Journal of Molecular and Cellular Cardiology 82 (2015) 13–21

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

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

Original article

Natriuretic peptide receptor 3 (NPR3) is regulated by microRNA-100

Lee Lee Wong [a,b], Abby S.Y. Wee [a,b], Jia Yuen Lim [a,b], Jessica Y.X. Ng [a,b], Jenny P.C. Chong [a,b], Oi Wah Liew [a,b], Shera Lilyanna [a,b], Eliana C. Martinez [a,c], Matthew Andrew Ackers-Johnson [a,b], Leah A. Vardy [d,e], Arunmozhiarasi Armugam [f], Kandiah Jeyaseelan [f,g], Tze P. Ng [a,b,h], Carolyn S.P. Lam [a,b,h], Roger S.Y. Foo [a,b], Arthur Mark Richards [a,b,h,i], Yei-Tsung Chen [a,b,*]

[a] Cardiovascular Research Institute, National University Health System, Singapore
[b] Department of Medicine, Yong Loo Lin School of Medicine, National University of Singapore, Singapore
[c] Department of Surgery, Yong Loo Lin School of Medicine, National University of Singapore, Singapore
[d] A*STAR Institute of Medical Biology, Singapore
[e] Department of Biological Sciences, Nanyang Technological University, Singapore
[f] Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore
[g] Department of Anatomy and Developmental Biology, School of Biomedical Sciences, Faculty of Medicine, Nursing and Health Sciences, Monash University, Victoria, Australia
[h] Cardiac Department, National University Health System, Singapore
[i] Christchurch Heart Institute, University of Otago, Christchurch, New Zealand

ARTICLE INFO

Article history:
Received 10 July 2014
Received in revised form 18 February 2015
Accepted 21 February 2015
Available online 28 February 2015

Keywords:
miR-100
NPR3
Hypoxia
Myocardial infarction
Heart failure

ABSTRACT

Natriuretic peptide receptor 3 (NPR3) is the clearance receptor for the cardiac natriuretic peptides (NPs). By modulating the level of NPs, NPR3 plays an important role in cardiovascular homeostasis. Although the physiological functions of NPR3 have been explored, little is known about its regulation in health or disease. MicroRNAs play an essential role in the post-transcriptional expression of many genes. Our aim was to investigate potential microRNA-based regulation of NPR3 in multiple models. Hypoxic challenge elevated levels of *NPPB* and *ADM* mRNA, as well as NT-proBNP and MR-proADM in human left ventricle derived cardiac cells (HCMa), and in the corresponding conditioned medium, as revealed by qRT-PCR and ELISA. NPR3 was decreased while NPR1 was increased by hypoxia at mRNA and protein levels in HCMa. Down-regulation of NPR3 mRNA was also observed in infarct and peri-infarct cardiac tissue from rats undergoing myocardial infarction. From microRNA microarray analyses and microRNA target predictive databases, miR-100 was selected as a candidate regulator of NPR3 expression. Further analyses confirmed up-regulation of miR-100 in hypoxic cells and associated conditioned media. Antagomir-based silencing of miR-100 enhanced NPR3 expression in HCMa. Furthermore, miR-100 levels were markedly up-regulated in rat hearts and in peripheral blood after myocardial infarction and in the blood from heart failure patients. Results from this study point to a role for miR-100 in the regulation of NPR3 expression, and suggest a possible therapeutic target for modulation of NP bioactivity in heart disease.

1. Introduction

The cardiac natriuretic peptides (NPs) comprising atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP), play an important role in the regulation of cardiovascular and renal function. NPs are fundamental contributors to pressure and volume homeostasis in both health and disease. Their broad spectrum of bioactivity includes immediate vasodilation, hemoconcentration, natriuresis, diuresis and suppression of renin–angiotensin–aldosterone activity [1]. In addition to trophic effects opposing vascular and cardiac hypertrophy and fibrosis, NPs suppress sympathetic nerve activity [2]. Genetically modified mice lacking functional genes for NPs or their receptors, exhibit salt responsive hypertension, cardiac hypertrophy, cardiac fibrosis and reduced longevity [1,3–5], indicating the compensatory effects of NP in response to any form of acute or chronic cardiac stress or injury. Hence the NPs constitute an endogenous cardioprotective system essential to normal cardiovascular health. Therefore, regulation of plasma and tissue levels of NPs is crucial to both normal circulatory homeostasis and in provision of an optimal compensatory response to cardiac disease.

ANP and BNP are synthesized and secreted into the circulation from atrial and ventricular cardiomyocytes, primarily in response to the degree of cardiomyocyte stretch and secondary to cardiac transmural distending pressures with additional modulation from neurohormones, cytokines and hypoxia [6,7]. The physiological functions of ANP and BNP are mediated by cyclic guanosine monophosphate (cGMP) upon binding with guanylyl cyclase-coupled natriuretic peptide receptor

* Corresponding author at: Cardiovascular Research Institute, Department of Medicine, Yong Loo Lin School of Medicine, National University of Singapore, #08-01, MD6 Centre for Translational Medicine, 14 Medical Drive, 117599, Singapore. Tel.: +65 81872886; fax: +65 67759715.
E-mail address: mdccyt@nus.edu.sg (Y.-T. Chen).

http://dx.doi.org/10.1016/j.yjmcc.2015.02.019
0022-2828/

(NPR1). Both peptides are rapidly removed from circulation by binding to the "clearance" receptor, NPR3, with subsequent internalization and degradation via neutral endopeptidase [8]. In most tissues, NPR3 constitutes the large majority of NP receptors. In both anesthetized rats and conscious intact sheep, blockade of NPR3 with C-ANF (4-23) promptly elevates circulating ANP and BNP concentrations with associated enhanced NP bioactivity [9,10]. This indicates NPR3 is a key determinant of the functional bioactivity of circulating NPs. Furthermore, evidence indicates CNP/NPR3 signaling has a role in regulating coronary blood flow and offering protection against cardiac ischemia–reperfusion injury [11]. Mouse models with loss of functional NPR3 exhibit reduced clearance of infused NPs, loss of urine concentrating function, volume depletion and lower blood pressure than the wild type [12]. Mechanisms which regulate the expression of NPR3 are relevant to the overall expression and bioactivity of the NP system in both health and disease. In fact, a genome-wide association study revealed the association of NPR3 with blood pressure, suggesting its importance in modulating blood pressure responses [13]. By modulating local NP concentrations, a beneficial effect of NPR3 down-regulation in cardiovascular system has been suggested by one study on NPR3 knockout and heterozygous mice [12]. A recent study of a tissue-specific knockout animal model further demonstrated the importance of CNP/NPR-3 signaling in preserving vascular homeostasis [14]. To date, most of the signal transduction studies in relation to downstream signaling of NPR3 have been conducted using vascular endothelial and smooth muscle cells [15,16].

BNP and its co-secreted congener amino-terminal proBNP (NT-proBNP) are diagnostic and prognostic in acute and chronic ischemic heart disease and in other cardiomyopathies [17,18]. The literature is replete with numerous basic and applied studies of BNP dating from its discovery; however, relatively little is known about the regulation of NPR3 in health or heart disease. It was found that exposure of rat aortic smooth muscle cells to labeled ANP reduces NPR1 but not NPR3 expression [19]. Conversely, angiotensin II caused the down-regulation of NPR3 but not NPR1 in the vascular tissue of a rat hypertensive model [20].

