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ARTICLE

Melatonin interrupts osteoclast functioning and suppresses tumor-secreted RANKL expression: implications for bone metastases

Po-I Liu[1,2] · An-Chen Chang[3] · Jiun-Lin Lai[1] · Tien-Huang Lin[4,5] · Chun-Hao Tsai[6,7] · Po-Chun Chen[3,8,9] · Ya-Jing Jiang[1] · Liang-Wei Lin[7] · Wei-Chien Huang[1] · Shun-Fa Yang[10,11] · Chih-Hsin Tang[1,7,12,13]

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Abstract

Cancer-related bone erosion occurs frequently in bone metastasis and is associated with severe complications such as chronic bone pain, fractures, and lower survival rates. In recognition of the fact that the darkness hormone melatonin is capable of regulating bone homeostasis, we explored its therapeutic potential in bone metastasis. We found that melatonin directly reduces osteoclast differentiation, bone resorption activity and promotes apoptosis of mature osteoclasts. We also observed that melatonin inhibits RANKL production in lung and prostate cancer cells by downregulating the p38 MAPK pathway, which in turn prevents cancer-associated osteoclast differentiation. In lung and prostate bone metastasis models, twice-weekly melatonin treatment markedly reduced tumor volumes and numbers of osteolytic lesions. Melatonin also substantially lowered the numbers of TRAP-positive osteoclasts in tibia bone marrow and RANKL expression in tumor tissue. These findings show promise for melatonin in the treatment of bone metastases.

Introduction

In postmortem studies, between 75 and 84% of patients who die of breast or prostate cancer have evidence of metastatic bone disease [1, 2]; incidence rates are considerably lower for other primary cancers (e.g., thyroid cancers 50%, lung cancer 44%, and renal cancers 37%) [2]. Bone metastases occur in every type of cancer, but are most frequently diagnosed in patients with prostate, breast, or lung cancer [3].

Amongst the lesion classifications of purely osteolytic or osteoblastic, or mixed osteolytic/osteoblastic, osteolytic lesions are characterized by destruction of normal bone and pathologic fracture [4]. Metastatic bone cancer cells secrete osteolytic factors including tumor necrosis factor alpha,

These authors contributed equally: Po-I Liu, An-Chen Chang

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✉ Shun-Fa Yang
ysf@csmu.edu.tw

✉ Chih-Hsin Tang
chtang@mail.cmu.edu.tw

[1] Graduate Institute of Biomedical Science, China Medical University, Taichung, Taiwan

[2] Department of General Thoracic Surgery, Asia University Hospital, Taichung, Taiwan

[3] Translational Medicine Center, Shin-Kong Wu Ho-Su Memorial Hospital, Taipei, Taiwan

[4] Department of Urology, Buddhist Tzu Chi General Hospital Taichung Branch, Taichung, Taiwan

[5] School of Post-Baccalaureate Chinese Medicine, Tzu Chi University, Hualien, Taiwan

[6] Department of Orthopedic Surgery, China Medical University Hospital, Taichung, Taiwan

[7] School of Medicine, China Medical University, Taichung, Taiwan

[8] Department of Medical Research, China Medical University Hospital, China Medical University, Taichung, Taiwan

[9] Department of Biotechnology, College of Health Science, Asia University, Taichung, Taiwan

[10] Institute of Medicine, Chung Shan Medical University, Taichung, Taiwan

[11] Department of Medical Research, Chung Shan Medical University Hospital, Taichung, Taiwan

[12] Chinese Medicine Research Center, China Medical University, Taichung, Taiwan

[13] Department of Biotechnology, College of Health Science, Asia University, Taichung, Taiwan

interleukin, and receptor activator of nuclear factor-kappa B ligand (RANKL), all of which enhance osteoclast bone resorption and enable the release of immobilized growth factors from the bone matrix [5]. Communication between cancer cells and osteoclasts promotes an aggressive tumor phenotype with potential for metastatic spread and bone erosion [6]. Interrupting osteoclastic resorption has formed the basis for bone metastasis treatments, such as bisphosphonates and denosumab [7, 8]. However, although these drugs markedly reduce the amount of osteoclasts and extent of bone resorption, associated complications are of great concern [9]. For instance, osteonecrosis is a severe complication that frequently develops during high-dose denosumab and bisphosphonate treatment for cancer [10, 11]. We therefore sought a novel, safer approach for treating bone metastasis.

