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In Vitro Differentiation of Endometrium Stem Cells into Cardiomyocytes: The Putative Effect of miR-17-5p, miR-26b-5p, miR-32-5p, and SMAD6

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Abstract

Background: The important role of SMAD6 and several microRNAs (miRNAs), such as miR-17-5p, miR-26b-5p, and miR-32-5p, has been demonstrated in controlling the proliferation and differentiation of cardiomyocytes (CMs). Hence, this study was designed to assess the role of these regulatory factors in cardiac cell generation from human endometrium-derived mesenchymal stem cells (hEMSCs).

Methods: To induce transdifferentiation into CMs, hEMSCs were treated with a cardiac-inducing medium containing 5-azacytidine and bFGF for 30 days. Immunofluorescence staining and qRT-PCR, respectively, were used to measure the protein levels of SMAD6 and the mRNA expression of SMAD6 and the three miRNAs every six days.

Results: Our findings demonstrated the mesenchymal stem cell properties of hEMSCs and their ability to differentiate into various types of mesenchymal stem cells. The differentiated hEMSCs exhibited morphological features resembling CMs. During the induction period, the number of positive cells for SMAD6 protein and the expression level of miR-26b-5p increased and peaking on days 24 and 30, while the expression levels of miR-17-5p and miR-32-5p decreased. The Pearson correlation coefficients revealed that SMAD6 level is inversely correlated with miR-17-5p and miR-32-5p and directly correlated with miR-26b-5p.

Conclusion: Our results indicate that miR-17-5p, miR-26b-5p, miR-32-5p, and SMAD6 are potentially involved in the molecular signaling pathways of transdifferentiation of hEMSCs to CMs.

Keywords: Endometrium-derived mesenchymal stem cells (EMSCs), SMAD6, miR-17-5p, miR-26b-5p, and miR-32-5p.

Introduction

Heart or blood vessel dysfunctions that lead to cardiovascular diseases (CVDs) are the primary cause of mortality globally (1). The heart's ability to produce functional

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cardiomyocytes (CMs) is highly limited (2). To address this issue in the treatment of CVDs, a large body of research has been dedicated to assessing stem cell therapy using mesenchymal stem cells (MSCs), especially bone marrow-derived MSCs (BMSCs) (3-5). To improve cardiac impairments via MSCs, researchers are pursuing an appropriate cell source that can generate functional CMs in the damaged regions (6). According to recent studies, due to several advantages such as lack of tumorigenicity and high capacity for transdifferentiation, human endometrium-derived stem cells (hEMSCs) may be a suitable source of MSCs for regenerative medicine in CVDs through the generation of functional CMs (7-9). In vertebrates, the differentiation of CMs from the precursor cells has a certain pattern and can be regulated by several transcription factors, including TBX5, GATA4, GATA6, and Nkx-2-5 (10, 11). Furthermore, the pivotal role of transforming growth factor beta (TGF)-β superfamily ligands such as bone morphogenetic proteins (BMPs) has been revealed in CM regeneration by regulating the commitment of cardiac stem cells (12). TGF-β family signal transduction is associated with SMAD proteins such as SMAD6 as intracellular transducer molecules (13). SMAD6 can influence the BMP molecular pathway and is critically involved in cardiac cell development by regulating specific cardiac markers, including Myosin Light Chain-2v (MLC-2v), cardiac muscle troponin T (*TNNT2* which encodes the cTnT protein), and T-box transcription factor (TBX5) (14, 15). Additionally, several important post-transcriptional gene expression regulatory factors named microRNAs (miRNAs) can control the proliferation and differentiation of CMs by targeting many molecular pathways such as STAT-3, Wnt/β-catenin, and Notch (16, 17). Currently, the exact molecular mechanism of hEMSC transdifferentiation into CMs is not well understood.

Considering the fundamental function of miRNAs in the differentiation of stem cells into CMs and the targeting of SMAD6 by miR-17-5p, miR-26b-5p, and miR-32-5p (according to miRTarBase: https://mirtarbase.cuhk.edu.cn/, TargetScan: https://www.targetscan.org/vert_80/, and TarBase v.8: https://dianalab.e-ce.uth.gr/html/diana/web/index.php?r=tarbasev8), this study was conducted to assess the expression levels of SMAD6 and the three aforementioned miRNAs before and after in vitro hEMSC differentiation into CMs.

