Cross-family Signaling: PDGF Mediates VEGFR Activation and Endothelial Function

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Abstract

A recently discovered but unexplored mechanism of angiogenesis regulation is cross-family binding between platelet-derived growth factors (PDGFs) and vascular endothelial growth factor receptors (VEGFRs), which suggests a novel therapy for addressing vascular dysregulation. This study elucidates the role of PDGFs in endothelial cell (EC) signaling and functions, focusing on VEGFR activation. Using human dermal microvascular ECs (HDMECs) with double knockout of PDGFRα/β and human brain microvascular ECs (HBMECs), we show three key findings: (1) PDGF-AA and -BB induced VEGFR1 phosphorylation, peaking at 2-fold increases at low concentrations (0.5 ng/mL), while PDGF-AB stimulated a 2-fold rise in VEGFR2 phosphorylation. (2) Downstream effectors PLCγ1, Akt, and FAK were activated by all three PDGFs at levels comparable to VEGF-A, achieving approximately 70% of VEGF-A's effects. (3) PDGF-BB significantly enhanced EC proliferation (up to 240%) and migration (up to 170%), with lower PDGF concentrations (0.5–5 ng/mL) eliciting stronger effects than higher concentrations (50–100 ng/mL). Overall, PDGF subtypes differentially induce VEGFR phosphorylation, downstream effector activation, and angiogenic hallmarks such as proliferation and migration, revealing novel mechanisms for regulating endothelial function.

Introduction

Angiogenesis, the formation of new blood vessels from existing vascular systems, is central to several physiological conditions and supports tissue growth in development, wound healing, or cancer [1]. The process is primarily regulated by vascular endothelial growth factors (VEGFs) and platelet-derived growth factors (PDGFs), which bind to their respective receptors, VEGFRs and PDGFRs, which we describe as a uni-family model, initiating downstream angiogenic signaling. Dysregulated angiogenesis can lead to over 70 vascular diseases and cancers [2, 3]. Current therapies for excessive angiogenesis targeting VEGFs have demonstrated limited efficacy [4]. The anti-VEGF-A antibody resulted in transitory improvements, such as tumor stasis and shrinkage. However, after a period of weeks to months, the tumors typically resume their path of growth, and the disease progresses [5]. Surprisingly, tumors resistant to anti-VEGF treatment showed PDGF overexpression [4–6]. This finding suggests the involvement of other molecules in the pathological process beyond the commonly recognized uni-family VEGF-VEGFR signaling axis.

A novel paradigm of cross-family signaling has been proposed and identified between VEGF and PDGF families [7]. The two families share similarities structurally and functionally for both ligands (VEGFs and PDGFs) and receptors (VEGFRs and PDGFRs) [8–11]. The ligands bind to and activate their respective receptors in a similar way, followed by dimerization and tyrosine phosphorylation [12–14]. Studies in literature have established evidence of cross-family signaling. The bindings of VEGF-to-PDGFR and PDGF-to-VEGFR were both discovered with high affinities [7], indicating a complex interplay between these growth factor families. VEGF-A has

shown the ability to activate PDGFRs in a dose-dependent manner, independently of VEGFRs [15]. Investigations in human dermal fibroblasts lacking VEGFRs have revealed that VEGF-A can modulate surface PDGFR levels and inhibit PDGF ligand interactions [15, 16]. Furthermore, PDGF-CC, a homodimer structurally similar to VEGF-A [17], has displayed the capacity to stimulate EC migration at a level comparable to VEGF-A [18]. Treatment of ECs with PDGFs has been associated with reduced VEGFR2 expression [19]. Computational models have predicted that PDGFs contribute significantly to VEGFR2 ligation in both healthy conditions and breast cancer [7]. Taken together, these data underscore the potential impact of VEGF-PDGF cross-family signaling in angiogenesis regulation. However, the resulting functional effects of PDGF-to-VEGFR binding have not been fully elucidated.

In this study, we aimed to reveal the role of PDGFs in regulating endothelial signaling and function. To achieve this, we used ECs that do not express PDGFRs on the plasma membrane to exclude the effects of PDGF signaling through PDGFRs. As comparative control, we also used HDFs lacking VEGFRs but expressing PDGFRs to elucidate the functional effects of PDGF signaling through VEGFRs and PDGFRs. To exclude the possible VEGF upregulation in the treatment medium, we validated the VEGF-A protein concentrations in the cell culture supernatant followed by PDGF stimulations compared with untreated conditions. Our comprehensive investigation assessed the activation of VEGFRs and their downstream effectors, i.e., PLCγ1, Akt, and FAK, and examined the resulting functional roles in angiogenesis, such as cell proliferation and migration. Through our research, we aim to shed light on alternative mechanisms that could potentially address drug resistance issues observed in anti-VEGF therapies, offering new insights into overcoming vascular dysregulation.

Material and Methods

Cell culture

Primary human brain microvascular endothelial cells (HBMECs) (Cell Systems, ACBRI 376) were cultured in Complete Classic Medium (4Z0-500) supplemented with CultureBoost and Bac-Off Antibiotic (Cell Systems, 4Z0-644). Primary human dermal microvascular endothelial cells (HDMECs) (ATCC, CRL-4060) *PDGFRA* and *PDGFRB* double knock out (DKO) (to eliminate PDGFRα and β expression, respectively) via CRISPR/Cas9 at the Genome Engineering & Stem Cell Center at Washington University in St. Louis, MO. The efficiency of DKO was validated by next-generation sequencing (NGS) analysis (cite my qFlow paper). HDMECs ($PDGFRA^{-/-}$ and $PDGFRB^{-/-}$) were cultured in Vascular Cell Basal Medium (ATCC, PCS100030) supplemented with microvascular endothelial cell growth kit -VEGF (i.e., with VEGF; ATCC, PCS110041) and penicillin streptomycin (10,000 U/mL) (Thermo Fisher Scientific, 15-140-122). Human dermal fibroblasts (HDFs) were purchased from ATCC (PCS-201-010) and cultured in DMEM (Thermo Fisher Scientific, 11995065) with 5% FBS (Sigma-Aldrich, 12306C) and Penicillin Streptomycin (10,000 U/mL) (Thermo Fisher Scientific, 15-140-122). Cells in passages 3–6 were used and maintained in a humidified incubator at 37 °C and with 5% CO_2. Low-serum medium used in this study for cell starvation was made from EBM-2 (Lonza, CC-3156) supplemented with 1% FBS, Bac-Off Antibiotic (Cell Systems, 4Z0-644), and necessary components minus growth factors (hydrocortisone, ascorbic acid, GA-1000, heparin).

