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Urothelial bladder cancer is a major cause of morbidity and mortality worldwide, causing an estimated 150,000 deaths per year. Whilst non-muscle-invasive bladder tumours can be effectively treated, with high survival rates, many tumours recur, and some will progress to muscle-invasive disease with a much poorer long term prognosis. Thus there is a pressing need to understand the molecular transitions occurring within the progression of bladder cancer to an invasive disease. Tumour invasion is often associated with a down regulation of E-cadherin expression concomitant with a suppression of cell: cell junctions and decreased levels of E-cadherin expression have been reported in higher grade urothelial bladder tumours. We find that expression of E-cadherin in a panel of bladder cancer cell lines correlated with the presence of cell: cell junctions and the level of PAK5 expression. Interestingly exogenous PAK5 has recently been described to be associated with cell: cell junctions and we now find that endogenous PAK5 is localised to cell junctions and interacts with an E-cadherin complex. Moreover, depletion of PAK5 expression significantly reduced junctional integrity. These data suggest a role for PAK5 in maintaining junctional stability and we find that in both our own patient samples and a commercially available datasets that PAK5mRNA levels are reduced in human bladder cancer compared to normal controls. Taken together this study proposes that PAK5 expression levels could be used as a novel prognostic marker for bladder cancer progression.

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Summary statement : We report high PAK5 expression in bladder cancer cells that are connected with significantly reduced expression in individual cells. Moreover, we localise PAK5 to cell: cell junctions. Our data points to the importance of PAK5 for junctional integrity.

Short title: PAK5 localises to cell: cell junctions in bladder cancer cells

Key words: PAK5, Bladder, E-cadherin

Abstract

Urothelial bladder cancer is a major cause of morbidity and mortality worldwide, causing an estimated 150,000 deaths per year. Whilst non-muscle-invasive bladder tumours can be effectively treated, with high survival rates, many tumours recur, and some will progress to muscle-invasive disease with a much poorer long term prognosis. Thus there is a pressing need to understand the molecular transitions occurring within the progression of bladder cancer to an invasive disease. Tumour invasion is often associated with a down regulation of E-cadherin expression concomitant with a suppression of cell: cell junctions and decreased levels of E-cadherin expression have been reported in higher grade urothelial bladder tumours. We find that expression of E-cadherin in a panel of bladder cancer cell lines correlated with the presence of cell: cell junctions and the level of PAK5 expression. Interestingly exogenous PAK5 has recently been described to be associated with cell: cell junctions and we now find that endogenous PAK5 is localised to cell junctions and interacts with an E-cadherin complex. Moreover, depletion of PAK5 expression significantly reduced junctional integrity. These data suggest a role for PAK5 in maintaining junctional stability and we find that in both our own patient samples and a commercially available datasets that PAK5mRNA levels are reduced in human bladder cancer compared to normal controls. Taken together this study proposes that PAK5 expression levels could be used as a novel prognostic marker for bladder cancer progression.

Introduction

Urothelial bladder cancer (UCB) is a major cause of morbidity and mortality worldwide, causing an estimated 150,000 deaths per year [1]. Approximately 75% of patients with bladder cancer present with non-muscle-invasive bladder cancer (NMIBC) that is either confined to the mucosa (stage Ta, CIS) or to the submucosa (stage T1), while further 25% of patients present with muscle-invasive bladder cancer (MIBC) [2]. Non-muscle-invasive bladder tumours can be effectively treated in a bladder-conservative approach by transurethral resection and adjuvant intravesical therapy. Patients with non-muscle-invasive bladder cancer have good prognosis, with survival rates of up to 94% [3]. However, as many as 50-70% of these superficial tumours can recur, and 20-30% progress to muscle-invasive disease within 5 years of treatment [4, 5]. Further research to understand the cellular and molecular behaviour of urothelial cells leading to MIBC holds the promise of more effective markers and therapeutic targets for bladder cancer.

Tumour invasion and progression in bladder cancer appear to be parts of multifactorial process, promoted by intracellular and micro-environmental changes that include the upregulation of N-Cadherin and the corresponding downregulation of E-cadherin [6-9] often referred to as a cadherin switch. Loss of E-cadherin expression is also associated with epithelial to mesenchymal transition (EMT) a biological process during embryologic development where epithelial cells down regulate expression of E-cadherin and/or disassemble cell: cell junctions and become more migratory as they assume a mesenchymal cellular phenotype [10, 11]. Adoption of EMT pathways has long been associated with tumour invasion, migration and metastasis [12, 13 14]. Decreased E-cadherin immune-reactivity was first described in bladder

cancer in 1993 [15]. A number of studies then followed, which demonstrated cadherin switching in the setting of bladder cancer, associated with late stage, high grade disease [9, 16-19]. A detailed study using on cadherin switching using pT1 and T2-T3 bladder tumours [19] demonstrated that N-Cadherin expressing bladder cancer progressed more rapidly, and the majority of T2-T3 tumours demonstrated no expression of E-cadherin. Furthermore, Bryan et al [9] investigated P-Cadherin in 153 human bladder cancer specimens, and observed that in high grade, muscle invasive tumours, E-Cadherin expression was decreased, P-Cadherin expression was increased, and this cadherin switching was associated with worse bladder cancer specific survival.

The presence of adherens junctions is a defining feature of all epithelial tissue [20, 21]. The prototypical protein that forms the transmembrane core of adherens junctions is E-cadherin. Whilst the extracellular domain of E-Cadherin is responsible for homotypic, calcium-dependent interactions with E-cadherins on the surface of adjacent cells, the cytoplasmic domain of E-Cadherin interacts with the actin cytoskeleton via catenins [22, 23]. Rho family of GTPases; Rho, Rac and Cdc42 are well-known regulators of actin dynamics with important roles in adherens junctions involving cadherin-catenin complexes in epithelial cells [24]. Activated Rho-GTPases bind to a variety of effectors that regulate these cytoskeletal dynamics, and p21-activated kinases (PAKs) are one family of such effectors [25]. In the decade following the cloning and characterisation of PAK1, additional members of the human PAK-family began to emerge with the identification of PAK-isoforms 2-6. PAK family of kinases are architecturally similar and their structure can be divided into three main domains: an N-terminal PBD (p21-GTPase binding domain), a central region and a

highly conserved C-terminal serine/threonine kinase domain [26, 27]. PAKs are subdivided into two groups based on structural and functional features [28, 29]; group-1 consists of PAK1, PAK2 and PAK3, while group-2 consists of PAK4, PAK5 and PAK6. Although PAK1 has been implicated in the recurrence of urothelial cancer of the bladder [30] and progression of upper tract urothelial cancer [31], the role of other PAKs have not been well characterised in urothelial oncogenesis.

Evidence for the role of group-2 PAKs in cell junctions is emerging, albeit poorly understood. One of the studies has demonstrated that Cdc42 regulates the apical junction formation in human bronchial epithelial cells through PAK4 [32]. PAK6, which was initially identified to be an androgen receptor protein, and has recently be reported to be involved in cell: cell dissociation in response to HGF, independent of androgen receptor (AR) signalling [33] and is thought to drive colony escape [34]. Mechanistically, a complex of PAK6/E-cadherin/IQGAP1 was identified as the cells dissociate in response to HGF[35]. Group 2 member PAK5 has been shown to interact with p120-Catenin, an important component of the cadherin-catenin-complex at adherens junctions [36]. In the study, PAK5 was shown to phosphorylate p120-catenin on Serine 288, but the effect of this interaction on the adherens junctions has not been assessed. Recent reports do suggest that GFP-tagged PAK5 can be localised to cell : cell junctions [34, 37], and it was suggested that PAK5 might regulate junctional integrity, however neither study considered bladder cancer cells. This study aims to establish the expression profile of PAK family proteins in bladder cancer samples and elucidate whether PAK5 plays a specific functional role in bladder cancer cells.

