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An SRF/miR-1 axis regulates NCX1 and Annexin A5 protein levels in the normal and failing heart

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Aims The expression of the sodium/calcium exchanger NCX1 increases during cardiac hypertrophy and heart failure, playing an important role in Ca^{2+} extrusion. This increase is presumed to result from stress signalling induced changes in the interplay between transcriptional and post-transcriptional regulations. We aimed to determine the impact of the SRF transcription factor known to regulate the NCX1 promoter and microRNA genes, on the expression of NCX1 mRNA and protein and Annexin A5 (AnxA5), a Ca^{2+}-binding protein interacting with NCX1 and increased during HF.

Methods and results NCX1 mRNA was decreased while the protein was increased in the failing heart of the cardiomyocyte-restricted SRF knock-out mice (SRF^{HKO}). The induction of NCX1 mRNA by the pro-hypertrophic drug phenylephrine observed in control mice was abolished in the SRF^{HKO} though the protein was strongly increased. AnxA5 protein expression profile paralleled the expression of NCX1 protein in the SRF^{HKO}. MiR-1, a microRNA regulated by SRF, was decreased in the SRF^{HKO} and repressed by phenylephrine. *In vitro* and *in vivo* manipulation of miR-1 levels and site-directed mutagenesis showed that NCX1 and AnxA5 mRNAs are targets of miR-1. AnxA5 overexpression slowed down Ca^{2+} extrusion during caffeine application in adult rat cardiomyocytes.

Conclusion Our study reveals the existence of a complex regulatory loop where SRF regulates the transcription of NCX1 and miR-1, which in turn functions as a rheostat limiting the translation of NCX1 and AnxA5 proteins. The decrease of miR-1 and increase of AnxA5 appear as important modulators of NCX1 expression and activity during heart failure.

Keywords Annexin • Cardiomyopathy • microRNA • NCX1 • SRF

1. Introduction

In response to increased haemodynamic stress and neuroendocrine activation, the heart responds by hypertrophic remodelling through major changes in the gene expression program.[1] In rodent models, pathological hypertrophy is characterized by the re-expression in the ventricular myocardium of genes that are normally expressed in foetal heart like the atrial natriuretic factor, skeletal α-actin, and cardiac-slow β-myosin heavy chain. In addition, the sodium/calcium exchanger-1 (NCX1) expression level is increased in the context of cardiac hypertrophy and during heart failure (HF).[2] Because of its role as the major path for Ca^{2+} ions efflux out of the cell during diastole, NCX1 increase is thought to compensate for the decrease of the cardiac sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA2a) expression level that plays a major role in the reduction of sarcoplasmic reticulum (SR) Ca^{2+} load and increased diastolic $[Ca^{2+}]_i$ in failing cardiomyocytes.[3] However, whether this increase is directly translated into a rise of NCX1 activity remained controversial since results varied depending on the methodological approaches and the animal models used.[2]

At the post-translational level, NCX1 activity could be modulated by phosphorylation of residues located in the intracytoplasmic loop

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although their role in mammalian NCX1 is still a matter of debate.[4] NCX1 activity could also be modulated through the interaction with various proteins including14-3-3 proteins, creatine kinases, and calcineurin.[5–8] Annexin A5 (AnxA5), a Ca^{2+}-binding and phospholipid-binding protein has also been shown to interact with the cytoplasmic intracellular loop of NCX1 in a trimolecular complex with Caveolin-3 although the functional consequence on NCX1 activity remained undetermined.[9] Interestingly, Anxa5 is increased in failing human heart or hypertensive patients with left-ventricular hypertrophy and high expression levels are correlated with impaired systolic functions.[9–11]

At the transcriptional level, the promoter of the *Slc8a1* gene encoding NCX1 protein has been shown to contain functional binding sites for the serum-response factor (SRF), a transcription factor of the MADS box family.[12] SRF is a regulator of genes involved in the development and pathogenesis of many organs through its control on actin cytoskeleton, transcription factors, and cell signalling.[13] During cardiac development, SRF has been shown to act as an upstream regulator of several microRNA (miR) genes including miR-1 and miR-133, further extending the regulatory network controlled by this transcription factor.[14,15] In the adult heart, miR-1 and miR-133a are down-regulated in various models of pressure overload hypertrophy and are thought to blunt the hypertrophic response through the repression of pro-hypertrophic genes.[16–18] MiR-1 down- or up-regulation has been shown to promote arrhythmogenesis through the repression of mRNAs encoding ion-channel proteins or their transcriptional regulators.[19–21] Recently, miR-1 overexpression or inhibition was shown to have a strong impact on Ca^{2+} transients in cardiomyocytes and miR-1 was shown to repress Sorcin, a modulator of Ca^{2+} signalling and excitation–contraction coupling.[22] NCX1 was also recently identified as a target of miR-1 in an elegant study by Kumarswamy *et al.* [23] showing that Serca2a gene therapy restored miR-1 expression and repressed NCX1 in the failing heart through an Akt/FoxO3A signalling pathway. These studies highlighted the fact that miR-1 regulation is playing a pivotal role in the mechanisms regulating Ca^{2+} homeostasis in the cardiomyocytes.

