Reduced blood pressure after smooth muscle EFNB2 deletion and the potential association of EFNB2 mutation with human hypertension risk

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Running title: EFNB2 deletion results in lower blood pressure

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Abstract

Ephrin B2 (EFNB2) is a ligand for erythropoietin-producing hepatocellular kinases (EPH), the largest family of receptor tyrosine kinases. It has critical functions in many biological systems, but is not known to regulate blood pressure. We generated mice with a smooth muscle cell-specific deletion of EFNB2 and investigated its roles in blood pressure regulation and vascular smooth muscle cell (VSMC) contractility. Male *Efnb2* knockout (KO) mice presented reduced blood pressure, while female KO mice had no such reduction. Both forward signaling from EFNB2 to EPHs and reverse signaling from EPHs to EFNB2 were involved in regulating VSMC contractility, with EPHB4 serving as a critical molecule for forward signaling, based on crosslinking studies. We also found that a region from aa 313 to aa 331 in the intracellular tail of EFNB2 was essential for reverse signaling regulating VSMC contractility, based on deletion mutation studies. In a human genetic study, we identified 5 SNPs in the 3' region of the *EFNB2* gene which were in linkage disequilibrium and which were significantly associated with hypertension for male but not female subjects, consistent with our findings in mice. The coding (minor) alleles of these 5 SNPs were protective in males. We have thus discovered a previously unknown blood pressure-lowering mechanism mediated by EFNB2 and identified *EFNB2* as a gene associated with hypertension risk in humans.

Key words: ephrin B2, blood pressure, vascular smooth muscle cells, sex hormone, single nucleotide polymorphism

Introduction

Erythropoietin-producing hepatocellular kinases (EPH) are the largest family of receptor tyrosine kinases. Based on sequence homology, they are divided into A and B subfamilies[1]. Their ligands, called ephrins (EFNs), are also cell surface molecules[2]. EFNs are also divided into A and B subfamilies, based upon the way they anchor on the cell surface: the A subfamily anchors on the cell surface through glycophosphatidylinositol, and the B subfamily, through a transmembrane domain[2]. The interactions between EPH kinases and EFNs are promiscuous, but EPHA kinases preferably interact with EFNA ligands, and EPHB kinases with EFNB ligands, which have 3 members, EFNB1, EFNB2 and EFNB3[2]. Although EPH members and EFN members share homology with their respective members, each member has its distinct function in different cellular processes[3-7]. In general, the EPH kinases interact with their EFN ligands on neighboring cells, because EPHs and EFNs are all cell surface molecules[2]. These molecules could be cleaved from the cell surface by enzymes such as ADAM10[8,9], an unspecified matrix metalloproteinase[10], or γ-secretase[11]; therefore, it is possible that the shed soluble fragments of EPH and EFN might be able to influence cells and tissues at a distance by blocking the interaction of EPHs and EFNs there.

EPH kinases and EFNs are expressed in a wide range of tissues and cells, and play vital roles in the development and function of different organs and systems[3,4,12-22]. They are also vital in many biological processes[5,6,23,24]; however, until our recent publications, there were no studies investigating the roles of these molecules in blood pressure (BP) regulation.

Recently, we reported novel observations that deletion of EFNB1 and its receptor EPHB6 leads to

increased BP[25,26]. In an additional study, we demonstrated that EPHB4 deletion leads to reduced BP[27]. As EFNB2 is the preferred ligand of EPHB4, we thus asked the question whether its deletion in vascular smooth muscle cells (VSMCs) will have a similar BP reduction effect. The results of our investigation are reported here.

Materials and Methods

Generation of smooth muscle cell-specific Efnb2 and Ephb4 gene knock-out (KO) mice We have previously reported on the generation of *Efnb2* floxed mice[14]. They were backcrossed with C57BL/6J for 10 generations and then mated with smooth muscle myosin heavy chain promoter-driven *Cre* transgenic mice (smMHC-Cre-IRES-eGFP) in the C57BL/6J background[12] to obtain smooth muscle cell-specific *Efnb2* gene KO mice. The ages of the KO and WT mice for the *in vivo* study were described in given experiments. Cells from mice at 3 to 6 months of age were used for *in vitro* studies.

In some experiments, VSMCs from smooth muscle-specific *Ephb4* gene KO mice were used. The generation and characterization of these mice are described in our recent publication[27].

Reverse transcription/quantitative-PCR (RT/qPCR), immunofluorescence microscopy, BP measurement by radiotelemetry, VSMC isolation, measurement of VSMC contractility, Ca^{++} flux measurement, lentivirus preparation and infection These methods and primers (S. Table 1) used are described in Supplementary Materials.

