Stimulation of soluble guanylate cyclase by riociguat attenuates heart failure and pathological cardiac remodelling

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Material and Methods

Gene expression analysis (NanoString Technologies®) All experiments were conducted in accordance with the manufacturer's protocol nCounter Vantage RNA Assays (NanoString Technologies®). The amount of 100 ng isolated RNA (miRNeasy Mini Kit. Qiagen) from left ventricle heart tissue or neonatal cardiomyocytes was used to run the assay. nCounter Elements Tag set design can be seen in Table 1. All procedures associated with mRNA quantification, including sample preparation, hybridization, detection and scanning, were carried out as recommended by NanoString Technologies, Inc. The analysis of gene expression was performed by using nSolver4 Analysis Software (Nano String Technologies®). In both experiments, the geometric mean of 5 housekeeping genes (Rpl4, Polr2a, Rpl32, Hprt1, Gapdh) was used for normalization.

Valdiation RNA Seq (mouse)

Gene	Accession number	Position
Rpl4	NM_024212.4	1323-1422
Polr2a	NM_009089.2	2221-2320
Rpl32	NM_172086.1	61-160
Hprt1	NM_013556.2	31-130
Gapdh	NM_001001303.1	891-990
Nppa	NM_008725.2	691-790
Nppb	NM_008726.4	177-276
Acta1	NM_001272041.1	172-271
Myh7	NM_080728.2	1133-1232
Col1a1	NM_007742.3	216-315
Col3a1	NM_009930.1	4371-4470
Timp1	NM_011593.2	437-536
Postn	NM_015784.2	760-859
Rcan1	NM_001081549.1	1061-1160

Gene expression NMCs (rat)

Gene	Accession number	Position
Rpl4	NM_022510.1	315-414
Polr2a	XM_343922.5	3376-3475
Rpl32	NM_013226.2	385-484
Hprt	NM_012583.2	21-120
Gapdh	NM_017008.2	851-950
Nppa	NM_012612.1	76-175
Nppb	NM_031545.1	263-362
Myh7	NM_017240.1	1891-1990
Tgfb1	NM_021578.2	1471-1570

Microarray Analysis

Total RNA was isolated from the tissue powder of each left ventricle using QIAzol (Qiagen) and miRNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) according to manufacturer's instructions for total RNA isolation from animal tissues. RNA concentration was measured with a Nanodrop ND-1000 (NanoDrop Technologies, Inc. Wilmington, USA) and RNA quality was assessed using a RNA 6000 Nano LabChip® on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The RNA integrity number (RIN) was generated by 2100 Expert Software (Version B02.05.SI360, Agilent Technologies).

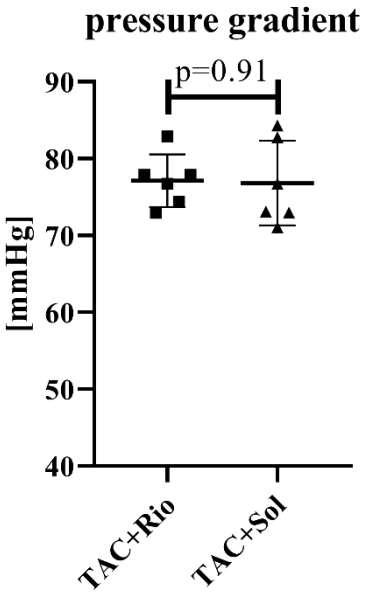
For microarray analysis, RNAs of the left ventricles of 6 TAC- and sham-mice were pooled. Transcriptional profiling of the pooled samples was performed with GeneChip® Mouse Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA, USA) according to manufacturer's protocol. For probe set extraction and normalization, expression raw data were transferred to Rosetta Resolver® 7.2 (Ceiba Solutions, Seattle, WA, USA). This system applies Rosetta Biosoftware's proprietary error models to yield quality statistics for gene expression measurement. Differences in gene expression were considered to be significant when the p-value was <0.05 and the fold change between TAC and sham group exceeded 1.5.

Cell culture solution and media ingredients

NCM culture medium (NCM-CM) for NCM:	
DMEM GlutaMax (4.5 g/l D-Glucose)	400 ml
M199	100 ml
Heat-inactivated horse serum	10 %
Fetal bovine serum (FBS)	5 %
4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)	5 mM
Penicillin/streptomycin 10,000 U/ml	100 U/ml
Ara C (cytosin-β-D-arabinofuranosid) (10 mM)	10 µM
Panserin (minimal medium) for NCM:	
Panserin	
Penicillin/streptomycin 10 000 U/ml	100 U/ml
Ara C (cytosin-β-D-arabinofuranosid) (10 mM)	10 µM
DMEM suppl.:	
DMEM GlutaMax (4.5 g/l D-Glucose)	
FBS	10 %
Penicillin/streptomycin	100U/ml:100µg/ml
Digestion solution:	
DPBS (without Mg^{2+} and Ca^{2+})	
Pancreatin	10%
Collagenase Type 2	125 U/ml

