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Isolation of human multipotent mesenchymal stem cells from second-trimester amniotic fluid using a novel two-stage culture protocol

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BACKGROUND: The aim of this study was to isolate mesenchymal stem cells (MSCs) from amniotic fluid obtained by second-trimester amniocentesis. METHODS: A novel two-stage culture protocol for culturing MSCs was developed. Flow cytometry, RT–PCR and immunocytochemistry were used to analyse the phenotypic characteristics of the cultured MSCs. Von Kossa, Oil Red O and TuJ-1 stainings were used to assess the differentiation potentials of MSCs. RESULTS: Amniotic fluid-derived MSCs (AFMSCs) were successfully isolated, cultured and enriched without interfering with the routine process of fetal karyotyping. Flow cytometry analyses showed that they were positive for SH2, SH3, SH4, CD29, CD44 and HLA-ABC (MHC class I), low positive for CD90 and CD105, but negative for CD10, CD11b, CD14, CD34, CD117, HLA-DR, DP, DQ (MHC class II) and EMA. Importantly, a subpopulation of Oct-4-positive cells was detectable in our cultured AFMSCs. Under specific culture conditions, AFMSCs could be induced to differentiate into adipocytes, osteocytes and neuronal cells. CONCLUSIONS: We demonstrate that human multipotent MSCs are present in second-trimester amniotic fluid. Considering the great potential of cellular therapy using fetal stem cells and the feasibility of intrauterine fetal tissue engineering, amniotic fluid may provide an excellent alternative source for investigation of human MSCs.

Key words: amniotic fluid cells/mesenchymal stem cells/Oct-4 expression

Introduction

Human mesenchymal stem cells (MSCs) are thought to be multipotent cells that have the potential to differentiate into multiple lineages including bone, cartilage, muscle, tendon, ligament fat and a variety of other connective tissues (Pittenger *et al.*, 1999; Minguell *et al.*, 2001). Morphologically, MSCs in their undifferentiated state are spindle shaped and resemble fibroblasts. MSCs have been identified mostly in adult bone marrow, while recent reports have shown that MSCs are also present in both adult and fetal peripheral blood, fetal liver, fetal spleen, placenta and in term umbilical cord blood (Campagnoli *et al.*, 2001; Hu *et al.*, 2003; Romanov *et al.*, 2003).

To date, little is known about whether MSCs are present in human amniotic fluid or not, despite culturing of amniotic fluid cells being a well-established routine procedure in cytogenetic laboratories. Multiple approaches have been used to characterize and classify the cell types of amniotic fluid, yet many questions concerning the nature and *in vivo* origin of these cells have not been entirely resolved (Prusa and Hengstschläger, 2002). Recently, some reports mentioned that amniotic fluid contained a variety of human stem cells, which were shed from embryonic and extra-embryonic tissues during the process of fetal development and growth. Human amniotic fluid epithelial cells could transform into neurons, astrocytes and oligodenrocytes, and the cells might be a possible candidate for transplantation therapy of neurodegenerative diseases (Kakishita *et al.*, 2003). Amniotic fluid is an attractive source of MSCs for therapeutic transplantation (In 't Anker *et al.*, 2003). Furthermore, Oct-4-expressing cells were present in human amniotic fluid, and it was proposed that human amniotic fluid might be a new source for pluripotent stem cells without raising any ethical concerns associated with human embryonic stem cell research (Prusa *et al.*, 2003).

In this study, we show that human MSCs can be isolated from second-trimester amniotic fluid without interfering with the process of fetal karyotyping using a novel two-stage culture protocol. The cells isolated by this culture method have the capacity to differentiate into multiple cell types *in vitro*.

