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Effect of miR-18a-5p, miR-19a-3p, and miR-20a-5p on *In Vitro* Cardiomyocyte Differentiation of Human Endometrium Tissue-Derived Stem Cells Through Regulation of Smad4 Expression

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Abstract

Background: Smad4 regulates the expression of the genes required for heart homeostasis. Regarding the central role of microRNAs in cardiac biology, we investigated the expression of the three Smad4-targeting miRNAs, namely miR-18a-5p, miR-19a-3p, and miR-20a-5p, as well as Smad4 during differentiation of human endometrium-derived mesenchymal stem cells (hEMSCs) into cardiomyocytes (CMs).

Methods: To evaluate mesenchymal phenotype and multi-lineage differentiation ability of hEMSCs, immunophenotyping by flow cytometry and differentiation into osteoblasts and adipocytes were performed, respectively. For transdifferentiation into CMs, hEMSCs were exposed to a cardiomyogenic medium composed of 5-aza and bFGF for 30 days. The comparison between transcriptional expression levels of Nkx2-5, GATA4, Smad4, TNNT2, TBX5, miR-18a-5p, miR-19a-3p, and miR-20a-5p by qRT-PCR, as well as protein levels of Nkx2-5, Smad4, and cTnT by immunofluorescence staining, was conducted in every 6 days.

Results: *In vitro*, the mesenchymal stem cell phenotype of hEMSCs and their potency for differentiation into other MSCs were confirmed. Differentiated hEMSCs had morphological characteristics of CMs. The percentage of positive cells for Nkx2-5, Smad4, and cTnT proteins was increased following induction and culminated on the 24th day. Also, mRNA levels of Nkx2-5, GATA4, Smad4, TNNT2, and TBX5 exhibited the same trend. The expression of investigated miRNAs was significantly decreased sequentially. A significant negative correlation between expressions of Smad4 and investigated miRNAs was observed.

Conclusions: Our results indicate that miR-18a-5p, miR-19a-3p, and miR-20a-5p are involved in the cardiac differentiation propensity of hEMSCs potentially by regulation of Smad levels. Although, more mechanistic experiments are required to confirm this idea.

Keywords: Endometrium-derived mesenchymal stem cells (EMSCs), miR-18a-5p, miR-19a-3p, miR-20a-5p, Smad4.

Introduction

Cardiovascular diseases are the leading cause of death worldwide, emanating from heart or blood vessel impairments (1). Despite the enhancement of pharmacological and surgical

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interventions for preventing progression and managing symptoms, they still, give rise to the deaths of about one in three people (2). Over the past two decades, many pre-clinical and clinical trials have been conducted to assess the safety and efficacy of stem cell therapy for heart diseases (3). Mesenchymal stem cells (MSCs) have attracted inordinate attention for regenerative cell therapy in the failing heart (4). Bone marrow-derived MSCs (BMSCs) have been the most investigated MSCs candidate cell type for repairing heart tissue in experimental and clinical trials, nonetheless, these cells represent disadvantages (5, 6). Regarding that physiological angiogenesis actively occurs in the endometrium of premenopausal women, where decidual tissue is generated by periodical division and differentiation of uterine stem/progenitor cells and, on the other hand, angiogenesis is a critical process in the recovery of cardiomyocytes (CMs) function, human endometrium-derived MSCs (hEMSCs) have been proposed as ideal candidate cells for cardiac repair (7).

MicroRNAs (miRNAs; miRs) with approximately 18-25 nucleotides are the most important subtype of short non-coding RNAs with crucial regulatory functions in a wide constellation of biological programs (8). Intriguingly, a large set of them plays a major role in cardiogenesis and myocardium regeneration (9). Concerning the miR-17~92 cluster that harbors six miRNAs-encoding genes (miR-17-5p, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a-1), Ventura et al, for the first time, unraveled that targeted deletion of miR-17~92 cluster in mice results in severe ventricular septal defects in the hearts, emphasizing the pivotal role of this cluster during heart development (10). Later, Chen and coworkers indicated the implication of this cluster in the regulation of CM proliferation in embryonic, postnatal, and adult periods (11). Moreover, they demonstrated that transgenic enforced expression of members of this cluster, in particular miR-19a, is sufficient to provoke CM proliferation *in vitro* (11).

