Comparative Analysis of Mesenchymal Stem Cells from Bone Marrow, Cartilage, and Adipose Tissue

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Mesenchymal stem cells (MSCs) isolated from bone marrow (BM), cartilage, and adipose tissue (AT) possess the capacity for self-renewal and the potential for multilineage differentiation, and are therefore perceived as attractive sources of stem cells for cell therapy. However, MSCs from these different sources have different characteristics. We compared MSCs of adult Sprague Dawley rats derived from these three sources in terms of their immunophenotypic characterization, proliferation capacity, differentiation ability, expression of angiogenic cytokines, and anti-apoptotic ability. According to growth curve, cell cycle, and telomerase activity analyses, MSCs derived from adipose tissue (AT-MSCs) possess the highest proliferation potential, followed by MSCs derived from BM and cartilage (BM-MSCs and C-MSCs). In terms of multilineage differentiation, MSCs from all three sources displayed osteogenic, adipogenic, and chondrogenic differentiation potential. The result of real-time RT-PCR indicated that these cells all expressed angiogenic cytokines, with some differences in expression level. Flow cytometry and MTT analysis showed that C-MSCs possess the highest resistance toward hydrogen peroxide -induced apoptosis, while AT-MSCs exhibited high tolerance to serum deprivation-induced apoptosis. Both AT and cartilage are attractive alternatives to BM as sources for isolating MSCs, but these differences must be considered when choosing a stem cell source for clinical application.

Introduction

THERE HAS BEEN AN explosion of reports on human stem cells isolated from a variety of sources including embryonic, fetal, and adult tissues over the last 10 years. Mesenchymal stem cells (MSCs) can be found in various adult tissues and may contribute to tissue repair or regeneration. Compared with stem cells from the embryo or fetus, adult MSCs are easy to obtain and handle. Moreover, MSCs are suggested to be immunoprivileged, such that allogeneic MSCs can be transplanted when necessary.

Bone marrow (BM) has been extensively investigated as a source of adult stem cells. Because MSCs are multipotent and readily expandable in vitro, these cells have already been employed in early clinical studies, including the treatment of human myocardial infarction, osteogenesis imperfecta, and graft versus host disease [1–4]. However, MSCs constitute only a small proportion of the cells in BM (0.01–0.001% of nucleated cells) and their number, frequency and differentiation capacity correlate inversely with age.

Subsequently, MSCs from other tissues having a similar immunophenotype have been isolated and investigated.

Adipose tissue (AT) is emerging as a source of stem cells that can be obtained by a less invasive method and in larger quantities than from BM. These cells can be isolated from human lipoaspirates and, like MSCs, can differentiate toward osteogenic, adipogenic, myogenic, chondrogenic, and especially neurogenic lineages [5].

Several groups have demonstrated that the cells derived from cartilage meet the criteria for MSCs, including high rates of proliferation, clonogenicity, multipotentiality, and MSC marker phenotype. These cells were called "cartilage-derived stromal cells" [6], "dedifferentiated chondrocytes" [7], and "articular-derived dedifferentiated chondrocytes (ADDCs)" [8]. We name these cells as cartilage-derived MSCs in our study.

When these MSCs were considered for potential therapeutic applications, various mechanisms for improving regeneration and functional repair were proposed. In addition

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to the effects caused by possible differentiation of these cells, one of the potential mechanisms for functional improvement in ischemic regions is the promotion of angiogenesis by means of the production of angiogenic cytokines [9,10]. Therefore, the expression level of angiogenic cytokines by these cells assessed in vitro may reflect their potential in angiogenesis.

The survival capacity of MSCs in host tissues in conditions of ischemia or ischemic reperfusion is another important property to be considered. The use of an MSC graft approach is limited by the fact that most of the transplanted MSCs are readily lost, potentially triggered by the ischemic or ischemia-reperfusion environment in vivo [11,12]. In our study, we investigated the anti-apoptosis ability of these MSCs toward oxidative stress induced by hydrogen peroxide (H_2O_2) or serum deprivation.

The aim of this study was to compare MSCs from BM, cartilage, and AT for their immunophenotype characterization, proliferation capacity, potential for multi-lineage differentiation, expression of angiogenic cytokines, and resistance to apoptosis.

Materials and Methods

Isolation and culture of BM-MSCs, C-MSCs, and AT-MSCs

MSC cells were harvested from 3-week-old male SD rats killed by cervical dislocation. Rats were obtained from the Beijing Animal Administration Center and the animal experiments were approved by the Animal Care and Use Committee of Peking University. Limb bones, articular cartilage, and AT from the inguinal groove were isolated and washed extensively with excess volumes of phosphate-buffered saline (PBS) to remove blood cells.

BM was harvested by flushing the tibial and femoral marrow cavities with PBS and cultured as described elsewhere [13]. The articular cartilage and AT were separately digested with 0.2% collagenase II (Sigma) with intermittent shaking at 37°C for 2 h and 0.1% collagenase I (Sigma) at 37°C for 30 min. Enzyme activity was terminated by dilution with Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA). The floating cells were separated from the mesenchymal cell fraction by centrifugation (150g) for 5 min. The pellets were resuspended in normal culture medium (DMEM, 10% FBS, and 100 U/ml penicillin/streptomycin) and filtered through a 200 µm nylon mesh to remove undigested tissue.

The primary cells were cultured in 9 cm² falcon culture plates for 4–5 days until they reached confluence and were defined as passage 0. The cells were passaged at a ratio of 1: 3. The cells used in subsequent experiments were between passage 3 and 6.

Immunophenotypic analysis

BM-MSCs, C-MSCs, and AT-MSCs at passages 3 and 6 were trypsinized into single cell suspension and stained with fluorescein (FITC)-labeled antibodies including anti-rat CD34, CD45, CD44, and CD90 (Becton Dickinson) for flow cytometric analysis. Nonspecific anti-rat IgG-FITC was

used as an isotype control. CD73 was examined by indirect immunofluorescence. The first antibody was mouse anti-rat CD73 (Becton Dickinson), and the secondary antibody was goat anti-mouse-FITC (Zhongshan Biochemical, China). Isotype control was established by eliminating the secondary antibody.

Proliferation characteristics

Growth curves. BM-MSCs, C-MSCs, and AT-MSCs at passage 3 were seeded in a 24-well plate with 1×10^4 cells per well in triplicate. Cells were collected from each well 1–7 days after seeding and counted microscopically to produce cell growth curves.

Cell cycle analysis. BM-MSCs, C-MSCs, and AT-MSCs at passages 3 and 6 ($n = 5$ each) were harvested respectively by trypsinization (0.25% trypsin-EDTA) and fixed in 70% cold ethanol, stored at 4°C and treated with 1 mg/ml RNase (TaKaRa, Japan) for 30 min at 37°C. DNA was labeled with 20 µg/ml propidium iodide (PI, Sigma) in the dark for 30 min at 4°C and DNA content was assessed by flow cytometry Calibur (Becton Dickinson) using the ModiFit LT v2.0 software. Each group was analyzed in triplicate.

Telomerase activity. The telomerase activity of BM-MSCs, C-MSCs, and AT-MSCs at passages 3 and 6 ($n = 5$ each) was detected using the telomeric repeat amplification protocol (TRAP) assay (Roche, Germany).

Induction of multilineage differentiation

In vitro differentiation was performed at passages 3 and 6 for all three cell sources. For osteogenic and adipogenic induction, cells were seeded at 2×10^5 cells/well in 6-well plates and each group was analyzed in triplicate. The control groups were cultured with normal culture medium. Chondrogenic induction was performed differently.

