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## The role of RANK and RANKL in hormone-driven breast cancer

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| Verfasserin / Verfasser: | Verena Sigl |
| Matrikel-Nummer: | 0304132 |
| Studienrichtung /Studienzweig (lt. Studienblatt): | A441 – Genetik |
| Betreuerin / Betreuer: | Prof. Dr. Josef M. Penninger |


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Worldwide, breast cancer is one of the most common cancers and a leading death cause amongst women (Ferlay et al., 2007; Jemal et al., 2009). Many factors contribute to the development and progression of breast cancer. Interestingly, sex hormones such as estrogen and progesterone are well known factors critically involved in mammary tumorigenesis and recently it has been shown that women undergoing combined hormonal replacement therapy (HRT) have an increased risk of developing breast cancer. In particular, synthetic progesterone derivatives like medroxyprogesterone acetate (MPA) which are widely used by millions of women in the form of HRT and contraceptives appear to markedly increase the risk of developing breast cancer (Beral, 2003; Rossouw et al., 2002). However, the mechanism how MPA is linked to breast cancer on a molecular level is still poorly understood.

Receptor activator of NFκB (RANK) and its ligand RANKL are essential and central regulators of osteoclast differentiation and osteoclast function (Kong et al., 1999b). The discovery of these molecules has unquestionably significantly contributed to the understanding of the molecular mechanisms of normal bone metabolism and bone disease. Moreover RANK and RANKL are also crucial in pregnancy-dependent mammary gland formation (Fata et al., 2000). Embedded in a hormonal regulatory network the RANK/RANKL pathway initiates proliferation and differentiation of terminal end buds to lobuloalveolar structures via activation of the Id2 pathway (Kim et al., 2006) and the NFκB – CyclinD1 axis (Cao et al., 2001). Intriguingly, we here report that *in vivo* MPA administration results in massive upregulation of RANKL expression in female mice. Most importantly, we show that tissue specific inactivation of RANK in mammary epithelial cells results in a significantly delayed onset of breast cancer in response to MPA/DMBA treatment. On a molecular level it appears that loss of RANK leads to impaired NFκB and Id2 activation resulting in a block in proliferation. Moreover we observed that MPA-induced RANKL expression protects of γ-irradiation-induced cell death, thus providing a prerequisite for the development of cancer. These data provide the first demonstration that RANK/RANKL control the development of hormone-driven breast cancer.

Weltweit ist Brustkrebs eine der häufigsten Krebsarten und zählt unter Frauen zu einer der führenden Todesursachen (Ferlay et al., 2007; Jemal et al., 2009). Viele Faktoren sind bekannt, die zur Entstehung und zur Progression von Brustkrebs beitragen. Auch Geschlechtshormone, wie Östrogene und Progesteron spielen eine kritische Rolle in manchen Brustkrebsarten. Kürzlichen Berichten zufolge ist die Wahrscheinlichkeit für Frauen, die sich einer Hormonersatztherapie unterziehen, grösser an Brustkrebs zu erkranken. Im speziellen werden syntethische Progesterone-Derivate (Progestine) wie Medroxyprogesteron Acetat (MPA) welches Millionen Frauen in Form von Hormonersatztherapeutika und hormonelle Kontrazeptiva zu sich nehmen, mit diesem erhöhten Brustkrebsrisiko in Verbindung gebracht. Trotzdem sind die genauen molekularen Mechanismen, welche Rolle Progestine in Brustkrebs spielen, noch wenig verstanden.

Rezeptor Aktivator von NFκB (RANK) und sein Ligand RANKL sind essentielle Regulatoren für die Differenzierung und Funktion von Osteoklasten. Unfraglich hat die Entdeckung dieser Moleküle erheblich dazu beigetragen die molekularen Mechanismen im Knochenstoffwechsel und von Knochenerkrankungen besser zu verstehen. Außerdem sind RANK und RANKL auch wesentliche Faktoren für die der Entwicklung der Brustdrüsen während der Schwangerschaft. Über hormonelle Regulation und die Aktivierung von Id2 (Kim et al., 2006), NFκB und CyclinD1 (Cao et al., 2001) induzieren RANK und RANKL die Proliferation und Differenzierung von terminalen Endknospen zu lobuloalveolaren Strukturen. Interessanterweise berichten wir hier, dass die *in vivo* Verabreichung von Medroxyprogesteron Acetat zu einer Hochregulierung der RANKL-Expression in weiblichen Mäusen führt. Außerdem zeigen wir, dass die gewebsspezifische Inaktivierung von RANK in Brustepithelzellen nach Progesteron/DMBA Adminsitration zu einer signifikant späteren Entstehung von Brusttumoren führt. Auf molekularer Ebene scheint es, als ob die Inaktivierung von RANK in einer Beeinträchtigung von Id2 und NFκB und somit in einem Proliferationsblock resultiert. Weiters konnten wir beobachten dass die MPA-induzierte Expression von RANKL Brustepithelialzellen vor dem programmierten Zelltod induziert durch γ-Bestrahlung schützt. Dies stellt eine Prärequisite für die Entstehung von Krebs dar. Zusammenfassend zeigen wir hier zum ersten Mal, dass RANK und

RANKL, bekannt als Schlüsselmoleküle im Knochenmetabolismus auch die Entstehung von hormonabhänigigen primärem Brustkrebs kontrollieren.

# 1. Introduction

## 1.1. RANK, RANKL and OPG – an overview

´ Over the course of our whole lifetimes bone is continuously being remodelled through the balanced activity of bone depositing osteoblasts and bone resorbing osteoclasts (Boyle et al., 2003; Karsenty and Wagner, 2002; Theill et al., 2002). Any perturbations which cause an imbalance between these dynamic processes of osteoblast and osteoclast activity can lead to severe skeletal diseases such as osteoporosis and osteopetrosis (Boyle et al., 2003; Karsenty and Wagner, 2002; Theill et al., 2002). Osteopetrosis, a state of abnormally increased bone density mainly results from very rare heritable conditions. By contrast, bone loss or osteoporosis is much more prevalent and hundreds of millions of people worldwide are affected by this devastating disease (Wada et al., 2006). Most frequently osteoporosis is observed in postmenopausal women, rheumatoid arthritis, immobilized patients, early childhood leukaemia, periodontitis and even astronauts as a consequence of zero gravity (Boyle et al., 2003; Theill et al., 2002; Wronski and Morey, 1983).

A new era in bone research has just started a decade ago with the discovery of three essential and central molecules of bone turnover. These key molecules are the receptor activator of NFκB (RANK), its ligand RANKL and the RANKL-decoy receptor osteoprotegerin (OPG). Binding of RANKL to its receptor RANK initiates the development and activation of osteoclasts whereas OPG effectively inhibits osteoclastogenesis by functioning as a molecular decoy receptor for RANKL (Simonet et al., 1997; Yasuda et al., 1998b). Unquestionable, the discovery of RANK, RANKL and OPG has significantly contributed to our understanding of the molecular processes underlying bone diseases. Most importantly, RANK, RANKL and OPG have become principal targets in the development of new therapies which might be used one day to treat hundreds of millions of people suffering diseases associated with bone loss (Leibbrandt and Penninger, 2008).

In the late 1990ies the Tumor necrosis factor (TNF) family member cytokine RANKL, also known as TRANCE (TNF-related activation-induced cytokine), ODF (osteoclast differentiation factor) and OPGL (OPG ligand) was discovered almost simultaneously by four independent groups (Anderson et al., 1997; Lacey et al., 1998; Wong et al., 1997b; Yasuda et al., 1998b). Whereas Anderson et al. and Wong et al. associated RANKL with essential functions in the immune system (Anderson et al., 1997; Wong et al., 1997b), Lacey et al. and Yasuda et al. identified RANKL as a ligand for OPG, inducing osteoclastogenesis (Lacey et al., 1998; Yasuda et al., 1998b). The RANKL gene encodes a type II transmembrane protein consisting of a membrane-anchoring domain, a connecting stalk and a receptor-binding ectodomain. Human und murine RANKL are highly conserved and are synthesized as approximately 316 amino acid, either membrane bound or soluble, glycoproteins. The soluble form is either derived from proteolytic cleavage or alternative splicing (Wada et al., 2006). All described RANKL isoforms can mediate osteoclastgenesis *in vitro* (Ikeda et al., 2003; Suzuki et al., 2004). Interestingly, it has been reported that co-expression of different isoforms seems to provoke inhibitory effects. Thus, the control of osteoclastogenesis might also require regulated expression of RANKL isoforms (Ikeda et al., 2003; Suzuki et al., 2004). RANKL is highly expressed in skeletal and primary and secondary lymphoid tissues; but RANKL expression can also be detected in keratinocytes of the skin, epithelial cells of the mammary gland, heart, skeletal muscle, lung, stomach, placenta, thyroid gland and brain (Leibbrandt and Penninger, 2008).

1997, RANK, the signaling receptor for RANKL was identified by several groups as a receptor involved in osteoclastogenesis, enhanced T-cell growth and survival of dendritic cells (Anderson et al., 1997; Hsu et al., 1999; Nakagawa et al., 1998). RANK encodes a type I transmembrane glycoprotein consisting of a short signal peptide, an extracellular domain, a transmembrane domain and a large cytoplasmic domain. Like many Tumornecrosis factor receptor (TNFR) members, RANK assembles into a trimeric complex on the cell surface prior to ligand binding (Chan et al., 2000; Locksley et al., 2001). RANK message is detected in skeletal muscle, thymus, liver, colon, small intestine, adrenal gland, osteoclasts, mammary epithelial cells prostate and pancreas (Leibbrandt and Penninger, 2009).

The third molecule of the triade, Osteoprotegerin (OPG), inhibits RANK/RANKL signaling effectively by competing with RANK for RANKL binding

(Simonet et al., 1997; Yasuda et al., 1998a). Therefore, OPG is also referred to as protector of the bone, as it prevents RANK/RANKL mediated bone degradation. OPG is a member of the TNFR-family and is synthesized as a 55-kDa glycoprotein. Unlike other TNFR-family members, OPG does not assemble into a trimeric molecule but is secreted as a 110-kDa homodimer. OPG is most abundantly expressed in the brain, liver, lung, heart, kidney, skeletal muscle, skin, intestines, calvaria, stomach testis and placenta (Tan et al., 1997).

### 1.2. The classical role of the RANKL-RANK-OPG axis in bone metabolism

Genetic manipulation of RANKL, RANK and OPG in mice contributed greatly to our understanding of its functions in the skeleton. Unambiguously, the first breakthrough was to show that the RANK-RANKL-OPG axis is essential for normal and pathologic bone metabolism. OPG was the first molecule of the triade to be shown to be critically required for the maintenance of postnatal bone mass *in vivo*. Mice overexpressing OPG and mice treated with recombinant OPG suffer from severe osteopetrosis (Simonet et al., 1997), whereas mice lacking OPG in the germline develop early-onset osteoporosis (Bucay et al., 1998). However, at this time it was only speculation that OPG might inhibit an activator of osteoclastogenesis. But only a few months later, targeted deletion of RANKL and RANK in mice provided the link between these three molecules and bone metabolism. Both, RANKL (Kong et al., 1999b) and RANK knockout mice (Dougall et al., 1999; Li et al., 2000) exhibit severe osteopetrosis and impaired tooth eruption due to a complete lack of osteoclasts. Thus, it was shown that whereas RANK and RANKL are positively regulating osteoclastogenesis, OPG is essential for counterbalancing this pathway by functioning as a natural decoy receptor. RANK, RANKL and OPG are therefore the key molecules driving osteclast induced bone turnover (Figure 1).

RANK/RANKL-functions underly various regulatory mechanisms. In particular, many cytokines influence the RANK/RANKL-system in bone metabolism. Macrophage colony-stiumlating factor (M-CSF) is a cytokine essential for RANKL-induced osteoclastogenesis (Arai et al., 1999). M-CSF is the most important survival factor for osteoclast-progenitor cells and enables the commitment of precursor cells into the osteoclast lineage (Arai et al., 1999).

Moreover, RANKL-dependent osteoclastogenesis has also been shown to be induced by the Transforming growth factor (TGF) family members TGFβ1 and activin A *in vitro* (Koseki et al., 2002). Furthermore, also Tumor necrosis factor alpha (TNFα), a cytokine which is abundant at sites of inflammation considerably influences RANKL-mediated bone turn over (Zhang et al., 2001). Therefore, osteoclastogenesis is enhanced under pro-inflammatory conditions due to RANKL and TNFα cooperation. Evidently, chronic inflammation is often associated with bone loss. Thus, the production of cytokines is one of the reasons for enhanced bone resorption during inflammatory events (Theill et al., 2002).

Another very powerful regulatory mechanism of the RANK/RANKL system are hormones. In particular, RANK activation is under a strong influence of estrogens and androgens. Both sex hormones suppress RANKL-induced osteoclast differentiation by interfering with the JNK-c-Jun pathway (Syed and Khosla, 2005). Moreover, estrogens and androgens also induce the expression of the decoy recptor OPG, thus directly controlling RANKL-mediated bone turnover (Syed and Khosla, 2005). Therefore, the loss of estrogen production in the ovaries of postmenopausal women often results in progressive bone loss and osteoporosis. Previously, it has been shown that estrogen specifically induces apoptosis in trabecular bones via upregulation of Fas ligand expression in mice (Nakamura et al., 2007). Thus, one plausible explanation for the osteoprotective function of estrogen is that it regulates the life span of mature osteoclasts via induction of the death-inducing Fas/FasL pair (Nakamura et al., 2007).

The functional role of the RANK-RANKL-OPG axis in osteoclastogenesis and bone remodelling is conserved in humans (Wada et al., 2006). Therefore, RANK/RANKL is relevant to many human bone diseases. For example, autosomal-recessive osteopetrosis (ARO) is a rare heritable bone disease which is usually associated with defects in bone resorption either due to a defect in osteoclastogenesis or due to non-functional mature osteoclasts (Sobacchi et al., 2007). Recently it has been shown that impaired RANK/RANKL signaling is associated with a new osteoclast-poor subset of ARO. All 6 individuals suffering from this ARO-subtype harbour mutations in RANKL (Sobacchi et al., 2007). Moreover, two siblings from a Turkish consanguineous family and 6 more patients from unrelated families suffering ARO were identified to

harbour a homozygous mutation in the RANK gene, thus clearly emphasizing the clinical importance of RANK mutations in ARO patients (Guerrini et al., 2008). Another bone disease resulting from an autosomal recessive osteopathy is known as Juvenile Paget disease. Patients suffering from this disease are characterized by rapid remodelling of woven bone, osteopenia, fractures and progressive deformation of the skeleton (Ralston, 2008). Intriguingly, these patients have several mutations affecting the ligand binding domain of OPG, thereby linking the RANK/RANKL system to this disease (Hughes et al., 2000).

However, compared to ARO and Juvenile Paget diseases, both of which are characterized by increased bone mass, pathologically decreased bone mass is far more prevalent. Diseases like postmenopausal osteoporosis, periodontitis, rheumatoid arthritis, childhood leukaemia or chronic infections are associated with extensive bone loss resulting from deregulated RANK/RANKL signaling (Leibbrandt and Penninger, 2008).

Moreover, RANK and RANKL have been shown to play a critical role in metastasis to bone (Jones et al., 2006). Metastases are a frequent complication of many cancers causing severe pain and increased disease burden. The microenvironment of the host tissue plays a considerable role in the metastatic behaviour and preferred migration sites of cancer cells (Joyce and Pollard, 2009). Intriguingly, it was shown that RANKL triggers the migration of human epithelial cancer cells and melanoma cells expressing the receptor RANK. Moreover, the inhibition of RANKL by osteoprotegerin significantly reduces the tumor burden in bone but not in other organs *in vivo*. Thus, RANKL production in bone tissue provides a very fertile milieu for metastases (Jones et al., 2006). Since RANK was also found to be expressed on breast cancer cells of patients, RANKL has also an important role in cell migration and tissue specific-metastatic behaviour in humans (Jones et al., 2006).

Importantly, extensive research during the last years has enabled the development of denosumab (AMG 162), a monoclonal IgG2 antibody to human RANKL which has just passed phase three of clinical trials (Cummings et al., 2009; Smith et al., 2009, Bone et al., 2008; Cohen et al., 2008; Lipton et al., 2007). Thus, in the near future a promising treatment for postmenopausal osteoporosis, cancer-induced bone diseases, metastasis to bone and rheumatoid arthritis might be available.

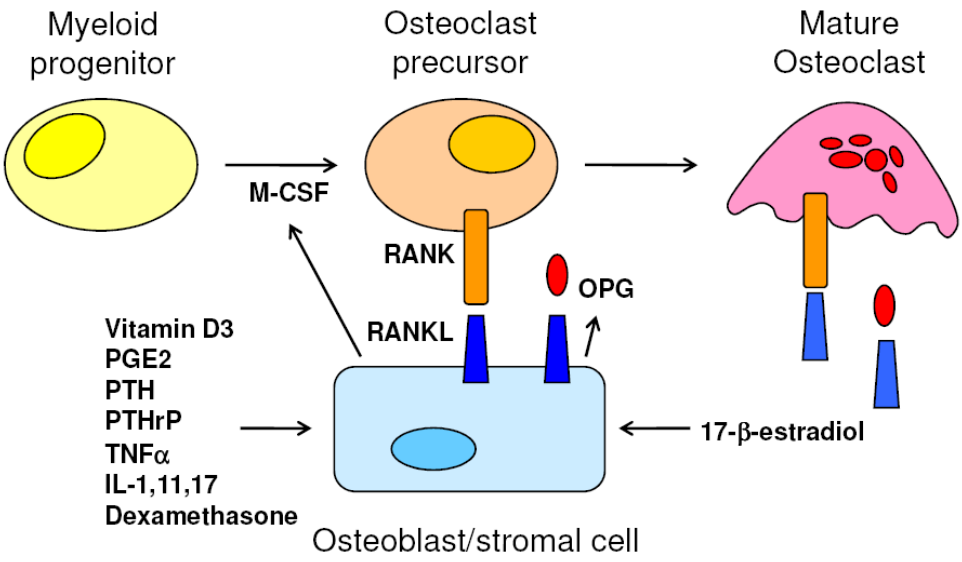



**Figure 1. Regulation of osteoclast formation in bones. RANKL expression in osteoclasts can be induced by calciotropic factors such as Vitamin D3, Prostaglandin E2 (PGE2), Parathyroid hormone (PTH), Parathyroid related-hormone peptide (PTHrP), Tumor necrosis factor alpha (TNFα), Interleukines 1, 11 and 17 (IL-1, 11 ,17) and the synthetic glucocorticoid Dexamethasone. Binding of RANKL to its receptor RANK on the surface of haematopoietic progenitor cells induces osteoclast differentiation in the presence of the survival factor M-CSF (Macrophage-colony stimulating factor). In mature osteoclasts binding of RANKL to RANK stimulates bone resorbing activity and promotes survival of the osteoclasts. The decoy receptor OPG is produced by osteoblasts and effectively inhibits osteoclastogenesis and osteoclast activation by binding to RANKL in the bone environment. Moreover, estrogen can accelerate OPG production on osteoblasts, which might, in part, explain postmenopausal osteoporosis.**

### 1.3. RANK(L) activated signaling pathways

Given the broad expression pattern and the pleiotropic *in vivo* functions of RANK and RANKL it is evidently of great interest to study RANK/RANKL signaling pathways. Most importantly, knowledge about RANK/RANKL downstream targets is crucial to identify new therapeutic targets for severe diseases involving impaired RANK/RANKL functions (Figure 2).