Transforming growth factor (TGF)-β superfamily, including TGF-βs and bone morphogenetic proteins (BMPs), are cytokines that execute their diverse array of roles in a broad range of cellular processes (12). The fundamental role of TGF-β/BMP signaling cascades in CM biology is well recognized. In this regard, Smad4 as a transducer of this cascade translocates into the nucleus, where it regulates the transcription of CM lineage-specific marker genes such as homeobox protein 2-5 (*Nkx2-5*), cardiac muscle troponin T (*TNNT2* encoding cTnT protein), T-box transcription factor (*TBX5*), and GATA binding protein 4 (*GATA4*) (13, 14). In several experiments, the results of conditional knockout of *Smad4* in CM of adult mouse underscored the importance of CM-Smad4–dependent TGF-β/BMP signaling in preserving cardiac function, and CM survival (15-17). According to miRTarBase, Smad4 is a computationally and experimentally validated target of miR-18a-5p, miR-19a-3p, and miR-20a-5p (18) (https://mirtarbase.cuhk.edu.cn/). Hence, in this study, we aimed to evaluate the putative effect of these three miRNAs on the TGF-β/BMP signaling pathways via regulation of Smad4 expression during *in vitro* transdifferentiation of hEMSCs into CMs.

Materials and Methods

hEMSCs collection, cultivation, and identification

hEMSCs of passage 3 were purchased from Avicenna Research Institute of Shahid Beheshti University, Tehran, Iran. The cells were subsequently cultured in T75 flasks (Fisher Scientific, USA) containing Dulbecco's Modified Eagle Medium (DMEM)-F12 (Gibco, USA) supplemented with 1% penicillin (100 U/ml)/streptomycin (100 µg/ml), and 10% fetal bovine serum (FBS) (all from Inoclon, IRAN) in a humidified atmosphere of 5% CO2 at 37 °C. The medium was changed every 2 days thereafter. When the cells reached 80% confluence, they were subcultured using 0.25% trypsin-EDTA (Inoclon, IRAN) and re-expanded. To characterize the hEMSCs,

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immunophenotyping was performed by flow cytometry on 2×10^4 cells (for each marker) of the fourth passage to evaluate the cell surface markers for mesenchymal (CD73, CD90, and CD105) and hematopoietic (CD34, CD45, and HLA-DR) stem cell lineages. For this purpose, cells were washed twice with sterile phosphate-buffered saline (PBS) and detached with 0.25% trypsin-EDTA (all from Inoclon, IRAN). The detached cells were re-suspended in PBS and incubated with each of the primary monoclonal antibodies against CD34, CD73, CD105, and HLA-DR (BD-PharMingen, USA) for 15 min at 4-8 °C. Finally, the cells were quantified using a FACS Calibur cytometer (Becton Dickinson, USA). The expression of cell surface markers was determined by comparison with isotype-matched control on a histogram plot.

Adipogenic and osteogenic differentiation of hEMSCs

To examine the multi-lineage differentiation ability of hEMSCs into other MSCs, they were differentiated into osteoblasts and adipocytes. To this end, hEMSCs of passage 3 were plated in a 24-well dish in the growth medium supplemented with 10% FBS. When the cells reached 80 confluency, the media was changed to each adipogenic and osteogenic induction media separately and the media was changed every 2–3 days for 21 days. Control cells were cultured in completed DMEM media without the differentiation stimuli. The composition of osteogenic and adipocyte induction media and interrogation of the differentiation of hEMSCs into bone and adipocytes by, respectively, alizarin red and Oil Red O staining corresponded to two previous works (19, 20)