The darkness hormone melatonin (*N*-acetyl-5-methoxytryptamine) is synthesized from the essential amino acid tryptophan or the naturally occurring chemical *N*-acetylserotonin and secreted principally by the pineal gland under the direct control of the suprachiasmatic nucleus in the hypothalamus [12]. Melatonin is also identified in various extrapineal tissues such as the brain, lung, liver, spleen, gut, and bone marrow, amongst other organs [13, 14]. Some evidence suggests that local melatonin synthesis and release are even more important than pineal-derived melatonin [15, 16]. In the bone marrow microenvironment, local melatonin regulates the renewal and differentiation of hematopoietic stem and progenitor cells (HSPCs) to maintain the bone marrow stem cell pool [15, 16]. Immune cells, including monocytes, macrophages, and T lymphocytes in the spleen and bone marrow, produce melatonergic biosynthetic enzymes that synthesize melatonin and respond as appropriate to immune signals [17]. In reaction to an acute inflammatory response, nuclear factor-kappa B (NF-κB) switches melatonin synthesis from pinealocytes to activated macrophages and microglia, then back to pineal melatonin synthesis after resolution of the acute inflammation [18]. This backward and forward switching of melatonin synthesis and coordination of systemic interactions are termed the immune-pineal axis [18]. Melatonin is known to contribute to many physiological and pathological functions, such as those involved in circadian rhythm and regulation of immune-mediated disorders such as multiple sclerosis, rheumatoid arthritis, and osteoarthritis [19–22]. Most importantly, melatonin displays antitumor effects [23]. The secretion of melatonin in the tumor environment depends on the enzyme acetylserotonin O-methyltransferase converting *N*-acetylserotonin into melatonin and the metabolizing of melatonin by the hepatic cytochromes (primarily CYP1A1 and CYP1A2) into 6-hydroxymelatonin [18, 24]. Genomic analyses that have characterized the tumor melatonergic system by rates of melatonin synthesis and metabolism have classified different cancer samples as high or low, according to mutational burden and neoantigen abundance [24]. The low melatonin synthesis/metabolism Index subgroup is associated with poor patient survival and greater tumor aggressiveness [18, 24]. In clinical applications, melatonin has been widely used in the treatment of insomnia [25] and as an antioxidative agent for more than 20 years [26]. Human studies have shown that short-term melatonin use is safe, with only mild adverse effects associated with high doses [27].

In vivo and in vitro investigations have established that melatonin influences bone formation and development by regulating the balance between osteoclasts (bone resorptive cells) and osteoblasts (bone formative cells) [28, 29]. By increasing the expression of osteocalcin, alkaline phosphatase, and bone sialoprotein in human osteoblasts, melatonin promotes osteoblast proliferation and bone mineralization [29]. Moreover, melatonin inhibits osteoclast differentiation [30]. At pharmacological doses of 1, 5, or 50 mg/kg, melatonin has been found to significantly increase trabecular bone mass and bone mineral density, and inhibit osteoclastic resorption in young growing mice [31]. The majority of melatonin-induced physiological effects are regulated by the high-affinity G protein-coupled receptors MT_1 ($pK_i = 10.09$) and MT_2 ($pK_i = 9.42$) [32]. Melatonin activation of homomers and heteromers of MT_1 and MT_2 receptors allows for Gi/o and Gq signaling; the heteromerization of MT_1 with GPR50 inhibits the binding of melatonin and simultaneously blocks downstream signaling cascades [33]. Clinical and preclinical effects of targeting MT1 and MT2 melatonin receptors have been investigated for the treatment of insomnia, circadian sleep disorders, and major depression [25]. Other research has shown that melatonin suppresses lung cancer metastasis by inhibiting epithelial–mesenchymal transition through the MT_1 but not the MT_2 receptor [34]. Melatonin-induced increases in MT_1 receptor expression have antiproliferative effects in prostate and breast cancer cell lines [25]. These observations confirm the contention that melatonin may be effective for treating bone-wasting disease and that the $MT_{1/2}$ receptor may play an important role in melatonin-regulated antitumor effects.

In this study, we demonstrate that melatonin not only downregulates osteoclast differentiation and bone resorption activity, but also promotes apoptosis in mature osteoclasts. Moreover, we found that melatonin reduces RANKL expression via the receptor MT_1/p38 mitogen-activated protein kinase (MAPK) signaling pathway, which in turn blocks cancer-related osteoclast differentiation in vitro and reduces the development of osteolytic lesions in vivo. Our evidence indicates that melatonin may be an effective and safe treatment for bone metastasis via the inhibition of osteoclastic bone resorption.

