Targeting of Receptor Activator of Nuclear Kappa B (RANK) in PC-3 Cells Increases Cell Proliferation and Matrix Adhesion *In Vitro*

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Abstract. *Background: In Western societies, prostate cancer is the most frequently diagnosed cancer amongst men. Efforts to improve diagnosis and treatment remain a major focus and have been proven beneficial in the approach to localised disease. However, currently, metastatic disease management still remains palliative. Receptor activator of nuclear kappa B (RANK) has been extensively studied in bone biology and immunology, whilst several links have been made between RANK-positive breast cancer cells and disease progression. Its role in prostate cancer biology remains poorly understood, therefore the aim of this study was to explore the functional role of endogenously produced RANK in metastatic PC-3 prostate cancer cells in isolation and in response to hepatocyte growth factor (HGF). Materials and Methods: RANK expression was targeted using hammerhead ribozyme technology in PC-3 prostate cancer cells, and verified by polymerase chain reaction and western blot. A variety of in vitro functional assays were conducted, including cell proliferation and matrix adhesion in the presence of HGF. Results: Suppression of RANK expression was successfully targeted with anti-RANK hammerhead ribozyme transgenes, as verified by PCR and western blot. Reduced RANK expression resulted in significantly increased PC-3 cell proliferation ($p<0.01$) and cell-matrix adhesion ($p<0.05$) compared to control cells. Conclusion: Previous work into RANK and prostate cancer has focused on its interaction with the bone environment, particularly with regard to its receptor RANK ligand. This study has shown that endogenous RANK expression changes might also influence prostate cancer cell behaviour. Further work is now required to elucidate the signaling pathways involved in these processes.*

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Prostate cancer remains the most commonly diagnosed cancer in males in the UK and, due to increasing life expectancy and prostate cancer predominately being linked with aging, better diagnosis and treatment options remain a major goal of healthcare systems worldwide. A better understanding in early prostate cancer aetiology has resulted in treatment progress in the past few decades, however, the same cannot be said for metastatic prostate cancer treatment options (1). Once prostate cancer progresses to an androgen-independent metastatic state it has a predisposition to form secondary tumours in the bone (2). Prostate cancer bone metastases are predominately osteoblastic in nature and commonly affect the axial skeleton, resulting in significant morbidity, including debilitating pain, impaired mobility, hypercalcaemia, and spinal cord and nerve compression (3). Current clinical interventions are limited to palliative options. Prostate cancer-associated bone metastases are predominately categorized as osteoblastic due to their immature woven structures detected on x-rays, although there is some evidence that approximately 10% of patients exhibit mixed lesion phenotypes, which include evidence of osteolytic (dysregulated bone destruction) (4).

In the late 1990s, with the discovery of the receptor activator of nuclear kappa B (RANK)–RANK ligand (RANKL)–osteoprotegerin (OPG) axis which controls bone turnover, major steps forward have been taken in bone-associated disorders, including rheumatoid arthritis (5), however, this progress has not directly translated to effective anticancer therapies (6). RANK, RANKL and OPG are all members of the tumour necrosis factor (TNF) ligand superfamily. RANK is expressed on osteoclasts and RANKL on osteoblasts, whilst OPG is a secreted inhibitory molecule acting on RANKL (7). RANKL expression is successfully targeted within cancer treatment as demonstrated by the licensing of denousamab, a neutralising monoclonal antibody, predominately used in the treatment of bone metastases associated with solid tumours, particularly of breast cancer (8). Its ligand, RANK has been detected on haematopoietic

Table I. *Receptor Activator of Nuclear Kappa B primers designed for ribozyme synthesis.*

RANK ribozyme	RANKRIBF	CTGCAGCTGGCATCTTCGCCTTGTGCGTAGGCTGATGAGTCCGTGAGGA
	RANKRIBR	ACTAGTGTCAGGGCACATGTGTAGGAGGTGGTTTCGTCCTCACGGACT

Table II. *Primers for conventional and real time for Receptor Activator of Nuclear Kappa B.*