Like all TNF-family members RANK does not posses any kinase activity but initates downstream signaling via recruiting various adaptor molecules (Wajant et al., 2003). The binding of Tumor necrosis factor related adaptor proteins (TRAFs) is common to all members of the TNF-family (Inoue et al., 2000). In its cytoplasmic tail RANK contains binding sites for TRAF2, 5 and 6. Functionally, it has been shown that TRAF6 is the major downstream transducer of RANK activation (Darnay et al., 1998; Darnay et al., 1999; Wong et al., 1998). Thereto, TRAF6 knockout mice exhibit severe osteopetrosis due to a partial block in osteoclast development and impaired osteoclast activation (Lomaga et al., 1999). By contrast, TRAF2 and TRAF5 appear to play a minor role in RANK/RANKL-mediated osteoclastogenesis (Kanazawa et al., 2003; Kanazawa and Kudo, 2005).

GRB2 associated binder 2 (GAB2) is another adaptor molecule that functions downstream of RANK activation. In particular, GAB2 is important for RANK-induced NFκB, Akt/PKB and JNK signaling (Wada et al., 2005). GAB2 knockout mice exhibit severe osteopetrosis due to defective osteoclast differentiation. Moreover, it was shown that inactivation of GAB2 is also critical in the differentiation of human progenitor cells into osteoclastogenesis. Thus, GAB2 is a key molecuclar scaffold protein that controls osteoclastogenesis and bone homeostasis in mice and human (Wada et al., 2005).

Nuclear factor kappa B (NFκB) is a major regulator of cell fates and strongly activated upon RANK stimulation (Karin and Lin, 2002). In particular, TRAF6 association is essential for RANKL-induced NFκB signaling (Darnay et al., 1999; Wong et al., 1998). Mammals express five Rel (NFκB) proteins referred to as RelA (p65), c-Rel, RelB, NFκB1 (p105) and NFκB2 (p100). Whereas RelA, c-Rel and RelB are synthesized as mature products, p105 and p100 are proteolytically processed into mature p50 and p52 NFκB proteins (Karin and Lin, 2002). Two Rel proteins, namely NFκB1 and NFκB2 have been shown to be required for osteoclastogenesis *in vivo* and

mice lacking both p50 and p52 exhibit severe osteopetrosis (Iotsova et al., 1997). Furthermore, IκB kinases (IKKα, IKKβ and IKKγ) have also been implicated in RANK/RANKL signaling. Whereas IKKβ is required for RANK/RANKL-induced osteoclastogenesis *in vitro* and *in vivo*, the role of IKKα seems to be marginal *in vivo* (Ruocco et al., 2005). Importantly in human patients, IKKγ mutations are associated with osteopetrotic patients suffering X-linked osteopetrosis, lymphedema, anhidrotic ectodermal dysplasia and immunodeficdiency (Doffinger et al., 2001). Therefore the NFκB pathway is also relevant for activation and function of osteoclasts in humans.

Stimulation of RANK also results in the activation of members of the mitogen activated protein kinase family (MAPK family). MAPK signaling is an evolutionarily conserved mechanism of cells to respond to environmental stresses, thereby controlling cell survival and adaptation. The family of MAPKs consist of p38-MAPKs, c-Jun N-terminal kinases (JNKs) and extracellular signal-regulated kinases (ERKs) (Chang and Karin, 2001). Pharmogological inhibiton of the p38-MAPK isoforms p38α and p38β blocks RANKL-induced osteoclast differentiation (Matsumoto et al., 2000). Thus the p38-MAPKs appear to be mediators of RANKL-induced osteoclast development from haematopoietic precursor cells. Moreover, it has also been shown that JNKs and their direct upstream kinase MKK7 are required for osteoclastogenesis *in vitro* (David et al., 2002; Yamamoto et al., 2002). In addition, JNKs control members of the AP-1 family of transcription factors such as c-Jun, JunB, C-Fos and Fra all of which have been shown to play roles in bone metabolism (David et al., 2002; Kenner et al., 2004; Wagner, 2002). Furthermore, RANK activation results in the induction of ERK which also increases osteoclastogenesis (Hotokezaka et al., 2002). However, since the NFκB and the MAPK pathways are common to many other signaling processes in different organs the suitability of these molecules as therapeutic targets is rather questionable.

One key transcription factor in RANKL-induced osteclastogenesis is the nuclear factor of activated T cells, calcineurin dependent 1 (NFATc1) (Takayanagi et al., 2002a). NFATc1 was identified in a genome-wide screen for genes that are specifically linked to osteoclast differentiation. In the presence of RANKL the expression of NFATc1 is significantly upregulated leading to terminal differentiation of osteoclasts (Takayanagi et al., 2002a). Targeted deletion of NFATc1 in mice leads to a significant defect in osteoclastogenesis thus resulting in osteopetrosis (Aliprantis et al., 2008). Importantly, RANKL-mediated activation of NFATc1 requires the calcium-dependent

phosphatase calcineurin (Takayanagi et al., 2002a). The RANK/RANKL pathway is coupled to calcium signaling by the phosphatidylinsoitol-related enzyme phospholipase C (PLC). The activation of PLCγ via Tec-kinase family members results in the mobilization of calcium from intracellular stores, thus allowing the activation of the essential transcription factor NFATc1 via calcineurin ( Mao et al., 2006, Komarova et al., 2003; Takayanagi, 2005; Takayanagi et al., 2002a, ).

Furthermore, RANKL-induced osteoclastogenesis also requires the activation of the anti-apoptotic serine/threonine proteinkinase B (Akt/PKB) via the proto-oncogene c-src and the inositol lipid metabolism (Leibbrandt and Penninger, 2008). Targeted disruption of c-src or the phosphoinositide phosphatase SHIP1 in mice leads to osteopetrosis, thus emphasising the critical role of this pathway in bone metabolism. Most importantly, c-src might be a suitable target for future treatment of osteoporosis and bone loss in arthritis.

Besides the major RANK/RANKL-activated signaling pathways fine tuning of RANKL-induced osteoclastogenesis and bone turnover requires the interaction with many cytokines. The T cell derived cytokine, Interferon gamma (INFγ) reduces RANK signaling by accelerating the degradation of the essential adaptor protein TRAF6. Moreover, it has been reported that RANKL-induced Interferon beta (INFβ) expression in osteoclast precursor cells also inhibits RANK-activity via interfering with c-Fos (Takayanagi et al., 2002b). Importantly, the interactions between bone and cytokines can also result in a variety of pathologic effects (Lee et al., 2008). Thus, the understanding of the roles of cytokines in bone metabolism can lead to the development of better therapeutic strategies.

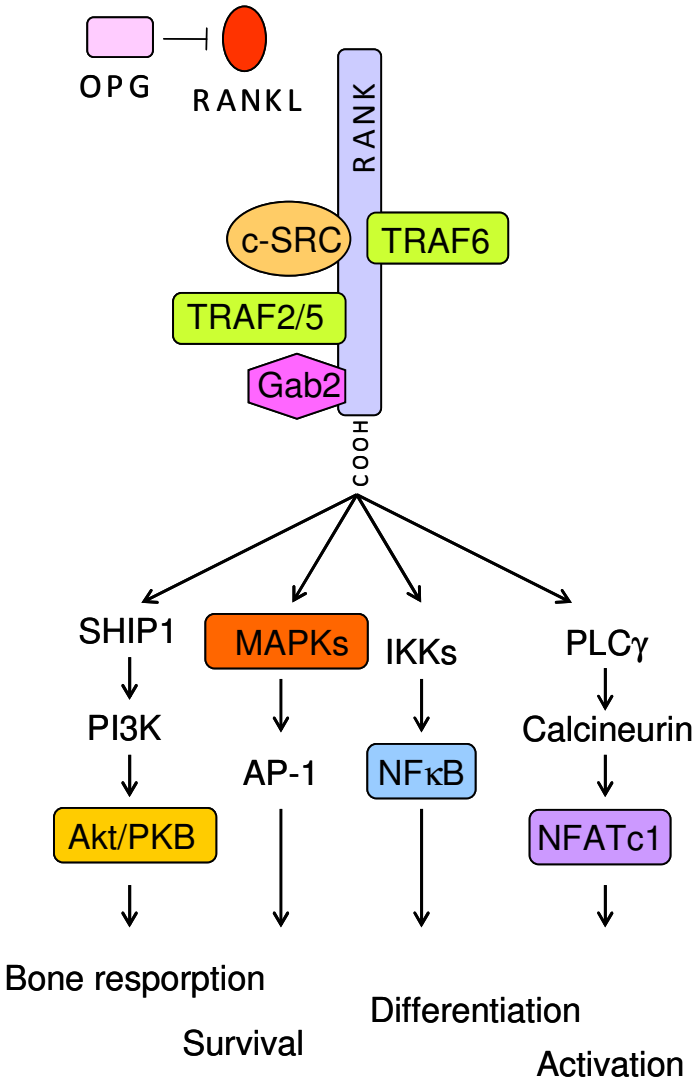



**Figure 2. RANK/RANKL downstream signaling pathways. Schematic illustration of known signaling cascades downstream of RANK/RANKL. RANKL binds and activates its receptor RANK, thus leading osteoclast differentiation and activation and survival of mature osteoclasts which ultimately results in bone resorption. OPG inhibits RANK activation by efficiently competing for RANKL binding. RANKL-stimulation induces the activation of Akt/PKB, MAPKs and NFκB through recruitment of adaptor molecules such as TRAFs, GAB2 and c-SRC. RANK/RANKL-mediated phosphatidylinositol signaling results in the activation of PKB and SHIP1 and the anti-apoptotic kinase Akt/PKC. Activation of MAPK family members induces the expression of AP1 family members such as Jun, Fos and Fra that are involved in osteoclastogenesis.**

### 1.4. Beyond bones

Surprisingly, detailed analysis of $RANK^{-/-}$ and $RANKL^{-/-}$ mice revealed another entirely unexpected phenotype. In addition to the severe bone disorder, both mutant mouse strains lack all lymph nodes but display normal development of Peyer's patches (Kong et al., 1999b). Since it had been assumed that lymph node organogenesis and the development of Peyer's patches is genetically coupled (Futterer et al., 1998) these findings provided the first evidence that these two developmental events underlie different genetical regulations. The biogenesis of primordial lymph nodes depends on the concerted activity of several cell lineages such as fibroblasts, macrophages, reticular cells and endothelial cells. Thereafter, these primordial lymphnodes populated with T and B cells and $CD4^{+}CD3^{-}LT\beta^{+}$ cells that differentiate into natural killer (NK) cells, antigen presenting cells (APCs) and follicular cells in order to generate mature compact nodes. So far, several studies have suggested that impaired lymph node development in $RANK^{-/-}$ and $RANKL^{-/-}$ mutant mice is neither due to restricted expression of RANK and RANKL in secondary lymphoid organs (Boyle et al., 2003; Simonet et al., 1997) nor due to cellular homing defects of RANKL deficient lymphocytes (Kong et al., 1999b). However, there is some indication that RANKL is involved in the development of lymph node morphogenesis by regulating the colonization and cluster formation of $\alpha_4\beta_7^{+}CD45^{+}CD4^{+}CD3^{-}$ in lymph nodes (Kim et al., 2000). But still, the exact molecular mechanisms how RANK and RANKL regulate lymph node formation as well as the link between lymph node and Peyer's patch development remain undefined.

Furthermore, RANK and RANKL are also important regulators of interactions between dendritic cells (DCs) and T cells. Dendritic cells are hematopoietic cells, specialized to initiate T cell immunity. Immature DCs reside in peripheral tissues waiting to capture and process antigen. In response to antigen contact and inflammatory stimuli they undergo maturation and migrate to T cell sites in secondary lymphoid organs where they present antigen to naïve T cells. After the event of antigen-presentation DCs are rapidly eliminated in order to avoid excessive immune responses (Banchereau and Steinman, 1998; Ingulli et al., 1997; Leibbrandt and Penninger, 2008). For example, it has been shown that prolonged DC survival is critical in patients with autoimmune lymphoproliferative syndrome (ALPS) (Wang et al., 1999). RANK and RANKL interactions between DCs and active T cells appear to positively regulate DC survival by upregulating the expression of the essential T cell co-stimulation factor

CD40 and of the anti-apoptotic factor Bcl-XL (Anderson et al., 1997; Josien et al., 2000; Wong et al., 1997a). Moreover it has been observed that OPG can bind to the TNF-family molecule TRAIL which is produced by activated T cells in order to induce death of DCs *in vitro* (Emery et al., 1998; Yun et al., 1998). Whether OPG-TRAIL interactions are also relevant *in vivo* still needs to be elucidated. Since modification DC survival could be useful in anti-tumor therapy and treatment of autoimmune diseases further research in this direction is ongoing.

In addition, epidermal RANKL which is strongly upregulated upon UV-irradiation in the skin has been shown to control peripheral homeostasis of $CD4^+CD25^+$ regulatory T-cells via activating dendritic cells (Loser et al., 2006). Regulatory $CD4^+CD25^+$ T cells (Tregs) are important for the avoidance of autoimmunity by suppressing autoreactive T cells (Sakaguchi, 2005). Thus, by controlling regulatory T cell numbers cutaneous RANK and RANKL appear to play a crucial role in the suppression of autoimmunity (Loser et al., 2006). Therefore, new therapeutic approaches for allergies and systemic autoimmunity might be based on the RANK/RANKL system.

Importantly, RANK and RANKL control also the development of $AIRE^+$ thymic epithelial cells in the thymus (Rossi et al., 2007). $AIRE^+$ cells are a subtype of thymic meduallary epithelial cells (mTECs) important for the maintenance of self-tolferance (Anderson et al., 2002). RANKL which is expressed on a $CD4^+3^-$ lymphoid tissue inducer cell population is required to mediate the development of $CD80^-Aire^-$ mTECs to $CD80^+Aire^+$ mTECs (Rossi et al., 2007) (Akiyama et al., 2008; Hikosaka et al., 2008). Thus, the RANK/RANKL system is also involved in regulating central tolerance.

The link between RANK/RANKL in bones and in the immune system lead to the emerge of a new research field referred to as osteoimmunology (Rho et al., 2004). Since activated T cells express RANKL the question arouse whether T-cell derived RANKL can also regulate osteoclast development and activity. Indeed it turned out the T cells are able to mediate bone turnover *in vitro* and *in vivo* (Kong et al., 1999a). Although still being debated, T cells are most likely not required for normal bone homeostasis (Kong et al., 1999b). However, systemic activation of T cells triggers bone loss thus, indicating a critical role of T cells in pathologic bone loss *in vivo*. Nowadays, many data strongly indicate that bone remodelling associated with systemic T cell

activation, viral infections, local bone inflammations and joint inflammation in arthritis is caused by RANKL-producing T cells (Theill et al., 2002).

Since T cells produce INFγ, an inflammatory cytokine which suppresses osteoclastogenesis (Gowen and Mundy, 1986; Takayanagi et al., 2000) it has been unclear for a long time how T cells can mediate bone destruction (Sato et al., 2006). By which mechanism T cells enhance bone resporption has been clarified in 2006 with the identification of Th17 which link T cell activation to bone destruction (Sato et al., 2006). Th17 cells are a specialized inflammatory subset of $CD4^+$ T cells producing Interleukin 17 (IL 17) which has been reported to induce RANKL expression and osteclastogenesis *in vitro* (Sato et al., 2006). Moreover, IL 23, which is essential for expanding the population of Th17 cells is also critically involved in T-cell mediated enhancement of osteoclastogenesis (Sato et al., 2006). Thus, the IL 17-IL 23 axis plays a crucial role in autoimmune inflammation and in the development of rheumatoid arthritis by acting as an osteoclastogenic helper (Sato et al., 2006).

Most recently our lab could show a novel and surprising new function of RANK/RANKL in the central nervous system (Hanada et al., 2009). RANK/RANKL are essential players in inflammatory fever response and in the regulation of female body temperature (Hanada et al., 2009). Mechanistically it appears that RANK/RANKL mediate fever in response to inflammation via the COX2-PGE(2)/EP3R pathway, whereas the regulation of female body temperature by RANK/RANKL is under the influence of sex hormones (Hanada et al., 2009). Since postmenopausal osteoporosis is often associated with hot flashes RANK and RANKL might now provide a plausible explanation for this symptom.

### 1.5. RANKL, RANK, OPG and the link to mammary gland formation

Besides of the lack osteoclasts, lymph nodes and $AIRE^+$ mTECs both RANK and RANKL knockout mice exhibt an additional phenotype. Both mouse strains fail to develop a functional lactating mammary gland during pregnancy resulting in the death of newborn pubs due to malnutrition (Fata et al., 2000).

The development of the mammary gland proceeds in distinct steps in the context of sexual development and reproduction (Hennighausen and Robinson, 1998). Unlike most organs, mammary gland development predominantly takes place postpartum (Hennighausen and Robinson, 2001). In newborn mice, rudimentary mammary anlagen are present which elongate moderately into the mammary fat pad during prepuberty. At the onset of puberty progesterone, estrogens and growth hormones induce further ductal extension and branching (Hennighausen and Robinson, 2001). Furthermore, bulbous terminal end buds form at the end of the immature ducts and differentiate into quiescent alveolar buds (Oakes et al., 2008). During pregnancy, massive side branching, proliferation and differentiation of ductal and alveolar epithelilum results in the development of milk secreting lobuloalveolar structures. After pregnancy and lactation the lobuloalveolar buds undergo apoptosis and the mammary gland is remodelled back towards a virgin state, ready for the next pregnancy (Brisken et al., 1999; Hennighausen and Robinson, 2001) (Figure 3).

