Importance of osteoprotegrin and receptor activator of nuclear factor κB in breast cancer response to hepatocyte growth factor and the bone microenvironment *in vitro*

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Abstract. Osteoprotegrin (OPG), receptor activator of nuclear factor κB (RANK) and RANK ligand (RANKL) are signal transducers which have pleiotropic actions. Each tumour necrosis factor receptor superfamily member has unique structural attributes which directly couples them to signalling pathways involved in cell proliferation, differentiation and survival. Previous studies have clinically linked OPG, RANK and RANKL to increasing tumour burden, metastatic bone involvement and estrogen status. This study aimed to establish the potential implications of targeting endogenously produced OPG and RANK in the osteotropic breast cancer cell line MDA-MB-231 *in vitro*. Subsequently this study also aimed to explore the potential links between these molecules with regards to hepatocyte growth factor (HGF) signalling and extracted bone proteins (BME). OPG and RANK expression was successfully suppressed using hammerhead ribozyme technology. Subsequently effects were explored in MDA-MB-231 cell proliferation, matrix adhesion, migration and invasion *in vitro* function assays. Reduced OPG expression resulted in increased breast cancer cell migration and invasion. These increases, particularly invasion, appeared to however be reduced under the influence of the exogenous stimuli (HGF and BME). In contrast, suppression of RANK in MDA-MB-231 breast cancer cells resulted in decreased cancer cell proliferation, matrix-adhesion, motility and invasion with little cumulative effect being noted after the addition of exogenous stimuli. The complexity of the bone environment underpins the vast number of soluble factors and signalling pathways which can influence osteotropic cancer behaviour and progression. Further work into elucidating all the pathways affected could potentially lead to better identification of those patients most at risk.

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Introduction

Despite advances in breast cancer care and regimens it still imposes a large burden on health care systems around the world, especially when metastatic disease is detected. Breast cancer is associated with latent disease and high relapse rate which can often present clinically as bone metastases (1). The majority of breast cancer related bone metastases present as the osteolytic phenotype, which is identified by loss of bone density accompanied by an increase in osteoclast numbers (2).

The variability in metastatic cancer patterns is undoubtedly influenced by the molecular and cellular characteristics of both the tumour cells and the tissue in which they invade (3). It was Stephen Paget, through autopsy data, who first established a link between breast cancer metastases and the bone, giving rise to his 'seed and soil' hypothesis (4). As this theory has evolved the metastatic cascade has been shown to be a highly inefficient multistep process which involves a wide variety of factors including integrins, matrix metalloproteinases and tumour secreted factors (5,6). Invasion into the bone results in the release of a variety of factors, in addition to those produced by the tumour cells, which generate a feedback loop to the tumour cells enhancing tumour cell dormancy, survival and growth in the bone marrow and the microenvironment (7). However, much still remains unknown of how these factors interact with each other and the disseminating tumour cells to culminate in bone metastases and how best these can be targeted in therapies.

Members of the tumour necrosis factor receptor superfamily (TNFRSF) osteoprotegrin (OPG), receptor activator of nuclear κB (RANK) and RANK ligand (RANKL) have been shown to be integral molecular regulators in the bone remodelling cycle. The RANKL:OPG ratio is a major determinant of bone mass, both physiologically and patho-physiologically (8). Osteoblasts have been shown to incorporate both pro- and anti-bone resorptive signals and thus control the bone remodelling response by altering the expression of RANKL and secretion of its inhibitor OPG (9,10). RANK, expressed on the surface

of osteoclasts, through binding to RANKL, expressed on the surface of osteoblasts, promotes osteoclast differentiation and maturation, thus promoting bone resorption. OPG, a soluble decoy receptor for RANKL, secreted by osteoblasts, inhibits RANK interaction thus promoting osteoblast survival and hence bone formation. However, OPG, RANK and RANKL have also been linked to tumourigenesis in a variety of cancers which have a predisposition to form bone metastases. Both circulating RANKL and OPG have previously been identified as novel biomarker candidates for predicting bone metastases in breast cancer patients (11,12). Quantitative PCR and immunohistochemistry have shown negative correlations between estrogen receptor status and levels of OPG, RANK and RANKL (13).

There has also been some *in vitro* evidence to suggest that endogenously produced OPG, from breast cancer cells or bone marrow stromal cells, can also promote breast cancer cell survival through inhibition of TNF related apoptosis inducing ligand (TRAIL) (14,15). This inhibition occurs as OPG acts as a decoy receptor for the TRAIL receptor, though with less affinity than that seen with RANKL, therefore blocking the apoptotic pathway. This prevention of apoptosis through TRAIL inhibition has also been shown, in the MDA-MB-231 breast cancer cell line, to result in the up regulation of RANKL thus contributing to the 'vicious' bone cycle between tumour cells and bone cells by further enhancing osteolysis and the release of growth factors which can further enhance tumour growth (16).

The bone microenvironment is a complex combination of cells, growth factors and cytokines. Trying to isolate the factors which are crucial components in facilitating the establishment of bone metastases is a substantial challenge. One of the factors, which have been shown to influence tumourigenesis traits and cancer progression is hepatocyte growth factor (HGF), also known as scatter factor (17-19). Despite its discovery 30 years ago its wide and complex influences on cancer cells, the metastatic cascade and tumour microenvironments remain under intense investigation for potential new targeted therapies (20,21).

In the present study the targeting of OPG and RANK in bone metastasis derived breast cancer cells (MDA-MB-231 cells) was explored. These manipulated cells were then exposed to the influences of HGF and a bone protein-like environment to explore the potential implications on HGF signalling thus potentially altering disease progression.

Materials and methods

Ethics statement. All research involving human tissue was carried out under the Panel B Bro Taf Research Ethics Committee for the Bro Taf Health Board, Cardiff, UK. All data were analysed anonymously and informed written consent was given (Bro Taf Health Board, 2007).

Cell lines and treatments. Human breast cancer MDA-MB-231 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). MDA-MB-231 cells were maintained in Dubecco's modified Eagle's medium (DMEM) (PAA Laboratories Ltd., Somerset, UK) supplemented with penicillin, streptomycin and 10% foetal calf serum (PAA Laboratories Ltd.) and incubated at 37˚C, 5% CO_2 and 95% humidity. Hepatocyte growth factor was a kind gift from Dr T. Nakamura (Osaka University Medical School, Osaka, Japan). Bone proteins were extracted from fresh human bone tissues collected immediately after hip replacement under the local health board ethics committee guidelines. Bones were crushed at ice cold temperatures and subsequently processed in a Bioraptor sonicator (Wolf Laboratories, York, UK) to extract matrix proteins (22). Throughout this study HGF was used at a final concentration of 40 ng/ml, whilst the BME extract from the femoral heads was used at a final concentration of 50 μg/ml.