Osteogenic differentiation. When they reached 80–90% confluence, BM-MSCs, AT-MSCs, and C-MSCs were induced to osteogenic differentiation with osteogenic culture medium (DMEM, 10% FBS, 10 mM-glycerophosphate, 0.01 µM 1,25-dihydroxyvitamin D3, 50 µM ascorbate-2-phosphate, and 100 U/ml penicillin/streptomycin) for defined time points.

The expressions of osteogenic markers Runx 2, collagen I (COL I) and osteocalcin (OCN) were assessed by real-time RT-PCR at 1, 3, 7, 10, and 14 days after induction. Osteogenic differentiation was also confirmed by alkaline phosphatase (ALP) expression by histochemical staining and ALP activity analysis. ALP activity was measured with the ALP assay kit (Zhongsheng Biochemical, Beijing, China) at 0, 3, 7, 10, and 14 days. The results were normalized against the protein concentration and expressed as U/g/min.

Adipogenic differentiation. When they reached 80–90% confluence, BM-MSCs, AT-MSCs, and C-MSCs were induced to adipogenic differentiation with adipogenic induction medium (DMEM, 10% FBS, 1 mM dexamethasone, 0.5 mM 3-isobutyl-1-methyl-xanthine, 10 µg/ml recombinant human insulin and 100 U/ml penicillin/streptomycin) for 7 days.

The expressions of peroxisome proliferator activated receptor γ (PPARγ) and lipoprotein lipase (LPL) were

analyzed at 0, 3, and 7 days after induction by real-time RT-PCR. Adipogenic differentiation was confirmed by the formation of neutral lipid-vacuoles stainable with Oil Red O (Sigma-Aldrich) [14]. In brief, MSCs at day 7 after induction were fixed with 4% paraformaldehyde, washed and stained with 0.18% Oil Red O for 5 min. The nuclei were counterstained with hematine solution. The proportion of adipogenic differentiation was evaluated by measuring the average photodensity of Oil Red O staining areas to the total area occupied by cells with a Leica Q550 CW microscope and Qwin image acquisition software.

Chondrogenic differentiation. BM-MSCs, AT-MSCs, and C-MSCs at passages 3 and 6 were induced to chondrogenesis. Centrifugation of 2.5×10^5 cells was done at $200g$ for 5 min in a 15 ml polypropylene tube. There were two groups in this study: one group was cultured in chondrogenic differentiation medium containing 10 ng/ml TGF-β1 (Peprotech, USA), 6.25 g/ml insulin, 1% antibiotic/antimycotic, and the control group was cultured with normal medium (DMEM, 10% FBS).

Cell pellets were harvested in Trizol reagent for the isolation of mRNA at 0, 7, and 14 days after induction. The expression of collagen II (COL II), aggrecan, and fibromodulin (Fmod) was assessed by real-time RT-PCR.

Fourteen days after induction, the cell pellets were fixed with 4% paraformaldehyde, embedded in paraffin and cut into 5 μm sections [15]. Sections were stained with 1% toluidine blue (Sigma, USA) at pH 2.5 for 30 min and rinsed with tap water. Morphometric analysis of images in histological sections was carried out with an Olympus IX-70 microscope (Tokyo, Japan).

COL II expression was examined by indirect immunofluorescence. After antigen repair and 5% goat serum blocking, the sections were incubated with a polyclonal antibody against COL II (Santa Cruz Biotechnology), followed by anti-goat FITC-conjugated antibody (Zhongshan Biochemical, China) at a dilution of 1:200. After rinsing three times with PBS, sections were visualized by fluorescence confocal microscopy.

Quantitative real-time RT-PCR

Total RNA of BM-MSCs, AT-MSCs, and C-MSCs ($n = 3$ each, repeated at least three times) was extracted with Trizol reagent (Life Technologies) and quantified by ultraviolet spectroscopy at assigned time points post-induction. cDNA synthesis was performed using total RNA (1 μg) as a template by oligo(dT) priming using the Superscript First Strand Synthesis System for RT-PCR (Invitrogen). Real-time RT-PCR was performed with an optional continuous fluorescence detection system (MJ research, MA); 1 μl of reverse transcribed product and 1$\times$ SYBR green (Molecular Probes, Eugene, OR) were included in 25 μl reaction mixture (10 mM Tris-HCL, pH 8.3, 50 mM KCL, 1.5 mM MgCl$_2$, 200 μM of dNTP mix, 0.2 μM of each primer and 1 unit of Taq DNA polymerase). Real-time RT-PCR oligonucleotide primers were designed using Oligo 6 primer analysis software. PCR primers were as follows (5′–3′): Runx-2: Fw AACCC ACGAATGCACTATCCA, Rev CTTCCATCAGCGTCAAC ACCA; COL I: Fw GGAGAGAGTGCCAACTCCAG, Rev CCACCCCAGGGATAAAAACT; OCN: Fw AACGGTG GTGCCATAGATGC, Rev AGGACCCTCTCTCTGCTCAC; PPARγ: Fw TGGAGCCTAAGTTTGAGTTTGC, Rev TGACAATCTGCCTGAGGTCTG; LPL: Fw GAGATT TCTCTGTATGGCACA, Rev CTGCAGATGAGAAACTT TCTC; COL II: Fw CACCGCTAACGTCCAGATGAC, Rev GGAAGGCGTGAGGTCTTCTGT; aggrecan-1: Fw CCA CTGGAGAGGACTGCGTAG, Rev GGTCTGTGCAAGTGA TTCGAG; Fomd: Fw ACGTCTACACCGTCCCTGACA, Rev CCTGCAGCTTGGAGAAGTTCA; VEGF: Fw ACTGGACCCTGGCTTTACTG, Rev ACGCACTCCA GGGCTTCATC; IGF: Fw GCATTGTGGATGAGT GTTGC, Rev GGCTCCTCCT ACATTCTGTA; PDGF: Fw AAGCATGTGCCGGA GAAGCG, Rev TCCTCTAACCT CACCTGGAC; HGF: Fw TATTTACGGCTGGGGCTACA, Rev ACGACCAGGAACAATGACAC; bFGF: Fw AAGC GGC TCTACTGCAAG, Rev AGCCAGACATTGGAAGAA ACA; TGF-α: Fw TGTGCTGATCCACTGCTGTCA, Rev AGCAG GCAGTCCTTCCTTTCA; Ang-1: Fw TCGCTGCCATTCT GACTCAC, Rev TCTGGGCCATCTCCGACTTC; SCF: Fw TGGTGGCATCTGACACTAGTGA, Rev CTTCCAGTATA AGGCTCCAAAAGC. Each cycle consisted of 30 s denaturation at 94°C, 45 s annealing at 60°C, and 45 s extension at 72°C. Levels of mRNA were normalized against GAPDH using the comparative cycle threshold (CT) method. PCR primers for GAPDH (5′–3′) were: Fw GAAAAGCTGTGGC GTGATGG-3, Rev GTAGGCCATGAGGTCCACCA.

Apoptosis induction and detection

2 mmol/L H$_2$O$_2$-induced apoptosis. BM-MSCs, AT-MSCs, and C-MSCs at passage 3 ($n = 6$ each) were seeded at a density of 5×10^4/cm^2 in a 24-well plate, cultured for a further 24 h and then changed to apoptosis-inducing medium (2 mmol/L H$_2$O$_2$, DMEM and 10%FBS). After 90 min, obvious apoptosis was observed under confocal microscopy using In Situ Cell Death Detection Kit (Roche, Germany) and Annexin-v-FITC Apoptosis Detection Kit (Biosea, Beijing, China). Apoptotic cell percentage was detected by flow cytometry with Annexin-v-FITC Apoptosis Detection Kit.

