Neuroimmune guidance cues in vascular (patho)physiology

Vreeken, D.

Chapter 5

Functional analysis of rare genetic variants in ligand-receptor pair EphrinB2-EphB4 in the pathophysiology of atherosclerosis

Dianne Vreeken
Caroline Suzanne Bruikman
Monique Jongbloed
Gerard Kornelis Hovingh
Anton Jan van Zonneveld
Janine Maria van Gils

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Abstract

The Eph family of Eph receptors (Ephs) and ephrin ligands are important guidance molecules during development of neuronal and vascular networks. Moreover, they serve important roles in endothelial and immune cell function postnatally, suggesting possible involvement of Eph family proteins in cardiovascular disease. Here, we describe a cohort of patients with premature atherosclerosis with genetic variations in Eph family members and experiments to validate the causality of observed genetic variations in ephrinB2 and EphB4 in human atherosclerosis. Identification of genetic variants in Eph family members revealed two genetic variations in a phosphorylation site and the ligand-binding domain of the ligand-receptor pair ephrinB2 and EphB4. While overexpression of ephrinB2, wildtype or the genetic variant c.C791T, in primary endothelial cells had no effect on endothelial function, binding assays of ephrinB ligands to the different variants of EphB4 revealed a marked impaired ligand binding to the EphB4-mutant receptor. Overexpression of EphB4, wildtype or the genetic variant c.G118A, did not alter normal pericyte function. However, co-culture of pericytes overexpressing EphB4 with endothelial cells improved barrier function and enhanced pericyte-endothelial cell contact when wildtype EphB4 was overexpressed. These changes were less prominent when there was overexpression of the genetic variant of EphB4. The current study shows that the novel genetic variant c.G118A in the EphB4 receptor has an impact on pericyte-endothelial cell interactions. These alterations might result in impaired vascular integrity and subsequent (premature) atherosclerosis. However additional in depth characterization of the precise effect of the c.G118A substitution in EphB4 is needed to confirm this.

1. Introduction

Atherosclerosis, the main underlying cause of cardiovascular disease (CVD), is a chronic inflammatory disease of the arteries. It is characterized by endothelial dysfunction, monocyte recruitment into the vessels wall and excessive deposition of low-density lipoprotein (LDL) cholesterol within the vessel wall. Continuous inflammation in the area promotes further growth of the atherosclerotic plaque resulting in a necrotic lipid core covered by a fibrous cap. Ultimately, rupture of (unstable) plaques or occlusion of an artery by the atherosclerotic plaque results in myocardial infarction or stroke (1,2). The development of atherosclerosis can often be attributed to one or more traditional risk factors such as dyslipidemia, age, smoking, diabetes mellitus, hypertension, obesity or a family history of cardiovascular disease. More recently inflammation has emerged as a risk factor for atherosclerosis as well, however, a substantial amount of environmental and genetic risk factors remain unaccounted for (3).

The Eph family of receptor tyrosine kinases is a large family of proteins consisting of 14 erythropoietin-producing hepatocellular receptors (Ephs) and 8 Eph receptor interacting protein (ephrin) ligands. Binding of an ephrin ligand to an Eph receptor induces not only receptor signaling (forward signaling) but also ligand signaling (reverse signaling) (4). The most renown ephrin family members are the ephrinB2 ligand and its cognate receptor Eph receptor B4 (EphB4), which are known to demarcate arterial and venous vessels respectively. This distinction is important for proper organization of the vascular system during embryonic development, but remains present in postnatal vasculature (5). Besides expression in endothelial cells, ephrins and their receptors are also expressed in cells that support the endothelial cells, such as pericytes and vascular smooth muscle cells.

Ephrin signaling induces several downstream pathways that predominantly regulate the cellular cytoskeleton and thereby attribute to cellular processes like cell proliferation, migration and cell-cell interactions (4). In the process of atherosclerosis, alterations in the expression profiles of several Eph receptors and ephrin ligands under pro-inflammatory conditions are described (6,7). Also, several Eph family members are found in human atherosclerotic lesions (8-10), suggesting involvement of these proteins in atherosclerosis. In addition, ephrinB2 and EphB4 have been found to modulate several atherosclerosis-related processes such as leukocyte migration and vascular integrity via regulation of endothelial cell-pericyte interactions (2,4,11,12). Therefore, we aimed to investigate the effect of genetic variations in the ephrinB2 ligand and EphB4 receptor genes.

2. Materials and methods

2.1 Patient cohort

Our patient cohort comprised a total of 87 patients with premature atherosclerosis, defined by the occurrence of an cardiac event before the age of 55 or 65 years in men and women respectively (30). These events occurred in the setting of absence or near absence of traditional risk factors for cardiovascular disease. Genomic DNA extraction, whole exome sequencing and candidate variant selection was done as described previously (31).The study is in compliance with the Declaration of Helsinki and the protocol was approved by the Institutional Review Board of the Amsterdam UMC, location Academic Medical Centre (METC-2004_236). All participants provided written informed consent. Functional consequences of a total of 13 genetic variants detected in the Eph family were evaluated with the Combined Annotation Dependent Depletion (CADD) software tool that quantitatively prioritizes functional, deleterious and disease causal variants across a wide range of functional categories, effect sizes and genetic architectures (13).

2.2 Primary cells, cell lines and media

2.2.1 Endothelial cells

Primary human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords as described previously (8). Cells were cultured on gelatin (1%) coated surfaces in EGM2 medium of Promocell (C-22211 supplemented with C-39211 and 1% antibiotics). The human immortalized endothelial cell line ECRF was cultured under similar conditions as primary endothelial cells.

2.2.2 Human brain vascular pericytes

Human brain vascular pericytes were obtained from Science Cell (#1200). Cells were cultured in complete classic medium (Cell Systems, 4ZO-500) supplemented with 1% antibiotics.