Materials and Methods

Cell culture

Bladder cancer cell lines: RT4, RT112, T24, TCCSUP and 253J were gifts from Professor John Masters, University College London. Cell line authentication by STR analysis was performed on these cell lines. Bladder cancer cell lines RT4, RT112, T24, TCCSUP and 253J were cultured in RPMI-1640 medium supplemented with L-glutamine and NaHCO3, 10% foetal bovine serum and 1% penicillin/streptomycin (100U/ml penicillin and 100□g/ml streptomycin). Human embryonic kidney 293 (HEK293) cells were cultured in DMEM supplemented with glucose (4500mg/L), L-glutamine, NaHCO3, pyridoxine HCl, 10% foetal bovine serum and 1% penicillin/streptomycin (100U/ml penicillin and 100□g/ml streptomycin). Cells were incubated at 37 0C in a humidified atmosphere of 5% CO2. Bladder cancer cell lines were transfected using X-tremeGENE HP (Sigma) according to the manufacturers protocol. HEK293 cells were transfected using a calcium phosphate according to the manufacturers protocol (Sigma).

Antibodies and siRNA

Control and PAK5 specific siRNA oligonucleotides were transfected into RT4 cells using Lipofectamine RNAiMAX (Invitrogen). The PAK5 targeting sequences and control siRNA were purchased from Qiagen (Qiagen, Skelton House, UK). PAK5 =ATGGTGTGCACGTTTCATTAA or ATGATCTGGATCCGTATTATA; mouse anti-E-cadherin was purchased from Zymed USA, mouse anti-GAPDH was purchased from Sigma, mouse andti-N-cadherin was purchased from Transduction Labs , USA, mouse anti-GFP was purchased from Roche, rabbit anti-PAK1 was purchased from Cell Signalling Technology, USA, rabbit anti-PAK6 from Gentex, USA, rabbit anti-HA

and mouse anti-HSP90 were purchased from Santa Cruz Technology USA, In house rabbit anti-PAK4 has been previously described [38]. In house rabbit anti- PAK5 was raised using peptide inoculation (Eurogentec Ltd.) using a synthetic peptide with the following sequence -YREKSLYGDDLDPYY- corresponding to aa146-160 of PAK5 protein - this antibody specifically recognises PAK5 and does not cross react with PAK4 and PAK6 as tested by western analysis using overexpressed proteins (Figure S1A).

Western blotting

Cells were washed twice in PBS and lysed on ice in NP-40 lysis buffer: 0.5% v/v NP-40, 30mM sodium pyrophosphate, 50 mM Tris-HCL [38] supplemented with a Protease inhibitor cocktail: 50 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml lysates were centrifuged at 13000 x g for 10 minutes to remove cell debris. Gel sample buffer was added to the supernatant the sample boiled at 90ºC for 3 minutes. Samples were resolved by SDS-PAGE as previously described [38]. Nitrocelluloase were incubated with primary antibodies at appropriate concentrations and HRP-conjugated secondary antibodies (Dako Ltd). Autoradiographs were quantified using ImageJ software.

Immunoprecipitation

Cells were lysed as previously described, [38]. For immunoprecipitation experiments, cell lysates were pre-cleared with IgG coupled Protein A or G sepharose beads (GE healthcare) for 1 h at 4˚C. The pre-cleared lysates were then mixed with primary antibody overnight at 4˚C followed by 1 h incubation with protein A or G-sepharose

beads. Alternatively lysates were incubated with GFP-Trap®_A and purified according to the manufacturers protocol (ChromTek, Germany) The immune complexes were washed three times with lysis buffer and resuspended in 2x SDS loading buffer. Proteins were resolved by SDS-PAGE as previously described [38]. Autoradiographs were quantified using Image J software.

Immunofluorescence

Cells on coverslips were fixed with 4% PFA in PBS at room temperature and washed 3 times with PBS. The cells were then permeabilised with 0.2% Triton X-100: PBS and washed 3 times with PBS. The cells were then blocked with 3% BSA in PBS and washed 3 times with PBS. Cells were then incubated with the required primary antibody for 2 hours, and washed 3 times with PBS. Finally, cells were incubated with Alexa Fluor® 488 or 568, (Mouse/Rabbit Dako Ltd.) as required. Actin co-staining was achieved using Phalloidin of complementary fluorescent emission (Trit-C/Rhodamine/488/633 Invitrogen , UK). For nuclear staining, DAPI (1:10000 in PBS) was added following secondary antibody incubation and the coverslips were incubated for further 5 minutes. Cells were then washed 2 times with PBS and once in ddH_2O before being mounted on glass slides using 10□L FluorSave™ reagent. Cells were imaged on an Olympus IX71 inverted microscope system or Nikon A1R Confocal Microscope system.

Generation of PAK5 expression constructs

Polymerase chain reaction amplification of PAK5 cDNA was performed using the Gateway™ Technology system (Invitrogen, UK). PAK5 wild-type plasmid (kind gift of Jonathan Chernoff, Fox Chase Cancer Centre) was used as the DNA template in the

production of PAK5 DNA flanked by *att*B sequences (the addition of *att*B sequences was required to allow for subsequent cloning into Gateway™ vectors) as described in the manufacturers protocol to generate PAK5 entry clone . The PCR reaction was performed using Accuprime PFX Supermix DNA polymerase. In order to generate GFP-, RFP-, myc-, and HA-PAK5 expression plasmids, Gateway™ LR recombination reactions were performed between the pENTR (PAK5) entry clone and the selected destination vectors including modified pEGFP-C1 (Clontech, UK), pDEST™ mRFP, and pDEST™ myc (pRK5-myc) in accordance with the manufacturer's instructions.

Image processing and cell shape analysis

Image J software was used to elucidate the morphology of each cell by manually drawing around individual cells and then processing the data to give a circularity score. The autoradiographs were saved as TIF files in Adobe Photoshop CS5 and ImageJ software was used to quantify desired protein levels. In this analysis system it was assumed that 0 is black and the maximal value is 255 at 8 bits per pixel. These values were then used to calculate the mean fold value.

Real-time reverse transcription quantitative PCR

Real-time quantitative PCR, based on the Amplifluor™ technology, was to quantify the mRNA expression level of Aurora kinases from above cDNA samples of breast tissues following the method previously reported[39]. The pairs of Q-PCR primers were designed with Beacon Designer software which include complementary sequence to universal Z probe (Intergen Inc., Oxford, United Kingdom). The pair Q-PCR primers sequences are shown in X. Real-time PCR was carried out using an IcyclerIQ™ (Bio-Rad, Hemel Hempstead, UK) kit with the following cycling conditions: 94°C for

12 minutes, 60 cycles of 94°C for 15 seconds, 55°C for 40 seconds (the data capture step) and 72°C for 20 seconds. The transcripts levels were generated from an internal standard which was simultaneously amplified with the samples.

Results

Differential morphology of bladder cancer cell lines in vitro

Many bladder cancer cell lines have been characterised and matched to represent clinical tumour stages and grades. The characterisation was based on their tumour of origin, cell morphology in culture, gene and protein expression, tumorigenicity and xenograft morphology [40-45]. A number of cell lines were selected to represent urothelial cell lines at different stages of tumour progression, corresponding to well-differentiated, moderately-differentiated and poorly-differentiated primary urothelial tumour. RT4 is a paradigm for well-differentiated bladder carcinoma. This cell line was established from primary bladder tumour [46], where the histological appearance of the original tissue cultured was of a well differentiated, low grade urothelial carcinoma of the bladder. RT112 is a moderately-differentiated urothelial carcinoma cell line which originated from a histology grade-2 (G2) papillary bladder tumour. In steady state, RT112 cells retain the epithelial-like morphology, in which the cells grow in distinct colonies to form an epithelial sheet in 2D culture [47, 48]. T24 and TCCSUP cell lines both originated from poorly differentiated, high grade primary urothelial tumours [49-51]. The cell line 253J originated from metastatic retroperitoneal lymph node of a male patient with high grade (historically grade-4) bladder tumour with lymph node, bone and cerebral metastasis [49, 51]. RT112 and RT4 cells both grew in distinct colonies which can form an epithelial sheet in 2-D, where the archetypal epithelial cell-cell junctions were conserved. Accumulation of F-actin at the margins of cell-cell junctions was prominent in these cell lines **(Figure 1A),** which is also a phenotype commonly associated with epithelial cells [52]. Actin-based membrane protrusions such as filopodia or lamellapodia were rarely observed in RT4 cells when cultured in basal growth condition. T24, TCCSUP and 253J cells

grow as detached cells in 2-D culture (Figure 1A). Distinct formation of cell: cell adhesion characterised by accumulation of F-actin at cell contact margins was not seen in these cells lines, even when the margins of adjacent cell were in contact with another. The differences in cell morphology suggested bladder tumour cells (T24, TCCSUP and 253J) had lost their epithelial morphology. We would predict that these cells have undergone epithelial to mesenchymal transition, whereas RT4 and RT112 cells still retained their epithelial morphology when cultured in basal growth conditions. Consistent with the epithelial cell morphology seen previously, RT4 and RT112 cells retained the protein expression of E-cadherin, with no detectable expression of N –cadherin under basal growth conditions **(Figure 1B)**. Conversely, T24, TCCSUP and 253J cells have lost the protein expression of E-cadherin, and consistent with the archetypal cadherin switch model, have gained the expression of N-cadherin **(Figure 1B).**

