Biochemical and Biophysical Research Communications 463 (2015) 699–705

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Ascorbic acid promotes the direct conversion of mouse fibroblasts into beating cardiomyocytes

Mahmood Talkhabi [a, b], Sara Pahlavan [a], Nasser Aghdami [a, **], Hossein Baharvand [a, b, *]

[a] Department of Stem Cells and Developmental Biology at Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

[b] Department of Developmental Biology, University of Science and Culture, ACECR, Tehran, Iran

ARTICLE INFO

Article history:
Received 13 May 2015
Accepted 31 May 2015
Available online 3 June 2015

Keywords:
Fibroblasts
Cardiomyocytes
Direct conversion
Small molecules
Ascorbic acid

ABSTRACT

Recent advances in the direct conversion of fibroblasts to cardiomyocytes suggest this process as a novel promising approach for cardiac cell-based therapies. Here, by screening the effects of 10 candidate small molecules along with transient overexpression of Yamanaka factors, we show ascorbic acid (AA), also known as vitamin C, enhances reprogramming of mouse fibroblasts into beating cardiomyocytes. Immunostaining and gene expression analyses for pluripotency and cardiac lineage markers confirmed beating patches were derived from non-cardiac lineage cells without passing through a pluripotent intermediate. Further analysis revealed that AA also increased the size of the beating areas and the number of cardiac progenitors. Immunostaining for cardiac markers, as well as electrophysiological analysis confirmed the functionality of directly converted cardiomyocytes. These results illustrate the importance of AA in direct conversion of fibroblasts to cardiomyocytes and may open new insights into future biomedical applications for induced cardiomyocytes.

1. Introduction

Heart diseases, particularly myocardial infarction, are a leading cause of death worldwide [1]. Although by now, several cell types have been studied for the treatment of heart diseases (for review see Ref. [2]), there has also been great interest in the development of alternative strategies for obtaining functional cells. Recent studies showed a cellular alchemy-like method, known as trans-differentiation or direct conversion to directly produce desired cells such as cardiomyocytes from fibroblasts [3–5]. Transdifferentiation defined as the direct conversion of one somatic mature cell type into another functional mature cell type without passing through a pluripotent intermediate [6]. This novel approach circumvents the teratoma risk associated with the use of induced pluripotent stem cells (iPSCs) and offers a short cut to generate cardiomyocytes. These induced cardiomyocyte-like cells (iCMs) have been obtained via two different strategies. First strategy relies on the cardiac-related factors and, to this end, various combinations of cardiac-specific transcription factors (TFs) and microRNAs have been used to convert human and mouse fibroblasts to iCMs in vitro and in vivo (for review see Ref. [7]). In the second strategy, called the epigenetic instability approach, iPSC-specific TFs are temporarily overexpressed in conjunction with lineage inducing signals to reprogram somatic cells into cardiomyocytes without first entering to a fully pluripotent state [3]. The latter strategy has been used to generate a variety of desired cells such as neurons [8] and hepatocytes [9]. To this end, various cytokines, growth factors and small molecules (SMs) have been found to modulate signaling pathways involved in cardiogenesis and thereby enhance the efficiency of cardiomyogenic differentiation [10].

In the current study we attempted to locate small molecules (SMs) that promote the in vitro conversion of mouse fibroblasts into cardiomyocytes by an epigenetic instability approach. Candidate SMs were chosen from a literature review and according to their molecular mechanisms involved in cardiogenesis and reprogramming. We found that ascorbic acid (AA) was a competent inducer during direct cardiac conversion and its promoting effect was further boosted in the presence of BMP4.

* Corresponding author. Department of Stem Cells and Developmental Biology at Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, P.O. Box 19395-4644, Tehran, Iran.
** Corresponding author.
E-mail addresses: Nasser.Aghdami@RoyanInstitute.org (N. Aghdami), Baharvand@RoyanInstitute.org (H. Baharvand).

