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Enhanced differentiation of rat MSCs into cardiomyocytes with 5-azacytidine/collagen I nano-molecules

Yi-Jhen Wu, Shu Ying Chen, Shwu Jen Chang, Shyh Ming Kuo

Abstract—**This study was to investigate the enhancement ability of 5-azacytidine (5-aza) and collagen I nano-molecules treatment to the differentiation of rat mesenchymal stem cells (MSCs) towards a cardiomyocytes in vitro. The results demonstrated that the size of the cells increased significantly and connecting with adjoining cells by forming myotube-like structures. Also, additional treatment of the MSCs with collagen I nano-fibrils significantly increased two transcription factors GATA-4 and Nkx2.5 expressions and three expressions of cardiac genes of troponin I, β-myosin heavy chain and cardiac α-actin compared with MSC groups treated only with 5-aza at early 3 d culturing(all, $P<0.01$ or better). These results indicate that culturing MSCs with collagen I nano-molecules, which could act as scaffolds or soluble protein ingredients, leads to alterations in gene expression and affects the differentiation fate induced with 5-aza.**

Keywords: 5-azacytidine, collagen I, nano particles, MSC

I. Introduction

Many efforts have been developed to facilitate MSCs to differentiate into cardiomyocytes, such as chemicals and growth factor supplements or changes to the composition of the culture medium [1-2]. Among of these, 5-aza has been used as a useful demethylation agent to study the roles of DNA methylation on gene activation and cell differentiation. MSCs can exhibit cardiomyocyte-like phenotypes following 5-aza treatment using different experimental procedures, and changes in delivery frequency, dose and incubation duration all would affect the outcome of cardiomyogenic differentiation [3- 4]. Furthermore, many research results have demonstrated that the cell-culture scaffold architecture, including the geometry and dimension of the scaffolds may influence cell proliferation and differentiation. Recent in vitro studies have also indicated that culturing cells with various nano-sized scaffolds led to alterations in gene expression and differentiation. Recently, we have successfully fabricated nano-sized collagen particles by using a high-voltage electrostatic field system under controllable experimental settings [5]. Effects of collagen I nano-molecules (nano-particle or nano-fibril) together with a 5-aza treatment on the differentiation of MSCs into cardiomyocytes by examination of cellular morphology and the protein and gene expression of the induced cardiomyocytes was presented in this study.

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Yi-Jhen Wu is with the Department of Biomedical Engineering, I-Shou University, Kaohsiung city, Taiwan, ROC (e-mail: purple19900104@yahoo.com.tw).

Shu Ying Chen is with the Department of Biomedical Engineering, I-Shou University, Kaohsiung city, Taiwan, ROC (email: shufentant@yahoo.com.tw).

Shwu Jen Chang is with the Department of Biomedical Engineering, I-Shou University, Kaohsiung city, Taiwan, ROC (email: sjchang@isu.edu.tw).

Shyh Ming Kuo is with the Department of Biomedical Engineering, I-Shou University, Kaohsiung city, Taiwan, ROC (Tel.:886-7-6151100 ext.7469; email: smkuo@isu.edu.tw).

II. Materials and Methods

A. Production of collagen I nano-molecules

A high-voltage electrostatic field system was constructed to produce nano-sized collagen particles. The strength of the electrostatic field was provided by a DC power supply. The whole system was placed inside a thermal control chamber and set to a designated temperature (4°C). A plastic Petri-dish with 1 mL of iced collagen I solution was placed at the center between the two plate electrodes. The applied strength of electrostatic field was set at 2.5 $kVcm^{-1}$. The concentration of collagen I was 0.1 mg/mL, and the reaction time was set to 1 h. The nano-sized collagen fibrils were produced by deactivating the power applied and increasing the reaction temperature to 37°C to initiate the spontaneous reconstitution of collagen.

