Supplemental Data

BIOLOGICAL ACTIVITIES OF PIGMENT EPITHELIUM-DERIVED FACTOR AND ITS PHOSPHOMIMETIC MUTANT ARE DIFFERENTIALLY REGULATED BY p38 AND JNK

By

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Supplemental Experimental Procedures:

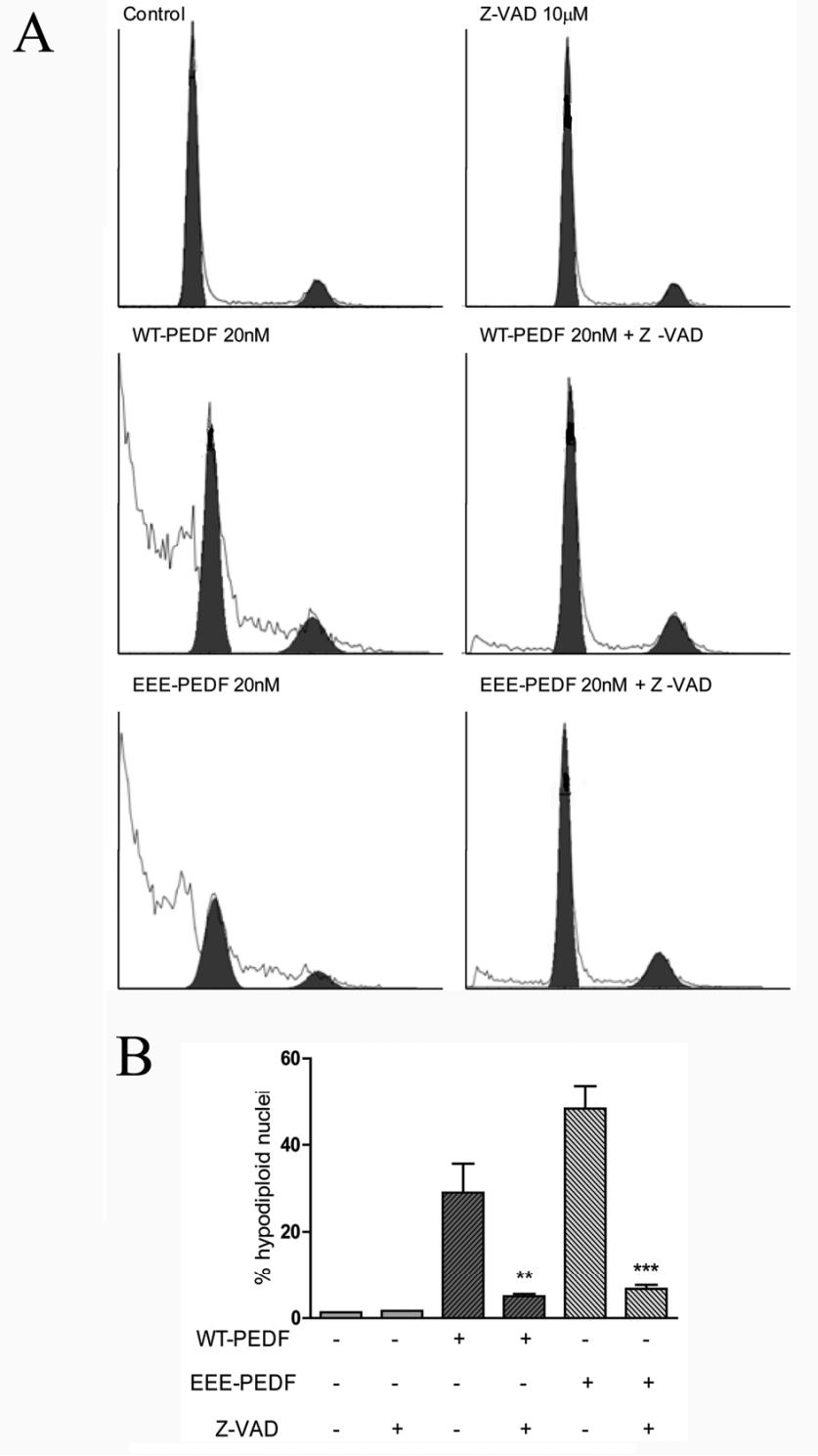
Materials - Propidium iodide was purchased from Sigma-Aldrich (St Louis, MO, USA). SB202190, RWJ67567, and BI78D3, inhibitors were from Tocris Bioscience (Ellisville, MO, USA). Anti pSer473-AKT (pAKT), general–AKT (gAKT) and GST antibodies were from Cell Signaling (Beverly, MA, USA).

