MicroRNA-133a inhibits norepinephrine-induced cardiac hypertrophy via suppression of PKC signaling

Jae Youn Moon

Department of Medicine

The Graduate School, Yonsei University

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Directed by Professor Namsik Chung

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This certifies that the Doctoral Dissertation of Jae Youn Moon is approved.

Thesis Supervisor: Namsik Chung

Ki-Chul Hwang: Thesis Committee Member#1

Young-Geun Kwon: Thesis Committee Member#2

Seung-Hyuk Choi: Thesis Committee Member#3

Sungha Park: Thesis Committee Member#4

The Graduate School
Yonsei University

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ABSTRACT

MicroRNA-133a inhibits norepinephrine-induced cardiac hypertrophy via suppression of PKC signaling

Jae Youn Moon

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Namsik Chung)

Cardiac hypertrophy is associated with the development of heart failure and has been known as a predictor for cardiovascular morbidity and mortality. A recent study unveiled potential regulatory roles for microRNA-133a (miR-133a) in cardiac hypertrophy. However, it has not been studied to elucidate the connection between miR-133a and norepinephrine-induced cardiomyocyte hypertrophy. Here, we investigated the expression and functional role of miR-133a in a norepinephrine-induced hypertrophic cardiomyocyte and determined the target of miR-133a in hypertrophic signaling. Neonatal rat cardiomyocytes were isolated, and cardiac hypertrophy was induced by treatment with 10 μM norepinephrine. In this study, we determined that miR-133a plays a pivotal role in the regulation of norepinephrine-induced cardiac hypertrophy. miR-133a expression is inversely related to cardiac hypertrophy, and the treatment with miR-133a mimics before norepinephrine management prevents norepinephrine-induced cardiac hypertrophy. Second, we found that the direct target of miR-133a is Protein Kinase C δ (PKCδ), which was confirmed by both Luciferase assay and Western blot analysis. We found no relationship between miR-133a and G protein. Finally, after transfection with miR-133a, MEK and ERK, which are in the downstream pathway, and proto-oncogenes such as c-fos, c-myc and c-jun were down-regulated in hypertrophic cardiomyocytes, suggesting that PKCδ is a novel target of miR-133a in cardiomyocytes, and that administration of or treatment with miR-133a mimics could be used in the future as a therapeutic application in the clinical setting.

Key words: microRNA-133a, Cardiac hypertrophy, Protein Kinase C δ, norepinephrine

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I. INTRODUCTION

Heart failure is the predominant global cardiovascular disorder. Cardiac hypertrophy is one of the major factors contributing to heart failure and leading to morbidity and mortality.[1] Pathological cardiac growth is known to occur due to neurohormonal factors such as epinephrine, norepinephrine, angiotensin II, and aldosterone.[2-6]

It is well known that α1-adrenergic agonists may induce cardiac hypertrophy via α1-adrenoceptors (ARs), members of the G-protein coupled receptor (GPCR) superfamily, regardless of regulation by small GTPases (Ras, RhoA, and Rac).[7] Cardiac hypertrophy results from signaling pathways that are initiated by the activation of α1-ARs, including phospholipase C (PLC), D, and A2, protein kinase C (PKC), Ca^{2+} channels, and mitogen-activated protein (MAP) kinases.[8] The activation of PKC and MAP kinases has been reported to be associated with stimulated cardiac growth.[9-12] PKC, which is a family of serine/threonine kinases, may play an important role in cardiovascular diseases including ischemic heart disease, cardiac hypertrophy, heart failure, hypertension and atherosclerosis. The activation of PKCδ has been reported to cause

nonpathological cardiac hypertrophy via MAPK signaling during cardiomyocyte hypertrophy.[13] Cardiomyocyte hypertrophy is associated with up-regulated changes in proto-oncogenes such as c-fos, c-jun, and c-myc.[14, 15]

MicroRNAs (miRNAs) are short non-coding RNAs (21-25 nucleotides) that regulate gene expression at the post transcriptional level either by degrading the target mRNA or by direct translational inhibition.[16] miRNAs negatively regulate gene expression via translation repression, mRNA degradation, or deadenylation binding to partially complementary sequences in the 3'untranslated region (3' UTR).[17] Recent studies have suggested that miRNAs are expressed in the failing myocardium and play an important role in progression of heart failure by targeting genes in the cardiac remodeling process including hypertrophy, excitation-contraction coupling, increased cardiomyocyte loss, and myocardial fibrosis.[18, 19] Among the several miRNAs involved, microRNA-133a (miR-133a) is known as a regulator of cardiomyocyte hypertrophy and failure. Several studies have revealed that several growth-related genes, including RhoA and Cdc42, act as miR-133a target genes, suggesting a potential regulator of miRNAs in cardiac hypertrophy.[20] Although anti- and pro-hypertrophic miRNAs have been studied in cardiac hypertrophy, the roles of miRNA-133a in a norepinephrine-induced cardiac hypertrophy model are not clearly understood.

Here, we examined the expression and functional role of miR-133a in a norepinephrine-induced cardiomyocyte hypertrophy model and investigated the target of miR-133a in hypertrophic signaling.