Materials and Methods

Isolation and identification of hEMSCs

The third passage of the hEMSCs was obtained from Avicenna Research Institute of Shahid Beheshti University of Medical Sciences, Tehran, Iran. Endometrial stem cells were cultured using cell culture medium [DMEM-F12; Gibco, Cat#: 12400024; Pen/Strep and fetal bovine serum (FBS) 10% from Inoclon, Iran; CO_2 5%; 37 °C temperature]. The cell culture media were refreshed every two days, and the cells were subsequently subcultured (in 80% confluence with 0.25 percent trypsin-EDTA purchased from Inoclon, IRAN). After the subculture process, the cells were expanded. Flow cytometry was used for immunophenotyping hEMSCs (3×104 cells, 4th passage). This technique was used to assess cell surface markers as follows: MSCs' lineage, including CD73, CD90, and CD105, and hematopoietic stem cell lineage, comprising CD34, CD45, and HLA-DR. In the first step, sterile phosphate-buffered saline (PBS) was used to wash the hEMSCs. Then, trypsin EDTA (0.25%) was used to detach the cells (all from Inoclon, Iran). Afterward, sterile PBS was utilized to re-suspend the detached cells. Then, hEMSCs were incubated with primary monoclonal antibodies for CD34, CD73, CD105, and HLA-DR (15 min, 4-8 °C; BD-PharMingen, San Diego, California). Ultimately, a FACS Calibur cytometer (Becton Dickinson, Lincoln Park, NJ) and specific software

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(CELLQUEST Pro) were used to quantify the hEMSCs. To determine the surface marker expression in the cells, a comparison was performed with isotype-matched controls (on a histogram plot).

Assessment of the osteogenic and adipogenic transdifferentiation of hEMSCs

The transdifferentiation ability of hEMSCs into other cell lineages, such as osteoblasts and adipocytes, was evaluated. For this purpose, the third passage of the hEMSCs were transferred to a 24-well dish containing growth medium (supplementary materials with 10% FBS). At 80% confluency, the cell medium was removed and osteogenic induction media (10 mM DMEM, beta-glycerol phosphate, 50 µg ascorbate-2-phosphate, and 10 nmol dexamethasone; Sigma, USA), as well as adipogenic induction medium (Sigma, USA), was added into the dishes separately. Over 21 days, the cell medium was refreshed every 2-3 days. Adipogenic induction medium was used for three days to differentiate hEMSCs into adipocytes (10% DMEM with FBS, 0.2 mM indomethacin, 1 µM dexamethasone, 4.5 g/L glucose, 0.5 mM 3-isobutyl-1-methylxanthine, insulin, and 1.7 µM Pen/Strep). Additionally, for one day, the cells were incubated with an adipogenesis maintenance medium containing DMEM, supplemented with 10% FBS, 1.7 µM insulin, 4.5 g/L glucose, and Pen/Strep, which was then replaced with the previous medium. The cells of the control group were treated with a DMEM cell culture medium, without any differentiation agents. After 21 days, staining by Oil Red O and alizarin-red was conducted to investigate the transformation of hEMSCs into adipocyte and bone cells, respectively. In the Oil Red O staining method, 4% formalin and 70% alcohol were used to fix the target cells, 60 min, room temperature. In the staining step, the 5% Oil Red was used in an 99% isopropanol solution for 15 min. Afterwards, the color solution was eliminated, the cells were washed with 70% alcohol, and finally, an inverted microscope was used for the observation of the cells. ImageJ software was used for the measurement of the differentiation rate (%). The alizarin-red staining method was as follows: PBS was used for washing the cell monolayer, methanol for fixation (10 min), staining step, color solution, 2 min using 1% alizarin-red in 25% ammonia water, washing by distilled water, drying, and, finally, observation with an inverted microscope.