	Primer Name	Primer sequence (5'-3')	Optimal annealing temperature	Product size (bp)
PCR	RANKF	CAGAGCACAGTGGGTTCAGA	55˚C	462
	RANKR	GATGATGTCGCCCTTGAAGT		
qPCR	RANKF	TCTGATGCCTTTTCCTCCAC	55˚C	119
	RANKZR	**ACTGAACCTGACCGTACA**TGGCAGAGAAGAACTGCAAA		
PCR	GAPDHF	AGCTTGTCATCAATGGAAAT	55˚C	593
	GAPDHR	CTTCACCACCTTCTTGATGT		
qPCR	GAPDHF	CTGAGTACGTCGTGGAGTC	55˚C	93
	GAPDHZR	**ACTGAACCTGACCGTACA**CAGA GATGATGATGACCCTTTTG		

ACTGAACCTGACCGTACA – ZR probe sequence, attached to the 5' end of each of the reverse primers.

osteoclast precursors, as well as on several tumour cell types, including prostate cancer (9). RANK was first identified by Anderson *et al*. on dendritic cells as a 616 amino-acid protein with homology to the extracellular domain of the TNF superfamily (10). Several previous studies demonstrated that activation of RANK on cancer cells, including of breast and prostate, by extracellular RANKL results in metastatic phenotypes and behaviours (11, 12). Links with breast cancer initiation and disease progression are particularly strong given the evidence of RANK-positive breast cancer epithelial cells and the subsequent activation of the RANK signaling pathway.

Hepatocyte growth factor (HGF) and its receptor cMET have also been shown to influence cancer cell behaviour, including cell motility, migration and invasion (13). This study, therefore, aimed to explore the effects of targeting endogenously produced RANK in PC-3 prostate cancer cells and subsequently how exposing these cells to the pro-oncogenic factor HGF might further affect that behaviour.

Materials and Methods

Cell line. Human prostate PC-3 cells were purchased from the American Type Culture Collection (Rockville, MD, USA). PC-3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (FCS) and antibiotic-antimycotic solution (containing penicillin, streptomycin and amphotericin B) (Sigma Aldrich, Poole, Dorset, UK) and incubated at 37˚C, with 5% CO_2 with 95% humidity.

Treatment. HGF was a kind gift from Dr. T. Nakamura (Osaka University Medical School, Osaka, Japan). For the duration of this study HGF was used at a final concentration of 40 ng/ml.

Generation of RANK ribozyme transgenes. Ribozyme transgene technology has previously been used (14). In brief, hammerhead ribozyme transgenes targeting *RANK* were designed using Zuker's RNA mFold programme based on the secondary predicted structure of RANK and generated by Sigma Aldrich (Table I). Ribozymes were subsequently cloned into a pEF6/V5-His-TOPO plasmid vector (Invitrogen, Paisley, UK). Both control pEF6 plasmids, containing no insert, and plasmids containing the *RANK* ribozyme transgene were transfected separately into PC-3 prostate cancer cells using electroporation set at 310 V (Easyjet; Flowgene, SLS, Hessel, Yorkshire, UK). Following transfection, these cells underwent a selection period for 10 days with blasticidin and subsequent verification of *RANK* knockdown. Cells containing the ribozyme transgenes were termed PC-3^{RANKKD} and were compared throughout the study to control PC-3 cells containing the closed control plasmid, termed PC-3^{pEF6}.

RNA isolation, cDNA synthesis and quantitative PCR (qPCR). Total RNA isolation was carried out using the TRIzol reagent kit, as described by Sigma Aldrich. Reverse transcription was completed using a high capacity cDNA reverse transcription kit (Applied Biosystems, Manchester, UK). RNA was standardised to 500 ng prior to reverse transcription using the Implen Nanophotometer (Munich, Germany). Following cDNA synthesis, sample quality and uniformity were assessed and normalised using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene primers (Table II).

