



Denosumab inhibits MCF-7 cell line-induced spontaneous osteoclastogenesis via the RANKL/MALAT1/miR-124 axis

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Background: Denosumab is an inhibitor of receptor activator of NF- κ B ligand (RANKL), which inhibits bone metastasis (BM) in breast cancer (BC), but does not completely control cancer cell BM in some BC patients. This study was designed to study whether denosumab inhibits human BC cells (MCF-7) cell line-induced spontaneous osteoclastogenesis via RANKL/metastasis-associated lung adenocarcinoma transcript 1 (MALAT1)/miR-124 axis.

Methods: We established a co-culture system of MCF-7-induced spontaneous osteoclastogenesis in RAW 264.7 cells, and denosumab is added into the co-culture system to inhibit RAW 264.7 cell differentiation into osteoclasts. Real-time PCR (RT-PCR), immunofluorescence and western blotting analysis were used to detect gene expression, while tartrate-resistant acid phosphatase (TRAP) staining was used to assess osteoclast formation.

Results: Denosumab inhibits MCF-7 cell line-induced spontaneous osteoclastogenesis, and the inhibition of denosumab was found to be more pronounced after MALAT1 downregulation and miR-124 overexpression. However, MALAT1 knockdown or miR-124 overexpression did not alter RANKL protein expression. Moreover, the dual luciferase gene reporter system showed that miR-124 targeted the inhibition of MALAT1, while si-MALAT1 upregulated miR-124 expression. miR-124-mimics were able to decrease the expression of Rab27a, IL-11, activated T-cell nuclear factor 1 (NFATc1) and TARP protein.

Conclusions: Denosumab inhibits MALAT1 expression by inhibiting RANKL, thereby upregulating miR-124 expression, which ultimately inhibits MCF-7 cell line-induced pseudo osteoclastogenesis.

Keywords: Denosumab; osteoclastogenesis; receptor activator of NF- κ B ligand (RANKL); metastasis-associated lung adenocarcinoma transcript 1 (MALAT1); miR-124

Submitted Sep 11, 2019. Accepted for publication Dec 12, 2020.

doi: 10.21037/tcr.2020.03.17

View this article at: <http://dx.doi.org/10.21037/tcr.2020.03.17>

Introduction

Breast cancer (BC) is a common malignant tumor in women, accounting for most malignant tumors in women. Approximately 1.2 million new BC patients are diagnosed and around 500,000 people die of BC each year worldwide (1,2). Clinical studies have shown that the incidence of bone metastases (BMs) in BC is highest in two common tumor

metastatic organs, the lung and liver (3,4). Since the BM mechanism of BC has not been fully elucidated, BM cannot be cured, resulting in a high mortality rate (5,6).

Denosumab targets the inhibition of receptor activator of NF- κ B ligand (RANKL), which inhibits the activation of osteoclasts and decreases bone absorption and destruction (7). It has been widely used to treat or prevent BM of solid tumors, such as BC, kidney cancer and urinary

system tumors, and has been proven to exert a therapeutic effect (8). However, for some BM patients, the effect of denosumab treatment on the prevention of BM is extremely low (9,10), but the specific cause is unknown, and we suspect that this may be related to the expression of effector molecules downstream of RANKL. Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) was found to be highly expressed in BC tissues and promoted the invasion and migration of BC cells (11,12). In addition, previous studies have found that RANKL can promote the expression of MALAT1 in human osteoblasts, thereby regulating the biological characteristics of human osteoblasts (13). miR-124 has been shown to be downregulated in BC tissues and inhibits EMT and metastasis in BC (14,15), and regulates MALAT1 expression (16).

This study aimed to determine whether the downstream molecular mechanism of denosumab inhibition of BM in BC proceeds by inhibiting RANKL and if it is related with MALAT1 and miR-124 expression. It was also determined whether MALAT1 and miR-124 exert a feedback effect on denosumab. In this study, we constructed a BM model by establishing a MCF-7 and RAW 264.7 non-contact co-culture system, and altered the expression of MALAT1 and miR-124 in RAW 264.7 cells to explore the effect of MALAT1 and miR-124 on the inhibition of MCF-7-induced osteoclastogenesis *in vitro* caused by denosumab. We found that denosumab inhibits MALAT1 expression by inhibiting RANKL, thereby upregulating miR-124 expression, and ultimately inhibiting MCF-7 cell line-induced pseudo osteoclastogenesis.