Serum deprivation-induced apoptosis. BM-MSCs, AT-MSCs, and C-MSCs at passage 3 were seeded at 2×10^3 cells per well in a 96-well plate and then subjected to 24 to 72 h exposure to serum free medium. Apoptosis was detected by MTT and flow cytometry analysis (as described above). The MTT method is based on the ability of living cells to reduce MTT tetrazolium salt to MTT formazan with the mitochondrial enzyme succinate-dehydrogenase. Briefly, cells were incubated for 2 h with MTT solution (0.5 mg/ml PBS) and MTT formazan was then extracted in DMSO. Measurement of optical density was performed at 560 nm with a microplate reader. The optical density of the control (cell culture without any treatment) corresponds to 100% MTT reduction. Results were expressed as a percentage of the control and data were presented as mean values $\pm$ SD ($n = 3$).

Statistical analysis

All experiments were repeated a minimum of three times. Data are presented as mean $\pm$ SD. The One-Way

ANOVA test was used to analyze results of flow cytometry, real-time RT-PCR, MTT and comparison of multilineage induction at different time points. Differences between the experimental and control groups were regarded as statistically significant when $p < 0.05$. The SPSS software package (version 13.0; SPSS Inc., USA) was used for the statistical tests.

Results

Isolation and proliferation characteristics of BM-MSCs, C-MSCs, and AT-MSCs

Cells isolated from limb bones, articular cartilage, and AT were initially plated in 9-cm^2 falcon culture plates. After 3–4 days culture, 6–10 cell colonies were observed in the BM-MSC and C-MSC plates, whereas AT-MSCs reached 100% confluence.

There were mixtures of cells at the original passage. During passaging to the third generation, cells derived from the three different sources became more uniform and grew in a monolayer with typical fibroblast morphology. BM-MSCs were larger than the others morphologically (Fig. 1A).

To further characterize these cells, cell surface markers were examined by flow cytometry. MSCs from all three sources were negative for the hematopoietic lineage markers CD34 and CD45, indicating that they were of non-hematopoietic origin. The percentage of CD44 positive cells in BM-, C- and AT-MSCs was 94.16%, 95% and 98.4%; CD73 was 84.07%, 60.63% and 96.98%; and CD90 (Thy-1) was 93.38%, 61.91% and 95.61%, respectively (Fig. 1B).

Expansion characteristics

Among the three sources of MSCs, AT-MSCs grew at the highest speed and kept almost the same growth speed throughout ten generations whereas C-MSCs showed replicative senescence as indicated by a loss of proliferation after the eighth generation. Compared with AT- and C-MSCs, BM-MSCs appeared to grow at a relatively slow but constant speed until the tenth generation. The population doubling time of BM-MSCs, C-MSCs, and AT-MSCs was 61.2, 51.47, and 45.2 h, respectively, based on the logarithmic growth phase. AT-MSCs possessed the lowest population doubling numbers at passages 3. BM-MSCs reached 100% confluence in 5 days, partly because the area of a single BM-MSC is larger than that of C-MSC or AT- MSC (Fig. 1C).

Flow cytometric analysis of the cell cycle showed different percentages of cell populations in each phase. The results indicated that AT-MSCs possessed the greatest proliferation capacity with the highest proportion in S phase, followed by BM-MSCs and C-MSCs (Fig. 1D). G1, G2/M, S proportions of MSCs at passage 3 and 6, respectively, are shown in Table 1. Significant differences in S phase were observed among the three MSC types (*$p < 0.05$).

The telomerase activity results indicated differences among BM-MSCs, AT-MSCs and C-MSCs at the same passage. There was no significant difference between passage 3 and passage 6 for the same type of cell (Fig. 1E).

Multilineage differentiation potential

We investigated the potential of BM-MSCs, C-MSCs, and AT-MSCs to differentiate toward osteogenic, adipogenic and chondrogenic lineages.

Osteogenic differentiation capacity. ALP staining showed that the three types of MSC all possessed the capacity for osteogenic differentiation 2 weeks after induction (Fig. 2A). No ALP aggregates were observed in control groups (not shown).

The expression of osteogenic genes was assessed at 1, 3, 7, 10, and 14 days after induction by real-time RT-PCR. The genes included Runx 2, a transcription factor at the downstream end of bone morphogenetic protein (BMP) signaling pathways, collagen I (COL I), and OCN, an extracellular matrix protein and a marker of mature osteoblasts. Upregulated mRNA expression of all three osteogenic genes was observed in induced MSCs but with different patterns. Generally, upregulation of all these genes peaked at 3 or 7 days in BM-MSCs, well ahead of AT-MSCs and C-MSCs (Fig. 2B–D).

Moreover, the ALP activity assay showed that BM-MSCs possess higher ALP activity than the other MSCs. The ALP activity of BM-MSCs markedly increased at days 10 and 14, and remained at a higher level than C-MSCs and AT-MSCs thereafter. There was no significant difference between the AT-MSC and C- MSC groups (Fig. 2E).

Adipogenic differentiation capacity. Adipogenic differentiation was demonstrated by the accumulation of neutral lipid vacuoles indicated by the Oil Red O stain (Fig. 3A). No red staining was detected in control groups (not shown).

The expressions of PPARγ and LPL were analyzed at 0, 3, and 7 days after induction by real-time RT-PCR. PPARγ, a lipocyte-specific transcription factor, and LPL, a lipid exchange enzyme, were upregulated during adipogenesis. The basic expressions of PPARγ and LPL were initially detected in AT-MSCs and their expression levels reached a peak at day 3 after induction. At the same time, compared with BM-MSCs, the expressions dramatically increased in C-MSCs and reached a peak at day 3 (Fig. 3B).

In order to quantify the ratio of lipogenic differentiation, additional slides stained with Oil Red O 7 days after induction were prepared for densitometric analysis. The ratio of red staining area to the total area of cells was significantly higher in C-MSC and AT-MSC samples than that of BM-MSCs ($p < 0.01$) (Fig. 3C).

Chondrogenic differentiation capacity. Compared with control groups, chondrogenic differentiation of BM-, C- and AT-MSCs was confirmed by the formation of sphere-like pellets and the secretion of cartilage-specific proteoglycans stainable with toluidine blue. Specially, C-MSCs were able to form pellets that stained more intensely with toluidine blue, not only in the induction groups but also in the non-induction groups (Fig. 4A).

