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PRH/Hhex inhibits the migration of breast and prostate epithelial cells through direct transcriptional regulation of Endoglin.

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Running title: **PRH/Hhex inhibits cell migration**

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ABSTRACT

PRH/Hhex (Proline Rich Homeodomain protein) is a transcription factor that controls cell proliferation and cell differentiation in a variety of tissues. Aberrant subcellular localisation of PRH is associated with breast cancer and thyroid cancer. Furthermore, in blast crisis chronic myeloid leukaemia, and a subset of acute myeloid leukaemias, PRH is aberrantly localised and its activity is down-regulated. Here we show that PRH is involved in the regulation of cell migration and cancer cell invasion. We show for the first time that PRH is expressed in prostate cells and that a decrease in PRH protein levels increases the migration of normal prostate epithelial cells. We show that a decrease in PRH protein levels also increases the migration of normal breast epithelial cells. Conversely, PRH over-expression inhibits cell migration and cell invasion by PC3 and DU145 prostate cancer cells and MDA-MB-231 breast cancer cells. Previous work has shown that the TGF-β co-receptor Endoglin inhibits the migration of prostate and breast cancer cells. Here we show that PRH can bind to the Endoglin promoter in immortalised prostate and breast cells. PRH over-expression in these cells results in increased Endoglin protein expression whereas PRH knockdown results in decreased Endoglin protein expression. Moreover, we demonstrate that Endoglin over-expression abrogates the increased migration shown by PRH knockdown cells. Our data suggest that PRH controls the migration of multiple epithelial cell lineages in part at least through the direct transcriptional regulation of Endoglin. We discuss these results in terms of the functions of PRH in normal cells and the mislocalisation of PRH seen in multiple cancer cell types.

KEY WORDS: HHex, PRH, cell migration, invasion, breast cancer, prostate cancer

INTRODUCTION

The transcription factor PRH (Proline Rich Homeodomain/HHex) is essential for formation of the vertebrate body axis and the development of most organs including the heart, thyroid, pancreas, vasculature and haematopoietic compartment (1). PRH can activate or repress transcription of its target genes and it can also control gene expression at the post-transcriptional level via a protein-protein interaction with eIF4E (2-5). In the mouse, retroviral expression of PRH leads to a T-cell leukaemia (6). Moreover, in human T-cell leukaemias associated with the aberrant expression of the LMO2 oncogene, elevated PRH expression is necessary for development of the disease (7, 8). However, more generally, it is the disruption of PRH activity that is associated with a variety of diseases states (9-12). Aberrant subcellular localisation of PRH with loss of nuclear PRH is associated with blast crisis chronic myeloid leukaemia (CML) and some subtypes of acute myeloid leukaemia (AML) (12). Furthermore in one human AML the only characterised genetic change is a fusion of the prh gene with the nucleoporin gene Nup98 and this is thought to decrease the activity of endogenous PRH (11). We have shown that in a CML cell line, BCR-ABL activity indirectly results in the down-regulation of PRH transcriptional repression activity and the derepression of several PRH target genes including Vegfa, Vegfr-1, and Vegfr-2 (13). The repression of these genes in CML K562 cells by PRH results in decreased VEGF autocrine signalling and decreased cell survival (13). Conversely, inactivation or down-regulation of PRH and the consequent de-repression of these genes results in increased cell survival. Significantly, decreased nuclear PRH protein is also associated with breast and thyroid tumours (9, 10).

Endoglin is a TGF-β coreceptor that modulates TGF-β-dependent cellular responses (14, 15). Most studies on Endoglin have focused on its pro-angiogenic role in endothelial cells, its involvement in vascular remodelling and its role as a marker of the tumour

vasculature, but it also plays a direct role in tumourigenesis (16). Several studies have concluded that decreased Endoglin expression is associated with prostate cancer cell migration. For example, Endoglin expression was found to be lower in multiple cancer cell lines than in immortalised normal prostate cells and down-regulation of Endoglin expression was shown to increase prostate cancer cell migration and invasion (17). More recent data have shown that decreased Endoglin levels in prostate cancer cells result in increased metastasis and increased tumour size (18). The inhibition of prostate cell migration by Endoglin occurs through the activation of TGF-β co-receptor signalling and the consequent phosphorylation of Smad1, as well as through a Smad1-independent pathway (19, 20). Endoglin has also been shown to inhibit invasion and colony formation by esophageal epithelial cells (21) and to suppress cancer formation by skin epithelial cells (22). Moreover, Endoglin inhibits the migration and invasion of breast tumour cells *in vivo* by modulating cytoskeletal remodeling rather than through TGFβ co-receptor modulation and low Endoglin expression correlates with poor prognosis in a panel of invasive breast tumours (23).

Interestingly, PRH over-expression has been demonstrated to inhibit angiogenesis but to increase Endoglin mRNA and protein levels in human endothelial cells (24). However, it is not known whether Endoglin is a direct target for transcriptional regulation by PRH in these cells. It is also unknown whether the regulation of Endoglin by PRH occurs in other cell types and whether this could be important in tumourigenesis. Here we show that PRH regulates the migration of normal prostate and breast epithelial cells and that PRH over-expression inhibits migration and invasion of prostate and breast tumour cells. Our findings suggest that in prostate cancer cells and breast cancer cells the regulation of Endoglin by PRH may be attenuated resulting in increased cell migration and cell invasion.

RESULTS

PRH regulates cell migration

PNT2-C2 cells are a normal immortalised prostate epithelial cell line (25, 26). Western blotting reveals that PNT2-C2 cells express PRH (Fig.1A). To determine whether PRH plays an important role in these cells we performed a PRH knockdown experiment using specific shRNAs. PNT2-C2 cells were transfected with plasmids expressing PRH shRNA and selected in puromycin for 10 days. Western blotting confirms that PRH protein levels are significantly reduced in the PRH knockdown cells (PNT2-C2 KD cells) compared to cells expressing a control shRNA (Fig.1A and Fig.1B). To determine whether the PNT2-C2 PRH KD cells exhibit increased cell motility we performed wound closure assays. Confluent layers of PNT2-C2 KD cells and control cells were wounded with a pipette tip and wound closure monitored over time using microscopy (Fig.1C). Interestingly, PNT2-C2 KD cells migrate into the wound significantly faster than control cells (Fig.1D). This experiment was performed in the presence of hydroxyurea to inhibit cell division and ensure that any effects of PRH knockdown on cell proliferation could not account for changes in cell migration. To investigate the effects of PRH on chemotaxis, we placed PNT2-C2 KD cells and control transfected cells in Boyden chambers and determined the number of cells that migrate towards high serum. After 4 hours significantly more PNT2-C2 KD cells than control cells migrate towards high serum (Fig.1E). Taken together these data show that PRH is expressed in prostate epithelial cells and that PRH knockdown increases cell migration.

PRH over-expression inhibits prostate cancer cell migration and invasion

To determine whether PRH also influences the migration of tumour cells we over-expressed PRH in PNT2-C2 cells and two well-characterised prostate cancer cell lines, PC3 cells and DU145 cells. We transfected each cell line with a vector expressing GFP or with a GFP expression vector in combination with a PRH expression vector and we used GFP as a marker to follow the migration of transfected cells. In each case over-expression of PRH brings about a significant reduction in cell migration (Fig.2A). We also examined the effects of PRH over-expression on the migration of DU145 cells using live cell imaging with time-lapse video microscopy. DU145 cells transfected with GFP and PRH expression vectors migrated less distance than cells transfected with a GFP expression vector alone (Fig.S1A). They also showed a significant reduction in migration velocity and displacement (Fig.S1B and S1C, respectively). To determine whether the transcriptional activity of PRH is required to inhibit cell migration we expressed two PRH mutants in these cells. PRH N187A contains a mutation in the PRH homeodomain and is unable to bind DNA (4, 27). PRH F32E contains a mutation that blocks the binding of PRH to co-repressor proteins belonging to the TLE family (3, 27). Both of these mutated proteins show a significantly reduced ability to inhibit cell migration compared to wild type PRH (Fig.2B) although all three proteins are expressed at roughly equivalent levels (Fig.2C). This suggests that PRH inhibits cell migration via the transcriptional regulation of PRH-target genes.