Generation of MDA-MB-231 breast cancer cells with suppressed OPG or RANK expression. OPG and RANK expression were targeted in human MDA-MB-231 breast cancer cells using ribozyme transgenes specifically generated to target and cleave each transcript. This methodology has been previously reported (23,24). Briefly, ribozyme transgene sequences were designed based on Zukers predicted secondary mRNA structure using Zukers RNA Mfold program (25) and were synthesised by Sigma-Aldrich (Poole, Dorset, UK) (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 relevant ribozyme transgene were transfected separately into MDA-MB-231 breast cancer cells using electroporation. Following transfection, these cells underwent a selection period and subsequent verification of OPG or RANK knockdown. Cells containing the ribozyme transgenes were termed MDA-MB-231^{OPGKD} or MDA-MB-231^{RANKKD} and were compared throughout the study to control MDA-MB-231 cells containing the closed control plasmid, termed MDA-MB-231^{pEF6}.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Cells were grown to confluence in a 25-cm^2 flask before RNA was extracted using total RNA isolation (TRI) reagent (Sigma) in accordance with the supplied protocol. RNA was subsequently quantified using a spectrophotometer (Implen Nanophotometer, Muchen, Germany) configured to detect single strand RNA (μg/μl). RNA was standardised to 500 ng and used as a template to generate cDNA using high capacity cDNA reverse transcription kit (Applied Biosystems, Manchester, UK). Following cDNA synthesis, sample quality and uniformity was normalised against GAPDH expression (primer details in Table II). The amplifluor system (Intergen Inc., New York, NY, USA) was utilised with qPCR Master Mix (ABgene, Surrey, UK). Conditions for qPCR were; 15 min initial 95˚C period followed by 60 cycles of 95˚C for 15 sec, 55˚C for 60 sec and 72˚C for 20˚C sec.

SDS-PAGE and western blotting. Protein was extracted from a confluent 75-cm^2 tissue culture flask of MDA-MB-231 cells. Cells were detached and lysed in a buffer comprising 50 mM Tris-base, 5 mM EGTA, 150 mM NaCl, 1% Triton X-100, 100 μg/ml PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 mM sodium vanadate and 50 mM sodium fluoride on a rotor wheel for 1 h before removal of insolubles through centrifugation at 13,000 g. The Bio-Rad DC protein assay kit (Bio-Rad Laboratories, CA, USA) was used to quantify protein levels in

Table I. Primers designed for ribozyme synthesis.

Target	Ribozyme	Primer name	Primer sequence (5'-3')
		T7F	TAATACGACTCACTATAGGG
		RBBMR	TTCGTCCTCACGGACTCATCAG
		RBTPF	CTGATGAGTCCGTGAGGACGAA
OPG	OPG ribozyme 1	OPGRIB1F	CTGCAGCTCCTTGCACACGGGGCTGCAGTATACT GATGAGTCCGTGAGGA
		OPGRIB1R	ACTAGTACACAGACAGCTGGCACACCAGTGACGA GTGTTTCGTCCTCACGGACT
	OPG ribozyme 2	OPGRIB2F	CTGCAGACACTGCAATTTGTGTGTTTTCTACTGGG TGCTTTACTGATGAGTCCGTGAGGA
		OPGRIB2R	ACTAGTTCTTCTCAAATGAGACGTCATTTCGTCCT CACGGACT
	OPG ribozyme 3	OPGRIB3F	CTGCAGGGTAACATCTATTCCACATTTTGAGTTCTG ATGAGTCCGTGAGGA
		OPGRIB3R	ACTAGTTCCGGAAACAGTGAATTTCGTCCTCACGG ACT
RANK	RANK ribozyme 1	RANKRIB1F	CTGCAGCGCGCGGGGCCATGGCGCGGCTGATGA GTCCGTGAGGA
		RANKRIB1R	ACTAGTGCCGCGGCGCCGCCAGCCTGTTTCGTCC TCACGGACT
	RANK ribozyme 2	RANKRIB2F	CTGCAGCTCATAATGCTTCTCACTGGCTGATGAGT CCGTGAGGA
		RANKRIB2R	ACAGTCTTTGCAGATCGCTCCTCCATGTTTCGTCC TCACGGACT
	RANK ribozyme 3	RANKRIB3F	CTGCAGGTACTTTCCTGGTTCACATTTGTCTGATG AGTCCGTGAGGA
		RANKRIB3R	ACTAGTAGCATTATGAGCATCTGGGACGGTGCTGT TTCGTCCTCACGGACT
	RANK ribozyme 4	RANKRIB4F	CTGCAGTGCTGACCAAAGTTTGCCGTGTGTGCTG ATGAGTCCGTGAGGA
		RANKRIB4R	ACTAGTGGAGTCCTCAGGTGACAGTTGTGTCAGTT TCGTCCTCACGGAC
	RANK ribozyme 5	RANKRIB5F	CTGCAGCTGGCATCTTCGCCTTGTGCGTAGGCTG ATGAGTCCGTGAGGA
		RANKRIB5R	ACTAGTGTCAGGGCACATGTGTAGGAGGTGGTTTC GTCCTCACGGACT

each sample and samples were subsequently standardised to 2 mg/ml and diluted in 2X concentrate Laemmli sample buffer (Sigma) before being boiled for 5 min. Samples were loaded onto a 10% acrylamide gel and separated electrophoretically. Following separation the proteins were blotted onto a PVDF membrane (Merck-Millipore, Feltham, UK). Proteins were detected using the Merck-Millipore SNAP i.d. protein detection system. OPG expression was detected using anti-OPG antibody [R&D Systems, Abingdon, UK (BAF805)], RANK expression was detected using anti-RANK antibody [Santa Cruz Biotechnology, Inc., CA, USA (sc-9072)]. To assess uniformity of the samples GAPDH expression was also detected using anti-GAPDH antibody [Santa Cruz Biotechnology, Inc. (sc-32233)]. Following binding of the primary antibody, the membranes were probed with peroxidase conjugated anti-goat (OPG), anti-rabbit (RANK) or anti-mouse (GADPH) secondary antibodies (Sigma). Expression was visualised using the Luminata chemiluminescence detection kit (Merck-Millipore) and detected using a UVIProChem camera system (UVItec Ltd., Cambridge, UK).

