Retention of Multilineage Differentiation Potential of Mesenchymal Cells during Proliferation in Response to FGF

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Mesenchymal stem cells (MSC) that can differentiate to various connective tissue cells may be useful for autologous cell transplantation to defects of bone, cartilage, and tendon, if MSC can be expanded *in vitro*. However, a short life span of MSC and a reduction in their differentiation potential in culture have limited their clinical application. The purpose of this study is to identify a growth factor(s) involved in self-renewal of MSC and the maintenance of their multilineage differentiation potential. Fibroblast growth factor-2 (FGF-2) markedly increased the growth rate and the life span of rabbit, canine, and human bone marrow MSC in monolayer cultures. This effect of FGF-2 was more prominent in low-density cultures than in highdensity cultures. In addition, all MSC expanded *in vitro* with FGF-2, but not without FGF-2, differentiated to chondrocytes in pellet cultures. The FGF(1) MSC also retained the osteogenic and adipogenic potential throughout many mitotic divisions. These findings suggest that FGFs play a crucial role in selfrenewalof MSC.

Key Words; mesenchymal stem cell; FGF; multilineage differentiation potential; life span.

Introduction

Embryonic tissues contain mesenchymal cells that differentiate to osteoblasts, chondrocytes, adipocytes, hematopoietic cells, endothelial cells, and muscle cells. Adult bone marrow, as well as the periosteum, adipose tissue and peripheral blood, also contain ﾒ mesenchymal stem cells (MSC)ﾋ (1 ﾝ 3). The relationship between embryonic and adult MSC is differentiate to osteoblasts, chondrocytes, tenocytes, adipocytes, muscle cells or nerve cells *in vitro* and/or *in vivo* (1 ﾝ 6). MSC can easily be obtained repeatedly by bone marrow aspiration. Thus transplantation of bone marrow MSC may provide a new method for treatment of osteoporosis, arthritis, periodontal diseases, intrinsic muscular dystrophies, cardiac diseases, and degenerative nerve diseases (5). However, isolation of marrow aspirates in great volume causes damage and pain, and it is difficult to isolate from the bone marrow 107 ﾝ 108 MSC that are required for regeneration of large injured tissues. Thus the expansion of MSC *in vitro* is a prerequisite for autologous cell transplantation. In other words, identification of growth factors that stimulate the proliferation of MSC and support their multilineage differentiation potential is a critical step towards the clinical application of MSC.

Previous studies have shown that FGF is a potent mitogen for some connective tissue cells including osteoblasts and chondrocytes (8). Furthermore, chondrocytes grown with FGF-2 *in vitro* but not without FGF-2 maintained the capability for the phenotypic expression after several mitotic divisions, even though direct addition of FGF-2 in confluent cultures markedly suppressed the phenotypic expression (8). These findings prompted us to examine the effect of FGF-2 on the proliferation and differentiation of MSC. The results showed that FGF-2 is a potent mitogen for MSC, and that incubation with FGF-2 maintains the multilineage differentiation potential of MSC throughout many mitotic divisions.

Materials and Methods

Cell culture.

