

EPHA4 regulates vascular smooth muscle cell contractility

and is a sex-specific hypertension risk gene in individuals with type 2 diabetes

Short title: EPHA4 is a hypertension risk gene

*Zeqin ZHANG^a, *Johanne TREMBLAY^a, John RAELSON^a, Tamar SOFER^b, Lizhong DU^c, Qiang FANG^d, Francois-Christophe MAROIS-BLANCHET^a, Yu WANG^c, Lingling YAN^c, John CHALMERS^e, Mark WOODWARD^e, Stephen HARRAP^f, Pavel HAMET^a, Hongyu LUO^a, and Jiangping WU^a

From the ^aResearch Centre, Centre hospitalier de l'Université de Montréal (CRCHUM), Montréal, Québec, Canada H2X 0A9; ^b Brigham and Women's Hospital, Harvard Medical School, Boston, the U.S.A. MA02115; ^cThe Children's Hospital, Zhejiang University School of Medicine, Hangzhou, China 320052; ^dThe Intensive Care Unit, The First Affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, China 310005; ^eThe George Institute for Global Health, University of Sydney, Sydney, New South Wales, Australia 2006 and The George Institute for Global Health, University of Oxford, Oxford, United Kingdom OX1 3BD; ^fDepartment of Physiology, University of Melbourne, Victoria, Australia 3010

Address correspondence to: Dr. Hongyu Luo CRCHUM, 900 Saint-Denis Street, Room R12.426, Montréal, Québec, Canada H2X 0A9, Telephone: (514) 890-8000 Extension 25319, Fax: (514) 412-7944, e-mail: hongyu.luo@umontreal.ca; or Dr. Jiangping Wu, CRCHUM, 900 Saint-Denis Street, Room R12.428, Montréal, Québec, Canada H2X 0A9, Telephone: (514) 890-8000 Extension 25164, Fax: (514) 412-7944, e-mail: jianping.wu@umontreal.ca

Footnote: *Z.Z. and J.T. contributed equally to this work.

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Conflict of interest

The authors declare no conflict of interest.

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Abstract

Objective: This study investigated the association of genetic variants of *EPHA4*, a receptor tyrosine kinase, with hypertension, and the role of *EPHA4* in vascular smooth muscle cell (VSMC) contractility.

Methods: Data from two human genetic studies, ADVANCE and HCHS/SOL, were analysed for association of *EPHA4* single nucleotide variants (SNVs) with hypertension risks. The effect of *EPHA4* signalling and sex hormones on mouse vascular smooth muscle cells contractility was assessed.

Results: We identified a single-nucleotide variant (rs75843691 hg19 chr2:g.222395371 C>G), located in the 3rd intron of *EPHA4* gene, as significantly associated with hypertension in human female subjects (p -value = 8.3×10^{-4} , below the *Bonferroni*-corrected critical p -value) but not male subjects with type 2 diabetes from the ADVANCE clinical trial. We found that *EPHA4* was expressed in VSMCs and its stimulation by solid-phase anti-*EPHA4* antibody led to reduced VSMC contractility triggered by phenylephrine. Estrogen enhanced the contractility-lowering effect of *EPHA4* stimulation. Conversely, small interfering RNA knockdown of *Epha4* expression in VSMCs resulted in increased contractility of VSMCs from female but not from male mice.

Conclusion: *EPHA4* is a sex-specific hypertension risk gene. Forward *EPHA4* signalling reduces VSMC contractility, and estrogen is a modifier of this effect. The effect of *EPHA4* on VSMCs contractility explains the association of *EPHA4* gene with hypertension risks in females.

Condensed Abstract

We found through a human genetic study that a variant in the *EPHA4* gene was associated with hypertension risks in females. In mice, EPHA4 signalling plus estrogen reduced vascular smooth muscle contractility. Thus, EPHA4 is a new hypertension risk gene and likely exerts its function in blood pressure control via VSMC contractility.

Key words: EPHA4, vascular smooth muscle cells, estrogen, single-nucleotide polymorphism, gene variants, *ADVANCE*; hypertension

Introduction

Erythropoietin-producing hepatocellular receptor kinases (EPHs) are the largest family of receptor tyrosine kinases. They are divided into A and B subfamilies, according to their sequence homology¹. Their ligands, ephrins (EFNs), are cell surface molecules². They are also divided into A and B families, based on the way they anchor on the cell membrane: EFNA members anchor to the cell membrane via glycosylphosphatidylinositol, while EFNB members are transmembrane proteins. EPHs receive stimulation from EFNs and transduce signals into cells, via a so-called canonical forward signalling. Although they are ligands, EFNs can also respond to stimulation from EPHs and transmit signals into cells via non-canonical reverse signaling². EPHs interact with EFNs promiscuously, but generally EPHAs bind preferentially to EFNAs, and EPHBs bind to EFNBs.

EPHs and EFNs are expressed in many tissues and organs. These kinases and their ligands play important roles in the central nervous system², the immune system³⁻⁹, the digestive system¹⁰, bone metabolism^{11,12}, angiogenesis¹³ and several other processes¹⁴⁻¹⁶. In a series of recent publications¹⁷⁻²³, we reported that some of EPHB and EFNB family members are implicated in blood pressure (BP) regulation. The vascular smooth muscle cells (VSMCs) are the major target cells of these molecules. In mice, EPHB6, EFNB1 and EFNB3 deletion leads to increased BP^{17,19,20}, while EPHB4 and EFNB2 deletion lowers it^{18,21}. Our human genetics studies have provided corroborating evidence that 5 single-nucleotide variants (SNVs) in the *EFNB2* gene²¹ and 2 SNVs in the *EFNB3* gene are significantly associated with human hypertension²³. Therefore, these EPH receptors and EFN ligands can be seen as yin and yang regulators of BP.

So far, the involvement of EPHA and EFNA family members in BP regulation is largely unknown. Although EPHA4 is an EPHA family member, it can interaction with EFNB family ligands such as EFNB1 and EFNB3. Further, Sallstrom *et al.*²⁴ generated mice with EPHA4 intracellular tails replaced by enhanced green fluorescence protein. These mice have high incidence of congenital hydronephrosis and those that have it manifest hypertension. These features of EPHA4 prompted us to investigate its role in hypertension.

Our human genetic study showed that a variant in the 3rd intron of *EPHA4* was significantly associated with hypertension in females but not in males. In mice, EPHA4 forward signalling controlled VSMC contractility in an estrogen-dependent manner, and this is likely one of the mechanisms by which EPHA4 regulates BP.

Materials and Methods

The ADVANCE (Action in Diabetes and Vascular Disease: Preterax and Diamicron-MR Controlled Evaluation) cohort

The *ADVANCE* cohort consisted of 3,368 type 2 diabetic (T2D) patients of Caucasian origin from *ADVANCE* trial, a factorial, multi-center, randomized, controlled clinical trial of 11,140 participants recruited from 215 centers in 20 countries^{25,26}. All individuals were T2D subjects age 65 years or older, or age 55 years or older if they had 1 of the following: a history of major macrovascular disease, a history of major microvascular disease, a diagnosis of T2D over 10 years

prior to entry into the study, or presence of another major risk factor for vascular disease, including smoking, dyslipidemia and microalbuminuria.

Among the 3,368 genotyped patients, there were 1,828 hypertensive and 402 normotensive males and 942 hypertensive and 196 normotensive females. Hypertension as was defined as having a physician's diagnosis of hypertension with a history of treatment with hypertension medications (calcium channel blockers, angiotensin II receptor antagonists, angiotensin-converting-enzyme inhibitors, beta-blockers, or diuretics such as thiazide, thiazide-like or others) or having systolic BP (SBP) >140 mmHg and diastolic BP (DBP) >90 mmHg at study entry.

Genotyping of the ADVANCE cohort

The 3,368 patients were genotyped on Affymetrix Genome-Wide Human SNV Array 5.0 or on Array 6.0 at the CRCHUM Genomic Platform. Only those SNPs with greater than 90% call rate were included in the analysis and only those SNPs that were in Hardy-Weinberg equilibrium at a p -value $> 10^{-3}$ were kept for analysis. Additional SNPs were imputed for each array using the IMPUTE2 program²⁷ and the 100 genome CEU linkage disequilibrium (LD) reference panel. Only those SNPs with an imputation score greater than 0.80 were retained. A subset (147,088) of the genome wide genotyped SNPs that were not in LD (defined as $r^2 \leq 0.8$) was selected to perform a principal component (PC) analysis with Eigenstrat software²⁸ to test for population stratification within the Caucasian population samples. The first 2 independent PCs from this analysis (PC1 and PC2), were subsequently considered as covariates in association analysis to correct for population stratification.

Association analysis of the ADVANCE cohort

Association analysis was performed separately for Array 5.0 and 6.0 datasets and for male and female subjects separately for the genotyped and imputed variants within the *EPHA4* gene region using PLINK software²⁹. Samples genotyped on the two different Affymetrix chips were combined by meta-analysis separately for males and females, using the meta-analysis subroutine of PLINK 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.

The association with hypertension was tested for 414 SNVs, both genotyped and imputed, which were common to both Array 5 and 6 sub-analyses, and which fell within the *EPHA4* gene or within additional regions 10 kb 5' and 3' of the gene and located within a 174.3-kb region between positions 222,272,747 and 222,447,010 on chromosome 2 (Build 37, hg/19). Only SNVs with minor allele frequency (MAF) greater or equal to 10% were analyzed, as association analysis has reduced power for SNPs with minor allele frequency below 10%. The MAF filter was applied after across-the-board genotyping, calculated over all samples.

Differences in several candidate covariate phenotypes between hypertensive and normotensive subjects were calculated and tested for significance using either a *t* test for quantitative variables or a χ^2 test for qualitative data. The values of age, body mass index (BMI) and chronic kidney disease in the cases are significantly higher than that in the controls (Supplementary Table 1 (S-Table 1)). As these factors could be causative to hypertension, we include them as covariates in the logistic regression analysis, to eliminate their influence on the results. Accordingly, a logistic regression model was used to perform association analysis, in which additive genotype coding, PC1 and PC2

from the stratification analysis, BMI and presence or absence of chronic kidney disease (CKD; defined as an estimated glomerular filtration rate less than 60 ml/min/1.73 m²) were used as covariates,

Qualitative analysis was conducted separately for 1,828 hypertensive and 402 normotensive males and 942 hypertensive and 196 normotensive females using logistic models both with the inclusion of the covariates. Genotypes were coded as number of copies of the reference allele, which is equivalent to an additive genetic model.

Differences in odds ratios (ORs) between males and females for the most significant SNVs were also tested for significance according to the method proposed by Altman and Bland³⁰.

Although case-control qualitative analysis is a more appropriate method for these data, we conducted genetic analysis of quantitative blood pressure measurements as well, to see whether we could obtain additional information. The *ADVANCE* sample consists of diabetics being studied for diabetes complications. Eighty two percent of the males and 82.8% of the females were affected by hypertension, and 96% of these hypertensive patients were being treated for hypertension to lower blood pressure. Ten mmHg were added to DBP measurements and 15 mmHg were added to SBP measurements for each treated subject as suggested by Tobin and coworkers³¹ and as was done for the International Blood Pressure Consortium hypertension meta-analysis³². Linear regression analysis was performed for these modified quantitative measurements against the additive genetic coding for each SNV, with and without the covariates described above. Effect sizes are reported as β coefficient for the genotype term.

Significance of associations of the ADVANCE cohort

The number of tag SNVs, proxy for all SNVs tested for associations within the above-described 174.3-kb *EPHA4* gene region, was determined employing the Tag SNV selector on the National Institutes of Health SNPinfo website³³, using a minimum r^2 value >0.8 and a minimum MAF of 0.10. Forty tag SNVs were identified and were considered to represent 40 conservatively independent LD blocks within the 174.3-kb test region covered by the analyzed SNVs around *EPHA4* gene. Subsequently, all p -values were corrected for multiple testing for 40 independent LD blocks, giving a *Bonferroni*-corrected p -value of 1.25×10^{-3} or a critical $-\log_{10} p$ -value of 2.90.

Plots of associations between SNVs and hypertension across the *EPHA4* gene region for males-only and females-only samples were constructed with LocusZoom³⁴.

The HCHS/SOL (Hispanic Community Health Study/Study of Latinos) cohort

HCHS/SOL is a community-based cohort study of 16,415 self-identified Hispanic/Latino individuals aged 18-74 years and selected from households in predefined census-block groups across four US field centers (Chicago, IL; Miami, FL; Bronx, NY; and San Diego, CA)^{35,36}. Most study individuals identified as one of the six background groups: Central American, Mexican, South American, Cuban, Dominican, or Puerto Rican. The *HCHS/SOL* baseline clinical examination occurred between 2008 and 2011 and included comprehensive biological, behavioural, and sociodemographic assessments. Initially, 12,803 participants consented for genetic studies and were genotyped. Later, some of the individuals withdrew their consent and

therefore did not participate in the current analysis. The current study included 12,126 participants who consented for genetic studies and were eligible.

