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RANKL inhibition: a promising novel strategy for breast cancer treatment

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Abstract The cytokine RANKL and its receptor RANK, key proteins in bone remodelling and bone metastasis, are essential for mammary gland development in mice. RANK absence or overexpression results in a lactation defect and a non-functional mammary gland. RANKL signalling mediates progesterone-induced proliferation and expansion of the stem cell compartment in the mouse mammary gland. RANK overexpressing mammary epithelial acini show hallmarks of transformation in a RANKL-dependent manner. Complementary gain- and loss-of-function approaches (RANK transgenic and knock-out mouse models and pharmacological RANKL inhibition) define a direct contribution of this pathway to progestin-driven mammary cancer. Moreover, decreased RANKL signalling attenuates preneoplasic lesions and lung metastasis in the spontaneous model of mammary tumorigenesis MMTV-neu, suggesting that RANK pathway promotes mammary tumorigenesis and metastasis in a wider tumour spectrum and beyond its established role in bone metastasis. In this review, we summarise the role of the RANKL pathway in mammary gland development, breast cancer and metastasis, and discuss the potential application of RANKL inhibition for breast cancer treatment.

Keywords RANK · RANKL · Osteoprotegerin · Progesterone · Her2/neu · Mammary gland development

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Introduction

Breast cancer is the most common malignancy among females in the Western world, resulting in approximately half a million deaths annually, mainly due to metastatic disease. Luminal tumours characterised by the expression of oestrogen and progesterone receptors (ER and PR, respectively) constitute 70% of breast cancer and constitute the subtype with a better prognosis. Overexpression or amplification of Her2 (ErbB2) is detected in 20% of breast tumours [1]. The remaining 10% are the so-called triple negative breast cancers (TNBCs), as they lack the expression of ER, PR and Her2. They are enriched in tumours with a basaloid expression genotype and, as Her2 tumours, have a particularly poor prognosis [2].

Current therapies for breast cancer include local treatments (surgery and radiation) and systemic treatments, mainly chemotherapy and directed therapies against hormones or the ErbB2/neu/Her2 signalling pathway [3]. Despite the progress achieved, the reality is that many tumours from all subtypes are not eradicated by local treatments and systemic adjuvant therapies because of their intrinsic or acquired resistance, and relapse, often leading to metastatic disease that kills the patient. Thus, it is essential to find new therapeutic targets and elucidate the resistance mechanisms to current therapies in breast cancer.

Novel results demonstrate that RANKL signalling through its receptor RANK, is the main mediator of progesterone-induced proliferation of mammary epithelial cells and that activation of this pathway promotes mammary tumorigenesis and lung metastasis in mice, suggesting that anti-RANKL treatment could be a novel therapeutic approach for prevention and treatment of breast cancer and metastatic disease. In this review we provide an overview of the RANK signalling pathway and the recently pub-

lished results that highlight the impact of RANKL inhibition on mammary tumorigenesis. Finally, we comment on the current knowledge of this receptor/ligand system in human breast cancer and the putative therapeutic impact of RANKL inhibition, given that an anti-RANKL drug is already in the clinic for the treatment of osteoporosis and bone metastasis.

Introducing RANK, RANKL and OPG

Interaction of RANK (Receptor of Activated Nuclear factor Kappa) and its ligand, RANKL, a cytokine member of the tumour necrosis factor (TNF) superfamily, is essential for osteoclast formation, function and survival. RANK signalling can be attenuated by osteoprotegerin (OPG), an endogenous soluble decoy receptor for RANKL that blocks its binding to RANK, acting as a dominant negative in physiological conditions. RANK is expressed in osteoclasts and osteoclast precursors, whereas RANKL and OPG are expressed in osteoblasts and other stromal cells. RANKL is the only known ligand of RANK and can exist as a transmembrane or soluble protein, probably resulting from alternative splicing, and shedding [4]. Functional implications of each form need further evaluation, as both are able to activate the receptor.