We next set out to determine whether PRH inhibits cancer cell invasion and extravasation. PNT2-C2 cells are normal immortalised cells that cannot invade a Matrigel layer, however, PC3 cells and DU145 cells expressing GFP alone are able to migrate through a Matrigel layer. In contrast tumour cells expressing both GFP and PRH show a significant reduction in invasion (Fig.2D). Furthermore, in extravasation assays, PC3 cells expressing

PRH show a significant reduction in their ability to traverse a layer of endothelial cells placed over Matrigel (Fig.2E).

PRH directly activates transcription of Endoglin

To determine whether PRH regulates Endoglin expression in normal prostate cells and prostate cancer cells we used quantitative RT-PCR to measure Endoglin mRNA levels in PNT2-C2 cells and PC3 cells. We infected the cells with an empty adenovirus or with an adenovirus that expresses Myc-tagged PRH (Ad PRH) (28) . In both PNT2-C2 cells and PC3 cells infection with Ad PRH brings about a significant increase in Endoglin mRNA levels (Fig.3A). Endoglin protein levels are also increased in cells over-expressing PRH (Fig.3B). Conversely, Endoglin protein levels are significantly reduced in PNT2-C2 cells in which PRH has been knocked down using shRNA (Fig.3C). Interestingly, Endoglin protein levels are significantly reduced in both prostate cancer cell lines compared to PNT2-C2 cells (Fig.S2A and S2B). The Endoglin promoter contains several putative PRH binding sites both proximal and distal to the transcription start site. To determine whether PRH binds to the Endoglin promoter we performed chromatin immunoprecipitation (ChIP) experiments. PNT2-C2 cells were infected with Ad PRH and chromatin from these cells was isolated and sheared by sonication. ChIP using a Myc antibody was then used to isolate DNA fragments bound to Myc-PRH. PCR with a primer pair specific for Endoglin promoter sequences from -1042 to -551 relative to the first exon (Fig.3D) indicates that PRH binds at or near this region (P1 in Fig.3D). This region contains three putative PRH binding sequences as defined by the presence of 5'TAAT3' motifs. Promoter sequences flanking a previously characterised enhancer (29, 30) located 8kb upstream of first exon also contain multiple putative PRH binding sites. Primer pairs specific for promoter sequences from -7352 to -6914 and -8857 to

-8398 (which contain 1 and 6 putative PRH binding sites respectively) show that PRH can also bind in these regions in PNT2-C2 cells (P3 and P4 in Fig.3D). This binding is unlikely to be an artefact of PRH over-expression since a primer pair specific for promoter sequences from -1547 to -1353 that lacks putative PRH sites fails to produce a product (P2 in Fig.3D). Moreover the PRM2 gene is highly expressed in tumour cells and primer pairs for this locus were used as a control to show that there is little non-specific enrichment of sequences from elsewhere in the genome using these conditions.

Reporter assays using a series of Endoglin promoter fragments cloned upstream of the luciferase gene confirm that sequences between -851 and -400 are responsive to PRH over-expression (Fig.S3B). However, unexpectedly, PRH over-expression represses the activity of Endoglin reporter constructs that contain this PRH-responsive region. This suggests that activation of the endogenous Endoglin promoter by PRH is complex and that it might require the presence of chromatin/chromatin associated factors or that activation is mediated by the promoter distal PRH binding sites identified above using ChIP.

Endoglin over-expression inhibits the increased migration of PRH knockdown cells

The data shown above suggest that PRH inhibits cell migration by the direct transcriptional regulation of Endoglin. To investigate this in more detail we over-expressed Endoglin in prostate cells (Fig.S4A). As expected, over-expression of Endoglin inhibits migration (Fig.S4B) and invasion by PC3 cells and DU145 cells (Fig.S4C). As mentioned earlier, PNT2-C2 PRH knockdown cells show increased cell migration. Over-expression of Endoglin in PNT2-C2 PRH knockdown cells completely abrogates the increased migration of these cells in wound closure assays (Fig.4A). Moreover, although over-expression of Endoglin has little or no effect on chemotaxis by PNT2-C2 cells (Fig.4B), Endoglin over-

expression completely abolishes the increased chemotaxis shown by PNT2-C2 PRH knockdown cells (Fig.4B, lanes 3 and 4). We conclude that the effects of Endoglin on cell migration are downstream from PRH. This suggests that PRH does indeed inhibit cell migration through the direct regulation of Endoglin expression.

To investigate whether TGFβ signalling is involved in the control of cell migration by PRH, we made use of a specific inhibitor that targets TGFβ superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. The inhibition of TGFβ signalling abrogates the negative effects of PRH over-expression on the migration of DU145 cells (Fig.4C). Furthermore, inhibition of TGFβ signalling also abrogates the negative effects of Endoglin over-expression on the migration of these cells (Fig.4C). As expected, the levels of phosphorylated Smad3, a downstream target for TGF-β-induced phosphorylation, are reduced in the treated cells (Fig4D). This indicates that both PRH and Endoglin inhibit prostate cell migration through the down-regulation of TGFβ signalling.

PRH inhibits the migration of normal breast epithelial cells and breast cancer cells

Since PRH is known to be aberrantly localised in breast cancer cells (9), we next set out to determine whether this protein also controls the migration of normal breast epithelial cells and breast cancer cells**.** We knocked down PRH in normal immortalised breast epithelial MCF 10A cells using a lentivirus expressing an IPTG-inducible shRNA against PRH (Fig.5A). Interestingly three independent PRH knockdown cell lines show a significant increase in chemotaxis compared to controls (Fig.5B). To investigate the effects of PRH on breast cancer cell migration and cell invasion we made use of two well characterised breast

cancer cell lines: MCF-7 cells, an estrogen receptor (ER) positive breast adenocarcinoma cell line, and MDA-MB-231 cells, a hormone-independent breast adenocarcinoma cell line. We infected these cells with Ad PRH or empty adenovirus and examined the effects on cell migration in scratch wound assays. In both cases cells infected with Ad PRH show a decrease in wound closure (Fig.S5A). In chemotaxis experiments infection with Ad PRH results in a significant reduction in cell migration in both cell lines (Fig.5C). To confirm that this reduction in cell migration is not due to adenoviral infection we also examined the migration of MCF-7 PRH knockdown cells (Fig.5A, left). Three independent MCF-7 cell lines transduced with lentiviruses expressing PRH shRNA exhibit a significant increase in wound closure (Fig.5D). We next transiently transfected MDA-MB-231 cells with a vector expressing GFP or with GFP and PRH expression vectors and performed invasion assays. As in PC3 cells and DU145 cells, over-expression of PRH dramatically reduces the ability of MDA-MB-231 cells to invade Matrigel (Fig.5E).

