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Reduction in tumour cell invasion by pigment epithelium-derived factor is mediated by membrane type-1 matrix metalloproteinase downregulation

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Prostate cancer and breast cancer are major killers among males and females respectively. In this study, pigment epithelium-derived factor (PEDF) was examined for its effect on commonly used human prostate cancer and human breast cancer cell lines. PEDF increased adhesion of cells to collagen-I, with decreased expression of phosphorylated focal adhesion kinase (p-Fak) consistent between the two cell types. Invasion of both tumour cell types through collagen-I was also reduced by PEDF, with decreased expression of membrane type-1 matrix metalloproteinase (MT1-MMP). These results were confirmed with specific antibodies to MT-MMP1. This study provides some vital clues as to which molecular players are perturbed by PEDF treatment of human prostate and breast cancer cells, raising hope that PEDF can in future be trialled against these major cancers in attempts to procure safer yet effective therapies for cancer.

1. Introduction

Prostate cancer is the most frequently diagnosed cancer in men (American Cancer Society 2011). Prostate cancer has an osteotropic predilection as a site for metastasis (Bruera and Sweeney 2003). Residence of these tumours in bone is usually accompanied by severe and debilitating pain. The 15-year survival rate from prostate cancer is 79%, mostly due to better diagnostic tools available today. However, there is a need for better forms of therapy, and the hunt for biological therapy, where endogenous molecules are used, is an active area of research and development.

Breast cancer is estimated to have affected more than a million women every year, with at least half of the sufferers dying from the disease, representing 14% of female cancer deaths (Parkin and Fernandez 2006). Once the leading female cancer in most Western countries, breast cancer incidences have since doubled or tripled in Asia and become more widespread globally (Parkin and Fernandez 2006; Anderson 2006). A leading cause of breast cancer mortality is metastasis to distant organs such as the bones (Pantel and Brakenhoff 2004). In addition, breast cancer treatment is further complicated and varied depending on the tumour type, grade, tumour size, lymph node involvement, steroid hormone receptor expression and HER-2 status (Weigelt et al. 2006). Among these clinical and pathological features, there has been emerging evidence of other biomarkers associated with the progress of breast cancer.

Of these biomarkers, a protein called pigment epithelium-derived factor (PEDF) is of interest, due to increasing evidence reporting that decreased expression of PEDF correlated with enhancement of breast cancer tumour growth and angiogenesis (Cai et al. 2006; Zhou et al. 2010), and progression of prostate cancer (Qingyi et al. 2009). PEDF is a 50-kDa secreted glycoprotein first discovered as a factor secreted by the pigment epithelium of the human foetal eye (reviewed by Manalo et al. 2011). It is the most potent of any known endogenous inhibitors of angiogenesis, being twice that of angiostatin and seven times that of endostatin (Dawson et al. 1999). PEDF induces its anti-angiogenic signals by activating endothelial cell apoptosis through the Fas/FasL death pathway and through disruption of the balance of pro- and anti-angiogenic factors, thereby decreasing the expression of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) (Cai et al. 2006; Dawson et al. 2011; Volpert et al. 2012). PEDF-null mice are born alive and healthy, although an increased stromal microvessel density in the pancreas, kidney and prostate is observed (Doll et al. 2003). Clinically, decreased expression of PEDF correlates with a higher intratumoral microvessel density (MVD) and the propensity to develop metastatic disease (Halin et al. 2004; Sidle et al. 2005). PEDF's anti-angiogenic activity targets the neovasculature without affecting the pre-existing vessels, which makes it an exciting and promising candidate for targeted cancer therapy (reviewed by Broadhead et al. 2010).

PEDF is also involved in the cell cycle, as well as the induction of both apoptosis and tumour cell differentiation (Abe et al. 2004; Crawford et al. 2001; Filleur et al. 2005; Tan et al. 2010). A previous study (Filleur et al. 2005) has demonstrated that PEDF was able to induce prostate cancer cell apoptosis directly, due to a distinct functional epitope on the PEDF protein. However, the molecular conduits responsible for PEDF activity were not elucidated. PEDF significantly reduced tumour cell proliferation in both human melanoma and osteosarcoma cell lines (Abe et al. 2004; Ek et al. 2007a,b). To date, PEDF has been examined in the context of prostate, ovarian and pancreatic cancers, melanoma, glioma and osteosarcoma (reviewed by Broadhead et al. 2010). In this paper, the effects of PEDF as a potential anti-cancer agent on the human prostate cancer cell line – PC3, and the human breast cancer cell line – MDA-MB231 - were closely evaluated, using cell-based assays and immunoblotting for markers cell cycling, adhesion and invasion.

