SUPPLEMENTARY MATERIAL

Pigment Epithelium-Derived Factor and its Phosphomimetic Mutant Induce JNK-Dependent Apoptosis and P38-Mediated Migration Arrest

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Supplemental Figures

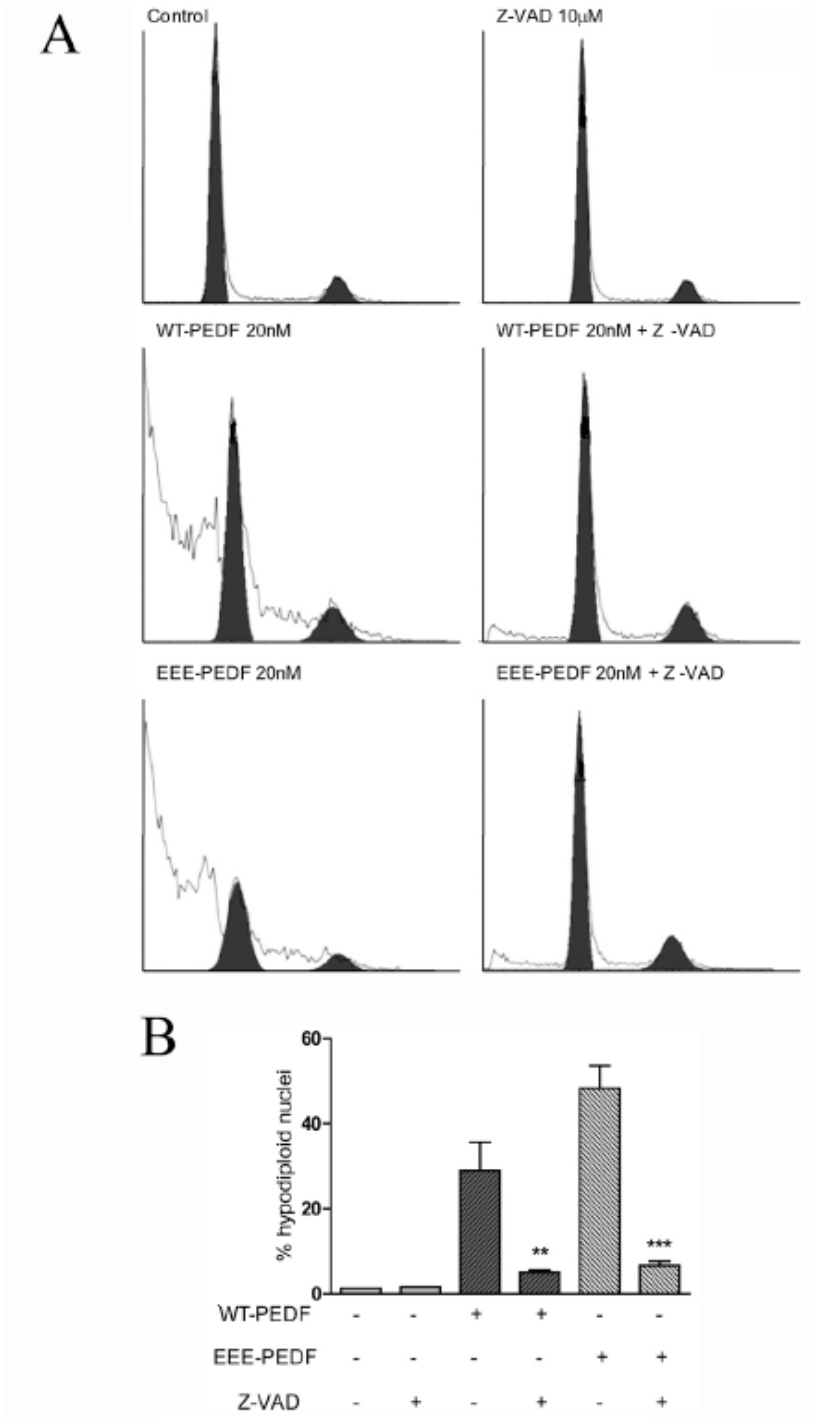

Fig. S1. FACS analysis confirms the apoptotic effect of WT-PEDF and EEE-PEDF on BAEC. (A) BAEC were either pretreated with the pan-caspases inhibitor Z-VAD.fmk (10 μM, for 30 minutes) or left untreated, and then stimulated with WT-PEDF or EEE-PEDF (20 nM, 48 h). Then the cells were stained with propidium 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.