Human genetic Study The patient cohort consisted of 3409 European, Australian, Canadian and

New Zealander Caucasians (S. Table 2) who had been ascertained to be suffering from type 2 diabetes and at high risk for macrovascular or microvascular diabetes complications and who were recruited for the *ADVANCE* (Action in Diabetes and Vascular Disease: Peterax and Diamicron-MR Controlled Evaluation) clinical study[28,29]. Patients were divided into male and female groups and genetic association studies for a hypertension phenotype were performed for male and female groups separately. A detailed explanation of the methodology of the association studies is presented in the supplementary materials.

Results

Smooth muscle cell-specific deletion of EFNB2 in mice The floxed *Efnb2* mice[14] in the C57BL/6J background were crossed with transgenic mice expressing smooth muscle myosin heavy chain promoter-driven Cre recombinase (also in the C57BL/6J background[12] to achieve smooth muscle cell-specific deletion of EFNB2. The deletion of *Efnb2* at the mRNA level in vascular smooth muscles, but not in the spleen, heart, brain or liver, was confirmed by RT-qPCR (Fig. 1A and Supplementary Figure 1 (S-Fig. 1)). The deletion EFNB2 at the protein level in VSMCs was further confirmed by immunofluorescence (Fig. 1B) and immunoblotting (Fig. 1C). These mice with smooth muscle cell-specific deletion of EFNB2 were called KO mice. There was no compensative upregulation of other EPHB and EFNB subfamily members in VSMCs after EFNB2 deletion (S. Fig. 2). The small artery structure of the KO mice was comparable to that of the wild type (WT) mice in terms of media thickness and lumen sizes (S. Fig. 3). The KO and WT VSMCs had similar proliferative rates *in vitro* culture (S. Fig. 4), and had similar levels of α-actin expression, a smooth muscle cell marker (S. Fig. 5). Although EFNB2 deletion was achieved by smooth muscle myosin heavy chain promoter-driven Cre, and is not specific to SMC

in the vascular system, we did not notice gross morphological and functional abnormalities in the digestive system, respiratory system and reproductive system in the KO mice.

BP phenotype of EFNB2 KO mice The BP of KO and WT mice was recorded every 2 min for a period of 24 h. All the time points were analyzed with a mixed-effects linear model, using genotype, individual mouse, sex and time as qualitative factors, and the mean + SEM of the values are presented in Figure 2. Male EFNB2 KO mice presented reduced BP [systolic pressure (SP), diastolic pressure (DP) and mean arterial pressure (MAP)] in general (the values of the WT parameters minus that of the KO were positive in Fig. 2C), compared to their WT counterparts, with SP and MAP differences (WT vs KO) reaching significance (Figs. 2A). Female KO mice, on the other hand, presented slightly increased BP parameters (the values of the difference were negative in Fig. 2C) compared to their WT counterparts, with DP difference reaching significance (Figs. 2B). The disparity (means + SEM of WT values minus KO values) of BP parameters between WT and KO mice are shown in Fig. 2C. The males and females were significantly different for the disparity of DP and MAP (Fig. 2C). The heart rates (HR) between WT and KO mice were not significantly different, and there was no sex differences (Fig. 2D).

Both forward and reverse signaling between EFNB2 and its receptors are responsible for regulating VSMC contractility EFNB2 and its receptors (mainly EPHB subfamily kinases) are all expressed in VSMCs (S. Fig. 2)[25], providing a molecular basis for their bi-directional signaling, *i.e.* from EFNB2 to EPHBs: forward signaling; from EPHBs to EFNB2: reverse signaling. We investigated which direction was functional in regulating VSMC contractility. We demonstrated that when WT VSMCs were cultured in wells coated with recombinant EFNB2-Fc tagged with human IgG Fc (EFNB2-Fc), which could crosslink EPHBs and initiate forward signaling, they

manifested significantly augmented contractility upon phenylephrine stimulation, compared to the VSMCs cultured on wells coated with normal human IgG, which was employed as a control (Fig. 3A). The augmentation could be neutralized by soluble EPHB4-Fc, which is a preferred receptor of EFNB2, suggesting that among different EPHB subfamily members, EPHB4 largely mediates forward signaling to achieve increased VSMC contractility. To further prove the essential role of EPHB4 in forward signaling and VSMC contractility, we cultured VSMCs from smooth muscle cell-specific EPHB4 KO mice[27] on EFNB2-Fc coated wells. As shown in Figure 3B, solid-phase EFNB2-Fc could no longer enhance contractility of EPHB4-deleted VSMCs, again indicating that solid phase EFNB2's effect on VSMC contractility is largely via EPHB4. To assess whether reverse signaling from EPHBs to EFNB2 also played a role in VSMC contractility, we cultured WT VSMCs on wells coated with anti-EFNB2 Ab to crosslink EFNB2, mimicking the EPHB binding. Such crosslinking could significantly increase VSMC contractility upon phenylephrine-stimulation (Fig. 3C). Moreover, the augmentation could be neutralized by soluble EFNB2-Fc, confirming the specificity of anti-EFNB2 Ab. Thus, we have proven that both forward signaling from EFNB2 to EPHB4 and reverse signaling from EPHBs to EFNB2 could enhance VSMC contractility.

