

Recent Progress in the Genetics of Spontaneously Hypertensive Rats

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Received June 13, 2013

Accepted July 24, 2013

Summary

The spontaneously hypertensive rat (SHR) is the most widely used animal model of essential hypertension and accompanying metabolic disturbances. Recent advances in sequencing of genomes of BN-Lx and SHR progenitors of the BXH/HXB recombinant inbred (RI) strains as well as accumulation of multiple data sets of intermediary phenotypes in the RI strains, including mRNA and microRNA abundance, quantitative metabolomics, proteomics, methylomics or histone modifications, will make it possible to systematically search for genetic variants involved in regulation of gene expression and in the etiology of complex pathophysiological traits. New advances in manipulation of the rat genome, including efficient transgenesis and gene targeting, will enable *in vivo* functional analyses of selected candidate genes to identify QTL at the molecular level or to provide insight into mechanisms whereby targeted genes affect pathophysiological traits in the SHR.

Key words

Spontaneously hypertensive rat • Recombinant inbred strains • Intermediary phenotypes • Transgenic • Gene targeting

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Introduction

Metabolic syndrome is a cluster of several risk factors for type 2 diabetes and cardiovascular disease, including obesity, hypertension, insulin resistance,

and dyslipidemia. These pathological conditions are determined multifactorially by many genes and their interactions with environmental effects. Genome wide association studies (GWAS) in humans, which are based on the “common variants – common diseases” hypothesis, identified only a minor proportion of the total heritability of complex traits so far (Manolio *et al.* 2009). Statistically significant variants (SNPs – single nucleotide polymorphisms) are typically associated with a miniscule phenotypic variability without meaningful clinical effects, for instance, with less than 1 mm Hg of blood pressure (Kurtz 2010). In addition, the associations of practically all significant variants with complex traits are based only on statistical evidence and most likely do not represent causal alleles, especially when they are often located within non-coding regions. Studies in animal models of human complex diseases can provide a useful alternative. Experiments with rat models can control for both genetic background and environmental effects as well as enable genetic manipulation of experimental animals. Although it cannot be expected that the individual predisposing genes themselves might be conserved between rats and humans, it is likely that the networks and pathways of genes leading to disease susceptibility will be conserved across species. Therefore, the identification of networks and pathways of genes underlying the cellular pathology of disease phenotypes in the rat could provide insight into the pathogenesis and treatment of the corresponding human diseases.

The spontaneously hypertensive rat (SHR) is the most widely used animal model of human essential hypertension and left ventricular hypertrophy and under

special environmental conditions (for instance, when fed a high-fructose or folate-deficient diets) also develops disturbances of lipid and glucose metabolism that are typical for metabolic syndrome (Pravenec *et al.* 1999, 2013, Pravenec and Kurtz 2010). Similar to humans, these hemodynamic and metabolic disturbances in the SHR are also determined multifactorially. To identify genetic determinants of such complex traits, we used a combination of linkage and correlation analyses with intermediary phenotypes in the BXH/HXB recombinant inbred (RI) strains and follow-up *in vivo* functional testing in SHR congenic and SHR transgenic or knockout lines.

Intermediary phenotype data sets in the BXH/HXB recombinant inbred strains

The BXH/HXB recombinant inbred (RI) strains were derived from reciprocal crosses between BN-*Lx* and SHR progenitors (Pravenec *et al.* 1989). For genetic dissection of complex pathophysiological traits in RI strains, it is possible to take the advantage of accumulated genotypes and intermediary phenotypes. Intermediate phenotypes have simpler genetic architectures and can be used for connecting variability at the DNA level with complex pathophysiological traits. For instance, the abundance of mRNA in tissues is a highly heritable trait (Petretto *et al.* 2006) and represents very useful intermediary phenotype since it is possible to identify *cis*- and *trans*-regulated expression quantitative trait loci (eQTL) as candidate genes for complex traits. The most promising candidate genes are those that are (1) located close to the peak of QTL linkages of pathophysiological traits, (2) whose expression is regulated in *cis*, and (3) that correlate with the pathophysiological complex traits (Morrissey *et al.* 2011). The availability of genome sequences of both progenitor strains, the SHR and BN-*Lx*, enables rapid screening for functional variants of such candidate genes (Attanur *et al.* 2010, Simonis *et al.* 2012). We used this approach to identify the first QTL at the molecular level in the SHR, including mutant *Ogn* (osteoglycin) (Petretto *et al.* 2008) and *Endog* (endonuclease G) (McDermott-Roe *et al.* 2011) genes that predispose to left ventricular hypertrophy, deletion variant of *Cd36* (fatty acid translocase) that is associated with metabolic disturbances and hypertension (Aitman *et al.* 1999, Pravenec *et al.* 2008) or *Ebi2* (Epstein-Barr virus induced gene 2) gene that is associated with an inflammatory gene network and its human ortholog with