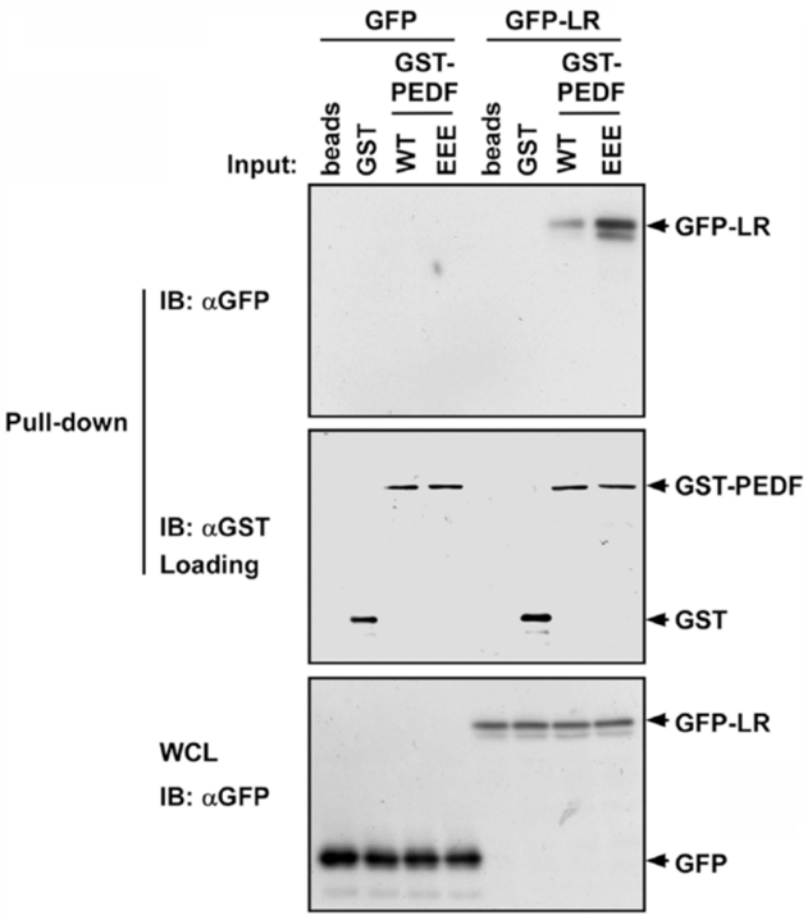

Fig. S2. Binding of WT-PEDF and EEE-PEDF to recombinant LR in GST-pull down assay. COS-7 cells were transfected with GFP-LR and, 48 h after transfection, cells were lysed and aliquots of cell lysates were incubated with 0.5 μg of GST-WT-PEDF, GST-EEE-PEDF or GST alone. Following incubation, protein complexes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

