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BASIC SCIENCE

Signal transducer and activator of transcription 3-mediated regulation of miR-199a-5p links cardiomyocyte and endothelial cell function in the heart: a key role for ubiquitin-conjugating enzymes

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Aims Mice with a cardiomyocyte (CM)-restricted knockout of signal transducer and activator of transcription 3 (STAT3-KO) develop spontaneous heart failure. We investigated the impact of STAT3-mediated regulation of microRNAs for pathophysiological alterations in the heart.

Methods and results MicroRNAchip and qRT–PCR analysis revealed elevated cardiac expression of miR-199a in STAT3-KO mice. Lentiviral shRNA-mediated STAT3-knock-down in neonatal rat CMs markedly increased miR-199a promoter activity and miR-199a levels indicative of a suppressive effect of STAT3 on miR-199a transcription.

Up-regulated miR-199a in CM by pre-miR-199a transfection (pre-miR-199a-CM) reduced expression of components of the ubiquitin-proteasome system (UPS), i.e. the ubiquitin-conjugating enzymes Ube2g1 (mRNA and protein) and Ube2i (protein). Pre-miR-199a-CM or CM with siRNA-mediated down-regulation of Ube2i and Ube2g1 (siRNA-Ube2i/2g1-CM) displayed massive down-regulation of α- and β-myosin heavy chain expression associated with disrupted sarcomere structures. In addition, protein arginine methyltransferase I (PRMT-I) expression and asymmetric dimethylarginine (ADMA) synthesis were increased in pre-miR-199a-CM or in siRNA-Ube2i/2g1-CM. Increased ADMA in cell culture supernatant (SN) from pre-miR-199a-CM or siRNA-Ube2i/2g1-CM lowered nitric oxide (NO) bioavailability of rat cardiac endothelial cells while lowering ADMA concentration in CM SNs by the PRMT inhibitor arginine methyltransferase inhibitor 1 (AMI-1) (100 μM) improved NO bioavailability. In STAT3-KO hearts Ube2i and Ube2g1 expression were markedly reduced. Human terminal failing hearts harbouring low STAT3 protein levels displayed increased miR-199a levels and decreased Ube2g1 expression.

Conclusion This study identifies a novel pathophysiological circuit in the heart between reduced STAT3 protein levels, increased miR-199a expression, and subsequent impairment of the UPS that disrupts CM sarcomere structure and impairs via the release of ADMA endothelial cell function.

† Helmut Drexler died on 13 September 2009.

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Keywords STAT3 • MicroRNA • ADMA • Ubiquitin-proteasome system • Heart • Sarcomere

Introduction

The signal transducer and activator of transcription 3 (STAT3) protein is expressed in the post-natal heart. Experimental studies showed that cardiomyocytes (CMs) STAT3 expression is a prerequisite for maintenance of cardiac integrity, function, and perfusion throughout life since CM-restricted deletion of STAT3 in mice [signal transducer and activator of transcription 3 knockout (STAT3-KO)] caused heart failure in response to physiological (age and pregnancy) or pathophysiological (ischaemia/reperfusion, myocardial infarction, anthracycline toxicity) stress.[1–4] Potential clinical relevance for these experimental findings is provided by the observation that STAT3 expression and activation is down-regulated in human failing hearts.[5]

Recent data indicate that microRNAs (miRNAs), small molecules that modify gene expression by inhibiting the translation or promoting the degradation of target mRNAs, play important roles in the onset of cardiac disease.[6,7] In this regard, it has been shown that miRNAs either in CMs or the non-myocyte fraction modulate cardiac pathophysiology.[8,9]

We discovered that miR-199a-5p (miR-199a) expression is suppressed by STAT3 in post-natal CMs and appeared up-regulated in STAT3-KO hearts before the onset of heart failure. We therefore hypothesized that a link between reduced STAT3 protein levels and increased miR-199a expression may be responsible for pathophysiological alterations contributing to and promoting the development of heart failure.

Materials and methods

The cell culture medium was from Biochrome, leukaemia inhibitory factor (LIF) and the protein arginine methyltransferase (PRMT) inhibitor arginine methyltransferase inhibitor 1 (AMI-1) were from Calbiochem, and all other chemicals were from Sigma-Aldrich (Germany).

Animal experiments. Mice with a CM-restricted deletion of STAT3 (α-MHC–Cre$^{tg/-}$; STAT3$^{flox/flox}$) have been described previously.[2] Only 3-month-old male mice were included. All animal procedures were approved by the Institutional Animal Care and were performed in accordance with the guidelines of the local animal ethics commission and German laws.

Cell culture and analysis of CM morphology. Isolation and culturing of neonatal rat CMs and cell culture conditions for rat heart endothelial cells (RHE-A), antagomir and miRNA transfection, transfection of luciferase reporter plasmids, collection of conditioned supernatants (SNs), and morphological analysis are described in Supplementary material online.

RNA isolation; qRT–PCR, miR-qRT–PCR, and pri-miRNA quantification; primer sequences. RNA isolation, primer sequences, and PCR conditions are described in Supplementary material online.

Microarray for miRNAs. Total left ventricle (LV) RNA pooled from five wild-type (WT) and five STAT3-KO mice was prepared by Miltenyi Biotec. PIQORTM miRXplore Microarray Miltenyi and miChip analysis[10] were performed on the same RNA pools (see Supplementary material online).

Lentiviral over-expression of shRNAs and miRNAs. The generation of lentiviral constructs, preparation of recombinant lentiviral SNs, and lentiviral transduction are provided in Supplementary material online.

miRNA target verification. Cloning of the predicted target sequence of miR-199a-5p in the rat Ube2g1 mRNA (NM_022690) and the mouse Ube2i mRNA (NM_011665) in the psiCHECK2 plasmid (Promega, Heidelberg) and their *in vitro* mutagenesis are described in Supplementary material online.

Cloning of miR-199a-5p promoter region and luciferase activity assay. The cloning of the miR-199a-5p promoter region (nucleotides −686 to −45, relative to the first nucleotide of the pre-miR-199a) into the pGL4-2.1 luciferase reporter vector (Promega) is described in Supplementary material online.

Electron spin resonance (ESR) spectroscopy analysis of superoxide production and nitric oxide (NO) production. Superoxide production and NO production of RHE-A cells were performed by ESR using a MiniScope ESR spectrometer (Magnettech; Berlin, Germany) as described previously (see Supplementary material online).[11]

Western blotting and immunohistochemistry. Protein isolation, western blotting, and immunohistochemistry were performed according to standard protocols (see Supplementary material online).[2] The following antibodies were used: anti-STAT3 antibody and anti-PRMT-I (cell signalling), anti-dimethylarginine dimethylaminohydrolase II (DDAH II) antibody, anti-Ube2i antibody, anti-Ube2g1 antibody (Santa Cruz Biotechnology), anti-myosin heavy chain (MHC) antibody (Abcam), anti-Troponin-T, anti-Tropomyosin, anti-actin, and sarcomeric α-actinin (Sigma-Aldrich).

Electron microscopy. Cardiomyocytes were fixed with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate (pH 7.4) for 30 min, rinsed in PBS, and embedded in Araldite (see Supplementary material online).[12]

Measurement of asymmetric dimethylarginine (ADMA). Concentrations of ADMA in cell culture SNs were determined by gas chromatography-tandem mass spectrometry (GC-tandem MS) as previously reported[13] (see Supplementary material online) and by ADMA ELISA (DLD Diagnostika GmbH). The metabolism of ADMA was analysed as described recently using d3-ADMA (at 1 μM) as the internal standard (see Supplementary material online).[13]

Statistical analyses. Data are presented as mean ± SD. Differences between groups were analysed by Student's *t*-test or ANOVA followed by Bonferroni *post hoc* analyses as appropriate (GraphPad PRISM, Version 5.0a). A two-tailed *P*-value of <0.05 was considered statistically significant.

