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▶ **To cite this version:**

Kanji Mori, K. Ando, Dominique Heymann, Françoise Rédini. Receptor activator of nuclear factor-kappa B ligand (RANKL) stimulates bone-associated tumors through functional RANK expressed on bone-associated cancer cells?. Histology and Histopathology, 2009, 24 (2), pp.235-42. inserm-00667499

HAL Id: inserm-00667499

https://inserm.hal.science/inserm-00667499

Submitted on 7 Feb 2012

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Histol Histopathol (2009) 24: 235-242

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Histology and Histopathology

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Review

Receptor activator of nuclear factor-kappa B ligand (RANKL) stimulates bone-associated tumors through functional RANK expressed on bone-associated cancer cells?

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Summary. Primary and secondary bone tumors clearly deteriorate quality of life and the activity of daily living of patients. These undesirable diseases become a major social and economic burden. As both primary and secondary bone tumors develop in the unique bone tissue, it is therefore necessary to understand bone cell biology in tumor bone environment. Recent findings of the Receptor Activator of Nuclear Factor-κB ligand (RANKL)/RANK/osteoprotegerin (OPG) molecular triad, the key regulators of bone remodeling, opened new era of bone research. Although RANK is an essential receptor for osteoclast formation, activation and survival, functional RANK expression has been recently identified on several bone-associated tumor cells. When RANK is expressed on secondary bone tumor cells, it is implicated in tumor cell migration, whereas this is not the case for primary bone tumors. In any case, RANK is not involved in RANK-positive cell proliferation or death. In two models of bone metastases secondary to melanoma or prostate carcinoma, *in vivo* neutralization of RANKL by OPG resulted in complete protection from paralysis, due to metastases of vertebral body, and a marked reduction in tumor burden in bones, but not in other organs. OPG also decreased tumor formation and tumor burden in a mouse model of primary bone tumor, osteosarcoma. In all these models, tumor cells express RANK. These data revealed that local differentiation factors, such as RANKL, play an important role in cell migration in a metastatic tissue-specific manner. These findings substantiate the novel direct role of RANKL/RANK in bone-associated tumors, and its capability of representing new therapeutic targets.

Key words: RANK, RANKL, Bone metastasis, Migration

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Introduction

Cancer is one of the major causes of death all over the world. Bone is a well-known target organ of cancer metastasis, resulting in severe pain, pathological fractures, nerve palsy and hypercalcemia (Coleman, 1997). Cancers induce marked deterioration of quality of life (QOL) and the activity of daily living (ADL) of patients. Thus, the social and economic burden of this disease is progressively increasing. As a result of the improved survival of patients with cancers by means of significant advances in primary cancer site control, much attention has been paid to cancer bone metastases.

Primary bone tumors such as osteosarcoma are also significant concerns, as well as metastatic ones. Aggressive chemotherapy has improved survival of osteosarcoma patients compared with the pre-chemotherapeutic era. Overall survival of non-metastatic patients at 5 years has improved to 60-70%, but has reached a plateau since the mid-1980s (Meyers et al., 2005). As the response of the tumor cells to chemotherapy drugs governs the survival of patients with osteosarcoma, multi-drug resistance is a major problem in osteosarcoma treatment. Thus, a breakthrough in the treatment of both primary and secondary bone tumors is really needed.

Since the late nineteenth century, it has been thought

that the microenvironment of the local host tissue actively participates in the propensity of certain cancers to metastasize to specific organs (Paget, 1889), but the specific factors involved are unknown.

Because bone tumors develop in a unique tissue, bone, bone cell biology must be taken into account. Bones are constantly remodelled throughout life by two complementary processes: bone matrix formation being regulated by osteoblasts and bone resorption by osteoclasts. The precise inter-relation between osteoblasts and osteoclasts leading to osteoclastogenesis is still partly unknown. However, the discovery of key factors involved in the control of osteoclastogenesis has moved bone research into a new era. Notably, factors belonging to the tumor necrosis factor (TNF)/TNF receptor family are: Receptor Activator of Nuclear Factor-κB (RANK/TNFRSF11A), its ligand RANKL/TNFSF11 and a decoy receptor for RANKL, osteoprotegerin (OPG/TNFRSF11B) (Anderson et al., 1997; Simonet et al., 1997; Lacey et al., 1998; Yasuda et al., 1998). Consequently, RANK expressed on osteoclasts/osteoclast precursor cells is an essential signaling receptor for osteoclastogenesis (Nakagawa et al., 1998). RANKL has been shown to both mediate osteoclastogenesis and activate mature osteoclasts, whereas OPG negatively regulates RANKL binding to RANK and reduces the half-life of membranous RANKL, therefore inhibiting bone resorption induced by osteoclasts (Tat et al., 2006).