II. MATERIALS AND METHODS

1. Isolation of neonatal rat cardiomyocytes

Neonatal rat cardiomyocytes were isolated and purified by enzymatic methods. Briefly, hearts of one- to two-day-old Sprague–Dawley rat pups were dissected, and the ventricles were treated with Dulbecco's phosphate-buffered saline solution (pH 7.4, Gibco BRL, Grand Island, NY) free from Ca^{2+} and Mg^{2+}. Using micro-dissecting scissors, the hearts were minced until the pieces were approximately 1 mm^3 and were then treated with 10 ml of collagenase II (0.8 mg/ml, 262 U/mg, Gibco BRL) for 5 min at 37℃. The supernatant was removed, and the tissue was treated with fresh collagenase II solution for an additional 5 min. The cells in the supernatant were transferred to a tube containing cell culture medium (α-MEM containing 10% fetal bovine serum, Gibco BRL). The tubes were centrifuged at 2000×g for 3 min at room temperature, and the cell pellet was resuspended in 5 ml cell culture medium. The above procedures were repeated seven to nine times. Cell suspensions were collected and incubated in 100-mm tissue culture dishes for 2 h to reduce fibroblast contamination. Non-adherent cells were collected and seeded to achieve a final concentration of 5×10^5 cells/ml. After incubation for 4–6 h, cells were rinsed twice with cell culture medium, and 100 μM BrdU was added. Cells were then cultured in a CO_2 incubator at 37℃. For stimulation with norepinephrine (10^{-5}M), the confluent cells were rendered quiescent by culturing them for 12 h in 0.1% (v/v) FBS.

2. Immunocytochemistry

Cells were grown on four-well plastic dishes, washed twice with PBS, and fixed with 4% paraformaldehyde in 0.5 ml of PBS for 30 minutes at room

temperature. The cells were washed again with PBS and then permeabilized for 30 minutes in PBS containing 0.2% Triton. The cells were blocked in PBS containing 10% goat serum and incubated for 1 hour with primary antibodies. The cells were washed with PBS three more times for 10 minutes and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG as the secondary antibody for 1 hour. All images were produced using an excitation filter under reflected light fluorescence microscopy and transferred to a computer equipped with MetaMorph software ver. 4.6 (Universal Imaging Corp., Marlow, U.K.).

3. Immunoblot analysis

Cells were washed once in PBS and lysed in lysis buffer (Cell Signaling Technology, Beverly, MA, USA) containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM Na_2-EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Protein concentrations were determined using the Bradford Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Proteins were separated in a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidenedifluoride membrane (Millipore, Billerica, MA, USA). After blocking the membrane with Tris-buffered saline-Tween 20 (TBS-T, 0.1% Tween 20) and 5% nonfat dried milk for 1 hour at room temperature, the membrane was washed twice with TBS-T and incubated with primary antibody for 1 hour at room temperature or overnight at 4℃. The membrane was washed three times with TBS-T for 10 minutes and incubated for 1 hour at room temperature with horseradish peroxidase-conjugated secondary antibody. After extensive washing, bands were detected using an enhanced chemiluminescence reagent (ECL) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Band intensities were quantified

using the Photo-Image System from Molecular Dynamics (Sunnyvale, CA, USA).

4. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted using TRIzol (Sigma, 500 μL/60 mm-diameter plate). Chloroform (100 μL) was added to the extract, and each sample was vortexed for about 10 sec. Next, centrifugation at 12,000×g, at 4℃ for 15 min caused three layers to separate, and the transparent upper layer was collected in a new tube. Each sample received 250 μL 2-propranolol and was vortexed for about 30 sec, followed by centrifugation at 12,000×g, at 4℃ for 10 min. The supernatant was discarded, and the pellet was washed in 75% (v/v) ethanol admixed with 0.1% diethylpyrocarbonate (DEPC; Sigma) solution. The sample was then centrifuged at about 7,500×g, at 4℃ for 5 min. The supernatant was discarded, and the pellet was dried at room temperature for about 7 min. Finally, each pellet was dissolved in 30 μL nuclease-free water (NFW). The quality and quantity of RNA were estimated by calculation of OD260/OD280 ratio using a DU 640 spectrophotometer (Eppendorf, Hamburg, Germany).

Complementary DNA was generated with the Promega Reverse Transcription System according to the manufacturer's instructions. Briefly, 1 μg of total RNA was reverse-transcribed in a 20 μl reaction volume containing 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 9.0 at 25℃), 50 mM KCl, 0.1% Triton X-100, 1 mM dNTP mix, 20 U of RNase inhibitor, 0.5 μg oligo-(dT) 15 primer, and 10 U of reverse transcriptase for 15 minutes at 42℃. The reaction was terminated by heating at 99℃ for 5 minutes. The PCR mix contained 10 mM of each primer, together with 200 mM Tri-HCl (pH 8.8), 100 mM KCl, 1.5 mM $MgSO_4$, 1% Triton X-100, 0.1 mM dNTPs mix, and 1.25 U of Taq polymerase in a total volume of 25 μl. PCR conditions consisted of denaturing at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94℃ for 30 seconds, annealing at 49℃ for 30

seconds, and extension at 72℃ for 2 minutes before a final extension at 72℃ for 10 minutes. RT-PCR products were separated by electrophoresis on a 1.2% agarose gel (BioRad), and a Gel-Doc (BioRad) was used to visualize the bands after staining with ethidium bromide.

5. MicroRNA Transfection

Mature miR-133a mimics and anti-miR-133a were purchased (Genolution Pharmaceuticals, Inc., Korea) and used at final concentrations of 100 nM (miR-133a) and 20 nM (anti-miR-133a). Transfections with miRNA-133a mimics were performed using siLentFect™ Lipid reagent (Life Science Research, CA, USA). After transfection with miR-133a for 4hr in CO_2 incubator maintaining 37℃, the medium was changed, and the cells were treated with norepinephrine.

6. Quantification of Total protein and DNA from cardiomyocytes

Total protein content/DNA ratio was measured in cardiomyocytes after lysis of the cells in 1 N NaOH at 37℃ for 30 min. Protein content was examined using BCA protein reagent (Pierce Biotechnology, Inc. Rockford, USA) with a bovine albumin standard following the manufacturer's directions. For the quantitative measurement of DNA, cells were lysed by adding SDS and proteinase K, and extraction of DNA was performed using phenol. The absorbance of purified DNA was measured at 260 nm.