COL II and aggrecan mRNA reached a maximum at about 7 days in C-MSCs, whereas mRNA levels increased up to 14 days in BM-MSCs and AT-MSCs. Fibromodulin (Fmod) levels exhibited a time-dependent increase up to 2 weeks after induction in all three type of cells (Fig. 4B).
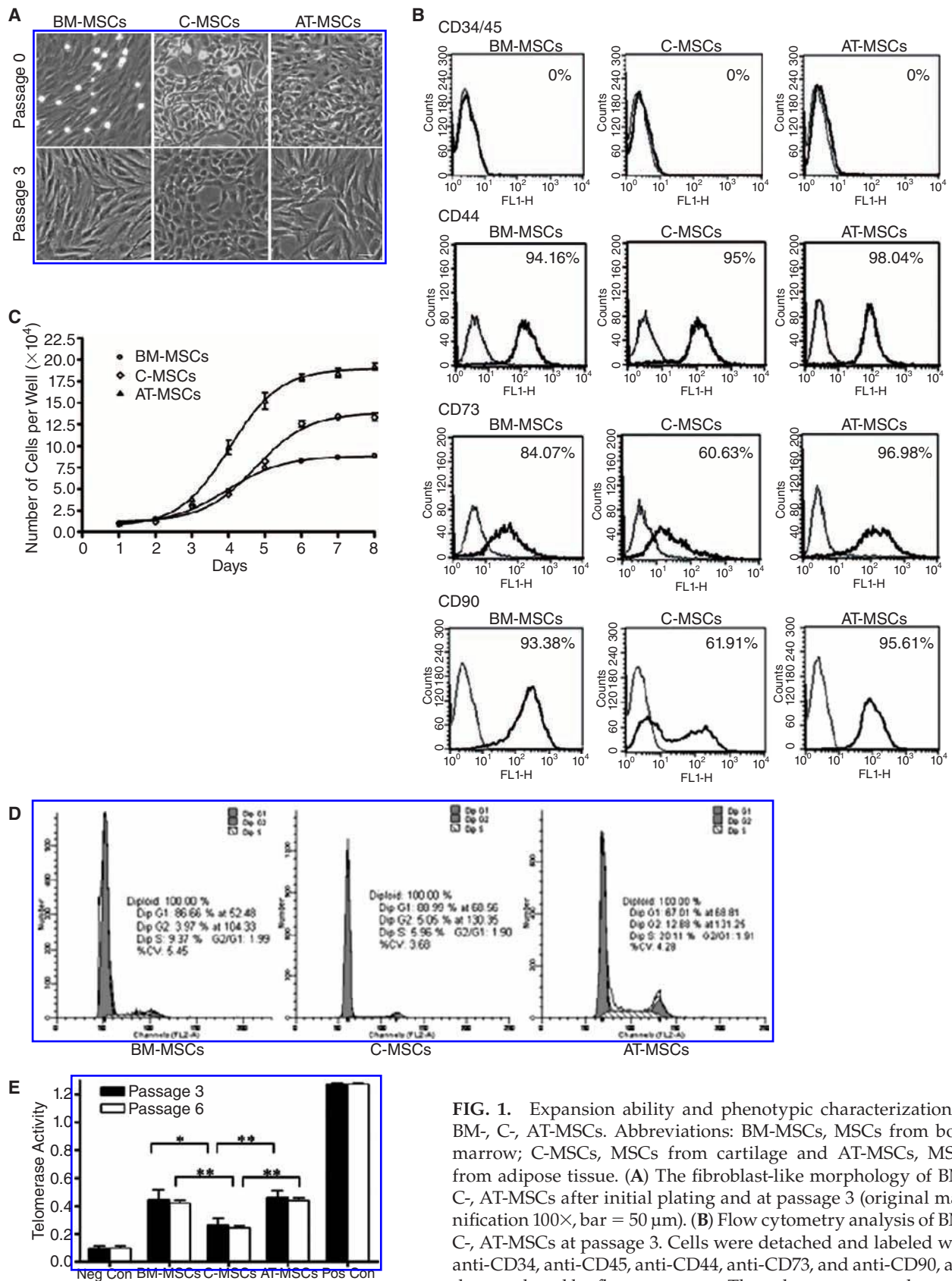
FIG. 1. Expansion ability and phenotypic characterization of BM-, C-, AT-MSCs. Abbreviations: BM-MSCs, MSCs from bone marrow; C-MSCs, MSCs from cartilage and AT-MSCs, MSCs from adipose tissue. (**A**) The fibroblast-like morphology of BM-, C-, AT-MSCs after initial plating and at passage 3 (original magnification 100×, bar = 50 μm). (**B**) Flow cytometry analysis of BM-, C-, AT-MSCs at passage 3. Cells were detached and labeled with anti-CD34, anti-CD45, anti-CD44, anti-CD73, and anti-CD90, and then analyzed by flow cytometry. The values represent the mean percentage of all assessed cells positively stained by the respective antibodies. (**C**) Growth curve of BM-, C-, AT-MSCs at passage 3 ($n = 3$, mean ± SD). (**D**) Flow cytometry analysis of cell cycle of BM-, C-, and AT-MSCs. (**E**) Telomerase activity of MSCs examined by Telomeric repeat amplification reaction. Extract of Hela cells was used as a positive control and corresponding extracts treated with RNase was used as negative controls ($n = 5$ each, mean ± SD, *$p < 0.05$, **$p < 0.01$).

TABLE 1. CELL CYCLE ANALYSIS OF MESENCHYMAL STEM CELLS FROM BONE MARROW,
CARTILAGE AND ADIPOSE TISSUE BY FLOW CYTOMETRY

	BM-MSCs		C-MSCs		AT-MSCs	
	Passage 3	Passage 6	Passage 3	Passage 6	Passage 3	Passage 6
G1 (%)	82.24 ± 3.43	89.57 ± 3.2	90.61 ± 2.49	93.19 ± 3.08	73.96 ± 2.43	75.44 ± 3.13
G2 (%)	6.44 ± 3.53	2.3 ± 2.06	3.74 ± 2.36	3.33 ± 2.32	0.003 ± 0.005	4.90 ± 3.18
S (%)*	11.3 ± 1.24	8.13 ± 1.18	5.64 ± 0.28	3.49 ± 0.86	26.22 ± 2.67	19.66 ± 0.94

The table shows the mean values of the percentage of cells at each phase (mean ± standard deviation). Significant differences in S phase were observed between every two groups (*$p < 0.05$).

A high level of COL II expression was also observed after chondrogenic induction of C-MSCs using confocal microscopy after immunofluorescent staining. In the control group, though no mature chondrocytes were shown in the C-MSC group, the intensity of COL II green fluorescence was significantly higher than in the parallel control groups of BM-MSCs and AT-MSCs (Fig. 4C).

Angiogenic cytokine expressions detected by real-time RT-PCR

The expression levels of various angiogenesis factors in BM-, C-, and AT-MSCs at passages 3 and 6 were measured by real-time RT-PCR. These factors included the following: vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), transforming growth factor-β1 (TGF -β1), angiopoetin-1 (Ang-1), and stem cell factor (SCF).

The results indicated basic expression of those angiogenic cytokines in original culture and varied from passage 3 to passage 6. In general, expression levels of these factors were higher in passage 3 than in passage 6. The expressions of VEGF (Fig. 5A) and IGF (Fig. 5B) were higher in BM-MSCs than other types of MSCs, whereas PDGF (Fig. 5C) and HGF (Fig. 5D) were highest in C-MSCs. The expression of bFGF (Fig. 5E) in AT-MSCs showed an incredibly high level at passage 3, which decreased dramatically by passage 6. Moreover, TGF-β1 exhibited lower level expression in C-MSCs than BM-MSCs and AT-MSCs (Fig. 5F). Ang-1, a strong angiogenesis factor, was expressed at a relatively high level at passage 3 of all three types of MSCs but decreased at passage 6 (Fig. 5G). High level expression of SCF was observed in BM-MSCs at passage 3 but not in C-MSCs and AT-MSCs (Fig. 5H).

Anti-apoptosis ability

Apoptosis triggered by 2 mmol/L H_2O_2. After 90 min of 2 mmol/L H_2O_2 induction, obvious morphology changes in the BM-MSCs, C-MSCs, and AT-MSCs were observed by light microscopy. AT-MSCs showed the most sensitive reaction to oxidative stress in that most of cells detached from the plate. In contrast to AT-MSCs and BM-MSCs, C-MSCs showed superior tolerance to oxidative stress with the least morphological change. Apoptosis was confirmed through confocal microscopy using Tunel staining (Fig. 6A) and Annexin-V and PI double staining (Fig. 6B).

Quantitative analysis of apoptosis was also conducted by FACS and confirmed that C-MSCs possessed the highest tolerance to oxidative stress, followed by BM-MSCs (Fig. 6C). Significant differences were observed among the three kinds of MSCs (Fig. 6D).

Apoptosis induced by serum deprivation. To evaluate the survivability of MSCs from the three different sources in response to serum-free culture, cells were analyzed by MTT. Within 60 h, proliferation ability was inhibited to different extents and the morphology of cells had changed. C-MSCs showed inferior tolerance to serum-free culture than the other MSCs (Fig. 6E).