Induction of hEMSCs differentiation into CMs

To induce transdifferentiation of hEMSCs into CMs, the cells were exposed to CM-differentiation induction media according to the modified format of a previously published protocol (21). For this purpose, 4th passage cultured cells were seeded at a concentration of approximately 3×10^5 in T25 flasks (for expression analysis of targeted mRNAs and miRNAs) and 3×10^4 in a 24-well cell culture plate (for immunofluorescent staining). Cells in 80% confluency were treated with DMEM-F12 cardiomyogenic medium (Inoclon, IRAN) supplemented with 5ng/ml basic Fibroblast Growth Factor (bFGF) (CellGS, USA) and 5% FBS for 24 hours. On the second day, non-adherent cells in the supernatant were removed carefully and treatment of the cells for 3 days was conducted by Iscove's Modified Dulbecco's Medium (IMDM)/F12 differentiation medium (Caisson, USA) containing 2% horse serum (Gibco, USA), 0.1% Insulin Transferrin Selenium (ITS) (Gibco, USA), 0.1% non-essential amino acids (Inoclon, IRAN), 1% penicillin/streptomycin, 1% Glutamax (Inoclon, IRAN), and 20 nM 5-azacytidine (5-aza) (CellGS, USA). After that, the medium was replaced by a 5-aza-free induction medium, and a change of medium was carried out every 72 hours for 30 days. During the period of differentiation induction, the morphological alterations of the cells were observed utilizing an inverted Nikon microscope (Don Santo Corp., USA). The comparison between transcriptional expression levels of Nkx2-5 (Gene ID: 1482), GATA4 (Gene ID: 2626), Smad4 (Gene ID: 4089), TNNT2 (Gene ID: 7139), TBX5 (Gene ID: 6910), miR-18a-5p (Gene ID: 609417), miR-19a-3p (Gene ID: 609418), and miR-20a-5p (Gene ID: 609420), as well as protein levels of Nkx2-5, Smad4, and TNNT2, was accomplished in zero (undifferentiated hEMSCs without exposure to differentiation medium as the control/basal group), 6th, 12th, 18th, 24th, and 30th days.

Assessment of Nkx2-5, Smad4, and cTnT protein levels

Immunofluorescence staining was exploited to measure the percentage of cells expressing Nkx2-5, Smad4, and cTnT proteins in six (basal and cardiogenic induced groups) 24-well cell culture plates with virtually 5×10^4 induced cells in each well. In detail, after fixation of cells for 20 min with 4% paraformaldehyde (Sigma, Japan) at 2-8 ºC, the samples were washed in PBS (Sigma,

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Japan) three times for 5 min and incubated at room temperature for 20 min. After washing with PBS, the cell membranes were permeabilized by exposure to 0.3% Triton X-100 (Sigma, Japan) for 30 min and blocked in 10% goat-serum (Sigma, Japan) for 30 min at 4˚C. The cells were then incubated with mouse monoclonal primary antibodies against human Nkx2-5 (Biorbyt, UK) Smad4 (Santa Cruz Biotechnology Inc., USA) and cTnT (Biorbyt, UK) for 24 hours at 2-8 ºC. After being washed with PBS in four steps at 5 minutes, the cells were incubated with secondary antibody (Biorbyt, UK) for 60 minutes at 37 ºC. Subsequently, the samples were washed in PBS three times and then the cell nuclei were stained with 4'-6-diamidino-2-phenylindole (DAPI) (Sigma, Japan). After 20 minutes, the samples were again washed in PBS. Coverslips were mounted onto the microscopic slides. The cells were visualized using a fluorescence microscope Olympus AX70 (Olympus, USA). Photographs were taken with the DP manager and DP controller (Olympus Life Science, USA), and the mean positive/negative numbers were calculated by ImageJ software. This experiment was done in duplicate.

mRNA and miRNA expression profiling

Total RNA, including mRNAs and miRNAs, was extracted employing the Hybrid-R miRNA-mini kit (GeneAll, South Korea) according to the manufacturer's recommendations. Quantity and quality of extracted RNA were assessed by NanoDrop spectrometer (Thermo Scientific, USA). For cDNA synthesis, two separate reactions were undertaken with HyperScriptTM RT master mix kit (GeneAll, South Korea), one with random hexamer primers for targeted mRNAs comprising Nkx2-5, GATA4, TNNT2, TBX5, and Smad4 and the other with RT Stem-Loop miRNA primers for miR-18a-5p, miR-19a-3p, and miR-20a-5p (Ana Cell, Iran). Finally, quantitative reverse transcription PCR (qRT-PCR) was done recruiting RealQ Plus 2X master mix Green High ROX kit (Amplicon, Denmark). The PCR cycles consisted of an initial denaturation step at 95 °C for 1 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s, and final dissociation stage (a cycle composed of 95 °C for 15 s, 60 °C for 30 s. The levels of investigated mRNAs and miRNAs were normalized against GAPDH and snRNA Snord47, respectively. The sequences of primers are represented in Table 1. All reactions were implemented in triplicate by using ABI Step One Plus (ABI, USA) instrument. The expression level of target mRNAs and miRNAs relative to housekeeping genes was determined using the $2^{-\Delta\Delta Ct}$ method (22).

