Universidade de Lisboa
Faculdade de Medicina de Lisboa

Analysis of the effect of the over-expression of receptor activator of NF-κB (RANK) in estrogen receptor-positive breast cancer cells over tumour behaviour *in vivo*

Inês André Correia

Orientador(a):
Prof.ª Doutora Sandra Cristina Cara de Anjo Casimiro

Dissertação especialmente elaborada para obtenção do grau de Mestre em Oncobiologia

Novembro 2016

Universidade de Lisboa
Faculdade de Medicina de Lisboa

Analysis of the effect of the over-expression of receptor activator of NF-κB (RANK) in estrogen receptor-positive breast cancer cells over tumour behaviour *in vivo*

Inês André Correia

Orientador(a):
Prof.ª Doutora Sandra Cristina Cara de Anjo Casimiro

Dissertação especialmente elaborada para obtenção do grau de Mestre em Oncobiologia

Novembro 2016

Todas as afirmações efetuadas no presente documento são da exclusiva responsabilidade do seu autor, não cabendo qualquer responsabilidade à Faculdade de Medicina de Lisboa pelos conteúdos nele apresentados.

A impressão desta dissertação foi aprovada pelo Conselho Científico da Faculdade de Medicina de Lisboa em reunião de 20 de Dezembro de 2016.

AGRADECIMENTOS

Para dar como concluído com êxito este percurso, não poderia deixar de agradecer a todos aqueles que tanto contribuíram para o meu sucesso.

Em primeiro lugar, quero expressar a minha gratidão à Prof.ª Doutora Sandra Casimiro, que me guiou e orientou desde o primeiro dia, por toda a partilha e transmissão de conhecimentos, pela confiança que sempre depositou em mim e, acima de tudo, pela paciência e amizade com que sempre me acolheu. Ao Prof. Doutor Luís Costa, pela disponibilidade em me receber na sua equipa e por todos os ensinamentos que tanto interesse e motivação me despertaram para querer continuar a contribuir para os avanços em Oncologia. Ao longo da vida profissional que ambiciono perseguir irei com certeza ter a oportunidade de aplicar todos os conhecimentos que me foram transmitidos pelos dois, por isso um grande obrigado.

Um enorme obrigado à Raquel, a minha verdadeira companheira de armas durante este último ano, pela amizade, por todas as conversas e risadas e pela preciosa ajuda, em todos os níveis. Fazer este percurso sem ti não teria, sem dúvida, sido a mesma coisa, minha pequenina. Às minhas colegas da Unidade LCosta, Irina e Marta, por toda a ajuda e partilha de conhecimentos. Um especial agradecimento à Inês Pinto e à Ana Duarte, que também comigo percorreram este caminho, por toda a partilha de ideias e pelo carinho e amizade que já se mantêm há tantos anos.

Aos grandes pilares da minha vida, os meus (gigantescos!) amigos: à Cláudia, Aninhas, Raquel, amizades de longa data que ainda hoje me aquecem o coração; à Margarida, Joana, ao Simão, Gabi, Gil, Bernardo, Gonçalo e tantos outros, por nunca permitirem que o meu sorriso desaparecesse, nem mesmo nos momentos mais difíceis. Um especial agradecimento ainda ao Diogo, pela enorme amizade, pelo apoio e incentivo incomparáveis, por surgir no momento certo e por me devolver o brilho.

Por fim, o maior obrigado de todos é para a minha família, sem os quais nada disto teria sido possível. Aos meus pais, por todo o apoio incondicional, por me incutirem os seus ideais e fazerem de mim a pessoa perseverante, lutadora e ambiciosa que hoje sou. Aos meus irmãos, Ricardo e Filipe, por, indiretamente, fazerem com que nunca desistisse de procurar ser melhor (ou 'A melhor'). À minha tia Carla e ao meu tio Francisco, por todo o carinho e encorajamento desde sempre. Aos meus avós, mesmo àqueles que já cá não estão, por contribuírem para a minha boa formação. À minha piolha, Filipinha, por involuntariamente fazer com que eu própria me tentasse elevar ao ponto de conseguir estar à altura de ser um exemplo a seguir.

Um final obrigado a todos os que igualmente contribuíram para a conclusão com sucesso desta nova etapa e que não estão aqui discriminados. Estarei eternamente grata pois, sem qualquer um de vós, tudo teria sido tão mais difícil.

INDEX

LIST OF ABREVIATIONS

Akt – Serine/threonine protein kinase

BC – Breast cancer

BPs – Bisphosphonates

BRCA1/2 – Breast cancer type 1 susceptibility gene 1/2

cDNA – Complementary deoxyribonucleic acid

CNA – Copy number aberrations

CNV – Copy number variations

CSC – Cancer stem cell

CTL – Cytotoxic T lymphocyte

DC – Dendritic cell

DMEM – Dulbecco´s Modified Eagle Medium

DNA – Deoxyribonucleic acid

DFS – Disease-free survival

DSS – Disease-specific survival

DTC – Disseminated tumour cell

ECM – Extracellular matrix

EGF – Epidermal growth factor

EGFR – Epidermal growth factor receptor

EMT – Epithelial-to-mesenchymal transition

ERK – Extracellular signal-regulated kinase

FFPE – Formalin-fixed paraffin-embedded

GFP – Green fluorescent protein

HRT – Hormone replacement therapy

HE – Hematoxylin & Eosin

IGF – Insulin-like growth factor

IHC – Immunohistochemistry

IL – Interleukin

KD – Knockdown

Luc – Luciferase

MCF-7 – Michigan Cancer Foundation-7 (Breast Cancer Cell Line)

MDA-MB-231 – M.D.Anderson – Metastatic Breast 231 (Breast Cancer Cell Line)

MaSC – Mammary stem cell

MMP – Matrix metalloproteinase

mRNA – Messenger ribonucleic acid

NF-κB – Nuclear factor kappa B

OE – Overexpression

OPG – Osteoprotegerin

OS – Overall survival

PCR – Polymerase chain reaction

PDGF – Platelet-derived growth factor

PDGFR – Platelet-derived growth factor receptor

PTHrP – Parathyroid hormone-related peptide

RANK – Receptor activator of nuclear factor kappa B

RANKL – Receptor activator of nuclear factor kappa B ligand

RFP – Red fluorescent protein

RNA – Ribonucleic acid

RT-qPCR – Quantitative reverse transcription PCR

SNP – Single nucleotide polymorphism

TF – Transcription factor

TNBC – Triple negative breast cancer

TNFRSF11A – Tumour necrosis factor receptor superfamily member 11

TNFSF11 – Tumour necrosis factor ligand superfamily member 11

LIST OF TABLES AND FIGURES

List of Tables:

List of Figures:

Supplementary Figures:

RESUMO

O cancro da mama é o segundo tipo de cancro mais frequente no mundo e o mais frequente na mulher. Segundo os últimos dados do GLOBOCAN, quase 2 milhões de casos de cancro da mama foram diagnosticados mundialmente em 2012. Em Portugal, o cancro da mama é o terceiro tipo de cancro com maior incidência e mortalidade, quando considerados ambos os sexos, apenas superado pelo cancro colo-rectal e cancro da próstata, sendo igualmente o mais frequente no sexo feminino. Com o desenvolvimento de novas terapêuticas e métodos de rastreio para deteção precoce, têm havido melhorias significativas nos *outcomes* clínicos de doentes com cancro da mama. Contudo, este continua a ser o quinto tipo de cancro com maior taxa de mortalidade a nível mundial, tendo sido responsável por 522 000 mortes em 2012. A principal causa de morte por cancro da mama deve-se ao desenvolvimento de metástases em locais secundários, sendo que 20 a 30% das mulheres que são diagnosticadas com cancro da mama em estadio inicial irão desenvolver metástases no curso da sua doença.

O cancro da mama é uma doença bastante heterogénea, quer ao nível clínico, quer ao nível morfológico e molecular. De acordo com características moleculares específicas, nomeadamente a expressão de recetores hormonais (RH) e HER2, o cancro da mama pode ser dividido em três subtipos principais, com diferentes implicações clínicas (prognósticas e terapêuticas): cancro da mama HR-positivo (luminal), que compreende cerca de 60% dos casos; cancro da mama HER2-positivo ($HER2^+$), com aproximadamente 20% dos casos; cancro da mama triplo negativo (basal ou *Claudin-low*), com cerca de 30% dos casos. Entre os diferentes subtipos, o cancro da mama do tipo luminal apresenta o melhor prognóstico. Contudo, cerca de 30 a 40% das doentes com cancro da mama luminal desenvolvem metástases no prazo de 15 anos após o diagnóstico, sobretudo metástases ósseas (em 70% dos casos).

No contexto do cancro da mama, a via de sinalização do recetor ativador da via do NF-κB (RANK) – ligando do RANK (RANKL) tem sido alvo de inúmeros estudos dado o seu potencial terapêutico. Esta via é extremamente importante em três processos fisiológicos distintos: na remodelação óssea; no desenvolvimento da glândula mamária; e na ativação funcional de células do sistema imunitário, nomeadamente células dendríticas (DCs). Por outro lado, a via RANK-RANKL desempenha um papel fundamental na tumorigénese mamária e progressão tumoral de cancro da mama.

Estudos anteriores *in vitro* demonstraram que a ativação da via de sinalização RANK-RANKL afeta a capacidade de invasão de células de cancro da mama, induzindo transição epitélio-mesênquima (TEM) e expressão de marcadores de células estaminais tumorais. Em estudos com amostras clínicas de cancro da mama, a sobreexpressão de RANK tem sido principalmente associada a tumores triplo negativos. No entanto, estudos anteriores do nosso grupo permitiram demonstrar que a sobreexpressão do RANK numa linha celular de cancro da mama do tipo luminal (HR^+), MCF-7, que expressa níveis endógenos baixos de RANK, leva à promoção da TEM, migração e invasão celulares, e aquisição de características de estaminalidade, aumentando a população de células $CD44^+/CD24^{-/low}$.

Com base nestes resultados, interrogámo-nos se a sobreexpressão do RANK em células de cancro da mama do tipo luminal poderá condicionar um fenótipo mais agressivo *in vivo*, sendo a resposta a esta questão o principal objetivo do presente estudo.

Assim, neste estudo foi utilizado um modelo animal de xenoenxerto ortotópico de células de cancro da mama do tipo luminal, por forma a podermos analisar o efeito da sobreexpressão do RANK em células MCF-7 no crescimento tumoral e invasibilidade do tumor. Foi utilizada a linha celular MCF-7$^{GFP+Luc+}$, que permite seguir o crescimento tumoral *in vivo* por análise de bioluminescência, e células MCF-7 com sobreexpressão de RANK (MCF-7 RANK OE) foram derivadas da linha parental por transdução lentiviral. Foi ainda derivada uma linha de células MCF-7 RANK OE^{RFP+}, também por transdução lentiviral, por forma a podermos visualizar simultaneamente as duas populações em co-cultura ou co-inoculação.

As células tumorais, parentais ou RANK OE, foram inoculadas na glândula mamária de ratinhos NSG e, uma vez que o nosso principal objetivo era verificar se as células RANK OE seriam mais agressivas e invasivas, foi ainda inoculada uma mistura (1:1) dos dois tipos de células na mesma glândula mamária (grupo Mix). Cinco semanas após inoculação foi colhido sangue para identificação de células tumorais circulantes (CTCs). Os ratinhos foram sacrificados oito semanas após inoculação, os tumores foram seccionados e congelados ou fixados em formol e incluídos em parafina. Foram ainda colhidos sangue e medula óssea para identificação de CTCs e células tumorais disseminadas (DTCs), respetivamente, bem como órgãos viscerais (pulmões, fígado e baço) para deteção de metástases.

Por análise de bioluminescência verificámos que os tumores RANK OE e Mix apresentaram um menor crescimento do que os tumores do grupo parental ($p<0.001$), com um crescimento idêntico entre si. O menor crescimento deveu-se a um índice proliferativo inferior, medido pela imunodeteção de Ki67, dado corroborado pelos resultados *in vitro*, que demonstram que as células com sobreexpressão do RANK têm uma taxa de proliferação inferior à das células parentais.

Após análise das células constituintes dos tumores por citometria de fluxo, observámos que os tumores do grupo Mix eram predominantemente constituídos por células com sobreexpressão do RANK (média ~60% do total de células), o que sugere que células RANK OE apresentam uma vantagem adaptativa sobre células com baixa expressão deste recetor (parentais).

Apesar dos tumores serem mais pequenos, foram identificadas mais CTCs em ratinhos inoculados com tumores RANK OE ou Mix, cinco semanas após inoculação, quando comparando com ratinhos inoculados com células parentais ($p<0.05$). Para além disso, é de notar que as CTCs identificadas em ratinhos do grupo Mix foram maioritariamente RANK OE, sugerindo uma maior capacidade invasiva neste tipo de células. A hipótese de que as células RANK OE são mais invasivas foi também corroborada *in vitro* através de ensaios de migração, onde as células MCF-7 RANK OE apresentaram maior capacidade de migração do que células parentais, mesmo em co-cultura ($p<0.001$). Estes resultados são consistentes com o facto de os tumores com sobreexpressão do RANK terem expressão aumentada de marcadores mesenquimatosos (Vimentina, Snail e N-caderina), quando comparando com tumores parentais. Este resultado deverá ser confirmado por análise da expressão dos

marcadores de EMT diretamente no tecido tumoral dos ratinhos (imunohistoquímica ou imunofluorescência).

Por último, observámos ainda que os tumores RANK OE e Mix apresentam um elevado grau de desmoplasia ($p<0.05$), estroma denso rico em colagénio, avaliado histologicamente por análise de secções coradas com Tricrómio de Masson.

Em suma, os resultados deste estudo sugerem a existência de uma correlação positiva entre a sobreexpressão do RANK em células de cancro da mama do tipo luminal e a aquisição de características mais agressivas, como maior desmoplasia e invasibilidade, para além de possuírem uma menor taxa de proliferação, que lhes poderá conferir resistência à quimioterapia.

Coexistindo num tumor "heterogéneo" com células com baixa expressão de RANK, as células que apresentam sobreexpressão deste recetor demonstram ter uma vantagem adaptativa, um resultado que necessita ainda de ser clarificado. Trabalhos futuros envolverão a co-cultura dos dois tipos de células e medição do crescimento celular específico, ou ainda a análise do crescimento celular em esferoides compostos pelos dois tipos celulares numa matriz 3D. Será ainda efetuado um estudo de sobrevivência e análise de CTCs e taxa metastização em modelo animal, comparando o efeito da inoculação das células RANK OE e parentais na mesma glândula mamária ou em glândulas contra-laterais.

Uma vez que a via de sinalização RANK-RANKL tem emergido como potencial alvo terapêutico em contexto adjuvante em cancro da mama, para além do seu papel como inibidor da reabsorção óssea em contexto metastático, é importante clarificar a contribuição desta via nos diferentes subtipos. Já foi demonstrado que a via RANK-RANKL está correlacionada com piores *outcomes* clínicos, desde sobrevivência global a doença livre de progressão, contudo está ainda por clarificar se a inibição desta via em cancro da mama poderá ter algum benefício para além do que possui no contexto da doença metastática do osso. Estudos futuros serão necessários para compreender de que forma a sobreexpressão de RANK em cancro da mama do tipo luminal se poderá correlacionar com a progressão tumoral, metastização e resistência à quimioterapia.

Palavras-chave:

Cancro da mama; Via de sinalização RANK-RANKL; Metastização; Células tumorais circulantes (CTCs); Invasibilidade

ABSTRACT

Breast cancer (BC) is the second most common cancer in the world and the most frequent cancer among women. Most frequent BC are hormone receptor positive (HR^+), commonly designated by luminal BC. Bone metastasis (BM) are the most frequent amongst metastatic BC and occur in about 70% of all HR^+ BC cases, significantly decreasing the overall survival.

The receptor activator of NF-κB (RANK) pathway not only controls bone remodelling, mammary gland development and activity of dendritic cells, but is also involved in the BC onset and progression, as well as in the preferential metastasis of BC cells to bone. Despite clinical data points to overexpression of RANK mostly in HR^- BC, previous studies have shown that RANK overexpression (OE) in luminal MCF-7 cells resulted in a mesenchymal transformation and increased stemness and migration potential. Therefore, we hypothesize that $RANK^+$ luminal BC cells could have an enhanced invasion ability and that metastasis may be enriched for this subpopulation of cells.

In this study we used an orthotopic xenograft mouse model of luminal BC to study how RANK OE correlates with tumour burden and invasiveness. MCF-7 parental, RANK OE or both cell-types were inoculated in the mammary fat pad of NSG mice. RANK OE and Mix tumours exhibited a similar decreased growth rate, but an increase in CTCs, desmoplasia and epithelial-to-mesenchymal transition (EMT)-related genes. Predominant CTCs in the blood of Mix tumour-bearing mice and predominant cells in the Mix tumours were RANK OE cells.

Our results suggest that RANK OE cells present a more aggressive and invasive phenotype and that, when coexisting in the same tumour with cells with low RANK expression, RANK OE cells present an adaptive advantage. Although further studies are still required to elucidate RANK's contribution in luminal-type BC, these findings can have high relevance since RANK pathway is emerging as a major target in BC treatment.

Key-words:

Breast cancer; RANK-RANKL signalling pathway; Metastization; Circulating Tumour Cells (CTCs); Invasiveness

1. Breast cancer

1.1. Etiology and epidemiology

Breast cancer (BC) is the second most common cancer in the world, just after lung cancer, and the most frequent among women, with almost 1,7 million new cases diagnosed worldwide in 2012[1]. Despite improved outcomes, mostly due to earlier detection and development of new therapies, BC still remains as the fifth most lethal cancer, responsible for 522 000 deaths in 2012, an increase of 18% in comparison to 2008. Current predictions and statistics suggest that BC incidence and mortality will continue to rise.

In Portugal, BC is the third most common cancer, following colorectal and prostate cancers, and the most common in women. In 2012, 6 088 new cases of BC were diagnosed in Portugal, and 1 570 patients died with this malignancy[1].

Several features, either genetic or lifestyle-based, have been correlated to BC risk over the years. Age is a major risk factor for BC and the estimated average probability of a 30-year-old woman to develop BC over a 10-year period is 0.43%, about 10 times less than that for a 70-year-old woman (3.74%)[2]. Overweight has also been associated with higher risk of developing BC[3], which can be correlated to the fact that obesity can lead to increased circulating levels of estrogen[4]. In fact, long exposure to endogenous (early menarche and late menopause) and exogenous (contraceptives and hormone-replacement therapies (HRT)) estrogens is also a risk factor for developing BC[5,6].