MicroRNAs (miRNAs) are an emerging class of molecules whose altered expression in the disease state leads to dysregulation of multiple gene expression responses. These small non-coding nucleotides bind to the 3′ untranslated region (3′UTR) of target genes and subsequently modulate gene expression [21]. A growing body of evidence has revealed that dysregulation of fetal cardiac miRNAs, such as miR-21, miR-129 and miR-212, might contribute to alterations in gene expression in the failing human heart [22] and the potential use of miRNAs as diagnostic/prognostic biomarkers for heart failure and acute myocardial infarction has been increasingly recognized [23–27]. Here, we hypothesized that NPR3 down-regulation fosters high compensatory levels of NPs during hypoxia and that the underlying mechanisms may involve miRNA-induced interruption of NPR3 expression.

2. Materials and methods

2.1. Cell culture

Human left ventricle derived cardiac cells (HCMa) were from ScienCell™ Research Laboratories (Carlsbad, CA, USA). HCMa cells were cultured in cardiac myocyte medium (CMM, ScienCell Research Laboratories) supplemented with cell-specific growth factors according to the manufacturer's protocol. Cells were cultured at 37 °C in an incubator maintained at 95% air and 5% CO_2 and propagated according to the protocol supplied by the manufacturer.

2.2. Hypoxic challenge

Cells were incubated at 37 °C in a controlled chamber maintained with 0.2% O_2, 5% CO_2, and 94.8% N_2 using OXc system (BioSpheric, Lacona, NY, USA) for the indicated time points in experiments.

2.3. Rat model of myocardial infarction (MI)

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the National University of Singapore and carried out in accordance with established guiding principles for animal research. Male Wistar Rats (250–300 g; Comparative Medicine, National University of Singapore) were used. Left-thoracotomy and pericardectomy followed by left anterior descending coronary artery (LAD) ligation were performed as previously described [28]. Anesthesia was induced and maintained with inhalational isoflurane (3%; Baxter, Deerfield, IL, USA). Rats were intubated with a 16G catheter attached to a ventilator (Harvard Apparatus, MA, USA) throughout the procedure. Animals that underwent thoracotomy and pericardectomy without ischemic injury were used as controls. Carprofen (5 mg/kg/day, SC) and enrofloxacin (25 mg/kg/twice daily, SC) were administered for 5 days post-surgery. MI and Sham animals were euthanized 2, 7 and 14 days later (Sham group, $n = 6$ and MI group, $n = 6$, respectively at each time point). For euthanasia, a terminal injection of pentobarbitone (150 mg/kg, IP) was administered followed by cardiac puncture and blood retrieval. Heart tissues from the infarct and peri-infarct regions were harvested, snap frozen in liquid nitrogen and stored at −80 °C until processing. Tissues from corresponding left ventricular areas were collected from sham animals.

2.4. Heart Failure Clinical Cohort

Patients with heart failure (HF) were identified from an ongoing cohort study of consecutive in- and outpatients with clinically confirmed HF, the "SHOP" (Singapore Heart Failure Outcomes and Phenotypes) study [29,30]. Control subjects without any history of coronary artery disease or HF were randomly recruited from the general community through an ongoing epidemiological study of aging in Singapore, the Singapore Longitudinal Ageing Study (SLAS) study at the National University Hospital (NUH) [30]. All patients and controls underwent comprehensive 2-dimensional echocardiography as recommended by the American Society of Echocardiography (ASE) guidelines. Demographic parameters and medical history were obtained (Table 2). The study conformed to the Declaration of Helsinki. All patients and control subjects provided written informed consent and the cohort study protocol was approved by the National Health Group Domain Specific Review Board (NHG DSRB Reference Code: 2010/00114) and Institutional Review Board of National University of Singapore (NUS-IRB Reference Code: 04-140) respectively.

2.5. NT-proBNP level measurement in conditioned medium and plasma samples

NT-proBNP concentrations (pg/ml) in conditioned media and plasma samples were measured using electrochemiluminescent immunoanalysis (Elecsys proBNP II assay) on an automated Cobas e411 analyzer according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). Measurements were made as singlicate tests and the quality controls (high and low) measured in each batch run were within 2 SD values, meeting the acceptance criteria for a valid assay as specified by the manufacturer.

2.6. Mid-region adrenomedullin (MR-proADM) measurement in conditioned medium and plasma samples

MR-proADM concentrations (nmol/l) in conditioned media and plasma samples were measured using Kryptor analyzer according to the manufacturer's instructions (BRAHMS GmbH, Hennigsdorf, Germany).

2.7. MiRNA microarray

MiRNA profiling was performed using LNA™-modified oligonucleotide (Exiqon, Denmark) probes (Sanger miRBase ver 12.0), [31] and analyzed on Mapix® Ver4.5 software (Innopsys). Details of the procedure are provided in the supplemental materials. Results of the microarray data have been deposited into GEO database under the accession number GSE55387.

2.8. RT-PCR and quantitative stem-loop RT-PCR

Taqman High Cap cDNA Rev Trans Kit (Applied Biosystems, Foster City, CA, USA), Taqman miRNA RT kit (Applied Biosystems), and Taqman Universal MMIX II with UNG (Applied Biosystems) were utilized to quantify the level of genes or miRNAs of interest. For mRNA endogenous control (GUSB and B2M), target gene, miRNA endogenous control (U6 and 4.5sRNA), and target miRNAs were as follows: GUSB (4326320E), β2 microglobulin (B2M) (Rn00560865_m1), BNP (Hs00173590_m1), NPR1 (Hs00181445_m1), NPR2 (Hs00241516_m1), NPR3 (Hs00168558_m1), NPR3 (Rn00563495_m1), U6 (001973), 4.5sRNA (001716) and miR-100 (000437). Expression levels for mRNAs and miRNAs were calculated based on their CT values using housekeeping genes, glucuronidase beta (GUSB) (4326320E) and U6 (001973) respectively. For rat tissue, B2M was used as housekeeping gene. RT PCR results were processed and analyzed using the comparative CT method ($2^{-\Delta\Delta CT}$) method [32,33]. Details of the procedure are provided in the supplemental materials.

2.9. Western blot analysis

Antibodies used were: NPR3 (ab123957, clone 11H6; 1:2000; Abcam Inc., Cambridge, MA, UK), BNP (ab92500, clone: EPR3735; 1:2000 – 20,000; Abcam Inc.), inositol 1,4,5-triphosphate receptor, IP3R, (#3763;1:1000; Cell Signaling Technology, MA, USA), IP3R (Ser1756) (#8548;1:1000; Cell Signaling Technology, MA, USA), and β-actin (sc-47778, clone C14; Santa Cruz Biotechnology Inc, CA, USA). Corresponding secondary antibodies, Goat anti-Mouse-IgG conjugated to HRP (Sigma) and Goat anti-rabbit-IgG conjugated to HRP (Sigma) were used at a dilution of 1:2000. The relative protein expression levels of NPR3 were normalized against β-actin. The band intensities were quantified by densitometric analysis. Details of the procedure are provided in the supplemental materials.

2.10. Luciferase-NPR3 3′UTR reporter constructs

The 3′UTR of NPR3 was directionally cloned into the psiCHECK-2 vectors (Promega, Madison, WI, USA) immediately downstream of the stop codon of the luciferase gene. The psiCHECK-2-NPR3-3′UTR luciferase reporter plasmid was generated to perform co-transfection and luciferase assays to validate the binding affinity of candidate miRNAs to the 3′UTR sites of putative targets (predicted by various predictive programs/algorithms). The luciferase activity in cell lysate was determined using the Dual-Luciferase Reporter Assay System (DLR assay system, Promega) according to the manufacturer's protocol. Details of the procedure are provided in the supplemental materials.

2.11. Transfection with antagomiR-100

AntagomiR-100 (Ambion) and its negative control, scrambled miRNAs were transfected into HCMa using Lipofectamine™ 2000 Reagent (Invitrogen) following manufacturer's instructions.