7. Luciferase assay

The predicted target gene of miR-133a was retrieved using a publicly available database (TargetScan, www.targetscan.org). Portions of the PKCδ 3' UTR were

cloned into the pmiR-GLO vector, and the recombinant pmiR-GLO vectors were transfected into HeLa cells. HeLa cells were plated at $2.5x10^4$ cells/well in 24-well. After 48 h, the pmirGLO vector containing the PKCδ binding site for miR-133a was co-transfected with the negative control using Lipofectamine 2000 (Invitrogen, CA, USA). Renilla luciferase was used to normalize cell number and transfection efficiency. Luciferase activity was measured by the Dual Luciferase assay (Promega) using a luminometer (Promega) according to the manufacturer's instructions. Each assay was repeated 3 times.

8. Real-time polymerase chain reaction (PCR)

Total RNA was isolated using Trizol® reagent (Sigma), according to the manufacturer's conditions. In brief, 100 ng purified total RNA was used for reverse transcriptase (Taqman® MicroRNA Reverse Transcriptase Kit, Applied Biosystems) in combination with Taqman® MicroRNA Assays for quantification of specific miRNAs and U6 control transcripts. Amplification and detection of specific products were performed in a TaqMan small RNA Assay kit (AB Applied Biosystems) at 95℃ for 10 min, followed by 40 cycles of 95℃ for 15 seconds and 60℃ for 60 seconds. The threshold cycle (Ct) of each target gene was automatically defined, located in the linear amplification phase of the PCR, and normalized to the control RNU19 (ΔCt value). The relative difference in expression levels of each miRNA was calculated (DDCt) and is reported as fold induction (2-DDCt). The sequences of miRNA primers were:

miR-1: 5'-UGGAAUGUAAAGAAGUGUGUAU-3',
miR-26a: 5'- UUCAAGUAAUCCAGGAUAGGCU-3',
miR-133a: 5'- UUUGGUCCCCUUCAACCAGCUG -3',
miR-143: 5'-UGAGAUGAAGCACUGUAGCUCA-3',
miR-145: 5'-GUCCAGUUUUCCCAGGAAUCCCU-3',

miR-181: 5’- AACAUUCAACGCUGUCGGUGAGU-3’
miR-210: 5’-CUGUGCGUGUGACAGCGGCUGA-3’.

9. Statistical analysis

Data are expressed as means ± SEM. The significance of differences between groups was assessed by Student’s t-test. More than two groups were compared using one-way ANOVA with a Bonferroni correction. P-values<0.05 were considered significant.

III. RESULTS

1. Norepinephrine induces cardiac hypertrophy

Cardiomyocyte hypertrophy was stimulated in response to norepinephrine, which is an agonist of the α_1-adrenoceptor. The cell surface area of the cardiomyocytes increased by 130% after treatment with 10 μM norepinephrine for 72 hr, and the cellular protein content increased by 40% compared to the control. Myosin light chain 2v (MLC 2v) expression level as a hypertrophic marker increased to 200% in norepinephrine-treated cardiomyocytes at 24 hr in a time-dependent manner (Fig. 1).

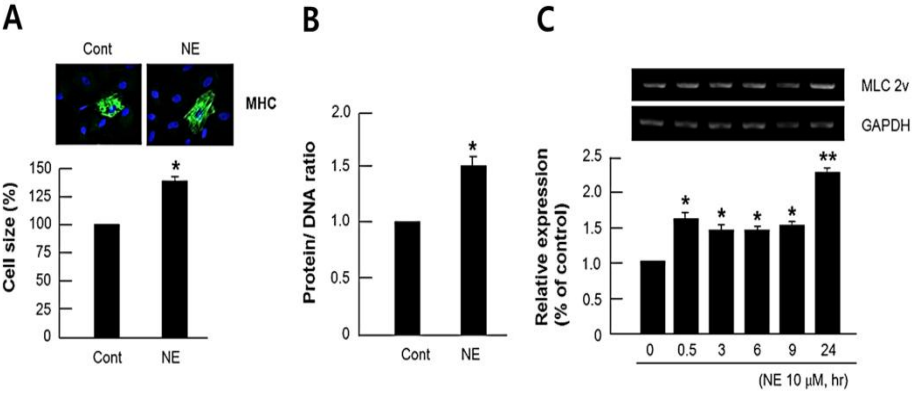

Fig. 1. The induction of cardiac hypertrophy by norepinephrine. **(A, B)** After 24 hr of treatment with norepinephrine (10 μM), cardiomyocyte cell size was observed by immunofluorescence, and total cellular protein content was estimated. **(C)** MLC 2v mRNA expression was observed by RT-PCR (*p<0.01, **p<0.05). (Cont, Control; NE, Norepinephrine; MHC, Myosin Heavy Chain; MLC 2v, Myosin light chain 2v; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase)

2. The change of microRNAs level in norepinephrine-induced cardiac hypertrophy

miRNAs, non-coding RNA molecules, are known to regulate diverse processes including cardiac hypertrophy. To examine changes in expression of miRNAs, real-time PCR was conducted, and the results showed that miRNA-1, miRNA-26, and miRNA-133a were decreased, whereas miRNA-143, miRNA-145, miRNA-181, and miRNA-210 were increased in cardiomyocyte hypertrophy induced by norepinephrine. We found that miR-133a level was decreased in response to cardiomyocyte hypertrophy (Fig. 2).[27] Previously, it was reported that miR-133a expression decreases in human and mouse models of cardiomyocyte hypertrophy.[20] Real-time PCR in our study demonstrated a time-dependent reduction in miR-133a expression after treating with 10 μM norepinephrine for 24 hr (Fig. 2B).

