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online data

SUPPLEMENTARY ONLINE DATA

Neuropilin-1 mediates PDGF stimulation of vascular smooth muscle cell migration and signalling via p130^{Cas}

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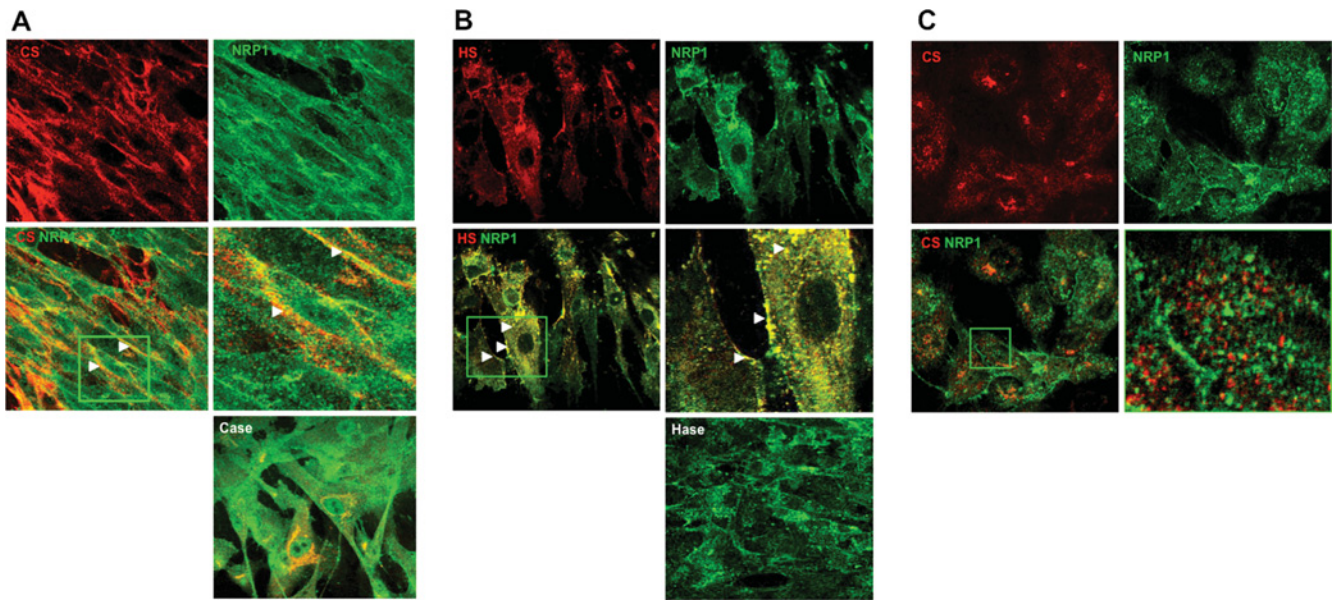

Figure S1 Immunofluorescent localization of NRP1 and CS and HS in HCASMCs and HUVECs

HCASMCs (**A** and **B**) and HUVECs (**C**) were fixed, permeabilized and then immunostained for NRP1 and co-stained with antibody for either CS (**A** and **C**) or HS (**B**). NRP1 is located in the cytoplasm and also at the membrane of the cells (**A** and **B**) where it co-localizes (indicated by white arrowheads) with CS (**A**) and HS (**B**). In HUVECs (**C**), such co-localization was not observed and rather distinct signals are seen for CS and NRP1. Parallel cultures of HCASMCs (**A** and **B**) were also treated with chondroitinase (Case) and heparitinase (Hase) before fixation and immunofluorescent staining. Results are representative of at least three independent experiments.