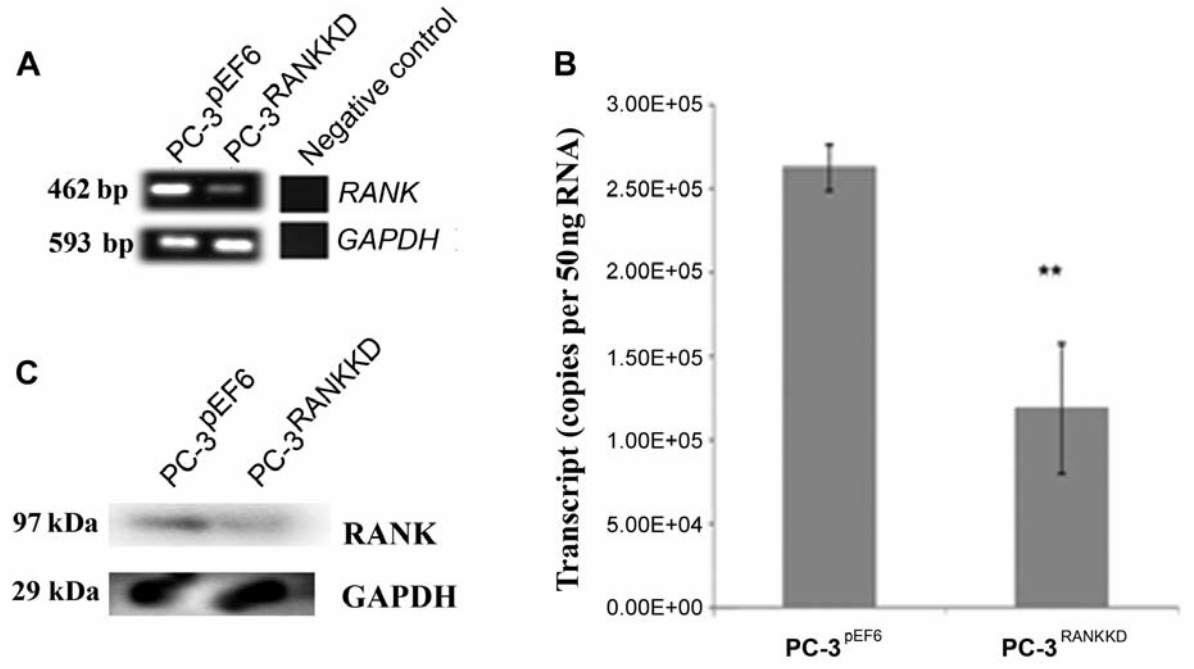

Figure 1. *Verification of ribozyme transgene knockdown of Receptor Activator of Nuclear Kappa B (RANK) in PC-3 cells. Reduced RANK expression (PC-3^{RANKKD}) was confirmed in PC-3 cells at the transcript level using conventional polymerase chain reaction (A) and quantitative polymerase chain reaction (B) compared to the pEF6 control cell line. Western blotting was used to confirm successful RANK knockdown (C) normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH). **p≤0.01. Data are the mean of n=4, error bars show SEM.*

All amplifluor system qPCRs were performed and normalised against *GAPDH*. In brief, the amplifluor probe contained a 3' region specific to the Z sequence (ACTGAACCTGACCGTACA) of the target reverse primer sequence (Table II) and a 5' hairpin structure labelled with a fluorophore (FAM). The amplifluor system (Intergen Inc., New York, USA) utilised qPCR Master Mix (ABgene, Loughborough, Leicestershire, UK) and the conditions used were: an initial 95˚C period for 15 min, followed by 60 cycles of 95˚C for 15 sec, 55˚C for 60 sec and 72˚C for 20˚C sec.

Protein isolation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. Cells were detached and lysed in a buffer comprising of 50 mM TRIS-base, 5 mM EGTA, 150 mM NaCl, 1% Triton X-100, 100 μg/ml phenylmethulsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 mM sodium vanadate and 50 mM sodium fluoride on a rotorary wheel for 1 h before removal of insolubles through centrifugation at 13,000 × *g* for 5 min. The Bio-Rad DC Protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) was used to quantify protein levels in each sample and samples were subsequently standardized to 2 mg/ml and diluted in 2× concentrate Laemmli sample buffer (Sigma Aldrich) before being boiled for 5 minutes. Samples were separated on a 10% acrylamide gel and subsequently transferred onto a polyvinylidene difluoride (PVDF) membrane. Proteins were detected using the Merck-Millipore SNAP i.d. protein detection system (Feltham, UK). RANK expression was detected using antibody to RANK (sc-9072; Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) and sample uniformity assessed using GAPDH expression, detected using anti-GAPDH (sc-32233; Santa Cruz Biotechnology). Samples were visualized using ECL chemiluminescence kit (Geneflow, Lichfield, UK) and photographed using UVIProChem camera system (UVItec Ltd, Cambridge, UK).

In vitro function assays. Cell proliferation: PC-3 transfectants were seeded at 3×10^3 cells/well into 96-well plates in triplicate and incubated for 1, 3 and 5 days. Following incubation, cells were fixed in 4% formalin (v/v) and stained with 0.5% crystal violet (v/v). Subsequently, crystal violet stain was extracted from the cells using 10% acetic acid (v/v) and the absorbance at 540 nm was determined using a spectrophotometer.