2.3 Site-directed mutagenesis

Using the Q5-site-directed mutagenesis kit (New England Biolabs, E0554S), vectors containing the selected patient variations were created from wildtype EphrinB2 and wildtype EphB4 vectors that were obtained from VectorBuilder (Vectorbuilder ID; VB161025-1120ysu and VB161108-1008udv respectively). Site-directed mutagenesis was performed according manufacturer's protocol with the primers indicated in the table below. Vectors were checked for the correct variation using Sanger Sequencing (LGTC facility, Leiden).

Gene	Forward primer	Reverse primer
EphrinB2	*GCCGCAGCACATGACCACGCTGT*	*GAGTGCTTCCTGTGTCTCCTCCG*
EphB4	*TCAGGTGGACAGGCAGTGGGA*	*GGGAATGTCACCCACTTCAG*

2.4 Lentiviral transduction

Overexpression of EphrinB2 or EphB4 (wildtype or mutant) was induced by transducing endothelial cells and/or pericytes with lentiviral particles containing an EphrinB2-wildtype overexpression vector, EphrinB2 overexpression vector containing the patient variant c.C791T, EphB4-wildtype overexpression vector EphB4 overexpression vector containing the patient variant c.G118A or a control vector. Selection of transduced cells was achieved using puromycin (Gibco, A113803) at a concentration of 2 µg/ml for endothelial cells and 5 µg/ml for pericytes.

2.5 Quantitative PCR

Total RNA was isolated using TRIzol (Invitrogen, 15596018) and RNeasy Mini Kit (Qiagen, 74106) according to manufacturer's instructions. Total RNA was reverse transcribed using M-MLV Reverse Transcriptase Kit (Promega, M1701). qPCR analysis was conducted using SYBR Select Master Mix (Applied Biosystems, 4472908) and the forward and reverse primers as indicated in the table below. The PCR cycling conditions were: Initial denaturation at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec, followed by a final extension step at 72°C for 10 minutes. mRNA expression was normalized to expression of GAPDH and represented as fold change compared to untreated cells.

Gene	Forward primer	Reverse primer
GAPDH	*TTCCAGGAGCGAGATCCCT*	*CACCCATGACGAACATGGG*
EphrinB1	*GAGGCAGACAACACTGTCAAG*	*AGCTTCAGTAGTAGGACCGTC*
EphrinB2	*AACTGTGCCAAACCAGACCA*	*TGTGGGTATAGTACCAGTCTTG*
EphrinB3	*TCGGCGAATAAGAGGTTCCA*	*GTCCCCGATCTGAGGGTACA*
EphB1	*TACGGCAAGTTCAGTGGCAA*	*AGGACACAACGAACACGACC*
EphB2	*GCTTCGAGGCCGTTGAGAAT*	*GAAGTGGTCCGGCTGTTGAT*
EphB3	*GTCATCGCTATCGTCTGCCT*	*AAACTCCCGAACAGCCTCATT*
EphB4	*CGCACCTACGAAGTGTGTGA*	*GTCCGCATCGCTCTCATAGTA*
EphB6	*CGACCAGACCAATGGGAACA*	*GGGTGAAGGAGTGGGATTCG*

2.6 Proliferation assay

Cells were seeded at a density of 20.000 cells/well in 12-wells plates in triplicate. On indicated days after seeding, cells were incubated with Methylthiazolyldiphenyl-tetrazolium bromide (MTT) solution (500 µg/ml in PBS) for 30 minutes at 37°C. After

incubation, MTT suspension was removed and cells were lysed using isopropanol/0.04M HCl. Lysates were transferred to a 96-wells plate and absorbance at 562 nm was measured using the Spectramax M2 plate reader. Proliferation rates are expressed as fold change in absorbance compared to baseline measurement at day 0.

2.7 Migration assay

Cells were seeded at a density of 21.000 cells/well into 2 well-culture inserts (Ibidi, 80209) placed in 24-wells plates in quadruplo. When confluent, inserts were removed and cells were placed on low serum medium (EBM2 with 0,5% FCS and 1% Pen/Strep) for endothelial cells or complete medium for pericytes. At different time intervals images were taken and gap closure was assessed using ImageJ. Wound healing is expressed as percentage open area compared to baseline at time=0 set at 100%.

2.8 Electrical cell-substrate impedance sensing (ECIS)

Electric cell-substrate impedance sensing system (ECIS Zθ, Applied Biophysics) was used for monitoring barrier formation and cell interactions. ECIS plates (96W20idf PET, Applied Biophysics) were pretreated with L-Cystein and coated with 1% gelatin. After taking baseline measurements, endothelial cells with and without knockdown were added to the plate. In case of co-culture experiments, equal amounts of endothelial cells and pericytes with or without knockdown were added to the plate. Multiple frequency/time (MFT) mode enabled real-time assessment of the endothelial-pericyte barrier formation. Results are corrected for baseline resistance and expressed as relative resistance at frequency of 4000 Hz.

2.9 Ligand-receptor binding assay

A confluent monolayer of EphB4 overexpressing cells were serum starved for 4 hours before incubation with recombinant ephrinB2 (R&D systems, 7397-EB) or ephrinB1 ligand (R&D systems, 7654-EB) at a concentration of 250 ng/ml for 60 minutes. After incubation, non-bound ephrin ligand was washed away and cells were fixed with 4% PFA. After permeabilization with 0.1% Triton and blocking with 5% BSA for 1 hour, cells were incubated with HRP-labelled IgG antibody (Biorad, 172-1050, 1/125.000) overnight to detect bound recombinant ephrin ligand that contains an Fc-fragment. Accordingly, the HRP signal was quantified with incubation with TMB substrate solution, addition of H_2SO_4 (1 M) after 30 minutes and measurement of the absorbance at 450nM using the Spectramax M2 plate reader. Results are expressed as fold change of absorbance compared to control cells.