Current findings have revealed that the RANKL/RANK/OPG molecular triad represents the key regulator, not only for normal, but also for pathological bone metabolism (Brown et al., 2001; Goltzman, 2001; Chen et al., 2006). The prevention of metastatic bone tumors by RANKL inhibitors [i.e., OPG or soluble RANK(sRANK)/RANK-Fc] in animal models of bone metastases highlights the critical role of this triad in cancer-induced bone manifestations (Zhang et al., 2001, 2003; Corey et al., 2005; Whang et al., 2005; Canon et al, 2007; Mountzios et al., 2007). These anti-tumor effects were only observed in bone, not in other tissues, such as subcutaneous models (Zhang et al., 2001, 2003). Thus, it has been believed that the anti-tumor effect induced by blocking RANKL/RANK interaction was the result of an indirect effect *via* osteoclasts.

Interestingly, functional RANK expression was recently reported in some bone-associated tumors and tumor cells (Jones et al., 2006; Wittrant et al., 2006; Mori et al., 2007a,b; Armstrong et al., 2008). As the migration of RANK-positive tumor cells was induced by RANKL stimulation (Jones et al., 2006; Mori et al., 2007c; Armstrong et al., 2008), the direct effect of RANKL on RANK-positive tumor cells has attracted special attention.

In this review, we update the accumulating evidence of RANK expression on cancer cells and its capability to be considered as a new therapeutic target to block RANKL induced bone resorption and therefore tumor progression.

RANK expression

RANK expression and its critical role in osteoclast formation, activation and survival are common knowledge. However, its expression has also been detected in other tissues, including mammary gland, lung, kidney and skeletal muscle (Theoleyre et al., 2004). Genetically, RANK is essential for the development of the lactating mammary gland during pregnancy (Fata et al., 2000) and for lymph node organogenesis in mouse embryos (Kong et al., 1999). However the role of RANK in other tissues is far from being completely understood.

The results of recent studies have opened the door to a new stage of RANK-related research. Namely, ourselves (Wittrant et al., 2006; Mori et al., 2007a,b) and other groups (Jones et al., 2006; Armstrong et al., 2008) have disclosed functional RANK expression on several bone-related tumor cells.

Primary bone tumors

Osteosarcoma

Osteosarcoma, the most frequent primary malignant bone tumor which originates from mesenchymal cells is characterized by a direct malignant osteoid and/or woven bone formation by tumor cells (Herzog, 2005), with approximately 1000 new cases per year both in North America and in Europe (Stiller et al., 2006).

Formerly, several studies reported RANK expression on osteoblastic cells at the transcript level without corresponding protein expression and functionality. However, the results were controversial (Huang et al., 2000; Miyamoto et al., 2002; Bu et al., 2003; Rifas et al., 2003). We first reported the expression of functional RANK on the POS-1 murine osteosarcoma cell line (Wittrant et al., 2006). Subsequently, we also revealed positive functional RANK expression in several human osteosarcoma cell lines, including MG-63, Saos-2 and MNNG/HOS, but not in U-2 OS (Mori et al., 2007b). This discrepancy might be explained, at least, by the heterogeneous population of osteosarcoma cell lines used which can lose some of their properties when cultured for a long time *in vitro* (Narita et al., 1996).

Furthermore, RANK is also detected in 11 out of 19 (57%) Caucasian osteosarcoma specimens, with a preferential expression in bad responders to standard chemotherapy as evaluated by Huvos score (Mori et al., 2007b). Although RANK is a membranous receptor, three types of localization were observed: nuclear, cytoplasmic and concomitantly in the nucleus and cytoplasm (Fig. 1). This is the first report on RANK distribution. Among 11 specimens of RANK-positive human osteosarcoma, RANK staining was localized to the cytoplasm (27%), nucleus (27%) or both compartments (46%). No apparent correlation was revealed between RANK status and age, sex, histological type and tumor localization (Mori et al.,

2007b).