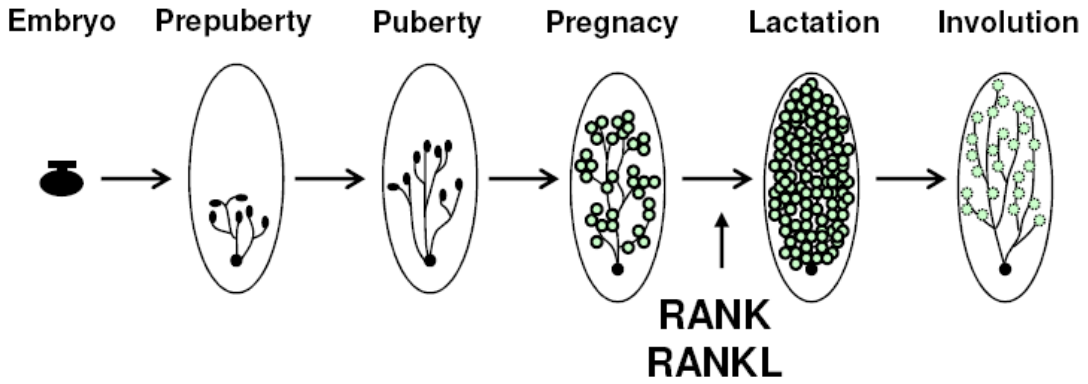


**Figure 3. Stages in murine mammary gland development. Small mammary gland anlagen are established in the embryo. During puperty, mammary gland anlagen undergo only moderate proliferation.. During puperty, sex hormones induce further proliferation and extension of the mammary epithelial tree. At the onset of pregnancy pregnancy hormones such as progesterone and lactogenic hormones such as prolactin drive massive proliferation and differentiation to lobuloalveolar milk-secreting structures. After lactation involution resets the mammary gland into a virgin state. RANK and RANKL act during pregnancy, inducing the differentiation and proliferation of lobuloalveolar, milk-secreting structures. Figure adapted from (Hennighausen and Robinson, 2001).**

Mammary gland formation up to mid-pregnancy occurs normally in $RANK^{-/-}$ and $RANKL^{-/-}$ mice, the proliferation and terminal differentiation of alveolar buds to mature lobuloalveoli is completely arrested (Fata et al., 2000). Whereas RANKL expression in mammary epithelial cells is induced by pregnancy hormones, RANK is constitutively expressed on these cells (Fata et al., 2000). Importantly, RANK/RANKL signaling in the mammary gland is regulated in a very strict spatial and temporal manner. In a virgin gland RANKL mRNA is absent but increases gradually during pregnancy reaching a peak at P16.5 to then decrease again to low levels at the beginning of lactation (Gonzalez-Suarez et al., 2007). In contrast, RANK is constitutively expressed on mammary epithelial cells, though the expression is weak in virgin glands (Gonzalez-Suarez et al., 2007). RANK is also upregulated during pregnancy starting at P14.5. After reaching a maximum at day P15.5 in the alveolar buds, expression levels decrease again when the alveoli are about to secrete milk (Gonzalez-Suarez et al., 2007). Importantly, RANK/RANKL mediates pregnancy induced mammary gland formation in an autocrine manner (Fata et al., 2000) and the transplantation of mammary glands $RANK^{-/-}$ or $RANKL^{-/-}$ into the cleared fat pads of wild type mice cannot rescue the phenotype (Fata et al., 2000).

In 2001, it was reported that the subsequent activation of IKKα, NFκB and Cyclin D1 is crucial for RANKL-induced proliferation of the alveolar buds during pregnancy (Cao et al., 2001) (Figure 4). Indeed, $IKK\alpha^{AA/AA}$ knockin females, in which the activation domain of IKKα is mutated, and $CyclinD1^{-/-}$ mice display a severe lactation defect which can be rescued by transgenic CyclinD1 expression. Further studies revealed that DNA-binding protein inhibitor 2 (Id2) is also a crucial RANK/RANKL downstream target involved in the proliferation and differentiation of

the mammary gland during pregnancy (Kim et al., 2006) (Figure 4). Of note, Id2-null mice also exhibit impaired lobuloalveolar development during pregnancy, though the mammary gland defects do not resemble those of CyclinD1$^{-/-}$ mice (Mori et al., 2003; Mori et al., 2000). In line with these phenotypic differences, the mammary gland defects of Id2$^{-/-}$ mice cannot be rescued through forced CyclinD1 expression (Mori et al., 2003). Thus, the question whether these two pathways act independent from one another still remains to be solved.

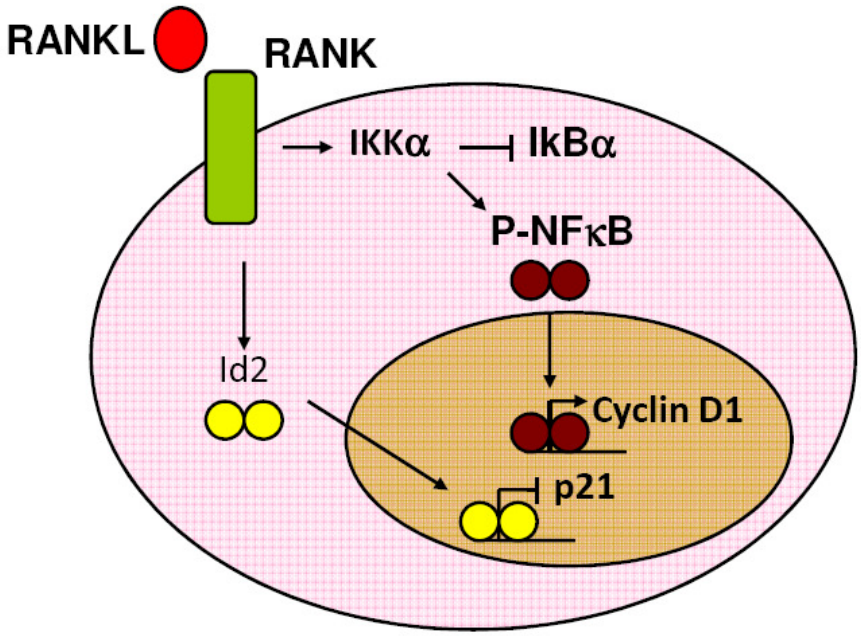



**FIGURE 4. RANK/RANKL signaling in mammary epithelial cells. RANK/RANKL signaling in mammary epithelial cells results in activation of the Id2 and NFκB pathways. RANKL expression in mammary epithelial cells triggers marked nuclear translocation of Id2 which is essential for cell cycle progression through the inhibition of p21 expression. Upon RANK activation IKKα induces the nuclear translocation of NFκB dimers by mediating the degradation of the IkBα. In the nucleus, NFkB leads to the expression of the cell cycle regulator Cyclin D1. All indicated pathways have been shown to be important by genetic experiments (Cao et al., 2001; Kim et al., 2006).**

### 1.6. Sex hormones in mammary gland development and tumorigenesis

RANKL expression in mammary epithelial cells is induced by the sex and pregnancy hormones Progesterone, Prolactin (PRL) and Parathyroid hormone-related protein (PTHrP) (Fata et al., 2000). The steroid hormone Progesterone plays a pivotal role in all aspects of female reproductive activity. Its physiological effects are mediated by the progesterone receptor (PR) which belongs to the nuclear receptor superfamily (Conneely et al., 2003a). Upon binding of progesterone a PR-ligand complex is formed followed by translocation to the nucleus (Conneely et al., 2003a). In mammals there are two structurally and functionally distinct nuclear receptors referred to as PR-A and PR-B. Besides female infertility and other reproductive abnormalities, mice lacking both isoforms of the progesterone receptor (PRKO) display severe defects in mammary gland development during pregnancy (Conneely et al., 2003a). In particular, PRBKO but not PRAKO knockout mice fail to undergo pregnancy induced ductal side-branching and lobuloalveolargenesis. Intriguingly, this defect mainly results from dramatically reduced RANKL levels (Mulac-Jericevic et al., 2003), thus strongly emphasizing a tight connection between RANK/RANKL and progesterone.

Progesterone has not only been associated with normal breast development but also with an increased risk of developing breast cancer. Both human and mouse data support a critical role for progesterone signaling in mammary carcinogenesis (Conneely et al., 2003b; Ross et al., 2000; Sitruk-Ware, 2002). Whereas progesterone is known to decrease the risk of developing endometrial cancer (Sitruk-Ware, 2002), overexposure to progesterone is a known risk factor for breast cancer (Mc Cormack et al., 2007). As reported by the Million women and the Womens Health Initiative studies, women receiving a combined HRT of progesterone and estrogen were 2 fold more likely to develop breast cancer compared to women receiving estrogen alone (Beral, 2003; Rossouw et al., 2002). Of note, it has also been reported that pregnancy and low-dose estrogen and progesterone treatment prevents chemically induced carcinogenesis (Jemal et al., 2009). However, the exact regulatory mechanisms by which progesterone is linked to mammary tumorigenesis are still unclear.

The lactogenic hormone PRL can also induce RANKL expression in the mammary gland (Fata et al., 2000). PRL is not only closely related to mammary gland development during pregnancy but also has been associated with mammary tumorigenesis (Llovera et al., 2000; Oakes et al., 2007). PRL is produced by the

pituitary gland but can also be secreted locally by mammary epithelial cells (Oakes et al., 2008). The prolactin receptor (PRLR) belongs to the superfamily of class I cytokine receptors. Upon prolactin binding PRLR dimerizes and activates a variety of downstream signaling cascades (Oakes et al., 2008). For instance, activation of PRLR results in the phosphorylation and activation of the Janus kinase 2 (JAK2) which is followed by recruitment and activation of STAT5 (Signal transducer and activator of transcription 5). Phosphorylated and dimerized STAT5 then translocates to the nucleus where it activates the transcription of genes required for alveolar morphogenesis (Oakes et al., 2008).

PRL and PRLR knockout mice exhibit a defect in mammary gland development already prior to pregnancy (Brisken et al., 1999, Ormandy, 1997 #138). In wild type animals the mammary gland undergoes ductal side branching and differentiation to quiescent alveolar buds as soon as the terminal end buds reach the periphery of the fat pad during puperty. This process is completely arrested in $PRL^{-/-}$ and $PRLR^{-/-}$ mice (Horseman et al., 1997). Similar defects in ductal brancing and differentiation of terminal end buds can also be observed in STAT5 knockout mice (Liu et al., 1998). As $PRLR^{-/-}$ mice are infertile, mammary gland development during pregnancy could not be assessed in knockout females but only in heterozygous $PRLR^{+/-}$ mice (Ormandy et al., 1997). These heterozygous $PRLR^{+/-}$ mice undergo normal mammary gland development up to mid pregnancy but then exhibit impaired lobuloalveoar development (Brisken et al., 1999). Thus, the phenotype of $PRLR^{+/-}$ mice is similar to the phenotype observed in RANK, RANKL and PR-knock out mice suggesting a potential link between these pathways.

In women, elevated serum PRL levels correlate with a twofold increase in the risk of developing breast cancer, and especially of invasive ER-α positive and PR-positive breast cancers (Oakes et al., 2007, Arendt, 2009). Overexpression of PRL in mice leads to the development of mammary cancer indicating that high levels of PRL alone are sufficient to induce mammary tumors (Oakes et al., 2007). Further, PRLR-deficient mice display delayed tumor onset in response to oncogenic stimuli (Oakes et al., 2007). PRL modulates carcinogenesis within the mammary epithelium directly as well as through the actions of estrogen and progesterone (Oakes et al., 2007). Mechanistically, PRL stimulation induces increased proliferation and CyclinD1 expression predominantly mediated by the JAK2/STAT5 signalling pathway (Schroeder

et al., 2002). Thus, the induction of Cyclin D1 expression by PRL suggests another potential link between RANK/RANKL and PRL-signaling.

### 1.8. Project outline

RANK-RANKL signaling is known as a very important survival pathway in mammary gland development during pregnancy. In addition, RANKL expression can be induced by the sex hormones progesterone, prolactin and PTHrP which are also known to be involved in mammary tumorigenesis, suggesting a plausible role for RANK-RANKL signaling in breast cancer development. The aim of my study therefore, was to investigate the role of RANK-RANKL and its downstream targets in hormonal driven primary breast cancer in mice. In order to do this I specifically inactivated the RANKL receptor RANK in mammary epithelial cells ($RANK^{\Delta mam}$). Intriguingly, $RANK^{\Delta mam}$ mice develop breast cancer significantly later than wild type littermate controls in response to progesterone/DMBA treatment. Furthermore, by generating $IKK\alpha^{\Delta mam}$, $TRAF6^{\Delta mam}$ and $NFATc1^{\Delta mam}$ mice, I was able to show that whereas these effectors are required for RANK-RANKL effects on osteoclastogenesis, only IKKα appears to be critical for the regulation of breast cancer onset. Thus, our findings provide the first direct evidence that RANK-RANKL, through activation of NFκB signaling, controls development of hormone-dependent breast cancer.

## 2. Results

In addition to our first knockout experiments (Fata et al., 2000), two overexpression-studies have suggested a potential role of RANK/RANKL in mammary epithelial cell growth. Gonzalez-Suarez et al. reported that MMTV-driven expression of RANK triggers massive proliferation of the mammary epithelium upon pregnancy (Gonzalez-Suarez et al., 2007). Moreover, mammary epithelial cells derived from these transgenic mice exhibit increased proliferation when put in 3-dimensional culture (Gonzalez-Suarez et al., 2007).

Similarly, Fernandez-Valdivia et al. could show that nulliparous MMTV-RANKL transgenic mice develop mammary hyperplasia with age (Fernandez-Valdivia et al., 2009). Importantly, sex hormones such as prolactin and progesterone which are crucial in RANKL-induced mammary gland development during pregnancy (Fata et al., 2000) are also related to breast cancer (Llovera et al., 2000; Oakes et al., 2007). Thus, we speculated that RANK/RANKL is involved in hormone-driven mammary tumorigenesis.

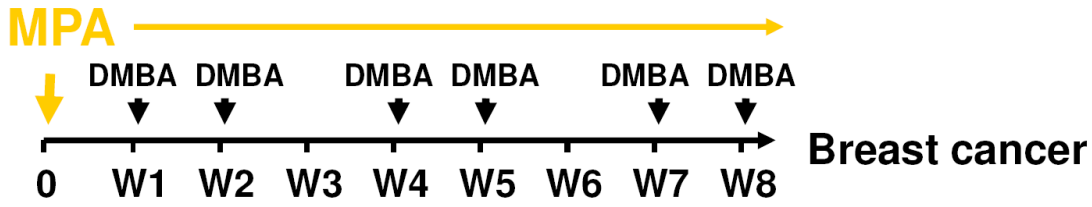
## 2.1. MPA/DMBA treatment leads to the induction of mammary tumors in wildtype mice

Synthetic progesterone derivatives (progestins) such as medroxyprogesterone acetate (MPA) are used in millions of women for hormonal replacement therapy (HRT) and contraceptives (Rossouw et al., 2002; Schwallie and Mohberg, 1977). A positive effect of using progesterone is that it decreases the risk of developing estrogen-driven endometrial cancer (Beral et al., 2005). But progestins are also known to be critically involved in mammary tumorigenesis (Mc Cormack et al., 2007). Moreover, according to the Women`s Health Initiative (WHI) and the Million Women Study the use of progestins, in particular MPA appears to markedly increase the risk of developing breast cancer (Beral, 2003; Rossouw et al., 2002).

A method to model progestin-driven breast cancer in female mice has been described (Aldaz et al., 1996, Cao et al., 2007). In this model the subcutanous implantation of slow release MPA pellets accelerates the development and increases the incidence of short latency mammary tumors in response to the DNA damaging carcinogen dimethylbenz(a)anthracene (DMBA). Of note, the course of the developing mammary tumors resembles that of human breast cancers (Escrich, 1987a; Escrich, 1987b).

To set up the protocol, we implanted MPA pellets (50mg 90-day release MPA pellets, Innovative Research of America NP-161) subcutaneously in the right flanks of 6 week old nulliparous BalbC wildtype mice followed by oral gavage of a DMBA-containing solution (Figure 5a). All female mice treated according to the scheme developed mammary tumors with 100% penetrance. The median tumor free survival is about 10 days after the last DMBA treatment (Figure 5b). Thus, I was able to successfully establish this progestin-driven breast cancer model in our laboratory.

**a**




**b**

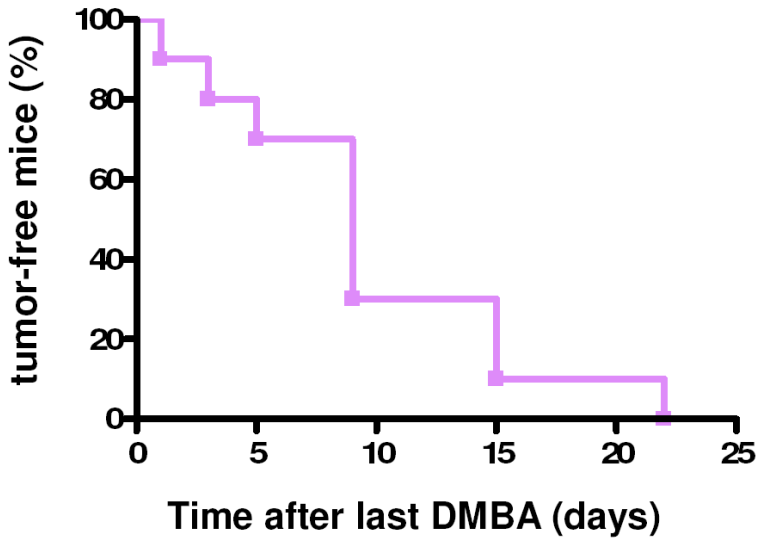
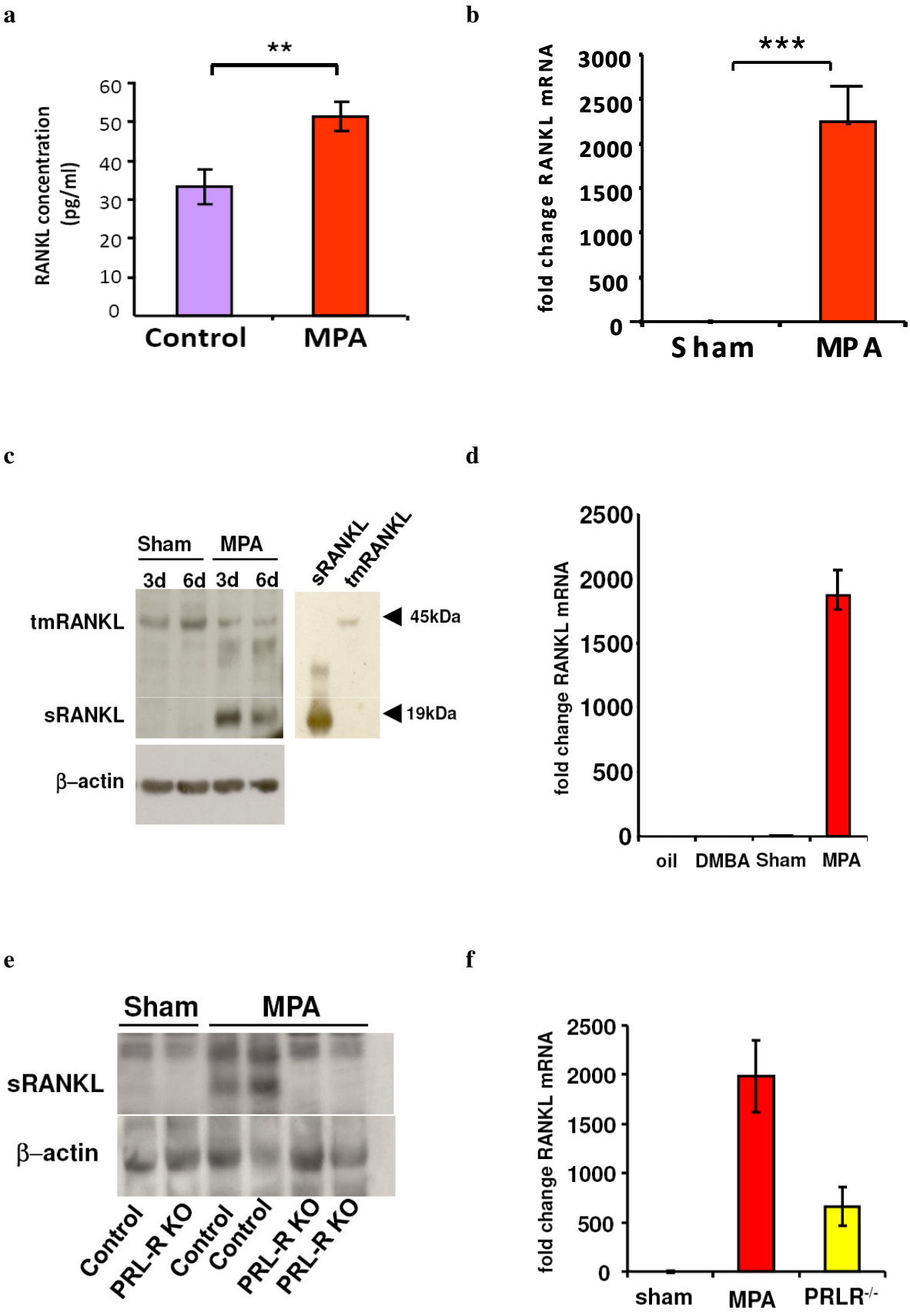



