Biotechnol Lett (2010) 32:1745–1752
DOI 10.1007/s10529-010-0344-3

ORIGINAL RESEARCH PAPER

Multilineage differentiation of muscle-derived stem cells from GFP transgenic mice

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Received: 3 April 2010 / Accepted: 28 June 2010 / Published online: 15 July 2010
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Abstract It is unclear whether green fluorescent protein (GFP) expression is maintained during the course of multilineage differentiation of muscle-derived stem cells (MDSCs). We isolated MDSCs from GFP-transgenic mice and transferred them to chondrogenic, neurogenic or myogenic media. Multilineage differentiation was examined by morphological observation, histological staining, immunocytochemical staining, real-time RT-PCR and Western blot. Both differentiated cells and non-differentiated cells maintained stable GFP expression until the cells exhibited a senescent phenotype. Thus, MDSCs from GFP-transgenic mice have multilineage potential in vitro and that GFP expression does not influence the multilineage potential of MDSCs (or vice versa).

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Keywords Chondrogenic · Green fluorescent protein · Muscle-derived stem cells · Myogenic cells · Neurogenic cells

Introduction

Regenerative medicine is proving to be an increasingly attractive technique to repair and restore the function of damaged or diseased tissues and organs. For cell-based therapeutic approaches, stem cells derived from various tissues have been actively investigated. Tissue-specific stem cells have been isolated from a variety of organs and tissues, including bone marrow, neural tissue, skin, cord blood, adipose tissue and intestine. Recently, stem cells derived from skeletal muscle have received considerable attention as a potential source of adult stem cells. The data indicate that these muscle-derived stem cells (MDSCs) are a potential autologous cell source that may contribute to tissue repair or regeneration of damaged or diseased musculoskeletal tissues, and also might be useful in tissue engineering (Alessandri et al. 2004; Bueno et al. 2009).

Since GFP-positive cells can be tracked and visualized without complex or destructive procedures (Kim et al. 2010), GFP-transgenic mice have facilitated detailed research on cell transplantation and the physiological behavior of stem cells (Payer et al. 2006). However, the ability of MDSCs from GFP-transgenic mice to differentiate into multiple lineages

has not been verified. It remains unclear whether GFP expression is maintained throughout MDSC multilineage differentiation. In the present study, MDSCs isolated from GFP-transgenic mice were incubated in media promoting differentiation toward chondrogenic, neurogenic or myogenic lineages. Specific characterization of these lineages was performed by morphological examination, immunohistochemical staining, and Western blot analysis. The results indicate that GFP-positive MDSCs can differentiate into chondrogenic, neurogenic and myogenic lineages in vitro. Moreover, we revealed that the specific differentiation processes did not influence expression of endogenous GFP in cultured MDSCs.

Materials and methods

Isolation and expansion of MDSCs

Six 5-week-old GFP transgenic mice [C57BL/6-Tg(ACTB-EGFP)1Osb/J,The Jackson Laboratory, USA] were used for this study. Their use complied with the international guide principles for animal research and was approved by the animal care committee at Guiyang Medical College. Following the pre-plate technique (Qu-Petersen et al. 2002), the animals were sacrificed with CO_2, and the hind limb muscles were removed, cleaned of all connective tissue and fat, minced and washed three times with phosphate-buffered saline (PBS). The muscle tissues were then enzymatically dissociated at 37°C for 1 h in 0.2% collagenase XI and for 45 min in 1 mg dispase/ml, followed by incubation for 30 min in 0.1% trypsin/EDTA. After digestion, muscle cells were passed through 50 μm nylon mesh to remove muscle fibers and debris, then centrifuged at 400×*g* for 5 min. The pellet was washed once in PBS, centrifuged and resuspended in growth medium containing Dulbecco's Modified Eagle Medium (DMEM), 10% (v/v) horse serum (HS), 10% (v/v) fetal bovine serum (FBS), 0.5% chick embryo extract, 100 units penicillin/ml and 100 mg streptomycin/ml (all reagents purchased from Gibco). The cells were plated on collagen-coated flasks for 1 h. Unattached cells were then transferred to fresh collagen-coated flasks. After 24 h, unattached cells were collected, centrifuged and replated. The subsequent pre-plates were repeated every 24 h for 5 days to eliminate most of the fibroblasts and endothelial cells. The medium was replaced every 2 days throughout the study. The cells were passaged using 0.1% trypsin (Sigma) five times prior to differentiation.

