Tumor and Stem Cell Biology

Cancer Research

RANK Signaling Blockade Reduces Breast Cancer Recurrence by Inducing Tumor Cell Differentiation

Guillermo Yoldí1, Pasquale Pellegrini1, Eva M. Trinidad1, Alex Cordero1, Jorge Gomez-Miragaya1, Jordi Serra-Musach2, William C. Dougall3, Purificación Muñoz1, Miguel-Angel Pujana2, Lourdes Planelles4, and Eva González-Suárez1

Abstract

RANK expression is associated with poor prognosis in breast cancer even though its therapeutic potential remains unknown. RANKL and its receptor RANK are downstream effectors of the progesterone signaling pathway. However, RANK expression is enriched in hormone receptor negative adenocarcinomas, suggesting additional roles for RANK signaling beyond its hormone-dependent function. Here, to explore the role of RANK signaling once tumors have developed, we use the mouse mammary tumor virus-Polyoma Middle T (MMTV-PyMT), which mimics in human breast adenocarcinomas. Complementary genetic and pharmacologic approaches demonstrate that therapeutic inhibition of RANK signaling drastically reduces the cancer stem cell pool, decreases tumor and metastasis initiation, and enhances sensitivity to chemotherapy. Mechanistically, genome-wide expression analyses show that anti-RANKL therapy promotes lactogenic differentiation of tumor cells. Moreover, RANK signaling in tumor cells negatively regulates the expression of Ap2 transcription factors, and enhances the Wnt agonist Rspo1 and the Sca1-population, enriched in tumor-initiating cells. In addition, we found that expression of TFAP2B and the RANK inhibitor, OPG, in human breast cancer correlate and are associated with relapse-free tumors. These results support the use of RANKL inhibitors to reduce recurrence and metastasis in breast cancer patients based on its ability to induce tumor cell differentiation. Cancer Res; 76(19); 5857-69. ©2016 American Association for Cancer Research.

Introduction

Multiple lines of evidence support the existence of tumor-initiating cells (TICs) or cancer stem cells (CSC) in breast cancer (1). Recent efforts to develop CSC-related therapies explored elimination of the CSC population, removal of their self-renewal capability, and forced terminal differentiation. The first differentiaton agent successfully used in the clinic was all-trans retinoic acid in acute promyelocytic leukemia (2). Retinoid signaling also regulates breast CSC self renewal and differentiation (3).

RANK ligand (RANKL) is expressed in progesterone receptor-positive (PR+) mammary epithelial cells and acts as a paracrine mediator of progesterone in mouse and human mammary epithelia (4–9). Overexpression of RANKL's receptor, RANK in mammary epithelial cells enhances proliferation, impairs lactation, and induces the accumulation of mammary stem cells (MaSC) and progenitors (9–12). In human adenocarcinomas, RANK is predominantly expressed in hormone receptor-negative (HR−) tumors, supporting a progesterone-independent role. In contrast to RANK, RANKL is rarely expressed on tumor cells, but it is expressed in tumor-infiltrating lymphocytes (7, 11, 13). RANK expression in human adenocarcinomas is associated with reduced overall survival (13, 14). However, the mechanisms underlying these aggressive tumor phenotypes and the therapeutic potential of RANKL inhibition once tumors have developed remain unexplored.

The MMTV-PyMT breast cancer mouse model displays widespread transformation of the mammary gland and a high incidence of lung metastasis (15, 16). Tumor cells of invasive PyMT adenocarcinomas do not express hormone receptors or RANKL, but do express high levels of RANK (7, 9). RANKL inhibitors are currently used for the treatment of bone-related pathologies, osteoporosis, and bone metastasis. Here we demonstrate that inhibition of RANK signaling acts as a differentiation therapy in breast cancer, depleting the cancer stem cell population and reducing recurrence and metastasis.

1Cancer Epigenetics and Biology Program, Bellvitge Biomedical Research Institute, IDIBELL, Barcelona, Spain. 2Program Against Cancer Therapeutic Resistance (ProCURE), Breast Cancer and Systems Biology Lab, Catalan Institute of Oncology, IDIBELL, Barcelona, Spain. 3Therapeutic Innovation Unit, Amgen Inc., Seattle, Washington. 4Centro Nacional de Biotecnología/CSIC, UAM Cantoblanco, Madrid, Spain.

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G. Yoldí, P. Pellegrini, and E.M. Trinidad contributed equally to this work.

Current address for P. Pellegrini: Gladstone Institutes, San Francisco, California; current address for W.C. Dougall: Department of Immunology in Cancer and Infection, QIMR Berghofer Medical Research Institute, Herston, QLD, Australia; current address for A. Cordero: Department of Neurological Surgery Feinberg School of Medicine, Northwestern University Chicago, IL.

