STAT3-mediated regulation of miR-199a-5p links cardiomyocyte and endothelial cell function in the heart: A key role for ubiquitin-conjugation enzymes

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Short title: miR-199a-5p links cardiomyocyte and endothelial function
Patient population
LV myocardium was obtained from patients undergoing heart transplantation due to end-stage heart failure (n=7). As control LV tissue from organ donors not suited for transplantation (NF: n=5) was used.

Isolation of primary cardiomyocytes and cell culture conditions
Neonatal rat cardiomyocytes (CM) were isolated by enzymatic disassociation of neonatal rat hearts as described previously. Rat endothelial cells (RHE-A cell line) was cultured in DMEM supplemented with 10% FCS.

Transfections of cultured neonatal cardiomyocytes with antagomirs, miRNAs or siRNAs and collection of conditioned supernatants
CM were transfected with antagomirs and miRNAs: Anti-miR-199a-5p, contr.-anti-miR (scrambled AntimiR), pre-miR-199a and contr.-pre-miR (100 nmol/L each; Ambion) or siRNAs: control siRNA, siRNA for Ube2i and siRNA for Ube2g1 (100 nmol/L each; Santa Cruz) by a liposomal-based method (Lipofectamine™2000, Invitrogen) according to the manufacturers instruction. Cellular uptake was monitored using Cy3-labeled antagomirs (Ambion) by fluorescence microscopy (Carl Zeiss Gmb H). Transfection was performed for 24h, then the cells were washed and kept in serum-free medium for additional 96 h, after that conditioned supernatants (SN) were harvested and centrifuged (1000 g, 10 min) to remove the cells and were used for gas chromatography-tandem mass spectrometry, ADMA ELISA (DLD Diagnostika GmbH).

Analysis of cardiomyocyte morphology
Surface dimensions of CM were measured 96 h after transfection with the use of the AxioVison Rel 4.1 package (Carl Zeiss Gmb H). At least 50 cells/culture dish from randomly selected fields were digitalized (Axiovert 200M, Carl Zeiss) and cell width and length were measured. CM from three dishes per cell isolation were analyzed for each experimental group and a total of 3 to 5 separate CM isolations were performed.

Electron microscopy
All cardiomyocytes were fixed with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate (pH 7.4) for 30 minutes, rinsed in PBS and embedded in Araldite as described previously. Alternating semi-thin (0.8 mm) and ultrathin sections (100nm) were cut with a Leica Ultracut UCT microtome (Nussloch, Germany). Semithin sections were stained with 1% toluidine blue (pH 9.0). Adjacent ultrathin sections were contrasted with 5% aqueous uranyl acetate for 5 minutes, followed by a 2-minute staining step with lead citrate.
Light microscopy pictures were taken with an Olympus DP71 camera mounted on an Olympus microtome BH-2, and documented by the computer-assisted analysis system (Soft imaging system GmbH, Münster, Germany). Ultrathin sections were viewed in a Philips EM420 electron microscope documented by the Digital Biomedical Imaging System (Pforzheim, Germany).

**Microarray for miRNAs**
Total RNA from adult hearts (male mice, 3 months of age, pooled from 5 wild types vs 5 STAT3-KO mice) was prepared by Miltenyi Biotec who subsequently performed a custom made PIQORTM miRXplore Microarray (Miltenyi). A second miCHIP on the same total RNA pools was performed by Castoldi and colleagues. For this, total RNA (1 µg) was labeled with a Cy3-conjugated RNA-linker and hybridized to miChip as described. miChip is based on Locked Nucleic Acid (LNA) technology whereby LNA-modified, Tm normalized capture probes (miRCURY, Exiqon) are printed onto Codelink (GE, healthcare) slides. Images were acquired using an Axon scanner (4000B) with identical photomultiplier settings and processed and analyzed with Genepix 6 (Axon Instruments) and Excel software.

