No evidence of Gremlin1-mediated activation of VEGFR2 signalling in endothelial cells

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Running Title: *GREM1 does not activate VEGFR2 phosphorylation*

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ABSTRACT

Canonical Gremlin1 (GREM1) signaling involves binding to and sequestering bone morphogenetic proteins (BMPs) in the extracellular matrix, preventing the activation of cognate BMP receptor signaling. Exquisite temporospatial control of the GREM1–BMP interaction is required during development, and perturbation of this balance leads to abnormal limb formation and defective kidney development. In addition to inhibition of BMP signaling, several other noncanonical signaling modalities of GREM1 have been postulated. Some literature reports have suggested that GREM1 can bind to and activate vascular endothelial growth factor receptor-2 (VEGFR2) in endothelial cells, human kidney epithelial cells, and others. These reports suggest that the GREM1→VEGFR2 signaling can drive angiogenesis both *in vitro* and *in vivo*. We report here that, despite exhaustive attempts, we did not observe GREM1 activation of VEGFR2 in any of the cell lines reported by the above-mentioned studies. Incubation of endothelial colony–forming cells (ECFCs) or human umbilical vein endothelial cells (HUVECs) with recombinant VEGF triggered a robust increase in VEGFR2 tyrosine phosphorylation. In contrast, no VEGFR2 phosphorylation was detected when cells were incubated with recombinant GREM1 over a range of time points and concentrations. We also show that GREM1 does not interfere with VEGF-mediated VEGFR2 activation, suggesting that GREM1 does not bind with any great affinity to VEGFR2. Measurements of ECFC barrier integrity revealed that VEGF induces barrier function disruption, but recombinant human GREM1 had no effect in this assay. We believe that these results provide an important clarification on the potential interaction between GREM1 and VEGFR2 in mammalian cells.

Bone morphogenetic proteins (BMPs) regulate a wide range of biological processes such as lower limb formation and kidney development (1, 2) The physiological action of BMPs is regulated by a number of secreted, protein antagonists such as Gremlin1 (GREM1), Noggin and Chordin that bind to BMPs in the extracellular space (1, 3).This formation of BMP-BMP antagonist complex prevents the activation of the BMPRI/II complex, preventing phosphorylation of R-SMAD 1/5/9 and resulting SMAD-dependent gene expression (4). GREM1 binding to BMP4 has been shown to play a key role in mammalian kidney formation (5–7). In addition, GREM1 has been identified as a pathogenic mediator of diabetic kidney disease (8–10), pulmonary hypertension (11–13), pancreatitis (14) and a range of human cancers including mesothelioma, glioma and colorectal cancer

(15–21). The mechanism of GREM1-mediated fibrosis or oncogenic signalling has not yet been definitively identified. Several groups have suggested that non-canonical signalling mechanisms for GREM1 via Slit proteins (22), ROBO (23) and fibrillin (24). Two reports in Blood in 2007 and 2010 identified GREM1 as a novel agonist of the proangiogenic receptor VEGFR2 (25, 26). These reports suggest that exposure of human endothelial vein endothelial cells (HUVECs) to GREM1 (approx. 3 nM) induces phosphorylation of VEGFR2 on Tyr1175, the site associated with PLCγ/ERK activation (27). This group also published several follow-up reports on the requirement of αvβ3 integrins for GREM1-mediated VEGFR2 activation (28) and the ability of monomeric GREM1 to act as an antagonist of VEGFR2 (29). Sporadic reports in the literature have suggested similar GREM1→VEGFR2 signalling in ARPE-19 retinal cells (30), HK-2 kidney tubule epithelial cells (31, 32), HaCaT skin keratinocytes and primary skin fibroblasts (33). In contrast, a recent paper suggests that GREM1 blocks VEGF signalling in the pulmonary microvascular endothelium (34). Given the wealth of new data implicating GREM1 signalling in human diseases such as cancer (21, 35) fibrosis of the kidney (36) and lung (11) as well as rheumatoid arthritis (37), we believe it is critical to define the precise signalling mechanisms of GREM1 signalling in mammalian cells. To that end, we report here that despite extensive effort, we failed to demonstrate that GREM1-mediated activation of VEGFR2 in endothelial and other cells. Given the current model of GREM1 signalling in the literature, our data challenge the current dogma that GREM1 activate VEGFR2 phosphorylation, and sheds new light on the likely signalling mechanisms engaged by GREM1 during development and disease.

Results

Endothelial colony-forming cells (ECFCs) are late outgrowth endothelial progenitor cells which can form new endothelial cells *in vivo* (34). ECFCs demonstrate a clear activation of VEGFR2 phosphorylation on both Tyr1175 (which binds Shc/Grb2/SOS leading to PLCγ and ERK activation) and Tyr951 (which binds Shc/Grb2/Gab1 leading to PI3K/Akt activation (27) in response to 25 ng/ml VEGF (Fig. 1). In contrast, GREM1 treatment for 15 min had no effect on phospho-VEGFR2 levels when 25 ng/ml (approx. 2.5 nM) or a supraphysiological concentration of 1 µg/ml (100 nM) were used (Fig. 1). A time-course of GREM1 treatment using 5, 10, 15, 30, 60 min and 16 h treatment also failed to demonstrate increased VEGFR2 phosphorylation (Supp. Fig. 1). Consistently, Akt phosphorylation was detected in ECFCs in response to VEGF after 15 min, with no increase evident in GREM1-treated cells (Fig. 1). No changes in total VEGFR2 or Akt were detected in any of the treatment groups. There was no difference in experimental outcome when we ECFCs were grown on either collagen or fibrinogen to trigger co-activation of αvβ3, which was reported to enhance GREM1-mediated VEGFR2 phosphorylation (Fig. 1, 2A, (28). Using immunofluoresence as a readout, increased phospho-VEGFR2 was detected at the plasma membrane of ECFCs (Supp. Fig. 2). Consistently, no increase was detected in GREM1-treated cells compared to vehicle (Supp. Fig. 2). These data suggest that in a VEGF-responsive cell where VEGFR2 phosphorylation is clearly evident, rhGREM1 cannot activate VEGFR2 phosphorylation despite the reports in the literature.

