·英文专栏·

Adipose tissue is an ideal stem cell source for tissue reconstruction of bone, cartilage, and soft tissue defects*

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Abstract: Background The use of stem cells for bone, cartilage, and adipose tissue engineering is promising for hard and soft tissue reconstruction. Bone marrow contains several cell populations, including mesenchymal stem cells (MSCs) that are capable of differentiating into adipogenic, osteogenic and chondrogenic cells. However, harvesting of stem cells from bone marrow has potential limitations such as low cell yield, patient discomfort and high cost. Consequently, alternative sources of autologous adult stem cells, obtainable in large quantities, under local anesthesia and with minimal patient discomfort are currently under investigation. Human adipose tissue-derived stromal cells (hADSCs), may be one such potential source as large quantities can be easily obtained by liposuction. **Objective** This study aims to verify whether hADSCs can differentiate along an osteoblastic, chondrocytic and adipocytic lineage, which could then potentially be used to restore bone, cartilage and soft tissue defects, respectively. **Methods** Osteogenic, chondrogenic, and adipogenic differentiation of hADSCs were induced by osteogenic medium (Dulbecco's modified Eagle's medium + 10% fetal bovine serum + dexamethasone + ascorbate + β-glycerophosphate), chondrogenic medium (Dulbecco's modified Eagle's medium + 1% fetal bovine serum + insulin + ascorbate + transforming growth factor-β1), or adipogenic medium (Dulbecco's modified Eagle's medium + 10% fetal bovine serum + dexamethasone + insulin + indomethacin + isobutyl-methylxanthine) respectively for 1 ~ 3 weeks. Osteogenic differentiation was assessed by von Kossa and alkaline phosphatase staining, while chondrogenic and adipogenic differentiation were assessed by Alcian blue staining and Oil Red O staining respectively. Expression of osteoblast specific genes, chondrocyte specific genes, and adipocyte specific genes were confirmed by RT-PCR. **Results** In the presence of lineage-specific induction factors, hADSCs differentiated in vitro into osteogenic, chondrogenic, and adipogenic cells. Conclusions hADSCs contain multipotent cells and may represent an ideal stem cell source for use in hard and soft tissue engineering.

Key words: human adipose tissue-derived stromal cells; osteoblasts; chondrocytes; adipocytes; differentiation

脂肪组织是骨、软骨、软组织缺损组织工程再建的理想干细胞来源

刘云松 周永胜 谭建国

摘要：目的 验证人脂肪基质细胞是否具有向成骨细胞、软骨细胞、脂肪细胞分化的能力，从而为骨、软骨、软组织再建寻找一种理想的干细胞来源。**方法** 分别用成骨向分化培养基(DMEM + 10% FBS + 地塞米松 + 维生素 C + β-甘油磷酸)、软骨向分化培养基(DMEM + 1% FBS + 胰岛素 + 维生素 C + 转化生长因子 β1)及脂肪向分化培养基(DMEM + 10% FBS + 地塞米松 + 胰岛素 + 吲哚美辛 + 异丁基甲基黄嘌呤)诱导人脂肪基质细胞向成骨细胞、软骨细胞及脂肪细胞分化。用 von Kossa 和碱性磷酸酶染色鉴定成骨细胞分化，而软骨细胞分化和脂肪细胞分化分别用 Alcian blue 染色和油红 O 染色显示。成骨细胞、软骨细胞以及脂肪细胞特异相关或标志基因的表达用 RT-PCR 检测。**结果** 体外实验表明，人脂肪基质细胞在定向分化诱导剂的作用下可分别向成骨细胞、软骨细胞及脂肪细胞分化。**结论**

* This Study was supported by a grant from the National Natural Science Foundation of China (No.30200319).