Table II. Primers for conventional RT-PCR and real-time qPCR.

Gene	Primer name	Primer sequence (5'-3')	Optimal annealing temperature (˚C)	Product size (bp)
OPG	OPGF8	GAACCCCAGAGCGAAATACA	55	509
	OPGR8	CGGTAAGCTTTCCATCAAGC		
	OPGF1	GTTCTGCTTGAAACATAGGAG	55	115
	OPGZR1	**ACTGAACCTGACCGTACA**CGTCT CATTTGAGAAGAACC		
RANK	RANKF9	CAGAGCACAGTGGGTTCAGA	55	462
	RANKR9	GATGATGTCGCCCTTGAAGT		
	RANKF2	TCTGATGCCTTTTCCTCCAC	55	119
	RANKZR2	**ACTGAACCTGACCGTACA**TGGCA GAGAAGAACTGCAAA		
RANKL	RANKLF9	GACTCCATGAAAATGCAGAT	55	500
	RANKLR9	TCCTTTCATCAGGGTATGAG		
	RANKLF1	AAGGAGCTGTGCAAAAGGAA	55	74
	RANKLZR1	**ACTGAACCTGACCGTACA**ATCCA CCATCGCTTTCTCTG		
GAPDH	GAPDHF10	AGCTTGTCATCAATGGAAAT	55	593
	GAPDHR10	CTTCACCACCTTCTTGATGT		
	GAPDHF	CTGAGTACGTCGTGGAGTC	55	93
	GAPDHZR	**ACTGAACCTGACCGTACA**CAGAG ATGATGATGACCCTTTTG		
PDPL	PDPLF	GAATCATCGTTGTGGTTATG	55	
	PDPLZR	**ACTGAACCTGACCGTACA**CTTTCA TTTGCCTATCACAT		

ACTGAACCTGACCGTACA represents the Z sequence.

In vitro cell proliferation assay. An *in vitro* cell proliferation assay was used to examine the impact of OPG or RANK suppression on cell proliferation. Cells were seeded into two 96-well plates at a seeding density of $3x10^3$ cells/well with or without treatment and incubated for 1 and 5 days. Following incubation, cells were fixed in 4% formaldehyde (v/v) and stained with 0.5% crystal violet (w/v). Subsequently, 10% acetic acid (v/v) was used to extract the crystal violet stain and cell density determined through spectrophotometric analysis using a Bio-Tek Elx800 multi-plate reader (Bio-Tek Instruments Inc., VT, USA).

In vitro Matrigel matrix adhesion assay. Cell-matrix adhesion was assessed using a modified *in vitro* Matrigel adhesion assay (26). In brief, wells in a 96-well plate were pre-coated with 5 μg of Matrigel (BD Matrigel matrix, Matrigel basement membrane matrix, Biosciences). Cells were seeded at $4.5x10^4$ cells/well with or without treatment and left to adhere to the Matrigel for 40 min at 37˚C with 5% CO_2. Adherent cells were fixed in 4% formaldehyde (v/v) and stained with 0.5% crystal violet (w/v). Subsequently, adherent cells were visualised under the microscope and representative images captured for analysis.

In vitro cell motility assay. Cell motility was assessed using a cytodex-2 bead motility assay as previously described (27,28). In brief, $1x10^6$ cells were incubated in 10 ml of complete medium supplemented with 20 mg of cytodex-2 beads. The following day, beads were washed twice with complete medium before being resuspended, added to the 96-well plate with or without treatment and incubated for 4 h at 37˚C with 5% CO_2. Migrated cells were fixed in 4% formaldehyde (v/v) and stained with 0.5% crystal violet (w/v). Subsequently, adherent cells were visualised under the microscope and representative images captured for analysis.

In vitro Matrigel cell invasion assay. Cell invasiveness was assessed using an *in vitro* Matrigel invasion assay modified from refs. 29,30. In brief, transwell inserts containing 8-μm pores (Falcon, 24-well format, Greiner Bio-One, Germany) were placed in a 24-well plate (Nunc, Greiner Bio-One) and coated with 50 μg of Matrigel (BD Matrigel matrix, Matrigel