Table 1. Primer sequences used for qRT-PCR.

Gene	Forward primer	Reverse primer
GATA4	5'-ACATAATCACTGCGTAATCTTC-3'	5'-GTTGTTGTTCTGGAGTCATT-3'
TNNT2	5'-CTATAACTTGGAGGCAGAGA-3'	5'-TCTTGGAGACTTTCTGGTTAT-3'
GAPDH	5'-GGACTTCCTCGGTGATAC-3'	5'-CGGTGACTGTAGCCATAT-3'
Nkx2-5	5'-CCTCAATCCCTACGGTTAT-3'	5'-TCACGAAGTTGTTGTTGG-3'
TBX5	5'-CATCAACCAGCCACATTC-3'	5'-GACTCCAACTACGCACTA-3'
Smad4	5'-CCACCAAGTAATCGTGCATCG-3'	5'-TGGTAGCATTAGACTCAGATGGG-3'

Statistical analysis

The Shapiro-Wilk test was used to test the normality of the data. All data exhibited a normal/Gaussian distribution. To compare between control (zero-day) and experimental groups (6th, 12th, 18th, 24th, and 30th days) and between experimental groups, a one-way analysis of variance (ANOVA) was harnessed followed by Tukey's post hoc test. The data are represented

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as mean ± SEM. Also, Pearson correlation coefficients were used to evaluate relationships between the expression of Smad4 and three investigated miRNAs. All the analysis was performed by SPSS version 20.0 (IBM Corp, USA), and P≤ 0.05 was considered statistically significant.

Results

Flow cytometry data showed that hEMSCs were positive for mesenchymal stem cell markers CD73, CD90, and CD105, but negative for hematopoietic lineage cell markers CD34, CD45, and HLA-DR. Quantification of the percentage of positive cells for CD73 and CD105 surface markers demonstrated hEMSCs being 99.3% positive for CD73 and CD105, 97.2% for CD90, 1.22% for HLA-DR, 0.55% for CD34, and 1.30% for CD45, thereby meeting the criteria identifying them as MSCs. Mineralization of calcium in hEMSCs that differentiated into osteoblasts and formation of oil droplets in hEMSCs that differentiated into adipocytes corroborated that these cells had osteogenic and adipogenic differentiation abilities (Fig. 1).