Methods

Cell culture and treatment

RAW 264.7 (SC-6003, ATCC, USA) and MCF-7 cells (CRL-10781) were all cultured in Dulbecco's Modified Eagle Medium (DMEM) solutions (31600091, Gibco, USA), into which 10% fetal bovine serum (10099-141, Gibco, USA) was added, while RAW 264.7 cells were co-cultured with MCF-7 cells in a non-contact system (Figure 1A). Denosumab (Amgen, USA) was added into the co-culture system for 5 days at a concentration of 0.1 mg/mL.

Tartrate-resistant acid phosphatase (TRAP) staining

Cells in cell culture suspension and urine were collected

through centrifugation, and phosphate buffer saline (PBS) was used to resuspend the sediment. Then, the cells were cytospun onto slides, and TRAP staining was performed using a TRAP staining solution kit (Solarbio, G1942, China).

Cell transfection

The miR-negative control (miR-NC) and mimic of miR-124, and si-NC and si-MALAT1 were purchased from Sangon Biotech (Shanghai, China), and were directly transferred into cells using LipofectamineTM 2000 transfection reagent (11668019, Invitrogen, CA, USA). The wild type and mutated mRNA 3'-UTR of MALAT1 were first connected to pISCHECK2 (Promega, WI, USA) before being transfected into cells as miRNAs.

Real-time quantitative PCR (RT-qPCR)

RT-qPCR was used to detect mRNA, miRNA and lncRNA expression, as previously described (17), with the PCR primers used given in Table 1.

Western blotting analysis

Levels of Rab27a, IL-11, activated T-cell nuclear factor 1 (NFATc1), TRAP and GAPDH protein were analyzed using western blotting analysis, as previously described (17). The primary antibodies used were Rab27a (ab55667, 1:500), IL-11 (ab187167, 1:1,000), NFATc1 (ab25916, 1:1,000), TRAP (ab65854, 1:1,500) and GAPDH (ab8245, 1:3,000), which were all purchased from Abcam.

Cellular immunofluorescence

Cells in cell culture suspension and urine were collected through centrifugation, and PBS was used to resuspend the sediment. Then, the cells were cytospun onto slides, and fixed using 4% paraformaldehyde for 0.5 hours at room temperature. Thereafter, they were incubated with a primary body, RANKL (ab45039, 1:100, ABCAM, UK), at 4 °C and left overnight. Goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody (A11008, 1:500, Invitrogen, USA) was used as the secondary antibody at room temperature for 1 hour, followed by incubation with 5 ug/mL 4',6-diamidino-2-phenylindole (DAPI) (D8417, Sigma, USA) at room temperature for 5 minutes.

Table 1 Primers for RT-qPCR

Gene	Primer sequence (5'-3')
<i>MALAT1</i>	Forward: AAAGCAAGGTCTCCCCACAAG Reverse: GGTCTGTGCTAGATCAAAGGCA
<i>miR-124</i>	Forward: ACACTCCAGCTGGGCGTGTTCACAGCGGAC Reverse: TGGTGTCTGGAGTCG
<i>Cathepsin K</i>	Forward: CTGAAGATGCTTCCCATATGTGGG Reverse: GCAGGCGTTGTTCTTATTCCGAGC
<i>CAII</i>	Forward: CTTCAGGACAATGCAGTGC Reverse: ATCCAGGTCACACATTCCAGC
<i>Integrin av</i>	Forward: GCCAGCCCATTGAGTTTGATT Reverse: GCTACCAGGACCACCGAGAAG
<i>Integrin β3</i>	Forward: TTACCCCGTGGACATCTACTA Reverse: AGTCTTCCATCCAGGGCAATA
<i>RANK</i>	Forward: ACCTCCAGTCAGCAAAGAAGT Reverse: TCACAGCCCTCAGAATCCAC
<i>TRAP</i>	Forward: ACACAGTGATGCTGTGTGGCAACTC Reverse: CCAGAGGCTTCCACATCTATGCTGG
<i>TRAF6</i>	Forward: AGCCCACGAAAGCCAGAAGAA Reverse: CCCTTATGGATTTGATGATGC
<i>MMP-9</i>	Forward: CGAGTGGACGCGACCGTAGTTGG Reverse: CAGGCTTAGAGCCACGACCATACAG
<i>U6</i>	Forward: CTCATAGGGTTGTTGCTCGG Reverse: AACGCTTACGAATTTGCGT
<i>GAPDH</i>	Forward: TAGCGGCTAGCGGTAT Reverse: CGGGGCTATGGCTAGCTAGCTTTC