**miR-qRT-PCR, and pri-miRNA quantification**
Expression of mature miRNAs (miR-199a-5p, miR-214, miR-199a-3p, Applied BioSystems) was determined in duplicate using miR-qRT-PCR on an ABI7500 cycler (Applied Biosystems, Foster City, USA) and was normalized using the 2-DDCT method relative to snoRNA-202 and miR-16, respectively.

Primers and probes to determine expression from rno-pri-miR-199a, mmu-pri-miR-199a-1, and mmu-pri-miR-199a-2 are:
- rno-miR-199a-FP: 5’-GACAGGCTCTCCCCAGTC-3’
- rno-miR-199a-RP: 5’-CCAGCCGTCCATGGCGT-3’
- mmu-miR-199a-1-FP: 5’-CTGGCCTGTACCATGGCC-3’
- mmu-miR-199a-1-RP: 5’-CAGACTACTGTACATGTCCC-3’
- mmu-miR-199a-2-FP: 5’-GACAGGCTCTCCCCAGGC-3’
- mmu-miR-199a-2-RP: 5’-GACAGGCTCTCCCCAGTC-3’
- rno-miR-199a and mmu-miR-199a-2: 5’-FAM-AGATCCTGCTCCGTCGCCC-MGB-3’
- mmu-miR-199a-1: 5’-FAM-ACAGCCATCCCCGCCCATCCCA-MGB-3’

normalized to rodent GAPDH expression (Applied BioSystems).

**RNA Isolation, primer sequences and qRT-PCR**
Isolation of total RNA from mouse LV tissue, human LV tissue and CM, cDNA synthesis and qRT-PCR were performed according to standard procedures. Quality control for total RNA
for microarray and miChip analysis was performed by Agilent 2100 bioanalyzer platform (Agilent Technologies). qRT-PCR measurement was performed using the Stratagene MX3005p QPCR System with a SYBR green dye method (Brilliant SYBR Green Mastermix-Kit; Stratagene). Results were expressed as percent of control (control at 100%). Specific primers used for real-time PCR were:

Rat:
Ube2i: 5'-GAGGCTTGTTCAAGCTACGG-3'; 5'-CTCTGCTTGAGCTGGGTCTT-3'
Ube2g1: 5'-ATGCTGCCAGATCCTAATGG-3'; 5'-GGTGGGTAGAGTGCAGGAAA-3'
STAT3: 5'-CAATACCATTGACCTGCCAT-3'; 5'-GACTCAAACTCCCTCTCTG-3'
α-MHC: 5'-GGAAGAGCGACGGGCATCAAGG-3' 5'-CTGCTGGACAGGTTATTCTCA-3'
β-MHC: 5'-CAAGTCCGCAAGGTGC-3' 5'-AAATTGCTTTATTGTGTCTT-3'
GAPDH: 5'-ACCACCATGGAGAAGGCTGG -3' 5'-CTCAGTGTAGCCCGAGATGC -3'

Human
Ube2i: 5'-CCAGCCATCAACATCACAACA-3' 5'-CACAAGGTCGCTGCTTATGA-3'
Ube2g1: 5'-CATGAGCCTGGGGAAGATAA-3' 5'-CTTACACAGCGGGCAACTTT-3'
GAPDH: 5'-ACCACCATGGAGAAGGCTGG -3' 5'-CTCAGTGTAGCCCGAGATGC -3'

**Protein isolation, Western blotting and immunohistochemistry**

Isolation of total cellular proteins, Western blotting and immunohistochemistry were performed according to standard protocols.1 The following primary antibodies were used for Western blotting: a rabbit polyclonal anti-STAT3 antibody, a rabbit polyclonal anti-PRMT I (1:1000; Cell Signalling), a rabbit polyclonal anti-DDAH II antibody, a rabbit polyclonal anti-Ube2i antibody, a goat polyclonal anti-Ube2g1 antibody (1:250; Santa Cruz Biotechnology) or a mouse monoclonal anti-MHC antibody (1:1000; Abcam) and equal protein loading was verified by reprobing the membrane with a rabbit polyclonal anti-actin (1:10000; Sigma-Aldrich). Indirect immunofluorescence was performed using the mouse monoclonal antibody directed against sarcomeric α-actinin (1:200; Sigma) as primary antibody and FITC donkey anti-mouse IgG as secondary antibody (1:250; Jackson ImmunoResearch).

