Université de Montréal

Eph kinases and their ligands ephrins act in concert with sex hormones in regulating blood pressure

par

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Résumé

Les Erythropoietin-producing hepatocyte (EPH) sont la plus grande famille de récepteurs tyrosine kinase. Leurs ligands, les éphrines (EFNs), sont aussi des molécules exprimées à la surface cellulaire. Les EPH/EFNs sont impliqués dans de nombreux processus biologiques.

L'hypertension artérielle (PA) est une maladie chronique qui, aujourd'hui, est devenue un problème médical critique dans le monde entier et un enjeu de santé publique. La découverte de nouvelles thérapeutiques de l'hypertension sont d'une grande importance pour la santé publique. Jusqu'à tout récemment, il existe seulement quelques études concernant le rôle de l'axe EPH/EFNs sur la fonction des cellules musculaires lisses vasculaires (CMLV). Dans nos études précédentes, nous avons montré qu'EPHB6 et EFNB1, de concert avec les hormones sexuelles, régulent la PA.

Dans la présente étude, nous avons constaté que les différents membres de la famille EPH/EFN peuvent réguler soit positivement, soit négativement, la contractilité des CMLV et la PA: tandis que EPHB4 et EFNB2 appartiennent à la première catégorie, EFNB1, EFNB3 et EPHB6 appartiennent à la deuxième.

In vivo, des souris males, mais non pas des femelles, porteuses d'une mutation EPHB4 (KO) spécifique du muscle lisse présentent une PA diminuée, comparée aux souris témoins (WT). Les CMLV de souris EPHB4 KO, en présence de testostérone, ont montré une contractilité réduite lors de la stimulation par la phényléphrine (PE). Au niveau moléculaire, la phosphorylation de la protéine kinase II dépendante de Ca2+/calmoduline et de la kinase de la chaine légère de la myosine (CLM) est augmentée, tandis que la phosphorylation de la kinase de la CLM est réduite dans les CMLV KO lors de la stimulation par PE, par rapport au WT CMLV. Cela fournit une base moléculaire à la réduction de la PA et de la contractilité des CMLV chez les souris EPHB4 KO.

EFNB2 est le ligand majeur de l'EPHB4. Comme attendu, les souris EFNB2 KO spécifique du muscle lisse avaient un phénotype de PA semblable, quoique non identique, aux souris EPHB4 KO. Les souris mâles EFNB2 KO, mais pas femelles, sous régime régulier ou riche en sel, présentent une PA réduite, par rapport à leurs homologues WT. Au niveau cellulaire, les CMLV des souris KO ont montré une contractilité réduite lors de la stimulation par PE par rapport aux témoins WT. Une région de l'acide aminé (aa) 313 à l'aa 331 dans la partie intracellulaire

d’EFNB2 est essentielle pour la signalisation inverse qui régule la contractilité des CMLV, selon des études de mutation-délétion. Dans une étude de génétique humaine, nous avons identifié, dans le gène EFNB2, six SNP qui étaient associées significativement au risque d'hypertension artérielle, de façon dépendante du sexe, ce qui corrobore nos résultats chez les souris.

En revanche, la délétion du gène EFNB3 (KO) chez les souris femelles aboutit à une PA élevée et à une augmentation des résistances des petites artères in vivo, améliore la contractilité des petites artères ex-vivo et augmente la contractilité des CMLV in vitro. Les souris mâles KO ont une PA normale, mais la castration conduit à une augmentation significative de la PA dans les souris KO, mais pas dans les souris WT. Les CMLV des souris KO femelles ont montré une phosphorylation accrue de la CLM et une phosphorylation réduite de la kinase de la CLM, ce qui fournit à nouveau une base moléculaire aux phénotypes de PA et de contractilité des CMLV observés. Ce changement de signalisation est attribuable à une protéine adaptatrice Grip1. En effet, dans une étude d'association pan génomique par le Consortium International pour la Pression Sanguine, un SNP dans le gène GRIP1 a approché le seuil de significativité de la valeur p pour son association avec la pression diastolique.

Nos recherches, pour la première fois, ont révélé que EPH/EFNs sont de nouveaux composants dans le système de régulation de la PA. Les membres de la famille EPH/EFN peuvent agir comme des forces Yin et Yang pour régler finement le tonus des vaisseaux pour assurer l'homéostasie de la PA et de sa régulation. Ces effets de EPH/EFNs dépendent du sexe et des niveaux d’hormones sexuelles. À partir de ces nouvelles connaissances, nous pourrions développer une nouvelle thérapie personnalisée pour l’hypertension artérielle, utilisant des antagonistes d'hormones sexuelles ou des thérapies de remplacement d'hormones sexuelles, selon les niveaux d'hormones sexuelles des patients et les mutations dans les gènes de l'EPH/EFN.

Mots-clés: Erythropoietin-producing hepatocyte (EPH), éphrines, cellules musculaires lisses vasculaires, hypertension artérielle, hormones sexuelles

Abstract

Erythropoietin-producing hepatocyte (EPH) kinases are the largest family of receptor tyrosine kinases. Their ligands, ephrins (EFNs), are also cell surface molecules. Ephs/EFNs are implicated in many biological processes.

Hypertension is a chronic medical condition of high arterial blood pressure (BP). New hypertension therapeutic treatments are of great importance for public health. Until recently, there are only a few studies related to the role of EPHs/EFNs in vascular smooth muscle cell (VSMC) function. In our previous studies, we have found that EPHB6 and EFNB1 function in concert with sex hormones to regulate BP.

In the present investigation, we found that different EPH/EFN family members can either positively or negatively regulate the VSMC contractility and BP: while EPHB4 and EFNB2 belong to the former category, EFNB1, EFNB3 and EPHB6, the latter.

In vivo, male but not female smooth muscle-specific EPHB4 knockout (KO) mice presented decreased BP, compared to WT controls. VSMCs from EPHB4 KO mice in the presence of testosterone showed reduced contractility.

EFNB2 is the major ligand of EPHB4. As expected, smooth muscle-specific EFNB2 KO mice had a similar although not identical BP phenotype as EPHB4 KO mice. Male but not female EFNB2 KO mice on regular or high-salt diet presented reduced BP, compared to WT counterparts. At the cellular level, the KO VSMCs showed reduced contractility compared to WT controls. In a human genetic study, we identified in the EFNB2 gene six SNPs that were significantly associated with hypertension risk in a sex-dependent way, corroborating our findings in mice.

On the other hand, EFNB3 gene KO in female mice resulted in elevated BP and small artery resistance *in vivo*, enhanced small arterial contractility *ex vivo*, and augmented VSMC contractility *in vitro*. Male KO mice had normal BP, but castration led to significant BP elevation in KO but not in WT mice. VSMCs from female KO mice showed heightened MLC phosphorylation and reduced MLC kinase phosphorylation. This signaling change was mediated through an adaptor protein Grip1. Indeed, in a genome-wide association study by the International Consortium for Blood Pressure, an SNP in the GRIP1 gene approached the significant threshold *p*-value for its association with diastolic BP.

Our research for the first time revealed that EPHs/EFNs are novel components in the BP regulation system. Members of the EPH/EFN family may act as Yin and Yang forces to finely tune the vessel tone for BP homeostasis and regulation. Such effects of EPHs/EFNs depend on sex and sex-hormone levels. Based on this new knowledge, we could develop novel personalized hypertension therapy using sex hormone antagonists or sex hormone replacement therapy, depending on the sex hormone levels of the patients and mutations in EPH/EFN genes.

Keywords: Erythropoietin-producing hepatocyte (EPH), ephrins, vascular smooth muscle cells, hypertension, sex hormones

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11β-HSD2	11b-hydroxysteroid Dehydrogenase Type 2
ABL	Abelson Murine Leukemia Viral Oncogene Homolog
ACE	Angiotensin Converting Enzyme
ACTH	Adrenocorticotropic Hormone
ADAM	A Disintegrin and Metalloprotease
AICD	Activation-induced Cell Death
ARG	Arginine
BP	Blood Pressure
CAMKII	Ca2+/calmodulin-dependent Protein Kinase II
CAMP	3'5'-Cyclic Adenosine monophosphate
CBL	Casitas B-lineage Lymphoma
CC	Colon Carcinoma
CGMP	3'5'-Cyclic Guanosine Monophosphate
CKD	Chronic Kidney Disease
CV	Cerebral Vasospasm
DP	Diastolic Pressure
EGF	Epidermal Growth Factor
EMAX	Maximal Tension
EPHS	Erythropoietin Hepatoma Kinases
ERK	Extracellular-signal Regulated Kinase
ES	Embryonic Stem

GDI	Gdp Dissociation Inhibitors
GEF	Gdp Exchange Factors
GPER	Membrane-associated G-protein
GRA	Glucocorticoid-remediable Aldosteronism
HCSCS	Human Cardiac Stem Cells
HIC1	Hypermethylated In Cancer 1
HSP	Heat Shock Proteins
IP3	Inositol 145-trisphosphate
JNC	Joint National Committee
JNK	Jun N-terminus Kinase
LRRK2	Leucine-rich Repeat Kinase 2 Gene
MAPK	Mitogen-activated Protein Kinase
MAPKK	Mapk Kinase
MLC	Myosin Light Chain
MLCK	Myosin Light Chain Kinase
MLCP	Myosin Light Chain Phosphatase
MMP2	Matrix Metalloproteinase 2
MVD	Microvessel Density
NHANES	Us National Health And Nutrition Examination Survey
NHIGG	Normal Human Igg
NO	Nitric Oxide
PHAII	Pseudohypoaldosteronism Type II
PKA	Protein Kinase A

PTEN	Phosphatase and Tensin Homolog
PUFAS	Polyunsaturated Fatty Acids
PYK2	Proline-rich Tyrosine Kinase 2
RAAS	Renin - Angiotensin - Aldosterone System
RAS	Renin-angiotensin System
RHOGEF	Rhoa Guanine Nucleotide Exchange Factor
ROS	Reactive Oxygen Species
RTKS	Receptor Tyrosine Kinases
SAH	Subarachnoid Hemorrhage
SAM	Sterile Alpha Motif
SDF-1A	Stromal Cell-derived Factor
SGN	Spiral Ganglion Neurons
SH	Src Homology
SHR	Spontaneously Hypertensive Rats
SNPS	Single Nucleotide Polymorphisms
SOCS	Suppressors Of Cytokine Signaling
SP	Systolic Pressure
TCR	T-cell Receptor
TEC	Thymic Epithelial Cell
TP	Thromboxane Prostanoid
VEGFR	Vascular Endothelial Growth Factor Receptors
VSMC	Vascular Smooth Muscle Cell
VCAM-1	Vascular Wall Adhesion Molecule-1

WNK	With No Lysine
WT	Wild Type

Liste des abréviations

AB	Antibody
ARG	Arginine
CAM	Calmodulin
E2	17β-estradiol
EFNs	Ephrins
KO	Knockout
PE	Phenylephrine

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Here is a statement regarding the contribution of co-authors and myself to the four papers included in this thesis.

Chapter 2:

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Y.W., Z.W., J.W., conceived and designed the experiments. Y.W., Z.W., H.L, S.Q., performed the experiments. Y.W., Z.W., J.T., JL.L., J.P., analyzed the data. J.W., initiated and guided the project, and participated in experimental design and manuscript writing.

Chapter 3:

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Y.W., J.R., Z.W. H.L., W.J., conducted experiments; E.T. conducted vessel contraction studies; P.H. contributed to experiment design and manuscript writing; J. T., J. L., and J. P. performed telemetry; S.Q. carried out castration and ovariectomy; F.M., J.C., M.W., S.H., C.L. carried out human genetic studies; J.W. initiated and guided the project, and participated in experimental design and manuscript writing.

Chapter 4:

Wang Y, Wu Z, Thorin E, Tremblay J, Lavoie J, Luo H, Peng J, Qi S, Wu T, Chen F, Shen J, Hu S, Wu J. Estrogen and testosterone in concert with EFNB3 regulate vascular smooth muscle cell contractility and blood pressure. ***Am J Physiol Heart Circ Physiol.*** 2016 Apr 1;310(7):H861-72.

Y.W., and Z.W. conducted experiments in VSMC and contributed to experimental design and manuscript writing; E.T. conducted vessel contraction studies and contributed to manuscript writing; J. T., J. L., and J. P. performed telemetry; H. L. participated in KO characterization; S.Q. carried out castration and ovariectomy; T.W., F.C. J.S., and S.H. carried out human vessel studies; J.W. initiated and guided the project, and participated in experimental design and manuscript writing.

Appendix-1: Wang Y, Wu Z, Luo H, Peng J, Raelson J, Ehret GB, Munroe PB, Stoyanova E, Qin Z, Cloutier G, Bradley WE, Wu J. The role GRIP1 and EFNB3 signaling in blood pressure control and vascular smooth muscle cell contractility. ***Plos One*** (submitted after revision)

Y.W., Z.W. H.L., J. P. conducted experiments; J.R., G.E., P.M., E.S., Z.Q., G.C., conducted human genetic experiments; W.B., contributed to experiment design; S.Q. carried out castration and ovariectomy. J.W., initiated and guided the project, and participated in experimental design and manuscript writing.

CHAPTER 1 INTRODUCTION

Part I. Function and signal pathways of Eph/Ephrins

Receptor tyrosine kinases (RTKs) are a large group of cell surface molecules with an intracellular tail that contains tyrosine kinase activity. They function as a sensor to detect extracellular signals and transmit them to the cells (Yarden & Ullrich, 1988). The effects of many growth factors are known to be mediated by high-affinity RTKs (Fantl, Johnson, & Williams, 1993).

Erythropoietin-producing hepatocyte (EPHs) form the largest family of RTKs, their corresponding ligands being named ephrins (Efn). About 20 years ago, the first member of the Eph kinase family was identified by Hirai et al.(Hirai, Maru, Hagiwara, Nishida, & Takaku, 1987) and was called Eph. Multiple other members were subsequently identified (Beckmann et al., 1994; Bennett et al., 1995; Davis et al., 1994; Drescher et al., 1995; Lackmann et al., 1996; Shao, Lou, Pandey, Pasquale, & Dixit, 1994; Winslow et al., 1995). Within a few years of the identification of EPHs, their ligands ephrins were identified (Bartley et al., 1994; Beckmann MP, 1994). Since then, active research has been carried out to understand their functions.

Structure of Eph/ephrin family members

Both EPHs and ephrins are membrane-bound proteins. According to sequence similarity, Eph receptors and the ligands are classified into A and B subfamilies. There are nine members of the EphA subfamily, EphA1-8 and EphA10, and five members of the EphB subfamily, EphB1-4 and EphB6. EphrinAs (A1–A6) anchor into the cell membrane by a glycosylphosphatidylinositol moiety, while ephrinBs (B1–B3) pass through the plasma

membrane by a short cytoplasmic tail. There is a PDZ-binding domain at ephrinBs' C-terminus in the cytoplasmic tail (Figure 1) (Arvanitis & Davy, 2008).

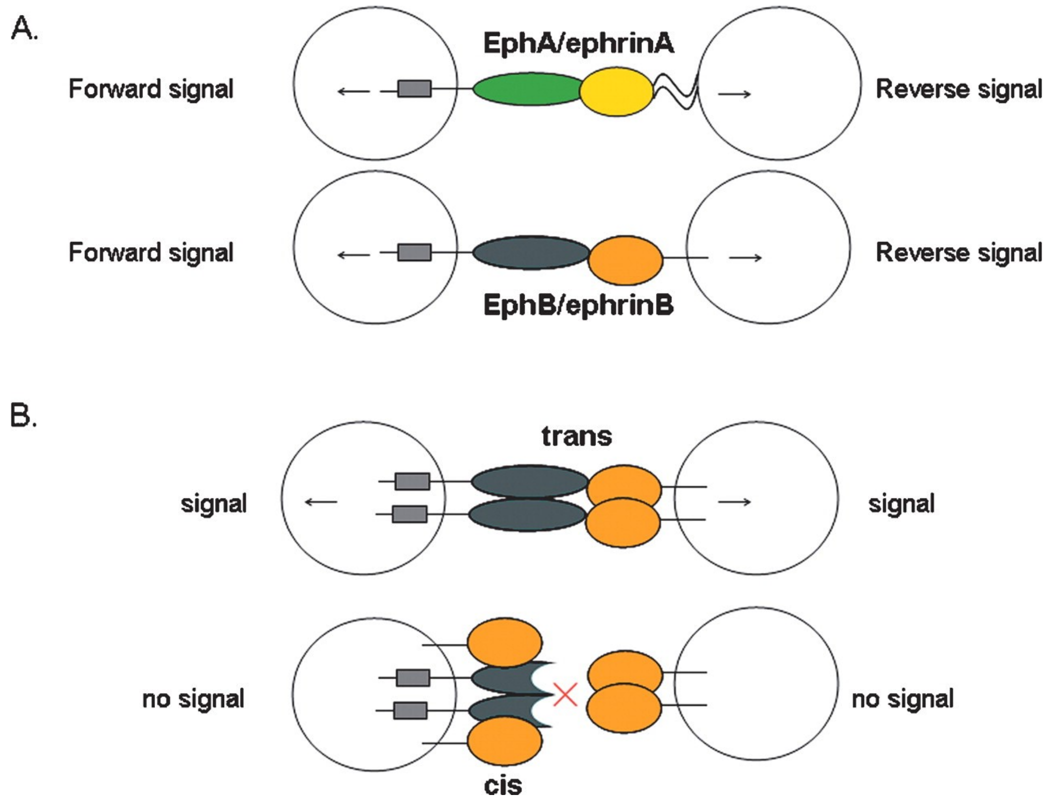

Figure 1. Features of Eph/ephrin signaling

Both types of Eph receptors (EphAs and EphBs) contain an extracellular region, a single transmembrane spanning domain, and an intracellular region which comprises a tyrosine kinase domain, a sterile alpha motif (SAM), and PDZ-binding motif (Surawska, Ma, & Salgia, 2004). The extracellular domain comprises a ligand-binding domain, including a highly conserved N-terminal globular domain, a cysteine-rich domain, and two fibronectin type III repeats. The A-type receptors preferentially bind A-type ligands while the B-class receptors bind B-type ligands, but there are exceptions. For example, EphA4 can bind both A and B-type ephrins. One of the unique features of Eph/ephrin signaling is the fact that both receptors and ligands are competent

to transduce signals into the cells upon interaction (Lisle, Mertens-Walker, Rutkowski, Herington, & Stephenson, 2013).

Functions of Eph/ephrin family members

Signaling pathways

Both EPHs and their corresponding ephrin ligands are membrane-bound proteins, they require direct cell-cell interactions for Eph receptor activation. The interactions between Eph receptors and ephrins can happen in *trans* (between two opposing cells) or in *cis* (within the same cell), and both can result in either cell-to-cell adhesion or de-adhesion. The Eph receptors are activated by ephrin ligands though forward signaling while the ephrin ligands are activated by Eph receptors through reverse signaling. Such bidirectional signalings are the characteristics of Eph/Ephrin signaling.

The interaction of the extracellular domains of Eph receptors and ephrins was described by Himanen et al.(Himanen et al., 2001a) on EphB2 and ephrinB2. This interaction forms a crystal structure on the cell surface. In the crystals, there are two different structures of bi-directional signaling presenting through a multiple-step process: dimeric and tetrameric. First, pre-clustered unbound ephrins form low-affinity ephrin-ephrin homodimers. Then Eph receptors bind to the ephrins in 1:1 ratio, forming high affinity and specificity heterodimers. Next, the dimer pairs of Eph-ephrin join into tetrameric complexes. The tetrameric complex contains two ligands interacting with two receptors and forming a circular structure (Himanen, Henkemeyer, & Nikolov, 1998). This ring-like tetrameric structure is necessarily required by the functional bidirectional signaling (Himanen & Nikolov, 2003). It allows the membrane-associated ephrins and Eph receptors to interact with the surfaces of adjacent cells (Drescher, 2002).

In solution, the range of EPHs and ephrins concentration can vary widely from 10 nM to 20 mM. At moderate concentration, EPHs and ephrins usually form heterodimers at a 1:1 stoichiometry, while, throughout higher concentration, those dimer pairs can form high-order tetramers (Himanen et al., 2001b). The Eph tyrosine kinase can then trans-phosphorylate each other on the tyrosine kinase domain and initiate forward and signalings via the tetramers, which can also mediate the phosphorylation of other proteins and associate with the receptor of various effector proteins. Importantly, the formation of the heterotetramers not only modulates the forward signaling but also repositions the ephrin transmembrane and cytoplasmic domains, converting them to a signaling-competent configuration. Tyrosine phosphorylation of the ephrin cytoplasmic tail follows, initiating reverse signaling. These reverse signaling acts through two types of residuals: the tyrosine/serine phosphorylation sites and the intracellular PDZ domain-binding motif (Himanen, Saha, & Nikolov, 2007). Besides, the extracellular cysteine-rich domain and the intracellular SAM domain of the EphB receptors also play a significant role in Eph–ephrin interaction by binding to the Ephrin carboxy-terminal PDZ domain binding motif (Himanen et al., 2001a; Holder & Klein, 1999; Stapleton, Balan, Pawson, & Sicheri, 1999).
The main downstream signaling pathway of Eph/Ephrin is mediated by G-proteins (Noren & Pasquale, 2004). There is a multiple, critical connection between these cell surface proteins and small GTPases of the Ras and Rho families. Eph/Ephrin work like a switch to regulate the GTPases cycled between the inactive state and active state (Van Aelst & D'Souza-Schorey, 1997). These components are modulated by some adhesion molecules and growth factor receptors (Noren & Pasquale, 2004), resulting in cell morphologic and cell behavioral regulation.

Rho proteins are key regulators of actin cytoskeleton dynamics in cells (Ramachandran, Patil, Combrink, Sharif, & Srinivas, 2011). EphrinB reverse signaling also activates Rho GTPases. It

performs in a phosphotyrosine-independent docking mechanisms by the Src family tyrosine kinases (Van Aelst & D'Souza-Schorey, 1997).

The Eph/ephrin signaling pathway is also connected to the Ras/ mitogen-activated protein kinase (MAPK) cascade. Once the Eph receptors are activated, adaptor molecules associated with them will transmit signals into the cell. Then the extracellular-signal regulated kinase (ERK) / MAPK pathway will be suppressed (Figure 2)(Coulthard et al., 2012). Consequently, the communication from the cell surface to the DNA in the nucleus of the cell and the activity of the transcription factors are suppressed (Chang et al., 2003; Miao et al., 2001).

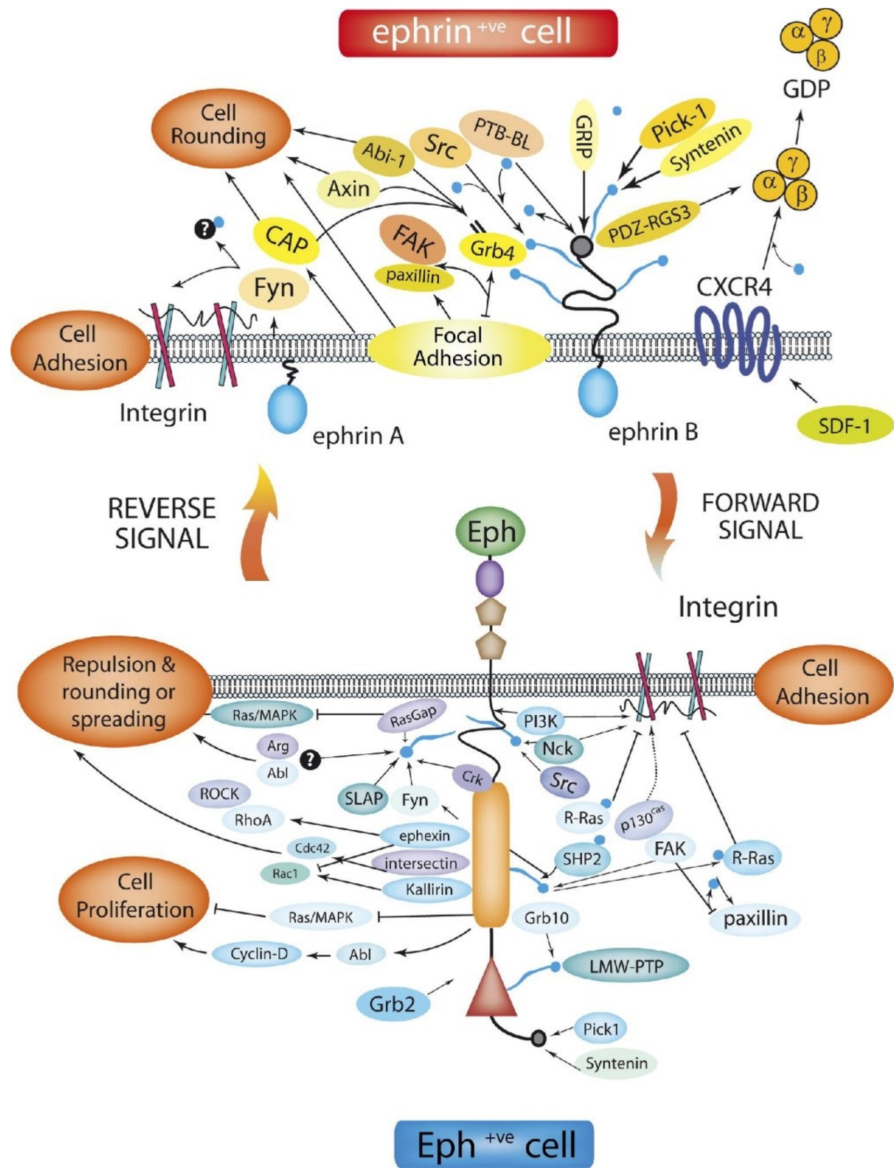

Figure 2. Eph/Ephrin bidirectional pathways

Beside the classical bidirectional signal pathway, Eph receptor and ephrins could also interact with other cell-surface communication systems, independent of receptor-ligand association. For example, recent studies have shown that members of the epidermal growth factor (EGF) receptor family can cooperate with EphA2 and promote cell motility and proliferation(Brantley-Sieders et al., 2008); ephrinBs could be phosphorylated on their intracellular tyrosine residues in response to platelet-derived growth factor receptor activation(Arvanitis & Davy, 2008).

Moreover, there are still many other proteins working in concert with Eph/Ephrins, such as Src Homology 2(SH2) and SH3 adaptor proteins(C. A. Cowan & Henkemeyer, 2001; Holland et al., 1997); phosphatidylinositol 3-kinase(Guan et al., 2010); focal adhesion kinase(Krupke & Burke, 2014); low-molecular-weight protein tyrosine phosphatase(Park, Warner, Mood, Pawson, & Daar, 2002); Abelson murine leukemia viral oncogene homolog (Abl) and Arginine (Arg) kinases(H. H. Yu, Zisch, Dodelet, & Pasquale, 2001); chemokine receptors(Zlotnik & Yoshie, 2000) and some adhesion molecules(Huynh-Do et al., 1999).

The function of EPHs/ephrins in different biological processes and systems

A great number of studies about Eph/ephrin functions have been carried out. EPHs/ephrins are implicated in many biological processes such as embryonic development, tissue boundary formation and cell migration. Additionally, they also play critical roles in angiogenesis (Cheng, Brantley, & Chen, 2002) and stem cell differentiation (Genander & Frisen, 2010).

EPHs and EPHRINs are expressed in many tissues and organs. They play important roles in the central nervous system(Dumas et al., 2000; Xin, Deng, Rishniw, Ji, & Kotlikoff, 2002), immune system(H. Luo, Charpentier, et al., 2011; H. Luo, Wan, Wu, & Wu, 2001; H. Luo, Wu, et al., 2011; H. Luo, Yu, Tremblay, & Wu, 2004; Hongyu Luo, Yu, Wu, & Wu, 2002; J. Wu & Luo, 2005; G. Yu, Luo, Wu, & Wu, 2003a, 2003b, 2004; G. Yu, Mao, Wu, Luo, & Wu, 2006), digestive system(Batlle et al., 2002), bone metabolism (Davy, Bush, & Soriano, 2006; C. Zhao et al., 2006), cardiovascular system (H. U. Wang, Chen, & Anderson, 1998) and other systems (Dravis et al., 2007; Hashimoto et al., 2007; Konstantinova et al., 2007).

EPHs/ephrins in the nervous system

The first described function of EPHs and ephrins was to regulate cell-cell communication in the central nervous system (Himanen et al., 2007). Later, more and more important functions of EPHs/Ephrins in the nervous system were found.

During neuron development, axons first need to find their appropriate target regions, and then have to recognize the correct area to form specific connections. During this process, many pieces of evidence showed that the EPHs/ephrins are critical in axon guidance (Flanagan & Vanderhaeghen, 1998).

GPI-anchored ephrinAs are involved in axon repulsion. Before cell–cell contact, A-Disintegrin-And-Metalloprotease (ADAM) will associate with ephrinA2 on the same cell. When encountering a second cell expressing EphA3 receptors, the interaction between ephrinA2 and EphA3 leads to the formation of higher-order ligand–receptor clusters, which help the ADAM10 to cleavage ephrin ectodomain (Egea & Klein, 2007), terminating the ephrinA2/EphA4 interaction, hence the repulsion.

Another report demonstrated that EPHs/ephrins cause axon guidance through the Rho family GEF Vav. During axon guidance, Vav-dependent endocytosis triggers EPHs and ephrins to form a ligand-receptor complex, leading to the generation of growth cone collapses, which is necessary for axon guidance. This endocytosis is blocked when Vav protein is absent, suggesting that Rho family GEF Vav can be a key molecular in Eph/ephrin signaling pathways relevant to axon guidance (C. W. Cowan et al., 2005).

Neural crest cells come from the neural epithelium. They can migrate to particular destinations, and differentiate into many cell types, including most neurons and glia cell of the peripheral nervous system, and most of the connective and skeletal tissue in the head. These cells migrate

following a particular pattern, either repulsive or attractive. EphrinB ligands are one set of repulsive cues, controlling the segmental migration of trunk and branchial neural crest cells (Wilkinson, 2001).

EphA4 is required for typical fasciculation of spiral ganglion neurons (SGN) axons in their fringe directions. *In vivo* study in mice demonstrated that a lack of EphA4 in SGN peripheral axons causes significantly smaller fascicles. A similar phenotype also happens when ephrinB2 is mutated (Cramer & Gabriele, 2014).

EPHs/Ephrins also help to maintain the functional plasticity in neuronal circuits. Some members of Eph receptor family, such as EphB1–3 and EphA4, are expressed in the subventricular zone while their ligands ephrinBs are expressed in astrocytes. Their interaction will disrupt the migration of neuroblasts and increase cell proliferation (Conover et al., 2000; Lledo, Alonso, & Grubb, 2006).

EPHs/ephrins in cancer

As EPHs/Ephrins play key roles in the regulation of cell migration and adhesion, they are strongly related to tumorigenesis. They are upregulated essentially in all types of cancer cells (Garber, 2010). Eph signaling in tumor suppressor activities has been reported in many different types of tumors, including colorectal, breast, prostate, and skin tumors (Pasquale, 2008).

Both ephrinBs and their receptors play a role in the growth and development of colon carcinoma (CC). In the intestinal epithelium, the activation of EphBs will enhance cell sorting and compartmentalization of CC cells (Cortina et al., 2007), and control the cell differentiation through E-cadherin–mediated adhesion (Herath et al., 2006). One particular pair of

EPHs/ephrins in CC is EphB4 and ephrin B2. Both of them are overexpressed in the epithelium of the intestinal luminal surface in CCs, regulating the tumor angiogenesis and vasculogenesis (Stephenson, Slomka, Douglas, Hewett, & Hardingham, 2001).

EPHs/Ephrins are overexpressed in ovarian cancers (Herath et al., 2006). EphrinA2 in tumor cells or endothelial cells has been observed in >75% of ovarian cancers. It is associated with increased microvascular density and increased expression of ovarian cancer special markers such as matrix metalloproteinases 2, 9, and 14 (Y. G. Lin et al., 2007). Overexpression of EphA2 is correlated to increased microvessel density (MVD), which is strongly associated with critical factors involved in angiogenesis and tumor invasion (Y. G. Lin et al., 2007).

EphA2 inhibitors are especially effective in treating ovarian cancers when used in combination with taxanes (a type of antimicrotubule agent), indicating that EphA2 is an attractive target for ovarian cancer therapy (Landen, Kinch, & Sood, 2005).

There are different types of EPHs expressed in breast cancers. EphB4 functions as a tumor suppressor in breast cancer. In breast cancer cells, EphB4 activates an anti-oncogenic pathway involving Abl family tyrosine kinases and the Crk adaptor protein. This Abl-Crk pathway downregulates the pro-invasive MMP2, not only inhibits breast cancer cell viability and proliferation, but also affect the motility and invasion,(Noren, Foos, Hauser, & Pasquale, 2006). In some breast cancers, the tumor suppressor gene HIC1 (hypermethylated in cancer 1) restricts ephrinA1 expression, the default function of which is to reduce tumor growth (W. Zhang et al., 2010).

The existing data show that EphA2 receptor overexpression and phosphorylation play a role in the progression of malignant prostate cancer, involving processes of cell adhesion, motility, invasion, and formation of metastases. EphA2 kinase deficient in the prostate tumor cells are correlated to cell rounding, retraction fiber formation, de-adhesion from the extracellular matrix, RhoA and Rac1 GTPase regulation, three-dimensional matrix invasion, and in vivo metastasis(Taddei et al., 2009).

Beside EphA2, EphB4 protein overexpression is also found in most prostate tumors. Such overexpression is induced by a loss of phosphatase and tensin homolog (PTEN) or p53, or induced by epidermal growth factor/epidermal growth factor and insulin-like growth factor-I. One report indicates that knockdown of the EphB4 protein by siRNA or antisense significantly inhibits cell viability, migration and invasion, and induces apoptosis in prostate cancer cell lines(Xia et al., 2005).

There seems to be a competition amongst Eph kinase family members in prostate cancer cells, in regulating tumor cell locomotion and invasiveness. Many members, such as EphB3, EphB4 and ephrin-B2, join in this competition. The balance between the different members leads to the switch of the migration of cancer cells from restrained to invasive (Astin et al., 2010).

Increased EphB4 and ephrin-B2 expression may reflect increased potential for growth and tumorigenicity in endometrial carcinomas. Furthermore, the EphB4 receptor kinases may modulate the biological behavior of endometrial carcinomas through autocrine and paracrine activation. That's caused by Efnb2 ligand which expresses on the same or adjacent cells surface (Takai, Miyazaki, Fujisawa, Nasu, & Miyakawa, 2001).

The expression of EphA1 in nonmelanoma skin cancers is augmented. It seems to be a marker of the normal differentiated epidermis. The downregulation of EphA1 in nonmelanoma skin cancer may contribute to carcinogenesis of this cancer (Hafner, Becker, Landthaler, & Vogt, 2006).

The expression of EphA2 and its ligand, ephrinA1, is significantly increased in carcinomas of the urinary bladder (Abraham et al., 2006).

EPHs/ephrins in the immune system

Eph/ephrin signaling is incorporated with inflammation disorders actuated by infection, injury, and inflammation.

First, EPHs/ephrins are involved in the activation of T cells. A prominent Eph function in T cells relates to T-cell costimulation. With the solid-phase antibody against EphB6 or solid-phase EPHRINB1-3 Fc, T-cell responses to suboptimal T-cell receptor (TCR) ligation could increase(H. Luo et al., 2004; G. Yu et al., 2003a, 2003b).

Normally, the TCR signaling strength could be positively or negatively modulated by certain EPHs, leading to different thymocyte selection results (Freywald, Sharfe, Rashotte, Grunberger, & Roifman, 2003).

Stimulation of Eph receptors on human CD4+ T cells by ephrinA1 enhances their migration (Hjorthaug & Aasheim, 2007). EphA receptor activation induces rapid tyrosine phosphorylation of the Casitas B-lineage Lymphoma (c-Cbl) proto-oncogene. In T cells that express EphA1 and EphA4 receptors, Cbl phosphorylation is observed. The following signaling events involve adaptors Crk-L and Crk-II (Sharfe, Freywald, Toro, & Roifman, 2003), resulting regulation of T cell migration into inflamed tissues (Y. Huang et al., 2015).

Another pathway through which ephrinA1 regulates T cell function is via proline-rich tyrosine kinase 2 (PYK2). After the binding of ephrinA1 to its receptors, the phosphorylation and kinase activity of the Src kinase family member Lck will be induced. This activates focal adhesion-like kinase Pyk2 (Aasheim, Delabie, & Finne, 2005), which has an essential role in T cell activation and polarized secretion of cytokines (Sancho et al., 2002).

Chemotaxis is a necessary process in T cell migration. Ephrin-A1 strongly inhibits chemotaxis by altering the balance of activities of different small G proteins. In the same time, Ephrin-A1 also prevented Rho activation, lead to stromal cell-derived factor (SDF)-1α induced activation of cdc42. The cdc42 activation can further increase calcium flux and activation of MAPK (Sharfe, Freywald, Toro, Dadi, & Roifman, 2002). As we mentioned before, EPHs/Ephrins activate MAPK strongly, downstream of which are Jun N-terminus kinase (JNK), p38 and Erk. All those molecules play crucial roles in cell migration (C. Huang, Jacobson, & Schaller, 2004).

Secondly, Eph/ephrin signals are involved in thymus development. During thymus development, different signals drive precursors to mature into functional T lymphocytes. The Eph family members are implicated in this process by regulating cytoskeleton function and cell adhesion (Munoz et al., 2002). For example, mice deficient of EphB in thymocytes experience a significant reduction of thymocyte subsets (Alfaro et al., 2008). The reduced EphA4 expression results in decreased numbers of double-positive (CD4+ CD8+) thymocytes (Munoz et al., 2006). Besides, the development and organization of thymic epithelial cell (TEC) was also influenced by EphB2 and EphB3 (Munoz et al., 2011). The migration of the thymic primordium into the thoracic cavity requires ephrinB2 expression (Foster et al., 2010).

Thirdly, EPHs's function is not restricted to the enhancement of the strength of TCR signaling. They might modulate T-cell survival and death by modulating immune cell apoptosis,

depending on their activation status (J. Wu & Luo, 2005). After soluble EphrinA1 stimulation of CD4+ T cells, the IL-2 expression is reduced. So is IL-4 but not IFN-γ. As activation-induced cell death (AICD) depends on IL-2 and IL-4 cytokines and their receptors (Zubiaga, Munoz, & Huber, 1992), EphrinA1 could thus prevent AICD (Wohlfahrt et al., 2004).

EPHs/ephrins in Glucose Homeostasis

In order to maintain glucose homeostasis in the body, the glucose levels in the blood are controlled by insulin from β cells in the pancreas. The underlying molecular mechanisms of how β cells modulate insulin secretion is not entirely understood. Recently, some studies demonstrated that both Eph/ephrins A and B family members are expressed in mouse and human islets, and their signaling is strongly related to the β cell function.

A recent study shows that certain communication between β cells is via EphA receptors and ephrinA ligands both *in vitro* and *in vivo.* EphA forward signaling will inhibit insulin secretion while ephrinA reverse signaling could enhance insulin secretion. Those functions lead to a two-way autoregulation in pancreatic cells. When blood sugar level is low, EphA forward signaling can be activated, leading to increasing insulin secretion; when blood glucose level is high, EphA receptor is dephosphorylated, leading to downregulation of insulin secretion (Kulkarni & Kahn, 2007).

A new hypothesis is that EphA receptors not only exist on the cell surface but also in the intracellular insulin secretory granules. This suggests that EphA levels on the plasma membrane, and, therefore, EphA-ephrinA complexes, increases upon insulin release. This causes a feedback loop that negatively limits insulin secretion through increased EphA signaling. On the other hand, there is a positive feedback loop: when glucose levels are low, the ephrinA signaling will upregulate to increase insulin secretion (Konstantinova et al., 2007).

One animal experiment showed that ephrinA5 KO mice are mildly glucose intolerant. When subjected to the same concentration of glucose stimulation, compared with the control group, the significant reduction in their insulin secretion. In contrast, the insulin content of these islets was not significantly changed. Also, knockdown of ephrinA5 in MIN6 cells reduces glucose-stimulated insulin secretion (Konstantinova et al., 2007; Pasquale, 2008).

Another relevant peptide for glucose homeostasis is glucagon. When the inhibition of glucagon secretion is out of control, types 1 and 2 diabetes happen. However, the molecular mechanisms that regulate glucagon secretion in normal and diabetic states are not fully understood. A new study shows that the tonic stimulation of α-cell EphA receptors between neighboring islet cells could negatively regulate glucagon secretion. The effect of EphA is mediated by juxtacrine. Sorted α-cells lacking endogenous stimulation of EphA forward signaling from neighboring cells will result in enhanced basal glucagon secretion and the elimination of glucose-induced inhibition of glucagon secretion. In these cells, both normal basal glucagon secretion and glucose-induced inhibition of glucagon secretion can be recapitulated by restoration of EphA forward signaling. In vivo. α-cell-specific EphA4-/- mice exhibit abnormal glucagon dynamics (Hutchens & Piston, 2015).

EPHs/ephrins in bone maintenance and homeostasis

Bone homeostasis strictly maintains balance, this maintenance in the cell largely depends on the cellular communication between osteoclasts and osteoblasts, and closely related to the coupling between bone resorption and bone formation. A range of pathologic disorders, including osteoporosis and cancer-induced bone diseases, disrupt this coupling and cause subsequent alterations in bone homeostasis.

Eph receptors and their associated ligands, ephrins, are also expressed in the bone marrow microenvironment, including osteoclasts and osteoblasts. More and more evidence suggests that this receptor family is closely related to regulating normal and pathological bone remodeling (Edwards & Mundy, 2008).

Some skeletal malformations are caused by the developmental deficiencies in EphB/ephrinB signaling, including cleft palate, defective development of the skull vault, craniosynostosis, and other bone abnormalities. The mutations of EphA2, EphA3 or ephrinB1 in mice may cause the X-linked developmental disorder of craniofrontonasal syndrome (Davy & Soriano, 2005; Pasquale, 2008).

Genetic and another evidence supports a model in which EphB/ephrinB1 bidirectional signaling at the ectopic boundaries that form between ephrinB1-positive and negative osteoblast precursors leads to impaired gap junction communication, which inhibits osteoblast differentiation and delays ossification of developing calvarial bones(Pasquale, 2008).

EphB4 receptors are expressed on osteoblasts, whereas osteoclasts express the ligand ephrinB2. Forward signaling through EphB4 into osteoblasts enhances osteogenic differentiation. Reverse signaling through ephrinB2 into osteoclast precursors suppresses osteoclast differentiation. Cytokines produced by osteoblasts activate the transcription factors c-Fos and NFATc1 in osteoclast precursors, and then promote osteoclast differentiation and increase ephrinB2 expression. EphB4 / EphrinB2 protein signaling complex mediated a significant bone remodeling process: activating bone formation while inhibiting bone resorption (Pasquale, 2008; Raggatt & Partridge, 2010).

Besides these developmental roles, EphB/ephrinB bidirectional signaling between osteoblasts and osteoclasts has been implicated in the regulation of bone homeostasis in the adult (C. Zhao et al., 2006).

EPHs/ephrins in the cardiovascular system

The studies about EPHs/ephrins in the cardiovascular system are limited, and those studies are mainly focused on EphrinAs.

In the adult human myocardium, cardiomyocytes preferentially express ephrinA1 ligand. Human cardiac stem cells (hCSCs) express EphA2 receptor (Goichberg et al., 2010). EphrinA1 promotes the motility of EphA2-positive hCSCs, enhancing cardiac repair. Thus, in stem cell therapy, ephrinA1 stimulation to hCSCs before their myocardial delivery can improve cell targeting to the sites of injury (Goichberg et al., 2011).

EphrinA1is also a regulatory factor in cardiac valve formation. A loss of ephrinA1 results in thickened aortic and mitral valves in newborn and adult animals. Animal study shows that deficient of ephrinA1 may cause congenital heart defects (Frieden et al., 2010).

Decreased ephrinB1 is associated with molecular/functional cardiac defects. EphirnB1 acts as a stabilizer of the cardiomyocyte morphology and the overall cardiac tissue cohesion, and may represent a new factor in the pathogenesis of heart failure (Genet et al., 2012).