Multilineage differentiation

The fifth-passage MDSCs were trypsinized and replated onto 100 mm^2 tissue culture plates at 10^5 cells per plate. The same growth medium described above was used for the control culture medium. After incubation in the control medium for 1 day, the medium was replaced with either chondrogenic medium, containing DMEM, 1% (v/v) FBS, 10 ng rh-TGFβ_1/ml, 50 mg ascorbic acid/l, 6.25 mg insulin/ml, 10^{-7} M dexamethasone, 100 units penicillin/ml, 100 mg streptomycin/ml and 12 μM L-glutamine; neurogenic medium, containing DMEM, 5 mM β-mercaptoethanol, 100 units penicillin/ml, 100 mg streptomycin/ml and 12 μM L-glutamine (Woodbury et al. 2000); or myogenic medium, containing DMEM, 1% (v/v) FBS, 5% (v/v) HS, 50 μM hydrocortisone, 100 units penicillin/ml, 100 mg streptomycin/ml and 12 μM L-glutamine (Zuk et al. 2002) (all from Sigma). Culture media were replaced every 3 days. Cells were fixed and assessed at several time points spanning 2–14 days after multilineage differentiation.

Histological staining

After 7 days of chondrogenic differentiation, cell monolayers were washed with PBS, fixed in 4% (v/v) paraformaldehyde for 30 min and rinsed with PBS. Cells grown in the control culture medium were prepared for the staining in the same way at the same time points. For Toluidine Blue staining, the cells were incubated in 0.5% potassium permanganate for 2 min, washed in 2% potassium metabisulfite in water, then incubated for 10 min with 0.1% Toluidine Blue in 0.1 M sodium acetate buffer (pH 4.0) to visualize tissue proteoglycans. For Safranin O staining, the cells were incubated in 1% (v/v) acetic acid and then immediately exposed to Safranin O for 3 min. After a water rinse, cells were viewed by microscopy and photographed.

Immunohistochemical staining

After 3 days of neurogenic or myogenic differentiation, cell monolayers were washed with PBS and

fixed in 4% (v/v) paraformaldehyde. Cells grown in the control culture medium were also prepared for the staining for the same time points. Endogenous peroxidase activity was quenched by incubation with 3% (v/v) H_2O_2 for 15 min. Non-specific binding was blocked by incubation in PBS with 10% (v/v) horse serum for 30 min. Slides were incubated overnight at 4°C with goat polyclonal antibodies against neuron-specific enolase (NSE) for neurogenic cells, or with mouse polyclonal antibodies against α-smooth muscle actin (α-SMA) for myogenic cells. The samples were subsequently incubated with secondary donkey anti-mouse biotinylated antibodies and horseradish peroxide-conjugated streptavidin to detect the primary antibodies. After using 3,3-diaminobenzidine tetrahydrochloride to develop the peroxidase reaction, images were viewed using microscopy and photographed. (Antibodies and streptavidin conjugate were purchased from Santa Cruz.)

