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RESEARCH ARTICLE

Nestin regulates prostate cancer cell invasion by influencing the localisation and functions of FAK and integrins

Claire L. Hyder[1,2], Glorianne Lazaro[1,2,*], Joanna W. Pylvänäinen[1,2,*], Maxwell W. G. Roberts[1,2], Susanna M. Qvarnström[1,2] and John E. Eriksson[1,2,‡]

ABSTRACT

Nestin, an intermediate filament protein and marker of undifferentiated cells, is expressed in several cancers. Nestin is important for neuronal survival and is a regulator of myogenesis but its function in malignancy is ambiguous. We show that nestin downregulation leads to a redistribution of phosphorylated focal adhesion kinase (pFAK, also known as PTK2) to focal adhesions and alterations in focal adhesion turnover. Nestin downregulation also leads to an increase in the protein levels of integrin $\alpha5\beta1$ at the cell membrane, activation of integrin $\beta1$ and an increase in integrin clustering. These effects have striking consequences for cell invasion, as nestin downregulation leads to a significant increase in pFAK- and integrin-dependent matrix degradation and cell invasion. Our results indicate that nestin regulates the localisation and functions of FAK and integrin. Because nestin has been shown to be prevalent in a number of specific cancers, our observations have broad ramifications for the roles of nestin in malignant transformation.

KEY WORDS: Intermediate filaments, Nestin, Integrins, Invasion, Migration, Prostate cancer, Subject category, Cancer

INTRODUCTION

Nestin is a member of the cytoskeletal intermediate filament family of proteins. The intermediate filaments not only provide structural functions and protection against mechanical stress, they also have essential functions in signal transduction, motility, cellular stress, organelle positioning and transport. Nestin is regarded as a marker of stem cells and differentiation, but its cellular functions remain unclear. Nestin is expressed in early development, and is induced during the regeneration of neuronal (Frisén et al., 1995; Lin et al., 1995; Shibuya et al., 2002) and muscle tissues (Čížková et al., 2009; Vaittinen et al., 2001). During differentiation, nestin is expressed at the early stages; as differentiation proceeds, nestin is downregulated and replaced by other cell-type-specific intermediate filaments, such as vimentin or desmin (Vaittinen et al., 1999). Nestin is a potent regulator of myogenesis, during which it regulates the pace of differentiation. It also functions as a scaffold for Cdk5 and regulates the cellular localisation and activation of Cdk5 by p35 and p25 during differentiation (Pallari et al., 2011; Sahlgren et al., 2006).

Nestin forms heteropolymers and its function requires a tissue-specific binding partner. One of these is vimentin, a protein commonly known to be a marker of cells and tissues of mesodermal origin and highly invasive and motile cells (Ivaska, 2011; Mendez et al., 2010). Vimentin has been identified as a regulator of integrin dynamics and cell motility (Ivaska et al., 2005; Rizki et al., 2007). Integrins form a family of transmembrane heterodimeric proteins consisting of α and β subunits, which are activated by bidirectional signalling. First, they can be activated by either ligand binding (in the case of non-adherent cells) or by increased affinity for the extracellular matrix (ECM) proteins to which adherent cells are attached. Activation instigates a downstream signalling cascade known as 'outside-in' signalling. Second, integrins can be activated through inside-out signalling, which involves the binding of proteins such as talin, which induces the integrin dimers to adopt an active conformation. The initial stages of integrin activation are accompanied by clustering of the integrin pairs, which acts to potentiate downstream signalling, resulting in changes in cell adhesion, motility and invasion (Shattil et al., 2010).

Nestin is expressed in a number of cancers including osteosarcoma, glioma, melanoma, pancreatic and prostate cancer, as well as in tumour vasculature (Ishiwata et al., 2011a; Krupkova Jr et al., 2010). Much evidence of the function of nestin in cancer is correlative, suggesting that nestin expression might have prognostic value for some cancer types and that expression correlates with tumour severity, particularly in more aggressive and invasive cell types.

Nestin expression has been found to correlate with increased cell motility and invasiveness in astrocytoma (Rutka et al., 1999), and to directly promote cell motility in prostate cancer (Kleeberger et al., 2007). Nestin and vimentin are binding partners, and vimentin regulates the cell membrane expression and recycling of integrins. Considering this, we wanted to investigate the role of nestin in cell motility and invasion, focussing on the regulation of integrin dynamics and signalling. Defining a role for nestin in cell migration and integrin dynamics in cancer cells will give insight into why nestin is expressed only in some cancers and what its functional roles are.

RESULTS

Nestin regulates the localisation of pFAK

The intermediate filament proteins are integrators of cell attachment, adhesion and movement. Several studies have suggested that the intermediate filament protein vimentin is associated with focal adhesions and with the regulation of focal adhesion size and stabilisation of ECM–cell adhesion sites

[1]Turku Centre for Biotechnology, University of Turku and Åbo Akademi University, POB 123, FIN-20521, Turku, Finland. [2]Department of Biosciences, Åbo Akademi University, Tykistökatu 6A, FI-20520, Turku, Finland.
*These authors contributed equally to this work

‡Author for correspondence (john.eriksson@abo.fi)

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(Bhattacharya et al., 2009; Tsuruta and Jones, 2003). Because vimentin and nestin are binding partners, we wanted to study whether nestin was also associated with focal adhesions and what the implications of this are.

Nestin is an intermediate filament protein that can only form heteropolymers (for example, with vimentin), and it has a perinuclear cytoplasmic localisation in PC-3 prostate cancer cells. Initially, we downregulated nestin in the PC-3 prostate cancer cell line using nestin-targeting siRNA, labelled the cells for the focal adhesion marker phosphorylated focal adhesion kinase (pFAK, phosphorylated at Y397) (Fig. 1A) and visualised the cells using confocal microscopy. The mean level of nestin downregulation that was achieved was ~75% (supplementary material Fig. S1). Y397 pFAK is a marker for the initial activation of pFAK, which is phosphorylated upon integrin engagement and clustering (Calalb et al., 1995; Michael et al., 2009). Nestin-downregulated cells displayed prominent punctate pFAK labelling at the periphery of the cells (Fig. 1A) but little in the underlying cell body. To identify whether the pFAK structures were adhesion sites, we looked at integrin localisation and co-labelling with pFAK. Integrin α5 is expressed in the PC-3 cell line and, along with its partner, integrin β1, is one of the most studied integrin heterodimer pairs in the cancer literature. In control cells, integrin α5 showed a distinctive punctate pattern throughout the cell (Fig. 1B). It was concentrated in ruffles at the edges of the cell and was frequently associated with pFAK at the cell edges (Fig. 1B, left panel, merge, inset). In nestin-downregulated cells, integrin α5 was more evenly distributed and was clearly less associated with pFAK (Fig. 1B, right panel, merge, inset). Colabelling of pFAK and actin (Fig. 1C) did not show any gross morphological changes in nestin-downregulated cells compared with control cells. However, some pFAK clusters were associated with the ends of actin filaments at the cell periphery (Fig. 1C, right panel, merge, inset). Quantification showed a significant increase in the percentage of cells with pFAK clusters (Fig. 1D). Colabelling of control and nestin-downregulated cells for pFAK and two markers of focal adhesions, talin and vinculin (supplementary material Fig. S1C,D), showed that they do localise to the same areas of the cell and display a similar pattern.

To see whether FAK signalling was altered by nestin downregulation, as would be indicated by a change in pFAK protein levels compared with FAK, western blots were quantified by densitometry. Intriguingly, nestin downregulation did not affect the protein levels or the ratio of pFAK to FAK (Fig. 1E–G), indicating that nestin downregulation regulates the cellular distribution of pFAK, not its initial activation. We blotted for Src, a downstream effector of FAK, as well as its activated form, pSrc (Y416), and the focal adhesion proteins talin and vinculin, but there were no changes in the protein levels (Fig. 1H).

Nestin inhibits VASP turnover but not zyxin at focal adhesions

The increased number and size of the focal adhesions would suggest that focal adhesion turnover and maturation is altered. Fluorescent recovery after photobleaching (FRAP) analysis was performed with vasodilator-stimulated phosphoprotein (VASP)–GFP and zyxin–GFP, which are markers of more mature focal adhesions (Fig. 2). Surprisingly, we found that VASP recovery was significantly faster in nestin-downregulated cells (Fig. 2B,D), whereas there was no change in the rate of zyxin recovery (Fig. 2C,E), indicating that nestin downregulation has specific effects on focal adhesion turnover and maturation.

Nestin regulates the protein level of integrins at the cell membrane

The changes in the localisation of pFAK upon nestin-downregulation were striking. Integrin function is primarily mediated through changes in protein levels and activity at the cell membrane. Because pFAK is an effector of integrin signalling, we investigated whether nestin downregulation induced changes in the integrins at the cell membrane. Using antibodies recognising integrin extracellular domains, we employed flow cytometry to investigate how nestin modulates integrin protein levels at the cell membrane (Fig. 3). Integrins regulate the adherence and spreading of cells on a substrate, so we performed a timecourse assay of integrin protein levels at the cell membrane. The proportion of integrin α5 at the cell membrane was significantly and consistently increased upon nestin downregulation both in the static cell (Fig. 3A) and in the timecourse assay (Fig. 3B; supplementary material Fig. S2A).

The use of antibodies recognising only the active conformation of β1 integrin and another recognising the β1 integrin extracellular domain showed that the proportion of active integrin β1 expressed at the cell membrane increased significantly (Fig. 3C) compared with the total level of integrin β1 at the cell membrane upon nestin downregulation (Fig. 3D). The timecourse assay showed that nestin downregulation significantly increased active β1 integrin cell membrane protein levels (Fig. 3E; supplementary material Fig. S2B). In the control, the decline in levels of active integrin β1 at the cell membrane occurs almost immediately from the 0 hour timepoint. In the nestin-downregulated cells, the cell membrane protein levels of active β1 integrin peak at 30 minutes and then decrease. This could reflect a nestin-dependent change in the integrin dynamics and activation state of β1 integrin during adhesion and spreading. Similarly to Fig. 3D, total cell membrane levels of β1 integrin are not significantly different during the timecourse assay (Fig. 3F; supplementary material Fig. S2C).

Quantitative analysis of western blots (supplementary material Fig. S2D) showed no increase in the protein levels of α5 integrin (supplementary material Fig. S2D,E) or β1 integrin (supplementary material Fig. S2D,F). Taken together with the data shown in Fig. 3, these results suggest that although total integrin levels are unaffected by nestin downregulation, α5 and β1 integrins are increasingly targeted to the cell membrane and there is an inherently higher level of active α5β1 in nestin-downregulated cells.

Nestin does not affect integrin trafficking or degradation

Cell membrane levels of integrins are tightly controlled through the trafficking of integrins throughout the cell by endocytosis and recycling pathways. Active trafficking maintains pools of active and inactive integrins that are used by the cell during adhesion and motility (Arjonen et al., 2012; Margadant et al., 2011). Vimentin, a binding partner of nestin, regulates the integrin recycling pathway (Fortin et al., 2010; Ivaska et al., 2005; Kim et al., 2010). Accordingly, we investigated whether nestin regulates integrin trafficking. We analysed endocytosis and net trafficking of α5 integrin (Fig. 4A). Because cells are constantly endocytosing and recycling receptors, we treated some samples with the recycling inhibitor primaquine (0.6 nM), to assess both the net trafficking and endocytosis alone. Although the results were not statistically significant, the internalisation of integrin α5 was slightly increased by nestin downregulation. Interestingly, in the nestin-downregulated primaquine-treated samples, endocytosis seemed to completely halt after 5 minutes.