As shown in S-Table 2, there were 4,972 males and 7,154 females in the current analysis. The two sex groups have similar characteristics, with females being two years older than males on average (mean age 47 and 45, respectively), the same proportion of hypertensive individuals (28%), but slightly lower quantitative measures of BP (5 mmHg lower mean SBP and 2 mmHg lower mean DBP).

The *HCHS/SOL* cohort was also stratified based on BMI and age (BMI ≥ 25 and age > 50 years old). The patient number and all the relevant parameters of this smaller sub-cohort are shown in S-Tables 3.

Genotyping, quality control and imputation of the HCHS/SOL cohort

Consenting *HCHS/SOL* participants were genotyped at Illumina on the *HCHS/SOL* custom 15041502 B3 array. The custom array comprised the Illumina Omni 2.5M array (HumanOmni2.5–8v.1-1) ancestry-informative markers, known GWAS hits and drug absorption, distribution, metabolism, and excretion markers, and additional custom content including ~150,000 SNVs selected from the CLM (Colombian in Medellin, Colombia), MXL (Mexican Ancestry in Los Angeles, California), and PUR (Puerto Rican in Puerto Rico) samples in the 1000 Genomes phase 1 data to capture a greater amount of Amerindian genetic variation³⁷.

We applied standardized quality-assurance and quality-control (QA/QC) methods³¹ to generate recommended SNV- and sample-level quality filters. Samples were checked for sex discrepancies, gross chromosomal anomalies, relatedness and population structure, missing call rates, batch effects, and duplicate-sample discordance. SNVs were checked for Hardy-Weinberg equilibrium, MAF, duplicate-probe discordance, Mendelian errors, and missing call-rates. A total of 12,803 unique study participants passed QC and met specific clinical inclusion criteria. A total of 2,232,944 SNVs passed filters for both quality and informativeness (polymorphic and unduplicated) and were carried forward for imputation and downstream association analyses.

Genome-wide imputation was carried out with the full, cosmopolitan 1000 Genomes Project phase 1 reference panel (n=1,092). The *HCHS/SOL* samples were imputed together with genotyped SNVs passing the quality filter and representing unique genomic positions on the autosomes and non-pseudoautosomal portion of the X chromosome. Genotypes were first pre-phased with SHAPEIT2 (v.2.r644) and then imputed with IMPUTE2 (v.2.3.0). All imputed genotypes in the present study had imputation quality score ‘info’ reported by IMPUTE2 ≥ 0.9 .

Association analysis of the HCHS/SOL cohort

We conducted qualitative analysis of the association of SNVs with hypertension, which was defined by an indication of antihypertensive drug use, or by either SBP ≥ 140 mmHg or DBP ≥ 90 mmHg. We also carried out quantitative analysis of the association of SNVs with SBP and DBP. For those under anti-hypertension medication, 15 mmHg was added to their SBP, and 10 mmHg, to their DBP for the quantitative analysis. For all the 3 analyses, we excluded 95 individuals with inconsistencies in their measured SBP or DBP (Omron mean and mean of raw measures difference

≥ 5 mmHg), 19 individuals with high degree of Asian ancestry, 328 individuals with missing covariates or outcomes, and 70 individuals with either SBP<80 or DBP<50, and finally 162 individuals who withdrew consent for genetic studies.

To study the association between genotypes and any trait of interest within sex groups, while controlling for population structure, we use mixed models, fitted in each of the sex groups separately, either logistic for qualitative analysis, or linear for the quantitative analysis of SBP and DBP³⁸. All models were adjusted for sex, age, age squared, study center, BMI, background group, sampling weights (to prevent potential selection bias resulting from the study design), and the 5 first principal components (estimated from the autosomal chromosome) as fixed effects. We used a random effect for genetic relatedness (kinship), and random effects accounting for environmental correlations corresponding to household and community (block unit). For the quantitative traits, we used a heterogeneous variances model, which allowed for different residual variance for each of the background groups.

Mice

In these experiments, 10- to 15-week-old C57BL/6J mice were housed in specific pathogen-free rooms with 12-h light and 12-h dark cycles. All experimental protocols were approved by the Animal Protection Committee of the Research Centre, Centre hospitalier de l'Université de Montréal.

VSMC isolation

Mouse VSMCs were isolated as described by Golovina and Blaustein³⁹, with modifications¹⁷. Briefly, the aorta and mesenteric arteries, including their secondary branches, were digested with type 2 collagenase (255 units/ml; LS004177, Worthington Biochemical Corporation, Lakewood, NJ, USA). The tissues were cut into small pieces (around 0.1 mm³) and subjected to a second digestion with type 2 collagenase (255 units/ml) and type IV elastase (from porcine pancreas; 6 units/ml, E0258, Sigma-Aldrich, Oakville, ON, Canada). Dissociated cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum (FBS) and penicillin (100 IU/ml)/streptomycin (100 µg/ml) at 37°C in 5% CO₂ atmosphere for 4-5 days before experimentation.

In some experiments, cell culture plates were coated with goat anti-mouse EPHA4 antibody (αEPHA4-Ab, AF641, R&D Systems, Minneapolis, MN, USA; 2 µg/ml during coating), normal goat IgG (NGIgG; 2 µg/ml during coating), recombinant mouse EPHA4 tagged with human IgG Fc (EPHA4-Fc, 641-A4, R&D Systems; 10 µg/ml during coating) or normal human IgG (NHlgG; 10 µg/ml during coating) overnight at 4°C with gentle shaking.

Immunofluorescence microscopy

Isolated mouse VSMCs, cultured on cover slips for 4-5 days, were washed twice with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. For cell surface EPHA4 staining, the cells were first blocked with 10% FBS in PBS for 1 h on ice, followed by incubation overnight at 4°C with goat anti-mouse EPHA4 Ab (15 µg/ml; R&D Systems, Minneapolis, MN, USA). The cells were then reacted with FITC-conjugated donkey anti-goat Ab (705-095-147, Jackson ImmunoResearch Laboratories, West

Grove, PA, USA; 1.5 µg/ml) for 2 h at room temperature. For intracellular α -actin staining, they were processed in permeabilization buffer (554715, BD Biosciences, San Diego, CA, USA) on ice for 20 min, incubated with mouse anti-human α -actin Ab (sc-32251, Santa Cruz Biotechnology, Mississauga, ON, Canada; 200 ng/ml) for 2 h at room temperature, followed by exposure to rhodamine-conjugated goat anti-mouse Ab (115-026-003, Jackson ImmunoResearch Laboratories; 1.4 µg/ml) for 2 h at room temperature. Finally, the cells were mounted with ProLong® Diamond Antifade Mountant with DAPI (P36966, Thermo Fisher Scientific, Burlington, ON, Canada) and examined under a Zeiss microscope (Axio Observer 3, Carl Zeiss Canada, Toronto, ON, CA), equipped with a fluorescence illuminator (X-Cite series 120Q, Lumen Dynamics Group Inc, Mississauga, ON, CA). The images were taken by an AxioCam MRm CCD camera driven by AxioVision v4.8.1.0 software (Carl Zeiss) through a 40X oil immersion objective.

Human mesenteric arteries including intima, tunica media and adventitia were paraffin-embedded and then sectioned. Five-micrometer thick sections were de-paraffinized in xylene and rehydrated in graded ethanol concentrations to PBS, followed by antigen retrieval. Double immunofluorescence staining was performed with rabbit anti-human EPHA4 Ab (ab5389, Abcam, Cambridge, MA USA; 0.014 µg/µl) at 4°C overnight, followed by Cy3-conjugated goat anti-rabbit Ab (Servicebio, GB21303, WuHan, China; diluted 1:300) for 1 hour at room temperature. Normal rabbit IgG at the same concentration as that of anti-EPHA4 Ab was used as an isotypic control of the first Ab. All sections were counterstained with DAPI. Images were acquired by a Nikon Eclipse C1 microscope (Nikon, Tokyo, Japan) and were with 3D Histech software (3DHISTECH Ltd, Budapest, Hungary).

Measurement of VSMC contractility

VSMC contractility was measured as described previously^{17,18}. Briefly, primary VSMCs were cultured for 4-5 days. In some experiments, estradiol (100 ng/ml, Sigma-Aldrich) or testosterone (6.5 ng/ml, Sigma-Aldrich) was added at the beginning of the culture. The plates were viewed in the chamber of a Zeiss microscope with environmental controls (37°C and 5% CO₂). The cells were stimulated with (R)-(-)-phenylephrine hydrochloride (PE, 20 µM, P6126, Sigma-Aldrich), and images were captured continuously for 15 min at 1 frame per min. Fifteen or more cells were selected randomly with their length measured at the time points indicated, using Carl Zeiss Axiovision software. The results are reported as contraction percentages (% contraction) calculated as follows:

$$\% \text{ contraction} = 100 \times (\text{cell length at time 0} - \text{cell length at time X}) / \text{cell length at time 0}.$$

Means and standard error of the means (SEM) of contraction percentages of 15 or more cells were presented.

Reverse transcription real time-quantitative polymerase chain reaction (RT-qPCR)

Mouse *Epha1-8*, *Epha10*, *Ephb1-4* and *Ephb6* mRNA levels were measured by RT-qPCR as described previously¹⁷. Total RNA from VSMCs was extracted by TRIzol Reagent (15596018, Life Technologies, Burlington, ON, Canada) and then reverse-transcribed with iScript™ Reverse Transcription Supremix for RT-qPCR (1708841, Bio-Rad Laboratories, Mississauga, ON, Canada). The PCR conditions were: 3 min at 95°C, followed by 45 cycles of 10 s at 94°C, 20 s at 58°C, and 20 s at 72°C. β-actin mRNA levels served as internal controls. The results are expressed

as signal ratios of test gene mRNA/ β -actin mRNA. S-Table 4 lists primer sequences and amplified fragment sizes.

Human *Epha4* and *Ephb6* mRNA expression in mesenteric artery smooth muscle cells were similarly measured by RT-qPCR. Mesenteric arteries were obtained from organ donors. Adventitia was removed from the vessels with scissors, and endothelium of the vessels was scrapped off with knife blades. The cleared vessels were then stored at -80°C until use. Total RNA was extracted from the frozen samples according to RNeasy protocol (Axygen, Union City, CA, USA). RNA was reverse transcribed with a Takara reverse transcription kit (Takara, Kusatsu, Japan). Real-time quantitative PCR was performed with StepOnePlus Real-Time PCR System following the Takara SYBR-Green protocol (Takara). The primers used and fragment size are listed in S-Table 5. Samples were assayed in triplicate. The PCR conditions were: 30s at 95°C, followed by 40 cycles of 5 s at 95°C, 30 s at 60°C. Two- $\Delta\Delta$ CT was calculated. β -actin was used as an internal control. The results are expressed as mean \pm SE of signal ratios of test gene mRNA/ β -actin mRNA.

Ca²⁺ influx measurements

PE-triggered Ca²⁺ influx in VSMCs was measured as described elsewhere¹⁷ with some modifications. Briefly, VSMCs were incubated in DMEM containing 15% FBS and 5 μ M Fura-2-AM (F1201, Life Technologies) for 60 min at 37°C. The cells were washed twice in warm DMEM containing 15% FBS to remove extracellular dye, and then cultured in warm Hank's balanced salt solution (HBSS) with Ca²⁺ (1.26 mM). They were placed in the chamber of a Zeiss microscope with environmental controls (37°C and 5% CO₂), stimulated with PE (20 μ M), and imaged at 1

frame per 3 s for 2 min. Excitation wavelengths were switched between 340 nm and 380 nm, and emission was recorded at 510 nm. Signals from more than 15 randomly-selected cells were analyzed, and the results are reported as ratios of fluorescence intensity at 510 nm excited at 340 nm versus 380 nm.

Small interfering RNA (siRNA) transfection

S-Table 6 shows the sequences of 2 sets of *Epha4* siRNAs (Integrated DNA Technologies, Coralville, IA, USA) tested in this study. The control siRNA set was also purchased from the same company. VSMCs were cultured for 2 days in DMEM with 15% FBS, then in antibiotic-free medium and transfected with a mixture of 2-sets of *Epha4* siRNA or 1-set of control siRNA (final concentration at 40 nM) and Lipofectamine[®] 2000 Transfection Reagent (11668-027, Invitrogen, Burlington, ON, Canada). Cell contractility was measured after 48 h.

Ethics statement

The human genetics study was approved by respective institutional review boards of participating investigators. Human mesenteric arteries were obtained from organ donors, and this *in vitro* study was approved by the Ethics Committee of the First Affiliated Hospital of Zhejiang University and informed consent was obtained from donors' families. Mouse studies were approved by the Animal Protection Committee of CHUM Research Center.

