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Pluripotency potential of human adipose-derived stem cells marked with exogenous green fluorescent protein

Yunfeng Lin,[1,3] Lei Liu,[1] Zhiyong Li,[1] Ju Qiao,[2] Ling Wu,[1,3] Wei Tang,[1] Xiaohui Zheng,[1] Xizhe Chen,[1] Zhengbin Yan[1] and Weidong Tian[1,3]

[1]*Department of Oral and Maxillofacial Surgery, West China College of Stomatology, Sichuan University, Chengdu 610041, P.R. China;* [2]*Department of Orthodontics, West China College of Stomatology, Sichuan University, Chengdu 610041, P.R. China;* [3]*Key Laboratory of Oral Biomedical Engineering, Ministry of Education. Sichuan University*

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Abstract

Musculoskeletal tissues regeneration requires rapid expansion of seeding cells both *in vitro* and *in vivo* while maintaining their multilineage differentiation ability. Human adipose-derived stem cells (ASCs) are considered to contain multipotent mesenchymal stem cells. Monolayer cultures of human ASCs were isolated from human lipoaspirates and passaged 3 times and then infected with replication-incompetent adenoviral vectors carrying green fluorescent protein (Ad/GFP) genes. Then, Ad/GFP infected human ASCs were transferred to osteogenic, chondrogenic, adipogenic, and myogenic medium. The morphological characterization of induced cells was observed using phase-contrast microscopy and fluorescence microscopy. The expression of marker proteins or genes was measured by immunocytochemical and RT-PCR analysis. Osteopontin (OPN), and osteocalcin (OCN) were positive in osteogenic lineages, aggrecan and SOX9 were positive in chondrogenic ones, peroxisome proliferator-activated receptor (PPAR-γ2) and lipoprotein lipase (LPL) were positive in adipogenic ones, and myogenin and myod1 was positive in myogenic ones. At the same time, the results of fluorescence microscopic imaging proved that the high level of GFP expression during ASCs differentiation maintained stable nearly 2 months. So the exogenous GFP and multilineage potential of human ASCs had no severe influences on each other. Since the human ASCs can be easily obtained and abundant, it is proposed that they may be promising candidate cells for further studies on tissue engineering. Imaging with expression of GFP facilitates the research on ASCs physiological behavior and application in tissue engineering during differentiation both *in vitro* and *in vivo*. (Mol Cell Biochem **291:** 1–10, 2006)

Key words: human adipose-derived stem cells, green fluorescent protein, pluripotency

Introduction

The regeneration medicine developed fast in recent years. The purpose of tissue regeneration has developed to repair and restore the function of damaged or diseased tissues [1, 2]. The fundamental factor of tissue engineering involves an ideal seeding cell source and a biocompatible scaffold to produce an artificial environment that mimic the *in vivo* condition [3, 4]. Recently, it has been reported that human adipose tissue-derived stem cells (ASCs) can be induced to express

Address for offprints: W. Tian, Department of Oral and Maxillofacial Surgery, West China College of Stomatology, Sichuan University, Chengdu 610041, P.R. China (E-mail: dentisttian@hotmail.com)

genes and protein markers associated with the chondrocyte, adipocyte, osteoblast, endothlium and myocyte [5–8]. Adipose tissue is derived from the embryonic mesenchyme and contains a stroma structure which is similar to the bone marrow stem cells (BMSCs), and research have proved the ASCs can be induced into multiple lineages in specific culture system similar to BMSCs [9, 10].

Compared with BMSCs, ASCs are much more abundant and easier to obtain, carrying relatively lower donor site morbidity. There is much less cell heterogeneity in ASCs, than in BMSCs, which is caused by the mixture of hemopoietic and mesenchymal stem cells [10–12]. So it is believed the ASCs represent an ideal substitute for BMSCs used in cell-based tissue-engineering strategies. However, limited availability of markers for human ASCs greatly hampered the further studies.

New techniques involving implantation of marked cells and tissue-engineered constructs are being developed to improve musculoskeletal tissue repair [13, 14]. The GFP is responsible for the green bioluminescence from the jellyfish Aequorea Victoria [15, 16]. The GFP transgenic animal is an excellent model because all the cells from the transgenic animal express endogenous GFP which is easy to observe and isolate. But the transgenic animal is very expensive and difficult to obtain, and it's impossible to be used in the human cells research. With the evolution of genetically encoded GFP, normal human ASCs infected with adenoviral vectors carrying green fluorescent protein (Ad/GFP) can also achieve the purpose of fluorescent imaging temporally. In view of the GFP expression by adenoviral vectors, they are preferably taken as the model to detect the durability of the desired gene expression in adenovirus infected cells [17]. Several months' stable expression is enough for *in vitro* and in temporary *in vivo* research.