**Preparation of recombinant lentiviral supernatants and lentiviral transduction**

VSV.G-pseudotyped lentiviral particles were generated by calcium phosphate co-transfection of 293T cells (maintained in Dulbecco’s modified Eagle’s medium (DMEM), 10% FCS and 2 mM L-glutamine), and viral supernatants were concentrated as previously described.5 dcH1-STAT3shRNA-SR and dcH1-GL4-SR (control) lentiviral preparations were titrated in triplicate by serial dilutions of the concentrated vector stocks on 1x10⁵ K562 cells (maintained RPMI
1640 with 10% FCS) in 24-well plates. The number of RFP- or GFP-positive cells was analysed 72h post-transduction by FACS-analysis (FACS-Calibur, Becton-Dickinson, Heidelberg, Germany). The titers were averaged and typically ranged between 1-5 x 10^8 IU/ml. Lentiviral supernatants expressing anti-STAT3 shRNAs and control shRNA were used to transduce NRCF with an MOI of approximately two. Lentiviral supernatants overexpressing miR-199a-5p and control-miRNA were used to transduce HL-1 cells and to generate stable clones of HL-1/S-miR-199a-5p-IEW, and HL-1/S-miR-ctrl-IEW, respectively.

Lentiviral over-expression of shRNAs and miRNAs
Anti-c-STAT3-shRNAs corresponding to position 823 to 841 of the murine c-STAT3 gene (Gene bank accession no. NM-213659), lentiviral transgene plasmids pdc-SR, and shRNA controls were cloned as described. The numbering of the first nucleotide of the shRNA refers to the ATG start codon. All lentiviral constructs encode RFP (red fluorescent protein) as reporter gene. Pre-miR-199a-5p (miRBase ID: M10000941), lentiviral transgene plasmids pdc-SEW, and miRNA-controls were cloned as described. All lentiviral constructs encode EGFP (enhanced green fluorescent protein) as reporter gene.

Cloning of miR-199a promoter region and luciferase activity assay
The miR-199a promoter region (nucleotides -686 to -45, relative to the first nucleotide of the pre-miR-199a-5p) was amplified by PCR from murine genomic DNA using the primer pairs described by. The amplified fragment was cloned using TA-Cloning Kit (Invitrogen), verified by sequencing and successively cloned by blunt-ends ligation into the EcoRV site of the pGL4-2.1 vector (Promega) upstream of the firefly luciferase reporter gene. 96 h after lentiviral transduction with anti-STAT3 sh-RNA or control sh-RNA GL4, NRCF cells in 35 mm plates were co-transfected using 6 µl of Lipofectamine (Lipofectamine™2000, Invitrogen) with 2 µg of pGL4-1.2-miR-199a-5p-Promoter construct or pGL4-1.2 empty vector and 1 µg of pRL-SV40 renilla luciferase reporter vector for normalization. Luciferase activities were measured 48h after transfection using the Dual-Glo Luciferase Assay System (Promega).

The miR-199a promoter contains 3 putative STAT binding motives:
Position: -339 TTACACGAA
        -383 TTGAGGGAA
        613 TTAAAAAAA

miRNA target verification
One DNA oligonucleotide corresponding to a predicted target of miR-199a-5p of the sequence of the rat Ube2g1 mRNA (NM_022690), and one DNA oligonucleotide
corresponding to a predicted target of miR-199a-5p of the sequence of the mouse Ube2i mRNA (NM_011665), were chemically synthesized including overhang sequences from a 5' XhoI- and a 3' NotI- restriction site:

