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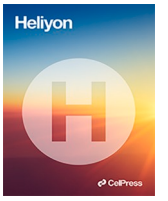

Research article

Assessment of the feasibility of human amniotic membrane stem cell-derived cardiomyocytes in vitro

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A B S T R A C T

Myocardial infarction (MI) is a leading cause of death worldwide, resulting in extensive loss of cardiomyocytes and subsequent heart failure. Inducing cardiac differentiation of stem cells is a potential approach for myocardial regeneration therapy to improve post-MI prognosis. Mesenchymal stem cells (MSCs) have several advantages, including immune privilege and multipotent differentiation potential. This study aimed to explore the feasibility of chemically inducing human amniotic membrane MSCs (hAMSCs) to differentiate into cardiomyocytes in vitro. Human amniotic membrane (AM) samples were obtained from routine cesarean sections at Far Eastern Memorial Hospital. The isolated cells exhibited spindle-shaped morphology and expressed surface antigens CD73, CD90, CD105, and CD44, while lacking expression of CD19, CD11b, CD19, CD45, and HLA-DR. The SSEA-1, SSEA-3, and SSEA-4 markers were also positive, and the cells displayed the ability for tri-lineage differentiation into adipocytes, chondrocytes, and osteoblasts. The expression levels of MLC2v, Nkx2.5, and MyoD were analyzed using qPCR after applying various protocols for chemical induction, including BMP4, ActivinA, 5-azacytidine, CHIR99021, and IWP2 on hAMSCs. The group treated with 5 ng/ml BMP4, 10 ng/ml Activin A, 10 μM 5-azacytidine, 7.5 μM CHIR99021, and 5 μM IWP 2 expressed the highest levels of these genes. Furthermore, immunofluorescence staining demonstrated the expression of α-actinin and Troponin T in this group. In conclusion, this study demonstrated that hAMSCs can be chemically induced to differentiate into cardiomyocyte-like cells in vitro. However, to improve the functionality of the differentiated cells, further investigation of inductive protocols and regimens is needed.

1. Introduction

Myocardial infarction (MI) is a major global cause of mortality, resulting in the permanent loss of cardiomyocytes [1]. Despite advances in medical therapy and mechanical reperfusion, MI still poses a significant challenge to public health due to the extensive loss of myocardial tissue and subsequent heart failure that often follow. These outcomes persist even with contemporary treatments, highlighting the need for further research and innovative therapeutic strategies to address the impact of MI.

In recent years, research has demonstrated the potential of stem cells in myocardial regeneration therapy following MI [2–4]. While pluripotent stem cells possess high differentiation abilities, they are often associated with tumorigenicity and bioethical concerns. In contrast, multipotent mesenchymal stem cells (MSCs) are emerging as popular candidates for cell therapy due to their low immunogenicity and tumorigenicity [5,6]. Numerous studies have highlighted the therapeutic potential of MSCs in promoting myocardial regeneration and reducing the risk of heart failure in post-MI patients [7].

hAMSCs are promising source of MSCs that can be harvested from the AM during daily cesarean sections. As medical waste, the AM

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provides an abundant and ethically uncontroversial source of MSCs. Previous studies have shown that hAMSCs express some pluripotent markers such as Oct-4, SSEA-3, and SSEA-4, and have high telomerase activity and long telomeres [8,9]. Moreover, hAMSCs have been shown to have low HLA-A,B,C expression levels, which makes them an ideal candidate for allogeneic transplantation, and they possess the ability to differentiate into various cell types [10,11]. These characteristics make hAMSCs a promising candidate for stem cell-based therapy for myocardial regeneration following MI.

The Wnt pathway plays a biphasic role in cardiac specification, promoting cardiogenesis in the early stage but inhibiting it in the late stage [12]. Recent studies have demonstrated that chemical modulation of the Wnt pathway is an effective strategy for inducing cardiomyogenic differentiation of pluripotent or multipotent stem cells. Commonly used chemicals for this purpose include CHIR99021 and IWP, which are Wnt pathway modulators. They succeeded to induce human iPSCs to differentiate into cardiomyocytes which possessed electrophysiological activities [13,14]. BMP4 and Activin A, members of the transforming growth factor β (TGF-β) superfamily, have also been shown to play a role in cardiogenesis and are implicated in the cardiac differentiation of stem cells in previous studies [15–18]. 5-azacytidine (5-aza), a nucleotide-like chemical, inhibits DNA methylation and has been used to induce cardiac differentiation of MSCs [19–22]. Collectively, these findings suggest that the Wnt pathway and TGF-β superfamily members, in addition to 5-aza, represent promising targets for improving the efficiency of cardiac differentiation in stem cells.

This study aims to investigate the feasibility of inducing cardiac differentiation in hAMSCs using a combination of chemicals, including 5-AZA, CHIR99021, IWP2, BMP4, and Activin A. The results of this study may shed light on the potential of these chemical inducers for the development of stem cell-based therapies for myocardial regeneration following MI.

2. Material and methods

2.1. Isolation and expansion of hAMSCs

The study was conducted with approval from the institutional review board of Far Eastern Memorial Hospital (certificate number: FEMH-106163-F). Prior informed consent was obtained from all tissue donors to ensure they understood the study's purpose and potential implications.