We tested the hypothesis that GREM1 may bind to and enhance or inhibit VEGF-mediated receptor activation. Preincubation of GREM1 with VEGF ligand did not inhibit VEGF-mediated VEGFR2 phosphorylation or Akt phosphorylation at the 15 min time point (Fig. 2A). We also explored the possibility that GREM1 may be acting as a partial agonist by binding and inhibiting VEGFR2 phosphorylation. Preincubation of ECFCs with GREM1, followed by VEGF did not affect VEGF-mediated VEGFR2 or Akt phosphorylation (Fig. 2A). Similar data was obtained using three independent ECFC clones (data not shown). These data suggest that GREM1 is not acting as an antagonist or partial agonist at VEGFR2 and does not affect VEGF signalling in these cells. Importantly, the rhGREM1 used can completely block BMP2-mediated pSMAD1/5/9 phosphorylation in HEK293T cells, confirming the integrity of the rhGREM1 used in our experiments (Fig. 2B). In addition to ECFCs, we have been unable to demonstrate GREM1-mediated activation of VEGFR2 in human umbilical vascular endothelial cells (HUVEC, Suppl. Fig. 3), human kidney epithelial cells (HK-2), HEK293

cells or human retinal pigment epithelial ARPE-19 cells (as reported by other authors (38, 39), data not shown).

VEGF has previously been demonstrated to reduce barrier integrity in endothelial cells (40–44). We tested the ability of GREM1 to alter ECFC barrier function compared to VEGF as a functional readout of VEGFR signalling. Using the XCELLigence system, the ability of rhVEGF and rhGREM1 to reduce the integrity of ECFC monolayer by decreasing cell impedence was assessed. Addition of rhVEGF (25 ng/ml) caused a transient reduction in ECFC barrier function within 30 min, which recovered after 60 min (Fig. 3). In contrast, rhGREM1 (100 ng/ml, ~ 10 nM) had no effect on ECFC cell impedence and did not compromise ECFC monolayer integrity (Fig. 3). Addition of higher concentrations of rhGREM1 (1 µg/ml, ~ 100 nM) also had no significant effect on ECFC cell impedence (data not shown). These data further support our conclusion that GREM1 does not signal via VEGFR2 or equivalent pathways that are engaged by VEGF in endothelial cells.

Discussion

In summary, we cannot demonstrate that GREM1 signals via the VEGFR2 in a range of cell lines that have been reported in the literature. We have considered the possibility that GREM1 treatment may be altering the expression levels of VEGFR2 in cells. However, quantitative PCR demonstrated no change in VEGFR2 mRNA levels in cells treated with GREM1 at 4, 8 and 16 h (Supp. Fig. 3). Our previous data demonstrating grossly normal development of the vasculature in retina and other tissues in *Grem1*$^{-/-}$ mice (36) also suggests that GREM1 signalling does not contribute a major physiological pro-angiogenic drive. We believe this is an important issue to resolve for the field, as GREM1 signalling via VEGR2 has been suggested as an important non-canonical signalling modality for GREM1 that may contribute to pathophysiological signalling in kidney fibrosis and cancer. We believe that our data shed important new light on this inconsistency and allow clarification in the field of GREM1/BMP signalling.

Experimental Procedures

Cell culture

Endothelial colony forming cells (ECFCs) were isolated by Dr Reinhold Medina, Queen's University Belfast and grown in Endothelial Cell Growth Medium 2 (EGM-2, PromoCell, Heidelberg, Germany) supplemented with 12% FBS, and were grown in t75 cm^2 flasks pre-coated with rat tail collagen type 1 (BD Biosciences) at 37°C, 5% CO_2 and 95% air. Human embryonic kidney cells 293T (HEK293T) were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 1 g/L glucose (Gibco, UK) supplemented with 10% FBS and 100 µg/mL Primocin (Invitrogen, UK). Cells were grown in $t75^2$ flasks at 37°C, 5% CO_2 and 95% air and plated on 60 mm dishes for treatments.

VEGFR2 Activation Assay

ECFCs were first seeded in 60 mm dishes coated with 2 µg/ml fibrinogen (Sigma-Aldrich, UK) to achieve 70% confluency on the day of the experiment. Cells were serum-deprived in 2% FBS medium overnight followed by a further 4 h in serum-free medium. Cells were treated with 25 ng/ml rhVEGF (R&D Systems) or 25 ng/ml rhGREM1 (R&D Systems) for 5 or 15 min. In parallel, cells were treated with 25 ng/ml rhVEGF and 25 ng/ml rhGREM1 pre-incubated at 37°C for 15 min and then added to the cells for 15 min. Cells were also pre-treated with 25 ng/ml rhGREM1 for 15 min followed by 25 ng/ml rhVEGF for 5 or 15 min.

