Genetic variations, DNA methylation patterns and protein expression in RANK contribute to breast cancer cell behaviour in bone

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Introduction

The outcomes of breast cancer patients depend on the development of distance metastases (1). Bone is one of the most common locations for metastasis, and bone metastasis occurs in up to 70% of patients with advance breast cancer (2). Bone metastasis is associated with a number of skeletal complications including, pain, hypercalcemia and pathological fractures. Breast cancer cells commonly cause osteolytic metastases, a process that is at least partly dependent on osteoclast-mediated bone resorption (3,4). Osteoclasts the bone resorbing cells and their precursors express RANK, while osteoblast the bone forming cells express RANK ligand. Receptor activator of NF-kappa-B (RANK) and its ligand (RANKL) are members of the TNF receptor super family. These molecules do not only play a role in the pathogenesis of bone disorders but also have a central role in mammary gland biology (5). Recent studies reported that RANK is involved in cancer and metastasis (6). However, the exact role of RANK in cancer induced bone cell activity and osteolysis is not fully understood. The present study, therefore, investigated the role of RANK receptor in breast cancer metastatic cell behaviour in bone using different approaches.

Objective

The aim of this study was to investigate the role of RANK in breast cancer induced bone cell activity and osteolysis *in vitro* and *in vivo*.

RANK is highly expressed in human bone tropic MDA-231 (MDA^{BT}) breast cancer cells

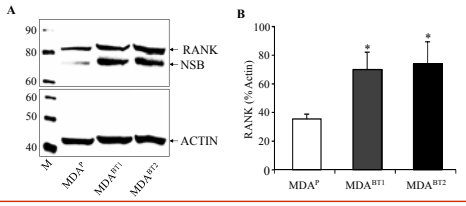

Figure 1. RANK expression in breast cancer cells were assessed by Western blot analysis (A). The quantification of RANK expression from the blot is shown in (B) and expressed as a ratio of actin (n=3).

Human bone tropic MDA breast cancer cells express altered forms of the RANK mRNA

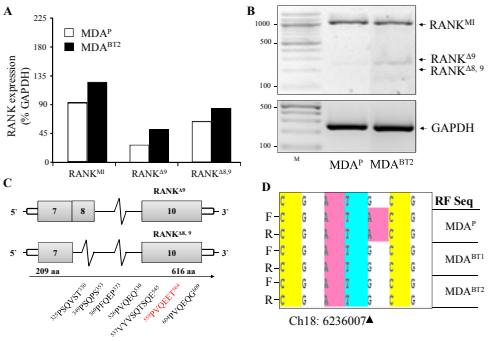

Figure.2: RANK isoforms were detected in breast cancer cell lines using RT-PCR (A). Different expression patterns were observed (B). A specific region of RANK (exon7-10) is also indicated (C). Genetic variation analysis revealed an amino acid change (p.Ala192Thr) in parental MDA^P (D). This alteration could affect RANK protein, as predicted by SIFT and PolyPhen analysis.

RANK ligand stimulates P38 and enhances the spreading and migration of bone tropic MDA cells

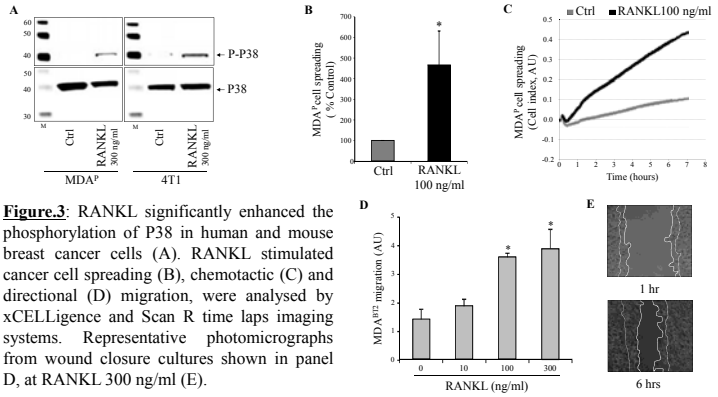

Figure.3: RANKL significantly enhanced the phosphorylation of P38 in human and mouse breast cancer cells (A). RANKL stimulated cancer cell spreading (B), chemotactic (C) and directional (D) migration, were analysed by xCELLigence and Scan R time laps imaging systems. Representative photomicrographs from wound closure cultures shown in panel D, at RANKL 300 ng/ml (E).