Growth factors in inducing medium had side effects to other cells or tissue and their influences were limited because of short biological half-lives. In respect of clinical application, adenoviral vector is one of the best choices to achieve short-lived effect and transient infection. Adenoviral-mediated gene transfer is the most reliable and efficient methods for introducing genes into mammalian cells because infection by adenovirus is not cell-cycle dependent [14, 17]. Desired gene is transiently expressed at high levels since many cells receive multiple copies of the recombinant adenoviral DNA, which does not integrate into the cellular genome.

In this study, human ASCs were infected with Ad/GFP and incubated in different inducing medium [6, 17]. Specific regulator and matrix molecules in mutilineage induced cells were assessed at mRNA and protein levels. It is suggested that the ASCs marked with GFP will be very useful in future study. By virtue of the exogenous GFP, we can observe the cells in living organisms and confirm the origin and migration of implanted cells. Furthermore, Microscopic imaging of GFP expression in Ad/GFP infected ASCs makes it easy to determine whether the level of desired gene maintain in the course of differentiation and how long it could last in differentiated ASCs. Also it can offer the information about the optimization of multiplicities of infection (MOI) for ASCs.

Materials and methods

Isolation and culture of human ASCs

The isolation and culture of human ASCs is similar to our precious methods [18]. Briefly, human adipose tissue was obtained from 20 healthy donors (age range, 20 to 35 years) during the liposuction procedure. All procedures were approved by the Human Subject Protection Committee (Protocol HSPC 98-08-011-01). Raw lipoaspirates (about 15 ml) were washed extensively with sterile phosphate-buffered saline (PBS) and incubated with 0.075% type I collagenase (Sigma-Aldrich, St. Louis, MO) for 40 min at 37 °C with gentle agitation. Cells released by the enzymatic digestion were filtered and collected by centrifugation at 1200 g for 10 min. Then, the pellet was resuspended, washed 3 times with medium, and seeded on the plastic tissue culture dishes in control medium containing DMEM/F12 (1:1), 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. Human ASCs were maintained in a humidified atmosphere of 5% CO_2 at 37 °C and passaged 3 times prior to Ad/GFP infection [17, 18].

Construction of a GFP adenoviral vector

The cDNA encoding GFP was isolated from plasmid (pEGFP-C1) and inserted into shuttle plasmid in pAdEasy-1 system which is provided by Stratagene Corp. Recombinant adenoviral vectors were constructed by the method of homologous recombination in *E. coli* BJ5183 and selected on kanamycin plates. Expression cassette of Ad/GFP was verified by restriction analysis and the adenoviral vectors were amplified in transforming *E. coli* DH5α. In order to obtain adenoviral particles, Ad/GFP was packaged by transfected into human embryonic kidney (HEK) 293 cells. Replication-incompetent adenovirus is conveniently harvested when HEK293 cells were lysed and filtered by a 0.45 micron filter (Corning, NY). MOI was determined by the plaque forming unit (PFU) and the viral stocks were frozen in single use aliquots at −80 °C [17].

Cell infection and examination of GFP expression

Infection of cells with Ad/GFP was performed in 1.0 ml of serum-free control medium for 4 h at 37 °C at various

Table 1. Lineage-specific differentiation induced by medium supplementation

Lineage	Medium	Serum	Supplementation
Osteogenesis	DMEM/F12	FBS (10%)	50 μM ascorbate-2-phosphate, 10 mM β-glycerophosphate, 0.01 μM 1,25-dihydroxyvitamin D3, 1% antibiotic/antimycotic
Chondorgenesis	DMEM/F12	FBS (10%)	10 ng/ml TGF-β1, 100 nM dexamethasone, 6.25 μg/ml insulin, 50 nM ascorbate-2-phosphate, 110 mg/L sodium pyruvate, 1% antibiotic /antimycotic
Adipogenesis	DMEM/F12	FBS (10%)	1 μM dexamethasone, 10 μM insulin, 200 μM indomethacin, 0.5 mM isobutyl-methylxanthine (IBMX), 1% antibiotic/antimycotic
Myogenesis	DMEM/F12	FBS (10%), HS (5%)	50 μM hydrocortisone, 1% antibiotic/antimycotic
Control	DMEM/F12	FBS (10%)	1% antibiotic/antimycotic

MOI (10–1000). Following infection, the remaining supernatant was removed and replaced with control medium supplemented with 10% fetal bovine serum. The expression of GFP in cells was assessed 48 hr after infection through fluorescence microscopic imaging. The percentage of the cells expressing GFP was determined by visual inspection.