The results from the recycling assay also showed a peculiar trend (Fig. 4B). Unlike in the endocytosis assay, where an

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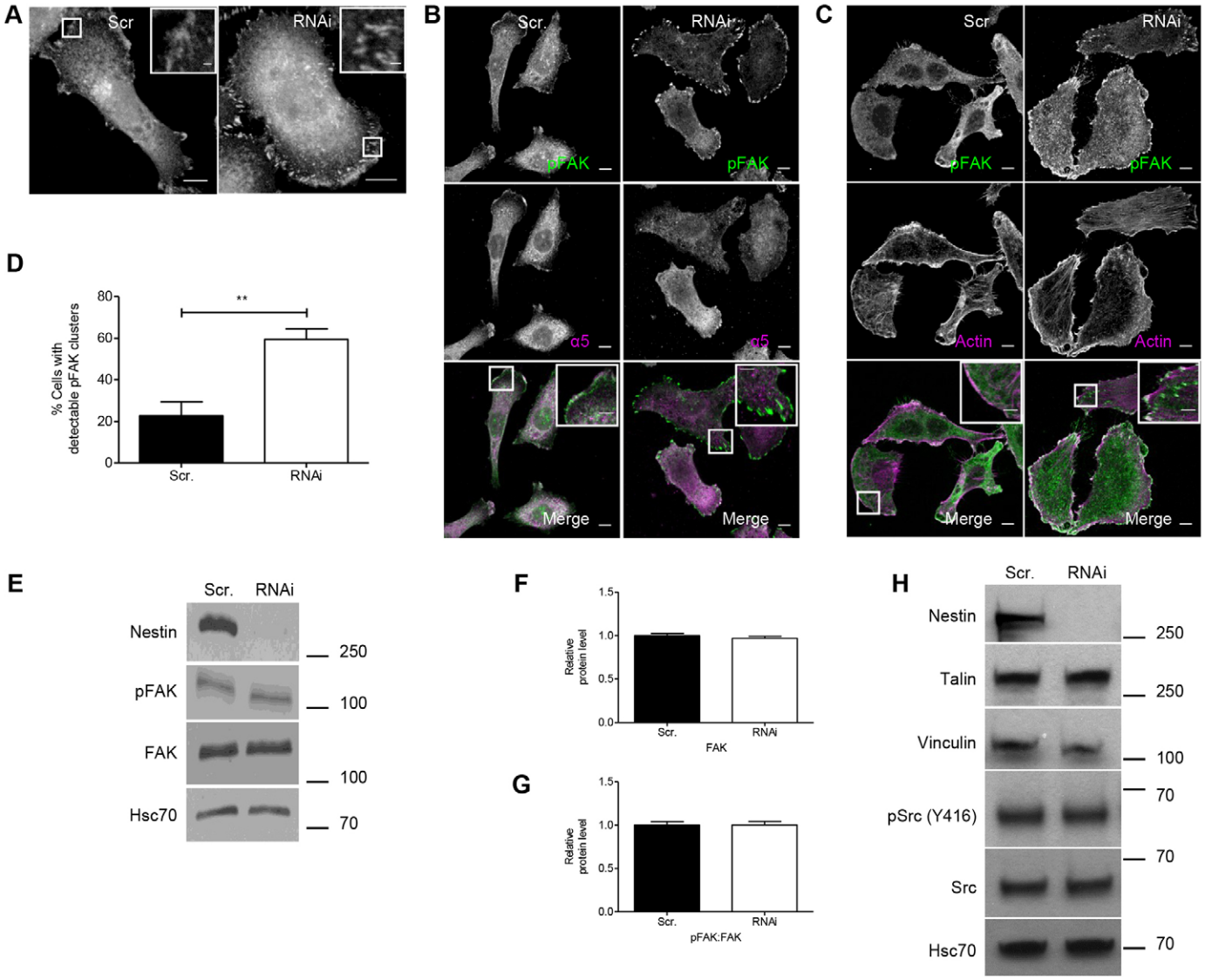

Fig. 1. Nestin downregulation causes relocalisation of pFAK. PC-3 cells were transfected with either control (Scr.) or nestin siRNA (RNAi). Each image in A–C is a sum intensity projection of the bottom region of the cell (between 5 and 13 individual *z*-stacks). Single-protein-labelled images are 8-bit greyscale, merged images are shown in colour as indicated. Protein colocalisation appears as white on the merged images. Insets show a magnified area (20 μm×20 μm) of the image as indicated by the white square in the merged image. Image analysis, including linear brightness and contrast adjustments, was performed using ImageJ. Images are representative of three independent experiments. Scale bars: 10 μm (main images), 5 μm (insets). (A) Nestin downregulation causes the localisation of pFAK to distinct clusters at the cell periphery. Cells were labelled for pFAK and imaged by confocal microscopy to identify how the downregulation of nestin affects pFAK localisation. (B) Nestin downregulation alters the localisation of α5 integrin in focal adhesions. Cells were colabelled for pFAK (green) and α5 integrin (magenta), and imaged using confocal microscopy. In nestin-downregulated cells, pFAK is redistributed to clusters at the cell periphery. These clusters appear more abundant and larger in RNAi cells compared with controls. The distribution of cytoplasmic α5 integrin in the cell appears more punctate in RNAi cells. α5 integrin localises with pFAK in control cells but is less associated with pFAK clusters in nestin-downregulated cells. (C) Nestin downregulation does not affect the localisation of actin in the cell or at the cell edge. Cells were colabelled for pFAK (green) and actin using fluorescently conjugated phalloidin (magenta). In both control and nestin-downregulated cells, actin colocalises to some extent with pFAK. (D) Quantification of the number of cells with pFAK clusters. The results are shown as a percentage of cells with detectable pFAK clusters, $n=3$. (E) Representative western blot to show the total protein levels of pFAK and FAK compared to an Hsc70 loading control. (F) Quantification of FAK protein levels in control and RNAi cells compared to Hsc70 shows that the total cellular protein level of FAK remains unchanged. (G) Quantification of the ratio of pFAK to FAK shows that the activation of pFAK is unchanged in nestin-downregulated cells. (H) Representative western blot to show that nestin does not affect the protein levels of integrin interaction partners and signalling effectors. Data show the mean±s.e.m.; $^{**}P<0.01$ (unpaired *t*-test).

increase in signal over time is expected, in the recycling assay the signal should decrease. There appeared to be little or no decrease in the internalised integrin α5, suggesting that the recycling pathway does not function efficiently, or at all, in PC-3 cells.

Using a modified version of the biotin assay, we looked at integrin degradation to see why α5 integrin was increased on the cell membrane in nestin-downregulated cells (Fig. 4C). There was a reduction in integrin α5 during this assay; however, nestin had no influence on this. Unlike vimentin, nestin has a marginal contribution to integrin trafficking, but it would need to be analysed in a cell line with more consistent endocytosis and recycling pathways. These studies suggest that nestin must affect

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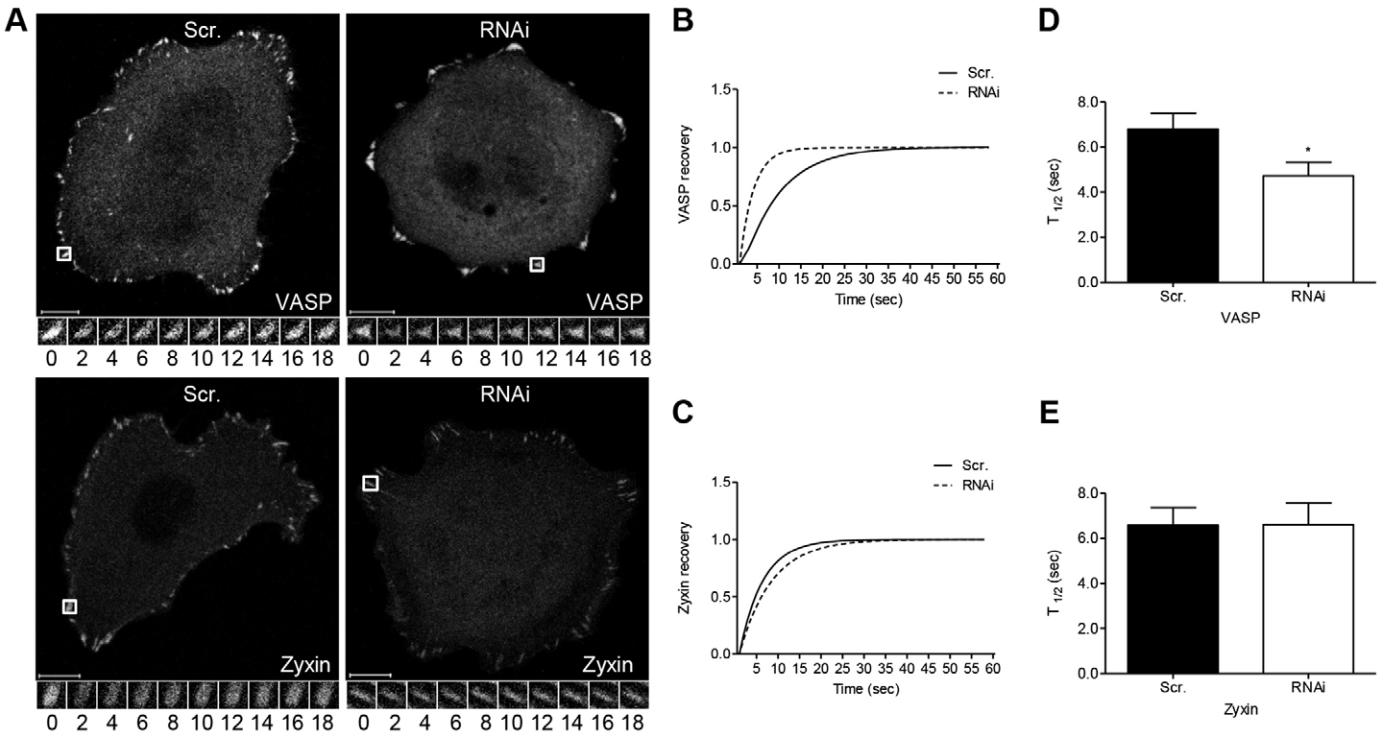

Fig. 2. Nestin downregulation increases the turnover of VASP but not zyxin at focal adhesion. PC-3 cells were co-transfected with either Cy3-tagged control (Scr.) or nestin siRNA (RNAi) and GFP-tagged zyxin or VASP. Focal adhesion turnover was studied by photobleaching a chosen focal adhesion site using a 488-nm laser at 100% with 20 iterations. Recovery was followed every second until the intensity reached a steady plateau. The recovery is presented as relative recovery. (A) Transfected PC-3 cells were imaged before bleaching of focal-adhesion-containing regions of interest (ROIs, white squares). The time-lapse sequence under each large image (times given in seconds) shows the recovery of fluorescence in the bleached area. The image at 0 seconds shows the fluorescence of the area prior to bleaching. Images of the whole cells are maximum intensity projection of the basal region of the cell. Scale bars: 10 μm. (B,C) Kinetics of recovery of GFP–VASP (B) and GFP–zyxin (C) in focal adhesions after photobleaching. Black line, the recovery of Scr. cells; dashed line, the recovery of RNAi cells. (D,E) Recovery half times (seconds) of GFP–VASP (D) and GFP-zyxin (E) in focal adhesions after photobleaching. Data show the mean±s.e.m; *$P<0.05$.

the levels of cell membrane integrin protein by some alternative process, such as modulation of integrin post-translational modifications. For example, glycosylation is known to affect α5β1 targeting to the cell membrane (Isaji et al., 2006).

Nestin modulates integrin clustering

Nestin regulates the redistribution of pFAK (Fig. 1). Labelling of α5 integrin showed no obvious redistribution of this protein and no association with the pFAK clusters; however, these experiments were performed without any substrate, which would activate integrins. pFAK is recruited to integrin tails in response to integrin activation, which occurs by outside-in signalling or by inside-out signalling (Tadokoro et al., 2003). Because integrin clustering is the common denominator of these two signalling pathways, we induced specific clustering of α5 integrin to determine whether nestin affects integrin activation.

Nestin-downregulated and control PC-3 cells were incubated with an anti-integrin-α5 antibody and the clustering of α5β1 was stimulated by the addition of a fluorescently conjugated secondary antibody and imaged using confocal microscopy (Fig. 5A,B). Nestin-downregulated cells exhibited more clustering of α5 integrin, and these clusters were larger than in the control (Fig. 5B). Quantification showed that the size of α5β1 clusters (Fig. 5C) and the number of clusters per cell (Fig. 5D) was significantly increased in nestin-downregulated cells, demonstrating that nestin downregulation enhances the clustering of activated α5β1 integrin. By using a FAK inhibitor, we looked at whether the effect of nestin on clustering was dependent on FAK activation (Fig. 5E–G). Although the FAK activity had a small effect on cluster volume (Fig. 5E), nestin-dependent clustering was unaffected.

Nestin does not affect the other components of the cytoskeleton

Microfilaments, microtubules and other intermediate filaments, such as vimentin, also have prominent effects on focal adhesion dynamics and the regulation of integrins (Ivaska et al., 2007; Kaverina and Straube, 2011; Rottner and Stradal, 2011; Yamaguchi and Condeelis, 2007). Immunofluorescence and western blotting showed that vimentin, tubulin and actin did not show changes in their localisation (Fig. 6A) or protein level (Fig. 6B) upon nestin-downregulation. Neither did nestin downregulation alter vimentin solubility (Fig. 6C). Taken together, these data demonstrate that the changes in focal adhesion formation and in integrin activation and levels at the cell membrane are dependent on nestin and not on alterations of other cytoskeletal proteins.