B. Differentiation of MSCs into cardiomyocytes

Bone marrow was aspirated from the femur and tibia bones of 3-week-old Sprague–Dawley rats after dislocation [6]. The marrow was collected and diluted with 4 mL of Dulbecco's modified Eagle's medium (DMEM, GIBCO). Second passaged MSCs (density of 1×10^5 cells/mL) were cultured in a DMEM medium containing collagen I nano -particles or nano-fibrils with a final concentration of 1×10^{-3} mg/mL or without the addition of collagen I nano-molecules. These MSCs were incubated at 37°C in a 5% CO_2 incubator for 24 h, and 10 μM 5-aza was then added for the induction of cardiomyogenic differentiation. Untreated MSCs served as the negative control group. After incubation for another 24 h, the cells were washed twice with PBS solution, and the medium was changed to a medium without 5-aza. The medium was changed every 3 days.

C. Immunofluorescence staining

To identify whether MSCs induced by 5-aza differentiated to cardiomyocytes, cells were fixed, permeabilized, and blocked with 5% albumin for 30 min before incubation with cardiac specific antibodies, anti-troponin I, anti-cardiac α-actin and anti-connexin 43 for 1 h. The cells were then incubated for 1 h with Rodamin-conjugated or FITC-conjugated secondary antibodies. Nuclei were visualized by staining with a DAPI solution for 30 min. Cell images were taken at 200 magnification viewed under a fluorescence microscope (Olympus IX-71, Japan).

D. Real-Time PCR analysis

The total RNA was extracted from MSCs with the Micro-to-Midi Total RNA Purification System (Invitrogen) according to the manufacturer's protocol. The RNA was dissolved in RNase-free water and stored at –80°C until use. A

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total of 5 μg RNA was reverse transcribed into cDNA using a SuperScriptTM III One-step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen). An ABI TaqMan® Gene Expression assays probe and gene-specific primers were used in this study. Amplification of cDNA was performed using the T StepOneTM Real-Time PCR System (Applied Biosystems, ABI). The relative quantity (RQ) of specific gene expression was determined using the $2^{-\triangle\triangle Ct}$ method [7].

E. Statistical analysis

The results are presented as the mean ± SD. Data were compared using an ANOVA statistical analysis, and a P value of less than 0.05 was considered statistically significant.

III. RESULTS AND DISCUSSION

Figure 1 shows the transmission electron microscope images of the prepared nano-sized collagen I particles and fibrils. As shown, the collagen I monomers could coil together and form stable spherical particles under specific preparation settings. When the temperature was increased to 37°C, more fibrous structures from the collagen I monomers were observed with similar experimental settings (Fig.1)

