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

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SUPPLEMENTARY MATERIALS AND METHODS

Plasmids

Entry vector pENTR223.1 (Genycell) or pENTR221 (Invitrogen) containing full length human RANK was utilized to create lentiviral constructs. RANK was subcloned into the destination vectors pLV409 that contains luciferase (Amgen) or pLenti6/V5-DEST (Invitrogen) or using Gateway technology. pWPT-GFP and pLenti6/V5-DEST+lacZ were used to control the infection efficiency.

Antibodies

Western blot: hRANK (R&D AF683), phospho IκBα (Cell Signaling), total IκBα (Sta Cruz), phospho-p65, total-p65, phospho-Akt Ser473, total Akt, phospho-p38 and total p38 (Cell Signaling), phospho ERK, total ERK ½, β-actin and tubulin (Sigma). Immunofluorescence/IHC: Fibronectin (BD Biosciences), Vimentin (V9, invitrogen), E-cadherin (BD Biosciences), ki67 (Zymed), cleaved caspase 3 (Cell signaling). Flow cytometry: directly conjugated antibodies include, CD44-FITC (G44-26), CD44-APC (G44-26), CD24-PE (ML5), EpCAM-APC (EBA-1), EpCAM-FITC (EBA-1), CD10-

PECy5 (HI10a) all from BD Biosciences, CD133-PE (AC-133, Miltenyi) and CD49f–APC (GoH3, R&D).

Culture of human mammary epithelial cells

All cells were cultured at 37º in 5% CO2. MCF10A and HMECs immortalized with telomerase were cultured in growth media in plastic (DMEM/F12, 5% Horse serum (Invitrogen), 20ng/ml EGF (Sigma), 500ng/ml Hydrocortisone (Sigma), 10μg/ml Insulin (Sigma), 1X Penicillin/Streptinomicin (PAA Laboratories) and L-Glutamate (Gibco). Assay media for MCF10A were as described in (12) (DMEM/F12, 2% Horse serum, 5ng/ml EGF, 500ng/ml Hydrocortisone, 10 μg/ml Insulin, 1X Penicillin/Streptinomicin and L-Glutamate). MDA-MB-436, HCC1937, UACC3199 and 293FT were cultured in DMEM high glucose containing 10% FBS (Gibco), L-Glutamate (Gibco) and Penicillin/Streptinomicin (PAA Laboratories). H14 differentiation media as (25), was used in 3D cultures. H14 differentiation media was used for MDA-MB-436, HCC1937 and UACC3199 in 3D cultures (DMEM/F12, 1% FBS, 1X Insulin-Transferrin-Selenium (ITS) cocktail, 10^{-10}M Estradiol, 1,4x10^{-6}M Hydrocortisone, 5ug/ml Prolactin, 1X Penicillin/Streptinomicin and L-Glutamate).

Lentiviral infection

Lentiviral infection was done following the manufacturer´s indications (Invitrogen). Briefly 293FT cells were used for the production of the virus. 293FT cells (5x10^6) were transfected with lentivirus and packaging (gag-pol, vsvg, rev) plasmids (Addgene) by calcium phosphate method. Viral production was induced by adding 10mM Na Butyrate the next day. Virus was harvested 72h post transfection and concentrated by centrifugation. RANK lentiviral stocks were titered using colony forming assay (Hela cells). MCF10A or BRCA1-defective cells were transduced with pLV409-RANK

(MOI:1), pLV417-control (MOI:2), or pLenti6/V5-DEST-RANK or -tubGFP with 8ug/ml of polybrene. Plates were centrifuged 1 hour at 1.000 rpm at 37ºC to improve the infection. Selection started with the corresponding amount of blasticidin/puromycin antibiotic for each cell line (blasticidin: 5µg/ml for MCF10A and UACC3199 and 7,5µg/ml for HMECs, MDA-MB-436 and HCC1937; puromycin: 0,75µg/ml). The resulting stable cell lines infected were maintained with 5µg/ml of blasticidin and 0,75 µg/ml of puromycin for pLV409-MCF10A and 1 µg/ml of blasticidin for MCF10A, HMECS, MDA-MB-436, HCC1937 and UACC3199.

