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# SERCA2a gene therapy restores microRNA-1 expression in heart failure via an Akt/FoxO3A-dependent pathway

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**Aims** Impaired myocardial sarcoplasmic reticulum calcium ATPase 2a (SERCA2a) activity is a hallmark of failing hearts, and SERCA2a gene therapy improves cardiac function in animals and patients with heart failure (HF). Deregulation of microRNAs has been demonstrated in HF pathophysiology. We studied the effects of therapeutic AAV9.SERCA2a gene therapy on cardiac miRNome expression and focused on regulation, expression, and function of miR-1 in reverse remodelled failing hearts.

**Methods and results** We studied a chronic post-myocardial infarction HF model treated with AAV9.SERCA2a gene therapy. Heart failure resulted in a strong deregulation of the cardiac miRNome. miR-1 expression was decreased in failing hearts, but normalized in reverse remodelled hearts after AAV9.SERCA2a gene delivery. Increased Akt activation in cultured cardiomyocytes led to phosphorylation of FoxO3A and subsequent exclusion from the nucleus, resulting in miR-1 gene silencing. *In vitro* SERCA2a expression also rescued miR-1 in failing cardiomyocytes, whereas SERCA2a inhibition reduced miR-1 levels. *In vivo*, Akt and FoxO3A were highly phosphorylated in failing hearts, but reversed to normal by AAV9.SERCA2a, leading to cardiac miR-1 restoration. Likewise, enhanced sodium–calcium exchanger 1 (NCX1) expression during HF was normalized by SERCA2a gene therapy. Validation experiments identified NCX1 as a novel functional miR-1 target.

**Conclusion** SERCA2a gene therapy of failing hearts restores miR-1 expression by an Akt/FoxO3A-dependent pathway, which is associated with normalized NCX1 expression and improved cardiac function.

**Keywords** FoxO3A • Gene therapy • Heart failure • MicroRNA-1 • SERCA2a • NCX1

## Introduction

Sarcoplasmic reticulum calcium ATPase 2a (SERCA2a) drives myocardial diastolic calcium ($Ca^{2+}$) cytoplasmic clearance and relaxation after systolic $Ca^{2+}$ release from the sarcoplasmic reticulum (SR). Sarcoplasmic reticulum calcium ATPase 2a expression and activity are impaired in failing hearts, suggesting SERCA2a as a potential therapeutic target.[1] Indeed, viral SERCA2a gene transfer to failing hearts improves cardiac function and reduces arrhythmias *in vivo*,[1,2] and potential efficacy in heart failure (HF) patients has been recently reported.[3] MicroRNAs (miRNAs; miR) are a class of small RNA molecules that regulate mRNA/protein expression by

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inducing transcript degradation or translational inhibition. Recent studies have shown that miRNAs play important roles in cardiac physiology and pathology.[4,5] However, the mechanism(s) underpinning their regulation, expression, and functional role in chronic HF and after SERCA2a gene therapy-mediated ventricular reverse remodelling has never been explored.

# Methods

## Rat heart failure model

Rats were subjected to chronic post-myocardial infarction HF as described previously.[1] Briefly, adult male Sprague–Dawley rats weighing 250–300 g underwent proximal coronary ligation ($n = 17$) to induce chronic myocardial infarction. Sixteen weeks later, a subgroup ($n = 9$) received intravenous AAV9.SERCA2a gene therapy (see below). Age-matched non-infarcted rats ($n = 8$) served as healthy non-failing controls (CTR). In the untreated HF and CTR arms, animals were anaesthetized (2% isoflurane) and hearts were explanted at 16-week post-infarction. The left ventricular myocardium was separated, immediately flash frozen in liquid nitrogen, and stored at −80°C until protein or RNA isolation.

## Gene therapy

SERCA2a gene therapy was carried out as described previously.[2] Heart failure animals were randomized to AAV9.SERCA2a gene therapy or placebo. Nine HF rats received a 300 μL tail vein injection of AAV9.SERCA2a ($2 \times 10^{11}$ DNase-resistant particles) at least 16-week post-infarction. We have previously reported the improvement in parameters of left ventricular systolic and diastolic function measured by pressure–volume analysis after AAV9.SERCA2a gene therapy in this model.[2] Four to six weeks post-AAV9.SERCA2a injection, hearts were explanted from HF + SERCA rats and left ventricular myocardium isolated, frozen, and stored as described above.

## MicroRNA profiling

MicroRNA profiling in left ventricular tissue from healthy control rats (CTR), rats with HF 16 weeks after myocardial infarction (HF), and HF rats treated with intravenous AAV9.SERCA2a (HF + SERCA)[1] was performed using the rat miRNome Sanger miRBase microRNA Profiler Set (System Biosciences). Expression of 391 rat microRNAs (based on Sanger miRbase version 14) was analysed as per the manufacturer's instructions.

## Adult cardiomyocyte adenoviral sarcoplasmic reticulum calcium ATPase 2a transduction

Details about the isolation and culture of adult cardiomyocytes are given in the Supplementary material online, Methods. A subset of freshly isolated cardiomyocytes from CTR and HF hearts were resuspended in M199 and transfected with adenoviral SERCA2a.GFP vector *in vitro* (multiplicity of infection: 100). After 48 h in culture with Ad.SERCA2a.GFP, with efficacy of *in vitro* transduction confirmed by GFP fluorescence, myocytes were spun down at 300*g* for 5 min, resuspended in 350 μL Trizol reagent, and frozen at −80°C for polymerase chain reaction (PCR) studies.