http://dx.doi.org/10.1016/j.bbrc.2015.05.127
0006-291X/

2. Materials and methods

2.1. Direct cardiac conversion

24 h after retroviral transduction, MEFs were trypsinized and seeded in plates coated with Matrigel at 4.5×10^4 cells/well of a six-well plate (5×10^4 cells for the experiments carried out with the lentiviral inducible system, were seeded per well). Cells were subsequently cultured in reprograming medium (RM) consisting of knockout DMEM (Invitrogen) with 5% knockout serum replacement (KSR, Invitrogen), 15% ES-FBS (Invitrogen), 1% Glutamax (Invitrogen), 1% nonessential amino acids (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), and 0.5 μM JAK inhibitor (JI1, EMD). For those experiments carried out through the lentiviral inducible system, 5 μg/ml doxycycline (Sigma–Aldrich) was applied for the first 5 days (Fig. 1A). After 6 days, RM was discarded and cells were washed with PBS and, then, cultured for 3 days in differentiation priming medium (DPM) consisting of knockout DMEM (Invitrogen) with 1% FBS, 14% KSR, 1% Glutamax, 1% nonessential amino acids, 0.1 mM β-mercaptoethanol and 0.5 μM JI1. For the next 5 days, cell were cultured in cardiomyocyte differentiation medium (CDM) that consisted of RPMI-1640 supplemented with 0.5 × N2, 1 × B27 (without vitamin A) (Invitrogen), 0.05% BSA-fraction V (Sigma), 0.5% Glutamax, 0.1 mM β-mercaptoethanol, and 20 ng/ml BMP4 (Stemgent). Then, BMP4 was removed from the medium and cells were cultured with CDM until the end of the experiment (day 25). To promote the direct conversion of MEFs to cardiomyocytes, the candidate SMs listed in (Table 1) were used from days 6–9 (hereafter called pretreatment step) and/or days 9–14 (hereafter called treatment step) in the presence or absence of BMP4 (Fig. 1A). The SMs tested in this study were purchased from Sigma (Ascorbic acid, Icariin, SB431542, Cardiogenol C and NOC-18), Stemgent (RG-108, Sodium Butyrate and CHIR99021) and Tocris (5-Azacytidine and XAV939). Cells were visualized daily under an inverted microscope (Olympus CKX41) to identify the day beating initiated, cell morphology, and to calculate the percentage of beating patches. The total numbers of patches and the numbers of beating patches were counted visually at day 25 to determine direct conversion efficiency in the presence of each SM. To compare the morphology of patches formed in each group, photos were taken at different time points using an inverted microscope connected to a camera (Olympus DP72).

2.2. Immunostaining, electrophysiology, virus preparation and cell transduction, quantitative RT-PCR, and flow cytometry analysis

The materials and methods of these techniques were described in details in supplementary materials and methods.

3. Results

3.1. Screening for small molecules that promote direct cardiac conversion

To screen for cardiogenic fate-promoting SMs during direct conversion into cardiac fate, we selected 10 candidate SMs based on previous reports that have shown an enhancement of cardiomyocyte differentiation from pluripotent stem cells or an increase in reprogramming to pluripotency (Table 1). The fibroblasts were retrovirally transduced with reprogramming genes encoding the four TFs *Oct4*, *Sox2*, *Klf4* and *c-Myc*. The SMs were applied at days 9–14, the most effective time for cardiac induction [3] (Fig. 1A). Daily observation of cells showed that small molecules SB431542 (SB43), XAV939, Sodium Butyrate (S.B.), RG108, CHIR, Icariin, NOC-18, and 5-Aza not only induce beating patches but also inhibit cardiac induction from mouse fibroblasts in the presence of BMP4 (Fig. 1B). Cardiogenol C (CC) alone did not significantly promote formation of beating patches. In contrast, AA alone promoted

