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A single nucleotide polymorphism of *Spp2* confers sex-specific effects on blood pressure and bone health

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Hypertension is a complex polygenic disease caused by a combination of genetic and environmental factors. Rat models serve as tools to dissect and prioritize genetic factors as candidate genes causing hypertension[1]. One such candidate gene prioritized through systematic linkage and substitution mapping is Secreted Phosphoprotein 2 (*Spp2*) [2]. A non-synonymous G/T single nucleotide polymorphism (SNP) between the Dahl Salt-Sensitive (S) rats and Spontaneously Hypertensive Rats (SHR) at the *Spp2* locus was prioritized as a candidate quantitative trait nucleotide responsible for the reduction in blood pressure (BP) and bone mass observed in the S.SHR congenic strain spanning the *Spp2* locus[2]. Hence, we hypothesized that CRISPR/Cas9 precision-engineering-guided replacement of the G allele at the *Spp2* locus with a T allele would lower BP and bone mass of the S rat.

Methods

Animals

All animal procedures and protocols performed in this study were approved by the University of Toledo Institutional Animal Care and Use Committee. The procedure for the generation of a CRISPR/Cas9 targeted knock-in rat model has been previously described [3]. The non-founder S rat is a control derived from generating the knock-in. The nonfounder and the *Spp2* knock-in rat strains were from stocks maintained at the University of Toledo animal facility. nonfounder and knock-in were not litter mates but were concomitantly bred for experimental procedures. Rats were weaned at 28 to 30 days of age

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Disclosures
None

and fed a low-salt diet (0.3% NaCl, TD 7034, Harlan Teklad). High-salt diet (2% NaCl, TD 94217, Harlan Teklad) was started at 6 weeks of age for all experiments.

BP Measurement

BP was recorded and analyzed using equipment from Data Sciences International, as previously described[4].

Microcomputed Tomography analysis

Trabecular bone architecture was evaluated in proximal tibia by assessing total volume (TV), bone volume (BV), BV to TV ratio, connectivity, Structure Model Index, trabecular number, trabecular thickness and trabecular spacing using micro CT. Image acquisition and analysis were conducted using μCT 35 system and Evaluation Program V6.5–3 (Scanco Medical AG, Bruettisellen, Switzerland) according to recommended guidelines[5].

Results and Discussion

Using CRISPR/Cas9 precision-engineering technology, a novel knock-in rat strain was generated, whereby the G nucleotide of the S rat was replaced with the T nucleotide of the SHR rat at the location, Rnor 6.0, Chr9:95506318 (Figure [A]). This non-synonymous SNP between the S and SHR rat is located in exon 4 of the *Spp2* locus. The G/T polymorphism translates to an amino acid change from Alanine in the S rat to Serine in the Spp2 knock-in rat at amino acid 125 of Spp2 (Figure [A]). Radiotelemetry was performed with *Spp2* knock-in rats using S rat as control. Systolic BP of the *Spp2* knock-in male rats was significantly lower, 192 ± 4 mmHg, compared with that of the S rats, 211 ± 7 mmHg, P=0.026 (Figure [B] left). However, there was no change in systolic BP of the *Spp2* knock-in female rats, 190 ± 5 mmHg, compared with S rats,183 ± 4 mmHg, P=0.268 (Figure [B] right). These data indicate that the T to G non-synonymous SNP in the *Spp2* locus exhibits sexual dimorphism because it lowers systolic BP only in male rats.

We performed micro CT analysis to observe differences in bone microarchitecture and bone mass that was previously shown in the male rat of the S.SHR congenic strain spanning the *Spp2* locus [2]. Comparison of trabecular bone between nonfounder and knock-in males showed that *Spp2* knock-in had zero effect on any of the structural parameters resulting in no effect in BV to TV ratio, P=0.657, (Figure [C]). There was also no observable structural difference between nonfounder and knock-in (Figure [D]). Analysis of trabecular bone in nonfounder and knock-in females showed that knock-in was associated with significant reduction of bone size and bone mass resulting in lower BV to TV ratio, P=0.033, (Figure [E]) stemming from lower trabecular thickness and increased trabecular spacing which is seen in (Figure [F]). Moreover, increase in structure model index in knock-in females indicated that knock-in results in a shift from plate- to rod-like trabeculae that is linked to lower bone quality.

Trabecular bone structure in nonfounder males and females differ significantly that sets “background” bone quality at significantly different start points with regard to the downstream effect of the knock-in. In females, TV is smaller by 40% but bone mass is 60% higher resulting in 3-fold higher BV to TV ratio, as compared with males. Higher bone mass

in females results from higher trabecular thickness and reduced trabecular spacing. Moreover, female bone shows 5-fold higher connectivity and significantly lower structure model index indicating higher number of plates versus rods. This means that trabecular bone in females, although smaller in size, is superior with regard to the microarchitecture, and in consequence presenting higher bone quality. These differences suggest that mechanisms regulating bone growth and structure are more robust in nonfounder females, and possibly more sensitive to the effect of *Spp2* knock-in in this particular rat model.

