Silence of LncRNA GAS5 Protects Cardiomyocytes H9c2 against Hypoxic Injury via Sponging miR-142-5p

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The regulatory role of long noncoding RNA (lncRNA) growth arrest-specific transcript 5 (GAS5) in both cancerous and noncancerous cells have been widely reported. This study aimed to evaluate the role of lncRNA GAS5 in heart failure caused by myocardial infarction. We reported that silence of lncRNA GAS5 attenuated hypoxia-triggered cell death, as cell viability was increased and apoptosis rate was decreased. This phenomenon was coupled with the down-regulated expression of p53, Bax and cleaved caspase-3, as well as the up-regulated expression of CyclinD1, CDK4 and Bcl-2. At the meantime, the expression of four heart failure-related miRNAs was altered when lncRNA GAS5 was silenced (miR-21 and miR-142-5p were up-regulated; miR-30b and miR-93 were down-regulated). RNA immunoprecipitation assay results showed that lncRNA GAS5 worked as a molecular sponge for miR-142-5p. More interestingly, the protective actions of lncRNA GAS5 silence on hypoxia-stimulated cells were attenuated by miR-142-5p suppression. Besides, TP53INP1 was a target gene for miR-142-5p. Silence of lncRNA GAS5 promoted the activation of PI3K/AKT and MEK/ERK signaling pathways in a miR-142-5p-dependent manner. Collectively, this study demonstrated that silence of lncRNA GAS5 protected H9c2 cells against hypoxia-induced injury possibly via sponging miR-142-5p, functionally releasing TP53INP1 mRNA transcripts that are normally targeted by miR-142-5p.

Keywords: H9c2 cell, heart failure, hypoxia, lncRNA GAS5, miR-142-5p

INTRODUCTION

Heart failure is a complex clinical syndrome caused by systolic and diastolic dysfunction, resulting in a mismatch between demand and supply of oxygenated blood (Vucicevic et al., 2018). It represents a debilitating disorder, affecting approximately 26 million people worldwide, and leading to more than 1 million hospitalizations in United States and Europe (Ambrosy et al., 2014). What’s worse, heart failure is associated with high morbidity and mortality as itself increases the risk of stroke (Kim and Kim, 2018). Recently, cardiac function can be significantly improved by medical treatment and instrument therapies. However, heart failure remains the major cause of death worldwide (Ziaeian and Fonarow, 2016) due to the current management is limited in improving symptoms and preventing disease progression. This phenomenon calls for a better understanding of heart failure, which will be helpful for improving the development of novel treatment strategies.

Noncoding RNAs (ncRNAs) are a class of RNAs without protein-coding capacity. ncRNAs were initially considered as “junk DNAs”. But recent decades, researchers found that
approximately 98% of the human genome are ncRNAs (Mattick, 2001), and they have regulatory functions that effectively feedback into a larger communication network (Adams et al., 2017). Long ncRNAs (lncRNAs) and microRNAs (miRNAs) are two main groups of ncRNAs that have gained widespread attention recently. It is believed that lncRNAs and miRNAs are key regulators in modulation of cell proliferation, cell-cycle progression, differentiation, apoptosis, migration, etc (Hagan et al., 2017; Mathieu et al., 2014; Montes and Lund, 2016; Peng and Croce, 2016). Besides, lncRNA may act as an endogenous sponge for miRNA, and thus prevent the target mRNA from degradation by miRNA (Ballantyne et al., 2016).

lncRNA growth arrest specific transcript 5 (GAS5) belongs to the 5’ terminal oligopyrimidine class and is a small nucleolar RNA (C/D box snoRNA genes) host gene similar to U22 to the 5’ terminal oligopyrimidine class and is a small nucleolar RNA (C/D box snoRNA genes) host gene similar to U22. GAS5 has been widely reported as a tumor suppressive gene in a wide range of cancers, such as bladder cancer (Wang et al., 2018), osteosarcoma (Wang and Kong, 2018), colorectal cancer (Li et al., 2018), and ovarian cancer (Li et al., 2017). Since the importance of lncRNA GAS5 in cancer has been well-established, several researchers focused on investigating the role of lncRNA GAS5 in noncancerous cells. For instance, knockdown of lncRNA GAS5 was found to promote endodermal differentiation of mouse embryonic stem cells (Tu et al., 2018). Likewise, knockdown of lncRNA GAS5 promoted the proliferation and differentiation, but repressed apoptosis of retinal ganglion cells (Xu and Xing, 2018). In this study, we studied the function of lncRNA GAS5 in hypoxia-injured H9c2 cells (a rat embryonic ventricular cardiomyocyte line). To this end, the expression of lncRNA GAS5 in H9c2 cells was silenced by shRNA transfection. This study demonstrated that knockdown of lncRNA GAS5 protected H9c2 cells against hypoxia-induced injury, and the expression of miR-142-5p was involved in the protective mechanism.