Results

miR-199a expression is up-regulated in signal transducer and activator of transcription 3 knockout hearts

The overlap of two independent microarray platforms, PIQORTM miRXplore Microarray and miChip analysis,[10] identified miR-199a up-regulated in LVs of young (3-month-old) asymptomatic

STAT3-KO mice[2] (RNA pool $n = 5$) when compared with age-matched WT mice (RNA pool $n = 5$), a feature that was confirmed by miR-qRT–PCR analysis on additional $n = 5$ individuals per genotype (*Figure 1A*). Two-fold up-regulation ($P = 0.043$) of miR-199a was also observed in freshly isolated CMs from STAT3-KO mice ($n = 3$) compared with WT mice ($n = 3$). Two genes in the mouse genome, *miR-199a-1* (chromosome 9) and *miR-199a-2* (chromosome 1), encode mature miR-199a but only the product of *miR-199a-2* was up-regulated as indicated by the up-regulation of its respective pri-miRNA (*Figure 1A*). *miR-199a-2* is part of a putative miR-199a/214 cluster,[14] but no difference in the expression of mature miR-214 was detected between WT and STAT3-KO LVs (*Figure 1A*).

STAT3 suppresses promoter activity of the *miR-199a-2 gene* in cardiomyocytes

Down-regulation of STAT3 by transduction of neonatal rat CMs with anti-STAT3 shRNA encoding lentivirus (*Figure 1B*) increased expression of the pri-miR-199a-2 rat homologue as well as mature miR-199a while miR-214 was not altered compared with control shRNA (GL4) lentivirus transduced CM (*Figure 1C*). miR-199a-2 gene promoter activity in a luciferase reporter plasmid assay was markedly enhanced in STAT3-knock-down CM (*Figure 2D*), indicating that STAT3 suppresses transcription of pri-miR-199a in CM. There are three putative STAT binding sites present in the miR-199a promoter (see Supplementary material online results), but activation of STAT3 by LIF (20 ng/ml) did not affect miR-199a promoter activity (luciferase activity in control: 100 ± 10%, in LIF: 109 ± 11%, $n = 3$, $P = 0.329$).

miR-199a affects sarcomere structure and expression of sarcomeric myosin heavy chain

Up-regulation of miR-199a by transfection of CM with pre-miR-199a (pre-miR-199a-CM) resulted in thinning of CM width and extension of CM length (*Figure 2A*) associated with disrupted sarcomere structure (*Figure 2A, B*). In turn, suppression of the miR-199a by anti-miR-199a (anti-miR-199a-CM) attenuated CM growth and only slightly altered sarcomere organization (*Figure 2A*). At the molecular level, pre-miR-199a-CM displayed

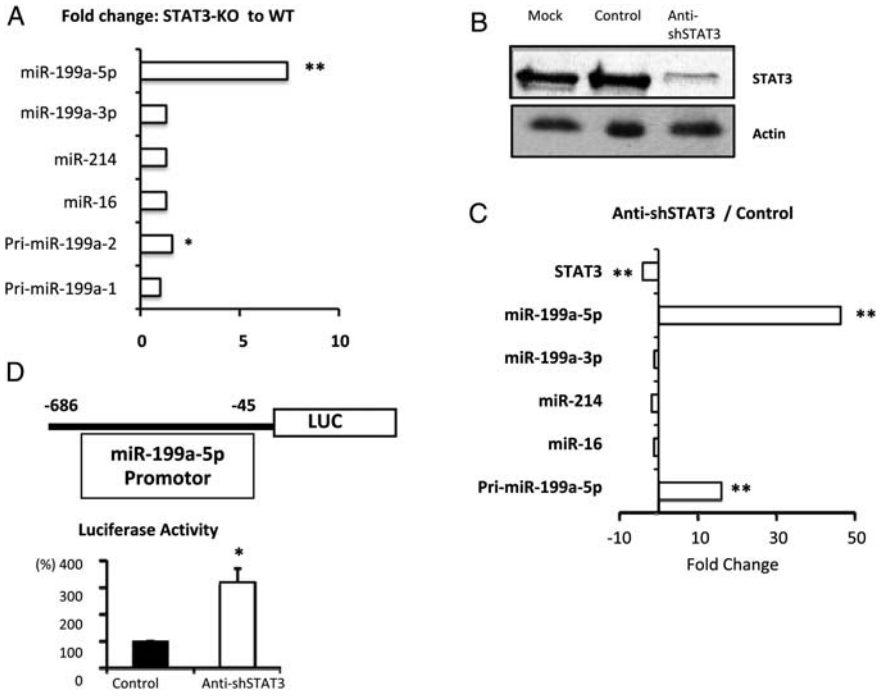

Figure 1 miR-199a is up-regulated in left ventricle (LV) tissue from signal transducer and activator of transcription 3 knockout (STAT3-KO) mice and in cardiomyocytes with lentiviral-mediated shRNA knock-down of signal transducer and activator of transcription 3 (STAT3). (*A*) Bar graph displaying expression levels of pri-miR-199a-1 and pri-miR-199a-2 [*$P = 0.0333$ vs. wild-type (WT)] and mature forms of miR-199a-5p (**$P = 0.0008$ vs. WT), miR-214, miR-199a-3p, and miR-16 as measured by miR-qRT–PCR in LVs from WT ($n = 5$) and STAT3-KO ($n = 5$) mice. (*B*) Western blot depicting efficient lentiviral-mediated anti-STAT3-shRNA knock-down of STAT3 in cardiomyocyte (CM) compared with GL4-shRNA lentivirus (control); Actin served as loading control. (*C*) Bar graph summarizing pri-miR-199a (**$P = 0.0001$), miR-199a-5p (**$P = 0.0001$), miR-214, miR-199a-3p, and miR-16 levels determined by miR-qRT–PCR in anti-STAT3-shRNA vs. control. (*D*) Bar graph summarizing *Firefly* luciferase activities in CM, normalized to *Renilla* luciferase from a reporter plasmid containing the putative rat miR-199a promoter (−686 to −45) that was co-transfected in CM transduced with anti-STAT3-shRNA or control (GL4 shRNA, *$P = 0.0220$). Experiments in (*C*) and (*D*) derived from 3–5 different cell isolations, and were performed in duplicate.