## Measurement of calcium transients

Adult cardiomyocytes were loaded with Fluo-4AM (1 mmol/L) with 0.16% pluronic acid F127 to visualize intracellular $Ca^{2+}$. Myocytes were spun down at 300 RPM, the supernatant removed, and the pellet resuspended in 1 mL of Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Life Technologies) + 100 μmol/L EGTA to wash. This was repeated twice and then the cells were resuspended in 2 mL of DMEM + EGTA and left for 30 min at room temperature (RT) to allow de-esterification of the fluorescent indicator. The cells were continuously superfused at a rate of 2–3 mL/min with normal Tyrode's (NT) solution (37°C) containing (in mmol/L): NaCl 140, KCl 6, $MgCl_2$ 1, $CaCl_2$ 2, glucose 10, and HEPES 10, pH 7.40 ± 0.01. Cells were field-stimulated at 1 Hz using a pair of platinum electrodes. To assess the SR $Ca^{2+}$ content, cells were superfused (15 mL/min) with NT containing 10 mmol/L caffeine producing a fast application of the substance—a 'caffeine spritz'. Caffeine releases SR $Ca^{2+}$, so the size of the caffeine-induced transient can be used as a qualitative index of the SR $Ca^{2+}$ content. The acquisition software pCLAMP was used and data analysed with Clampfit.

The effects of thapsigargin on $Ca^{2+}$ transients of cardiomyocytes were observed after cells had been preloaded with fura-2 (1.5 μM dissolved in DMSO stock solution). Thapsigargin (100 μM) dissolved in cardiomyocyte solution (117 mM NaCl, 5.7 mM KCl, 1.2 mM $NaH_2PO_4$, 0.66 mM $MgSO_4 \cdot 7H_2O$, 10 mM glucose, 5 mM Na-pyruvate, 10 mM creatine, 20 mM HEPES, and 1.25 mM $CaCl_2 \cdot 2H_2O$) was flushed into the cell chamber while measuring continuously fluorescence. Cells were alternately quiescent and stimulated (1 Hz) for periods of ~1–2 min. In order to block thapsigargin effects, cells were pre-incubated with the $Ca^{2+}$ scavenger 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA; 100 μM) for 5 min before changing to thapsigargin/BAPTA solution.

## RNA and protein detection

RNA from frozen heart tissue or from cell cultures was isolated using TRIzol reagent as per the manufacturer's instructions. miR-1 expression was quantified by using TaqMan assays (Applied Biosystems) and normalized to the small RNA Rnu6b as a control. Messenger RNA expression was quantified by the SYBR-green method using the following primers: sodium-calcium exchanger 1 (NCX1)—5′-GTG GAG GTC TGG GAA GGA TT-3′ (forward) and 5′-GCT TCC TCG TCA TCT TGG TC-3′ (reverse); GAPDH—5′-GAAGGGCTCATGACCACAGT-3′ (forward) and 5′-GGA TGC AGG GAT GAT GTT CT-3′ (reverse). Protein from heart tissue or cultured cells was isolated using cell lysis buffer (Cell Signaling) and separated on a 10% SDS–PAGE and protein expression was detected by Western blotting using specific antibodies stated in Supplementary material online, Methods.

## Immunofluorescence microscopy

Adult rat cardiomyocytes were transfected with pre-miR-1 or anti-miR-1 using lipofectamine and treated with or without phenylephrine (PE) for 48 h. After the treatment, cells were fixed in 4% paraformaldehyde for 15 min at RT followed by 3× washes with PBS for 5 min each. Cells were then blocked with 5% donkey serum (in PBS) for 30 min at RT and incubated overnight in the presence of primary antibody against α-actinin (1:600 in blocking buffer). At the end of incubation, cells were 3× washed with PBS incubated with FITC-labelled secondary antibody (1:500 in blocking buffer, 1 μL/mL DAPI from 5 mg/mL stock) for 30 min at RT, washed again 3× with PBS, and then observed under the fluorescence microscope.

## Luciferase gene reporter assay

Targeting of NCX1 3′UTR by miR-1 was validated by the luciferase reporter assay. The putative target sequence in the NCX1 3′UTR was cloned into pMIR-REPORT vector (Ambion) between *Spe*I and

*Hin*dIII sites using 5′-AAA ACT AGT GCA ACT CCT GAG TGG AAA GG-3′ (forward) and 5′-AAA AAG CTT CAG GGA GGC TCA GAG AAG TG-3′ (reverse) primers. Luciferase reporter (0.2 μg/well) was co-transfected along with pre-miR-1 (100 nM) and β-galactosidase-expressing plasmid (0.2 μg/well) into HEK293 cells using lipofectamine (Invitrogen) in a 48-well plate. Luciferase and β-galactosidase enzyme activity was measured after 48 h of transfection and luciferase values were normalized to β-galactosidase readings.

### Data analysis

Statistical analyses were performed by one-way ANOVA followed by Bonferroni's multiple comparison test (for comparison of more than two groups) or Student's *t*-test (for comparison of two groups) (GraphPad Prism). Data are given as mean ± SD or mean ± SEM as indicated in the respective legends. A *P*-value (two-sided) of $<0.05$ was considered significant.