Although nuclear RANK localization has not been formerly reported, this finding is not unprecedented. In fact, other membrane-associated growth factor receptors have been recently reported to function in the nucleus. This is the case for EGF (Lin et al., 2001) and FGF (Peng et al., 2002) receptors and other TNF receptor family members, including TNF-related apoptosis inducing ligand (TRAIL) receptors (Zhang et al., 2000) and the CD40 receptor (Lin-Lee et al., 2006). The signalosome associated with the TNF family has been also observed in the nucleus [e.g. TRAFs, NF-κB proteins, etc. (Min et al., 1998; Anest et al., 2003)], suggesting the potential nuclear migration of signaling pathway components after the binding of ligands from the TNF family to their membrane receptors.

Secondary (metastatic) bone tumors

Breast cancer

Breast cancer is the major cause of death among women worldwide. Bone metastasis occurs in 16-73% of breast cancer patients. Breast cancer keenly metastasizes to bone to form osteolytic lesions, which are mediated either directly by tumor cells or indirectly by osteoclasts (Guise, 2000; Käkönen and Mundy, 2003). However the factors favoring breast cancer growth in bone remain to be resolved.

Expression of RANK at the cell surface of three different human breast cancer cell lines (MDA-MB-231, MCF-7 and Hs578T) was detected by flow cytometry analysis (Jones et al., 2006) and by immunohistochemistry on human breast cancer cells at the site of the primary tumor and in lymph node metastases (Jones et al., 2006). Since RANK is expressed in normal mammary gland and has an essential role in the development of the lactating mammary gland during pregnancy (Fata et al., 2000), it could be hypothesized that malignant breast cancer cells also express RANK.

Prostate cancer

Prostate cancer is the most common cancer diagnosed in men, currently the major leading cause of cancer death (Landis et al., 1999; Mundy, 2002). Prostate cancer metastases to bone are observed in approximately 80% of patients and are associated with significant morbidity and mortality (Landis et al., 1999; Mundy, 2002). Unlike other solid tumors that are associated with osteolytic bone metastases, prostate cancer bone metastases stimulate an overall increase in both bone remodeling and bone volume (Kawano et al., 1989). Recent findings also strongly suggest the importance of osteoclast function in osteoblastic tumors (Zhang et al., 2001, 2003; Corey et al., 2005; Whang et al., 2005). However, the mechanisms underlining these processes are not fully determined.

RANK expression was analyzed by flow cytometry or immunohistochemistry on the cell surface of six prostate cancer cell lines established from a variety of disease stages: primary tumor (22Rv1), lymph node (LNCaP), brain (DU145) or bone (PC3, MDAPCa2b and C42B) metastases (Jones et al., 2006; Mori et al., 2007c; Armstrong et al., 2008). Four out of six cell lines (PC3, DU145, LNCaP and C42B) were RANK-positive both at the transcript and protein levels, whereas 22Rv1 and MDAPCa2b were RANK-negative at the mRNA level (Armstrong et al., 2008). As the RANK-positive cell lines were isolated from both androgen-dependent (LNCaP) and -independent (PC3, DU145 and C42B) tumors, it appeared that RANK expression on prostate cancer cells is not related to androgen-dependency.

Malignant melanoma

Melanoma now accounts for 5% of all cancers diagnosed. According to the American Cancer Society,