2.10 Endothelial-pericyte co-culture

Black glass bottom 96-wells plates were coated with gelatin and seeded with 25.000 endothelial cells combined with 25.000 transduced pericytes in 200 µl of endothelial growth medium. Cells were pelleted together by centrifugation at 300xg for 30 seconds, after which plates were incubated for 7 days. Medium was refreshed on day 1 and day 4 and co-cultures were terminated at day 7 by fixation of the cells with methanol for 10 minutes. Covered by PBS, cells were kept at 4 degrees until staining.

For staining, cells were blocked with 3% BSA, 1% FCS and 1% NGS in PBS for 60 minutes. Accordingly cells were incubated with an antibody mix comprised of 1:25 FITC-labeled anti-CD31 (55545, BD pharming) 1:200 Cy3-labeled anti-α-SMA (CD6198, Sigma) and 1:5000 Hoechst (33258, Molecular Probes) in blocking buffer for 3 hours at room temperature. Cells were mounted in DABCO glycerol and imaged using the SP8 Confocal WLL microscope (Leica).

2.11 Statistical analyses

The differences between two groups were analyzed with unpaired t-tests. Two-way repeated measures ANOVA tests were used to test the difference between two groups over time. One way ANOVA test with post-hoc multiple comparisons by the Tukey method were performed to test differences between >2 groups. Two-sided P-values of <0.05 were considered statistically significant. All statistical analysis were performed with Graphpad Prism 8.

3. Results

3.1 Genetic variant in ephrinB2 and EphB4 in a family with premature atherosclerosis

Exome sequencing was performed in men and women who suffered from an cardiovascular event before the age of 55 or 65 respectively without showing the classical risk factors for cardiovascular disease, such as e.g. hyperlipidemia. Genetic variants within the Eph family of receptor tyrosine kinases were selected (a total of 13 variants) and revealed one genetic variation in the ephrinB2 gene that was predicted to be highly deleterious with a CADD score of 34.8 (ranging: 1-40) (13). The index case was a male patient that suffered a cardiac event at the age of 27. The patient was a non-smoking, non-diabetic with plasma lipid levels within the normal range and did not suffer from hypertension (Supplemental table 1). The genetic variation c.C791T (Figure 1A), results in a threonine-to-methionine substitution at position 264 (Figure 1B). This threonine residue is highly conserved between species (Supplemental figure 1A) and has been assigned as potential phosphorylation site. Loss of this residue would potentially result in disruption of ephrinB2 reverse signaling (Figure 1C). In addition to a

genetic variant in the arterial marker ephrinB2, a variant was also observed in the cognate ephrinB2-receptor, EphB4. This genetic variation c.G118A (Figure 1D) had a CADD score of 27.4 and induces a glycine-to-arginine substitution at position 40, which is also conserved between species (Figure 1E, Supplemental figure 1B). As this residue resides within the ligand binding domain of the receptor, the observed variant could potentially alter ligand-receptor interactions and therewith receptor forward signaling (Figure 1F). As both genes had relatively high CADD scores, detectable expression and an indication to functionality in the vasculature, the potential effect of these genetic variants were studied in more detail.

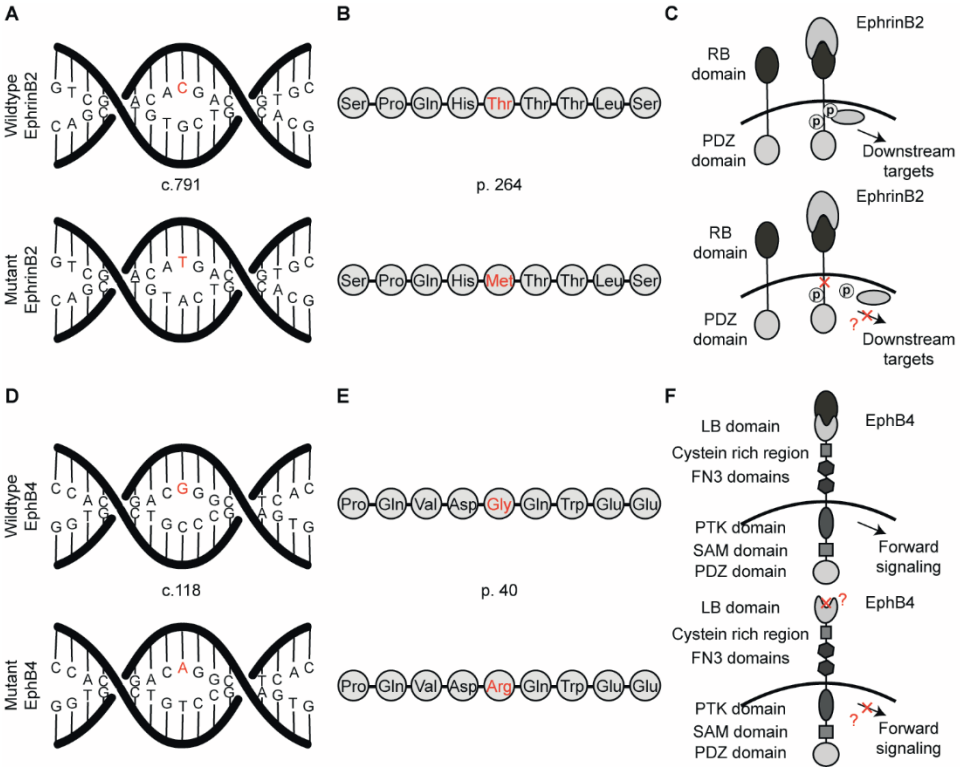

Figure 1 Genetic variants in EphrinB2 and EphB4 in a premature atherosclerosis patient cohort. (A) Exome sequencing revealed a high impact genetic variant in the ephrinB2 gene of a premature atherosclerosis patient (B) resulting in a threonine-to-methionine substitution (C) at a phosphorylation site within the ephrinB2 ligand. (D) High impact genetic variant detected in premature atherosclerosis patient in the EphB4 receptor (E) resulting in a glycine-to-arginine substitution (F) within the ligand binding domain of the EphB4 receptor. RB = Receptor Binding, LB = Ligand binding, FN3 = Fibronectin type III domain, PTK = Protein Tyrosine Kinase Domain, SAM = Sterile Alpha Motif.

