# RANK signaling, a master regulator of mammary stemness and cancer

Characterization of RANK and RANKL pathway in mammary stem cell fate, tumorigenesis and metastasis

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# RESUME

RANK signaling is essential for mammary gland development and progesterone induced tumorigenesis. Here we show that RANK controls mammary stem cell fate; increased RANK signaling results in accumulation of stem and progenitor cells leading to spontaneous tumor formation.
RANK signaling interferes with alveolar commitment through depletion of CD61+ alveolar progenitors and regulation of Elf-5/STAT5 pathway.
Using pharmacological and genetic approaches we show that RANK signaling promotes tumor initiation, progression and metastasis in spontaneous breast cancer models and expands tumor initiating cells and metastasis initiating cells. RANK pathway mediates the crosstalk between tumor cells and their microenvironment modulating the tumor immune response.
RANK expression correlates with aggressiveness and metastasis in human tumors. Altogether our data support that RANK signaling could be a novel therapeutic target in breast cancer.

# RESUMEN

La vía de RANK es esencial para el desarollo de la glándula mamaria y la tumorigénesis inducida por progesterona. Demostramos que RANK controla la especificación de linajes mamarios, y que un aumento de señalización a través de RANK induce una acumulación de células madre y progenitoras de mama con la consiguiente formación espontánea de tumores. RANK bloquea la diferenciación alveolar a través de la disminución de progenitoras alveolares CD61+ y la regulación de la via Elf-5/STAT5.
Utilizando estrategias genéticas y farmacológicas demostramos que RANK promueve la formación, progresión tumoral y la metástasis de modelos de tumorigenesis espontánea de mama, y expande las poblaciones de células iniciadoras de tumores y células iniciadoras de metastasis. La vía de RANK media además la interacción entre las células tumorales y su entorno, modulando la respuesta inmune tumoral.
El aumento en la expresión de RANK se correlaciona con la agresividad de tumores humanos y la metástasis. Estos resultados sugieren que la vía de RANK puede ser una nueva diana terapéutica en cáncer de mama.

# PROLOGUE

Breast cancer is one of the most common malignancies in the western world. As in many others cancer types, current therapies are often unable to completely eliminate tumor cells: tumors acquire resistance, relapse and metastasize to other organs resulting in an inescapable and lethal desease. Therefore new therapeutic targets and a more profound understanding of the cancer cell homeostasis are needed.

The thesis is focused on RANK/RANKL pathway as during the last years it has emerged as a key pathway in mammary development and tumorigenesis. RANK overexpression or deletion impairs mammary gland differentiation in mice (Fata JE et al., 2000; Gonzalez-Suarez E et al., 2007). It has been demonstrated that RANKL is downstream of progesterone and mediates proliferative stimuli of this hormone. Indeed, pharmacological inhibition of RANKL prevents tumor formation in a model of mammary gland carcinogenesis that combines a mutagen with progestin (Gonzalez-Suarez E et al., 2010).

Our first effort aimed to understand how RANK signaling is regulating mammary differentiation. We demonstrated that RANK regulates different populations in mammary epithelial hierachy. RANK expands mammary stem cells and intermediate progenitors but reduces alveolar progenitors and downregulates Elf-5/STAT5 signaling at midgestation. Thus, we shed light on the molecular origin of the lactation defect previously described and we integrated the progesterone-RANK signaling in a "big picture" where different molecular mechanisms orchestrate mammary gland development.
These results are described in the peer-reviewed article:

Pellegrini P*, Cordero A *, Gallego MI, Dougall WC, Muñoz P, Pujana MA and Gonzalez-Suarez E. Constitutive activation of RANK in the mammary gland disrupts mammary cell fate leading to tumorigenesis. Stem Cells. 2013 Sep;31(9):1954-65. *both authors contributed equally.

and ANNEX 1:

Pellegrini P, Cordero A and González Suarez E. RANK signaling at midgestation impairs Elf-5 induced alveolar cell fate. Manuscript in preparation.

In order to evaluate the clinical relevance of targeting RANK signaling in established tumors, we focused on two mouse models of spontaneous and metastatic breast cancer: MMTV-neu and MMTV-PyMT. We demonstrated that RANK signaling is important not only in tumor initiation, as previously described, but also in progression of advanced tumors. This suggests that targeting RANK could be beneficial for women with diagnosed breast cancer.

As we discovered that RANK is a master regulator of mammary stem cell fate we investigated whether RANK signaling is playing a role in the so-called cancer stem cells. We demonstrated that blocking RANK signalling with RANK-Fc reduces the cancer stem cell pool resulting in decreased tumor relapse. Characterization of tumor immune infiltrates revealed new roles of RANK in the regulation of the tumor immune response.
We generated a new model of MMTV-PyMT with genetic deletion of RANK and showed that RANK is important in tumor formation and mediates the metastatic potential of mammary tumor cells. Characterization of the role of RANK signaling in spontaneous breast cancer models is described in the following study:

Pellegrini P, Cordero A, Yoldi G and González Suarez E. RANK signaling expands the pool of tumor and metastasis initiating cells and attenuates the anti-tumor immune response in advanced breast cancer. Manuscript in preparation.

We also demonstrated the relevance of RANK signaling in human breast cancer as RANK expression correlates with poor prognosis. These results are included in the following peer-reviewed study:

Palafox M, Ferrer I, Pellegrini P, Vila S, Hernandez-Ortega S, Urruticoechea A, Climent F, Soler MT, Muñoz P, Viñals F, Tometsko M, Branstetter D, Dougall WC, González-Suárez E. RANK induces epithelial-mesenchymal transition and stemness in human mammary epithelial cells and promotes tumorigenesis and metastasis. Cancer Res. 2012 Jun 1;72(11):2879-88.

The characterization of RANK function in human breast cancer cells validates some of the many roles of RANK that we demonstrated in this thesis and will further support RANK as a novel therapeutic target in breast cancer.

# INDEX

# INTRODUCTION

# INTRODUCTION

# 1. MAMMARY GLAND BIOLOGY AND BREAST CANCER

## 1.1 The Mammary gland: anatomy and physiology

The mammary gland is a type of exocrine gland specialized in the production of milk for feeding of the newborn (Medina D et al., 1996), a unique feature of mammals. The mammary gland consists of two primary components: the parenchyma, composed of branching ducts from which secretory acini develop, and adipose stroma, that provides a substrate in which parenchyma develops (Medina D et al., 1996); (Figure 1).

The functional units of mammary gland are the acini. An inner (luminal) and outer (basal) layer compose the acinar epithelium. Luminal cells are organized with the apical cytoplasm toward the lumen, in which they secrete milk during pregnancy (Rosen et al., 2008). Basal layer is composed of contractile myoepithelial cells that tightly surround the luminal layer and have properties of smooth muscle cells (Hassiotou F et al., 2013). Myoepithelial cells surround luminal cells and have contractile properties in order to push milk from the alveoli toward the ducts. Ducts transport milk secreted from alveolar lobules out the tip of the nipple. The adipose stroma includes essentially adipocytes in which the ductal network is embedded; vascular endothelial cells, which make up the blood vessels; stromal cells, including fibroblasts and a variety of immune cells (Watson CJ et al., 2008); (Figure 2).

Unlike most of organs that develop to relatively mature state during embryonic life, mammary gland reaches its mature state in adult female, during pregnancy and lactation. There are three main stages of mammary gland development both in rodents and humans: embryonic, pubertal and adult/pregnant. Hormones and growth factors regulate these different stages of mammary development (Watson CJ et al., 2008).

Mammary gland development starts during fetal phase in which there is only a primordial epithelial tree, originating from nipple that remains undeveloped until puberty. Once the organism goes through puberty, sex hormones finely orchestrate a radical process of development and differentiation. Estrogen and progesterone induce respectively epithelial ductal tree elongation and side branching into the mammary fat pad or stroma in order to form an adult gland. During pregnancy, mammary gland goes through a second stage of growth and differentiation: progesterone is essential for epithelial cell proliferation and formation of alveolar buds along epithelial branches. Lactogenic switch occurs at the end of gestation; mammary gland is completely differentiated and ready for nursing the pups: alveolar buds differentiate into milk secreting structures under prolactin (PRL) stimuli (Oakes SR et al., 2006). At the same time myoepithelial cells that surround epithelial layer acquire contractility in order to mechanically stimulate milk secretion (Gudjonsson T et al., 2005). After lactation, mammary gland returns to its non lactating stage by a process called involution; this process is characterized by a high grade of apoptosis, re-development of mammary adipose tissue and tissue remodeling (Lund LR et al., 1996); (Figure 1).

Several cellular pathways are involved in mammary gland development; many of them are downstream of sex hormones. Genetic deletion of putative genes in mouse models revealed that a tight regulation of each pathway is essential for a correct and functional development of the mammary gland (Topper YJ et al., 1980). Progesterone (P4), WNT and other pathways control ductal elongation and side branching during puberty (Hennighausen L et al., 2001). When mammary gland goes through gestation, PrlR/JAK-2/STAT5, RANKL, transcription factor C/EBP and cell cycle proteins Cyclin D1 and ID2

regulate most of the differentiation and proliferation events. Finally secretion of milk from lobulo-alveoli structures during lactation is controlled by PRL through STAT5 (Hennighausen L et al., 2001).

Differential cytoskeletal keratin pattern provides an accurate identification of the basal and luminal cluster: in mouse epithelium, basal cells express cytokeratins 14 and 5, while luminal cells express cytokeratin 8 and 18. Existence of a bi-potent progenitor that express both luminal and basal cell cytokeratins has been described (Li Z et al., 2007; Chakrabarti R et al., 2012).

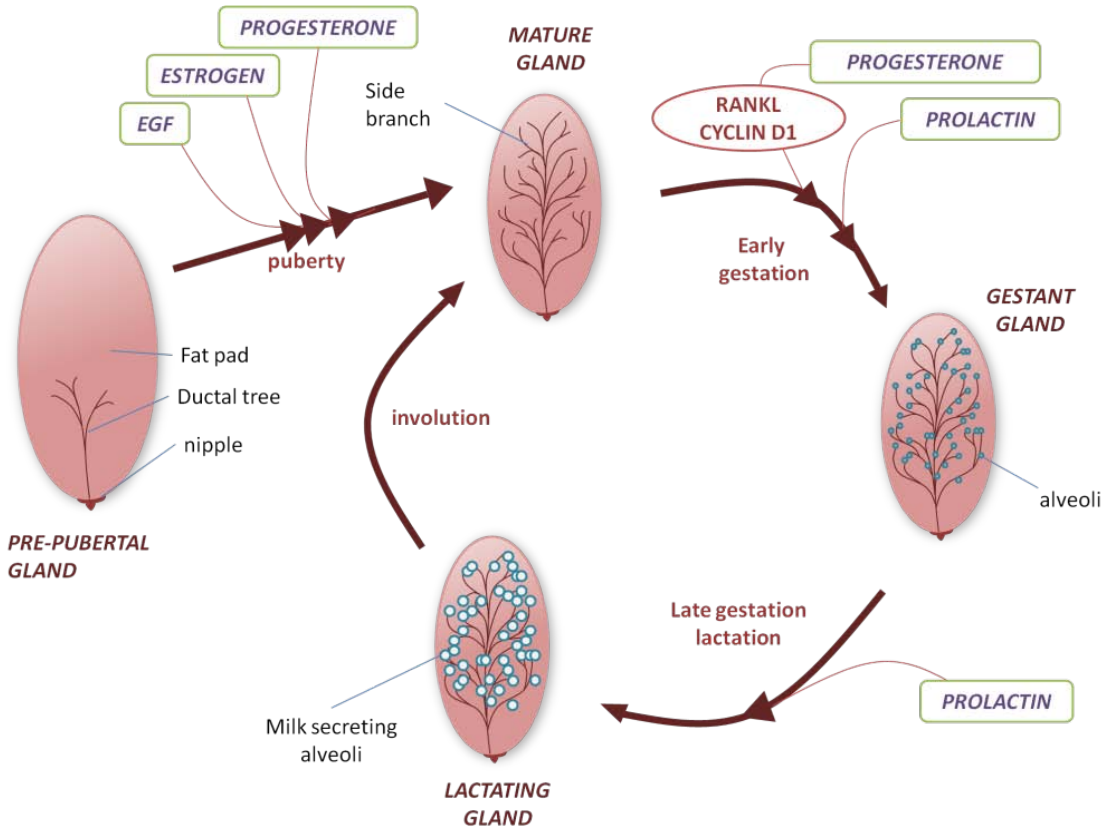


**Figure 1**. Mammary gland development. Schematic representation of gestation-lactation-involution cycle. A small ductal tree is visible in pre-pubertal mammary gland arising from the nipple. During PUBERTY ductal tree invades the mammary fat pad under hormonal stimuli. Alveoli formation occurs during EARLY GESTATION upon progesterone and prolactin stimulus. During LATE GESTATION prolactin induces alveolar differentiation into milk secreting structures in order to prepare the mammary gland for LACTATION. After this process mammary goes through epithelium remodeling/apoptosis and returns in the original "virgin-like" state in a process called INVOLUTION.

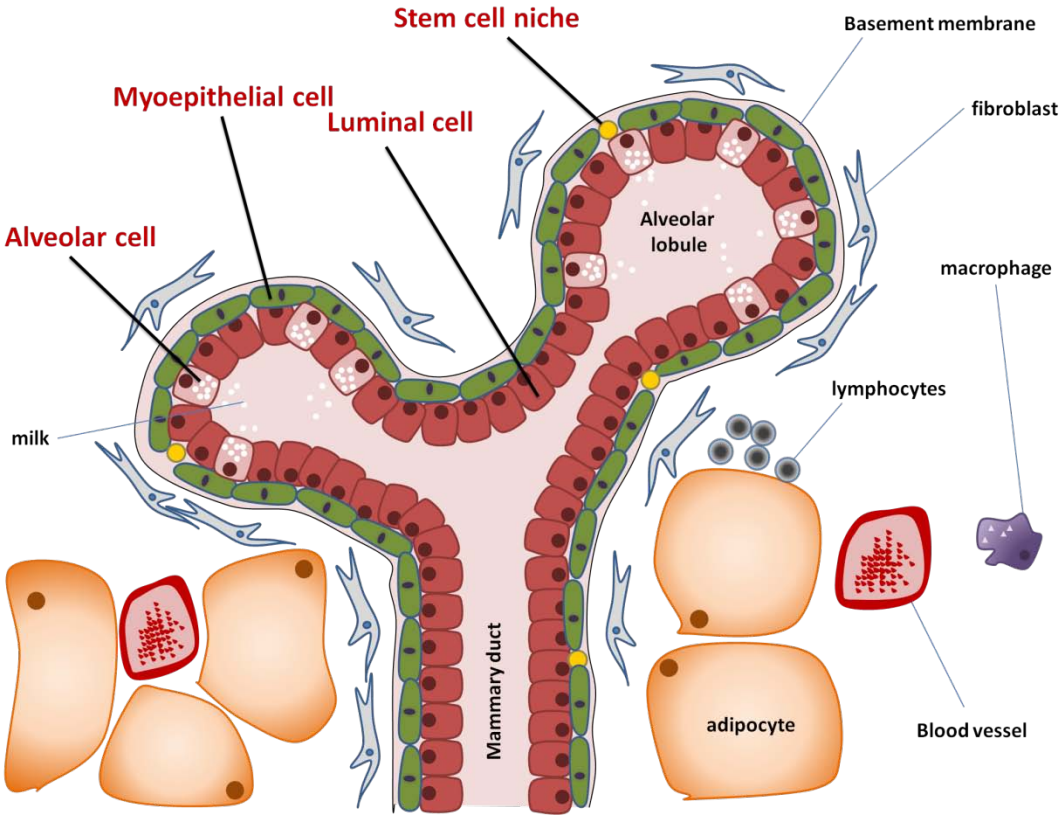


**Figure 2**. Mammary gland anatomy. Diagram of a section of mouse mammary gland. Epithelial ductal tree is visible with LUMINAL (red) and BASAL (layer). Luminal layer contains ALVEOLAR CELLS (pink) that, differentiate during gestation and secrete milk in the ALVEOLAR LOBULE. Basal layer is mostly composed of MYOEPITHELIAL CELLS and contains the MAMMARY STEM CELL NICHE. A continuous BASEMENT MEMBRANE separes epithelial cells from stroman non-epithelial cells such as FIBROBLASTS, ADIPOCYTES, BLOOD CELLS and cells of the IMMUNE SYSTEM.

## 1.2 Mammary epithelium hierarchy: mammary stem cells (MaSC)

The epithelium of the mammary glands exists in a highly dynamic state as it is able to differentiate during puberty and go through continuous cycles of pregnancy-involution in adult life. The plasticity of the epithelial structure was long cited as evidence for the existence of mammary stem cells. The mammary reconstitution assay through *in-vivo* transplantation is considered as the "gold-standard" for characterization of the mammary stem cell (Deome KB et al., 1959). Explants of fragments randomly resected from the epithelial tree reconstitute functional mammary glands when implanted in a recipient mouse, demonstrating the existence of a mammary repopulating pool (Hoshino K et al., 1967; Daniel CW et al., 1968). Epithelial outgrowths can be serially transplanted in multiple generations of recipient mice, evidencing the auto-renovation ability of mammary stem cells (Daniel CW et al., 1971). In order to investigate the clonal origin of mammary stem cells, isolated cell suspensions were labeled by infection with Mammary Tumor Virus (MMTV) and implanted in limiting dilutions. Serial transplantation of pieces of the clonally derived outgrowths produced second generation glands possessing the same viral insertion sites of the MMTV confirming the monoclonal origin of the outgrowth: the original stem cell has self-renewal ability (Kordon EC et al., 1998).

Isolation of epithelial cells by short enzymatic treatments, sorting by flow citometry and subsequent mammary repopulation assay legitimated the existence of stem cell enriched populations in the mammary epithelium (Shackleton M et al., 2006; Stingl J et al., 2006). A multipotent mammary stem cell is defined as a cell able to reconstitute an entire and functional mammary gland that contains all cell lineages of basal and luminal clusters. Stem cell enriched populations are isolated based on differential distribution of integrins and adhesion molecules (Table 1). These cells injected in limiting dilutions in mice have a higher mammary repopulating ability when compared with resting epithelial cells (Figure 3).

CD24 glycoprotein is heterogeneously expressed in the mammary gland. M. Smalley group identified three distinct cell populations: $CD24^{high}$, $CD24^{low}$ and $CD24^{negative}$; cytokeratin characterization demonstrated that these populations represented luminal epithelial, myoepithelial (basal) and non-epithelial cells, respectively. Mammary repopulation assays revealed that the $CD24^{low}$ population is enriched for mammary stem cells (Sleeman KE 2007).

Charaterization of β-1 integrin (CD29) and α-6 integrin (CD49f) distribution allowed a better identification of the mammary stem cell enriched population. Indeed, mammary repopulation ability was increased in cells derived from the basal $CD24^{low}$ cluster that express high levels of β-1 integrin (CD29) and α-6 integrin (CD49f) (Shackleton M et al., 2006; Stingl J et al., 2006). Therefore the mammary stem cell enriched population is considered $CD24^{low}CD29^{high}CD49f^{high}$ (Table 1). MaSC enriched population characterized by surface markers allowed the identification of single cells able to reconstitute an entire mammary gland *in-vivo* (Shackleton M et al., 2006). (Figure 3)

| | STEM CELL | BASAL PROGENITOR | MYOEPITHELIAL CELL | LUMINAL PROGENITOR | ALVEOLAR PROGENITOR | DUCTAL LUMINAL CELL | ALVEOLAR CELL |
|---|---|---|---|---|---|---|---|
| CD24 | LOW | LOW | HIGH | HIGH | HIGH | HIGH | HIGH |
| ALPHA 6 INTEGRIN (CD49f) | VERY HIGH | HIGH | HIGH | LOW | LOW | LOW | LOW |
| BETA 1 INTEGRIN (CD29) | VERY HIGH | HIGH | HIGH | LOW | LOW | LOW | LOW |
| ALPHA 2 INTEGRIN (CD49b) | | LOW | LOW | HIGH | | | |
| Sca-1 | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE | POSITIVE | |
| BETA-3 INTEGRIN (CD61) | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE | POSITIVE | NEGATIVE | NEGATIVE |
| HORMONE RECEPTORS (ER PR) | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE | POSITIVE OR NEGATIVE | NEGATIVE |
| PROMININ-1 (CD 133) | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE | NEGATIVE | POSITIVE | |

**Table 1**. Markers used in the fractionation of mammary epithelial cells.

## 1.3 Mammary epithelium hierarchy: luminal progenitors

Cells in the luminal CD24$^{high}$ compartment have lower expression of β-1 and α-6 integrins than basal cells. The so-called mammary colony forming cells (Ma-CFC) refer to those progenitors that produce discrete colonies of mammary CK8+CK5- cells in low-cell density adherent cultures, these cells have been described to reside mostly into the luminal CD24$^{high}$CD29$^{low}$CD49f$^{low}$ compartment (Stingl J et al., 2006) and have low mammary repopulation ability compared to MaSC (Figure 3).

The luminal population is hormone responsive as contains a subset of cells that express progesterone and estrogen receptors (PR and ER) (Petersen OW et al., 1987). Cells that express ER and PR are considered mature luminal cells as they showed low proliferation level in the adult mammary gland. Proliferating luminal cells are negative for hormone receptor suggesting that hormones act by paracrine signaling (Clarke RB et al., 1997; Russo J et al., 1999; Conneely OM et al., 2003).

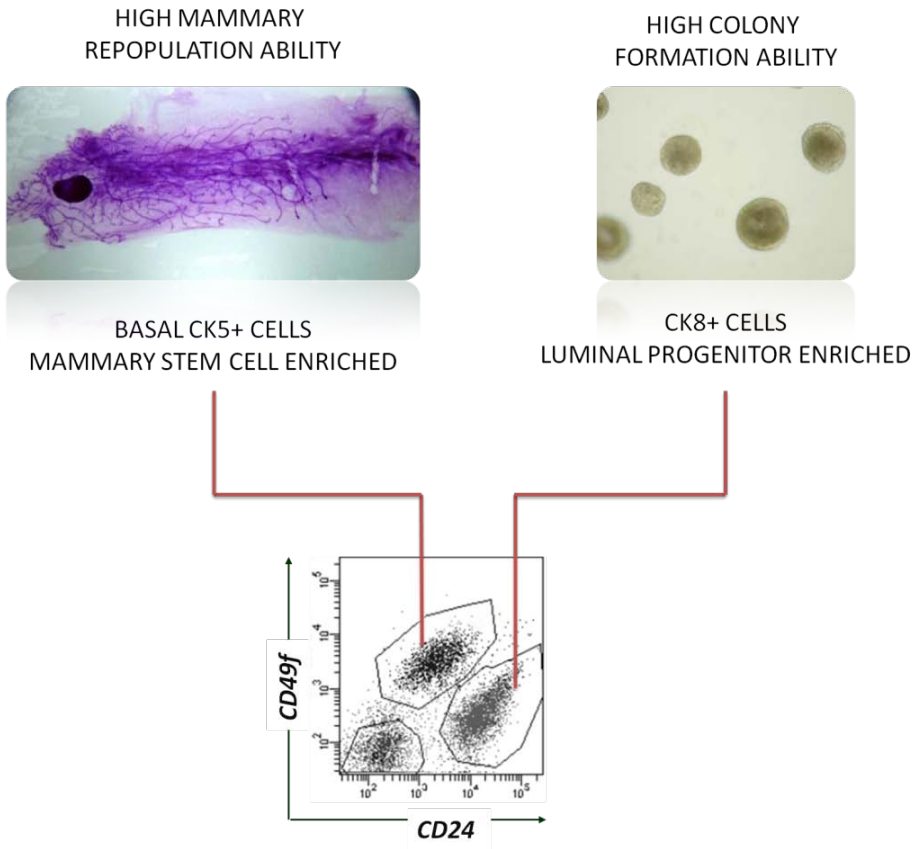


**Figure 3**. Luminal progenitors and mammary stem cells. Schematic representation of *in-vitro* and *in-vivo* assays to quantify mammary progenitor cell functionality. Mammary stem cells isolated from basal CD24$^{low}$CD49f $^{high}$ population show high mammary repopulation ability *in-vivo* as compared with other epithelial populations. Luminal progenitors, isolated from luminal CD24$^{high}$CD49f$^{low}$ population show high colony formation ability *in-vitro*.

The subset of luminal ER-PR- cells showed a higher colony forming ability *in-vitro* compared with luminal ER+PR+ cells suggesting that this population is enriched in a

subset of progenitor lineage-committed cells called luminal progenitors (Sleeman KE et al., 2007). These progenitors coincide with the previously called Ma-CFC.

Discovery of additional surface markers by flow citometry revealed a complex hierarchy within the luminal compartment (Table 1). Prominin-1 (CD133) and Sca-1 identify a population that express high levels of hormone receptors (ER, PR and PRL receptor) in the luminal $CD24^{high}$ cluster suggesting to be markers of luminal mature/hormone sensor cells (Sleeman KE et al., 2007). β-3 integrin (CD61) and α-2 integrin (CD49b) have been identified for the positive selection of luminal progenitors (Asselin-Labat ML et al., 2007; Li W et al., 2009). In fact $CD24^{high}CD49b^{+}$ cells and $CD24^{high}CD61^{+}$ showed an increased colony forming ability compared to luminal cells that do not express these markers. In particular CD61 identifies a subpopulation of luminal progenitors that during pregnancy is able to differentiate to alveolar $CD61^{-}$ cells; these cells express high levels of Elf-5, a key transcription factor of alveolar commitment (Oakes SR et al., 2008). Based on these discoveries it has been proposed a model of mammary epithelial hierarchy (Figure 4).
The process of mammary differentiation and preservation of mammary stem cell pool is regulated by different pathways. Notch signaling, Hedgehog signaling and polycomb proteins, which are common regulators of stem cell fate in many tissues, regulate MaSC pool self renewal and differentiation (Moraes RC et al., 2007; Bouras T et al., 2008; Pietersen AM et al., 2008).

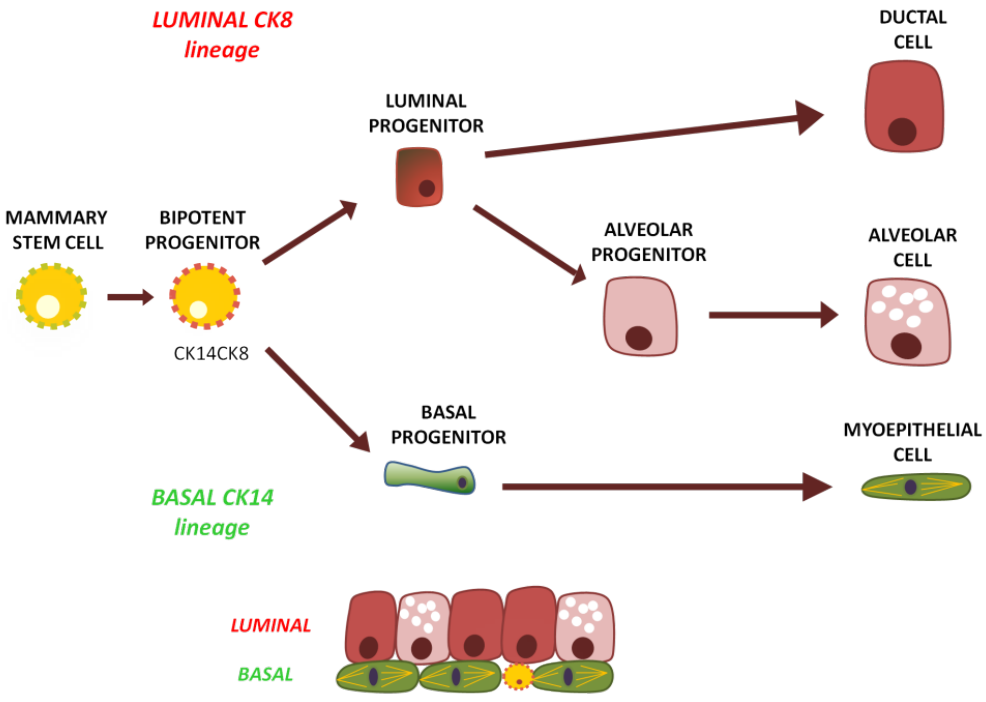


**Figure 4**. Mammary epithelium hierarchy. Model of the differentiation hierarchy in the mammary epithelium. Existence of a CK14 CK5 expressing MAMMARY STEM CELL in pre-natal mammary gland has been demonstrated. Differentiated DUCTAL, ALVEOLAR (gestation-specific lineage) and MYOEPITHELIAL CELLS compose an adult mammary epithelium. Lineage committed progenitors mediate the differentiation of the MAMMARY STEM CELL.

## 1.4 Hormonal and transcriptional regulation during pregnancy

Pregnancy is a process characterized by a rapid expansion of both epithelial and non-epithelial compartments and subsequent differentiation in order to prepare the mammary gland for milk secretion. Early gestation (days 2.5 to 6.5) is mostly a proliferative phase: serum levels of progesterone and PRL increase in order to stimulate massive proliferation of epithelial cells (Brisken C et al., 2002). During early pregnancy, PRL ensures the secretion of progesterone and moderate levels of estrogen through maintenance of the *corpus luteum*. Moreover PRL binds to its receptor in mammary epithelial cells and directly stimulates mammary proliferation and differentiation (Hennighausen L et al., 2005).

Common targets of progesterone and PRL signaling are Elf-5/STAT5, Gata-3, RANKL, WNT-4, amphiregulin and ID4 (Fernandez-Valdivia R et al., 2008). Elf-5 is a member of the Elf subfamily of Ets transcription factors and is expressed specifically in the mammary luminal epithelium. Elf-5 expression drastically increases during pregnancy (Harris J et al., 2006) and induces the expression of the milk specific-whey acidic protein WAP (Thomas RS et al., 2000) starting the alveolar switch.

At mid-gestation (days 14.5-15.5), when alveolar switch occurs, proliferation of the mammary epithelium decreases and a subset of luminal cells differentiate in alveolar cells. At this stage, progesterone plays a negative regulation on mammary differentiation. From mid to late gestation, a decrease in progesterone levels is associated with increased milk secretion and terminal differentiation of the alveolar cells (Barcellos-Hoff MH et al., 2000; Ismail PM et al., 2002). During late stages of gestation, the milk protein β-casein is expressed under PRL and glucocorticoids stimuli (Doppler W et al., 1990). Activated STAT5, interacts with β-casein promoter and enhancer (Groner B et al., 1994; Rosen JM et al., 1999). *In-vitro*, progesterone is able to interfere with PRL signaling, by physical interaction with STAT5 (Buser AC et al., 2011) leading to inhibition of β-casein expression.

After lactation, mammary gland returns to a virgin-like state. Although post-pregnancy mammary gland, morphologically resembles a virgin gland, it has been demonstrated that there are permanent alterations in gene expression pattern (Russo J et al., 2005) providing a molecular basis of pregnancy associated breast cancers (Schedin P et al., 2006).

## 1.5 Breast cancer

Breast cancer is the most diagnosed form of cancer and the second cause of death in women in the Western world (Weigelt B et al., 2005). Prognosis of breast cancer varies depending on cancer type, stage and treatment.

In 5% of cases, breast cancer is due to a strong inherited risk (Malone KE et al., 1998), harmful mutation of BRCA1 or BRCA2 genes increase from 60% to 80% the risk of developing breast cancer (Malone KE et al., 1998). In the majority of cases, breast cancer can be caused by multiple factors: environmental, genetic and hormonal.

Classification of breast cancers follows several different criteria: histopathology, clinical stage, histological grade, receptor status and molecular profile. Based on histology classification, the most common type is invasive ductal carcinoma. Invasive ductal carcinoma accounts for 50 to 75 percent of all breast cancers. Invasive lobular carcinoma is the next most common type and accounts for about 10 to 15 percent of cases . Tubular carcinoma and mucinous carcinoma are less common types of invasive breast cancer that tend to have a good prognosis (Dillon DA et al., 2010; Grube BJ et al., 2010).

Hormone receptor status is essential for characterization of breast cancer; breast cancer cells may or may not have three important receptors: ER, PR, and Her2. 70% of breast cancers the so-called luminal tumors contain cells that express ER and PR. Around 30% of breast tumors showed over-expression or amplification of Her2 (ErbB2) receptor and are called Her2+ tumors. Finally the remaining 10-15% of tumors lack in expression of ER, PR and Her2, these tumors are called triple-negative. Treatment of breast cancer includes various strategies such as radiotherapy, chemotherapy and hormone therapies. Luminal tumors tend to have the best prognosis; as these tumors express hormone receptors, treatments often include endocrine therapies. Her2+ tumors are prone to frequent metastasis and recurrence and can be treated with trastuzumab, a monoclonal antibody that targets Her2 receptor (McKeage K et al., 2002). Triple negative breast cancer have a poor prognosis targeted therapies do not exist; these tumors are treated with systemic chemotherapy (Sorlie T et al., 2003; Sørlie T et al., 2006).