RNA isolation and quantitative RT-PCR analysis

Total RNA was isolated with TRIzol reagent (Invitrogen) according to manufacturer's instructions. 20ng/ml of mRNA were pretreated with DNase I (Ambion), reverse transcribed using Applied Biosystems TaqMan reverse transcriptase reagents and random hexamer priming. Amplifications were done using LightCycler 480® machine. UPL Probes were used for hRANK (probe number #25), hRANKL (probe number #17) and PP1A as a normalizer (probe number #48) (Roche). All other genes were analyzed by SYBR. Amplifications were done using LightCycler 480® machine. 95º 10s, 58º 30s, 72º 20s, for 45 cycles. All other genes were analyzed by SYBR and the, 95º 10s, 60º 20s, 72º 15s for 50 cycles.

Oligos (Sigma): For each sample amplifications were done in triplicate. Analysis was performed using LightCycler 480® Software release 1.5.0 SP4.

For UPL probes:

hRANKL: Fw: 5´- TGATTCATGTAGGAGAATTAAACAGG -3´

Rv: 5´- GATGTGCTGTGATCCAACGA -3´

hRANK: Fw: 5´- GCAGGTGGCTTTGCAGAT-3´

Rv: 5′-GCATTTAGAAGACATGTACTTTCCTG-3′

hPP1A: Fw: 5′- ATGCTGGACCCAACACAAAT-3′

Rv: 5′- TCTTTCACTTTGCCAAACACC -3′

For SYBR

hPP1A: Fw: 5′-ATGGTCAACCCCACCGTGT-3′

Rv: 5′-TCTGCTGTCTTTGGGACCTTG-3′

hFIBRONECTIN: Fw: 5′- CCGCCGAATGTAGGACAAGA -3′

Rv: 5′- TGCCAACAGGATGACATGAAA -3′

hVIMENTIN: Fw: 5′- CAACCTGGCCGAGGACAT -3′

Rv: 5′- ACGCATTGTCAACATCCTGTCT -3′

hN-CADHERIN: Fw: 5′- GACGGTTCGCCATCCAGAC -3′

Rv: 5′- TCGATTGGTTTGACCACGG -3′

hE-CADHERIN: Fw: 5′- CAAGCTATCCTTGCACCTCAG -3′

Rv: 5′- GCATCAAGAGAACTCCTATCTTG -3′

hSNAIL: Fw: 5′- GCTGCAGGACTCTAATCCAGAGTT -3′

Rv: 5′- GACAGAGTCCCAGATGAGCATTG -3′

hSLUG: Fw: 5′- GCGATGCCCAGTCTAGAAAA -3′

Rv: 5′- GCAGTGAGGGCAAGAAAAAG -3′

hTWIST: Fw: 5′- GGAGTCCGCAGTCTTACGAG -3′

Rv: 5′- TCTGGAGGACCTGGTAGAGG -3′

hNANOG: Fw: 5′- CAGCTGTGTGTACTCAATGATAGATTT -3′

Rv: 5′- ACACCATTGCATTTCTTCGGCCAGTTG -3′

hOCT4: Fw: 5′- GACAACAATGAAAATCTTCAGGAG -3′

Rv: 5′- CTGGCGCCGGTTACAGAACCA -3′

hSOX2: Fw: 5′- GCACATGAACGGCTGGAGCAACG -3

Rv: 5′- TGCTGCGAGTAGGACATGCTGTAGG -3′

hZEB1: Fw: 5′- GCCAATAAGCAAACGATTCTG -3′

Rv: 5′- TTTGGCTGGATCACTTTCAAG -3′

hZEB2: Fw: 5′- CCCTTCTGCGACATAAATACG -3′

Rv: 5′- TGTGATTCATGTGCTGCGAGT -3′

hRPL38: Fw: 5′- TGGGTGAGAAAGGTCCTGGTCC - 3′

Rv: 5′- CGTCGGGCTGTGAGCAGGAA - 3′

hCK18: FW: 5′-TCAGCAGATTGAGGAGAGCA-3′

Rv: 5′-GAGCTGCTCCATCTGTAGGG-3′

hCD45: Fw: 5′- AGTTATTGT TAT GCTGACAGAACTGAA -3′

Rv; 5′- TGCTTTCCTTCTCCCCAGTA-3′

Protein isolation and Western blot analysis

Cells were lysed with RIPA buffer (50 mM Tris-HCl at pH 7.6, 150mM NaCl, 1% NP-40, 0,5% Sodium deoxycholate, 0,1% SDS, 5mM EDTA plus proteases and phosphatases inhibitors) 30 min at 4ºC. Proteases and phosphatases inhibitors (Roche) were added freshly to the lysis buffer. Total protein quantification was performed with BCA Protein Pierce Assay Reagent following manufacturer's instructions (Thermo Scientific). A total of 30µg of protein were separated on 10% SDS-PAGE at 30mA and transferred to nitrocellulose membranes during 1 hour at 400mA. Membranes were hybridized with primary antibodies indicated. Secondary antibodies marked with HRP (1:1000; DAKO) were used and proteins were detected with the Enhanced Chemiluminescence Western Blotting Detection System (Amersham Biosciences).

