Enhancing induced pluripotent stem cell toward differentiation into functional cardiomyocytes

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Abstract

Background: Heart diseases, especially myocardial ischemia, remain one of the leading causes of mortality worldwide and usually result in irreparable cardiomyocyte damage and severe heart failure. Recent advances in induced pluripotent stem cell (iPSC) technologies for applied regenerative medicine and stem cell research, especially for iPSC-derived cardiomyocytes have increased the hope for heart repair. However, the driver molecules of myocardial differentiation and the functional reconstruction capacity of iPSC-derived cardiomyocytes are still questionable.

Methods: Herein, we established a rapid differentiated platform that is involved in cardiomyogenic differentiation and maturation from iPSCs in vitro. Functional analysis is performed in miR-181a-transfected iPSC-derived cardiomyocyte (iPSC-cardio/miR-181a) under a time-lapse microscope. In addition, we calculated the beating area and frequency of iPSC-cardio/miR-181a cells in the presence of HCN4 shRNA or miR-181a SPONGE.

Results: miR-181a enhanced the beating area and maintained the beating frequency of iPSC-derived cardiomyocytes by enhancing HCN4 expression.

Conclusion: miR-181a would play a key role on maintaining proper beating function in iPSC-derived cardiomyocytes.

Keywords: Cardiomyocyte; Induced pluripotent stem cell (iPSC); Myocardial differentiation

1. INTRODUCTION

Ischemic heart diseases remain the top cause of mortality worldwide and are frequently characterized by damage of cardiomyocytes that is difficult to repair. The irreparable damage to cardiomyocytes is largely due to the cells' low regenerative activity—following ischemic syndrome is the major obstacle for patients to overcome. Because postnatal cardiomyocytes have little or no regenerative capacity, current therapeutic approaches are limited. Although the adult heart contains cardiac stem cells that can be differentiated into mature cardiomyocytes and are, therefore, a candidate source for cardiac cell transplantation therapy,[1] recently, reprogrammed cells, such as induced pluripotent stem cells (iPSCs), have been shown to acquire self-renewal properties and to modulate the oxidative stress pathway, thus creating a rejuvenated state that is capable of avoiding cellular senescence and acquiring pluripotent differentiation potential.[2,3] MicroRNAs (miRNAs) are short noncoding RNAs that negatively regulate gene expression at the posttranscriptional level by inhibiting mRNA translation or enhancing mRNA degradation. There is evidence of miRNA deregulation in heart diseases such as cardiac hypertrophy,[4] congestive heart failure,[5] and myocardial infarction.[6] Several miRNAs that are involved in the inflammatory macrophage response, such as miR-21, miR-34a, miR-210, and miR-146, were upregulated in atherosclerotic plaques.[7] This study thus developed an iPSC-derived myocardial differentiation technique. The findings indicated that miRNAs, particularly miR-181a, are upregulated during myocardial cell differentiation. Overexpression of miR-181a efficiently increased the beating area of cultured iPSC-differentiated cardiomyocytes without affecting the beating frequency, leading

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to enhance cardiomyocyte differentiation. With these results and knowledge, we may be able to provide a method to efficiently generate functional cardiomyocytes that may improve the development of cell therapy and tissue engineering in the treatment of patients suffering from ischemic heart diseases.

2. METHODS

2.1. iPSC culture and in vitro myocardiogenic induction

As followed the previous protocol, the iPSCs derived from mouse strain was generated from mouse embryonic fibroblasts (MEFs) derived from 13.5-day-old embryos of C57/B6 mice. In brief, iPSC was first reprogrammed by transduction with retroviral vectors encoding 4 transcription factors (Oct-4/Sox2/Klf4/c-Myc) as the standard protocol.[8] The undifferentiated iPSCs were routinely cultured and expanded on mitotically inactivated MEFs (50 000 cells/cm^2) in 6-well culture plates (BD Falcon) with iPSC medium consisting of DMEM (Sigma-Aldrich, Invitrogen) with 15% fetal bovine serum (FBS; Sigma-Aldrich, Invitrogen), and 100 mM minimal essential medium nonessential amino acids (FBS; Sigma-Aldrich, Invitrogen).[9] Before the myocardiogenic induction, 2 × 10^3 embryonic stem cells (ESCs) or iPSCs were hanging drops to form the embryoid body (EB) for 3 days. The differentiation medium was composed of Iscove's modified Dulbecco's medium (Sigma-Aldrich, Invitrogen) and was supplemented with 5% fetal calf serum, 0.2 mmol/L l-glutamine, 0.1 mmol/L β-mercaptoethanol, and 0.1 mmol/L nonessential amino acid stock. Each EB outgrowth was examined daily for spontaneously beating areas.[10]

2.2. Digital ratio imaging for intracellular calcium

The intracellular calcium was measured as described previously using ratio imaging.[8] Differentiated cells were treated with 5 L M Fura-2 AM in the loading buffer for 30 minutes at 37°C. The coverslips were washed 3 times in the loading buffer and then mounted in a recording chamber on the platform of