Real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) analysis

To further evaluate the degree of multilineage differentiation, real-time RT-PCR was performed after 3, 7 and 14 days of differentiation. Total RNA was extracted from monolayer cultures using Trizol solution (TaKaRa), and 1 μg total RNA from each differentiation class was reverse transcribed into complementary cDNA using a reverse transcription system kit (Promega) according to the manufacturer's instructions. Cells cultured in non-differentiation medium were used as a control. The primer sequences of genes selected for real-time PCR were obtained from published literature (Shen et al. 2008; Goetsch et al. 2003; Reddy et al. 2009): Collagen type I (Col I), 5′-TTGTTCTCCTGGTAAAGATGGT-3′, 5′-CAG TATCACCAGGTTCACCTTTC-3′; Collagen type II (Col II), 5′-TCCAGGATCTGCACTGAATG-3′, 5′-TCTG CCCAGTTCAGGTCTCT-3′; Myogenic regulatory factor 5 (Myf5), 5′-CTGTCTGGTCCCGAA AGAAC-3′, 5′-AAGCAATCCAAGCTGGACAC-3′; Myogenic regulatory factor 6 (Myf6), 5′-AATTCTT GAGGGTGCGGATT-3′, 5′-ATGGAAGAAAGGCG CTGAAG-3′; neuronal nuclei (NeuN), 5′-GGCAAT GGTGGGACTCAAAA-3′, 5′-GGGACCCGCTCCT TCAAC-3′; glial fibrillary acidic protein (GFAP), 5′-CCAGCTTCGAGCCAAGGA-3′, 5′-GAAGCTC CGCCTGGTAGACA-3′; and beta-Actin, 5′-ACGGC CAGGTCATCACTATTC-3′, 5′-AGGAAGGCTGGA AAAGAGCC-3′. Real-time PCR was run in a Light-Cycler Instrument (Roche) using the SYBR Green Master Mix kit (Promega) according to the manufacturer's recommendations. Data analysis was carried out using the GeneAmp 5700 SDS software (Applied Biosystems). All measurements were performed in triplicate. The C_T value for each sample was defined as the cycle number at which the fluorescence intensity reached a threshold where amplification of each target gene was within the linear region of the reaction amplification curves. The relative expression level for each gene of interest was normalized by the C_T value of house-keeping gene GAPDH and determined using the $\Delta\Delta C_T$ method.

Western blot analysis

Cells cultured under various conditions were washed twice with PBS and lysed in buffer containing 50 mM Tris/HCl, 0.1% SDS, 100 mg PMSF/l, 1 mg aprotinin/l, 1% Tween 20 and 150 mM NaCl. After centrifugation at 18,000×g for 20 min, the supernatant was collected, and protein concentrations were determined by the bicinchoninic acid assay. Equal amounts of protein extracts were fractionated using 10% (v/v) SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane (Bio-Rad). Membranes were blocked with 5% (v/v) fat-free milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T, pH 7.6) for 2 h, then incubated overnight at 4°C with primary antibodies against Col II, aggrecan, GFAP, nestin, myogenic determination factor (Myod) (all from Santa Cruz) and β-actin (Sigma) at a dilution of 1:1,000 in the blocking buffer. Membranes were washed, and probed with horseradish peroxidase conjugated secondary antibodies (Sigma) diluted at 1:2,000 for 1 h. The bands were scanned and evaluated using Quantity One software (Bio-Rad).

Statistical analysis

The data are expressed as means ± standard error of the mean. Statistical comparisons between non-differentiated cells and differentiated cells were performed using Student–Newman–Keuls (SPSS Inc., Version 12) multiple ranges tests. P values <0.05 were considered statistically significant.

Results and discussion

Here, we show that MDSCs isolated from GFP transgenic mice were capable of differentiating into chondrogenic, neurogenic and myogenic cells, and that the differentiation process had no significant influence on GFP expression.

Primary culture

We obtained a considerable number of MDSCs in culture suggesting that MDSCs can be easily isolated from GFP-positive muscle tissues and are relatively tolerant of ex vivo manipulation. The primary MDSCs were spindle-like in shape (Fig. 1a, b), proliferated in the control medium and reached confluence after 12 days. After three passages, the cells approached confluence quickly at 8–9 days and exhibited a more spindle-shaped, fibroblastic morphology. Endogenous GFP expression in the passaged MDSCs was maintained at a high level without any decline until the cells entered senescence or apoptosis after 14 weeks, as revealed under fluorescent microscopy. As stable GFP expression over several months is sufficient for most in vitro and in vivo research, this model is suitable for studies involving, for example, the tracking of cells in tissue-engineered constructs used to repair lesions.