Immunofluorescence in monolayer cultures

50.000 cells per well were seeded in 8 wells-chambers slides in growth medium and stimulated with RANKL for 30 min as indicated. At 80% of confluence the medium was removed and the cells were fixed with PFA 2%, 20 minutes at RT, permeabilized with Triton-X100 0.1% during 15 minutes at RT and washed three times with PBS-Glycine. Blocked with PBS-goat serum 10% during 1h at RT. Primary antibody 1,5 h at RT and secondary antibody 45 minutes at RT. Finally, the slides were coverslipped with Prolong® Gold antifade reagent with DAPI (Invitrogen) and dried over-night at RT.

Culture and immunofluorescence analysis in 3D cultures from human cells

Cell organoids were seeded in matrigel (BD Biosciences) in eight-well chamber slides (LabTek) with growth medium (5.000 cells/well for MCF10A and UACC3199, 2.500 cells/well for MDA-MB-436 and 10.000 cells/well for HCC1937). After 24 h, the medium was changed to differentiation medium (Assay medium for MCF10A and HMECs as (12) and H14 for BRCA1 mutated cell lines as (25) with or without human RANKL-LZ (100 ng/ml, Amgen Inc.). Medium was changed every 3-4 days. Medium was removed and acini were fixed in 2% paraformaldehyde and permeabilized using 0.5% Triton X-100 before blocking. Cells were incubated overnight at room temperature with the primary antibodies indicated and then with Alexa-488 -568 conjugated secondary antibodies (1:500; Molecular Probes) for 45 min at room temperature and DRAQ5 (1:5000; Alexis) or DAPI for nuclear staining. Slides were mounted with Prolong Gold Antifade Reagent (Molecular Probes

Flow cytometry

MCF10A cells were washed once with PBS 1x and twice with PBS-EDTA 2 mM, 15 minute each, prior to trypsinization. Cells (10^5) were re-suspended in 25ul of PBS

containing 2% FBS and 2mM EDTA and blocked with human blocking reagent (2.5ul per 100.000 cells in 25 µL, Miltenyi Biotec) during 10 minutes at 4ºC. Cells were then incubated with the corresponding anti-body combination during 30 minutes at 4ºC in dark, washed with PBS-FBS-EDTA and in the case of hRANK (R&D, 0.1 µg /100.000 cells or 0.004 µg/µl.) or H2K-biotin (BD) incubated with the secondary antibodies alexa-488 donkey anti-goat IgG (0.2 µg g/100.000 cells or 0.01 µg /µl, Molecular probes) or SA-PECy7 (BD Biosciences) After washing, cells were re-suspended in PBS-FBS-EDTA, stained with 7AAD (1ug/ml, AnaSpect) during 10 minutes at 4ºC.

Wound healing Assay

MCF10A cells were seeded in triplicates at 80% of confluence with growth medium. After 24h, a wound of approximately 20 µm was made, cells were washed with PBS and Mitomicin C 1ng/mL and stimuli were added: growth medium, starving medium (without EGF and 5% Serum), EGF (Sigma, 20 ng/mL) and/or RANKL (Amgen, 100 ng/mL). Photographs in the same points were taken at indicated times and the average of the distance that the cells have moved in five different points of the healing was calculated. Assay was done in triplicates.

Growth in soft agar

Cells (5x 10^4) were suspended in 0.35% agarose growth medium, disposed onto a solidified base of growth medium for MCF10A containing 0.5% agarose. Plates were cooled during 15 minutes at 4ºC and then incubated at 37ºC 5%CO2. After 30 days the number of foci of each cell line was counted.

Growth curve

A time course curve of parental and hRANK-expressing cells was generated by seeding $3x10^4$cells in 35mm dishes in triplicate samples. After 24h (day 0) and every 2 days (for 10 days), cells were fixed with glutaraldehyde 0.5% and stored in PBS at 4ºC. The culture medium was replaced every 3 days. When all samples were fixed, cells were stained with crystal violet 1%. After extensive washing, crystal violet was resolubilized in 15% acetic acid and quantified at 595 nm as a relative measure of cell number. Values were referred to cell growth of cells growing at day 0. Data presented were the mean from triplicate samples and bars:±SD.