At 60 h after induction of serum deprivation, BM-MSCs, C-MSCs, and AT- MSCs were stained with Annexin-V and PI and assessed by FACS. The ratio of apoptosis was consistent with the result of MTT. BM-MSCs and AT-MSCs have superior anti-apoptosis capacity toward serum-free culture (Fig. 6F and G).

We summarize our observations in Table 2.

Discussion

Transplantation of autologous or allogeneic MSCs represents a novel form of stem cell therapy which shows substantial promise in the treatment of a number of human diseases. In order to provide a foundation for further biological characterization, we analyzed MSCs from rat BM and two alternative sources, cartilage and AT. As potential seed cells for stem cell transplantation, their ease of isolation, survival ability and expansion potential, capacity for differentiation, and potential to enhance repair in vital tissues are among their most important properties [5,16,17]. This study, to our knowledge, presents for the first time a systematical and all-round comparison of MSCs from BM, cartilage, and AT for the purpose of setting up an experimental evaluation system to help choose a better cell source for further clinical therapies. Our observations could provide some experimental evidences on choosing a suitable cell source for a particular therapeutic purpose.

Our findings indicate that: (1) AT-MSCs are a promising source due to their high proliferation ability; (2) C-MSCs possess superior capacity toward chondrogenic differentiation and therefore might be a good seed cell source for cartilage tissue engineering; (3) MSCs from BM, cartilage, and AT all express angiogenic cytokines; (4) C-MSCs possess the highest resistance toward H_2O_2-induced apoptosis, while AT-MSCs exhibit high tolerance to serum deprivation-induced apoptosis.
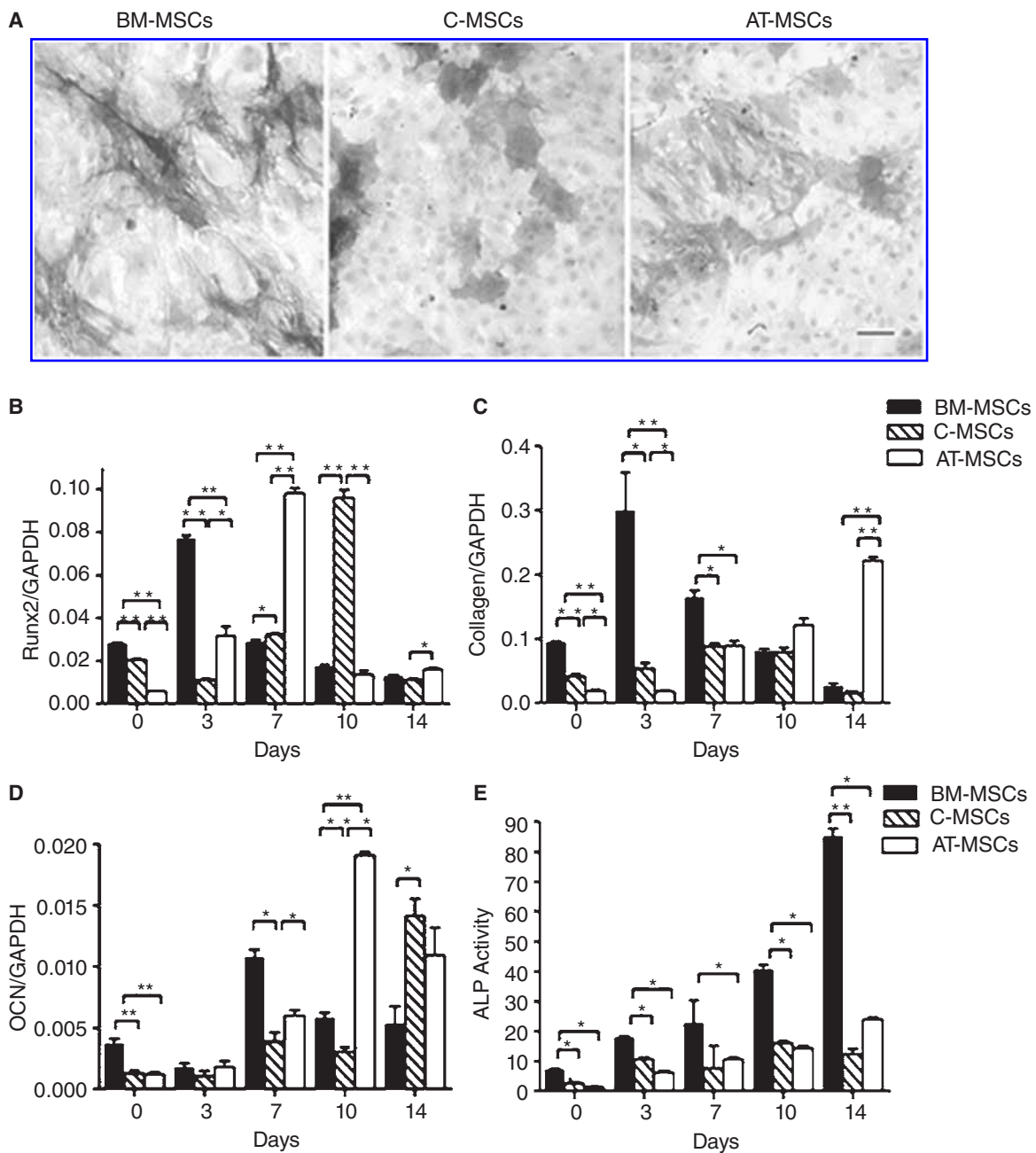
FIG. 2. Comparative analysis of the osteogenesis differentiation capacity of BM-, C-, and AT-MSCs. (**A**) Osteogenesis was demonstrated by enhancement of alkaline phosphatase activity 14 days after induction (original magnification 100×, bar = 50 µm). Upregulation of the expression of specific osteogenic genes, runt related transcription factor 2 (Runx-2) (**B**), collagen I (COL I) (**C**), and osteocalcin (OCN) (**D**), were evaluated by real-time RT-PCR at 0, 3, 7, 10, and 14 days post-induction (mean ± SD, $n = 3$, *$p < 0.05$, **$p < 0.01$). (**E**) Analysis of ALP activity in the lysates of BM-, C-, AT-MSCs at 0, 3, 7, 10, and 14 days. ALP activity in BM-MSCs was significantly higher than C- and AT-MSCs. The results of real-time RT-PCR and ALP activity were analyzed by One-Way ANOVA test (mean ± SD, $n = 3$ each, *$p < 0.05$, **$p < 0.01$).

A promising alternative source to BM-MSCs, MSCs from human AT can be obtained by a less invasive method and harvested in larger quantities than from other sources [5,18]. According to the growth curve analysis, the population doubling time of AT-MSCs is 45.2 h, significantly shorter than BM-MSCs (61.2 h), and C-MSCs (51.47 h). The percentage of AT-MSCs in S phase was 26.22 ± 2.67 % at passage 3 and

19.66 ± 0.94 h at passage 6, indicating that AT-MSCs possess high self-propagating potential, which was verified by their high telomerase activity.