RT-qPCR, real-time quantitative PCR.

Statistical analysis

Data were analyzed using SPSS 20.0 software. Data between two groups were compared using student's *t*-test, and data between multiple groups were compared using one-way ANOVA, with Duncan test as the post hoc test. A P value of

<0.05 was considered to indicate a significant difference.

Results

Denosumab inhibits spontaneous osteoclastogenesis of RAW 264.7 cells

First, we established a non-contact co-culture system (*Figure 1A*), in which MCF-7 cells were cultured in the upper chamber and RAW 264.7 cells were cultured in the lower chamber. As shown in *Figure 1B*, the TRAP⁺ osteoclast of the control group was significantly higher than that of the denosumab group, which was added with denosumab to the co-culture system to prevent osteoclast differentiation of RAW 264.7 cells, induced by MCF-7 cells. Moreover, we also measured the expression of bone resorption genes, such as cathepsin K, CAII, integrin av and integrin β3, and the expression of osteoclast phenotype genes, such as RANK, TRAP, TRAF6 and MMP-9. We found that the mRNA expression levels of bone resorption genes (*Figure 1C*) and osteoclast phenotype genes (*Figure 1D*) in control group were all significantly higher than that of the denosumab group.

MALAT1 knockdown enhances denosumab function

We transferred si-MALAT1 into RAW 264.7 cells to knockdown MALAT1, and then the MALAT1 knockdown cells were placed in the lower chamber of the co-culture system to be cultured. TARP staining was used to measure TARP⁺ osteoclast. We found that (*Figure 2A*) the TRAP⁺ osteoclast in control group was highest, while the TRAP⁺ osteoclast in si-MALAT1 group was the lowest, and the results of RT-qPCR confirmed that (*Figure 2B*) the level of MALAT1 in the si-MALAT1 group was the lowest. However, immunofluorescence detection of RANKL in the si-MALAT1 group found that its expression was not significantly different from that of the denosumab group (*Figure 2C*).

miR-124 overexpression enhances denosumab function

We transfected the miR-124-mimic into RAW 264.7 cells to upregulate miR-124, and then the resulting miR-124