FACS analysis - Cells were plated in 60mm dish, starved overnight in 1% FCS media and then either preincubated with Z-VAD.fmk (BD biosciences; 10µM, 30 min) or left untreated. Cells were then treated with PEDFs (WT-PEDF and EEE-PEDF, 20nM) for 48 h, after which they were harvested in Buffer W 0.1% sodium citrate, 0.1% Triton X-100. Cell cycle profiles and apoptosis were evaluated by DNA staining with 500 µl solution of 50 µg/ml propidium iodide in Buffer W for 30 minutes at 23°C. Samples were then analyzed with a FACScan flow cytometer (LSR II, Becton Dickinson), using Mod FitLT program.

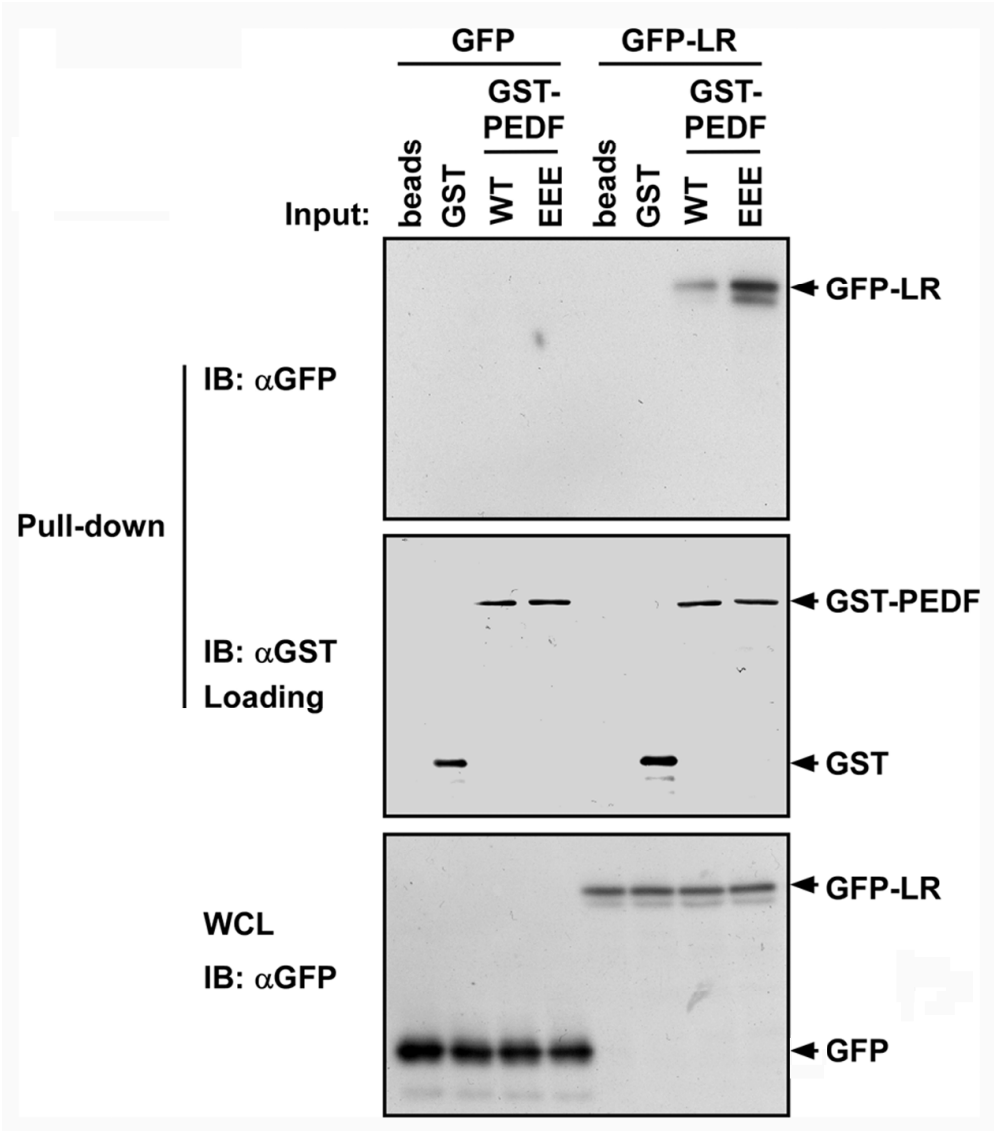
GST-PEDF purification - WT-PEDF and EEE-PEDF were cloned from pBlueScriptSKII(+)/PEDF, and pcDNA3/EEE-PEDF (17,18) respectively into pGJ41-GST-8His-TEV (provided by Dr. Ghil Jona, Protein Purification Unit, Weizmann Institute of Science) and expressed in *E. coli* BL21-pLysS. Bacterial cells were grown at 37°C to $OD_{600\ nm}$ = 0.5-0.6, and the expression of recombinant proteins was induced by 0.5 mM IPTG for 4 h. Pelleted bacterial cells were lysed in ice-cold PBS supplemented with 10 µg/ml leupeptin, 1 mM PMSF, 1 mM DTT and 1 mg/ml lysozyme followed by sonication. Lysates were then cleared by centrifugation at 12,000Xg at 4°C for 15 min. Purification of recombinant proteins was performed using glutathione sepharose beads (GE Healthcare) according to the manufacturer's protocol. The identity of recombinant PEDF constructs from elution fractions was verified by immunoblotting with anti PEDF antibody and mass spectroscopy.