The impact of SRF on the regulation of NCX1 expression has never been tested *in vivo*. We took advantage of a previously generated Cre-loxP mouse model of cardiomyocyte-specific tamoxifen-inducible inactivation of *Srf* gene (SRF^{HKO}) to address this question. SRF^{HKO} mice develop progressive dilated cardiomyopathy (DCM) starting at 3 weeks after SRF inactivation leading to overt HF between weeks 6–8. This phenotype is associated with defects in actin polymerization and energy flux to the myofibrils because of the combined down-regulation of the cardiac α-actin and muscle creatine kinase SRF-regulated genes.[24,25] Interestingly, AnxA5, a protein able to bind to NCX1, was among the up-regulated proteins identified by a 2D-DIGE proteomic screen performed on SRF^{HKO} failing hearts.[25]

Here, we used this temporally controlled model of DCM to determine the contribution of SRF to miR-1 and NCX1 expression during the establishment of HF and in response to pathological hypertrophic stimulus in the adult heart. We show that NCX1 and AnxA5 are direct targets of miR-1 that are increased concomitantly during HF.

2. Methods

2.1 Transgenic mice

All experiments with animals conformed to the Directive 2010/63/EU of the European Parliament and approved by the local Paris-Île de France Region Ethics Committee no. 3 (agreement p3/2007/012). Tamoxifen injections were performed in double transgenic mice bearing the α-MHC-MerCreMer transgene and/or SRF floxed (Sf/Sf) alleles.[25] (Sf/Sf) mice were used as control. Doxicycline inducible transgenic mice overexpressing miR-1 in the heart were generated as described in details elsewhere.[26] Induction of cardiac-specific expression of miR-1 in Tg mice was obtained by administration of doxycycline (dox) in their food pellet (400 mg/kg). Tet/CMV-miR-1 negative/ α-MHC rtTA positive mice fed with dox were used as controls.

2.2 Phenylephrine and antagomiR administration

Phenylephrine (PE) was administrated at 80 mg/kg/day for 15 days with osmotic micropumps implanted in the back skin of adult mice. Cholesterol-linked antagomiRs were injected at 20 mg/kg in 12-week-old CR57BL6/N mice by a single injection in the saphenous vein. Surgery was performed under 2% Isoflurane, 2% oxygen. After 5 min, absence of pain reaction was verified by repeated pinching of the limb pad.

2.3 Echocardiography

Echocardiography was performed under light anaesthesia [≈1% Isofurane] as previously described.[24] Formula for cardiac parameters are given in Supplementary material online.

2.4 Left-ventricular tissue RNA and protein extraction

Control and SRF^{HKO} 12-week-old mice were killed by cervical dislocation and hearts were dissected immediately after sacrifice. RNA and proteins were extracted as previously described.[25] For NCX1 western blot, proteins were denatured in Laemli buffer at 37°C for 30 min instead of the usual 5 min at 95°C to avoid proteolysis.

2.5 Q-RT–PCR and microRNA expression

For microRNA, 200 ng of total RNA was reverse transcribed using miScript Reverse Transcription Kit (Qiagen). For mRNA, 1 μg of RNA was reverse-transcribed using M-MuLV Reverse Transcriptase (Fermentas). Quantitative PCR was performed with LightCycler® 480 SYBR Green (Roche) for mRNA and miScript SYBR Green PCR Kit (Qiagen) for microRNA analysis.

2.6 Isolation and culture of neonatal mouse cardiac myocytes

One-day-old C57Bl6/N neonate mice were euthanatized by decapitation and cardiomyocytes were isolated and cultured as previously described.[25]

2.7 Isolation of adult rat cardiomyocytes

Male Wistar rats (250–300 g) were subjected to anaesthesia by intraperitoneal injection of pentobarbital (100 mg/kg body weight) and hearts were excised rapidly. Individual adult rat ventricular myocytes were obtained by retrograde perfusion of the heart with collagenase as previously described.[27]

2.8 Cell-line culture

H9c2 cardiac cells were cultured in D-MEM/10% FBS. Forty-eight hours prior to transfection experiments, cells were seeded at a density of 75 000 cells/mL and cultured in antibiotics-free medium 24 h prior to transfection.

2.9 Cloning, co-transfection, and Dual-Luciferase Assay

3′UTR sequences were amplified by PCR from mouse genomic DNA and cloned into PsiCHECKTM-2 vector (Promega). Transfection was performed using Lipofectamine 2000 reagent (Invitrogen) with duplicates for each experimental condition. RNAiMAX reagent (Invitrogen) was used when transfecting oligos only.

2.10 Mutagenesis of 3′UTRs

Deletion of the miR-1 seed sequence was carried out using Phusion Site-directed Mutagenesis Kit (Finnzymes).

2.11 Western blot

Protein samples were analysed by SDS–PAGE western blot. Primary antibodies against Anxa5 (rabbit monoclonal, Origene), and NCX1 (rabbit polyclonal, Swant) were used with anti-GAPDH (rabbit polyclonal, Santa Cruz Biotechnology) as internal control. Bands were revealed with HRP coupled secondary antibody and ECL reaction. Signal was quantified using image J software (National Institute of Health).

2.12 Adenoviral constructions and infections

Anxa5 cDNA (Origene clone RG205619) was amplified by PCR and inserted into Adeno-dsRED viral vector (Clontech). Adult rat cardiomyocytes were infected at a multiplicity-of-infection of 500 pfu/cell. All experiments were performed 48 h after plating.

2.13 Measurements of Ca^{2+} transients and sarcomere shortening

Isolated cardiomyocytes were loaded with 5 μM Fura-2 AM (Invitrogen) at RT for 15 min and then washed with external Ringer solution. The loaded cells were field stimulated (5 V, 4 ms, 0.5 Hz). Sarcomere length and Fura-2 ratio were simultaneously recorded using an IonOptix System (Milton, MA, USA).