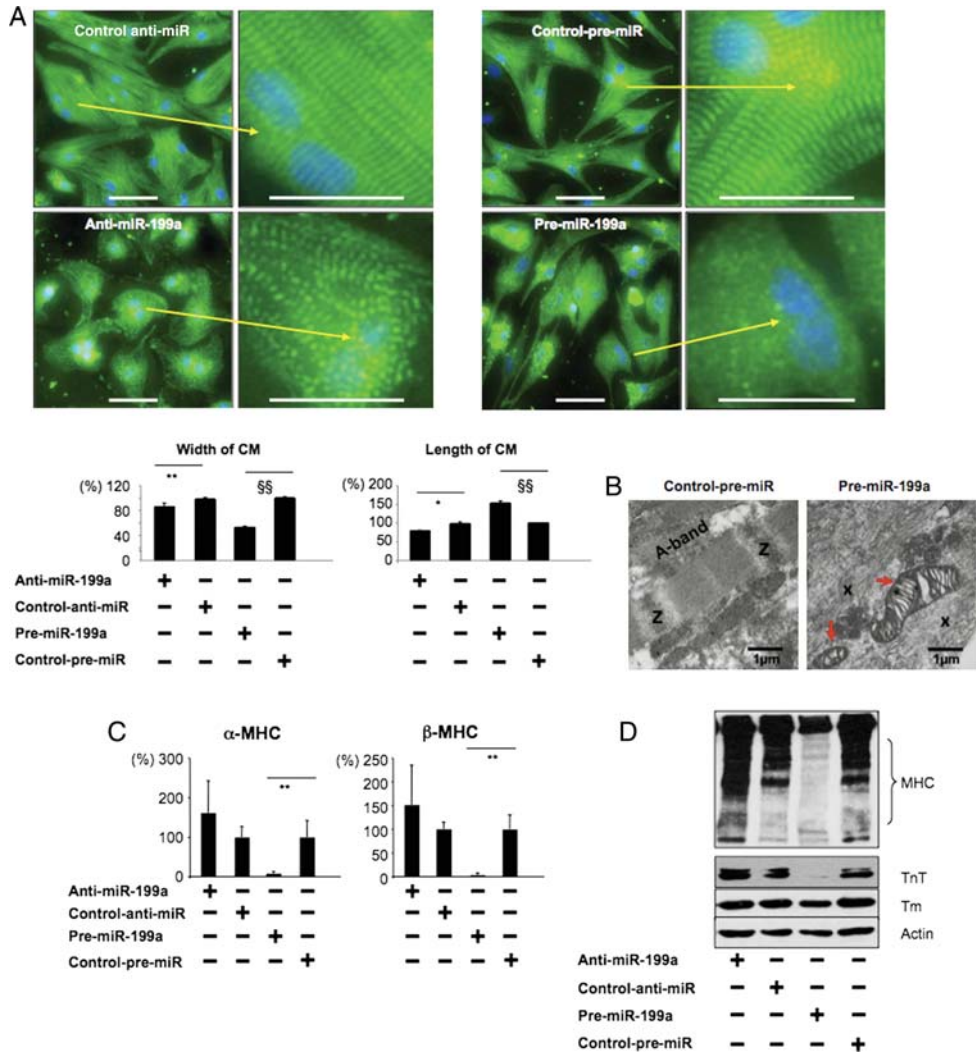

Figure 2 Effect of pre-miR-199a or anti-miR-199a transfection on cardiomyocytes. (*A*) Sarcomeric α-actinin staining (green) and nuclear stain with DAPI (blue) of anti-miR-199a-CM, control-anti-miR-CM, pre-miR-199a-CM, and control-pre-miR-CM. Bar graph summarizing cardiomyocyte dimensions in control-anti-miR-CM vs. anti-miR-199a-CM (width: $^{**}P = 0.0027$; length: $^{*}P = 0.0102$) and control-pre-miR-CM vs. pre-miR-199a-CM (width: $^{\S\S}P = 0.0023$; length: $^{\S\S}P = 0.0043$). (*B*) Electron microscopy revealed deranged sarcomeres (black crosses) in pre-miR-199a-CM with enlarged mitochondria (red arrows) compared with control-pre-miR-CM with defined Z-lines and A-bands. (*C*) Bar graphs summarize α-myosin heavy chain (α-MHC) ($^{**}P = 0.0021$) and β-myosin heavy chain (β-MHC) ($^{**}P = 0.0068$) mRNA levels determined by qRT–PCR and (*D*) representative western blot displays sarcomeric MHC, Troponin-T (TnT), and Tropomyosin (Tm) in CM transfected with anti-miR-199a, control-anti-miR, pre-miR-199a, and control-anti-miR. Experiments derived from 3–5 different cell isolations, and were performed in triplicate.

reduced levels of α- and β-MHC mRNA (*Figure 2C*) and total MHC protein (*Figure 2D*: decrease of sarcomeric MHC in pre-miR-199a-CM vs. control-miR-CM: $-72 \pm 10\%$, $P = 0.0006$). In addition, pre-miR-199a-CM displayed reduced protein levels of Troponin-T (TnT: $-90 \pm 8\%$, $P = 0.0002$) and a tendency to lower Tropomyosin (Tm: $-20 \pm 14\%$, $P = 0.0663$) levels while total cellular Actin content was not affected (*Figure 2D*). In contrast, in anti-miR-199a-CM no significant difference in α- and β-MHC mRNA expression or MHC protein content compared with anti-miR-controls was observed (*Figure 2C*, *D*).

miR-199a suppressed expression of ubiquitin-conjugating enzymes Ube2i and Ube2g1 in cardiomyocytes

Computational prediction programs (MIRANDA) and screening by RNA22 software analysis revealed that ubiquitin-conjugating enzyme Ube2g1 from mouse, rat, and humans harbour conserved miR-199a seed sequences in the 3′UTR as six out of seven nucleotides match complementary to the miR-199a seed sequence (*Figure 3A*). Conserved miR-199a seed sequences were present