Corresponding Author: Eva González-Suárez, Bellvitge Biomedical Research Institute (IDIBELL), Av. Gran Via de L’Hospitalet, 199-203, L’Hospitalet de Llobregat, Barcelona 08908, Spain. Phone: 932607347; Fax: 932607219; E-mail: egsuarez@idibell.cat
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Materials and Methods

Animals, RANKL, RANK-Fc, and docetaxel treatments

All research involving animals was performed at the IDIBELL animal facility in compliance with protocols approved by the IDIBELL Committee on Animal Care and following National and European Union regulations. MMTV-PyMT (FVB/N-Tg(MMTV-PyVT)634Mul) were obtained from the Jackson Laboratory (15). MMTV-PyMT−/−;RANK−/− mice were obtained by backcrossing the MMTV-PyMT (FvB/N) strain with RANK+/− mice into the C57BL/6 background (17). RANKL (1 mg/kg, Amgen) and RANK-Fc (10 mg/kg, Amgen) were injected subcutaneously three times a week (7, 10). Docetaxel (Actavis) was administered at 25 mg/kg intraperitoneally twice per week.

Figure 1.

RANKL decreases MMTV-PyMT tumor cell differentiation. A, representative images of RANK and RANKL protein expression. Note that in carcinomas, RANKL is not expressed in tumor cells but in tumor-infiltrating lymphocytes. B, Rank and Rankl mRNA expression relative to K8 or β-actin in seven WT, eight MMTV-PyMT tumors, and three draining lymph nodes of MMTV-PyMT tumor-bearing mice. C, Rank and Rankl mRNA expression relative to Rpl38 in FACS-sorted (Supplementary Fig. S2) tumor cells (TUM) CD45−, macrophages (TAM) CD45+, CD11b+, F4/80+, Gr1−, CD4+, and CD8+ T lymphocytes (CD45+CD11b−CD3+CD4+ or CD8+) from four MMTV-PyMT tumors. D, schematic overview of short-term (2 weeks) RANKL treatment in MMTV-PyMT tumor-bearing females. E, tumor volume normalized to the first day of treatment of five MMTV-PyMT tumor-bearing mice undergoing RANKL treatment and controls. F, percentage of secretory areas relative to total tumor area in (3–5) MMTV-PyMT primary tumors after RANKL treatment. G, representative images of hematoxylin and eosiin and milk protein staining in MMTV-PyMT primary tumors. B, E, F, mean, SEM, and t test probabilities are shown (*, 0.01 < P < 0.05; **, 0.001 < P < 0.01; ***, 0.0001 < P < 0.0001).
Tumor cell isolation, tumor and metastasis initiation assays

Tumor cells were isolated as described (18). For orthotopic transplants and tumor-limiting dilution assays (LDA), tumor cells were mixed 1:1 with Matrigel matrix (BD Biosciences) and orthotopically implanted in the inguinal mammary gland of 6- to 10-week-old syngeneic females. For metastasis assays, tumor cells resuspended in cold PBS were injected intravenously in 6- to 10-week-old Foxn1nu females.

Tissue histology and immunostaining

Tissue samples were fixed in formalin and embedded in paraffin. Three-micrometer sections were cut for histologic analysis and stained with hematoxylin and eosin. Entire lungs were step sectioned at 100 μm and 15 cuts per lung were quantified. Antigen heat retrieval with citrate was used for PR (DAKO), SMA-1 (Sigma-Aldrich), mRANKL (R&D Systems), KI67 (Thermo Scientific), cleaved caspase-3 (Cell Signaling) antibodies, and rabbit antimilk serum (kindly provided by Prof. Nancy E. Hynes). mRANK (R&D Systems) immunostaining was performed, pretreating sections with Protease XXIV 5 U/mL (Sigma-Aldrich), mRANKL (R&D Systems), Ki67 (Thermo Scientific), cleaved caspase-3 (Cell Signaling) antibodies, and rabbit antimilk serum (kindly provided by Prof. Nancy E. Hynes). mRANK (R&D Systems) immunostaining was performed, pretreating sections with Protease XXIV 5 U/mL (Sigma-Aldrich) for 15 minutes at room temperature. All antibodies were incubated overnight at 4°C, detected with biotinylated secondary antibodies and streptavidin horseradish peroxidase (Vector), and revealed with DAB substrate (DAKO).

Figure 2.

Inhibition of RANK signaling depletes the pool of MMTV-PyMT TICs. A, schematic overview of RANK-Fc treatments in orthotopic MMTV-PyMT tumors. One million cells isolated from one MMTV-PyMT carcinoma were orthotopically injected into syngeneic WT mice (FVB), which were randomized 1:1 for RANK-Fc (10 mg/kg, 3 times per week, 4 weeks) or mock treatment starting 24 h later (passage 1). Cells isolated from three tumors from each treatment arm were pooled and orthotopically injected into WT (passage 2) mice in limiting dilutions and randomized 1:1 for additional RANK-Fc or mock treatment (2 weeks). Total number of tumors was scored after 26 weeks. B, tumor growth of passage 1 tumors. C, percentage of positive cleaved caspase-3 cells in passage 2 tumors. D, TIC frequencies (with confidence intervals), χ² values, and associated probabilities. E, number of secondary tumorspheres formed by RANK-Fc-treated MMTV-PyMT tumors. Each bar represents data from four tumors plated in triplicates. B, C, E, mean, SEM, and t test statistics are shown (*, 0.01 < P < 0.05).
Tumorsphere culture