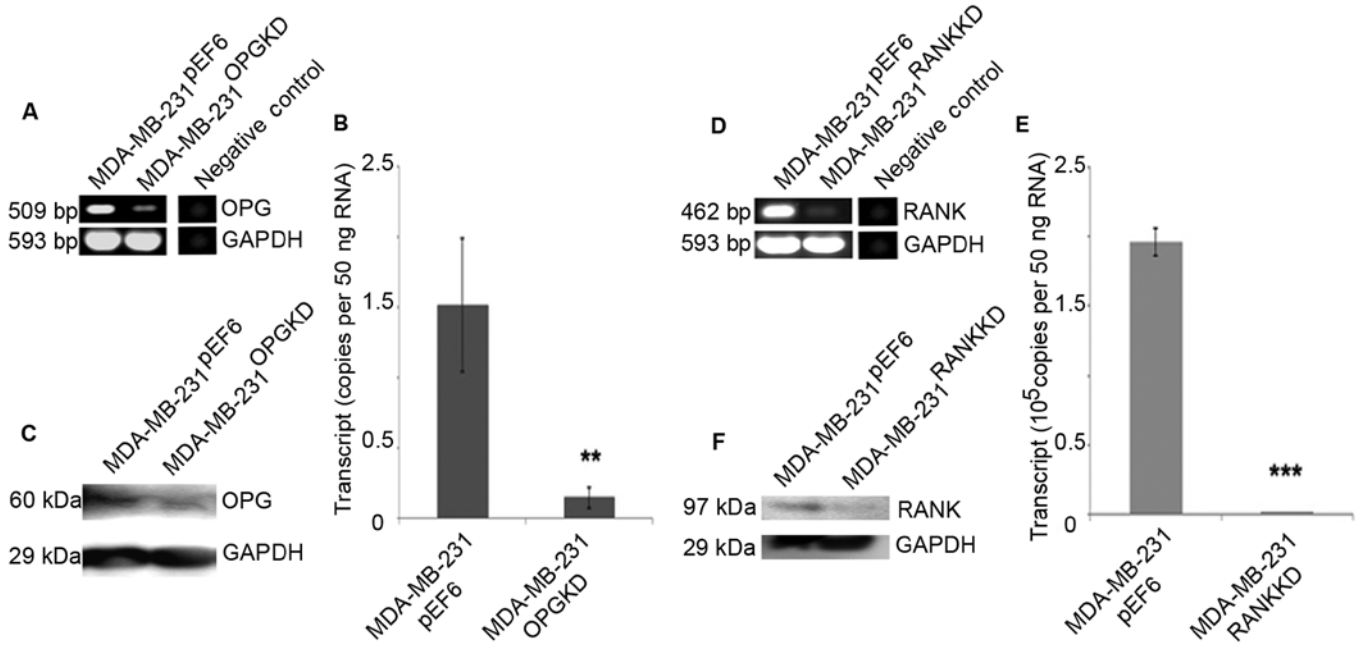

Figure 1. Verification of ribozyme transgene knockdown of OPG and RANK in MDA-MB-231 cells. Reduced expression of OPG and RANK were confirmed at a transcript level using RT-PCR (A and D) and qPCR (B and E) compared to the control cell line. Western blotting was used to confirm knockdown of OPG and RANK at a protein level (C and F respectively). PCR and western blotting were normalised against GAPDH. Representative images and data shown. $^{**}p\leq0.01$, $^{***}p\leq0.001$.

basement membrane matrix, Biosciences). Subsequently $2x10^4$ cells/insert were added to the insert and 1 ml of medium was added to the bottom of the 24-well plate to sustain any invaded cells. The plate was incubated for 3 days at 37˚C with 5% CO_2 after which inserts were cleaned to remove any non-invaded cells, before invaded cells were fixed in 4% formaldehyde (v/v) and stained with 0.5% crystal violet (w/v). Subsequently, invaded cells were visualised under the microscope and representative images captured for analysis.

Statistical analysis. The Sigma plot 11.0 statistical software package was used to assess statistical differences between the OPG or RANK suppressed MDA-MB-231 cells compared to the pEF6 vector control MDA-MB-231 cells using the Student's two tailed t-test or non-parametric Mann-Whitney U test. Experimental procedures were repeated a minimum of 3 independent times. Data represent mean values ± SEM, p-values of ≤0.05 were regarded as statistically significant.

Results

Expression of OPG and RANK has previously been established in three breast cancer cell lines (31). There are also potential links between the expression profiles of OPG, RANK, the pro-tumourigenic stimuli HGF and the bone microenvironment.

Suppression of molecules of interest using ribozyme transgenes. OPG or RANK expression was successfully targeted in MDA-MB-231 breast cancer cells following transfection with anti-OPG or anti-RANK ribozyme transgenes contained within a pEF6 plasmid. Reduced OPG transcript expression was seen in MDA-MB-231^{OPGKD} cells compared to the MDA-MB-231^{pEF6} cells using both RT-PCR and qPCR (Fig. 1A and B). The result was subsequently confirmed at a protein level using western blotting (Fig. 1C).

Reduced RANK transcript expression was seen in MDA-MB-231^{RANKKD} cells compared to the MDA-MB-231^{pEF6} cells using both RT-PCR and qPCR (Fig. 1D and E). This was subsequently confirmed at a protein level using western blotting (Fig. 1F).

Impact on MDA-MB-231 breast cancer cell proliferation. MDA-MB-231 breast cancer cell proliferation over 5 days was not significantly altered after suppression of OPG compared to the control cells (Fig. 2A). Suppression of RANK in MDA-MB-231 breast cancer cells resulted in a statistically significant decrease in cell proliferation after 5-day incubation compared to the control cells (Fig. 2B, p=0.029). Individual 40 ng/ml HGF and 50 μg/ml BME treatments significantly increased MDA-MB-231^{pEF6} cell proliferation after 5-day incubation compared to the untreated control (Fig. 2C, p=0.029). A similar pattern was seen after incubation with a combined 40 ng/ml HGF and 50 μg/ml BME treatment; however, this did not reach statistical significance. MDA-MB-231^{OPGKD} (Fig. 2D) and MDA-MB-231^{RANKKD} (Fig. 2E) cell proliferation was less responsive to HGF and BME treatments compared to those observed in the MDA-MB-231^{pEF6} cells. No statistically significant changes were observed in either of the suppressed cell lines compared to their respective untreated controls.

Impact on MDA-MB-231 breast cancer cell-matrix adhesion. Suppression of OPG in MDA-MB-231 breast cancer cells did not appear to affect cell-matrix adhesion (Fig. 3A). In contrast,