The majority of BC is sporadic, driven by genetic alterations on somatic cells, but occasionally these alterations can occur in the germline and therefore be inherited, leading to an increase in susceptibility to the disease. In fact, genetic predisposition is known to be one of the most important risk factors for BC, being present in 20% of BC patients who have a positive family history of BC[7]. Breast cancer susceptibility genes 1 and 2 (*BRCA1* and *BRCA2*) were identified in the '90s as associated with inherited susceptibility to both breast and ovarian cancers[8–10]. Intense studies on this subject have culminated in the application of such knowledge in the clinical practice, with the implementation of genetic screening for germline mutations in these genes in women with a family history of both breast and ovarian cancers.

It is extremely important to understand and address BC risk factors to improve BC prevention and decrease its incidence and mortality.

1.2. Classification and prognosis

BC is a very heterogeneous disease, both at the molecular and clinical level, and it can be subdivided into different subtypes, according to histopathological and molecular characteristics. BC classification into subtypes not only allows the stratification of patients according to prognosis, but also defines the best therapeutic approach for each one.

Histologically, BC can be classified as ductal carcinomas, if originated in the mammary ducts; or lobular carcinomas, if originated in the lobules[11,12]. Ductal BC are the most frequent, representing approximately 80% of BC cases, and have decreased disease-specific survival (DSS) when comparing to lobular carcinomas[13]. Independently of histological sub-type, BC can be classified as *in situ*, when the tumour mass is delimitated by the basal membrane, or invasive, if cancer cells already invade the basal membrane. Demonstrating already a more aggressive potential, invasive BC has worse prognosis.

Breast tumours are also classified according to specific molecular characteristics into three major subtypes with clinical implications: hormone receptor (HR) positive BC (HR^+ BC); HER2 amplified BC ($HER2^+$ BC); and triple negative BC (TNBC).

However, the molecular portraits of BC have being refined over the last 15 years, essentially due to the huge technologic advances in gene expression-related techniques. In the first seminal work by Perou *et al.* it was possible to correlate the phenotypic diversity observed in BC and specific gene expression patterns[14]. Importantly, the different patterns of gene expression were correlated with biologic features, like variation in growth rate, in the activity of specific signalling pathways, and in the cellular composition of the tumours. It was also shown that the gene expression clusters reflected two different epithelial origins, from basal (and/or myoepithelial) or luminal epithelial cells. The gene expression cluster characteristic of the luminal cells was anchored by the previously noted cluster of transcription factors (TF) that included the estrogen receptor (ER), and segregated from HR^-HER2^+ tumours or basal-like, mostly TNBC. This led to the current denomination of BC intrinsic sub-types into three groups that are related to different molecular features of mammary epithelial biology: ER^+/luminal-like, basal-like, and $HER2^+$.

The ER-positive (ER^+)/luminal group of BC is not only the biggest, representing almost 60% of all BC, but also the most diverse one, and in general has the better prognosis[11,15,16]. Patients with ER^+ BC are likely to benefit from hormone-based therapies, which inactivate the ER signalling, either by blocking (e.g. Tamoxifen) or inducing the degradation of ER (e.g. Fulvestrant); or suppress the estrogen biosynthesis (e.g. aromatase inhibitors)[17,18]. Luminal BC can be subdivided into luminal A and luminal B. Luminal A breast tumours represent approximately 40% of all BC and are characterized by expression of HRs and low proliferation rate. Luminal B are less common than luminal A (~20% of all BC) and also express HRs, although at lower levels, but present a variable expression of HER2 and a relatively higher proliferation rate[15]. These molecular and biologic characteristics reflect a poorer prognosis of luminal B BC, when comparing to luminal A BC[16].

The $HER2^+$ BC are the second most prevalent, accounting for approximately 20% of all BC cases. Although the amplification of HER2 *per se* is associated with tumour aggressiveness and poor prognosis, the development of targeted anti-HER2 therapies (e.g. Trastuzumab and Pertuzumab), has significantly improved the overall survival (OS) for these patients[12,19,20].

TNBCs have the worst prognosis[16], due to the higher tumour aggressiveness and lack of targeted-therapies, rendering chemotherapy as the only possible therapeutics. Two major molecular subtypes are included into the TNBC group: basal-like and claudin-low. Basal-like BC represents about 15% of all BC and is characterized by expression of

basal-epithelial cells-related genes, such as keratins 5/6, and no expression of neither HRs or HER2[21]. Claudin-low BC, on the other hand, is characterized by high expression levels of epithelial-to-mesenchymal transition (EMT)-associated genes, cancer stem cell-like features, immune response genes and low to absent expression of luminal differentiation markers[22].

From the work of Perou *et al.* it also came out a fourth subgroup of BC, named "normal-like BC", which presented a similar gene expression pattern to the normal breast, with high expression of genes characteristic from non-epithelial cells[14].

The characteristics of each molecular BC subtype are resumed in Table 1.

Table 1 – Molecular classification of breast cancer subtypes. [12,14,15,22,21]

Molecular subtype		Common characteristics	Frequent mutations	Prevalence
Luminal	***A***	ER^+ and/or PR^+ $HER2^-$ Low Ki67 $Ck8/18^+$	PIK3CA (49%) TP53 (12%) GATA3 (14%) MAP3K1 (14%)	30-40%
	B	ER^+ and/or PR^+ HER2 overexpression/amplification and/or high Ki67	TP53 (32%) PIK3CA (32%) MAP3K1 (5%)	20%
HER2-positive		ER^- and PR^- HER2 amplification High Ki67	TP53 (75%) PIK3CA (42%) PIK3R1 (8%)	20%
TNBC	***Basal-like***	ER^- and PR^- $HER2^-$ High Ki67 $Ck5/6^+$	TP53 (84%) PIK3CA (7%) BRCA1 EGFR1	15%
	Claudin-low	EMT-associated genes (vimentin, fibronectin) Stem-cell like genes ($CD44^+/CD24^-$) CXCL12	Non-applicable	10%
Normal-like		High expression of genes from non-epithelial cell types (e.g. Adipose tissue)	Non-applicable	Unknown

More recently, Curtis *et al*. analysed the relative influence of single nucleotide polymorphisms (SNPs), copy number variations (CNVs) and copy number aberrations (CNAs) on tumour expression architecture of a collection of over 2,000 primary fresh-frozen BC specimens from tumour biobanks in the UK and Canada[23]. This study led to the stratification of BCs into ten integrative subgroups, clustered by well-defined CNAs, presenting different clinical outcomes, opening new doors in BC research.

1.3. Metastization patterns

BC distant recurrence remains common and incurable, being responsible for 15% of cancer deaths in women[1]. Most deaths due to cancer still result from the progressive growth of metastases that are resistant to the current available therapies. Even though many improvements have been made in BC treatment, 20 to 30% of patients diagnosed with early BC will have disease recurrence at secondary sites on the course of their disease[24].

BC metastasizes typically to bone, lungs, liver, soft tissue and brain[25]. Bone is the most common site of metastization in both luminal and $HER2^+$ BC[24]. Compared to luminal A BC, luminal B and $HER2^+$ subtypes show higher rates of lung and liver metastasis. On the other side, the most common metastatic sites for TNBC are the brain, lung and distant nodes, having low rates of relapse in liver and bone.

Since about 70% of all metastatic luminal BC cases have bone metastases (BM), bone is the most frequent site of relapse in BC. Patients with BM will develop skeletal-related events (SREs), such as pain (and consequent radiotherapy), spinal cord compression, pathologic fractures, and hypercalcemia, that will cause severe morbidity and significantly decrease OS[24,26]. Patients with BM-only have a more indolent disease in terms of progression than patients with bone and visceral metastasis[27].

Results from previous studies suggest that tumour expression profiles could help to predict the first site of metastasis[25,28–32]. However, there are still no clear predictive signatures of organ-specific BC tropism.

2. RANK-RANKL signalling pathway

The receptor activator of NF-κB (RANK) is a transmembrane protein from the tumour necrosis factor receptor (TNFR) superfamily, initially identified in dendritic cells (DC)[33]. The RANK ligand (RANKL) is expressed by osteoblasts, T cells and stromal cells and can be present either in its transmembrane or soluble form[33–36].

Upon binding to RANKL, RANK triggers the downstream activation of several signalling pathways involved in proliferation, survival and differentiation. RANK-RANKL pathway is known to be involved in DC survival and activity[33], osteoclastogenesis[37] and mammary gland development[38]. It has also been shown that RANK-RANKL pathway is crucial for proliferation and differentiation of hair follicular cells, thymic modullary cells and microfold intestinal cells[39,40].

When RANK signalling is triggered by RANKL, several TNFR-associated factor (TRAF) proteins, namely TRAF2, 5 and 6, interact with different RANK motifs in its cytoplasmic domain: motif 1 ($PFQEP^{369\text{-}373}$), motif 2 ($PVQEET^{559–564}$) and motif 3 ($PVQEQG^{604–609}$), being essential for downstream activation[41] (Figure 1). TRAF6 interaction with RANK's motif 1 is essential to functional activation of osteoclasts[42], leading to activation of NF-κB and MAPK (JNK, ERK, p38) pathways. Interaction between RANK-TRAF6 strongly induces cFos and NFATc1 transcription in osteoclast precursor cells, which is fundamental to osteoclastogenesis[43–45]. PI3K/AKT pathway is activated in DCs through the formation of a complex that includes RANK-RANKL, TRAF6 and c-Src[46]. Functional significance of RANK interaction with TRAF2 and 5 remains elusive.

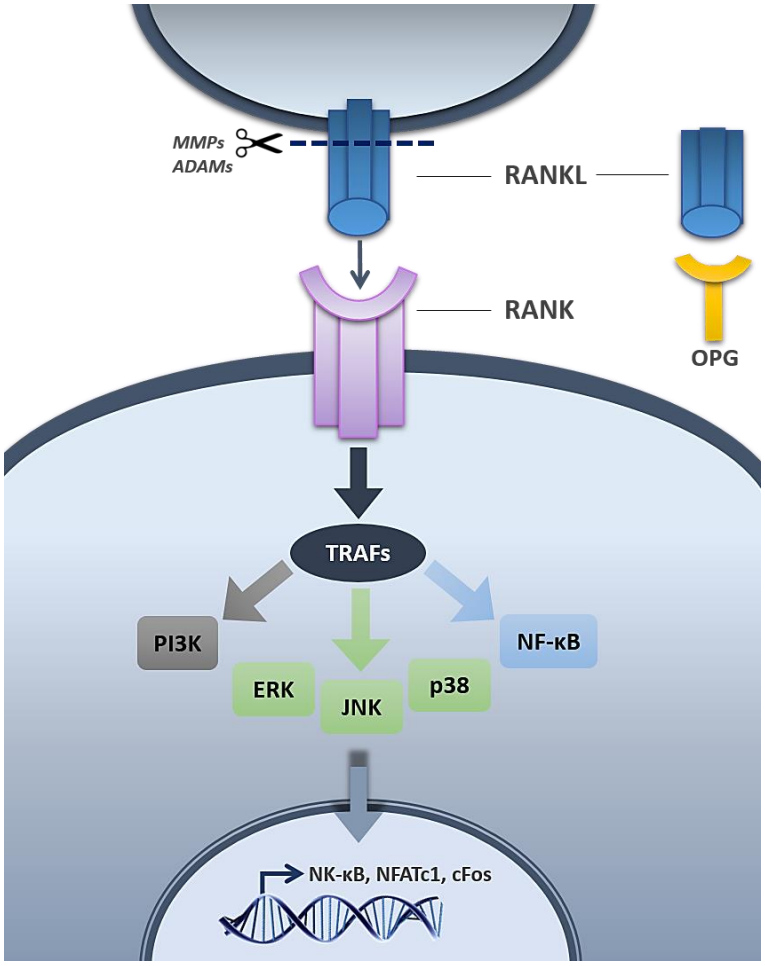

Figure 1 - Schematic representation of RANK-RANKL signalling pathway.

RANKL, both in its transmembrane or soluble form, binds to RANK, triggering its trimerization. Recruited TRAFs bind to TRAF-binding motifs present in the receptor and downstream targets are activated (PI3K/AKT, NF-κB and MAPK pathways). Osteoprotegerin (OPG) is a decoy receptor for RANKL, controlling RANK-RANKL signalling. *Adapted from [47]*

2.1. RANK signalling in bone remodelling

Physiologic bone remodelling cycle involves constant bone resorption and formation, a process depending on several factors, but tightly controlled by RANK-RANKL-osteoprotegerin (OPG) axis[37,48]. RANK is expressed by the osteoclast precursors and mediates osteoclast differentiation, activation and survival, while RANKL is expressed by osteoblasts and other stromal cells[37,49].

Osteoclasts are multinucleated cells that derive from hematopoietic precursors from the monocytic lineage[37,50] and are the major resorbing cells in the bone environment. RANK pathway activation in monocytic precursors leads to TRAF6 recruitment and activation[42], which mediates the activation of several downstream targets, such as c-Src kinase, PI3K, and AKT/PKB, as previously mentioned[46]. Downstream NFATc1 and cFos transcription is induced, which is fundamental for osteoclast functional activation. RANK-deficient ($RANK^{-/-}$) mice are characterized by profound osteopetrosis, resulting from block in osteoclast differentiation, elucidating the importance of RANK signalling for osteoclast function[37].

Bone remodelling process is controlled by OPG, a secreted decoy receptor for RANKL that lacks transmembrane domains[51]. By blocking RANK-RANKL interaction, it inhibits osteoclastogenesis, attenuating bone resorption and permitting bone formation. Overexpression of OPG in mice also results in osteopetrosis, due to defective osteolysis[51].

2.2. RANK signalling in mammary gland development

Mammary gland morphogenesis is a multistep process that is controlled by sex and pregnancy hormones, beginning with extensive proliferation, ductal elongation and side branching of the mammary epithelium and ending with the formation of complex lobulo-alveolar structures[52]. Several studies have shown that RANK signalling is involved in mammary epithelial cell differentiation and mammary stem cells (MaSCs) expansion, being sufficient to elicit ductal side-branching and alveologenesis in the mammary gland of the virgin mouse[53].

RANKL mediates the expansion of mammary epithelia that occurs during pregnancy, acting through RANK to deliver proliferative and survival signals that promote the development of lobulo-alveolar structures and, thus, formation of lactating mammary glands[38]. In fact, RANK expression is strictly regulated in a spatial and temporal manner during mammary gland development[54], and it has been demonstrated that $RANK^{-/-}$ female mice show disrupted mammary gland development during pregnancy and impaired lactation, with absolute absence of lobulo-alveolar development[38,54].

RANK pathway not only interferes with mammary epithelial differentiation, but also mediates the major proliferative response of mammary epithelium to progesterone and progesterone-driven expansion of MaSCs[55]. RANKL expression is induced by progesterone, prolactin and parathyroid hormone-related peptide (PTHrP)[38] interaction within luminal epithelial cells that express ER and progesterone receptor (PR), conducting either in an autocrine or paracrine way to the activation of RANK expressed in myoepithelial cells ($ER^{-}PR^{-}$), leading to their proliferation and survival[56,57] (Figure 2).

In mammary epithelia, RANK activation triggers the downstream intermediate NF-κB, whose expression is strongly regulated during mammary gland development[57,58]. IKKα and NF-κB activation are required for optimal cyclin D1 expression, a known downstream target of RANK signalling in mammary cells that promotes cell proliferation[52,57]. Inhibitor of DNA binding protein 2 (ID2) is activated by RANK pathway and leads to downregulation of the cell cycle inhibitor p21[57,59]. This suggests that this process can be linked to the emergence of TNBC.

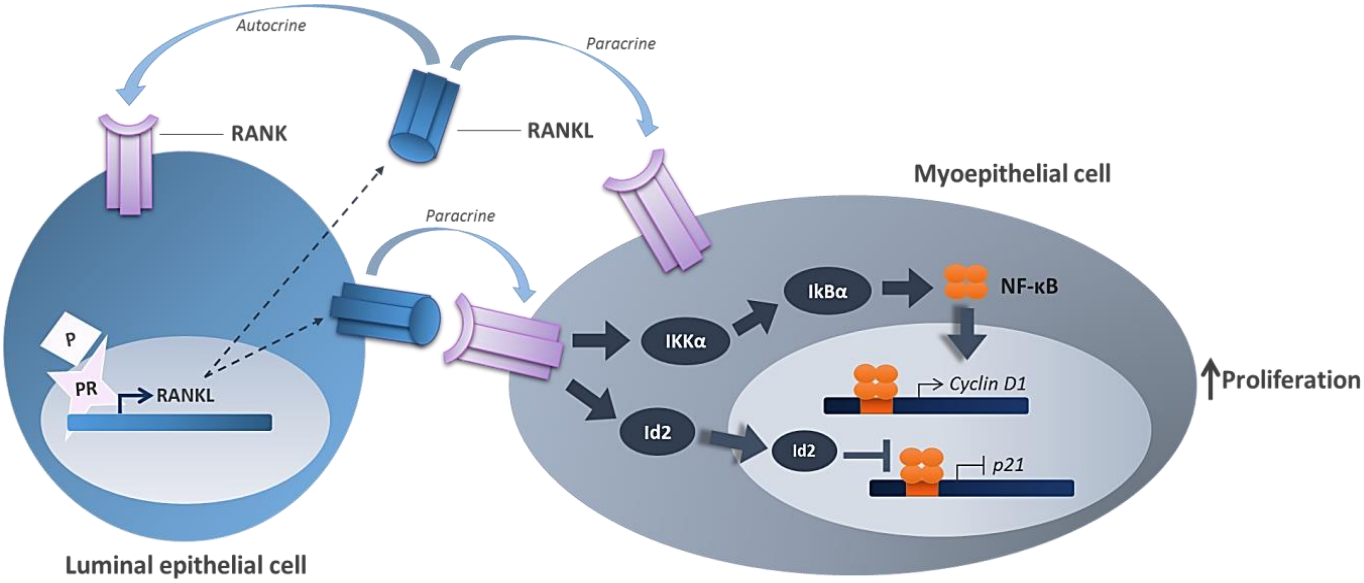

Figure 2 - RANK-RANKL signalling pathway in mammary epithelial cells.

Activation of progesterone receptor (PR) in luminal epithelial mammary cells, by progesterone/progestin (P), leads to an increase in RANKL expression. This will potentiate paracrine RANK activation in myoepithelial cells, triggering NF-κB pathway and consequently cyclin D1 transcription activation, promoting cell survival and proliferation. Intracellular activation of Id2 is involved in p21 transcription blockage. RANKL also activates RANK pathway in luminal epithelial cells, in an autocrine loop. *Adapted from [57]*

2.3. RANK signalling in immune system

Several members of the TNFR superfamily are involved in the regulation of the immune response, namely CD40, that increases the functional activity of DCs[60,61]; and RANK, a regulator of DCs' survival and activity[33,34,62] (Figure 3). Mature bone marrow and spleen DCs express high levels of RANK on their surface, suggesting an important role for RANK in the regulation of their activity[46]. Previous studies have demonstrated that stimulation of DCs with RANKL leads to enhancement of mature DCs' survival and allostimulatory activation of T cells[33,34,40,62].