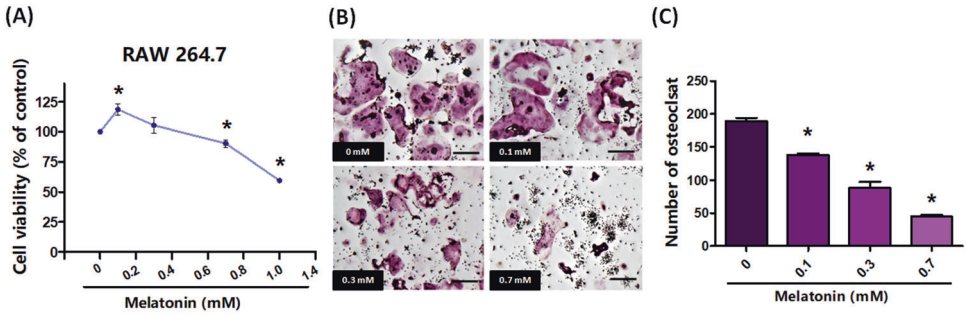


Fig. 1 Melatonin regulates osteoclast differentiation. A RAW 264.7 cells were incubated with melatonin (0, 0.1, 0.3, 0.7, or 1 mM) for 24 h, and cell viability was examined by MTT assays. **B**, **C** RAW 264.7 cells were co-treated with melatonin (0, 0.1, 0.3, or 0.7 mM) and RANKL (50 mg/mL) for 5 days to observe osteoclast differentiation. Mature osteoclasts were visualized by the TRAP assay, and numbers of TRAP-positive osteoclasts were quantified (scale bars: 200 μm). All data are expressed as the mean ± the S.D. of triplicate samples. *$p <$ 0.05 compared with the control group.

Results

Melatonin inhibits osteoclast differentiation

In view of the fact that melatonin negatively regulates osteoclast differentiation [30], we initially sought to confirm how this process occurs. RAW 264.7 cells were treated with different concentrations of melatonin to measure cell viability. The MTT assay showed that low levels of melatonin (0.1 and 0.3 mM) had no significant cytotoxicity, while high levels of melatonin (0.7 and 1 mM) slightly inhibited cell viability (Fig. 1A). We therefore selected the melatonin concentrations of 0.1–0.7 mM to study RANKL-dependent osteoclast differentiation. The RANKL-induced osteoclast differentiation in vitro model is the most commonly used model in osteoclastogenesis studies. After treating RAW 264.7 cells with melatonin and RANKL for 5 days, multinucleated cells (identified as mature osteoclasts) were measured by the osteoclast marker TRAP. Low numbers of TRAP-positive multinucleated cells were identified in the melatonin-treated group and an MT_1 antagonist (S26131) reversed melatonin-induced downregulation of osteoclastogenesis (Fig. 1B, C and Supplementary Fig. 1A), indicating that melatonin inhibits RANKL-dependent osteoclastogenesis and that the MT_1 receptor is involved in this mechanism.

Melatonin promotes apoptosis and reduces bone resorption activity in mature osteoclasts

We also investigated whether melatonin regulates cellular functions in mature osteoclasts. In the presence of RANKL (50 ng/mL) for 5 days, precursor cells differentiated into TRAP-positive mature osteoclasts and then were co-treated with RANKL and melatonin for 2 days, to study the effects of melatonin. TRAP staining revealed that melatonin significantly decreased the lifespans of mature osteoclasts (Fig. 2A, B). Moreover, F-actin and DAPI staining demonstrated that melatonin promotes apoptosis (Fig. 2C). The essential function of a mature osteoclast is to resorb bone [35]. We therefore used the pit formation assay to examine whether melatonin regulates bone resorption activity in vitro. We found that melatonin effectively inhibits bone resorption activity in mature osteoclasts (Fig. 2D, E). These results show that melatonin is associated with two important anti-osteoclastogenic effects: (1) melatonin reduces the differentiation of osteoclast precursors into mature osteoclasts; and (2) melatonin promotes cell apoptosis and reduces bone resorption activity in mature osteoclasts (Fig. 2F).