2.12. Cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) measurements

cGMP and cAMP measurements from cell lysates were performed using cGMP and cAMP complete ELISA kits (Enzo Life Sciences ELS AG, Postfash Lausen, Switzerland) respectively, according to the manufacturer's instructions. Details of the procedure are provided in the supplemental materials.

2.13. Statistical analysis

Relative expression of *NPPB*, *ADM*, *NPR1*, *NPR2*, *NPR3*, NT-proBNP, MR-proADM and miR-100 at different time points of hypoxic treatment, MI vs sham, as well as between clinical samples of heart failure patients and controls were all compared by Student's *t*-test. Significance set at $P < 0.05$. All in-vitro assays were performed on samples from at least 3 independent experiments. All data were expressed as mean ± SD.

3. Results

3.1. Elevations of NPPB/NT-proBNP and ADM/MR-proADM in human cardiac derived in-vitro model

NT-proBNP and MR-proADM were elevated in conditioned media in human cardiomyocyte (HCMa) cultures after hypoxic (0.2% oxygen) challenge (Fig. 1A and B). Although differences in cell numbers were observed after hypoxia, TUNEL assay revealed no significant difference in the level of DNA fragmentation between hypoxic and normoxic groups (data not shown). Using stem–loop real-time PCR (qRT-PCR), *NPPB* and *ADM* mRNA levels were found to be elevated in a time-dependent manner during hypoxia when compared to normoxic control cells (Fig. 2A). The level of *NPR1* mRNA, but not *NPR2*, increased significantly in hypoxia (Fig. 2B and C). Conversely, expression of *NPR3* mRNA was decreased after hypoxia when compared with controls (Fig. 2D). Western blot analysis confirmed NPR3 protein levels declined steadily over the duration of hypoxia (Fig. 3A). Quantitative densitometric analyses reveal that NPR3 decreased in a time-dependent fashion in hypoxia treated HCMa ($r = 0.984$; Fig. 3B).

3.2. MiR-100 as a candidate suppressor of NPR3

Using miRNA microarray analyses and multiple web-based miRNA-mRNA prediction algorithms (DIANAmT miRanda, miRWalk, PICTAR5, and RNA22), a cluster of up-regulated miRNAs were first selected as potentially targeting the 3′UTR of NPR3 and subjected for further qPCR analyses. Consistent with miRNA microarray result comparing miRNAs profiles in HCMa with or without hypoxic challenge (Supplementary Table 1), up-regulation of miRNAs of interest was revealed by qRT-PCR verification at different time points (Table 1). MiR-100 was selected for further investigation by cross comparison with miRNA microarray data generated from the human HF cohort. Fig. 4A illustrates the number of predicted target sites from different algorithms and the possible complementarity of miR-100 to the 3′UTR of NPR3. Further qPCR analysis revealed that the expression of miR-100 in hypoxia-treated HCMa cells increased in a time-dependent manner ($r = 0.969$; $P < 0.01$) (Table 1 and Fig. 4B). In addition to intracellular content, the levels of miR-100 were elevated 1.6 fold in HCMa conditioned medium after 120 h of hypoxia compared to normoxic controls (Fig. 4C). Since no miR-100 was found in fresh culture medium, the extracellular miR-100 detected after hypoxia originated from the cultured cells.

3.3. Regulatory effect of miR-100 on NPR3 expression

A reduction in relative luciferase activity in Hela cells containing NPR3-LUC and miR-100 constructs was observed when compared to controls (Fig. 4D), indicating interaction between miR-100 and its target site on *NPR3* 3′UTR. Further corroborating this observation, HCMa cells were transfected with negative control scrambled miRNA and antagomiR-100 inhibitor to test the regulatory effect of miR-100 on the expression of NPR3 in normoxic and hypoxic conditions. An increase in *NPR3* at

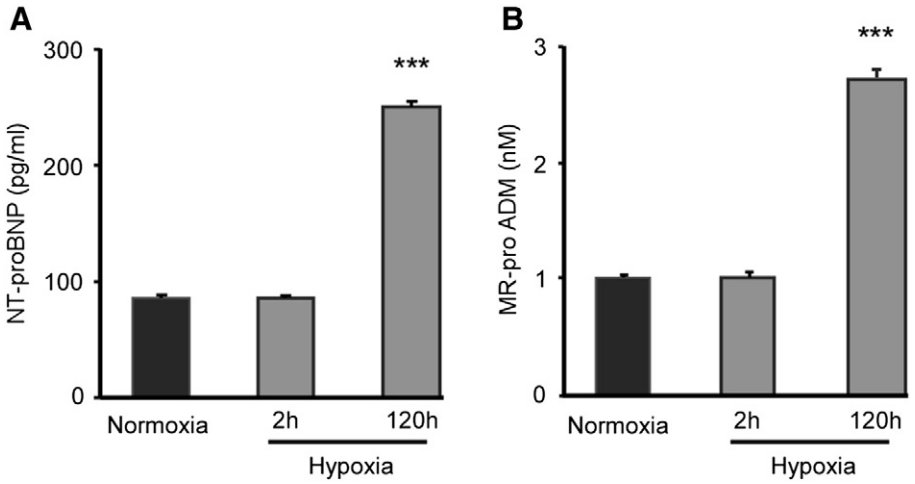

Fig. 1. The effect of hypoxia on NT-proBNP, MR-proADM levels in HCMa conditioned medium. (A) The levels of NT-proBNP in conditioned medium are enhanced by more than 2 folds ($p < 0.001$) after 120 h hypoxic treatment. (B) The levels of MR-proADM in conditioned medium are enhanced by more than 2 folds ($p < 0.001$) after 120 h hypoxic treatment. Similar results were obtained from at least 3 independent experiments. Data are presented as mean ± SD. $n = 3$, Student's *t*-test. $^{***}p < 0.001$.

mRNA and protein levels was observed in antagomiR-100 treated cells under both normoxic and hypoxic conditions (Fig. 5A–C). The regulatory effect of miR-100 on NPR3 expression was further corroborated by an increase in NPR3 mRNA level in primary rat left ventricular adult cardiomyocytes with antagomiR-100 treatment under hypoxic conditions (Supplemental Figure 1). Hence, the combined results from antagomiR-100 experiments provided strong evidence that NPR3 level is regulated by miR-100.

3.4. Levels of miR-100 and NPR3 in MI rat model

In left ventricular heart tissue harvested from rats undergoing myocardial infarction (MI) induced by left anterior descending (LAD) coronary artery ligation, the transcriptional expression of miR-100 and *NPR3* was examined at days 2, 7, and 14 after MI. In MI rats, reduction in ejection fraction (EF%) was observed at day 7 (Supplemental Figure. 2A) and day 14 (Supplemental Figure. 2B) by 25% and 49% respectively