Induction of hEMSCs for transdifferentiation into CMs

A CM differentiation medium that was verified by Xu et al. was used for the induction of hEMSCs (18). To reach this objective, the cultured hEMSCs (fourth passage) were treated in T25 flasks (2×10^5 cell concentration) and 24-well plates for the assessment of gene expression and immunofluorescence staining, respectively. In sufficient confluency (80%), the cells were treated with cardiomyogenic medium (DMEM-F12 from Inoclon, IRAN) containing FBS (5%) and basic Fibroblast Growth Factor (bFGF) (5 ng/cc) (CellGS, USA) for 24 hours. The non-adherent cells were eliminated precisely from the supernatant on the second day with care, and then treated with the differentiation medium (Iscove's Modified Dulbecco's Medium /F12, with 0.1% Insulin Transferrin Selenium, 2% horse serum, 0.1% non-essential amino acids, 1% Glutamax, 20 nM 5-azacytidine, and 1% Pen/Strep, all from Caisson, USA for three days. Then, the cell medium was changed with the 5-azacytidine-free induction cell medium. The changing the cell culture media continued every three days for 30 days. The changes in cell morphology during the differentiation induction process were assessed by an inverted Nikon microscope (Don Santo Corp., USA). On days 0 (undifferentiated hEMSCs as a control group), 6, 12, 18, 24, and 30, the expression levels of GATA-6 (Gene ID: 2627), SMAD6 (Gene ID: 4091), miR-17-5p

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(Gene ID: 609416), miR-26b-5p (Gene ID: 612152), and miR-32-5p (Gene ID: 609355) were compared.

Evaluation of the expression levels of SMAD6 at the protein level

For the assessment of the protein expression levels of SMAD6 in control and cardiogenic-induced cells, immunofluorescence staining was performed. To this end, paraformaldehyde (4%, 20 min) (Sigma, Japan) was used for fixation and PBS was used (3 times, 5 min) (Sigma, Japan) for washing. Then, Triton X-100 (0.3%) (Sigma, Japan) in PBS was used for 30 min as well as goat-serum (10%) (Sigma, Japan) for blocking (45 min, 4 ºC). In the next step, a primary mouse monoclonal antibody against SMAD6 (Invitrogen, Cat#: PA1-41026) was employed. Afterward, PBS solution was used for washing (five times, 5 min) and the target cells were treated with the secondary antibody (90 min, 37 ºC) (1:200; Biorbyt, Cambridge, UK, Cat#: orb868321). Then, PBS solution was used for washing (3 times). In the next step, 4'-6-diamidino-2-phenylindole (DAPI) (D9542-Sigma) was used for staining the cell nuclei, and the specimens were washed using PBS (after 20 min). Finally, the samples were mounted and observed under a microscope, and the figures were prepared. The mean number of positive and/or negative target factors was analyzed. The test was conducted in duplicates.

Transcriptional expression profiling

According to the manufacturer's protocol, total RNAs were obtained using the Hybrid-R miRNA-mini kit (GeneAll, South Korea). A Nano-Drop spectrometer (Thermo Scientific, USA) was used to assess the quality and quantity of the prepared RNAs. In the next step, cDNA synthesis was performed using an RT master mix kit (GeneAll, South Korea). The two separate reactions were accomplished as follows: I) With random hexamer primers (GATA6 and SMAD6), and II) with RT Stem-Loop miRNA primers (miR-17-5p, miR-26b-5p, and miR-32-5p). Ultimately, quantitative reverse transcription PCR (qRT-PCR) was performed with RealQ Plus 2X master mix Green High ROX kit (Amplicon, Denmark) as follows: Initial denaturation at 95 °C (1 min), followed by 40 cycles of 95 °C (5 sec) and 60 °C (30 sec). The levels of target RNAs were normalized according to a previous study using GAPDH and Snord47 as the reference genes (19). The sequences of the target primers are depicted in Table 1.

Table 1. Primer sequences used for qRT-PCR.

