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Differentiation of cardiomyocytes from amniotic fluid-derived mesenchymal stem cells by combined induction with transforming growth factor β1 and 5-azacytidine

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Abstract. As a novel type of seed cell, amniotic fluid-derived mesenchymal stem cells (AFMSCs) are promising for the regeneration of myocardial cells. A focus of cardiovascular regenerative medicine is to improve the efficiency of AFMSC differentiation. The present study replaced the traditional method of AFMSC differentiation with a combined induction method, in order to improve the efficiency of directional differentiation. AFMSCs were obtained from rabbit amniotic fluid samples, and western blot analysis was performed to analyze the expression of octamer-binding transcription factor 4 (OCT4), and tumorigenicity experiments were conducted. AFMSCs were divided into the following 4 groups: Induction with transforming growth factor β1 (TGFβ1); induction with 5-azacytidine (5Aza); induction with TGFβ1 and 5Aza combined; and untreated controls. Reverse transcription-quantitative polymerase chain reaction was performed to analyze the expression of cardiac-specific GATA binding protein 4 (GATA4), and immunofluorescence was employed to analyze the expression of cardiac troponin T (cTnT). In addition, western blotting was performed to analyze the expression of connexin 43, and transmission electron microscopy was used to observe the ultrastructure of the differentiated cells. AFMSCs exhibited positive OCT4 expression and were not observed to induce tumor development in nude mice. The expression levels of GATA4, cTnT, and connexin 43 in the combined induction group were markedly higher when compared with the remaining groups. Transmission electron microscopy analysis revealed that differentiated cells exhibited myocardial cell characteristics. In conclusion, AFMSCs are multipotent, non-tumorigenic cells that are capable of differentiating into cardiomyocyte-like cells. This combined induction method may improve the efficiency of directed differentiation.

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Introduction

Specific differentiation protocols induce mesenchymal stem cells (MSCs) to differentiate into various types of mature target cells, and MSCs are a suitable option for adult stem cell transplantation (1). MSCs have been used in numerous studies that involve the regeneration of cardiac cells post-myocardial infarction, and are capable of enhancing myocardial perfusion and cardiac function in the ischemic hearts of patients with acute or chronic heart diseases (2-4). However, the culture of MSCs from the bone marrow of humans or animals, or embryonic and adult tissues has a number of limitations, including the possibility of carcinoma, short survival time and difficulties in isolation. Therefore, the identification of additional types of suitable stem cells is required. Amniotic fluid-derived MSCs (AFMSCs) exhibit a higher proliferation rate, are easier to isolate and extraction process does not injure the mother or the embryo. Therefore, AFMSCs may be a favorable source of cardiomyocytes due to their differentiation potential and characteristics (5). A focus of cardiovascular regenerative medicine is to improve the efficiency of directed differentiation of AFMSCs. In a previous preliminary study, high-quality AFMSCs were successfully isolated (6). To the best of our knowledge, the present study provides novel results demonstrating that combined transforming growth factor β1 (TGFβ1) and 5-azacytidine (5Aza) treatment may be used to improve the efficiency of AFMSC differentiation.

Materials and methods

Isolation and culture of AFMSCs. Female New Zealand white rabbits (weight: 4.5-5.5 kg, age: 1-1.5y; Song Lian Laboratory Animal Farms, Shanghai, China; production license no. SCXK2007-0011) at 16-18 days gestation were narcotized via the ear vein with pentobarbitone (concentration: 30 mg/ml, dosage: 2 ml/kg), the uterus excised,

the muscle removed and amniotic fluid obtained with an injector. This was then centrifuged at 152.9 x g) for 15 min at 37˚C, resuspended in amniocyte-specific medium (AmnioMAX-C100; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and plated in 6-cm dishes at 37˚C and 5% CO_2 in a fully humidified atmosphere. Following 7-10 days of culture, the specific medium was replaced with normal medium DMEM (cat. no. 31600034; Invitrogen; Thermo Fisher Scientific, Inc.) + 10% fetal bovine serum to remove non-adherent cells and this was refreshed every 3 days thereafter. When cells reached 80% confluence, they were treated with 0.25% trypsin (Invitrogen; Thermo Fisher Scientific, Inc.) for 1 min. Trypsin digestion was then inhibited and cells were subcultured at a ratio of 1:2, and cultured under the same aforementioned conditions. Animal experiments performed in the present study were approved by the Ethics Committee of Xinhua Hospital (Affiliated Hospital of Shanghai Jiaotong University, Shanghai, China).