To identify the EFNB2 intracellular sequence critical for controlling the VSMC contractility during reverse signaling EFNB2 has a short intracellular tail (73 aa), which has no enzymatic activity. EFNB2 reverse signaling depends on the association of its intracellular tail with adaptor proteins, which in turn link to various signaling pathways. We conducted step-wise deletion of the EFNB2 intracellular tail from the C-terminus (illustrated in Fig. 4A) and used lentiviruses to overexpress these deletion mutants in VSMCs from mice with smooth muscle cell-specific EFNB2 deletion. The infected VSMCs were then cultured in wells coated with anti-EFNB2 Ab.

In this system, the absence of endogenous EFNB2 in VSMCs reduced constitutive reverse signaling from EPHB to EFNB2 occurring among neighboring cells, hence reducing the background noise. The overexpression of the exogenous full-length EFNB2 and its deletion mutants were confirmed by RT-qPCR (Fig. 4B). The titers of the lentivirus were adjusted such that the deletion mutant mRNA expression was at least the same or less than 15% higher than the full-length *Efnb2* mRNA. Overexpression of full-length EFNB2 (336 aa long with 73 aa in the intracellular tail) in EFNB2 KO VSMCs significantly augmented their contractility upon PE-stimulation (Fig. 4C). The deletion of the last 5 aa in EFNB2 intracellular C-terminus (EFNB2-Δ2Y), which removed the PDZ domain-binding motif plus the last two of the total 5 conserved tyrosine residues (Y333 and Y334), did not reduce VSMC contractility compared to the full-length EFNB2 (Fig. 4D). However, an additional deletion of 19 aa, which contains two additional tyrosine residues at Y314 and Y319 (EFNB2-Δ4Y), caused a drastic drop of VSMC contractility, which was reduced to the level of control virus-infected VSMCs (Fig. 4E). Further deletion of 7 aa, which contains the 5th conserved tyrosine residue Y307 (EFNB2-Δ5Y), did not cause more changes compared to EFNB2-Δ4Y (Figure 4F). These data suggest that the EFNB2 intracellular sequence between aa 313 and aa 331 containing Y314 and Y319 harbors a critical element(s) that could associate with other signaling proteins and regulate VSMC contractility.

Contractility-related signaling events in EFNB2 KO VSMCs We assessed several key signaling events in EFNB2 KO VSMC to further understand the cause of these cells' reduced contractility. VSMCs from male and female KO mice showed mRNA levels of α1-adrenoreceptor expression comparable to those from WT counterparts (S. Fig. 6A). VSMCs from male KO mice had comparable phenylephrine-stimulated Ca^{2+} influx to VSMCs from WT mice (S. Fig. 6B). Crosslinking EFNB2 with solid-phase anti-EFNB2 Ab, which enhanced WT VSMC contractility,

did not change phenylephrine-triggered Ca^{2+} flux in VSMCs from male mice (S. Fig. 6C). This indicates that EFNB2 does not affect Ca^{2+} flux in VSMCs.

The reduced contractility could then be caused by the sensitivity of KO VSMCs to Ca^{2+} flux. We investigated this possibility by examining KO VSMC myosin light chain phosphorylation, which is known to increase the Ca^{2+} sensitivity. Indeed, when stimulated with phenylephrine, VSMCs from male but not female KO mice manifested significantly lower myosin light chain (MLC) phosphorylation at ser19 (S. Fig. 6D), consistent with reduced BP in male but not female KO mice (Figs 2A and 2B). Myosin light chain is dephosphorylated by myosin phosphatase. Phosphorylation of myosin phosphatase target protein-1 (thr696), a process that decreases myosin light chain phosphatase activity[30], was not influenced by EFNB2 deletion (S. Fig. 6E). ERK activation can enhance smooth muscle activation through several proposed modes of action: 1) it might activate MLC kinase through a so-far un-delineated pathway[31,32] or 2) it might phosphorylate the actin-binding protein, caldesmon, which removes caldesmon's inhibitory effect on smooth muscle contraction[33] . In both male and female KO VSMCs, ERK phosphorylation, a surrogate marker of ERK activation, was significantly reduced upon phenylephrine stimulation (S. Fig. 6F), implicating ERK as a link in EFNB2 deletion-caused hypocontractility of VSMCs. The lack of sex difference in ERK phosphorylation suggests that although it might contribute to reduce VSMC contractility in KO mice, it is not responsible for the observed sex difference.