Cells isolated from primary tumors were resuspended in serum-free DMEM F12 mammosphere medium containing 20 ng/mL of EFG, 1× B27, and 4 μg/mL heparin (Sigma-Aldrich), as previously described (19) with 2% of growth factor–reduced matrigel. Primary tumorspheres were derived by plating 20,000 cells/mL in 2 mL of medium onto cell-suspension culture plates. After 14 days, tumorspheres were isolated by 5 min treatment with PBS-EDTA 1 mmol/L and 5 min of trypsin at 37°C and plated for secondary tumorsphere formation at a concentration of 5,000 cells/mL in triplicate. Individual spheres from each replicate well were counted under a microscope.

Flow cytometry

Single cells were resuspended and blocked with PBS 2% FBS and IgG blocking reagent for 10 min on ice and incubated for 30 min on ice with CD45-APC-Cy7 (30-F11), CD4-PE-Cy7 (RM4-5), CD11b-APC (M1/70), CD8-PE or CD8-FITC (53-6.7), Gr1-FITC (RB6-8C5), F4/80-PE (BM8), CD49b-Alexa 647 (1HMa2), CD45-PECy7 or –APCCy7 (30-F11), and CD31-PECy7 (390), all from Biolegend, CD24-FITC (M1/69), CD61-FITC (2C9.G2), Sca-1-APC (Ly-6A/E) from BD Pharmingen, CD90-PE (HIS51; Bioscience) and CD49f-AF647 (GoH3; R&D Systems). FACS analysis was performed using FACS Canto, FACS Aria (Becton Dickinson) and Diva software. Cells were sorted using MoFlo (Beckman Coulter) at 25 psi (172 kPa) and a 100-μm tip.

Figure 3.

RANKL inhibition induces differentiation of tumor cells into milk secreting cells. A, expression profile in mammary gland development of differentially expressed genes between RANK-FC-treated tumors and controls (21). Genes further validated by reverse transcription-PCR are shown in red. B, GSEA graphical output for the association between lactation overexpressed genes and RANK-Fc treatment. The top genes contributing to this association are listed. C, mRNA expression levels of indicated genes relative to HPRT. Each bar is representative of three tumors. D, representative images of milk staining in RANK-Fc-treated tumors. E, fold change of mRNA expression levels of indicated genes in RANKL-treated acinar cultures of MMTV-PyMT tumor cells relative to untreated controls. Three tumors were analyzed. B and E, mean, SEM, and t test P values are shown (*, 0.01 < P < 0.05).
RNA labeling and hybridization to Agilent microarrays
Hybridization to SurePrint G3 Mouse Gene Expression Microarray (ID G4852A; Agilent Technologies) was conducted following manufacturer’s protocol (Two-Color Microarray-Based Gene Expression Analysis v. 6.5; Agilent Technologies), and dye swaps (Cy3 and Cy5) were performed for RNA amplified from each sample. Microarray chips were washed and scanned using a DNA Microarray Scanner (Model G2505C; Agilent Technologies).

Microarray analysis
Microarray data were feature extracted using Feature Extraction Software (v. 10.7) available from Agilent, using the default variables. Outlier features on the arrays were flagged by the same software package. Data analysis was performed using Bioconductor package, under R environment. Data preprocessing and differential expression analysis was performed using limma and RankProd package, and latest gene annotations available was used. Raw feature intensities were background corrected using normexp background correction algorithm. Within-array normalization was done using spatial and intensity-dependent loess. Aquantile normalization was used to normalize between arrays. The expression of each gene is reported as the base 2 logarithm of ratio of the value obtained of each condition relative to controls. A gene is considered differentially expressed if it displays a pfp (proportion of false positives) less than 0.05 by nonparametric test. The GSEA was run using default values for all parameters. Raw microarray data has been deposited in GEO, access number GSE66085. The mature luminal and stem cell gene sets were taken from the original publication (20). The differentially expressed genes between lactation and pregnancy were identified using the GEO GSE8191 dataset (21) and the TFAP2C-regulated genes in breast cancer cells using the GEO GSE8640 dataset (22).

Statistical analysis
Differences were analyzed with a two-tailed Student t test or an F test, one sample t test against a reference value of 1. Two-way analysis of variance was used to compare tumor growth curves. The Mantel–Cox test was used for tumor-free survival studies. Frequency of tumor initiating was estimated using the extreme limiting dilution software (ELDA; ref. 23). The statistical significance of difference between groups is indicated by asterisks (*, 0.01 < P < 0.05; **, 0.001 < P < 0.01; ***, 0.001 < P < 0.0001; ****, P < 0.0001).