Human bone marrow MSC were obtained from Bio-Whittaker Inc. (secondary cultures, Walkersville, MD) or patents as part of a protocol approved by ethical authorities. Marrow aspirates were also obtained from three 4- or 12-week-old male Japan White rabbits. The cells, including erythrocytes (0.1 ml aliquots of the aspirates), were seeded at 2 3 108 cells per 100-mm tissue culture dish and maintained in 10 ml of Dulbecco ± s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (medium A). Three days after seeding, floating cells were removed and the medium was replaced by flesh medium A. Thereafter attached cells were fed with fresh medium A every three days. Passages were performed when cells were approaching confluence. Cells were seeded at 1 or 5 3 103 cells per cm2 in 100-mm dishes. Chondrogenic, osteogenic or adipogenic conversion of MSC was determined according to the procedures reported by Pittenger *et al.* with some modifications (1). For chondrogenic differentiation, cells were seeded at 2 3 105 cells per 15 ml plastic centrifuge tube, and maintained in 0.5 ml of serum-free a-MEM (high glucose) supplemented with 6.25 mg/ml insulin, 6.25 mg/ml transferrin, 6.25 ng/ml selenite, 5.33 mg/ml linolate, 1.25 mg/ml bovine serum albumin, 10 ng/ml TGF-b-1, 100 nM dexamethasone, and 50 mg/ml ascorbic acid ² 2-phosphate (Wako, Tokyo, Japan). The cultures were fed with 0.5 ml of the medium until 4 days after seeding. Thereafter, the cultures were fed with 1 ml of the medium every other day. For osteogenic differentiation, cells were seeded at 104 cells per 9-mm-dish, and maintained for 28 days in DMEM supplemented with 10 mg/ml insulin, 10 mM b-glycerophosphate, 100 nM dexamethasone, and 50 mg/ml ascorbic acid ² 2-phosphate. For adipogenic differentiation, cells were seeded at 4 3 104 cells per 9-mm dish and maintained in DMEM containing 10% fetal bovine serum and antibiotics for 3 days. The cells were exposed to DMEM (high glucose) supplemented with 10 mg/ml insulin, 0.2 mM indomethacin, 1 mM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 10% fetal bovine serum for 25 days. Lipid was stained with oil-red O.

Glycosaminoglycan, alkaline phosphatase activity, and calcium.

The glycosaminoglycan content was determined as described previously (9). Alkaline phosphatase activity in pellet cultures was determined by the method of Bessey *et al.* (10). The calcium content of monolayer cultures in 11-mm dishes was determined by the method of Gitelman (11).

RT-PCR.

Total RNA was extracted using Isogen (Nippon Gene, Tokyo, Japan). The first-strand cDNA was synthesized from 1 mg of total RNA using the Superscript II RNase H2 reverse transcriptase (Life Technologies, Inc.,

Rockville, MD). Using the cDNAs as a template, PCR was carried out under the following conditions: denaturation at 94° C for 30 s and primer extension at 65° C for 1.5 min in 25, 30, 30, 30, 25, 30, 30 cycles for type II collagen and type X collagen, GAPDH, bone sialoprotein, osteopontin, osteocalcin and PPAR-g2, respectively. Pairs of nucleotides, 59-CATACCGGTAAGTGGGGCAAGA CTG-39 and 59-TGCCCAGTTCAGGTCTCTTA-39 for rabbit type II collagen, 59-CCCAACACCAAGACACAGTT-39 and 59-ATCACCTTTGATG CCTGGCT-39 for rabbit type X collagen, and 59-GTCAAGGCCGA GAATGGGAA-39 and 59-GCTTCACCACCTTCTTGATG-39 for rabbit and human GAPDH, 59-CATTTTGGGAATGGCCTGTG-39 and 59-ATTGT CTCTCCGCTGCTGC-39 for human bone sialoprotein, 59-CTAGGCATCA CCTGTGCCATACC-39 and 59-CAGTGACCAGTTCATCAGATTCATC-39 human osteopontin, 59-CCACCGAGACACCATGAGAG-39 and 59-CCATA GGGCTGGGAGGTCAG-39 for human osteocalcin, and 59-CATTCTGGCCC ACCAACTT-39 and 59-CCTTGCATCCTTCACAAGCA-39 human PPAR-g2 were used as primers for RT-PCR. Obtained PCR products were separated on 1% agarose gels, and stained with ethidium bromide.

Results

FGF-2 increased the growth rate and the life span of rabbit and human MSC from the ilium and/or tibia in monolayer cultures in the presence of 10% fetal bovine serum (Fig. 1). FGF-2 had greater effects in lowdensity cultures (seeding density, 1000 cells/cm2) than in high-density cultures (5000 cells/cm2) in all series of studies (data not shown). Similar results were obtained with human alveolar bone MSC and canine ilium bone marrow MSC (data not shown).