Even though breast cancer is one of the best known cancers in terms of prevention, diagnosis and treatment, 30% of patients under treatment show lethal metastatic disease (Jemal A et al., 2010). Failure in treatment is due to the intrinsic heterogeneity of cancers and multiple mechanisms involved in tumor biology (Perou CM et al., 2000). For this reason a broad knowledge of molecular mechanisms underlying cancer is essential for the selection of new therapeutic targets and biomarkers.

## 1.6 Mouse models of metastatic breast cancer

The knowledge of metastatic breast cancer has greatly profited from the use of mouse models. Most of research studies in mammary gland biology are focused on mouse models because of their evolutional similarity to humans, short life span and easy handling. Mice have been genetically engineered to emulate molecular alterations found in human breast cancers, including inactivation of RB, TP53, BRCA1, over-expression of MYC and HER2/ERBB2/NEU and many others. Several strategies have been used to generate mammary tumors in mice, such as treatments with chemical carcinogens, targeted disruption of tumor suppressor genes, expression of dominant interfering proteins and over expression of oncogenes (Hennighausen L et al., 2000).

Mouse Mammary Tumor Virus (MMTV) promoter is used to target expression of the gene of interest in the mammary gland because of the natural tissue-specificity of this virus. MMTV promoter is hormonally regulated by glucocorticoids, progesterone and dihydrotestosterone (Otten AD et al., 1988). As these hormones act in mammary development, they activated the transgene specifically in the mammary epithelium (Muñoz B et al., 1989). The MMTV-LTR is active throughout mammary development and its transcriptional activity increases during pregnancy (Pattengale PK et al., 1989).

Comparisons of gene expression profiles between primary breast tumors and oncogene-driven mouse tumor models revealed similar mechanisms (Desai KV et al., 2002). However, no single mouse model recapitulated all the expression features of a given human subtype, probably because of species-specific pathway differences (Herschkowitz JI et al., 2007). In this paragraph, we will focus on the well characterized MMTV-neu and MMTV-PyMT models.

Neu is the rat ortholog of the tyrosine kinase receptor Her2(ErbB2); amplification of ErbB2 has been observed in invasive human ductal carcinoma (Allred DC et al., 1992) and is used as a clinically useful prognostic marker. Continuous tyrosine kinase activity in MMTV-neu mice leads to uncontrolled cell proliferation; tumors occur at median age of 8-

11 months (Bargmann CI et al., 1986) with frequent lung metastasis (Muller WJ et al., 1988; Guy CT et al., 1996) in pure-strains (fully inbred in FvB background). However, tumor latency can increase up to 18 months if mice have a mixed FvB x C57BL/6 genetic background (Rowse GJ et al., 1998).

MMTV-PyMT mouse model express the middle T protein of SV40 virus; this protein is able to activate multiple pathways such as RAS/MAPK and PI3K that are essential for regulation of cell proliferation, apoptosis, adhesion and migration. This leads to an aggressive multifocal tumor formation and metastatic lesions in lymph nodes and lungs (Guy CT et al., 1992). Tumor latency of MMTV-PyMT mice is very short, adenomas are observed in 3-weeks old mice (Guy CT et al., 1992). Based on the histological and molecular features, four distinctly identifiable stages of tumor progression occur in MMTV-PyMT mice: hyperplasia, adenoma/MIN (mammary intraepithelial neoplasia), early carcinoma and late carcinoma. These stages share many similarities with human tumorigenesis as tumor progression is accompanied by loss of ER and β1-integrin, increased expression of Her2/neu gene and Cyclin D1 (Maglione JE et al., 2001; Lin EY et al., 2003).

The MMTV-PyMT transgenic mice exhibits a high incidence of pulmonary metastasis. A unique feature of this tumor model is that the primary mammary tumor usually develops as a single focus on the ducts connected to the nipples (Lin EY et al., 2001). Afterwards PyMT protein expression on the whole mammary gland induces formation of multiple tumoral foci resulting in formation of heterogeneous tumors with different stages of progression.

Although end-stage MMTV-PyMT and MMTV-neu adenocarcinomas do not express hormone receptors ER and PR, cytokeratin profile and DNA microarrays revealed that these tumors are highly similar to human luminal tumors (Herschkowitz JI et al., 2007).

# 2. DISCOVERY OF RANK/RANKL SIGNALING

## 2.1 RANK and RANKL pathway in bone metabolism

Receptor Activator of Nuclear Factor ϰ-B (RANK), also known as TRANCE receptor, is a type I homo-trimeric trans-membrane protein which is ubiquitously expressed in many organs such as skeletal muscle, thymus, liver, colon, small intestine, adrenal gland, osteoclast, mammary gland, prostate and pancreas (Anderson DM et al., 1997; Yasuda H et al., 1998; Fata JE 2000; Theill LE et al., 2002; Boyle WJ et al., 2003).

Receptor Activator of Nuclear Factor ϰ-B ligand (RANKL) is a type II homo-trimeric trans-membrane protein that is expressed as a membrane-bound and a secreted protein. The latter is derived from the membrane form as a result of either proteolytic cleavage or alternative splicing (Ikeda T et al., 2001). RANKL is the only known ligand of the receptor RANK. It is highly expressed in lymph nodes, thymus and lung; low levels of RANKL can be detected in spleen, bone marrow, peripheral blood, leukocytes, heart, placenta, skeletal muscle, stomach and thyroid (Anderson DM et al., 1997; Wong BR et al., 1997; Yasuda H et al., 1998; Theill LE et al., 2002; Boyle WJ et al., 2003). Osteoprotegerin (OPG) is a member of the Tumor Necrosis Factor (TNF) receptor superfamily that binds to RANKL and functions as decoy receptor (Yasuda H et al., 1998).

RANK, RANKL and OPG play an essential role in bone metabolism and, in particular, in osteoclastogenesis (Kong Y et al., 1999; Dougall WC et al., 1999; Karsenty G et al., 2002). Mice with a disruption in either RANK or RANKL show severe osteopetrosis and defective tooth eruption resulting from a complete lack of osteoclasts, while OPG null mice are osteoporotic (Bucay N et al., 1998). These and other studies lead to the formulation of a model of bone turnover regulated by RANK/RANKL/OPG. RANKL, is expressed and secreted from osteoblasts (cells responsible for bone formation), binds to its receptor RANK, expressed on osteoclast precursors that differentiate into multinucleated bone resorping cells. OPG, secreted from osteoblasts, can bind to RANKL and compete for the activation of RANK receptor; by this way OPG can block osteoclast activation.

## 2.2 RANK/RANKL in bone metastasis

Given the essential role of RANK, RANKL and OPG on bone remodeling this pathway is deregulated in pathologic processes such as postmenopausal osteoporosis or cancer-induced bone destruction.

After menopause, decreased estrogen is associated with increased production of RANKL. The resulting increased osteoclast activity leads to increased resorption and decreased bone mass (Boyle WJ et al., 2003). Over time, this process leads to osteoporosis: compromised bone strength and increased risk of fracture throughout the skeleton. Bone is a relatively easy environment for metastatic colonization. Bone is rich of blood vessels for circulating metastatic cells; adhesive molecule on tumor cells can interact with stromal cells and production of bone-resorbing cytokines can result in a creation of metastatic niches. Many cancers metastasize to bone, among them, multiple myeloma, prostate and breast cancer have the highest rate of bone metastasis (Coleman R 1997).

Osteolytic lesions are the most common features of multiple myeloma, prostate cancer and breast cancer (Coleman et al., 1997). One of the most studied factors secreted by metastatic breast tumor cells is parathyroid hormone-related protein (or PTHrP). This hormone leads

to increased RANKL expression causing osteoclast activation and bone degradation. Bone matrix degradation releases growth factors that perpetuate tumor activity. This "vicious cycle" promotes tumor-cell growth, survival, angiogenesis, invasion and metastasis (Paget et al., 1989).

RANKL pathway plays a role in benign Giant Cell tumor: misbalanced expression of RANKL versus OPG resulted in a massive tissue destruction operated by osteoclast-like tumor cells (Huang L et al., 2000).

Based on the critical role of RANK/RANKL in osteoclastogenesis, a monoclonal antibody against RANKL, called Denosumab was developed and approved for clinical use by the Food and Drug Administration in 2010 (Yasuda H et al., 2013). The antibody is currently used to reduce risk of osteoporosis in postmenopausal women, for the prevention of skeleton-related events in patients with bone metastases from solid tumors (breast cancer and prostate cancer) and for treatment of unresectable giant cell tumor of bone.

# 3. RANK SIGNALLING IN MAMMARY DEVELOPMENT AND TUMORIGENESIS

## 3.1 RANK pathway in mammary gland development

In addition to the crucial function in bone metabolism, RANK/RANKL play an essential role in mammary morphogenesis. Progesterone up-regulates RANKL expression in virgin mice suggesting that RANKL expression is regulated by sex hormones during gestation (Fata JE et al., 2000).

Characterization of expression profile of RANK/RANKL revealed a strict regulation of the spatial and temporal distribution of RANK pathway during gestation. RANK and RANKL expression is relatively low in virgin mammary glands. RANKL expression increases at early gestation, the proliferative-phase of pregnancy in which progesterone signaling drives mammary epithelial expansion and morphogenesis; then RANKL gradually decreases during late gestation (Fata JE et al., 2000; Srivastava S et al., 2003). RANKL is expressed mostly in isolated luminal cells that are responsive to progesterone (Mulac-Jericevic B et al., 2003). RANK expression significantly increases during gestation, reaching a peak at day 15.5 and then decreases until day 1 of lactation (Gonzalez-Suarez E et al., 2007). Histological analysis of a midgestant (day 15.5) mammary gland revealed that RANK protein is expressed mostly in lobulo-alveolar structures. The first evidence for RANK pathway being essential in mammary gland formation came from deletion of either RANK or RANKL in mice. RANK or RANKL null mice showed normal glands at birth, that developed at puberty without any apparent defect (Fata JE et al., 2000). Nevertheless, during gestation RANK or RANKL null mammary glands showed a strong defect in mammary epithelial cell proliferation, survival, and development. This resulted in a complete failure in alveoli differentiation and milk production (Fata JE et al., 2000).

When RANK is over-expressed under the MMTV promoter, gestant mammary glands proliferate aberrantly and show a block in differentiation of lobulo-alveolar structures (Gonzalez-Suarez E et al., 2007). RANKL over-expression, resulted in an aberrant "gestant-like" phenotype in pubertal mice: mammary glands showed an extensive ductal side branching and alveoli (Fernandez-Valdivia R et al., 2009). Mouse models and expression analysis showed how a fine regulation of RANKL signaling is essential for a correct mammary development during pregnancy.

## 3.2 The RANKL-progesterone axis in mouse mammary gland development

The phenotype observed in RANK-KO and RANKL-KO mice shares many similarities with the PR knockout, PRL knockout and PRL receptor knockout mammary phenotypes (Lydon JP et al., 1995; Horseman et al., 1997; Brisken C et al., 1999; Oakes SR et al., 2008). All these models showed a lack in formation of a milk secreting mammary gland, evidencing that RANK signaling together with PRL and progesterone are essential for mammary cell differentiation during gestation. RANKL is a key mediator of progesterone signaling. Exogenous progesterone induces RANKL in the mammary gland (Fata et al., 2000). Interestingly, induced expression of RANKL rescues the lack of mammary ductal side branching and alveologenesis in PR knockout mice (Mukherjee A et al., 2010), legitimating RANKL as a mediator of progesterone induced mammary proliferation and

differentiation. These findings were further strengthened by the characterization of RANK signaling during gestation: as mentioned above, RANK expression increases during early to mid-pregnancy (Srivastava S et al., 2003; Gonzalez-Suarez E et al., 2007; Fernandez-Valdivia R et al., 2008) when progesterone stimulates proliferation and differentiation of mammary epithelial cells.

Moreover, it has been demonstrated that when RANKL is induced under progesterone stimulus, its expression is confined in ER+PR+ cells that are located near responder cells that express Cyclin D1 and proliferate (Mulac-Jericevic B et al., 2003; Fernandez-Valdivia R et al., 2009).

Progesterone elicits mammary epithelial cell proliferation by two mechanisms: direct and indirect (Beleut M et al., 2010). Direct mechanism occurs 24h after hormone stimuli: progesterone binds to its receptor on PR+ cells and induces proliferation by up-regulation of cyclin D1. Indirect mechanism occurs 72h after hormone stimuli. During this time progesterone induces expression of RANKL in PR+ cells. RANKL, by paracrine signaling binds to its receptor RANK on PR- cells leading to proliferation of these cells (Figure 5). A similar regulation occurs in the human mammary epithelium in specimens from healthy donors, RANKL is required for progesterone-induced mammary epithelial proliferation (Tanos T et al., 2013).

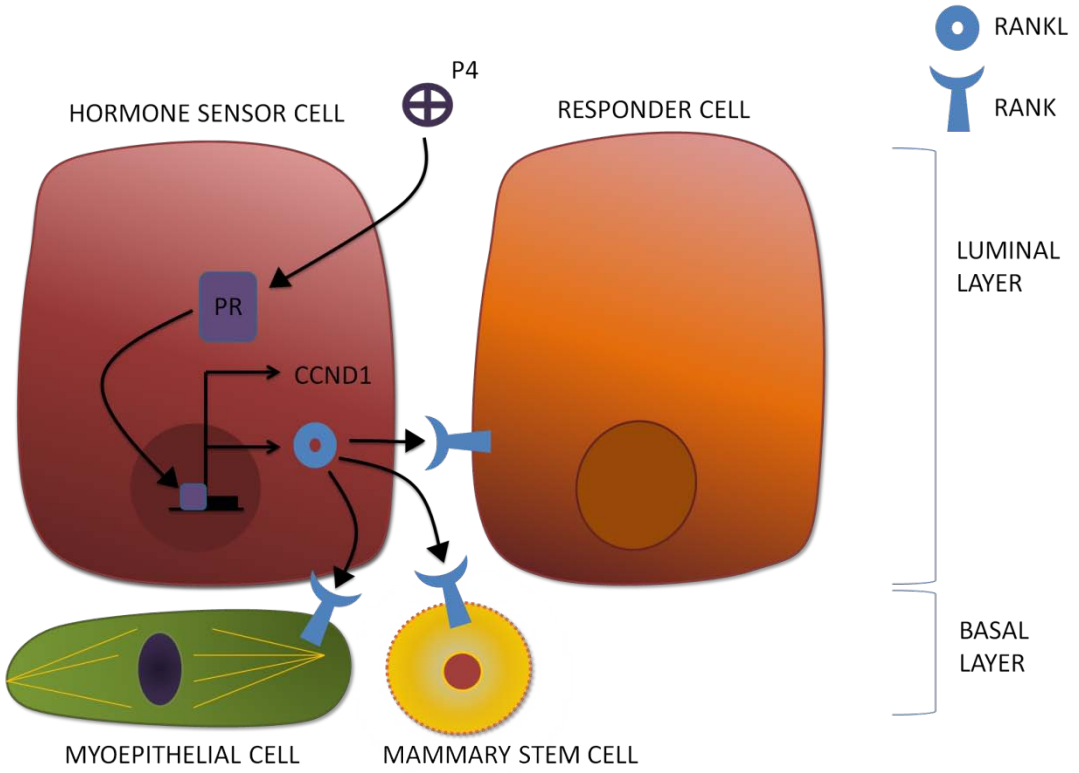


**Figure 5**. PR-RANKL signaling. Accredited model of paracrine signaling of RANKL under progesterone stimulus. Progesterone (P4) binds to its receptor (PR) in the mammary cytoplasm of a hormone sensor luminal cell. PR traslocate to the nucleus and lead the expression of target genes such as Cyclin D1 (CCND1) and RANKL. CCDN1 leads to proliferation of hormone sensor cell. RANKL binds to its receptor located to the proximal PR- luminal cells or to the basal cells (including Mammary Stem Cells) activating proliferation by paracrine effect.

## 3.3 The RANKL-progesterone axis in mouse mammary tumorigenesis.

The role of progesterone in inducing tumor formation has been elucidated during the last decade. PR deficient mice have less incidence of tumors than WT because mammary glands are not responsive to mitogenic signals of progesterone (Lydon JP et al., 1999). This supported that progesterone-driven mitogenicity is essential not only for normal mammary development but also in the promotion of tumorigenesis.

Evidence of a role of RANK/RANKL signaling in mammary cell transformation was first demonstrated *in-vitro*. RANKL stimulation of 3D acinar culture isolated from MMTV-RANK mice mammary cells resulted in increased proliferation, loss of milk secretion, impaired cell polarization and lumen formation (Gonzalez-Suarez E et al., 2007; Gonzalez-Suarez E et al., 2010).

To study the role of RANK/RANKL pathway in hormone-induced tumorigenesis, a carcinogen-induced murine mammary tumor model was used: carcinogenic protocol included an analogous of progesterone, Medroxyprogesterone (MPA) and the carcinogen 7,12-dimethylbenz[α]anthracene (DMBA) (Aldaz CM et al., 1996). The MPA, significantly increased RANKL expression in the ER+/PR+ "transmitter" cell population in the WT mammary epithelium as well as in pre-neoplastic lesions and hyperplasias (Gonzalez-Suarez E et al., 2010; Schramek D et al., 2010).

MMTV-RANK mice under DMBA-MPA treatment showed a decreased latency and higher incidence of ductal hyperplasias, MIN and adenocarcinomas as compared to wild-type mice. On the other hand, inhibition of RANKL signaling by treatment with RANK-Fc (competitive inhibitor of RANKL) decreased tumor incidence and increased tumor latency in MMTV-RANK mice under MPA-DMBA treatment (Gonzalez-Suarez E et al., 2010), (Figure 6A).

Strikingly, pharmacological inhibition of RANKL signaling by RANK-Fc treatment prevents formation of adenocarcinomas in wild-type mice under DMBA+MPA treatment (Figure 6B). Similarly to RANK-Fc treatment, specific deletion of RANK in the mammary gland, results in increased tumor latency and incidence of DMBA-MPA induced tumors (Schramek D et al., 2010). MPA-DMBA induced mammary adenocarcinomas in RANK deleted mice had impaired NF-κB pathway, and IKK-α deletion *in-vivo*, resulted in a decreased tumor latency, similarly to RANK deleted mice (Schramek D et al., 2010).
Anti-RANKL treatment reduces proliferation in normal mammary epithelia and pre-neoplastic lesions (hyperplasia); whereas in adenocarcinomas it results in increased apoptosis (Gonzalez-Suarez E et al., 2010). The impact of RANK/RANKL signaling in cell survival was further investigated by inducing cell apoptosis with γ-irradiation. MPA treatment has a protective effect against γ-irradiation induced apoptosis. Conversely RANK deleted mice abrogated this effect leading to a significant increase of cell death (Schramek D et al., 2010).

The role of RANKL signaling in spontaneous mammary tumors has been investigated in MMTV-neu mice. These tumors arise in absence of exogenous hormone stimuli and, as mentioned above, lack expression of ER and PR. RANK expression increases during tumor progression suggesting that RANK pathway may play a role in this process; however RANKL expression is undetectable in tumor cells in accordance with the loss of PR (Gonzalez-Suarez E et al., 2010). RANKL expression is mostly detected in the surrounding stroma and is described to play a role in tumor progression (Tan W et al., 2011). The role of RANK signaling in MMTV-neu tumor initiation and progression has been investigated treating adult mice with RANK-Fc before tumor formation. Blocking RANK signaling do

not affect to tumor latency, however it does significantly decrease the number of pre-neoplastic lesions, tumors and lung metastasis (Gonzalez-Suarez E et al., 2010). Conversely, RANKL increases the incidence of lung metastasis in MMTV-neu mice (Tan W et al., 2011).

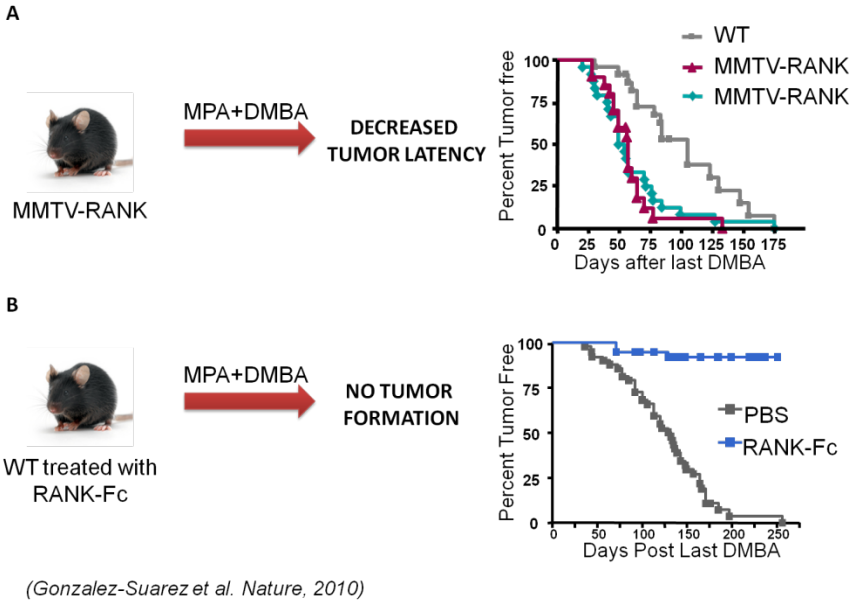


**Figure 6**. RANK signaling mediates progesterone-induced tumorigenesis. Kaplan-Meyer curves of tumor latency.
A. Tumor latency after DMBA-MPA treatment in WT mice and two independent strains of MMTV-RANK mice.
B. Tumor latency after MPA-DMBA treatment in WT mice treated with RANK-Fc or with PBS.

## 3.4 RANKL signaling in human breast cancer

RANK expression has been detected in several human cancer cell lines such as MDA-MB-231, MCF-7, T47D (Thomas RJ et al., 1999; Schramek D et al., 2010). *In-vitro*, RANKL promotes migration of human and mouse epithelial cells (Jones DH et al., 2006). Treatment with RANKL in SKBR3 cell line resulted in marked protection from cell death in response to γ-irradiation and doxorubicin (Schramek D et al., 2010).
In breast tumors, mutations of RANK/RANKL have not been identified so far, however a genetic variant of RANK gene has been strongly associated with breast cancer risk in patients carrying mutation of BRCA2 (Bonifaci N et al., 2011). Expression levels of RANK and RANKL proteins in human breast cancer are controversial probably because commercial antibodies are not fully specific and because of the lack of negative/positive controls. RANK expression was detected in most of primary breast cancers, in local lymph node metastasis and in bone metastasis (Bhatia P et al., 2005; Jones DH et al., 2006). However, Gonzalez-Suarez and collaborators reported high levels of RANK in only 6% of primary breast tumors (Gonzalez-Suarez E et al., 2010). RANKL expression in tumor cells was reported in 11% of tumors analyzed. Similarly to mouse models RANKL was often detected in sporadic infiltrating mononuclear cells in 67% of tumor stroma and in fibroblast-like cells in the stroma of rare tumors (Gonzalez-Suarez E et al., 2010). Within the stroma, RANKL expression is higher in invasive ductal carcinomas (55%) than in carcinomas in situ (8%) or lymph node positive tumors (22%) (Tan W et al., 2011). Expression of RANKL in epithelial compartment is inversely correlated with tumor

progression, in correlation with loss of PR expression in carcinomas . RANKL is expressed in 90% of normal breast, in 62% of non-metastatic intraductal carcinomas, 31% of metastatic intraductal carcinomas and 2% of bone metastasis (Bhatia P et al., 2005).

# 4. The role of RANKL signaling in mammary stem cell fate and tumor initiation

## 4.1 RANKL signaling and hormone regulation of mammary stem cell fate

MaSCs are highly responsive to hormones. During mouse estrous cycle and pregnancy, when sex hormones orchestrate mammary epithelial remodeling, mammary stem cell population increases 14 fold and 11 fold, respectively (Asselin-Labat ML et al., 2010). MaSC population decreases significantly after resection of mice ovaries, due to hormone depletion (Asselin-Labat ML et al., 2010; Joshi PA et al., 2010).

RANK is expressed in both basal and luminal compartments, although more abundantly in the basal compartment (Asselin-Labat ML et al., 2010; Joshi PA et al., 2010). RANKL expression, as mentioned above, is restricted to the ER+PR+ luminal cells (Beleut M 2010).

MaSCs enriched cells do not express ER and PR and reside in the basal/myoepithelial compartment while ER+PR+ cells reside in the luminal compartment (Asselin-Labat ML et al., 2006; Joshi PA et al., 2010). When RANK is deleted in mammary gland, progesterone stimuli does not induce basal cell expansion (Schramek D et al., 2010) suggesting that RANK/RANKL pathway is mediating basal cell proliferation. Anti-RANKL antibody treated mammary epithelial basal cells isolated from mid-gestant mice showed decreased colony formation suggesting that RANK signaling controls mammary stem cell function (Asselin-Labat ML et al., 2010).

The most accredited hypothesis is that RANKL derived from luminal hormone sensor cells acts as a paracrine mediator to the basal PR- MaSC enriched compartment similarly to the paracrine signal described in the luminal compartment (Beleut M et al., 2010; Joshi PA et al., 2010), (Figure 5).

## 4.2 Tumor initiating cells: cancer could be a stem cell disease

The cancer stem cell (CSC) model postulates a hierarchical organization of tumor cells such that a small subset is characterized by stem-cell like characteristics such as self-renewal and ability to regenerate a new tumor *in-vivo*. The hypothesis that cancer is caused by a population of cells with stem-cell characteristics called tumor-initiating cells (cancer stem cells) has recently attracted a great deal of attention. Identification of cancer stem cells relies on different strategies: expression of surface markers, *in-vitro* assays such as anchorage-independent growth assays (tumorspheres assays) and limiting dilution assays *in-vivo*. In human breast cancer, existence of a small subset of cells with a high tumor initiation ability (Al-Hajj M et al., 2003) oriented research toward identification of breast tumor initiating cells. CSC have been reported to resist to chemotherapy and radiotherapy (Bao S et al., 2006); current therapies, generally target highly replicating cancer cells, that constitute the bulk of the tumor and may not kill CSC.

RANKL pathway mediates mammary gland development and tumor formation driven by progesterone (Gonzalez-Suarez E et al., 2010; Schramek D et al., 2010) and might mediate MaSC expansion (Asselin-Labat ML et al., 2010). Breast cancer cells from MPA and DMBA-treated RANK-defective mice formed primary but not secondary tumospheres,

suggesting that a loss of RANK expression markedly impairs the self-renewal capacity of cancer stem cells (Schramek D et al., 2010). Furthermore, blockage of NFκB activity, which is the main downstream effector of RANK/RANKL pathway, leads to a decrease in tumor formation ability and anchorage independent growth *in-vitro* in MMTV-neu mice (Cao Y et al., 2007). These findings suggest that RANK/RANKL signaling could be important for tumor-initiating cell expansion and activation; further studies will be needed to elucidate these questions.

# 5. Tumor immunology

Tumor microenvironment is an important factor in tumor biology and modulates tumor initiation, progression and response to therapy. Cells and molecules of the immune system play a key role in tumor microenvironment. Myeloid and lymphoid cells can exist in different bioactive state and influence each other in a not fully understood way. Tumor cells are also able to modulate the immune response and its progression or regression will depend on the balance between pro-tumorigenic and anti-tumorigenic environment (Figure 7).

## 5.1 Lymphoid cells

Historically leukocyte tumor infiltrates were considered as an attempt of the immune system to block tumor progression. In fact cytotoxic CD8+ T lymphocytes and natural killer cells can eliminate tumor cells. However other immune cell types and molecules can modulate and antagonize the anti-tumor activity, allowing tumor progression (Ostrand-Rosenberg S et al., 2008). Indeed T helper CD4+ cells, macrophages, B cells, dendritic cells and mast cells can play multiple roles based on their differentiation state (Stout RD et al., 1989; Watkins S et al., 2007; Ostrand-Rosenberg S et al., 2008), (Figure 7).

Unlike the cytotoxic anti-tumor response of CD8+ T lymphocytes, CD4+ T helper lymphocytes can infiltrate tumors and foster anti-tumor o pro-tumor response. CD4+ T lymphocytes are heterogeneous populations that play a key role in orchestrating the adaptive immune response by activating macrophages, controlling CD8+ cytotoxic activity and B cell antibody class switching. Classically CD4+ T lymphocytes stay in an anergic (inactive) Th0 state and can differentiate in Th1 or Th2 commitments based to the exposure to interleukins IL-12 and IL-4 respectively. Th1 cells secrete pro-inflammatory cytokines IFN-γ, TNF-α, IL-2 and IL-12 (Munk et al., 1995). This cytokine profile results in a increased immune surveillance: up-regulating antigen presentation by antigen presenting cells (APCs), activation of cytotoxic response of macrophages and direct elimination of tumor host by high levels of IFN-γ and TNF-α (Romagnani S et al., 1997). Therefore Th1 cell infiltrates induce an anti-inflammatory and anti-tumor program. Conversely, Th2 T lymphocytes express anti-inflammatory cytokines IL-4, IL-5, IL-6, IL-10 and IL-13. These molecule induce T cell anergy, reduce cytotoxic activity of T cells and macrophages and induce humoral immune responses by B cells (Parker DC et al., 1993; Pollard JW et al., 2004). Th2 are considered anti-inflammatory cells that can foster tumor growth. *In-vivo*, high ratio of Th2 to Th1 correlates with increased tumor progression in breast cancer (Chin Y et al., 1992). (Figure 7)

In addition Th17 is a subset of CD4 T helper cells which differentiation is induced by IL-6 and TGF-β exposure and secrete IL-17, IL-21 and IL-22 (Weaver CT et al., 2006). Th17 cell role in tumorigenesis is controversial as it has been associated with pro-tumorigenic and anti-tumorigenic effects (Zou W et al., 2010).

CD4+CD25+FoxP3+ Tregulatory (Treg) lymphocytes are also known as T suppressor cells for their ability to maintain tolerance to self-antigen and avoid autoimmune disease. Immunosuppressive activity of Treg cells can facilitate tumor escape from immune response and is generally associated with poor prognosis in human cancers (Wilke CM et al., 2010). Treg cells suppress cytotoxic responses of CD8 T cells, natural killers and reduce antigen presentation of dendritic cells (Trzonkowski P et al., 2004; Strauss L et al., 2007) by secreting immunosuppressive cytokines TGF-β, IL-10 and IL-35.

## 5.2 Myeloid cells

Cells of myeloid origin such as granulocytes (neutrophils, basophils and eosinophils), dendritic cells, macrophages and natural killer cells are involved in tumor response and they can play opposite roles inducing or antagonizing tumor progression by an intricate cytokine trafficking with tumor cells and lymphoid cells.

Tumor associated macrophages (TAMs) can enhance angiogenesis and metastasis of mammary tumors (Qian B et al., 2009), secrete IFN-γ and other factors that directly kill mammary tumor cells (Stout RD et al., 1989). Macrophages can exist in two bioactive states stimulated by T helper cells: Th1 lymphocytes can activate classical macrophages (M1) while Th2 lymphocytes can induce alternative macrophages (M2) activation (Ostrand-Rosenberg S et al., 2008). M1 macrophages secrete high levels of IL-12 and low levels of IL-10, M1 macrophages enhance cytotoxic activity, produce proinflammatory cytokines and increase antigen presentation capacity (Ostrand-Rosenberg S et al., 2008). M2 macrophages, also called tumor associated macrophages, produce high levels of IL-10 and they stimulate invasion and suppression of immune responses (Mantovani A et al., 2007).

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of cells, expanded in pathological conditions such as cancer and inflammation, that share the expression of Gr1 marker. These cells suppress immune response by many different molecular mechanisms. Most of immunosuppressive activity of MDSCs has been associated with the secretion of Arginase-1 and TGF-β (Gabrilovich DI et al., 2009).