Nestin does not affect the migration of PC-3 cells

Nestin is expressed in a number of cancers (Ishiwata et al., 2011a); however, little is understood about its function during malignancy. Because alterations in focal adhesion dynamics and

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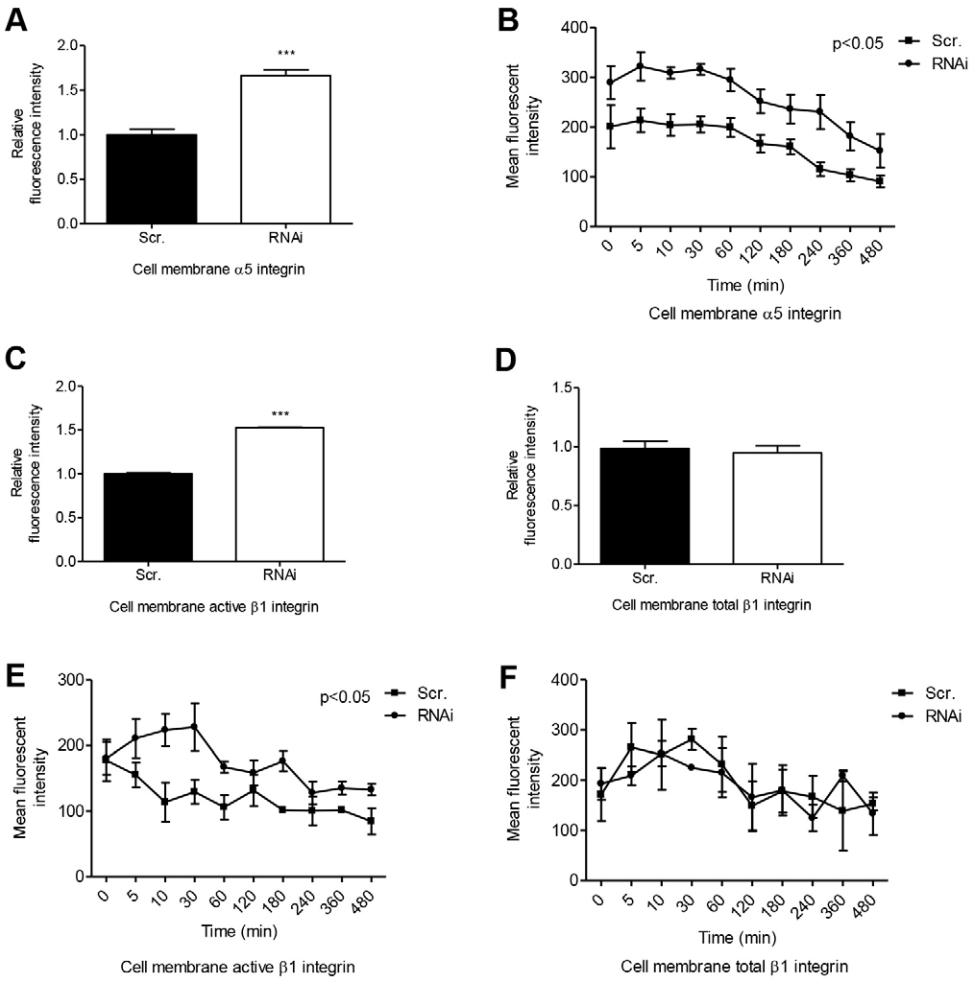

Fig. 3. Nestin modulates integrin protein levels at the cell membrane. PC-3 cells were transfected with either control (Scr.) or nestin siRNA (RNAi). The amount of cell-membrane-associated integrin was analysed by flow cytometry. Results were normalised and are presented as normalised fluorescent intensity; $n=3$–4. In B,E and F, cells adhered to fibronectin-coated plates for the indicated times before analysis. The amount of surface-labelled integrin detected is indicated by quantification of the observed relative fluorescence intensity. (A) Nestin downregulation increases the levels of cell-membrane-associated α5 integrin in steady-state cells. Cells were fixed and labelled with an antibody recognising the extracellular domain of α5 integrin. (B) Nestin downregulation leads to consistently increased surface levels of α5 integrin in actively adhering and spreading cells. At the indicated timepoints cells were fixed and labelled with antibody against extracellular domain of α5 integrin. (C) Nestin downregulation increases the level of cell-membrane-associated active β1 integrin in steady-state cells. Cells were labelled with an antibody recognising the active conformation of β1 integrin. (D) Nestin downregulation does not alter the total level of cell-membrane-associated β1 integrin in steady-state cells. Cells were labelled with an antibody recognising the extracellular domain of β1 integrin, both active and inactive conformations. (E) Downregulation of nestin protein levels increases the level of active β1 integrin at the cell membrane in actively adhering and spreading cells. At the indicated timepoints, cells were fixed and labelled with antibody against active β1 integrin. (F) Nestin protein levels do not affect total levels of cell-membrane-associated β1 integrin in actively adhering and spreading cells. At the indicated timepoints, cells were fixed and labelled with antibody against the extracellular domain of β1 integrin. Data show the mean±s.e.m.; ***$P<0.001$ (paired *t*-test).

integrins are often correlated with changes in cellular motility, and considering that nestin has a role in cell migration (Ishiwata et al., 2011a), we tested chemotactic migration (supplementary material Fig. S3A), performed a wound-healing assay (supplementary material Fig. S3B) and analysed time-lapse videos to investigate random migration parameters, including velocity and persistence (supplementary material Fig. S3C–E), and found no effect of nestin-downregulation on cell motility.

Nestin downregulation increases the invasion and matrix degradation of PC-3 prostate cancer cells in a FAK- and integrin-dependent manner

Although we saw no differences in cell migration, the profound effect that nestin downregulation had on pFAK localisation, VASP turnover, integrin levels at the cell membrane and integrin clustering must have some physiological relevance. Invasion and migration use similar signalling and mechanisms; the key difference is that, during invasion, the cell needs to penetrate the ECM to relocate. Integrins are potent mediators of cell invasion, so we performed an inverted invasion assay to investigate the invasive capacity of nestin-downregulated cells (Caswell et al., 2007). The resulting *z*-projections (Fig. 7A) show that nestin-downregulated cells invaded ~1.5 times further into the Matrigel than the control cells (Fig. 7B). For further quantification, we used Matrigel-coated invasion chambers, and allowed control and nestin-downregulated cells to invade for 24 hours. Nestin downregulation increased invasion twofold (Fig. 7C). *In vitro* migration and invasion assays occasionally

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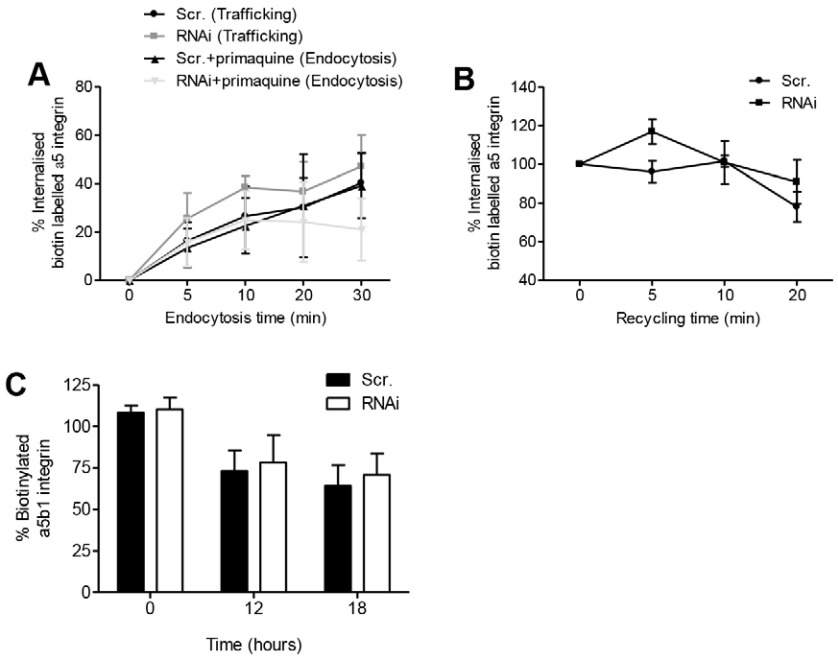

Fig. 4. Nestin does not affect trafficking of integrins or their degradation from the cell membrane. PC-3 cells were transfected with either control (Scr.) or nestin siRNA (RNAi). Cells were biotin labelled and allowed to internalise biotin-labelled cell membrane proteins for the times indicated. For the trafficking, endocytosis and recycling experiment, internalised protein levels were measured using capture ELISA with α5 integrin. For the degradation experiments, cell membrane proteins were biotin labelled and, at the times indicated, whole-cell lysate samples were collected. Levels of biotin-labelled integrin were analysed with capture ELISA. (A) Nestin does not affect endocytosis and net trafficking of α5 integrin. Internalisation of biotin-labelled α5 integrin was analysed at the timepoints specified in the presence or absence of the recycling inhibitor primaquine bisphosphate (0.6 nM), $n=3$. (B) Nestin does not affect recycling of α5 integrin. Internalisation and accumulation of biotin-labelled α5 integrin was allowed to occur for 30 minutes before samples were collected at the indicated timepoints following the initial internalisation, $n=6$. (C) Nestin does not alter degradation of α5 integrin from the cell membrane. Quantification of the levels of biotin-labelled α5 integrin remaining in the cell after the timepoints indicated, $n=3$. Data show the mean±s.e.m (two-way repeated measures ANOVA).

yield false-positive results due to changes in cell proliferation; to control for this we examined the protein levels of the proliferation marker Ki67 and found that they remained unchanged (supplementary material Fig. S3F). Overexpression of nestin inhibited invasion in the same assay (supplementary material Fig. S4A,B). Furthermore, downregulation and overexpression studies in A172 glioma (supplementary material Fig. S4C,D) and DU145 prostate cancer cells also showed that nestin inhibits invasion (supplementary material Fig. S4B,E).

The invasion assays (Fig. 7A–C; supplementary material Fig. S4) showed that nestin impedes invasion. We used a matrix-degradation assay to investigate whether the increased invasion in nestin-downregulated cells was a result of increased matrix proteolysis. Because pFAK localisation is altered in nestin-downregulated cells and it is known that FAK influences cell invasion (Chan et al., 2009; Hauck et al., 2002; Hsia et al., 2003), we combined nestin downregulation with FAK inhibition. The results of experiments in which nestin was downregulated or overexpressed clearly showed that nestin inhibits matrix degradation (Fig. 7D,E; supplementary material Fig. S4F). Although there were not statistically significant differences, increased matrix degradation by nestin-downregulated cells was dependent on FAK activity (Fig. 7D,E). We also showed that, in nestin-downregulated cells, FAK inhibition abrogated any increase in invasion (supplementary material Fig. S4G). In order to invade, cells produce structures known as invadopodia, which are cortactin- and actin-rich (Murphy and Courtneidge, 2011). Analysis of the number and size of invadopodia showed that neither nestin downregulation nor FAK inhibition had any effect on these parameters (supplementary material Fig. S4H,I). Proteolysis of the ECM is facilitated by the matrix metalloproteinases (MMP). Western blotting showed that there was no change in the amount of secreted MMP9 or MT1-MMP (also known as MMP14; data not shown).

Nestin downregulation increases the level of integrin protein at the cell membrane (Fig. 3) and modulates integrin clustering (Fig. 5). To determine whether the effect of nestin on invasion is a result of its effect on integrin activation, we combined inhibition of the function of integrins with the Matrigel–Boyden-chamber assay. Nestin-downregulated and control cells were incubated with specific antibodies to block integrin function. The antibodies used were against α5β1, α3β1 and αVβ3, with IgG included as an antibody control (Fig. 7F). Similar to the data shown in Fig. 7A–C, nestin downregulation significantly increased invasion in the IgG control. In control cells in which the function of any of the tested integrin heterodimers was blocked, there was no significant change in invasion. Nestin downregulation combined with the inhibition of integrin function significantly inhibited invasion for all integrins tested. These results imply that nestin modulates which integrin heterodimers are required for PC-3 cells to invade. To test this hypothesis further, invasion assays were performed with a combination of nestin and integrin β1 downregulation (Fig. 7G,H). The results showed that the increased invasion in nestin-downregulated cells is β1-dependent.

