Ephrin-B2 controls PDGFRβ internalization and signaling

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B-class ephrins, ligands for EphB receptor tyrosine kinases, are critical regulators of growth and patterning processes in many organs and species. In the endothelium of the developing vasculature, ephrin-B2 controls endothelial sprouting and proliferation, which has been linked to vascular endothelial growth factor (VEGF) receptor endocytosis and signaling. Ephrin-B2 also has essential roles in supporting mural cells (namely, pericytes and vascular smooth muscle cells [VSMCs]), but the underlying mechanism is not understood. Here, we show that ephrin-B2 controls platelet-derived growth factor receptor β (PDGFRβ) distribution in the VSMC plasma membrane, endocytosis, and signaling in a fashion that is highly distinct from its role in the endothelium. Absence of ephrin-B2 in cultured VSMCs led to the redistribution of PDGFRβ from caveolin-positive to clathrin-associated membrane fractions, enhanced PDGF-B-induced PDGFRβ internalization, and augmented downstream mitogen-activated protein (MAP) kinase and c-Jun N-terminal kinase (JNK) activation but impaired Tiam1–Rac1 signaling and proliferation. Accordingly, mutant mice lacking ephrin-B2 expression in vascular smooth muscle developed vessel wall defects and aortic aneurysms, which were associated with impaired Tiam1 expression and excessive activation of MAP kinase and JNK. Our results establish that ephrin-B2 is an important regulator of PDGFRβ endocytosis and thereby acts as a molecular switch controlling the downstream signaling activity of this receptor in mural cells.

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Cell surface receptors integrate numerous signals from the tissue environment and can thereby induce fundamental changes in cellular behavior, which are the basis of numerous growth, migration, and tissue patterning processes. Increasing evidence indicates that endocytosis not only regulates receptor levels and bioavailability in the plasma membrane but can also influence the strength and identity of downstream signal transduction events (Abella and Park 2009; Hansen and Nichols 2009; Kumari et al. 2010). The concept that receptor internalization and trafficking can regulate signal transduction events has been initially proposed for the epidermal growth factor receptor (Vieira et al. 1996), but, subsequently, similar findings have been reported for the receptors binding insulin growth factor, transforming growth factor β, Wnt, or vascular endothelial growth factor (VEGF) (Chow et al. 1998; Di Guglielmo et al. 2003; Yu et al. 2007; Finger et al. 2008; Lanahan et al. 2010; Sawamiphak et al. 2010; Wang et al. 2010; Morcavallo et al. 2012).

Platelet-derived growth factor receptor β (PDGFRβ), a receptor tyrosine kinase [RTK] activated by PDGF, suppresses the differentiation and promotes the proliferation of vascular smooth muscle cells [VSMCs] and other mesenchymal cell types (Andrae et al. 2008; Olson and Soriano 2011). Signals induced by PDGF binding to PDGFRβ include the activation of Ras-mitogen-activated protein [MAP] kinase, phosphoinositide 3-kinase, the small GTPase Rac1, and c-Jun N-terminal kinase [JNK] (Andrae et al. 2008). Different membrane domains and endocytic pathways have been proposed to regulate
PDGFRβ function [Liu et al. 1996; Sundberg et al. 2009]. Treatment of cultured human fibroblasts with dynamin inhibitors, which block clathrin-dependent endocytosis, argues for both dynamin-dependent and -independent PDGFRβ internalization processes [Sadowski et al. 2013]. Caveolin-1 can interfere with PDGF-induced signal transduction [Fujita et al. 2004; Yamamoto et al. 2008], which suggests that lipid rafts or caveolae might harbor a passive RTK pool devoid of signaling activity. Recently, it also has been proposed that PDGFRβ endocytosis in cultured cells can be mediated by macropinocytosis involving dorsal, clathrin-containing membrane ruffles [Moes et al. 2012]. However, the precise roles of different membrane domains for PDGFRβ function, the underlying molecular processes, and the regulation of downstream signal transduction responses remain incompletely understood.

Ephrins, membrane-anchored ligands for Eph family RTKs, are emerging as key regulators of endocytosis and trafficking processes [Bethani et al. 2010; Pitulescu and Adams 2010]. In the developing cardiovascular system, ephrin-B2, a transmembrane protein belonging to the B-class ephrin subfamily, is an important regulator of endothelial cell behavior and blood vessel growth. This function was recently linked to the regulation of ligand-induced VEGFR receptor [VEGFR] internalization from the plasma membrane, which enhances certain downstream signaling events such as MAP kinase activation [Lanahan et al. 2010; Sawamiphak et al. 2010; Wang et al. 2010]. Ephrin-B2, like other ephrins, not only activates Eph RTKs termed “forward” signaling on adjacent cells but also has receptor-like (“reverse”) signal transduction capacity that contributes to its role in VEGFR endocytosis (Sawamiphak et al. 2010; Wang et al. 2010; Nakayama et al. 2013). In addition to its functions in the endothelial monolayer of blood vessels, ephrin-B2 also controls the behavior of pericytes and VSMCs in the blood vessel wall. Targeting of ephrin-B2 [encoded by the gene Efnb2] in these perivascular cell types led to the formation of unstable blood vessels, hemorrhaging, and perinatal lethality [Foo et al. 2006]. Many of these features resembled macroscopic defects observed in mutant mice lacking PDGFR or PDGFRβ [Lindahl et al. 1997; Tallquist et al. 2003]. However, the exact mechanistic role of ephrin-B2 in perivascular cells and possible links to PDGF signaling have not been explored so far.

Results

Vessel wall and signaling defects in VSMC-specific ephrin-B2 mutants

To circumvent the previously reported perinatal lethality of general mural cell-specific ephrin-B2 mutants [Foo et al. 2006], probably a consequence of pericyte defects, and inactivate the ligand specifically in VSMCs, we interleaved mice carrying a loxP-flanked version of the Efnb2 gene [Efnb2floxed] [Grunwald et al. 2004] and SM22α-Cre transgenics [Lepore et al. 2005]. While a fraction of the resulting Efnb2A(SMC) mutants were viable, reached adulthood, and were fertile, only 42% of the expected number ([i.e., 10.7% instead of 25%] was obtained at weaning age [Supplemental Fig. 1A]. The absence of ephrin-B2 protein in mutant VSMCs was confirmed by immunostaining of Efnb2A(SMC) aorta sections [Fig. 1A]. The body weight of 30- to 60-wk-old mutants was reduced compared with age-matched littermates [Supplemental Fig. 1B]. In addition, adult Efnb2A(SMC) mice showed significant dilatation of the aorta accompanied by reduced thickness of the aortic VSMC layer, which was most obvious for the aortic arch region [Fig. 1B,C; Supplemental Fig. 1C,D]. The mutant tunica media appeared flattened, and the elastic lamella failed to show the wavy morphology observed in control aortae [Supplemental Fig. 1E]. Consistent with the reduced thickness of the adult Efnb2A(SMC) vessel wall, VSMC number and proliferation were reduced in mutants at postnatal day 8 [P8] [Fig. 1D–F], whereas the number of apoptotic cells was not significantly changed [data not shown]. Our previous characterization of mural cell-specific Efnb2 mutants had shown that loss of ephrin-B2 resulted in impaired association of pericytes and VSMCs with perinatal blood vessels [Foo et al. 2006]. In addition to the aorta, VSMC-specific Cre activity in SM22α-Cre transgenic mice is detectable in the embryonic dermis and postnatal retinal vasculature [Supplemental Fig. 1F; data not shown]. Accordingly, arterial smooth muscle cell coverage was reduced and irregular in Efnb2A(SMC) embryonic day 17.5 [E17.5] skin and P8 retina [Supplemental Fig. 1G,H; data not shown]. In contrast, the [untargeted] pericytes in Efnb2A(SMC) mutants showed no overt differences from control littermates [Supplemental Fig. 1H; data not shown].