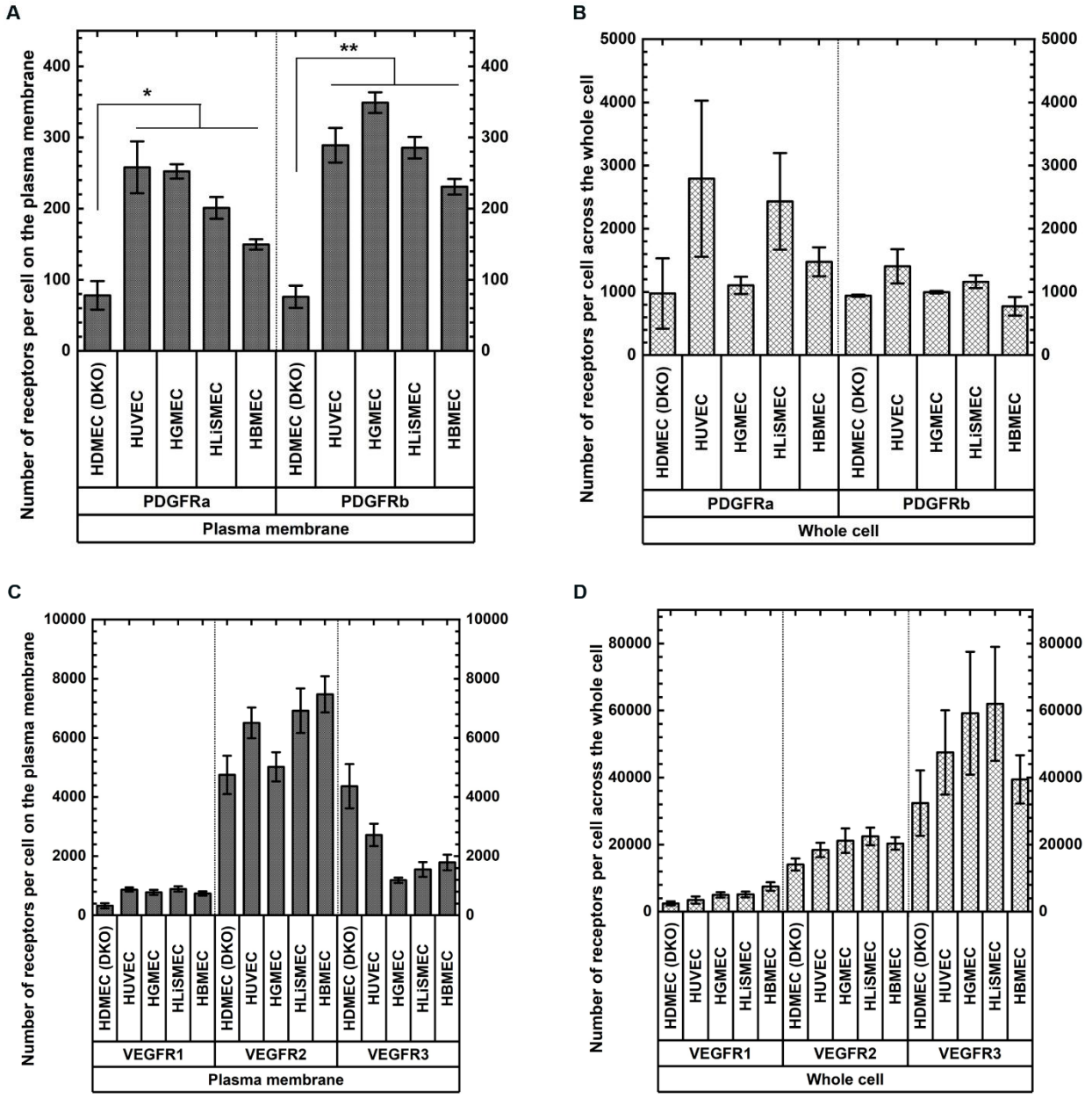

Figure 1. ECs expressed few to no PDGFRα or PDGFRβ on either plasma membrane (A) and low levels in the whole cell (B). The numbers of PDGFRs resulted from nonspecific binding between PDGFR antibodies and PDGFRs. Error bars represent the standard error of mean (SEM). (*p<0.05, **p<0.01, ***p<0.001 as determined by one-way ANOVA and multi-comparison post-hoc Tukey's test.) The same method was used to calculate all p-values in this study.

Proliferation assay

Cells were seeded in a 96-well plate and grown to approximately 50% confluence and serum-starved overnight using low-serum medium. Cells were stimulated with specific ligands, PDGF-AA, -AB, -BB, or VEGF-A (R&D Systems, 221-AA-050, 222-AB-050, 220-BB-050, 293-VE-050/CF; same materials were used throughout the study) at specific concentrations for 24 hours. XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) (Invitrogen, X6493) was dissolved in EBM-2 (Lonza, CC-

3156) at 1 mg/mL and activated by PMS (N-methyl dibenzopyrazine methyl sulfate) (0.0075 mg/mL). The reagent was added to each well and mixed with pipetting. The plates were incubated at 37 °C for 3 hours in the dark. The absorbance was read at 450 and 650 nm. The final OD value is [Abs_{450nm}(Test) – Abs_{450nm}(Blank)] – Abs_{660nm}(Test) and normalized to the untreated control.

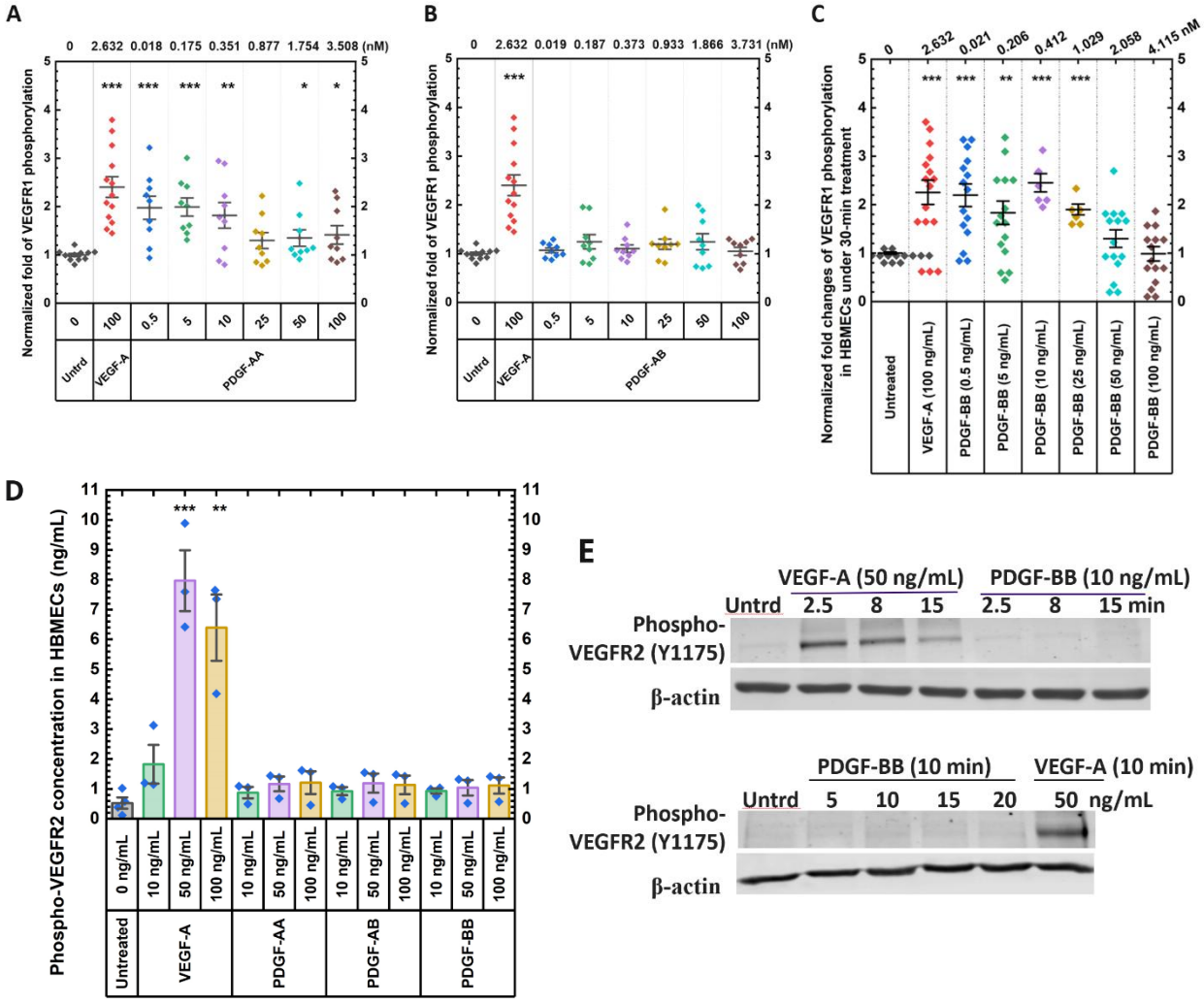

Figure 2. VEGFR1 was significantly phosphorylated by low concentrations of PDGF-AA and –BB in HBMECs, whereas no PDGFs induced VEGFR2 phosphorylation (Ty1175) in HBMECs. HBMECs were starved with low-serum medium for 6 hours and stimulated with growth factors at the indicated concentrations for 30 min (VEGFR1) or 10 min (VEGFR2). The receptor activation was picked up either by ELISA detecting the pan-tyrosine phosphorylation of VEGFR1 **(A–C)** or VEGFR2 **(D)**, or by immunoblotting and probed with primary antibody targeting VEGFR2 phosphorylation at tyrosine site 1175 **(E)**.