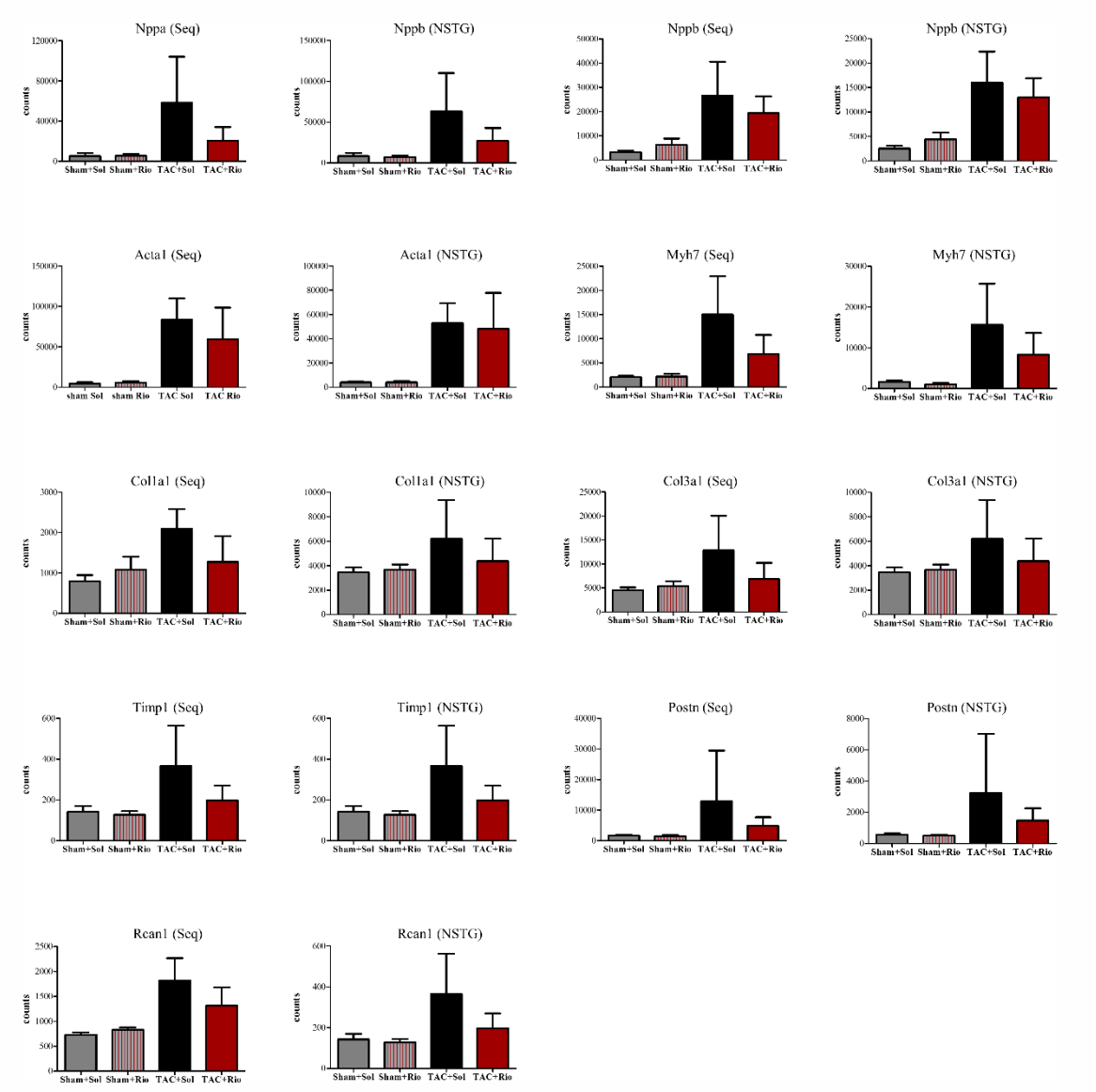
Hypertrophy experiment in HL-1 cells

HL-1 cells were cultured according to Claycomb protocol (Proc Natl Acad Sci U S A. 1998 Mar 17;95(6):2979-84) in a gelatine/fibronectin pre-coated 48-well-plate (6000 cells per well). After 24 hours starvation in Claycomb Minimal Medium, cells were stimulated for 48 hours with either 10 nM of angiotensin II (Ang II), 100 nM of riociguat (Sigma-Aldrich) or the combination of both. Then cells were fixed with 2 % paraformaldehyde (PFA) and stained with Phalloidin and 4′-6-diamidino-2-phenylindole (DAPI). Five random pictures per well (3 wells per condition) were taken with the Keyence BZ-9000 fluorescence microscope and analysed by BZ II Analyzer Software. The total area was divided by the number of cells to receive the mean size of the cells.