Human cartilage has been reported to contain multipotent stem cells that possess functional capacity both for self-renewal and multipotential differentiation [7,8]. In our research, we harvested a population of stem cells from rat
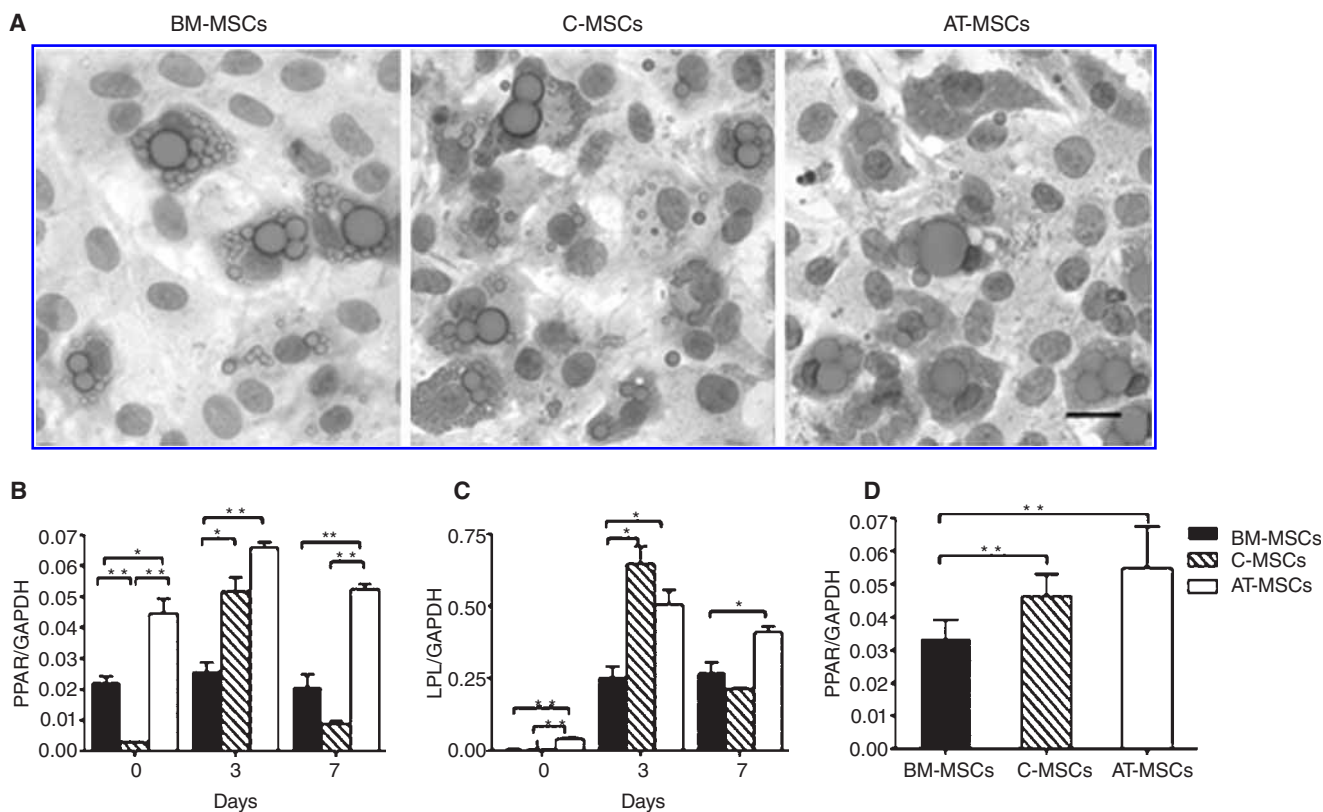
FIG. 3. Comparative analysis of adipogenesis differentiation capacity of BM-, C-, and AT-MSCs. (**A**) Adipogenesis as detected by the formation of neutral lipid vacuoles stainable with Oil Red O 7 days after induction (original magnification 200×, bar = 30 μm). The expression of specific adipogenic genes, peroxisome proliferator-activated receptor γ (PPARγ) (**B**) and lipoprotein lipase (LPL) (**C**), evaluated by real-time RT-PCR at 0, 3, 7, days post-induction. The results were analyzed by One-Way ANOVA test (mean ± SD, $n = 3$, $*p < 0.05$, $**p < 0.01$). (**D**) Densitometric analysis of Oil Red O staining of BM-, C-, and AT-MSCs 7 days after induction. The ratio of red-stained area to the total area of cells was calculated by image analysis and represented the ratio of adipogenic differentiation.

cartilage, which proved to have self-renewal and meso-dermal differentiation capacity. Furthermore, this population of cells was negative for CD34 and CD45, and positive for CD44, CD73 and CD90. These results are similar to those of previous reports [18,19].

Multi-lineage differentiation potential has been considered an important quality of stem cells. In the present study, MSCs from BM, cartilage, and AT were verified to possess osteogenic, adipogenic, and chondrogenic potential. Meanwhile, the differences in differentiation propensity illustrated that the capacity for differentiation should be evaluated. Depending on the expression of lineage-specific markers, BM-MSCs exhibited superior capacity to osteogenic differentiation but inferior capacity to adipogenic differentiation. Compared with BM- and AT-MSCs, C-MSCs have the greatest potential for chondrogenesis based on the formation of cartilage matrix and the expression of COL II, indicating that C-MSCs may be a good cell source for cartilage tissue engineering.

Why do such differences exist? One possibility is that MSCs are composed of different types of precursor cells rather than having a pure cell population [5]. This is supported by the fact that non-differentiated MSCs expressed multiple osteogenic, adipogenic, and chondrogenic genes as shown by the results of real-time RT-PCR. However, our investigations were limited to studying mesodermal differentiation. The spectrum of differentiation of MSCs does not seem to be restricted to these lineages. MSCs derived from BM and AT have been shown to differentiate into other mesodermal lineages and into endo- and ectodermal lineages as well [20–22].