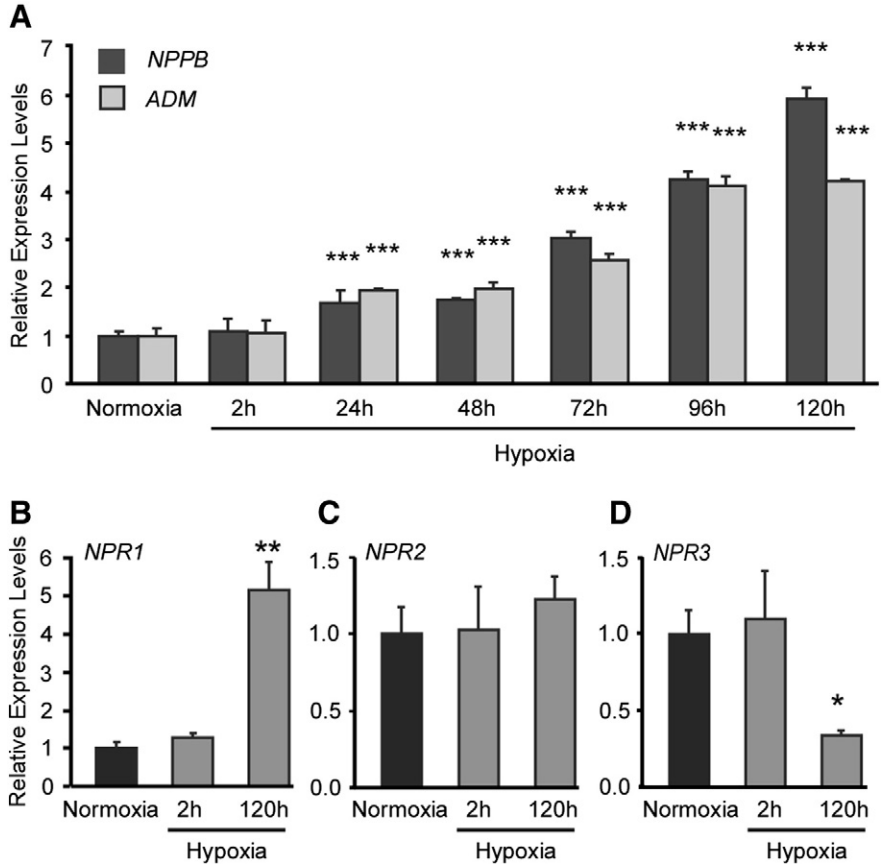

Fig. 2. The effect of hypoxia on *NPPB*, *ADM* and *NPRs* mRNA levels in HCMa cells. (A) Hypoxic challenge increases *NPPB* and *ADM* mRNA expressions in a time-dependent fashion. Alterations of mRNA levels of (B) *NPR1*, (C) *NPR2* and (D) *NPR3* in HCMa after hypoxic treatment. Similar results were obtained from at least 3 independent experiments. Data are presented as mean ± SD, $n = 3$, Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

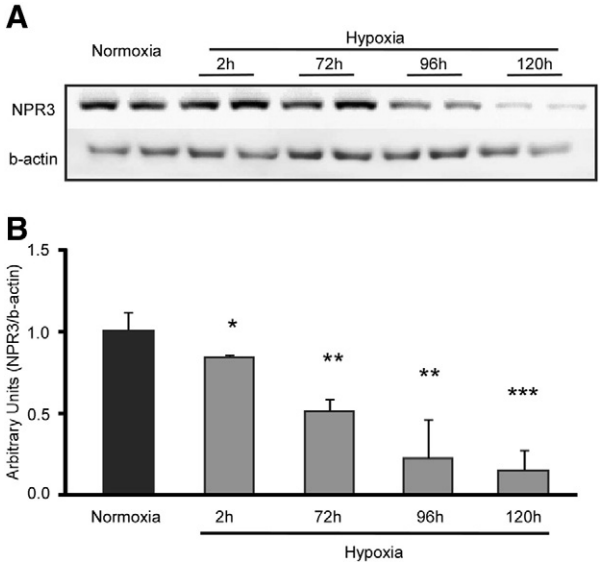

Fig. 3. Down-regulation of NPR3 level after hypoxic treatment in cells. (A) The protein level of NPR3 is decreased in HCMa after hypoxic treatments. (B) Densitometric analysis of immunodetected NPR3 bands on the western blot using G Box Chemi XL1.4 software. Band intensities were normalized against those of the corresponding housekeeping beta actin protein band. Similar results were obtained from at least 3 independent experiments. Data are presented as mean ± SD, $n = 2$, Student's t-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

when compared to sham controls. Masson's trichrome staining revealed 40% of LV cross-sectional area contained fibrotic tissues at days 7 and 14 after MI (Supplemental Figure. 2C). Consistent with *in-vitro* results, the expression of NPR3 in the infarct and peri-infarct area of MI tissues at day 2, 7, and 14 after MI was reduced by 68.9%, 24.5% and 39.3%, respectively, compared with sham controls (Fig. 6A). The expressions of miR-100 in MI tissues were increased by 8.3 and 1.6 fold at day 7 and day 14, respectively (Fig. 6B). However, the expression of miR-100 in the MI group at day 2 was down-regulated compared with sham controls (Fig. 6B). It has been reported that miRNAs may exert paracrine effects after being exported into the circulation [34]. To test whether the elevation of miR-100 level could be found in the circulation, levels of miR-100 in the peripheral blood were examined. MiR-100 was significantly increased by 3.9 fold in the rat blood at day 7 after MI when compared with sham controls, with weaker trends (not significant) towards increased levels at days 2 and 14 post-MI groups (Fig. 6C). Since up-regulation of miR-100 was found in the MI heart tissue, the elevated level of circulating miR-100 observed after MI in rats may in part originate from the injured heart.

3.5. MiR-100 level in patients with heart failure

To corroborate *in vivo* and *in vitro* findings, the expression of miR-100 in the circulating peripheral blood obtained from the clinical heart failure (HF) cohort was examined. The characteristics of the study cohort are summarized in Table 2. As expected, higher NT-proBNP and MR-proADM levels in HF plasma were observed when compared to controls (Table 2). In this cohort, the level of miR-100 in peripheral blood in the HF group ($n = 42$) was 1.7 fold higher than in healthy controls ($n = 54$), $p = 0.024$ (Fig. 6D).

4. Discussion

Our study suggests that down-regulation of NPR3 by miR-100 may be a compensatory mechanism by increasing the levels of bioactive NPs under conditions of cardiac stress. NPR3 is down-regulated in our *in vitro* and *in vivo* models of cardiac hypoxic challenge in the presence of elevated levels of miR-100. Reporter assays indicate direct inhibition of NPR3 expression by miR-100. Blood levels of miR-100 are elevated in our clinical cohort with failure predominantly of ischemic etiology. The current findings provide a possible basis for developing therapeutics focused on the NPs/NPR3 signaling network.

Since the identification of NP in 1981, numerous studies have provided substantial evidence for the roles of NPs in heart diseases [35]. Measurements of plasma concentrations of ANP, BNP and NT-proNPs have established roles as diagnostic and prognostic markers in various cardiomyopathies [17,18]. Physiological functions of NPs provide a beneficial compensatory response ameliorating the adverse consequence of cardiac injury [36]. Hence any alteration in NPR expression or function that leads to chronic changes in the levels of NPs is likely to be relevant in the net response to cardiac injury.

To simulate elevations of BNP/NT-proBNP and/or ADM/MR-proADM, typically seen in heart failure or under hypoxic conditions, cardiac cell lines derived from the human left ventricle were evaluated by biochemical analyses after subjecting the cells to hypoxia. The HCMa cell line used in the current study displayed characteristic elevated BNP/*NPPB* and *ADM* responses at both the transcriptional and translational levels. Furthermore, the observation that no ANP expression in the HCMa cells further indicates its ventricular originity. In

Table 1
Relative miRNA expression profiles in HCMa with hypoxic treatment.