Gene	Forward primer	Reverse primer
SMAD6	5'-CCACATTGTCTTACACTGAA-3'	5'-CTGAGGTAGGTCGTAGAAG-3'
GATA6	5'-AACAACACTTTACTACCTAACG-3'	5'-ACACTATACAGACTTCATCAGA-3'
GAPDH	5'-GGACTTCCTCGGTGATAC-3'	5'-CGGTGACTGTAGCCATAT-3'

Statistical analysis

The data normality was assessed using the Shapiro-Wilk test, normal/Gaussian distribution. A one-way analysis of variance (ANOVA) with Tukey's *post-hoc* test was performed to compare the control and treatment groups. The Pearson correlation coefficient was used to assess the relationships between the expression of SMAD6 and the investigated miRNAs. The results are depicted as mean ± SEM. For the data analysis, SPSS software version 20.0 (SPSS, Inc., Chicago, IL, United States) was used. The significance was considered at $P \leq 0.05$.

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Results

The results of flow cytometry indicated that hEMSCs were positive for MSC markers including CD73, CD90, and CD105. Additionally, these cells were negative for hematopoietic lineage cell markers consisting of CD34, CD45, and HLA-DR. Furthermore, the measurement of the number of positive cells for surface markers such as CD73 and CD105 demonstrated that hEMSCs were positive for CD73 and CD105 (99.3%), CD90 (97.2%), HLA-DR (1.22%), CD34 (0.55%), and CD45 (1.30%), thus fulfilling the criteria for MSC characteristics. The multi-lineage differentiation ability of hEMSCs into osteoblasts and adipocytes was concluded, respectively, from calcium mineralization and oil droplet formation, as shown in our previous study (19).

In the primary cell culture, hEMSCs were able to adhere to the flask surface, and the microscopy assessment showed spindle-shaped fibroblastic cells. The data indicated that 5-azacytidine detached and killed some hEMSCs and the remaining cells adhered, proliferated, and differentiated themselves. An assessment of the morphological features on the sixth-day post-induction showed that the cells were stretched and lined up; however, some cells were still star-shaped, and very few were forming gap junctions. On the 24th day of induction, the number of enlarged, elongated, stretched, branched, and stick-like cells with myotube-like structures that formed gap junctions with each other gradually increased and culminated. The data demonstrated no statistical difference between the morphological features of the cultured hEMSCs on days 24 and 30 In the primary cell culture, hEMSCs were able to adhere to the flask surface, and the microscopy assessment showed spindle-shaped fibroblastic cells. The data indicated that 5-azacytidine detached and killed some hEMSCs and the remaining cells adhered, proliferated, and differentiated themselves. An assessment of the morphological features on the sixth day post-induction showed that the cells were stretched and lined up; however, some cells were still star-shaped, and very few were forming gap junctions. On the 24th day of induction, the number of enlarged, elongated, stretched, branched, and stick-like cells with myotube-like structures that formed gap junctions with each other gradually increased and culminated. The data demonstrated no statistical difference between the morphological features of the cultured hEMSCs on days 24 and 30 (19).

The data from the immunofluorescence technique showed that the mean expression levels of SMAD6 protein were elevated during induction (Fig. 1). The mRNA levels of SMAD6 and GATA6 significantly increased during differentiation ($P \leq 0.001$). The data from the miRNAs assessment showed that the expression levels of miR-32-5p and miR-17-5p significantly decreased, while miR-26b-5p (20) was up-regulated during the induction period ($P \leq 0.001$). Overall, the expression levels of miRNAs and SMAD6 (at the mRNA and protein levels) showed a completely oscillating differentiation. Nonetheless, the expression of these miRNAs and SMAD6 was not different between the 24th and 30th days of induction ($P > 0.05$) (Fig. 2.).

Pearson's correlation coefficient test showed an inverse correlation between the SMAD6 protein and the two miRNAs (R=-0/943 and -0/527 for miR-17-5p and miR-32-5p, respectively), and a direct correlation between the SMAD6 protein and miR-26b-5p (R=0/947).