Results
RANKL stimulation promotes tumor growth in MMTV-PyMT primary tumor cells
MMTV-PyMT preneoplastic lesions and adenocarcinomas expressed high levels of RANK (Fig. 1A and B). RANKL expression

Figure 4.
Constitutive deletion of RANK increases tumor latency, decreases tumor incidence, and prevents lung metastasis of MMTV-PyMT tumors. A, kinetics of palpable tumor onset with age in 18 PyMT;RANK+/+ and 10 PyMT;RANK−/− mice. Log-rank test (***, P < 0.0001). B, number of palpable lesions detected at necropsy in 17 PyMT;RANK+/+ and 10 PyMT;RANK−/− mice. C, number of preneoplastic regions per mammary gland detected in mammary whole mounts of 9 PyMT;RANK+/+ and 5 PyMT;RANK−/− mice 15 to 22 weeks old. Each dot represents one mammary gland. D, percentage of PyMT;RANK+/+ (n = 6) and PyMT;RANK−/− (n = 7) mice with lung metastasis. The total number of metastatic foci per mouse is indicated. χ² = 6.96, as calculated by contingency 2 × 2, P = 0.01. B and C, mean, SEM, and t test P values are shown (*, 0.01 < P < 0.05).
**A**

LATENCY (days)

- **RANK+/+ Tumor in WT host**
- **RANK–/– Tumor in WT host**
- **RANK–/– Tumor in RANK–/– host**
- **RANK+/+ Tumor in RANK–/– host**

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**B**

![Image](image.png)

- **RANK+/+ Tumor in WT**
- **RANK–/– Tumor in WT**

50 μm

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**C**

Non-viable areas

- % Nervous areas

- % Caspase-3/nuclei

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**D**

Docetaxel response

- **RANK+/+ in WT**
- **RANK–/– in WT**
- **RANK+/+ in WT docetaxel**
- **RANK–/– in WT docetaxel**

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**E**

Tumorspheres

- **RANK+/+ Tumor in WT host**
- **RANK–/– Tumor in WT host**
- **RANK+/+ Tumor in RANK–/– host**
- **RANK–/– Tumor in RANK–/– host**

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**F**

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**H**

- **N of metastatic foci**
- **RANK+/+**
- **RANK–/–**

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Inhibiting RANKL signaling decreases the frequency of tumor-initiating cells

No significant changes in tumor growth, tumor cell proliferation, or apoptosis were observed after 2 weeks of RANKL treatment in vivo in tumor-bearing MMTV-PyMT mice (Fig. 1D–E and Supplementary Fig. S3E; ref. 10), but tumor cell density was higher in RANK-deleted lesions, in contrast to control mice, where extensive areas of dilated ducts and hyperplasias full of milk secretions were observed (Fig. 1F–G). These results indicate that short-term in vivo activation of RANK signaling is not sufficient to change the growth of established tumors, but appears to prevent secretory differentiation of tumor cells.

The putative benefit of pharmacologic RANKL inhibition with RANK-Fc in tumor recurrence was interrogated (Fig. 2A; ref. 7). No significant differences in tumor growth or the frequency of apoptotic cells were observed after RANK-Fc treatment (Fig. 2B and C). However, LDAs revealed that tumor cells pretreated with RANK-Fc at passage 1 showed a 10-fold decrease in tumor-initiating ability, whereas RANK-Fc treatment only at passage 2 did not significantly change tumor-initiating cell (TIC) frequency (Fig. 2D). Concomitantly, the ability to form secondary tumorspheres was significantly impaired in cells derived from the RANK-Fc-pretreated pool (Fig. 2E), consistent with a reduction in the CSC population (19). These results demonstrate that pretreatment with RANK-Fc reduces tumor-initiating ability, and suggests that in clinics, RANKL inhibition may reduce the risk of relapse by depleting the population of CSCs.

Pharmacological inhibition of RANK signaling induces lactogenic differentiation of tumor cells

To investigate the molecular mechanism underlying the reduction in tumor-initiating ability, we analyzed global gene expression profiles from all RANK-Fc treatment arms. Genes induced by RANK-Fc included milk proteins such as Pip, caseins, Wap, or Lpl, which are expressed during differentiation of mammary cells into milk-secreting alveoli (21) and multiple members of the secretoglobin family (Scgb1b27, Scgb1b30, Scgb2b2), which are associated with differentiation and low risk of relapse in human breast cancer (Table S1 and Fig. 3A; refs. 3, 27). In fact, genes upregulated during lactation (21) were significantly overexpressed in the RANK-Fc-treated tumors (Fig. 3A–B). Upregulation of Csn2, Pip, Scgb1b27, and Scgb2b27 mRNA was confirmed in the RANK-Fc-treated tumors (Fig. 3C). Immunostaining with an anti-milk antibody confirmed that RANKL inhibition induced differentiation of late-adenocarcinoma cells into milk secreting cells (Fig. 3D). Conversely, tumor acini cultured with RANKL showed lower Csn2, Pip, and Scgb2b27 expression (Fig. 3E), in correlation with the reduced milk protein found in vivo (Fig. 1F–G). These results demonstrate that pharmacological inhibition of RANK signaling in PyMT tumor-bearing mice promotes tumor cell differentiation into an apocrine, milk-secreting phenotype that mimics mammary lactogenic differentiation, concomitantly with the reduction in tumor-initiating ability.