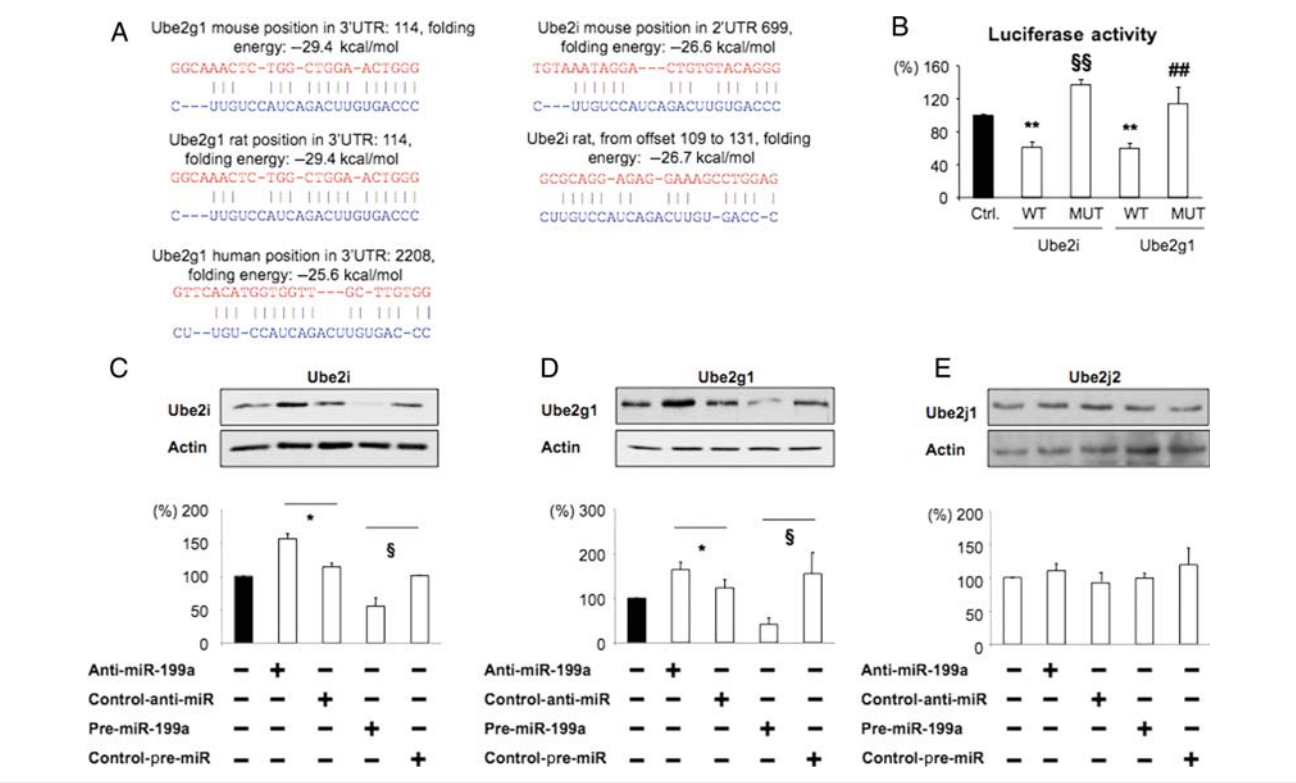

Figure 3 miR-199a targets Ube2i and Ube2g1 in cardiomyocytes. (*A*) Putative miR-199a seed sequences in the 3′UTR of the mouse (NM_025985.4), rat (NM_0022690.2), and human (NM_003342.4) Ube2g1 gene and the Ube2i mRNA of mouse (NM_001177609.1) and rat (NM_013050.1). Blue: miR-199a consensus; blue: corresponding 3′UTR seed sequence. The folding energy and the position are indicated. (*B*) Bar graph summarizing luciferase reporter assays in stably transfected HL-1/S-miR-199a-IEW co-transfected with psiCHECK vector either empty (Ctrl) or harbouring the potential miR-199a binding sites of Ube2i or Ube2g1 (Ube2g-wt, Ube2i-wt), respectively, the corresponding mutated sequence (Ube2g1-mut, Ube2i-mut), $^{**}P = 0.007$ Ube2i-wt vs. empty psiCHECK, $^{§§}P = 0.003$ Ube2i-mut vs. Ube2i-wt, $^{**}P = 0.0001$ Ube2i-wt vs. empty psiCHECK, $^{\#\#}P < 0.0098$ Ube2g1-mut vs. Ube2g1-wt. Western blots and bar graphs depicting protein levels of Ube2i $^{*}P = 0.0120$, anti-miR-199a vs. control-anti-miR; $^{§}P = 0.0240$ pre-miR-199a vs. control-pre-miR (*C*), Ube2g1 $^{*}P = 0.0401$, anti-miR-199a vs. control-anti-miR; $^{§}P = 0.0480$ pre-miR-199a vs. control-pre-miR (*D*), and Ube2j2 (not significant, $P = 0.3981$) (*E*). Actin served as loading control. Experiments were performed in triplicate from at least three independent cell preparations.

in mouse and rat Ube2i but were not detected in the human Ube2i gene (*Figure 3A*).

Reporter plasmids (psiCHECK) containing the potential miRNA binding sites of mouse Ube2i and Ube2g1 at the 3′-position of the luciferase reporter gene (Ube2i-wt and Ube2g1-wt) or containing a mutated sequence of the miRNA binding site (Ube2i-mut and Ube2g1-mut) were transfected into the cardiomyogenic cell line HL-1 stably over-expressing miR-199a. Ube2i-wt and Ube2g1-wt but not their respective mutant miR-199a seed sequences displayed significantly decreased luciferase activity compared with the empty vector or their respective mutated constructs (*Figure 3B*), indicating functionality of putative Ube2i and Ube2g1 3′UTR miR-199a seed sequences. Western blot analyses showed that pre-miR-199a-CM display decreased and anti-miR-199a-CM increased Ube2i and Ube2g1 protein levels compared with their respective controls (*Figure 3C, D*). mRNA level of Ube2g1 was decreased in pre-miR-199a-CM (−50% vs. control-pre-miR, $n = 4$, $P = 0.030$) and increased in anti-miR-199a-CM (+47% vs. control-pre-miR, $n = 5$, $P = 0.0070$) while mRNA level of Ube2i remained unaltered (anti-miR: 14% $n = 5$, $P = 0.121$, pre-miR: −25%, $n = 5$, $P = 0.438$ vs. respective controls). Protein levels of Ube2j2, an ubiquitin-conjugating enzyme that harbours no specific seed sequence for miR-199a were not altered in pre-miR-199a-CM or anti-miR-199a-CM (*Figure 3E*).

miR-199a up-regulation in signal transducer and activator of transcription 3 knockout hearts is associated with decreased Ube2i and Ube2g1 expression

Increased miR-199a expression in STAT3-KO LVs ($n = 4$) was associated with reduced protein levels of Ube2i and Ube2g1 compared with WT ($n = 6$, *Figure 4A*).

miR-199a is up-regulated and Ube2g1 is down-regulated in failing human hearts with low STAT3 protein levels

We previously described a collective of patients with end-stage failing hearts [dilated cardiomyopathy (DCM)] that displayed a substantial reduction of LV STAT3 protein levels compared with non-

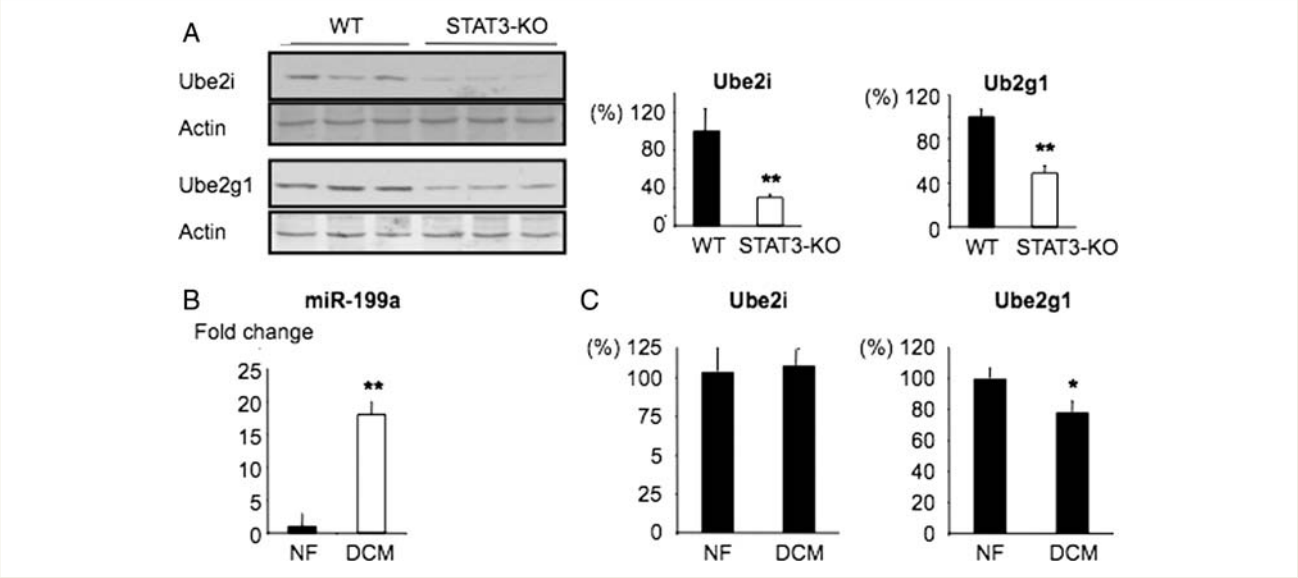

Figure 4 Expression of miR-199a, Ube2i, and Ube2g1 in STAT3-KO and in human hearts. (*A*) Western blot and bar graphs depict decreased Ube2i (**$P = 0.0009$) and Ube2g1 (**$P = 0.0050$) protein levels in LVs from STAT3-KO ($n = 4$) compared with WT ($n = 6$) mice. (*B*) Bar graph summarizes miR-199a levels by miR-qRT–PCR in non-failing ($n = 3$) and terminal failing human hearts (dilated cardiomyopathy (DCM): $n = 7$, **$P < 0.0001$. (*C*) Bar graphs summarizing Ube2i ($P = 0.7540$) and Ube2g1 (**$P = 0.0015$) mRNA levels by qRT–PCR in LVs from non-failing heart (NF) ($n = 3$) and DCM ($n = 6$).

failing hearts.[5] In LV tissue samples of the same hearts we observed markedly increased miR-199a levels (*Figure 4B*) associated with reduced Ube2g1 mRNA levels, while Ube2i mRNA levels were not altered (*Figure 4C*).

