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miR-30c and miR-181a synergistically modulate p53–p21 pathway in diabetes induced cardiac hypertrophy

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Abstract p53–p21 pathway mediates cardiomyocyte hypertrophy and apoptosis and is upregulated in diabetic cardiomyopathy (DbCM). We investigated role of microRNAs in regulating p53–p21 pathway in high glucose (HG)-induced cardiomyocyte hypertrophy and apoptosis. miR-30c and miR-181a were identified to target p53. Cardiac expression of microRNAs was measured in diabetic patients, diabetic rats, and in HG-treated cardiomyocytes. Effect of microRNAs over-expression and inhibition on HG-induced cardiomyocyte hypertrophy and apoptosis was examined. Myocardial expression of p53 and p21 genes was increased and expression of miR-30c and miR-181a was significantly decreased in diabetic patients, DbCM rats, and in HG-treated cardiomyocytes. Luciferase assay confirmed p53 as target of miR-30c and miR-181a. Over-expression of miR-30c or miR-181a decreased expression of p53, p21, ANP, cardiomyocyte cell size, and apoptosis in HG-treated cardiomyocytes. Concurrent over-expression of these microRNAs resulted in greater decrease in cardiomyocyte hypertrophy and apoptosis, suggesting a synergistic effect of these microRNAs. Our results suggest that dysregulation of miR-30c and miR-181a may be involved in upregulation of p53–p21 pathway in DbCM.

Keywords Apoptosis · Cardiac hypertrophy · Diabetic cardiomyopathy · miR-30c · miR-181a · p53

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Introduction

Diabetes is known to be associated with increased risk of cardiovascular morbidity and mortality even in the absence of hypertension and coronary atherosclerosis [1] and diabetic patients are at greater risk of ventricular dysfunction and heart failure [2]. Diabetes-induced cardiomyopathy (DbCM) is characterized by cardiomyocyte hypertrophy, myocyte apoptosis, and cardiac fibrosis [3]. Hyperglycemia has been shown to promote cardiomyocyte apoptosis by activation of p53 and several effector responses involving the local renin angiotensin system (RAS) [4]. The increased cardiomyocyte loss is suggested to induce compensatory hypertrophy of the remaining viable cardiomyocytes [5]. Increased p53 levels have been reported in DbCM and in various other models of cardiac hypertrophy [6–16]. Mönkemann et al. proposed that deregulation of p53–p21 axis was involved in the diabetes-induced myocyte apoptosis; they observed that p53-induced p21 (WAF1/CIP1) gene binds to and inhibits a broad range of cyclin–cyclin-dependent kinase complexes [17]. Golubnitschaja et al. showed that increased levels of p53 in cardiomyocytes in early diabetes resulted in induction of p21 and 14-3-3 σ genes, which in turn triggered cell-cycle arrest and DNA repair of these cells, resulting in their accumulation in the G1 and G2 phases. They proposed that deregulated p53–p21 pathway leads to decreased proliferative activity and tissue degeneration in diabetic myocardium in later stages of diabetes [18]. However, molecular mechanisms

resulting in dysregulation of this pathway in diabetic heart are not well studied.

microRNAs have emerged as one of the key players of gene regulation, and dysregulated expression of several microRNAs targeting genes involved in DbCM has been reported in several different studies [19–21]. miR-30 and miR-181a have been recently shown to regulate p53 expression in cardiomyocytes; for example, miR-30 was found to suppress the expression of p53 and its downstream target Drp1 in cardiomyocytes [22]. Forini et al., showed p53 as a direct target of miR-30 [23]. In a recent study, Cheah et al. 2014 reported that miR-181a binds to TP53 gene and inhibits its expression, decreasing the synthesis of p53 in head and neck cancer [24]. However, the role of these microRNAs in regulating p53–p21 axis in DbCM is lacking. Since p53–p21 axis has been found to play an important role in DbCM pathophysiology, we investigated if these microRNAs were involved in regulating the expression of p53, p21 in DbCM. Our results show a significantly decreased expression of miR-30c and miR-181a in DbCM hearts, and over-expression of these miRNAs in high glucose (HG)-treated cardiomyocytes decreased expression of p53 and its downstream target p21 synergistically and attenuated HG-induced cardiomyocytes apoptosis and hypertrophy. To the best of our knowledge, this is the first report showing involvement of synergistic regulation of p53–p21 pathway by miR-30c and miR-181a in DbCM.

Materials and methods

Animal model of diabetic cardiomyopathy

An animal model of diabetic cardiomyopathy was developed by high-fat diet (HFD) and two low-dose Streptozotocin (STZ) as described previously [25]. Briefly, male Wistar rats were fed HFD for 4 weeks, followed by two injections of STZ (i.p. 30 mg/kg body weight), a week apart. Sex- and body weight-matched control rats were given an equal volume of citrate buffer. DbCM was confirmed by echocardiography, morphological, histopathological examination, and cardiac expression of hypertrophic markers (ANP & β-MHC). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This study was performed in compliance with the "Guide for the Care and Use of Laboratory Animals," published by the National Institutes of Health (National Institutes of Health publication 85-23, revised 1985). The study protocol was approved (Approval number: 62/IAEC/358) by the Animal Experimentation Committee of the Post Graduate Institute of Medical Education and Research, Chandigarh India.

Archived tissue samples

The formalin-fixed paraffin-embedded cardiac tissue samples were obtained from the Department of Histopathology PGIMER, Chandigarh. The samples were stratified into DbCM ($n = 5$) based on the following inclusion criteria: Cases with history of Diabetes Mellitus for more than 5 years with clinical symptoms related to heart failure. Patients with history of hypertension, coronary artery disease, or myocardial infarction and angina were excluded from the study. In addition, patients with history of chronic renal disease were also excluded from the study. Detailed examination of the heart autopsy was done both grossly and microscopically and the cases selected did not show any coronary artery occlusion or any evidence of old or fresh myocardial infarction. The control group ($n = 5$) included patients without history of diabetes mellitus and cardiovascular diseases. The study was approved by the institutional Ethical Review Committee, and was conducted in accordance with guidelines of the Declaration of Helsinki.

Cell culture

Rat cardiomyocyte cell line H9c2 was procured from NCCS, Pune and cells were cultured in low glucose Dulbecco's Modified Eagle's Medium (DMEM; Cat No-31600-034; Life Technologies), supplemented with 10 % fetal bovine serum (FBS). For High glucose treatment, cells were starved in low glucose DMEM containing 0.1 % FBS for 24 h and incubated with either 30 mM D-glucose (HG) or 5.5 mM (Normal) or 30 mM Mannitol (Osmotic control, OC) for 48 h [26].

Phalloidin staining and morphometric analysis

Filamentous actin was stained with Alexa fluor® 488 phalloidin (Molecular Probes™ Invitrogen detection technologies) and cardiomyocyte cross section area was measured as described previously [26].

RNA extraction and real-time PCR (qRT-PCR)

To measure cardiac expression of various genes, total RNA was extracted with mirVana™ microRNA isolation kit (Ambion, USA) according to the protocol of the manufacturers followed by DNase I treatment 1unit/uL (Sigma-Aldrich; AMPD1). Total RNA (1 μg) was used for cDNA synthesis with RevertAid™ reverse transcriptase by using RevertAid first strand cDNA synthesis kit (Fermentas Life Sciences, EU). qRT-PCR was performed using the Real-Time PCR 7500 (Applied Biosystem). For a final reaction volume of 20 μL, the following reagents were added:

10 μL SYBR Green Taq ReadyMix (Applied Biosystem), 0.5 μL of each forward and reverse primer (Primer list, Table 1), 8 μL dH_2O, and 1 μL cDNA. Melting curve analysis was used to determine melting temperature (Tm) of specific amplification products and primer dimers. The data were normalized to 18S RNA to account for differences in reverse transcription efficiencies and amount of template in the reaction [26].

microRNA analysis by qRT-PCR

Total RNA was isolated from cells or tissues using the mirVana™ microRNA isolation kit (Ambion, USA) according to the protocol of the manufacturers followed by DNase I treatment 1unit/uL (Sigma-Aldrich; AMPD1). microRNA-specific cDNA was generated with high specificity microRNA 1st strand cDNA synthesis kit (Agilent Technlogies, USA). qRT-PCR was performed using 10 μL SYBR Green Taq Ready Mix (Applied Biosystem), and 0.5 μL forward primer for miR-30c (TGT AAA CAT CCT ACA CTC TCA GC), miR-181a (AAC ATT CAA CGC TGT CGG TGA GT) and 0.5 μL universal reverse primer (Agilent Technologies, USA). The data were normalized to 18S RNA [26].