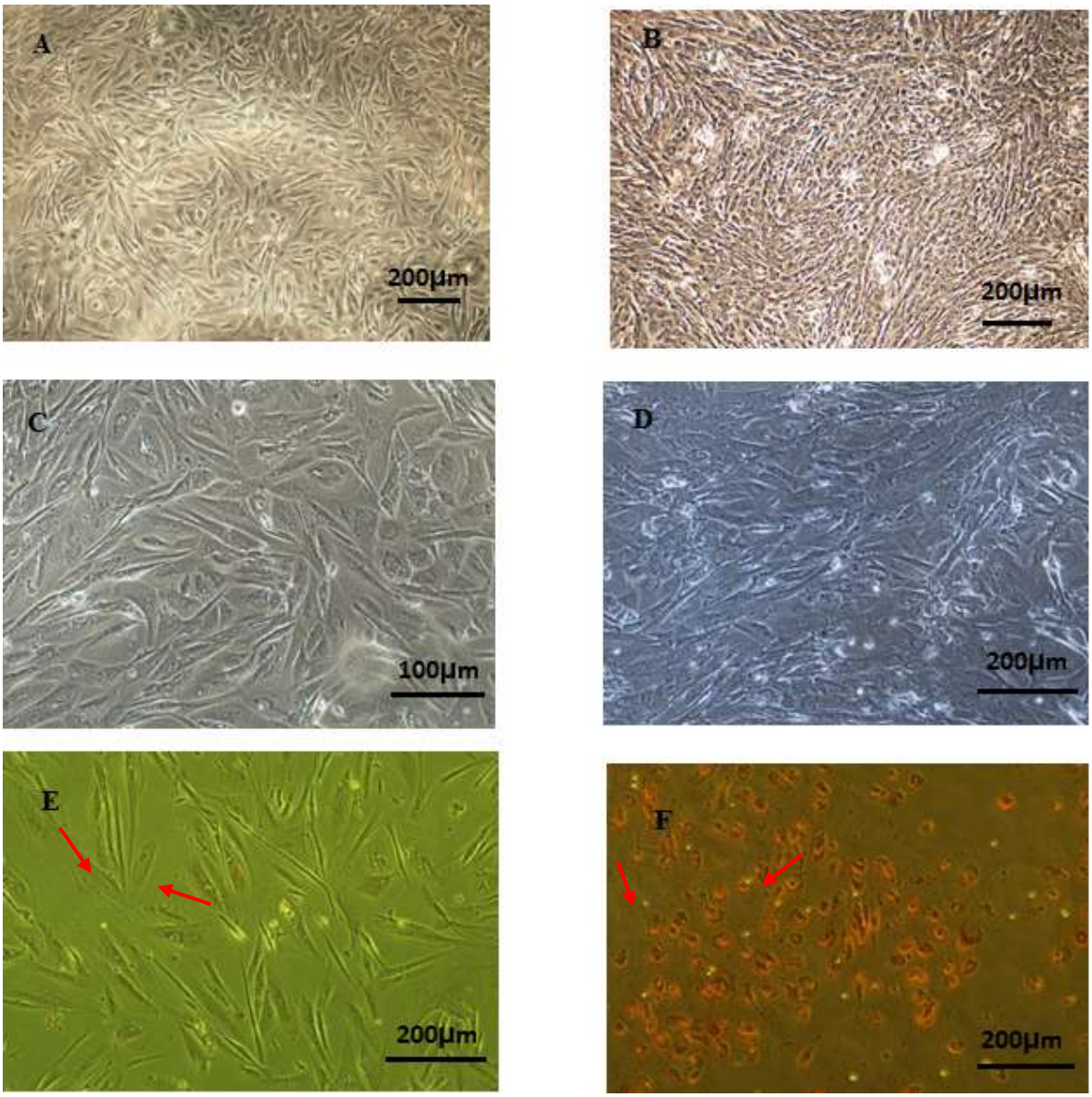

Fig. 1. Evaluation of multi-lineage differentiation potential of hEMSCs. A and C, representative micrographs of differentiated adipocytes from hEMSCs, and E, Oil red O staining of oil vacuoles (arrows). B and D, representative micrographs of differentiated osteoblasts from hEMSCs, and F, Alizarin red staining of calcium deposits (arrows).

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Before differentiation, hEMSCs adhered to the plastic surface in primary culture and looked like spindle-shaped fibroblastic cells. Following the addition of 5-aza, a few cells died and detached from the bottom of the flask, whereas the remaining cells adhered and began proliferation, differentiation, and change in their shape (Fig. 2). Of note, there was no difference between the morphology of the differentiated cells between the 24th and 30th days of differentiation.