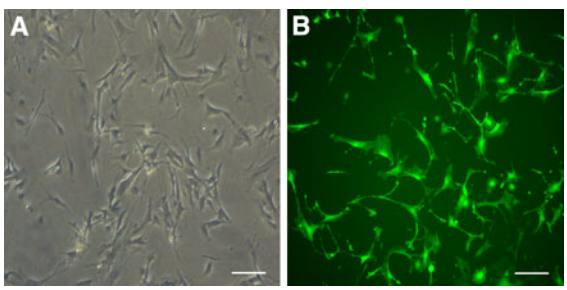

Fig. 1 Primary culture of MDSCs from GFP transgenic mice. **a** Light microscopy. **b** Fluorescence microscopy. The cells show a fibroblastic appearance with concomitant GFP fluorescence. *Bar* represents 100 μm

Chondrogenesis

After 3 days of chondrogenic differentiation, the cells changed morphology from a spindle-like to a polygonal shape. These changes became more marked over time (Fig. 2a), and the chondrogenic cells maintained stable GFP expression after 14 days of culture (Fig. 2d). To confirm that the MDSCs had committed to the chondrogenic lineage, we performed Toluidine Blue staining, Safranin O staining, and Western blot analysis. Differentiated cells were stained positive with toluidine blue and safranin O at day 7 (Fig. 2b, c), confirming the secretion of sulfated proteoglycans and glycosaminoglycans into the cellular matrix. In contrast, control cultures failed to show any notable staining with either toluidine blue (Fig. 2e) or safranin O (Fig. 2f).

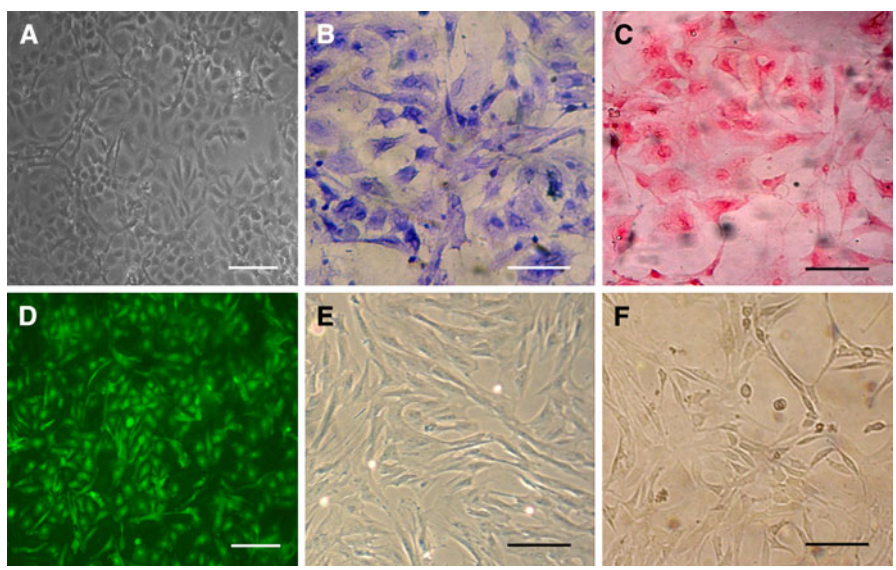

Fig. 2 Chondrogenic differentiation of MDSCs. **a** Cell morphology changed from fibroblast-like to polygonal after culture in chondrogenic medium for 14 days. **b** Chondrogenic cells maintained stable GFP expression after 14 days of differentiation. Cells were stained positive with **c** toluidine blue and **d** safranin O after 7 days of chondrogenic differentiation. Control cells did not stain with either **e** toluidine blue or **f** safarin O. *Bar* represents 100 μm

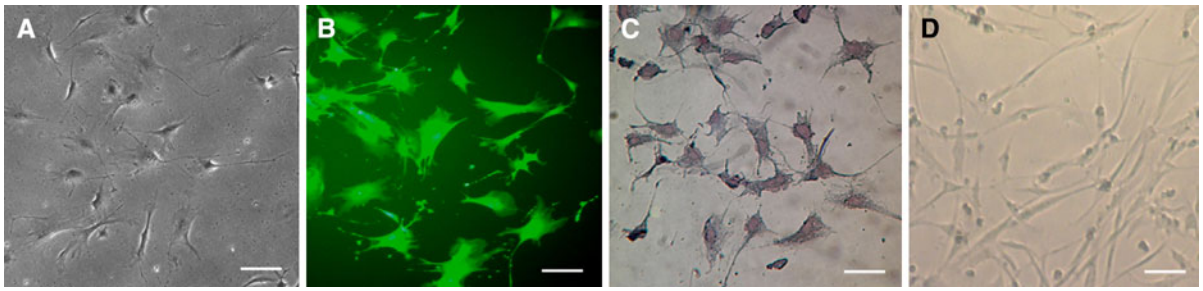

Fig. 3 Neurogenic differentiation of MDSCs. **a** After 3 days of culture in neurogenic medium, the cell bodies contracted and presented extended cytoplasmic extensions. **b** Neurogenic cells displayed a high level of GFP expression at day 7. **c** Immunocytochemical staining of NSE was positive after 3 days of neurogenic differentiation, but negative for the control cells (**d**). *Bar* represents 100 μm

