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# Differentiation of amniotic membrane mesenchymal stem cells to cardiomyocytes and the expression of Nkx2.5 and C-TNT genes

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## Abstract

Cardiovascular disease (CVDs) continues to account for more deaths globally than any other single disease. Currently, significant advances have been made in the field of cardiac diseases, and stem cell transplantation-based therapies have emerged as a hopeful therapeutic tool for improving cardiac regeneration and function. Mesenchymal stem cells (MSCs) represent a promising source to be used by regenerative medicine. MSCs have potential to self-renew and discriminate into several cell types. In this study, MSCs were isolated from the amniotic membrane, and their surface markers were identified using flow cytometry. The cells were differentiated to osteoblasts, adipocytes and cardiomyocytes using differentiation medium. RealTime-PCR, and immunochemistry assays were used to assess the expression of cardiac marker genes (Nkx2.5 and C-TNT) and protein. The results confirmed the differentiation of MCSs to cardiomyocytes. NKX2.5 and C-TNT expression level strikingly increased in cardiomyocyte cells compared to hAM-dMSCs. The results of the present study suggested that differentiated human amniotic MSC possessed some characteristics of cardiomyocytes.

**Keywords:** Cardiomyocyte, Mesenchymal stem cells, Differentiation, NKX2.5, CTNT

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# 1 Introduction

Cardiovascular diseases (CVDs) are a group of disorders of the heart and blood vessels and contain coronary heart disease, cerebrovascular disease, rheumatic heart disease and other conditions (Ou et al., 2020). CVDs are a leading cause of death in the world. Cardio metabolic, behavioral, environmental, and social risk agents are main drivers of CVD. Currently available treatments for heart failure include drug treatment, cardiac resynchronization therapy, mechanical support devices, and heart transplantation (Zanjanizadeh Ezazi et al., 2020). The last treatment option is organ transplantation, which has many impediments due to donor shortage, donor adaptation, and complications during and after transplant surgery (Jennings et al., 2018). Therefore, the search for new and low-risk treatments is essential. One of the latest approaches which stand a possibility for being used for the treatment of the disease is cellular cardiomyoplasty, use of stem cells or progenitor cells for myocardial regeneration to improve cardiac function and decrease heart failure (Tavakoli et al., 2013).

Mesenchymal stem cells (MSCs) are stromal cells and are extracted from different sources: bone marrow, adipose tissue, placenta, umbilical cord, heart, and amniotic membrane (AM) (Bagno et al., 2018). They are multipotent cells and can differentiate to osteogenic, chondrogenic, and adipogenic lineages *in vitro* (Naji et al., 2019). MSCs have increased popularity for their use in myocardial regeneration (Kobayashi and Suzuki, 2018) and may expand a new therapeutic strategy. Amniotic membrane-generated MSCs (AM-MSCs) can express the surface markers such as CD73, CD29, CD90, and CD105, and do not express the CD34 and CD45. MSCs have been reported to be able to repair damaged tissue by secreting different growth factors and anti-inflammatory molecules (Barrett et al., 2019). AM-MSCs are located in an avascular stroma. AM membrane is considered postpartum tissue, which can be used for cell therapy by anti-inflammatory effects, low immunity, antimicrobial, and anti-apoptotic and anti-angiogenesis properties.

In tissue regeneration based on animal studies, human amniotic stem cells (hAMSCs) showed the shortest regeneration time in comparison with other sources (Yusoff et al., 2015; Prieto et al., 2016). As hAMSCs have immunomodulatory properties, paracrine actions, and potential applications in regenerative medicine, they have gained much attention (Abou-ElNaga et al., 2020).

On the other hands, the mammalian NKX2.5 gene was discovered to be vital for cardiac growth and the NKX2.5 protein has been categorized as a Class I NK-2 homeodomain protein. Also, cardiac muscle troponin T (cTnT) is a protein that is encoded by the TNT2 gene in humans and is expressed in cell cytoplasm. Troponin complex is a component of skeletal and cardiac muscle thin filaments. It consists of three subunits - troponin I, T, and C, and it plays a critical role in muscle activity, connecting changes in intracellular Ca2+ concentration with generation of contraction (Tong, 2016). Due to the importance of these genes in heart disease and the clinical application of AM-MSCs in the area of cell therapy for heart failures, in this study, we investigated the changes of NKX2.5 and CTNT in mesenchymal stem cells differentiated into myocardial cells.

