SUPPLEMENTARY MATERIALS

Supplementary Methods

Reverse transcription/quantitative-PCR (RT/qPCR)

The *Efnb1, Efnb2 Efnb3, Ephb1, Ephb2, Ephb3, Ephb4, Ephb6,* and α1-adrenoreceptor and *iNOS* mRNA levels in VSMCs were measured by RT-qPCR. Total RNA from mesenteric arteries, cultured VSMCs or the spleen was extracted with TRIzol® (Invitrogen, Burlington, Ontario, Canada) and reverse-transcribed with iScriptTM cDNA Synthesis Kit (BIO-RAD, Mississauga, Ontario, Canada). The primers used are listed in Supplementary table 1. Conditions for the qPCR reactions were as follows: 2 min at 50 °C, 2 min at 95 °C followed by 40 cycles of 10 s at 94 °C, 20 s at 58 °C, and 20 s at 72 °C. β-actin mRNA levels were used as internal controls. The qPCR signals between 22-30 cycles were analyzed. Samples were tested in triplicate, and data were expressed as signal ratios of Efnb2 RNA/β-actin mRNA.

Immunofluorescence microscopy

VSMCs were cultured in 24-well plates with cover glass placed at the bottom of the wells. After 4 to 5 days, the cells were washed twice with PBS and fixed with paraformaldehyde (4%) for 20 min. For cell surface EFNB2 staining, cells were blocked with 10% goat IgG in PBS for 20 min and then incubated with goat anti-mouse EFNB2 Ab (2 µg/ml: R&D System, Minneapolis, MN) overnight at 4°C. Cells were then reacted with rhodamine-conjugated donkey anti-goat Ab (0.15 µg/ml, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) overnight at 4°C. For intracellular β-actin staining, the cells were permeabilized with permeabilization buffer (BD Biosciences, San Jose, CA) for 20 min at 4°C, and then incubated with mouse anti-human α-actin mAb (2 µg/ml; Santa Cruz Biotechnology); Cells were then washed and reacted with FITC-conjugated goat anti-mouse IgG (0.2 µg/ml; Bethyl Laboratories, Montgomery, TX) at room temperature for 2 h, and imbedded with ProLong® Gold anti-fade reagent (Invitrogen). The stained cells were examined under a Zeiss microscope.

Blood pressure, heart rate and activity measurement by radiotelemetry

Mice were anesthetized with isoflurane and implanted surgically with TA11PA-C10 radiotelemetry sensors (Data Sciences International, St. Paul, MN, USA) in the left carotid artery for direct measurement of systolic pressure (SP), diastolic pressure (DP) and heart rate (HR) in conscious free-moving mice, as described previously[1]. Measurements were conducted at least 9 days after the radiotelemetry sensor implantation and 2 days after any post-operational care. Individual 10 s waveforms of SP, DP, mean arterial pressure (MAP), HR and activity were sampled every 2 min and the data were recorded continuously for 24h with the Dataquest acquisition 3.1 system (Data Sciences International). The mice were on a normal diet unless specified. The raw data were processed with the Dataquest A.R.T-Analysis program[2]. The daily blood pressure and heart rate are presented as bar graphs (means + SEM). Parameters of all the time points in a 24-h period were analyzed by a mixed-effects linear model, with genotype, individual mouse, sex and time as qualitative factors. The statistical

significance (*p<0.05 or **p<0.01) is indicated.

VSMC isolation

VSMCs were isolated from male KO or WT mice, as described by Golovina and Blaustein[4] with modifications[1,2]. Briefly, the aorta and mesenteric arteries, including their secondary branches, were isolated by fine forceps under sterile conditions. The isolated vessels were digested for 20 min in HBSS containing collagenase type II (347 U/ml) (Worthington Biochemical Corporation, Lakewood, NJ, USA). After the first digestion, the blood vessels were cut into small pieces (1 mm^3) and further digested at 37°C for 20 min with both collagenase type II (347 U/ml) and elastase type IV (6 U/ml) (Sigma-Aldrich Corporation, St. Louis, MO, USA) in HBSS. The dissociated cells were cultured at 37ºC in Dulbecco's modified Eagle's medium (Wisent, St-Bruno, Quebec, Canada) supplemented with 15% fetal bovine serum for 3 to 4 days before experimentation. At such a time, about 80% of the cells were positive for β-actin and capable of efficient contraction on phenylephrine stimulation.

Measurement of VSMC contractility

VSMC contractility was measured as described before[1,2]. Briefly, the VMSCs were washed once with Ca^{2+}-free HBSS, and then cultured in the same solution at 37°C with 5% CO2 under a Zeiss microscope. The cells were stimulated with phenylephrine (20 μM) and photographed continuously for 15 min at a rate of 1 picture per min. Fifteen or more cells were randomly selected, and their lengths at each time point were measured with Zeiss Axiovision software. The percentage contraction was calculated as follows:

% contraction = 100 x (cell length at time 0 – cell length at time X) / cell length at time 0.