Molecular changes by PEDF in prostate cancer cells have not been documented apart from alterations in PAI-2 (Guan et al. 2007) and NFκB (Smith et al. 2008) as determined by

immunoblotting. A recent study failed to find anti-proliferation effects of PEDF in breast cancer cells (Konson et al. 2010). As yet, aside from treatment based on HER-2 status, there are no published reports of molecular-based treatment alternatives for breast cancer. Thus, the present study sheds light along several molecular pathways that PEDF may choose to activate in curbing both prostate and breast cancer growth. Some of these may be useful in designing of future experiments testing the efficacy of PEDF in clinically-relevant models of prostate and breast cancer.

2. Investigations and results

2.1. PEDF promotes tumour cell adhesion

Previous studies have reported a trend showing that decreased levels of PEDF is associated with an increased incidence of metastatic spread and a poorer prognosis for osteosarcoma and other tumours (reviewed by Manalo et al. 2011). Prostate cancer and breast cancer both metastasise to the bone, causing severe pain and debilitation in advanced cases (Bruera and Sweeney 2003), and thus PEDF treatment can be considered a preventative measure against metastatic osteotropic cancer spread if results *in vitro* looked promising. To directly evaluate this, the effect of 100 nM PEDF on human prostate cancer PC3 and human breast cancer MDA-MB231 cellular adhesion and invasion of collagen-1-coated surfaces was attempted. Collagen-1 was chosen as it is usually used for bone tumour cell biological evaluation (Ek et al. 2007a,b; Dass et al. 2006) as it represents the major protein in mature bone.

Treatment with PEDF (100 nM) increased PC3 cell adhesion to collagen-1 coated surfaces by 137% compared to control untreated cells (Fig. 1). PEDF treatment increased MDA-MB231 cell adhesion to collagen-1 coated surfaces significantly by 63%, with accompanying microscope images clearly showing the difference (Fig. 1). Following a 90 min treatment of MDA-MB231 cells with PEDF, treated cells displayed significant adhesion to collagen-1 coated plates while there was almost no adhesion of control cells. In PEDF-treated PC3 cells, p-FAK decreased in expression, while other common adhesion molecules such as β-integrin, Cdc42, RhoA and Rac-1 levels remained at baseline after PEDF treatment (Fig. 2). On examination of the immunoblotting results for MDA-MB231 cells, expression of p-FAK decreased while other markers were left unperturbed.

2.2. PEDF-mediated decrease in cell invasion is dependent on MT1-MMP

Adhesion and migration are two critical elements leading to tumour cell metastasis. MT1-MMP was decreased in expression following 100 nM PEDF treatment in both human tumour cell lines (Fig. 3). All the other invasion markers remained unaltered post-PEDF treatment, except uPAR which decreased in MDA-MB231 cells. On investigation of the effect of PEDF treatment on PC3 cell invasion, results showed a 40% decrease in cell invasion of PEDF-treated cells as compared to control cells (Fig. 4). An antibody to PEDF abrogated this decrease in invasion effected by PEDF. Antibody to MT1-MMP alone on either cell line caused a similar decrease in invasion as that by PEDF treatment, confirming the importance of MT1-MMP on cell invasion through collagen-1. These results were mirrored in the MDA-MB231 cells.

2.3. Conclusion

The adhesion and migration assays both showed evidence of increased adhesion and decreased invasion with PEDF treatment of the human prostate cancer cell line. The study highlighted several markers for adhesion and invasion processes, the majority of which are novel findings. Thus, the use of PEDF as a novel treatment agent for prostate cancer is justified, and future studies evaluating efficacy of this protein against orthotopic tumour models is warranted. Likewise, treatment of human breast cancer MDA-MB231 cells with PEDF increased adhesion and decreased migration of PEDF-treated cells, as evidenced through the adhesion assay and immunoblotting of adhesion and migration-related molecules. PEDF has displayed great potential as a novel anti-cancer agent for breast cancer and should be further investigated in preclinical studies.