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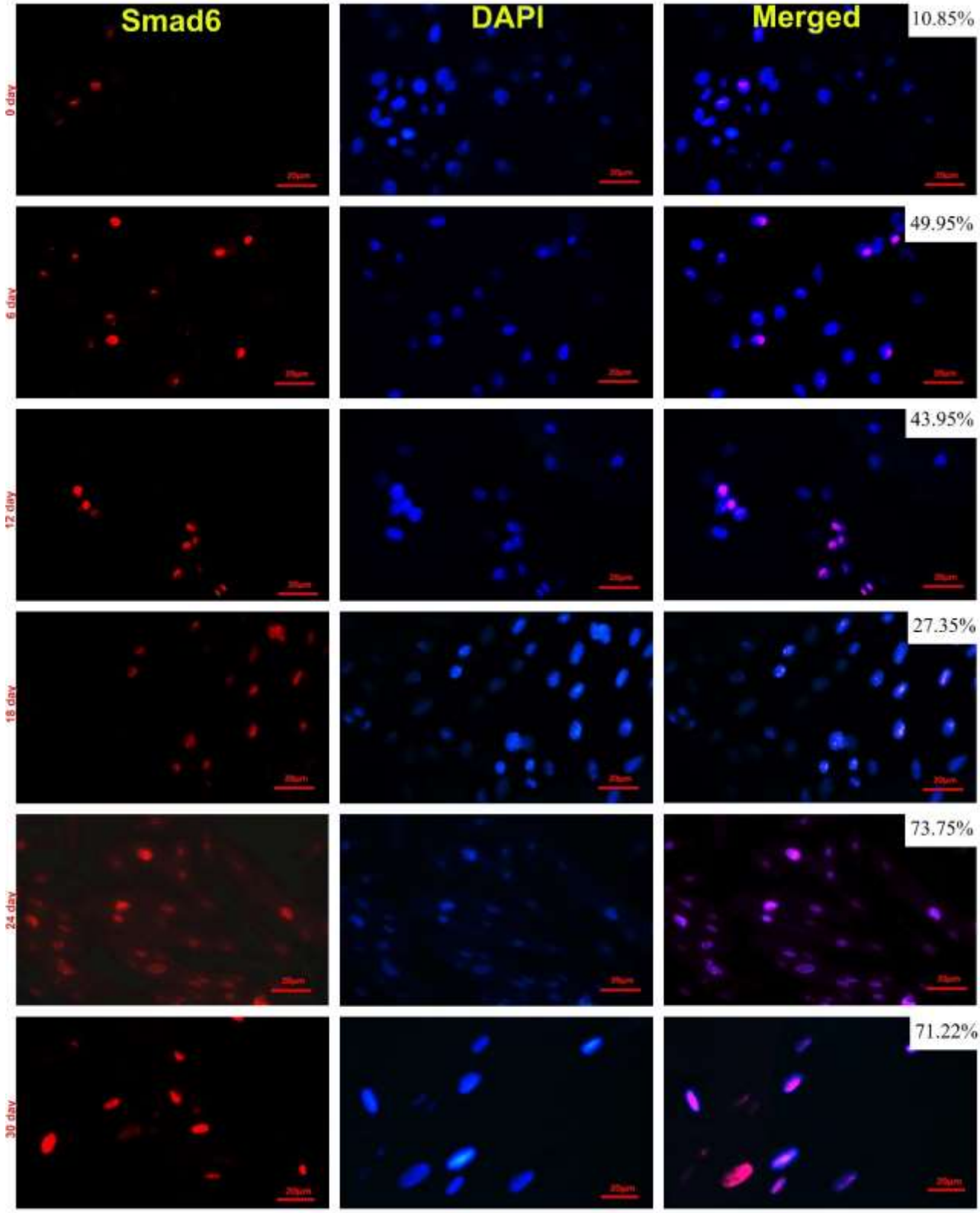

Fig. 1. Immunofluorescent staining of differentiated hEMSc. Expression of SMAD6 in differentiated hEMSc. Nuclei were counterstained with DAPI (blue); Scale bar: 20 μm; %: of positive cells; d: days.