Similar to the results seen in prostate cells, Endoglin protein levels are higher in normal breast MCF 10A cells than in either MCF-7 cells or MDA-MB-231 cells (Fig.S5B and S5C). To examine whether the ability of PRH to influence the migration of MCF 10A cells and breast cancer cells also involves the regulation of Endoglin we measured Endoglin mRNA levels in MCF-7 and MCF 10A cells over-expressing PRH. In both cases PRH over-expression results in a significant increase in Endoglin mRNA (Fig.5F). Moreover, Endoglin protein levels are decreased in PRH knockdown MCF 10A cells (Fig.5G). The effects of PRH on Endoglin mRNA and protein expression appear to be direct since ChIP of Myc-tagged PRH pulls down Endoglin promoter sequences in both MCF-7 and MCF 10A cells (Fig.S5D). These data suggest that PRH inhibits the migration of normal breast cells and breast cancer cells via transcriptional regulation of Endoglin expression.

DISCUSSION

The ability of PRH/HHex to control cell proliferation and cell differentiation in multiple cell types is well documented. PRH can control cell proliferation via the post-transcriptional regulation of cyclin D mRNA transport (2). In addition, our previous work has shown that in leukaemic cells, PRH directly represses multiple genes that encode proteins involved in VEGF signalling including Vegfa and Vegfr-1 (31). In these cells VEGF acts as an autocrine growth factor and the transcriptional regulation of these VEGF signalling genes by PRH controls cell survival. The extent to which these modes of cell survival control operate in other cell types is not known, although in breast cancer MCF-7 cells, PRH regulates the transcription of Vegf receptor genes and a PRH knockdown increases cell growth (31). In endothelial cells PRH over-expression also controls VEGF signalling genes and alters cell migration and invasion (24). In breast and thyroid cancer cells PRH subcellular localisation is altered resulting in cytoplasmic and nucleolar accumulation of PRH (9, 10). However, the ability of PRH to regulate other aspects of cancer cell behaviour relevant to tumourigenesis, including cell motility and cell invasion, has not been investigated.

Here we have shown that PRH knockdown increases the motility of immortalised prostate and breast epithelial cells and increases chemotaxis by these cells. Conversely, PRH over-expression decreases cell migration by prostate and breast cancer cells and inhibits the ability of these cells to invade a Matrigel layer. Moreover, PRH over-expression decreases the ability of prostate cancer cells to travel though a layer of endothelial cells in extravasation assays. These findings suggest that the aberrant localisation of PRH seen in breast cancer cells and thyroid cancer cells could contribute to their increased migration and cell invasion as well as having effects on cell proliferation. Moreover, these results show that PRH activity is important in the control of these aspects of cell behaviour in normal epithelial cells and

they suggest that PRH may also be aberrantly localised or otherwise misregulated in prostate cancer cells.

Knockout of PRH in embryoid bodies results in down-regulation of Endoglin mRNA (32) and PRH over-expression has been shown to up-regulate Endoglin mRNA and protein levels in endothelial cells (24). We have shown that PRH associates directly with the Endoglin promoter and up-regulates transcription of Endoglin in normal prostate and breast epithelial cells and prostate and breast cancer cell lines. PRH binds to DNA sequences within the Endoglin proximal promoter located around 500bp upstream of the transcription start point and upstream of a number of important transcription factor binding sites including sites for regulation by Sp1, HIF-1, Smad proteins, KLF6 and ETS family members (30, 33-35). PRH also binds to sequences in the distal promoter near a previously characterised Enhancer (29, 30) around 8kb upstream of the transcription start point. These regions both contain multiple copies of the core PRH binding site 5‘TAAT’3. Other PRH target genes including Goosecoid, VegfR1 and Vegfa also contain arrays of PRH core binding sites and at these promoters PRH also binds over extensive regions (36, 37). This suggests that PRH oligomers bind to these promoters in a similar manner in order to control promoter architecture. However, at other promoters PRH binds in conjunction with other transcription factors and in these cases extensive direct contacts between PRH and the DNA may not be required (1). Surprisingly, although the promoter proximal binding sites in the Endoglin promoter confer responsiveness to PRH over-expression in luciferase assays, PRH appears to repress Endoglin transcription when bound at these sites. Presumably, the promoter distal PRH binding sites are responsible for Endoglin transcription activation by PRH. Alternatively, the presence of chromatin/chromatin associated factors may be important for activation of the Endoglin promoter by PRH just as they are for repression by PRH at the Vegfr-1 promoter (31).

Further experiments will be required to determine the mechanisms that PRH uses in order to activate transcription at this promoter.

Importantly, the increased cell migration shown by PRH knockdown cells is completely abolished by Endoglin over-expression. We conclude that in both breast and prostate cells PRH regulates Endoglin expression and that the effects of PRH on cell migration are in part at least through the control of this target gene. Endoglin has been shown to inhibit cell migration, cell invasion and tumour growth by prostate cancer cells and cell migration and invasion of breast cancer cells (18, 23). However, it has been reported that Endoglin expression is higher in metastatic cancer epithelial cells and prostatic intraepithelial neoplasia compared to benign or normal prostate cells (38). Moreover, in highly metastatic breast cancer cells increased Endoglin expression is associated with invasion and metastasis (39). It would seem likely that the effects of Endoglin over-expression may depend on the cell type and/or the over-expression level as well as interplay between the tumour and the stroma and inputs from multiple signalling pathways. However, we have observed decreased Endoglin expression in prostate tumour cells and breast tumour cells relative to immortalised cells. Henry et al also reported decreased Endoglin expression in most breast tumour cell lines relative to immortalised cells and detected Endoglin expression in only a minority of primary tumours (23). We expect that the regulation of Endoglin by PRH is disrupted in breast cancer cells. Further experiments will be required to determine whether PRH activity in prostate cancer cells is disrupted by changes in PRH localisation as has been observed in breast cancer cells (9) or by some other mechanism. However, it would seem likely that in both of these cancer types and in other cancer cells in which PRH is aberrantly localised, the loss of PRH activity may contribute to increased cell invasion, increased cancer metastasis and ultimately, decreased patient survival in part at least through a decrease in Endoglin expression. Loss of PRH activity would also be expected to derepress VEGF signalling genes

resulting in increased cell survival and/or increased neoangiogenesis. This combination of outcomes arising from the loss of PRH activity suggests that this protein plays a critical role in tumourigenesis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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MATERIALS AND METHODS

Expression vectors and reporters

pMUG1-Myc-PRH expresses human PRH tagged with the Myc9E10 epitope (3). pMUG1-Myc-PRH N187A and pMUG1-Myc-PRH F32E express mutated PRH proteins that fail to bind DNA and TLE respectively (3). The plasmids shRNAPRH49 and shRNAPRH51 and control shRNA plasmid were obtained from Origene. The Endoglin expression vector pcDNA3.1-Endoglin (long isoform) was a gift from Professor Clare Isacke. The recombinant adenoviral construct expressing Myc-PRH has been described previously (28). pCD105(-2450/+350), pCD105(-851/+350) and pCD105(-400/+350) containing Endoglin promoter sequences cloned upstream of the firefly luciferase gene were a gift from Professor Carmelo Bernabeu (34, 35). pRL-CMV expressing Renilla luciferase was purchased from Promega. Lentiviral constructs expressing IPTG-inducible PRH shRNA or a control shRNA were obtained from Sigma.