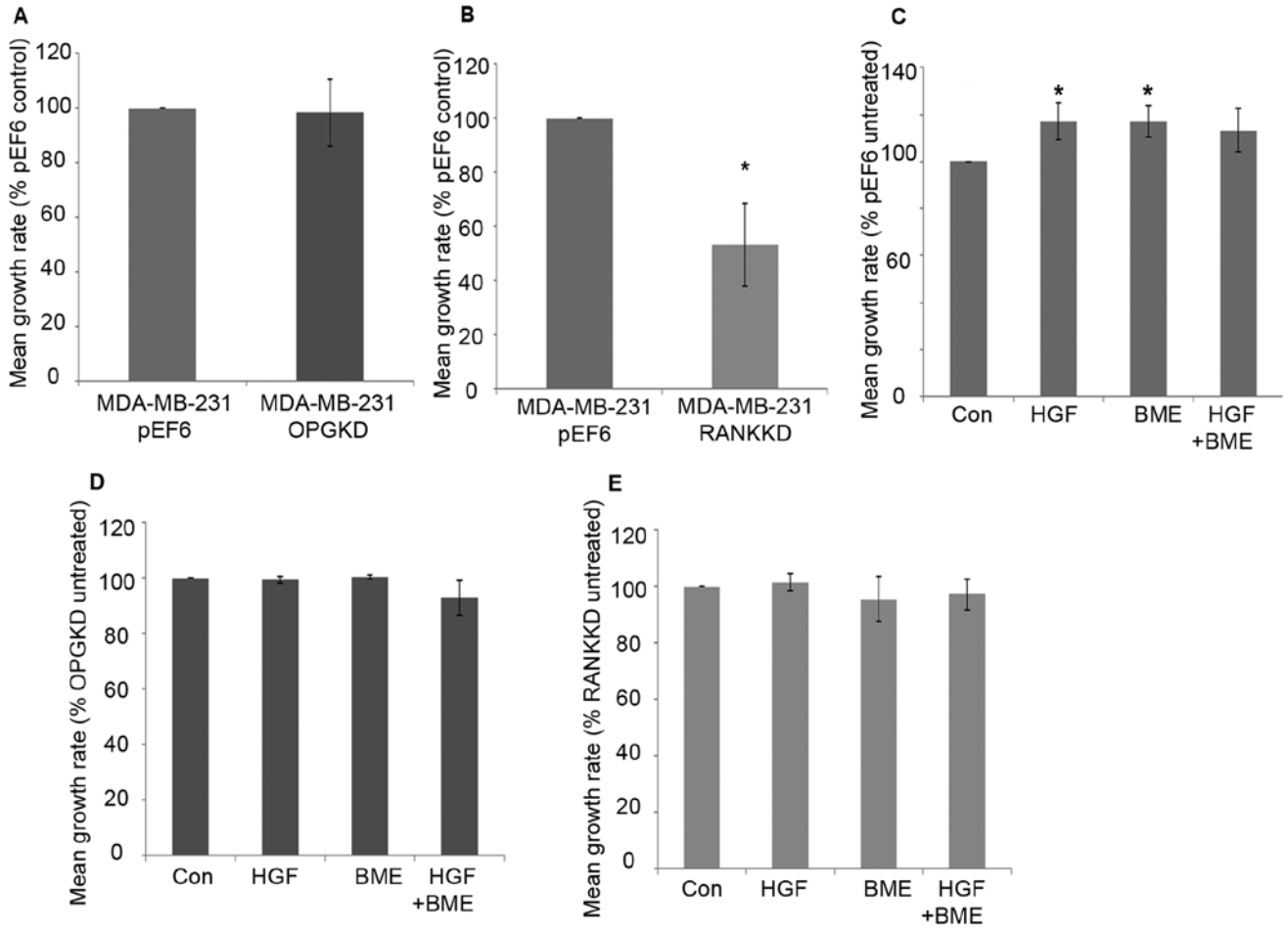

Figure 2. Impact of OPG and RANK knockdown on MDA-MB-231 cell proliferation *in vitro*. Suppression of OPG expression had no effect on MDA-MB-231 cell proliferation after 5-day incubation compared to MDA-MB-231^{pEF6} control cells (A). Reduced RANK expression in MDA-MB-231 cells resulted in a significant decrease in cell proliferation after 5-day incubation compared with control cells (B). Treatment of the MDA-MB-231^{pEF6} control cell line with 40 ng/ml HGF, 50 μg/ml BME or a combination of 40 ng/ml HGF and 50 μg/ml BME resulted in a significant increase in cell proliferation after 5-day incubation compared to the untreated cells (C). After 5-day treatment of MDA-MB-231^{OPGKD} cells or MDA-MB-231^{RANKKD} cells with 40 ng/ml HGF, 50 μg/ml BME or a combination of 40 ng/ml HGF and 50 μg/ml BME no changes were seen compared to the respective untreated cells (D and E). Data represent the mean of 4 independent repeats, error bars represent SEM. $^{*}p\leq0.05$.

suppression of RANK in MDA-MB-231 breast cancer cells resulted in a statically significant decrease in cell-matrix adhesion *in vitro* (Fig. 3B, p=0.029). MDA-MB-231^{pEF6} cell-matrix adhesion appeared unaffected under the influence of treatment with 40 ng/ml HGF and/or 50 μg/ml BME (Fig. 3C). A similar trend was seen in the MDA-MB-231^{OPGKD} cells treated individually with 40 ng/ml HGF or 50 μg/ml BME (Fig. 3D), however, when these treatments were combined cell-matrix adhesion was significantly reduced compared to the untreated cells (p=0.024). In the MDA-MB-231^{RANKKD} cells treatment with 40 ng/ml HGF and/or 50 μg/ml BME did not appear to significantly affect cell-matrix adhesion (Fig. 3E).

Impact on MDA-MB-231 breast cancer cell motility. Suppression of OPG expression in MDA-MB-231 cells resulted in significantly increased cell motility *in vitro* compared to the control cells (Fig. 4A, p=0.029). In contrast, suppression of RANK in MDA-MB-231 breast cancer cells resulted in significantly decreased cell motility *in vitro* (Fig. 4B, $p\leq0.001$). When MDA-MB-231^{pEF6} cells were treated with 40 ng/ml HGF cell motility was increased, however, this did not pass the statistical threshold (Fig. 4C). However, no noticeable effect was seen on MDA-MB-231^{pEF6} cell motility when cells were treated with 50 μg/ml BME. When both treatments were combined MDA-MB-231^{pEF6} cell motility was significantly increased compared to the untreated control cells (Fig. 4C, p=0.029). In contrast, treatment of MDA-MB-231^{OPGKD} cells with 40 ng/ml HGF or 50 μg/ml BME did not appear to impact MDA-MB-231 cell motility compared to the untreated control (Fig. 4D). When these treatments were combined increased cell motility was observed compared to the untreated control cells, however, this trend also failed to reach the statistically significant threshold. Similar responses to the exogenous HGF and BME treatments were also seen in the MDA-MB-231$^{RANK\ KD}$ cells, in which cell motility was only marginally affected by these factors (Fig. 4E).

Impact on MDA-MB-231 breast cancer cell invasion. Suppression of OPG resulted in significantly increased cell invasiveness compared to the control cells (Fig. 5A, p=0.037).