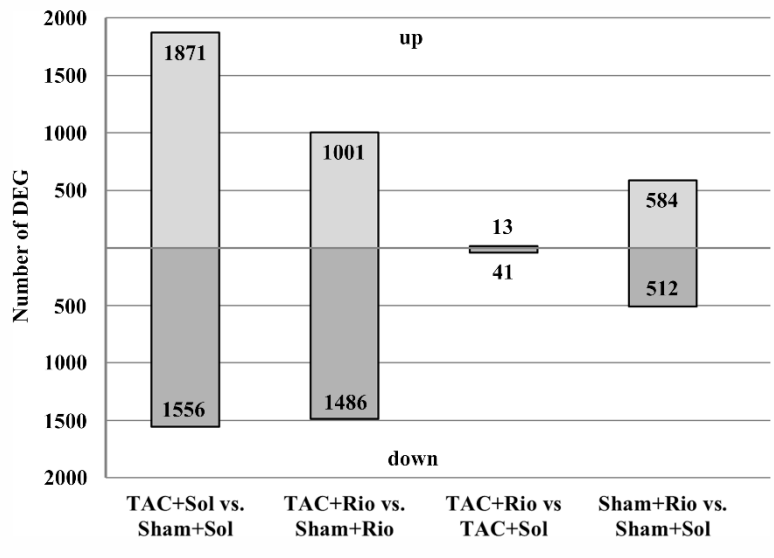


Supplemental Figure S1. Pressure gradient in mice after TAC

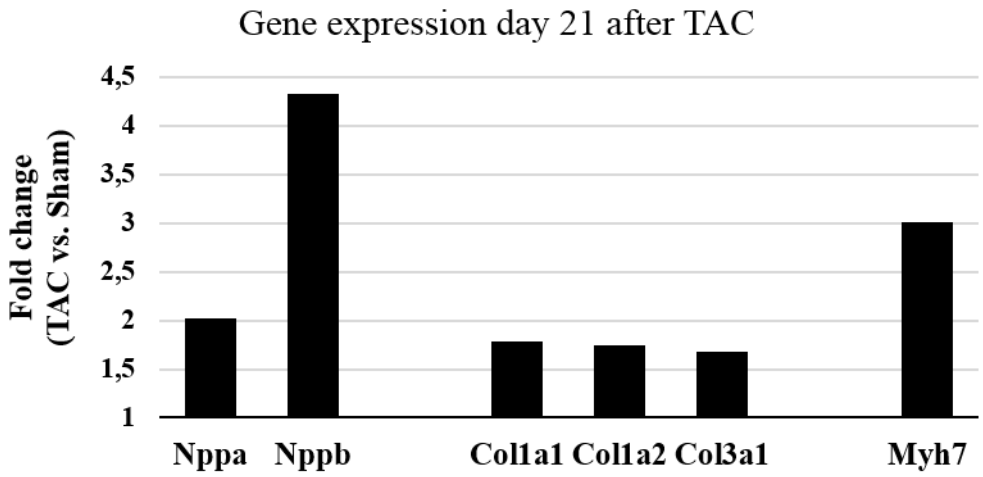
The mean pressure gradients after TAC were similar in both groups. The mice of the TAC+Rio group showed a mean pressure of 77.13±3,45 mmHg (mean±SD) and the TAC+Sol group 76.83±5.53 mmHg (mean±SD). N=6 per group