Multilineage differentiation of human ASCs

Allowed to recover for 5 days, human ASCs infected with Ad/GFP were transferred to the osteogenic, adipogenic, neurogenic, myogenic and control medium (Table 1). The GFP marked human ASCs were trypsinized and reseeded onto 100-mm^2 tissue culture plates at a density of 10^5 cells. The cells were incubated in the control medium for a day to adhere to the plates and then the medium was replaced by specific medium contained the following components listed in Table 1. The medium was replaced every 3–4 days. When differentiated cells were confluent, most of they were harvested for studies. We performed more than 3 independent sets of the experiments, and each experiment was run at least 3 times. Differentiated phenotype of variant lineages was viewed and compared by phase-contrast microscopy and fluorescence microscopic imaging during culture [16].

Histochemical staining

The mineralization nodules in osteogenic lineages was stained with alizarin red S (AR-S). The cells on slides were rinsed in phosphate-buffered saline and incubated with 40 mM AR-S (pH 4.2) with rotation for 10 min. Then they were rinsed 5 times with water followed by a 15-min washing with PBS with rotation to reduce nonspecific AR-S stain. The stained nodules were observed through microscope.

The adipogenic cells were assessed using Oil Red O staining for accumulation of cytoplasmic lipid droplets. The cells on slides were rinsed in phosphate-buffered saline and fixed in 4% paraformaldehyde for 15 min at room temperature. Briefly, cell layers were stained with 1% oil-red O in 60% isopropanol for 10 min. The stain was differentiated with 60% isopropanol and excess stain was removed by washing with 70% ethanol. Followed several times washing in distilled water, the stained slides were covered for microscopy.

Immunocytochemical analysis

Monolayer slides of multilineage differentiated human ASCs were prepared for immunocytochemical analysis and fixed in 4% buffered paraformaldehyde. Fixed glass slides were incubated with 3% hydrogen peroxide in methanol for 30 min to inhibit endogenous peroxidase activity. After washed with PBS, they were blocked in 1% bovine serum albumin and 1.5% normal goat serum at room temperature for 30 min. Slides were then incubated overnight at 4 °C with mouse monoclonal antibodies against aggracan (Santa Cruz) for chondrogenic cells, and mouse monoclonal antibodies against α-SMA (Dako; dilution, 1:200) for myogenic cells. Sequentially, slides were then incubated with secondary biotinylated antibodies and horseradish peroxide conjugated streptavidin to detect the primary antibodies. The peroxidase reaction was developed using 3, 3′-diaminobenzidine

Table 2. Specific primers were designed following the cDNA sequences of each gene in GenBank. The upstream primer is shown above the downstream primer in all cases. The expected fragments size upon amplification is given with optimal annealing temperature

Gene	Primers	Annealing temperature (°C)	Fragment (bp)	Cycle no.	GenBank no.
β-actin	5′-ACTCTTCCAGCCTTCCTTCC-3′ 5′-ACTCGTCATACTCCTGCTTGC-3′	55	313	30	BC013835
OCN	5′-AGGGCAGCGAGGTAGTGA-3′ 5′–CCTGAAAGCCGATGTGGT-3′	56	150	30	NM_199173
OPN	5′-AAATTCTGGGAGGGCTTGG-3′ 5′-TTCCTTGGTCGGCGTTTG-3′	55	117	30	NM_000582
Aggrecan	5′-GCAGAGACGCATCTAGAAATTG-3′ 5′-GGTAATTGCAGGGAACATCATT-3′	55	504	31	NM_013227
SOX9	5′-GAACGCACATCAAGACGGAG-3′ 5′-TCTCGTTGATTTCGCTGCTC-3′	60	631	28	NM_000346
PPAR-γ2	5′-AGGAGCAGAGCAAAGAGG-3′ 5′-CCTCGGATATGAGAACCC-3′	51	219	30	NM_005037
LPL	5′AGTTGTACTTCCAGTGCGTCTC3′ 5′TACTTTCACAGTCGGGTT3′	48	366	30	NM_000237
Myogenin	F: 5′-ATCTGCTCACGGCTGACCCTACA-3′ R: 5′- ACAATCTCAGTTGGGCATGGTTTC- 3′	56	125	30	NM_002479
Myod1	F: ′-CCTTTGCCACAACGGACGACTTCT-3′ R: ′-TGCGAGTGCTCTTCGGGTTTCAG-3′	60	127	30	NM_002478

tetrahydrochloride (DAB) as chromogens. After rinsing in distilled water, slides were dehydrated in ascending ethanol solutions, cleared in xylene, and covered with slips for microscopy. The human ASCs which is not marked with AD/GFP were taken as control group.

RNA isolation and reverse transcription-polymerase chain reaction

Total RNA was extracted from all the specimens using the TRIzol Reagent (Life Technologies, Rockville, MD) according to the protocol. About 1 μg of total RNA was reversed transcribed by murine leukemia virus reverse transcriptase (TaKaRa, Jap) and PCR amplification of target message RNA was performed by TaKaRa PCR kit (TaKaRa, Jap). PCR oligonucleotide primers and annealing temperature were listed in Table 2. All primers were determined through established GenBank sequences and amplification of β-actin was used as a control for assessing PCR efficiency. The products were electrophoresed on 1.5% agarose gels, stained with Ethidium Bromide and visualized with Quantity One software (BIO-RAD).