RANK deletion increases tumor latency, decreases tumor incidence, and impairs lung metastasis in MMTV-PyMT mice

Genetic deletion of RANK in the MMTV-PyMT background significantly delayed tumor onset and reduced tumor incidence (Fig. 4A–B and Supplementary Fig. S4A). In accordance with their multifocal origin (24), PyMT;RANK−/− palpable lesions showed multiple stages of tumor progression, whereas one predominant stage was found throughout the whole PyMT;RANK−/− palpable mass (Supplementary Fig. S4B-C). Accordingly, the number of preneoplastic lesions quantified in mammary gland whole mounts was significantly reduced in PyMT;RANK−/− compared with control mice (Fig. 4C). PyMT;RANK−/−−/− lesions contained extensive areas of early and/or late carcinoma, indicating that tumors can progress to the invasive stage in the absence of RANK. However, most of PyMT;RANK−/− mice were devoid of lung metastasis, whereas all PyMT;RANK−/−−/− mice with early/late carcinomas developed lung metastasis, and several showed 30 to 200 metastatic foci per lung (Fig. 4D). Thus, RANK deletion increases tumor latency, decreases tumor incidence, and impairs lung metastasis in the MMTV-PyMT tumor-prone model.
RANK loss in tumor cells depletes the tumor and metastasis-initiating cell pools and increases apoptosis and sensitivity to docetaxel. To rule out the progesterone/RANKL-mediated effects acting in early tumorigenesis (7) and the influence of the RANK-null microenvironment (17), PyMT;RANK−/− and PyMT;RANK+/− tumors, isolated from established carcinomas were orthotopically implanted in syngeneic wild-type (WT) females. PyMT;RANK−/− tumors showed a significantly longer latency to tumor formation than did PyMT;RANK+/− tumors, indicating a tumor cell autonomous defect (Fig. 5A). Longer latency was also observed when PyMT;RANK+/− tumors were implanted in RANK null mice compared with WT, but no synergic effect after implantation of PyMT;RANK−/− in RANK null hosts was found (Fig. 5A).

PyMT;RANK−/− tumors growing in WT hosts contained more apoptotic cells and extensive nonviable areas relative to PyMT;RANK+/− tumors (Fig. 5B–C). No significant differences in tumor cell survival were observed when the same tumor, either PyMT;RANK−/− or PyMT;RANK+/−, was implanted on WT and RANK null hosts supporting a tumor cell intrinsic mechanism (Fig. 5C). This demonstrates that tumor cell survival is impaired in the absence of RANK, which may contribute to the delayed tumor formation observed. Moreover, the absence of RANK on tumor cells sensitized tumors to docetaxel (Fig. 5D).

Next, we aimed to determine whether loss of RANK signaling exclusively on tumor cells reduced the CSC pool as observed after RANKL inhibition. PyMT;RANK−/− tumor cells gave rise to less tumospheres than controls, independently of the initial host (Fig. 5E), highlighting an extenuation of a self-renewal capability that is tumor cell-autonomous. LDA in WT hosts also revealed a decrease in the frequency of metastasis-initiating cells (MIC) was significant in WT hosts supporting a tumor cell intrinsic mechanism (Fig. 5C). Longer latency was also observed with very few metastatic foci (Fig. 5G), implying that RANK expression in tumor cells is determinant for metastasis. TFAP2B expression analyses confirmed that Spdef was positively regulated by Tfap2b (Supplementary Fig. S5C). Analyses of PyMT tumor acini cultured for 2 weeks revealed that RANKL led to an increase on acini size irrespectively of Tfap2b expression (Supplementary Fig. S6A–B). However, in Tfap2b-overexpressing PyMT acini treated with RANKL, Rspo1 mRNA expression was 30% lower than in RANKL-treated control acini. Conversely, in shTfap2b PyMT acini treated with RANKL, Rspo1 expression was 50% higher than in controls, demonstrating that Tfap2b interfered with RANKL-driven increase in Rspo1 (Fig. 6H). Rspo1 expression decreased, whereas Tfap2b and Spdef expression increased in PyMT tumor cells infected with shRANK, further supporting that RANK pathway negatively regulates luminal differentiation (Supplementary Fig. S3D).

Figure 6.
RANK loss or inhibition induces the expression of AP2 transcription factors, drivers of luminal differentiation, and induces Rspo1.