GST pull-down assay - COS-7 cells were transiently transfected with 0.5 µg of either GFP-LR plasmid DNA using PEI (polyethylenimine). Forty eight hours after transfection cells were lysed and aliquots of cell lysates were combined with 0.5 µg of recombinant GST-WT-PEDF, GST-EEE-PEDF or GST alone in PBS. Samples were incubated overnight at 4°C with rotation. Thereafter, equal amounts of glutathione sepharose beads were added to each sample and incubated for additional 2 h at 4°C with rotation. Beads washed three times with washing buffer (10mM Tris pH 7.4, 1mM EDTA, 1mM EGTA pH 8.0, 150mM NaCl, 0.5% Triton X-100), resuspended in 1XSB and boiled. Resolved protein complexes were analyzed by immunoblotting with anti GFP antibody. To confirm equal amounts of GST-WT-PEDF, GST-EEE-PEDF or GST in each sample, membrane was then re-probed with anti GST antibody.

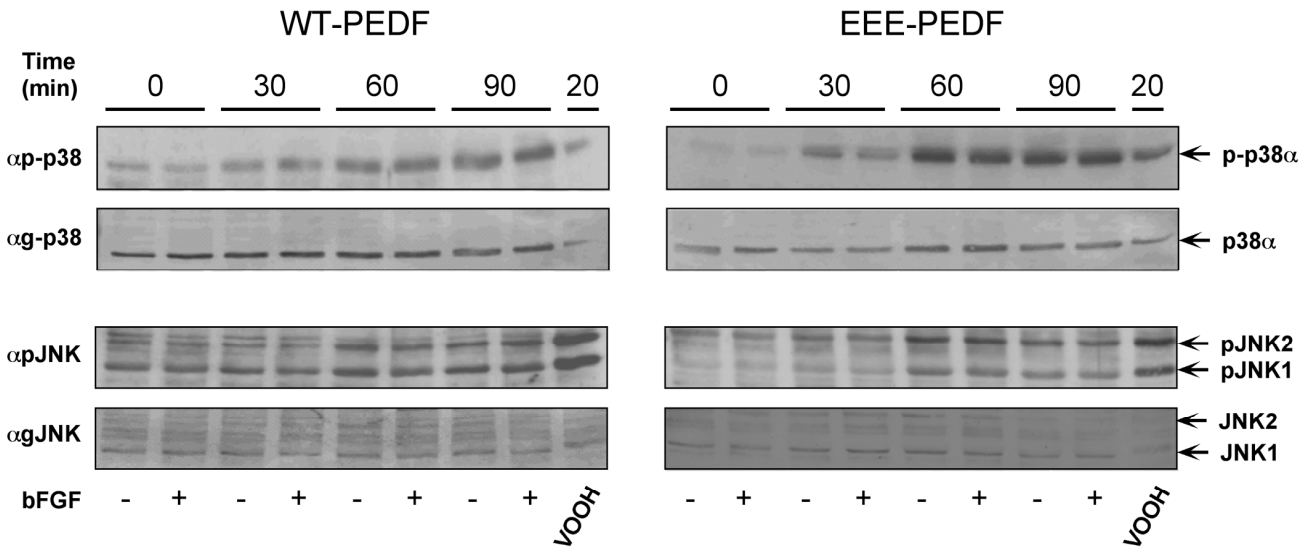
Supplemental Figures



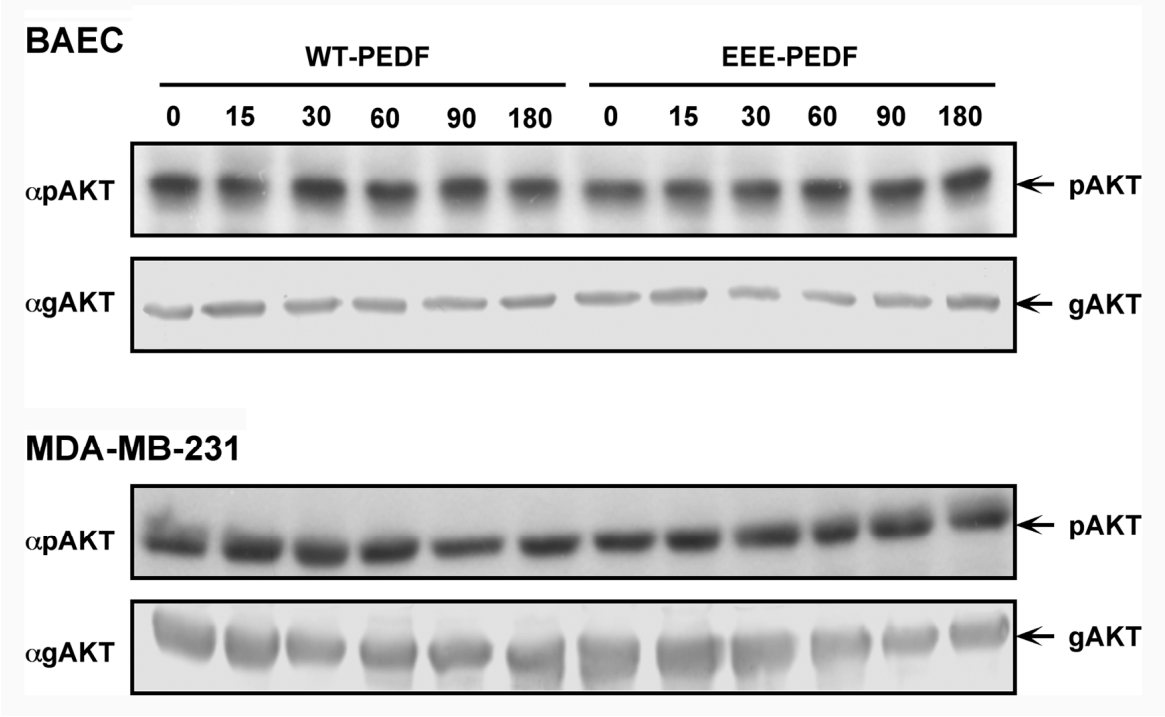
