Transcriptomic Analysis of Left Ventricle Myocardium in an SHR Congenic Line With Ameliorated Cardiac Fibrosis

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Summary

Metabolic syndrome and one of its manifestations, essential hypertension, is an important cause of worldwide morbidity and mortality. Morbidity and mortality associated with hypertension are caused by organ complications. Previously we revealed a decrease of blood pressure and an amelioration of cardiac fibrosis in a congenic line of spontaneously hypertensive rats (SHR), in which a short segment of chromosome 8 (encompassing only 7 genes) was exchanged for a segment of normotensive polydactylous (PD) origin. To unravel the genetic background of this phenotype we compared heart transcriptomes between SHR rat males and this chromosome 8 minimal congenic line (PD5). We found 18 differentially expressed genes, which were further analyzed using annotations from Database for Annotation, Visualization and Integrated Discovery (DAVID). Four of the differentially expressed genes (*Per1*, *Nr4a1*, *Nr4a3*, *Kcna5*) belong to circadian rhythm pathways, aldosterone synthesis and secretion, PI3K-Akt signaling pathway and potassium homeostasis. We were also able to confirm Nr4a1 2.8x-fold upregulation in PD5 on protein level using Western blotting, thus suggesting a possible role of Nr4a1 in pathogenesis of the metabolic syndrome.

Key words

Spontaneously hypertensive rats • Congenic strain • Metabolic syndrome • Insulin resistance • Hypertension • Cardiac fibrosis • Nr4a1 • Nr4a3 • Per1 • Kcna5

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Introduction

Dissection of the genetic background of the most prevalent multifactorial diseases including the metabolic syndrome, and its components, such as insulin resistance, dyslipidemia, obesity and hypertension, is becoming one of the major issues in current genomic research. This is not surprising considering the ever rising prevalence, morbidity, mortality and treatment costs associated with the above mentioned diseases. For this reason, numerous methods have been developed, the main being the hypothesis driven candidate gene studies (knock-out) and the hypothesis-free genome-wide association studies (GWAS). Potential candidate genes for the multifactorial diseases have been identified on every chromosome of the most commonly used model organisms. (Perusse *et. al.* 2005). Genetic background of multifactorial diseases was traditionally described as polygenic. However, as indicated by some authors, the homogeneous coverage of the genome by signals from genome-wide association studies suggest an "omnigenic" hypothesis (Boyle *et al.* 2017). On the other hand, stressing that some loci may have much larger effects than others led to an oligogenic hypothesis), (Hamet *et al.* 2005, Šeda *et al.* 2005, Loos *et al.* 2003). Anyway, identification of causative alleles and their respective role in the pathophysiology proves to be a real challenge. However, the combination of genetically defined model organisms in combination with high-throughput technologies can undoubtedly enable us to characterize transcriptomic changes and help us to understand the genetic background of multifactorial diseases.

Spontaneously hypertensive rat (SHR hereafter),

is probably the most commonly used rodent model for essential hypertension (Loos *et al.* 2003, Pravenec *et al.* 2013). We previously described an SHR derived minimal congenic counterpart SHR.PD-(D8Rat42-D8Arb23)/Cub (Rat Genome Database ID: 1641851; PD5 hereafter), (Šeda *et al.* 2005). PD5 strain has been established by introgression of a small segment of chromosome 8 from the PD/Cub strain (an inbred model of metabolic syndrome without hypertension, Šedová *et al.* 2000), on the genetic background of SHR by repeated backcrossing. Using the high definition marker-assisted approach the congenic segment was identified as containing 788 kbp (chr8:51,897,776-52,685,422 according to the rat reference genome version 3.4), encompassing 7 genes: *Plzf*, *Htr3a*, *Htr3b*, *Usp28*, *Zw10*, *Tmprss5*, and *Drd2*. *Drd2* is represented in the segment only by its promoter, first noncoding exon, and part of the first intron. Sequencing the congenic segment a deletion in the noncoding sequence (with possible enhancer function) of *Plzf* was revealed (Liška *et al.* 2014). From the phenotypic point of view PD5 displays significantly lower blood pressure, heart weight (Křen *et al.* 1997), lesser tendency to myocardial fibrotization (Liška *et al.* 2014) and lower triacylglycerol and cholesterol levels (Krupková *et al.* 2014), when compared to SHR.

In this study, we compared the gene expression profiles of hearts of SHR and PD5 male rats to determine to what extent does the minimal congenic segment of PD5 affect gene expression and possibly identify the significantly differentially expressed genes.

Methods

Rat strains

All animal related experiments were performed in agreement with the Animal Protection Law of the Czech Republic and were approved by the ethics committees of the First Faculty of Medicine, Charles University, Prague. We used SHR/OlaIpcv (SHR hereafter; RGD ID: 631848) and its minimal congenic strain SHR.PD (D8Rat42-D8Arb23)/Cub (RGD ID: 1641851, PD5 hereafter). Both strains were bred in-house. Rats were kept under 12 h light-dark cycle, fed standard laboratory chow and given access to water *ad libitum*. At 2 months of age rats were anesthetized and decapitated. Complete organs were weighted, left ventricular myocardium was snap frozen in liquid nitrogen, and used for further analysis.