Cell culture

PNT2-C2, PC3, DU145, MCF-7 and MDA-MB-231 cells were cultured in RPMI-1640 supplemented with 10% FBS, 2mM L-glutamine and 1% Penicillin/Streptomycin. MCF 10A cells were cultured in DMEM:F12 (Sigma) supplemented with 5% Horse Serum Heat-Inactivated (Sigma), 20ng/ml EGF (Peprotech) 0.5ug/ml Hydrocortisone (Sigma), 100ng/ml Cholera toxin (Sigma), 10μg/ml Insulin (Sigma) and 1% Penicillin/Streptomycin. Human umbilical vein endothelial cells were cultured in DMEM:F12 supplemented with 5ng/ml EGF (Peprotech), 10ng/ml bFGF, 20μg/ml Heparin, 1μg/ml Hydrocortisone, 250ng/ml Insulin, 1% Penicillin/Streptomycin and 2% FCS. All cells were maintained in a humidified atmosphere at 37°C and 5% CO_2.

Transient transfection

PNT2-C2 cells, PC3 cells and DU145 cells were transfected using TransIT® (Mirus). MCF-7 cells and MDA-MB-231 cells were transfected using Lipofectamine 2000 (Invitrogen). PC3 cells were transfected for luciferase assays using electroporation (250 V, 975 μF).

Luciferase assays

PC3 cells were transiently transfected with reporter plasmids and either pMUG1-Myc-PRH or empty pMUG1 vector. After 24 hours at 37°C and 5% CO_2 the cells were lysed and assayed for luciferase activity using a dual luciferase assay system (Promega) and a Berthold Technologies luminometer. Renilla luciferase activity was used as an internal control for transfection efficiency.

PRH knockdown

Knockdown of PRH in PNT2-C2 cells was performed as described previously (31). Knockdown of PRH in MCF-7 and MCF 10A cells was performed using an IPTG-inducible PRH shRNA lentiviral construct (Sigma). Cells were infected with the PRH shRNA lentiviral construct or a control scrambled shRNA lentivirus and after 48 hours transduced cells were selected using puromycin. Stably transduced cell lines were grown in the presence of 1mM IPTG for 7 days to induce shRNA expression.

Cell migration assays

Cell monolayers on microscope coverslips were produced plating cells infected with Ad PRH or empty adenovirus (MOI 50) and incubating the cells for 24 hours at 37°C and 5% CO_2. After the addition of 1μM hydroxyurea (Sigma) to inhibit cell division a wound was

created using a P1000 pipette tip. Pictures were taken using a Leica DMIRBE microscope with Hamamatsu CCD camera or a AMG EVOS XL CORE AMEX 1200 and the width and area of the wound was quantified using ImageJ software (40).

Chemotaxis assays were performed by seeding cells onto 8μm Boyden chambers (Greiner Bio-One) in RPMI with 2% FBS. The chambers were placed into 24 well plates containing RPMI with 10% FBS to create a serum gradient. In some experiments cells, were seeded in the presence of 3μM ALK 4/5/7 kinase inhibitor SB-431542 (Sigma) or DMSO. At the time points indicated the cells were fixed with 4% paraformaldehyde (Fisher) and stained with 2μg/ml bisbenzimide (Sigma). Cells on the top and bottom of the membrane were counted using a Leica Q550 inverted epifluorescence microcope or Zeiss axioplan 2.

Invasion assays were performed as above except that 50% Matrigel (BD Biosciences) was added to the Boyden chambers and left at 37°C for 1 hour to solidify prior to seeding the cells. Extravasation assays were performed as described by Ma and Wang (41) except that PC3 cells were dissociated using Cell Dissociation Solution (Sigma) prior to seeding on the HUVEC layer.

Quantitative reverse transcriptase-mediated PCR (RT-qPCR)

RNA was purified 48 hours post-infection as described previously (31, 42). Quantitative PCR was performed in triplicate with Endoglin and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (shown below). Data were analysed using Rotorgene 6 software (Corbett Research; Rotorgene RG-3000) with GAPDH mRNA as an internal control.

Endoglin 5’ GCCGTGCTGGGCATCACCTT 3’ 5’ CGCTTGCTGGGGGAACCTGG 3’, annealing at 60°C.

GAPDH 5’ TGATGACATCAAGAAGGTGGTGAAG 3’ 5’ TCCTTGGAGGCCATGT GGGCCAT 3’, annealing at 55°C.

Western blotting

Whole cell extracts were prepared using TES buffer (1% SDS, 2mM EDTA, 20mM Tris-HCl pH 7.4) as described previously (27). A rabbit polyclonal antibody was used to detect Endoglin (Abcam). PRH antibodies have been described previously (3, 42). Lamin A/C and Tubulin antibodies were from Santa-Cruz. Densitometry was performed using Quantity One 4.6 software (BioRad). Phosphorylated Smad3 (Ser423/425) was detected using a rabbit monoclonal antibody (Cell Signaling Technology).

Chromatin immunoprecipitation

Cells were infected with Ad PRH or control virus (MOI 50) and incubated for 24 hours at 37°C and 5% CO_2 before fixation in 1% formaldehyde for 8 minutes at 21°C. Glycine was added to a final concentration of 125mM, then the cells were pelleted by microcentrifugation at 4°C, resuspended in 130µl lysis buffer (50mM Tris-Cl pH 8.0, 10mM EDTA, 1% SDS, 1mM PMSF and protease inhibitor cocktail (Roche)) and sonicated in a Biorupter (Diagenode) for 20 minutes on high power at 4°C. Dynabeads® protein A magnetic beads (Invitrogen) were incubated with normal mouse IgG (Santa Cruz) or Myc9B11 (Cell Signalling Technology) in RIPA buffer (10mM Tris-Cl pH 8.0, 140mM NaCl, 1% v/v Triton X-100, 1mM EDTA, 0.5mM EGTA, 0.1% w/v SDS, 0.1% sodium deoxycholate) for 2 hours at 4°C on a rotary wheel. Chromatin lysates were incubated with antibody:bead complexes overnight at 4°C on a rotary wheel. Beads were washed x3 with 1ml RIPA buffer, 1ml RIPA containing 500mM NaCl, 1ml RIPA containing 0.5% NP-40 and twice with 1 ml TE buffer (10mM Tris-Cl pH 8.0, 1mM EDTA pH 8.0) and resuspended in

elution buffer (20mM Tris-Cl pH 7.5, 50mM NaCl, 5mM EDTA, 1% SDS). After digestion with proteinase K for 2 hours at 68°C DNA was obtained by phenol chloroform isoamyl alcohol extraction and precipitated prior to resuspension in TE. Binding was analysed by PCR (95°C for 1 min, 60°C for 1 min, 72°C for 1 min) using the following Endoglin primer pairs: P1 5' CAGGAAGGCATCGTGCCCCA 3' 5' TCACCGACAAAACACAGCTCCA 3', P2: 5'CTCTGCCAGCGTCCTTCTGCTC 3' 5' AGGGTGCCAGACTAAGC AAAGCAAC 3', P3 5' GGGTTGCCATGGTGGGAATATA 3' 5' TATGGGTGTT GGGGGCATTC 3', P4 5' AGCTAATAGCCCGTGTGCAA 3' 5' AGGGGGAGAG TGGTCCTAGA 3'. The prm2 gene was used as a negative control: prm2 primers 5' TGTACAGGCAGCAGTTGCATGG 3' 5' CTCCTTCGAGAGCAGTGTCTGC 3' (annealing temperature 62°C, 33 cycles).