Supplemental Fig. S1. FACS analysis confirms the apoptotic effect of WT-PEDF and EEE-PEDF on BAEC. (A) BAEC were either pretreated with the pan-caspases inhibitor Z-VAD.fmk (10 μM, for 30 minutes) or left untreated, and then stimulated with WT-PEDF or EEE-PEDF (20 nM, 48 h). Then the cells were stained with propidium iodide and analysed by FACS as described under Supplemental Experimental Procedures. (B) Apoptosis of BAEC treated as described in (A) Quantification of hypodiploid nuclei from the experiment in A. Data shown are mean ± SD (n =3) **, $P < 0.01$, WT-PEDF+Z-VAD vs WT-PEDF; ***, $P < 0.001$, EEE-PEDF+Z-VAD vs. EEE-PEDF.



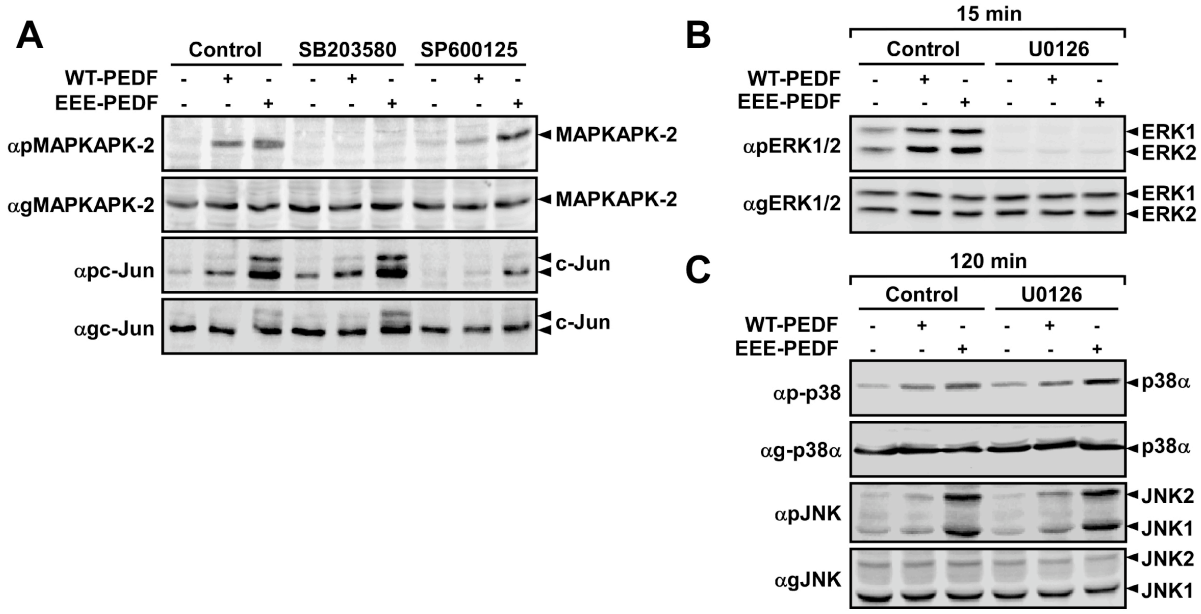
Supplemental Fig. S2. Binding of WT-PEDF and EEE-PEDF to recombinant LR in GST-pull down assay. COS-7 cells were transfected with GFP-LR and, 48 h after transfection, cells were lysed and aliquots of cell lysates were incubated with 0.5 µg of GST-WT-PEDF, GST-EEE-PEDF or GST alone. Following incubation, protein complexes were pulled-down using glutathione sepharose beads. GFP-LR pulled down with different PEDF constructs was analyzed by immunoblotting with anti GFP antibody.