To gain insight into the molecular changes associated with the vessel wall defects in Efnb2A(SMC) mutants in vivo, we investigated the activation status of key signaling pathways in adult mutant and littermate control aorta lysates. Activation of MAP kinase Erk1/2, JNK, and p27kip1, an inhibitor of G1-to-S-phase transition in the cell cycle [Bond et al. 2008], whereas the active, GTP-associated form of the small GTPase Rac1 was strongly reduced [Fig. 1H–J]. Thus, loss of ephrin-B2 in vascular smooth muscle led to vessel wall defects and altered activation of multiple key signaling pathways in vivo.

Regulation of Tiam1–Rac1 signaling by ephrin-B2

To gain further insight into the functional role of ephrin-B2 in VSMCs, we compared the gene expression profiles of previously generated control and Efnb2 knockout VSMCs [Foo et al. 2006] by Affymetrix microarray analysis. This revealed that loss of ephrin-B2 led to pronounced changes in gene expression [Supplemental Fig. 2A,B]. One of the top down-regulated genes [49-fold reduced compared with control] was Tiam1, which encodes T-cell lymphoma invasion and metastasis-inducing protein 1, a key regulator of cell morphology and polarity [Mertens et al. 2006]. As Tiam1 is guanine nucleotide exchange factor [GEF] and thereby an activator of Rac1, we reasoned...
Figure 1. Vessel wall defects in smooth muscle cell-specific ephrin-B2 mutants. (A) Confocal images showing ephrin-B2 (green) and α-smooth muscle actin (α-SMA; red) immunostaining on sections of adult (30 wk) control and Efnb2ΔSMC mutant aortae. (*) Vessel lumen. (B) Dilation affecting freshly isolated adult Efnb2ΔSMC aortic arches (right) compared with control littermates (left). Arrows indicate vessel diameter. (C) Quantitation of relative aortic arch diameter of adult mice (>30 wk). P-value was calculated using two-tailed Student’s t-test (n = 4). Error bars indicate SD. (D–F) 5-Ethynyl-2'-deoxyuridine (EdU) labeling (2-h pulse; red) of proliferating cells in control and mutant P8 aorta. (Green) α-SMA; (blue) nuclei (DAPI). (*) Vessel lumen. Quantitation of total α-SMA-positive cells (E) and EdU-labeled VSMCs (F). P-values were calculated using two-tailed Student’s t-test (n = 3). Error bars indicate SD. (G) Western blot analysis of total and phosphorylated JNK (p-JNK), Erk1/2 (p-Erk1/2), and PDGFRβ (p-PDGFRβ) in control and Efnb2ΔSMC aorta lysate, as indicated. Tubulin is shown as a loading control. Molecular weight markers (in kilodaltons) are indicated. (H, I) Strongly decreased levels of active Rac1 (GTP-Rac1) in Efnb2ΔSMC aorta lysate relative to control [shown in H]. (I) Tiam1 protein was nearly undetectable in mutant samples, whereas amounts of p27kip1 were elevated. Tubulin is shown as a loading control. Molecular weight markers are indicated. (J) Quantitative analysis of band intensities in the Western blots shown in H and I and replicates. P-values were calculated using two-tailed Student’s t-test (n = 3). Error bars indicate SD.
that its down-regulation might be causally linked to the reduced Rac1 activity in Efnb2<sup>±/±</sup> VSMCs (Fig. 1H). Western blot analysis confirmed reduced Tiam1 protein levels in Efnb2<sup>±/±</sup> aorta lysate (Fig. 1I) as well as in cultured murine Efnb2 knockout VSMCs (Fig. 2A).

As both MAP kinase and Rac1 signaling play important roles in the regulation of smooth muscle cell proliferation (Zou et al. 1998; Bond et al. 2008), we next investigated the interplay between these two pathways. Inhibition of Rac1 by administration of the small compound NSC23766 significantly reduced proliferation of cultured VSMCs and led to up-regulation of p27<sup>kip1</sup> (Fig. 2B,C). VSMC proliferation was also slightly reduced and p27<sup>kip1</sup> proliferation was increased after overactivation of MAP kinase (Fig. 2B,C), which was achieved by expressing a Raf/estrogen receptor (ER) fusion protein [ΔRaf1-ER] with tamoxifen-inducible kinase activity (Thiel et al. 2009). Further reduction of VSMC mitosis was obtained after combined NSC23766 administration and ΔRaf1-ER activation (Fig. 2B), which resembled the low Rac1 activity but elevated phospho-Erk1/2 observed in Efnb2<sup>±/±</sup> aorta lysates (Fig. 1G,H). We reported previously that cultured Efnb2 knockout VSMCs display spreading defects (Foo et al. 2006), which can be mimicked by Efnb2 knockout cells was rescued by re-expression of full-length Tiam1 (Fig. 2D,E), which also significantly restored the proliferation of ephrin-B2-deficient VSMCs (Fig. 2F). Thus, ephrin-B2 is a critical regulator of Tiam1/Rac1 and thereby controls VSMC spreading and mitosis.

Next, we investigated the regulation of Tiam1 expression by upstream signals. When control or Efnb2 knockout cells were treated with the Erk1/2 inhibitor U0126, Tiam1 expression was significantly increased at both the mRNA and protein levels (Fig. 2G,H). Conversely, Erk1/2 activation with tamoxifen-inducible ΔRaf1-ER reduced Tiam1 mRNA and protein in murine VSMCs (Fig. 2I,J). The regulation of Tiam1 by ephrin-B2 does not appear to be regulated by acute reverse signal transduction. While the stimulation of VSMCs with recombinant EphB4/Fc fusion protein led to detectable phosphorylation of B-class ephrins after 15 and 30 min, there was no appreciable change in Tiam1 protein levels under the same conditions (Supplemental Fig. 2D). We showed previously that prolonged EphB4/Fc stimulation triggers pronounced internalization and degradation of ephrin-B2 after 2.5 and 6 h (Foo et al. 2006). The strong reduction of ephrin-B2 at these time points was accompanied by down-regulation of Tiam1 protein (Fig. 2K). This reduction of Tiam1 was prevented by the addition of the proteasome inhibitor MG132 (Fig. 2K,L), which indicates a role of protein degradation in this process.

Together, these data strongly argue for positive regulation of Tiam1 in smooth muscle cells by ephrin-B2, whereas MAP kinase activity reduces Tiam1 expression. Accordingly, the combination of absent ephrin-B2 expression and elevated phospho-Erk1/2, as observed in mutant aorta lysates, can explain the lost expression of Tiam1 and low Rac1 activity in Efnb2<sup>±/±</sup> VSMCs.