Association of EFNB2 with hypertension in subjects with diabetes We tested the association of 55 DNA markers falling within the *EFNB2* gene or within additional approximately 10 kb 5' and 10 kb 3' of the gene (located within a 58.72-kb region between positions 107134861 and

107,193,584 on chromosome 13 (Build 37/hg19) with hypertension in a type 2 diabetes patient cohort; males and females were tested separately. Five SNPs were found to be significantly associated with hypertension for the male-only samples (Table 1), using a *Bonferroni*-corrected critical *p*-value of 3.33×10^{-3} for 15 independent tests, corresponding to 15 independent ($r^2 \leq 0.80$) LD blocks within the region. The locations of the 5 significant SNPs (encircled) with respect to the LD structure around the 3' end of the *EFNB2* gene are shown in S. Figure 7A. These SNPs fall just downstream (within 1 kb) of the 3' untranslated region (UTR) of the gene. The 5 SNPs, with the most significant SNP, rs7328882, as a representative, whose allele frequencies are depicted in S. Figure 7B, are all in high LD ($r^2 \geq 0.80$), and so are likely to all be proxy to one underlying functional polymorphism located within the 3' region of the gene or just 3' downstream of the gene.

The coding (minor) allele of these 5 significantly associated SNPs in male subjects was protective, as their odds ratios were below 1.0, and the 95% confidence intervals of the odds ratios did not include 1.0 (Table 1). When chronic kidney disease (CKD), defined as estimated glomerular filtration rate < 60 ml/min/1.73 m^2, was used as a covariate in the logistic association model, the *p*-values were slightly increased, although the general pattern of association remained the same; however, only rs7328882, which was the most significant SNP for the model without CKD as a covariate, remained significant at the *Bonferroni*-corrected critical *p*-value (Table 1). No SNPs were nominally significant for the female-only samples in either the case of inclusion or exclusion of CKD as a covariate (Table 1). The confidence intervals of odds ratios of SNPs for the females-only samples all included 1.0. These confidence intervals were wider (~0.45) for females than for the male samples (~0.25), likely reflecting lower statistical power due to a

smaller female sample size. The differences in odds ratios between male and female subjects calculated using the Altman-Bland test[34] for SNP, rs7328882, was not significant ($p = 7.98x10^{-2}$ ($<1x10^{-2}$ but $>5x10^{-2}$), most likely due to lack of power in the female samples.

Discussion

BP is a vital physiological parameter and is highly regulated with multiple compensatory mechanisms including blood volume, cardiac output and/or vascular tone regulation that maintain it within a normal range. EFNB2 deletion in VSMCs likely leads to a default phenotype of reduced VSMC contractility, and consequently reduced BP. This phenotype is pronounced in males, with SP and MAP reaching statistical significance. However, in female KO mice, BP parameters (SP, DP and MAP) were not reduced, with DP being significantly increased. This suggests that one or more of the above-mentioned compensation mechanisms is at work, and sex/sex hormone might be an additional modifier which could even push the final outcome to a different direction. The nature of the sex/sex hormone-dependent modulation of BP in the absence of EFNB2 remains to be investigated. Regardless, the sex difference in the BP phenotype of the KO mice is true and significant, according to analysis with a mixed-effects linear model (Fig. 2C).

Nakayama et al. reported that smooth muscle-specific deletion of EFNB2 using SM22Δ-Cre results in lower body weight, reduced VSMC proliferation, thinner arterial vessel wall and enlarged arterial diameter[35] . However, none of these phenotypes was observed in our KO mice, in which EFNB2 was deleted using smMHC-Cre. There could be several possible explanations for these discrepancies. 1) The mouse genetic background might heavily influence these

phenotypes: Nakayama's mice are in a 129sv X C57BL/6J mixed background, while ours are in a pure C57BL/6J background. 2) Different smooth muscle-specific promoters might result in different degrees of EFNB2 deletion in target organs/tissues and of leaky deletion in untargeted organs/tissues. 3) Cell proliferation was assessed at different time points (postnatal day 8 in Nakayama's study versus adult in our study). While Nakayama's study focuses on EFNB2's role in PGDFRβ signaling in VSMCs related to their proliferation, we are focusing on VSMCs' functionality and their signaling during contractile stimulation. Naturally, these two aspects have different time windows. Nevertheless, these two studies agree that EFNB2 plays a critical role in VSMC biology, which has not been previously appreciated.

