



# Neuropilin-1 mediates PDGF stimulation of vascular smooth muscle cell migration and signalling via p130<sup>Cas</sup>

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NRP1 (neuropilin-1) is a co-receptor for members of the VEGF (vascular endothelial growth factor) family in endothelial cells, but is increasingly implicated in signalling induced by other growth factors. NRP1 is expressed in VSMCs (vascular smooth muscle cells), but its function and the mechanisms involved are poorly understood. The present study aimed to determine the role of NRP1 in the migratory response of HCASMCs (human coronary artery smooth muscle cells) to PDGF (platelet-derived growth factor), and to identify the signalling mechanisms involved. NRP1 is highly expressed in HAoSMCs (human aortic smooth muscle cells) and HCASMCs, and modified in VSMCs by CS (chondroitin sulfate)-rich O-linked glycosylation at Ser<sup>612</sup>. HCASMC migration induced by PDGF-BB and PDGF-AA was inhibited by NRP1 siRNA (small interfering RNA), and by adenoviral overexpression of an NRP1 mutant lacking the intracellular domain (Ad.NRP1ΔC). NRP1 co-immunoprecipitated with PDGFRα (PDGF receptor α), and immunofluorescent staining indicated that NRP1 and PDGFRα co-localized in VSMCs. NRP1 siRNA also inhibited

PDGF-induced PDGFRα activation. NRP1-specific siRNA, Ad.NRP1ΔC and removal of CS glycans using chondroitinase all inhibited PDGF-BB and -AA stimulation of tyrosine phosphorylation of the adapter protein, p130<sup>Cas</sup> (Cas is Crk-associated substrate), with little effect on other major signalling pathways, and p130<sup>Cas</sup> knockdown inhibited HCASMC migration. Chemotaxis and p130<sup>Cas</sup> phosphorylation induced by PDGF were inhibited by chondroitinase, and, additionally, adenoviral expression of a non-glycosylatable NRP1S612A mutant inhibited chemotaxis, but not p130<sup>Cas</sup> phosphorylation. These results indicate a role for NRP1 and NRP1 glycosylation in mediating PDGF-induced VSMC migration, possibly by acting as a co-receptor for PDGFRα and via selective mobilization of a novel p130<sup>Cas</sup> tyrosine phosphorylation pathway.

**Key words:** chemotaxis, glycosylation, neuropilin-1 (NRP1), p130<sup>Cas</sup>, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF).

## INTRODUCTION

NRP1 (neuropilin-1) is a transmembrane co-receptor with essential roles in neural pathfinding and cardiovascular development [1–3]. NRP1 is a receptor for Sema3A (semaphorin 3A), a member of a family of proteins involved in axonal guidance [4,5], and for VEGF (vascular endothelial growth factor)-A<sub>165</sub> and other members of the VEGF family of angiogenic cytokines, including the heparin-binding PIGF-2 (placental growth factor 2) isoform, VEGF-B and VEGF-E [6,7]. NRP2 has a similar domain structure to that of NRP1 with 44% amino acid identity, and is also expressed in endothelial, tumour and neuronal cells, but exhibits a distinct expression pattern in the developing nervous system [5,8,9]. Studies of NRP1 function in endothelial cells are generally indicative of a key role of NRP1 in VEGF-dependent cell migration. NRP1 is thought to act as a co-receptor for VEGF by forming complexes with the VEGF protein tyrosine kinase receptor, VEGFR2 (VEGF receptor 2)/KDR (kinase insert domain-containing receptor), resulting in enhanced

VEGFR2 activation, intracellular signalling, cell migration and angiogenesis [10–13].

Various evidence indicates that NRP1 plays a role in the functions of VSMCs (vascular smooth muscle cells). NRPs are expressed and up-regulated by FGF-2 (fibroblast growth factor 2) and PDGF (platelet-derived growth factor)-BB in VSMCs [14–16]. It was also reported that NRP1 can be co-immunoprecipitated with PDGF-BB [15]. However, the role of NRP1 in PDGF-regulated functions in VSMCs is poorly characterized, and the mechanisms through which NRP1 regulates PDGF actions are not known. In the present study, we examined the expression of NRP1 in VSMCs and its role in VSMC migration. NRP1 is highly expressed in HAoSMCs (human aortic smooth muscle cells) and HCASMCs (human coronary artery smooth muscle cells), both as the more usual full-length form of 130 kDa and as a high-molecular-mass species modified by O-linked glycosylation [16]. Importantly, we show that inhibition of NRP1 function significantly decreases the migratory response to PDGF-BB. Adenoviral overexpression of an NRP1 mutant

Abbreviations used: Ad.GFP, adenovirus expressing green fluorescent protein; Ad.NRP1ΔC, adenovirus expressing neuropilin-1 mutant lacking the intracellular domain; Ad.NRP1S612A, adenovirus expressing S612A mutant neuropilin-1; Ad.NRP1WT, adenovirus expressing wild-type neuropilin 1; Cas, Crk-associated substrate; CS, chondroitin sulfate; ERK, extracellular-signal-regulated kinase; FBS, fetal bovine serum; GAG, glycosaminoglycan; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HAoSMC, human aortic smooth muscle cell; HCASMC, human coronary artery smooth muscle cell; HCAEC, human coronary artery endothelial cell; HS, heparan sulfate; HUVEC, human umbilical vein endothelial cell; NRP1, neuropilin-1; PAE cell, porcine aortic endothelial cell; PAE/NRP1 cell, PAE cell expressing NRP1; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2; VSMC, vascular smooth muscle cell.

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lacking the C-terminal intracellular domain also inhibited PDGF-BB-induced migration, suggesting an important role for NRP1-dependent intracellular signalling in this role. Furthermore, we show that NRP1 associates with PDGFR (PDGF receptor)  $\alpha$  in HCASMCs, and that NRP1 inhibition reduces PDGF-BB signalling through p130<sup>Cas</sup> (Cas is Crk-associated substrate), an adaptor protein with important roles in cell migration [17], and which we recently showed to be regulated by NRP1 overexpression in the invasion of U87MG glioma cells [18]. PDGF-induced migration was also inhibited by expression of a non-glycosylatable NRP1S612A mutant. These findings indicate a novel role for NRP1 and NRP1 O-linked glycosylation in the VSMC chemotactic response to PDGF-BB and suggest that NRP1 is critical for optimal PDGF signalling through p130<sup>Cas</sup> which is important for migration.

## MATERIALS AND METHODS

### Materials

siRNAs (small interfering RNAs) were purchased from Ambion and an electroporation kit (Amaxa) was used for VSMC transfection. Chondroitinase ABC and heparinase III (heparitinase) were from Sigma, and the following antibodies were from Santa Cruz Biotechnology: anti-NRP1 (C-19), anti-NRP2 (C-9), anti-Flk-1 (N-19), anti-synectin/GIPC [GAIP ( $G_{\alpha}$ -interacting protein)-interacting protein C-terminus] (C-1158), PDGFR $\beta$  (PDGF receptor  $\beta$ ), PDGFR $\alpha$  (C-20), GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (V-18) and secondary antibodies against goat, rabbit and mouse. Anti-p130<sup>Cas</sup> (total) antibody was from BD Biosciences; antibodies against phospho-p130<sup>Cas</sup> (pTyr<sup>410</sup>), phospho- and total Akt and phospho- and total ERK (extracellular-signal-regulated kinase) 1/2 were from Cell Signaling Technology; antibody against the NRP1 N-terminal domain was from R&D Systems, and antibodies against CS (chondroitin sulfate) and HS (heparan sulfate) were from Sigma and Seikagaku respectively.

### Cell culture

HCASMCs and HCAECs (human coronary artery endothelial cells), HAoSMCs and HUVECs (human umbilical vein endothelial cells) (all from TCS CellWorks), were cultured in Human VSMC basal medium containing growth supplements plus 2.5% (v/v) FBS (fetal bovine serum), or large vessel endothelial basal medium containing 10% (v/v) FBS, gentamycin (25  $\mu$ g/ml) and amphotericin B (50  $\mu$ g/ml). PAE (porcine aortic endothelial) cells were cultured in Ham's F12 medium containing 10% (v/v) FBS, supplemented for PAE/NRP1 cells (PAE cells expressing NRP1) with 25  $\mu$ g/ml hygromycin B. Cells used in experiments were up to passage six for endothelial cells and passage five for VSMCs.

### Cell treatments

All treatments with growth factors (e.g. PDGF) were performed in serum-free medium and in the absence of other growth factors unless stated otherwise, and were also performed after incubation of cells for 16 h (overnight) in serum-free medium. Cells were incubated with 5  $\mu$ g/ml tunicamycin for 16 h, or with chondroitinase and heparitinase at 1 unit/ml for 2 h at 37°C. Cells were then lysed with RIPA buffer (30 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate and 2 mM EDTA), and VSMCs were lysed with lysis buffer (20 mM

sodium phosphate, pH 7.4, 150 mM NaCl, 1% Triton X-100 and 5 mM EDTA), supplemented with protease and phosphatase inhibitors. NRP1 immunoprecipitates were treated overnight at 37°C with 1 unit/ml chondroitinase or heparitinase in 1% Nonidet P40, 0.15 M NaCl, 0.05 mM CaCl<sub>2</sub> and 20 mM Tris/HCl (pH 7.2). Immunoprecipitates were then washed three times with lysis buffer and resuspended in 2 $\times$  electrophoresis sample buffer for protein analysis.