Migration assay

Cells were cultured in T-75 flasks until they reached approximately 90% confluence. Subsequently, the cells were serum-starved overnight using low-serum medium. Transwell inserts (Falcon, 353097) were coated with 2.45 µg/mL Collagen type I (Corning, 354236) in PBS and incubated overnight at 4°C. After starvation, the cells were detached and seeded in the top chamber of the transwell inserts at a density of 1

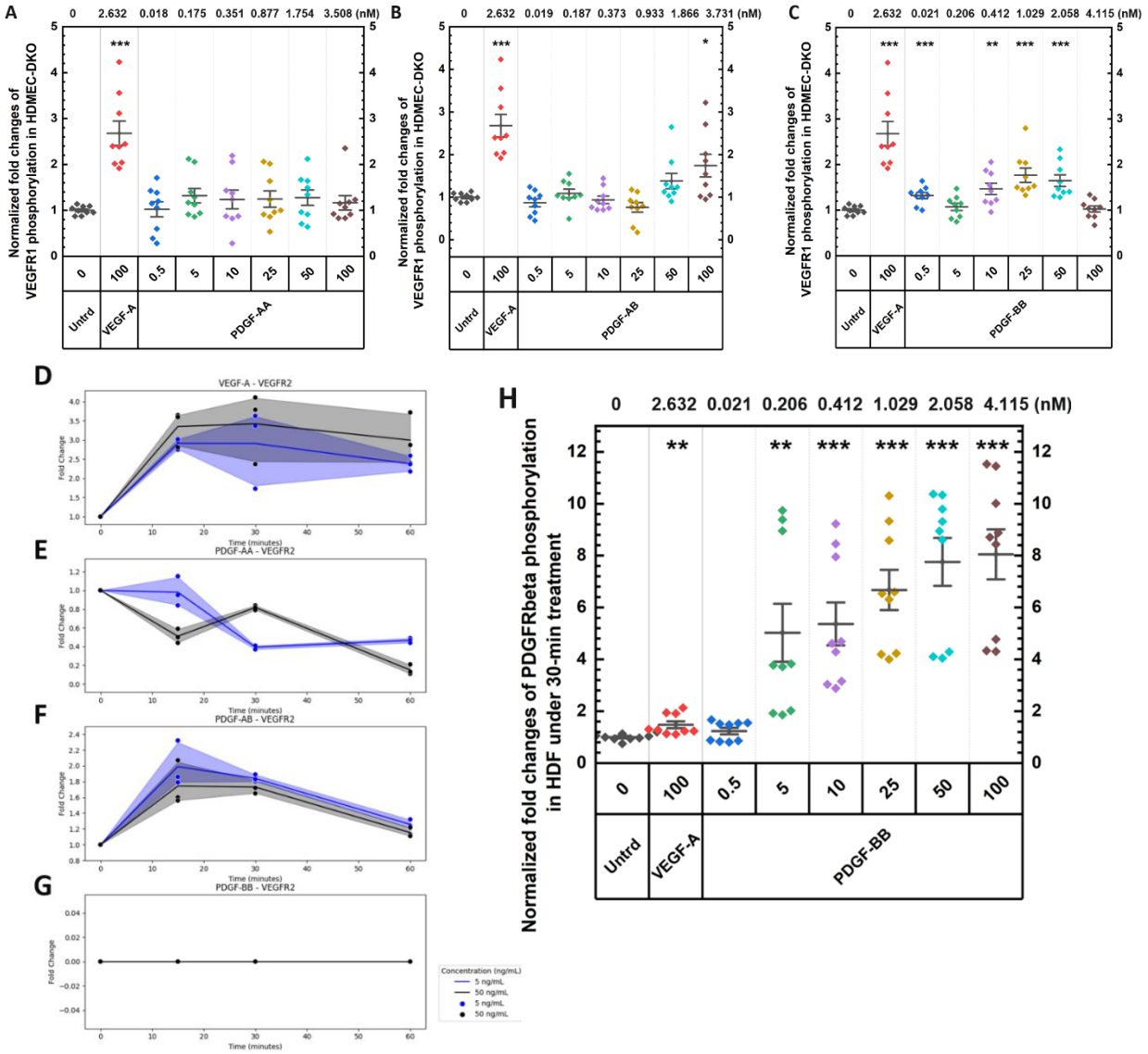

Figure 3. High concentrations of PDGF-BB induced VEGFR1 phosphorylation in HDMECs (*PDGFRA*$^{-/-}$ and *PDGFRB*$^{-/-}$), whereas low concentrations of PDGF-AB activated VEGFR2. (A–G) HDMEC (*PDGFRA*$^{-/-}$ and *PDGFRB*$^{-/-}$) were starved with low-serum medium for 6 hours and stimulated with growth factors at the indicated concentrations for 30 min (VEGFR1) or 10 min (VEGFR2). **(A–C)** The fold changes of VEGFR1 phosphorylation induced were assessed via ELISA. **(D–G)** The fold changes of VEGFR1 phosphorylation induced were assessed via immunoblotting. **(H)** The fold changes of PDGFRβ phosphorylation in HDFs induced by 30-min VEGF-A or PDGF-BB treatment were measured via ELISA.

$\times 10^5$ cells/well. The bottom chamber contained growth factors at the specified concentrations in low-serum medium. The cultures were then incubated for 24 hours at 37 °C with 5% CO_2. Following incubation, the unmigrated cells on the upper surface of the chamber were gently removed using a cotton swab. The inserts were subsequently washed with PBS. The cells that had migrated through the membrane to the lower side of the insert were fixed with 4% paraformaldehyde, permeabilized with 0.1% Tween-20, and stained with a 0.01% Hoechst 3342 solution (Invitrogen, H3570). After a final wash with PBS, the inserts were imaged

under the DAPI channel (EVOS M5000). Cell migration was quantified using ImageJ (1.52a) as the number of migrated cells to the downside of the inserts.

Enzyme-Linked Immunosorbent Assay (ELISA) / Immunology for phosphorylation detection

Cells were cultured till approximately 90% confluence and processed serum starvation for 6 hours using low-serum medium. The indicated growth factors were added into the medium and incubated for 30 minutes for detecting phosphor-VEGFR1 and phosphor-PDGFRβ or 10 minutes for detecting phosphor-VEGFR2. Ttreatment medium was aspirated to terminate the stimulation, and the plates were washed with ice-cold phosphate-buffered saline (PBS). For ELISA, cell lysates were collected with lysis buffer (R&D Systems, DYC002) with protease and phosphatase inhibitor cocktail (Sigma-Aldrich, P8340). After being briefly vortexed and centrifuged at 12,000×*g* for 10 min at 4 ℃, the cell lysates went through the protocol of Human Phospho-VEGFR1/Flt-1 DuoSet IC ELISA kit (R&D Systems, DYC4170), Human Phospho-VEGFR2/KDR DuoSet IC ELISA kit (R&D Systems, DYC1766), or Human Phospho-PDGFRβ DuoSet IC ELISA kit (R&D Systems, DYC1767) to assess the phosphorylation levels of VEGFR1, VEGFR2, or PDGFRβ, respectively. The protein mass was quantified through bicinchoninic acid assay (BCA) (Pierce Biotech, 23227). The results were normalized to untreated control samples and statistically analyzed.

For immunoblotting, the cells were scraped in ice-cold 1× RIPA lysis buffer (EMD Millipore, 20-188) supplemented with protease and phosphatase inhibitor cocktails (Sigma-Aldrich, 11836170001 and 4906837001). The cell lysates were cleared by centrifuge at 12,000×*g* for 10 min at 4 ℃, mixed with 6 × SDS buffer (Boston BioProducts, BP-111R), denatured at 95 ℃ for 10 min, resolved on 4–15% SDS-PAGE gels (Bio-Rad, 4561084), and transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked with 5% non-fat dry milk dissolved in 1× Tris Buffered Saline (TBS) for 1 hour at room temperature before adding primary antibodies (Supplementary Table 1) overnight at 4 ℃. Secondary antibodies were added and incubated for 20 min at room temperature. Protein signals were detected using the LI-COR Odyssey CLx Imager (LI-COR Biosciences) and quantified using ImageJ Software (2.14.0/1.54f) or Image Studio 6.0 (LI-COR Biosciences). The protein mass was quantified through bicinchoninic acid assay (BCA) (Pierce Biotech, 23227).

Enzyme-Linked Immunosorbent Assay (ELISA) for VEGF-A secretion detection

Cells were cultured till approximately 90% confluence and processed serum starvation for 6 hours using low-serum medium. The indicated growth factors were added into the medium and incubated for 10 min, 30 min, or 24 hours. The cell culture supernatant was collected and centrifuged at 1000×*g* for 5 min. The concentration of VEGF-A in the cell culture supernatant was detected with Human VEGF ELISA Kit - Quantikine (R&D Systems, SVE00) following the instructions. The results were normalized to untreated control samples and statistically analyzed.

Quantitative flow cytometry

ECs were seeded in T-75 flasks and grown to approximately 80% confluence. The cells were washed with Ca^{2+}/Mg^{2+}-free PBS, harvested in Corning CellStripper solution, a nonenzymatic cell-dissociation solution, and incubated for 15 min at 37 ℃ and 5% CO_2. The disassociation was stopped by adding Ca^{2+}/Mg^{2+}-free PBS, and the cell suspension was centrifuged twice at 400×*g* for 5 min at 4 ℃. The cells were resuspended at 2–4 ×10^6 cells/mL in ice-cold stain buffer (PBS, bovine serum albumin, sodium azide). We added 25-μL aliquots of a single-cell suspension for plasma membrane staining and 50 μL for whole-cell staining to 96-well deep-well plates. The appropriate amount of human VEGFR1 phycoerythrin (PE)-conjugated antibody (R&D Systems, FAB321P), VEGFR2 PE-conjugated antibody (BioLegend, 359904), VEGFR3 PE-conjugated antibody (R&D Systems, FAB3492P), PDGFRα PE-conjugated antibody (R&D Systems,

FAB1264P), or PDGFRβ PE-conjugated antibody (R&D Systems, FAB1263P) was added to each sample well except the fluorescence-minus-one (FMO) control. The saturating concentrations of PE-conjugated antibodies to detect receptors on the plasma membrane were previously determined at 14 µg/mL for VEGFR1, 2, and 3; and 9.4 µg/mL for PDGFRα and PDGFRβ [16, 20]. For whole-cell receptor detection, the saturating concentration of VEGFR1 and VEGFR2 antibodies was determined at the beginning of the study to be 20 ug/mL for both. Samples were incubated in dark for 40 min at 4 °C and washed twice with stain buffer by centrifugation at 400×*g* for 5 min at 4 °C. Samples for plasma membrane receptor measurement were washed and resuspended in 100 µL stain buffer before analysis.