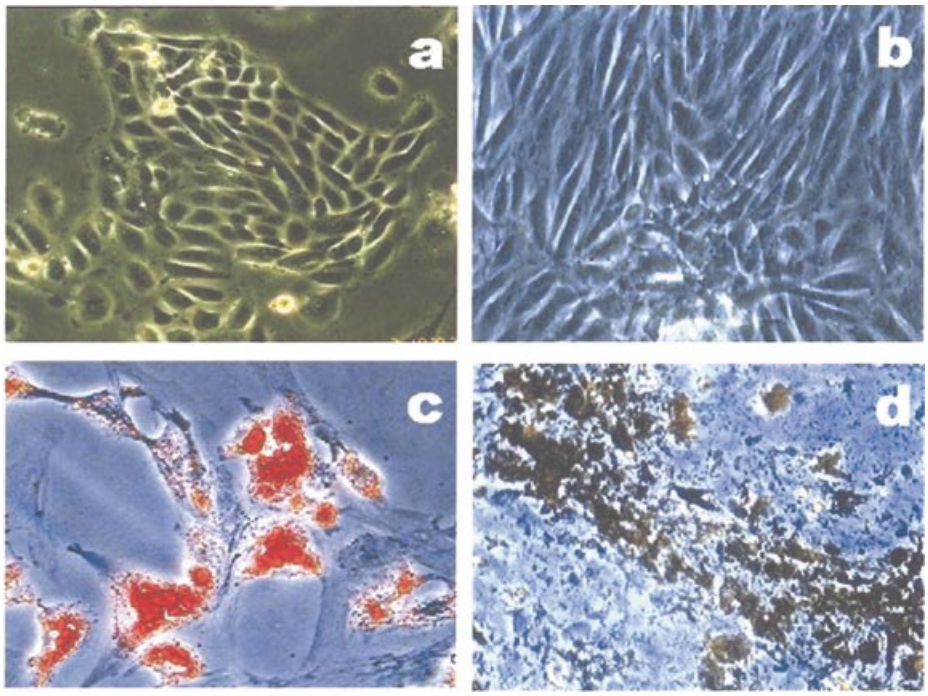

Figure 1. Amniotic fluid-derived mesenchymal stem cells and their multilineage differentiation staining. (**a**) The appearance and growth of MSC-like cell colonies on the fifth day of culturing. (**b**) The fibroblastic-like AFMSC colonies grew to confluence in the first-passage culture. (**c**) Adipogenic differentiation was demonstrated by Oil Red O staining. (**d**) Osteogenic differentiation was demonstrated by von Kossa staining.

Materials and methods

Culture of MSC from amniotic fluid

Twenty amniotic fluid samples (20 ml) were obtained by amniocentesis performed between 16 and 20 weeks of gestation for fetal karyotyping. We developed a novel two-stage culture protocol for isolating MSCs from amniotic fluid. For culturing amniocytes (first stage), four primary *in situ* cultures were set up in 35 mm tissue culture-grade dishes using Chang medium (Irvine Scientific, Santa Ana, CA). Microscopic analysis of Giemsa-stained chromosome banding was performed, and the rules for metaphase selection and colony definition were based on the basic requirements for prenatal cytogenetic diagnosis in amniocytes (Moertel *et al.*, 1992). For culturing MSCs (second stage), non-adhering amniotic fluid cells in the supernatant medium were collected on the fifth day after the primary amniocytes culture and kept until completion of fetal chromosome analysis. The cells then were centrifuged and plated in 5 ml of α-modified minimum essential medium (α-MEM; Gibco-BRL) supplemented with 20% fetal bovine serum (FBS; Hyclone, Logan, UT) and 4 ng/ml basic fibroblast growth factor (bFGF; R&D systems, Minneapolis, MN) in a 25 cm^2 flask and incubated at 37°C with 5% humidified CO_2 for MSC culture. This protocol has been approved by the Institutional Review Board (IRB) of Cathay General Hospital and each patient signed a written informed consent.

Differentiation assay for MSCs

Amniotic fluid-derived mesenchymal stem cells (AFMSCs) were cultured to confluence and shifted to osteogenic medium (α-MEM supplemented with 10% FBS, 0.1 μmol/l dexamethason, 10 mmol/l β-glycerol phosphate, 50 μmol/l ascorbate) and adipogenic medium (α-MEM supplemented with 10% FBS, 1 μmol/l dexamethasone, 5 μg/ml insulin, 0.5 mmol/l isobutylmethylxanthine and 60 μmol/l indomethacin) for 3 weeks. The differentiation potential for osteogenesis was assessed by the mineralization of calcium accumulation by von Kossa staining. For adipogenic differentiation, intracellular lipid droplets could be observed under the microscope and confirmed by Oil Red O staining. For differentiation of neural cells, AFMSCs were incubated with α-MEM supplemented with 20% FBS, 1 mmol/l β-mercaptoethanol, 5 ng/ml bFGF (Sigma, St Louis) for 24 h, and then treated with serum depletion for 5 h. Immunocytochemical stain with neuron-specific class III β-tubulin (TuJ-1) was used to assess the capacity of neuronal differentiation.

Flow cytometry analysis

The specific surface antigens of AFMSCs in the cultures of passage 4–8 were characterized by flow cytometry analyses. The cells in culture were trypsinized and stained with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies against CD10, CD11b, CD34, CD90, HLA-A,B,C, HLA-DR,DP,DQ (BD PharMingen); CD14, CD29, CD44, CD105, CD117 (Eurolone); and SH2, SH3, SH4 (American Type Culture Collection, Manassas, VA). Thereafter, the cells were analysed using a Becton Dickinson flow cytometer (Becton Dickinson, San Jose, CA).