Differential integrity of E-cadherin mediated cell: cell junctions in bladder cancer cell lines

Our expression and morphological analysis would predict that RT4 and RT112 cells would have E-cadherin mediated cell: cell junctions. We therefore sought to localise E-cadherin in RT112 and RT4 cells under basal growth conditions. In RT4 cells, the e-cadherin staining at cell-cell junction was uniform and evenly distributed to form distinct E-cadherin plaques along the areas of cell-cell contact margin **(Figure 1C).** However, the e-cadherin staining in RT112 cells displayed a more disrupted or ‘zippered’ appearance, where multiple E-cadherin-containing punctae cluster at varying intensities along the contact margins **(Figure 1C).** For cells forming epithelial sheets, three stages of cell-cell adhesion and colony formation have been

proposed [22, 52, 53]. Stage 1 corresponded with immature junction which loosely hold cells together. At stage 2, E-cadherin plaques develop at the edges of the contact, and at stage 3, the E-cadherin plaques cinch together to form multi-cellular vertices, further condensing the cell colonies, which indicated mature and stable cell-cell adherens junctions. The fluorescence of E-cadherin signal was plotted to represent immunofluorescence intensity at the region of cell-cell contact in both these cell lines **(Figure 1Ci)**. The formation of continuous of E-cadherin plaques at the margins of cell-cell contact in RT4 cells were consistent with more established and mature cell-cell junctions (stages 2-3), whereas the interrupted staining pattern in RT112 cells suggested less mature formation of epithelial cell-cell junctions(stages 1-2).

Bladder cancer cells express multiple Group II PAKs with differential expression levels.

Having characterised our panel of bladder cancer cell lines we then sought to establish the profile of group II PAK expression. Previous work has demonstrated that high expression of PAK1 at both the gene transcription and protein expression levels was associated with highest grade bladder cancer. Thus as a control we initially tested our panel of cells lines for the presence of PAK1 protein . Immunoblotting with a PAK1 specific antibody revealed expression of PAK1 with high total protein levels in RT4 and TCCSUP cells compared to the other cell lines examined **(Figure 2A)**. However, whilst PAK1 was differentially expressed, the protein levels of PAK1 *per se* did not show any direct correlation with the epithelial differentiation of the cell lines within this panel. We next tested for PAK4, PAK5 and PAK6 expression using isoform specific antibodies. Interestingly, whilst the expression profile of PAK4 and PAK6 across the cell lines was ubiquitous, there was

no differentiation between the cell lines of highly invasive origin and low grade papillary origin **(Figure 2B&C)**. In contrast using an in house PAK5 specific antibody **(Figure S1A)** we found that PAK5 expression **(Figure 2D)** was restricted to those cells of epithelial morphology RT4 and RT112 that still expressed e-cadherin **(Figure 1B)** and exhibited junctional E-cadherin localisation **(Figure 1C).** Moreover, relative PAK5 expression was highest in RT4 cells which contain extremely mature junctions **(Figure 1D).** Furthermore we have observed the same expression profile in breast and pancreatic cancer cell lines where PAK5 is expressed in those cells with E-cadherin positive junctions but not in those cells that do not form cell: cell contacts **(Figure S1B).**

Endogenous PAK5 is localised at cell: cell junctions in bladder cancer cells

Our data suggests that cells with higher levels of PAK5 expression are more likely to be colony forming. Interestingly it was recently reported that PAK5 could be localised to cell: cell junctions [34, 37] although this localisation was based on exogenous overexpression. Using our PAK5-specific antibody **(Figure S1A)** we were able to detect endogenous PAK5 at cell: cell junctions in both RT4 **(Figure 3A)** and RT112 cells **(Figure S1C)**. Indeed, line scans revealed a strong correlation between PAK5 staining and E-cadherin staining in these cells **(Figure 3B).**

PAK5 interacts in the E-cadherin complex in bladder cancer cells

The localisation of endogenous PAK5 suggest that PAK5 may be an integral cell junction protein. We therefore tested whether PAK5 and E-cadherin were in a protein complex in RT4 bladder cancer cells. We were able to demonstrate that endogenous E-cadherin is present in an endogenous immunoprecipitation of PAK5 but not in the

control sample. Equally we were able to demonstrate that endogenous PAK5 is present in an immunoprecipitation of endogenous E-cadherin **(Figure 3C).** Moreover, we could also demonstrate this interaction in RT112 cells **(Figure S2A)** Having established that PAK5 and E-cadherin are in a complex we proceeded to dissect the interaction in more detail by establishing whether E-cadherin binds to the PAK5 N- or C- terminal. HA-tagged full length PAK5, HA-tagged PAK5-N-terminal and HA-tagged C-terminal PAK5 were co-expressed with GFP-E-cadherin in the HEK293 cell line to maximise protein production. GFP-E-cadherin was isolated from cell lysates using GFP-TRAP and the isolate tested for the presence of PAK5 variants using an anti-HA antibody. Whilst full length PAK5 and N-terminal PAK5 were clearly present in the E-cadherin isolate there was no evidence to suggest that C-terminal PAK5 could interact with E-cadherin.

Depletion of PAK5 expression promotes junctional disruption

The differential expression level of PAK5 in cells which formed colonies, taken together with the localisation of PAK5 at cell: cell junctions and the interaction between PAK5 and E-cadherin lead us to speculate that PAK5 might play a role in junctional integrity. To test this hypothesis we depleted PAK5 expression in RT4 cells using two different siRNA oligos. Maximal knockdown of PAK5 expression was achieved 48 hours post treatment **(Figure 4A)** and PAK5 levels could be seen to be recovering at 72 hours (data not shown). Thus experiments were conducted within the 48 hour window. To ascertain the impact on junctional formation of loss of PAK5 expression cell colonies were stained for E-cadherin **(Figure 4B)** and scored for the presence of less than or more than 50% adherent contact as defined in **Figure 4C.** There was significant reduction in cell: cell contact when RT4 cells were depleted of

PAK5 expression using two specific siRNA oligos. Moreover, PAK5 depleted cells exhibited an increased circularity **(Figure 4E)** that would be expected from the definition criteria **(Figure 4C).** Importantly re-expression of siRNA resistant PAK5 decreased circularity (Figure 4E and Figure S2C arrow versus arrowhead). Whilst we detected a loss of junctional integrity in our PAK5 depleted cells we did not see any evidence that PAK5 transcriptionally regulates E-cadherin expression. Global overexpression PAK5 does not elevate E-cadherin levels (Figure S2D). Moreover, exogenous expression of PAK5 in T24 cells did not promote E-cadherin expression nor the establishment of E-cadherin mediated junctions in cells of close proximity (Figure S2E arrowhead).

PAK5 transcripts are significantly reduced in invasive disease.