Melatonin suppresses tumor-associated osteoclastogenesis via the ERK and p65 pathways

Malignancies commonly metastasize to bone, resulting in pain and fracture during the process of bone destruction. Substantial evidence shows that bone metastatic cancer cells stimulate osteolysis [36]. We investigated whether melatonin inhibits cancer-related bone erosion in prostate and lung cancer cells, as these cancers have a high likelihood of bone metastasis. The osteolytic prostate cancer cell line (PC-3) and lung cancer cell line (A549) were treated with different concentrations of melatonin for 24 h. According to MTT data, cell viability was only slightly inhibited by a high concentration of melatonin (0.7 mM) (Fig. 3A, B). Both cell lines were then stimulated with or without melatonin and the CC-CMs were collected to assess the effects upon osteoclastogenesis. TRAP staining detected that whereas CC-CM induced the differentiation of osteoclast precursors into mature osteoclasts, melatonin treatment inhibited osteoclast differentiation in CC-CM; RANKL served as a positive control (Fig. 3C, D). Results with additional cell lines (prostate cancer DU145 and lung cancer CL1-5) were the same as those obtained using PC-3 and A549 cell lines (Supplementary Fig. 1B).

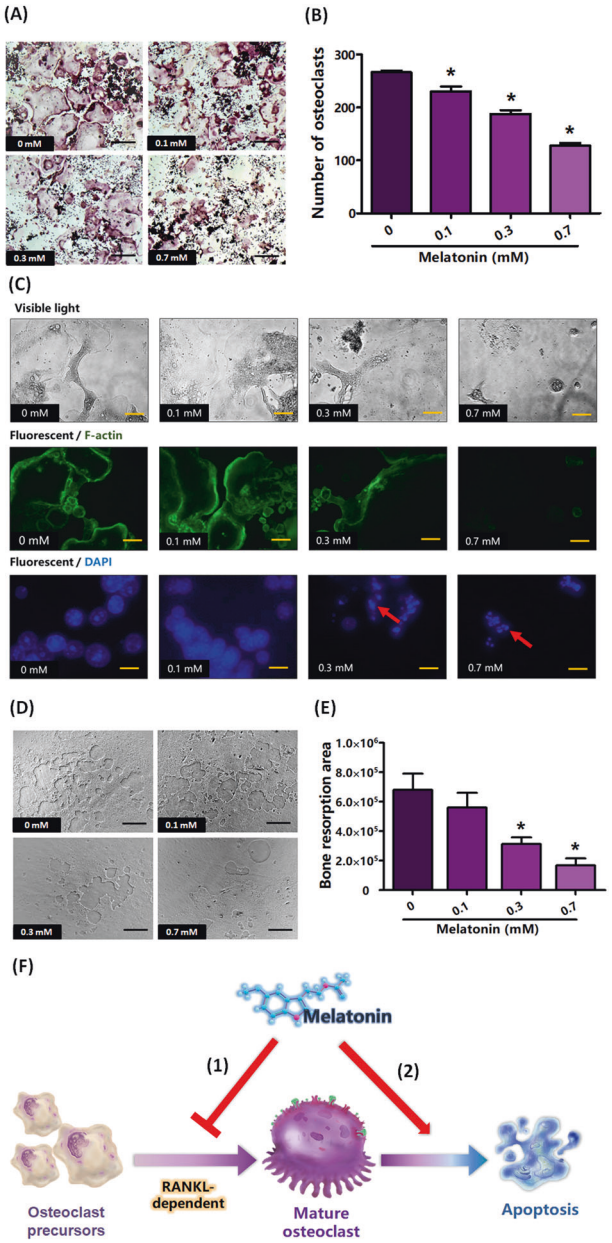


Fig. 2 Melatonin affects the functions of mature osteoclasts. RAW 264.7 cells were inoculated onto 24-well plates or the Osteo Assay Plate and administered RANKL (50 ng/mL) for 5 days to ensure differentiation into mature osteoclasts, followed by 2 days of treatment with RANKL (50 ng/mL) and different concentrations of melatonin (0, 0.1, 0.3, or 0.7 mM). **A**, **B** Mature osteoclasts were visualized by the TRAP assay, and the numbers of TRAP-positive osteoclasts were quantified (scale bars: 200 μm). **C** Phase-contrast micrographs of the control- and melatonin-treated groups. Osteoclast activity was performed by F-actin staining, and apoptosis in osteoclasts (red arrow) was determined by DAPI staining (scale bars: 10 μm). **D**, **E** Bone resorption areas were observed with an inverted microscope and quantified using ImageJ software (scale bars: 200 μm). **F** Melatonin primarily regulated osteoclast functioning in two ways: (1) by inhibiting RANKL-dependent osteoclast differentiation; (2) by causing apoptosis in mature osteoclasts. All data are expressed as the mean ± the S.D. of triplicate samples. $*p < 0.05$ compared with the control group.