RT–PCR procedure

Total RNA was extrated from the cultured cells by using Tri Reagent (MRC Inc., Cincinnati, OH) according to the manufacturer's instructions. RT–PCR is performed using the OneStep RT–PCR kit (QIAGEN Inc., Valencia, CA) using specific DNA primers as follows: Oct-4 (247 bp) sense, 5′-CGTGAAGCTGGAGAAGGAGAAGCTG-3′, and antisense, 5′-CAAGGGCCGCAGCTTACACATGTTC-3′; and β-actin (396 bp) sense, 5′-TGGCACCACACCTTCTAC-AATGAGC-3′, and antisense, 5′- GCACAGCTTCTCCTTAA-TGTCACGC-3′. RT–PCR was performed initially at 50°C for 30 min and 95°C for 15 min for reverse transcription, then followed by 35 cycles, with each cycle consisting of denaturion at 94°C for

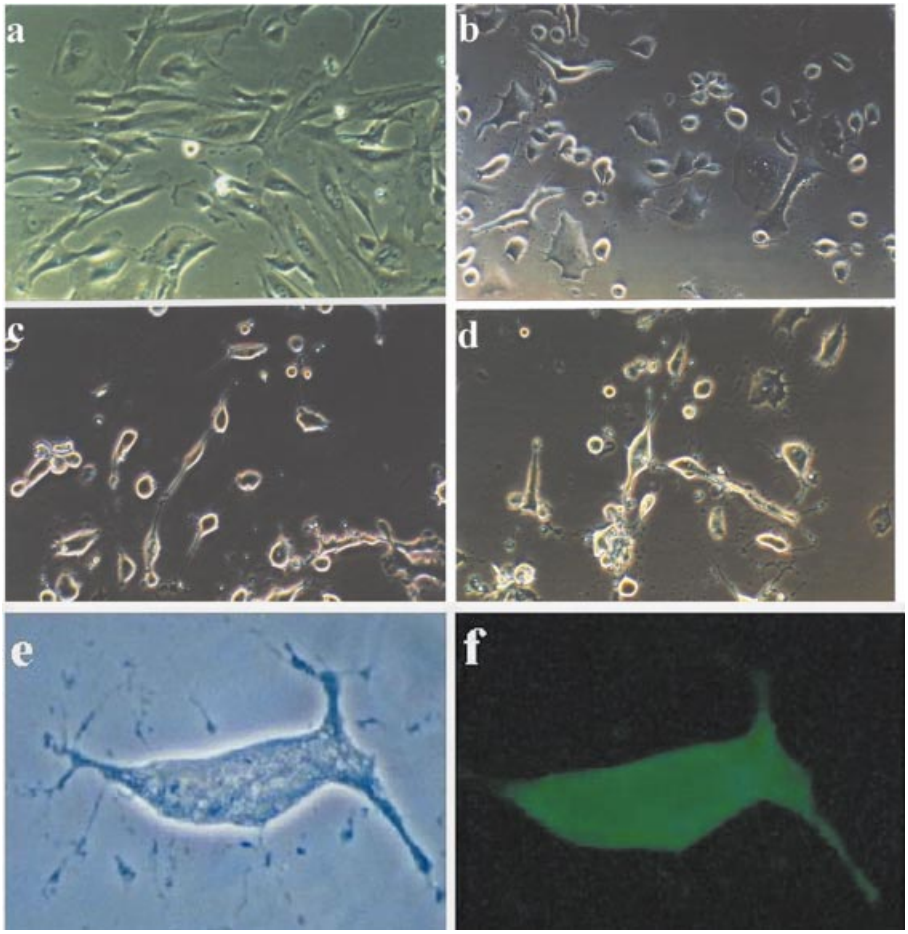

Figure 2. AFMSCs differentiate into neurons. (**a**) AFMSCs at the fifth-passage culture before neuronal induction. (**b**) The cytoplasm of the cells retracts toward the nucleus and forms a rounded cell body after 6 h of pre-treatment. (**c**) An apparent neuron-like cell with a long axon-like process was observed after 4 h of serum-free induction. (**d**) Typical neuron-like cell with multiple neurites after 4.5 h of serum-free induction. (**e**) A neuron-like cell before staining. (**f**) Positive immunofluorescence stain of TuJ1, a marker for neuronal differentiation.