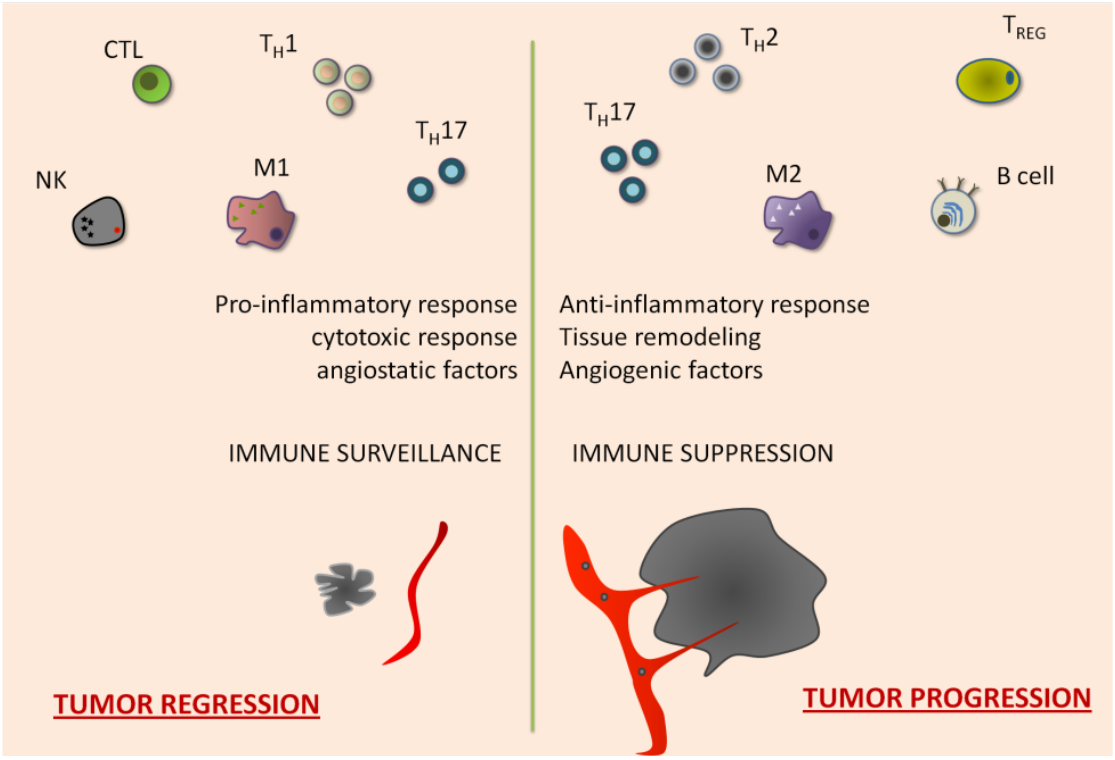


**Figure 7.** Cancer immunology. Schematic classification of immune cell types based on tumor immune response. NK: Natural Killer, Th1: T helper 1, Th2: T helper 2, M1 classical macrophages, M2: alternative macrophages, Th17: T helper 17, Treg: Tregulatory cells, CTL: cytotoxic T lymphocytes.

# OBJECTIVES

# OBJECTIVES

Based on the relevance of RANK pathway in mammary development and progesterone dependent tumor formation, the objectives of this thesis are:

1. Characterize the role of RANK in mammary stem cell fate and alveolar commitment.

2. Investigate the role of RANK in spontaneous tumor prone mouse models MMTV-PyMT and MMTV-neu.

3. Investigate the role of RANK in Tumor Initiating Cell pool (TICs).

4. Analyze RANK expression in tumor samples of breast cancer patients.

# ARTICLE 1

“Constitutive activation of RANK in the mammary gland disrupts mammary cell fate leading to tumorigenesis”

## PEER REVIEWED ARTICLE

Pellegrini P, Pasquale P, Cordero A, Alex C, Gallego MI, Marta Ines G, et al. Constitutive activation of RANK disrupts mammary cell fate leading to tumorigenesis. Stem Cells. 2013 Sep;31(9):1954–65. DOI: 10.1002/stem.1454

## AUTORSHIP DECLARATION

Authors Pasquale Pellegrini and Alex Cordero Casanovas equally contributed to the study "Constitutive activation of RANK disrupts mammary cell fate leading to tumorigenesis" (Stem Cells. 2013 Sep;31(9):1954-65). Alex Cordero characterized the amplification of the mammary epithelium observed in MMTV-RANK model by whole mount analysis and cytokeratin quantification; Pasquale Pellegrini characterized the anomalies observed in distribution of epithelial cytokeratins. Afterwards both authors characterized mammary progenitor enriched populations by flow cytometry, and the functional activity of mammary progenitors using colony forming assays in-vitro and mammary repopulation assays in-vivo. Alex Cordero focused on the characterization of the MMTV-RANK driven tumorigenesis analyzing tumor histological heterogeneity and distribution of epithelial surface markers by flow citometry. Pasquale Pellegrini evaluated the impact of RANK signaling in physiological conditions by characterizing the correlation between RANK expression and accumulation of mammary progenitors in aged WT mammary glands and in WT tumors.

# ANNEX 1

“RANK signaling at midgestation impairs Elf-5 induced alveolar cell fate”

# RANK signaling at midgestation impairs Elf-5 induced alveolar cell fate

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AUTHOR CONTRIBUTIONS. Pasquale Pellegrini: Collection and/or assembly of data, data analysis and interpretation, manuscript writing. Alex Cordero: Collection and/or assembly of data; data analysis and interpretation; final approval of manuscript. Eva Gonzalez-Suarez: Conception and design, financial support, collection and/or assembly of data, data analysis and interpretation, manuscript writing and final approval of manuscript.

CONFLICT OF INTEREST
Authors declare no conflict of interest.

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KEY WORDS: RANK, RANKL, Elf-5, alveolar cell, prolactin, progesterone

# ABSTRACT

RANK signaling is essential for the differentiation of the mammary gland and in particular for the alveolar commitment. We demonstrate that increased RANK signaling during pregnancy results in a significant reduction of Elf5 expression, the transcription factor that controls the alveolar switch, in accordance with the lactation failure observed in MMTV-RANK mice. Conversely, blockage of RANK signaling at midgestation in WT mice results in precocious alveolar differentiation, increased expression of Elf5 and the milk protein WAP. Overall, we demonstrate that RANK signaling impairs secretory differentiation during pregnancy through Elf5 downregulation.

# RESULTS AND DISCUSSION

Most of mammary gland development occurs in puberty and pregnancy. The pituitary hormone prolactin (PRL) is a master regulator in driving pregnancy and lactation. Prolactin and progesterone both orchestrate the proliferation and differentiation of the mammary gland during gestation (Hennighausen L et al., 2001). Differentiation of a secretory mammary gland depends on the prensence of Prolactin receptor (PrlR) and the downstream JAK2-STAT5 pathway (Hennighausen L et al., 2001). PRL binds to its receptor and activates JAK2/STAT5 signaling, phosphorylated STAT5 binds to consensus DNA binding sites inducing expression of milk proteins encoding genes β-casein and WAP (Oakes SR et al., 2008). Elf-5 transcription factor is expressed in mammary luminal cells and specifies the differentiation of CD61+ progenitors to establish the secretory alveolar lineage during pregnancy (Oakes SR et al., 2008). Expression of Elf-5 is regulated by prolactin signaling and increases during gestation and lactation (Harris J et al., 2006). Elf-5-null and heterozygous mammary glands show reduced STAT5 expression/phosphorylation and impaired alveologenesis (Choi YS et al., 2009; Zhou J et al., 2005) whereas Elf-5 overexpression results in secretory alveoli and milk production in virgin mice. Deletion of STAT5 in the mammary gland results in a failure of alveologenesis and lactation (Liu X et al., 1995). As MMTV-RANK mice lack alveolar secretory differentiation during mid-gestation (Fata JE et al., 2000; Gonzalez-Suarez E et al., 2007) we investigated the mechanisms underlying the observed defect in alveologenesis.

We have recently reported that luminal $CD24^{hi}CD49f^{low}$ cells extracted from virgin MMTV-RANK mammary glands have decreased mRNA levels of Elf-5 (Pellegrini P et al., 2013). Given the relevance of Elf-5 in the alveolar switch, expression of Elf-5 was analyzed in the glands of MMTV-RANK pregnant females and the corresponding controls. In contrast to WT (Oakes SR et al., 2008) no expression of Elf-5 protein was found in MMTV-RANK mammary glands at G16,5 in correlation with the lack of secretory differentiation (Figure 1) suggesting that over-activation of RANK signaling at mid-gestation disrupts alveolar cell fate through Elf-5. Next, we investigated the role of RANK pathway in the alveolar switch under physiological conditions. Hence, RANK signaling was inhibited at specific time points of gestation in WT mice using the pharmacologic inhibitor of RANKL, RANK-Fc (Gonzalez-Suarez E et al., 2010). RANKL expression is significantly upregulated at midgestation (Gonzalez-Suarez E et al., 2007; Srivastava S et al., 2003) therefore, pregnant WT mice were injected with RANK-Fc at gestation day G9.5 and G13.5 and mammary glands were harvested and analyzed 24h later (Fig. 2A).

Histological analysis of the mammary glands showed that inhibition of RANKL signaling induced precocious alveolar secretory differentiation at G10,5 and enhanced secretory alveologenesis at G14,5, as compared to mock injected animals (Fig. 2B).

Blockage of RANKL led to premature (G10.5) or enhanced (G14.5) expression of the milk-specific protein WAP, consistent with the morphological changes observed (Fig. 3B). Colony-forming ability of mammary cells from G10,5 RANK-Fc injected animals was reduced when compared to control cells (Fig. 3A) suggesting that luminal cells were in a more differentiated state.

A significant increase in Elf-5 mRNA expression was observed 24h after RANK-Fc treatment at time-points G10.5 and G14.5 as compared to control mice at the same stage while STAT5 mRNA expression was slightly higher in RANK-Fc-treated mice than in controls at G14.5 (Fig. 3B). Accordingly, analysis of protein expression confirmed increased Elf-5 protein expression in the mammary cells from RANK-Fc treated mice than in corresponding controls (Fig. 3C). These results indicate that inhibition of RANK signaling at mid-gestation is required for the induction of Elf-5 that initiates alveolar secretory differentiation and milk production.

We had previously shown that secretory alveolar differentiation is impaired in MMTV-RANK mice leading to lactation failure (Gonzalez-Suarez E et al., 2007; Srivastava S et al., 2003). Our findings indicate that the decline in the CD61+ alveolar progenitors (Pellegrini P et al., 2013), together with the defective induction of Elf-5 at midgestation contributes to the failure of alveologenesis and lactation in MMTV-RANK mice.

Recently it has been demonstrated that in virgin mammary glands RANKL mediates the progesterone induced Elf-5 expression in CD61+ luminal cells, leading to luminal differentiation (Lee HJ et al., 2013).

In contrast, here we demonstrate that at midgestation, inhibition of RANKL signaling in WT mice induces terminal alveolar differentiation and milk production through up regulation of ELF-5. These results shed further light on the role of RANK signaling in mammary alveolar differentiation and provide a rationale for the apparently paradoxical phenotypes of impaired alveolar differentiation and lactation failure in RANK KO and MMTV-RANK mice (Fata JE et al., 2000; Gonzalez-Suarez E et al., 2007).

This dual role of RANKL in virgin and midgestant mice is similar to the essential role of progesterone in early pregnancy: it drives expansion of stem cells by paracrine signals to generate progenitors required for side branching and alveologenesis; however, during mid-to-late pregnancy, progesterone suppresses secretory activation mediated in part by crosstalk between PR and prolactin/STAT5 (Buser AC et al., 2007). In fact progesterone can suppress PrlR expression in late pregnancy mammary glands (Nishikawa S et al., 1994). This evidence demonstrates a role for progesterone in repressing functional alveologenesis and milk protein gene expression during pregnancy (Neville MC et al., 2001).

We concluded that progesterone-RANKL axis during early pregnancy is essential for mammary epithelial proliferation and side branching and probably for the generation of the intermediate progenitors that will later differentiate into secretory cells, whereas at mid-gestation the pathway must be shut down to allow Elf-5 induction and the final secretory differentiation. These observations extend the known connections between RANKL and PR signaling in mammary epithelium beyond mitogenesis and mammary stem cell fate.

# MATERIALS AND METHODS

## Mice

All research involving animals was performed at the IDIBELL animal facility and complied with protocols approved by the IDIBELL Committee on Animal Care. MMTV-RANK mice (FvB) and RANK-Fc were obtained through collaboration with Dr Bill Dougall (Oncology Research-AMGEN). RANK-Fc (10 mg/Kg of mouse, AMGEN) was injected 24 hours before sacrifice.

## Tissue section histology and Immunostaining

Mammary glands and tumors were fixed in 4% PFA (paraformaldehyde) or formalin and embedded in paraffin. For histological analysis, 3 μm sections were cut and stained with hematoxylin and eosin (H&E). Immunostaining was performed on 3 μm mammary gland/tumor sections. Antigen heat retrieval with Tris-EDTA was used for Elf-5 (N-20, sc-9645, Santa Cruz) primary antibody was incubated overnight at 4ºC. The antigen antibody complexes were detected with streptavidin horseradish peroxidase (PK-6100 Vector Laboratories) Peroxidase was finally revealed with DAB (00035 DAKO).

## Colony forming assays

For colony-forming assays, sorted cells were plated in matrigel cultures as previously described (Stingl J et al., 2006) in growth medium (Stingl J et al., 2006) that contains B27, 5% FBS, EGF (10 ng/mL), Hydrocortisone 0.5 μg/mL, Insulin 5 μg/mL, Cholera toxin 100 ng/mL and Penicilin/Streptomiciyn and RANKL (1 μg/ml; Amgen Inc) as indicated.

## RNA preparation and Quantitative RT–PCR

Total RNA of mammary glands was prepared with Tripure Isolation Reagent (11667165001 Roche) in accordance with the manufacturer's instructions. Single-stranded cDNA was produced by reverse transcription using 1 μg of RNA in a 20-μL reaction (N8080234 Applied Biosystems). Quantitative PCR was performed using the TaqMan probe-based system WAP (Mm00839913_m1) ELF-5 (Mm00468732_m1) and STAT5 (Mm00839861_m1) assays on demand were used on the ABI 7900HT (Applied Biosystems).

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# FIGURES

## Figure 1

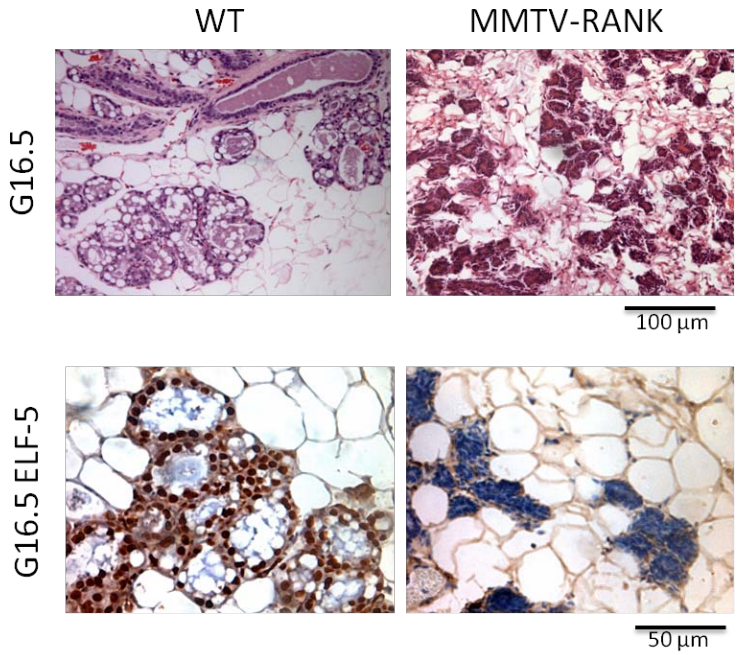


**Figure 1**. MMTV-RANK mice do not express Elf-5 at mid-gestation. Representative H&E and ELF-5 immunostaining images at G16.5 of WT and MMTV-RANK mammary glands.

## Figure 2

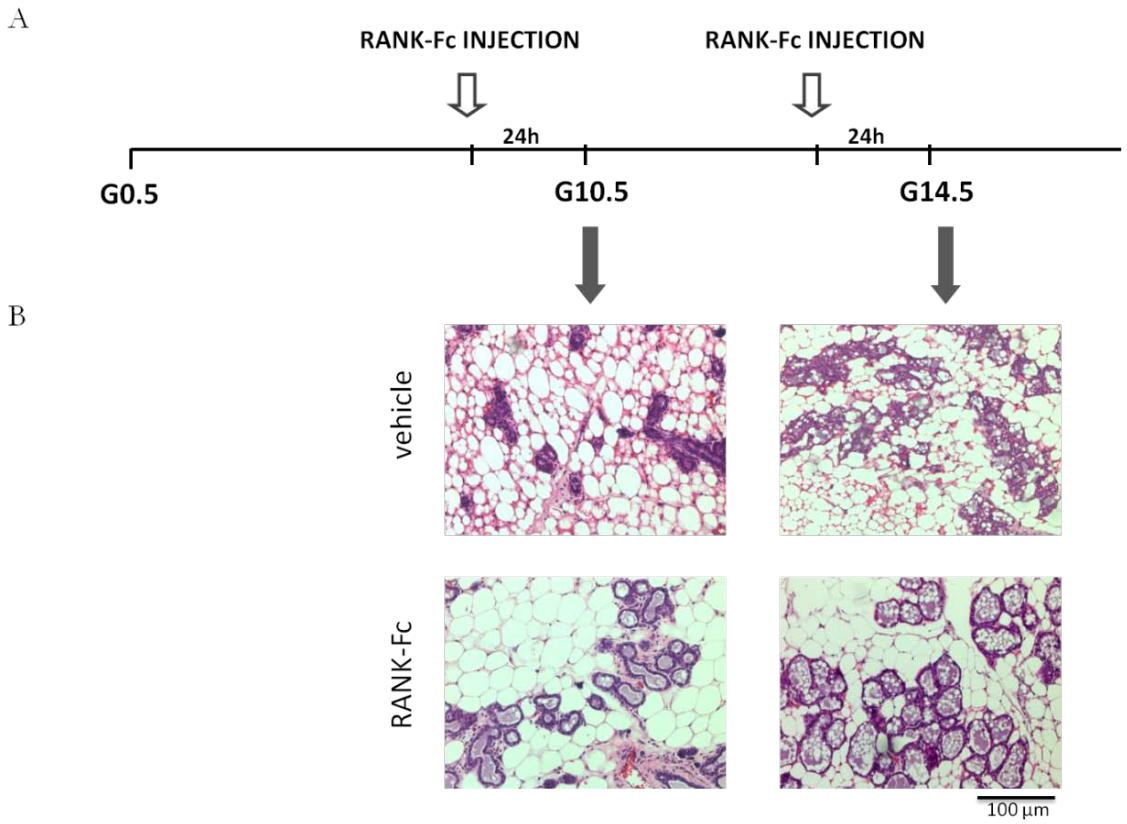


**Figure 2**. Inhibition of RANK signaling induces alveolar differetiation

A. Inhibition of RANK signaling induces alveolar differentiation. Schematic representation of RANK-Fc experiment.

B. Representative images of H&E of WT mice at G10.5 and G14.5 24h after treatment with RANK-Fc or serum (vehicle). At least three mice per treatment were analyzed.

## Figure 3

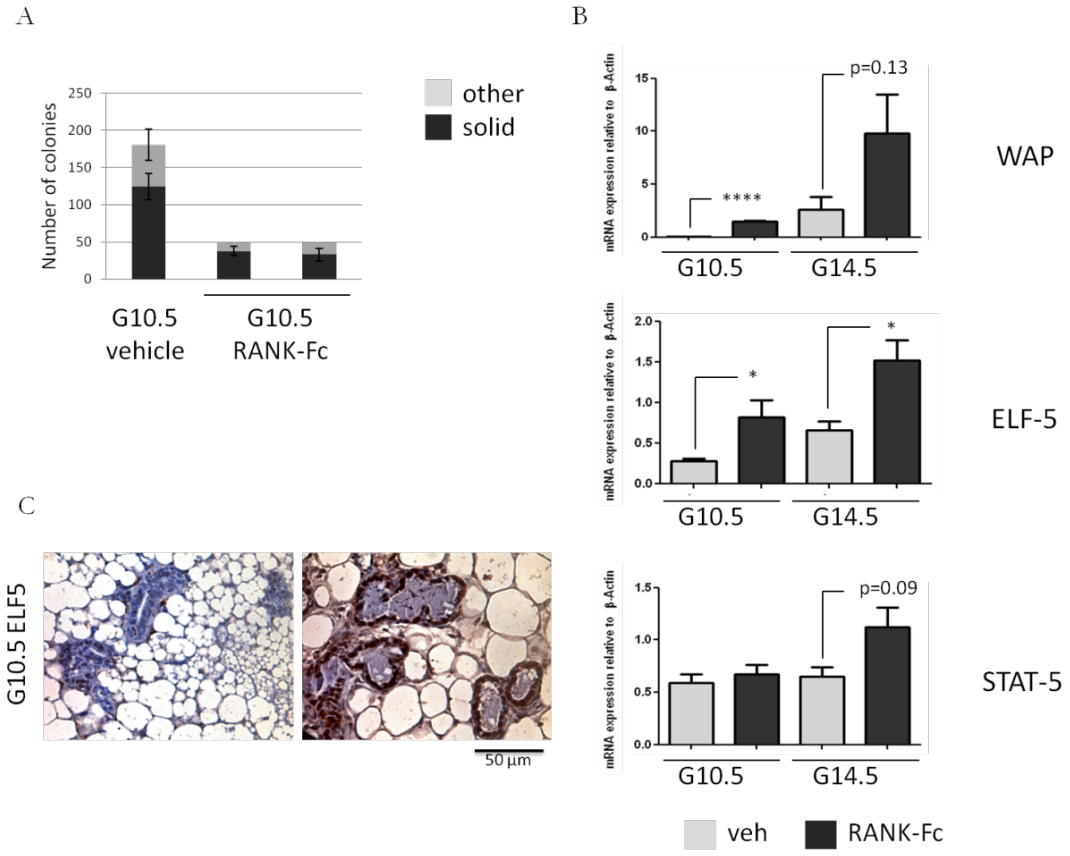


**Figure 3.** RANK-Fc induces alveolar differentiation through Elf-5.
A. Number of colonies in matrigel formed by G10.5 MECs 24h after treatment with serum (vehicle) or RANK-Fc.
B. Wap, Elf-5 and STAT5 mRNA expression relative to β-actin in G10.5 and G14.5 WT mammary glands, 24h after treatment with serum (vehicle) or RANK-Fc. Each bar represents mean values for three mice; SEM and p values are shown. Measurements for each sample were performed in triplicate and mean values were used.
C Representative images of ELF-5 immunostaining at G10.5 in RANK-Fc treated WT mice and controls.

## ARTICLE 2

“RANK signaling expands the pool of tumor and metastasis initiating cells and attenuates the anti-tumor immune response in advanced breast cancer”

Per motiu de l’acord de confidencialitat subscrit amb les entitats participants en aquesta tesi s'han eliminat de la versió electrònica les pàgines 82, 83, 104, 120, 121 i 122

# RANK signaling expands the pool of tumor and metastasis initiating cells and attenuates the anti-tumor immune response in advanced breast cancer

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AUTHOR CONTRIBUTIONS.
Pasquale Pellegrini: Collection and/or assembly of data, data analysis and interpretation, manuscript writing. Alex Cordero and Guillermo Yoldi: Collection and/or assembly of data; data analysis and interpretation; final approval of manuscript. Eva Gonzalez-Suarez: Conception and design, financial support, collection and/or assembly of data, data analysis and interpretation, manuscript writing and final approval of manuscript.

CONFLICT OF INTEREST
Authors declare no conflict of interest.

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KEY WORDS: RANK, RANKL, MMTV-PyMT, MMTV-neu, tumor initiating cell, metastasis.

# ABSTRACT

RANK is a key pathway of mammary differentiation and mediates progesterone induced mammary tumorigenesis in mice. However, the therapeutic impact of targeting RANK signaling in advanced tumors is unknown. We demonstrate that RANK is highly expressed in tumors from two widely used models of spontaneous tumorigenesis: MMTV-neu and MMTV-PyMT. Stimulation of RANK signaling in cells derived from MMTV-neu and MMTV-PyMT established tumors promotes tumor cell proliferation, survival and invasion.
Given that activation of RANK signaling expands mammary stem cells and induces spontaneous tumorigenesis in the mammary gland, we investigate the impact of pharmacological inhibition of of RANK pathway in cancer stem cells and the tumor microenvironment and we demonstrate that neoadjuvant inhibition of RANK signaling reduces the tumor initiating ability and modulates immune response in favor of an anti-tumor response.
Genetic deletion of RANK in MMTV-PyMT tumor model delays tumor onset, decreases tumor incidence, attenuates growth rate and blocks lung metastasis. Limiting dilution assays demonstrate a significant reduction in the metastasis initiating cell pool of tumor cells lacking RANK.
In summary, RANK signaling expands the pool of tumor and metastasis initiating cells and attenuates the anti-tumor immune response in advanced breast cancer. Our results extend the use of RANKL inhibitors for therapeutic intervention in breast cancer beyond the management of bone metastasis disease and suggest that RANKL inhibition in the neoadjuvant setting may reduce the risk of relapse and metastasis.

# INTRODUCTION

RANK signaling plays an essential role in the control of proliferation and differentiation of the mammary epithelia during mouse pregnancy (Fata JE et al., 2000; Gonzalez-Suarez E et al., 2007). RANKL mediates the mytogenic effects of progesterone in mouse (Fata JE et al., 2000; Beleut M et al., 2010) and human mammary epithelia (Tanos T et al., 2013). RANK overexpression in the mammary epithelial cells results in increased proliferation, impaired mammary alveolar differentiation and spontaneous tumor formation after multiple pregnancies (Gonzalez-Suarez E et al., 2007; Pellegrini P et al., 2013).

It has been recently shown that RANK signaling regulates mammary stem cell fate. RANKL mediates progesterone induced expansion of the mammary stem cell enriched population in mice (Schramek D et al., 2010; Asselin-Labat ML et al., 2010). Characterization of mammary populations in MMTV-RANK mice revealed an accumulation of MaSC and intermediate progenitors, that could underlie the spontaneous mammary tumor development observed in these mice (Pellegrini P et al., 2013). In human breast cell lines, RANK over-expression increases the frequency of $CD44^{+}CD24lo/^{-}$, a phenotype ascribed to human breast cancer stem cells (Palafox M et al., 2012).

We and others have shown that RANK/RANKL pathway mediates progesterone induced mammary tumorigenesis in mice (Gonzalez-Suarez E et al., 2010; Schramek D et al., 2010). After DMBA (dimethylbenz(a)anthracene) and MPA (medroxiprogesterone) treatment MMTV-RANK mice show a shorter latency and a higher incidence of preneoplasic lesions and adenocarcinomas as compared to wild-type (WT) mice, whereas mice with genetic deletion of RANK in the mammary gland show the opposite phenotype (Gonzalez-Suarez E et al., 2010; Schramek D et al., 2010). Moreover, pharmacological treatment with RANK-Fc, which binds to RANKL and blocks RANK/RANKL signaling, similarly to OPG, attenuates mammary tumorigenesis in transgenic MMTV-RANK mice and completely blocks the occurrence of tumors in WT mice. High levels of RANK induces hallmarks of transformation and invasion in untrasformed human cell line MCF10A (Palafox M et al., 2012), while RANK overexpression in cells with not functional BRCA1 increased tumor growth and metastasis (Palafox M et al., 2012). In primary human breast cancer, expression of RANK is associated with reduced overall survival, accelerated bone metastasis formation (Santini D et al., 2011) and aggressive tumor phenotypes (Palafox M et al., 2012).

MMTV-neu mice bear an amplification of the oncogene neu, the rat ortholog of the human Her2 (ErbB2) that results in mammary tumor formation and lung metastasis (Muller WJ et al., 1988). Her2 is amplified in more than one fourth of human breast cancers resulting in poor prognosis (Slamon DJ et al., 1987). Preventive treatment with RANK-Fc decreased the number of tumoral foci and lung metastasis in MMTV-neu mice (Gonzalez-Suarez E et al., 2010).

Mammary specific expression of the polyoma virus middle T protein in the MMTV-PyMT mouse model results in aggressive widespread transformation of the mammary gland with multifocal adenocarcinoma and high lung metastasis incidence (Guy CT et al., 1992). MMTV-PyMT is clinically relevant because it resembles the luminal B subtype of breast cancer, based on its genomic profile (Herschkowitz JI et al., 2007). Nevertheless advanced carcinomas in this model are negative to sex hormones receptors, similarly to carcinomas in MMTV-neu mice (Maglione JE et al., 2001).

Cancer stem cells are a generally small proportion of tumor cells with stem cell-like characteristics such as auto-renovation and ability to differentiate in different cancer cell types (Reya T et al., 2001). Such cells are proposed to persist in tumors as a distinct

population and cause relapse and metastasis by giving rise to new tumors (Merlos-Suarez A et al., 2001; Overdevest JB et al., 2011). The identification of tumor initiating cells in MMTV-PyMT and MMTV-neu mice remains elusive despite recent efforts (Asselin-Labat ML et al., 2011; Malanchi I et al., 2011; Schwab LP et al., 2012; Lo PK et al., 2013).

Microenvironment plays an important role in tumor progression; immune cells do not always play against tumor cells. Although some subsets of leukocytes certainly exhibit antitumor activity, such as cytotoxic T lymphocytes (CTLs), other leukocytes, most notably mast cells, B cells, dendritic cells, granulocytes, and macrophages, exhibit more bipolar roles, by virtue of their capacity to either hinder or potentiate tumor progression (de Visser KE et al., 2006; Mantovani A et al., 2007). In particular, a Th1 subset of T cells, secrete cytokines that induce inflammation resulting in cytotoxic anti-tumor response; Th2 subset secretes anti-inflammatory cytokines that induce immune suppression resulting in a pro-tumor response (DeNardo DG et al., 2007). In addition to T helper response, T regulatory cells can induce suppression of immune responses allowing tumor progression (Wilke CM et al., 2010).

In breast cancer, increased infiltration of B cells, T regulatory cells (Treg) and high ratio of Th2/Th1 lymphocytes correlates with higher tumor grade/stage and poor overall survival (Coronella-Wood JA et al., 2003; Kohrt HE et al., 2005; Bates GJ et al., 2006). In the MMTV-PyMT model depletion of macrophages delays tumor progression and dramatically reduces metastasis whereas an increase in macrophage infiltration by transgenic means remarkably accelerated these processes (Lin EY et al., 2001).

Signaling through RANK and its ligand RANKL are important for the development and activation of osteoclasts, activation of monocytes and macrophages, survival of dendritic cells, lymphocyte costimulatory signals and Treg- induced self-tolerance (Dougall WC et al., 1999; Josien R et al., 2000; Padigel UM et al., 2003; Seshasayee D et al., 2004; Biswas SK et al., 2010). RANK signaling is required for lymph node development as RANK and RANKL null mice lack lymph nodes (Kong et al.,. 1999; Dougall WC et al., 1999).

These data have prompted us to evaluate the hypothesis that RANK could enhance the activity or survival of the tumor- or metastasis-initiating cells using MMTV-neu and MMTV-PyMT established tumors and we investigate the impact of RANK signaling in the tumor microenvironment. Our results legitimated RANK signaling as a novel pathway in regulation of the cancer stem cell pool and tumor immune responses.

# MATHERIALS AND METHODS

## Animals

All research involving animals was done at IDIBELL animal facility and complied with protocols approved by the IDIBELL Committee on Animal Care. SCID/Beige, NOD/SCID and FvB/N mice were provided by Charles River, *Foxn1$^{nu}$* were provided by Harlan. MMTV-PyMT mice were provided by the Mouse repository (National Cancer Institute). MMTV-neu mice were provided by Jackson Laboratory.

MMTV-PyMT$^{-/+}$;RANK$^{-/-}$ mice were obtained by crossing MMTV-PyMT (FvB/N) strain with RANK$^{-/+}$ (C57BL/6) (Dougall WC et al., 1999) mice as shown in Sup. Fig. 12A. Genotyping was performed by PCR analysis of ear biopsy genomic DNA. RANKL-LZ, OPG and RANK-Fc reagents and RANK$^{-/+}$ (C57BL/6) mice were obtained through collaboration with Dr Bill Dougall (AMGEN Inc). Animals bearing tumors with diameter bigger than 1 cm were considered as endpoint criteria for sacrifice.

## Mammary cell isolation

Single cells were isolated from tumors as previously described (Smalley MJ et al., 2010). Briefly, fresh tissues were mechanically dissected with McIlwain tissue chopper and enzymatically digested with appropriate medium (DMEM F-12, 0.3% Collagenase A, 2.5U/mL dispase, 20 mM HEPES, and antibiotics) 30 minutes at 37°C. Samples were washed with Leibowitz L15 medium 10% fetal bovine serum (FBS) between each step. Erythrocytes were eliminated by treating samples with hypotonic lysis buffer (10-548E, Lonza Iberica). Single epithelial cells were isolated by treating with trypsin (L11-004; PAA Laboratories) 2 minutes at 37°C. Cell aggregates were removed by filtering cell suspension with 40 μm filter and counted.

## Colony forming assays

Isolated tumor cells were plated as previously described (Shackleton M et al., 2006) for colony forming assays on irradiated 3T3-L1 fibroblasts; briefly, 500 epithelial cells/well were resuspended in growth medium that contains 5% FBS, EGF 10 ng/mL (E9644, Sigma-Aldrich), hydrocortisone 0.5 μg/mL (H-0888, Sigma-Aldrich), insulin 5 μg/mL (I-1882, Sigma-Aldrich), cholera toxin 100 ng/mL (C-8052 Sigma-Aldrich) and penicillin/streptomycin (15070-063, Invitrogen). After 6 days of culture, colonies were fixed with PFA 2% and stained with hematoxylin-eosin for quantification.