Western blotting

Cardiomyocyte cells H9c2 were lysed in RIPA lysis solution [50 mM Tris–HCl pH 7.6, 150 mM NaCl, 1 mM EGTA, 1 % Triton NP-40, 1 % sodium deoxycholate, 0.1 % SDS, 50 mM NaF and 1X Protease inhibitor] and protein concentration was estimated by quick start™ Bradford dye reagent 1X (BIO-RAD USA). Equal amounts of protein lysate (50 μg/lane) were resolved by SDS-PAGE and transferred onto PVDF membrane at 30 V for 8 h/4 °C. PVDF membrane was blocked in 5 % nonfat dry milk in TBST for overnight at 4 °C. Primary antibodies for the detection of p53 (**Cat no: sc-6243,** Santa Cruz Biotechnology, Dallas, Texas, USA), p21 (**Cat no: sc-397,** Santa Cruz Biotechnology, Dallas, Texas, USA), ANP (**Cat no: sc-18811,** Santa Cruz Biotechnology, Dallas, Texas, USA), and β-tubulin (**Cat no: sc-5274,** Santa Cruz Biotechnology, Dallas, Texas, USA) were used in the blocking buffer for 2 h at 37 °C under agitation on a rotary shaker. Target protein reactive with primary antibodies was detected with HRP-conjugated secondary antibodies for 2 h at 37 °C and the bands were visualized by Clarity™ western ECL substrate (BIO-RAD USA). The data were normalized to β-tubulin [26].

Table 1 Primer sequences for qRT-PCR

Gene	Sequence
p53	F 5′-GTCTACGTCCCGCCATAAAA-3′
	R 5′-AGGCAGTGAAGGGACTAGCA-3′
p21	F 5′-GAAAACGGAGGCAGACCAG-3′
	R 5′-TTCAGGGCTTTCTCTTGCAG-3′
18S	F 5′-CGCGGTTCTATTTTGTTGGT-3′
	R 5′-AGTCGGCATCGTTTATGGTC-3′
miR-30c	F 5′-TGTAAACATCCTACACTCTCAGC-3′
miR-181a	F 5′-AACATTCAACGCTGTCGGTGAGT-3′

microRNA over-expression/inhibition studies

For microRNA over-expression, cardiomyocytes were transfected with 40 nmol/L of microRNA mimics (Ambion, USA) using lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. microRNA mimics was complexed with the transfection reagent in Opti-MEM I reduced serum medium (Invitrogen) and added directly to cells. After 6 h, 1 % FBS was added along with high glucose treatment for 48 h. To inhibit microRNA expression, cardiomyocytes were transfected with 40 nmol/L of microRNA inhibitor (Ambion, USA), using lipofectamine 2000 transfection reagent (Invitrogen). Transfection using a scrambled sequence anti-miR (Ambion, USA) was used as a negative control [26].

Luciferase reporter assay

Cardiomyocytes were seeded in 96-well plates and co-transfected with luciferase plasmid 145-pGL3ctrl-3′ UTR (Addgene plasmid # 28175) which is a gift from Michael Kastan [27], pRL-SV40P (Addgene plasmid # 27163) renilla luciferase plasmid a gift from Ron Prywes [28] and miR-30c mimics or miR-181a mimics or control using Lipofectamine 2000 (Invitrogen). After 48-h incubation, luciferase activity was measured using a noncommercial dual luciferase enzyme assay system. Renilla luciferase was used as internal control and the firefly luciferase activity was calculated as the mean ± SD after being normalized by Renilla luciferase activity [29].

microRNA co-transfection

To over-express microRNA expression, cardiomyocytes were transfected with 20 nmol/L each of miR-30c mimics (pre–miR-30c; ID PM11060; Ambion) and miR-181a mimics (pre-miR-181a-5p; ID 10421; Ambion), using lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. miR-30c mimics and miR-181a mimics were complexed with the transfection reagent in Opti-MEM I reduced serum medium (Invitrogen) and added directly to cells. After 6 h 1 % FBS was added along with HG treatment for 48 h. Transfection using a scrambled sequence anti-miR (Ambion, USA) was used as a negative control.

Cell apoptosis assay by flow cytometry

H9c2 cells were seeded in 12-well plates at a suitable density and grown to 40 % confluency. miR-30c and miR-181a mimics were complexed with the transfection reagent in Opti-MEM I reduced serum medium (Invitrogen) and added directly to cells. After 6 h 1 % FBS was added along with HG treatment for 48 h. Finally, the cells were applied to apoptosis analysis. For flow cytometry analysis, an Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Invitrogen) was used and apoptotic rate was

Fig. 2 miR-30c and miR-181a directly target p53. **a** Schematics of the ▶ miR-30c and miR-181a putative binding site in rat p53 gene. **b** Luciferase reporter activities of chimeric vectors carrying the luciferase gene and a fragment of the p53 3′-UTR containing the binding sites of miR-30c or miR-181a in control, mimic, and NC groups. **c** p53 levels in HG-treated cardiomyocytes transfected with miR-30c mimic, miR-30c inhibitor or scrambled sequence. **d** p53 levels in HG-treated cardiomyocytes transfected with miR-181a mimic, miR-181a inhibitor or scrambled sequence. [# significantly different from 5.5 mM D-glucose; †significantly different from 30 mM D-glucose, mRNA levels are expressed as a ratio to 18S; protein levels are expressed as a ratio to β-tubulin and normalized to 5.5 mM D-glucose. $*P < 0.05$ considered significant. Results are expressed as mean ± SD. All data shown are results from minimum of three independent experiments, run in triplicate]