# 2 Materials and Methods

Most of the chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

### 2. 1. Flow cytometry

The human amniotic membrane was purchased from Iranian biological resource center. After isolating, the cells were cultured in DMEM-LG, 10% FBS and 5% penicillin/streptomycin. Thereafter, cells were trypsinized with 0.25%Trypsin/EDTA, centrifuged, washed twice with BSA/PBS and incubated for 30 min with antibodies against CD105, CD73, CD29 and CD45, CD34. They were conjugated with primary antibodies with PE, FITC and percP Flow-cytometry was performed with BD FACS Calibur instrument. The cells were analyzed by flowjo 7.6 (Ghaneialvar et al., 2018).

### 2. 2. Differentiation into osteoblast and adipocyte

Cells in passage 6 were prepared for differentiation into osteoblast and adipocytes. In osteoblast differentiation plate, collagen was first added, and after 24 h, it was removed. AM-MSC cells were added to osteogenic and adipogenic culture medium. After 21 days of differentiation, the cells in osteogenic culture medium were stained with Alizarin red and the cells in adipogenic culture medium were stained with oil red (all from Sigma) (Yu et al., 2021; Li et al., 2021).

### 2. 3. Differentiation to cardiomyocytes

Mesenchymal stem cells (passage 5) were transferred to culture medium containing 10% fetal bovine serum (FBS) and 20% Chang's medium including 5μM butyric acid (BA), 2mg hyaluronic acid (HA) and 1μM retinoic acid (RA) to differentiate to cardiomyocytes (Yong et al., 2016).

### 2. 4. Extraction of RNA

Differentiated cardiac cells were used to extract RNA on day 14 using RNA extraction YTA Total RNA purification Mini Kit (Favorgen Biotech Corp., Kaohsiung, Taiwan). The primers were designed for the NKX2.5 and C-TNT genes (Table 1). cDNA was synthesized from RNA using the cDNA synthesis kit according to the manufacturer's instructions.

Table 1. Primer sequences and size

| Gene | Primer sequence (5'→3') | Size(bp) |
|---|---|---|
| C-TNT(forward) | GGCAGCGGAAGAGGATGCTGAA | 150 |
| C-TNT(reverse) | GAGGCACCAAGTTGGGCATGAACGA | 150 |
| NKX2.5(forward) | CTGCCGCCGCCAACAAC | 136 |
| NKX2.5(reverse) | CGCGGGTCCCTTCCCTACCA | 136 |
| GAPDH(forward) | ATGGGGAAG GTGAAGGTCG | 70 |
| GAPDH(reverse) | TAAAAGCAGCCCTGGTGACC | 70 |

**2. 5. Real-Time Polymerase Chain Reaction**

RT-PCR analysis was carried out to measure the changes in the expression of NKX2.5 and CTNT by differentiated cells compared with mesenchymal stem cells as control cells. In this method, the fluorescence intensity curve versus the number of cycles shows a linear relationship in the logarithmic phase in PCR, through which it is possible to compare the DNA/cDNA product with the internal standard. The expression of the NKX2.5 and C-TNT was tested using the Real-time PCR. Samples were compared based on the expression of GAPDH gene (Yang et al., 2020).

**2. 6. Immunofluorescence**

We performed immunocytochemistry technique to confirm the differentiation of mesenchymal stem cell to cardiomyocyte. Mesenchymal stem cells were used as control and the cells was fixed with 4% formaldehyde for 20 min at 4˚C and washed with PBS. In this staining, 5% Tritone X-100 and the primary antibodies of connexin43, hematoxin, and PBS were used. Then, the cells were placed in a refrigerator, set at 2˚C to 8˚C overnight, and washed with PBS. The secondary antibodies were added and incubated for 1h at 37˚C and for 30 min in a dark room. After that, DAPI was used and checked by a fluorescence microscope (Olympus BX51, Japan).

**2. 7. Statistical analysis**

Data have been expressed as mean values ± standard error. SPSS v. 23 software (SPSS Ins, Chicago, IL, USA) was used to determine the statistically significance differences between groups. Multiple groups were analyzed by one-way ANOVA. All experiments were performed at least in triplicates and $p<0.05$ was considered significant in comparison to the control group.

# 3 Results and Discussions

**3. 1. Cell Morphology**

MSCs acted as adherent cells and they fixed to the floor of the cell flask. These cells were spindle shaped. As well, they had a large and rounded nucleus with long and short cellular debris (Fig. 1).