### Immunoprecipitation

HCASMCs were extracted in lysis buffer, and lysates were incubated with primary antibodies overnight at 4°C, washed three times in lysis buffer, and captured with Protein A/G Plus-agarose beads (Santa Cruz Biotechnology) for 3 h at 4°C. Immunoprecipitates were washed and subjected to various treatments as described, before solubilization in 2 $\times$  sample buffer and analysis by Western blotting.

### Western blotting

Cell lysates were separated by electrophoresis on 4–12% Bis-Tris polyacrylamide gels, and electrotransferred on to PVDF membranes. Membranes were blocked with 5% (w/v) non-fat dried milk in PBS containing 0.1% Tween 20, incubated with primary antibodies overnight, washed five times in PBS containing 0.1% Tween 20, incubated for 1 h with horseradish peroxidase-labelled IgG, and proteins were detected using the ECL Plus<sup>TM</sup> Western blotting detection system and Hyperfilm ECL (both Amersham Biosciences).

### Flow cytometry

HUVECs and HCASMCs were dissociated using non-enzymatic dissociation buffer, resuspended in blocking buffer (1% BSA/Hepes with FcR-blocking reagent, Miltenyi Biotech), and incubated with primary antibodies for 20 min at 4°C. All antibodies were conjugated to phycoerythrin, used at a 1:10 dilution, and antibodies of the same isotype (mouse IgG<sub>1</sub>) were used as controls. Cells were then washed with PBS containing 0.2% BSA, pelleted by centrifugation at 1000 g for 5 min and resuspended in PBS, 1% BSA, 20 mM Hepes and 7AAD (7-aminoactinomycin D) (1:100 dilution; Sigma-Aldrich). Cells were then analysed using a Becton Dickinson FACScan flow cytometer with CellQuestPro software.

### Immunofluorescent staining

Cells were fixed for 15 min in 4% (w/v) paraformaldehyde, permeabilized for 10 min with 0.1% Triton X-100, then incubated overnight at 4°C with primary antibodies in 0.1% Tween 20 and 1% BSA in PBS, and then incubated for 1 h in the dark with Alexa Fluor<sup>®</sup> 488-conjugated donkey anti-goat IgG and/or Alexa Fluor<sup>®</sup> 555-conjugated donkey anti-mouse IgG (Invitrogen Molecular Probes). Cells were then rinsed three times with PBS, and images were acquired using a Leica TCS SP2 confocal microscope (excitation at 488 nm and 543 nm).

### Transfection with siRNAs

Transfection of HCASMCs was performed using a Nucleofector<sup>TM</sup> kit for mammalian smooth muscle cells (Amaxa). A total of 10<sup>6</sup> cells were resuspended in 100  $\mu$ l of the proprietary transfection reagent supplied with the Amaxa kit with

200 nM siRNA, and transfected according to the manufacturer's instructions. Cells were transferred to six-well plates and used experimentally 72 h after transfection.

### Mutagenesis and adenoviruses

Adenoviruses expressing wild-type NRP1 (Ad.NRP1WT), NRP1 lacking the intracellular domain (Ad.NRP1 $\Delta$ C) and NRP1S612A (Ad.NRP1S612A) were generated using the Gateway<sup>®</sup> system (Invitrogen). NRP1 open reading frames were subcloned into the pENTR/D-TOPO vector by PCR amplification with primers designed according to the manufacturer's instructions (forward, 5'-CACCATGGAGAGGGGGCTGCC-3'; reverse, 5'-TCATGCCTCCGAATAAGTACTCTGTG-3') using TOPO cloning (Invitrogen). NRP1 adenoviral expression vectors (pAd/CMV/V5-DEST) (Invitrogen) were generated by recombination, and adenovirus was produced by transfection into host HEK (human embryonic kidney)-293A cells (Invitrogen). Viral particles were purified by caesium chloride centrifugation, the titre was determined by immunoassay (QuickTiter Adenovirus Titer Immunoassay kit, Cell Biolabs), and adenoviruses were stored at  $-20^{\circ}\text{C}$ .

### PDGFR activity assay

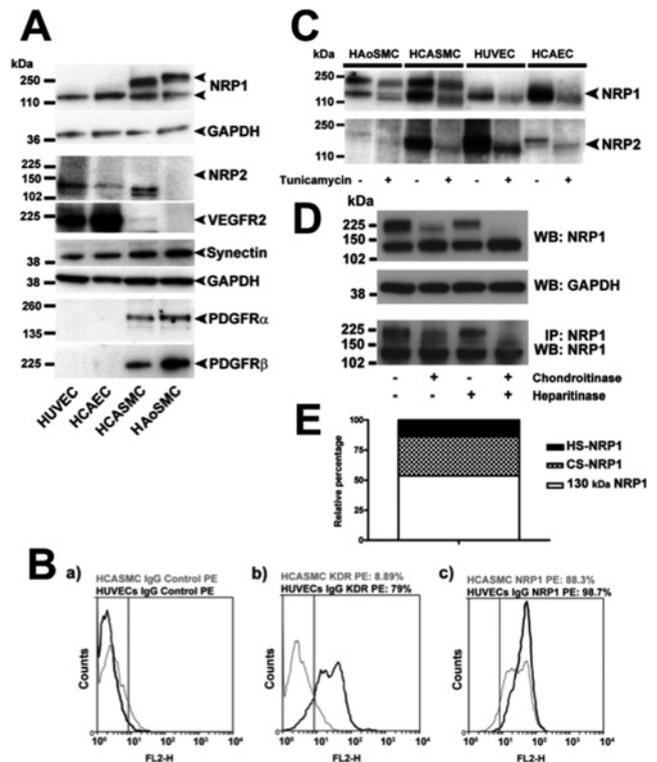
Intact HCASMCs were treated with growth factors as described in the Results and Figure legends and lysed, and activities of PDGFR $\alpha$  and PDGFR $\beta$  were determined in cell lysates using specific DuoSet IC ELISAs (R&D Systems) for tyrosine-phosphorylated PDGFR $\alpha$  and PDGFR $\beta$  according to the manufacturer's instructions. See the Supplementary Online Data at <http://www.BiochemJ.org/bj/435/bj4350609add.htm> for further details.

### Migration assay

A transwell assay was used to assess cellular migration through a porous membrane. Before migration assays, HCASMCs were treated with siRNAs, adenoviruses or as indicated. Transwells were coated with collagen (0.01%, Sigma) overnight at  $4^{\circ}\text{C}$ . HCASMCs were grown to 80% confluence, detached with non-enzymatic cell dissociation buffer and resuspended in serum-free VSMC medium before transfer into the transwells ( $8\ \mu\text{m}$  pore size, 6.4 mm diameter, VWR) at a density of  $1.5 \times 10^5$  cells/ml. Chemoattractants were placed in the bottom of the transwell, and cells were allowed to migrate for 4 h, after which non-migrated cells were removed from the upper surface of the transwell membrane using a cotton bud, and migrated cells were stained with Reastain Quick-Diff Kit (Reagen), and counted under brightfield microscopy ( $\times 200$  magnification). Each experiment was performed at least three times ( $n = 3$ ), with each treatment performed in duplicate.

### Surface protein biotin labelling and affinity selection

HCASMC surface membrane proteins were isolated using a surface protein isolation kit (Pierce) according to the manufacturer's instructions. Briefly, intact adherent HCASMCs were labelled at  $4^{\circ}\text{C}$  for 30 min with a cell-impermeable cleavable biotinylation reagent that covalently links functional amines of proteins. Biotinylation was then stopped by addition of quenching solution, cells were washed once with ice-cold PBS, and then lysed and sonicated. The cell lysate was incubated with NeutrAvidin Agarose for 60 min at room temperature ( $23^{\circ}\text{C}$ ), followed by solubilization of the labelled surface proteins with



**Figure 1** Neuropilin expression and glycosylation in human VSMCs and endothelial cells

(A) Whole-cell lysates of HUVECs, HCAECs, HCASMCs and HAoSMCs were immunoblotted for NRP1, NRP2, VEGFR2, synectin, PDGFR $\alpha$ , PDGFR $\beta$  and GAPDH. NRP1 bands of approximately 130 kDa and  $>250$  kDa are indicated. (B) Cell-surface expression of NRP1 was examined in HUVECs and HCASMCs using flow cytometry as described in the Supplementary Online Data at <http://www.BiochemJ.org/bj/435/bj4350609add.htm>. KDR, kinase insert domain-containing receptor (VEGFR2); PE, phycoerythrin. (C) Confluent cultures of the cells indicated were pre-treated with (+) or without ( $-$ )  $5\ \mu\text{g/ml}$  tunicamycin for 16 h. Lysates were then prepared and immunoblotted with an antibody against NRP1 or NRP2. (D) Top and middle: HCASMCs were treated with chondroitinase, heparitinase or both enzymes combined (each at 1 unit/ml) for 4 h, and lysates were prepared and immunoblotted with an antibody against NRP1 or GAPDH. Bottom: NRP1 immunoprecipitates prepared from HCASMCs were incubated for 4 h with chondroitinase or heparitinase, and then immunoblotted with anti-NRP1 antibody. WB, Western blot; IP, immunoprecipitate. Results shown in (A)–(D) are representative of at least three independent experiments. Molecular masses are indicated in kDa. (E) Amounts of GAG-modified NRP1 after chondroitinase or heparitinase treatments in the Western blots shown in (D) were quantified by scanning densitometry, and used to calculate the relative levels of unmodified NRP1, HS-GAG–NRP1 and CS-GAG–NRP1 in HCASMCs. Results are mean percentages of total NRP1 immunoreactivity (non-GAG-modified 130 kDa NRP1 plus GAG-modified  $>250$  kDa NRP1).

sample buffer and heat treatment for 5 min at  $95^{\circ}\text{C}$  before SDS/PAGE (4–12% gradient gels) and Western blotting.