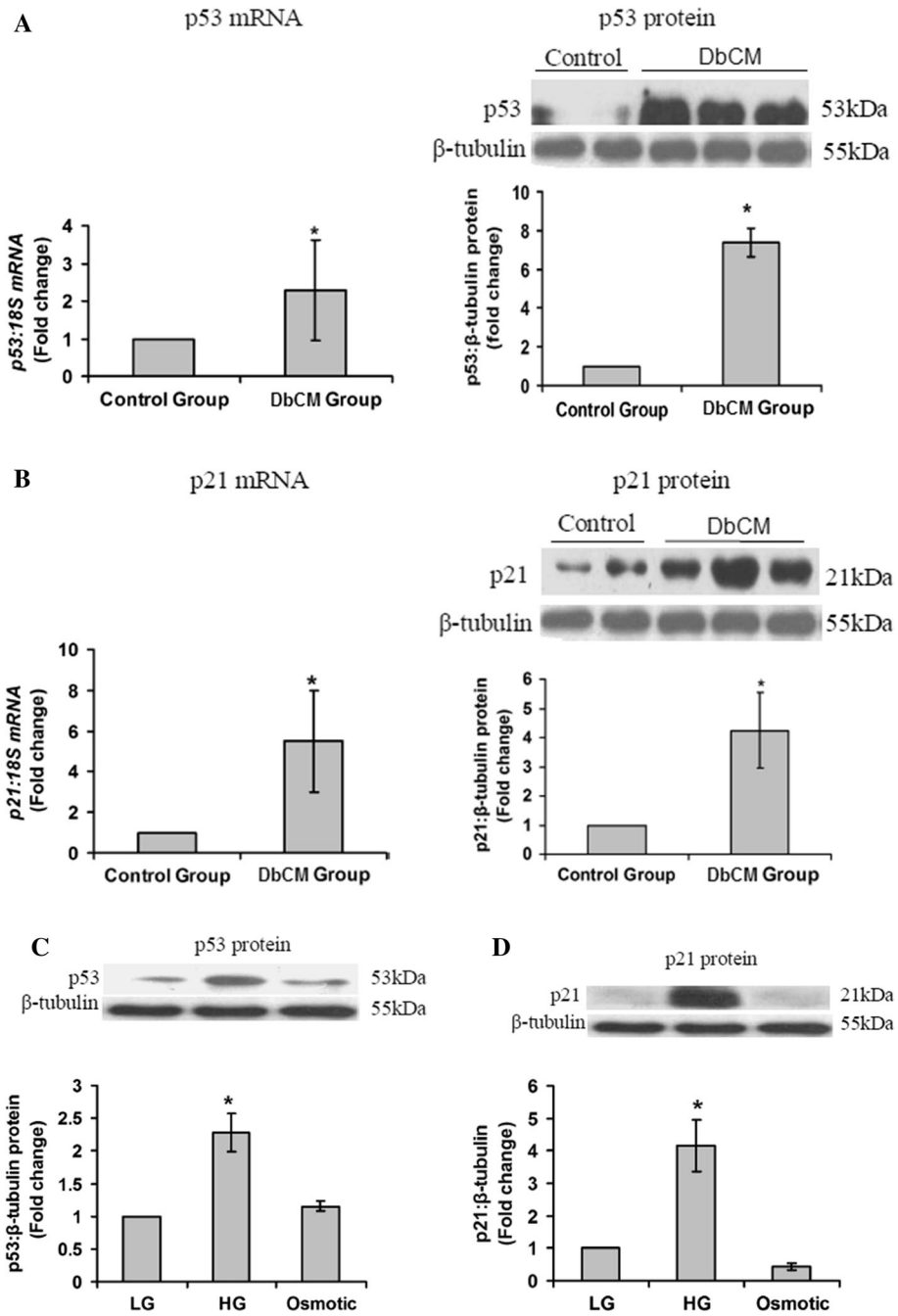
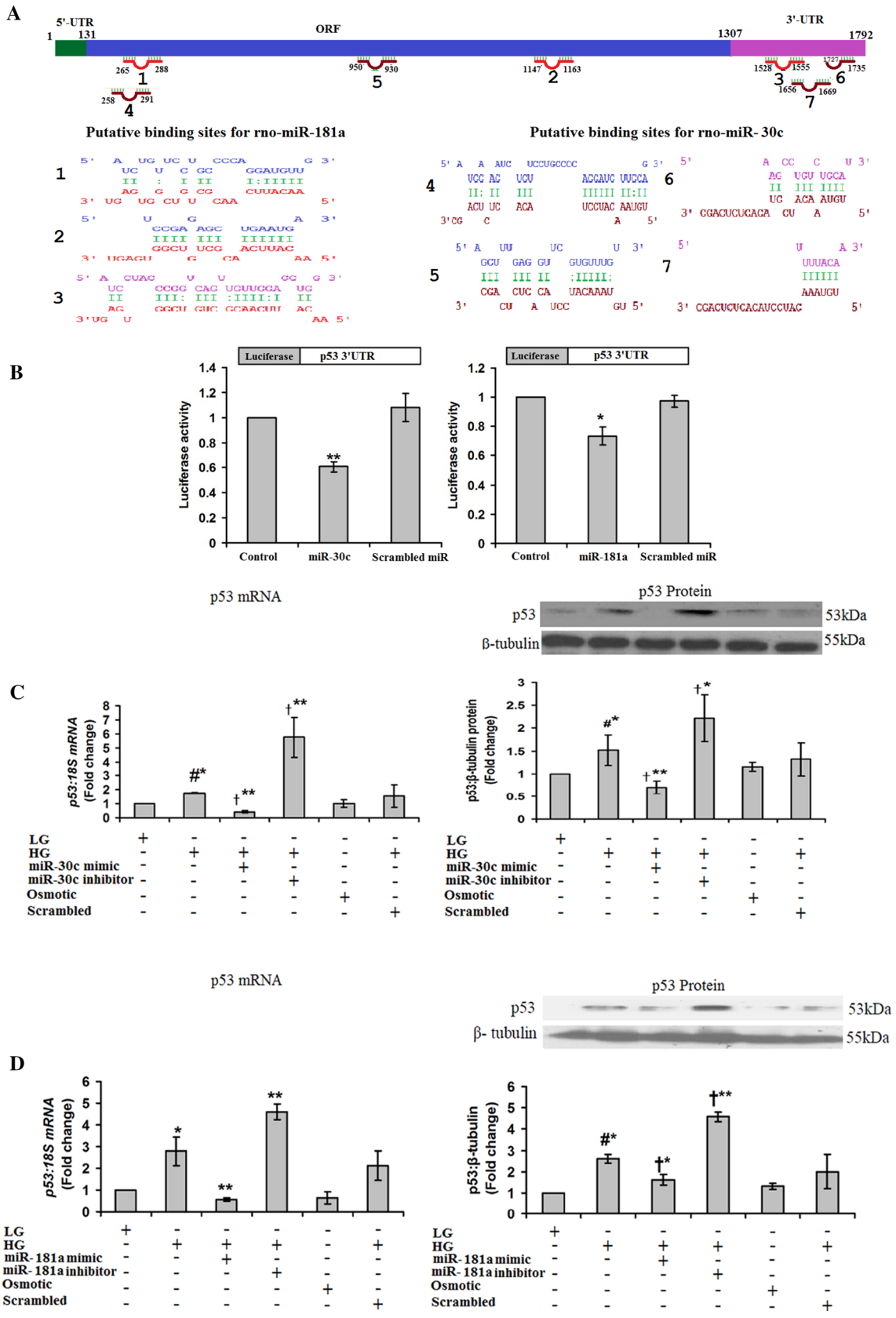


Fig. 1 p53 and p21 expression are increased in DbCM. **a** p53 expression (mRNA and protein) in DbCM and in control group, **b** p21 expression (mRNA and protein) in DbCM and in control group, ($n = 6$/group). Results are expressed as mean ± SD. $*P < 0.05$ compared to control group. **c** p53 and **d** p21 protein levels in HG and mannitol-treated cardiomyocytes. [Protein levels are expressed as ratio of target protein to β-tubulin, normalized to 5.5 mM D-glucose. $*P < 0.05$ compared to 5.5 mM D-glucose. Data are expressed as mean ± SD. Representative Western blot from three independent experiments, run in triplicate]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Putative binding sites for rno-miR-181a
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p53:18S mRNA (Fold change)
p53:β-tubulin protein (Fold change)
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miR-30c inhibitor
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miR-181a mimic
miR-181a inhibitor
β- tubulin

analyzed by flow cytometry (FACS CantoTM II, Becton–Dickinson) [30].

Statistical analysis

All results are shown as mean ± SD and sample sizes are mentioned in the figure legends. Student's t test or ANOVA was performed to compare the difference between means. P value <0.05 was considered significant.

Results

p53 and p21 expression in Cardiomyocytes was increased in high glucose-treated cardiomyocytes and in DbCM hearts

High glucose-treated cardiomyocytes showed significantly increased p53 and p21 protein expression ($P < 0.05$, Fig. 1C and D) as compared to LG (Normal Glucose; 5.5 mM D-glucose)-treated cells. Cardiac p53 and p21 levels were also significantly increased in DbCM rats as compared to control group ($P < 0.05$) (Fig. 1a, b).

p53 holds conserved binding sites for miR-30c and miR-181a: synergistic effect

RNAhybrid program showed that 3′-UTR of p53 mRNA has 'seed' sequences and flanking nucleotides complementary to both miR-30c and miR-181a. The ΔG values was calculated and found to be −16.9 kcal/mol and −17.3 kcal/mol for the complementary binding between miR-30c with 3′-UTR of p53 mRNA and −20.8 kcal/mol for miR-181a with 3′-UTR of p53 mRNA, respectively, suggesting this molecule to be the potential target of miR-30c and miR-181a (Fig. 2a).