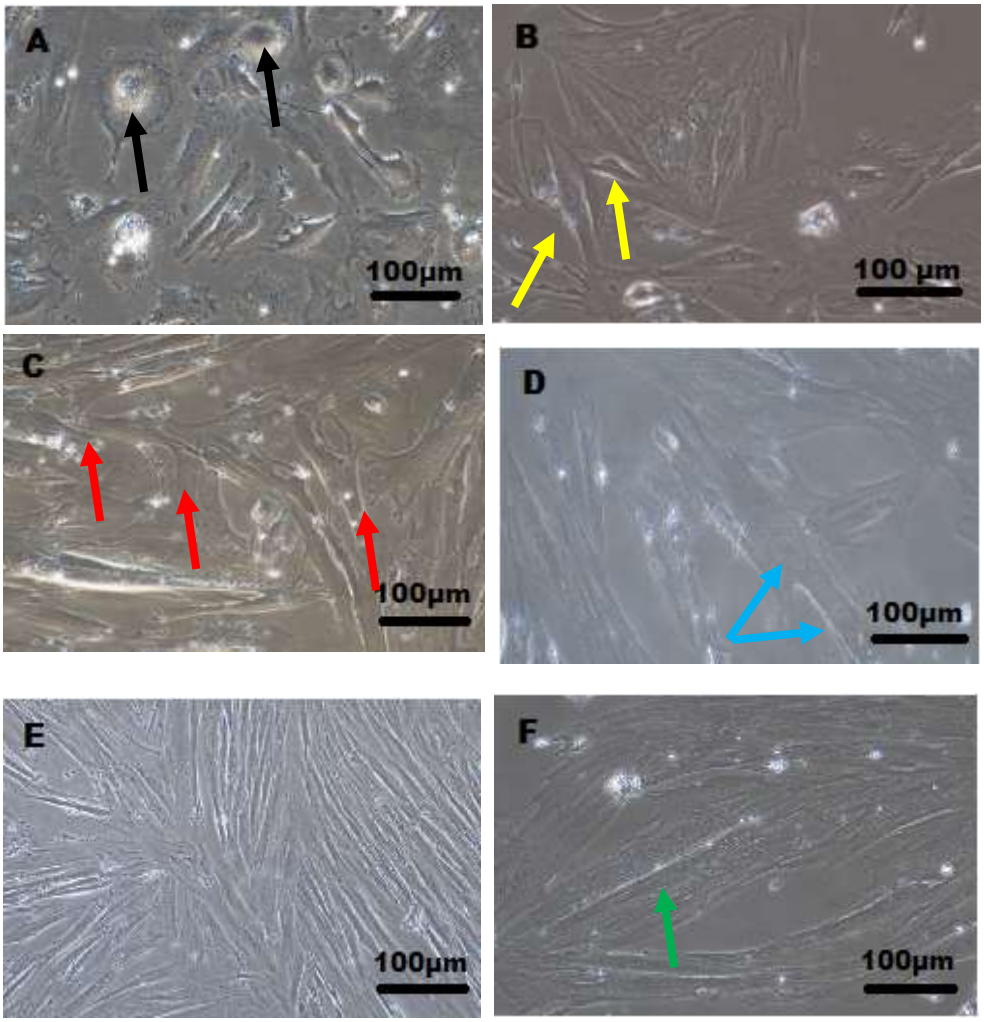

Fig. 2. Examination of morphological changes of hEMSCs during differentiation into cardiomyocytes by inverted microscope. (A) On day 0, before the addition of the differentiation medium, hEMSCs were star-shaped with large nuclei (black arrows); (B) On day 6, hEMSCs showed typical spindle-shaped fibroblast-like morphology (yellow arrows); (C) On day 12. cells were pulling together and created intercalated discs and syncytium (red arrows); (D). On day 18, more cells merged with each other to develop intercalated discs and multinucleated cells were observed (blue arrows); (E). On day 24, a unified and coherent structure of functional syncytium formed. (F). On day 30, sarcomere structures were observed indicating the maturity of cardiomyocytes and their contractile ability (green arrows).

Immunofluorescent staining indicated that the mean level of Nkx2-5, Smad4, and cTnT protein-expressing cells steadily increased during the differentiation process ($P \leq 0.001$) (Fig. 3). However, the expression of these three proteins was not different between the 24th and 30th days of induction ($P>0.05$). mRNA levels of Smad4 grew markedly from 1±0.000 in the control group on day zero to 91.51±3.00 at the end of day 24 ($P \leq 0.001$). Moreover, significant upper levels of mRNA of TNNT2 and early-stage cardiac

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transcription factors, Nkx2-5, GATA4, and TBX5, were observed in differentiated hEMSCs at the day of 24 relative to undifferentiated hEMSCs (94.97±0.98, 74.93±2.20, 83.36±2.03, and 73±00 for TNNT2, Nkx2-5, GATA4, and TBX5, respectively; P≤ 0.001). Compared to uninduced hEMSCs, miR-18a-5p, miR-19a-3p, and miR-20a-5p were considerably down-regulated following cardiogenic induction until 24th day (0.43±0.02, 0.2497±0.01, and 0.5434±0.01 at the day of 24, respectively, for miR-18a-5p, miR-19a-3p, and miR-20a-5p; P≤ 0.001). This difference did not reach significance between 24th and 30th days (P> 0.05). Finally, correlation analysis revealed an inverse association between the expression of Smad4 and three investigated miRNAs (P≤ 0.001). Pearson correlation coefficient for relationship between expression of miR-18a-5p, miR-19a-3p, and miR-20a-5p was -0.974, -0.971, and -0.973, respectively.