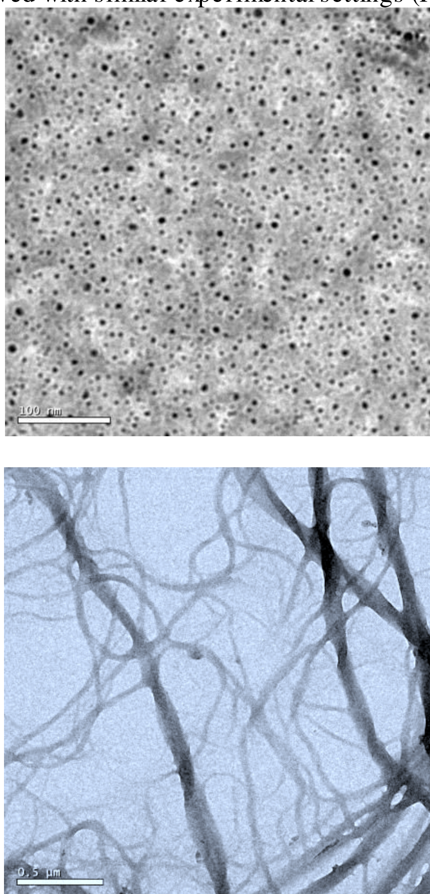
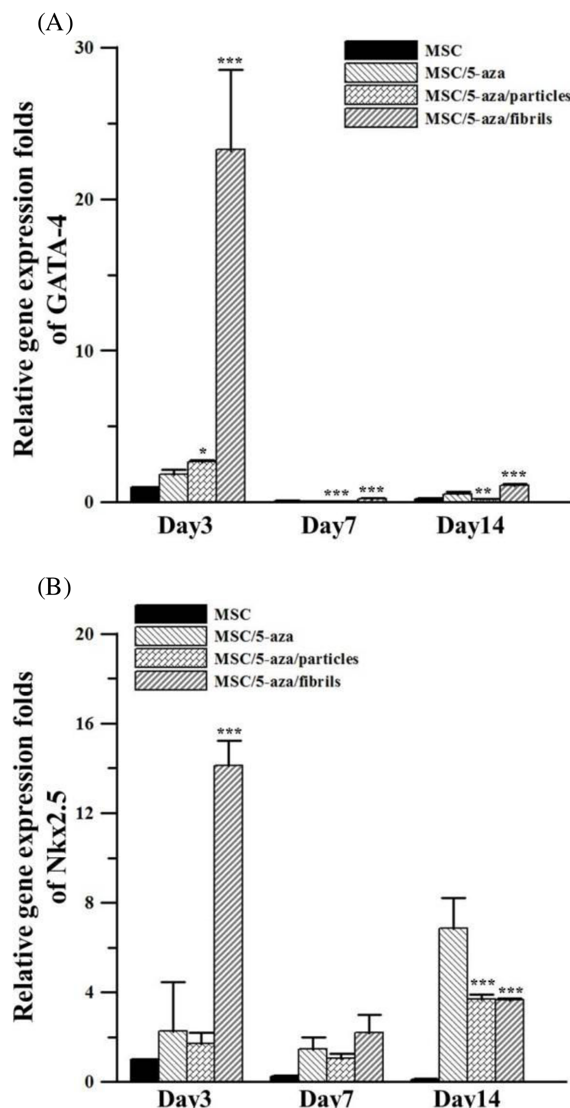


Figure1. Transmission electron micrographs of nano-sized collagen I molecules prepared from high-voltage electrostatic field system.

From the initial cultures, MSCs displayed a spindle-like fibroblastic shape. As growth continued, the MSCs size increased significantly and acquired a flattened, triangular-shaped morphology after treatment with collagen I nano-molecules and 5-aza (data not shown). These cells exhibited a distinct morphology compared with the non-induced MSCs post-treatment and connected with adjoining cells, forming myotube-like structures.

MSCs can differentiate into cardiomyocytes under stimulus of some chemicals, such as 5-aza, retinoic acid or TGF-β. Here we used 5-aza as the stimulant and assess the enhancement ability of collagen I nano-molecules on the gene expressions of important transcription factors and proteins of cardiomyogenesis of 5-aza inducing MSCs, the expression of two transcription factors (GATA-4 and Nkx2.5) and three cardiac-specific markers (troponin I, β-myosin heavy chain and cardiac α-actin) was evaluated by real-time RT-PCR. As shown in Fig. 2, the results showed that two transcription factors GATA-4 and Nkx2.5 expressed the highest gene expressions at MSCs with pretreatment of collagen I nano-fibrils at 3 d post-induction. Furthermore, MSCs pretreated with collagen I nano-fibrils significantly increased the other three cardiac-specific gene expressions, with a 5.8-fold increase in troponin I, 10.3-fold increase in β-myosin heavy chain and 25.4-fold increase in cardiac α-actin compared with expressions in the group treated only with 5-aza (at day 14).