How did MSCs affect repair and regeneration? In animal models of cardiac ischemia, a large body of evidence indicates that administration of angiogenic cytokines can augment reperfusion and enhance neovascularization through paracrine mechanisms. Several growth factors have angiogenic activity, such as VEGF, bFGF, PDGF, HGF, IGF, TGF, and Ang-1 [13,23–25]. SCF was considered to conduct the mobilization of BM stem cells [26]. However, the basic expression of angiogenic cytokines was not clear. We compared the expression of angiogenic cytokines in BM-, C-, and AT-MSCs at different passages under normal growth conditions by quantitative RT-PCR. The results demonstrated that expression levels of angiogenesis factors differed among
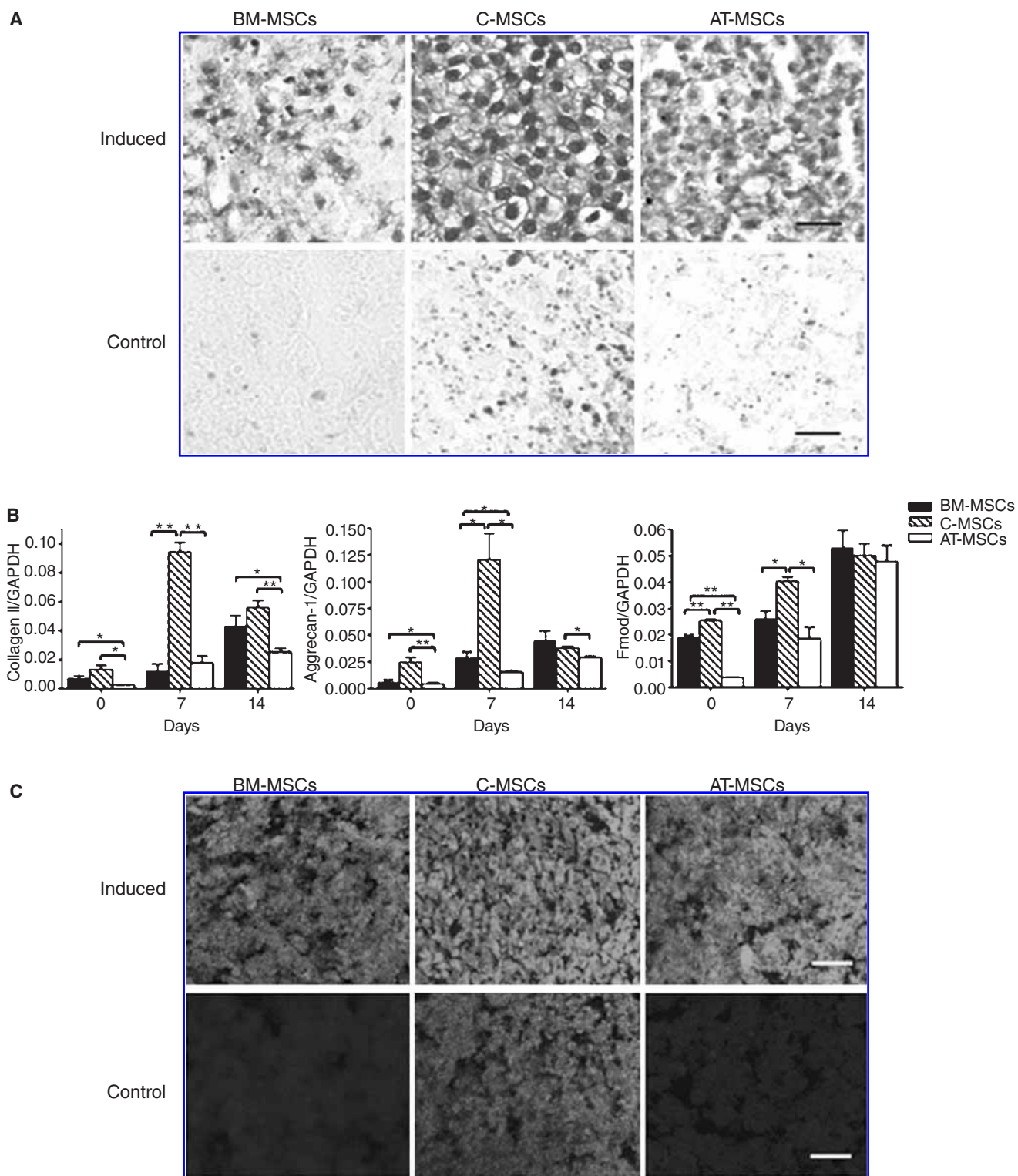
FIG. 4. Comparative analysis of chondrogenesis differentiation capacity of BM-, C-, and AT-MSCs. (**A**) Chondrogenesis was indicated by toluidine blue staining in cryosections from all three sources 14 days after induction. The upper group was induced with classic induction medium and the control group was cultured with non-inductive medium. (**B**) The expression of specific chondrogenic genes collagen II (COL II), aggrecan-1, and fibromodulin (Fmod) were evaluated by real-time RT-PCR at 0, 7, and 14 days post-induction. The results were analyzed by One-Way ANOVA test (mean ± SD, $n = 3$ each, $p < 0.05$, $p < 0.01$). (**C**) The expression of COL II was examined by indirect immunofluorescence (original magnification 400×, bar = 20 μm).
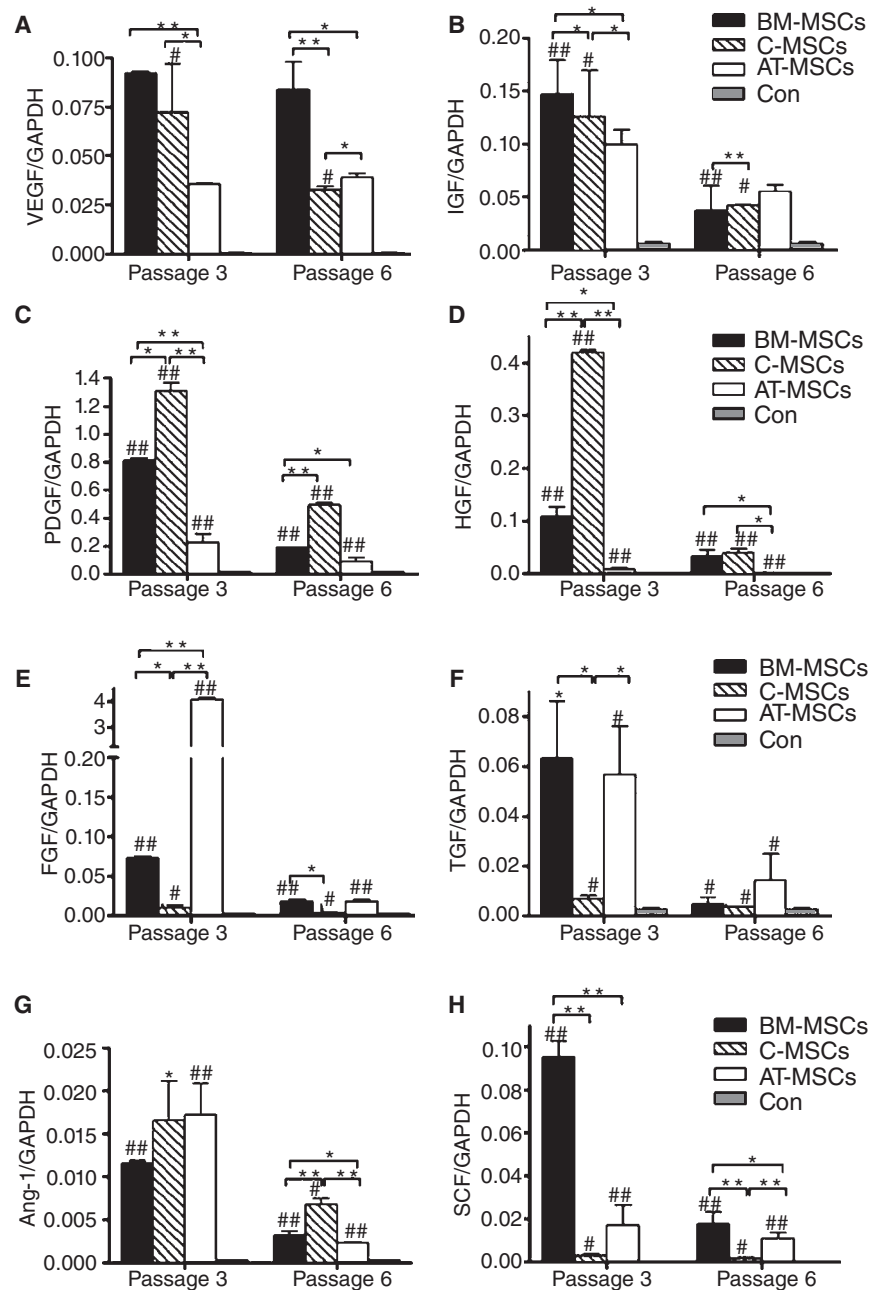
FIG. 5. mRNA expression of angiogenesis factors in BM-, C-, and AT-MSCs examined by real-time RT-PCR. (**A**) Vascular endothelial growth factor (VEGF), (**B**) insulin-like growth factor (IGF), (**C**) platelet-derived growth factor (PDGF), (**D**) hepatocyte growth factor (HGF), (**E**) basic fibroblast growth factor (bFGF), (**F**) transforming growth factor-β1 (TGF-β1), (**G**) angiopoietin-1 (Ang-1), and (**H**) stem cell factor (SCF). The results indicated the relative level of angiogenesis factor secreted by MSCs. Control cells are Rat-2, cells of rat fibroblast lineage. The results were analyzed by One-way ANOVA test (mean ± SD, $n = 6$ each, #$p < 0.05$ vs. different passage of the same MSCs, ##$p < 0.01$ vs. different passage of the same MSCs *$p < 0.05$, **$p < 0.01$).

the three sources of MSCs and decreased with passaging as shown by comparison of MSCs at passage 3 and passage 6. The different types of MSC show differential expression of the various angiogenic cytokines. Though the actual expression levels of these genes in vivo may differ from the baseline in vitro, the basic expression level is a possible way of investigating the angiogenic effects. How these angiogenic cytokines function in vivo requires further investigation.