Results

Results of ADVANCE human genetic studies

In the ADVANCE cohort, 37 SNVs were found to be associated with hypertensive status at nominally significant level of $p \leq 0.01$ in the smaller female-only sample based on qualitative logistic regression analysis with covariates (Table 1). This dataset was deposited into GWAS Central (<http://www.gwascentral.org/study/HGVST1850>). P -values, odds ratios (ORs) of association and 95% confidence intervals (95% CI) for the odds ratios of the 37 SNVs from males-only and females-only samples are reported in this table. No SNVs were associated at the nominally significant level of $p \leq 0.01$ in the larger male-only sample. One SNV, *rs75843691* was significantly associated with hypertension in females-only samples at a *Bonferroni*-corrected significant p -value of 8.3×10^{-4} .

According to the GAS power calculator by University of Michigan Center for Statistical Genetics⁴⁰, with a sample size of 940 female hypertensives and 200 female normotensives in our ADVANCE study, and with relative risk of 1.79 and a p -value of 0.00083, we have 92.5 % statistical power.

Figure 1 presents LocusZoom plots illustrating association plots of all 363 SNVs from males-only (A) and females-only (B) samples. The LD in LocusZoom plots always refers to the most highly associated variant. In the males-only plot a different variant, *rs57493772*, happens to have the lowest (probably randomly as it is not significant) p -value. This SNV is in low LD with all of the others. There are many variants in LD with the most significant variant *rs75843691* in the females-only plot. This SNV is located in the 3rd intron in the 5' region of *EPHA4*, in high LD with many nominally ($p < 0.01$) significantly associated variants across the 3rd intron, suggesting a functional variant(s) located somewhere within in this 5' region of the gene. There was a second independent

signal suggesting modest association in the 3' region of the gene. However, none of the associations in this region were significant after *Bonferroni* correction.

The results for the Bland-Altman tests³¹ of significant differences in ORs for variant *rs75843691* between male and female subjects are shown in S-Table 7. The 95% CI of ORs for association in the males-only samples were between 0.76 and 1.20. The 95% CI of ORs in the females-only sample were between 1.27 and 2.52, which was significantly different from that of the males-only sample. ORs indicated by the Altman-Bland test are the ratios of the male to female ORs. The expected value was 1.0, which lay outside the CI of 0.36 to 0.81, and the *p*-value for the differences in ORs was 3.0×10^{-3} , indicating that the association for females was statistically different from that for males.

A quantitative linear regression analysis of SNVs with BP measurements after adjusting for medication was performed, but none of the queried SNVs were significantly associated with SBP or DBP in either males-only or females-only groups (data not shown). The possible reasons are explained in the section of Discussion.

Results of the HCHS/SOL genetic study

The 37 *EPHA4* SNPs that were found to be associated with hypertension at nominally significant level of $p \leq 0.01$ in the ADVANCE cohort were also analyzed for their association with hypertension in the *HCHS/SOL* cohort. In a qualitative analysis of the whole cohort of 12,126 individuals, none of the SNVs were associated with hypertension even at a nominally significant level of $p < 0.05$ (data not shown). Quantitative analysis of SBP and DBP showed that only one

SNV rs13035425 had a nominal p -value < 0.05 for association with DBP (detailed data now shown), but the p -value was of course not significant after *Bonferroni* correction.

To mimic the composition of *ADVANCE* cohort, which is composed of individuals at least more than 55 years old and with T2D, we stratified the *HCHS/SOL* cohort and selected those with BMI>25 and age>50 years old, and obtained a much smaller 4,512-individual sub-cohort. Qualitative analysis of this sub-cohort did not reveal any significantly associated SNVs (data not shown). However, quantitative analysis showed that 4 SNVs and 2 SNVs had association with SBP and DBP, respectively, at p -values below the nominal significant level of < 0.05 (Tables 2 and 3). Interestingly, the association started to show sex bias: these SNVs were significant in males-only but not females-only group, as was the case in the *ADVANCE* study. A more detailed comparison of the results of the two cohorts was provided in the section of Discussion.

Expression of EPHA4 in VSMCs

We first demonstrated, by immunofluorescence, that EPHA4 was expressed in mouse VSMCs. Anti-EPHA4 Ab stained VSMCs (Fig. 2A, green), while isotypic control Ab gave no apparent signals (Fig. 2B). EPHA4-positive cells assayed were all α -actin-positive (Fig. 2C), and the co-expression of EPHA4 and α -actin is evident in the merged micrograph (Fig. 2D). EPHA4 expression in mouse VSMCs provided a molecular basis for its function in these cells.

EPHA4 expression in human VSMCs was also assessed. Figure 3A shows *EPHA4* mRNA expression in human mesenteric artery smooth muscle cells, with *EPHB6* mRNA expression included for comparison. *EPHA4* mRNA was expressed in this tissue, albeit at a level much lower

than EPHB6 mRNA. EPHA4 expression at the protein level in human mesenteric artery endothelial cells (Fig. 3B, panel **a**) and smooth muscle cells (Fig. 3B, panels **a** and **c**) was shown according to immunofluorescence microscopy. Isotypic control staining of the same tissues showed near nil signal (Fig. 3B, panels **b** and **d**). These results indicate the relevance of our *in vitro* mouse study to human physiology.

EPHA4 forward signalling decreases VSMC contractility with estrogen as a modifier

Whether EPHA4 affected VSMC contractility was explored. To test whether forward signalling altered contractility, VSMCs were stimulated with solid phase anti-EPHA4 Ab (Ab coated on wells). Such stimulation decreased PE-triggered VSMCs contractility in both male and female mice (Figs. 4A and 4B), although more so in female than male VSMCs. This sex difference in the presence of EPHA4 signalling (caused by solid phase anti-EPhA4 on the wells) was significant as shown in Fig. 4C. However, male and female VSMCs without EPHA4 signalling (cultured in isotypic Ab-coated wells) presented similar contractility (Fig. 4D), indicating that the sex difference is not intrinsic but depends on EPHA4 forward signalling.

Our previous study demonstrated that sex hormones act in concert with EPHB/EFNB to regulate VSMC contractility. Whether this was also the case with EPHA4 stimulation was, therefore, assessed. To avoid carry-over of sex hormones inside cells from the *in vivo* environment to *in vitro* experiments, male VSMCs were treated with estrogen (Figs. 4E and 4G), and female VSMCs with testosterone (Figs. 4F and 4H). Estrogen enhanced the contractility-lowering effect of EPHA4 forward stimulation in male VSMCs (cultured in wells coated with anti-EPHA4 Ab) in that the contractility of these cells became even lower (Fig. 4E). Such effect depended on the presence of

EPHA4 forward signalling. In its absence (in male VSMCs cultured in wells coated with normal goat IgG), estrogen did not significantly alter the contractility (Fig. 4G). On the other hand, testosterone had no obvious effect on female VSMC contractility, either in the presence (Fig. 4F) or absence (Fig. 4H) of EPHA4 forward stimulation.

We also added testosterone or vehicle to male VSMCs cultured with solid phase anti-EPHA4, but their contractility showed no significant difference (Supplementary Figure (S-Fig.) 1A), confirming that testosterone does not act in concert with EPHA4 to control the contractility. When estrogen or vehicle was added to female VSMCs in the presence of solid-phase anti-EPHA4, no difference in the contractility was observed neither (S-Fig. 1B), suggesting that the endogenous estrogen carried over from *in vivo* is already sufficient to act with EPHA4 to control the contractility.

To exclude the possibility that solid-phase anti-EPHA4 Ab altered the expression of other *Epha* family members, the mRNA of all *Ephas* (including *Epha4*) was analyzed after 4-day solid-phase anti-EPHA4 Ab stimulation (S-Figs. 2A and 2B). No significant differences in expression were observed in these cells, either from male or female mice, compared to those cultured in wells coated with normal goat IgG. The expression of *Ephbs* in these stimulated cells had no significant alternation neither (S-Figs. 2C and 2D). These results indicate that EPHA4 stimulation does not affect VSMC contractility by altering the expression of these molecules.

Ca²⁺ influx is a major signalling event that initiates VSMC contractility. However, no differences in Ca²⁺ influx between anti-EPHA4 Ab-stimulated and normal goat IgG-stimulated VSMCs were detected (S-Fig. 3). These data suggest that forward signalling (*i.e.*, from EFNs to EPHA4) via EPHA4 reduces PE-induced VSMC contractility, probably by altering the Ca²⁺ sensitivity of these cells rather than by altering Ca²⁺ influx.

We assessed whether EPHA4 reverse signalling (*i.e.*, from EPHA4 to EFNs) had a similar effect on VSMCs. For this purpose, male (Fig. 5A) or female (Fig. 5B) VSMCs were stimulated with solid-phase EPHA4 (EPHA4-Fc-coated on wells). Such stimulation had no effect on PE-triggered contractility. We can thus conclude that EPHA4 forward but not reverse signalling reduces VSMC contractility.

To further confirm the role of EPHA4 in VSMC contractility, EPHA4 expression was knocked down by *siRNA*. Reduced expression at the mRNA level in male and female VSMCs was confirmed by RT-qPCR (Figs. 5C and 5D). The knockdown of EPHA4 at the protein level was confirmed by immunofluorescence (Figs. 5E and 5F). It is to be noted that male and female VSMCs had no significant difference in EPHA4 expression at the protein level without or with *siRNA* knockdown (data now shown). Female (Fig. 5H) but not male (Fig. 5G) VSMCs with EPHA4 knockdown presented significantly increased PE-triggered contractility, compared to control *siRNA*-transfected cells.

Discussion

The present study evaluated whether EPHA4 is a hypertension risk factor. Our *ADVANCE* human genetic study identified a significant association of SPV rs75843691, which is in the 3rd intron of EPHA4, with hypertension in T2D females. This finding was supported by mouse studies: EPHA4 KO led to increased BP; EPHA4 knockdown in VSMCs from female mice resulted in augmented contractility.

We have reported previously that several EPH **B** family members EPHB6 and EPHB4, and their ligands EFNB1, EFNB2 and EFNB3, are implicated in the modulation of VSMC contractility and, consequently, BP¹⁷⁻²¹. The current study is the first to demonstrate that EPHA4, a member of the EPH **A** family is also associated with hypertension risks via the regulation of VSMC contractility. This is not surprising, as EPHA4, in addition to interacting with EFNA family ligands such as EFNA1 and EFNA3^{41,42}, can also interact with EFNB family members such as EFNB2⁴³ and EFNB3⁴⁴. Our previous study has shown that in mice EFNB3 deletion in VSMCs leads to increased contractility²⁰, and SNVs of the *EFNB3* gene is associated with hypertension risks in a sex-specific way²³. This is consistent with the current findings that EPHA4, one of the receptors of EFNB3, has a similar effect.

EPHA4 and its EFN ligands can transduce signals in both directions (*i.e.*, forwardly from EFNs to EPHA4, or reversely from EPHA4 to EFNs). We demonstrated that only forward signaling was capable of decreasing VSMC contractility. As EFNB3 is one of the EPHA4 ligands, this suggests the increased VSMC contractility and BP in EFNB3 knockout (KO) mice, documented in our

previous study²⁰, is possibly caused, at least in part, by loss of EFNB3 to EPHA4 forward signaling.

Our qualitative analysis of the *ADVANCE* cohort revealed that a SNV in the 3rd intron of *EPHA4* was significantly associated in females. However, No *EPHA4* SNVs were shown to be associated with BP in several large-scale genome-wide association studies (GWAS)⁴⁴⁻⁵¹, including a most recent one by Warren et al.⁵². GWAS require large sample size that can be reached by combining multiple subgroups, often with different clinical phenotypes, ethnical backgrounds or environmental conditions that increase genetic and phenotypic heterogeneity. Also, GWAS suffer from large multiple-testing penalties as millions of SNVs are tested. The present study was more focused, with defined inclusion criteria and a small multiple-testing penalty of 40 (for 40 independent LD blocks in the *EPHA4* gene). This could explain the different outcome of ours versus most other GWAS. One caveat of our study is that the *ADNAVCE* cohort comprises T2D patients, and our conclusion has to be restricted to such individuals until we identify the exact factor of this cohort that enhances the detection of the association.

It is to be noted that in the *ADVANCE* qualitative analysis, only SNV *rs75843691* is really significant after *Boferroni* correction with an OR of 1.79 in females. The OR applies specifically to the reference allele, which is G in the case of *rs758436* (OR > 1). One would expect a group of SNVs in the same LD block to be all associated with a similar OR. The allele frequency of the associated allele gives a clue to the likely presence of an LD block. There is a cluster of SNVs in the region with the allele frequency of the reference allele around 0.133 like that of *rs7583691*. All these SNVs have OR above 1. These are all probably located on the same positively associated

haplotype. There are other SNVs in the region with reference alleles that have a frequency of around 0.52, and these all have odds ratios < 1 . Possibly they represent a second haplotype association in this region that does not reach *Bonferroni* significance.