Knock-down of Ube2i and Ube2g1 impairs cardiomyocyte ultrastructure and reduces expression of sarcomeric myosin heavy chain

siRNA-mediated knock-down reduced Ube2i by 90% (siRNA-Ube2i-CM, $P = 0.0001$) and Ube2g1 by 80% (siRNA-Ube2g1-CM, $P = 0.0001$) and resulted in loss of sarcomere organization, thinning of CM width and extension of CM length in single knock-downs or in combined siRNA-Ube2i/Ube2g1-CM (*Figure 5A, B*) associated with a profound reduction in α- and β-MHC mRNA expression (*Figure 5C*) and protein levels of sarcomeric MHC, TnT, and Tm content compared with controls (total MHC: $-85 \pm 15\%$, $P = 0.0001$; TnT $-95 \pm 5\%$, $P = 0.0001$; Tm $-97 \pm 3\%$, $P = 0.0001$), while the content of total cellular Actin was not affected (*Figure 5D*).

miR-199a in cardiomyocytes affects nitric oxide and reactive oxygen species production of cardiac endothelial cells in a paracrine fashion

STAT3 regulates paracrine factors affecting endothelial cell proliferation.[2] We therefore analysed whether up-regulation of miR-199a in CMs has a paracrine effect on endothelial cells. Cell culture SN from pre-miR-199a-CM, anti-miR-199a-CM, and their respective controls were collected and applied to cultured RHE-A. As shown in *Figure 6A* SN from pre-miR-199a-CM reduced the NO bioavailability and increased the production of reactive oxygen species (ROS) in RHE-A cells compared with SN from control-pre-miR-CM. In contrast, SN from anti-miR-199a-CM increased NO bioavailability and reduced ROS production in RHE-A cells compared with SN from control-anti-miR-CM (*Figure 6A*).

miR-199a over-expression increases levels of the endothelial nitric oxide synthase inhibitor asymmetric dimethylarginine in cardiomyocyte supernatant

Asymmetric dimethylarginine acts as a potent inhibitor of the endothelial nitric oxide synthase (eNOS), thereby impairing NO production in endothelial cells.[15] Indeed, levels of ADMA were increased in SN of pre-miR-199a-CM and decreased in SN of anti-miR-199a-CM compared with respective controls (*Figure 6B*). Furthermore, miR-199a specifically affects ADMA synthesis since pre-miR-199a-CM displayed significantly increased and anti-miR-199a-CM decreased generation of ADMA (*Figure 6C*), while neither pre-miR-199a-CM ($P = 0.112$) nor anti-miR-199a-CM ($P = 0.264$) displayed alterations in the ADMA elimination rate of exogenously applied synthetic heptadeutero-asymmetric dimethylarginine (d_7-ADMA) over a period of 24 h (*Figure 6D*). In line with increased ADMA generation, western blot analysis showed enhanced expression of PRMT-I, the enzyme that mediates methylation of arginines and ADMA generation.[15] In pre-miR-199a-CM (+115% vs. control pre-miR, $n = 4$, $P = 0.041$) while reduced expression of PRMT-I

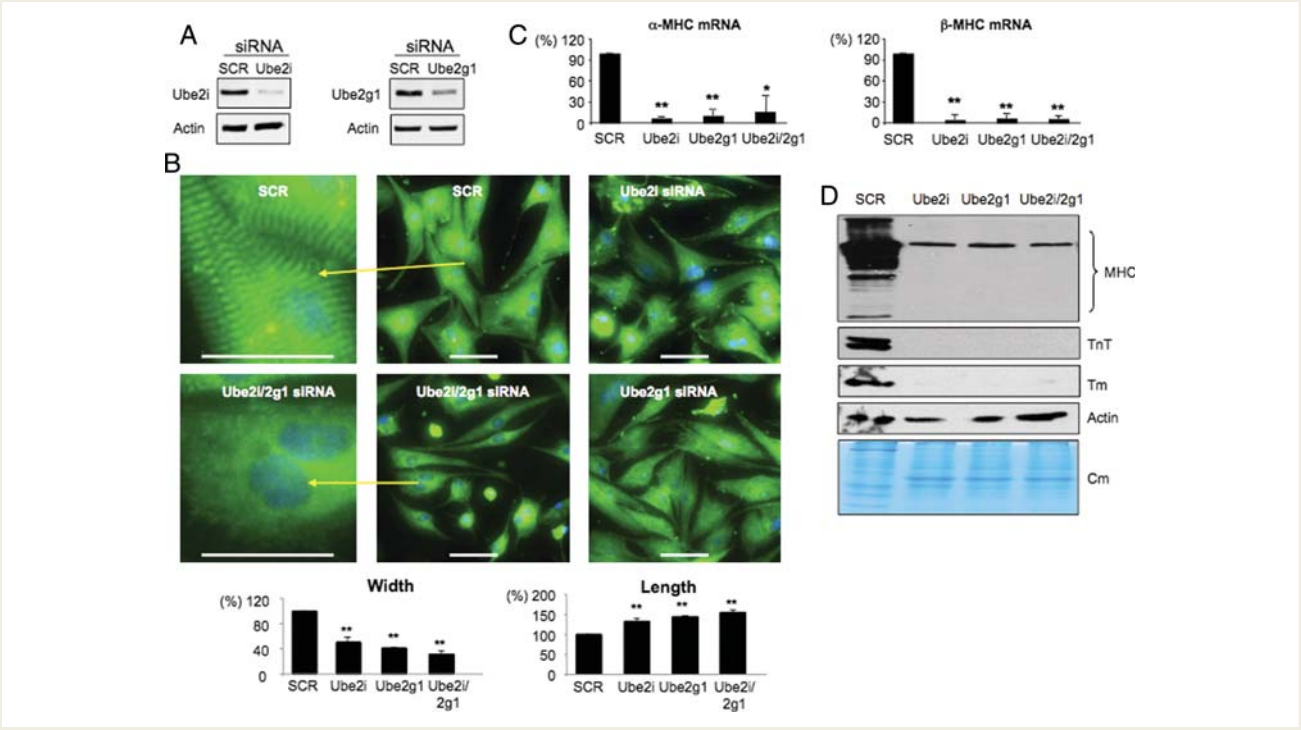

Figure 5 siRNA-mediated knock-down of Ube2i and/or Ube2g1 in cardiomyocytes. (*A*) Western blots depict efficient siRNA-mediated knock-down of Ube2i and/or Ube2g1 compared with scrambled (SCR) siRNA, data from $n = 3$ experiments are summarized in the bar graphs. (*B*) Sarcomeric α-actinin staining (green) and nuclear stain (DAPI, blue) depict sarcomeric structure in CM with Ube2i, Ube2g1, or Ube2i/Ube2g1 siRNA-knock-downs compared with SCR. The bar graph summarizes length and width from respective CM ($n > 50$), $^{**}P = 0.0001$ SCR vs. Ube2i, $^{**}P = 0.0001$ SCR vs. Ube2g1, $^{**}P = 0.0001$ SCR vs. Ube2i/2g1. (*C*) Bar graphs summarize α- and β-MHC mRNA levels determined by qRT–PCR (siRNA-Ube2i-CM α-MHC: $^{**}P = 0.0002$, β-MHC: $^{**}P = 0.0016$, vs. SCR; siRNA-Ube2g1-CM: α-MHC: $^{**}P = 0.0021$, β-MHC: $^{**}P = 0.001$ vs. SCR; α-MHC: $^{*}P = 0.0237$, β-MHC: $^{**}P = 0.0006$ vs. SCR). (*D*) Representative western blots show protein levels of total sarcomeric MHC, TnT, Tm, and Actin. Coomassie (Cm) stained polyacrylamide gel displays pattern from total cellular proteins.

was observed in anti-miR-199a-CM (−45% vs. control anti-miR, $n = 4$, $P = 0.045$) (*Figure 6E*). The expression of the major ADMA degradation enzyme, DDAH II[15] was not affected (pre-miR-199a-CM: $n = 4$, $P = 0.355$; anti-miR-199a-CM: $n = 4$, $P = 0.244$, *Figure 6E*). Pharmacological blockade of PRMT-I activity by the PMRT inhibitor AMI-1 (100 μM) decreased ADMA levels in SN from pre-miR-199a-CM (*Figure 6F*).