predisposition to Type 1 diabetes (Heinig *et al.* 2010). Using similar approach, additional candidate genes have been identified, including genes associated with cardiac microvascular remodeling (Mancini *et al.* 2013), catecholamine synthesis and secretion (Jirout *et al.* 2010, Friese *et al.* 2013) or preference to alcohol (Tabakoff *et al.* 2009). In addition to *cis*-regulated eQTL, systems-level approaches combining radiotelemetry blood pressures and transcriptome data revealed conservation of *trans*-regulated genes in the rat and genetic determinants of blood pressure in humans (Langley *et al.* 2013).

Additional intermediary phenotypes including quantitative proteomics determined by the SILAC (Stable isotope labeling by amino acids in cell culture) method (Nielsen *et al.*, unpublished results), metabolomics (Le Ven *et al.* 2013), variability in microRNA abundance (Grunz *et al.* 2011), methylomics (Johnson *et al.* unpublished results), or histone modifications (Rintisch *et al.* 2011) are becoming available thanks to an international collaborative effort within the EURATRANS Integrated Project of the European Union (Abbott 2009). Analyses of these data sets will enable to identify networks of genes, transcripts, proteins, and metabolites that underlie pathophysiology of complex traits in the SHR.

New technologies for derivation of genetically modified SHR lines

Recently, new highly efficient techniques become available for derivation of transgenic or knockout rats for *in vivo* functional studies of candidate genes for QTL, for analyses of genes with unknown function or for derivation of new rat models of human diseases. These techniques include transposon-mediated transgenesis using microinjections of zygotes with mixes containing Sleeping Beauty constructs and mRNA of hyperactive SB100X transposase. The rate of transgenesis is 14-72 % and in most cases only a single insertion outside coding regions is detected (Ivics *et al.* 2014, Katter *et al.* 2013). Until recently, gene targeting was impossible in the rat due to the absence of embryonic stem cells. Currently, several new techniques are available for gene targeting that are not based on the use of embryonic stem cells. These techniques include ZFN (Zinc finger nuclease) method (Jacob *et al.* 2010), TALEN (Transcription activator-like effector nuclease) method (Qiu *et al.* 2013) or CRISPR/Cas (Clustered regularly interspaced short palindromic repeats/(CRISPR-associated (Cas)) method

(Wang *et al.* 2013). Recently, we derived multiple SHR transgenic lines using the Sleeping Beauty transposon constructs and mRNA of SB100X transposase, for instance lines expressing *Venus*, *Nrf2*, *Dbh*, *Pnmt* or *Endog* cDNA under the control of universal and tissue-specific promoters (Fig. 1A). In addition, we were able to obtain the first transgenic rat using the recombinase-mediated cassette exchange (RMCE) method when green fluorescent protein *Venus* has been exchanged for red fluorescent protein *Cherry* in a defined position on

chromosome 2 (Fig. 2) (Pravenec, Landa, Mátés, Izsvák, unpublished results). We have also derived SHR lines with disrupted *Tmem70* gene using ZFN technology (Pravenec, Landa, Houštěk *et al.*, unpublished results) or knocked out *Plzf* gene with the TALEN technique (Pravenec, Landa, Liška, Mátés, Izsvák, unpublished results) (Fig. 1B). Examples demonstrating usefulness of gene manipulation in the SHR for analysis of complex cardiometabolic traits are given below.

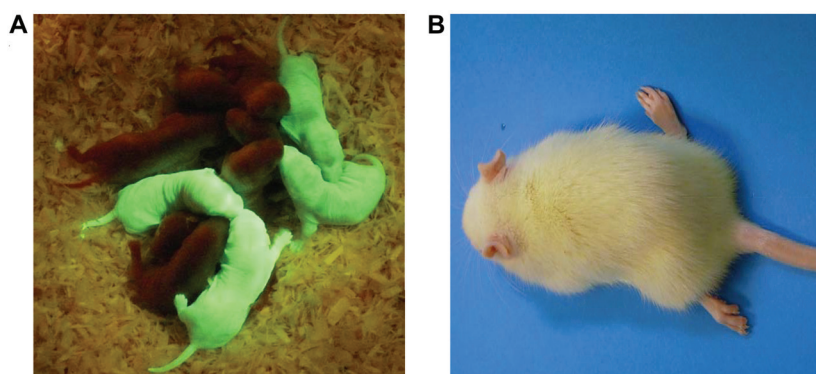


Fig. 1. Transposon-mediated transgenesis and TALEN method for gene targeting in the SHR. **A.** Two day old transgenic SHR rats with the *Venus* green fluorescent protein and their nontransgenic siblings. **B.** *Plzf* knockout SHR rat prepared by TALEN mediated mutagenesis with polydactyly.