SUPPLEMENTARY METHODS

Receptor binding assay in intact cells

Confluent HCASMCs, PAE cells and PAE/NRP1 cells cultured in 24-well plates were washed twice with PBS. Binding medium (Dulbecco's modified Eagle's medium and 25 mM Hepes, pH 7.3, containing 0.1 % BSA) was added, followed by addition of 0.1 nM ^{125}I-PDGF-BB (1825 Ci/mmol; Perkin-Elmer) or ^{125}I-VEGF-A$_{165}$ (1200–1800 Ci/mmol; GE Healthcare). After 2 h of incubation at 4 °C, the medium was aspirated and cells were washed four times with ice-cold PBS. The cells were then lysed with 0.25 M NaOH and 0.5 % SDS solution, and radioactivity in the lysates was measured in a γ-counter. Non-specific binding was determined in the presence of a 100-fold excess of unlabelled PDGF-BB or VEGF-A$_{165}$ (R&D Systems).

PDGFR assay

In these assays of PDGFRs, an immobilized capture antibody specific for human PDGFR binds both tyrosine-phosphorylated and unphosphorylated PDGFR. After washing away unbound material, a biotinylated detection antibody specific for total PDGFR is used to detect both tyrosine-phosphorylated and unphosphorylated receptor, utilizing streptavidin–HRP (horseradish peroxidase), whereas an HRP-conjugated detection antibody specific for phosphorylated tyrosine is used to detect only tyrosine-phosphorylated receptor, utilizing HRP. The capture antibody was diluted to a working concentration of 4 μg/ml in PBS, without carrier protein and 100 μl was immediately added to a 96-well microplate for overnight incubation at room temperature. The next day, each well was washed four times with washing buffer (0.05 % Tween 20 in PBS, pH 7.2) and

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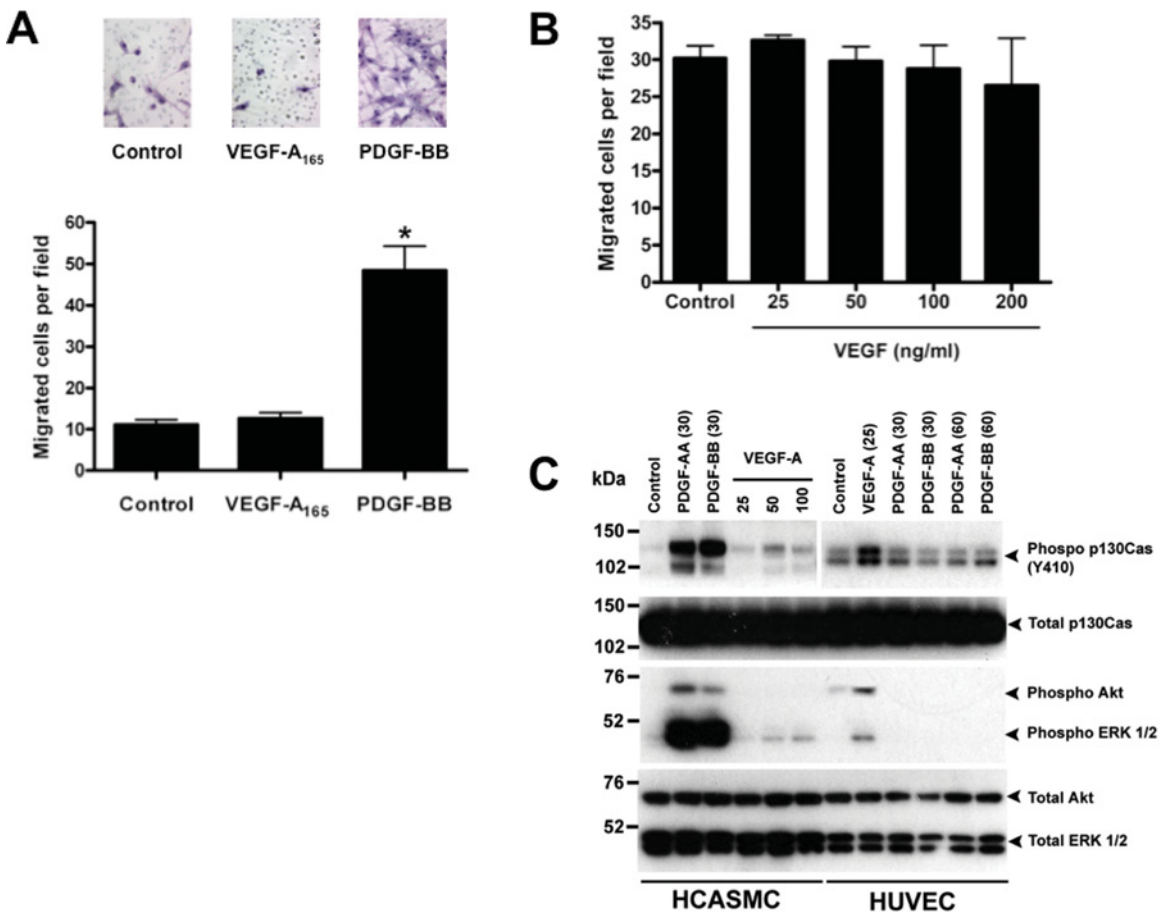

