Supplementary Information

Preserved heart function after left ventricular pressure overload in adult mice subjected to neonatal cardiac hypoplasia

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Supplementary Materials and Methods

Western Blot Analyses

Left ventricular tissue samples were homogenized in RIPA buffer supplemented with protease (Complete Protease Inhibitor Cocktail Tablets, Roche) and phosphatase inhibitors (10 mM sodium fluoride and 1 mM sodium orthovanadate). 40 µg proteins were separated using denaturing polyacrylamide gels (SDS-PAGE) and blotted onto nitrocellulose membranes (GE Healthcare). Membranes were blocked in 5% dry milk in TBS-T and incubated with the following primary antibodies at 4°C over night: phospho-STAT3 Tyr705 (#9145), total STAT3 (#4904), phospho-p38 MAPK Thr180/Tyr182 (#4511), total p38 MAPK (#8690), phospho-Akt Ser473 (#4060), total Akt (#4691), phospho-p44/42 MAPK Thr202/Tyr204 (#4370), total p44/42 MAPK (#4695), phospho-p70S6K (#9234), total p70S6K (#2708) (all from Cell Signaling). An antibody against vinculin (Sigma V9131) was used for loading control. Secondary detection was performed using HRP-conjugated antibodies (Cell Signaling) and an enhanced chemiluminescence (ECL) reaction followed by detection of bands using a BIO-RAD ChemiDoc XRS+ imaging system. Intensity of protein bands was quantified by densitometry using ImageJ.

Quantitative real time PCR

Left ventricular tissue samples were homogenized in TRIzol reagent (Invitrogen) and total RNA was isolated according to the manufacturer´s instructions. RNA was subsequently purified using RNeasy spin columns (Qiagen) and reverse transcribed using M-MuLV reverse transcriptase (New England BioLabs) and random hexamer primers. Quantitative real time PCR was performed using the ABsolute QPCR SYBR Green Fluorescein Mix (Thermo Scientific) on the iCycler iQ Real-Time PCR detection system (BIO-RAD). Primers were obtained from BioTeZ (Berlin) with the following sequences: *Nppa* forward 5´-CATCATGGGCTCCTTCTCCAT-3´, *Nppa* reverse 5´-TGTACACAGGATTTGGTCCAATATG-3´; *Nppb* forward 5´-AGGACCAAGGCCTCACAAAA-3´, *Nppb* reverse 5´-TTGAGATATGTGTCACCTTGGAATTT-3´; *Myh7* forward 5´-CCCTCCTCACATCTTCTCCA-3´, *Myh7* reverse 5´-CTGGGGTCTGGTCCTTCTTG-3´; *Gapdh* forward 5´-AGGTTGTCTCCTGCGACTTCA-3´, *Gapdh* reverse 5´-CCAGGAAATGAGCTTGACAAAGTT-3´. Samples were analyzed in triplicate and target gene expression was normalized against *Gapdh*. Relative expression differences between groups were determined using the ΔΔCT method.

Supplementary Discussion

Developmental programming of cardiovascular disease has been proposed to involve the renin angiotensin system (RAS). [2] Sex-specific hypersensitivity towards Ang II mediated increase in blood pressure has been demonstrated in growth restricted rats (Supplementary Reference 1 & 2), whereas little is known about direct effects on the IUGR heart. We have previously shown that adult *cHccs*$^{+/-}$ hearts are specifically sensitive to Ang II, evident as an accelerated and overshooting hypertrophic response detectable at the organ as well as cardiomyocyte level after 2 weeks Ang II infusion. [21] Importantly, this exaggerated growth is transient and partially reversed upon long term (i.e. 4 weeks) Ang II treatment without causing LV dysfunction in *cHccs*$^{+/-}$ females. Given that Ang II impacts on the heart by increasing systemic blood pressure as well as by directly acting on cardiomyocytes (Supplementary Reference 3), our previous study could not differentiate between these two effects. Here we show that pressure overload itself does not induce an exaggerated hypertrophic response in *cHccs*$^{+/-}$ versus control hearts after 2 weeks. These data therefore suggest that overshooting cardiomyocyte hypertrophy in Ang II treated *cHccs*$^{+/-}$ hearts is primarily driven by its direct action on cardiomyocytes but not systemic blood pressure regulation. In regard of such interpretation, cardiomyocyte size was not studied after 2 weeks AAC, so we cannot exclude a transient peak in cell size. Nevertheless, such scenario seems unlikely as it is not reflected by echocardiography measurements of LV wall thickness and mass, two parameters that were clearly increased in *cHccs*$^{+/-}$ hearts compared to controls after 2 weeks Ang II. [21] In summary, our data support the idea that IUGR and fetal programming alter the expression and activity of various RAS components in the heart (Supplementary Reference 4 & 5), thereby inducing hypersensitivity towards Ang II.