## Results

### Sarcoplasmic reticulum calcium ATPase 2a gene therapy restores muscle-specific miR-1 expression in failing hearts

We previously reported a rat model of advanced HF 16-week post-myocardial infarction[6] and rescue of the HF phenotype after AAV9.SERCA2a gene therapy.[1] Specifically, SERCA2a restored systolic and diastolic function, reduced arrhythmia generation, and normalized cardiomyocyte $Ca^{2+}$ transients and SR load–leak relationships.[1] Here, we examined left ventricular samples from the same study, given the profound reverse remodelling changes observed, to explore whether reversal of myocardial miRNome changes observed in failing hearts could account for some of these beneficial changes previously reported. We profiled miRNA expression of left ventricular tissue from rats with HF with (HF + SERCA) and without (HF) AAV9.SERCA2a gene therapy (*Figure 1A*). Fifty-two miRNAs were deregulated during chronic HF (*Figure 1A* and *B*; see Supplementary material online, *Table S1*). Of importance, array results detected normalization of 20 (38.4%) deregulated miRNAs by SERCA2a gene therapy. In particular, among the normalized miRNAs, only miR-1 was cardiomyocyte-specific, and this was validated by qRT–PCR analyses (*Figure 1B*). We thus focused on miR-1 and next determined the potential molecular mechanisms responsible for this observation.

### Akt activation negatively regulates cardiomyocyte miR-1 expression *in vitro* and in failing hearts *in vivo*, and this is reversed in sarcoplasmic reticulum calcium ATPase 2a treated hearts

To assess successful miR-1 regulation and function in a cardiomyocyte *in vitro* model, we first measured miR-1 expression in adult rat cardiomyocytes 48 h after PE treatment. miR-1 expression was down-regulated after PE treatment (*Figure 2A*). miR-1 overexpression significantly attenuated PE-induced cardiomyocyte hypertrophy, demonstrating functionality of miR-1 modulation *in vitro* (*Figure 2B* and *C*; efficiency of pre- or anti-miR-1 molecules to modulate miR-1 expression in adult rat cardiomyocytes is shown in *Figure 2D* and *E*, respectively). We next assessed the upstream miR-1 regulation in this cardiomyocyte *in vitro* model.

The miR-1 promoter has consensus sequences for the FoxO3a transcription factor and a dominant active form of FoxO3A (non-phosphorylated) up-regulates miR-1 in isolated cardiomyocytes.[7] Transcriptional activity of FoxO3a depends on its phosphorylation status, and once phosphorylated, e.g. by the serine/threonine protein kinase Akt, FoxO3a is shifted from the nucleus to the cytosol.[8] We detected a strong increase in phosphorylated (active) Akt levels in failing hearts, which were completely restored to normal levels after SERCA2a gene therapy (*Figure 3A* and *B*). Consistent with this, expression of phospho-FoxO3A paralleled phospho-Akt levels (*Figure 3A* and *B*). To examine whether Akt activation leads to FoxO3A phosphorylation, cultured cardiomyocytes were treated with the Akt activator platelet-derived growth factor (PDGF). This led to increased Akt and FoxO3A phosphorylation based on time-dependency studies (*Figure 3C*). To verify whether FoxO3a phosphorylation leads to decreased nuclear FoxO3A content, cultured cardiomyocytes were treated with PDGF, and cytosolic and nuclear fractions separately investigated for FoxO3a levels. Indeed, Akt activation resulted in reduced nuclear FoxO3a content in cardiomyocytes (*Figure 3D*). Pharmacological SERCA2a inhibition using thapsigargin also resulted in increased Akt and FoxO3A phosphorylation in cultured cardiomyocytes (*Figure 3E*). Under these conditions, thapsigargin-mediated SERCA2a inhibition led to a significant repression of miR-1 (*Figure 3F*). These results suggest that Akt-mediated phosphorylation of FoxO3A results in its exclusion from the nucleus and subsequent down-regulation of miR-1 in failing cardiomyocytes, and this is reversed by SERCA2a gene therapy.

To test the influence of SERCA2a gene therapy on miR-1 expression directly in cardiomyocytes, we performed further *in vitro* studies. We acutely overexpressed SERCA2a in freshly isolated cardiomyocytes from adult rats with normal and failing hearts using an adenoviral vector. There was no significant effect of SERCA2a transduction in healthy cultured cardiomyocytes on miR-1 expression. However, miR-1 expression was reduced in failing cardiomyocytes and normalized upon SERCA2a transduction (*Figure 3G*). To further assess the effects of $Ca^{2+}$ availability on cardiac miR-1 expression, we treated adult healthy cardiomyocytes with the SERCA2a inhibitor thapsigargin, which raised diastolic $Ca^{2+}$ levels and also resulted in reduced miR-1 expression levels. This thapsigargin-dependent miR-1 reduction could be attenuated by concomitant treatment with the cell membrane permeable $Ca^{2+}$ scavenger BAPTA (*Figure 3H* and *I*). As high diastolic intracellular $Ca^{2+}$ levels activate the $Ca^{2+}$/calmodulin-dependent protein kinase kinase (CaMKK), which further contributes to Akt activation,[9] we additionally tested involvement of CaMKK in $Ca^{2+}$-mediated miR-1 modulation. Indeed, miR-1 down-regulation upon SERCA inhibition was attenuated by CaMKK inhibition (*Figure 3K*). In summary, the combined results from our *in vivo* and *in vitro* experiments suggest that SERCA2a gene therapy especially in failing cardiomyocytes with high intracellular $Ca^{2+}$ concentrations normalizes miR-1 expression by normalization of cellular $Ca^{2+}$ concentrations and an Akt–FoxO3a-dependent mechanism.