Taken together our data supports a role for PAK5 in stabilising cell: cell junctions via an interaction with the E-cadherin complex. We might therefore speculate that a loss of PAK5 expression might correlate with disease progression in bladder cancer. To test this hypothesis we investigated a small cDNA library of human bladder tissue samples from Cardiff University which consisted of 34 samples of tumour tissue and 18 samples of bladder tissue without any malignancy. We screened for expression of PAK1 and PAK5. The mRNA expression of PAK1 has been previously studied in bladder cancer and was shown to be associated with increased risk of tumour recurrence [30]. For quantitative analysis of PAK1 and PAK5 transcripts we employed a quantitative real-time polymerase chain reaction qRT PCR assay. The levels of the transcripts shown here are the ratios of respective PAK transcripts normalised to the level of GAPDH **(Figure 5A).** The values obtained from the assay indicated that in human patient samples, PAK1 transcripts were expressed at higher levels in

malignant urothelium, compared to non malignant epithelium. Excitingly, the expression of PAK5 mRNA was lower in malignant epithelium compared to non-malignant bladder tissue **(Figure 5A).** However, it was noted that the mRNA expression obtained in this assay varied greatly between each individual samples, even those within the subgroup (malignant/non-malignant), as indicated by the large error. Due to this variation, the differential expression of PAK1 and PAK5 detected whilst strongly suggesting an opposing trend did not achieve statistical significance. As the mRNA expression profile for PAK5 obtained from bladder tissue samples was underpowered **(Figure 5A)** we turned to a publicly available microarray data which included PAK5 expression in bladder cancer tissue on Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) to expand our studies. One set of data was available which included the mRNA expression of PAK5 (referred to as PAK7 in the search) in 60 bladder tissue specimens with complete histopathological staging and grading of the tumour. The microarray data was linked to a publication on carcinoma in situ (CIS) of the bladder [54], in which the association between PAK5 and CIS was not identified, or discussed. The data in the microarray (GEO: GPL96, 213990_s_at (ID_REF), GDS1479, 57144) had been normalised using the Robust Multi-array Analysis (RMA). First, the data were analysed by comparing the mRNA transcription of PAK5 in normal urothelial specimen in this population (including normal urothelium obtained from patients with past history of urothelial cancer), and specimen from malignant urothelium of all grades and stages. PAK5 expression was higher in normal bladder tissue, and was downregulated in tumour tissues **(Figure 5B),** similar to the finding of PAK5 mRNA expression in Cardiff cohort **(Figure 5A).** In addition, the difference was statistically significant. To further analyse of PAK5 expression in this dataset, we subdivided the tumours into the following 4 groups to indicate the

risk of bladder cancer progression, and progressively poorer prognosis: Normal bladder tissue, Low risk non-muscle invasive bladder tumours (stage: pTa, grade: 2, no CIS), Moderate to high risk non-muscle invasive bladder tumours (stage pTa-1 grade 2 with CIS, CIS, all stage pTa-1 grade 3 tumours), Muscle invasive bladder tumours (tumour stage T2 and above). Analysis of PAK5 mRNA levels according to these subgroups also indicated that PAK5 mRNA transcription were lower in tumours of all subgroups, compared to normal bladder tissues **(Figure 5C)** Statistical analysis comparing the expression levels between the 4 subgroups (one-way analysis of variance, ANOVA) detected significant difference in PAK5 mRNA expression in bladder cancer compared to normal tissue **(Figure 5C).**

Discussion

We describe here a specific role for PAK5 in junctional integrity. We find that there is a positive correlation between PAK5 and E-Cadherin protein expression in well and moderately differentiated urothelial cancer cell lines (RT4 and RT112) which form cell-cell adherens junctions. Conversely, low or undetectable protein expression of PAK5 was associated bladder cancer cell lines with mesenchymal phenotype, lacking in cell-cell adherens junctions (T24, TCCSUP and 253J). We report that endogenous PAK5 is co-localised with E-Cadherin at cell-cell adherens junctions. Furthermore, we find that PAK5 directly interacts with the E-Cadherin complex and functions to stabilise junctions in colony forming cell lines.

The clinical outcomes related to cancer diagnosis and treatment for many cancers have improved over the last decades but not so for bladder cancer, for which mortality has changed very little in the last few decades in England and Wales [55]. Most patients with MIBC will still succumb to the disease associated with metastasis despite radical treatment regimens [56, 57], while patients with NMIBC are faced with lifelong surveillance due to the characteristic high recurrence rate of urothelial cancer [58]. Although the molecular events that characterise urothelial carcinoma are increasingly defined and our understanding of the relevant pathways and networks has evolved, it is disappointing that no significant markers either for diagnosis of MIBC or NMIBC, let alone a marker able to risk stratify recurrence or relative prognosis have gained widespread acceptance and consistent validity. One of the proteins that can potentially differentiate urothelial tumours into low or high risk of disease progression is E-Cadherin. E-Cadherin is the major mediator of cell-cell junctions in epithelial tissues, and is expressed by most epithelial cells [59]. Decreased

E-cadherin immune-reactivity was first described in bladder cancer in 1993 [15]. A number of studies then followed, which demonstrated cadherin switching in the setting of bladder cancer, associated with late stage, high grade disease [9, 16-19]. A detailed study using on cadherin switching using pT1 and T2-T3 bladder tumours [19] demonstrated that N-Cadherin expressing bladder cancer progressed more rapidly, and the majority of T2-T3 tumours demonstrated no expression of E-cadherin. In our study we find that PAK5 expression is high in cell lines with junctional formation but is almost entirely lost from those cells that no longer express E-cadherin. Moreover we find differential levels of PAK5 mRNA expression in human normal bladder tissue compared to carcinoma samples. Our findings are not in agreement with some previous reports that suggest PAK5 expression increases with malignant disease in colorectal [60, 61], gastric [62] and hepatocellular carcinoma [63]. However, on reviewing these publications, the sensitivity and specificity antibodies used to detect PAK5 could have contributed significantly to the conflicting results. For example in the colorectal study [61], the isoform specificity of the polyclonal antibody has not been well described. The epitope for antibody recognition was generated against 224-amino acids N-terminal sequence of PAK5, and cross reaction with PAK4 was excluded. This could be significant as a recent classification of bladder cancer sub typing identified increased expression of PAK2 and PAK4 in high grade tumours of poor prognosis but not PAK5[64] in line with our findings. Moreover, a recent screen of 317 cancer cell lines for levels of PAK5 expression concluded that even those cells with relatively high PAK5 mRNA levels were low compared to PAK4 and PAK2 [37]. We now propose that loss of PAK5 expression can be considered as a potential novel prognostic marker of bladder cancer progression.

Our results clearly indicate a localisation and functional significance for PAK5 at adherens junctions . Indeed, GFP-tagged PAK5 was also reported to be localised at cell: cell junctions in canine kidney cells[37] and prostate cancer cells[34]. However the mechanism of function remains to be elucidated. We find that the N-terminal region of PAK5 preferentially interacts with E-cadherin. Interestingly it has recently been reported that the N-terminal Cdc42 binding region of PAK6 is required to localise this protein to cell: cell junctions. Thus, it may be the case that binding of Cdc42 also localises PAK5 to cell: cell junctions. We and others[34] , propose that PAK6 drives colony escape. Whilst work presented here supports a role for PAK5 in junction stabilisation. However, overexpression of GFP-tagged PAK5 was reported to disrupt cell: cell junctions in DU145 prostate cancer cells, thus opposing the role we propose here for PAK5[34]. It should be noted that DU145 cells are not thought to express detectable levels of endogenous PAK5[34] and thus overexpression in this system might emulate a PAK6 rather than PAK5 physiological function as the two family members are structurally related. In our work we have concentrated on working with endogenous PAK5. We found no evidence to suggest that PAK5 can regulate the expression level of E-cadherin. Rather, given that PAK5 is also reported to bind to p120 catenin we would predict a functional E-cadherin focussed unit at the cell junction that becomes destabilised when PAK5 is depleted**.** It would be of interest to monitor the localisation/mis-localisation of junctional proteins in PAK5 depleted cells. The presence of N-terminal PAK5 in a complex with E-cadherin would suggest that PAK5 performs a scaffolding function within this unit but we cannot rule out a possible C-terminal substrate interaction with a junctional protein. Further work is required to characterise the full interaction between PAK5 and adherens junction proteins.

Declarations of Interest

We declare we have no competing interests

Funding information

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Author contribution Statement

AFI conducted the majority of experiments. TAM and WGJ supervised the RT-PCR and provided samples. NB conducted western analysis. SO conducted some siRNA experiments. MDP conducted interaction studies. MLSK and PD supervised experiments and oversaw statistical analysis of database studies. CW and AFI conceived the experiments and wrote the paper.

Figure Legends

Figure 1: Characterisation of E-cadherin expression status in bladder cancer cell lines

A) Immunofluorescent phalloidin staining for F-actin in RT4, RT112, T24, 253J, and TCCSUP cells at 40-60% subconfluent monolayer. Cell co-stained for DAPI Scale bar = 10□m. **B)** Cell lysates were prepared from RT4, RT112, T24, 253J, and TCCSUP cells. Lysates were probed for expression of E-cadherin/N-cadherin and GAPDH was used as a loading control. **C)** RT4 and RT112 cells were stained for E-cadherin **Ci)** Immunofluorescence and line scan of E-cadherin staining at cell: cell junction of RT4 and RT112 cells. The boxes approximately register the edges of the contact plotted for fluorescence (arbitrary unit). Images shown are representative of at least 3 independent experiments. Bar = 10μm.