We also conducted quantitative analysis for the association of EPHA4 SNVs with BP measurement of the *ADVANCE* cohort. For those patients under anti-hypertensive treatment, 15 and 10 mmHg were added to their SBP and DBP, respectively. However, no SNVs, which were significant in the qualitative analysis, remained significantly associated with quantitative measures of SBP or DBP. The *ADVANCE* samples consisted T2D individuals being studied for complications. Eighty-two percent of the males and 82.8% of the females were cases affected by hypertension. This skewed case-control sample composition was a reflection of the fact that hypertension is common in diabetic samples. Furthermore, about 96% of the male and female cases had been treated for hypertension, so that the BP readings of approximately 79% of all subjects were artificially raised. Thus, the quantitative distribution of BP measurements was not representative of the normal population distribution and was highly skewed for artificially raised SBP and DBP values. This skewed distribution could be the cause of lack of significant association between SNVs and BP measurements in the qualitative analysis.

We queried a second cohort *HCHS/SOL*, which had a total of 12,126 individuals, much bigger than the *ADVANCE* cohort (a total of 3,368 individuals). None of the tested SNVs was found significantly associated with hypertension in this cohort, even at a nominal significant level of $p < 0.05$ in qualitative or quantitative analysis. However, after we restricted the cohort to individuals with BMI > 25 and age > 50 years to mimic the *ADVANCE* cohort, an interesting pattern

emerged. In spite of this much smaller sub-cohort of 4,512 individuals, there was an increase in the level of association between the SNVs and BP in the males-only analysis: 4 SNVs and 2 SNVs reached the nominal significant level of $p < 0.05$ for SBP and DBP, respectively, suggesting that EFNA4 might also play a role in BP regulation in this sub-cohort. By what mechanisms overweight/obesity and older age in both the *ADVANCE* cohort and *HCHS/SOL* sub-cohort improves the detection of the association remains to be investigated.

In the *ADVANCE* cohort, an EPHA4 SNV was associated with increased hypertension risks in females but not males. We have shown in mouse studies that EPHA4 forward signaling lowered VSMC contractility and this effect was enhanced by estrogen. Conversely, EPHA4 knockdown in the presence of estrogen augmented VSMC contractility, which would translate into increased BP. Hence, under a physiological condition, females benefit from EPHA4's BP lowering effect, but if they carry an allele harboring a loss-of-function EPHA4 mutation, they would have an increased risk hypertension, everything else (other genetic or environmental risk factors) being equal. For the males, as testosterone is not a co-modulator for EPHA4's effect on VSMC contractility and their estrogen level is very low, EPHA4's involvement in their BP regulation is not apparent.

The significant SNV rs75843691 (in the 3rd intron of the EPHA4 gene) identified in the *ADVANCE* study or other potentially significant SNVs revealed in the *HCHS/SOL* sub-cohort are not necessarily the actual functional mutations, which might lie in LD with the said SNVs and the real functional mutation could be in the coding region, or in the regulatory region at a long distance away from the marker SNVs we identified. One very surprising and unexpected result of the multiple GWAS studies done over the last decade or so is that most of the replicated significant

SNVs are not in exons. These initially unexpected results have led to a fundamental change in our model of what is a gene. Rather than confining the concept of a gene to a protein coding sequence, we are now discovering that there are a multitude of transcribed but untranslated RNA sequences (*e.g.*, micro RNAs, long intronic RNA sequences etc) that are now in the process of being confirmed as regulators of gene expression or exon splicing. In addition, it is known that enhancer/repressors can reside in introns⁵³, and such intronic enhancer/repressor can regulate gene expression levels. These possible reasons can well explain why intronic SNVs are significantly associated hypertension risks.

Additional genetic studies using reporter gene along with deletion or point mutations might be able pin-point these functional ones in the future.

There are 3 noted differences of the results from the two cohorts. 1) Those SNVs with nominal significant association in *HCHS/SOL* sub-cohort males were not the same as found in the *ADVANCE* cohort. 2) The nominally significant SNVs in *HCHS/SOL* sub-cohort males were negatively associated with hypertension risks, as they had negative β -coefficient (Tables 2 and 4). 3) In the *HCHS/SOL* sub-cohort, the nominally significant SNVs are found in males-only analysis, while in the *ADVANCE* cohort, the significant SNV is found in the females-only analysis. These differences could be due to different ethnic background of the two study populations. It is possible that in individuals with the Latino genetic background, there is a functional sex- or sex hormone-dependent gain-of-function mutation in LD with the marker SNVs, augmenting EPHA4 forward signalling, hence reducing VSMC contractility and hypertension risks in males, while in the Caucasian population, there is a different loss-of-function mutation in LD with the significant

SNV, causing an opposite effect in the female population. One thing in common in both cohorts is that EPHA4 is likely involved in BP regulation and hypertension risks.

The results of the present human and mouse studies are compatible with *in vivo* findings reported by Sallstrom *et al.*²⁴, who generated mice with the EPHA4 intracellular tail replaced by green fluorescence protein (hence, they become incapable of forward signaling), and found them to be hypertensive. Although these authors attribute the hypertensive phenotype to the hydronephrosis, it is entirely possible that VSMCs are also a target tissue of EPHA4, whose deletion increases VSMC contractility and this, in turn, contributes to the observed hypertensive phenotype in these genetically manipulated mice. Whether the BP increase in these mutant mice is sex- and sex-hormone-dependent has not been reported by Sallstrom *et al.*²⁴. It would be interesting to examine the BP of these KO mice of both sexes, with or without castration/ovariectomy.

Several questions related to sex hormones need to be addressed. If estrogen is crucial for EPHA4 function in VSMCs, why did EPHA4 knockdown in female VSMCs cause increased contractility, when no exogenous estrogen was added? When EPHA4 forward signaling in VSMCs was triggered by solid-phase anti-EPHA4 Ab, cell contractility in both male and female mice was depressed (Figs. 4A and 4B), while *siRNA* knockdown of EPHA4 expression resulted in increased VSMC contractility in female but not in male mice (Figs. 5G and 5H). Why did male VSMCs respond to EPHA4 stimulation and knockdown differently? If estrogen is required for EPHA4's effect on VSMC contractility, why was male VSMC contractility reduced after EPHA4 stimulation?

One possible explanation is that sex hormones have long half-lives, and some intracellular sex hormones remained (estrogen in VSMCs from female mice) after 4 days of culture, even in the absence of exogenous sex hormones. This explains why VSMCs from female mice presented increased contractility after EPHA4 knockdown (Fig. 5H). Secondly, it is possible that estrogen only acts as a modifier, and its effect could be overridden by strong EPHA4 signaling. In the case of *siRNA* knockdown, where VSMCs received EFN signaling from neighboring cells, signaling strength might not have been very strong because of low cell density and limited cell-cell contact in wells. Thus, estrogen carried over *in vivo* might have been sufficient to increase VSMC contractility after EPHA4 knockdown. When VSMCs were exposed to solid-phase anti-EPHA4 Ab (Figs. 4A and 4E), all cells were strongly stimulated, as half of their cell surface area (the bottom half) was in contact with anti-EPHA4 Ab. Such strong stimulation might have been sufficient to reduce male VSMC contractility but to a less extent, even without estrogen (Fig. 4A). However, estrogen could certainly enhance the contractility lowering effect of solid-phase anti-EPHA4 Ab. This is evidenced in Fig. 2C, which demonstrates that female VSMCs with carry-over intracellular estrogen were significantly lower in contractility compared to male VSMCs after anti-EPHA4 Ab stimulation. Further, male VSMCs in the presence of estrogen had their contractility further reduced after EPHA4 forward stimulation, compared to these without estrogen (Fig. 4E), proving that estrogen is indeed a modifier enhancing the effect of EPHA4 signaling. With that said, we cannot exclude the possibility that some genes coded by sex chromosomes might also be modifiers for the effect of EPHA4 in VSMC contractility.

EPHA4 and its cell surface ligands are in constant contact in VSMCs, and their control of VSMC contractility and BP does not serve a fast-acting purpose. Rather, EPHA4 signaling might function

to maintain homeostasis of VSMC contractility and BP in a fine-tuning capacity, for which estrogen is an effective modifier. Since the augmentation of VSMC contractility when EPHA4 is knocked down is estrogen-dependent, it follows that if females with EPHA4 loss-of-function mutations have high systemic estrogen levels due to hormonal replacement therapy or oral contraceptives, they will be at a higher risk of hypertension, even the exogenous estrogen level is only high during the peak of its pharmacokinetics. Indeed, a large-scale genetics study (40,000 cases) documented that hormone replacement therapy is associated with increased hypertension risks in menopause patients: about 3% of these patients develop *de novo* hypertension⁵⁴⁵³. Also, about 5% of females taking oral contraceptives incur *de novo* hypertension⁵⁵⁵⁴. It will be interesting to assess whether these subpopulations with *de novo* hypertension have *EPHA4* loss-of-function mutations.

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Figures and legends

A Males-only

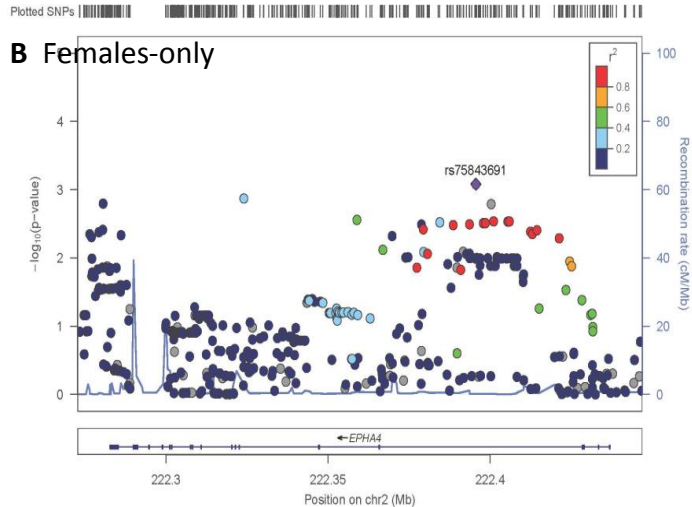
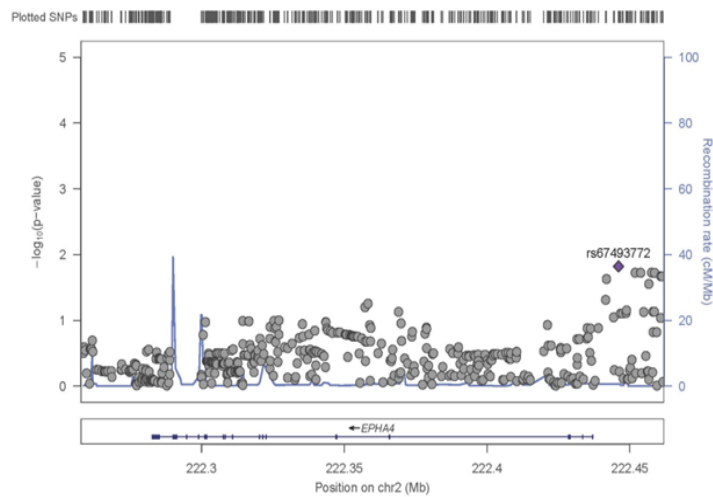


Figure 1. LocusZoom plots of $-\log_{10} p$ -values and r^2 values of EPHA4 SNVs in regions analyzed for association with hypertension and rates of recombination in the ADVANCE study

Symbols (circles and diamonds) and the left-hand vertical axis illustrate the $-\log_{10} p$ -values of SNPs. Lines and the right-hand vertical axis represent recombination rates in the chromosomal regions concerned. Symbol colors represent r^2 values. The horizontal axis indicates position in chromosomes. A. Males-only samples. B. Females-only samples.