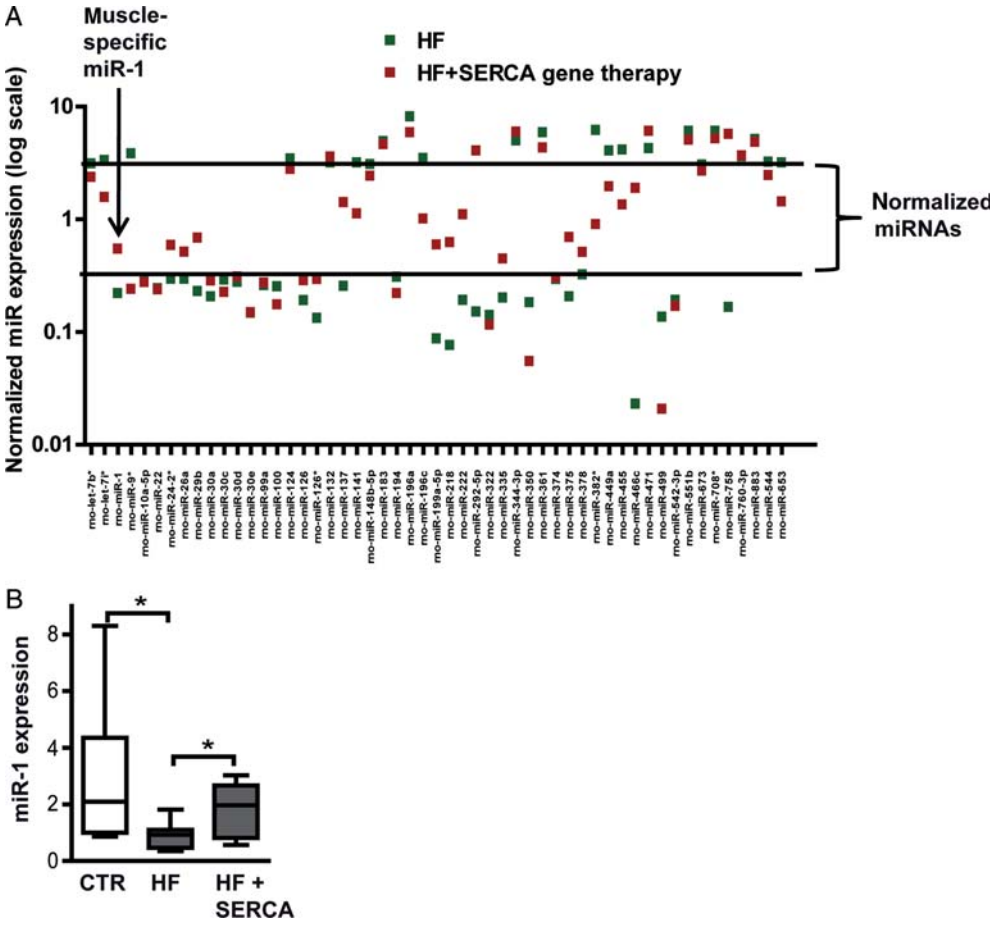


**Figure 1** (*A* and *B*) miRNA expression profiling in pooled RNA samples from left ventricular tissue from controls (CTR), rats with heart failure, and heart failure rats after sarcoplasmic reticulum calcium ATPase 2a gene therapy (HF + SERCA). MicroRNAs deregulated greater than three-fold (HF vs. CTR) are depicted in green and the effects on individual microRNA expression up on sarcoplasmic reticulum calcium ATPase 2a gene therapy in shown in red. (*B*) Validation of miR-1 expression in left ventricular tissue from controls (CTR), rats with heart failure, and heart failure rats after sarcoplasmic reticulum calcium ATPase 2a gene therapy (HF + SERCA). $n = 6–10$ experiments/animals per group for validation experiments. Triplicate polymerase chain reactions for each animal/sample were used. Data are mean ± SD. $^*P < 0.05$.

## miR-1 targets the sodium–calcium exchanger (NCX1)

The sodium–calcium exchanger NCX1 (also known as SLC8A1) is frequently up-regulated during HF.[10] Cardiac overexpression of NCX1 results in the depression of contractile function,[11] whereas NCX1 inhibition restores $Ca^{2+}$ handling in cardiomyocytes.[12] During chronic HF, NCX1 up-regulation may serve as a compensatory mechanism to improve diastolic $Ca^{2+}$ clearance in the setting of reduced SERCA2a levels and/or activity. However, the mechanism for this compensatory response is currently unknown. Within the 3′UTR of rat NCX1 gene, we identified a putative miR-1-binding sequence that is highly conserved throughout evolution (*Figure 4A*). miR-1 overexpression in cultured cardiomyocytes silenced NCX1 protein expression (*Figure 4B*). To validate NCX1 as a *bona fide* miR-1 target, we cloned a 241 nt length NCX1 3′UTR harbouring the miR-1-binding site to a luciferase coding sequence. Indeed, luciferase activity was decreased by more than 60% when miR-1 was overexpressed, confirming the direct targeting of NCX1 by miR-1 (*Figure 4C*).