Figure 2: PAK family expression profile in bladder cancer cell lines

Cell lysates were prepared from RT4, RT112, T24, 253J, and TCCSUP cells. Lysates were probed for expression of PAK1 (A) , PAK4 (B) and PAK6 (C) and PAK5 (D) in all experiments GAPDH was used as a loading control. Autoradiographs were scanned and quantified for expression from 3 independent experiments.

Figure 3:PAK5 is localised at cell: cell junctions

A) RT4 cells were stained for PAK5, DAPI and E-cadherin bar = 10mm B) co-localisation analysis of PAK5 and E-cadherin signal using confocal line scanning. **C)** immunoprecipitation experiments isolating endogenous E-cadherin or endogenous PAK5 and probing for presence of PAK5 or E-cadherin in RT4 cells. Whole cell

lysates (WCL) also probed for E-cadherin/PAK5 for size comparison. **D)** HEK293 cells were transfected with GFP-Ecadherin and HA- PAK5 derivatives as indicated. GFP-E-cadherin was isolated by GFP-TRAP reagents and probed for the presence of HA-tagged PAK5 derivatives. Whole cell lysates of input were also probed (input) to confirm double transfection Heat Shock protein 90 (HSP90) was used as a loading control.

Figure 4: Loss of PAK5 expression modulates cell junction integrity

A) RT4 cells were treated with control or PAK5 specific siRNA oligos (si63 and si70). Cell lysates were harvested at 48 hours post treatment and probed for PAK5 and GAPDH as a loading control. Autoradiographs were scanned and quantified for expression from 3 independent experiments. **B)** RT4 cells were treated with control or PAK5 specific siRNA 70 oligos fixed 48 hours post treatment and stained for E-cadherin and DAPI. Arrows indicate cells with <50% membranous cell: cell adherent contact **C)** illustration of scoring criteria for junctional integrity. **D)** Cell images from RT4 cells treated with control or PAK5 specific siRNA oligos were scored as described in (C) **E)** individual cells control , treated with si63 or si70 or treated with si70 and transfected with siRNA resistant GFP-PAK5 were measured for circularity using ImageJ software.

Figure 5:Differnetial PAK5 mRNA levels in patient samples

A) Quantitative RT-PCR of human normal and bladder cancer samples expression levels were normalised to the GAPDH signal. 34 samples of tumour tissues and 18 samples from bladder tissues without any malignancy were screened for expression of PAK1 and PAK5 using primers detailed in Figure S2B. **B)** Data in the microarray

(GEO: GPL96, 213990_s_at (ID_REF), GDS1479, 57144) dataset were analysed by comparing the mRNA transcription of PAK5 in normal urothelial specimen in this population (including normal urothelium obtained from patients with past history of urothelial cancer), and specimen from malignant urothelium of all grades and stages. **C)** Dataset analysis comparing the expression levels between the 4 subgroups indicated (one-way analysis of variance, ANOVA).

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Abbreviations

Urothelial bladder cancer (UCB)

non-muscle-invasive bladder cancer (NMIBC)

muscle-invasive bladder cancer (MIBC)

epithelial to mesenchymal transition (EMT)

p21-activated kinases (PAKs)

PBD (p21-GTPase binding domain)

androgen receptor (AR)

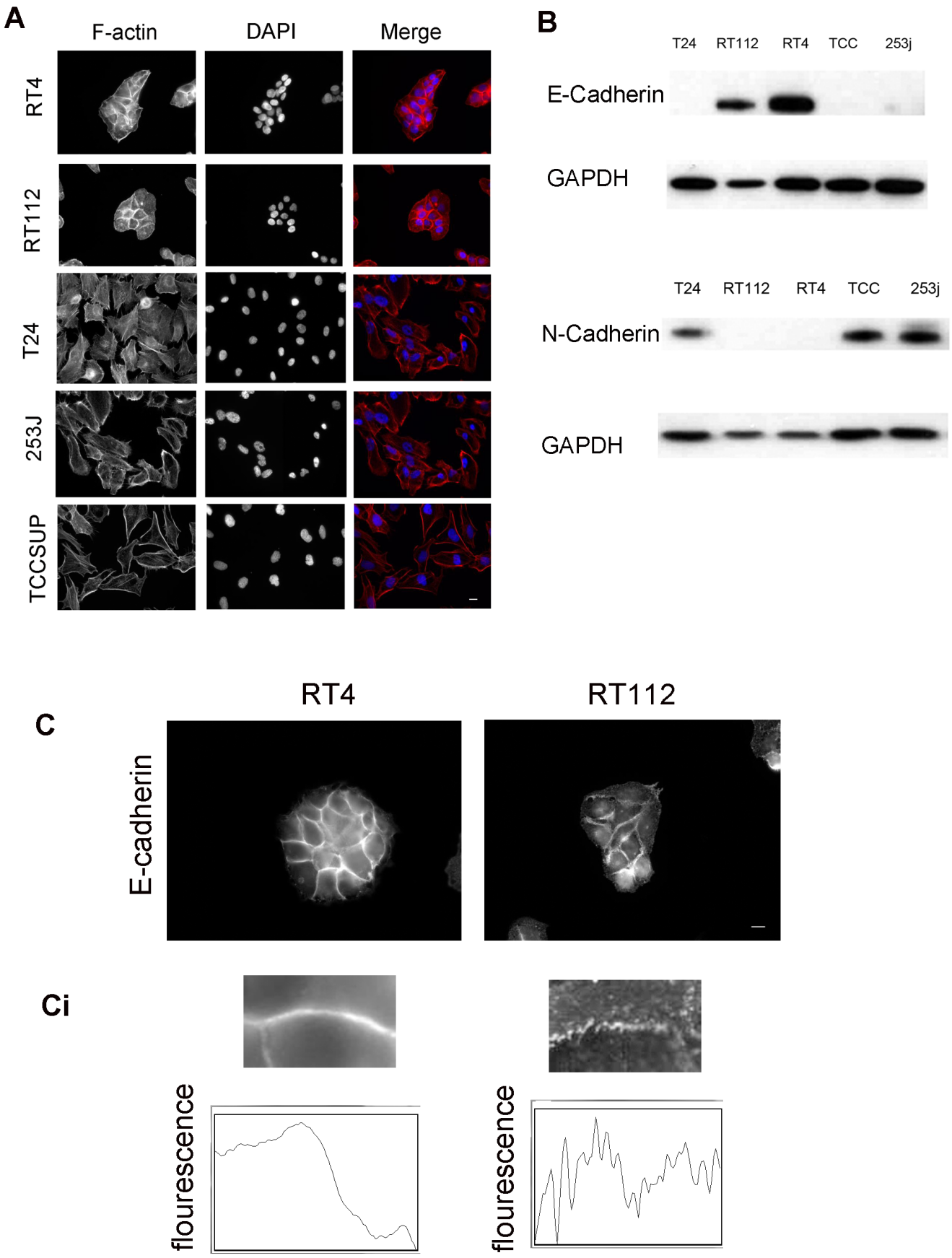
grade-2 (G2)

carcinoma in situ (CIS)

one-way analysis of variance (ANOVA)

Human embryonic kidney 293 (HEK293)