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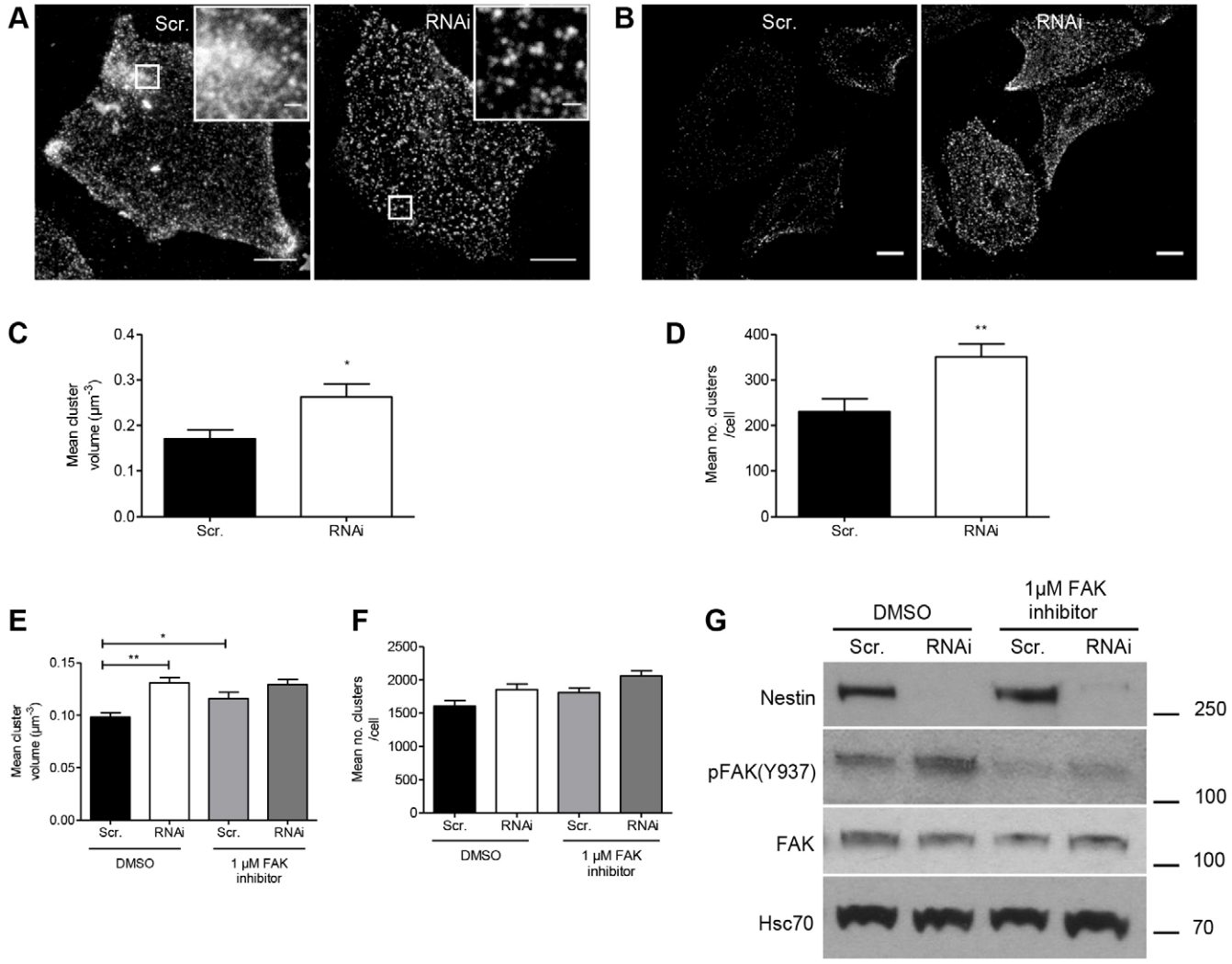

Fig. 5. Nestin modulates integrin clustering. PC-3 cells were transfected with either control (Scr.) or nestin siRNA (RNAi). (A) Nestin downregulation increases clustering of α5 integrin at the cell membrane. Transfected cells were incubated with an antibody recognising the extracellular domain of α5 integrin. Clustering of the α5 integrin receptors was achieved using an Alexa-Fluor-488-conjugated secondary antibody. Cells were then fixed and mounted, and imaged with confocal microscopy. (B) A representative confocal image of several cells showing that nestin downregulation increases clustering of α5 integrin. Scale bars: 10 μm. (C) Quantification of the mean cluster volume (μm^3) in Scr- and RNAi-treated cells. (D) Quantification of the mean number of clusters per cell in Scr- and RNAi-treated cells. (E) Quantification of the mean cluster volume (μm^3) in Scr- and RNAi-treated cells, showing the influence of a DMSO control and 1 μM FAK inhibitor II on cluster size. (F) Quantification of the mean number of clusters per cell in Scr- and RNAi-treated cells, showing the influence of a DMSO control and 1 μM FAK inhibitor II on cluster number. Data show the mean±s.e.m; *$P<0.05$, **$P<0.01$ [unpaired *t*-test, 20 cells per experiment, $n=3$ (for C and D); one-way ANOVA with Bonferroni post-test, 25 cells per experiment, $n=2$ (for E and F)]. (G) Western blot to show a representative example of the degree of inhibition of FAK activity as measured by the protein levels of phosphorylated (Y937) FAK compared with FAK.

DISCUSSION

Elevated expression of nestin in a number of different cancers is a well-known phenomenon, but only recently has the function of nestin in cancer gained more attention. Nestin has been implicated in cancer cell invasion and metastasis in pancreatic cancer, glioma and prostate cancer, and is a tentative marker for cancer progression (reviewed in Ishiwata et al., 2011a). It is expressed at the tumour invasion front in pancreatic cancer (Kawamoto et al., 2009) and was hypothesised to promote tumour invasion. To date, there is no evidence showing the mechanistic basis of the function of nestin in cancer migration.

This study provides novel data showing that nestin significantly influences the dynamics of key regulators of cell adhesion and motility. Initial observations showed that nestin downregulation caused relocalisation of pFAK to focal adhesions, indicating that more and larger focal adhesions are formed. FAK is known to positively regulate focal adhesion turnover and, as such, the targeting of pFAK to focal adhesions could lead to increased focal adhesion turnover as indicated by the faster recovery of VASP. VASP is a known negative regulator of cell motility (Bear et al., 2000), and it stabilises focal adhesions through its actin-related functions (Huttenlocher and Horwitz, 2011). Thus, if VASP turnover is faster, the focal adhesions are less stable, which could contribute to the increased invasion of nestin-downregulated PC-3 cells.

A previous study has shown that FAK localisation regulates cell invasion (Hsia et al., 2003). We hypothesised that nestin could affect FAK directly, in the same way that synemin, an intermediate filament protein that is structurally similar to nestin, binds to zyxin, thereby affecting its localisation at focal adhesions

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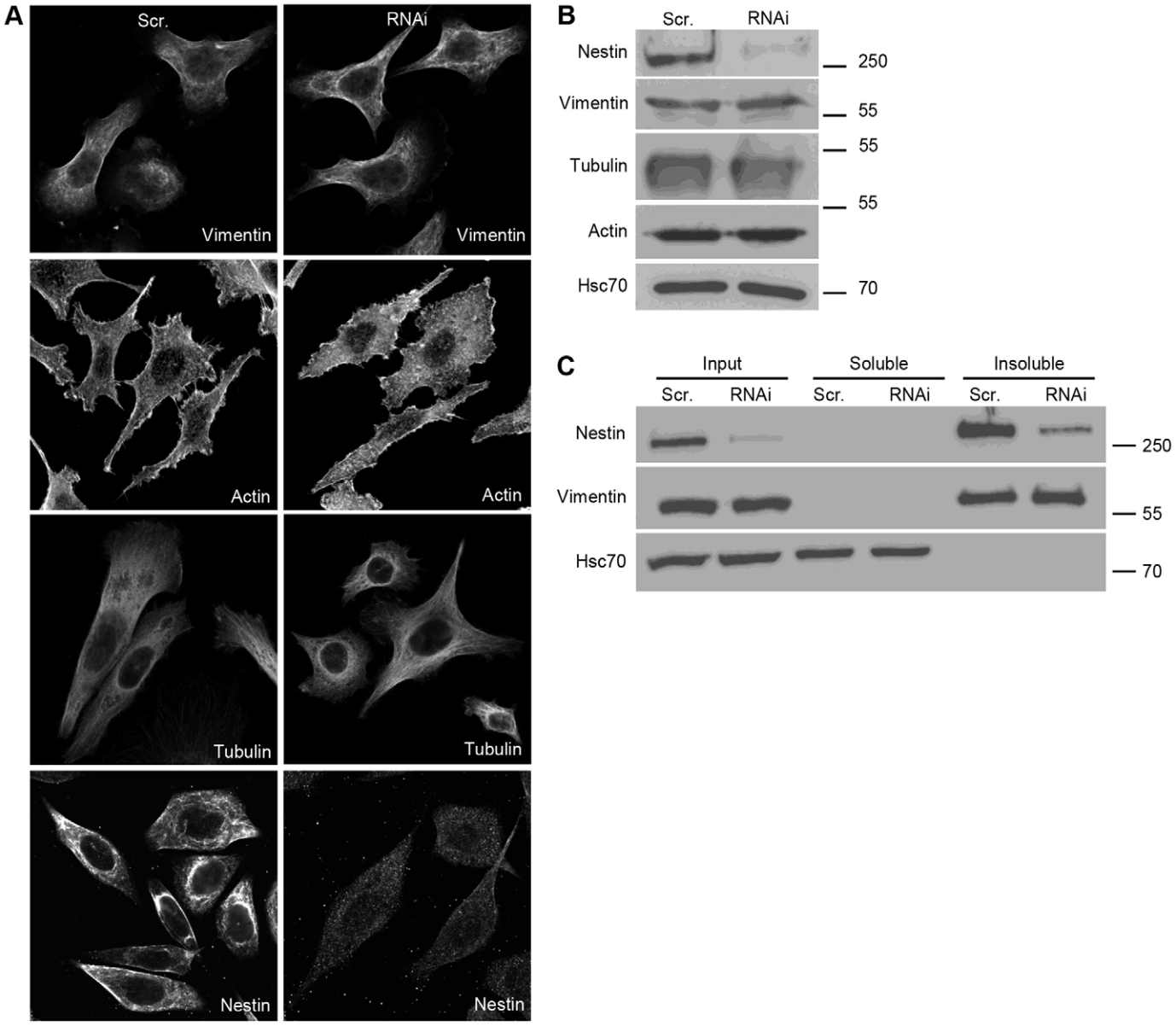

Fig. 6. Nestin downregulation does not affect other components of the cytoskeleton. PC-3 cells were transfected with either control (Scr.) or nestin siRNA (RNAi). (A) Nestin downregulation does not affect cytoskeleton organisation. Representative images from three independent experiments. The images of individual protein labels are 8-bit greyscale maximum-projection confocal images. Cells were labelled with vimentin, phalloidin (actin) and tubulin. Nestin-labelled images indicate the degree of downregulation achieved. Scale bars: 20 μm. (B) Nestin downregulation does not affect cytoskeletal protein levels. Representative western blots of vimentin, tubulin or actin compared to the Hsc70 loading control. (C) Nestin downregulation does not affect the solubility and therefore assembly of vimentin. Cells were separated into detergent soluble and insoluble fractions. Filamentous nestin and vimentin appear as part of the insoluble fraction. Hsc70 was used to indicate the soluble fraction.

(Sun et al., 2010). Nestin has been shown to have the propensity to act as a cytoskeletal signalling regulator, as it regulates Cdk5, with consequences for Cdk5 activation and localisation in neural progenitor cells, myoblasts and at the neuromuscular junctions (Mohseni et al., 2011; Pallari et al., 2011; Sahlgren et al., 2006; Yang et al., 2011). In agreement with these reports, our current results suggest that nestin also has the capacity to act as a cytoskeletal regulator of FAK organisation and activity.