Next, we compared NCX1 protein expression in HF and HF + S hearts and found that consistent with miR-1 down-regulation, its target NCX1 was up-regulated in failing hearts but normalized after SERCA2a gene therapy (*Figure 4D*). To investigate whether inhibition of miR-1 (and subsequent NCX1 up-regulation) would enhance $Ca^{2+}$ extrusion from the cell, anti-miR-1-transfected cardiomyocytes were treated with a caffeine pulse to rapidly unload SR $Ca^{2+}$ stores and challenge the cardiomyocyte $Ca^{2+}$ extrusion systems, including NCX1. Decay of the caffeine-induced cytoplasmic $Ca^{2+}$ transient was significantly faster in miR-1-silenced cells compared with scrambled control-transfected cells, showing a direct functional link of altered miR-1 expression to cardiomyocyte $Ca^{2+}$ handing (*Figure 4E*).

In conclusion, we here propose an explanation of the beneficial effects of SERCA2a gene therapy partly based on normalized $Ca^{2+}$

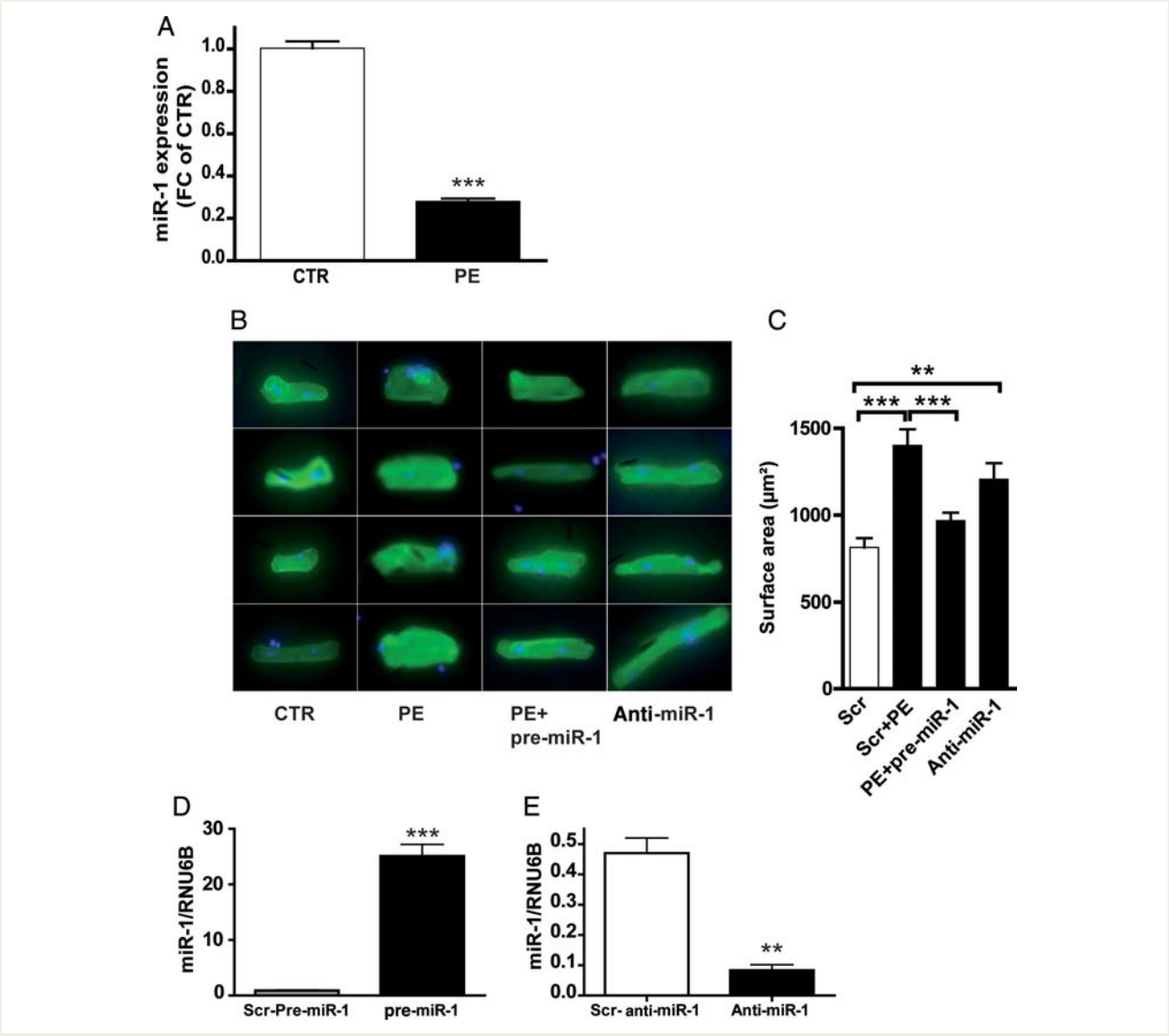


**Figure 2** (*A*) miR-1 expression 48 h after treatment of adult rat cardiomyocytes with phenylephrine (PE, 100 μM) or placebo (CTR). (*B*) Adult rat cardiomyocytes were transfected with scrambled control, pre-miR-1, or anti-miR-1 and treated with phenylephrine (100 μM) as indicated. Cells were fixed with paraformaldehyde and stained for α-actinin. (*C*) Cardiomyocyte surface area was measured using NIS-Elements BR software (version 3.2). Cell size of 13–15 cardiomyocytes was counted for each condition. miR-1 expression in adult rat cardiomyocytes 48 h after transfection with scrambled control, pre-miR-1 (*D*), or anti-miR-1 (*E*) (each 100 nM). RNU6b served as a housekeeping control. $^{**}P < 0.01$, $^{***}P < 0.001$.

homeostasis and an Akt–FoxO3a-dependent mechanism leading to restored miR-1 expression (*Figure 5*).

