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Akt1 mediates prostate cancer cell microinvasion and chemotaxis to metastatic stimuli via integrin β_3 affinity modulation

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BACKGROUND: Activation of Akt and increased expression of integrin β_3 are the two most important changes that have been linked to the attainment of metastatic potential by prostate cancer cells. However, a direct link between Akt activity and inside-out activation of integrin β_3 in mediating prostate cancer cell metastatic properties is not established.

METHODS: Using functional and biochemical approaches, we examined the role of Akt1 in the affinity modulation of integrin β_3 in prostate cancer cells.

RESULTS: Although expression of murine TRAMP and human PC3 cells with constitutively active Akt1 (CA-Akt1) enhanced their affinity for integrin $\alpha_v\beta_3$ specific ligands and motility on various extracellular matrix proteins, the reverse was observed with the expression of dominant-negative Akt1 (DN-Akt1). Although enhanced motility and transendothelial migration of CA-Akt1-expressing cells were blunted by co-expression with DN-integrin β_3 or upon pre-treatment with integrin β_3-blocking antibodies (LM 609), impaired motility and transendothelial migration of DN-Akt1-expressing cells were rescued by pre-treatment of prostate cancer cells with integrin β_3-activating antibodies, AP7.4.

CONCLUSION: Our data is the first to demonstrate a link between Akt1 activity and affinity modulation of integrin β_3 in the regulation of prostate cancer cell motility, transendothelial migration and chemotaxis to metastatic stimuli.

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Molecular Diagnostics

Treatment of confined, localised and slow-growing solid tumours, such as prostate cancer, is often associated with good prognosis. However, highly invasive and metastatic prostate cancer is difficult to treat mainly because of our poor understanding of the molecular mechanisms regulating these processes. Invasion and metastasis of different types of cancers are highly complex and coordinated events. These steps include detachment of cancer cells from primary tumour, directional migration, invasion into the micro-vasculature (micro-metastasis), extravasation and finally spreading of tumours to distant tissues (Porkka and Visakorpi, 2004). The initial, as well as, the rate-limiting step in this process is the cellular recognition of various extracellular matrix (ECM) proteins, resulting in the spreading and acquisition of migratory properties in response to chemotactic stimuli. Previous studies have established a link between the migratory properties of cancer cells and their invasive and metastatic potential (Clarke *et al*, 2009). Cancer cells respond to cues from the extracellular environment in the regulation of various functions including motility (Gertler and Condeelis, 2011). These stimuli include stroma, growth factors, cytokines, matrix metalloproteases (MMPs) and ECM proteins (Briest *et al*, 2012). Tumour cells have the ability to modulate stromal cells to their benefit (Stewart *et al*, 2004). Thus, stromal secreted factors and fragmented ECM fibrils facilitate the migration and invasion of tumour cells.

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Cellular adhesion to ECM and migration is mediated through heterodimeric ECM receptors called integrins (Ramsay *et al*, 2007; Schneider *et al*, 2011; Shin *et al*, 2012). There are 18α and 8β subunits that form 24 known combinations in various cell types (Plow *et al*, 2000). Not all integrins are present in all cell types (Hynes, 1992). Abnormal expression of many of these integrins and changes in their activity levels with respect to interactions with several ECM proteins are among the major alterations described in prostate cancer (Fornaro *et al*, 2001). This abnormal repertoire of integrins in conjunction with the aberrant changes in ECM as a result of enhanced MMP activities has profound effects on cancer progression and metastasis *in vivo*. Among these, expression of integrin β_3 in prostate cancer cells is noteworthy. Normal prostatic epithelial cells do not express integrin β_3 (Zheng *et al*, 1999). Upon oncogenic transformation, prostate cancer cells start to express integrin β_3, which is further elevated in highly metastatic prostate cancer (Zheng *et al*, 1999), thus resulting in enhanced tumour invasion and metastasis (McCabe *et al*, 2007, 2008). This process may be corroborated with the downregulation of a number of other integrins such as α_2 (Nagle *et al*, 1994) and β_{1C} (Fornaro *et al*, 1999; Perlino *et al*, 2000).

Integrins are transmembrane receptors where the extracellular domain binds to a wide variety of ECM proteins and the intracellular cytoplasmic domain anchor to the cytoskeletal proteins. Thus, integrins allow a bidirectional transmission of biochemical signals across the plasma membrane (Hynes, 1992). At basal levels, integrins normally attain low affinity to the respective ECM proteins needed to activate them. Upon growth-factor stimuli, activation of an array of the intracellular signalling pathways induces conformational change in

the integrin cytoplasmic domain that transmits to the extracellular domain. Thus, integrins are transformed from a low- to a high-affinity ligand-binding state. Such inside-out regulation of integrin affinity states is distinct from the outside-in signalling observed upon activation of most other transmembrane receptors for growth factors and is independent of changes in their expression levels in cells.

The mechanisms regarding differential response of tumour cells to various ECM proteins to develop invasive and metastatic potential are not clearly defined. Akt (protein kinase B) is central to many cellular functions in normal and cancer cells (Kandel and Hay, 1999; Somanath *et al*, 2006). We previously showed that Akt is activated downstream of integrin–matrix interactions (outside-in signalling) in many cell types and, in turn, is necessary for inside-out activation of integrins that increases its affinity towards its ligand (Chen *et al*, 2005; Somanath *et al*, 2007; Somanath and Byzova, 2009). Changes in Akt1 activity in cells modulate physiological (Somanath *et al*, 2008) and tumour angiogenesis (Chen *et al*, 2005) and oncogenic transformation (Somanath *et al*, 2009b). Previously, we demonstrated that Akt1 is necessary for many integrin-dependent functions by prostate cancer cells (Goc *et al*, 2011) and that pharmacological inhibition of Akt by simvastatin resulted in impaired cell function and prostate tumour growth in a nude mouse xenograft model (Kochuparambil *et al*, 2011). With the support of our previous findings, we hypothesised that Akt1 mediates integrin affinity modulation in prostate cancer cells, leading to directional migration, invasion and acquisition of metastatic potential. The current study was designed to provide novel insights on the cooperation between Akt1 and integrins in the regulation of prostate cancer cell motility on various integrin ligands such as vitronectin, laminin, osteopontin (bone sialoprotein) and osteonectin (SPARC), which are ECM proteins abundantly present in the prostate, vasculature and bone. Our study reveals the ability of Akt1 to modulate integrin function, integrin $\alpha_v\beta_3$ in particular, in response to interactions with various ECM proteins, consequently, mediating the invasion, transendothelial migration and metastasis of prostate cancer cells.

MATERIALS AND METHODS

Cells, reagents and antibodies

Human PC3 cells were obtained from ATCC. Murine TRAMP (TR-C2D, TR-C2 and TR-C2N) cells were gifted by Dr Barabara Foster, Baylor College of Medicine, TX, USA. Cells were maintained in DMEM with 10% FBS, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin in a 5% CO_2 atmosphere at 37°C. A total of 50 μM of epidermal growth factor (EGF; R&D, Minneapolis, MN, USA) was prepared in DMSO. LY294002, SH-5 and rapamycin were purchased from EMD Biosciences (Billerica, MA, USA) and were dissolved in DMSO. Anti-phospho-Akt^{S473} and anti- phospho-GSK-3$^{S9/21}$ were purchased from Cell Signaling (Danvers, MA, USA). Anti-Integrin β_3 antibodies were obtained from Santa Cruz biotechnology (Santa Cruz, CA, USA). LM 609 and anti-vitronectin antibodies were purchased from Millipore (Billerica, MA, USA). AP7.4 clone of integrin antibodies and WOW-1 were kindly gifted by Dr Thomas Kunicki and Dr Sanford Shattil, respectively (Scripps Research Institute, La Jolla, CA, USA). Primary antibodies against β-actin were purchased from Sigma (St Louis, MO, USA). All secondary antibodies were obtained from Bio-Rad (Hercules, CA, USA). Fibronectin and vitronectin were purchased from BD Biosciences (San Jose, CA, USA), laminin from Sigma and osteopontin and osteonectin from R&D. Anti-mouse Alexa-488 antibodies and Alexa 488-labelled fibronegen were purchased from Life Technologies (Carlsbad, CA, USA).