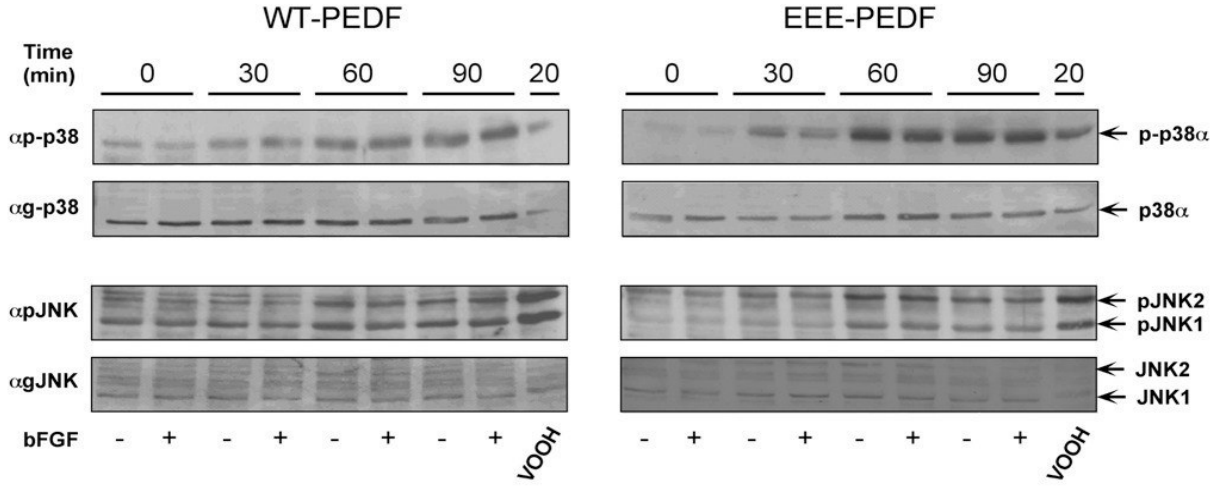

Fig. S3. The effect of bFGF on WT-PEDF and EEE-PEDF treated BAEC. BAEC were treated with or without bFGF (20 ng/ml) and either WT-PEDF or EEE-PEDF (20 nM) at the indicated time points. P38α and JNK1/2 phosphorylation was detected by immunoblotting with the indicated antibodies.

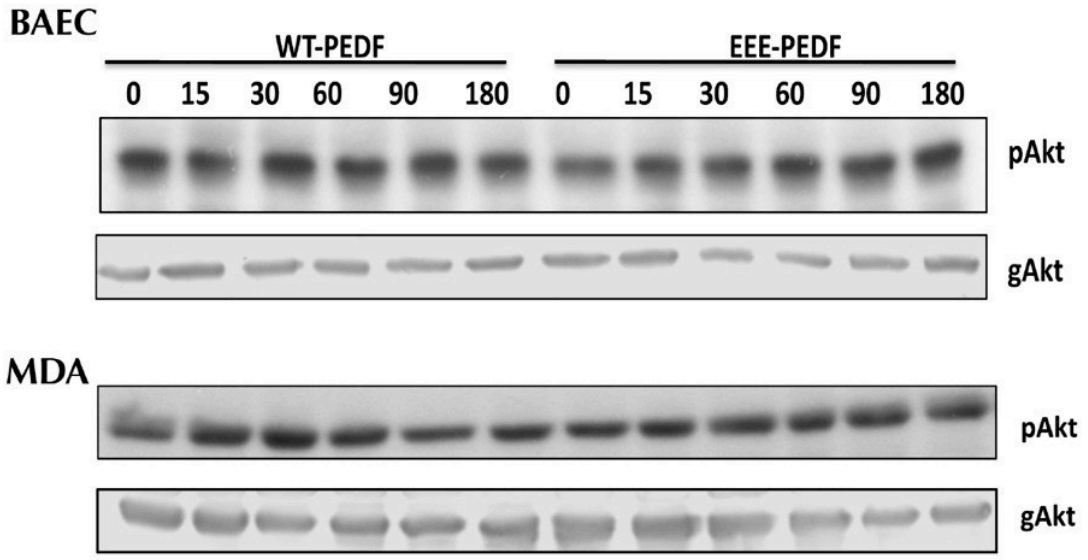

Fig. S4. The effect of WT-PEDF and EEE-PEDF on the activity of Akt in BAEC and MDA-MB-231 cells. The cells were treated with either WT-PEDF or EEE-PEDF (20 nM) at the indicated time points. Phosphorylation was detected by immunoblotting with anti pSer473-AKT (pAKT) and general–AKT (gAKT) antibodies as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