## Discussion

In this report, we demonstrate that reverse remodelling by therapeutic SERCA2a gene delivery normalizes expression of a number of miRNAs including myocyte-specific miR-1 within the failing heart. miR-1 is one of the most abundant miRNAs in the heart[13] and regulates several target proteins that function as transcription factors,[14,15] receptor ligands,[16,17] apoptosis regulators,[18] and ion channels.[19] Although miR-1 has been found to be overexpressed in individuals with coronary artery disease[19] and to exacerbate arrhythmogenesis when overexpressed,[19,20] several studies indicate that miR-1 plays a protective role against cardiac hypertrophy or HF by regulating several hypertrophy-associated genes including HCN2,[21] calmodulin 1, 2, Mef2a,[15] IGF1, and IGF1R.[7] miR-1 was previously found to be down-regulated in different models of cardiac hypertrophy and failure including transverse aortic constricted mice or transgenic mice with selective cardiac overexpression of a constitutively active mutant of the Akt kinase.[22]

We found SERCA2a-mediated restoration of miR-1 expression to be dependent on an Akt–FoxO3a-regulated mechanism, which led to a normalization of cardiac sodium–calcium exchanger

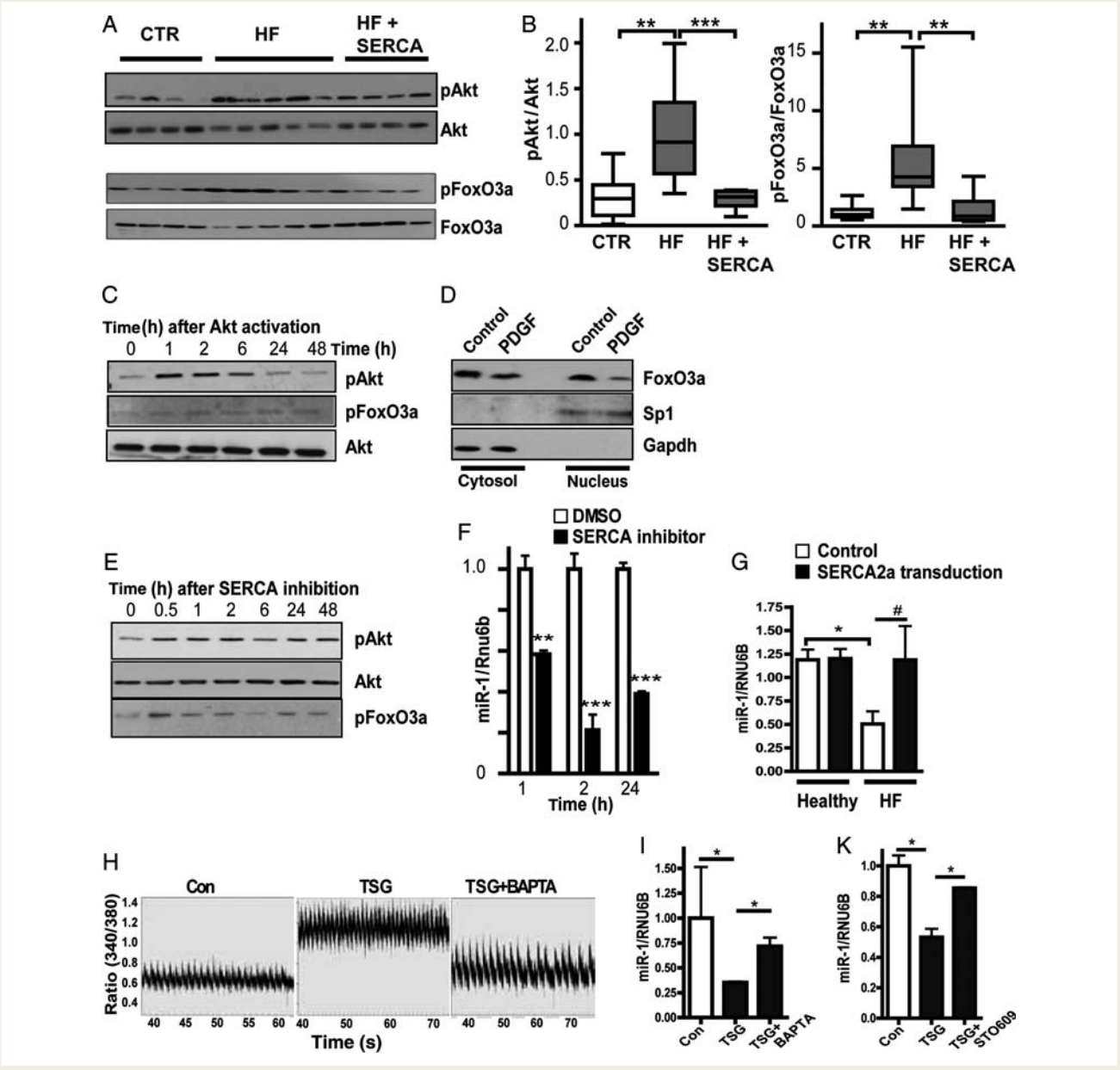


**Figure 3** (*A* and *B*) Akt, p-Akt, FoxO3a, and p-FoxO3a protein expression in left ventricular tissue from controls (CTR), rats with heart failure, and heart failure rats after sarcoplasmic reticulum calcium ATPase 2a gene therapy (HF + SERCA). See *Figure 4D* for housekeeping control GAPDH. (*C*) Time course of p-Akt and p-FoxO3a expression after Akt activation (PDGF, 100 ng/mL, 24 h) in cultured cardiomyocytes. (*D*) FoxO3a total levels in cytosolic and nucleic fractions of adult cardiomyocytes 24 h after Akt stimulation (PDGF, 100 ng/mL). Gapdh (cytosolic localization) and Sp1 (nuclear localization) levels served as controls. (*E*) Time course of p-Akt and p-FoxO3a