MATERIALS AND METHODS

Cell culture
Rat embryonic ventricular cardiomyocyte H9c2 (CRL-1446, ATCC, USA) was routinely cultured in DMEM (Sigma-Aldrich, USA) containing 10% fetal bovine serum (Gibco, USA). The cells were maintained at an atmosphere with 95% air and 5% CO2 at 37℃.

To make hypoxic injury, H9c2 cells were incubated in a hypoxic incubator containing 94% N2, 5% CO2, and 1% O2. The cells incubated in normoxic condition (with 21% O2) was performed for testing miR-142-5p levels. For AGO-RIP assay, the cells were transfected with miR-142-5p mimic or miR-NC. AGO2 antibody (Abcam) was used and the level of AGO2 was analyzed by the cytometry (Beckman Coulter, USA). Apoptotic cells (FITC-annexin V-positive and PI-negative) were analyzed by using FlowJo software (TreeStar, USA).

RNA immunoprecipitation (RIP) assay
For testing the endogenous association between IncRNA GAS5 and miR-142-5p, MS2-RIP and AGO-RIP assays were carried out as previously described (Li et al., 2015). In brief, GAS5 wild type (wt) or GAS5 mutant type (mut) was respectively inserted into pcDNA3.1-MS2 plasmid (Addgene, USA). These plasmids were co-transfected with pMS2-GFP into H9c2 cells with the help of lipofectamine 3000 reagent (Life Technologies Corporation). Empty pcDNA3.1-MS2 plasmid was transfected as a blank control. At 48 h of transfection, cells were used for RIP by using Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA) and GFP antibody (Abcam, USA). IgG antibody purchased from Abcam was used as a negative control. Finally, qPCR was performed for testing miR-142-5p levels. For AGO-RIP assay, the cells were transfected with miR-142-5p mimic or miR-NC. AGO2 antibody (Abcam) was used and the level of IncRNA GAS5 was tested by qPCR.

Dual-luciferase reporter assay
The 3'UTR fragment of TP53INP1 was inserted into pmirGLO Dual-luciferase miRNA Target Expression Vector (Promega, USA) for construction of TP53INP1-wt plasmid. TP53INP1-mut for expression of TP53INP1 3'UTR with mutant type was constructed as a control reporter vector. These vectors were co-transfected with miR-142-5p mimic or miR-NC into H9c2 cells with the help of lipofectamine 3000 reagent (Life Tech-
After transfection, Dual-Luciferase Reporter Assay System (Promega) was used to test luciferase activity according to the instructions recommended.

**RT-qPCR**

After transfection and hypoxia exposure, the cells in 24-well plates were washed twice with ice-cold PBS. Total RNA in cell was extracted by using the Trizol reagent (Life Technologies Corporation, USA). The purity and concentration of RNA in the extracts was tested by UV spectrophotometry. To test the expression of lncRNA GAS5 and TP53INP1, First Strand cDNA Synthesis Kit (GeneCopoeia, USA) and 2xAllinOneTM-Q-PCRmix (GeneCopoeia) were used. To test the expression of miR-378, miR-21, miR-30b, miR-93 and miR-142-5p, One Step PrimeScript® miRNA cDNA Synthesis Kit and Takara SYBR® PrimeScript™ RT-PCR Kit (both from TaKaRa Biotechnology, China) were used. β-actin was used as an internal control for lncRNA GAS5 and TP53INP1, and U6 for miRNAs. Fold changes were calculated by the 2^−ΔΔCt method.