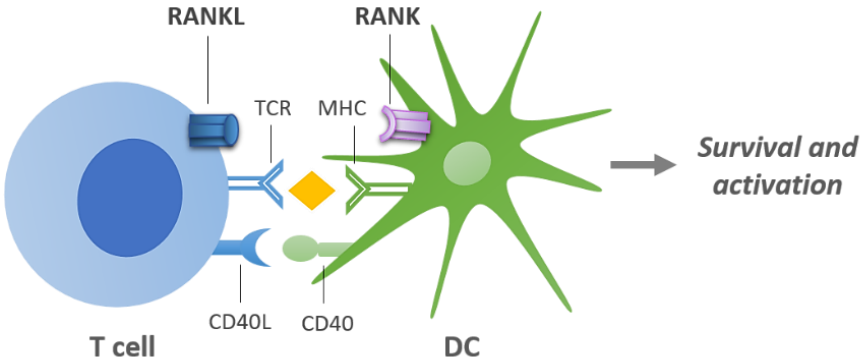

Figure 3 - RANK-RANKL signalling pathway in immune response.

Mature DCs express high levels of RANK in their surface, while T cells express its ligand RANKL. Not only is RANK-RANKL signalling involved in survival and activation of DCs by itself, but CD40L-CD40 and RANK-RANKL interactions seem to enhance T cell activation by DCs. *Adapted from [65]*

RANK-RANKL pathway has been shown to enhance DCs' tumour antigen immunogenicity, by increasing effector and memory cytotoxic T-lymphocyte (CTL) responses[63]. In another study, RANKL blockade *in vivo* has been shown to increase tumour-specific effector T cells persistence, increasing anti-tumour immunity[64].

Additionally, it has also been shown that RANK is essential for lymph node organogenesis[37,65]. RANK-deficient mice lacked all peripheral lymph nodes, retaining only musical-associated lymphoid tissues, and had impaired B-cell development[37].

The crosstalk between cancer cells and the immune system and its impact on tumour progression remains far from being completely understood. It is important to further clarify the role of RANK signalling pathway in this process.

3. RANK-RANKL signalling pathway in cancer

The relevance of RANK-RANKL signalling pathway in different physiologic processes described above, suggests that it may also be implicated and/or dysregulated in cancer. RANK-RANKL relevance in the context of bone metastatic disease has been addressed for long, but in fact, over the past years it was found that RANK is expressed in different types of tumour cells, affecting tumour invasiveness and stemness; and also that it has a key role in mammary tumorigenesis. Therefore, RANK-RANKL pathway may impact all stages of tumour progression, from initiation to metastization, including immune response (Figure 4).

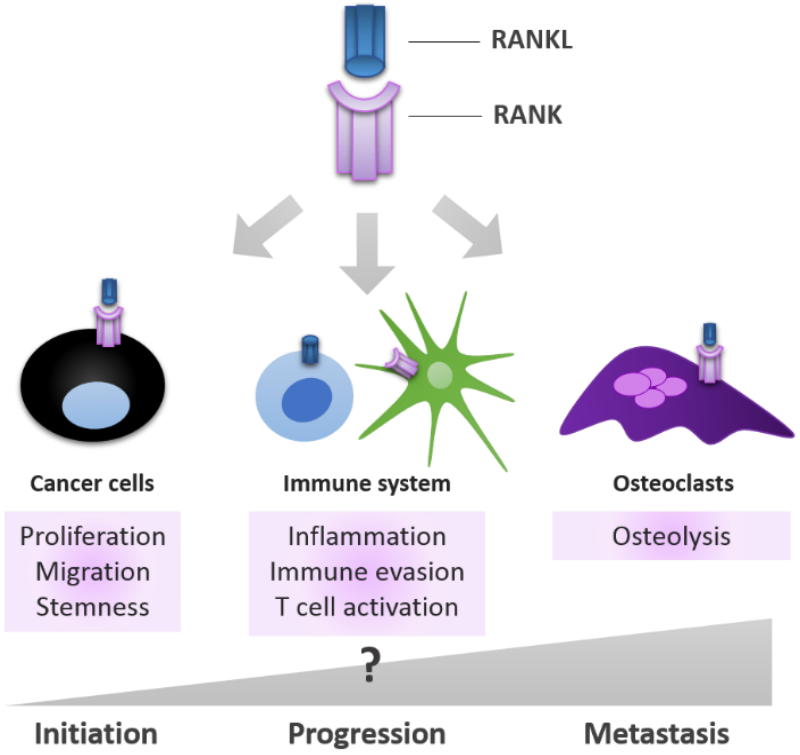

Figure 4 – RANK-RANKL signalling pathway is involved in all stages of cancer progression.

RANK signalling is involved in three different contexts during tumour progression: in cancer cells, it influences their capability to proliferate, migrate and invade; in osteoclasts, it enhances their functional activation, contributing to the 'vicious cycle' of bone metastasis, where bone resorption is increased; in the immune cells, it can influence immune responses and even be involved in the acquired capability of the tumour cells to evade these responses. *Adapted from [47]*

3.1. RANK signalling in breast cancer

A decade ago, a seminal paper by Jones *et al.* has shown that different epithelial tissues, human primary breast tumours, and breast and prostate cancer cell lines expressed functional RANK in their surface[66]. It has been previously shown that several BC cell lines expressed the receptor but its function was unknown[67]. Jones *et al*. have demonstrated that, in cell lines, RANK activation by RANKL induced downstream activation of PI3K/AKT and ERK pathways, and increased cell migration[66]. Posteriorly, different studies have shown that activation of RANK pathway in several *in vitro* models leads to a more aggressive phenotype, with increased migration and invasion ability[66–70]. After that the importance of RANK signalling in BC has been deeply investigated, and several studies demonstrated its role in BC onset[71–74].

It has been demonstrated that RANK pathway activation promotes proliferation and aberrant survival of mammary epithelial cells, by inhibiting their terminal differentiation[54]. In a transgenic mice model for RANK overexpression, mouse mammary tumour virus (MMTV)-RANK mice not only have increased mammary epithelial proliferation, impaired differentiation of the lobulo-alveolar structures and deficient lactation[54], but also spontaneous development of mammary tumours[75]. Moreover, mice with constitutive activation of RANK-RANKL pathway spontaneously developed high-grade invasive tumours with evidence of pulmonary metastasis[75]. These tumours were extensively heterogeneous, originated from different populations of basal or luminal cells or alternatively from multipotent progenitors.

RANK pathway is also involved in the development of progestin-driven mammary cancer. Several studies clearly demonstrated that RANKL is involved in the promotion of the expansion of MaSCs by progesterone[76,77] and that it may also mediate the critical role of progesterone in the promotion and growth of breast tumours[78]. MMTV-Cre/rank$^{floxed/\Delta}$ mice transgenic model, which lacks RANK in mammary epithelial cells, show attenuate tumour development when comparing with WT mice[79]. Additionally, Beleu *et al*. demonstrated that a large number of PR^{-} cells proliferate in a RANKL-dependent mechanism, through paracrine stimulation[80]. All these results suggest that RANKL inhibition could not only reduce BC cells' proliferation and delay tumorigenesis, but also mediate the reduction in MaSCs. Interestingly, it is known that the risk associated with HRT is greater for estrogen–progesterone combination than for estrogen alone, either in continuous or sequential therapy[81], which is in accordance to these findings.

In this context, the use of anti-RANKL therapies may prove to be effective in the prevention of BC. In fact, it has already been shown that inhibition of RANK signalling reduces the accumulation of MaSCs in invasive mammary tumours by inducing tumour cell differentiation, and decreased metastasis-initiating cell pools[82].

Importantly, these studies were expanded to the human setting, and it was shown that overexpression of RANK in untransformed MCF10A cells induced changes associated with both stemness and transformation, such as mammary gland reconstitution, EMT, increased migration and anchorage-independent growth[66,68]. Later it was also shown that MCF-7 ER^{+} BC cells that express low endogenous levels of RANK undergone EMT upon RANK overexpression[83]. In our group we have also shown that

RANK overexpression in MCF-7 cells resulted in increased EMT markers, migration, and $CD44^{+}/CD24^{-/low}$ subpopulation, known markers for MaSC[84].

Recently, RANK-RANKL signalling pathway has also been implicated in progenitor cell expansion and tumorigenesis in inherited BC. It was shown that genetic inactivation of RANK in the mammary epithelium markedly delayed onset, reduced incidence, and attenuated progression of BRCA1:p53 mutation-driven mammary cancer[85]. Additionally, long-term pharmacological inhibition of RANKL in mice abolished the occurrence of BRCA1 mutation-driven pre-neoplastic lesions. Another study has shown in the clinical setting that the progesterone–RANKL/OPG system is in fact dysregulated in BRCA-mutation carriers, with lower OPG levels in serum and tissue[86].

Despite all the evidences from basic and translational studies, clinical evidences for the prognostic role of RANK expression in BC are still sparse[72–74,87]. High levels of RANK, either mRNA or protein, were found in human primary BC ER^{-}/PR^{-}, with high pathologic grade and high Ki67[88]. In this study, high RANK mRNA expression was significantly associated with metastatic occurrence. It has also been demonstrated, using 295 primary BC patients microarray datasets, that low levels of RANK and higher OPG expression correlates with both longer OS and disease-free survival (DFS)[71].

Altogether these results support the hypothesis that inhibition of RANKL–RANK axis may offer a promising therapeutic target for preventing tumour progression and metastization, beyond its use for management of SREs that emerge with BM.

3.2. RANK signalling and bone metastases

Bone is the most common site of relapse in BC. BM are associated with increased morbidity and decreased OS, urging the need to find therapeutic options that prevent SREs but also that impact on tumour growth.

BM fit perfectly in the 1889 Stephen Paget's 'seed and soil' hypothesis to explain cancer relapse: "When a plant goes seed, it seeds are carried in all directions, but they can only live and grow if they fall on congenial soil"[89]. Metastatic tumour cells seeded in bone secrete growth factors and cytokines that alter the normal balance of bone remodelling, driving further activation of osteoclasts in a 'vicious cycle' of bone resorption[90,91]. PTHrP secreted by tumour cells leads to osteoclasts' differentiation and activation, via RANKL up-regulation, and ultimately increases bone resorption. This will lead to the release of mitogenic growth factors that induce tumour growth (Figure 5).

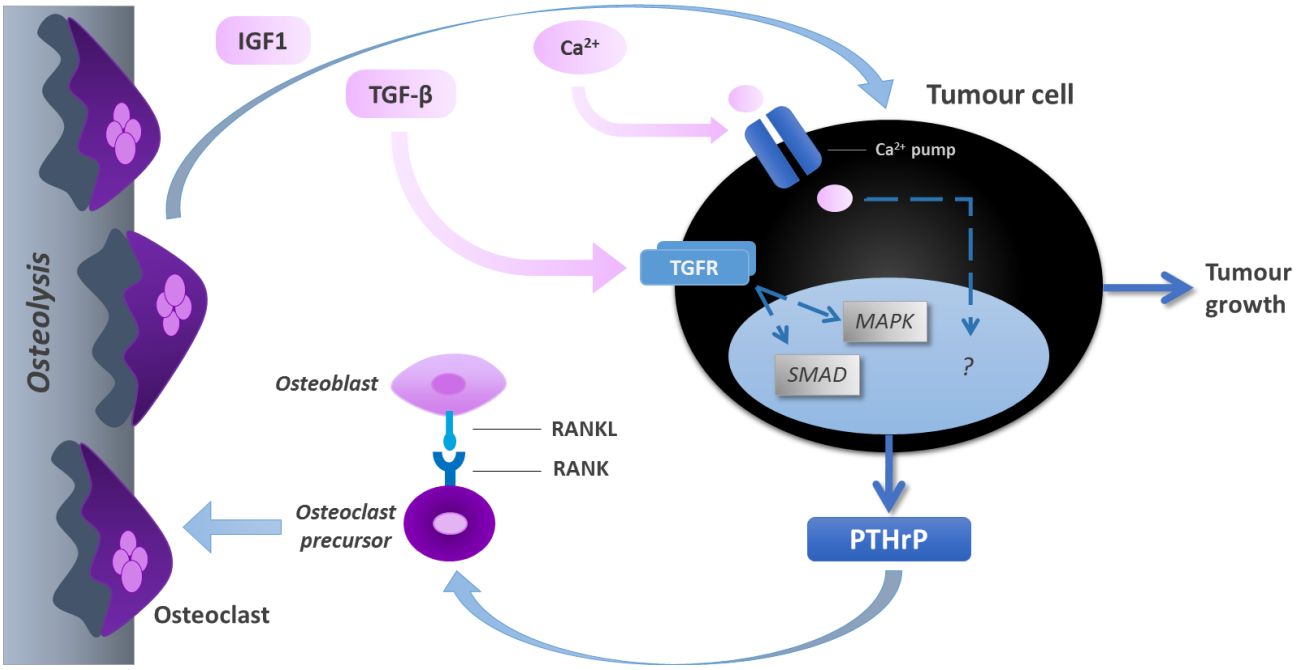

Figure 5 – 'Vicious cycle' of bone metastases.
Cancer cells in bone secrete high amounts of PTHrP, which induces osteoclasts' differentiation and activation via up-regulation of RANKL, promoting osteolysis. Bone disruption leads to the liberation of several cytokines and growth factors, such as IGF-1 and TGF-β, which in turn enhance tumour proliferation. *Adapted from [92]*

RANK and RANKL not only control bone remodelling, as previously mentioned, but are also involved in this preferential metastasis of BC cells to the bone[93]. In fact, deregulation of RANK-RANKL signalling pathway is known to be involved in the establishment of BM and consequent pathological conditions, such as osteoporosis[94]. Being essential for osteoclast differentiation and activation and also being expressed in many tumour cell types, it was suggested that RANK pathway could play a direct role on tumour cells. Armstrong *et al.* demonstrated that RANK is expressed in prostate cancer cells, another type of cancer that preferentially metastasizes to bone, and promotes invasion in a RANKL-dependent manner, by activating several downstream events that lead to cellular proliferation, invasion and migration[70]. Other previous *in vitro* and *in vivo* studies have indicated that RANKL can promote distant metastasis via direct effects on RANK-expressing cells, either tumour or progenitor cells. It was proven that RANKL inhibition not only protects against bone destruction but also inhibits BM progression and delays the formation of *de novo* BM in cancer models[56].

RANK expression was associated with accelerated bone metastasis formation[71,93]. In fact, RANK overexpression alone seems to be sufficient to confer a significantly greater metastatic growth in bone[93]. Previous studies have also elucidated an important role of RANK signalling in the upregulation of MMP-1, a metalloproteinase that contributes to bone metastases formation and osteoclastogenesis[69]. High levels of MMP-1 are significantly associated with decreased OS of patients with BM, and in a mouse model MMP-1 knockdown (KD) resulted in smaller x-ray osteolytic lesions and osteoclastogenesis and decreased tumour burden[95].

3.3. RANK as a therapeutic target in breast cancer

RANK-RANKL pathway has been implicated in several contexts of BC disease, from early tumorigenesis to progression and metastasis. As already mentioned above, both *in vitro* and *in vivo* evidences point RANK-RANKL axis as a promising therapeutic target for prevention of BC onset, progression and metastization. But the importance of RANK-RANKL pathway in the context of BM has been addressed for long. The use of denosumab, a fully humanized anti-RANKL monoclonal antibody that decreases bone resorption, was approved by FDA in 2010 for the prevention of SREs in patients with BM; and in 2011 for the treatment of bone loss in patients with BC undergoing hormone ablation therapy. In fact, adjuvant denosumab in postmenopausal BC patients receiving aromatase inhibitors has shown to reduce the risk of clinical fractures[96].

Inhibition of bone resorption makes bone a less favourable microenvironment for tumour cells to growth. Since the host microenvironment is essential for cancer colonization at secondary sites, there is a rational to the adjuvant use of therapies that decrease bone resorption (antiresorptive therapies) in patients with BC. Currently, denosumab is being studied on a phase III clinical trial to determine whether adjuvant RANKL inhibition prolongs BM-free survival and DFS in BC[97].

Moreover, it has been demonstrated that RANK-RANKL pathway directly contributes to increased proliferation and survival of preneoplastic breast cells, promoting tumour growth, as well as to an increased expansion of MaSCs population[75–79]. Further clinical studies are required in order to determine the potential benefit of RANK-RANKL targeted therapies in prevention of BC in high risk groups. Importantly, RANK pathway seems to be implicated in BRCA1-mutation driven mammary cancers[85,86], which opens doors to the investigation of a new possibility of BC prevention in women that have this inherited mutation.

4. Objectives

Over the past years, the role of RANK-RANKL signalling pathway in BC development and progression has been widely assessed. RANK is expressed by cancer cells and is involved in the promotion of mammary tumorigenesis. Several *in vitro* studies have correlated RANK expression with EMT, increase in migration and invasion potentials, and stemness.

Although RANK overexpression has been mostly associated to TNBC, previous studies suggest that RANK overexpression in luminal BC cells also induces a more invasive and *stem-like* phenotype. Therefore, we hypothesize that RANK-positive luminal BC cells could have enhanced invasion ability, and so forth metastases may be enriched for this particular population of cells.

The present study aimed to contribute to test this hypothesis by determining the effect of RANK overexpression in luminal BC cells in tumour burden and invasiveness *in vivo*.

Cell Culture

Human breast carcinoma cell lines MDA-MB-231$^{GFP+Luc+}$ and MCF-7$^{GFP+Luc+}$ were obtained by Sérgio Dias Lab (IMM), through lentiviral transduction with GFP-Luciferase lentiviral particles and cell sorting of pure GFP$^+$ cell populations. MDA-MB-231$^{GFP+Luc+}$ cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco), 1% (v/v) Penicillin/Streptomycin (10,000 U/mL Penicillin, 10,000 µg/mL Streptomycin, Gibco). MCF-7 $^{GFP+Luc+}$ cells were cultured in the same medium, additionally supplemented with 0,01mg/mL insulin (Gibco). Cells were maintained at 37°C with 5% CO_2.

RANK overexpression and knockdown

For lentiviral transduction, MDA-MB-231$^{GFP+Luc+}$ and MCF-7$^{GFP+Luc+}$ cells were seeded in 24-well plates, at a density of 4×10^4 cells/well. 24 hours after seeding, medium was replaced by fresh medium containing 8µg/ml Polybrene (Sigma). RANK lentiviral activation particles (h) (30µl/well; sc-400559-LAC) or control lentiviral particles (15µl/well; sc-437282); and RANK shRNA (h) lentiviral particles (15-20µl/well; sc-42960-V) or control shRNA lentiviral particles (15-20µl/well; sc-108080) were used (Santa Cruz). For RANK activation, cells were selected with 0,5µg/mL Puromycin dihydrochloride (Sigma), 5µg/ml Blasticidin S HCl (Santa Cruz) and 200µg/mL Hygromycin B (Santa Cruz), starting three days after transduction. For RANK KD, cells were selected with 0,5µg/mL Puromycin dihydrochloride (Sigma) starting three days after transduction. RANK overexpression (OE) and KD was confirmed by RT-qPCR and flow cytometry.