To examine the chondrogenic potential, MSC grown with FGF-2 (FGF(1) MSC) were transferred into the chondrogenic medium containing TGF-band insulin in pellet cultures (Fig. 2A). The chondrogenic medium did not contain FGF-2. Chondrocyte differentiation occurs at a much higher level in pellet cultures than in monolayer cultures (12). In the pellet cultures of FGF(1) MSC, spherical cells (chondrocytes) appeared near the surface of the pellet on day 4 even in the absence of FGF-2 (Fig. 2B). The chondrocytes were surrounded by cartilage-characteristic proteoglycan that stained metachromatically with toluidine blue. On day 8, all cells in FGF(1) MSC cultures (Fig. 2C) became spherical. However, the chondrogenic differentiation was markedly delayed in cultures of FGF(2) MSC (Fig. 2D). The chondrogenic potential of FGF(1)

MSC was examined as a function of the passage number. FGF(1) MSC from the 3rd, 6th, 9th, and 12th passage cultures (Figs. 2E, 2H, respectively) were maintained in pellet cultures for 16 days. Almost all FGF(1) MSC from the 3rd through 9th passage cultures reorganized into a cartilage-like tissue by day 16 (Figs. 2D, 2G). However, toluidine blue stained cartilage proteoglycan decreased with the increase in the passage number. A few FGF(1) MSC from the 12th passage cultures became chondrocytes (Fig. 2H). The glycosaminoglycan and alkaline phosphatase levels decreased with the increase in the passage number in the FGF(1) and FGF(2) MSC cultures.

However, at each passage number, the glycosaminoglycan (Fig. 3A) and alkaline phosphatase levels (Fig. 3B) were much higher in FGF(1) MSC cultures than in FGF(2) MSC cultures. Furthermore, the levels of type II collagen and type X collagen mRNAs, markers for chondrocytes, were higher in FGF(1) MSC from the 9th passage cultures than in FGF(2)MSC from the 9th passage cultures (Fig. 3C). Next FGF(1) and FGF(2) MSC grown in high- or low-density cultures for many generations were transferred into the osteogenic medium. When MSC were passaged at a high initial cell density (5000 cells/cm2), FGF(1) and FGF(2) MSC from the 3rd, 6th, and 9th passage cultures showed similar calcium and alkaline phosphatase levels, irrespective of the passage number or the presence or absence of FGF-2 (Figs. 4A and 4B).

When MSC were passaged at a low initial cell density (1000 cells/cm2), FGF(1) MSC showed a higher calcium content than FGF(2) MSC, and the calcium content decreased with the increase in the passage number (Fig. 4C). The expressions of bone sialoprotein, osteopontin and osteocalcin mRNAs in

cultures of FGF(1) MSC from the 9th passage cultures were higher than in cultures of FGF(2) MSC from the 9th passage cultures (Fig. 4D).

These observations suggest that FGF-2 maintains the osteogenic potential of MSC. FGF(1) and FGF(2) MSC showed similar adipogenic differentiation, which was estimated by oil-red O staining (Figs. 5A and 5B). The level of PPAR-g2 mRNA, a marker of adipocyte differentiation, in the FGF(1) and FGF(2) MSC cultures was almost constant, at least until the 9th passage (Fig. 5C).

Discussion

The use of leukemia inhibitory factor (LIF) and feeder cells allowed the proliferation of embryonic stem cells (ES cells) *in vitro*. The availability of the cultured ES cells greatly contributed to developmental biology. Similarly the availability of large amounts of MSC expanded with FGF-2 *in vitro* will facilitate the clinical application of MSC and basic studies on MSC.

The chondrogenic potential of adult MSC markedly decreased with the increase in the passage number, even when the cells were seeded at a high initial cell density. Incubation of MSC with FGF-2 maintained the chondrogenic potential during the expansion of MSC *in vitro*. However, even in the presence of FGF-2, the chondrogenic potential gradually decreased with the passage number. Additional factors seem to be required for the maintenance of the chondrogenic potential. In contrast, the osteogenic potential of MSC was maintained at a high level throughout many mitotic divisions, when MSC were seeded and grown in high-density cultures in the presence of FGF-2. The osteogenic potential decreased when MSC were seeded and grown in low-density cultures in the absence of FGF. The adipogenic potential of MSC was maintained at a high level even in the absence of FGF-2 throughout many mitogenic divisions. These observations suggest that the chondrogenic potential of MSC is unstable throughout mitotic divisions *in vitro,* as compared to the osteogenic and adipogenic potential. FGF-2 markedly suppressed the decline in the chondrogenic/osteogenic potential of MSC, when it enhanced cell proliferation. The mechanism by which FGF-2 maintains the differentiation potential of MSC is unknown.