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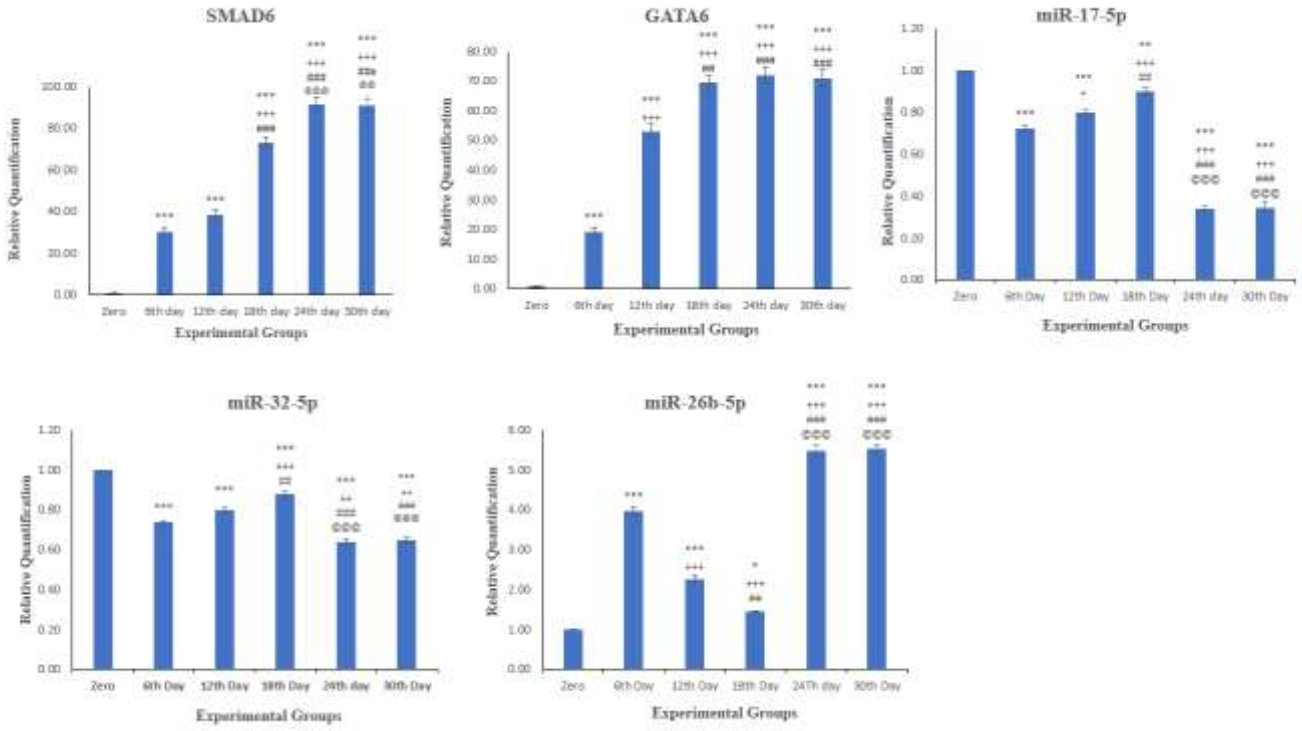

Fig. 2. Expression analysis of SMAD6, GATA6, miR-17-5p, miR-32-5p, and miR-26b-5p during differentiation of hEMSc into cardiomyocyte. ***P<0.001 and **P≤0.01 vs Day zero as a control, +++P≤ 0.001 and + P≤ 0.05 vs 6th day, ### P≤0.001 and # P≤0.05 vs 12th day, ***P≤0.001, **P≤0.01 and *P≤0.05 vs 18th.

Discussion

Our findings indicated that the decreased levels of miR-32-5p and miR-17-5p, along with the increased expression of miR-26b-5p, are potentially linked to the differentiation of hEMSCs into cells resembling CMs, possibly through the regulation of SMAD6 expression. Previous studies have highlighted the functional significance of these three miRNAs in cardiac processes and their positive impact on stem cell therapy for cardiac injury (21-23). Similarly, numerous studies have presented findings on how the three examined miRNAs regulate the TGF-β pathway in various cell types (24-26). Based on the current study, the investigated miRNAs in conjunction with SMAD6 can potentially control cardiac cell differentiation through the regulatory pathways (11, 27, 28). Additionally, we showed the role of GATA6 in the differentiation of pacemaker cells. Our morphological studies have demonstrated the existence of a series of cells with different appearances specific to pacemaker cells.