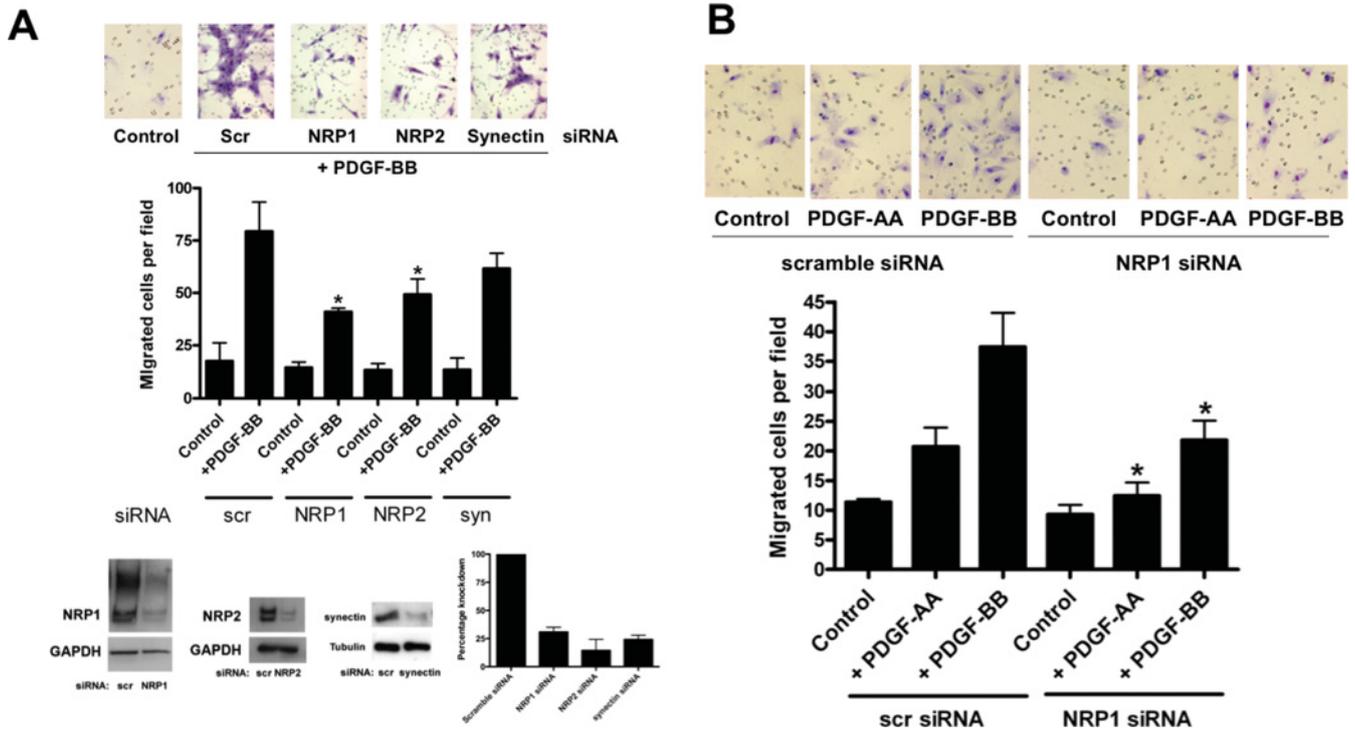
### Statistical analysis

Results are presented as means  $\pm$  S.E.M. Statistical analysis was performed by one-way ANOVA with Bonferroni post test. Statistical significance was validated at  $P < 0.05$ .

## RESULTS

### Expression of neuropilins

NRP1 and the neuropilin-interacting protein, synectin, were expressed in HCASMCs, HAoSMCs, HCAECs and HUVECs (Figure 1A). In contrast, the major signalling VEGF receptor, VEGFR2, was expressed at very low levels in HCASMCs and in



**Figure 2** NRP1 plays a role in PDGF-induced VSMC chemotaxis

HCASMCs were transfected with siRNA to NRP1, NRP2 or synectin (syn), or with control non-targeted scrambled siRNA (Scr), and 3 days later were either transferred to transwells and allowed to migrate for 4 h in response to 30 ng/ml PDGF-BB (**A** and **B**), 30 ng/ml PDGF-AA (**B**) or serum-free medium (Control), or were used to assess expression of targeted proteins by immunoblotting of whole-cell lysates (**A**, bottom). Results are mean  $\pm$  S.E.M. ( $n = 3$ ) numbers of migrated cells obtained from multiple independent experiments. \* $P < 0.05$  compared with Scr siRNA plus PDGF-BB or PDGF-AA. Representative transwell filters showing stained migrated cells are shown above the histogram. Protein expression 3 days after siRNA transfection was quantified by scanning densitometry of blots from three independent experiments (**A**, bottom right).

HAoSMCs in comparison with both endothelial cell types. NRP2 was expressed in HCASMCs, but was barely detectable in HAoSMCs (Figure 1A). In addition to the normal full-length NRP1 band of 130 kDa, a high-molecular-mass (>250 kDa) NRP1 band was prominent in HCASMCs and HAoSMCs, but not detectable in endothelial cells (Figure 1A). A high-molecular-mass NRP1 species was previously reported in VSMCs and in tumour cell lines [16,18], but anti-NRP2 antibody did not recognize a similar band (Figure 1A). Flow cytometry verified that NRP1 was expressed in HCASMCs and HUVECs at the cell surface, whereas surface VEGFR2 expression was very low in HCASMCs, but high in HUVECs (Figure 1B). In contrast, PDGFR $\alpha$  and PDGFR $\beta$  were expressed in HAoSMCs and HCASMCs, with higher expression of both receptors in HAoSMCs, but were not detected in endothelial cells (Figure 1A).

### VSMC neuropilins are O- and N-glycosylated

Treatment with tunicamycin, which blocks addition of carbohydrates to asparagine residues, caused a small reduction in the apparent molecular mass of both the >250 kDa and 130 kDa NRP1 bands, and similarly decreased the apparent molecular mass of NRP2, indicating that NRP1 and NRP2 are modified by N-glycosylation (Figure 1C). Removal of O-linked GAGs (glycosaminoglycans) containing CS by treatment of intact cells or NRP1 immunoprecipitates with chondroitinase markedly reduced expression of the >250 kDa NRP1 band, and concomitantly increased expression of the 130 kDa band, whereas heparitinase, which removes HS-GAGs, had less effect, and chondroitinase and heparitinase together completely removed the

>250 kDa band (Figure 1D). These results indicate that the high-molecular-mass NRP1 band in HCASMCs results predominantly from post-translational modification by CS-GAGs with less addition of HS-GAGs (Figure 1E). Immunofluorescent staining of NRP1, CS and HS showed that, in HCASMCs, both CS and HS co-localized with NRP1 in the cytoplasm, particularly in perinuclear Golgi-like structures, and at the cell membrane (see Supplementary Figures S1A and S1B at <http://www.BiochemJ.org/bj/435/bj4350609add.htm>), whereas little co-localization of NRP1 and CS was observed in HUVECs (Figure S1C).

### Role of NRP1 in HCASMC migration

Since VEGF is reported to stimulate migration of VSMCs [16,19,20], we addressed the possibility that NRP1 could mediate cell migration induced by VEGF in HCASMCs. VEGF exhibited specific high-affinity binding to HCASMC cultures as determined by radiolabelled ligand binding (see Supplementary Table S1 at <http://www.BiochemJ.org/bj/435/bj4350609add.htm>). However, VEGF-A<sub>165</sub> at concentrations of 25–200 ng/ml, caused no significant increase in HCASMC migration, whereas the potent VSMC chemoattractant PDGF-BB induced a marked increase in HCASMC migration at 30 ng/ml (see Supplementary Figures S2A and S2B at <http://www.BiochemJ.org/bj/435/bj4350609add.htm>). In addition, whereas PDGF-AA and PDGF-BB induced a striking increase in ERK1/2 and Akt activation in HCASMCs, VEGF-A<sub>165</sub> in the concentration range 25–100 ng/ml had little, if any, significant effect on ERK and Akt activity (see Supplementary Figure S2C). In parallel experiments in HUVECs, VEGF-A<sub>165</sub>

induced strong ERK1/2 and Akt activation, whereas neither PDGF-AA nor PDGF-BB had any detectable effect in HUVECs (see Supplementary Figure S2C).