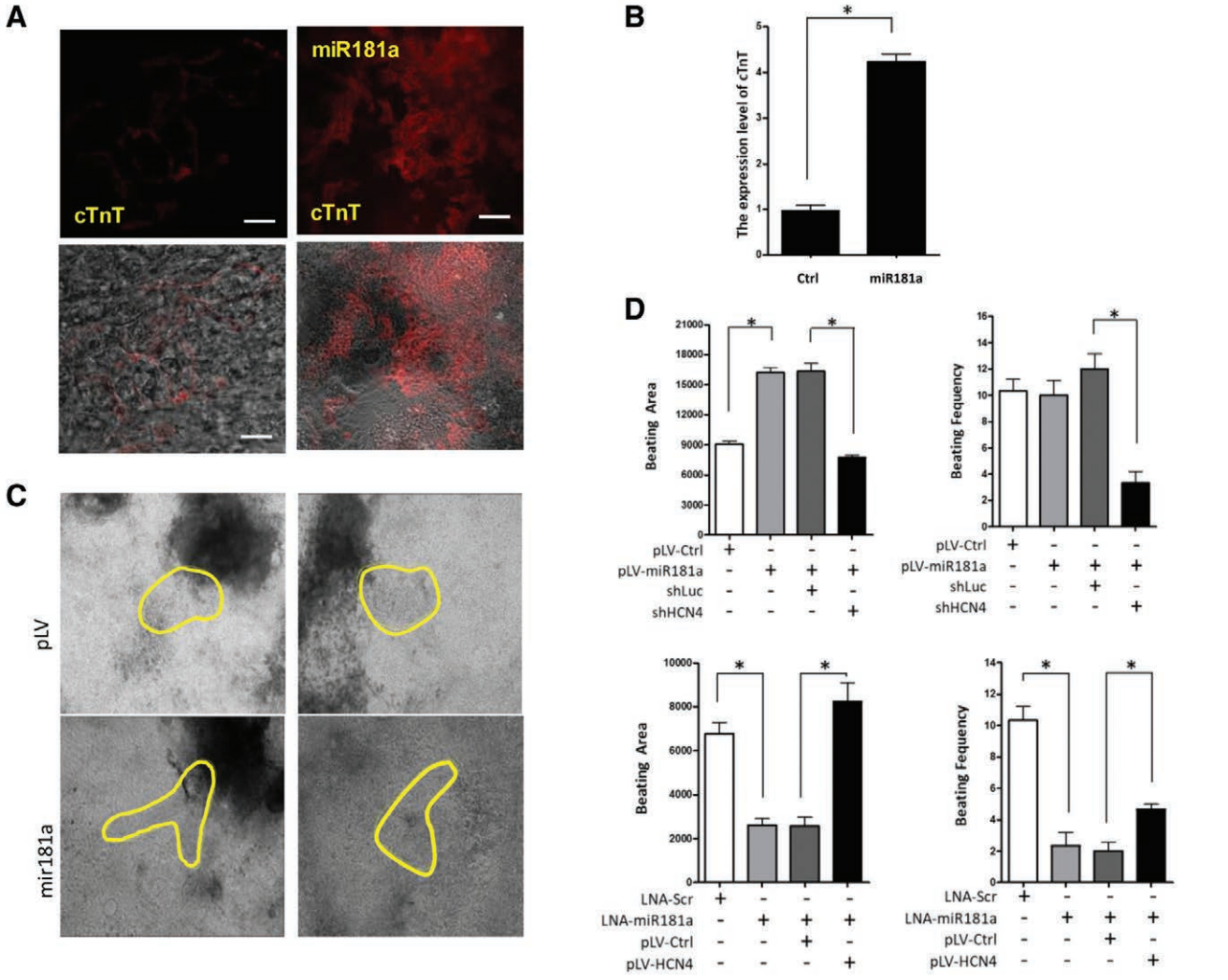

Fig. 1 Delivery of miR-181a into iPSC cells to promote the cardiogenic differentiation. A, Using immnunofluorecence assay to detect the protein expression of cTnT in the overexpressed miR-181a-treated iPSC-differentiated cells. Bar = 50 μm. B, Quantitative results of immunofluoresence were shown in (B). C, Evaluation of the beating ability and frequency with synchronized rhythm monitored by time-laps microscope. Using imaging recording with Image J software, quantification of the beating area of the overexpressed miR-181a iPS-cardio/miR-181a was significantly larger than those of iPS-cardio/pLV. D, Evaluation of the beating area and frequency in iPSC-derived cardiomyocytes with overexpression or knockdown of miR-181a, and treated with shHCN4 or pLV-HCN4, respectively. *$p < 0.05$. Data shown here are the mean ± SD of 3 independent experiments. iPSC = induced pluripotent stem cell.