Western blot analysis of octamer-binding transcription factor 4 (OCT4) expression. Stem cells at passage 3 were washed with phosphate-buffered saline (PBS) and protein extracts were obtained following treatment of cells with radioimmunoprecipitation assay (RIPA) lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology, Shanghai, China), then centrifuged at 4˚C, 152.9 x g for 15 min. A BCA kit (cat. no. P0010S; Beyotime Institute of Biotechnology) was used to quantify the protein samples. Samples (20 μl) were loaded onto a precast gel (15% SDS-PAGE kit; cat. no. P0012A, Beyotime Institute of Biotechnology) and run at 80 V, followed by an increase to 120 V on ice. Proteins were subsequently transferred to polyvinylidene difluoride membranes for 45 min at 280 mA in transfer buffer. Membranes were blocked for 2 h with 5% skimmed milk powder in PBS at room temperature and subsequently incubated overnight at 4˚C with mouse anti-rabbit OCT4 antibodies (cat. no. ab52110; 1:500; Abcam, Cambridge, UK). Following washing by PBS, the membranes were treated with goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (cat. no. ab6789; 1:1,000; Abcam) for 2 h at 37˚C. GAPDH (cat. no. ab9483; 1:1,000; Abcam) was used as the loading control. Membranes were subsequently washed and enhanced by BeyoECL Plus reagent (cat. no. P0018A; Beyotime Institute of Biotechnology) and used for protein identification by GelDocXR$^+$ system (model 1708195; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

AFMSC tumorigenicity experiment. A total of 20 female BALB/C nude mice (age: 3-4 weeks, 9.8-14.2 g weight, 12-h light/dark cycle, humidity: 45-55%, in rat cages, 32-34˚C) were divided into 2 groups at random, with 10 mice in the control group and 10 in the experimental group. OCT4-positive stem cells at passage 3 were treated with 0.25% trypsin, before digestion was inhibited and the cells were resuspended in PBS at a density of $5x10^6/cm^3$. A 0.2 ml cell suspension was injected subcutaneously into the necks of the mice in the experimental group, while 0.2 ml PBS was injected in the same manner in the control group. The 2 groups were observed for 8 weeks, then all mice were dissected and the neck, axillary cavity and groin examined for signs of tumor growth or hyperplasia in the injection site or lymphatic distribution area.

Induction of AFMSC differentiation. OCT4-positive cells were divided into 4 groups, including the control group (group A), TGFβ1-induced group (group B), 5Aza-induced group (group C), and the combined TGFβ1 and 5Aza-induced group (group D). When the cells reached 70% confluence, TGFβ1 (cat. no. 100-21, PeproTech, Suzhou, China) and/or 5Aza (cat. no. A3656, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) were added to the media of the appropriate groups as follows: PBS in group A for 24 h; 5 ng/ml TGFβ1 for 24 h in group B; 10 μmol/l 5Aza for 24 h in group C; and 5 ng/ml TGFβ1 + 10 μmol/l 5Aza for 24 h in group D. All groups were cultured at 37˚C and 5% CO_2 in a fully humidified atmosphere. Cells were harvested when they reached 80% confluence.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR analysis of GATA binding protein 4 (GATA4) expression. Following AFMSC differentiation, total RNA was extracted from cultured cells each week for 4 weeks using TRIzol reagent (Takara Bio, Inc., Otsu, Japan), according to the manufacturer's protocol. The sequences of the GATA4 primers (GeneBank Reference Sequence: XM_002717998.1; Sangon Biotech Co., Ltd., Shanghai, China) were as follows: Forward, cagtgagagccttcctcctac (5'-3'), and reverse, catagccttgtggggacag (5'-3'). The sequences of the GAPDH primers (GeneBank Reference Sequence: NM_001082253.1; Sangon Biotech Co., Ltd.) were as follows: Forward, atggtgaaggtcggagtgaa (5'-3'), and reverse, tgggtggaatcatactggaac (5'-3'). A cDNA synthesis kit (cat. no. 6110A, Takara Bio, Inc., Otsu, Japan) was used to reverse transcribe total RNA in accordance with the manufacturer's protocols. PCR was performed at 85˚C for 30 sec and 37˚C for 15 min to reverse transcribe to cDNA, then qPCR was subsequently performed with a SYBR Premix Ex Taq GC kit (cat. no. DRR041A; Takara Bio, Inc.) and 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) used to analyze the expression of GATA4 (7). Briefly, the reaction volume of 20 μl comprised SYBR Premix Ex Taq (10 μl), PCR Forward Primer (10 μM; 0.4 μl), PCR Reverse Primer (10 μM; 0.4 μl), ROX Reference Dye II (0.4 μl), cDNA (2 μl) and dH_2O (6.8 μl) mixed on ice, and was then used on the 7500 with the following steps: 1 cycle at 95˚C for 30 sec, 40 cycles at 95˚C for 5 sec and 60˚C for 34 sec, then 1 cycle at 95˚C for 15 sec, 60˚C for 1 h, and finally 95˚C for 15 sec.