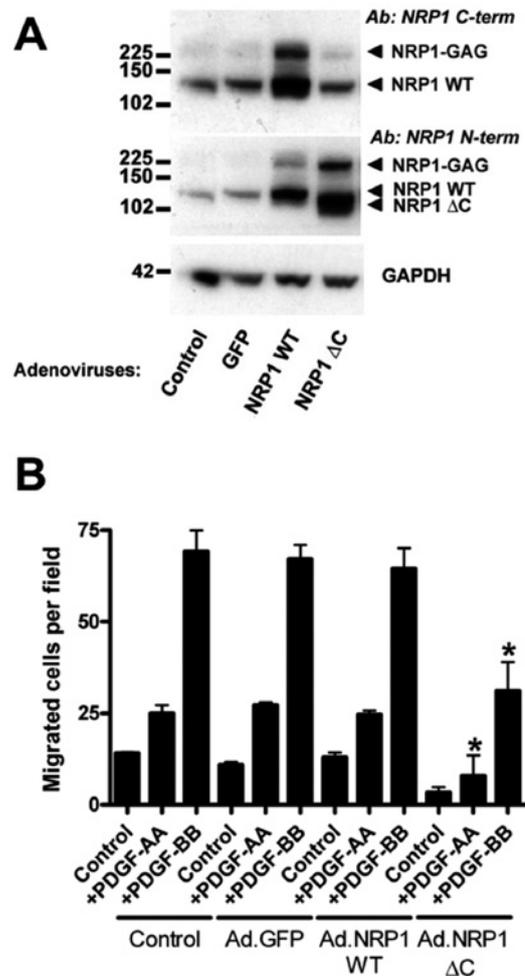
The role of NRP1 in the migratory response of HCASMCs to PDGF-BB was examined by siRNA-mediated knockdown of NRP1 and NRP2. Transfection of HCASMCs with siRNAs targeted to NRP1 and NRP2 significantly reduced NRP protein expression in HCASMCs (Figure 2A). When siRNA-transfected HCASMCs were used in migration assays, NRP1 knockdown caused a significant decrease in migration in response to PDGF-BB (Figure 2A). NRP2 siRNA significantly inhibited the migratory response to PDGF-BB, although somewhat less than did NRP1 (Figure 2A). Synectin knockdown caused a small and statistically insignificant reduction in the PDGF-BB response (Figure 2A). PDGF-AA-induced migration was also significantly reduced by NRP1 siRNA (Figure 2B).

The NRP1 cytosolic domain has been implicated in the functions of NRP1 in endothelial cells [21]. To substantiate further the role of NRP1 in the PDGF-BB migratory response in HCASMCs, we examined the effects of adenoviral overexpression of wild-type NRP1 and a mutant NRP1 lacking the cytosolic domain. Infection of HCASMCs with Ad.NRP1WT resulted in a significant increase in protein expression above the endogenous level (Figure 3A), but did not significantly alter the migration of HCASMCs towards a gradient of either PDGF-AA or PDGF-BB, compared with control cells infected with Ad.GFP (adenovirus expressing green fluorescent protein) (Figure 3B). In contrast, Ad.NRP1 $\Delta$ C infection, resulting in overexpression of NRP1 lacking the intracellular domain (Figure 3A), caused a significant inhibition of cell migration induced either by PDGF-AA or by PDGF-BB (Figure 3B), similar to the effect of NRP1 knockdown. It was also noted that Ad.NRP1 $\Delta$ C overexpression also reduced the basal level of HCASMC migration seen in the absence of PDGF stimulation.

PDGF-BB has been reported to co-immunoprecipitate with NRP1 [15], suggesting that PDGF-BB might bind directly to NRP1. We investigated this possibility by determining the specific ligand binding of  $^{125}$ I-PDGF-BB to PAE/NRP1 cells. Whereas  $^{125}$ I-PDGF-BB showed significant specific binding to HCASMCs,  $^{125}$ I-PDGF-BB exhibited little specific binding to PAE/NRP1 cells above the level observed in wild-type PAE cells not expressing NRP1 (see Supplementary Table S1). It was also noted that a high percentage of the total  $^{125}$ I-PDGF-BB binding to PAE and PAE/NRP1 cells was non-specific.

### PDGFR $\alpha$ and NRP1 associate in HCASMCs

As shown in Figure 1(A), HCASMCs express PDGFR $\alpha$  and PDGFR $\beta$ , either of which can mediate responses to PDGF-BB, whereas PDGFR $\beta$  does not recognize PDGF-AA with high affinity. A mechanism through which NRP1 could regulate migratory responses of HCASMCs to PDGF is the formation of heterocomplexes between PDGFRs and NRP1. To examine this possibility, we co-immunoprecipitated NRP1 with PDGFRs from HCASMC lysates. An NRP1 immunoblot of PDGFR $\alpha$  immunoprecipitates detected significant NRP1 compared with IgG controls, indicating that PDGFR $\alpha$  associated with NRP1 (Figure 4A). Treatment of HCASMCs with PDGF did not significantly affect the level of NRP1 co-immunoprecipitation with PDGFR $\alpha$ . Co-immunofluorescent staining of NRP1 and PDGFR $\alpha$  in HCASMCs also revealed significant co-localization of these molecules at the cell membrane, consistent with the conclusion that they associate (Figure 4B). We could not detect NRP1 in PDGFR $\beta$  immunoprecipitates (results not shown), and co-staining of NRP1 and PDGFR $\beta$  revealed little



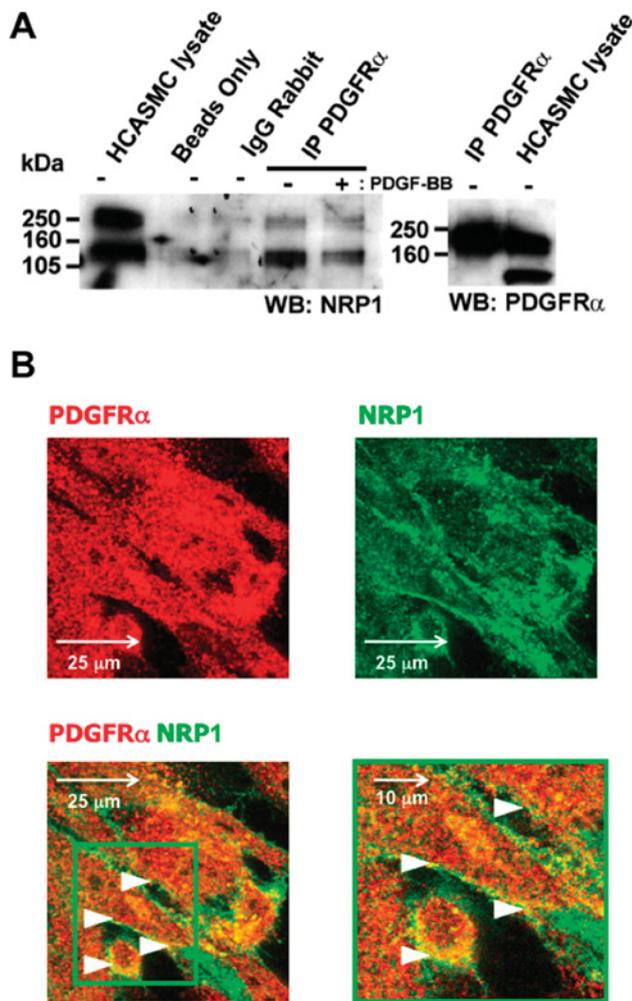
**Figure 3** Overexpression of NRP1 lacking the cytosolic domain inhibits VSMC chemotaxis

HCASMCs were either uninfected (Control) or infected with Ad.GFP, Ad.NRP1WT or Ad.NRP1 $\Delta$ C. At 3 days after infection, cell lysates were prepared and immunoblotted with antibodies specific for the NRP1 C- or N-terminus (A), or were transferred to transwells and allowed to migrate for 4 h in response to serum-free medium (control) or 30 ng/ml PDGF-AA or PDGF-BB (B). Results are mean  $\pm$  S.E.M. ( $n = 3$ ) numbers of migrated cells obtained from multiple independent experiments. \* $P < 0.05$  compared with Ad.NRP1WT plus PDGF-AA or PDGF-BB. Molecular masses in kDa are indicated in (A).

significant co-localization (see Supplementary Figure S3 at <http://www.BiochemJ.org/bj/435/bj4350609add.htm>).