Signaling defects in ephrin-B2-deficient VSMCs are linked to PDGFRβ

An increasing body of evidence connects the Eph/ephrin system to the modulation of other cell surface receptors, such as VEGFRs in endothelial cells (Bethani et al. 2010; Pitulescu and Adams 2010). Given the prominent role of PDGFR signaling in VSMCs and other cell types of the mesenchymal lineage (Andrae et al. 2008; Olson and Soriano 2011), we next investigated the role of ephrin-B2 in the regulation of PDGFRβ. Stimulation of murine control VSMCs with PDGF-B led to transient autophosphorylation of PDGFRβ, which was accompanied by JNK and Erk1/2 activation (Fig. 3A). In line with findings in Efnb2<sup>±/±</sup> aorta lysates (Fig. 1G), PDGFRβ tyrosine phosphorylation as well as phospho-JNK and phospho-Erk1/2 levels were substantially increased in Efnb2 knockout VSMCs at 5 and 15 min after PDGF-B treatment (Fig. 3A). In contrast, other factors triggering MAP kinase activation in VSMCs, such as insulin-like growth factor 1 (IGF-1) and tumor necrosis factor α (TNF-α) (Hayashi et al. 1999, Yoshimura et al. 2005), led to comparable Erk1/2 phosphorylation in control and Efnb2 knockout VSMCs (Supplemental Fig. 3A). Thus, the modulatory role of ephrin-B2 is confined to certain growth factors but not others. Consistent with the strongly reduced Tiam1 expression in Efnb2 knockout cells, PDGF-B-induced activation of Rac1 was impaired in comparison with control VSMCs (Fig. 3B). However, NSC23766 treatment of cultured VSMCs did not result in appreciable alterations in PDGF-B-induced JNK and Erk1/2 phosphorylation, suggesting that the up-regulation of these signals in Efnb2 knockout cells is not a direct consequence of Tiam1/Rac1 defects (Supplemental Fig. 3B).

Since ephrin-B2 positively regulates VEGFR internalization and thereby promotes the downstream activation of MAP kinase and Rac1 in endothelial cells (Sawamiphak et al. 2010; Wang et al. 2010; Nakayama et al. 2013), we next investigated whether PDGFRβ function is modulated by ephrin-B2. Immunostaining of surface PDGFRβ in nonpermeabilized murine VSMCs and surface biotinylation experiments indicated that PDGF-B-induced uptake of the receptor was accelerated in Efnb2 knockout cells relative to controls (Fig. 3C–F). The faster internalization of PDGFRβ in ephrin-B2-deficient cells was accompanied by more rapid degradation of the RTK (Fig. 3E). Moreover, strongly enhanced association of PDGFRβ with the clathrin heavy chain (CHC) suggested that clathrin-mediated endocytosis of this receptor might be enhanced in Efnb2 knockout VSMCs as well as in Efnb2<sup>±/±</sup> aorta lysate (Fig. 3G–I).

Ephrin-B2 controls PDGFRβ distribution in the plasma membrane

PDGFRβ distribution and endocytosis have been assigned to a number of different membrane domains, including clathrin-positive membrane ruffles, macropinosomes, and caveolae (Liu et al. 1996; Sundberg et al. 2009; Moes et al. 2012). To study the association of PDGFRβ with caveolin-1-positive or clathrin-containing membrane
Figure 2. Regulation of VSMC proliferation by Tiam1–Rac1 and MAP kinase. (A) Western blot showing strongly decreased Tiam1 protein levels in *Efnb2* knockout VSMCs. Tubulin is shown as a loading control. Molecular weight markers are indicated. (B) Cell proliferation (measured by the detection of cleaved tetrazolium salts) was reduced by Rac1 inhibition ([ΔRaf1:ER]+tamoxifen [TMX]). *P*-values were calculated using ANOVA with Tukey’s post-hoc test (*n* = 3). Error bars indicate SD. (C) Levels of the cell cycle inhibitor p27kip1 were increased by Rac1 inhibition and were not restored by Erk1/2 activation ([ΔRaf1:ER]+TMX) in cultured murine VSMCs. Tubulin is shown as a loading control. Densiometric readings for p27kip1 bands and molecular weight markers (in kilodaltons) are indicated. (D) Images of automatic cell shape analysis. Phalloidin-stained VSMCs (blue) and DAPI-stained nuclei (green) were segmented. Arrows mark *Efnb2* knockout cells that express pEGFP-Tiam1 (yellow); arrowheads in the images at the right indicate rejected cells due to undetectable GFP expression. (E) Box-and-whiskers diagram of shape factor data of D. A dot marks the median, the box spans 30% of the values, and whiskers span 50% of the values. *P*-values were calculated using ANOVA and Tukey’s post-hoc test (*n* = 263; knockout [KO], *n* = 263; KO+pEGFP Tiam1, *n* = 232). Smaller shape factor of *Efnb2* cells was rescued by re-expression of Tiam1. (F) Re-expression of Tiam1 restored cell proliferation defects (measured by 2 h of EdU incorporation) in *Efnb2* knockout smooth muscle cells. *P*-values were calculated using ANOVA (*n* = 3). Error bars indicate SD. (G) Western blot showing increased Tiam1 protein after Erk1/2 inhibition (U0126) for 24 h. Total Erk1/2 and p-Erk1/2 bands, which were strongly reduced by U0126, are shown below. (H) Quantitative PCR (qPCR) of Tiam1 expression in control or *Efnb2* knockout VSMCs incubated with U0126 for 24 h. *P*-values were calculated using two-tailed Student’s *t*-test (*n* = 3). Error bars indicate SD. (I,J) Western blot (I) and qPCR results (J) showing reduced Tiam1 levels after Erk1/2 activation ([ΔRaf1:ER]+TMX) for the indicated times. *P*-values were calculated using two-tailed Student’s *t*-test (*n* = 3). Error bars indicate SD. (K) Tiam1 and ephrin-B2 protein levels were decreased after stimulation of VSMCs with EphB4/Fc for the indicated times (left), which was strongly reduced after administration of MG132 (right). (L) Densiometric analysis of data shown in K.
fractions, cultured murine VSMCs were analyzed by sucrose density gradient centrifugation. In the 11 fractions collected from control cells, ephrin-B2 and caveolin-1 were found together in fractions 3–6, which also contained the vast majority of PDGFRb [Fig. 4A]. In Efnb2 knockout VSMCs, the distribution of caveolin-1 appeared unaltered,
Figure 4. PDGFRβ membrane distribution depends on ephrin-B2. (A,B) Sucrose density gradient centrifugation of membrane fractions from control and Efnb2 knockout cells (fractions 1–11, top to bottom of gradient). (A) Note redistribution of PDGFRβ from caveolin-1-positive into CHC-containing fractions in Efnb2 knockout VSMCs. (B) Quantitation of PDGFRβ signals in fractions 1–11 (densiometric readings). Error bars indicate SD. (C) Coimmunoprecipitation of PDGFRβ with ephrin-B2 (in sucrose gradient fractions 3–6) was enhanced after stimulation with PDGF-B (5') from control but not Efnb2 knockout cells. (D) Immunofluorescence of ephrin-B2 (detected by EphB4/Fc; red), PDGFRβ (green), and caveolin-1 (blue). Individual channels of the insets are shown below the top panels. Arrowheads indicate colocalization of ephrin-B2, PDGFRβ, and caveolin-1. (E) Sucrose density gradient fractionation of PDGF-B-stimulated control and Efnb2 knockout VSMCs. Note the predominant distribution of p-PDGFRβ bands in fractions 8–11, which was enhanced in the absence of ephrin-B2, whereas weak phosphorylation was associated with the bulk of PDGFRβ in fractions 4–6. (F,G) The PDGFRβ immunosignal in VSMCs surrounding P8 retinal arteries is reduced in Efnb2ΔSMC mutants relative to littermate controls, whereas comparable signals were seen in capillary perivascular cells (shown in F), which are not targeted by SM22α-Cre transgenics. (G) Quantitation of PDGFRβ immunosignals. P-values were calculated using two-tailed Student’s t-test (n = 3). Error bars indicate SD.
while a significant portion of PDGFRβ had shifted to the bottom fractions [8–11] containing the CHC [Fig. 4A,B]. Indicating association of ephrin-B2 and PDGFRβ, both proteins were communoprecipitated from the pooled sucrose gradient fractions 3–6 of cultured control but not Efnb2 knockout VSMCs [Fig. 4C]. Arguing further for an ephrin-B2-dependent sequestering of PDGFRβ into caveolin-1-containing membrane fractions, immunofluorescence showed that signals for ephrin-B2 [detected by binding of EphB4/Fc], PDGFRβ, and caveolin-1 overlapped in spot-like structures in cultured control VSMCs [Fig. 4D]. In contrast, EphB4/Fc binding was absent in ephrin-B2-deficient cells, and little or no overlap was seen between PDGFRβ and caveolin-1 signals [Supplemental Fig. 4A].