Immunofluorescence analysis of cardiac troponin T (cTnT) expression. Cells in the 4 experimental groups were cultured in 6-well culture plates ($5x10^6/cm^3$), and the cytomembranes were permeabilized for 30 min with 0.3% Triton X-100 at 37˚C. Following fixation with 4% paraformaldehyde for 12 h at 4˚C, cells were blocked with 5% bovine serum albumin (FBS+PBS) for 2 h at room temperature, and incubated overnight at 4˚C with mouse anti-rabbit cTnT antibodies (1:200; cat. no. ab10214; Abcam, Cambridge, UK). Subsequent to washing with PBS, cells were incubated with goat anti-mouse antibodies (cat. no. ab6789; 1:1,000; Abcam) for 2 h in the

dark at room temperature. They were then washed with PBS and stained with DAPI for 5 min in the dark at room temperature, and subsequently observed under a fluorescence microscope.

Western blot analysis of connexin 43 expression. Cells ($5x10^6/cm^3$) in each experimental group were washed with PBS and protein extracts were obtained following treatment with RIPA lysis buffer. Samples, prepared as for OCT4, above, were loaded onto a 15% precast gel and run at 80 V followed by an increase to 120 V. Proteins were then transferred to polyvinylidene fluoride membranes for 45 min at 280 mA in transfer buffer. Membranes were subsequently blocked with 5% skimmed milk powder in PBS for 2 h at room temperature and incubated overnight at 4˚C with mouse anti-rabbit connexin 43 primary antibodies (cat. no. ab79010; 1:500; Abcam). GAPDH (cat. no. ab9483; 1:1,000; Abcam) was used as the loading control. Following washing with PBS, membranes were treated with HRP-conjugated goat anti-mouse secondary antibodies (dilution, 1:1,000; cat. no. ab6789; Abcam). The membranes were washed and ECL reagent (cat. no. P0018A; Institute of Biotechnology) was used for protein imaging with the GelDocXR+ system (model 1708195; Bio-Rad Laboratories, Inc.) was used for analysis and the number of replicates per group was 28.

Transmission electron microscopy. Following AFMSC differentiation, cells were treated with 0.25% trypsin, washed with PBS, fixed with 4% glutaraldehyde and 1% osmic acid at 4˚C for 12 h and embedded in Epon 812 epoxy resin following graded ethanol dehydration at room temperature. Samples were cut at 60-70 nm with an ultramicrotome and double-stained with uranyl acetate for 5-10 min and lead citrate for 5 min all on ice. A transmission electron microscope was used to observe the ultrastructure of differentiated cells in each group.

Statistical analysis. Data were presented as mean + standard deviation and differences between any 2 groups were compared using one-way analysis of variance by SPSS version 19.0 (IBM Corp., Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Isolation and culture of AFMSCs. Amniotic fluid samples were first obtained from rabbits. Following AFMSC isolation for 7-10 days, the proliferative capacity of cells increased (data not shown) and the cells were elongated and formed numerous discontiguous colonies that displayed a circinate alignment pattern and began to proliferate more rapidly (Fig. 1A). Red blood cells disappeared from the culture medium following several media changes.

Western blot analysis of OCT4 expression. Western blot analysis demonstrated positive OCT4 expression in AFMSCs (Fig. 1B). AFMSCs exhibited characteristics similar to embryonic stem cells; the cell colonies displayed a circinate alignment pattern and began to proliferate more rapidly (Fig. 1A).

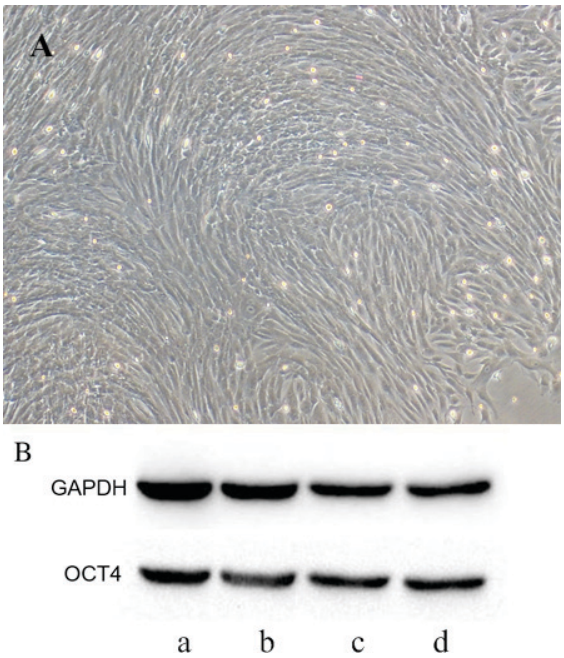

Figure 1. Morphology of AFMSCs and western blot analysis of OCT4 expression at 7-10 days post-isolation. (A) AFMSCs displayed a circinate alignment pattern without spaces between the cells (magnification, x5). (B) Cells in the 4 groups (a, untreated control; b, TGFβ1-treated group; c, 5Aza-treated group; and d, TGFβ1 + 5Aza-treated group) were OCT4-positive as determined by western blot analysis. AFMSCs, amniotic fluid-derived mesenchymal stem cells; OCT4, octamer-binding transcription factor 4.

AFMSC tumorigenicity experiment. The tumorigenicity experiment demonstrated that nude mice in the control and OCT4-positive AFMSC-injected groups (Fig. 2A and B) remained alive and did not develop tumors (Fig. 2C).