We have previously shown that adaptive cardiomyocyte hypertrophy in *cHccs*$^{+/-}$ hearts upon Ang II stress is dependent on STAT3 signalling. [21] STAT3 is activated 2 weeks after initiation of Ang II infusion and inhibition of JAK/STAT signalling impairs cardiomyocyte growth and LV function in Ang II stressed *cHccs*$^{+/-}$ females. Here we show that STAT3 activity is not different in *cHccs*$^{+/-}$ hearts compared to controls 4 weeks after pressure overload. These data argue for a direct activation of JAK/STAT3 signalling by Ang II via its respective receptors expressed on *cHccs*$^{+/-}$ cardiomyocytes (Supplementary Reference 6), rather than activation secondary to increased blood pressure. We did not measure STAT3 activation after 2 weeks AAC, however, such that we cannot exclude a transient activation as observed upon Ang II stress. [21]

Supplementary References

1. Ojeda NB, Royals TP, Black JT, et al. Enhanced sensitivity to acute angiotensin II is testosterone dependent in adult male growth-restricted offspring. *Am J Physiol Regul Integr Comp Physiol.* 2010; 298, R1421-1427.
2. Ojeda NB, Intapad S, Royals TP, et al. Hypersensitivity to acute ANG II in female growth-restricted offspring is exacerbated by ovariectomy. *Am J Physiol Regul Integr Comp Physiol.* 2011; 301, R1199-1205.
3. Schlüter KD, Wenzel S. Angiotensin II: a hormone involved in and contributing to pro-hypertrophic cardiac networks and target of anti-hypertrophic cross-talks. *Pharmacol Ther.* 2008; 119, 311-325.
4. Xue Q, Dasgupta C, Chen M, Zhang L. Foetal hypoxia increases cardiac AT(2)R expression and subsequent vulnerability to adult ischaemic injury. *Cardiovasc Res.* 2011; 89, 300-308.
5. Goyal R, Galffy A, Field SA, et al. Maternal protein deprivation: changes in systemic renin-angiotensin system of the mouse fetus. *Reprod Sci.* 2009; 16, 894-904.
6. Tsai CT, Lai LP, Kuo KT, et al. Angiotensin II activates signal transducer and activators of transcription 3 via Rac1 in atrial myocytes and fibroblasts: implication for the therapeutic effect of statin in atrial structural remodeling. *Circulation.* 2008; 117, 344-355.