Fig. 4 Effect of miR-30c and miR-181a over-expression and inhibition on p21 expression and cardiomyocyte hypertrophy. **a** p21 levels in HG-treated cardiomyocytes transfected with miR-30c mimic, miR-30c inhibitor or scrambled sequence. **b** p21 levels in HG-treated cardiomyocytes transfected with miR-181a mimic, miR-181a inhibitor or scrambled sequence. **c** ANP levels in HG-treated cardiomyocytes transfected with miR-181a mimic, miR-181a inhibitor or scrambled sequence. **d** Representative bright field image of cardiomyocytes and morphometric analysis in HG-treated cardiomyocytes with miR-181a mimics, miR-181a inhibitor or scrambled sequence. [#significantly different from 5.5 mM D-glucose; † significantly different from 30 mM D-glucose. *$P < 0.05$ considered significant. Results are expressed as mean ± SD. All data shown are results from minimum of three independent experiments] ▶

Luciferase assay validated p53 as a direct target miR-30c and miR-181a

To validate whether p53 was a direct target of miR-30c and miR-181a, cardiomyocytes were co-transfected with a 3′-UTR luciferase reporter vector along with either miR-30c mimic or miR-181a mimic, and 3′-UTR luciferase assays were performed. Luciferase activity was significantly reduced in cells co-transfected with miR-30c or miR-181a and 3′-UTR Luciferase vector as compared with cells transfected with luciferase vector alone (Fig. 2b). In order to determine whether miR-30c and miR-181a were regulating p53 expression in cardiomyocytes in hyperglycemic milieu, we modulated cellular expression of miR-30c or miR-181a, in vitro, by transfecting HG-treated cardiomyocytes with either miR-30c- or miR-181a-specific mimic or inhibitor. HG-treated cardiomyocytes transfected with miR-30c or miR-181a mimic showed a significantly decreased mRNA and protein expression of p53 as compared to HG-treated cardiomyocytes ($P < 0.05$, Fig. 2c, d), whereas transfection with miR-30c or miR-181a inhibitor resulted in significantly increased mRNA and protein expression of p53 levels in these cells. These

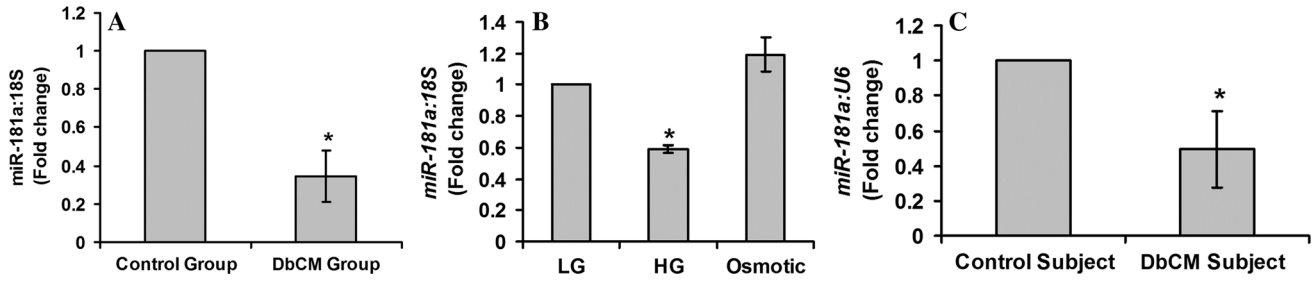
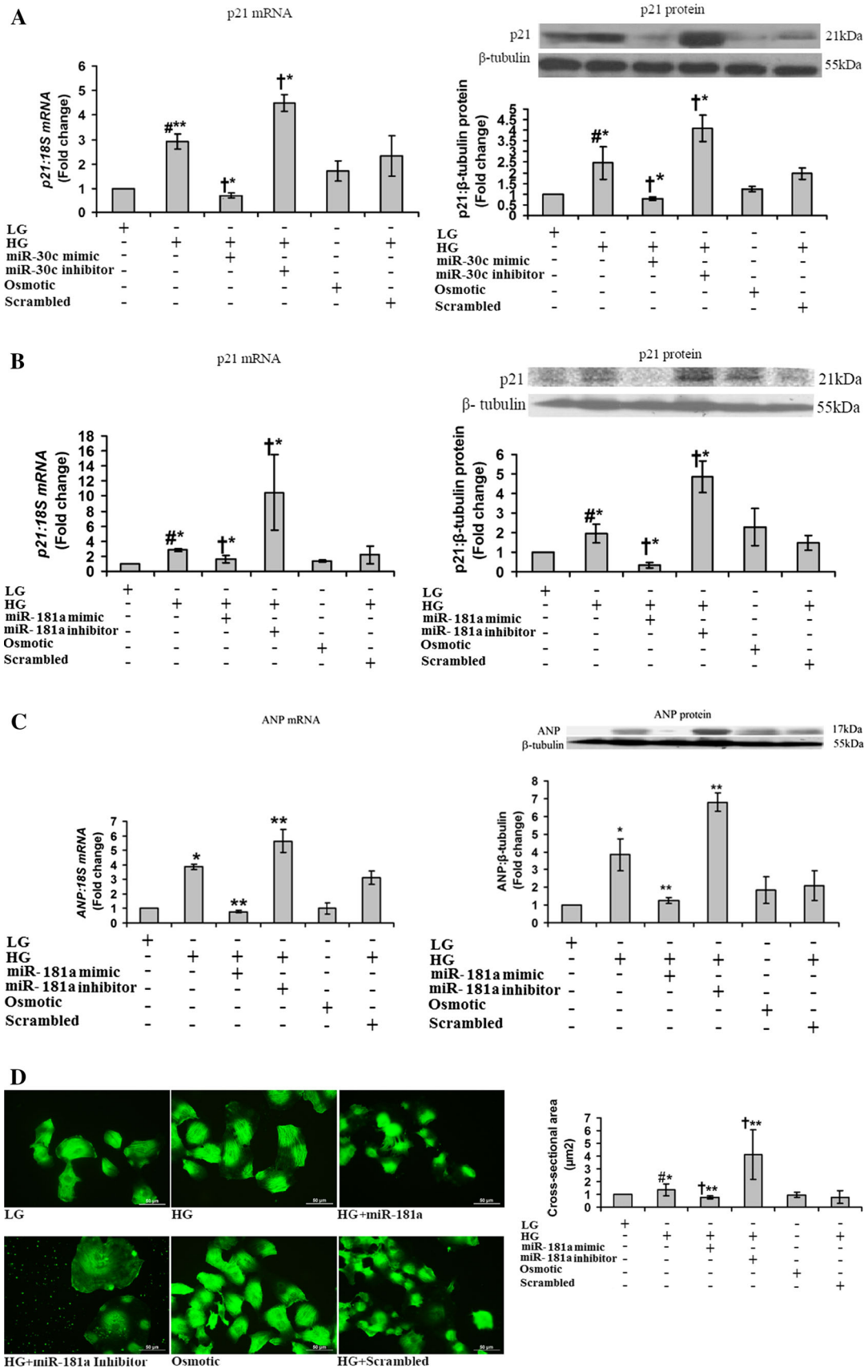