**Figure 5. MPA/DMBA treatment induces mammary tumors in wildtype mice. a, MPA/DMBA carcinogenesis scheme. Nulliparous, six-week old female mice were s.c. implanted with slow release Medroxyprogesterone acetate (MPA) pellets and treated orally with Dimethylbenz(a)anthracene DMBA 6 times throughout 8 weeks (W) as indicated. b, Onset of palpable tumors in wild type mice (n=10) treated with MPA and DMBA as indicated in Fig. 5a. Data are shown as percentage of tumor free mice after the last DMBA challenge.**

## 2.2. *In vivo* progestine administration leads to massive RANKL expression via the Prolactin receptor

The RANK/RANKL system in the mammary gland is tightly linked to hormonal regulation and previously it has been shown that RANKL expression can be induced by progesterone stimulation in nulliparous female mice (Fata et al., 2000). In order to examine whether the synthetic progesterone-derivative MPA also affects RANKL expression in the mammary glands we implanted MPA pellets into the right flanks of 6 week old estrus-synchronized female wild type mice. We observed a mareked upregulation of RANKL expression in the serum (Figure 6a). Moreover, in isolated mammary epithelial cells RANKL mRNA was upregulated up to 2000 times three days and six days after MPA stimulation (Figure 6b). Interestingly, we did not detect an increase in expression of the 45 kDa membrane bound RANKL isoform but we observed elevated levels of the 19kDa soluble form of RANKL (Figure 6c), which can be derived either from alternative splicing or cleavage of the membrane bound form (Ikeda et al., 2001). Of note, neither sham-operated nor DMBA treated mice exhibited an increase of RANKL mRNA levels in isolated mammary gland epithelial cells (Figure 6d). Thus, MPA treatment results in a systemic and mammary gland specific upregulation of RANKL expression.

Recently, it has been reported that prolactin can directly induce RANKL expression via the JAK2/STAT5 pathway (Srivastava et al., 2003). Interestingly, MPA can also induce the transcription of prolactin (Reis et al., 1999) as well as the prolactin receptor in breast cancer cells (Chaudhury et al., 1977; Ormandy et al., 1992). Therefore we speculated that MPA induces RANKL expression in mammary epithelial cells via the prolactin receptor. Analysis of prolactin receptor knockout mice (Ormandy et al., 1997) revealed that RANKL protein expression was not induced three days after MPA pellet implantation whereas in littermate controls we observed a significant upregulation of RANKL expression upon MPA stimulation (Figure 6e). Intriguingly, whereas RANKL protein was completely undetectable in prolactin receptor mutants we could still observe an increase of RANKL mRNA in isolated mammary epithelial cells (Figure 6f). Thus, one could speculate that the absence of the PRL not only affects RANKL expression but could also interferes with RANKL mRNA stability. Taken together, these results suggest that MPA triggers massive RANKL expression in mammary epithelial cells, at least in part, via the prolactin receptor *in vivo*.


a
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RANKL concentration (pg/ml)
60
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40
30
20
10
0
Control
MPA
b
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fold change RANKL mRNA
3000
2500
2000
1500
1000
500
0
Sham
MPA
c
Sham
MPA
3d
6d
3d
6d
sRANKL
tmRANKL
tmRANKL
sRANKL
β–actin
45kDa
19kDa
d
fold change RANKL mRNA
2500
2000
1500
1000
500
0
oil
DMBA
Sham
MPA
e
Sham
MPA
sRANKL
β–actin
Control
PRL-R KO
Control
Control
PRL-R KO
PRL-R KO
f
fold change RANKL mRNA
2500
2000
1500
1000
500
0
sham
MPA
PRLR-/-

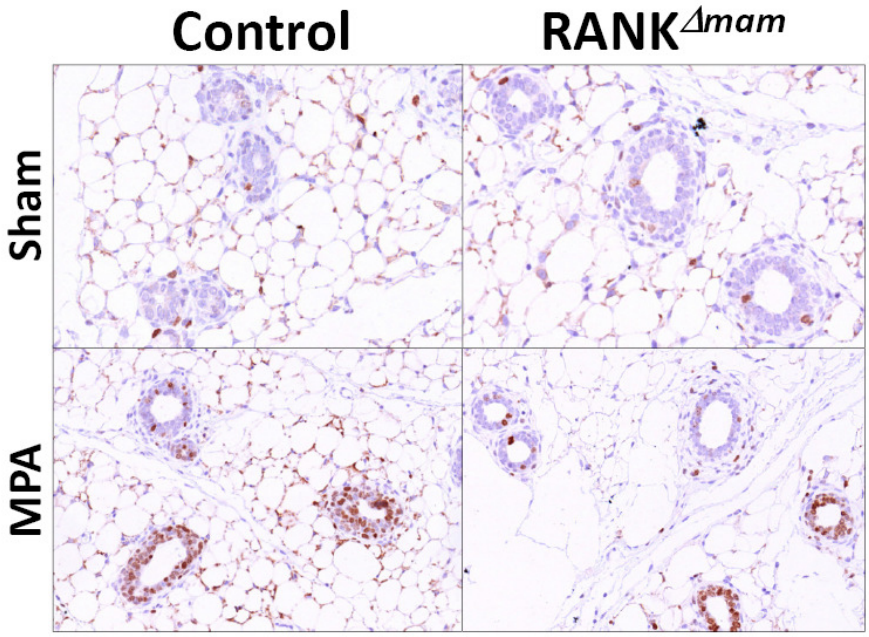
**Figure 6. The progesterone-derivative MPA triggers *in vivo* RANKL expression via the prolactin system. a-d, Induction of RANKL expression by the progesterone-derivative MPA. Nulliparous wild type females were subcutaneously implanted with slow-release MPA pellets or treated with surgery sham. a, Systemic RANKL levels were determined in serum 3 days after MPA pellet implantation by ELISA. Data are shown as pg RANKL/ml serum (mean values, +/- sem; n=3 mice per group). ** P > 0.005 (Student`s t-test). b, Total mRNA was isolated from purified mammary epithelial cells 3 days after MPA implantation. RANKL mRNA was determined by qRT-PCR relative to β-actin levels. Data are shown as fold change compared to sham treatment (mean values, +/- sem; n=3 mice per group). *** P > 0.0005 (Student`s t-test). c, Expression of transmembrane (tm) and soluble (s) RANKL protein was assayed on isolated mammary gland epithelial cells by Western blot 3 and 6 days after MPA pellet implantation or sham surgery. β-actin is shown as a loading control. Recombinant sRANKL and tmRANKL protein are shown for molecular size comparison in the right panels. Data are representative of 8 animals tested. d, MPA but not oil-vehicle, DMBA or surgery sham induces RANKL expression in mammary epithelial cells. Total mRNA was isolated from purified mammary epithelial cells 3 days after oil-gavage, DMBA-treatment, surgery sham and MPA-implantation. RANKL mRNA was determined by qPCR relative to β-actin levels. Data are shown as fold change compared to sham treatment +/- sem (n=3 mice per group) e, Induction of soluble RANKL protein assayed on isolated mammary gland epithelial cells in control, but not in prolactin receptor mutant (PRLR KO) females on day 3 after s.c. MPA implantation. Mammary glands from sham operated control littermates and PRL-R KO females are shown as controls (n=3 animals per group) f, Total mRNA was purified from isolated mammary epithelial cells of PRLR KO mice and control females 3 days after MPA pellet implantation and sham-surgery. RANKL expression relative to β-actin levels was determined by qRT-PCR. Data are shown as fold change compared to sham treatment +/- sem (n=3 mice per group). * P > 0.05 (Student`s t-test).**

## 2.3. MPA induced RANKL expression triggers massive proliferation of mammary epithelial cells *in vivo*

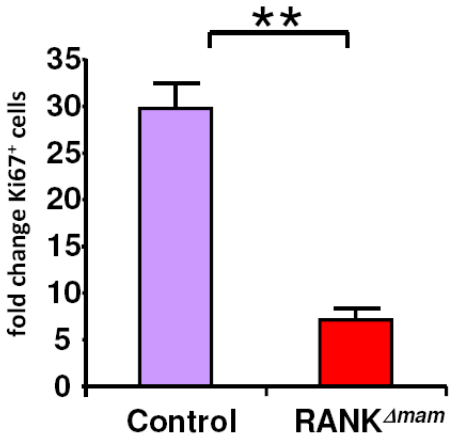
During pregnancy RANKL expression induces proliferation of mammary epithelial cells (Fata et al., 2000). Therefore we examined whether MPA-induced RANKL expression can also trigger proliferation of mammary epithelial cells *in vivo.* Using Ki67 as a proliferation marker we determined proliferation rates in nulliparous female mice in which we have deleted RANK specifically in mammary epithelial cells ($RANK^{\Delta mam}$) and littermate controls upon MPA-treatment. The generation of $RANK^{\Delta mam}$ mutant mice will be discussed in chapter 2.4. Indeed, three days after MPA implantation we could detect a strong induction of proliferation in the mammary glands of nulliparous control females as defined by $Ki67^+$ cells (Figures 7a, b).

In $RANK^{\Delta mam}$ nulliparous female mice induction of proliferation of mammary epithelial cells in response to MPA was significantly lower than in control mice (Figures 7a, b). Up to 80% of acini of control mice showed signs of medium to high proliferation. Intriguingly, in more than 60 % of all acini of $RANK^{\Delta mam}$ females analyzed we observed only very few proliferating cells (Figure 7c). The slight induction of proliferation in $RANK^{\Delta mam}$ mutant mice could be explained with incomplete RANK-deletion and proliferation of remaining wildtype cells. Of note, proliferation was not induced in sham operated female mice (Figure 7d). Importantly, since proliferation in mammary glands varies during the course of the estrus (Fata et al., 2001) all mice used in this experiment were synchronized for the estrus cycle. To test whether MPA induced RANKL can directly induce proliferation in mammary epithelial cells we next injected RANKL i.p. into control and $RANK^{\Delta mam}$ mice (5 g/mouse). As expected, we could observe massive proliferation in mammary epithelial cells of control mice whereas $RANK^{\Delta mam}$ female mice showed significantly reduced induction of proliferation in response to RANKL (Figure 7e). Thus, we conclude that the massive proliferation of mammary epithelial cells upon MPA stimulation is largely controlled by RANK/RANKL signaling.

a


Control
RANKΔmam
Sham
MPA

b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fold change Ki67+ cells
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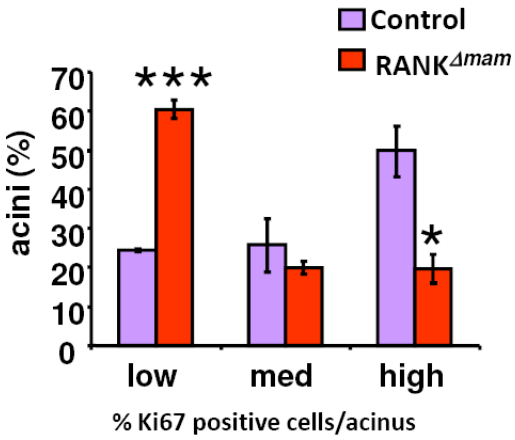
Control
RANKΔmam

c


Control
RANKΔmam
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acini (%)
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40
30
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10
0
low
med
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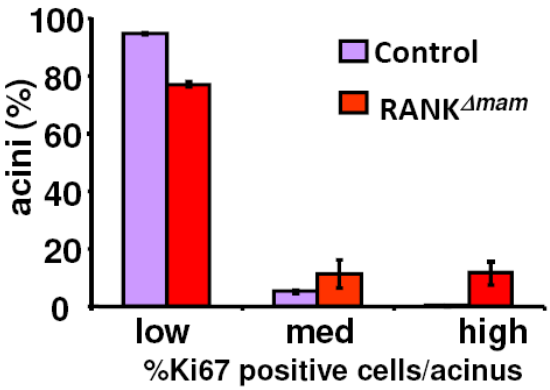
% Ki67 positive cells/acinus

d


Control
RANKΔmam
acini (%)
100
80
60
40
20
0
low
med
high
%Ki67 positive cells/acinus

e

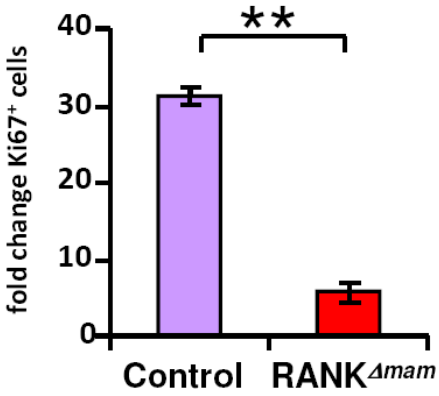
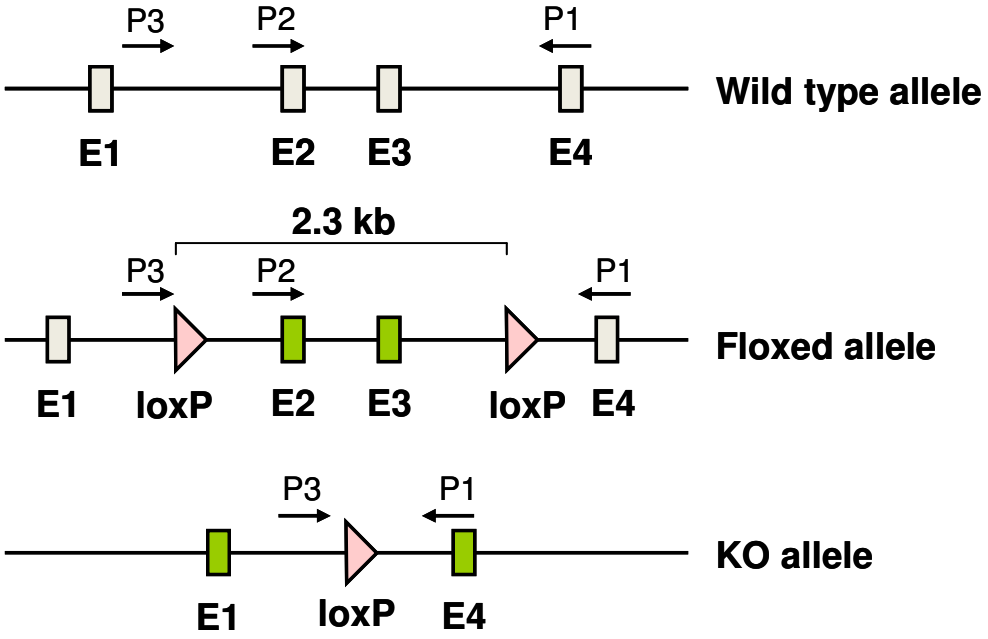

**
fold change Ki67+ cells
40
30
20
10
0
Control
RANKΔmam

**Figure 7. MPA-induced RANKL expression triggers massive proliferation of mammary epithelial cells *in vivo*. a, Epithelial proliferation was detected by *in situ* Ki67 staining of mammary gland sections of estrus-synchronized RANK$^{\Delta mam}$ females and control mice 3 days after MPA treatment. b, Quantification of Ki67$^{+}$ cells shows the relative changes in proliferation of mammary epithelial cells three days after MPA treatment. MPA treated mice were compared to sham-operated females of the respective genotype. Data are shown as mean value +/- sem, n=3 mice per genotype. ** P > 0.005 (Student's t-test). c, Quantification of Ki67$^{+}$ mammary epithelial cells after 3 days of MPA treatment, n=3 mice per genotype. * P > 0,05; ** P > 0.005; *** P > 0.001 (Student`s t-test) d, Quantification of Ki67+ mammary epithelial cells of oestrus-cycle synchronized wild type and RANK$^{\Delta mam}$ females 3 days after surgery sham, n=3 mice per genotype. Data in c, and d, are shown as percentage of acini/ducts with low (<20% of epithelial cells per acinus/duct are Ki67$^{+}$), medium (20-80% of epithelial cells per acinus/duct are Ki67$^{+}$) and high (>80% of epithelial cells per acinus/duct are Ki67$^{+}$) numbers of proliferating cells +/- sem. e; Epithelial proliferation after i.p. RANKL injection (5mg/mouse); control females were injected with PBS. Data are shown as relative fold change of Ki67$^{+}$ cells 3 days after RANKL/PBS injection, mean values +/- sem. n = 3 mice per genotype. ** P > 0.005; (Student's t-test). For b, c, d and e at least 1000 mammary gland epithelial cells were counted per mouse.**

## 2.4. Generation of RANK$^{\Delta mam}$ mice

In order to reveal the potential functions of RANK/RANKL in the development of mammary tumors we generated a mouse line in which the receptor of RANKL, RANK, is specifically deleted in mammary epithelial cells. To this aim, we crossed mice harboring a conditional RANK allele to a MMTV-*cre* deleter line which mediates the recombination of the conditional allele in mammary epithelial cells (Wagner et al., 2001) (Figure 8a). These mice are hereafter termed as RANK$^{\Delta mam}$. RANK$^{\Delta mam}$ mice appear healthy and are born at normal Mendelian ratios. They don't display impaired breast development during puperty (Figure 8b). Moreover, lymphnode development is normal. RANK$^{floxed}$ mice have been recently published by our group and reproduce all phenotypes of RANK full body knockout mice when crossed to the ubiquitous deleter line β-actin-*cre* (Lewandoski et al., 1997). Thus, we have generated RANK$^{\Delta mam}$ mice that delete RANK in mammary epithelial cells.