Cell transfections

Stable TRAMP and PC3 cells expressing constitutively active Akt1 (CA-Akt1, myristoylated Akt) and dominant-negative Akt1 (DN-Akt1, Akt1-K179M) was made by retroviral transfections (pBabe-puro backbone) as done previously (Somanath *et al*, 2007) and selected with 2 μg ml^{-1} puromycin treatment. Control cells expressed empty vector (pBabe-Puro). Plasmids expressing wild-type (WT) and constitutively active integrin β_3 (in pcDNA3 backbone) were introduced into the PC3 cells using lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions.

Cell-migration assay

Actively growing TRAMP and PC3 cells were subjected for migration assays in the presence of 50 μM EGF in DMSO (0.1% DMSO in control) as described previously (Goc *et al*, 2011; Kochuparambil *et al*, 2011). Briefly, cells were grown in 12-well plates either directly on plastic or in wells pre-coated with respective ECM protein (500 ng per well) and was allowed to reach confluence. A scratch was made in the serum-starved confluent monolayer using a 1-ml pipet tip and cells were treated with 50 μM EGF in the presence/absence of integrin β_3 blocking (25 μg ml^{-1} LM609) or activating (5 μg ml^{-1} AP7.4) antibodies. Pictures were taken at 0, 12 and 24 h after EGF treatment. The rate of migration (wound recovery) was calculated using the following equation $(1 - T_{16}/T_0) \times 100$. The data are presented as mean ± s.d. ($n = 4$).

Electric cell substrate impedance sensing (ECIS) to measure transendothelial migration of tumour cells

Transendothelial migration of PC3 and TRAMP cells was measured using ECIS equipment according to the manufacturer's instructions with HUVEC plated on 8W10E+ array chips (Applied Biophysics, Troy, NY, USA). Following this, control TRAMP and CA-Akt1, dominant-negative Akt1 (DN-Akt1)- or empty vector-expressing cells were directly added onto HUVEC monolayer at a density of 2×10^5 cells per well in 100 μl medium. In another experiment, stable PC3 cells expressing CA-Akt1, DN-Akt1 or empty vector (pBabe-puromycin) were transiently co-transfected with control pcDNA3, WT or dominant-negative (S752P mutant) integrin β_3 plasmids (in pcDNA3 backbone) using lipofectamine 2000 added onto the ECIS arrays plated with confluent HUVEC. Cells were isolated using cell-dissociation buffer (20 mM EDTA in PBS, pH 7.4). Similar experiments were also carried out to study the effects of 5 μg ml^{-1} AP7.4 and 25 μg ml^{-1} LM 609 on transendothelial migration of tumour cells. Real-time measurements on the transendothelial migration were recorded by the ECIS. The data is presented as mean ± s.d. ($n = 4$).

Immunostaining and assay for fibrinogen and vitronectin binding

The analysis of fibrinogen binding was performed as previously described (Somanath *et al*, 2007). Briefly, PC3 cells, stably transfected with either pBabe empty vector, pBabe CA-Akt or DN-Akt, were plated on cell-culture chamber slides at the 70–80% of confluence and cultured for the next 12 h in serum-free medium. Cells were then fixed with 2% paraformaldehyde and blocked with 2% BSA for 1 h at room temperature. Cells were incubated either with primary antibody against vitronectin (dilution 1 : 250) overnight or Alexa Flour 488-labelled fibrinogen (200 nM) for 40 min. For vitronectin-binding assay, cells were then incubated with Alexa Fluor 488-labelled ant-rabbit secondary antibody for 1 h at room temperature (dilution 1 : 500). After three times washing with PBS, cells were mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame, CA, USA). Images were taken by Zeiss fluorescent microscope (Zeiss Axiovert100M, Carl Zeiss, Germany). Alternately, cells cultured in a 96-well plate (85–95% of confluence) were incubated with Alexa Flour 488-labelled fibrinogen as mentioned above and was subjected for quantification of fluorescence in a Synergy HT multi-plate reader

(Bio-Tek Instruments Inc., Winooski, VT, USA) with excitation wavelength at 484/20 nm and emission wavelength at 590/35 nm.

WOW-1-binding assay

Activation-dependent ligand WOW-1 Fab was used to determine the activation status of integrins (Somanath *et al*, 2007). To analyse WOW-1 binding, serum-starved PC3 cells expressing control vector (pBabe-Puro), CA-Akt1 or DN-Akt1 were cultured in cell-culture chamber slides (Fisher Scientific, Hampton, NH, USA) until they reach confluence and were incubated with WOW-1 Fab (30 $\mu g\,ml^{-1}$) in adherent conditions for 40 min. Wells were washed in PBS and further incubated with goat anti-mouse IgG labelled with Alexa Fluor 488 antibody for another 1 h. After washing, cells were fixed in 2% paraformaldehyde, mounted with vectashield containing DAPI. Images were taken by Zeiss fluorescent microscope. Alternately, cells cultured in a 96-well plate (85–95% of confluence) were incubated with WOW-1 and Alexa Flour 488-labelled secondary antibodies as mentioned above and was subjected for quantification of fluorescence in a Synergy HT multi-plate reader with excitation wavelength at 484/20 nm and emission wavelength at 590/35 nm.

ELISA for vitronectin binding

Integrin $\alpha_v\beta_3$ and vitronectin-binding analysis was performed using the standard ELISA. Briefly, PC3 cells, stably transfected with either pBabe empty vector, pBabe CA-Akt or DN-Akt, were plated on 96-well plates at the 85–95% of confluence. Cells were starved for 4 h, fixed with 1% paraformaldehyde and incubated with primary antibodies against vitronectin (dilution 1 : 250) overnight. After washing, cells were incubated with Alexa Fluor 488-labelled ant-rabbit secondary antibody for 1 h at room temperature (dilution 1 : 500). Cells were washed three times with 1 × PBS and the intensity of fluorescence signal was measured immediately using Synergy HT multi-plate reader with excitation wavelength at 484/20 nm and emission wavelength at 590/35 nm.

Western analysis

Western analyses were performed as described previously (Somanath *et al*, 2007). Briefly, whole-cell lysates were prepared using lysis buffer (50 mM Tris-HCl (pH = 7.4), 1% TritonX-100, 150 mM NaCl, 1 mM EDTA, 2 mM Na_3VO_4 and 1X Complete protease inhibitors (Roche, Indianapolis, IN, USA)). The protein concentration was measured by the D_c protein assay (Bio-Rad). Densitometry of the bands was analysed using NIH ImageJ software.

Statistical analysis

Mean ± s.d. were calculated from three to four independent experiments performed in quadruplicates. The Student's two-tailed *t*-test was used to determine significant differences between treatment and control values.

RESULTS

The PI3-kinase-Akt1 pathway regulates prostate cancer cell migration

Motility is one of the most important features that determine the ability of prostate cancer cells to undergo invasion, transendothelial migration and metastasis. Hence, we first attempted to verify if modulation of the PI3-Kinase-Akt pathway would affect the migration of prostate cancer cells in response to EGF stimulation. Murine TRAMP cells with shared genetic alteration, but different levels of Akt activity and tumorigenic potential such as the non-tumorigenic (TR-C2D), tumorigenic and non-metastatic (TR-C2), and tumorigenic metastatic (TR-C2N) cells were used. In the presence and absence of EGF treatment, TR-C2 and TR-C2N cells that express higher levels of activated Akt (Goc *et al*, 2011) exhibited significantly higher rate of cell migration compared with TR-C2D (Figures 1A and B). In order to study the precise role of Akt1 in the regulation of prostate cancer cell motility, we prepared stably transfected TR-C2D, TR-C2 and TR-C2N TRAMP cells overexpressing either myristoylated CA-Akt1 or dominant-negative Akt1 (DN-Akt1) K179M mutant. TRAMP cells expressing CA-Akt1 exhibited increased cell migration and those expressing DN-Akt1 exhibited impaired migration (Figure 1A). Interestingly, although TR-C2N (metastatic) cells expressing CA-Akt1 showed higher rate of migration compared with TR-C2 (non-metastatic) in the absence of EGF, the opposite was observed in the presence of EGF. In either case, rate of migration TR-C2 and TR-C2N were always higher compared with TR-C2D (Figure 1B).

