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Brief communication (Original)

Effects of mechanical stimulus on mesenchymal stem cells differentiation toward cardiomyocytes

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Background: Mesenchymal stem cells (MSCs) known to be sensitive to mechanical stimulus. This type of stimulus plays a role in cellular differentiation, so that it might affect MSCs differentiation toward cardiomyocytes.
Objectives: Investigate the effect of mechanical stimulus on MSCs differentiation toward cardiomyocytes.
Methods: The adipose tissue-derived MSCs were induced to differentiate with 5-azacytidine, and stimulated by one Hz mechanical stretching up to 8%. After 10 days, the cell's cardiac markers and cardiogenesis-related genes were detected by immumohistochemistrical staining and reverse transcriptase-polymerase chain reaction, and the cell's ATPase activity was detected.
Results: The cyclic mechanical stretching enhanced the expression of cardiogenesis-related genes and cardiac markers, and stimulated the activity of Na^+-K^+-ATPase and Ca^{2+}-ATPase in the MSCs treated with 5-azacytidine. Without 5-azacytidine pre-treatment, cyclic mechanical stretch alone has little effect.
Conclusion*:* Mechanical stretch combined with 5-azacytidine treatment could accelerate MSCs differentiation toward cardiomyocytes.

Keywords: 5-azacytidine, cardiomyocyte, differentiation, mechanical stretch, mesenchymal stem cells

Adipose tissue-derived mesenchymal stem cells (AT-MSCs) have multilineage differentiation potential. The cells can differentiate into osteoblast, chondrocyte, adipocyte, muscle, and endothelial cells similarly to bone marrow-derived mesenchymal stem cells (BM-MSCs) [1, 2].

Previous studies have shown that AT-MSC can differentiate into cardiomyocyte or cardiomyocyte-like cells *in vitro* and *in vivo* [3-5]. AT-MSCs are easily harvested with minimal morbidity, and the proportion of AT-MSCs in adipose tissue is much higher than that of BM-MSC in bone marrow [6]. Therefore, AT-MSCs may be important for the development of cell therapy and tissue engineering.

Mechanical stimulus on cells plays an essential role in their differentiation and growth. Mesenchymal stem cells are highly sensitive to mechanical strain. For example, such mechanical strain can stimulate early osteo-chondrogenic response [7]. Mechanical stretch is also believed to be a physiological stimulus required for cardiac development and growth [8-10]. It is supposed that mechanical stimulus affects AT-MSC differentiating toward cardiomyocytes, and some kinds of mechanical stretch can accelerate their differentiation. However, little is known about the effect of mechanical stimulus on MSCs or AT-MSCs differentiation toward cardiomyocytes.

In this study, we treated AT-MSCs with 5-azacytidine and submitted the cells to mechanical stretch. We examined the effect of this chemical and mechanical treatment on cell differentiation toward cardiomyocytes.

Materials and methods

This study was approved by the Ethics Committee of Tianjin Institute of Medical Equipment. All experimental animals were cared for following the rules defined in the Guide for the Care and Use of Laboratory Animals, National Institute of Health, USA.

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Isolation and culture of AT-MSCs

Cells were isolated from the fat tissue as described previously [11], with some modifications. Briefly, inguinal and interscapular adipose tissue from Wistar rats was washed extensively with sterile phosphate-buffered saline (PBS) to remove contaminating debris and red blood cells, then digested at 37°C, on a shaker, in PBS containing 1% bovine serum albumin (BSA) and 2 mg/mL collagenase A (Roche), for 45 minutes. After filtration through 40 μm mesh filters (Falcon, Bedford, USA) and centrifugation, isolated cells were re-suspended in DMEM medium (Invitrogen) containing 10% fetal bovine serum (FBS) (Invitrogen, USA), glutamine (2 mM), penicillin G (100 U/mL), and streptomycin (100 μg/mL). Cells were then introduced into 25 cm^2 flask (Corning, USA) and cultured in 95% air and 5% CO, at 37°C. Medium was replaced every three days. Non-attached cells were discarded and adherent cells retained. The plastic-adherent cells were designated AT-MSCs.