2.14 Statistical analysis

We used two-way ANOVA for independent samples for comparisons between experimental animal groups with or without treatment for gene expression data, followed by Tukey's HSD for post-ANOVA comparisons. We used the Student's unpaired *t*-test for comparisons between the two groups. The data shown are means ± SEM. *P*-values of $***P < 0.001$, $**P < 0.01$, and $*P < 0.05$ were considered statistically significant.

Detailed protocols and sequences of oligonucleotides used in the study are provided in the Supplementary material online.

3. Results

3.1 Differential regulationNCX1 mRNA and protein and miR-1 in the absence of SRF

To examine the requirement of SRF for the expression of NCX1 mRNA and protein during pathological cardiac remodelling in the adult heart, we sacrificed SRF^{HKO} mutant mice at early asymptomatic stage [day (D) 8], onset of DCM (D25) and overt HF (D50) or after PE treatment from D10 to D25 to activate hypertrophic pathways. We performed echocardiography analyses at baseline (D0, prior to tamoxifen injection), and at Day23 post-tamoxifen in untreated mice or PE-treated mice (Supplementary material online, *Figure S1*). There was no difference at baseline between the two groups of mice. SRF^{HKO} displayed only a mild reduction of ejection fraction and the left-ventricular end-diastolic diameter was unchanged. At this stage, PE treatments led to a similar increase of ventricular weight/body weight ratio in control and SRF^{HKO} mice, but did not alter in a major way the cardiac parameters confirming that we are focusing on an early step of cardiac hypertrophy (Supplementary material online, *Figure S1*). SRF inactivation in the adult heart was maintained all along the period and resulted in a decrease of NCX1 mRNA from D8 to D50 (*Figure 1A* and *B*). The induction of NCX1 mRNA by PE observed in control mice was abolished in SRF^{HKO} mutant. SRF expression level was not modified by PE treatment. The profile of NCX1 protein expression in the same conditions was strikingly different from the mRNA. NCX1 protein was unchanged until D25 and strongly increased at D50 in the SRF^{HKO} heart (*Figure 1C* and

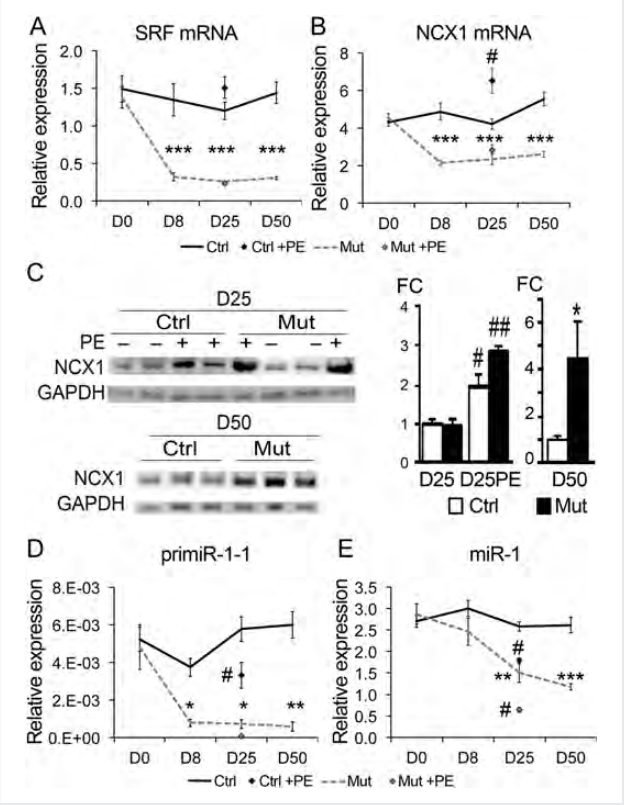

Figure 1 Opposite regulation of NCX1 mRNA and protein and decrease of miR-1 in the failing heart of SRF^{HKO}. (*A* and *B*) RT–q-PCR analysis of SRF (*A*) and NCX1 (*B*) mRNA levels. RNAs were extracted in control and SRF^{HKO} hearts at Day (D) 0 (baseline), D8, D25, and D50 after tamoxifen injection and at D25 after phenylephrine (PE) delivery by osmotic minipumps from D10 to D25. (*C*) Western blot for NCX1 protein at D25 (± PE) and D50. NCX1 migrated at an apparent MW160. Loading control: GAPDH migrating at apparent MW38. Right graph: quantification of NCX1/GAPDH ratio ($n = 4$ for each control groups, $n = 5$ for each mutant group). (*D* and *E*) RT–q-PCR for primary miR-1-1 transcripts (*E*) and mature miR-1 (*F*). RT–q-PCR data are represented as means ± SEM and expressed as relative expression to Hprt for SRF, NCX1, and pri-miR-1 and to miR-16 for miR-1. Western blot quantifications are given as fold change (FC) expression over time-matched controls. $n \geq 5$ for each group for RT–q-PCR analyses. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ mutant vs. control. $\#P < 0.05$ in PE treated vs. non-treated animals.

Supplementary material online, *Figure S2*). Moreover, the increase in NCX1 protein triggered by PE treatment was stronger in the heart of SRF^{HKO} mice than in controls.

These changes in NCX1 gene and protein expression were paralleled by a rapid decrease of miR-1-1 primary transcript (*Figure 1D*). The level of mature MiR-1 microRNA was decreased in the heart of SRF^{HKO} although the decline was slower than for the NCX1 mRNA reaching 40% of control levels at D50 (*Figure 1E*). In addition, we found that PE triggered a 35% decrease of miR-1 levels in control mice and further decreased miR-1 to very low levels in the SRF^{HKO} mutant hearts. However, the percentage of miR-1 repression between PE-treated and untreated SRF^{HKO} mutant was similar than in control mice (−40%) suggesting that this repression is taking place independently from the presence of SRF on the miR-1 *cis*-regulatory regions.