Supplementary Figures

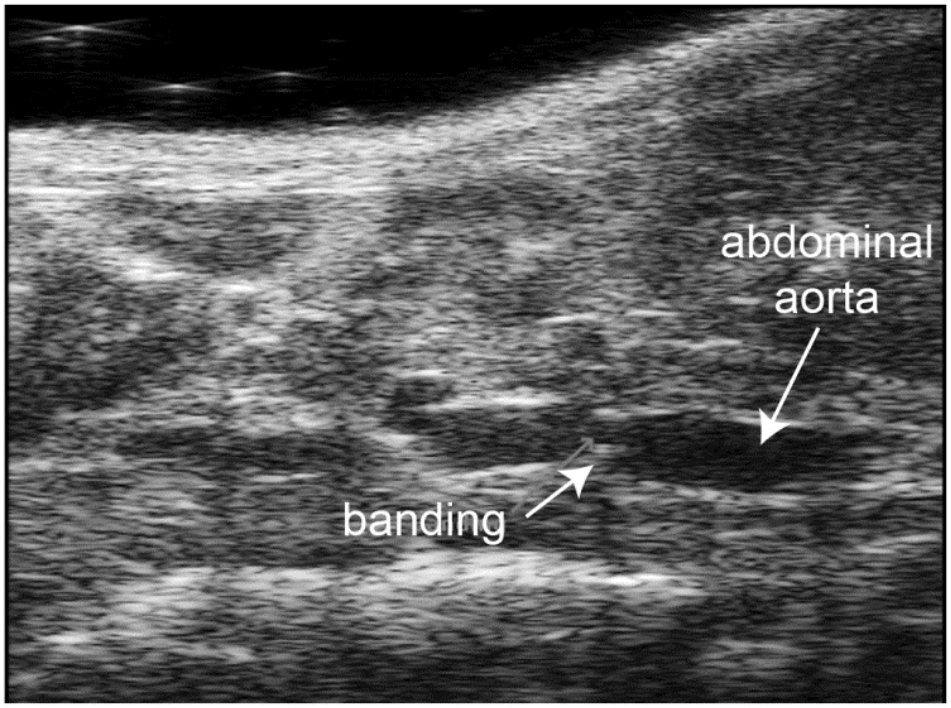

Fig. S1: Validation of abdominal aortic constriction.
Representative ultrasound image of the abdominal aorta showing constriction of the vessel lumen at the site of banding.

Supplementary Tables

Ten week old *cHccs*$^{+/-}$ and control (*Hccs*$^{+/+}$) female mice underwent abdominal aortic constriction (AAC) or sham operation. Echocardiography was performed prior to the intervention (baseline) as well as 2 and 4 weeks after surgery.

		n=	IVS dia (mm)		LVPW dia (mm)		IVS sys (mm)		LVPW sys (mm)	
			Mean±SEM	95% CI	Mean±SEM	95% CI	Mean±SEM	95% CI	Mean±SEM	95% CI
Sham										
Baseline	*Hccs*$^{+/+}$	3	0.74±0.03	0.61-0.87	0.75±0.03	0.64-0.87	0.98±0.03	0.86-1.11	1.05±0.04	0.88-1.21
	cHccs$^{+/-}$	3	0.83±0.06	0.56-1.11	0.83±0.07	0.54-1.12	1.14±0.09	0.75-1.53	1.14±0.08	0.80-1.48
2 weeks	*Hccs*$^{+/+}$	3	0.89±0.10	0.46-1.33	0.93±0.10	0.50-1.37	1.28±0.14	0.69-1.87	1.36±0.14	0.77-1.96
	cHccs$^{+/-}$	3	0.77±0.10	0.35-1.19	0.79±0.09	0.40-1.18	1.17±0.14	0.56-1.77	1.22±0.14	0.62-1.82
4 weeks	*Hccs*$^{+/+}$	3	0.81±0.04	0.66-0.96	0.81±0.04	0.62-1.0	1.13±0.03	1.01-1.24	1.14±0.04	0.97-1.30
	cHccs$^{+/-}$	3	0.83±0.03	0.71-0.96	0.80±0.03	0.67-0.93	1.15±0.04	0.99-1.31	1.13±0.04	0.94-1.32
AAC										
Baseline	*Hccs*$^{+/+}$	6	0.78±0.07	0.60-0.97	0.76±0.07	0.57-0.95	1.11±0.11	0.84-1.38	1.06±0.07	0.88-1.25
	cHccs$^{+/-}$	6	0.84±0.05	0.70-0.97	0.83±0.03	0.75-0.92	1.21±0.08	1.02-1.40	1.21±0.08	1.02-1.40
2 weeks	*Hccs*$^{+/+}$	6	0.90±0.04	0.81-0.99	0.89±0.03	0.81-0.96	1.23±0.08	1.03-1.43	1.21±0.05	1.08-1.34
	cHccs$^{+/-}$	6	0.99±0.06	0.84-1.15	0.98±0.05	0.85-1.11	1.36±0.08	1.15-1.58	1.29±0.08	1.09-1.48
4 weeks	*Hccs*$^{+/+}$	6	0.81±0.05	0.69-0.94	0.80±0.04	0.71-0.90	1.04±0.05¶	0.92-1.17	1.01±0.05¶	0.88-1.15
	cHccs$^{+/-}$	6	1.01±0.04*	0.90-1.12	1.02±0.04*$^{\#§}$	0.92-1.11	1.46±0.06*$^{\#§}$	1.29-1.62	1.44±0.07*$^{\#§}$	1.28-1.61