3.2 No effect of c.C791T variant in ephrinB2 on endothelial proliferation, migration and barrier function

As a marker for arterial endothelial cells, ephrinB2 has been described to modulate endothelial function and therewith could influence atherosclerosis. As the genetic variant observed in ephrinB2 potentially could disrupt ephrinB2 reverse signaling, its effect on endothelial (dys)function was investigated. Primary endothelial cells were transduced with lentiviral vectors containing either wildtype ephrinB2, the mutant variant of ephrinB2 or a control vector. After selection, cells with a ~14 fold increase of ephrinB2 wildtype or mutant expression compared to control cells, were obtained. Gene expression of other ephrinB family members was not significantly altered by

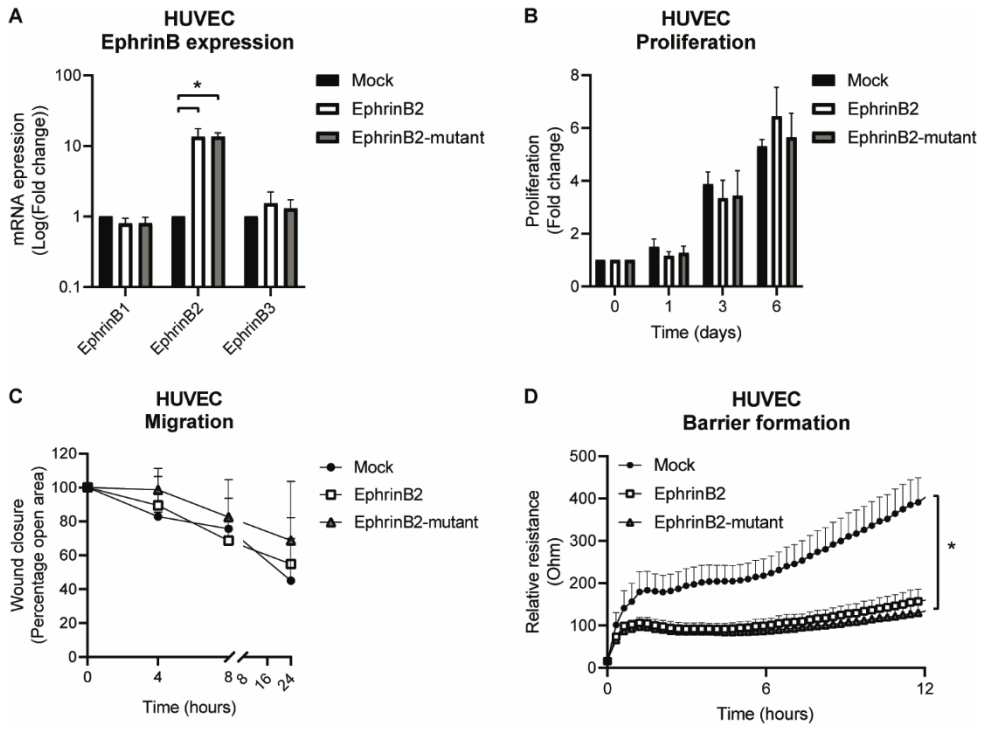

Figure 2 c.C791T variant in ephrinB2 has no effect on endothelial function. (A) mRNA expression levels of ephrinB1, ephrinB2 and ephrinB3 in primary endothelial cells transduced with a control vector (Mock), ephrinB2 wildtype overexpression vector or ephrinB2 mutant overexpression vector. Results are expressed as log(10) of the fold change in mRNA expression. Mean ± S.E.M. of N=5, *P<0.05. (B) Proliferation rates of ephrinB2 (wildtype and mutant) overexpressing cells and control (Mock) cells. Results are expressed as fold change to day 0 set at 1. Mean ± S.E.M. of N=3. (C) Migration rates of ephrinB2 (wildtype and mutant) overexpressing and control cells. Results are presented as percentage of open wound area. Mean ± S.E.M. of N=3. (D) Transendothelial electrical resistance of ephrinB2 (wildtype and mutant) overexpressing and control (mock-treated) endothelial cells over time. Mean ± S.E.M. of N=5, *P<0.05.

transduction of the endothelial cells with the ephrinB2 overexpression vectors (Figure 2A). Accordingly, these cells were used to investigate the effect of ephrinB2, wildtype or mutated, on endothelial proliferation, migration and barrier function. No significant differences in both proliferation and migration rates were observed between ephrinB2 overexpressing and control cells (Figure 2B/C). In contrast, measuring transendothelial electrical resistance showed significant impairment of tight endothelial barrier formation in endothelial cells that overexpressed ephrinB2 but no difference was observed between overexpression of wildtype ephrinB2 compared to mutant ephrinB2 (Figure 2D).

3.3 Impaired receptor-ligand binding and pericyte-endothelial contact due to c.G118A variant in EphB4 in pericytes

The *c.G118A* variant observed in EphB4 was predicted to impair ligand-receptor binding. Therefore, first the binding capacity of ephrinB2 to wildtype or mutated EphB4 was analyzed. In cells that overexpress EphB4, a significantly increased binding of ephrinB2 was detected (Figure 3A). Binding of ephrinB2 to mutant EphB4 was significantly less then binding to wildtype EphB4. A similar trend was observed for ephrinB1 binding (Figure 3B) however, overall binding of EphrinB1, depicted as absolute absorbance, was less then observed for EphrinB2 (Figure 3C).