Results

Morphological features of primary cultures

Approximately 5×10^6 nucleated cells were yielded from the lipoaspirates of each donor. When isolated and primarily cultured as monolayer, most of the cells that attached to the culture dish surface exhibited a fibroblast-like spindle shape at first (Fig. 1A). They proliferated quickly in control medium to form colonies that grew and merged to a uniform confluent cell monolayer after 2 weeks. The cultures were passaged 3 times for expansion.

Generation of GFP adenoviral expression vector

Ad/GFP was constructed by inserting the GFP cDNA into the pAdEasy-1 backbone. For verification, Ad/GFP was digested with Pac I and the result of electrophoresis showed fragments with expected length. Ad/GFP particles were harvested from lysates of HEK 293 cells and purified by CsCl gradient centrifugation, and the viral titer in stocks was 10^{12}/ml calculated by plaque forming unit assay. According to our previous works, with a MOI of 50 to 100, nearly 70% of ASCs were positive for GFP expression and no toxicity was observed. When the MOI was over 100, there were serious cytopathic effect and obvious decline in cellular viability. So in this experiment the MOI of 100 is used for infection. [17]

Expression of GFP in human ASCs

ASCs infected with Ad/GFP produced elevated amounts of GFP as determined by fluorescence microscopic imaging. With 100 MOI after 48 hr, exogenous GFP expression indicated initially. It reached the peak level after 7 days and lasted for about 8 weeks (Fig. 1 B, C). But the Ad/GFP expression declined stepwise irreversibly henceforward.

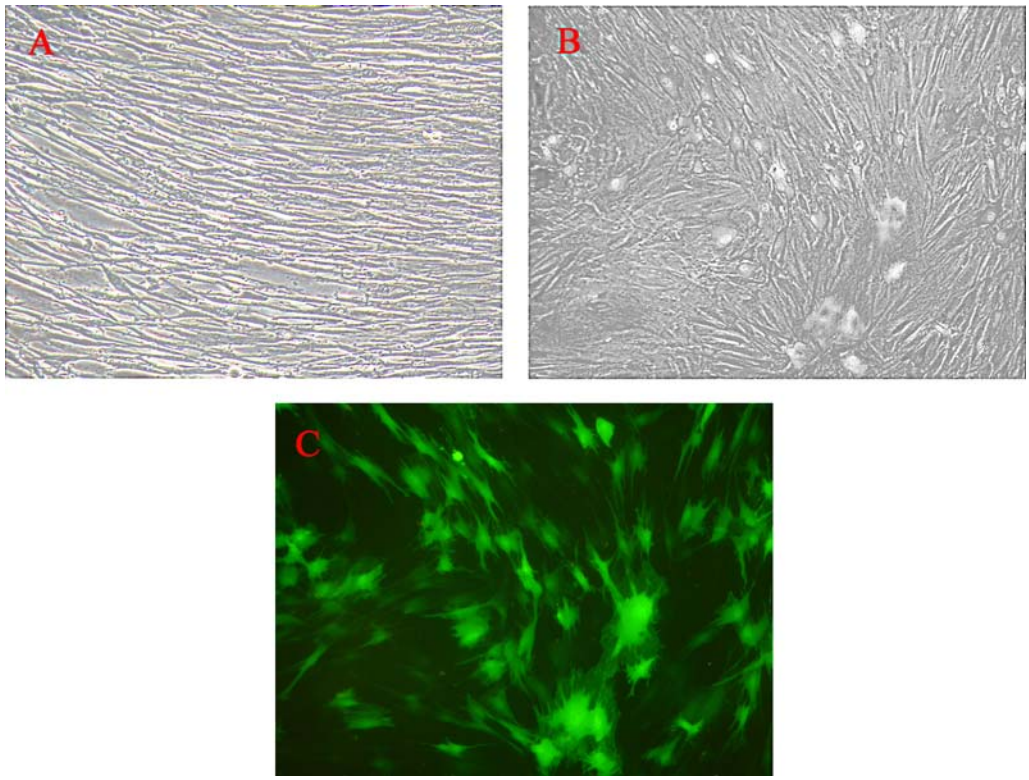

Fig. 1. (A)ASCs harvested from raw lipoaspirates were incubated in a control medium for 10 days and showed an elongated fibroblastic appearance. Primary culture of AD/GFP infected ASCs for 48 h. (B) Phase-contrast microscopic images. (C) Fluorescence microscopic images. (Original magnification of all images $\times$200).