Ube2g1-FP:
5'TCGAGGACTTCTGTGGTAAACAAGTTGGCAAAGACTCTGGCTGAGAGCTGGC4TAAACATGAGCCAGTGCA3',
Ube2g1-RP:
5'GGCCGCACTGCGCATGGTTTTATTGCAGGCCAGTTCCAGCGCAAGGTTTTGCGAACTTGTATCAACAGAAGTAGC3',
Ube2i-FP:
5'TCGAGTGCAGCTCTCTTCATCTGTTGTTTGTAAATAGGACTGTGTACAGGGGAACAAGACAAGAGGGGTGGC3',
Ube2i-RP:
5'GGCCGCGGTGGGGAGAACAGAACACATCCCTGTACACAGTCTCTATTTAACAAGAACCAGATGAAGATGGAGCTGCAC3'.

Annealed oligonucleotides were directionally cloned into the XhoI / NotI-digested psiCHECK2 plasmid (Promega, Heidelberg). For each plasmid the correct sequence and insertion was confirmed by DNA sequencing. Site-directed mutagenesis of the miR-199a-5p target sites in the Ube2g1- and Ube2i-3'UTR was carried out using the Quick-change mutagenesis kit (Stratagene, Heidelberg, Germany) in which the corresponding wildtype 3'UTR was used as a template.

HL-1 cells stably expressing miR-199a-5p and control miRNA (30% confluence) in 24-well plates were transfected with reporter genes by use of Lipofectamine (Lipofectamine™2000, Invitrogen). Cell extracts were prepared 96 h after transfection and the luciferase activity was determined using the Dual-Glo Luciferase Assay System (Promega) and Mithras LB 940 luminometer (Berthold technologies).

**Electron spin resonance (ESR) spectroscopy analysis of superoxide production and nitric oxide (NO) production**

Analysis of RHE-As pretreated with the indicating conditioned supernatans for 24h was performed by electron spin resonance spectroscopy (ESR) using a MiniScope ESR spectrometer (Magnettech; Berlin, Germany) as described previously. Cellular O$_2^-$ production was determined by following the oxidation of CP-H to paramagnetic 3-carboxy-proxyl (CP). ESR instrumental settings were as follows: field sweep 108G, microwave frequency 9.78GHz, microwave power 20mW, modulation amplitude 2G, 4096 points resolution and receiver gain 1 x 105 (74dB). Results were expressed as percent of control (untreated cells at 100%).
For measurement of the NO production, samples were pre-incubated with Fe(DETC)$_2$ (285µM) at 37°C for 60 min. Results were expressed as percent of control (untreated cells at 100%).

**Measurement of ADMA**

The concentration of endogenous ADMA in cell culture supernatants was determined by gas chromatography-tandem mass spectrometry (GC-tandem MS) as previously reported for circulating ADMA using newly synthesized trideutero-ADMA (d3-ADMA) as the internal standard at a final concentration of 1 µM with minor modifications.$^{9,10}$ In order to investigate the metabolism of ADMA by cardiomyocytes, synthetic heptadeutero-ADMA (d7-ADMA) was used as a substrate at an initial concentration of 1 µM. The concentration of d7-ADMA was determined by GC-tandem MS as described recently using d3-ADMA (at 1 µM) as the internal standard.$^{11}$

**Supplementary Figures**

Supplementary Figure 1

Bar graph depicting dose-dependent effects of ADMA on NO-bioavailability (*p=0.0322, **p=0.0001) and ROS production (*p=0.0260, **p=0.0050) in RHE-A cells vs cell culture medium alone. All experiments derived from 3-5 different cell isolations, and were performed in triplicates.
Supplementary Figure 2
(A) Effect of lentiviral-mediated shGL4 or shSTAT3 knock-down cardiomyocyte dimensions in NRCM. The bar=100µm. (B) The bar graphs summarize cardiomyocyte dimensions (width and length) of 50 cardiomyocytes per stimulation in three separate cell isolations, *p<0.05.

Literature