Name	Microarray relative fold change Hypoxia/normoxia	RT qPCR relative fold change Hypoxia/normoxia		
		2 h	24 h	120 h
hsa-miR-100	1.47	1.63 ± 0.14 $p = .054$	1.51 ± 0.22 $p = .063$	3.01 ± 0.21 $p = .011$
hsa-miR-149*	2.04	0.71 ± 0.19 $p = .257$	2.16 ± 0.20 $p = .056$	2.82 ± 0.07 $p = .002$
hsa-miR-186	2.12	1.52 ± 0.03 $p = .196$	1.06 ± 0.26 $p = .808$	2.99 ± 0.21 $p = .013$
hsa-miR-222	1.51	1.38 ± 0.25 $p = .273$	3.39 ± 0.14 $p = .012$	4.00 ± 0.07 $p = .022$
hsa-miR-487a	1.30	1.88 ± 0.14 $p = .026$	1.58 ± 0.25 $p = .149$	2.67 ± 0.13 $p = .006$
hsa-miR-498	1.39	2.17 ± 0.15 $p = .034$	2.12 ± 0.17 $p = .046$	3.31 ± 0.05 $p = .001$
hsa-miR-500	2.37	0.93 ± 0.17 $p = .683$	1.82 ± 0.04 $p = .015$	2.21 ± 0.11 $p = .039$
hsa-miR-643	1.57	1.80 ± 0.20 $p = .085$	2.08 ± 0.23 $p = .024$	4.06 ± 0.15 $p = .008$

List of 8 up-regulated miRNAs that are predicted to targeting 3′UTR of *NPR3* transcripts by at least two microRNA target site predictive algorisms. Expression data presented are the relative fold changes as compared to normoxia control. Microarray results were obtained from pooled samples, normoxia, $n = 3$; hypoxia, $n = 3$. RT qPCR results represent the mean ± SD from 3 samples; each condition was replicated by at least two independent experiments. 2 h, 24 h, and 120 h indicate the duration (hour) of hypoxic treatment (0.2% oxygen).

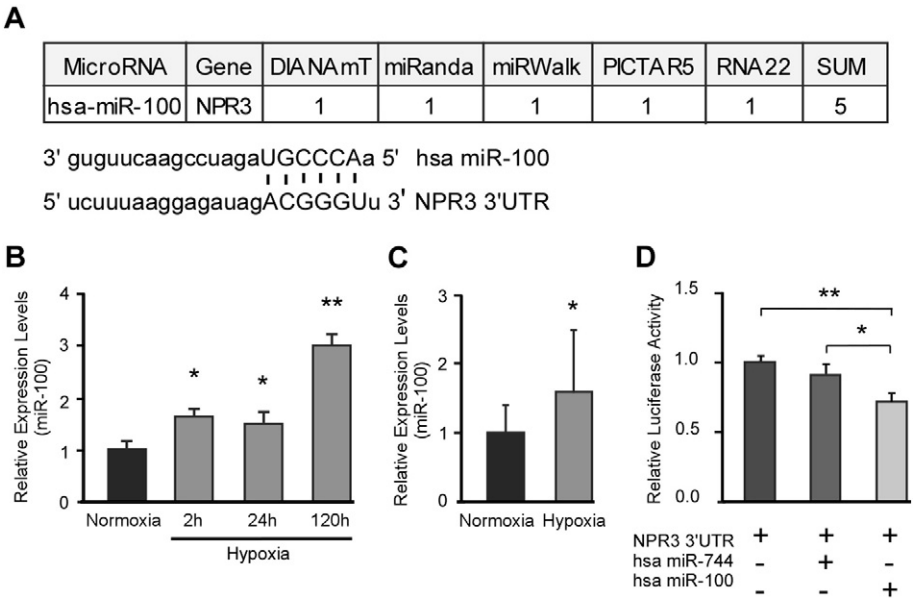

Fig. 4. Identification of miR-100 targeting the NPR3 3′UTR. (A) The numbers of miR-100 predicated target sites on NPR3 3′UTR from 5 web-based microRNA target predictive algorithms and predicated alignment between seed sequence of miR-100 and the 3′UTR of human NPR3. (B) Expression of miR-100 in HCMa after 2 h, 24 h and 120 h hypoxic treatments. (C) Up-regulation of miR-100 level in the conditioned medium of HCMa subjected to 120 h hypoxic treatment. (D) miR-100 reduces the luciferase activity of the NPR3 3′UTR LUC construct. Similar results were obtained from at least 3 independent experiments. Data are presented as mean ± SD, $n = 3$, Student's t-test. $^{*}p < 0.05$, $^{**}p < 0.01$.

HCMa, hypoxic treatment returns more robust BNP and ADM responses than mechanical deformation (15%–20% deformation, 1 Hz, 60 min), suggesting the expression of BNP and ADM in this particular cardiac derived cell line might be more sensitive to hypoxic challenge. The potential role of hypoxia in regulating the expression of NPs has been suggested in several studies. The elevation of BNP/*NPPB* levels in ischemic patients without impaired left ventricular ejection fraction provides some evidence that stimuli other than myocyte stretch, such as hypoxia, might be involved in regulating *NPPB* expression [37]. Additionally, hypoxia induced NP responses have been reported in other model systems. In a rat atrial derived cell line, AT-1, ANP expression is transcriptionally regulated as evidenced by increased activity in the *NPPA* promoter region [38]. In a fusion cell line, AC-16, it was demonstrated that increased transcriptional activity of hypoxia-inducible factor-1 (HIF-1) subsequently enhanced the expression of *NPPB* in cells [19]. The roles of NPs in the adaptation to hypoxic challenge have also been indicated by several *in vivo* or *ex vivo* studies. In isolated rodent hearts and in anesthetized animals, acute hypoxia exposure enhances cardiac ANP secretion [39]. In animals exposed to chronic hypoxia with elevated pulmonary arterial pressure, *NPPA* gene expression is increased [40]. Elevations in both plasma and ventricular tissue, BNP and NT-proBNP levels were observed after acute hypoxic exposure in a porcine model and these authors also demonstrated that hypoxic challenge increased *NPPB* mRNA level in cultured porcine cardiac cells [41]. A number of *cis*-acting elements have been reported in the *NPPB* promoter region [9] but no hypoxic response element has been reported. Our results provide evidence that expression of *NPPB* and *ADM* is stimulated by oxygen deprivation. This model is of

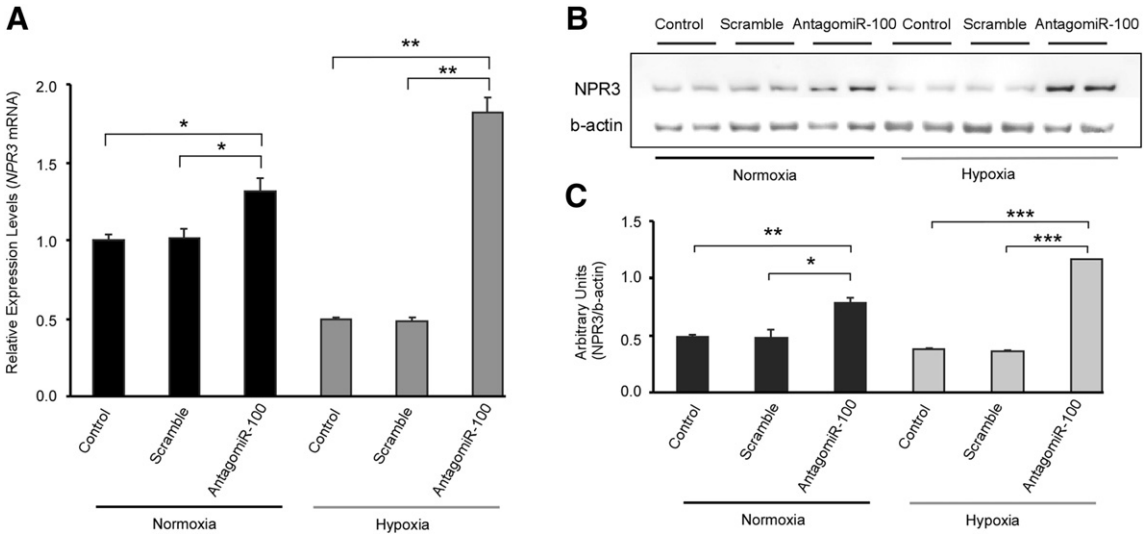

Fig. 5. NPR3 level is negatively regulated by miR-100. (A) The expression of *NPR3* mRNA in HCMa cells after treatment with scrambled miRNA and antagomiR-100, and exposed to normoxia or hypoxia for 72 h. (B) The protein level of NPR3 in HCMa after treatment with scrambled miRNA and antagomiR-100, and subjected to normoxia or hypoxic treatment. (C) Relative expression of NPR3 normalized against beta actin. The immunodetected NPR3 and beta actin bands were imaged and quantified using G Box Chemi XL1.4 software. Similar results were obtained from at least 3 independent experiments. Data are presented as mean ± SD. $n = 3$. Student's t-test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

