



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

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Canonical Gremlin1 (GREM1) signaling involves binding to and sequestering bone morphogenetic proteins (BMPs) in the extracellular matrix, preventing the activation of cognate BMP receptor. 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 of the potential interaction between GREM1 and VEGFR2 in mammalian cells.

Bone morphogenetic proteins (BMPs)⁴ regulate a wide range of biological processes, such as lower-limb formation and kid-

ney 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 a BMP-BMP antagonist complex prevents the activation of the BMPRI/II complex, preventing phosphorylation of R-SMAD1/5/9 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 signaling has not yet been definitively identified. Several groups have suggested noncanonical signaling mechanisms for GREM1 via Slit proteins (22), ROBO (23), and fibrillin (24). Two reports in *Blood* (25, 26) in 2007 and 2010 identified GREM1 as a novel agonist of the proangiogenic receptor VEGFR2. These reports suggest that exposure of human endothelial vein endothelial cells (HUVECs) to GREM1 (~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 signaling in ARPE-19 retinal cells (30), HK-2 kidney tubule epithelial cells (31, 32), HaCaT skin keratinocytes, and primary skin fibroblasts (33). In contrast, another paper (46) suggests that GREM1 blocks VEGF signaling in the pulmonary microvascular endothelium. Given the wealth of new data implicating GREM1 signaling 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 signaling mechanisms of GREM1 signaling in mammalian cells. To that end, we report here that despite extensive effort, we failed to demonstrate GREM1-mediated activation of VEGFR2 in endothelial and other cells. Given the current model of GREM1 signaling in the literature, our data challenge the current dogma that GREM1 activates VEGFR2 phosphorylation and sheds new light on the likely sig-

cellular signal-regulated kinase; ECFC, endothelial colony-forming cell; PI3K, phosphatidylinositol 3-kinase; VEGF, vascular endothelial growth factor; EGM-2, endothelial cell growth medium 2; FBS, fetal bovine serum; HEK, human embryonic kidney; HRP, horseradish peroxidase.

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This article contains Figs. S1–S4.

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⁴ The abbreviations used are: BMP, bone morphogenetic protein; HUVEC, human umbilical vein endothelial cell; PLCγ, phospholipase Cγ; ERK, extra-

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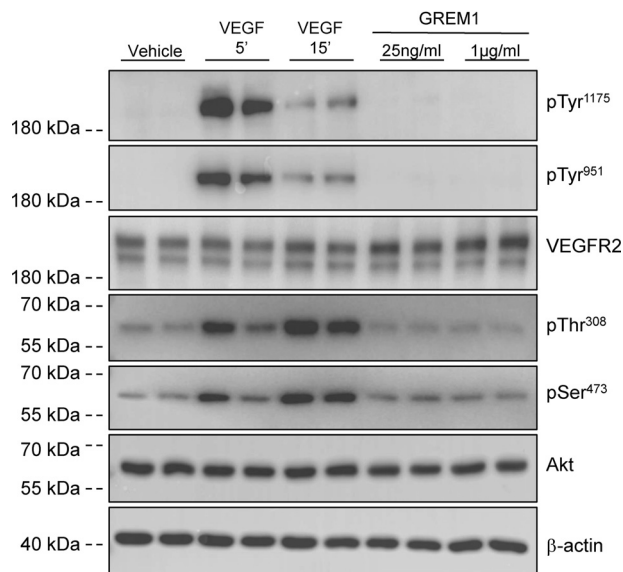


Figure 1. Gremlin1 does not activate VEGFR2 phosphorylation in ECFCs.

ECFCs isolated from human blood were seeded on fibrinogen (2 $\mu\text{g}/\text{ml}$ in double-distilled H_2O) and serum-reduced in 2% FBS EGM-2 medium overnight. On the day of the 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, or rhGREM1 (25 ng/ml and 1 $\mu\text{g}/\text{ml}$; R&D Systems) for 15 min. Protein lysates were run on SDS-PAGE and probed with the indicated antibodies reactive to pTyr¹¹⁷⁵ and pTyr⁹⁵¹ on VEGFR2, total VEGFR2, pThr³⁰⁸ and pSer⁴⁷³ phospho-Akt antibodies, total Akt, and β -actin.

nal mechanisms engaged by GREM1 during development and disease.