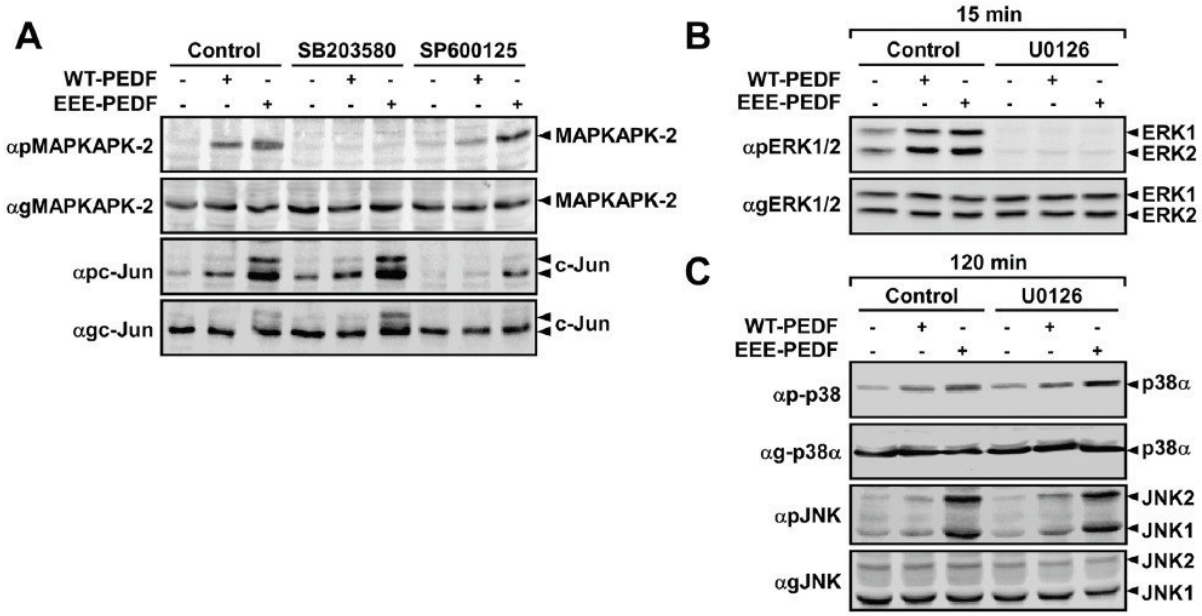

Fig S5. Specificty of p38α/β, JNK1-3 and MEK1/2 inhibitors. (A) BAEC were pretreated with p38α/β (SB203580, 10 μM) or JNK1-3 (SP600125, 5μM) inhibitors for 1 h followed by incubation with WT-PEDF or EEE-PEDF (20 nM) for additional 2 h. Cells were lysed and phosphorylation level of the respective p38a and JNK1/2 substrates, c-Jun and MAPKAPK2 was analyzed by immunoblotting with anti phospho-specifci antibodies. (B) BAEC were pretreated with MEK1/2 (U0126, 5μM) inhibtor 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, 5μM) inhibtor for 1 h followed by incubation with WT-PEDF or EEE-PEDF (20 nM) for additional 2 h. Cells were lused and p38a and JNK1/2 phosphorylation was analyzed by immunoblotting with anti phospho-specific antibodies.