In osteoclasts RANKL-RANK signalling activates a variety of downstream pathways. RANK, as other members of the tumour necrosis factor receptor (TNFR) superfamily, assembles into functional trimers. Various TNF-receptor (TNFR)-associated factor (TRAF) proteins associate with the cytoplasmic domain of RANK and mediate ligand-induced signalling. RANKL-RANK induces activation of the transcription factor, NF-kB, mediated by the IKK complex [5, 6]. Mitogen-activated protein kinase (MAPK) family members including, p38-MAPKs, c-Jun N-terminal kinases and extracellular signal-regulated kinases (ERKs) are activated downstream of RANK [7, 8]. RANK also induces activation of the anti-apoptotic serine/threonine kinase Akt/PKB [9]. Activation of Akt by Src requires the lipid kinase phosphatidylinositol 3-kinase (PI3-kinase) [10]. Given the variety of signalling cascades activated by RANKL-RANK, it is important to identify the specific pathways activated in each cell type and biological process, and understand their cross-talk with other receptor/ligand systems.

Genetic ablation of RANK and RANKL in mice results in the absence of osteoclasts and significant osteopetrosis, suggesting that the RANKL pathway is a key mediator of bone resorption [11, 12]. In contrast, OPG depletion results in severe osteoporosis [13, 14]. Subsequent data have supported the role of this pathway in bone-related pathologies including bone metastases [15]. Bone metastasis is a common complication of breast cancer (and other tumours such as prostate, multiple myeloma and lung tumours) and results in bone pain, fractures, and increased morbidity and mortality in patients. The major driver of these skeletal complications is increased osteoclast activity driven by the RANKL pathway. Numerous lines of evidence indicate that blocking RANK/RANKL interaction effectively prevents or reduces osteoporosis and tumour-induced bone lesions [13].

Expression of RANK and/or RANKL has been found in a wide variety of tissues outside the bone including lymphoid tissues, skeletal muscle, thymus, liver, colon, small intestine, heart, brain, adrenal gland and mammary gland [16]. Moreover, a functional role for this pathway has been demonstrated in the embryonic development of peripheral lymph nodes, dendritic cell survival and T-cell communication, establishment of central tolerance and central nervous system, autoimmunity, and post-natal mammary gland development [17–19].

A tight regulation of the RANK pathway is essential for mammary gland development

Mammary glands of the RANK- and RANKL-deficient mice develop normally during sexual maturation, but fail to form lobuloalveolar structures during pregnancy due to defective proliferation and increased apoptosis of the mammary epithelium [20]. In transgenic mice that constitutively express RANK in the mammary gland (under the MMTV promoter), increased proliferation at midgestation and impaired alveolar differentiation is observed [21]. Thus, RANK absence or overexpression impairs lobuloalveolar development and results in a lactation defect and a non-functional mammary gland. This stage-specific defect correlates with a selective and strict regulation of the RANK/RANKL pathway in mammary epithelium during pregnancy by breast mitogens such as progesterone, prolactin and PTHrP [20–22]. RANKL expression is induced by progesterone specifically in cells that express ER/PR. These cells are segregated from a subset of PR-negative cells that express cyclin D1 and proliferate in response to progesterone [23], providing support for a mediator role of RANKL in mammary progesterone action. Indiscriminate RANKL overexpression in the mammary epithelia results in increased and sustained proliferation, spatially disorganised ductal branching and alveologenesis, giving rise to hyperplasic mammary glands later in life [24]. Only when RANKL expression is specifically targeted to the ER/PR positive cells, an ordered ductal arborisation and alveolar development is observed [25]. It has been shown that RANKL signalling pathway is responsible for the major proliferative response of mouse mammary epithelium to progesterone during mammary lactational morphogenesis [26] and that the catalytic subunit IKKα is required for NF-κB activation in mammary epithelial cells in response to RANKL [27].

Altogether, these data demonstrate that a strict spatial and temporal regulation of RANK/RANKL expression is

essential for mammary epithelial proliferation and morphogenesis.