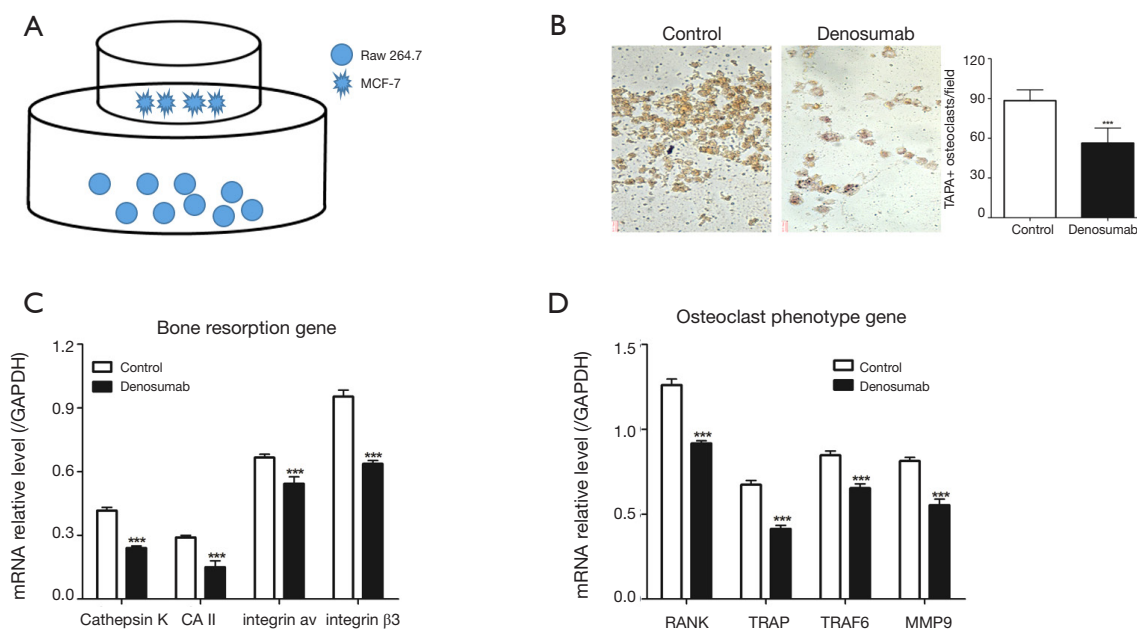


Figure 1 Denosumab inhibits MCF-7-induced spontaneous osteoclastogenesis of RAW 264.7 cells. (A) Co-culture system for spontaneous osteoclastogenesis of RAW 264.7 cells induced by MCF-7 cells; (B) representative field of co-culture after TRAP staining and statistics of TRAP+ osteoclastogenesis; (C,D) the mRNA expression of bone resorption gene (C) and osteoclast phenotype gene (D). Three times for each experiment and ***, $P < 0.001$ vs. control group. Scale bar = 20 μ m. TRAP, tartrate-resistant acid phosphatase.

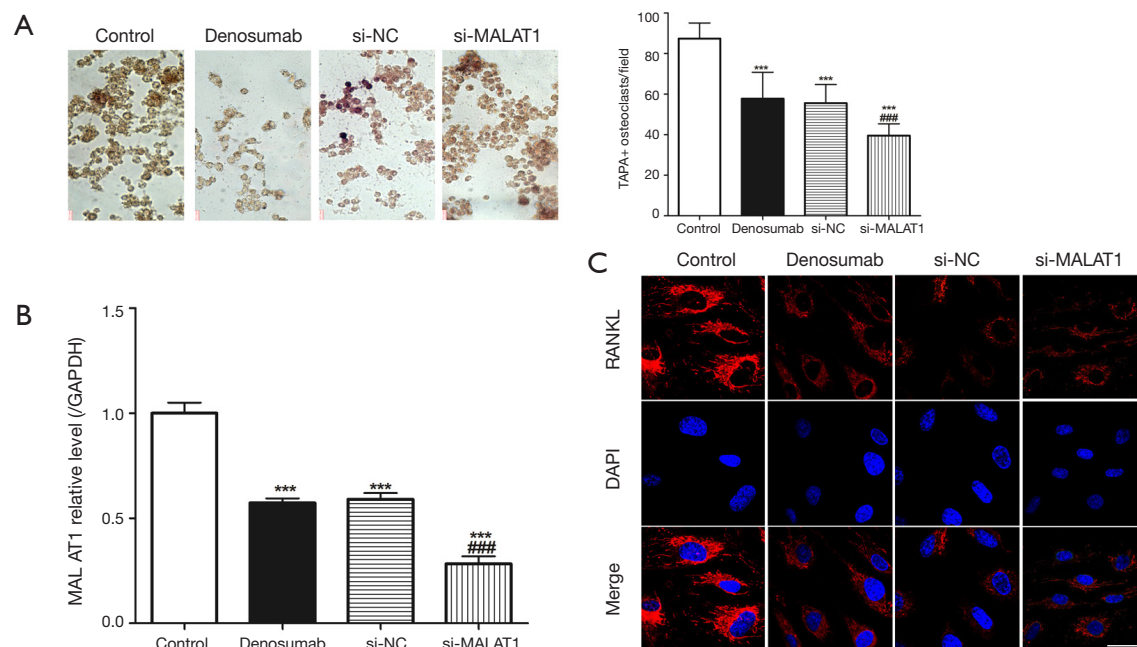


Figure 2 MALAT1 regulates spontaneous osteoclastogenesis of RAW 264.7 cells inhibited by denosumab. (A) Representative field of co-culture after TRAP staining and statistics of TRAP+ osteoclastogenesis; (B) MALAT1 expressed in each group; (C) immunofluorescence staining of RANKL in RAW 264.7 cells of each group. Three times for each experiment; ***, $P < 0.001$ vs. control group, and ###, $P < 0.001$ vs. denosumab group. Scale bar = 50 μ m. TRAP, tartrate-resistant acid phosphatase; RANKL, receptor activator of NF- κ B ligand.