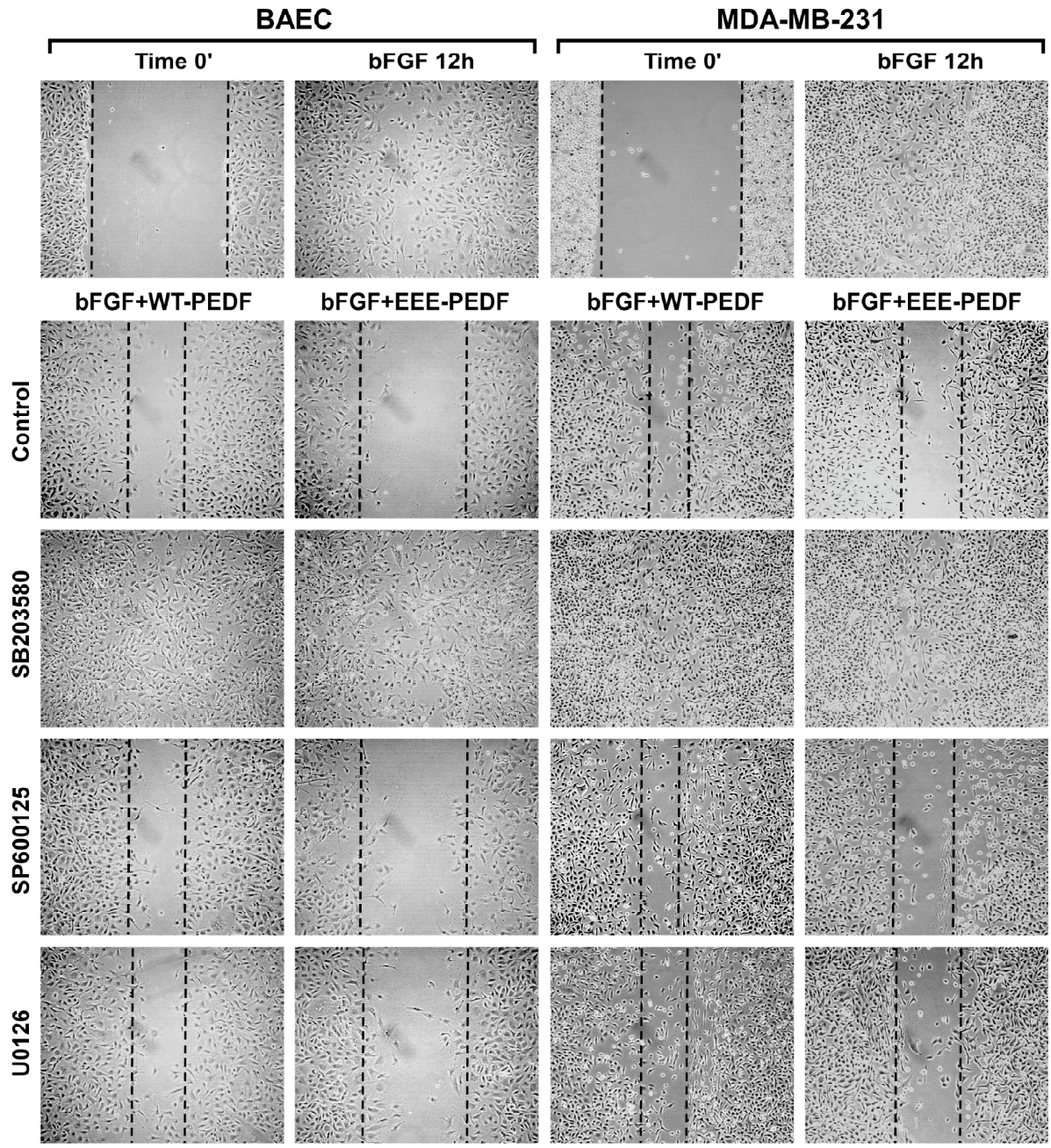
Supplemental Fig. S3. The effect of bFGF on WT-PEDF and EEE-PEDF treated BAEC. BAEC were treated with or without bFGF (20 ng/ml) and either WT-PEDF or EEE-PEDF (20 nM) at the indicated time points. P38α and JNK1/2 phosphorylation was detected by immunoblotting with the indicated antibodies.