Matrigel adhesion: A 96-well plate was coated with 5 μg/well of Matrigel (Corning, St Davids Park, Flintshire, UK) and left to dry. PC-3 transfectants were seeded at 4.5×10^4 cells/well and left to adhere for 45 minutes before being fixed in 4% formalin (v/v) and stained with 0.5% crystal violet (v/v). Four representative images were captured for each well and subsequently counted using Image J software (National Institutes of Health, USA).

Cell migration: The cytodex bead motility assay was used to assess PC-3 cell motility [adapted from (15)]. Briefly, 1×10^6 cells in 10 ml of medium were left to incubate with cytodex-2 beads (20 mg/ml) overnight. The following day, cells were washed twice with fresh medium before being re-suspended in 1.5 ml medium and added to a 96-well plate in triplicate (100 μl/well). Cells were incubated for 4 h, after which the plate was washed, fixed in 4% formalin (v/v) and stained with 0.5% crystal violet (v/v). Four representative images were captured for each well and subsequently counted using Image J software.

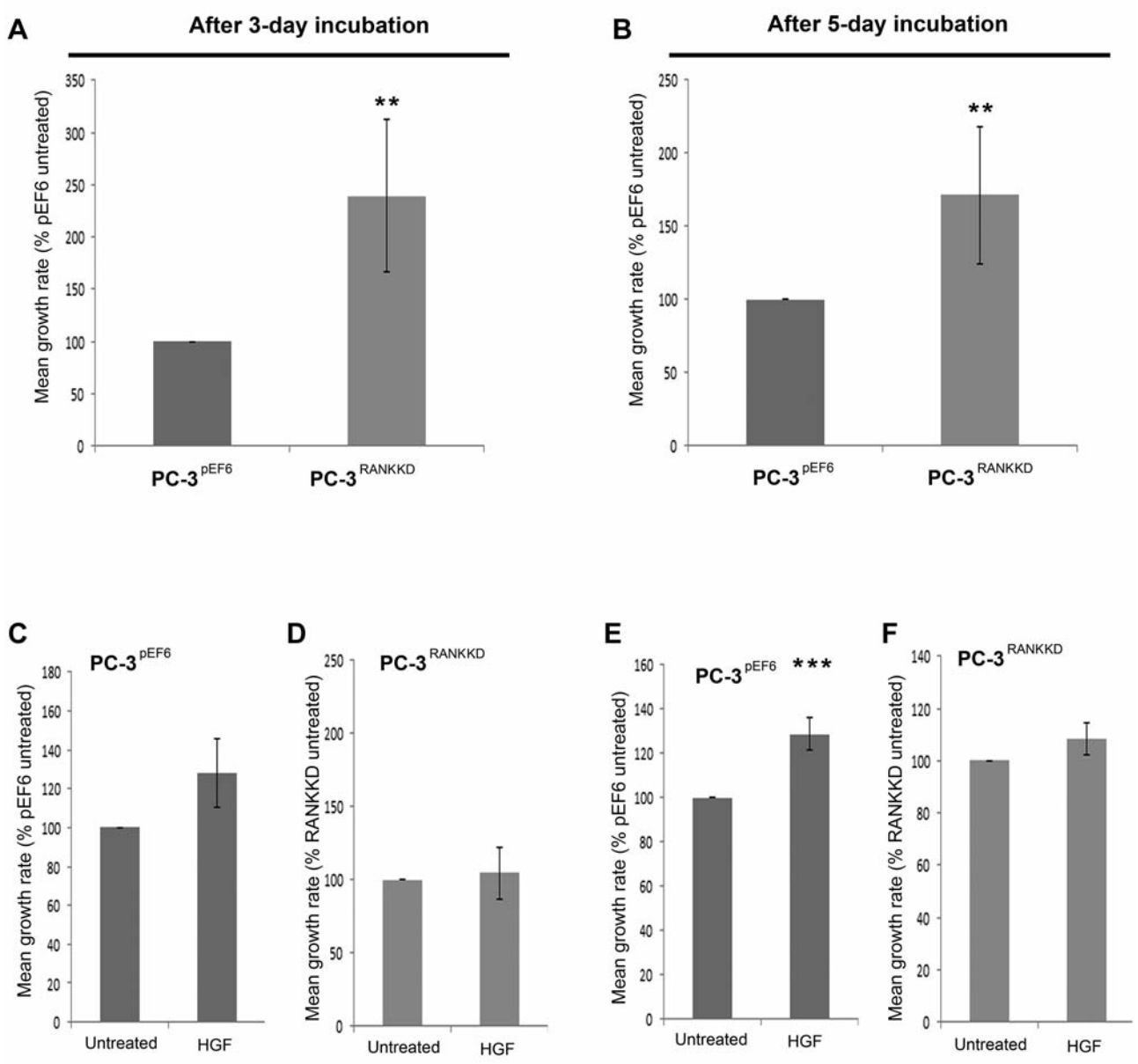

Figure 2. *Impact of Receptor Activator of Nuclear Kappa B (RANK) knockdown on PC-3 cell proliferation. Suppression of RANK expression in PC-3 cells (PC-3^{RANKKD}) resulted in a significant increase in PC-3 cell proliferation after 3 (A) and 5 (B) days compared to control cells. When control PC-3 (C) and PC-3^{RANKKD} (D) cells were treated with 40 ng/ml hepatocyte growth factor (HGF) for 3 days, no significant increase in PC-3 cell proliferation was seen. However, after 5 days' incubation, control cells treated with HGF showed significantly increased cell proliferation (E), a trend that was not seen in RANK-suppressed cells when compared to untreated RANK-suppressed control cells (F). **p≤0.01, ***p≤0.001. Data are the mean of n=3, error bars show SEM.*

Matrigel cell invasion: Transwell inserts (8-μm pore size) were coated with 50 μg/insert of Matrigel and dried before PC-3 cell transfectants were seeded (2×10^4/100 μl) into each insert and incubated for 3 days. Following incubation, invading cells were fixed in 4% formalin (v/v) and stained with 0.5% crystal violet (v/v). Five representative images were captured per transwell insert and subsequently counted using Image J software.

Results

Confirmation of ribozyme transgene knockdown of RANK expression in PC-3 prostate cells. Expression of *RANK* was successfully targeted in PC-3 prostate cancer cells following transfection with an anti-*RANK* ribozyme transgene, as