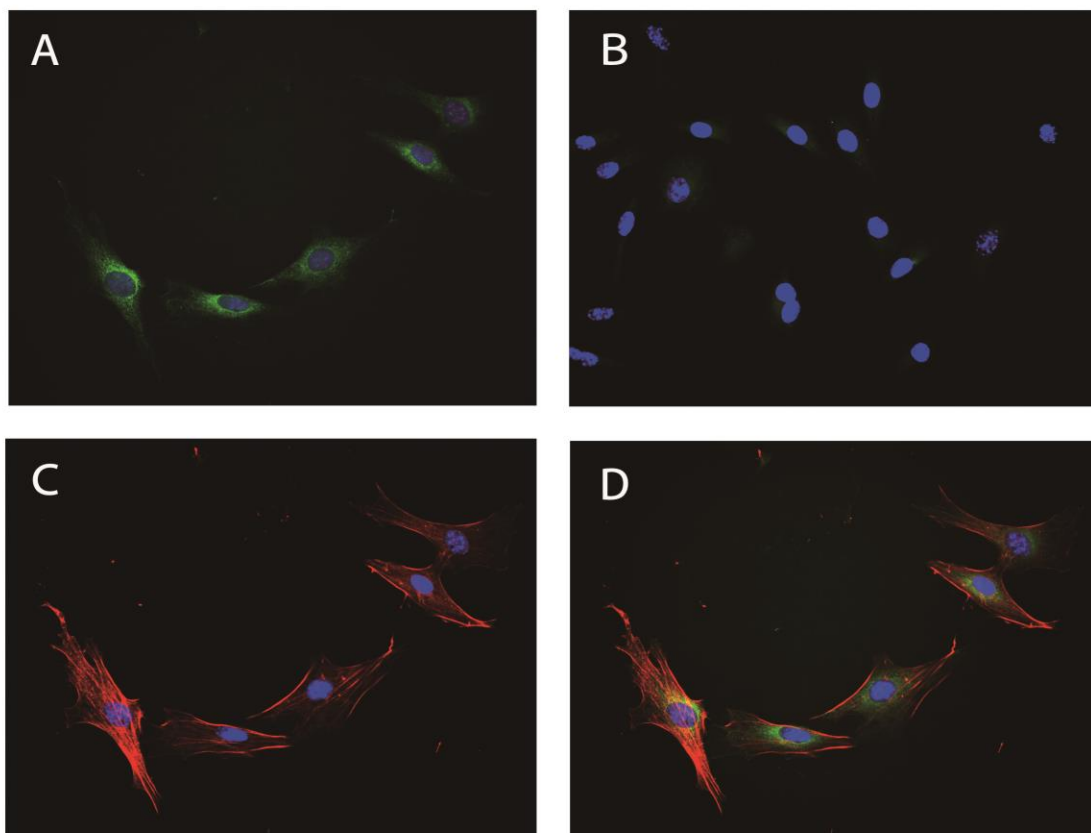


Figure 2. EPHA4 protein expression in mouse VSMCs according to immunofluorescence microscopy

Wild type VSMCs from male C57BL/6 mice were cultured for 4-5 days and stained with goat anti-EPHA4 Ab (**A**) or normal goat IgG (**B**) (both at 15 $\mu\text{g/ml}$), followed by FITC-conjugated donkey anti-goat secondary Ab (1.5 $\mu\text{g/ml}$, in pseudo green). For α -actin staining (**C**), the cells were permeabilized and stained with mouse anti-human α -actin Ab (200 ng/ml), followed by rhodamine-conjugated goat anti-mouse Ab (1.4 $\mu\text{g/ml}$, in pseudo-red). Nuclei were identified by DAPI staining (in pseudo-blue). The images in **A** and **C** are merged in **D** to show EPHA4 and α -actin staining at the same time. The experiments were repeated 3 times, and representative results are shown.

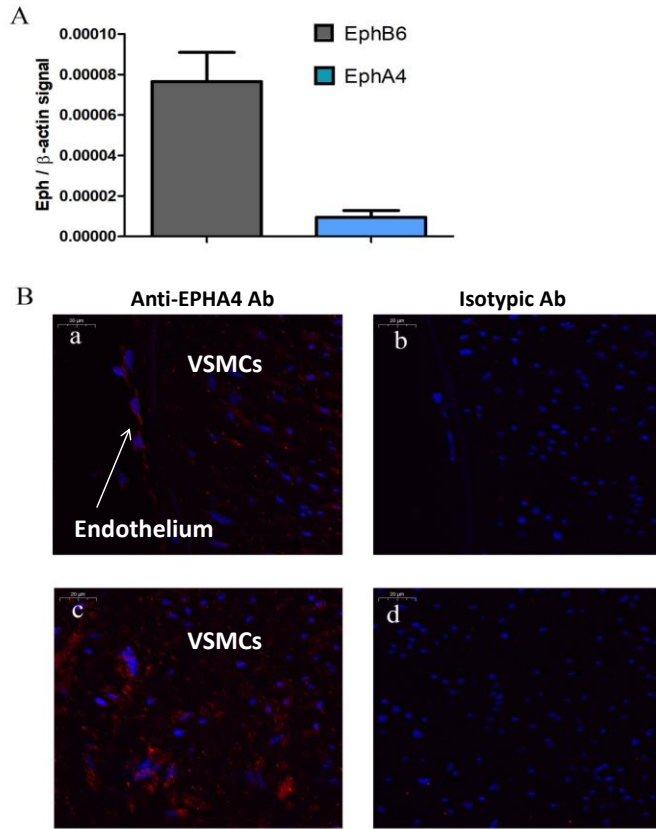


Figure 3. Expression of EPHA4 in human mesenteric arteries

A. EPHA4 mRNA expression in VSMCs from human mesenteric arteries according to RT-qPCR

Adventitia and endothelium of human mesenteric arteries were stripped off, and *EPHA4* mRNA expression in the remaining VSMCs was measured by RT-qPCR. Samples were in triplicate in RT-qPCR measurement. *EPHB6* mRNA expression was included as a reference to show the relative expression level of EPHA4. The results of 3 independent experiments are pooled and are expressed as mean \pm SE of ratios of EPH versus β -actin signals.

B. EPHA4 protein expression in human mesenteric arteries according to immunofluorescence microscopy

Human mesenteric arteries were cryo-sectioned, and stain with rabbit anti-human EPHA4 Ab (**A** and **C**; pseudo-red). Normal rabbit IgG was used as isotypic control Ab (**B** and **D**). Nuclei were stained with DAPI (pseudo-blue). Both endothelium (arrow) and VSMC stainings are shown in **A**, and VSMC staining is shown in **B**. Representative images of similar ones obtained in 3 independent experiments are shown.

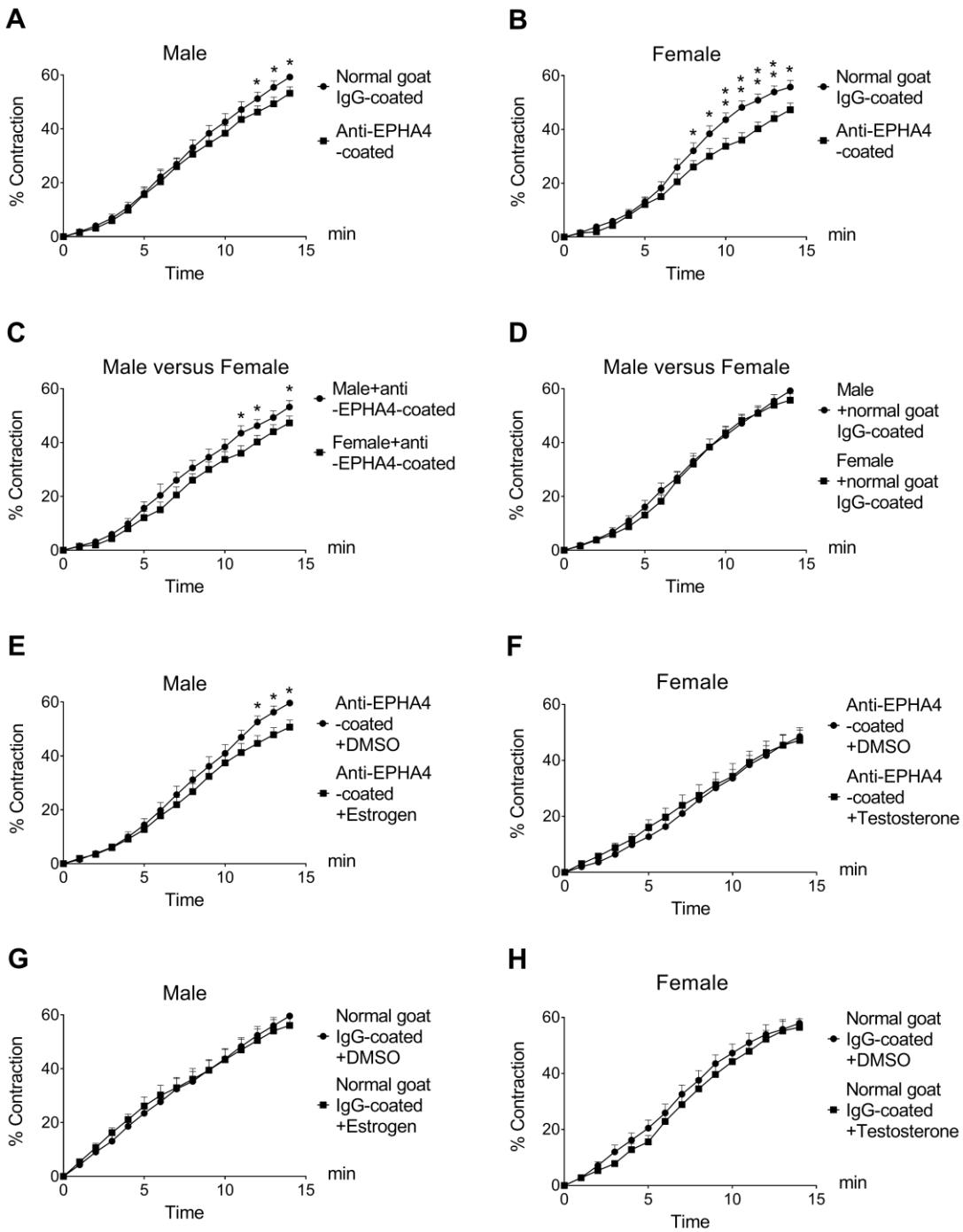


Figure 4. EPHA4 forward signaling regulates VSMC contractility in concert with estrogen

VSMCs from male and female C57BL/6J mice (as indicated) were cultured in wells coated with goat anti-mouse EPHA4 Ab or control normal goat IgG (NGIgG; both at 2 µg/ml during coating) for 4 days, in the absence or presence of estrogen (100 ng/ml) or testosterone (6.5 ng/ml). The cells were then stimulated with PE (20 µM) and imaged continuously for 15 min at 1 frame per min in a controlled environment (37°C in 5% CO₂). Images of 15 or more cells were selected randomly, with their length measured at the time point indicated. The results are expressed as means \pm SEM of contraction percentage (% contraction), calculated as follows: % contraction = 100 x (cell length at time 0 - cell length at time X) / cell length at time 0. * p <0.05; ** p <0.01: 2-tailed Student's t tests. The experiments were repeated 3 times and representative results are reported.

A and B. Reduced contractility of male (A) and female (B) VSMCs when stimulated with solid-phase anti-EPHA4 Ab

C. Lower contractility of female VSMCs stimulated with solid-phase anti-EPHA4 Ab compared to that of their male counterparts

D. Similar contractility of male and female VSMCs without solid-phase anti-EphA4 Ab stimulation

E. Estrogen enhances the contractility lowering effect of solid-phase anti-EphA4 Ab in male VSMCs

F. Testosterone has no effect on the contractility of female VSMCs stimulated by anti-EPHA4 Ab

G and H. Estrogen (G) and testosterone (H) have no effect on VSMC contractility from male and female mice, respectively.