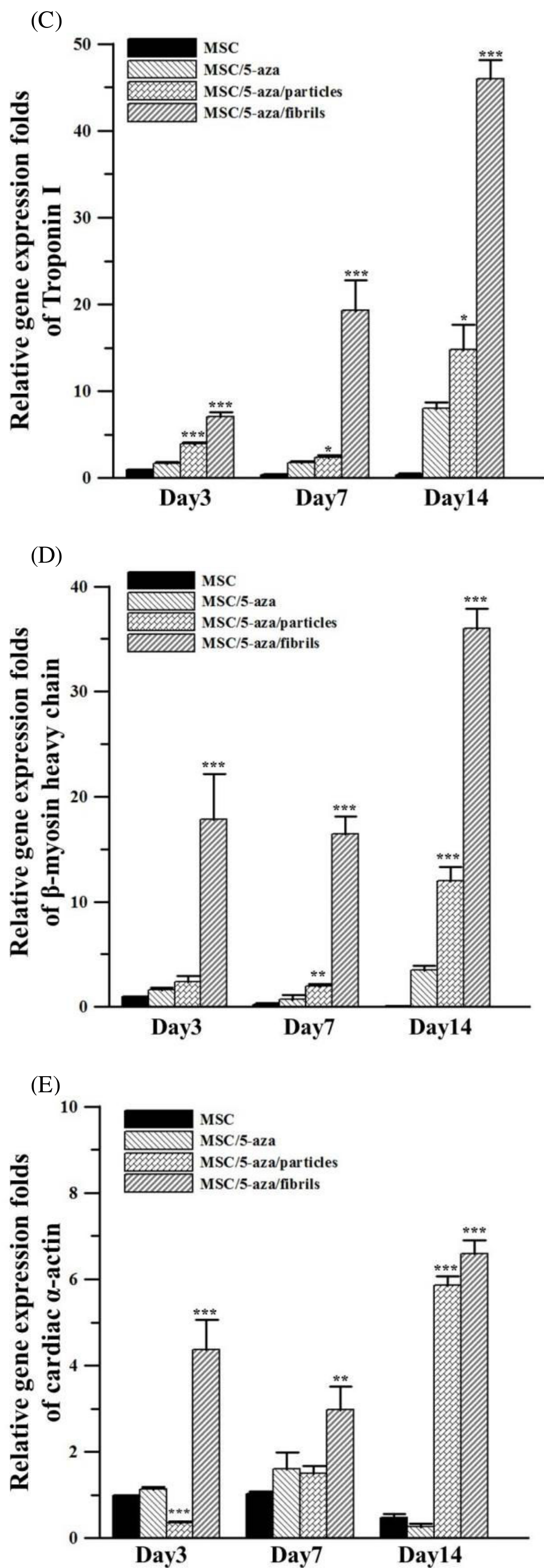


Figure 2. Effects of collagen I nano-molecules on the 5-azacytidine induced of MSC differentiation into cardiomyocytes. After 3,7 and 14 d incubation, the relative quantity (RQ) of specific gene expression was detected by quantitative RT-PCR and analyzed by statistic software. (A)GATA-4, (B)Nkx2.5, (C)troponin I, (D)β-myosin heavy chain, (E)cardiac α-actin. P values are based on comparisons with MSC/5-aza group (at each time point). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

To elucidate the effect of collagen I nano-molecules and the 5-aza treatment process on the differentiation of MSCs into cardiomyocytes, specific markers of cardiomyogenic differentiation were also examined by immunoblot analysis (Figure 3). It was found that MSCs pretreated with collagen I nano-molecules and the 5-aza treatment had greatly increased Nkx2.5 and Troponin I protein levels of cardiomyogenic differentiation markers compared with the MCS control group at 14 d post-induction. However, the levels of connexin 43 were not consistent changes with time dependent compared with other groups.

TABLE I. PRIMERS FOR REAL-TIME RT- PCR

	Sequences (5'-3')	Accession No.	Product
α-Actin	Forward: CCCAGCACCATGAAGATTAAGATTATTG Reverse: CCTCATCGTACTCTTGCTTGCTGATC	NM_019183.1	146 bp
Arbp	Forward: GTACCATTGAAATCCTGAGCGATGTG Reverse: GATGCTGCCATTGTCAAACACCTG	NM_022402.2	130 bp
GATA4	Forward: GTCCCAGACATTCAGTACTGTGTCCG Reverse: GTGACAGGAGATGGATAGCCTTGTGG	NM_144730.1	99 bp
β-MHC	Forward: CACAGATGCCGCCATGATGG Reverse: CGATCTGCTCTGCCTCGTCCAG	NM_017240.1	134 bp
NKX2.5	Forward: CCAAGTGCTCTCCTGCTTTCC Reverse: CGCACAGCTCTTTCTTATCCG	NM_053651.1	105 bp
Troponin I	Forward: CCATGATGCAGGCACTACTGGG Reverse: GGTTTTCCTTCTCAATGTCCTCCTTC	NM_017144.1	99 bp