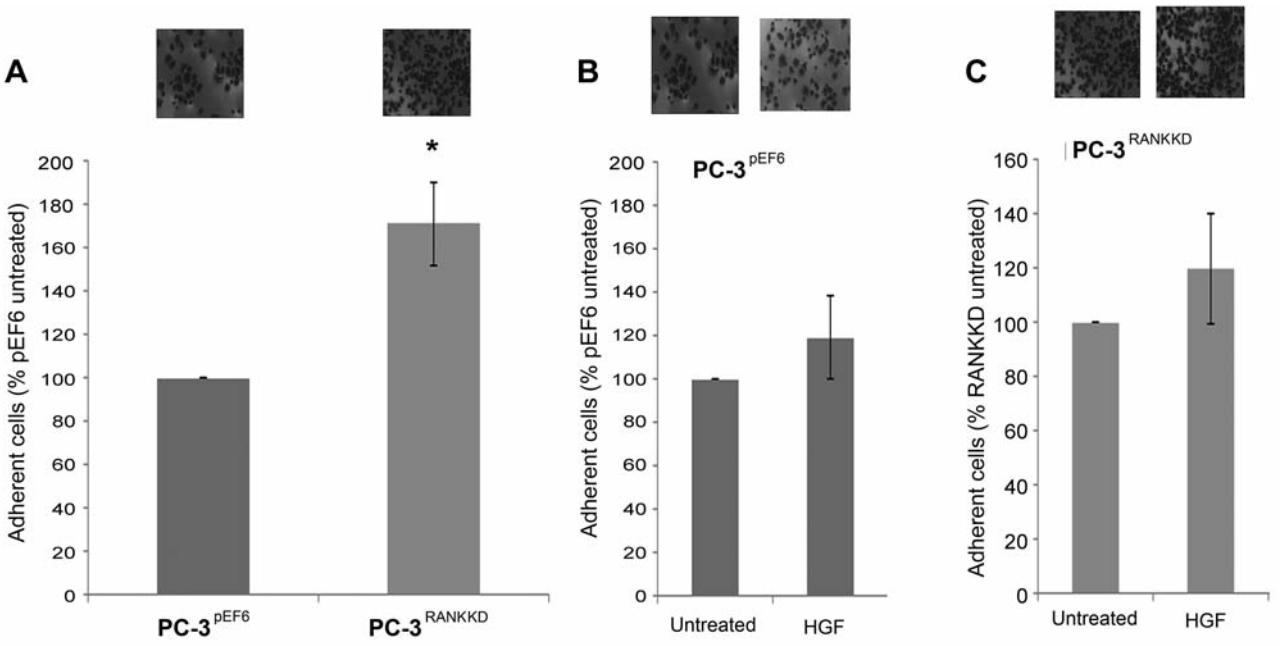

Figure 3. *Impact of Receptor Activator of Nuclear Kappa B (RANK) knockdown on PC-3 cell-matrix adhesion. Suppression of RANK expression in PC-3 cells (PC-3^{RANKKD}) resulted in a significant increase in cell-matrix adhesion (A) compared to control cells. When control (B) and PC-3^{RANKKD} (C) cells were treated with hepatocyte growth factor (HGF), no significant changes in cell-matrix adhesion were seen. *p≤0.05. Data are the mean of n=3, error bars show SEM.*

verified by RT-PCR and qPCR and western blot compared to the matched control transfected PC-3 cells (Figure 1).

RANK suppression enhances PC-3 cell proliferation. Suppression of RANK expression significantly enhanced PC-3 prostate cancer cell proliferation after 3 days' incubation (166% of control, p=0.008; Figure 2A) and after 5 days' incubation (165% of control, p=0.008; Figure 2 B) compared to PC-3^{pEF6} control cells.

Treatment of PC-3^{pEF6} control cells with 40 ng/ml HGF resulted in increases in proliferation of PC-3^{pEF6} control cells compared to untreated cells after 3 days' incubation (Figure 2C) and 5 days' incubation, which reached statistical significance (134% of control, p=0.004 compared to untreated control; Figure 2E).

When PC-3^{RANKKD} cells were incubated for 3 days with 40 ng/ml HGF, no further increase in cell proliferation was observed (Figure 2D). After 5 days' incubation, there appeared to be no difference in proliferation rates between the PC-3^{RANKKD} cells treated with 40 ng/ml HGF compared to the untreated control (Figure 2F).

RANK suppression enhances cell-matrix adhesion by PC-3 prostate cancer cells. Suppression of *RANK* expression in PC-3 prostate cancer cells significantly increased cell-matrix adhesion compared to PC-3^{pEF6} control cells (171% of control, p=0.02; Figure 3A).

Treatment of PC-3^{pEF6} control cells with 40 ng/ml HGF appeared to increase cell-matrix adhesion (119% of untreated control); however, this change was not deemed significant (Figure 3B). Cell-matrix adhesion by the PC-3^{RANKKD} cells appeared to be further increased following treatment with 40 ng/ml HGF (119% of untreated control), however, this also did not reach significance compared to the untreated PC-3^{RANKKD} cells.

RANK suppression enhances PC-3 prostate cancer cell motility. PC-3 cells with suppressed RANK expression appeared to exhibit increased cell motility compared to the PC-3^{pEF6} control cells (206% of control, Figure 4A). This increase was of borderline significance (p=0.057), possibly due to the large standard error. The most notable change observed in the PC-3^{RANKKD} cells was the higher level of aggregation they appeared to undergo compared to control PC-3pEF6 cells. When the PC-3^{pEF6} control cells were treated with 40 ng/ml HGF, cell motility appeared to be increased compared to the untreated PC-3^{pEF6} control cells (136% of untreated control; Figure 4B), although again not quite passing the significance threshold (p=0.057).