PRMT-I-mediated ADMA production is responsible for endothelial cell dysfunction induced by supernatant of pre-miR-199a treated cardiomyocytes

Improved NO bioavailability and attenuated ROS production in RHE-A cells were obtained if SN derived from pre-miR-199a-CM treated with AMI-1 or if the eNOS substrate L-Arginine (0.5 mM) was added to pre-miR-199a-CM SN when compared with effects of pre-miR-199a-CM SN alone (*Figure 6G*). Addition of ADMA to the cell culture medium in concentrations measured in SN from pre-miR-199-CM significantly reduced NO bioavailability and increased ROS production in RHE-A cells (see Supplementary material online, *Figure S1*). These data indicate that modulation of NO and ROS production of endothelial cells upon treatment with pre-miR-199a-CM SN derived mainly from ADMA.

Pharmacological inhibition of the ubiquitin-proteasome system or knock-down of Ube2i and Ube2g1 increased asymmetric dimethylarginine levels via protein arginine methyltransferase while their effect on down-regulation of α- and β-myosin heavy chain mRNA is independent of protein arginine methyltransferase

Protein arginine methyltransferase-I protein levels were up-regulated in CM with global inhibition of the ubiquitin-proteasome system (UPS) by MG-132 (0.5 μM) or in siRNA-Ube2i/Ube2g1-CM (*Figure 7A*, *B*). Asymmetric dimethylarginine was up-regulated in MG-132 treated CM (*Figure 7C*). Elevated ADMA levels in siRNA-Ube2i/Ube2g1-CM could be attenuated with the PRMT blocker AMI-1 (*Figure 7D*). α- and β-MHC mRNA levels were markedly down-regulated in MG-132 treated

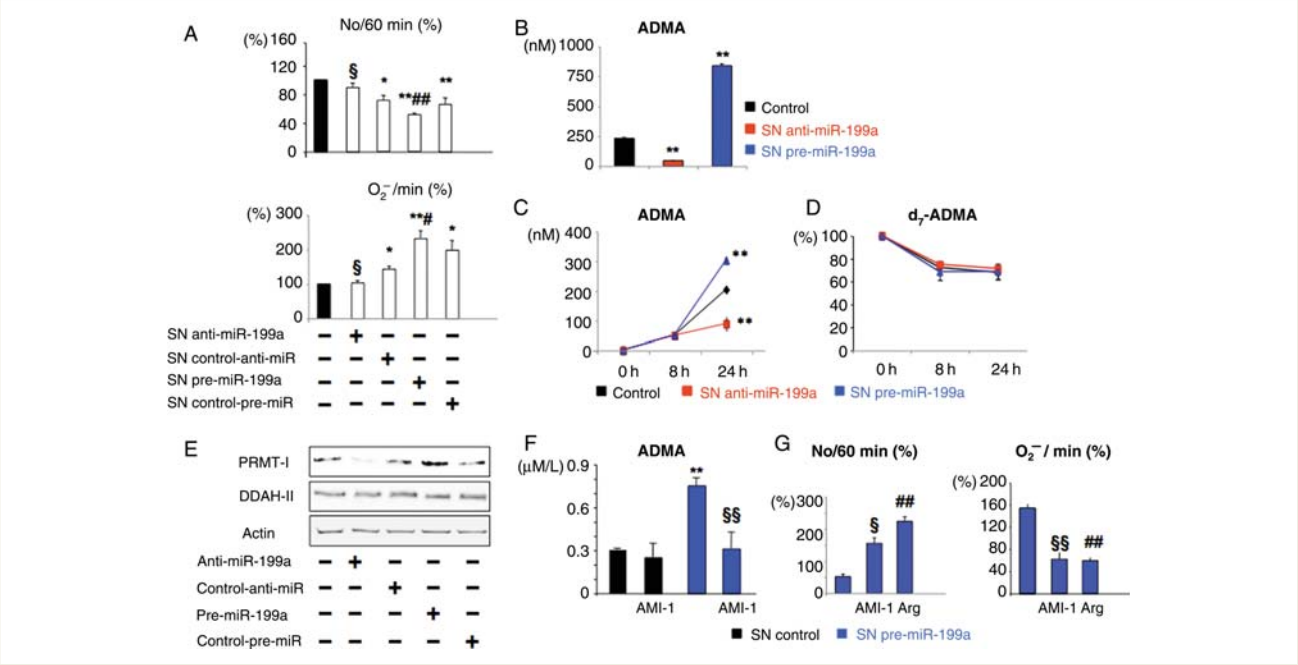

Figure 6 Asymmetric dimethylarginine (ADMA) synthesized in dependence of miR-199a in cardiomyocyte supernatant effects on endothelial cells. (*A*) Bar graphs depicting effects of supernatant (SN) from anti-miR-199a-CM and pre-miR-199a-CM and respective controls on RHE-A cultivated without addition of cardiomyocyte supernatant (control) were set at 100% on nitric oxide (NO) bioavailability: $^{*}P = 0.0289$ SN control-anti-miR-CM vs. control; $^{\S}P = 0.0327$ SN anti-miR-199a-CM vs. SN control-anti-miR-CM; $^{**}P = 0.0005$ SN pre-miR-199a-CM vs. SN control-pre-miR-CM; $^{**}P = 0.0005$ SN control-pre-miR-CM vs. control; $^{\#\#}P < 0.0012$ SN pre-miR-199a-CM vs. SN control-pre-miR-CM. Reactive oxygen species (ROS) production: $^{*}P = 0.0179$ SN control-anti-miR-CM vs. control; $^{\S}P = 0.0139$ SN anti-miR-199a-CM vs. SN control-anti-miR-CM; $^{**}P = 0.0051$ SN pre-miR-199a-CM vs. SN control-pre-miR-CM; $^{**}P = 0.0276$ SN control-pre-miR-CM vs. control; $^{\#\#}P = 0.0444$ SN pre-miR-199a-CM vs. SN control-pre-miR-CM. (*B*) Gas chromatography-tandem mass spectrometry displays ADMA levels in SN of pre-miR-199a-CM, anti-miR-199a-CM, and controls (ADMA levels in supernatant of mock, control-anti-miR-CM and control-pre-miR-CM were similar and were pooled as 'controls': $^{**}P = 0.0007$ pre-miR-199a vs. controls; $^{**}P = 0.0004$ anti-miR-199a and controls). (*C*) Bar graphs depicting ADMA synthesis in SN of controls, pre-miR-199a-CM ($^{**}P = 0.002$), and anti-miR-199a-CM ($^{**}P = 0.0003$) and (*D*) degradation capacity determined by elimination rate of exogenously applied synthetic heptadeutero-asymmetric dimethylarginine (d_7-ADMA) over a period of 24 h. (*E*) Western blot displays protein levels of protein arginine methyltransferase I (PRMT-I) and dimethylarginine dimethylaminohydrolase II (DDAH II) in anti-miR-199a-CM, pre-miR-199a-CM, or their respective controls. (*F*) Bar graph depicting ADMA levels in supernatant from control and pre-miR-199a-CM with or without addition of arginine methyltransferase inhibitor 1 (AMI-1) (AMI: 100 µM), $^{**}P = 0.0002$ vs. control-pre-miR, $^{\S\S}P = 0.0019$ AMI-1/pre-miR-199a vs. pre-miR-199a. (*G*) Bar graph summarizing NO bioavailability and ROS production of RHE-A cells exposed to SN of pre-miR-199a-CM without or with AMI-1 (100 µM, NO bioavailability: $^{\S}P = 0.0311$, ROS production: $^{\S\S}P = 0.0042$) or where L-Arginine (Arg: 0.5 mM, NO bioavailability: $^{\#\#}P = 0.0040$, ROS production: $^{\#\#}P = 0.0002$) was added to SN of pre-miR-199a-CM prior to addition to RHE-A cells. All experiments derived from 3–5 different cell isolations, and were performed in triplicate.