Figure 1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F-actin
DAPI
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RT4
RT112
T24
253J
TCCSUP
B
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E-Cadherin
GAPDH
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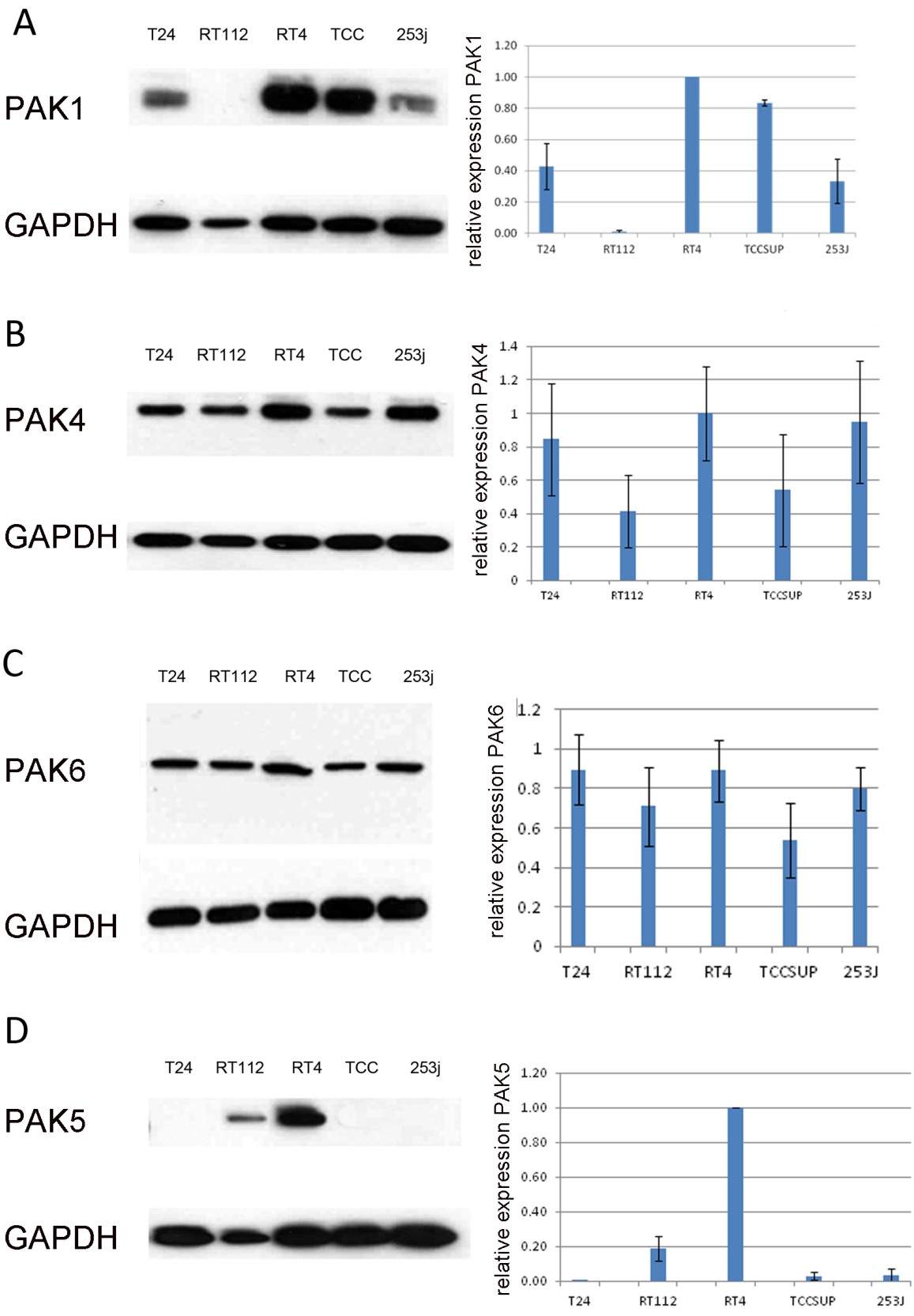
N-Cadherin
GAPDH
C
RT4
RT112
E-cadherin
Ci
flourescence
flourescence

Figure 2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PAK1
GAPDH
relative expression PAK1
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0.60
0.40
0.20
0.00
T24 RT112 RT4 TCCSUP 253J
B
T24 RT112 RT4 TCC 253j
PAK4
GAPDH
relative expression PAK4
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1.2
1
0.8
0.6
0.4
0.2
0
T24 RT112 RT4 TCCSUP 253J
C
T24 RT112 RT4 TCC 253j
PAK6
GAPDH
relative expression PAK6
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1
0.8
0.6
0.4
0.2
0
T24 RT112 RT4 TCCSUP 253J
D
T24 RT112 RT4 TCC 253j
PAK5
GAPDH
relative expression PAK5
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0.40
0.20
0.00
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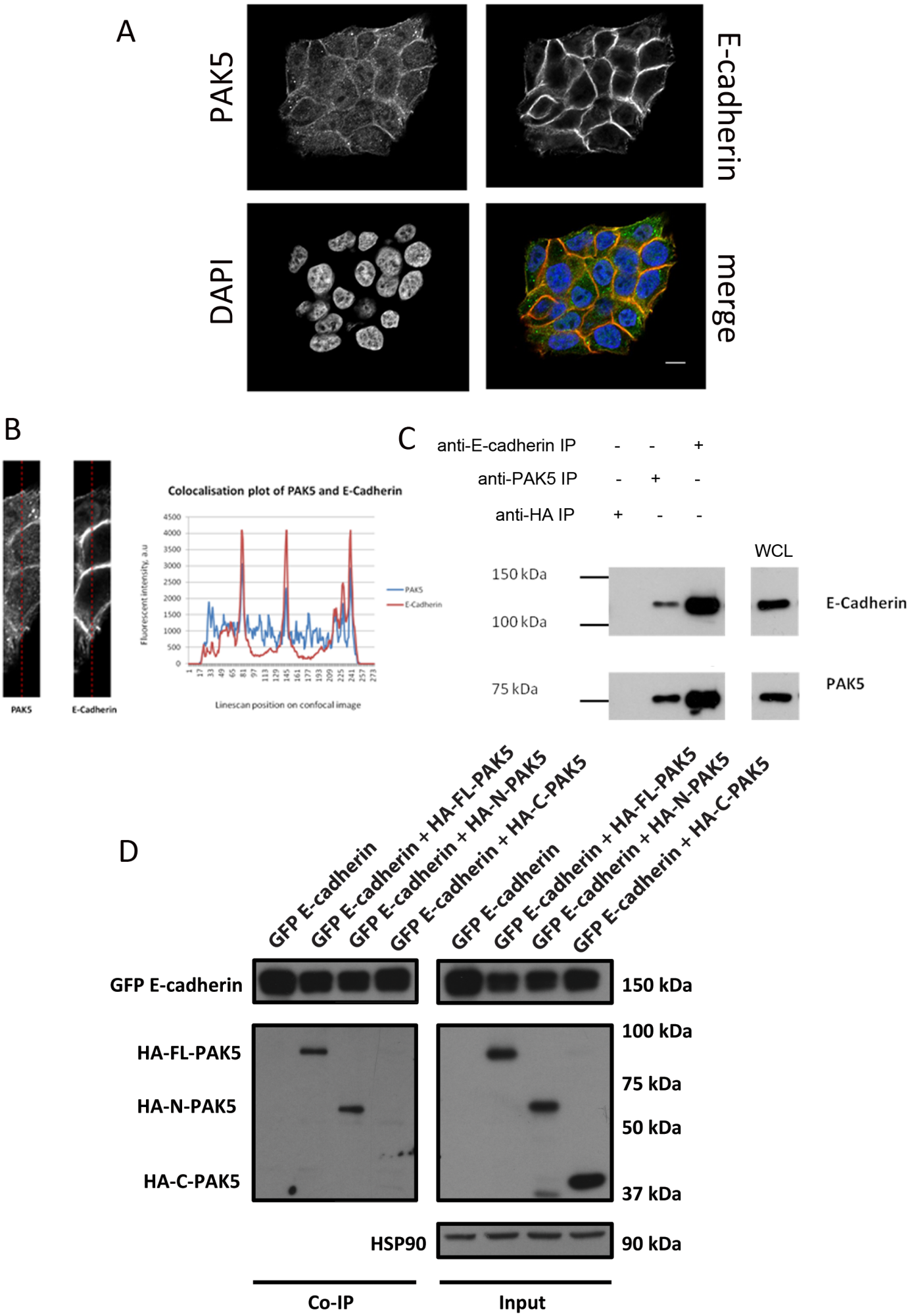
Figure 3