For 3D acinar cultures, isolated cells (10.000/well) were plated on top of 45 μL of Growth-Factor-reduced (GFR) matrigel (354230, BD Biosciences), in growth medium for 24h and then changed to differentiation medium that contains DMEM F-12, prolactin 3μg/mL (L6520, Sigma-Aldrich), hydrocortisone 1 μg/mL, ITS (insulin, transferring, selenium; I3146, Sigma-Aldrich), cholera toxin 100 ng/mL and penicillin/Streptomycin as previously described (Hathaway HJ 1996). Medium was replenished three times a week and maintained in culture for 15 days. 200.000 cells on 350 μL/well of GFR matrigel were cultured in a 6-well plate for RNA isolation and *in-vivo* assays. Acini diameter was quantified with ImageJ software (Wayne Rasband, NIH).

## Tumorsphere culture

RANK-Fc treated or serum treated MMTV-PyMT tumors were digested and filtered to obtain single cells. Single cells were resuspended in serum-free DMEM F12 mammosphere medium containing 20ng/mL of EFG, 1x B27 and 4μg/mL heparin (H3149, Sigma-Aldrich) as previously described (Dontu G et al., 2005). Primary tumorspheres were derived by plating 30.000 cells/ mL in 4mL of medium into cell-suspension culture plates. After 20 days, tumorspheres were isolated by 5 min treatment with PBS-EDTA 5 mM + 5 min of trypsin at 37C° and plated for secondary tumorsphere at a concentration of 5.000 cells/ mL in triplicate in 6-well suspension cultures. Individual spheres from each replicate well were counted under a microscope and t-tests with SEM were calculated.

## *In-vivo* short term treatments and growth/metastasis assays from acinar cultures

For short-term experiments RANKL (25μg/mouse, Amgen Inc, Thousand Oaks, CA, USA) or RANK-Fc (10 mg/Kg; Amgen Inc, Thousand Oaks, CA, USA) were injected subcutaneously 3 times a week for 2 weeks in tumor bearing MMTV-PyMT females (10-14 weeks old) and MMTV-neu females (33-77 weeks-old). Treatments started when tumors were approximately 14.1 $mm^3$ (calculated as $4/3^* n r^3$) in MMTV-PyMT mice and 65.4 $mm^3$ in MMTV-neu mice. Mice were sacrificed and tumors were recovered 24h after last injection for sampling and cell isolation.

For *in-vivo* assays with MMTV-PyMT acinar cultures, matrigel was dissolved by treatment with cold PBS-EDTA 5mM for 25 min on ice, washed with PBS and tumor cells were obtained after digestion with trypsin 5 minutes at 37°C. 100.000 tumor cells were diluted 1:1 in matrigel matrix (254234, BD Biosciences) and injected in the 4$^{th}$ mammary fat pad of 4 weeks old *Foxn1$^{nu}$* and SCID/Beige mice for growth assays; animals were palpated twice weekly until euthanasia. For metastatic assays 100.000 isolated MMTV-PyMT tumor cells were diluted in 200uL of cold PBS and injected in the tail vein of 4-weeks old *Foxn1$^{nu}$* mice.

## Limiting dilution assays

For all tumor limiting dilution assays mammary epithelial cells were isolated and diluted 1:1 in matrigel matrix (254234, BD Biosciences) and injected in a final volume of 40 μL in the inguinal mammary fat pad.

For RANK-Fc experiments in orthotopic tumors, $10^6$ MMTV-PyMT (FvB/N) isolated tumor cells were injected in 18 (12-weeks old) syngenic WT FvB/N females (P1). 24h after tumor cell injection, mice were randomized 1:1 and injected 3 times a week (4 weeks in total), with either RANK-Fc (10 mg/Kg) or with serum (control). 24h after last RANK-Fc injection, tumors were surgically excised; cells derived from 4 serum or RANK-Fc treated tumors were isolated, mixed in two pools (serum and RANK-Fc treated pool) diluted in matrigel and injected in mammary glands of 6-weeks old FvB/N females (P2). Injections were performed with limiting dilution number of cells (100.000, 10.000, 1.000 and 100); for each dilution 6 mice randomized 1:1 were injected with either RANK-Fc (10 mg/Kg) or with serum 3 times a week for 2 weeks in total. Mice were monitored for tumor formation during 38 weeks.

Limiting dilution assay of MMTV-PyMT$^{-/+}$;RANK$^{-/-}$ and MMTV-PyMT$^{-/+}$;RANK$^{+/+}$ (FvB/N; C57BL/6) tumor cells was done in 4 weeks old *Foxn1$^{nu}$* mice. Cells isolated from two primary tumors of two mice per genotype were pooled and injected in limiting dilution (10.000, 1.000, 100 and 10 cells) in mammary fat pad of *Foxn1$^{nu}$* mice (4 mice per dilution).

In all assays tumor initiating potential was defined as the ability to form palpable, growing tumors of at least >2mm of diameter.

For metastasis assays tumor cells isolated from MMTV-PyMT$^{-/+}$;RANK$^{-/-}$ and MMTV-PyMT$^{-/+}$;RANK$^{+/+}$ females (1 mouse per genotype) were resuspended in 200 μL of cold PBS and injected intravenously in limiting dilution (100.000, 10.000, 1.000 and 100 cells) in 4-weeks old *Foxn1$^{nu}$* mice (4 mice per dilution). Mice were sacrificed 8 weeks after cell injection and lungs recovered for histological analysis.

## Gene expression analysis

Total RNA of tumors and matrigel cultures was prepared with Tripure Isolation Reagent (11667165001 Roche); RNA was purified in accordance with the manufacturer's instructions. Matrigel cultures were dissolved with cold PBS-EDTA (5 mM) on ice for 25 min. Matrigel-free cell suspensions were then pelleted at maximum speed and resuspended in TriPure Isolation Reagent for RNA isolation.

Frozen tumor tissues were fractionated using glass beads, (G1152, Sigma-Aldrich) and PrecCellys® 24 tissue homogenizer (Berting Technologies) with the following protocol: 2 cycles 30s 5500rpm, pause 30s. Homogenized tissue was then resuspended in Tripure Isolation Reagent.

cDNA was produced by reverse transcription using 1 μg of RNA in a 35-μL reaction following kit instructions (N8080234, Applied Biosystems). Quantitative PCR was performed using the TaqMan probe-based system on the ABI 7900HT (Applied Biosystems) for mRANK (Mm00437135_m1) and mRANKL (Mm00441908_m1). Q-PCR of remaining genes was performed on Lightcycler 480 II (Roche) using LightCycler® 480 SYBR green MasterMix (04707516001, Roche). Primer sequences are indicated in **TABLE 1**.

**TABLE 1**

| NAME | | SEQUENCE 5'→3' |
|---|---|---|
| BIRC2 | FWD | GAAGAAAATGCTGACCCTACAGA |
| | REV | CATGACGACATCTTCCGAAC |
| BIRC3 | FWD | AGAGAGGAGCAGATGGAGCA |
| | REV | TTTGTTCTTCCGGATTAGTGC |
| BCL2A1B | FWD | ATACGGCGGAATGGAGGT |
| | REV | TCCCAGAACTGTCCTGTCATC |
| ICAM1 | FWD | CCCACGCTACCTCTGCTC |
| | REV | GATGGATACCTGAGCATCACC |
| VCAM1 | FWD | TGGTGAAATGGAATCTGAACC |
| | REV | CCCAGATGGTGGTTTCCTT |
| MMP9 | FWD | ACGACATAGACGGCATCCA |
| | REV | GCTGTGGTTCAGTTGTGGTG |
| TGF-β | FWD | TCACTGGAGTTGTACGGCAGTG |
| | REV | TCCCGTTGATTTCCACGTG |

| | | |
|---|---|---|
| IL12 | FWD | AAGAACGAGAGTTGCCTGGCT |
| | REV | TTGATGGCCTGGAACTCTGTC |
| IL13 | FWD | CAGCATGGTATGGAGTGTGGA |
| | REV | GGTCCTGTAGATGGCATTGCA |
| FoxP3 | FWD | AGAAGCTGGGAGCTATGCAG |
| | REV | GCTACGATGCAGCAAGAGC |
| TNF-α | FWD | CCAGACCCTCACACTCAGATC |
| | REV | CACTTGGTGGTTTGCTACGAC |
| iNOS | FWD | CAGCTGGGCTGTACAAACCTT |
| | REV | CATTGGAAGTGAAGCGTTTCG |
| Arginase-1 | FWD | CTCCAAGCCAAAGTCCTTAGAG |
| | REV | AGGAGCTGTCATTAGGGACATC |
| IL-1β | FWD | CAACCAACAAGTGATATTCTCCATG |
| | REV | GATCCACACTCTCCAGCTGCA |
| IL-2 | FWD | CCTGAGCAGGATGGAGAATTACA |
| | REV | TCCAGAACATGCCGCAGAG |
| IFN-γ | FWD | CACGGCACAGTCATTGAAAG |
| | REV | CCATCCTTTTGCCAGTTCCTC |
| IL6 | FWD | TGATGGATGCTACCAAACTGG |
| | REV | TTCATGTACTCCAGGTAGCTATGG |
| IL10 | FWD | CAGAGCCACATGCTCCTAGA |
| | REV | TGTCCAGCTGGTCCTTTGTT |
| Bmi-1 | FWD | CCGGGATCTTTTATCAAGCA |
| | REV | ATGAAGTACCCTCCACACAGG |
| Sox-9 | FWD | TCCGGCATGAGTGAGGTG |
| | REV | TCAGATCAACTTTGCCAGCTT |
| Axin-2 | FWD | TGTGAGATCCACGGAAACAG |
| | REV | GTGGCTGGTGCAAAGACATA |
| Hif-1α | FWD | TCATATCTAGTGAACAGAATGGAACG |
| | REV | CCACTCTCATCCATTGACTGC |
| Gata-3 | FWD | GAAACTCCGTCAGGGCTAC |
| | REV | TCCAGCCAGGGCAGAGAT |
| Notch-1 | FWD | TGACCTGCTCACTCTCACAGA |
| | REV | ATTGGCACAGGGGTTGGA |
| Hey-1 | FWD | CCGACGAGACCGAATCAATA |
| | REV | AGGTGATCCACAGTCATCTGC |
| CK14 | FWD | TGAGAGCCTCAAGGAGGAGC |
| | REV | TCTCCACATTGACGTCTCCAC |
| CK8 | FWD | ATTGACAAGGTGCGCTTCCT |
| | REV | CTCCACTTGGTCTCCAGCATC |
| Sma-1 | FWD | TGATCACCATTGGAAACGAACG |
| | REV | TGGTTTCGTGGATGCCCGCT |
| Fibronectin | FWD | CTGGGACTGTACCTGCATCG |
| | REV | CTCCACTTGTCGCCAATCTT |
| Vimentin | FWD | CATTGAGATCGCCACCTACAG |
| | REV | AGGAGTGTTCTTTTGGAGTGG |
| PP1A | FWD | CAAATGCTGGACCAAACACAAACG |
| | REV | GTTCATGCCTTCTTTCACCTTCCC |
| RPL38 | FWD | AGGATGCCAAGTCTGTCAAGA |
| | REV | TCCTTGTCTGTGATAACCAGGG |
| r18S | FWD | GTAACCCGTTGAACCCCATT |
| | REV | GGAGTTGCTGTTGAAGTCGC |

## Tissue histology and immunostaining

Tissue samples were fixed in formalin and embedded in paraffin. 3 μm sections were cut for histological analysis and stained with hematoxylin and eosin. For characterization of MMTV-PyMT; $RANK^{+/+}$ and MMTV-PyMT; $RANK^{-/-}$ tumors stage, hematoxilin-eosin stained tumor sections were classified as previously described (Lin EY et al., 2003). Histological areas were quantified with ImageJ and normalized to the whole section excluding non-epithelial areas (muscle, adipose tissue and lymph nodes).

Lung metastasis were detected and counted based on nuclear morphology and similarity with primary tumors. 15-16 cuts per lung were quantified in limiting dilution assays and in primary MMTV-PyMT;$RANK^{-/-}$ or MMTV-PyMT;$RANK^{+/+}$ tumors. 3 cuts per lung were quantified in metastasis assays of matrigel cultures, as macroscopic metastases were evident at necropsy. For immunostaining, 3 μm tissue sections were used. Antigen heat retrieval with citrate was used for PR (Maximum pressure: 25min), Sma-1 (Maximum pressure: 25min), RANKL (Maximum pressure: 2min), Ki67 (Maximum pressure: 10 min) and Cleaved Caspase-3 (Maximum pressure: 15 min) antibodies. mRANK immunostaining was performed pre-treating sections with Protease XXIV 5U/mL (P8038, Sigma-Aldrich) for 15 minutes at room-temperature. mRANK (AF692; R&D Systems) and mRANKL (AF462; R&D Systems) antibodies were diluted 1:100 and 1:200 respectively, in 5% horse serum-PBS. Sma-1 (A2547, Sigma-Aldrich), Ki67 (RM9106S1, Thermo Scientific) and PR (A0098, DAKO) were diluted 1:6.000, 1:500 and 1:1.000, respectively in 5% goat serum-PBS. Cleaved Caspase-3 (9661, Cell Signaling) antibody was diluted 1:100 in 3% BSA 0.5% Tween-20 in PBS. All antibodies were incubated over-night at 4°C. The antigen-antibody complexes of Cleaved-Caspase-3 and PR antibodies were detected with biotynilated anti-rabbit antibody (BA-1000, Vector); RANK and RANKL antibodies were detected with biotynilated anti-goat antibody (BA9500, Vector); Sma-1 was detected with biotynilated anti-mouse antibody (BA-9200, Vector) and Ki67 was detected with Polyclonal anti-rabbit-HRP (P0448, DAKO). Biotynilated secondary antibodies were diluted 1:1000 in blocking buffer and incubated 40 min at RT. Anti-rabbit-HRP was diluted 1:200 and incubated 1h at RT. Biotynilated antibodies, were detected with streptavidin horseradish peroxidase (PK-6100, Vector). All immunostainings were revealed with DAB substrate (00035, DAKO). Intensity of RANK and RANKL staining were quantified at the microscope and classified as indicated in Sup. Fig. 1.

## Immunofluorescence

Immunofluorescence of Ki67 (1:150) and cleaved caspase-3 (1:600) antibodies in acinar cultures were performed as previously described (Debnath J et al., 2003). Briefly, acini were fixed in 2% paraformaldehyde (20min), permeabilized with PBS containing 0.5% Triton X-100 (15 min), washed with PBS-Glycine 100mM (3 washes of 15 min each). Antigens were blocked with IF buffer (PBS, 7.7 mM $NaN_3$, 0.1% bovine serum albumin, 0.2% Triton x-100, 0.05% Tween-20)+10% goat serum, 1 hour and then with IF buffer+goat serum+ 20 μg/mL F(ab') fragment (115-006-006, Jackson ImmunoResearch) for 30 min. Primary antibodies were incubated O.N. in humid chamber. Antibody-antigen complexes were detected using Alexa-488 conjugated anti-rabbit (A21441, Invitrogen) diluted in IF buffer+10% goat serum 1:500 and incubated 40 minutes. Acini were then washed with IF buffer and nuclei stained with DAPI. Confocal analysis was carried out using a Leica confocal microscopy. Images were captured using LasAF software (Leica). Percentage of Ki67 or Caspase-3+ cells was calculated with ImageJ software.

## Flow citometry

Single cells were resuspended at a density of up to 40.000 cells/ μL in PBS and blocked with 2% of Fetal Bovine Serum, 0.05% $NaN_3$ and IgG blocking reagent (0.01 μg /μL) for 10 min on ice. Cells used for analysis of lymphoid and myeloid tumor infiltrates, were incubated 30 min on ice with antibodies against CD45-APC-Cy7 (0.125 μg /mL; 103115, 30-F11, Biolegend), CD4-PE-Cy7 (2 μg /mL; 100527, RM4-5, Biolegend), CD11b-APC (2.5 μg /mL; 101211, M1/70, Biolegend), CD8-PE (1 μg /mL; 100707, 53-6.7, Biolegend), CD25-APC (2 μg /mL; 102011, PC61, Biolegend), Gr1-FITC (2 μg /mL; 108405, RB6-8C5, Biolegend). For detection of FoxP3, cells were stained with antibodies for cell surface molecules, and then were fixed and permeabilized following FoxP3 kit instructions; briefly cells were fixed 20 minutes in fixation buffer, washed twice with permeabilization buffer and left 15 minutes in permeabilization buffer. Cells were then stained with Alexa 488-conjugated anti-Foxp3 (2 μg /mL; 126411, MF14, Biolegend) or isotype control antibody. A population of 15.000 CD45+ cells was captured in all fluorescence-activated cell sorting (FACS) experiments.

For analysis of epithelial markers, 4.000/μL cells were blocked as described and then incubated 25min on ice with the following antibodies: CD24-FITC (5 μg /mL; 553261, M1/69, BD Pharmingen), D49f-a647 (2.5 μg /mL; 562473, GoH3, R&D Systems), CD61-FITC (2.5 μg /mL; 553346, 2C9.G2, BD Pharmingen), Sca-1-APC (0.5 μg /mL; Ly-6A/E, BD Pharmingen) and CD49b-Alexa 647 (1.25 μg /Ml; 103511, HMa2, Biolegend). Lymphocytes and endothelial cells were excluded in flow cytometry using CD45-PECy7 (0.125 μg /mL; 103114, 30-F11, Biolegend) or CD45-APC Cy7 (0.125 μg /mL; 30-F11, 103115, Biolegend) and CD31-PECy7 (0.5 μg /mL; 102418, 390, Biolegend) antibodies, respectively. A population of 10.000 CD45-CD31-CD24+ cells were captured. FACS analysis was performed using FACS Canto, FACS Aria (Becton Dickinson) and Diva software package.

## Statistical analysis

Statistical analyses were performed using GraphPad Prism. Analysis of the differences between two mouse cohorts or conditions was performed with a two-tailed Student's t-test. Two-way analysis of variance (ANOVA) was used for analysis of tumor growth curves. Mantel–Cox test was used for tumor-free survival studies. Estimation of tumor initiating cells in limiting dilutions was calculated using the Extreme limiting dilution assay E.L.D.A. (Hu Y et al., 2009).

# RESULTS AND DISCUSSION

## 1. Characterization of RANK and RANKL expression in tumor prone mouse models MMTV-neu and MMTV-PyMT

We focused on MMTV-neu and MMTV-PyMT to understand the role of RANK signaling in spontaneous mammary carcinogenesis and established tumors. The expression profile of RANK and RANKL in MMTV-neu and MMTV-PyMT at different steps of tumorigenesis was investigated. We detected a 6-fold and 4-fold increase of RANK mRNA in MMTV-Neu and MMTV-PyMT tumors respectively, as compared to wild-type mammary glands (Fig. 1A, left panel). In contrast, RANKL mRNA was detected in wild-type and MMTV-neu mammary glands at lower levels than MMTV-neu and MMTV-PyMT tumors (Fig. 1A, right panel).

RANK protein is expressed focally in MMTV-neu normal glands similarly to wild-type (Fig. 1B-C). We could not collect normal mammary glands from MMTV-PyMT adult mice for RT-PCR analyses as this model shows extensive hyperplasias before 4 weeks of age (Guy CT et al., 1992) however immunohistochemical staining revealed focal expression of RANK in residual normal epithelium (Sup. Fig. 1). Mammary hyperplasias and intraepithelial neoplasias of both MMTV-neu and MMTV-PyMT showed a significant increase of RANK protein expression, compared to WT normal glands (Fig.1 B-C, Sup. Fig. 1). High levels of RANK protein were detected in both MMTV-neu and MMTV-PyMT carcinomas in accordance with mRNA expression profile (Fig. 1B-C, Sup. Fig. 1).

MMTV-neu normal mammary epithelia showed focal staining of RANKL comparable to wild type normal glands (Fig. 1B-C). Tumor progression of both MMTV-neu and MMTV-PyMT models is characterized by the loss of expression of estrogen and progesterone receptors (Lin EY et al., 2003) and it is described that RANKL is expressed in PR+ cells (Fernandez-Valdivia R et al., 2009). In accordance with loss of hormone responsive cells, we detected very low or undetectable levels of RANKL in preneoplastic and neoplastic MMTV-neu and MMTV-PyMT lesions (Fig. 1C).

These results concur with two recent studies (Gonzalez-Suarez E et al., 2010; Schramek D et al., 2010), in which RANK expression was detected in MMTV-PyMT and MMTV-neu tumors. In both mouse models we observed that RANK expression increases in pre-neoplastic lesions and carcinomas as compared to normal mammary glands suggesting a role for RANK pathway in tumor progression.

## 2. RANKL induces hallmarks of survival and invasion in MMTV-neu and MMTV-PyMT tumor cells

RANKL is an essential mediator of progesterone-induced tumour initiation (Gonzalez-Suarez E et al., 2010; Schramek D et al., 2010). However, the role of RANK pathway in established tumors remains unknown.

To address this question, tumor cells were isolated from advanced MMTV-PyMT and MMTV-neu carcinomas, cultured in matrigel in differentiation media and treated for two weeks with RANKL or OPG, a decoy receptor of RANKL that inhibits RANK signaling (Kostenuik PJ et al., 2005). Isolated tumor cells gave rise to tumor acini similarly to those of mammary epithelial cells (Barcellos-Hoff MH et al., 1989). MMTV-neu and MMTV-

PyMT tumoral acini express RANK, similarly to primary tumors; RANKL expression was higher in MMTV-PyMT acini as these tumors usually contained preneoplasic lesions that conserve PR expression (Lin EY et al., 2003), (Sup. Fig. 2B). RANKL stimulation during two weeks resulted in increased acinar size and cell density in both MMTV-neu and MMTV-PyMT acini (Fig. 2A-B) and further increased RANKL mRNA expression (Sup. Fig. 2B). Proliferation quantified as frequency of Ki67+cells in the tumor acini was significantly increased under RANKL stimuli in 2/3 MMTV-neu independent tumors (Fig. 2C). In MMTV-PyMT a modest increase in Ki67 was observed but it did not reach significance (Sup. Fig.2A). Expression levels of cleaved caspase-3 decreased in 1/3 MMTV-neu and 2/3 MMTV-PyMT tumors under RANKL stimuli in cultures (Sup. Fig. 2A). However, expression profile of putative genes involved in cell survival revealed that RANKL treatment significantly upregulates mRNA levels of apoptosis inhibitors BIRC2, BIRC3 and the survival gene Bcl2A1b in MMTV-neu and MMTV-PyMT tumor acini (Fig. 2D). The pro-survival effect of RANKL in primary tumor cells agrees with the notion that RANKL treatment plays an anti-apoptotic effect under γ-irradiation or doxorubicin in RANK expressing cell lines (Schramek D et al., 2010). In addition, RANKL treated MMTV-neu and MMTV-PyMT acinar cultures have an "invasive-like" phenotype: isolated cells are visible (arrows) and are apparently migrating out of the acini (Fig. 2F). Expression profile of genes involved in cell invasion revealed that ICAM-1, VCAM-1 and MMP9 proteins were upregulated upon RANKL stimulation in both MMTV-PyMT and MMTV-neu (Fig. 2G). No significant changes in the expression of any gene analyzed were observed upon OPG treatment (Fig 2D, 2G).

In order to evaluate the functional impact of these changes *in-vivo*, tumor cells from RANKL and control MMTV-PyMT acini were injected in the mammary gland of *Foxn1*$^{nu}$ mice. Remarkably, a significant increase of growth rate in tumors that arise from 2 week-RANKL treated acini, compared to controls was detected (Fig. 2E).

To determine the metastatic potential of RANKL or OPG treated MMTV-PyMT acini, tumor cells were isolated and injected in tail vein of NOD/SCID mice. Lung metastasis of RANKL/OPG treated cells were analyzed and compared to their controls. 80% of RANKL treated tumor cells had lung metastasis, as compared to 60% in the group of controls and OPG treated tumor cells (Sup. Fig. 3A). Mice injected with RANKL treated cells showed a modest increase in the number of lung metastatic foci as compared with those injected with control and OPG treated acini (Sup. Fig. 3B).

Together these data demonstrate that RANKL exposure increased proliferation, survival and induced and invasive phenotype suggesting that RANK-expressing tumor cells are responsive to RANKL, and that activation of RANK signaling promotes tumorigenesis and metastasis in established tumor cells.

RANK signaling is known to mediate progesterone dependent mammary tumor initiation as both, exogenous (Gonzalez-Suarez E et al., 2010; Schramek D et al., 2010) and pregnancy associated (Gonzalez-Suarez E et al., 2010; Pellegrini P et al., 2013) progesterone stimuli increase tumor incidence legitimating activation of RANK signaling as an early event in tumorigenesis. We demonstrated that activation of RANK signaling can increase aggressiveness of carcinoma derived MMTV-neu and MMTV-PyMT cells *in-vitro* and *in-vivo*, as previously observed in human cancer cell lines (Palafox M et al., 2012). Absence of a clear effect with OPG treatment is probably due to the low expression of RANKL in these tumor cells (Sup. Fig. 2B), similar to the absence of RANKL expression in MMTV-neu and MMTV-PyMT advanced carcinomas. *In-vivo* RANKL can be provided by tumor infiltrates or by surrounding non-transformed or preneoplasic mammary epithelia that conserves progesterone/RANKL expression. Breast cancer patients are already receiving a RANKL inhibitor (Denosumab) for the treatment of bone metastasis and skeletal related events

(Yasuda H et al., 2013). Our results provide evidence that anti-RANKL treatment could also have anti-tumoral effects in patients with breast cancer as RANKL promotes tumor growth and metastasis.

## 3. *In-vivo* short-term treatment with RANKL does not affect tumor growth but increases colony forming ability of tumor cells

In order to investigate the impact of RANK activation or inhibition *in-vivo*, tumor bearing MMTV-neu (30-50 weeks old) and MMTV-PyMT mice (10-13 weeks old) were treated *in-vivo* for 2 weeks with RANKL or RANK-Fc (Fig. 3A). Treatments started once tumors were already palpable

No significant changes in tumor growth rates were detected after 2 weeks of RANKL or RANK-Fc treatment in MMTV-neu mice, nor in MMTV-PyMT mice (Fig. 3B). Quantification of Ki67 and cleaved caspase-3 in MMTV-neu and MMTV-PyMT treated carcinomas confirmed that RANKL or RANK-Fc treatment did not significantly alter tumor proliferation or apoptosis during this time (Sup. Fig. 4). A modest increase in apoptosis under RANK-Fc treatment was observed in MMTV-PyMT carcinomas, but it did not reach significance. Analyses of gene expression profile revealed increased mRNA expression of the pro-survival genes, BIRC2, BIRC3 and Bcl2a1b, as well as ICAM-1, VCAM-1 and MMP9 in tumors from RANKL-treated MMTV-neu mice (Sup. Fig. 5), corroborating the *in-vitro* data. In contrast, in MMTV-PyMT-tumors no changes in the expression of these genes were detected between different treatments (Sup. Fig. 5) maybe due to the histological heterogeneity found in these tumors (Fig. 3D) (Lin EY et al., 2003). RANK expression decreased upon exogenous RANKL treatment *in-vivo* (Sup. Fig. 5) probably because of a negative-feedback previously reported (Fernandez-Valdivia R 2009) which could attenuate RANKL driven responses. Modulation of RANK and RANKL expression by RANKL treatment is very different between the acini and the tumors which could explain discrepancies between the *in-vitro* and the *in-vivo* results (Sup. Fig. 2B, 5). RANKL expression was higher in MMTV-PyMT as compared to MMTV-neu tumors, probably because these tumors contain non-tumorigenic epithelium and hyperplasia surrounding the tumor bulk (Lin EY et al., 2003). Indeed, analysis of MMTV-PyMT palpable lesions revealed histological variable areas at different stages of tumorigenesis including hyperplasias, adenomas and carcinomas, confirming the polyclonal origin of these palpable lesions (Lin EY et al., 2003). Tumor histological heterogeneity of MMTV-PyMT tumors may be the cause of such different behavior of RANKL treated acinar cultures as compared with RANKL treated mice in terms of gene expression. In contrast, MMTV-neu palpable lesions were very homogeneous which may result in a higher degree of correlation between *in-vitro* and *in-vivo* gene expression changes.

It has been previously shown that RANK signaling mediates the expansion of mammary stem and progenitor cells (Asselin-Labat ML et al., 2010; Pellegrini P et al., 2013). RANKL increases colony forming ability of MMTV-RANK mammary cells (Pellegrini P et al., 2013) and treatment with an anti-RANKL antibody *in-vitro* decreases colony forming ability of mammary stem and progenitor cells (Asselin-Labat ML et al., 2010). In order to determine whether *in-vivo* RANKL or RANK-Fc treatment may have similar effects on tumor cells we tested the colony forming ability of tumor cells derived from RANKL or RANK-Fc treated MMTV-neu and MMTV-PyMT mice. RANKL treatment *in-vivo* increased the colony forming ability of MMTV-neu and MMTV-PyMT tumor cells: in MMTV-PyMT

tumor cells a moderate increase in number of colonies is observed, while in MMTV-neu tumor cells, the number of colonies increased 3-fold after RANKL treatment (Fig. 3C).

Blocking RANKL signaling with RANK-Fc, didn't affect colony formation ability in both mouse models. However, morphological analyses of RANK-Fc treated MMTV-PyMT lesions revealed extensive areas of secretive hyperplasia that were less abundant in RANKL treated lesions (Fig. 3D). This phenotype resembled the RANK-Fc treated mid-gestant mammary gland (Pellegrini P et al., 2014-ANNEX1): we speculate that RANK-Fc treatment could induce differentiation of preneoplastic lesions.

Altogether these results demonstrate that *in-vivo* short-term treatment with RANKL or RANK-Fc did not modify tumor proliferation and survival of established tumors; maybe longer treatments or combination with other therapies such as γ-irradiation or doxorubicin (Schramek D et al., 2010) are required to observe the pro-tumorigenic or pro-survival role of RANK signaling in tumor bearing mice. However, clonogenic ability of isolated tumor cells upon RANKL treatment increased suggesting that activation of RANK signaling could expand the cancer stem cell pool, as previously reported in the non-transformed mammary epithelia (Schramek D et al., 2010; Pellegrini P et al., 2013) or human breast cancer cell lines (Palafox M et al., 2012).

## 4. Neoadjuvant inhibition of RANKL signaling decreases the frequency of tumor-initiating cells

The link between the role of RANK signaling in MaSC expansion (Schramek D et al., 2010; Asselin-Labat ML et al., 2010) and increased tumor incidence (Pellegrini P et al., 2013), together with the increased colony forming ability observed in MMTV-neu and MMTV-PyMT tumor cells after short term treatment with RANKL, led us to investigate whether RANK signaling plays a role in tumor initiating ability. Based on tumor kinetics we decided to focus on MMTV-PyMT tumors. *In-vivo* treatment of orthotopically transplanted tumors may be a useful tool to evaluate the therapeutic role of blocking RANKL *in-vivo* and to avoid confounding effects due to MMTV-PyMT tumor heterogeneity. Cells isolated from one single MMTV-PyMT carcinoma were injected in the inguinal fat pads of 18 syngenic WT mice ($1 \times 10^6$ cells per gland) and mice were randomized 1:1 for RANK-Fc or mock treatment (P1). RANK-Fc treatment (3 times per week) started 24h after tumor cell injection. After 4 weeks, tumors were surgically removed and cells isolated for functional assays (Fig. 4A). To determine whether RANK-Fc could directly impact tumor initiating ability, cells isolated from 3 RANK-Fc treated MMTV-PyMT tumors and corresponding controls (P1) were pooled and injected in the fat pad of FvB recipients (P2) at limiting dilutions, and again, mice from both groups were randomized 1:1 for additional RANK-Fc or mock treatment that lasted for 2 weeks (Fig. 4A). This treatment scheme aims to mimic the clinical procedure and allow us to evaluate the therapeutic benefit of neoadjuvant and adjuvant treatment with Denosumab. Strikingly, tumor cells that have been pre-treated with RANK-Fc during 4 weeks gave rise to tumors with less frequency than controls (Fig. 4B). ELDA (Extreme Limiting Dilution Analysis) test revealed a 10-fold decrease in the tumor initiating ability of RANK-Fc pre-treated tumors (Fig. 4B). The estimated number of tumor initiating cells/cancer stem cells in the control group was 1 out of 206, whereas in the RANK-Fc pretreated pool was only 1 out of 1929. Additional 2 weeks RANK-Fc treatment (pre&post-RANK-Fc) reduced the tumor initiating cell frequency to 1 out of 2353 (Fig. 4B).

Treatment with RANK-Fc in P2 (post-RANK-Fc, 2 weeks), without RANK-Fc treatment in P1, decreased virtual tumor initiating cell frequency from 1/206 to 1/466, although differences did not reach statistical significance. Together these results indicate that neoadjuvant RANK-Fc treatment (P1) reduces the risk of relapse by depleting the population of TICs; whereas adjuvant RANK-Fc treatment after surgery (P2) still decreases tumor initiating cell pool but less markedly than neoadjuvant treatment. Unexpectedly RANK-Fc treatment decreased tumor latency and increased 1.7 fold Cyclin D1 expression in P1 orthotopic tumors (Sup. Fig. 6A). A similar trend was observed in post RANK-Fc treatment in P2 tumors (Sup. Fig. 6B). According to the decreased tumor latency observed, RANK-Fc treatment increased mRNA expression of Cyclin D1 (Sup. Fig. 6C).