A discrepancy between the current null effect of the Knock-In on bone health in males and dramatic effect previously observed with male congenic rats suggests that the congenic segment around the *Spp2* locus harbors other polymorphisms which operate in males to regulate bone physiology. A potential limitation of our study is a lack of temporal analysis, whereby, it is not clear whether the effects on bone precede the BP effect or vice versa. Taken together these data provide conclusive evidence for a SNP within the *Spp2* gene as a quantitative trait nucleotide (QTN) responsible for the sex-dependent inheritance of BP and bone health.

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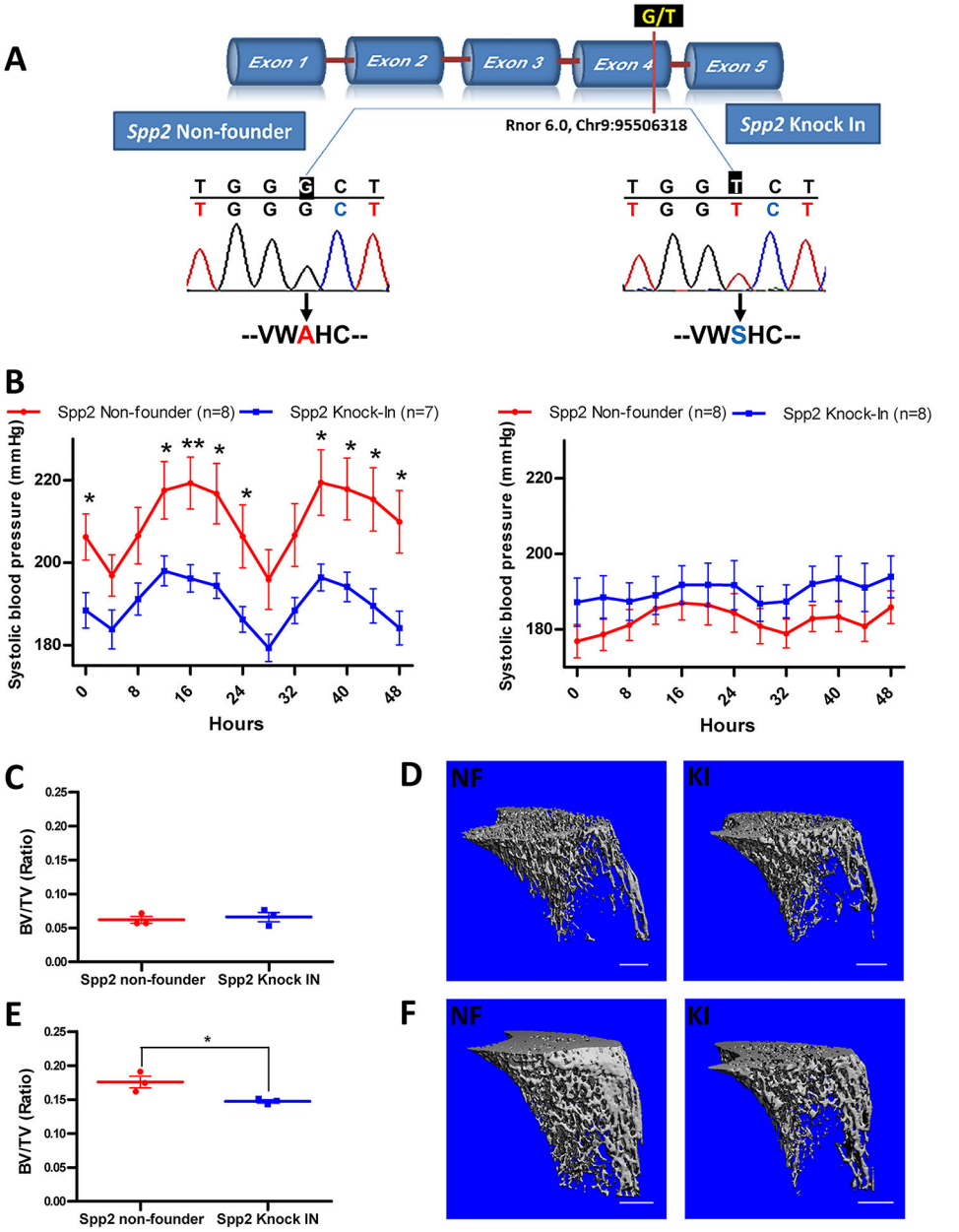

Single nucleotide polymorphism in *Spp2* leads to lower systolic blood pressure in male and lower bone volume in female rats. A) DNA sequencing results from the PCR products for both *Spp12* non-founder and knock-in at the location Chr9:95506318 (Rnor 6.0) within exon 4 of *Spp2*. B) Radiotelemetry systolic BP measurements of *Spp2* non-founder (N=8) vs. *Spp2* knock-in (N=7) males on the left and *Spp2* non-founder (N=8) vs. *Spp2* knock-in (N=8) females on the right. *Spp2* non-founder- red and *Spp2* knock-in- blue. **P*<0.05, ***P*<0.01. Proximal tibia trabecular bone BV/TV from *Spp2* non-founder vs. *Spp2* knock-in (N=3) in males (C) and females (E) *P< 0.05. 3D renderings of trabecular bone in proximal tibia from *Spp2* non-founder and knock-in (N=3) in males (D) and females (F). Bar on micro CT renderings represents 1 mm.