Immunoblotting

VSMCs from the aorta and mesenteric arteries of WT and KO mice were cultured for 3-4 days. They were lysed by radioimmunoprecipitation assay buffer, which contained PhosSTOP and protease inhibitor mixture (Roche Applied Science, Meylan Cedex, France)[5,6]. For some experiments, VSMCs were stimulated with phenylephrine (20 μM) for 3 s before being lysed. Twenty to 80 micrograms of proteins per sample were resolved in 12% SDS-PAGE. Proteins from the gel were transferred to PVDF membranes (Life Technologies, Burlington, Ontario, Canada), which were incubated in blocking buffer containing 5% (w/v) skim milk (for MLC, 5% BSA was used in the blocking buffer) for 1 h at room temperature, and then hybridized overnight at 4ºC with goat anti-mouse EFNB2 Ab (R & D Systems, Minneapolis, MN, USA), rabbit anti-mouse β-actin Ab, mouse anti-mouse phospho-myosin light chain mAb and rabbit anti-mouse myosin light chain Ab (both from Cell Signaling Technology, Danvers, MA), rabbit anti-mouse phospho-myosin phosphatase target subunit 1 Ab (from Cell signaling), and rabbit anti-mouse phospho-ERK Ab (from Life Technologies). The Abs were used at the manufacturers' recommended dilutions or at 1:1000. The membranes were washed 3 times and reacted with corresponding second Abs, *i.e.*, horseradish peroxidise-conjugated rabbit anti-goat IgG Ab (R & D Systems), horseradish peroxidise-conjugated donkey anti-rabbit IgG Ab (GE healthcare, Baie d'Urfé, Quebec, Canada), or horseradish peroxidise-conjugated sheep anti-mouse IgG Ab (GE Healthcare) for 90 min. The signals were detected with

SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA).

Human genetic study

Patients for the human genetic study The patient cohort consisted of 3409 European, Australian, Canadian and New Zealander Caucasians (See supplementary 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, a factorial, multi-center, randomized controlled clinical trial of 11,140 participants recruited from 215 centers in 20 countries[3,4]. All individuals were type 2 diabetes subjects age 65 or older, or they were type 2 diabetes subjects age 55 or older who were diagnosed at age 30 or older, and had one of the following: a history of major macrovascular disease; a history of major microvascular disease; diagnosis of type 2 diabetes over 10 years prior to entry into study; presence of another major risk factor for vascular disease including: smoking, dyslipidemia, microalbuminuria.

The 3409 patients were qualitatively classified as normotensive or hypertensive, which was defined as having a physician diagnosis of hypertension with a history of treatment with hypertension drugs (calcium antagonists; angiotensin II receptor blockers; angiotensin-converting-enzyme inhibitors; perindopril; beta-blockers; other antihypertensive agents, such as thiazide or thiazide-like diuretics or other diuretics) or having a measurement of systolic pressure > 140 mm Hg or diastolic pressure > 90 mm Hg at entry into the study. The cohort included 1,789 hypertensive and 417 normotensive males, and 996 hypertensive and 206 normotensive females. There were 4.5 times as many hypertensive as normotensive diabetic subjects and 1.8 times as many males as females.

The 3409 individuals were genotyped for either 440,794 SNPs on the Affymetrix Genome-Wide Human SNP Arrays 5.0 or 906,600 SNPs on the 6.0 Array at the genomic platform of the CHUM Research Center. An additional 4,547,420 SNPs were imputed for array 5.0 and 4,768,948 for array 6.0 independently using the program IMPUTE2[5]. The data related to the current study are submitted to GWAS Central[6].

Association analysis was performed separately for the array 5.0 and array 6.0 data sets using the R statistical package software[7]. The final *p*-values for the combined sample over both arrays were then determined using meta-analysis of the 5,045,528 SNPs common to both arrays using the meta-analysis subroutine of R with a fixed effects model. This method corrected for any possible effect of cases and controls not being randomly distributed across the different genotype arrays. A subset (147,088) of the genotyped SNPs that were in Linkage equilibrium (defined as $r^2 \leq 0.80$) was selected to perform a principal component analysis, using the Eigenstrat software[8] in order to test for population stratification within the European and Canadian Caucasian samples. The first two independent principal components from this analysis (PC1 and PC2), which account for the majority of the covariance among genotypes due to population structure, were able to clearly separate samples according to geographic origin (ancestral regions of origin for Canadian, New Zealand and Australian

samples) and were subsequently used as covariates in the association analysis in order to correct for population stratification. The association with hypertension was tested for 55 SNPs both genotyped and imputed, that were common to both the Array 5 and 6 sub analysis, and that fell within EFNB2 or within an additional region 10 kb 5' and 3' of the gene and located within a 58.72 kb region between positions 107,134, and 107,193, on chromosome 13 (Build 37, hg/19). Only SNPs or markers with a minor allele frequency (MAF) greater or equal to 10.0 and imputed SNPs with imputation quality scores greater than 0.80 were analyzed. Even though there were allele frequency differences noted between eastern and western European samples, this MAF filter was applied after genotyping across-the-board calculated over all samples.