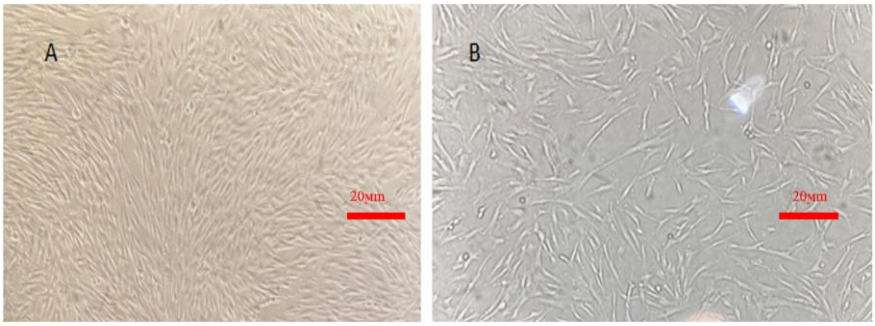


Figure 1. (A) Characterization of control cell or mesenchymal stem cells (MSCs) in passage 4, (B) morphology of MSCs in passage 2 (magnification, 40×).

### 3. 2. Characterization of cultured hAM-MSC

Results showed that amniotic MSCs can express the cluster of differentiation (CD) markers such as CD29 (98.4%), CD105 (100%), CD73 (99.3%), CD45 (0.27%) and CD3 (40.76%). The hAM-MSCs were confirmed during this experiment (Fig. 2).

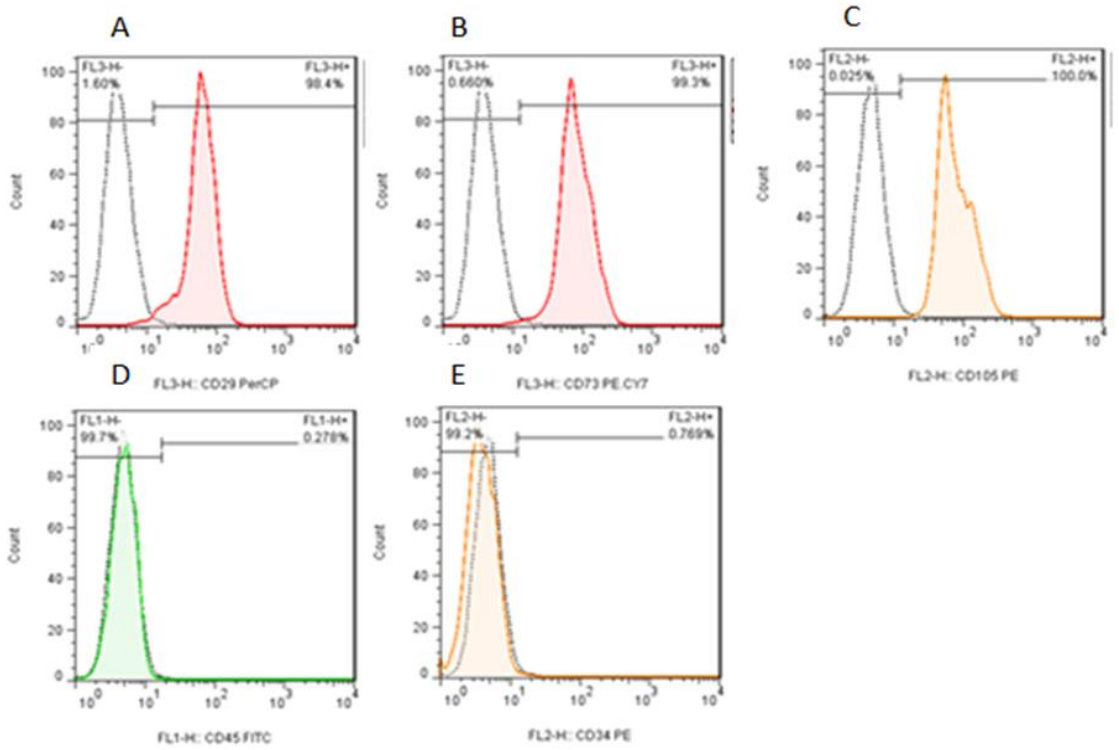


Figure 2. Flow cytometry characterization of human amniotic stem cells. CD marker analysis of mesenchymal stem cells (MSCs) in passages 3, (A) CD29 (98.4%), (B) CD73 (99.3%), (C) CD105 (100%), (D) CD45 and (E) CD34 were 0.27%, 0.76%.