3.2 An SRF/MiR-1 regulatory loop tunes the level of NCX1 mRNA and protein in cardiomyocytes

The opposite regulation of the NCX1 mRNA and protein suggested the existence of a strong post-transcriptional regulation that compensates for the down-regulation of the NCX1 mRNA in the absence of SRF. Examination of the NCX1 3′-UTR using TargetScan[28] revealed the presence of a miR-1 binding site, which was conserved between mouse and rat (*Figure 2A*) and human (data not shown). This site was identical to the site present in a 214 bp fragment of the NCX1 3′UTR that was recently shown to be responsive to miR-1-mediated repression in a luciferase assay.[23] To establish whether this site is primordial for miR-1 repression in the context of the full length NCX1 3′UTR, we cloned a 1418

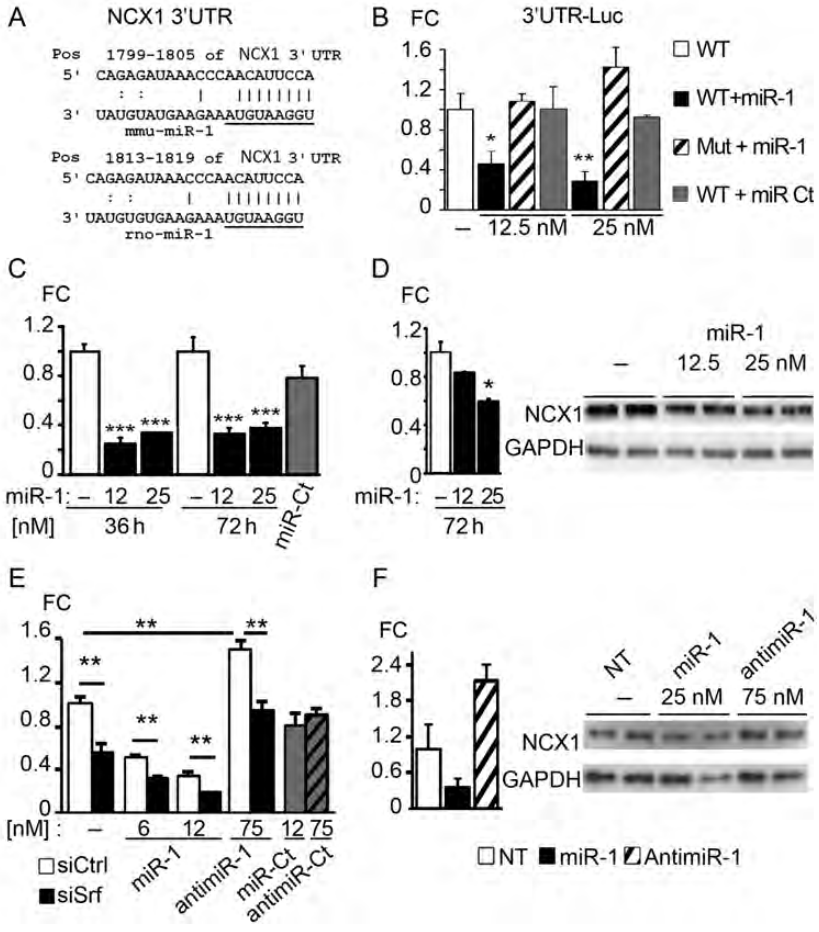

Figure 2 Regulation of NCX1 expression by miR-1 and SRF in cardiomyocytes. (*A*) MiR-1 target sequence in the 3′UTR of mouse (top) and rat (bottom) NCX1 mRNA. (*B*) Luciferase reporter activity for the 3′ UTR of NCX1 cloned in the psicheck2 luciferase vector and transfected in H9c2 cardiomyoblast cells. WT, wild-type 3′UTR; mut: mutation of miR-1-binding site. Ct: control miR; $n = 4$ for each condition. (*C*) RT–q-PCR analysis and (*D*) Western blot for NCX1 expression after miR-1 transfection and in non-transfected (NT, RNAiMax only) or control miR (miR-Ct) transfected H9c2 cardiomyoblasts. (*E*) RT–q-PCR analysis for NCX1 mRNA in neonatal mouse cardiomyocytes (NMCs) transfected with either control siRNA (white bars) or siRNA directed against SRF (black bars), with or without miR-1 or anti-miR-1 at indicated concentration. $n = 4$ for each condition. (*F*) Western blot for NCX1 expression in NMC after miR-1 and anti-miR-1 transfections. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ transfected vs. control. Data are expressed as in *Figure 1*.

nucleotides long fragment of the mouse NCX1 3′UTR into the psiCheck2-Luciferase reporter vector and we performed site-directed mutagenesis to delete the complementary sequence to the miR-1 seed sequence. MiR-1 strongly repressed NCX1 3′-UTR-luciferase reporter expression and the repression was abolished by the deletion of the miR-1-binding site (*Figure 2B*). Negative control miR without binding site in the 3′UTR had no effect on luciferase expression. Overexpression of miR-1 repressed endogenous NCX1 expression both at the mRNA level and at the protein level in H9c2 foetal cardiomyocytes (*Figure 2C* and *D*). SiRNA-mediated SRF down-regulation in neonatal mouse cardiomyocytes (NMCs) resulted in a 56% decrease of NCX1 mRNA (*Figure 2E*) and 41% decrease of miR-1 (Supplementary material online, *Figure S3*). Co-transfecting miR-1 in the context of SRF knock-down further decreased NCX1 mRNA levels (*Figure 2E*). On the contrary, anti-miR-1, which lowered the amount of mature miR-1 in NMCs (Supplementary material online, *Figure S3*), increased NCX1 expression at the mRNA and protein level (*Figure 2E* and *F*). Inhibition with anti-miR-1 of the residual miR-1 still present in siSRF transfected cells (≈60% control level) was sufficient to abolish the decrease of NCX1 mRNA that is normally seen after SRF knock-down (*Figure 2E*). Hence NCX1 protein level in cardiomyocytes is tuned by SRF activity through direct transcriptional activation of the NCX1 gene and indirect, miR-1-mediated, repression of NCX1 mRNA.