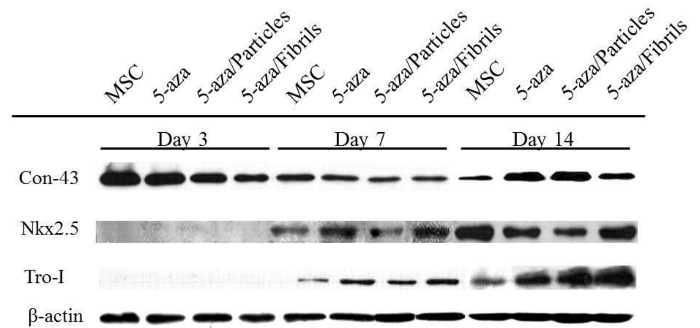


Figure 3. Western blot analysis showed that the expressions of cardiac specific proteins connexin-43, Nkx2.5 and Troponin I cardiac α-actin after 3, 7 and 14 d incubation.

Figure 4 showed the immunofluorescence analysis on the presence of cardiac-specific proteins in differentiated cells. As shown, the cells were positively immune-stained with cardiac α-actin, connexin-43, Desmin and troponin I after 3 d, 7d and 14d of cultivation. We found that these cardiac-specific proteins were localized at all groups that demonstrated most of the differentiated cells expressed markers for cardiomyocytes.

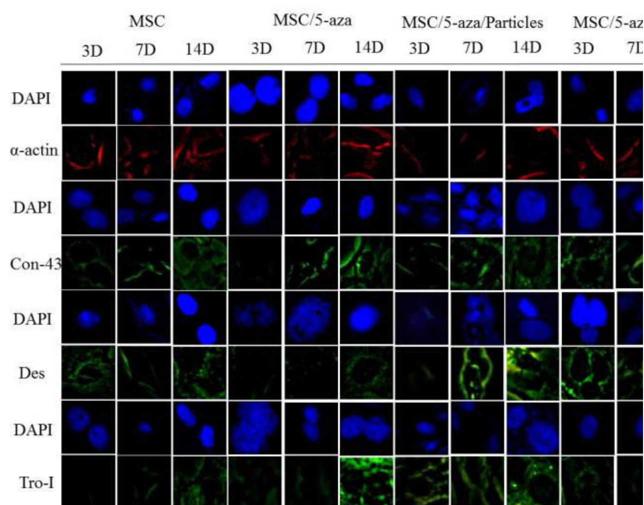


Figure 4. Immunostaining analysis of effects of collagen I nano-molecules on the 5-aza induced of MSC differentiation into cardiomyocytes. Positive staining for cardiac α-actin (red), connexin 43 (green), desmin (green) and troponin I (green) were seen at 3,7 and 14 d of cultivation (×200). All nuclei were stained with DAPI.

IV. CONCLUSION

In the present study, the ability of MSCs to differentiate towards cardiomyocytes was enhanced by combining collagen I nano-molecules and 5-aza treatment compared with relatively short 3-d cultivation. The most of important is that ,these extra treatments with collagen I nano-fibrils on MSCs significantly increased the GATA-4, Nkx2.5, troponin I, β-myosin heavy chain and cardiac α-actin expressions compared with treatments with collagen I nano- particles or 5-aza alone. However, the roles and contributions of the collagen I nano-molecules, acting as scaffolds from fibrils or supplying nutrients or chemical cues from the particles still need to be examined. Furthermore, the influences of the timing of the treatment and concentration of collagen I nano-molecules added are being evaluated in our laboratory. We hope to create new directions for cell culture and tissue engineering by using collagen I nano-molecules with extraordinary capabilities. However, the detail of the mechanism is under evaluation in our laboratory.

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