### Role of NRP1 in PDGF-BB chemotactic signalling via p130<sup>Cas</sup>

In order to understand further the mechanism through which NRP1 regulates PDGF-BB chemotactic signalling, we investigated the contribution of NRP1 to PDGF-BB-stimulated chemotactic cell signalling in HCASMCs. We first examined whether NRP1 is required for activation of PDGFRs in VSMCs, by determining the effect of NRP1 knockdown on PDGF-induced receptor activation in a quantitative ELISA specific for phosphorylation of PDGFR $\alpha$  and PDGFR $\beta$ . The results indicate that NRP1 knockdown markedly inhibited PDGF-AA stimulation of PDGFR $\alpha$  activity and significantly reduced PDGF-BB induction of PDGFR $\alpha$  activity, although the effect of NRP1 siRNA on the response to PDGF-BB was noticeably smaller than for PDGF-AA (Figure 5A, upper panel). In contrast, NRP1

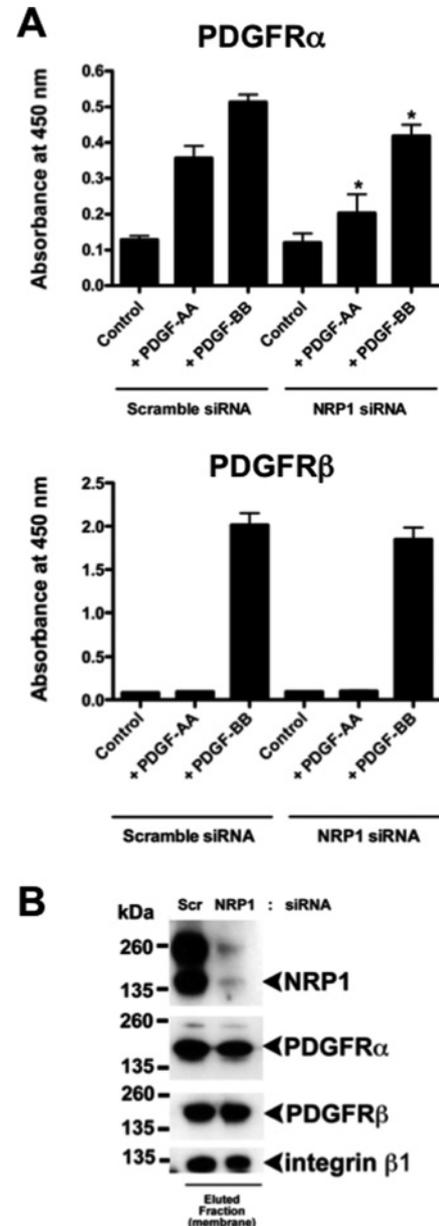


**Figure 4** NRP1 associates with PDGFR $\alpha$  in HCASMCs

(A) HCASMCs were incubated in serum-free medium overnight, then treated with (+) or without (-) 30 ng/ml PDGF-BB for 10 min. Cells were then lysed and PDGFR $\alpha$  was immunoprecipitated (IP). In parallel, lysates were incubated with either secondary antibody alone (IgG), or agarose beads alone. Immunoprecipitates were then immunoblotted (WB) with anti-NRP1 or anti-PDGFR $\alpha$  antibodies. Results are representative of at least three independent experiments. Molecular masses are indicated in kDa. (B) HCASMCs were fixed, permeabilized and then immunostained for NRP1 (green) and PDGFR $\alpha$  (red). The lower left-hand panel shows a merge of immunostaining for NRP1 and PDGFR $\alpha$ , and an enlarged image of the region in the green square is shown in the lower right-hand panel. Areas of co-localization at the cell membrane are indicated by white arrowheads. The results shown are representative of at least three independent experiments.

siRNA had no significant effect on PDGF-BB-induced PDGFR $\beta$  phosphorylation (Figure 5A, lower panel). It was confirmed that PDGF-AA did not stimulate PDGFR $\beta$  phosphorylation. To determine whether effects of NRP1 knockdown were due to decreased surface receptor expression, we performed biotinylation of cell-surface receptors and their subsequent isolation using avidin-agarose beads. As shown in Figure 5(B), whereas NRP1 knockdown markedly reduced surface biotinylated NRP1, it had only a modest effect on surface PDGFR $\alpha$  expression and had little detectable effect on PDGFR $\beta$  expression.

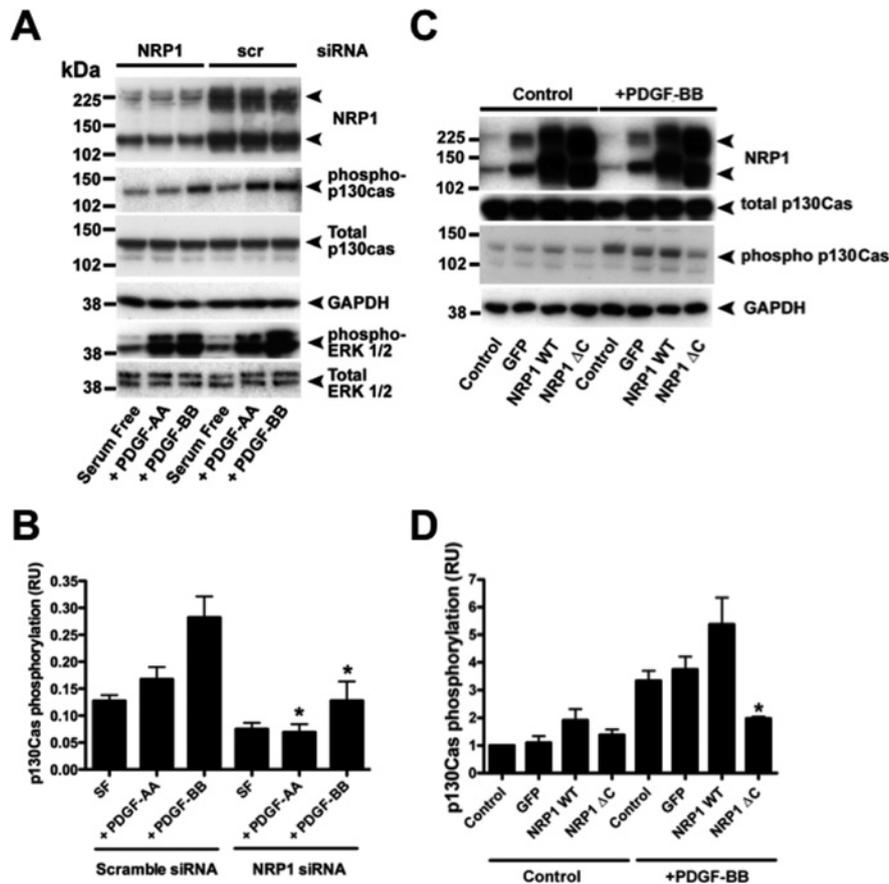
We reported previously that overexpression of NRP1 in U87MG glioma cells enhanced signalling through the adapter protein p130<sup>Cas</sup> [18], which is strongly implicated in cell migration and invasion [17]. PDGF-BB and PDGF-AA increased p130<sup>Cas</sup>



**Figure 5** NRP1 knockdown inhibits PDGFR $\alpha$  activation

(A) 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 10 min with serum-free medium (control), 30 ng/ml PDGF-AA or 30 ng/ml PDGF-BB. Cells were then lysed, and the activity of either PDGFR $\alpha$  (upper) or PDGFR $\beta$  (lower) was measured using a specific ELISA. Results are means  $\pm$  S.E.M. obtained from three independent experiments each performed in triplicate. \* $P < 0.05$  compared with Scr siRNA plus PDGF-BB or PDGF-AA. (B) HCASMCs were transfected with siRNA as indicated, and 3 days later, intact cells were surface labelled with biotin. Biotinylated surface membrane proteins were isolated and then immunoblotted as indicated. Molecular masses are indicated in kDa.

tyrosine phosphorylation in HCASMCs, consistent with this pathway playing a role in their chemotactic responses (Figure 6A). VEGF had little effect on p130<sup>Cas</sup> tyrosine phosphorylation in HCASMCs in a direct comparison with PDGF-BB (see Supplementary Figure S2). Depletion of NRP1 by siRNA-mediated knockdown significantly reduced p130<sup>Cas</sup> tyrosine phosphorylation stimulated by PDGF-BB or -AA, but had little effect on PDGF-induced activation of ERK1/2 or Akt



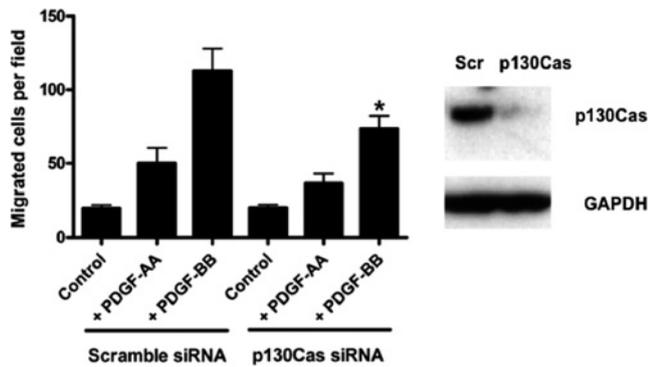
**Figure 6** PDGF-stimulated p130<sup>Cas</sup> phosphorylation is mediated via NRP1

(**A** and **B**) HCASMCs were transfected with control scrambled siRNA (Scr) or with NRP1 siRNA, and 3 days later, cells were incubated overnight in serum-free medium (SF) and then treated for 10 min with 30 ng/ml PDGF-BB or 30 ng/ml PDGF-AA. Cells were then lysed and immunoblotted with the antibodies indicated. Results are representative of at least three independent experiments. p130<sup>Cas</sup> tyrosine phosphorylation in three independent experiments similar to those in (**A**) were quantified by scanning densitometry (**B**). \**P* < 0.05 compared with Scr siRNA plus PDGFs. (**C** and **D**) HCASMCs were infected with Ad.GFP, Ad.NRP1WT or Ad.NRP1 $\Delta$ C. At 3 days after infection, cells were incubated overnight in serum-free medium and then treated for 10 min with either serum-free medium or with 30 ng/ml PDGF-BB for 10 min. Cells were then lysed and immunoblotted with antibodies specific for the NRP1 N-terminus, total and phosphorylated p130<sup>Cas</sup> and GAPDH as indicated. Results from three independent experiments similar to those in (**C**) were used for quantification of p130<sup>Cas</sup> tyrosine phosphorylation using scanning densitometry (**D**). Molecular masses in kDa are indicated in (**A**) and (**C**). RU, relative units.