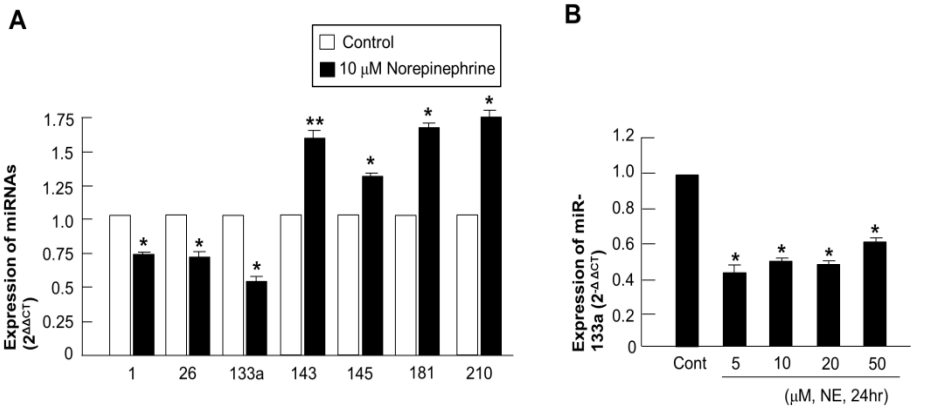

Fig. 2. Screening of cardiomyocyte miRNA levels in response to treatment with norepinephrine. **(A)** After treatment of cardiomyocytes with norepinephrine for 24 hr, the levels of miRNAs were measured by real-time PCR. Note that the levels of miR-1, miR-26, and miR-133a were significantly lower in the treated cells compared to the control. **(B)** Cardiac hypertrophy stimulated by norepinephrine induced a decrease in endogenous miR-133 expression (**p<0.05). (Cont, Control; miRNA, microRNA; NE, Norepinephrine)

3. miR-133a inhibits norepinephrine-induced cardiomyocyte hypertrophy

To investigate whether miR-133a inhibited cardiomyocyte hypertrophy, we transfected a miR-133a mimic into cardiomyocytes for 4 hr. The transfection efficacy of miR-133a was monitored until 48 hr by real-time PCR and remained higher than the control (Fig. 3A). miR-133a also regulated the cell size of cardiomyocytes induced by norepinephrine (Fig. 3B). In cardiomyocytes treated with norepinephrine and transfected with miR-133a, the cell size was decreased more so than in the control. To confirm the potential involvement of miR-133a in cardiac hypertrophy, we performed RT-PCR analysis. Decreased levels of myosin light chain 2 (MLC 2v) mRNA were observed in cardiomyocyte-transfected miR-133a mimic cells after treatment with norepinephrine (Fig. 3C). Cardiomyocyte-treated norepinephrine showed decreased level of miR-133a expression likewise cardiomyocyte-transfected miR-133a and -treated with norepinephrine decreased the miR-133a expression suggesting that miR-133a was regulated by norepinephrine (Fig. 3D)

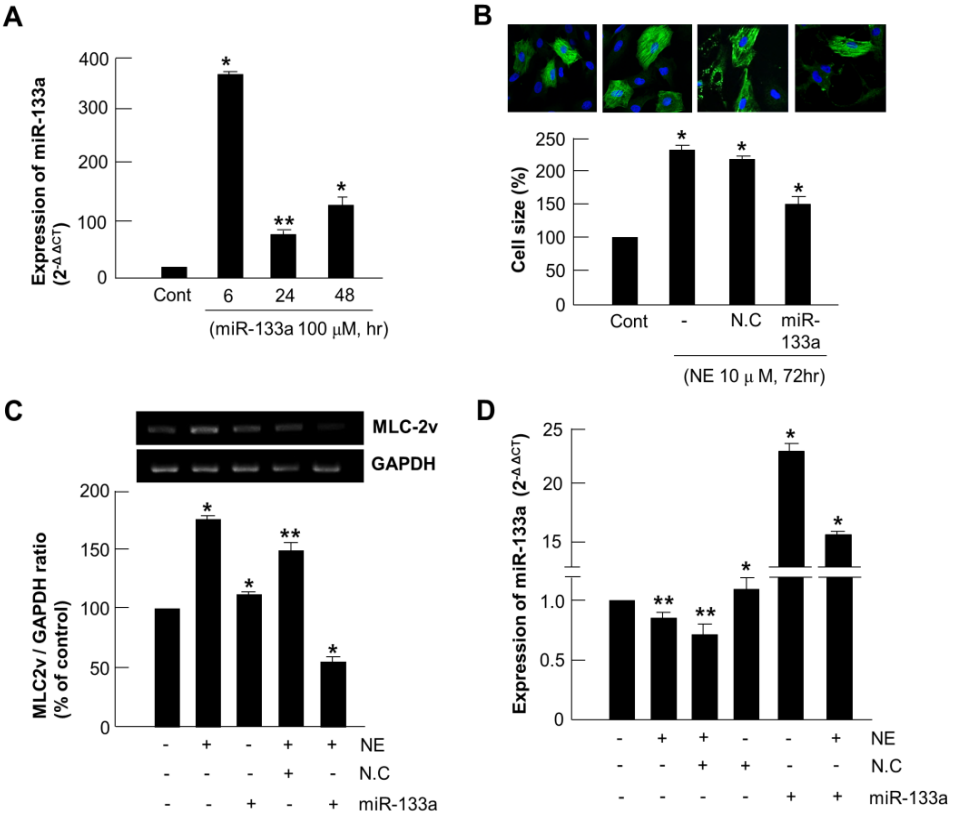

Fig. 3. The inhibitory effect of miR-133a in norepinephrine-stimulated cardiomyocytes. **(A)** The transfection efficiency was detected by real-time PCR in samples of lysed cardiomyocytes transfected with miR-133a at each harvest time (6, 24, and 48 hr). This experiment was repeated three times, and the level of miR-133a remained 100-fold higher than the control until 48 hr. **(B)** Cell size was induced in cardiomyocytes treated with norepinephrine for 72 hr (10 μM). The overexpression of miR-133a decreased the cell size compared with the control. **(C)** Semiquantitative RT-PCR showed a significant change in the level of myosin light chain 2v (MLC 2v). Cardiomyocytes treated with 10 μM norepinephrine were cultured for 24 hr after transfection with the miR-133a mimic. **(D)** In cultured primary cardiomyocytes treated with norepinephrine, the miR-133a level decreased more than in the control, and transfection with miR-133a reduced the miRNA-133a level. (*p<0.01, **p<0.05). (Cont, Control; N.C, Negative Control; miRNA, microRNA; NE, Norepinephrine, GAPDH, Glyceraldehyde 3-phosphate dehydrogenase)