Table S1: Echocardiographic measurement of left ventricular wall thickness during pressure overload.
IVS = interventricular septum, LVPW = left ventricular posterior wall, dia = diastole, sys = systole, CI = confidence interval. *P<0.05 versus *Hccs*$^{+/+}$ after 4 weeks AAC, $^{\#}P$<0.05 versus *cHccs*$^{+/-}$ sham animals (4 weeks), $^{§}P$<0.05 versus baseline, $^{¶}P$<0.05 versus 2 weeks

		n=	LV mass (mg)		LV mass/BW (mg/g)	
			Mean±SEM	95% CI	Mean±SEM	95% CI
Sham						
Baseline	*Hccs*$^{+/+}$	3	85.81±2.79	73.80-97.83	4.74±0.30	3.45-6.02
	cHccs$^{+/-}$	3	95.48±11.8	44.72-146.23	5.13±0.67	2.23-8.04
2 weeks	*Hccs*$^{+/+}$	3	102.98±10.6	57.39-148.58	5.31±0.53	3.02-7.61
	cHccs$^{+/-}$	3	99.48±13.4	41.80-157.15	5.03±0.60	2.43-7.63
4 weeks	*Hccs*$^{+/+}$	3	94.07±1.45	87.82-100.33	4.74±0.30	3.44-6.04
	cHccs$^{+/-}$	3	101.03±6.72	72.14-129.92	4.40±0.26	3.27-5.54
AAC						
Baseline	*Hccs*$^{+/+}$	6	97.63±9.77	72.53-122.73	4.74±0.36	3.82-5.66
	cHccs$^{+/-}$	6	109.22±6.65	92.13-126.31	5.30±0.46	4.11-6.48
2 weeks	*Hccs*$^{+/+}$	6	125.29±5.62§	110.83-139.75	6.07±0.32§	5.24-6.89
	cHccs$^{+/-}$	6	132.38±7.02§	114.34-150.42	6.34±0.21§	5.79-6.88
4 weeks	*Hccs*$^{+/+}$	6	112.25±6.67	95.12-129.38	5.47±0.36	4.55-6.40
	cHccs$^{+/-}$	6	141.79±4.17*$^{\#\S}$	131.08-152.50	6.31±0.41$^{\#}$	5.25-7.36

Table S2: Echocardiographic measurement of left ventricular mass during pressure overload.
LV = left ventricle, BW = body weight, CI = confidence interval. *P<0.05 versus *Hccs*$^{+/+}$ after 4 weeks AAC, $^{\#}P$<0.05 versus *cHccs*$^{+/-}$ sham animals (4 weeks), $^{\S}P$<0.05 versus baseline

		n=	LVID dia (mm)		LVID sys (mm)		FS (%)		EF (%)	
			Mean±SEM	95% CI	Mean±SEM	95% CI	Mean±SEM	95% CI	Mean±SEM	95% CI
Sham										
Baseline	*Hccs*$^{+/+}$	3	3.50±0.06	3.23-3.78	2.53±0.06	2.27-2.79	27.89±0.71	24.82-30.96	52.27±0.72	49.18-55.35
	cHccs$^{+/-}$	3	3.39±0.08	3.04-3.74	2.43±0.04	2.27-2.59	28.11±1.00	23.80-32.42	55.55±1.27	50.09-61.01
2 weeks	*Hccs*$^{+/+}$	3	3.28±0.15	2.63-3.94	2.26±0.21	1.37-3.15	31.54±3.07	18.31-44.77	61.00±5.18	38.73-83.27
	cHccs$^{+/-}$	3	3.69±0.11	3.21-4.16	2.43±0.13	1.86-3.01	33.98±3.24	20.04-47.92	62.28±5.62	38.09-86.46
4 weeks	*Hccs*$^{+/+}$	3	3.46±0.12	2.97-3.96	2.36±0.16	1.67-3.06	31.92±2.39	21.62-42.22	61.45±4.37	42.67-80.23
	cHccs$^{+/-}$	3	3.59±0.14	2.96-4.21	2.68±0.05	2.47-2.89	25.05±1.75	17.51-32.58	50.06±2.24	40.44-59.68
AAC										
Baseline	*Hccs*$^{+/+}$	6	3.69±0.13	3.36-4.01	2.68±0.17	2.24-3.11	27.50±3.40	18.76-36.23	53.69±4.88	41.16-66.22
	cHccs$^{+/-}$	6	3.71±0.18	3.25-4.16	2.57±0.20	2.04-3.09	31.13±2.43	24.88-37.38	58.63±3.76	48.98-68.29
2 weeks	*Hccs*$^{+/+}$	6	3.79±0.07	3.60-3.99	2.58±0.12	2.26-2.89	31.99±3.39	23.28-40.71	60.34±5.31	46.69-73.99
	cHccs$^{+/-}$	6	3.60±0.07	3.43-3.77	2.50±0.12	2.18-2.81	30.69±2.37	24.59-36.79	58.30±3.63	48.97-67.63
4 weeks	*Hccs*$^{+/+}$	6	3.87±0.12	3.55-4.19	3.06±0.19	2.57-3.55	21.24±3.40¶	12.50-29.98	43.39±5.09¶	30.31-56.48
	cHccs$^{+/-}$	6	3.67±0.09	3.43-3.92	2.45±0.14*	2.09-2.81	33.48±2.68*	26.60-40.36	61.66±3.47*	52.74-70.58