(Figures 6A and 6B and see Supplementary Figure S4 at <http://www.BiochemJ.org/bj/435/bj4350609add.htm>). We noted that the effect of PDGF-AA on p130<sup>Cas</sup> tyrosine phosphorylation was weaker than that of PDGF-BB (Figure 6B). The role of the NRP1 cytosolic domain in p130<sup>Cas</sup> signalling was examined by overexpressing the NRP1 $\Delta$ C mutant. As shown in Figure 6(C), Ad.NRP1 $\Delta$ C inhibited PDGF-BB-induced p130<sup>Cas</sup> tyrosine phosphorylation. Quantification of data from several independent experiments showed that Ad.NRP1 $\Delta$ C markedly and significantly reduced the stimulation of p130<sup>Cas</sup> tyrosine phosphorylation by PDGF-BB (Figure 6D). In contrast, it was noted that Ad.NRP1WT overexpression was associated with some enhancement of p130<sup>Cas</sup> tyrosine phosphorylation, although the effect of Ad.NRP1WT was not statistically significant. Whereas p130<sup>Cas</sup> is implicated in the mechanisms underlying cell migration and associated cellular processes such as cytoskeletal organization [17], the role of this adapter protein in VSMC migration has been little investigated. As shown in Figure 7, p130<sup>Cas</sup> siRNA significantly reduced the migratory response of HCASMCs to PDGF-BB by an amount similar to the effect of NRP1 knockdown, and also reduced the increase in migration induced

by PDGF-AA, although the latter effect was not statistically significant.

We also examined the possibility that NRP1 CS modification played a role in PDGF chemotactic signalling via p130<sup>Cas</sup>, by enzymatically removing CS-rich glycan moieties using chondroitinase. Chondroitinase treatment of intact cells significantly reduced the stimulation of VSMC chemotaxis by PDGF-BB (Figure 8A), and also reduced the induction of p130<sup>Cas</sup> tyrosine phosphorylation by PDGF-BB and PDGF-AA (Figure 8B). Removal of HS-rich glycans using heparitinase had no significant effect on PDGF-BB-induced migration (results not shown), and also had no detectable effect on stimulation of p130<sup>Cas</sup> tyrosine phosphorylation (Figure 8B). To examine the role of NRP1 CS-GAG modification specifically, we used an adenoviral vector expressing a non-glycosylatable NRP1S612A mutant. Ad.NRP1S612A resulted in overexpression of the 130 kDa NRP1 species to a level similar to that resulting from Ad.NRP1WT expression, but with no concomitant increase in the 250 kDa NRP1 CS-GAG species (Figure 8C). However, overexpression of Ad.NRP1S612A significantly inhibited migration induced by PDGF-AA and PDGF-BB compared with NRP1WT (Figure 8C).



**Figure 7** PDGF-BB-induced VSMC chemotaxis is mediated via p130<sup>Cas</sup>

HCASMCs were transfected with control scrambled siRNA (Scr) or with p130<sup>Cas</sup> siRNA, and 3 days later, cells were transferred to transwells and allowed to migrate for 4 h in response to 30 ng/ml PDGF-BB, 30 ng/ml PDGF-AA or serum-free medium (Control), or were used to assess expression of targeted proteins by immunoblotting of whole-cell lysates (right). Migration of transfected cells was measured as described in the Materials and methods section. Results are mean  $\pm$  S.E.M. numbers of migrated cells obtained from three independent experiments, each performed in triplicate. \* $P < 0.05$  compared with Scr siRNA plus PDGF-BB. Immunoblots are representative of three independent experiments.

We also examined the effect of the NRP1S612A mutant on PDGF stimulation of p130<sup>Cas</sup> tyrosine phosphorylation, but could detect no consistent or significant inhibition (results not shown).

## DISCUSSION

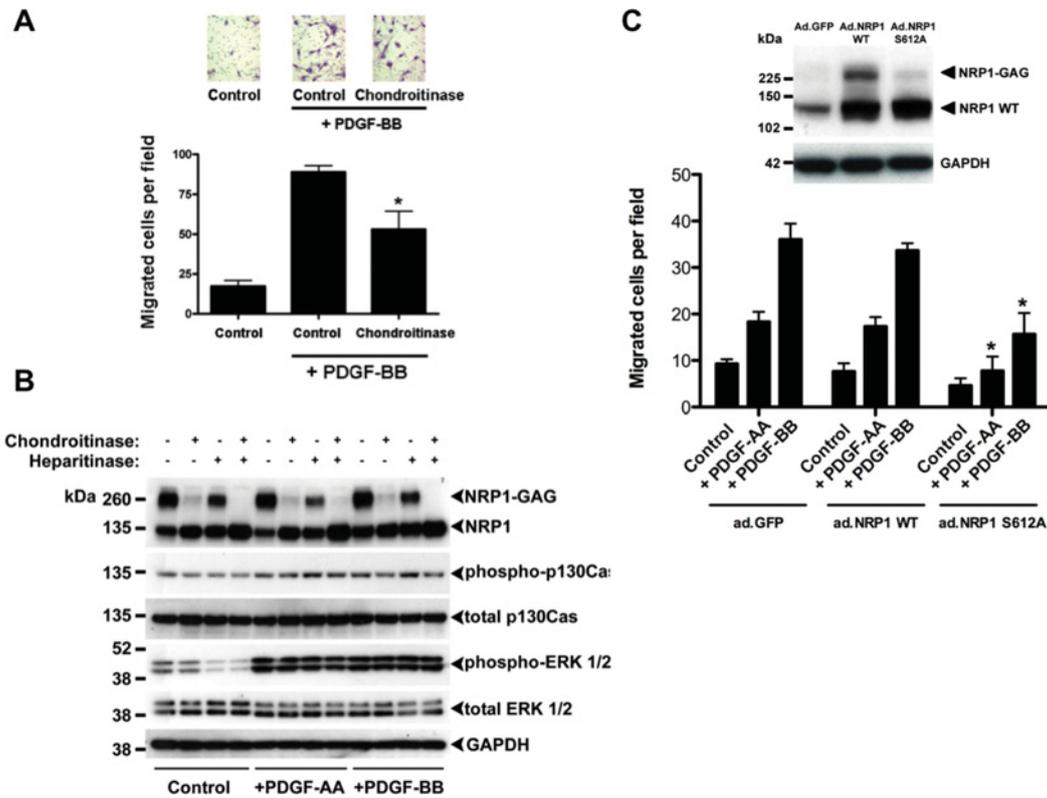
NRP expression has been reported in VSMCs and to play a role in VSMC migration [15], but the mechanism through which NRP1 regulates VSMC migration is poorly understood. The key findings of the present study are that NRP1 plays a significant role in the stimulation of human VSMC migration by the potent VSMC chemoattractants PDGF-AA and PDGF-BB, and in chemotactic PDGF signalling in VSMCs via p130<sup>Cas</sup>.

The conclusion that NRP1 is important for PDGF-BB-induced HCASMC migration is supported by the effects of NRP1 siRNA and mutant NRP1 overexpression: NRP1 knockdown and overexpression of NRP1 lacking the cytoplasmic domain (NRP1 $\Delta$ C) both significantly inhibited HCASMC migration induced either by PDGF-AA or by PDGF-BB. Since the NRP1 cytoplasmic domain is known to mediate protein–protein interactions, the fact that an NRP1 construct lacking this domain inhibited the migratory response to PDGF-BB suggests that NRP1 is required for optimal signalling important for migration downstream of PDGFRs. We noted that Ad.NRP1 $\Delta$ C expression had a more marked inhibitory effect on PDGF-induced HCASMC migration than did NRP1 siRNA, and also reduced the basal unstimulated level of migration, whereas NRP1 siRNA did not. These results are consistent with the conclusion that the NRP1 $\Delta$ C mutant exerts a dominant-negative effect, possibly via prevention of interactions between the NRP1 cytoplasmic domain and intracellular binding partners important for transducing chemotactic signals and/or sequestration in inactive complexes of PDGF, PDGFR and possibly other cellular components important for chemotactic signalling. The only known NRP1 cytosolic domain-binding molecule is synectin, and our findings show that synectin knockdown reduced PDGF-BB-induced cell migration, although this effect did not reach statistical significance. Other proteins may interact with the NRP1 cytoplasmic domain and contribute to PDGF-BB chemotactic signalling.