As tumor initiating cells are described to grow in anchorage-independent conditions, isolated cells from P2 tumors were cultured *in-vitro* for tumorsphere assay. These assays are described to enrich in tumor initiating cells as tumorsphere derived cells can form new tumors in-vivo (Dontu G et al., 2005). No differences were observed in primary tumorspheres between all groups of treatment (Fig. 4C), as previously observed in colony forming assays after RANK-Fc treatment (Fig. 3C). However, after dispersion into single cells, the ability to form secondary tumorspheres was significantly impaired in cells derived from the RANK-Fc pretreated pool (Fig. 4C). Our tumorsphere assays demonstrated that blocking RANKL decrease anchorage-independent growth.

So far RANK signaling has been described to mediate colony formation ability of normal mammary epithelial cells (Asselin-Labat ML et al., 2010) and enrich tumorsphere formation of the transformed cell line SKBR3 (Schramek D et al., 2010). Cancer stem cells or tumor initiating cells are described as the responsible of tumor recurrence in cancer (Pardal R et al., 2003). Neo-adjuvant treatment with RANK-Fc before injection of tumor cells in limiting dilution demonstrated that blocking RANKL could deplete the pool of tumor initiating cells and, as a consequence, tumor relapse. We speculate that targeting RANKL signaling in the neoadjuvant setting can be a useful strategy in preventing tumor recurrence after surgery in breast cancer patients.

## 5. Inhibition of RANK signaling increases the frequency of Sca-1+ cells in MMTV-PyMT tumors

In order to investigate the molecular mechanism mediating the decreased tumor initiating ability observed after RANK-Fc treatment in MMTV-PyMT tumor cells , we analyzed the mRNA expression levels of several genes that modulate self renewal or the tumor initiating capability of tumor cells in the breast or other tissues (Axin2, Gata3, Notch1, Hey-1, Hif1-α, Bmi-1, Sox-9, Fibronectin, Vimentin), (Liu S et al., 2006; Harrison H et al., 2010; Singh A et al., 2010; Takahashi-Yanaga F et al., 2010; Asselin-Labat ML et al., 2011; Wang J et al., 2012; Schwab LP et al., 2012) but no clear differences were observed between treatments in P1 tumors (Sup. Fig. 7A) and P2 tumors (data not shown), suggesting that the mechanism by which RANK signaling expands the population of TICs does not involve these pathways. The mRNA expression of genes involved in cell survival and invasion (BIRC2, BIRC3, Bcl2a1b, ICAM1, VCAM1 and MMP9) after 4 weeks of treatment with RANK-Fc in P1 tumors was similar to controls except for MMP9 that decreases modestly (Sup. Fig. 7B).

Surface markers have been used to identify populations of epithelial cells with stem/progenitor cell characteristics or increased tumor/metastasis initiating ability in the MMTV-PyMT mice and other tumor prone models (Liu S et al., 2006; Vaillant F et al.,

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frequent in MMTV-PyMT RANK$^{-/-}$ mice, as compared with their controls (Fig. 6C). At 17 weeks, 75% MMTV-PyMT$^{-/+}$;RANK$^{-/-}$ showed palpable tumors in all mammary glands against 50% of MMTV-PyMT$^{-/+}$;RANK$^{-/-}$ mice (Sup. Fig. 12B). Overall, homozygous RANK deletion decreases tumor incidence and growth rate and significantly increases tumor latency as previously observed in MMTV-neu mice treated with RANK-Fc in a preventive setting (Gonzalez-Suarez E et al., 2010). However, specific deletion of RANK in the mammary gland under a MMTV promoter did not affect tumor latency or incidence in MMTV-neuT mice (Schramek D et al., 2010), suggesting that inhibition of RANK signaling not only in the tumor cells, but also in the microenvironment, may be responsible of the anti-tumor phenotype. Previous evidences showing that RANK-Fc can interfere with immune cell infiltrates in orthotopic MMTV-PyMT tumors support this hypothesis. Mammary tumor initiation and progression can be affected not only by tumor cells but also by the microenvironment. Further investigations will clarify the contribution of the RANK deficient tumor cells or the RANK deficient stroma to the MMTV-PyMT;RANK$^{-/-}$ phenotype.

## 8. RANK deletion results in "monoclonal-like tumors" with decreased histological heterogeneity and accumulation of CD49b+ cells

Palpable lesions of MMTV-PyMT$^{-/+}$;RANK$^{-/-}$ mice and corresponding controls were morphologically analyzed: histological areas were identified and quantified based on histopathology and expression pattern of PR and Sma-1 (Sup. Fig. 13A-B) based on previous criteria (Lin EY et al., 2003). As shown in Sup. Fig. 13B, tumor progression of the MMTV-PyMT model can be separated in four distinct phases: hyperplasia, adenoma, early and late carcinoma. Hyperplastic area showed densely packed lobules and ducts, similar to normal epithelium, a single layer of luminal cells around the lumen and surrounded by a single, continuous layer of myoepithelial cells, as demonstrated by Sma-1 expression pattern; PR is expressed mostly in normal ducts. Adenoma is characterized of increased hyperplastic and non-invasive structures with frequent mammary intraepithelial neoplasias (MIN): cells are homogeneously packed into lobuloalveolar structures with no lumen. A mosaic expression of PR is detected although most MINs lack PR expression, a monolayer of Sma-1+ cells is visible although it is discontinuous in many areas. Early carcinoma is characterized by increased cytological atypia and invasion of the surrounding stroma, PR expression is almost completely lost and Sma-1 is clearly discontinuous although it keeps the lobular-like expression pattern. Late carcinoma is characterized by high cytologic atypia, reactive stroma, complete loss of PR expression, while Sma-1 expression is randomly distributed in mosaic (Sup. Fig. 13B).

MMTV-PyMT$^{-/+}$;RANK$^{+/+}$ palpable lesions are characterized by extensive areas of early and/or late carcinomas surrounded by adenomas and hyperplasic epithelia (Fig. 7A-B; Sup. Fig. 13A). In contrast, 70% of MMTV-PyMT$^{-/+}$;RANK$^{-/-}$ mice showed almost exclusively one predominant stage that was homogeneously distributed in the whole palpable mass suggesting that palpable lesions might arise from one or few tumoral foci, unlike MMTV-PyMT$^{-/+}$;RANK$^{+/+}$palpable lesions. The fact that constitutive deletion of RANK impairs mammary epithelial cell proliferation, resulting in a reduced ductal tree, (Fata JE et al., 2000) and no expansion of MaSC upon progesterone stimulation (Schramek D et al., 2010), may contribute to this phenotype. We concluded that epithelial targets of transformation by the PyMT oncoprotein were decreased in the absence of RANK resulting in less tumoral foci and lower histological heterogeneity. Evidence that RANK-Fc treatment

reduces the number of pre-neoplastic lesions in MMTV-neu mammary glands (Gonzalez-Suarez E et al., 2010) suggest that tumor histological homogeneity observed in MMTV-PyMT$^{-/+}$;RANK$^{-/-}$ mice may reflect the decreased tumor formation ability of the mammary gland in the absence of RANK signaling.

Expression profile of epithelial populations was analyzed by flow citometry in MMTV-PyMT$^{-/+}$;RANK$^{-/-}$ tumors and their controls. Whereas the levels of CD49f, CD61 and Sca-1, within the CD45-CD24+ cells were comparable between genotypes, CD49b+ cells constitute the 20% of tumor epithelial CD45-CD24+ cells in MMTV-PyMT;RANK$^{+/+}$ mice as compared to 50% in MMTV-PyMT;RANK$^{-/-}$ tumors (Fig. 7C). Interestingly a modest increase in CD49b was also observed in orthotopic MMTV-PyMT P2 tumor cells after RANK-Fc neoadjuvant treatment suggesting that RANK signaling negatively regulates the CD49b+ tumor cells population. However, the increase in Sca-1+ cells observed upon RANKL inhibition is not observed in MMTV-PyMT; RANK$^{-/-}$ tumors evidencing differences between pharmacological inhibition of RANK signaling and genetic deletion of RANK. CD49b+ cells are described to identify luminal progenitors in untransformed mammary gland (Shehata M et al., 2012). Functional characterization of tumoral CD49b+ cells is required to elucidate the role of CD49b in MMTV-PyMT tumors.

## 9. RANK deletion does not directly affect to TICs pool

RANK deletion delays PyMT driven tumor initiation and blocking RANKL with RANK-Fc treatment depletes the cancer stem cell population in orthotopic MMTV-PyMT tumors. For this reason we investigated whether RANK deletion decreases tumor initiation ability of PyMT tumor cells.

Cells were isolated from MMTV-PyMT$^{-/+}$;RANK$^{+/+}$ and MMTV- PyMT$^{-/+}$;RANK$^{-/-}$ tumors and injected in the mammary glands of *Foxn1$^{nu}$* nude mice in limiting dilutions (Sup. Fig. 15A). Immunodeficient mice were used as RANK$^{+/+}$ and MMTV-PyMT$^{-/+}$;RANK$^{-/-}$ and their controls are in a mixed genetic background. Quantification of tumor frequency by ELDA revealed no differences between the two groups indicating that TICs pool was not affected by RANK deletion in an immunodeficient environment. Different hypothesis may explain the controversial results on the TICs population after RANK-Fc treatment and RANK genetic deletion. First, genetic deletion of RANK in the MMTV-PyMT$^{-/+}$ tumor prone background may result in development of RANK independent MMTV-PyMT tumors. Unlike MMTV-PyMT TICs pool in the orthopic setting which depends on RANK signaling for their maintenance and expansion, RANK deficient MMTV-PyMT tumors probably rely on a different population of tumor initiating cells. In fact, the frequency of Sca-1+ is similar in both genotypes. In addition, we demonstrated that inhibition of RANK signaling in an immunocompetent environment decreases tumor formation ability and importantly induces an anti-tumor immune response. In contrast, in an immunodeficient environment no differences in the TICs population are observed deleting RANK in MMTV-PYMT tumor cells. This supports the hypothesis that pharmacological inhibition of RANK signaling can inhibit tumor initiation through modulation of the microenvironment towards an anti-tumor immune response. In the absence of a proper adaptive immune response (nude mice), tumors rely to their intrinsic tumor formation ability that may not be RANK dependent. As observed after RANK-Fc treatments, deletion of RANK in MMTV-PyMT tumors implanted in immunodeficient mice results in reduced tumor latency (Sup. Fig. 15B). Further characterization will clarify the mechanisms by which RANK signaling controls tumor latency.

## 10. Metastatic potential of primary MMTV-PyMT tumors is RANK dependent

Tumor heterogeneity is strictly associated with tumor cell plasticity and metastatic potential (Meacham CE et al., 2013). Several evidences support the role of RANK in metastasis of human and mice: pharmacological inhibition of RANK signaling in MMTV-neu decreases the incidence of lung metastasis (Gonzalez-Suarez E et al., 2010) and conversely RANKL promotes cell migration and invasion of mouse and human cells (Jones DH et al., 2006; Palafox M et al., 2012). We therefore, characterized the metastatic potential of primary MMTV-PyMT;$RANK^{-/-}$ tumors and their controls.

In MMTV-PyMT;$RANK^{+/+}$ F1 mice bearing early/late carcinomas (mixed FvB/C57BL6 background), the incidence of metastasis is 57%, whereas in F1 mice with heterozygous deletion of RANK the incidence drops to 31% (Fig. 8A). A 62% of MMTV-PyMT;$RANK^{+/+}$ F2 mice show lung metastasis; metastatic foci can be detected in mice bearing early carcinoma and all mice with late stage carcinoma show extensive metastasis with more than 300 metastatic foci per lung (Fig. 8B). Surprisingly, almost all F2 MMTV-$PyMT^{-/+}$; $RANK^{-/-}$ were devoid of lung metastasis independently from primary tumor stage (Fig. 8A-C). Only 1 out of 3 MMTV-$PyMT^{-/+}$; $RANK^{-/-}$ mouse bearing late stage carcinoma showed two small metastatic foci in the lungs, in contrast with the hundreds of metastasis found in MMTV-PyMT;$RANK^{+/+}$ controls (Fig. 8B). Interestingly, MMTV-$PyMT^{-/+}$;$RANK^{-/-}$ lungs have an accumulation of non-tumorigenic lymphatic cells around blood vessels. These cells are also found in the lungs of non tumor bearing $RANK^{-/-}$ mice in a $PyMT^{-/-}$ background (Sup. Fig. 14), suggesting that this abnormality could be consequence of the deficient immune system in these mice and not related with tumor growth (Dougall WC et al., 1999). This result demonstrates that deletion of RANK blocks the metastatic potential of the aggressive MMTV-PyMT tumor model, demonstrating that RANK is a key mediator of metastatic process as previously shown after RANK-Fc treatment in MMTV-neu mice (Gonzalez-Suarez E et al., 2010).

## 11. Metastatic potential of primary MMTV-PyMT tumors is tumor cell autonomus

Metastatic potential of MMTV-PyMT tumors depends not only on the tumor cells but also on immune cell infiltrates (Lin EY et al., 2001; DeNardo DG et al., 2009). It has been demonstrated that RANKL expressing T regulatory lymphocytes induce metastatic disease in RANK expressing MMTV-neu tumor cells (Tan W et al., 2011).

In order to investigate whether the absence of metastasis observed in the MMTV-PyMT;$RANK^{-/-}$ mice is tumor autonomous or it is related with the abnormalities of the RANK null immune system MMTV-PyMT;$RANK^{-/-}$ tumor cells and their correspondent controls were injected in the tail vein of *Foxn1*$^{nu}$ recipients in limiting dilution. MMTV-PyMT;$RANK^{+/+}$ tumor cells efficiently colonized the lung: virtual frequency of metastatic cells was 1 out of 2.952 cells (Fig. 8D). Strikingly MMTV-$PyMT^{-/+}$;$RANK^{-/-}$ tumors showed a 10 fold (1 out of 29.420) decrease in the frequency of metastasis initiating cells (Fig. 7D). Moreover, mice injected with $10^5$ WT cells showed between 18 to 133 metastatic foci, whereas in mice injected with the same number of $RANK^{-/-}$ cells, only 0 to 9 metastatic foci were found (Fig. 8E). Altogether these results demonstrate that RANK deletion in tumor cells impairs lung colonization and the metastatic ability of MMTV-PyMT tumor cells even in an immunodeficient background. Remarkably, the injection of

transformed MMTV-PyMT tumor cells in the blood stream reveals that the reduced metastatic potential of tumor cells was not a consequence of the reduced incidence of primary tumors (Gonzalez-Suarez E et al., 2010). Moreover, in the orthotopic model of MMTV-PyMT;RANK$^{-/-}$ tumor cells injected in limiting dilution in the fat pad of immunodeficient mice, the metastatic cell frequency decreased 6 fold, despite the unaltered tumor initiation ability (Sup. Fig. 15B-C). In conclusion, RANK deletion in tumor cells depletes the pool of metastasis initiating cells and severely reduces lung colonization.

RANK signaling plays a key role in the process of breast cancer metastasis as previously demonstrated in human cells and clinical samples (Palafox M et al., 2012). Impaired metastatic potential of MMTV-PyMT$^{-/+}$;RANK$^{-/-}$ tumors was more accused in endogenous primary tumors than in orthotopic transplants in nude mice suggesting that the RANK-deficient microenvironment also interferes with the metastatic potential of MMTV-PyMT;RANK$^{-/-}$ cells. Cells of the immune system are most likely influencing the RANK dependent metastatic potential of MMTV-PyMT mice as we observed that interfering RANK signaling with RANK-Fc modifies immune cell infiltrates. We could not exclude the contribution of tumoral stroma as it has been described to promote metastasis in MMTV-PyMT model (Malanchi I et al., 2011; Pickup MW et al., 2013; Wallace JA et al., 2013; Werbeck JL et al., 2013). Further investigations will clarify the contribution of RANK signaling in the immune infiltrates to the lack of metastasis observed in MMTV-PyMT;RANK$^{-/-}$ mice.

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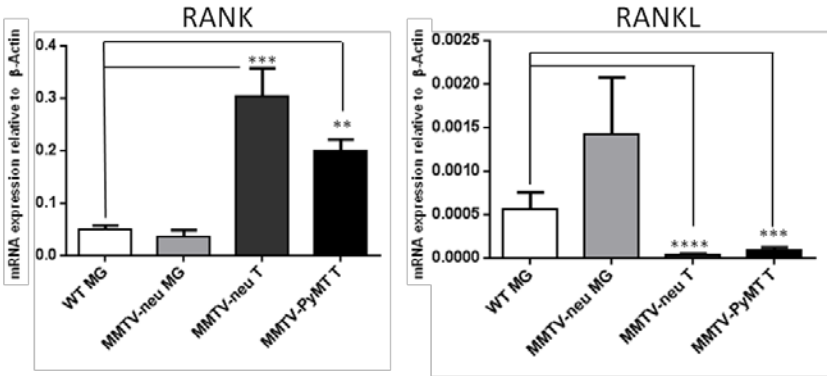
# FIGURES

## Figure 1

**A**

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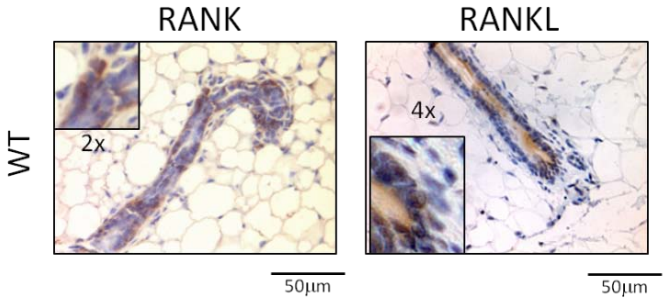


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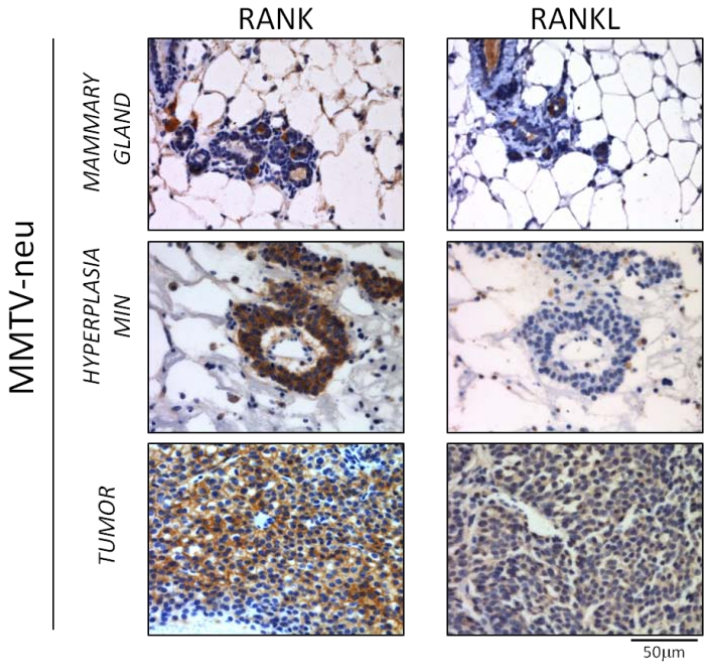


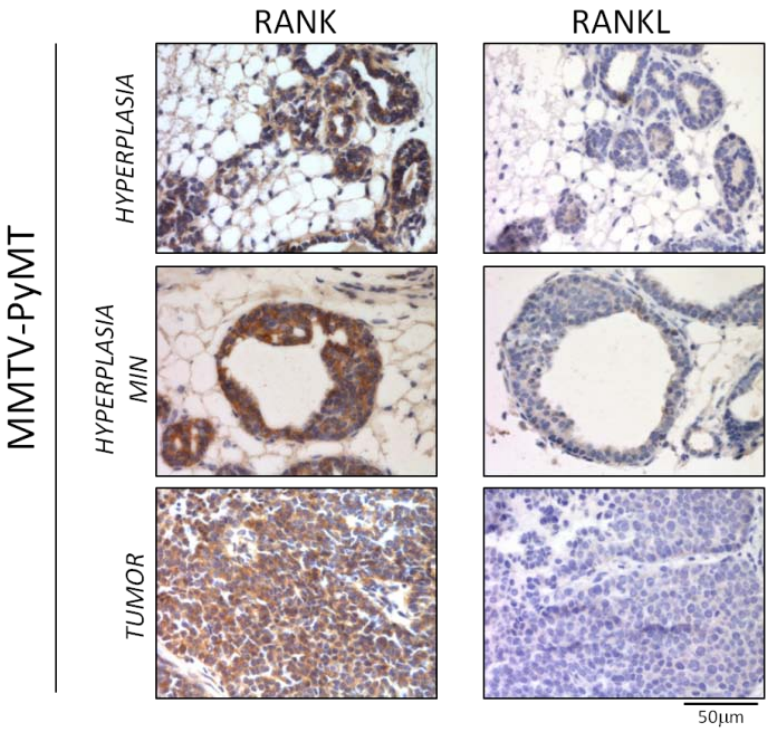


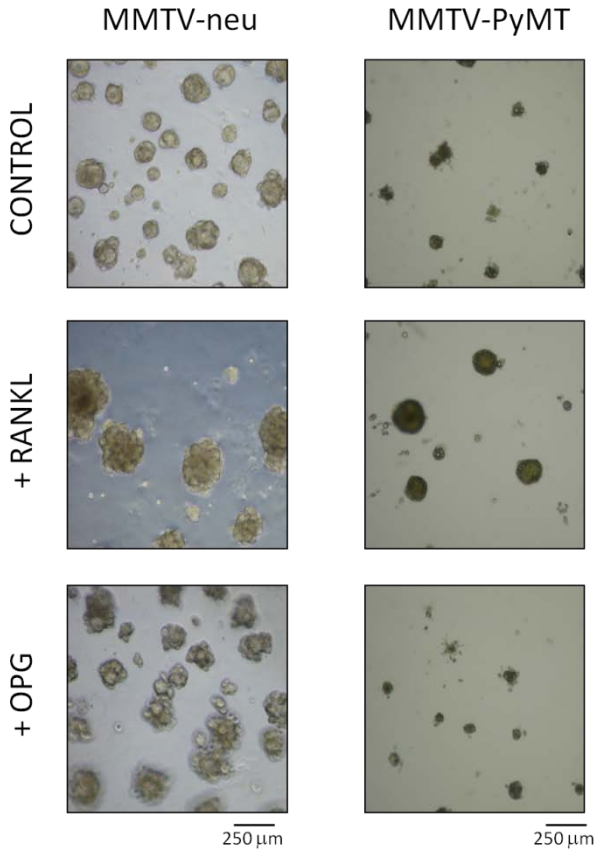

**Figure 1.** RANK expression increases during tumor progression in MMTV-neu and MMTV-PyMT spontaneous tumors

A. Relative mRNA expression of RANK and RANKL, normalized with β-actin in 7 WT, 8 MMTV-neu mammary glands (MG), 11 MMTV-neu tumors (T) and 8 MMTV-PyMT tumors. Mean, SEM and t-test with p values are shown. Quantifications were done in triplicate for each sample and mean values were used in the calculations.

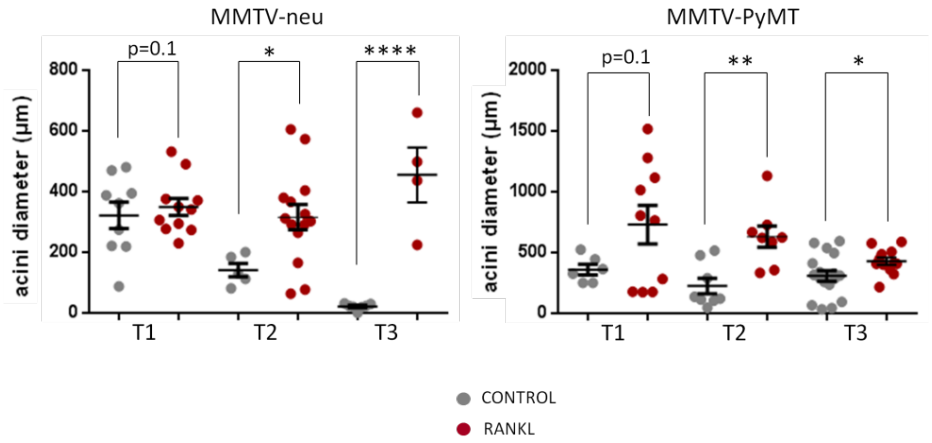
B,C. Representative images of RANK and RANKL protein expression as detected by IHC in WT mammary glands (B), MMTV-neu and MMTV-PyMT mammary glands, preneoplasic lesions and tumors (C).

## Figure 2

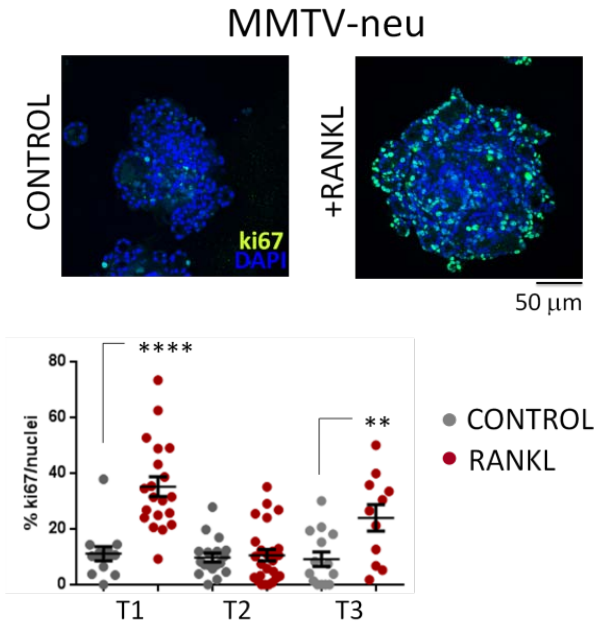
**A**


MMTV-neu
MMTV-PyMT
CONTROL
+ RANKL
+ OPG
250 µm
250 µm

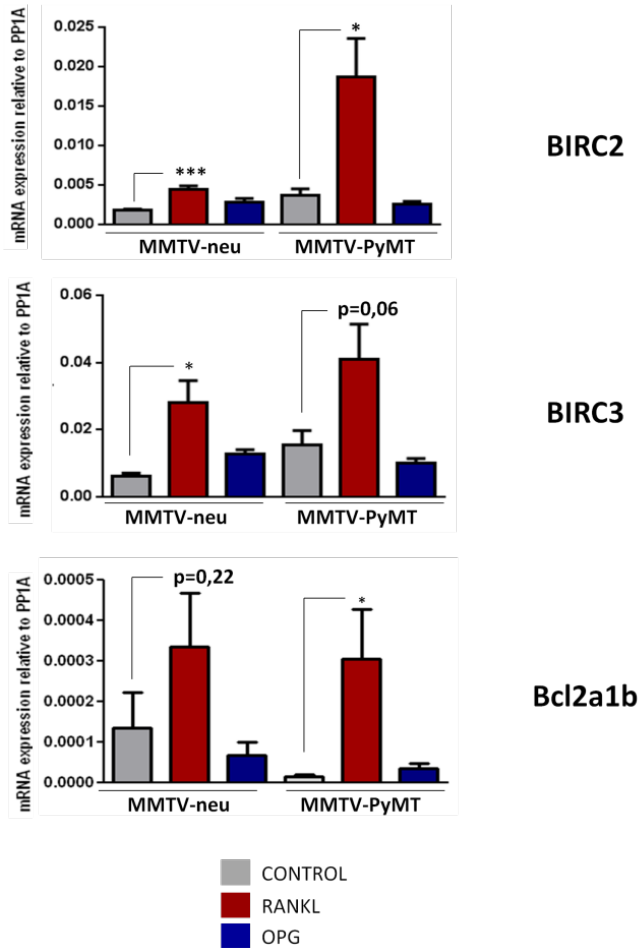
**B**


MMTV-neu
MMTV-PyMT
acini diameter (µm)
p=0.1
*
****
p=0.1
**
*
T1
T2
T3
CONTROL
RANKL

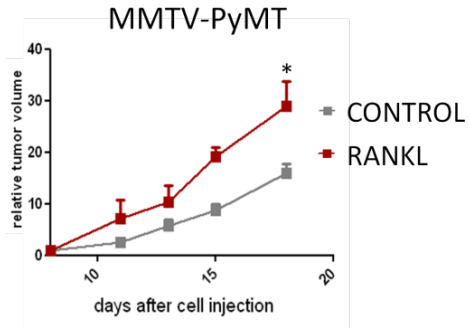
**C**


MMTV-neu
CONTROL
+RANKL
ki67
DAPI
50 µm
% ki67/nuclei
****
**
T1
T2
T3
CONTROL
RANKL

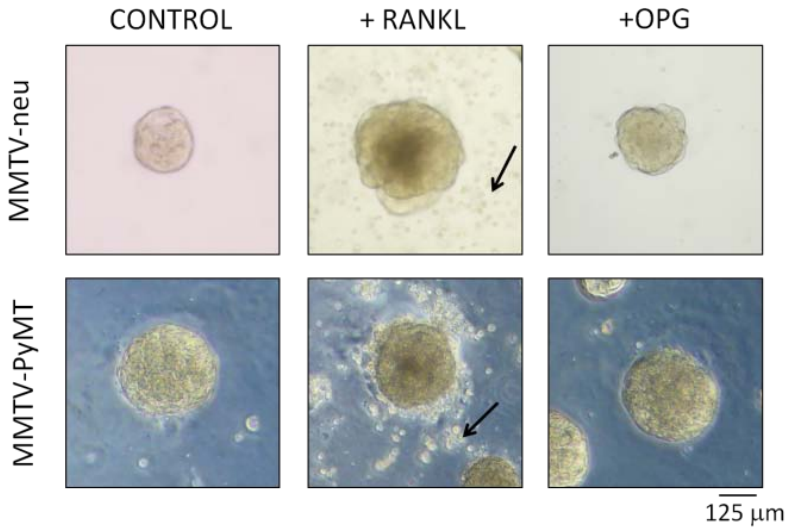
**D**


mRNA expression relative to PP1A
MMTV-neu
MMTV-PyMT
BIRC2
BIRC3
Bcl2a1b
p=0,06
p=0,22
CONTROL
RANKL
OPG

**E**


MMTV-PyMT
relative tumor volume
40
30
20
10
0
10
15
20
days after cell injection
*
CONTROL
RANKL

**F**


CONTROL
+ RANKL
+OPG
MMTV-neu
MMTV-PyMT
125 µm

**G**

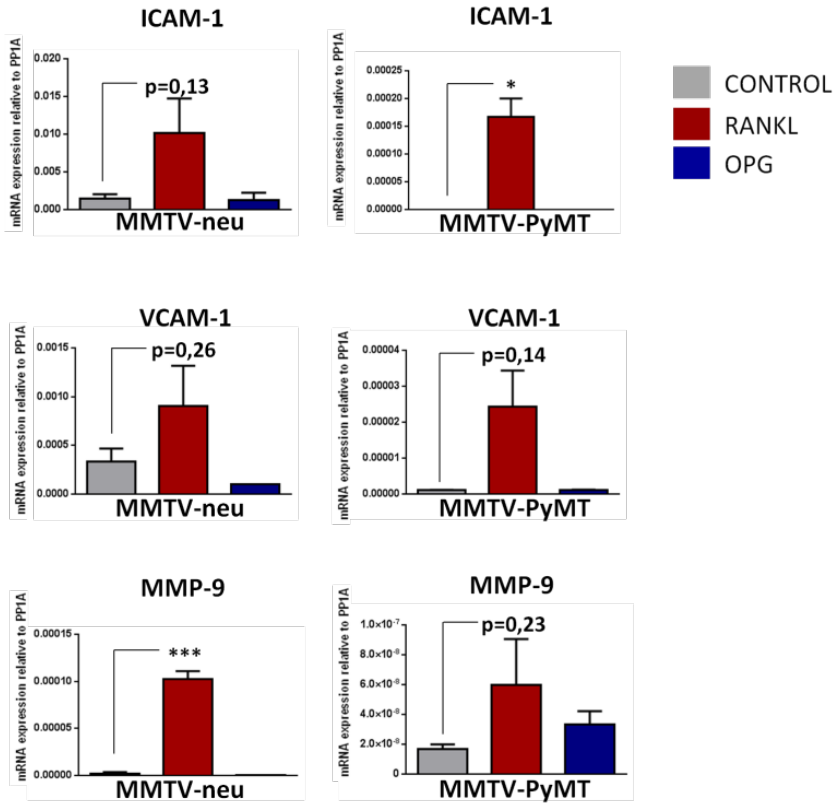
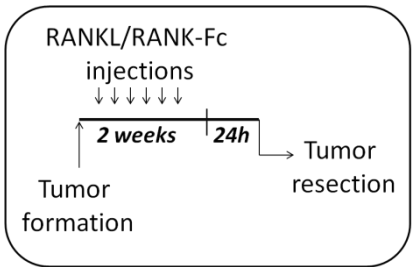

ICAM-1
mRNA expression relative to PP1A
p=0,13
MMTV-neu
ICAM-1
*
MMTV-PyMT
CONTROL
RANKL
OPG
VCAM-1
p=0,26
MMTV-neu
VCAM-1
p=0,14
MMTV-PyMT
MMP-9
***
MMTV-neu
MMP-9
p=0,23
MMTV-PyMT

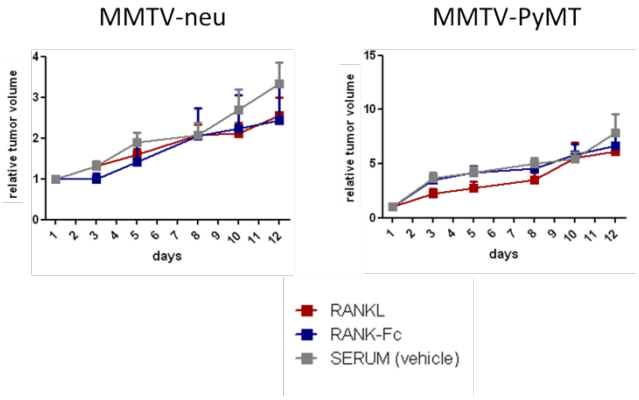
**Figure 2.** RANKL increases proliferation, invasion and up-regulates prosurvival genes in MMTV-neu and MMTV-PyMT derived tumor cells *in-vitro*.
A, F. Representative pictures of MMTV-neu and MMMTV-PyMT tumor acini cultured in matrigel with RANKL or OPG for 15 days. Arrows in (F) indicate the invasive phenotype under RANKL treatment.
B. Diameter (μm) of MMTV-neu and MMTV-PyMT tumor acini treated or not with RANKL during 15 days . Each dot represents one acini. SEM and t-test p values are shown.
C. Representative confocal images of Ki67 staining (green) in RANKL treated MMTV-neu acini and controls. Nuclei are stained with DAPI. Graph shows the percentage of proliferative cells (Ki67+) in MMTV-neu tumor (T1, T2, and T3) derived acinar cultures after 15 days treatment with RANKL. Each dot represents one acini. SEM and t-test p values are shown.
D,G. Relative mRNA expression of the indicated genes normalized to PP1A, in MMTV-neu and MMMTV-PyMT tumor acini cultured in matrigel with RANKL or OPG for 15 days. Means and SEM for 3 independent tumors per treatment are shown. t test p values between control and RANKL treated acini are shown. Quantifications were done in triplicate for each sample and mean values were used in the calculations.