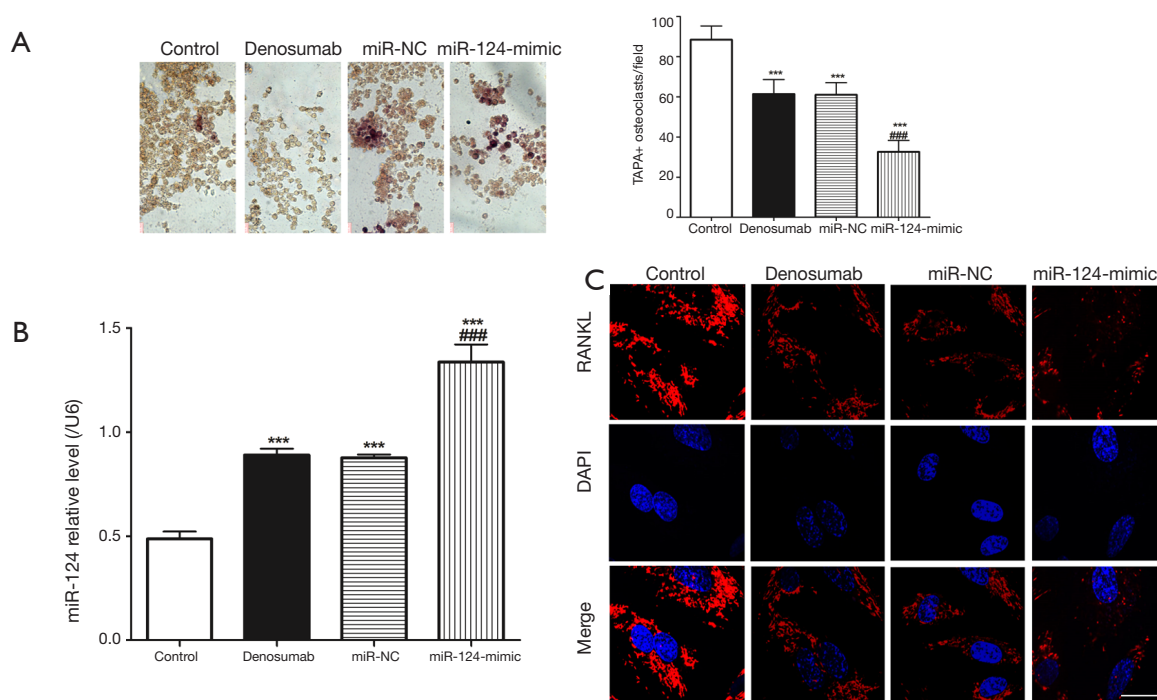


Figure 3 miR-124 regulates spontaneous osteoclastogenesis of RAW 264.7 cells inhibited by denosumab. (A) Representative field of co-culture after TRAP staining and statistics of TRAP+ osteoclastogenesis; (B) miR-124 expressed in each group; (C) immunofluorescence staining of RANKL in RAW 264.7 cells of each group. Three times for each experiment, ***, $P < 0.001$ vs. control group, and ###, $P < 0.001$ vs. denosumab group. Scale bar = 50 μm . TRAP, tartrate-resistant acid phosphatase; RANKL, receptor activator of NF- κB ligand.

overexpressing cells were placed in the lower chamber of the co-culture system to be cultured. TARP staining was used to measure TARP+ osteoclast. We found that the TRAP+ osteoclast in control group was the highest (Figure 3A), and that the TRAP+ osteoclast in miR-124-mimic group was the lowest, and the results of RT-qPCR confirmed that (Figure 3B) the level of miR-124 in the miR-124-mimic group was the lowest. However, immunofluorescence detection of RANKL in the miR-124-mimic group found that its expression was not significantly different from that of the denosumab group (Figure 3C).