EFNB2 can transduce signals in both forward and reverse directions. We have demonstrated that both directions are involved in VSMC contractility. EPHB4 is the preferred EPH, with which EFNB2 could associate. We have proved *in vitro* that only WT, but not EPHB4 KO VSMCs, could respond to EFNB2 stimulation and augment contractility (Fig. 3B), indicating that the predominant effect of EFNB2 forward signaling with regard to enhancing VSMC contractility was mediated by EPHB4. If this is the case, then smooth muscle cell-specific deletion of EPHB4 should have a similar BP phenotype to that seen in the EFNB2 KO mice. Indeed, our recent report reveals that EPHB4 KO mice present sex-dependent hypotension, with male KO being hypotensive while female KO being normotensive[27], corroborating our results from EFNB2 KO mice.

EFNB2 has a short intracellular tail (73 aa), which has no enzymatic activity. Its reverse signaling depends on adaptor proteins associating with the tail. EFNB2 intracellular tails are characterized by 2 major features: the C-terminal PDZ domain-binding motif and 5 conserved tyrosine

residues, which could be associated with PDZ-domain-containing proteins (*e.g.,* PDZ-RGS3, GRIP1/2, TIAM1 and DISHEVELED ([3,17,21,36,37], and SH2-domain-containing proteins (e.g. GRB4, STAT3 and CRK[38] , respectively. These 2 features are located in the last 31 aa, which are highly conserved. EFNB can also have PDZ- and SH2-independent functions[39], some of which presumably depend on the SH3 domain of associating proteins (*e.g.,* CRK and GRB4. By deleting different lengths of the EFNB2 intracellular tail, we found that the C-terminal PDZ-domain binding motif and the last 2 tyrosine residues were dispensable for EFNB2 reverse signaling with regard to VSMC contractility, but a 19-aa long region (*i.e.*, from aa 313 to aa 331) containing the 3rd and 4th conserved tyrosine residues (counting from the C-terminus) was critical. The 2 tyrosine residues (Y314 and Y319) in this region are good candidates responsible for reverse signaling as they could interact with SH2 domain-containing adaptor proteins mentioned above, although S328 should also be considered, as it may attract SH3 domain-containing adaptor proteins. It is possible that the molecules associating with these residues are connected to the VSMC contraction machinery, and the function of these associating molecules is differentially affected by sex hormones in the absence or presence of EFNB2. A search of the associated proteins that mediate EFNB2's effect and a study of how their effects are influenced by sex hormones with regard to VSMC contractility are warranted.

Our human genetic study revealed that 5 SNPs that are in high LD with each other in the 3' untranslated region of *EFNB2* were significantly associated with hypertension risk at a *p*-value below the Bonferroni-corrected *p*-value in the males-only samples but not in females-only samples. The minor alleles of each of the 5 significant SNPs were negatively associated with hypertension risk (*i.e.*, protective against hypertension, with odds ratios between 0.77 and 0.78)

in male human subjects. These SNPs are likely in LD with a functional mutation that may be linked to the effects of *EFNB2* on blood pressure through the 3' UTR; however, additional functional studies are required to investigate this possibility. This finding implies that the occurrence of a loss-of-function mutation in the *EFNB2* gene, in LD with the coding allele of the 5 associated SNPs, could result in reduced risk of hypertension in male patients, and this is not inconsistent with the effect of EFNB2 deletion in mice. However, it must be emphasized that a human gene mutation is not equivalent to the mouse EFNB2 deletion, which invariably leads to loss of function; a human mutation could be either a loss- or gain-of-EFNB2-function. A gain-of-function of mutation in human males could explain the association of increased hypertension risks with EFNB2 mutations in the alternative allele.

Our mouse study showed that in females, EFNB2 null-mutation did not lead to hypotension. Therefore, EFNB2 gene mutation in female humans should not impact on BP, or at least should not affect the BP in the same direction as it would in the males, if our mouse study is of any guidance. Indeed, we observed no association between *EFNB2* SNPs with hypertension in females in this study. The mechanisms responsible for the influence of sex/sex hormones in regulating BP jointly with EFNB2 are currently under investigation. With that said, our results of the Altman-Bland test show that there is no difference between males-only and females-only samples with regards to the odds ratios of the 5 SNPs (the *p*-value of the most significant SNPs rs7328882 was given in the section of Results; *p*-values of other SNPs are not shown). A possible explanation is that the female sample size is rather small; with a larger sample size, we might observe a significant difference after the Alman-Bland test, as would be expected.