To confirm GREM1 activity, rhBMP2 treatment of HEK293T cells was also carried out. After serum deprivation, serum-free media containing either vehicle (4 mM HCl), 5 ng/ml rhBMP2 (R&D Systems) or 5 ng/ml rhBMP2 (R&D Systems) and 25 ng/ml rhGREM1 were pre-incubated for 15 min at 37°C, before adding to the cells for 60 min at 37°C.

For protein extraction, cells were washed once with PBS and protein extracted with 150 µL ice-cold radioimmunoprecipitation assay (RIPA) buffer (50 Mm Tris-HCl (pH 7.4), 1% (v/v) Nonidet p-40 (NP-40), 0.5% (v/v) sodium deoxycholate, 150 mM sodium chloride (NaCl)

and 1 mM EDTA) supplemented with 250 µM sodium orthovanadate ($NaVO_4$, Sigma-Aldrich, UK), 40 mM β-glycerolphosphate (Sigma-Aldrich, UK), 1 mM sodium fluoride (NaF, Sigma-Aldrich, UK), 2 µM microsystin-LR (Enzo® Life Sciences, New York, USA), 1 mM phenylmethanesulfonyl fluoride (PMSF, Sigma-Aldrich, UK) and 1x protease inhibitor cocktail (Sigma-Aldrich, UK). Protein lysates were probed by Western blotting using antibodies (1:1000) reactive to pVEGFR2 (pY1175) (Cell Signalling), pVEGFR2 (pY951) (Cell Signalling) or total VEGFR2 (Cell Signalling) after blocking the membrane with 3% (w/v) BSA in TBS 0.1% (v/v) Tween 20 (TBS-T). Protein lysates were also probed with antibodies (1:1000) against pAkt (T308) (Cell Signalling), pAkt (S473) (Cell Signalling), total AKT (Cell signalling) and β-actin (Cell Signalling) as the loading control in 5% (w/v) non-fat milk TBS-T. Reactive bands were visualised using anti-rabbit or mouse-HRP using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Watford UK) and imaged on the G:BOX Chemi XX6 system (Syngene).

Barrier Function Analysis

To assess ECFC barrier function we used the xCELLigence® DP Real-Time Cell Analyser (ACEA Biosciences, Agilent San Diego CA, USA). Briefly, E-plate 16 composed of gold-film electrodes positioned on the bottom surface of the well were coated with rat tail collagen type 1 (Corning) as previously described (45). Background measurements were recorded by filling wells with EBM-2 medium (Lonza). Cord blood derived ECFCs suspended in EBM-2 were then added at a density of 20,000 cells/well and left at RT for 30 min to facilitate uniform attachment. Impedence/cell index measurements were then taken every 15 min. When a stable monolayer was formed, EBM-2 medium containing vehicle (PBS), rhVEGF (50 ng/ml, R&D Systems), rhGREM1 (100 ng/ml (~10 nM) or 1 µg/ml (~100 nM, R&D Systems) were then added to the appropriate wells and recordings taken. Changes in cell impedence values were normalised prior to treatment and measured for at least 5 hours post-treatment.

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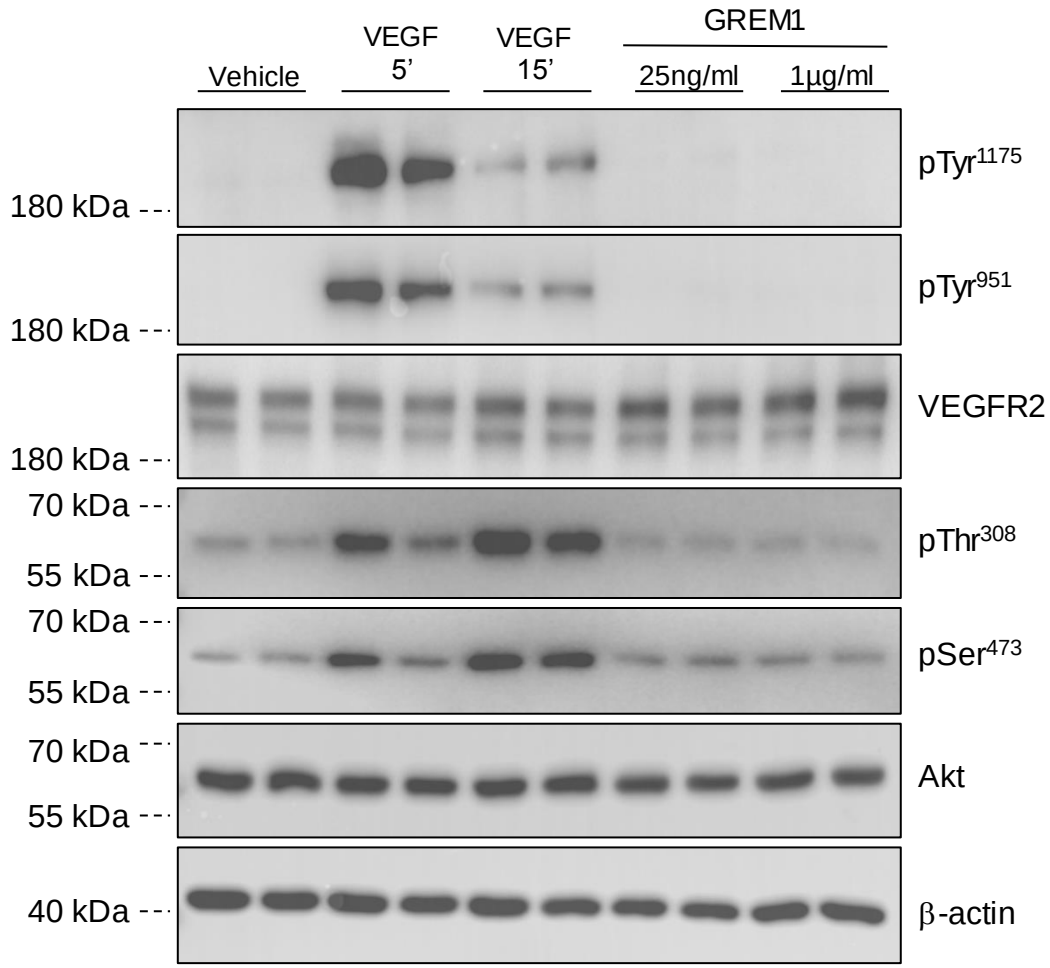