CM (*Figure 7E*). Down-regulation of α- and β-MHC mRNA could not be prevented by AMI-1 in siRNA-Ube2i/Ube2g1-CM (*Figure 7F*).

Discussion

The present study shows that failure-prone STAT3-KO mice[2] display up-regulated cardiac expression of miR-199a before the onset of heart failure. Further analysis revealed that STAT3 protein acts as a potent suppressor of miR-199a transcription in post-natal CMs. Up-regulated miR-199a promotes at least two different pathophysiological changes in CMs: (1) it lowers α- and β-MHC mRNA as well as protein levels of sarcomeric MHC and TnT, thereby impairing sarcomere composition, and (2) it promotes via up-regulation of PRMT the secretion of the eNOS inhibitor ADMA from CMs, thereby inducing endothelial cell dysfunction. Ube2i and Ube2g1 were identified as direct targets of miR-199a. Ube2i and Ube2g1 expression was reduced in miR-199a over-expressed CMs *in vitro* and in STAT3-KO mice *in vivo*. Down-regulation of Ube2i and/or Ube2g1 in CMs lowered α- and β-MHC mRNA levels as well as protein levels of sarcomeric MHC, TnT, and Tm and promoted PRMT-mediated synthesis of ADMA indicating that miR-199a-mediated pathophysiological changes in CMs derive at least in part from its suppressive effect on Ube2i and Ube2g1. Potential clinical importance of our findings is suggested by observations in the terminal failing human heart where low STAT3 protein levels[5] were associated with increased miR-199a levels and reduced Ube2g1 expression.

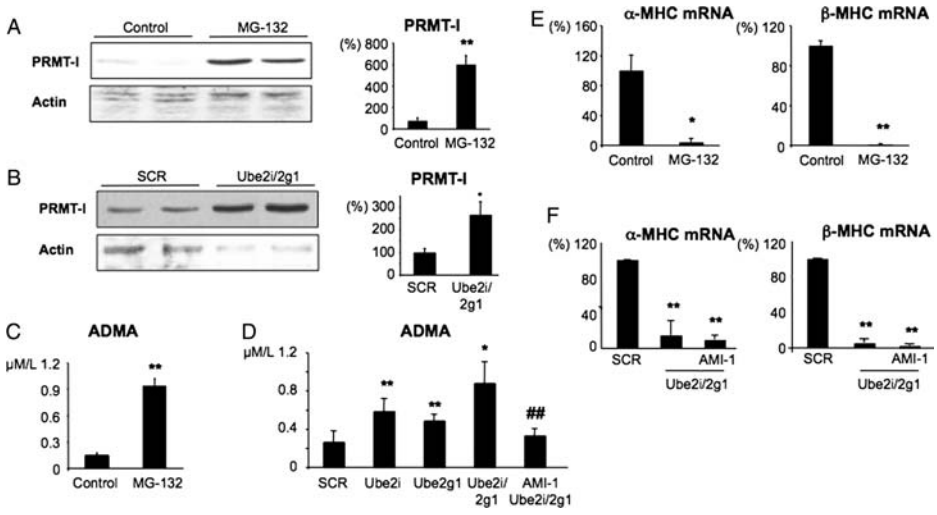

Figure 7 Impairment of the ubiquitin-proteasome system activity promotes ADMA secretion but does not affect MHC expression in cardiomyocytes. (*A*) Western blot and bar graphs depicting PRMT-I protein levels in CM treated with MG-132 (**$P = 0.0004$) or (*B*) in siRNA-Ube2i/Ube2g1-CM (**$P = 0.0316$ vs. SCR). (*C*) Bar graph summarizing ADMA levels in SN of CM with or without MG-132 (0.5 μM for 5 days), **$P = 0.0001$ vs. untreated control (Ctrl) and (*D*) in SN of with siRNA-Ube2i-CM (**$P = 0.00327$ vs. SCR), siRNA-Ube2g1-CM (**$P = 0.0093$ vs. SCR), and siRNA-Ube2i/Ube2g1-CM with (*$P = 0.0227$ vs. SCR) or without AMI-1 ($^{\#\#}P = 0.0004$ vs. untreated siRNA-Ube2i/Ube2g1-CM) compared with SCR-CM. (*E*) Bar graphs summarize α- and β-MHC mRNA levels by qRT–PCR in MG-132-treated CM (α-MHC: **$P = 0.0111$ vs. SCR, β-MHC: **$P = 0.0004$ vs. control) and (*F*) in siRNA-Ube2i/Ube2g1-CM with and without AMI-1 (α-MHC: **$P = 0.0003$ vs. SCR, β-MHC: **$P = 0.0001$ vs. SCR). Experiments were performed in triplicate from three independent cell preparations.

Taken together, these data suggest a novel pathophysiological circuit in the heart that links reduced STAT3 protein levels to increased miR-199a expression with subsequent down-regulation of Ube2i and Ube2g1, thereby promoting disruption of CM sarcomere structures and via the release of ADMA, endothelial cell dysfunction (*Figure 8*).

Our data suggest that *miR-199a-2* located on the opposite strand of the dynamin-3 gene is regulated by STAT3 in CMs since the pri-miR-199a-2 transcript is up-regulated in STAT3-KO mice and in isolated CMs with STAT3-knock-down. The STAT3-knock-down was associated with a strong increase in *miR-199a-2* promoter activity indicating that STAT3 suppresses *miR-199a-2* transcription in CMs. The *miR-199a-2* promoter contains three putative STAT3 binding sites. However, activation of STAT3 by LIF that would induce binding to these sites had no effect on *miR-199a-2* promoter activity suggesting that canonical STAT3 activation is not involved. Further studies to elucidate the role of STAT3 for miR-199a expression are currently conducted in our lab. Interestingly, STAT3 knock-down in CMs produced a similar phenotype with regard to myocyte thinning and elongation (see Supplementary material online, *Figure S2*), as observed in miR-199a over-expressing CMs confirming that at least some effects of STAT3 down-regulation seem to derive from up-regulated miR-199 expression. Surprisingly, STAT3 did not affect expression levels of *miR-214*, a gene belonging to a putative *miR-199l-214* gene cluster.[14] *miR-199a-2* and *miR-214* are separated by almost 7 kb leaving open the possibility that the two genes are regulated by independent promoter motives.

Low-level constitutive expression of miR-199a is present in the human heart even under normal condition,[7] suggesting that subthreshold levels of miR-199a do not induce obvious pathophysiological alterations. However, massive up-regulation of miR-199a has been associated with an eccentric hypertrophic phenotype in CMs.[7] Indeed, with increasing levels of miR-199a, as observed in STAT3-KO mice, the susceptibility towards manifestation of the disease phenotype slowly increases, a feature that can be observed in slightly older STAT3-KO mice starting to display signs of heart failure at 5 and 6 months of age.[2]

We observed that miR-199a markedly down-regulates α- and β-MHC transcript levels and total sarcomeric MHC and TnT protein content associated with substantial sarcomere disorganization implicating functional impairment of CMs. This effect was even more pronounced in siRNA-Ube2i/Ube2g1-CM and seemed to correlate directly with the amount of Ube2i and/or Ube2g1 protein levels present in CMs. A similar down-regulation of α- and β-MHC mRNA was also obtained in CM with global pharmacological inhibition of the UPS indicating that impairment of UPS activity due to reduced Ube2i/Ube2g1 expression massively down-regulates α- and β-MHC at the mRNA level. This observation explains why despite attenuated UPS-mediated protein degradation the amount of MHC protein levels is markedly reduced in pre-miR-199a-CM and in siRNA-Ube2i/Ube2g1-CM.