EPHs/ephrins in angiogenesis

Angiogenesis is a highly complex process, through which new blood vessels form from pre-existing vessels (Birbrair et al., 2014). It plays a critical role in normal development and the pathophysiology of multiple disease processes, including tumor neovascularization, ischaemic recovery, and wound healing (Makrilia, Lappa, Xyla, Nikolaidis, & Syrigos, 2009). More

recently, there have been many research on the participation of EPHs/Ephrins in angiogenesis. It has been revealed that EPHs/Ephrins play pivotal roles in modulating the angiogenic process in the cardiovascular system and tumor vascularization (J. Zhang & Hughes, 2006).

Most studies focused on the EphB/ephrinB sub-families, as they can induce capillary sprouting *in vitro* with a similar efficiency as VEGF, and contribute to the development and demarcation of the boundaries between arterial and venous vessels in the murine embryonic cardiovascular system (Adams et al., 1999; Kubis & Levy, 2003).

The interaction between EphrinB2 and EphB4 has been studied extensively with respect to angiogenesis. Recent reports show that the ephrinB2 ligand is an important artery endothelial marker in the initial stages of embryo angiogenesis, and its receptor EphB4 marks the embryo vein endothelium. These findings suggest that ephrinB2 and EphB4 are involved in establishing arterial versus venous identity and perhaps in anastomosing arterial and venous vessels at their junctions (Gale et al., 2001). One report demonstrates that ephrinB2 functions in the early embryonic stage as a typical instructive ligand to stimulate EphB4 receptor forward signaling during angiogenic remodeling(Yancopoulos, Klagsbrun, & Folkman, 1998). Because of defects in the embryonic vascular system reconstruction, mice lacking ephrinB2 die *in utero* before embryonic day 11.5(Adams et al., 1999; Y. Lin, Jiang, Ng, Jina, & Wang, 2014). However, the ephrinB2 not only express on the vascular endothelial cell surface, but also capable of playing a role in the adjacent mesenchymal cells. Furthermore, ephrinB2 expression in perivascular mesenchyme is not sufficient to compensate for the loss of ephrinB2 in these vascular cells (Gerety & Anderson, 2002).

The understanding of molecular and cellular mechanisms of EPHs/Ephrins governing vascular development is still poor. Some researchers claim that cell-bound ephrin ligands are the key

target molecules for vascular endothelial growth factor receptors (VEGFR-1 and VEGFR-2)(Adams & Klein, 2000). The EphB4 receptor binds to the membrane-anchored ligand ephrinB2(Gerety & Anderson, 2002), the ephrinb2 ligand binds to VEGF, Tie-1 and Tie-2 receptors, and the multimolecular complex then further bind to the angiopoietins, regulating angiogenesis.

EPHs/ephrins in vascular smooth cells

Until recently, there are only a few studies related to the role of EPHs/EPHRINs in vascular smooth muscle cell (VSMC) function. In our recent studies, we have found that EphB6, EphB4 and EphrinB1 in concert with sex hormones regulate blood pressure (BP). The interesting fact is that although they are all Eph/EphrinB family members, their functions in BP regulation are different. While EphB6 and EphrinB1 deletion positively regulate the VSMC contractility and blood pressure(H. Luo et al., 2012; Z. Wu et al., 2012), EphB4 deletion has an opposite effect in these regards(Y. Wang et al., 2015).

Part II. Hypertension

Hypertension is a chronic medical condition of high arterial blood pressure. In the arterial system, blood pressure is measured by two parameters, the systolic and diastolic pressures. The most recent diagnosis recommendation was established by the Eighth Report of the Joint National Committee (JNC): for the person aged 30 to 59 years old, normal blood pressure should be below 120/80mmHg; blood pressure between 120/80mmHg and 139/89mmHg is called "pre-hypertension; blood pressure staying at 140/90 mmHg or above means hypertension. For the

hypertensive persons aged 60 years or older, a BP goal of less than 150/90 mmHg will be suitable; while for hypertensive adults with diabetes or nondiabetic chronic kidney disease (CKD), the goal is recommended as for the general hypertensive population younger than 60 years(140/90 mmHg)(James et al., 2014).

Nowadays, hypertension has become a worldwide critical medical and public health issue. It was the third largest disease burden (4.5%) according to disability-adjusted-life-years (64 million) (Ezzati et al., 2002). Approximately more than one billion people have hypertension in the world. Hypertension is estimated to cause 7.1 million premature deathsper year (Whitworth & World Health Organization, 2003). Based on large-scale population-based studies of prevalence, the incidence of resistant hypertension, and associated risk factors carried out by US National Health and Nutrition Examination Survey (NHANES), the prevalence of resistant hypertension is 8–12% of adult patients with high blood pressure (6–9 million people in US). The increasing prevalence of resistant hypertension contrasts to the improvement in blood pressure control rates during the same period (Sarafidis, Georgianos, & Bakris, 2013).

With such high disease incidence, and very high attendant risks for cardiovascular and kidney diseases, hypertension always ends up with a high morbidity and mortality rate. So, hypertension treatment is of great importance to public health.

Symptoms and pathogenesis of hypertension

There is no common symptom of hypertension. Some patients may suffer from different degrees of a headache or vertigo, but these are not always serious. Hypertension is usually detected by regular health check (Kaplan & Opie, 2006).

There are two different types of hypertension, primary and secondary. Primary hypertension is also named essential, or idiopathic hypertension. It is defined as high BP without any known

causes. Primary hypertension is a polygenic disorder, accounting for 95% of all cases of hypertension. The diagnosis is made when no other causes for increased BP are found (Carretero & Oparil, 2000). Secondary hypertension is a type of hypertension with an underlying, potentially correctable cause, such as renovascular disease, aldosteronism, or other causes (Viera & Neutze, 2010).

Both environmental and genetic factors may contribute to regional and racial variations in BP and hypertension prevalence. Social studies show that urbanization, and the immigrants who were assimilated by the environment, will have some effect on blood pressure ("Hypertension: uncontrolled and conquering the world," 2007). Obesity and weight gain are strong, independent risk factors for hypertension. It has been estimated that 60% of hypertensives are >20% overweight (Messerli, Williams, & Ritz, 2007). Population studies show that the diet is important in the prevalence of hypertension, especially sodium intake. Age is also a risk factor for high blood pressure, when the elderly and high-salt diet coexist, the risk of developing hypertension could significantly increase. Diet low in calcium and potassium content also increases the risk of hypertension. The urine sodium-to-potassium ratio has a stronger correlation to blood pressure than is either sodium or potassium alone. Alcohol consumption, psychosocial stress, and low levels of physical activity may also contribute to hypertension.

A number of factors increase BP: e.g., obesity(Parati, Liu, & Ochoa, 2014), insulin resistance(Cutler, 1996; Grassi, 2005), alcohol and substance abuse (Chaturvedi, 2004), high salt intake (in salt-sensitive patients)(Gong & Hubner, 2006; Zozaya, 2000), aging, perhaps sedentary lifestyle, risky sexual behavior(Scott & Happell, 2011; Shimbo et al., 2013) stress(Oehme et al., 1987), low potassium intake(Elliott, 1991), or low calcium intake(Cormick,

Ciapponi, Cafferata, & Belizan, 2015). Furthermore, many of these factors are additive, such as obesity and alcohol intake (Oparil, Zaman, & Calhoun, 2003).

Pathology of hypertension

Pathology of primary hypertension

Pathophysiology

Cardiac output and peripheral resistance. The role of the circulatory system is to pump blood to various body systems. Blood flow arises because the heart muscle as a pumping organ of power, can generate a predetermined pressure difference. The relation between the pressure difference and flow can be described by the Ohm's Law for electrical current (Mayet & Hughes, 2003). The formula is as follows:

$$\Delta P = Q \times R$$

(Where ΔP=pressure difference; Q=bulk flow; R=resistance)

The relation between mean arterial pressure, cardiac output, and peripheral resistance (Mayet & Hughes, 2003), The formula is as follows:

$$MAP = CO \times PVR$$

(Where MAP=mean arterial pressure, CO=cardiac output (=stroke volume × heart rate), PVR=total peripheral vascular resistance)

An average blood pressure is maintained by the balance of the cardiac output and peripheral vascular resistance. The majority of essential hypertension are caused by raised peripheral resistance, which is determined by small arterioles.

Renin-angiotensin system. The most important players in the endocrine system for blood pressure controlling are in the renin-angiotensin system(Engeli, Negrel, & Sharma,

2000).(Figure 2) The renin-angiotensin-aldosterone concatenation, via two effector components, angiotensin II and aldosterone, simultaneously regulates body sodium and water content, arterial blood pressure and potassium balance(Laragh et al., 1972). Renin is responsible for converting angiotensinogen to angiotensin I, which is converted to angiotensin II in the lungs by angiotensin converting enzyme (ACE). Angiotensin II is a potent vasoconstrictor and thus causes a rise in blood pressure. The circulating renin/angiotensin are now thought to be directly responsible for the increase in BP in essential hypertension (Lavoie & Sigmund, 2003). They are usually regulated in response to glomerular under-perfusion or a reduced salt intake (G. Beevers, G. Y. Lip, & E. O'Brien, 2001).

Angiotensin II is crucial in maintaining the structural and functional integrity of the vessel wall (Touyz & Schiffrin, 2000), and it plays a significant role in vascular smooth muscle cell contraction. Also, Angiotensin II induces vascular wall adhesion molecule-1(Kranzhofer, Browatzki, Schmidt, & Kubler, 1999).

In addition to the effect on arterial pressure, angiotensin II also significantly contributes to the development and progression of hypertensive heart disease (Figure 3)(Ibrahim, 2006; Kobori, Ichihara, Miyashita, Hayashi, & Saruta, 1999).

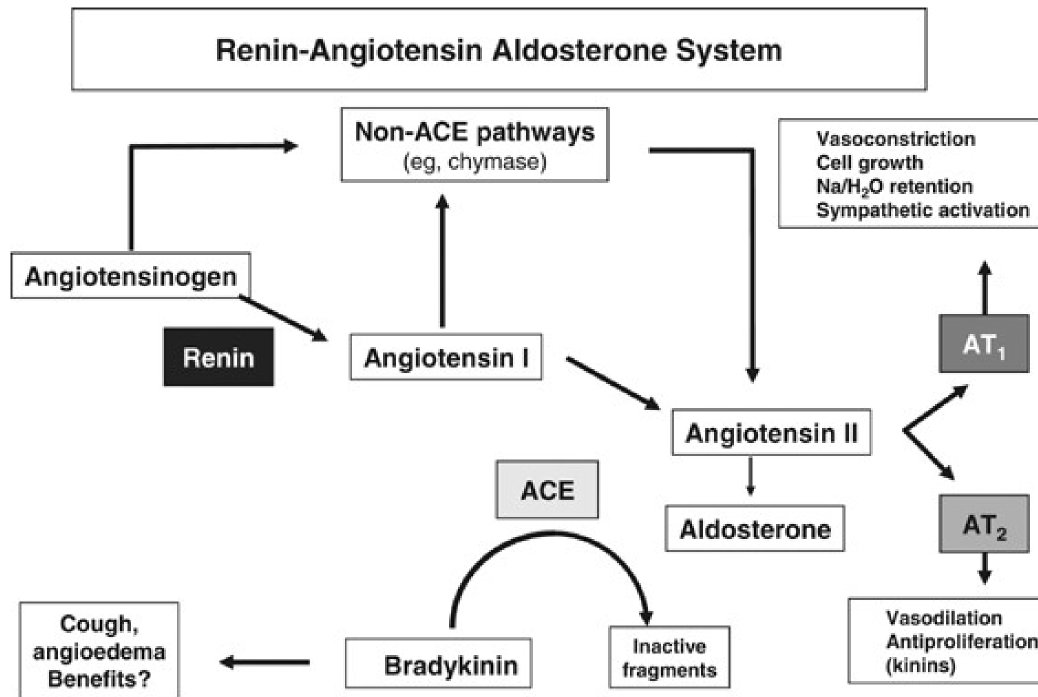

Figure 3. The renin–angiotensin system

Autonomic nervous system. Arteriolar constriction can also be caused by sympathetic nervous system stimulation, so the latter plays an important role in maintaining a normal blood pressure. Apparently, hemodynamics is changed in hypertension. The central nervous system seeks to maintain systemic blood pressure at a higher level (Julius, 1991). When vascular over-responsiveness sets in, the less sympathetic drive is needed to maintain neurogenic hypertension (Julius, 1990).

There is also an interaction between the autonomic nervous system and the renin-angiotensin system, both of which function together with sodium, circulating volume, and some of the more recently described hormones (Julius, Esler, & Randall, 1975).

Vascular endothelial cells. The vascular endothelium is a layer of squamous epithelial cells which directly contact with the blood. It tweaks the reactivity of vascular smooth muscles. The

function of vascular endothelium includes the follows. It intervenes a physical obstruction between the vascular smooth muscle and hormones and other vasoactive substances circling in blood. It concentrates or metabolically degrades vasoactive substances, such as norepinephrine, serotonin, and kinins, and subsequently averts or reduces their movement in the vascular smooth muscle. It secretes vasoactive substances, such as nitric oxide (NO)(Forstermann & Munzel, 2006; Moncada & Higgs, 2006), prostacyclin(Moller & Grande, 1999) and endothelin-1(Bohm & Pernow, 2007). It releases other so-far unidentified vasoactive inhibitory and excitatory factors (De Mey, Claeys, & Vanhoutte, 1982).

Nitric oxide is a vasodilative molecule (Nathan & Xie, 1994). It has been found to play a significant role in many parts of the body (Figure 4(Faraci & Heistad, 1998)). It, acts not only as a physiological intercellular messenger but also displays cytotoxic activity *in vivo* (Beckman & Koppenol, 1996; Kroncke, Fehsel, & Kolb-Bachofen, 1997). NO serves as biological agents in regulating BP. Abnormal increase of nitric oxide synthase could reduce acetylcholine levels, and leads to a rise in 3'5'-cyclic adenosine monophosphate (cAMP) and 3'5'-cyclic guanosine monophosphate (cGMP) levels, and ultimately vessel relaxation (Bredt & Snyder, 1994; Hirsch et al., 1993; Lowenstein, Dinerman, & Snyder, 1994).

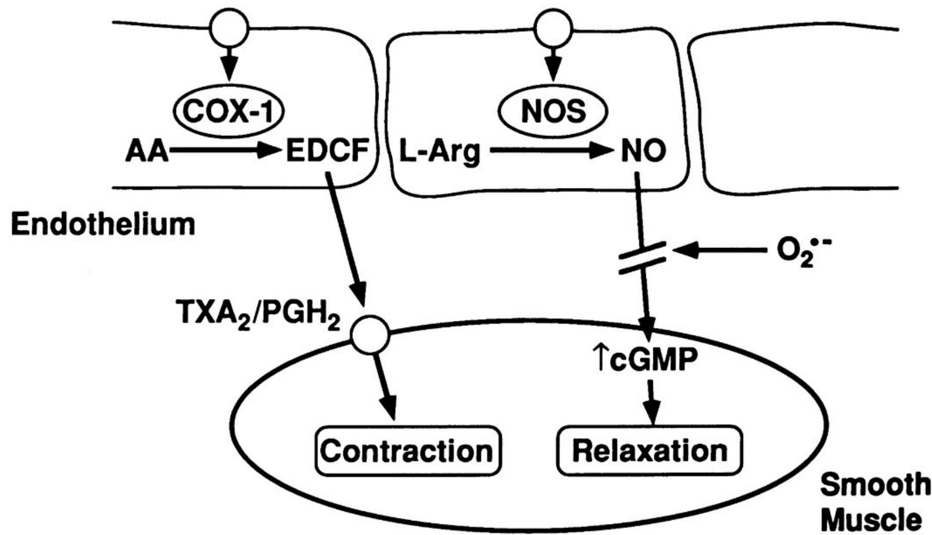

Figure 4. The mechanism of nitric oxide function in vessels

Endothelin is a powerful cytokine, which is a vasoconstrictive peptide, and may lead to a salt-sensitive rise in BP. It mainly modulates pulmonary BP (Galie, Manes, & Branzi, 2004). A functional isomer of endothelin, endothelin-1, is involved in essential hypertension. It modulates the autocrine feedback loops in vascular smooth muscle cells *in vitro.* It can down-regulate platelet-derived growth factor and transforming growth factor-beta (Hahn, Resink, Mackie, Scottburden, & Buhler, 1993). The endogenously generated endothelin-1 can contribute to the maintenance of peripheral vascular tone by regulating phosphoramidon and inhibitor of endothelin converting enzyme (Haynes & Webb, 1994). Besides, it can also activate local renin-angiotensin systems, and act as a mediator in cyclosporine A (CsA)-related renal vasoconstriction (Lanese & Conger, 1993).

Vasoactive substances. There are many other vasoactive systems and mechanisms. Some could affect sodium transport while some could maintain the vascular tone.

Atrial natriuretic peptide (ANP) is a hormone secreted from the atria of the heart. It is released by cardiac muscle cells in response to high blood volume (Song, Wang, & Wu, 2015). The role

of ANP is to reduce the retention of water, sodium and fat in the circulation, thus reducing blood pressure (Jeppesen et al., 2015).

Sodium-dependent glucose co-transporters are a family of glucose transporters. They contribute to renal glucose reabsorption (Wright, 2001). In renovascular hypertension, it may be upregulated by angiotensin II via the AT1 receptor, and then contribute to increased absorption of Na^+ and therefore to the development or maintenance of hypertension (Bautista et al., 2004; Ismael-Badarneh et al., 2015). Some reports showed that it also can interrelate the calcium transport across of the vascular smooth muscle cell walls (McCarty & O'Neil, 1992).

Hypertension-related pathological conditions

Hypercoagulability: Patients with hypertension demonstrate vascular endothelial dysfunction. At the same time, the levels of hemostatic factors, platelet activation, and fibrinolysis increase abnormally, and the blood flow reduces. All those conditions suggest that hypertension confers a prothrombotic or hypercoagulable state (G. Beevers, G. Y. H. Lip, & E. O'Brien, 2001; Lip & Li-Saw-Hee, 1998).

Insulin sensitivity: Insulin resistance syndrome (IRS) has the potential to explain a large group of common metabolic and cardiovascular disorders (DeFronzo, 1997). The insulin resistance plays a role in the pathogenesis of essential hypertension, through an antidiabetic agent such as Troglitazone (Ogihara, Rakugi, Ikegami, Mikami, & Masuo, 1995), sympathetic nerve activity, or increasing muscle blood flow stimulated by insulin (Scherrer & Sartori, 1997).

Diastolic dysfunction: cardiac diastolic dysfunction has been found to be common in patients with hypertension. It will slow the maximal rate of left ventricular filling in hypertension (Fouad, Slominski, & Tarazi, 1984). Isolated diastolic dysfunction very often accompanies hypertensive

heart disease, and is also associated with myocardial ischemia and fibrosis in hypertension(Slama, Susic, Varagic, & Frohlich, 2002).

Genetic disorders

Liddle's syndrome: Liddle's syndrome is an autosomal dominant genetic disorder. It leads to high blood pressure associated with low plasma renin activity, increased resorption of sodium, metabolic alkalosis, low blood potassium, normal to low levels of aldosterone and water in the renal collecting tubules (Palmer & Alpern, 1998).

Syndrome of apparent mineralocorticoid excess (AME): AME is an autosomal recessive disorder causing hypertension and hypokalemia (Levtchenko et al., 2007). It results from defective 11b-hydroxysteroid dehydrogenase type 2 (11b-HSD2), which is critical for mineralocorticoid synthesis. The deficiency of mineralocorticoid allows the unmetabolized cortisol to bind to the mineralocorticoid receptor, inducing sodium retention, hypokalemia, suppression hypertension (Palermo, Quinkler, & Stewart, 2004).

Glucocorticoid-remediable aldosteronism (GRA): GRA is an autosomal dominant disorder in which the increase in aldosterone secretion produced by adrenocorticotropic hormone (ACTH) is no longer transient. It is one of the most common monogenic causes of hypertension (Halperin & Dluhy, 2011). The syndrome is characterized by high levels of aldosterone, suppressed plasma renin activity, high incidence of intracranial hemorrhage, and paradoxical sensitivity to glucocorticoid therapy (Kamrath, Maser-Gluth, Haag, & Schulze, 2011; Pizzolo et al., 2005).

Pseudohypoaldosteronism type II (PHAII): PHAII is a group of genetic disorders, characterized by hypertension and hyperkalemia despite normal glomerular filtration rates. There are four types of PHAII, which are determined by the following gene mutations: WNK4 (PHA type IIB),

WNK1 (PHA type IIC), KLHL3 (PHA type IID), and CUL3 (PHA type IIE)(F. H. Wilson & Kahle, 1993).

Vascular Function

Vascular smooth muscle tone

The vessel tone is mediated by vascular smooth muscle cells signaling pathways. Generate tension leads to vasoconstriction and the release of tension is causing blood vessels to relax.(Woodrum & Brophy, 2001). The intrinsic tone of vascular smooth muscle is determined by the balance between contraction and relaxation signals, which in turn regulates the dynamic caliber of the blood vessel. This balance is regulated by some extracellular signals, including neural, humoral, ionic, and mechanical forces, which induce contraction or relaxation of the vascular smooth muscle. All those factors are thought to lead to an alteration in intracellular calcium concentration, resulting changes in vessel tone (Hill-Eubanks, Werner, Heppner, & Nelson, 2011).

Vascular smooth cell function

The function of the vascular smooth muscle cell (VSMC) is contraction/relaxation and the regulation of blood vessel tone-diameter, blood pressure, and blood flow distribution. The control of contraction and relaxation is dependent upon intracellular and extracellular signals(Clark & Pyne-Geithman, 2005). The contractile function of smooth muscle is modulated by the $Ca2^{+}$/calmodulin interaction to stimulate phosphorylation of the light chain of myosin. In order to maintain the force generated, the dephosphorylation of the myosin light chain by myosin phosphatase is inhibited by the RhoA/Rho kinase pathway (Jono et al., 2000).

Main signaling pathways in VSMCs

Intracellular Ca^{2+}: Changes in intracellular Ca^{2+} concentration play a vital role in the contraction of VSMCs. Calmodulin is one of the most important sensors of intracellular Ca^{2+} changes (Klee & Vanaman, 1982). Ca^{2+}–calmodulin complex could associate with many downstream kinases including myosin light chain kinase (MLCK)(Kamm & Stull, 2011).

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII): It is a serine/threonine-specific protein kinase that is regulated by the Ca^{2+}/calmodulin complex, and it has multiple functions. In our case, MLCK can be phosphorylated by CaMKII (Tansey et al., 1992), negatively modulating MLCK activity in VSMC (Van Riper, 1995). Thr-286 autophosphatase of CaMKII will decrease its sensitivity to Ca^{2+}, leading to a Ca^{2+}-independent function.

Protein kinases: A protein kinase usually causes a functional change of the target proteins by changing their enzyme activity or cellular location. Up to 30% of all human proteins may be modified by kinase activity(Manning, Whyte, Martinez, Hunter, & Sudarsanam, 2002). There are two protein kinases play a key role in VSMC contraction: myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP).

Myosin light chain kinase catalyzes the phosphorylation of 2 residues in the myosin light chain (MLC): serine 19 and threonine 18(Ikebe & Hartshorne, 1985). MLC phosphorylation requires relatively high concentrations of active myosin light chain kinase (Hathaway, Konicki, & Coolican, 1985; Nishikawa, Shirakawa, & Adelstein, 1985). This MLCK pathway is Ca^{2+}/calmodulin-dependented, and it can be modulated by some other kinases, such as p21-activated kinases (Sanders, Matsumura, Bokoch, & de Lanerolle, 1999; Wirth et al., 2003). Once the MLCK has been phosphorylating, its activity decreases (Gallagher, Herring, & Stull, 1997). Myosin light chain phosphatase plays a pivotal role in smooth muscle contraction by regulating myosin light chain phosphorylation (Hartshorne, Ito, & Erdodi, 1998), hence MLC Ca^{2+}

sensitivity. It is a G protein-mediated pathway, controlled by cGMP (Kitazawa, Eto, Woodsome, & Brautigan, 2000; Kitazawa, Masuo, & Somlyo, 1991). cGMP activates MLCP indirectly by the small GTPase Rho and its downstream target Rho kinase(Lee, Li, & Kitazawa, 1997; Parizi, Howard, & Tomasek, 2000; X. Wu, Somlyo, & Somlyo, 1996).

The small GTPase Rho and its target, Rho-associated kinase, are the major regulators of smooth muscle cell contract function. They mediate Ca^{2+} sensitization and are involved in the pathophysiology of hypertension (Uehata et al., 1997). Their function is to cause myosin II dephosphorylation mediated by MLCP and increase MLCK activity. The modulation of MLCP activity could be directly caused by Rho-associated kinase, independent of the Ca^{2+}/calmodulin-dependent myosin light chain kinase pathway(Kureishi et al., 1997; Somlyo & Somlyo, 2000, 2003).

Another important kinase family is protein kinase A (PKA). When smooth muscle cells are stimulated by platelet-derived growth factor (PDGF), mitogen-activated protein kinase (MAPK) and MAPK kinase (MAPKK) are activated rapidly. That will increase levels of cAMP and subsequently PKA activity (Graves et al., 1993).

The plasma membrane-associated, Ca^{2+}-sensitive form of protein kinase C can be stimulated by a rising of $Ca^{2+.}$ Furthermore, it would cause calmodulin-dependent activation of both myosin light chain kinase and the dissociation of caldesmon from the actin-caldesmon-tropomyosin-myosin fibrillar domain (Rasmussen, Takuwa, & Park, 1987; Takuwa, 1996). In most smooth muscles, PKC has contraction-promoting effects such as phosphorylation of Ca^{2+} channels or other proteins that regulate cross-bridge cycling.

Mitogen-activated protein (MAP) kinases, a family of serine/threonine protein kinases are involved in many different cell signaling pathways. A Recent study demonstrated that an MAP kinase-dependent pathway could mediate Ang II synthesis (Duff, Berk, & Corson, 1992).

Caldesmon and Ca^{+2}/calmodulin:

Caldesmon is a major calmodulin- and actin-binding protein found in smooth muscle cells. Current studies suggest a vital role for this protein in the regulation of smooth muscle contraction(Frid, Shekhonin, Koteliansky, & Glukhova, 1992). As a kind of regulatory contractile proteins, heavy caldesmon (h-caldesmon) and calponin were studied in SMCs development. In the actomyosin system, caldesmon inhibits the conversion of the chemical energy of ATP to mechanical force and inhibits the movement of actin filaments by myosin (Sobue & Sellers, 1991). It is also used a marker of VSMCs.

In general, various agonists bind to specific receptors to activate contraction in smooth muscle (Figure 5 (Webb, 2003)). After this binding, the prototypical response of the cell is to increase phospholipase C activity via coupling through a G protein. Phospholipase C produces an extraordinary potent second messenger, named inositol 1,4,5-trisphosphate (IP3). IP3 binds to specific receptors on the sarcoplasmic reticulum, causing the release of activator Ca^{2+}. Then Ca^{2+} further activates PKC, which phosphorylates specific target proteins. The activator Ca^{2+} binds to calmodulin, leading to the activation of MLCK. The concentration of free Ca^{2+} increase in sarcoplasmic under calmodulin (Calmodulin, CaM) participation. Then myosin light chain kinase (MLCK) is activated to catalyze the phosphorylation of myosin light chain, result in starting of the smooth muscle cross bridge cycling. However, the intracellular Ca^{2+} concentration increases shortly, contractile responses is mainly regulated by Rho kinase activity

through calcium sensitization mechanism, thereby inhibiting myosin phosphatase activity (Kienitz & Quinkler, 2008).

This calcium-sensitization mechanism and phospholipase C are activated at the same time, and this mechanism relates to the activation of small GTP-binding protein RhoA. According to the literature, RhoA guanine nucleotide exchange factor (RhoGEF) involves in the activation of RhoA, but the whole process of RhoA activation is not entirely clear. Then RhoA activation of Rho kinase activity can be increased, thereby inhibiting myosin phosphatase (Webb, 2003).

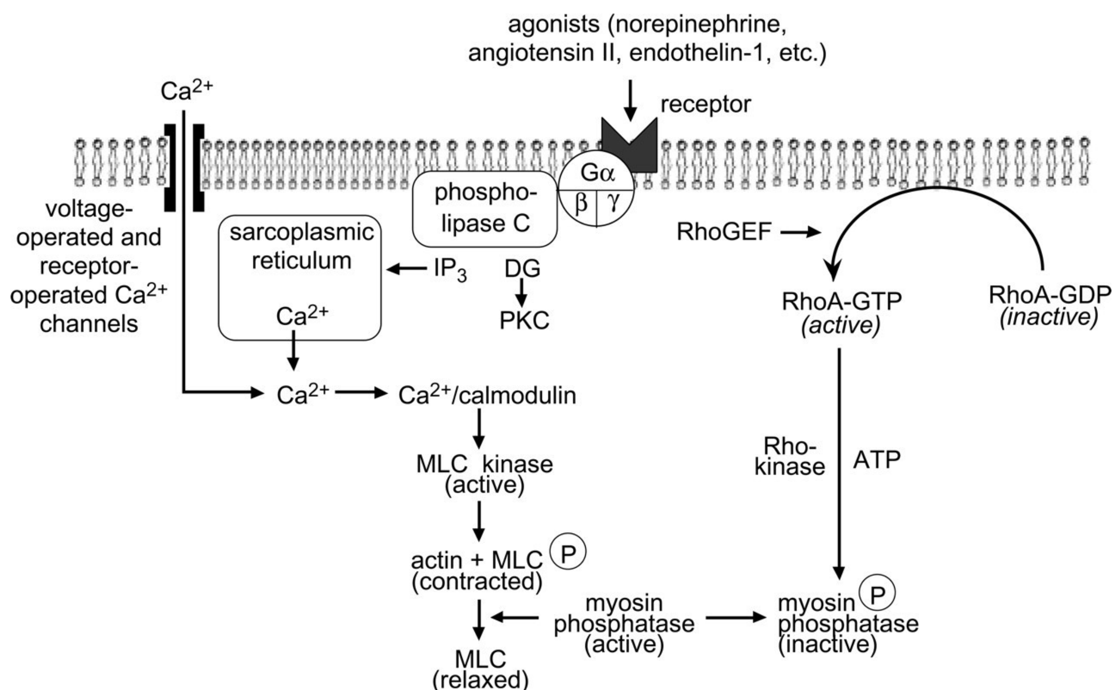

Figure 5. Regulation of smooth muscle contraction (Webb, 2003)

Other molecules

Free radicals and oxidative stress: At moderate concentrations, free radicals such as reactive oxygen species (ROS) play an important role as regulatory mediators in VSMC signaling processes. Many of the ROS-mediated responses protect the VSMC against oxidative stress. This include the regulation of vascular tone and erythropoietin production which is adjusted by

oxygen concentration, and various physiological processes of signal transduction from membrane receptors.(Droge, 2002). ROS and oxidant stress would be an inactive nitric oxide, and then modulate the vasomotor tone (Rocic, Seshiah, & Griendling, 2003). So they have the potential to be used a new specific therapy for hypertension patients with endothelial dysfunction (Cai & Harrison, 2000; Madamanchi, Vendrov, & Runge, 2005).

Cerebral vasospasm (CV): Vasospasm is recalcitrant to vasodilators. CV remains one of the main causes of the deferred neurological deficit and ischemic damage after subarachnoid hemorrhage (SAH).

Fatty acids: The major type of fatty acid with cardiovascular health benefits is long-chain n−3 polyunsaturated fatty acids (PUFAs). It has an effect on the vascular wall and the vascular endothelium. Since the endothelium plays a central role in the regulation and maintenance of vessel tone, fatty acids could have beneficial effects on the cardiovascular system (Abeywardena & Head, 2001).

Complications of hypertension

Patients with high blood pressure are more likely to have other components of metabolic syndrome. There are many clinical outcomes that result from hypertension: it is a risk factor for all clinical manifestations of atherosclerosis(Hollander, 1976); it is an independent risk factor for cardiovascular disease, especially heart failure(Kostis, 2003) and coronary artery disease(Hunt, 2006); it constantly leads to stroke risk; and it is also an important risk factor for renal diseases and peripheral arterial diseases.

Complications affecting the heart

As a major public health problem, congestive heart failure may develop as a result of long-standing hypertension and leads to a very high mortality (Kostis, 2003; Levy, Larson, Vasan, Kannel, & Ho, 1996). The major reason is the increased pressure load imposed on the heart, once the increased muscle mass can no longer cope with the load, the myocardial insufficiency develops. Another reason is the associated accelerated coronary atherosclerosis promoted by hypertension will induce myocardial hypertrophy (which often ends up with heart failure) (Kannel, Castelli, McNamara, McKee, & Feinleib, 1972).

Increased blood pressure can also make the blood vessel weak and stretch to form an aneurysm. If an aneurysm ruptures, it can be fatal(Krishnan & Todd, 2016).

Complications affecting the brain

Recent studies have demonstrated that there is a strong association between blood pressure and stroke (for both intracerebral hemorrhage and ischemic stroke). Hemorrhage is often caused by intracerebral vessel atherosclerosis. Higher blood pressure leads to lower blood flow in the small size vessels when lower cerebral blood flow occurs; ischemia happens. Sometimes, it may cause poor prognosis while sometimes the outcome will be less significant, such as troubles with memory and understanding, or ability to think and learn(Lawes, Bennett, Feigin, & Rodgers, 2004).

Complications affecting the eye

Frequently in the aged population, retinal microvascular develops in response to raised blood pressure, which will cause hypertensive retinopathy. Hypertension will also cause other retinal vascular diseases, such as ischemic optic neuropathy and retinal artery occlusion. Besides, hypertension is also involved in the diabetic retinopathy exacerbation. These are all important

hypertension-caused end-organ effects, which may result in vision loss. (Wong & Mitchell, 2007).

Complications affecting the kidneys

Hypertension-caused renal dysfunction has been investigated for many years. There are three stages of pathogenic determinants of hypertensive renal damage: the increase of systemic BP loading; the high loading transmitting the pressure to the renal vascular bed; barotrauma on the local tissue caused by the pressure. Once hypertension-related renal dysfunction occurs, the renin-angiotensin system (RAS) blockade will be damaged. The BP renoprotection provided by RAS blockade is diminished, and then higher BP ensues. This creates a vicious circle.

Complications associated with diabetes and hypertension

Metabolic syndrome: The metabolic syndrome considerably increases the risk of cardiovascular and renal events in hypertension(Redon et al., 2008). The symptoms include insulin resistance, obesity, and dyslipidemia. The pathophysiology of metabolic syndrome is complex, and many important factors are involved in the pathogenesis of this syndrome, such as sympathetic nervous system, insulin, endothelin-1, adipokines and tumor necrosis factor-α. The abnormal functions of these factors lead to a macro-microvascular malfunction, due to hypertension(Mendizabal, Llorens, & Nava, 2013).

Hypertension treatment

Currently, the hypertension medications can help patients to control their blood pressure with minor side effects. Different blood pressure medications work in different ways to lower blood pressure. Some remove extra fluid and salt from the body, others slow down the heartbeat or

relax and widen blood vessels. Most patients will require multiple drugs to achieve blood pressure goals, usually two or more medications work better than monotherapy.

Diuretics

Thiazide diuretics are the first effective oral antihypertensive agents. They reduce blood pressure by removing the extra fluid from the body, resulting in a reduction in both systolic and diastolic blood pressures. They are especially suitable for patients with low-renin or salt-sensitive hypertension. It could also enhance the efficacy of other antihypertensive agents, reduce the risk of cardiovascular events, and hypertension-related morbidity and mortality(Ernst, 2010).

β Blockers

Traditional β blockers are normally used for cardiovascular conditions, but it could also reduce blood pressure by decreasing cardiac output. However, the BP reducing effect is unstable, and may cause a high risk of reduced heart rates. Therefore, they are not used commonly as antihypertensive agents. On the other hand, vasodilatory β blockers could reduce blood pressure by reducing systemic vascular resistance. Furthermore, with beneficial effects on metabolic and lipid parameters, vasodilatory β blockers provide not only effective blood pressure control but also a treatment for the comorbid disease, including diabetes or coronary artery disease(Morgan, Anderson, & MacInnis, 2001; Ram, 2010).

Angiotensin-converting enzyme inhibitor (ACE Inhibitors)

ACE inhibitors are almost the first choice of antihypertensive agents. They reduce the activity of RAAS in the development of hypertension (Leonetti & Cuspidi, 1995; Morgan et al., 2001). They are suitable for patients with hypertension, type II diabetes mellitus, myocardial infarction and heart failure. However, ACE is a non-specific target for blocking the renin-angiotensin enzymatic cascade. The availability of orally active ACE inhibitors is a therapeutic breakthrough for hypertension, but more specific blockade always seems desirable (2015).

Angiotensin II receptor blockers

The effects of angiotensin II receptor blockers are almost equal to ACE inhibitors in hypertensive patients(Burnier & Brunner, 2000). It is an important new development for the treatment of hypertension and probably for congestive heart failure and chronic renal failure as well(Burnier & Brunner, 1998).

Calcium channel blockers

Calcium channel blockers are a class of structurally heterogeneous drugs, used widely to control blood pressure and manage symptoms of angina(Eisenberg, Brox, & Bestawros, 2004). There are two different types: dihydropyridines and nondihydropyridines. Dihydropyridines include amlodipine, felodipine, nicardipine, and nifedipine, whereas nondihydropyridines comprise agents such as diltiazem and verapamil(Luft & Haller, 1993).

Nervous system inhibitors

The activation of the sympathetic nervous system, triggered by reflex and humoral mechanisms(Dalakos, Streeten, Jones, & Obeid, 1974), is critical to the essential hypertensive state(Grassi, 1998). The importance of sympathetic activation in essential hypertension is now

well recognized. So nervous system inhibitors could powerfully reduce blood pressure, and potentially provide specific cardiovascular protection(Esler, 2000).

Vasodilators

Vasodilating drugs reduce the blood pressure by reversing the major hemodynamic abnormality. Vasodilation cause the blood pressure decrease by three aspects: cardiac output is increased by the reflex sympathetic activity, plasma renin activity increase, and sodium retention reduced plasma volume expansion.(Kincaidsmith, 1985).

Treatment of hypertension during pregnancy

Hypertension is a leading cause of maternal and fetal morbidity(Lindheimer, Taler, Cunningham, & American Society of, 2009). In the report of National High Blood Pressure Education Program working group, high BP in pregnancy has four categories: preeclampsia-eclampsia; chronic hypertension of any cause; preeclampsia superimposed on chronic hypertension; gestational hypertension(Zamorski & Green, 2001). The information about antihypertensive drugs during lactation remains limited. ACE inhibitors are important for treating proteinuria and diabetic patients while diuretics may decrease breast milk production and should be withheld(Lindheimer et al., 2009).

Children and Teens

Until recently, the incidence of persistent hypertension in children is in a range of 1% to 3%(Sinaiko, Gomez-Marin, & Prineas, 1989). However, due to obesity and other lifestyle factors, such as increased intake of high-calorie, high-salt foods, the tendency of hypertension in children is increasing. Hypertension in children is viewed as a significant risk factor for the development of cardiovascular disease in adulthood(Berenson et al., 1998).

The goal of treatment of hypertension in children is to reduce BP to a level below the ninetieth percentile and prevent the development of target-organ damage. However, few antihypertension drugs have been systematically tested in children. Thus little is known about the pharmacokinetics, the efficacy, or the safety of these agents in children (Mitsnefes, 2006). How to choose an appropriate antihypertension therapy during an appropriate age is still not been initiated; more clinical trials are still needed to establish the criteria for children (Kay, Sinaiko, & Daniels, 2001).

Part III. Sex hormones and blood pressure

Estrogen

Estrogen is an important regulator of blood pressure. It normally regulates metabolic syndrome, a collection of abnormalities including obesity, insulin resistance/glucose intolerance, hypertension, dyslipidemia, and inflammation, which together lead to increased risk of cardiovascular disease and diabetes(Roberts, Hevener, & Barnard, 2013).

The role of estrogens is mostly mediated by two nuclear receptors (ERα and ERβ) and a membrane-associated G-protein (GPR30 or GPER). Its function is not limited to the reproduction but extends to the skeletal, cardiovascular and central nervous systems. Various pathologic conditions such as cancer, inflammatory, neurodegenerative and metabolic diseases are often associated with dysfunctions of the estrogenic system. (Paterni, Bertini, Granchi, Macchia, & Minutolo, 2013).

Cardiovascular diseases, especially coronary heart diseases, are the leading cause of death among women in the US and exceed breast cancer mortality in women of all ages. Some animal and observational studies have suggested female ovarian sex hormones can protect against cardiovascular dysfunction(Yang & Reckelhoff, 2011). However, this is not a far-gone

conclusion, as 5% women take estrogen-containing contraceptives develop de novo hypertension(Chasan-Taber et al., 1996). The mechanism of how estrogen works in regulating BP remains under-studied. Therefore, the further mechanistic study of the effects of estrogen on the vascular system is warranted.

Estrogen in the cardiovascular system

Experimental evidence suggests that estrogens protect the premenopausal heart from hypertension and ventricular remodeling. Clinical and basic research has revealed that ovarian lower estrogen levels will affect the activation of renin - angiotensin - aldosterone system (RAAS), which is one of the pathogenesis of postmenopausal vasodilation dysfunction (Z. Zhao et al., 2014).

Obesity increases coronary artery disease caused by insulin resistance, and also increases the diabetes, hypertension, and dyslipidemia risk. After menopause, due to the lack of endogenous estrogen production, the probability of obesity greatly increases in woman(Meyer, Clegg, Prossnitz, & Barton, 2011). Estrogen and its receptors are important regulators of body weight and insulin sensitivity not only in women but also in men. (Meyer et al., 2011). On the other hand, overweight/obesity causes increased estrogen levels due to increased aromatase activity in the adipocytes(Cleary & Grossmann, 2009).

Other experiment showed that increased estradiol can stimulate the vessel to generate more vascular connective tissue, and also alters the proportions of collagen and elastin so that the vessel is more distensible(Fischer & Swain, 1977).

Estrogen Receptor

The third and non-genomic estrogen receptor has been identified, and it is called the G protein-coupled estrogen receptor (GPER/GPR30). Filardo et al. described that in breast cancer cell lines, the estrogenic activation of the mitogen-activated protein (MAP) kinases and extracellular signal-regulated kinases (ERK)-1/2 is dependent on GPER(Filardo, 2000). A known function of GPER is to regulate metabolic parameters associated with obesity and diabetes (Sharma et al., 2013).

17β-estradiol (E2) is the most potent human estrogen. It has several protective properties in the cardiovascular system: it activates a set of protective responses to protect cells from injury by activating signaling through the GPER cascade; influences the expression of protective heat shock proteins (HSP); protects the heart from ischemic injury(Booth, Flint, Lucas, Knittel, & Lucchesi, 2008; Knowlton & Korzick, 2014). However, the detailed mechanism is not elucidated.

Many questions remain unanswered about the blood pressure regulation of estradiol, and its non-genomic signaling cascade. Some research provided evidence showing that estrogen modulates the RAAS and NOS system and related intracellular signaling pathways. This modulation is partly via GPER(Z. Zhao et al., 2014).

GPER in vascular contraction

The cardiovascular role of estrogen is complex and far from fully understood with the identification of GPER(Figure 6(Prabhushankar, Krueger, & Manrique, 2014)). A better evaluation of this receptor may lead to more selective therapies to emulate the beneficial actions of estrogen in the cardiovascular system(Lindsey & Chappell, 2011). Activation of GPER exerts a protective effect on hypertension models and relaxes arteries *in vitro*. Some recent studies

showed that GPER-induced relaxation of small arteries is mediated via cAMP/PKA signaling. The experiment has also been carried out to determine downstream signals of the cAMP/PKA cascade and RhoA activity in cultured VSMCs and myosin light chain phosphatase (MLCP) activity in these artery rings. Once the RhoA activity was downregulated, GPER antagonist could restore it. In this case, localized cAMP/PKA signaling is involved in GPER-mediated vasodilation by activating MLCP via the inhibition of RhoA pathway(Prabhushankar et al., 2014; X. Yu, Li, Klussmann, Stallone, & Han, 2014).

There are two different conditions when GPER loses its function. a. Chronic GPER deficiency is associated with increased endothelial prostanoid-mediated vasoconstriction; GPER deletion could increase thromboxane prostanoid (TP) receptor-mediated contraction. b. Acute GPER blockade enhances endothelium-dependent contractions and reduces endothelial NO bioactivity(Meyer et al., 2012).