Related to its effects on FAK, nestin was shown to direct integrin organisation and turnover. Although nestin did not affect the protein levels of integrins in the cell, it affected the amount of integrin α5 and active integrin β1 at the cell membrane, implying that nestin regulates integrin activation at the cell membrane. Nestin also regulated the formation of α5β1 clusters, in a FAK-related fashion, as FAK activity regulated cluster volume in control cells, but had no effect on cluster size or number in nestin-downregulated cells. A recent paper describing a spatial model of integrin clustering (Welf et al., 2012) has shown that a small pool of active integrin is required to initiate integrin clustering, which triggers a positive-feedback loop to increase integrin binding and activation. In this context, it is plausible that the increase in integrin activity as a result of nestin downregulation could trigger

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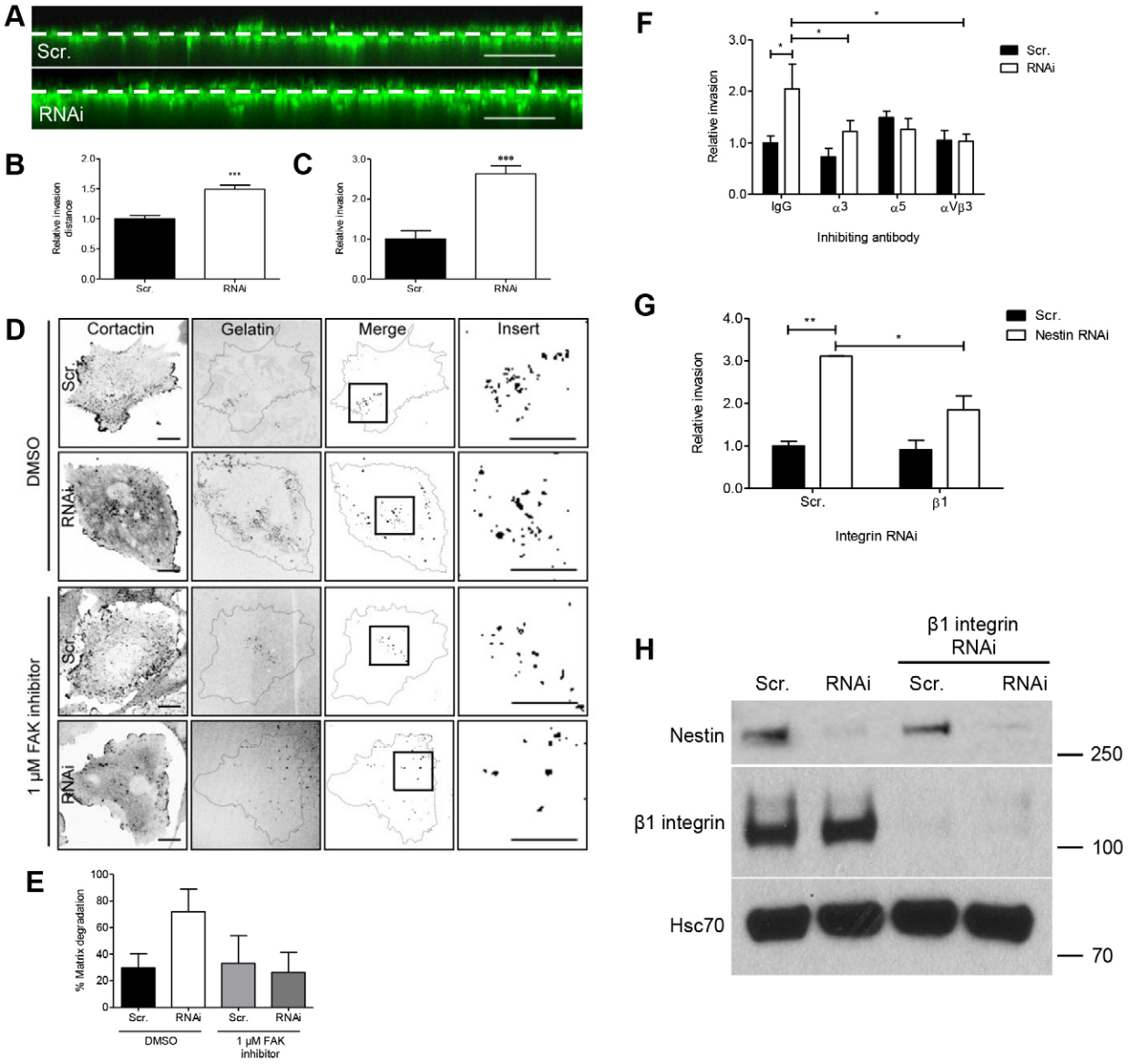

Fig. 7. Nestin downregulation inhibits matrix degradation in a FAK-dependent manner and increases the integrin-dependent invasion of PC-3 cells. PC-3 cells were transfected with either control (Scr.) or nestin siRNA (RNAi). Image analysis was performed using ImageJ. (A) Nestin downregulation increases the invasion of PC-3 cells in an inverted invasion assay. RNAi- and Scr-transfected PC-3 cells invaded a thick Matrigel–fibronectin matrix for 6 days. PC-3 cells were fixed and labelled for F-actin with phalloidin (green) and viewed by confocal microscopy. The maximum *z*-projection shows the degree to which cells invaded the 3D Matrigel–fibronectin matrix. White dashed line, the position of the membrane. Scale bars: 100 μm. (B) Quantification of the relative distance invaded by the Scr- and RNAi-treated cells into the 3D Matrigel–fibronectin matrix from eight randomly chosen fields in each of three different experiments equivalent to those shown in A. Distance invaded from the membrane is represented as a fold change relative to Scr., $n=3$. (C) Nestin downregulation increases invasion in the Matrigel–Boyden-chamber assay. PC-3 cells invaded through the Matrigel layer for 24 hours. Invaded cells were stained with Crystal Violet, and the number of cells that had invaded through the membrane was counted manually. Quantification indicates the relative invasion of the RNAi cells compared to Scr., $n=6$. (D) Nestin downregulation increases matrix degradation. Matrix degradation in Scr or RNAi cells was measured using a fluorescent-gelatin-degradation assay. Cells were plated on fluorescently conjugated gelatin and allowed to adhere for 24 hours in the presence of 1 μM FAK inhibitor II or a DMSO control. The cells were labelled with cortactin, an invadopodia marker. Dashed grey line, the cell periphery. Merge image shows the areas where cortactin labelling overlaps areas of matrix degradation, indicating the presence of active invadopodia. Inserts show a magnified area of the image, indicated by the black square on the merged image. Scale bars: 10 μm. (E) Increased matrix degradation by nestin-downregulated cells is FAK-activity dependent. Quantification of the amount of degraded matrix compared to the area covered by the cells, $n=3$. (F) Nestin downregulation alters the integrins required by PC-3 cells for invasion in the Matrigel–Boyden-chamber model. Detached cells were pre-treated for 30 minutes with integrin-function-blocking antibodies, as indicated, before invading the Matrigel layer for 24 hours. Cells that invaded were fixed and stained with Crystal Violet, and the number of cells that invaded through the membrane was counted manually. Quantification indicates the normalised invasion of the integrin-antibody-treated cells compared to the Scr-IgG control, $n=4$. (G) Increase in nestin-downregulation-mediated invasion is β1 integrin dependent. Cells were double transfected (total 150 nM siRNA) with: control siRNA + control siRNA, control siRNA + nestin siRNA, β1 integrin siRNA + control siRNA or β1 integrin siRNA + nestin siRNA for 48 hours prior to the assay. Cells invaded the Matrigel layer in a Matrigel–Boyden chamber for 24 hours. Cells that invaded were fixed and stained with Crystal Violet, and the number of cells that invaded through the membrane was counted manually. Quantification indicates the normalised invasion of the RNAi-treated cells compared to the control, $n=2$. (H) Representative western blot to show the level of β1-integrin-downregulation in the invasion assays. Quantitative data show the mean±s.e.m.; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. [paired *t*-test (for B and C); one-way ANOVA with Bonferroni correction (for E–G)].

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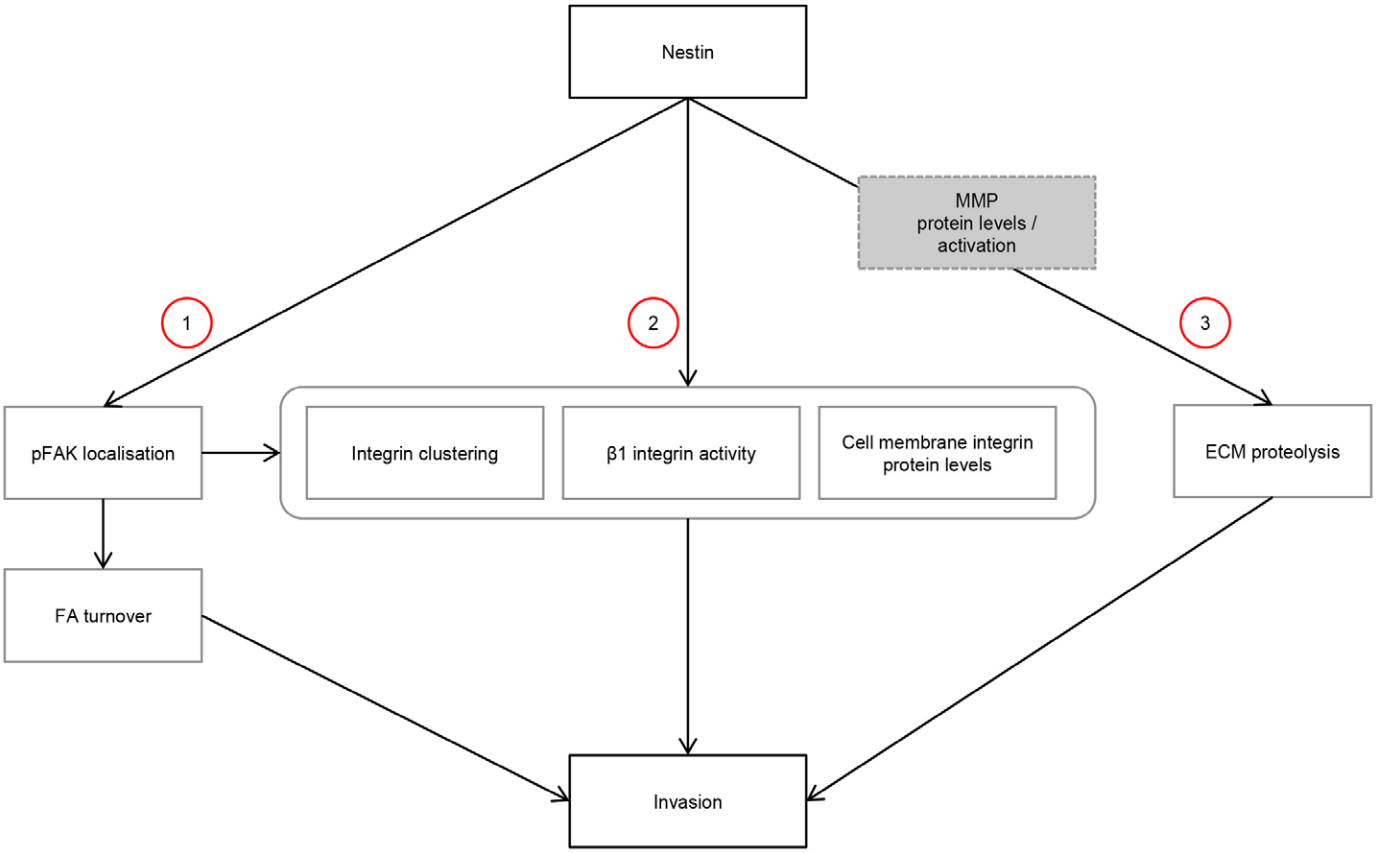

Fig. 8. Nestin regulates pFAK localisation, integrin cell membrane dynamics and ECM proteolysis with consequences for cell invasion. Nestin influences cell invasion by three pathways. In pathway 1, when nestin is downregulated in PC-3 cells, pFAK localisation is altered, which impacts on focal adhesion (FA) turnover. In pathway 2, nestin downregulation increases integrin clustering, integrin activation and integrin protein levels at the cell membrane. The box outlined in grey in pathway 2 is used to group the integrin functions together. In pathway 3, nestin downregulation might also influence MMP activation and organisation at the cell membrane, with consequences for ECM proteolysis.

a positive-feedback loop that would, in turn, increase integrin clustering. Clustering is a requirement for integrin signalling, and the degree of clustering achieved will affect the strength and type of the downstream signal (Comisar et al., 2011; Miyamoto et al., 1995; Yauch et al., 1997). Changes in the clustering of integrin when nestin is downregulated could be explained by changes in the recruitment of pFAK to the site of integrin activation. This might explain why we see alterations in the localisation of pFAK in nestin-downregulated cells. Integrin clustering is considered to be the initial step in cell adhesion, and it stimulates the recruitment of adaptor proteins (such as talin, FAK and possibly other kinases) to the integrin tails to form focal adhesions. We did not detect any global changes in FAK activation or in activation of Src, which is downstream of FAK. The function of FAK is spatially rather than globally regulated (Cai et al., 2008; Casaletto and McClatchey, 2012). Consequently, we think that the spatial localisation of active FAK, and possibly of Src, rather than global changes in cell signalling, is more important for nestin-mediated effects on integrin activation, clustering and invasion.

Additionally, we showed that nestin downregulation alters the integrins required for invasion and that invasion is dependent on β1 integrin and the activity of α3, α5 and αVβ3 integrins. β1 integrin activity positively correlates with cell invasion in PC-3 cells (Pellinen et al., 2012). Integrin organisation at the cell membrane is also correlated with cell invasion (Van Slambrouck et al., 2009). Different classes of integrin heterodimers at the cell membrane might have specific effects on focal adhesion formation, with consequences for cell motility (Schiller et al., 2013). Thus, a nestin-mediated change in integrin composition and activity could conceivably impact on cell invasion. If nestin is regulating integrin clustering and organisation at the cell membrane this could lead to spatial and temporal changes in downstream signalling, with consequences for cell invasion.