3.3 MiR-1 represses Annexin A5 and NCX1 *in vivo*

We previously identified Anxa5 as an increased protein in the SRF^{HKO} cardiac tissues at the onset of HF.[25] Because AnxA5 was shown to bind NCX1,[9] we thought to further characterize the expression profile and regulation of this protein in parallel to our characterization of NCX1 and miR-1 expression profiles. We validated the increase of AnxA5 protein in the SRF^{HKO} by western blot at D25 and D50 (*Figure 3A*) while the mRNA expression level was increased only at D50 in the SRF^{HKO} heart and was not changed in response to PE (*Figure 3B*). We identified an miR-1-binding site in the mouse AnxA5 3′-UTR which was conserved in rat sequence (*Figure 3C*). Mir-1, but not a negative control miR, repressed the wild-type Anxa5 3′-UTR-luciferase reporter plasmid, but not when the miR-1-binding site was mutated (*Figure 3D*). MiR-1 overexpression in H9c2 rat cardiomyocytes and NMCs repressed AnxA5 at the protein level (*Figure 3E* and *F*). Anti-miR-1 increased the level of AnxA5 in NMCs (*Figure 3G* and *H*) and an oligonucleotide protecting the mouse AnxA5 3′UTR against the binding of miR-1 abolished the repressive effect of miR-1 (*Figure 3H*).

To confirm the impact of miR-1 on NCX1 and AnxaA5 expression *in vivo* independently of the inactivation of *Srf* gene, we analysed their expression in mice with overexpression or knock-down of miR-1 level (*Figure 4*). MiR-1 overexpression started at D2 following doxycycline injection in transgenic mice bearing a cardiac-specific inducible miR-1 transgene (TG-MiR-1) (*Figure 4A*). It triggered a 30% reduction of NCX1 mRNA while the AnxA5 mRNA level was not affected (*Figure 4B* and *C*). NCX1 and AnxA5 protein levels were lower in the heart of TG-MiR-1 mice (*Figure 4D* and *E*). Conversely, a partial knock-down of miR-1 by antagomiR-1 injection led to a modest increase of NCX1 and AnxA5 protein levels at D5 after injection (Supplementary material online, *Figure S4*).

3.4 AnxA5 overexpression slows down Ca^{2+} extrusion

The above results and the reported interaction of AnxA5 with NCX1[9] suggested that AnxA5 could regulate the activity of this exchanger in cardiomyocytes. In order to test this hypothesis, AnxA5 was cloned into an Adenovirus-dsRed backbone and overexpressed in isolated adult rat ventricular cardiomyocytes (ARVCs) (*Figure 5A*). Ca^{2+} transients and sarcomere shortening were simultaneously recorded in Fura-2-loaded cells. The average basal sarcomere length and Fura-2 ratio, as well as peak height of Ca^{2+} transients and sarcomere shortening triggered by electrical stimulation, were identical between control Ad-dsRed and Ad-dsRed-AnxA5 infected cells (*Figure 5B* and *C*). However, the decay kinetics of the Ca^{2+} transients was slightly but significantly slower in AnxA5-overexpressing cells than in controls ($P < 0.01$, *Figure 5B*) and the same tendency was observed on sarcomere relaxation (*Figure 5C*, $P = 0.09$). To evaluate the impact of AnxA5 on SR Ca^{2+} content and on Ca^{2+} extrusion, cardiomyocytes were exposed to rapid and sustained application of caffeine (10 mM), which opens the ryanodine receptors and cancels the Ca^{2+} pump action of SERCA2a. Fast caffeine application resulted in a strong increase in cytoplasmic Ca^{2+}, which was not different between control and AnxA5-overexpressing cells, indicating a similar SR Ca^{2+} load (*Figure 5D* and *E*). However, in the presence of caffeine, Ca^{2+} failed to return to baseline in about one-third of Anxa5-overexpressing cells (data not shown). In addition, the remaining cells displayed a slower return to diastolic Ca^{2+} level compared with control cells (*Figure 5D* and *E*). These results indicate that AnxA5 overexpression impairs Ca^{2+} extrusion in ARVCs.

4. Discussion

In the present study, we uncovered a complex feedback regulatory loop in the heart in which SRF controls the transcription of *NCX1* and of miR-1, the latter blunting the level of NCX1 protein expression and associated AnxA5 protein. The impact of this regulatory loop on NCX1 and AnxA5 expression in basal and pathological context is schematized in *Figure 6*.

4.1 An SRF/miR-1 axis regulates the level of NCX1 protein in hypertrophic and dilated hearts

In SRF^{HKO} mice, the down-regulation of miR-1 allows the maintenance and even increase of NCX1 protein despite the down-regulation of the mRNA. This could be relevant in the context of HF when SRF is degraded.[29] Conversely, this regulatory loop can also contribute to the increase of NCX1 protein level in response to PE, because PE stimulates the transcription of the *NCX1* gene through SRF-dependent mechanisms (this study and previous studies[14,15]) while it triggers a 35–40% reduction of miR-1. This repressive effect of PE on miR-1 expression is also taking place in the SRF^{HKO} heart even though miR-1 basal levels are already low in the mutant, suggesting that PE repress miR-1 expression, at least in part, through other transcriptional regulators.