Activation of RANKL signalling expands the mammary stem cell compartment

The massive expansion of the mammary epithelium during puberty and gestation, together with the cell renewal capacity during the successive reproductive cycles, supports the presence of mammary stem cells (MaSC). There are two main compartments within the mammary gland, the luminal and the basal. The luminal compartment is formed by two cell types that give rise to ducts and alveoli and can be positive or not for the expression of the hormone receptors ER and PR. The basal compartment is mainly formed by contractile and specialised cells, localised in the basal area of the epithelium, the myoepithelial cells, and also contains the MaSC population. MaSC, also called mammary reconstituting units (MRU), are cells capable of reconstituting a whole mammary gland *in vivo* and give rise to all mammary lineages [28–30]. Within the luminal compartment, there are cells with a high capacity to form colonies *in vitro* but lacking reconstituting potential *in vivo*, which are called colony-forming cells (CFC) and are likely intermediate progenitors [28, 30, 31], and mature luminal cells, where expression of ER and PR is mostly confined [32, 33].

Mouse MaSC are highly responsive to steroid hormone signalling despite lacking ER and PR. Ovariectomy markedly reduces the number and outgrowth potential of MaSC *in vivo*, whereas MaSC activity notably increases in mice treated with oestrogen plus progesterone. Accordingly, the MaSC pool transiently increases during maximal progesterone levels at the luteal dioestrous phase and during pregnancy [32, 34]. RANKL expression is restricted to the luminal compartment where the ER/PR-positive cells localise. RANK expression, although more abundant in the basal, is detected in both the basal and luminal compartments [32, 34]. It has been postulated that paracrine signalling through RANKL is responsible for the expansion of the MaSC cell compartment driven by progesterone. In correlation, genetic inactivation of RANK in the mammary epithelia impaired the expansion of the MaSC-enriched population upon progesterone treatment [35].

There is increasing evidence that some neoplasms, including breast tumours, can originate from stem and progenitor cells. Progenitor cells reside in the tissue for long periods of time and can accumulate the mutations required for transformation. In fact, tumours are highly heterogeneous and only one concrete population with stem cell properties (self-renewal and pluripotency) can give rise to new tumours upon transplantation that recapitulate the heterogeneity found in the initial tumour [36]. The expansion of the progenitor population induced by progesterone through RANKL may therefore have implications for breast cancer initiation as it provides a greater pool of cells that could be targets of transformation.

RANKL mediates progestin-induced mammary preneoplasic lesions and adenocarcinomas in mice

Recent results from several groups demonstrate that the RANK pathway is implicated in mammary tumour development in mice [35, 37]. RANKL treatment of three-dimensional (3D) acinar cultures isolated from MMTV-RANK mice results in hallmarks of transformation, including increased proliferation, loss of milk secretion, and impaired luminal cavitation and polarisation [21, 37]. After multiple pregnancies, MMTV-RANK females spontaneously develop mammary preneoplastic lesions and adenocarcinomas, and after dimethylbenz(a)anthracene (DMBA) and medroxyprogesterone (MPA) treatments they show a shorter latency and a higher incidence of ductal hyperplasia (HP), mammary intraepithelial neoplasia (MIN) and adenocarcinomas as compared to wild-type (WT) mice. Reciprocally, pharmacological treatment beginning at early timepoints with RANK-Fc, an antibody that binds to RANKL and blocks the pathway similarly to OPG, decreases the incidence and increases the latency of palpable tumours in transgenic MMTV-RANK mice treated with MPA and DMBA. Moreover, RANKL inhibition almost completely blocks the occurrence of palpable mammary tumours driven by progestin and carcinogen in WT mice that have physiological doses of RANK. The reduction in tumorigenesis upon RANKL inhibition in both models is preceded by a reduction in preneoplasias as well as rapid and sustained reductions in hormone- and carcinogen-induced mammary epithelial proliferation and cyclin D1 levels [37]. Similarly, specific deletion of RANK in the mammary epithelium decreases the incidence and delays the onset of MPA-driven mammary cancer, as does IKKα deletion, suggesting that NF-κB pathways may be the downstream mechanism responsible for RANKL impact on tumour initiation [35]. RANK and RANKL are expressed in preneoplasias and tumours in both WT and MMTV-RANK mice in this model [21] and co-localisation of PR and RANKL is observed in all lesions. RANK and RANKL usually show a reciprocal pattern of expression, probably due to a RANKL-driven suppression of its receptor [21, 38].