Although PDGF-BB has been reported to coimmunoprecipitate with NRP1 [15], we did not find convincing

evidence that PDGF-BB binds directly to NRP1 either in co-immunoprecipitation, or in studies of radiolabelled PDGF-BB binding to PAE/NRP1 cells. These data indicate that PDGF-BB may not be able to bind to the NRP1 extracellular domain with high affinity, although our results do not preclude this possibility. NRP1 regulation of PDGF-BB-induced migration may involve protein–protein associations between NRP1 and PDGFRs, since NRP1 co-immunoprecipitated with PDGFR $\alpha$  in HCASMCs. However, we were unable to detect similar complex formation between PDGFR $\beta$  and NRP1, indicating a preferential association with PDGFR $\alpha$ . Since PDGFR $\alpha$  is a ligand for PDGF-BB and PDGF-AA homodimers as well as PDGF-AB heterodimers, our results suggest a possible mechanism in which PDGF-BB and PDGF-AA act through PDGFR $\alpha$ –NRP1 complexes, resulting in optimal chemotactic signalling. NRP1 siRNA-mediated knockdown also caused a marked inhibition of PDGFR $\alpha$  activation in response to PDGF-AA, and caused a significant, but more modest, reduction in PDGF-BB-induced PDGFR $\alpha$  activation, whereas NRP1 knockdown did not affect PDGFR $\beta$  activity. Part of the inhibition of PDGFR $\alpha$  could be due to a modest decrease in surface PDGFR $\alpha$  expression resulting from NRP1 knockdown, although we think this unlikely to wholly explain our results. These results suggest that the constitutive association between NRP1 and PDGFR $\alpha$  may contribute to receptor activity and at least partly account for the role of NRP1 in PDGF responses in VSMCs. The mechanism underlying the greater effect of NRP1 knockdown on PDGF-AA-induced PDGFR $\alpha$  activity compared with PDGF-BB is unclear, but PDGF-BB-induced PDGFR $\alpha$  activation may reflect PDGF-BB binding to PDGFR $\alpha\beta$  heterodimers as well as PDGFR $\alpha\alpha$  homodimers, and heterodimer activation may be independent of effects of NRP1 depletion. Effects of PDGF-BB and PDGF-AA on PDGFR $\alpha$  activation can be differentially regulated, for example by heparin [22]. It is of note that selective NRP1 inhibition in endothelial cells, using either antibodies or antagonistic peptides that specifically block VEGF binding to NRP1 [13,23], only partially reduces VEGFR2 activity, indicating that NRP1 is not required for VEGFR2 activation in endothelial cells.

In the present study, we have shown that PDGF-BB stimulates p130<sup>Cas</sup> tyrosine phosphorylation and that this response is partially inhibited by both NRP1 knockdown and by expression of the NRP1 $\Delta$ C mutant. PDGF-AA also stimulated p130<sup>Cas</sup> tyrosine phosphorylation and this effect was also reduced by NRP1 knockdown. In contrast, NRP1 siRNA had little significant effect on other PDGF-BB-stimulated signalling pathways, including activation of ERK and Akt. p130<sup>Cas</sup> is an adapter protein, phosphorylated at multiple tyrosine residues, strongly implicated in the migration of cancer cells and other cell types, and known to associate with several proteins implicated in cell migration [17]. In support of the conclusion that p130<sup>Cas</sup> is a key mediator of PDGF-BB-dependent chemotactic signalling, p130<sup>Cas</sup> knockdown inhibited migration in response to PDGF-BB. We have also found that PDGF-BB induces p130<sup>Cas</sup> tyrosine phosphorylation via an NRP1-dependent mechanism in U87MG glioblastoma cells, further supporting this conclusion [24]. We are unaware of other reports showing PDGF-BB regulation of p130<sup>Cas</sup> tyrosine phosphorylation in VSMCs or other cell types; indeed, PDGF-BB was shown to increase tyrosine phosphorylation of the Cas family member HEF-1 (human enhancer of filamentation 1), but not p130<sup>Cas</sup>, in glioblastoma cells [25]. These findings suggest that NRP1 is selectively important for optimal PDGFR chemotactic signalling via tyrosine phosphorylation of p130<sup>Cas</sup>. It remains unclear exactly how NRP1 is able to regulate p130<sup>Cas</sup> phosphorylation, and investigation of



**Figure 8** Role of NRP1 O-linked glycosylation in PDGF-induced VSMC chemotaxis

(A and B) HCASMCs were incubated in serum-free medium overnight and then treated with chondroitinase, heparitinase or both enzymes combined (each at 1 unit/ml) for 2 h. Cells were then either transferred to transwells and allowed to migrate for 4 h (A) in response to either 30 ng/ml PDGF-BB or serum-free medium (Control), or were stimulated with PDGF-AA or PDGF-BB for 10 min and lysates were immunoblotted with the indicated antibodies (B). (C) HCASMCs were infected with Ad.GFP, Ad.NRP1WT or Ad.NRP1S612A. At 3 days after infection, cell lysates were prepared and immunoblotted with antibodies against NRP1 and GAPDH (upper), or were transferred to transwells and allowed to migrate for 4 h in response to serum-free medium (Control) or 30 ng/ml PDGF-AA or PDGF-BB (lower). The values shown are mean±S.E.M. ( $n=3$ ) numbers of migrated cells obtained from multiple independent experiments. \* $P < 0.05$  compared with Ad.NRP1WT plus PDGF-AA or PDGF-BB. Molecular masses are indicated in kDa.

the molecular mechanisms involved warrants further experimental work.

Similar to previous studies [16,18], NRP1 is expressed in VSMCs as a high-molecular-mass protein resulting from O-linked glycosylation. Our results showed that enzymatic removal of CS-GAGs in VSMCs reduced PDGF-induced migration and p130<sup>Cas</sup> tyrosine phosphorylation, and expression of an NRP1S612A mutant non-modifiable by O-linked glycosylation inhibited PDGF-induced cell migration. Since chondroitinase is non-selective in removing CS-GAG moieties, its overall impact on cell migration and signalling is likely to be the complex outcome of possibly both positive and negative effects, not attributable only to NRP1 deglycosylation. The inhibition of migration by NRP1S612A probably reflects a dominant-negative effect due to the utilization of an overexpressed non-modified NRP1 by PDGF rather than endogenous modified NRP1. Since NRP1S612A did not inhibit p130<sup>Cas</sup> phosphorylation, we conclude that the role of NRP1 CS-GAG modification in migration is distinct from the role of NRP1 in PDGF signalling. It is possible that NRP1 CS-GAG participates in interactions with matrix components of other cell-associated molecules important for cell migration.

The findings of the present study indicate an important and hitherto unrecognized role of NRP1 in PDGF cell signalling in HCASMCs, and also identify a novel role of p130<sup>Cas</sup> in PDGF signalling mediating HCASMC migration. While this work was under review, Ball et al. [26] reported that NRP1

associates with PDGFR $\alpha$  and mediates PDGF signalling in mesenchymal stem cells, further consistent with our findings. Our results suggest that NRP1-dependent mobilization of p130<sup>Cas</sup> tyrosine phosphorylation is a key mechanism in the chemotactic response of VSMCs to PDGF, implicating this pathway in the pathophysiological accumulation of VSMCs in early atherosclerotic lesion formation, restenosis after balloon angioplasty and other instances of neointimal thickening contributing to vasculoproliferative pathologies. Further studies should therefore examine the role of NRP1 in neointimal VSMC accumulation *in vivo*.

#### AUTHOR CONTRIBUTION

Caroline Pellet-Many designed and performed experiments, interpreted results and wrote the paper. Paul Frankel generated adenoviral constructs, interpreted results and wrote the paper. Ian Evans performed experiments. Birger Herzog generated adenoviral constructs. Manfred Jünnemann-Ramírez performed experiments. Ian Zachary designed experiments, interpreted results and wrote the paper.

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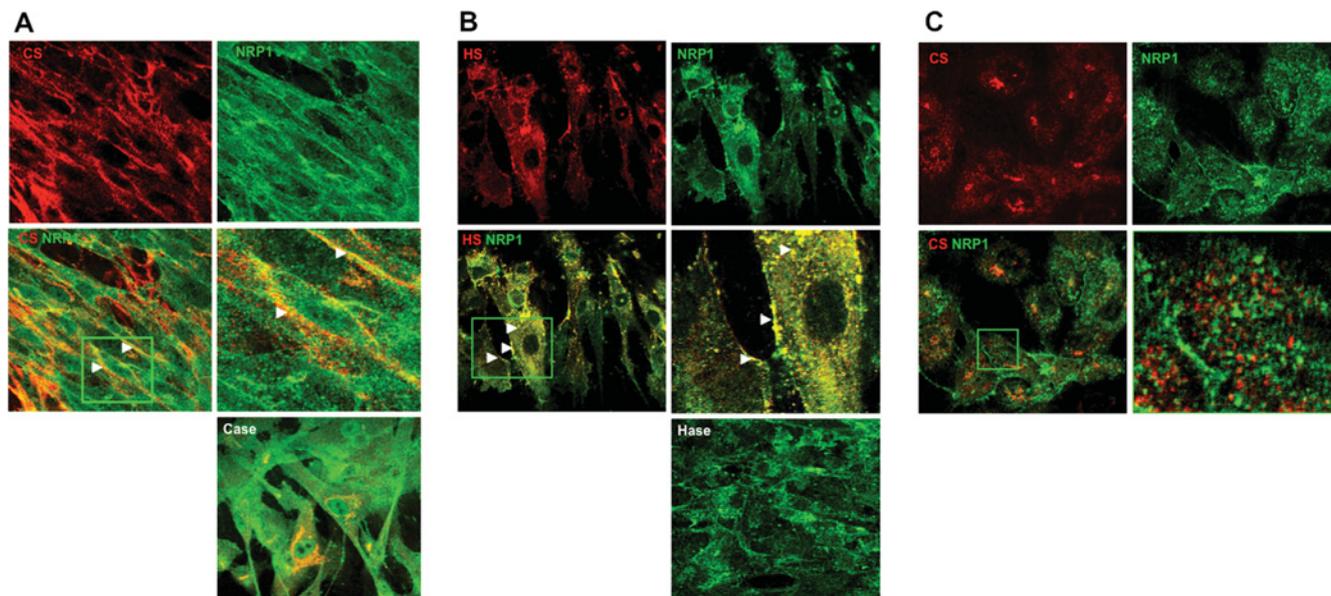
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## SUPPLEMENTARY ONLINE DATA