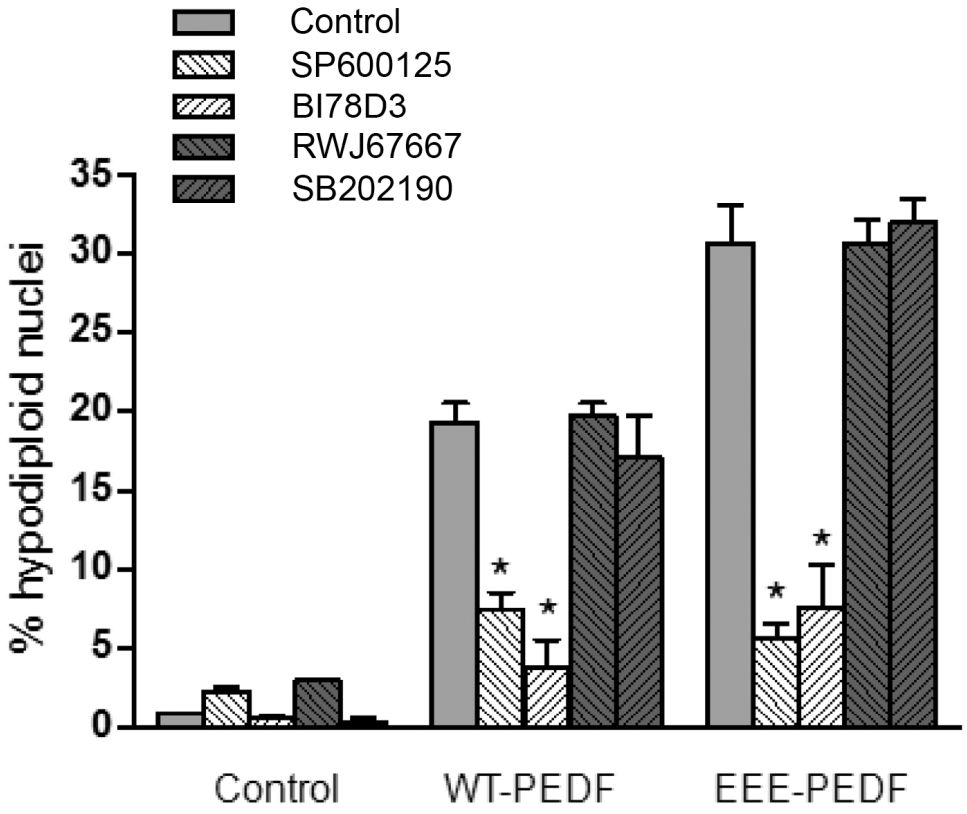
Supplemental Fig. S4. The effect of WT-PEDF and EEE-PEDF on the activity of Akt in BAEC and MDA-MB-231 cells. The cells were treated with either WT-PEDF or EEE-PEDF (20 nM) at the indicated time points. Phosphorylation was detected by immunoblotting with anti pSer473-AKT (pAKT) and general–AKT (gAKT) antibodies as indicated.