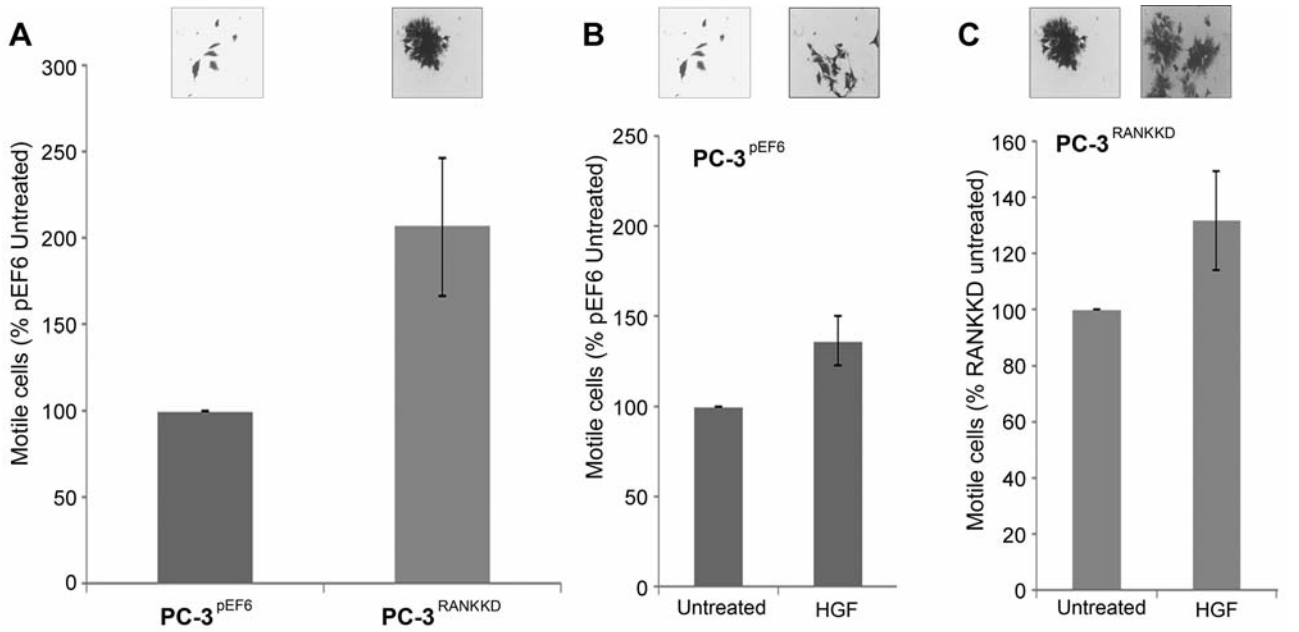

Figure 4. *Impact of Receptor Activator of Nuclear Kappa B (RANK) knockdown on PC-3 cell motility. Suppression of RANK expression in PC-3 cells (PC-3^{RANKKD}) did not affect cell motility (A) compared to the control cells. When control (B) and PC-3^{RANKKD} cells (C) were treated with hepatocyte growth factor (HGF), no significant changes in cell motility were seen. Data are the mean of n=3, error bars show SEM.*