## Figure 3

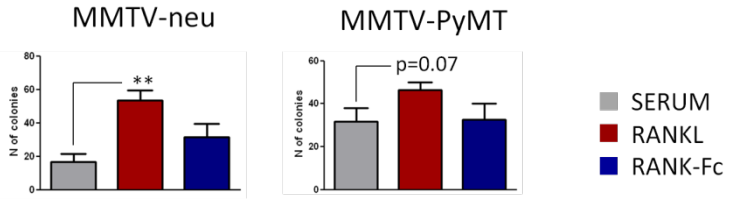
**A**




**B**




**C**




**D**

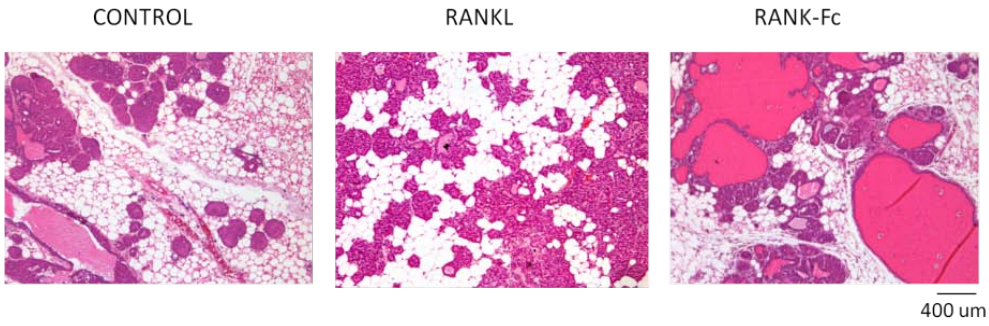



**Figure 3**. No changes in MMTV-neu & MMTV-PymT tumor growth after short-term RANKL/RANK-Fc *in vivo* treatment

A. Schematic overview of short-term RANKL (25 μg/mouse),RANK-Fc (10 mg/Kg) or serum (vehicle) treatment in tumor bearing MMTV-neu and MMTV-PyMT females. Treatment started when growing tumors reached a volume (4/3 π·r$^3$) of approximately 14.1 mm$^3$ in MMTV-PyMT mice and 65.4 mm$^3$ in MMTV-neu mice. 5 mice per treatment arm were included.

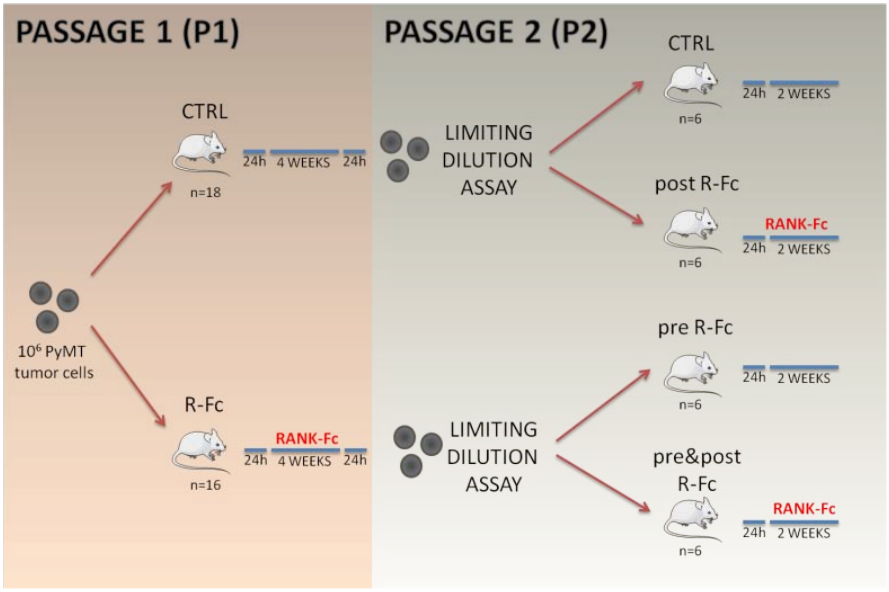
B.Tumor volume of MMTV-neu and MMTV-PyMT mice under RANKL or RANK-Fc treatment. Means and SEM of 5 mice per treatment are shown. Tumor volume is normalized to the first tumor measurement (day 1).

C. Colony formation ability of isolated tumor cells derived from RANKL or RANK-Fc treated MMTV-neu and MMTV-PyMT mice as compared with controls. Tumor cells from at least 3 mice/tumors per treatment were seeded in triplicates and quantified. Mean, SEM and t-test p values are shown.

D. Representative images of hematoxylin-eosin staining of MMTV-PyMT tumors after 2 weeks of treatment with RANKL or RANK-Fc as compared with control.

## Figure 4

**A**




**B**

| GROUP | N cells | N fat pad | N tumors | TIC frequency |
|---|---|---|---|---|
| CTRL | 100.000 | 4 | 4 | 1/ 206 (C.I. 599-70,9) |
| | 10.000 | 6 | 6 | |
| | 1.000 | 6 | 5 | |
| | 100 | 6 | 6 | |
| post R-Fc | 100.000 | 6 | 6 | 1/ 466 (C.I. 1138-190,7) |
| | 10.000 | 6 | 6 | |
| | 1.000 | 6 | 4 | |
| | 100 | 6 | 4 | |
| Pre-R-Fc | 100.000 | 6 | 6 | 1/ 1929 (C.I. 5431-685,1) |
| | 10.000 | 6 | 5 | |
| | 1.000 | 6 | 5 | |
| | 100 | 6 | 2 | |
| pre&post R-Fc | 100.000 | 6 | 6 | 1/ 2353 (C.I. 6378-868,3) |
| | 10.000 | 6 | 5 | |
| | 1.000 | 6 | 3 | |
| | 100 | 6 | 3 | |

| GROUP | P value | $X^2$ |
|---|---|---|
| CTRL vs Post RANK-Fc | 0,13 | 2,27 |
| CTRL vs Pre RANK-Fc | $2,99^{-5}$ | 17,4 |
| CTRL vs Pre&Post RANK-Fc | $7,48^{-06}$ | 7,41 |
| Pre RANK-Fc vs Pre&Post RANK-Fc | 0,701 | 0,147 |

**C**

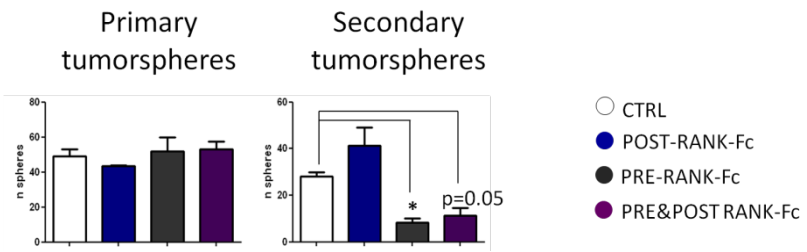



**Figure 4**. Inhibition of RANK signaling depletes the pool of MMTV-PyMT tumor-initiating cells.
A. Schematic representation of RANK-Fc treatments in orthotopic MMTV-PyMT tumors in passage 1 (P1) and passage 2 (P2). $10^6$ cells from one MMTV-PyMT tumor were implanted in the inguinal fat pads of FVB females (P1). Mice were randomized 1:1 between RANK-Fc treatment for 4 weeks or controls (serum). Isolated tumor cells derived from CONTROL and RANK-Fc treated tumors were injected in limiting

dilutions in the inguinal fat pads of FVB females (P2). Groups post RANK-Fc and pre&post RANK-Fc received additional 2 weeks long treatment with RANK-Fc; groups CONTROL and pre-RANK-Fc were treated with serum. Treatments started 24h after tumor injection and lasted for 4 weeks for P1 and 2 weeks for P2. Serum or RANK-Fc (10 mg/Kg) was injected subcutaneously 3 times per week.

B. Tumor frequency per N of fat pads injected/ cell dilution is shown for the indicated groups. Extreme limiting dilution analysis (ELDA) between indicated groups is shown. Tumor formation was monitored for 38 weeks. Virtual tumor initiating cell frequency calculated by ELDA, confidence intervals, p values and chi-square are indicated.

C. Number of primary and secondary tumor mammospherers derived from the indicated groups of treatment (P2). For primary and secondary mammospheres, each bar is representative of 4 tumors quantified in duplicate and triplicate, respectively. Mean, SEM and t-test p values are shown.

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## Figure 6

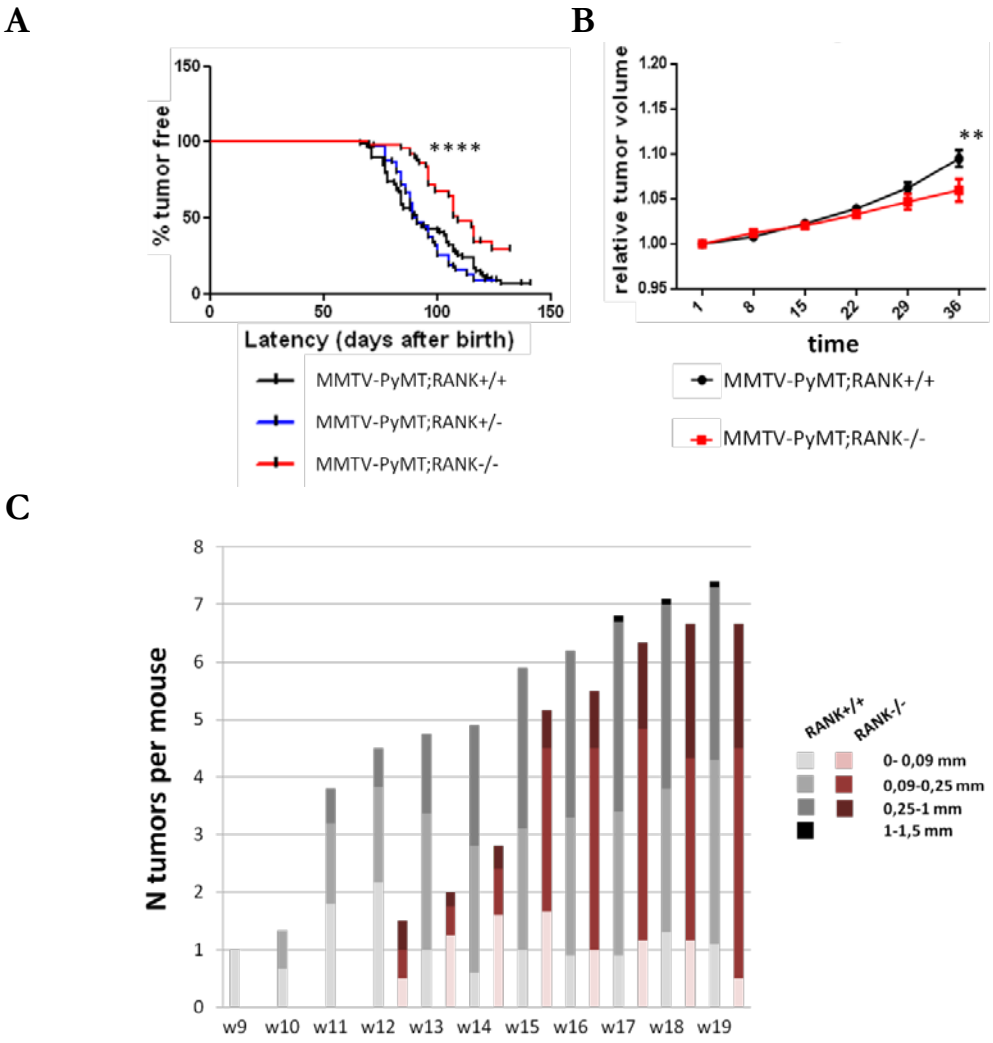



**Figure 6.** Constitutive deletion of RANK increases tumor latency, decreases growth rate and incidence of MMTV-PyMT tumors.

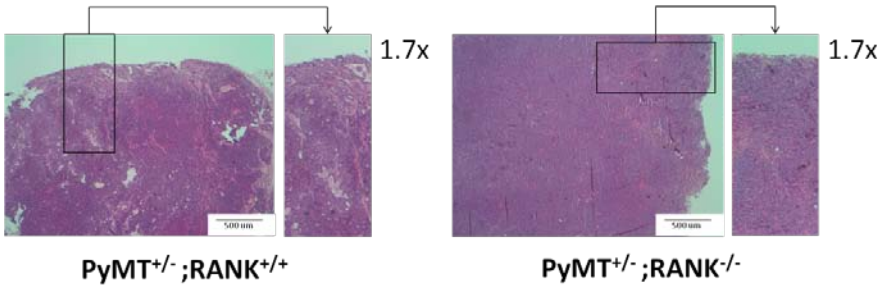
A. Kinetics of palpable tumor onset (Kaplan-Meyer curves) with age in the indicated genotypes. 10 MMTV-PyMT;RANK$^{+/+}$, 12 MMTV-PyMT;RANK$^{+/-}$ and 6 MMTV-PyMT;RANK$^{-/-}$ mice were analyzed. Only growing palpable lesions of at least 2 mm of diameter were considered. Statistical difference between MMTV-PyMT;RANK$^{-/-}$ and MMTV-PyMT;RANK$^{+/+}$ groups was observed as evaluated by logrank test.

B. Growth curves of MMTV-PyMT;RANK$^{-/-}$ and MMTV-PyMT;RANK$^{+/+}$ tumors relative to initial tumor volume. The mean ± SEM of tumor volume of 49 MMTV-PyMT;RANK$^{+/+}$ and 31 MMTV-PyMT;RANK-/- tumors are represented in each time point. p value of 2-way ANOVA test is shown.

C. Cumulative number of tumors per mouse in MMTV-PyMT;RANK$^{+/+}$(WT) MMTV-PyMT;RANK$^{-/-}$ (RANK$^{-/-}$) mice from week 9 (w9) to week 19 (w19) of age. Tumors were classified by diameter as indicated. 8 MMTV-PyMT;RANK$^{+/+}$mice and 4 MMTV-PyMT;RANK$^{-/-}$ mice (F2)were analyzed.

## Figure 7

**A**




**B**

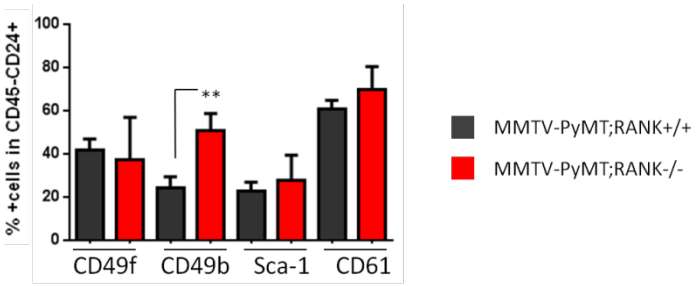
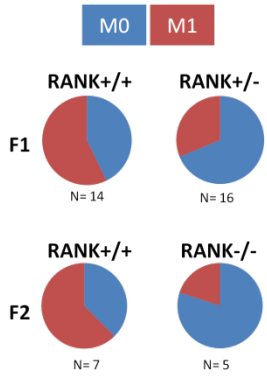



**Figure 7**. Constitutive deletion of RANK results in decreased histological heterogeneity and accumulation of CD49b+ cells in MMTV-PyMT tumors.
A. Pictures of hematoxylin eosin staining of representative MMTV-PyMT;RANK$^{-/-}$ and MMTV-PyMT;RANK$^{+/+}$ palpable lesions. Histological areas are magnified in the insets.
B. Quantification of the percentage of CD49f, CD49b, Sca1 and CD61 within the CD45-CD24+population in MMTV-PyMT;RANK$^{-/-}$ and MMTV-PyMT;RANK$^{+/+}$ tumors analyzed by flow citometry. Bars represent the mean and SEM of 6 MMTV-PyMT;RANK$^{+/+}$ and 4 MMTV-PyMT;RANK$^{-/-}$ tumors.

# Figure 8

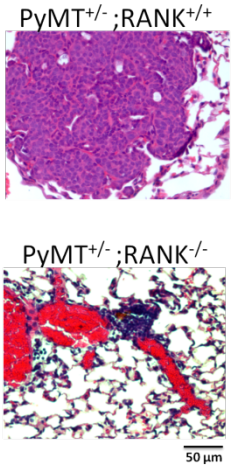
**A**




**B**

| mouse | PyMT | RANK | AGE | lung metastasis | PRIMARY TUMOR HISTOLOGY |
|---|---|---|---|---|---|
| 1 | -/+ | +/+ | 20,10 | 565,00 | late carcinoma |
| 2 | -/+ | +/+ | 18,00 | 354,00 | late carcinoma |
| 4 | -/+ | +/+ | 19,50 | 14,00 | late carcinoma |
| 3 | -/+ | +/+ | 19,60 | 29,00 | early carcinoma |
| 5 | -/+ | +/+ | 15,30 | 6,00 | early carcinoma |
| 6 | -/+ | +/+ | 19,10 | 0,00 | early carcinoma |
| 7 | -/+ | +/+ | 14,30 | 0,00 | early carcinoma |
| 8 | -/+ | +/+ | 15,00 | 0,00 | MIN/ adenoma |
| 1 | -/+ | -/- | 19,60 | 2,00 | late carcinoma |
| 2 | -/+ | -/- | 17,00 | 0,00 | late carcinoma |
| 3 | -/+ | -/- | 15,00 | 0,00 | late carcinoma |
| 4 | -/+ | -/- | 18,90 | 0,00 | early carcinoma |
| 5 | -/+ | -/- | 13,10 | 0,00 | early carcinoma |
| 6 | -/+ | -/- | 18,60 | 0,00 | MIN/ adenoma |

**C**




**D**

| GROUP | N cells | mice | Metastasis incidence |
|---|---|---|---|
| MMTV-PyMT;RANK+/+ | 100.000 | 4 | 4 |
| | 10.000 | 4 | 4 |
| | 1.000 | 4 | 0 |
| | 100 | 4 | 1 |
| MMTV-PyMT;RANK-/- | 100.000 | 4 | 3 |
| | 10.000 | 4 | 3 |
| | 1.000 | 4 | 1 |
| | 100 | 4 | 0 |

**METASTASIS INITIATING CELL FREQUENCY**

MMTV-PyMT;RANK+/+ : 1/ 2.952 (C.I. 93.261-9.281)
MMTV-PyMT;RANK-/- : 1/ 29.420 (C.I. 9.374-929)

**P value:** 0,00262 $\chi^2$: 9,06

**E**

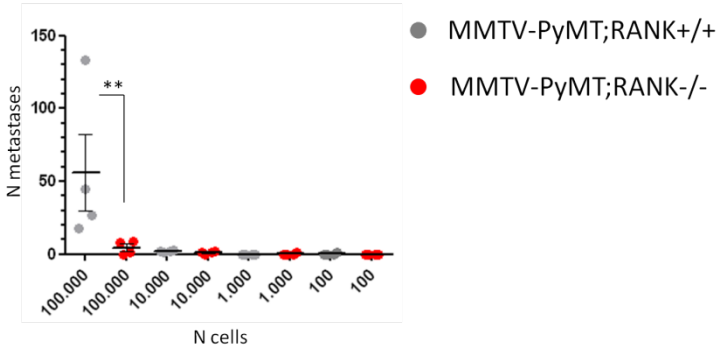
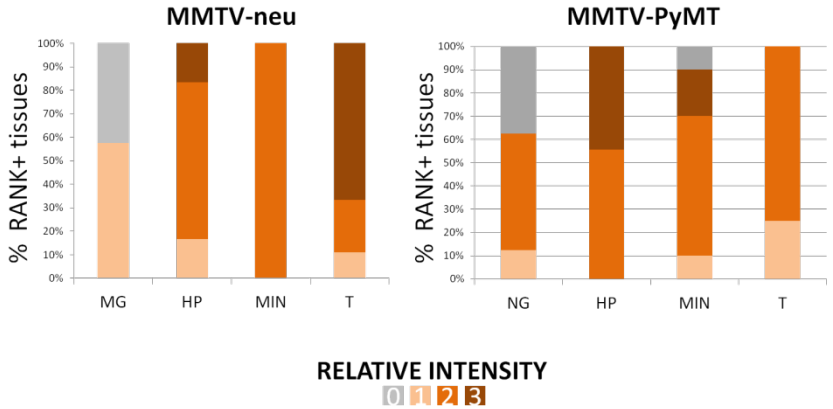



**Figure 8.** Metastatic potential of primary MMTV-PyMT tumors is RANK dependent
A. Pie charts showing lung metastasis incidence in F1 MMTV-PyMT;RANK$^{+/+}$ and MMTV-PyMT;RANK$^{+/-}$ and in F2 MMTV-PyMT;RANK$^{+/+}$ and MMTV-PyMT;RANK$^{-/-}$ mice. Only mice bearing early/late carcinomas were included. Presence (M1) or absence (M0) of lung metastasis is quantified. 3 histological sections were quantified for F1 tumors, 16 histological sections (whole lung) were quantified for F2 tumors.

B. Table showing genotype, age of the mouse, quantification of lung metastasis and histology of the most advanced tumor present in MMTV-PyMT;RANK$^{+/+}$ and MMTV-PyMT;RANK$^{-/-}$ mice.
C. Representative hematoxylin-eosin staining of lungs of tumor bearing MMTV-PyMT;RANK$^{+/+}$ and MMTV-PyMT;RANK$^{-/-}$ mice (late carcinoma) . Lung metastasis are detected in MMTV-PyMT;RANK$^{+/+}$ mice whereas in MMTV-PyMT;RANK$^{-/-}$ lungs only lymphoid infiltrates are found.
D. Table showing limiting dilution assay to test metastasis initiation ability of MMTV-PyMT;RANK$^{-/-}$ and MMTV-PyMT;RANK$^{+/+}$ tumor cells. Cells were injected intravenously in *Foxn1$^{nu}$* females and the presence of lung metastasis was scored after 8 weeks;16 histological sections of the whole lung were quantified. Metastatic cell frequency for each group calculated by ELDA with confidence intervals, p value and chi-square are shown.
E. Quantification of the absolute number of lung metastasis in nude mice which received intraveouns injection of MMTV-PyMT;RANK$^{-/-}$ and MMTV-PyMT;RANK$^{+/+}$ tumor cells in limiting dilutions. Each dot represents the lung of one mouse.

# SUPPLEMENTAL INFORMATION

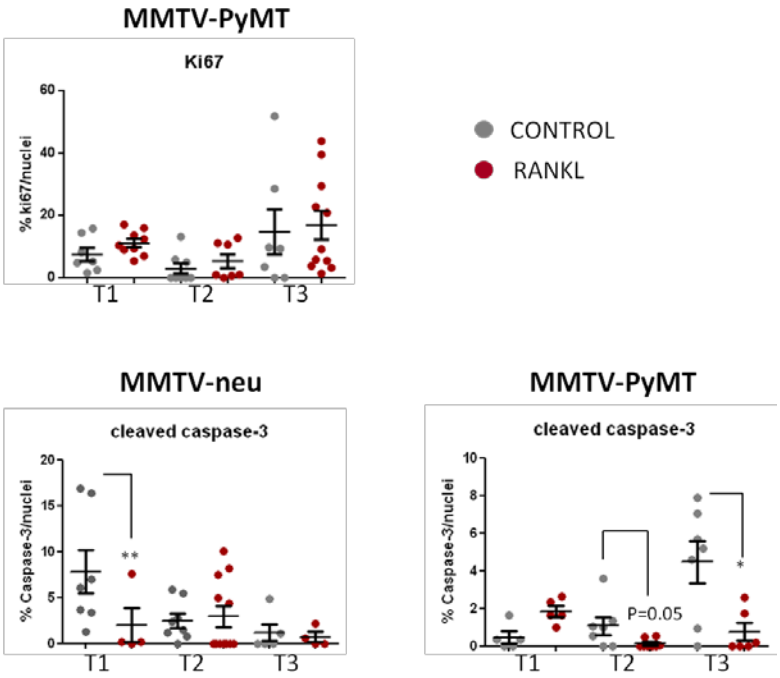
## Supplemental Figure 1



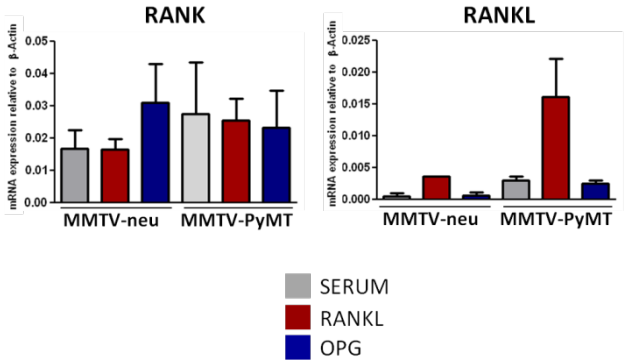

**Supplemental Figure 1**. Expression profile of RANK protein during MMTV-neu and MMTV-PyMT tumorigenesis.
Percentage of positive tissues and intensity of RANK protein expression during tumor progression in MMTV-neu and MMTV-PyMT mice as detected by immunohistochemistry. For MMTV-PyMT, 8 residual normal gland areas (NG), 9 hyperplasias (HP), 9 mammary intraepithelial neoplasias (MIN) and 9 tumors (T) were quantified. For MMTV-neu 33 NG, 6 HP, 6 MIN and 9 T were quantified. Intensity of staining was classified as strong (3), medium (2), weak (1) or undetectable (0).

## Supplemental Figure 2

**A**




**B**

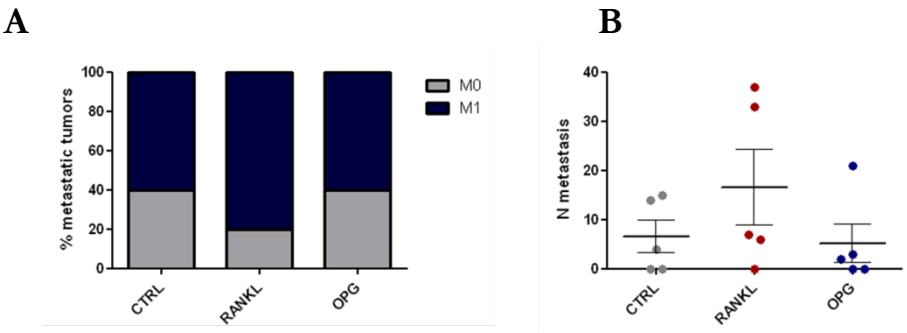



**Supplemental Figure 2**. Proliferation and apoptosis, RANK and RANKL expression in MMTV-neu and MMTV-PyMT acini.

A. Percentage of proliferative (Ki67) and apoptotic cells (cleaved Caspase-3) in MMTV-neu and MMTV-PyMT derived acinar cultures after 15 days of treatment with RANKL. Each dot represents one acini. Three tumors for each model were quantified (T1, T2, T3). Mean, SEM and significant t-test p values are shown.

B. mRNA expression of RANK and RANKL in MMTV-neu and MMTV-PyMT acinar cultures after 15-weeks treatment with RANKL or OPG. Mean and SEM for 3 mice per condition are shown. Quantifications were done in triplicate for each sample and mean values were used in the calculations.

## Supplemental Figure 3

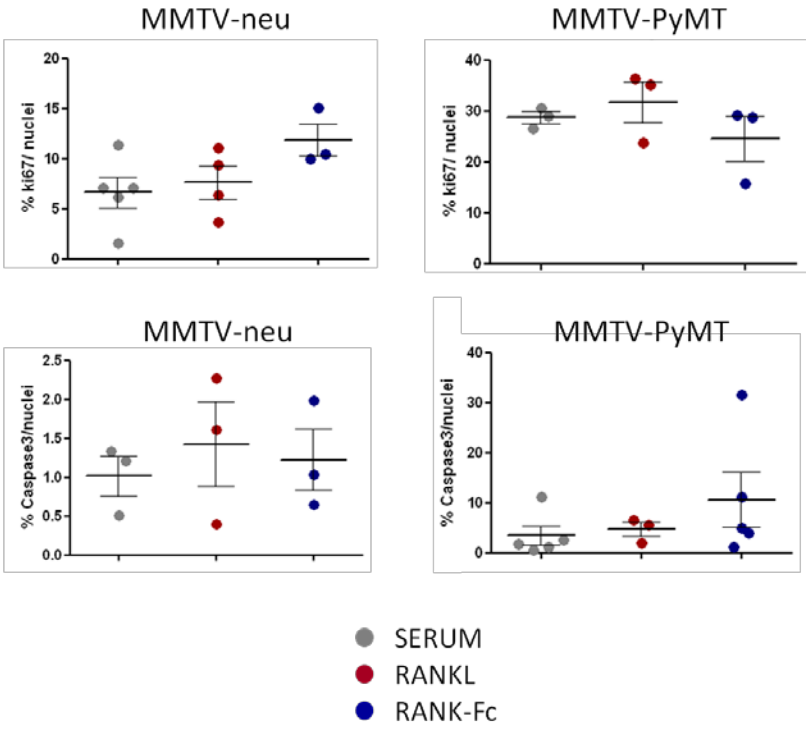



**Supplemental Figure 3**. Lung metastasis from OPG treated MMTV-PyMT acini show decreased cell density.

A. Metastasis incidence of MMTV-PyMT tumor cells isolated from RANKL or OPG treated matrigel cultures, injected in Nod/Scid mice intravenously as compared with non treated controls. 9 weeks after injections, lung metastasis were quantified and mice classified as metastatic (M1) or free of metastasis (M0). 5 mice per treatment were quantified.