FIGURE LEGENDS

Figure 1. PRH regulates cell motility. (A) PNT2-C2 cells were transfected with plasmids expressing PRH shRNAs or a control shRNA and grown for ten days in puromycin selection. Western blotting of day 10 whole cell extracts was performed using a monoclonal antibody that recognises PRH. The two bands detected represent hyper- and hypo-phosporylated PRH (42). Antibodies that recognise Tubulin confirm equal loading. (B) PRH protein levels in three independent experiments after 10 days in selection were quantified relative to Tubulin. The graph shows the average PRH level. Mean and standard deviation (M+SD), n=3. ** indicates $P<0.01$. (C) After 10 days in selection control cells (top) and PRH knockdown PNT2-C2 cells (bottom) were used to produce confluent monolayers. The monolayers were wounded with a pipette tip and imaged at 0, 6 and 18 hours. Representative images from n=4. (D) Wound width was measured at 5 locations and is plotted as percentage wound width remaining. M+SD, n=4. (E) Transwell chemotaxis assays using control cells (open bars) and PRH knockdown PNT2-C2 cells (shaded bars). The graph shows the percentage of cells migrated in ten fields of view at the time points shown. M+SD. n=2. *** indicates $P<0.001$.

Figure 2. PRH over-expression inhibits prostate cell migration and cancer cell invasion. (A) Transwell chemotaxis assays were performed using cells transiently transfected with a vector expressing GFP (open bars) or vectors expressing GFP and PRH (shaded bars). The graph shows the percentage of migrated cells in ten fields of view after 24 hours. M+SD. n=2. ** indicates $P<0.01$. (B) DU145 cells were transiently transfected with a vector expressing GFP or vectors expressing GFP and either wild type PRH, PRH N187A or PRH F32E. 24 hours post-transfection the cells were plated in transwell inserts and after a further 24 hours the number of migrated green cells was determined by counting cells in ten fields of

view. M+SD. n=5. * indicates P<0.05. (C) Myc-PRH protein levels in whole cell extracts prepared from the cells in part (B) were determined using western blotting. Lamin A/C was used as a loading control. (D) Transwell invasion assays were performed using cells transiently transfected with a vector expressing GFP (open bars) or vectors expressing GFP and PRH (shaded bars). The graph shows the percentage of invaded cells in ten fields of view after 24 hours. M+SD. n=2. *** indicates P<0.001. (E) *In vitro* extravasation assays were performed using HUVECs grown as a monolayer on Matrigel in Boyden chambers. PC3 cells were transiently transfected with a vector expressing GFP (open bars) or vectors expressing GFP and PRH (shaded bars) prior to seeding on the HUVEC layer. The graph shows the number of invaded cells in ten fields of view after 12 hours. M+SD. n=3. * indicates P<0.05.

Figure 3. PRH directly activates Endoglin expression. (A) Endoglin mRNA levels in PNT2-C2 cells and PC3 cells 48 hours post-infection with an empty adenoviral vector (open bars) or an adenoviral vector expressing PRH (shaded bars). mRNA levels were measured by RT-qPCR and compared to Gapdh mRNA levels. M+SD, n=3. *indicates P<0.05. (B) Top- Endoglin protein levels in whole cell extracts prepared from the cells described in (A) were determined using western blotting. Antibodies that recognise Tubulin confirm equal loading. Bottom- The graph shows Endoglin protein levels in the cells described above determined using densitometry. M+SD, n=3. * indicates P<0.01. (C) Top- Endoglin protein levels in whole cell extracts prepared from control PNT2-C2 cells (1) and PRH knockdown PNT2-C2 cells (2) at day 10 in selection were determined using western blotting. Antibodies that recognise Lamin A/C confirm equal loading. Bottom- The graph shows Endoglin protein levels in PRH knockdown PNT2-C2 cells compared to control PNT2-C2 cells. M+SD, n=4. ** indicates P<0.01. (D) Upper panel- a cartoon of the Endoglin promoter region, showing the transcription start point (bent arrow) and primers used for ChIP. Lower panels- Myc-PRH

was expressed in PNT2-C2 cells using Ad PRH. ChIP was then performed as described in the text. Each panel shows the result of PCR with primer pairs P1-P4 and input chromatin (1), chromatin precipitated with IgG (2), Myc antibody (3), or a no template control (4).

Figure 4. Endoglin over-expression abrogates the effects of a PRH knockdown on cell migration. (A) PNT2-C2 cells were transfected with plasmids expressing PRH shRNAs or a control shRNA and grown for ten days in puromycin selection. The cells were then transiently transfected with an Endoglin expression vector or a control empty vector and plated out to produce confluent monolayers as in Figure 1. The monolayers were wounded with a pipette tip and imaged at 0, 6, 18 and 24 hours. After imaging wound width was measured at 5 locations and is plotted as percentage wound width remaining. M+SD, n=3. (B) Control PNT2-C2 cells (open bars) and PRH knockdown PNT2-C2 cells (shaded bars) were grown for 10 days in puromycin selection and then transiently transfected with an Endoglin expression vector or empty vector as in (A). 24 hours post-transfection transwell chemotaxis assays were performed as in Figure 1. The graph shows the percentage of cells migrated in ten fields of view after 24 h. M+SD, n=2. ** indicates $P<0.01$. (C) DU145 cells were transiently transfected with vectors expressing GFP alone (1 and 2), GFP and PRH (3 and 4), or GFP and Endoglin (5 and 6). After 24 hours transwell chemotaxis assays were performed as above but in the absence (-) or presence (+) of 3μM SB-431542. The graph shows the percentage of cells migrated in ten fields of view after 24 hours. M+SD, n=2. * indicates $P<0.05$. (D) DU145 cells were treated with 3μM SB-431542 for 24 hours or left untreated. Western blotting was then performed using antibodies that recognise phosphorylated SMAD3 and antibodies that recognise Lamin A/C as a loading control.

Figure 5. PRH regulates breast cancer cell migration and invasion. (A) MCF 10A cells and MCF-7 cells were transduced using a control lentivirus that activates RISC and the RNAi pathway but does not target any known gene (1) or a lentivirus expressing PRH shRNA (2). shRNA expression was then induced using IPTG for 7 days. Western blotting of whole cell extracts was performed using a monoclonal antibody that recognises PRH and antibodies that recognise Tubulin or Lamin A/C. (B) Transwell chemotaxis assays were performed using control MCF 10A cells (open bars) and PRH knockdown MCF 10A cells (shaded bars). The graph shows the percentage of cells migrated in five fields of view after 24 hours. M+SEM, n=4. * indicates $P<0.05$. (C) MCF-7 cells and MDA-MB-231 cells were infected with empty Ad empty (open bars) or Ad PRH (filled bars). 24 hours post-infection transwell chemotaxis assays were performed as above. The graph shows the percentage of cells migrated in five fields of view after 24 hours. M+SEM, n=3. ** indicates $P<0.01$. (D) PRH knockdown MCF-7 cell lines and control cells were plated to produce confluent monolayers and then wounded as in Figure 1. After imaging wound area was measured in six fields of view in three independent clones and is plotted as percentage wound area remaining. M+SD, n=3. *** indicates $P<0.001$. (E) Transwell invasion assays were performed using MDA-MB-231 cells transiently transfected with a vector expressing GFP (open bar) or vectors expressing GFP and PRH (shaded bar). The graph shows the percentage of invaded cells after 24 hours. M+SEM, n=3. * indicates $P<0.05$. (F) Endoglin mRNA levels in MCF 10A cells and MCF-7 cells 48 hours post-infection with Ad empty (open bar) or Ad PRH (shaded bar). Endoglin mRNA levels were measured by RT-qPCR and compared to Gapdh mRNA levels. M+SD. n=4. * indicates $P<0.05$. (G) Western blotting for Endoglin in whole cell extracts prepared from control MCF 10A cells (1) or MCF 10A PRH knockdown cells (2). Tubulin was used as a loading control.