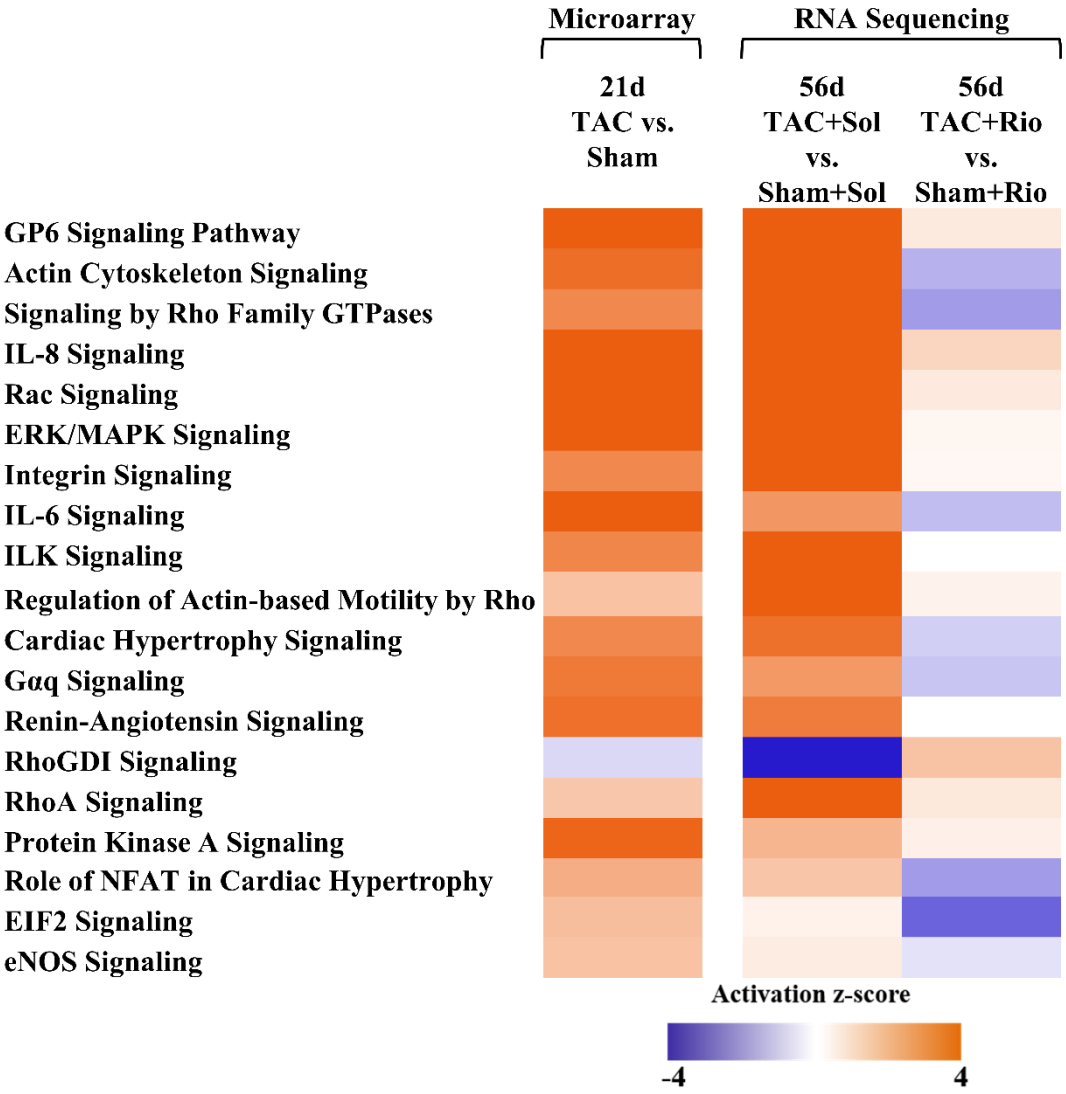
Supplemental Figure S2. Validation of RNA sequencing data in a subset of genes. Graphs show expression of *Nppa*, *Nppb*, *Acta1*, *Myh7*, *Col1a1*, *Col3a1*, *Timp1*, *Postn* and *Rcan1* using Illumina HiSeq4000 (Seq) and NanoString nCounter™ (NSTG) in heart tissue of the various treatment groups (n=6).



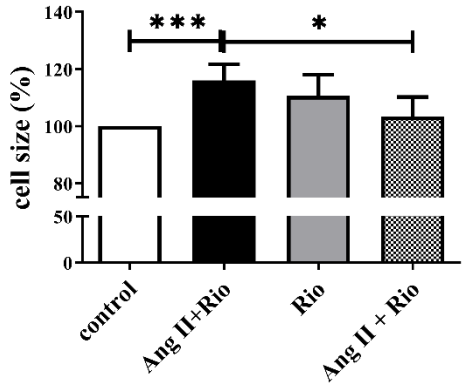
Supplemental Figure S3. The total number of up- and downregulated differentially expressed genes (DEG) in each condition.



Supplemental Figure S4. Microarray analysis of Heart Failure marker genes in left ventricle 3 weeks (21 days) after TAC. RNAs of the left ventricles of 6 TAC- and 6 sham-mice were pooled for microarray analysis. Compared to the sham-group, the expression of Nppa ANP), Nppb (BNP). Collagen and β-myosin heavy chain (Myh7) was already increased in TAC mice after 3 weeks of banding.



Supplemental Figure S5. Ingenuity® Pathway Analysis (IPA®) based canonical pathway analysis of differentially expressed genes 3 weeks (21 days) after TAC (TAC vs. Sham; data generated by microarray analysis) in comparison with RNA sequencing data of riociguat (Rio) and solvent (Sol) treated TAC mice after 8weeks (56 days). A positive z-score predicts activation (orange); a negative score (purple) predicts inhibition of the pathway.



Supplement Figure S6. Riociguat (100nM) prevents angiotensin II (10nM)-induced hypertrophy in HL-1 cells.