miR-124 negatively regulates osteoclast differentiation and mutual inhibition along with MALAT1

After analyzing the sequences of MALAT1 and miR-124, we found that MALAT1 and miR-124 have complementary sequences (Figure 4A). We validated the results using the luciferase gene reporter system and found that transfection with the miR-124-mimic significantly increased WT type 3'-UTR luciferase activity of RAW 264.7 cells, but did

not have the same effect on that of MUT (Figure 4B,C). In addition, we also found that MALAT1 knockdown by si-MALAT1 could increase miR-124 expression, and that miR-124 overexpression could decrease the expression of Rab27a, IL-11, NFATc1 and TARP proteins (Figure 4D,E).

Discussion

There are four basic elements required for the development of BM in malignant tumor cells: cancer cells, osteoblasts, osteoclasts and bone matrix (18). Metastatic cancer cells cannot directly destroy bone, and its metastases must first activate osteoclasts to differentiate and mature, and then only can osteoclasts mediate bone resorption to cause tumorous bone destruction for further local growth, which involves an important signal transduction pathway, the RANK/RANKL/OPG pathway (19). Normal bone metabolism maintains a dynamic balance between the osteogenic effects of osteoblasts and the bone resorption effect of osteoclasts, with the RANKL/RANK/OPG pathway being one of the main mechanisms of regulating

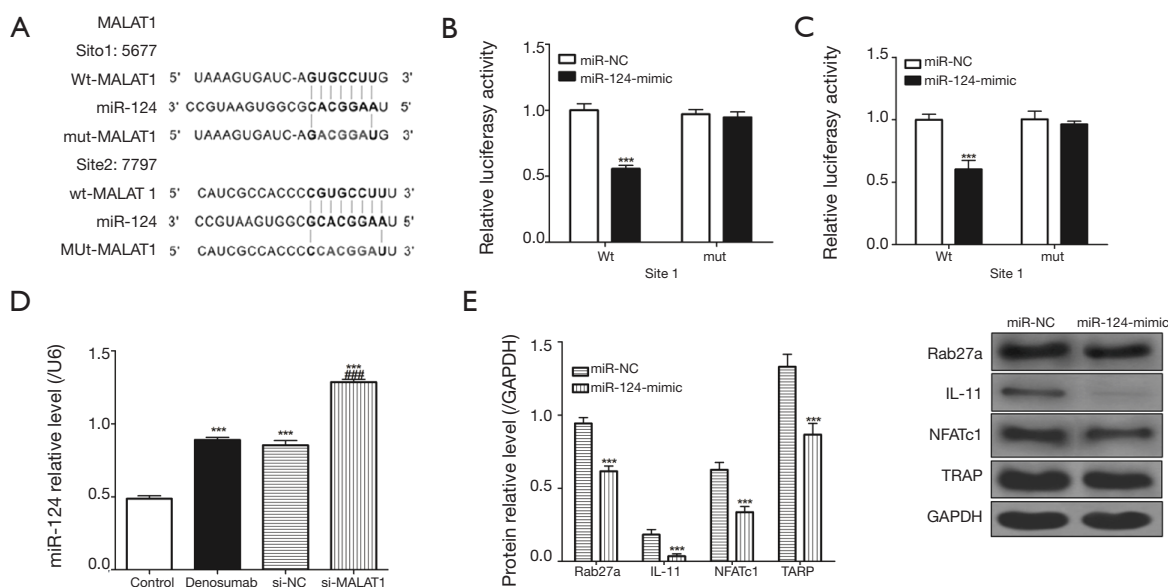


Figure 4 miR-124 and MALAT1 inhibit each other in RAW 264.7 cells. (A) Sequence in which MALAT1 and miR-124 bind to each other; (B,C) dual luciferase gene reporter system validates sequence of direct binding of MALAT1 to miR-124; (D) miR-124 expressed in each group; (E) levels of Rab27a, IL-11, NFATc1 and TARP protein in RAW 264.7 cells after transferring to miR-NC or miR-124-mimic. Three times for each experiment, ***, $P < 0.001$ vs. control group, and ###, $P < 0.001$ vs. denosumab group. MALAT1, metastasis-associated lung adenocarcinoma transcript 1; NFATc1, activated T-cell nuclear factor 1; miR-NC, miR-negative control; TRAP, tartrate-resistant acid phosphatase.