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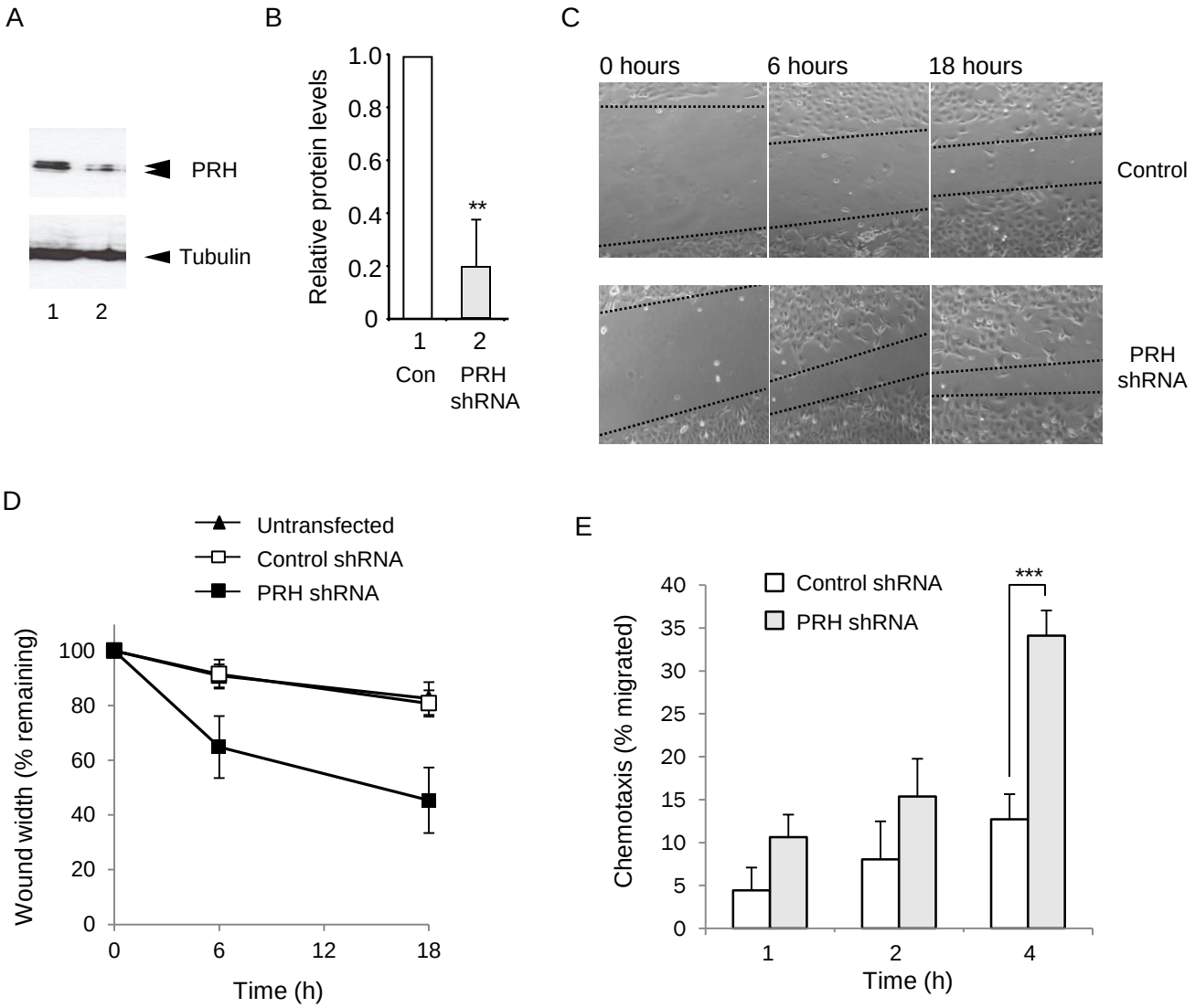
Figure 1


A
PRH
Tubulin
1
2
B
Relative protein levels
1.0
0.8
0.6
0.4
0.2
0
**
1
2
Con
PRH shRNA
C
0 hours
6 hours
18 hours
Control
PRH shRNA
D
Untransfected
Control shRNA
PRH shRNA
Wound width (% remaining)
100
80
60
40
20
0
0
6
12
18
Time (h)
E
Control shRNA
PRH shRNA
***
Chemotaxis (% migrated)
40
35
30
25
20
15
10
5
0
1
2
4
Time (h)