To isolate hAMSCs, human amnions from full-term births were collected immediately after Cesarean section. The amnions were washed and stored in 1x sterile HBSS (Gibco™, 14175095). The membrane was incubated in 0.05% (w/v) trypsin/EDTA at 37 °C for 1 h. The remaining tissue was transferred to digestion buffer and incubated on a rotor for 1 h at 37 °C. The resulting solution was centrifuged at 200×*g* for 5 min at 4 °C, and the resulting pellet was resuspended and plated at a density of up to 1×10^5 cells per cm^2.

hAMSCs were cultured in EFD medium at 37 °C in a humid air environment containing 5% CO2. EFD medium is composed of high glucose DMEM (Sigma, D5648), 1 × Sodium Pyruvate (Gibco™, 11360070), 3.7 g/L sodium bicarbonate (Sigma, S5761), 20% bovine fetal serum (Merck, TMS-013-BKR), 1 mM NEAA (Hyclone, AE29434713), 2 mM GlutaGO (Simply, CC516-0100), 1 × Antibiotic-Antimycotic (Gibco™, 15240062), 0.1 mM beta-mercaptoethanol (Gibco™, 21,985,023), and 10 ng/ml hEGF (Sigma, E9644). The medium was changed twice a week, and the cells were subcultured when they reached 80% confluency. The EFD medium was stored at 4 °C and warmed to 37 °C in a water bath before use.

2.2. Immunocytochemistry

To prepare the cells for immunofluorescence staining, hAMSCs were washed with 1x DPBS (Sigma, D5773) and fixed with 4% paraformaldehyde (EMPROVE®, Germany, 1,040,051,000) for 5 min at 4 °C. The cells were then washed again with 1x DPBS and permeabilized with 0.1% Triton-X100 in 1x DPBS for 5 min at 4 °C. The following primary antibodies were used for staining: anti-CD44 (1:50), anti-CD73 (1:200), anti-CD90 (1:100), anti-CD105 (1:100), anti-CD34 (1:250), anti-CD11b (1:250), anti-CD19 (1:250), anti-CD45 (1:250), and anti-HLA-DR (1:250) (Invitrogen™). Differentiating cells were stained with Sarcomeric alpha actinin monoclonal antibody (EA-53) (Invitrogen, MAI-22863, 1:500) and Anti-Cardiac Troponin T (abcam, ab8295, 1:1000) primary antibodies, Goat Anti-Mouse IgG H&L secondary antibody (abcam, ab150113, 1:1000), and DAPI (Biotium, 40043, 1:1000) to visualize nuclei. The antibodies were diluted in PBS containing 1% bovine serum albumin (Sigma, Germany, A2153) and cells were incubated with the primary antibodies overnight at 4 °C. The cells were then washed with PBS. To observe the results of immunofluorescence staining in cardiac genes, we used Leica DM2500 Optical Microscope (Leica Microsystems, Germany) equipped with a Canon EOS 7D camera (Canon, Japan). As part of the cardiac gene expression analysis, human cardiomyocytes (Promocell, C-12810) were used as positive control.

2.3. Flow cytometric analysis

We observed the results of immunofluorescence staining in MSC surface antigens through Flow cytometric analysis, which was conducted on the BD FACSAria III cytometer (BD Biosciences, USA). Data were analyzed using BD FACSDiva™ Software (BD Biosciences, USA).

2.4. Trilineage differentiation of hAMSCs

To induce trilineage differentiation, hAMSCs (cell passage = 4) were cultured using the StemPro™ Adipogenesis Differentiation Kit, StemPro™ Chondrogenesis Differentiation Kit, and StemPro™ Osteogenesis Differentiation Kit (Gibco™, USA, A1007001,

A1007101, A1007201). The cells were induced and cultured for 14, 21, and 28 days according to the manufacturer's instructions. After the differentiation period, the cells were stained with Oil Red O for adipogenic differentiation, Alcian Blue for chondrogenic differentiation, and Alizarin Red for osteogenic differentiation. We observed the results with the Leica DM2500 Optical Microscope (Leica Microsystems, Germany) equipped with a Canon EOS 7D camera (Canon, Japan).

2.5. RNA extraction and reverse transcription

Total RNA was extracted from hAMSCs by washing the cells with 1x DPBS and adding 1 mL Trizol (Geneaid, GZR100). The solution was transferred into a 1.5 mL tube after the cells were dissolved, followed by the addition of 100 μL 1-Bromo-3-chloropropane (Sigma, B9673). The tube was incubated at room temperature for 15 min, and then centrifuged for 15 min at 16,000×*g* and 4 °C. The supernatant was transferred into another 1.5 mL tube with an equal volume of isopropanol (Supelco, 109634) and incubated for 10 min at room temperature. After centrifugation for 8 min at 16,000×*g* and 4 °C, the supernatant was removed, and the pellet was washed three times with 75% ethanol. The pellet was then air-dried and dissolved in molecular biology grade water. RNA quality and concentration were assessed using a Nanodrop instrument. For reverse transcription, the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, 4,368,814) was used.

2.6. Detection of pluripotent genes in hAMSCs

To detect the expression of Oct-4, Nanog, and Rex1 (Table 1) in hAMSCs (cell passage = 3), 25 ng of cDNA was mixed with 10 μM of primers and 1x PowerAmp 2X PCRmix-Green (Bioman, RT008G), and PCR amplification was carried out using a PCR machine. The PCR program started with a 3-min denaturation step at 94 °C, followed by 45 cycles of amplification consisting of 30 s at 94 °C, 30 s at 57 °C, and 1 min at 72 °C. The reaction was terminated with a final extension for 1 min at 72 °C.

2.7. Agarose gel electrophoresis

The electrophoresis of PCR products were performed on a 1.5% agarose gel and stained with ethidium bromide at 100V, 40 min.

2.8. Cardiac differentiation

On day 0, passage 2 hAMSCs were seeded at a density of 3×10^4 cells/m^2 in 6-well plates. The culture medium was then changed to EFD medium supplemented with 10 μM 5-AZA (Sigma, A2385), 7.5 μM CHIR99021 (Sigma, SML1046), 10 ng/mL Activin A, 5 ng/mL BMP4, and 5 μM IWP2 (Sigma, 5.06072) on day 0 to Day 5, and with only 5 μM IWP2 on days 6 to Day 9 (Fig. 1). After day 9, the culture medium was changed to EFD medium without any supplements twice a week, and cells were cultured until day 17. To investigate the effects of each supplement, the experiments were divided into 16 groups. BMP4 and Activin A were combined as one condition to reduce the number of groups. The corresponding groups and the added chemicals are listed in Table 2. All supplements were stored at −20 °C, and 5-AZA (100 mM), CHIR99021 (12 mM), and IWP2 (10 mM) were dissolved in DMSO. BMP4 and Activin A were dissolved in 1x DPBS and diluted with EFD medium before use.

