Nanodiamond-based microRNA delivery system promotes pluripotent stem cells toward myocardiogenic reprogramming

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Abstract

Background: Gene therapy is the advanced therapeutics for supplying or replacing the genetic material in patients with inherited disorders. Recent clinical studies have made some progress in a wide range of applications, including monogenic disorders, neurodegenerative diseases, malignant tumors, and congenital diseases. 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.

Methods: Most advances in induced pluripotent stem cell (iPSC) technologies for promoting regenerative medicine and stem cell research. However, the driver molecules of myocardial-lineage differentiation and the functional reconstruction capacity of iPSC-derived cardiomyocytes are still an open question. Nanomedicine-based gene delivery provided a crucial platform to carry on the biogenomic materials for equipping functionalities and engineering the living organ environment. Nanodiamond (ND), a carbon-based nanomaterial, has been discovered and shown the high biocompatible and less toxicity for transporting protein, drug, and genomic plasmids.

Results: Here, we applied ND as a gene delivery vehicle to carry microRNA (miR-181a), and then transfected into iPS to promote cardiomyocyte-lineage differentiation. Notably, miR-181a plays a key role in iPS-derived cardiomyocyte differentiation which directly targets Hox-A11, leading to elevated MyoD expression and enhanced cardiomyocyte differentiation.

Conclusion: Our study demonstrated that miR-181a promotes iPSC differentiation into functional cardiomyocytes. Delivery of NANO-DIAMOND-miR-181a may host clinical potential to enhance the differentiation and recovery of the cardiogenic function in injured cardiomyocytes.

Keywords: Cardiomyocytes; Induced pluripotent stem cell; MicroRNA; Nanodiamond

1. INTRODUCTION

Gene therapy is a biological technology method that utilizes genes to treat genetic disorders or prevent diseases.[1] Moreover, this strategy is thought to permit medical doctors to treat a disorder by delivering a gene into patients' cells as an alternative method for medications or medical procedures.[2–4] Scientists are trying a few ways to deal with gene therapy, including replacing a mutated gene that causes disease with a normal copy of the gene. Knocking out is to take out the mutated gene that is working inappropriately, bringing another gene into the cell of an individual to help fight a genetic disorder. Even though gene therapy is a promising therapeutic alternative for various diseases (including inherited disorders, malignant cancers, and certain types of viral infections), the method stays hazardous is as yet under investigation to ensure that it will be safe and successful. Gene therapy is now being tested distinctly for diseases that cannot be cured by medicine.

It has been widely accepted that postnatal cardiomyocytes carry little or no regenerative potential. Therefore, damaged cardiomyocytes are generally difficult to be repaired in several lethal heart diseases, such as ischemic heart diseases. The development

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and progress of stem cell biology and regenerative medicine have drawn attention from clinical physicians and provided opportunities for cell therapy against heart diseases. Cardiac stem cells, somatic stem cells identified from the heart and can differentiate into cardiomyocytes with mature functions, may represent a source of cell therapy for heart diseases.[5] However, such cells were also found to exist only in a little amount and carry limited differentiation potential. Interestingly, Yamanaka and his research teams have demonstrated that forced expression of reprogramming factors Oct4/Sox2/Klf4/c-Myc can potentially drive somatic cells to be reprogrammed into induced pluripotent stem cells (iPSCs) that can proliferate unlimited and carry the ability for undergoing tridermal differentiation.[6,7] The progress of iPSC technologies and tissue engineering have rendered such pluripotent cells as excellent options for drug screening, in vitro disease modeling, and investigations of the signaling pathway. The potential of iPSCs to differentiate into functional cardiomyocytes also highlight their bioavailability as a candidate source in cell therapy for life-threatening heart diseases.

Nanodiamond (ND), a carbon-based nano-sized material, has been used to deliver drugs, biomaterials, and biochemicals.[8] Recent study progress showed that NDs could perform the carry on the genomics, including DNA and RNA.[9] However, it is still undetermined whether ND could carry on microRNA (miR) to promote stem cells toward cardiogenic reprogramming. In this study, we examined the effect of miR-181a on cardiogenic differentiation in iPSCs using ND as a nonviral miR delivery system. Meanwhile; however, the mechanisms for the miR-181a regulatory effect on cardiogenesis are largely unclear. Next, we investigated the mechanisms and the signaling molecules and regulatory pathways involved in the miR-181a-enhanced cardiogenesis.