4. miR-133a targets PKCδ mRNA

PKCδ is predicted as a target of miR-133a according to TargetScan (http://www.targetscan.org/). The conserved bases of the miR-133a target sequence were present in the 3'-untranslated region (3'UTR) of the PKCδ mRNA. We compared miR-133 nucleotide sequences with the sequences in the 3'UTR of PKCδ mRNA in rats and found that the 3'UTR of PKCδ mRNA contains sequences that were imprecisely complementary to miR-133. There are 6 nucleotides matching nucleotides 2 to 10 from the 5' end of miR-133. The matched base pairs and seed sequences (in bold) from different species (human, rat, and mouse) are shown in Fig. 4A. Western blotting was performed to confirm that PKCδ is a target of miR-133a (Fig. 4A). In miR-133a mimic-transfected cardiomyocytes, protein expression of PKCδ was significantly reduced compared with the control (Fig. 4B). Cotransfection of miR-133a with the luciferase reporter gene linked to the wild-type 3'UTR of PKCδ resulted in a significant decrease in luciferase activity. In contrast, no change in luciferase activity was observed when miR-133a was cotransfected with a construct in negative control seed sequences (Fig. 4C).

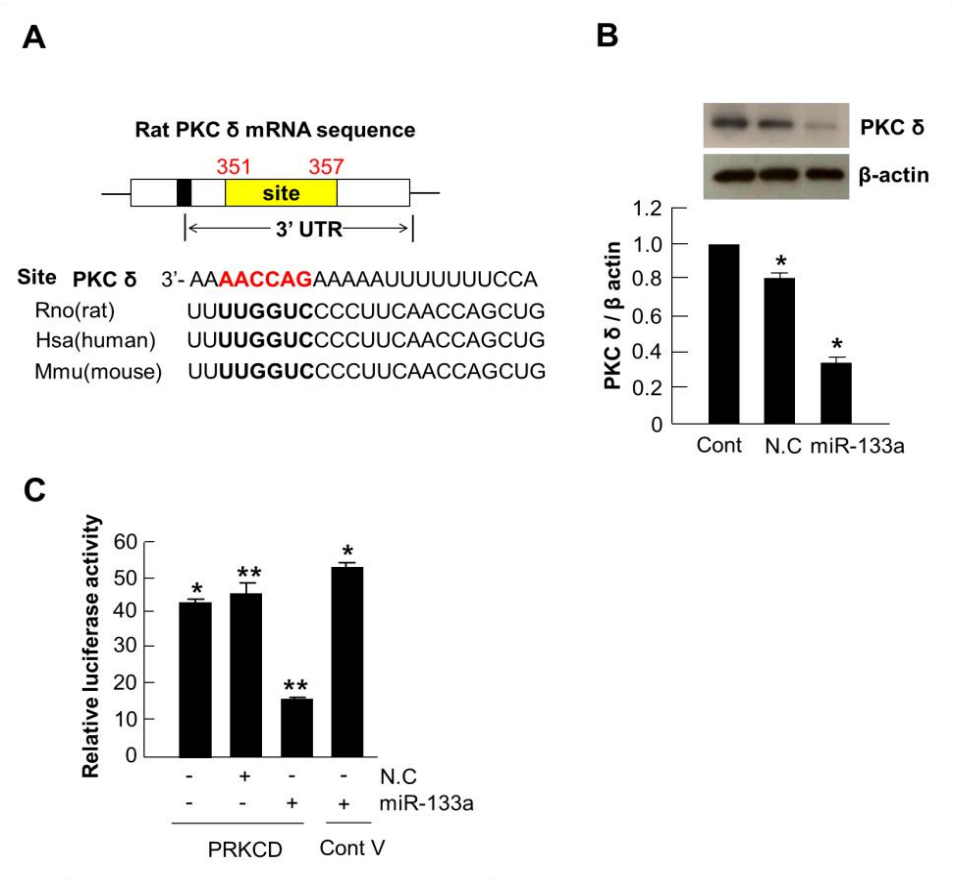

Fig. 4. PKCδ is a predicted target of miR-133a. **(A)** The sequence of the predicted target of hsa-miR-133a in rat PRKCD mRNA transcripts. **(B)** Western blot of cardiomyocytes after transfection with the miR-133a mimic. PKCδ is the predicted target, and protein levels of PKCδ decreased significantly. **(C)** Luciferase reporter assay from HeLa cells performed by cotransfection with the miR-133a mimic and a luciferase reporter gene linked to the PKCδ. (*p<0.01, **p<0.05). (Cont, Control; N.C, Negative Control; miRNA, microRNA).

5. miR-133a is not a regulator of G protein

A previous study showed that G_h is involved in ERKs activation by norepinephrine in cardiomyocytes. 10 μM norepinephrine for 24 hr the groups of miR-133a transfected with miR-133a mimic for 4 hr before treatment of

norepinephrine. The effect of miR-133a related to G proteins in cardiomyocytes was determined by RT-PCR (Fig. 5A). Cardiomyocytes treated with norepinephrine increased G_h and G_q expressions as compared to the control but did not increase G_i or G_s. In miR-133a-transfected cardiomyocytes, the levels of G_h, G_q, G_i as well as G_q were unchanged. The function of G_h as a receptor-coupled G protein depends on intracellular and extracellular environments (Fig. 5B). Therefore, G_h expression was measured in both the membrane and cytosol. Treatment with norepinephrine increased the membrane expression of G_h by 200% and the cytosol expression by 20%. Transfection with miR-133a (100 μM) did not show any change in either the membrane or the cytosolic environment.