Studies with FGF-2-knockout mice have shown that bone mass is smaller in FGF-2-knockout mice than control mice, and that bone marrow mesenchymal cells from FGF-2-knockout mice have poor osteogenic potential *in vitro* (13). These findings, taken together with our findings in the present study, suggest that FGF-2 plays a role in the maintenance of the osteogenic potential of MSC *in vivo*. Since the FGF family has many members (.23), it is difficult to determine which FGF family members play the most critical role in selfrenewal of MSC.

Oct-3/4 has been shown to be expressed at high levels in ES and EG (embryonic germ) cells and essential for the maintenance of the undifferentiated state of these cells (14). LIF activates STAT3 via LIF receptors and gp130 to maintain the undifferentiated stage of ES cells (15). The mechanism involved in the maintenance of the undifferentiated state or self-renewal of MSC remains unknown. However, MSC maintained in the presence of FGF may be useful for identification of signaling molecules and transcription factor(s) involved in self-renewal and the multilineage differentiation potential of MSC. We are investigating FGF-inducible genes in MSC using DNA arrays.

In preliminary studies, we transplanted rabbit MSC into 5-mm-diameter full-thickness defects in rabbit knee joints. MSC were obtained from the 3rd

passage cultures of 12-week-old rabbits, and expanded *ex vivo* with FGF-2, before transplantation. Nine weeks after transplantation, nontreated defects were not covered with a cartilaginous tissue, although a bone-like tissue was formed. In contrast, MSC-treated defects were completely covered with cartilage, which was indistinguishable from the surrounding original articular cartilage (data not shown). Transplantation of FGF(1) MSC will be useful for tissue regeneration *in vivo*.

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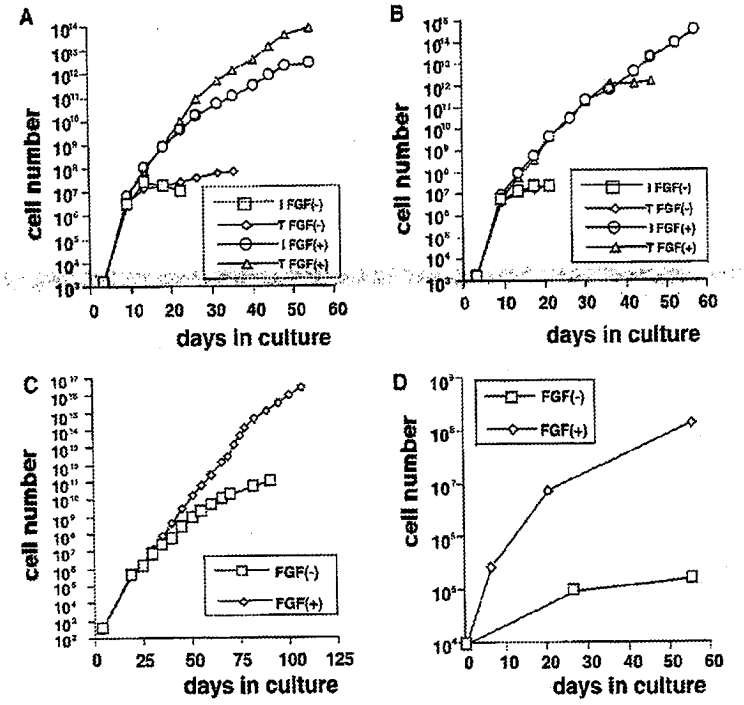

Fig. 1. Culture lifetime of rabbit (A, B) and human MSC (C, D) in monolayer cultures in the presence and absence of FGF-2.
MSC were isolated from the ilium (I) or tibia (T) of three rabbits in two independent studies (A, B) or from the ilium of two patients (C, D). MSC used in D were obtained from BioWhittaker Inc. MSC were seeded at 1 (D) or 5 3 103 cells (A-C) per cm2 and maintained in the medium containing 10% fetal bovine serum (A, C) or human serum (D) in the presence or absence of FGF-2 (1 ng/ml). Passages were performed when the cells were approaching confluence.