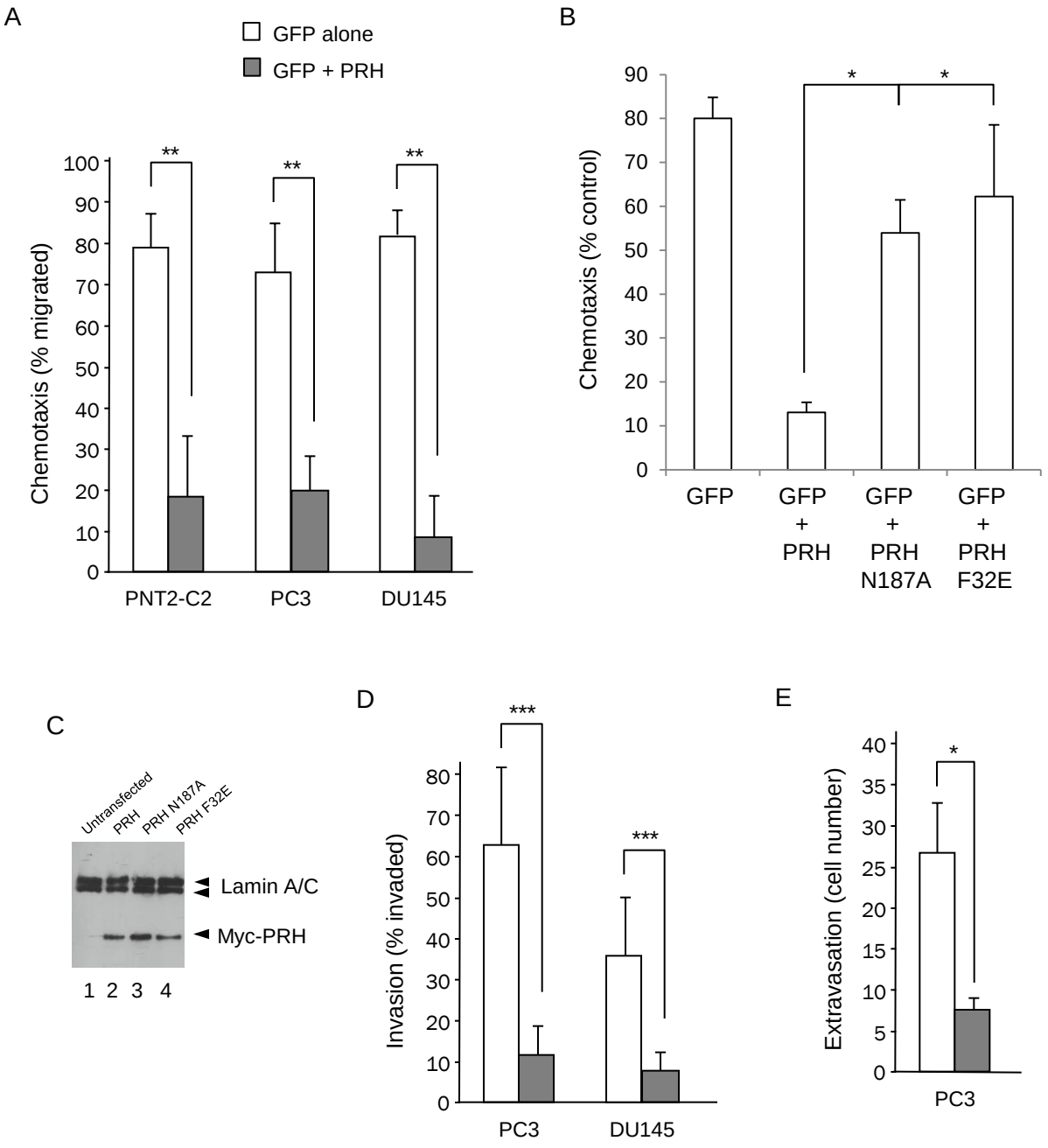

A
GFP alone
GFP + PRH
Chemotaxis (% migrated)
100
90
80
70
60
50
40
30
20
10
0
**
**
**
PNT2-C2
PC3
DU145
B
Chemotaxis (% control)
90
80
70
60
50
40
30
20
10
0
*
*
GFP
GFP + PRH
GFP + PRH N187A
GFP + PRH F32E
C
Untransfected
PRH
PRH N187A
PRH F32E
Lamin A/C
Myc-PRH
1 2 3 4
D
Invasion (% invaded)
80
70
60
50
40
30
20
10
0
***
***
PC3
DU145
E
Extravasation (cell number)
40
35
30
25
20
15
10
5
0
*
PC3

Figure 3

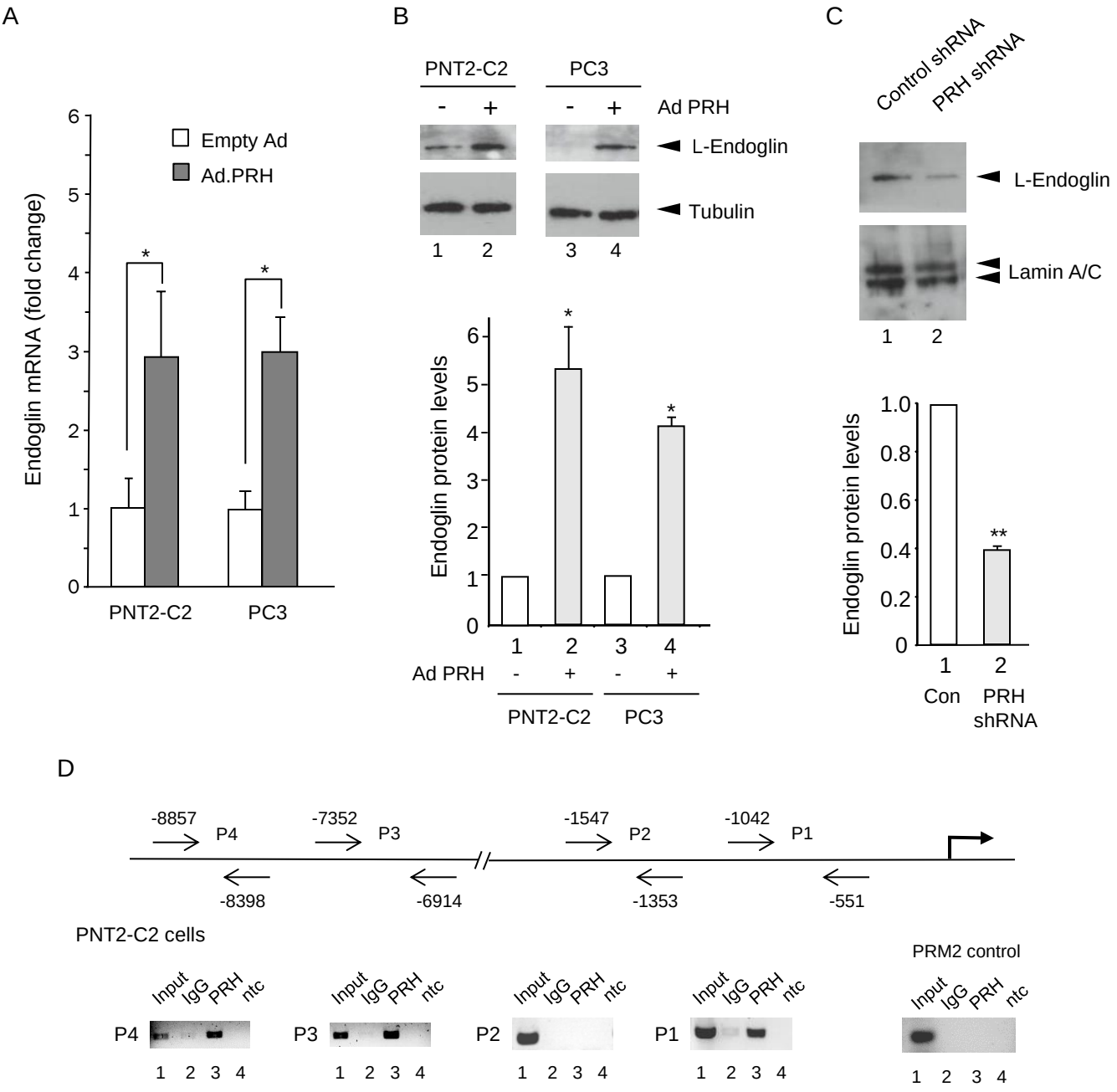
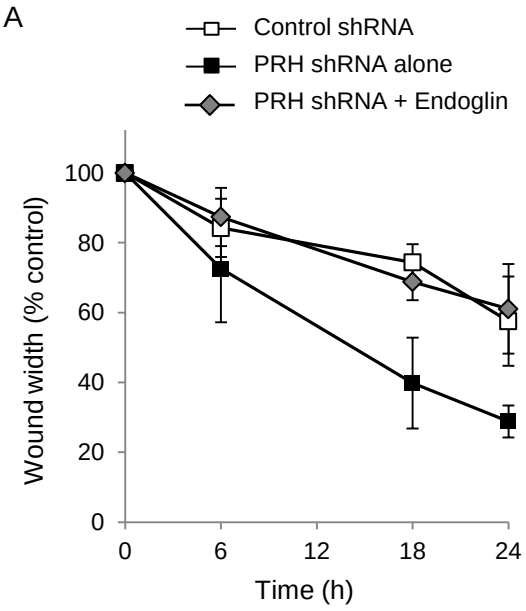
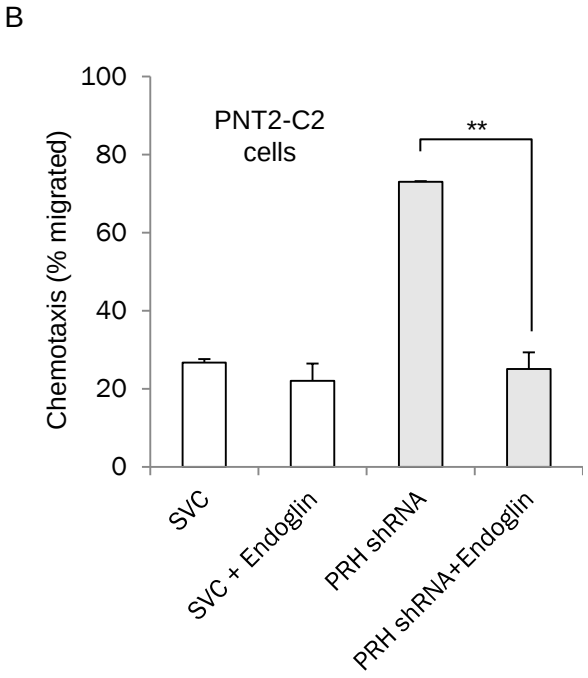
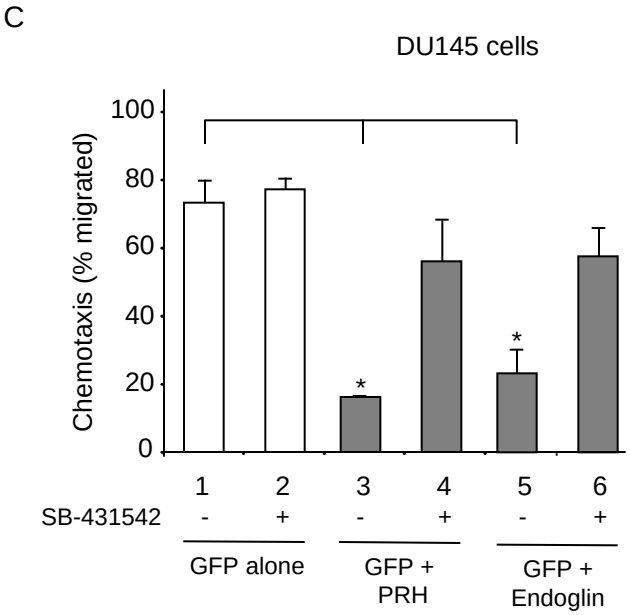