Samples for whole-cell receptor measurement were fixed using 2% paraformaldehyde for 20 min in the dark, washed, and permeabilized (0.5% Tween-20) for 20 min in the dark. Samples were washed twice with stain buffer containing 0.1% Tween-20. PE-conjugated antibodies were added to the samples for staining for 40 min in the dark, followed by three washes with stain buffer containing 0.1% Tween-20. Washed samples for whole-cell receptor measurement were resuspended in 50 µL stain buffer. The readouts were acquired by a Cytoflex S flow cytometer (Beckman Coulter, IN) and CytExpert software (Beckman Coulter, IN). Kaluza software (Beckman Coulter, IN) was used for data analysis. Quantibrite PE beads (BD Biosciences, 340495) were collected and analyzed under the same compensation and voltage settings as cell fluorescence data. Quantibrite PE beads are a mixture of beads with four different concentrations of conjugated PE molecules: low (474 PE molecules/bead), medium-low (5359 PE molecules/bead), medium-high (23,843 PE molecules/bead), and high (62,336 PE molecules/bead). A calibration curve that translated PE geometric mean to the number of bound molecules was determined using linear regression, $y = mx + c$, where y is $\log_{10}$ fluorescence and x is $\log_{10}$ PE molecules per bead. Receptor levels were calculated as described previously [20–23].

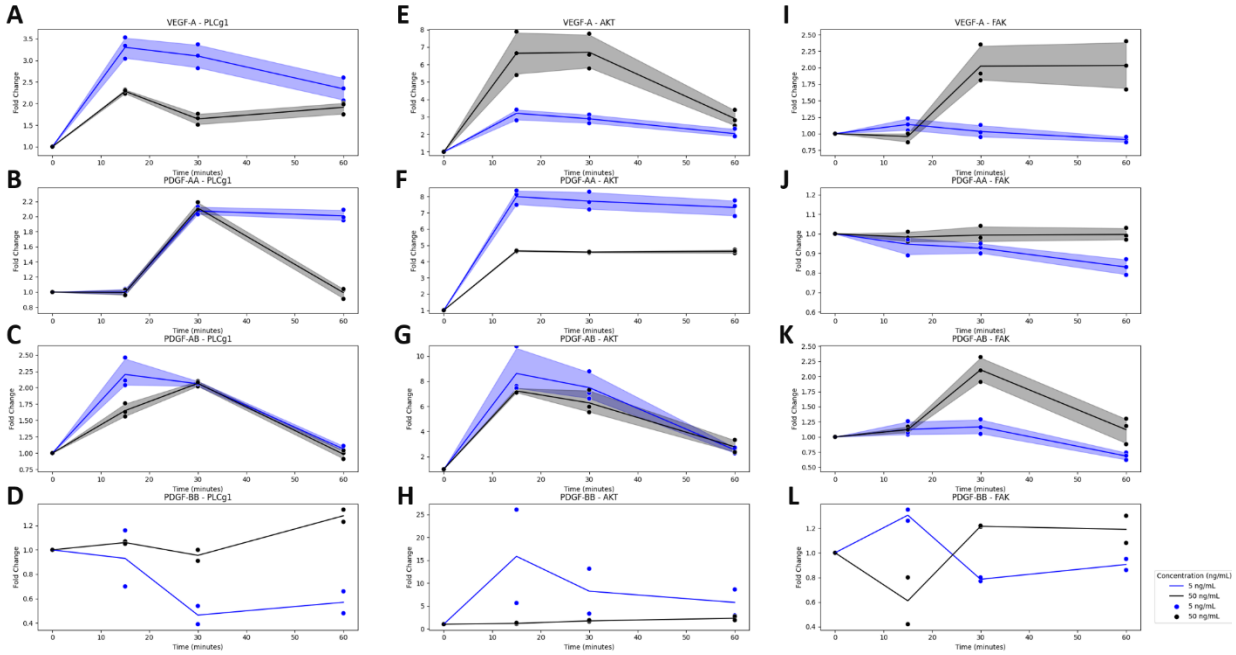

Figure 4. PDGFs activated adaptor proteins at levels comparable to those activated by VEGF-A. HDMECs (*PDGFRA*$^{-/-}$ and *PDGFRB*$^{-/-}$) were treated with PDGF-AA, -AB, or -BB at the concentrations of 5 and 50 ng/mL for 15 min. The effects of PDGF treatment on phosphorylating PLCγ1 at tyrosine site 783 **(A-D)**, Akt at serine site 473 **(E-H)**, and FAK at tyrosine site 397 **(I-L)** were assessed via immunoblotting.

Statistics

All data were acquired in at least three independent experiments. Ensemble averaged data are represented by mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA), multiple comparison post-hoc Tukey's test, and power analysis of pair-comparison were performed.

Results

Selection of VEGFR-expressing cell lines for investigating PDGF-induced signaling and functions

We selected human brain microvascular endothelial cells (HBMECs) to investigate the PDGF-induced VEGFR signaling and its effects on endothelial functions. This selection was based on our quantification of VEGFR expression across five human endothelial cell lines, which showed that HBMECs have the highest surface expression of VEGFR1 while exhibiting similar levels of VEGFR2 (Figure 1C). Further, to eliminate potential PDGF signaling through PDGFRs, we engineered a *PDGFRA* and *PDGFRB* double knockout (PDGFR-DKO) human dermal microvascular endothelial cell (HDMEC) line using CRISPR/Cas9. HDMECs (*PDGFRA*$^{-/-}$ and *PDGFRB*$^{-/-}$) expressed < 100 PDGFRα/cell and < 100 PDGFRβ/cell on the plasma membrane (Figure 1A), which likely resulted from nonspecific binding as described previously [22, 23]. Similarly, HBMECs exhibited negligible amounts of plasma membrane PDGFRs (< 300 PDGFRs/cell) when compared to HDMECs (*PDGFRA*$^{-/-}$ and *PDGFRB*$^{-/-}$) ($p < 0.05$). The whole-cell PDGFR expressions were about 10 times higher than the expression levels on the plasma membrane, likely because of increased nonspecific binding during whole-cell staining (Figure 1B). As a control, we performed the same PDGF treatments on human dermal fibroblast (HDF) cells, which naturally express PDGFRs but lack VEGFRs on their plasma membranes [24], to confirm the PDGF protein activity.

PDGF-AA and -BB induced VEGFR1 phosphorylation, whereas PDGF-AB phosphorylated VEGFR2

We initially assessed angiogenic receptor activation by quantifying VEGFR1 and VEGFR2 phosphorylation through ELISA and immunoblotting under growth factor stimulation. We treated HBMECs and HDMECs (*PDGFRA*$^{-/-}$ and *PDGFRB*$^{-/-}$) with PDGF-AA, -BB, and -AB at increasing concentrations from 0 to 100 ng/mL. HBMECs showed an overall descending trend as PDGF-AA and -BB treatment concentrations increased (Figure 2A, 2C). We observed that 0.5 ng/mL PDGF-AA and PDGF-BB treatments significantly increased VEGFR1 phosphorylation and presented a peak by a 1.8 and 2.2-fold increase, respectively, compared with untreated HBMECs ($p<0.001$), which was similar to the changes of VEGF-A treatment (Figure 2A, 2C). PDGF-AB did not have an effect on VEGFR1 phosphorylation (Figure 2B). We did not observe any PDGF-induced VEGFR2 phosphorylation in HBMECs, regardless of low or high concentrations of PDGF (Figure 2D, 2E).