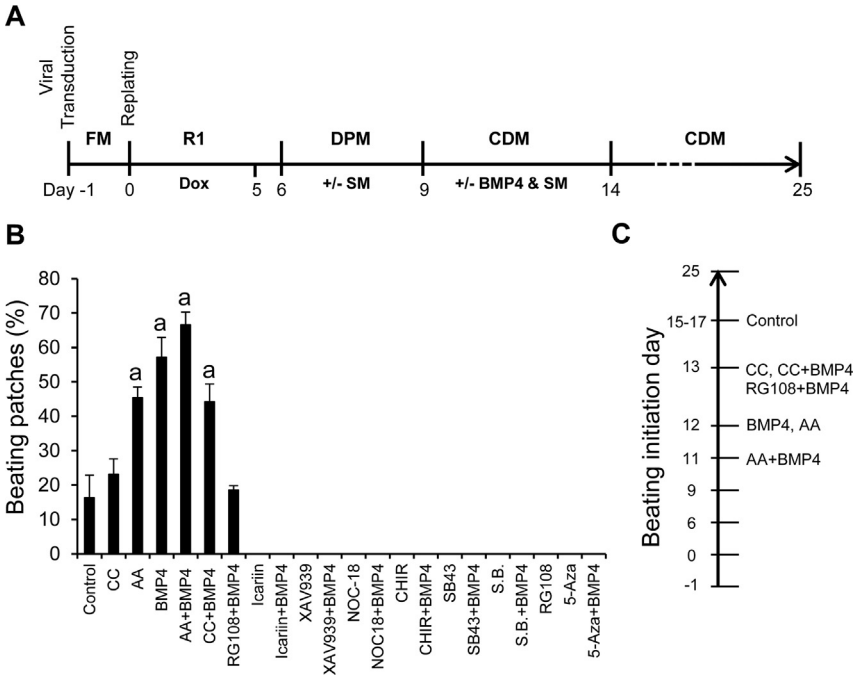

Fig. 1. Experimental design and screening for small molecules promoting the direct cardiac conversion in retroviral transduced fibroblasts. (A) Schematic representation of the small molecule-based strategy to convert mouse fibroblasts to cardiomyocytes. Dox was incorporated into the protocol when cells were transduced with the inducible polycistronic lentivirus. (B) The percentage of beating patches formed in the cells transduced with retroviruses. Chemical treatment was performed with candidate small molecule for 5 days (days 9–14). a: $P < 0.005$ vs. control. (C) The day of beating initiation related to the data presented in part B. Data are expressed as means ± SD.

Table 1
Candidate small molecules for direct cardiac conversion.

Name	Concentration	Characteristics	Ref.
Ascorbic acid	50 μg/ml	Antioxidant and cofactor for epigenetic modifiers	[11]
Cardiogenol C	5 μM	Activator of Wnt signaling and SIK1 and Smarce1	[18]
CHIR	3 μm	GSK3B inhibitor	[19]
Sodium Butyrate	2.5 mM	Histone Deacetylase (HDAC) inhibitor	[20]
SB431542	5 μm	Activin A receptor-like kinase ALK5/ALK4/ALK7 inhibitor	[19]
Icariin	100 nM	Inducer for ROS generation and p38MAPK activation	[21]
XAV939	1 μM	Wnt/β-catenin signaling inhibitor	[22]
RG108	5 μM	DNA methyltransferase inhibitor	[23]
NOC-18	1 μM	Nitric Oxide (NO) donor	[24]
5-Aza	3 μM	DNA methylation inhibitor	[25]

significantly the induction of beating patches and there was no significant difference between the percentages of beating patches formed following treatment with AA alone and BMP4 alone. Compared to AA alone, simultaneous treatment with both AA and BMP4 increased significantly the percentage of beating patches (Fig. 1B, $P < 0.005$). Temporally, the first beating was observed at day 11 in cells treated simultaneously with AA and BMP4, while other effective SMs led to a later onset of beating patches (days 12–13) even along with BMP4 (Fig. 1C). These data suggest that AA promotes direct cardiac conversion from mouse fibroblasts as efficiently as BMP4.

3.2. Ascorbic acid promotes direct cardiac conversion following temporal expression of OSKM

Based on the first screening using retrovirally transduced fibroblasts, AA was selected for additional analyses. To eliminate the possibility of fully pluripotent intermediate generation during direct conversion, an inducible polycistronic OSKM was expressed by adding Dox for 5 days (Fig. 1A). In order to determine the most appropriate time of AA, we treated the cells at days 6–9 and 9–14 (Fig. 1A). At 25 days after infection, beating analyses revealed that prolonged treatment (day 6–14) with AA increased the percentage of beating patches compared to short-term treatment (5 days, days 9–14, Fig. 2A, $P < 0.05$). Moreover, it was observed that pretreatment of cells with AA at days 6–9 followed by simultaneous treatment with both AA and BMP4 at days 9–14 led to the highest percentage of beating patches compared to the other groups (Fig. 2A, $P < 0.05$). We also observed that direct cardiac conversion by the inducible system was more efficient compared to that of retrovirally transduced fibroblasts. Surprisingly, in contrast to preliminary screening with retrovirally transduced cells, very few beating patches formed in the control (Fig. 2A). These data indicate that prolonged treatment (days 6–14) with AA following temporal expression of OSKM adequately promotes efficient direct cardiac conversion. Hence, we chose AA as the effective single SM for further experiments using the inducible system.