RANKL-induced proliferation is a critical step in tumour initiation (normal epithelia, HP), but in late lesions, MIN and adenocarcinomas, anti-RANKL treatment shows minimal effects on proliferation and instead results in increased apoptosis [37]. In correlation, RANKL treatment results in a marked protection from cell death in response to gamma-radiation and doxorubicin in mouse mammary epithelial cells and the RANK-expressing human cancer cell line, SKBR3 [35]. These results may have implications in the clinical setting as most chemotherapeutics are DNA-damaging agents.

Altogether, these results are important given the profound influence of steroid hormones on breast cancer risk. Breast cancer risk increases with the number of menstrual cycles a woman experiences [39]. Oestrogen plus progesterone results in a greater stimulatory effect on breast proliferation than oestrogen alone [40], consistent with the increased breast cancer incidence in women receiving both hormones in hormonal replacement therapies and contraceptives [41].

In summary, increased RANK signalling promotes mammary tumour initiation and progression in mice and RANKL is the main mediator of the protumorigenic effects of progesterone in the mouse mammary gland. These results suggest that increased RANK expression and RANKL signalling in mammary epithelial cells could be a risk factor in breast cancer and enhance resistance to chemotherapy in the clinical setting.

RANKL promotes spontaneous mammary tumorigenesis and lung metastasis in MMTV-neu mice

MMTV-neu mice spontaneously develop adenocarcinomas driven by the overexpression of the oncogene neu (rat orthologue to human Her2) in the absence of exogenous hormone requirement [42]. In contrast with MPA-DMBA-driven tumours, MMTV-neu adenocarcinomas, although luminal (express CK18), lack expression of the hormone receptors, ER and PR. RANKL expression decreases with a similar pattern to PR, whereas RANK expression increases during tumour progression, suggesting that RANK signalling may also play a role in this model [37].

RANK-Fc treatment, starting post-puberty (20 weeks of age), does not impact onset of tumour development. However, it does significantly decrease multiplicity of preneoplastic lesions and tumours, and, importantly, the incidence and total number of lung metastases in MMTV-neu mice [37]. In correlation, reduced RANK gene dosage decreases by half the incidence of lung metastasis in these mice without affecting tumour growth [43].

In contrast to RANK, which is highly expressed in adenocarcinomas, RANKL expression is mostly restricted to the surrounding stroma in several cell types [37]. It was reported that infiltrating macrophages and T lymphocytes express RANKL and promote metastasis in a transgenic mouse model of prostate cancer [44]. Similarly, in MMTV-neu mice tumour-infiltrating T-regulatory cells (T-reg) are a major source of RANKL, which stimulates metastasis of RANK-expressing tumour cells. Injection of recombinant RANKL also significantly increases the incidence and multiplicity of lung metastases in MMTV-neu mice, highlighting the relevance of the RANK pathway in this model [43]. RANK also enhances lung metastasis incidence and multiplicity in the RANK-expressing ZR-75-1 breast carcinoma cell line [43]. Importantly, RANKL stimulation allows SKBR3 breast cancer cells to grow in an anchorage-independent manner [35], facilitating survival of tumour cells once they leave the primary tumour and enter circulation. It was previously reported that RANKL triggers migration in several human RANK-expressing cancer cell lines, including breast, prostate and melanoma, and increases bone metastasis in these models [45].