Translocation of pFAK, increased integrin clustering and levels of integrin at the cell membrane suggested that cell adhesion and motility would be altered in nestin-downregulated cells. Interestingly, cell motility was unaffected. Instead, nestin downregulation significantly increased invasion and proteolysis of the ECM, which is a requirement for successful invasion. We could not observe any marked effects on MMP protein levels (data not shown), but MMP-regulated proteolysis depends not only on MMP protein levels, but also on MMP activity, which can be regulated in concert with integrin turnover (Gálvez et al., 2002; Shi and Sottile, 2011; Stefanidakis and Koivunen, 2006). The interrelationship between nestin, integrin turnover and localisation, MMP activity and ECM proteolysis provides a potential future avenue to investigate when dissecting the nestin-mediated effects on invasion. We have summarised our findings in Fig. 8.

Although our study presents nestin as an inhibitor of invasion, some studies indicate that nestin is a positive regulator of migration and metastasis (Ishiwata et al., 2011b; Kleeberger et al., 2007; Matsuda et al., 2011). Nestin might have contrasting effects on cell function depending on the cell type and transformation stage. During myogenesis, nestin is an inhibitor of differentiation (Pallari et al., 2011). In the adult, nestin has a role in promoting regeneration (Vaittinen et al., 2001), which involves acquisition of a motile phenotype. In acinar-ductal metaplasia, nestin expression was correlated with the accumulation of cells at an intermediate stage of metaplasia, implying that nestin inhibits progress through metaplasia (Miyamoto et al., 2003). It is also known that nestin positively regulates cell survival (Sahlgren et al., 2006). It might be the case that, although our study addresses the short-term effects of nestin downregulation, clearly pointing towards an inhibitory role, upon prolonged tumorigenesis the presence of nestin might positively affect invasiveness, as nestin would promote the survival of the tumour cells. Hence, there might be a positive selection among the surviving tumour cells for invading cells to overcome the inhibitory effect of nestin. In this respect, certainly the balance between nestin and vimentin would be a factor to take into account, as numerous studies have shown that vimentin promotes invasion. The upregulation of vimentin could overcome the inhibitory effect of nestin, the survival-promoting effect of which could still give a selection advantage for the tumour cells. As indicated by this and previous reports on nestin functions in cancer, nestin clearly has a multi-functional role in invasion and migration. This work provides novel data on how nestin might regulate cell invasion by regulating spatial FAK activity, integrin cell membrane localisation and dynamics, and ECM proteolysis. Future studies will expose the molecular details underlying these functions and how they are employed in different physiological and pathophysiological contexts.

MATERIALS AND METHODS

Cell lines and culture

The androgen-independent prostate cancer PC-3 cell line (DSMZ, Germany) was used. PC-3 cells were cultured in RPMI (Sigma-Aldrich, St Louis, MO) with 10% fetal calf serum (FCS), L-glutamine, penicillin and streptomycin at 37℃ under a 5% CO_2 humidified atmosphere until passage 15–18.

Transfection and FAK inhibition

Nestin was downregulated by transfection of a pool of four FlexiTube siRNA oligos (100 nM, Qiagen, Germany) against human nestin. β1 integrin was downregulated by using 50 nM custom-ordered siRNA (Eurofins MWG Operon, Germany) with the following sequence: 5′-AAUGUAACCAACCGUAGCA-3′. Allstars Negative Control siRNA (Qiagen) was used as a control. Fluorescently tagged siRNAs were custom ordered (Eurofins MWG Operon), and the sequences used were identical to the Qiagen siRNA pool. siRNA transfections were performed using Lipofectamine RNAiMAX (Invitrogen, UK). GFP and Nes640–GFP were as used previously (Pallari et al., 2011). GFP–zyxin and GFP–VASP were kind gifts from Maddy Parsons (King's College London, UK) (Worth et al., 2010). Lipofectamine LTX was used as the plasmid transfection reagent. Co-transfections were performed so that first the siRNA was transfected and, 6 hours later, plasmid DNA transfections were performed. The medium was changed the following day. All assays were performed at 48–72 hours post-transfection. FAK was inhibited for 24 hours prior to and during the relevant assays using 1 µM FAK Inhibitor II (Millipore, Billerica, MA), and DMSO was used as a control.

Immunofluorescence

Cells were fixed with 3.7% paraformaldehyde (PFA) for 10 minutes, permeabilised in phosphate-buffered saline (PBS) with 0.1% Triton X-100 and blocked for 1 hour in 1% bovine serum albumin (BSA) in PBS. The antibodies used are listed in supplementary material Table S1.

Confocal microscopy

Confocal microscopy was performed with a Leica SP5 confocal microscope with 63× magnification, 2048×2048 pixels. *Z*-stacks were taken according to Nyquist's Theorem for optimal sampling. All images were imported into ImageJ and Fiji, only linear adjustments were made to brightness and contrast. For focal adhesion images, *z*-stack projections were made between four and ten stacks from the bottom half of the cell. Otherwise, *z*-stack projections were made from *z*-stacks from the entire cell.

SDS-PAGE and western blotting

Whole-cell lysates were prepared by lysing cells in Laemmli buffer containing β-mercaptoethanol. Proteins were resolved using SDS-PAGE and then transferred to either nitrocellulose or PVDF membrane using wet transfer. Antibodies are listed in supplementary material Table S1. Blots were quantified using ImageJ.

FRAP analysis

RNAi and plasmid co-transfected cells were plated on 10 µg/ml fibronectin-coated live cell imaging dishes (MatTek, Ashland, MA) and allowed to adhere for 24–36 hours. The medium was replaced with Leibovitz L-15 (Invitrogen, UK) with 10% FCS before the experiment. FRAP experiments were performed using the Zeiss LSM780 confocal microscope. Cells were first imaged with a 543-nm laser to visualise siRNA transfection and with a 488-nm laser to visualise focal adhesions. Photobleaching of the focal adhesions was performed with the 488-nm laser at 100%, with 20 iterations. Recovery was followed with low laser power every second until the intensity had reached a steady plateau. For each timepoint the bleached area intensity was normalised to a corresponding unbleached area and background signal. The recovery signal was fitted with the ImageJ FRAP analyzer.

Flow cytometry

For integrin cell membrane expression analysis, cells were fixed with 3.7% PFA, washed with Tyrode's buffer and incubated with primary and then either Alexa-Fluor-488- or Alexa-Fluor-633-conjugated secondary antibodies. Antibodies are listed in supplementary material Table S1. The mean fluorescent intensity of 10^4 cells was analysed by using BD FacsCalibur and BD CellQuest software (BD Biosciences, San Jose, CA).

To measure the level of activated integrins over time, transfected cells were plated onto plates coated with 5 µg/ml human fibronectin (Gibco, Invitrogen, Carlsbad, CA) and collected over the indicated timepoints. Collected cells were fixed in 3.7% PFA and processed for flow cytometry analysis as described above.

Integrin recycling and trafficking assays

The integrin recycling and trafficking assays were performed as described previously (Arjonen et al., 2012; Ivaska et al., 2002; Roberts et al., 2001). To measure endocytosis as opposed to net trafficking, recycling was blocked with 0.6 nM primaquine bisphosphate (Sigma-Aldrich).

Integrin degradation assay

Serum-starved cells were cell-membrane labelled with 0.5 mg/ml EZ link-sulfo-NHS-SS biotin (Pierce, Thermo Scientific, Rockford, IL) in Hank's balanced salt solution on ice for 30 minutes. Cells were washed once with sterile PBS and then incubated for 0 hours, 12 hours and 18 hours. Samples were lysed and analysed by using an enzyme-linked immunosorbent assay (ELISA) as described for the integrin recycling assay.

Integrin clustering assay

Integrin clustering assay was performed as described previously (Jokinen et al., 2010) using anti-integrin-α5 (supplementary material Table S1) and Alexa-Fluor-488-conjugated mouse IgG antibodies (Invitrogen, UK). Cells were fixed in 3.7% PFA and mounted with Mowiol. Images were

taken with a Leica SP5 confocal microscope with a 63× oil objective. *Z*-stacks were collected randomly. To quantify cluster number, volume and intensity per cell, images were thresholded, segmented and analysed using BioImageXD (Kankaanpää et al., 2012).

Intermediate filament solubility assay

Soluble and insoluble intermediate filament fractions were separated as described previously (Sahlgren et al., 2006).

In vitro migration assay

Cells were serum starved overnight in serum-free medium. A total of 5×10^4 cells in complete medium were added to the top chamber of an uncoated 8.0-µm-pore transwell insert (Corning, NY), medium containing 10 ng/ml human stromal-derived factor 1α (SDF1-α, Peprotech, Rocky Hill, NJ) was added to the bottom chamber and cells were allowed to migrate for 24 hours. Cells that had not migrated were wiped from the top of the chamber. The chambers were fixed with 3.7% PFA and stained with 0.1% Crystal Violet (Reagecon, Ireland). The number of cells was counted manually from edge to edge using a Leica DMIL inverted light microscope with 20× magnification.

In vitro wound healing assay

Confluent monolayers were wounded by scratching a pipette tip from the top to the bottom of the well. Images were taken of the whole length of the wound at the indicated timepoints using an Olympus GK2 inverted microscope coupled to a digital camera (Canon PowerShot A510), and were analysed with PSRemote (Canon).

Random motility assay

Cells were imaged on glass-bottomed cell culture dishes (MatTek), and widefield images were taken every 10 minutes for 250 minutes using a Leica SP5 confocal microscope and a 10× air objective. The videos were analysed for individual cell speed, directionality and persistence using ImageJ with the MtrackJ plugin (Meijering et al., 2012). A total of 20 cells per video were analysed with three independent repeats.

Inverted invasion assay

Transfected cells were serum starved overnight in serum-free medium. The inverted invasion assay was performed as described previously (Caswell et al., 2007), except that the Matrigel was combined with 250 nM nestin siRNA (Qiagen FlexiTube siRNA) and Lipofectamine RNAiMAX (Invitrogen) before polymerisation. Cells were allowed to invade for 6 days, and the medium was changed every 2 days. To maintain the downregulation of nestin, 100 nM siRNA with Lipofectamine RNAiMAX was added during medium changes. Inserts were fixed with 3.7% PFA, permeabilised (with 3% Triton X-100, 2 mM $MgCl_2$ and 5 mM EGTA in PBS) and labelled with phalloidin. Inserts were visualised with a Leica SP5 Matrix, with *z*-intervals of 1.8 µm, 20× magnification, 1.0 optical zoom, 1048×1048 pixel resolution from eight randomly chosen fields. The distance invaded was measured using BioImage XD (Kankaanpää et al., 2012).

In vitro invasion assay

The *in vitro* invasion assay was performed similarly to the migration assay; however, cells migrated towards a 10% serum gradient for 24 hours. The number of cells was counted manually from the whole chamber using a Leica DMIL inverted light microscope with 20× magnification.

Integrin-function-blocking invasion assay

To block integrin function, cells were briefly serum starved and then the appropriate antibody was incubated with the cells for 30 minutes at 37℃, and the Matrigel–Boyden-chamber invasion assay was performed as described above, with the exception that the integrin-function-blocking antibodies were added to the medium in the upper chamber. The antibodies used are listed in supplementary material Table S1.

Fluorescent gelatin degradation assay

Eight-well µ-Slides (Ibidi, Germany) coated with cross-linked fluorophore (Cy3)-conjugated gelatin matrix (Millipore) were prepared according to the manufacturer's protocol. Cells transfected with Cy3-tagged nestin siRNAs and control siRNAs were cultured for 24 hours in the presence of 1 µM FAK inhibitor II (Millipore) or DMSO prior to the assay. siRNA, GFP and Nes640–GFP transfected cells were plated onto the gelatin and allowed to degrade the matrix for 24 hours. Cells were fixed with 3.7% PFA and labelled for cortactin. Gelatin matrix and cortactin-labelled PC-3 cells were imaged with a Zeiss LSM780 confocal microscope. Large 4×4 tile images were captured with a 10× objective to analyse the total degradation of the matrix by cells. Single-cell images were captured to study the invadopodia of single cells. Images were analysed using ImageJ.

Statistical analysis

Statistical significance was determined with Graphpad Prism 5 (GraphPad Software, La Jolla, CA). The relevant tests used are indicated in the figure legends.

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Competing interests

The authors declare no competing interests.

Author contributions

C.L.H. planned the experiments under the guidance of J.E.E., performed the majority of experiments with help from G.L., J.W.P., M.W.G.R. and S.M.Q., generated the figures with help from J.W.P. C.L.H., and wrote the article with J.E.E.