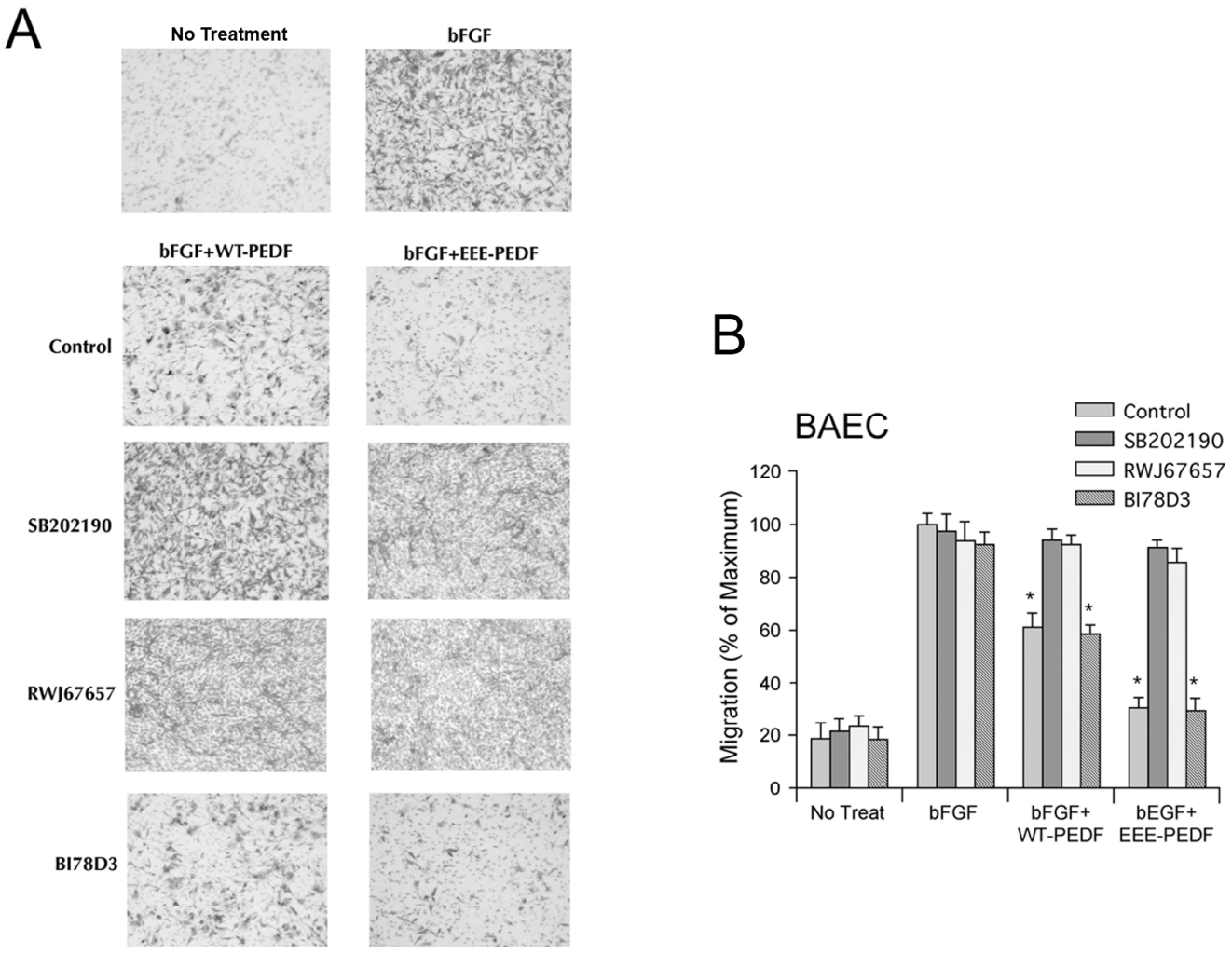
Supplemental Fig. S5. Specificity of p38α/β, JNK1-3 and MEK1/2 inhibitors. (A). BAEC were pretreated with p38α/β (SB203580, 10 μM) or JNK1-3 (SP600125, 5 μM) inhibitors for 1 h followed by incubation with WT-PEDF or EEE-PEDF (20 nM) for additional 2 h. Cells were lysed and phosphorylation level of the respective p38α and JNK1/2 substrates, c-Jun and MAPKAPK2 was analyzed by immunoblotting with anti phospho-specific antibodies. (B) BAEC were pretreated with MEK1/2 (U0126, 5 μM) inhibitor for 1 h followed by incubation with WT-PEDF or EEE-PEDF (20 nM) for additional 15 min. Cells were lysed and ERK1/2 phosphorylation was analyzed by immunoblotting with anti phospho ERK1/2 antibody. (C) BAEC were pretreated with MEK1/2 (U0126, 5 μM) inhibitor for 1 h followed by incubation with WT-PEDF or EEE-PEDF (20 nM) for additional 2 h. Cells were lysed and p38α and JNK1/2 phosphorylation was analysed by immunoblotting with anti phospho-specific antibodies.



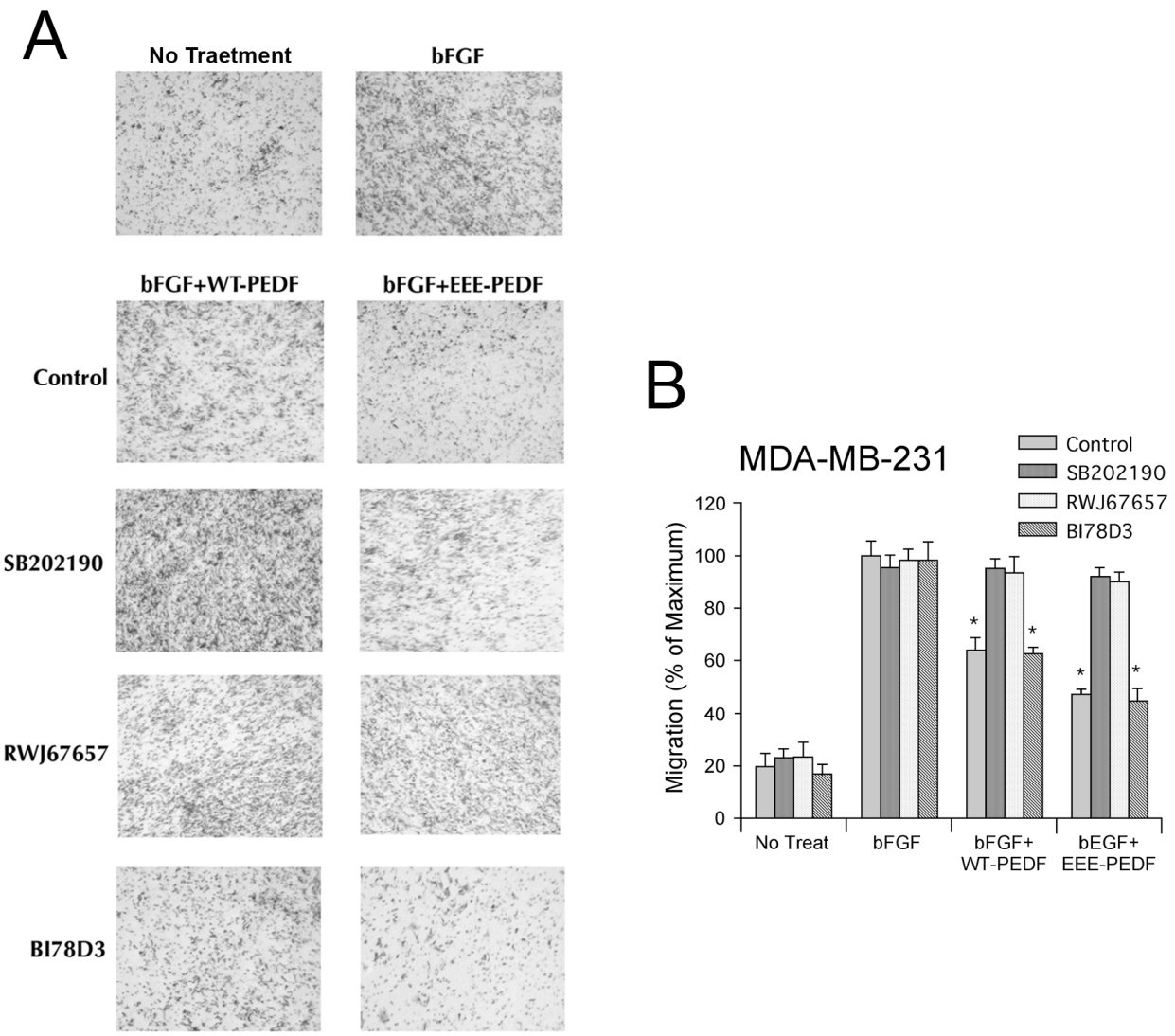
Supplemental Fig. S6. The involvement of p38, JNK1/2 and ERK1/2 in the antimigratory activity of WT-PEDF and EEE-PEDF. Migration of BAEC and MDA-MB-231 in the presence of bFGF (20 ng/ml) after pretreatment with p38α/β (SB203580, 10 μM), JNK1-3 (SP600125, 5 μM) or MEK1/2 (U0126, 5 μM) inhibitors for 1 h followed by incubation with either WT-PEDF or EEE-PEDF (20 nM) for 12 h was evaluated by "wound healing" assay.