Fig. 3 Cardiac miR-30c and miR-181a are down regulated in DbCM. **a** Cardiac miR-181a expression in the DbCM rats and controls ($n = 6$/group). Data are expressed as mean ± SD. *$P < 0.05$ compared to control group. **b** miR-181a expression in H9c2 cardiomyocytes treated with 30 mM D-glucose or 5.5 mM D-glucose (LG). [microRNAs levels were calculated as ratio of 18S and are normalized to 5.5 mM D-Glucose. Results are expressed as mean ± SD. *$P < 0.05$ compared to 5.5 mM D-glucose. All data shown are results from minimum of three independent experiments run in triplicate]. **c** Myocardial expression of miR-181a in DbCM patients, ($n = 5$/group). Data are expressed as mean ± SD. *$P < 0.05$ compared to control subjects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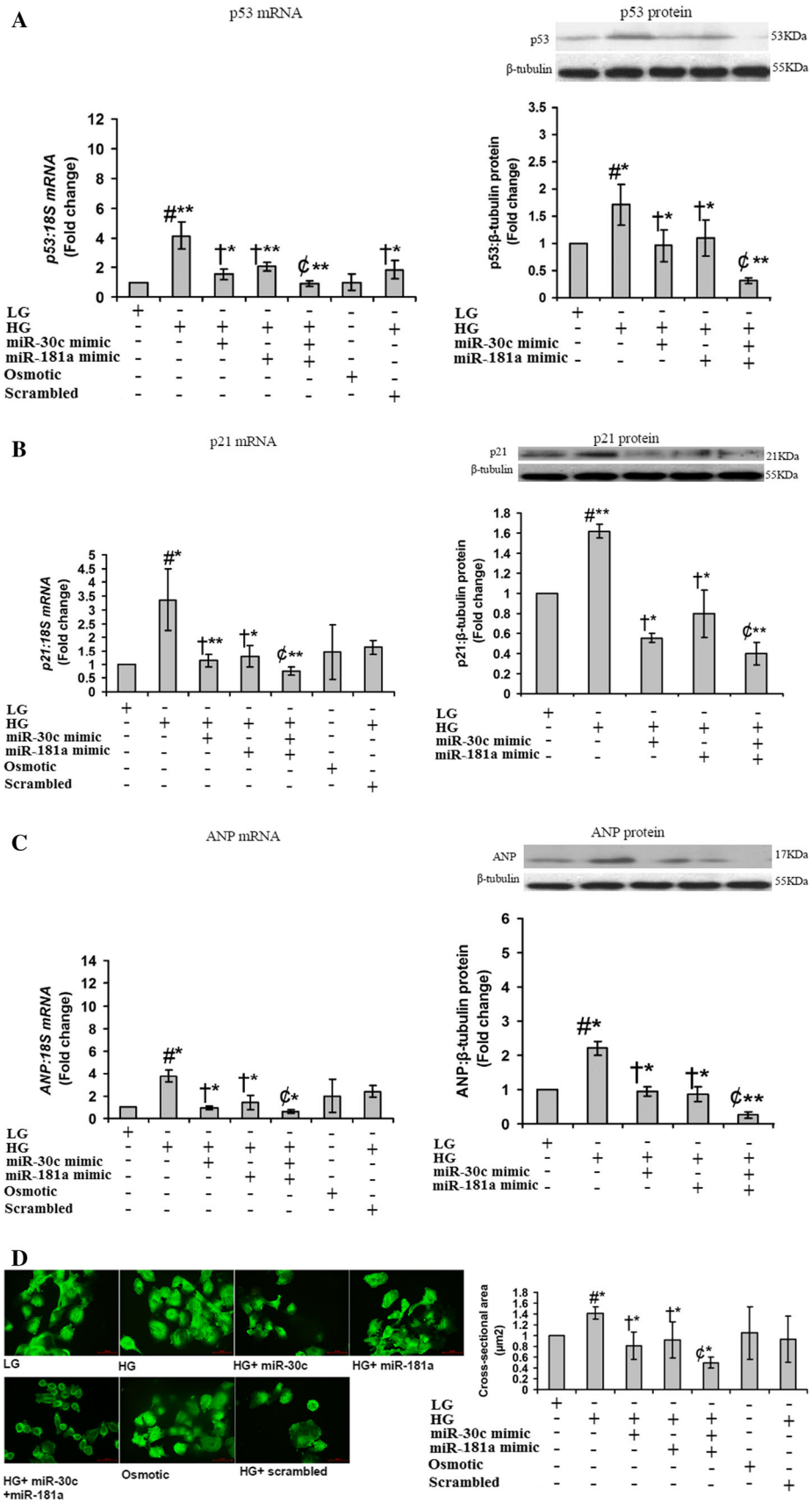

Fig. 5 Synergistic effects of miR-30c and miR-181a on p53–p21 axis and cardiomyocyte hypertrophy. **a** p53, **b** p21, and **c** ANP expression both at mRNA and protein in HG-treated cardiomyocytes co-transfected with miR-30c and miR-181a mimic. **d** Representative Phalloidin stained image of cardiomyocytes and morphometric analysis in HG-treated cardiomyocytes co-transfected with miR-30c mimics and miR-181a mimic. [#significantly different from 5.5 mM D-glucose; † significantly different from 30 mM D-glucose, ¢significantly different from either miR-30c or miR-181a alone. mRNA levels are expressed as a ratio to 18S; protein levels are expressed as a ratio to β-tubulin, normalized to 5.5 mM D-glucose. $*P < 0.05$ considered significant. Results are expressed as mean ± SD. All data shown are results from minimum of three independent experiments, run in triplicate]

results suggest that miR-30c and miR-181a directly regulate p53 expression by binding to p53 3′-UTR region.

Cardiac miR-30c and miR-181a were decreased in DbCM

Cardiac miR-181a expression was significantly decreased (3.1 fold) in DbCM as compared to control group ($P \leq 0.05$; Fig. 3a). HG-treated cardiomyocytes also showed decreased expression of miR-181a as compared to HG-treated cells (1.7 fold, $P < 0.05$) (Fig. 3b). Cardiac expression miR-181a was also found to be significantly decreased (2.3 fold, $P < 0.05$) in archived tissues of diabetic cardiomyopathy patients as compared to non-diabetic control subjects ($P < 0.05$) (Fig. 3c). Cardiac miR-30c expression was found to be significantly decreased in all three models of DbCM [26].

miR-181a and miR-30c over-expression decreased p21 expression and attenuated cardiomyocyte hypertrophy

HG-treated cardiomyocytes transfected with miR-30c mimic showed a significantly decreased expression of p21, whereas in vitro inhibition of miR-30c with miR-30 antagomir resulted in significantly increased expression of p21 ($P < 0.05$, Fig. 4a). Over-expression of miR-30c mimic resulted in significantly decreased expression of ANP and a significant decrease in cardiomyocyte cross-sectional area as compared to HG-treated cardiomyocytes, whereas transfection with miR-30c inhibitor resulted in significantly increased expression of ANP and cross-sectional area in these cells [26].

A significant decrease in p21 expression ($P < 0.05$, Fig. 4b), ANP expression, and cardiomyocyte cross-sectional area ($P < 0.05$, Fig. 4c, d) was seen in HG-treated cardiomyocytes transfected with miR-181a mimic as compared to HG-treated cardiomyocytes. The cardiomyocytes transfected with miR-181a inhibitor showed increase in p21 expression ($P < 0.05$, Fig. 4b), ANP expression and cell surface area in these cells ($P < 0.05$) (Fig. 4c, d).

miR-30c and miR-181a decreased HG-induced cardiomyocyte apoptosis

Cardiomyocyte apoptosis is a modulator of cardiomyocyte hypertrophy in diabetic cardiomyopathy, so we examined the effect of miR-30c and miR-181a on cardiomyocyte apoptosis under high glucose conditions, in vitro. We observed a significant decrease in number of apoptotic cells in HG-treated H9c2 cells transfected with either miR-30c or miR-181a mimics as compared to non-transfected HG-treated cells (11.9 vs. 18.3 %, and 10.5 vs. 18.3 %).

Concurrent over-expression of miR-30c and miR-181a inhibited p53–p21 pathway, cardiomyocyte apoptosis, and cardiomyocyte hypertrophy synergistically

Concurrent over-expression of miR-30c and miR-181a with miR-30c and miR-181a mimics resulted in greater decrease in p53 and p21 expression in HG-treated cardiomyocytes ($P < 0.05$) (Fig. 5a, b; Table 2). Concurrent over-expression of these microRNAs also resulted in 0.64 and 0.26 fold decrease in ANP mRNA and protein expression, as compared to miR-181a or miR-30c alone. It also led to greater decrease in cross-sectional area of cardiomyocytes (0.49 μm^2, $P < 0.05$) as compared to miR-181a or miR-30c alone (Fig. 5c, d; Table 2). Further, combined over-expression of miR-30c and miR-181a mimics resulted in greater decrease in apoptotic rate compared to either miR-30c or miR-181a alone (7.9 vs. 11.9 or 10.5 %) ($P < 0.05$; Fig. 6a, b; Table 2).

