RANK induces epithelial-mesenchymal transition and stemness in human mammary epithelial cells and promotes tumorigenesis and metastasis

Marta Palafox¹, Irene Ferrer¹, Pasquale Pellegrini¹, Sergi Vila¹, Sara Hernandez-Ortega¹, Ander Urruticoechea², Fina Climent³, Maria Teresa Soler³, Purificación Muñoz¹, Francesc Viñals⁴, Mark Tometsko⁵, Dan Branstetter⁶, William C. Dougall⁵ and Eva González-Suárez¹,*

¹Cancer Epigenetics and Biology Program, ²Catalan Institute of Oncology, ³University Hospital of Bellvitge, ⁴University of Barcelona, Bellvitge Biomedical Research Institute, (¹-⁴) IDIBELL, Barcelona, Spain; ⁵Department of Hematology/Oncology Research; ⁶Department of Pathology, (⁵-⁶) Amgen Inc, Seattle, WA, USA

*corresponding author: Eva González Suárez.


egsuarez@idibell.cat Phone: +34 932607253 Fax: +34 9326071

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ABSTRACT

Paracrine signaling through RANK pathway mediates the expansion of mammary epithelia that occurs during pregnancy and activation of RANK pathway promotes mammary tumorigenesis in mice. In this study we extend these previous data to human cells and demonstrate that the RANK pathway promotes the development of mammary stem cells and breast cancer. Overexpression of RANK (FL-RANK) in a panel of tumoral and normal human mammary cells induces the expression of breast cancer stem and basal/stem cell markers. High levels of RANK in untransformed MCF10A cells induce changes associated with both stemness and transformation including mammary gland reconstitution, epithelial-mesenchymal transition, increased migration and anchorage independent growth. In addition, spheroids of RANK-overexpressing MCF10A cells displayed disrupted acinar formation, impaired growth arrest and polarization, and luminal filling. RANK overexpression in tumor cells with non functional BRCA1, enhances invasiveness in acinar cultures and increases tumorigenesis and metastasis in immunodeficient mice. High levels of RANK were found in human primary breast adenocarcinomas that lack expression of the hormone receptors, estrogen and progesterone, and in tumors with high pathological grade and proliferation index; high RANK/RANKL expression was significantly associated with metastatic tumors. Together, our findings demonstrate that RANK promotes tumor initiation, progression and metastasis in human mammary epithelial cells by increasing the population of CD44+CD24- cells, inducing stemness and epithelial mesenchymal transition. These results suggest that RANK expression in primary breast cancer associates with poor prognosis.
INTRODUCTION

RANKL and its receptor RANK, members of the TNF ligand and receptor super-family respectively, are key regulators of bone remodeling and metastasis (1), and mammary gland development (2). Impaired mammary gland development of the RANK- and RANKL-null mice is due to defective proliferation and increased apoptosis of mammary epithelium (2). Conversely, overexpression of RANK or RANKL in the mammary gland leads to increased proliferation of the mammary epithelia (3, 4). It has been postulated that paracrine signaling through RANK/RANKL is responsible for the expansion of mammary stem cell (MaSC) observed during pregnancy and luteal cycles (5, 6).

We and others have recently shown that activation of RANK signaling promotes mammary tumorigenesis in mice (4, 7, 8). MMTV-RANK transgenic mice are prone to mammary tumors (4, 7). Reciprocally, pharmacological inhibition of RANKL or genetic ablation of RANK attenuates mammary tumor development (7, 8).

Epithelial to mesenchymal transition (EMT) involves the loss of E-cadherin-mediated cell–cell adhesion and apical–basal polarity, concomitantly with the acquisition of a motile behavior contributing to invasion and metastasis (9). Induction of EMT in immortalized human mammary epithelial cells also results in the expression of stem cell markers (10), suggesting that these two processes may be functionally linked. Since rodent studies have demonstrated that activation of RANK signaling promotes tumor initiation, progression and metastasis involving mechanisms including increased proliferation, survival (7, 8) and enhanced regenerative capacity of cancer stem cells (8), then we analyzed the contribution of the RANK pathway to human breast stem cells and EMT using a panel of normal and tumoral human mammary epithelial cells. Our results highlight the relevance of the RANK pathway in human mammary stem cells and breast cancer.
MATERIALS AND METHODS

Culture of human mammary epithelial cells.

All cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD), except UACC3199 which was obtained from the Arizona Cancer Center (Tucson, AZ). ATCC provides molecular authentication in support of their collection through their genomics, immunology, and proteomic cores, as described, by using DNA barcoding and species identification, quantitative gene expression, and transcriptomic analyses [ATCC Bulletin, 2010]. UACC3199 cells harbor a methylated BRCA1 promoter suppressing gene transcription (11). UACC3199 was authenticated by its ability to re-express BRCA1 after DNA demethylation treatment with 5-aza-2'-deoxycytidine. All lines were expanded and frozen within 2 weeks of purchase and used for a maximum of 2 months after resuscitation of frozen aliquots. MCF10A and HMECs immortalized with telomerase were cultured as described in (12). Other cell culture conditions are described in SM.

Lentiviral infection.

Lentiviral infection using pLV409-RANK, pLV417-control that contain a luciferase reporter, or pLenti6/V5-DEST-RANK, pLenti6/V5-DEST-tubGFP was done following the manufacturer’s indications (Invitrogen) and SM.

Protein isolation and Western blot analysis.

To evaluate activation of RANK signaling, MDA-MB-436, HCC1937 and UACC3199 cells were starved (S) with 0% FBS for 48h, MCF10A cells were starved in 2% serum without EGF overnight, and then stimulated with hRANKL (100ng/ml; Amgen). Proteins were isolated and analyzed following standard procedures and antibodies (SM).
3D cultures and immunofluorescence analysis from human cells.

Acinar structures were cultured and stained as described (12) and SM. Confocal analysis was performed using a Leica confocal microscopy system equipped with argon and HeNe lasers. Images were captured using LasAF software (Leica).

Flow cytometry

Cells were seeded at 50% of confluence in growth medium. After 24h 100 ng/mL hRANKL (Amgen) were added. 48h later medium was removed, MCF10A cells were washed and stained as reported in SM and analyzed with the FACS Canto BD Flow Cytometer.

Xenograft in immunodeficient mice.

FL-RANK or parental MCF10A, MDA-MB-436 or UACC 3199 (3x 10⁶) cells mixed with matrigel (50%) were injected into the fat pad or (10⁶) in the tail vein of SCID/Beige (Charles River) or athymic nude (Harlan). After 110-160 days, MCF10A-injected mice were injected with 150 μg/g luciferin substrate and mammary glands and lungs were scored for bioluminescence and cell growth using (IVIS, xenogen). Outgrowths were scored in mammary glands that were efficiently cleared. Other mice were observed for palpable tumors or health deterioration once a week. Lungs were collected at indicated times, sectioned every 100μm and scored for metastasis. All mice were bred and maintained in a specific pathogen-free AAALAC International accredited facility with controlled light/dark cycle, temperature and humidity. Cages, bedding, food and water were all autoclaved. Experimental procedures were approved by the Bellvitge Biomedical Research Institute (IDIBELL) ethics committee and were in accordance with Spanish and European regulations.
Human tumor samples

Samples from breast cancer patients for mRNA analysis were collected from the University Hospital of Bellvitge (details in SM), using protocols approved by the IDIBELL ethics committee and according to Declaration of Helsinki. Samples were collected immediately after surgery and subsequently frozen or fixed for RNA extraction or IHC.

Samples from consented breast cancer patients for IHC analysis were ethically collected and procured through various human biospecimen providers (Asterand, Ardais, Bio-Options, Cytomyx Origene and Zoion). Samples were obtained from providers through the Amgen Tissue Bank (ATB) and IHC analysis done at Amgen.

Immunohistochemistry

Anti-human RANK or vimentin immunohistochemistry was performed on sections from formalin-fixed, paraffin-embedded specimens using anti-human RANK monoclonal antibodies (N-1H8 and N-2B10; Amgen) as described in (7) or anti-human vimentin.