RFP lentiviral transduction

MCF-7$^{GFP+Luc+}$ cells overexpressing RANK (RANK OE$^{GFP+Luc+}$) were transduced with Cignal Lenti Positive Control (RFP) ready-to-transduce lentiviral particles (Quiagen). Cells were seeded in 96-well plates (10^4cells/well), and 24 hours after seeding, medium was replaced with fresh medium supplemented with 8µg/ml Polybrene and lentiviral particles were added at a multiplicity of infection (MOI) of 25 or 50. RFP+ cells were selected with 0,5µg/mL Puromycin dihydrochloride (Sigma). RFP positivity was confirmed by flow cytometry in a BD Accuri C6 flow cytometer and RFP+ cells were sorted in a FACS Aria III cell sorter (BD Biosciences) to obtain a RFP+ pure cell population.

RT-qPCR

For RT-qPCR analysis of mRNA expression, cells were grown up to 80-90% confluency and total RNA was extracted using the NZY Total RNA Isolation kit (Nzytech), according to manufacturer's instructions. Total RNA was quantified in a NanoDrop spectrophotometer (Thermo Ficsher Scientific).

Total RNA (500ng to 1µg) was treated with RQ1 RNase-free DNase I (Promega) for 30min at 37°C, according to manufacturer's instructions. DNase I-treated RNA was reverse transcribed using the NZY M-MuLV First-Strand cDNA Synthesis kit (Nzytech), and Oligo(dT)$_{20}$ primer, according to manufacturer's instructions.

cDNAs were amplified by real-time PCR using TaqMan Gene Expression Master Mix (Applied Biosystems), according to manufacturer's instructions, and specific primers for human *RANK*, *18S* and *GAPDH*; or Power SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies) and specific primers for *Vimentin*, *Fibronectin*, *E-cadherin*, *N-cadherin*, *Snail*, *Slug*, and *Twist*; in a Viia7 System (Applied Biosystems) (Table 2). Gene expression was normalized using the housekeeping genes 18S or GAPDH, and relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

List of primers used is resumed in Table 2.

Table 2 – List of primers used in qPCR.

Gene	Common name	Primer sequence (5'-3')
GAPDH	GAPDH	PPH00150F (SA Biosciences)
TNRFS11A	RANK	PPH01102C (SA Biosciences)
Vimentin	Vimentin	F: GAAAACACCCTGCAATCTT R: CCTGGATTTCCTCTTCGTG
FN1	Fibronectin	F: CAGTGGGAGACCTCGAGAAG R: TCCCTCGGAACATCAGAAAC
SNAI1	Snail1	F: CTCTTTCCTCGTCAGGAAGC R: GGCTGCTGGAAGGTAAACTC
SNAI2	Slug	F: CCAAACTACAGCGAACTGGA R: GTGGTATGACAGGCATGGAG
TWIST1	Twist	F: CCGGAGACCTAGATGTCATTG R: CCACGCCCTGTTTCTTTG

Flow Cytometry

To analyse RANK expression by flow cytometry, cells were grown up to 80-90% confluency, trypsinized, centrifuged at 200 g for 5min, and then ressuspended in FACS buffer (PBS 1X, 2%FBS). Cells were fixed with 1mL ice cold methanol, at -20°C for 10min. After washing with FACS buffer, cells were permeabilized with PBS 1X, 0,5% Tween-20, on ice for 10min. Cells were incubated with a specific mouse monoclonal antibody against human RANK (1µg/ml; clone 9A725; Enzo) for 30min at 4°C in the dark, washed with FACS buffer, and incubated with a goat anti-mouse secondary antibody conjugated with Alexa Fluor 633 (1:400; Life Technologies) for 20min at 4°C. After labelling cells

were washed twice with FACS buffer, ressuspended in 1mL FACS buffer and analysed on a BD Accuri C6 flow cytometer (BD Biosciences).

For RFP-positive cell sorting, cells were grown up to 80-90% confluency, trypsinized and ressuspended in FACS buffer. RFP-positive cells were sorted on a FACSorting Instrument – FACSAria III (BD Biosciences), and cultured under standard conditions.

Flow Cytometry data analysis was performed in FlowJo V10 software.

Western Blot

Activation of RANK pathway upon stimuli with RANKL was analysed by Western blot. For this purpose, $4x10^5$ cells were seeded in 6-well plates for 24h, and serum-starved in low-serum medium (0,1% FBS, 1% Pen/Step) for another 24h. Medium was replaced by fresh low-serum medium containing 1µg/mL human RANKL (PeproTech) and total cell lysates obtained at different time points. Total cell lysates were prepared with 200µl RIPA buffer per well (Santa Cruz), with 1:100 phenylmethane sulfonyl fluoride (PMSF, Santa Cruz), 1:100 protease inhibitor (PI, Santa Cruz), 1:100 sodium orthovanadate (NaOR, Santa Cruz) and 1:100 phosphatase inhibitor cocktail (Sigma), on ice for 30min. Samples were sonicated in a Soniprep 150 equipment (MSE) for approximately 1min and centrifuged at 10,000g for 20min, at 4°C. Supernatants were transferred to new tubes and total protein was quantified using Pierce BCA Protein Assay Kit (ThermoSicentific), according to manufacturer's instructions. Proteins were resolved by SDS-PAGE, using 10% polyacrylamide gels, and then transferred to nitrocellulose membranes using an iBlot® Gel Transfer Device (Invitrogen), according to manufacturer's instruction.

Membranes were blocked for 1h at room temperature (RT) in 5% Non-Fat Dry Milk (NFDM) in PBS-0.1% Tween (PBST) for β-actin; or in 5% bovine serum albumin (BSA) (Santa Cruz) for other antibodies. Membranes were incubated with the following specific antibodies, overnight at 4ºC: mouse anti-β Actin antibody (1:25,000; Ab6276; Abcam), rabbit anti –Akt1/2/3 (1:250; H-136), rabbit anti -pAkt (1:250; Ser-473), rabbit anti –ERK1/2 (1:1,000; C-14) and rabbit anti –pERK1/2 (1:500; Thr-202/Tyr-204), all from Santa Cruz. After washing with PBST, membranes were incubated with horseradish peroxidase-conjugated (HRP) specific secondary antibodies: donkey anti-mouse (1:2,000) or goat anti-rabbit (1:4,000) (Santa Cruz) for 2h at RT. Proteins were detected using a Novex® ECL HRP chemiluminescent substrate reagent kit (Invitrogen) according to the manufacturer's instructions, and x-ray films (Fujifilm) developed in a Curix 60 processor (AGFA).

Proliferation Assay

Cells were seeded in 96-well plates, at a density of 10^4 cells/well. Approximately 4 h after seeding, 1:20 Alamar blue (Invitrogen) was added to each well and fluorescence was measured 24 and 48h after incubation (excitation 560nm; emission 590nm) in an Infinite M200 microplate reader (Tecan).

Migration assay

MCF-7$^{GFP+Luc+}$ and MCF-7 RANK OE$^{GFP+Luc+}$ cells were grown to confluency in 24-well plates, and a straight wound was made across each well with a sterile 200µl pipette tip. After washing away loose cells with PBS 1X, cells were incubated with low-serum medium with or without 1µg/mL RANKL. Each well was digitally imaged using an inverted wide-field Zeiss microscope (Jena) at 0h and 24h after stimuli with RANKL.

MCF-7$^{GFP+Luc+}$ and MCF-7 RANK OE$^{GFP+Luc+RFP+}$ cells were plated 1:1 at confluence density in 6-well plates. Cells were left to adhere ON in low-serum medium and a straight wound was made across each well with a sterile 200 pipette tip. After washing loose cells, cells were incubated with low-serum medium for another 24h. Each well was digitally imaged using a Zeiss Axio Observer at 0h and 24h after scratch.

Migrated area was calculated using ImageJ 1.6.0 software.

Orthotopic xenograft model

In all studies involving animals, mice were handled and euthanized in accordance with approved institutional, national and international guidelines, applying the Principle of the 3Rs. This study was approved by the ethical Committee of the Rodents' facility of IMM and DGAV.

- Estradiol supplementation

Nine days prior to BC cell inoculation (day -9) 4 week-old female BALB/c nude or NOD scid gamma (NSG) mice were subcutaneous implanted with 17β-estradiol pellets (60-day release, 0.36mg/pellet, Innovative Research of America), inserted with a trochar in the mid-scapular region. For this procedure mice were anaesthetized with isoflurane.

- Tumour inoculation

MCF-$^{7GFP+Luc+}$, MCF-7 RANK OE$^{GFP+Luc+}$ and MCF-7 RANK OE$^{GFP+Luc+RFP+}$ cells were harvested in the exponential phase of growth, washed with PBS, trypsinized and counted with a haemocytometer. Cells were ressuspended in 50mL ice-cold PBS and washed twice by centrifugation at 200g, for 10min. Cells were ressuspended in ice-cold 50% phenol-red free Matrigel Matrix (in PBS) (BD Biosciences) to a final concentration of 2×10^5 cells/mL. Mice were injected unilaterally into the fourth abdominal (nude) or second thoracic (NSG) fat pad with a total of 2×10^4 MCF-7$^{GFP+Luc+}$, MCF-7 RANK OE$^{GFP+Luc+}$, or MCF-$^{7GFP+Luc+}$/MCF-7 RANK OE$^{GFP+Luc+RFP+}$ (1:1) cells.

- Tumour visualization

Every week (or starting at week four in the BALB/c nude mice experience) post tumour inoculation, mice were injected with 100µl/10g body weight (BW) XenoLight D-Luciferin - K+ Salt Bioluminescent Substrate (PerkinElmer) and after 4 min they were anesthetized with 75mg/KgBW Ketamine + 1mg/KgBW Medetomidine.

After approximately 6min luminescence was analysed in an IVIS Lumina, using Living Image 3.0 software (30s of exposure; field of view D 12,5cm; subject height 1,5cm), and mice recovered with 1mg/KgBW Atipamezole.

- Circulating tumour cells (CTCs) and disseminated tumour cells (DTCs) identification

For CTC analysis, venous blood was collected from the submandibular vein five weeks post tumour inoculation, or by cardiac puncture before sacrifice, into 1.5 mL centrifuge tubes with 5µl EDTA 0,5M (pH8.0). Erythrocytes were lysed by incubation with 1X RBC Lysis Buffer Multi-species (eBioscience) for 13min at RT. Cells were washed with FACS buffer and centrifuged for 3min at 2,000 rpm. The supernatant was discarded and cells were ressuspended in FACS buffer.

For DTC analysis, tibia and/or femur were removed and the epiphyses of the bones were cut off. Using a 1-cc syringe with a 26G needle (Omnifix® 100 Duo, B.Braun) filled with DMEM, bone marrow cells were flushed from both ends of the bone shafts onto a 15mL Falcon tube fitted with a sterile 70µm Nylon cell strainer (Falcon®).

Samples were analysed for GFP and/or RFP expression in a BD Accuri C6 flow cytometer.

- Sacrifice and necropsy

Mice were sacrificed eight weeks post tumour inoculation by administration of 0,25mg/KgBW Sodium Pentobarbital (Eutasil®). Necropsies were performed to identify macrometastases. Additionally, bone marrow was collected to detect disseminated tumour cells (DTCs) by flow cytometry.

Tumours were harvested and divided into four parts to be frozen in liquid nitrogen embedded in O.C.T. compound (Tissue-Tek, Sakura); frozen in RNA later solution (Ambion); formalin fixed and paraffin embedded (FFPE); and analysed by flow cytometry after cell disruption using a sterile 70µm Nylon cell strainer (Falcon®). Visceral organs (lung, liver, spleen) were collected and FFPE.

Hematoxylin-Eosin (H&E) and Masson's Trichrome staining were performed by the Histology and Comparative Pathology Service at IMM.

- Immunohistochemistry

5µm tissue sections from FFPE samples were stained by immunohistochemistry (IHC) for the detection of Ki67, in an AutostainerPlus (Dako Cytomation. Deparaffinization and antigen retrieval was performed in a PT Link Pre-Treatment Module for Tissue Specimens (Dako), using Antigen Retrieval Solution pH9.0, at 94ºC for 20min. Endogenous peroxidase was blocked with 3% H_2O_2 (Sigma) for 10min at RT, and total protein was blocked by incubation with Protein Block Solution (Dako), for 20 min at RT. Slides were incubated for 30min with rabbit anti-human Ki67 primary antibody (MIB-1, Dako), 1:100 in Antibody Diluent (Dako). Slides were incubated with EnVisionTM Detection System, rabbit/mouse (Dako), according to manufacturer's instructions, with 5min of incubation with DAB. Slides were counterstained with hematoxylin, dehydrated, mounted with Quick-D mounting medium (Klinipath) and visualized in a bright field microscope (LeicaDM750 with a Leica ICC50 HD camera). Imunoratio was obtained through the calculi of the percentage of DAB-stained nuclear area over total nuclear area (hematoxylin-stained nuclei regions) (5 fields, 400x, ImageJ software).

Statistical Analysis

Data was analysed using GraphPad Prism6 software. The number of replicates performed for each experiment is indicated. Statistics were performed by one-way ANOVA or unpaired t-test (*in vitro* experiments); two-way ANOVA (tumour burden and mice BW). Results are presented as mean with SEM and p-value <0.05 was considered significant.

1. RANK overexpression and knockdown

In this study we propose to address the effect of RANK overexpression (OE) in the behaviour of luminal BC cells *in vivo*. Therefore, we started by generating stable MCF-7 RANK OE cells by lentiviral transduction. We also generated non-luminal BC cells with RANK OE (MDA-MB-231 triple negative BC cells), or the respective counterparts with RANK knockdown (KD), to be used in control assays. To be able to monitor tumour growth *in vivo* we used $GFP^{+}Luc^{+}$ parental cells to derivate OE and KD clones.

RANK OE was obtained by transduction with RANK lentiviral activation particles, that consist in a dCas9 nuclease fused to a VP64 activation domain, in conjunction with a target-specific sgRNA. This synergistic activation mediator (SAM) maximizes the activation of endogenous RANK. Upon transduction, activated clones were selected with blasticidin, hygromycin and puromycin, and analysed for *RANK* mRNA expression (Figure 6A,B). Two MCF-7 OE clones and three MDA-MB-231 OE clones were obtained, with between 9 and 54-fold up-regulation of *RANK* expression, when comparing to cells transduced with control activation particles.

RANK KD was obtained by transduction of specific shRNA lentiviral particles. Upon transduction, KD clones were selected with puromycin, and analysed for *RANK* mRNA expression (Figure 6C,D). Five MCF-7 KD clones and four MDA-MB-231 KD clones were obtained, with between 0,1 and 0,4-fold down-regulation of *RANK* expression, when comparing to cells transduced with control shRNA particles.

We selected the clones MCF-7 OE1, KD1 and MDA-MB-231 OE2 and KD1 to perform the subsequent studies, and analysed *RANK* mRNA expression at different cell passages to confirm stable RANK OE or KD (Figure 6E,F).

RANK OE in MCF-$7^{GFP+Luc+}$ cells was also confirmed at the protein level by flow cytometry (Figure 6G,H).

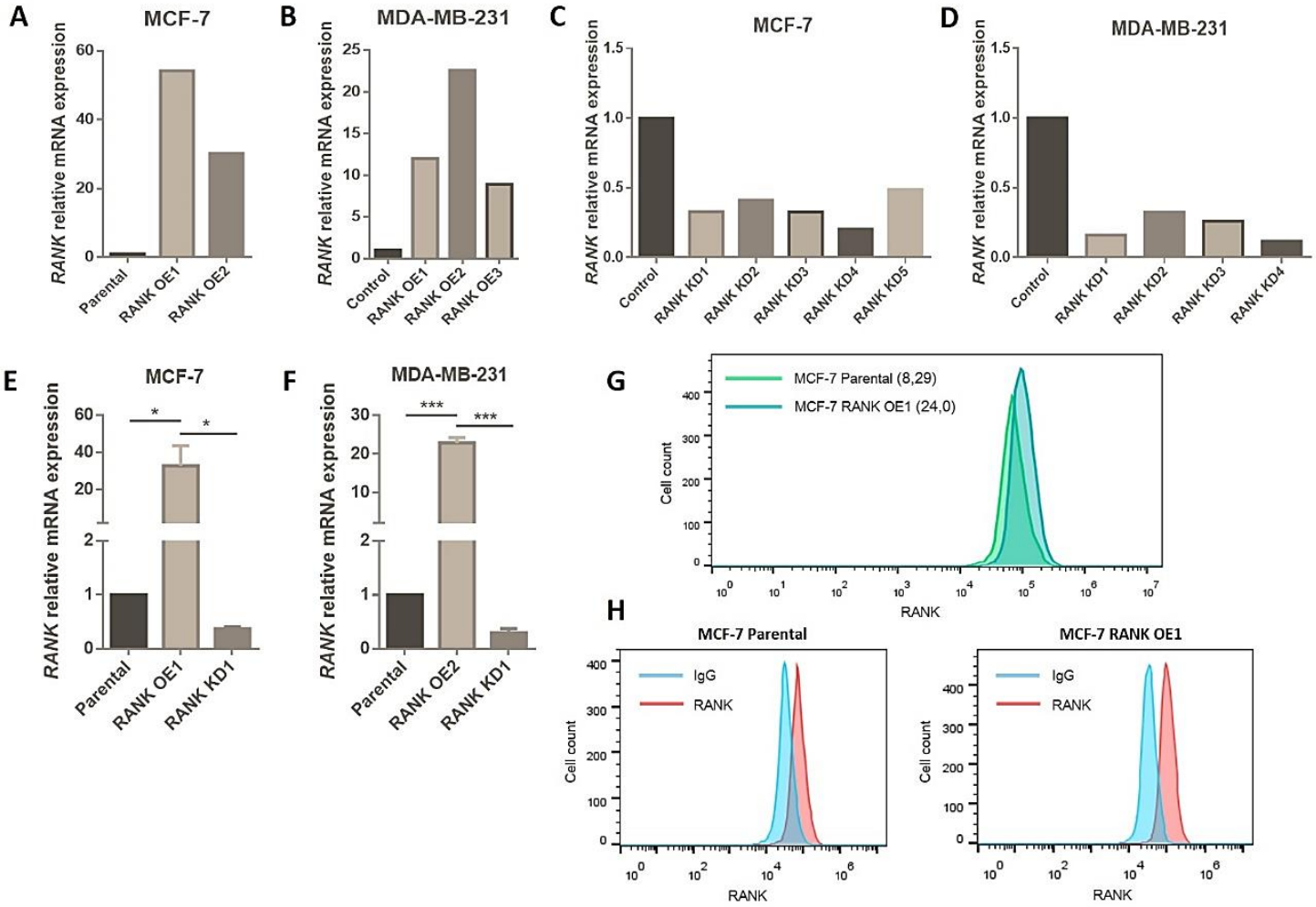

Figure 6 - RANK stable activation and knockdown by lentiviral transduction.