Chemical and mechanical stretch treatment of AT-MSCs

After three passages of subculture, the AT-MSCs were seeded onto silicone elastic membranes (Specialty MFG, Saginaw, USA), coated with collagen-IV (1 μg/cm^2) at density of $7.5x10^4/cm^2$. After 24 hours serum starvation, the cells were divided into four groups for treatment. In the first group (5-aza + stretch), where, after treatment with 10 μmol/L 5-azacytidine, the cells were stimulated by 1 Hz mechanical stretching up to 8%. In the second group (stretch), the cells were stimulated by identical mechanical stretching without 5-azacytidine. In the third group (5-aza), the cells were incubated with 10 mol/L 5-azacytidine but not subjected to mechanical stretch. In the fourth group (control), the cells were cultured under the same conditions but subjected neither to stretch nor to 5-azacytidine treatment.

To apply mechanical stretch *in vitro*, AT-MSCs were cultured on silicone rubber membrane in stretch chambers of a custom-made cell stretch apparatus, as described previously [12].

Immunofluorescence and immunocytochemical staining

The AT-MSCs on silicone membranes were rinsed with PBS, fixed with 3% paraformaldehyde, washed in PBS, permeabilized with 0.1% Triton X-100 for 15 minutes, and washed in PBS containing 0.01% Tween 20 (PBST). Proteins were blocked in PBST supplemented with 2% BSA. Primary and secondary antibodies were used at a 1:200 dilution in PBST/2% BSA and incubated for 30 minutes. Nuclei were counterstained with 10mg/mL 4',6-diamidino-2-phenylindole (DAPI) solution, or hematoxylin solution. Primary antibodies used were mouse anti-GATA4 (fluoresceinisothiocyanate-conjugated secondary antibody), mouse anti-α-sarcomeric actinin (Cy3-conjugated secondary antibody) and mouse anti-α-sarcomeric actin (horseradish peroxidase-conjugated secondary antibody), staining with diaminobenzidine (Santa Cruz, CA,USA). Photographs were taken with Olympus BX51 fluorescence microscope (Olympus, Tokyo, Japan), analyzed with the Image-Pro Plus 6.2 software (Media Cybernetics Inc, Bethesda, USA) and processed with Adobe Photoshop. The positive immunofluorescence or immunocytochemical staining area percentage and positive staining integral density value (IDV) were calculated.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RT-PCR was performed with Biort One Step RT-PCR kit (Hangzhou Bioer Technology Co, Hangzhou, China) and a 96-well thermal cycler (Mastercycler 5331, Eppendorf, Germany) following the manufacturers' protocols.

PCR primers were as follows:

1) the primer sequence of atrial natriuretic peptide (ANP), forward 5'-GGGCTCCTTCTCCATCAC-3', reverse 5'-TCCAATCCTGTCCTACC-3',

2) the primer sequence of Nkx2.5, forward 5'-CAGTGGAGCTGGACAAAGCC-3', reverse 5'-TAGCGACGGTTCTGGAACCA -3',

3) the primer sequence of α-myosin heavy chain (α-MHC), forward 5'-AGTATGTCACCAAGGGGC AG -3', reverse 5'-CGAACATGTGGTTGAAG -3'.

β-actin gene (forward primer 5'-ATCATGTTTG AGACCTTCAACA3' and reverse primer 5'-CATC TCTTGCTCGAAGTCCA3') served as an internal standard for RNA integrity and gel loading. The reverse transcription step was run for 30 minutes at 50°C, followed by PCR activation for 10 minutes at 95°C. Thirty amplification cycles were run, consisting of 50 seconds denaturation at 94°C, 40 seconds of

annealing at 56°C, and 40 seconds of extension at 72°C. Final extension was allowed to run for 10 minutes at 72°C. The PCR reaction products were separated by gel electrophoresis using 2% agarose gel, stained with ethidium bromide, and visualized under an Ultraviolet Transilluminator (Beijing Liuyi Instrument, Beijing, China). The IDV of each PCR band from electrophoresis image was analyzed using AlphaEase software (Alpha Innotech, Miami, USA). The relative expression of mRNA was calculated using IDV of each PCR production band normalized against the IDV of β-actin gene band.