Osteogenesis of GFP marked human ASCs

After 5 days recover, Ad/GFP infected ASCs were placed in osteogenic medium. The GFP marked human ASCs changed from a fibroblast-like appearance to a multilateral form after 5 days induction. After inducing culture for 2 week, the monolayer cells aggregated together just like islands of differentiated cells trapped in a matrix secreted from them. After the formation of nodules, cells migrated out from the nodules and organized more cells to become larger mineral nodules (Fig. 2A). Fluorescence imaging indicated that the stable expression GFP (Fig. 2B). The mineralized nodular structures were assessed by Alizarin Red-S staining, which localized specifically in the mineral nodules (Fig. 2C). Such changes were not observed in the control group (data not shown). To verify the osteogenesis, cells are also examined by RT-PCR for the expression of specific genes including OCN and OPN. Both OCN and OPN were observed in differentiated ASCs and which ware not observed in the controls (Fig. 4).

Chondrogenic differentiation

After 5 days recover, GFP marked human ASCs were placed into chondrogenic medium. The ASCs cells modulated from fibroblastic appearance to a polygonal shape after 7 days induction (Fig. 2D). Fluorescence imaging indicated the expression of GFP in chondrogenic lineages were stable and high (Fig. 2E). The immunostaining showed highly positive of aggrecan (Fig. 2F). To verify the chondrogenesis, cells were also examined by RT-PCR for the expression of specific genes including aggrecan and SOX9. Both aggrecan and SOX9 were observed in differentiated ASCs but not in the controls (Fig. 4).

Adipogenesis of GFP marked human ASCs

When the Ad/GFP infected ASCs recover for 5 days, they were transferred into adipogenic medium. The morphology of induced human ASCs modulated from an elongated to a rounder shape. Two weeks after initial induction, there were more and more lipid-filled cells (Fig. 3A). The GFP in differentiated cells expressed still high and stable (Fig. 3B). The adipogenic differentiation was evidenced by positive Oil Red O staining of lipid droplets present throughout the cytoplasm of the cells (Fig. 3C). Whereas, the cells growing in control media were not stained (data not shown). The expression of adipocyte specific gene PPAR-γ2 and LPL was also