2. METHODS

2.1. The cell culture for iPSC and cardiomyocyte-lineage differentiation

Mouse iPSCs were generated from the embryonic fibroblasts from 13.5-day old embryos of C57/B6 mice and reprogrammed via the introduction of retroviral factors encoding Oct4/Sox2/Klf4/c-Myc. Undifferentiated iPSCs were routinely cultivated and expanded on mitotically inactivated embryonic fibroblasts as described previously.[10] For the cardiogenic induction, iPSCs were hanging drops for embryoid body formation for 3 days. The composition of the differentiation medium included Iscove's modified Dulbecco's medium (Sigma-Aldrich, Invitrogen, Carlsbad, CA, USA), 5% fetal calf serum, 0.2 mM l-glutamine, 0.1 mM β-mercaptoethanol, and 0.1 mM nonessential amino acid stock. The spontaneous beating areas for each embryoid bodies were examined daily after the addition of the differentiation medium.[11]

2.2. Western blot analysis

The cells were fixed, washed once in cold phosphate buffered saline, scraped, lysed with extraction buffer, and centrifuged at 10 000 rpm (9730*g*) for 10 minutes to remove insoluble material. Protein concentrations were determined using a protein assay kit (Bio-Rad, Hercules, CA, USA). The cell extracts in the sample buffer were placed in boiling water for 5 minutes and then separated by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred onto a polyvinylidene difluoride membrane for immunoblotting. The membrane was blocked by incubation in nonfat milk at room temperature for 0.5 hours and was then incubated with Hox-A11 (1:1000; Cell Signaling Technology), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:10 000; Genetax), MyoD, Cx43 (1:2000), and pCx43 (1:2000) antibodies for 16 hours at 4°C; washed five times with tris-buffered saline tween-20 (TBST), and incubated at room temperature with horseradish peroxidase-conjugated secondary antibody for 2 hours. The membrane was washed six times with TBST, and specific bands were made visible by chemiluminescence (ECL, Santa Cruz).

2.3. Real-time reverse transcription-polymerase chain reaction

Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed as previously described.[12] For real-time RT-PCR, total RNA was extracted using an RNAeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Total RNA (1 μg) of each sample was reverse-transcribed in a 20-μL reaction using 0.5 μg of oligo-dT and 200 U Superscript II reverse transcriptase (Invitrogen). The amplification was performed in a total volume of 20 μL containing 0.5 μM of each primer, 4 mM $MgCl_2$, 2 μL of LightCycler (TM)–FastStart DNA Master SYBR green I (Roche Molecular Systems, Alameda, CA, USA), and 2 μL of 1:10-diluted cDNA. PCRs were performed using the ABI PRISM 7900HT Sequence Detection System and the ABI Prism 5700 SDS (Applied Biosystems). In each experiment, the GAPDH housekeeping gene was amplified as a reference standard. The reactions were prepared in duplicate and heated to 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 55°C for 5 seconds, and extension at 72°C for 20 seconds. All PCRs were performed in duplicate. Standard curves (cycle threshold values versus template concentration) were prepared for each target gene and for the endogenous reference (GAPDH) in each sample. To confirm the specificity of the PCR, the PCR products were electrophoresed through a 1.2 % agarose gel.

2.4. Statistical analysis

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

3. RESULTS

Recently, we have found miR-181a as an upregulating factor during the cardiogenic induction. We further used NANO-DIAMOND,[13–15] a nonviral delivery vector, to deliver miR-181a into iPSCs, leading to the enhanced cardiac differentiation from iPSCs to iPSC-derived cardiomyocytes (iPSC-CMs) (Fig. 1A). In this study, we first attempted to examine whether miR-181a can be also introduced into iPSCs by another nonviral vehicle carboxylated nanodiamond (cND) (Fig. 1B). ND particles were functionalized by the carboxylation of its surface and were covalently bonded with mCherry protein. The linear miR-181a DNA construct was then attached to the bound mCherry via the phosphoryl imidazole bond between the imidazole group on the 6 His-Tag tail of mCherry and the DNA phosphate group. After introducing the cND-mCherry/miR-181a and the cardiac induction cocktails to iPSCs, the cND-mediated delivery upregulated miR-181a and led to an expected miR-181a effect that both increased the cTnT expression and the beating areas in iPSC-CMs (data not shown), similar to the observations of miR-181a delivery by NANO-DIAMOND in our previous report.[16]