Table S3: Echocardiographic measurements of left ventricular diameter and contractility during pressure overload.

LVID = left ventricular internal diameter, FS = fractional shortening, EF = ejection fraction, dia = diastole, sys = systole, CI = confidence interval. *P<0.05 versus *Hccs*$^{+/+}$ after 4 weeks AAC, $^{¶}P$<0.05 versus 2 weeks

		Litters n=	IVS dia (mm)	LVPW dia (mm)	IVS sys (mm)	LVPW sys (mm)	LVID dia (mm)	LVID sys (mm)	FS (%)	EF (%)	LV mass (mg)	LV mass/ BW (mg/g)
Sham												
Baseline	***Hccs*$^{+/+}$**	3	0.74 ±0.03	0.75 ±0.03	0.98 ±0.03	1.05 ±0.04	3.50 ±0.06	2.53 ±0.06	27.89 ±0.71	52.27 ±0.72	85.81 ±2.79	4.74 ±0.30
	***cHccs*$^{+/-}$**	2	0.82 ±0.05	0.81 ±0.08	1.11 ±0.11	1.11 ±0.11	3.35 ±0.11	2.42 ±0.05	27.87 ±0.71	55.51 ±0.11	90.63 ±14.5	4.87 ±0.80
2 weeks	***Hccs*$^{+/+}$**	3	0.90 ±0.10	0.93 ±0.10	1.28 ±0.14	1.36 ±0.14	3.28 ±0.15	2.26 ±0.21	31.54 ±3.07	61.00 ±5.18	102.98 ±10.6	5.31 ±0.53
	***cHccs*$^{+/-}$**	2	0.73 ±0.12	0.75 ±0.11	1.11 ±0.16	1.16 ±0.18	3.69 ±0.01	2.50 ±0.19	32.38 ±4.81	59.49 ±8.36	93.14 ±18.9	4.77 ±0.78
4 weeks	***Hccs*$^{+/+}$**	3	0.81 ±0.04	0.81 ±0.04	1.13 ±0.03	1.14 ±0.04	3.46 ±0.12	2.36 ±0.16	31.92 ±2.39	61.45 ±4.36	94.07 ±1.45	4.74 ±0.30
	***cHccs*$^{+/-}$**	2	0.85 ±0.04	0.81 ±0.03	1.13 ±0.05	1.11 ±0.06	3.53 ±0.18	2.67 ±0.05	24.23 ±2.44	49.24 ±2.46	100.32 ±2.14	4.37 ±0.09
AAC												
Baseline	***Hccs*$^{+/+}$**	3	0.78 ±0.11	0.76 ±0.11	1.11 ±0.15	1.06 ±0.11	3.69 ±0.14	2.68 ±0.20	27.50 ±3.18	53.69 ±4.53	97.63 ±13.88	4.74 ±0.49
	***cHccs*$^{+/-}$**	6	0.84 ±0.05	0.83 ±0.03	1.21 ±0.07	1.21 ±0.07	3.71 ±0.18	2.57 ±0.20	31.13 ±2.43	58.63 ±3.76	109.22 ±6.65	5.30 ±0.46
2 weeks	***Hccs*$^{+/+}$**	3	0.90 ±0.03	0.89 ±0.03	1.23 ±0.02	1.21 ±0.01	3.79 ±0.09	2.58 ±0.11	31.99 ±2.97	60.34 ±5.69	125.29 ±2.33	6.07 ±0.33
	***cHccs*$^{+/-}$**	6	0.99 ±0.06	0.98 ±0.05	1.36 ±0.08	1.29 ±0.08	3.60 ±0.07	2.50 ±0.12	30.69 ±2.37	58.30 ±3.63	132.38§ ±7.02	6.34§ ±0.21
4 weeks	***Hccs*$^{+/+}$**	3	0.81 ±0.06	0.80 ±0.05	1.04 ±0.04	1.01 ±0.05	3.87 ±0.13	3.06 ±0.17	21.24¶ ±2.38	43.39¶ ±2.77	112.25 ±5.16	5.47 ±0.47
	***cHccs*$^{+/-}$**	6	1.01* ±0.04	1.02*§ ±0.04	1.46*§ ±0.06	1.44*§ ±0.07	3.67 ±0.09	2.45 ±0.14	33.48* ±2.67	61.66* ±3.47	141.79*§ ±4.17	6.31 ±0.41