3.3. Ascorbic acid promotes direct cardiac conversion without passing through a fully pluripotent state

We analyzed MEFs and emerging intermediates for the expressions of cardiac and pluripotency markers to confirm that AA promoted formation of beating patches by a direct conversion process. Immunostaining for cardiac lineage markers Nkx2.5, Mef2C, Isl1, cTnT and MHC indicated no positive cells for these markers in MEFs at day 0 (Supplementary Fig. 1). There were also no positive cells for the pluripotency marker Nanog at days 0, 6 and 9 (Supplementary Fig. 2A). Whereas, a few cells stained for Mef2C at day 9 (Supplementary Fig. 2B) indicating that cardiac induction occurred before emerging of Nanog expressing cells. To examine whether pluripotency genes were expressed before or after AA treatment, we performed qRT-PCR analysis at different time points until day 15. This was the end of cardiac induction where most patches showed spontaneous beating. Compared to mouse induced pluripotent stem cells (miPSCs), pluripotency genes *Nanog* and *Rex1* expressed at very low levels during direct cardiac conversion using AA alone or AA and BMP4 (Supplementary Fig. 3A).

In addition, we conducted a secondary analysis to exclude the possibility of generation of fully pluripotent cells during direct cardiac conversion. Mouse iPSC colonies were treated with converting media containing AA alone or both AA and BMP4 (Supplementary Fig. 3B). No beating colonies formed from mouse iPSCs, whereas the cultured MEFs in converting media that contained AA alone or both AA and BMP4 led to >60% beating patches (Fig. 2A).

These data indicate that the beating cardiac patches were derived from non-cardiac lineage cells in a direct conversion process without transition to fully pluripotent cells.

3.4. AA increases the size of beating patches and the number of cardiac progenitors

We visually investigated the sizes of the beating areas at day 25 in order to further determine the effects of AA on direct cardiac conversion. Only a very small part of the patches showed spontaneous beating in the control. Whereas cells pretreated (days 6–9) and treated (days 9–14) with AA alone or in conjunction with BMP4 (Supplementary Fig. 4A), as well as cells treated with BMP4 alone had larger beating areas compared to control. These beating areas comprised approximately the entire size of the patches (Supplementary Fig. 4B). Quantitatively, the sizes of beating areas in the cells pretreated and treated with AA were significantly larger than control (Fig. 2B, $P < 0.05$). Even it was further larger when cells were co-treated with BMP4.

Flow cytometry analysis showed that the number of cardiac lineage cells ($Flk1^+$, $GATA4^+$, and MHC^+ cells) was significantly higher in the presence of AA and BMP4 compared with those in the control (Fig. 2C, $P < 0.05$). $Flk1^+$ cardiac progenitors were clearly present at day 9 and comprised approximately 20%–30% of cells at day 14 in the cells pretreated with AA and treated with AA or BMP4. Pretreatment and treatment with AA alone led to the emergence of the highest percentage of $GATA4^+$ cells (~40%) at day 14. This result was significantly higher than that with control cells and to that of cells treated with BMP4 alone, as well as to that in the cells treated with both AA and BMP4. However, there were more MHC^+ cells in the group treated with both AA and BMP4 (Fig. 2C, $P < 0.05$). Totally, the percentage of all cardiac lineage cells in treated groups (BMP4, AA/AA and AA/BMP4+AA) was significantly higher than control and day 0, 6, and 9 (Fig. 2C). These data indicate that AA increases the direct cardiac conversion via increasing the number of cardiac lineage cells which leads to formation of larger beating areas.