PI3-Kinase-Akt1 signalling cooperates with cell-surface integrins in mediating prostate cancer cell migration

Tumour cell migration, invasion and metastasis depend on the type of integrins expressed and the ECM proteins present in the tumour microenvironment. We determined whether modulation of the PI3-Kinase-Akt pathway can induce changes in the function of different integrins expressed in prostate cancer cells in response to specific ECM proteins that often interact with them during invasion (vitronectin), transendothelial migration (laminin) and bone metastasis (vitronectin, osteopontin and osteonectin). Our data indicated that the rate of migration by TR-C2 and TR-C2N cells were significantly higher on all ECM proteins compared with TR-C2D. However, no significant differences were observed in the migration of each cell type between different ECM proteins (Figure 2A). We next treated the cells plated on various ECM proteins with inhibitors of PI3 kinase (LY294002), Akt (SH-5) and

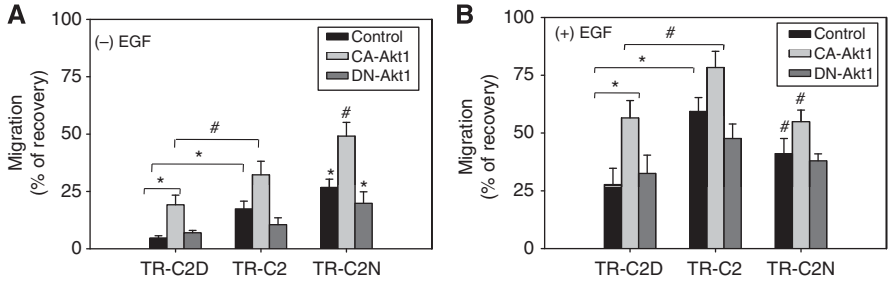

Figure 1 Akt1 modulates prostate cancer cell migration. (**A**, **B**) Stable TR-C2D, TR-C2 and TR-C2N cells expressing either control plasmid (pBabe-Puro), CA-Akt1 or DN-Akt1 were prepared by retroviral transfections, followed by puromycin (2 $\mu g\,ml^{-1}$) selection and were used for migration assays in the absence and presence of 50 μM EGF. Bar graph shows enhanced cell migration by TR-C2D, TR-C2 and TR-C2N cells with the expression of CA-Akt1 and impaired cell migration with the expression of DN-Akt1, compared with vector control. The data are presented as mean ± s.d. ($n = 3$ of quadruplicate experiments); $^{*}P < 0.001$ and $^{\#}P < 0.05$.

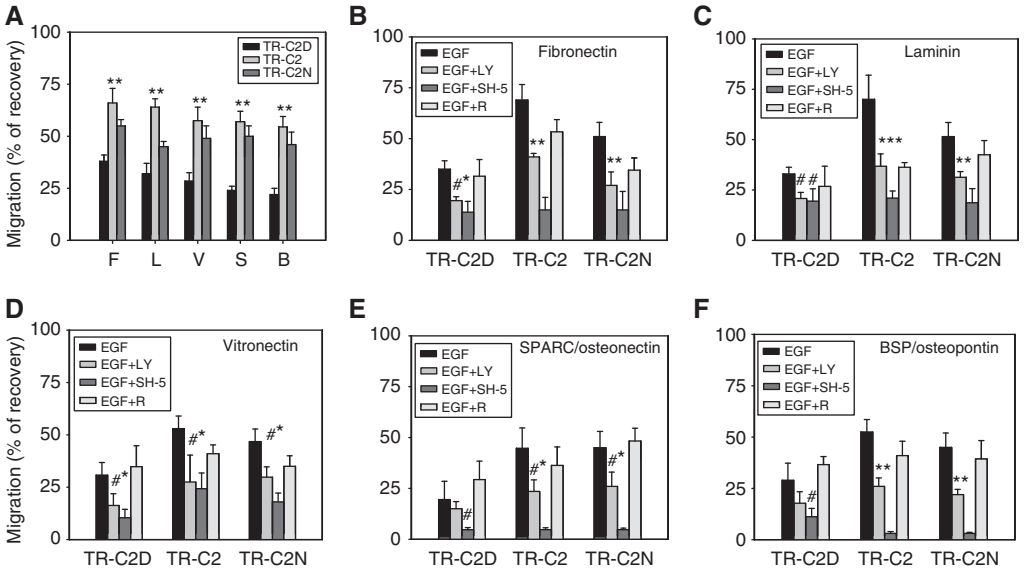

Figure 2 Pharmacological inhibition of PI3 kinase and Akt, but not mTOR, inhibits prostate cancer cell migration. (**A**) Bar graphs showing rate of migration of TR-C2D, TR-C2 and TR-C2N cells plated on different ECM proteins (500 ng per well of a 12-well plate) such as fibronectin, laminin, vitronectin, SPARC/osteonectin and bone sialoprotein/osteopontin, in response to 50 μM EGF (B). (**B**–**F**) Bar graphs showing rate of migration of TR-C2D, TR-C2 and TR-C2N plated on different ECM proteins, such as fibronectin, laminin, vitronectin, SPARC/osteonectin and bone sialoprotein/osteopontin, respectively, in response to 50 μM EGF and in the presence or absence of inhibitors of PI3 kinase (25 μM LY294002), Akt (10 μM SH-5) and mTOR (25 nM rapamycin). The data are presented as mean ± s.d. ($n = 3$ of quadruplicate experiements); $^{*}P < 0.001$ and $^{\#}P < 0.05$.

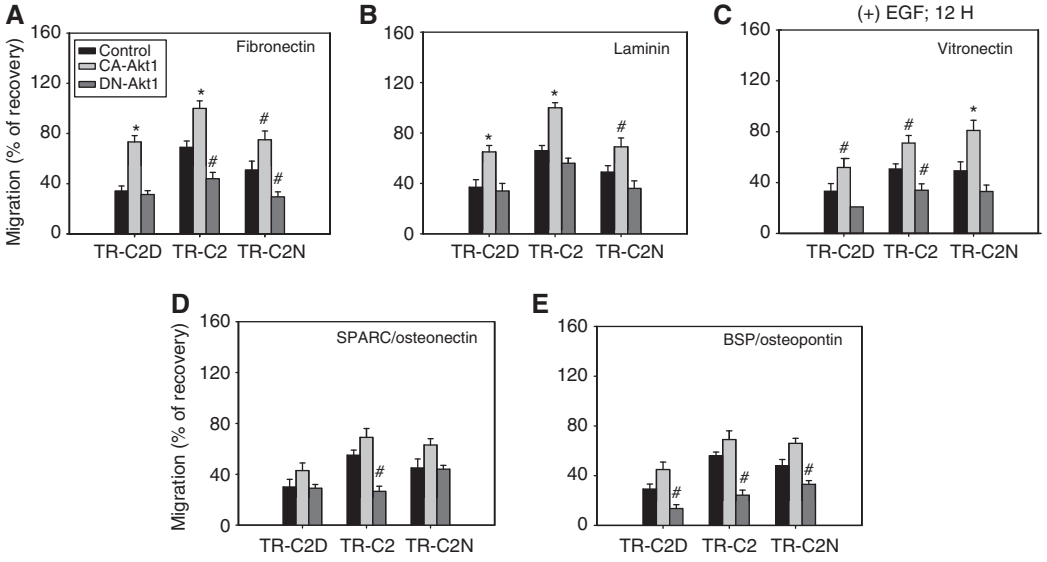

Figure 3 Rate of prostate cancer cell migration on specific ECM proteins is reliant on Akt1 activity. (**A**–**E**) Bar graphs showing rate of migration of TR-C2D, TR-C2 and TR-C2N cells with the stable expression of either control vector (pBabe-Puro), CA-Akt1 or DN-Akt1 plated on different ECM proteins (500 ng per well of a 12-well plate), such as fibronectin, laminin, vitronectin, SPARC/osteonectin and bone sialoprotein (BSP)/osteopontin, respectively, in response to 50 μM EGF. The data are presented as mean ± s.d. ($n = 3$ of quadruplicate experiments); $^{*}P < 0.001$ and $^{\#}P < 0.05$.

mTOR (rapamycin) and subjected them for cell-migration analyses. Our data indicated that these inhibitors, with the exception of rapamycin, significantly inhibited EGF-stimulated TRAMP cell migration on various ECM proteins (Figures 2B–F). Inhibition of Akt had the most profound effect, preferably on vitronectin, osteopontin and osteonectin (Figures 2B–F), indicating that Akt is necessary for integrin-mediated prostate cancer cell migration on ECM proteins.