Table 2 Combined effect of miR-181a and miR-30c on HG-induced cardiomyocyte Cell size and Apoptosis

Treatment	Genes/cell size/apoptotic rate							
	p53		p21		ANP		Cell size	Apoptotic rate %
	mRNA (fold)	Protein (fold)	Mrna (fold)	Protein (fold)	mRNA (fold)	Protein (fold)	Cross-sectional area (μm^2)	
Normal glucose (LG)	1	1	1	1	1	1	1	100
High glucose (HG)	4.14**	1.71*	3.36*	1.61**	3.82*	2.20*	1.41*	120*
HG+ miR-181a mimic (40 nM)	2.04**	1.09*	1.30*	0.79*	1.40*	0.86*	0.92*	69.33**
HG+ miR-30c mimic (40 nM)	1.53*	0.96*	1.14**	0.55*	0.95*	0.94*	0.81*	78.66*
HG+ miR-181a mimic (20 nM) + miR-30c mimic (20 nM)	0.88**	0.31**	0.74**	0.39**	0.64*	0.26**	0.49*	52*

* $P < 0.05$, ** $P < 0.01$

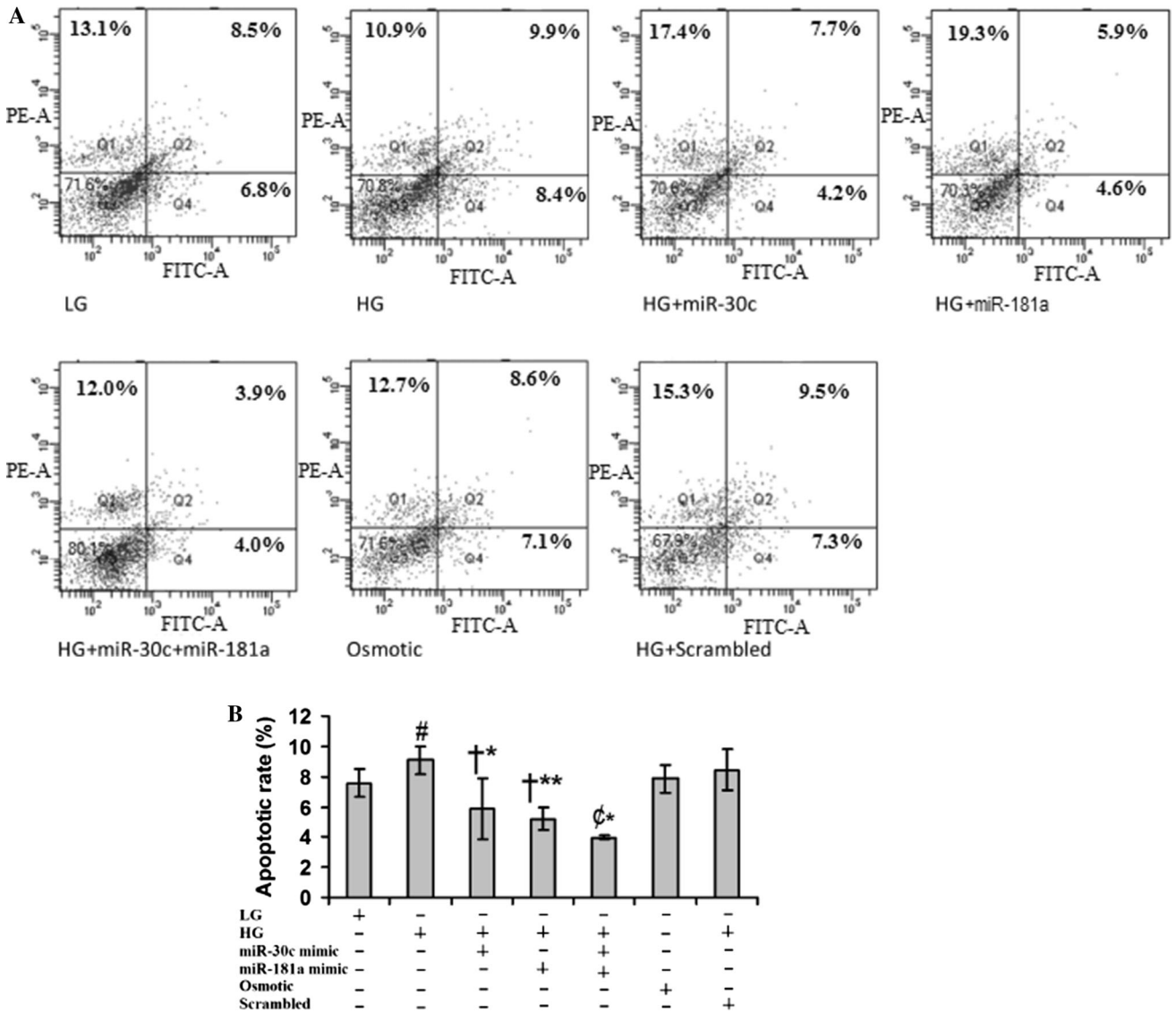

Fig. 6 Synergistic effects of miR-30c and miR-181a on apoptosis. **a** The combined effect of miR-30c and miR-181a on apoptosis was examined by FCM (flow cytometry) analysis. Cells were analyzed for apoptotic rate after staining with Annexin V-FITC and PI. [#significantly different from 5.5 mM D-glucose; †significantly different from 30 mM D-glucose, ¢significantly different from either miR-30c or miR-181a alone. $*P < 0.05$ considered significant. Results are expressed as mean ± SD. All data shown are results from minimum of three independent experiments, run in triplicate]. **b** One representative FCM analysis is shown in **b**

Discussion

Cardiac hypertrophy along with myocyte loss and interstitial fibrosis is an important pathological feature of diabetic cardiomyopathy. p53 has been shown to mediate oxidative stress-induced cardiac cell death via induction of p53–p21 pathway in diabetic cardiomyopathy [17]. However, the molecular mechanisms contributing to p53–p21 induction in DbCM remain obscure. In the present study, we provide evidence that down regulation of miR-30c and miR-181a may contribute to deregulation of p53–p21 pathway in diabetic cardiomyopathy. A significantly decreased expression of miR-30c and miR-181a in DbCM hearts and in HG-treated cardiomyocytes observed in the present study suggests that HG mediates down regulation of these microRNAs in cardiomyocytes. A decreased expression of miR-30c and miR-181a has been earlier reported in pathological heart failure such as myocardial infarction (MI), dilated cardiomyopathy (DCM), pathological left ventricular hypertrophy, cardiac fibrosis, apoptosis and human coronary artery SMCs [31–38]. Recently, Isserlin R et al. 2015 showed that apoptosis is a hallmark of multiple etiologies of heart failure, including dilated cardiomyopathy and silencing of miR-30c led to a

strong apoptotic phenotype in cell culture, suggesting they repress pro-apoptotic factors [39]. Feng et al., 2014 performed global microRNA profiling in Transverse aortic constriction (TAC) model of cardiac hypertrophy and observed decreased miR-181a expression in array [38]. Reddy et al. 2012 have also reported decreased miR-181a expression in hypertrophic cardiomyopathy [40]. However, there are conflicting reports on miR-30c in DCM and HCM; two recent studies have shown increased miRNA-30c expression in DCM [41], and increased miR-181a expression in cardiac tissue of hypertrophic cardiomyopathy patients with MYBPC3 mutations [42]. We observed that both miR-30c and miR-181a were down regulated in DbCM. These contradictory roles of miR-30 and 181-a may be due to different pathological triggers initiating the cardiac hypertrophic response and the different experimental systems employed. Moreover, the functional role of these microRNAs ascribed to till date is based on in vitro studies and its in vivo effects on the heart have not yet been clearly established. Thus, status of these microRNAs in heart failure still needs to be confirmed.