These results indicate that mechanisms other than progesterone may exist to deregulate and activate RANK pathway in breast cancer, extending its relevance not only to ER/PR positive tumours but also to other breast cancer subtypes, as RANKL can be delivered by infiltrating lymphocytes and it is active in a soluble form. They constitute the first direct evidence for a role of RANK/RANKL in metastasis to organs other than the bone.

RANK and RANKL are expressed in human breast tumours

The results discussed above clearly demonstrate the relevance of RANK signalling in mammary gland development, breast cancer and metastasis in mice. Given the need of novel targets for breast cancer treatment, it is essential to address the relevance of this pathway in the clinical setting. The first step is to evaluate the expression pattern of RANK, RANKL and OPG in the human mammary gland and during breast cancer progression. This apparently easy task has encountered some difficulties, given the lack of appropriate tools and the complexity of the pathway.

Immunostainings of human breast carcinomas revealed that RANKL is expressed in 11% of human breast tumours. As occurs in mouse, RANKL expression is not confined to the tumours and was often detected in sporadic infiltrating mononuclear cells present in most tumour stroma (67%) and in fibroblast-like cells in the stroma of rare tumours (5%) [37]. Within the stroma, RANKL expression is higher in invasive ductal carcinomas (IDC) (55%) than in ductal carcinomas *in situ* (8%) or lymph node positive tumours (22%) [43]. Bhatia et al. reported that expression of RANKL in the epithelial compartment is inversely correlated with tumour progression and metastasis in breast carcinoma, that is, RANKL is expressed in 90% of non-neoplastic breast, 62% of non-metastatic IDC, 31% of metastatic IDC and only 2% of breast cancer bone metastasis [46].

Specific detection of human RANK has proven to be even more difficult. RANK expression has been reported in most primary human breast tumour samples, as well as in cancer cells present in local lymph node metastasis and in bone metastasis [45, 46]. In contrast, in our study we only found high levels of expression of RANK in 6% of human breast tumours [37]. This controversy regarding RANK expression in human breast cancer is due to the lack of highly sensitive and specific antibodies, and appropriate negative controls. It is critical to precisely determine RANK expression on the tumour cells as this may allow the identification of the tumours that will respond to anti-RANKL treatment. Another level of difficulty is the

down-modulation of RANK driven by RANKL [21, 38]. Thus, absence of RANK expression in tumour cells might mean that RANKL is present and the pathway is activated. Unfortunately, specific downstream effectors of RANKL have not been found yet, as the pathways that it controls can be activated by other mechanisms. Very little is known about the role of OPG in the mammary epithelia, but given the ability of this protein to inhibit RANKL signalling, it is important to determine its expression in tumours. Ideally, expression of RANK, RANKL, OPG and downstream effectors should be addressed in human breast tumours.

Several studies have reported expression of these proteins in tumour and bone metastasis not only from breast, but also from prostate, lung, renal cancer and melanoma, as it has been shown that the RANK pathway is critically involved in metastasis formation in these tumours [45–49]. Again, these results need to be validated with appropriate tools.

Great advances are being made in the identification of different subpopulations of human mammary epithelial cells and the cell of origin of different breast cancers [50, 51]. Expression of RANK and RANKL can be analysed in these subpopulations to evaluate if they follow similar patterns to those observed in mice.

Better quality antibodies and immunostaining protocols are needed to evaluate the relevance of the RANK pathway in the clinical setting and to identify the group of patients that may benefit from anti-RANKL tumour therapy. However, alternative approaches such as RNA expression levels, in situ hybridisation and analyses of several integrants of the pathway will advance our knowledge of the relevance of RANKL signalling in human mammary gland and breast cancer development. More importantly, the functionality of the pathway can be directly evaluated on breast cancer patients, as discussed in the next section.

RANKL inhibition is already in the clinic

Inhibition of RANKL using OPG causes a greater suppression of bone resorption and hypercalcaemia than bisphosphonates (the current standard therapy for the treatment of bone loss) and less side effects are expected [52]. Moreover, a fully human anti-RANKL antibody has been developed that inhibits RANKL function. This humanised RANKL-inhibiting antibody, denosumab (Amgen), was approved by the US Food and Drug Administration and the European Commission in 2011 for osteoporosis treatment.