**Western blot**

After transfection and hypoxia exposure, the cells in 24-well plates were washed twice with ice-cold PBS. Total protein in cell was extracted by using RIPA lysis buffer (Santa Cruz Biotechnology, USA). The proteins were separated by SDS-PAGE and were transferred onto PVDF membranes (Millipore, MA). After blocking for 1 h at room temperature with 5% nonfat milk in Tris buffered saline-0.01% Tween 20 (Santa Cruz Biotechnology), the membranes were incubated with primary antibodies at 4℃ overnight for detection of p53 (ab26), CyclinD1 (ab16663), CDK4 (ab199728), Bax (ab32503), Bcl-2 (ab32124), pro-caspase-3 (ab32150), cleaved-caspase-3 (ab2302), PI3K (ab191606), p-PI3K (ab182651), AKT (ab8805), p-AKT (ab38449), MEK (ab32091), p-MEK (ab96379), ERK (ab32537), p-ERK (ab131438), β-actin (ab8227, Abcam, USA) and TP53INP1 (orb163035, Biorbyt, USA). After three washes with Tris buffered saline-0.01% Tween 20, the membranes were incubated with goat anti-mouse IgG (ab6785, Abcam) or goat anti-rabbit IgG (ab6721, Abcam) for 1 h at room temperature. The signals were developed by using the chemiluminescence detection kit (Pierce, USA), and the intensity was quantified by using Image Lab™ Software (Bio-Rad).

**Statistics**

All experiments were repeated three times in triplicate. All results were presented as mean ± SD. Statistical analyses were done in the SPSS 19.0 software (SPSS Inc., USA). Difference between groups was analyzed by Student t test or ANOVA with Duncan post-hoc. A p-value of < 0.05 was considered as a significant result.

**RESULTS**

**Hypoxia induces cell damage in H9c2 cells**

To start with, H9c2 cells were subjected to hypoxic condition for 0-24 h. We observed that the viability of H9c2 cells was significantly decreased in hypoxia group as compared to the normoxia control group (p < 0.01 or p < 0.001, Fig. 1A). It seems that hypoxia reduced H9c2 cells viability in a time-dependent fashion. Considering the viability of cells was declined to 52.5% following 6 h of hypoxia exposure, 6 h was selected as a hypoxia-stimulating condition for use in the follow-up experiments. Figs. 1B and 1C showed that the protein level of p53 was significantly increased (p < 0.05),

![Fig. 1. Effect of hypoxia on H9c2 cell growth.](image)

(A) Viability of H9c2 cells was detected by CCK-8 assay, after exposure of hypoxia for 0-24 h. The cells cultured in normoxic condition were used as control. Then, H9c2 cells were exposed to hypoxia for 6 h, after which (B-C) protein changes of p53, CyclinD1, and CDK4, (D) apoptosis rate, and (E-F) protein changes of apoptosis-related factors were assessed by flow cytometer and Western blot. *p < 0.05, **p < 0.01, ***p < 0.001 compared to the normoxic control group.
while the protein levels of CyclinD1 and CDK4 were significantly decreased (both $p < 0.05$) in hypoxia group when compared to the normoxia control group. Fig. 1D showed that hypoxia significantly induced apoptosis, as the apoptosis rate was increased from 3.2% to 14.2% ($p < 0.001$). This phenomenon was consistent with the results obtained from Western blot analysis. As shown in Figs. 1E and 1F, Bax was up-regulated ($p < 0.01$), Bcl-2 was down-regulated ($p < 0.05$), and caspase-3 was clearly cleaved ($p < 0.001$) in hypoxia group as compared to the normoxia control group. These data collectively indicated that hypoxia triggered cell damage in H9c2 cells.