Association testing was performed for males and females separately. A logistic regression model with additive genotype coding and with principal components, PC1 and PC2 from the stratification analysis, and with age, and body mass index as covariates was used to perform association tests. Analysis was also performed with and without the presence of chronic kidney disease (CKD), defined as estimated glomerular filtration rate (eGFRMDRD) less than 60 mL/min/1.73 m^2, as a covariate. Differences of odds ratios between males and females for the most significant SNPs were also tested according to the method proposed by Altman and Bland[9].

The number of tagging SNPs that proxy for all 61 markers tested for association within the gene region was determined using the LDSELECT program[10] using an r^2 value > 0.80 and a minimum minor allele frequency of 0.10. Fifteen tag SNPs were identified and were considered to represent 15 independent linkage disequilibrium (LD) blocks within the region around EFNB2 covered by the analyzed SNPs; subsequently, all *p*-values were corrected for multiple testing by the *Bonferroni* correction for 15 independent LD, giving a critical *p*-value of 3.33×10^{-3} or a critical -log10 *p*-value of 2.48.

1. Luo H, Wu Z, Tremblay J *et al*: Receptor tyrosine kinase Ephb6 regulates vascular smooth muscle contractility and modulates blood pressure in concert with sex hormones. *The Journal of biological chemistry* 2012; **287:** 6819-6829.

2. Lavoie JL, Lake-Bruse KD, Sigmund CD: Increased blood pressure in transgenic mice expressing both human renin and angiotensinogen in the renal proximal tubule. *American journal of physiology Renal physiology* 2004; **286:** F965-971.

3. Colagiuri S, Walker AE: Using an economic model of diabetes to evaluate prevention and care strategies in Australia. *Health Aff (Millwood)* 2008; **27:** 256-268.

4. Yu B, Barbalic M, Brautbar A *et al*: Association of genome-wide variation with highly sensitive cardiac troponin-T levels in European Americans and Blacks: a meta-analysis from atherosclerosis risk in communities and cardiovascular health studies. *Circ Cardiovasc Genet* 2013; **6:** 82-88.

5. Marchini J, Howie B: Genotype imputation for genome-wide association studies. *Nat Rev Genet* 2010; **11:** 499-511.

6. report GC-s: http://www.gwascentral.org/study/HGVST1838.

7. Purcell S, Neale B, Todd-Brown K *et al*: PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 2007; **81:** 559-575.

8. Patterson N, Price AL, Reich D: Population structure and eigenanalysis. *PLoS Genet* 2006; **2:** e190.

9. Altman DG, Bland JM: Interaction revisited: the difference between two estimates. *Bmj* 2003; **326:** 219.

10. LDSelect-Multipopulation: Download and Documentation. Available at: http://droog.gs.washington.edu/multiPopTagSelect.html. Accessed Nov, 2014.

Supplementary Tables

Supplementary Table 1. List of RT-PCR primers

Supplementary Table 1. RT-PCR primer sequences

Gene	Sense sequence	antisense sequence
β-actin	5'-TCGTACCACAGGCATTGTGATGGA-3'	5'-TGATGTCACGCACGATTTCCCTCT-3'
EphB1	5'-ACCATGAGGAGCATCACCTTGTCA-3'	5'-TAGCCCATCGATACGTGCTGTGTT
EphB2	5'-CCAGTGATGTGTGGAGCTATG-3'	5'-GGAGGTAGTCTGTAGTCCTGTT-3'
EphB3	5'-AGTTCGCCAAGGAGATCGATGTGT-3'	5'-TCAGCGTCTTGATAGCCACGAACA-3'
Ephb4	5'- CTACGTCTCTAACCTCCCATCT-3'	5'- GCTGGTCACCCTTTCTCTTT-3'
EphB6	5'-AAGCCATAGCAGTGCCTCAGAACA-3'	5'-TCCAGAGCTAGAACTGATGACCCT-3'
Efnb1	5'-TGCAACAAGCCACACCAGGAAATC-3'	5'-CAAGCTCCCATTGGACGTTGATGT-3'
Efnb2	5'-CCCTTTGTGAAGCCAAATCCAGGT-3'	5'-TCCTGATGCGATCCCTGCGAATAA-3'
Efnb3	5'-AGTTCCGATCCCACCACGATTACT-3'	5'-TCCATGGGCATTTCAGACACAGGT-3'
α1-AR	5'-TGCCCTTCTCTGCCATCTTTGAGA-3'	5'-AGCGGGTAGCTCACACCAATGTAT-3'
iNOs	5'-GGAATCTTGGAGCGAGTTGT-3'	5'-CCTCTTGTCTTTGACCCAGTAG-3'