Since male but not female EFNB2 KO mice had lower BP, logically this suggests that either testosterone is inductive to the lower BP when EFNB2 is absent, or else EFNB2 deletion has a default effect lowering BP but estrogen counteracts this effect. With this conceptual frame work, we attempt to explain the detection of association of EFNB2 SNPs with hypertension in males in the ADVANCE genetic study, in which all the cases and controls are type 2 diabetics.

The minimal age of male patients in the ADVANCE study was 55-years. Plasma total testosterone levels range from 270 to 1,070 ng/dL in "normal" males. Considering 346 ng/dL as a cut-off for the diagnosis of hypogonadism, as recommended by the International Society for the Study of the Aging Male, about 30% of men older than 40 years are hypogonadic[40] . Further, male patients with the metabolic syndrome are prone to hypogonadism[41]. Therefore, it is conceivable that more than 30% of the male patients in the ADVANCE study suffer from hypogonadism.

Further, the type 2 diabetes males are generally overweight or obese. Adipose tissues are rich in aromatase, a rate-limiting enzyme converting testosterone to estrogen[42]. Hence, overweight/obese males tend to have relatively higher estrogen levels locally in the perivascular adipose tissues or systemically.

The above-described special changes of sex hormone levels in the ADVANCE males might make them a unique subpopulation in which the association between EFNB2 mutations and hypertension risks becomes easier to detect, compared to the general population. Therefore, the observed sex difference in EFNB2 SNPs with regard to their association to hypertension might

exist in certain subpopulations based on sample selection, and further validation studies of this explanation is warranted.

The incidence of CDK (including diabetic nephropathy) is increased in diabetic patients [43]. Since CDK is a cause of hypertension, it is therefore logical to include CDK as a covariate, to exclude the possibility that EFNB2 is in association with hypertension via CDK. We registered the CDK incidence in our cohorts, and the data are presented in S. Table 4. Generally speaking, there are more subjects without CKD than with CKD, for both males and females (S. Table 4A). In the male cohort, 16% and 9% of the hypertensive subjects and normotensive subjects, respectively, have CKD (S. Table 4B), showing significant dependence between hypertension and CKD. In the female cohort, however, there is no significant dependence between hypertension and CKD (S. Table 4C). In our genetic study, 5 EFNB2 SNPs are significantly associated with hypertension in males but not females. Does such association depend on CKD? The answer is negative based on following considerations. 1) After CKD was used as a covariate, one SNP rs7328882 was still significantly associated with hypertension in males, and the rest 4 SNPs, although their p-values increased, but were just above the Bonferroni-corrected *p*-value. Considering the conservative nature of Bonferroni correction, and the high LD link of rs7328882 with the other 4 SNPs, it is reasonable to assume that the functional mutation related to this LD block is still significantly associated with hypertension even after CKD was used a covariate. 2) Although CKD could cause hypertension[44], the reverse is also true, *i.e.*, hypertension frequently leads to the development of CKD[45]. In our case, the introduction of CKD as a covariate in the statistical analysis reduces somewhat significance of the association between some EFNB2 SNPs and hypertension, but this does not necessarily mean that CKD is causative for the hypertension. Rather, since CKD and hypertension phenotypes are correlated and confounded, we might have

simply removed some weight of hypertension and reduced the power of the analysis. 3) Our human genetic study is corroborated by the KO mouse phenotype, which is caused by vascular defects but not kidney dysfunction. Taking together, we believe that the association of EFNB2 SNPs to hypertension is direct but not via CKD.

We have queried the International Consortium for Blood Pressure (ICBP) dataset[46], but no significant association of EFNB2 SNPs with BP is found. We should point out the differences in the two studies. The IBPC sample specifically excluded, cohorts of diabetic cases, hypertensive cases, and myocardial infarction cases, which is specifically the type of samples we analyzed. They analyzed general population samples that had incidentally been measured for systolic pressure (SP) and diastolic pressure (DP), and performed a quantitative analysis against DP and SP values. The advantage of their approach is that it allows the assembly of very large sample sizes to increase power; however, it also might introduce significant heterogeneity into their sample decreasing genotype relative risks. In our current study, we used a qualitative phenotype that defined cases and controls for hypertension. Also, multiple BP measurements were carefully made over multiple time points in the course of the *ADVANCE* study, and only consistently high readings were included as cases.

The 5 associated SNPs are in LD with each other and are located within a large LD block that includes the 3` UTR region of *EFNB2* and could reflect the presence a polymorphism within the 3' untranslated region (3' UTR) of the gene. 3'UTRs of various genes are known to contain regulatory elements that can control rates of translation and mRNA stability by serving as binding sites for various proteins or miRNAs. Many 3'-UTRs contain AU-rich elements which affect the stability or decay rate of transcripts in a localized manner or affect translation initiation. Also the

3'-UTR contains the sequence AAUAAA that directs addition of the poly-A tail which is involved in mRNA stability. By binding to specific sites within the 3'-UTR, miRNAs can decrease gene expression of various mRNAs by either inhibiting translation or directly causing degradation of the transcript. Discovery of the exact location and mechanism of the functional mutations in LD with the identified SNPs would require further extensive functional research.