RNA isolation

Total myocardial RNA was homogenized in TRIzol reagent (Invitrogen, Carlsbad, California). After removal of phenol by chloroform, the upper aqueous phase was applied to RNeasy columns (Qiagen) and RNA purified according to the manufacturer's instructions. RNA was quantified by spectrophotometry and its integrity was assessed using 2100 Bioanalyzer on RNA-6000 Nano-LabChip (Agilent, Böblingen, Germany). Only samples showing RNA integrity numbers (RIN) above 8 were used for further analysis.

Transcriptomic analysis

Four SHR and four PD5 RNA samples obtained from left ventricle myocardium were assessed using Affymetrix GeneChip® Rat Gene 2.0 arrays (Affymetrix, Santa Clara, CA, USA). Expression console software (Affymetrix, Santa Clara, CA, USA) was used to perform quality control. Differential gene expression between strains was determined using PARTEK Genomics Suite 6.6 (Partek, St. Louis, MO, USA) software. We considered a p value lower than 0.05 as well as fold change <-1.5 or >1.5 with subsequent false discovery rate (FDR) correction, applying Benjamini-Hochberg procedure, where α=0.05 (5 % FDR). The microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.* 2002) and are accessible through GEO Series accession number GSE126709.

RT PCR and qPCR

One µg total RNA was used to synthesize cDNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, California) and oligo-dT primers, according to manufacturer's recommendation. The resulting cDNAs were then used as template in quantitative real-time PCR (qPCR) reactions. Primers for qPCR reactions were designed using PrimerBLAST (Ye *et al.* 2012) to span at least one exon-exon junction and amplification was done in 7900HT (Applied Biosystems) in Power-up SYBRGreen master mix (Thermofisher). The primers were as follows:

Kcna5

F 5′GGAAGAACAAGGCAACCAGA 3′,
R 5′TGTTTCACGGCTAGTGTCCA 3′,

Nr4a3

F 5′CAGAAGCTGGGCAGAAAAGA 3′,
R 5′CAGGACAAGTCCATTGCAGA 3′,

Nr4a1

F 5′ATCTGCCTGGCAAACAAGGA 3′,

R 5′GGCTGCTTGGGTTTTGAAGG 3′.

For *Per1*, TaqMan® probe Rn01496761_g1 was used (Thermofisher). Cycle threshold (Ct) values were normalized against GAPDH (Taqman® chemistry, Applied Biosystems), and relative quantification was performed using the ΔΔCt method (Livak and Schmittgen 2001). Fold change values were calculated as the change in mRNA expression levels relative to the control. Statistical comparison was performed using Student′s two-tailed t-test on ΔΔCt values. Their normal distribution was assessed using normal probability plots (using STATISTICA13).

Nr4a1 protein expression determined by Western blotting

N-terminal rabbit monoclonal anti-Nra4a1 antibody (anti-Nur77; ab109180) was purchased from Abcam (Cambridge, UK). Monoclonal mouse anti-α-tubulin (B-5-2-1) was from Sigma-Aldrich (St. Louis, MO, USA). Membranes were incubated overnight at 4 °C with antibodies at final dilution 1:5000 (Nr4a1) or 1:15000 (α-tubulin), secondary HRP-conjugated antibody was from GE Healthcare Bio-Sciences (Little Chalfont, UK), and signal was detected using ECL Prime chemiluminiscent detection kit (GE Healthcare Bio-Sciences) and Hyperfilm ECL. Developed hyperfilms were scanned and densitometry performed in ImageJ. We made 3 technical replicates and used normalized average of Nr4a1/control protein density as an estimate of expression level of Nr4a1 in each rat. Theoretical molecular weight of unmodified Nr4a1 is 64 kDa. The observed signal was consistent with the expected value.

Bioinformatics

In silico analysis was performed using Ingenuity Pathway analysis (IPA; Ingenuity Systems; www.ingenuity.com; Redwood City, CA, USA) and Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.8), (Huang *et al.* 2009).

Results

Biometrical analysis of SHR and PD5

We compared 8 rats from each strain at the age of 2 months. We observed a proportional increase in whole heart and total body weight in SHR; however, heart weight relative to 100 g of total body weight was not significantly different in this comparison (Table 1).

Table 1. Biometrical analysis of PD5 and SHR strains. Absolute weight, weight relative to body weight, mean and standard deviation (SD) was measured and calculated for each organ. p values represent statistical significance calculated using Student's *t*-test.