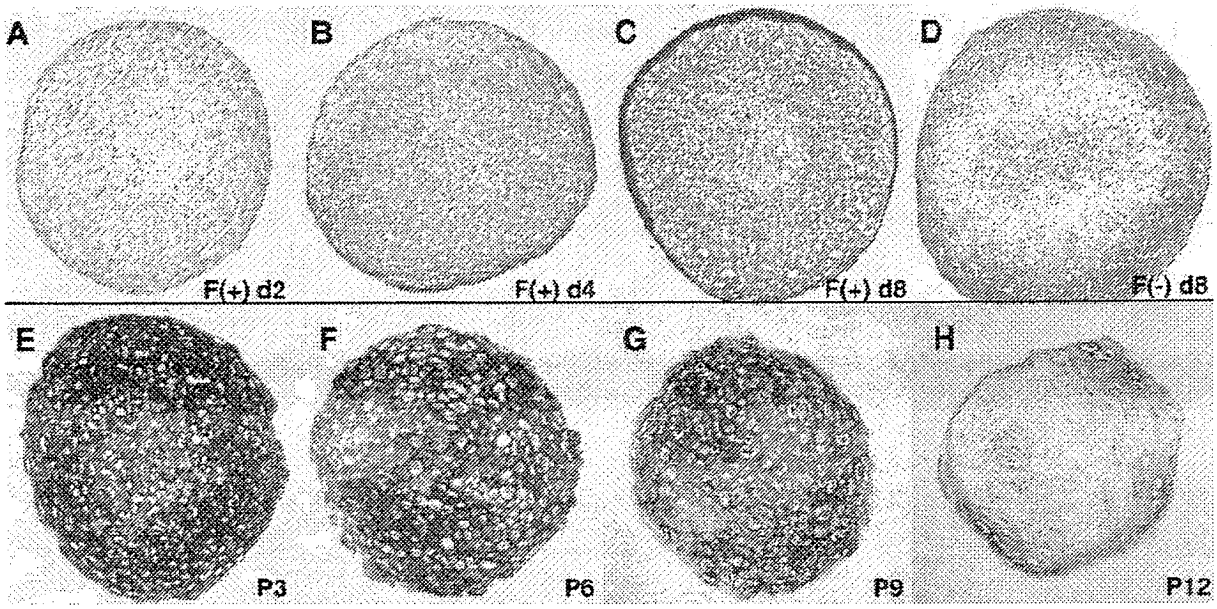

Fig. 2. Chondrogenic potential of MSC expanded *ex vivo* with FGF-2.
(A) MSC isolated from 4-week-old rabbits and grown in the presence (A, C) or absence (D) of FGF-2. The MSC obtained from the 3rd passage cultures were transferred into the chondrogenic medium in pellet cultures for 2 (A), 4 (B), and 8 days (C, D). FGF(1) MSC from the 3rd, 6th, 9th, and 12th passage cultures (E-H, respectively) were maintained in pellet cultures for 16 days.