2.9. qPCR of cardiac gene expression

In this study, the expression levels of MLC2v, Nkx2.5, and MyoD were detected in differentiating cells with primers list in Table 1, and β-actin was used as an internal control. For qPCR analysis, SYBR green, primers (10 μM), cDNA, and molecular biology grade water were mixed at a ratio of 10:6:1:3. The qPCR program was performed using the Fast SYBRTM Green Master Mix (Applied Biosystems, 4,385,612). qPCR was conducted and the Ct value were calculated by QuantStudio 3 (ThermoFisher, USA).

Table 1
The primer sequences.

	Forward (5'→3')	Reverse (5'→3')	Length (bp)
hOCT4 [26]	GAGGAGTCCCAGGACATGAA	GTGGTCTGGCTGAACACCTT	149
hNanog [26]	ACATGCAACCTGAAGACGTGTG	CATGGAAACCAGAACACGTGG	144
hREX1 [27]	TGAAAGCCCACATCCTAACG	CAAGCTATCCTCCTGCTTTGG	554
hREX1 (qPCR)	CGCAATCGCTTGTCCTCAGAGT	GCTCTCAACGAACGCTTTCCCA	130
hTERT [26]	AGAGTGTCTGGAGCAAGTTGC	CGTAGTCCATGTTCACAATCG	183
hβ-actin [26]	CGCACCACTGGCATTGTCAT	TTCTCCTTGATGTCACGCAC	206
MLC2v	TGAGAGACACCTTTGCTGCC	GGGTCCGCTCCCTTAAGTTT	139
Nkx2.5	CCTTCACCGGCCAAGTGTG	AATTTCAGGCTTTCTTTTCGGC	115
MyoD	GCCACAACGGACGACTTCTA	AGTGCTCTTCGGGTTTCAGG	118
β-actin	CGCCGCCAGCTCACC	CACGATGGAGGGGAAGACG	120

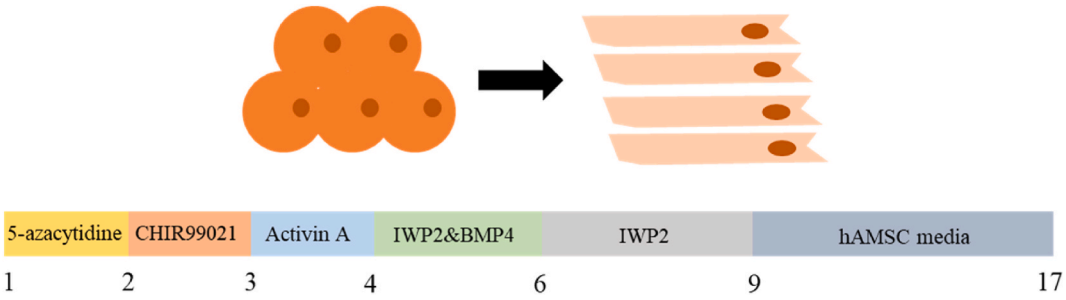

Fig. 1. The schedule of cardiac differentiation in hAMSCs.

Table 2
The groups correspond to the adding of chemicals.

Group No.	BMP4&ActivinA	5-azacytidine	CHIR99021	IWP2
1	–	–	–	–
2	–	+	–	–
3	–	–	+	–
4	–	–	–	+
5	–	+	+	–
6	–	+	–	+
7	–	–	+	+
8	–	+	+	+
9	+	–	–	–
10	+	+	–	–
11	+	–	+	–
12	+	–	–	+
13	+	+	+	–
14	+	+	–	+
15	+	–	+	+
16	+	+	+	+

2.10. Statistical analysis

All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, USA). Data are presented as mean ± standard error of the mean (SEM). $p < 0.05$ (calculated using a one-way ANOVA with Tukey's post hoc test) was considered statistically significant.

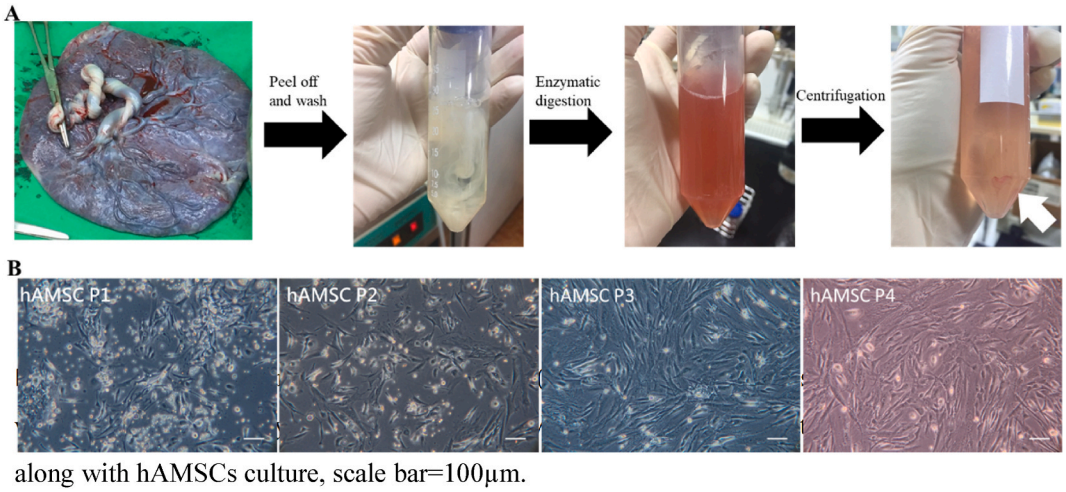

Fig. 2. The isolation and culture of hAMSCs. (A) The isolation process of hAMSCs. AM were digested by 10X trypsin and collagenase IV (B) Cell morphology at different passage along with hAMSCs culture, scale bar = 100 μm.