	SHR mean ± SD	**PD5 mean ± SD**	**p value**
Body weight (g)	189.72 ± 10.32	162.31 ± 12.54	3.33×10^{-4}
Heart absolute weight (g)	0.75 ± 0.06	0.63 ± 0.05	9.3×10^{-4}
Heart relative to 100 g body weight (g)	0.39 ± 0.02	0.39 ± 0.03	8.5×10^{-2}
Liver absolute weight (g)	9.35 ± 0.46	7.68 ± 0.66	6.5×10^{-5}
Liver relative to 100 g body weight (g)	4.94 ± 0.22	4.73 ± 0.17	6.0×10^{-2}
Kidneys absolute weight (g)	1.5 ± 0.09	1.19 ± 0.09	8.7×10^{-6}
Kidneys relative to 100 g body weight (g)	0.79 ± 0.02	0.74 ± 0.02	3.7×10^{-5}
Soleus muscle absolute weight (mg)	74.39 ± 11.25	60.85 ± 9.37	2.0×10^{-2}
Soleus muscle relative to 100 g body weight	$3.91 \times 10^{-4} \pm 4.51 \times 10^{-5}$	$3.75 \times 10^{-4} \pm 5.57 \times 10^{-5}$	5.5×10^{-2}

Dissection of differentially expressed genes

Four SHR heart samples and four PD5 heart samples were used for microarray experiments (see Materials and Methods). After Benjamini-Hochberg correction, 18 (about 0.008 % of 220,232) DNA probe sets were identified as significantly differentially expressed between the SHR and PD5. These probe sets represented 10 up-regulated (*Col9a1*, *E9PT29_RAT*, *Epha7*, *Ephb3*, *Nr4a1*, *Nr4a3*, *Ptprt*, *Reln*, *Smg6* and *Tiam1*) and 8 down-regulated (*Crlf1*, *Dusp2*, *Dvl1*, *Kcna5*, *LOC500300*, *Nfkbia*, *Pax3* and *Per1*) known genes or loci in PD5 compared to SHR.

Bioinformatic analysis

To obtain more in-depth understanding of the above mentioned differentially expressed genes as to their

function we performed a Gene ontology (GO function) enrichment of these 18 significantly differentially expressed genes using DAVID. Gene ontology terms were divided into 3 main GO categories which were GO molecular function, GO cellular compartment and GO biological process. Within the molecular function subgroup the top five enriched terms were as follows: GO:0005515 protein binding, GO:0051393 alpha-actinin binding, GO:0035259 glucocorticoid receptor binding, GO:0048365 Rac GTPase binding, GO:0005003 ephrin receptor activity. Within the biological process subgroup the top five enriched terms were as follows: GO:0016477 cell migration, GO:0048013 ephrin receptor signaling pathway, GO:0007411 axon guidance, GO:0045893 positive regulation of transcription, DNA-templated, GO:0071376 cellular response to corticotropin-releasing hormone stimulus. As far as the GO cellular compartment is concerned only three terms – GO:0043025 neuronal cell body, GO:0030425 dendrite, GO:0005737 cytoplasm were enriched. None of the GO terms reached statistical significance.

Confirmation of differentially expressed genes

To validate the trancriptomic data, we selected four differentially expressed genes for qPCR analysis (*Per1*, *Kcna5*, *Nr3a4* and *Nr3a1*). All the observed results were in a good concordance with the microarray data (Fig. 1). We were able to confirm Nr4a1 upregulation in PD5 compared to SHR also on protein level using Western blotting (Fig. 2).

Pathway analysis of differentially expressed genes

In silico analysis using Ingenuity Pathway analysis (IPA; Qiagen) was performed to get a better understanding of the mutual interactions among these transcripts (Fig. 3). This analysis uncovered a wide range of possible interactions of these genes and pathways responsible for blood pressure regulation and other components of metabolic syndrome. Particularly interesting is the interaction with eNOS signaling pathway, renin-angiotensin signaling, as well as VEGF signaling pathways.

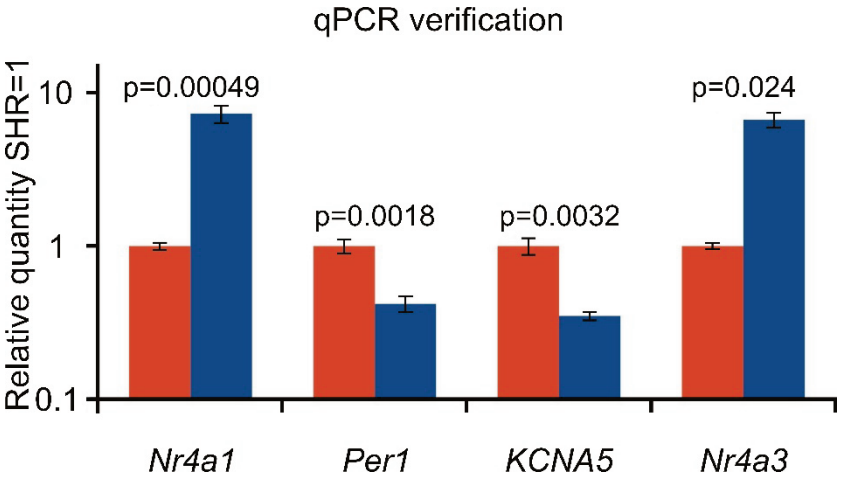

Fig. 1. qPCR validation of microarray results of heart tissue of SHR and PD5 rat strains: *Nr4a1* – nuclear receptor subfamily 4 group A member 1 (p=0.00049), *Nr4a3* – nuclear receptor subfamily 4 group A member 3 (p=0.024), *Kcna5* – potassium Voltage-Gated Channel Subfamily A Member 5 (p=0.0032) *Per1* – period circadian regulator 1 (p=0.004).