Statistical Analyses

To evaluate whether RANK/RANKL mRNA levels discriminate metastastatic tumors we used Multivariate Analyses of Variance (MANOVA) considering N0=lymph node negative (n=25); Ni+= isolated tumor cells in nodes; isolated groups of epithelial cells less than 0.2mm in diameter, only visible by immunohistochemistry (n=7); Nmi= lymph node metastases over than 0.2 mm but less than 2mm in diameter (n=5); N1=lymph node positive or distant metastasis (n=26). Analyses was adjusted by tumor subtype (S):ER+PR+Her2- (n=39); ER+PR+Her2+ (n=5); ER-PR-Her2+ (n=8); ER-PR-Her2- (n=10). Logaritm transformation was required to avoid data hetercedasticity.
Plasmids, antibodies and other methods are included in Supplementary Methods (SM).
RESULTS

RANK overexpression induces EMT in non-tranformed human mammary epithelial cells.

In order to evaluate the impact of RANK overexpression in human mammary epithelial cells we obtained stable MCF10A cells, immortal but non transformed cells (12), expressing high levels of the full length hRANK receptor (FL-RANK) and a control vector (PARENTAL) (Fig 1A). FL-RANK expression levels were maintained during serial passages, whereas endogenous RANK in parental cells was low or undetectable by our methodology. RANKL expression was very low and similar in both genotypes (Fig. 1A, S1A). Functionality of the protein was demonstrated by phosphorylation of IkBα and the p65 subunit of NF-kB upon RANKL stimulation (Fig S1B). Increased basal levels of RANK downstream targets, P-IkBα, P-p38, P-Erk and P-Akt were observed in FL-RANK when compared to parental cells (Fig S1C), indicating that the pathway is constitutively active. Parental cells showed highly organized cell-cell adhesion with cobble-stone like appearance at confluence, whereas FL-RANK MCF10A cells had an elongated appearance and loss of cell-cell contacts with a spindle-like fibroblast morphology, suggesting that the cells may be undergoing EMT (Fig 1B). In monolayer cultures parental MCF10A cells show high levels of the epithelial protein E-cadherin, low levels of Vimentin and no expression of Fibronectin (mesenchymal proteins). In contrast, undetectable E-cadherin and strong staining of Vimentin and Fibronectin was observed in FL-RANK MCF10A cells, supporting an EMT phenotype (Fig 1C). Quantitative real time RT-PCR analyses confirmed changes observed for Fibronectin, Vimentin and E-cadherin (73- and 25-fold increase, 13-fold decrease, respectively) and increased N-cadherin expression in FL-RANK cells (Fig 1D). We observed a clear increase in mRNA levels of transcription factors known as repressors of E-cadherin promoter activity (13, 14) including, Snail (23-fold), Twist (3-fold), Zeb1 (6-fold), Zeb2 (10-fold) and Slug.
(2-fold) in FL-RANK MCF10A (Fig 1D). The increased expression in EMT related genes was confirmed using independently-transduced pools of RANK expressing MCF10A and also in a distinct, non-transformed breast cell line, HMECS immortalized with telomerase (Fig S2A-C). Treatment of FL-RANK MCF10A cells with hRANKL for 48h led to further increases in EMT genes such as Vimentin (1,4 fold) or Snail (7-fold), suggesting that RANKL stimulation further induces the EMT characteristics of the FL-RANK cells (Fig S2D). In summary, analyses of morphological and molecular changes indicate that RANK overexpression causes EMT in human mammary epithelial cells.

RANK overexpression induces stemness in non-transformed human mammary epithelial cells.