Figure 1. Gremlin1 does not activate VEGFR2 phosphorylation in endothelial colony-forming cells (ECFCs). ECFCs isolated from human blood were seeded on fibrinogen (2 µg/ml in ddH_2O) and serum reduced in 2% FBS EGM-2 medium overnight. On the day of experiment, cells were serum starved for 3 h prior to treatment with vehicle (PBS), rhVEGF (25 ng/ml, R&D Systems) for 5 and 15 min, rhGREM1 (25 ng/ml and 1 µg/ml, R&D Systems) for 15 min. Protein lysates were run on SDS-PAGE and probed with the indicated antibodies reactive to pTyr 1175 and pTyr951 on VEGFR2, total VEGFR2, pThr308 and pSer473 phospho-Akt antibodies, total Akt and β-actin.

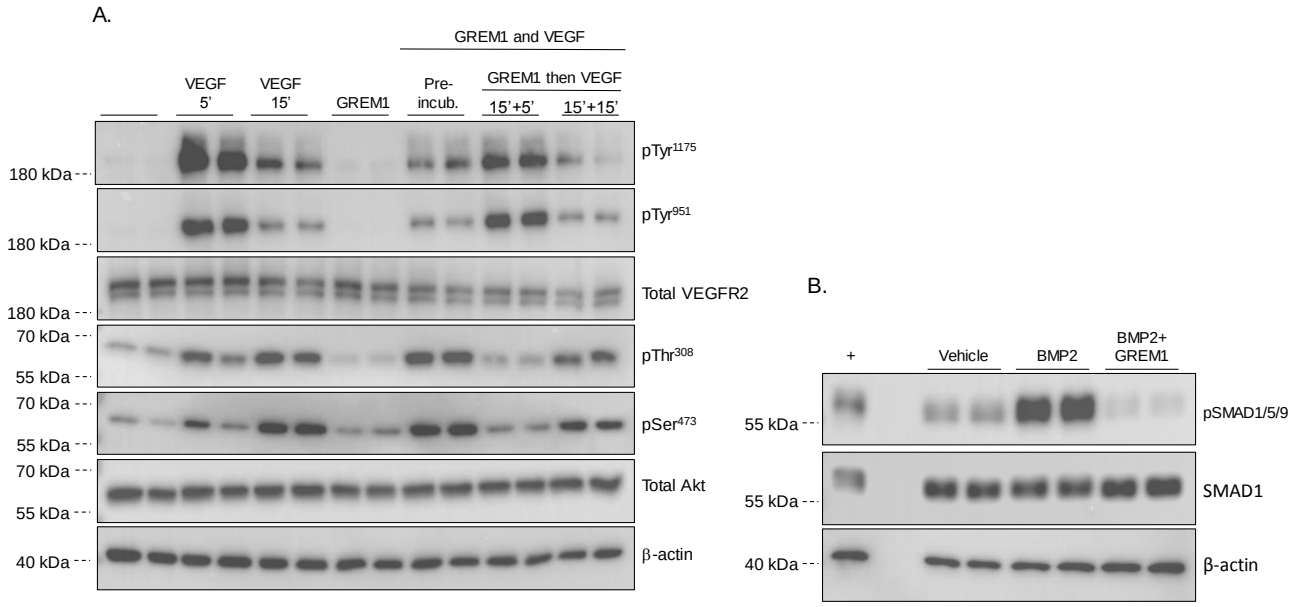


Figure 2. Gremlin1 does not inhibit VEGF signalling via the VEGFR2 receptor. A. ECFCs isolated from human blood were seeded on fibrinogen and serum reduced in 2% FBS EGM-2 medium overnight. On the day of experiment, cells were serum starved for 3 h prior to treatment with rhVEGF (25 ng/ml) for 5 and 15 min, rhGREM1 (25 ng/ml, R&D Systems) for 15 min. rhGREM1 (25 ng/ml) and rhVEGF (25 ng/ml) were also preincubated for 15 min prior to addition to cells for 15 min. In addition, rhGREM1 (25 ng/ml) were added to cells for 15 min prior to the addition of rhVEGF (25 ng/ml) for 5 and 15 min. Protein lysates were run on SDS-PAGE and probed with the indicated antibodies reactive to pTyr 1175 and pTyr951 on VEGFR2, total VEGFR2, pThr308 and pSer473 phospho-Akt antibodies, total Akt and β-actin as loading control. B. HEK293 cells were serum reduced in 1% FBS overnight, followed by serum-free medium for 3 h. Cells were treated for 60 min with vehicle (4 mM HCl), rhBMP2 (5 ng/ml) or rhBMP2 (5 ng/ml) and rhGREM1 (25 ng/ml) that had been preincubated for 15 min at 37 ºC. Protein lysates were run in SDS-PAGE and probed with the indicated antibodies reactive to pSmad1/5/9, total Smad1 and β-actin as loading control. "+" indicates positive control.