Discussion

Decreased cardiac fibrosis in PD5 compared to SHR was attributed to decreased expression of transcription factor *Plzf* (promyelocytic leukemia zinc finger, now called *Zbtb16* zinc finger and BTB domain containing protein 16), although the decrease was modest (Liška *et al.* 2014). The role of *Plzf* in cardiac fibrosis was confirmed using *Plzf* knockout heterozygotes (Liška *et al.* 2017). In current study, *Plzf* expression difference did not reach statistical significance. This can be attributed to the modest difference of expression level in the context of whole genome assay corrected to multiple comparisons. However, some of the differentially expressed genes in this study may be regulated by *Plzf*. Other source of differential expression of cardiac genes can be reactive, as a result of the hypertension development. PD5 has been shown to have lower blood pressure compared to SHR (Liška *et al.* 2014), but the difference is small, and was not confirmed in *Plzf* knock-out heterozygotes (Liška *et al.* 2017). In the cohort analyzed here, the relative heart weight in PD5 is not significantly different from controls, although the trend is in the direction previously described. This could be an advantage, since the reactive, non-primary, effects on gene expression are limited. Several of the differentially

expressed genes warrant further attention. The nuclear orphan receptors Nr4a1 and Nr4a3 display pleiotropic functions – functional regulation of cell differentiation, proliferation, apoptosis, and inflammation (Maxwell *et al.* 2006, Chao *et al.* 2008), furthermore several papers dissected its role in development of cardiovascular diseases (e.g. atherosclerosis, cardiac hypertrophy, and cardiac ischemia/reperfusion injury) (Hamers *et al.* 2012, Wang *et al.* 2013, Cheng *et al.* 2011). Nr4a1 has been proved to be regulated by angiotensin II in adrenocortical cells and cardiomyocytes and plays important roles in hypothalamic–pituitary–adrenal axis and development of cardiac hypertrophy (Wang *et al.* 2013). Furthermore, recent findings also suggest that overexpression of Nr4a1 could, *via* regulation of the *RLN3* expression, suppress apoptosis in the ventricular cardiomyocytes, thus attenuate cardiac fibrosis (You *et al.* 2018). This finding is in full accordance with our data.

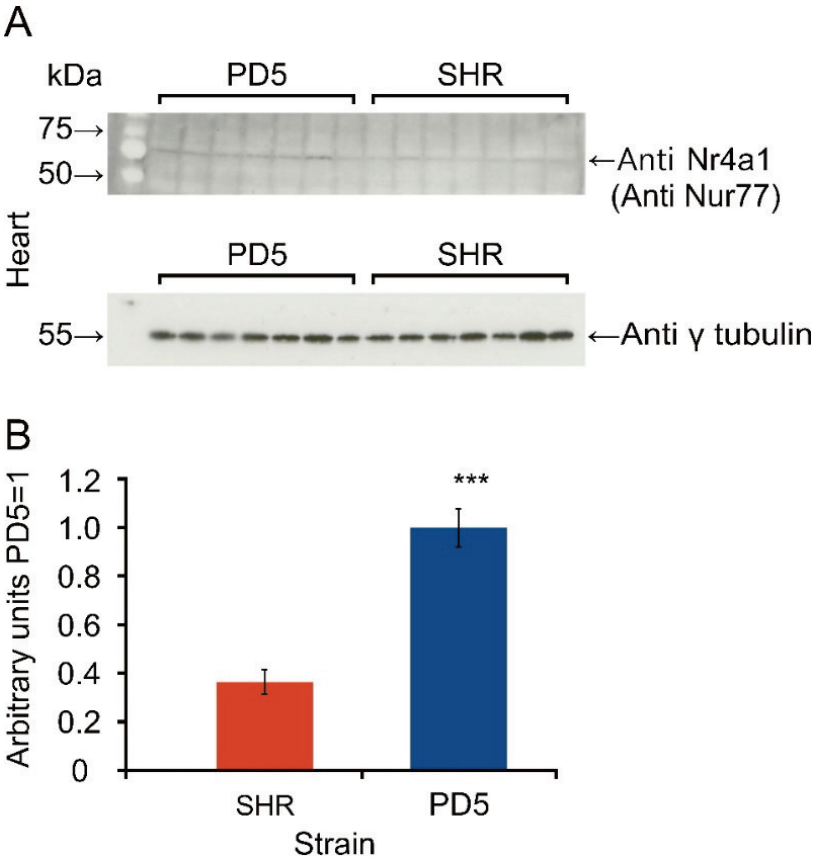

Fig. 2. Western blot of Nr4a1 protein (nuclear receptor 4 alpha 1, also known as Nur77) shows upregulation in the minimal congenic strain PD5 compared to SHR. Western blotting using N-terminal anti-Nur77 antibody in the heart (**A**), Densitometry data (representing means ± S.E.M, ***=p<0.001; n=7 for both SHR and PD5 rat strains (**B**). Theoretical molecular weight of unmodified Nr4a1 is 64 kDa. We observed signal consistently at slightly >60 kDa.