Induction of AFMSC differentiation. Following induction of AFMSC differentiation, no marked alterations in cell morphology were observed in group A cells, which maintained their morphology following passage 6 (Fig. 3A). However, cells in group B proliferated rapidly and were more elongated following TGFβ1 treatment when compared with group A cells (Fig. 3B). Cytomixis was observed in group C cells, which were elongated following 5Aza treatment when compared with group A cells (Fig. 3C). Following combined TGFβ1 and 5Aza treatment (group D), cytomixis was observed and cells were elongated and larger when compared with group A cells (Fig. 3D).

RT-qPCR analysis of GATA4 expression. RT-qPCR was performed to analyze GATA4 expression in AFMSCs among the 4 experimental groups and all groups were treated for 4 weeks. No increase in GATA4 expression was observed in group A at 4 weeks following treatment when compared with 1 week following treatment (Fig. 4). However, GATA4 expression was increased in group B cells at 4 weeks following treatment, in group C cells at 3 weeks following treatment, and in group D cells at 2 weeks following treatment when compared with 1 week following treatment. GATA4 expression was highest in group D and this difference was statistically significant when compared with the other groups (Fig. 4). These results indicated that GATA4 was expressed

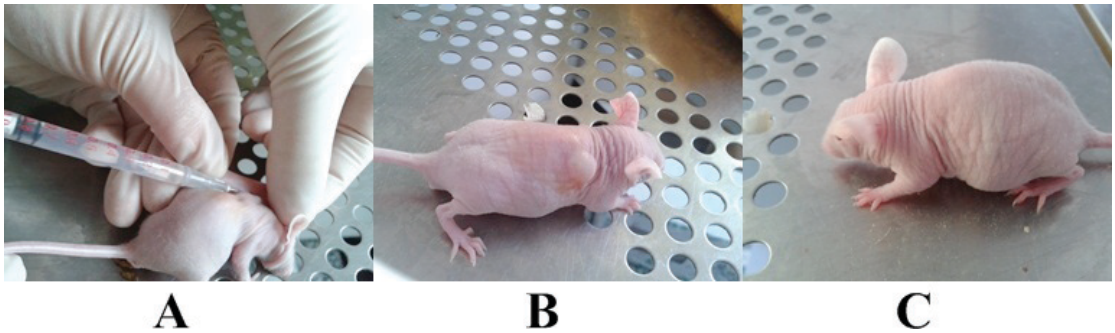

Figure 2. (A and B) Following injection with AFMSC, BALB/C nude mice remained alive and did not develop tumors in the neck or elsewhere (C).

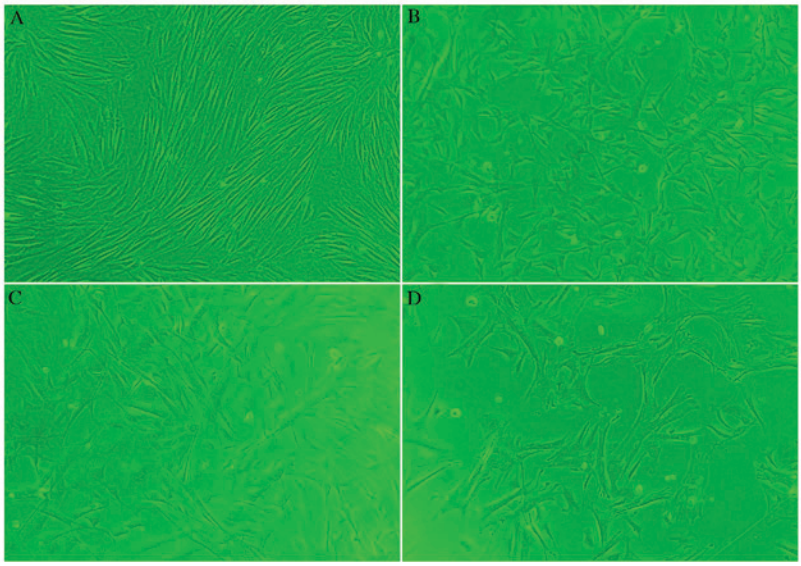

Figure 3. Morphology of AFMSCs in differentially-treated groups following induction of differentiation (magnification, x5). (A) Group A cells maintained their morphology, and kept their distribution characteristics. (B) Cells in group B proliferated rapidly and became elongated. (C) Cytomixis and elongation was observed in group C cells. (D) Cytomixis was observed in group D cells, which were elongated and larger in size compared with other groups. AFMSCs, amniotic fluid-derived mesenchymal stem cells; group A, untreated control; group B, TGFβ1-treated; group C, 5Aza-treated; group D, TGFβ1 + 5Aza-treated; TGFβ1, transforming growth factor β1; 5Aza, 5-azacytidine.

earlier and at a higher level in group D cells, which were treated with a combination of TGF1β and 5Aza (Fig. 4).