Long-term exposure of human MDA breast cancer cells enhances the ability of these cells to stimulate osteoclastogenesis and alters methylation in *RANK*

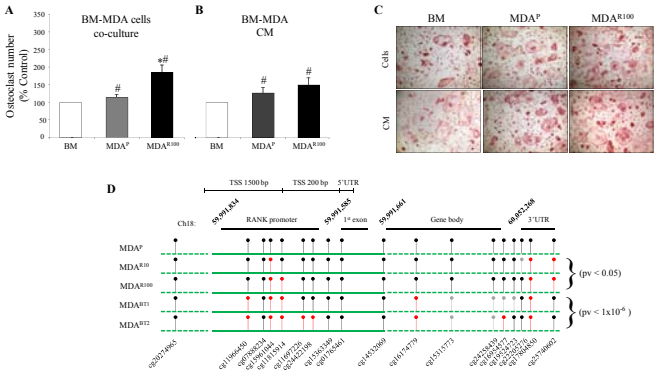

Figure.4: Long-term exposure of human parental MDA^P breast cancer cells to RANKL 100 ng/ml for up to 50 passages enhances their ability to stimulate osteoclastogenesis. Cancer-bone marrow (BM) cells co-culture A (cancer cells) and B (conditioned medium, CM). Images of the cultures (C). Methylation pattern of *RANK* in breast cancer cells was detected by 450K illumina DNA methylation system. Dots indicate hyper-(red) and hypo-methylated (grey) positions compared to parental (D).

Knockdown of *RANK* in bone tropic MDA breast cancer cells reduces cell migration and ability to stimulate osteoclastogenesis

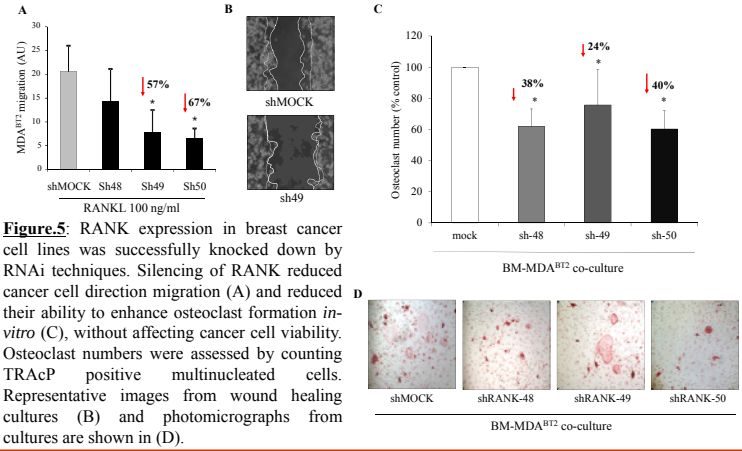

Figure.5: RANK expression in breast cancer cell lines was successfully knocked down by RNAi techniques. Silencing of RANK reduced cancer cell direction migration (A) and reduced their ability to enhance osteoclast formation *in-vitro* (C), without affecting cancer cell viability. Osteoclast numbers were assessed by counting TRAcP positive multinucleated cells. Representative images from wound healing cultures (B) and photomicrographs from cultures are shown in (D).

Conclusion

Silencing of the RANK receptor in bone tropic MDA-231 breast cancer cells reduces cell motility and ability to stimulate osteoclastogenesis via a mechanism dependent in part on P38 inhibition. Moreover, long-term exposure to RANKL did not significantly induces "*bony*" epigenetic changes in *RANK* but there were significant differences in *RNAK* methylation profile between parental and bone tropic breast cancer cells. The role of these effects in the regulation of breast cancer cell behaviour in bone will require further in vivo investigation.

References

(1) Armstrong AP, *et al.* The Prostate 2008;68:92-104.
(2) Tang ZN, *et al.* Oncology reports 2011;26:1243-50.
(3) Jones DH, *et al.* Nature 2006;440:692-6.
(4) Papanastasiou A, *et al.* Breast Cancer Res 2012;14:1-16.
(5) Schramek D, *et al.* Nature 2010;468:98-102.
(6) Sigl V, *et al.* Cytokine & Growth Factor Reviews 2014.

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