As far as hypertension is concerned, it was shown that Nr4a1 by down-regulation of β-catenin signaling in vascular smooth muscle cells (Cui *et al.* 2016) inhibits angiotensin induced phenotypic switch which leads to hypertension (Vukelic *et al.* 2014). Interestingly however, in our experiment we saw a significant overexpresion of *Nr4a1* on transcriptomic as well as on proteomic level in strain with ameliorated blood pressure. This might reflect its function as a factor preventing development of cardiac fibrosis; however in our experiment limited to whole heart left ventricle tissue, we cannot specifically dissect smooth vascular muscle cell expression. Regarding other aspects of metabolic syndrome, some authors showed protective role of Nr4a1 in atherosclerosis development (Hu *et al.* 2014). Nr4a1 is also capable of reducing hepatic cholesterol based on lipid overloading, and this may be due to the decrease in LDLR and HMG-CoA reductase (HMGCR) levels (Zhang *et al.* 2012). The enriched GO-terms for *Nr4a1* showed an involvement of *Nr4a1* in steroid receptor signaling, regulation of type B pancreatic cell proliferation, and fat cell differentiation. These processes are important for metabolic syndrome development; however, their significance for left ventricle myocardium is unknown.

Per1 is a key component of circadian loop, displaying regulatory effects on renal epithelial sodium channel gene expression (Gumz *et al.* 2012, Gumz *et al.* 2009). Lower levels of the *Per1* mRNA were associated with attenuated sodium channel expression, increased

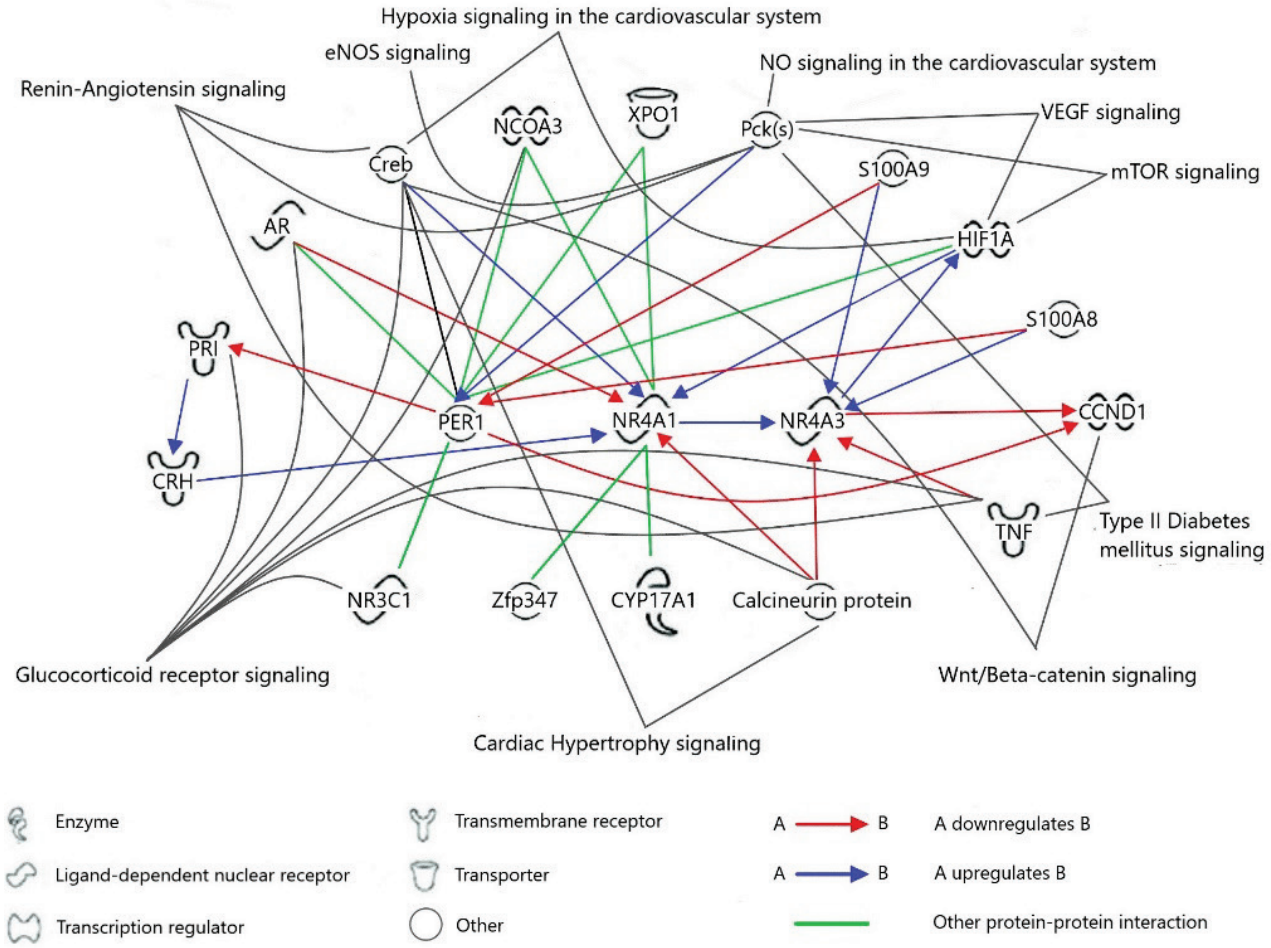

Fig. 3. The highest-score network derived using the set of transcripts verified by qPCR derived using Ingenuity Pathway Analysis, and showing significant STRAIN (PD5 * SHR) interaction.

sodium excretion, and low blood pressure (Richards *et al.* 2013). Interestingly, *Per1* knockout mice develop hypertension when given a long-lasting mineralocorticoid and a high salt diet, while wild type mice do not, suggesting that *Per1* gene expression may also be involved in sodium appetite and central control of blood pressure (Solocinski *et al.* 2017). However, apart from reports showing expression of *Per1* in the heart (Bonaconsa *et al.* 2014, Richards *et al.* 2014) its cardiac function has not been dissected.