PDGF-B-induced tyrosine phosphorylation and therefore activation of PDGFRβ was almost exclusively associated with the CHC-containing fractions 8–11, while the vast majority of total PDGFRβ protein remained in fractions 4–6 and showed only weak tyrosine phosphorylation [Fig. 4E]. In Efnb2 knockout cells, a significantly larger fraction of PDGFRβ was located in fractions 8–11, and only this pool showed substantial and, compared with the control, enhanced tyrosine phosphorylation [Fig. 4E]. Likewise, phospho-Erk1/2 and JNK were found in fractions 8–11 after sucrose gradient centrifugation [Supplemental Fig. 4B]. The notion that PDGFRβ signaling is associated with clathrin-mediated endocytosis was further supported by nystatin treatment of murine VSMCs. This inhibitor, which binds to cholesterol and disrupts caveolar-mediated endocytosis [Sakane et al. 2010], did not compromise PDGFRβ internalization but enhanced phosphorylation of PDGFRβ, JNK, and Erk1/2 in response to PDGF-B [Supplemental Fig. 4C,D].

Taken together, the above results argue that PDGFRβ signaling activity is primarily linked to the clathrin pathway in VSMCs. Ephrin-B2 interacts with PDGFRβ and directs the receptor into caveolin-1-containing membrane domains, which negatively control clathrin-mediated PDGFRβ endocytosis as well as downstream activation of JNK and MAP kinase. Further supporting that ephrin-B2 indeed counteracts clathrin-mediated PDGFRβ internalization and degradation not only in vitro [Fig. 3E,G], PDGFRβ immunostaining was significantly reduced in Efnb2ASMC retinal arterial smooth muscle cells in vivo [Fig. 4F,G]. Providing an internal control, anti-PDGFRβ signals in the untargeted pericytes were comparable in control and mutant retinas [Fig. 4F,G].

Given the important role of ephrin-B2 in internalization processes in endothelial cells, we tested whether the ligand might also direct the distribution of VEGF receptors to specific membrane domains. However, sucrose density gradient centrifugation of cultured murine endothelial cells failed to reveal overt alterations in VEGFR3 distribution in the absence of ephrin-B2 [Supplemental Fig. 4E,F].

Role of Eph–ephrin interactions
To investigate whether the loss of ephrin-B2 reverse signaling or the lack of ligand-induced [forward] EphB activation was responsible for the observed smooth muscle cell defects, we stimulated cultured VSMCs with ephrin-B2/Fc or EphB4/Fc fusion proteins. Remarkably, the stimulation of ephrin-B2 with EphB4/Fc not only triggered the internalization of ephrin-B2 but also led to pronounced clustering and endocytosis of surface PDGFRβ [Fig. 5A,B; Supplemental Fig. 5A]. In contrast, the stimulation of EphB receptors with recombinant ephrin-B2/Fc had no overt effect on PDGFRβ [Fig. 5A; data not shown]. The EphB4/Fc-induced internalization of PDGFRβ was not accompanied by appreciable tyrosine phosphorylation of the PDGFR and was largely unaffected by nystatin treatment [Fig. 5B,C]. Nystatin also had no substantial effect on EphB4/Fc-induced ephrin-B2 internalization, which argues against a crucial role of caveolae in this process [Fig. 5B,C].

PDGFRβ showed a substantial level of colocalization with EphB4/Fc fusion protein in VSMCs at different time points after stimulation [Fig. 5D; Supplemental Fig. 5B]. Further supporting a role in PDGFRβ internalization, a fraction of the EphB4/Fc-positive (i.e., ephrin-B2) and PDGFRβ-positive spots overlapped with EEA1, a marker of early endosomes [Fig. 5D]. Likewise, sucrose density gradient centrifugation confirmed that PDGFRβ protein was shifted into CHC-containing fractions [8–11] at 30 min after EphB4/Fc stimulation [Fig. 5E]. Following EphB4/Fc treatment and concomitant with the removal of ephrin-B2 from the cell surface, PDGF-B-mediated phosphorylation of PDGFRβ and Erk1/2 was gradually increased [Fig. 5F; Supplemental Fig. 5C], which resembled the enhanced activation of these molecules in Efnb2 knockout VSMCs [Fig. 3A]. In contrast, no appreciable alteration in Erk1/2 activation was seen in cultured cells treated with ephrin-B2/Fc [Supplemental Fig. 5D,E].

We showed previously that cultured and freshly isolated VSMCs express the receptors EphB2, EphB3, and EphB4, all of which can interact with ephrin-B2 [Foo et al. 2006]. The data above indicate that interactions with the local microenvironment, such as neighboring EphB receptor-presenting smooth muscle cells, can alter ephrin-B2 surface presentation and thereby strongly influence PDGFRβ internalization and signaling in VSMCs. Specifically, ephrin-B2-expressing cells that encounter high levels of EphBs in their direct environment would down-regulate Tiam1 and thereby Rac1 signaling [Fig. 2K], whereas PDGFRβ endocytosis as well as MAP kinase and JNK activation are concomitantly enhanced. We therefore propose that both the nature and quantity of signal transduction processes downstream from PDGFRβ are critically controlled by ephrin-B2 and its interactions with EphB receptors.