1 min, annealing at 57°C for 1 min, elongation at 72°C for 1 min, and the final extension at 72°C for 10 min. The amplified DNA fragments were visualized through 2% agarose gel electrophoreses, stained and photographed under UV light. NTERA-2 c1.D1 cells (ATCC CRL-1973, a pluripotent human testicular embryonic carcinoma cell line) and MRC-5 cells (human diploid lung fibroblast, ATCC CCL-171) were used as positive and negative control for Oct-4 RT–PCR expression analysis, respectively.

Immunocytochemical analyses

For immunofluorescence analyses of cellular Oct-4 expression, the cultured AFMSCs, MRC-5 cells (negative control) and NTERA-2 c1.D1 cells (positive control) were washed, fixed, and incubated with 1:100 mouse anti-human Oct-4 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Thereafter, the cells were washed and incubated with a secondary antibody of FITC-conjugated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) at room temperature for 1 h. Cell nuclei were counterstained with 1 μg/ml 4′,6-diamino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR) in phosphate-buffered saline (PBS) for 5 min and mounted in VECTASHIELD mounting medium (Vector Laboratories). For cellular neuron-specific class III β-tubulin analyses, the induced AFMSCs were washed, fixed, and incubated with 1:400 mouse anti-human β-tubulin III monoclonal antibody (Sigma, St Louis, MO) overnight at 4°C. Thereafter, the cells were washed, incubated with a secondary antibody of FITC-conjugated horse anti-mouse IgG (Vector Laboratories) at room temperature for 1 h and mounted in VECTASHIELD mounting medium (Vector Laboratories).

Results

Appearance of AFMSCs in culture

The colonies of MSC-like cells began to appear in the culture flask 5 days after plating the non-adhering amniotic fluid cells from primary amniocyte culture (Figure 1a). After 2 weeks culture, the cells were washed with PBS, treated with 0.05% trypsin-EDTA, and passed into a new 25 cm^2 flask for the first-passage culture. Then, when these cells grew to 90% confluence of the culture (3–7 days) (Figure 1b), they were reseeded into a 75 cm^2 flask for the second-passage culture. The third and subsequent passages were carried on in 75 cm^2 flasks under the same condition at a split ratio 1:4. Currently, the twelfth-passage culture has been completed in our laboratory and their chromosome analyses remain as a normal karyotype of either 46,XX or 46,XY. We have succeeded in culturing the MSC-like cells from every tested amniotic fluid sample (n = 20).

Differentiation potential of AFMSCs

To evaluate the differentiation potential of AFMSCs, cells at the third to fifth passage were induced to form adipocytes, osteocytes and neuronal cells. Adipogenic differentiation was apparent after 1 week of incubation with adipogenic induction medium. Between the culture periods of 2–3 weeks, almost all cells contained numerous Oil Red O-positive lipid droplets (Figure 1c). Similarly, after culturing AFMSCs with osteogenic induction medium for 14 days, most of the cells became alkaline phosphatase positive and showed the aggregates or nodules of calcium mineralization in the culture by von Kossa staining (Figure 1d). To induce neural differentiation, AFMSCs were incubated in a serum-containing medium for 24 h, and then treated with serum depletion for 5 h. Initially, cytoplasm in some of the AFMSCs retracted toward the nucleus and began to form a rounded cell body within several hours of pre-treatment (Figure 2b and c). After incubation in serum-free conditions, the morphological changes increased progressively and then the neurite outgrowth was observed at 2–3 h (Figure 2d). At 5 h (Figure 2e), the induced cells exhibited a positive immunofluorescence stain for neuron-specific class III β-tubulin (Figure 2f). Yet, non-treated control cultures did not show any of the above differentiations.

Phenotypic characterization of AFMSCs

Figure 3 shows the cell surface antigenic characteristics of the cultured AFMSCs at passage 4–8 by flow cytometry. The analyses revealed that the expression of surface antigens, such as SH2 (passage 8), SH3, SH4, CD29, CD44 and HLA-A,B,C (MHC class I) was strongly positive; SH2 (passage 4), CD90 and CD105 were low positive, but CD10, CD11b, CD14, CD34, CD117, HLA-DR,DP,DQ (MHC class II) and EMA were negative. Further characterization studies were performed using RT–PCR and immunocytochemical staining for the detection of Oct-4 expression, which is a transcription factor expressed in undifferentiated embryonic stem cells. In addition to Oct-4 mRNA expression detected in all 20 independent cases of AFMSCs (Figure 4), Oct-4 protein expression was also noted in a subpopulation of our cultured AFMSCs (Figure 5D).