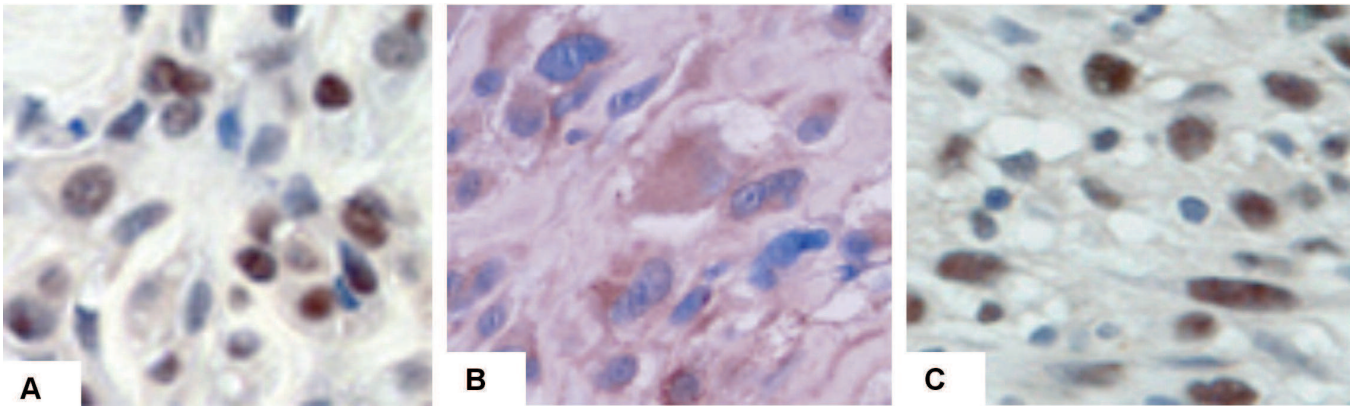

Fig. 1. Representative RANK immunostaining in human osteosarcoma samples. RANK can be localized in the nucleus **(A)**, cytoplasm **(B)** or concomitantly in both cytoplasmic and nuclear compartments **(C)** of tumor cells. Original magnifications, x 400

an estimated 59,940 new cases of melanoma were diagnosed in 2007, and approximately 8,110 patients will die of this disease (Jemal et al., 2007). In contrast to most metastatic bone tumors, including breast cancers, malignant melanoma metastasize to bone without stimulating osteoclastic bone resorption (Arguello et al., 1988). Notably, a mouse B16F10 melanoma subclone does not trigger osteoclast activation (Sanchez-Sweatman et al., 1997), but expresses high levels of RANK both at the mRNA and protein levels (Bakewell et al., 2003; Jones et al., 2006). Jones et al. therefore used this subclone in an *in vivo* study to demonstrate the involvement of RANK on the bone metastases process (Jones et al., 2006).

Downstream signal transduction of RANK

Downstream signal transduction pathways of RANK were analyzed using RANKL on RANK-positive bone-associated tumors (Jones et al., 2006; Mori et al., 2007b,c; Armstrong et al., 2008). RANKL treatment induced dose-dependent activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38, Jun-kinase (JNK), NF-κB, phospholipase C (PLC), protein kinase C (PKC), protein kinase B (PKB/AKT) and phosphatidylinositol 3-kinase (PI3-K) *via* their phosphorylation (Fig. 2). Furthermore, as RANKL-inhibitors (OPG or sRANK/RANK-Fc) blocked these activations, these signaling pathways were recognized as RANKL specific, reinforcing the hypothesis that RANK is functional on these tumor cells.

Biological function of RANK expressed on tumor cells

To summarize the knowledge from recent studies, it appears that the biological function of RANK expressed on tumor cells differs according to their origin (primary or secondary bone tumors).

RANKL-induced cell migration *in vitro* was confirmed in three different human breast cancer cell lines (MDA-MB-231, MCF-7 and Hs578T), two prostate cancer cell lines (PC3 and DU145) and a mouse B16F10 melanoma subclone (Jones et al., 2006; Mori et al., 2007c; Armstrong et al., 2008). *In vitro* migrations of cancer cells was evaluated in Boyden chambers (Jones et al., 2006; Armstrong et al., 2008) or by slit assay (Mori et al., 2007c). This RANKL-induced cell migration is concentration-dependent and is blocked by RANKL-inhibitors (OPG or sRANK/RANK-Fc). The specific signal transduction pathways responsible for RANK-positive tumor cell migration were determined using several specific inhibitors. Thus, ERK1/2, PLC, PKC and PI3-K signaling pathways were involved in RANKL-induced cell migration in RANK-positive tumor cells (Jones et al., 2006; Mori et al., 2007c; Armstrong et al., 2008) (Fig. 2).

Moreover, Armstrong et al. demonstrated that RANKL stimulation alters the gene expression that governs chemotaxis, migration and invasion of PC3 prostate cancer cells using microarray and quantitative RT-PCR (RT-qPCR) (Armstrong et al., 2008). Among 14,500 genes analyzed, 42 genes were significantly (greater than twofold, $p<0.05$) regulated by RANKL treatment (Armstrong et al., 2008).