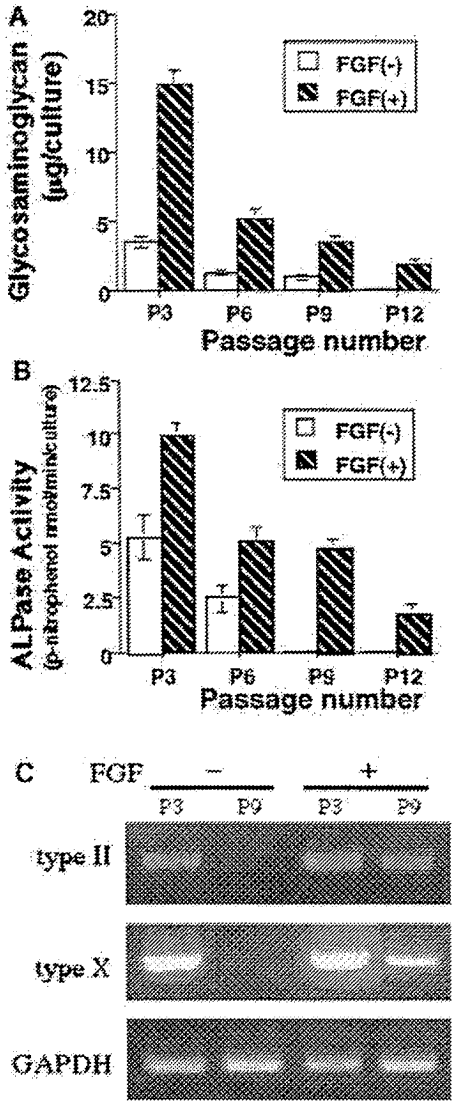

Fig. 3. The levels of glycosaminoglycan, alkaline phosphatase and cartilage-specific gene expression in pellet cultures of FGF(1) and FGF(2) MSC as a function of the passage number.

FGF(1) and FGF(2) MSC from the 3rd, 6th, 9th, and 12th passage cultures were maintained in pellet cultures. The glycosaminoglycan content (A), alkaline phosphatase activity (B), and type II and type X collagen mRNA levels (C) were determined 16 days after seeding. Values are averages 6 SD for four cultures.

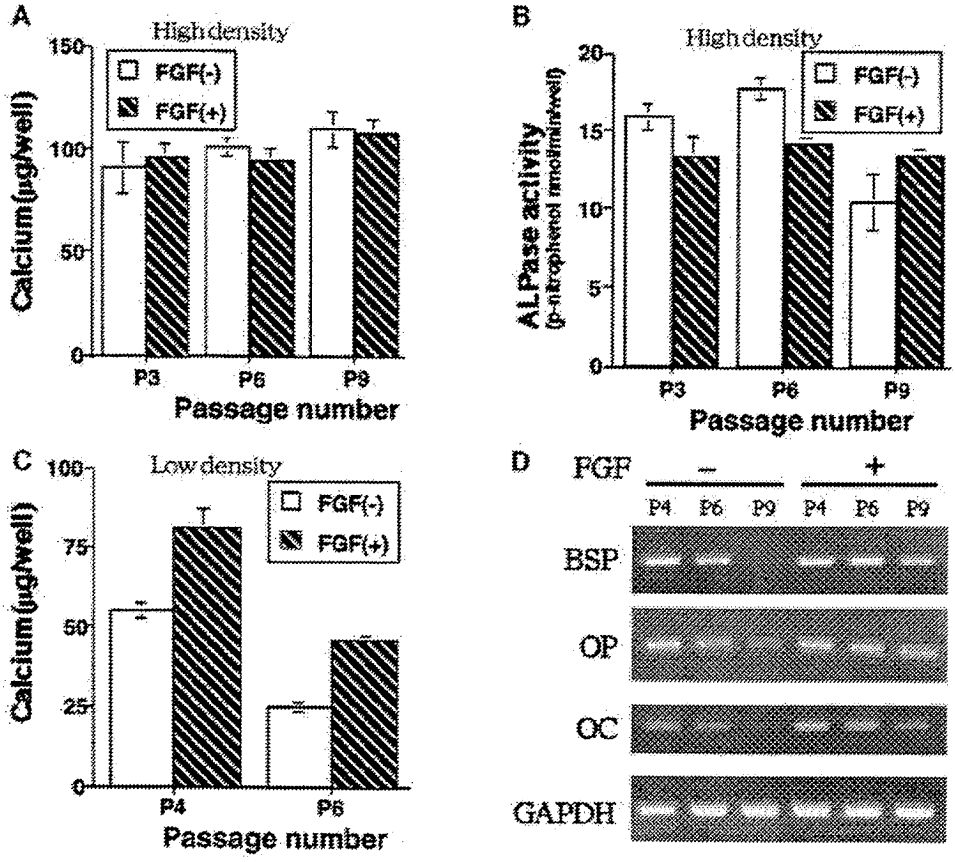

Fig. 4. Osteogenic potential of MSC expanded *ex vivo* with or without FGF-2.