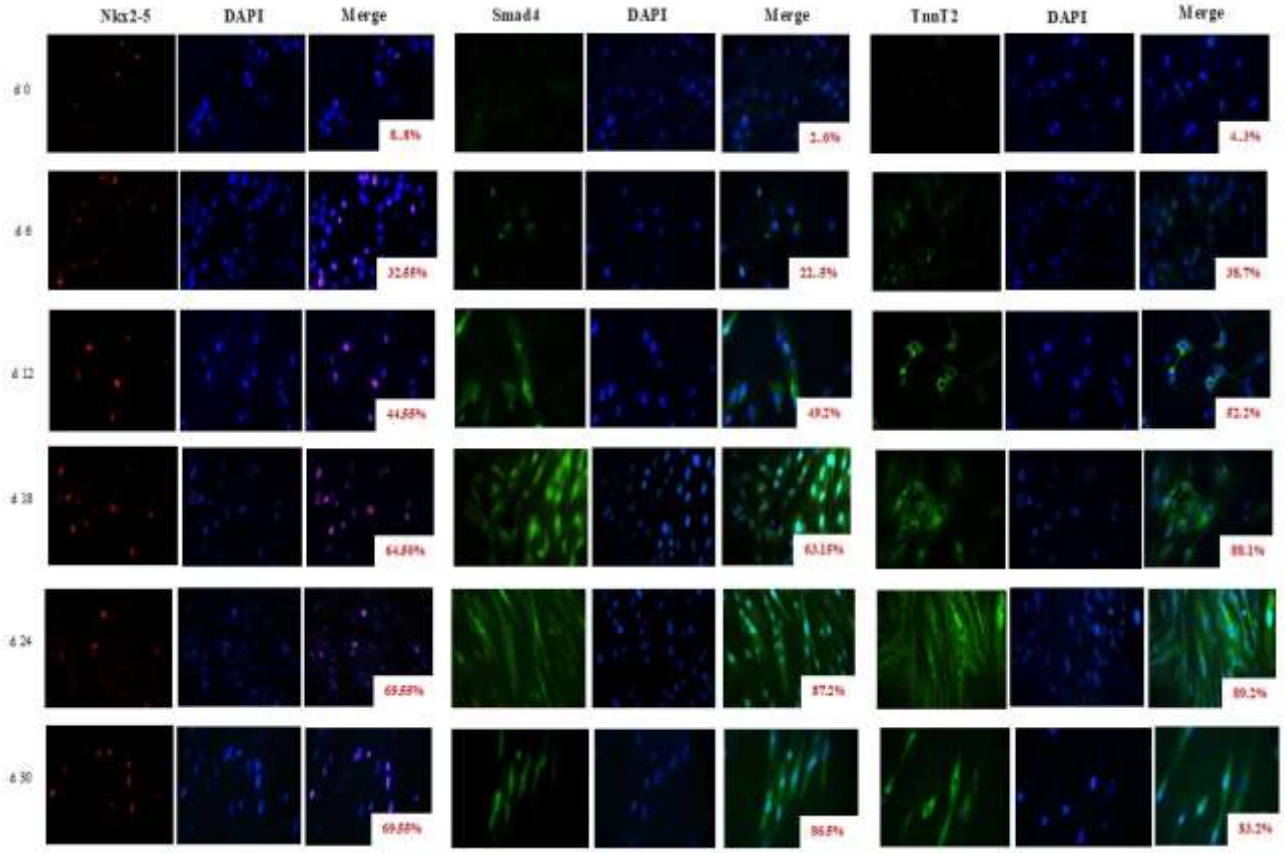

Fig. 3. Immunofluorescent staining of differentiated hEMSc. Expression of Nkx2-5, Smad4, and TTN2 (green) in differentiated hEMSc. Nuclei were counterstained with DAPI (blue); Scale bar: 20 μm; %: of positive cells; d: days.