3. Discussion

The studies performed herein utilised 100 nM PEDF, and this dose is very similar to the EC_{50} of conventionally used drugs such as doxorubicin for osteosarcoma (Broadhead et al. 2011; Ta et al. 2009; Tan et al. 2010). These results are comparable to PEDF cell biological activity in other human tumour cells such as osteosarcoma (Ek et al. 2007b) and chondrosarcoma (Tan et al. 2010). In human breast cancer cells, clinically-used agents such as Taxol exhibit an EC_{50} from 50 nM to 1 μM (Charles et al. 2001). In a clinically-relevant spontaneously-metastasising model for osteosarcoma, a dose of 0.2 nmol of PEDF/day was more effective at controlling tumour than doxorubicin administered at 5.2 nmol/3d (Broadhead et al. 2011).

PEDF significantly increased PC3 and MDA-MB231 cell adhesion to collagen-coated plates. This is a beneficial effect of PEDF as it may curb prostate and breast cancer metastasis to bone. Surprisingly, there are no reports of collagen-1 localisation in either prostate or breast tissue or tumours in the literature. If present, breast and prostate cancer cells movement through tumour stroma collagen-1 may be hindered by PEDF as well, in addition to metastasis to the bone. This observation concurred with decreased phosphorylation of the FAK protein in PEDF-treated PC3 and MDA-MB231 cells, leading to decreased invasiveness and motility as shown in colon cancer cells when FAK is activated by phosphorylation (Yu et al. 2006). Phosphorylation of FAK decreases adhesion in melanoma cells (Nishibaba et al. 2012), so our findings where PEDF downregulates FAK phosphorylation are in line with other findings. Previously, in human chondrosarcoma cells Tan et al. (2010), PEDF was able to downregulate RhoA, though no such alteration was noted in this study in either of the human tumour cell lines.

The invasion assay also demonstrated that PEDF treatment could potentially be anti-metastatic in prostate and breast cancer. Both these tumour types have a predilection towards spread and growth in the bone as a secondary lesion site where collagen-1 has to be degraded to allow cells to track through the bone (Bruera and Sweeney 2003). A significant lower number of PEDF-treated cells were able to invade through the PET membranes. This biological assay was further confirmed with immunoblotting which showed a decreased expression of uPAR in MDA-MB231 cells. The urokinase-type plasminogen activator (uPA) system, composed of uPA, its membrane-bound receptor uPAR, are critical components involved in tumour cell dissemination via capillaries and lymph nodes (Pillay et al. 2007). In osteosarcoma cells, treatment with PEDF altered the distribution of uPA and uPAR from the cell surface (Dass and Chong 2008). PEDF also represses the ability of VEGF to induce uPAR expression in endothelial cells (Yang et al. 2010). uPAR was downregulated when human chondrosarcoma cells were treated with PEDF (Tan et al. 2010).

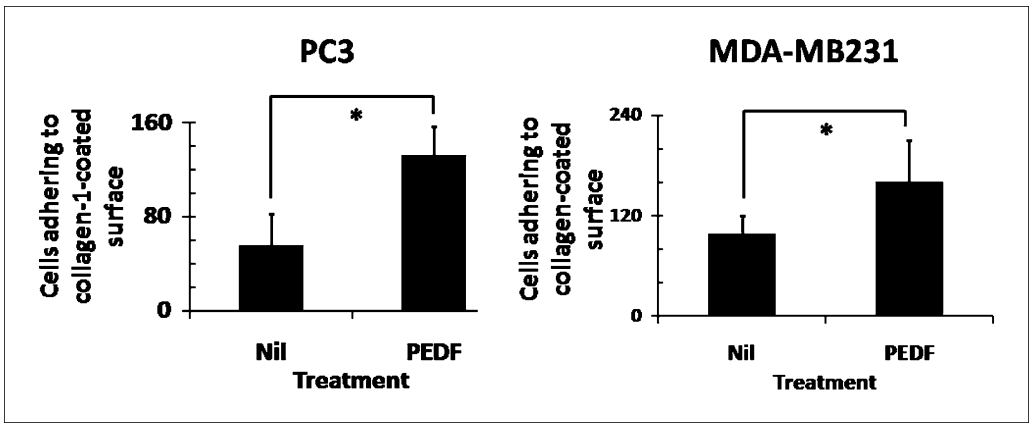

Fig. 1: PEDF increases the ability of cell to adhere to collagen-1-coated wells. PEDF (100 nM) increases the ability of human prostate cancer (PC3) cells to bind to collagen-1-coated plastic wells. PEDF (100 nM) increases the ability of human breast cancer (MDA-MB231) cells to bind to collagen-1-coated plastic wells. Graphs depict representative data from one of two independent studies, each study performed with replicates of $N > 3$. The statistical analyses were done separately for each independent study and was found to be consistent at * $p < 0.01$