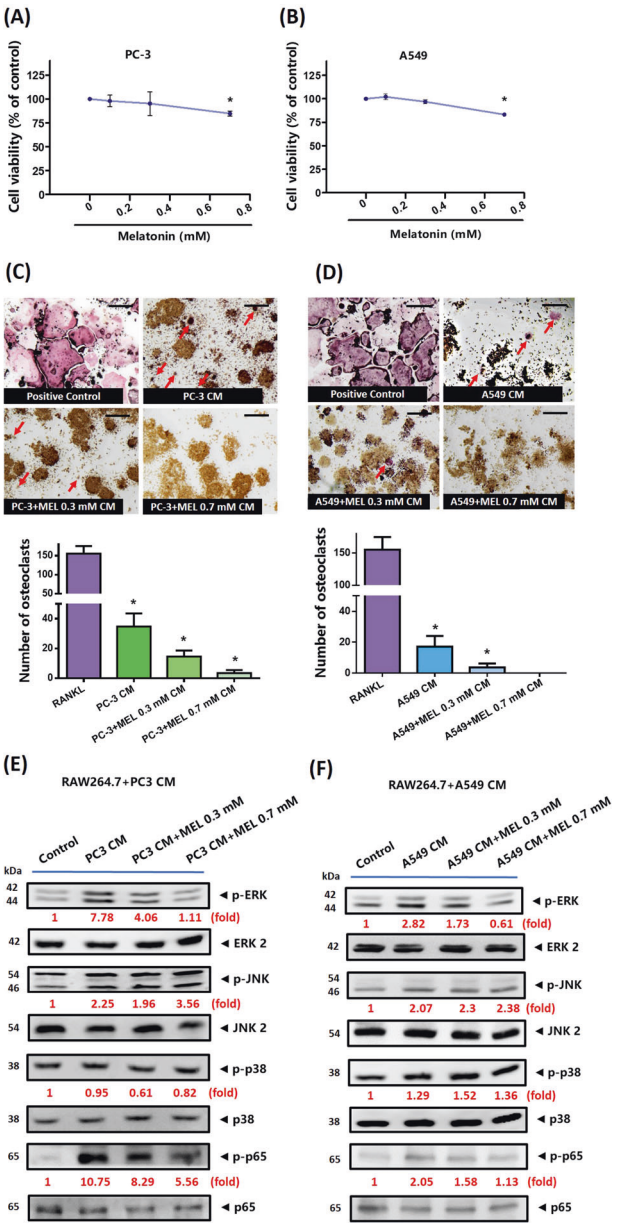


Fig. 3 Melatonin regulates tumor-associated osteoclastogenesis. A, **B** PC-3 and A549 cells were incubated with melatonin (0, 0.1, 0.3, or 0.7 mM) for 24 h. Cell viability was examined by the MTT assay. **C**, **D** PC-3 and A549 cells were incubated with melatonin (0, 0.3 or 0.7 mM) for 72 h, and the cancer-cell-derived conditioned medium (CC-CM) was collected. 15% of the obtained CC-CM was mixed with macrophage colony-stimulating factor M-CSF (20 ng/mL) and applied to the RAW 264.7 cells, then incubated for 7 days. At the end of 7 days, TRAP assay findings detected that the cells had differentiated into complex osteoclasts (red arrows) (scale bars: 200 μm). **E**, **F** 15% of the CC-CM was applied to RAW 264.7 cells and incubated for 15 min. A Western blot assay detected levels of protein expression in the osteoclast differentiation pathways. All data are expressed as the mean ± the S.D. of triplicate samples. $*p < 0.05$ compared with the RANKL group.

Osteoclast differentiation depends upon the MAPK and NF-κB signaling pathways [37]. We observed that melatonin-treated CC-CM reduced ERK and p65 phosphorylation in

RAW 264.7 cells, whereas there was no effect upon p38 or JNK activity (Fig. 3E, F and Supplementary Fig. 1C). It appears that cancer cells stimulate osteoclast differentiation via the ERK and NF-κB p65 pathways and melatonin reduces this phenomenon.