Immunofluorescence analysis of cTnT expression. Immunofluorescence was performed to analyze the expression of cTnT. At 2 weeks following treatment, low cTnT expression was observed in group A cells, while marginally higher levels were observed in groups B and C (Fig. 5A-C). However, cTnT expression in group D was increased when compared with groups A, B and C (Fig. 5D), with more red fluorescing cells in group D (P<0.05) when compared with groups A, B and C. After 2 weeks, the expression of cTnT in all groups was stable.

Western blot analysis of connexin 43 expression. Western blot analysis of connexin 43 expression revealed low expression in group A (Fig. 6). By contrast, a marked increase in connexin 43 expression was observed in groups B and C when compared with group A (Fig. 6). Notably, connexin 43 expression was significantly (P<0.05) higher in group D when compared with groups A, B and C (Fig. 6).

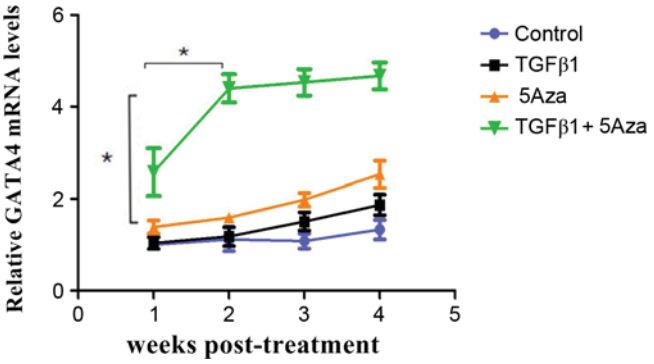

Figure 4. Detection of GATA4 expression levels by reverse transcription-quantitative polymerase chain reaction in groups of amniotic fluid-derived mesenchymal stem cells treated with TGFβ1 and/or 5Aza. GATA4 expression was higher in the TGFβ1 + 5Aza-treated group when compared with the other groups. *P<0.05. GATA4, GATA binding protein 4; TGFβ1, transforming growth factor β1; 5Aza, 5-azacytidine.

Transmission electron microscopy analysis. Following AFMSC differentiation, cells were observed under a

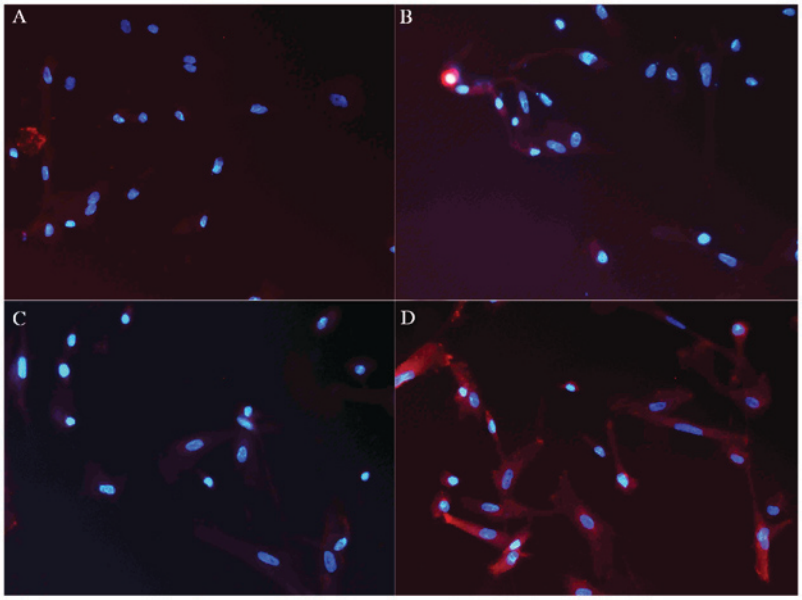

Figure 5. Detection of cTnT expression by immunofluorescence analysis of amniotic fluid-derived mesenchymal stem cells in the (A) untreated control, (B) TGFβ1-treated group, (C) 5Aza-treated group and (D) TGFβ1 + 5Aza-treated group. Blue, cell nucleus fluorescence signals and red, cTnT fluorescence signals. Expression of cTnT was higher in the TGFβ1 + 5Aza-treated group when compared with the other groups. cTnT, cardiac troponin T; TGFβ1, transforming growth factor β1; 5Aza, 5-azacytidine.

transmission electron microscope. A myofilament-like structure, which is a characteristic feature of cardiomyocytes, was observed in group D cells (Fig. 7), however this was not observed in the other groups.