Previously, NANO-DIAMOND-mediated delivery of miR-181a was found to putatively target Hox-A11 3′UTR.[17] Herein, we sought to use western blot to examine the signaling cascade

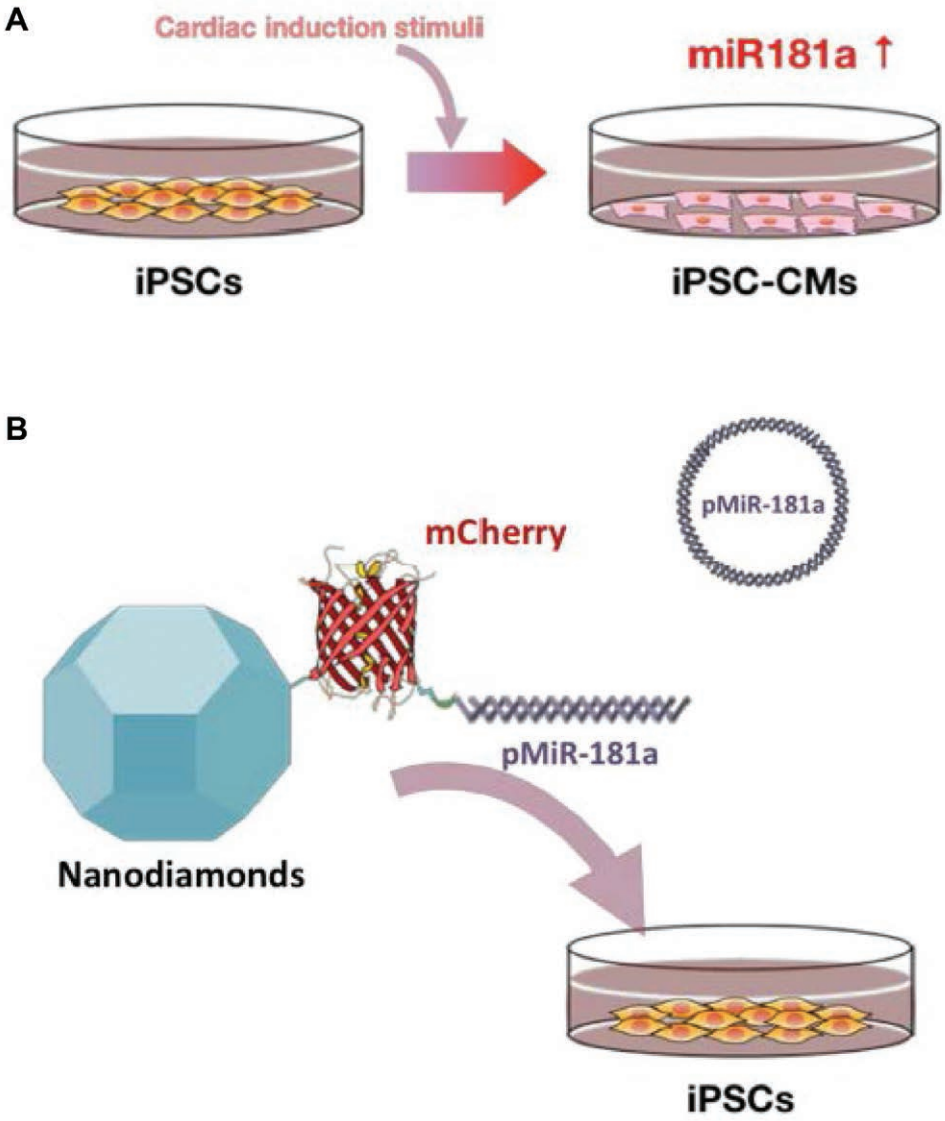

Fig. 1 MiR-181a as an upregulating factor during the cardiogenic induction. A, NANO-DIAMOND, a nonviral delivery vector, to deliver miR-181a into iPSCs. B, miR-181a can be also introduced into iPSCs by another nonviral vehicle carboxylated nanodiamond (cND).