There are also animal experiments supporting the role of GPER in vasocontraction. Results showed that left ventricles of male Spontaneously Hypertensive Rats (SHR) express higher levels of GPER compared to normotensive rats. In addition, the selective GPER agonist G-1 induces negative inotropic and lusitropic effects to a greater extent in isolated and Langendorff perfused hearts of male SHR compared to normal rats(De Francesco et al., 2013). These cardiotropic effects elicited by G-1 involved the GPER/eNOS transduction signaling. G-1 also induces activation of ERK1/2, AKT, GSK3b, c-Jun and eNOS, and reduces the expression of ACE and Ang II. Hence, GPER may be considered as a useful target for the treatment of essential hypertension(Han & White, 2014; X. Yu et al., 2014).

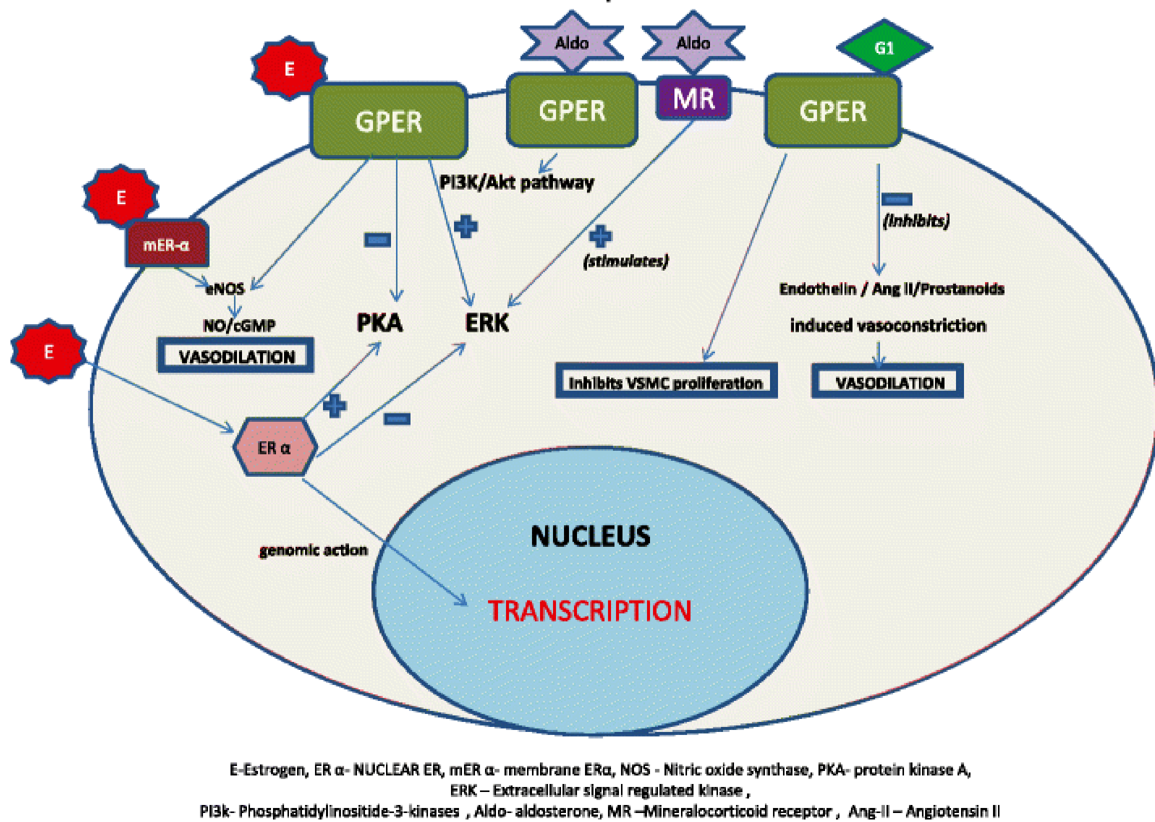

Figure 6. Effects of membrane estrogen receptor activation by estradiol, G1 agonist and aldosterone in vascular cells

Testosterone

There are many studies confirmed that in elderly men, the reduced plasma levels of testosterone might contribute to the increased arterial stiffness and blood pressure. The underlying mechanism is unclear. Further studies are needed to clarify the relationship between plasma testosterone level and BP. There are several possibilities: Testosterone induces vasodilation through an androgen receptor-independent and nongenomic pathway via ion channels (Deenadayalu, White, Stallone, Gao, & Garcia, 2001; Tep-areenan, Kendall, & Randall, 2002). Testosterone can promote norepinephrine synthesis by increasing tyrosine hydroxylase

activity(Kumai, Tanaka, Watanabe, & Kobayashi, 1994). Testosterone can also be involved in the adjustment of vascular connective tissue(Fischer & Swain, 1977). On the other hand, many animal studies indicated that testosterone causes increased BP. Possible mechanisms include upregulation of norepinephrine synthesis, Ang II expression and endothelin-1 action (Kienitz & Quinkler, 2008).

More researches are needed to resolve above-mentioned controversial findings regarding the role of testosterone in BP regulation and hypertension pathogenesis.

Concluding remarks

Eph kinases are the largest receptor tyrosine kinase family. Our recent research represents the very first attempt to elucidate the role of EPHBs and their ligand EphrinBs in BP regulation. We revealed that EPHBs and its EphrinBs ligands are implicated in VSMC, contractility in mice, leading to BP regulation, and such effects are sex hormone-dependent. Our human genetic studies have confirmed our findings in mice, demonstrating the relevance of the mouse results to human hypertension. Details of the results of our investigation are provided in the following four articles.

CHAPTER 2 ARTICLE-1

TITLE: EPHB4 expression in vascular smooth muscle cells regulates their contractility and EPHB4 deletion leads to hypotension in mice

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Running title: *Ephb4* Gene Knockout Leads to Hypotension

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Capsule

Background: EPHB4's role in blood pressure regulation was not previously known.

Results: Male but not female **s**mooth muscle-specific *Ephb4* gene knockout mice were hypotensive. Two directional signaling between EFNBs and EPHB4 modulated small artery contractility.

Conclusion: EPHB4 and sex hormones in concert regulates blood pressure.

Significance: A new mechanism of blood pressure regulation has been discovered.

Abstract

EPH kinases are the largest family of receptor tyrosine kinases, and their ligands, ephrins (EFNs), are also cell surface molecules. This work presented evidences that EPHB4 on vascular smooth muscle cells (VSMCs) is involved in blood pressure (BP) regulation. We generated gene knockout (KO) mice with smooth muscle-specific deletion of EPHB4. The male not the female KO mice were hypotensive. VSMCs from male KO mice showed reduced contractility compared to their WT counterparts. Signaling from EFNBs to EPHB4 (forward signaling) and from EPHB4 to EFNB2 (reverse signaling) could modulate VSMC contractility. At the molecular level, a lack of EPHB4 in VSMCs resulted in compromised signaling from CamKII to MLCK to MLC, the last of which controls the contraction force of motor molecule myosin. Near the cell membrane, an adaptor protein

GRIP1, which can associate with EFNB2, was found to be essential in mediating the EPHB4-to-EFNB reverse signaling, which regulated VSMC contractility, based on siRNA gene knockdown studies. Our research indicates that EPHB4 plays an essential role in regulating small artery contractility and BP.

Introduction

EPH kinases are the largest family of receptor tyrosine kinases. According to sequence homology, the EPHs can be classified into A and B subfamilies. There are 9 members in the EPHA subfamily, and 6 members in the EPHB subfamily. The ligands of EPH kinases, ephrins (EFNs), are also cell surface molecules("Unified nomenclature for Eph family receptors and their ligands, the ephrins. Eph Nomenclature Committee," 1997). There are 9 EFNs divided into A and B subfamilies. Interactions among EPHs and EFNs are promiscuous. One EPH can interact with multiple EFNs and vice versa. In general, EPHA members bind preferentially to EFNA members, and EPHB members to EFNB members(Pasquale, 2008; J. Wu & Luo, 2005). The preferred ligand of EPH4 is EFNB2, but the latter binds most EPHB subfamily members.

EPHs and EFNs play important roles in the development and function of many tissues and organs, such as the central nervous system(Flanagan & Vanderhaeghen, 1998; Pasquale, 2008), immune system(Luo, Charpentier, et al., 2011; Luo, Wan, Wu, & Wu, 2001; Luo, Wu, et al., 2011; Luo, Yu, Tremblay, & Wu, 2004; Luo, Yu, Wu, & Wu, 2002; J. Wu & Luo, 2005; Yu, Luo, Wu, & Wu, 2003a, 2003b, 2004; Yu, Mao, Wu, Luo, & Wu, 2006) and digestive system(Batlle et al., 2002). They are also vital in many biological processes, such as bone metabolism, angiogenesis, insulin secretion(Konstantinova et al., 2007), chemotaxis(Salvucci, de la Luz Sierra, Martina, McCormick, & Tosato, 2006), kidney glomerula filtration(Hashimoto

et al., 2007), ionic homeostasis of vestibular endolymph fluid in the inner ear(Dravis et al., 2007), etc. There are a few reports showing that some EPHs and EFNs are expressed in vascular smooth muscle cells (VSMCs) and play a role in VSMC migration (Foo et al., 2006; Gale et al., 2001; Shin et al., 2001). However, until our recent publications, there was no documented evidence that these molecules are involved in blood pressure regulation.

In 2012, we published 2 articles reporting novel observations that EphB6 and EFNB1 modulate BP in mice (Luo et al., 2012; Z. Wu et al., 2012). The salient findings are as follows: systolic pressure (SP) and diastolic pressure (DP) in male and female EPHB6 KO mice are comparable to those in their wild type (WT) counterparts, but castrated KO mice have higher SP and DP than WT(Z. Wu et al., 2012), indicating that sex hormones act in concert with EPHB6 to regulate BP. We have identified VSMCs as one of the target tissues of EPHB6 in regulating BP, as small arteries from castrated EPHB6 KO mice show increased contractility in response to phenylephrine (PE) stimulation(Z. Wu et al., 2012). Adrenal gland chromaffin cells are another target tissue on which EPHB6 and androgens exert their BP regulatory effects (Z. Wu et al., 2012). Smooth muscle-specific EFNB1 KO mice also have increased blood pressure in a sex hormone-dependent way, similar but not identical to EPHB6 KO mice (Luo et al., 2012). Stimulating EFNB1 in VSMCs with solid-phase Ab diminishes VSMC contractility, supporting the concept that EPHB6 reverse signaling reduces VSMC contractility.

Given the new finding that the EPHB6 and EFNB1 KO leads to hypertension based on our previous study, we wondered whether other members of the Eph/Ephrins system would play similar roles in blood pressure control. To address this question, we generated floxed *Ephb4*

mice, and subsequently bred them to achieve smooth muscle-specific deletion of EPHB4. To our total surprise, we found the male EPHB4 KO mice were *hypotensive.* A series of mechanistic studies at the cellular and molecular levels were conducted. The results and significance of our unexpected findings are presented and discussed here.

Materials and Methods

Generation of smooth muscle cell-specific Ephb4 gene knockout mice

A PCR fragment amplified with a primer set (5'-GCCCTTAAAGGACCGACTTC-3' and 5'-GCCTAACGCTGGAGAAAGTG-3') based on the *Ephb4* genomic sequence was used as a probe to isolate a genomic BAC DNA clone 4M20 from the 129/sv mouse BAC genomic library RPCI-22. The targeting vectors were constructed by recombination and routine cloning methods, using a 12-kb *Ephb4* genomic fragment from clone 4M20 (illustrated in Figure 1A). The final targeting fragments for *Ephb4* were excised from its cloning vector backbone by BamHI and electroporated into embryonic stem (ES) cells. After G418 selection, the FRT-flanked Neo/TK cassette was eliminated by subsequent transient transfection of the ES cells with a Flippase expression vector. The targeting scheme is shown in Figure 1A. These genetic engineering steps in ES cells resulted in two net insertions in the *Ephb4* gene: a 118-bp LoxP-containing sequence (5′-AGTACGGGCC CAAGCTGGCC GCCCTAGGGG CGCGCCTGCA GATAACTTCG TATAATGTAT GCTATACGAA GTTATGATAT CAAGCTTATC GATACCGTCG AAGCTTGCTA GCGGTACC-3′) at position 26061 (based on the sequence of AL671478.9 in the GenBank), and a 151-bp LoxP- plus FRT-containing sequence (5′-GGCCGCCCTA GGGGCGCGCC TGCAGATAAC TTCGATAATG TATGCTATAC GAAGTTATGG ATCGAAGTTC CTATTTCTAA AAAGTATAGG AACTTCTTAA GGCCACCGCG

GCCGAACGCT AGAGCTTGTC GACGGTACCT AACTTCCTAG G-3′) at position 28713, 756 bp upstream and 1285 bp downstream of the *Ephb4* Exon 1, respectively.

The targeted ES cell clones were injected into C57BL/6 blastocysts. Chimeric male mice were mated with C57BL/6 females to establish mutated *Ephb4* allele germ line transmission. Southern blotting with probes corresponding to 5' and 3' sequences outside the targeting region, as shown in Figure 1A (hatched squares), was used to screen and confirm the gene targeting and the successful removal of the Neo-TK cassette in ES cells and eventually in mouse tail DNA.

Mice with floxed *Ephb4* allele(s) were named $Ephb4^{f/f}$ (loxP insertions in both alleles). They were backcrossed with C57BL/6 for 10 generations and then mated with smooth muscle myosin heavy chain promoter-driven Cre transgenic (Tg) mice in the C57BL/6 background (*smMHC-Cre-IRES-eGFP* Tg mice(Xin, Deng, Rishniw, Ji, & Kotlikoff, 2002)) to obtain smooth muscle cell-specific EPHB4 KO mice. They are referred to as EPHB4 KO mice or KO mice in the text below.

PCR was used for routine genotyping of the floxed allele(s) and the *Cre* transgene. Primers 5′-GCCCT TAAAG GACCG ACTTC -3′ (forward) and 5'- GCCTA ACGCT GGAGA AAGTG-3′ (reverse) amplified a 271-bp fragment from the floxed allele and a 133-bp fragment from the WT allele. Primers (forward: 5'-CCA ATT TAC TGA CCG TAC ACC-3'; reverse: 5'-GTT TCA CTA TCC AGG TTA CGG-3') amplified a 310-bp fragment of the *Cre* transgene. The PCR cycling condition was as follows for both types of PCR: 4 min at 95℃, followed by 34 cycles of 15 s at 94℃, 30 s at 58 ℃, and 60 s at 72℃, and a final incubation at 72℃ for 10 min.

Reverse transcription/quantitative-PCR (RT/qPCR)

Ephb4, Grip1, PDZ-Rgs3 and *Disheveled* mRNA levels in VSMCs were measured by RT-qPCR. Total RNA from VSMCs was extracted with TRIzol® (Invitrogen, Camarillo, CA) and reverse-transcribed with iScriptTM cDNA Synthesis Kit (BIO-RAD, Mississauga, Canada). Primers used are listed in Table I. iQTM SYBR® Green Supermix (BIO-RAD) was employed in qPCR. The cycling condition for all 4 molecules was as follows: 2 min at 50°C, 2 min at 95°C followed by 25-35 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 an internal control. Samples were measured in duplicate, and the data are expressed as signal ratios of test gene mRNA/*β-actin* mRNA.

BP measurement by radiotelemetry

Mice were implanted surgically with TA11PA-C10 telemetry sensors (Data Sciences International, St. Paul, MN, USA) in the left carotid artery for direct measurement of arterial pressure and heart rate (HR), as described previously(Luo et al., 2012; Z. Wu et al., 2012). For radiotransmitter implantation and castration/ovariectomy, mice were anesthetized with isoflurane (2% isoflurane with 0.75L/min O_2 flow). Radiotelemetry was started 1 week after transmitter implantation. The raw data were processed with the Dataquest A.R.T-Analysis program(Lavoie, Lake-Bruse, & Sigmund, 2004) and are presented as daily means ± SE

Ex vivo vessel constriction

Mice between 18 to 20 weeks of age were used for all *in vitro* studies. The mice were euthanized with pentobarbital (400 mg/Kg body weight, *i.p.*) at the end of in vivo studies or for tissue

retrieval. Vessel constriction was studied ex vivo, as described previously (Luo et al., 2012; Z. Wu et al., 2012). Three measurements from 3 segments per mouse and 2 mice per group were pooled to construct concentration-response curves with SE.

VSMC isolation

Mouse VSMCs were isolated, as described by Golovina and Blaustein (Miller, Silver, & Stull, 1983), with modifications (Luo et al., 2012; Z. Wu et al., 2012).

Measurement of VSMC contractility

VSMC contractility was measured as described previously (Luo et al., 2012; Z. Wu et al., 2012). In some experiments, an EPHB4 kinase inhibitor NVP-BHG712 (0.5μM; Tocris Bioscience, Bristol, United Kingdom) was added at the last 4h of the culture.

Immunoblotting

VSMCs from the aorta and mesenteric arteries of WT and KO mice were cultured for 3-4 days. In some experiments, EPHB4 kinase inhibitor NVP-BHG712 (0.5μM) was added for the indicated duration. The VSMCs were stimulated with PE (20 μM) for 3 s, then lysed by immunoprecipitation assay buffer, which contained PhosSTOP and protease inhibitor mixtures (Roche Applied Science, Meylan, France). Immunoblotting for phosphorylated myosin light chain (MLC) and total MLC, and phosphorylated and total MLC kinase (MLCK) were described before[22, 23]. Phosphorylated and total MLC phosphatase (MYPT), phosphorylated and total Ca^{++}/calmodulin-dependent protein kinase II (CaMKII) of the VSMCs were measured using rabbit anti-MLCK mAb (clone EP1458Y; Abcam, Cambridge, UK), rabbit anti-phospho-

MLCK Ab (Invitrogen), rabbit anti-MYPT Ab (#2634; Cell Signalling, Danvers, MA), rabbit anti-phospho-MYPT Ab (#5163; Cell Signalling), rabbit anti-CaMKII (phospho T286) antibody(ab32678; Abcam), and rabbit anti-CaMKII antibody (ab52476; Abcam). For α1-adrenergic receptor detection, VSMCs were cultured and lyzed as described above without stimulation. Rabbit Ab against α1-AR (ab3462; Abcam) was used for blotting. All the Abs were used at the manufacturers' recommended dilutions. The signals were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Burlington, Ontario, Canada).

Small interfering RNA (siRNA) transfection

siRNAs of *Disheveled, PDZ-RGS3, Grip1* and negative control siRNAs were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Their sequences have already been described in our previous publication (Z. Wu et al., 2012). VSMCs were cultured in wells coated with recombinant EPHB4-Fc (2 μg/ml during coating) or normal human IgG (NHIgG; 2 μg/ml during coating) for 3 days, and then in medium free of antibiotics for an additional 24h. The cells were then transfected with a mix of 3 pairs of siRNAs of a particular gene (for each pair, the final concentration is 10 nM), as described before (Z. Wu et al., 2012).

Ethics statement

All studies were approved by the Animal Protection Committee (Le Comité institutionnel d'intégration de la protection des animaux) of the CHUM Research Center.

Statistical analysis

All results were expressed as means ± SEM. Data were statistically analyzed using Student's *t*-tests, or repeated-measures ANOVA. A *p*-value of <0.05 was considered statistically significant.

Results

Generation and characterization of SMC-specific Ephb4 conditional gene KO mice

To investigate the function of EPHB4 in BP regulation, we generated *Ephb4* floxed mice, with *LoxP* sites flanking exon 1 of *Ephb4* (Fig. 1A). The germline transmission of the mutated gene was confirmed by Southern blotting of tail DNA (Fig. 1B). With the 5' probe, the floxed alle showed a 9.1-kb EcoRV/BclI band, and the WT alle, an 11.4-kb EcoRV/BclI band. With the 3' probe, the floxed alle showed a 7.0-kb BamHI/EcoRI band, and the WT alle, a 12.6-kb BamHI/EcoRI band. These floxed mice were first backcrossed to the C57BL/6 background for 9 generations, and then crossed with Tg mice expressing smooth muscle myosin heavy chain promoter-driven Cre recombinase (*smMHC-Cre-IRES-eGFP* Tg mice(Xin et al., 2002)) to achieve smooth muscle-specific deletion of EPHB4. The deletion of *Ephb4* at the mRNA level in vascular smooth muscles, but not in spleen cells, was confirmed by RT-qPCR (Fig. 1C). The deletion of EPHB4 at the protein level in VSMCs, but not in spleen cells, was further confirmed by immunoblotting (Fig. 1D).

EPHB4 deletion in smooth muscle cells causes hypotension in males

Our previous study showed that BP is significantly heightened in castrated EPHB6 KO, but not in uncastrated or female EPHB6 KO mice, compared to their WT counterparts, indicating that EPHB6 acts in concert with sex hormones in BP regulation(Luo et al., 2012). We further demonstrated that one of the tissues responsible for this phenotype is VSMCs(Luo et al., 2012).

As EPHB4 is another notable member of the EPHB subfamily, we wondered whether SMC-specific deletion of EPHB4 would result in a similar BP phenotype. The BP and HR of EPHB4 KO mice were measured by radiotelemetry. The mean ± SE of daily SP, DP, MAP and HR of the mice are presented in Figure 2, and were analyzed by Student's *t* tests. The hourly BP and HR during the entire 3-day period were also analyzed by repeated-measures ANOVA. Most surprisingly, male KO mice presented significantly reduced SP (by both hourly and daily analysis) and MAP (on day 3, by daily analysis) with normal HR compared to WT mice (Fig. 2A). On the other hand, the BP parameters of the KO females were comparable to those of their WT counterparts, although the former showed reduced HR (Fig. 2B; by both hourly and daily analysis). BP and HR of both KO and WT mice, either males or females, all showed expected circadian fluctuations (data not shown).

Decreased vessel contractility of male EPHB4 KO mice ex vivo

BP is a function of cardiac output and blood vessel flow resistance, which is determined mainly by small arteries. As the KO mice only had EPHB4 deletion on SMC, we focused our attention on small arterial contractility, which determines small arterial tone.

The PE-triggered contractility of mesenteric arteries from KO and WT mice was assessed *ex vivo*. The vessel contractility of male KO (Figs. 3A and 3B) but not female KO (Figs. 3C and 3D) mice, whether with endothelium (Figs. 3A and 3C) or without endothelium (Figs. 3B and 3D), was significantly lower than that of their WT counterparts, corroborating the hypotensive phenotype in male KO mice. These results also indicate that the reduced contractility of male KO vessels is not caused by cytokines or nitric oxide produced by the endothelium.

Both forward and reverse signaling between EPHB4 and its ligands is responsible for regulating VSMC contractility

Since EPHB4 deletion is SMC-specific, and male mice had reduced BP and vasoconstriction, we conducted further experimentation at the cellular level to understand the underlying mechanisms. As shown in Figure 4A, VSMCs from male KO mice did indeed present decreased contractility upon PE-stimulation. VSMCs from male mice were then used in all subsequent cellular and molecular studies.

EPHB4 and its ligands (mainly EFNBs) are all expressed in VSMCs(Luo et al., 2012). EPHB4 and its ligands can trigger signaling in both directions, i.e. from the ligands to EPHB4 (forward) and from EPHB4 to EFNBs (reverse). We wondered which direction is essential in regulating VSMC contractility. When WT VSMCs were cultured in wells coated with recombinant EFNB2 [in the form of EFNB2 tagged with human IgG Fc (EFNB2-Fc) for triggering forward signaling], the preferred ligand of EPHB4, their contractility on PE-stimulation was significantly increased, compared to WT VSMCs cultured in wells coated with control normal human IgG (NHIgG) (Fig. 4B). On the other hand, EPHB4 KO VSMCs did not respond to solid phase EFNB2-Fc and their contractility was no different from that of WT VSMCs treated with NHIgG (Fig. 4B, thin line). This indicates that the major receptor receiving the solid phase EFNB2 stimulation is EPHB4, and that enhanced EPHB4 forward signaling increases VSMC contractility. To verify this finding using a different approach, we treated the WT male VSMCs with an EPHB4 inhibitor NVP-BHG712 at 0.5 μM for 4 h, and found that it could also inhibit VSMC contractility (Fig. 4C), rendering the WT VSMCs a phenotype similar to that of EPHB4 KO

VSMCs. Such inhibition is not due to general cytotoxicity of the inhibitor, as VSMCs cultured in 0.5 μM NVP-BHG712 up to 5 days proliferated at a similar rate as those cultured in vehicles (data not shown).

To assess whether reverse signaling through EPHB4 ligands could also regulate VSMC contractility, we cultured the WT VSMCs in wells coated with recombinant EPHB4-Fc, which could trigger reverse signaling via EFNBs. Such treatment also enhanced VSMC contractility on PE stimulation (Fig. 4D), and the enhancement could be specifically neutralized by soluble recombinant EFNB2-Fc, indicating that the effect is mainly reversely transmitted via EFNB2.

It is worth mentioning that cross-linked EPHB4-Fc or EFNB2-Fc by itself in the absence of PE had no effect on VSMC contractility (data not shown).

Contractility-related signaling events affected by EPHB4 in VSMCs

The reduced contractility of KO VSMCs in response to PE could in theory be due to reduced α1-adrenoreceptor (α1AR). However, based on immunoblotting, male WT and KO VSMCs had similar levels of α1AR (data now shown). Activation of AR leads to Ca^{++} flux, which is an event controlling contractility. Again, PE-triggered Ca^{++} flux showed no differences between male WT and KO VSMCs (data not shown).

We then suspected that the reduced contractility of male KO VSMCs was a result of decreased VSMC Ca^{++} responsiveness, which is regulated by MLC phosphorylation. We tested MLC phosphorylation at different time points after PE stimulation, and found that it occurred very

quickly within seconds and then subsided (data not shown). We thus chose 3 s as a testing point as it was the earliest time point technically possible. KO VSMCs from males did indeed manifest a significantly lower degree of MLC phosphorylation than their WT counterparts immediately (Fig. 5A). Inhibition of EPHB4 kinase activity in VSMCs by NVP-BHG712 also repressed MLC phosphorylation (Fig. 5B). MLC is phosphorylated by MLCK. This kinase is phosphorylated at ser1760 by calmodulin-dependent protein kinase II (CamKII) or protein kinase A, and such phosphorylation reduces its kinase enzymatic activity(Miller et al., 1983). We found that MLCK phosphorylation at ser1760 was increased in male KO VSMCs compared to that in WT counterparts (Fig. 5C). The kinase enzymatic activity of CamKII is controlled by its phosphorylation at ser286; the phosphorylated CamKII presented lower kinase activity MLCK(Pfitzer, 2001; Tansey et al., 1992). In male KO VSMCs, such CamKII phosphorylation was increased (Fig. 5D), corroborating the increased MLCK and reduced MLC phosphorylation. Thus, a deletion of EPHB4 compromised the signaling from CamKII to MLCK to MLC in VSMCs, with enhanced CamKII phosphorylation as the upstream event. On the other hand, in VSMCs, phosphorylation of MLC phosphatase, which also regulates MLC phosphorylation, was not influenced by EPHB4 deletion (data not shown).

Identification of GRIP1 as a component in the EPHB4-triggered reverse signaling leading to increased VSMC contractility

To understand how reverse signaling is transmitted through EFNBs after EPHB4 triggering in VSMCs, we used siRNAs to knock down the expression of 3 adaptor proteins, GRIP1, PDZ-RGS3 and DISHEVELED, which are known to associate with EFNBs(Bruckner et al., 1999; Lu, Sun, Klein, & Flanagan, 2001; Tanaka, Kamo, Ota, & Sugimura, 2003). The effectiveness

of mRNA knockdown was verified by RT-qPCR (Fig. 6A). It is to be noted that for PDZ-RGS3, although the knockdown reduced its mRNA levels, a statistical difference was not reached. VSMCs from WT males were cultured in wells coated with recombinant EPHB4-Fc to invoke EFNB reverse signaling. With *Grip1* siRNA but not *PDZ-Rgs3* or *Disheveled* siRNA transfection, the contraction enhancing effect of EFNB reverse signaling (mainly through EFNB2, as demonstrated in Fig. 3D) was eliminated (Fig. 6B). *Grip1* siRNA transfection in WT VSMCs without solid phase EPHB4-Fc stimulation did not affect their contractility (data not shown), indicating that GRIP1 is only functional when there is EPHB4-triggered reverse signaling. VSMCs cultured in wells with solid phase recombinant EPHB4-Fc with GRIP1 knockdown also manifested a significantly lower degree of CamkII phosphorylation than the controls (VSMCs transfected with control siRNA and cultured in wells coated with NHIgG; Fig. 6C), suggesting that GRIP1 is upstream of CamKII.

Discussion

Our recent work demonstrates that the deletion of several EPH/EFN members in VSMCs, such as EPHB6 and EFNB1, leads to hypertension in mouse models, revealing a previously unknown BP control system(Luo et al., 2012; Z. Wu et al., 2012). In support of our findings that deletion of certain members of EPH/EFN families could cause hypertension, a recent publication by Carlstrom et al. demonstrated that mice with deletion of EPHA4 intracellular tail were also hypertensive(Sallstrom et al., 2013). Quite unexpectedly, we found in the present study that deletion of a member of the EPH/EFN system, EPHB4, in VSMCs resulted in *hypotension*. Such a phenotype was only apparent in males, suggesting that the phenotype is affected by sex hormones. Obviously, a tremendous amount of mechanistic study needs to be conducted to

elucidate how the sex hormones in concert with EPHB4 modulate VSMC contractility. Our present work serves as a starting point but not a complete report for a full understanding of the complex interaction among EPHs/EFNs, sex hormones and blood pressure regulation.

The co-existence of opposing effects of these EPH/EFN molecules in VSMC contractility and BP regulation are reminiscent of the Yin and Yang of many other cellular and molecular events, such as kinases/phosphatases, osteoblasts/osteoclasts, calcium influx through L/T calcium channels and out flux through BK channels, etc. The co-existence of these cells/molecules with opposite functions likely results in better homeostatic control of different biological processes; in this case, the fine tuning of vascular tone and consequently, BP. The opposite effects will need to be modulated to achieve the fine tuning. We have found that sex hormones are one of these modulators with regard to EPHB4 functions in BP regulation. There is no evidence that sex hormones change the expression levels of EPHB4 and EFNB family members in VSMCs (unpublished observation). The exact underlying mechanisms for such sex hormone-mediated fine tuning are under investigation.

We previously found that the VSMC relaxation effect of EPHB6 and EFNB1 is mediated by reverse signaling through EFNBs, but not by forward signaling through EPHBs. For EPHB4, its effect on VSMC constriction could be achieved by both forward and reverse signaling, and EFNB2 is the main ligand mediating the two-way signaling, consistent with the fact that EFNB2 is the major ligand for EPHB4(Cheng, Brantley, & Chen, 2002). This indicates that the effect of EFNB2 in VSMCs is opposite to that of EFNB1, and implies that EFNB2 deletion in VSMCs might also lead to hypotension. Further experimentation by our group has proved that this

prediction is generally correct (unpublished observation). The bi-directional vasoconstriction-enhancing effect between EPHB4 and EFNB2 also implies that inhibiting EPHB4 kinase activity could achieve a blood pressure lowering effect. We have proved in principle *in vitro* that a Novartis EPHB4 kinase inhibitor NVP-BHG712 (Martiny-Baron et al., 2010; Wnuk et al., 2012) could effectively reduce VSMC contractility. Although NVP-BHG712 was developed as an EPHB4 kinase inhibitor, it is not mono-specific, but inhibits the kinase activity of other members of EPH kinase family members as well (Martiny-Baron et al., 2010). Therefore, the effect we observed in VSMCs is likely the sum of the effects it has on all the EPH kinases, some of which may have opposite effects to EPHB4 in terms of modulating VSMC contractility. With that said, we noticed that NVP-BHG712 did not further reduce the EPHB4 KO VSMC contractility (data not shown), suggesting that the major effect of this inhibitor is via EPHB4. It is worth mentioning that EPHB4 plays a critical role in angiogenesis (Salvucci & Tosato, 2012), and thus is a potential drug target in tumor therapy. Many pharmaceutical and biotech companies have been developing EPHB4 inhibitors for such an application (Martiny-Baron et al., 2010; Wnuk et al., 2012). They will certainly benefit from our current findings by becoming aware of the blood pressure lowering potential of EPHB4 inhibitors as possible side effects.

We noticed that in female KO mice, HR was significantly lower than their WT counterparts, although their BP was in the normal range. As BP is a tightly regulated event with multiple feedback regulations, it is not surprising that reduced HR does not translate into lower BP. The reason why HR is affected by SMC-specific EPHB4 deletion is not clear. One possible explanation is that there is leaky Cre expression in cells controlling the electric conductivity that dictates HR, or a leaky Cre expression in cardiomyocytes, which express quite a high level of

EPHB4 (unpublished observation); deletion of EPHB4 in these cells in turn affects the rhythm of their contractions. Further investigation of this aspect is warranted.

We have established that EPHB4 deletion compromises the CamKII/MLCK/MLC signaling cascade in VSMCs, with increased CamKII phosphorylation as the most-upstream culpable event. Close to the cell membrane, we have identified GRIP1, which is known to associate with EFNB2, as an essential component in the reverse signaling of VSMCs with regard to contractility. GRIP1 knockdown resulted in reduced VSMC contractility in the presence of EFNB2 crosslinking. The molecular events between GRIP1 and CamKII will need to be further elucidated for a full characterization of the signaling pathway from EFNB2 to motor molecules (myosin). We previously identified GRIP1 as a key molecule mediating EFNB1 reverse signaling: its knockdown results in increased VSMC contractility after EFNB1 crosslinking. This indicates that the function of GRIP1 is versatile, depending on the EFNBs it associates with. Under a physiological condition, it probably functions as a node receiving opposite inputs from various EFNBs, and its final function is based on the sum of these positive and negative inputs, with regard to VSMC contractility, and consequently, BP.

Our current findings have revealed the existence of a previously undocumented blood pressure lowering mechanism. Such new knowledge will lead to a better understanding of blood pressure regulation mechanisms. Moreover, as EPHB4 inhibitors are been developed as cancer drugs due to its anti-angiogenesis effect, our findings have raised a timely caution for this previously unknown effect on lowering blood pressure.

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Figure legends

Figure 1. Generation of mice with smooth muscle cell-specific Ephb4 null mutation

A. Scheme of Ephb4 $^{f/f}$ mouse generation

Bold lines represent the left and right arms of genomic sequences used in gene targeting. LoxP and FRT sites are represented by empty and gray small arrowheads respectively. The hatched box represents a genomic region from which probes were produced for Southern blot analysis.

B. Southern blot analysis of tail DNA of floxed Ephb4 mice

Tail DNA was digested with EcoRV/BclI, or BamHI/EcoRI, and hybridized with the 5' probe or 3' probe respectively. For the 5' probe (upper panel), the 11.4-kb band was derived from the WT allele, and the 9.1-kb band, from the mutated allele. For the 3' probe (lower panel), the 12.6-kb band was derived from the WT allele, and the 7.0-kb band, from the mutated allele.

C. Ephb4 mRNA deletion in mesenteric arteries of Ephb4 KO mice

RNA was extracted from mesenteric arteries and spleens from WT and *Ephb4* KO mice and analyzed by RT-qPCR for *Ephb4* mRNA levels. *β-actin* mRNA levels were used as an internal control. Samples in RT-qPCR were in triplicate, and means + SE of *Ephb4* signal/*β-actin* signal ratios are shown. The experiment was conducted twice; a representative one is shown.

D. EPHB4 protein deletion in EPHB4 KO VSMCs according to immunoblotting

VSMCs from *Ephb4* KO and WT mice were cultured for 4 days and then harvested. Cell lysates were analyzed for EPHB4 protein expression by immunoblotting. Spleens from KO and WT

mice were used as controls for tissue specificity. The experiment was conducted twice; a representative one is shown.

Figure 2. Male EPHB4 KO mice were hypotensive

BP and HR were measured for 72 h by radiotelemetry, starting at least 7 days after radiotransmitter implantation. The number per group and the mean ages of the mice at the time of BP measurement are indicated. The BP and HR values are expressed as means ± SE of h daily BP and HR during 48 to 72-h periods. SP: systolic pressure; DP: diastolic pressure; MAP: mean arterial pressure; HR: heart rate. The statistical significance of differences between the hourly BP and HR of the experimental groups was evaluated by repeated-measures ANOVA, and *p*-values were indicated. The daily values were additionally analyzed by Student's *t* test, and "*" indicates *p*-values <0.05, "**", <0.01; "***": <0.001.

A. BP and HR of males

B. BP and HR of females

Figure 3. Reduced contractility of mesenteric arteries from male EPHB4 KO mice

Segments of the third-order branch of the mesenteric artery with (A and C) or without (B and D) endothelium were stimulated with PE. A single cumulative concentration-response curve to PE (1 nM to 100 μM) was obtained. Maximal tension (Emax) was determined by challenging the vessels with physiological saline containing 127 mM KCl. Vessel contractility is expressed as the percentage of Emax. Data from 2 mice (with 3 arterial segments tested for each mouse, i.e., 6 determinants per group) were pooled, and means ± SE are reported. Contractility differences were analyzed by paired Student's *t* tests and *p*-values are indicated. *: $p<0.05$.

A. Contractility of mesenteric arteries with endothelium from male KO and WT mice

B. Contractility of mesenteric arteries without endothelium from male KO and WT mice

C. Contractility of mesenteric arteries with endothelium from female KO and WT mice

D. Contractility of mesenteric arteries without endothelium from female KO and WT mice

Figure 4. VSMCs stimulated by both EPHB4 forward and reverse signalings show increased contractility

VSMCs from male WT and KO mice were cultured for 4 days. In some experiments, the VSMCs were cultured in wells coated with EFNB2-Fc (2 μg/ml for coating), EPHB4-Fc (2 μg/ml for coating), or NHIgG (2 μg/ml for coating, as a control for EPHB4-Fc), as indicated. VSMCs were stimulated with 20 μM PE at 37℃ and imaged every min for 15 min. All experiments were conducted 3 times independently. Means + SE of percentage contraction of more than 15-30 cells of a representative experiment are shown. The data at 10 min and 15 min of 3 independent experiments were summarized and expressed as bar graphs (mean + SE) at left. The data were analyzed by paired Student's *t* tests (*: $p<0.05$).

A. Increased contractility of VSMCs from male EPHB4 KO mice

VSMCs were cultured in plain wells.

B. Forward signaling through EPHB4 increases WT VSMC contractility

WT VSMCs were cultured in wells coated with EFNB2-Fc. KO VSMCs in coated wells were used as a negative control. WT VSMCs cultured in wells coated with NHIgG were used as an

additional control, and their mean contractility is presented as a thin line without SE to facilitate viewing.

C. EPHB4 inhibitor NVP-BHG712 reduces VSMC contractility

WT VSMCs were cultured in plain wells in the presence of NVP-BHG712 (0.5 μM) for the last 4 hours of culture. The controls were treated with vehicle (DSMO) at the same dilution and same duration.

D. Reverse signaling triggered by EPHB4 increases WT VSMC contractility

WT VSMCs were cultured in wells coated with EPHB4-Fc. In one of the groups, soluble EFNB2-Fc (2 μg/ml) was added to the culture to block the interaction between solid phase EPHB4-Fc and cell surface EFNB2. WT VSMCs cultured in wells coated with NHIgG were used as an additional control, and their mean contractility is presented as a thin line without SE to facilitate viewing.

Figure 5. MLC, MLCK, MYPT and CamKII phosphorylation of VSMC from WT and KO mice
VSMCs from male KO and WT mice were cultured for 4 days, and then stimulated with 20 μM PE for 3 s and immediately lyzed. Total and phosphorylated MLC, MLCK, and CamKII were analyzed by immunoblotting. Three independent experiments were conducted. Immunoblotting images from representative experiments are illustrated. The signal ratios of phosphorylated (P-) versus total (T-) MLC, MLCK, CamKII and MYPT were quantified by densitometry. Densitometry data from the 3 independent experiments were pooled and are presented as bar

graphs (mean + SE of relative intensity) in the panels at the left. Paired Student's *t* tests were employed for statistical analysis. "*" indicates $p<0.05$.

A. Decreased MLC phosphorylation in VSMC from EPHB4 KO mice

B. Decreased MLC phosphorylation in VSMCs after EPHB4 inhibitor treatment

WT VSMCs were cultured in the presence of NVP-BHG712 (0.5 μM) for the last 4 hours of culture. The controls were treated with vehicle (DSMO) at the same dilution and same duration.

C. Increased MLCK phosphorylation in VSMC from EPHB4 KO mice

D. Increased CamKII phosphorylation in VSMC from EPHB4 KO mice

Figure 6. GRIP1 mediates EPHB4 reverse signaling in controlling VSMC contractility

A. Effective mRNA knockdown of Disheveled, PDZ-Rgs3 and Grip1 by siRNA

Cultured WT VSMCs were transfected with a mixture of 3 siRNAs of a particular gene or control siRNA, as indicated. After 24-h culture, the cells were harvested and the mRNA expression of each gene was determined by RT-qPCR in duplicate. The experiments were conducted twice, and the data (4 determinants for each group) were pooled and expressed as means ± SE of the ratios of the target gene signal versus the *β-actin* signal. The data were analyzed with Student's *t* tests. *: $p<0.05$.

B. GRIP1 knockdown by siRNAs partially reverses the enhancing effect of solid-phase EPHB4-Fc in VSMC contractility

VSMCs from WT males were cultured in wells coated with recombinant EPHB4-Fc (2 μg/ml during coating). After 2 days, they were transfected with siRNAs targeting *Disheveled, PDZ-*

Rgs3 or *Grip1* mRNA, or with control siRNA. On day 4 of culture, they were stimulated with PE (20 μM), and their percentage contraction was recorded. Means ± SE of percentages are reported. The thin lines represent the mean percentage contraction of VSMCs cultured in wells coated with NHIgG (2 μg/ml) without siRNA transfection (for a better visual effect, the SE of each time point in this control is omitted). All experiments were conducted 3 times independently. Means ± SE of percentage contraction of more than 15-30 cells of a representative experiment are shown. The data at 10 min and 15 min of the 3 independent experiments were summarized and expressed as a bar graph (mean ± SE) at left. The data were analyzed by paired Student's *t* tests (*: $p<0.05$).

C. Decreased CamKII phosphorylation in VSMC after GRIP1 knockdown

VSMCs from WT males were cultured in wells coated with recombinant EPHB4-Fc (2 μg/ml during coating) or NHIgG (2 μg/ml during coating). After 2 days, they were transfected with *Grip1* siRNA or control siRNA. On day 4 of culture, they were stimulated with PE (20 μM) for 3 s, and lyzed for immunoblotting. Phosphorylated CamKII and β-actin were analyzed by immunoblotting. The signal ratios of phospho-CamKII versus β-actin were quantified by densitometry. Densitometry data from two independent experiments were pooled and presented as a bar graph at left. Paired Student's *t* tests were employed to examine statistical significant differences. "*" indicates $p<0.05$.