NCX1 expression level has been shown to increase in most models of cardiac hypertrophy and HF.[2] In the mouse, targeted NCX1 gene inactivation dramatically alter the ability of the heart to cope with hypertrophic stimuli leading to death of the animals[30] while NCX1 overexpression is sufficient to trigger cardiac hypertrophy and

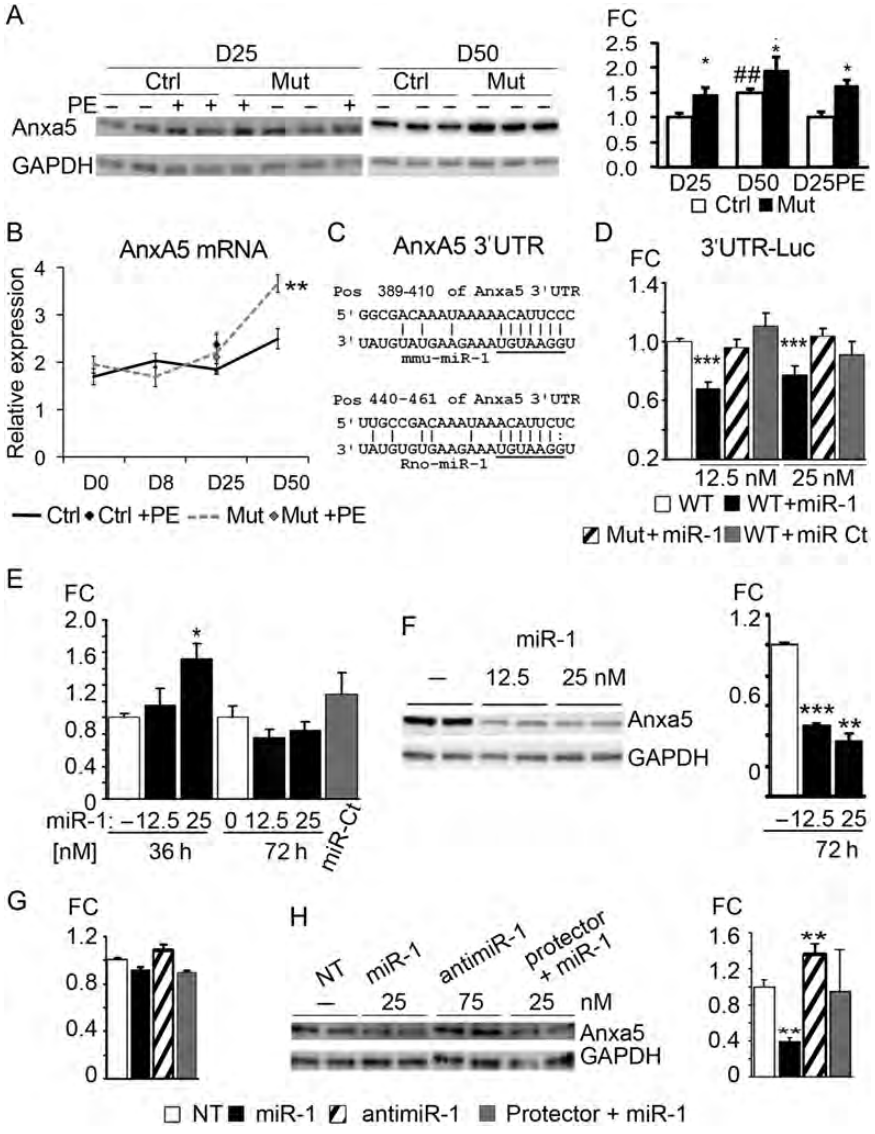

Figure 3 MiR-1 represses Anxa5 protein expression. (*A*) Western blot for AnxA5 (MW 35) in control and SRF^{HKO} hearts at D25 ± PE treatment and D50, GAPDH loading control. Right graph: Image J quantification of AnxA5/GAPDH ratio ($n = 4$ for each control groups, $n = 5$ for each mutant group). (*B*) RT–q-PCR analysis of AnxA5 mRNA expression in control and SRF^{HKO} hearts from D0 to D50. (*C*) MiR-1 target sequence in the mouse (top) and rat (bottom) Anxa5 3′UTR. (*D*) Luciferase reporter activity for the 3′ UTR of AnxA5 in H9c2 cells. Legend as in *Figure 2*. (*E–H*) RT–q-PCR analysis (*E* and *G*) and western blot (*F* and *H*) for AnxA5 in H9c2 cells (*E* and *F*) and neonatal mouse cardiomyocytes (NMC) (*G* and *H*). Cells were either non-transfected (NT, RNAiMAx only) or transfected with negative control miR (miR-Ct), miR-1, anti-miR-1, or miR-1 ± miR-protector. $n = 3$ for each condition. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ transfected vs. control. Data are expressed as in *Figure 1*.

failure.[31] Recently, it was shown that the level of NCX1 protein was also determined by the expression level of SERCA2a at the SR in a rat model of HF induced by chronic myocardial infarction.[23] Interestingly, rescue of SERCA2a expression by gene therapy restored the level of miR-1 via an Akt/FoxO3A-dependent pathway, and miR-1 was shown to repress NCX1 mRNA. Here we confirm and extend the latter observation by showing through site-directed mutagenesis that the miR-1-binding site is indeed required for miR-1 mediated repression. In addition, our study shows that SRF intervenes in the regulatory loops controlling NCX1 protein level in the context of HF. We also reported previously that SERCA2a gene expression was reduced at an early timepoint after SRF inactivation (D5) although it raised and lowered again at later timepoints (D30 and D60, respectively) suggesting a complex regulation of the SERCA2a gene in response to SRF deficiency.[24] Altogether, these observations raise the question whether restoring SRF activity in the context of HF would enforce the normalization of Ca^{2+} handling proteins expression and other target genes involved in contraction.