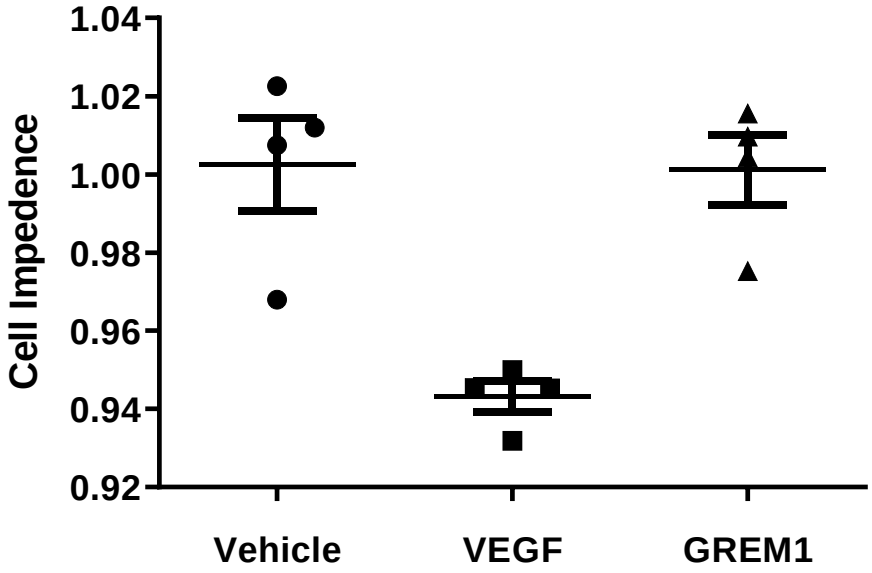


Figure 3. GREM1 does not alter ECFC barrier function. Endothelial colony-forming endothelial cells (ECFCs) were plated in E platescontaining XCELLigence® electrodes as described in Methods. Cell impedence levels were normalised prior to the addition of vehicle (PBS, filled circle), rhVEGF (25 ng/ml, filled square) or rhGREM1 (100 ng/ml, filled triangle). Changes in ECFC barrier function were measured for 60 min, and data plotted as mean cell impedence -/+ SD. Cell Index values were also taken after ligand-induced peak dip for at least 5 h. Data for an individual clone is shown, and is representative of data obtained xCELLigence® data obtained from 4 independent ECFC clones tested in quadruplicate.

Supporting Information

Quantitative PCR

Endothelial colony-forming cells (ECFCs) were seeded on 6-well plates coated with fibrinogen (2 mg/ml in ddH_2O) to be 70 % confluent the next day before serum reducing in 2 % FBS Endothelial Cell Growth Medium 2 (EGM-2, PromoCell, Heidelberg, Germany) overnight. Cells were then treated with vehicle (PBS) or rhGREM1 (100 ng/ml, R&D Systems) for 4, 8 and 16h in serum-free EGM-2. Total RNA was extracted using the RNeasy Mini Kit (QIAGEN) following manufacturer's instructions. The cDNA was synthesised from total RNA using the SuperScript III First-Strand cDNA Synthesis System for RT-qPCR (Invitrogen; Thermo Fisher Scientific, Inc.). QT-PCR was carried out using ThermoFisher TaqMan probesets (VEGFR2, assay ID Hs00911706_m1; B actin, and 18S) and ran on the Roche LightCycler 480. Relative quantification values were obtained according to the ΔΔCt method, using β-actin and 18S as housekeeping controls. Values were normalised to each time-matched vehicle control which was set to 1. Data were plotted as mean fold-change -/+ SD. Data were analysed using one way ANOVA with Bonferroni's multiple comparison test. No significant differences between the groups were detected.