A,B,C,D) *RANK* relative mRNA expression in MCF-7 and MDA-MB-231 clones was determined by RT-qPCR. Ct values were normalized against the *18S* gene and *RANK* expression was normalized against the respective empty vector control (or parental cell line in the MCF-7 overexpression subset). **E,F)** *RANK* expression was evaluated at different cell passages for MCF-7 OE1 and KD1, or MDA-MB-231 OE2 and KD1, and relative mRNA expression was normalized against the respective parental cell line. Data is presented as mean ± SEM. **G)** RANK expression in MCF-7 RANK OE1 clone was assessed by flow cytometry and compared to MCF-7 parental cells. **H)** Mouse IgG was used as a negative control. *p<0.05; ***p<0.001

Since one of our objectives was to follow *in vivo* the growth and behaviour of tumours containing both MCF-7 parental and RANK OE cells, we also derived RFP^{+} cells in MCF-7 RANK $OE^{GFP+Luc+}$ background, by lentiviral transduction and fluorescence-activated cell sorting (FACS) (Figure 7).

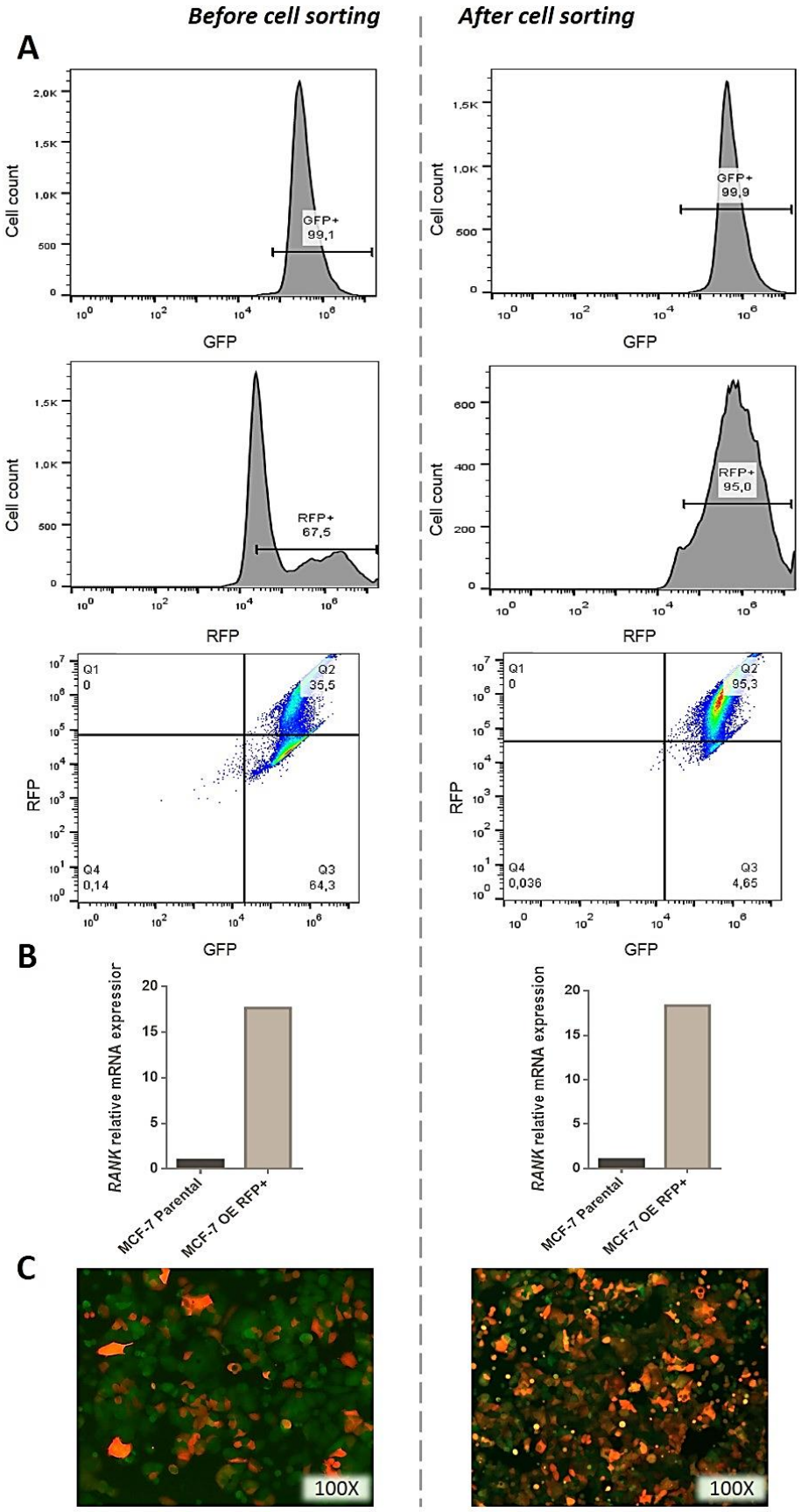

Figure 7 – Stable RFP expression in MCF-7 RANK OE$^{GFP+Luc+}$ cells by lentiviral transduction and cell sorting.

A) RFP expression was assessed by flow cytometry before and after fluorescence-activated cell sorting (FACS). **B)** *RANK* expression before and after FACS was confirmed by RT-qPCR. Ct values were normalized against *GAPDH* and relative mRNA expression was normalized to MCF-7$^{GFP+Luc+}$ (parental). **C)** Representative fluorescence-images.

Next, to confirm that RANK OE was functional, we analysed the phosphorylation status of known downstream proteins like ERK and AKT upon RANKL stimulus (Figure 8).

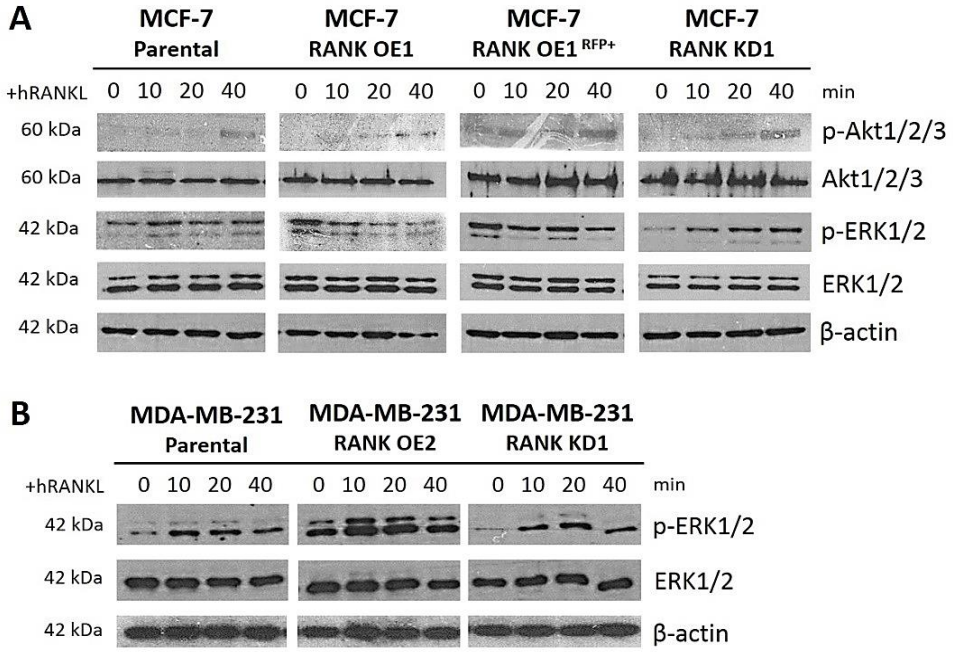

Figure 8 – RANK overexpression leads to functional activation of RANK signalling pathway.

RANK pathway activation was confirmed by western blot in **A)** MCF-7 cells and **B)** MDA-MB-231 cells. After 24h in low-serum medium, cells were stimulated with 1µg/mL hRANKL for 10, 20 and 40min. β-actin was used as loading control.

RANK OE cells show the expected time-dependent increase in p-AKT upon incubation with RANKL. We also observe RANKL-independent basal protein phosphorylation, probably due to the high degree of OE and ligand-independent receptor activation. In MCF-7 cells, p-ERK1 is predominant over p-ERK2 and decreases over time, whereas in MDA-MB-231 cells p-ERK2 is predominant and increases over time. These results are in accordance with what we observe in other RANK OE experiments, where we analysed the cells for longer time points and see that in fact in MCF-7 cells, but not in MDA-MB-231 cells, p-ERK2 is only observed upon p-ERK1 decrease and total ERK1 depletion.

To address if this difference could be linked to the BC cells sub-type we performed a pilot experiment. However, the analysis of other BC cell lines (SKBR-3 ($HER2^+HR^-$), ZR-75 ($HER2^-HR^+$), MDA-MB-435 ($HER2^-HR^-$) and MCF10A ($HER2^-HR^-$)) was not conclusive (Supplementary Figure 1).

2. RANK OE decreases tumour growth, but increases EMT, CTCs and desmoplasia

The main goal of this study was to address the effect of RANK OE in luminal BC behaviour *in vivo*. Therefore, we used an orthotopic xenograft mouse model to compare the behaviour of MCF-7 parental and RANK OE tumours.

First we inoculated MCF-7 parental or RANK OE cells in the fourth left inguinal mammary fat pad of female BALB/c nude mice, that were first supplemented with slow-release sub-cutaneous 17β-estradiol. However, in this strain the tumour uptake was very low (1/5 in both groups) and therefore no conclusions could be taken (Supplementary Figure 2).

To overcome this problem, we used a more immunocompromised strain, NSG mice, which we have previously used with 100% tumour uptake in a MCF-7 xenograft model (unpublished data). We inoculated MCF-7 parental or RANK OE cells in the second right thoracic mammary fat pad of female NSG mice, that were first supplemented with slow-release sub-cutaneous 17β-estradiol. We also included a third group, were we inoculated both parental and RANK OE (RANK $OE^{GFP+Luc+RFP+}$) cells (1:1), in the same mammary fat pad (Mix).

We monitored tumour growth weekly by luminescence analysis (Figure 9A,B). Luminescence analysis shows that tumour growth was decreased in both groups with RANK OE xenografts ($p<0.001$) (Figure 9B), which was posteriorly confirmed by tumour measurement at sacrifice (Figure 9D), and reflected a lower proliferative index as measured by Ki67 immunoscoring (Figure 9E,F).

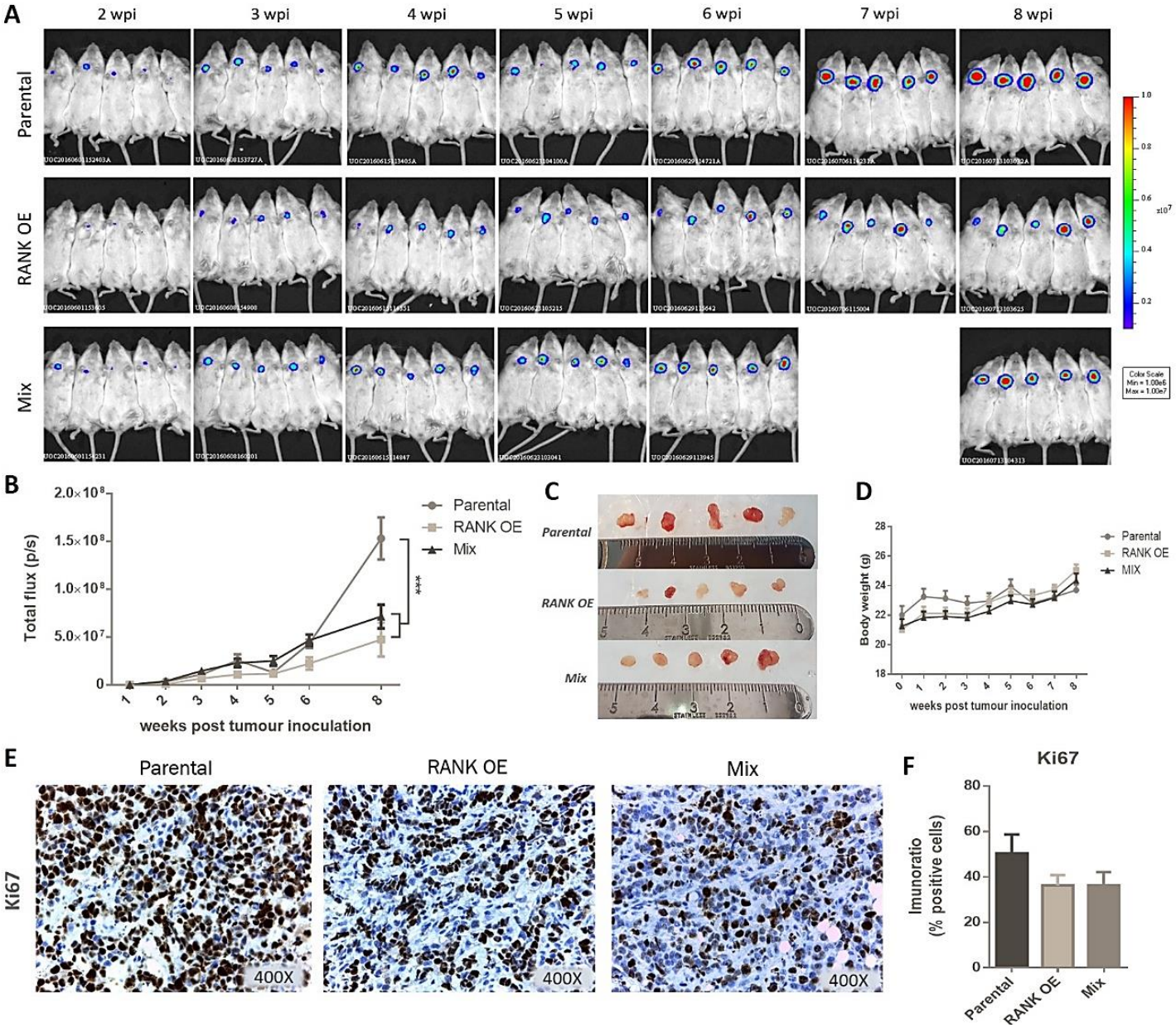

Figure 9 – RANK OE tumours have decreased proliferation rate in vivo.

Nod scid gama (NSG) mice were inoculated with MCF-7 $^{GFP+Luc+}$ (Parental), MCF-7 RANK OE $^{GFP+Luc+}$ (RANK OE) or MCF-7 $^{GFP+Luc+}$ and MCF-7 RANK OE $^{GFP+Luc+RFP+}$ cells (1:1) (Mix) (n=5/group). Inoculation was performed in the 2nd thoracic mammary fat pad. **A)** Tumours were imaged every week post tumour inoculation till the end of the experience (eight weeks). **B)** Total flux (p/s). **C)** Photographic images of tumours at sacrifice. **D)** Body weight motorization. **E)** FFPE sections of tumour tissues were immunostained for Ki67. **F)** Quantification of Ki67 (Imunoratio). Data is presented as mean ± SEM. ***p<0.001

In order to measure RANK expression in the tumours we used snapshot frozen tumours' tissues obtained at sacrifice and analysed RANK mRNA expression by RT-qPCR (Figure 11A). RANK expression was 8±2,4-fold higher in RANK OE tumours and 2±0,5-fold higher in the Mix group, when compared to parental tumours ($p<0.05$ for RANK OE tumours). RANK expression in the Mix group was lower than in the RANK OE group, probably reflecting the sum of the different expression levels in the two cell types.

Since tumours in RANK OE and Mix group achieved similar sizes, we next interrogated if tumours in the Mix group would be mostly comprised of RANK OE cells, despite we have inoculated the same proportion of both cell types. To address this question we analysed GFP and RFP expression in the tumours, at sacrifice, by flow cytometry (Figure 10B). Data shows that in fact GFP^+RFP^+ cells, which are RANK OE cells, are prevalent in comparison to GFP^+RFP^- parental cells ($p<0.001$).

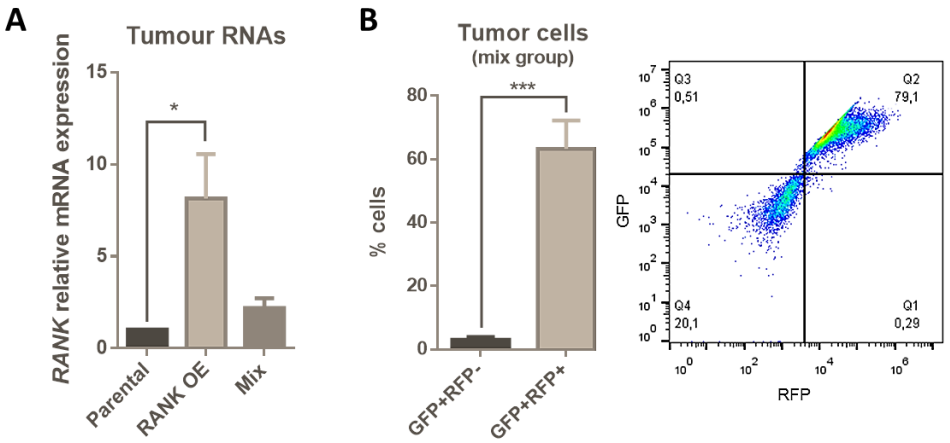

Figure 10 – RANK OE cells prevail over parental cells, eight weeks post tumour inoculation.

A) RANK relative mRNA expression in tumours was assessed by RT-qPCR. Ct values were normalized to GAPDH. **B)** Percentage of GFP^+RFP^- and GFP^+RFP^+ cells in Mix tumours was assessed by flow cytometry. *p<0.05; ***p<0.001

The observation of tissue sections also revealed that RANK OE and Mix tumours were enriched in stroma tissue when compared to parental tumours. To quantify the degree of desmoplasia we measured the collagen occupied area in tissue sections stained with Masson's trichrome, and confirmed that RANK OE and Mix tumours were significantly more desmoplasic ($p<0.05$) (Figure 11).