4.2 Relieve of AnxA5 inhibition by SRF/miR-1 drop modulates NCX1 functions

Although changes in NCX1 mRNA and proteins levels is well established in several models of cardiac remodelling, the consequences on

the level of NCX1 exchange activity is more complex to understand and some discrepancies were reported between different studies on similar models, sometimes showing reduced exchange activity despite increased or unchanged protein level.[2] This suggested that additional factors modulate NCX1 activity in the context of cardiac remodelling as discussed subsequently. Here we show that AnxA5 could be one of these factors. AnxA5 is a Ca^{2+}-binding protein, which can interact with phosphatidylserine phospholipids at the inner side of the plasma membrane. In the myocardium, AnxA5 is an abundant protein localized mainly in the T-tubules and sarcolemma of myocytes.[10] AnxA5 is increased in failing human heart[9,11] but its role in the pathogenesis of the disease and its impact on calcium flux in cardiomyocytes remained undefined. We show that AnxA5 overexpression in adult rat cardiomyocytes led to a slight but significant slowing of electrically evoked Ca^{2+} transients decay, while the basal and peak Ca^{2+} concentration and sarcomere shortening were not affected. This is at variance with what was previously observed in cardiomyocytes isolated from transgenic mice over-expressing AnxA6, which displayed decreased contraction as well as decreased basal and peak Ca^{2+} and shorter Ca^{2+} decay time.[32] These differences may reflect a different role of these two Annexins in the regulation of Ca^{2+} homeostasis and/or ability to interact with NCX1, or could be due to the different experimental models. In addition, we found that overexpression of AnxA5 slowed down the decay of caffeine-induced Ca^{2+} transients. In the presence of caffeine, the ryanodine receptors are open, the net SR Ca^{2+} uptake by SERCA2a is prevented while leaving the extrusion systems intact. This indicates that AnxA5 acts on another Ca^{2+} removal mechanism. NCX1 is

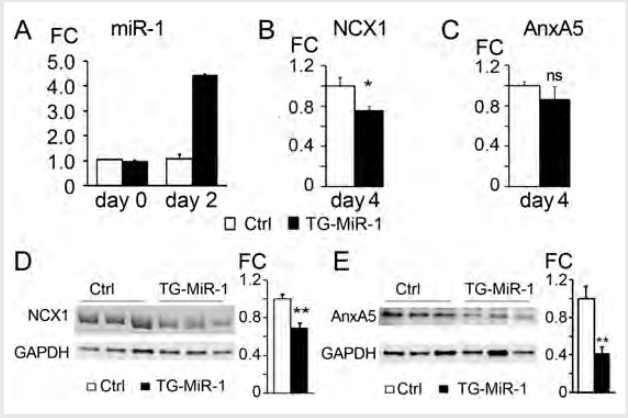

Figure 4 MiR-1 overexpression in the heart represses NCX1 and AnxA5 expression. (*A*–*E*) RT–q-PCR analysis (*A*–*C*) and western blot analysis (*D* and *E*) in control (white bars) and MiR-1 transgenic mice (black bars) at baseline (day 0) or after 2 or 4 days of doxycycline administration. ($n = 5$ for each group). Legends and data expressed as in *Figure* 2. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. respective controls.