**a**



**b**

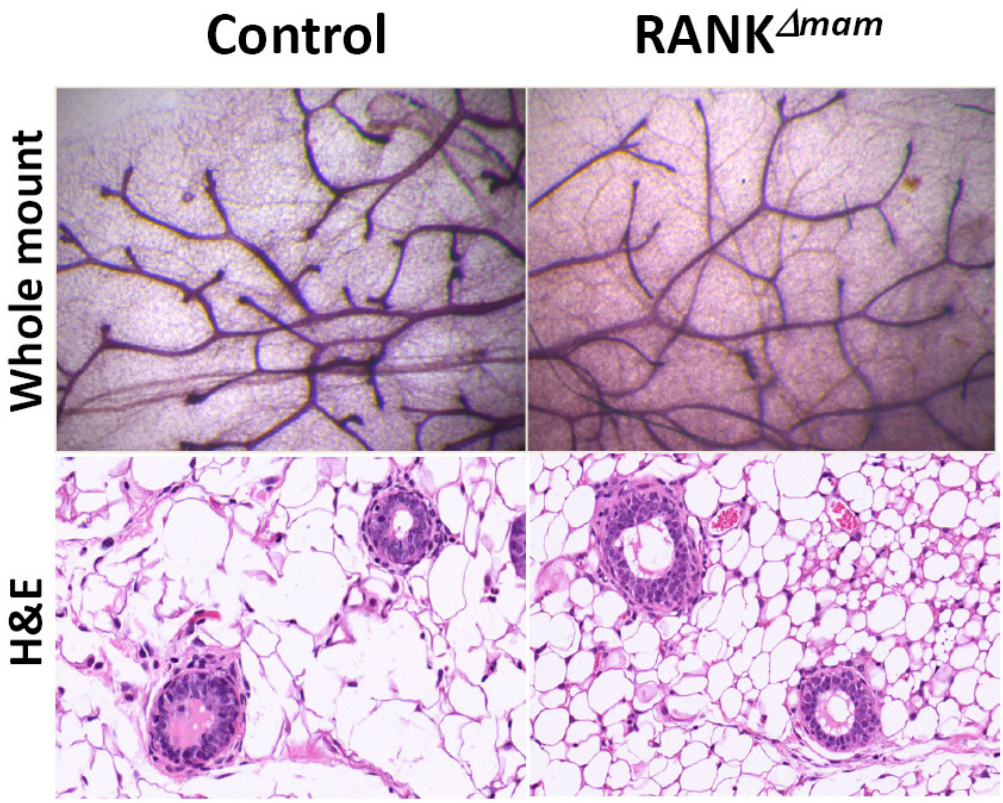
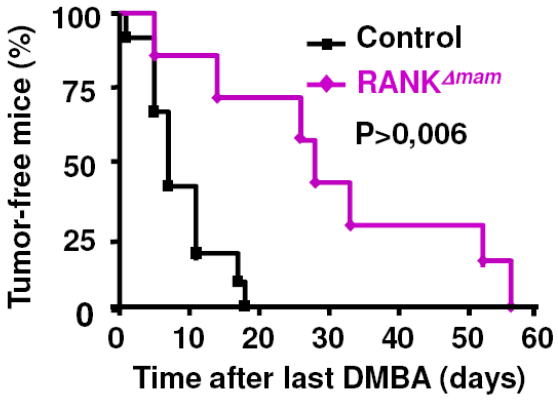



**Figure 8. Generation of RANK$^{\Delta mam}$ knockout mice. a, Gene targeting strategy for the generation of the conditional RANK mutant allele. Breeding with MMTV-*Cre* mice specifically induces deletion of RANK exons 3 and 4 in mammary epithelial cells. Genotyping primers are indicated as red arrowheads (P1, P2 and P3). Primers P1 and P2 detect the wild type allele and the floxed allele, respectively. Primers P1 and P3 detect the knockout (KO) allele. b, Nulliparous RANK$^{\Delta mam}$ female mice exhibit normal mammary gland development. Whole mount staining (upper panels) and H&E analysis (lower panels) of mammary glands of control and RANK$^{\Delta mam}$ females showing normal ductal side branching and apparently normal ductal/epithelial structures.**

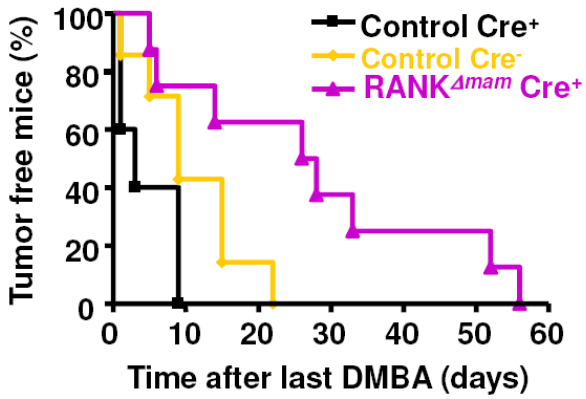
## 2.5. Tissue specific inactivation of RANK in mammary epithelial cells results in a delayed onset of primary breast cancer

We next induced progestin-driven breast cancer in 6 week old RANK$^{\Delta mam}$ females and littermate controls according to the scheme described in Figure 5a. Control mice rapidly developed mammary tumors in response to MPA/DMBA treatment. Intriguingly, RANK$^{\Delta mam}$ female mice exhibited a marked delay in the onset of MPA/DMBA-induced palpable mammary tumors (Figure 9a). Only one RANK$^{\Delta mam}$ female exhibited a palpable tumor at an early time point when all control mice had already developed tumors. The median time until the first detection of palpable tumors was 7 days after last DMBA treatment in control mice and 27 days in RANK$^{\Delta mam}$ females. Of note, the tumor onset was comparable between MMTV-*cre*-positive and MMTV-*cre*-negative control mice (Figure 9b). Histological analysis revealed that most of the occurring tumors in both RANK$^{\Delta mam}$ and littermate controls are typical ductal adenocarcinomas (Figure 9c). The absence of RANK in tumors that developed in RANK$^{\Delta mam}$ females was confirmed by Southern blotting (Figure 9d). Some residual wild type bands which we could still observe may be explained by the presence of other cell types like stromal cells and infiltrates and/or escaper cells. These data show that deletion of RANK in mammary epithelial cells significantly delays the onset of progestin-driven breast cancer.

**a**




**b**



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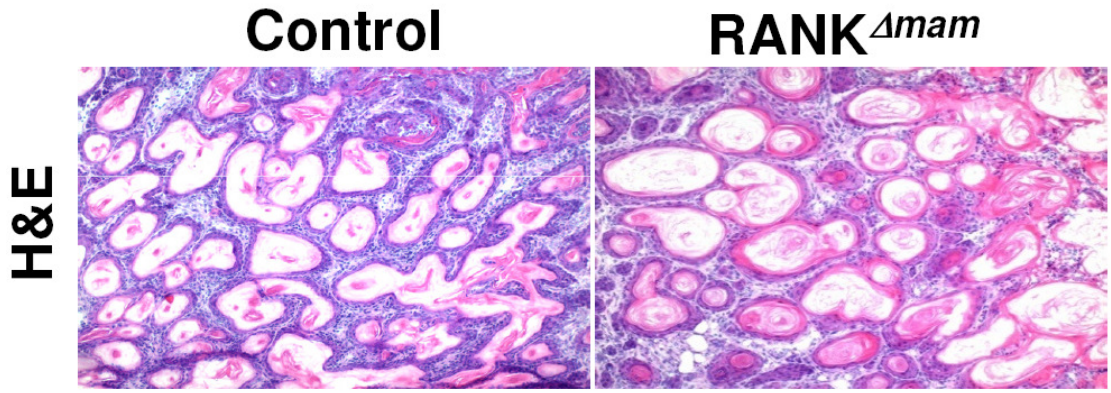



d

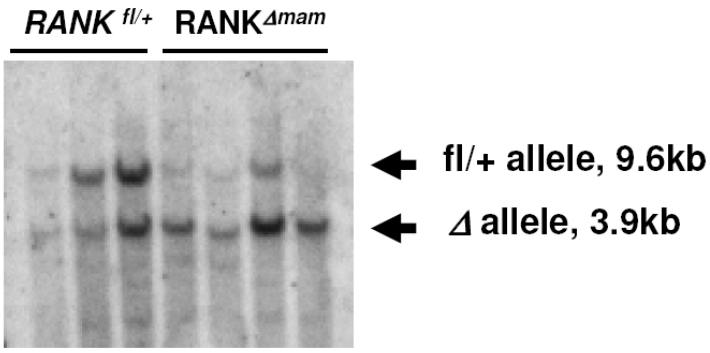



**Figure 9. RANK controls the incidence and onset of progestin-driven mammary cancer. a, RANK$^{\Delta mam}$ females (n=8) exhibited a markedly delayed onset of mammary tumors in response to MPA/DMBA callenge compared to age-matched littermate control females (n=12). b, Tumor onset in response to MPA/DMBA challenge is comparable between *cre*-positve (n=7) and *cre*-negative (n=5) control mice. Mice in a, and b, were treated with MPA pellets and DMBA as indicated in Figure 5a. Mammary tumor onset was determined by palpation. Data are shown as percentage of tumor free mice after the last DMBA challenge. c, H&E staining of representative histological sections of mammary tumors isolated from control littermate and RANK$^{\Delta mam}$ females 56 days after the last DMBA treatment showed typical features of adenocarcinomas. d, Southern blot analysis of mammary tumors derived from RANK$^{\Delta mam}$ and control females for confirmation of deletion in tumors. Genomic DNA was digested with BamHI and SphI. The floxed/+ and the deleted (Δ) alleles are indicated by arrowheads.**

### 2.6. The numbers of carcinomas *in situ* and invasive carcinomas is decreased in RANK$^{\Delta mam}$ females

Further analysis of mammary tumor incidence in response to MPA/DMBA at early stages emphasized the critical role of RANK/RANKL in breast cancer onset. All RANK expressing control females sacrificed only seven days after the last DMBA treatment already exhibited multiple highly proliferative *in situ* carcinomas and even invasive mammary tumors. By contrast, in RANK$^{\Delta mam}$ we detected only very few carcinomas *in situ* and never any invasive tumor one week after the last DMBA challenge (Figure 10a). Three weeks after last DMBA treatment the number of carcinomas *in situ* was similar between RANK$^{\Delta mam}$ females and control littermates however the incidence of invasive carcinomas in controls was significantly increased in the littermate controls (Figure 10b). Thus, the development of carcinoma in situ and invasive carcinomas in response to MPA/DMBA is delayed in RANK$^{\Delta mam}$ females.

**a** **b**

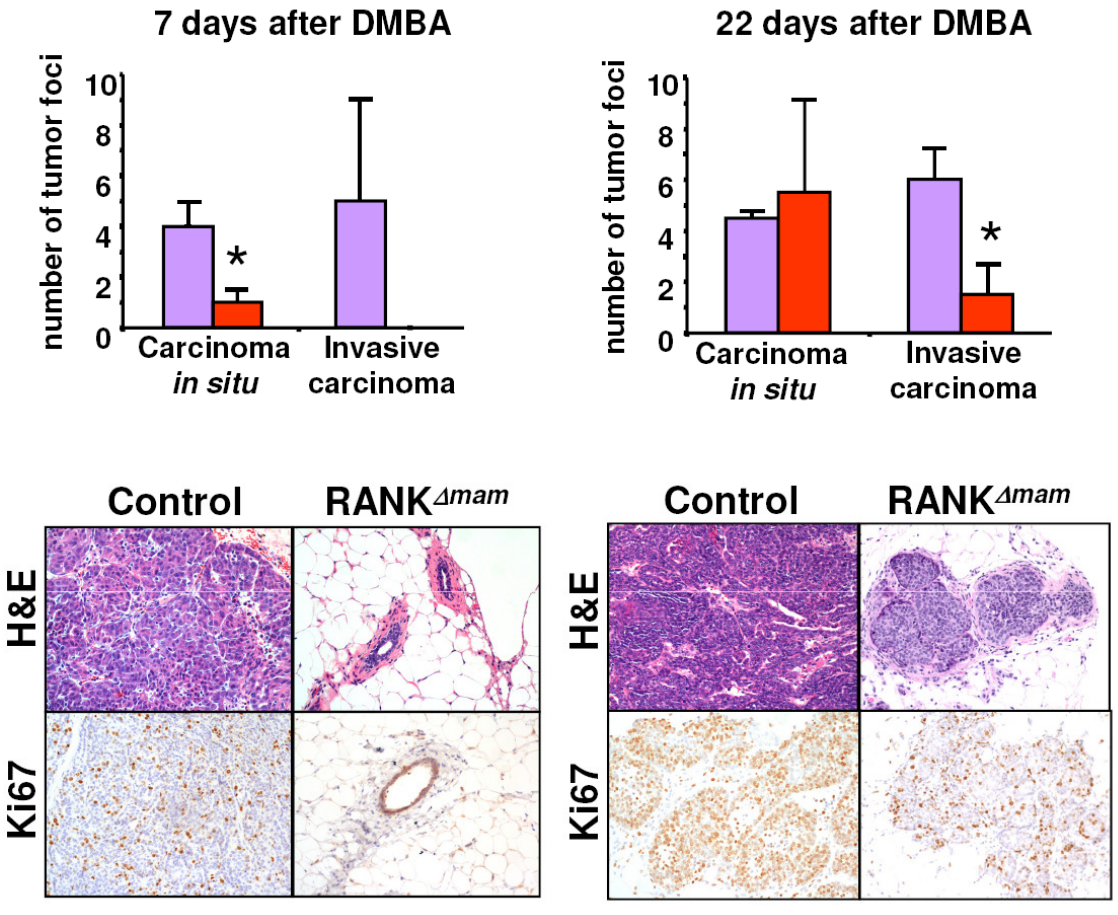
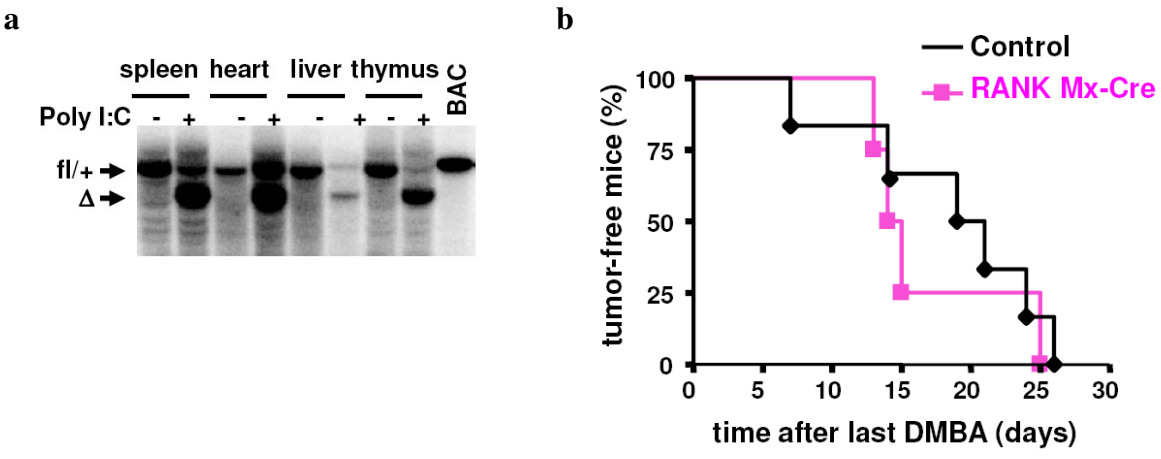


**Figure 10. Incidence of carcinomas in situ and invasive carcinomas is reduced in RANK$^{\Delta mam}$ females 7 and 22 days after final DMBA treatment. a and b, Numbers of carcinomas *in situ* and invasive mammary cancers in control and RANK$^{\Delta mam}$ females on day 7 (a) and day 22 (b) after the final DMBA treatment. Data are shown as mean values per mouse +/- sem (n= 3 mice per genotype). All 10 mammary glands were analysed for each mouse. * P > 0.05 (Student's t-test). The upper panels show H&E staining of representative histological sections. The lower panels represent immunostaining for the proliferation marker Ki67. Control females display typical invasive adenocarcinomas on days 7 and 22. For RANK$^{\Delta mam}$ female mice, normal acinar morphology (day 7) and a carcinoma in situ (day 22) are shown.**

## 2.7. Neither RANK Mx-*Cre* nor RANK$^{\Delta mam}$ NeuT-positve female mice display a delayed onset of breast cancer

It is known that the MMTV-*cre* transgene is not only expressed and active in mammary epithelial cells but also in other secretory organs as well as in B cells and T cells and in the female germline (Wagner et al., 2001). To rule out any contributions of deletion in other organs of RANK$^{\Delta mam}$ mice, we next examined MPA/DMBA-induced tumor onset in RANK Mx-*cre* mice. Importantly, Mx-*cre* mediated deletion of RANK in liver, heart, muscle and the haematopoietic system (Figure 11a) did not delay tumor onset (Figure 11b). This suggests that cell-autonomous RANK/RANKL signaling is critical for early events of malignant transformation in mammary epithelial cells.

Furthermore, one of the major genetic alternations observed in human mammary tumors is the mutation of the human epidermal growth factor 2 (ErbB2) which is also known under the acronyms Neu and Her2 (Ursini-Siegel et al., 2007). ErbB2-dependent breast cancer can be modeled with transgenic mice overexpressing an activated form of the Neu-oncogene (Ursini-Siegel et al., 2007). In order to investigate the role of RANK and RANKL in this genetical mammary tumor model we crossed our RANK$^{floxed}$ mice to a transgenic NeuT line. Interestingly, MMTV-driven deletion of RANK did not alter tumor incidence in the NeuT-positive female mice (Figure 11c). The deletion of RANK in tumors derived from RANK$^{\Delta mam}$ NeuT-positive female mice was confirmed by Southern blotting (Figure 11d). Thus these results indicate that RANK/RANKL is a critical mediator of progestin-induced breast cancer *in vivo*.