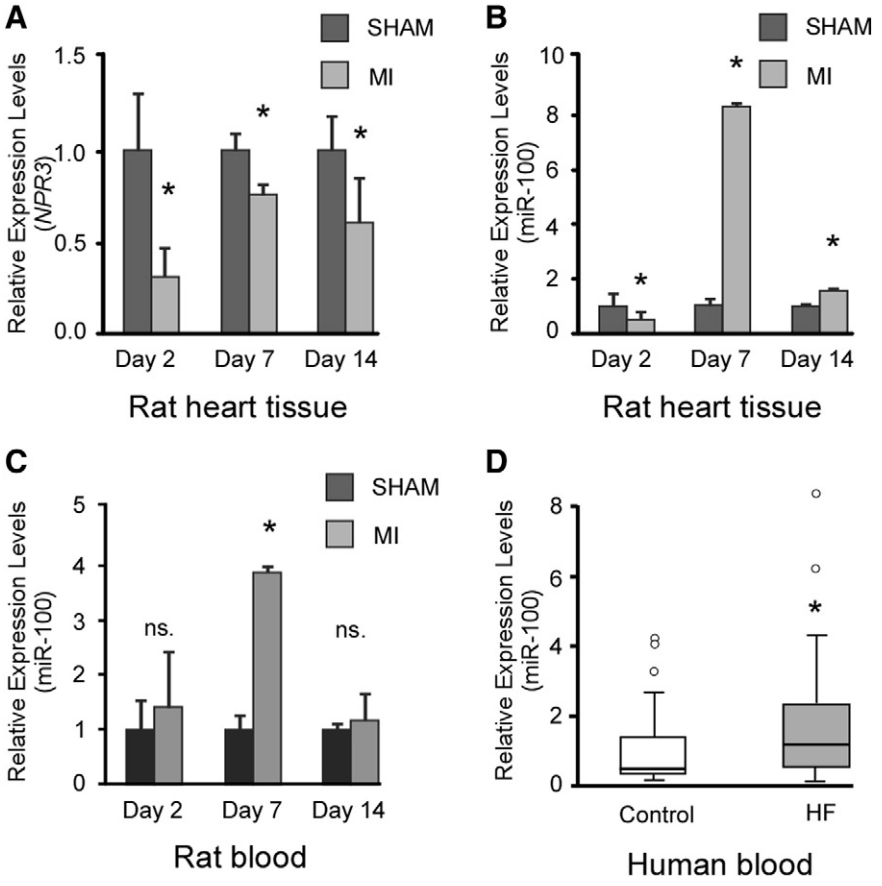

Fig. 6. Expression profiles of NPR3 and miR100 in rat MI specimens and peripheral blood from heart failure patients. (A) Expressions of NPR3 mRNA level in rat infarct and peri-infarct heart area (MI) at 2, 7, and 14 days post-surgery as compared to sham. (B) Expressions of miR-100 level in rat infarct and peri-infarct heart area (MI) at 2, 7, and 14 days post-surgery as compared to sham. (C) Expressions of miR-100 level in rat peripheral blood at 2, 7, and 14 days post-surgery as compared to sham controls. (D) Up-regulation of miR-100 in HF patients as compared to controls. Data in (A)–(C) are presented as mean ± SD, sample size, $n = 6$; in (D), center lines show the median values; box limits indicate the 25th and 75th percentiles as determined by BoxPlotR software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles; outliers are represented by opened dots. Control: $n = 54$, HF: $n = 42$. Student's t-test. $^{*}p < 0.05$; ns., non-significant.

particular interest because it replicates NT-proBNP and MR-proADM responses as seen in HF patients. The current *in vitro* platform will be useful in addressing the interaction of the transcriptional activity of the *NPPB* and hypoxia.

It is known that changes in the content of ligand might subsequently alter the expression of the corresponding receptors in cells. Previously, it was demonstrated that administration of NPs reduces the transcriptional activity of *NPR1* promoter in rat aortic smooth muscle cells [19]. Intriguingly, in the current platform, elevated expression of *NPPB*/BNP in cardiac cells did not lead to down-regulation of *NPR1*; instead, up-regulation of *NPR1* was observed. We further demonstrated that the induced NPR1 in cells is functionally intact as indicated by the elevation of cGMP level after BNP treatment (Supplemental Figure. 3). Given the anti-hypertrophic effect is one of the cardioprotective actions of *NPPB*, elevations of BNP and NPR1 may represent a compensatory mechanism countering the change in cell size. The observed down-regulation of NPR3 expression may provide an additional compensatory mechanism by prolonging the half-lives of bio-active ANP, BNP and CNP. Previous studies have demonstrated that the regulation of NPR3 is relevant to the bioactivity of NPs and subsequently modulate the circulatory function [9–16]. Intriguingly, our immunohistochemical analyses show that NPR3 reactivity is found ubiquitously in various cardiac cells with reduced levels after MI in all cardiac cells (Supplemental Figure. 4), suggesting the existence of a hypoxia-mediated trigger mechanism that globally down-regulates the expression of NPR3. It is plausible that in addition to the modulatory actions of miRNAs, regulation of NPR3 levels could be mediated via mechanisms that alter the transcriptional activity of the NPR3 promoter region or its protein half-life via ubiquitination-mediated processes.

Down regulation of cAMP and stimulation of IP3R have been suggested to be the secondary messenger events mediating NPR3 and NPR1/2 signaling cascades, respectively, and these molecules are involved in maintaining the homeostasis of intracellular calcium in

Table 2
Baseline characteristic of heart failure clinical SHOP and control SLAS study cohorts.

	Controls ($n = 42$)	Heart failure ($n = 54$)
Age, years	58.4 ± 7.7	59.6 ± 10.8
Male, %	43	72
Race % (Chinese, Malay, Indian)	78.5, 16.7, 4.8	38.9, 54.8, 6.3
BMI (kg/m^2)	23.98 ± 3.62	26.77 ± 5.43**
LVEF, %	63.24 ± 3.86	37.88 ± 17.20***
NYHA Class I–II/III–IV,%	100/0	83/17
Systolic BP, mm Hg	130.93 ± 19.54	119.35 ± 21.88**
Diastolic BP, mm Hg	76.52 ± 9.70	70.04 ± 12.95**
NT-proBNP (pg/ml)[a]	58.99 ± 112.89	3199.44 ± 3295.64***
MR-proADM (nmol/l)	0.43 ± 0.08	1.12 ± 0.43***

BMI, body mass index; LVEF, left ventricular ejection fraction; NYHA class, New York Heart Association classification; NT-proBNP, N-terminal pro brain natriuretic peptide, and MR-proADM, mid regional pro adrenomedullin. Values presented are mean ± SD, Student's t-test $^{**}p < 0.01$, $^{***}p < 0.001$.

smooth muscle cells [16]. The reduction in cAMP levels and elevation in phosphorylated IP3R levels in normoxic HCMa cells after antigomiR-100 treatment (Supplemental Figure. 5), is consistent with a regulatory effect of miR-100 to down-regulate NPR3 expression and enhance NPR1/2 mediated bioactivity.