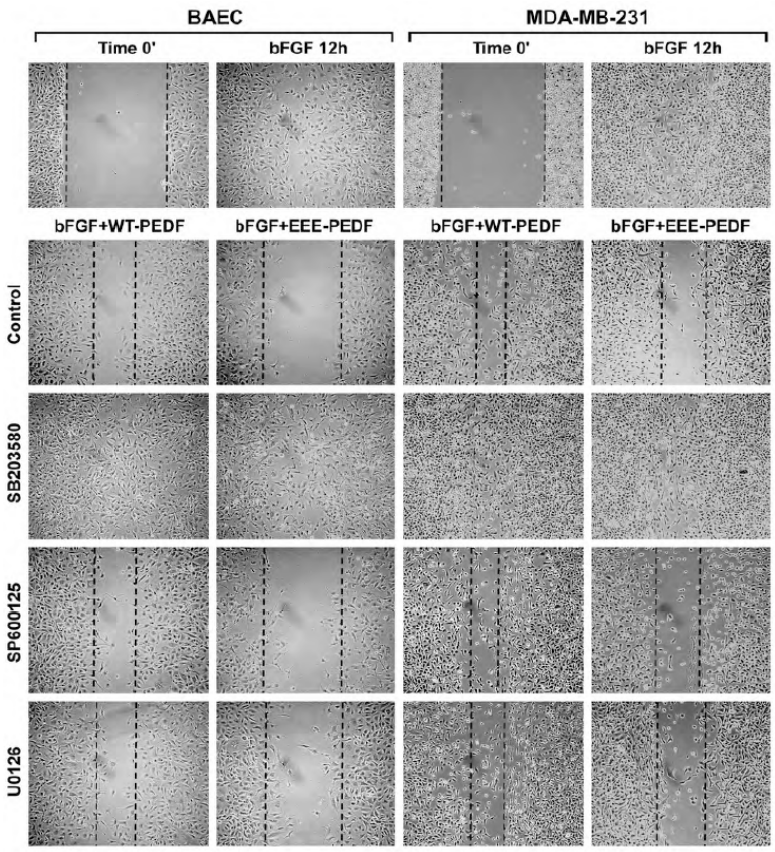

Fig. S6. The involvement of p38, JNK1/2 and ERK1/2 in the antimigratory activity of WT-PEDF and EEE-PEDF. Migration of BEAE and MDA-MB-231 in the presence of bFGF (20 ng/ml) after prestreatment with p38α/β (SB2013580, 10 μM), JNK1-3 9SP600125, 5μM) or MEK1/2 (U0126, 5 μM) inhibtors for 1 h, followed by incubation with either WT-PEDF or EEE-PEDF (20 nm) for 12 h was evaluated by "wound healing" assay.

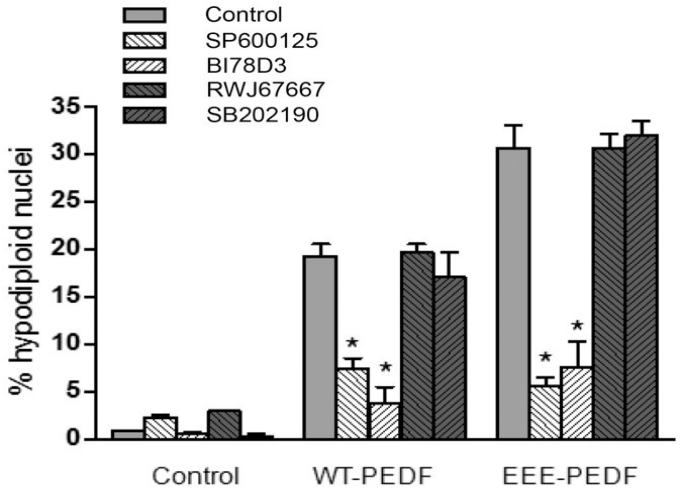

Fig. S7. Confirmation of JNK1-3, but not p38α/β, role in the pro-apoptotic activity of WT-PEDF and EEE-PEDF. BAEC cells were pretreated with either p38α/β (RWJ67567, 10 μM; SB202190, 10 μM) or JNK 1-3 (SP600125, 5 μM; BI78D3, 10 μM) inhibitors for 1 h, followed by incubation with WT-PEDF or EEE-PEDF (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.