expression and miR-1 expression (*F*) after sarcoplasmic reticulum calcium ATPase inhibition (thapsigargin, 100 μM) in cultured cardiomyocytes. (*G*) Effects of viral sarcoplasmic reticulum calcium ATPase 2a transduction of freshly isolated adult cardiomyocytes from healthy control rats (Healthy) and rats with heart failure due to myocardial infarction. (*H*) Typical examples of intracellular $Ca^{2+}$ levels (inferred from the ratio of fluorescence emitted at 510 nm after excitation at 340 nm and 380 nm) immediately after thapsigargin (TSG) addition in stimulated (1 Hz) adult rat cardiomyocytes. (*I*) miR-1 expression levels in adult rat cardiomyocytes treated with the sarcoplasmic reticulum calcium ATPase inhibitor thapsigargin (TSG, 100 μM, 24 h) or with TSG and the calcium scavenger BAPTA (100 μM, 24 h) or (*K*) TSG and the calmodulin-dependent protein kinase kinase inhibitor STO609 (10 μM, 24 h). $n = 3–10$ experiments/animals per group. Data are mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{\#}P = 0.06$.

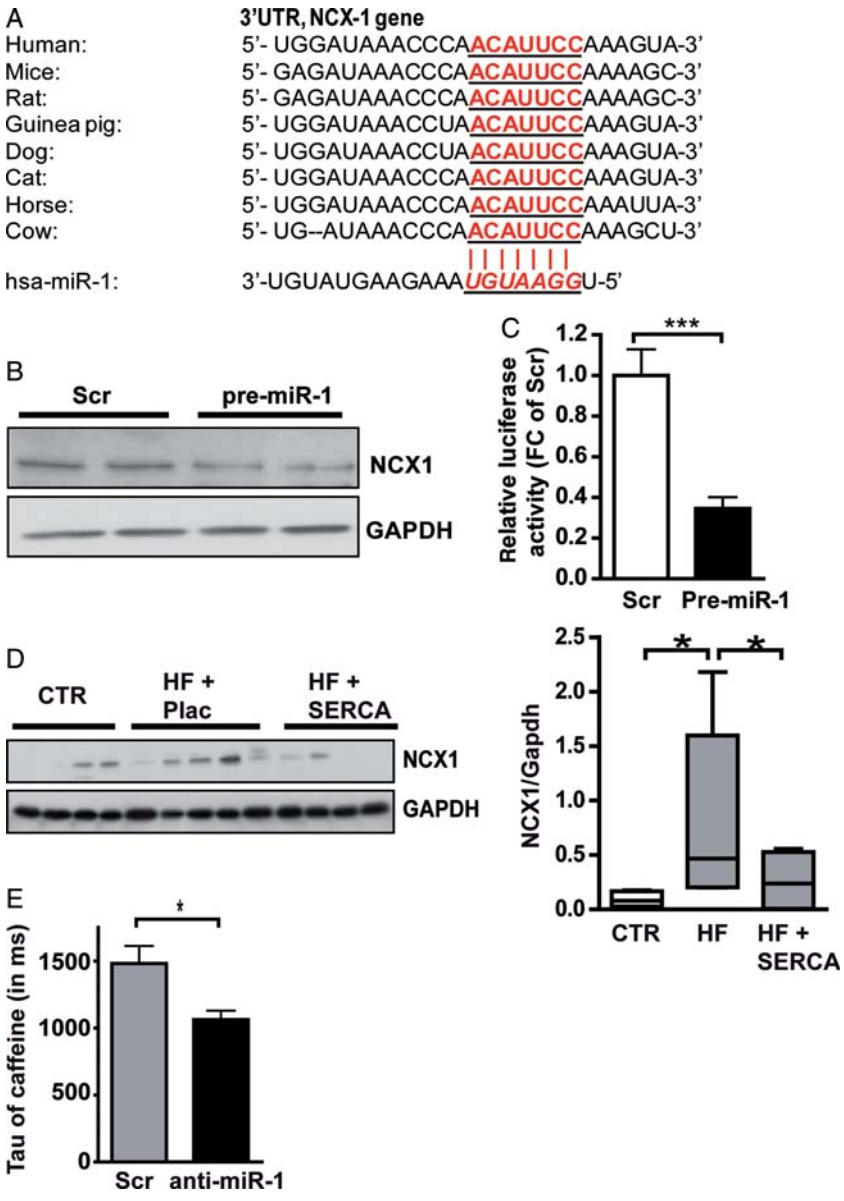


**Figure 4** (*A*) Evolutionarily conserved miR-1 binding sites in the 3′UTR of the NCX1 gene. (*B*) NCX1 protein expression 48 h after transfection of adult cardiomyocytes with miR-1 precursor molecules (pre-miR-1; 100 nM) or scrambled controls (Scr, 100 nM). (*C*) Luciferase gene reporter assay of a 3′UTR region of the NCX1 gene harbouring an miR-1-binding site and co-transfection with miR-1 or scrambled controls (Scr). (*D*) NCX1 protein expression in left ventricular tissue from controls (CTR), rats with heart failure, and heart failure rats after sarcoplasmic reticulum calcium ATPase 2a gene therapy (HF + SERCA). (*E*) Time constant of decay ($\tau$) of caffeine transients in cardiomyocytes transfected with miR-1 inhibitors (anti-miR-1) or scrambled controls (Scr). $n = 3-8$ experiments/animals per group. Data are mean ± SEM. $^{*}P < 0.05$; $^{***}P < 0.001$.