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人脂肪基质细胞中包含有多向分化能力的干细胞，可用于今后骨、软骨、软组织的组织工程再建。
关键词：人脂肪基质细胞；成骨细胞；软骨细胞；脂肪细胞；分化

The incidence of bone and soft tissue defects has progressively increased over the years. Defects result from a variety of conditions, including, tumor resections, trauma, extensive deep burns, and cartilage damage due to injury or osteoarthritis, and others. The use of stem cells for bone, cartilage and adipose tissue engineering is considered promising for future cell-based reconstruction for these tissues.[1, 2] Although studies suggest that embryonic stem cells (ESCs) may be ideal candidates for cell-based technologies,[3] potential regulatory limitations and ethical considerations may limit their use. Mesenchymal stem cells (MSCs) isolated from bone marrow stroma have been shown to possess adipogenic, osteogenic, chondrogenic, myogenic and neurogenic potential *in vitro*.[4, 5, 6] However, harvesting of MSCs from bone marrow for autologous use is a high-cost, time-consuming process potentially limited by low cell yield and patient discomfort. Consequently, alternative sources of adult stem cells, obtainable in large quantities and with minimal discomfort are being pursued. Recent studies suggest that human adipose tissue contains pluripotent stem cells similar to bone marrow-derived stem cells.[7] Adipose tissue, like bone marrow, is derived from the embryonic mesenchyme and contains a stroma that is easily isolated. When compared to cells harvested from bone marrow stroma, hADSCs are easier to obtain, carry relatively lower donor site morbidity and are available in large quantities of stem cells at harvest.[8] Because of their abundance and accessibility, hADSCs may prove to be a novel cell therapeutic for hard and soft tissue repair and regeneration with enormous potential for use in orthopeadics, plastic and reconstructive surgery, and so on. The purpose of this study is to verify whether hADSCs will differentiate along osteoblastic, chondrocyte and adipocyte lineage, for subsequent potential use in reconstruction of bone, cartilage, and soft tissue defects respectively.

METHODS

Materials

All materials were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. Dulbecco's modified Eagle's medium (DMEM) and Fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT, USA). TRIZOL Reagent and Superscript II reverse transcriptase were purchased from Invitrogen (Carlsbad, CA, USA), Oligo dT Primer and RNase-Free DNase I were purchased from Promega (Madison, WI, USA), Taq DNA polymerase and dNTP were purchased from GIBCO-BRL (Grand Island, NY, USA).

Cell Origin, Isolation and Culturing of hADSCs

Human adipose tissue was obtained with informed consent from 6 healthy patients (age 25 to 55) undergoing liposuction at the plastic surgery hospital affiliated with the Chinese Academy of Medical Science as approved by the ethical committee of Peking University Health Science Center. Patients with diabetes, hepatitis, metabolic diseases or other systemic complications were excluded from the study. Liposuction tissues were transported to the laboratory in saline solution within 2 hours postsurgery then washed at least three times with equal volumes of phosphate-buffered saline (PBS). The tissue was then digested with 0.075% type I collagenase (60 minutes, 37℃) with intermittent shaking. hADSCs were then isolated, incubated and passaged by the method of Zhou YS et al.[9]

Osteogenic Induction and confirmation of osteogenic differentiation of hADSCs

Passage 2 cells were trypsinized, replated onto 6-well culture plates (10^4 cells/well) and incubated in control medium for a day to adhere to the plates prior to osteogenic induction. Osteogenic differentiation was induced by culturing hADSCs for 7 to 21 days in osteogenic medium (OM) (control medium + 100 nmol/L dexamethasone + 0.2 mmol/L ascorbate + 10 mmol/L β-glycerophosphate). The media was changed every 2 ~ 3 days. The osteogenic differentiation was examined for alkaline phosphatase (ALP) activity by ALP staining and extracellular matrix calcification by von Kossa staining as described by Zuk PA et al.[7] Expression of osteoblast-associated genes such as Type I Collagen (COL I), bone sialoprotein (BSP), osteocalcin (OC), Runt-related

transcription factor-2/Core binding factor alpha 1 (Runx2/Cbfa1) and osterix (Osx) was confirmed by RT-PCR, 7 to 21 days after initial osteogenic induction. hADSCs maintained in untreated control medium were analyzed as negative control.