It has been shown that mammary cells undergoing EMT exhibit stem cell markers and properties of stem cells (10). We therefore looked at markers that have been related to stem cells and cancer stem cells in the human breast such as CD44, CD24, EpCAM, CD10, CD49f, CD133 (15-20). We found that most FL-RANK MCF10A cells (82,6 ± 2,9%) were CD44+CD24- (Fig 2A, S3A), compared with only 3,1 ± 0,15% parental cells. In correlation with their mesenchymal phenotype, most FL-RANK cells (99,6%) were negative for the luminal marker EpCAM, and 95,6% positive for the basal marker CD10, while parental cells were EpCAM+ (87,7%), CD10+ (64,4%), lower levels of CD49f and a slight increase in CD133 expression was observed in FL-RANK cells (Fig 2B and Fig S3A). Both phenotypes related with basal/stem like cells (16, 17, 20, 21). RANKL stimulation for 48h further enhanced the changes induced by RANK, including the increased frequency of CD44+CD24- (10% more) and CD10 cells, and the decreased EpCAM and CD49fhi (Fig S3B). FL-RANK cells also show significant higher levels of mRNA expression of SOX2, NANOG, and OCT4 (fold change 3x, 3x, 2x, respectively), transcription factors expressed.
in mammary stem cells and breast tumor cells (22) (Fig 2C). To evaluate the \textit{in vivo} stem cell activity we used the cleared fat pad transplant assay (23, 24). FL-RANK MCF10A cells, in contrast to parental, were detected in the fat pad four months after injection, as revealed by bioluminiscence analyses (Fig 2D); moreover, they formed small outgrowths demonstrating that, in correlation with their stem characteristics, FL-RANK MCF10A cells were able to repopulate the mammary epithelia \textit{in vivo} (Fig 2E,F; S3C). In summary, these results demonstrate that in human mammary cells RANK overexpression induces the expression of mammary stem cell markers and transcription factors and the ability to generate mammary outgrowths \textit{in vivo}, a function of stem cells.

\textbf{RANK overexpression increases migration and induces hallmarks of transformation in MCF10A cells.}

The main functional consequence of EMT is enhanced cell scattering and migration. Thus, in wound healing assays FL-RANK MCF10A cells migrated faster than the parental cells both in the presence or absence of EGF. RANKL significantly increased the motility of FL-RANK as compared to EGF, and the most motile cells were the FL-RANK cells treated with EGF plus RANKL (Fig. 3A).

We next asked whether RANK overexpression could induce transformation. MCF10A cells grown in matrigel with EGF, recapitulate several features of breast epithelium \textit{in vivo}, including the formation of acinus-like spheroids with a hollow lumen, apico-basal polarization and growth arrest (12, 25), whereas transformed mammary epithelial cells show a multiacinar phenotype, filled lumen and absence of proliferative arrest (25). In contrast to parental, FL-RANK MCF10A cells formed large and disrupted structures at 20 days of culture (Fig 3B). RANKL treatment resulted in aberrantly elongated tubular-like structures (day 2), that by day 20 looked like aggregates (Fig 3B). Confocal cross sections revealed in
FL-RANK cultures abnormal structures often composed of cells filling the luminal space and occasionally showing protrusions into the matrigel, characteristic of invasive cells (Fig 3C). In FL-RANK structures E-cadherin expression was hardly detected in the cell-cell contacts and decrease expression or mislocalization of the basal marker CD49f was often observed in FL-RANK cells indicating loss of polarization (Fig 3C). Unlike parental cells, FL-RANK acini/aggregates were not growth arrested after 20 days of culture as demonstrated by the presence of ki67 and activated caspase 3 (Fig S4A). Moreover, FL-RANK overexpressing cells cultured with RANKL formed acini with filled lumens in the absence of EGF whereas parental cells were unable to grow in these conditions (Fig S4B). Consistent with their non transformed phenotype, parental MCF10A cells failed to grow in soft agar whereas FL-RANK MCF10A cells formed numerous foci (Fig 3D) indicating that RANK expression confers the cells the ability to grow in the absence of anchorage, a characteristic of tumor or stem cells. We conclude that RANK overexpression in human mammary epithelial cells results in hallmarks of transformation, including, increased motility, inability to respond to growth arrest signals, impaired polarization, luminal filling, and growth in soft agar. Despite in vitro observations consistent with the RANK-dependent transformation of MCF10A cells, these cells although able to grow in vivo (Fig 2D, E) were non tumorigenic when injected in the cleared fat pad of immunodeficient mice and did not form lung metastasis when injected in the tail vein (Fig S4C).

RANK overexpression increases the frequency of CD44+CD24- cells in BRCA1 deficient cell lines.