In HDMECs (*PDGFRA*$^{-/-}$ and *PDGFRB*$^{-/-}$), VEGFR1 phosphorylation was induced to the greatest level to 180% by 25 ng/mL PDGF-BB treatment ($p < 0.001$) (Figure 3C). PDGF-AB demonstrated a 1.8-fold increase at 10 ng/mL ($p < 0.05$) (Figure 3B), whereas PDGF-AA did not show any significant effects (Figure 3A). Phosphorylation of VEGFR2 at tyrosine site 1175 was highly induced by 5 ng/mL PDGF-AB to 200% elevation after 15-min stimulation (Figure 3F), whereas the other two PDGFs showed no significant impacts (Figure 3E, 3G). VEGF-A induced the activation to a greater extent to 360%– 400% increase at a relatively high concentration of 50 ng/mL, aligning with the observations from ELISA conducted in HBMECs (Figure 3D, 2D).

In HDFs, PDGFRβ phosphorylation was assessed to technically validate PDGF treatment. PDGF-BB induced robust PDGFRβ phosphorylation (Figure 3H). The significant effects were detectable at 5 ng/mL and peaked at 100 ng/mL, where the phosphorylation level was 8-fold higher compared with untreated cells. In contrast, treatment with 100 ng/mL VEGF or 0.5 ng/mL PDGF-BB resulted in low PDGFRβ phosphorylation, approximately 1.5-fold higher than control levels (Figure 3H).

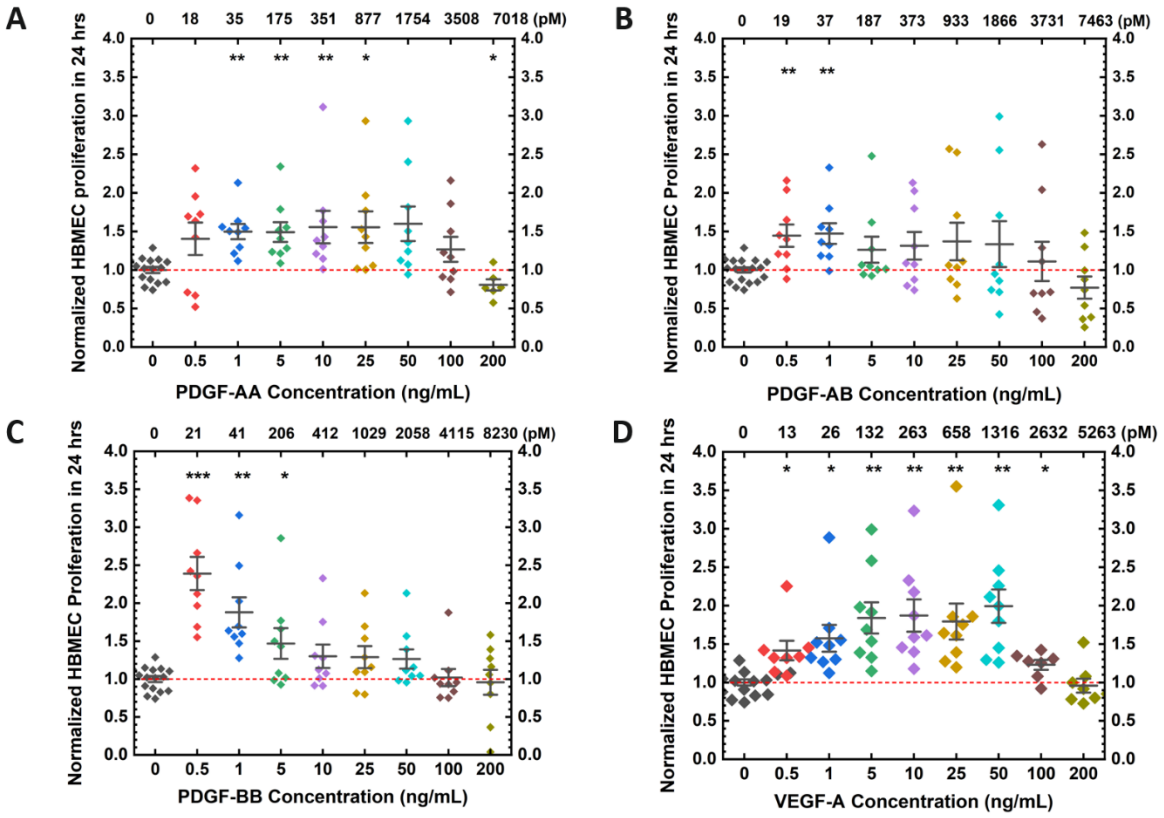

Figure 5. Low concentrations of PDGF significantly induced HBMEC proliferation up to 240%. HBMECs were treated with PDGF-AA, -AB, -BB, or VEGF-A with indicated concentration for 24 hours and subjected to proliferation assay. XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) was used to quantify cell numbers by detecting cell viability. The absorbance was read at 450 and 650 nm. OD value for each condition was normalized by untreated

PDGFs activated adaptor proteins in HDMECs to a level comparable to that of VEGF-A

The effects of PDGF-AA, -AB, or -BB (5 and 50 ng/mL) on downstream protein activation (i.e., PLC γ1, Akt, and FAK) were assessed in HDMECs (*PDGFRA*$^{-/-}$ and *PDGFRB*$^{-/-}$) via immunoblotting. Both PDGF-AA and -AB concentrations significantly phosphorylated PLCγ1 at tyrosine site 783 ($p < 0.001$) by similar fold changes to 200%–225% within 30-min treatment, which is comparable to the effects by 50 ng/mL VEGF-A (Figure 4A–4C). In contrast, 5 ng/mL of VEGF-A induced PLCγ1 phosphorylation to over 300% increase ($p < 0.001$) (Figure 4A). PDGF-BB, however, showed a subtle phosphorylation elevation to1.2-fold at 50 ng/mL after 60-min incubation ($p < 0.05$) (Figure 4D). The phosphorylation of Akt at serine site 473 showed the highest increase of all investigated growth factors to a 6–15-fold elevation ($p < 0.001$) (Figure 4E–4H). Notably, PDGF-AA and -AB at 5 ng/mL showed Akt activation at a similar level to VEGF-A at 50 ng/mL, whereas PDGF-BB showed the highest level to 15-fold elevation at 5 ng/mL after 15-min treatment (Figure 4E–4H). Growth factors showed the lowest impacts on the activation of FAK at tyrosine site 397 with a 120–200% increase (Figure 4I–4L). A high concentration of 50 ng/mL PDGF-AB displayed

a 2-fold increase in FAK phosphorylation ($p < 0.001$), comparable to that of 50 ng/mL VEGF-A after 30-min treatment ($p < 0.05$) (Figure 4I, 4K). PDGF-BB showed a minor increase at 1.2 times ($p < 0.05$), whereas PDGF-AA did not significantly phosphorylate FAK at the site ($p = 0.86$) (Figure 4J, 4L).

PDGFs showed significant capacities on stimulating EC proliferation comparable to VEGF-A

The activation of PLCγ1, Akt, and FAK has been found to modulate VEGF-induced EC proliferation, migration, and survival [25–28], which served as the foundation for exploring PDGF-induced EC proliferation. Serum-starved cells were treated with 0.5 ng/mL to 200 ng/mL VEGF-A or PDGF. Notably, all studied PDGFs (PDGF-AA, -AB, and -BB) significantly stimulated EC proliferation. HBMECs treated with PDGF-AA or -AB exhibited up to a 150% increase in proliferation when the concentrations were between 0.5 ng/mL and 50 ng/mL ($p < 0.01$) (Figure 5A, 5B), while PDGF-BB exhibited an inverse dose-response relationship (Figure 5C): the low PDGF-BB concentration, 0.5 ng/mL, induced the highest HBMEC proliferation, resulting in a remarkable 240% increase compared with untreated cells ($p < 0.001$) (Figure 5C). As expected, VEGF-A treatment served as a positive control, demonstrating a substantial increase in cell growth, reaching up to 200% at concentrations ranging from 0.5 ng/mL to 100 ng/mL (Figure 5D). HDMEC (*PDGFRA*$^{-/-}$ and *PDGFRB*$^{-/-}$) showed significant proliferation increases relative to