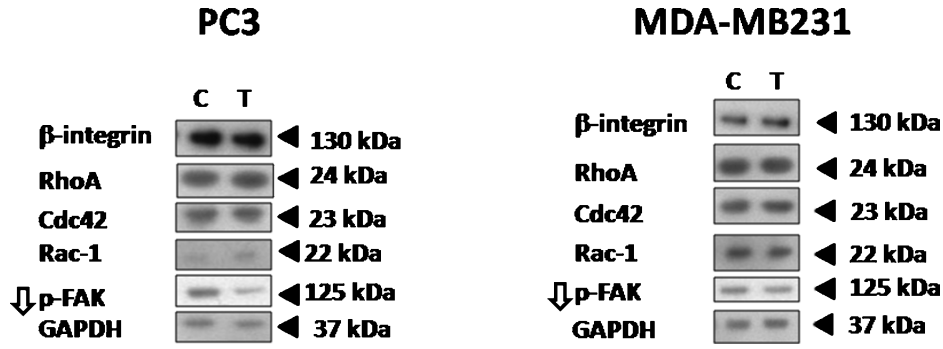

Fig. 2: PEDF decreases expression of p-FAK. Control and 100 nM PEDF-treated PC3 and MDA-MB231 cells probed for molecules involved in cell protrusion, adhesion, and traction, shown with corresponding molecular weights. GAPDH was used as housekeeping protein to normalise protein loading between sample lanes on the gel. Data was acquired from three independent studies, and representative bands from one of the studies is shown

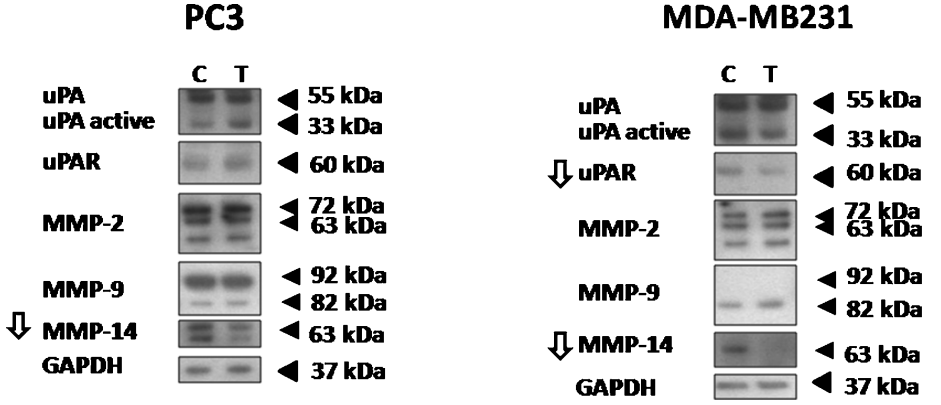

Fig. 3: PEDF decreases MT1-MMP protein levels in human prostate and human breast cancer cell lines. Control (C) and 100 nM PEDF-treated (T) human prostate cancer (PC3) and human breast cancer (MDA-MB231) cells probed for molecules involved in cell invasion, shown with corresponding molecular weights. GAPDH was used as housekeeping protein to normalise protein loading between sample lanes on the gel. Data was acquired from three independent studies, and representative bands from one of the studies is shown

A decreased expression of MT1-MMP in both cell lines further supports observations of decreased migration as MT1-MMP is a major protein responsible for directly cleaving extracellular matrix components and activating MMP-2, which serves to amplify aberrant proteolysis, facilitate the destruction of the matrix and promote invasion and migration (Chernov et al. 2009). In the present study, MMP-2 and MMP-9 were not upregulated by PEDF, though PEDF has been found to act as a substrate for MMP-2 and -9 activities (Notari et al. 2005). The downregulation of MT1-MMP by PEDF was previously noted in a human chondrosarcoma cell line (Tan et al. 2010).