bone homeostasis. OPG, RANKL and RANK are members of the tumor necrosis factor family, and OPG and RANKL are expressed in osteoblasts and bone stromal cells, while RANK is expressed on the surface of osteoclast precursors. During the process of bone destruction, RANKL acts as an activating factor that induces the maturation and activation of osteoclasts by binding to RANK on the surface of osteoclast precursors, which ultimately leads to bone resorption. However, OPG is a soluble RANKL inhibitor that binds to RANKL and inhibits the binding of RANKL to RANK, thereby inhibiting the differentiation and maturation of osteoclast precursors (20).

In this study, we found that MCF-7 could induce the spontaneous osteoclast differentiation of RAW 264.7 cells in the non-contact co-culture system of MCF-7 and RAW 264.7 cells, and that denosumab not only significantly inhibited the number of TARP osteoclasts, but also decreased the expression of bone resorption genes and osteoclast phenotype genes. This indicates that: (I) the non-contact co-culture system of MCF-7 and RAW 264.7 cells in this study can be used to demonstrate BM

mechanism of BC cells *in vitro*; (II) Denosumab can inhibit the spontaneous osteoclast differentiation of RAW 264.7 cells induced by MCF-7. As an inhibitor of RANKL, denosumab has been widely used in clinical settings to treat BM of cancer and has been shown to produce a good effect. Theoretically, denosumab can block the pathway of BM by interfering with the RANKL/RANK axis of bone, delay the progression of BM, decrease tumor burden and prolong patient survival time, and this theory has been confirmed in animal models (21). Canon and Gonzalez-Suarez found that denosumab can decrease tumor burden of bone in advanced BM animal models, hinder tumor progression, prolong the formation in mice and decrease the development of spontaneous lung metastases (22,23). However, denosumab did not prolong survival and BM time of patients with malignant tumors in clinical trials (24). Two theories that may explain the difference in these outcomes have been accepted. On the one hand, activation of osteoclasts not only involves OPG, RANKL and RANK, but involves many other related factors involved in the regulation of activation of bone cells, such as M-CSF and sRANKL (25,26). On the