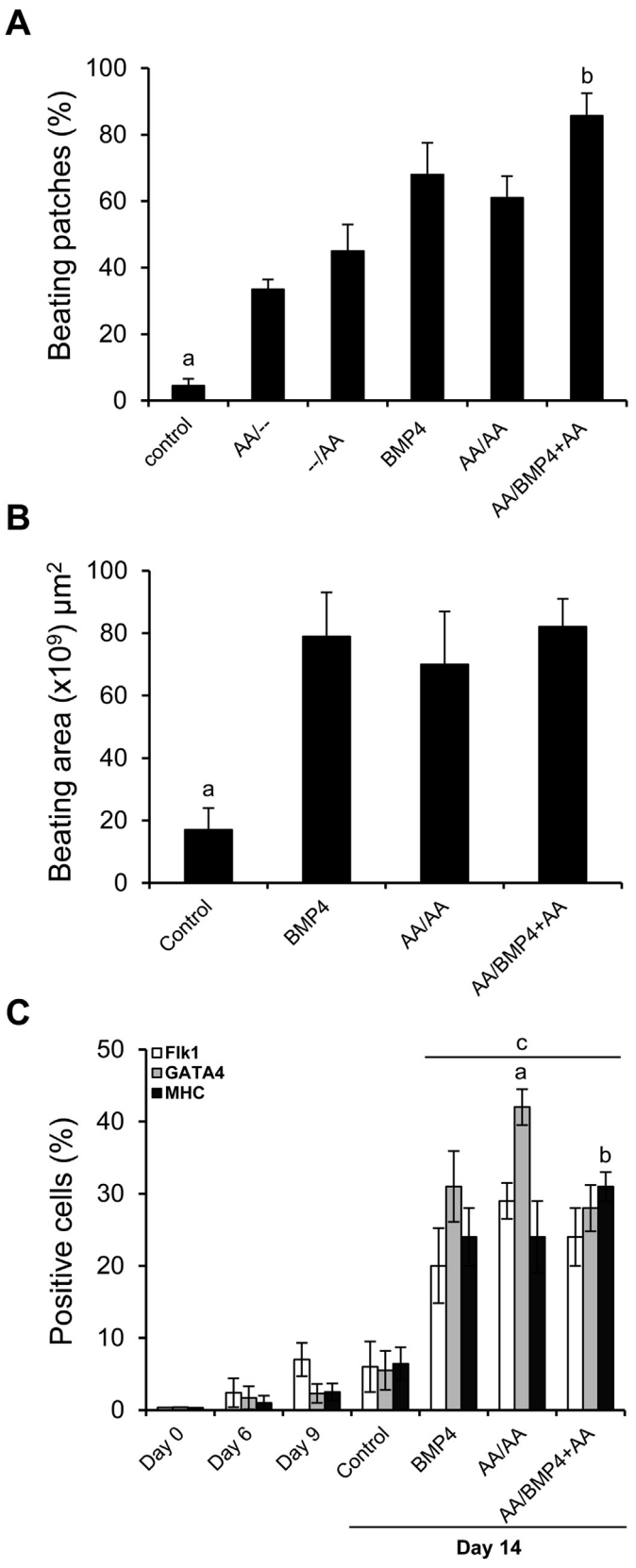

Fig. 2. Inducing role of AA in direct conversion of mouse fibroblasts to beating cardiomyocytes. (A) The efficiency of beating patches formed in the cells pretreated and/or treated with AA or BMP4. a: $P < 0.005$ vs. treated groups, b: $P < 0.005$ vs. other treated groups. (B) The size of beating patches formed in the presence of AA and BMP4. a: $P < 0.05$ vs. treated groups. (C) The percentage of cardiac lineage cells emerged during direct cardiac conversion promoted by AA and/or BMP4. a: $P < 0.005$ vs. other treated groups (BMP4 and AA/BMP4+AA), non-treated group (control) and other days (0, 6 and 9), b: $P < 0.05$ vs. other treated groups (BMP4 and AA/AA), control and other days, c: cardiac lineage cells in treated groups vs. control and days 0, 6 and 9 ($P < 0.005$). Slash sign between AAs or between AA and BMP4 represents day 9 of direct conversion. Data are expressed as means ± SD.

3.5. The direct converted cells exhibit a cardiac phenotype

In order to confirm the cardiomyocyte identity of directly converted cells, the beating patches formed following pretreatment with AA and treatment with AA and/or BMP4 were dissociated into single cells and analyzed. Morphology analyses under microscope revealed that most individualized cells exhibited spontaneous contractions. To determine if cardiac genes were enriched in beating patches derived following AA treatment, the expression of cardiac specific genes *Gata4*, *Nkx2.5*, *Myh6* and *Tnnt2* was determined using qRT-PCR. Beating patches formed following pretreatment with AA and treatment with AA and/or BMP4 showed an enrichment of their transcripts (Fig. 3A). However, in comparison with adult heart, direct converted cardiomyocytes expressed lower levels of *Myh6* (cardiac myosin heavy chain) and *Tnnt2* (cardiac muscle troponin T). Whereas, the expression of cardiac TFs was upregulated in the directly converted cardiac cells (Fig. 3A). Next, immunostaining analyses showed that beating cardiomyocytes expressed cardiac transcription factors Mef2c and Nkx2.5, as well as the structural proteins cTnT and MHC (Fig. 3B). Moreover, the expression of gap junction protein connexin-43 (Cx43) was also detected in the border of some cardiomyocytes, which indicates cellular communication between converted cardiomyocytes (Fig. 3B).