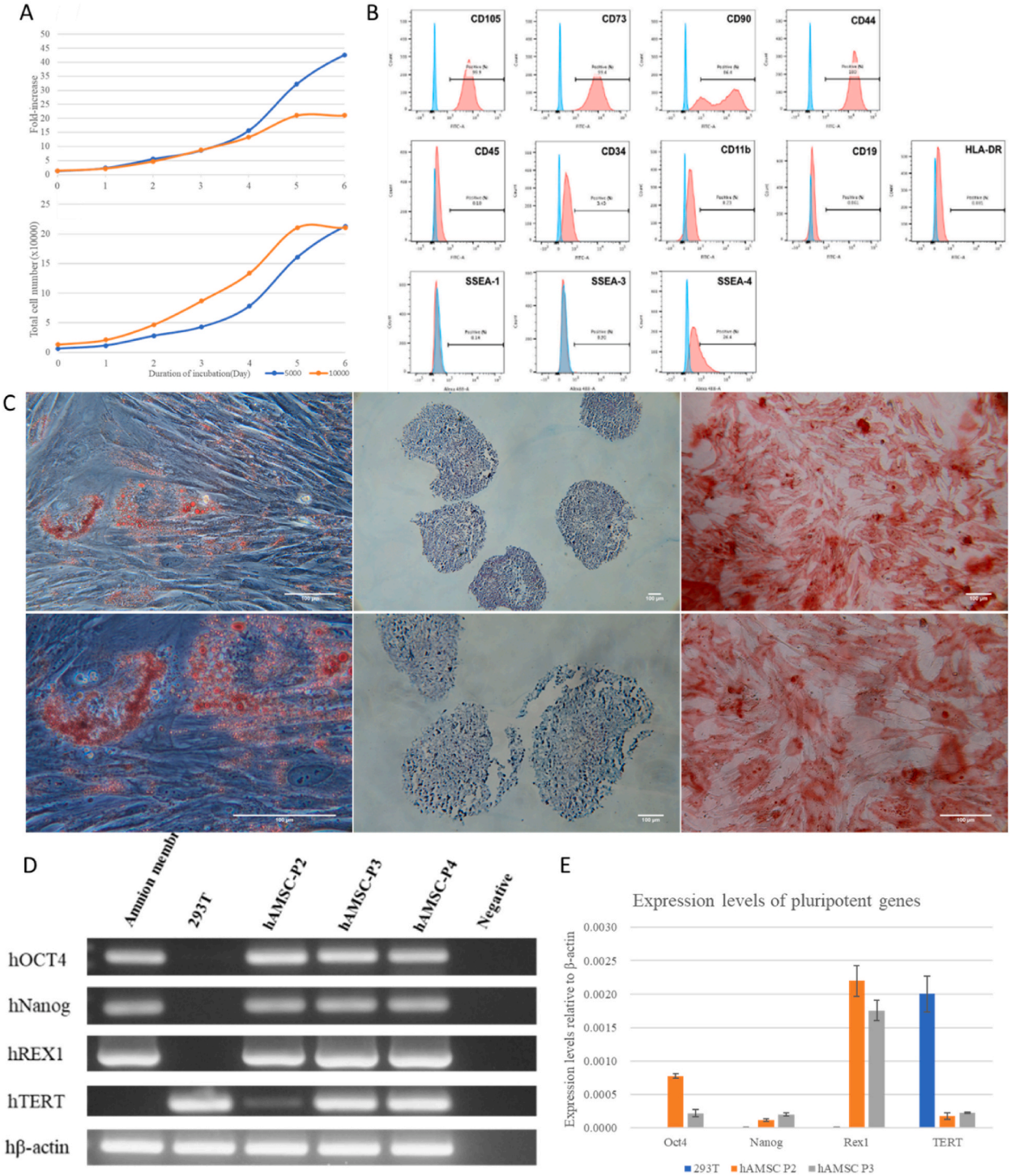

Fig. 3. The characterization of hAMSCs (A) The cell proliferation curve in total cell number and fold-increase (B) The surface antigens of isolated hAMSCs (C) Trilineage differentiation in adipocytes, chondrocytes and osteoblast, scale bar = 100 μm (D) (E)The pluripotent gene expression of hAMSCs, including hOCT4, hNanog, hREX1, hTERT. hβ-actin was used as an internal control. In this experiment, we utilized AM tissue, the 293T cell line, hAMSC passages 2 to 4, and dd water as a negative control for the samples.

3. Results

3.1. Isolation and characterization of hAMSCs

The protocol outlined in Fig. 2A was successful in isolating primary culture hAMSCs from the AM. Briefly, we peeled off the AM from placenta, and cut it into small pieces. Then, we digested the AM by trypsin and Collagenase, and collected the cell pellet. Finally, derived hAMSCs exhibited stable proliferation with a spindle-shaped morphology (Fig. 2B). To verify the cellular identity of our derived hAMSCs, the characterization experiments of these hAMSCs included a cell proliferation assay using CCK-8, surface antigen analysis, pluripotent gene expression assay, and trilineage differentiation were conducted. Firstly, in the cell CCK8 proliferation assay,