Supplementary Table 2. Characteristics of the ADVANCE cohort used in the human genetic study giving distribution of sexes by country of recruitment

Country of recruitment	Males	Females	Total
United Kingdom	489	184	673
Australia	420	150	570
Netherlands	231	134	365
New Zealand	180	90	270
Poland	143	114	257
Ireland	155	80	235
Canada	178	52	230
Germany	118	76	194
Slovakia	94	94	188
Hungary	84	96	180
Czech Republic	50	52	102
Russia	18	27	45
France	26	16	42
Estonia	12	25	37
Italy	6	5	11
Lithuania	2	8	10
Totals	2206	1203	3409

Supplementary Table 3. BP and HR of WT and KO mice analyzed by a mixed-effects linear model

	DP (mmHg)	*p*-value	SP (mmHg)	*p*-value	MAP (mmHg)	*p*-value	HR (beat/min)	*p*-value
Male WT	[a]91.23		120.06		104.83		590.12	
Male KO	85.16		111.85		97.76		558.86	
Female WT	81.79		118.09		103.34		641.55	
Female KO	91.14		120.63		104.90		635.64	
Difference (Male WT - Male KO)	[b]6.07	$5.05x10^{-2}$	8.21	[c]$4.1x10^{-3}$	7.06	$1.91x10^{-2}$	31.25	$1.25x10^{-2}$
Difference (Female WT - Female KO)	-9.35	$1.44x10^{-2}$	-2.55	$5.89x10^{-1}$	-1.56	$6.12x10^{-1}$	5.91	$6.13x10^{-1}$
Difference (Male WT - Female WT)	9.34	$8.0x10^{-4}$	1.98	$4.9x10^{-1}$	1.48	$5.66x10^{-1}$	69.26	$2.47x10^{-9}$
Difference (Male KO - Female KO)	4.44	$2.04x10^{-1}$	-8.78	$1.21x10^{-1}$	-7.08	$6.67x10^{-1}$	74.12	$4.08x10^{-5}$

[a]Means of DP, SP, MAP and HR of WT and KO mice of both sexes.
[b]The differences between the group means.
[c]P-values are determined by a mixed-effect linear model. Significant *p*-values are in red.

Supplementary Table 4. CKD incidence in ADVANCE case and control cohorts

A

	[a]CKD+ [c]N (%)	[b]CKD- N (%)	Ratio of CDK+/CDK-	Total CKD N
Males	313 (15)	1842 (85)	0.17	2155
Females	344 (29)	827 (71)	0.42	1171
		X^2 test of independence	105.6	
		p-value	$< 1x10^{-5}$	

B

	CKD+ N (%)	CKD- N (%)	Ratio +/- CDK+/CDK-	Total CKD N
Hypertensive Males	275 (16)	1467 (84)	0.19	1742
Normotensive Males	38 (9)	375 (91)	0.10	413
Total	313 (15)	1842 (85)	0.17	2155
		X^2 test of independence	11.7	

		p-value	6.4×10^{-4}	

C

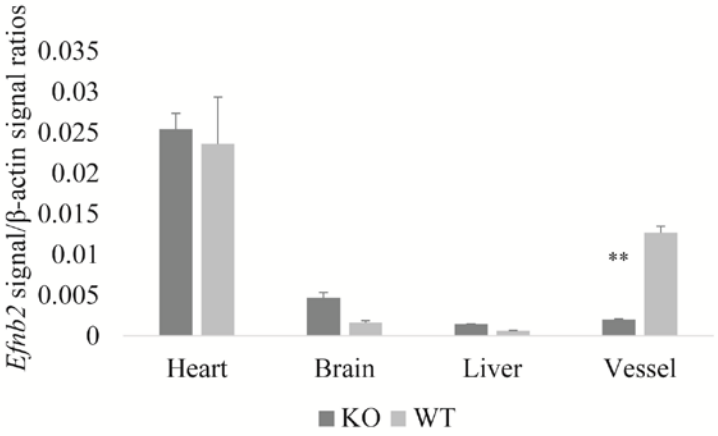
	CKD+ N (%)	CKD- N (%)	Ratio +/- CDK+/CDK-	Total CKD N
Hypertensive Females	295 (30)	673 (70)	0.44	968
Normotensive Females	49 (24)	154 (76)	0.32	203
Total	344 (29)	827 (71)	0.42	1171
		X^2 test of independence	3.3	
		p-value	NS	