We sought to determine if directly converted cardiomyocytes possessed functional electrophysiological properties. In this regard, extracellular field potentials from spontaneously beating patches formed following pretreatment with AA and treatment with AA and/or BMP4 were recorded before and after administration of the β-agonist Isoproterenol (Fig. 4A). β-adrenergic stimulation with 100 nM Isoproterenol (Iso) increased the spontaneous beating rate, as determined by decreased interspike intervals (Fig. 4B Left, $P < 0.0005$). cFPD in beating cells formed after pretreatment with AA and treatment with BMP4 was shorter than that of cells converted by AA alone (Fig. 4B Right, $P < 0.005$), suggesting that the direct cardiac conversion using both AA and BMP4 produced more mature cardiomyocytes. Taken together, these results demonstrated that the direct cardiac conversion promoted by AA alone or AA and BMP4 led to the production of cardiac cells which displayed properties of functional cardiac cells.

4. Discussion

Here we attempted to find SMs with ability to promote direct cardiac conversion from mouse fibroblasts during epigenetic instability approach by Yamanaka factors [3]. We selected 10 SMs based on their inducing roles during cardiogenesis and reprogramming. Specifically, the number of beating patches and the day of beating initiation indicated that AA was a robust cardiac inducer during direct cardiac conversion. Beating cardiomyocytes derived in the presence of AA showed mature cardiac properties such as electrophysiological activity and the expression of cardiac specific genes at mRNA and protein levels. Surprisingly, other SMs such as 5-Aza and Icariin, which are known for their cardiogenic effects at different cell types, could not efficiently promote direct cardiac conversion and even they suppressed the cardiogenic effects of BMP4. As we expected, BMP4 had a synergistic effect on direct cardiac reprogramming when added to AA. To find the best time