To further understand the molecular mechanism underlying tumor cell differentiation after RANKL inhibition, we focused on genes specifically induced in tumors that received RANK-Fc treatment at passage 1 such as Tfap2b (Supplementary Table S1). The AP2 transcription factor family is a set of retinoic acid inducible genes that governs the luminal epithelial phenotype in mammary development and carcinogenesis (28, 29) and whose expression is associated with survival (30, 31). Consistent with a tumor cell-luminal differentiation phenotype, GSEA analyses of the genes that characterize mammary differentiation hierarchy (20), revealed that the mature luminal upregulated set and the MaSC downregulated set, were overexpressed on the RANK-Fc-treated tumors (Fig. 6A). Spdef, which also promotes luminal differentiation (32) was the top gene in these associations (Fig. 6A). Genes upregulated by TFAP2C in human breast cancer cells (22) were significantly overexpressed in RANK-Fc-treated tumors (Fig. 6B). Moreover, an increase in Tfap2a and Tfap2b was observed in the RANK null mammary epithelia and RANKL treatment significantly reduced the Tfap2a, Tfap2b, and Tfap2c mRNA expression in PyMT cells (Fig. 6C–D and G).

Gene expression analysis in the pre-RANK-Fc-treated tumors confirmed upregulation of Tfap2b, the luminal genes Spdef and Fbp1 and cdh11a/p21 (known to be induced by Tfap2; ref. 29) and downregulation of the basal genes p63, Krt14. No significant changes were detected between groups in Krt8, Foa1, Gata3, Esr1, Elf5, and Rspo1 (Fig. 6E). Higher levels of Tfap2b, Tfap2c, and p21 lower levels of Krt14 and Rspo1 were found in PyMT;RANK−/− tumor cells isolated from transplants as compared to controls (Fig. 6F). R-spondin1 (Rspo1), a Wnt agonist that has been shown to be expressed on luminal progenitor cells and mediate RANK-driven expansion of mammary progenitors in the healthy gland (33, 34), was strongly induced by RANKL in PyMT acini cultures (Fig. 6G).

PyMT;RANK−/− tumors transplanted as mammary glands. Each bar is representative of three mammary glands. The AP2 transcription factor family is a set of retinoic acid inducible genes that governs the luminal epithelial phenotype in mammary development and carcinogenesis (28, 29) and whose expression is associated with survival (30, 31). Consistent with a tumor cell-luminal differentiation phenotype, GSEA analyses of the genes that characterize mammary differentiation hierarchy (20), revealed that the mature luminal upregulated set and the MaSC downregulated set, were overexpressed on the RANK-Fc-treated tumors (Fig. 6A). Spdef, which also promotes luminal differentiation (32) was the top gene in these associations (Fig. 6A). Genes upregulated by TFAP2C in human breast cancer cells (22) were significantly overexpressed in RANK-Fc-treated tumors (Fig. 6B). Moreover, an increase in Tfap2a and Tfap2b was observed in the RANK null mammary epithelia and RANKL treatment significantly reduced the Tfap2a, Tfap2b, and Tfap2c mRNA expression in PyMT cells (Fig. 6C–D and G).

Gene expression analysis in the pre-RANK-Fc-treated tumors confirmed upregulation of Tfap2b, the luminal genes Spdef and Fbp1 and cdh11a/p21 (known to be induced by Tfap2; ref. 29) and downregulation of the basal genes p63, Krt14. No significant changes were detected between groups in Krt8, Foa1, Gata3, Esr1, Elf5, and Rspo1 (Fig. 6E). Higher levels of Tfap2b, Tfap2c, and p21 lower levels of Krt14 and Rspo1 were found in PyMT;RANK−/− tumor cells isolated from transplants as compared to controls (Fig. 6F). R-spondin1 (Rspo1), a Wnt agonist that has been shown to be expressed on luminal progenitor cells and mediate RANK-driven expansion of mammary progenitors in the healthy gland (33, 34), was strongly induced by RANKL in PyMT acini cultures (Fig. 6G).