EphB4 is minimally expressed in arterial endothelial cells (14), but highly expressed in the cells supporting endothelial cells such as vascular smooth muscle cells and

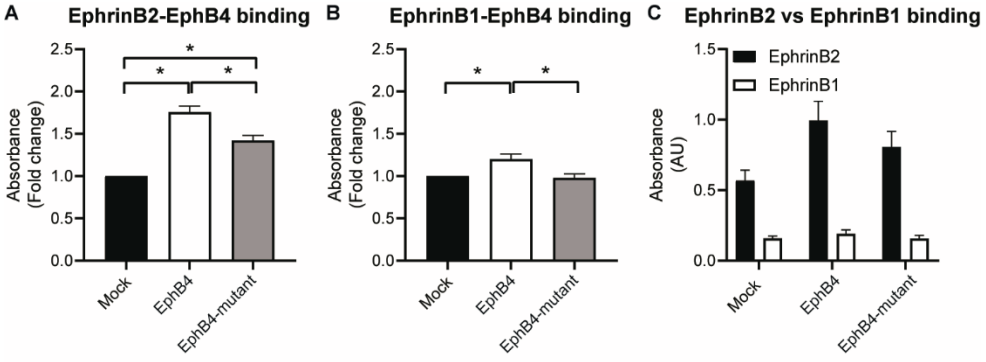

Figure 3 Impaired ligand binding to mutant EphB4 receptor. (A) EphrinB2 ligand binding to cells overexpressing wildtype or mutant EphB4 and (mock-treated) control cells. Results are expressed as the fold change in absorbance relative to control cells. Mean ± S.E.M. of N=5, *P<0.05. (B) EphrinB1 ligand binding to cells overexpressing wildtype or mutant EphB4 and (mock-treated) control cells. Results are expressed as the fold change in absorbance relative to Mock. Mean ± S.E.M. of N=3, *P<0.05. (C) Absolute absorbance of EphrinB1 in relation to EphrinB2 ligand-receptor interactions. Mean ± S.E.M. of N=3 or 5 respectively.

pericytes (15). Therefore, the effect of mutant EphB4 was investigated in pericytes. Human pericytes were transduced with lentiviral vectors containing either wildtype EphB4, the mutant variant of EphB4 or a control vector. After selection, pericytes with a ~25-fold increase of EphB4 expression compared to control cells were obtained (Figure 4A) and used for further experiments. Expression of other EphB receptors was not altered by transduction of the cells, except for EphB6. Transduction also increased, though not significant, the expression of EphB6 yet overall expression of EphB6 was less than EphB4. Functional assays revealed no changes in pericyte basic behavior as proliferation and migration rates were comparable between EphB4-wildtype, EphB4-mutant and mock-transduced cells (Figure 4B-D).

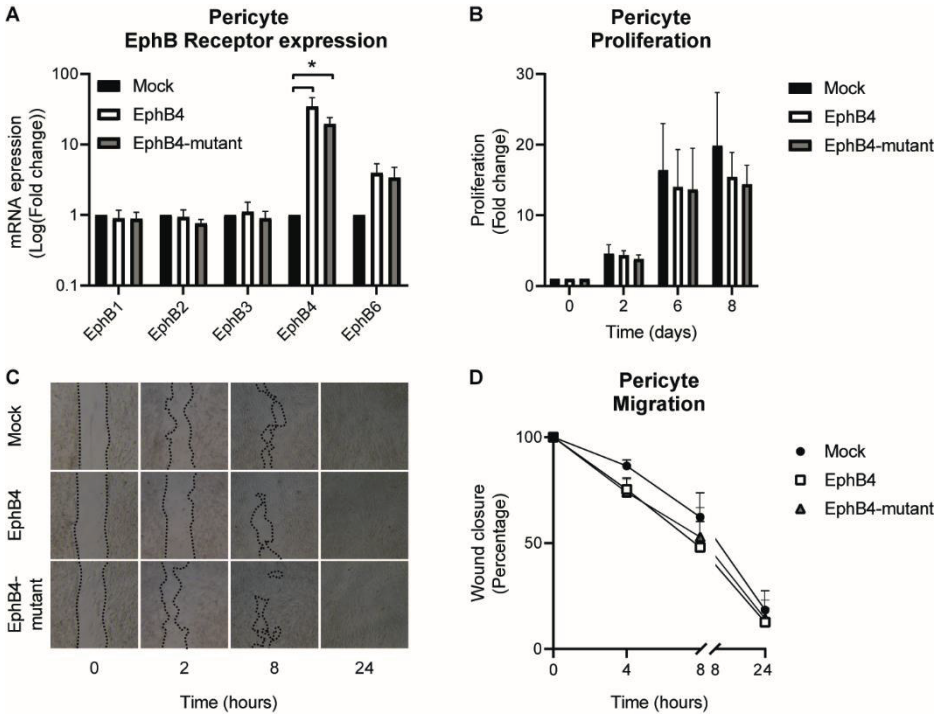

Figure 4 No effect of EphB4 overexpression on basic pericyte function. (A) mRNA expression levels of EphB1, EphB2, EphB3, EphB4 and EphB6 in pericytes transduced with a control vector (Mock), EphB4 wildtype overexpression or EphB4-mutant overexpression vector. Results are expressed as log(10) of the fold change in mRNA expression. Mean ± S.E.M. of N=5, *P<0.05. (B) Proliferation rates of EphB4 (wildtype and mutant) overexpressing cells and control (mock-treated) cells. Results are expressed as fold change to day 0 set at 1. Mean ± S.E.M. of N=4. (C) Representative overview pictures and (D) quantification of migration rates of EphB4 (wildtype and mutant) overexpressing and control (Mock) cells. Results are presented as percentage of open wound area. Mean ± S.E.M. of N=4.