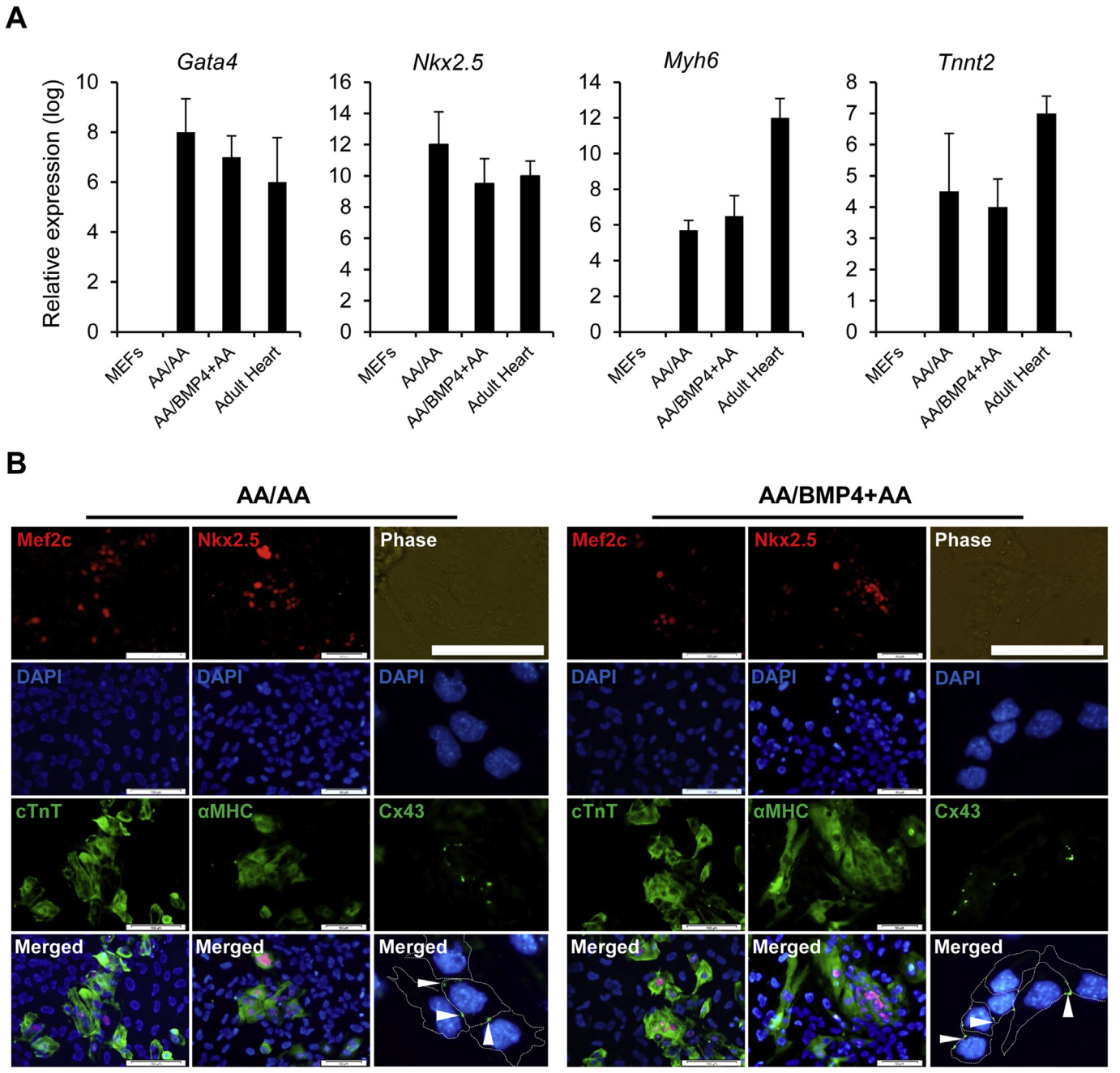

Fig. 3. Cellular and molecular characteristics of AA-promoted direct converted cardiomyocytes. (A) Quantitative gene expression analysis of cardiac markers in the directly converted cardiomyocytes at day 25. (B) Immunostaining of single cardiomyocytes dissociated from beating patches formed following pretreatment with AA and treatment with AA or BMP4. For those photos stained for Cx43, white lines indicate the cell borders and arrow heads show the Cx43 stained sites. Slash signs between AAs or between AA and BMP4 represent day 9 of direct cardiac conversion. Scale bars are 100 μM except for those stained for Cx43 that Scale bars indicate 50 μM.

point in which AA was most efficient, we began AA treatment from day 6.

It has been previously reported that AA induces cardiogenesis from pluripotent stem cells at different phases of differentiation including cardiac progenitor specification and proliferation and cardiomyocyte maturation at both the structural and functional levels [11].

Extending the exposure time increased the efficiency of direct conversion and was as efficient as direct conversion using cardiogenic growth factor BMP4. Since AA has been also shown to strongly enhance the efficiency and kinetics of reprogramming and to increase the quality of mouse iPSCs [12], the AA treatment was not started earlier than day 6. This cardiogenic effect of AA was consistent with the previous studies in cardiomyocyte differentiation from human and mouse pluripotent stem cells [11,13]. In comparison to cardiomyocyte differentiation from pluripotent stem cells in which a constant exposure to AA leads to an efficient differentiation [11], direct cardiac conversion could efficiently be achieved by a short time (8 days) treatment with AA alone and adding BMP4 synergistically augmented its promotional effect. We also observed that AA increased the size of beating areas and the number of cardiac progenitor cells, $Flk1^{+}$ and $Gata4^{+}$. AA could be