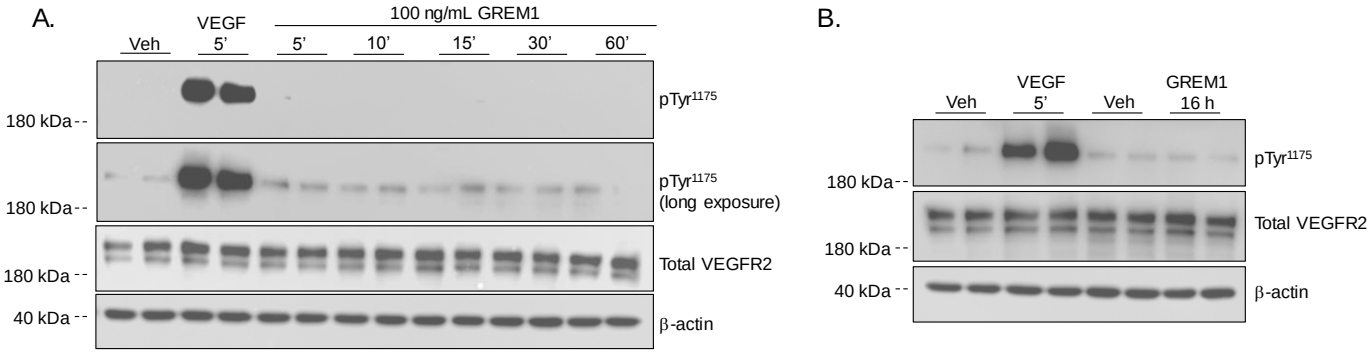
Immunocytochemistry

Endothelial colony-forming cells (ECFCs) were seeded on Ibidi µ-Slide 8-Well chambered coverslips coated with fibrinogen (2 mg/ml in ddH_2O) and serum reduced in 2 % FBS Endothelial Cell Growth Medium 2 (EGM-2, PromoCell, Heidelberg, Germany) overnight. Cells were further serum starved in serum-free media for 4 h, and then incubated with vehicle (PBS), rhVEGF (50 ng/ml for 5 min, R&D Systems) or rhGREM1 (25 ng/ml or 100 ng/ml for 15 min, R&D Systems). Cells were washed 1x with PBS, fixed in 4 % (w/v) paraformaldehyde at room temperature for 15 min, washed 3x 5 min with PBS and permeabilised with 0.1 % Tween-20 for 10 min. Cells were then blocked for 1 hour at room temperature with 5 % PBS/0.1 % Tween and incubated with anti-p1175 VEGFR2 antibody (Cell Signalling Laboratories, 19A10) at 1:200 dilution overnight at 4 °C in 1 % PBS/0.1 % Tween. Cells were washed with PBS 3 x and then incubated with donkey anti-rabbit Alexa Fluor 488 secondary (1:1000 dilution in 1 % PBS 0.1 % Tween. Abcam (ab150073)) for 60 min at RT, followed by 3 x washes in PBS-T. DAPI was added to the final wash to visualise cell nuclei. Cells were imaged using the Leica DMi8 microscope. Images were captured at 20 X magnification.