This study and our most recent publication show that the deletion of either of the receptor EPHB4 or its major ligand EFNB2 leads to a similar hypotensive phenotype in mice[27], corroborating the results of our human genetics study. Collectively, these novel findings reveal a previously unknown EPH/EFN-based mechanism for blood pressure regulation.

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Titles and Legends of Figures

Figure 1. Generation of mice with smooth muscle cell-specific Efnb2 null mutation

A. Efnb2 mRNA deletion in mesenteric arteries of EFNB2 KO mice RNA was extracted from the mesenteric arteries and spleens from WT and EFNB2 KO mice and analyzed by RT-qPCR for *Efnb2* mRNA levels. β-actin mRNA levels were used as an internal control. Samples were in triplicate, and means ± SEM of *Efnb2* signal/β-actin signal ratios are shown.

B. EFNB2 protein deletion in EFNB2 KO VSMCs according to immunofluorescence microscopy VSMCs from EFNB2 KO and WT mice were isolated from mesenteric arteries, and their EFNB2 (red, lower row) and α-actin (green, upper row) expression was detected by immunofluorescence microscopy.

C. EFNB2 protein deletion in EFNB2 KO VSMCs according to immunoblotting VSMCs from EFNB2 KO and WT mice were cultured for 4 days and then harvested. Cell lysates were analyzed for EFNB2 protein expression by immunoblotting.

The experiments in this figure were conducted three times; representative experiments are shown.

Figure 2. BP and HR of EFNB2 KO mice

The BP and HR were measured every 2 min by telemetry for 24-h. The mean ± SEM of daily BP and HR are shown. Parameters of all the time points in the 24-h period were evaluated by a mixed-effects linear model, with genotype, individual mouse, sex and time used as qualitative factors. *P*-values are indicated: * $p<0.05$; ** $p < 0.01$. SP: systolic pressure; DP: diastolic pressure; MAP: mean arterial pressure; HR: heart rate. The average age of mice of each group at the time of telemetry measurement and the number of each group (n) are indicated.

A. *Blood pressure of male KO and WT mice*

B. *Blood pressure and of female KO and WT mice*

C. *BP differences between KO and WT mice and between males and females*

   The differences of SP, DP, and MAP of KO versus WT mice (difference = WT value – KO value) are shown. Negative values mean that the KO values are larger than those of WT. The differences between sexes (according to a mixed-effects linear model), if statistically significant, are indicated.

D. *HR differences between KO and WT mice (left panel) and between males and females (right*

panel)

Figure 3. Both forward and reverse signaling by EFNB2 in VSMCs results in increased contractility Male VSMCs were cultured in wells coated with EFNB2-Fc, normal human IgG (NHIgG; as a control for EFNB2-Fc), goat anti-EFNB2 Ab, or goat IgG (as a control for anti-EFNB2 Ab; 2 μg/ml for coating for all the cases) as indicated. VSMCs were stimulated with 20 μM phenylephrine at 37℃ and imaged every min for 15 min. Means ± SEM of percentage contraction of 15-30 cells are shown. Data were assessed by one way ANOVA. *P*-values between groups with significant differences are indicated. All experiments were conducted 3 times independently. Data from representative experiments are presented.

A. Forward signaling triggered by EFNB2-Fc increases WT VSMC contractility Male WT VSMCs were cultured in wells coated with EFNB2-Fc or normal human IgG, the latter being presented as a thin line without SEM to facilitate viewing. In one group, soluble EPHB4-Fc (2 μg/ml) was added to the wells to block the interaction between solid phase EFNB2 and cell surface EPHB4.

B. EPHB4 is mainly responsible for EFNB2-triggered forward signaling for increased VSMC contractility Male WT or EPHB4 KO VSMCs were cultured in wells coated with EFNB2-Fc, and their contractility upon phenylephrine stimulation was measured. Male WT VSMCs cultured in normal human IgG-coated wells were used as an additional control; their mean contractility is presented as a thin line without SEM to facilitate viewing.

C. Reverse signaling mediated by EFNB2 increases WT VSMC contractility Male WT VSMCs were cultured in wells coated with goat anti-EFNB2 Ab or goat IgG. In one of the groups, soluble EFNB2-Fc (2 μg/ml) was added to the culture to block the interaction between solid phase anti-

EFNB2 Ab and cell surface EFNB2 for verification of the Ab specificity.