Clinically, PEDF has been noted to decrease in breast cancer patients (Cai et al. 2006; Zhou et al. 2010). In the earlier study by Cai et al. (2006), the authors determined through reverse transcription PCR (RT-PCR), real-time quantitative RT-PCR, immunohistochemisty, and enzyme-linked immunosorbent assay (ELISA) that PEDF mRNA expression was dramatically decreased in breast cancer. Application of exogenous PEDF in an endothelial cell tubule formation assay resulted in a significant decrease in tubule formation and provided evidence of PEDF treatment in preventing breast tumour angiogenesis. More recently, Zhou et al. (2010) further demonstrated the strong clinical correlation between decreased PEDF expression and the progression of breast cancer, including adverse prognostic factors such as lymph node metastasis and survival status. In addition, the authors reported a positive correlation between PEDF protein expression level, microvessel density and lesion size of breast carcinoma. These results

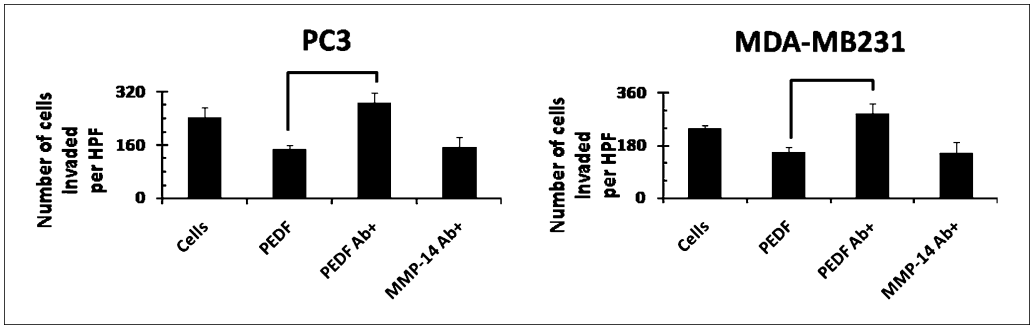

Fig. 4: PEDF-mediated decrease in invasion involves MT1-MMP. PEDF (100 nM) decreases invasion of PC3 and MDA-MB231 cells through collagen-1 matrix in a modified Boyden chamber, and this depends on MMP-14, as a specific antibody to this matrix metalloproteinase protein (MMP) abrogates the decreased invasion effected by PEDF. **(D)** PEDF (100 nM) decreases invasion of MDA-MB231 cells through collagen-1 matrix in a modified Boyden chamber, and this depends on MMP-14, as a MMP-14-specific antibody abrogates the decreased invasion effected by PEDF. Graphs depict representative data from one of two independent studies, each study performed with replicates of $N > 3$. The statistical analyses were done separately for each independent study and was found to be consistent at $* p < 0.01$

pointed to the role of PEDF in breast cancer and suggested that suppression of PEDF expression played a major role in promoting angiogenesis, poising it as a new drug target in the treatment of breast cancer. For prostate cancer, PEDF has been found to be downregulated in prostate cancer patients (Qingyi et al. 2009). Thus, akin to breast cancer, substantiated by our findings, it can be speculated that PEDF may have efficacy against these two major gender-based cancers.

To summarise our findings in the human breast cancer cell line MDA-MB231, PEDF can potentially decrease MDA-MB231 metastasis, a relief for many breast cancer sufferers as metastatic breast cancers often prove fatal (American Cancer Society 2011). Recently the decreased expression of FAK and p-FAK through the use of a FAK inhibitor has been found to decrease tumour growth and tumour metastasis from breast to lung in preclinical models (Notari et al. 2005). Immunoblotting results of other molecules commonly associated with migration, such as MT1-MMP, supported the observed trend of decreased migration of MDA-MB231 and human prostate PC3 cells. Decreased expression of MMP-14 provided further evidence of decreased migration as MT1-MMP is a major protein involved in cleaving the extracellular matrix components and activating MMP-2, whose downstream processes amplifies aberrant proteolysis, facilitates matrix destruction and promotes invasion and migration (Chernov et al. 2009). Collectively, these molecular findings will pave the way ahead for testing the efficacy of PEDF in preclinical models of prostate and breast cancer.