in cND-mediated miR-181a-overexpressing iPSC-CMs and investigate the role of Hox-A11 in the signaling. cND-mediated miR-181a overexpression was found to suppress Hox-A11 but increase MyoD protein amount in iPSC-CMs (Fig. 2A). Lentiviral overexpression of Hox-A11 led to a direct inhibition on MyoD content, whereas overexpression of MyoD showed no effect on Hox-A11 (Fig. 2A). These findings suggested that miR-181a directly targeted Hox-A11 3´UTR and suppressed its protein content, and subsequently promoted the downstream MyoD protein upregulation. To confirm this interpretation, we further used cND to deliver Sponge181a to knock down miR-181a, and examined the consequence of Hox-A11 or MyoD knockdown on these iPSC-CMs with reduced miR-181a expression (Fig. 2B). miR-181a knockdown largely increased Hox-All while abolished MyoD protein expression. Meanwhile, Hox-All knockdown restored the abolishment of MyoD protein induced by miR-181a knockdown (Fig. 2B). Lentiviral-mediated MyoD knockdown did not modify Hox-Al1 protein expression, indicating that Hox-A11 is the upstream regulator of MyoD.

Next, we further examined the interrelationship among miR-181a, Hox-A11, and MyoD at mRNA levels using quantitative real-time PCR (qRT-PCR). We hypothesized that in cardiomyocytes, miR-181a mediates the differentiation process through regulation of Hox-A11/MyoD axis. To test this hypothesis, we overexpressed miR-181a in iPSCs using the NANO-DIAMOND delivery method and subsequently differentiated the iPSCs into cardiomyocyte-like cells. Compared with the vector control, miR-181a overexpression suppressed the Hox-A11 protein level and elevated MyoD protein expression on day 6 post-induction of cardiomyocyte differentiation (Fig. 3). The elevated MyoD expression was reversed by co-overexpression of Hox-A11, whereas the co-overexpression of MyoD did not change the expression level of Hox-A11. To further confirm these results, we suppressed miR-181a expression in iPSCs using the SPONGE strategy before the induction of differentiation, which resulted in elevated Hox-A11 and suppressed MyoD expression 6 days after cardiomyocyte differentiation. This effect was reversed by the co-knockdown of Hox-A11 but not Myo-D. To investigate the causal effects of the expression of miR-181a, Hox-A11, MyoD, and cardiomyocyte-specific markers, we measured the mRNA levels of cTnT, MLC2a and HCN-4 in iPSCs

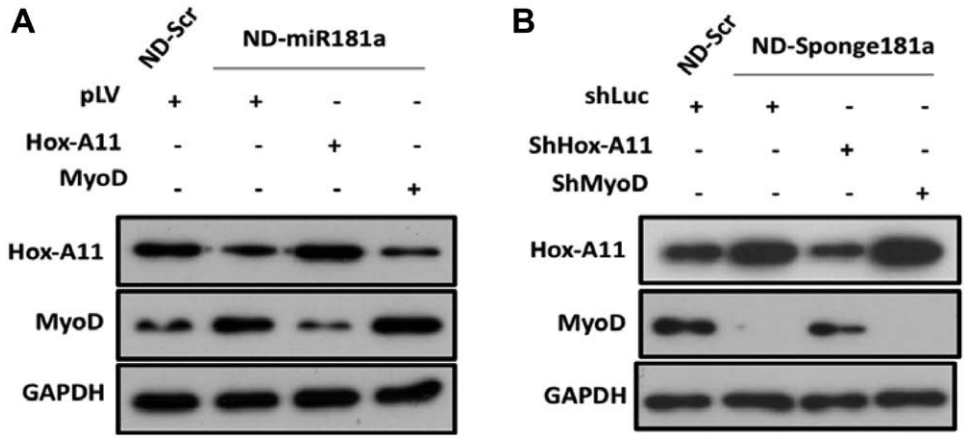

Fig. 2 Western blot to examine the signaling cascade in cND-mediated miR-181a-overexpressing iPSC-CMs and investigate the role of Hox-A11 in the signaling. A, cND-mediated miR-181a overexpression was found to suppress Hox-A11. B, cND to deliver Sponge181a to knock down miR-181a.