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Supplementary material

Supplementary material available online at
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Supplementary Figure 1: Nestin downregulation in PC-3 cells and effect on FA protein localisation

PC-3 cells were transfected with either control (Scr.) or nestin siRNA (RNAi). Whole cell laemmli lysate samples were taken from every experiment conducted in this study and checked for their level of nestin downregulation. Error bars indicate s.e.m.

A. Representative western blot to show the level of nestin downregulation achieved in this study compared to an Hsc70 loading control.

B. Quantification from representative western blots of nestin downregulation from experiments in this study. The results were analysed using an unpaired t-test, n=22.

C. Nestin-downregulation affects localisation of pFAK at the cell periphery and the formation of distinct clusters of pFAK. Cells were co-labelled for pFAK (green) and vinculin (magenta) in order to identify if the clusters of pFAK are focal complexes. There is some co-localisation of pFAK clusters and vinculin, a marker of FA.

D. Nestin-downregulation affects localisation pFAK at the cell periphery and the formation of distinct clusters of pFAK. Cells were co-labelled for pFAK (green) and talin (magenta) in order to identify if the clusters of pFAK are focal complexes. There is some co-localisation of pFAK clusters and talin, another marker of FA.

Supplementary Figure 2: Representative flow cytometry histograms showing the effect of nestin downregulation on the cell membrane labelling of integrins and total integrin protein level

Representative histograms of the flow cytometry data of control (light black lines) and RNAi cells (thick black lines) analysed in Figure 2. The X-axis (FL-1) represents the fluorescence on a logarithmic scale and the Y-axis indicates the number cells counted, each tick mark represents 20 cells. Quantification of western blot films was done using ImageJ and the results were analysed using an unpaired t-test.

A. Representative histograms of the flow cytometry data analysed in Figure 3B to show that nestin-downregulation increases CM levels of α5 integrin in actively adhering and spreading cells.

B. Representative histograms of the flow cytometry data analysed in Figure 3E to show that nestin-downregulation modulates CM levels of active β1 integrin in actively adhering and spreading cells.

C. Representative histograms of the flow cytometry data analysed in Figure 3F to show that nestin-downregulation does not affect total CM levels of β1 integrin in actively adhering and spreading cells.

D. Representative western blot to show that the protein levels of integrins β1 and α5 do not change upon nestin-downregulation compared to the Hsc70 loading control

E. Quantification of the total protein levels of α5 integrin, n=5.

F. Quantification of the total protein levels of β1 integrin, n=5.

Supplementary Figure 3: Nestin does not affect migration of PC-3 cells

PC-3 cells were transfected with either control (scr.), nestin siRNA (RNAi), as indicated. Error bars indicate s.e.m.

A. Nestin-downregulation does not affect chemotactic migration of PC-3 cells towards SDF-1α. Cells were allowed to invade through an uncoated boyden chamber for 24 hours towards SDF-1α. Migrated cells were fixed and stained with crystal violet and number of cells migrated through a cross-section of the whole membrane was counted manually. Quantification indicates the relative migration of the nestin RNAi cells compared to the control. The results were analysed using the paired t-test, n=3.

B. Nestin-downregulation does not affect the wound healing of PC-3 cells. Cells were grown as a monolayer and wounds were made into the monolayer with a pipette tip. The closure of the wound was monitored over a 24 hour period. The images of the wounds are representative, n=3.

C. Live cell tracking shows that nestin-downregulation does not affect the random migration of PC- 3 cells. Plots of the tracks of 12 randomly selected cells from 3 independent experiments. Cells were tracked for 4.5 hours and wide-field images were obtained every 10 minutes. Data analysis and tracking was done using ImageJ mTrackJ plugin. The intersection of the X and Y axis is the starting point for each cell. The axis values are in μM.

D. Quantification and analysis of the mean velocity of the tracked cells. The results were analysed using an unpaired t-test on 60 cells from 3 independent experiments.

E. Quantification and analysis of the directional persistence of the tracked cells. Directional persistence is a measure of much a cell will persist in travelling in a particular direction. It is able differentiate cells which are motile but remain within a small area to those which are motile and travel further distances from their starting point. The measure of directional persistence is acquired by dividing the furthest distance travelled from the starting point (D) by the total distance travelled (T) by the cell. The closer the value is to one, the more directionally persistent a cell is. The results were analysed using an unpaired t-test on 60 cells from 3 independent experiments.

F. Representative western blot to show that the protein levels of the proliferation marker Ki67 do not change upon nestin downregulation compared to an Hsc70 loading control.

Supplementary Figure 4: Nestin is an inhibitor of invasion and matrix degradation

PC-3 cells were transfected with either GFP control (GFP), nestin 640 GFP-tagged overexpression vactor (nestin640-GFP), control (Scr.) or nestin siRNA (RNAi), as indicated. Error bars indicate s.e.m. Result F was analysed using a paired t-test. Results G-I were analysed using a 2-way ANOVA with Bonferroni correction.

A. Overexpression of nestin inhibits invasion of PC-3 cells in the matrigel boyden chamber assay. PC-3 cells transfected with either the GFP or nestin640-GFP were allowed to invade through the matrigel layer for 24 hours. Invaded cells were stained with crystal violet and number of cells invaded through the whole membrane was counted manually. Quantification indicates the relative invasion of the nestin640-GFP cells compared to the GFP control, n=6.

B. Representative western blot to show to level of nestin overexpression in the invasion assays.

C. Downregulation of nestin inhibits invasion of A172 glioma cells in the matrigel boyden chamber assay. A172 cells transfected with either Scr. or RNAi were allowed to invade through the matrigel layer for 24 hours. Invaded cells were stained with crystal violet and number of cells invaded through the whole membrane was counted manually. Quantification indicates the relative invasion of the RNAi cells compared to the control, n=3.

D. Representative western blot to show to level of nestin downregulation in the invasion assays.

E. Overexpression of nestin inhibits invasion of DU145 prostate cancer cells in the matrigel boyden chamber assay. DU154 cells transfected with either the GFP or nestin640-GFP were allowed to invade through the matrigel layer for 24 hours. Invaded cells were stained with crystal violet and number of cells invaded through the whole membrane was counted manually. Quantification indicates the relative invasion of the nestin640-GFP cells compared to the control, n=2.

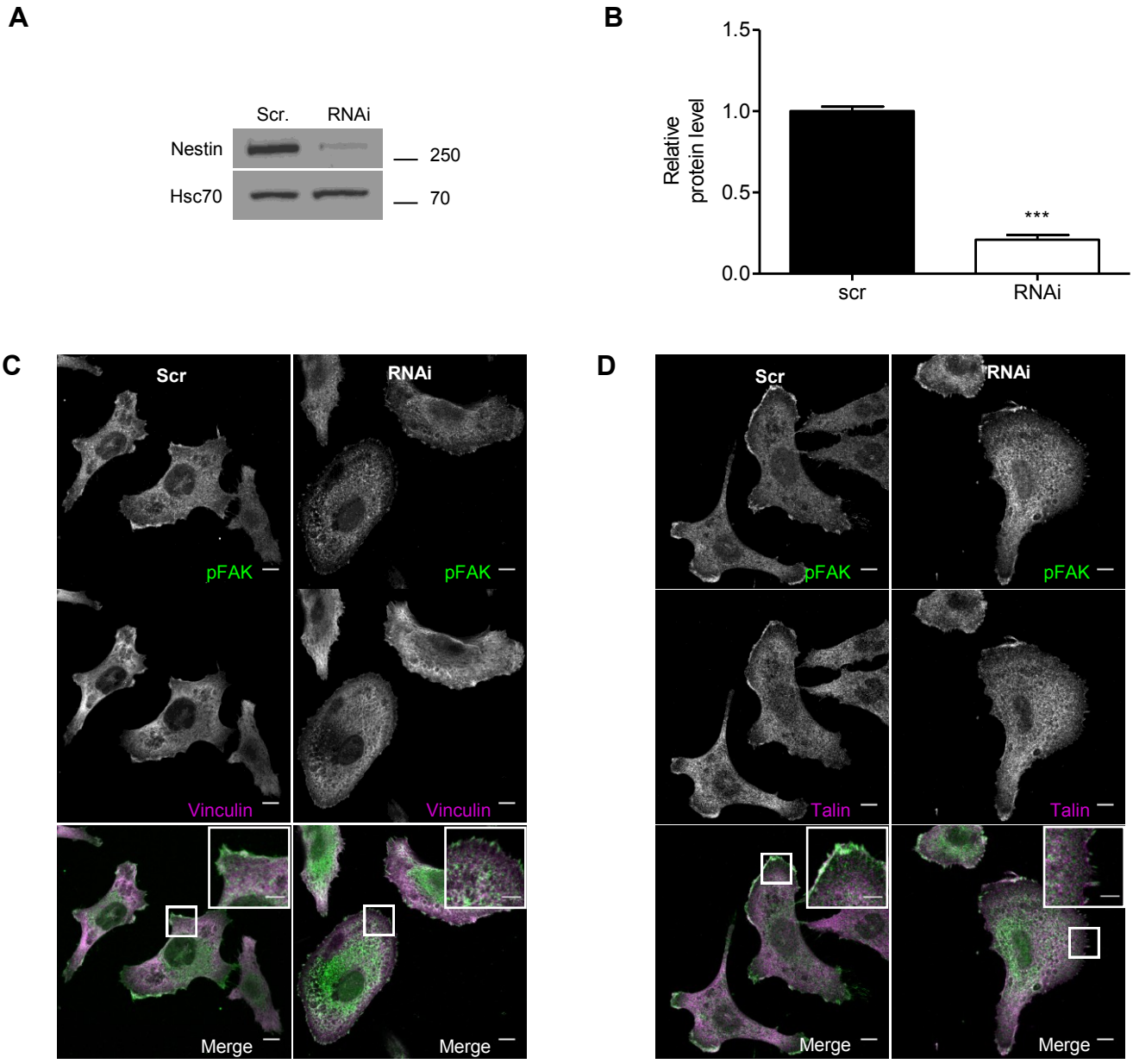
F. Nestin overexpression inhibits matrix degradation. Matrix degradation in GFP or Nes640-GFP cells using a fluorescent gelatin degradation assay. Cells were plated on fluorescently-conjugated gelatin and allowed to adhere for 24 hours. The cells were labelled with cortactin, an invadopodia marker. Images were analysed in the same way as for Fig. 7D. Increased matrix degradation by nestin-downregulated cells is FAK activity dependent. Quantification of the amount of degraded matrix compared to the area covered by the cells, n=4.

G. Inhibition of FAK abrogates the increase of invasion in nestin downregulated cells. PC-3 cells transfected with either scr. or RNAi were allowed to invade through the matrigel layer for 24 hours. Invaded cells were stained with crystal violet and number of cells invaded through the whole membrane was counted manually. Quantification indicates the relative invasion of the RNAi cells compared to the control, n=1.

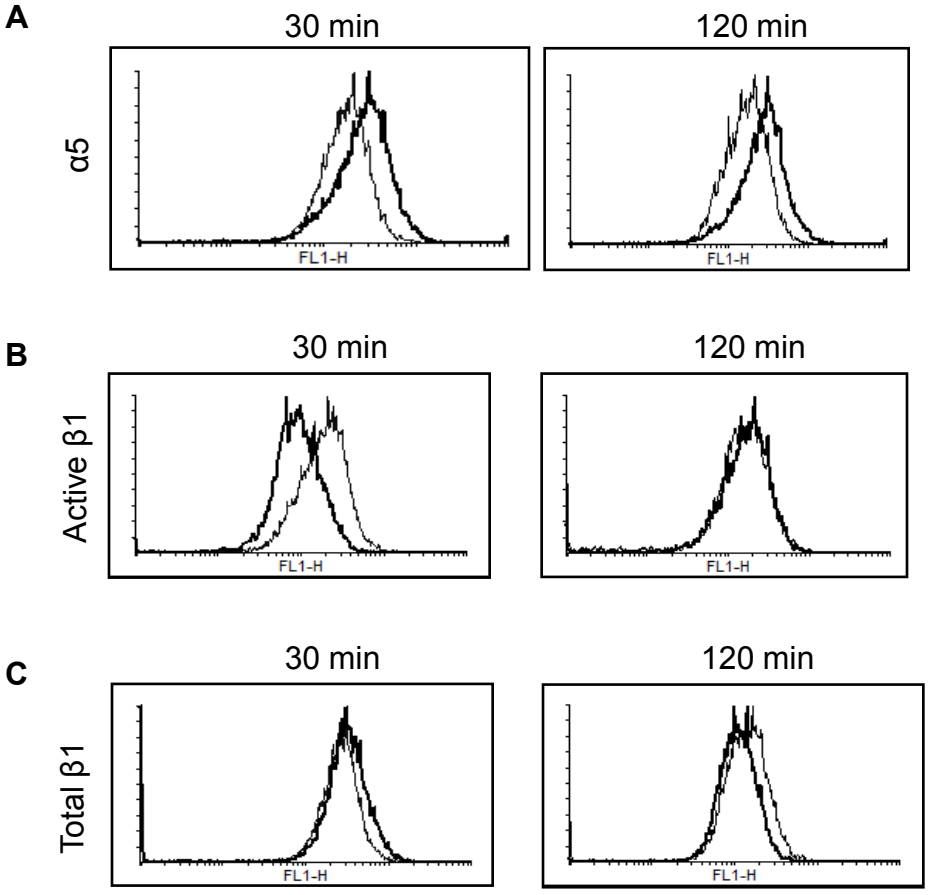
H. Nestin does not affect the number of invadopodia produced by PC-3 cells. Detailed analysis of number of invadopodia as characterized by overlap of cortactin and degraded matrix, n=3.