Chondrogenic Induction and confirmation of chondrogenic differentiation of hADSCs

For Chondrogenic differentiation, passage 2 cells were trypsinized and replated onto 100-mm^2 tissue culture plates by using the modified micromass culture technique[1]. Briefly, 10 μL of a concentrated hADSCs cell suspension (8×10^6 cells/mL) was plated at approximately 1 cm intervals on each plate and allowed to attach (37℃, 2 h). Chondrogenic medium (CM) (DMEM + 1% FBS + 100 units/mL penicillin + 100 μg/mL streptomycin + 6.25 μg/ml insulin + 10 ng/ml TGF-β1 + 50 nmol/L ascorbate) was gently overlaid so as not to detach the cell nodules. The media were changed every 2 ~ 3 days and the cultures were maintained in CM for 2 weeks prior to analysis. Chondrogenesis was confirmed using the histologic stain Alcian Blue at acidic PH as described by Zuk PA et al.[7] Additionally, expression of the cartilage-specific genes such as type II collagen isoform (COL II) and aggrecan were also examined by RT-PCR assays. hADSCs maintained in untreated control medium were analyzed as negative control.

Adipogenic Induction and confirmation of adipogenic differentiation of hADSCs

Passage 2 cells were trypsinized, replated onto 6-well culture plates (10^4 cells/well) then incubated in control medium for 6 ~ 10 days to allow cells to reach confluency prior to adipogenic induction. Adipogenic differentiation was then induced by culturing hADSCs for 2 weeks in adipogenic medium (AM) (control medium + 0.5 mmol/L isobutyl-methylxanthine + 1 μmol/L dexamethasone + 10 μmol/L insulin + 200 μmol/L indomethacin). Oil Red O staining was used to assess intracellular lipid accumulation as described by Zuk PA et al.[7] Expression of adipose tissue-specific genes such as peroxisome proliferator activated receptor γ2 (PPARγ_2), aP2 (an adipocyte-specific fatty acid binding protein) and lipoprotein lipase (LPL) was confirmed by RT-PCR 2 weeks after initial adipogenic induction. hADSCs maintained in untreated control medium were analyzed as negative control.

RNA isolation and Reverse transcription-polymerase chain reaction (RT-PCR)

Following osteogenic, chondrogenic, and adipogenic induction, treated and control culture layers were rinsed using cold PBS and immediately lysed into Trizol Reagent. Total RNA was isolated and treated by RNase-free DNase I and quantified by UV spectrophotometry. For RT-PCR analysis of mRNA expression, 1.0 μg of total RNA (in 20 μl reaction volume) was reverse transcribed using reverse transcriptase (Superscript II) and oligo-dT primers in a standard reaction. The resultant cDNA (1 μl) was then used as template for PCR amplification (in 25 μl reaction volume) of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), COL I, BSP, OC, Runx2/Cbfa1, Osx, PPARγ2, aP2, LPL, COL II and aggrecan. The primers used in this study are listed in Table. All primer sequences were determined through established GenBank sequences. Amplification of GAPDH was used as a control for assessing PCR efficiency. Aliquots (10 μl) of each reaction were evaluated by 2% agarose gel electrophoresis. Ethidium bromide-stained gels were digitally photographed (Kodak, Rochester, NY, USA).

Table Primers and cycling conditions for RT-PCR.

Target Gene (GenBank Accession No.)	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Product size (bp)
GAPDH (# BC013852)	TGGTATCGTGGAAGGACTCATGAC	ATGCCAGTGAGCTTCCCGTTCAGC	189
Type Ⅰ Collagen (# AB209597)	TGACGAGACCAAGAACTG	CCATCCAAACCACTGAAACC	599
OC (# X53698)	ATGAGAGCCCTCACACTCCTC	CGGGCCGTAGAAGCGCCGATA	297
BSP (# J05213)	GCTCAGCATTTTGGGAATGGC	CTGCATTGGCTCCAGTGACAC	614
Cbfa1 (# NM_004348)	GTGGACGAGGCAAGAGTTTCA	TGGCAGGTAGGTGTGGTAGTG	698
Osx (# AF477981)	CTTCAGTCTTCCCAACTTCTTACAC	ACAAATTGGGTTAGCTACATCTCTG	486
Type Ⅱ Collagen (# X13783)	TTTCCCAGGTCAAGATGGTC	CTTCAGCACCTGTCTCACCA	378
Aggrecan (# NM_001135)	TGAGGAGGGCTGGAACAAGTACC	GGAGGTGGTAATTGCAGGGAACA	350
PPARγ2 (# NM_015869)	TGGGTGAAACTCTGGGAGATTC	CATGAGGCTTATTGTAGAGCTG	381
aP2 (# BC003672)	GTACCTGGAAACTTGTCTCC	GTTCAATGCGAACTTCAGTCC	419
LPL (# BT006726)	GAGATTTCTCTGTATGGCACC	CTGCAAATGAGACACTTTCTC	276

All primer sequences were determined with established GenBank sequences.