Silence of lncRNA GAS5 attenuates hypoxia-mediated cell death in H9c2 cells

To explore the functional effects of lncRNA GAS5 on hypoxia-injured H9c2 cells, sh-GAS5 or sh-NC was transfected into cell. RT-qPCR data in Fig. 2A showed that, transfection with sh-GAS5 significantly reduced the expression of lncRNA GAS5 in H9c2 cells when compared to sh-NC transfection ($p < 0.01$), indicating lncRNA GAS5 was successfully silenced. Further functional assay results showed that cell damage triggered by hypoxia was attenuated when lncRNA GAS5 was silenced. As compared to Hypoxia + sh-NC group, cell viability was significantly increased ($p < 0.05$, Fig. 2B), protein level of p53 was down-regulated ($p < 0.05$), and protein levels of CyclinD1 and CDK4 were up-regulated ($p < 0.05$, Figs. 2C and 2D) in Hypoxia + sh-GAS5 group. Meanwhile, apoptosis rate was lower in Hypoxia + sh-GAS5 group than that in Hypoxia + sh-NC group ($p < 0.05$, Fig. 2E). And also, the expression changes of apoptosis-related proteins, including Bax, Bcl-2 and caspase-3 induced by hypoxia were partially flattened by silence of lncRNA GAS5 ($p < 0.05$ or $p < 0.01$, Figs. 2F and 2G).
LncRNA GAS5 works as a molecular sponge for miR-142-5p

LncRNA GAS5 works as a molecular sponge for miR-142-5p. miR-142-5p, miR-378 (Pinti et al., 2017), miR-21 (Xu et al., 2016), miR-30b (Ellis et al., 2013), miR-93 (Dickinson et al., 2013) and miR-142-5p (Voellenkle et al., 2010) have been previously reported as heart failure-related miRNAs. In order to reveal whether lncRNA GAS5 functioned to H9c2 cells in a miRNA-mediated signaling, the regulatory relationship between lncRNA GAS5 and these miRNAs was studied. RT-qPCR data in Fig. 3A showed that transfection of cells with sh-GAS5 significantly up-regulated miR-21 and miR-142-5p expression (p < 0.05), down-regulated miR-30b and miR-93 expression (p < 0.05), while had no impact on miR-378 expression (p > 0.05), when compared to sh-NC group. These data indicated that miR-21 and miR-142-5p were negatively regulated by lncRNA GAS5. Considering that the elevated level of miR-142-5p by sh-GAS5 was higher than miR-21 level, miR-142-5p was selected for the follow-up investigations. Bioinformatics analysis showed that lncRNA GAS5 contains a binding site of miR-142-5p (Fig. 3B). To validate whether lncRNA GAS5 could directly bind with miR-142-5p, a construct containing lncRNA GAS5 transcripts combined with MS2 was generated (Fig. 3C). MS2 RIP assay results showed that MS2-GAS5 was able to enrich miR-142-5p as compared to MS2 empty vector and MS2-GAS5-mut (p < 0.001, Fig. 3D). At the meantime, AGO RIP assay results showed that lncRNA GAS5 was also significantly enriched by miR-142-5p (p < 0.001, Fig. 3E). Together, these data suggested that lncRNA GAS5 worked as a molecular sponge for miR-142-5p.

Silence of lncRNA GAS5 protects H9c2 cells against hypoxia-induced cell damage via up-regulation of miR-142-5p

Next, we measured the expression changes of miR-142-5p in response to hypoxia. As shown in Fig. 3F, miR-142-5p was significantly down-regulated by hypoxia, when compared to normoxic control group (p < 0.01). Besides, the expression of miR-142-5p was significantly up-regulated by hypoxia in H9c2 cells transfected with sh-GAS5 (p < 0.01), implying miR-142-5p might be a downstream effector of lncRNA GAS5 in response to hypoxia.

To validate the abovementioned hypothesis, an inhibitor specific for miR-142-5p was transfected into H9c2 cells. RT-qPCR data in Fig. 4A indicated that, the expression of miR-142-5p was significantly decreased in miR-142-5p inhibitor group than that in the NC group (p < 0.01). Of note, cell viability was decreased (p < 0.05, Fig. 4B), p53 was up-regulated, and CyclinD1 and CDK were down-regulated (p < 0.05, Figs. 4C and 4D) in Hypoxia + sh-GAS5 + miR-142-5p inhibitor group, than those in Hypoxia + sh-GAS5 + NC group. Also, the apoptosis rate was increased (p < 0.01, Fig. 4E), Bax was up-regulated, Bcl-2 was down-regulated, and caspase-3 was remarkably cleaved (Figs. 4F and 4G) in hypoxia as compared to hypoxia + sh-GAS5 + NC group. Together, these results demonstrated that lncRNA GAS5 worked as a molecular sponge for miR-142-5p and regulated miR-142-5p expression in response to hypoxia and protected cells against hypoxia-induced cell damage via up-regulation of miR-142-5p.
Hypoxia + sh-GAS5 + miR-142-5p inhibitor group, than those in Hypoxia + sh-GAS5 + NC group. Collectively, it seems that the protective effects of lncRNA GAS5 are impeded when miR-142-5p is knocked down, suggesting lncRNA GAS5 confers its function via regulating miR-142-5p.