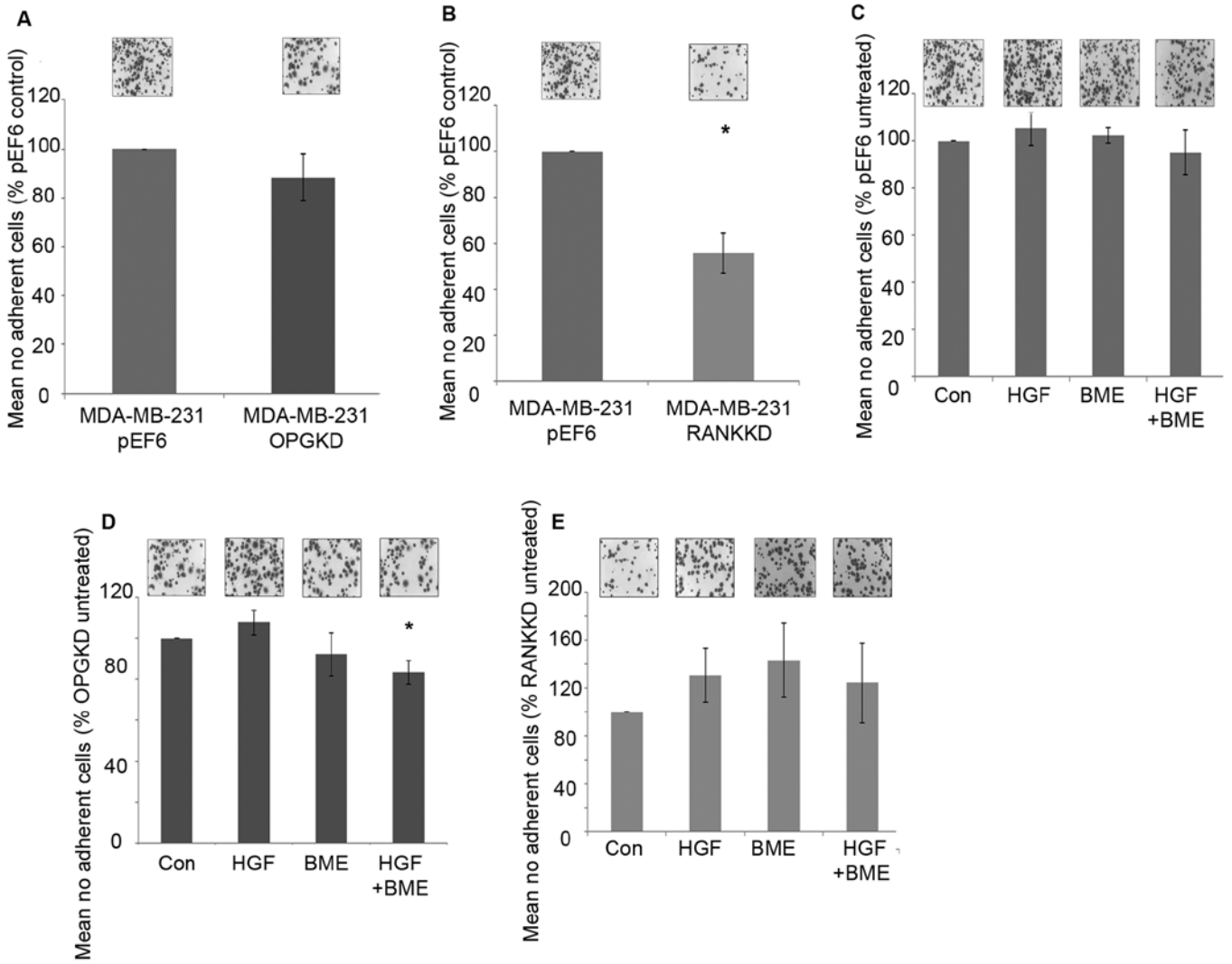

Figure 3. Impact of reduced OPG and RANK expression in MDA-MB-231 cells on cell-matrix adhesion *in vitro*. Reduced OPG expression did not alter MDA-MB-231 cell-matrix adhesion compared with control cells (A). Reduced RANK expression resulted in a significant decrease in MDA-MB-231 cell-matrix adhesion compared with control cells (B). When MDA-MB-231^{pEF6} control cells were treated with 40 ng/ml HGF or 50 μg/ml BME or a combination of 40 ng/ml HGF and 50 μg/ml BME did not significantly alter MDA-MB-231 cell-matrix adhesion (C). MDA-MB-231^{OPGKD} cells treated with 40 ng/ml HGF or 50 μg/ml BME also did not alter cell-matrix adhesion, however, a combined of HGF and BME treatment significantly decreased cell matrix adhesion (D). MDA-MB-231^{RANKKD} cells treated with 40 ng/ml HGF, 50 μg/ml BME or combined HGF and BME did not affect cell-matrix adhesion compared to the untreated cells (E). Data represent the mean of 4 independent repeats, error bars represent SEM. $^{*}p \leq 0.05$.

However, suppression of RANK in MDA-MB-231 cells resulted in a significant decrease in breast cancer cell invasion *in vitro* compared to the control cells (Fig. 5B, p=0.002). Treatment of MDA-MB-231^{pEF6} cells with 40 ng/ml HGF or 50 μg/ml BME increased cell invasion *in vitro* compared to untreated cells, however, these trends did not reach the statistically significant threshold (Fig. 5C). When these treatments were added in combination no noticeable effect on MDA-MB-231 cell invasion was observed. In contrast, when MDA-MB-231^{OPGKD} cells were treated with 40 ng/ml HGF or a combination of 40 ng/ml HGF and 50 μg/ml BME significant reductions in cell invasion were observed compared to the untreated cells (Fig. 5D, p=0.002 and 0.013, respectively). The largest reduction in cell invasion was observed under the individual 50 μg/ml BME treatment; however, this trend did not reach statistical significance. MDA-MB-231^{RANKKD} cell invasion under both individual exogenous treatments increased *in vitro* compared to the untreated control cells in a similar trend to that observed in the MDA-MB-231^{pEF6} control cells, though none of these changes reached a significant level (Fig. 5E). However, interestingly a combined treatment appeared to have little impact on breast cancer cell invasion.

Discussion

With the combined efforts of surgeons, oncologists and research, treatment options for primary breast cancer have improved. However, one aspect of the disease which still remains poorly understood and controlled is its metastatic spread, particularly to the bone. Through the recent licensing of Denosumab, the neutralising RANKL antibody, some progress has been achieved; however, there still remains no preventative measures or screening tools which can identify those most at risk.