Figure 6.
RANK loss or inhibition induces the expression of AP2 transcription factors and reduces Rspo1. A and B, GSEA graphical outputs for the association between mammary mature luminal (upregulated genes) and mammary stem (downregulated genes) cells gene sets (A) and TFAP2C upregulated genes sets in human breast cancer (B) and RANK-Fc treatment. The top genes contributing to the association are listed. C, fold changes in mRNA expression of indicated genes in PyMT tumor acini cultures treated with RANKL for 24 h relative to untreated cultures. Each bar is representative of three tumors. D, mRNA expression levels of indicated genes relative to Krt8 in PyMT RANK+/− and PyMT;RANK−/− mammary glands. Each bar is representative of three mammary glands. E, fold changes in mRNA expression of indicated genes in RANK-Fc treated PyMT tumors at passage I relative to expression in the other treatment arms. Each bar is representative of six tumors. F, fold changes in mRNA expression of indicated genes in PyMT;RANK−/− relative to expression in PyMT;RANK+/− sorted tumor cells. Each bar is representative of three-four-different tumors. G, fold changes of Rspo1 and Tfap2b mRNA expression levels in PyMT acini tumor acini cultured with RANKL for 3 and 14 days relative to untreated cultures. H, relative induction of Rspo1 mRNA expression in Tfap2b-knockdown or -overexpressing PyMT tumor acini cultured with RANKL for 14 days relative to the induction in RANKL-treated controls (normalized as 100%). Induction of Rspo1 mRNA in RANK-knockdown PyMT acini is included. I, association between TFAP2B and TNFRSF1B tumor expression and distant metastasis in lymph-node negative breast cancer patients (GSE20354). Graphs show the proportion of distant metastasis-free patients over time (months) and are stratified according to the first (low expression) or the third (high expression) tertiles. C, D, E, F, mean, SEM and t-test statistics are shown. (*, 0.01 < P < 0.05; **, 0.001 < P < 0.01; ***, 0.0001 < P < 0.0001; ****, P < 0.0001).
To investigate the clinical relevance of TFAP2B, we analyzed an expression dataset from lymph-node negative breast cancer patients that developed distant metastasis (35). The expression of TFAP2B was found to be significantly associated with the absence of distant metastasis: Cox regression HR = 0.25; 95% confidence interval (CI), 0.11–0.57; P = 0.001 (Fig. 6I). Similar results were observed in tumors with a luminal phenotype (ER+): HR = 0.24; 95% CI, 0.09–0.63; P = 0.004, and the same trend for (ER-) tumors: HR = 0.24; 95% CI, 0.04–1.29; P = 0.09. Consistent with the proposed cancer-promoting role for enhanced RANK signaling, associations with relapse free were observed for TNFRSF11B (OPG), the canonical negative regulator of the RANK pathway: HR = 0.49; 95% CI, 0.31–0.78; P = 0.002 (Fig. 6I); in luminal tumors (ER+): HR = 0.33; 95% CI, 0.17–0.62; P = 0.0006. Accordingly, TFAP2B and TNFRSF11B were found to be significantly coexpressed (Pearson’s correlation coefficient = 0.14, P = 0.018). Together these data indicate that RANKL inhibition leads to tumor differentiation, metastasis impairment, and good prognosis.

RANK signaling inhibition depletes the pool of Sca1+/ThCs

Next, we aimed to identify the CSC population regulated by RANK in our models, which remains elusive in the MMTV-PyMT model (36–38). The levels of CD49f, CD49b, CD61, and CD90 within epithelial cells were comparable for all RANK-Fc treatment arms; in contrast, Sca1+/hi cells were more abundant in tumors pretreated with RANK-Fc, which show a lower tumor-initiating ability (Fig. 7A and B). In the normal mammary gland, Sca1+ identifies a population enriched in ER+/PR+ luminal mature cells (37). However, we could not detect an increase in PR+ cells after RANK-Fc treatment (Fig. 7C). Similarly, an increase in the Sca1+/hi population, but none of the other markers, was found in PyMT;RANK−/− tumors as compared to controls (Fig. 7D). Secondary tumorspheres of Sca1+/lo tumor cells were larger and five times more numerous as those of Sca1+/hi cells (Fig. 7E–F). Strikingly, LDA assays revealed the TIC frequency is significantly enhanced by 200-fold in Sca1+/lo compared with Sca1+/hi tumor cells (Fig. 7G), indicating that the Sca1+/lo population is enriched in CSCs. Altogether these results demonstrate that RANK loss or RANKL inhibition reduced the frequency of the Sca1+/lo CSC population.

Discussion

The work presented here reveals a central role of RANK signaling promoting recurrence and metastasis in aggressive breast tumors, providing a rationale for additional therapeutic applications of RANK inhibitors beyond its current use for the management of skeletal-related events. We found that constitutive deletion of RANK in MMTV-PyMT mice increases tumor latency and decreases tumor and lung metastasis incidence, as observed in MMTV-neu mice upon RANK-Fc preventive treatment (7), reinforcing the role of RANK signaling in early stages of tumorigenesis (8).

Our previous data showed that enhanced RANK activation promotes stemness in human and mouse mammary epithelia, leading to the accumulation of MaSC and progenitors (11, 12, 39). Importantly, now we demonstrate that inhibition of RANK signaling reduces CSC in invasive mammary tumors decreasing recurrence and metastasis, and induces tumor cell differentiation. LDA assays aim to mimic occult disease that remains in breast cancer patients after surgery. Our results suggest that neoadjuvant RANKL inhibition may be more efficient in reducing recurrence and metastasis than adjuvant treatment, as a significant reduction in the CSC population was observed on tumors treated at passage 1. Along with our previous data demonstrating that overactivation of RANK signaling at midgestation disrupts lactogenesis (12, 39), current results suggest that RANK signaling regulates the balance between self-renewal and differentiation not only during mammary gland development but also in breast adenocarcinomas.

The impaired tumor and metastasis initiation ability observed in RANK null tumor cells growing in WT hosts demonstrates that tumor cell intrinsic mechanisms mediate the observed reduction in CSC. However, we cannot discard that tumor cell extrinsic mechanisms induced by RANK signaling inhibition in the microenvironment can also contribute to reduce recurrence (40).