[a]CDK+: subjects with chronic kidney diseases

[b]CDK-: subjects without chronic kidney diseases

[c]N: number

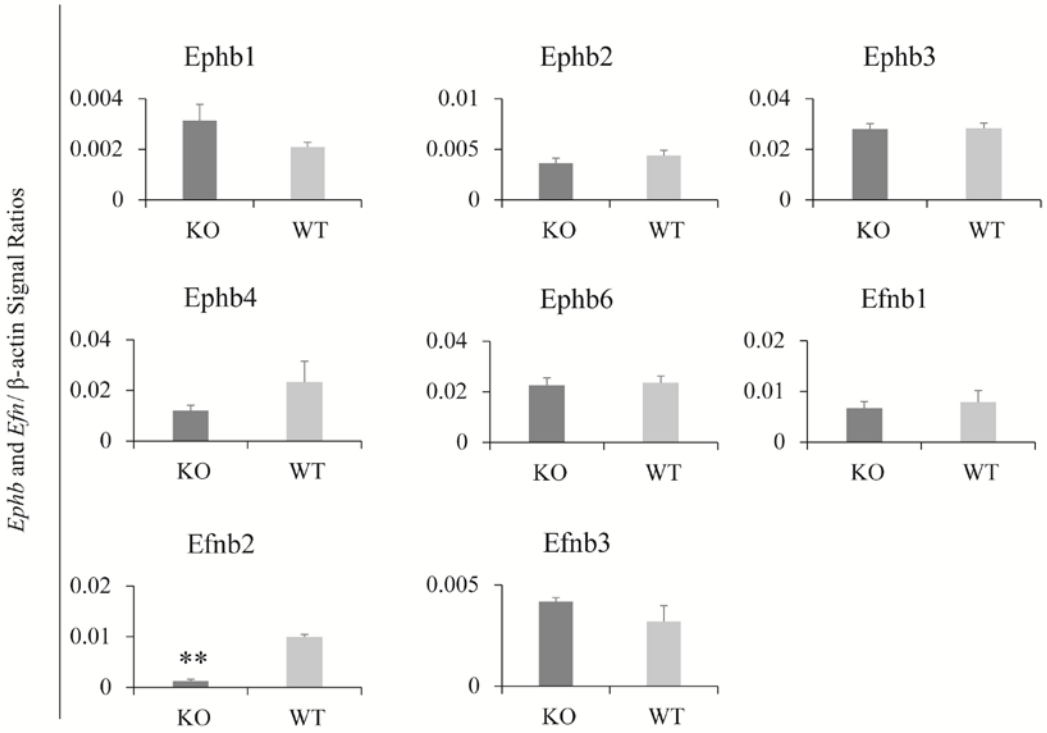
Supplementary Figures and legends



Supplementary Figure 1

Supplementary Figure 1. Vessel-specific deletion of Efnb2 mRNA

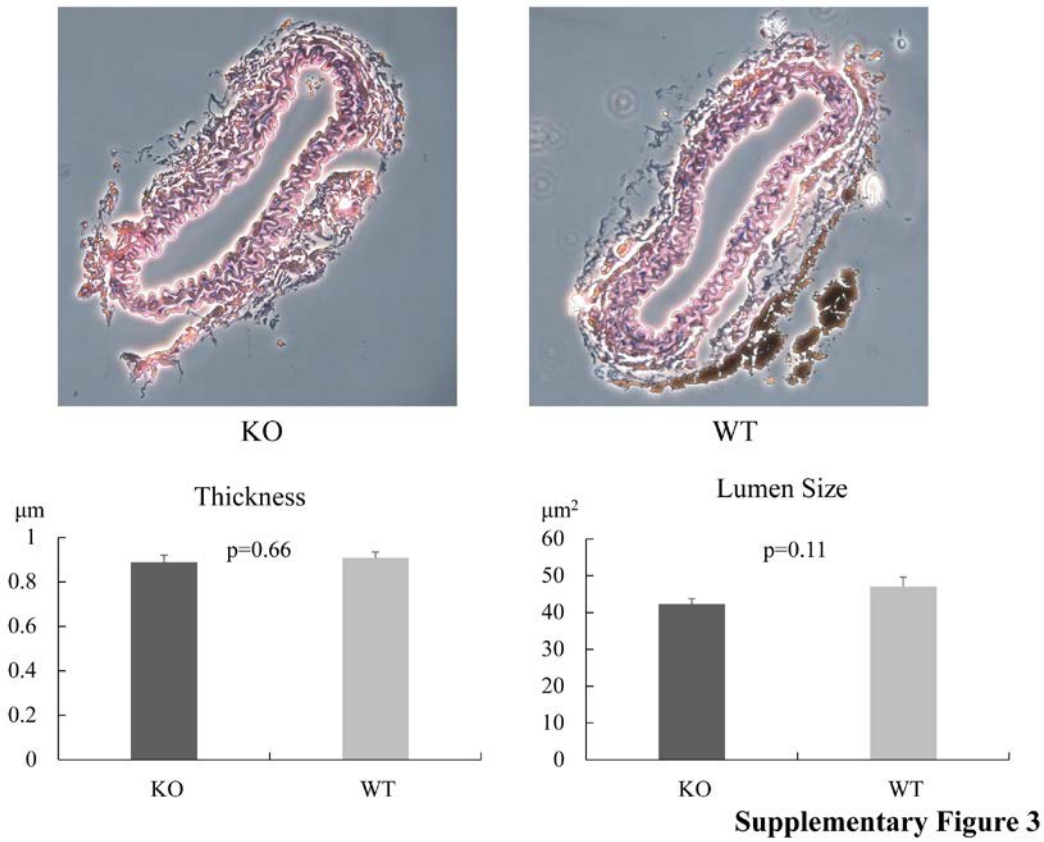
RNA was extracted from different organs and tissues from male WT and EFNB2 KO mice and analyzed by RT-qPCR for *Efnb2* mRNA levels. β-actin mRNA levels were used as an internal control. Samples were in duplicate, and means ± SEM of *Efnb2* signal/β-actin signal ratios are shown. Data represent the summary of 3 independent experiments. Statistical significant difference is only observed in the vessels but not in the spleen (Student's *t* test; **: $p<0.01$).