Discussion

Our results revealed that the reduced expression of miR-18a-5p, miR-19a-3p, and miR-20a-5p is putatively involved in turning out of hEMSCs into CM-like cells probably by modulation of SMAD4 expression. Different protocols have been proposed for the transdifferentiation of MSCs into CMs. To drive MSCs to a cardiac fate, the most studied substance has been 5-aza, either alone or in combination with other substances such as angiotensin II and TGF-β1(23-25). Given our findings, we suggest that, without utilizing 5-aza that can cause cell damage, knock-down of three investigated miRNAs in hEMSCs may individually induce differentiation of them toward CM. In this context, Shen et al. exhibited that overexpression of miR1-2 in mouse BMSCs leads to a lower apoptosis rate and a higher level of cardiac-specific markers genes consisting of *Nkx2.5*, *cTnI*, and *GATA4*

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compared to BMSCs being exposed to 5-aza, hinting that treatment of BMSCs with miR1-2 mimics may be more effective and less cytotoxic than 5-aza treatment in the induction of BMSCs differentiation into myocardial-like cells (26). Also, the role of miR-20a in the multi-differentiation capacity of MSCs has been reported. In this regard, miR-20a overexpression was demonstrated to promote *in vitro* bone formation from BMSCs through co-regulating BMP signaling (27) and adipose tissue-derived MSCs (AMSCs) (28). Another experiment showed that downregulation of miR-20a can favor CM differentiation of P19 cells independent of treatment by dimethyl sulfoxide (DMSO) (29). This strategy has also been successful in the programming of hEMSCs into preoligodendrocyte cells through overexpression of miR-219 and miR-338 (30, 31).

Previous studies have established 21-24 days of induction as the optimum duration for cardiogenic differentiation of different MSCs including AMSCs (32), human amniotic fluid-derived MSCs (33), menstrual blood-derived stem cells (34), and human first-trimester fetal MSCs (35). In the current study, we increased this time to 30 days, but no difference was observed between differentiated cells on the 21th day and 30th day in terms of morphological characteristics and expression of CM-specific gene markers. Thus, it can be concluded that the maximum level of differentiation occurs up to 24 days following induction.

Our study had some limitations that must be noted. First, the findings of this study only displayed the tendency of hEMSCs for differentiation into CM-like cells, not necessarily actual mature CMs because we did not determine cardiac-specific action potentials, beating, and contractile characteristics of differentiated cells by assays such as *in vitro* electrophysiological studies and *in vivo* echocardiography of animal myocardial infarction (MI) models after hEMSCs transplantation (36-39). Hence, transplantation of these differentiated cells into the cardiac muscle of the MI animal model is warranted. Secondly, for confirmation of the role of investigated miRNAs in CM differentiation of hEMSc, it would have been better that miRNA mimics were leveraged during the induction process to assess whether differentiation is blocked or not.

For future studies, exploration of the impact of other Smad4-targeting miRNAs would be contributory to the understanding of underlying mechanisms of hEMSCs transdifferentiation into CMs. Within this context, a luciferase reporter assay unveiled that Smad4 is a bona fide target of miR-34a (40), miR-146b (41), and miR-130a (42) in cardiac tissue; therefore, the putative role of these miRNAs in heart differentiation of hEMSCs could be a topic of considerable interest. Also, elucidation of the factors that regulate the expression of miR-18a-5p, miR-19a-3p, and miR-20a-5p during differentiation of hEMSCs into CMs e.g., long non-coding RNAs (lncRNAs) could be advantageous to decipher the reason of the downregulation of these three miRNAs during CM induction.

In conclusion, the findings of this study bring light to the role of miR-18a-5p, miR-19a-3p, and miR-20a-5p down-regulation in CM differentiation of hEMSCs potentially by modulation of Smad4 expression levels. Also, we indicated that treatment of hEMSCs via induction media for additional six days has not any more beneficial effects. Altogether, our results pave the way for future mechanistic experiments.

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Conflicts of Interest

None.

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