Several clinical trials have also demonstrated that denosumab is an effective inhibitor of bone metastasis [53, 54]. In fact, denosumab is superior to zoledronic acid in delaying or preventing skeletal-related events (SREs) in patients with breast cancer metastatic to bone [55]. Based on the results of this and other clinical trials, denosumab has also been approved by the FDA for the prevention of SREs in patients with bone metastases from solid tumours.

The results obtained in mice tumours and human cell lines are promising, suggesting that RANKL inhibition may have several therapeutic applications in the clinical setting of breast cancer. The impact of the RANK/RANKL pathway during tumour progression and the putative benefits of anti-RANKL therapy at different stages of tumorigenesis are schematically represented in Fig. 1.

Given the tumour attenuation observed in progestin- and carcinogen-treated mice after RANK-Fc treatment and in genetically modified mice lacking RANK expression [35, 37], we can speculate that denosumab may prevent or attenuate tumour initiation in women (Fig. 1). Interestingly, the bisphosphonate zoledronic acid had no effect in preventing mammary tumour development in mice after mutagen and progestin treatment, as compared to the almost complete prevention observed with RANK-Fc for tumour relapse (Fig. 1) [37].

Activation of RANKL signalling induced by progesterone results in the expansion of mammary progenitor cells and increases the survival of tumour cells growing in suspension [32, 34, 43]. Suspension cultures are enriched in cells with enhanced ability to seed new tumours or tumour-initiating cells [56]. Based on this evidence we can speculate that inhibition of RANKL signalling may reduce tumour recurrence, as tumour-initiating cells have been indicated as being responsible for tumour relapse of current chemotherapy drugs (Fig. 1).

Increased apoptosis is observed in tumours after RANK-Fc treatment, and RANKL activation protects against DNA damage induced by doxorubicin or gamma irradiation [35, 37]. Thus, RANKL inhibition used in the adjuvant setting may improve the efficiency of current chemotherapy drugs, as already demonstrated for bone metastasis [57–60] in preclinical trials.

RANK-Fc treatment or genetic ablation of RANK attenuates the incidence and multiplicity of lung metastasis in MMTV-neu mice [37]. It has been postulated that the potent prometastatic effect of tumour-infiltrating T-reg can be dismantled by anti-RANKL therapy [43]. Therefore, we can speculate that RANKL inhibition may be effective to prevent or attenuate not only bone metastasis but also metastasis to other organs (Fig. 1).

Conclusion

The RANK pathway has emerged as a putative novel target in breast cancer. RANKL is the main mediator of progesterone in mouse mammary epithelial cells and RANKL inhibition attenuates mammary tumour formation and metastasis to organs other than the bone in several models of mouse carcinogenesis. A RANKL-inhibiting antibody is already in the clinic for the treatment of osteoporosis and bone metastasis. The relevance of the RANK pathway in human breast cancer remains to be proven. However, given the promising results obtained in mouse models upon RANKL inhibition and the existence of a clinically approved anti-RANKL drug, we can only be optimistic that