Tables

Table 1

qPCR primer sequences

qPCR primer sequences			
Gene	Sense sequence	antisense sequence	PCR Product
β-actin	5'TCGTACCACAGGCATTGTGATGGA-3'	5'-TGATGTCACGCACGATTTCCCTCT-3'	200bp
Ephb4	5'- CTACGTCTCTAACCTCCCATCT-3'	5'- GCTGGTCACCCTTTCTCTTT-3'	100bp
Disheveled	5'-TCTCGGCTAGTTCGGGAAGCACAAA-3'	5'-TGATGTTCAGGGACATGGTGGAGT-3'	112bp
*Grip*1	5'-ACAAGTCCCGTCCGGTTGTGATAA-3'	5'TCTATCAGCAGCGTGGCTTCTTGT-3'	181bp
PDZ-Rgs3	5'-TGGCAACAGGAGGAACAGTCCTAT-3'	5'-ATGTCTTCCAGCAGGAATGGGTCA-3'	170bp

Figures

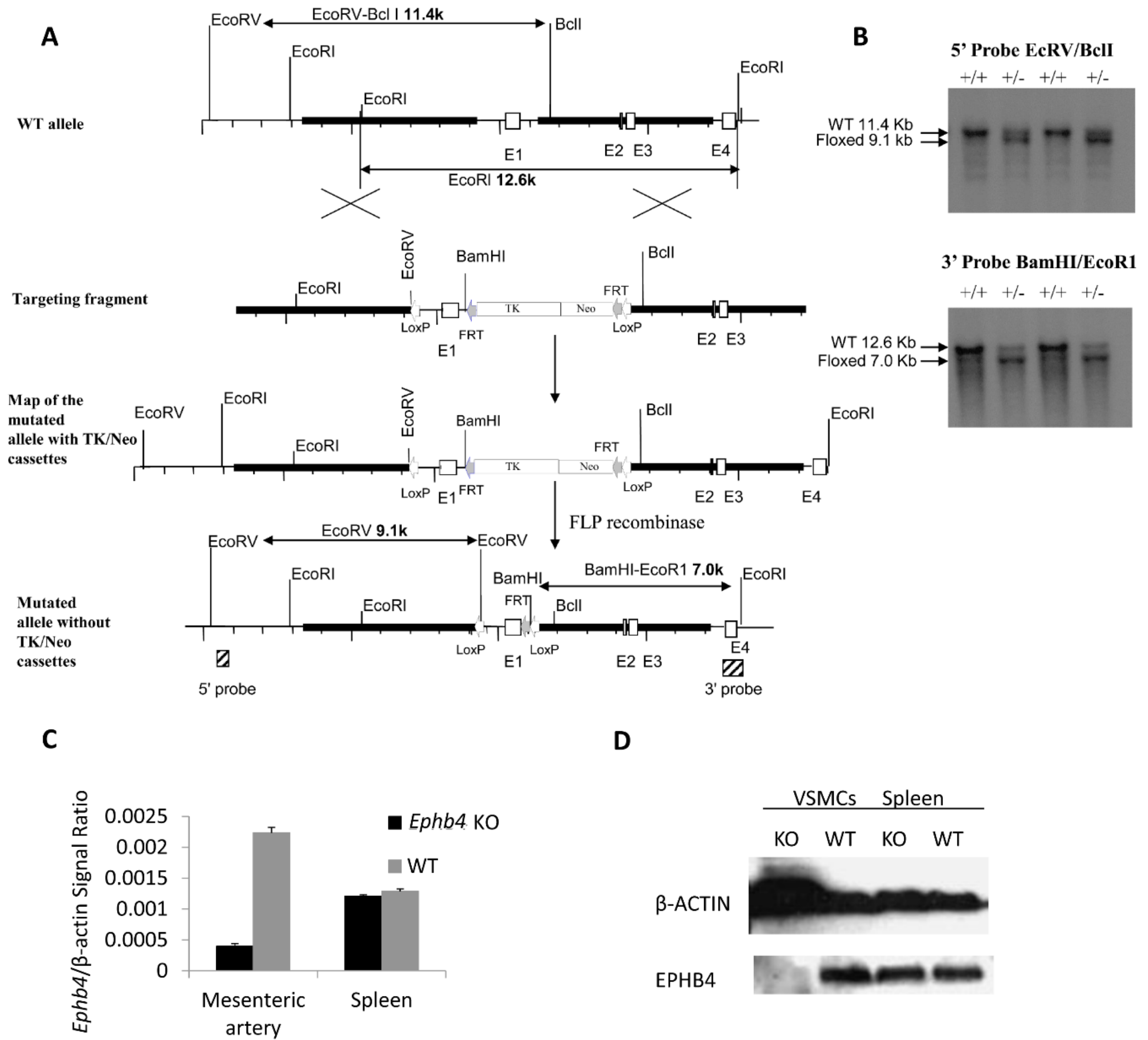

Figure 1

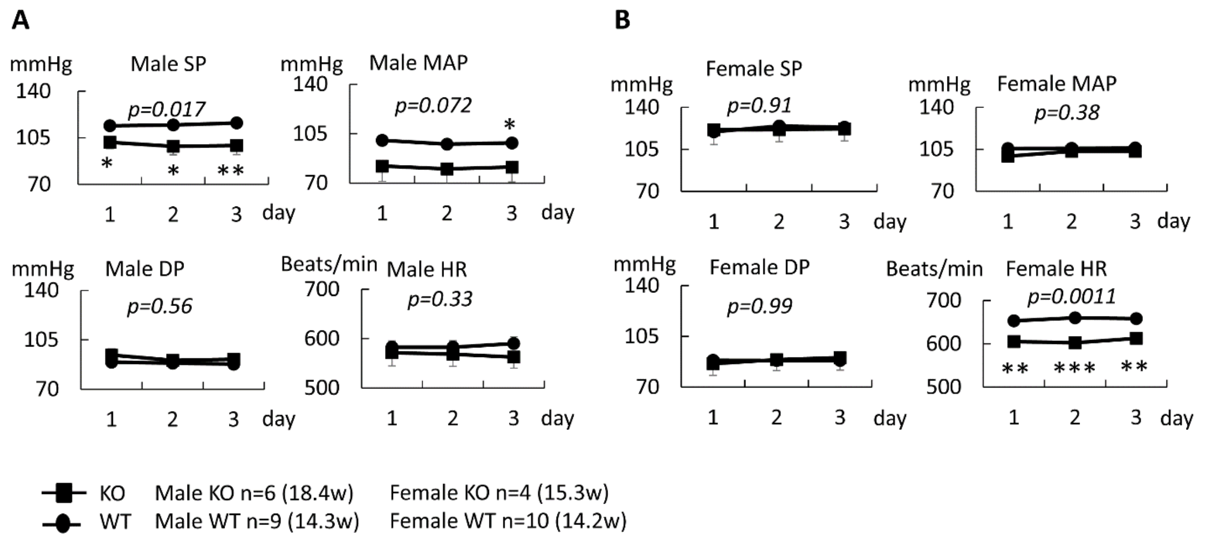

Figure 2

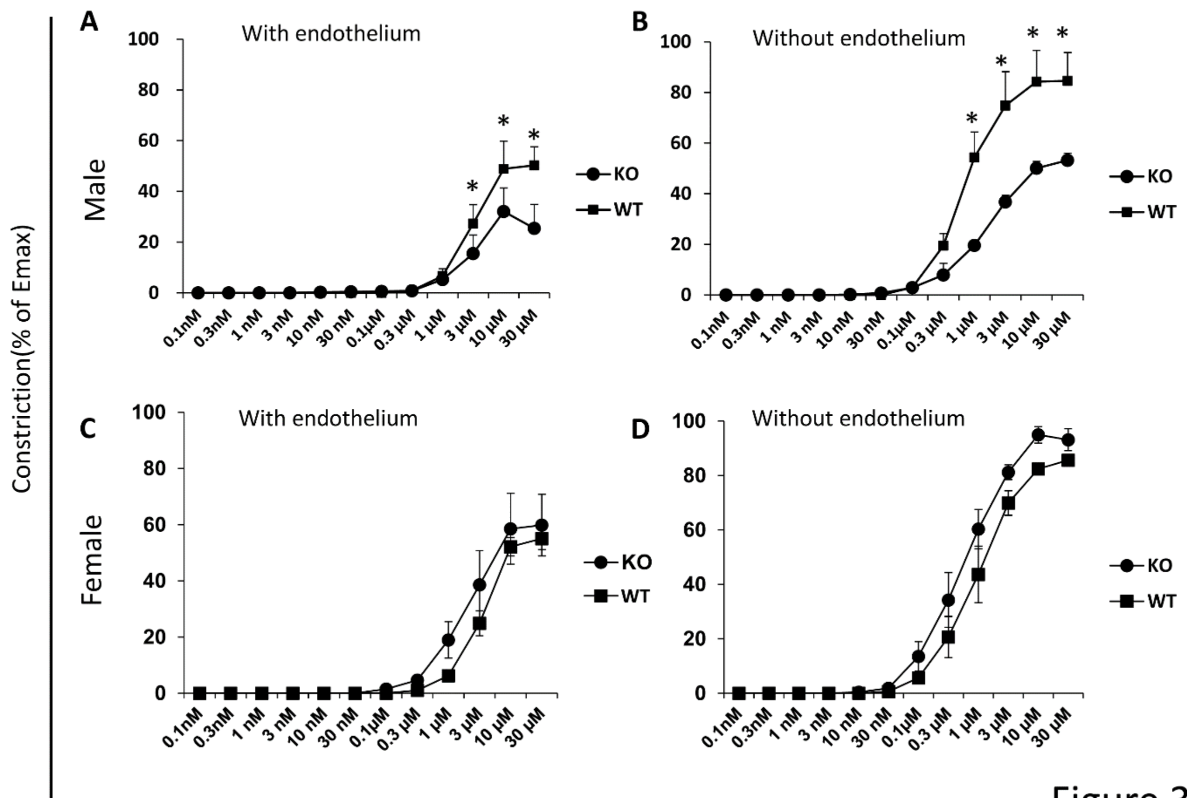

Figure 3

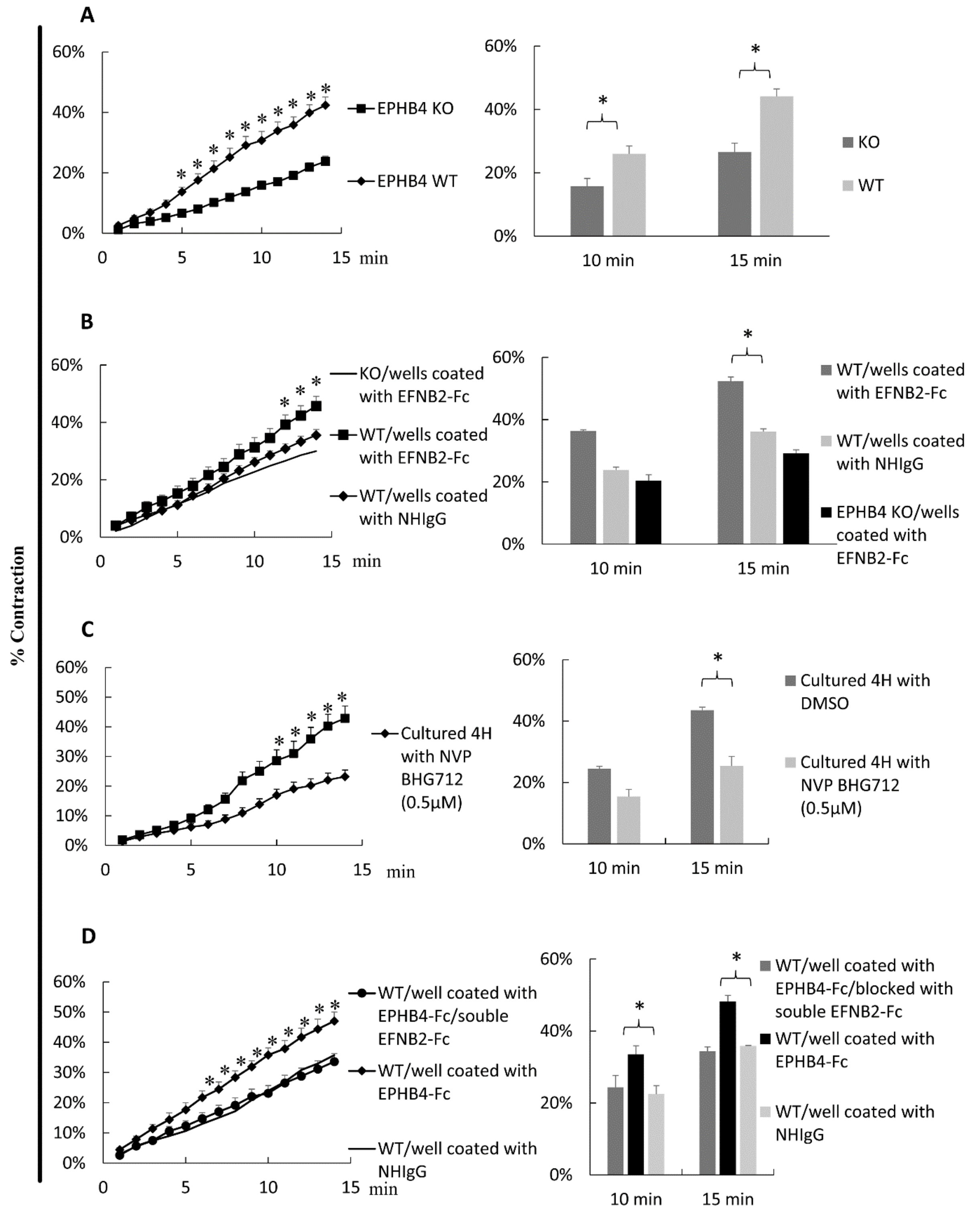

Figure 4

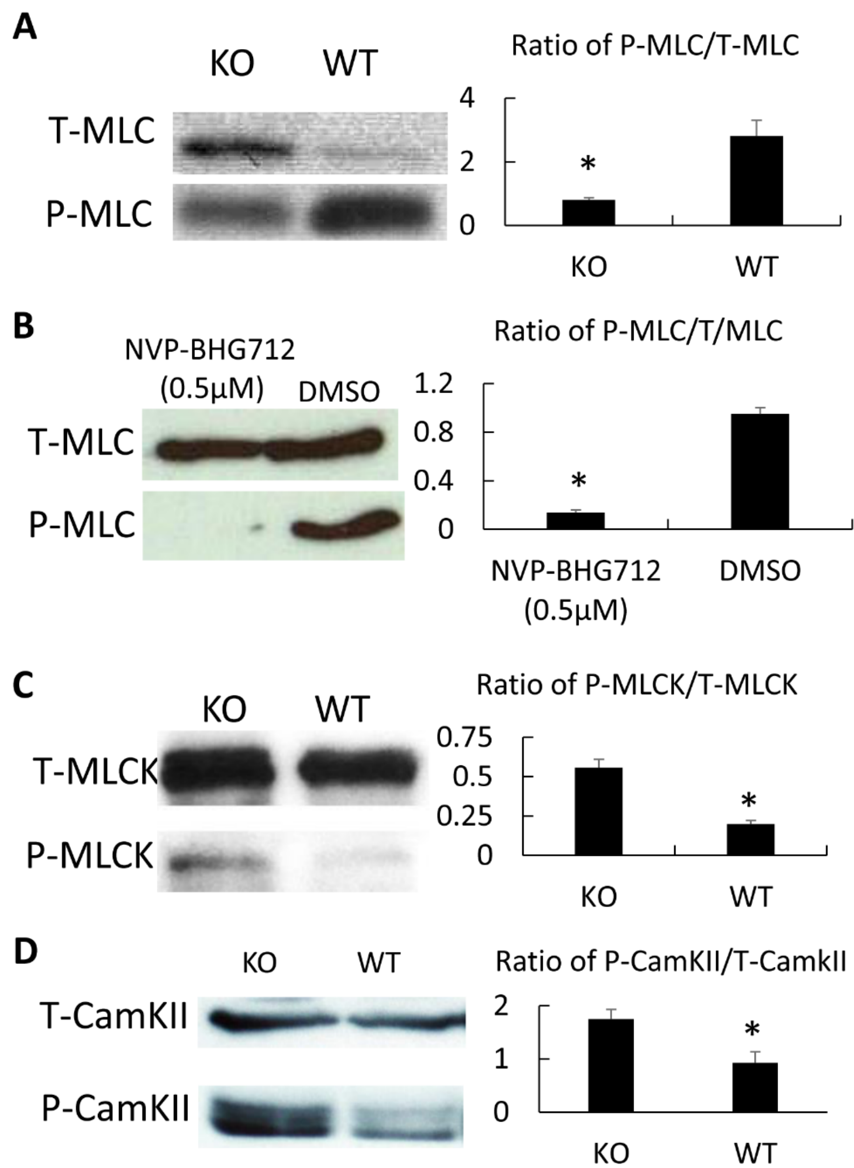

Figure 5

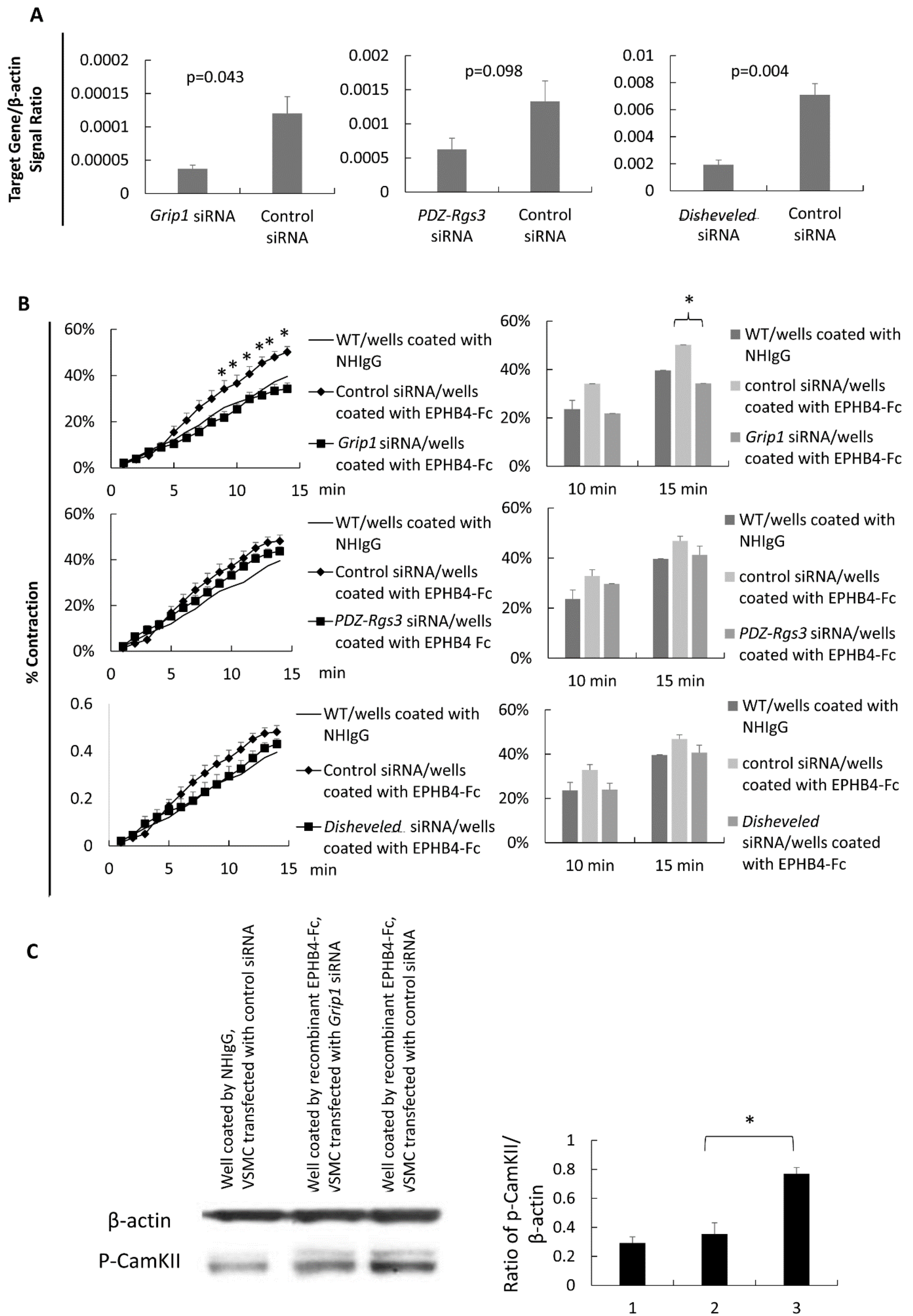

Figure 6

CHAPTER 3 ARTICLE-2

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

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Running title: EFNB2 deletion results in lower blood pressure

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Abstract

Ephrin B2 (EFNB2) is a ligand for erythropoietin-producing hepatocellular kinases (EPH), a family of receptor tyrosine kinases. It has critical functions in many biological systems, but is not known to regulate blood pressure. We generated mice with smooth muscle cell-specific deletion of EFNB2 and investigated its roles in blood pressure regulation and vascular smooth muscle cell (VSMC) contractility. Male EFNB2 knockout (KO) mice presented reduced blood pressure, while female KO mice had no consistent blood pressure phenotype. Both forward signaling from EFNB2 to EPHs and reverse signaling from EPHs to EFNB2 were involved in regulating VSMC contractility, with EPHB4 as a critical molecule for forward signaling, based on crosslinking studies. We also found that a region from aa 313 to aa 331 in the intracellular tail of EFNB2 was essential for reverse signaling regulating VSMC contractility, based on deletion mutation studies. In human genetic study, we identified 6 SNPs in the EFNB2 gene to significantly associate with hypertension risk in a sex-dependent way, consistent with our findings in mice. We have thus discovered a previously unknown blood pressure-lowering mechanism mediated by EFNB2 and identified it as a gene associated with hypertension risk in humans.

Key words: ephrinB2, blood pressure, vascular smooth muscle cells, sex hormone, single nucleotide polymorphism

Introduction

Erythropoietin-producing hepatocellular kinases (EPH) are the largest family of receptor tyrosine kinases. Based on sequence homology, they are divided into A and B subfamilies("Unified nomenclature for Eph family receptors and their ligands, the ephrins. Eph Nomenclature Committee," 1997). Their ligands, called ephrins (EFNs), are also cell surface molecules(Pasquale, 2008). EFNs are also divided into A and B subfamilies, based upon the way they anchor on the cell surface: the A subfamily anchors on the cell surface through glycophosphatidylinositol, and the B subfamily, through a transmembrane domain (Pasquale, 2008). The interactions between EPH kinases and EFNs are promiscuous, but EPHA kinases preferably interact with EFNA ligands, and EPHB kinases with EFNB ligands, which have 3 members, EFNB1, EFNB2 and EFNB3(Pasquale, 2008). Although EPH members and EFN members share homology with their respective members, each member has its distinct function in different cellular processes (Batlle et al., 2002; Hongyu Luo et al., 2016; Konstantinova et al., 2007; H. Luo, Yu, Tremblay, & Wu, 2004; Salvucci, de la Luz Sierra, Martina, McCormick, & Tosato, 2006). In general, the EPH kinases interact with their EFN ligands on neighboring cells, because EPHs and EFNs are all cell surface molecules(Pasquale, 2008). These molecules could be cleaved from the cell surface by enzymes such as ADAM10 (Kaushal & Shah, 2000; Primakoff & Myles, 2000), an unspecified matrix metalloproteinase(Georgakopoulos et al., 2006), or γ-secretase(Tomita, Tanaka, Morohashi, & Iwatsubo, 2006); therefore, it is possible that the shed soluble fragments of EPH and EFN might be able to influence cells and tissues at a distance by blocking the interaction of EPHs and EFNs there.

EPH kinases and EFNs are expressed in a wide range of tissues and cells, and play vital roles in the development and function of different organs and systems(Batlle et al., 2002; Dumas et al., 2000; H. Luo, Charpentier, et al., 2011; H. Luo, Wan, Wu, & Wu, 2001; H. Luo, Wu, et al., 2011; H. Luo et al., 2004; Hongyu Luo, Yu, Wu, & Wu, 2002; J. Wu & Luo, 2005; Xin, Deng, Rishniw, Ji, & Kotlikoff, 2002; Yu, Luo, Wu, & Wu, 2003a, 2003b, 2004; Yu, Mao, Wu, Luo, & Wu, 2006). They are also vital in many biological processes(Dravis et al., 2007; Hashimoto et al., 2007; Konstantinova et al., 2007; Salvucci et al., 2006); however, until our recent publications, there were no studies investigating the roles of these molecules in blood pressure (BP) regulation.

Recently, we reported novel observations that deletion of EFNB1 and its receptor EPHB6 leads to increased BP(H. Luo et al., 2012; Z. Wu et al., 2012). In an additional study, we demonstrated that EPHB4 deletion leads to reduced BP(Wang et al., 2015). As EFNB2 is the preferred ligand of EPHB4, we thus asked the question whether its deletion in vascular smooth muscle cells (VSMCs) will have a similar BP reduction effect. The results of our investigation are reported here.

Materials and Methods

Generation of smooth muscle cell-specific Efnb2 and Ephb4 gene knock-out (KO) mice We have previously reported on the generation of *Efnb2* floxed mice(H. Luo, Charpentier, et al., 2011). They were backcrossed with C57BL/6J for 10 generations and then mated with smooth muscle myosin heavy chain promoter-driven *Cre* transgenic mice (smMHC-Cre-IRES-eGFP) in the C57BL/6J background(Xin et al., 2002) to obtain smooth muscle cell-specific *Efnb2* gene KO

mice. The ages of the KO and WT mice for the *in vivo* study were described in given experiments. Cells from mice at 3 to 6 months of age were used for *in vitro* studies.

In some experiments, VSMCs from smooth muscle-specific *Ephb4* gene KO mice were used. The generation and characterization of these mice are described in our recent publication(Wang et al., 2015).

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

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("ADVANCE trial / Action in Diabetes and Vascular disease: PreterAx and Diamicron MR Controlled Evaluation. Available at: www.advance-trial.com. Accessed Nov, 2014," ; Colagiuri, 2008). 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 being currently treated for hypertension at baseline 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.

Genotyping 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(Marchini & Howie, 2010) and SHAPEIT for prephasing haplotypes[31].

Association analysis Association analysis was performed separately for the array 5.0 and array 6.0 data sets using the PLINK program(Purcell et al., 2007). 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 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. A subset (147,088) of the genotyped SNPs that were in Linkage equilibrium (defined as $r^2 \leq 0.8$) was selected to perform a principal component

analysis, using the Eigenstrat software(Price et al., 2006) 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 61 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 63.73 kb region between positions 107,133,382 and 107,197,119 on chromosome 13 (Build 37, hg/19). Only SNPs or markers with a minor allele frequency (MAF) greater or equal to 0.05 and imputed SNPs with imputation quality scores greater than 0.8 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 as association analysis has very little power for SNPs with a minor allele frequency below 0.05.

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.

Significance of associations The number of tagging SNPs that proxy for all 61 markers tested for association within the gene region was determined using the LDSELECT

program("LDSelect-Multipopulation: Download and Documentation. Available at: http://droog.gs.washington.edu/multiPopTagSelect.html. Accessed Nov, 2014,") using an r^2 value > 0.8 and a minimum minor allele frequency of 0.05. 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 blocks and 2 sexes = 30 independent tests, giving a critical p-value of 0.00167 or a critical -log10 *p*-value of 2.778.

Power calculation A power calculation was conducted for the female samples using the CaTS power calculator(Skol, Scott, Abecasis, & Boehnke, 2006).

Results

Smooth muscle cell-specific deletion of EFNB2 in mice The floxed *Efnb2* mice (H. Luo, Charpentier, et al., 2011) in the C57BL/6J background were crossed with transgenic mice expressing smooth muscle myosin heavy chain promoter-driven Cre recombinase (also in the C57BL/6J background (Xin et al., 2002) to achieve smooth muscle cell-specific deletion of EFNB2. The deletion of *Efnb2* at the mRNA level in vascular smooth muscles, but not in the spleen, heart, brain or liver, was confirmed by RT-qPCR (Fig. 1A and Supplementary Figure 1 (S. Fig. 1)). The deletion EFNB2 at the protein level in VSMCs was further confirmed by immunofluorescence (Fig. 1B) and immunoblotting (Fig. 1C). These mice with smooth muscle cell-specific deletion of EFNB2 were called KO mice. There was no compensative upregulation of other EPHB and EFNB subfamily members in VSMCs after EFNB2 deletion (S. Fig. 2). The small artery structure of the KO mice was comparable to that of the wild type (WT) mice in terms of media thickness and lumen sizes (S. Fig. 3). The KO and WT VSMCs had similar

proliferative rates *in vitro* culture (S. Fig. 4), and had similar levels of α-actin expression, a smooth muscle cell marker (S. Fig. 5). Although EFNB2 deletion was achieved by smooth muscle myosin heavy chain promoter-driven *Cre*, and is not specific to SMC in the vascular system, we did not notice gross morphological and functional abnormalities in the digestive system, respiratory system and reproductive system in the KO mice.

BP phenotype of EFNB2 KO mice The BP of KO and WT mice was recorded every 2 min for a period of 72 h. All the time points in each day were analyzed with a mixed-effects linear model, using genotype and time as qualitative factors (SPSS Statistics 19), and the mean + SEM of the daily values are presented in Figure 1. Male EFNB2 KO mice presented significantly reduced BP for all the parameters registered (*i.e.,* systolic, diastolic, and mean arterial pressure) in all the 3 days, compared to their WT counterparts (Fig. 2A). Female KO mice had no consistent change in BP. On day 1, the KO mice had lower systolic pressure and mean arterial pressure, but on day 2, their diastolic pressure was actually higher than that of the WT counterparts (Fig. 2B).Values of each parameter for different days for the males and females are presented in S. Figure 6 to give a different perspective.

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

EFNB2-Fc tagged with human IgG Fc (EFNB2-Fc), which could crosslink EPHBs and initiate forward signaling, they manifested significantly augmented contractility upon phenylephrine stimulation, compared to the VSMCs cultured on wells coated with normal human IgG, which was employed as a control (Fig. 3A). The augmentation could be neutralized by soluble EPHB4-Fc, which is a preferred receptor of EFNB2, suggesting that among different EPHB subfamily members, EPHB4 largely mediates forward signaling to achieve increased VSMC contractility. To further prove the essential role of EPHB4 in forward signaling and VSMC contractility, we cultured VSMCs from smooth muscle cell-specific EPHB4 KO mice (Wang et al., 2015) on EFNB2-Fc coated wells. As shown in Figure 3B, solid-phase EFNB2-Fc could no longer enhance contractility of EPHB4-deleted VSMCs, again indicating that solid phase EFNB2's effect on VSMC contractility is largely via EPHB4. To assess whether reverse signaling from EPHBs to EFNB2 also played a role in VSMC contractility, we cultured WT VSMCs on wells coated with anti-EFNB2 Ab to crosslink EFNB2, mimicking the EPHB binding. Such crosslinking could significantly increase VSMC contractility upon phenylephrine-stimulation (Fig. 3C). Moreover, the augmentation could be neutralized by soluble EFNB2-Fc, confirming the specificity of anti-EFNB2 Ab. Thus, we have demonstrated that both forward signaling from EFNB2 to EPHB4 and reverse signaling from EPHBs to EFNB2 could enhance VSMC contractility.

To identify the EFNB2 intracellular sequence critical for controlling the VSMC contractility during reverse signaling EFNB2 has a short intracellular tail (73 aa), which has no enzymatic activity. EFNB2 reverse signaling depends on the association of its intracellular tail with adaptor proteins, which in turn link to various signaling pathways. We conducted step-wise deletion of

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

Contractility-related signaling events in EFNB2 KO VSMCs We assessed several key signaling events in EFNB2 KO VSMC to further understand the cause of the reduced contractility of these cells. VSMCs from male and female KO mice showed mRNA levels of α1-adrenoreceptor expression comparable to those from WT counterparts (S. Fig. 7A). VSMCs from male KO mice had comparable phenylephrine-stimulated Ca^{2+} influx to VSMCs from WT mice (S. Fig. 7B). Crosslinking EFNB2 with solid-phase anti-EFNB2 Ab, which enhanced WT VSMC contractility, did not change phenylephrine-triggered Ca^{2+} flux in VSMCs from male mice (S. Fig. 7C). This indicates that EFNB2 does not affect Ca^{2+} flux in VSMCs.

The reduced contractility could then be caused by the sensitivity of KO VSMCs to intracellular Ca^{2+}. We investigated this possibility by examining KO VSMC myosin light chain phosphorylation, which is known to increase the Ca^{2+} sensitivity. Indeed, when stimulated with phenylephrine, VSMCs from male but not female KO mice manifested significantly lower myosin light chain (MLC) phosphorylation at ser19 (S. Fig. 7D), consistent with reduced BP in male but not female KO mice (Figs 2A and 2B). Myosin light chain is dephosphorylated by myosin phosphatase. Phosphorylation of myosin phosphatase target protein-1 (thr696), a process that decreases myosin light chain phosphatase activity (Ramachandran, Patil, Combrink, Sharif, & Srinivas, 2011), was not influenced by EFNB2 deletion (S. Fig. 7E). ERK activation can enhance smooth muscle activation through several proposed modes of action: 1) it might activate MLC kinase through a so-far un-delineated pathway (Klemke et al., 1997; Roberts, 2004) or 2) it might phosphorylate the actin-binding protein, caldesmon, which removes caldesmon's inhibitory effect on smooth muscle contraction(Hedges et al., 2000) . In both male and female KO VSMCs, ERK phosphorylation, a surrogate marker of ERK activation, was

significantly reduced upon phenylephrine stimulation (S. Fig. 7F), implicating ERK as a link in EFNB2 deletion-caused hypocontractility of VSMCs. The lack of sex difference in ERK phosphorylation suggests that although it might contribute to reduce VSMC contractility in KO mice, it is not responsible for the observed sex difference.

Association of EFNB2 with hypertension in males in diabetic individuals We tested the association of 61 DNA markers falling within the *EFNB2* gene or within additional approximately 10 kb 5' and 10 kb 3' of the gene (located within a 63.74-kb region between positions 107,133,382 and 107,197,119 on chromosome 13 (Build 37/hg19) with hypertension in a type 2 diabetes patient cohort; males and females were tested separately. Two SNPs were found to be significantly associated with hypertension for the male-only samples (Table 1, and S. Fig. 8A) using a *Bonferroni*-corrected critical *p*-value of 0.00167 for 30 independent tests, corresponding to 15 independent ($r^2 \leq 0.80$) LD blocks within the region and the 2 sexes tested. No SNP was nominally significant for the female-only sample (Table 1 and S. Fig. 8B). A power calculation(Skol et al., 2006) for 1000 cases and 210 controls (our sample had 996 female hypertensives and 206 normotensives) using a prevalence of 0.83 for hypertensives (prevalence among our diabetic subjects) a major allele frequency of 0.646 and a bonferroni critical *p*-value of 0.0017 determined that the female sample had 95% power to detect a genotype relative risk of 1. Although this is a small dataset to have full power at a genome-wide association study (GWAS), it is sufficient to detect association at the level of multiple testing that we are using. The fact that the female sample is not significant for any SNPs is a reflection of the lack of any genetic association not lack of power. The odds ratios in females were 1.001 and 1.01 for the

two significant SNPs in males which have odds ratios of 1.3 for the major alleles, indicating that *EFNB2* is associated with hypertension only in males.

The locations of the 2 significant SNPs (encircled) with respect to the LD structure around the 3' end of the *EFNB2* gene are shown in S. Figure 8C. These SNPs fall just downstream (within 1 kb) of the 3' untranslated region (UTR) of the gene but within an LD block that covers the region just 3' of the gene but also the 3' untranslated region (3'UTR) the gene. The two significant SNPs are in very high LD in our sample ($r^2 = 0.977$). These SNPs may well be in LD with a functional polymorphism which may reside within the 3' UTR region of the gene.

The case/control distributions for male and females of the A and G alleles for SNP rs7329357, the most significantly associated SNP, within the male sample are presented as an example in Table 2 along with the odds ratios (ORs) of their case-control distributions and the 95% confidence intervals for these ORs. Since the other significant SNP rs7328882 is in very high LD with rs7329357, the results are similar. The results of *chi*-square tests of independence between major and minor allele distributions and sexes for cases and controls are presented in Table 3. The frequencies of the minor A allele in male and female cases are similar and not statistically significant (0.343 and 0.349 respectively); however, the frequency of the A allele is significantly higher among male controls (0.473) than among female controls (0.352) (p = 0.000045), suggesting that the minor A allele is protective against hypertension in the presence of diabetes, but only in males.

Discussion

BP is a vital physiological parameter and is highly regulated with multiple compensatory mechanisms including blood volume, cardiac output and/or vascular tone regulation to maintain it within a normal range. EFNB2 deletion in VSMCs likely leads to a default phenotype of reduced VSMC contractility, and consequently reduced BP. However, this phenotype is pronounced in males but not in female due to one or more of the above-mentioned compensatory mechanisms, which in this case seemed to be additionally influenced by sex hormones. These sex hormone-dependent compensation mechanisms likely have masked the default lower blood pressure in female KO mice. The nature of the sex hormone-dependent compensation remains to be investigated.

Nakayama *et al.* reported that smooth muscle-specific deletion of EFNB2 using SM22Δ-Cre results in lower body weight, reduced VSMC proliferation, thinner arterial vessel wall and enlarged arterial diameter[40]. However, none of these phenotypes was observed in our KO mice, in which EFNB2 was deleted using smMHC-Cre. There could be several possible explanations for these discrepancies. 1) The mouse genetic background might heavily influence these phenotypes: Nakayama's mice are in a 129sv X C57BL/6J mixed background, while ours are in a pure C57BL/6J background. 2) Different smooth muscle-specific promoters might result in different degrees of EFNB2 deletion in target organs/tissues and of leaky deletion in untargeted organs/tissues. 3) Cell proliferation was assessed at different time points (postnatal day 8 in Nakayama's study *versus* adult in our study). While Nakayama's study focuses on EFNB2's role in PGDFRβ signaling in VSMCs related to their proliferation, we are focusing on VSMCs'

functionality and their signaling during contractile stimulation. Naturally, these two aspects have different time windows. Nevertheless, these two studies agree that EFNB2 plays a critical role in VSMC biology, which has not been previously appreciated.

EFNB2 can transduce signals in both forward and reverse directions. We have demonstrated that both directions are involved in VSMC contractility. EPHB4 is the preferred EPH, with which EFNB2 could associate. We have proved *in vitro* that only WT, but not EPHB4 KO VSMCs, could respond to EFNB2 stimulation and augment contractility (Fig. 3B), indicating that the predominant effect of EFNB2 forward signaling with regard to enhancing VSMC contractility was mediated by EPHB4. If this is the case, then smooth muscle cell-specific deletion of EPHB4 should have a similar BP phenotype to that seen in the EFNB2 KO mice. Indeed, our recent report reveals that EPHB4 KO mice present sex-dependent hypotension, with male KO being hypotensive while female KO being normotensive (Wang et al., 2015), corroborating our results from EFNB2 KO mice.

EFNB2 has a short intracellular tail (73 aa), which has no enzymatic activity. Its reverse signaling depends on adaptor proteins associating with the tail. EFNB2 intracellular tails are characterized by 2 major features: the C-terminal PDZ domain-binding motif and 5 conserved tyrosine residues, which could be associated with PDZ-domain-containing proteins (*e.g.,* PDZ-RGS3, GRIP1/2, TIAM1 and DISHEVELED) (Bruckner, Pasquale, & Klein, 1997; Cowan & Henkemeyer, 2001; H. Luo et al., 2004; Hongyu Luo et al., 2002; Yu et al., 2004), and SH2-domain-containing proteins (e.g. GRB4, STAT3 and CRK(Arvanitis & Davy, 2008), respectively. These 2 features are located in the last 31 aa, which are highly conserved. EFNB

can also have PDZ- and SH2-independent functions (Lu, Sun, Klein, & Flanagan, 2001), some of which presumably depend on the SH3 domain of associating proteins (*e.g.,* CRK and GRB4). By deleting different lengths of the EFNB2 intracellular tail, we found that the C-terminal PDZ-domain binding motif and the last 2 tyrosine residues were dispensable for EFNB2 reverse signaling with regard to VSMC contractility, but a 19-aa long region (*i.e.,* from aa 313 to aa 331) containing the 3rd and 4th conserved tyrosine residues (counting from the C-terminus) was critical. The 2 tyrosine residues (Y314 and Y319) in this region are good candidates responsible for reverse signaling as they could interact with SH2 domain-containing adaptor proteins mentioned above, although S328 should also be considered, as it may attract SH3 domain-containing adaptor proteins. It is possible that the molecules associating with these residues are connected to the VSMC contraction machinery, and the function of these associating molecules is differentially affected by sex hormones in the absence or presence of EFNB2. A search of the associated proteins that mediate the effect of EFNB2 and a study of how their effects are influenced by sex hormones with regard to VSMC contractility are warranted.

Our human genetic study revealed that 2 SNPs that are in high LD with each other and with the 3, untranslated region of *EFNB2* were associated with hypertension risk at a p-value below the *Bonferroni*-corrected $p \leq 0.00167$ in the males-only sample. The minor alleles of each of the 2 SNPs were negatively associated with hypertension risk (*i.e.,* protective against hypertension) in male human subjects, while the major (more frequent) alleles were associated with increased hypertension risk. This supports our finding in mice that the lower BP phenotype was pronounced in males but female KO mice. The occurrence of a loss-of-function mutation in the *EFNB2* gene, in LD with the minor alleles of the 2 associated SNPs, could result in reduced risk

of hypertension which is not inconsistent with the effect of EFNB2 deletion in mice. However, it must be emphasized that human gene mutation is not equivalent to mouse EFNB2 deletion, which invariably leads to loss of function, whereas a mutation could either lead to a loss- or gain-of-EFNB2-function. A gain-of-function of mutation in human males could explain the association of increased hypertension risks with EFNB2 mutations of the major allele.

Our mouse study showed that in females, EFNB2 null-mutation did not lead to apparent hypotension. Therefore, EFNB2 gene mutation in female humans should not impact on BP, if our mouse study is of any guidance. Indeed, we observed no association with hypertension in females in this study. The mechanisms responsible for the influence of sex hormones regulate blood pressure jointly with EFNB2 are currently under investigation.

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

The minimal age of male patients in the ADVANCE study was 55-years. Plasma total testosterone levels range from 270 to 1,070 ng/dL in “normal” males. Considering 346 ng/dL as a cut-off for the diagnosis of hypogonadism, as recommended by the International Society for the Study of the Aging Male, about 30% of men older than 40 years are hypogonadic (Traish, Miner, Morgentaler, & Zitzmann, 2011) . Further, male patients with the metabolic syndrome

are prone to hypogonadism(Zitzmann, 2009). Therefore, it is conceivable that more than 30% of the male patients in the ADVANCE study suffer from hypogonadism.

Further, the type 2 diabetes males are generally overweight or obese. Adipose tissues are rich in aromatase, a rate-limiting enzyme converting testosterone to estrogen(Nelson & Bulun, 2001). Hence, overweight/obese males tend to have relatively higher estrogen levels locally in the perivascular adipose tissues or systemically.

The above-described special changes of sex hormone levels in the ADVANCE males might make them a unique subpopulation in which the association between EFNB2 mutations and hypertension risks becomes easier to detect, compared to the general population.
We queried the International Consortium for Blood Pressure (ICBP) dataset(International Consortium for Blood Pressure Genome-Wide Association et al., 2011), but no significant association of EFNB2 SNPs with BP was found. We should point out the differences in the two studies. The IBPC sample specifically excluded, cohorts of diabetic cases, hypertensive cases, and myocardial infarction cases, which is specifically the type of samples we analyzed. They analyzed general population samples that had incidentally been measured for systolic pressure (SP) and diastolic pressure (DP), and performed a quantitative analysis against DP and SP values. The advantage of their approach is that it allows the assembly of very large sample sizes to increase power; however, it also might introduce significant heterogeneity into their sample decreasing genotype relative risks. We used a qualitative phenotype that defined cases and controls for hypertension. Also, multiple BP measurements were carefully made over multiple

time points in the course of the *ADVANCE* study, and only consistently high readings were included as cases.

The two associated SNPs are in LD with each other and are located within a large LD block that includes the 3` UTR region of *EFNB2* and could reflect the presence a polymorphism within the 3' untranslated region (3' UTR) of the gene. 3'UTRs of various genes are known to contain regulatory elements that can control rates of translation and mRNA stability by serving as binding sites for various proteins or miRNAs. Many 3'-UTRs contain AU-rich elements which affect the stability or decay rate of transcripts in a localized manner or affect translation initiation. Also the 3'-UTR contains the sequence AAUAAA that directs addition of the poly-A tail which is involved in mRNA stability. By binding to specific sites within the 3'-UTR, miRNAs can decrease gene expression of various mRNAs by either inhibiting translation or directly causing degradation of the transcript. Discovery of the exact location and mechanism of the associated polymorphism would require further extensive functional research.

This study and our most recent publication show that the deletion of either of the receptor EPHB4 or its major ligand EFNB2 leads to a similar hypotensive phenotype in mice(Wang et al., 2015), corroborating the results of our human genetics study. Collectively, these novel findings reveal a previously unknown EPH/EFN-based mechanism for blood pressure regulation.

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Titles and Legends of Figures

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

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

B. EFNB2 protein deletion in EFNB2 KO VSMCs according to immunofluorescence microscopy VSMCs from EFNB2 KO and WT mice were isolated from mesenteric arteries, and their

EFNB2 (red, lower row) and α-actin (green, upper row) expression was detected by immunofluorescence microscopy.