Melatonin inhibits RANKL release from cancer cells

In the bone microenvironment, the secretion of osteolytic factors from metastatic cancer cells stimulate osteoclast differentiation and increase bone erosion [38]. RANKL, also known as TNF superfamily member 11, is crucial for osteoclast formation [39]. RANKL is produced as a membrane-bound protein that can be cleaved by matrix metalloproteinase or disintegrin and metalloproteinase (ADAM) [40] and is released as a soluble form in the tumor microenvironment [41]. When we analyzed data from the Oncomine database (35107_at), we found high levels of RANKL expression in prostate and lung cancer tissues compared with healthy tissue samples (Fig. 4A, B). Our in vitro study found reductions in RANKL mRNA and protein expression following melatonin treatment in different prostate and lung cancer cell lines (Fig. 4C–F and Supplementary Fig. 2A). Numerous oncostatic properties of melatonin are mediated through the activation of the specific melatonin receptors, MT_1 and MT_2 [42, 43]. It would be of great interest to determine how exactly melatonin regulates RANKL expression (through which of its receptors and which intracellular signaling pathway). Transfection of cancer cells with MT_1 shRNA reversed melatonin-mediated inhibition of RANKL (Fig. 4G, H). To confirm whether the expression of RANKL is inhibited by melatonin through the downstream G protein signaling pathway of the MT_1 receptor, we co-treated cancer cells with melatonin and either the Gi inhibitor (PTX) or Gq inhibitor (YM-254890). The data revealed that melatonin-induced inhibition of RANKL mRNA expression was rescued by Gi and Gq inhibitors (Supplementary Fig. 2B), indicating that melatonin suppresses RANKL expression through the MT_1 receptor and its downstream Gi and Gq proteins.

Moreover, we found that melatonin significantly repressed p38 protein activation, but did not affect the levels of ERK and JNK expression in either cell line (Fig. 4I, J and Supplementary Fig. 2C), while melatonin-regulated inhibition of RANKL expression was reversed by treatment with the p38 activator U-46619 (Fig. 4K, L). The evidence indicates that melatonin-inhibited RANKL expression is regulated by the MT_1 receptor and p38 signaling pathway.

Next, we sought to determine whether soluble RANKL (sRANKL) is involved in cancer-related osteoclast differentiation. The data showed that CC-CM induced osteoclast differentiation; pretreating CC-CM with anti-RANKL antibody interrupted this phenomenon (Fig. 4M, N and Supplementary Fig. 2D). Thus, melatonin reduces the release of osteolytic factor sRANKL from cancer cells via the MT_1 receptors and p38 signaling pathway, and interrupts sRANKL-induced osteoclast differentiation. Figure 4O summarizes our study findings regarding the primary functions of melatonin in cancer-related osteoclastogenesis.

Melatonin inhibits in vivo bone metastasis

To confirm the role of melatonin in the suppression of tumor growth and bone lesions, we injected PC-3 and A549 cells stably expressing pLenti CMV V5-Luc (labeled as PC-3-Luc and A549-Luc, respectively) into the right tibias of nude mice (Fig. 5A). The extremely fast growth of bone tumors during weeks 3 and 4 was significantly slowed by melatonin, according to in vivo bioluminescence imaging (Fig. 5B, C) and manual measurements of tumor size (Fig. 5D, E). X-radiography observed the phenomena of tumor penetration from the tibia to the femur among animals in the PC-3 and A549 groups; melatonin treatment inhibited tumor expansion and bone erosion in both the tibia and the femur (Fig. 5F, G). H&E staining revealed the osteolytic nature of the bone lesions formed by PC-3 and A549 cells and their attenuation by melatonin treatment (Fig. 5H). We therefore conclude that melatonin inhibits tumor growth and bone erosion during bone metastasis.

Histopathological examination of RANKL expression revealed lower levels in tissue from mice treated with melatonin compared with those that were not (Fig. 5I, J). Moreover, TRAP staining revealed abundant quantities of TRAP-positive osteoclasts in the non-treated mice and markedly reduced amounts in melatonin-treated mice (Fig. 5K, L). Clearly, melatonin suppresses cancer-related RANKL expression and has anti-osteoclastogenic effects in in vivo analysis.

Discussion

An increasing body of research has begun to expose the vast clinical potential for melatonin in the treatment of Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease, stroke, and brain trauma [44]. Metastatic bone disease is associated with deregulation of bone homeostasis, with increased bone resorption and diminished bone synthesis [45]. Our findings reveal new clinical potential for melatonin in the treatment of bone metastasis.