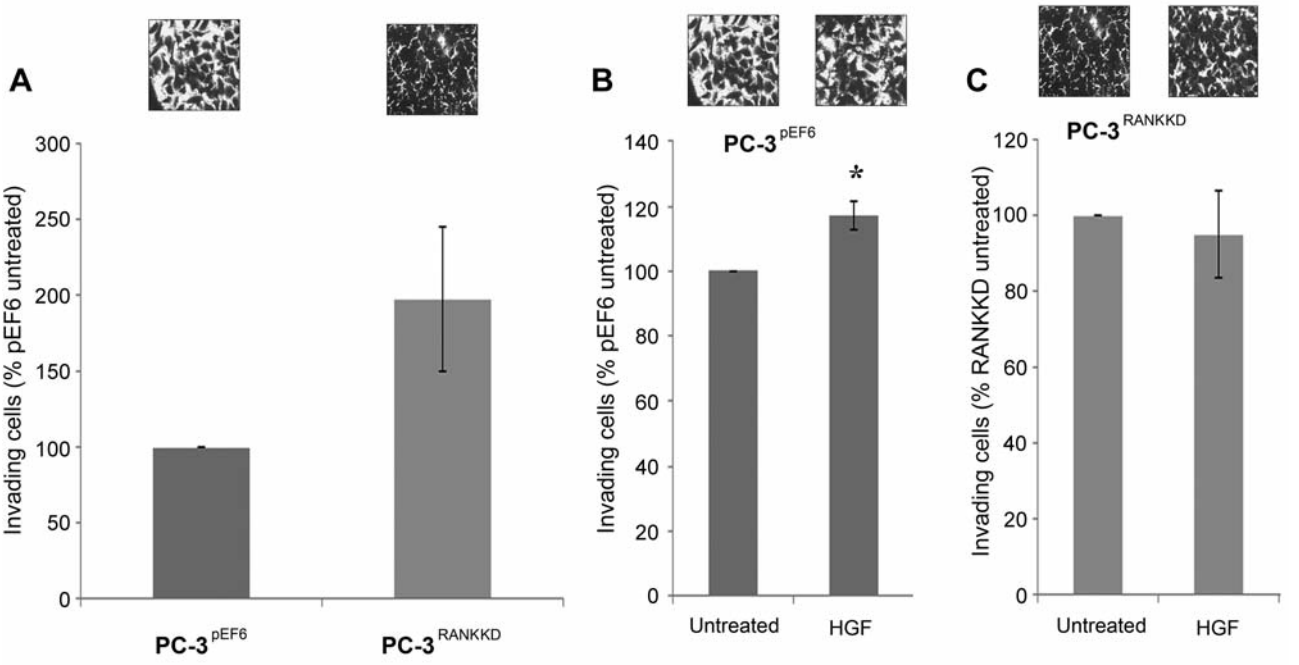

Figure 5. *Impact of Receptor Activator of Nuclear Kappa B (RANK) knockdown on PC-3 cell invasion. Suppression of RANK expression in PC-3 cells did not affect cell invasion (A) compared to control cells. When control cells (B) were treated with hepatocyte growth factor (HGF), cell invasion significantly increased compared to the untreated control, a trend which was not seen in RANK-suppressed PC-3 cells (C). *p≤0.05. Data are the mean of n=3, error bars show SEM.*

When *RANK*-suppressed PC-3 cells were exposed to treatment with 40 ng/ml HGF, PC-3 cell motility appeared to be enhanced compared to the untreated control but this trend did not reach significant level (Figure 4C).

RANK suppression did appear to increase *in vitro* PC-3 cell invasion, however, due to the large standard error, this trend did not reach significance (Figure 5A). As noted in all previous functional assays conducted in this study, the addition of 40 ng/ml HGF to *RANK*-suppressed PC-3 cells did not alter their invasive potential, a trend that was seen in the control cells (Figure 5B and C).

Discussion

In this study, after successful transgene targeting of *RANK* expression in PC-3 prostate cancer cells, both cell proliferation and matrix adhesion were significantly increased. Of further note from this study was that targeting of *RANK* expression also made these cells less responsive to the pleiotropic growth factor HGF. Previous work studying the role of RANK in PC-3 prostate cancer cells has focused on its interaction with stromal RANKL, especially as immunohistochemical staining of RANK has shown that these two bone markers may offer prognostic potential for metastatic disease (16, 17). Interestingly, Casimiro *et al*. reported that in response to RANKL the metastatic phenotype of PC-3 cells was increased through up-regulation of matrix metalloproteinase-1 (18). Furthermore, they demonstrated that knocking-down *RANK* using siRNA inhibited RANKL-induced c-Jun N-terminal kinases' (JNK) phosphorylation. However, there was no comment on how this transient targeting of *RANK* in