We aim to evaluate whether RANK can cooperate with other mutations relevant in breast cancer such as BRCA1 deficiency (26) during tumorigenesis and metastasis. We therefore overexpressed RANK in breast cancer cells defective for BRCA1, MDA-MB-436,
HCC1937, and UACC3199 cells (27), and confirmed its functionality (Fig. 4A and S5A,B). RANK expression in the parental lines was low or undetectable by our methods; RANKL expression was low and comparable between both phenotypes (Fig 4A, S5A;). As in MCF10A cells, RANK overexpression increased the frequency of CD44+CD24- cells in all three cell lines due to reduced CD24 levels as compared to parental (Fig. 4B, S5C,D); a further reduction in CD24 expression was observed after RANKL stimulation in most cells (Fig S5C). Lower frequency of EpCAM+ (13% reduction) and CD49fhi (17% reduction) cells in FL-RANK MDA-MB-436, and higher of CD10+ in UACC3199-FL-RANK, as compared to the correspondent parental lines was observed (Fig S5C,D). Consistent with their mesenchymal morphology, MDA-MB-436 cells express fibronectin, vimentin, N-cadherin, and the transcription factors, Snail, Twist, Zeb1 and Zeb2 and even higher levels upon RANK overexpression (Fig 4C, S5E). In FL-RANK HCC1937 and UACC3199 the expression levels of EMT mesenchymal markers or transcription factors, and the frequency of EpCAM+ was similar to the corresponding parental lines (Fig 4C, S5D,E). These results demonstrate that RANK induces CD44+CD24- phenotype in breast cancer cell lines concomitantly with or separately from EMT.

**RANK overexpression increases invasiveness and promotes tumorigenesis and metastasis in BRCA1 deficient cell lines.**

We next investigated the functional consequences of RANK overexpression in BRCA1-defective breast cancer cell lines. Monolayer cultures revealed a modest increase in growth of the three FL-RANK BRCA1 defective cell lines (Fig S6A). Protusions into the matrigel, indicative of an invasive phenotype were often observed in MDA-MB-436 FL-RANK acini but not in parental and even more prominent in the presence of RANKL (Fig S6B). Quantification of proliferation in acinar cultures revealed a small increase upon RANK
overexpression in some cell lines. FL-RANK cells showed lower levels of apoptosis when cultured in the presence of RANKL (Fig S6C).

To investigate whether RANK expression in BRCA1-defective cells promotes tumorigenesis and metastasis in vivo, we used the MDA-MB-436 cells where EMT was enhanced upon RANK overexpression and UACC3199 cells where RANK increased the frequency of CD44+CD24- cells but no EMT was observed. After injection in the mammary fat pad, FL-RANK MDA-MB-436 and UACC3199 cells gave rise to tumors with faster growth and/or a shorter latency than parental cells (Fig. 5A,B). We next determined whether RANK overexpression would enhance the metastatic properties of these cells. 6.5 weeks after tail vein injection frequency and size of lung metastasis were higher in mice inoculated with FL-RANK cells as compared to parental MDA-MB-436 cells (Fig. 5C). By 12 weeks metastatic lesions in mice injected with FL-RANK MDA-MB-436 cells have colonized most of the lung area as compared to smaller metastatic foci formed by parental cells (Fig. 5D; S6D). UACC3199 cells were poorly metastatic as compared to MDA-MB-436, in correlation with the absence of EMT in these cells. Micrometastases were observed in 50% mice injected with FL-RANK UACC3199 cells (up to 56 metastatic foci), and in 33% (n=6) mice injected with the parental UACC3199 (up to 3 lesions per mouse) (Fig S6D). In summary, RANK overexpression enhances tumorigenesis and metastasis in BRCA1 deficient cells.

High RANK/RANKL expression levels are found in aggressive and metastatic adenocarcinomas.

To investigate the potential clinical relevance of RANK pathway in human breast cancer we analyzed RANK and RANKL mRNA expression levels in adenocarcinomas considering their pathological characteristics. We found significantly higher levels of mRNA RANK in human tumors that lack expression of the hormonal receptors for estrogen and progesterone.
(ER and PR) as compared to (ER+PR+) tumors that usually bear a better prognosis, and in tumors with high proliferation index (more than 40% of ki67+ cells) and high pathological grade (Fig 6A). Similar epithelial and leucocyte content was found within all groups (Fig S7A). To confirm the mRNA results, we analyzed RANK protein expression by IHC in a second collection of breast carcinomas. Consistent with our previous study (7), RANK protein expression in found within the carcinoma element of some tumors and a subset of infiltrating macrophages but is not observed on stromal cells, fiboblasts or lymphocytes (Fig 6B). A higher incidence of RANK protein expression was observed in ER-/PR- (50%) vs ER+/PR+ (18%), and in grade III (32%) vs. grade I (13%) or grade II (18%) (Fig 6C).