Both miR-30 and miR-181a have been reported to regulate p53 expression; for example, Li et al. 2010 showed that miR-30 suppressed p53 and its downstream target Drp1 in cardiomyocytes [22] and Cheah et al. 2014 have reported miR-181a to suppress p53 expression in head and neck cancer [24]. Our bioinformatics data showed that both miR-30c and miR-181a could potentially regulate p53 gene expression: 3′-UTR and ORF region of p53 mRNA showed 'seed' sequences and flanking nucleotides complementary to both miR-30c and miR-181a, suggesting this molecule to be potential target of miR-30c and miR-181a. We carried out luciferase reporter gene assay to further validate p53 to be target of these microRNAs and our results showed miR-30c and miR-181a directly regulated p53 expression by binding to p53 3′-UTR region, thus confirming p53 to be target gene of these microRNAs.

To explore the role of these microRNAs in DbCM, we increased the cellular expression of these microRNAs individually and also concurrently in HG-treated cardiomyocytes and found that their over-expression resulted in decreased expression of p53, p21 and attenuated HG-induced cardiomyocyte hypertrophy and as evidenced by decreased expression of hypertrophic marker (ANP) and myocyte cell size. These results indicate that both miR-30c and miR-181a may mediate HG-induced cardiomyocyte hypertrophy by regulating p53–p21 axis.

We observed that concurrent over-expression of miR-30c and miR-181a resulted in significantly decreased number of apoptotic cells in HG-treated cardiomyocytes indicating an anti-apoptotic response. miR-30c has been earlier shown to regulate cardiomyocytes apoptosis through suppressing the expression of p53 and targeting the mitochondrial fission machinery [22]. We also found that cardiomyocytes over-expressing both these microRNAs either individually or together showed significant decrease in p53 expression; however, when these microRNAs were co-expressed together, the decrease in p53 was more pronounced as compared to either miR-30c or miR-181a. Zhu et al., have also reported high synergy interaction between these two microRNAs in cardiovascular diseases [43]. Our results further confirm that miR-30c and miR-181a act synergistically to regulate p53 in cardiomyocytes. Thus, our results suggest that HG-induced decrease in miR-30c and miR-181a results in increased p53 expression leading to increased cardiomyocyte apoptosis. The increased apoptosis of cardiomyocytes has been reported to result in cardiomyocyte cell loss leading to cell hypertrophy as a compensatory mechanism [5]. We observed a significant decrease in the cardiomyocytes cell size and expression of hypertrophy marker, ANP in HG-treated cardiomyocytes suggesting a role for these microRNAs in HG-induced cardiomyocyte hypertrophy.

In conclusion, our results suggest down regulation of cardiac miR-30c and miR-181a in diabetic milieu. These microRNAs act synergistically in up-regulation of p53–p21 pathway in cardiomyocytes, thereby contributing to cardiomyocytes loss and hypertrophy in DbCM. Hence, appropriate manipulation of the expression of miR-30c and miR-181a in cardiomyocytes might help prevent the initiation and progression of diabetic cardiomyopathy.

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Compliance with ethical standards

Conflict of Interest The authors declare they have no conflict interests.

References

1. Avogaro A, Vigili de Kreutzenberg S, Negut C, Tiengo A, Scognamiglio R (2004) Diabetic cardiomyopathy: a metabolic perspective. Am J Cardiol 93:13A–16A
2. Anguita Sánchez M (2002) Prevention and treatment of congestive heart failure in diabetic patients. Rev Esp Cardiol 55:1083–1087
3. Nunes S, Soares E, Fernandes J, Viana S, Carvalho E, Pereira FC, Reis F (2013) Early cardiac changes in a rat model of prediabetes: brain natriuretic peptide overexpression seems to be the best marker. Cardiovasc Diabetol 12:44
4. Fiordaliso F, Leri A, Cesselli D, Limana F, Safai B, Nadal-Ginard B, Kajstura J (2001) Hyperglycemia activates p53 and p53-regulated genes leading to myocyte cell death. Diabetes 50:2363–2375