In order to determine if Akt1 has direct effects on integrin-activity modulation in prostate cancer cells, we plated TRAMP cells with stable expression of CA-Akt1 or DN-Akt1 to migrate on various ECM proteins. Our data show that in the presence or absence of EGF, expression with CA-Akt1 significantly increased the rate of migration in non-tumour forming TR-C2D cells on all ECM proteins, except osteonectin and osteopontin (Figures 3A–E and Supplementary Figure 1). However, tumorigenic TR-C2 and TR-C2N cells exhibited enhanced cell migration on all ECM proteins (Figures 3A–E). In contrast, expression with DN-Akt1 significantly impaired migration of tumorigenic TR-C2 and TR-C2N cells on vitronectin, osteonectin and osteopontin, but no

significant differences were observed on fibronectin and laminin. However, non-tumorigenic TR-C2D cells expressing DN-Akt1 did not exhibit inhibition of cell migration on any ECM proteins compared with the control cells, except on osteopontin in the presence of EGF (Figures 3A–E). Our data suggest that Akt1 expression and/or activation may be directly linked to the invasive potential of prostate cancer cells.

Akt1 regulates transendothelial migration of prostate cancer cells

The vascular or the lymphatic endothelium and the basement membrane are the initial barriers for the prostate cancer cell intravasation and extravasation. We determined whether Akt1 activity is necessary for the prostate cancer cells to cross the endothelial barrier. In order to do that, we utilised 'ECIS' that measures endothelial barrier impedance in a monolayer in response to the invasive potential of TRAMP cells with stable expression of either CA-Akt1 or DN-Akt1. Metastatic TR-C2N cells showed the highest invasive potential on HUVEC monolayer compared with TR-C2D and TR-C2 as evidenced by the reduced impedance/resistance of the endothelial monolayer (Figures 4A–C). In all the cell types, expression with CA-Akt1 significantly enhanced transendothelial migration. In contrast, significantly impaired transendothelial migration with the stable expression of DN-Akt1 was specifically observed in highly metastatic TR-C2N cells (Figure 4D). Overall, our data indicate that Akt1 is necessary for transendothelial migration of prostate cancer cells.

Akt1 mediates inside-out activation of integrin $\alpha_v\beta_3$ in prostate cancer cells

To test whether Akt1 activity is important for the inside-out activation (affinity modulation) of integrin $\alpha_v\beta_3$, we developed stable lines of metastatic human PC3 cells expressing control vector (pBabe-Puro), CA-Akt1 or DN-Akt1 and subjected them for multiple ligand-binding assays by fluorescence microscopy and ELISA. First, we subjected the PC3 cells containing plasmids expressing active and inactive variants of Akt for vitronectin- or fibrinogen-binding assays, two highly specific ligands for integrin $\alpha_v\beta_3$. Our results indicated that although expression with CA-Akt1 resulted in enhanced binding of PC3 cells to both vitronectin and fibrinogen compared with control vector-expressing cells, expression with DN-Akt1 significantly inhibited this process (Figures 5A–C). In a separate experiment, we performed a WOW-1-binding assay, which is a clone of Fab fragments of antibodies that bind only to active form (high affinity) of integrin β_3. Our analysis indicated that, similar to fibrinogen binding, expression with CA-Akt1 resulted in enhanced binding of WOW-1 to PC3 cell surface compared with control vector. In contrast, expression with DN-Akt1 significantly inhibited WOW-1 binding to PC3 cells (Figures 5A and D), indicating that Akt1 is necessary for the affinity modulation of integrin β_3.

Akt1-mediated affinity modulation of integrin $\alpha_v\beta_3$ is necessary for the directional migration of PC3 cells on different ECM proteins

To study the role of integrin $\alpha_v\beta_3$ in Akt1-mediated cell migration and invasion, we utilised stable lines of metastatic human PC3 cells expressing CA-Akt1 and transiently transfected them with control, WT and DN-integrin β_3 (inactive S752P mutant) plasmids (Supplementary Figure 2). Although expression with CA-Akt1 significantly enhanced the rate of EGF-stimulated PC3 cell migration on vitronectin, osteonectin and osteopontin (Figures 6A–C), co-expression with DN-integrin β_3 blunted these effects (Figures 6A–C). Although expression of PC3 cells with WT integrin β_3 significantly enhanced migration on vitronectin, osteopontin

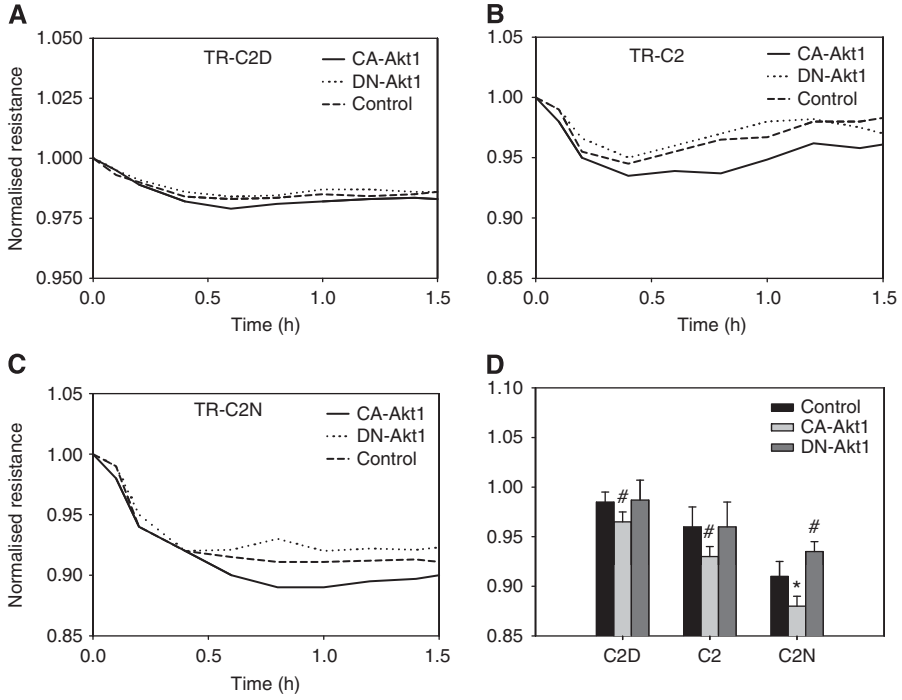

Figure 4 Akt1 activity modulates transendothelial migration of prostate cancer cells. (**A**–**C**) Illustration of electric resistance by the endothelial monolayer measured as an index of rate of transendothelial migration by TR-C2D, TR-C2 and TR-C2N cells with the stable expression of either control vector (pBabe-Puro), CA-Akt1 or DN-Akt1 as determined by the ECIS equipment showing reduced endothelial resistance upon administration of CA-Akt1-expressing cells and increased endothelial cell resistance upon administration of DN-Akt1-expressing cells, compared with vector control expressing cells. (**D**) Bar graph showing quantification of the above data at 1 h time point. The data are presented as mean ± s.d. ($n=4$ of quadruplicate experiments); $^{*}P<0.001$ and $^{\#}P<0.05$.