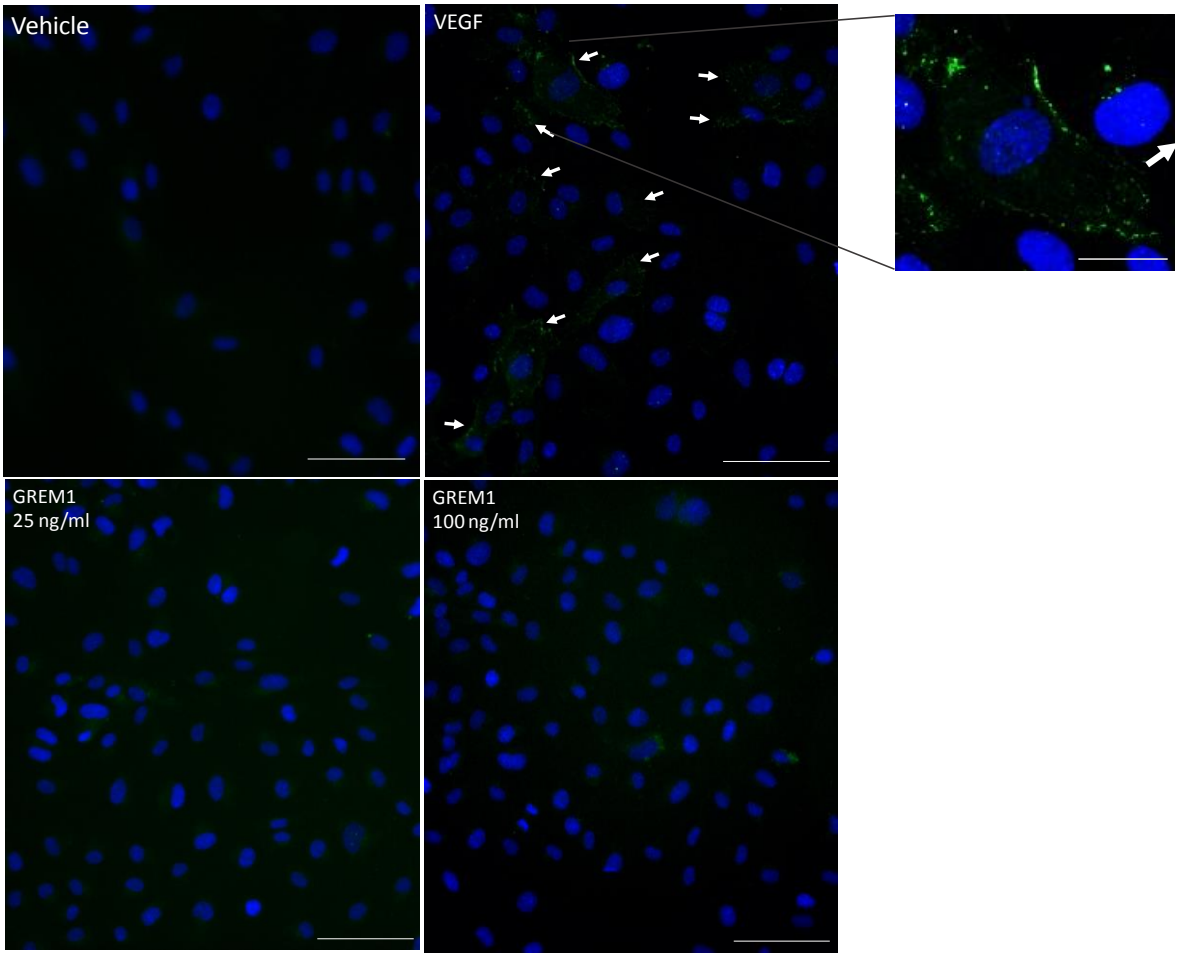
HUVEC Treatment

Human umbilical vein endothelial cells (HUVECs) were maintained in Endothelial Cell Growth Medium MV 2 (PromoCell) supplemented with Endothelial Cell Growth Medium MV2 SupplementMix (PromoCell) to final concentrations of 5 ng/mL Epidermal Growth Factor (recombinant human), 10 ng/mL Basic Fibroblast Growth Factor (recombinant human), 20 ng/mL Basic

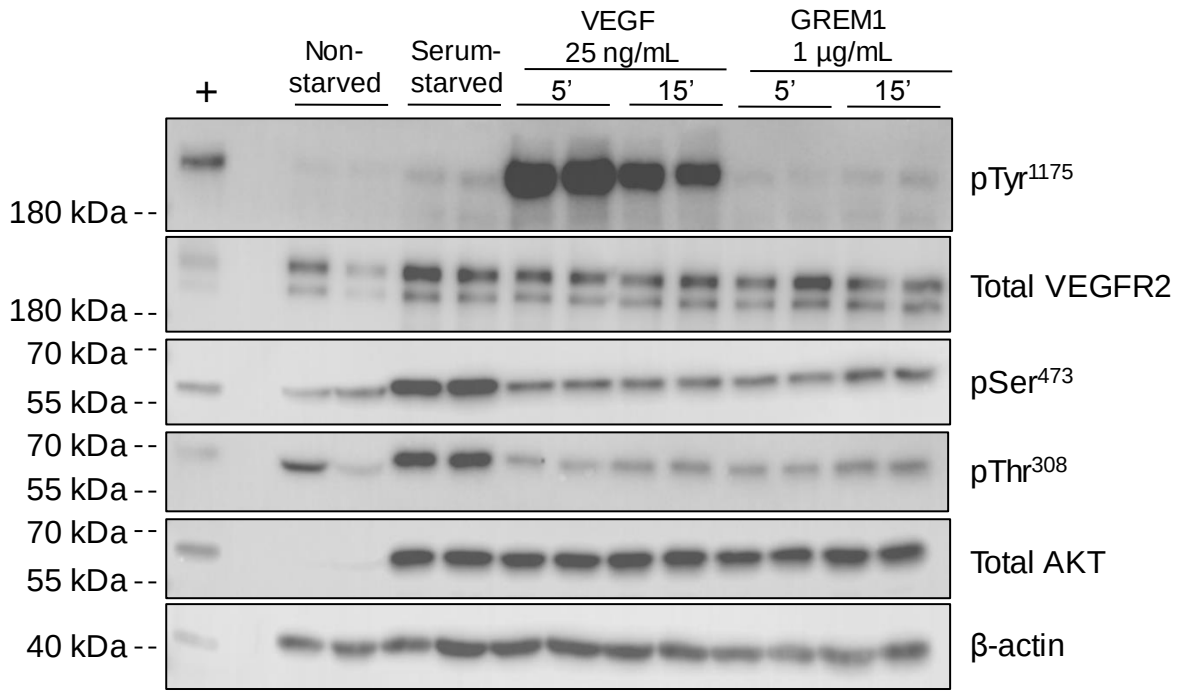
Fibroblast Growth Factor (recombinant human), 0.5 ng/mL Vascular Endothelial Growth Factor 165 (recombinant human), 1 µg/mL Ascorbic Acid, 0.2 µg/mL Hydrocortisone and further supplemented with 10% FBS. Cells were grown in gelatin-coated T75 cm^2 flasks at 37°C, 5% CO_2 and 95% air and plated on 60 mm gelatin-coated dishes for treatments.