Kcna5 gene encodes the Kv1.5 channel crucial for carrying the ultra-rapid potassium current (IKur) (Christophersen *et al.* 2013). Since the importance of normal electrophysiological environment for myocardium, any alterations within the biophysical properties of IKur are associated with higher incidence of large-scale and multi-faceted electrical and structural remodeling (Workman *et al.* 2001, González de la Fuente *et al.* 2012), thus leading to atrial fibrillation (Christophersen *et al.* 2013, Caballero *et al.* 2010) and other potentially life threatening dysrhythmias, increasing the risk of heart failure development and sudden cardiac arrest (Tomaselli *et al.* 1994). There was no indication of arrhythmias during the telemetric blood pressure recording in PD5 and SHR (Liška *et al.* 2014, unpublished data). Whether the channel upregulation in SHR can contribute to fibrosis and hypertrophy without causing arrhythmias is unknown.

To our knowledge, no direct connection among our differentially expressed genes and the genes present within the congenic segment has been reported so far, especially *Zbtb16*, which is apparently the quantitative trait gene (Liška *et al.* 2017). This may lead to the assumption that the observed differences in expression might be a secondary effect of the phenotype. However, the rats in our experiment were just two months old and the phenotypic differences in the heart weights between the groups were not yet expressed to the extent to which they normally are in older rats – therefore we suggest that the difference in expression could be a primary effect,

although there is no evidence of *Zbtb16* (*Plzf*) causation. Interestingly, *Plzf* and *Nr4a1* have been shown to be part of mutual exclusive pathways responsible for T-cell maturing process (O´Hagan *et al.* 2015) or *Per1* (Leigh *et al.* 2016). The relation of *Zbtb16* and metabolic syndrome was recently reviewed (Seda *et al.* 2017).

In conclusion, using microarray approach following qPCR verification we revealed several differentially expressed genes in the heart of PD5 compared to SHR. *Nr4a1*, upregulated in PD5 in comparison to SHR both on RNA and protein level, may play a role in the differential cardiac phenotypes of PD5 and SHR, i.e. amelioration of cardiac fibrosis in PD5.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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References

BONACONSA M, MALPELI G, MONTARULI A, CARANDENTE F, GRASSI-ZUCCONI G, BENTIVOGLIO M: Differential modulation of clock gene expression in the suprachiasmatic nucleus, liver and heart of aged mice. *Exp Gerontol* **55**: 70-79, 2014.

BOYLE EA, LI YI, PRITCHARD JK: An expanded view of complex traits: from polygenic to omnigenic. *Cell* **169**: 1177-1186, 2017.

CABALLERO R, DE LA FUENTE MG, GOMEZ R, BARANA A, AMOROS I, DOLZ-GAITON P, OSUNA L, ALMENDRAL J, ATIENZA F, FERNANDEZ-AVILES F, PITA A, RODRIGUEZ-RODA J, PINTO A, TAMARGO J, DELPON E: In humans, chronic atrial fibrillation decreases the transient outward current and ultrarapid component of the delayed rectifier current differentially on each atria and increases the slow component of the delayed rectifier current in both. *J Am Coll Cardiol* **55**: 2346-2354, 2010.

CHAO LC, BENSINGER SJ, VILLANUEVA CJ, WROBLEWSKI K, TONTONOZ P: Inhibition of adipocyte differentiation by Nur77, Nurr1, and Nor1. *Mol Endocrinol* **22**: 2596-2608, 2008.

CHENG Z, VOLKERS M, DIN S, AVITABILE D, KHAN M, GUDE N, MOHSIN S, BO T, TRUFFA S, ALVAREZ R, MASON M, FISCHER KM, KONSTANDIN MH, ZHANG XK, HELLER BROWN J, SUSSMAN MA: Mitochondrial translocation of Nur77 mediates cardiomyocyte apoptosis. *Eur Heart J* **32**: 2179-2188, 2011.

CHRISTOPHERSEN IE, OLESEN MS, LIANG B, ANDERSEN MN, LARSEN AP, NIELSEN JB, HAUNSO S, OLESEN SP, TVEIT A, SVENDSEN JH, SCHMITT N: Genetic variation in KCNA5: impact on the atrial-specific potassium current IKur in patients with lone atrial fibrillation. *Eur Heart J* **34**: 1517-1525, 2013.