Discussion

In this study, we demonstrated that multipotent MSCs are present in human amniotic fluid and we developed a novel two-stage culture method to isolate, culture and enrich MSCs from second-trimester amniotic fluid obtained by routine amniocentesis for prenatal diagnosis. There are at least four major advantages of this culture protocol for fetal MSCs. First, it does

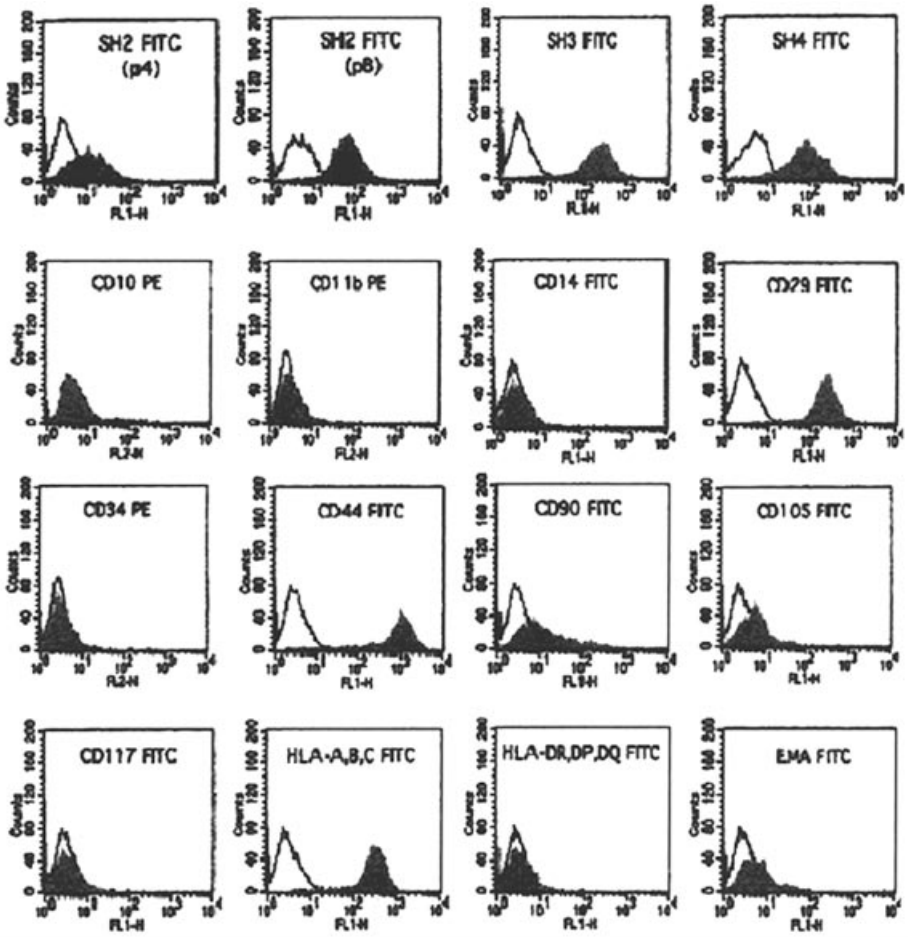

Figure 3. Phenotypic characteristics of AFMSCs. Flow cytometry analyses revealed that their expression of surface antigens such as SH2 (passage 8), SH3, SH4, CD29, CD44 and HLA-A,B,C was strongly positive; SH2 (passage 4), CD90 and CD105 were low positive, but CD10, CD11b, CD14, CD34, CD117, HLA-DR,DP,DQ and EMA were negative.

not interfere with the normal culture process for fetal karyotyping. Secondly, it does not raise any ethical issues that are associated with human embryonic stem cell research. Thirdly, it provides an unprecedented and abundant source for research on human MSCs. Fourthly, it opens a new avenue for autologous intrauterine fetal gene and cellular therapies without inducing tissue rejection.

Our results are consistent with the recent report (In 't Anker *et al.*, 2003) which demonstrated that MSCs could be isolated and expanded from 2 ml of second-trimester amniotic fluid, which was collected transcervically from terminations of pregnancy and showed multilineage potential for differentiation into adipocytes and osteocytes. However, there are several disadvantages in collecting amniotic fluid through the cervix compared with through the abdomen by routine second-trimester amniocentesis: first, it might possibly encourage illegal termination of pregnancy. Secondly, the transcervical approach could increase the possibility of contamination. Thirdly, the amount of MSCs isolated from 2 ml of amniotic fluid culture is far less than that from our two-stage culture protocol.