Results

Endothelial colony-forming cells (ECFCs) are late outgrowth endothelial progenitor cells that 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 PLC γ 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 (~ 2.5 nM) or a supraphysiological concentration of 1 $\mu\text{g}/\text{ml}$ (~ 100 nM) was used (Fig. 1). A time course of GREM1 treatment using 5-, 10-, 15-, 30-, and 60-min and 16-h treatment also failed to demonstrate increased VEGFR2 phosphorylation (Fig. S1). 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 ECFCs were grown on either collagen or fibrinogen to trigger co-activation of $\alpha\beta 3$, which was reported to enhance GREM1-mediated VEGFR2 phosphorylation (Figs. 1 and 2A) (28). Using immunofluorescence as a readout, increased phospho-VEGFR2 was detected at the plasma membrane of ECFCs (Fig. S2). Consistently, no increase was detected in GREM1-treated cells compared with vehicle (Fig. S2). 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 were 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 signaling in these cells. Importantly, the rhGREM1 used can completely block BMP2-mediated pSMAD1/5/9 phosphorylation in human embryonic kidney 293T (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 HUVECs (Fig. S3), 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 with VEGF as a functional readout of VEGFR signaling. Using the XCELLigence system, the ability of rhVEGF and rhGREM1 to reduce the integrity of the ECFC monolayer by decreasing cell impedance was assessed. The 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 impedance and did not compromise ECFC monolayer integrity (Fig. 3). The addition of higher concentrations of rhGREM1 (1 $\mu\text{g}/\text{ml}$, ~ 100 nM) also had no significant effect on ECFC cell impedance (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 (Fig. S4). Our previous data demonstrating grossly normal development of the vasculature in retina and other tissues in *Greml1*^{-/-} mice (36) also suggest that GREM1 signaling does not contribute a major physiological pro-angiogenic drive. We believe this is an important issue to resolve for the field, as GREM1 signaling via VEGFR2 has been suggested as an important noncanonical signaling modality for GREM1 that may contribute to pathophysiological signaling 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 signaling.

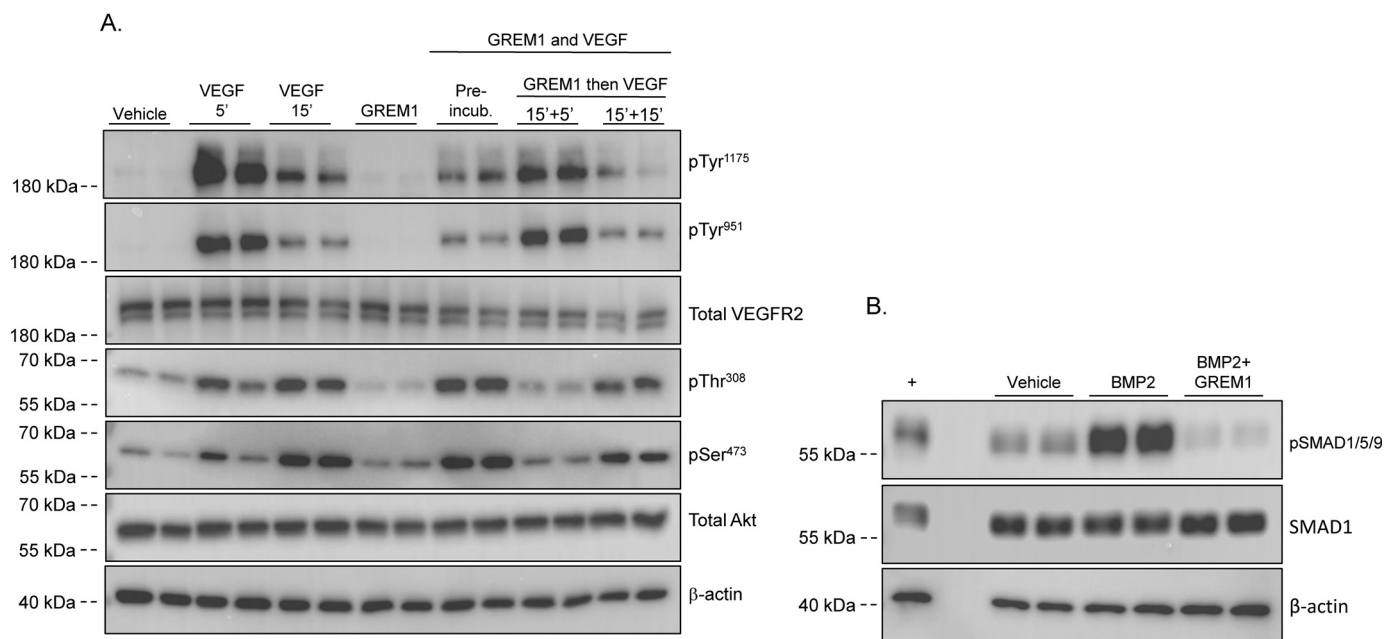


Figure 2. Gremlin1 does not inhibit VEGF signaling via the VEGFR2 receptor. *A*, ECFCs isolated from human blood were seeded on fibrinogen and serum-reduced in 2% FBS EGM-2 medium overnight. On the day of the experiment, cells were serum-starved for 3 h prior to treatment with vehicle (PBS), rhVEGF (25 ng/ml) for 5 and 15 min or 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 the addition to cells for 15 min. In addition, rhGREM1 (25 ng/ml) was 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 pTyr¹¹⁷⁵ and pTyr⁹⁵¹ on VEGFR2, total VEGFR2, pThr³⁰⁸ and pSer⁴⁷³ phospho-Akt antibodies, total Akt, and β-actin as loading control. *B*, HEK293T 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 °C. 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. +, positive control.