Since no miR-100 was found in the fresh culture medium, our results indicate that miR-100 is transcribed and exported from cardiac cells after hypoxia and conceivably may exert paracrine or autocrine effects on recipient cells. The possible regulatory effect of miR-100 on the expression of NPR3 was supported by our *in vitro* antagomirR-100 experiment, as well as the cardiac tissue expression patterns of miR-100 and NPR3 from the MI rat model. In our rat MI model, the expression of NPR3 and miR-100 was examined on days 2, 7 and 14 following LAD ligation. In this model, day 2 reflected early post-MI deterioration of cardiac function response to injury, day 7 reflected the early post-MI deterioration of cardiac function and day 14 represented the progression to fully developed heart failure. Consistent with cellular findings, an inverse correlation between miR-100 and NPR3 expression levels was observed in infarct and peri-infarct tissues in data from days 7 and 14 post-MI.

Intriguingly, down-regulation of miR-100 in heart tissue was seen at day 2 post-MI with concurrent down-regulation of NPR3. Since the evidence from our antagomiR-100 study (Fig. 5) point to the regulatory effect of miR-100 on the expression of NPR3, the down-regulation of miR-100 at early MI stage might then be attributed to a compensatory feedback loop mechanism to attenuate the suppression of NPR3 level. This hypothesis is further supported by the down-regulation of miR-100 observed in HCMa cells after treatment with small interfering NPR3 RNA (siNPR3) in siRNA-knockdown experiments (Supplemental Figure. 6). It is also plausible that the reciprocal relationship between miR-100 and NPR3 is unmasked in the later stages of ischemic insult (day 7 and day 14 post-MI) than in the very early phase (day 2 post MI) when complex factors from inflammatory responses as well as cardiac repair and remodeling are involved [42]. Taken together, results from siNPR3 knockdown studies and examination of MI tissues at early and late post-MI stages indicate that the level of miR-100 alters over time and may have its main effect upon NPR3 in the subacute, rather than the acute or chronic phase of myocardial ischemic injury.

The potential clinical significance of mir-100-NPR3 interactions is suggested by the elevation of miR-100 in the peripheral blood of patients with HF predominantly of ischemic etiology. It has been postulated that circulating miRNAs are not only potential biomarkers but also key regulators of gene expression and could be therapeutic targets for drug development. For instance, blockade of miR-208a has been proven to improve cardiac function and prevent hypertrophy in a rat model of cardiac pressure overload [43]. Elevation of miR-100 in blood of MI animals and HF patients suggests that the expression of NPR3 might be systemically down-regulated in multiple tissues which might prolong NP bioactivity systemically, as well as enhancing the tissue-specific cardioprotective effects of NPs.

Single miRNAs, including mir-100 may modulate the expression of multiple target genes [44]. Conceivably changes in miR-100 levels interfere with expression of other targets aside from NPR3. For instance, in hepatocarcinoma, miR-100 was found to inhibit the expression of rapamycin (mTOR) kinase with consequent effects on regulation of autophagy [44]. It would be interesting to further explore the putative targets of miR-100 in addition to NPR3. Results from our study provide the first evidence that miR-100 may play a role in the cardiovascular system via regulation of NPR3 expression. Modulation of miR-100 could be a possible adaptive mechanism whereby its up-regulation in stressed or injured cardiac cells/tissues subsequently suppresses the expression of NPR3 and prolongs the half-life of NPs in blood and tissues (Fig. 7). Further *in vivo* studies such as manipulation of mir-100 in the rat myocardial infarction will be necessary to further elucidate the function of miR-100/NPR3 in the response to cardiac ischemic injury.

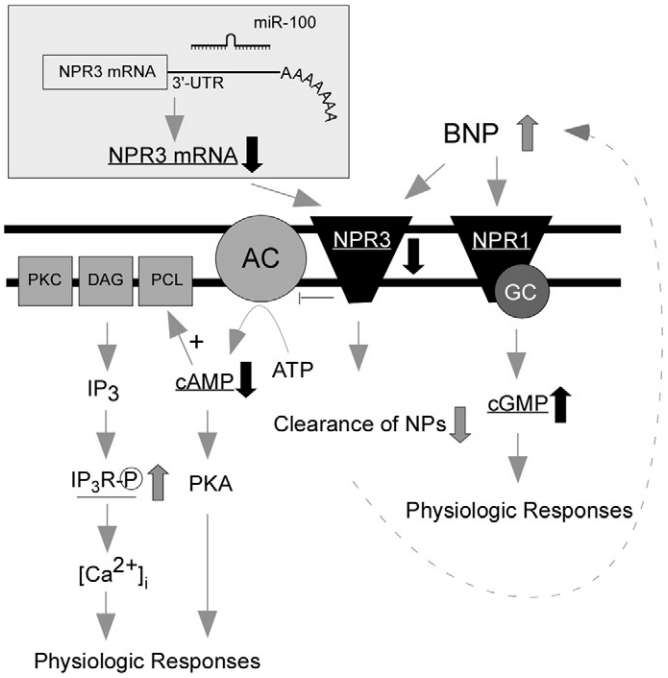

Fig. 7. Proposed role of miR-100 regulating NPR3 during cardiac overload/injury. The schematic diagram proposes that miR-100 might play a role in the cardiovascular system via regulation of NPR3 expression. Modulation of miR-100 could be a possible adaptive mechanism whereby its up-regulation in stressed or injured cardiac cells/tissues subsequently suppresses the expression of NPR3 and prolongs the half-life of NPs in blood and tissues. Grey arrows denote the regulation.

5. Conclusion

Our study suggests that down-regulation of NPR3 by miR-100 may be a compensatory mechanism by increasing the levels of bioactive NPs under conditions of cardiac hypoxic stress. Current findings might be useful for developing therapeutics focused on NPs/NPR3 signaling.

Sources of funding

This research was supported by NUS-CVRI start-up grant (R-172-000-206-733).

Conflict of interest

None declared.

Acknowledgments

We would like to acknowledge Yhee Cheng Chng, Yu Pei Peh, Yiqing Li and Dawn S. Koh for their contributions to this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2015.02.019.

References

[1] Potter LR, Abbey-Hosch S, Dickey DM. Natriuretic peptides, their receptors, and cyclic guanosine monophosphate-dependent signaling functions. Endocr Rev 2006;27:47–72.
[2] Floras JS. Sympathoinhibitory effects of atrial natriuretic factor in normal humans. Circulation 1990;81:1860–73.
[3] Tamura N, Ogawa Y, Chusho H, Nakamura K, Nakao K, Suda M, et al. Cardiac fibrosis in mice lacking brain natriuretic peptide. Proc Natl Acad Sci U S A 2000;97:4239–44.