Our results with cell lines indicate that activation of RANK pathway enhances migration and metastasis. In human breast adenocarcinomas using a multivariant anova analyses we found that RANK/RANKL mRNA expression levels were able to discriminate between non metastatic (N0) and metastatic tumors (N1) (either to the lymph nodel or other sites) (Fig 6D; Fig S7B-E). These results indicate that high RANK expression levels correlate with tumor aggressiveness and that RANK/RANKL expression in the primary tumor may indicate its metastatic behaviour.
DISCUSSION

The RANK/RANKL signaling pathway, although dispensable for the initial development of the mammary gland in mice (2), controls the expansion of the stem cell compartment in adults (5, 6). Similarly, it has been recently shown that RANK is expressed in several stem cells of the skin, and that RANK signaling activate the hair cycle and epidermal growth (28). The mechanism responsible of this RANK-induced expansion of stem cells remains unknown. We show that RANK overexpression in human MCF10A cells results in constitutive activation of the pathway demonstrated by the increased levels of RANK downstream targets (P-IκBα, P-p38, P-Akt and P-Erk). It is well established that overexpression of any TNF receptor family member leads to ligand-independent receptor oligomerization and ligand-independent activation of signal transduction pathways (29-32). RANK overexpression generates a EpCAM-/CD10+/CD49f lo phenotype, markers used to identify populations enriched in mammary stem cells in the healthy breast (15-17) and provides the MCF10A with the ability to reconstitute the cleared fat pad of immunodeficient mice in contrast to parental MCF10A that are unable to grow in vivo (33). Moreover, RANK overexpression increases the frequency of CD44+CD24lo/- , a phenotype ascribed to human breast cancer stem cells, in MCF10A and in breast cancer cell lines with non functional BRCA1. Plasticity of breast cancer cells with respect to CD24, CD44, EpCAM, CD49f has been reported (18, 34, 35). Although we cannot rule out that RANK is promoting proliferation or survival of stem cells, the rapid acquisition of stem markers by the entire MCF10A population upon RANK overexpression suggests that activation of RANK signaling may induce a de-differentiation process.

An association between RANK expression and EMT has been reported in prostate cancer (36). Here we demonstrate that RANK overexpression directly induces a strong EMT phenotype in MCF10A cells (and HMECs). EMT induced by twist/snail generates cells with
cancer stem cell properties (10), and usually CD44+CD24- cells have a mesenchymal phenotype as compared to the luminal phenotype of CD24+ cells, leading to the believe than CSC and EMT are interchangeable events. However, despite the gain in CD44+CD24- phenotype induced by RANK in all BRCA1-deficient cell lines, an increase in the mesenchymal markers is observed only in MDA-MB-436 cells, similarly to results reported with urokinase receptor signaling (37). Thus, RANK overexpression induces CD44+CD24- phenotype in non transformed mammary epithelial cells and in breast cancer cell lines that can be accompanied by EMT depending on the cell of origin.

We have previously shown that RANK overexpression promotes mammary tumorigenesis in mice (7) but the relevance of this pathway in human breast disease remained largely unknown, except for a SNP in the locus of the TNFRSF11A gene (RANK), that associates with breast cancer risk (38). Now we show that in non-transformed mammary epithelial cells RANK expression enhances migration, disrupts acinar formation and allows growth in soft agar, a classical transformation assay. Changes observed in FL-RANK MCF10A acini are consistent with those described in mouse MMTV-RANK MECs (4, 7) and may reflect an imbalance response between proliferation and apoptosis, that together with the impaired polarization results in increased organoid size and luminal filling. Despite these changes associated with transformation, FL-RANK MCF10A cells do not form tumors or metastasis when injected in immunodeficient mice. Similar results have been observed with several oncogenes, such as ErbB2/neu that are able to disrupt acinus formation and transform MCF10A or HMLE in vitro (39) but fail to convey tumor growth as xenografts (10, 40). It has been shown that RANK signaling induces migration and bone metastasis in human breast cancer lines (41). Here, we provide a putative mechanism by which RANK promotes tumorigenesis, migration and metastasis. The enhanced motility and ability to seed tumors in other locations provided by the CD44+CD24- phenotype in cells with non functional
BRCA1, together with the EMT phenotype when existing, indicates that metastasis may not be restricted to the bone. In fact, pharmacological inhibition of RANKL or genetic ablation of RANK significantly reduced the incidence and multiplicity of lung metastasis in MMTV-neu mice (7, 42). These findings demonstrate that RANK overexpression in human breast cancer cells increases their aggressiveness and may result in poorer clinical outcome, as recently shown (43). In fact, we observe that RANK expression progressively increases with pathological grade and significantly associates with a high proliferative index in clinical samples of breast cancer patients. Higher levels of RANK mRNA expression were found in (ER- PR-) tumors which was consistent with the higher incidence of RANK protein expression in these tumors. ER-PR- tumors are more aggressive, show a higher incidence of metastasis and worse prognosis than luminal tumors and contain a higher frequency of CD44+/CD24- cells (21, 44). Moreover, RANK/RANKL mRNA expression levels allow discrimination between metastatic and non metastatic adenocarcinomas.