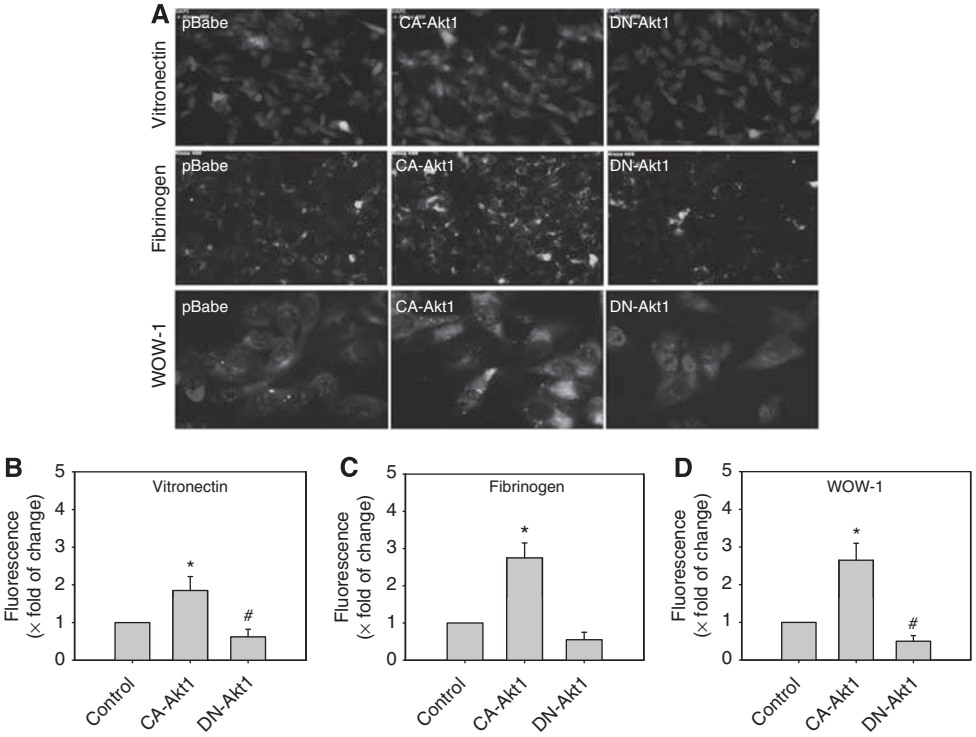

Figure 5 Akt1 activity modulates integrin $\alpha_v\beta_3$ affinity modulation in prostate cancer cells. (**A**) Microscopic pictures of PC3 cells expressing control vector (pBabe-Puro), CA-Akt1 or DN-Akt1 showing bound vitronectin, fibrinogen and WOW-1, respectively. (**B**–**D**) Bar graph showing quantification of vitronectin (1 : 250 dilution), fibrinogen (200 nM) and WOW-1 binding (30 μg ml^{-1}), respectively by PC3 cells expressing either control vector, CA-Akt1 or DN-Akt1 using ELISA protocol. The data are presented as mean ± s.d. ($n = 4$ of quadruplicate experiments); $^{*}P < 0.001$ and $^{\#}P < 0.05$.

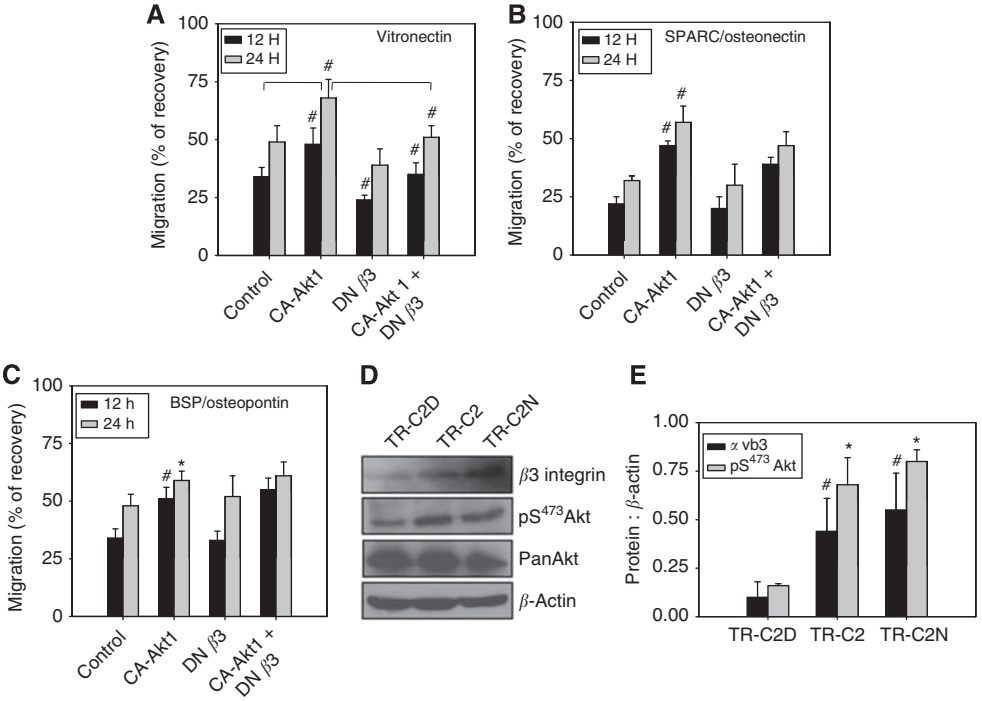

Figure 6 Akt1 involves integrin $\alpha_v\beta_3$ in the modulation of prostate cancer cell migration on ECM proteins. (**A**–**C**) Bar graphs showing rate of migration of human metastatic PC3 cells with the stable expression of either control vector (pBabe-Puro), CA-Akt1 or DN-Akt1 with the co-expression of either control vector (pcDNA3) or DN-integrin β_3 plated on different integrin $\alpha_v\beta_3$ ligands, such as vitronectin, SPARC/osteonectin and bone sialoprotein (BSP)/osteopontin, respectively, in response to 50 μM EGF. (**D**) Western data showing increased expression of integrin β_3 and phosphorylated Akt in TR-C2 and TR-C2N compared with TR-C2D cells. (**E**) Bar graph showing the quantification and significance level of the above western data. The data are presented as mean ± s.d. ($n = 4$ of quadruplicate experiments); $^{*}P < 0.001$ and $^{\#}P < 0.05$.

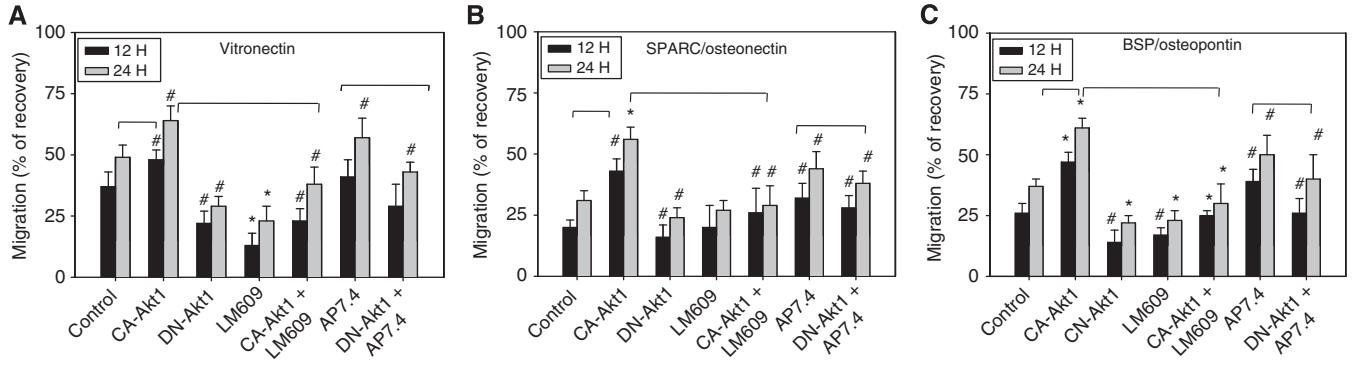

Figure 7 Cooperation between Akt1 and integrin $\alpha_v\beta_3$ regulates prostate cancer cell migration on vascular and bone matrix proteins. (**A**–**C**) Bar graphs showing rate of migration of human metastatic PC3 cells with the stable expression of either control vector (pBabe-Puro), CA-Akt1 or DN-Akt1 treated with and without integrin β_3-activating and -blocking antibodies (5 μg ml^{-1} AP7.4 and 25 μg ml^{-1} LM609, respectively) plated on different integrin $\alpha_v\beta_3$ ligands, such as vitronectin, SPARC/osteonectin and bone sialoprotein (BSP)/osteopontin, respectively, in response to 50 μM EGF. The data are presented as mean ± s.d. ($n = 4$ of quadruplicate experiments); $^{*}P < 0.001$ and $^{\#}P < 0.05$.