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

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

Figure 2. BP and heart rate of EFNB2 KO mice

The BP and heart rate were measured every 2 min by telemetry for 72-h. The mean + SEM of daily BP and heart rate are shown. Parameters of all the time points in each 24-h period were evaluated by a mixed-effects linear model, with genotype and time as qualitative factors. *P*-values are indicated: * $p<0.05$; ** $p < 0.01$. SP: systolic pressure; DP: diastolic pressure; MAP: mean arterial pressure; HR: heart rate. The average age of mice of each group at the time of telemetry measurement and the number of each group (n) are indicated.

A. *Blood pressure and heart rate of male KO and WT mice*
B. *Blood pressure and heart rate of female KO and WT mice*

Figure 3. Both forward and reverse signaling by EFNB2 in VSMCs results in increased contractility

Both forward and reverse signaling by EFNB2 in VSMCs results in increased contractility Male VSMCs were cultured in wells coated with EFNB2-Fc, normal human IgG (NHIgG; as a control for EFNB2-Fc), goat anti-EFNB2 Ab, or goat IgG (as a control for anti-EFNB2 Ab; 2 µg/ml for coating for all the cases) as indicated. VSMCs were stimulated with 20 µM phenylephrine at

37℃ and imaged every min for 15 min. Means ± SEM of percentage contraction of 15-30 cells are shown. Data were assessed by one way ANOVA. P-values between groups with significant differences are indicated. All experiments were conducted 3 times independently. Data from representative experiments are presented.

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

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

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

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

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

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

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

Tables

Table 1. SNPs, alleles, allele frequencies, p-values and Odds Ratios for association with hypertension in the ADVANCE study for males-only and females-only samples

SNP	Position Chromosome 13 (hg19/Build 37)	Major Allele	Minor Allele	Males			Females		
				Minor Allele Frequency	[a]Association p-value	[b]Odds Ratio	Minor Allele Frequency	[a]Association p-value	[b]Odds Ratio
rs72656488	107139203	G	A	0.156	0.118	1.186	0.153	0.272	0.831
rs1407584	107139790	C	A	0.367	0.323	1.086	0.354	0.068	0.801
rs9555251	107140587	C	T	0.349	0.00226	1.285	0.341	0.976	1.004
rs7322914	107140910	C	T	0.349	0.00226	1.284	0.341	0.970	1.004
rs7328698	107141349	G	C	0.348	0.00208	1.286	0.342	0.915	1.013
rs7328882	**107141433**	**G**	**A**	**0.349**	**0.00133**	1.299	0.339	0.993	1.001
rs7329357	**107141705**	**G**	**A**	**0.354**	**0.00111**	1.308	0.349	0.913	1.013

rs56077446	107142499	A	G	0.076	0.688	0.939	0.075	0.350	0.805
rs9520087	107144320	G	A	0.431	0.00333	1.269	0.427	0.646	0.949
rs3742160	107145829	A	T	0.265	0.200	1.124	0.256	0.575	1.076

[a]*p*-values that are ≤ critical *Bonferroni*-corrected *p*-value of 0.00167, corrected for 30 independent tests, are in bold.

[b]Odds Ratios are calculated using major allele as reference.

Table 2. Case-control distribution of alleles and odds ratios for males and females for SNP rs7329357, the most significantly associated SNP with males

		Allele			Odds Ratio [b](95% C.I.) calculated for minor (A) Allele
		[a]A	[a]G	Total	
Female Cases	N	694.8	1297.2	1992	
	Frequency	0.349	0.651	1	0.984
Female Controls	N	145.2	266.8	412	(0.79-1.23)

	Frequency	0.352	0.648	1	
Male cases	N	1227.4	2350.6	3578	
	Frequency	0.343	0.657	1	0.581
Male Controls	N	394.7	439.3	834	(0.50-0.68)
	Frequency	0.473	0.527	1	

[a] The Allele counts are not integers for this imputed SNP, as imputation gives probabilitlies of genotypes and these totals reflect the sums of the probabilities.

[b]95% CI = 95% confidence interval of odds ratio.

Table 3. Chi-Square contingency tables for tests of independence between sexes, and allele distributions for cases and controls for SNP rs7329357

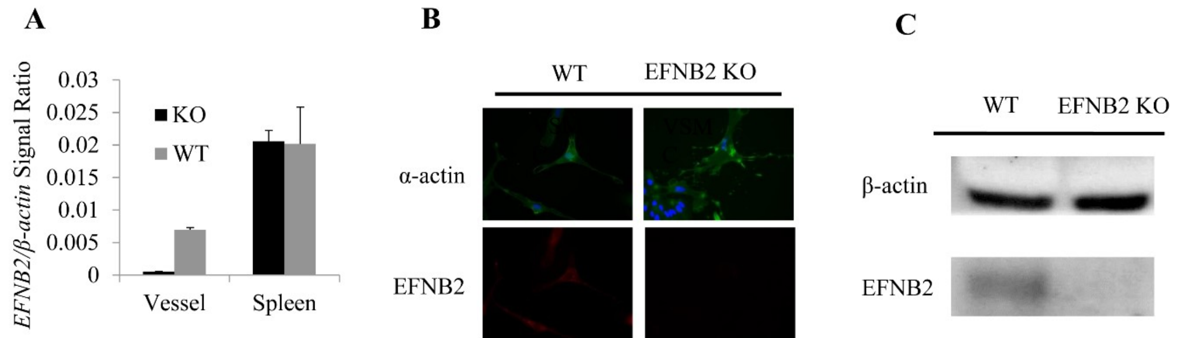
		Allele		Chi-Square Test
		A	G	of Independence
Cases				
Male	*N	1227.4	2350.6	$\chi^2 = 0.202$

	Frequency	0.34304	0.657	*p* = 0.653
Female	*N	694.8	1297.2	
	Frequency	0.349	0.651	
Controls				
Male	*N	394.7	439.3	$\chi^2 = 16.627$
	Frequency	0.473	0.527	$p = 0.000045$
Female	*N	145.2	266.8	
	Frequency	0.352	0.648	

* Distribution counts of imputed SNPs are not necessarily integers. Counts have been rounded to nearest integer for performing Chi-Square test of independence.

Figures

Figure 1



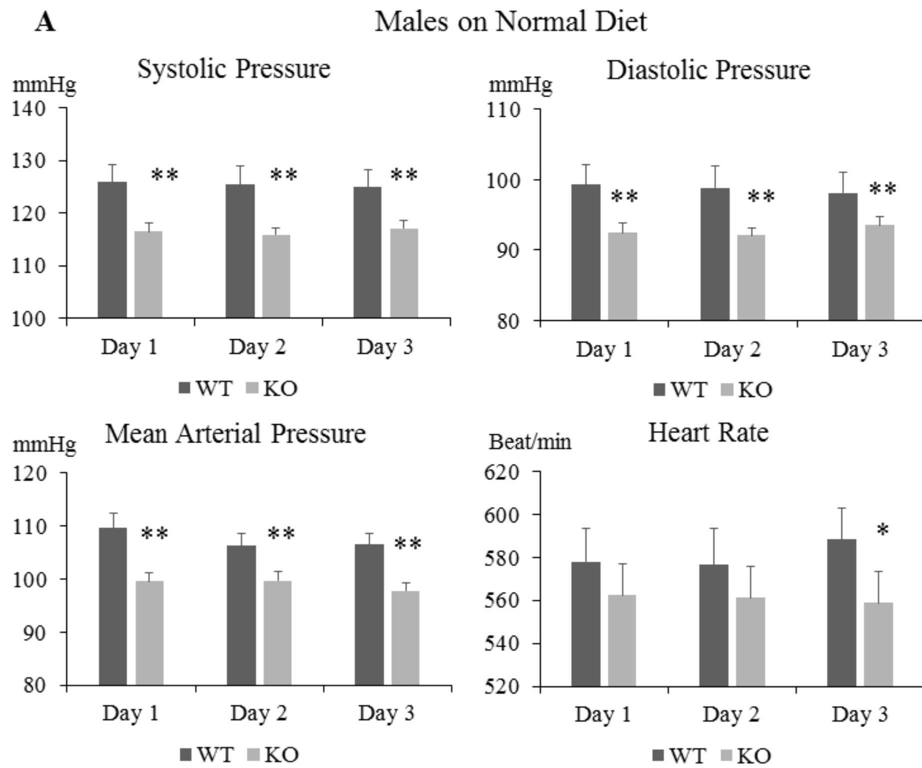
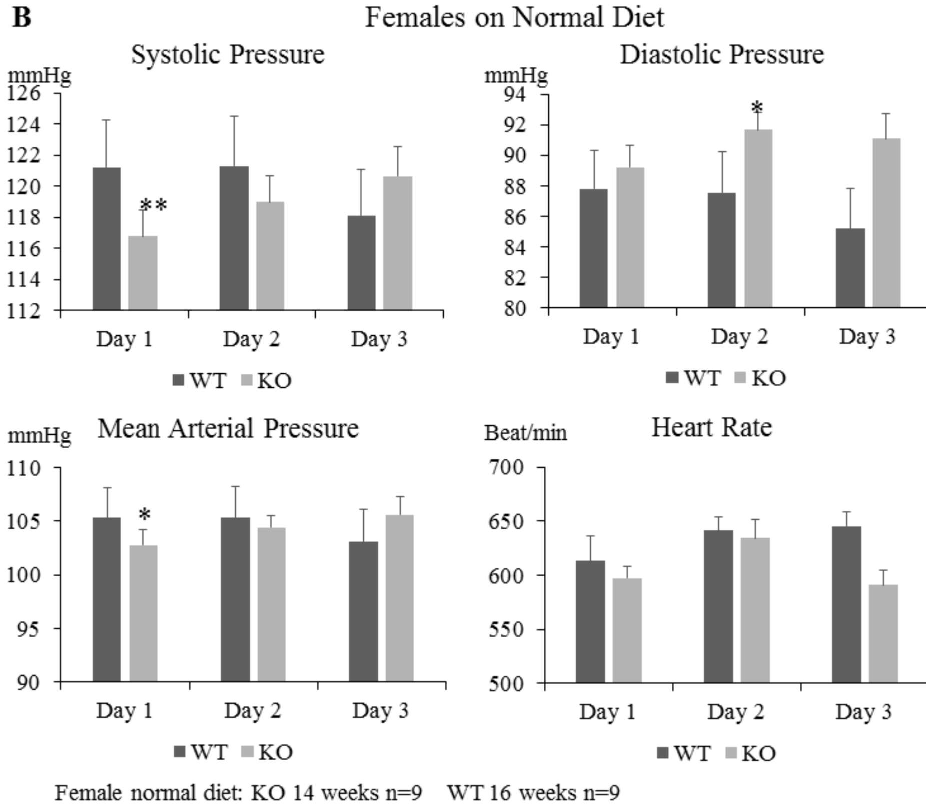


Figure 2



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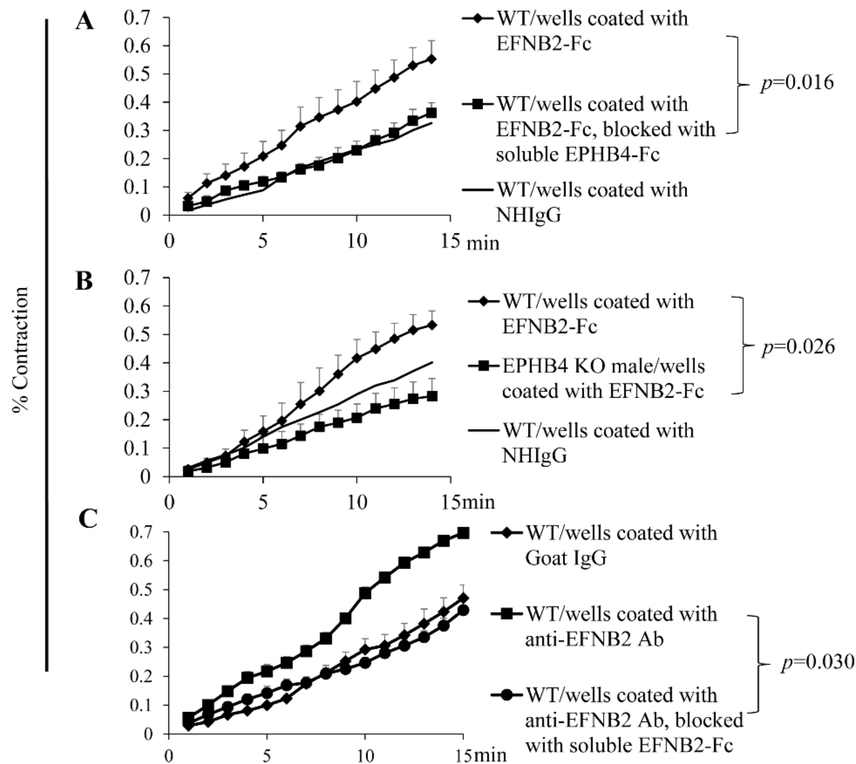

Figure 3

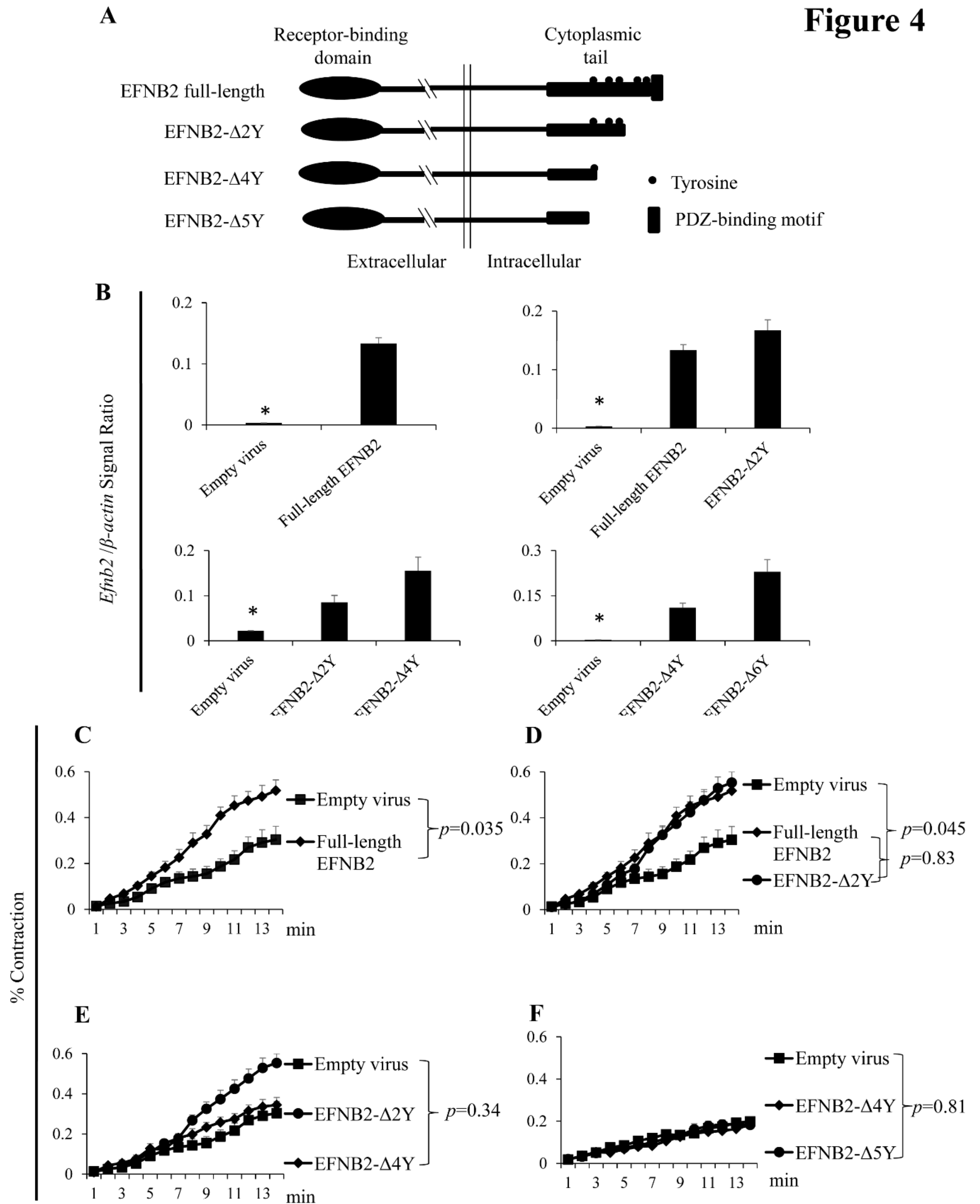

Figure 4
A
Receptor-binding domain
Cytoplasmic tail
EFNB2 full-length
EFNB2-Δ2Y
EFNB2-Δ4Y
EFNB2-Δ5Y
Tyrosine
PDZ-binding motif
Extracellular
Intracellular
B
Efnb2 /β-actin Signal Ratio
Empty virus
Full-length EFNB2
EFNB2-Δ2Y
EFNB2-Δ4Y
EFNB2-Δ6Y
C
D
E
F
% Contraction
min
Empty virus
Full-length EFNB2
p=0.035
p=0.045
p=0.83
p=0.34
p=0.81

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 4ºC. 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(Luo et al., 2012). Measurements were conducted at least 7 days after the radiotelemetry sensor implantation. 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 3 days with the Dataquest acquisition 3.1 system (Data Sciences International). The mice were on a normal diet unless specified. For some experiments, the mice were on a high-salt diet (containing 8% NaCl) for 3 weeks before the telemetry. In some experiments, the mice were immobilized in a restraining device (IITC Life Science, Woodland Hills, CA)(Dumas et al., 2000), and blood pressure and heart rate were similarly measured continuously every minute during a 30 min period. The raw data were processed with the Dataquest A.R.T-Analysis program (Lavoie, Lake-Bruse, & Sigmund, 2004). The daily blood pressure and heart rate are presented as bar graphs (means + SEM). Parameters of all the time points in any given 24-h period were analyzed by a multiple linear model, with

genotype and time as qualitative factors. The statistical significance ($p<0.05$ or $p<0.01$) is indicated.

*VSMC isolatio*n

VSMCs were isolated from male KO or WT mice, as described by Golovina and Blaustein[4] with modifications (Lavoie et al., 2004; Luo et al., 2012). 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 α-actin and capable of efficient contraction on phenylephrine stimulation.

Measurement of VSMC contractility

VSMC contractility was measured as described before(Lavoie et al., 2004; Luo et al., 2012). Briefly, the VMSCs were washed once with $Ca2^+$-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 4°C with goat anti-mouse EFNB2 Ab (R & D Systems, Minneapolis, MN, USA), rabbit anti-mouse α-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).

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Luo, H., Wu, Z., Tremblay, J., Thorin, E., Peng, J., Lavoie, J. L., . . . Wu, J. (2012). Receptor tyrosine kinase Ephb6 regulates vascular smooth muscle contractility and modulates blood pressure in concert with sex hormones. *J Biol Chem, 287*(9), 6819-6829. doi:10.1074/jbc.M111.293365

Supplementary Tables

Supplementary Table 1. List of RT-PCR primers

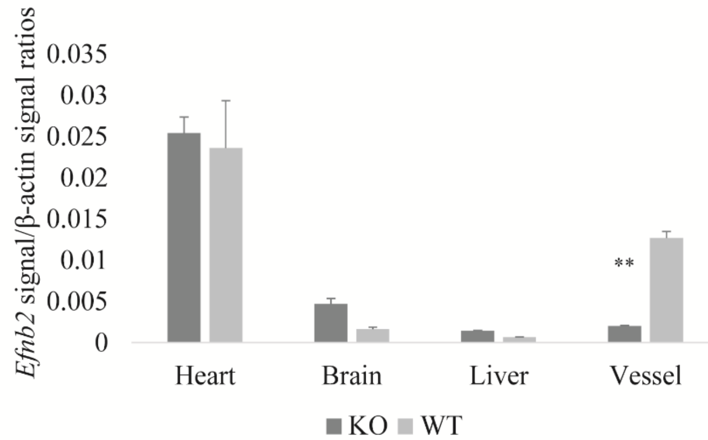
Supplementary Table 1. RT-PCR primer sequences

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

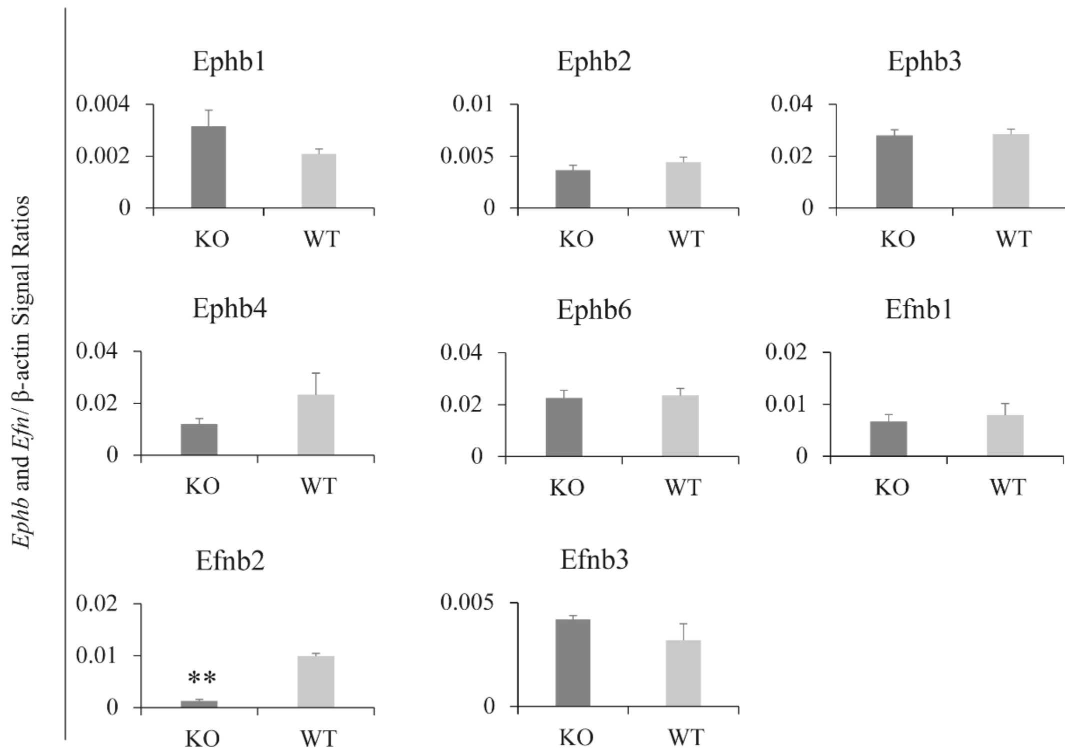
Supplementary Figures and legends



Supplementary Figure 1

Supplementary Figure 1. Vessel-specific deletion of Efnb2 mRNA

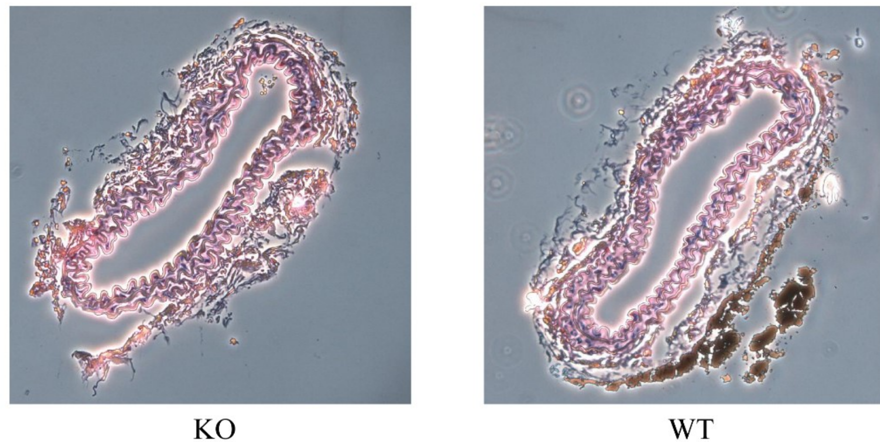
RNA was extracted from different organs and tissues from male WT and EFNB2 KO mice and analyzed by RT-qPCR for *Efnb2* mRNA levels. β-actin mRNA levels were used as an internal control. Samples were in duplicate, and means ± SEM of *Efnb2* signal/β-actin signal ratios are shown. Data represent the summary 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).

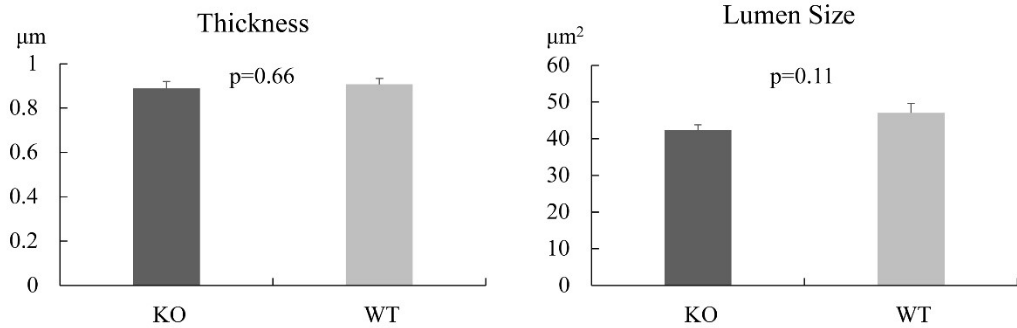


Supplementary Figure 2

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

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.

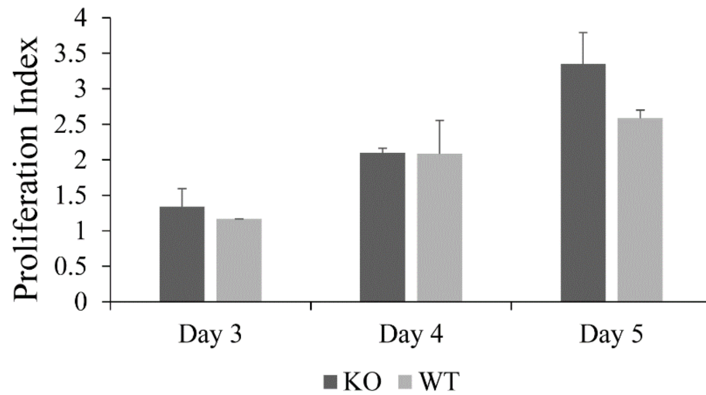
A. Carotid arteries from male KO and WT mice show comparable histology

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

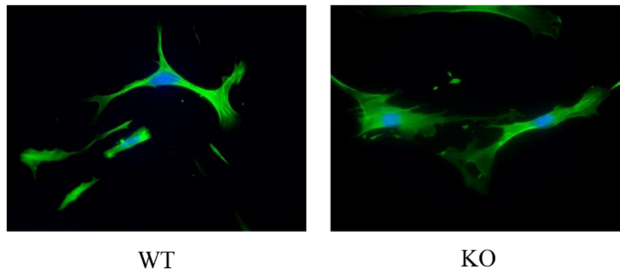
Supplementary Figure 4. Normal proliferation of VSMCs from EFNB2 KO mice

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.

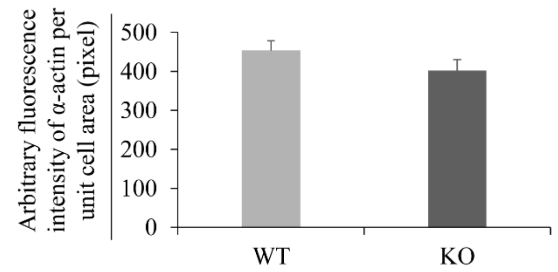
Proliferation Index = VSMC cell number after culture / VSMC cell number on day 1 (the beginning of the culture)

A. Immunofluorescence of α-actin



WT KO

B



Supplementary Figure 5

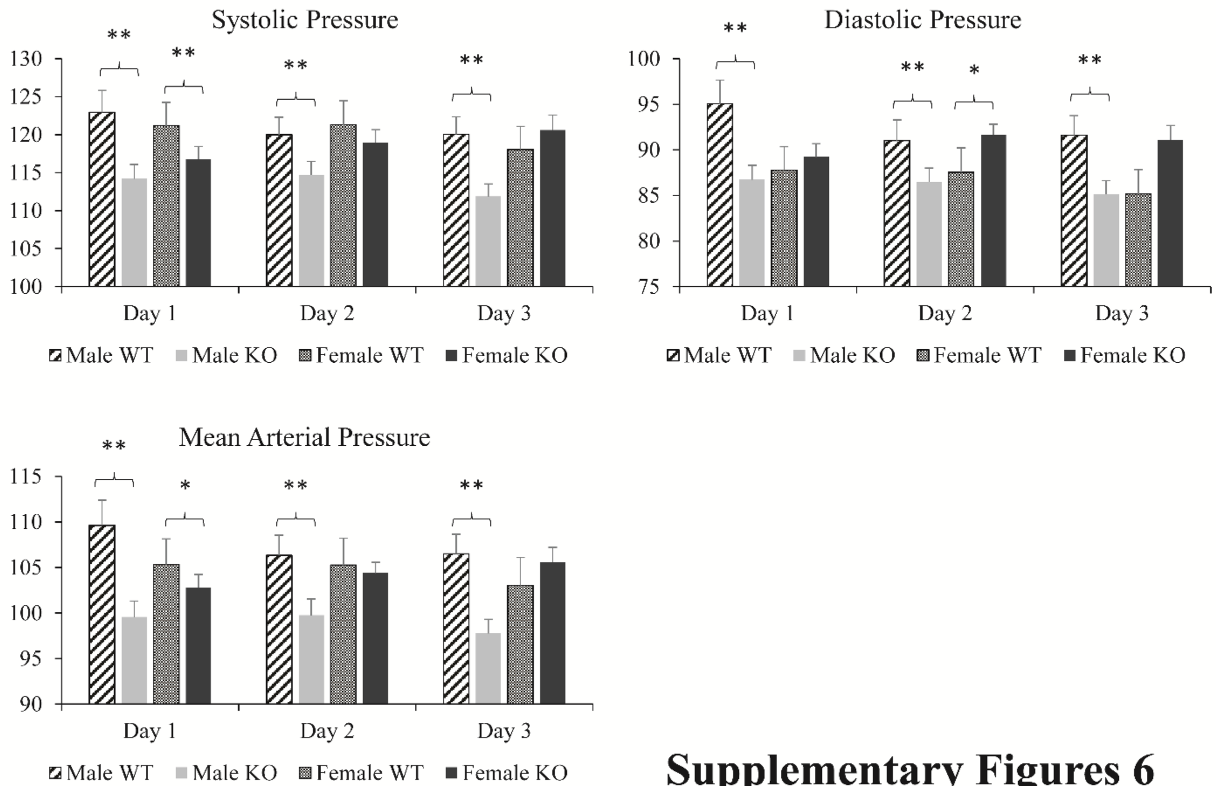
Supplementary Figure 5. Similar SMC marker a-actin expression in KO and WT VSMCs

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

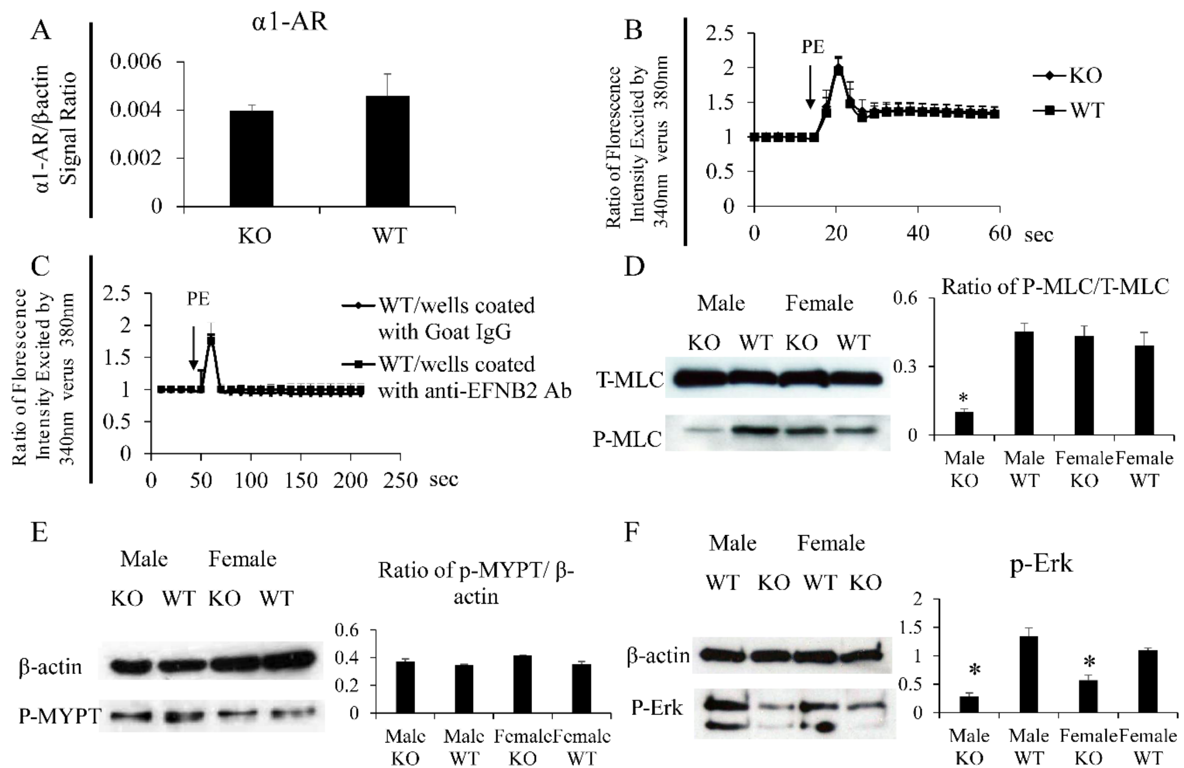
B. Quantitative assessment of the intensity of a-actin signal in male WT and KO VSMC

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



Supplementary Figure 6. KO and WT mouse BP parameters grouped according to SP, DP, and MAP with male and female data shown in the same panel

The blood pressure and heart rate were measured every 2 min by telemetry. The daily mean ± SEM of daily blood pressure and heart rate during a 72-h period are shown. The daily values were evaluated by a multiple linear model, with genotype and time as qualitative factors, and significant *p*-values are indicated: * $p<0.05$; ** $p<0.01$. The average age of mice of each group at the time of telemetry measurement and the number of each group (n) are indicated.



Supplementary Figure 7. The effect of EFNB2 deletion on signaling events of VSMCs

A. Normal adrenoreceptor 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 α1-adrenoreceptor (α1-AR) mRNA expression by RT-qPCR. Pooled data from 3 independent experiments were presented as mean ± SEM of α1-adrenoreceptor versus α-actin signal ratios.

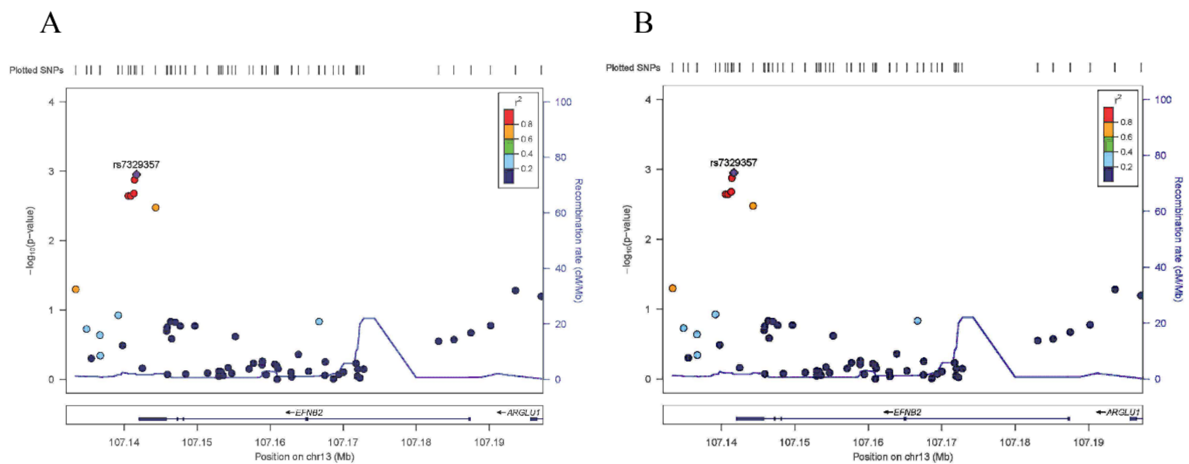
B and C. Normal Ca^{2+} flux in EFNB2 KO VSMCs or in WT VSMCs stimulated by solid phase EFNB2-Fc

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 µg/ml for coating; C). The 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 α-actin was used as loading controls. Three independent experiments were conducted. Immunoblottings from representative experiments are illustrated; ratios of phosphorylated and total protein (or α-actin) signals according densitometry of the 3 independent experiments are presented as bar graphs on the right.



C

Supplementary Figure 8. Association of SNPs within human EFNB2 gene and its adjacent regions with hypertension in the ADVANCE study

A. Distribution of SNP association -log *p*-values for males

B. Distribution of SNP association -log *p*-values for females

C. Location of Associated SNPs in Males with respect to LD structure in 3’ region of EFNB2

CHAPTER 4 ARTICLE-3

TITLE: Estrogen and testosterone in concert with EFNB3 regulate vascular smooth muscle cell contractility and blood pressure

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Running title: EFNB3 regulates blood pressure

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Key words: ephrinb3, estrogen, blood pressure

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Abstract

EPH kinases and their ligands, ephrins (EFNs), have vital and diverse biological functions, although their function in blood pressure (BP) control has not been studied in detail. In the present study, we report that Efnb3 gene knockout (KO) led to hypertension in female but not male mice. Vascular smooth muscle cells (VSMCs) were target cells for EFNB3 function in BP regulation. The deletion of EFNB3 augmented contractility of mesenteric arteries and VSMCs from female but not male KO mice, compared to their WT counterparts. Estrogen augmented VSMC contractility while testosterone reduced it in the absence of EFNB3, although these sex hormones had no effect on the contractility of VSMCs from WT mice. The effect of estrogen on KO VSMC contractility was via a non-genomic pathway involving GPR30, while that of testosterone was likely via a genomic pathway, according to VSMC contractility assays and GPR30 knockdown assays. The sex hormone-dependent contraction phenotypes in KO VSMCs were reflected in BP *in vivo*. Ovariectomy rendered female KO mice normotensive, while castration, male KO mice hypertensive. At the molecular level, EFNB3 KO in VSMCs resulted in reduced MLC kinase phosphorylation, an event enhancing sensitivity to Ca^{++} flux in VSMCs. Our investigation has revealed previously unknown EFNB3 functions in BP regulation and shown that EFNB3 is a hypertension risk gene in certain individuals.

New and noteworthy

We have revealed EFNB3 as a novel component in blood pressure regulation. Its deletion caused hypertension in a sex-dependent fashion. Vascular smooth muscle is the target tissue mediating

such an effect of EFNB3. This finding may lead to sex hormone level-specific treatment of hypertension in individuals with EFNB3 mutations.

Introduction

EPH erythropoietin-producing human hepatocellular carcinoma receptor kinases are the largest family of receptor tyrosine kinases. They are divided into A and B subfamilies according to sequence homology ("Unified nomenclature for Eph family receptors and their ligands, the ephrins. Eph Nomenclature Committee," 1997). Ephrins (EFNs), which are also cell surface molecules, are ligands of EPHs. EFNs are also classified into A and B subfamilies; members of the A subfamily attach to the cell surface through glycosylphosphatidylinositol anchoring, whereas members of the B subfamily attach through transmembrane tails (Flanagan & Vanderhaeghen, 1998; Pasquale, 2008; Wilkinson, 2000). Interactions among EPHs and EFNs are promiscuous, but in general, EPH A members interface preferentially with EFN A family members, and EPH B members with EFN B family members (Flanagan & Vanderhaeghen, 1998; Pasquale, 2008; Wilkinson, 2000). EFNs can stimulate EPH receptors, and this is called forward signaling. Interestingly, EPHs are also capable of stimulating EFNs which then transmit signaling reversely into cells, a phenomenon known as reverse signaling.

EPHs and EFNs are expressed in many tissues and organs. They play important roles in the central nervous system (Flanagan & Vanderhaeghen, 1998; Pasquale, 2008; Wilkinson, 2000), immune system (Luo, Charpentier, et al., 2011; Luo, Wan, Wu, & Wu, 2001; Luo, Wu, et al.,

2011; Luo, Yu, Tremblay, & Wu, 2004; Luo, Yu, Wu, & Wu, 2002; J. Wu & Luo, 2005; Yu, Luo, Wu, & Wu, 2003a, 2003b, 2004; Yu, Mao, Wu, Luo, & Wu, 2006), digestive system (Batlle et al., 2002), bone metabolism (Davy, Bush, & Soriano, 2006; Zhao et al., 2006), angiogenesis (H. U. Wang, Chen, & Anderson, 1998) and other processes (Dravis et al., 2007; Hashimoto et al., 2007; Konstantinova et al., 2007).

Until recently, limited studies assessed the role of EPHs and EFNs in vascular smooth muscle cell (VSMC) function. VSMCs with an EFNB2 deletion show compromised migration(Foo et al., 2006). In cultured rat and human VSMCs, EFNA1 triggers EPHA4 signaling and actin stress fiber assembly (Ogita et al., 2003). Whether such signaling elicits changes in VSMC contractility has not been investigated.

We recently reported that EPHB6, in concert with sex hormones, is crucial in VSMC contraction and blood pressure (BP) regulation (Luo et al., 2012). Male but not female *Ephb6* gene knockout (KO) mice are hypertensive. VSMC are targets through which EPHB6 exerts its effect on BP control. Since EPHB6 and all its major ligands of the EFN B family, *i.e.,* EFNB1, EFNB2 and EFNB3, are expressed in VSMC (Luo et al., 2012), there exists a molecular framework for their function in these cells. We showed that while solid-phase recombinant EPHB6 reduces VSMC contraction in response to phenylephrine (PE) stimulation, solid-phase anti-EPHB6 antibody (Ab) does not (Luo et al., 2012). Since anti-EPHB6 Ab serves as an EPHB6 agonist surrogate, this indicates that reverse signaling from EPHB6 to EFNBs, but not forward signalling from EFNBs to EPHB6 is responsible for dampening VSMC contractility. Deletion of *Efnb1* in smooth muscle cells also elicits hypertension in mice (Z. Wu et al., 2012), while deletion of

Ephb4 in these cells results in hypotension (Y. Wang et al., 2015). Therefore, multiple members of the EPHB/EFNBs family compose a previously unknown BP regulation system.

So far, there is no report on the regulation of EPH/EFN function by sex hormones, except a study by Nikolova showing that estrogen could modulate EPHB4 and EFNB2 expression in mammary glands (Nikolova, Djonov, Zuercher, Andres, & Ziemiecki, 1998). The classic estrogen receptor (ER) is an intracellular protein. ER has α and β subunits. They form heterodimers (αβ) or homodimers (αα or ββ) upon binding of estrogen, which enter the cells passively. The estrogen/ER complex then enters the nucleus serving as transcription factors, regulating target gene expression (Levin, 2005). In addition to the classical intracellular ERs, there is also GPR30, a G protein-coupled cell membrane estrogen receptor, which mediates fast nongenomic responses to estrogens (Prossnitz & Barton, 2014). Androgen receptors are also intracellular proteins, and when they bind to androgens, they will translocate into the nuclei, and serve as DNA-binding transcription factors, regulating genes with androgen-responsive elements (Kerkhofs, Denayer, Haelens, & Claessens, 2009). There are also cell membrane androgen receptors, which are less well-defined and might also be G-protein-associated receptors (Benten et al., 1999), mediating fast non-genomic actions.

In the present study, we discovered that female but not male *Efnb3* KO mice were hypertensive. Castration rendered male KO mice hypertensive, and ovariectomy, female KO mice normotensive. VSMCs were the major cell type responsible for these phenotypes. Non-genomic effect of estrogen via GPR30 promoted KO VSMC contractility, while genomic effect of testosterone reduced it.