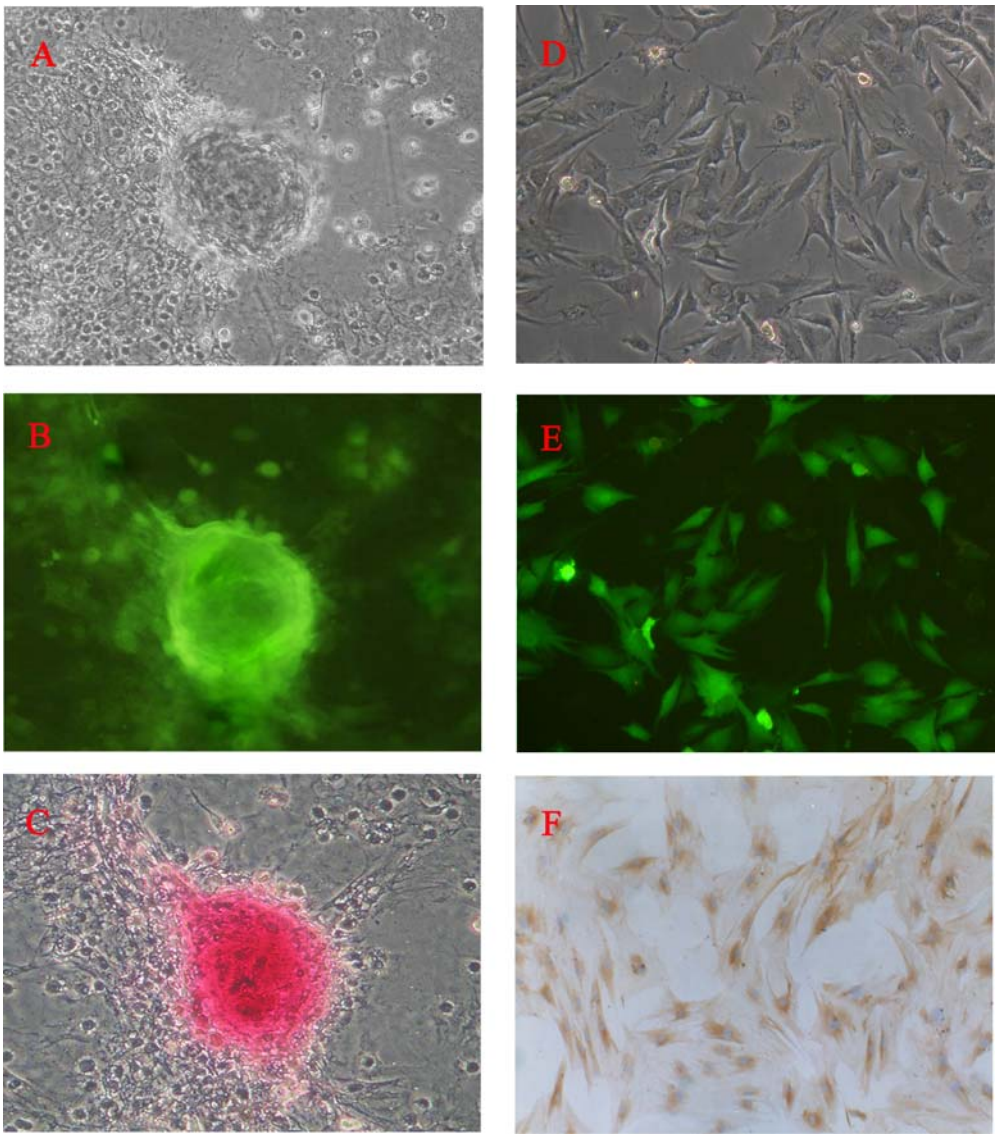

Fig. 2. Osteogenic *sseogenic* differentiation of GFP marked ASCs, 14 days after the osteogenic induction. (A) The morphology changed from fibroblast-like to a multilateral and cuboidal form, which showed a tightly packed arrangement, the mineral nodules were observed. (B) AD/GFP infected ASCs after induction with stable osteogenic phenotype still expressed GFP. (C) Mineral nodules were stained positively with AR-S. (Original magnification of all images $\times 100$). *Chondrogenic* differentiation of GFP marked ASCs. (D) After placed into chondrogenic medium for 10 days, the ASCs exhibited changes in cell shape, modulating from an elongated fibroblastic appearance to a polygonal shape. (E) High level expression of GFP in the chondrogenic lineages. (F) Positive immunoreactivity with the antibody to aggrecan after 10 days induction. (Original magnification of all images $\times 200$).

examined using RT-PCR. The level of PPAR-$\gamma 2$ and LPL in the adipogenic ASCs was high at 2 weeks since induction, but they were negative in the controls (Fig. 4).

Myogenesis of GFP marked human ASCs

After 5 days recover, the Ad/GFP infected ASCs were placed into myogenic medium for 3 days. The ASCs became larger and flatter like myofibroblasts in morphological features. With culture time increasing, this trend became more obvious at 7 days (Fig. 3D). Simultaneously, myogenic ASCs also possessed the high expression of GFP (Fig. 3E). The majority of the flattened cells expressed α-SMA, a well-characterized smooth muscle marker (Fig. 3F). In contrast, the cells maintaining without myogenic supplements were stained negatively with antibody to α-SMA. RT-PCR analysis of myogenin and myod1 mRNA showed that the induced ASCs had high level expression, but no expression was observed in the control group (Fig. 4).