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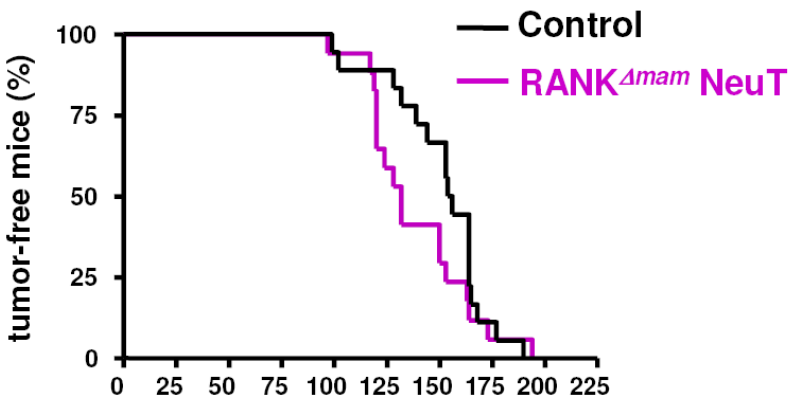


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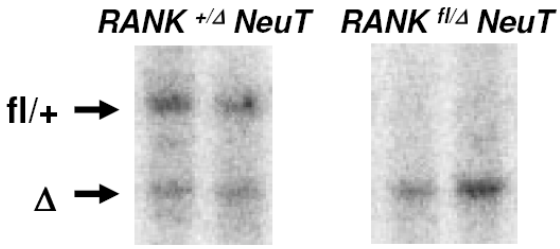
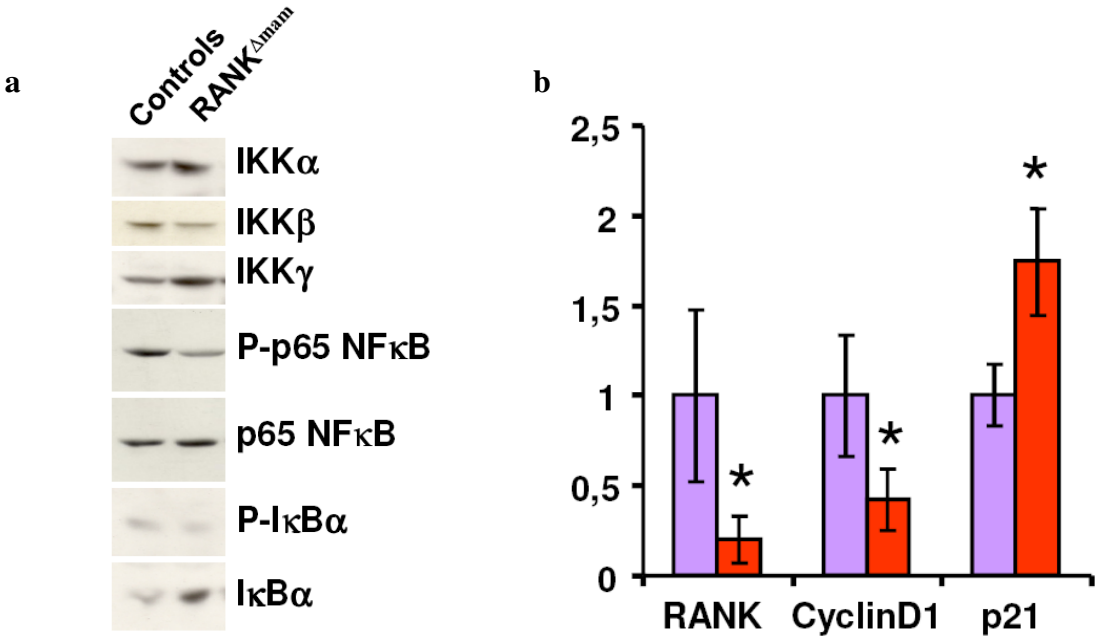



**Figure 11. Neither RANK Mx-*Cre* nor RANK$^{\Delta mam}$ NeuT-positive female mice display a delayed onset of breast cancer. a, Southern blot analysis on various tissues of the RANK$^{floxed}$ allele before (fl/+) and after Mx-*Cre*-induced deletion (fl/Δ). Mx-*Cre* mediated deletion was induced by 4 consecutive injections of poly I:C over the course of 8 days. Genomic DNA was digested with BamHI and Sph1. Floxed/+ and delta (*Δ*) alleles are indicated with arrowheads. A BAC vector was loaded as a control for the floxed and + alleles. b, Survival of Mx-*Cre* RANK$^{fl/\Delta}$ females (n=4) and age-matched littermate controls (n=6) after MPA/DMBA challenge as indicated in Figure 5a. Data are shown as percentage of tumor free mice after last DMBA treatment. c, Onset of palpable tumors in RANK$^{\Delta mam}$ NeuT-positive females and littermate controls. Data are shown as percent of tumor free mice after birth. d, Southern blot analysis of tumors derived from RANK$^{\Delta mam}$ NeuT-positive female mice (n=13) and RANK$^{+/\Delta}$ NeuT-positive controls (n=14) confirming RANK deletion. Genomic DNA was digested with BamHI and Sph1. Floxed/+ and delta (*Δ*) alleles are indicated with arrowheads.**

## 2.8. RANK/RANKL activate the NFκB pathway in primary mammary epithelial and breast cancer cells

Previously it has been shown that RANK/RANKL signals proliferation and differentiation in the mammary gland via the the Id2 pathway (Kim et al., 2006) and the IKKα – NFκB – CyclinD1 axis (Cao et al., 2001). To analyze the activation status of the Id2 and the NFκB pathways in MPA/DMBA-induced mammary adenocarcinomas we pooled tumors derived from RANK$^{\Delta mam}$ females and control littermates, respectively. By Western blotting we found that tumors which grew out in RANK$^{\Delta mam}$ female mice exhibited impaired NFκB signaling indicated by increased protein levels of the inhibitor of NFκB, IκB, decreased p65 NFκB phosphorylation (Figure 12a) and reduced mRNA levels of the NFκB target Cyclin D1. Moreover, we could also detect an increase of p21 mRNA in RANK$^{\Delta mam}$ tumors (Figure 12b). p21 is a negative regulator of cell cycle progression (Gartel et al., 1996) being transcriptionally suppressed by the Id2 pathway (Kim et al., 2006). Furthermore, RANKL stimulation of primary mouse mammary epithelial cells also resulted in upregulation of NFκB signaling as indicated by elevated p65 NFκB and IkBα phosphorylation (Figure 12c). We also observed induction of the NFκB pathway in the human breast tumor cell line SKB3 in response to RANKL stimulation (Figure 12d). Thus, RANK/RANKL induces NFκB as well as Id2 signaling in normal and transformed mammary epithelial cells.



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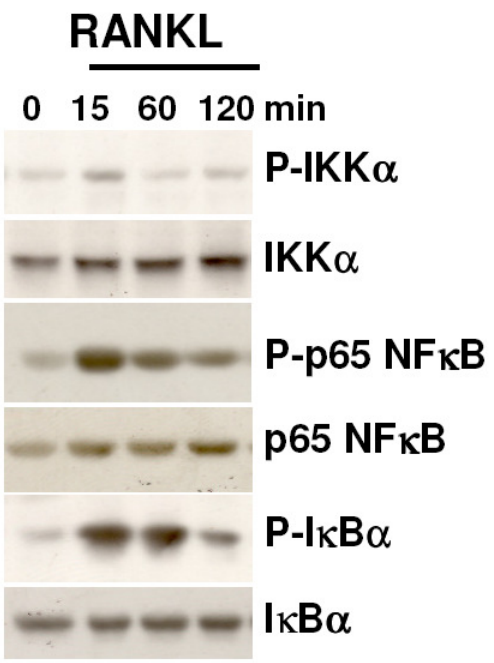


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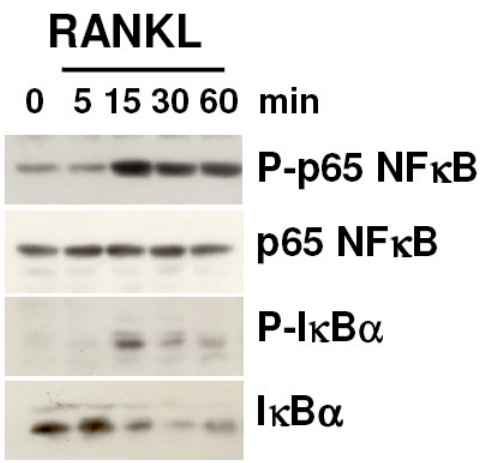
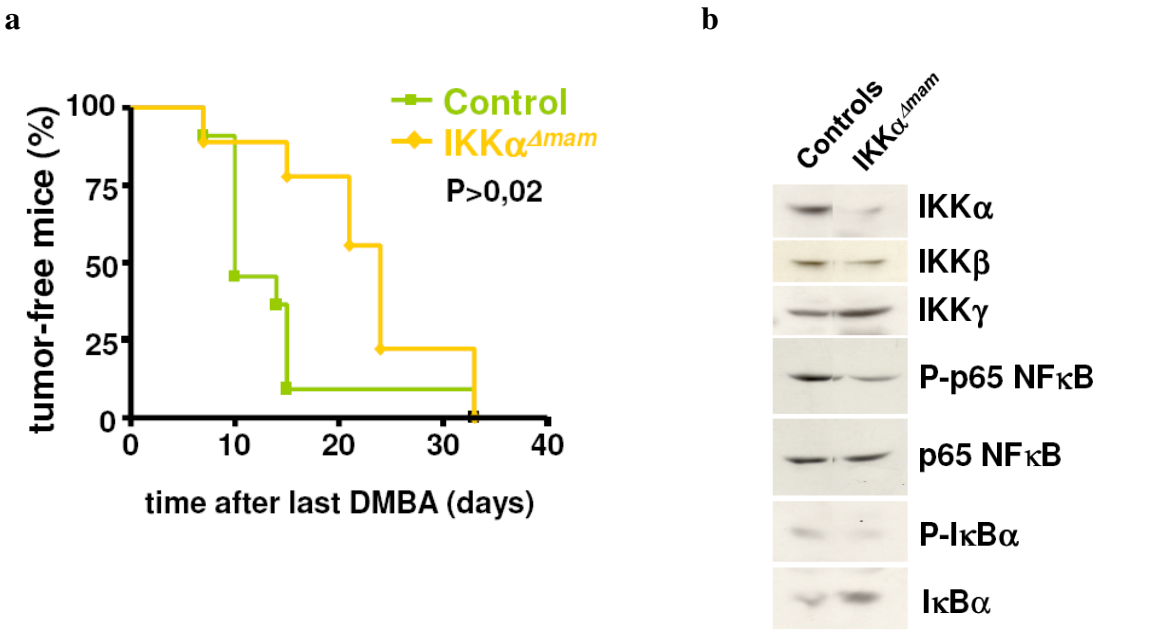



**Figure 12. RANK/RANKL activates the NFκB pathway in mammary epithelial cells and breast cancer cells. a, NFκB pathway activation status in pooled late stage tumors derived from RANK$^{\Delta mam}$ females and control littermates. Tumors isolated from RANK$^{\Delta mam}$ females (n=4) and tumors from control mice (n=4) were pooled and levels of IKKα, β, γ, phosphorylated (P) p65 NFκB, total p65 NFκB, phosphorylated (P) IκBα and total IκBα were assessed by Western blotting. b, Relative expression levels of RANK, CyclinD1 and p21 were determined by qRT-PCR on total mRNA isolated from tumors of RANK$^{\Delta mam}$ females and control littermates. Data are shown as mean values +/- sem (n=4 for each genotype). * P > 0.05 (Student's t-test). c, Western blotting on total protein extracts of mammary epithelial cells for phosphorylated (P) IKKα, total IKKα, phosphorylated (P) p65 NFκB, total p65 NFκB, phosphorylated (P) IκBα and total IκBα untreated (0 min) and in response to RANKL stimulation (1 g/ml) after 15, 60 and 120 minutes. d, Western blotting for phosphorylated (P) p65 NFκB, total p65 NFκB, phoshphorylated (P) IκBα and total IκBα in untreated (0 min) SKB3 human breast cancer cells and SKB3 cells in response to RANKL stimulation (1 g/ml) after 5, 15, 30 and 60 minutes.**

## 2.9. Mammary epithelial cell specific inactivation of IKKα but not TRAF6 and NFATc1 delays the onset of primary breast cancer

RANK/RANKL induced osteoclast differentiation and activation essentially requires NFκB signaling (Franzoso et al., 1997; Iotsova et al., 1997) and TRAF6 which is the major adaptor molecule for NFκB activation downstream of RANK/RANKL (Darnay et al., 1998; Darnay et al., 1999). Moreover, RANK/RANKL mediated osteoclastogenesis also requires the calcineurin-NFATc1 signaling pathway (Takayanagi et al., 2002a). Importantly, NFATc1 can also be regulated by the Id2 pathway (Lee et al., 2006). To assess whether these key-downstream pathways of RANK/RANKL are also important in tumor onset of progestin-driven mammary tumors we generated MMTV-Cre *Nfatc1*$^{floxed/floxed}$ (NFATc1$^{\Delta mam}$), Traf6$^{floxed/floxed}$ (TRAF6$^{\Delta mam}$) and *Ikkα*$^{floxed/floxed}$ (IKKα$^{\Delta mam}$) conditional knockout mice in which NFATc1, TRAF6 and IKKα are specifically deleted in mammary epithelial cells. All mutant mouse lines were born healthy. Interestingly, IKKα$^{\Delta mam}$ mice exhibited a markedly delayed onset of mammary tumors in response to MPA/DMBA treatment (Figure 13a). The median time of palpable mammary tumor onset in littermate controls was 10 days and 24 days in IKKα$^{\Delta mam}$ mutant mice. Deletion of IKKα in tumors derived from IKKα$^{\Delta mam}$ mice was confirmed by Western blotting (Figure 13b). Analysis of pooled tumors from IKKα$^{\Delta mam}$ mice confirmed downregulation of the NFκB pathway. The expression of IKKβ and IKKγ is not changed (Figure 13b). Of note, our results are supported by the previous finding that mutation of the IKKα kinase domain in IKKα$^{A/A}$ knock-in mice results in a delayed onset in both the MPA/DMBA and NeuT mammary cancer models (Cao et al., 2007). Surprisingly, neither TRAF6$^{\Delta mam}$ nor NFATc1$^{\Delta mam}$ mutant female mice exhibited delayed onset of mammary tumors in response to MPA/DMBA (Figure 13c, d). These results indicate that RANK/RANKL in mammary glands controls the onset of mammary tumors in mice at least in part by activating the NFκB pathway.



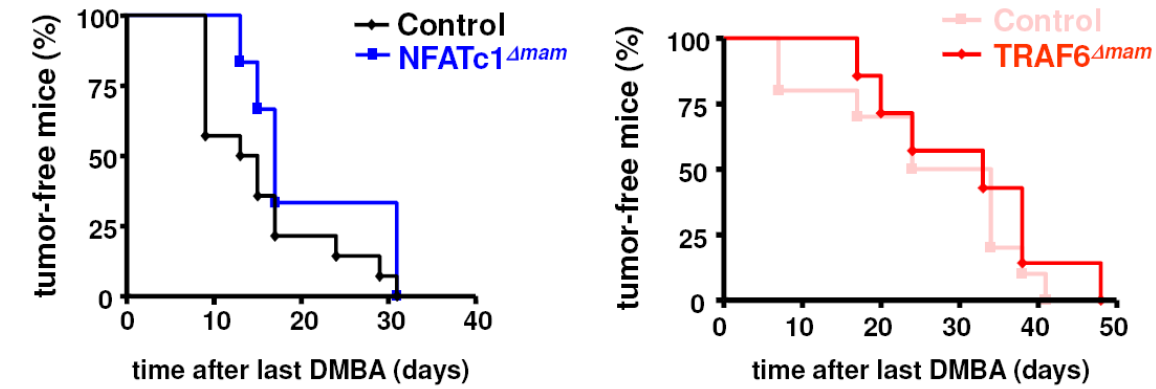



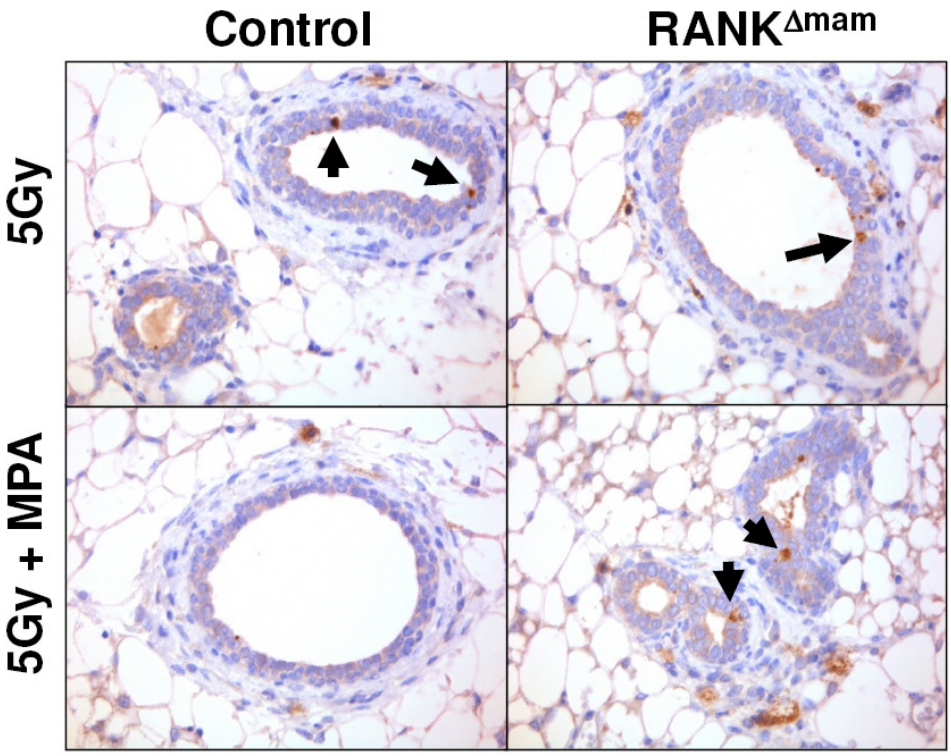

**Figure 13. Deletion of IKKα in mammary epithelial cells delays the onset of mammary tumors in response to MPA/DMBA. a, c, and d, Onset of palpable mammary tumors in IKKα$^{\Delta mam}$ (a), NFATc1$^{\Delta mam}$ (c) and TRAF6$^{\Delta mam}$ females (d) and age-matched littermate controls in response to MPA/DMBA treatment as indicated in Figure 5a. Data are shown as percentage of tumor free mice after last DMBA treatment. (IKKα$^{\Delta mam}$ n=9, IKKα control n=11; TRAF6$^{\Delta mam}$ n=7, TRAF6 control n=10; NFATc1$^{\Delta mam}$ n=6, NFATc1 control n=14) b, Western blotting for IKKα, β, γ, phosphorylated (P) p65 NFκB, total p65 NFκB, phosphorylated (P) IκBα and total IκBα on pooled tumors isolated from IKKα$^{\Delta mam}$ and control females, n=4 mice per genotype.**

## 2.10. RANK/RANKL protects mammary epithelial cells form γ-irradiation induced cell death

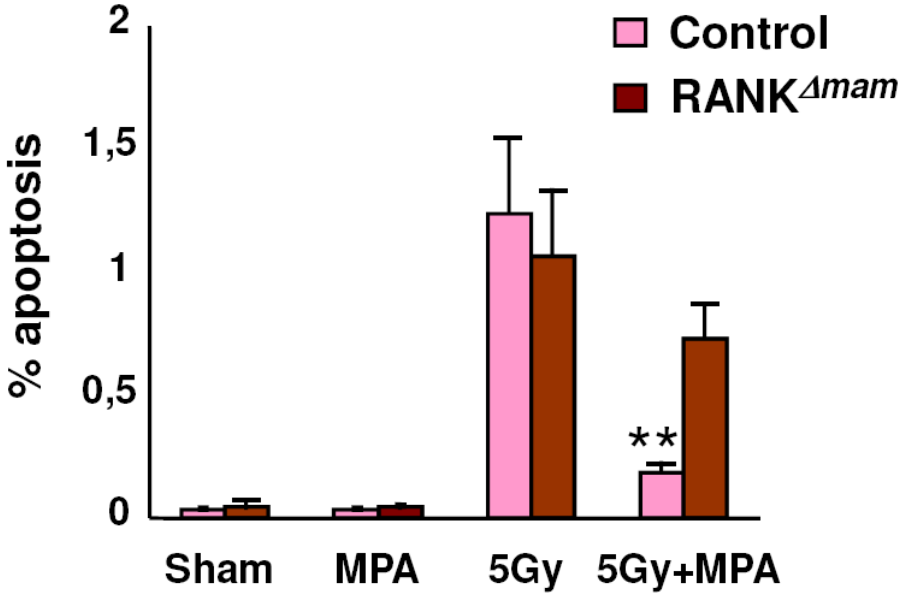
Apoptosis in response to genotoxic stress is essential for the survival of an organism (Jackson and Bartek, 2009). A failure in activating the death machinery upon severe DNA damage has fatal consequences and provides a prerequisite for the initiation of cancer (Hanahan and Weinberg, 2000). The DNA damaging agent DMBA which we used in our progestin-driven cancer model induces genotoxic stress by randomly introducing a large number of point mutations (Aldaz et al., 1996). As RANK$^{\Delta mam}$ female mice exhibited a markedly delay in tumor onset in response to MPA/DMBA challenge we were wondering whether RANK/RANKL also protects mammary epithelial cells from genotoxic stress-induced programmed cell death. Thus, we performed subcutanous implantation of MPA pellets in RANK$^{\Delta mam}$ females and control mice in order to stimulate RANKL expression. Using active caspase3 as a marker for cell death (Mazumder et al., 2008) we then analyzed γ-irradiation induced apoptosis in the mammary glands *in vivo*. γ-irradiation triggered a marked increase of apoptotic mammary epithelial cells in both RANK$^{\Delta mam}$ and control females. Intriguingly, γ-irradiation after three days of MPA-stimulation resulted in a significantly reduced number of apoptotic mammary epithelial cells in control females compared to MPA-treated RANK$^{\Delta mam}$ mice (Figures 14a, b). Therefore we conclude that MPA markedly protects from γ-irradiation-induced cell death dependent on RANKL expression.