Silence of lncRNA GAS5 promotes the activation of PI3K/AKT and MEK/ERK signaling pathways via up-regulation of miR-142-5p

Next, the expression changes of core proteins in the PI3K/AKT and MEK/ERK signaling pathways were determined by Western blot analysis. Results in Figs. 5A and 5B indicated that, hypoxia exposure significantly increased the phosphorylation levels of PI3K and AKT (p < 0.001 and p < 0.05), but have no significant impacts on the phosphorylation of MEK and ERK (p > 0.05). The phosphorylation levels of PI3K, AKT, MEK and ERK were significantly higher in Hypoxia + sh-GAS5 + NC group, than those in Hypoxia + sh-GAS5 + NC group (p < 0.01 or p < 0.001). However, the phosphorylation levels of these four kinases were significantly lower in Hypoxia + sh-GAS5 + miR-142-5p inhibitor group, than those in Hypoxia + sh-GAS5 + NC group (p < 0.01 or p < 0.001). Thus, we preliminarily conclude that silence of lncRNA GAS5 promotes the activation of PI3K/AKT and MEK/ERK signaling pathways possibly via up-regulation of miR-142-5p.

TP53INP1 is a target gene of miR-142-5p

Finally, by using the TargetScan online database, TP53INP1 was predicted as a target gene of miR-142-5p (Fig. 6A). Then, dual-luciferase reporter assay was carried out to verify the predicted results. Data in Fig. 6A showed that the relative luciferase activity was significantly suppressed by co-transfection with TP53INP1-wt and miR-142-5p mimic, when compared to co-transfection with TP53INP1-wt and miR-NC (p < 0.01). Besides, the mRNA (Fig. 6B) and protein (Figs. 6C and 6D) levels of TP53INP1 were significantly down-regulated by miR-142-5p mimic (p < 0.01), and were up-regulated by miR-142-5p inhibitor (p < 0.05 or p < 0.01). These data suggested TP53INP1 as a target gene of miR-142-5p.
DISCUSSION

The hypothesis of the present study was that silence of lncRNA GAS5 could protect H9c2 cells against hypoxia-mediated cell damage. The major findings are the following.

First, silence of lncRNA GAS5 attenuated hypoxia-triggered cell damage by controlling cell proliferation and apoptosis. Second, lncRNA GAS5 worked as a molecular sponge for miR-142-5p. Third, the protective actions of lncRNA GAS5 silence on hypoxia-stimulated cells were eliminated by miR-142-5p suppression together with lncRNA GAS5 silence.
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142-5p suppression. Fourth, silence of LncRNA GAS5 promoted the activation of PI3K/AKT and MEK/ERK signaling pathways also in a miR-142-5p-dependent manner. Fifth, TP53INP1 was a target gene of miR-142-5p.

A growing number of lncRNAs have been linked to various kinds of cardiovascular diseases. For instance, increased expression of lncRNA Kcnna Antisense RNA (Kcna2 AS) led to an increased incidence of ventricular arrhythmias in association with heart failure (Long et al., 2017). LncRNA UCA1 was able to promote the progression of cardiac hypertrophy, a condition associated with a series of cardiovascular diseases, including heart failure (Zhou et al., 2018). In this study, we explored the functional role of LncRNA GAS5, a widely reported tumor suppressive gene (Li et al., 2017; 2018; Wang et al., 2018; Wang and Kong, 2018), in hypoxia-injured H9c2 cells, aiming to evaluate the importance of LncRNA GAS5 in heart failure caused by myocardial infarction. We found that silence of LncRNA GAS5 significantly attenuated hypoxia-induced cell loss. These findings were consistence with a previous study performed in hypoxic/ischemic-injured neonatal brain and hippocampal neurons (Zhao et al., 2018), suggesting LncRNA GAS5 might contribute to hypoxia-induced cell death.