### 3. 3. Differentiation of MSCs to osteoblasts and adipocytes

The red area in the osteoblast cell showed calcium accumulation and the red area in the adipocytes showed lipid vesicles (Fig. 3) demonstrating the differentiation of MSCs to osteoblasts and adipocytes, respectively.

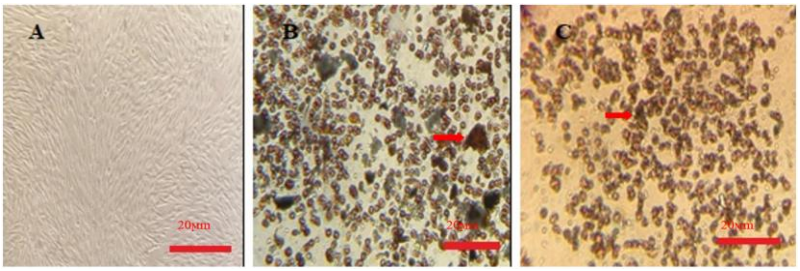


Figure 3. (A) Characterization of mesenchymal stem cells (MSCs) and differentiation of MSCs to (B) adipocytes stained by oil red O, and (C) osteoblasts stained by alizarin red. Scale bar 20µm, (magnification: 40×).

### 3. 4. Differentiation of MSCs to cardiomyocytes

Mesenchymal stem cells differentiated into myocardial cells using Chang differentiation medium (Fig 4). Immunocytochemistry was used to identify differentiated mesenchymal stem cells to the cardiomyocyte and to detect connexin-43. Connexin 43 (Cx43) is the major connexin protein in ventricular cardiomyocytes. Immunofluorescence showed differentiation of MSCs to cardiomyocytes using primary antibody to Gonnexin-43 with the positive reaction of 50% r (Fig. 5 and 6).

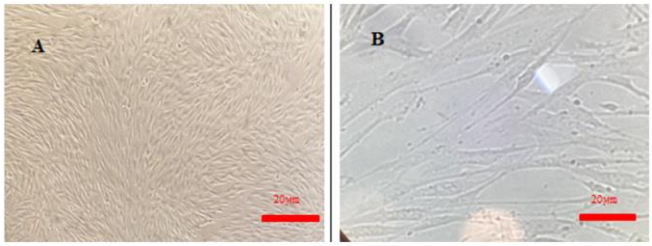


Figure 4. (A) The image was taken after 14 days of differentiation of mesenchymal stem cells using Chang medium, (B) mesenchymal stem cells in passage 2. Scale bar 20μm, (magnification: 40×).