**Figure 14. MPA-induced RANKL expression protects mammary epithelial cells of γ-irradiation induced cell death. a, Immunostaining of mammary glands sections with the apoptotic marker active caspase3. Arrows indicate apoptotic mammary epithelial cells in sham-operated (upper panels) and MPA treated (lower panels) RANK$^{\Delta mam}$ females and littermate controls isolated 6 hours after γ-irradiation (5 Gray). b, Quantification of active caspase3 positive nuclei of mammary epithelial cells of sham-operated, MPA-treated, γ-irradiated and MPA-treated plus γ-irradiated RANK$^{\Delta mam}$ females and littermate controls 6 hours after γ-irradiation. A minimum of 5000 nuclei was counted for each mouse. Results shown are mean values +/- sem, n = 3 mice per group. * P > 0.05; ** P > 0.02 (Student's t-test).**

**a**


Control
RANKΔmam
5Gy
5Gy + MPA

**b**


Control
RANKΔmam
% apoptosis
2
1,5
1
0,5
0
**
Sham
MPA
5Gy
5Gy+MPA

## 2.11. Generation and characterization of MMTV-RIC mice

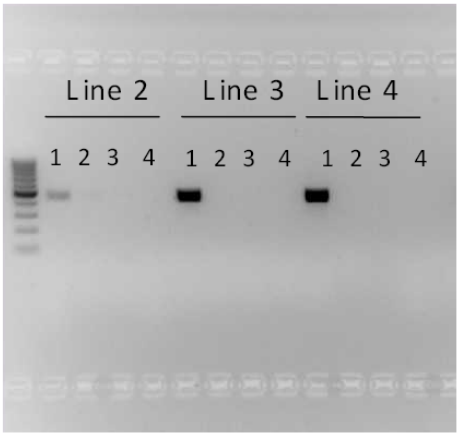
Mice overexpressing RANK specifically in mammary epithelial cells display a severe lactation defect due to impaired lobuloalevolar differentiation during pregnancy (Gonzalez-Suarez et al., 2007). Moreover, *in vitro* culture of mammary epithelial cells isolated from these mice hyperproliferate in response to RANKL stimulation (Gonzalez-Suarez et al., 2007). Now we report, that deletion of RANK in mammary epithelial cells of female mice results in a significant delay of mammary tumor onset in response to MPA/DMBA. Taken together, all these results clearly emphasize a critical role for RANK/RANKL in hormone driven mammary carcinogenesis. Therefore we decided to generate new transgenic mouse lines which overexpress RANK and *cre*-recombinase under the control of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) (MMTV-RIC-Ires-CRE) (Figure 15a) in order to further dissect the RANK/RANKL downstream signaling pathways in mammary tumorigenesis.

In total 6 founder lines harboring the MMTV-RANK-Ires-CRE transgene were generated by pronuclear injection. Ires is an internal risbome entry site (Ires) which allows the 5'-cap independent initiation of *Cre*-expression (Hellen and Sarnow, 2001). These mice will hereafter be termed as MMTV-RIC lines 1 to 6. 5 out of the 6 MMTV-RIC lines successfully transmitted the transgene to their progeny, thus confirming germline transmission. In order to verify transgene expression we isolated total RNA of mammary epithelial cells from offsprings of lines 2, 3 and 4. All lines analyzed express the MMTV-RIC mRNA as shown by Cre-PCR on total cDNA isolated from mammary epithelial cells (Figure 15b). Furthermore, we confirmed by qRT-PCR that carriers of line 3 express the highest levels of the transgene in isolated mammary epithelial cells (Figure 15c). We next determined the tissue specificity of transgene expression. qRT-PCR revealed that high levels of transgene mRNA are expressed in mammary epithelial cells, spleen, liver and lung while moderate MMTV-RIC expression was detected in the ovaries. Surprisingly, in line 3 the thymus was the site of highest transgene mRNA expression (Figure 15d).

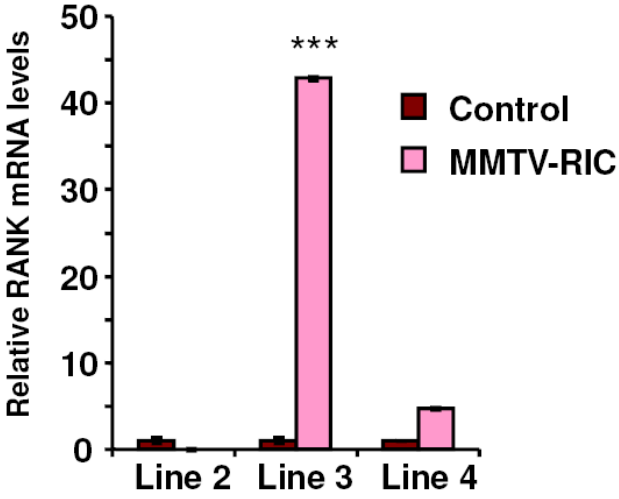
**a**


MMTV
RANK
CRE
IRES
SV40 pA

**b**


Line 2
Line 3
Line 4
1 2 3 4 1 2 3 4 1 2 3 4

**c**


Relative RANK mRNA levels
50
40
30
20
10
0
***
Control
MMTV-RIC
Line 2
Line 3
Line 4

**d**

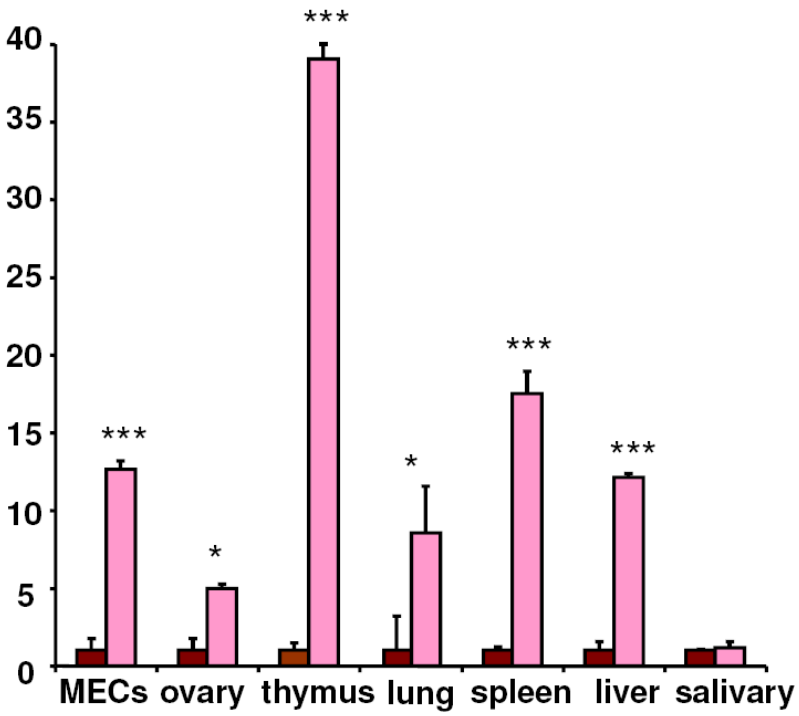
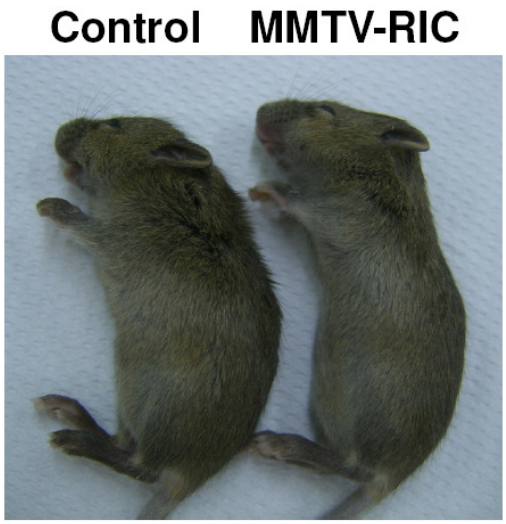

40
35
30
25
20
15
10
5
0
***
*
***
*
***
***
MECs
ovary
thymus
lung
spleen
liver
salivary

**Figure 15. Generation of MMTV-RIC transgenic mice. a, Schematic of the linearized MMTV-RANK-Ires-Cre construct used for pronuclear injection. b, PCR on total mRNA isolated from mammary epithelial cells of offsprings from MMTV-RIC lines 2, 3 and 4. Lane 1 = cDNA MMTV-RIC. Lane 2 = non RT-control MMTV-RIC. Lane 3 = cDNA control. Lane 4 = non RT control. c, Quantification of mammary epithelial cell specific MMTV-RIC transgene expression in offsprings from lines 2, 3, and 4. Expression levels were determined by qRT-PCR on total mRNA isolated from mammary epithelial cells. Data are shown relative to endogenous RANK levels in control mice, n=2 mice per group. d, RANK qRT-PCR was conducted on total mRNA isolated from multiple tissues obtained from females of line 3. Note the high level of transgene expression in the thymus. n=3 mice per group. In c, and d, Primers binding endogenous and transgenic RANK mRNA were used. Data were normalized to b-actin and are shown relative to endogenous RANK levels of age-matched control mice, mean values +/- sem. * P > 0,05; ** P > 0.005; *** P > 0.001 (Student`s t-test)**

The offsprings of all MMTV-RIC mice are born healthy and do not display any overt defects (Figure 16a). Whole mount staining of mammary glands isolated from nulliparous female mice revealed that line 3 carriers display a less dense ductal network compared to age-matched littermate controls, which needs to be further analyzed. However, we could not observe a difference between nulliparous mammary glands of the progeny from MMTV-RIC lines 2 and 4 and control littermates. (Figure 16b). None of the five founder lines exhibited an obvious lactation defect even after several rounds of pregnancy.

We are currently crossing these transgenic mice to mice with conditionally targeted alleles of TRAF6, NFATc1 and IKKα. Since RANK expression is coupled to *Cre*-recombinase activity we are able to overexpress RANK and simultaneously delete one of these putative downstream molecules. Afterwards these mice will then be challenged with MPA/DMBA to trigger breast cancer. Thus, the MMTV-RIC mice will be very useful for understanding the complexity of RANK/RANKL downstream signaling in hormone-driven breast cancer in more detail.

**a**




**b**

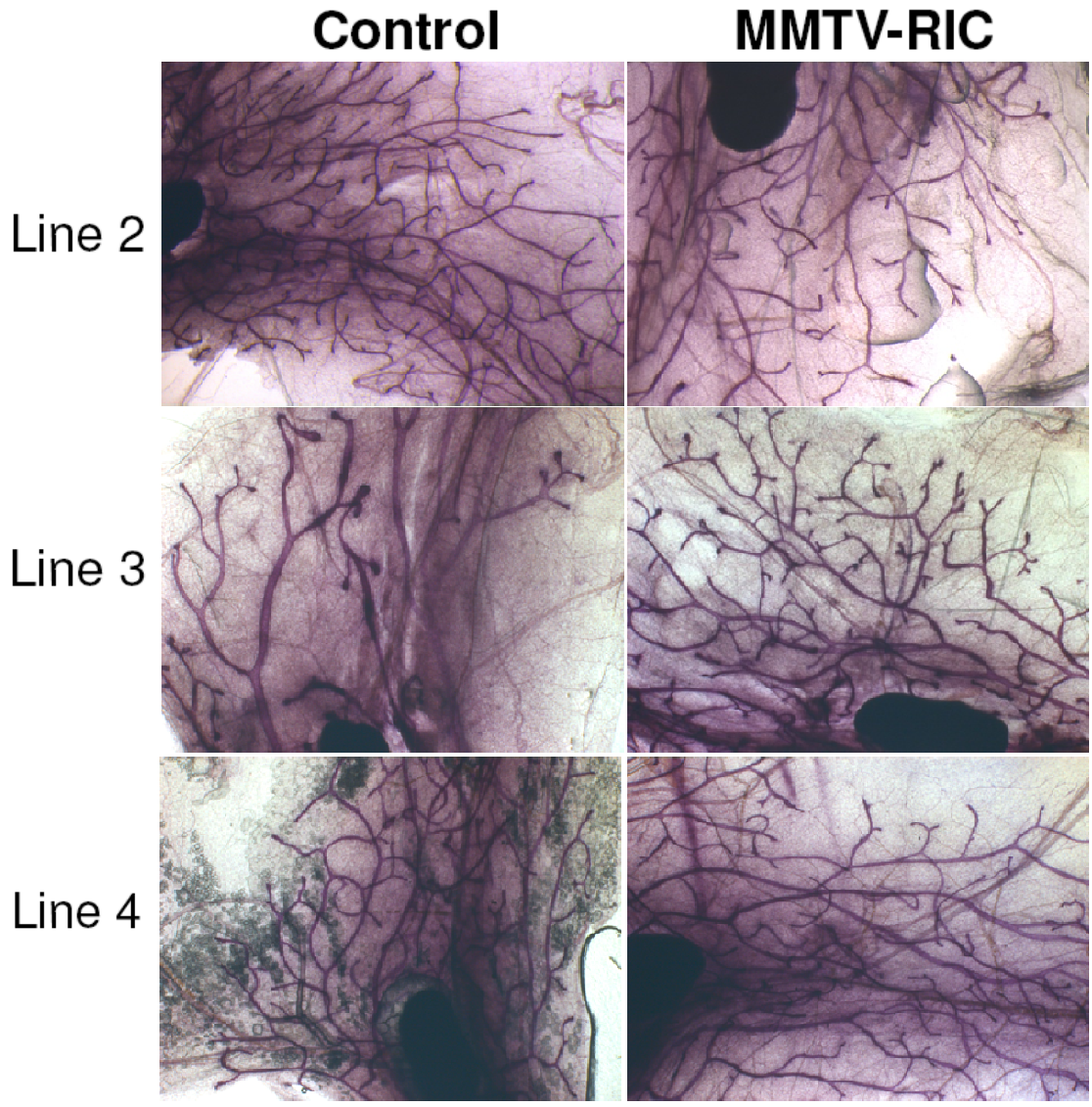


**Figure 16. a, Female offspring of MMTV-RIC Line 2 are shown at the age of 10 days. b, Whole mount staining of mammary glands isolated from 6 week old nulliparous offsprings of MMTV-RIC Lines 2, 3 and 4. Ductal sidebranching and expansion in the fat pad is comparable between transgenic females of Lines 2 and 4 and age-matched littermate controls. Line 3 nulliparous females display decreased expansion of the epithelial ducts in the fat pad.**

All experiments shown here were done by me, except the following: Andreas Leibbrandt generated the $RANK^{floxed}$ mice and carried out the RANK-NeuT experiment. The Progesterone receptor knockout experiment was carried out by Heather Lee (Garvan Institute, Australia) and Daniel Schramek. Daniel Schramek did all the Western Blots and cell culture experiments and helped with qRT-PCR, the γ-irradiation experiment and the cloning of the MMTV-RIC construct. Histological pictures of tumors were taken by Lukas Kenner. Lukas Kenner also did the quantification of Carcinomas *in situ* and Invasive carcinomas.

## 3. Discussion

Initially RANKL and its receptor RANK were identified as key regulators of osteoclastogenesis (Kong et al., 1999b). RANK/RANKL signaling is not only of utmost importance for bone metabolism but also crucial in pathologic bone remodeling (Leibbrandt and Penninger, 2008). Osteoporosis is one of the diseases which is caused by unbalanced RANK/RANKL activity. Especially postmenopausal women who have stopped estrogen production in the ovaries are prone to develop osteoporosis, thus indicating that RANK/RANKL is strongly influenced by sex hormones (Leibbrandt and Penninger, 2008). Surprisingly RANK/RANKL signaling also turned out to be required for the development of a lactating mammary gland during pregnancy (Fata et al., 2000). By activating the NFκB (Cao et al., 2001) and Id2 (Kim et al., 2006) signaling pathways, RANK and RANKL induce the differentiation of terminal endbuds to milk-secreting loculoalveolar structures (Fata et al., 2000).

Mammalian evolution may provide a rational reason for linking bone metabolism to mammary gland development. The formation of a lactating mammary gland allows the mother to provide the offspring with calcium rich milk. Essentially, the mothers' milk is the main calcium resource for newborns and a lack of calcium has severe consequences including rickets and heart and brain defects (Theill et al., 2002). To meet the increased calcium demand lactating females mobilize internal calcium resources by demineralization of their skeletons via the activation of osteoclasts (Cross et al., 1995). Thus, around 200 million years ago when the first mammals appeared RANK/RANKL might have overtaken the role of a common master regulator for bone turnover and pregnancy dependent mammary gland development (Theill et al., 2002). Recently Hanada et al. reported that hormone regulated RANK/RANKL signaling controls female body temperature, a new function which might explain hot flashes in older women undergoing hormonal change (Hanada et al., 2009). Thus, it appears that the master regulators of bone metabolism are embedded in the tight regulatory network of female sex hormones and reproduction.