Materials and Methods

Efnb3 KO mice

Efnb3 KO mice (referred to KO mice) were generously provided to us by Regeneron Pharmaceuticals (Kullander et al., 2001b). They had been backcrossed to the C57BL/6J background for more than 10 generations. Age- (16-26 weeks) and sex-matched WT mice were served as controls and are referred to as WT mice. The animals were housed in specific pathogen-free rooms with 12-h light and 12-h dark cycles. Females were not monitored for their estrous cycles. For radiotransmitter implantation and castration/ovariectomy, mice were anesthetized with isoflurane (2% isoflurane with 0.75L/min O_2 flow). In some experiments, the mice with radiotransmitter implantation were castrated or ovariectomized, and telemetry was conducted at least 3 weeks after the gonadectomy. The mice were euthanized with pentobarbital (400 mg/Kg body weight, *i.p.)* at the end of *in vivo* studies or for tissue retrieval.

VSMC isolation

Mouse VSMCs were isolated, as described by Golovina and Blaustein (Golovina & Blaustein, 2006) with modifications (Luo et al., 2012; Z. Wu et al., 2012). The aorta and mesenteric arteries, including their secondary branches, were pooled and digested with collagenase type II (347 U/ml) (Worthington Biochemical Corporation, Lakewood, NJ, USA). They were further digested with both collagenase type II (347 U/ml) and elastase type IV (6 U/ml) (Sigma-Aldrich Corporation, St. Louis, MO, USA). 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 4 to 5 days before experimentation.

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

Efnb3 and *GRP30* mRNA levels in VSMCs were measured by RT-qPCR (Luo et al., 2012). For *Efnb3*, the 5' primer was 5'-AGTTCCGATCCCACCACGATTACT-3', and the 3' primer was 5'-AGAAGCACCTTCATGCCTCTGGTT-3'. For *GPR30*, the 5' primer was 5'-CATCATTGGCCTCTGCTACTC-3', and the 3' primer was 5'-GAAGATCATCCTCAGGGCTTTC -3'. *β-actin* mRNA served as an internal control, and primers for *β-actin* was described previously(Z. Wu et al., 2012). The following qPCR condition was employed: 2 min at 50 °C and then 2 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. Signals between 20-30 cycles were analyzed. Samples were tested in triplicate, and the data are expressed as signal ratios of test gene mRNA/*β-actin* mRNA.

BP measurement

Mice were implanted surgically with TA11PA-C10 radiotransmitters (Data Sciences International, St. Paul, MN, USA) in the left carotid artery as described previously (Lavoie, Lake-Bruse, & Sigmund, 2004). At least 7 days were allowed for recovery before the measurement of systolic, diastolic and mean arterial pressure (MAP), systolic pressure (SP), diastolic pressure (DP) and heart rate (HR).

BP and HR in conscious, free-moving mice were then recorded continuously for 3 days with the Dataquest acquisition 3.1 system (Data Sciences International). Individual 10-s waveforms of SP, DP, MAP and HR were sampled every 2 min throughout the monitoring period. The raw data were processed with the Dataquest A.R.T-Analysis program (Lavoie et al., 2004), and

means + SEM of hourly BP and HR are calculated and presented. ANOVA was used to compare the 72 hourly values of each group (area under the curve) for statistical significance.

Ex vivo vessel constriction

Third order mesenteric arteries were employed in these vessel constriction experiments. In some experiments, the endothelium was removed by scrapping the luminal surface of the arterial segments with a human hair. The lack of endothelium-dependent relaxation induced by acetylcholine (1 μM) was confirmed on a test-contraction induced by phenylephrine (10 μM). The artery rings of 2 mm were mounted on 20-μm tungsten wires in microvessel myographs (IMF, University of Vermont, Burlington, VT) as described previously(Luo et al., 2012; Thorin, Huang, Fishman, & Bevan, 1998). An optimal basal tension of ~ 300 mg was determined for vessels while no differences in basal tension were observed among groups (data not shown). The mounted vascular ring was equilibrated for 30 to 45 minutes, after which the contractility of each arterial ring was determined by a 40 mM KCl-PSS solution, followed by two washout periods; then, vessels were allowed to further equilibrate for 30 to 45 minutes. Contraction curves to phenylephrine (0.1 nM to 10 μM) were obtained.

During equilibration and experiment, vascular segments were bath in a physiological salt solution at pH 7.4 containing 119 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO_4, 1.17 mM $MgSO_4$, 24.9 mM $NaHCO_3$, 1.6 mM $CaCl_2$, 0.023 mM EDTA, and 10 mM glucose, aerated with 12% O2 / 5% CO_2 / 83% N_2 at 37°C. At the end of the protocol, maximal tension (Emax) was determined by changing the PSS to a solution containing 127 mM KCl. The data are expressed as percentages of Emax.

Contractility of 3 segments of mesenteric arteries per mouse was measured and data from 3 independent experiments per group were pooled to construct mean concentration-response curves with SEM. ANOVA is performed to compare concentration-response curves using the percentage of the Emax.

VSMC contractility

VSMC contractility was measured as described previously(Luo et al., 2012; Z. Wu et al., 2012). Briefly, primary VSMCs were cultured for 3-4 days and then stimulated with PE (20 μM). They were photographed continuously for 15 min at a rate of 1 picture per min. In experiments testing the effect of sex hormones, the VSMC were cultured charcoal-stripped sera in the presence of human 5α-dihydrotestosterone (6.49 ng/ml), cell membrane impermeable testosterone-3-(O-carboxymethyl)oxime-BSA (Aviva Systems Biology Corp., San Diego), or 17-β-estradiol (100 ng/ml) for periods indicated.

Fifteen or more cells were randomly selected, and their length was measured at each time point with Zeiss Axiovision software. Percentage contraction was calculated as follows:

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

Immunofluorescence microscopy

VSMC surface EFNB3, α1-adrenergic receptor (α1-AR) and GPR30 expression was assessed by immunofluorescence microscopy (Luo et al., 2012). Cells were blocked with 10% goat IgG

in PBS for 20 min and then incubated with rabbit anti-mouse EFNB3 Ab (OAAF01784, Aviva Systems Biology Corporation, San Diego, CA) rabbit anti-mouse α1-AR Ab, (ab3462, Abcam Inc., Cambridge, MA) or rabbit anti-mouse GPR30 Ab (ab39742, Abcam Inc., Cambridge, MA). Cells were then washed and reacted with FITC-conjugated sheep anti-rabbit IgG Ab (0.2 µg/ml; Chemicon International, Temecula, CA) at room temperature for 2 h, and imbedded with ProLong® Gold anti-fade reagent (Invitrogen). The stained cells were examined under a Zeiss microscope. The total fluorescence intensity of a cell and cell size were measured using AxioVision software from Zeiss; results from measurements of more than 15 cells are then presented as fluorescence intensity per arbitrary unit of cell area (1 pixel).

Immunoblotting

VSMCs from WT and KO mice were isolated and cultured for 3-4 days. VSMCs were stimulated with PE (20 µM) for 3 s and then lyzed. Total and phosphorylated MLC kinase (MLCK) and MLC phosphatase (MYPT) of the VSMCs were measured similarly, using rabbit anti-mouse MLCK mAb (clone EP1458Y; Abcam, Cambridge, UK), rabbit anti-mouse phospho-MLCK Ab (Invitrogen, Camarillo, CA), rabbit anti-mouse MYPT Ab (#2634; Cell Signaling, Danvers, MA), and rabbit anti-mouse phospho-MYPT Ab (#5163; Cell Signaling).

Measurement of Ca^{++} flux

PE-stimulated Ca^{++} flux in VMSC was measured by immunofluorescence microscopy(Golovina & Blaustein, 2006; Luo et al., 2012) Briefly, VSMC were culture in medium containing Ca^{++} for 4 days. They were then loaded with Fura-2-AM, stimulated with PE (20 µM) at 37ºC in medium without Ca^{++}, as most of the Ca^{++} flux in VSMC was from sarcro-endoplasmice

reticulum. The cells were imaged for 60 s at a rate of 1 picture per 3 s. Excitation wavelengths were switched between 340 nm and 380 nm, and emission wavelength was 510 nm. Signals from more than 15 randomly-selected cells were recorded, and the results expressed as ratios of fluorescence intensity at 510 nm excited by 340 nm versus 380 nm.

Small interfering RNA (siRNA) transfection

Gpr30 siRNAs and negative control siRNAs were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Their sequences are presented in supplementary table 1 (Table 1). VSMCs were cultured for 40 h in regular medium. They were then transfected with a mix of 3 pairs of *Gpr30* siRNAs (for each pair, the final concentration was 10 nM), or control siRNA, and cultured in medium free of antibiotics, as described before (Z. Wu et al., 2012). Forty eight hours after the siRNA transfection, VSMC contractility upon PE stimulation was assessed.

Urinary catecholamine measurement

Twenty four-hour urine under a fasting condition was collected by placing 15- to 17-week old KO and WT mice individually in metabolic cages. Urinary catecholamines were quantified by competitive enzyme immunoassays (3-CAT Research ELISA kits) according to the manufacturer's instructions (Labor Diagnostika Nord GmbH & Co. Nordhorn, Germany). The reaction was monitored at 450 nm. Urine samples were measured in duplicate.

Ethics statement

All animal studies were performed according to guidelines of Canadian Council for Animal Protection and were conducted according the protocols approved by CRCHUM Animal Protection Committee.

Statistics

Statistical significances were determined using a one-way ANOVA or paired Student's *t tests*.

Results

Efnb3 KO deletion in VSMCs

RT-qPCR demonstrated that *Efnb3* mRNA was virtually absent in endothelium-stripped mesenteric arteries from *Efnb3* KO mice (Fig. 1A). *Efnb3* KO was also confirmed at the protein level in isolated EFNB3 KO VSMCs by immunofluorescence microscopy (Fig. 1B).

EFNB3 deletion caused hypertension in females but not males

BP of the aged-matched *Efnb3* KO and WT mice was measured by radiotelemetry. The MAP, SP, and DP of male KO mice showed no significant differences from their WT controls, although the HR of the KO mice was higher (Fig. 2A). Females KO had increased MAP, SP and DP compared to their WT counterparts, while their HR were normal (Fig. 2B). These results clearly show that EFNB3 deletion caused hypertension in a sex-dependent way.

Increased contractility of small arteries and VSMCs from female KO mice

The *ex vivo* contractility of mesenteric arteries from KO and WT mice was assessed after stimulation with PE. Female but not male KO vessels showed greater contractility than their WT counterparts (Figs. 3A and 3B), corroborating the BP phenotype in KO mice. This contractility phenotype remained unchanged in the presence or absence of endothelium, indicating that VSMCs but not the endothelium were responsible for the phenotype, and that nitric oxide or endothelin-1 produced by the endothelium was not required to enhance female vessel contractility in the absence of EFNB3. In this experiment, WT and KO vessels had no significant difference in their EC50 to PE stimulation (data not shown).

When isolated VSMCs were stimulated with PE, those derived from female *Efnb3* KO mice but not male KO mice showed increased contractility compared to their WT counterparts (Figs. 3C and 3D), consistent with the findings in *ex vivo* vessel contraction studies.

The increased contractility of female KO VSMCs upon PE stimulation might be due to heightened adrenoreceptor signaling strength. However, we found no difference in α1-AR expression in KO and WT VSMCs, regardless of the sex, according to either immunofluorescence (Figs. 4A).

Ca^{++} flux (Fig. 4B) in KO VSMCs was comparable to that of the WT counterparts, whether they were from males or females. Thus, the increased contractility of VSMCs from female KO mice was likely not due to increased Ca^{++} flux but augmented Ca^{++} responsiveness, which is regulated by MLCK. When MLCK is phosphorylated at ser1760 by calmodulin-dependent protein kinase II or protein kinase A, its MLCK enzymatic activity is reduced (Giembycz & Newton, 2006;

Miller, Silver, & Stull, 1983; Raina, Zacharia, Li, & Wier, 2009). Conversely, reduced phosphorylation at ser1760 will lead to increased VSMC contractility. We assessed MLCK phosphorylation at ser1760 and observed that VSMCs from female but not male KO mice had significantly lower values upon PE stimulation than WT controls (Fig. 4C). This result implies higher MLCK activity and hence higher Ca^{++} responsiveness in VSMCs from female KO mice, compatible with the finding that female VSMCs presented augmented contractility. VSMC contractility is also modulated by MYPT, which dephosphorylates MLC and reduces VSMC contractility (Kawano et al., 1999). MYPT phosphorylation at T695 prevents its binding to MLC and thus diminishes its phosphatase activity (Muranyi et al., 2005). We investigated the outcome of EFNB3 deletion on MYPT phosphorylation in VSMCs. After PE stimulation (3 s), MYPT phosphorylation at T695 in VSMCs from both female and male KO mice was similar to that of their WT counterparts (Fig. 4D). This observation suggests that MYPT is not influenced by EFNB3 deletion and does not participate in the contractility upregulation of female KO arteries.

Nongenomic effect of estrogen augments while genomic effect of testosterone reduces KO VSMC contractility

The default function of EFNB3 signaling is to reduce VSMC contractility, and hence EFNB3 KO deletion led to the increased VSMC contractility (Fig. 3D). However, such an increase only occurred in VSMCs from female but not male KO mice, suggesting that either estrogen is permissive for this phenotype, or androgen antagonizes it, or both.

To assess the role of estrogen in this regard, we cultured VSMCs from male KO mice in the presence of a physiological concentration of estrogen (17β-estradiol). VSMCs from male but

not female mice were used in order to minimize intracellular estrogen carry-over from *in vivo* exposure. The fetal calf sera used in these sex hormone studies were absorbed by charcoal to remove residual sex hormones in the sera.

We found that after 3-4 day culture in the presence of 17β-estradiol, the contractility of male KO VSMCs, which had no difference from WT counterparts in the absence of estrogen, was increased compared to vehicle controls, while estrogen did not affect the contractility of male WT VSMCs (Fig. 5A).

In the above-described experiments, estrogen was present in the culture for 3-4 days. So its effect could be either genomic or nongenomic, or both. A short-term estrogen treatment was employed to discern these two possibilities. When VSMCs from male KO mice were incubated with 17β-estradiol, their contractility were increased within as short as 15 min, while the contractility of WT VSMCs were not influenced by such a treatment (Fig. 5B). The speed of the estrogen effect suggests that the non-genomic pathway is involved in the effect of estrogen on male KO VSMCs.

To assess whether androgen has protective effect against the augmented VSMC contractility after EFNB3 deletion, we cultured VSMCs from KO females in the presence of physiological concentration of 5α-dihydrotestosterone for 3-4 days. The female VSMCs but not male VSMCs were used in this experiment to minimize the intracellular androgen carry-over from the *in vivo* environment. 5α-dihydrotestosterone effectively reduced the augmented female KO VSMC contractility to the WT level (Fig. 5C), while had no effect on female WT VSMC. However, short-term (15 min) 5α-dihydrotestosterone (6.49 ng/ml) treatment (Fig. 5D) or a long-time (3

days) treatment of a cell membrane-impermeable BSA-conjugated testosterone (testosterone-3-(O-carboxymethyl)-oxime-BSA (testosterone-BSA; Aviva Systems Biology Corporation, San Diego, CA) used at an equal molar concentration (1.1 μg/ml) as 5α-dihydrotestosterone (6.49 ng/ml) could not reduce the female KO VSMC contractility (Fig. 5E). These results suggest that the genomic but not non-genomic effect of testosterone is responsible for countering the VSMC contractility-augmenting effect of EFNB3 deletion, and explains the normal contractility of VSMCs from male KO mice, and the normal BP of those mice.

The above *in vitro* study showed that in the absence of EFNB3, estrogen promoted VSMC contractility, while testosterone reduced it. Is this conclusion valid *in vivo* where VSMC contractility is reflected in BP? Indeed, when male KO mice were castrated, they became hypertensive (Fig. 5F), and when females KO mice were ovariectomized, they lost the hypertensive phenotype (Fig. 5G), in agreement with our *in vitro* results.

GPR30 mediates the non-genomic effect of estrogen in KO VSMC contractility

GPR30 is a G-protein-associated cell surface molecule mediating the nongenomic effects of estrogen(Evans, Bayliss, & Reale, 2014). We found that its mRNA (Fig. 6A) and protein expression (Fig. 6B), as detected by RT-qPCR and immunofluorescence, respectively, was significantly augmented KO VSMCs, compared to the WT counterparts, consistent with the obvious non-genomic effect in KO VSMC.

To further prove GPR30's role in mediating estrogen's effect in VSMC contractility, we transfected VSMCs with *Gpr30* siRNA. The knockdown *Gpr30* expression at the mRNA level

was confirmed by RT-qPCR (Fig. 6C). Such knockdown resulted in failed augmentation of male KO VSMC contractility by estrogen (Fig. 6D).

Investigation of the effect of EFNB3 KO on the sex hormone levels and levels of BP-related endocrine molecules

The serum testosterone and estrogen levels of males and females, respectively, of KO and WT mice were measured, but no apparent difference between the KO and WT mice was observed (data not shown).

We showed that in male or female mice, EFNB3 KO did not alter 24-h urine catecholamine contents (Fig. 7). Serum AngII levels in KO and WT mice, whether males or females, were comparable (data not shown).

Discussion

Our data indicate that VSMCs are a target tissue for EFNB3 function in BP regulation. In the absence of EFNB3, the VSMCs contracted stronger, hence increased BP. However, such phenotype was only revealed under permissive conditions, *i.e.*, the presence of estrogen or absence of testosterone.

In our *in vivo* BP measurement, in some test and control groups, there was 3 to 5-week age difference. The BP difference between the KO and WT mice is not caused by such age

difference, as we have confirmed that the BP in mice with 10- to 20-week age difference are similar (data not shown).

We demonstrated EFNB3 can modulate VSMC contractility by reverse signalling. EFNB3 is a transmembrane protein without enzymatic activity in its intracellular tail. How does it regulate VSMC contractility? EFNB intracellular tails are known to associate with GRIP1 (Dong et al., 1997), Dishevelled (Tanaka, Kamo, Ota, & Sugimura, 2003), PDZ-RGS3 (Lu et al., 2012), TIAM1 (Tanaka, Kamata, Yanagihara, & Sakai, 2010) and GRB4(Segura, Essmann, Weinges, & Acker-Palmer, 2007). We have demonstrated that the association of EFNB3 with GRIP1 is critical for its function in regulating VSMC contractility (Yujia Wang et al.). In VSMCs, EFNB3 might also associate with other so-far unidentified binding proteins. During reversesignalling, some of these proteins might interact with and modulate the functions of other pathways that control VSMC contractility, the MLCK pathway being one of them. As shown in Figure 4C, in female KO VSMCs, MLCK phosphorylation was reduced compared to that of the WT counterparts. Reduced MLCK phosphorylation augments the MLCK activity, and then MLC phosphorylation, which promotes Ca^{++} sensitivity of VSMCs, hence increased contraction. MLCK can be phosphorylated by Cam-kinase II, kinase A or kinase C, resulting in its inactivation. In our previous publication related to the role of EphB4 in VSMC contractility, we reported that EphB4 deletion modulates Cam-kinase II (Y. Wang et al., 2015). As EphB4 interacts with EFNB2, a molecule belonging to the same subfamily as EFNB3 does, it is possible that EFNB3 deletion also modulates Cam-kinase II. The intermediate signalling molecules between EFNB3/GRIP1 and Cam-kinase II/MLCK/MLC remain to be identified. In addition, EFNB3 could also modulate MLC function through other pathways. For example, Zip kinase in

the Rho kinase pathway could directly activate MLC(Brozovich, 2002). Putative connection between EFNB3 and the Rho pathway is quite relevant, as among EFNB-associating proteins, Tiam1 is a guanine exchange factor for Rho GTPase(Tanaka et al., 2010).

The default function of EFNB3 with regard to VSMC contractility should be an inhibitory one, but such a default function was only revealed in the presence of estrogen or absence of testosterone. What are possible mechanisms by which sex hormones in concert with EFNB3 modulate VSMC contractility? A possible one is that, in the absence of EFNB3, sex hormones regulate the expression of certain cell surface receptors or their signalling involved in VSMC contractility. These receptors include vasoconstrictor receptors, such as type 1a α-adrenoreceptor, AT1 receptor (the major angiotensin II receptor) and ETα/ETβ2 (endothelin-1 receptors for constriction). Another possible mechanism is that that some EFNB3-associating proteins start to respond to sex hormones only when EFNB3 is deleted, with regard to their downstream connection/modulation of VSMC contracting machinery. In other words, although they are capable of associating with EFNB3, they still regulate VSMC contractility in the absence of EFNB3, but such regulation is now being modulated by sex hormones. The above-described two mechanisms are not mutually exclusive.

The HR of male but not female KO mice was significantly higher than that of the WT counterparts, especially during night when the mice were active (Fig. 2A). The *Efnb3* KO mice used in this study have a general deletion of the gene. One of the possible reasons of the increased HR is that EFNB3 has a functional role in heart rhythm pacing cells, and such a role is sex-dependent. Another possibility is that as EFNB3 has known function in the central

nervous system(Kullander et al., 2001a) , it might influence the circadian neural outflow to the pacing cells. These possibilities are under investigation. However, such a HR increase did not influence BP in males, likely due to multiple BP compensation mechanisms. Such HR phenotype did not appear in KO females, and therefore, does not contribute to the increased BP in them. In castrated KO males, the HR also increased and such an increase might contribute to the higher BP. However, increased VSMC contractility remains a significant contributor to the elevated BP, as evidence by our *ex vivo* and *in vitro* data.

Estrogen was required to manifest the hypertensive phenotype in KO females, while androgen suppressed this phenotype, as proved by KO mice with ovariectomy and castration (Figs. 2A, 2B, 5F and 5G). If the mouse results are relevant to humans,, for men with loss-of-function mutations in the EFNB3 gene, a decrease of testosterone level will increase their hypertension risks, and menopause will confer a protective effect against hypertension for the affected females. Male hypogonadism has high prevalence: about 30% males above age of 40 years are hypogonadic(Zitzmann, 2009). Reports overwhelmingly indicate that hypogonadism in men, whether evoked by ageing, diabetes, chemical castration or other unidentified factors, correlates with hypertension(Garcia-Cruz et al., 2012; Liu, Death, & Handelsman, 2003; Reckelhoff & Granger, 1999; Svartberg et al., 2004; Torkler et al., 2011; Traish, Miner, Morgentaler, & Zitzmann, 2011; Zitzmann, 2009). We discovered that contractility of male KO VSMCs could be enhanced by the nongenomic effect of estrogen. This is consistent with the finding that KO VSMCs presented augmented GPR30 expression(Huang, Jacobson, & Schaller, 2004; Prossnitz & Barton, 2014; Roman-Blas, Castaneda, Largo, & Herrero-Beaumont, 2009). With that said,

our results could not rule out the involvement of the genomic effect of estrogen on KO VSMC contractility.

There is existing literature related to the inhibitory effect of estrogen on vessel contraction. However, most of such data come from rat or rabbit experiments(Gui, Zheng, Zheng, & Walsh, 2008; Koga, Hirano, Nishimura, Nakano, & Kanaide, 2004), and such effect is often attributed to the regulation of nitric oxide production by endothelium(Farhat, Lavigne, & Ramwell, 1996). Controversies remain in this regard. For example, α2-AR antagonist-induced mouse tail artery constriction is not affected by estrogen(Eid et al., 2007) ; estrogen has no effect on the PE-induced constriction in aortic rings from iNOS KO mice(Zhu et al., 2002) . Further, in women taking estrogen-containing contraceptives, about 5% will develop *de novo* hypertension. Therefore, we regard the effect of estrogen on BP regulation and VSMC contractility as unresolved controversy. In our model, VSMCs are totally devoid of endothelial cells. Possible explanations for such controversy include species differences, and difference in how completely the endothelial cells are removed from the vessels during experimentation.

The effect of estrogen on enhancing male KO VSMC contractility might have relevance to our current health problems: estrogen levels in males tend to increase in industrialized countries. This is caused by 2 factors. Firstly, adipose tissue is a major periphery source of aromatase activity, which converts testosterone into estrogen(Nelson & Bulun, 2001), hence increased estrogen levels locally (particularly in the perivascular adipose tissue) or systemically in overweight/obese individuals. Secondly, our exposure to environmental xenoestrogens (chemical compounds mimicking endogenous estrogen) in industrialized world is on the

rise(Danzo, 1998). Therefore, for male patients with loss-of-function EFNB3 mutation, if their GPR30 expression is increased as is the case in mice, they will have increased hypertension risk if they are overweight or obese, as would be expected in metabolic syndrome. Similarly, due to exposure to xenoestrogens, living in industrialized countries also poses increased risks of hypertension for males carrying EFNB3 mutations.

Our additional human genetic study revealed that 5 single nucleotide polymorphisms (SNPs) in the EFNB3 gene present higher frequency in hypertensive patients than in normotensive ones, and are thus significantly associated with hypertension risks (data not shown). Such data corroborate our findings in the EFNB3 KO mice and demonstrate the relevance of the data from our animal studies to human hypertension. Based on this, we could propose the following therapeutic options. For those men with hypertension caused by a combination of EFNB3 loss-of-function plus hypogonadism, testosterone might be a safe and effective personalized medicine. Elevated estrogen levels in males might represent a l hypertension risk if they carry the EFNB3 mutations, and estrogen antagonists might be an effective therapy. For females with loss-of-fucntion EFNB3 mutations, avoiding estrogen replacement therapy after menopause might reduce their risk of hypertension. Of course, a large amount of clinical study will be needed to validate our proposed therapeutic strategies.

Our previous work has shown that several members of the Eph/EFN family (*e.g.,* EphB6 and EFNB1) have default negative impacts on VSMC contractility, as their deletion leads to hypertension(Luo et al., 2012; Z. Wu et al., 2012) . This study has added a new member to this category. On the other hand, certain other members of the family, such as EphB4 has defaults

positive impacts on VSMC contractility and its deletion results in reduced VSMC contractility and lower BP(Y. Wang et al., 2015). Collectively, our research has revealed a previously unknown Eph/EFN-based mechanism for blood pressure regulation. An interesting question is why there needs to have opposing forces from members of the same families to regulate blood pressure. In the biological systems, such opposing forces are widely present. Examples are: protein phosphorylation versus dephosphorylation; calcium influx versus outflux in a cell; sympathetic versus parasympathetic neurological activities, etc. Such opposing forces, which can also be called Yin and Yang, provide a mechanism for achieving the rejuvenation or homeostasis of biological processes and systems to cope with ever-changing external environments. Co-existing positive and negative regulatory effects on VSMC contractility by different members of the EPH/EFN family likely provide an additional layer of stabilizing force to maintain blood pressure within an acceptable range. EFN and Eph members are constitutively expressed on VSMCs and engaged with each other constantly. Therefore, they are probably not for rapid regulation of blood pressure or for rejuvenation of each other's signalling pathways, but rather for some chronic fine-tuning of vascular tone.

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DISCLOSUSRE

None declared.

AUTHOR CONTRIBUTIONS

Y.W., and Z.W. conducted experiments in VSMC and contributed to experimental design and manuscript writing; E.T. conducted vessel contraction studies and contributed to manuscript writing; J. T., J. L., and J. P. performed telemetry; H. L. participated in KO characterization; S.Q. carried out castration and ovariectomy; T.W., F.C. J.S., and S.H. carried out human vessel studies; J.W. initiated and guided the project, and participated in experimental design and manuscript writing.

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Tables

Table 1.

Gpr30 siRNA sequences

Gene	Sense sequence	antisense sequence
Gpr30	5’-GCUCUGUUAAUCUAACGAUCAGACUUA-3’	5’-CGAGACAAUUAGAUUGCUAGUCUGA-3’
	5’- UUGAGAGUAUGACAUAGCUUAGCCCAC-3’	5’- AACUCUCAUACUGUAUCGAAUCGGG-3’
	5’-AGAUCCAUUUACAGCUCGCUGUUCCCA -3’	5’-TCUAGGUAAAUGUCGAGCGACAAGG-3’

Figures and Legends

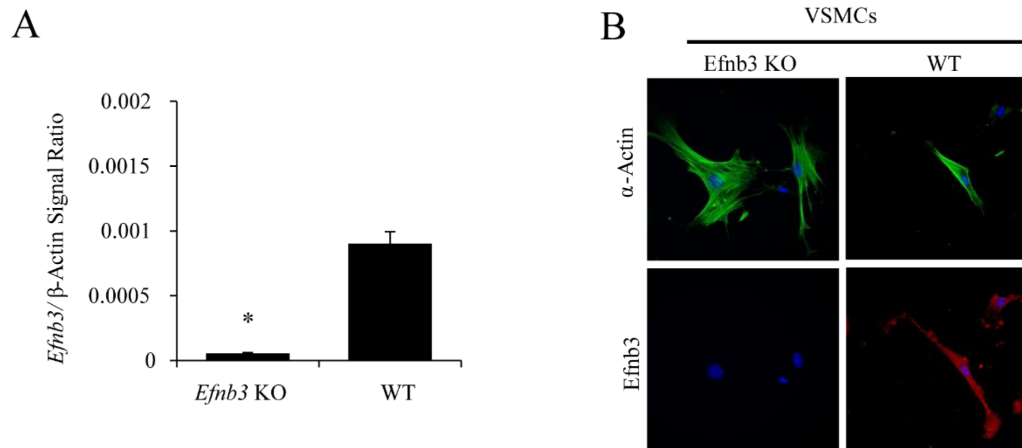

Figure 1

Figure 1. Efnb3 deletion in vascular cells from Efnb3 KO mice

The experiments were conducted twice, and data from representative experiments are reported.

A. Efnb3 mRNA deletion in mesenteric arteries of Efnb3 KO mice

Efnb3 mRNA expression in mesenteric arteries from WT and *Efnb3* KO mice was measured by RT-qPCR with *β-actin* mRNA as internal control. Samples in RT-qPCR were in triplicate, and means $\pm$ SEM of *Efnb3* signal/β-actin signal ratios are reported.

B. EFNB3 deletion in VSMC

WT and EFNB3 KO VSMC cultured for 4-5 days were stained with mouse anti-human α-actin mAb (in pseudo-green color) followed by Rhodamine-conjugated goat anti-mouse IgG Ab, and goat anti-mouse-EFNB3 Ab followed by Rhodamine-conjugated bovine anti-goat IgG Ab (in pseudo-red color). Nuclei were identified by DAPI staining.

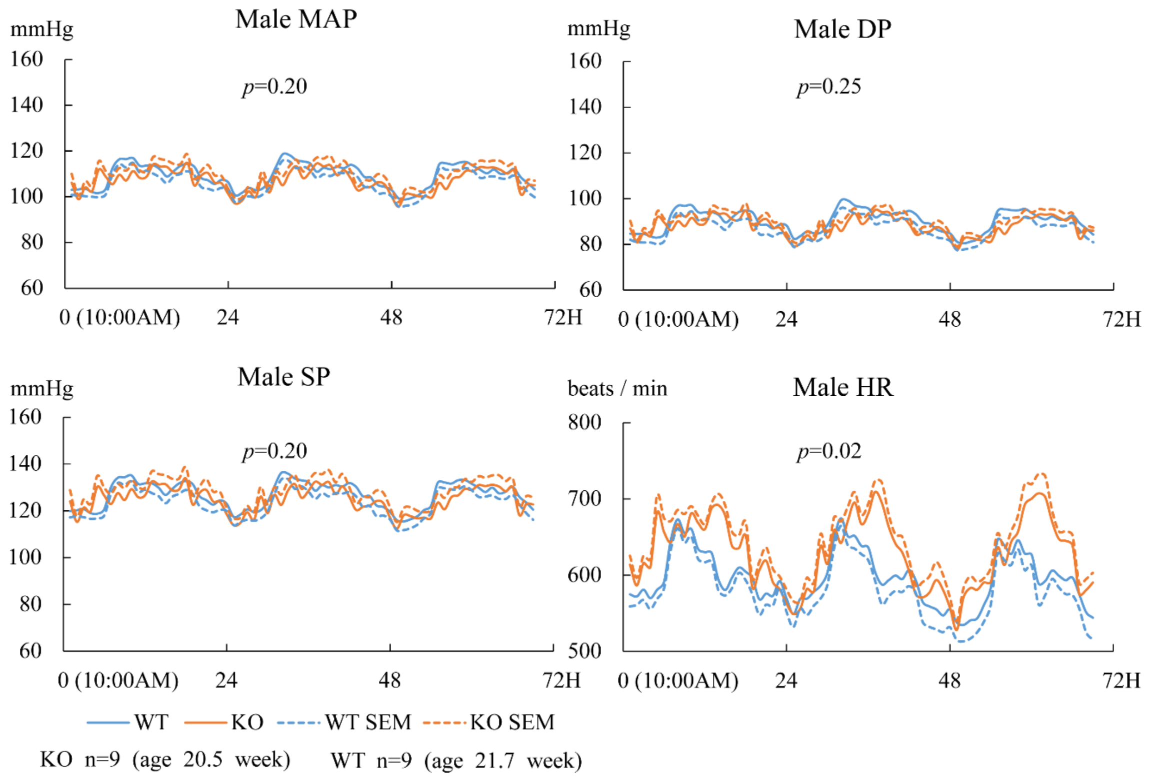

Figure 2A

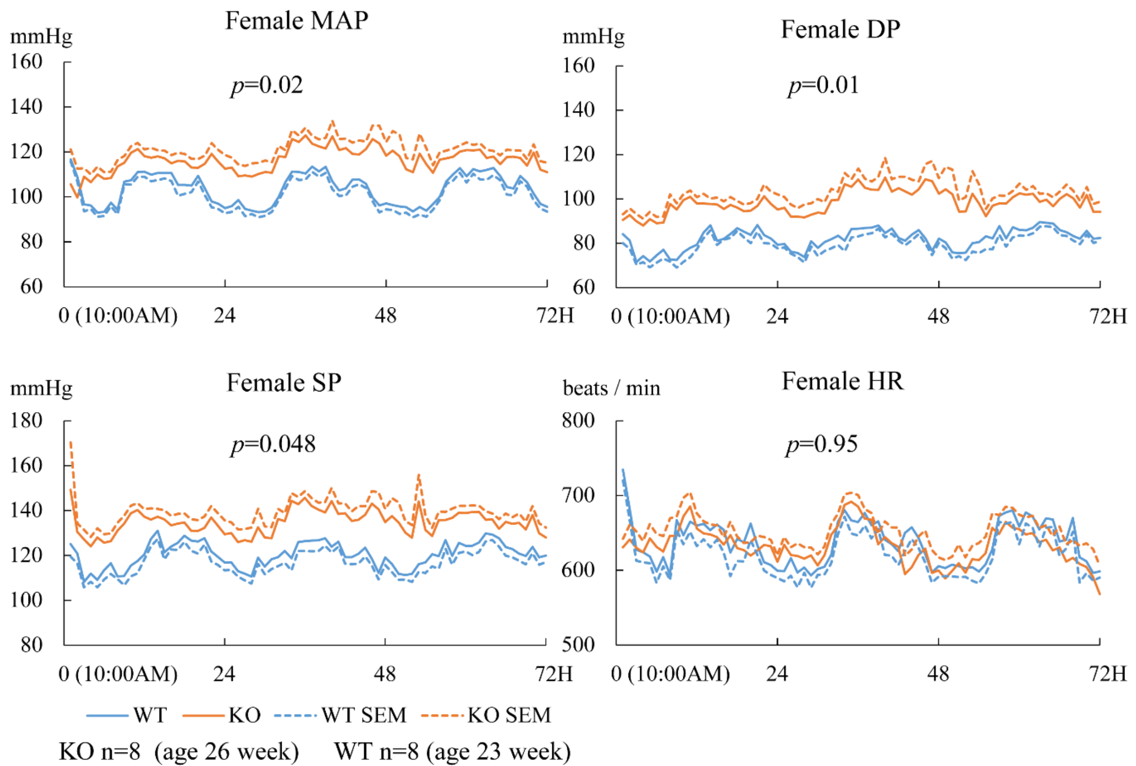

Figure 2B

Figure 2. Increased BP in female Efnb3 KO mice

BP and HR of male (A) and female (B) KO and WT mice were measured by radiotelemetry starting at least 7 days after transmitter implantation. Number per group and the mean ages of the mice at the time of BP measurement are indicated. The BP and HR of all mice were measured for 72 h. Means ± SEM of hourly BP and HR are plotted. MAP: mean arterial pressure; SP: systolic pressure; DP: diastolic pressure; HR: heart rate. The 72 hourly values were used for statistical analysis (ANOVA). *P*-values are indicated.

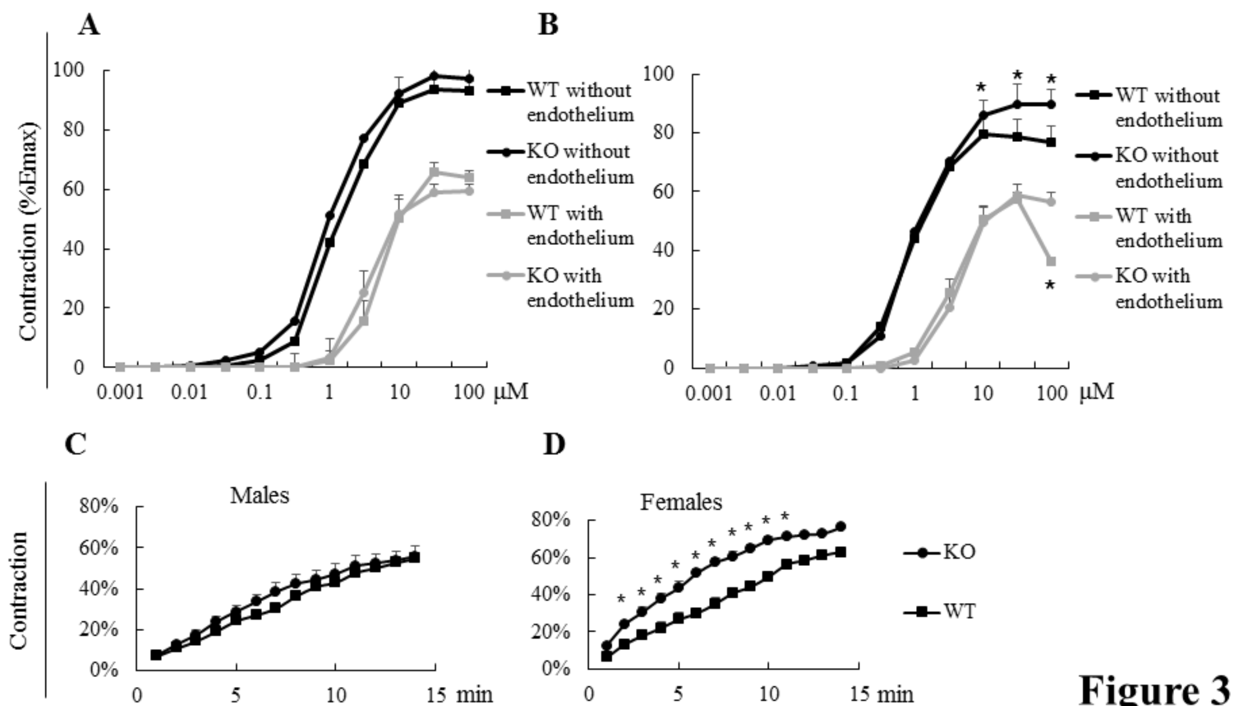

Figure 3. Female but not male VSMC contractility is augmented by EFNB3 deletion

All experiments were conducted 3 times independently. Data from representative experiments are presented.

A and B. Contractility of mesenteric arteries from Efnb3 KO mice

Segments of the third-order branch of the mesenteric artery with or without endothelium from male (A) or female (B) KO and WT mice were stimulated with PE. A single cumulative concentration-response curve to PE (1 nmlo/L to 100 μmol/L) was obtained. Maximal tension (Emax) was determined by challenging the vessels with physiological saline containing 127 mmol/L KCl. Vessel contractility is expressed as the percentage of Emax. Data from 3 pairs of mice (WT versus KO; tested independently in 3 separate days) per group were pooled, and means + SEM are reported. Statistically significant differences were assessed by Student's t test. *: $p<0.05$.

C and D. Increased contractility of VSMCs from female but not male Efnb3 KO mice

VSMCs were isolated from the mesenteric arteries and aorta of male (C) or female (D) WT and KO mice, and cultured for 4 days. They were then stimulated with 20 μM PE at 37°C and imaged every min for 15 min. Means $\pm$ SEM of percentage contraction of more than 15 cells per group are reported. The experiment was conducted 3 times, using 3 pairs of WT and KO mice in 3 different days, and data from a representative one are shown. Statistically significant differences were assessed by Student's *t* test. *: $p<0.05$.

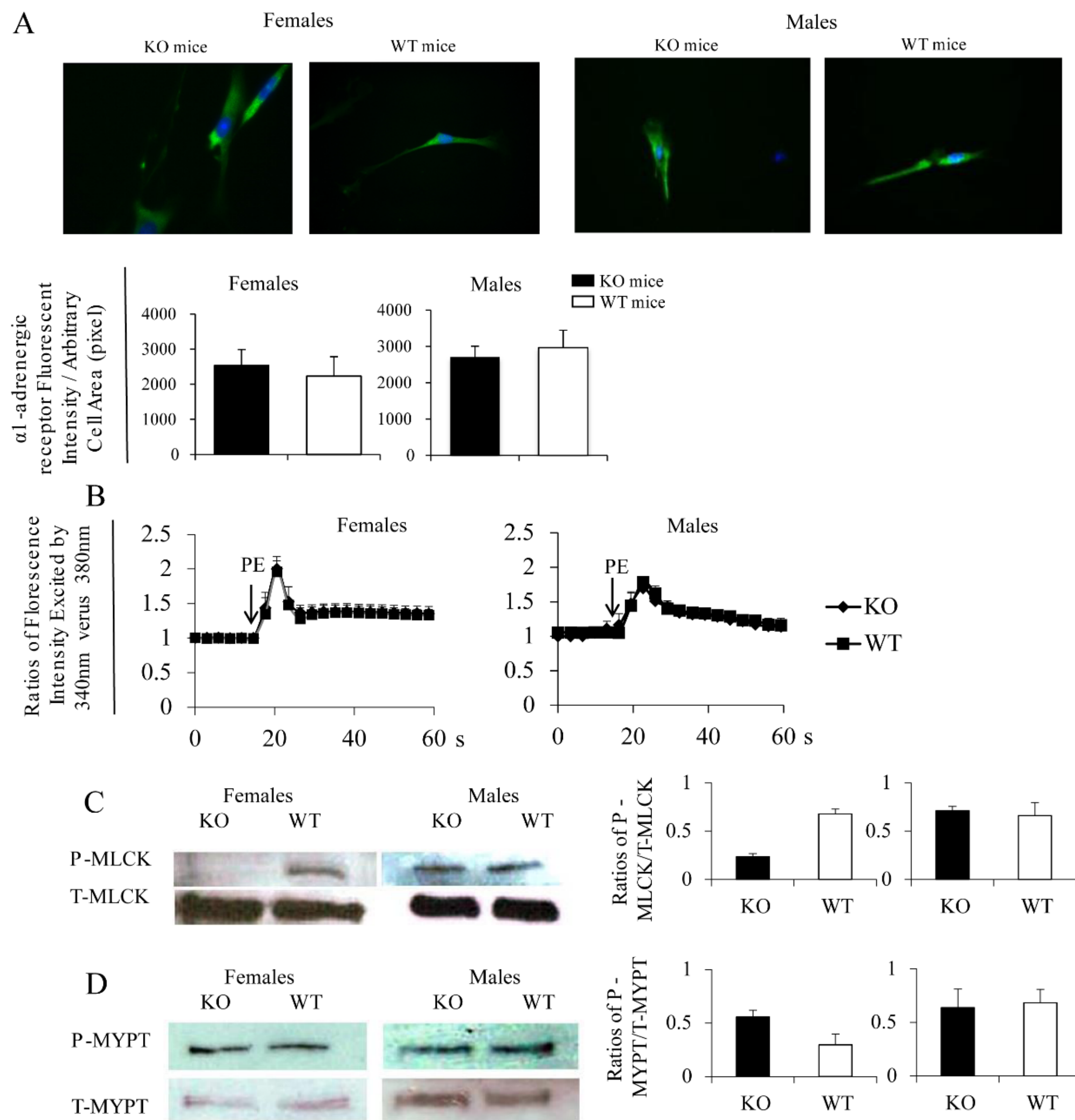

Figure 4. Signalling events related to contractility of KO and WT VSMCs

A. Normal α1-AR expression in Efnb3 KO VSMCs according to immunofluorescence microscopy

VSMCs from female (left panel) and male (right panel) *Efnb3* KO or WT mice were cultured for 4 days, then stained with Ab against α1-AR and α actin. For each experiment, more than 15 α-actin-positive cells were randomly selected, and their total α1-AR immunofluorescence intensity and cell size were measured. The upper panels are the micrographs of α1-AR

immunofluorescent staining. The lower panels present means + SEM of α1-AR fluorescence intensity per unit of arbitrary cell area (pixel) of all cells examined in a group of a representative experiment. The data were analyzed by paired Student's *t* test, but no significant difference was found. The experiment was performed three times independently and data of a representative one are shown.