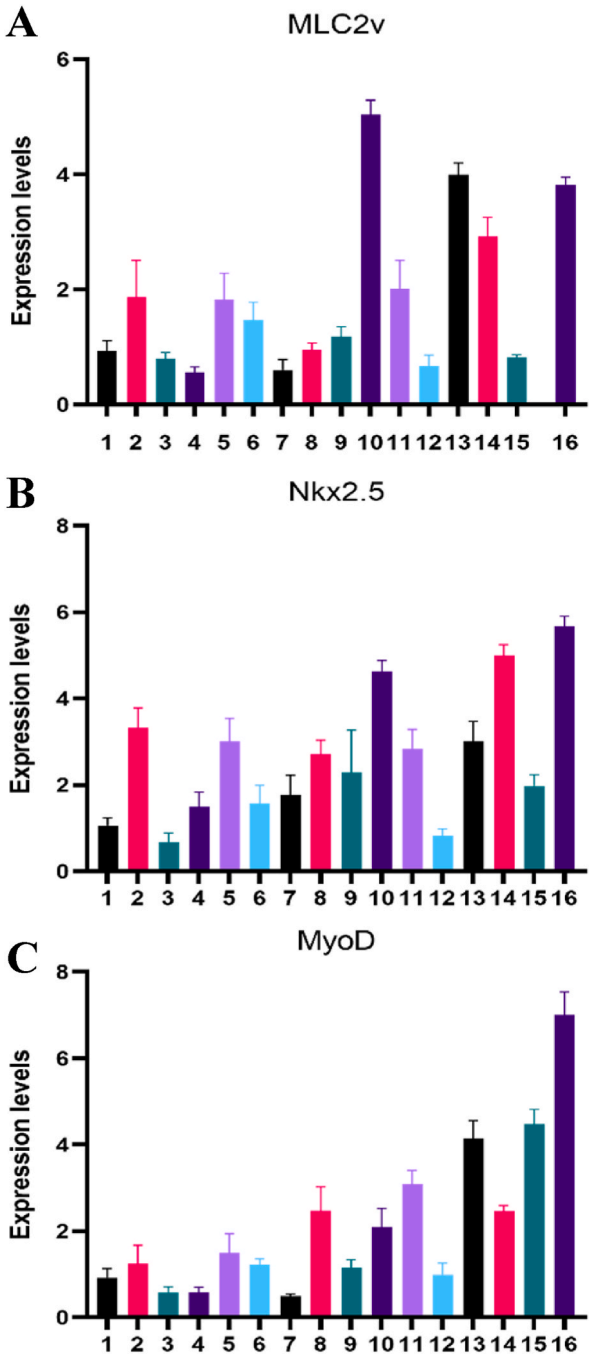

Fig. 4. The expression levels of cardiomyocyte-related genes. (A) MLC2v (B)Nkx2.5 (C) MyoD.

$5 \times 10^3$and 1×10^4 hAMSCs(cell passage = 3) in 500 μL medium were seeded in 24-well plate. When 24 h after seeding, 50 μL CCK-8 was added and incubated for 3 h. Then, we measured the absorbance at 450 nm by a microplate reader. The results indicated an approximately eightfold increase in the number of hAMSCs within three days. Furthermore, this cell proliferation assay demonstrated that the isolated hAMSCs grew quickly and steadily. (Fig. 3A). To identify the isolating cells as MSCs, we performed immunocytochemistry staining to screen the expression of cell markers of varied cell types with corresponding antibodies, flow cytometric analysis was conducted following the immunocytochemistry staining for result quantification. Analysis of surface antigens of MSCs by Flow cytometry showed that these hAMSCs (cell passage = 4) expressed positive markers for MSCs, including CD105, CD90, CD73, and CD44, while lacking expression of hematopoietic cell markers such as CD11b, CD34, CD45, CD19, and HLA-DR. The expression patterns of the isolating hAMSCs in this study presented a similarity to original MSCs. It meant that we succeed in isolating hAMSCs from AM. Furthermore, the hAMSCs were found to express SSEA-1, SSEA-3, and SSEA-4 (Fig. 3B). Passage 4 hAMSCs were able to