Whole mounts

Number 4 mammary glands were collected, imaged for luciferase activity and fixed in Carnoy´s for 1-3h, stained in carmine alum overnight at 4ºC and then dehydrated and stored in methyl-salycilate.

Human tumor samples

Bellvitge series included 73 patients with a median age of 59 years (range 42-78). After surgical excision of breast tumors and axillary clearance, when node metastases where detected, patients followed standard adjuvant treatment with chemotherapy in node positive patients and high risk node negative patients (mostly sequential regimens based on anthracyclines and taxanes). Estrogen and progesterone receptor positivity was defined as the presence of at least 10% of tumor cells positively stained by immunohistochemistry.Her2 positivity was defined as three plus in immunohistochemistry or amplified by FISH. Pathological tumor grade was established using Nottingham classification. All hormone receptor positive patients received state of the art endocrine therapy. All Her2 positive patients received adjuvant trastuzumab.

Patients undergoing breast conserving surgery and/or presenting extensive axillary involvement received complementary radiotherapy.

The breast tumor samples for IHC analysis included 86 total patients with a median age of 61.6 (range 39-85). Estrogen and progesterone receptor positivity was defined exactly as described for the Bellvitge series above.

Human RANK and vimentin immunohistochemistry

Anti-human RANK IHC was performed on sections prepared with heat retrieval in Diva buffer (formalin fixed). The primary antibody was anti-human RANK (N-1H8 and N-2B10) followed with goat anti mouse secondary antibody. A formalin fixed giant cell tumor of the bone and transfected cell lines were used as positive controls for the anti-RANK antibodies, as described (7). The anti-hRANK antibodies recognize distinct epitopes within the extracellular domain of RANK. There was precise concordance in the staining pattern of breast tumor samples using these two distinct anti-huRANK antibodies. A tumor was scored positive for RANK expression if IHC staining was observed at any intensity within membrane and/or cytoplasm of the epithelial component of tumors. Anti human vimentin IHC was performed on Carnoys´ or formalin fixed mammary glands. After carmine staining tissues were embedded in paraffin and sectioned. Antigen retrieval was done in citrate (pH=6), the primary antibody was anti vimentin clon V9, invitrogen followed by mouse Envision (Dako) and DAB. A tumor from MDA-MB-436 cells was used as positive control and a mouse mammary gland was used as negative control.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. RANK is active in FL-RANK MCF10A cells.

A. qRT-PCR showing the expression of hRANK and hRANKL mRNA in MCF10A cells infected with a lentivirus containing FL-RANK or control vector at passage 10 and 20 after infection as indicated.

B. Western blot showing phospho-IκBα and phospho-p65 in FL-RANK and parental cells starved (S) and treated with RANKL for the indicated times. Total IκBα, p65 and tubulin are shown as loading controls.

C. Western blot analyses of the indicated proteins in FL-RANK and parental MCF10A cells growing in complete media. Tubulin is used as a loading control.

Supplementary Figure 2. RANK expression induces EMT in MCF10A using a second lentiviral construct and in HMECS immortalized with telomerase.

A-B. qRT-PCR (A) and western blotting (B) showing the expression of RANK in MCF10A and HMECs cells infected with a second lentiviral vector containing FL-RANK and a control vector (PARENTAL).

C. mRNA expression of EMT proteins and transcription factors in MCF10A and HMECs infected with a second lentiviral construct determined by qRT-PCR. In all qRT-PCR mRNA expression of the indicated genes relative to PP1A or RPL38 is shown. Determinations were done in triplicate and standard deviations are shown.

D. RANKL stimulation increases mRNA expression of vimentin and Snail in FL-RANK but not in parental MCF10A cells as measured by qRT-PC. Results are representative of 2 independent experiments. Relative expression to PP1A is shown. Untreated cells (-) or RANKL treated cells (+RL, 100 ng/ml; 48h) are indicated. Determinations were done in triplicate and standard deviations are shown.

Supplementary Figure 3. RANK overexpression induces mammary stem markers

A. Flow cytometry analyses (histograms) showing expression patterns of CD44, CD24, CD133 and RANK in FL-RANK and parental MCF10A. Numbers indicate the frequency of positive cells based on the negative population (in bold) or the mean of the histograms (in italic).