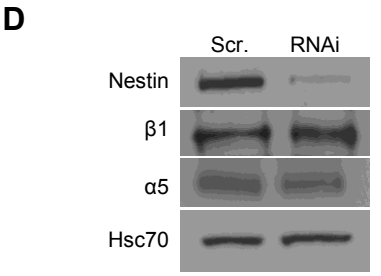
I. Nestin does not affect the size of invadopodia produced by PC-3 cells. The mean area of individual invadopodia (μm^2) was calculated from the total area covered by invadopodia by the number of invadopodia per cell, n=3.

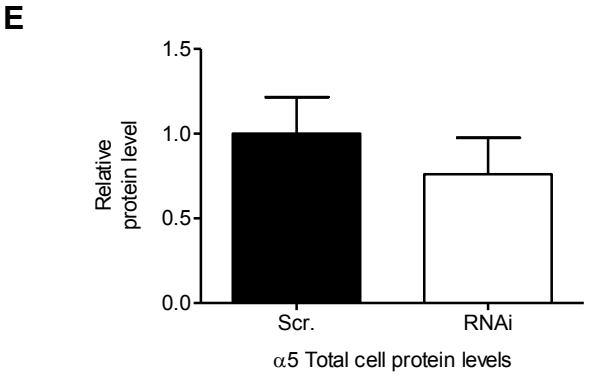
Supplementary Figure 1: Nestin downregulation in PC-3 cells and effect on FA protein localisation

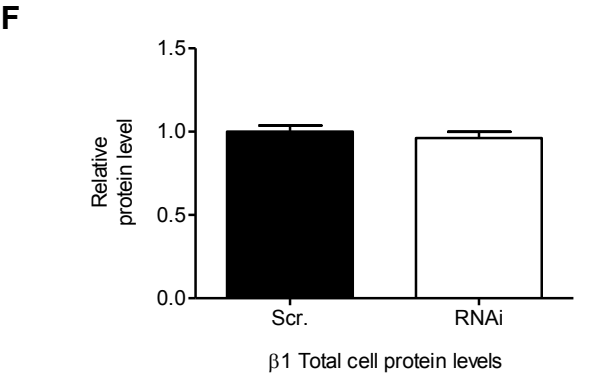


Supplementary Figure 2: Representative flow cytometry histograms and western blot quantification showing the effect of downregulation on the surface labelling of integrins and total integrin protein level

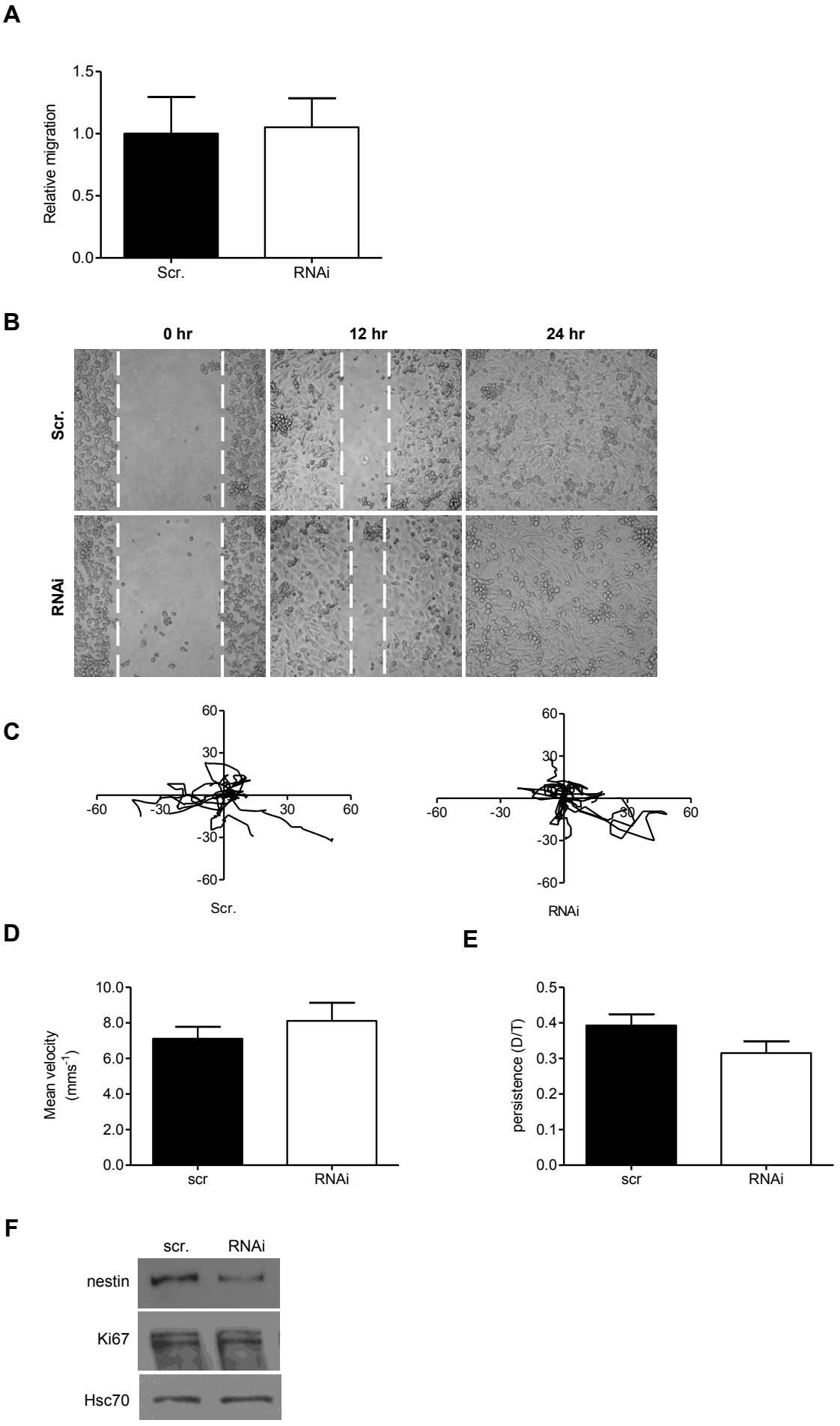




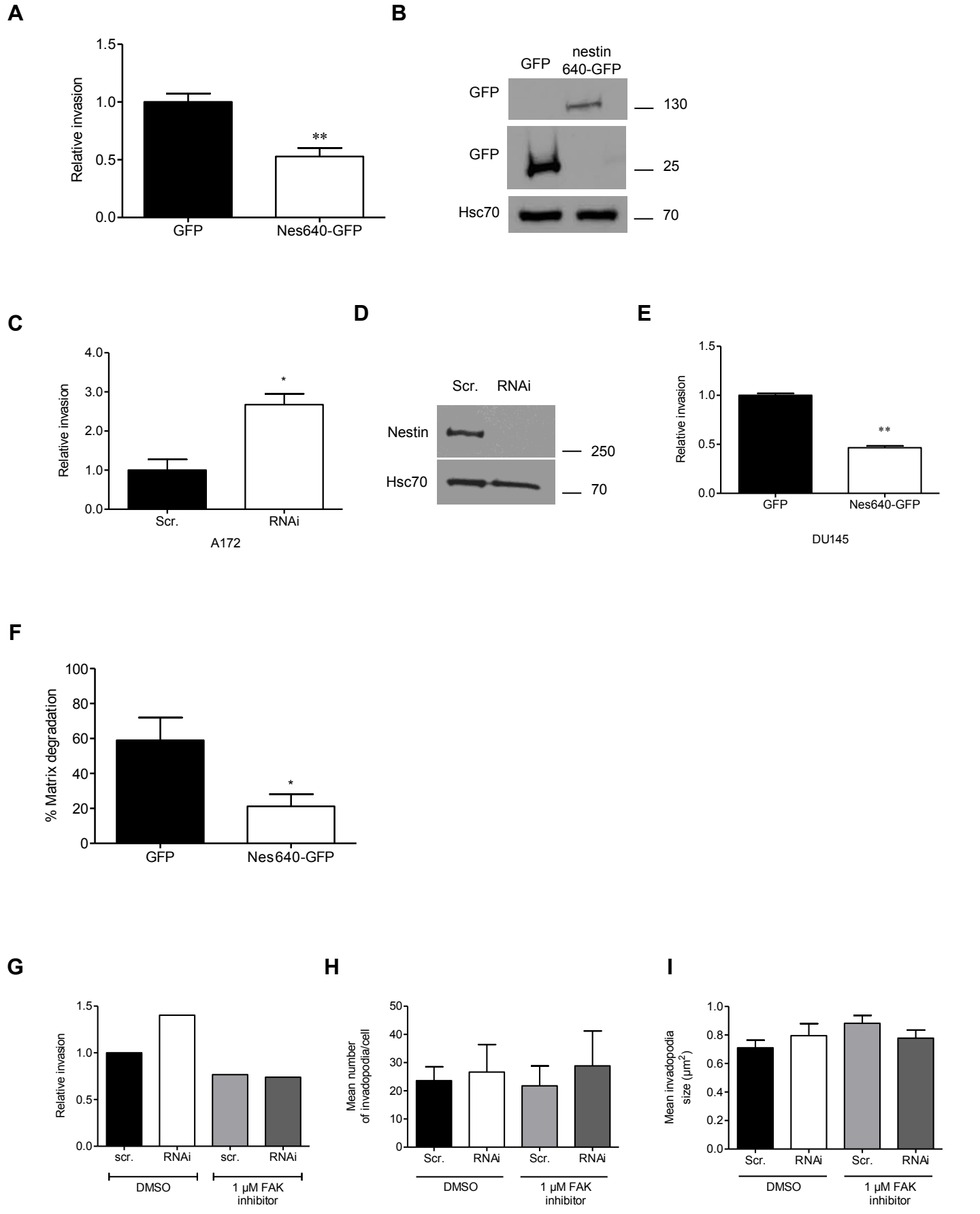




Supplementary Figure 3: Nestin does not affect migration of PC-3 cells



Supplementary Figure 4: Nestin is an inhibitor of invasion and matrix degradation



Supplementary Table 1: Antibodies used in article

Targeted protein	Clone	Company	Assay
Actin	AC-74	Sigma-Aldrich, USA	Western blotting
Cortactin	0.T.21	Abcam, UK	Immunofluorescence
Focal adhesion kinase (FAK)	77	BD Transduction	Western Blotting
phosphorylated Y397 FAK		Invitrogen, UK	Immunofluorescence
Hsc70	SPA-815	Stressgen, USA	Western blotting
Integrin α3	P1B5	Millipore, USA	Function blocking
Integrin α5	P-19	Santa Cruz Biotechnology,Germany	Immunofluorescence
Integrin α5		Millipore, USA	Western blotting
Integrin α5	JBS5	AbD Serotec, UK	Integrin clustering Flow cytometry Function blocking
Integrin αVβ3	LM609	Millipore, USA	Function blocking
Integrin α5	VC5	BD Pharmingen,	ELISA
Integrin β1	N29	Millipore, USA	Western blotting
Integrin β1	CD29-FITC	Beckman-Coulter, France	Flow cytometry
Integrin β1 (active)	12G10	Abcam, UK	
Ki67		Abcam, UK	Western blotting
Nestin	10C2	Millipore, USA	Immunofluorescence Western blotting
Nestin	MCA-4D11	EnCor Biotechnology	Western blotting
Alexafluor488-conjugated phalloidin		Invitrogen, UK	Immunofluorescence

Rabbit anti-mouse IgG		Invitrogen, UK	Function blocking
Src	GD11	Millipore, USA	Western blotting
Phosphorylated Src (Y416)		Cell Signaling Technology, USA	Western blotting
β-tubulin	DM1B	Abcam, UK	Immunofluorescence Western blotting
Talin	8D4	Sigma-Aldrich, USA	Immunofluorescence
Vimentin	V9	Sigma-Aldrich, USA	Immunofluorescence Western blotting
Vinculin	7F9	Millipore, USA	Immunofluorescence