RESULTS

Primary culture of hADSCs

hADSCs, isolated from adult human adipose tissue, exhibited spindle-shaped fibroblast-like morphology (Fig. 1). They normally reached confluence after 7 ~ 10 days culturing.

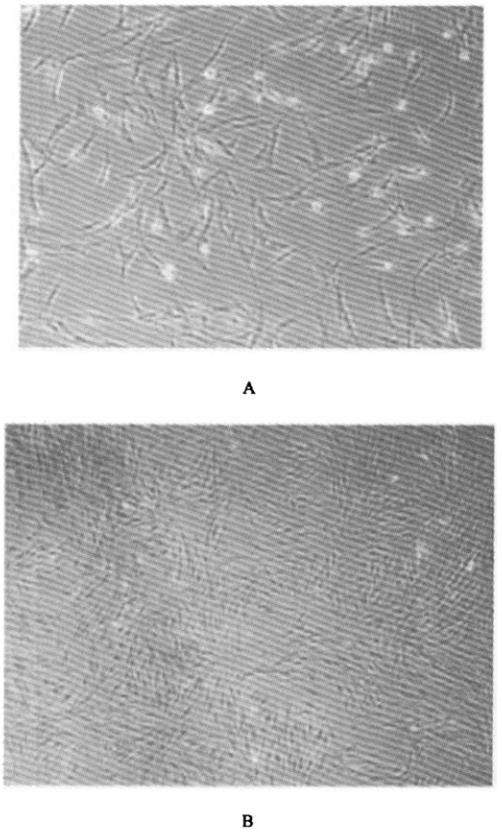

Fig.1 The cell morphology and proliferation of uninduced or undifferentiated hADSCs: (A) Cells exhibited spindle-shaped fibroblastic appearance (100×); (B) hADSCs during proliferation (40×).

Osteogenic differentiation of hADSCs

hADSCs induced by osteogenic media exhibited cell morphology changes after 3 days in culture. Cell appearance changed from spindle-shaped fibroblastic to a rounder, more cuboidal shape and cells formed an extensive network of dense, multilayered nodules. The induced cells also demonstrated positive staining for membrane-bound ALP activity after 7 days of culture in OM and the staining became more intense after 14 days of culture (Fig.2). In contrast, uninduced hADSCs did not show evidence of ALP expression at any time point. Extracellular mineralization capacity of induced cell layers was confirmed by von Kossa staining after 14 and