5. Letonja M, Petrovič D (2014) Is diabetic cardiomyopathy a specific entity? World J Cardiol 6:8–13
6. Abid S, Houssaïni A, Mouraret N, Marcos E, Amsellem V, Wan F, Dubois-Randé JL, Derumeaux G, Boczkowski J, Motterlini R, Adnot S (2014) p21-dependent protective effects of a carbon monoxide-releasing molecule-3 in pulmonary hypertension. Arterioscler Thromb Vasc Biol 34:304–312
7. Chatterjee A, Mir SA, Dutta D, Mitra A, Pathak K, Sarkar S (2011) Analysis of p53 and NF-κB signaling in modulating the cardiomyocyte fate during hypertrophy. J Cell Physiol 226:2543–2554
8. Das B, Young D, Vasanji A, Gupta S, Sarkar S, Sen S (2010) Influence of p53 in the transition of myotrophin-induced cardiac hypertrophy to heart failure. Cardiovasc Res 87:524–534
9. Hernández JS, Barreto-Torres G, Kuznetsov AV, Khuchua Z, Javadov S (2014) Crosstalk between AMPK activation and angiotensin II-induced hypertrophy in cardiomyocytes: the role of mitochondria. J Cell Mol Med 18:709–720
10. Ikeda S, Hamada M, Hiwada K (1999) Cardiomyocyte apoptosis with enhanced expression of P53 and Bax in right ventricle after pulmonary arterial banding. Life Sci 65:925–933
11. Jiang FL, Leo S, Wang XG, Li H, Gong LY, Kuang Y, Xu XF (2013) Effect of tanshinone IIA on cardiomyocyte hypertrophy and apoptosis in spontaneously hypertensive rats. Exp Ther Med 6:1517–1521
12. Kimura TE, Jin J, Zi M, Prehar S, Liu W, Oceandy D, Abe J, Neyses L, Weston AH, Cartwright EJ, Wang X (2010) Targeted deletion of the extracellular signal-regulated protein kinase 5 attenuates hypertrophic response and promotes pressure overload-induced apoptosis in the heart. Circ Res 106:961–970
13. Sano M, Minamino T, Toko H, Miyauchi H, Orimo M, Qin Y, Akazawa H, Tateno K, Kayama Y, Harada M, Shimizu I, Asahara T, Hamada H, Tomita S, Molkentin JD, Zou Y, Komuro I (2007) p53-induced inhibition of Hif-1 causes cardiac dysfunction during pressure overload. Nature 446:444–448
14. Tsukamoto O, Minamino T, Okada K, Shintani Y, Takashima S, Kato H, Liao Y, Okazaki H, Asai M, Hirata A, Fujita M, Asano Y, Yamazaki S, Asanuma H, Hori M, Kitakaze M (2006) Depression of proteasome activities during the progression of cardiac dysfunction in pressure-overloaded heart of mice. Biochem Biophys Res Commun 340:1125–1133
15. Vahtola E, Storvik M, Louhelainen M, Merasto S, Lakkisto P, Lakkisto J, Tikkanen I, Kaheinen P, Levijoki J, Mervaala E (2011) Diabetic cardiomyopathy and post-infarct ventricular remodelling: effects of levosimendan in a rodent model of type II diabetes. Basic Clin Pharmacol Toxicol 109:387–397
16. Vahtola E, Louhelainen M, Merasto S, Martonen E, Penttinen S, Aahos I, Kytö V, Virtanen I, Mervaala E (2008) Forkhead class O transcription factor 3a activation and Sirtuin1 overexpression in the hypertrophied myocardium of the diabetic Goto-Kakizaki rat. J Hypertens 26:334–344
17. Mönkemann H, De Vriese AS, Blom HJ, Kluijtmans LA, Heil SG, Schild HH, Golubnitschaja O (2002) Early molecular events in the development of the diabetic cardiomyopathy. Amino Acids 23:331–336
18. Golubnitschaja O, Moenkemann H, Trog DB, Blom HJ, De Vriese AS (2006) Activation zf genes inducing cell-cycle arrest and of increased DNA repair in the hearts of rats with early streptozotocin-induced diabetes mellitus. Med Sci Monit 12:BR68–BR74
19. Kuwabara Y, Horie T, Baba O, Watanabe S, Nishiga M, Usami S, Izuhara M, Nakao T, Nishino T, Otsu K, Kita T, Kimura T, Ono K (2015) MicroRNA-451 Exacerbates Lipotoxicity in Cardiac Myocytes and High-Fat Diet-Induced Cardiac Hypertrophy in Mice Through Suppression of the LKB1/AMPK Pathway. Circ Res 116:279–288
20. Zheng D, Ma J, Yu Y, Li M, Ni R, Wang G, Chen R, Li J, Fan GC, Lacefield JC, Peng T (2015) Silencing of miR-195 reduces diabetic cardiomyopathy in C57BL/6 mice. Diabetologia 58:1949–1958
21. Li X, Du N, Zhang Q, Li J, Chen X, Liu X, Hu Y, Qin W, Shen N, Xu C, Fang Z, Wei Y, Wang R, Du Z, Zhang Y, Lu Y (2014) MicroRNA-30d regulates cardiomyocyte pyroptosis by directly targeting foxo3a in diabetic cardiomyopathy. Cell Death Dis 5:e1479
22. Li J, Donath S, Li Y, Qin D, Prabhakar BS, Li P (2010) miR-30 regulates mitochondrial fission through targeting p53 and the dynamin-related protein-1 pathway. PLoS Genet 6:e 1000795
23. Forini F, Kusmic C, Nicolini G, Mariani L, Zucchi R, Matteucci M, Iervasi G, Pitto L (2014) Triiodothyronine prevents cardiac ischemia/reperfusion mitochondrial impairment and cell loss by regulating miR30a/p53 axis. Endocrinology 155:4581–4590
24. Cheah YK, Cheng RW, Yeap SK, Khoo CH, See HS (2014) Analysis of TP53 gene expression and p53 level of human hypopharyngeal FaDu (HTB-43) head and neck cancer cell line after microRNA-181a inhibition. Genet Mol Res 13:1679–1683
25. Zhang M, Lv XY, Li J, Xu ZG, Chen L (2009) The characterization of high-fat diet and multiple low-dose streptozotocin induced type 2 diabetes rat model. Exp Diabetes Res 2008:704045
26. Raut SK, Kumar A, Singh GB, Nahar U, Sharma V, Mittal A, Khullar M (2015) miR-30c Mediates Upregulation of Cdc42 and Pak1 in Diabetic Cardiomyopathy. Cardiovasc Ther 33:89–97
27. Chen J, Kastan MB (2010) 5′–3′-UTR interactions regulate p53 mRNA translation and provide a target for modulating p53 induction after DNA damage. Genes Dev 24:2146–2156
28. Chen X, Prywes R (1999) Serum-induced expression of the cdc25AGene by relief of E2F-mediated repression. Mol Cell Biol 19:4695–4702
29. Ganesan J, Ramanujam D, Sassi Y, Ahles A, Jentzsch C, Werfel S, Engelhardt S (2013) MiR-378 controls cardiac hypertrophy by combined repression of mitogen-activated protein kinase pathway factors. Circulation 127:2097–2106
30. Wang JX, Zhang XJ, Feng C, Sun T, Wang K, Wang Y, Li PF (2015) MicroRNA-532-3p regulates mitochondrial fission through targeting apoptosis repressor with caspase recruitment domain in doxorubicin cardiotoxicity. Cell Death Dis 6:e1677
31. Roca-Alonso L, Castellano L, Mills A, Dabrowska AF, Sikkel MB, Pellegrino L, Jacob J, Frampton AE, Krell J, Coombes R, Harding SE, Lyon AR, Stebbing J (2015) Myocardial MiR-30 downregulation triggered by doxorubicin drives alterations in β-adrenergic signaling and enhances apoptosis. Cell Death Dis 6:e1754
32. Duisters RF, Tijsen AJ, Schroen B, Leenders JJ, Lentink V, van der Made I, Herias V, van Leeuwen RE, Schellings MW, Barenbrug P, Maessen JG, Heymans S, Pinto YM, Creemers EE (2009) miR-133 and miR-30 regulate connective tissue growth factor Implications for a role of microRNAs in myocardial matrix remodeling. Circ Res 104:170–178
33. Liu Q, Du GQ, Zhu ZT, Zhang C, Sun XW, Liu JJ, Li X, Wang YS, Du WJ (2015) Identification of apoptosis-related microRNAs and their target genes in myocardial infarction post-transplantation with skeletal myoblasts. J Transl Med 13:270
34. Hirt MN, Werner T, Indenbirken D, Alawi M, Demin P, Kunze AC, Stenzig J, Starbatty J, Hansen A, Fiedler J, Thum T, Eschenhagen T (2015) Deciphering the microRNA signature of pathological cardiac hypertrophy by engineered heart tissue- and sequencing-technology. J Mol Cell Cardiol 81:1–9
35. Balderman JA, Lee HY, Mahoney CE, Handy DE, White K, Annis S, Lebeche D, Hajjar RJ, Loscalzo J, Leopold JA (2012) Bone morphogenetic protein-2 decreases MicroRNA-30b and MicroRNA-30c to promote vascular smooth muscle cell calcification. J Am Heart Assoc 1:e003905

36. Abonnenc M, Nabeebaccus AA, Mayr U, Barallobre-Barreiro J, Dong X, Cuello F, Sur S, Drozdov I, Langley SR, Lu R, Stathopoulou K, Didangelos A, Yin X, Zimmermann WH, Shah AM, Zampetaki A, Mayr M (2013) Extracellular matrix secretion by cardiac fibroblasts role of MicroRNA-29b and MicroRNA-30c. Circ Res 113:1138–1147
37. Carolina G, Claudia K, Elena C, Francesco M, Milena R, Laura M, Letizia P (2013) miR-29a and miR-30c negatively regulate DNMT 3a in cardiac ischemic tissues: implications for cardiac remodelling. MicroRNA Diagn Ther 1:2084–6843
38. Feng HJ, Ouyang W, Liu JH, Sun YG, Hu R, Huang LH, Xian JL, Jing CF, Zhou MJ (2014) Global microRNA profiles and signaling pathways in the development of cardiac hypertrophy. Braz J Med Biol Res 7:361–368
39. Isserlin R, Merico D, Wang D, Vuckovic D, Bousette N, Gramolini AO, Bader GD, Emili A (2014) Systems analysis reveals down-regulation of a network of pro-survival miRNAs drives the apoptotic response in dilated cardiomyopathy. Mol BioSyst 11:239–251
40. Reddy S, Zhao M, Hu DQ, Fajardo G, Hu S, Ghosh Z, Rajagopalan V, Wu JC, Bernstein D (2012) Dynamic microRNA expression during the transition from right ventricular hypertrophy to failure. Physiol Genomics 44:562–575
41. Wijnen WJ, van der Made I, van den Oever S, Hiller M, de Boer BA, Picavet DI, Chatzispyrou IA, Houtkooper RH, Tijsen AJ, Hagoort J, van Veen H, Everts V, Ruijter JM, Pinto YM, Creemers EE (2014) Cardiomyocyte-Specific miRNA-30c Over-Expression Causes Dilated Cardiomyopathy. PLOS ONE 9:e96290
42. Kuster DW, Mulders J, Ten Cate FJ, Michels M, Dos Remedios CG, da Costa Martins PA, van der Velden J, Oudejans CB (2013) MicroRNA transcriptome profiling in cardiac tissue of hypertrophic cardiomyopathy patients with MYBPC3 mutations. J Mol Cell Cardiol 65:59–66
43. Zhu W, Zhao Y, Xu Y, Sun Y, Wang Z, Yuan W, Du Z (2013) Dissection of Protein Interactomics Highlights MicroRNA Synergy. PLoS ONE 8:e63342