 months after the injection of the MSCs into the spleen. Bone formation in the liver is probably due to transfer of donor cells via portal vein from the spleen to the liver.

mice showed predominant targeting of donor cells to the lung, where clusters of donor cells were seen in lymphatic vessels and in the vessel walls. At 4 weeks following transplantation, very few cell clusters remained, and some EGFP-positive cells covered the vessel lumens completely (Fig. 5A and B). Although EGFP-positive cells in the lung were negative for the vascular endothelium marker anti-CD31 at 1 week, they had formed tube-like structures 4 weeks after intravenous injection, suggesting that they had differentiated into smooth muscle cells or pericytes. Only a small portion of stromal cells in the interstitial space of the lung expressed EGFP 12 weeks after the grafting (Fig. 5C and D). No obvious tissue-specific differentiation was observed. In addition, no tumorous growth was seen in any tissue throughout the experiment.

Small numbers of EGFP-positive cells were also seen in the brain, thymus, uterus, and kidney throughout these experiments (data not shown). Although few EGFP-positive cells were present in the stomach and small intestine in the early phase after both intravenous and intramuscular transplantation, many donor cells were recognized in these organs at 12 weeks following injection (Fig. 5E and F).

We examined the dose escalation of MSC injection in the skeletal muscle. Donor mesenchymal stem cells differentiated along a similar time course into endothelial cells and myocytes in skeletal muscle as in heart. By 1 week after implantation, the donor cells had differentiated into endothelial cells and formed vessels in the graft sites. A skeletal myocyte phenotype was first observed in cells derived from the donor cells at 3 months after transplantation (Fig. 6A and B). When a megadose of cells (1×10^8 cells/mouse) was implanted into one site in skeletal muscles, no endothelial cells or skeletal myocytes emerged at any observation time point. However, we detected a massive hard mass in the thigh, and hematoxylin and eosin staining indicated that the mass was newly formed bone (Fig. 6C and D). Transplantation into the spleen also led to bone formation in the spleen and liver (Fig. 6E and F).

Discussion

This experiment demonstrated that MSCs retain their plasticity in the adult body in addition to being able to differentiate into mesoderm-derived cells in the fetus [15]. Immortalized cells, including MSCs, have specific patterns of DNA methylation, and because the established methylation pattern is subsequently maintained with considerable fidelity [16–18], the cell phenotype remains in its original state in the immortalized cells. The silenced genes are stably inherited throughout the culture period, and such genes can be demethylated and reactivated by a demethylating agent [19]. 5-Azacytidine, a cytosine analog, has a remarkable effect on transdifferentiation of cells and has been shown to induce differentiation of mesenchymal cells into cardiomyocytes, skeletal myocytes, adipocytes, and chondrocytes [7,20].

5-Azacytidine is incorporated into DNA and has been shown to cause extensive demethylation. The demethylation is attributable to covalent binding of DNA methyltransferase to 5-azacytidine in the DNA [21], with the subsequent reduction of enzyme activity in cells resulting in dilution out and loss of methylation at many sites in the genome. This may in turn account for reactivation of cardiomyogenic "master gene(s)," such as MEF-2C, GATA4, dHAND, TEF-1 and Nkx2.5/Csx, by 5-azacytidine, leading to transdifferentiation of MSCs into cardiomyocytes.

Isolated adult MSCs could differentiate into the progeny in a tissue-specific manner according to their ultimate destination. Nonphysiological cells, such as osteocytes, chondrocytes, or adipocytes, were not generated in this experimental setting in 41 transplants, despite that these donor cells have the potential to generate a variety of mesoderm-derived cells in vitro. Although there were some EGFP-positive cells in the interstitial spaces, no newly formed matrices were observed. There was no significant inflammatory reaction, consistent with the long survival of these donor stem cells in the heart. In this transplant procedure into the heart that we have been performing since 1992, no lethal arrhythmia has ever been recorded [13].

It has been generally accepted that cardiac myocytes are unable to divide once cell proliferation ceases shortly after birth in the mammalian heart, because mitotic figures have not been detected in myocytes [22]. Although genetically altered cardiomyocytes induced DNA synthesis in vivo and in vitro [23,24], it remained unclear whether normal or diseased cardiac myocytes can synthesize DNA. Adult hearts often exhibit a polypoid structure, which results from stochastic accumulation of mutations as cells pass through cell cycle check points [25]. However, the hypothesis that the life span of terminally differentiated cells corresponds to that of the body may contradict the concept of cellular aging. Recently, there are a number of lines of evidence that suggest that there is some cell proliferation in the adult heart. A mitotic index was calculated in patients with heart failure and 11 myocyte nuclei per 1 million cells exhibited mitotic images [26], and karyokinesis and cytokinesis were observed by confocal microscopy in adult normal subjects and patients with dilated cardiomyopathy [27]. Our data together with a report [28] that bone marrow-derived cells provided cardiomyocytes and skeletal muscle in dystrophic mdx mice support this hypothesis.

The potential for engraftment of bone marrow-derived stromal cells following transplantation has been debated in mice [29] and humans [30]. Since stromal cells are estimated to account for only around 1% of total nucleated marrow cells [31], it is very difficult to monitor engraftment of stromal cells in bone marrow transplantation. But, transplantation of a portion enriched for stromal cells from crude human bone marrow showed engraftment into SCID mice. Murine mesenchymal cells were distributed in the lung,