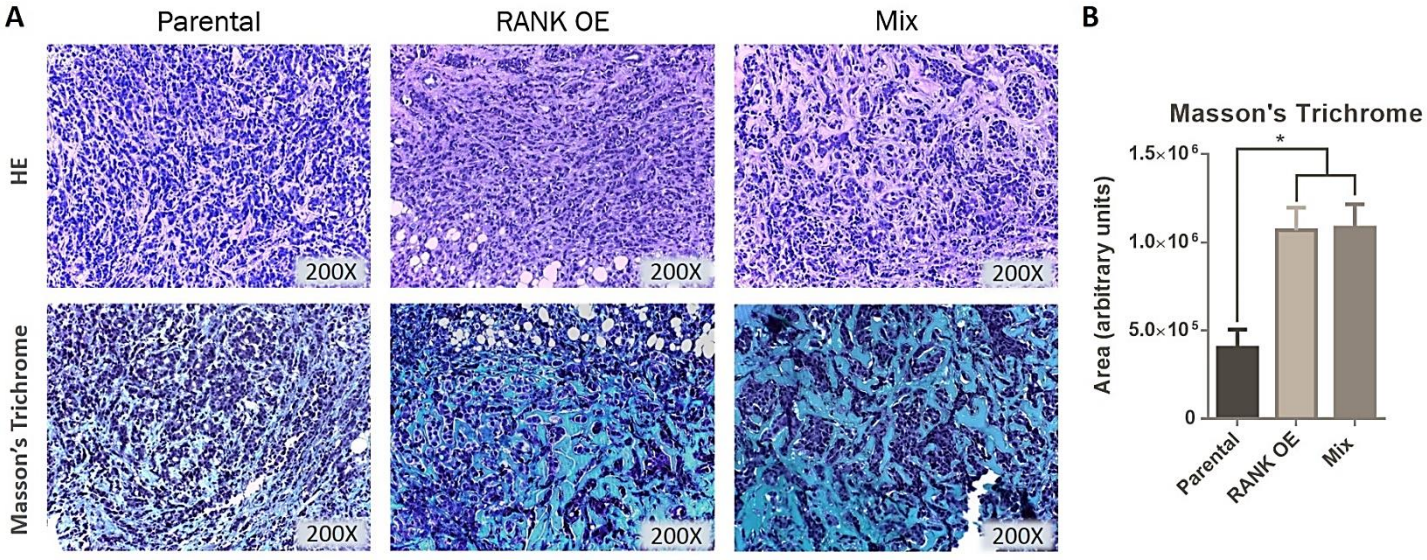

Figure 11 – RANK OE tumours are more desmoplasic.

A) FFPE sections of tumour tissues were stained with hematoxylin and eosin (HE) and Masson's trichrome. **B)** Quantification of collagen area on Masson's stained slides was performed using ImageJ software. Data is presented as mean ± SEM. *p<0.05

We were also interested in analysing the invasive potential of RANK OE cells. Based on our previous studies, we hypothesize that RANK OE cells can be more invasive. In this study our data show that RANK OE tumours have decreased proliferation rate and higher desmoplasia, characteristics that can be related with higher invasiveness. Therefore, we analysed the presence of CTCs and DTCs in tumour bearing mice.

CTCs were quantified by GFP and RFP analysis of venous blood collected five weeks post tumour inoculation (wpi) and at sacrifice. The percentage of GFP^+ cells in both OE and Mix tumour bearing mice was higher at five but not at eight wpi, when comparing with parental tumours' bearing mice, although this was not statistically significant (Figure 12A). In the Mix tumour bearing mice, the CTCs were mainly GFP^+RFP^+ (RANK OE) cells ($p<0.05$) (Figure 12B). We could not detect DTCs by flow cytometry analysis of bone marrow collected at sacrifice.

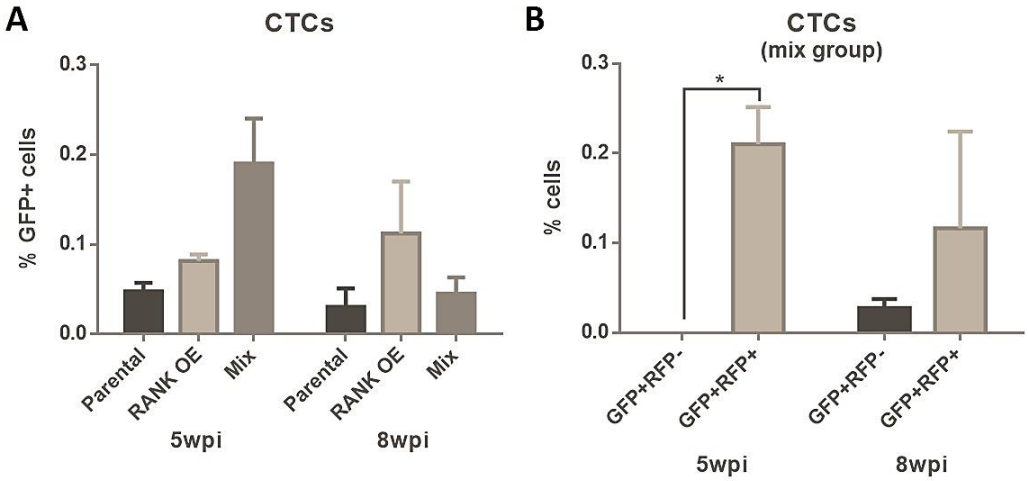

Figure 12 – RANK overexpression increases the number of circulating tumour cells (CTCs).

Blood was collected from the submandibular vein at 5th week post tumour inoculation and by cardiac puncture at sacrifice (8th week). CTCs were quantified by flow cytometry. Data analysis was performed using FlowJo V10 software. **A)** Percentage of GFP^+ cells in blood. **B)** Percentage of GFP^+RFP^- cells and GFP^+RFP^+ cells in blood from mice inoculated with Mix cells. Data is presented as mean ± SEM. *p<0.05

Since increased motility and invasiveness of cancer cells are reminiscent of EMT, we evaluated the expression of EMT-related genes by RT-qPCR and found that tumours with RANK OE have an up-regulation of Vimentin, Snail and N-cadherin (Figure 13).

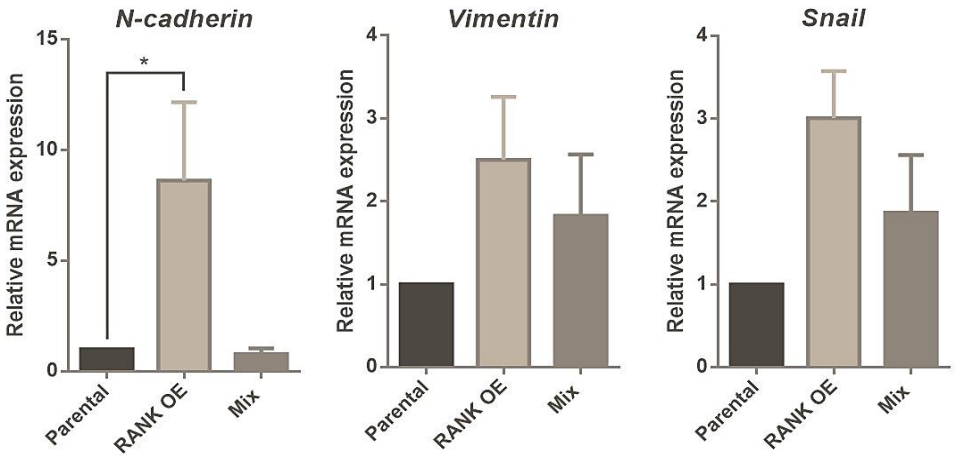

Figure 13 – RANK overexpression up-regulates the expression of epithelial-to-mesenchymal transition (EMT)-related genes.

Relative mRNA expression in tumours was assessed by RT-qPCR. Ct values were normalized to GAPDH. *p<0.05

3. RANK OE decreases MCF-7 cells' proliferation and increases migration *in vitro*

Since we observed *in vivo* that MCF-7 RANK OE tumours have decreased proliferation rate but lead to higher number of CTCs, we next analysed *in vitro* the proliferation and migration of the same clones. We observed that MCF-7 RANK OE cells have decreased proliferation, when compared to parental cells ($p<0.05$) (Figure 14A).

To analyse cell migration we performed a wound healing assay, with and without RANKL stimulus (Figure 14B,C). Although statistically not significant in this assay, RANK OE cells showed increased migration ability when compared to parental cells, and RANKL increased cell migration as expected.

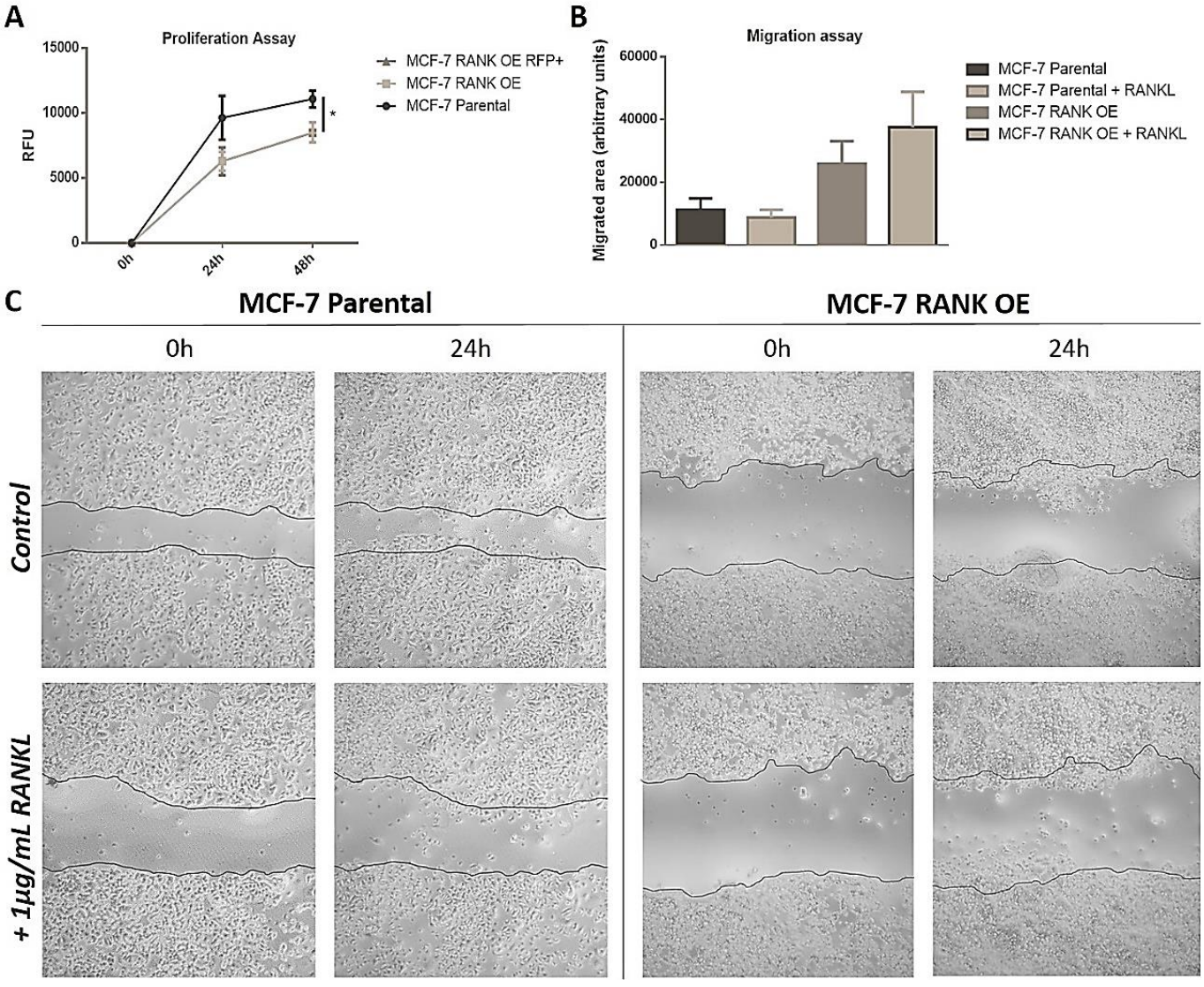

Figure 14 – MCF-7 RANK OE cells proliferate less, but migrate more than MCF-7 parental cells.

A) Proliferation was assessed by Alamar blue for 24h and 48h. Experiment was performed in triplicate. **B)** Migration was assessed by scratch wound assay, in low-serum medium, with or without 1µg/ml hRANKL. Migrated area was quantified using ImageJ software. **C)** Representative images are presented (50X magnification). Data is presented as mean ± SEM. *$p<0.05$

We also tested the migration of MCF-7$^{GFP+Luc+}$ (parental) and MCF-7 RANK OE$^{GFP+Luc+RFP+}$ in co-culture (1:1), and RANK OE cells show consistently increased migration ability (Figure 15).

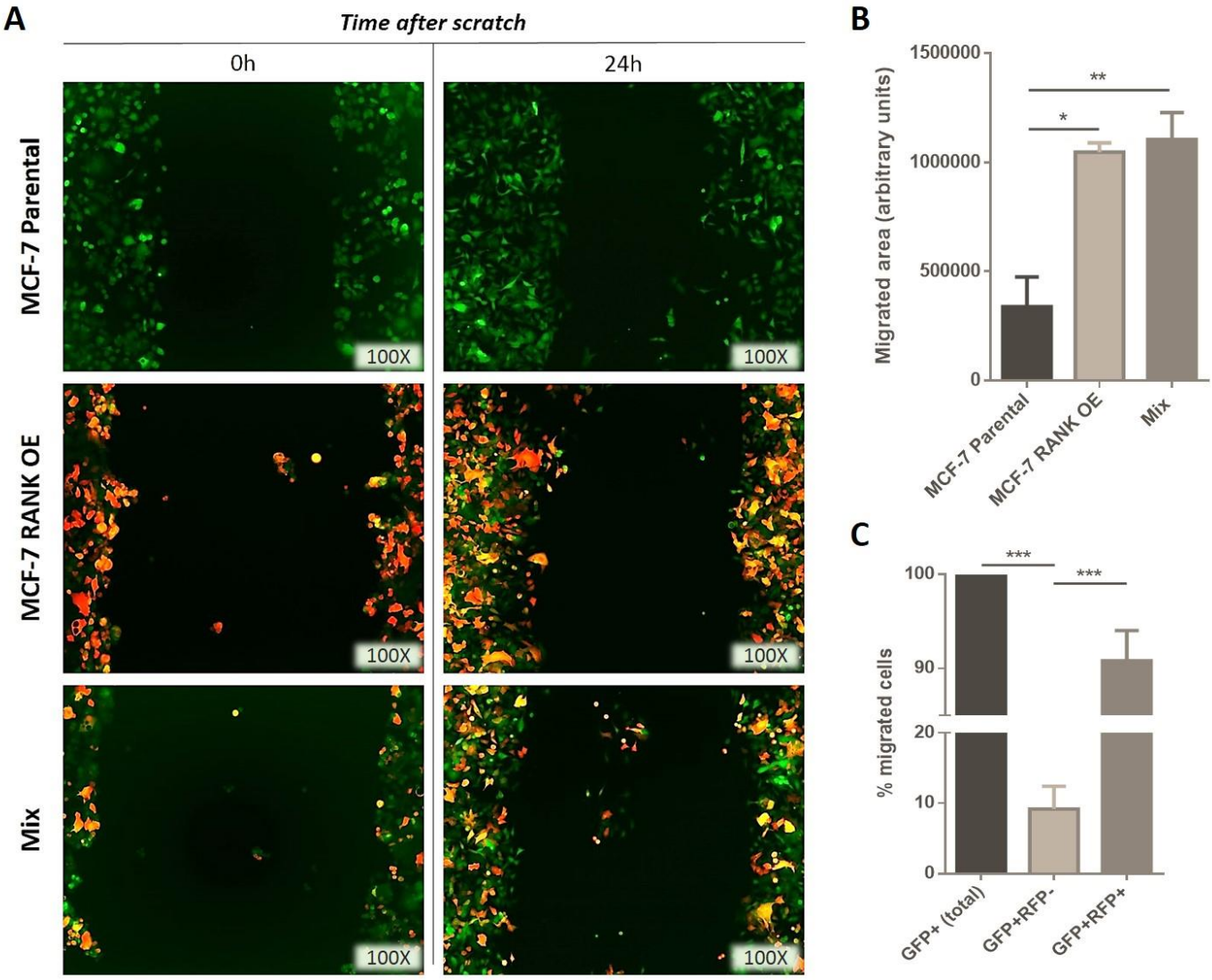

Figure 15 – MCF-7 RANK OE cells migrate more than MCF-7 parental cells.

Migration was assessed by scratch wound assay, in low-serum medium. **A)** Representative fluorescence microscopy images. **B)** Migrated area was quantified using ImageJ software ($Area_{0h}$-$Area_{24h}$). **C)** Percentage of $GFP^{+}RFP^{+}$ migrated cells was obtained through quantification of migrated area in each channel (red/green). Data is presented as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001

BC is the most frequent type of cancer among women and, although many improvements have been made in BC treatment, 20 to 30% of patients diagnosed with early BC will develop metastases at secondary sites in the course of their disease[24].

Amongst the different subtypes of BC, the luminal BC (HR^+) has the better prognosis. Nevertheless, about 30% of patients with luminal A BC, and 40% of patients with luminal B BC will have relapsed within 15-years of initial diagnosis[24]. Bone is the preferential site for metastization of luminal BC, being affected in about 70% of all metastatic luminal BC.

In the context of BC, RANK-RANKL signalling pathway can be considered a recent therapeutic target. The role of this pathway as the key determinant of bone remodelling[37,42,48,49] led to the development of denosumab, an anti-RANKL antibody, as a bone-targeted agent. The use of denosumab was approved by FDA in 2010 for the prevention of SREs in patients with bone metastases from solid tumours including BC; and in 2011 for the treatment of bone loss in patients with BC undergoing hormone ablation therapy.

However, RANK-RANKL signalling pathway has been also widely dissected in the past years, for its implications in BC onset and progression. Not only it has been demonstrated that RANK is functionally expressed by tumour cells[66,69,83,98–100], but it also plays a key role in mammary tumorigenesis[78,79], and affects tumour invasiveness, inducing EMT and stemness[68,83,84].

In vitro studies have shown that overexpression of RANK in untransformed MCF10A cells induced EMT, and increased migration and stemness ($CD44^+CD24^{-/low}$)[68]. In this study, overexpression of RANK in a panel of tumoral BRCA1 defective, HR^- cell lines also induced the expression of BC basal/stem cell markers, and EMT-related genes. Posteriorly, it was shown that also in luminal BC cell line MCF-7, which expresses low levels of endogenous RANK, RANKL stimuli was able to promote EMT, migration, and invasion[83]. Based on this data we have also priory investigated RANK-pathway derived phenotypes of luminal BC cells, and found that RANK overexpression and activation by RANKL induced EMT in MCF-7 cells, resulting in a mesenchymal phenotype with increased migration potential[84]. Moreover, we also observed an increase in $CD44^+/CD24^{-/low}$ population of cancer stem cells (CSCs) in response to RANKL.

Therefore, we further interrogated if RANK overexpressing luminal BC cells could also have a more aggressive phenotype *in vivo*, justifying further studies in the clinical setting. So far, RANK expression in human samples has been found to be mostly up-regulated in TNBC[88].