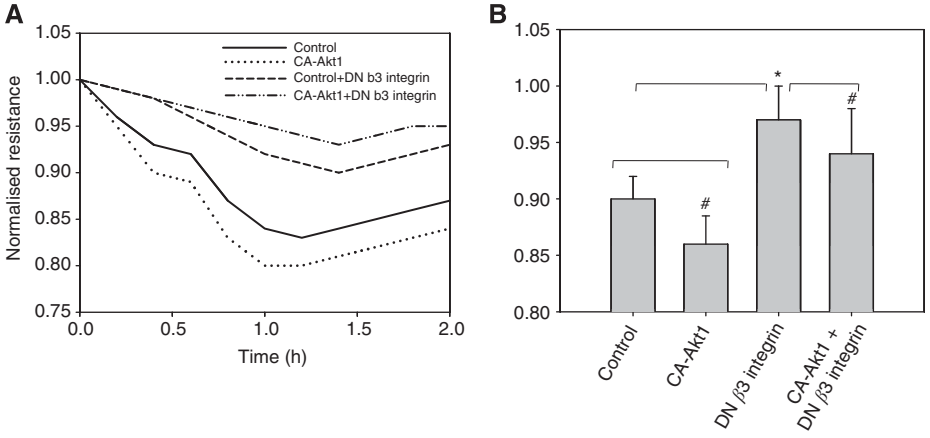

Figure 8 Akt1 involves integrin $\alpha_v\beta_3$ in the modulation of transendothelial migration of prostate cancer cells. (**A**) Illustration of electric resistance by the endothelial monolayer measured as an index of rate of transendothelial migration by PC3 cells with the stable expression of either control vector (pBabe-Puro) or CA-Akt1 with the co-expression of either control vector (pcDNA3) or DN-integrin β_3 as determined by the ECIS equipment showing reduced endothelial resistance upon administration of CA-Akt1-expressing cells as compared with vector control expressing cells, which is inhibited by co-expression with DN-integrin β_3. (**B**) Bar graph showing quantification of the above data at 1 h time point. The data are presented as mean ± s.d. ($n = 4$ of quadruplicate experiments); $^{*}P < 0.001$ and $^{\#}P < 0.05$.

and oseteonectin, co-expression of WT integrin β_3-expressing cells with DN-Akt1 significantly inhibited these effects (Supplementary Figures 3A–C).

We next determined whether there is any correlation between enhanced phosphorylation of Akt, enhanced expression of integrin β_3 and metastatic potential of prostate cancer cells. Our data show that TR-C2 and TR-C2N cells expressed higher levels of integrin β_3, which was proportional to the increased levels of Akt phosphorylation, compared with TR-C2D (Figures 6D and E). Using a more sensitive approach, we attempted to characterise the causal relationship between Akt1 and integrin $\alpha_v\beta_3$ in prostate cancer cells. On one end, we prepared stable PC3 cells expressing CA-Akt1 or vector control and stimulated cell migration with EGF treatment in the presence and absence of integrin β_3-blocking antibodies (LM609). On the other end, we prepared stable PC3 cells expressing DN-Akt1 or vector control and stimulated cell migration with EGF treatment in the presence and absence of integrin β_3-activating antibodies (AP7.4). Our study indicated that treatment with LM609 significantly inhibited cell migration stimulated by EGF in control cells plated on vitronectin and osteopontin (Figures 7A and C). Enhanced cell migration observed in CA-Akt1-expressing PC3 cells on vitronectin, osteopontin and osteonectin was blunted by co-treatment with LM609 antibodies (Figures 7A–C). In contrast, treatment with AP7.4 augmented EGF-stimulated PC3 cell migration on vitronectin, osteonectin and osteopntin (Figures 7A–C). Furthermore, inhibition of cell migration by DN-Akt1-expressing PC3 cells on vitronectin, osteonectin and osteopontin were significantly rescued by co-treatment with AP7.4 (Figures 7A–C), thus demonstrating that Akt1-mediated affinity modulation of integrin β_3 is necessary for prostate cancer cell migration.

Akt1-mediated affinity modulation of integrin $\alpha_v\beta_3$ is necessary for the transendothelial migration of PC3 cells

Next, we investigated whether modulation of integrin $\alpha_v\beta_3$ has any effect on transendothelial migration of prostate cancer cells. HUVECs were grown to confluence on ECIS arrays. Control and CA-Akt1-expressing PC3 cells, followed by transient transfections by control vector or DN-integrin β_3, were introduced into the array wells. PC3 cells expressing CA-Akt1 exerted significantly increased endothelial barrier disruption as evidenced by the lowered endothelial barrier resistance (Figures 8A and B). In contrast, PC3 cells expressing DN-integrin β_3 were less effective compared with the control in disrupting the endothelial barrier (Figure 8B). Interestingly, co-expression of PC3 cells, CA-Akt1 and DN-integrin β_3,

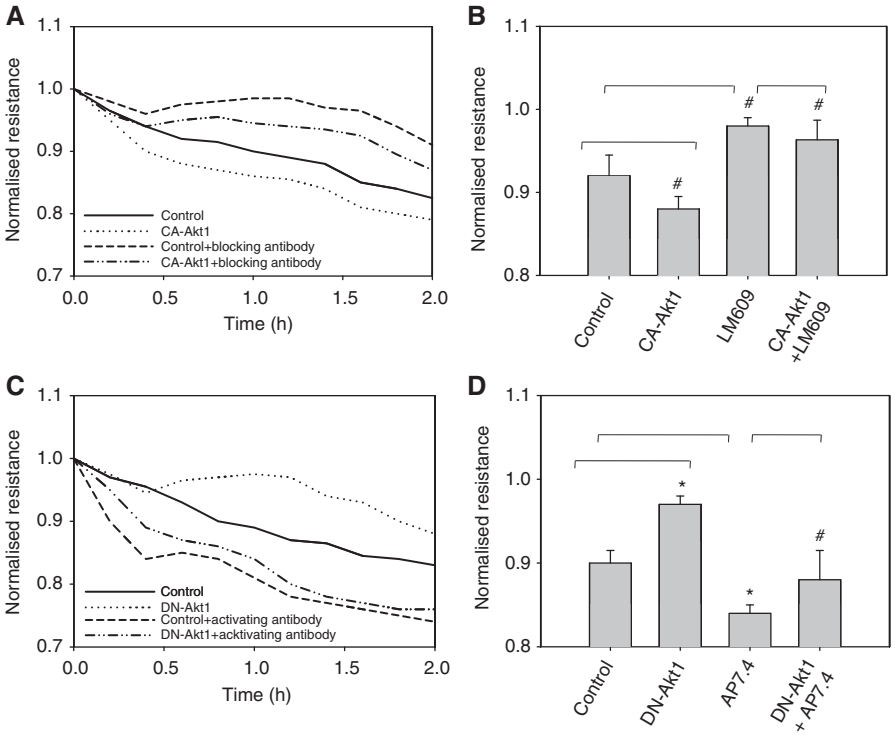

Figure 9 Cooperation between Akt1 and integrin $\alpha_v\beta_3$ regulates transendothelial migration of prostate cancer cells. (**A**) Illustration of electric resistance by the endothelial monolayer measured as an index of rate of transendothelial migration by PC3 cells with the stable expression of either control vector or CA-Akt1, treated with and without integrin β_3-blocking antibodies (25 μg ml^{-1} LM609), as determined by the ECIS equipment. (**B**) Bar graph illustrating quantification of the above data showing that enhanced transendothelial migration of PC3 cells expressing CA-Akt1 is blunted by pre-treatment with integrin β_3-blocking antibodies LM609. (**C**) Illustration of electric resistance by the endothelial monolayer measured as an index of rate of transendothelial migration by PC3 cells with the stable expression of either control vector or DN-Akt1, treated with and without integrin β_3-activating antibodies (5 μg ml^{-1} AP7.4), as determined by the ECIS equipment. (**D**) Bar graph illustrating quantification of the above data showing that impaired transendothelial migration of PC3 cells expressing DN-Akt1 was rescued by pre-treatment with integrin β_3-activating antibodies (AP7.4). The data are presented as mean ± s.d. ($n = 4$ of quadruplicate experiments); $^{*}P < 0.001$ and $^{\#}P < 0.05$.