Figure S2 VEGF does not induce migration or signalling in HCASMCs

(**A** and **B**) HCASMCs transferred to transwells were stimulated to migrate for 4 h in response to serum-free medium (Control), 30 ng/ml PDGF-BB or 30 ng/ml VEGF-A_{165} (**A**), or in response to the indicated VEGF concentrations (**B**). Cells were stained and counted as described in the Materials and methods section of the main text. Results are mean+S.E.M. ($n = 3$) numbers of migrated cells obtained from multiple independent experiments. *$P < 0.05$ compared with control. (**C**) Confluent HCASMCs or HUVECs were treated for 10 min with the indicated concentrations (all ng/ml) of VEGF-A_{165}, PDGF-AA or PDGF-BB. Lysates were prepared and blotted with the antibodies indicated. Results are representative of three independent experiments. Molecular masses are indicated in kDa.

blocked for 2 h with 300 μl of PBS containing 1 % BSA. Wells were washed again three times before adding 100 μl of lysate prepared in the following diluent: 1 % Nonidet P40, 20 mM Tris/HCl (pH 8.0), 137 mM NaCl, 10 % glycerol, 2 mM EDTA and 1 mM activated sodium orthovanadate. The same diluent without protein was used as a blank. The plate was left to incubate for 2 h at room temperature and washed again. Then, 100 μl of detection antibody diluted to the manufacturer's recommendations in 20 mM Tris/HCl, 137 mM NaCl, 0.05 % Tween 20 and 0.1 % BSA (pH 7.2) was added directly to the well, before incubation for a further 2 h. When measuring total PDGFR levels, the following extra step was required: after another three washes, streptavidin–HRP was diluted according to the manufacturer's recommendations in the same buffer as the detection antibody and 100 μl was added to each well for 20 min at room temperature. Finally, 100 μl of substrate solution was added to each well for 20 min, followed by 50 μl of stop solution. The absorbance of each well was determined immediately, using a microplate reader set to 450 nm with wavelength correction of 595 nm. The values obtained for phospho-PDGFRs were normalized using the values obtained for total PDGFRs.