As EphrinB2-EphB4 signaling has been shown to contribute to the assembly of vascular structures (15,16), the effect of the c.G118A variant on endothelial-pericyte interactions was studied. Endothelial cells and pericytes were co-cultured and barrier function was assessed. Co-cultures with pericytes overexpressing wildtype EphB4 showed an increase in barrier function compared to co-cultures with control pericytes (Figure 5A-B). This became visible after approximately 2 hours of endothelial cell-pericyte co-culture, but became more evident and significant over time. When pericytes overexpressed mutant EphB4, barrier function also increased but not as strong as with wildtype EphB4 and did not became significantly different from control cells (Figure 5A-B). In addition to impedance measurements of endothelial-pericytes co-cultures, immunostainings on co-cultured cells were performed. We observed striking differences in morphology of control versus EphB4 or EphB4-mutant overexpressing pericytes co-cultured with endothelial cells. Cells that overexpress EphB4 were more flattened and spread-out compared to control cells that were elongated and stretched (Figure 5C). Quantification of alpha smooth muscle actin (α-SMA), as a marker of pericytes in contact with endothelial cells, revealed an increase in positive area (Figure 5D), though not significant, in EphB4 overexpressing cells. Again, in EphB4-mutant cells the increase in positive area was less pronounced compared to EphB4-wildtype pericytes. Mean fluorescent intensity did not differ between conditions (Figure 5E).

4. Discussion

In this study, we identified two genetic variants in the Eph family of guidance cues in patients with premature atherosclerosis. Given the anticipated deleterious effect based on prediction models, we investigated the impact of two variants found in the ligand-receptor pair EphrinB2 and EphB4 in different aspects of vascular (atherosclerotic) biology.

The Eph family can signal bidirectional by both receptor signaling (forward signaling) as well as ligand signaling (reverse signaling). EphrinB reverse signaling occurs via its PDZ-binding domains and phosphorylation sites (17). Besides the often-described serine and tyrosine residues, ephrinB2 also contains threonine residues that can be phosphorylated. Clear roles for these threonine residues in ephrinB2 have not been described so far. One of the observed genetic variations in our patient cohort, was located at one of these threonine phosphorylation sites in the ephrinB2 gene, which led to the hypothesis that this variation could potentially disrupt ephrinB2 reverse signaling and endothelial function.

While EphB4 forward signaling in endothelial cells has been described to be anti-proliferative and -migratory (18-21), little is known on the role of ephrin reverse

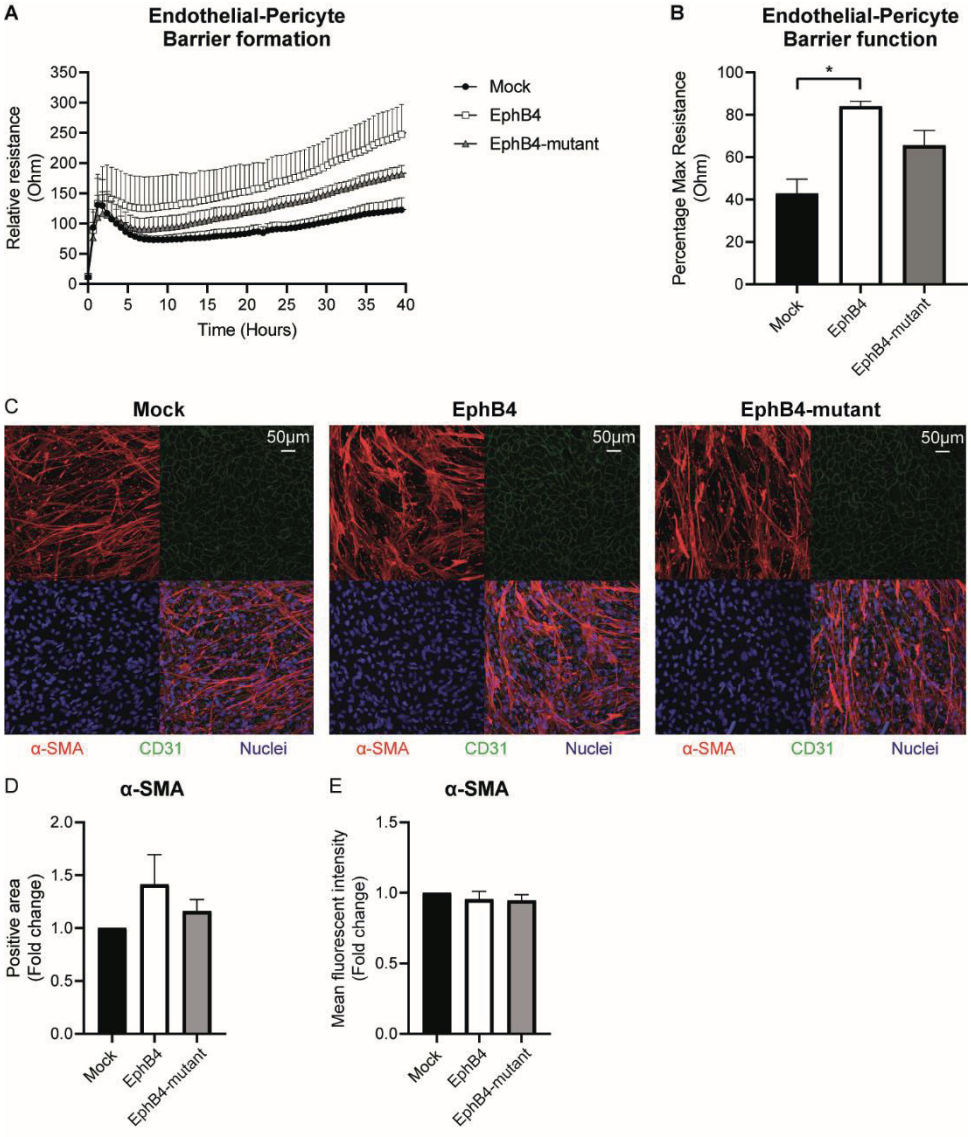

Figure 5 Reduced effect of EphB4 overexpression in EphB4-mutant overexpressing pericytes on endothelial-pericyte contact. (A, B) Real-time barrier resistance measurement of endothelial cells co-cultured in a 1:1 ratio with mock-treated, EphB4 or EphB4-mutant overexpressing pericytes. Results are presented as (A) relative resistance over time or (B) percentage of maximum resistance over the last hour. Mean ± S.E.M. of N=4. (C) Representative overview pictures of control (mock-treated), EphB4 overexpressing or EphB4-mutant overexpressing pericytes co-cultured with endothelial cells for 7 days. (D, E) Quantification of α-SMA (D) positive area or (E) mean fluorescent intensity. Results are expressed as fold change compared to (mock-treated) control cells. Mean ± S.E.M. of N=3.