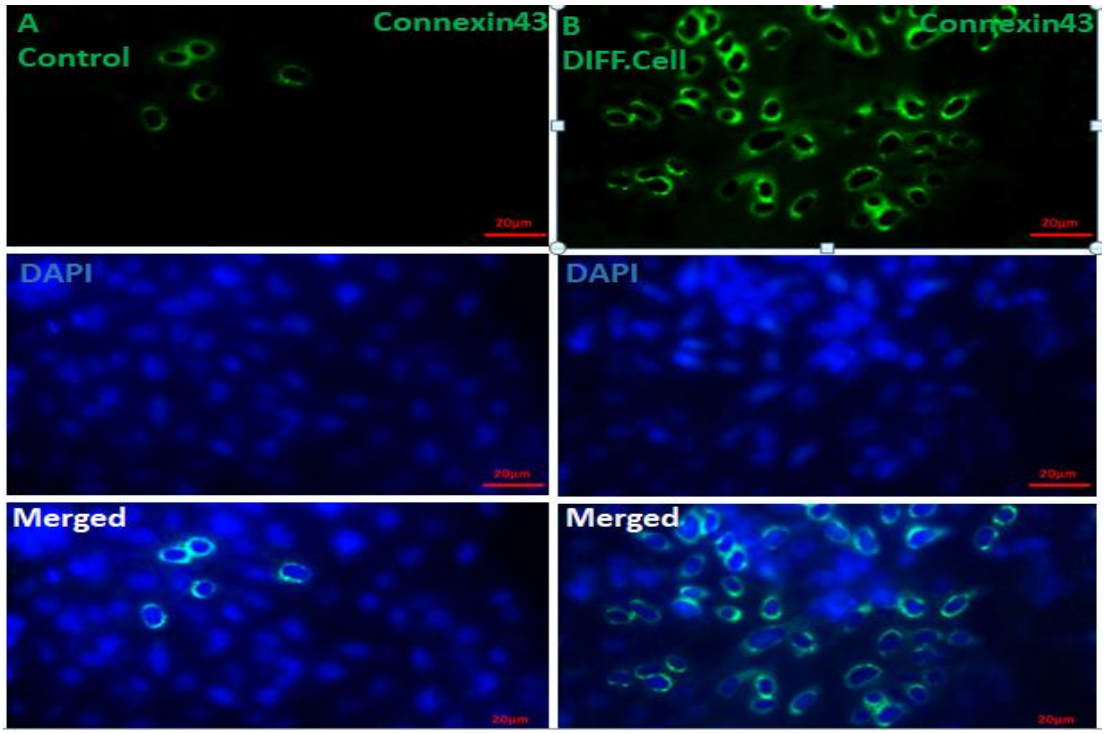


Figure 5. Differentiation of mesenchymal stem cells (MSCs) to cardiomyocytes. (A) MSCs as Control cell using primary antibody connexin-43 positive reaction= 8%, (B) differentiation of MSCs to cardiomyocytes using primary antibody connexin-43 positive reaction = 50%, (C) nuclei stained of MSCs by DAPI (D) nuclei stained of cardiomyocyte by DAPI, (E) merge of A and C.(F) merge of B and D. Scale bar 20 μm. (Magnification, 400×).

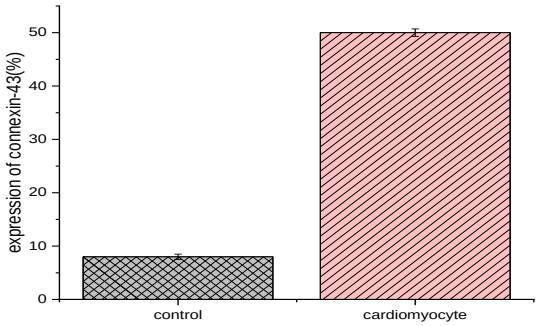


Figure 6. The expression level of Connexin-43 was assessed in the control group (MSCs)=8% and differentiated cells to cardiomyocyte=50%. The difference was significant (P≤0.05).

### 3. 5. Expression of NKX2.5 and C-TNT genes by RT-PCR

RT-PCR was applied to analyze the expression of NKX2.5 and C-TNT cardiac markers in differentiated and undifferentiated cells (Fig. 7). As shown in Fig. 7, expression level of the genes strikingly increased in cardiomyocyte cells compared to AM-MSCs. Approximately, transcript levels of NKX2.5 and C-TNT in cardiomyocyte cells were 5 and 6.5 times higher than that in AM-MSCs.

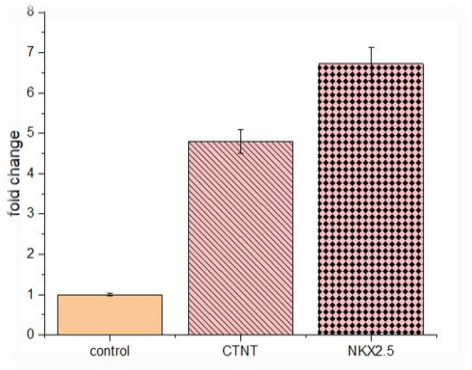


Figure 7. The expression level of C-TNT and NKX2.5 in cardiomyocyte cells compared to mesenchymal stem (control) cells. The difference was significant compared to control group (P≤0.05).

Currently, great advances have been made in the field of cardiac diseases and stem cell transplantation-based therapies have emerged as a promising therapeutic tool for improving cardiac function and regeneration. Mesenchymal stem cells have been used for the treatment or restoration of damaged tissue. These cells are multipotent, which have self-renewal ability and can differentiate into other tissues. AM-MSCs have unique immunological properties and don't decrease in the capacity and the amount of MSCs with increase in age despite other sources and also because of the greater self-renewal ability and differentiation of MSCs from this source compared to others, this cell is proper for the study of cell therapy ( Cui et al., 2015). MSCs are able to reduce the scar size of heart and improve heart function. MSCs perform these functions by stimulating of endogenous repair mechanisms, along with regulating of immune responses, tissue perfusion, inhibition of fibrosis and proliferation of damage heart cells (Bagno et al., 2018; Li et al., 2021). Numerous researches have represented that special surface antigens, such as

CD29, CD44, CD90, and CD105, detected on MSCs that originate from umbilical cord blood, bone marrow and adipose tissue. In this study, flow cytometry results and surface CD markers confirmed the mesenchymal homogenous cells isolated from the amniotic membrane tissue. Our result showed that the tested cells were positive for MSCs markers, such as CD 105, CD73, and CD 29. On the other hand, tested cells were negative for HSCs markers, such as CD34 .