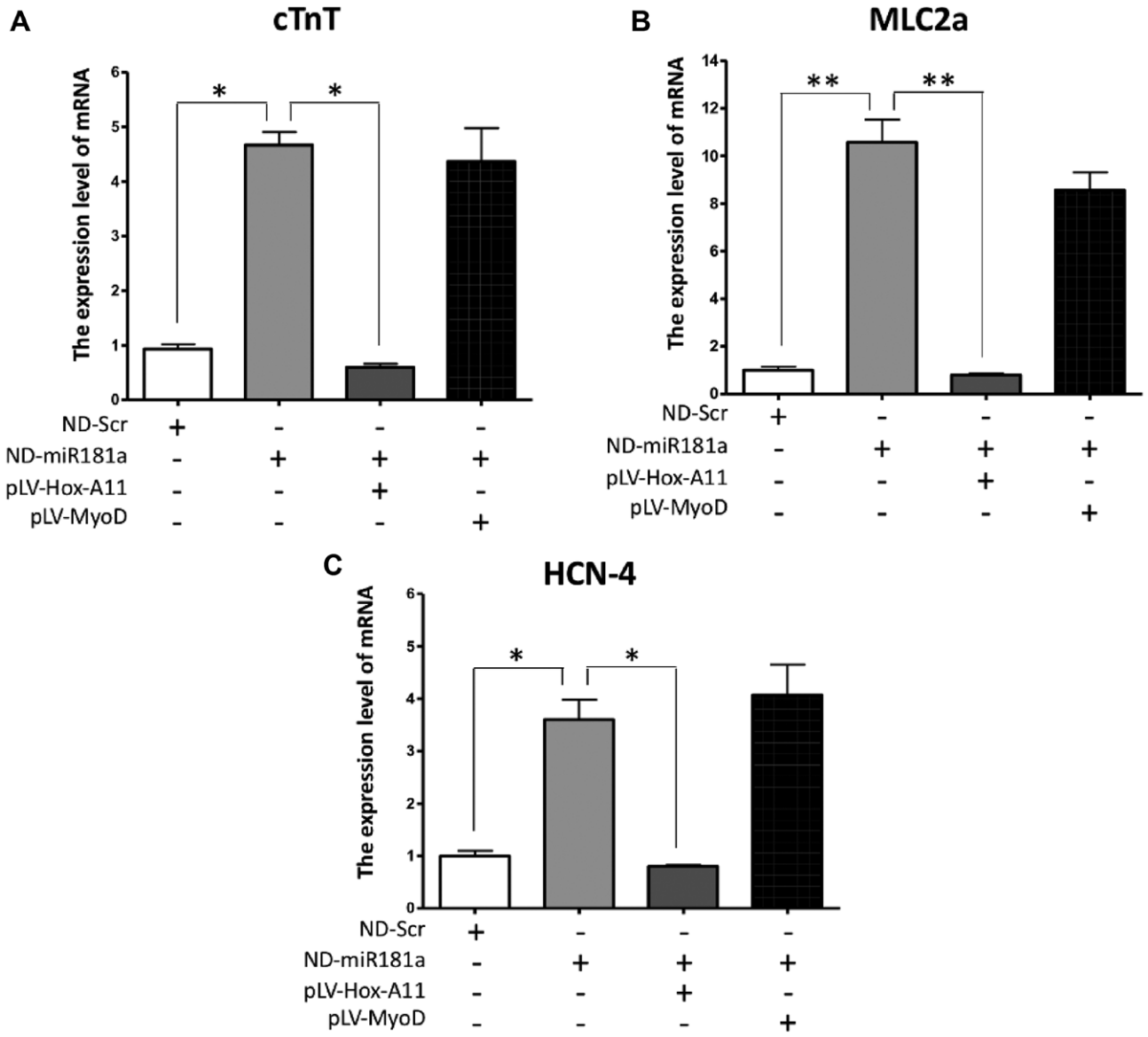

Fig. 3 The interrelationship among miR-181a, Hox-A11, and MyoD at mRNA levels using quantitative real-time polymerase chain reaction (qRT-PCR). The mRNA levels of A, cTnT, B, MLC2a and C, HCN-4 in iPSCs transfected with miR-181a, Hox-A11, and MyoD.

transfected with miR-181a, Hox-A11, and MyoD (Fig. 3A–C). As shown in Fig. 3, all three markers were elevated by miR-181a. Co-overexpression of Hox-A11, but not MyoD, suppressed the mRNA levels of these markers down to control levels. These data indicated that miR-181a negatively regulates Hox-A11 and consequently leads to elevated MyoD expression during cardiomyocyte differentiation, which may partially explain how miR-181a positively regulates cardiac differentiation.