Supplementary Figure 2

Supplementary Figure 2. Ephb1, Ephb2, Ephb3, Ephb4, Ephb6, Efnb1 and Ephb3 mRNA expression in EFNB2 KO VSMCs are comparable to those of WT counterparts according to RT-qPCR

RNA was extracted from VSMCs of male KO and WT mice and analyzed by RT-qPCR for *Ephb1*, *Ephb2*, *Ephb3*, *Ephb4*, *Ephb6*, *Efnb1*, *Efnb2 and Ephb3* mRNA levels. *β-actin* mRNA levels were used as an internal control. Samples were in duplicate, and means ± SEM of *Ephb and Efnb* signal/*β-actin* signal ratios are shown. Data represent the summary of 3 independent experiments. **: p<0.01 (Student's *t* test).



Supplementary Figure 3. Histology, media thickness and lumen size of resistance arteries in EFNB2 KO mice are comparable to those of WT mice

Right and left carotid arteries at comparable positions in the neck of male WT and KO mice were isolated, sectioned and stained by hematoxylin and eosin.

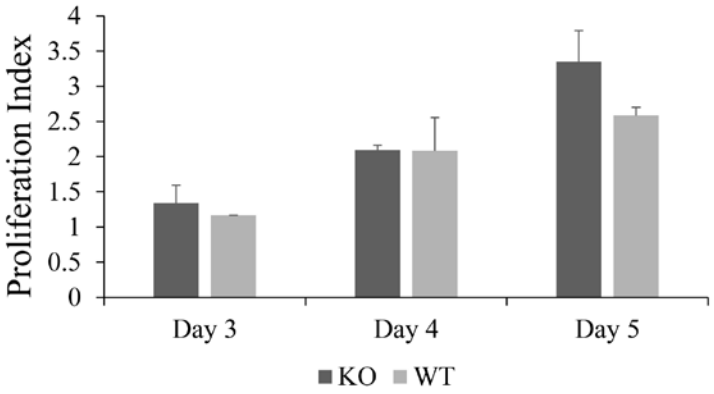
A. Carotid arteries from male KO and WT mice show comparable histology

Representative hematoxylin and eosin-stained male KO and WT carotid artery sections are shown.

B. Media thickness and lumen size of carotid arteries from male KO and WT mice are comparable

Means ± SEM of tunica media thickness (left panel) and lumen size (right panel) of male KO

and WT carotid arteries at comparable locations (3 randomly selected sections per each of the right and left carotid arteries per mouse, and 3 mice per group; *i.e.*, 18 determinants per group) are presented in a bar graph. No statistical significant difference is observed (Student's *t* test).

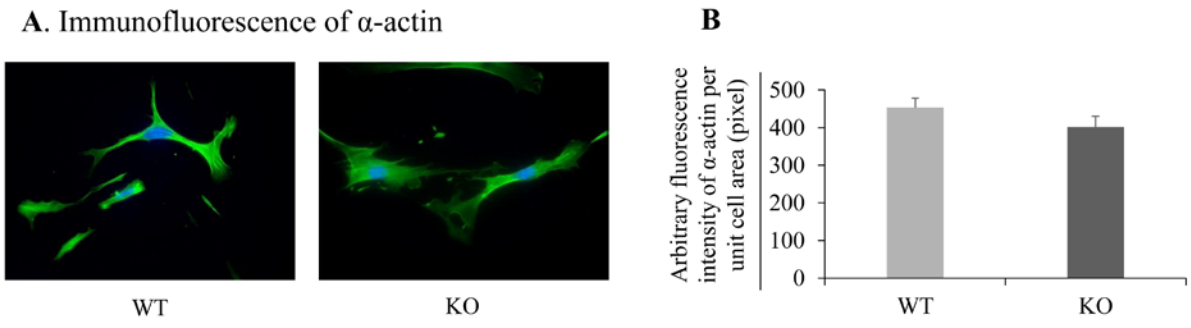


Supplementary Figure 4

Supplementary Figure 4. Normal proliferation of VSMCs from EFNB2 KO mice
VSMCs from male KO and WT mice were cultured for 3, 4 and 5 days, and means ± SEM of their proliferation indices of on these days are calculated based on pooled results of 2 independent experiments, with samples of each experiment in duplicate. No statistical significant difference is observed (Student's *t* test).