A
PAK5
E-cadherin
DAPI
merge
B
PAK5
E-Cadherin
Colocalisation plot of PAK5 and E-Cadherin
Fluorescent intensity, a.u
Linescan position on confocal image
PAK5
E-Cadherin
C
anti-E-cadherin IP - - +
anti-PAK5 IP - + -
anti-HA IP + - -
WCL
150 kDa
100 kDa
75 kDa
E-Cadherin
PAK5
D
GFP E-cadherin
GFP E-cadherin + HA-FL-PAK5
GFP E-cadherin + HA-N-PAK5
GFP E-cadherin + HA-C-PAK5
GFP E-cadherin
GFP E-cadherin + HA-FL-PAK5
GFP E-cadherin + HA-N-PAK5
GFP E-cadherin + HA-C-PAK5
GFP E-cadherin
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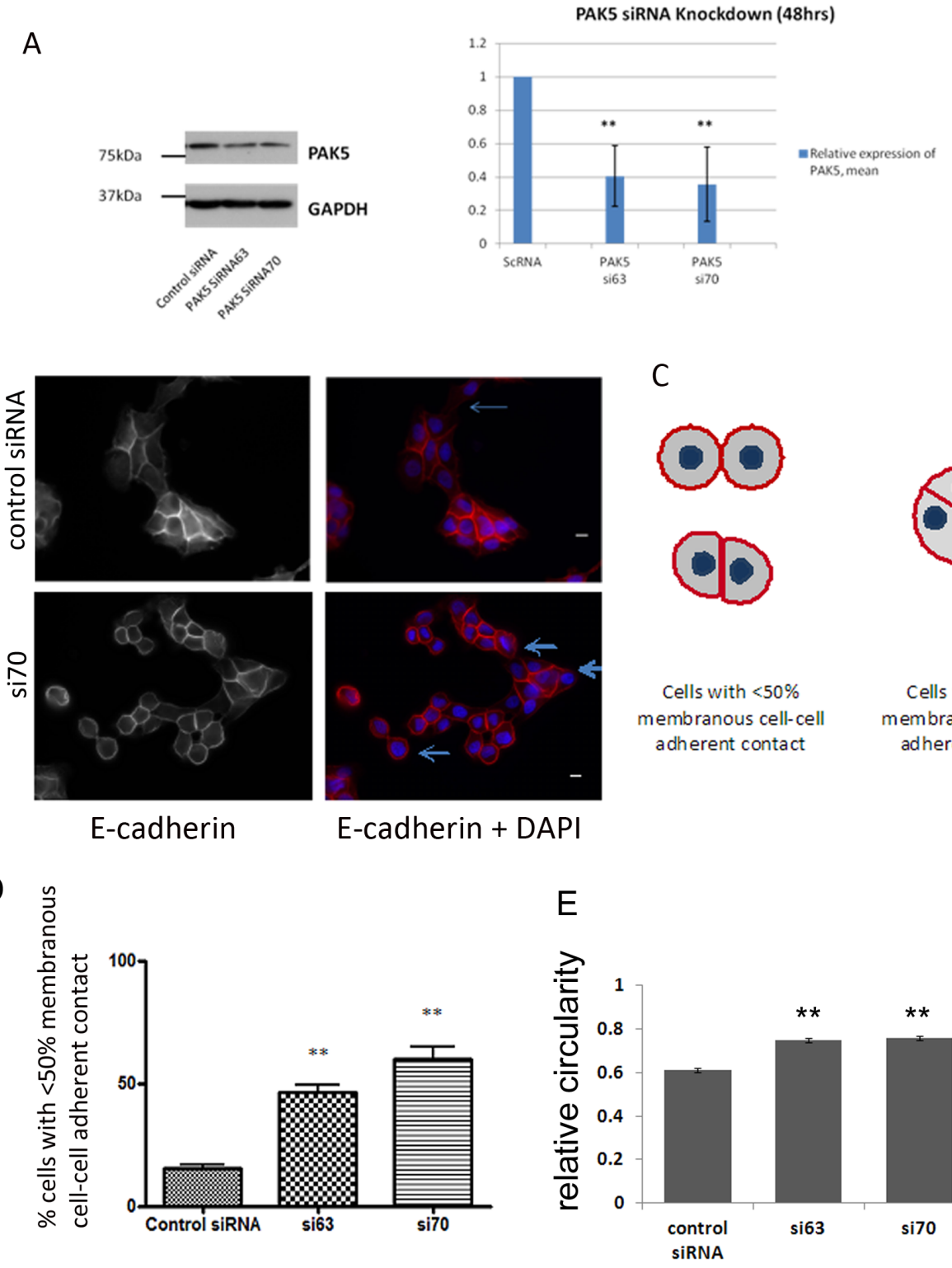
75 kDa
50 kDa
37 kDa
90 kDa
Co-IP
Input

Figure 4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PAK5
37kDa
GAPDH
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PAK5 SiRNA70
PAK5 siRNA Knockdown (48hrs)
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1
0.8
0.6
0.4
0.2
0
**
**
ScRNA
PAK5 si63
PAK5 si70
Relative expression of PAK5, mean
B
control siRNA
si70
E-cadherin
E-cadherin + DAPI
C
Cells with <50% membranous cell-cell adherent contact
Cells with >50% membranous cell-cell adherent contact
D
% cells with <50% membranous cell-cell adherent contact
100
50
0
**
**
Control siRNA
si63
si70
E
relative circularity
1
0.8
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0.4
0.2
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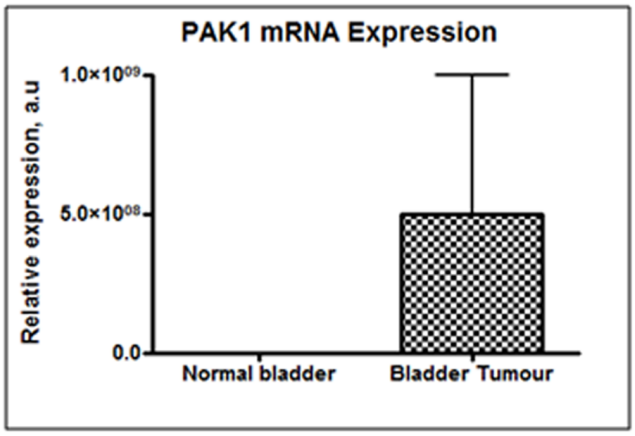
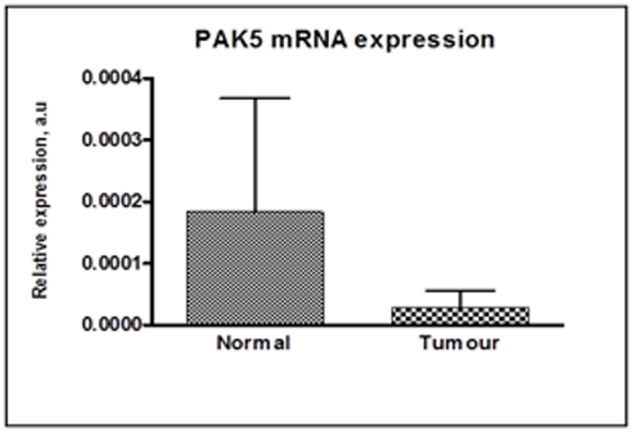
**
control siRNA
si63
si70
si70+PAK5r

Figure 5

A





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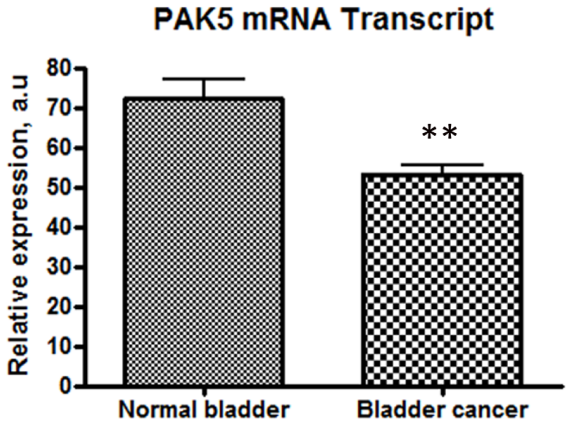

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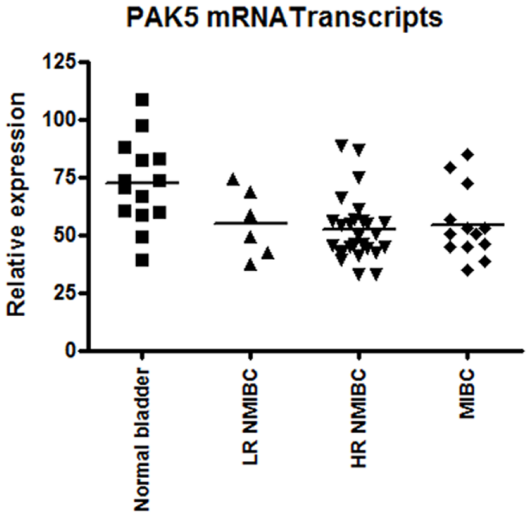