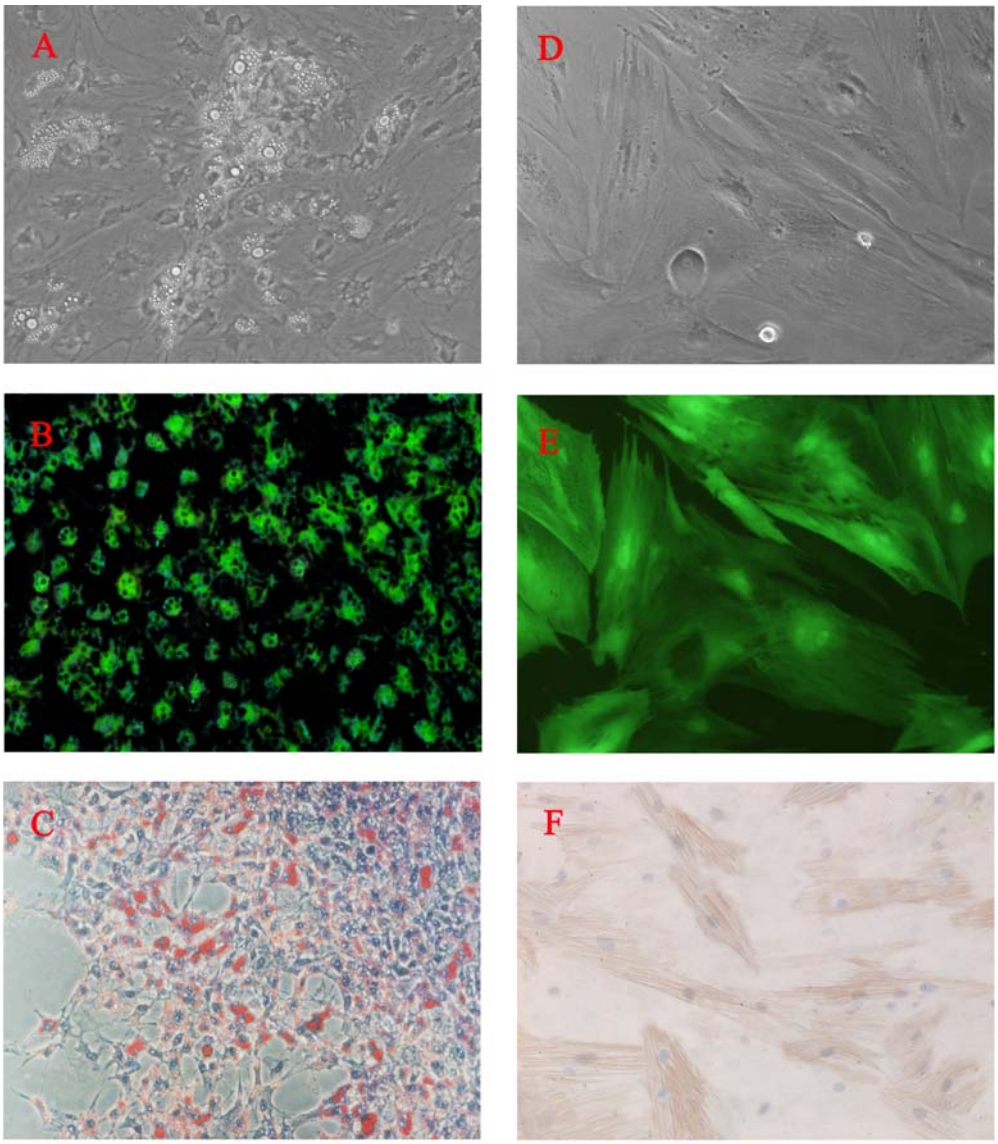

Fig. 3. *Adipogenic* differentiation of GFP marked ASCs. (A) GFP marked ASCs were incubated two weeks in inductive medium and lipid-filled cells could be observed through light microscopic images. (B) The lipids showed black on fluorescence microscopic images. (C) With the accumulation of lipid in cytoplasm, adipogenic cells were stained positively with Oil Red O. (Original magnification of all images $\times 100$). *Myogenic* differentiation of GFP marked ASCs. (D) Cells induced with myogenic medium for 7 days became flattened and larger. (E) High level of GFP in the myogenic lineages at the same time. (F) After 7 days, parts of the ASCs differentiated into smooth muscle cells which were stained positively with antibody to α-SMA. (Original magnification of all images $\times 400$).

Durability of GFP expression

When the GFP marked human ASCs were placed in the different inducing medium, the differentiated lineages kept high level of GFP paralleling with phenotypic modulation during induction. When the variant lineages turned to the terminal differentiation, their exogenous GFP expression declined in fluorescence intensity and cell proportion. This happened in osteogenic lineages after 9 weeks, chondrogenic lineages after 10 weeks, and myogenic lineages after 7 weeks respectively (data not shown). The declination of GFP expression in adipogenic lineages was not observed because of the floating of the mature adipose cells.

Discussion

According to previous works, adipose tissue from the lipoaspirates contains a supportive stroma and ASCs are easily isolated. In these adipose-derived cells, there is not only

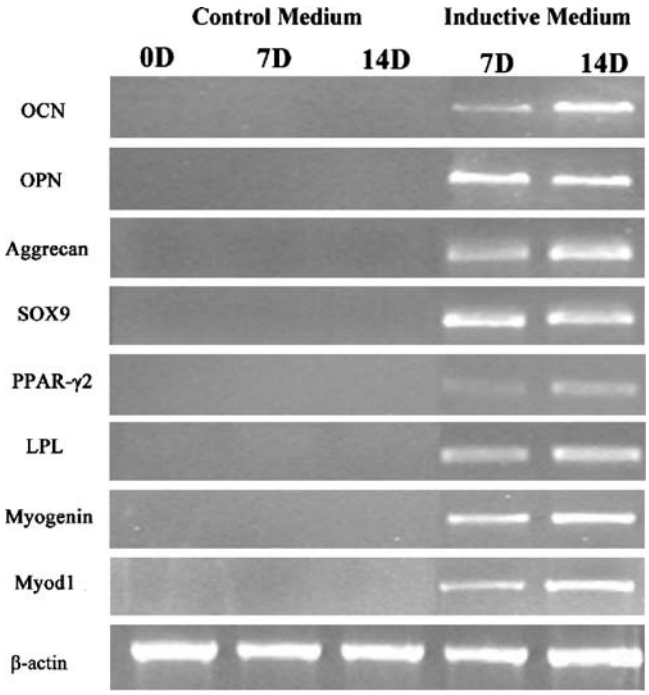

Fig. 4. RT-PCR analysis of variant tissue specific molecules. All the differentiated GFP marked ASCs revealed upregulated expression of the specific genes compared to untreated control cultures.

the population of preadipocytes but pluripotent stem cells with the potential toward several musculoskeletal lineages [19, 20]. ASCs harvested from the human raw lipoaspirates were fibroblastic in appearance in primary culture. And they indicated high proliferation rate in monolayer culture conditions *in vitro* [21]. Based on this, adipose tissue may represent a source of stem cells that could have far-reaching effects on several fields, particularly the tissue engineering for clinical application [21, 22].