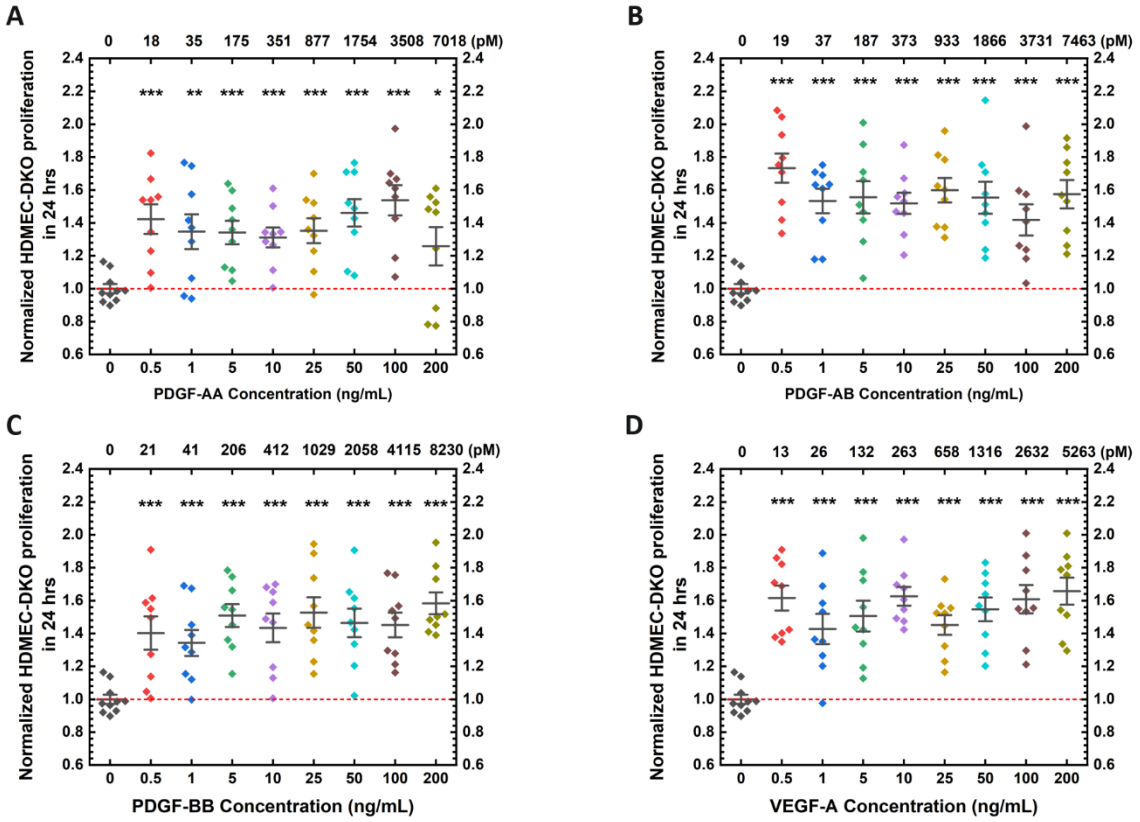

Figure 6. HDMEC (*PDGFRA*$^{-/-}$ and *PDGFRB*$^{-/-}$) showed a comparable increase in cell proliferation to around 150% induced by PDGF or VEGF-A across all investigated concentrations.

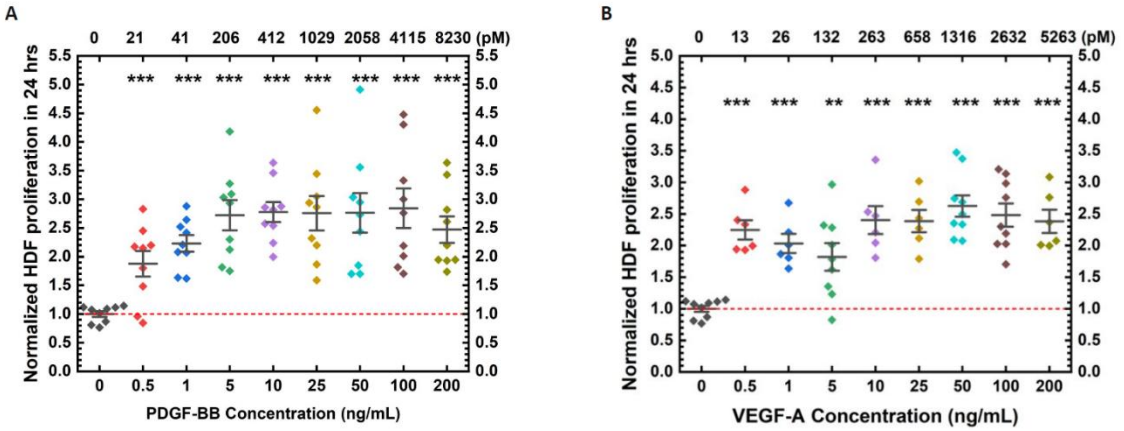

Figure 7. HDF exhibited a more remarkable proliferation increase to 280% by PDGF-BB or 250% by VEGF-A at higher concentrations.

untreated conditions across all investigated concentrations of PDGF or VEGF-A ($p < 0.001$). Notably this increased proliferation was similar across all concentrations (Figure 6). While PDGFs can induce EC proliferation, the PDGF-BB-mediated proliferation response in HDFs was more pronounced ($p < 0.001$) with up to a 280% increase in proliferation relative to the untreated control across the 5 ng/mL to 100 ng/mL concentration range (Figure 7A). Notably, VEGF-A mediated a significant proliferation response in HDFs ($p < 0.001$), relative to the untreated control, at concentrations between 10 ng/mL to 200 ng/mL, promoting proliferation by 250% (Figure 7B).

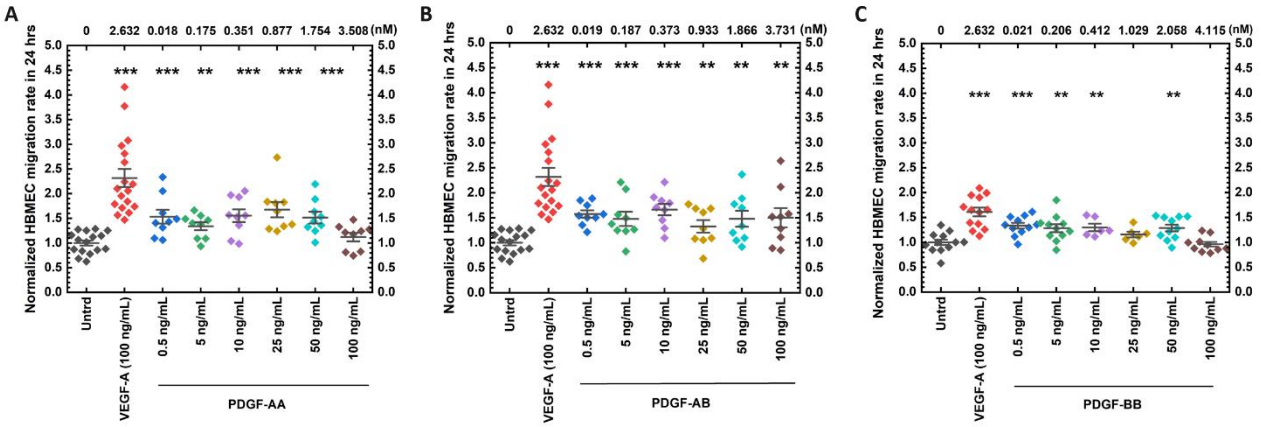

Figure 8. PDGFs induced significant and comparable enhancement in HBMEC migration across a wide range of concentrations. HBMECs were seeded in the top chamber of transwell inserts and stimulated with PDGF-AA, -AB, -BB, or VEGF-A at the indicated concentrations in low-serum medium in the lower chamber for 24 hours. The cells that migrated through the membrane to the downside of the insert were fixed, permeabilized, stained, imaged, and counted on the following day.

PDGF induced EC migration to 70% of the level stimulated by VEGF-A

To investigate the impact of PDGF on cell migration, we quantified the number of migrated cells through the transwell inserts stimulated by growth factors across the 0.5 ng/mL to 100 ng/mL concentration range for 24 hours. All investigated PDGF significantly triggered EC migration. In HBMECs, we observed that 25 ng/mL PDGF-AA or 10 ng/mL PDGF-AB induced the highest level of migration to 170% and 160% compared with untreated cells, respectively ($p < 0.001$) (Figure 8A, 8B). PDGF increased HBMEC

migration to 130% at 0.5 ng/mL ($p < 0.001$) (Figure 8C). In HDMEC (*PDGFRA*$^{-/-}$ and *PDGFRB*$^{-/-}$), low concentrations of PDGF-AA and -AB resulted in the most remarkable migration with a 130% enhancement relative to the untreated control at 5 ng/mL and 0.5 ng/mL, respectively ($p < 0.01$ and $p < 0.001$, respectively) (Figure 9A, 9B). PDGF-BB exhibited less migration with increasing concentrations in both EC types, with the lowest concentration, 0.5 ng/mL PDGF-BB resulting in a significant, 1.3-fold increase in migration relative to the untreated control in HDMECs (*PDGFRA*$^{-/-}$ and *PDGFRB*$^{-/-}$) ($p < 0.001$) (Figure 9C). Conversely, increasing PDGF-BB concentrations correlated with increasing migration of HDFs, starting from 5 ng/mL and peaking with a 2.8-fold increase in migration at 100 ng/mL ($p < 0.001$) (Figure 9D). VEGF-A served as a positive control with a substantial increase of up to 230% in both ECs ($p < 0.001$) but failed to show significant changes in HDFs ($p = 0.09$).