Discussion
Ephrin-B2 modulates PDGFRβ activity
RTKs can activate a variety of downstream signal transduction processes, leading to very different cellular behaviors, such as proliferation, migration, differentiation, or cell shape modulation. Previous work has shown that coreceptors, which interact with RTKs at the cell surface,
Figure 5. Eph–ephrin binding triggers PDGFRβ internalization. (A) Indirect immunofluorescence of PDGFRβ [green] in cultured murine VSMCs stimulated with IgG/Fc, ephrin-B2/Fc, or EphB4/Fc for 2 h, as indicated. (Red) Actin (phalloidin), (blue) nuclei [DAPI]. Note accumulation of PDGFRβ in perinuclear structures resembling vesicles after EphB4/Fc but not ephrin-B2/Fc treatment. The panels at the right show higher magnification of the insets. (B) Western blot showing reduction of biotinylated (surface) ephrin-B2 and PDGFRβ in cultured murine VSMCs treated with EphB4/Fc. Nystatin treatment had a mild effect on EphB4/Fc-induced PDGFRβ and ephrin-B2 internalization. (C) Densitometric analysis of biotinylated (surface) PDGFRβ shown in B. P-values were calculated using two-tailed Student’s t-test (n = 3). Error bars indicate SD. (D) Immunofluorescence of ephrin-B2 [detected by EphB4/Fc binding; red], PDGFRβ [green], and EEA1 [blue] in murine VSMCs at 0.5 and 2.5 h after EphB4/Fc treatment. Higher magnifications of the insets in the left images are shown in the other panels. Arrowheads indicate colocalization (white) of ephrin-B2, PDGFRβ, and EEA1 in early endosomes; arrows mark ephrin-B2⁺ and PDGFRβ⁺ but EEA1⁻ structures. (E) Sucrose density gradient centrifugation of VSMCs at 30 min after stimulation with IgG/Fc, EphB4/Fc, or EphB4/Fc+PDGF-B, as indicated. EphB4/Fc triggered redistribution of PDGFRβ from fractions 4–7 into fractions 8–11. Active PDGFRβ [p-PDGFRβ] at 5 min after stimulation with PDGF-B was associated with fractions 8–11. Molecular weight markers [in kilodaltons] are indicated. (F) Western blot showing that Erk1/2 and PDGFRβ phosphorylation in PDGF-B-stimulated VSMCs [10 min] was enhanced by pretreatment with EphB4/Fc for the indicated times. Total Erk1/2 and PDGFRβ levels and molecular weight markers [in kilodaltons] are shown. Statistical analysis of p-Erk1/2 is provided in Supplemental Fig. 5C.
can strongly influence their activity and integrate different signals from the local tissue environment. For example, low-density lipoprotein (LDL) receptor-related protein 1 (LRP1)—a transmembrane protein that can bind ligands as diverse as lipoproteins, proteases, growth factors, cytokines, and matrix proteins—associates with PDGFRβ and modulates its expression, internalization, and signaling (Boucher et al. 2003; Lehti et al. 2009; Muratoglu et al. 2010). Some controversy surrounds the exact role of LRP1, another member of the LDL receptor-related protein family, can also interact with PDGFRβ, promote its degradation, and reduce PDGF-induced smooth muscle cell proliferation. LRP6, a coreceptor for VEGFs in endothelial cells, has been found in VSMCs, where it promotes PDGF-induced smooth muscle cell migration. This is thought to involve an interaction with PDGFRα, the second member of the PDGF family (Pellet-Many et al. 2011). Here, we identified ephrin-B2 as a novel interaction partner of PDGFRβ, which modulates the membrane distribution, internalization from the cell surface, and signaling activity of this RTK (Fig. 6). Our data suggest that PDGFRβ-expressing cells can have different signaling outputs in response to PDGF depending on the presence and expression levels of ephrin-B2. In particular, the absence of ephrin-B2 strongly enhances PDGF-B-induced MAP kinase and JNK activation, whereas Tiam1/Rac1 signaling, a pathway critical for cell migration, proliferation, and spreading, gets diminished. Thus, ephrin-B2 can act as a molecular switch for different downstream signals induced by PDGF-B/PDGFRβ.

**Ephrin-B2 and surface receptor internalization**

Members of the large Eph/ephrin gene families appear to be expressed in virtually all cell types and tissues, which raises the question of how a large but nevertheless limited set of ligand–receptor interactions can be translated into highly diverse biological responses. An increasing body of evidence indicates that the functional versatility of Eph/ephrin molecules is achieved by molecular cross-talk with receptors from other families and, in particular, the modulation of their surface availability, clustering, trafficking, and endocytosis (Bethani et al. 2010; Pitulescu and Adams 2010). Among the surface molecules that are controlled by ephrin-B2, the VEGFRs VEGFR2 and VEGFR3 are most similar to PDGFRβ because all three RTKs share a similar organization of several extracellular immunoglobulin domains and a single cytoplasmic kinase domain and are evolutionarily closely related to Drosophila FVR (PDGF/VEGF receptor). While previous work has revealed that ephrin-B2 controls VEGFR2 and VEGFR3 internalization and signaling activity in vitro and in vivo, it is remarkable that these processes are positively regulated by the ephrin, which is the opposite of what we observed for PDGFRβ. It is currently unclear whether these differences reflect distinct molecular properties of PDGFRs and VEGFRs, cell type-specific features distinguishing endothelial cells and VSMCs, or the influence of ephrin-associated or independently acting additional regulators. Therefore, it will be important to identify the relevant molecular interaction partners of ephrin-B2 and PDGFRβ in caveolin-positive membrane domains as well as in the clathrin uptake machinery. Our findings also highlight that the precise role of ephrin-B2 in the modulation of other surface receptors can be complex and follow distinct mechanistic principles.

**Potential disease relevance**

Our data indicate that ephrin-B2 is a critical regulator of PDGF-B-induced signaling responses in vascular smooth muscle. While the phosphorylation of JNK and MAP kinase were strongly enhanced in *Efnb2* mutant aortae and cultured cells, the expression of the GEF Tiam1 and activation of Rac1 were diminished. These changes were associated with diminished VSMC proliferation and vessel wall defects in *Efnb2* mutant aortae and cultured cells. The expression of the GEF Tiam1 and activation of Rac1 were diminished. These changes were associated with diminished VSMC proliferation and vessel wall defects in *Efnb2* mutant aortae and cultured cells. While future work will have to address whether ephrin-B2 might be relevant in the context of VSMC-associated human diseases such as aortic aneurysms, our results suggest that the levels or availability of ephrin-B2 at the cell surface could potentially affect a variety of pathobiological processes involving PDGFs and their receptors (Ostman and Heldin 2007; Andrae et al. 2008). Examples include the autocrine and cell-autonomous PDGF-B/PDGFRβ signaling that promotes glioma growth and invasiveness in the brain (Uhrbom et al. 1998) or the PDGF-B overexpression in experimental cancer models, which induces the recruitment of vessels and stromal fibroblasts (Forsberg et al. 1993). High levels of PDGF expression and PDGFR signaling have been also associated with atherosclerotic lesions and the administration of neutralizing antibodies and...
aptamers can reduce smooth muscle cell proliferation and neointima formation in mice (Raines 2004; Andrae et al. 2008). PDGF-B/PDGFRβ signaling in hepatic stellate cells, renal fibroblasts, and myofibroblasts has been linked to fibrosis in the liver, kidney, and skin, respectively (Andrae et al. 2008). As these examples highlight the great clinical relevance of the PDGF pathway, it should be worthwhile to investigate whether the presence or absence of ephrin-B2, related B-class ephrins, or the corresponding EphB receptors might alter PDGFRβ surface availability, internalization, and signaling in pathological settings. Likewise, it remains to be resolved whether therapeutic modulation of ephrin-B2 expression might be beneficial in disease processes involving dysregulated PDGF-B/PDGFRβ activity.