Here we used an orthotopic xenograft mouse model of luminal BC to address if RANK overexpression in MCF-7 cells was correlated with tumour burden and invasiveness. Since MCF-7 cells express low endogenous levels of RANK, we overexpressed RANK by lentiviral transduction, and used a RANK OE stable clone with 24-fold up-regulation of RANK for *in vivo* assays (Figure 6).

We confirmed the functional activation of RANK pathway in MCF-7 RANK OE cells by measuring the phosphorylated levels of ERK and AKT (Figure 8). It is known that

RANKL binding induces RANK trimerization and activation, triggering the recruitment of TRAFs and phosphorylation of downstream targets such as the TF NF-κB and members of the mitogen-activated protein (MAP) kinase family including MAPK, c-Jun N-terminal kinase (JNK), and p38[101]. In cancer cells it was also shown that RANK stimulation results in enhanced activation of AKT and ERK1/2[66,69].

To evaluate *in vivo* the behaviour of MCF-7 RANK OE cells, in comparison with the MCF-7 parental cell line, we inoculated each cell line in the mammary fat pad of NSG mice. Since we hypothesize that RANK OE cells will be more aggressive and invasive we also inoculated a mixture of cells, parental and RANK OE, in a 1:1 proportion (Mix group).

Initial tumour growth was similar amongst different xenografts, but from week six post tumour inoculation onwards tumours from both RANK OE and Mix groups exhibited a decreased growth rate when compared to parental cell tumours (Figure 9). Moreover, predominant cells in the tumours of the Mix group eight weeks post inoculation (at sacrifice) were RANK OE cells; and in fact the growth of Mix tumours was identical to the growth of RANK OE tumours. The decreased tumour rate was due to a lower proliferative index of RANK OE and Mix tumours, measured by Ki67 expression (Figure 9). We confirmed *in vitro* that RANK OE cells have a decreased proliferation rate (Figure 14), therefore our data suggest that in the Mix tumours the slower growing sub-population of RANK OE cells exhibited an advantage over the faster growing parental cells. It will be interesting to co-culture both cell types *in vitro* and measure specific cell growth, and to address cell growth in mixed spheroids growing in a 3D matrix. Both soluble factors and contact-mediated mechanisms may be implicated, and this needs to be further addressed. Additionally, since RANK OE cells were less proliferative *in vivo* it is necessary to investigate a putative role of RANK in resistance to chemotherapy. In fact, a recent study has already proven that RANK inhibition leads to an increase in the sensibility to chemotherapy[82].

An interesting feature common to both RANK OE and Mix tumours was the high degree of desmoplasia, when compared to parental tumours (Figure 11). Desmoplasia consists in the presence of a dense collagenous stroma, often seen in breast tumours, but its role in BC progression remains unclear, as well as which are the events that induce this response. However, it was suggested that PDGF secreted by BC cells is the major initiator of tumour desmoplasic responses[102,103] and, interestingly, it has already been demonstrated that high PDGF receptor (PDGFR) expression is associated with a significantly shorter recurrence-free survival and BC specific survival[104]. It is also known that mammographic density, that reflects collagen content of the breast stroma, is associated with an increased risk of BC[105,106]. Therefore, it will be interesting to analyse PDGF/PDGFR expression in the xenografts' tissue, and to perform *in silico* analysis of available datasets, interrogating for the correlation between RANK expression and desmoplasia related markers.

Despite tumours being smaller, we identified a higher number of CTCs in both RANK OE and Mix tumour-bearing mice, comparing to parental tumour-bearing mice (Figure 12). Moreover, CTCs in Mix tumour-bearing mice were predominantly RANK OE cells. This suggests that cells that overexpress RANK are more invasive. Our previous data has shown that RANK OE cells have increased migration ability[84], a feature that was confirmed in this work (Figures 14 and 15). Therefore, we next analysed the expression of EMT related genes in the tumours.

It is acknowledged that, in order to evade the tumour and metastasize, tumour cells stop proliferating and acquire an invasive phenotype through genetic/epigenetic alterations. Increased motility and invasiveness of cancer cells are reminiscent of EMT, a process of cell transformation associated with increased metastatic potential. In fact, EMT has been closely associated with the acquisition of aggressive traits by cancer cells, and loss of E-cadherin, an epithelial adhesion molecule, and switching to N-cadherin represents a key step in the acquisition of an invasive phenotype[107]. Transcription factors Snail, Slug and Twist are known to regulate this down-regulation of E-cadherin[108–110]. Previous studies have demonstrated that RANKL induces EMT in BC cells through activation of RANK signalling pathway, leading to increased migration and invasion potentials[83,84]. We identified higher Vimentin, Snail and N-cadherin expression in tumours with RANK OE, in comparison with the parental tumours (Figure 13).

Altogether, our results suggest that, in a "heterogeneous" tumour where cells with low and high RANK expression coexist, the ones that overexpress this receptor acquire an adaptive advantage. Further studies are required to answer the questions raised by this study. We plan to perform an *in vivo* survival experiment, where RANK OE and parental cells will be either inoculated in the same mammary gland or in contra-lateral glands, and we will follow tumour growth and CTCs. To be able to conduct such an experiment we will replace sub-cutaneous 17β-estradiol by 17β-estradiol provided in drinking water *ad libitum*, to overcome the side effects that compromise long course experiments with MCF-7 xenografts. To study tumour metastization we will include paired groups where we will excise tumours before they reach humanitarian endpoint size.

One interesting observation we made in this work was that RANK OE in MCF-7 cells induced a time-dependent increase of p-AKT but a time-dependent decrease in pERK1/2, although the activation pattern in RANK OE and parental MDA-MB-231 cells was as expected. Interestingly, we observed in independent RANK OE experiments that in fact in MCF-7 and MDA-MB-231 cells, the phosphorylation of ERK is different upon RANK activation. Whereas in MDA-MB-231 cells mostly p-ERK2 is induced by RANKL, in MCF-7 cells is the ERK1 that is phosphorylated at earlier time points. p-ERK1 will decrease over time, ERK1 will be depleted and only then p-ERK2 is activated (Supplementary Figure 2). Whether ERK1 and ERK2 are functionally different or redundant is still an open debate, and it is hypothesized that is total ERK quantity that affects ERK function[111]. It will be interesting to further analyse if the different ERK phosphorylation pattern we observe between luminal and TNBC cells is biologically meaningful. We performed a simple analysis of ERK1/2 phosphorylation upon RANKL stimuli in other cell lines, wondering if we could detect a pattern related to HR status, but results were inconclusive (Supplementary Figure 2).

Overall, this study has shown that RANK overexpression in luminal-type BC leads to a more aggressive and invasive phenotype *in vivo*.

The inhibition of RANK pathway with denosumab, an anti-RANKL antibody, is the current standard of care treatment for patients with BC and BM; and interim data from adjuvant phase III clinical trials points to its effectiveness in preventing BC relapse in bone. Therefore, it is important to clearly characterize the contribution of RANK pathway in different BC sub-types.

CONCLUSIONS AND FUTURE PERSPECTIVES

RANK-RANKL signalling pathway has emerged as a major target for BC treatment over the past years. Denosumab has been shown to improve clinical outcomes in BC patients with BM and SREs, but it is necessary to have in consideration that RANK-RANKL signalling pathway has many roles besides the critical one that it presents in the 'vicious-cycle' of BM. Addressing this will open new doors onto the use of denosumab outside its current use in SREs' management, namely in prevention of relapse in high risk groups.

In fact, this pathway has been already correlated with poor clinical outcomes, such as a decreased OS, DFS. Accumulating evidences highlight the critical role of this pathway during all stages of tumour progression, from initiation to metastasis formation. However, it is still to be demonstrated if RANK pathway inhibition in BC could have benefits beyond the context of bone metastatic disease. Interim data from adjuvant phase III clinical trials points to its effectiveness in preventing BC relapse in bone. Therefore, it is important to dissect the contribution of RANK signalling in different types and scenarios of BC.

We have priory demonstrated that RANK overexpression in luminal MCF-7 cells induces EMT, stemness and increased migration potential. The results from the present study suggest a positive association between RANK overexpression and the acquisition of more aggressive traits in luminal-BC cells, such as higher desmoplasia and invasiveness. Moreover, when coexisting in the same tumour with cells that express low levels of RANK, RANK-overexpressing cells seem to acquire an adaptive advantage, a result that needs further clarification. Further studies will focus on understanding how RANK expression is correlated with metastasis and resistance to chemotherapy.

REFERENCES

1. Cancer Today. GLOBOCAN2012. http://globocan.iarc.fr/.

2. Toriola, A. T. & Colditz, G. A. Trends in breast cancer incidence and mortality in the United States: implications for prevention. *Breast Cancer Res. Treat.* **138,** 665–673 (2013).

3. Tretli, S. Height and weight in relation to breast cancer morbidity and mortality. A prospective study of 570,000 women in Norway. *Int. J. Cancer* **44,** 23–30 (1989).

4. Verkasalo, P. K., Thomas, H. V., Appleby, P. N., Davey, G. K. & Key, T. J. Circulating levels of sex hormones and their relation to risk factors for breast cancer: a cross-sectional study in 1092 pre- and postmenopausal women (United Kingdom). *Cancer Causes Control CCC* **12,** 47–59 (2001).

5. Brinton, L. A., Hoover, R. & Fraumeni, J. F. Reproductive factors in the aetiology of breast cancer. *Br. J. Cancer* **47,** 757–762 (1983).

6. Colditz, G. A. *et al.* Prospective study of estrogen replacement therapy and risk of breast cancer in postmenopausal women. *JAMA* **264,** 2648–2653 (1990).

7. Easton, D. F., Ford, D. & Bishop, D. T. Breast and ovarian cancer incidence in BRCA1-mutation carriers. Breast Cancer Linkage Consortium. *Am. J. Hum. Genet.* **56,** 265–271 (1995).

8. Miki, Y. *et al.* A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* **266,** 66–71 (1994).

9. Hall, J. M. *et al.* Linkage of early-onset familial breast cancer to chromosome 17q21. *Science* **250,** 1684–1689 (1990).

10. Wooster, R. *et al.* Localization of a breast cancer susceptibility gene, BRCA2, to chromosome 13q12-13. *Science* **265,** 2088–2090 (1994).

11. Bertos, N. R. & Park, M. Breast cancer — one term, many entities? *J. Clin. Invest.* **121,** 3789–3796 (2011).

12. Rivenbark, A. G., O'Connor, S. M. & Coleman, W. B. Molecular and Cellular Heterogeneity in Breast Cancer. *Am. J. Pathol.* **183,** 1113–1124 (2013).

13. Wasif, N., Maggard, M. A., Ko, C. Y. & Giuliano, A. E. Invasive Lobular vs. Ductal Breast Cancer: A Stage-Matched Comparison of Outcomes. *Ann. Surg. Oncol.* **17,** 1862–1869 (2010).

14. Perou, C. M. *et al.* Molecular portraits of human breast tumours. *Nature* **406,** 747–752 (2000).

15. Kittaneh, M. & Gluck, S. in *Current Cancer Treatment - Novel Beyond Conventional Approaches* (ed. Ozdemir, O.) (InTech, 2011).

16. Sorlie, T. *et al.* Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc. Natl. Acad. Sci.* **98,** 10869–10874 (2001).

17. Jordan, V. C. & Brodie, A. M. H. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. *Steroids* **72,** 7–25 (2007).

18. Onitilo, A. A., Engel, J. M., Greenlee, R. T. & Mukesh, B. N. Breast Cancer Subtypes Based on ER/PR and Her2 Expression: Comparison of Clinicopathologic Features and Survival. *Clin. Med. Res.* **7,** 4–13 (2009).

19. Slamon, D. J. *et al.* Use of Chemotherapy plus a Monoclonal Antibody against HER2 for Metastatic Breast Cancer That Overexpresses HER2. *N. Engl. J. Med.* **344,** 783–792 (2001).

20. Smith, I. *et al.* 2-year follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer: a randomised controlled trial. *The Lancet* **369,** 29–36 (2007).

21. Carey, L. A. *et al.* Race, Breast Cancer Subtypes, and Survival in the Carolina Breast Cancer Study. *JAMA* **295,** 2492 (2006).

22. Prat, A. *et al.* Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. *Breast Cancer Res.* **12,** R68 (2010).

23. Curtis, C. *et al.* The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. *Nature* (2012).

24. Kennecke, H. *et al.* Metastatic Behavior of Breast Cancer Subtypes. *J. Clin. Oncol.* **28,** 3271–3277 (2010).

25. Sihto, H. *et al.* Breast cancer biological subtypes and protein expression predict for the preferential distant metastasis sites: a nationwide cohort study. *Breast Cancer Res.* **13,** R87 (2011).

26. Coleman, R. E. Metastatic bone disease: clinical features, pathophysiology and treatment strategies. *Cancer Treat. Rev.* **27,** 165–176 (2001).

27. Fontanella, C., Fanotto, V., Rihawi, K., Aprile, G. & Puglisi, F. Skeletal metastases from breast cancer: pathogenesis of bone tropism and treatment strategy. *Clin. Exp. Metastasis* **32,** 819–833 (2015).

28. Minn, A. J. *et al.* Distinct organ-specific metastatic potential of individual breast cancer cells and primary tumors. *J. Clin. Invest.* **115,** 44–55 (2005).

29. Smid, M. *et al.* Genes Associated With Breast Cancer Metastatic to Bone. *J. Clin. Oncol.* **24,** 2261–2267 (2006).

30. Bos, P. D. *et al.* Genes that mediate breast cancer metastasis to the brain. *Nature* **459,** 1005–1009 (2009).

31. Minn, A. J. *et al.* Lung metastasis genes couple breast tumor size and metastatic spread. *Proc. Natl. Acad. Sci.* **104,** 6740–6745 (2007).

32. Kang, Y. *et al.* A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell* **3,** 537–549 (2003).

33. Anderson, D. M. *et al.* A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature* **390,** 175–179 (1997).

34. Wong, B. R. *et al.* TRANCE (tumor necrosis factor [TNF]-related activation-induced cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor. *J. Exp. Med.* **186,** 2075–2080 (1997).

35. Yasuda, H. *et al.* Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proc. Natl. Acad. Sci. U. S. A.* **95,** 3597–3602 (1998).

36. Lacey, D. L. *et al.* Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* **93,** 165–176 (1998).

37. Dougall, W. C. *et al.* RANK is essential for osteoclast and lymph node development. *Genes Dev.* **13,** 2412–2424 (1999).

38. Fata, J. E. *et al.* The osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development. *Cell* **103,** 41–50 (2000).

39. Duheron, V. *et al.* Receptor activator of NF- B (RANK) stimulates the proliferation of epithelial cells of the epidermo-pilosebaceous unit. *Proc. Natl. Acad. Sci.* **108,** 5342–5347 (2011).

40. Akiyama, T. *et al.* The Tumor Necrosis Factor Family Receptors RANK and CD40 Cooperatively Establish the Thymic Medullary Microenvironment and Self-Tolerance. *Immunity* **29,** 423–437 (2008).

41. Darnay, B. G., Haridas, V., Ni, J., Moore, P. A. & Aggarwal, B. B. Characterization of the intracellular domain of receptor activator of NF-kappaB (RANK). Interaction with tumor necrosis factor receptor-associated factors and activation of NF-kappab and c-Jun N-terminal kinase. *J. Biol. Chem.* **273,** 20551–20555 (1998).

42. Armstrong, A. P. *et al.* A RANK/TRAF6-dependent Signal Transduction Pathway Is Essential for Osteoclast Cytoskeletal Organization and Resorptive Function. *J. Biol. Chem.* **277,** 44347–44356 (2002).

43. Huang, H. *et al.* Induction of c-Fos and NFATc1 during RANKL-stimulated osteoclast differentiation is mediated by the p38 signaling pathway. *Biochem. Biophys. Res. Commun.* **351,** 99–105 (2006).

44. Takayanagi, H. *et al.* Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. *Dev. Cell* **3,** 889–901 (2002).

45. Darnay, B. G., Ni, J., Moore, P. A. & Aggarwal, B. B. Activation of NF-kappaB by RANK requires tumor necrosis factor receptor-associated factor (TRAF) 6 and NF-kappaB-inducing kinase. Identification of a novel TRAF6 interaction motif. *J. Biol. Chem.* **274,** 7724–7731 (1999).

46. Wong, B. R. *et al.* TRANCE, a TNF family member, activates Akt/PKB through a signaling complex involving TRAF6 and c-Src. *Mol. Cell* **4,** 1041–1049 (1999).

47. González-Suárez, E. & Sanz-Moreno, A. RANK as a therapeutic target in cancer. *FEBS J.* **283,** 2018–2033 (2016).

48. Kong, Y. Y. *et al.* OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* **397,** 315–323 (1999).

49. Hsu, H. *et al.* Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. *Proc. Natl. Acad. Sci. U. S. A.* **96,** 3540–3545 (1999).

50. Suda, T., Takahashi, N. & Martin, T. J. Modulation of osteoclast differentiation. *Endocr. Rev.* **13,** 66–80 (1992).

51. Simonet, W. S. *et al.* Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* **89,** 309–319 (1997).

52. Cao, Y. *et al.* IKKalpha provides an essential link between RANK signaling and cyclin D1 expression during mammary gland development. *Cell* **107,** 763–775 (2001).

53. Fernandez-Valdivia, R. *et al.* The RANKL signaling axis is sufficient to elicit ductal side-branching and alveologenesis in the mammary gland of the virgin mouse. *Dev. Biol.* **328,** 127–139 (2009).

54. Gonzalez-Suarez, E. *et al.* RANK Overexpression in Transgenic Mice with Mouse Mammary Tumor Virus Promoter-Controlled RANK Increases Proliferation and Impairs Alveolar Differentiation in the Mammary Epithelia and Disrupts Lumen Formation in Cultured Epithelial Acini. *Mol. Cell. Biol.* **27,** 1442–1454 (2007).

55. Tanos, T. *et al.* Progesterone/RANKL Is a Major Regulatory Axis in the Human Breast. *Sci. Transl. Med.* **5,** 182ra55-182ra55 (2013).

56. Dougall, W. C., Holen, I. & González Suárez, E. Targeting RANKL in metastasis. *BoneKEy Rep.* **3,** (2014).

57. Schramek, D., Sigl, V. & Penninger, J. M. RANKL and RANK in sex hormone-induced breast cancer and breast cancer metastasis. *Trends Endocrinol. Metab.* **22,** 188–194 (2011).

58. Brantley, D. M. *et al.* Dynamic expression and activity of NF-kappaB during post-natal mammary gland morphogenesis. *Mech. Dev.* **97,** 149–155 (2000).

59. Kim, N.-S. *et al.* Receptor activator of NF-kappaB ligand regulates the proliferation of mammary epithelial cells via Id2. *Mol. Cell. Biol.* **26,** 1002–1013 (2006).

60. Caux, C. *et al.* Activation of human dendritic cells through CD40 cross-linking. *J. Exp. Med.* **180,** 1263–1272 (1994).