Another aspect we considered an important property for seed cells is survival capacity. To imitate conditions in vivo, MSCs was exposed to superoxide stress and serum deprivation. Surprisingly, C-MSCs were found to possess superior anti-apoptotic ability under H_2O_2 conditions but inferior survival ability toward serum deprivation. AT-MSCs showed the opposite, with higher sensitivity to superoxide stress, which usually accompanies ischemia-reperfusion damage.
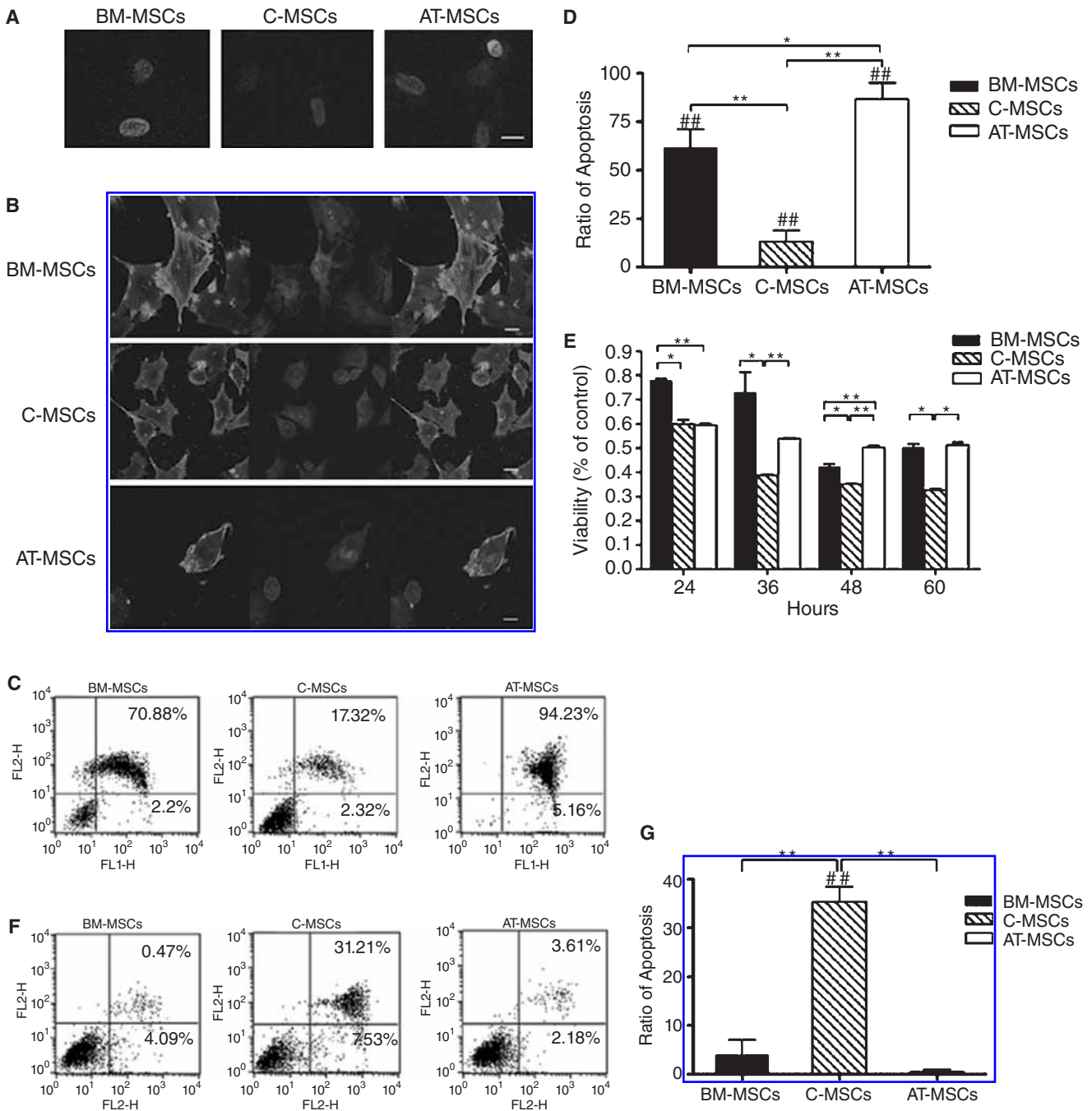
FIG. 6. H_2O_2 and serum deprivation triggered apoptosis in BM-, C-, and AT- MSCs. Apoptosis in MSCs were identified by nuclear positive staining with terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick end labeling (TUNEL) after 90 min H_2O_2 induction (**A**) (original magnification 600×, bar = 10 μm). Annexin V and propidine iodide (PI) staining (**B**) (original magnification 600×, bar = 20 μm). Apoptosis was then quantified by FACS analysis after staining with Annexin V and PI. Viable cells are Annexin V−/PI−. The Annexin V+/PI− cells are in the early apoptotic process, whereas the Annexin V+/PI+ cells have lost cell membrane integrity and have entered the late phase of apoptosis. Necrotic cells show Annexin V−/PI+. Representative examples are shown in (**C**). Serum deprivation induced a reduction in viability determined by MTT method (**E**) and apoptosis 60 hours post-induction was quantified by FACS analysis after staining with Annexin V and PI (**F**). The results are presented as the ratio of apoptosis induced by 2 mmol/L H_2O_2 and serum deprivation compared with corresponding control cells shown in (**D**) and (**G**) (mean ± SD, n = 3 each, *p < 0.05, **p < 0.01, ##p < 0.01 vs. control group).

Table 2. Characteristics Comparison of MSCs from Bone Marrow, Cartilage and Adipose Tissue

| | Proliferation | Differentiation potentials | | | Angiogenic cytokines | Anti-apoptosis | |
		Osteogenic	Adipogenic	Chondrogenic		H_2O_2	SD**
BM-MSCs	++	++	+	+	*	++	++
C-MSCs	+	+	++	++	*	+++	+
AT-MSCs	+++	+	++	+	*	+	+++

The table shows the comparative results of MSCs from BM, C, and AT. Significant differences were shown as "+++" "++" and "+." "+++" shows the highest ability among the three kinds of MSCs; "++" shows moderate ability;"+" shows the least ability. *The basal expressions of different angiogenic cytokines were varied in greater degrees and thus we did not attempt to make a general rating. **SD stands for serum deprivation.

These results indicate that the optimal method for and timing of cell transplantation needs to be considered with a view to the potential clinical application.

The findings that MSCs from different sources behave differently toward H_2O_2-induced or serum deprivation-induced apoptosis are extremely interesting. Some studies indicated that serum deprivation-induced apoptosis in MSCs occurred through the mitochondrial apoptotic pathway by inducing Bax protein translocation to the mitochondria, loss of $\Delta\psi m$, release of cytochrome c, and activation of caspase cascades, but in a p53-independent manner [11].While the mechanism of H_2O_2-induced apoptosis in MSCs is still not well understood. It was reported that H_2O_2-induced apoptosis in neural cells was mediated by p53 pathway [27]. If this mechanism is also involved in MSCs, the different behavior of the three sources of MSCs toward oxidative stress and serum deprivation might be explained. To fully elucidate the mechanisms, further studies are needed.

Taking into account the advantages and disadvantages of the three stem cell sources discussed above, clinical applications may be based on their differentiation capacity, but more likely on the abundance, frequency, and expansion potential of the cells. AT-MSCs could be a good resource as an alternative to BM-MSCs, and C-MSCs might be a good candidate for cartilage tissue engineering.

Acknowledgments

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