A
Endoglin mRNA (fold change)
Empty Ad
Ad.PRH
PNT2-C2
PC3
B
PNT2-C2
PC3
Ad PRH
L-Endoglin
Tubulin
Endoglin protein levels
C
Control shRNA
PRH shRNA
L-Endoglin
Lamin A/C
Endoglin protein levels
Con
PRH shRNA
D
-8857
P4
-7352
P3
-1547
P2
-1042
P1
-8398
-6914
-1353
-551
PNT2-C2 cells
PRM2 control
Input
IgG
PRH
ntc

Figure 4


A
Control shRNA
PRH shRNA alone
PRH shRNA + Endoglin
Wound width (% control)
100
80
60
40
20
0
0
6
12
18
24
Time (h)


B
PNT2-C2 cells
**
Chemotaxis (% migrated)
100
80
60
40
20
0
SVC
SVC + Endoglin
PRH shRNA
PRH shRNA+Endoglin


C
DU145 cells
Chemotaxis (% migrated)
100
80
60
40
20
0
*
*
1
2
3
4
5
6
SB-431542
-
+
-
+
-
+
GFP alone
GFP + PRH
GFP + Endoglin

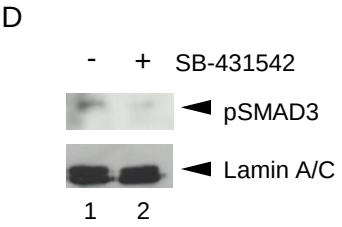
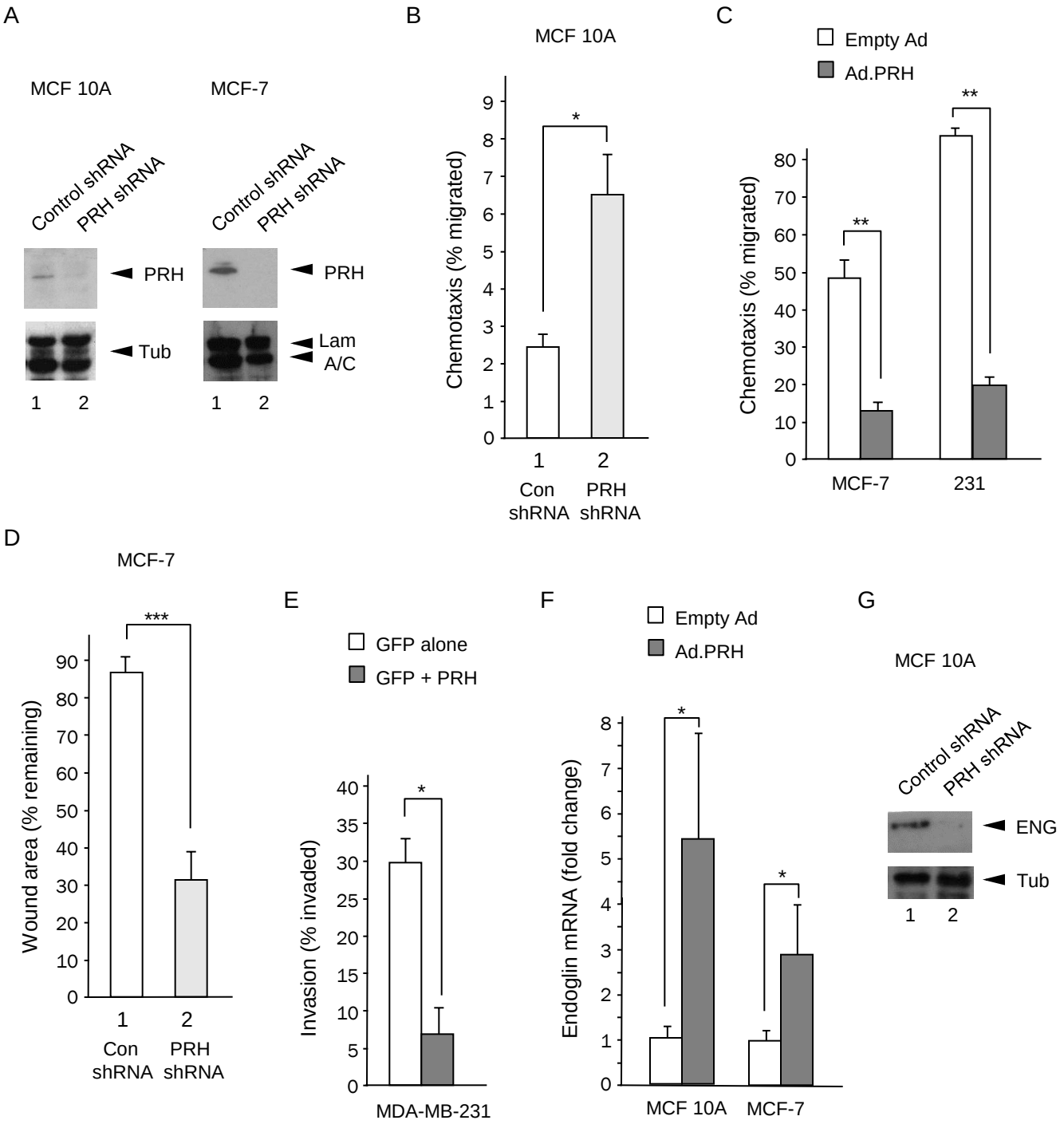

D
-
+
SB-431542
pSMAD3
Lamin A/C
1
2

Figure 5


A
MCF 10A
MCF-7
Control shRNA
PRH shRNA
Control shRNA
PRH shRNA
PRH
PRH
Tub
Lam A/C
1 2
1 2
B
MCF 10A
Chemotaxis (% migrated)
Con shRNA
PRH shRNA
C
Empty Ad
Ad.PRH
Chemotaxis (% migrated)
MCF-7
231
D
MCF-7
Wound area (% remaining)
Con shRNA
PRH shRNA
E
GFP alone
GFP + PRH
Invasion (% invaded)
MDA-MB-231
F
Empty Ad
Ad.PRH
Endoglin mRNA (fold change)
MCF 10A
MCF-7
G
MCF 10A
Control shRNA
PRH shRNA
ENG
Tub
1 2