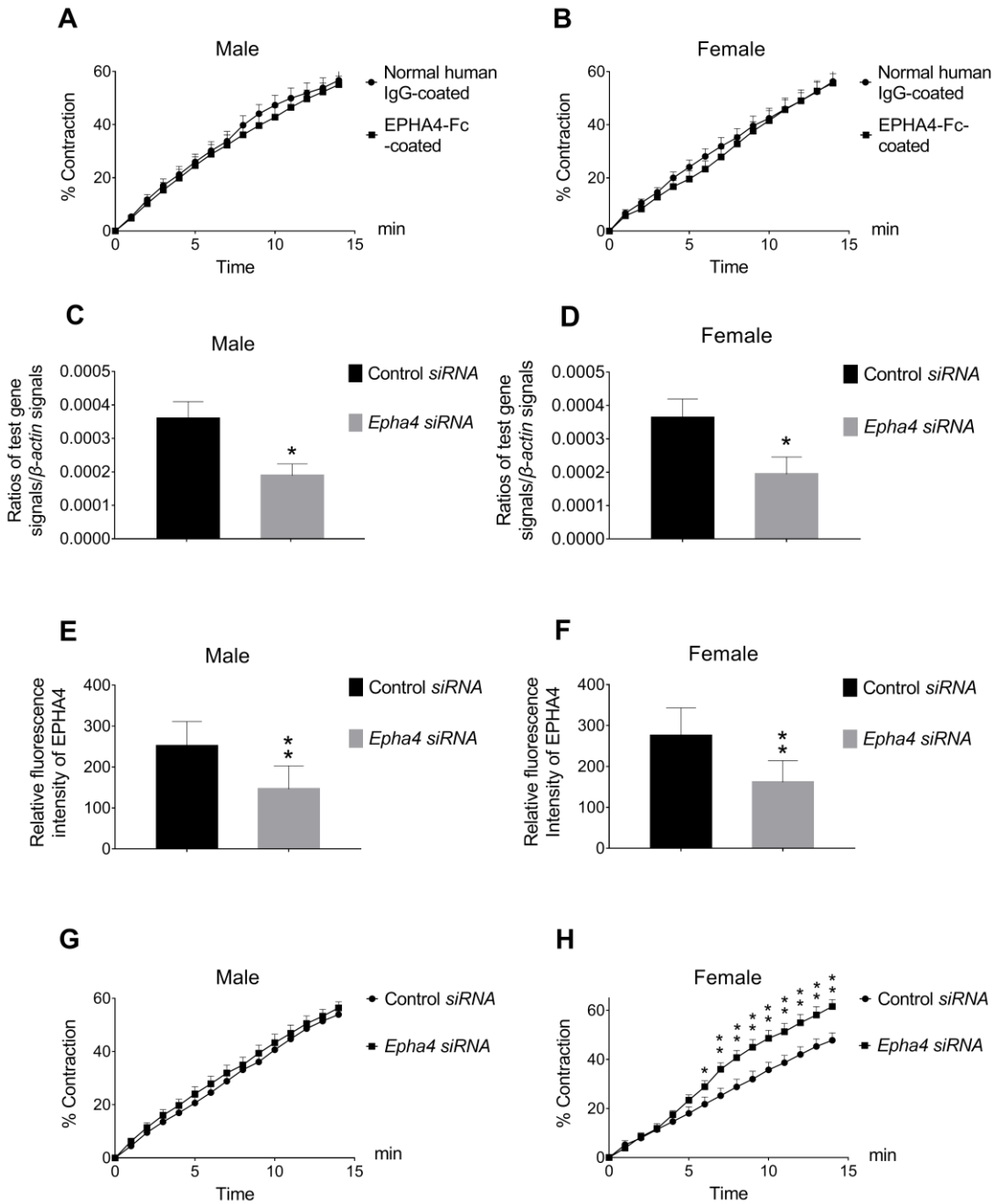


Figure 5. Effect of EPHA4 reverse signaling and knockdown in VSMC contractility

A-B. Reverse signaling from EPHA4 to EFNs does not affect VSMC contractility

VSMCs from male (A) and female (B) C57BL/6J mice were cultured in wells coated with EPHA4-Fc or NHIgG (both at 10 μ g/ml for coating). PE (20 μ M)-stimulated contractility was

ascertained after 4-5 days of culture. The experiments were repeated 3 times, and representative results are presented as means \pm SEM of contraction percentage of 15 randomly-selected cells. No significant difference between EPHA4-Fc and NHIgG-stimulated cells is evident (2-tailed Student's *t* test).

C-H. Increased contractility in female but not male VSMCs after EPHA4 knockdown

VSMCs from male and female C57BL/6J mice (as indicated) were cultured in uncoated wells. After 48 h, the cells were cultured in antibiotic-free media and transfected with *Epha4* siRNA (2 pairs of siRNA each at 20 nM) or control siRNA (40 nM). After another 48 h, *Epha4* mRNA levels in *Epha4* siRNA- and control siRNA-transfected cells were quantified by RT-qPCR. Pooled results from 3 independent experiments (mean \pm SD of ratios of *Epha4* signals versus β -actin signals) are shown in C and D. EPHA4 protein knockdown by siRNA in male and female VSMCs were shown in E and F according to immunofluorescence, in which means and SD of immunofluorescence intensity of more than 15 randomly selected cells in a representative experiment are presented. Background signal (isotypic Ab staining) was deducted from the anti-EPHA4 Ab staining signals. PE (20 μ M)-triggered contractility of these siRNA-transfected cells was measured at 48 h post-transfection (G and H). The experiments were repeated 3 times, and representative results are shown. The result was expressed as means \pm SEM of contraction percentage. **p*<0.05; ***p*<0.01: 2-tailed Student's *t* test.

Tables

Table 1. All SNPs associated at p -values ≤ 0.01 in either male or female ADVANCE subjects

SNV	Position Chrom 2 (Build 37/hg 19)	Coding Allele	Alternate Allele	Frequency Coding Allele	Males only				Females only			
					p -value	Odds Ratio	*Lower 95% CI	**Upper 95% CI	*** p -value	Odds Ratio	*Lower 95% CI	**Upper 95% CI
rs2052941	222276477	G	A	0.293	4.53E-01	0.94	0.79	1.11	4.48E-03	0.70	0.55	0.90
rs10432450	222277000	A	G	0.293	4.68E-01	0.94	0.79	1.12	4.93E-03	0.71	0.55	0.90
rs12989105	222279289	A	G	0.293	5.43E-01	0.95	0.80	1.13	4.18E-03	0.70	0.55	0.89
rs12996275	222280501	T	C	0.279	5.51E-01	0.95	0.79	1.13	3.86E-03	0.70	0.55	0.89
rs13013934	222280602	T	A	0.287	5.85E-01	0.95	0.80	1.14	1.59E-03	0.68	0.53	0.86
rs1036025	222286133	A	G	0.279	6.29E-01	0.96	0.80	1.14	4.63E-03	0.70	0.55	0.90
rs72963139	222324012	G	A	0.109	4.12E-01	0.90	0.69	1.16	1.34E-03	1.85	1.27	2.70
rs10498114	222358909	G	A	0.132	1.18E-01	0.83	0.66	1.05	2.76E-03	1.66	1.19	2.32
rs16862787	222366918	G	A	0.120	2.78E-01	0.87	0.68	1.12	7.58E-03	1.62	1.14	2.30
rs13035425	222369776	C	T	0.347	6.16E-01	0.96	0.81	1.13	4.76E-03	1.40	1.11	1.78
rs2710502	222373951	C	G	0.428	5.68E-01	0.95	0.82	1.12	9.84E-03	1.34	1.07	1.67
rs2710503	222374699	C	G	0.428	6.15E-01	0.96	0.82	1.12	9.75E-03	1.34	1.07	1.68
rs1529572	222378815	G	A	0.507	1.34E-01	0.88	0.75	1.04	3.23E-03	1.40	1.12	1.76
rs10498116	222379392	A	G	0.134	7.41E-01	0.96	0.77	1.21	3.80E-03	1.65	1.18	2.32
rs2710506	222379489	C	A	0.431	4.87E-01	0.95	0.81	1.11	8.16E-03	1.35	1.08	1.69
rs72963194	222380681	G	A	0.122	9.28E-01	0.99	0.77	1.26	8.67E-03	1.62	1.13	2.33
rs2710511	222384480	C	A	0.556	5.08E-01	1.06	0.90	1.24	3.00E-03	0.71	0.57	0.89
rs11900971	222388614	G	A	0.136	7.03E-01	0.96	0.76	1.20	3.29E-03	1.66	1.18	2.33
rs10932916	222389971	A	G	0.519	2.55E-01	1.10	0.94	1.29	9.66E-03	0.75	0.60	0.93
rs7575507	222392742	A	C	0.521	3.44E-01	1.08	0.92	1.27	8.77E-03	0.75	0.60	0.93
rs4613266	222393635	C	T	0.521	3.42E-01	1.08	0.92	1.27	8.63E-03	0.75	0.60	0.93
rs72965011	222393646	A	G	0.134	7.84E-01	0.97	0.77	1.22	3.20E-03	1.66	1.19	2.33
rs75843691	222395621	G	C	0.133	6.99E-01	0.96	0.76	1.20	8.30E-04	1.79	1.27	2.52
rs2680848	222395890	G	T	0.521	3.50E-01	1.08	0.92	1.26	9.99E-03	0.75	0.60	0.93
rs16862804	222397892	C	T	0.134	8.17E-01	0.97	0.78	1.22	3.08E-03	1.67	1.19	2.34
rs16862806	222398572	T	C	0.134	8.01E-01	0.97	0.77	1.22	3.08E-03	1.67	1.19	2.34
rs16862811	222401086	T	G	0.135	7.08E-01	0.96	0.76	1.20	2.90E-03	1.67	1.19	2.35
rs2680852	222402104	G	C	0.520	3.40E-01	1.08	0.92	1.27	9.70E-03	0.75	0.60	0.93
rs958165	222405460	C	T	0.134	8.01E-01	0.97	0.77	1.22	2.93E-03	1.67	1.19	2.34
rs960201	222405497	G	A	0.134	8.01E-01	0.97	0.77	1.22	2.93E-03	1.67	1.19	2.34
rs76514686	222405948	A	G	0.134	8.00E-01	0.97	0.77	1.22	2.91E-03	1.67	1.19	2.35
rs10498117	222412452	T	C	0.137	8.08E-01	0.97	0.77	1.22	4.11E-03	1.64	1.17	2.29
rs952162	222413051	C	T	0.137	6.82E-01	0.95	0.76	1.20	4.45E-03	1.63	1.16	2.28
rs142514650	222414463	C	T	0.138	7.14E-01	0.96	0.76	1.20	3.92E-03	1.64	1.17	2.30
rs75126815	222421316	T	C	0.138	6.19E-01	0.94	0.75	1.19	5.14E-03	1.62	1.15	2.26
rs991921	223061305	C	G	0.511	9.33E-01	0.99	0.85	1.16	9.13E-03	1.34	1.08	1.68
rs13017777	223069625	C	T	0.510	9.98E-01	1.00	0.86	1.17	9.47E-03	1.34	1.07	1.68

Coding Allele is the allele whose allele counts are entered into the X variable of the association analysis logistic regression equation. It is therefore, the reference allele for the odds ratios. *: Lower 95% CI of ORs; **: Upper 95% CI of ORs. SNV with *Bonferroni* significant p -value is grey-shaded and the p -value appears in boldface.

Table 2. Association of EPAA4 SNVs with SBP of individuals in the HCHS/SOL sub-cohort