Intriguingly, we now could identify RANK/RANKL signaling as a critical mediator of hormone dependent breast cancer. Assessing the role of RANK/RANKL in the context of MPA/DMBA-driven breast cancer we observed that mice lacking RANK, the receptor of RANKL, in mammary epithelial cells developed mammary tumors markedly later than control littermates. Thus, the deletion of RANK significantly

protects female mice from hormone-induced breast cancer. Mechanistically, our data suggest that the progestin derivative MPA triggers massive RANKL expression in the mammary gland. Importantly, RANKL expression requires also the expression of the prolactin receptor which is already known to critically drive breast cancer (Llovera et al., 2000; Oakes et al., 2007). As a consequence of elevated RANKL levels mammary epithelial cells enter the cell cycle resulting in markedly increased proliferation rates. Moreover, RANKL appears to protect mammary epithelial cells from γ-irradiation induced apoptosis. Since proliferation and evading programmed cell death are hallmarks of malignant tissue transformation (Hanahan and Weinberg, 2000), our results suggest that RANK/RANKL provides mammary epithelial cells with these prerequisites for the development of hormone-driven breast cancer. Importantly, clinical data also confirm the implication of RANK/RANKL in breast cancer in humans. Analysis of serum taken from women over many years revealed that the manifestation of breast cancer but not of other cancers can be associated with elevated OPG levels. Simultaneously, soluble RANKL levels in most individuals tested declined with the manifestation of breast cancer. Thus, according to these data in a prospective human cohort altered OPG-to-sRANKL levels correlates with new-onset breast cancer.

There a many questions which still remain to be answered. So far it appears that RANK/RANKL signals via the NFκB pathway in MPA/DMBA-induced carcinogenesis. Indeed this mechanism seems plausible since NFκB can induce the expression of the cell cycle promoter Cyclin D1 (Cao et al., 2001). However, we cannot exclude the contribution of other downstream targets in RANK/RANKL controlled mammary tumorigenesis. Therefore the MMTV-RIC transgenic mice which we generated should be very helpful to better understand the complexity of RANK/RANKL downstream signaling pathways. Moreover, additional experiments are required to define the role of hormonal pathways in RANK/RANKL signaling in more detail.

Interestingly, high estrogen levels as well as enhanced local production of estrogens are considered as critical primary mediators of breast cancer development in obese postmenopausal women (Cleary and Grossmann, 2009). Therefore it would be very interesting to test whether RANK/RANKL can also be associated with mammary tumors resulting from increased body weight. Another intriguing aspect of RANK/RANKL signaling in breast cancer is the following: micro-calcifications and anormal tissue density constitute two of the most important markers for early

mammographic diagnosis of malignant lesions of the breasts (Mortier et al., 1997). Importantly, our data indicate that RANK and RANKL are involved in early events of breast carcinogenesis including hyperplasia. Moreover, radiographic mammary calcifications occur in 30-50 % of all breast cancer cases (Morgan et al., 2005). Since RANK/RANKL control bone mineralization and early events in malign transformations of the mammary tissue we predict a reasonable connection between RANK/RANKL and micro-calcifications in mammary glands. Although we could not observe a difference in mammary tumor onset in NFATc1$^{\Delta mam}$ and TRAF6$^{\Delta mam}$ mice it might be worth testing whether these signaling pathways could be involved in microcalifications of the breasts.

In conclusion we have uncovered a major and novel pathway which appears to be required and sufficient to mediate hormone-induced breast cancer. We postulate that our data have some far-reaching implications. Millions of women use progesterone derivates in contraceptives and for hormonal replacement therapy. Epidemiologically, hormones and in particular progestins like MPA have been linked to an increased risk to develop breast cancer (Beral, 2003). For instance, breast cancer is much more prevalent in industrial countries were the use of HRT is very frequent and a decrease of breast cancer incidence since 1999 is likely to correlate with the decrease of use of HRT (Jemal et al., 2009). Since we now suggest for the first time that progestins drive mammary carcinogenesis via the RANK/RANKL system new ways for preventing and/or treating breast cancer might have been revealed.

## 4. Acknowledgements

Upon finishing this thesis I would like to give thanks to all the people who helped so much and who made it possible that I could complete this project.

I want to thank Daniel Schramek and Andreas Leibbrandt who always had an open ear to my questions and who were there for me whenever I needed help.

Moreover I am thankful to Josef Penninger for giving me the opportunity to be in his lab, which is a fantastic and inspiring place to work. I also would like to thank all the members of the Penninger lab for the great atmosphere they provide. Although there were also difficulties along the way, these moments were quickly forgotten due to the fun and joy of working with the Penninger group.

Without any doubt, my endless gratefulness goes to my parents for their love, for their belief in me and for their constant support.

Last but not least I would like to thank my friends, my brother and Matthias who mean so much to me and I know how lucky I am to spend my time with them.

## 5. Materials and Methodes

**Mice.** *Rank*$^{floxed}$ mice have been recently generated in our laboratory (Hanada et al., 2009). In order to generate a *Rank*-null allele (*Rank*$^{\Delta}$ allele) *Rank*$^{floxed}$ mice were crossed to ubiquitous deleter mice expressing *cre*-recombinase under control of the β-actin promoter (Lewandoski et al., 1997). For mammary gland specific deletion mice carrying the *Rank*$^{floxed}$ and *Rank*$^{\Delta}$ alleles were crossed to MMTV-Cre mice. Rank$^{floxed}$, Rank$^{\Delta}$ as well as MMTV-Cre mice were backcrossed seven times onto a BALBc background before generating MMTV-Cre RANK$^{\Delta/fl}$ mice. MMTV-Cre mice (Wagner et al., 2001) (stock # 00553) and Mx-Cre mice (Kuhn et al., 1995) (stock # 003556) were obtained from the Jackson Laboratory. IKKα$^{floxed}$ mice (Gareus et al., 2007), TRAF6$^{floxed}$ mice and NFATc1$^{floxed}$ mice (Aliprantis et al., 2008) have been previously described. Genotyping was performed using the following primers:

**Rank:**

Forward Primer: 5'GGCAG AACTC GGATG CACAG ATTGG3'
Reverse Primer: 5'AGTGT GCCTG GCATG TGCAG ACCTT3'
Mutant allele: 5'CTGGT GGTTG TTCTC CTGGT GTCAT3'
PCR conditions: 95°C/10s, 60°C/20s, 72°C/32s, 35 cycles

**TRAF6:**

Forward Primer: 5'AGGGC ATATT TGAAG TCCATT3'
Reverse Primer: 5'TCTTG CTCTT CAAGA GGATCG3'
Mutant allele: 5'TTCAT GGCGT GGTGC TATATA3'
PCR conditions: 94°C/30s, 55°C/30s, 72°C/30s, 34 cycles

**IKKα:**

Forward Primer: 5'CGCTT AGTGT GACTG AGGAA C3'
Reverse Primer: 5'ATGAG CCCAA CATTT AATCT T3'
Mutant allele: 5'GGCAT CAGAG TCCGT GGGT3'
PCR conditions: 94°C/30s, 55°/30s, 72°C 60s, 35 cycles

**NFATc1:**

Forward Primer: 5'AAGGA ATTAC TGGGA AGCCT GGCA3',
Reverse Primer: 5'AGGGA CTATC ATTTG GCAGG GACA3'
Mutant allele: 5'ACAGG AAACA GCTCT GTTCC ACAC3'
PCR conditions: 95°C/10s, 60°C/15s, 72°C/40s, 40 cycles.

Animal experiments were approved by an ethics committee. Mice were housed according to institutional guidelines.

**MPA/DMBA-induced mammary carcinogenesis.** 6-week old female mice were anesthetized with ketamine-xylamine and surgically implanted with slow-release Medroxyprogesterone Acetate pellets (50 mg, 90 days of release, Scientific research of America, # NP-161) subcutaneously on the right flank. 200µl DMBA (5mg/ml cottonseed oil) was administered by oral gavage 6 times throughout the following 8 weeks as outlined in Figure 5a. Mammary tumor onset was determined by palpation.

**Histology, whole mount and immunohistochemistry.** Mammary glands were isolated and fixed over night in Rotihistofix 4%. After dehydration 5 µm paraffin sections were cut and stained for hematoxylin and eosin (H&E). Whole mount staining of glands was performed as previously described (Fata et al., 1999). Briefly, the fourth inguinal fat pads of virgin mice were dissected, placed on a microscope slide and fixed over night in 4% PFA. The adipose tissue was removed in acetone for 3 hours. For rehydration, glands were put in 100% ethanol and 95% ethanol for 1 hour respectively. Glands were stained in Hematoxylin for 3 hours and afterwards transferred into slightly basic tap water for 2 hours followed by 2 hours of destaining in 50% ethanol acidified with 25ml 1.0M HCl/liter. After dehydrating in ascending concentrations of ethanol (70%, 95% and 100% ethanol, incubation for 1 hour, respectively), glands were stored and xylene. For immunoperoxidase staining paraffin sections were incubated with rabbit polyclonal anti-Ki67 (Novocastra # 6000224) and anti-active Caspase 3 (Cell Signaling #9661) and visualized using 1 to 200 diluted peroxidase-conjugated anti-rabbit IgG (DAKO, # E0432). For calculation of proliferation and apoptosis positive epithelial cells were divided by the total number of epithelial cells. For Ki67 stainings no fewer than 1000 nuclei and for active Caspase 3 staining no fewer than 5000 cells were counted per section.

**Isolation of primary mammary epithelial cells.** Mammary glands from female wildtype mice were isolated, minced under sterile conditions and incubated in a collagenase solution (2 mg/ml Collagenase A, Sigma # C274; 5 % FCS; 0,2 % Trypsin, 50 µg/ml Gentamycin; 5 µg/ml Insulin) for 30 minutes at 100 rpm, 37°C. For all following steps pipettes and tubes were precoated in 5% BSA. Fatty layer was removed. Pellet was resuspended in 10 ml DMEM/F12 (Invitrogen) and centrifuged for 10 min at 1500 rpm. Pellet was resuspended in 4 ml DMEM/F12 and centrifuged at1550 rpm to remove fibroblasts. Cleared pellets were resuspended in growth medium and put in culture.

**Western blotting.** Primary non/transformed mouse mammary epithelial cells and the human epithelial breast tumor cell line SKB3 (Mo and Reynolds, 1996) were stimulated using 1µg recombinant RANKL (Oriental yeast # NIB47197900). Cells were lysed in RIPA buffer plus phosphatase and protease inhibitor. Samples were loaded onto 8 % gels and run using SDS running buffer. Separated proteins were transferred onto a membrane using SEMI-DRY Transfer Cell (BIORAD). Blots were blocked with 5% BSA in 1xTBS 0.1% Tween-20 (TBST) for 1 hour followed by incubation with primary antibody overnight at 4$^{o}$C (diluted in TBST according to the manufactures protocol). Primary antibodies reactive to mouse β-actin (Sigma), phosphorylated (P) NFκB (#4767), phosphorylated (P) IκBα (1:1000, # 2859), IkBα (1:1000, # 4814), phosphorylated (P) IKKα (1:1000, # 2618), IKKβ (1:1000, # 2678), IKKγ (1:1000, # 2685), (all from Cell Signaling), were used. Blots were washed in TBST for 30 minutes with 2 times of solution change, incubated with HRP-conjugated 2$^{nd}$ antibodies (1:2000, Promega, diluted in 3% milk) for 1 hour at room temperature, washed in TBST for 30 minutes and visualized using ECL.

**qRT-PCR:** Total RNA of tumors and of the indicated tissues of MMTV-RIC females was extracted using RNeasy Mini Kit (Qiagen # 74104). 2 µg of total RNA was converted to cDNA by using Ready to go Beads (GE Healthcare, Cat. No. 27-9264-01) and used for quantitative (q)RT-PCR analysis. The following primers were used:

β-actin:

Forward Primer: 5'GCTCA TAGCT CTTCT CCAGGG3'
Reverse Primer: 5'CCTGA ACCCT AAGGC CAACCG3'

RANKL:

Forward Primer: 5'CTGAG GCCCA GCCAT TTG3'
Reverse Primer: 5'GTTGC TTAAC GTCAT GTTAG AGATC TTG3'

RANK:

Forward Primer: 5'CTTGG ACACC TGGAA TGAAG3'
Reverse Primer: 5'CAGCA CTCGC AGTCT GAGTT3'

Cyclin D1:

Forward Primer: 5'CTGTG CGCCC TCCGT ATCT TA3'
Reverse Primer: 5'GGCGG CCAGG TTCCA CTTGAG3'

p21 (Cdkn1a):

Forward Primer: 5'GTGGC CTTGT CGCTG TCTT3'
Reverse Primer: 5'GCGCT TGGAG TGATA GAAAT CTG3'

***In vivo* cell death.** RANK$^{\Delta mam}$ and littermate controls were γ-irradiated with a total dose of 5 Gray (Gy). After six hours, mice were sacrificed and mammary glands were isolated. Cell death was determined by immunostaining using the apoptotic marker active Caspase 3 (Cell Signaling #9661).

**Generation of MMTV-RANK-Ires-CRE transgenic mouse lines.** The full length murine RANK (Tnfrsf11a) cDNA (GenBank accession number: AF019046) was directionally inserted into the HindIII-EcoRI sites of pMMTV-SV40. The Ires-Cre cDNA was excised from pMMTV-PMT-Ires-Cre as an EcoRI fragment and inserted into a corresponding unique EcoRI site 3' of MMTV-LTR-RANK and 5' of SV40. The vector inserts were sequenced to exclude mutations in the coding regions of the construct. Prior to pronuclear injection the MMTV-RANK-Ires-CRE transgene was isolated with OliI and SphI from the pBabe cloning vector. The injection fragment was recovered by electroelution (QIAquick Gel extraction kit, # 28704) and injected into male pronuclei of single-cell (FVB/N) embryos. Viable eggs were subsequently transferred to pseudopregnant B6CBAF1 recipients. Founders were identified by PCR with the following PCR primers for *cre*-recombinase 5'TCGCG ATTAT CTTCT ATATC TTCAG3' (forward) and 5'GCTCG ACCAG TTTAG TTACCC3' (reverse), 95°C/10s, 58°C/10s, 72°C/30s, 34 cycles.

## 6. Abbreviations

| | |
|---|---|
| AIRE | Autoimmune regulator |
| Akt/PKB | Protein kinase 2 |
| ALPS | Autoimmune lymphoproliferative syndrome |
| APC | Antigen presenting cell |
| ARO | Autosomal recessive osteopetrosis |
| Bcl-XL | B-cell lymphoma-extra large |
| c-Fos | V-fos FBJ murine osteosarcoma viral oncogene homolog |
| c-Jun | Jun antigen |
| COX2 | Cyclooxygenase 2 |
| DC | Dendritic cell |
| DMBA | Dimethylbenz(a)anthracene |
| ErbB2 | Human epidermal growth factor receptor 2 |
| EP3R | Prostaglandin E receptor 2 |
| ERK | Extracellular signal-regulated kinase |
| Fra | Fos related antigen |
| GAB2 | GRB2 associated binder 2 |
| GRB2 | Growth factor receptor bound protein 2 |
| HRT | Hormonal replacement therapy |
| Id2 | Inhibitor of DNA binding 2 |
| IkB | Inhibitor kappa B |
| IKK | IkB kinase |
| INF | Interferon |
| JAK2 | Janus kinase 2 |
| JNK | c-Jun N-terminal kinase |
| JunB | Jun B proto-oncogene |
| kDa | kilo Dalton |
| LTβ | Lymphotoxin beta |
| MAPK | Mitogen activated protein kinase |
| M-CSF | Macrophage colony stimulating factor |
| MKK7 | Mitogen activated protein kinase kinase 7 |

| | |
|---|---|
| MPA | Medroxyprogesterone Acetate |
| mTEC | Medullary thymic epithelial cell |
| NFATc1 | Nuclear factor of activated T-cells, calcineurin dependent 1 |
| NFkB | Nuclear factor kappa B |
| NK cells | Natural killer cells |
| ODF | Osteoclast differentiation factor |
| OPG | Osteoprotegerin |
| OPGL | OPG ligand |
| PGE2 | Prostaglandin E2 |
| PLC | Phospholipase C |
| PR | Progesterone receptor |
| PRAKO | Progesterone receptor-A knockout |
| PRBKO | Progesterone receptor-B knockout |
| PRKO | Progesterone receptor knockout |
| PRL | Prolactin |
| PTHrP | Parathyroid hormone-related peptide |
| RANK | Receptor activator of NFkB |
| RANKL | Receptor activator of NFkB ligand |
| SHIP1 | SH2 (Src homology 2)-containing inositol phosphatase-1 |
| STAT5 | Signal transducer and activator of transcription 2 |
| TGF | Transforming growth factor |
| TNF | Tumor necrosis factor |
| TRAF | Tumor necrosis factor related adaptor protein |
| TRAIL | TNF-related apoptosis-inducing ligand |
| TRANCE | TNF-related activation-induced cytokine |
| Treg | Regulatory T cell |
| UV | Ultraviolet |

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## Curriculum Vitae

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|---|---|
| **Personal Information** | |
| Surname / First name | Sigl Verena |
| Addresses | Hehenberg 10<br>5142 Eggelsberg<br>Austria |
| Telephone | (+43) 650 9364117 |
| e-mail | a0304132@unet.univie.ac.at |
| Nationality | Austrian |
| Date of birth | 17/04/1984 |
| Place of birth | Oberndorf b. Salzburg |
| **Research experience** | |
| Dates | 01.07.2008 - present |
| Postition | Diploma student |
| Responsibilites | Examination of the role of RANK/RANKL in MPA/DMBA-induced mammary carcinogenesis in mice |
| Employer | IMBA – Institute of Molecular Biotechnology GmbH<br>Austrian Academy of Science<br>Prof. Dr. Josef Penninger<br>Dr. Bohrgasse 3<br>1030 Vienna; Austria |
| Dates | 01.01.2008 – 31.3.2008 |
| Position | Student |
| Responsibilites | "The role of MKK7 in cancer"<br>"Genome-wide RNAi screen in Drosophila Melanogaster for genes involved in fat or glucose metabolism" |
| Employer | IMBA – Institute of Molecular Biotechnology GmbH<br>Austrian Academy of Science<br>Prof. Dr. Josef Penninger<br>Dr. Bohrgasse 3<br>1030 Vienna; Austria |

| | |
|---|---|
| **Education** | |
| Dates | 10/2004 – present |
| | Student of the field of Genetics |
| Principal Subjects | Genetics and molecular pathology, Immunbiology, Cell- and Developmental Genetics |
| University | University of Vienna |
| International Classification | Masters Degree |
| | |
| Dates | 1998-2003 |
| School | Secondary School Braunau |
| International Classification | A-level |
| | |
| **Language skills** | |
| Mother Tongue | German |
| Other Languages | English, French |