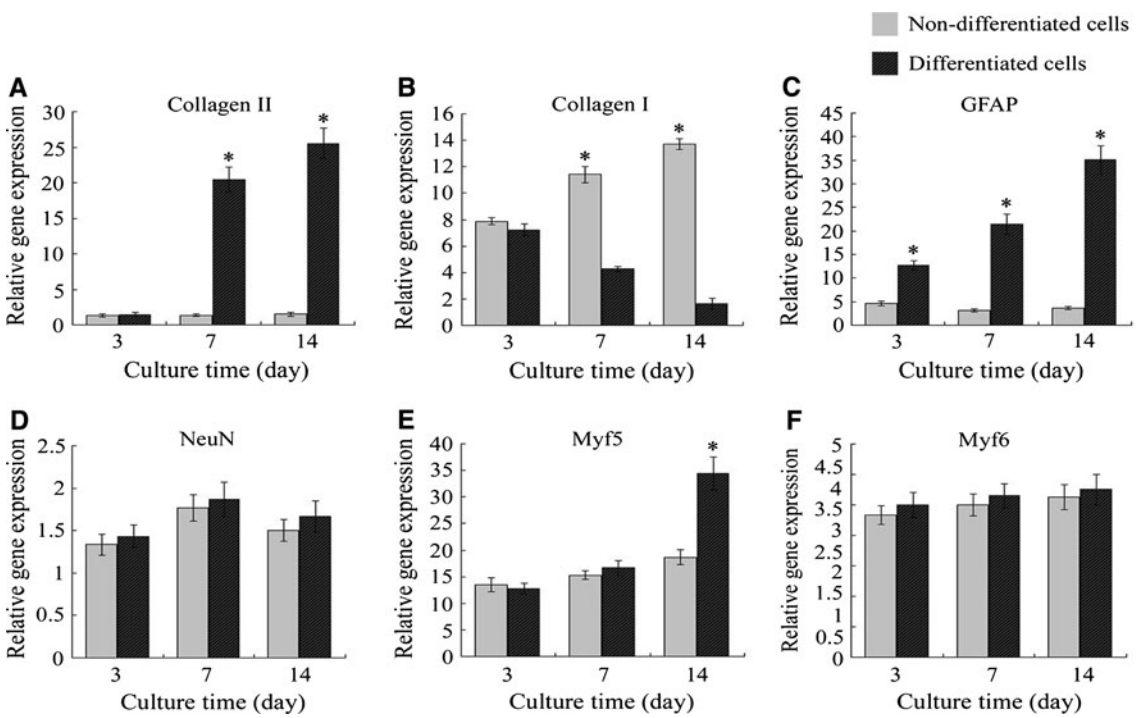

Fig. 4 Real-time RT-PCR analysis comparing gene expression profiles between non-differentiated and differentiated cells. **a** Col II. **b** Col I. **c** GFAP. **d** NeuN. **e** Myf5. **f** Myf6. * $P < 0.05$ between the two cell types

To further investigate the chondrogenic phenotype, we went onto examine the expression of genes involved in chondrogenesis in differentiated cells by using real-time RT-PCR after 3, 7 and 14 days of differentiation. The mRNA level of the chondrocyte-specific gene Col II (Fig. 4a) was significantly increased in differentiated cells compared to non-differentiated cells ($P < 0.05$). Col I mRNA levels increased in the non-differentiated cells but decreased in the differentiated cells during the 14-day differentiation period ($P < 0.05$) (Fig. 4b). In addition, chondrocyte-specific proteins Col II and aggrecan expressed in the differentiated cells but not in the cells cultured in control medium, as revealed by Western blot (Fig. 5). Together, these findings strongly suggest that MDSCs will undergo chondrogenesis in response to chondrogenic stimuli.