PDGF-induced EC responses are independent of VEGF-A upregulation

To ascertain the potential for VEGF-A protein to have induced the PDGF-changes observed via PDGF-mediated upregulation of VEGF-A [4,43], we conducted an ELISA to quantify VEGF-A concentrations in the cell culture supernatant following varying durations of PDGF-BB treatment. Our analysis revealed no statistically significant changes in VEGF-A levels between the untreated control and PDGF-BB-treated experimental cohorts (Figure 10B). Interestingly, within the VEGF-A positive subgroup, a progressive decline in VEGF-A concentration was noted over the course of incubation (Figure 10A). The trajectory of VEGF-A concentration reduction was accurately delineated by a regression curve (Figure 10C), with a calculated half-life of 32.33 minutes, suggesting the temporal stability of VEGF-A in the culture media after VEGF-A treatment.

Discussion

Our study elucidates the effects of cross-family PDGF-to-VEGFR binding in regulating endothelial signaling and functions. We found that (1) PDGFs played a critical role in augmenting EC angiogenic hallmarks, i.e., receptor and downstream protein phosphorylation, cell proliferation, and migration, through non-PDGF receptors, to a similar level compared to VEGF-A. (2) Low concentrations of PDGF-BB induced the angiogenic capacity of ECs to the greatest extent, whereas high concentrations showed a more potent effect in HDF. Other PDGFs did not exhibit such trends. (3) The angiogenic effects of PDGF-BB in HBMECs were more prominent than in HDMECs (*PDGFRA*$^{-/-}$ and *PDGFRB*$^{-/-}$). Other PDGFs showed similar impacts between EC types. (4) VEGF-A protein upregulation was not observed upon PDGF-BB stimulation, further validating the effects of PDGF-to-VEGFR signaling in ECs.

PDGF-induced endothelial signaling and function

Cross-family interaction has been identified between PDGF and VEGFR as a novel angiogenic mechanism [7], the physiological functions are, however, yet unexplored. Here, we examined the angiogenic hallmarks of cross-family PDGF-to-VEGFR binding in regulating endothelial signaling and functions, i.e., receptor and effector activation, cell proliferation, and migration. We employed ECs that lack PDGF receptors (PDGFRs) on their plasma membranes to isolate the effects of possible PDGF-BB signaling through VEGFR2 from its signaling through PDGFR and also employed human fibroblasts that lack VEGFRs but have PDGFRs on their plasma membranes as a comparative control. To exclude the possible effects of upregulated VEGF-A transcription induced by PDGF treatment [29, 30], we observed no significant changes in VEGF-A protein concentrations in the cell culture supernatant after varying durations of PDGF-

BB treatment, further confirming that the observed effects resulted from PDGF signaling through non-PDGFRs.

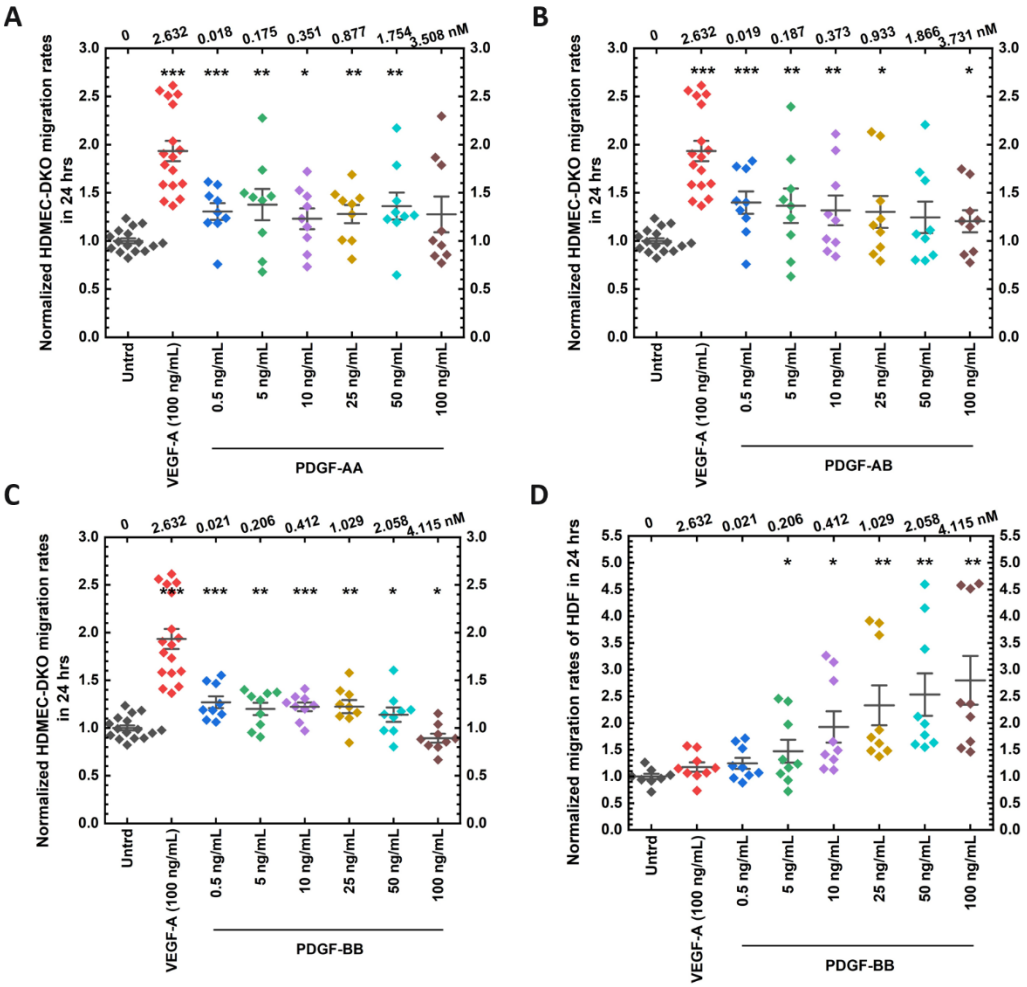

Figure 9. Migrative effects of PDGFs in HDMECs (*PDGFRA*$^{-/-}$ and *PDGFRB*$^{-/-}$) and HDF. (A-C) Low concentrations of PDGF significantly induced HDMEC (*PDGFRA*$^{-/-}$ and *PDGFRB*$^{-/-}$) migration by approximately 1.3-fold, comparable to HBMEC. **(D)** Higher concentrations (> 5 ng/mL) of PDGF-BB promoted a significant increase in HDF migration by 2.8-fold.

Low concentrations of PDGF showed more pronounced effects

Our detailed analyses revealed that low concentrations of PDGFs induced EC functional changes at a higher level. Specifically, treatment of 0.5 ng/mL PDGF-AA or -BB induced the highest VEGFR1 phosphorylation concentration by approximately 2-fold in HBMECs, whereas high concentrations at 50–100 ng/mL yielded the lowest receptor activation. Around 5 ng/mL of PDGF-AA and -AB activated downstream proteins, PLCγ1, Akt, and VEGFR2 at the investigated sites at a higher level than the concentration of 50 ng/mL. A low concentration of PDGF-BB at 0.5 ng/mL exhibited stronger activation of Akt and FAK compared to a high concentration at 50 ng/mL. Similar results were observed in the examinations of angiogenic hallmarks, where the highest impacts of PDGF treatment appeared at relatively low concentrations. Notably, PDGF in human blood serum is measured to be 17.5 ng/mL [31],

approximately 35-fold higher than 0.5 ng/mL, the concentration producing the most substantial angiogenic effects, which may explain why the functions of PDGF were long overlooked.