The proliferation index is calculated as follows.

Proliferation Index = VSMC cell number after culture / VSMC cell number on day 1 (the beginning of the culture)

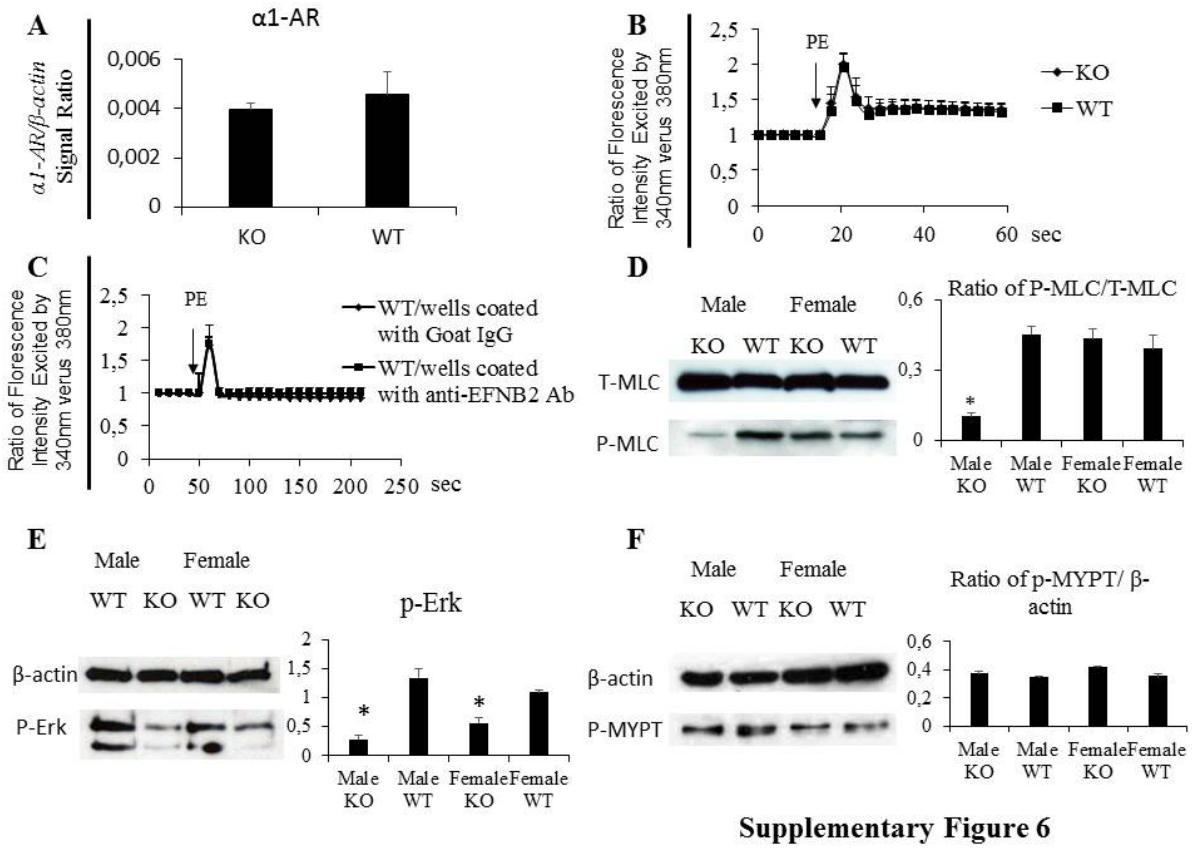


Supplementary Figure 5

Supplementary Figure 5. Similar SMC marker α-actin expression in KO and WT VSMCs
Male KO and WT VSMCs were cultured for 4 days, and then stained by FITC-conjugated anti-α-actin Ab.

A. Typical immunofluorescence staining of male WT and KO VSMC for smooth muscle cell marker -actin

B. Quantitative assessment of the intensity of a-actin signal in male WT and KO VSMC
More than 15 VSMCs per group were randomly selected and quantified for their levels of *a*-actin signals. Means ± SEM of *a*-actin signal intensity per unit cell area (1 pixel) of the *a*-actin-positive cells are shown. No statistical significant difference is observed (Student's *t* test).