Previously, GFP expression levels in transgenic adipose tissue-derived stem cells were shown to remain stable up to 3 months after chondrogenic

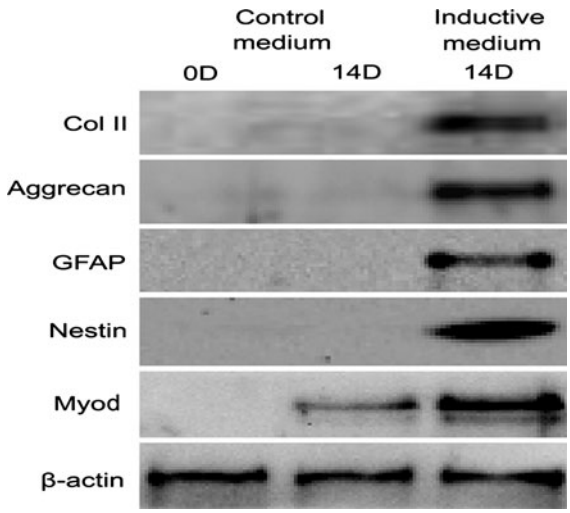

Fig. 5 Western blot analysis of chondrogenic, neurogenic and myogenic-specific proteins. Col II, Aggrecan, GFAP and Nestin were expressed in differentiated MDSCs but not in control cultures; Myod was expressed more strongly in differentiated MDSCs than in controls

differentiation, when the differentiated cells were undergoing phenotypic modulation and apoptosis (Lin et al. 2005). Since muscle-derived stem cells and adipose tissue-derived stem cells are the two main kinds of adult mesenchymal stem cells and may share similar properties, we postulate that chondrogenic MDSCs may have a similar duration of GFP expression to that of adipose tissue-derived stem cells. As expected, our study showed that the MDSCs cultured in the chondrogenic medium maintained stable GFP expression over the 14-day chondrogenic differentiation.

Neurogenesis

The MDSCs cultured in the neurogenic medium underwent a morphological transition from a spindle-like appearance to contracted cell bodies with extended cytoplasmic extensions. In particular, after 1 day of neurogenic differentiation, the cytoplasm retracted toward the nucleus, forming contracted cell bodies with extended cytoplasmic extensions. After 3 days of differentiation, the cell bodies shifted toward an increasingly spherical appearance, and the cells became longer (Fig. 3a). These changes became more significant over time, suggesting that the MDSCs were differentiating toward the neurogenic lineage. Meanwhile, the cells maintained high levels of GFP expression during the process of differentiation (Fig. 3b).

To further confirm that the MDSCs were differentiating along the neurogenic lineage, we performed immunochemical staining of the neuronal protein NSE, real-time RT-PCR analysis of the neuronal markers GFAP and NeuN, and Western blot analysis of GFAP and nestin. NSE synthesis in the differentiated MDSCs was revealed with immunochemical staining at day 3 (Fig. 3c) but was not evident in the control cultures (Fig. 3d). Real-time RT-PCR showed that GFAP gene expression increased with differentiation time (Fig. 4c), and that no remarkable difference in NeuN mRNA expression was found between differentiated cells and non-differentiated cells ($P > 0.05$) (Fig. 4d). Western blot assays demonstrated that the neuroblast-specific proteins GFAP and nestin were expressed in the differentiated cells but not in the cells cultured in the control medium at day 14 (Fig. 5).

These findings indicate that stem and progenitor cells from GFP mouse muscles retain the capacity of differentiating toward the neurogenic lineage. However, the absence of the mature neuronal marker NeuN in the differentiated MDSCs may reflect inadequacies of the differentiation conditions used or the need for a prolonged differentiation time.

Myogenesis

To determine whether MDSCs could also undergo myogenesis, cells were cultured for 2 weeks in the presence of β-mercaptoethanol. After 3 days of myogenic differentiation, the cells became larger and flatter, which are morphological features reminiscent of myofibroblasts (Fig. 6a, b). This pattern of morphological changes became more obvious at day 14.