Human MSC were seeded at a high (5000 cells/cm2) (A, B, D) or low (1000 cells/cm2) density (C). The MSC from the 3rd, 4th, 6th, and 9th passage cultures with or without FGF-2 were transferred into the osteogenic medium for 28 days, and the calcium content (A, C) and alkaline phosphatase activity (B) of the cell-matrix layers were determined. Values are averages 6 SD for four cultures. (D) RNA was isolated on day 28. RT-PCR analysis of osteoblast-specific gene expression (bone sialoprotein, osteopontin, and osteocalcin) in cultures of FGF(1) or FGF(2).

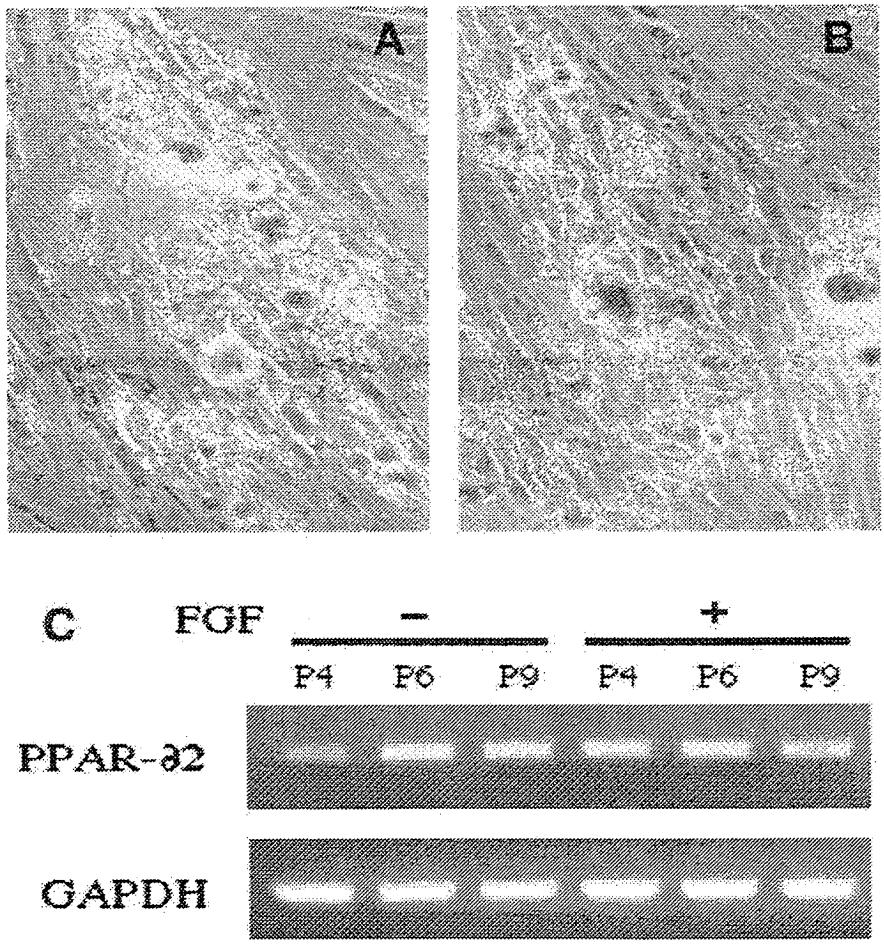

Fig. 5. Adipogenic potential of MSC expanded *ex vivo* without (A) or with FGF-2 (B).

Human MSC were seeded at 5000 cells/cm2. The FGF(1) and FGF(2) MSC obtained from the 6th (A, B) and the 4th, 6th, and 9th passage cultures (C) were maintained under the adipogenic conditions for 28 days. RT-PCR analysis of adipocyte-specific gene expression (PPAR-g2) in cultures of FGF(1) or FGF(2) MSC on day 28 (C).