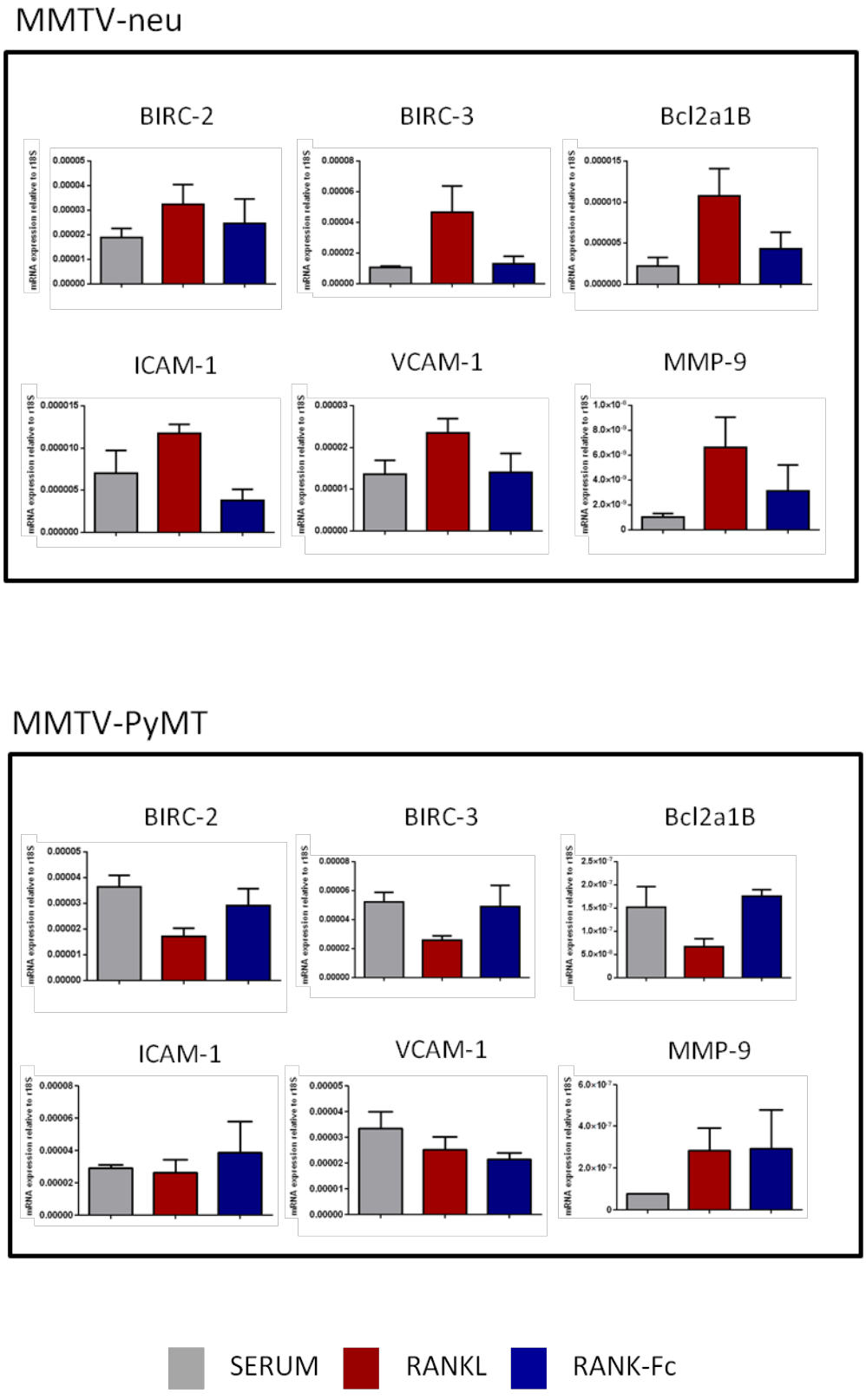
B. Quantification of lung metastatic foci in RANKL or OPG treated as compared with their control. Each dot represents one mouse.

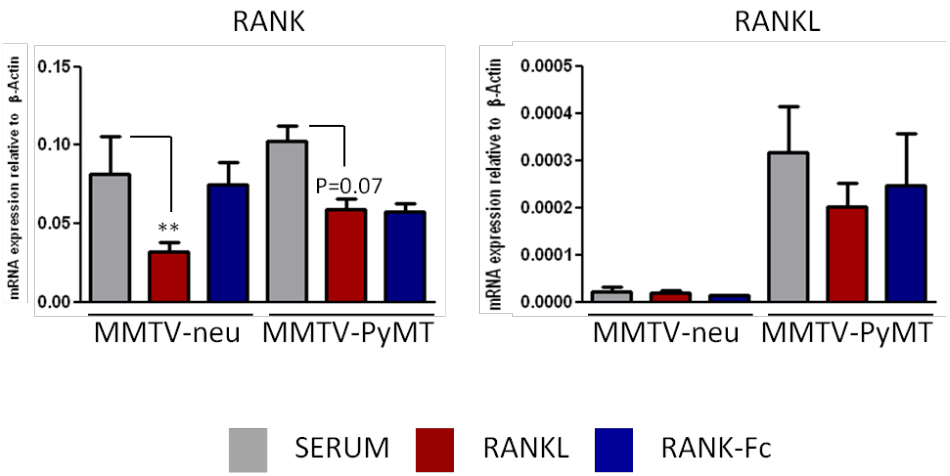
## Supplemental Figure 4




**Supplemental Figure 4.** Short-term treatment with RANKL and RANK-Fc in MMTV-neu and MMTV-PyMT mice does not modify tumor cell proliferation and apoptosis.
Tumor proliferation quantified as percentage of Ki67+ cells and apoptosis quantified as percentage of cleaved Caspase-3+ cells after 2-weeks of treatment with RANKL or RANK-Fc in tumor bearing MMTV-neu and MMTV-PyMT mice. Each dot represents one tumor of one mouse. 6 sections per tumor were quantified and mean values are represented. Mean and SEM for each group is shown.

## Supplemental Figure 5

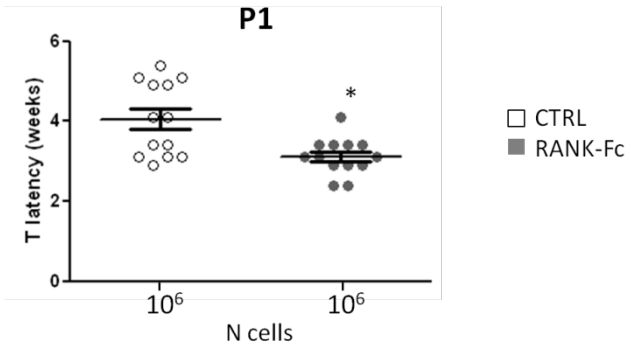




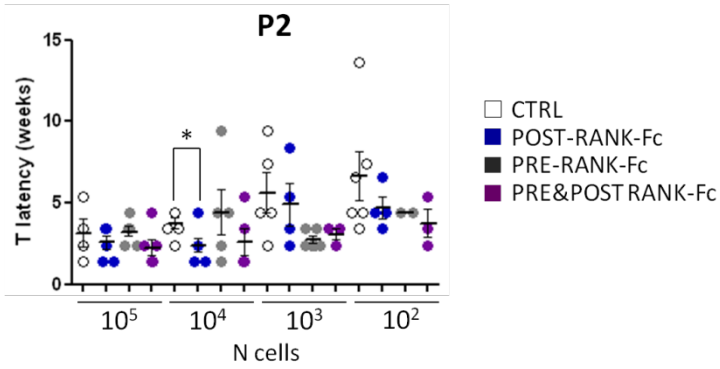

**Supplemental Figure 5.** RANKL treated MMTV-neu tumors show similar expression profile to acinar cultures, unlike MMTV-PyMT tumors.
Relative mRNA expression levels of the indicated genes in tumors derived MMTV-neu and MMTV-PyMT mice treated 2 weeks with RANK-Fc or RANKL. Each bar represents the mean of at least 3 tumors and SEM is represented. Quantifications for each sample were done in triplicates and mean values were used in the calculations. Note that basal levels of RANKL are higher in MMTV-PyMT than in MMTV-neu acini and increase further after RANKL treatment.

## Supplemental Figure 6

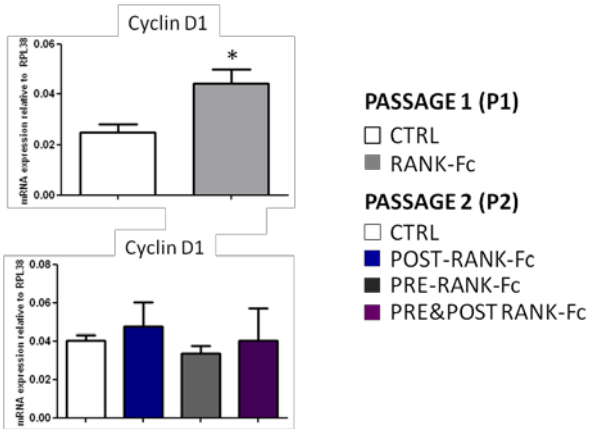
**A**




**B**



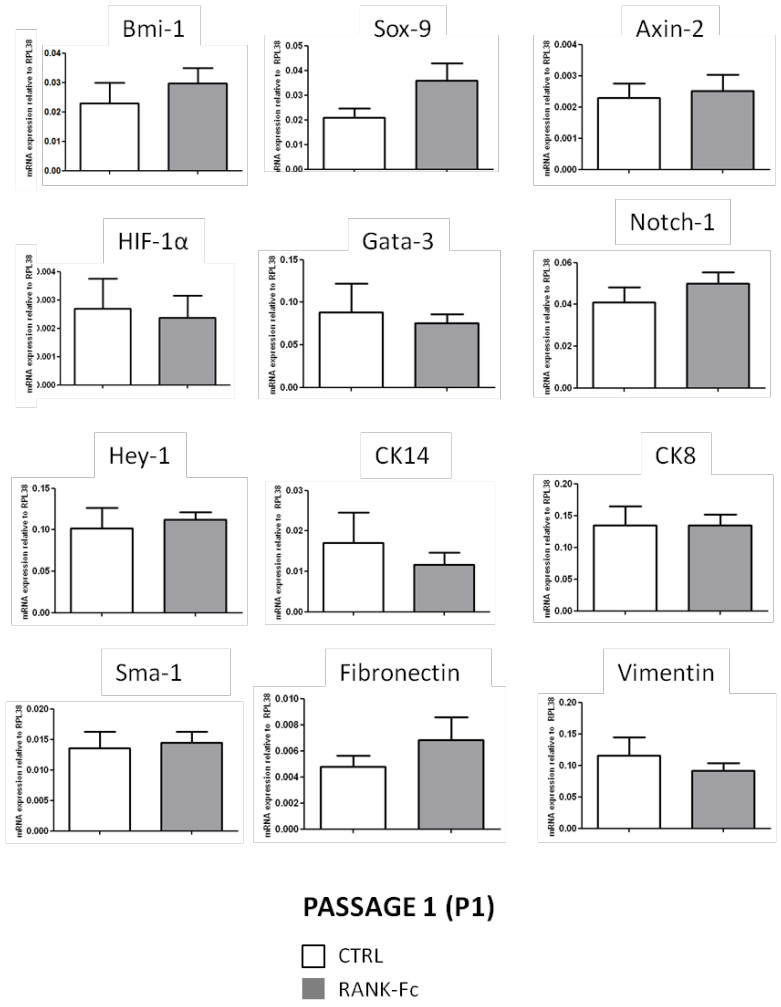

**C**



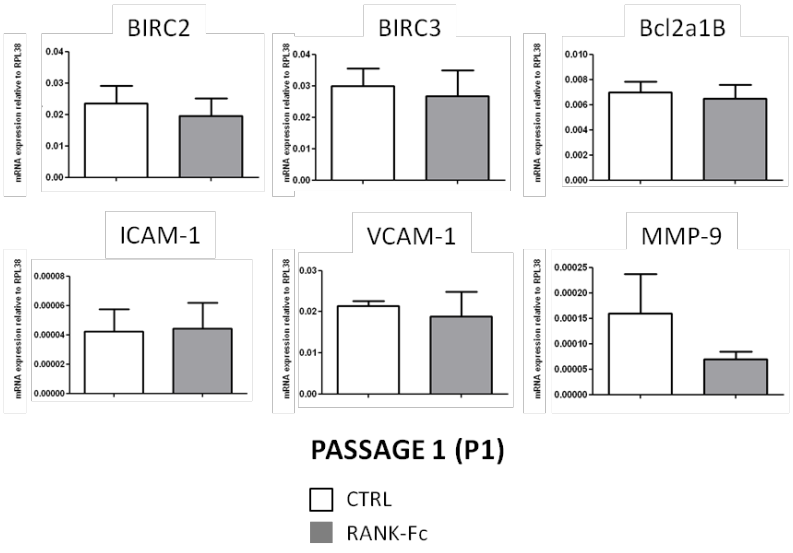

**Supplemental Figure 6**. RANK-Fc treatment decreases tumor latency in MMTV-PyMT orthotopic model.
A, B. Tumor latency of RANK-Fc treated MMTV-PyMT orthotopic tumors for P1 (A) and P2 (B). Each dot represents one tumor. Mice were considered to be tumor-free until a growing palpable mass of at least 2 mm of diameter was observed. Mean, SEM and significant t-test p-values are indicated.
C. mRNA expression of Cyclin D1 in P1 and P2 tumors . Each bar represents the mean of at least 7 tumors in P1 and 3 tumors in P2. SEM and t-test are represented. Quantifications for each sample were done in triplicate and mean values were used in the calculations.

## Supplemental Figure 7

**A**



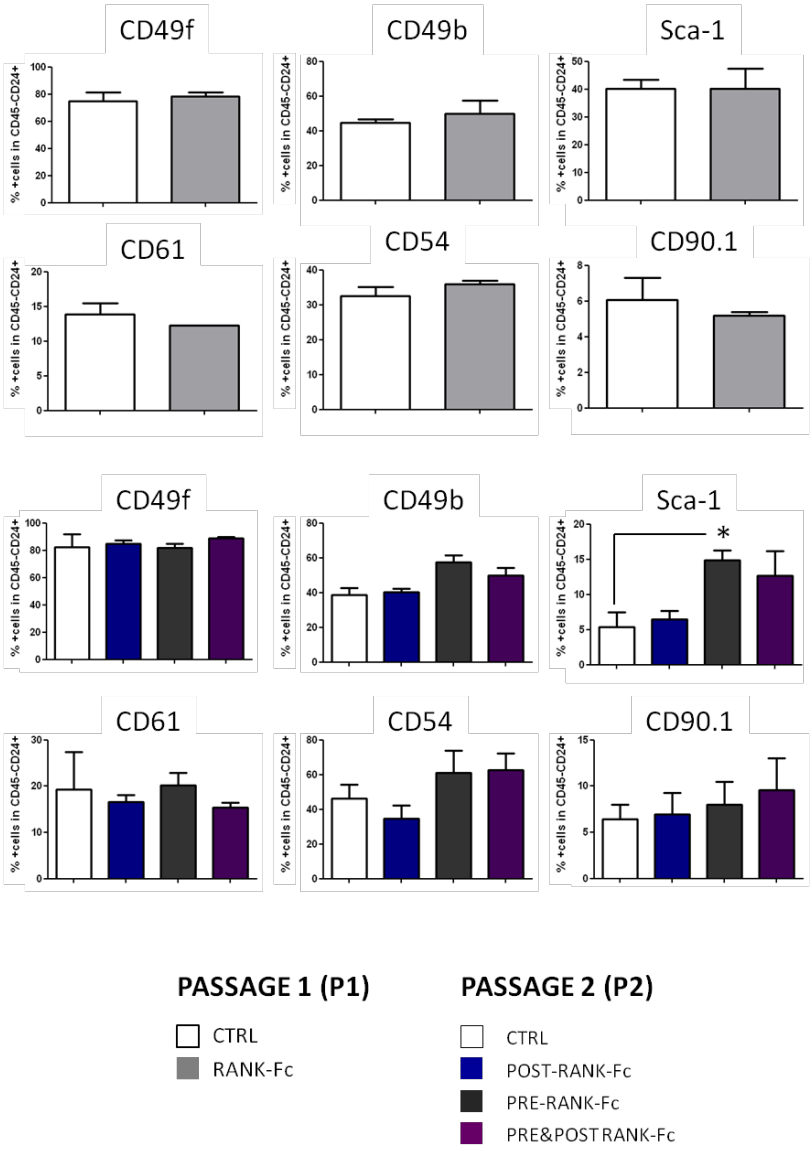

**B**




**Supplemental Figure 7**. Expression profile of genes of tumor epithelial cells in RANK-Fc treated MMTV-PyMT tumors.

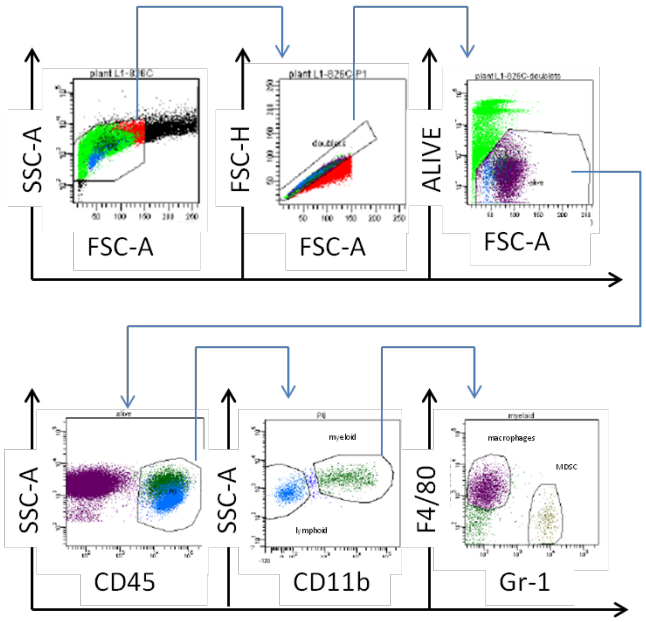
A. Relative mRNA expression levels of the indicated genes in P1 MMTV-PyMT orthotopic tumors after 4 weeks of RANK-Fc treatment. Gene expression levels are normalized to RPL38. Each bar represents the mean and SEM of 4 tumors. Quantifications for each sample were done in triplicates and mean values were used in the calculations.
B. Relative mRNA expression levels of the indicated genes in P1 MMTV-PyMT orthotopic tumors after 4 weeks of RANK-Fc treatment. Each bar represents the mean and SEM of at least 9 tumors. Quantifications for each sample were done in triplicates and mean values were used in the calculations.

## Supplemental Figure 8




**Supplemental Figure 8**. RANK-Fc neo-adjuvant treatment increases the CD24+Sca1+ population. Frequency of the indicated populations within the CD45-CD24+ tumor cells as analyzed by flow cytometry in P1 and P2 RANK-Fc treated MMTV-PyMT orthotopic tumors. Each bar represents the mean and SEM of 2 tumors in P1 and 4 in P2. Analysis of Sca-1, CD49f and CD61 in P2 tumors is representative of two independent experiments.

## Supplemental Figure 9




**Supplemental Figure 9**. Lymphoid and myeloid infiltrates are in MMTV-PyMT orthotopic tumors
Dot blots representing the hierarchy defined for the selection of lymphoid and myeloid tumor infiltrates in MMTV-PyMT orthotopic tumors. A representative tumor from P2 CTRL group is shown.

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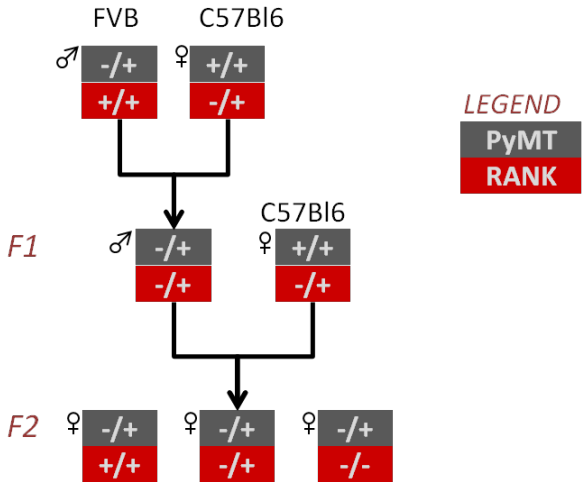
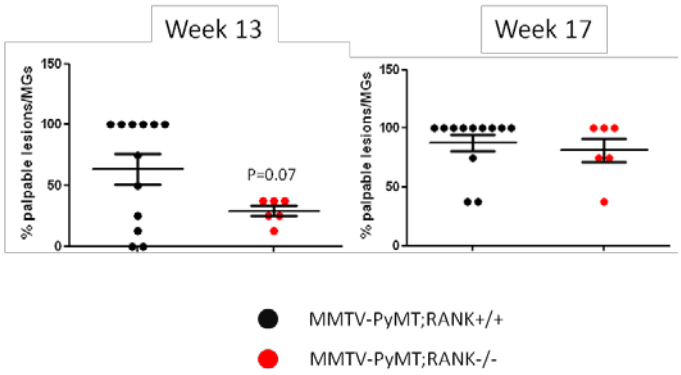
Pàgina eliminada de la versió electrònica per motiu de l'acord de confidencialitat subscrit amb les entitats participants en la tesi

## Supplemental Figure 12

**A**




**B**



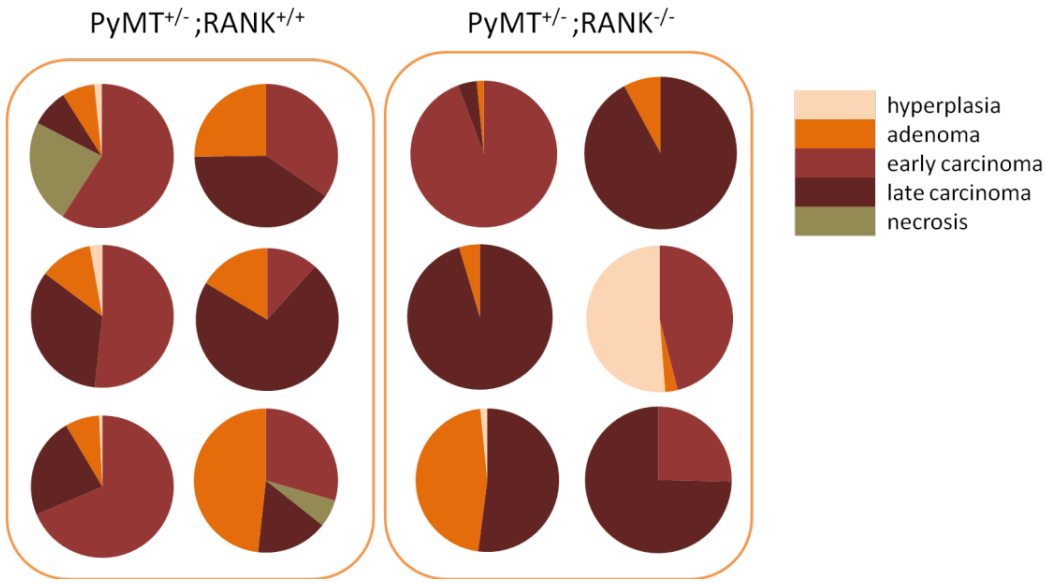

**Supplemental Figure 12**. Genetic deletion of RANK delays tumor onset and decreases tumor incidence in MMTV-PyMT mice

A. Schematic representation of the generation of MMTV-PyMT;RANK$^{-/-}$ mice. MMTV-PyMT;RANK$^{+/+}$ mice from the same genetic background/generation were used for comparison.

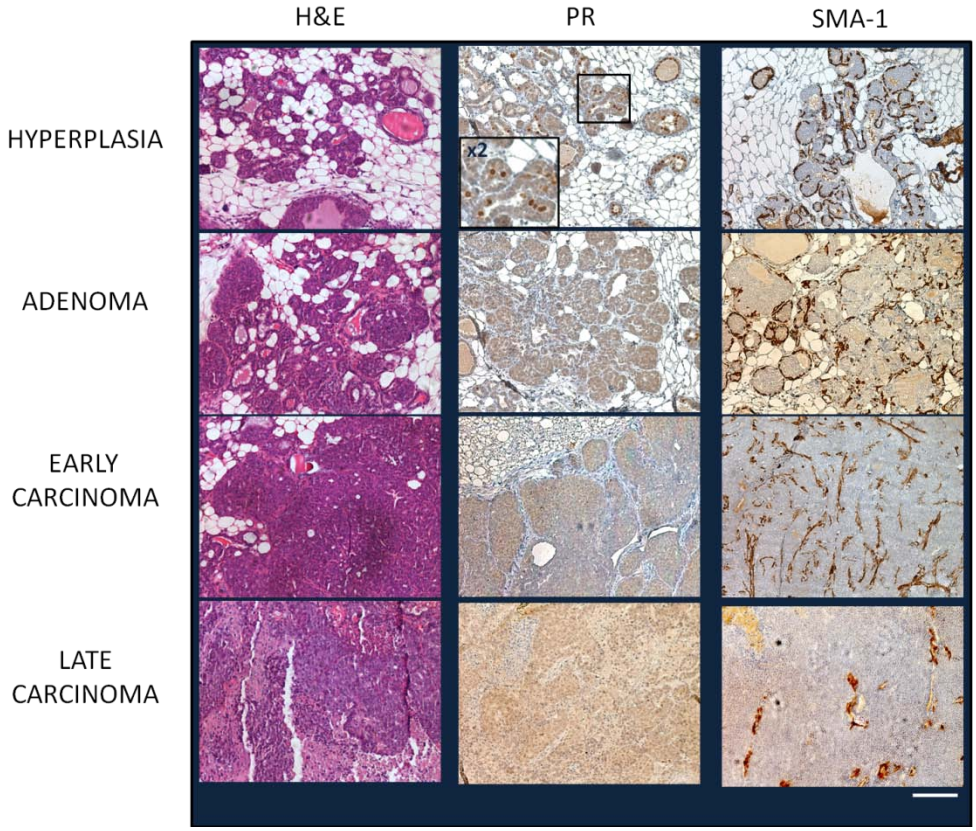
B. Incidence of palpable lesions per mouse in MMTV-PyMT;RANK$^{-/-}$ and their controls at 13 and 17 weeks of age. Each dot represents percentage of palpable lesions in one mouse, considering a total of 8 sites (mammary gland #2 and #3 were considered as 1 site).

## Supplemental Figure 13

**A**

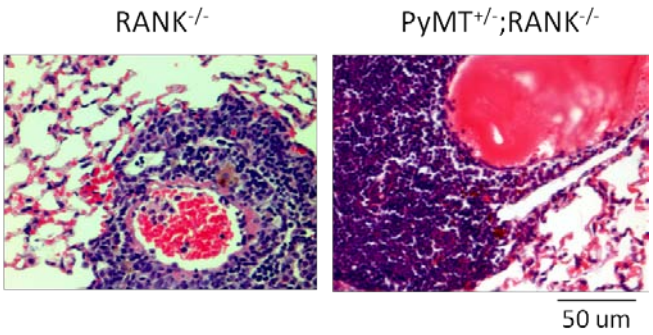



**B**




**Supplemental Figure 13**. Constitutive deletion of RANK results in decreased histological heterogeneity
A. Pie charts representing quantification of histological areas of MMTV-PyMT;RANK$^{-/-}$ and MMTV-PyMT;RANK$^{+/+}$ tumors. Classification was done based on H&E, Sma-1 and PR staining.
B. Representative pictures of hematoxylin eosing, PR and Sma-1 protein expression in MMTV-PyMT palpable lesions by immunostaining.

## Supplemental Figure 14




**Supplemental Figure 14**. Genetic deletion of RANK results in an accumulation of lymphoid infiltrates in the lungs independently of tumor growth
Representative histology (H&E) of accumulation of lymphatic cells in the lungs of tumor bearing MMTV-PyMT;RANK$^{-/-}$ mice compared to RANK$^{-/-}$ mice (C57Bl6) in the absence of tumor development.

## Supplemental Figure 15

**A**

| GROUP | N cells | TICs | | MICs | |
|---|---|---|---|---|---|
| | | N fat pads | Tumor incidence | N mice | Metastasis incidence |
| MMTV-PyMT; RANK+/+ | 10.000 | 8 | 8 | 4 | 4 |
| | 1.000 | 8 | 6 | 4 | 3 |
| | 100 | 8 | 6 | 4 | 2 |
| | 10 | 8 | 0 | 4 | 2 |
| MMTV-PyMT; RANK-/- | 10.000 | 8 | 8 | 4 | 3 |
| | 1.000 | 8 | 6 | 4 | 3 |
| | 100 | 8 | 6 | 4 | 3 |
| | 10 | 8 | 1 | 4 | 1 |

**TICs: TUMOR INITIATING CELL FREQUENCY (ELDA)**

**MMTV-PyMT;RANK+/+ :** 1/ 22,5 (C.I. 45,2-11,2)
**MMTV-PyMT;RANK-/- :** 1/ 20,8 (C.I. 41,7 -10,3)

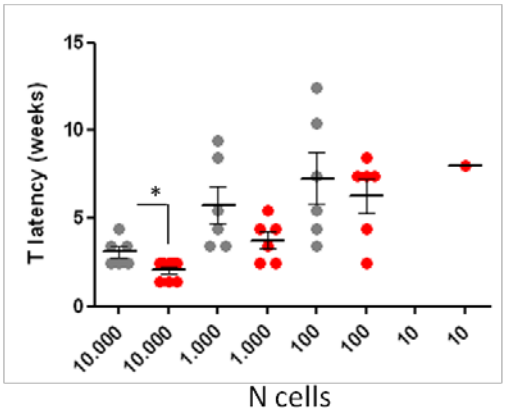
**P value:** 0.823 $\chi^2$: 0,049

**MICs: METASTASIS INITIATING CELL FREQUENCY (ELDA)**

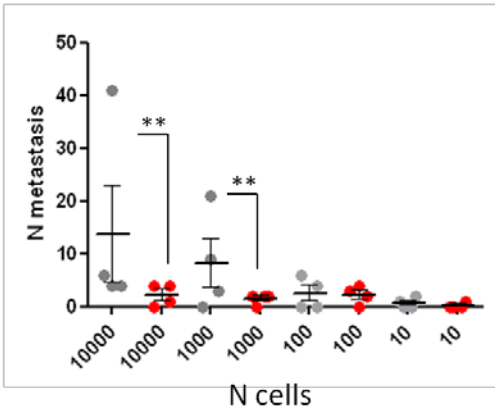
**MMTV-PyMT;RANK+/+ :** 1/ 303 (C.I. 954 - 95.9)
**MMTV-PyMT;RANK-/- :** 1/ 1790 (C.I. 6382 - 502.2)

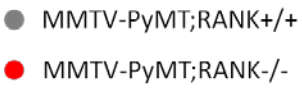
**P value:** 0.00296 $\chi^2$: 8.83

**B**




**C**




**Supplemental Figure 15**. Constitutive deletion of RANK in othotopic MMTV-PyMT tumors does not alter tumor initiating cells but decreases tumor latency and impairs lung metastasis

A. limiting dilution assay of MMTV-PyMT;RANK$^{-/-}$ and MMTV-PyMT;RANK+/+ tumors in *Foxn1*$^{nu}$ female recipients. Tumor cells from 2 tumors/mice from each genotype were pooled for injections in limiting dilution. 4 nude mice per each cell dilution were injected in both inguinal mammary glands and tumor growth was monitored. The presence of lung metastasis was scored at necropsy. Mice were sacrificed when tumors reach 1 cm of diameter or after 45 weeks. Tumor initiating cell frequency and metastasis initiating cell frequency for each group as calculated by ELDA is indicated with confidence intervals; p-value and chi-square values are shown.

B. Tumor latency in *Foxn1*$^{nu}$ females injected with MMTV-PyMT;RANK$^{-/-}$ and MMTV-PyMT;RANK+/+tumor cells in limiting dilutions. Each dot represents one tumor. Mice were considered to be tumor free until a growing palpable mass of at least 2 mm of diameter was observed. Significant t-test p -values are indicated.

C. Quantification of lung metastatic foci in *Foxn1*$^{nu}$ females injected with MMTV-PyMT;RANK$^{+/+}$ and MMTV-PyMT;RANK$^{-/-}$ tumor cells in the inguinal mammary glands. Each dot represents one mouse.

# ARTICLE 3

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

## PEER REVIEWED ARTICLE

Palafox M, Ferrer I, Pellegrini P, Vila S, Hernandez-Ortega S, Urruticoechea A, Climent F, Soler MT, Muñoz P, Viñals F, Tometsko M, Branstetter D, Dougall WC, González-Suárez E. RANK induces epithelial-mesenchymal transition and stemness in human mammary epithelial cells and promotes tumorigenesis and metastasis. Cancer Res. 2012 Jun 1;72(11):2879-88.

## AUTORSHIP DECLARATION

Contribution of the author Pasquale Pellegrini is confined to the characterization of the expression of RANK in clinical samples by performing immunohistochemistry, data analysis, correlation of RANK, RANKL mRNA expression levels with tumor subtype and pathological grade and final approval of manuscript.

# SUPPLEMENTAL 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,  -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, 10-10M Estradiol, 1,4x10-6M 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 (5x106) 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 □g/ ml of blasticidin for MCF10A, HMECS, MDAMB-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 (105) 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 20m 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 104) 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 3x104cells 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.

# FINAL REMARKS

# FINAL REMARKS

RANK in mammary gland biology has emerged as a key pathway in the interplay of mammary stem cell fate, differentiation, tumor initiation and metastasis.