B. RANKL stimulation increases the frequency of CD44+CD24- cells in FL-RANK MCF10A. Gain or loss in the percentages of the indicated populations analyzed by flow cytometry after 48h of stimulation with RANKL (100 ng/ml) is shown for one representative experiment out of 3.

C. BLI (bioluminescence) values (Avg Radiance [p/s/cm²/sr]) in mammary glands of scid/beige mice injected with parental or FL-RANK MCF10A cells.

Supplementary Figure 4. FL-RANK cells grow in the absence of EGF and do not form tumors *in vivo*.

A. Percentage of ki67 and caspase 3 positive nuclei in parental, and FL-RANK at day 20 of culture. Note that no proliferation is detected in the parental acini, as by 20 days of culture they are fully differentiated and arrested. P-values vs parental cells are included.

B. Representative phase contrast and confocal pictures of FL-RANK and parental growing in matrigel in the absence of EGF (cyan=nuclei). Results are representative of three independent experiments.

C. Table indicates the number of intra-fat pad ($3x10^6$ cells) or tail vein ($1x10^6$cells) cell inoculations done with FL-RANK and parental MCF10A cells. As indicated no tumors or metastasis were observed after 110-160 days, respectively.

Supplementary Figure 5. RANK expression induces a CD44+CD24- phenotype in breast cancer cells with non functional BRCA1 and enhances the mesenchymal markers in MDA-MB-436 cells.

A. Relative expression of hRANK and hRANKL mRNA in the indicated cells infected with a hRANK expressing vector (FL-RANK) and a control vector (PARENTAL) as determined by qRT-PCR.

B. Western blot showing phospho-IκBα in FL-RANK, and parental cells with non-functional BRCA1 treated with RANKL for the indicated times. Total IκBα and tubulin are shown as loading controls.

C. Analyses by flow cytometry of CD24, EpCAM, CD49f and CD10 in the indicated cells cultured with or without RANKL (100 ng/ml) for 48h. Numbers indicate the frequency of positive cells based on the negative population (in bold) or the mean of the histograms (in italic).

D. Frequency of CD24, EpCAM and CD10 positive cells in parental and FL-RANK non-functional BRCA1 cells analyzed by flow cytometry. Values for one representative experiment out of 4 are shown.

E. qRT-PCR showing mRNA expression of the indicated genes relative to PP1A in parental and FL-RANK cells with non functional BRCA1. Determinations were done in triplicate and standard deviations are included. Note that the mesenchymal markers are already expressed in MDA-MB-436 cells in correlation with their mesenchymal morphology.

Supplementary Figure 6. RANK overexpression promotes growth, invasive changes and metastasis in cells with non-functional BRCA1.

A. Growth curves of MDA-MB-436, HCC1937 and UACC3199 cells in complete media. Growth is represented as relative to day 0. p-values are shown (t-test)

B. Phase contrast images of MD-MB-436 acinar cultures with or without RANKL (day 20). Note the protrusions observed in FL-RANK cells.

C. Percentages of ki67 and cleaved caspase 3 in parental and FL-RANK MDA-MB-436, HCC1937 and UACC3199 acini cultured with or without RANKL at day 20. Significant p-values are included.

D. Representative H&E pictures of lung metastasis formed by FL-RANK and parental MDA-MB-436 (12 weeks) and UACC 3199 (9 weeks) cells injected in the tail vein. Arrows indicate metastatic foci.

Supplementary Figure 7. RANK/RANKL expression significantly associates with tumor subtype and lymph node metastasis.

A. Box and whiskers graphs showing expression of CK18 (epithelia) and CD45 (lymphocytes/leukocytes) mRNA relative to PP1A in human adenocarcinomas classified by ER, PR, status, tumor grade (gr) or proliferative index. Determinations were done in triplicates and means were used.

B-C. Scatterplot log(RANK) vs log(RANKL) by subtype and N stage (see Methods). RANK and RANKL mRNA expression values measured by quantitative PCR were considered.

D-E. Multivariate analyses of variance (MANOVA). (D) RANKlog/RANKLlog expression significantly associate with the two different variables considered: lymph node metastasis (N) and tumor subtype (S). (E) MANOVA analyses considering RANK and RANKL separately and comparing N0 (no metastasis) vs N1 (metastasis). Tumor subtype was considered as a variable in the model.