Discussion

The development of molecular biological techniques and stem cell research has provided promising progress in the regeneration of necrotic myocardium. In 2001, Orlic *et al* (8) were the first to report that bone marrow cells were capable of regenerating infarcted myocardium. This previous study demonstrated that bone marrow cells were able to regenerate myocardial cells, which may improve the prognosis of coronary artery disease. These results were the first to demonstrate that stem cells may be used to regenerate infarcted myocardium, thus representing a novel approach for the regeneration and therapy of myocardial infarction. Therefore, the focus of research into stem cell treatment and regenerative medicine, is to identify an alternative source of stem cells. Recently, AFMSCs have been used for several applications, including prenatal diagnostics, tissue engineering, gene therapy, cell transplantation, as well as additional cell-based procedures, such as neuro-regeneration, and myocardial infarction therapy (3,9). Researchers have recently started to investigate the differentiation of AFMSCs into myocardial cells. In 2007, Chiavegato *et al* (4) assayed the phenotypic conversion of AFMSCs using cardiovascular-specific induction media or co-culture with rat neonatal cardiomyocytes. AFMSCs exhibited a cardiomyocyte phenotype following co-culture with rat neonatal cardiomyocytes. In 2011, Guan *et al* (10) demonstrated that human MSCs may be a potential source of cells for cardiac cell therapy. This previous study induced the differentiation of hAFS along the cardiac lineage by incubation with 5Aza for 24 h. Evidence for this differentiation included morphological alterations, upregulation

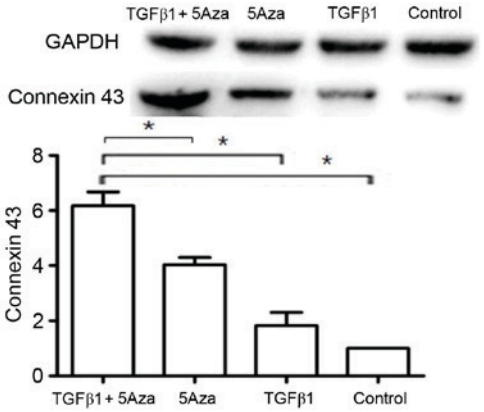

Figure 6. Expression of connexin 43 as determined by western blot analysis. Connexin 43 expression was higher in the TGFβ1 + 5Aza-treated group when compared with the other groups. *P<0.05. TGFβ1, transforming growth factor β1; 5Aza, 5-azacytidine.

of cardiac-specific genes and redistribution of connexin 43. Thus, hMSC differentiated into a cardiomyocyte-like phenotype and established functional communication when co-cultured with neonatal rat cardiomyocytes.

Once suitable cells for seeding are obtained, controlling the direction of differentiation and improving the efficiency of differentiation are important. 5Aza promoted the generation of myocardial sarcomeres and the production of myosin and muscle protein (11). Xing *et al* (12) demonstrated that mouse bone marrow MSCs transformed into cardiomyocyte-like cells following induction with 5Aza *in vitro*. However, the differentiation cycle induced by 5Aza alone was lengthy (13), and such a long duration of treatment reduces the rate of cell proliferation (14). Therefore, an alternative and effective method for the induction of stem cell differentiation is required.

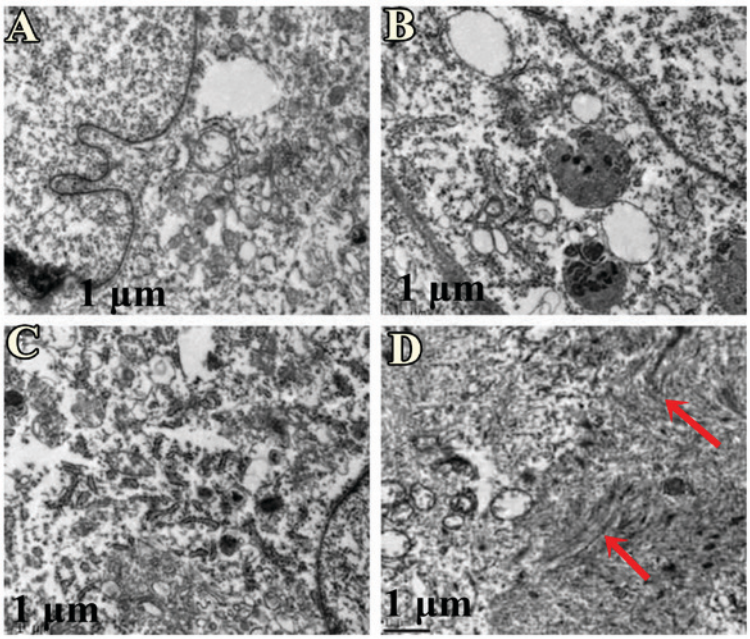

Figure 7. Transmission electron microscopy analysis of differentially-treated groups of amniotic fluid-derived mesenchymal stem cells. (A) myofilament-like structure, (as indicated by red arrows), was observed in the cells treated with transforming growth factor β1 + 5-azacytidine (D), but not found in the (A) untreated control, (B) TGFβ1-treated group and (C) 5Aza-treated group.