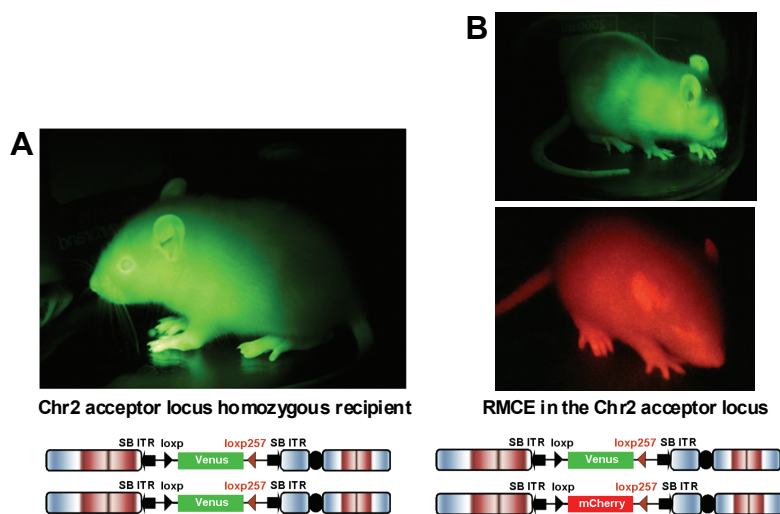


Fig. 2. Transposon-coupled RMCE in pronuclear microinjection in the SHR. **A.** SHR homozygous line for the acceptor locus on chromosome 2. **B.** Successful single allele RMCE on chromosome 2 in SHR rat born from zygotes microinjected with a mix containing *mCherry* plasmid and *Cre* mRNA.

Transgenic rescue experiments in the SHR

To obtain definitive evidence for the identity of a candidate gene with a QTL that is associated with complex trait such as blood pressure, it is necessary to perform *in vivo* functional tests. For candidate genes with downregulated expression of mRNA or protein or with mutated nonfunctional protein, transgenic rescue experiments can provide such evidence. The SHR harbors a deletion mutation of the *Cd36* gene that is associated

with clustering of several cardiovascular risk factors, including predisposition to hypertension (Aitman *et al.* 1999, Pravenec *et al.* 1999, 2001). The mutated *Cd36* protein is expressed, but its fatty acid translocase activity is significantly reduced (Hajri *et al.* 2001). In addition, renal expression of *Cd36* is regulated in *cis* and genome-wide quantitative trait transcript analysis in the BXH/HXB RI strains performed by searching for correlations between renal expression of *cis*-acting eQTLs and direct measurements of arterial pressure

revealed that *Cd36* showed the strongest correlation with diastolic blood pressure. The renal expression of *Cd36* correlated inversely with blood pressure and RI strains with the SHR *Cd36* allele had significantly higher blood pressure when compared to RI strains with the BN-Lx allele. To investigate whether mutant *Cd36* in the kidney might be sufficient to promote increased blood pressure, we carried out renal transplantation experiments using donor kidneys from either the SHR progenitor that lacks wild-type *Cd36* or from the SHR-TG19 transgenic strain with robust renal expression of wild-type *Cd36*. We found that blood pressure of recipients that received a donor kidney with mutant *Cd36* was significantly greater than the blood pressure of recipients that received a “transgenic” kidney expressing wild-type *Cd36* (Pravenec *et al.* 2008).