# Neuropilin-1 mediates PDGF stimulation of vascular smooth muscle cell migration and signalling via p130<sup>Cas</sup>

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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

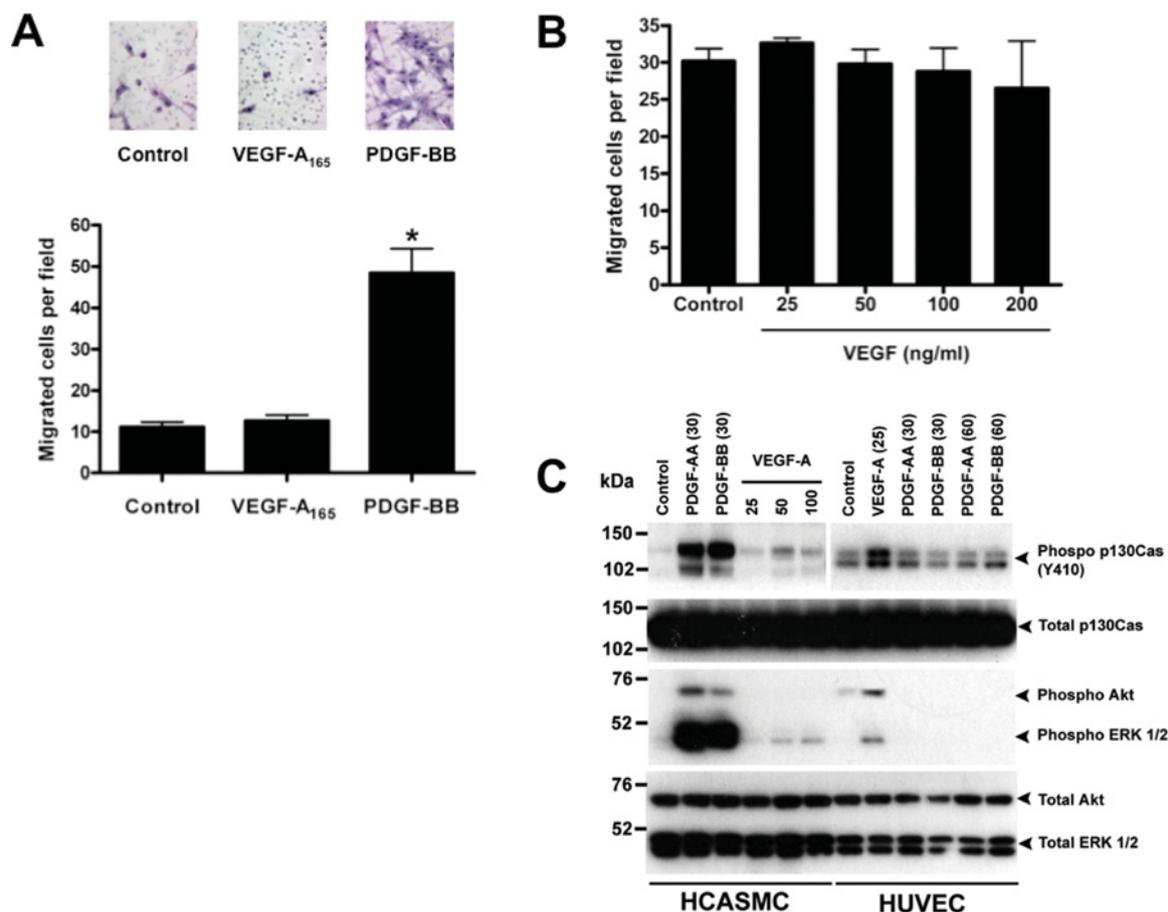
Confluent HCASMCs, PAE cells and PAE/NRP1 cells cultured in 24-well plates were washed twice with PBS. Binding medium (Dulbecco's modified Eagle's medium and 25 mM Hepes, pH 7.3, containing 0.1% BSA) was added, followed by addition of 0.1 nM <sup>125</sup>I-PDGF-BB (1825 Ci/mmol; Perkin-Elmer) or <sup>125</sup>I-VEGF-A<sub>165</sub> (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  $\gamma$ -counter. Non-specific binding was determined in the presence of a 100-fold excess of unlabelled PDGF-BB or VEGF-A<sub>165</sub> (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  $\mu$ g/ml in PBS, without carrier protein and 100  $\mu$ l 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

<sup>1</sup> Birger Herzog and Paul Frankel are employees of Ark Therapeutics Ltd, and Ian Zachary is a consultant to Ark Therapeutics Ltd. Ark Therapeutics Ltd have a commercial interest in developing neuropilin-1 antagonists as therapeutic agents.

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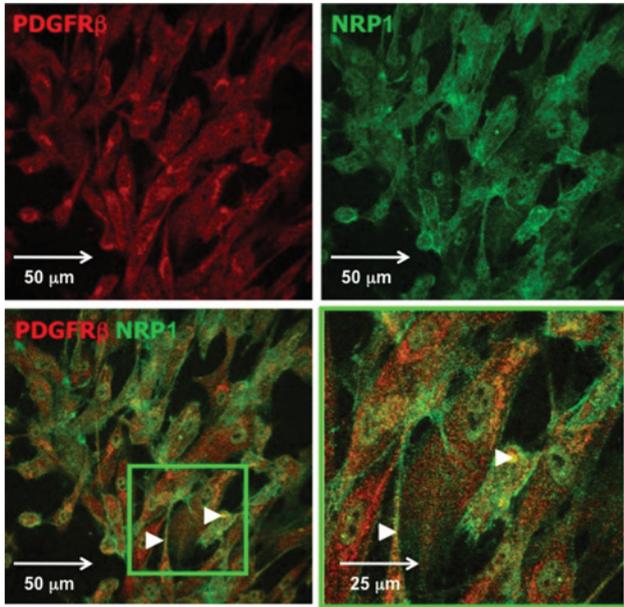


**Figure S2 VEGF does not induce migration or signalling in HCASMCs**

(**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<sub>165</sub> (**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<sub>165</sub>, 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  $\mu$ l of PBS containing 1% BSA. Wells were washed again three times before adding 100  $\mu$ l 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  $\mu$ l 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  $\mu$ l was added to each well for 20 min at room temperature. Finally, 100  $\mu$ l of substrate solution was added to each well for 20 min, followed by 50  $\mu$ l 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** PDGFR $\beta$  and NRP1 do not co-localize in HCASMCs

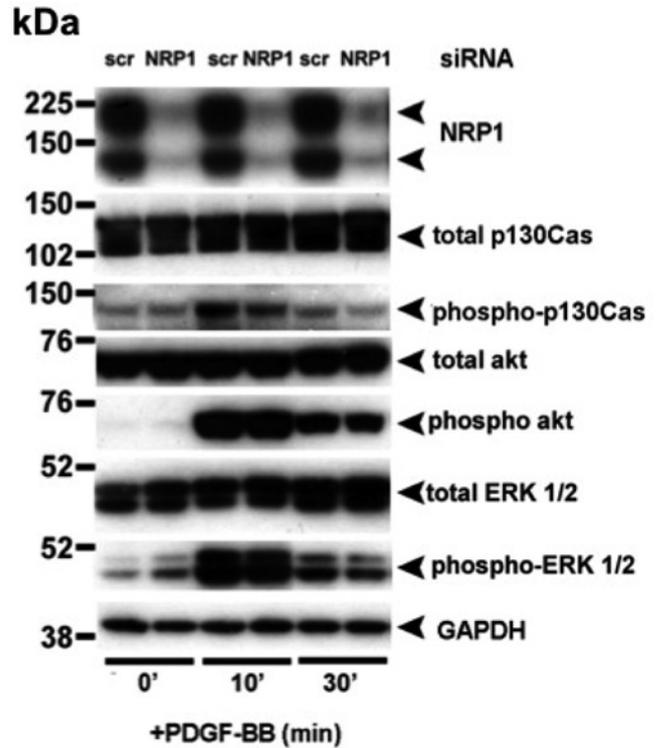
HCASMCs were fixed, permeabilized and then immunostained for NRP1 (green) and co-stained with antibody for PDGFR $\beta$  (red). NRP1 is located in the cytoplasm and also at the membrane of the cells, and in the merge shows little co-localization with PDGFR $\beta$  in the membrane (bottom panels). Some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

**Table S1** Binding of  $^{125}\text{I}$ -PDGF-BB or  $^{125}\text{I}$ -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.

Growth factor	Cell type	$^{125}\text{I}$ -ligand binding (c.p.m.)		
		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<sup>Cas</sup> 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.