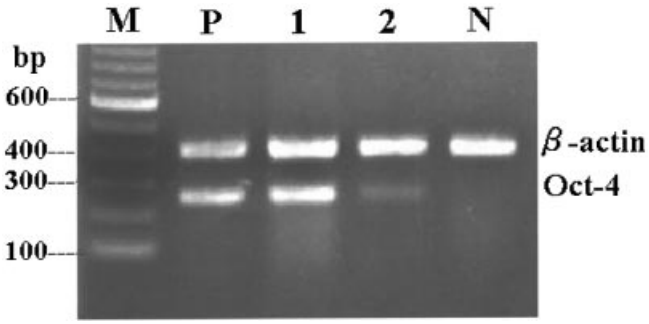

Figure 4. AFMSCs express Oct-4 mRNA. RT–PCR analyses showed that Oct-4 mRNA expression was detectable in our cultured AFMSCs, and the PCR products of β-actin served as an internal control (column P, positive control with NTERA-2 c1.D1 cells; columns 1 and 2, two independent samples of cultured AFMSCs; column N, negative control with MRC-5 cells).

It has long been proposed that stem cells of all three germ layers (ectoderm, mesoderm and endoderm) can be detected in human amniotic fluid, while very little is known about their lifespan throughout pregnancy. Under routine culture conditions for fetal karyotyping, amniotic fluid cells (AFCs) can be divided into two major categories: adhering and non- adhering cells. Furthermore, AFCs can also be classified according to their morphological aspects and growth characteristics into three groups: epitheloid E-type cells; amniotic fluid-specific AF-type cells; and fibroblastic F-type cells (Milunsky, 1992). The fibroblastic F-type cells were considered to originate from mesenchymal tissue and usually appear late during routine AFC cultivation (Prusa and Hengstschläger, 2002). We propose that the AFMSCs isolated by our two-stage culture protocol are most likely to be the fibroblastic F-type cells in the amniotic fluid and confirm that they are mesenchymal in origin.

AFMSCs have phenotypic characteristics similar to those of MSCs derived from other sources, such as term umbilical cord blood and first-trimester fetal tissues (blood, liver and bone

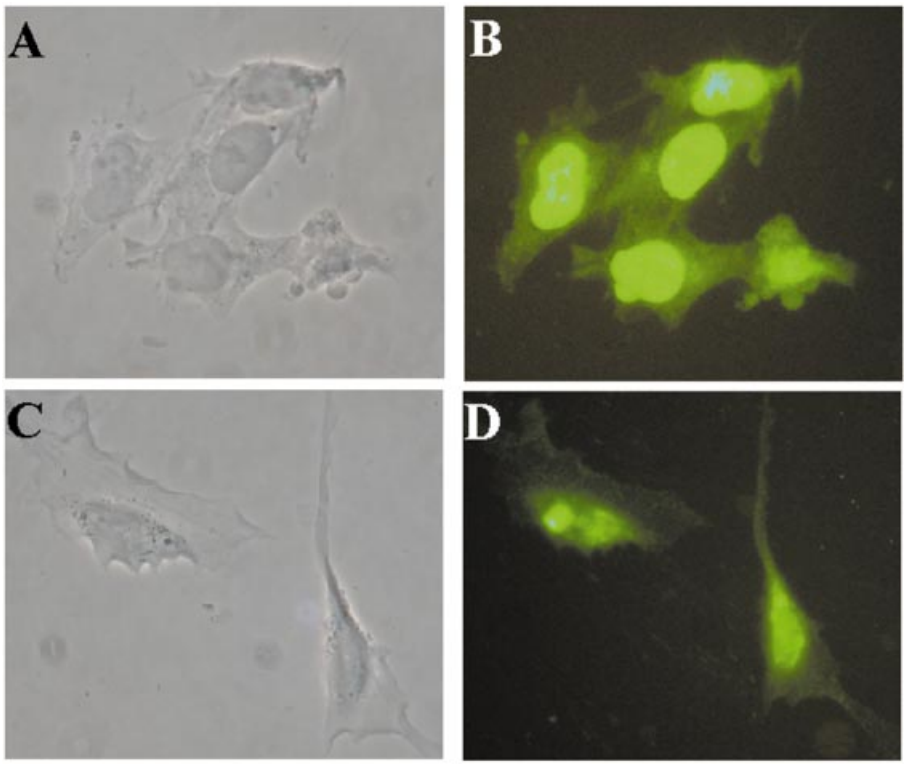

Figure 5. Immunocytochemical analyses revealed that a subpopulation of Oct-4-expressing cells could be found in the culture of AFMSCs. (**A**) Light phase of NTERA-2 c1.D1 cells. (**B**) Oct-4 immunocytochemical staining of NTERA-2 c1.D1 cells. (**C**) Light phase of AFMSCs. (**D**) Immunocytochemical staining of Oct-4-positive cells in cultured AFMSCs.