Mechanistically, we demonstrate that RANK signaling negatively regulates the AP2 transcription factor family that can mediate retinoic acid responsiveness (41, 42). TFAP2A functions as a tumor suppressor in several solid tumors including breast cancer (43, 44). Overexpression of Tfp2a and Tfp2c mimics the mammary phenotype of RANK null mice (5, 45, 46). Tfp2a and Tfp2c maintain the luminal phenotype (28, 29) and negatively regulate cancer stem cell markers (28). Although little is known about TFAP2B in mammary epithelia, our results suggest that, similarly to other members of the family, TFAP2B promotes luminal differentiation and is associated with good prognosis. The positive correlation between the RANKL inhibitor, OPG, and TFAP2B expression in human breast tumors and their association with metastasis-free phenotype support the clinical implication of our findings.

Although enhanced Tfp2b expression alone cannot prevent RANKL-driven increased in acinar size, it interferes with the induction of the Wnt agonist Rspo1. Rspo1 together with Wnt4 promote MaSC self-renewal and Rspo1 rescues some of the mammary developmental defects in RANK null mice (33, 34). Similarly to our previous results on mammary epithelial cells at midgestation where RANKL induces the expression of Rspo1, leads to the expansion of basal and bipotent cells and prevents lactogenic differentiation (39), we now observe that on PyMT tumor acini, RANK pathway also enhances Rspo1 and interferes with differentiation. Our results evidence a complex regulatory loop between RANK, Tfp2, and Rspo1 underlying the reduction in the CSC pool observed upon RANK pathway inhibition (Fig. 7H). Further experiments will be required to clarify their contribution to the protumorigenic role of RANK in cancer. Sca-1/Ly6A is found in the luminal differentiated ER+/PR+ cell cluster and according to our data it is likely to be induced by Tfp2a, whereas Rspo1 is expressed on luminal Sca1− progenitor cells (33, 34). The decrease in Sca1− cells upon RANK loss or inhibition and their enhanced mammosphere-forming and tumor-initiating potential demonstrate that this population is enriched in CSCs in the PyMT tumors, as shown in the MMTV-wnt model (47). A negative regulation of Sca1− by RANK has been observed during mammary gland development (12, 34). The relevance of Sca1/Ly6a as a CSC marker in human luminal adenocarcinomas deserves further investigation.

Mortality in breast cancer is due to tumor recurrence and metastasis, which is driven by surviving CSC. RANKL inhibitors, although unable to reduce tumor growth, can be used as differentiation therapy of CSC (Fig. 7H). Moreover, RANK null tumor cells are more susceptible to taxanes than RANK-expressing tumor...
Figure 7.
RANK-Fc pretreatment reduces the Sca1^τ tumor cell population. A, frequency of the indicated populations within tumor CD45^-CD31^-CD24^+ cells. Each bar represents data from four mice in two independent experiments. B, representative histograms of Sca1^τ/hi and Sca1^-/lo populations. C, representative images of PR immunostaining. D, frequency of the indicated populations within tumor cell transplants. Each bar is representative of 3-5 tumors. E and F, representative images (E) and number (F) of tumorspheres derived from FACS-sorted Sca1^τ/hi and Sca1^-/lo tumor cells. Each bar is representative of two tumors quantified in triplicates. G, tumor-initiating frequency (with confidence intervals), $\chi^2$ values, and associated probabilities. H, graphical abstract indicating the multiple effects observed after therapeutic inhibition of RANK pathway in MMTV-PyMT tumors. A, D, F, mean, SEM and t-test statistics are shown (*, 0.001 < P < 0.01).
cells, supporting the use of neoadjuvant RANKL inhibitors in the clinical setting to reduce the frequency of tumor relapse and metastasis and to increase sensitivity to chemotherapy. FDA-approved RANKL inhibitors are currently in clinic for the management of skeletal-related events, therefore patients may quickly benefit from this therapeutic strategy to combat advanced breast cancer.

Disclosure of Potential Conflicts of Interest

W.C. Dougall has ownership interest (including patents) in a patent. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: G. Yoldi, P. Pellegrini, E.M. Trinidad, A. Cordero, E. González-Suárez


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): G. Yoldi, P. Pellegrini, E.M. Trinidad, A. Cordero, J. Gomez-Miragaya, E. González-Suárez


Writing, review, and/or revision of the manuscript: G. Yoldi, P. Pellegrini, E.M. Trinidad, A. Cordero, J. Gomez-Miragaya, W.C. Dougall, P. Muñoz, M-A. Pujana, L. Planelles, E. González-Suárez

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P. Pellegrini, J. Serra-Musach, E. González-Suárez

Study supervision: E. González-Suárez

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RANK Signaling Blockade Reduces Breast Cancer Recurrence by Inducing Tumor Cell Differentiation

Guillermo Yoldi, Pasquale Pellegrini, Eva M. Trinidad, et al.

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