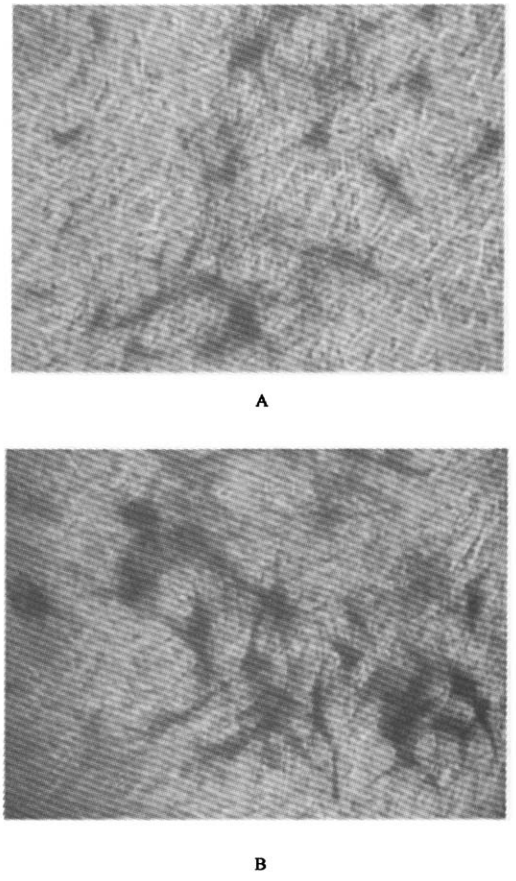

Fig.2 ALkaline Phosphatase staining for induced hADSCs. Osteogenesis was confirmed with ALP staining. The cells stained positively for endogenous ALP activity after 7, or 14 days of culture in osteogenic media containing DEX: (A) hADSCs induced by DEX for 7 days (100×); (B) hADSCs induced by DEX for 14 days (100×).

21 days of culture in OM (Fig.3). To confirm osteogenesis, the cells were also examined by RT-PCR for the expression of several osteoblast-related genes including COL I, BSP, OC, Runx2/Cbfa1 and Osx (Fig.4). COL I and BSP were observed on day 7, day 14, and day 21 after induction in the differentiated hADSCs. OC expression was only observed on day 21 in the differentiated hADSCs. Runx2/Cbfa1 was observed at all time points post-induction in the differentiated cells and Osx was observed on day 7 and day 14 post-induction. None of the genes, with the exception of GAPDH, were

seen in control cells.

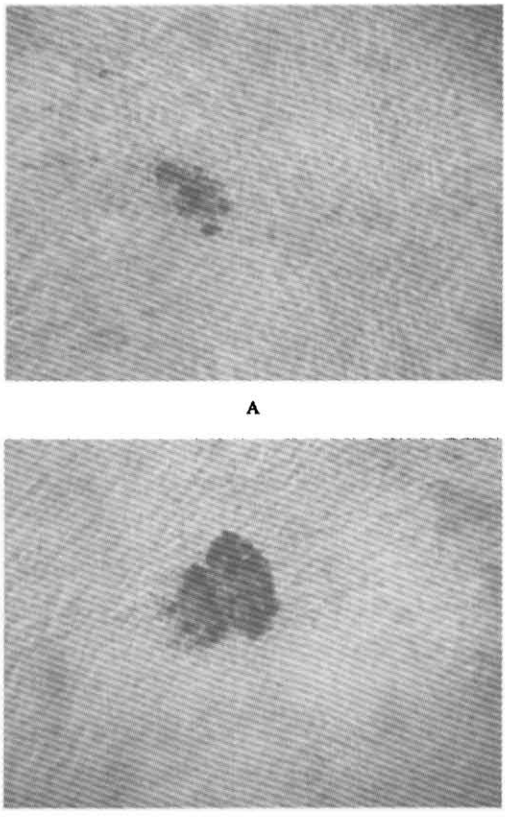

Fig.3 von Kossa staining for induced hADSCs. Osteogenesis was also examined by von Kossa staining. Secretion of a calcified extracellular matrix was observed as black nodules: (A) hADSCs induced by DEX for 14 days (100 ×); (B) hADSCs induced by DEX for 21 days (100 ×).

Chondrogenic differentiation of hADSCs

High-density micromass cultures of hADSCs incubated in chondrogenic media condensed into small spheroids after initial chondrogenic induction. Over the course of 14 days, the number of cartilaginous nodules observed increased. No nodules were observed in cell cultures placed in nonchondrogenic control media. Nodules that formed in the micromass cultures supplemented with CM stained positively for Alcian blue at pH 1.0, which is specific for the highly sulfated proteoglycans of cartilage matrices (Fig.5). Expression of chondrocyte-specific genes, including aggrecan and type II collagen, were also detected in the induced cells (Fig. 6), but not in cells incubated in the control medium.

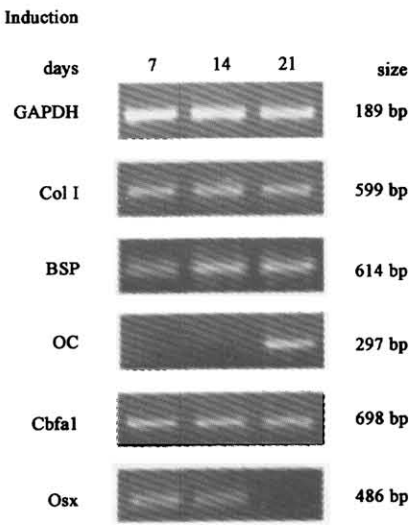

Fig.4 RT-PCR of osteogensis-related genes. To confirm osteogenesis, induced cells are also exmamined by RT-PCR for the expression of several genes including type I collagen, bone sialoprotein, osteocalcin, Runx2/Cbfa1, Osx. Primers of GAPDH were used as a control. These genes except GAPDH were not detected in uninduced control cells (data were not shown).