CUI M, CAI Z, CHU S, SUN Z, WANG X, HU L, YI J, SHEN L, HE B: Orphan nuclear receptor Nur77 inhibits angiotensin ii-induced vascular remodeling via downregulation of beta-catenin. *Hypertension* **67**: 153-162, 2016.

EDGAR R, DOMRACHEV M, LASH AE: Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res* **30**: 207-210, 2002.

GONZÁLEZ DE LA FUENTE M, BARANA A, GÓMEZ R, AMORÓS I, DOLZ-GAITÓN P, SACRISTÁN S, ATIENZA F, PITA Á, PINTO A, FERNÁNDEZ-AVILÉS F, CABALLERO R, TAMARGO J, DELPÓN E: Chronic atrial fibrillation up-regulates beta1-Adrenoceptors affecting repolarizing currents and action potential duration. *Cardiovasc Res* **97**: 379-388, 2013.

GUMZ ML, POPP MP, WINGO CS, CAIN BD: Early transcriptional effects of aldosterone in a mouse inner medullary collecting duct cell line. *Am J Physiol Renal Physiol* **285**: F664-F673, 2003.

GUMZ ML, STOW LR, LYNCH IJ, GREENLEE MM, RUDIN A, CAIN BD, WEAVER DR, WINGO CS: The circadian clock protein Period 1 regulates expression of the renal epithelial sodium channel in mice. *J Clin Invest* **119**: 2423-2434, 2009.

HAMERS AA, VOS M, RASSAM F, MARINKOVIC G, KURAKULA K, VAN GORP PJ, DE WINTHER MP, GIJBELS MJ, DE WAARD V, DE VRIES CJ: Bone marrow-specific deficiency of nuclear receptor Nur77 enhances atherosclerosis. *Circ Res* **110**: 428-438, 2012.

HAMET P, MERLO E, SEDA O, BROECKEL U, TREMBLAY J, KALDUNSKI M, GAUDET D, BOUCHARD G, DESLAURIERS B, GAGNON F, ANTONIOL G, PAUSOVA Z, LABUDA M, JOMPHE M, GOSSARD F, TREMBLAY G, KIROVA R, TONELLATO P, ORLOV SN, PINTOS J, PLATKO J, HUDSON TJ, RIOUX JD, KOTCHEN TA, COWLEY AW JR: Quantitative founder-effect analysis of French Canadian families identifies specific loci contributing to metabolic phenotypes of hypertension. *Am J Hum Genet* **76**: 815-832, 2005.

HU YW, ZHANG P, YANG JY, HUANG JL, MA X, LI SF, ZHAO JY, HU YR, WANG YC, GAO JJ, SHA YH, ZHENG L, WANG Q: Nur77 decreases atherosclerosis progression in apoE(-/-) mice fed a high-fat/high-cholesterol diet. *PLoS One* **9**: e87313, 2014.

HUANG DA W, SHERMAN BT, LEMPICKI RA: Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* **37**: 1-13, 2009.

HUANG DA W, SHERMAN BT, LEMPICKI RA: Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* **4**: 44-57, 2009.

KREN V, PRAVENEC M, LU S, KRENOVA D, WANG JM, WANG N, MERRIOUNS T, WONG A, ST LEZIN E, LAU D, SZPIRER C, SZPIRER J, KURTZ TW: Genetic isolation of a region of chromosome 8 that exerts major effects on blood pressure and cardiac mass in the spontaneously hypertensive rat. *J Clin Invest* **99**: 577-581, 1997.

KRUPKOVA M, LISKA F, SEDOVA L, KRENOVA D, KREN V, SEDA O: Pharmacogenomic analysis of retinoic-acid induced dyslipidemia in congenic rat model. *Lipids Health Dis* **13**: 172, 2014.

LEIGH R, MOSTAFA MM, KING EM, RIDER CF, SHAH S, DUMONCEAUX C, TRAVES SL, MCWHAE A, KOLISNIK T, KOOI C, SLATER DM, KELLY MM, BIEDA M, MILLER-LARSSON A, NEWTON R: An inhaled dose of budesonide induces genes involved in transcription and signaling in the human airways: enhancement of anti- and proinflammatory effector genes. *Pharmacol Res Perspect* **4**: e00243, 2016.

LISKA F, LANDA V, ZIDEK V, MLEJNEK P, SILHAVY J, SIMAKOVA M, STRNAD H, TRNOVSKA J, SKOP V, KAZDOVA L, STARKER CG, VOYTAS DF, IZSVAK Z, MANCINI M, SEDA O, KREN V, PRAVENEC M: Downregulation of Plzf gene ameliorates metabolic and cardiac traits in the spontaneously hypertensive rat. *Hypertension* **69**: 1084-1091, 2017.

LISKA F, MANCINI M, KRUPKOVA M, CHYLIKOVA B, KRENOVA D, SEDA O, SILHAVY J, MLEJNEK P, LANDA V, ZIDEK V, D'AMATI G, PRAVENEC M, KREN V: Plzf as a candidate gene predisposing the spontaneously hypertensive rat to hypertension, left ventricular hypertrophy, and interstitial fibrosis. *Am J Hypertens* **27**: 99-106, 2014.