## 1. RANK pathway is a master regulator of mammary stem cell fate and alveolar commitment.

Genetically modified mouse models evidence RANK pathway as an essential regulator of mammary differentiation (Fata JE et al., 2000; Gonzalez-Suarez E et al., 2007). RANK signaling mediates the major proliferative response of mouse mammary epithelium to progesterone during mammary morphogenesis (Asselin-Labat ML et al., 2010; Beleut M et al., 2010; Joshi PA et al., 2010). Accordingly, we observed that virgin MMTV-RANK mice showed increased mammary proliferation and side branching, a phenotype that mimics the acute progesterone stimuli that occurs during pregnancy (Fernandez-Valdivia R et al., 2012). MMTV-RANK mammary glands showed a lack of alveologenesis as previously reported (Gonzalez-Suarez E et al., 2007) suggesting that RANK signaling regulates mammary stem cell fate interfering with differentiation.

Previous studies suggested that RANKL pathway could mediate the expansion of the mammary stem cell population regulated by progesterone (Asselin-Labat ML et al., 2010; Joshi PA 2010). Schramek et al. supported this hypothesis demonstrating that deletion of RANK in the mammary epithelia results in a decrease of self-renewal ability *in vitro* in mammary stem cells under progesterone stimuli (Schramek D et al., 2010). Based on these evidences we showed an unambiguous role of RANK signaling in mammary stem cell expansion, as enhanced RANK signaling results in increased mammary repopulating ability in-vivo. Similarly, our laboratory demonstrated that overexpression of RANK leads to acquisition of a stem-like phenotype in human cell lines (Palafox M et al., 2012).

RANK is expressed in both basal and luminal cells, while RANKL is expressed only in a subset of luminal cells that are ER+PR+. The ability of RANK to expand the mammary stem cell pool gives strength to the hypothesis that progesterone could induce expansion of the mammary stem cells by paracrine signaling: progesterone induces expression of RANKL in PR+ cells (Beleut M et al., 2010) and RANKL could induce accumulation of RANK+ basal mammary stem cells with consecutive expansion of mammary epithelium.

MMTV-RANK mice overexpress RANK in both basal and luminal compartments. Even though MMTV-RANK mammary gland showed abnormal expansion of mammary stem cells and proliferation of the whole epithelium, there is a block in alveolar differentiation during gestation (Gonzalez-Suarez E et al., 2007). We identified several defects in the luminal compartment that could be responsible of defective alveologenesis. First, we demonstrated that activation of RANK results in an expansion of the luminal population. Moreover, luminal progenitors increased in MMTV-RANK mice, evidenced by their higher colony formation ability when stimulated with RANKL as compared to WT cells. When MMTV-RANK mice go through gestation, the expansion of the luminal population was accentuated, thus excluding the hypothesis that the lack in alveologenesis would be caused from a reduced volume of luminal cells, in favour of basal cells, and pointed to a specific defect in alveolar commitment. Characterization of lineage-specific keratins also supported that constitutive activation of RANK leads to an accumulation of intermediate progenitors. MMTV-RANK mice showed an accumulation of CK5+CK8+ and CK14+CK8+ cells. Similarly, genetic deletion of Elf5 leads to an accumulation of CK14+CK8+ cells (Chakrabarti R et al., 2012). Expression of CK14 has been detected in both basal and luminal populations (Shackleton M et al., 2006) suggesting that these cells could be bipotent progenitors that differentiate into basal or luminal lineages. CK5 has been

detected only in basal specific populations suggesting that CK5+CK8+ progenitors are more upstream in the differentiation hirearchy.

We showed that the markers of luminal differentiation, Sca-1 and PR, were decreased in MMTV-RANK mammary glands. Whereas an increase in CD24+CD49b+ and CD24+Sca1- populations was observed within the luminal MMTV-RANK cells. It has been previously shown that these populations show higher colony forming ability and therefore are enriched in luminal progenitors (Sleeman KE et al., 2007; Shehata M et al., 2012).
In contrast, a dramatic drop of the CD61+ alveolar progenitors was detected into the luminal population. Elf-5 is a transcription factor highly expressed in luminal CD61+ progenitor population that specifies the alveolar-cell fate (Oakes SR et al., 2008). We showed that luminal cells derived from virgin MMTV-RANK mammary glands have decreased expression of Elf-5 in accordance with the reduced luminal CD61+ population. In MMTV-RANK pregnant glands a severe reduction in Elf5 expression is observed, in concordance with the absence of WAP expression and alveologenesis. The loss of CD61+ luminal progenitors did not reduce the colony formation ability in vitro (Asselin-Labat ML et al., 2007), evidencing the existence of several luminal progenitor populations within the mammary gland.
We therefore demonstrated that, MMTV-RANK mammary glands have an abnormal accumulation of luminal progenitors and luminal cells. Moreover, the luminal alveolar compartment is severely reduced, providing a molecular basis for the alveolar defect observed during gestation in MMTV-RANK mice (Gonzalez-Suarez E et al., 2007).

Inhibition of RANKL during WT gestation confirmed that RANK signaling is a direct negative regulator of the Elf-5/STAT5 pathway in physiological conditions, as RANK-Fc treatment resulted in increased expression of Elf5 and STAT5 forcing differentiation of luminal cells towards a secretory phenotype. The reduced colony forming ability observed is in accordance with a more differentiated phenotype. Mammary glands under RANK-Fc showed a premature lactating phenotype with increased expression of the milk protein WAP. Previous results demonstrated that progesterone induces Elf5 expression and precocious mammary gland differentiation, through a paracrine signaling mediated by RANKL (Lee HJ et al., 2013). Lee's study and ours are only apparently in contrast. Indeed progesterone plays both a positive and negative role in mammary development. At early steps of gestation, progesterone (and the downstream effector RANKL as demonstrated by Lee et al.) can induce mammary gland proliferation and differentiation. During midgestation progesterone (and RANKL as we demonstrated) interferes with alveolar differentiation and lactation (Buser AC et al., 2007).
Altogether our results demonstrate that RANK signaling expands basal and mammary stem cells as previously described, and also luminal progenitors, but impairs differentiation to the alveolar lineage through regulation of Elf5/STAT5 pathway.

## 2. Enhanced activation of RANK pathway induces spontaneous tumorigenesis.

RANK-driven increased epithelial proliferation and expansion of luminal and basal compartments resulted in many anatomical abnormalities such as accumulation of multiple layers of luminal cells and alteration of myoepithelial basal cell structure. These alterations of the epithelial architecture can be considered as early pre-neoplastic lesions, as later

confirmed by spontaneous mammary tumor development in multiparous MMTV-RANK mice. MMTV-RANK spontaneous tumors show inter- and intra-tumor heterogeneity containing frequent CK14+CK8+ cells, establishing a direct connection in solid tumors between alterations of the mammary stem cell fate (accumulation of intermediate progenitors) and tumor initiation.
RANK/RANKL signaling is the main mediator of protumorigenic role of progesterone in the mammary gland (Gonzalez-Suarez E et al., 2010; Schramek D et al., 2010). We hypothesized that the acute progesterone exposure at gestation would give the proliferative booster to activate a tumorigenic process in MMTV-RANK mammary glands. Physiological levels of progesterone were probably not sufficient to elicit mammary tumorigenesis as virgin MMTV-RANK mice show extensive hyperplasias (Gonzalez-Suarez E et al., 2007), but do not develop tumors (Pellegrini P et al., 2013). In accordance with the evidence that progesterone strongly upregulates RANKL expression (Fata JE et al., 2000) one possibility is that increased levels of RANKL would over-activate RANK signaling (Junko GT et al., 2002) and that would be the starter event of tumorigenesis. Alternatively, expansion of the mammary gland during gestation would have generated new pregnancy specific populations that would be targets of transformation (Matulka LA et al., 2007).
Analyses of the WT mammary gland revealed that the expression levels of RANK increased with age and parity; preneoplasic lesions and tumors from old WT mice that underwent multiple gestations showed high levels of RANK and an accumulation of CK14+CK8+ progenitors. As these features share similarities with MMTV-RANK model we can conclude that RANK driven-phenotypes are reproduced in a WT organism and could have clinical relevance for human breast cancer. We detected two different factors that, associated with RANK signaling contributed to mammary tumorigenesis: age and reproductive history, recognized risk factors of human breast cancer (Bernstein L et al., 2002). Association between RANK expression, age and parities could be a candidate biomarker for prediction of risk.

MMTV-RANK derived tumors are very heterogeneous in terms of clinical and molecular features. We hypothesized that accumulated luminal, basal and intermediate progenitors observed in MMTV-RANK mammary gland would be targets of transformation leading to tumor heterogeneity.
Tumor latency of MMTV-RANK model is longer than other tumor prone mouse models such as MMTV-neu or MMTV-PyMT suggesting that RANK is not acting as a potent oncogene but cooperates with oncogenic events. Whether cancer stem cell theory is correct and cancer is a stem cell disease, we could conclude that MMTV-RANK is a model of stem/progenitor cell disease. Expanded stem/progenitor cells would probably stay in a quiescent state until they receive the proliferative boost of pregnancy associated hormones (progesterone as the first candidate).
The identity of the cells that originate MMTV-RANK tumors is unknown, high molecular heterogeneity suggest that tumors might derive from poorly differentiated cells in the epithelial hierarchy; these cells would then differentiate in different epithelial tumorigenic cell types. We cannot exclude that expanded cell lineages in the MMTV-RANK mammary gland, could be all susceptible targets of transformation resulting in heterogeneous tumors. Whether the tumor initiation derived from one poorly differentiatied cell or multiple cells or both hypotheses cannot be elucidated with characterization of advanced tumors: different cell populations may exhibit differentiation plasticity during tumor progression, and they may not reflect the properties of the cell of origin. Tracing different cell lineages during mammary gland development and tumor initiation could clarify the cell or cells of origin in the MMTV-RANK model.

The role of RANK signaling in spontaneous pregnancy-independent tumorigenesis, with physiological levels of progesterone has been recently investigated in MMTV-neu mouse model. Blockade of RANKL before tumors arise decreased tumor incidence and lung metastasis (Gonzalez-Suarez E et al., 2010). We investigated the role of RANK signaling in a model of aggressive, multifocal and metastatic mammary tumorigenesis: MMTV-PyMT mice. We demonstrated that constitutive deletion of RANK results in a delay in tumor formation, decreased growth rate and blockage of lung metastasis. Moreover, tumoral foci per mammary gland were decreased: MMTV-PyMT;$RANK^{-/-}$ mice showed one predominant and homogeneous tumor while controls showed multiple pre-neoplastic or neoplastic foci in all the mammary glands. $RANK^{-/-}$ mice have reduced mammary epithelium due to low proliferation and survival (Fata JE et al., 2000): as MMTV promoter drives PyMT expression in mammary epithelium we especulate that deletion of RANK could decrease the number of epithelial cells targets of transformation and this would restrict the PyMT multifocal tumorigenesis. Other, more intriguing explanation would be that deletion of RANK would interfere with early steps of multifocal tumor initiation resulting in monoclonal tumor formation.
MPA-DMBA derived tumors are PR+ and RANK signaling was therefore associated to this cancer subtype (ER+PR+). Our data revealed that RANK is also important for tumor initiation of spontaneous mammary tumors that do not express ER and PR such as MMTV-PyMT and spontaneous MMTV-RANK tumors. Therefore, targeting RANK could prevent both hormone dependent and independent tumors.

## 3. RANK induces tumor progression of estabilished mammary tumors and expands the tumor initiating cell pool.

The role of RANK signaling as a positive regulator of tumor initiation in MPA/DMBA driven tumors or in spontaneous tumorigenesis was clearly established (Gonzalez-Suarez E et al., 2010; Schramek D et al., 2010; Pellegrini P et al., 2013); these results support the use of Denosumab in a preventive setting, which can be justified in individuals with high risk of developing breast cancer for example in. BRCA1 or BRCA2 carriers.
We have also investigated the therapeutic potential of RANKL inhibition in breast cancer, this is, whether inhibition of RANK signaling could also be beneficious once tumors are established in patients already diagnosed with breast cancer. We demonstrate that RANKL facilitates tumor progression and metastasis, enhancing proliferation, survival and invasion in RANK+ tumor cells derived from MMTV-neu and MMTV-PyMT carcinomas and up-regulates the expression of genes related with survival and invasion. Similarly, RANKL treatment results in a marked protection from cell death in response to γ-radiation and doxorubicin in mouse mammary epithelial cells and the RANK-expressing human cancer cell line, SKBR3 (Schramek D et al., 2010). Our laboratory also demonstrated that RANK overexpression increases tumor growth and metastasis of transformed human cell lines (Palafox M et al., 2012).

As RANK signaling expands mammary stem cells and progenitors leading to tumor formation, we investigated the role of this pathway in cancer stem cells of MMTV-PyMT model.
The pathways involved in MMTV-PyMT tumor initiation have been recently investigated by deletion of putative genes involved in stemness, such as Hif-1α and CSF-1 (Lin EY et al., 2001; Malanchi I et al., 2011; Schwab LP et al., 2012). However, identity and clinical relevance of the cell of origin in this model remains undefined. We decided to investigate

the effect of pharmacological inhibition of RANKL (RANK-Fc) in MMTV-PyMT tumors as it may have a higher clinical relevance than constitutive genetic deletion of RANK. Both *in-vivo* and *in-vitro* studies showed that tumor initiating cell or cancer stem cell population under RANK-Fc treatment was significantly decreased. Tumor cells that have been treated with RANK-Fc were less able to initiate tumors in a new host than untreated cells, suggesting that neoadjuvant inhibition of RANKL could decrease the frequency of tumor relapse in the clinical setting. No significant differences in pathways/genes previously related with self-renewal or TICs, such as Notch, Wnt, Gata-3 and Hif-1α, were found between RANK-Fc treated tumors and controls, suggesting that activation of RANK signaling may directly mediate the expansion of TICs. Analyses of surface markers commonly used to identify mammary stem cells and cancer stem cells revealed a significant increase in Sca-1+ cells after RANK-Fc neoadjuvant treatment, suggesting that Sca-1 expression is preferently associated to bulk tumor cells and not to cancer stem cells.
Cancer stem cells are a subpopulation of tumor cells that share some characteristics with adult stem cells, such as self renewal, ability to differentiate and quiescence; they are resistant to chemotherapy that mostly targets proliferating cells and are therefore responsible for tumor relapse and metastasis (Jordan CT et al., 2006). Tumor cells under RANK-Fc treatment show higher proliferation rates, evidenced by increased levels of cyclin D1 expression and shorter latency to tumor formation. This apparently contradictory phenotypes, may be explained by the loss of low proliferating CSC under RANK-Fc treatment and suggest that in the clinical setting RANKL inhibition should be combined with conventional therapies that target the buk of proliferating cells such as chemotherapy.
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It has been reported that dissemination of metastatic cells is an early event in MMTV-neu and MMTV-PyMT tumor progression (Hüsemann Y et al., 2008). Thus, the decrease in lung metastasis observed in MMTV-neu mice treated with RANK-Fc or in MMTV-PyMT;$RANK^{-/-}$ mice could be a consequence of the reduced tumor incidence observed (Gonzalez-Suarez E et al., 2010). However, the significant reduction of metastasis initiating cells observed in MMTV-PyMT;$RANK^{-/-}$ tumors cells compared to MMTV-PyMT;$RANK^{+/+}$, when injected in limiting dilution in the fat pad or in the blood stream of immunodeficient mice demonstrates that RANK is essential for the intrinsic metastatic potential of tumor cells, independently of primary tumor incidence.

MMTV-PyMT tumors are PR negative, and importantly RANKL negative, therefore blocking RANKL could be a novel therapy to treat human ER-PR- or ER+PR+ tumors, even if they do not express RANKL, as long as they express RANK.

## 4. The role of RANK in tumor microenvironment, tumor initiation and metastatic disease

Tumor microenvironment plays a key role in tumor progression (Swartz MA et al., 2012). As MMTV-PyMT tumor cells are RANKL negative we hypothesized that RANK-Fc treatment would be targeting RANKL derived from stroma, including immune cells and fibroblasts contributing to the decrease in the tumor initiating ability (decreased TICs frequency). RANK is expressed not only in tumor cells but also in several immune cells such as macrophages, dendritic cells, monocytes and natural killers, whereas RANKL is expressed in activated T lymphocytes (Josien R et al., 2000; Seshasayee D et al., 2004; Schmiedel BJ et al., 2013). These cells will be susceptible to the effects of RANKL inhibition.
Immune infiltrates such as macrophages, CD4+ T lymphocytes and CD8+ cytotoxic lymphocytes are abundant in MMTV-PyMT tumors and they increase with tumor

progression (DeNardo DG et al., 2009). It has been clearly shown that tumor microenvironment drastically affects the metastatic potential of MMTV-PyMT cells (Lin EY et al., 2001; DeNardo DG et al., 2009; Malanchi I et al., 2011). In particular CD4+ T lymphocytes stimulate indirectly metastatic desease by recruiting M2 macrophages that secrete anti-inflammatory cytokines and EGF (deNardo et al., 2009). Macrophage derived CSF-1 factor induce invasive and metastatic phenotype of MMTV-PyMT tumors (Lin EY et al., 2001).
We demonstrated that in our orthotopic model of MMTV-PyMT tumorigenesis, RANK-Fc treatment increased the cytotoxyic CD8+ T infiltrates and decreased CD4+ T helper suggesting that an anti-tumor response is occurring. In addition, RANK-Fc treatment results in a decrease in T regulatory lymphocytes. These cells secrete high amounts of RANKL (Tan W et al., 2011) and induce immune tolerance in favor of tumor progression (Merlo A et al., 2009). RANK is expressed on dendritic cells and influence their capacity to activate CD4+ T cells (Josien R et al., 2000) suggesting that RANK-Fc treatment is interfering with expansion or activation of CD4+ T cells and T regulatory cells or their recruitment to the tumor and promoting an anti-tumor response that could contribute to the reduction in tumor initiating ability of MMTV-PyMT tumor cells after RANK-Fc treatment.
In contrast with an anti-tumor response, we observed a slight increase in the expression of TGF-β and expansion of the Myeloid Derived Suppressor Cell (MDSC) population, an heterogeneous cell type that can secrete TGF-β and many other factors inducing immune suppression (Gabrilovich DI et al., 2009). MDSC may contribute to the decreased tumor latency observed in tumors treated with RANK-Fc. TGF-β might also derive from the tumor cells: epithelial cell derived TGF-β has tumor-suppressive effects in normal and premalignant-lesions while in carcinoma TGF-β has pro-tumorigenic effects by activating epithelial to mesenchymal transition (Massagué J et al., 2008). However, it is possible that RANK signaling could affect to tumor latency in a TGF-β independent way as it has been demonstrated that interference of TGF-β signaling affects to migration and metastasis of MMTV-PyMT mice but not tumor latency (Muraoka RS et al., 2002).

Based on limiting dilution assays in immunodeficient mice the TICs pool of MMTV-PyMT tumor cells (mixed C57Bl6 and FvB background) seems unaltered after deletion of RANK (MMTV-PyMT;$RANK^{-/-}$). This is apparently in contrast with the observation that RANK-Fc decreases TICs of MMTV-PyMT (FvB background). As RANK-Fc induce profound effects not only in the tumor cells but also in the anti-tumor immune response we speculated that immunodeficient mice would not benefit of these effects resulting in an unaltered TICs frequency between MMTV-PyMT;$RANK^{-/-}$ and their controls. Alternatively, TICs of MMTV-PyMT;$RANK^{-/-}$ tumors might be different from those of MMTV-PyMT;$RANK^{+/+}$ and they might form tumors as efficiently as controls. We cannot rule out the effect of tumor plasticity and genetic background (FvB or C57Bl6). In fact, no differences in Sca1 expression were detected between MMTV-PyMT;$RANK^{-/-}$tumor cells and their controls, in contrast with the increased expression of Sca1+ observed in MMTV-PyMT tumors after neoadjuvant RANK-Fc treatment.

The significant increase in tumor latency and decreased tumor incidence observed in MMTV-PyMT;$RANK^{-/-}$ mice as compared to their controls, and the depletion in the frequency of TICs after RANK-Fc treatment in immunocompetent mice, contrasts with the lack of effect on tumorigenesis in MMTV-NeuT tumors with specific deletion of RANK in the mammary epithelia (Schramek D et al., 2010), highlighting the contribution of the microenvironment to anti-tumoral effects of RANK inhibition.

RANK constitutive deletion results in a significant reduction in the frequency of metastasis initiating cells but not of tumor initiating cells in immunodeficient hosts. These results suggest that whereas the decreased tumor initiating capability may be a consequence of RANK signaling inhibition in the tumor-immune interactions and that tumors can originate in the absence of RANK, the significant reduction in metastasis initiating cells is an intrinsic defect of tumor cells lacking RANK. RANK is essential for lung colonization as revealed by the lower incidence of metastasis and the smaller and reduced number of metastatic foci detected in the absence of RANK in both intravenous and orthotopic LDA assays.
Defective immune microenvironment in $RANK^{-/-}$ mice probably contributes to the reduced tumor and metastasis incidence: MMTV-PyMT;$RANK^{-/-}$ tumors are free of lung metastasis, but when implanted orthotopically in nude mice they do form metastasis even though metastatic potential is lower than their controls. Complete loss of metastatic potential in endogenous MMTV-PyMT;$RANK^{-/-}$ mouse models might be related to alterations in the immune system observed in $RANK^{-/-}$ mice (Dougall WC et al., 1999). Further investigations are ongoing to characterize tumor microenvironment and unravel how the tumor-stroma interactions could be affected by anti-RANKL therapies or RANK deletion.
Until now the pro-tumorigenic role of RANK signaling was associated only with its proliferative effects as mediator of progesterone as demonstrated by MPA/DMBA protocols and pregnancy-dependent spontaneous tumorigenesis; our results demonstrate that RANK signal plays a more complex role in tumorigenesis affecting not only proliferation, but also the self-renewal and differentiation ability of the cancer stem cell population, colonization of the metastatic niche, and importantly modulating tumor-stroma interactions, including the anti-tumor immune response.

## 5. RANK in human breast cancer: future perspectives.

Given the relevance of RANK signaling in tumorigenesis and tumor initiating cells we investigated the role of RANK signaling in human cancer. We observed that increased RANK mRNA expression correlates with markers of tumor aggressiveness such as tumor grade, cell proliferation and metastasis (Palafox M et al., 2012). These results are in accordance with a recent study where increased RANK expression has been correlated with decreased overall survival and increased bone metastasis incidence (Santini D et al., 2011). Interestingly, we detected an increased expression of RANK mRNA in ER-PR- tumors that are more aggressive than other subtypes of breast cancer and contain a higher frequency of the human breast cancer stem cells enriched CD44+/CD24- population (Park SY et al., 2010; Ricardo S et al., 2011). In fact, RANK could expand the cancer stem cell population in human cell lines (Palafox M et al., 2012) as it does in MMTV-PyMT mouse model. In accordance with these results our Gene Set Enrichment Analysis (GSEA) tool revealed that high expression of RANK in breast tumors correlates with high expression of genes of mammary stem cells and luminal progenitors and low expression of genes representative of luminal differentiation (Pellegrini P et al., 2013).
A broad analysis of RANK and RANKL protein expression in breast cancer is needed in order to evaluate the prognostic and therapeutic value of RANK pathway in the clinics.

Altogether we demonstrated that RANK signaling is a master regulator of mammary gland development, cancer stem cells, and tumor-immune crosstalk and targeting RANK signaling in tumorigenesis could reduce tumor progression, relapse and metastatic disease

(Figure 1). These results have a very high clinical relevance as an inhibitor of RANKL has been approved for the treatment of tumor derived bone metastasis. Therefore, a drug is already available in clinics and, if these results are confirmed, patients could quickly benefit from this new therapeutic strategy.

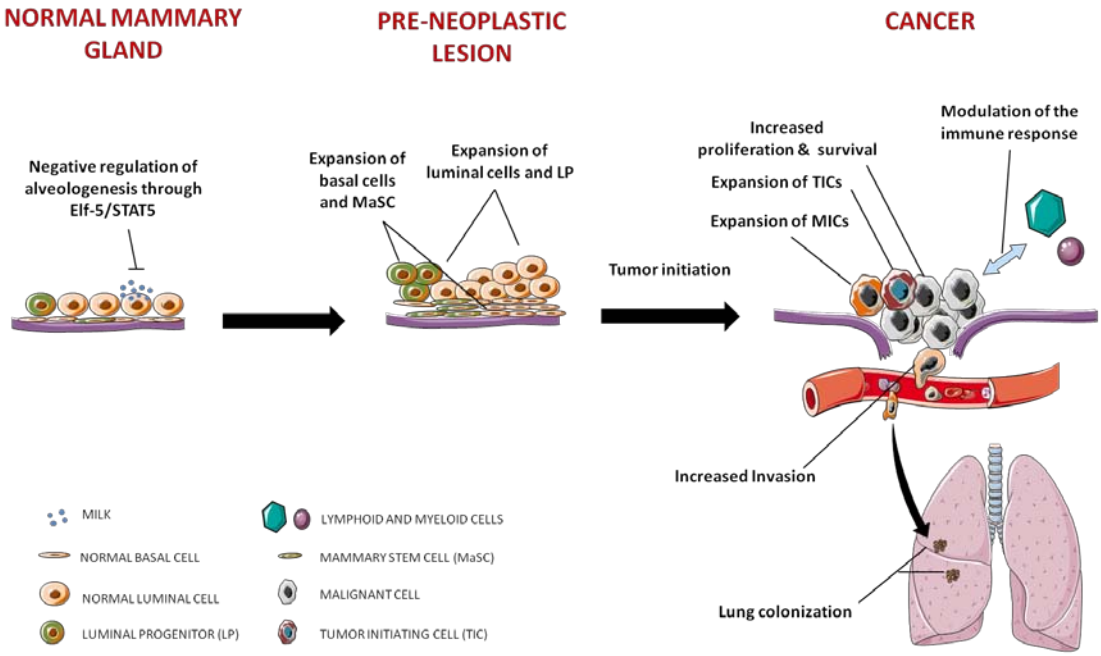


**Figure 1**. Effects of RANK signaling in mammary gland development and tumorigenesis.

# CONCLUSIONS

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1. RANK signaling is a positive regulator of mammary stem cell pool, bipotent K14+/K8+ cells and luminal progenitors.

2. RANK overexpression leads to alterations in different luminal populations in virgin mammary glands.

3. Lack of alveologenesis in MMTV-RANK mice is a consequence of the decrease in CD61- alveolar progenitors and Elf-5 expression in the virgin mammary gland.

4. RANKL impairs alveolar differentiation by inhibiting Elf-5/STAT5 signaling at midgestation not only in MMTV-RANK but also in WT mice.

5. RANK overexpression leads to an aberrant organization of the mammary epithelium that affects both basal (CK14+ and CK5+) and luminal (CK8) lineages.

6. Multiple gestations lead to spontaneous tumor formation in aged MMTV-RANK mice. Tumors are highly heterogeneous and contain bipotent CK14+CK8+ cells.

7. Expression of RANK and accumulation of CK14+CK8+ cells increases with age and reproductive history in WT mammary epithelia.

8. Spontaneous tumors derived from MMTV-neu and MMTV-PyMT models, express high levels of RANK but not RANKL.

9. RANKL treatment in MMTV-neu and MMTV-PyMT tumor cells increases proliferation and invasiveness, and enhances expression of pro-survival and prometastatic genes, resulting in increased tumor growth and metastasis.

10. Neoadjuvant inhibition of RANKL (RANK-Fc) decreases tumor initiating cells in an orthotopic model of MMTV-PyMT tumors. Adjuvant RANKL inhibition decreases tumor latency and increases proliferation.

11. Pharmacological inhibition of RANKL modulates the proportion of immune cell infiltrates in MMTV-PyMT tumors in promoting an anti-tumorigenic program.

12. Deletion of RANK increases tumor latency, decreases tumor growth and incidence and blocks lung metastasis in the MMTV-PyMT mouse model.

13. RANK deletion in MMTV-PyMT tumors decreases the pool of MICs but not TICs in an immunodeficient environment. RANK mediates the intrinsic metastatic potential of MMTV-PyMT tumor cells.

# ABBREVIATIONS

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-/-: homozygous
-/+: heterozygous
+/+: wild type
24h: 24 hours
-a647: Alexa 647
ANOVA: Analysis of variance
-APC: -Allophycocyanin
APC: Antigen Presenting Cell
Axin-2: Axis Inhibition protein 2
Bcl2a1b: B Cell Leukemia2 related Protein A1b
BIRC2: Baculoviral IAP repeat-containing protein 2
BIRC3: Baculoviral IAP repeat-containing protein 3
Bmi-1: B cell-specific Moloney murine leukemia virus Integration site 1
BRCA1: Breast Cancer 1
BrdU: 5-Bromo-2'-deoxyurdine
C/EBP: CCAAT/enhancer binding protein
CCND1: Cyclin D1
CD: Cluster of Differentiation
cDNA: Complementary DNA
CK: Cytokeratin
CSC: Cancer Stem Cell
CTL: Cytotoxic T Lymphocyte
CTRL: Control
-Cy7: Cyanine 7
DAB: 3,3'-Diaminobenzidine
DMBA: 7,12-Dimethylbenz[α]anthracene
DMEM: Dulbecco's modified Eagle's medium
DNA: Deoxyribonucleic Acid
EDTA: Ethylenediaminetetraacetic Acid
EGF: Epidermal Growth Factor
ELDA: Extreme Limiting Dilution Analysis
Elf-5: E74-Like Factor 5
EMT: Epithelial–Mesenchymal Transition
ER: Estrogen Receptor
F1: Filial 1
F2: Filial 2
FACS: Fluorescence-Activated Cell Sorting
FBS: Fetal Bovine Serum
-FITC: Fluorescein Isothiocyanate
FOXN1: Forkhead Box N1
FoxP3: Forkhead Box P3
FSC-H: Forward Scatter
G16.5: Gestation day 16.5
GFR: Growth Factor Reduced
GSEA: Gene Set Enrichment Analysis
H&E: Hematoxylin & Eosin
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2: v-erb-b2 Avian Erythroblastic Leukemia Viral Oncogene Homolog 2
Hey-1: Hairy/Enhancer-Of-Split Related With YRPW Motif 1

Hif1α: Hypoxia-Inducible Factor 1α
HP: Hyperplasia
HRP: Horseradish Peroxidase
ICAM-1: Intercellular Adhesion Molecule 1
ID2: Inhibitor Of DNA Binding 2
IFN-γ: Interferon γ
IL: Interleukin
iNOS: Inducible Nitric Oxide Synthase
ITS: Insulin, Transferring, Selenium
Jak-2: Janus Kinase 2
KO: Knock-Out
LDA: Limiting Dilution Assay
M1/M2: Macrophage 1/Macrophage 2
Ma-CFC: Colony Forming Cells
MAPK: Mitogen-Activated Protein Kinase
MaSC: Mammary Stem Cell
MCF7: Michigan Cancer Foundation-7
MDSC: Myeloid Derived Suppressor Cell
MICs: Metastasis Initiating Cells
MIN: Mamamary Intra-epithelial Neoplasia
MMP-9: Matrix Metallopeptidase 9
MPA: Medroxyprogesterone
mRNA: Messenger Ribonucleic Acid
MYC: v-myc Avian Myelocytomatosis Viral Oncogene Homolog
NFkB: Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B Cells
NG: Normal Gland
NK: Natural Killer
NOD: Nonobese Diabetic
OPG: Osteoprotegerin
P1, P2: Passage 1, Passage 2
P4: Progesterone
P53: Tumor Protein p53
PBS: Phosphate Buffered Saline
-PE: Phycoerythrin
PFA: Paraformaldehyde
PI3K: Phosphoinositide-3-Kinase
PP1A: Protein Phosphatase 1
PR: Progesterone Receptor
PRL: Prolatin
PrlR: Prolactin Receptor
PTHrP: Parathyroid Hormone 1 Receptor
r18S: 18S Ribosomal RNA
RANK: Receptor Activator of Nuclear Factor ϰ B
RANKL: Receptor Activator for Nuclear Factor ϰ B Ligand
RAS: Rat Sarcoma Viral Oncogene
RB: Retinoblastoma 1
R-Fc: RANK-Fc
RNA: Ribonucleic Acid
ROCK: Rho-Associated Coil Kinase
RPL38: Ribosomal Protein L38
Sca-1: Stem Cell Antigen-1

SCID: Severe Combined Immunodeficiency
SEM: Standard Error Of The Mean
Sma-1: Smooth Muscle Actin-1
Sox-9: SRY (sex determining region Y)-Box 9
SSC-A: Side Scatter
STAT5: Signal Transducer and Activator of Transcription 5A
SV0: Simian Vacuolating Virus 40
TAM: Tumor Associated Macrophage
TCGA: The Cancer Genome Atlas
TGF-β: Transforming Growth Factor β
Th1: T helper 1
Th2: T helper 2
TICs: Tumor Initiating Cells
TNF: Tumor Necrosis Factor
TRANCE: TNF-Related Activation-Induced Cytokine
Treg: T Regulatory cells
Tris: Tris(Hydroxymethyl)Aminomethane
VCAM-1: Vascular Cell Adhesion Molecule 1
WAP: Whey Acidic Protein
WNT-4: Wingless-Type MMTV Integration Site Family, Member 4
WT: Wild-Type

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