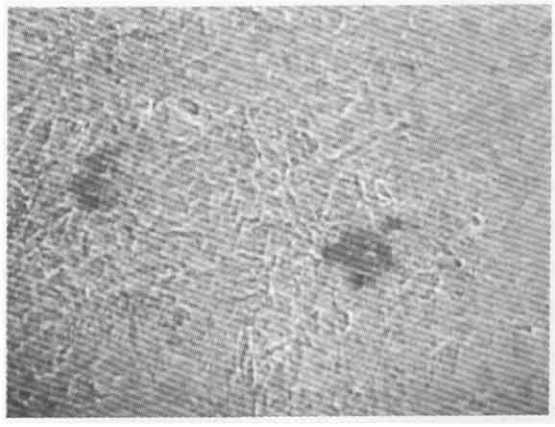

Fig.5 Alcian blue staining. The nodules that formed in the micromass cultures supplemented with chondrogenic media stained positively for Alcian blue at pH 1, which is specific for the highly sulfated proteoglycans of the cartilae matrix. For the uninduced control cells, negative staining were observed (date were not shown).

Adipogenic differentiation of hADSCs

Adipogenic induction of hADSCs resulted in an expanded cell morphology and a significant fraction of the cells contained multiple, intracellular lipid-filled droplets that accumulated Oil Red O (Fig.7), consistent with the

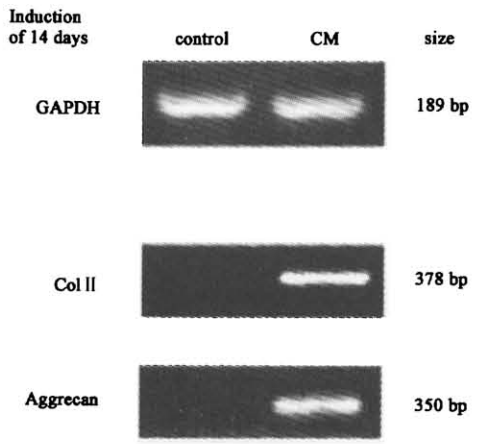

Fig.6 RT-PCR of chondrogenesis-specific genes. Expression of chondrocyte-specific enes, including type Ⅱ collagen and aggrecan were also observed. These genes were not observed in control cells.

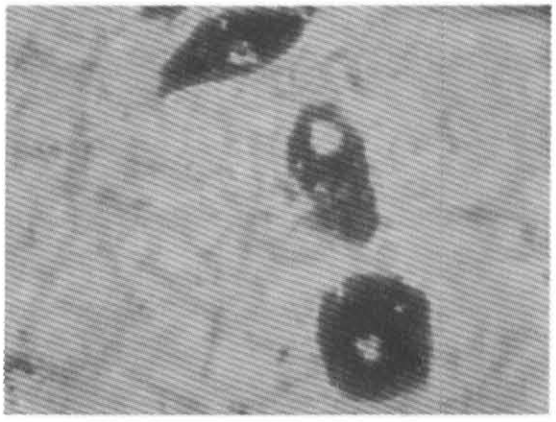

Fig.7 Oil Red O staining. The induced hADSCs by adipogenic media form and accumulate intracellular lipid-filled droplets that can be positively stained by Oil Red O. For the uninduced control cells, no lipid-filled droplets and negative staining were observed (date were not shown).

phenotype of mature adipocytes. During the induction course, detachment of 20% differentiated cells from the culture plate was also observed. No lipid droplets were observed in undifferentiated hADSCs. Adipogenic induction of hADSCs also resulted in the expression of lineage-specific genes (Fig.8). Induction of hADSCs with AM resulted in expression of the adipose-specific transcription factor PPARγ2. Additionally, expression of the adipogenic genes LPL and aP2 was also detected in induced hADSCs. No expression of these genes was observed by RT-PCR in the non-induced controls.