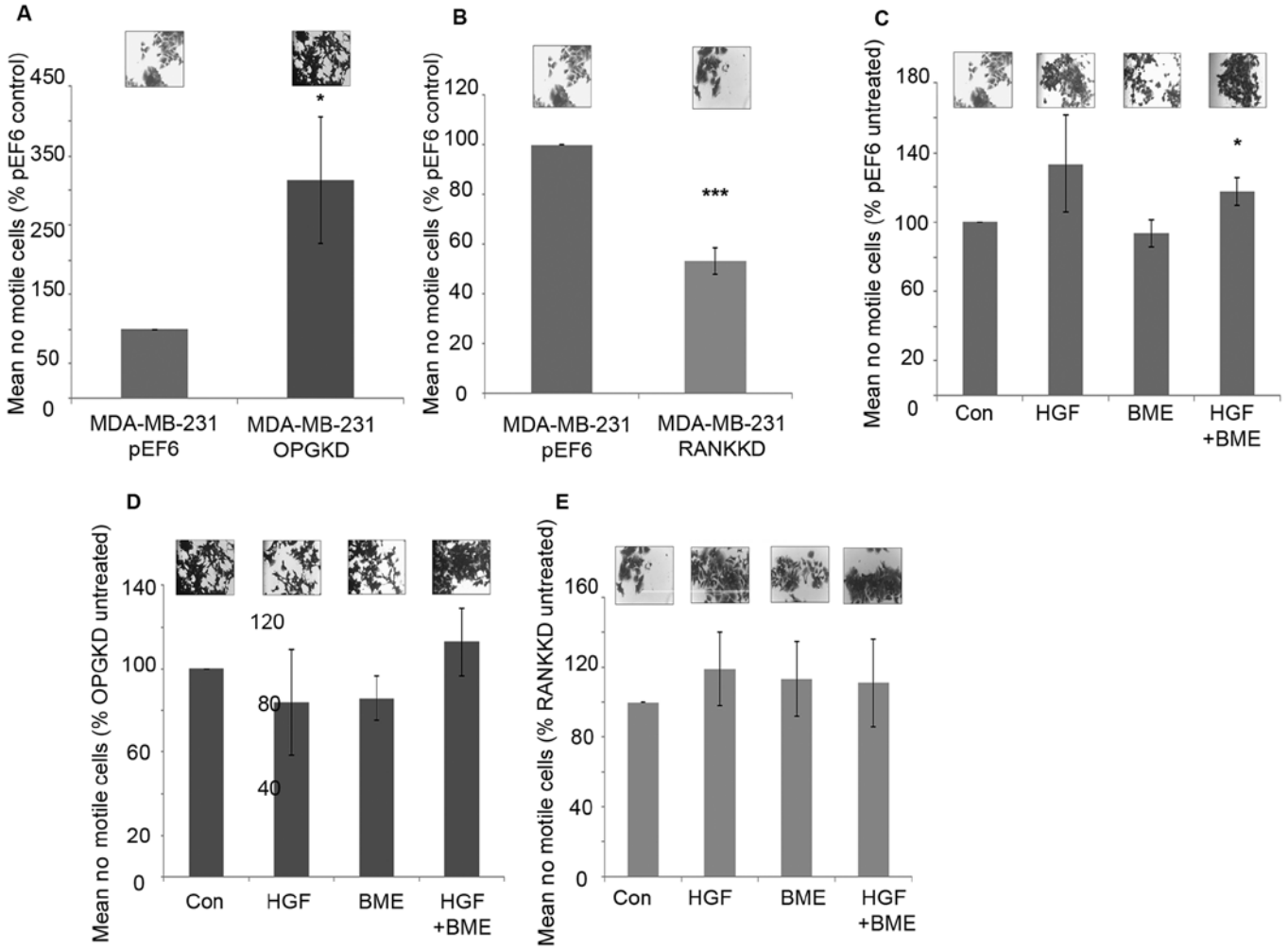

Figure 4. Effect of OPG and RANK knockdown on MDA-MB-231 cell motility. MDA-MB-231^{OPGKD} cells showed significantly increased motility compared with MDA-MB-231^{pEF6} control cells (A). Reduced RANK expression resulted in a significant decrease in MDA-MB-231 cell motility compared to MDA-MB-231^{pEF6} control cells (B). Treatment of MDA-MB-231^{pEF6} control cells with 40 ng/ml HGF or 50 μg/ml BME did not significantly alter cell motility. However, a combination of 40 ng/ml HGF and 50 μg/ml BME significantly increased cell motility compared to the untreated control (C). Treatment of MDA-MB-231^{OPGKD} or MDA-MB-231^{RANKKD} cells with 40 ng/ml HGF and/or 50 μg/ml BME did not significantly impact cell motility (D and E, respectively). Data represent the mean of 4 independent repeats, error bars represent SEM. $^{*}p\leq0.05$, $^{**}p\leq0.01$, $^{***}p\leq0.001$.

Whilst many previous studies have considered the role OPG plays in the inhibition of TRAIL and thus apoptosis (15), few reports consider the molecular implications in breast cancer progression to the bone. Of interest from this study was the lack of proliferation response to the exogenous HGF and BME treatments in the OPG suppressed cells which had been seen in the control cells. Of similar interest was that this lack of response to the exogenous stimuli (HGF and BME) was also seen in the cell-matrix adhesion assays. This study has also highlighted the potential roles suppression of OPG may have in increasing breast cancer cell motility and invasion in addition to its role in preventing apoptosis. Suppression of OPG resulted in significantly more motile MDA-MB-231 breast cancer cells compared to the untreated control. However, also of interest was that the exogenous treatments did not appear to have any further effect on this cell function. Though suppression of OPG resulted in significantly increased cell invasion, of note was that all the exogenously added treatments resulted in decreased cell invasion. This is of particular interest because, though not reaching statistical significance in the control cell line, treatment with HGF or BME resulted in increases in cell invasion. This highlights that OPG may play an integral role in breast cancer cells homing to the bone environment. This present data therefore suggest that expression of OPG may result in the suppression of these aggressive cancer cell traits and may also contribute to the regulation of the MDA-MB-231 breast cancer cell response to various environmental stimuli, including HGF, once bone metastases have already been established. This study implies *in vitro* the targeting of OPG also results in a response suppression to the oncogenic factor HGF. This is an interesting observation given a number of reports which demonstrate the potential prognosis effect of its receptor, c-MET expression and its phosphorylated version could have on breast cancer survival (32). However, an *in vivo* study by Zinonos *et al* (33) suggested that pharmacological inhibition of OPG though beneficial for bone health (reduction in osteolysis) also resulted in an increase in formation of soft tissue metastases. This was supported by Weichhaus *et al* (34) demonstrating that suppression of OPG in a chick embryo model reduced metastasis. These data and that from