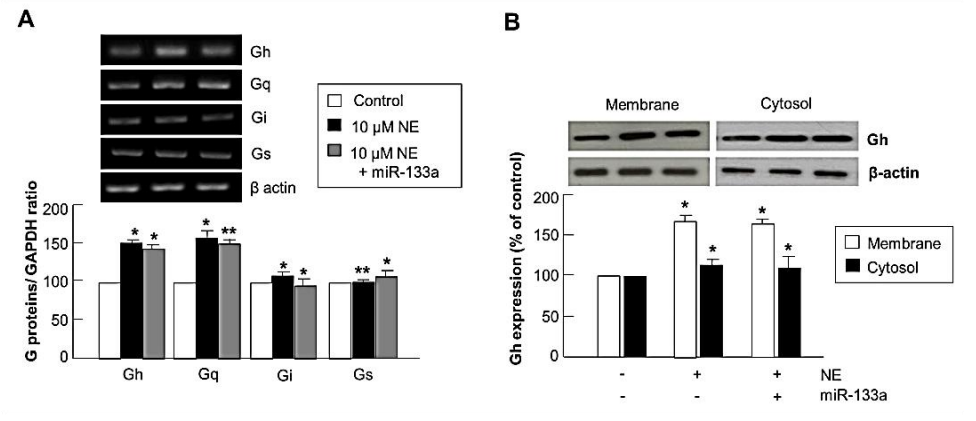

Fig. 5. No change in miR-133a with regard to G protein levels in norepinephrine-stimulated cardiomyocytes. **(A)** G protein expression was determined by RT-PCR after transfection with miR-133a or treatment with norepinephrine. **(B)** To determine translocation of G_h proteins by treatment with norepinephrine or transfection with miR-133a, the expression of G_h was analyzed by Western blotting. (*p<0.01, **p<0.05). (Cont, Control; miRNA, microRNA; NE, Norepinephrine, GAPDH, Glyceraldehyde 3-phosphate dehydrogenase)

6. miR-133a downregulates PKC signaling and proto-oncogene expression in cardiomyocytes

Norepinephrine increased the level of phosphorylated ERKs, which are hypertrophic cardiomyocyte markers, but miRNAs generally regulate target protein expression via degradation of target mRNA and translation control. PKCδ is a target of miR-133a (Fig. 4), and miR-133a regulates the cascade of MEK and ERK proteins downstream of PKC. With norepinephrine, prazosin (PRA), α1-AR blocker, and propranolol (PRO), β1-AR blocker-treated cardiomyocytes did not experience expression level changes of PKC, p-MEK or p-ERK. However, cardiomyocytes transfected with miR-133a showed decreased expressions of PKC, p-MEK and p-ERK regardless of treatment with prazocin or propranolol (Figs. 6A-C). To determine whether miR-133a affected the immediate early genes, proto-oncogenes such as c-jun, c-fos, and c-myc, mRNA levels were examined by RT-PCR in norepinephrine-stimulated cardiomyocytes. In response to treatment of norepinephrine stimulation, c-jun, c-fos, and c-myc increased, unlike the levels of the three proto-oncogenes when treated with prazosin or propranolol. However, transfection with miR-133a regulates the mRNA levels of these protooncogenes in cardiomyocytes treated with norepinephrine, prazosin, or propranolol (Figs. 6D-F).