Since the mouse B16F10 melanoma subclone used by Jones et al. does not trigger osteoclast activation, it allowed the authors to uncouple the direct effects of RANKL on tumor metastasis from osteoclast-mediated effects (Jones et al., 2006). *In vivo* inhibition of RANKL with the decoy receptor OPG resulted in a marked reduction of the melanin-producing B16F10 cancer foci

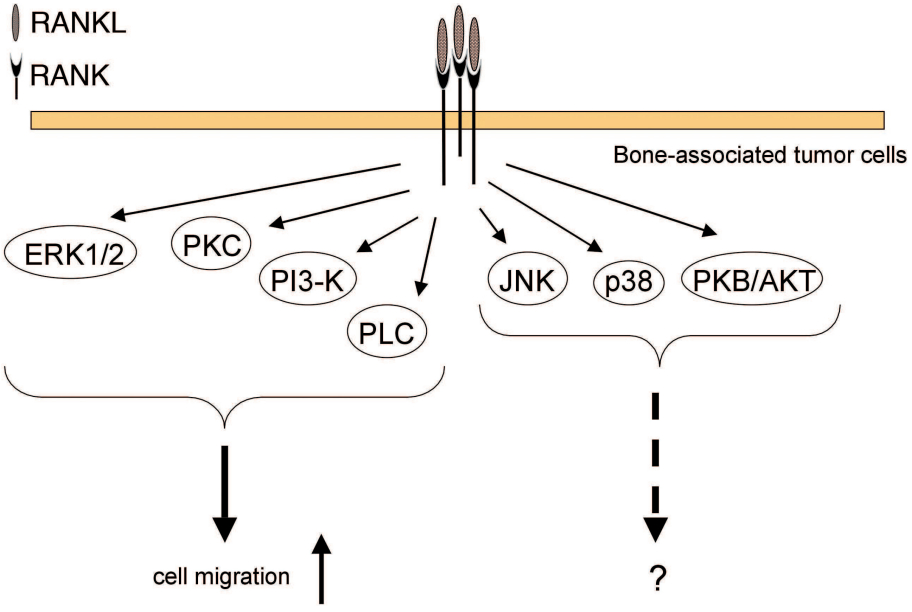

Fig. 2. Schematic presentation of the downstream signal transduction pathways activated by RANKL in RANK-positive cells. Extracellular signal-regulated kinases 1 and 2 (ERK1/2), phospholipase C (PLC), protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3-K) are involved in RANKL-induced RANK-positive tumor cell migration. Jun-kinase (JNK), p38 and protein kinase B (PKB/AKT) are involved in so far unknown mechanisms.

and tumor burden in a bone-specific fashion, as the tumor burden and metastasis of B16F10 melanoma cells into other tissue, including ovaries, adrenal glands and the brain were not attenuated by OPG treatment. In addition, OPG treatment reduced the tumor burden in vertebrae, which resulted in spinal cord invasion followed by clinical paralysis, and none of the OPG-treated mice developed clinical paralysis. Moreover, control mice injected with B16F10 cells showed high morbidity compared to OPG-treated mice. Thus, *in vivo* inhibition of RANKL by OPG can abrogate metastasis and tumor burden of B16F10 melanoma cells selectively in bones.

In contrast to these tumor cells, cell migration of RANK-positive osteosarcoma cells was not triggered by RANKL (Mori et al., 2007b). In RANK-positive POS-1 cells, RANKL induced expression of bone morphogenetic protein (BMP)-2 and slightly inhibited osteosarcoma cell proliferation (Wittrant et al., 2006). As a substitute, we also revealed a direct gene modulation by RANKL on RANK-positive Saos-2 human osteosarcoma cells, which was confirmed by RT-qPCR (Mori et al., 2007a). Briefly, 69 genes out of 6,864 cancer-related genes analyzed showed significantly different levels of expression in RANKL-treated Saos-2 cells compared to the control group: 48 were down-regulated, whereas the remaining 21 were up-regulated. The down-regulated group involved some genes implicated in protein metabolism, nucleic acid metabolism, intracellular transport, cytoskeleton organization and biogenesis, as well as apoptosis and signaling cascade. In the up-regulated group, the main genes affected by RANKL as referred to ontology biological processes were nucleic acid and protein metabolisms. Some genes shifted towards osteosarcoma development, whereas others towards its suppression. Further experiments are needed to determine the balance between pro- and anti-tumor activities of RANKL in osteosarcoma.

Alternatively, OPG treatment in the mouse POS-1 osteosarcoma model not only prevented osteosarcoma-induced osteolysis, but also indirectly inhibited tumor development, leading to a longer survival of OPG-treated animals (Lamoureux et al, 2007). Since POS-1 cells express RANK but not RANKL, these results indicate that RANKL has a propensity for osteosarcoma development in bone tumor environment.

RANKL-induced downstream signal transduction pathways of RANK, such as PKB/AKT, ERK1/2, p38, are frequently involved in cell proliferation and growth. However, RANKL had no apparent effects on proliferation or death of RANK-positive tumor cells (Jones et al., 2006; Mori et al., 2007b,c; Armstrong et al., 2008).