signaling on endothelial function. Using overexpression of ephrinB2, wildtype or mutant, in endothelial cells we studied endothelial proliferation, migration and barrier function. Our studies showed no effect of ephrinB2 on endothelial proliferation nor migration. While our study focused on the overall migration of cells from a monolayer, overexpression of ephrinB2 has been shown to be promigratory when tracking the migration of individual endothelial cells over a shorter time interval (22). In addition, we showed that endothelial barrier function was significantly diminished when endothelial cells overexpressed ephrinB2. Strikingly, the overexpression of ephrinB2 itself induced a decrease in the endothelial barrier. In contrast, while not investigating endothelial barrier function directly, another study has shown that activation of endothelial ephrinB2 signaling, via exposure of endothelial cells to EphB4, had no effect on adherens junction proteins, suggesting no effect on endothelial barrier function (23,24). While both approaches are assumed to increase ephrinB2 reverse signaling, this suggests that overexpression of ephrinB2 activates different pathways compared to exposure to EphB4, resulting in different effects on endothelial function. This stresses the importance of more comparable and physiological methods to enable better insights and understanding of ephrinB/EphB reverse signaling and its delicate balance in relation to cell function.

Our results showed that the c.C791T genetic variant in the ephrinB2 gene did not impact endothelial cell function, which suggests that this threonine residue is not crucial for ephrinB2 reverse signaling in endothelial cells. Other signaling modules like tyrosine and serine phosphorylation sites and the PDZ-binding domain are probably of more importance for ephrinB2 reverse signaling in endothelial cells. Despite the fact that we observed no clear effect of the c.C791T genetic variant in endothelial cells, we cannot fully exclude a role of this variant in atherosclerosis. Future studies investigating the effects of this ephrinB2 variant upon interaction of endothelial cells with other cell types involved in vascular pathophysiology, such as pericytes, vascular smooth muscle cells and/or leukocytes, could provide alternative ways for this variant to effect atherosclerosis. Besides, ephrinB2 is expressed in other cell types as well and studying the ephrinB2 variant in these other cell types might reveal a role for threonine phosphorylation in ephrinB2 which could provide better insights in ephrinB2 reverse signaling in general. However, with the data presented in this study no causative role for the c.C791T variant in ephrinB2 and the phenotype of premature atherosclerosis patients can be made.

The EphB4 receptor primarily binds to ephrinB2. This in contrast to other EphB receptors which promiscuously bind to a number of ephrin ligands (25). A variant in the EphB4 receptor, identified in a patient with unexplained premature atherosclerosis and

predicted to be highly deleterious, was attracting our attention as a potential genetic predisposition to (premature) atherosclerosis. As mentioned before, EphB4 is barely expressed in endothelial cells in atheroprone regions, but is present in mural cells (14,15). Mural cells, including pericytes and vascular smooth muscle cells (vSMCs), provide support to the vascular endothelium, and are involved in guiding vascular innervation (26). Interactions between mural cells and endothelial cells enable the formation of stable and functional vascular structures with little vascular remodeling and low permeability, preventing vascular leakage of solutes and cells. Though the specific molecular mechanisms of mural cell-endothelial cell interactions are still unclear, several pathways including the TGF-β, angiopoietins, PDGF-B, S1P, and Notch signaling pathways have been shown to be important (15,26,27). In addition, some studies show a role for EphB4-ephrinB2 signaling in endothelial-pericyte interactions (15,16). Therefore we aimed to investigate EphB4 in pericyte function and pericyte-endothelial interactions and the potential implications of the c.G118A genetic variant.

Our initial results showed that the genetic variation in the ligand binding domain of the EphB4 receptor, indeed decreased ephrinB ligand binding to cells overexpressing the mutant form of EphB4 compared to overexpression of the wildtype form. However, ligand binding is not completely prevented by the mutation as binding of ephrinB2 to the mutant form of EphB4 was shown to be increased compared to control cells. Perhaps the increased amount of receptors present, due to overexpression of EphB4, and the excessive availability of ephrinB ligand enabled receptor-ligand interactions under less favorable conditions. It would be of interest to define ephrinB2-EphB4 mutant ligand-receptor binding affinities under more physiological conditions. Besides, as differences in receptor-ligand binding affinity might lead to the formation of different signaling complexes and activation of different downstream pathways, characterization of downstream targets of the EphB4 variants could yield interesting new insights in EphB4 forward signaling.

A small number of studies described that activation of EphB4 signaling, using recombinant ephrinB2, in vascular smooth muscle cells decreased migration of the cells, while proliferation was unaffected (28,29). How these findings relate to pericyte function remained elusive. This study adds to our understanding of the role of EphB4 on pericytes, that overexpression of EphB4 had no effect on pericyte basal function as measured by proliferation and migration. Co-culture experiments, however, did show a beneficial effect on endothelial cells by the presence of EphB4 on pericytes. Previous studies indicated the importance of ephrinB2-EphB4 signaling in the assembly and stabilization of vascular structures (15,16). The study of Foo and colleagues showed that mural cell-specific inactivation of ephrinB2 in mice results in defective

incorporation of mural cells into the vessel wall and vascular defects (15). However, whether these observations are due to loss of ephrinB2 reverse signaling in mural cells or due to diminished autocrine EphB4 signaling remains to be elucidated. Using a novel co-culture model for endothelial cells and pericytes within the ECIS system, we showed improvement of endothelial barrier function in the presence of pericytes overexpressing EphB4. In addition, pericytes overexpressing EphB4 and co-cultured with endothelial cells appear more spread out and have slightly increased levels of α-SMA. These effects were less pronounced when the mutant form of EphB4 was present, indicating that ephrinB2-EphB4 signaling is distorted in the EphB4 mutant. Similar to the diminished, but not entirely blocked binding of ephrinB2 to EphB4, the EphB4-effect on pericyte-endothelial cell interaction is not entirely prevented by the EphB4 genetic variation. However, the non-physiological expression of EphB4 could have enabled ligand-receptor binding under unfavorable conditions, yielding some of the effect observed for wildtype EphB4. In addition, regular expression of the wildtype EphB4 receptor in pericytes is not inhibited and could still contribute to the observed effects.