completely blunted the transendothelial migratory effects induced by CA-Akt1 (Figure 8). In addition, PC3 cells expressing WT integrin β_3 showed significantly enhanced transendothelial migration compared with control cells, which was blunted by co-expressing PC3 cells with DN-Akt1 (Supplementary Figures 4A and B). Co-expressing PC3 cells with CA-Akt1 and WT integrin β_3 showed significantly higher rate of transendothelial migration, compared with cells expressing either of the CA-Akt1 or WT integrin β_3 plasmids alone (Supplementary Figures 4C and D).

To further investigate and characterise the link between Akt1 and integrin $\alpha_v\beta_3$ in transendothelial migration of prostate cancer cells, we performed ECIS assays utilising AP7.4 or LM609. Our results show that treatment with LM609 resulted in significant impairment in the ability of PC3 cells for transendothelial migration compared with control cells (Figures 9A and B). Additionally, enhanced transendothelial migration of PC3 cells expressing CA-Akt1 was completely blunted by pre-treatment with LM609 (Figures 9A and B). Although control cells pre-treated with AP7.4 significantly enhanced transendothelial migration of PC3 cells, defective transendothelial migration by DN-Akt1-expressing PC3 cells were significantly rescued by pre-treatment with AP7.4 (Figures 9C and D). Overall, our data indicate that Akt1-mediated affinity modulation of integrin β_3 is necessary for transendothelial migration of prostate cancer cells.

DISCUSSION

The PI3-kinase-Akt pathway, the major mediator of cell survival and a plethora of other key cellular functions, is the most common pathway deregulated in many cancers (Grant, 2008; Kinkade *et al*, 2008), including prostate cancer (Schmitz *et al*, 2007). Our previous reports assert this and further document that increased expression and phosphorylation of Akt mediates oncogenic transformation (Somanath *et al*, 2009b) and correlates with the tumorigenic and metastatic potential of prostate cancer cells (Goc *et al*, 2011). The precise role of Akt and its downstream signalling pathway in any cancer cells with respect to the development of invasive and metastatic potential is not clearly understood. Integrin $\alpha_v\beta_3$ is a subset of heterodimeric receptors for ECM proteins, such as vitronectin, entactin, osteonectin and osteopontin, which are abundant in blood vessels and bone matrix, is upregulated during the advanced stages of prostate cancer and has been implicated in mediating prostate tumour invasion and bone metastasis (Cooper *et al*, 2002; McCabe *et al*, 2007, 2008). In the current study, we focused on characterising the cooperation between Akt1, major isoform of Akt in prostate cancer cells (Goc *et al*, 2011), with integrin $\alpha_v\beta_3$, in the regulation of its affinity for ECM proteins in mediating ECM-specific modulation of cell migration and transendothelial migration.

The major finding from our current study is that Akt1 activity is necessary for the affinity modulation of integrin $\alpha_v\beta_3$ in prostate cancer cells in order to adapt to its constantly changing microenvironments and to attain invasive and metastatic potential. First, we identified that different level of Akt activity and integrin $\alpha_v\beta_3$ expression in various non-tumorigenic, tumorigenic and metastatic murine prostate cancer (TRAMP) cells correlated with their ability to migrate on various ECM proteins. Second, modulation of Akt1 activity in TRAMP cells, in turn, modulated its affinity for integrin $\alpha_v\beta_3$ ligands, such as vitronectin, fibrinogen and WOW-1,

as well as transendothelial migration. Third, Akt1-stimulated human PC3 cell migration on ECM proteins, such as vitronectin, osteopontin and osteonectin, as well as transendothelial migration, was significantly inhibited by either co-expression of the cells with DN-integrin β_3 (β3 S752P) or treatment with integrin-blocking antibodies (LM609). In contrast, impaired migration of PC3 cells expressing DN-Akt1 (Akt1-K179M) on these ECM proteins and transendoethelial migration was significantly rescued by treatment with integrin β_3-activating antibodies (AP7.4). Overall, our study suggests that Akt1 is a major regulator of integrin–ECM interactions in prostate cancer cells in mediating invasion, transendothelial migration and metastasis.

Integrin–ECM interactions are regulated bidirectionally through distinctly different mechanisms (Hynes, 1992). Interaction of growth factors with their receptors or engagement of ECM proteins with their respective integrins, both cross-activate each other via outside-in signalling (avidity modulation) (Muller *et al*, 2008). These signalling changes activate integrins back via inside-out signalling (affinity modulation) by inducing conformational changes in the cytoplasmic domain of the β member (Somanath *et al*, 2009a). One of the major pathways activated downstream of outside-in signalling in mammalian cells is the PI3 kinase-Akt pathway, which is necessary for the anchorage-dependent survival of the cells (Somanath *et al*, 2007). In the absence of anchorage to the substratum, adherent cells exhibit impaired Akt signalling and undergo apoptosis, a process known as Anoikis (Shiojima and Walsh, 2002). Whether or not Akt is important for the affinity modulation of integrins that mediate their switch to the invasive and metastatic cells is not yet known. Our data on PC3 and TRAMP cell migration on various ECM proteins, as well as the effect of changes in Akt1 activity in modulating the affinity of PC3 cell-surface integrin $\alpha_v\beta_3$ on its specific ligands, such as vitronectin, fibrinogen and WOW-1, demonstrate that Akt1 activity is necessary for the affinity modulation of integrin $\alpha_v\beta_3$. An interesting observation from these studies was that control and CA-Akt1-expressing metastatic TR-C2N cells migrated slower than tumorigenic/non-metastatic TR-C2 cells in the presence of EGF. This surprising observation could be due to the role of Akt1 in establishing cell polarity through modulation of Rac and p21 activated kinase signalling (Chung *et al*, 2001; Somanath and Byzova, 2009). Although Rac1 is known to establish cell polarity by inducing cytoskeletal changes specifically in the leading edge, cell polarity is perturbed at higher levels of Rac1 activity as a result of cytoskeletal changes induced in the entire cell (Pankov *et al*, 2005). Hence, significantly higher levels of Akt1 or rac1 activation for a longer period of time may abolish leading- and lagging-edge characteristics resulting in the inhibition of directional migration.