In summary, we demonstrate that RANK overexpression in human mammary epithelial cells generates a "stem" phenotype in non transformed basal human mammary epithelial cells and human breast cancer cells that can be accompanied by EMT. Our results highlight the relevance of RANK signaling pathway in human mammary tumorigenesis, and human mammary stem cells in correlation with the results previously obtained in mice and suggest that RANK promotes tumor initiation, progression and metastasis in breast cancer.
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FIGURE LEGENDS

Figure 1. RANK overexpression induces EMT in MCF10A.
A. Western blot showing RANK expression in MCF10A cells infected with lentivirus containing FL-RANK and control vectors (PARENTAL).
B-C. Pictures showing representative morphology (B) and immunofluorescence analyses of epithelial and mesenchymal proteins (C) of FL-RANK and parental MCF10A cells.
D. mRNA expression levels of EMT proteins and transcription factors in FL-RANK and parental MCF10A cells measured by qRT-PCR relative to PP1A. Determinations were done in triplicate and standard deviations are shown.

Figure 2. RANK overexpression induces stemness in MCF10A.
A-B. Flow cytometry analyses (A dot plots; B histograms) showing expression of indicated proteins in FL-RANK and parental MCF10A. Numbers indicate (A) the frequency of CD44+/CD24- cells or (B) the frequency of positive cells based on the negative population (in bold) or the mean of the histograms (in italic).
C. qRT-PCR showing the expression of Sox2, Nanog and Oct4 relative to PP1A in FL-RANK and parental MCF10A. Determinations were done in triplicate and standard deviations are shown.
D. Bioluminescence (BLI) images of mammary glands of scid/beige mice 4 months after injection with FL-RANK or parental MCF10A cells.
E. Schematic representation of engraftment. Each circle represents one mammary gland with the percentages of engraftment.
F. Representative image of outgrowth (top panel). Human specific staining (vimentin) confirmed the human origin of the FL-RANK outgrowths (bottom panel).
Figure 3. FL-RANK MCF10A cells show increased migration and hallmarks of transformation.

A. Wound healing assays: Parental and FL-RANK MCF10A cells were EGF-starved overnight and then stimulated with EGF (20 ng/ml), RANKL (100 ng/ml) or both. Quantification of the distance invaded at different time points is shown. Assay was done in triplicates and SD and p-values for indicated comparisons are included. One representative experiment out of 5 is shown.

B-C. Representative phase contrast (B) and confocal pictures (C) with magnified insets of FL-RANK and parental acini at the indicated days (cyan=nuclei). Data is representative of 3 independent experiments.

D. Anchorage independence assay. Foci number in FL-RANK or parental cells after 4 weeks of culture in soft agar are represented for 3 independent experiments.

Figure 4. RANK overexpression increases the frequency of the CD44+ CD24-population in cells with non functional BRCA1.

A. Western blot showing RANK protein expression in MDA-MB-436, HCC1937 and UACC3199 cells infected with lentivirus expressing FL-RANK or a control vector (PARENTAL).

B. Frequency of CD44+/CD24- cells in parental and FL-RANK non-functional BRCA1 cells analyzed by flow cytometry. Values for one representative experiment out of 4 are shown.