Recent studies indicate that lncRNAs can interact with miRNAs and these interactions play significant roles in the determination of cell fate (Cao et al., 2017; Duval et al., 2017). Herein, we focused on the regulation between LncRNA GAS5 and miRNAs, in order to reveal a possible underlying mechanism of which LncRNA GAS5 protected H9c2 cells against hypoxia. RT-qPCR data showed that miR-21 and miR-142-5p were up-regulated, miR-30b and miR-93 were down-regulated, and miR-378 was unaffected by silencing of LncRNA GAS5. These data suggested that miR-21 and miR-142-5p were negatively regulated by LncRNA GAS5, while miR-30b and miR-93 were positively regulated by LncRNA GAS5. Considering that the negative regulation between LncRNAs and miRNAs may due to binding effects, we inferred that miR-21 and miR-142-5p might be two downstream targets for LncRNA GAS5. Actually, several previous studies have confirmed miR-21 was a target for LncRNA GAS5 (Liu et al., 2018; Tao et al., 2017; Zhang et al., 2013). However, the present work for the first time demonstrated that miR-142-5p could also bind with LncRNA GAS5. Our further rescue assay showed that the protective actions of LncRNA GAS5 silence on hypoxia-injured H9c2 cells were eliminated by miR-142-5p suppression. It seems that silence of LncRNA GAS5 protects H9c2 cells against hypoxia-mediated cell death via up-regulation of miR-142-5p.

TP53INP1, a stress-induced tumor suppressor gene, has been identified as a key partner in regulating p53-mediated apoptosis (Okamura et al., 2001). In this study, p53 was sensitively regulated by LncRNA GAS5 silence and miR-142-5p suppression, suggesting an involvement of p53 in LncRNA GAS5 and miR-142-5p-mediated cell death in H9c2 cells. Besides, we found that TP53INP1 was a target gene for miR-142-5p. These data provided in vitro evidence that miR-142-5p modulated hypoxia-triggered cell damage via targeting TP53INP1. Together with the above mentioned hypothesis, we preliminary draw a conclusion that LncRNA GAS5 worked as a molecular sponge for miR-142-5p, in having miR-142-5p exhausted by binding effects, and subsequent preventing TP53INP1 from degradation by miR-142-5p.

PI3K/AKT and MEK/ERK signaling pathways are known to play key regulatory roles in numerous cellular functions, including proliferation, cell-cycle progression, apoptosis, differentiation and neoplastic transformation (Bader et al., 2005; Chang et al., 2003; Peyssonnaux and Eychene, 2001). The importance of these two signaling in heart failure has been widely revealed. For instance, Chinese medicine Qishenkel could prevent cardiomyocytes apoptosis through modulation of PI3K/AKT signaling pathway in animal and H9c2 cell model of heart failure (Chang et al., 2017). Advanced glycation end products caused cardiac hypertrophy via the MEK/ERK pathway (Ko et al., 2013). Under hypoxic condition, p-AKT is decreased, while the total level of AKT is unchanged (Hirai et al., 2003). Besides, AKT is a downstream effector of PI3K, and PI3K is importance for the activation of MEK and ERK (Schmidt et al., 2004). In the present study, we found that hypoxia exposure for 6 h significantly decreased p-PI3K and p-AKT levels, while have no impacts on the levels of p-MEK and p-ERK. More importantly, silence of LncRNA GAS5 activated PI3K/AKT and MEK/ERK signaling pathways, and the activation of these two signaling induced by LncRNA GAS5 silence was attenuated by miR-142-5p suppression. These results suggested that LncRNA GAS5 silence activated PI3K/AKT and MEK/ERK signaling pathways also in a miR-142-5p-dependent manner.

To sum up, we demonstrated that silence of LncRNA GAS5 protected H9c2 cells against hypoxia-induced injury via sponging miR-142-5p, functionally releasing TP53INP1 mRNA transcripts that are normally targeted by miR-142-5p. The findings in this study enlarged our understanding of LncRNA GAS5 in noncancerous cells.

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