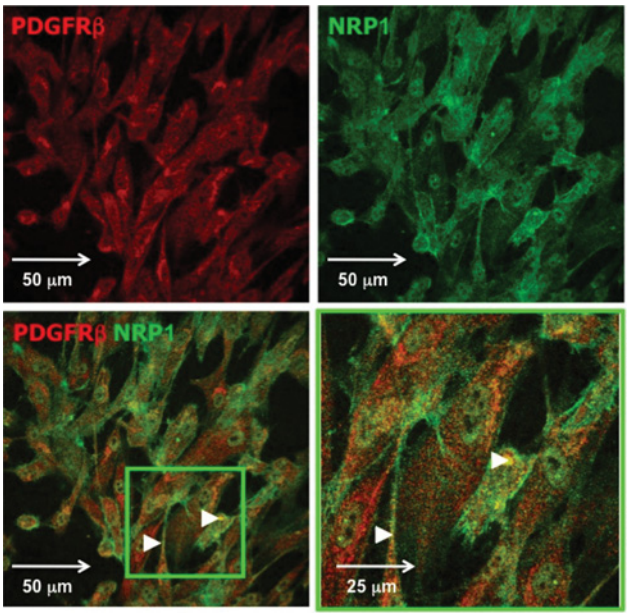

Figure S3 PDGFRβ and NRP1 do not co-localize in HCASMCs

HCASMCs were fixed, permeabilized and then immunostained for NRP1 (green) and co-stained with antibody for PDGFRβ (red). NRP1 is located in the cytoplasm and also at the membrane of the cells, and in the merge shows little co-localization with PDGFRβ in the membrane (bottom panels). Some co-localization can be observed in the cytoplasm (white arrowheads).

Table S1 Binding of ^{125}I-PDGF-BB or ^{125}I-VEGF-A_{165} was determined in intact confluent cultures of HCASMCs, PAE cells or PAE/NRP1 cells in the presence or absence of a 100-fold excess of unlabelled PDGF-BB or VEGF

Non-specific binding was defined as that which is not competed by an excess of unlabelled ligand. Specific binding was calculated by subtracting non-specific binding from total counts. Results are mean counts (c.p.m.) obtained from three independent experiments, with each experiment being performed in triplicate wells. Percentages of total binding are indicated in parentheses.

		^{125}I-ligand binding (c.p.m.)		
Growth factor	Cell type	Total	Non-specific	Specific
PDGF-BB	HCASMCs	6542 (100)	2600 (40)	3942 (60)
	PAE cells	13899 (100)	11857 (85)	2042 (15)
	PAE/NRP1 cells	13363 (100)	10626 (80)	2737 (20)
VEGF	HCASMCs	1595 (100)	726 (46)	868 (54)
	PAE/NRP1 cells	4137 (100)	688 (17)	3448 (83)

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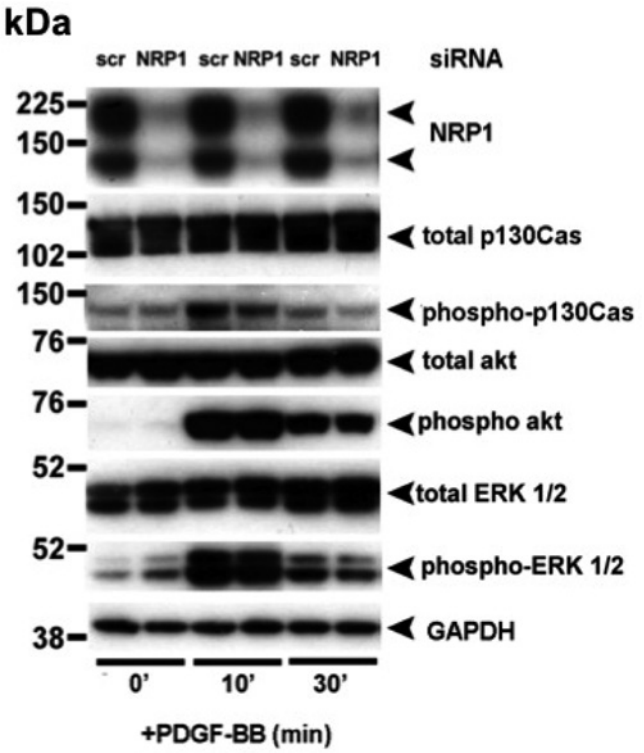

Figure S4 PDGF-stimulated p130^{Cas} phosphorylation is mediated via NRP1

HCASMCs were transfected with control scrambled siRNA (scr) or with NRP1 siRNA, and 3 days later, cells were incubated overnight in serum-free medium and then treated for the times indicated (in min) with 30 ng/ml PDGF-BB. Cells were then lysed and immunoblotted with the antibodies indicated. The results shown are representative of at least three independent experiments. Molecular masses are indicated in kDa.