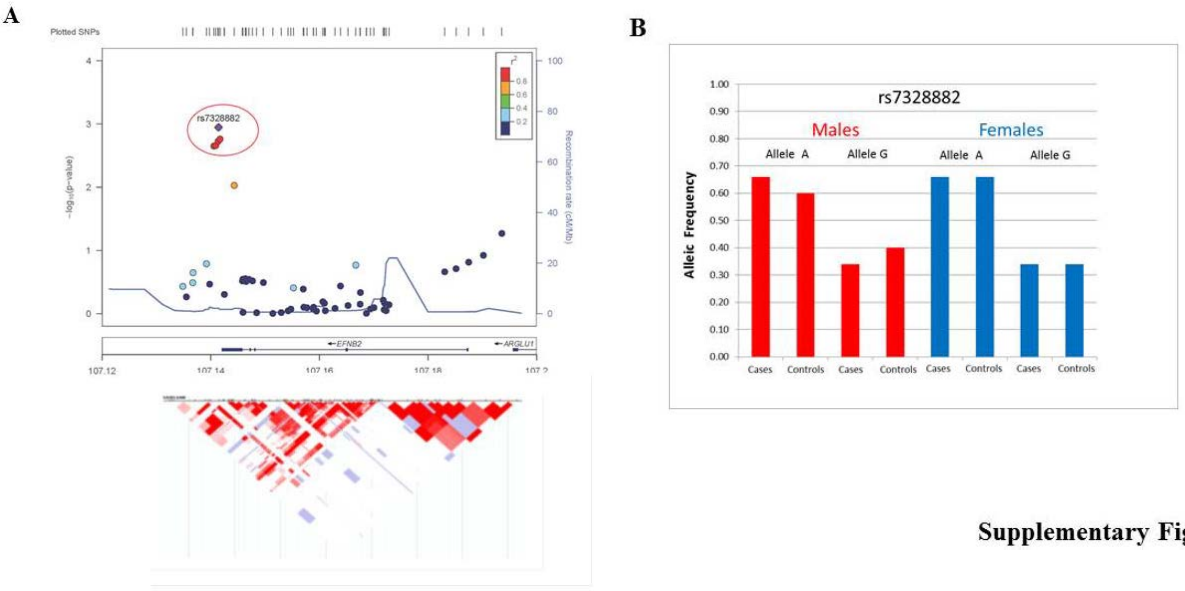
Supplementary Figure 6

Supplementary Figure 6. The effect of EFNB2 deletion on signaling events of VSMCs
A. Normal adreno-receptor mRNA expression in male EFNB2 KO VSMC according to RT-qPCR
VSMCs from male EFNB2 KO and WT mice were cultured for 4 days and then harvested. Cell lysates were analyzed for -adrenoreceptor (α1-AR) mRNA expression by RT-qPCR. Pooled data from 3 independent experiments were presented as mean ± SEM of *a*1-adrenoreceptor versus α-actin signal ratios.

B and C. Normal Ca^{2+} flux in EFNB2 KO VSMCs or in WT VSMCs stimulated by solid phase EFNB2-Fc
VSMCs from male EFNB2 KO or WT mice were cultured for 4 days (B). VSMCs from male

WT VSMCs were also cultured in wells coated with EFNB2-Fc (2 □g/ml for coa
cells were then loaded with Fura2. They were then placed in HBSS and stimulated with phenylephrine (20 μM). The arrow indicates the time point at which phenylephrine was added. The ratio of emissions at 510 nm triggered by 340 nm versus 380 nm excitation in each cell was registered every 3 s for 60 sec. The experiments were conducted 3 times. Means ± SEM of the ratio of more than 15 randomly selected VSMCs of a representative experiment are illustrated. No statistically significant differences were found between the KO and WT groups according to ANOVA.

D-F. MLC, MPTK and ERK phosphorylation of VSMCs from WT and KO mice
VSMCs from male KO and WT mice were cultured for 4 days and then stimulated with 20 μM phenylephrine for 3 s and immediately lysed. Phosphorylated (P-) MLC (D), MPKT (E) and ERK (F) were analyzed by immunoblotting. Total (T) MLC or β-actin was used as loading controls. Three independent experiments were conducted. Immunoblottings from representative experiments are illustrated; ratios of phosphorylated and total protein (or β-actin) signals according densitometry of the 3 independent experiments are presented as bar graphs on the right.



Supplementary Figure 7

Supplementary Figure 7. Association of SNPs within human EFNB2 gene and its adjacent regions with hypertension in the ADVANCE study

A. Positions of SNPs tested in males-only and females-only samples
The six significantly associated SNPs are encircled. The location of the *EFNB2* gene in relation to the SNP positions is depicted at the bottom. The LD plot was taken from a screen capture of the region presented in the HapMap browser.

B. Frequency of A and G alleles for SNP, rs7328882 in hypertensive cases and normotensive controls for male and female sample