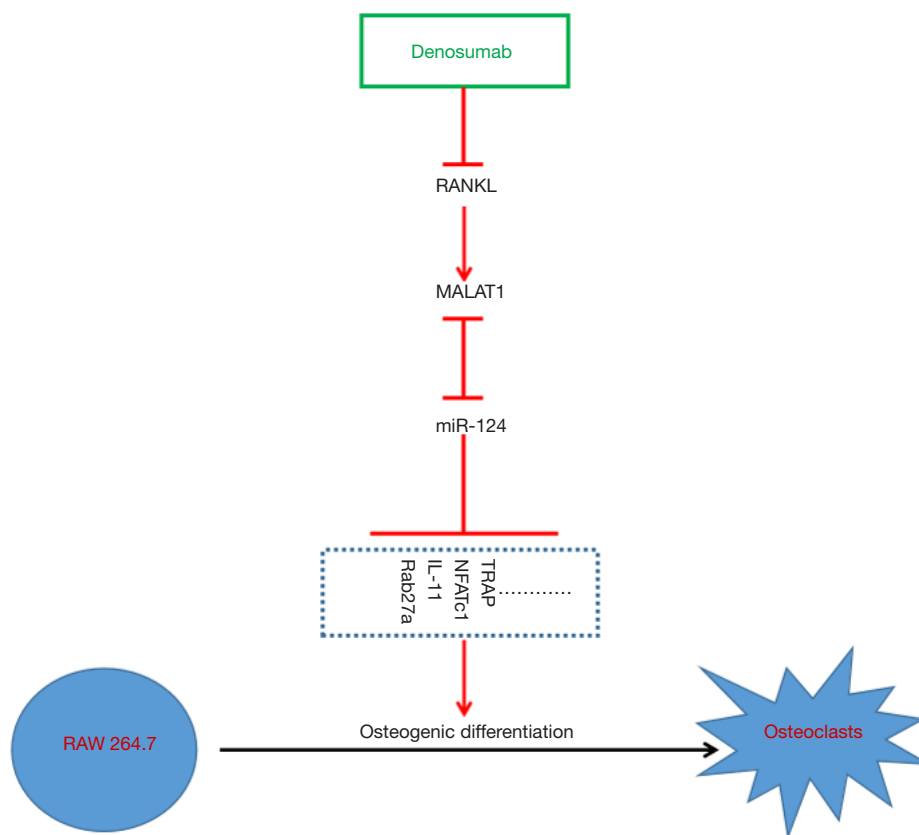


Figure 5 Molecular mechanism of denosumab inhibiting MCF-7-induced spontaneous osteoclastogenesis. RANKL, receptor activator of NF- κ B ligand; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; TRAP, tartrate-resistant acid phosphatase; NFATc1, activated T-cell nuclear factor 1.

other hand, the OPG/RANKL/RANK pathway regulated osteoclast activation also requires downstream effector molecules, which undergo various changes in BM.

In this study, we found that inhibition of MALAT1 expression or upregulation of miR-124 expression in RAW 264.7 cells did not alter RANKL expression, but significantly increased denosumab induced inhibition of spontaneous osteoclast differentiation caused by MCF-7 cells, while denosumab could also inhibit MALAT1 expression and increase miR-124 expression.

MALAT1 is also named nuclear-enriched autosomal transcript 2, and is an important member of the lncRNA family that was discovered in NSCLC tissues in 2003 (27). Many studies have found that MALAT1 is abnormally expressed in multiple tumor tissues (28,29). Previous research has found that MALAT1 can promote the proliferation, metastasis and invasion of tumor cells (30,31) through the recruitment of specific SR protein family

members (32,33), and is involved in epigenetic regulation (34,35) and cell cycle regulation (36). In BC, MALAT1 is considered as an oncogene and was found to be highly expressed in BM tumor tissues, and the high expression of MALAT1 was found to promote the proliferation, migration and invasion of BC cells. In addition, MALAT1 has been shown to promote cancer cell BM in non-small cell lung cancer (37). In human mature bone cells, RANKL promotes the expression of MALAT1. Combined with the results of the present study, MALAT1 may be considered as a downstream effector molecule of RANKL, and high expression of MALAT1 may play a role in promoting BM of BC cells.

miR-124 is a miRNA that is closely involved with malignant tumors, and many studies have shown that miR-124 is downregulated in a variety of malignant tumor tissues, including BC, and *in vitro* and *in vivo* experiments have confirmed that miR-124 is a tumor suppressor gene

that inhibits the proliferation, invasion and migration of many malignant tumor cells, including BC cells. Moreover, previous research has also found that miR-124 can inhibit osteoclast differentiation (38). In this study, we found that miR-124 targeted the inhibition of MALAT1 expression, but its expression was also inhibited by MALAT1, and miR-124 could significantly inhibit the expression of Rab27a, IL-11, NFATc1 and TARP proteins. Previous studies have found that miR-124 could inhibit the osteoclastic differentiation of RAW 264.7 cells, induced in BC cell lines through the targeted inhibition of IL-11 (39), Rab27a (40) and NFATc1 (41).

Conclusions

Taken together, the results of this study found that denosumab inhibits MALAT1 expression by inhibiting RANKL, thereby upregulating miR-124 expression, which ultimately inhibits the pseudo osteoclastogenesis caused by the MCF-7 cell line (Figure 5). High expression of MALAT1 and low expression of miR-124 in BC tumor tissues may be reasons why denosumab cannot be used to effectively treat and prevent BM in BC patients.

Acknowledgments

Funding: None.

Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr.2020.03.17>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by the Ethics Committee of The Fourth Hospital of Hebei Medical University (No. 2019123).

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Cite this article as: Feng Q, Wang D, Feng J, Guo P, Geng C. Denosumab inhibits MCF-7 cell line-induced spontaneous osteoclastogenesis via the RANKL/MALAT1/miR-124 axis. *Transl Cancer Res* 2020;9(4):2482-2491. doi: 10.21037/tcr.2020.03.17