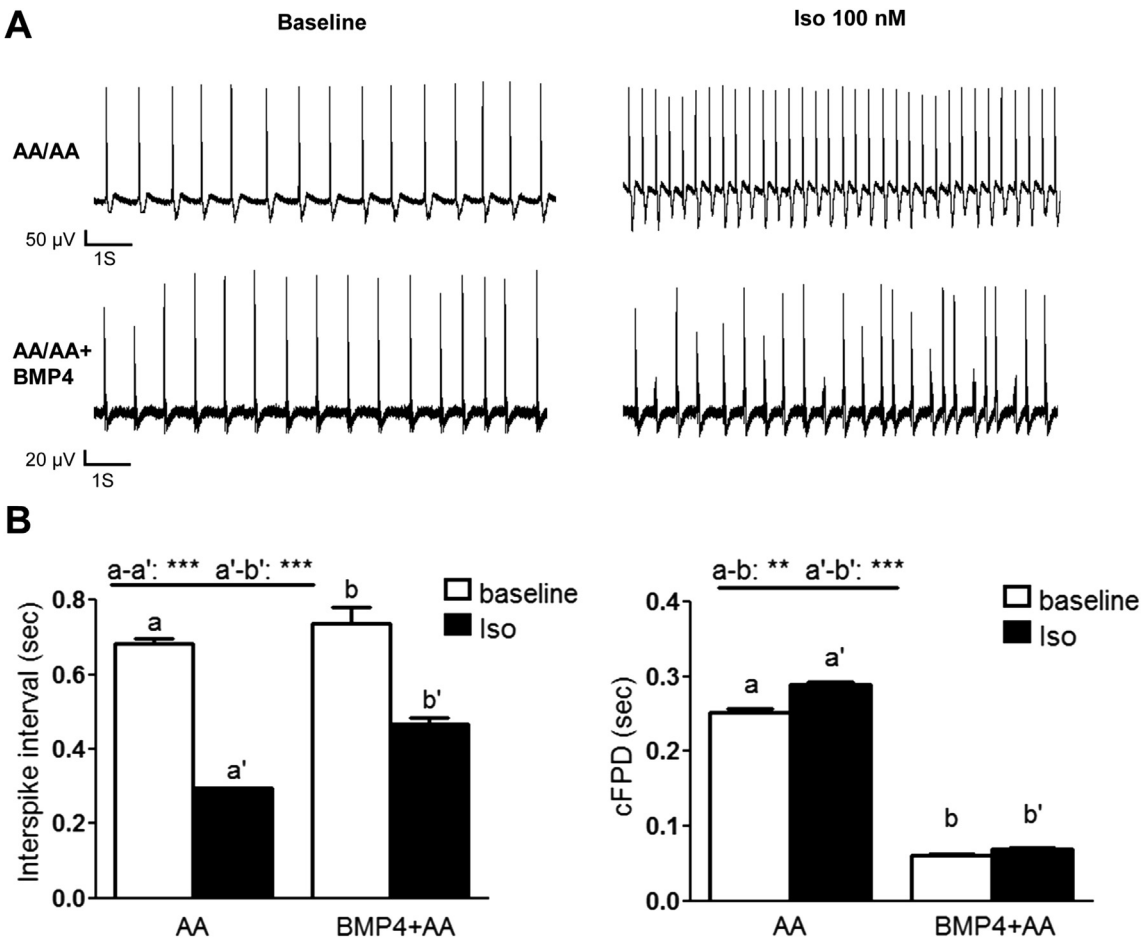

Fig. 4. Electrophysiological properties of beating patches using microelectrode array (MEA). (A) Extracellular field potential (FP) recordings of directly converted cardiomyocytes following isoproterenol (Iso) treatment. (B) Changes in electrophysiological parameters in the presence of Iso. Treatment of directly converted cardiomyocytes with Iso caused a decrease in the interspike intervals (left). The corrected field potential duration (cFPD) in directly converted cardiomyocytes derived following simultaneous treatment with AA and BMP4 was evidently smaller than that of AA treated cardiomyocytes (right). **: $P < 0.005$, ***: $P < 0.0005$.

further exploited to derive long-term proliferative cardiac progenitors rather than mature beating cardiomyocytes, for potential in vivo and in vitro applications.

Compared to BMP4 or other cardiogenic growth factors, AA presents an inexpensive approach for cellular differentiation and reprogramming. AA is a natural compound that can easily be used to enhance the rate of in vivo cardiac reprogramming. The rate of generated beating cardiomyocytes by using AA is higher compared to cardiac reprograming with cardiac specific TFs or microRNAs [14]. On the other hand, it was demonstrated that SB431542, a TGFβ inhibitor, increased the efficiency of direct cardiac conversion of mouse fibroblasts [15]. SB431542 was included in our list as well; however it did not promote beating patch formation and was excluded from further experiments. Moreover, recently a small molecule-based method by SB431542, CHIR99021, Parnate and Forskolin (referred as SCPF) in conjunction with *Oct4* and cardiogenic growth factor BMP4 for cardiac reprogramming from mouse fibroblasts was developed [5].

The molecular mechanism by which AA promoted cardiac reprogramming is not clear. It was reported that AA exerts its cardiogenic effects by increasing ROS levels [16], activating the MEK-ERK1/2 pathway [11], and epigenetic modification in cellular reprogramming. Ascorbic acid functions as a cofactor for H3K36 histone demethylases Jmjd1a and Jmjd1b to enhance the efficiency of iPSCs reprogramming [17]. Besides, AA works as a cofactor for Tet enzymes, which thereby causes a genome-wide DNA hypomethylation and a global decrease in the repressive H3K9me2 and H3K9me3 marks in embryonic stem cells and nascent iPSCs, respectively [17]. These new findings propose that AA might exert its roles via different mechanisms in various cell types.

In conclusion, the AA enhanced direct conversion of fibroblasts into beating cardiomyocytes, and our finding also suggests a vital role for AA in direct cardiac reprograming of human cells.

Conflict of interest

The authors have declared that no potential conflict of interest exists.

Acknowledgments

This study was funded by grants provided from Royan Institute, Iranian Council of Stem Cell Research and Technology and the Iran National Science Foundation (INSF).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.127.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.127.

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