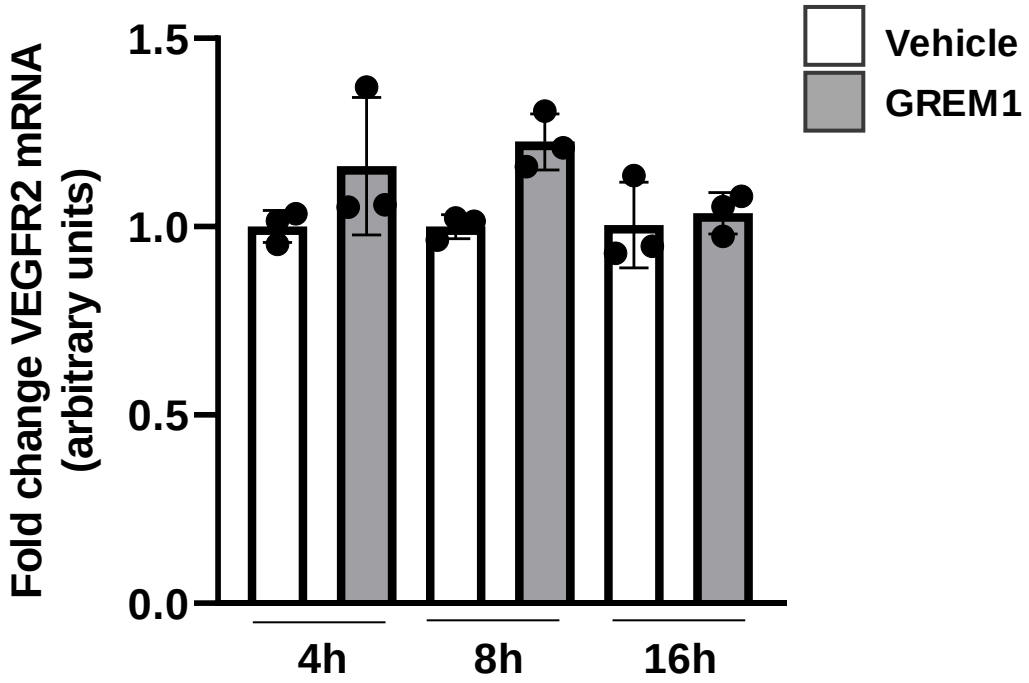
Supporting Figure 1. GREM1 does not activate VEGFR2 phosphorylation over a range of time-points. Endothelial colony-forming cells (ECFCs) isolated from human blood were seeded on fibrinogen (2 mg/ml in ddH_2O) and serum reduced in 2 % FBS EGM-2 medium overnight. On the day of experiment, cells were serum starved for 4 h, and then incubated with vehicle (PBS) or rhGREM1 (100 ng/ml) for 5, 10, 15, 30, 60 min (A) or 16 h (B). Separate cells were incubated with vehicle (Veh, PBS) or rhVEGF (25 ng/ml) for 5 min as positive control. Protein lysates were analysed in duplicate by SDS-PAGE using antibodies reactive to pTyr1175 on VEGFR2, total VEGFR2 and β-actin as loading control. Both short and long exposure panels for pTyr1175 VEGFR are included to maximise the detection of GREM1-induced phosphorylation.



Supporting Figure 2. Increased VEGFR2 phosphorylation at the plasma membrane is detected with VEGF, but not GREM1 treatment of ECFCs. Endothelial colony-forming cells (ECFCs) isolated from human blood were seeded on fibrinogen (2 mg/ml in ddH_2O) and serum reduced in 2 % FBS EGM-2 medium overnight. On the day of experiment, cells were serum starved for 4 h, and then incubated with vehicle (PBS), rhVEGF (50 ng/ml for 5 min) or rhGREM1 (25 ng/ml or 100 ng/ml for 15 min). Cells were washed 1 x with PBS, fixed in 4 % (w/v) paraformaldehyde, permeabilised with 0.1 % Tween-20 and then incubated with anti-p1175 VEGFR2 antibody (Cell Signalling Laboratories, 19A10) at 1:200 dilution overnight at 4 ^{o}C. Cells were washed and then incubated with donkey anti-rabbit Alexa Fluor 488 secondary (1:1000 dilution) for 60 min at RT, followed by washing in TBS-T. DAPI was added to the final wash to visualise cell nuclei. Cells were imaged using a Lecia DMi8 microscope. Images were captured at 20 X magnification, with the insert image captured at 40 X magnification. Scale bars represent 100 μm for the four main panels and 160 μm for the insert panel at 40 X. Arrows indicate areas of positive staining for phosphorylated VEGFR2.



Supporting Figure 3. GREM1 does not activate VEGFR2 phosphorylation in HUVECs. Human umbilical vein endothelial cells (HUVECs) were grown in complete medium ("non-starved"), or medium without FBS overnight ("starved") as indicated. Starved cells were treated with recombinant hVEGF (25 ng/ml) for 5 or 15 min, or recombinant hGREM1 (1 µg/ml) for 5 or 15 min. Protein lysates (duplicates) were run on SDS-PAGE and probed with the indicated antibodies reactive to pTyr 1175 on VEGFR2, total VEGFR2, pThr308 and pSer473 phospho-Akt antibodies, total Akt and β-actin as loading control. "+" indicates positive control.



Supporting Figure 4. GREM1 does not alter *VEGFR2* mRNA levels over a range of time-points. Endothelial colony-forming cells (ECFCs) isolated from human blood were seeded on fibrinogen (2 mg/ml in ddH_2O) and serum reduced in 2 % FBS EGM-2 medium overnight. On the day of experiment, cells were treated with vehicle (PBS, empty bars), or rhGREM1 (100 ng/ml, grey bars) for 4, 8 or 16 h. mRNA was extracted, cDNA generated and levels of *VEGFR2* were measured using ThermoFisher TaqMan probeset (assay ID Hs00911706_m1). Relative quantification values were obtained according to the DDCt method, using b-actin and 18S as housekeeping controls. Values were normalised to each time-matched vehicle control which was set to 1. Data were plotted as mean fold-change -/+ SD. Data were analysed using one way ANOVA with Bonferroni's multiple comparison test. No significant differences between the groups were detected.