TGFβ is a multifunctional cytokine that regulates cell growth, differentiation and death, and has emerged as an important factor in the self-renewal and maintenance of stem cells (15). There are 3 subtypes of TGFβ, and the TGFβ1 signaling pathway serves an important role in the regulation of cell growth, differentiation, tissue repair and carcinogenesis. TGFβ1 has been previously implicated in a number of cardiac diseases, such as post-myocardial infarction ventricular remodeling, and it stimulates the proliferation of mouse and human myofibroblasts (16). Huntgeburth *et al* (17) reported that transgenic mice lacking TGFβ1 displayed increased fibrosis and myocyte hypertrophy. An additional study indicated that TGFβ1 affected the regulation of MSCs at transcriptional and post-transcriptional levels, and promoted MSC differentiation (1).

Improving the efficiency of directional differentiation of AFMSCs is one of the technological challenges for cardiovascular stem cell therapy. Therefore, the present study used 5Aza combined with TGFβ1 to induce AFMSC differentiation more effectively. Taking the results of previous studies into account (18-20), the optimum concentration was determined to be 10 μmol/l for 5Aza and 5 ng/ml of TGFβ1, with each group treated for 24 h, and the effectiveness of induction was subsequently observed.

GATA4 is a cardiac marker that is highly expressed in cardiac muscle cells throughout embryonic development, postnatal growth and adulthood. During this process, it functions as a critical regulator of cardiac differentiation (19), and regulates the expression of genes that are critical for cardiac contraction. GATA4 controls the expression of genes involved in cardiac structure and is essential for the cardiovascular system; the quantity of its expression has a decisive effect (21). Thus, the present study selected GATA4 as a target to investigate whether AFMSCs transformed into cardiomyocytes.

Cardiac troponin includes proteins T, I, and C. cTnT is considered to be a reliable biomarker with sufficient sensitivity and specificity for cardiac injury in the majority of laboratory animals (22), and it is the 'gold-standard' biomarker of myocardial injury in humans (23).

Connexin 43 controls the migration and proliferation of smooth muscle cells, and the expression of Connexin 43 is increased according to the synthetic state of these cells, which develop in early atherosclerotic lesions (24). Delmar and Makita (25) demonstrated that connexin 43 serves an important role in cardiac conduction and heart disease, and Thimm *et al* (26) reported that connexin 43 was functionally associated with calcium, which regulated its open/closed conformations. Antunes *et al* (27) demonstrated that connexin 43 was an important component of ventricles and cardiac muscle. Therefore, the present study selected cTnT, connexin 43 and GATA4 as indicators of AFMSC-to-cardiomyocyte differentiation.

In the present study, AFMSCs were successfully isolated and cultured from amniotic fluid using the direct adherence method. Western blot analysis demonstrated the expression of OCT4, which confirmed that AFMSCs are capable of multipotent differentiation. Furthermore, the tumorigenicity experiment demonstrated that AFMSCs were not tumorigenic. Following combined treatment with 5Aza and TGFβ1, AFMSCs exhibited positive expression of GATA4, cTnT and connexin 43, and a myofilament-like structure was observed under transmission electron microscopy. The results of the present study demonstrated that AFMSCs exhibited cardiomyocyte-like characteristics following differentiation, indicating that transformation into cardiomyocyte-like cells had occurred. A small number of cardiomyocyte-like cells were observed following treatment with 5Aza or TGFβ1 alone, however, an increased number of cardiomyocyte-like

cells were observed following combined treatment with 5Aza and TGFβ1. These results indicated that combined induction may improve the directional differentiation efficiency of AFMSCs. The current study provides an efficient and practical method for the directional differentiation of AFMSCs, increases the effectiveness of the transformation of cardiomyocyte-like cells *in vitro,* and presents a promising strategy for the regeneration of myocardial cells.