We have previously shown that defects in STAT3-KO mice are mainly present in the non-CM compartment and seem to derive from paracrine effects of STAT3-KO CMs on endothelial cells and fibroblasts.[2] There we analysed effects of miR-199a on the

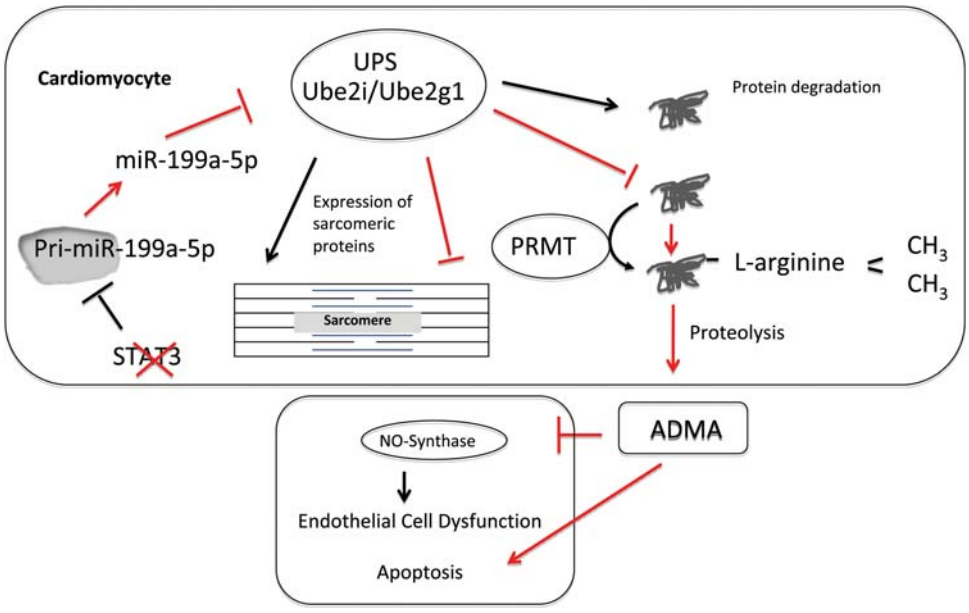

Figure 8 Scheme displaying the link between STAT3-regulated miR-199a expression and the Ube2i and Ube2g1 enzymes that impacts on two different pathophysiological mechanisms in the heart: (1) the cardiomyocyte sarcomere composition with consequences for the cardiomyocyte function and (2) the cardiomyocyte secretome with consequences for endothelial cell function and survival. Red arrows indicate the situation in STAT3 knock-down or knockout conditions.

CM secretome. Indeed, miR-199a-mediated down-regulation of Ube2i and Ube2g1 enhanced the synthesis of the endogenous eNOS inhibitor ADMA by CMs that subsequently induces endothelial cell dysfunction. Similar to effects on MHC mRNA levels, the effect on ADMA production depends on impaired UPS activity, since not only down-regulation of Ube2i and Ube2g1 but also global pharmacological inhibition of the UPS increases ADMA synthesis in CM. Responsible for increased ADMA synthesis in CMs appears to be the up-regulation of PRMT-I, an enzyme that induces methylation, mainly at arginine residues, on proteins that are not properly degraded by the UPS, thereby leading to UPS-independent protein proteolysis and subsequent synthesis of ADMA.[15] This notion is supported by the observations that PRMT-I is up-regulated in pre-miR-199a-CM, in siRNA-Ube2i/Ube2g1-CM, and in CM with UPS inhibition and that the PRMT inhibitor AMI-1 prevented ADMA generation in pre-miR-199a-CM and in siRNA-Ube2i/Ube2g1-CM. In contrast, the PRMT inhibitor had not effect on the down-regulation of α- and β-MHC mRNA in pre-miR-199a-CM and in siRNA-Ube2i/Ube2g1-CM, indicating that PRMT is not linked to this UPS-mediated process.

Asymmetric dimethylarginine acts as an endogenous inhibitor of NO production in endothelial cells by uncoupling eNOS, thereby promoting the generation of reactive nitrogen species such as peroxynitrite.[15,16] Cell culture SN from pre-miR-199a-CM as well as from siRNA-Ube2i/Ube2g1-CM impaired endothelial cell function with respect to reduced NO availability and increased ROS production. These effects on endothelial cells seem mainly caused by increased ADMA levels because they were not observed if SN derived from CMs treated with the PRMT inhibitor AMI or if the NO donor L-Arginine was added to CM SN prior to addition to endothelial cells. These experiments identify CMs as a source of biologically active ADMA, that if up-regulated by miR-199a-mediated Ube2i and Ube2g1 down-regulation is sufficient to induce endothelial cell dysfunction.

These novel findings broaden the biological spectrum of STAT3 from a CM survival factor that promotes cardiac angiogenesis[17] to novel functions involving regulation of the CM sarcomere composition, a feature that fits well with the DCM phenotype observed in older STAT3-KO hearts.[2] Furthermore, the previously reported decreased capillary density in STAT3-KO mice may be caused at least in part by ADMA-mediated endothelial cell apoptosis.[18] In addition, the early metabolic impairment[2] in response to catecholamine stress in young STAT3-KO mice has been explained so far by the slightly reduced capillary density. The new findings in this study, however, suggest that endothelial cell dysfunction caused by ADMA may also contribute to impaired blood supply under conditions of increased energy demand.

Taken together, the consistence between knock-down of STAT3 in CMs *in vitro*, and hearts from STAT3-KO mice *in vivo* with regard to up-regulation of miR-199a expression, reduction of Ube2i and Ube2g1 expression, and subsequent impairments in the sarcomeric gene expression and the CM secretome implicates an important role of a novel regulatory circuit involving STAT3, miR-199a, and Ube2i and Ube2g1 for adaptive vs. maladaptive cardiac remodelling processes, and is likely to contribute to the predisposition of STAT3-KO mice to develop heart failure.[2] Moreover, it is conceivable that it promotes progression of heart failure in diseased hearts harbouring reduced STAT3 expression.

Especially the latter possibility is supported by the notion that terminally failing human hearts display low STAT3 protein levels[5] which, as observed in the present study, is associated with increased miR-199a levels and decreased Ube2g1 expression, thereby confirming a link between low STAT3, up-regulated miR-199a, and subsequent impairment of the UPS in failing human hearts.

In conclusion, the present study confirms the important role of STAT3 as a factor controlling post-natal cardiac integrity. As a novel aspect we describe a link between STAT3-regulated miR-199a expression and the UPS that is controlling both the composition of the CM sarcomere and the CM secretome (*Figure 8*). As targets of miR-199a and thereby also as indirect targets of STAT3, the two ubiquitin-conjugating enzymes Ube2i and Ube2g1 emerged as necessary for adequate expression of sarcomeric α- and β-MHC genes and for controlling the generation of the eNOS inhibitor ADMA. Their miR-199a-dependent down-regulation link maladaptive structural alterations of CMs with endothelial cell dysfunction in an early stage of cardiomyopathy, a feature that seems important for the progression of the disease. Therefore, miR-199a, specific components of the UPS, and ADMA may emerge as novel therapeutic targets in heart failure.

Supplementary material

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

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