C. qRT-PCR showing mRNA expression of the indicated genes in parental and FL-RANK cells with non functional BRCA1 relative to PP1A. Determinations were done in triplicate and standard deviations are shown.
Figure 5. RANK overexpression promotes tumorigenesis and metastasis population in cells with non functional BRCA1.

A-B. FL-RANK and parental MDA-MB-436 (1x 10^6) (A) or UACC3199 (3x10^6) cells (B) were implanted in the fat pad of immunodeficient mice. Tumor volume \( \frac{\text{length}\times\text{width}\times\text{height}}{6} \) in mm\(^3\) is shown at the indicated days after implantation, SEM and p values are included for one representative experiment out of 2.

C. Number of metastatic lesions 6.5 weeks after tail vein injection of 1x10^6 FL-RANK or parental MDA-MB-436 for one representative experiment out of 2. Total number of metastatic foci in 3 representative sections was quantified. Each bar represents one mouse.

D. Representative H&E pictures of lung metastasis formed by FL-RANK and parental MDA-MB-436 cells 12 weeks after tail vein injection.

Figure 6. Aggressive and metastatic adenocarcinomas of the human breast express high levels of RANK/RANKL

A. Box and whiskers graph showing expression levels of RANK mRNA relative to PP1A in clinical samples of breast cancer patients measured by qRT-PCR. Determinations for each sample were done in triplicates and means are used. Samples were classified by expression of ER, PR, ki67, and pathological grade (p values are indicated).

B. hRANK protein expression (determined by IHC) in ER-PR- human adenocarcinomas, showing a representative tumor with RANK positive epithelial carcinoma cells (left panel) and a tumor in which the epithelial carcinoma cells do not express RANK (right panel). RANK protein was also detected on a subset of infiltrating macrophages in both tumor types.

C. Frequency of RANK protein expression (determined by IHC) in epithelial carcinoma cells in tumors classified by ER, PR expression and pathological grade (gr). The degree of
RANK-positive macrophage infiltration was not substantially different between cohorts (73% for ER-PR- and 82% for ER+PR+). p values for chi square test are indicated.

D. MANOVA test of logRANK/logRANKL expression and metastasis. N0= lymph node negative; N1=lymph node positive or distant metastasis.
Figure 1

A

B

C

VIMENTIN

FIBRONECTIN

E-CADHERIN

200μm

FL-RANK

PARENTAL

FL-RANK

PARENTAL

100μm

D

FIBRONECTIN, VIMENTIN, N-CADHERIN, E-CADHERIN

Relative expression (Corrected 2^ΔΔCT)

SNAIL, TWIST, ZEB 1, ZEB 2, SLUG

FL-RANK □ PARENTAL
Figure 2

A

B

C

D

E

F
Figure 3

A

B

C

D

E-CADHERIN

CD49f

0 5 10 15 20 25 30 35 40 45

p<0.001

p=0.0018

p=0.001

0

1

2

3

4

5

6

7

8

FL-RANK

FL-RANK+RL

PARENTAL

200 μm

p<0.0001

p<0.0001

p<0.0001

PARENTAL

FL-RANK

FL-RANK+RL

PARENTAL

3x

3x

3x

3x

3x

3x

3x

50 μm

no. of foci per well

120

100

80

60

40

20

0

FL-RANK

PARENTAL

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Research.
Figure 4

A) Western blot analysis of hRANK and Tubulin in FL-RANK PARENTAL cells. 


C) Relative expression of FIBRONECTIN, E-CADHERIN, and SNAIL in FL-RANK PARENTAL cells.
Figure 5

A. MDA-MB-436

- FL-RANK n=8
- PARENTAL n=8

B. UACC3199

- FL-RANK n=8
- PARENTAL n=8

C. Number of metastasis

- Big (≥100 cells)
- Medium (15-100 cells)
- Small (≤15 cells)

D. MDA-MB-436

- FL-RANK
- PARENTAL
Figure 6

A) Relative expression of RANK in different groups. 

B) Histological images showing expression of RANK.

C) Frequency distribution of RANK expression in different groups.

D) Log(RANK) vs Log(RANKL) by N (MANOVA)

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Marta Palafox, Irene Ferrer, Pasquale Pellegrini, et al.

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