“Humanized” SHR-CRP transgenic rats – a new model for testing the role of C-reactive protein in the pathogenesis of metabolic syndrome

Inflammation has been implicated in the pathogenesis of obesity, metabolic disturbances, diabetes mellitus, and cardiovascular disease. C-reactive protein (CRP) is a well-known biomarker of inflammation associated with increased risk for cardiovascular disease and diabetes. Whether or not CRP is a mediator or just a marker of disease pathogenesis remains highly controversial. The recent results from the JUPITER trial showed that statin therapy reduces cardiovascular risk more in patients who achieved substantial reductions in both CRP and LDL-cholesterol than in those who achieved substantial reductions in LDL-cholesterol without substantial reductions in CRP levels (Ridker *et al.* 2008). However, epidemiological studies and randomized trials of statins in relationship to CRP and coronary heart disease (CHD) cannot provide the evidence for causal relationship because of confounding effects of other risk factors for CHD and/or reverse causality. In addition, negative results in transgenic mice expressing human CRP did not also support a direct role of CRP in atherosclerosis development. However, it has been demonstrated that the mouse may not be a suitable genetic animal model for studying the biologic effects of human CRP. CRP is not an acute phase reactant in mice and human CRP fails to activate mouse complement in the presence of endogenous CRP ligands such as modified forms of cholesterol. Contrary to mice, human

CRP activates rat complement. Therefore we decided to test the effects of transgenic expression of human CRP in the SHR. The expression of human CRP specifically in the liver was associated with inflammation and oxidative tissue damage, insulin resistance and increased blood pressure (Pravenec *et al.* 2011a). These findings are consistent with the hypothesis that increased CRP is more than just a marker of inflammation and can directly promote multiple features of the metabolic syndrome. The humanized CRP transgenic SHR represents a new model for investigating mechanisms whereby increased CRP levels may promote multiple components of the metabolic syndrome and could be further used to search for genetic factors that might influence susceptibility to the adverse metabolic effects of human CRP. This transgenic SHR model should also be of interest for testing the therapeutic effects of statins or novel CRP inhibitors and a variety of other drugs such as antioxidants, anti-inflammatory agents, etc. Recently, we treated the SHR-CRP transgenic rats with rosuvastatin and observed significantly reduced inflammation and oxidative stress which was associated with amelioration of insulin resistance and dyslipidemia (Šilhavý *et al.* 2012). These findings provide support for the important pleiotropic effects of statins that are beyond LDL cholesterol lowering effects (Hayward *et al.* 2012).

Autocrine effects of resistin in the pathogenesis of insulin resistance

The adipokine resistin has been originally identified as a possible link between obesity and insulin resistance (Steppan *et al.* 2001). To analyze mechanisms of prodiabetic effects of resistin, we derived transgenic SHR expressing mouse resistin (*Retn*) cDNA under the control of aP2 promoter. Transgenic mouse resistin is expressed specifically in adipose tissue but is not secreted into circulation (Pravenec *et al.* 2003). Transgenic SHR-*Retn* strain thus represents a unique model to analyze autocrine effects of resistin. SHR-*Retn* transgenic rats showed moderate expression of the resistin transgene in adipose tissue but had serum resistin levels similar to control SHR and undetectable levels of transgenic resistin in the circulation. Older transgenic rats displayed marked glucose intolerance in association with a near total resistance of adipose tissue to insulin-stimulated glucose incorporation into lipids. These results suggest that with increasing age, the autocrine effects of resistin in fat tissue may predispose to diabetes in part by impairing

insulin action in adipose tissue (Pravenec *et al.* 2011b). Recently, brown adipose tissue (BAT) has been suggested to play an important role in the pathogenesis of metabolic disturbances by its ability to dissipate energy excess (Bartelt *et al.* 2011). Accordingly, we analyzed autocrine effects of transgenic resistin on BAT glucose and lipid metabolism in the SHR-*Retn* versus nontransgenic SHR controls. We observed that interscapular BAT isolated from SHR-*Retn* transgenic rats when compared to SHR controls showed lower relative weight, significantly reduced both basal and insulin-stimulated incorporation of palmitate into BAT lipids, and significantly decreased palmitate oxidation and glucose oxidation. These results provide compelling evidence that autocrine effects of resistin in BAT might play an important role in the pathogenesis of insulin resistance in the rat (unpublished results).

Conclusions

During several past years, major advances have been made in the genetic analysis of the SHR. The

availability of genome sequences of BN-*Lx* and SHR progenitors of RI strains, the accumulation of genome-wide intermediary phenotypes, as well as development of new statistical tools, enabled system-level approach for identification of genetic determinants of complex traits at the molecular level. In addition, recent advances in manipulation of the rat genome provide new tools for studying mechanisms whereby genes regulate complex pathophysiological traits.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This work was supported by grants LL1204 (within the ERC CZ program), LH12061, and LH11049 from the Ministry of Education, Youth and Sports of the Czech Republic, grants P303/10/0505, P301/10/0290, P301/12/0696, and 13-04420S from the Czech Science Foundation, grant NT/14325 from the Ministry of Health of the Czech Republic, and grant TA02010013 from the Technological Agency of the Czech Republic.

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