SNV	Position Chrom 2 (Build 37/hg 19)	Coding Allele	Alternate Allele	Frequency Coding Allele	Males only				Females only			
					p-value	Beta	Lower 95% CI	Upper 95% CI	p-value	Beta	Lower 95% CI	Upper 95% CI
rs2052941	222276477	G	A	0.254	1.54E-02	-1.81	-3.27	-0.35	9.15E-01	0.07	-1.15	1.28
rs10432450	222277000	A	G	0.247	5.00E-02	-1.49	-2.99	0.00	8.40E-01	-0.13	-1.36	1.10
rs12989105	222279289	A	G	0.249	4.77E-02	-1.50	-2.99	-0.01	9.51E-01	0.04	-1.19	1.26
rs12996275	222280501	T	C	0.243	5.40E-02	-1.48	-2.98	0.03	5.41E-01	0.38	-0.84	1.61
rs13013934	222280602	T	A	0.241	6.49E-02	-1.41	-2.91	0.09	8.01E-01	0.16	-1.07	1.39
rs1036025	222286133	A	G	0.246	2.04E-02	-1.75	-3.22	-0.27	4.27E-01	0.49	-0.73	1.71
rs72963139	222324012	G	A	0.065	2.04E-01	1.89	-1.03	4.80	3.17E-01	-1.19	-3.51	1.14
rs10498114	222358909	G	A	0.085	3.99E-01	0.99	-1.31	3.28	9.18E-02	-1.63	-3.52	0.26
rs16862787	222366918	G	A	0.070	5.24E-01	0.83	-1.72	3.39	2.43E-01	-1.26	-3.38	0.86
rs13035425	222369776	C	T	0.291	8.71E-01	0.12	-1.29	1.53	4.27E-01	-0.49	-1.70	0.72
rs2710502	222373951	C	G	0.348	8.80E-01	0.10	-1.22	1.42	8.42E-01	-0.11	-1.23	1.00
rs2710503	222374699	C	G	0.348	9.14E-01	0.07	-1.25	1.39	8.65E-01	-0.10	-1.21	1.02
rs1529572	222378815	G	A	0.549	6.56E-01	-0.30	-1.61	1.01	7.37E-01	0.19	-0.92	1.30
rs10498116	222379392	A	G	0.087	8.03E-01	0.30	-2.03	2.62	4.62E-01	-0.70	-2.58	1.17
rs2710506	222379489	C	A	0.342	9.05E-01	0.08	-1.24	1.40	9.53E-01	0.03	-1.08	1.15
rs72963194	222380681	G	A	0.081	9.78E-01	-0.03	-2.42	2.36	2.81E-01	-1.09	-3.06	0.89
rs2710511	222384480	C	A	0.576	5.96E-01	-0.35	-1.63	0.94	9.60E-01	0.03	-1.05	1.10
rs11900971	222388614	G	A	0.070	5.95E-01	0.67	-1.80	3.13	1.07E-01	-1.70	-3.78	0.37
rs10932916	222389971	A	G	0.546	4.36E-01	-0.50	-1.75	0.76	7.10E-01	0.20	-0.86	1.26
rs7575507	222392742	A	C	0.545	4.75E-01	-0.46	-1.71	0.79	6.59E-01	0.24	-0.82	1.30
rs4613266	222393635	C	T	0.545	4.61E-01	-0.47	-1.72	0.78	6.43E-01	0.25	-0.81	1.31
rs72965011	222393646	A	G	0.069	5.95E-01	0.67	-1.81	3.15	1.13E-01	-1.69	-3.78	0.40
rs75843691	222395621	G	C	0.071	4.08E-01	1.01	-1.38	3.39	1.37E-01	-1.56	-3.61	0.49
rs2680848	222395890	G	T	0.545	5.09E-01	-0.42	-1.67	0.83	6.65E-01	0.23	-0.82	1.29
rs16862804	222397892	C	T	0.072	4.22E-01	0.97	-1.40	3.35	1.24E-01	-1.60	-3.64	0.44
rs16862806	222398572	T	C	0.071	4.24E-01	0.97	-1.41	3.35	1.35E-01	-1.56	-3.60	0.49
rs16862811	222401086	T	G	0.072	4.04E-01	1.01	-1.36	3.37	1.29E-01	-1.58	-3.62	0.46
rs2680852	222402104	G	C	0.545	5.14E-01	-0.42	-1.67	0.84	6.79E-01	0.22	-0.83	1.28
rs958165	222405460	C	T	0.070	6.50E-01	0.56	-1.87	3.00	8.99E-02	-1.79	-3.85	0.28
rs960201	222405497	G	A	0.069	6.41E-01	0.59	-1.88	3.06	1.14E-01	-1.68	-3.76	0.40
rs76514686	222405948	A	G	0.071	4.24E-01	0.97	-1.41	3.35	1.35E-01	-1.56	-3.60	0.49
rs10498117	222412452	T	C	0.086	6.35E-01	0.55	-1.71	2.80	3.80E-01	-0.85	-2.76	1.05
rs952162	222413051	C	T	0.084	9.93E-01	-0.01	-2.34	2.32	2.62E-01	-1.10	-3.02	0.82
rs142514650	222414463	C	T	0.084	9.80E-01	0.03	-2.29	2.35	2.56E-01	-1.11	-3.04	0.81
rs75126815	222421316	T	C	0.085	5.80E-01	0.64	-1.62	2.89	4.02E-01	-0.81	-2.72	1.09
rs991921	223061305	C	G	0.433	3.51E-01	-0.62	-1.92	0.68	6.24E-01	0.27	-0.81	1.35
rs13017777	223069625	C	T	0.400	4.28E-01	-0.54	-1.86	0.79	4.50E-01	0.42	-0.67	1.52

The 37 SPVs which had nominal significance ($p < 0.05$) in association with hypertension in the *ADVANCE* cohort were analyzed for their association with SBP of individuals in the *HCHS/SOL* sub-cohort (BMI > 25 and age > 50 years), using a linear mixed model for the quantitative analysis.

Beta: β co-efficiency. SPVs with nominal significant association with SBP are shaded in grey.

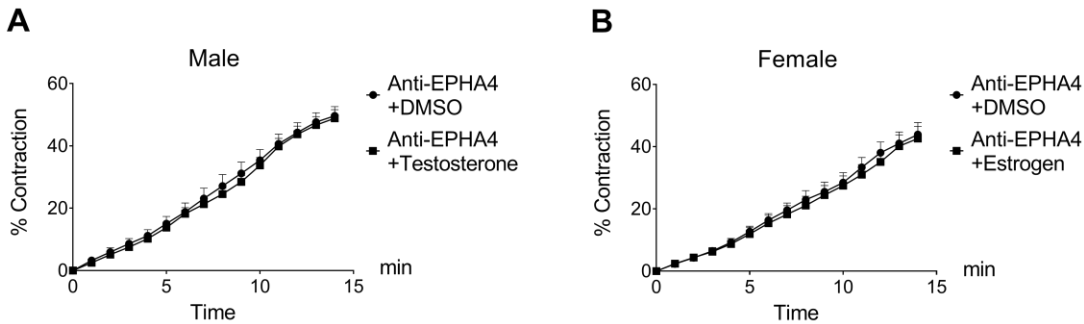
Table 3. Association of EPAA4 SNVs with DBP of individuals in the HCHS/SOL sub-cohort

SNV	Position Chrom 2 (Build 37/hg 19)	Coding Allele	Alternate Allele	Frequency Coding Allele	Males only				Females only			
					<i>p</i> -value	Beta	Lower 95% CI	Upper 95% CI	<i>p</i> -value	Beta	Lower 95% CI	Upper 95% CI
rs2052941	222276477	G	A	0.254	1.61E-02	-1.07	-1.94	-0.20	8.47E-01	-0.06	-0.72	0.59
rs10432450	222277000	A	G	0.247	6.11E-02	-0.85	-1.74	0.04	7.63E-01	-0.10	-0.76	0.56
rs12989105	222279289	A	G	0.249	5.80E-02	-0.86	-1.74	0.03	9.12E-01	-0.04	-0.69	0.62
rs12996275	222280501	T	C	0.243	8.89E-02	-0.78	-1.67	0.12	7.31E-01	0.12	-0.54	0.77
rs13013934	222280602	T	A	0.241	7.43E-02	-0.81	-1.71	0.08	9.79E-01	0.01	-0.65	0.67
rs1036025	222286133	A	G	0.246	2.65E-02	-1.00	-1.87	-0.12	6.46E-01	0.15	-0.50	0.81
rs72963139	222324012	G	A	0.065	5.89E-01	0.48	-1.26	2.22	2.11E-01	-0.80	-2.04	0.45
rs10498114	222358909	G	A	0.085	7.61E-01	0.21	-1.15	1.58	2.70E-01	-0.57	-1.59	0.45
rs16862787	222366918	G	A	0.070	9.79E-01	0.02	-1.50	1.54	4.86E-01	-0.40	-1.54	0.73
rs13035425	222369776	C	T	0.291	4.87E-01	0.30	-0.54	1.14	1.61E-01	-0.46	-1.11	0.18
rs2710502	222373951	C	G	0.348	5.70E-01	0.23	-0.56	1.01	4.08E-01	-0.25	-0.85	0.35
rs2710503	222374699	C	G	0.348	6.04E-01	0.21	-0.58	0.99	4.10E-01	-0.25	-0.85	0.35
rs1529572	222378815	G	A	0.549	4.96E-01	0.27	-0.51	1.05	7.93E-01	-0.08	-0.68	0.52
rs10498116	222379392	A	G	0.087	9.96E-01	0.00	-1.38	1.37	6.72E-01	-0.22	-1.22	0.79
rs2710506	222379489	C	A	0.342	5.56E-01	0.24	-0.55	1.02	4.52E-01	-0.23	-0.83	0.37
rs72963194	222380681	G	A	0.081	6.58E-01	-0.32	-1.74	1.10	5.06E-01	-0.36	-1.41	0.70
rs2710511	222384480	C	A	0.576	4.48E-01	-0.30	-1.06	0.47	5.41E-01	0.18	-0.40	0.76
rs11900971	222388614	G	A	0.070	6.74E-01	0.31	-1.15	1.78	2.95E-01	-0.59	-1.70	0.52
rs10932916	222389971	A	G	0.546	2.60E-01	-0.43	-1.18	0.32	8.66E-02	0.50	-0.07	1.07
rs7575507	222392742	A	C	0.545	2.76E-01	-0.41	-1.16	0.33	8.79E-02	0.50	-0.07	1.06
rs4613266	222393635	C	T	0.545	2.69E-01	-0.42	-1.17	0.32	8.33E-02	0.50	-0.07	1.07
rs72965011	222393646	A	G	0.069	6.37E-01	0.35	-1.12	1.83	2.84E-01	-0.61	-1.73	0.51
rs75843691	222395621	G	C	0.071	5.52E-01	0.43	-0.99	1.85	2.86E-01	-0.60	-1.69	0.50
rs2680848	222395890	G	T	0.545	2.64E-01	-0.43	-1.17	0.32	8.67E-02	0.50	-0.07	1.06
rs16862804	222397892	C	T	0.072	5.49E-01	0.43	-0.98	1.84	2.80E-01	-0.60	-1.70	0.49
rs16862806	222398572	T	C	0.071	5.49E-01	0.43	-0.98	1.84	3.04E-01	-0.57	-1.67	0.52
rs16862811	222401086	T	G	0.072	5.13E-01	0.47	-0.94	1.87	3.04E-01	-0.57	-1.67	0.52
rs2680852	222402104	G	C	0.545	2.54E-01	-0.43	-1.18	0.31	8.41E-02	0.50	-0.07	1.07
rs958165	222405460	C	T	0.070	7.00E-01	0.28	-1.16	1.73	2.11E-01	-0.71	-1.81	0.40
rs960201	222405497	G	A	0.069	7.29E-01	0.26	-1.21	1.72	2.63E-01	-0.64	-1.75	0.48
rs76514686	222405948	A	G	0.071	5.49E-01	0.43	-0.98	1.84	3.04E-01	-0.57	-1.67	0.52
rs10498117	222412452	T	C	0.086	5.35E-01	0.43	-0.92	1.77	5.44E-01	-0.32	-1.34	0.71
rs952162	222413051	C	T	0.084	8.70E-01	0.12	-1.27	1.50	4.99E-01	-0.36	-1.39	0.68
rs142514650	222414463	C	T	0.084	8.23E-01	0.16	-1.22	1.54	4.99E-01	-0.36	-1.39	0.68
rs75126815	222421316	T	C	0.085	3.72E-01	0.61	-0.73	1.96	4.91E-01	-0.36	-1.38	0.66
rs991921	223061305	C	G	0.433	5.54E-01	-0.23	-1.01	0.54	7.36E-01	0.10	-0.48	0.68
rs13017777	223069625	C	T	0.400	7.81E-01	-0.11	-0.90	0.68	8.50E-01	0.06	-0.53	0.65

The 37 SPVs which had nominal significance ($p < 0.05$) in association with hypertension in the *ADVANCE* cohort were analyzed for their association with DBP of individuals in the *HCHS/SOL* sub-cohort (BMI > 25 and age > 50 years), using a linear mixed model for the quantitative analysis.

Beta: β co-efficiency. SPVs with nominal significant association with SBP are shaded in grey.

Supplementary Materials

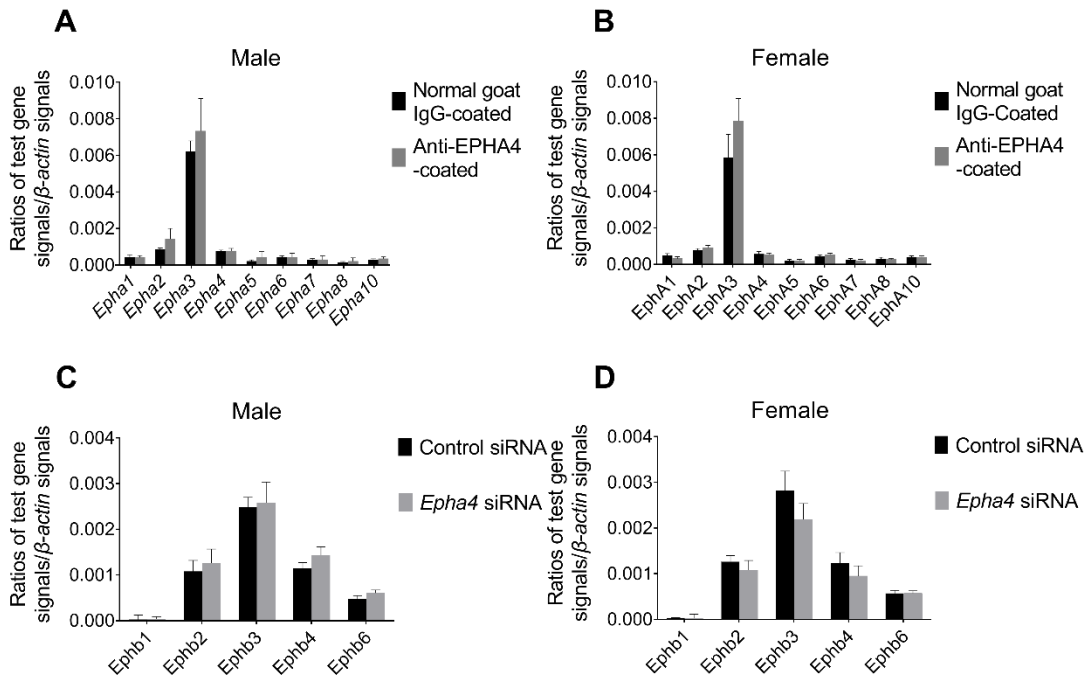


Supplementary Figure 1. No apparent effect on male and female VSMC contractility by testosterone and estrogen, respectively