Materials and methods

Loss-of-function genetics

SM22a-Cre (Lepore et al. 2005) mice were bred with Efnb2 conditional mice (Grunwald et al. 2004). CreERT2;Efnb2 lox/lox males and females were subsequently crossed with Efnb2 lox/lox females for experimental breedings. Cre-negative littermates were used as controls. Animals were in a mixed 129 X C57BI/6 genetic background. For examination of Cre activity, SM22a-Cre mice were bred to Rosa26-EYFP Cre reporter transgenics (Srinivas et al. 2001) and analyzed at the indicated stages. All animal experiments were performed in compliance with the relevant laws and institutional guidelines and were approved by local animal ethics committees.

Tissues and sections

For staining of retinas, eyes were removed and, for staining of paraffin sections of aorta, sections were deparaffinized and incubated in 1% hydrogen peroxide for 10 min after antigen unmasking. Then, sections were blocked with 5% goat serum in PBS (30 min at room temperature) prior to incubation with Cy3-conjugated anti-α-SMA antibody (1:400, Sigma, A2547) diluted in the blocking solution in combination with TO-PRO3 (1:1000, Invitrogen). Elastin was detected by autofluorescence on paraffin after staining with Cy3-conjugated anti-α-SMA antibody. Detection of ephrin-B2 on paraffin sections was performed with goat anti-ephrin-B2 antibodies (1:200, R&D Systems, AF496) as previously described (Battle et al. 2002) in combination with Cy3-conjugated anti-α-SMA antibody (1:400, Sigma, A2547). For the labeling of proliferating cells, P8 pups received intraperitoneal injections with 5-ethyl-2'-deoxyuridine (EdU, dissolved 2 mg/mL in PBS; Invitrogen). After 2 h, pups were humanely sacrificed, and aortas were dissected and fixed for 1 h on ice and paraffin-embedded. After anti-α-SMA-Cy3 staining, EdU was detected with a Click-it Edu Alexa Fluor 647 imaging kit (Invitrogen) according to the manufacturer’s instructions and counterstained with DAPI.

For Western blotting, aorta lysates were prepared in lysis buffer (0.1% SDS, 1% Triton X-100, 150 mM NaCl, 25 mM Tris-HCl at pH 7.5, 5 mM EDTA-NaOH at pH 8.5, 100 mM NaF, 10 mM Na2P2O7, 1 mM Na3VO4, protease inhibitor cocktail [1:100, Sigma, P2714]) by using a tissue homogenizer (Ultra-Turrax, IKA). The following antibodies were used: phospho-SAPK/JNK [1:1000; Cell Signaling, 9251], SAPK/JNK [1:1000; Cell Signaling, 9252], PDGFRβ [1:1000; Cell Signaling, 3169], phospho-PDGFRβ [Tyr751, 1:1000; Cell Signaling, 3161], phospho-PDGFRβ [Tyr716, 1:1000], ABNOVA, PAB1241), phospho-p42/44 MAPK [1:1000; Cell Signaling, 9106], p44/42 MAPK [1:1000, Cell Signaling, 9102], p27kip1 [1:1000; Becton Dickinson, 610242], Tiam1 [1:200; R&D Systems, AF5038], and tubulin [1:2000, Sigma, T3526].

Immunofluorescence

Control and Efnb2 knockout cells were plated on glass coverslips, washed in PBS, fixed in 4% PFA for 10 min on ice, washed in PBS, permeabilized with blocking buffer containing 0.1% Triton X-100 for 10 min, and incubated in blocking buffer (1% BSA in PBS) for 1 h at room temperature. Primary antibodies in blocking buffer were incubated overnight. Antibodies used were directed against PDGFRβ [1:50 dilution; R&D Systems, AF1042], caveolin-1 [1:250, Becton Dickinson, 610493], or EE1A [1:250; Abcam, ab2900]. The cells were then washed three times for 10 min with PBS and incubated with appropriate secondary antibodies covalently linked to Alexa Fluor 488 or Alexa Fluor 546 [1:500] for 1 h at room temperature in PBS and counterstained with Alexa Fluor 546-conjugated anti-phalloidin antibody and/or DAPI (1:1000, Sigma). To detect PDGFRβ on the surface of cultured VSMCs, cells were incubated with anti-PDGFRβ antibody without permeabilization. The number of PDGFRβ signals in 1 μm² was calculated from five cells from each group, three independent experiments were performed. To visualize ephrin-B2 in cultured VSMCs, 5 mg/mL EphB4/Fc (R&D Systems, 466-B4) was incubated with blocking solution. After washing with PBS, cells were incubated with Cy3-conjugated anti-human IgG/Fc [1:500, Jackson Laboratories, 109-165-008]. For the labeling of proliferating cells, cultures were incubated with Edu [dissolved 10 μM in culture medium, Invitrogen] for 2 h before staining, Edu was detected with a Click-it Edu Alexa Fluor 647 imaging kit (Invitrogen) according to the manufacturer’s instructions.
Images were recorded with a Leica SP5 confocal microscope and Volocity software [Improvision].

Cell stimulation experiments

Control [Efnb2 lox/lox] and Efnb2 knockout cells were seeded at a density of 3.0 × 10^5 in 6-cm dishes and incubated for 24 h. After overnight serum starvation, cells were then incubated in serum-free growth medium containing 10 ng/mL PDGF-B [R&D Systems, catalog no. 520-BB], 2 ng/mL LIF [Peprotech, 100-11], or 10 ng/mL TNF-α [R&D Systems, 2279-BT] at 37°C for the indicated times; collected with 300 μL of 1× SDS sample buffer; and subjected to immunoblotting with antibodies binding phosphoprotein A and G beads [GE Healthcare] and incubated with antibodies for 1 h at 4°C. Immunocomplexes were captured by adding 100 μL of prewashed protein A/G beads [GE Healthcare] and incubated for a further 2 h at 4°C on an orbital shaker. The beads were then collected by centrifugation, the supernatant was discarded, and beads were washed three times with lysis buffer. The beads were resuspended in 50 μL of sample buffer.

Rac1 pull-down assay

Cells were lysed in buffer (50 mM Tris/HCl at pH 7.5, 500 mM NaCl, 1 mM EGTA, 1% NP-40, 100 mM NaF, 10 mM Na3VO4, 1 mM Na4P2O7, protease inhibitor cocktail [1:100], Sigma, P2714]), and centrifuged. Aortae were lysed in buffer (0.1% SDS, 1% Triton X-100, 150 mM NaCl, 25 mM Tris/HCl at pH 7.5, 5 mM EDTA-NaOH at pH 8.5, 100 mM NaF, 10 mM Na3VO4, 1 mM Na4P2O7, protease inhibitor cocktail [1:100], Sigma, P2714]) by using a tissue homogenizer [Ultra-Turrax, IKA] and centrifuged. Supernatants were preclarified with uncoupled protein A or G beads [GE Healthcare] and incubated with antibodies for 1 h at 4°C. Immunocomplexes were captured by adding 100 μL of prewashed protein A/G and incubating for a further 2 h at 4°C on an orbital shaker. The beads were then collected by centrifugation, the supernatant was discarded, and beads were washed three times with lysis buffer. The beads were resuspended in 50 μL of sample buffer.