LOOS RJ, KATZMARZYK PT, RAO DC, RICE T, LEON AS, SKINNER JS, WILMORE JH, RANKINEN T, BOUCHARD C, STUDY HF: Genome-wide linkage scan for the metabolic syndrome in the HERITAGE Family Study. *J Clin Endocrinol Metab* **88**: 5935-5943, 2003.

MAXWELL MA, MUSCAT GE: The NR4A subgroup: immediate early response genes with pleiotropic physiological roles. *Nucl Recept Signal* **4**: e002, 2006.

O'HAGAN KL, ZHAO J, PRYSHCHEP O, WANG CR, PHEE H: Pak2 Controls acquisition of NKT cell fate by regulating expression of the transcription factors PLZF and Egr2. *J Immunol* **195**: 5272-5284, 2015.

PERUSSE L, RANKINEN T, ZUBERI A, CHAGNON YC, WEISNAGEL SJ, ARGYROPOULOS G, WALTS B, SNYDER EE, BOUCHARD C: The human obesity gene map: the 2004 update. *Obes Res* **13**: 381-490, 2005.

PRAVENEC M, KREN V, LANDA V, MLEJNEK P, MUSILOVA A, SILHAVY J, SIMAKOVA M, ZIDEK V: Recent progress in the genetics of spontaneously hypertensive rats. *Physiol Res* **63** (Suppl 1): S1-S8, 2014.

RICHARDS J, CHENG KY, ALL S, SKOPIS G, JEFFERS L, LYNCH IJ, WINGO CS, GUMZ ML: A role for the circadian clock protein Per1 in the regulation of aldosterone levels and renal Na+ retention. *Am J Physiol Renal Physiol* **305**: F1697-F1704, 2013.

RICHARDS J, WELCH AK, BARILOVITS SJ, ALL S, CHENG KY, WINGO CS, CAIN BD, GUMZ ML: Tissue-specific and time-dependent regulation of the endothelin axis by the circadian clock protein Per1. *Life Sci* **118**: 255-262, 2014.

SEDA O, LISKA F, KRENOVA D, KAZDOVA L, SEDOVA L, ZIMA T, PENG J, PELINKOVA K, TREMBLAY J, HAMET P, KREN V: Dynamic genetic architecture of metabolic syndrome attributes in the rat. *Physiol Genomics* **21**: 243-252, 2005.

SEDA O, LISKA F, SEDOVA L, KAZDOVA L, KRENOVA D, KREN V: A 14-gene region of rat chromosome 8 in SHR-derived polydactylous congenic substrain affects muscle-specific insulin resistance, dyslipidaemia and visceral adiposity. *Folia Biol (Praha)* **51**: 53-61, 2005.

SEDA O, SEDOVA L, VCELAK J, VANKOVA M, LISKA F, BENDLOVA B: ZBTB16 and metabolic syndrome: a network perspective. *Physiol Res* **66** (Suppl 3): S357-S365, 2017.

SEDOVA L, KAZDOVA L, SEDA O, KRENOVA D, KREN V: Rat inbred PD/cub strain as a model of dyslipidemia and insulin resistance. *Folia Biol (Praha)* **46**: 99-106, 2000.

SOLOCINSKI K, HOLZWORTH M, WEN X, CHENG KY, LYNCH IJ, CAIN BD, WINGO CS, GUMZ ML: Desoxycorticosterone pivalate-salt treatment leads to non-dipping hypertension in Per1 knockout mice. *Acta Physiol (Oxf)* **220**: 72-82, 2017.

TOMASELLI GF, BEUCKELMANN DJ, CALKINS HG, BERGER RD, KESSLER PD, LAWRENCE JH, KASS D, FELDMAN AM, MARBAN E: Sudden cardiac death in heart failure. The role of abnormal repolarization. *Circulation* **90**: 2534-2539, 1994.

VUKELIC S, GRIENDLING KK: Angiotensin II, from vasoconstrictor to growth factor: a paradigm shift. *Circ Res* **114**: 754-757, 2014.

WANG RH, HE JP, SU ML, LUO J, XU M, DU XD, CHEN HZ, WANG WJ, WANG Y, ZHANG N, ZHAO BX, ZHAO WX, SHAN ZG, HAN J, CHANG C, WU Q: The orphan receptor TR3 participates in angiotensin II-induced cardiac hypertrophy by controlling mTOR signalling. *EMBO Mol Med* **5**: 137-148, 2013.

WORKMAN AJ, KANE KA, RANKIN AC: The contribution of ionic currents to changes in refractoriness of human atrial myocytes associated with chronic atrial fibrillation. *Cardiovasc Res* **52**: 226-235, 2001.

YOU X, GUO ZF, CHENG F, YI B, YANG F, LIU X, ZHU N, ZHAO X, YAN G, MA XL, SUN J: Transcriptional up-regulation of relaxin-3 by Nur77 attenuates beta-adrenergic agonist-induced apoptosis in cardiomyocytes. *J Biol Chem* **293**: 14001-14011, 2018.

ZHANG P, HU Y, YANG J, ZHENG L, WANG Q: The orphan nuclear receptor Nur77 regulates hepatic cholesterol metabolism through the suppression of LDLR and HMGCR expression. *Mol Med Rep* **5**: 1541-1547, 2012.