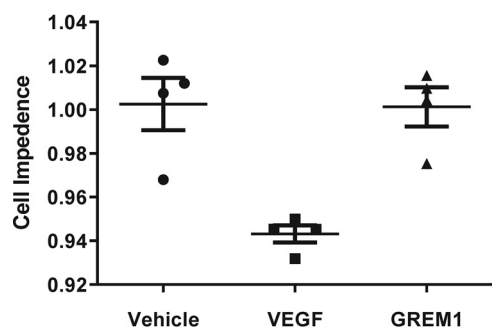


Figure 3. GREM1 does not alter ECFC barrier function. ECFCs were plated in E-plates containing XCELLigence® electrodes as described under "Experimental procedures." Cell impedance levels were normalized prior to the addition of vehicle (PBS; filled circles), rhVEGF (25 ng/ml; filled squares), or rhGREM1 (100 ng/ml; filled triangles). Changes in ECFC barrier function were measured for 60 min, and data are plotted as mean cell impedance ± S.D. (error bars). Cell index values were also taken after ligand-induced peak dip for at least 5 h. Data for an individual clone are shown and are representative of xCELLigence® data obtained from four independent ECFC clones tested in quadruplicate.

Experimental procedures

Cell culture

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 10% FBS and were grown in t75 cm² flasks precoated with rat tail collagen type 1 (BD Biosciences) at 37 °C, 5% CO₂, and 95% air. HEK293T cells were maintained in Dulbecco's modified Eagle's medium containing 1 g/liter glucose (Gibco) supplemented with 10% FBS and 100 μg/ml Primocin (Invitrogen).

Cells were grown in t75² flasks at 37 °C, 5% CO₂, 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) to achieve 70% confluence 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 preincubated at 37 °C for 15 min and then added to the cells for 15 min. Cells were also pretreated 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 preincubated 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 of ice-cold radioimmuno-precipitation assay buffer (50 mM Tris-HCl (pH 7.4), 1% (v/v) Nonidet P-40, 0.5% (v/v) sodium deoxycholate, 150 mM sodium chloride (NaCl), and 1 mM EDTA) supplemented with 250 μM sodium orthovanadate (NaVO₄; Sigma-Aldrich), 40 mM β-glycerolphosphate (Sigma-Aldrich), 1 mM sodium fluoride (NaF; Sigma-Aldrich), 2 μM microcystin-LR (Enzo® Life Sciences, New York), 1 mM phenylmethanesul-

ACCELERATED COMMUNICATION: *GREM1* does not activate *VEGFR2*

fonyl fluoride (Sigma-Aldrich), and 1× protease inhibitor mixture (Sigma-Aldrich). Protein lysates were probed by Western blotting using antibodies (1:1000) reactive to pVEGFR2 (pTyr¹¹⁷⁵) (Cell Signaling), pVEGFR2 (pTyr⁹⁵¹) (Cell Signaling), or total VEGFR2 (Cell Signaling) 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 (Thr³⁰⁸) (Cell Signaling), pAkt (Ser⁴⁷³) (Cell Signaling), total AKT (Cell Signaling), and β-actin (Cell Signaling) as the loading control in 5% (w/v) nonfat milk TBS-T. Reactive bands were visualized 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 Analyzer (ACEA Biosciences, Agilent, San Diego, CA). 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 described previously (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 room temperature for 30 min to facilitate uniform attachment. Impedance/cell index measurements were then taken every 15 min. When a stable monolayer was formed, EBM-2 medium containing vehicle (PBS), rhVEGF (25 ng/ml; R&D Systems), or rhGREM1 (100 ng/ml (~10 nM) or 1 μg/ml (~100 nM); R&D Systems) was then added to the appropriate wells, and recordings were taken. Changes in cell impedance values were normalized prior to treatment and measured for at least 5 h post-treatment.

Author contributions—L. R. D. and D. P. B. data curation; L. R. D. formal analysis; L. R. D., C. L. O., and R. J. M. investigation; L. R. D. and D. P. B. writing-review and editing; C. L. O., R. J. M., and D. P. B. conceptualization; C. L. O. and R. J. M. methodology; D. P. B. funding acquisition; D. P. B. project administration.

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