PAK5	
One-way analysis of variance	
P value	0.0024
P value summary	**
Are means significantly different? (P < 0.05)	Yes
Number of groups	4

Supplementary Figure Legends

Figure S1

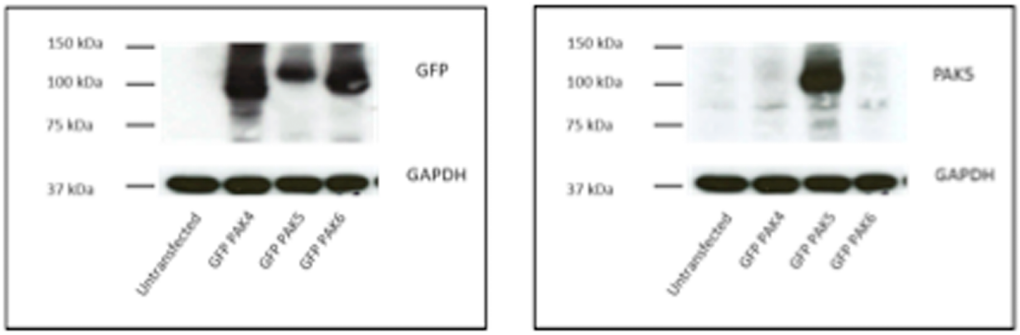
A) The in house PAK5 antibody was tested against whole cell lysates overexpressing GFP-PAK4, GFP-PAK5 and GFP-PAK6. Only GFP-PAK5 was detected (panel 2) despite high levels of Expression (panel 1) **B)** Cell lysates form cells derived from other tissue types as indicated were probed for E-cadherin, PAK5 and GAPDH expression. **C)** RT112 cells were fixed and stained for endogenous PAK5 , E-cadherin and DAPI bar = 10μm

Figure S2

A) immunoprecipitation experiments isolating endogenous E-cadherin or endogenous PAK5 and probing for presence of PAK5 or E-cadherin from RT112 cells. Whole cell lysates (WCL) also probed for E-cadherin/PAK5 for size comparison. **B)** primer sequences used in quantitative RT-PCR (Figure 5) **C)** HEK293 cells were transfected with GFP alone or GFP-PAK5 and whole cell lysates probed for E-cadherin, PAK5 and GAPDH as a loading control. **D)** RT4 cells were treated with si70 siRNA specific to PAK5 and subsequently transfected with GFP-PAK5 si70 resistant. Cells were fixed and stained for F-actin. GFP-positive cells (arrow) are less circular than si70 treated cells (arrow head) and regain cell: cell contact length. **E)** T24 cells were transfected with GFP-PAK5 , fixed and stained for F-actin and E-cadherin. Arrow head indicates region of contact with lack of E-cadherin positive staining Bar =10μm

Figure S1

A



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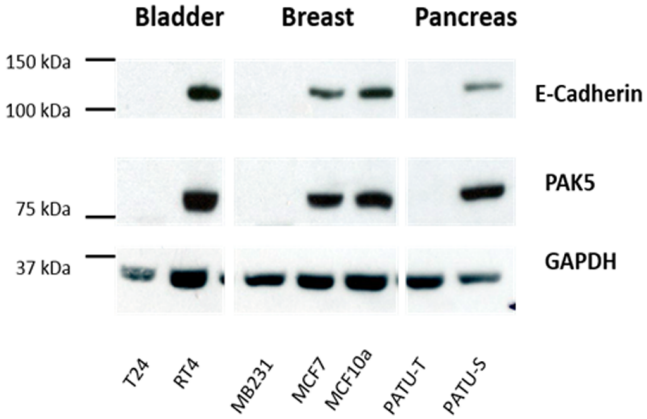

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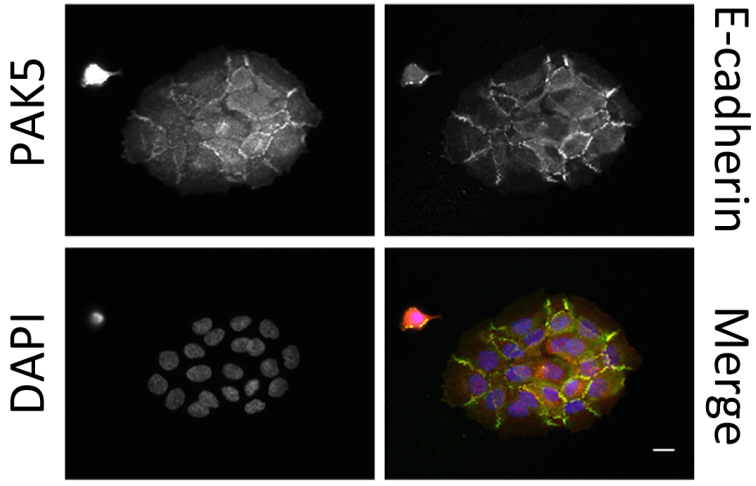
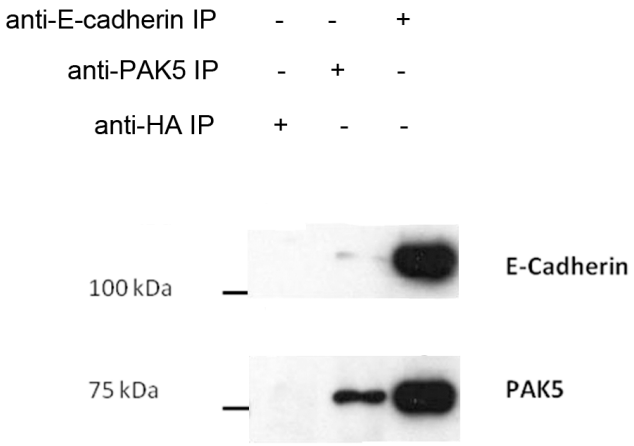


Figure S2

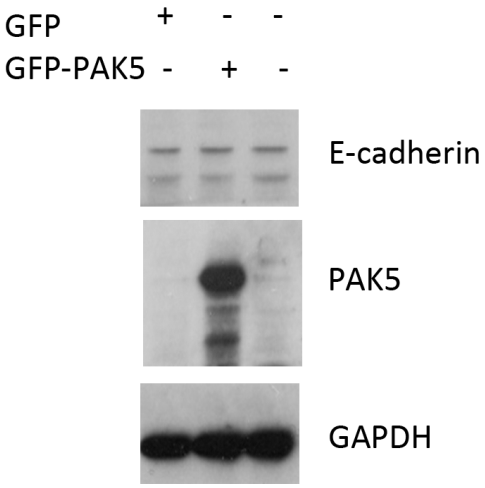
A



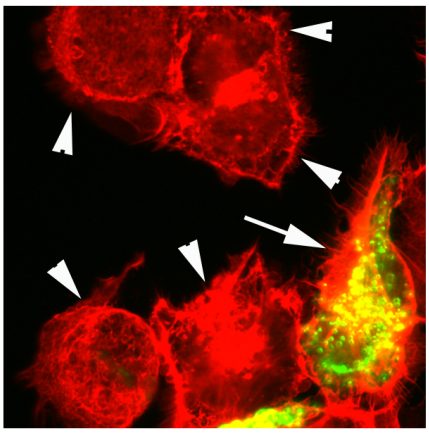
B

Target	Sequence	
GAPDH	F	GTGAAGGTCGGAGTCAACG
	R	ACTGAACCTGACCGTACA-TGAGGTCAATGAAGGGGTC
PAK1	F	CCAGTGATTGCTCCACGCCCA
	R	ACTGAACCTGACCGTACA-GGGAGATGTAGCCACGTCCCGA
PAK5	F	CCTCAGCCTCTCATCCAGCACC
	R	ACTGAACCTGACCGTACA-AGGGCCGCCCGAAACTGTTC
PAK6	F	ACAAGCAGATGCCGTGGCCC
	R	ACTGAACCTGACCGTACA-CGCTGCGAGGCACCCTGAAA

D



C



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