B. Normal Ca^{++} flux in VSMCs from Efnb3 KO mice

VSMCs from male (right panel) and female (left panel) *Efnb3* KO or WT mice were cultured for 4 days and loaded with Fura2. They were then placed in HBSS at 37ºC and stimulated with PE. Arrows indicate the time points at which PE (20 μM) 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 1 min. The experiments were conducted 3 times independently. Means + SEM of the ratio of more than 15 randomly selected VSMC of a representative experiment are illustrated. No statistical significant difference between the KO and WT groups are found according to ANOVA.

C-D. Phosphorylation of MLCK and MTPT in VSMCs from KO and WT mice

VSMC from female and male KO and WT mice were cultured for 4 days, then stimulated with 20 μM PE for 3 s and immediately lysed. Total (T-) and phosphorylated (P-) MLCK (C) and MYPT (D) were analyzed by immunoblotting, which was conducted twice. Immunoblotting from representative experiments are illustrated. Data from densitometry of all the immunoblots are presented as bar graphs with mean + SD shown.

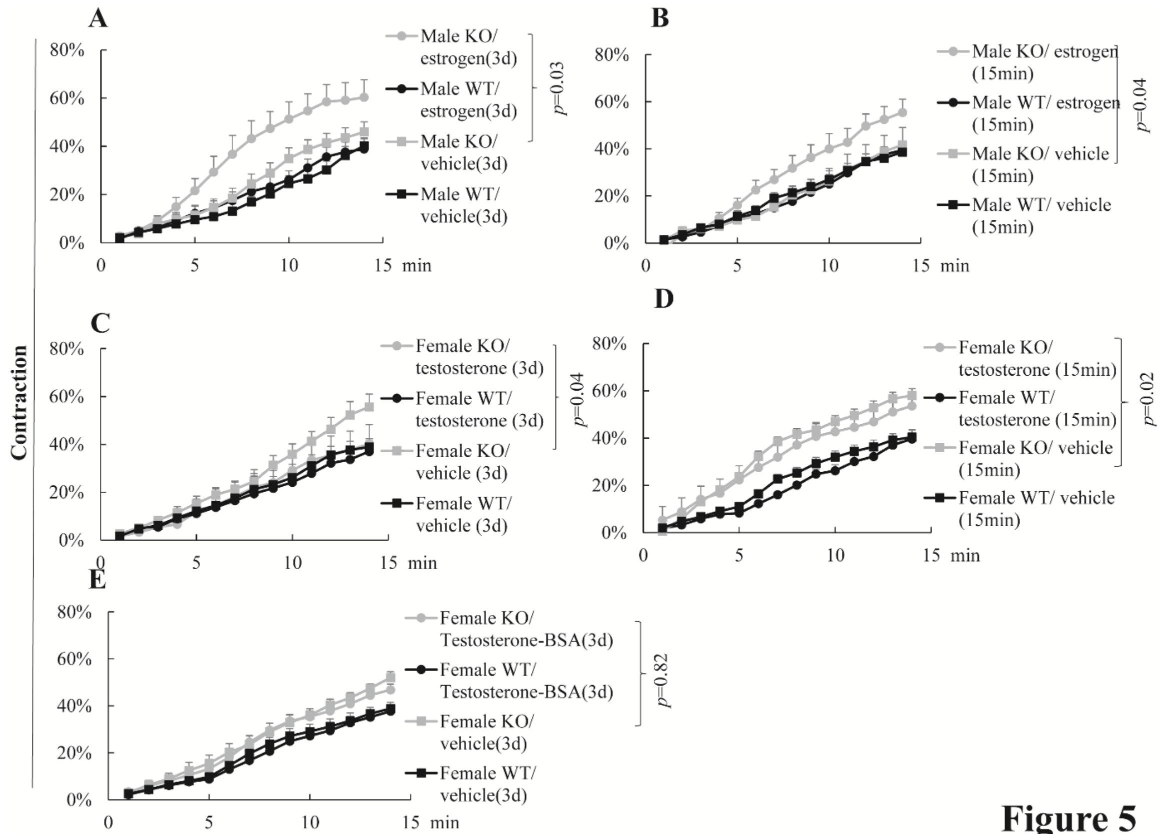

Figure 5

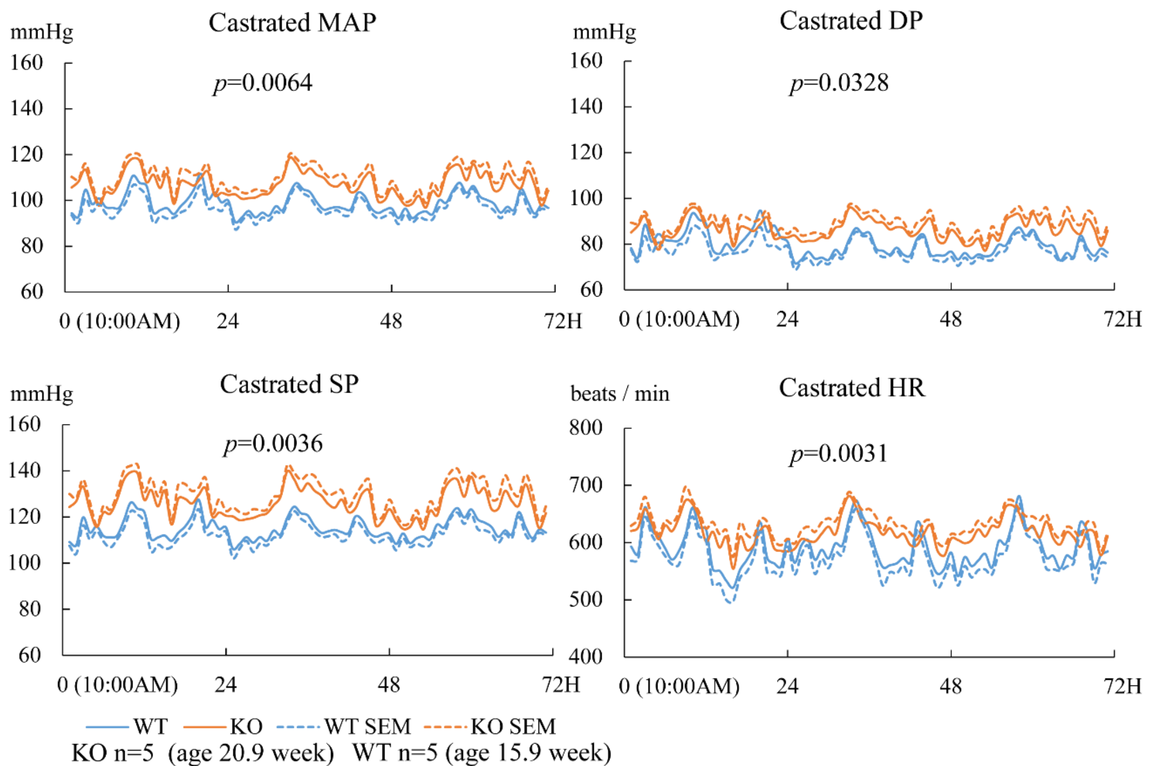

Figure 5F

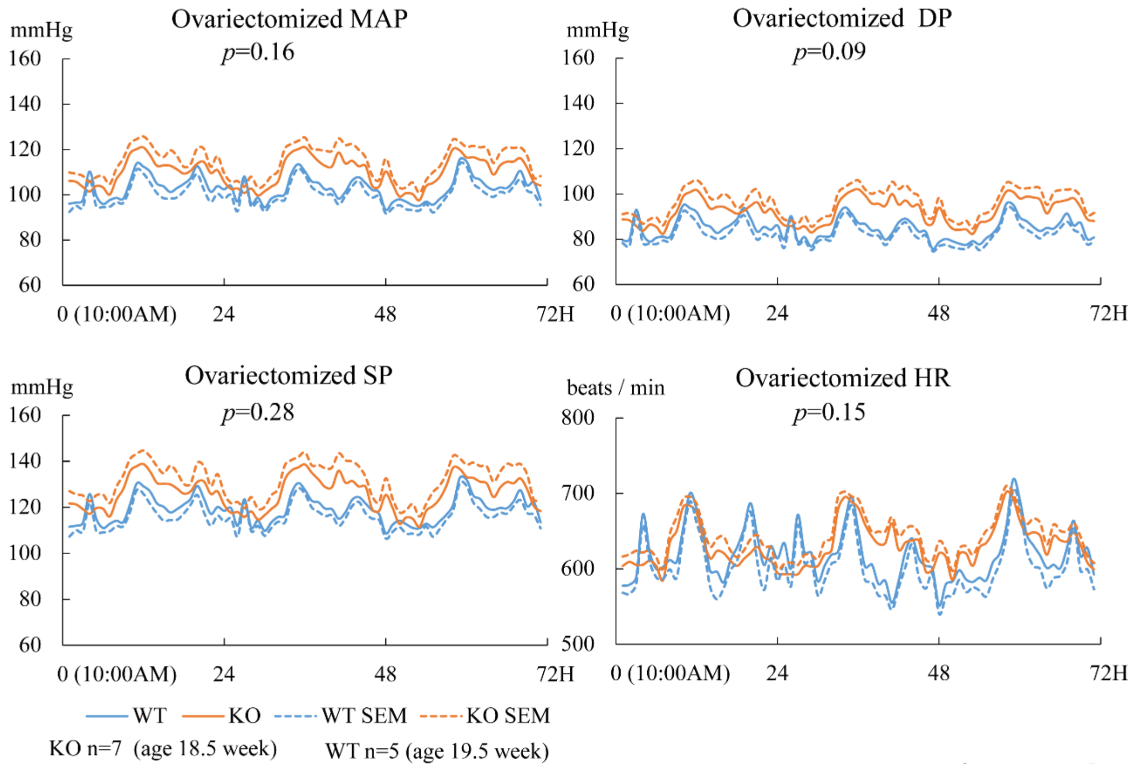

Figure 5. Estrogen enhances but testosterone suppresses and VSMC contractility and hence BP in the absence of EFNB3

VSMCs were cultured in medium containing charcoal-stripped FCS for 3-4 days. Means + SEM of percentage contraction of more than 15 cells per group are registered. Statistically significant differences were assessed by ANOVA followed by *post hoc* examination. P-values between groups with significant differences are indicated. All experiments were conducted 3 times independently. Data from representative experiments are presented.

A. Long-term estrogen treatment augments contractility of VSMCs from KO but not WT mice

VSMCs from WT and KO males were cultured for 3-4 days in the presence of 17β-estradiol (100 pg/ml) or vehicle, and then stimulated with PE (20 μM). Cell contractility was registered.

B. Short-term estrogen treatment augments contractility of VSMCs from KO but not WT male mice

VSMCs from male KO or WT mice were cultured for 3-4 days and then stimulated with 17β-estradiol (100 pg/ml) or vehicle for 15 min. In the same last 15 min of culture, PE (20 μM) was also added and VSMC contractility was recorded.

C. Long-term testosterone treatment suppresses contractility of VSMCs from female KO but not WT mice

VSMC from WT and KO females were cultured for 3-4 days in the presence of 5α-dihydrotestosterone (6.49 ng/ml) or vehicle, and then stimulated with PE (20 μM). VSMC contractility was recorded.

D. Short-term testosterone treatment has no effect on contractility of VSMCs from female KO or WT mice

VSMCs from female KO or WT mice were cultured for 3-4 days and then stimulated with 5α-dihydrotestosterone (6.49 ng/ml) or vehicle for 15 min. In the last 15 min of culture, PE (20 μM) was also added and VSMC contractility was recorded.

E. Cell membrane-impermeable testosterone-3-(O-carboxymethyl)-oxime-BSA has not effect on contractility of VSMCs from female KO or WT male mice

VSMCs from female KO or WT mice were cultured for 3-4 days in the presence of cell membrane-impermeable testosterone-3-(O-carboxymethyl)-oxime-BSA (1.1 μg/ml) and then stimulated with PE (20 μM). VSMC contractility was recorded.

F and G. Castrated male KO mice are hypertensive and ovariectomized female KO mice are normotensive

BP and HR of castrated male (F) and ovariectomized female (G) KO and WT mice were measured by radiotelemetry as described in Figure 2. Telemetry transmitters were implanted in the mice, and after at least 1 month, they were castrated or ovariectomized. Telemetry was conducted 3 weeks after gonadectomy. Number per group and the mean ages of the mice at the time of BP measurement are indicated. The BP and HR of all mice were measured for 72 h. The 72 hourly values were used for statistical analysis (ANOVA). *P*-values are indicated.

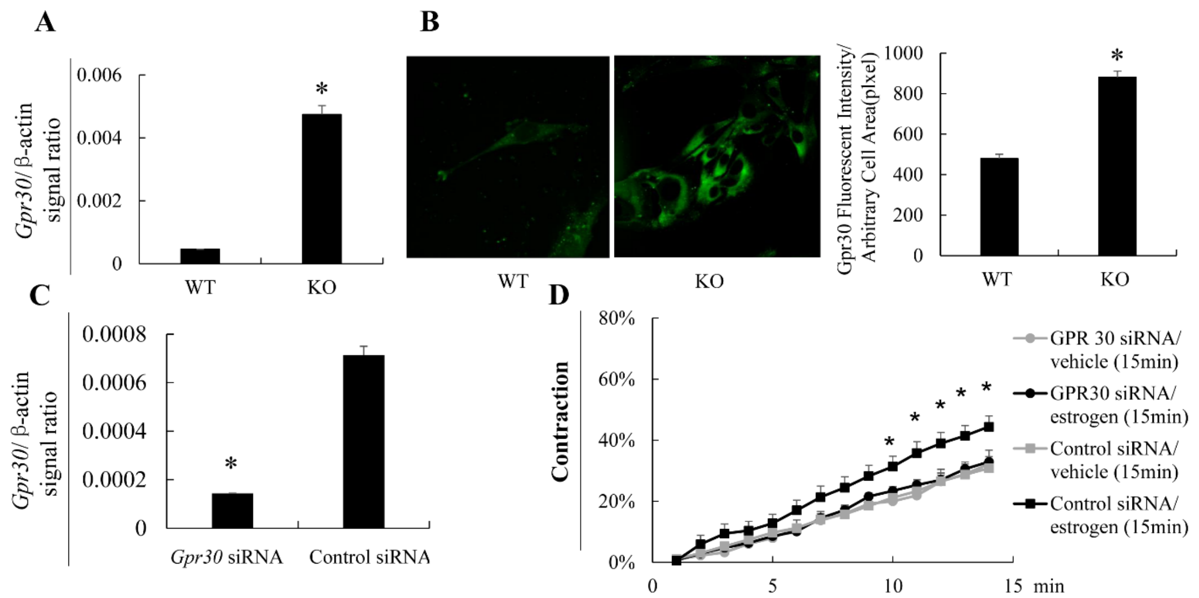

Figure 6. EFNB3 deletion enhances GPR30 expression and results in increased VSMC contractility in the presence of estrogen

A. EFNB3 KO VSMCs present enhanced Gpr30 mRNA expression according to RT-qPCR

VSMCs from male KO or WT mice were cultured in medium containing charcoal-stripped FCS for 4 days, and their *Gpr30* mRNA levels were measured by RT-qPCR. The samples were in

triplicate. Data from 3 independent experiments are pooled and mean + SD of signal ratios of *Gpr30* versus β-actin are shown. The data were analyzed by Student's *t* test. *: $p<0.05$.

B. EFNB3 KO VSMCs present enhanced GPR30 protein expression according to immunfluoscence

VSMCs from male KO or WT mice were prepared as described in A, and their GPR30 protein expression was detected by immunofluorescence. Micrographs are shown at left. For each experiment, more than 15 α-actin-positive cells per type of VSMCs were randomly selected, and their total immunofluorescence intensity and cell size were recorded AxioVision software. Means + SEM of fluorescence intensity per unit of arbitrary cell area (pixel) of all cells examined in a given type of VSMCs of 3 independent experiments are pooled and summarized in a bar graph at right. The data were analyzed by Student's *t* test. *: $p<0.05$.

C and D. GPR30 knockdown by siRNA abolishes estrogen-promoted KO VSMC contractility

VSMCs from male KO and WT mice were cultured for 40 h in regular medium containing charcoal-stripped FCS. They were then transfected with a mix of 3 pairs of *Gpr30* siRNAs (for each pair, the final concentration was 10 nM), or control siRNA, and cultured in medium free of antibiotics, as described before (Z. Wu et al., 2012). Forty eight hours after the siRNA transfection, VSMCs were treated with 17β-estradiol (100 pg/ml) or vehicle for 15 min. In the same last 15 min of culture, PE (20 μM) was also added and VSMC contractility was assessed (D). The cells were then harvested, their *Gpr30* mRNA levels were determined by RT-qPCR (C). The experiment was conducted 3 times. Data (mean + SEM) of a representative one are shown. *: $p<0.05$ (Student's *t* test) between cells treated with *Gpr30* siRNA plus estrogen versus control siRNA plus estrogen.

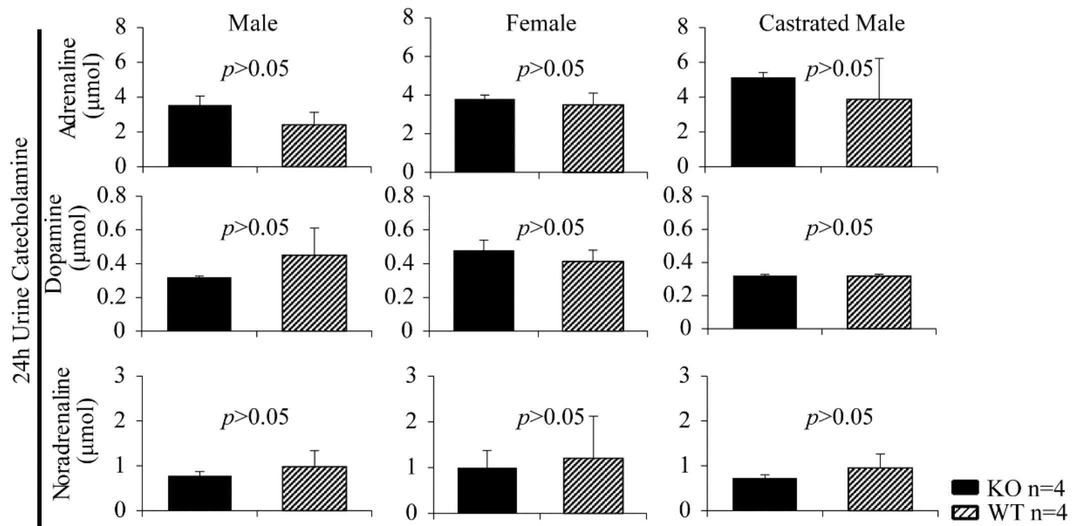

Figure 7. Normal 24-hour urinary catecholamine levels in Efnb3 KO mice

Male and female *Efnb3* KO and WT mice were placed in metabolic cages. Urine was collected during a 24-h fasting period. Urinary catecholamines were measured by competitive enzyme immunoassay. Means + SEM of hormones excreted during the 24-h period and mouse number per group (n) are presented. No statistically significant differences were found between KO and their WT counterparts (unpaired Student's *t* test).

CHAPTER 5 ARTICLE-4

TITLE: The role GRIP1 and EFNB3 signaling in blood pressure control and vascular smooth muscle cell contractility

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Short title: The role of GRIP1 and EFNB3 in blood pressure regulation

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Abstract

Several EPHB receptor tyrosine kinases and their ligands, ephrinBs (EFNBs), are involved in blood pressure regulation in animal models. We selected 522 single nucleotide polymorphisms (SNPs) within the genes of *EPHB6, EFNB2, EFNB3* and *GRIP1* in the EPH/EFN signalling system to query the International Blood Pressure Consortium dataset. A SNP within the *GRIP1* gene presented a *p*-value of 0.000389, approaching the critical *p*-value of 0.000302, for association with diastolic blood pressure of 60,396 individuals. According to echocardiography, we found that *Efnb3* gene knockout mice showed enhanced constriction in the carotid arteries. *In vitro* studies revealed that in mouse vascular smooth muscle cells, siRNA knockdown of GRIP1, which is in the EFNB3 reverse signalling pathway, resulted in increased contractility of these cells. These data suggest that molecules in the EPHB/EFNB signalling pathways, specifically EFNB3 and GRIP1, are involved blood pressure regulation.

Key words: GRIP1, EFNB3, blood pressure, SNPs

Introduction

EPH kinases are the largest family of receptor tyrosine kinases. They are divided into A and B subfamilies according to sequence homology("Unified nomenclature for Eph family receptors and their ligands, the ephrins. Eph Nomenclature Committee," 1997). Ephrins (EFNs), which are also cell surface molecules, are ligands of EPHs. EFNs are classified as A and B subfamilies. EFNAs attach to the cell surface through glycosylphosphatidylinositol anchoring, whereas EFNBs attach through transmembrane sequences(Flanagan & Vanderhaeghen, 1998; Pasquale, 2008; Wilkinson, 2000). Interactions among EPHs and EFNs are promiscuous but, in general, EPHA members interface preferentially with EFNAs, and EPHBs with EFNBs(Flanagan & Vanderhaeghen, 1998; Pasquale, 2008; Wilkinson, 2000). Such redundancy suggests that these kinases are crucial in various biological contexts. EFNs can stimulate EPH receptors, and this is called forward signalling. Interestingly, EPHs are also capable of stimulating EFNs which then transmit signalling reversely into cells, a phenomenon known as reverse signalling.

EPHs and EFNs are expressed in many tissues and organs. They play important roles in the central nervous system(Flanagan & Vanderhaeghen, 1998; Wilkinson, 2000), immune system(Luo, Charpentier, et al., 2011; Luo, Wan, Wu, & Wu, 2001; Luo, Wu, et al., 2011; Luo, Yu, Tremblay, & Wu, 2004; Luo, Yu, Wu, & Wu, 2002; J. Wu & Luo, 2005; Yu, Luo, Wu, & Wu, 2003a, 2003b, 2004; Yu, Mao, Wu, Luo, & Wu, 2006), digestive system (Batlle et al., 2002), bone metabolism (Davy, Bush, & Soriano, 2006; Zhao et al., 2006), angiogenesis (H. U. Wang, Chen, & Anderson, 1998)and other processes (Dravis et al., 2007; Hashimoto et al., 2007; Konstantinova et al., 2007).

We recently reported that EPHB6, in concert with sex hormones, is crucial in VSMC contraction and blood pressure (BP) regulation(Luo et al., 2012). *Ephb6* gene knockout *(*KO) mice after

castration manifest higher blood pressure that their wild type (WT) counterparts(Luo et al., 2012). Vascular smooth muscle cells (VSMC) are a target tissue through which EPHB6 exerts its effect on BP control. Since EPHB6 and all its major ligands of the EFNB family, *i.e.,* EFNB1, EFNB2 and EFNB3, are expressed in VSMCs(Luo et al., 2012), there is a molecular framework for their function in these cells. We showed that while solid-phase recombinant EPHB6 reduces VSMC contraction in response to phenylephrine (PE) stimulation, solid-phase anti-EPHB6 antibody (Ab) does not(Luo et al., 2012), indicating that reverse signalling from EPHB6 to EFNBs but not forward signalling from EFNBs to EPHB6 is responsible for dampening VSMC contractility. In support of this notion, we have shown that deletion of EFNB1, a ligand of EPHB6, results in a hypertensive phenotype in mice(Z. Wu et al., 2012). Therefore, we identified EPHB6 and, by logical extension and with certain experimental evidence, its ligands (EFNBs) as novel BP regulatory factors in animal models.

In order to establish the relevance of our findings in mice to human blood pressure regulation, we selected 522 single nucleotide polymorphisms (SNPs) within the genes of *EPHB6, EFNB2, EFNB3* and *GRIP1*, which is a key molecule in EFNB reverse signalling, to query the International Blood Pressure Consortium (IBPC) dataset, which contains SNP information on 69,395 individuals. We found that a SNP in the *GRIP1* gene approached statistical significance for association with diastolic blood pressure in humans. Additional animal studies revealed roles of EFNB3 and GRIP1in regulating arterial tone and VSMC contractility, providing phenotypic evidence supporting the genetic findings in humans.

Materials and Methods

Meta-analysis of SNPs in EPHB and EFNB genes and a related gene GRIP1 for association with BP phenotypes in humans

The *p*-values for association with diastolic pressure (DP) and systolic pressure (SP) were calculated for a total of 528 SNPs found within the regions of 4 genes (*EPHB6, EFNB2, EFNB3* and *GRIP1)* and within 10 kb 5' and 3' of these genes, employing the LocusZoom genome browser(http://csg.sph.umich.edu/locuszoom/) to query the IBPC dataset(International Consortium for Blood Pressure Genome-Wide Association et al., 2011), which contains SNP information on 69,395 individuals of European ancestry in 29 general population-based cohorts.

These 528 SNPs represented 166 independent linkage disequilibrium (LD) blocks, as determined by the Tagger program(de Bakker et al., 2005) on the HapMap website(http://hapmap.ncbi.nlm.nih.gov.). Table 1A lists the genes and regions in which the SNPs are located. Query of 166 independent LD blocks resulted in a Bonferroni-corrected critical *p*-value of 0.0003012 for a given BP phenotype (systolic or diastolic pressure). Looking for the best results between systolic or diastolic pressure phenotypes would require a lower critical *p*-value; however, correcting for 2 X 166 tests (p=0.000151) would be overly conservative as these 2 measurements are not independent.

Efnb3 KO mice

Efnb3 KO mice were produced by Regeneron Pharmaceuticals, as described previously(Kullander et al., 2001), and generously provided to us. They had been backcrossed to the C57BL/6 background for more than 10 generations. Age- and gender-matched wild type (WT) littermate mice served as controls and are referred to as WT mice. The mice were housed

in ambient temperature (22°C) with 12-h light and dark cycles. For all the *in vivo* experiments, mice were housed one per cage.

Echocardiography

Transthoracic echocardiography was undertaken in mice lightly anesthetized with isoflurane. The experiments were always conduced around noon time. The oestrus cycles of the females were not monitored. Their carotid vessels and heart were imaged with a high-resolution ultrasound biomicroscope (Vevo770; Visualsonics, Toronto, ON, Canada) equipped with a 100% bandwidth 30-MHz central frequency transducer (RMV-707, 12.7 mm focal length, 6 mm aperture). Lateral and axial resolutions with this probe are ~115 μm and ~55 μm, respectively(Zhou et al., 2004). Preheated ultrasound transmission gel (Aquasonic 100, Parker Laboratories, Orange, NJ, USA) was placed on regions of interest to provide acoustic coupling medium between the transducers and animals. The left and right common carotid arteries were imaged longitudinally in B mode to guide recordings of Doppler time-varying flow velocities for 2 s. Doppler sample volume was positioned 1-2 mm prior to the carotid bifurcation at a 60° angle. Acquired angle-corrected Doppler data were analyzed to measure the mean Pourcelot index (PI) over 10 consecutive cardiac cycles, according to Stoyanova et al.(Stoyanova, Trudel, Felfly, Garcia, & Cloutier, 2007). The PI is a dimensionless echocardiographic parameter that characterizes vascular hemodynamics downstream of a measurement point. It depends on both arterial compliance and downstream vascular resistance.

The heart was also imaged in B mode via the parasternal long-axis view to assess aortic hemodynamics and cardiac output (CO). The M-mode sampling line was positioned perpendicularly to the ascending aorta, 0.5-1.5 mm downstream of the aortic valve, and time-

varying tracings tracked changes in aortic diameter (AoD). Mean AoD was assessed over 5 consecutive cardiac cycles. Doppler velocity waveforms were then recorded in the ascending aorta by positioning sampling volume at the exact same location where M-mode tracings were obtained. The envelope of angle-corrected (60°) Doppler tracings was delineated manually to compute the velocity time integral (VTI), which was averaged over 10 cardiac cycles. Assuming a parabolic velocity profile in the ascending aorta(Stoyanova et al., 2007), stroke volume (SV) was calculated (in ml) as $\frac{1}{2}\,(AoD / 2)^2 \times \pi \times VTI$, and CO (in ml/min) was estimated as SV × heart rate (HR), where HR was mean HR of the animals.

Left ventricle (LV) dimensions (in mm) at end-systole and end-diastole were finally assessed to quantify fractional shortening, ejection fraction and LV mass. B-mode parasternal long-axis viewing guided the capture of M-mode tracings through the anterior and posterior LV walls at the level of the papillary muscle. For each mouse, LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), LV end-diastolic posterior wall thickness (LVEDPW) and intra-ventricular septum dimension at end-diastole (IVSED) were quantified, and LV mass ascertained in mg with the following equation(Pollick, Hale, & Kloner, 1995; Yang et al., 1999):

$$\text{LV mass (mg)} = 1.055 \times [(LVEDD + LVEDPW + IVSED) - LVEDD]$$

A visual illustration of methods of mouse cardioechography can be found in a video publication by Respress and Wehren(Respress & Wehrens, 2010).

VSMC isolation

Mouse VSMC were isolated from aortic and mesenteric arteries, including their secondary branches as described before(Luo et al., 2012; Z. Wu et al., 2012).

siRNA transfection and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The sequences of *Grip1, Pdg-rgs3, and Disheveled* siRNA and siRNA transfection procedures are described in our previous publication(Luo et al., 2012). Their mRNA levels in VSMCs were measured by RT-qPCR; the primer sequences and qPCR cycling program are detailed before(Luo et al., 2012). -actin mRNA served as internal control. Samples were tested in triplicate, and the data were expressed as signal ratios of test gene mRNA/α-actin mRNA.

VSMC contractility

VSMC contractility was measured as described before(Luo et al., 2012; Z. Wu et al., 2012). Briefly, the cells were cultured in wells coated with goat anti-mouse EFNB3 Ab (sc-7281, Santa Cruz Biotechnology, Dallas, Texas) or control goat IgG (2 μg/ml during coating). After 3-4 days, they were stimulated with PE (20 μmol/L), and photographed continuously for 15 min at a rate of 1 picture per min. Fifteen or more cells were randomly selected, and their length was measured at each time point with Zeiss Axiovision software. Percentage contraction was calculated as follows:

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

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Committee on Animal Care of Research Centre, Centre hospitalier de l'Université de Montréal (Permit Number: 4I14033JWs). All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

Results

Association of SNPs in the EPHB/EFNB system with BP phenotypes

Our previous studies(Luo et al., 2012; Z. Wu et al., 2012) along with some of our unpublished observations indicate that molecules in the EPHB and EFNB families (*e.g.,* EPHB6, EFNB1, EFNB2, and EFNB3) and a certain adaptor protein (GRIP1) within their signalling pathways are novel factors that can modulate BP in mice. The relevance of these molecules in human BP regulation was investigated. The IBPC conducted a meta-analysis of genome-wide association scanning (GWAS) in 69,395 individuals of European ancestry in 29 cohorts from European and North American countries(International Consortium for Blood Pressure Genome-Wide Association et al., 2011). Two and a half million genotyped or imputed SNPs were tested for their association with SP and DP in these individuals. We queried the results of this meta-analysis for association of 528 SNPs in *EPHB6, EFNB2, EFNB3* and *GRIP1* genes with systolic or diastolic pressure in these individuals. *EFNB1* was not included in the analysis because it is an X-linked gene, and its SNP information is not available in the IBPC dataset. The *p*-values of these SNPs for their association with systolic or diastolic pressure are illustrated in Supplementary Figure 1 (S. Fig. 1). Table 1B summarizes the names of SNPs with the most significant association and their *p*-values. The minimum observed *p*-value for any association (0.000389) was for the association of SNP rs1495496 located within the *GRIP1* gene with diastolic pressure. This value approaches the critical *p*-value of 0.000302. For the other 3 genes analyzed*, i.e.,* EPHB6, EFNB2 and EFNB3, the minimum *p*-values of their SNPs did not approach the critical *p*-value. The implications of these findings are elaborated in the Discussion.

Table 1. Association of SNPs in the EPHB6/EFNB system with BP phenotypes in 69,396 human subjects

A. Locations of 4 genes for which 528 SNPs were tested

	Build 36		Build 37	
Gene	Location	Size (kb)	Location	size (kb)
EPHB6	chr7: 142,252,914 - 142,288,969	36.06	chr 7: 142,542,792 - 142,578,847	36.06
EFNB2	chr13: 105,930,097-105,995,338	65.24	chr 13: 107,132,079-107,197,388	65.31
EFNB3	chr17: 7,539,245-7,565,418	26.17	chr 17: 7,598,520-7,624,693	26.17
GRIP1	chr12: 65,019,066-65,369,020	349.95	chr 12: 66,731,211-67,082,925	351.71

B. Minimum p-values from IBPC meta-analysis among SNPs examined within EPHB6, EFNB2, EFNB3 and GRIP1 genes

Gene	Number of SNPs examined	LD Blocks r2=0.8	Diastolic Pressure		Systolic Pressure	
			SNP	p-value	SNP	p-value
EPHB6	48	4	rs1009848	0.0373	rs2299557	0.404

EFNB2	54	24	rs2057408	0.077	rs9520087	0.218
EFNB3	6	6	rs3744258	0.201	rs3744258	0.191
GRIP1	420	132	rs1495496	0.000389	rs1495496	0.00144
Total	528	166	Critical p value: 0.05/166 = 0.0003012			

Small artery resistance in EFNB3 KO mice in vivo

EFNB3 is an EPHB6 ligand, and its function might contribute to the BP phenotype observed in *Ephb6* KO mice(Luo et al., 2012). We assessed several BP related parameters in *Efnb3* KO mice (called KO mice hereafter) using echocardiography.

BP is a function of CO, blood volume and blood vessel flow resistance. Echocardiography was employed to examine CO, the PI (a parameter reflecting blood vessel flow resistance) of carotid arteries, and LV mass in live KO and WT mice. Due to technical limitations, we were not able to conduct echocardiography in smaller arteries. CO in male (Table 2A) and female (Table 2B) KO mice was comparable to that in WT controls, indicating that this parameter, which could affect BP, is not under the influence of EFNB3. However, the left carotid PI in female KO (Table 2B) but not male KO (Table 2A) mice was significantly higher than that of WT counterparts ($p=0.0218$), reflecting heightened resistance of these small arteries. LV mass increased significantly in female but not in male KO mice, compared to their WT counterparts ($p=0.0397$, Table 2B). This hypertrophy might be the result of augmented cardiac workload in female KO mice to overcome the heightened blood flow resistance of their small arteries.

Table 2. Echocardiographic analysis of CO, carotid artery resistance and left ventricle mass of Efnb3 KO and WT mice

BP-related echocardiographic parameters of individual *Efnb3* KO and WT mice are reported. Means $\pm$ SD are shown at the end of each group, and *p* values (unpaired one-way Student's *t* test) are indicated at the bottom of Tables 2A and 2B. PI: Pourcelot index; LV: left ventricle.

A. Echocardiographic parameters of male Efnb3 KO mice

Mouse Type	Age	Cardiac output(ml/min)	Left Carotid PI	Right Carotid PI	LV Mass(mg)
KO male	15wk	14.65	0.75	0.72	103.7
KO male	15wk	21.46	0.87	0.68	125.8
KO male	15wk	24.79	0.73	0.78	154.1
KO male	15wk	17.23	0.74	0.70	91.7
KO male	15wk	14.93	0.78	0.78	89.7
KO male	14wk	18.2	0.73	0.72	133.6
KO mean		**18.54**	**0.77**	**0.73**	**116.4**
SD		3.939	0.0533	0.041	25.65
WT male	14wk	21.5	0.83	0.84	116.0
WT male	15wk	15.33	0.74	0.70	140.5
WT male	15wk	23.52	0.75	0.77	115.6
WT male	15wk	18.21	0.73	0.72	133.6
WT male	14wk	31.85	0.77	0.86	115.0

WT male	14wk	28.57	0.77	0.81	145.1
WT male	14wk	24.19	0.74	0.76	152.7
WT male	14wk	30.95	0.79	0.83	175.6
WT mean		**24.265**	**0.77**	**0.78**	**126.4**
SD		5.92	0.030	0.047	12.57
p value (t test)		0.0637	0.9075	0.08200	0.4967

B. Echocardiographic parameters of female Efnb3 KO mice

Mouse Type	Age	Cardiac output(ml/min)	Left Carotid PI	Right Carotid PI	LV Mass(mg)
KO female	15wk	21.89	0.75	0.78	112.3
KO female	15wk	16.29	0.77	0.8	92.5
KO female	14wk	20.16	0.81	0.81	121.1
KO female	15wk	27.57	0.76	0.77	143.3
KO female	15wk	20.54	0.94	0.76	130.5
KO female	14wk	27.71	0.75	0.73	134.3
KO female	15wk	19.13	0.8	0.77	135.8
KO female	14wk	18.38	0.81	0.82	122
KO mean		**21.45**	**0.79**	**0.78**	**123.9**

SD		4.153	0.062	0.029	16.04
WT female	14wk	33.49	0.72	0.72	109
WT female	14wk	18.92	0.69	0.67	118.1
WT female	14wk	29.03	0.71	0.8	110.9
WT female	14wk	28.06	0.81	0.79	95.9
WT female	14wk	15.96	0.68	0.76	114.6
WT female	14wk	22.46	0.7	0.74	72.2
WT mean		**24.65**	**0.71**	**0.74**	**93.4**
SD		6.664	0.047	0.048	29.98
***p* value(t test)**		0.289	**0.0218**	0.132	**0.0397**

GRIP1 knockdown by siRNA cancelled the effect of solid phase anti-EFNB3 Ab on the contractility of WT VSMCs

The echocardiography results suggest that the default function of EFNB3 is vessel relaxation, and hence the EFNB3 KO carotid artery manifested increased resistance. To prove this at the cellular level, we triggered EFNB3 reverse signalling by incubating WT VSMCs in wells coated with anti-EFNB3 Ab and tested their contractility. Such solid phase anti-EFNB3 Ab serves as an agonist to EFNB3, because it acts at the bottom of the cells and will not block putative EFNB3/EPH interaction which might exist at the vertical side of neighboring VSMCs. Further, during to the low density used in culture, such fraternal cell interaction is rare. A similar strategy of solid phase anti-EFNB1 Ab as an agonist to trigger EFNB1 reverse signalling in VSMCs has

been previously published (Z. Wu et al., 2012). As shown in Figures 1A and 1B, WT VSMCs crosslinked with anti-EFNB3 Ab presented significantly reduced contractility, compared to those cultured in wells coated with normal goat IgG. On the other hand, KO VSMC contractility was not affected by anti-EFNB3 crosslinking, as expected, because they have no EFNB3 expression (data not shown). These results corroborate that of echocardiography and confirm that EFNB3 reverse signalling leads to reduced VSMC contractility. The solid phase anti-EFNB3 Ab indeed triggered signalling in VSMCs in that their ERK phosphorylation upon PE stimulation was increased(S. Fig. 2).

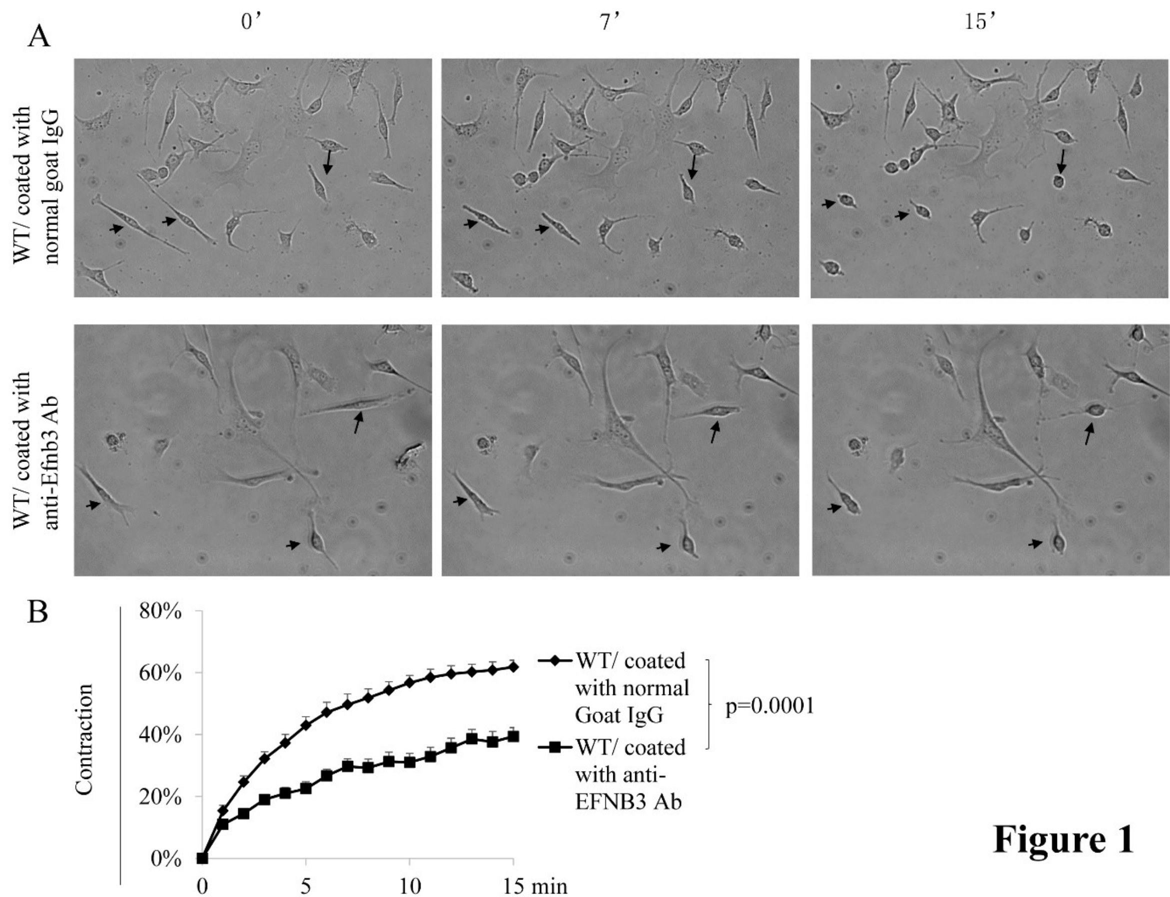

Figure 1. Crosslinking EFNB3 on VSMCs results in decreased contractility

WT VSMCs were cultured in wells coated with goat anti-mouse EFNB3 Ab or control goat IgG (2 µg/ml during coating) for 4 days The cells were then stimulated with PE (20 µmol/L), and their percentage contraction was recorded by microscopy.

A. Micrographs showing the contraction of WT VSMCs in the presence or absence of solid phase anti-EFNB3

Upper row: WT VSMCs cultured in wells coated with control goat IgG (20 g/ml for coating). Lower row: WT VSMCs cultured in wells coated with goat anti-mouse EFNB3 Ab. The images were taken at 0, 7 and 15 min after PE stimulation. Arrows mark the same cells in each row at different time points, to show the shortening of the cells.

B. Reduced contractility of WT VSMCs cultured in the presence of solid phase anti-EFNB3 Ab

WT were cultured in wells coated with normal goat IgG or goat anti-mouse EFNB3 Ab for 4 days. The cells were then stimulated with PE, and were imaged at one frame per min for 15 min. Means $\pm$ SD of the percentage contraction during a 15-min period are reported. The percentage contraction is calculated as follows.

% contraction = length of cells at a given time point/length of the cells at time 0.

The data were analyzed with one-way ANOVA, and *p*-value is indicated. The experiment in this figure was repeated three times, and representative data are shown.