Consistent with the above observation that the MDSCs were differentiating along the myogenic lineage, immunohistochemical staining showed that α-SMA was expressed at day 3 in the cells exposed to β-mercaptoethanol (Fig. 6c) but not in the control group (Fig. 6d). Furthermore, an abundant expression of the myogenic-specific protein Myod was detected in the myogenic cells by Western blot; however, a low level of Myod was also observed in the undifferentiated MDSCs (Fig. 5). Additionally, there

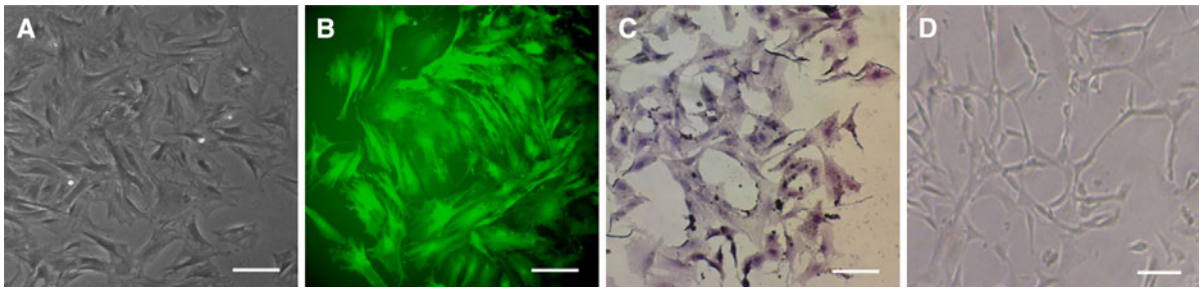

Fig. 6 Myogenic differentiation of MDSCs. **a** After 3 days of culture in neurogenic medium, the cells became larger and flatter, resembling myofibroblasts. **b** Myogenic cells displayed a high level of GFP expression at day 3. **c** Immunocytochemical staining of α-SMA was positive after 3 days of myogenic differentiation, but negative for the control cells (**d**). *Bar* represents 100 μm

was no difference regarding Myf5 mRNA expression between differentiated cells and non-differentiated cells at day 3 or 7 ($P < 0.05$); however, its mRNA expression increased significantly in the differentiated cells at day 14 as compared with the control cells ($P < 0.05$) (Fig. 4e). In contrast, no significant difference in Myf6 mRNA expression was noted between differentiated cells and non-differentiated cells throughout the 14 days of culture ($P < 0.05$) (Fig. 4f). This finding is in agreement with the study by McKarney et al. (1997) who found that Myf6 expression was upregulated at later stages of myogenic differentiation than the other three myogenic regulatory factors. Conversely, Myf5 expression is initiated at earlier stages of myogenesis than Myf6 (Buckingham 1997; Haldar et al. 2008). In this study, the lack of a significant difference in Myf6 expression between differentiated and non-differentiated cells suggests that the differentiation time we used here may not be long enough to allow these cultures progress to the terminal stage of myogenic differentiation.

GFP expression remained high and stable during myogenic differentiation, indicating that this process does not influence the expression of GFP in transgenic MDSCs.

Conclusions

The differentiation conditions we used here and the progress toward terminal differentiation have no significant influence on GFP expression in the MDSCs. Considering the mounting interest in MDSCs for both biological and therapeutic applications, the ability to monitor in vivo the functional states (e.g., proliferation and differentiation) of the cells seeded in scaffolds is highly desirable. Stem cells that express GFP provide an excellent model to visualize cells in vitro and in vivo. By taking advantage of improvements in optical imaging modalities, we can observe cells in living organisms at high resolution and confirm cellular origins directly (Olivo et al. 2008). It is feasible that this non-destructive method can be used to evaluate the cellularity of tissue-engineered constructs in vitro and to track cells after implantation, which may provide insights into the fate of MDSCs in vivo as well as the physiological behaviors of MDSCs. We believe that these methods and potential outcomes will be beneficial for future applications involving MDSCs in stem cell biology and regenerative medicine.

Acknowledgments We thank Dr. Xie Qiji, Li Xinsheng and Pan Zen for experimental support. This work was supported by grants from Department of Science and Technology (20092156, G20107015), Department of Education (2009143) and Department of Public Health (G2009-2), Guizhou Province, China.

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