ATPase activity assay

The AT-MSCs were digested with 0.2% trypsin and centrifuged. The cells were lysed by brief sonication on ice in a lysis buffer (10 mmol/L HEPES, 250 mmol/L sucrose, 5 mmol/L Tris-HCl, 1mmol/L EDTA, 0.01% TritonX-100, pH 7.5). The protein concentration of the cell lysates was measured with BCA Protein Assay Kit (Beijing Applygen Genetechnology, Beijing, China), following the procedure provided by the manufacturer. The ATPase (Na^+-K^+-ATPase and Ca^{2+}-ATPase) activity of the cell lysates was assayed with ATPase activity assay Kit (Nanjing Jiancheng Biotechnology, Nanjing, China). The ATPase activity was expressed in the inorganic phosphor production from the decomposition per mg protein per hour (mol Pi per mg protein per h, mol Pi/mg.h).

Statistical analysis

Results were presented as mean±S.D. Statistical significance was evaluated using one-way ANOVA, followed by Student's t-test (SPSS 8.0, Chicago, USA). A value of $p < 0.05$ was considered statistically significant.

Results

Immunofluorescence and immunocytochemical staining

After AT-MSCs stimulation with the mechanical stretch (1 Hz) up to 8% for 10 days, the immunofluorescence staining result for GATA4 was nearly negative in both the group "stretch" and control group. In the cells treated with 5-azacytidine, the positive stained area percentage and positive staining IDV for GATA4 were seven and six times higher, respectively, in the cells stimulated with mechanical stretch for 10 days (group "5-aza + stretch") than for AT-MSCs not submitted to mechanical stretch (group "5-aza") as shown in **Figure 1**. Interestingly, after mechanical stretch stimulation for 10 days, the immunofluorescence staining of α-sarcomeric actinin in group "stretch" and control group was very weak. However, in the cells treated with 5-azacytidine, the positive stained area percentage and positive staining IDV of the cells stimulated with mechanical stretch (group"5-aza + stretch") were 23% and 16% higher, respectively, than in the "5-aza" group ($p < 0.05$). In addition, the immunocytochemical staining results of -sarcomeric actin in the four groups were similar to those for α-sarcomeric actinin and GATA4. The positive stained area percentage and positive staining IDV of the cells in group "5-aza + stretch" were 143% and 186% higher than in group "5-aza" ($p < 0.01$).

RT-PCR of ANP, Nkx2.5 and -MHC

Gene expression was examined using RT-PCR technique (**Figure 2**). The ANP gene was expressed at a significantly lower level in the latter case, compared with group "5-aza + stretch" and for group "5-aza" ($p < 0.05$). The ANP expression was not detected in either group "stretch" or "control". Expression patterns for Nkx2.5 were similar to those of ANP gene; Nkx2.5 in group "5-aza+stretch" was expressed at a higher level than in group "5-aza" ($p < 0.05$). The expression of α-MHC was detected only in group"5-aza+stretch".

ATPase activity assay

After submitting the cells (not treated with 5-azacytidine) to the mechanical stretch for 10 days, the activities of Na^+-K^+-ATPase and Ca^{2+}-ATPase in the cells were changed little, compared with control group. In the groups treated with 5-azacytidine, the activities of Na^+-K^+-ATPase and Ca^{2+}-ATPase of the cells stimulated with mechanical stretching were enhanced remarkably, compared with the cells which did not undergo mechanical stretching ($p < 0.05$) as shown in **Figure 3**.

Discussion

MSCs are highly sensitive to mechanical stimulus, and mechanical strain plays an important role in their differentiation and growth. Many studies have explored the effect of mechanical stimulus on the differentiation. For example, cyclic mechanical strain (5% or 2000 microseconds, 1 Hz) promoted or induced osteogenic differentiation of MSCs *in vitro* [13, 14].

Cyclic compressive strain (10% or 15% at frequency of 1 Hz) enhanced chondrogenic differentiation of MSCs *in vitro* [15, 16]. Mechanical stimulation *in vitro* also induced the selective differentiation of MSCs into a ligament cell lineage [17]. In addition, cyclic mechanical strain strongly affected the differentiation of MSCs into smooth muscle cells [18, 19]. However, most of these studies did not use adipose tissue-derived MSCs. Mechanical stimulus effect on differentiation of MSCs into cardiomyocytes was not examined, but Rangappa et al. [20] demonstrated that mechanical stretch (1Hz, 4% or 8%) could induce the expression of early cardiac genes (Cx43, bone morphogenetic protein-2, etc.) in bone marrow-derived MSCs.