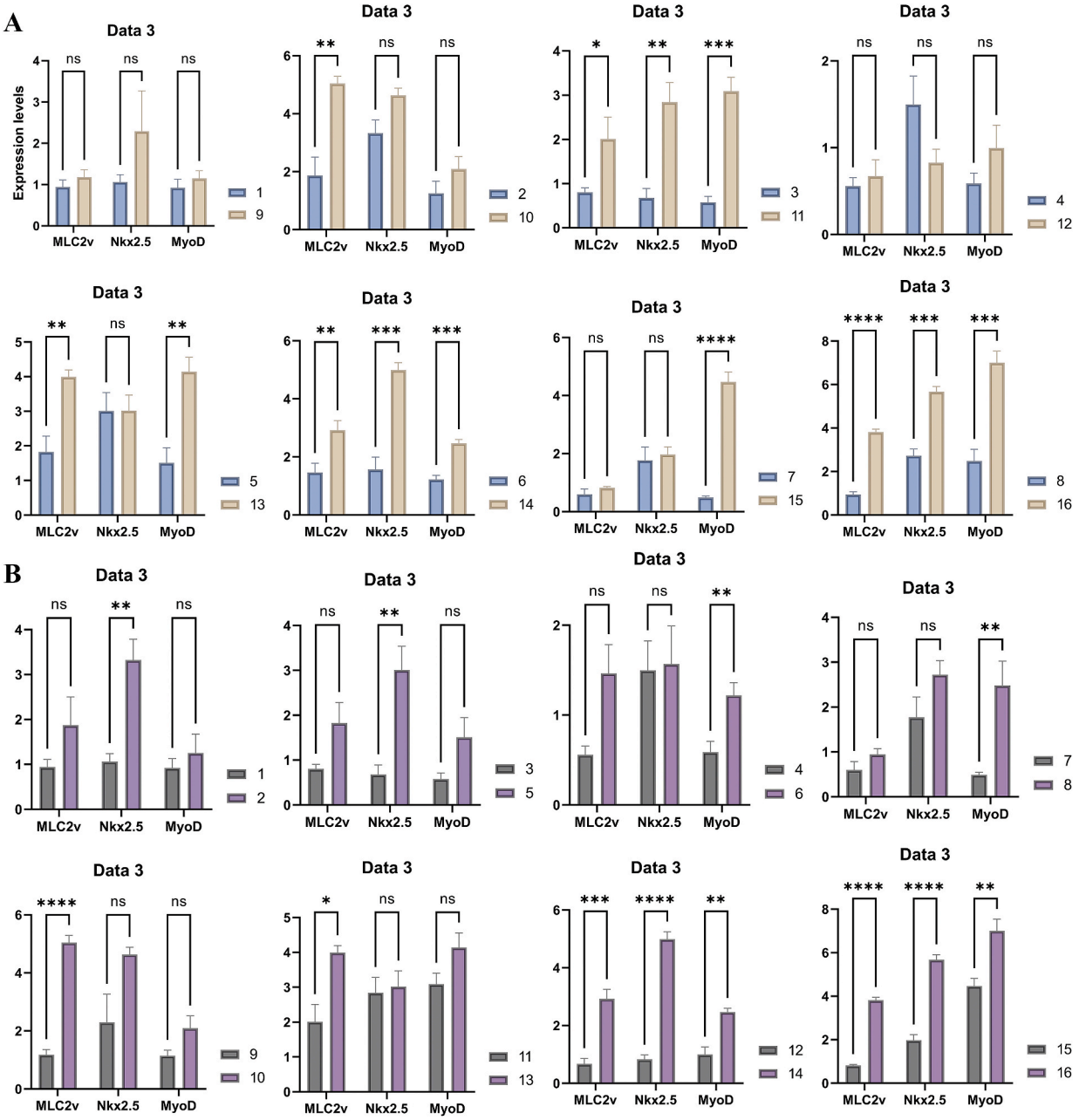

Fig. 5. The difference of the expression levels of cardiomyocyte-related genes between the groups with or without (A)BMP4 and Activin A (B)5-azacytidine (C)CHIR99021 (D)IWP2. All data are mean ± SEM.*P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001 (one-way ANOVA with LSD test; n = 3).

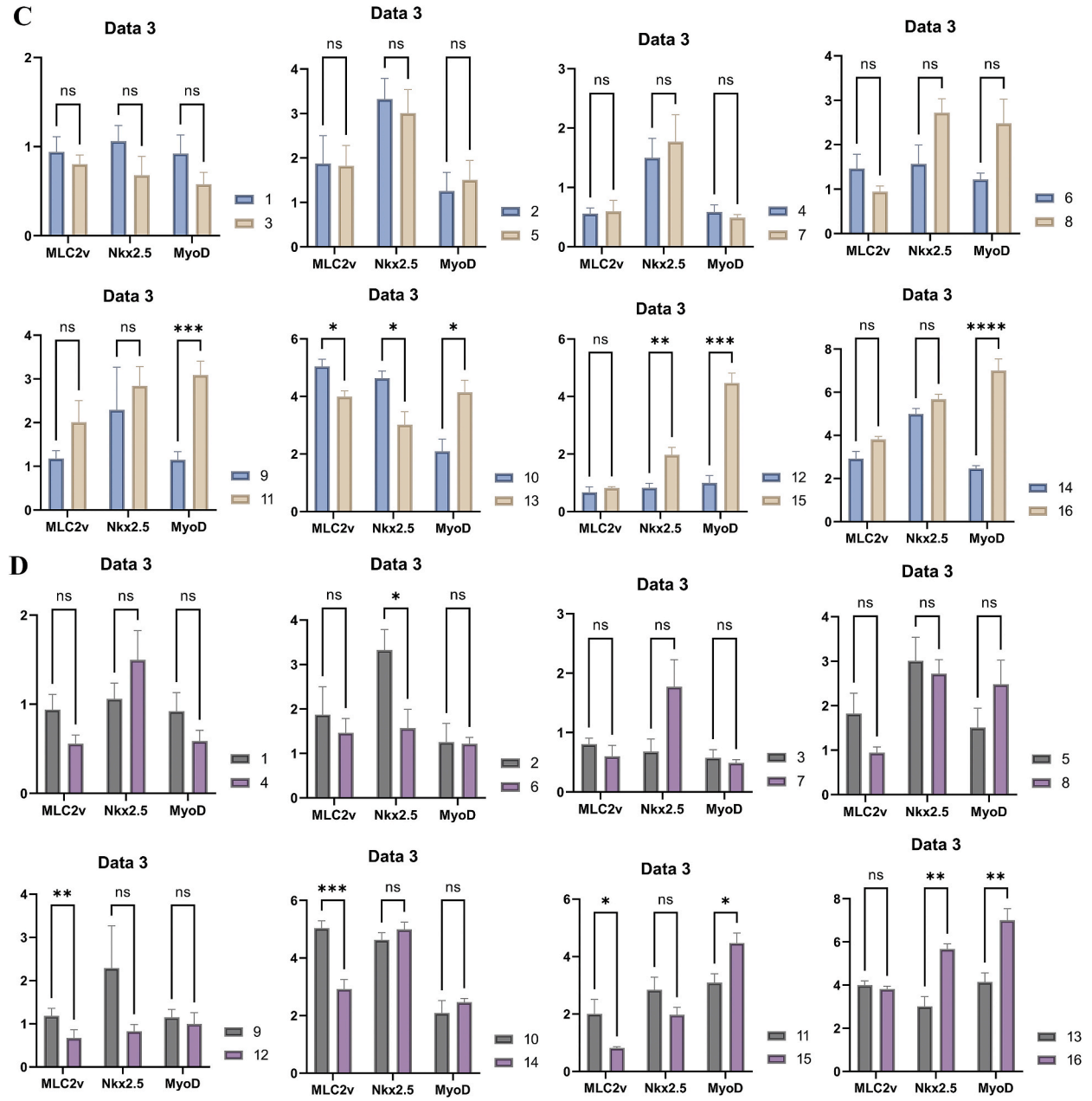

Fig. 5. (*continued*).

differentiate into adipocytes, chondrocytes, and osteocytes (Fig. 3C). Although untreated cells were not shown within the image, we found no autonomous differentiation if no induction was involved in our culture condition. Furthermore, P2–P4 hAMSCs expressed Oct-4, Nanog, TERT, and Rex1, similar to the expression profile found in the amniotic membrane tissue (Fig. 3D–E). These results suggest that hAMSCs exhibit pluripotent characteristics to a certain extent and have the ability to proliferate rapidly, although they are not immortal. Taken together, these findings indicated that the isolation and culture of hAMSCs were successful in this study.