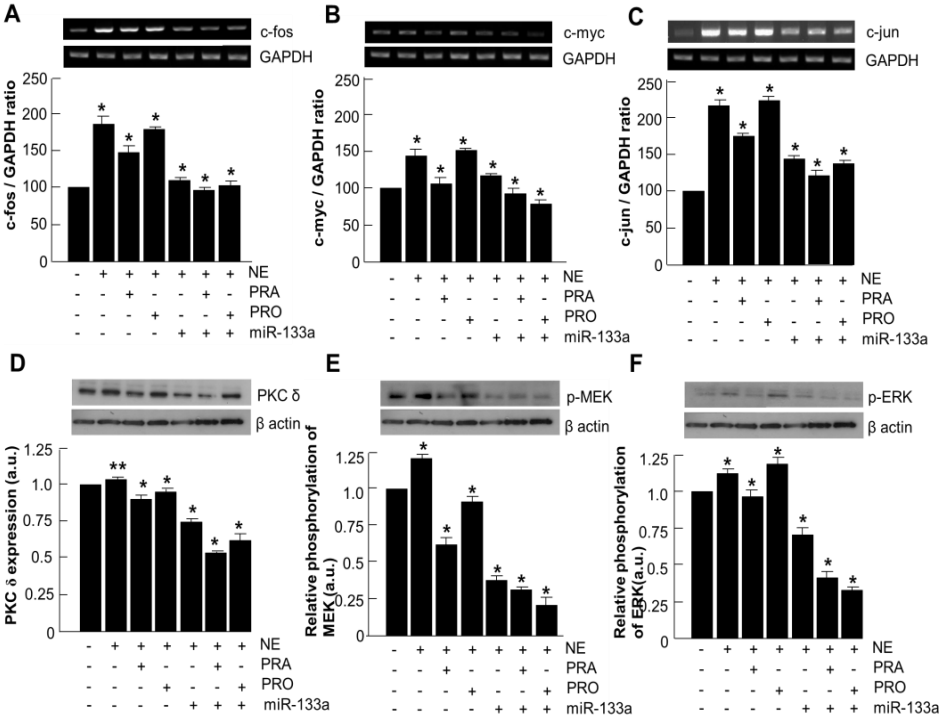

Fig. 6. Down-regulation of PKC signaling and proto-oncogene expression by miR-133a. **(A-C)** The expression levels of PKC δ, MEK, and ERKs were decreased by transfection with the miR-133a mimic (100 nM, 4 hr) regardless whether the cells were treated with prazosin (100 nM) or propranolol (2 μM) for 30 min. **(D-F)** Significant increases in c-jun, c-fos, and c-myc were observed after norepinephrine (10 μM, 24 hr) stimulation; however, transfection with miR-133a regulated the mRNA expression of these proto-oncogenes. (*p<0.01, **p<0.05). (PKC δ, Protein Kinase C; MEK, Mitogen-activated ERK Kinase; ERK, Extracellular signal-Regulated Kinases, miRNA, microRNA; NE, Norepinephrine; PRA, Prazosin; PRO, Propranolol; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase)

7. Inhibitory effect of anti-miR-133a in norepinephrine-stimulated cardiomyocytes

To further confirm whether miR-133a acts as regulator in norepinephrine-induced cardiomyocytes, anti-miR-133a was transfected into cardiomyocytes after treatment with norepinephrine, prazosin, or propranolol. The expressions of MEK and ERK decreased in miR-133a-transfected cardiomyocytes, whereas the expressions of these two molecules were unchanged in anti-miR-133a-transfected cardiomyocytes (Fig. 7).

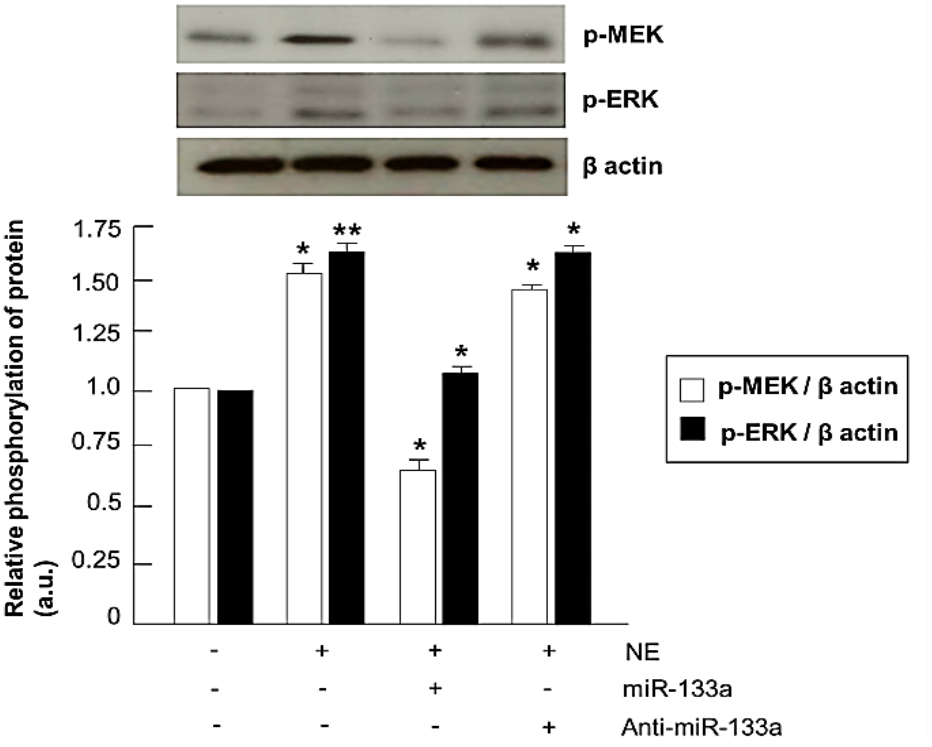

Fig. 7. The effect of anti-miR-133a in norepinephrine-stimulated cardiomyocytes. The expression levels of MEK and ERKs were down-regulated by miR-133a (100 nM, 4 hr); however, anti-miR-133a (20 nM, 4 hr) showed no changed compared to cardiomyocytes stimulated by norepinephrine. (*p<0.01, **p<0.05). (MEK, Mitogen-activated ERK Kinase; ERK, Extracellular signal-Regulated Kinases, miRNA, microRNA; NE, Norepinephrine)

IV. DISCUSSION

Cardiac hypertrophy is related to a risk of cardiac arrhythmia, diastolic dysfunction, congestive heart failure, and death. It is well-known that norepinephrine, depending on its concentration, is able to cause hypertrophy and apoptosis in several tissues such as the heart through interactions with GPCR and PLC.[21-23] Norepinephrine is released from the sympathetic nervous system and plays a pivotal role in cardiac performance with the stimulation of α1-adrenoceptor. α1-adrenoceptors interact with various signaling moieties including PLC, PLD, PKC, calcium channels, and MAPK by activating G proteins.[24, 25] PKC is one of the largest protein families in the human genome and has a critical role in diverse signaling pathways, differentiation, proliferation, cardiac hypertrophy, and death. Numerous studies have shown that PKCδ modulates the expression of collagen genes, and increased PKCδ has been associated with the development of pathologic tissue fibrosis.[26] While it is known that PKCδ is activated in myocardial ischemia, little known about its role in cardiac hypertrophy. In this study, we found that the 3'UTR of PKCδ mRNA contains sequences that are imprecisely complementary to miR-133. The conserved bases of the miR-133a target sequence are present in the 3'UTR of PKCδ. There are 6 nucleotides matching the nucleotides 2 to 10 from the 5' end of miR-133. Therefore, we suspected an important relationship between miR-133a and PKCδ in the signaling pathway of hypertrophy.