Makino et al. [21] used 5-azacytidine to induce marrow stromal cells differentiation into cardiomyocytes [21]. Since then, 5-azacytidine has become a typical treatment used to stimulate cardiomyocyte differentiation, and it was reported that similar treatment transforms AT-MSCs into cardiomyocytes [22]. Hence, we used the same compound to induce AT-MSCs differentiation to cardiomyocytes. In our study, in all four experimental groups described above, immunocytochemical staining results for α-skeletal myosin were negative. This suggests that, although 5-azacytidine might induce AT-MSCs differentiation to cardiomyocytes, it does not promote differentiation to skeletal muscle cells (data not shown).

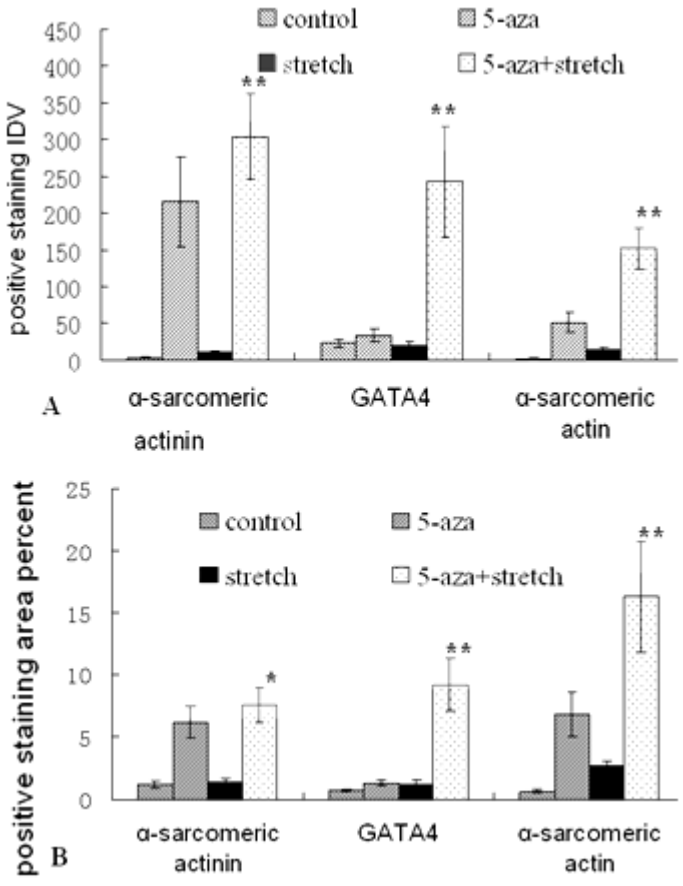

Figure 1. Quantitative analysis of the immunofluorescence/immumohistochemical staining results in AT-MSCs. **A:** Positive stained area percentage for α-sarcomeric actinin, GATA4 and α-sarcomeric actin. The positive stained area percentage for the cells in group "5-aza+stretch" was higher than for group "5-aza". *p <0.05, group "5-aza+ stretch" versus group "5-aza", **p <0.01, group "5-aza+ stretch" versus group "5-aza". **B:** Positive staining IDV for -sarcomeric actinin, GATA4 and α-sarcomeric actin. The positive staining IDV of the cells in group "5-aza+stretch" was higher than for group "5-aza". (*p <0.05, group "5-aza+ stretch" versus group "5-aza", **p <0.01, group "5-aza+ stretch" versus group "5-aza").