3.2. *Induction and characterization of hAMSC-derived cardiomyotcytes*

To achieve the goal of accessing the ability of hAMSCs (cell passage = 2) in cardiac differentiation, we conducted a differentiation induction assay. Five components have been used for inducing the cardiac differentiation in other stem cell types that were chosen for the induction assay in hAMSCs. After induction, we used Myosin light chain 2 (MLC2v; ventricular-specific marker of cardiac myocardium), Nkx2.5 (precursor cardiac cell marker) and MyoD (related to myogenic), these cardiomyocyte markers to examine the

differentiating condition of these cells. At the endpoint analysis (Day 17), Group 16 exhibited the highest expression levels of MLC2v and Nkx2.5, which meant cells in this group had the highest differentiating ratio. (Fig. 4). The qPCR results also allowed us to explore the effects of each inducing factor on cardiac differentiation. Further confirmation can be obtained using factorial design (Fig. 5). We observed that the addition of BMP4 and Activin A demonstrated the potential to enhance the expression levels of cardiac-related markers when compared to groups without these supplements (Fig. 5A). 5-AZA also exhibited the capability to elevate cardiac-related gene expression levels, particularly when combined with other chemicals (Fig. 5B). Although the impact of CHIR99021 was not immediately evident (Fig. 5C), it appeared to influence cardiac-related gene expression in the presence of BMP4 and Activin A. The interplay among these three factors remains unclear, but there may be underlying connections worth exploring. Notably, the contribution of IWP2 was not clearly discernible, and the results did not entirely align with previous research findings (Fig. 5D). Consequently, we propose the existence of a potential interaction between CHIR99021 and IWP2. It appears that solely modulating the Wnt pathway might not be sufficient for hAMSCs to differentiate into cardiomyocytes. Further investigation is warranted to elucidate the intricate relationships among these factors.

To further confirm the cardiac characteristics, immunostaining assays were performed on Group 16 using α-actinin and Troponin T. Secondary antibodies labeled with Alexa Fluor® 488 were employed to detect the primary antibodies for α-actinin and Troponin T. The presence of differentiated cells was confirmed through the observation of green fluorescent protein expression emanating from Alexa Fluor® 488. This outcome substantiates the acquisition of cardiac features by the cells in Group 16. (Fig. 6A). Additionally, the cell morphology differed between GFP-positive and GFP-negative cells. GFP-positive cells, which expressed α-actinin or Troponin T,