NCX1 expression. Thus, the beneficial effects of SERCA2a gene therapy are at least in part mediated by normalization of miR-1. Indeed, the Akt–FoxO3a pathway seems to play important role in regulation of miR-1 levels. Although acute activation of Akt is cardio-protective *in vitro* and *in vivo* due to its ability to inhibit apoptosis,[23,24] chronic Akt activation in the heart has been shown to be detrimental[25,26] and higher levels of phosphorylated-Akt have been reported in chronically failing human hearts.[27] In this study, we show that Akt phosphorylation in chronic failing hearts is accompanied by FoxO3a phosphorylation, which is responsible for decreased miR-1 expression. This miR-1 repression in failing hearts could be normalized by AAV9.SERCA2a treatment via reversal of the phosphorylated Akt–FoxO3a axis. *In vitro*, we showed that acute SERCA2a overexpression normalized miR-1 levels in cardiomyocytes from failing hearts, while miR-1 levels in healthy cardiomyocytes remained unchanged. Conversely, acute selective SERCA2a inhibition lowered miR-1 expression in a manner dependent upon the rise in diastolic $Ca^{2+}$ and CaMKK activation. These observations are in agreement

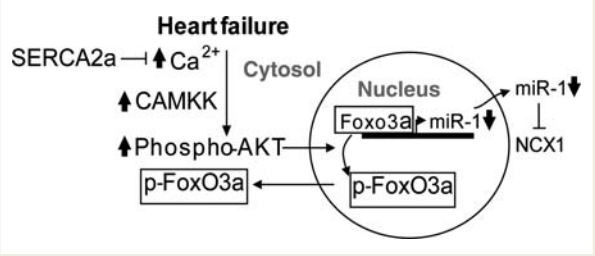


**Figure 5** Scheme of the proposed mechanism: high intracellular cytoplasmatic calcium ($Ca^{2+}$) concentrations during heart failure lead to a $Ca^{2+}$/calmodulin-dependent protein kinase kinase (CaMKK)-dependent activation of Akt, which phosphorylates FoxO3a, thus affecting miR-1 expression. FoxO3a phosphorylation leads to FoxO3a export from the cardiomyocyte nucleus, thus resulting in decreased miR-1 transactivation. This leads to a de-repression of the direct miR-1 target sodium–calcium exchanger 1 (NCX1). These detrimental mechanisms are reversed by sarcoplasmic reticulum calcium ATPase 2a gene therapy.

with a direct effect of SERCA2a levels and activity upon miR-1 expression levels, rather than the miR-1 recovery observed *in vivo* being solely an indirect reflection of beneficial reverse remodelling. Indeed, this recovery of miR-1 expression may provide one explanation for the beneficial effects of SERCA2a gene therapy in rescuing advanced chronically failing hearts.

Next to SERCA2a silencing, NCX1 up-regulation during HF is frequently observed[28–30] and this combination is proarrhythmic.[1,31] In the present study, we identified NCX1 as a direct miR-1 target and found that selectively reducing miR-1 levels in adult cardiomyocytes resulted in accelerated cytoplasmic $Ca^{2+}$ removal. Sarcoplasmic reticulum calcium ATPase 2a-mediated normalization of cardiac miR-1 expression levels may explain normalization of NCX1 levels via an Akt–FoxO3a–miR-1-dependent negative feedback circuit. Hence, two critical proteins for cardiomyocyte diastolic $Ca^{2+}$ clearance, SERCA2a and NCX, are intimately regulated with SERCA2a activity directly influencing NCX expression in a reciprocal manner via the Akt–FoxO3a–miR-1 pathway. Therapeutically, this SERCA2a–miR-1-dependent mechanism of NCX1 down-regulation in failing hearts may contribute to the reduced incidence of delayed after depolarizations and arrhythmias previously reported after SERCA2a gene therapy.[1] This may also aid the contractile improvements, as improving the SERCA2a:NCX ratio, in the context of restored SERCA2a levels, will allow more $Ca^{2+}$ to be re-sequestered into the SR and available for subsequent systolic release, without influencing the kinetics of relaxation which are predominantly SERCA2a-dependent. The influence and effects resulting from the normalization of the other non-exclusively myocyte expressed miRNAs observed during SERCA2a gene therapy on cardiac reverse remodelling remains to be determined.

In conclusion, our results show that miR-1 is down-regulated in a chronic HF model and its expression is restored to normal levels during reverse remodelling by SERCA2a gene therapy, which was mediated at least in part by modulation of intracellular $Ca^{2+}$ handling and an Akt–FoxO3a-dependent mechanism.

## Supplementary material

Supplementary material is available at *European Heart Journal* online.

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**Conflict of interest:** T.T. filed and licensed patents about diagnostic and therapeutic use of microRNAs. R.J.H. is the scientific founder of Celladon Inc. which is developing AAV1.SERCA gene therapy for therapeutic purposes.

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