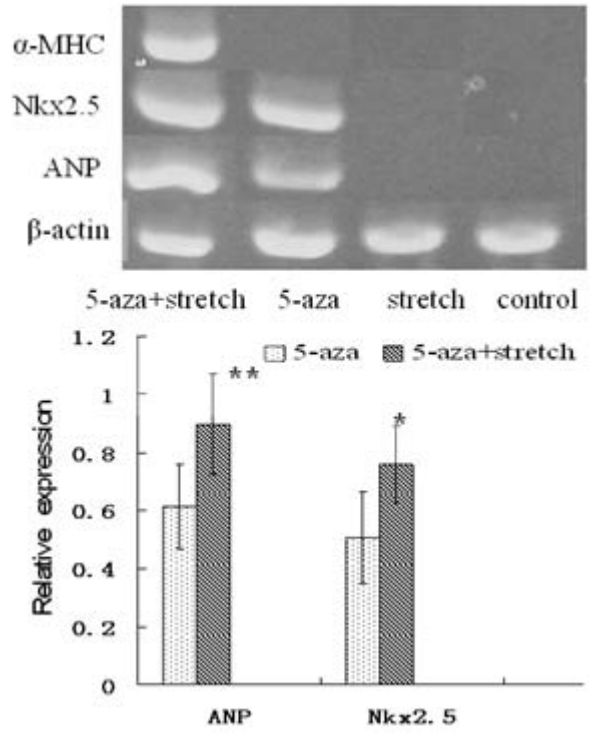

Figure 2. RT-PCR analysis of mRNA expression for ANP, Nkx2.5 and α-MHC in AT-MSCs. **A:** RT-PCR of ANP, Nkx2.5, α-MHC and β-actin. **B:** Relative expression of ANP and Nkx2.5 mRNA. The relative expression for group "5-aza+ stretch" is higher than for group "5-aza" (*p <0.05, group "5-aza+ stretch" versus group "5-aza").

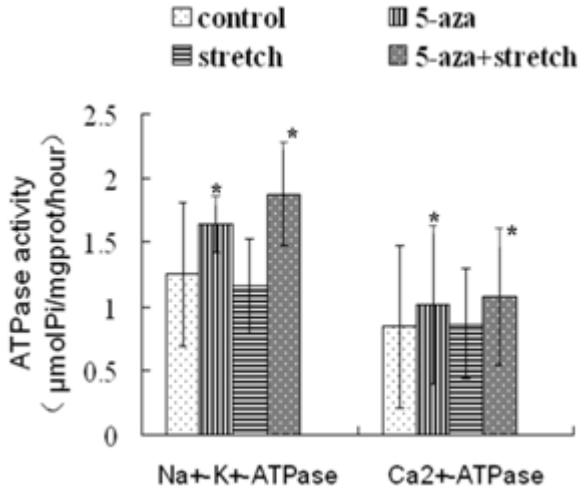

Figure 3. ATPase activity of AT-MSCs. Compared with control group, the activity of Na^+-K^+-ATPase and Ca^{2+}-ATPase in group "stretch" has changed very little; that in group "5-aza+ stretch" is enhanced remarkably compared with group "5-aza" (*p <0.05, group "5-aza+ stretch" versus group "5-aza").

Some transcription factors, such as Nkx2.5 and GATA4, are essential in cardiogenesis and cardiac differentiation. Nkx2.5 is of crucial importance in early cardiac differentiation and the normal growth of the embryonic myocardium. GATA family genes, including GATA4, expressed in the developing heart, have been implicated as key regulators of cardiogenesis in several model systems [23]. α-sarcomeric actin and α-MHC are important for motor-unit contractile properties of muscle, and are the markers of cardiomyocyte and

skeletal muscle cells. ANP is produced, stored, and released by cardiac myocytes of the atria of the heart. It is released in response to atrial stretching, and a variety of other signals induced by hypervolemia, exercise, or caloric restriction [24]. Our study showed that mechanical stretch (1Hz, 8%) could improve the growth and metabolism of cardiac myocytes *in vitro*.

In this study, mechanical stretch significantly elevated the expression of α-sarcomeric actin, α-sarcomeric actinin and GATA4 in AT-MSCs (as shown by immunocytochemical staining and immunofluorescence staining) and increased mRNA expression for ANP, Nkx2.5 and α-MHC (shown using RT-PCR). In addition, mechanical stretching enhanced the ATPases (Na^+-K^+-ATPase and Ca^{2+}-ATPase) activity in the cells. It is well known that cardiomyocyte ATPase activity is higher than that in other cell types. Our data show that mechanical stretch applied to adipose tissue-derived MSCs stimulates their differentiation toward cardiomyocytes.

However, our research also showed that in absence of 5-azacytidine the mechanical stretch had little effect on the expression of cardiogenesis-related genes and cardiac markers, and ATPase activity. We demonstrated that, in the case of AT-MSCs, the cyclic mechanical stretch on its own hardly affects the differentiation toward cardiomyocytes.

In conclusion, applying mechanical stimulus on its own has little or no effect on adipose tissue-derived mesenchymal stem cells differentiation toward cardiomyocytes, but accelerates the differentiation of the cells treated with 5-azacytidine.

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The authors have no conflict of interest to declare.

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