Acknowledgements

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References

1. Zhao L and Hantash BM: TGF-β1 regulates differentiation of bone marrow mesenchymal stem cells. Vitam Horm 87: 127-141, 2011.
2. Mohanty S, Bose S, Jain KG, Bhargava B and Airan B: TGFβ1 contributes to cardiomyogenic-like differentiation of human bone marrow mesenchymal stem cells. Int J Cardiol 163: 93-99, 2013.
3. Zhang S, Liu X, Goldstein S, Li Y, Ge J, He B, Fei X, Wang Z and Ruiz G: Role of the JAK/STAT signaling pathway in the pathogenesis of acute myocardial infarction in rats and its effect on NF-κB expression. Mol Med Rep 7: 93-98, 2013.
4. Chiavegato A, Bollini S, Pozzobon M, Callegari A, Gasparotto L, Taiani J, Piccoli M, Lenzini E, Gerosa G, Vendramin I, *et al*: Human amniotic fluid-derived stem cells are rejected after transplantation in the myocardium of normal, ischemic, immuno-suppressed or immuno-deficient rat. J Mol Cell Cardiol 42: 746-759, 2007.
5. Benthien JP and Behrens P: Reviewing subchondral cartilage surgery: Considerations for standardised and outcome predictable cartilage remodelling: A technical note. Int Orthop 37: 2139-2145, 2013.
6. Fei X, Jiang S, Zhang S, Li Y, Ge J, He B, Goldstein S and Ruiz G: Isolation, culture and identification of amniotic fluid-derived mesenchymal stem cells. Cell Biochem Biophys 67: 689-694, 2013.
7. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
8. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, *et al*: Bone marrow cells regenerate infarcted myocardium. Nature 410: 701-705, 2001.
9. Dubuis C, May L, Alonso F, Luca L, Mylonaki I, Meda P, Delie F, Jordan O, Déglise S, Corpataux JM, *et al*: Atorvastatin-loaded hydrogel affects the smooth muscle cells of human veins. J Pharmacol Exp Ther 347: 574-581, 2013.
10. Guan X, Delo DM, Atala A and Soker S: In vitro cardiomyogenic potential of human amniotic fluid stem cells. J Tissue Eng Regen Med 5: 220-228, 2011.
11. Aseem O, Barth JL, Klatt SC, Smith BT and Argraves WS: Cubilin expression is monoallelic and epigenetically augmented via PPARs. BMC Genomics 14: 405, 2013.
12. Xing Y, Lv A, Wang L and Yan X: The combination of angiotensin II and 5-azacytidine promotes cardiomyocyte differentiation of rat bone marrow mesenchymal stem cells. Mol Cell Biochem 360: 279-287, 2012.
13. Balana B, Nicoletti C, Zahanich I, Graf EM, Christ T, Boxberger S and Ravens U: 5-Azacytidine induces changes in electrophysiological properties of human mesenchymal stem cells. Cell Res 16: 949-960, 2006.
14. Zhang Y, Chu Y, Shen W and Dou Z: Effect of 5-azacytidine induction duration on differentiation of human first-trimester fetal mesenchymal stem cells towards cardiomyocyte-like cells. Interact Cardiovasc Thorac Surg 9: 943-946, 2009.
15. Kim YM, Kim J, Heo SC, Shin SH, Do EK, Suh DS, Kim KH, Yoon MS, Lee TG and Kim JH: Proteomic identification of ADAM12 as a regulator for TGF-β1-induced differentiation of human mesenchymal stem cells to smooth muscle cells. PLoS One 7: e40820, 2012.
16. Chen LX, Yang K, Sun M, Chen Q, Wang ZH, Hu GY and Tao LJ: Fluorofenidone inhibits transforming growth factor-beta1-induced cardiac myofibroblast differentiation. Pharmazie 67: 452-456, 2012.
17. Huntgeburth M, Tiemann K, Shahverdyan R, Schlüter KD, Schreckenberg R, Gross ML, Mödersheim S, Caglayan E, Müller-Ehmsen J, Ghanem A, *et al*: Transforming growth factor β_1 oppositely regulates the hypertrophic and contractile response to β-adrenergic stimulation in the heart. PLoS One 6: e26628, 2011.
18. Wan Safwani WK, Makpol S, Sathapan S and Chua KH: 5-Azacytidine is insufficient for cardiogenesis in human adipose-derived stem cells. J Negat Results Biomed 11: 3, 2012.
19. Rosca AM and Burlacu A: Effect of 5-azacytidine: Evidence for alteration of the multipotent ability of mesenchymal stem cells. Stem Cells Dev 20: 1213-1221, 2011.
20. Zhang S, Zhang M, Goldstein S, Li Y, Ge J, He B and Ruiz G: The effect of c-fos on acute myocardial infarction and the significance of metoprolol intervention in a rat model. Cell Biochem Biophys 65: 249-255, 2013.
21. Xu M, Millard RW and Ashraf M: Role of GATA-4 in differentiation and survival of bone marrow mesenchymal stem cells. Prog Mol Biol Transl Sci 111: 217-241, 2012.
22. Wada R, Muraoka N, Inagawa K, Yamakawa H, Miyamoto K, Sadahiro T, Umei T, Kaneda R, Suzuki T, Kamiya K, *et al*: Induction of human cardiomyocyte-like cells from fibroblasts by defined factors. Proc Natl Acad Sci USA 110: 12667-12672, 2013.
23. de Lemos JA: Increasingly sensitive assays for cardiac troponins: A review. JAMA 309: 2262-2269, 2013.
24. Wildi K, Reichlin T, Twerenbold R, Mäder F, Zellweger C, Moehring B, Stallone F, Minners J, Rubini Gimenez M, *et al*: Serial changes in high-sensitivity cardiac troponin I in the early diagnosis of acute myocardial infarction. Int J Cardiol 168: 4103-4110, 2013.
25. Delmar M and Makita N: Cardiac connexins, mutations and arrhythmias. Curr Opin Cardiol 27: 236-241, 2012.
26. Thimm J, Mechler A, Lin H, Rhee S and Lal R: Calcium-dependent open/closed conformations and interfacial energy maps of reconstituted hemichannels. J Biol Chem 280: 10646-10654, 2005.
27. Antunes E, Borrecho G, Oliveira P, Brito J, Águas A and Martins dos Santos J: Immunohistochemical evaluation of cardiac connexin43 in rats exposed to low-frequency noise. Int J Clin Exp Pathol 6: 1874-1879, 2013.