Integrin signalling has been shown to be deregulated in several types of cancer (Fornaro *et al*, 2001; Goel *et al*, 2009). Extracellular matrix in the neoplastic prostate tissue, vascular basement membrane and the sites of metastasis, such as bone, are all different from the normal prostate tissue (Sikes *et al*, 2004; Stewart *et al*, 2004). Thus, depending upon the site of growth, prostate tumour cells are known to possess different profile of integrins, which may be functionally relevant and contribute to aberrant intracellular signalling. Among the integrin subsets highly expressed in different stages of prostate cancer include integrin $\alpha_2\beta_1$, $\alpha_6\beta_1$, $\alpha_4\beta_6$ and $\alpha_v\beta_3$ (Fornaro *et al*, 2001; Goel *et al*, 2009). Integrin $\alpha_2\beta_1$ is known to interact with fragmented collagens and fibronectins and hence may facilitate the tumour invasion (Murant *et al*, 1997). Integrins $\alpha_6\beta_1$ and $\alpha_4\beta_6$ bind to laminin and have been reported to aid attachment of invasive tumour cells to the basal lamina in the brain through their hemidesmosomes (Sroka *et al*, 2011). However, the most important among these appears to be integrin $\alpha_v\beta_3$ (Cooper *et al*, 2002; McCabe *et al*, 2007, 2008). Although integrin α_v is abundantly expressed in normal prostatic epithelial cells, integrin β_3 is normally absent (Zheng *et al*, 1999). However, prostate tumour cells start to express integrin β_3 (Zheng *et al*, 1999) and our data indicates that the expression level of integrin β_3 is directly linked to its tumorigenic and metastatic potential. Hence, integrin β_3 is emerging as one of the most reliable markers to diagnose disease progression in prostate cancer patients. Over the years, research has demonstrated the importance of integrin $\alpha_v\beta_3$ in prostate cancer cells, the major reason being the ability of integrin $\alpha_v\beta_3$ to interact with ECM proteins such as vitronectin, osteopontin and osteonectin so on (Plow *et al*, 2000), which is necessary to mediate prostate tumour invasion and metastasis. Our results indicate that Akt1 enhances the ability of prostate cancer cells to recognise the ECM proteins in the microenvironment, invade, transmigrate across the endothelial barrier and metastasise to bone mainly via the affinity modulation of integrin $\alpha_v\beta_3$. Because Akt1-mediated effects on PC3 cells can be reversed upon modulation of integrin β_3 affinity either using blocking/activating antibodies or expression with inactive mutant of integrin β_3, our data demonstrate that enhanced prostate cancer cell migration and transendothelial migration by Akt1 activation is mediated through inside-out activation of integrin $\alpha_v\beta_3$.

Intravasation and extravasation are prerequisites for the cancer cells to penetrate the circulation and metastasise to distant tissues (Cooper *et al*, 2002). Prostate cancer cells secrete proteases to degrade the ECM proteins in the basement membrane and attach to vascular wall by binding to laminin, probably involving integrins $\alpha_6\beta_4$ and/or $\alpha_6\beta_1$ (Plow *et al*, 2000; Sroka *et al*, 2010), likely involving Akt1 in the process. Although integrin $\alpha_v\beta_3$ has been shown to be highly expressed in metastatic prostate cancer cells (McCabe *et al*, 2007, 2008), specific function of integrin $\alpha_v\beta_3$ in prostate cancer metastasis is not clearly understood. A recent report from our group has shown that cell-surface integrin $\alpha_v\beta_3$ is necessary for even bone-marrow-derived cells and inflammatory cells to undergo transendothelial migration (Feng *et al*, 2008). In prostate cancer cells, integrin $\alpha_v\beta_3$ may be necessary for directly interacting with the endothelial cells through binding to cellular adhesion molecules, such as ICAM1, via a heterophilic mode of interaction, a mechanism similar to the known pericellular diapedesis of inflammatory cells across the endothelial monolayer (Zhang *et al*, 2011). A working model based on our findings on the collective role of the Akt1-integrin $\alpha_v\beta_3$ pathway in mediating prostate cancer cell motility, invasion, transendothelial migration and bone metastasis is depicted in Figure 10.

One important aspect that our manuscript does not address is the molecular mechanisms by which Akt mediate affinity modulation of integrins in prostate cancer cells. Several mechanisms have been proposed for the Akt-mediated cell migration in mammalian cells. Although an earlier report claimed that Akt is necessary for the phosphorylation of threonine 753 on the integrin β_3 cytoplasmic domain in platelets, the impact of this phosphorylation on integrins and cellular function was never identified (Kirk *et al*, 2000). Many studies have also shown that integrin-linked kinase mediate cytoskeletal changes and cell migration via activation of Akt (Qian *et al*, 2005) and that Akt is necessary for the recycling of integrins from the lagging edge to the leading edge (Roberts *et al*, 2004), facilitating cell adhesion and migration. However, the first report on the role of Akt in integrin affinity modulation with respect to a particular function came from our laboratory. We showed that Akt is necessary for the migration of normal endothelial cells and fibroblasts in the regulation of angiogenesis and ECM remodelling via inside-out activation of integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$, respectively (Chen *et al*, 2005; Somanath *et al*, 2007; Somanath and Byzova, 2009). Later, Akt was identified to promote cell migration by phosphorylating a cytoskeletal protein called girdin (Enomoto *et al*, 2005; Jiang *et al*, 2008).

In summary, our study is the first to establish a direct link between Akt1 activity and affinity modulation of integrin $\alpha_v\beta_3$ in the regulation of the ECM recognition, chemotaxis, invasion and transendothelial migration of prostate cancer cells. Our data project Akt1-integrin $\alpha_v\beta_3$ signalling axis as a potential target for therapeutic interventions for prostate cancer. However,

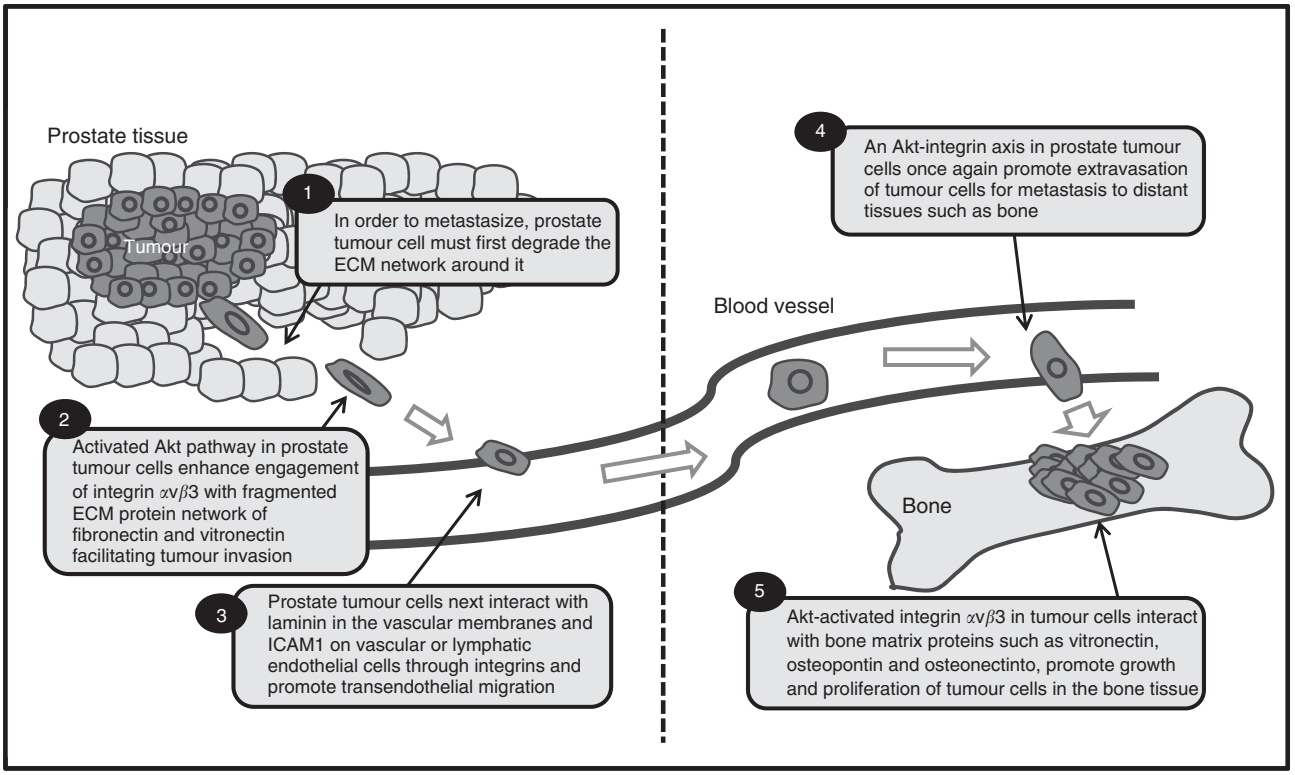

Figure 10 Working hypothesis on the cooperation between Akt1 and integrin $\alpha_v\beta_3$ in the regulation of prostate cancer cell migration and transendothelial migration to chemotactic stimuli during tumour invasion and metastasis.

the mechanisms by which Akt mediate this process appears to be complex and require further investigation.

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