Sucrose density gradient centrifugation

Cells were dissolved in 0.8 mL of 500 mM sodium carbonate (pH 11) and homogenized with a 20-gauge needle and three 20-sec bursts of a sonicator. The sucrose concentration in cell extracts was adjusted to 45% by the addition of 0.8 mL of 90% sucrose prepared in MBS [25 mM Mes at pH 6.5, 0.15 M NaCl], and the extracts were placed at the bottom of an ultracentrifuge tube. A 5%–35% discontinuous sucrose gradient was formed above (4 mL of 35% sucrose/4 mL of 5% sucrose, both prepared in MBS containing 250 mM sodium carbonate) and centrifuged at 45,000 rpm for 16 h at 4°C in a SW55 Ti rotor (Beckman). From the top of each gradient, a total of 11 fractions (0.4 mL of each) were collected. Fractions were analyzed by immunoblotting with antibodies detecting caveolin-1 [1:500 dilution, Becton Dickinson, catalog no. 610493], CHC [1:1000], Becton Dickinson, 610500], EEA1 [1:1000, Novus Biologicals, NB50-0562], PDGFβ [1:1000], R&D Systems, 3169], phospho-PDGFRβ [1:1000, R&D System, 3161], ephrin-B2 [1:1000, Sigma, H6A008999], or VEGFR3 [1:1000, eBioscience, 45-5989].

Immunoprecipitation

Cells were lysed in 800 μL of buffer (25 mM Tris/HCl at pH 7.5, 150 mM NaCl, 1 mM EGTA, 1% NP-40, 100 mM NaF, 10 mM Na3VO4, 1 mM Na4P2O7, protease inhibitor cocktail [1:100], Sigma, P2714]), and centrifuged. Aortae were lysed in buffer [0.1% SDS, 1% Triton X-100, 150 mM NaCl, 25 mM Tris/HCl at pH 7.5, 5 mM EDTA-NaOH at pH 8.5, 100 mM NaF, 10 mM Na3VO4, 1 mM Na4P2O7, protease inhibitor cocktail [1:100], Sigma, P2714]) by using a tissue homogenizer [Ultra-Turrax, IKA] and centrifuged. Supernatants were preclarified with uncoupled protein A or G beads [GE Healthcare] and incubated with antibodies for 1 h at 4°C. Immunocomplexes were captured by adding 100 μL of prewashed protein A/G and incubating for a further 2 h at 4°C on an orbital shaker. The beads were then collected by centrifugation, the supernatant was discarded, and beads were washed three times with lysis buffer. The beads were resuspended in 50 μL of sample buffer.

Automatic cell shape analysis

Electroporation was performed by using the Amaxa Nucleofection system. Nonconfluent cells were trypsinized, washed in medium, and resuspended at a concentration of 1 × 10^6 cells per 100 μL in an Amaxa Basic Nucleofector kit. Two micrograms of plasmid DNA was then mixed with 100 μL of cells and placed in a cuvette. After electroporation by using setting T-030, cells were directly transferred to warm medium. Control or pEGFP-tagged full-length Tiam1 transfected cells were seeded on six-well dishes, fixed, and stained with Alexa Fluor 546 phallolidin [Invitrogen, 1:500], Alexa Fluor 488-coupled anti-CFP antibody [Invitrogen, 1:500], and DAPI. The images were automatically analyzed by a custom-developed image-processing journal detecting the DAPI-stained nuclei followed by detection of cell outlines (using the Alexa Fluor 488 image) separating touching outlines [using the Alexa Fluor 488 image] separating touching outlines.
cells with watershed lines. Cells touching the border were rejected. A shape factor \(4 \times \pi \times \text{area}/\text{perimeter}^2\) was calculated for each cell.

**Microarray and quantitative PCR analysis**

RNA was isolated from control and Efnb2 knockout cells after 3 d of cultivating in IFN-γ-free cell medium using the RNeasy kit (Qiagen) following the manufacturer’s guidelines. Generation of gene expression profiles for control and Efnb2 knockout cells was carried out by the GeneChip microarray service at the Paterson Institute for Cancer Research using Affymetrix moe430A 2.0 chips.

For real-time PCR, cDNA synthesis was achieved from 500 ng of total RNA by using the iScript cDNA synthesis kit (Bio-Rad) following the manufacturer’s guidelines. Then, TaqMan gene expression assays (Applied Biosystems) for murine GAPDH and Tiam1 were used in combination with TaqMan gene expression master mix.

**Quantification and image processing**

Velocity (Improvision), Photoshop CS, and Illustrator CS (Adobe) software was used for image processing without distorting or misrepresenting results. Data were based on at least three independent experiments or three mutant and control animals for each stage and result shown.

**Competing interest statement**

The authors declare that they have no competing financial interests.

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**References**

Abella JV, Park M. 2009. Breakdown of endocytosis in the oncogenic activation of receptor tyrosine kinases. Am J Physiol Endocrinol Metab 296: E973–E984.

Andrae J, Gallini R, Betsholtz C. 2008. Role of platelet-derived growth factors in physiology and medicine. Genes Dev 22: 1276–1312.

Batlle E, Henderson JT, Beghtel H, van den Born MM, Sancho E, Huls G, Meeldijk J, Robertson J, van de Wetering M, Pawson T, et al. 2002. β-Catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. Cell 111: 251–263.

Bethani I, Skanland SS, Dikic I, Acker-Palmer A. 2010. Spatial organization of transmembrane receptor signalling. EMBO J 29: 2677–2688.

Bond M, Wu YJ, Sala-Newby GB, Newby AC. 2008. Rho GTPase, Rac1, regulates Skp2 levels, vascular smooth muscle cell proliferation, and intima formation in vitro and in vivo. Cardiovasc Res 80: 290–298.

Boucher P, Gotthardt M, Li WP, Anderson RG, Herz J. 2003. LRP: Role in vascular wall integrity and protection from atherosclerosis. Science 300: 329–332.

Chow JC, Condorelli G, Smith RJ. 1998. Insulin-like growth factor-I receptor internalization regulates signaling via the Shc/mitogen-activated protein kinase pathway, but not the insulin receptor substrate-1 pathway. J Biol Chem 273: 4672–4680.

Di Guglielmo GM, Le Roy C, Goodfellow AF, Wrana JL. 2003. Distinct endocytic pathways regulate TGF-β receptor signaling and turnover. Nat Cell Biol 5: 410–421.

Finger EC, Lee NY, You HJ, Blobе GC. 2008. Endocytosis of the type III transforming growth factor-β (TGF-β) receptor through the clathrin-independent/lipid raft pathway regulates TGF-β signaling and receptor down-regulation. J Biol Chem 283: 34808–34818.

Foo SS, Turner CJ, Adams S, Compagni A, Aublyn D, Kogata N, Lindblom P, Shani M, Zicha D, Adams RH. 2006. Ephin-B2 controls cell motility and adhesion during blood-vessel-wall assembly. Cell 124: 161–173.