[4] Oliver PM, Fox JE, Kim R, Rockman HA, Kim HS, Reddick RL, et al. Hypertension, cardiac hypertrophy, and sudden death in mice lacking natriuretic peptide receptor A. Proc Natl Acad Sci U S A 1997;94:14730–5.
[5] Lopez MJ, Wong SK, Kishimoto I, Dubois S, Mach V, Friesen J, et al. Salt-resistant hypertension in mice lacking the guanylyl cyclase-A receptor for atrial natriuretic peptide. Nature 1995;378:65–8.
[6] Hunt PJ, Espiner EA, Nicholls MG, Richards AM, Yandle TG. Differing biological effects of equimolar atrial and brain natriuretic peptide infusions in normal man. J Clin Endocrinol Metab 1996;81:3871–6.
[7] Kuwahara K, Nakao K. Regulation and significance of atrial and brain natriuretic peptides as cardiac hormones. Endocr J 2010;57:555–65.
[8] Maack T, Suzuki M, Almeida FA, Nussenzveig D, Scarborough RM, McEnroe GA, et al. Physiological role of silent receptors of atrial natriuretic factor. Science 1987;238:675–8.
[9] Almeida FA, Suzuki M, Scarborough RM, Lewicki JA, Maack T. Clearance function of type C receptors of atrial natriuretic factor in rats. Am J Physiol 1989;256:R469–75.
[10] Charles CJ, Espiner EA, Nicholls MG, Richards AM, Yandle TG, Protter A, et al. Clearance receptors and endopeptidase 24.11: equal role in natriuretic peptide metabolism in conscious sheep. Am J Physiol 1996;271:R373–80.
[11] Hobbs A, Foster P, Prescott C, Scotland R, Ahluwalia A. Natriuretic peptide receptor-C regulates coronary blood flow and prevents myocardial ischemia/reperfusion injury: novel cardioprotective role for endothelium-derived C-type natriuretic peptide. Circulation 2004;110:1231–5.
[12] Matsukawa N, Grzesik WJ, Takahashi N, Pandey KN, Pang S, Yamauchi M, et al. The natriuretic peptide clearance receptor locally modulates the physiological effects of the natriuretic peptide system. Proc Natl Acad Sci U S A 1999;96:7403–8.
[13] International Consortium for Blood Pressure Genome-Wide Association S, Ehret GB, Munroe PB, Rice KM, Bochud M, Johnson AD, et al. Genetic variants in novel pathways influence blood pressure and cardiovascular disease risk. Nature 2011;478:103–9.
[14] Moyes AJ, Khambata RS, Villar I, Bubb KJ, Baliga RS, Lumsden NG, et al. Endothelial C-type natriuretic peptide maintains vascular homeostasis. J Clin Invest 2014;124:4039–51.
[15] Palaparti A, Li Y, Anand-Srivastava MB. Inhibition of atrial natriuretic peptide (ANP) C receptor expression by antisense oligodeoxynucleotides in A10 vascular smooth-muscle cells is associated with attenuation of ANP-C-receptor-mediated inhibition of adenylyl cyclase. Biochem J 2000;346(Pt 2):313–20.
[16] Anand-Srivastava MB. Natriuretic peptide receptor-C signaling and regulation. Peptides 2005;26:1044–59.
[17] Kragelund C, Gronning B, Kober L, Hildebrandt P, Steffensen R. N-terminal pro-B-type natriuretic peptide and long-term mortality in stable coronary heart disease. N Engl J Med 2005;352:666–75.
[18] Bibbins-Domingo K, Ansari M, Schiller NB, Massie B, Whooley MA. B-type natriuretic peptide and ischemia in patients with stable coronary disease: data from the Heart and Soul study. Circulation 2003;108:2987–92.
[19] Cao L, Wu J, Gardner DG. Atrial natriuretic peptide suppresses the transcription of its guanylyl cyclase-linked receptor. J Biol Chem 1995;270:24891–7.
[20] Yoshimoto T, Naruse M, Naruse K, Shionoya K, Tanaka M, Tanabe A, et al. Angiotensin II-dependent down-regulation of vascular natriuretic peptide type C receptor gene expression in hypertensive rats. Endocrinology 1996;137:1102–7.
[21] Pasquinelli AE. MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. Nat Rev Genet 2012;13:271–82.
[22] Thum T, Galuppo P, Wolf C, Fiedler J, Kneitz S, van Laake LW, et al. MicroRNAs in the human heart: a clue to fetal gene reprogramming in heart failure. Circulation 2007;116:258–67.
[23] Condorelli G, Latronico MV, Cavarretta E. microRNAs in cardiovascular diseases: current knowledge and the road ahead. J Am Coll Cardiol 2014;63:2177–87.
[24] Leite-Moreira AM, Lourenco AP, Falcao-Pires I, Leite-Moreira AF. Pivotal role of microRNAs in cardiac physiology and heart failure. Drug Discov Today 2013;18:1243–9.
[25] Li C, Pei F, Zhu X, Duan DD, Zeng C. Circulating microRNAs as novel and sensitive biomarkers of acute myocardial Infarction. Clin Biochem 2012;45:727–32.
[26] Kumarswamy R, Thum T. Non-coding RNAs in cardiac remodeling and heart failure. Circ Res 2013;113:676–89.
[27] Fiedler J, Thum T. MicroRNAs in myocardial infarction. Arterioscler Thromb Vasc Biol 2013;33:201–5.
[28] Martinez EC, Wang J, Lilyanna S, Ling LH, Gan SU, Singh R, et al. Post-ischaemic angiogenic therapy using in vivo prevascularized ascorbic acid-enriched myocardial artificial grafts improves heart function in a rat model. J Tissue Eng Regen Med 2013;7:203–12.
[29] Santhanakrishnan R, Ng TP, Cameron VA, Gamble GD, Ling LH, Sim D, et al. The Singapore Heart Failure Outcomes and Phenotypes (SHOP) study and Prospective Evaluation of Outcome in Patients with Heart Failure with Preserved Left Ventricular Ejection Fraction (PEOPLE) study: rationale and design. J Card Fail 2013;19:156–62.
[30] Santhanakrishnan R, Chong JP, Ng TP, Ling LH, Sim D, Leong KT, et al. Growth differentiation factor 15, ST2, high-sensitivity troponin T, and N-terminal pro brain natriuretic peptide in heart failure with preserved vs. reduced ejection fraction. Eur J Heart Fail 2012;14:1338–47.
[31] Karolina DS, Tavintharan S, Armugam A, Sepramaniam S, Pek SL, Wong MT, et al. Circulating miRNA profiles in patients with metabolic syndrome. J Clin Endocrinol Metab 2012;97:E2271–6.
[32] Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 2008;3:1101–8.
[33] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. Methods 2001;25:402–8.
[34] Gupta SK, Bang C, Thum T. Circulating microRNAs as biomarkers and potential paracrine mediators of cardiovascular disease. Circ Cardiovasc Genet 2010;3:484–8.
[35] de Bold AJ, Borenstein HB, Veress AT, Sonnenberg H. A rapid and potent natriuretic response to intravenous injection of atrial myocardial extract in rats. Life Sci 1981;28:89–94.
[36] Lee CY, Burnett Jr JC. Natriuretic peptides and therapeutic applications. Heart Fail Rev 2007;12:131–42.
[37] Goetze JP, Christoffersen C, Perko M, Arendrup H, Rehfeld JF, Kastrup J, et al. Increased cardiac BNP expression associated with myocardial ischemia. FASEB J 2003;17:1105–7.
[38] Chen YF, Durand J, Claycomb WC. Hypoxia stimulates atrial natriuretic peptide gene expression in cultured atrial cardiocytes. Hypertension 1997;29:75–82.
[39] Baertschi AJ, Adams JM, Sullivan MP. Acute hypoxemia stimulates atrial natriuretic factor secretion in vivo. Am J Physiol 1988;255:H295–300.
[40] Jin H, Yang RH, Chen YF, Jackson RM, Oparil S. Atrial natriuretic peptide attenuates the development of pulmonary hypertension in rats adapted to chronic hypoxia. J Clin Invest 1990;85:115–20.
[41] Goetze JP, Gore A, Moller CH, Steinbruchel DA, Rehfeld JF, Nielsen LB. Acute myocardial hypoxia increases BNP gene expression. FASEB J 2004;18:1928–30.
[42] Frangogiannis NG. The inflammatory response in myocardial injury, repair, and remodelling. Nat Rev Cardiol 2014;11:255–65.
[43] Montgomery RL, Hullinger TG, Semus HM, Dickinson BA, Seto AG, Lynch JM, et al. Therapeutic inhibition of miR-208a improves cardiac function and survival during heart failure. Circulation 2011;124:1537–47.
[44] Ge YY, Shi Q, Zheng ZY, Gong J, Zeng C, Yang J, et al. MicroRNA-100 promotes the autophagy of hepatocellular carcinoma cells by inhibiting the expression of mTOR and IGF-1R. Oncotarget 2014;5:6218–28.