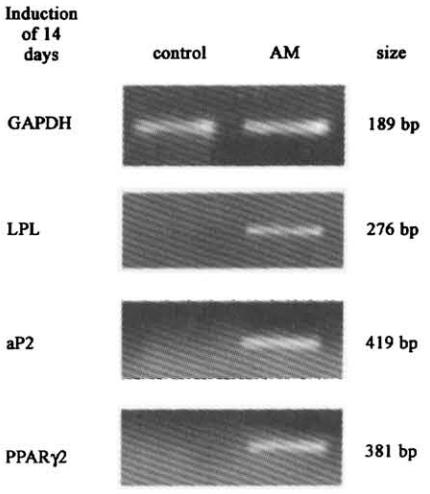

Fig.8 RT-PCR of adipogenesis-specific genes. Expression of adipocyte-specific genes, including LPL, aP2, PPARγ2 were also observed. These genes were not observed in control cells.

DISCUSSION

Osteogenic Capacity of hADSCs

Differentiation of MSCs into osteoblasts is normally characterized by ALP enzyme activity, extracellular mineralization and expression of osteoblast-associated genes.[6, 9, 10] ALP, as a marker enzyme, is one of the most frequently used parameters for identification of osteoblastic differentiation and osteogenic property.[11] After OM treatment for 7 and 14 days, the induced hADSCs formed an extensive network of dense nodules that stained positively for ALP. The upregulation of ALP, which is reported to lead to mineral formation subsequently,[12] is a critical process to confirm the osteogenesis of the induced hADSCs.

To further verify the osteogenic capacity of hADSCs, the expression of several osteoblast-related genes including COL I, BSP, OC were examined by RT-PCR assays. COL I, an early osteogenic marker, is a major component of extracellular matrix (ECM) protein secreted by osteoblasts and is an absolute requirement for progression of osteoblast differentiation and subsequent mineralization of the matrix.[13] COL I expression in induced hADSCs began 7 days post-induction and was observed until day 21. Its expression is strongly suggestive of

osteogenesis.[13] Compared with COL I, BSP and OC are more specific for osteoblastic differentiation.[4, 6, 14, 15] BSP expression marks a middle to late stage of osteoblastic differentiation and represents an early stage of matrix mineralization due to its capacity to nucleate hydroxyapatite deposition.[14, 15] Our data suggest induced hADSCs expressed BSP on day 7, 14, and 21 post-induction. Its expression paralleled that of COL I and occurred just before the matrix mineralization. OC, as a marker of mature osteoblast, is the most specific gene expressed only in terminally differentiated osteoblasts[14, 15]. Our results suggest that very late expression of OC was seen on day 21 post-induction, potentially indicating the matrix maturation.

To further investigate the osteogenetic potential of hADSCs, osteoblast-specific transcription factors, such as Runx2/Cbfa1 and Osx, were also characterized by RT-PCR. Runx2/Cbfa1 is the predominant transcriptional activator of osteoblast associated genes such as OC, BSP, and Col I.[16] This indicates that its expression is crucial for osteoblast differentiation and bone formation.[16] Osx, a zinc-finger-containing transcription factor, also regulates osteogenic differentiation in vitro and *in vivo*. It acts downstream of Runx2/Cbfa1 in mesenchymal cells.[17, 18] In Osx null mice, osteoblastic differentiation is impaired and no bone forms[18]. It can induce an increase in the expression of osteogenesis-associated markers including ALP, BSP, OC.[18] These findings indicate that one possible mechanism leading to the osteogenesis of hADSCs may be attributed, at least in part, to the upregulation of Runx2/Cbfa1 and Osx. In the current study, we noted upregulation of both Cbfa1 and Osx during the osteogenic induction of hADSCs. These data, combined with the positive expression of OC, BSP and COL I, indicates that the two transcription factors play crucial roles in the osteogenesis of hADSCs.