As mentioned above, the very common nature of breast and prostate cancer, the poor outcomes and significant treatment morbidities reinforce the importance of why examining a molecule like PEDF is so important. PEDF has many of the characteristics of other treatment agents used in breast and prostate cancer such as the antimetastatic activities highlighted here, but is less toxic. Indirectly, PEDF may also be beneficial for osteotropic tumours due to its antiosteoclastic nature, as our previous study shows that this is one mechanism whereby PEDF controls primary bone tumour – osteosarcoma – growth and metastasis (Akiyama et al. 2010). Recently, PEDF has been shown to be efficacious when tested in an orthotopic model of cancer using osmotic pumps (Broadhead et al. 2011), as has PEDF-based peptides (Broadhead et al. 2012). Thus, studies to test the efficacy of PEDF against metastasis to the bone by prostate cancer and breast cancer are warranted.

4. Experimental

4.1. Cell culture

Human prostate cancer cell line PC3 and the human breast cancer cell line MDA-MB231 (American Tissue Culture Collection, VA, USA) were cultured in α-MEM/GlutaMAX (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 1% antibiotic/antimycotic (Invitrogen), at 37 °C in a humidified 5% CO_2 atmosphere. PEDF was obtained as lyophilised powder (BioProducts MD, Middletown, MD, USA) and re-constituted in 1x phosphate buffered saline (PBS) pH 7.2 (Invitrogen) to desired test concentrations. For assay setup, exponentially-growing cells below passage number 20 were used. **C**ells were trypsinised and viable cells quantified using the trypan blue dye exclusion assay (Sigma Aldrich, St Louis, MO, USA).

4.2. Collagen-I adhesion assay

24-well plates were coated with 0.2% rat-tail collagen-1 (BD Biosciences, USA) for one hour at 37 °C [26]. Excess collagen was gently removed before PC3 and MDA-MB231 cells were seeded at a density of 2×10^4 cells/ml ± 100 nM PEDF and allowed to incubate at 37 °C/5% CO_2 for 90 min. Wells were then washed twice gently with PBS to remove loose cells and debris before observation and photography. Final cell count was enumerated using ImageJ software.

4.3. Collagen-I invasion assay

Tumour cell invasiveness was examined using cell culture inserts with polyethylene terephthalate (PET) track-etched membranes. 8.0 μM pore size (BD Biosciences) inserted into 24-well plates and coated with 2 mg/ml rat-tail collagen-I (Dass et al. 2006). PC3 and MDA-MB231 cells were seeded onto each insert at 2.5×10^5 cells/ml with α-MEM/0.5% FBS and allowed to incubate at 37 °C/5% CO_2 for 4 days in wells filled with supplemented media. Cells that had invaded through the collagen-coated pores onto the PET membranes were quantified after staining with QuickDip (Fronine Laboratory Supplies, Sydney, Australia). Studies examining the effect of blocking PEDF or MMP-14 were performed with 1 μg/mL antibody (Santa Cruz Biotechnology, CA).

4.4. Western blot analysis

PC3 and MDA-MB231 cells were grown to 90–95% confluency in the presence of 100 nM PEDF, harvested and total cellular proteins extracted using modified RIPA buffer (150 nM NaCl, 50 mM Tris pH 8, 1 mM EDTA, 0.1% SDS and 1% Triton X-100) with protease inhibitor cocktail (Roche, Mannheim, Germany). Protein lysates were electrophoresed through 4–20% NuPAGE® Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) and transferred onto polyvinylidene diflouride (PVDF) membranes for probing with antibodies (Fahmy et al. 2003). Antibodies were visualised using the ECL-Plus chemiluminescence system (Amersham BioSciences, Buckinghamshire, UK). PVDF membranes were stripped in 65 mM Tris-HCl pH 6.7/10% SDS at 55 °C for one hour and reprobed with GAPDH for protein loading normalisation.

The following antibodies were acquired from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA): β-integrin, cdc42, c-Jun, c-Fos, Chk2, GAPDH, JNK1, NFκB, MMP-2, MMP-9, MMP-14, p63, p73, p-Erk, p-Fak, uPA, uPAR, Rac-1, RhoA. The following antibodies were acquired from Cell Signalling Technology, Inc (Danvers, MA, USA): Akt, p-Akt and Chk1. The following antibody was acquired from BD BioSciences (San Jose, CA, USA): pan-Erk.

4.5. Statistical analyses

Means and standard deviations of the means were calculated for each study. All data were analysed using the two-way student's t-test with unequal variances. A *p*-value of less than 0.025 was considered significant.

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