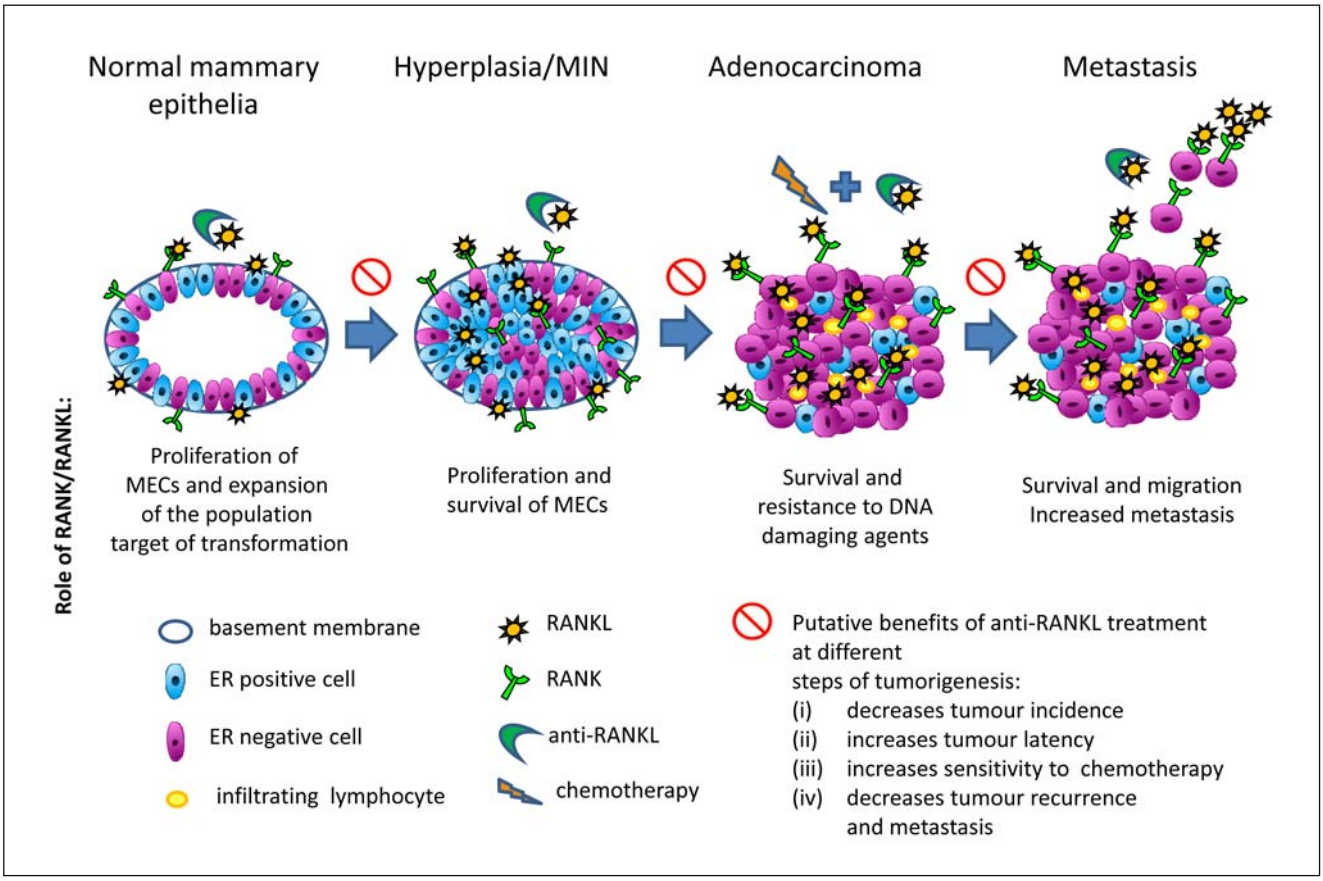

Fig. 1 The putative impact of anti-RANKL treatment at different steps of tumour progression. For simplicity the basal compartment is not shown. Anti-RANKL treatment may have a preventive and therapeutic value alone or in combination with current chemotherapy. RANKL promotes tumour initiation by expanding the compartment that can be the target of transformation and by increasing proliferation of mammary epithelial cells. Then, it increases survival of tumour cells and resistance to DNA-damaging agents, such as current therapeutics, and promotes metastasis by increasing migration and survival of tumour cells in circulation

it will soon be elucidated whether RANKL inhibition is an effective therapy for breast cancer treatment.

Thousands of patients all around the world are already taking denosumab for the treatment of osteoporosis or bone metastasis. Properly designed clinical trials are urgently needed to evaluate the impact of RANKL inhibition on breast cancer incidence and the effects on the primary mammary tumour.

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