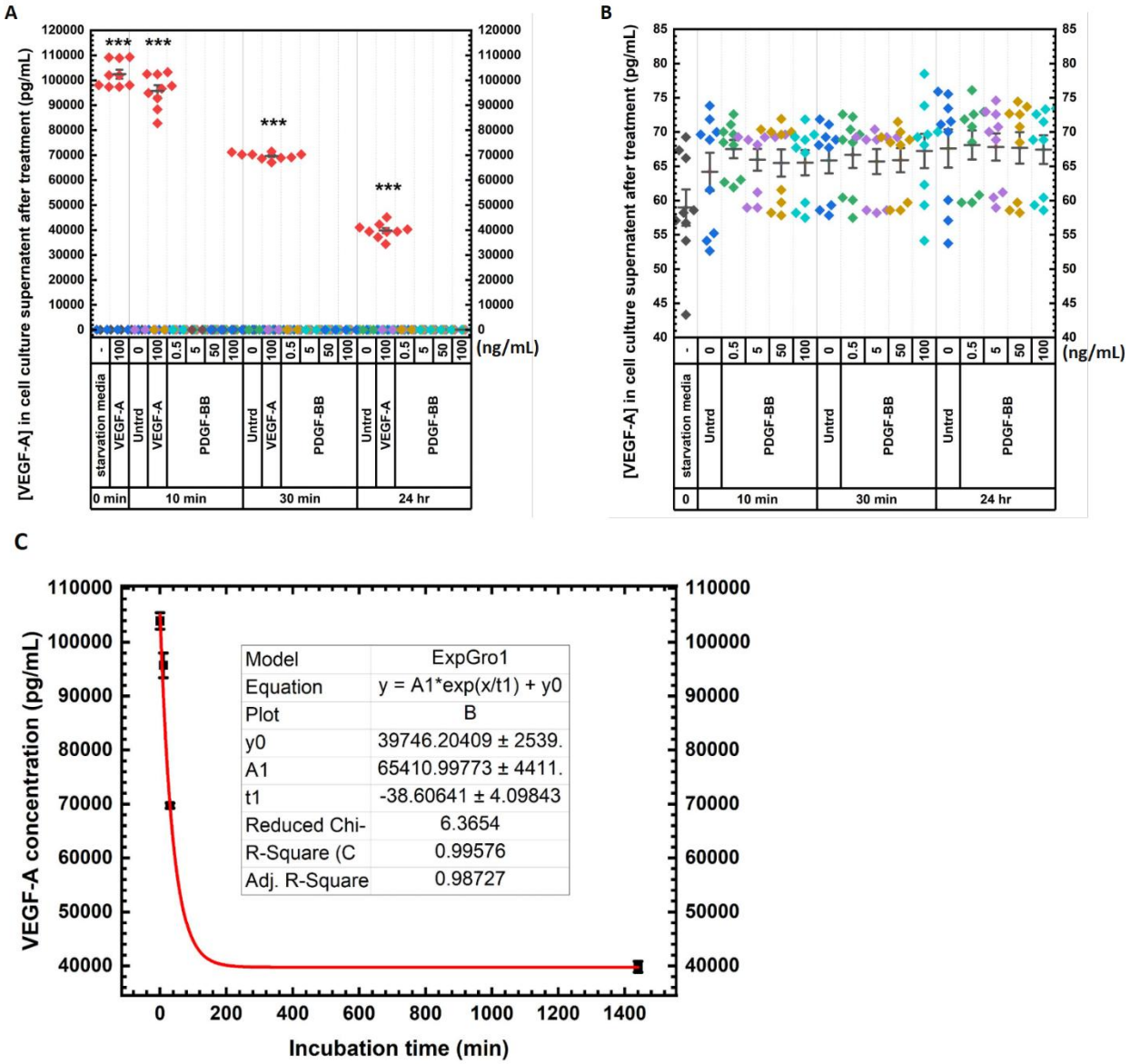

Figure 10. The concentration of VEGF-A exhibited no significant alteration upon PDGF-BB stimulation. (A and B) Subsequent to a 6-hour period of serum deprivation (1% FBS), HBMECs underwent treatment with growth factors as detailed. Post-incubation, the cell culture supernatant underwent centrifugation and analysis as per the protocols outlined in the Human VEGF ELISA Kit – Quantikine (R&D Systems, SVE00). **(C)** A fitting curve was generated to depict the correlation between VEGF-A concentration and in-vitro incubation duration. The determination of VEGF-A half-life time was computed utilizing the formula $t = -t1 \cdot \ln 2$, yielding a value of 26.76 minutes.

PDGF-to-VEGFR1 signaling

Our observations suggest a compelling link between PDGF-induced signaling through VEGFRs, downstream proteins, and EC angiogenic hallmarks. PLCγ1, Akt, and FAK are downstream proteins after VEGFR2 activation induced by VEGF [25]. They are found to involve the signaling that regulates EC

proliferation, survival, and migration [25–28]. In this study, we found that PDGFs could significantly activate VEGFRs and these proteins and induced EC angiogenic hallmarks. It is suggested that PDGF may signal through VEGFR1 and downstream proteins to regulate EC angiogenesis.

Our observations suggest that ECs may engage in novel signaling pathways between PDGF and non-PDGF receptors on the plasma membrane. PDGF-BB was suggested to induce VEGFR1 and VEGFR2 phosphorylation through VEGFR2 binding, since PDGF-BB-to-VEGFR1 binding was not previously observed, but PDGF-BB-to-VEGFR2 binding was seen [7]. In addition, VEGFR1 primarily heterodimerizes with VEGFR2 to regulate VEGF activity [13, 32]. Most VEGFR1 are in heterodimer forms with VEGFR2 on the plasma membrane of ECs [33]. These findings suggest the need to explore VEGFR1-VEGFR2 heterodimerization in PDGF-mediated cross-family interactions.

VEGFR1 has been typically considered a "decoy" receptor in angiogenesis due to its high binding affinity but limited kinase activity [34, 35]. However, an association between increased VEGFR1 levels and angiogenesis hallmarks was recently found [21, 24, 36–38], challenging the decoy paradigm. VEGFR1 is upregulated in tumors and ischemia, but VEGFR2 remains stable [21, 38]. Here, the VEGFR activity on ECs induced by PDGFs is the first time shown. PDGFs could significantly activate and phosphorylate VEGFR1 and VEGFR2 in ECs.

PDGFs function variously in different cells

PDGF-BB showed more pronounced angiogenic effects in HBMECs compared with HDMECs (*PDGFRA*$^{-/-}$ and *PDGFRB*$^{-/-}$). Treatment with PDGF-BB led to a 220% increase in VEGFR1 phosphorylation in HBMECs, contrasting with a 180% increase in HDMECs (*PDGFRA*$^{-/-}$ and *PDGFRB*$^{-/-}$). Moreover, cell proliferation in HBMECs surged by 240% compared to 150% in HDMECs following PDGF-BB treatment, potentially influenced by the twofold higher concentration of VEGFR1 on the plasma membrane of HBMECs (700 ± 70 VEGFR1/cell) compared to HDMECs (300 ± 40 VEGFR1/cell) [cite]. Moreover, heparin can inhibit growth factor effects on cell proliferation [39]. The medium culturing HDMECs (*PDGFRA*$^{-/-}$ and *PDGFRB*$^{-/-}$) contain a significant amount of heparin (0.0015 mg/mL).

The more remarkable effects of PDGF at higher concentrations in HDFs were observed compared with the results in ECs with PDGF-BB at lower concentrations. PDGF-BB increased VEGFR1 phosphorylation by around 2-fold at 0.5 ng/mL and 8-fold at 100 ng/mL in ECs and HDFs, respectively. Cell proliferation rate was elevated by 240% in HBMECs at 0.5 ng/mL compared with 280% in HDFs by 100 ng/mL of PDGF-BB treatment. Cell migration was promoted to 130% enhancement by 0.5 ng/mL PDGF-BB in HBMECs and HDMECs (*PDGFRA*$^{-/-}$ and *PDGFRB*$^{-/-}$), whereas HDFs reached the peak migration capacity by 100 ng/mL PDGF-BB of 2.8-fold increase. The data agreed with previous publications that strong cell proliferation induction only occurs with PDGF concentrations > 5 ng/mL and arises as the concentration increases [40]. PDGF-BB at 25 ng/mL was found to increase fibroblast proliferation by approximately 2-fold [41], comparable to our findings. The cell migration response is negligible at low concentrations and arises starting from 1 ng/mL [40]. PDGF-BB at 10 ng/mL increases fibroblast migration to around 200% [42], similar to our result. The less remarkable PDGF-induced impacts in ECs aligned with the differential receptor expression on these cells, as HDFs express PDGFRs on the plasma membrane, but ECs do not. In such case, PDGF displayed less activity on ECs than HDFs due to the lack of plasma-membrane PDGFRs.

Our study highlighted significant VEGF-A-induced effects in HDFs lacking VEGFRs. VEGF-A significantly phosphorylated VEGFR1 and enhanced cell proliferation of HDFs, but not on cell migration. The validated impacts of VEGF-A in HDFs that lack VEGFRs but express PDGFRs on the plasma

membrane, agreed with the published studies that VEGF-A could signal through PDGFRs [15] and demonstrated the effects of cross-family signaling between VEGF-A to PDGFRβ.

Conclusion

By uncovering the angiogenic functions of PDGF-VEGFR interactions, we can better understand the cross-family angiogenic pathway, which may reveal alternate mechanisms for addressing vascular dysregulation. The pathways that downregulate VEGFR1 may be responsible for limiting excessive angiogenesis (cite). Proteomics is a reasonable attempt to systematically explore the underlying mechanisms. Our findings also provide essential data for computational modeling and systems biology to refine the signaling pathways.

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