Nkx2–5 homeobox transcription factor is essential for early heart development. The transcription factor is responsible for the activation of variety of specific genes of heart and is essential for migratory cardiac mesoderm (Markmee et al., 2020). With NKX2.5 in steering, germ layer plays a critical role within the development of viscus tissue and activates the assembly of several cardiac transcription factors (especially GATA, T-Box, and Mef2) (Dobrzycki et al., 2020). The C-TNT gene (150bp) is used to measure troponin in the kidneys and heart. Examination of stroke areas can be estimated based on troponin measurements taken within 72 h (Markmee et al., 2020). In addition, GATA4 and NKX2.5 are the greatest transcription factors that have major regulatory roles in the cardiogenic differentiation of MSCs.

In this study, mesenchymal stem cells differentiated into myocardial cells using Chang differentiation medium, and Connexin-43 was dtected by immunocytochemistry method. Connexins are the primary components of gap junctions, providing direct links between cells under many physiological processes (Soltani and Mahdavi, 2021). Connexin-43 is a protein coded in humans using the GJA11 gene on chromosome 6. Gap junction is an intercellular channel that connects adjacent cells to the exchange molecules of low relative molecular mass, like small ions and secondary messages, and is employed to keep up homeostasis of the heart. It plays a key role within the heart (Atala et al., 2006; Falleni et al., 2021). Also, our results showed that the expression level of NKX2.5 and C-TNT genes noticeably increased in cardiomyocyte cells compared to AM-MSCs. In this regard, Abou-ElNaga et al. studied the differentiation of mesenchymal stem cells to cardiomyocyte with 5-azacytidine medium. They reported that gene expression of NKX2.5 and TNNT1 increased in cardiomyocyte cells than control group (Abou-ElNaga et al., 2020). Their study detected also the progress of myocardial differentiation of MSCs during the expression of these genes at the RNA level to conclude the best differential protocol in a time-dependent manner (one and three weeks). However protocols of differentiation confirmed a considerably higher expression of these cardiac-specific genes in comparison with undifferentiated cells. TNNT1 expression increased gradually by time, whereas NKX2.5 is an early marker that elevated in the first week and peaked after three weeks from the starting of induction (Abou-ElNaga et al., 2020). NKX2.5 is highly expressed in early heart progenitor cells as they assign to the cardiac lineage during embryogenesis, where it continues to be expressed in the heart throughout adulthood. Indeed, humans with mutations in Nkx2.5 have congenital abnormalities characterized by aberrant ventricular septation and atrioventricular node and conduction anomalies (Ruan et al., 2019). Also, Ruan et al reported that overexpression of Nkx2.5 significantly promotes the differentiation of human umbilical cord drived mesenchymal stem cells into cardiomyocytes and increases the expression of cTnI, Desmin and GATA-4 (Ruan et al., 2016). Li et al. investigated differentiated mesenchymal stem cells to cardiomyocyte in four groups, 1 control, 2 with FGF2, 3 Lenti-FGF-2-GFP lentivirus transfection, and 4 Lenti-control-GFP lentiviral transfers using Real-time PCR, immunocytochemical staining, immunofluorescence staining, and Western blot. Expression

levels of GATA-4 and Nkx2.5 genes in Lenti-FGF-2-GFP were higher than other groups. The expressions of cTnI, cTnT, Cx43, and Desmin were detected by immunocytochemical staining and immunofluorescence staining showing that the expression of this protein significantly increased in Lenti-FGF-2-GFP groups. The results in this study also indicated a strong correlation with the early evaluation of cardiogenic differentiation of AM-MSCs and corresponded with previous studies, which reported that AM-MSCs can be extended *in vitro* and can be differentiated into cardiomyocytes with a strong expression of cardiac specific genes (Li et al., 2021).

## 4 Conclusion

Many researchers have been done to investigate the efficient therapy of cardiac diseases, and stem cells transplanting has been a promising therapeutic strategy. Stem cells could be induced into cardiomyocytes, and then migrated to damaged location to play the therapeutic effect. Among different types of stem cells, MSCs are a strong candidate to be used by regenerative medicineand to investigate cellular differentiation. This result can be critical in treatment by cell treatment in the future. Be that as it may, this field is comprehensive and needs more inquire about.

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## Conflict of interests

The authors declare that they have no conflicts of interests regarding the publication of this paper.

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