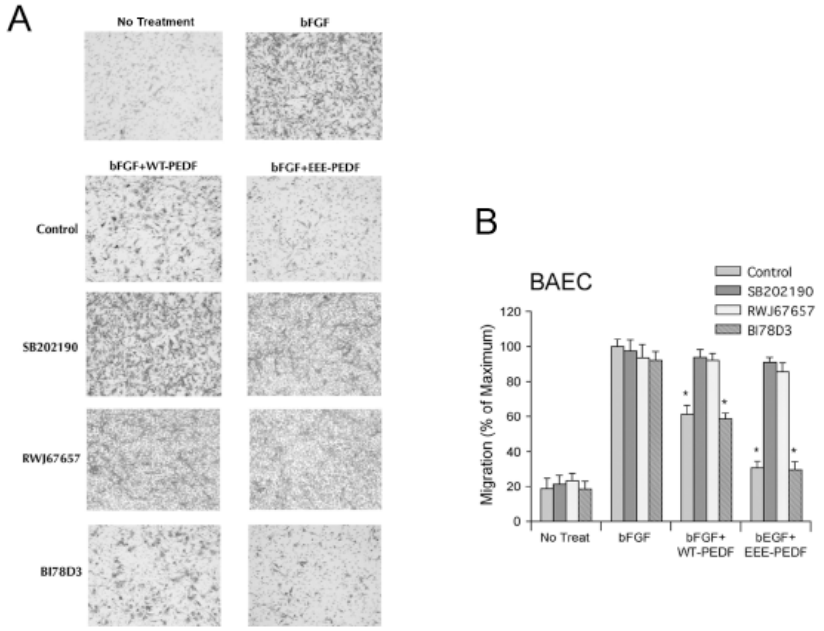

Fig. S8. Confirmation of p38α/β, but not JNK, role in bFGF-stimulated BAEC migration. BAEC were subjected to a transwell migration assay in the presence of bFGF (20 ng/ml). After pretreatment with either inhibitors for p38α/β (SB202190, 10μM and RWJ67657, 10μM), JNK (BI78D3, 10μM) or mock control for 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. (B) 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.

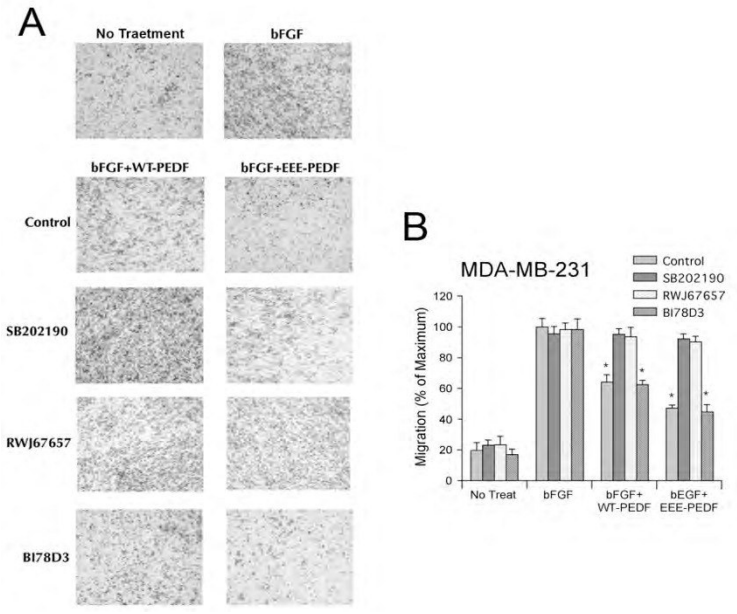

Fig. S9. Confirmation of p38α/β, but not JNK, role in bFGF-stimulated MDA-MB-231 cell migration. MDA-MB-231 cells were subjected to a transwell migration assay as in Fig S8. (A) Shown are representative photographs of crystal violet stained X20 fields of migrated cells. (B) Migration was quantified by 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.