marrow), which are positive for SH2, SH3, SH4, CD29, CD44 and HLA-A,B,C, and negative for CD10, CD11b, CD14, CD34, CD117, EMA and HLA-DR,DP,DQ (Pittenger *et al.*, 1999; Colter *et al.*, 2001; Young *et al.*, 2001). Most importantly, AFMSCs express Oct-4 mRNA and Oct-4 protein, a transcription factor expressed in embryonic carcinoma cells, embryonic stem cells and embryonic germ cells, reflecting a key role in the maintenance of pluripotency of mammalian stem cells both *in vivo* and *in vitro* (Henderson *et al.*, 2002; Jiang *et al.*, 2002; Cogle *et al.*, 2003). This finding confirms the recent report (Prusa *et al.*, 2003) which described that there were Oct-4-expressing cells in human amniotic fluid and suggested that human amniotic fluid might contain stem cells of high potency.

The AFMSCs described here can be expanded rapidly and they maintain the capacity to differentiate into multiple cell types *in vitro*. Aside from the common mesenchymal lineages (adipocytes and osteocytes), they also have been differentiated successfully into neuron-like cells. These findings are quite encouraging and similar to several recent reports, which described that marrow stromal cells could be differentiated into multiple mesodermal cell types and neuron-like cells that expressed neuronal markers, suggesting that MSCs may be capable of overcoming germ layer commitment (Kopen *et al.*, 1999; Reyes and Verfaillie, 1999; Hofstetter *et al.*, 2002; Hung *et al.*, 2002). Whether AFMSCs have the ability to differentiate into lineages of endoderm *in vitro* is an issue that is worth investigating.

An increasing number of reports have shown that MSCs represented an attractive population for cellular therapy protocols, such as repairing infarcted myocardium, improving angiogenesis of stroke and repairing bone damage (Mitka, 2001; Sekiya *et al.*, 2002; Barbash *et al.*, 2003; Chen *et al.*, 2003; Davani *et al.*, 2003; Korbling and Estrov, 2003). Traditionally, the main sources of MSCs are isolated from postnatal origins, including adult bone marrow, umbilical cord blood, peripheral blood and various mesenchymal tissues (muscle, bone, cartilage, tendon, vessels and adipose). Current evidence suggests that aside from being difficult to obtain, adult stem cells have disadvantages of markedly restricted differentiation potential, decreasing number with age, more DNA damage and shorter life span compared with pluripotent stem cells derived from embryos or fetal tissue (Steindler and Pincus, 2002). Since there are many ethical concerns with human embryonic stem cells, looking for a feasible way to obtain fetal pluripotent stem cells has generated a great deal of interest from researchers. Although MSCs have been isolated successfully from first-trimester fetal blood, liver, spleen and bone marrow (Campagnoli *et al.*, 2001; Hu *et al.*, 2003), their use in research and therapeutics is also encumbered with ethical considerations and difficult access with minute quantities. In contrast, the AFMSCs obtained by our two-stage culture protocol may be a superior alternative source for MSC research without the limitations mentioned above. However, it remains a very interesting issue to be elucidated in the near future whether AFMSCs have greater therapeutic potential than that of the current adult MSCs.

In conclusion, we demonstrate that MSCs can be successfully isolated and expanded from second-trimester amniotic fluid obtained by routine amniocentesis, and they maintain the capacity to differentiate not only into mesodermal cell types but also into ectodermal neuron cells *in vitro*. Considering the easy access to samples for stem cell culture, the greater potential of cellular therapy using fetal stem cells and the feasibility of intrauterine fetal tissue engineering, amniotic fluid may provide an excellent alternative source for investigation of human MSCs and their potential therapeutic applications.

Acknowledgement

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References

Barbash IM, Chouraqui P, Baron J, Feinberg MS, Etzion S, Tessone A, Miller L, Guetta E, Zipori D, Kedes LH *et al.* (2003) Systemic delivery of bone marrow-derived mesenchymal stem cells to the infarcted myocardium: feasibility, cell migration, and body distribution. Circulation 108,863–868.

Campagnoli C, Roberts IA, Kumar S, Bennett PR, Bellantuono I and Fisk NM (2001) Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. Blood 98,2396–2402.