an inverted epifluorescence microscope. The cells were stimulated with high K^+ buffer (150 mM) at the indicated conditions. When measuring $[Ca^{2+}]^i$ using the ratio imaging method as described above, micropipetting with a tip 2.l-m diameter opening was used for the delivery of very small amounts (picoliter to nanoliter) of reagents locally by a 15-psi ejection pulse for 0.5 s (Picospritzer II; General Valve, Fairfield, NJ, USA). A $[Ca^{2+}]^i$ ratio image with excitations of 340 and 380 nm was collected every 0.5 s using an imaging system with a high-speed cooled CCD camera (Micro-MAX: 782YHS, Princeton Instruments, Roper Scientific, Trenton, NJ, USA) and a xenon lamp within a monochromator as an excitation light source (Polychrome II, T.I.L.L. Photonics, Germany). Images were acquired with the software Axon Image Workbench 2.0 (Axon Instruments, Foster City, CA, USA).

2.3. Statistical analysis

The results are expressed as mean ± SD. Statistical analyses were performed using the *t*-test for comparing two groups, and 1-way or 2-way ANOVA, followed by Bonferroni's test, was used to detect differences among 3 or more groups. The correlation between expression levels and age was analyzed by Pearson's correlation coefficient and unpaired Student *t* test. Results were considered statistically significant at $p < 0.05$. All analyses were performed using SPSS 12.0.

3. RESULTS

Recent studies have shown that iPSCs are capable of differentiation into functional cardiomyocytes after incubation in cardiac differentiation medium. We generated mouse iPSCs by overexpressing four genes Oct4/Sox2/Klf4/c-Myc (mRe-7 iPSC clone). At 2 weeks postdifferentiation, immunofluorescence staining further showed that cTnT was increased by 4-fold at the cell junctions after miR-181a delivery (Fig. 1A, B). The beating capacity is one of the most crucial functions of heart muscle and requires a synchronized beating rhythm without random frequency. To examine the effect of miR-181a delivery on this crucial cardiomyocyte characteristic, we further compared the miR-181a-transfected iPSC-derived cardiomyocyte-like cells (iPSC-cardio/miR-181a) with vector control-delivered cells (iPSC-cardio/Ctrl) under a time-lapse microscope. As shown in Figure 1C, the beating area (enclosed by yellow lines) of the iPSC-cardio/miR-181a cells was larger that in the iPSC-cardio/Ctrl cells.

The potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4) is prominently expressed in the pacemaker region of the mammalian heart.[11] Cardiac HCN4 channels are essential for normal heart impulse generation and conduction.[11] Patients with bradycardia and sick sinus syndrome have been reported to express mutated HCN4.[12,13] To determine whether HCN4 is involved in the miR-181a-mediated elevation of the myocardiac beating function, we calculated the beating area and frequency of iPSC-cardio/miR-181a cells in the presence of shRNA specifically against HCN4 (Figure 1D, upper). Consistent with our hypothesis, HCN4 knockdown reduced the miR-181a-increased beating function; both the beating area and frequency were decreased (Fig. 1D, upper). However, miR-181a knockdown by SPONGE decreased the beating area and frequency, while HCN4 overexpression increased both parameters in cardiomyocytes (Fig. 1D, lower). Taken together, these data demonstrated that miR-181a enhanced the beating area and maintained the beating frequency of iPSC-derived cardiomyocytes by enhancing HCN4 expression, possibly via an indirect regulatory mechanism.

4. DISCUSSION

It is the key issue to improve of induction rates of cardiomyogenic differentiation from pluripotent stem cell. Although pluripotent stem cells, including ESC and iPSCs, have been recently shown to be successfully differentiated into liver and neuronal cells under well-defined culture conditions, revealing the potential application of iPSC-derived cardiomyocytes for heart tissue repair, the induction efficiency of iPSC-derived tissue is still low. Moreover, the driver molecules of myocardial differentiation and the functional reconstruction capacity of iPSC-derived cardiomyocytes are still questionable. Herein, we demonstrated that mouse iPSC-differentiated cardiomyocytes. Using an in-vitro differentiation model, we identified miR-181a as being upregulated during the myocardiac differentiation process. Functional analysis indicated that miR-181a increased the beating area of cultured iPSC-differentiated cardiomyocytes without altering the beating frequency, indicating that miR-181a may not only promote cardiomyocyte differentiation but may also enhance the reconstructed beating function. These data suggested the importance of miR-181a in maintaining proper beating function. Interestingly, quantitative RT-PCR analysis revealed that miR-181a overexpression increased the HCN4 mRNA expression level, whereas HCN4 overexpression did not alter the miR-181a level (data not shown). Importantly, our findings supported that miR-181a would be a key play to largely enhance the beating area and promote the beating frequency of iPSC-derived cardiomyocytes by simultaneously increasing HCN4 expression. Therefore, the further exploration of delivery of miR-181a may have clinical potential for enhancing the efficiency of heart-lineage differentiation from iPSCs, as well as may promote the beating function of maturing cardiomyocytes and heart capabilites.

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