All data combined, our results show an effect of the genetic variation c.G118A on ligand-receptor binding and endothelial cell-pericytes interactions, however the precise mechanisms remain unknown. It is possible that distorted ligand-receptor interactions prevent EphB4 forward signaling in pericytes, modulating their ability to migrate and interact with endothelial cells. However, the distorted ligand-receptor interactions could also prevent ephrinB2 reverse signaling in endothelial cells, directly affecting endothelial cells. Additional co-culture experiments, e.g. co-culture within Matrigel monitoring cell-cell interactions during tube formation or co-culture experiments in microfluidic 3D culture plates, might further elucidate the contributions of EphB4-ephrinB2 signaling in pericyte-endothelial interactions. In addition, more in depth investigation of Eph-ephrin signaling, e.g. by detection of ephrinB2 or EphB4 phosphorylation or modulation of receptor or ligand signaling domains, could clarify whether forward or reverse signaling or combined is of most importance for endothelial-pericyte interactions. Similar to ephrinB2, EphB4 expression is not restricted to pericytes. For example, EphB4 is expressed in leukocytes and ephrinB2-EphB interactions have been shown to be involved in monocyte trans-endothelial migration (8,24). Studying this genetic variation in EphB4 in other cell types could provide more insight as to why this variant could contribute to premature atherosclerosis. Overall, the data presented here showed some interesting observations suggesting a potential causative connection of c.G118A variation in EphB4

and the phenotype of premature atherosclerosis. Additional studies are needed to confirm the involvement of this variant in premature atherosclerosis.

In summary, we have identified two genetic variants in the receptor-ligand combination EphB4-ephrinB2 in a cohort of patients with premature atherosclerosis. The c.G118A substitution in the EphB4 gene affected ligand-receptor binding and pericyte-endothelial cell interactions. These alterations might result in impaired vascular integrity, and possibly the development of (premature) atherosclerosis, but more in depth characterization of the precise effect of the c.G118A substitution in EphB4 is necessary.

Author contributions

Conceptualization, D.V., C.S.B., and J.M.G.; investigation, D.V., C.S.B., and J.M.G.; writing—original draft preparation, D.V.; writing—review and editing, C.S.B., M.R.M.J., G.K.H., A.J.Z., and J.M.G.; supervision, M.R.M.J., A.J.Z., and J.M.G.; funding acquisition, G.K.H., A.J.Z., and J.M.G. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

G.K.H. has served as consultant and speaker for biotech and pharmaceutical companies that develop molecules that influence lipoprotein metabolism, including Regeneron, Pfizer, MSD, Sanofi, and Amgen. Until April 2019, G.K.H. has served as PI for clinical trials conducted with A.O. Amgen, Sanofi, Eli Lilly, Novartis, Kowa, Genzyme, Cerenis, Pfizer, Dezima, Astra Zeneca. The Department of Vascular Medicine receives the honoraria and investigator fees for sponsor studies/lectures for companies with approved lipid lowering therapy in the Netherlands. Since April 2019, GKH is partly employed by Novo Nordisk and the AMC. GKH has no active patents. The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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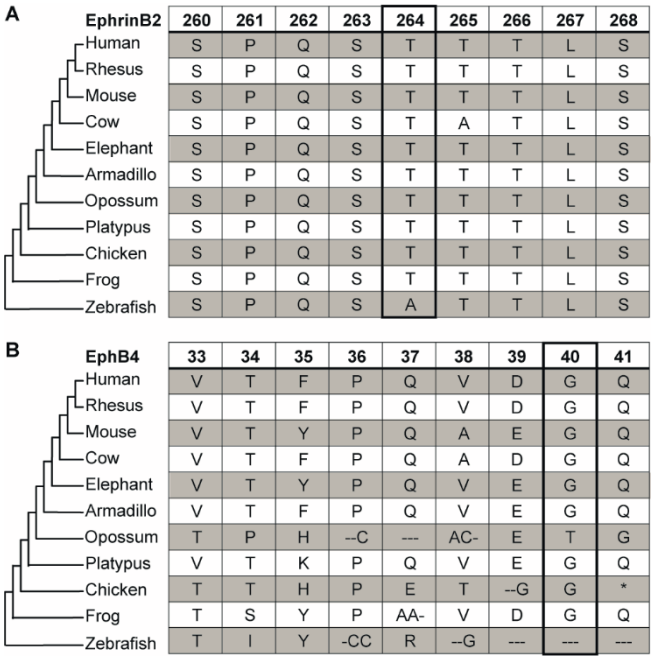
Supplemental data

Supplemental table 1: Clinical characteristics of premature atherosclerosis patients with a genetic variant in the ephrinB2 or EphB4 gene:

Gene	Genetic variant	CADD score	Gender	Age of cardiac event	Diabetes Mellitus	Hyper-tension	Smoking	HDL (mmol/L)	LDL (mmol/L)
EphrinB2	c.C791T	34,8	Male	27	No	No	No	1,57	1,04
EphB4	c.G118A	27,4	Male	39	No	No	Yes	2,9	1,1

Supplemental figure 1: Domain conservation between species within the ephrinB2 and EphB4 gene.



(A) Conservation of the threonine residue at position 264 in ephrinB2 between species. (B) Conservation of the glycine residue at position 40 in EphB4 between species.