Chen F, Hui JHP, Chan Wk and Lee EH (2003) Cultured mesenchymal stem cell transfers in the treatment of partial growth arrest. J Pediatr Orthop 23,425–429.

Cogle CR, Guthrie SM, Sandes RC, Allen WL, Scott EW and Petersen BE (2003) An overview of stem cell research and regulatory issues. Mayo Clin Proc 78,993–1003.

Colter DC, Sekiya I and Prockop DJ (2001) Identification of a subpopulation of rapidly self-renewing and multipotentail adult stem cells in colonies of human marrow stromal cells. Proc Natl Acad Sci USA 98,7841–7845.

Davani S, Marandin A, Mersin N, Royer B, Kantelip B, Herve P, Etievent JP and Kantelip JP (2003) Mesenchymal progenitor cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a rat cellular cardiomyoplasty model. Circulation 108,253–258.

Henderson JK, Draper JS, Baillie HS, Fishel S, Thomson JA, Moore H and Andrews PW (2002) Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. Stem Cells 20,329–337.

Hofstetter CP, Schwarz EJ, Hess D, Widenfalk J, Manira AE, Prockop DJ and Olson L (2002) Marrow stromal cells form guiding strands in the injured spinal cord and promote recovery. Proc Natl Acad Sci USA 99,2199–2204.

Hu Y, Liao L, Wang Q, Ma L, Ma G, Jiang X and Zhao RC (2003) Isolation and identification of mesenchymal stem cells from human fetal pancreas. J Lab Clin Med 141,342–349.

Hung SC, Cheng H, Pan CY, Tsai MJ, Kao LS and Ma HL (2002) In vitro differentiation of size-sieved stem cells into electrically active neural cells. Stem Cells 20,522–529.

In'tAnker PS, Scherjon SA, Kleijburg-van der Keur C, Noort WA, Claas FH, Willemze R, Fibbe WE and Kanhai HHH (2003) Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation. Blood 102,1548–1549.

Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M *et al.* (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418,41–49.

Kakishita K, Nakao N, Sakuragawa N and Itakura T (2003) Implantataion of human amniotic epithelial cells prevents the degeneration of nigral dopamine neurons in rats with 6-hydroxydopamine lesions. Brain Res 980,48–56.

Kopen GC, Prockop DJ and Phinney DG (1999) Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astocytes after injection into neonatal mouse brains. Proc Natl Acad Sci USA 96,10711–10716.

Korbling M and Estrov Z (2003) Adult stem cells for tissue repair—a new therapeutic concept? N Engl J Med 349,570–582.

Milunsky A (1992) Genetic Disorders and the Fetus: Diagnosis, Prevention, and Treatment, 3rd edn. Johns Hopkins University Press, London, pp. 101–117.

Minguell JJ, Erices A and Conget P (2001) Mesenchymal stem cells. Exp Biol Med 226,507–520.

Mitka M (2001) Amniotic cells show promise for fetal tissue engineering. J Am Med Assoc 286,2083.

Moertel CA, Stupca PJ and Dewald GW (1992) Pseudomosaicism, true mosaicism, and maternal cell contamination in amniotic fluid processed with in situ culture and robotic harvesting. Prenat Diagn 12,671–683.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S and Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284,143–147.

Prusa A.R and Hengstschläger M (2002) Amniotic fluid cells and human stem cell research: a new connection. Med Sci Monit 8,253–257.

Prusa AR, Marton E, Rosner M, Bernaschek G and Hengstschläger M (2003) Oct-4-expressing cells in human amniotic fluid: a new source for stem cell research? Hum Reprod 18,1489–1493.

Reyes M and Vertfaillie CM (1999) Turning marrow into brain: generation of glial and neuronal cells from adult bone marrow mesenchymal stem cells. Blood 94,377a.

Romanov YA, Svintsitskaya VA and Smirnov VN (2003) Searching for alternative sources of postnatal human mesenchymal stem cells: candidate MSC-like cells from umbilical cord. Stem Cells 21,105–110.

Sekiya I, Larson BL, Smith JR, Pochampally R, Cui JG and Prockop DJ (2002) Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. Stem Cells 20,530–541.

Steindler DA and Pincus DW (2002) Stem cells and neuropoiesis in the adult human brain. Lancet 359,1047–1054.

Young HY, Steele TA, Bray RA, Hunson J, Floyd JA, Hawkins K, Thomas K, Austin T, Edwards C, Cuzzourt J *et al*. (2001) Human reserve pluripotent mesenchymal stem cells are present in the connective tissues of skeletal muscle and dermis derived from fetal, adult, and geriatric donors. Anat Rec 264,51–62.

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