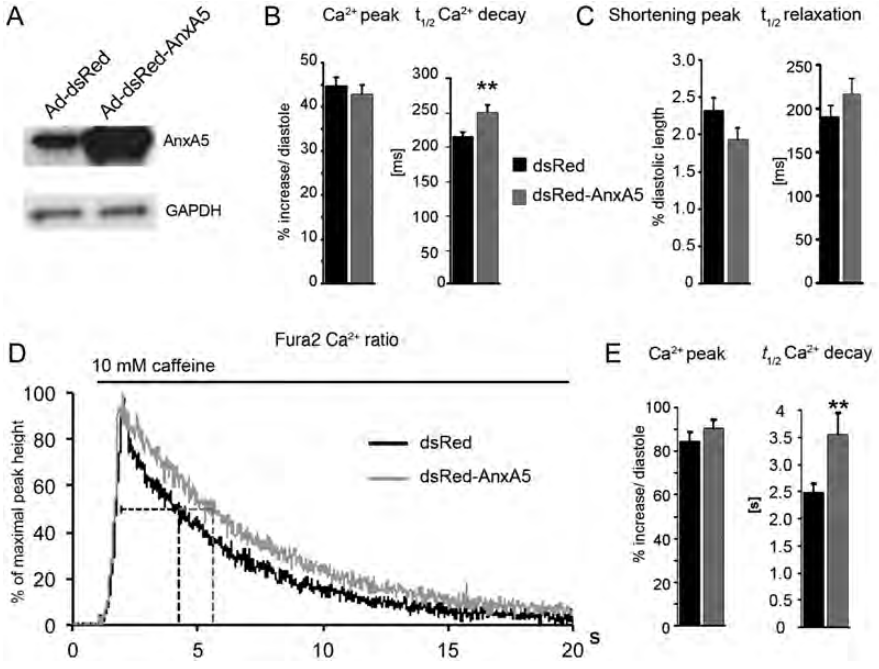

Figure 5 Anxa5 overexpression prolongs Ca^{2+} transients in ARVCs. (*A*) Western blot for Anxa5 expression in ARVCs infected with control recombinant adenovirus (Ad-dsRed) or adenovirus expressing AnxA5 (Ad-dsRed-AnxA5). Proteins were extracted 48 h after adenoviral infection. GAPDH was used a loading control. (*B*) Summary of Ca^{2+} transient characteristics in Fura-2-loaded, electrically paced (0.5 Hz, 4 ms, 5 V) ARVCs infected with Adeno-dsRed as controls (black bars, $n = 34$) or Adeno-dsRed-Anxa5 (grey bars, $n = 37$). Left: systolic Ca^{2+} peak ratio (% increase of diastolic ratio); Right: decay time to 50% of the Ca^{2+} peak during diastole. (*C*) Sarcomere shortening triggered by electric pulse as in (*B*). Left: systolic sarcomere length shortening expressed as % of diastolic sarcomere length; Right: relaxation time to 50% length. (*D*) Typical Ca^{2+} transient triggered by fast caffeine perfusion. Values are represented as percentage of the maximal Fura-2 ratio (= 100%). Dotted lines indicate $t_{1/2}$ Ca^{2+} decay for control dsRed(black) and ds-Red-AnxA5 (grey). (*E*) Average amplitude and decay kinetics of caffeine-induced Ca^{2+} transient in Ad-Anxa5-dsRed ($n = 7$) and Ad-dsRed ($n = 14$) infected cells. $^{**}P < 0.01$ Adeno-dsRed-Anxa5 vs. Adeno-dsRed.

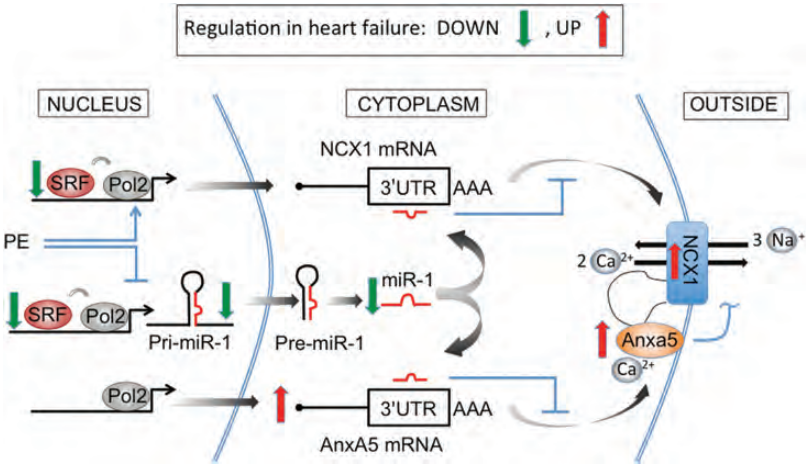

Figure 6 Model of NCX1 and Anxa5 regulation by the SRF/miR-1 axis. In the healthy adult heart, SRF transcription factor maintains the transcription level of NCX1 mRNA and pri-miR1-1 gene through direct binding to *cis*-regulatory sequences.[12,15] AnxA5 transcription is not regulated by SRF. Mature miR-1 limits the translation level of NCX1 protein (this study and a previous study[23]) and Anxa5 associated protein (this study). In response to PE, NCX1 transcription is increased through SRF-dependent mechanisms (this study and previous studies[12,34]) while PE represses pri-miR-1 transcription through SRF-independent pathways (this study and previous studies[16–18]) leading to the upregulation of NCX1 and AnxA5. Decrease of SRF activity as in SRF^{HKO} (this study) or in end-stage failing heart[29,35] will result in lower transcription level of NCX1 mRNA and pri-miR-1-1 RNA (this study). The down-regulation of mature miR-1, delayed in time compared with pri-miR-1-1, ultimately alleviates the repression on NCX1 mRNA, overcoming the impact of lower NCX1 mRNA amount and resulting in a net increase in NCX1 protein. AnxA5 mRNA and proteins also increase at end-stage HF. The calcium and phosphatidylserine phospholipid-binding protein AnxA5 is known to bind the cytoplasmic intracellular loop of NCX1,[9] and can modulate the Ca^{2+} extrusion activity of NCX1 at the sarcolemma.

the major route to extrude Ca^{2+} in cardiomyocytes[33] and AnxA5 was shown to bind directly to the intracytoplasmic loop of NCX1 together with Caveolin-3,[9] making NCX1 the most likely candidate for AnxA5 regulation. An interesting hypothesis is that AnxA5 could locally decrease the amount of Ca^{2+} available for NCX1, changing the thermodynamic equilibrium that drives NCX1 activity. In this way, AnxA5 could slow down Ca^{2+} extrusion by NCX1 during diastole. More experiments will be required in the future to fully understand the impact of AnxA5 overexpression on Ca^{2+} transients in the failing heart.

Altogether, our results shed new light on the ways SRF exerts an integrative control on the expression level of miR-1, NCX1, and AnxA5 in the heart and the kinetics of this regulation during the establishment of HF. Indeed, an important issue in the future will be to understand the impact of the differences in processing kinetics, storage, and stability between microRNAs and mRNAs that are co-regulated by transcription factors like SRF. The complexity of these regulatory loops and the fact that, like many other adaptive mechanisms in the heart, they may be both a necessity and an issue for the maintenance of cardiac functions highlight the need for further research in this field to better understand their role in cardiac physiopathology.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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