Forsberg K, Valyi-Nagy I, Heldin CH, Herlyn M, Westermark B. 1993. Platelet-derived growth factor (PDGF) in oncogenesis: Development of a vascular connective tissue stroma in xenotransplanted human melanoma producing PDGF-BB. Proc Natl Acad Sci 90: 393–397.

Fujita Y, Maruyama S, Kogo H, Matsuo S, Fujimoto T. 2004. Caveolin-1 in mesangial cells suppresses MAP kinase activation and cell proliferation induced by bFGF and PDGF. Kidney Int 66: 1794–1804.

Grunwald IC, Korte M, Adelmann G, Plueck A, Kullander K, Lindahl P, Shani M, Zicha D, Trillos G, Lifton RP, Mani A. 2011. Wild-type LRP6 inhibits, whereas atherosclerosis-linked LRP6E611C increases PDGF-dependent vascular smooth muscle cell proliferation. Proc Natl Acad Sci 108: 1914–1918.

Kumari S, Mg S, Mayor S. 2010. Endocytosis unplugged: Multiple ways to enter the cell. Cell Res 20: 256–275.

Lanahan AA, Hermans K, Claes F, Kerley-Hamilton JS, Zhuang ZW, Giordano FJ, Carmeliet P, Simons M. 2010. VEGF receptor 2 endocytic trafficking regulates arterial morphogenesis. Dev Cell 17: 713–724.

Lehti K, Rose NF, Valavaara S, Weese SJ, Keski-Oja J. 2009. MT1-MMP promotes vascular smooth muscle dedifferentiation through LRPI processing. J Cell Sci 122: 126–135.

Leopore JJ, Cheng L, Min Lu M, Mericko PA, Morrisey EE, Parmacek MS. 2005. High-efficiency somatic mutagenesis in mice. Proc Natl Acad Sci 102: 1713–1720.

Lindahl P, Johansson BR, Leveen P, Betsholtz C. 1997. Pericyte recruitment and turnover. Trends Cell Biol 7: 33–40.

Mertens AE, Pegtel DM, Collard JG. 2006. Tiam1 takes PARt in cell polarity. Trends Cell Biol 16: 308–316.
Moes JJA, Zhou Y, Boomstra J. 2012. Co-localization of the PDGF β-receptor and actin during PDGF stimulation in mouse fibroblasts. ISRN Cell Biology 2012: doi: 10.5402/2012/568104.

Morcavallo A, Genua M, Palummo A, Kletvikova E, Jiracek J, Brzozowski AM, Izollo RV, Belfiore A, Morrione A. 2012. Insulin and insulin-like growth factor II differentially regulate endocytic sorting and stability of insulin receptor isoform A. J Biol Chem 287: 11422–11436.

Muratoglu SC, Mikhailenko I, Newton C, Migliorini M, Strickland DK. 2010. Low density lipoprotein receptor-related protein 1 (LRP1) forms a signaling complex with platelet-derived growth factor receptor-β in endosomes and regulates activation of the MAPK pathway. J Biol Chem 285: 14308–14317.

Nakayama M, Nakayama A, van Lessen M, Yamamoto H, Hoffmann S, Drexler HC, Itoh N, Hirose T, Breier G, Vestweber D, et al. 2013. Spatial regulation of VEGF receptor endocytosis in angiogenesis. Nat Cell Biol 15: 249–260.

Olson LE, Soriano P. 2011. PDGFRb signaling regulates mural cell plasticity and inhibits fat development. Dev Cell 20: 815–826.

Ostman A, Heldin CH. 2007. PDGF receptors as targets in tumor treatment. Adv Cancer Res 97: 247–274.

Pellet-Many C, Frankel P, Evans IM, Herzog B, Junemann-Ramirez M, Zachary IC. 2011. Neuropilin-1 mediates PDGF stimulation of vascular smooth muscle cell migration and signalling via p130Cas. Biochem / 435: 609–618.

Pitulescu ME, Adams RH. 2010. Eph/ephrin molecules—a hub for signaling and endocytosis. Genes Dev 24: 2480–2492.

Raines EW. 2004. PDGF and cardiovascular disease. Cytokine Growth Factor Rev 15: 237–254.

Sadowski L, Jastrzebski K, Kalaidzidis Y, Heldin CH, Hellberg C, Miaczynska M. 2013. Dynamin inhibitors impair endocytosis and mitogenic signaling of PDGF. Traffic 14: 725–736.

Sakane H, Yamamoto H, Kikuchi A. 2010. LRP6 is internalized by Dkk1 to suppress its phosphorylation in the lipid raft and is recycled for reuse. J Cell Sci 123: 360–368.

Sawamiphak S, Seidel S, Essmann CL, Wilkinson GA, Pitulescu ME, Acker T, Acker-Palmer A. 2010. Ephrin-B2 regulates VEGFR2 function in developmental and tumour angiogenesis. Nature 465: 487–491.

Srinivas S, Watanabe T, Lin CS, William CM, Tanabe Y, Jessell TM, Costantini F. 2001. Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev Biol 1: 4.

Sunberg C, Friman T, Hecht LE, Kuhl C, Solomon KR. 2009. Two different PDGF β-receptor cohorts in human pericytes mediate distinct biological endpoints. Am J Pathol 175: 171–189.

Tallquist MD, French WJ, Soriano P. 2003. Additive effects of PDGF receptor β signaling pathways in vascular smooth muscle cell development. PLoS Biol 1: E52.

Thiel G, Ekici M, Rossler OG. 2009. Regulation of cellular proliferation, differentiation and cell death by activated Raf. Cell Commun Signal 7: 8.

Uhbröm L, Hesselager G, Nister M, Westermark B. 1998. Induction of brain tumors in mice using a recombinant platelet-derived growth factor B-chain retrovirus. Cancer Res 58: 5275–5279.

Veira AV, Lamaze C, Schmid SL. 1996. Control of EGF receptor signaling by clathrin-mediated endocytosis. Science 274: 2086–2089.

Wang Y, Nakayama M, Pitulescu ME, Schmidt TS, Bochenek ML, Sakakibara A, Adams S, Davy A, Deutsch U, Luthi U, et al. 2010. Ephrin-B2 controls VEGF-induced angiogenesis and lymphangiogenesis. Nature 465: 483–486.

Yamamoto H, Sakane H, Michiue T, Kikuchi A. 2008. Wnt3a and Dkk1 regulate distinct internalization pathways of LRP6 to tune the activation of β-catenin signaling. Dev Cell 15: 37–48.

Yoshimura K, Aoki H, Ikeda Y, Fujii K, Akiyama N, Furutani A, Hoshii Y, Tanaka N, Ricci R, Ishihara T, et al. 2005. Regression of abdominal aortic aneurysm by inhibition of c-Jun N-terminal kinase. Nat Med 11: 1330–1338.

Yu A, Rual JE, Tamai K, Harada Y, Vidal M, He X, Kirchhausen T. 2007. Association of Dishevelled with the clathrin AP-2 adaptor is required for Frizzled endocytosis and planar cell polarity signaling. Dev Cell 12: 129–141.

Zhou L, Takayama Y, Boucher P, Tallquist MD, Herz J. 2009. LRP1 regulates architecture of the vascular wall by controlling PDGFRβ-dependent phosphatidylinositol 3-kinase activation. PLoS ONE 4: e6922.