61. Cella, M. *et al.* Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J. Exp. Med.* **184,** 747–752 (1996).

62. Wong, B. R. *et al.* TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. *J. Biol. Chem.* **272,** 25190–25194 (1997).

63. Wiethe, C., Dittmar, K., Doan, T., Lindenmaier, W. & Tindle, R. Enhanced Effector and Memory CTL Responses Generated by Incorporation of Receptor Activator of NF- B (RANK)/RANK Ligand Costimulatory Molecules into Dendritic Cell Immunogens Expressing a Human Tumor-Specific Antigen. *J. Immunol.* **171,** 4121–4130 (2003).

64. Khan, I. S. *et al.* Enhancement of an anti-tumor immune response by transient blockade of central T cell tolerance. *J. Exp. Med.* **211,** 761–768 (2014).

65. Cheng, M. L. & Fong, L. Effects of RANKL-Targeted Therapy in Immunity and Cancer. *Front. Oncol.* **3,** (2014).

66. Jones, D. H. *et al.* Regulation of cancer cell migration and bone metastasis by RANKL. *Nature* **440,** 692–696 (2006).

67. Thomas, R. J. *et al.* Breast Cancer Cells Interact with Osteoblasts to Support Osteoclast Formation [1]. *Endocrinology* **140,** 4451–4458 (1999).

68. Palafox, M. *et al.* RANK Induces Epithelial-Mesenchymal Transition and Stemness in Human Mammary Epithelial Cells and Promotes Tumorigenesis and Metastasis. *Cancer Res.* **72,** 2879–2888 (2012).

69. Casimiro, S. *et al.* RANKL/RANK/MMP-1 Molecular Triad Contributes to the Metastatic Phenotype of Breast and Prostate Cancer Cells In Vitro. *PLoS ONE* **8,** e63153 (2013).

70. Armstrong, A. P. *et al.* RANKL acts directly on RANK-expressing prostate tumor cells and mediates migration and expression of tumor metastasis genes. *The Prostate* **68,** 92–104 (2008).

71. Santini, D. *et al.* Receptor Activator of NF-kB (RANK) Expression in Primary Tumors Associates with Bone Metastasis Occurrence in Breast Cancer Patients. *PLoS ONE* **6,** e19234 (2011).

72. Santini, D. *et al.* Expression pattern of receptor activator of NFκB (RANK) in a series of primary solid tumors and related bone metastases. *J. Cell. Physiol.* **226,** 780–784 (2011).

73. Owen, S., Ye, L., Sanders, A. J., Mason, M. D. & Jiang, W. G. Expression profile of receptor activator of nuclear-κB (RANK), RANK ligand (RANKL) and osteoprotegerin (OPG) in breast cancer. *Anticancer Res.* **33,** 199–206 (2013).

74. Bhatia, P., Sanders, M. M. & Hansen, M. F. Expression of receptor activator of nuclear factor-kappaB is inversely correlated with metastatic phenotype in breast carcinoma. *Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res.* **11,** 162–165 (2005).

75. Pellegrini, P. *et al.* Constitutive activation of RANK disrupts mammary cell fate leading to tumorigenesis: RANK Disrupts Mammary Cell Fate Leading to Cancer. *STEM CELLS* **31,** 1954–1965 (2013).

76. Joshi, P. A. *et al.* Progesterone induces adult mammary stem cell expansion. *Nature* **465,** 803–807 (2010).

77. Asselin-Labat, M.-L. *et al.* Control of mammary stem cell function by steroid hormone signalling. *Nature* **465,** 798–802 (2010).

78. Gonzalez-Suarez, E. *et al.* RANK ligand mediates progestin-induced mammary epithelial proliferation and carcinogenesis. *Nature* **468,** 103–107 (2010).

79. Schramek, D. *et al.* Osteoclast differentiation factor RANKL controls development of progestin-driven mammary cancer. *Nature* **468,** 98–102 (2010).

80. Beleut, M. *et al.* Two distinct mechanisms underlie progesterone-induced proliferation in the mammary gland. *Proc. Natl. Acad. Sci.* **107,** 2989–2994 (2010).

81. Narod, S. A. Hormone replacement therapy and the risk of breast cancer. *Nat. Rev. Clin. Oncol.* **8,** 669–676 (2011).

82. Yoldi, G. *et al.* RANK Signaling Blockade Reduces Breast Cancer Recurrence by Inducing Tumor Cell Differentiation. *Cancer Res.* **76,** 5857–5869 (2016).

83. Tsubaki, M. *et al.* Activation of NF-κB by the RANKL/RANK system up-regulates snail and twist expressions and induces epithelial-to-mesenchymal transition in mammary tumor cell lines. *J. Exp. Clin. Cancer Res.* **32,** 62 (2013).

84. Henriques, S. Luminal-type breast cancer: prognostic value of RANK+ luminal breast cancer (in vitro approach). (Faculdade de Medicina da Universidade de Lisboa).

85. Sigl, V. *et al.* RANKL/RANK control Brca1 mutation-driven mammary tumors. *Cell Res.* **26,** 761–774 (2016).

86. Widschwendter, M. *et al.* Osteoprotegerin (OPG), The Endogenous Inhibitor of Receptor Activator of NF-κB Ligand (RANKL), is Dysregulated in BRCA Mutation Carriers. *EBioMedicine* **2,** 1331–1339 (2015).

87. Chen, G. *et al.* Expression of RANKL/RANK/OPG in primary and metastatic human prostate cancer as markers of disease stage and functional regulation. *Cancer* **107,** 289–298 (2006).

88. Pfitzner, B. M. *et al.* RANK expression as a prognostic and predictive marker in breast cancer. *Breast Cancer Res. Treat.* **145,** 307–315 (2014).

89. Paget, S. The distribution of secondary growths in cancer of the breast. 1889. *Cancer Metastasis Rev.* **8,** 98–101 (1989).

90. Mundy, G. R. Mechanisms of bone metastasis. *Cancer* **80,** 1546–1556 (1997).

91. Casimiro, S., Ferreira, A., Mansinho, A., Alho, I. & Costa, L. Molecular Mechanisms of Bone Metastasis: Which Targets Came from the Bench to the Bedside? *Int. J. Mol. Sci.* **17,** 1415 (2016).

92. Weinberg, R. A. *The Biology of Cancer, 2nd Edition*. (Garland Science).

93. Blake, M. L., Tometsko, M., Miller, R., Jones, J. C. & Dougall, W. C. RANK expression on breast cancer cells promotes skeletal metastasis. *Clin. Exp. Metastasis* **31,** 233–245 (2014).

94. Lacey, D. L. *et al.* Bench to bedside: elucidation of the OPG–RANK–RANKL pathway and the development of denosumab. *Nat. Rev. Drug Discov.* **11,** 401–419 (2012).

95. Costa, L. *et al.* Expression of matrix metalloproteinase 1 (MMP1) and dickkopf-1 (DKK1) in bone metastatic tissue (BMT) and in serum of patients with bone metastases (BM) from different solid tumors. *Bone* **48,** S25–S26 (2011).

96. Gnant, M. *et al.* Adjuvant denosumab in breast cancer (ABCSG-18): a multicentre, randomised, double-blind, placebo-controlled trial. *The Lancet* **386,** 433–443 (2015).

97. Goss, P. E. *et al.* Denosumab versus placebo as adjuvant treatment for women with early-stage breast cancer at high risk of disease recurrence (D-CARE): an international, randomized, double-blind, placebo-controlled phase 3 clinical trial. *Cancer Res.* **72(24 Suppl): Abstract number OT2-3-03**

98. Mori, K. *et al.* Human osteosarcoma cells express functional receptor activator of nuclear factor-kappa B. *J. Pathol.* **211,** 555–562 (2007).

99. Tometsko, M., Armstrong, A., Miller, R., Jones, J. & Chaisson, M. RANK ligand directly induces osteoclastogenic, angiogenic, chemoattractive and invasive factors on RANK-expressing human cancer cells MDA-MB-231 and PC3. *J. Bone Miner. Res.* **19,** (2004).

100.Zhang, L. *et al.* C-Src-mediated RANKL-induced breast cancer cell migration by activation of the ERK and Akt pathway. *Oncol. Lett.* **3,** 395–400 (2012).

101.Darnay, B. G., Besse, A., Poblenz, A. T., Lamothe, B. & Jacoby, J. J. in *Madame Curie Bioscience Database [Internet]* (Landes Bioscience, 2000).

102.Shao, Z.-M., Nguyen, M. & Barsky, S. H. Human breast carcinoma desmoplasia is PDGF initiated. *Oncogene* **19,** 4337–4345 (2000).

103.Walker, R. A. The complexities of breast cancer desmoplasia. *Breast Cancer Res. BCR* **3,** 143–145 (2001).

104.Paulsson, J. *et al.* Prognostic Significance of Stromal Platelet-Derived Growth Factor β-Receptor Expression in Human Breast Cancer. *Am. J. Pathol.* **175,** 334–341 (2009).

105.McCormack, V. A. Breast Density and Parenchymal Patterns as Markers of Breast Cancer Risk: A Meta-analysis. *Cancer Epidemiol. Biomarkers Prev.* **15,** 1159–1169 (2006).

106.Boyd, N. F. *et al.* Mammographic densities as a marker of human breast cancer risk and their use in chemoprevention. *Curr. Oncol. Rep.* **3,** 314–321 (2001).

107.Hazan, R. B., Qiao, R., Keren, R., Badano, I. & Suyama, K. Cadherin switch in tumor progression. *Ann. N. Y. Acad. Sci.* **1014,** 155–163 (2004).

108.Batlle, E. *et al.* The transcription factor Snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat. Cell Biol.* **2,** 84–89 (2000).

109.Hajra, K. M., Chen, D. Y.-S. & Fearon, E. R. The SLUG zinc-finger protein represses E-cadherin in breast cancer. *Cancer Res.* **62,** 1613–1618 (2002).

110.Mironchik, Y. Twist Overexpression Induces In vivo Angiogenesis and Correlates with Chromosomal Instability in Breast Cancer. *Cancer Res.* **65,** 10801–10809 (2005).

111.Buscà, R., Pouysségur, J. & Lenormand, P. ERK1 and ERK2 Map Kinases: Specific Roles or Functional Redundancy? *Front. Cell Dev. Biol.* **4,** (2016).

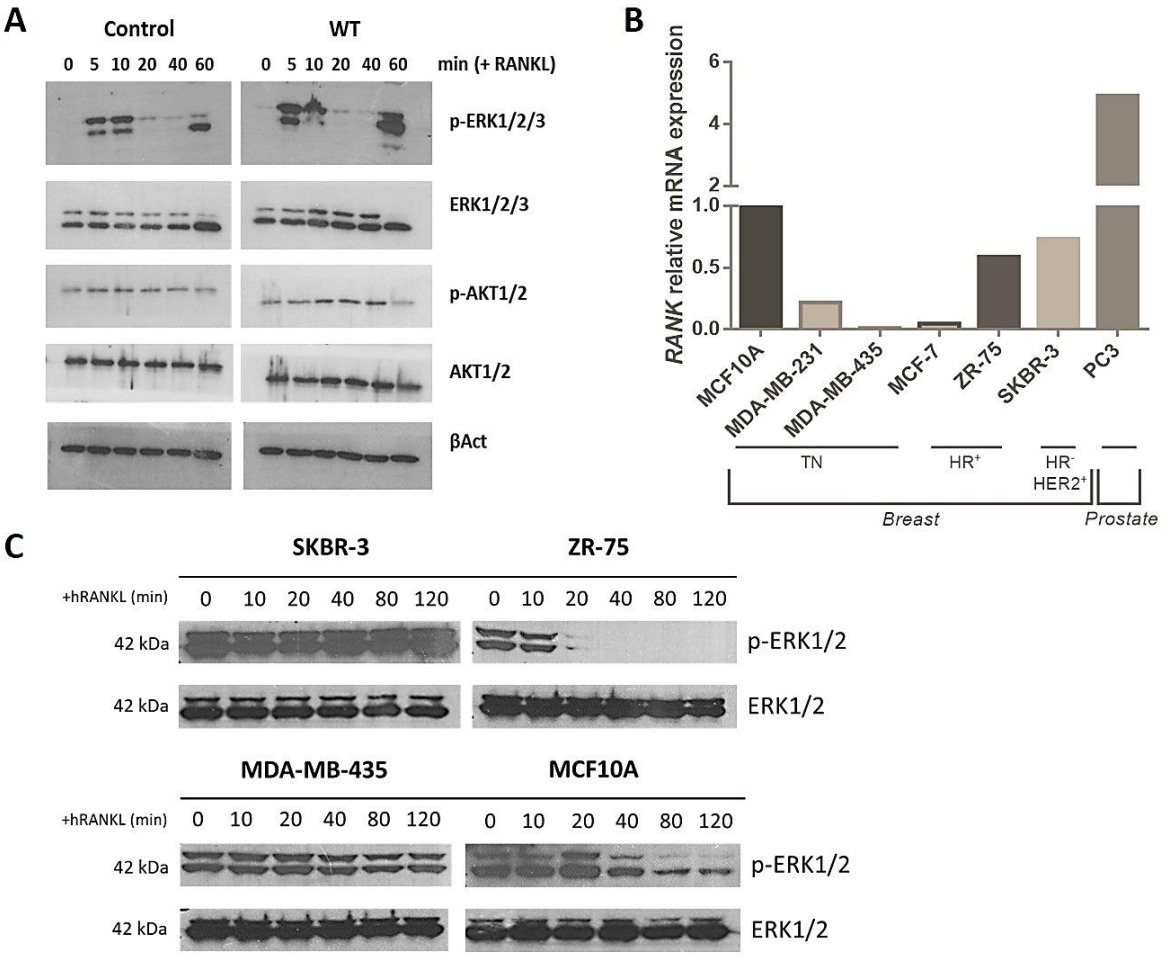

Figure S1 – RANK signaling pathway has distinct ERK phosphorylation patterns in different BC cell lines.

RANK pathway activation was confirmed by western blot. **A)** Functional assay in MCF-7 cells: Control – empty vector; WT – transient RANK overexpression generated using CRISP/cas9 methodology (Santa Cruz). After 24h in low-serum medium, cells were stimulated with 1µg/mL hRANKL for 5, 10, 20, 40 and 60min. β-actin was used as loading control. **B)** Relative mRNA RANK expression levels in different cell lines was normalized against MCF10A and CT values were normalized against GAPDH. **C)** After 24h in low-serum medium, cells were stimulated with 1µg/mL hRANKL for 10, 20, 40, 80 and 120 min.

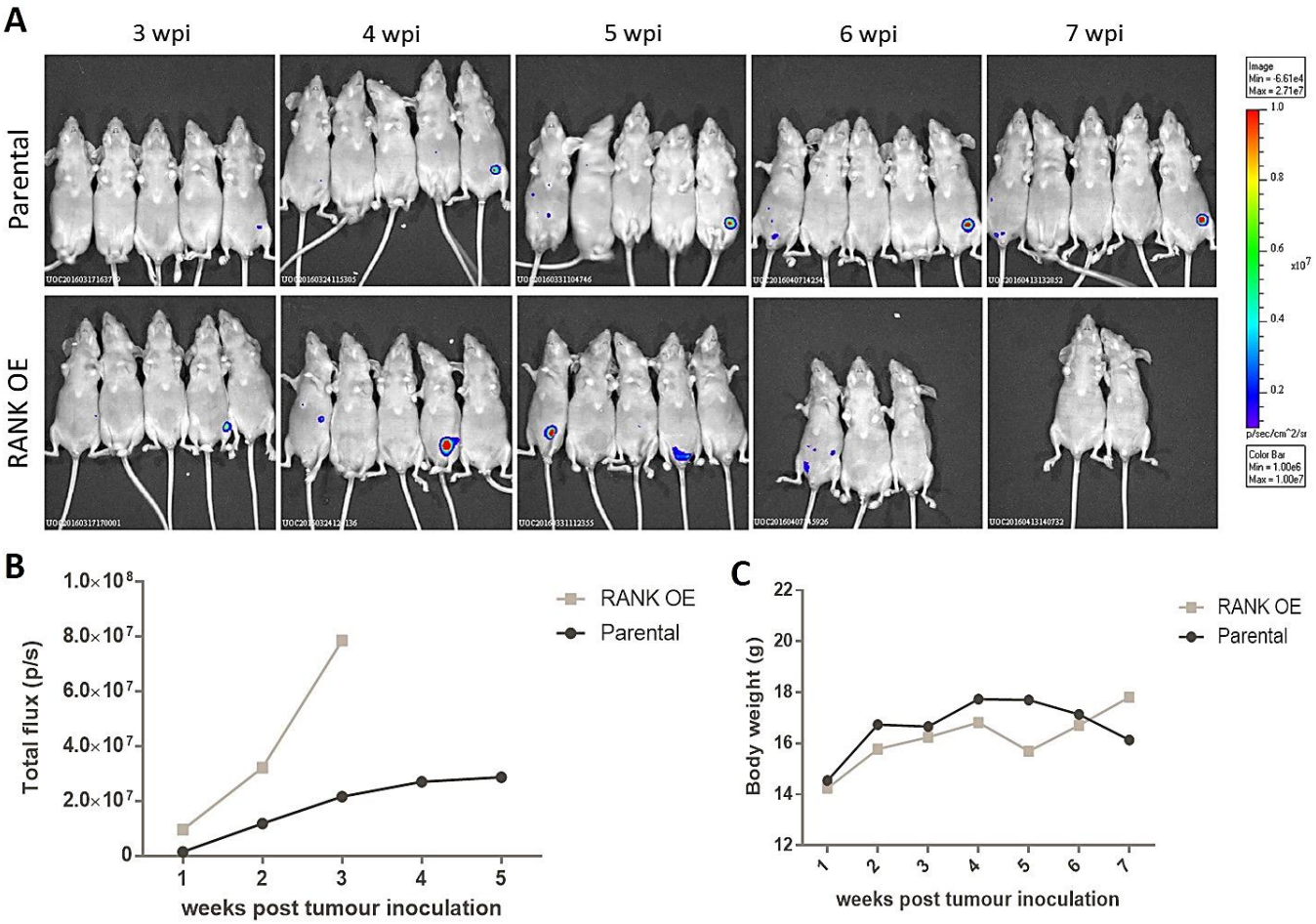

Figure S2 – MCF-7 cells have low engraftment rate in BALB/c nude mice.

Groups of five BALB/c nude mice were inoculated with MCF-7 $^{GFP+Luc+}$ (Parental), MCF-7 RANK OE $^{GFP+Luc+}$ (RANK OE) Inoculation was performed into the 4th inguinal mammary fat pad. **A)** Tumours were imaged from third week post tumour inoculation till the end of the experience. Images from the 1st week are not represented since there was still no tumours. **B)** Total flux (p/s) was calculated using IVIS Lumina software. **C)** Body weight motorization.