During the research of tissue engineering, there is an important factor is the rate of seed cell turnover and survival in the end. The most common cell label methods are Brdu, GFP, LacZ *et al*. The advantage of BrdU over thymidine autoradiography is that the marked cells can be observed by immunocytochemistry. By this method, a more accurate estimate of the number of new cells can be obtained. The merit of LacZ over other colorimetric indicators is that prolonged staining time can visualize relatively low levels of expression. Microscopic imaging of GFP florescence develops an ideal method, as it clearly traces the stem cells seeded on the scaffold. We can observe the migration of the implanted cells and the local cells. Since the cell population infected with Ad/GFP also can be easily observed and isolated, it should be an ideal approach to produce large number of labeled ASCs for further research [17, 23, 24].

Our present work consisted of two parts. The first one was to determine whether human ASCs marked with exogenous GFP still could be induced to multilineage differentiation in vitro. The second was aimed at the optimization of adenoviral MOI for human ASCs and the duration of exogenous gene expression. Based on the obtained results, we got the valuable information about the optimization of adenoviral MOI for human ASCs and the duration of exogenous gene expression controlled by adenovirus in infected ASCs. Parts of the results have been reported in our previous works [16, 17]. Additionally, we got more detailed information on the durability of GFP expression, the multi-differentiation potential of the exogenous GFP marked human ASCs which had not been mentioned in others before.

When the infected human ASCs cultured in osteogenic lineages for 1 or 2 weeks, mineralized nodules could be observed. The nodule formation seemed to be the result of a locally higher proliferation rate of cells, associated with a higher synthesis of ECM [25–27]. The nodules showed a highly organized structure, displaying cell types at variant level of differentiation and reminding the different cell types present in bone [26, 27]. In order to confirm the formation of mineral nodules, histological staining and RT-PCR were used to investigate the calcium aggradations and related marker gene expression. The mineral nodules formation and bands

of OCN and OPN in RT-PCR confirmed the osteogenesis [28, 29]. OCN is a late bone marker only secreted by osteoblasts and it also signals terminal osteoblast differentiation. Its expression after a longer time of inductive culture made sure that osteogenesis occurred [30].

Chondrogenic differentiation was assessed by immunochemical staining and RT-PCR analysis of cartilage specific genes. Positive immunostaining of aggrecan suggested the chondrogenesis. Simultaneously, the high expression of the aggrecan and SOX9 in ASCs after induction confirmed chondrogenic differentiation in these ASCs [31, 32]. There was no expression of aggrecan and SOX9 in non-induced ASCs.

When cultured in adipogenic medium, human ASCs gradually changed into multilocular adipogenic cells *in vitro*. Stained positively with Oil Red O, these multilocular cells were considered as preadipocytes. With the obvious increase of lipid accumulation, small lipid droplets in the preadipocytes fused together and became enormous. At the end, the cells grew unilocular and filled with lipid like mature adipocytes [33, 34]. Moreover, expression of PPAR-$\gamma 2$ and LPL at mRNA level confirmed the adipogenic differentiation of ASCs in comparison with no transcription in the controls [35]. At the same time, the GFP expression was stable till the mature adipocyte floated. In further studies, we will continue this approach in fat tissue engineering *in vivo* and it is convenient to follow the adipogenic differentiation by imaging the GFP [34, 36–38].

After 10 days of myogenic differentiation, the obvious positive immunostaining was observed. It resulted in a heterogeneous staining of the cells: staining intensity was high in cells with more mature myogenesis, whereas it was absent in control group [39]. Simultaneously, myogenic differentiation was assessed using RT-PCR analysis of muscle specific genes. The amplification of myogenin and myod1 mRNA indicated the myogenesis. The morphological changes showed that human ASCs marked with GFP underwent the similar myogenic differentiation as the normal adipose stem cells. During the differentiation process, the GFP expressed very high and stable.

Based on these works, much valuable information was obtained about the GFP marked human ASCs differentiation and the duration of GFP expression. In summary, the exogenous GFP expression on human ASCs doesn't block the multilineage differentiation in appropriate conditions. The multilineage capability of human ASCs supports the feasibility of tissue engineering multiphasic constructs using a single cell source. In view of this, we can trace the human ASCs through GFP expression, we can analyze the physiological course of differentiation in the implanted ASCs within constructs, and determined whether the local mesenchymal cells replace the seed cells or not. These method and information will be very useful for the further application of human ASCs in the regeneration medicine.

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