Others have suggested that osteogenic differentiation of hADSCs should also be examined by matrix mineralization, reported to be the last phase in the developmental sequence of bone.[19] The identification of a mineralized ECM in culture layers is normally examined by von Kossa staining.[6, 9] Mineralization regions appear as black calcified nodules within the cell layers. We identified several black nodules, indicative of a calcified or mineralized ECM, in hADSCs layers treated for 14 and 21 days in OM. This is consistent with osteogenesis. Taken together, the expression of ALP, COL I, BSP, OC, Runx2/Cbfa1 and Osx by induced hADSCs, as well as the production of a calcified ECM, strongly suggest that hADSCs can be induced toward the osteoblastic lineage. Therefore, these data provide basis for the future use of hADSCs in bone tissue engineering and treatment of bone loss or resorption.

Chondrogenic Capacity of hADSCs

Stem cell-based tissue engineering approaches presently under development, represent a promising alternative for the repair of cartilage defects caused by osteoarthritis and other joint diseases, as articular cartilage exhibits little intrinsic repair capacity.[20] Although MSC is the major stem cell source for cartilage tissue engineering in much of the current research,[21] the limited output, complications related to marrow procurement and the high cost of harvesting and processing limit their clinical use. hADSCs might be an ideal stem cell source and represent a promising approach for cartilage reconstruction and cartilage tissue engineering. The micromass culture technique, mimics the cellular condensation process, has been previously demonstrated to verify the capacity of chondrogenic differentiation.[22] During the induction course, media supplementation with TGF-β1, was a key factor in the regulation of cell proliferation and differentiation, especially in cartilage formation[21] and resulted in the condensation of hADSCs into three-dimensional aggregates, a critical first event of chondrogenesis.[23] These condensed cells were stained positively for Alcian blue, which specifically confirmed the presence of the highly sulfated proteoglycans within the matrix.[24] These results are strongly suggestive of chondrogenesis. Upregulation in the expression of the cartilaginous marker collagen type Ⅱ and the cartilage-specific proteoglycan aggrecan, was also detected by RT-PCR in the induced hADSCs, results also suggestive of chondrogenesis.[6, 22, 24] Therefore, the findings in this study confirm the chondrogenic capacity of hADSCs. The induced hADSCs demonstrate properties in agreement with native chondrocytes, which may make these cells an appropriate system for future cartilaginous tissue engineering strategies.

Adipogenic Capacity of hADSCs

Resection of tumors in the head and neck as well as trauma and congenital abnormalities in these regions often result in contour defects due to loss of soft tissue, largely composed of subcutaneous adipose tissue. The defects lead to abnormal physical appearance impairing the function and affecting the emotional health of patients.[25] Autologous fat transplantation yields poor results, with unpredictable partial reduction in graft volume.[26] Although numerous natural, synthetic and hybrid materials have been used to act as adipose surrogates, many of these materials possess severe limitations, such as unpredictable outcomes, fibrous capsule contraction, allergic reactions, sub-optimal mechanical properties, distortion, migration and long-term resorption.[25] In the search for new alternatives, adipose tissue engineering, based on multipotent stem cells, is seen as a feasible source to solve soft tissue defects and greatly reduce patient discomfort and post-transplantation resorption resulting from direct fat grafting.[27] Given that adipose tissue can be obtained easily and in large quantities, stem cell procurement from this tissue becomes more attractive to both the surgeons and patients. In the present study, AM-induced hADSCs not only accumulated lipid droplets intracellularly, but also expressed several adipocyte-specific genes like PPARγ2, LPL, aP2 that are involved in lipid biosynthesis and storage.[7] Uninduced hADSCs did not form or accumulate lipid intracellularly, nor did they express any adipocyte-specific genes. These findings suggest that hADSCs, harvested from adipose tissue, cannot spontaneously differentiate along adipogenic lineage; only lineage specific induction conditions can induce hADSCs to differentiate into specific lineage orientation. In this regard, hADSCs are quite similar to MSCs[6] and should be considered an ideal source for adipose tissue engineering.

Based on the findings in the current study, hADSCs might represent an optimal choice for progenitor cell-based strategies for tissue reconstruction of bone, cartilage, or soft tissue defects.

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（收稿日期：2006-03-09）