VSMCs from male and female C57BL/6J mice (as indicated) were cultured in wells coated with goat anti-mouse EPHA4 Ab (both at 2 μ g/ml during coating) for 4 days. Testosterone (6.5 ng/ml) was added to male VSMCs (A), and estrogen (100 ng/ml) was added to female VSMCs (B) during this period. DMSO was added as vehicle control for the sex hormones. The cells were then stimulated with PE (20 μ M) and imaged continuously for 15 min at 1 frame per min in a controlled environment (37°C in 5% CO₂). Images of 15 or more cells were selected randomly, with their length measured at the time point indicated. The results are expressed as means \pm SEM of contraction percentage (% contraction), calculated as follows:

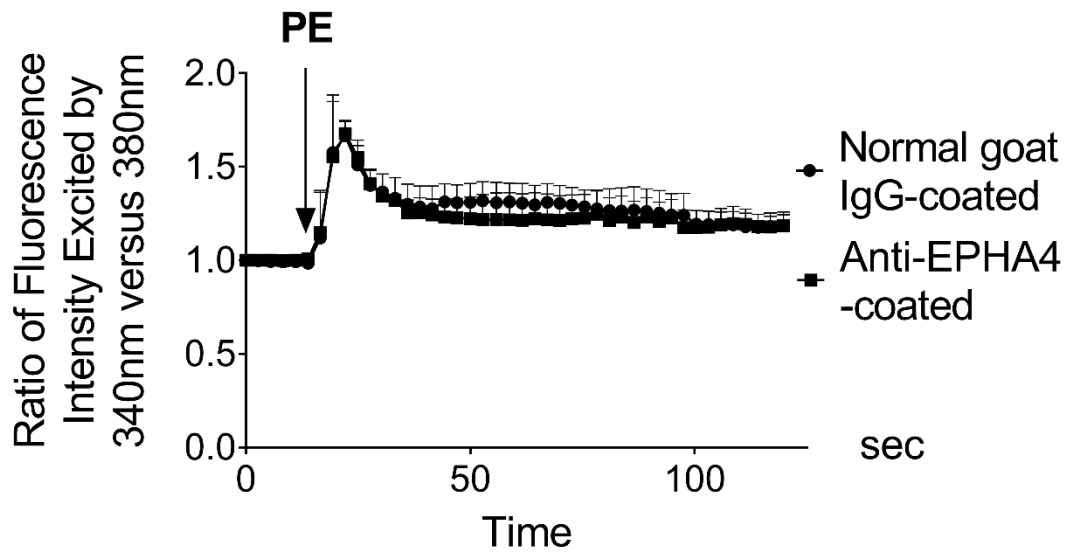
$$\% \text{ contraction} = 100 \times (\text{cell length at time 0} - \text{cell length at time X}) / \text{cell length at time 0}.$$

The experiments were repeated 3 times and representative results are reported. No statistically significant difference was observed in sex hormone- and vehicle-treated counterparts (2-tailed Student's *t* tests).



Supplementary Figure 2. No alteration of *Epha* and *Ephb* mRNA expression of members in *EPHA4*-stimulated VSMCs

VSMCs from male (A and C) and female (B and D) mice were cultured in wells coated with goat anti-mouse EPHA4 Ab or control goat IgG (both at 2 μ g/ml during coating) for 4 days. The expression of *Epha* (A and B) and *Ephb* (C and D) family members at the mRNA level in the cells from male and female mice was measured by RT-qPCR. The pooled data (means \pm SD of ratios of test gene signals versus β -actin signals) from 3 independent experiments are shown. No statistically significant difference was observed between VSMCs with and without solid-phase anti-EPHA4 Ab stimulation (2-tailed Student's *t* tests).



Supplementary Figure 3. Normal Ca^{2+} influx in EPHA4-stimulated VSMCs.

PE-triggered Ca^{2+} influx in VSMCs cultured in wells coated with anti-EPHA4 Ab is presented. VSMCs were loaded with Fura-2 (5 μM), then cultured at 37°C in HBSS containing Ca^{2+} (1.26 mM) and stimulated with PE (20 μM). The arrow indicates the time point at which PE was added. Emission ratios at 510 nm triggered by 340 nm versus 380 nm excitation were registered at 1 image per 3 s for 2 min. Ratios from 15 or more randomly-selected VSMCs were pooled and expressed as means \pm SEM. The experiments were repeated 3 times, and representative results are presented. No significant difference in Ca^{2+} influx was found between VSMCs cultured on anti-EPHA4 Ab- and normal goat IgG-coated wells (2-tailed Student's t test).

Supplementary Table 1. Mean values or percent frequencies and statistical significance of differences between hypertensive and normotensive ADVANCE subjects for covariates used in association analysis

Covariate	Hypertensive subjects (n=2710) mean (SD)	Normotensive subjects (n=615) mean (SD)	p-value
Age	67.4 (6.7)	66.52 (6.44)	* 3.30 X 10 ⁻³
PC1	0.06 (1.03)	-0.25 (0.83)	* 3.18X 10 ⁻¹⁵
PC2	-0.02 (0.96)	0.1 (1.17)	* 1.33 X 10 ⁻²
BMI	30.3 (5.0)	29.1 (5.25)	* 9.35 X 10 ⁻⁸
CKD: N (%)	570 (21.0)	87 (14.1)	**1.30 X 10 ⁻⁴

BMI: body mass index; CKD: chronic kidney disease. PC1 and PC2, principal components from population stratification analysis, BMI, body mass index, CKD, presence of chronic kidney disease. *: *p*-value from *t*-test. **: *p*-value from χ^2 test.

Supplementary Table 2. Parameters of the HCHS/SOL cohort without stratification

	Overall	Males_normal	Males_HT	Females_HT	Females_normal
n	12126	3575	1397	2016	5138
Mean age (SD)	46 (14)	41 (13)	55 (11)	56 (9)	43 (13)
Mean BMI (SD)	30 (6)	29 (5.2)	30 (5.4)	32 (6.6)	30 (6.2)
Mean SBP (SD)	125 (20.2)	119 (9.5)	149 (17.2)	148 (18.5)	113 (12)
Mean DBP (SD)	75 (11.9)	72 (8.2)	88 (11.4)	86 (11)	70 (8.5)
T2D (%)	2383 (19.7)	420 (11.7)	522 (37.4)	822 (40.8)	619 (12)
CKD (%)	5829 (48.1)	1845 (51.6)	497 (35.6)	762 (37.8)	2725 (53)

HT: hypertensive; T2D: type 2 diabetes.

Supplementary Table 3. Parameters of HCHS/SOL sub-cohort restricted to overweight/obese and aged individuals (BMI \geq 25 and \geq 50 years old)

	Overall	Males_normal	Males_HT	Females_HT	Females_normal
n	4512	831	875	1408	1398
age	59 (6)	57 (6)	60 (7)	60 (7)	57 (6)
BMI	31 (4.9)	30 (3.6)	31 (4.3)	33 (5.8)	31 (4.6)
SBP	136 (21.1)	122 (9.9)	151 (17.5)	150 (19)	120 (11)
DBP	79 (11.7)	73 (7.8)	87 (11.1)	85 (10.9)	72 (7.9)
T2D	1532 (34)%	179 (21.5)%	390 (44.6)%	644 (45.7)%	319 (22.8)%
CKD	1467 (32.5)%	287 (34.5)%	272 (31.1)%	459 (32.6)%	449 (32.1)%

Supplementary Table 4. RT-qPCR primer sequences and product sizes for mouse EphA1-8, EphA10, Ephb1-4, Ephb6, and β -actin mRNA quantification

RT-qPCR primer sequences			
Genes	Sense sequences	Antisense sequences	PCR products
<i>Epha1</i>	5'-GCCTGTCTACTGGTTTCTATC-3'	5'-CCTCAGACTCTGCTATGCTATG-3'	85 bp
<i>Epha2</i>	5'-GAGCAGGAGGGTAGAAGTTGT-3'	5'-CATCAGGTCCCACCCTTTG-3'	262 bp
<i>Epha3</i>	5'-TTGGACTCTCTCGGGTACTT-3'	5'-TCTGGTGATGTCCACCTTATTG-3'	88 bp
<i>Epha4</i>	5'-ACAGCCCTTCCTGTTGATTAC-3'	5'-GGCAGAAGAGAAGCCAAAGA-3'	96 bp
<i>Epha5</i>	5'-CTCTCTCTGTCTCTCTCTCTTT-3'	5'-TTCAGTCTTCAGTGCCGATATG-3'	97 bp
<i>Epha6</i>	5'-ATGAACACAATAGGCCCATACA-3'	5'-GCATCACGAGAGATCCAGTTAG-3'	97 bp
<i>Epha7</i>	5'-GGATGAGAACTACACTCCGATAAG-3'	5'-CCTTTGTGCGTTGCCTTTAG-3'	109 bp
<i>Epha8</i>	5'-GTGTCTACGCCGAGATCAAAT-3'	5'-CTCCAGGTAGTGAAGGTTGAA-3'	98 bp
<i>Epha10</i>	5'-AGAGGCAGGAGTAGAAGTAGAG-3'	5'-TGCGTCCTTAATATGGGATCAG-3'	111 bp
<i>Ephb1</i>	5'-ACCATGAGGAGCATCACCTTGTC-3'	5'-TAGCCCATCGATACGTGCTGTGTT-3'	153 bp
<i>Ephb2</i>	5'-CCAGTGATGTGTGGAGCTATG-3'	5'-GGAGGTAGTCTGTAGTCCTGTT-3'	124 bp
<i>Ephb3</i>	5'-AGTTCGCCAAGGAGATCGATGTGT-3'	5'-TCAGCGTCTTGATAGCCACGAACA-3'	135 bp
<i>Ephb4</i>	5'-CTACGTCTCTAACCTCCCATCT-3'	5'-GCTGGTCAACCCTTTCTCTTT-3'	100 bp
<i>Ephb6</i>	5'-CTTTGCCTTTGTTACCGAGCACT-3'	5'-AGCAAGGAACTTGAACCCTGAGGA-3'	111 bp
<i>β-actin</i>	5'-TCGTACCACAGGCATTGTGATGGA-3'	5'-TGATGTCACGCACGATTTCCTCT-3'	200 bp

Supplementary Table 5. RT-qPCR primer sequences and product sizes for human EPHA4, EPHB6 and β -actin mRNA quantification

Genes	Sense sequences	Antisense sequences	PCR products
<i>EPHA4</i>	5'-AGTCCTTCTGGTCTCTGTCTC-3'	5'-CTTCATCCGCTTCTTGTTC-3'	116bp
<i>EPHB6</i>	5'-TGCTGGTGAATAGCCACTTG-3'	5'-CGGAACCTCTGCTCTATTGC-3'	235bp
β -actin	5'-AGTTGCGTTACACCCTTTCTTGAC-3'	5'-GCTCGCTCCAACCGACTGC-3'	165bp

Supplementary Table 6. Epha4 siRNA sequences

Genes	Sense sequences	Antisense sequences
<i>Epha4</i> set 1	5'-rArGrCrUrArUrArArCrArUrCrArArArUrCrArGrGrGrArATT-3'	5'-rArArUrUrCrCrCrUrGrArUrUrGrArUrGrUrUrArUrArGrCrUrUrA-3'
<i>Epha4</i> set 2	5'-rArCrCrUrUrGrArUrCrArUrCrArGrArArUrUrArArArCrCTG-3'	5'-rCrArGrGrUrUrUrArArUrUrCrUrGrArUrGrArUrCrArArGrGrUrCrA-3'

Supplementary Table 7. Alman-Bland test for Significance of difference in odds ratios between male and female samples for SNP, rs75843691

Males only				Females only				Alman-Bland test for differences in odds ratios between males and females			
<i>p</i> -value	Odds Ratio	*Lower 95% CI	**Upper 95% CI	<i>p</i> -value	Odds Ratio	*Lower 95% CI	**Upper 95% CI	<i>p</i> -value	***Odds Ratio	*Lower 95% CI	**Upper 95% CI
6.99 X 10 ⁻¹	0.96	0.76	1.20	8.30 X 10⁻⁴	1.79	1.27	2.52	3.0 X 10⁻³	0.54	0.36	0.81

*: Lower 95 confidence interval for odds ratio; **: Upper 95% confidence interval for odds ratio;

***: Odds ratio for difference test is the ratio of odds ratios for males and females; if not equal to 1, then the difference is significant.