Supplemental Fig. S7. Confirmation of JNK1-3, but not p38α/β, role in the pro-apoptotic activity of WT-PEDF and EEE-PEDF. BAEC cells were pretreated with either p38α/β (RWJ67567, 10 μM; SB202190, 10 μM) or JNK 1-3 (SP600125, 5 μM; BI78D3, 10 μM) inhibitors for 1 h, followed by incubation with WT-PEDF or EEE-PEDF (20 nM) for 24 hours. Cells were then stained with propidium iodide as described under Supplemental Experimental Procedures. Apoptosis is shown as % of hypodiploid nuclei. Data shown are mean ± SD (n =3) *, $P < 0.05$, WT-PEDF and EEE-PEDF + JNK1/2 inhibitors vs. WT-PEDF and EEE-PEDF alone.



Supplemental Fig. S8. Confirmation of p38α/β, but not JNK, role in bFGF-stimulated BAEC migration. BAEC were subjected to a transwell migration assay in the presence of bFGF (20 ng/ml). After pretreatment with either inhibitors for p38α/β (SB202190, 10μM and RWJ67657, 10μM), JNK (BI78D3, 10μM) or mock control for 1 h, the cells were incubated with either WT-PEDF or EEE-PEDF (20 nM) for 24 h and analysed as described under Experimental Procedures. (A) Shown are representative photographs of crystal violet stained X20 fields of migrated cells taken from the bottom side of the polycarbonate membranes. (B) Following visualization, stain was extracted and migration was quantified by OD_{540} measurement, and calculated as mean ± SD (n =3). *, WT-PEDF and EEE-PEDF and WT-PEDF and EEE-PEDF + JNK1/2 inhibitors vs. WT-PEDF and EEE-PEDF alone.



Supplemental Fig. S9. Confirmation of p38α/β, but not JNK, role in bFGF-stimulated MDA-MB-231 cell migration. MDA-MB-231 cells were subjected to a transwell migration assay in the presence of bFGF (20 ng/ml). After pretreatment with either inhibitors for p38α/β (SB202190, 10μM and RWJ67657, 10μM), JNK (BI78D3, 10μM) or mock control for 1 h, the cells were incubated with either WT-PEDF or EEE-PEDF (20 nM) for 24 h, and analysed as described under Experimental Procedures. (A) Shown are representative photographs of crystal violet stained X20 fields of migrated cells taken from the bottom side of the polycarbonate membranes. (B) Following visualization, stain was extracted and migration was quantified by OD_{540} measurement, and calculated as mean ± SD (n =3). *, $P < 0.05$ *, WT-PEDF and EEE-PEDF and WT-PEDF and EEE-PEDF + JNK1/2 inhibitors vs. WT-PEDF and EEE-PEDF alone.