Various methods have been suggested for transforming MSCs into CMs. One of the earliest methods involves co-culturing with murine fetal or adult CMs, replicating the natural physiological environment and offering essential cues to guide the site-specific differentiation of MSCs into CMs (29, 30). The most extensively studied compound for inducing MSCs to the cardiac fate has been 5-azacytidine, either used alone or in conjunction with other compounds such as angiotensin II and TGF-β1 (31-33). Rahimi et al. found that the use of a combination of 5- azacytidine and bFGF is more effective than 5-azacytidine alone in promoting the differentiation of MSCs, particularly endometrial MSCs (EMSCs), into heart tissue (34). Additionally, in a separate study, they showcased the *in vitro* cardiogenic differentiation of menstrual blood-derived EMSCs through co-culture with native CMs (35). Another study by Hasani et al. reported that bFGF along with BMP4 as a promising

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combination for enhancing the CM differentiation of adipose tissue-derived MSCs (AMSCs) (36). Based on our results, we propose that instead of using potentially cell-damaging 5-azacytidine, manipulating miR-26b-5p down-regulation and overexpressing miR-32-5p and miR-17-5p in EMSCs individually may drive their differentiation towards CMs. In this context, Shen et al. demonstrated that elevating miR1-2 levels in mouse BMSCs results in reduced apoptosis and increased expression of cardiac-specific marker genes like Nkx2.5, cTnI, and GATA4 compared to BMSCs treated with 5-azacytidine, suggesting that using miR1-2 mimics could be more effective and less harmful than 5-azacytidine in prompting BMSCs to differentiate into myocardial-like cells (37). Additionally, the significance of miR-20a in the multi-differentiation potential of MSCs has been noted, where miR-20a overexpression was found to enhance bone formation in both BMSCs by co-regulating BMP signaling (38, 39). Moreover, reducing miR-20a levels was shown to promote CM differentiation of P19 cells without the need for dimethyl sulfoxide (DMSO) treatment (40). This approach has also proven successful in converting EMSCs into pre-oligodendrocyte cells through miR-219 and miR-338 overexpression (41, 42).

Prior works have determined that a 21- to 24-day induction period is most effective for the cardiogenic differentiation of various MSCs, including AMSCs (36), human amniotic fluid-derived MSCs (43), menstrual blood-derived stem cells, menstrual blood-derived stem cells (34), and human first-trimester fetal MSCs (44). In the present study, we extended this duration to 30 days, but we did not observe any differences between the morphological features and expression of CM-specific gene markers in differentiated cells on the 24th day compared to those on the 30th day. Therefore, it can be inferred that the highest level of differentiation occurs within the first 24 days following induction.

Our study had certain limitations that should be acknowledged. Firstly, the results presented in this study only indicated the inclination of EMSCs to differentiate into CM-like cells, rather than fully mature CMs, as we did not assess cardiac-specific action potentials, beating behavior, and contractile properties of the differentiated cells through methods such as *in vitro* electrophysiological studies or *in vivo* echocardiography in animal models of myocardial infarction following the transplantation of EMSCs. Therefore, further research involving the transplantation of these differentiated cells into the cardiac tissue of myocardial infarction animal models is necessary. Secondly, to confirm the role of the studied miRNAs in the CM differentiation of hEMSCs, it would have been preferable to introduce miRNA mimics or antisense oligonucleotides during the induction process to determine whether differentiation is impeded or not.

In future research, investigating the effects of additional miRNAs that target SMAD6 could enhance our understanding of the mechanisms involved the transformation of hEMSCs into CMs. Furthermore, uncovering the factors that control the expression of miR-17-5p, miR-26b-5p, and miR-32-5p during the differentiation of hEMSCs into CMs -such as long non-coding RNAs (lncRNAs)- could help clarify why these three miRNAs are either downregulated or upregulated during CM induction.

In conclusion, exposure to 5-azacytidine for 30 days prompted the cardiomyogenic differentiation of hEMSCs, as evidenced by the morphological changes resembling cardiomyocytes (CMs) and the increased expression of specific genes involved in cardiomyocyte muscle development. This study sheds light on how the downregulation of miR-17-5p and miR-32-5p and the upregulation of miR-26b-5p may play a role in the CM differentiation of hEMSCs, potentially through regulating SMAD6 expression levels. Additionally, our research suggests that extending the induction period of hEMSCs by six more days does not yield additional benefits. In summary, these findings set the stage for future in-depth mechanistic investigations.

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Conflicts of Interest
None.

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