The expression of miRNAs in human disease is regarded as an important issue, especially in the cardiovascular system. miR-133a, which is enriched in cardiac and skeletal muscle, is involved in cell specification, differentiation, and development.[27] According to a previous study, miR-133a is downregulated during cardiac hypertrophy, and it has been suggested that miR-133a may play a key role in the development of hypertrophy[20]. It was also reported that the

over-expression of miR-133a diminished agonist-induced cardiac hypertrophy by repressing levels of the Rho kinase family, including RhoA, Cdc42 and NelfA.[20, 28] In vitro over-expression of miR-133a suppressed cardiac hypertrophy, whereas inhibition of miR-133a by "decoy" sequences stimulated cardiac hypertrophy.[20] These studies showed that miR-133a is necessary for controlling cardiac hypertrophy. There are some validated targets of miR-133a.

Recently, both calcineurin/nuclear factor of activated T cells pathway and miR-133a were shown to play critical roles in cardiac hypertrophy. The reciprocal repression between miR-133a and calcineurin regulation of cardiac hypertrophy was documented in a previous report.[29] Interestingly, the target of miR-133a was not clearly documented in that model of norepinephrine-induced hypertrophy.

The data from these previous studies together with those from our study suggest a key role for miR-133a in the regulation of norepinephrine-induced cardiac hypertrophy. First, miR-133a expression was inversely related to cardiac hypertrophy in the model of norepinephrine-induced cardiac hypertrophy. Second, the direct target of miR-133a in the norepinephrine-induced cardiac hypertrophy model is PKCδ. We performed luciferase reporter assays in HeLa cells to test whether miR-133a targets PKCδ. Compared with the negative control using chimeric constructs, the cotransfection of miR-133a with the luciferase reporter gene linked to the wild-type 3'UTR of PKCδ resulted in a significant decrease in luciferase activity. To our knowledge, this result is the first report demonstrating an inhibitory role of miR-133a via suppression of PKC signaling in norepinephrine-stimulated cardiac hypertrophy. Third, we found no relationship among microRNA-133a, G proteins and membrane translocation of Gh protein. G proteins are known to be important in the activation of PKCδ and ERK, thus we performed RT-PCR to determine whether G protein expression was affected

by miR-133a in our norepinephrine-induced cardiac hypertrophy model but found no correlations between G proteins and miR-133a treatment. Finally, after treatment with miR-133a, proto-oncogenes such as c-fos, c-myc and c-jun, which are downstream of the hypertrophic signaling, were down-regulated in hypertrophic cardiomyocytes.

In this study, miR-133a was shown to regulate the protein expression of PKCδ belonging to a cascade of hypertrophic signaling pathways that is regulated by norepinephrine. Moreover, proto-oncogene expression, ERKs, and MEK were also shown to be regulated by miR-133a. Our results suggest that PKCδ is a novel target of miR-133a in hypertrophic cardiomyocytes. Recently, a selective PKCδ inhibitor was studied as an anti-cancer drug or an anti-inflammatory drug because it had been associated with the development of pathologic tissue fibrosis.[30] However, to our knowledge, there are no studies regarding selective PKCδ inhibitors and cardiomyocyte hypertrophy including heart failure or hypertrophic cardiomyopathy.

V. CONCLUSION

This study demonstrates that miR-133a inhibited cardiomyocyte hypertrophy, and that treatment with miR-133a mimics before norepinephrine management prevents norepinephrine-induced cardiac hypertrophy. We also found that PKCδ is a novel target of miR-133a in our model of norepinephrine-induced cardiomyocyte hypertrophy. After treatment with miR-133a, MEK and ERK, which are in the downstream pathway, the proto-oncogenes c-fos, c-myc and c-jun were down-regulated in hypertrophic cardiomyocytes. The suppression of PKCδ expression by miRNAs and the overexpression of dominant-negative PKCδ are potential therapeutic modalities. Therefore, our findings support the possibility that the administration of or treatment with miR-133a mimics could be used in future clinical therapeutic applications.

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ABSTRACT(IN KOREAN)

miR-133a에 의한 노에피네프린으로 유도된 심근비대 억제효과

<지도교수 정 남 식>

연세대학교 대학원 의학과

문 재 연

심장비대는 심부전 그리고 심혈관 질환으로 인한 사망을 예측할 수 있는 인자로 알려져 있다. 심비대를 유발하는 많은 기전 중 최근에는 microRNA-133a (miR-133a) 의 역할이 많이 연구되고 있다. 본 연구에서는 노에피네프린에 의해서 발생되는 심근비대 과정에서 mR-133a의 발현과 기능적인 역할을 확인하고 심근비대 신호전달 체계에서 miR-133a의 작용 표적을 알아보고자 하였다. 쥐의 심근세포를 분리하고 여기에 10μM 농도의 노에피네프린을 처리하여 실험을 진행하였다. 본 연구에서는 miR-133a가 노에피네프린에 의해서 발생되는 심근비대 과정에서 중요한 역할을 하고 있음을 증명하였다. 첫째, miR-133a의 발현 정도는 심근비대 발생과 반비례 관계에 있으며, miR-133a mimics 를 미리 투여하면 노에피네프린에 의한 심근비대를 예방할 수 있었다. 둘째, miR-133a의 심근비대 신호전달 체계에서의 직접 표적은 Protein Kinase C δ (PKCδ)로 나타났으며, 이는 luciferase assay 와 western blot 방법으로 확인되었다. 셋째, miR-133a 와 G 단백질은 서로 관련이 없었다. 넷째, miR-133a 의 투여는 하위 경로에 있는 MEK 와 ERK, proto-oncogene 으로 알려져 있는 c-fos, c-myc, c-jun의 발현을 감소시켰다. 위 결과들은 PKCδ가 miR-133a의 새로운 작용 표적임을 확인하였으며, miR-133a의 투여가 심근비대 (심비대)의 발생을 억제할 수 있는 미래의 치료제로 개발될 가능성을 제시한다.

핵심되는 말: microRNA-133a,심근비대, Protein Kinase C δ, 노에피네프린