PC-3 cells affected cell behaviour. The current study appears to show that targeting *RANK* expression with transgenes significantly alters PC-3 cell proliferation and matrix adhesion. It was also interesting to observe that the PC-3^{RANKKD} cells appeared to be more aggregated during the motility assay compared to the PC-3^{pEF6} control cells, although more investigation into this is needed.

Given the wealth of data that has demonstrated the pro-metastatic influence of cMET in a wide variety of solid tumour types, including prostate cancer, HGF as its activator therefore also continues to be scrutinised as a potential target for cancer therapies. Chu *et al*. showed that RANKL-expressing prostate cancer cells influenced other localised cells and that RANK- and cMET-mediated signaling also played crucial roles in this recruitment process (19). It is, therefore, interesting to note that in this study, direct targeting of *RANK* expression on prostate cancer cells appeared to de-sensitise them to the tumourigenic effects of HGF. This poses the question of what advantageous influence RANK expression might also have on prostate cancer itself.

The complexities of the bone microenvironment and the dynamic interactions between the diverse range of cells and disseminating prostate cancer cells is only now starting to be understood. The discovery of theRANK–RANKL–OPG axis provided a direction which has uncovered potential therapeutic benefit, as demonstrated by the licensing of denosumab, a neutralising monoclonal to RANKL. Whilst other studies have demonstrated the promise of combined approaches in murine models (20), more recent focus has been placed on the role of RANK in prostate cancer progression, given its interactions with RANKL.

In summary, the current study has highlighted the potential that suppression of *RANK* expression results in prostate cancer cells losing their sensitivity to HGF stimulation. Further work is now required to elucidate how these two molecules interact and influence prostate cancer progression.

Acknowledgements

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