Conclusion

There is considerable experimental evidence that bone microenvironment provides reciprocal feedback leading to the formation of a vicious cycle that supports tumor growth and occurs in both osteolytic and osteoblastic bone metastases (Roodman, 2004). This vicious cycle has been proposed to explain tumor development in bone sites. Indeed, current studies have disclosed that blocking RANKL, RANK interaction prevents the progression of both breast and prostate cancer in bone by preventing bone degradation due to osteoclasts (Zhang et al., 2001, 2003; Corey et al., 2005; Whang et al., 2005; Canon et al., 2008; Mountzios et al., 2007).

In metastatic bone tumors, RANKL might have a propensity for bone-metastases development by synergistic effect on osteoclast activity, acting as a "soil" factor in bone environment. On the contrary, the exact

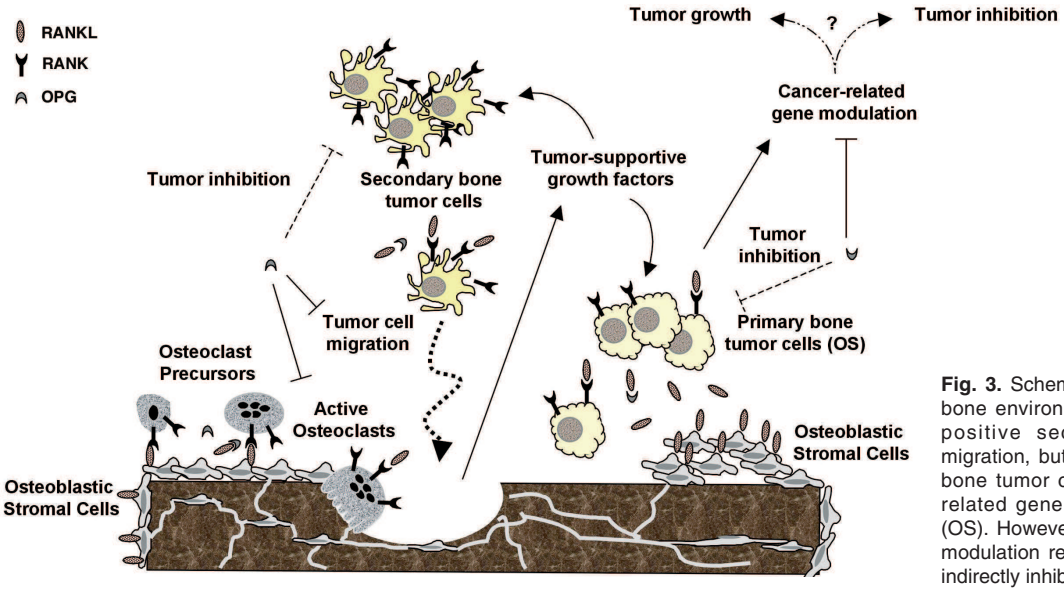

Fig. 3. Schematic presentation of the tumor bone environment. RANKL induces RANK-positive secondary bone tumor cells migration, but not in RANK-positive primary bone tumor cells. RANKL induced cancer-related gene modulation in osteosarcoma (OS). However, the exact effect of this gene modulation remains to be determined. OPG indirectly inhibits bone tumor growth.

role played by RANKL is not clear in primary bone tumors such as osteosarcoma.

RANKL triggered directional migration of mature osteoclasts towards a RANKL source (Jones et al., 2006). Moreover, a positive correlation between constant RANK expression with decreased/absent expression of RANKL and a high metastatic phenotype has been reported in breast carcinoma (Bhatia et al., 2005). In this context, it appears that RANK-positive cells are starving RANKL and are preferentially attracted to a RANKL rich bone environment. It could therefore be hypothesized that RANK expressed on tumor cells represents a specific marker of bone metastasis.

The term 'osteoimmunology' was recently coined to identify the collaboration between the field of bone biology and immunology (Arron and Choi, 2000). Thus, osteoimmunology is becoming increasingly important to understand the pathogenesis and to develop new therapeutic strategies affecting both systems. Alternatively, RANKL could act as a protector of tumor development, as it plays a role as a potent immune activator by inhibiting dendritic cell apoptosis (Wong et al., 1997). Further studies are needed to disclose the full potential of RANK/RANKL in bone oncology as therapeutic targets.

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Accepted June 25, 2008