4. DISCUSSION

Hox-A11, a homeobox transcription factor that regulates uterine development, is required for female fertility.[18] Recently, Naguibneva and colleagues demonstrated that Hox-A11 is a direct target of miR-181a during mammalian myoblast differentiation.[19] These investigators demonstrated the upregulation of MyoD, an essential factor for muscle differentiation, through the miR-181a-mediated suppression of Hox-A11.[19,20] However, the role of miR-181a-Hox-a11 signaling in cardiomyocytes is still unclear.

Investigations of the mechanism for this miR-181a activity revealed the differentiation inhibitor Hox-A11 as a direct target of miR-181a; miR-181a-mediated suppression of Hox-A11 led to elevated MyoD expression and enhanced cardiomyocyte differentiation. In a mouse model, orthotropic injection of NANO-DIAMOND-miR-181a in I/R hearts showed promising repair efficacy of damaged heart tissue. Therefore, delivery of NANO-DIAMOND-miR-181a may have clinical potential for enhancing the efficiency of cardiac differentiation from iPSCs and may increase the beating function of both maturing cardiomyocytes and ischemic cardiomyocytes. Here, we showed that in iPSC-derived cardiomyocytes, miR-181a directly targets the 3′UTR region of Hox-A11 and releases MyoD from the inhibition of Hox-A11 (Fig. 4). In addition to MyoD, the expression of several cardiac progenitor markers, such as cTnT, MHC, MCK, and Cx43, were all elevated by miR-181a but were suppressed by Hox-A11 co-overexpression, which indicated the involvement of miR-181a-Hox-A11 in cardiomyocyte differentiation. Moreover, elevated HCN4 mRNA levels were also detected in NANO-DIAMOND-miR-181a-delivered cells (Fig. 4). HCN4 is expressed in the pacemaker region of mammalian hearts and is involved in the autonomic control of the heartbeat rate.[21–24] Constitutive HCN4 knockout mice, either global or cardiac-specific, die in utero at embryonic days 10.5-11.5.[24] We showed that overexpression of miR-181a increased HCN4 expression, whereas Hox-A11 suppressed it; correspondingly, miR-181a knockdown resulted in reduced HCN4 expression, suggesting the involvement of miR-181a-Hox-A11 signaling in the control of heartbeats. In support of this observation, the functional analysis indicated a larger beating area in cultured NANO-DIAMOND-miR-181a-delivered cardiomyocytes than in vector control-delivered cells (Fig. 4). How miR-181a-Hox-A11 signaling regulates HCN4 is still unclear, though our data indicate that delivery of NANO-DIAMOND-miR-181a not only enhances cardiomyocyte differentiation but also reconstructs the beating function of the cells.

In this report, we investigated the regulatory mechanism of miRNA that is involved in cardiomyogenic differentiation and maturation from iPSCs; further, we developed a method to reconstruct myocardial functions in iPSC-derived cardiomyocytes by applying the NANO-DIAMOND delivery system. 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.

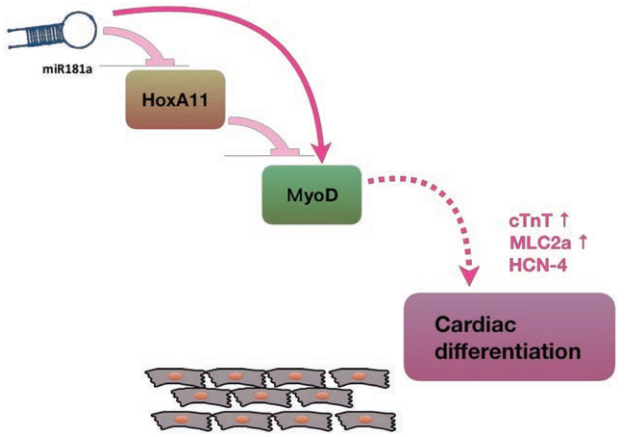

Fig. 4 iPSC-derived cardiomyocytes, miR-181a directly targets the 3′UTR region of Hox-A11 and releases MyoD from the inhibition of Hox-A11.

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