Figure 4. Critical regions in EFNB2 intracellular tail for regulating VSMC contractility

A. Illustration of EFNB2 deletion mutants the general structures [extracellular domain, intracellular tails, intracellular tyrosine residues (dots) and PDZ-domain-binding motif (rectangles)] of EFNB2 are depicted. Different deletion mutants and their nomenclatures are illustrated.

B. mRNA levels of EFNB2 in VSMCs infected with lentiviruses expressing different EFNB2 deletion mutants Male WT VSMCs were infected with lentiviruses expressing different EFNB2 deletion mutants or the full length EFNB2, and the mRNA overexpression of these molecules was measured by RT-qPCR, which detected an undeleted region in the EFNB2 sequence. Male WT VSMCs infected with empty viruses were used as a control. Data were expressed as the mean + SEM of ratios of *Efnb2* versus β-actin mRNA signals. The experiment was conducted 3 or more times, and representative results are shown. *: $p<0.05$ (Student's *t* test). The cells tested in B were the same ones used in C-F.

C-F. Identification of a critical region in the EFNB2 intracellular tail for regulating VSMC contractility Male EFNB2 KO VSMCs were infected with lentiviruses expressing the full-length EFNB2 or its deletion mutants, and the VSMCs were cultured in wells coated with anti-EFNB2 Ab. VSMC contractility upon phenylephrine stimulation (20 μM) was measured. Means + SEM of percentage contraction of 15-30 cells are shown. Data were assessed by ANOVA. P-values between groups with significant differences are indicated. Data from representative experiments are presented.

Table 1. SNP rs numbers, alleles, allele frequencies, p-values, odds ratios and 95% confidence intervals of odds ratios for SNPs associated with hypertension in the ADVANCE study with normal p-values ≤ $5.00x10^{-2}$ for male-only or female only samples with chronic kidney diseases (CKD) included and not included in the covariates for the analysis.

SNP	Position chrome 13 (Build37/ hg19)	Coding Allele[a]	Alternate Allele	Frequency of Coding Allele	Males				Females			
					Association *p*-value[b]	Odds Ratio	Lower 95% CI[c]	Upper 95% CI[c]	Association *p*-value	Odds Ratio	Lower 95% CI[c]	Upper 95% CI[c]
Without CKD as a covariate												
rs9555251	107140587	T	C	0.35	**$2.23x10^{-3}$**	0.78	0.66	0.91	$8.77x10^{-1}$	0.98	0.78	1.24
rs7322914	107140910	T	C	0.35	**$2.19x10^{-3}$**	0.78	0.66	0.91	$8.69x10^{-1}$	0.98	0.78	1.23
rs7328698	107141349	C	G	0.35	**$1.88x10^{-3}$**	0.77	0.66	0.91	$8.02x10^{-1}$	0.97	0.77	1.22
rs7328882	107141433	A	G	0.35	**$1.13x10^{-3}$**	0.76	0.65	0.90	$8.78x10^{-1}$	0.98	0.78	1.23
rs7329357	107141705	A	G	0.35	**$1.73\ x10^{-3}$**	0.77	0.65	0.91	$8.62x10^{-1}$	0.98	0.78	1.23
rs9520087	107144320	A	G	0.43	$9.35x10^{-3}$	0.81	0.69	0.95	$7.69x10^{-1}$	1.03	0.83	1.29
With CKD as a covariate												
rs9555251	107140587	T	C	0.35	$4.62x10^{-3}$	0.79	0.67	0.93	$6.29x10^{-1}$	0.94	0.74	1.20
rs7322914	107140910	T	C	0.35	$4.57x10^{-3}$	0.79	0.67	0.93	$6.21x10^{-1}$	0.94	0.74	1.19
rs7328698	107141349	C	G	0.35	$4.12x10^{-3}$	0.79	0.67	0.93	$5.60x10^{-1}$	0.93	0.74	1.18
rs7328882	107141433	A	G	0.35	**$2.54x10^{-3}$**	0.78	0.66	0.91	$6.27x10^{-1}$	0.94	0.75	1.19
rs7329357	107141705	A	G	0.35	$3.91x10^{-3}$	0.78	0.66	0.92	$6.99x10^{-1}$	0.95	0.76	1.21
rs9520087	107144320	A	G	0.43	$1.78x10^{-2}$	0.82	0.70	0.97	$9.03x10^{-1}$	1.01	0.81	1.28

[a] Coding allele is reference allele for calculation of odds ratio. If odds ratio is < 1.0, then coding allele is protective against hypertension.

[b] *p*-values ≤ Bonferroni critical p-value of $3.33x10^{-3}$ are indicated in bold.

[c] 95%CI: 95% confidence interval for odds ratio.