Table S4: Echocardiographic data during left ventricular pressure overload adjusted for independent litters/pregnancies.
To account for potential effects on cardiac outcome caused by intrauterine or postnatal conditions specific to certain pregnancies or litters, echocardiography data of mice from the same litter was averaged (as recommended in Dickinson H et al., 2016).[22] That way the sample size (n) represents litter rather than individual mice. Given that *Hccs*$^{+/+}$ sham animals and *cHccs*$^{+/-}$ ACC animals were all from different litters, data and sample size are the same as in Table S1-S3. Data is slightly different and sample size is smaller in the other groups, for which two mice per litter were included in the study. The latter results in n=2 for *cHccs*$^{+/-}$ sham litters precluding meaningful statistical analyses with this group, such that data is presented only. For abbreviations see Tables S1-S3. Data are shown as mean ± SEM. *P<0.05 versus *Hccs*$^{+/+}$ after 4 weeks AAC, $^{§}P$<0.05 versus baseline, $^{¶}P$<0.05 versus 2 weeks

		Litters n=	CSA (μm^2)	Ki67 positive nuclei (%)	Fibrosis in LV myocardium (%)	TUNEL positive nuclei (%)
Sham						
4 weeks	***Hccs*$^{+/+}$**	3	348.23 ±12.37	1.32 ±0.13	1.46 ±0.22	0.0135 ±0.0029
	***cHccs*$^{+/-}$**	2	398.41 ±12.62	N/A	1.50 ±0.34	0.0128 ±0.0020
AAC						
4 weeks	***Hccs*$^{+/+}$**	3	445.85* ±17.01	1.44 ±0.18	5.70* ±1.53	0.0139 ±0.0027
	***cHccs*$^{+/-}$**	5	466.97 ±21.34	1.28 ±0.15	4.90 ±0.37	0.0191 ±0.0045

Table S5: Histological data after 4 weeks left ventricular pressure overload adjusted for independent litters/pregnancies.
To account for potential effects on cardiac histology caused by intrauterine or postnatal conditions specific to certain pregnancies or litters, data of mice from the same litter was averaged (as recommended in Dickinson H et al., 2016).[22] That way the sample size (n) represents litter rather than individual mice. Given that *Hccs*$^{+/+}$ sham animals and *cHccs*$^{+/-}$ ACC animals were all from different litters, data and sample size are the same as in Figure 4-6, whereas both are different in the other groups, for which two mice per litter were included in the study. The latter results in n=2 for *cHccs*$^{+/-}$ sham litters precluding meaningful statistical analyses with this group, such that data is presented only. CSA = cardiomyocyte cross sectional area. Data are shown as mean ± SEM. *P<0.05 versus *Hccs*$^{+/+}$ sham