EFNBs had no enzymatic activity. However, EFNBs engage adaptor proteins to link their intracellular tails to various signalling pathways. *Grip1* siRNA was used to knock down the expression of this adaptor protein in the WT and KO VSMCs, as it is known to associate with EFNBs(Bruckner et al., 1999; Lu, Sun, Klein, & Flanagan, 2001; Tanaka, Kamo, Ota, &

Sugimura, 2003). The effectiveness of mRNA knockdown was verified by RT-qPCR (Fig. 2A). EFNB3 expression in the WT VSMCs was not affected by the knockdown (S. Fig. 3). WT VSMCs were cultured in wells coated with anti-EFNB3 Ab to invoke EFNB3 reverse signalling, and KO VSMCs were used as controls. In WT VSMCs, such treatment (Fig. 2B) dampened VSMC contractility of WT VSMCs transfected with control siRNA, but such dampening effect was reversed by *Grip1* siRNA (Fig. 2B). KO VSMCs cultured in wells coated with anti-EFNB3 Abs had a higher contraction, as they had no EFNB3 and were not affected by anti-EFNB3-evoked reverse signalling, which could reduce contractility. As expected, *Grip1* siRNA knockdown did not affect KO VSMC contractility, because GRIP1 was not utilized by the KO VSMCs. The effect of GRIP1 knockdown on the augmentation of WT VSMC contractility depended on the existence of EFNB3 reverse signalling, as *Grip1* siRNA had no effect on the contractility of WT VSMCs cultured in wells without anti-EFNB3 Ab coating (Fig. 2C). DISHEVELLED AND PDZ-RGS3 are two other proteins capable of associating with the EFNB3 intracellular tail [36,37]. However, their knockdown by siRNA had no effect on WT VSMC contractility (Fig. 3), suggesting that they were not involved in EFNB3 reverse signalling in VSMCs.

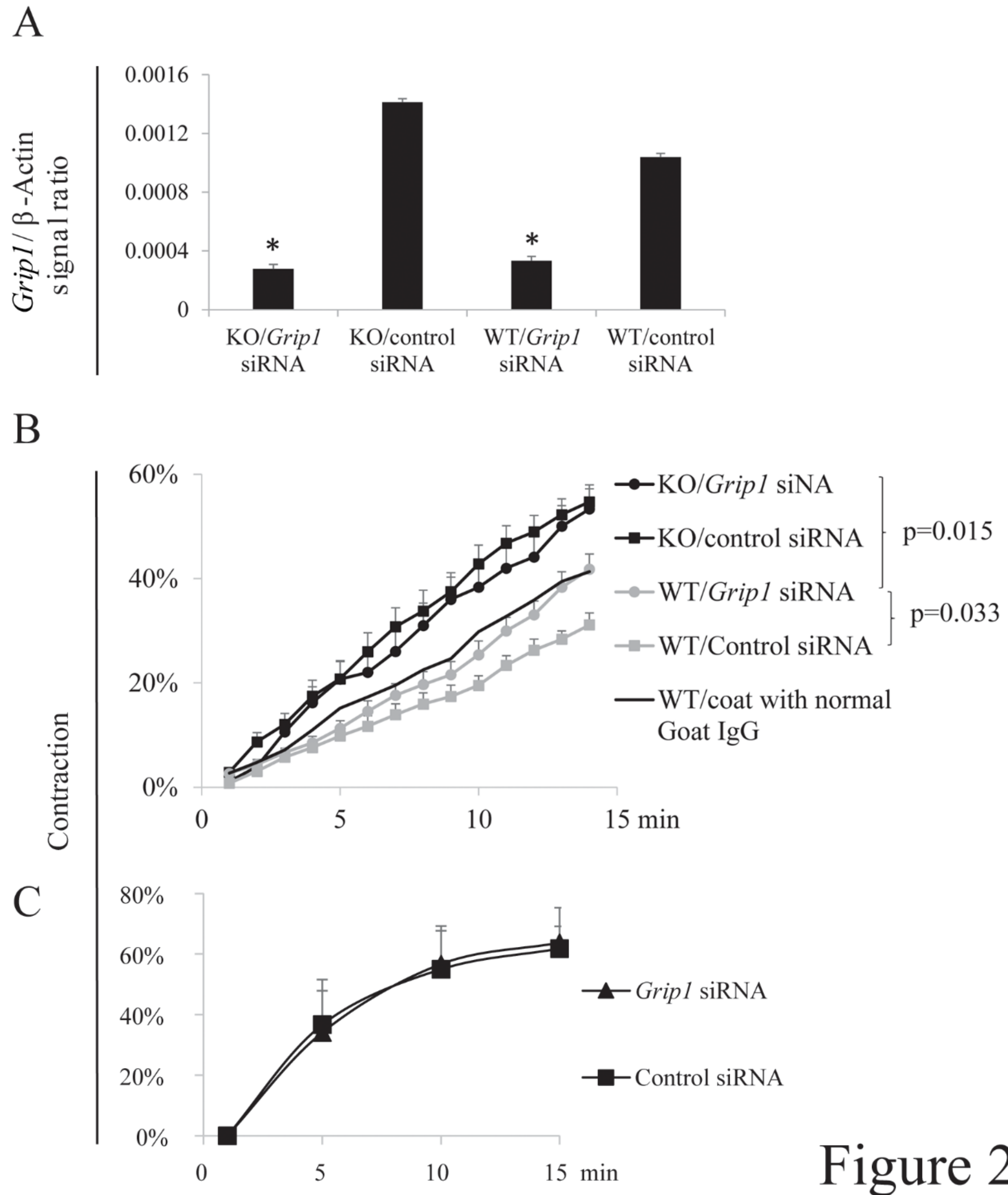

Figure 2. GRIP1 in the EFNB3 reverse signaling pathway in VSMCs

Experiments in this figure were repeated more than twice, and representative data are shown.

A. Effective mRNA knockdown of Grip1 by siRNA

Cultured WT VSMC were transfected with siRNAs of a particular gene or control siRNA, as indicated. After 24-h culture, the cells were harvested and the mRNA expression of each gene was determined by RT-qPCR. The data are expressed as means $\pm$ SD of the ratios of the target gene signal versus the α-actin signal. The data were analyzed by one-way Student's *t* test. *: $p < 0.05$.

B. GRIP1 knockdown by siRNAs reverses the effect of solid-phase anti-EFNB3 Ab on reducing VSMC contractility

VSMC from WT and KO mice were cultured in wells coated with goat anti-mouse EFNB3 Ab (2 µg/ml during coating). After 2 days, they were transfected with siRNAs targeting *Grip1*, or with control siRNA. On day 4 of culture, they were stimulated with PE (20 µmol/L), and their percentage contraction was recorded. Means $\pm$ SD of the percentage are reported. The thin line represents mean contractility of VSMCs cultured in well without coating of anti-EFNB3 Ab as an additional control; for better viewing, SD was not added to the line. The data were analyzed with one-way ANOVA followed by *ad hoc* analysis, and *p*-values of significant difference are indicated.

C. Grip1 siRNA in the absence of EFNB3 reversing signaling had no effect on VSMC contractility

WT VSMCs were cultured in plain wells without Ab coating. They were transfected with *Grip1* or control siRNA and then stimulated with PE as described in A. Mean $\pm$ SD of percentage contraction are shown. The data were analyzed with one-way ANOVA but not statistically significant difference between the test and control groups is found.

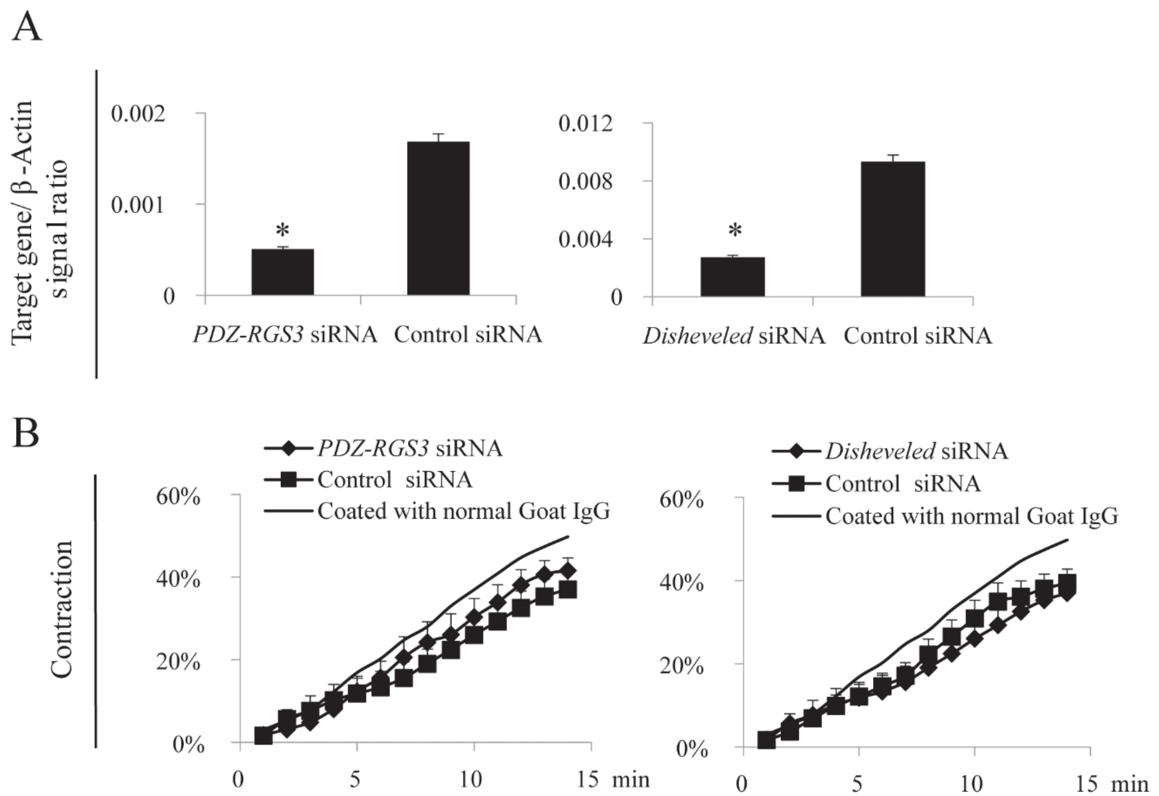

Figure 3

Figure 3. DISHEVELLED AND PDZ-RGS3 ARE NOT in the EFNB3 reverse signaling pathway in VSMCs

Experiments in this figure were repeated more than twice, and representative data are shown.

A. Effective mRNA knockdown of Disheveled and PDZ-RGS3 by siRNA

Cultured WT VSMC were transfected with siRNAs of *Disheveled and PDZ-RGS3* as described in Figure 2. The mRNA expression of each gene was determined by RT-qPCR. The data are expressed as means ± SD of the ratios of the target gene signal versus the -actin signal. The data were analyzed by Student's *t* test. *: $p < 0.05$.

B. Dishevelled and PDZ-RGS3 knockdown by siRNAs had no effect on WT VSMC contractility

Contractility of VSMCs from WT mice were assessed in the presence of EFNB3 reverse signalling and DISHEVELLED/PDZ-RGS3 knockdown, as described in Figure 2B. The data were analyzed with one-way ANOVA but no significant difference was observed.

These results corroborate the finding of the human genetic study, indicating that GRIP1 is involved in EFNB3 reverse signalling and VSMC contractility regulation, and it is hence a factor modulating blood pressure.

Discussion

EPHs and EFNs perform critical functions in many tissues and organs. In our previous studies, we found that *Ephb6* KO and *Efnb1* KO mice have augmented BP(Luo et al., 2012; Z. Wu et al., 2012). In the current investigation, we provided additional human genetic evidence showing that the mutations in GRIP1, a molecule in the EPHB6/EFNB signalling pathway and an adaptor protein capable of binding to EFNBs, were associated with human blood pressure phenotype. We also provided mouse experimental data showing that the default function of EFNB3, one of the ligands of EPHB6, was to reduce arterial tone, and such function depended on signalling via GRIP1, corroborating the human genetic findings.

As of today, no genes in the EPHB/EFNB family have been identified as hypertension risk genes in several large-scale GWAS(Adeyemo et al., 2009; Guo et al., 2012; Hiura et al., 2010; International Consortium for Blood Pressure Genome-Wide Association et al., 2011; Levy et al., 2009; Org et al., 2009; Padmanabhan et al., 2010; Slavin, Feng, Schnell, Zhu, & Elston, 2011; Wellcome Trust Case Control, 2007). There are a couple of possible reasons. 1) The

contribution of genes in the EPHB/EFNB family to the BP phenotype might be relatively small, and the possible association is rendered undetectable due to heavy statistical penalties of multiple testing in GWAS. 2) In order to reduce the effect of type 2 error resulting from multiple-testing correction in genome-wide association studies, the IBPC study has assembled very large samples by combining cohorts from many sub-studies. The hypothesis is that increasing sample size will increase power, allowing the decrease in both type 1 and type 2 errors. However, *p*-value is controlled by both sample size and effect size (odds ratio) and, of the two, a slight decrease in effect size has a more dramatic impact on *p*-value. Assembly of large disparate cohorts does increase sample size but it also introduces increased heterogeneity, both phenotypic and genetic. Such heterogeneity may reduce the average effect size.

We conducted a more focused query of the IBPC dataset, using only 4 genes in the EPHB/EFNB signalling pathway, and discovered that a SNP, rs1495496, located between exons 22 and 23 of GRIP1, had a *p*-value of 0.000389 for its association with DP, which approaches the Bonferroni corrected critical *p*-value 0.000302. This finding is very promising since Bonferroni correction by itself was deemed to be quite conservative. Considering our observation that EPHBs/EFNBs likely influence vessel tone in mice, the possible association of this SNP in *GRIP1* gene with DP seems logical. Why does only a SNP from *GRIP1* approach significance and not those from 3 *EPHB6/EFNBs* queried? GRIP1 is situated in a node of EPHB6, EFNB1 and EFNB3 reverse signalling pathway; it thus probably carries more weight than each individual EPHB or EFNB member and, hence, is easier to detect for its associations with BP phenotypes. Our recent published results(Luo et al., 2012; Y. Wang et al., 2015) and unpublished observations in animal models indicate that the effect of EPHBs and EFNBs on blood pressure regulation is not only sex-dependent, but also sex hormone level-dependent. It is possible that in a cohort stratified by

sex and sex hormone levels, more significant association of BP phenotypes with SNPs from *GRIP1* and *EPHB/EFNB* family members will be detected in humans, and this will fully establish the relevance of our findings from animal models to human blood regulation.

Our cardioechogrphy data using EFNB3 KO mice suggest a default sex/sex hormone-dependent vasolaxative role of EFNB3 in that its deletion led to increased blood flow resistance in the carotid artery of female KO but not male KO mice, compared to their WT counterparts. Such increased resistance is not due to developmental changes of the arteries in the KO mice, as we found no abnormalities in the aorta and mesenteric arteries in terms of histology and diameters (S. Figure 4).

Using telemetry, we have recently revealed that female but not male KO mice have increased BP, but after ovariectomy, the female KO BP returns to the normal range(Y et al.). This finding corroborates the data from cardioechography, suggesting that after EFNB3 deletion, increased vascular tone results in increased BP, but such effect is sex- and sex hormone level-dependent.

We demonstrated EFNB3 can modulate VSMC contractility by reverse signalling. EFNB3 is a transmembrane protein without enzymatic activity in its intracellular tail. How does it regulate VSMC contractility? We have demonstrated that the association of EFNB3 with GRIP1 is critical for its function in regulating VSMC contractility. In VSMCs, EFNB3 might also associate with other so-far unidentified binding proteins. During reverse signaling, some of these proteins might interact with and modulate the functions of other pathways that control VSMC contractility, the MLCK pathway being one of them. The intermediate signalling molecules between EFNB3/GRIP1 and MLCK/MLC remain to be identified.

What is the possible mechanism by which estrogen in concert with EFNB3 modulates VSMC contractility? We have demonstrated that estrogen augments KO but not WT VSMC contractility, and this is mediated by its nongenomic effect via cell surface receptor GPR30. GRP30 can directly regulate ERK activity (Gros et al., 2011), which in turn controls VSMCs contractility (Bhattacharya & Roberts, 2003). As EFNB3 reverse signalling can activate ERK as shown in S. Figure 2, ERK or molecules in this category which modulates VSMC activity both in the presence or absence of EFNB3, can function as a node on which EFNB3 and GPR30 signalling pathways converge. This could be a possible mechanism by which EFNB3 and estrogen act in concert in regulating VSMC contractility.

Our research on the roles of EPHBs/EFNBs in VSMC contractility has revealed a group of previously unknown molecules capable of regulating vascular tone and blood pressure. Among this group of molecules, we have shown that the default function of EPHB6, EFNB1 and EFNB3 is to reduce vascular tone (Luo et al., 2012; Z. Wu et al., 2012; Y et al.), and thus their deletion leads to increased vessel contractility and blood pressure. Our most recent finding indicates that among the EPHBs and EFNBs, there are another group of molecules such as EPHB4 and possibly its major ligand EFNB2, whose default function is opposite, *i.e.*, to enhance the vascular tone (Y. Wang et al., 2015); consequently, their deletion lead to reduced blood pressure. These opposing forces of different member of EPHBs and EFNBs are like Yin and Yang and probably play a role for the fine tuning of the vascular tone. A better understanding of the physiological relevance and mechanisms of action of these molecules for their role in vascular contractility and blood pressure regulation will afford us a novel personalized therapeutic approach to blood pressure management. For example, female hypertensive patients with

EFNB3 mutations could be identified by genetic testing, and for this subpopulation of patients, avoidance of oral contraceptive/hormone replacement will reduce their hypertension risks.

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DISCLOSURES

The authors declare no conflict of interests.

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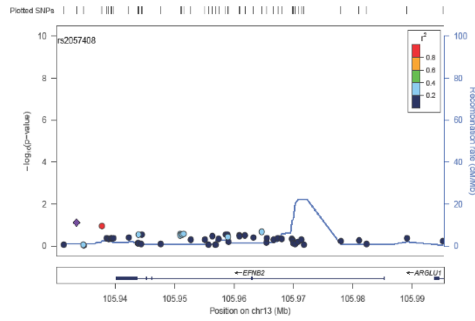
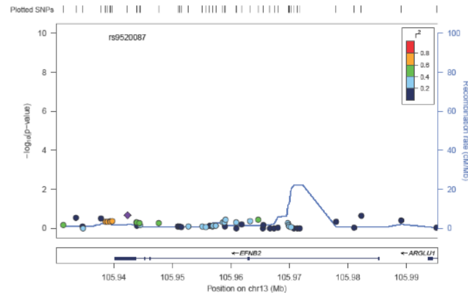
SUPPORTING INFORMATION

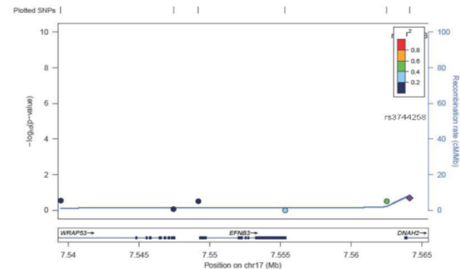
A. EFNB2 region for diastolic BP



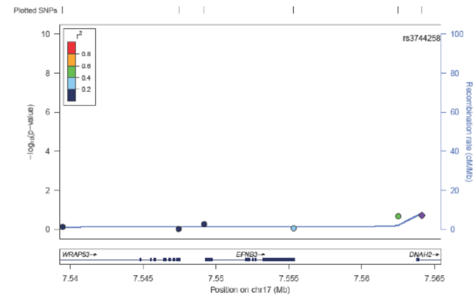
B. EFNB2 region for systolic BP



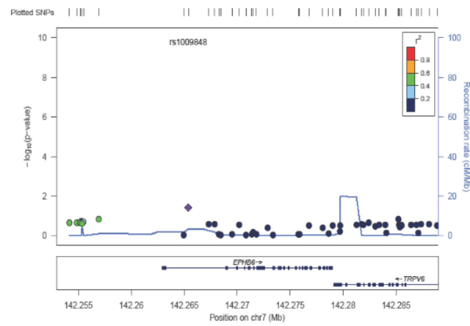
C. EFNB3 region for diastolic BP



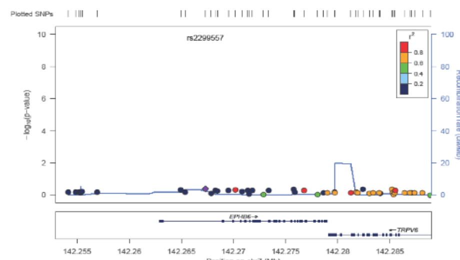
D. EFNB3 region for systolic BP



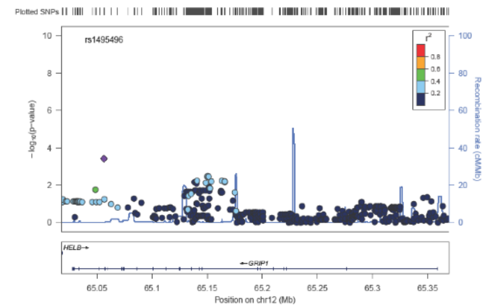
E. EPHB6 region for diastolic BP



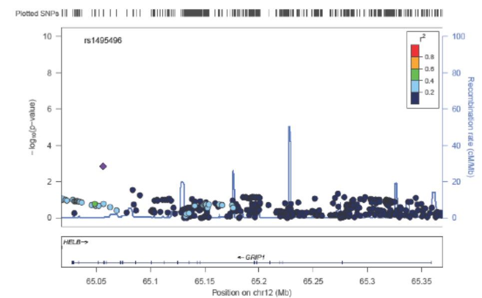
F. EPHB6 region for systolic BP



G. GRIP1 region for diastolic BP

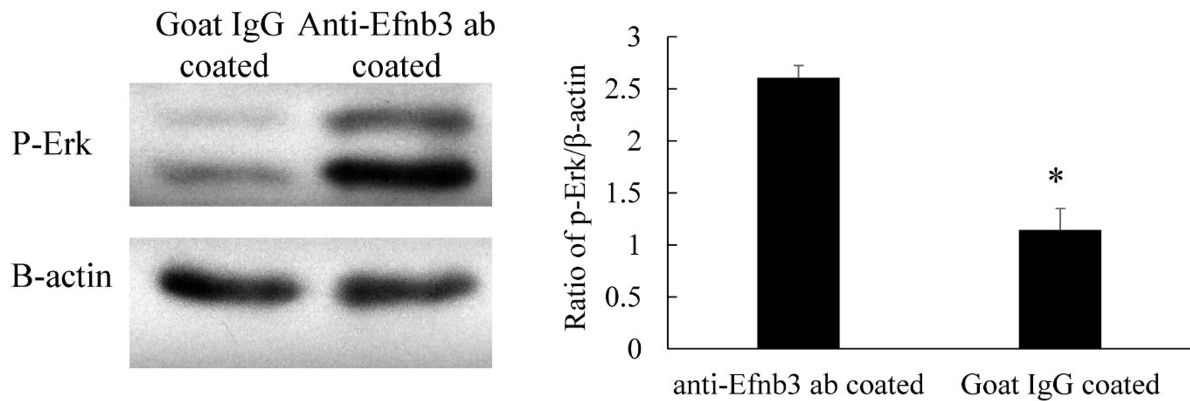


H. GRIP1 region for systolic BP



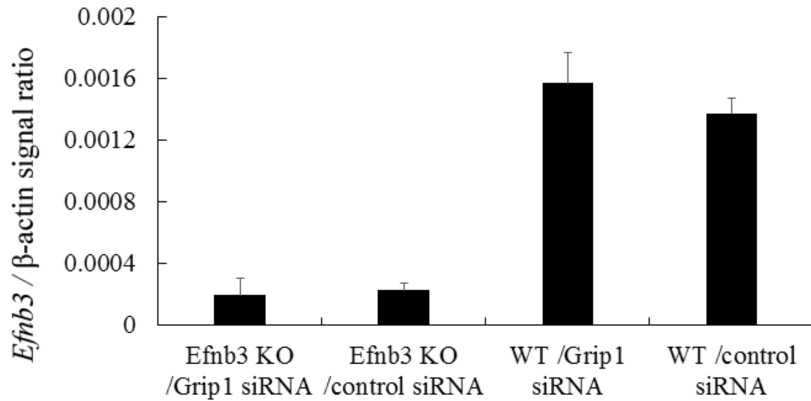
Supplementary Figure 1. LocusZoom plots of -$\log_{10}$ p-values (left hand vertical axis) from IBPC meta-analysis of specific queried genes

The positions of all SNPs in the query are indicated at the top of the plot as short, thin vertical lines, and as diamonds and circles in the plots. Diamonds represent reference SNPs with the highest -$\log_{10}$ p-value in the region, and their names are indicated above the diamonds. R^2 refers to LD between the reference SNP and respective SNPs within the region. Blue lines refer to recombination rates (right hand vertical axis) in centimorgans/megabase at each position. Thus, peaks in blue lines indicate low LD regions of high recombination rates or recombination hotspots.



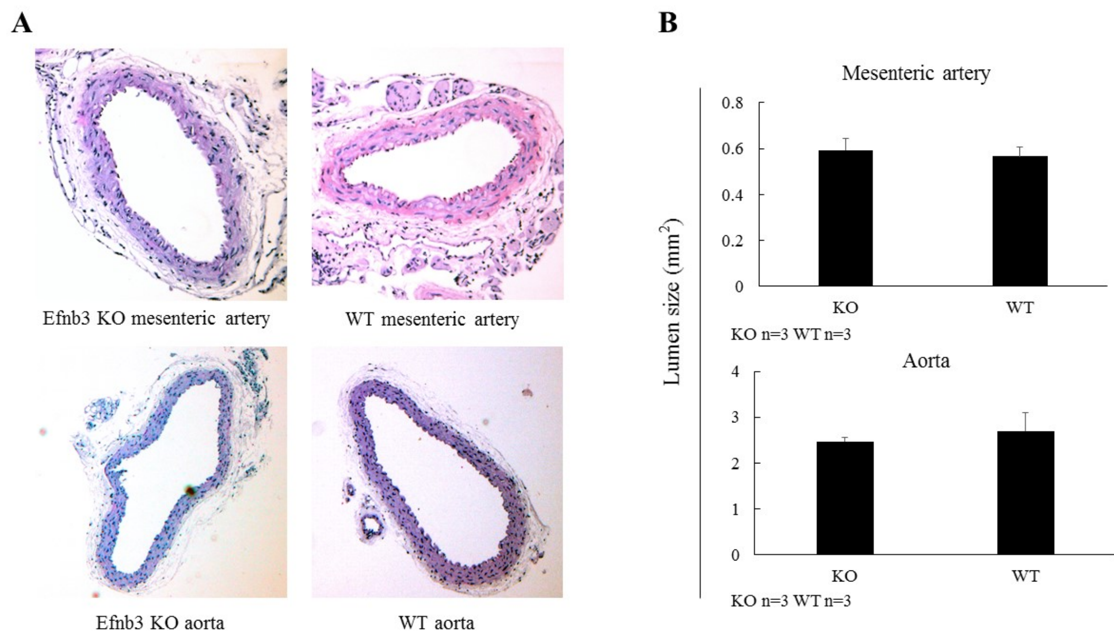
Supplementary Figure 2. Reduced ERK phosphorylation in WT VSMCs stimulated with solid phase anti-EFNB3 Ab

WT VSMCs were cultured in wells coated with anti-EFNB3 Ab, and then stimulated with PE, as described in Figure 1. The cells were harvested 3 sec after PE stimulation, and their ERK phosphorylation was determined by Western blotting. The experiment was conducted 3 times and a representative result is shown.



Supplementary Figure 3. Unaltered EFNB3 mRNA expression in WT VSMCs transfected with Grip1 siRNA

WT VSMCs were transfected with control and EFNB3 siRNA. After 48 h, their EFNB3 mRNA expression was determined by RT-qPCR. The mean + SD of the ratios between EFNB3 and -actin signals in two independent experiments are presented. No significant difference is observed between control and EFNB3 siRNA transfected samples (Student's *t*-test).



Supplementary Figure 4. Histology and lumen diameters of KO and WT vessels

A. HE-stained sections of the aorta, and mesenteric of WT and KO mice

B. Lumen sizes of WT and KO vessels

The lumen sizes of the aorta, and mesenteric artery of WT and KO mice were measured using Image J and mean + SD of data from 3 WT and 3 KO mice of similar age are presented. No significant difference is found (Student's *t* test).

CHAPTER 6 General Discussion

Yin and Yang of the novel BP regulation system

One of the first reports on EPH was published by Hirai et al. about 20 years ago (Hirai et al., 1987). Since then, many works have been carried out, studying the function of EPHs and their ligands EFNs. Recent studies have demonstrated that EPHs and EFNs have critical functions in many systems. Investigations on the roles of EPH/EFN in the cardiovascular system (except angiogenesis) are limited. Based on our studies, several types of EPHs and EFNs were discovered as being essential for BP regulation. The general findings are summarized in Table 1 below. While EPHB6, EFNB1 and EFNB3 deletion lead to increased BP, EFNB2 and EphB4 deletion results in a BP decrease. Moreover, such regulation is often sex hormone level-dependent.

Table 1.

The phenotypes of blood pressure in various EPHB and EFNB KO mice of different sexes

	EPHB4		EPHB6		EFNBb1		EFNB2		EFNB3	
	M*	F*	M	F	M	F	M	F	M	F
BP	↓*	–*	–	–	–	n/a*	↓	–	–	↑
HR	↑	↑*	↑	↑	–	n/a	–	↓	↑	–
Vasocontractility	↓	↑	↓	↑	↓	n/a	↓	–	–	↑

	EPHB4		EPHB6		EFNB1		EFNB2		EFNB3	
	C*	O*	C	O	C	O	C	O	C	O
BP	−	−	↑	↑	−	n/a	−	−	↑	−
HR	−	−	−	−	−	n/a	−	−	↑	−
Vasocontractility	n/a	n/a	↑	−	↑	n/a	n/a	n/a	−	−
Role in maintaining BP	EphB4 + testosteron → increase BP		EphB6 + testosteron →increase BP		EFNB1 + testosteron →reduce BP		EFNB2 + testosteron →increase BP		EFNB3 + estrogen →reduce BP	

*: F: female; M: male; C: castrated; O: ovariectomized; −: no difference between KO and WT; ↑: KO increased; ↓: KO decreased; n/a: not applicable (Efnb1 is an X-linked gene, and null mutation of Efnb1 causes embryonic lethality. So only male smMHC-Cre-Efnb1 KO mice could be generated.)

These functions of EPHBs and EFNBs are mainly mediated by VSMCs, based on our studies using smooth muscle-specific gene deletion and using isolated VSMCs. It is interesting to observe that different members of EPHBs and EFNBs have opposing effects in BP regulation. Such opposing forces, which may also be called Yin and Yang, are widely present in biological systems. The coexistence of positive and negative regulatory effects on VSMC contractility by different members of the EPH/EFN family likely controls homeostasis of vascular tone and consequently blood pressure. As EFN and EPH members are constitutively expressed on VSMCs and are engaged with each other constantly, they are probably not for rapid regulation of BP. The EPH and EFN signaling are more likely to achieve some fine-tuning of the vascular tone and help the individuals to adapt to the gradually changing environment. The physiological

and pathophysiological factors that are capable of modulating the Yin and Yang of EPH/EFN signaling in VSMCs will be the topic of our next phase of research.

EPH/EFN-related downstream signaling pathways

Changes in intracellular Ca^{2+} concentration play a vital role in the contraction of VSMCs. Calmodulin is one of the most important sensors of intracellular Ca^{2+} changes (Klee & Vanaman, 1982). Ca^{2+}–calmodulin complex could associate with many downstream kinases including myosin light chain kinase (MLCK) (Kamm & Stull, 2011). In our study, both female and male EPH/EFN KO mice presented normal Ca^{2+} flux. This suggests that the change of contractility in these cells is achieved in a Ca^{2+} flux-independent way.

Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) is a serine/threonine-specific protein kinase that is regulated by the Ca^{2+}/calmodulin complex. In our case, MLCK can be phosphorylated by CaMKII (Tansey et al., 1992), resulting negative modulation of MLCK activity in VSMCs (Van Riper, 1995). With Thr-286 autophosphorylation of CaMKII, its sensitivity to Ca^{2+} activation is decreased (Shifman, Choi, Mihalas, Mayo, & Kennedy, 2006). Our study on EPHB4 and EFNB2 showed increased CaMKII and MLCK phosphorylation in male KO VSMCs, and this led to decreased activity of MLCK. On the other hand MLCK phosphorylation was reduced in EFNB3 female KO VSMCs. These signaling changes finally led to the modulation of myosin light chain (MLC) phosphorylation, which is the major modulatory event cause for VSMC contractility.

Another important protein for regulating the contraction of smooth muscle is myosin light chain phosphatase (MLCP), which promotes contraction by dephosphorylating myosin light-chain proteins (D. P. Wilson, Susnjar, Kiss, Sutherland, & Walsh, 2005). MLCP is present in smaller amounts compared with MLCK. In our experiments of EPHB4, EFNB2 and EFNB3, the MLCP

phosphorylation levels remained unchanged in KO VSMCs with no sex difference. This result suggests that MLCP is not involved in VSMC contractility in the absence of EPHBs and EFNBs that are examined. (Figure 1).

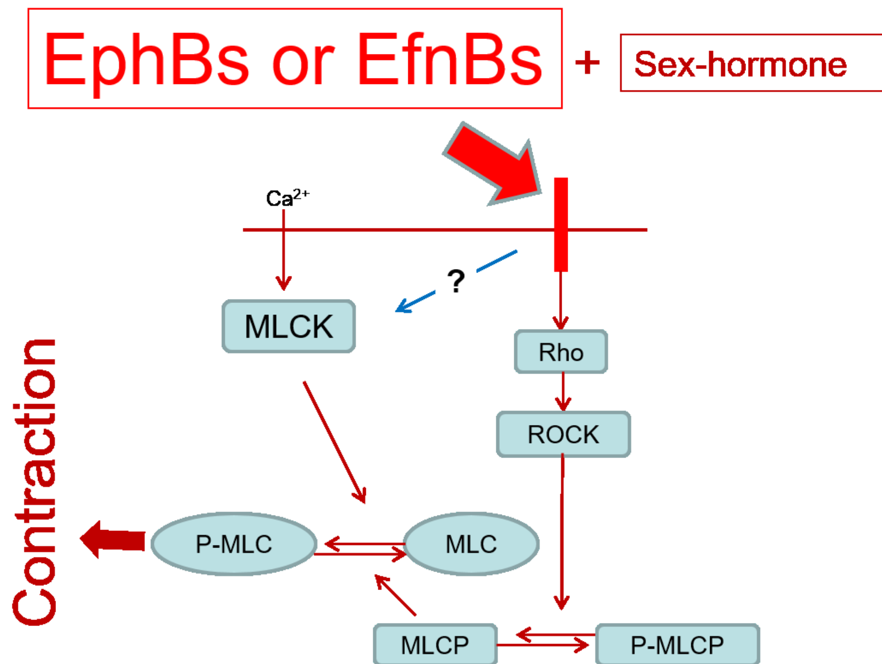


Figure 1. Possible signaling pathways downstream of EPHBs/EFNBs

EFNB2 and EFNB3 are two important EPHB kinase ligands; they have short intracytoplasmic tails with no enzymatic activity. There are five conserved tyrosine phosphorylation sites in the tails in addition to a PDZ-binding motif at the C-terminus. EFNB2 and EFNB3 are capable of binding to several proteins, such as GRIP1, RGS3 and DISHEVELLED (Bruckner et al., 1999; Lu, Sun, Klein, & Flanagan, 2001; Tanaka, Kamo, Ota, & Sugimura, 2003). Among these three associating molecules, we discovered that knocking down GRIP1 but not Dishevelled or PDZ-RGS3 expression in VSMCs by siRNAs partially reversed the KO contraction phenotype, suggesting that GRIP1 is mediating EFNB2 and EFNB3 reverse signaling, leading to the altered contractile phenotype of the KO VSMCs.

GRIP1 has 5 PDZ domains that can associate with signaling molecules that contain PDZ-binding domains and mediate EFNB2/EFNB3 reverse signalling (Figure 2A). Therefore, GRIP1 is situated in a node of signaling pathways emanating from the EphB/ephrinB family of molecules concerning their roles in BP regulation. GRIP1 can associate with a Rho-GDI (Su, Wang, & Garabedian, 2002) and a Ras-GEF (Ye et al., 2000) through its PDZ domains. It is possible that the remaining PDZ domains in GRIP1 might interact with modulators of RhoA activity such as GDP dissociation inhibitors (GDI) or GDP exchange factors (GEF) and influence their functions, which will, in turn, regulate RhoA activity. The net effect of GRIP1 could be positive or negative regarding VSMC contractility, depending on the context of signaling pathways it mediates in EFNB2 versus EFNB3. Such dual effect of signaling molecules does exist in other systems, such as suppressors of cytokine signaling (SOCS) have dual effects of inhibition and restoration on growth hormone signal transduction (Favre, Benhamou, Finidori, Kelly, & Edery, 1999).

We conducted analysis of the International Blood Pressure Consortium dataset, which contains SNP information on a large number of healthy individuals, and found a tag SNP in GRIP1 gene having a strong association with diastolic BP (Figure 2B).This finding corroborates our data in mice and shows the relevance of our mouse results to human BP control.

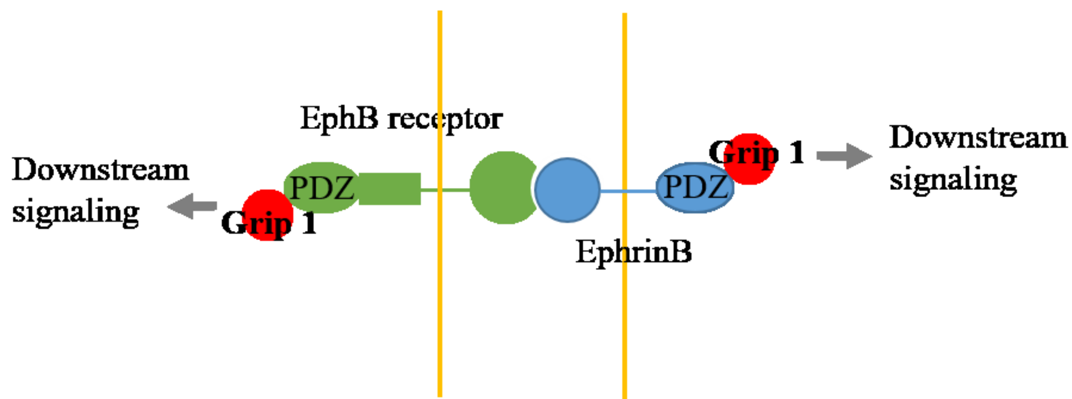


Figure 2. GRIP1 is in the EPH/EFN signaling pathways leading to VSMC contractility

The mutations identified in human genetics studies could be loss-of-function, or gain-of-function or alternation-of-function mutations

Although genetic mutation can lead to a loss of function, it is not always the case. Sometimes, the mutations may cause enhancement of the original function, or even evoke functions unrelated to the original function. For example, mutations in the leucine-rich repeat kinase 2 gene (LRRK2) could enhance GTP-binding and kinase activities to neuronal toxicity(West et al., 2007).

In our EFNB2 human genetic study, we revealed that 6 SNPs, apparently in high linkage disequilibrium with each other, were associated with hypertension risks in males-only samples. This in contrast to our finding in mice that the hypotension phenotype of the KO mice was more pronounced in males. While the major allele in humans is associated with hypertension risk, the minor alleles are protective against hypertension. On the other hand, there is no positive or negative association of these EFNB2 SNPs to hypertension in females in this study. The results, in general, match the findings of our mouse study: EFNB2 null-mutation manifested low BP phenotype in males but not females under normal condition. How do we explain the fact that the major allele of EFNB2 SNPs is associated with hypertension risks? As mentioned above, it is possible that the mutation in the major allele leads to a gain of function, enhancing the default function of EFNB2, *i.e.,* augmenting VSMC contractility, hence increased BP.

We also conducted human genetic studies on EFNB3. The results from several large-scale GWAS (Adeyemo et al., 2009; Guo et al., 2012; Hiura et al., 2010; International Consortium for Blood Pressure Genome-Wide Association et al., 2011; Levy et al., 2009; Org et al., 2009; Padmanabhan et al., 2010; Slavin, Feng, Schnell, Zhu, & Elston, 2011; Wellcome Trust Case

Control, 2007) datasets do not reveal a significant association of t EFNB3 to BP and hypertension. There are several possible reasons such as high heterogeneity of the samples; the relatively small contribution of EFNB3 to the BP phenotype; and the heavy statistical penalties due to genome-wide SNP analysis.

Why is then a SNP of GRIP1 found to be almost significantly associated to BP? The possible reason might be that GRIP1 is involved in signaling of multiple EPHBs and EFNBs. Thus the statistical weight of GRIP1 is probably much heavier than each EPHB or EFNB member alone, and consequently the association of GRIP1 to BP is easier to detect.

Sex hormones differentially influence the BP of males and females

We revealed that the function of EPH and EFNs in BP regulation was strongly related to sex and sex hormone level. Androgen deficiency, also known as hypoandrogenism or hypogonadism in males, is caused primarily by dysfunction, failure, or absence of the gonads. As determined by the International Society for the Study of the Aging Male, about 30% of men older than 40 years are hypogonadism(Traish, Miner, Morgentaler, & Zitzmann, 2011). Further, male patients with the metabolic syndrome are prone to hypogonadism (Zitzmann, 2009). There are two common etiologies of abnormal estrogen levels in males and females. In males, overweight/obesity may lead to increased estrogen levels. As adipose tissue is an important peripheral source of aromatase activity, which converts testosterone to estrogens (Nelson & Bulun, 2001). In females, the leading cause of estrogen level change is menopause, which leads to a rapid reduction of estrogen.

Subpopulation of people with abnormal sex hormone levels will have increased or decreased hypertension risks if they also have some mutations in EFNBs or EFNBs

Genetic diagnosis and hormone therapy

Our observations confirm that EPHBs and EFNBs are involved in blood pressure regulation. Base on that finding, several diagnostic and treatment methods can be generated.

First, from the perspective of prevention, it is valuable to develop genetic tests based on our current polymorphism studies. If the presence of Eph/ephrins mutations is found, then the sex hormone levels will be assessed. For the patients with both genetic mutations in EPHB and EFNB genes and sex hormone changes, further intervention can be performed. For example, for male hypertensive patients with hypogonadism or elevated estrogen levels, combines with EFNB3 mutations, testosterone replacement therapy or estrogens blockers (such as tamoxifen) can be used. In contrast, the reduced estrogen levels in old females after menopause could have a protective effect against hypertension if they happen to have EFNB3 mutations. For this subpopulation, avoidance of hormone replacement therapy will be a prudent measure to reduce hypertension risks. In subpopulations males with EPHB4 and/or EFNB2 loss-of-function mutations, maintaining sufficiently high testosterone levels should have protective effects against hypertension. These are personalized treatment/management of hypertension. As Eph are kinases, small molecule kinase inhibitors could also be used regulate BP. NVP-BHG712 is a small molecule synthesized based on a rational design. It is a potent EPHB4 inhibitor, although it also inhibitor several other EPH kinases. (Martiny-Baron et al., 2010; Wnuk et al., 2012). In our study, NVP-BHG712 was shown to be able to significantly reduce VSMC contractility. We found that this EPHB4 inhibitor could drastically reduce BP in mice (data not shown), consistent with the EPHB4 KO BP phenotype. Thus, EPHB4 inhibitors might be a new category of BP regulation agents.

A brief summary Nowadays, hypertension has become a worldwide critical medical and public health issue. Approximately more than one billion people have hypertension in the world. Currently, research on the pathogenesis of hypertension has made significant progress. Although we are still far away from a full understanding of the mechanisms underlying the disease. Our research has revealed that Eph/ephrins are previous unknown components of BP regulation. This has opened a new area of investigation into the pathogenesis of hypertension and hypertension treatment.

Contribution to sciences and future research directions

This research for the first time revealed that in the Eph/ephrin system, there were components whose deletion led to reduced BP. We also established the relevance of our mouse results to human hypertension according to human genetic studies. We elucidated certain aspects of signaling pathways by which sex hormones in concert with EFNs regulated VSMC contractility. We need to study further the detailed signaling events from Eph/ephrin in the cell surface to the contraction machinery of VSMCs, and the interaction of sex hormone signaling with the Eph/ephrin signaling.

We have found that EFN and EPH deletion also affects heart rates. The mechanisms by which these molecules control the rhythm of the pacing cells will also be an interesting aspect of future research.

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