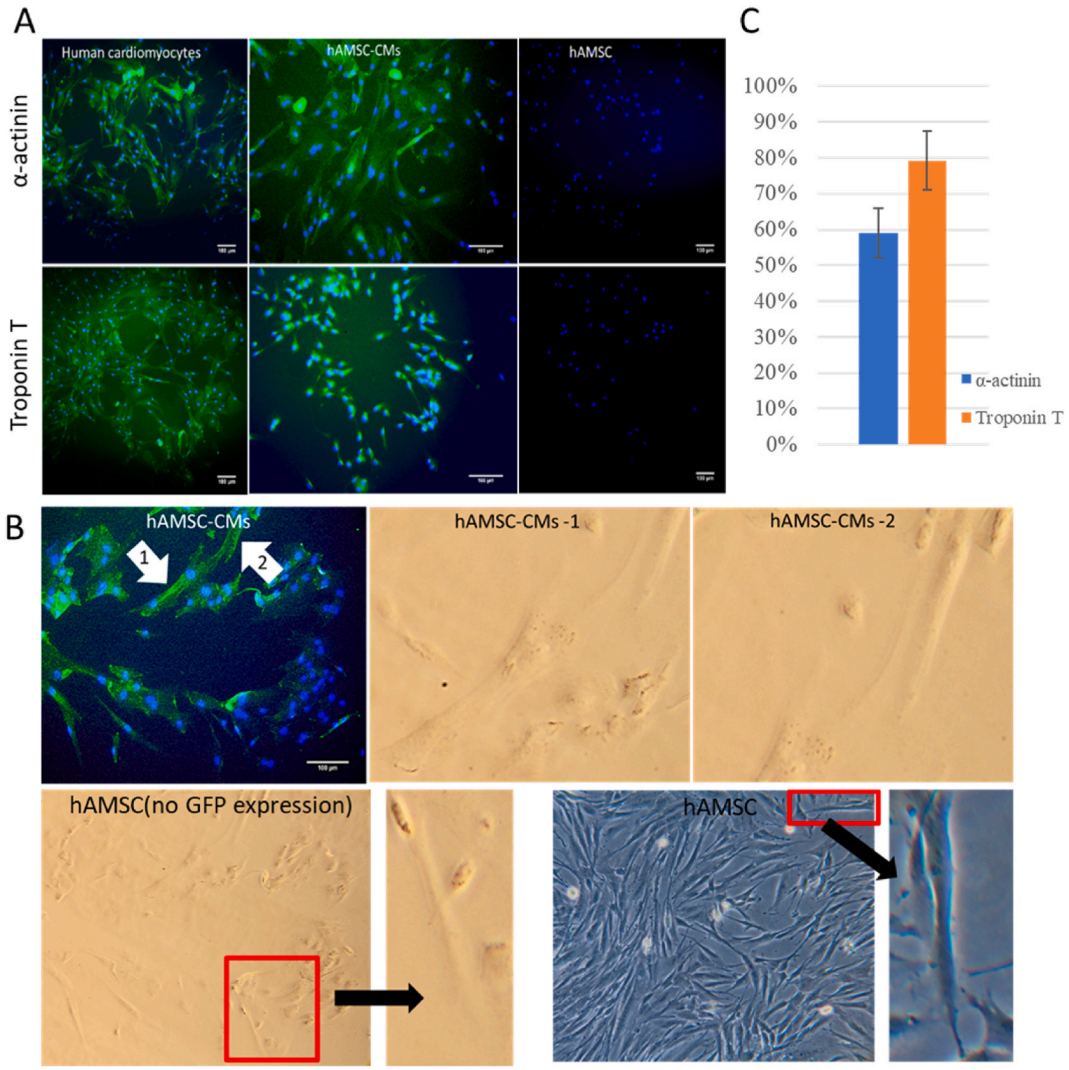

Fig. 6. (A)The Immunofluorescence staining of α-actinin and Troponin T in human cardiomyocytes, treated hAMSC and untreated hAMSC. Scale bar = 100 μm (B)The difference of cell morphology between cells with or without inductive differentiation. The differentiating cells exhibit a rod-shaped morphology, distinct from the spindle-shaped morphology of non-differentiating cells. (C)The percentage of cells expressing α-actinin and Troponin T after differentiation.

exhibited a rod-shaped morphology, whereas GFP-negative cells resembled untreated hAMSCs (Fig. 6B). These findings suggest that some cells in Group 16 had differentiated into cardiomyocyte-like cells. The average differentiation rates for α-actinin and Troponin T in the figure were manually calculated from immunofluorescence staining images, resulting in 64% and 79%, respectively (Fig. 6C)

4. Discussion

Recent studies have demonstrated the feasibility of differentiating human amniotic mesenchymal stem cells (hAMSCs) into cardiac cells, both in vitro and in vivo [1]. In vivo differentiation has been a popular research topic in the past decades, and these studies have shown that transplanting hAMSCs can effectively recondition heart function after MI by secreting paracrine factors. However, the long-term and immediate effects of these transplanted cells have yet to be fully evaluated, as they have only been traced for up to 4 weeks. Therefore, several studies have aimed to induce cardiac differentiation of hAMSCs in vitro, using strategies such as coculturing with xenogenic cardiomyocytes and treating with chemicals. Through coculture, hAMSCs have been shown to differentiate into cardiomyocytes, which can contract spontaneously [11].

While coculture with xenogenic cardiomyocytes has shown promise, there are concerns around biosafety and bioethics. As a result, many studies have explored alternative methods to induce differentiation of hAMSCs into cardiomyocytes, such as chemical treatments. These differentiated cells have shown the potential to not only compensate for lost cardiomyocytes, but also improve heart function through the secretion of paracrine factors, similar to transplanted hAMSCs. In addition to their therapeutic potential, these differentiated cells can also be used in transplantation, cardiac drug testing, and scientific research, making them a valuable tool in the field of regenerative medicine.

Although 5-aza has shown potential in inducing cardiac differentiation, the resulting differentiated cells have been found to be unfunctional in terms of electrophysiological activities. In contrast, CHIR and IWP2 have been shown to be effective in inducing cardiac differentiation of induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs). Also, 5-aza are widely used in in vitro cardiac differentiation of MSCs. Given that MSCs have lower differentiating potential than pluripotent stem cells, we initially attempted to induce differentiation of AMSCs using 5-aza, CHIR, and IWP2. However, these induced cells did not exhibit detectable cardiac-related markers. Subsequently, we turned to modify the differentiation protocol. Our experimental design was influenced by the observation that, despite numerous signaling pathways implicated in cardiac differentiation, limited noteworthy results have been reported in multipotent stem cells. Therefore, we decided to combine the Wnt, BMP pathways and 5-aza to enhance the differentiating efficiency in AMSCs. The results demonstrated that this chemical combination could induce the cardiac differentiation and all five chemicals played important roles in inducing cardiac differentiation in AMSCs. Moreover, we successfully demonstrated the in vitro feasibility of transforming cardiomyocytes from hAMSCs using readily available and cost-effective materials. Although it is true that some publications reported the cytotoxicity of 5-azacytidine, there is evidence demonstrated that its cytotoxicity may not always manifest under certain conditions. For example, nearly 100% cell viability was found after treated with 10 μM 5-azacytidine for 24 h [23], the same duration as our experiment. Also, observed that few cells died after 5-azacytidine treatment (10 μM, 24hr), but cells proliferated afterward [24,25]. In our experiment, we did not observe significant cell death after 5-azacytidine treatment, and the results showed that cells with 5-azacytidine exposure had higher expression levels in cardiac genes. Therefore, we suggest that low dose of 5-azacytidine was beneficial with apoptosis prevention and induction of cardiac differentiation. These contents were also added to the discussion section of the manuscript.

The functionality of induced cardiomyocytes can be determined by conducting electrophysiological activity assays. However, the chemicals used in inductive differentiation and the cell culture environment can also influence the results of these assays, indicating a need for further research to fully understand their effects. If the functionality of cardiomyocyte-like cells derived from hAMSCs can be improved in the future, it may provide a safer and more effective therapy for autograft heart regeneration. This potential advancement underscores the importance of ongoing research efforts to develop improved methods for cardiac differentiation of stem cells, with the ultimate goal of improving clinical outcomes for patients with heart disease.

CRediT authorship contribution statement

Hsiu-Man Shih: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Yi-Chen Chen:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Yen-Ting Yeh:** Writing – original draft, Resources, Conceptualization. **Fu-Shiang Peng:** Resources. **Shinn-Chih Wu:** Supervision, Resources, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Shinn-Chih Wu reports financial support was provided by National Science and Technology Council. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e28398.

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