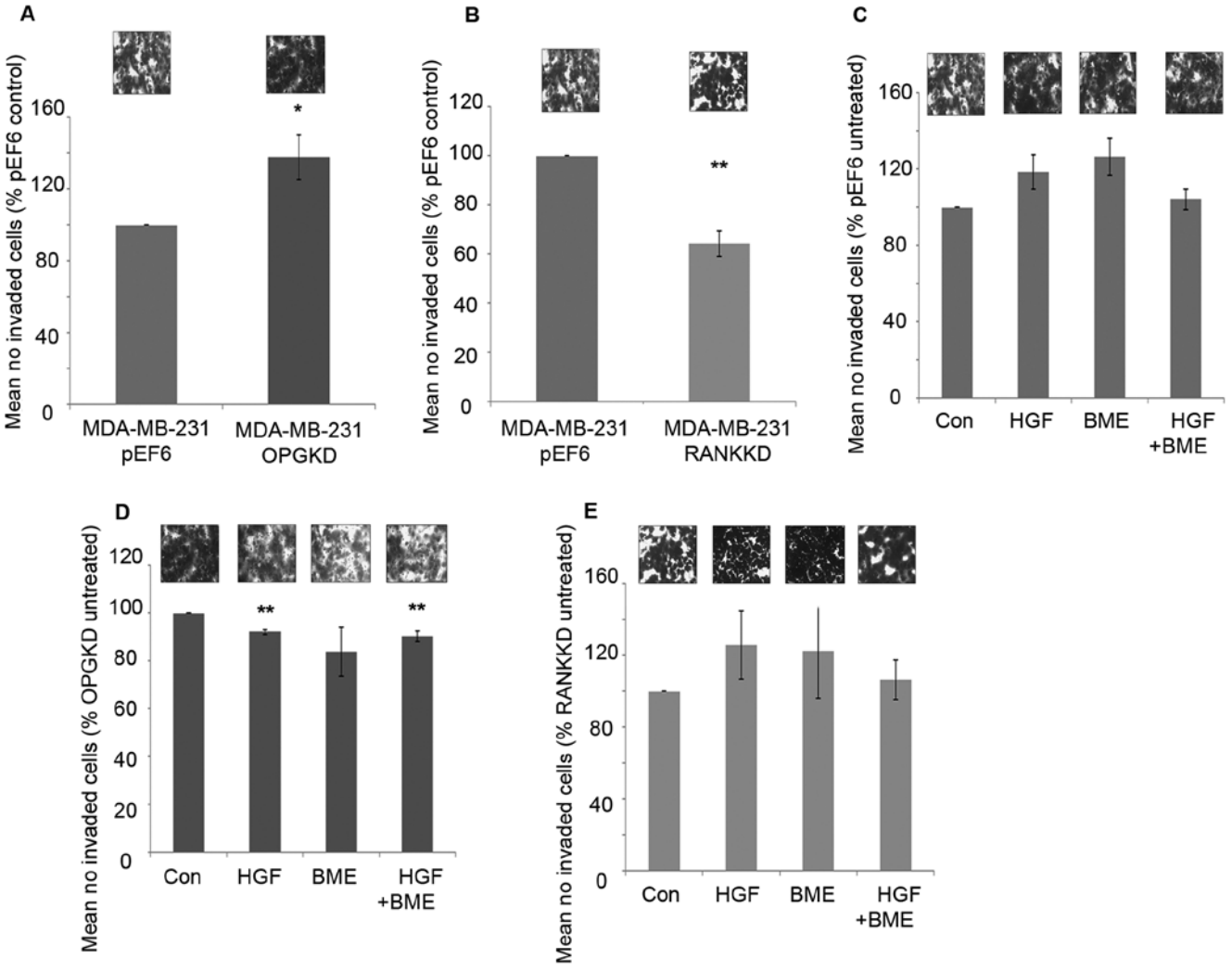

Figure 5. Impact of reduced OPG and RANK expression on MDA-MB-231 cell invasion *in vitro*. MDA-MB-231^{OPGKD} cells showed significantly increased cell invasion compared with MDA-MB-231^{pEF6} control cells (A). MDA-MB-231^{RANKKD} cells showed reduced cell invasion compared with MDA-MB-231^{pEF6} control cells (B). Treatment of MDA-MB-231^{pEF6} control cells with 40 ng/ml HGF, 50 μg/ml BME or a combination of 40 ng/ml HGF and 50 μg/ml BME do not impact cell invasion (C). Treatment of MDA-MB-231^{OPGKD} cells with 40 ng/ml HGF or a combination of 40 ng/ml HGF and 50 μg/ml BME resulted in a significant decrease in cell invasion (D). MDA-MB-231^{RANKKD} cells treated with 40 ng/ml HGF, 50 μg/ml BME or combined 40 ng/ml HGF and 50 μg/ml BME showed non-significant increases in cell invasion (E). Data represent the mean of 3 independent repeats, error bars represent SEM. *p=≤0.05, **p≤0.01.

elsewhere in the scientific literature therefore suggest that the targeting of OPG in breast cancer may be a double edged sword (35,36).

In contrast, the suppression of RANK in MDA-MB-231 breast cancer cells resulted in decreased responses in all the traits studied. Cell proliferation was significantly decreased after 5-day incubation compared to the control. Similar lack of response to the exogenous treatments was seen in the RANK suppressed cells. Interestingly, individual HGF or BME treatments increased cell-matrix adhesion and cell invasion in the RANK suppressed cells, though these did not pass the threshold for statistical significance. Most previous studies have overexpressed RANK in breast cancer models and reported increases in aggressive cell behaviour, including increased cell migration and invasion as well as greater metastatic bone colonisation (37). Casimiro *et al* (38) in their study found a link between bone-seeking RANK positive subclones of MDA-MB-231 cells and increased cell migration and invasion through the RANKL JNK and ERK 1/2 signalling pathway. This demonstrates that the three molecules, OPG, RANK and RANKL, originally linked to regulation of bone turnover have other roles, potentially even pro-metastatic ones in breast cancer. The data reported here suggest that the targeting of RANK affects breast cancer cell behaviour associated with a metastatic phenotype (i.e., migration and invasion) in its own right, changes which subsequently remained unaltered when exposed to a bone-like environment. This therefore opens the possibility to explore the combination of dual therapies which combine targeting of breast cancer cell expressed RANKL (Denosumab) and RANK.

The human body is an intricate combination of a variety of cells and factors which could never be replicated in a 2-D model, possibly accounting for the disparity between the *in vitro* results and our previously published clinical data. Isolating OPG and RANK in this model system has demonstrated, particularly with OPG that they may play roles in bone metastases associated with breast cancer. Further scientific study is now necessary to fully understand the downstream

molecules of OPG which influence this tumourigenic behaviour beyond the inhibition of TRAIL-induced apoptosis.

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