Favorable effects of melatonin have been observed in the bone microenvironment [15, 16, 46]. First, melatonin is a key player involved in the kinetics of light–dark cycles to maintain the reservoir of bone marrow HSPCs. The onset of light triggers high levels of norepinephrine and TNF

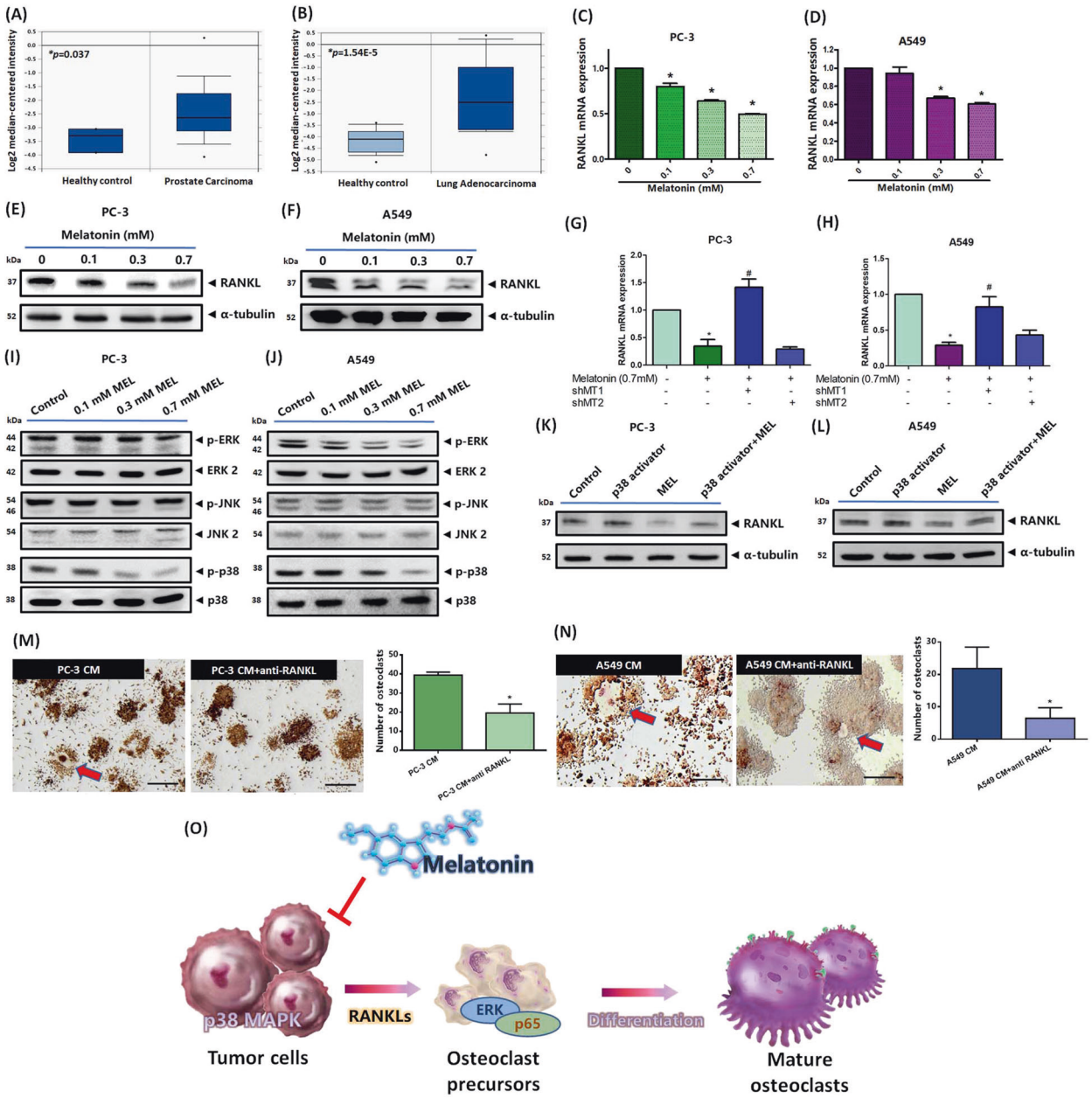
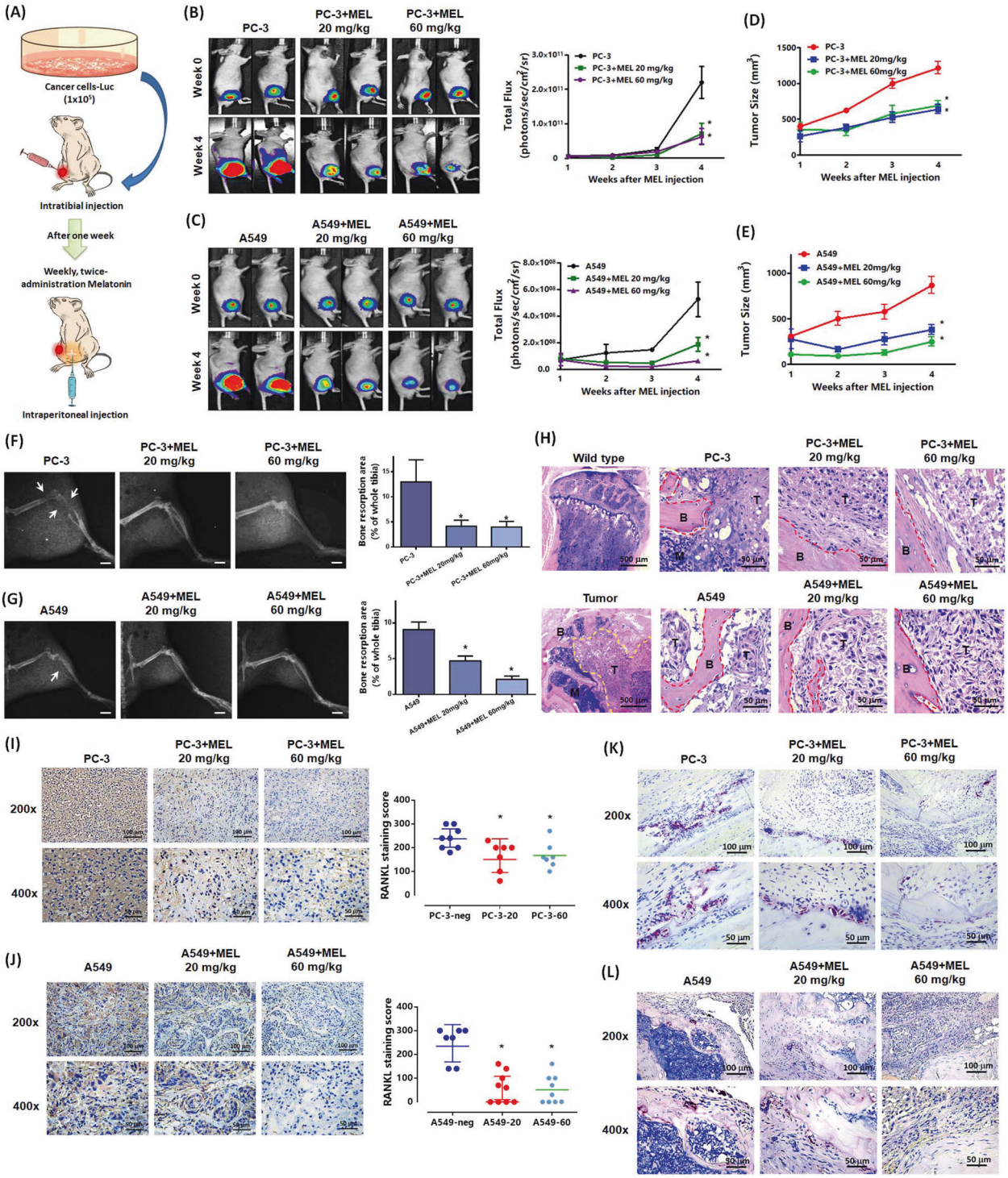


Fig. 4 Melatonin regulates RANKL production in cancer cells. A, **B** RANKL expression in prostate or lung adenocarcinoma tissue samples from the Oncomine database. **C–F** PC-3 and A549 cells were incubated with melatonin (0, 0.1, 0.3, or 0.7 mM) for 24 h. qPCR and Western blot assays were used to detect RANKL mRNA and protein expression, respectively. **G**, **H** PC-3 and A549 cells were transfected with MT_1 or MT_2 shRNA for 24 h with or without melatonin 0.7 mM for another 24 h. The qPCR assay was used to detect RANKL mRNA. **I, J** PC-3 and A5459 cells were incubated with melatonin (0.1, 0.3, or 0.7 mM) for 24 h. The Western blot assay was used to detect levels of ERK, JNK, and p38 protein expression. **K**, **L** PC-3 and A549 cells were pretreated with a p38 activator (U-46619) for 30 min with or without melatonin 0.7 mM for 24 h. The Western blot assay was used to detect the levels of RANKL protein expression. α-Tubulin served as the positive control. **M**, **N** After incubating CC-CM with or without RANKL antibody for 30 min, 15% of the mixture was applied with M-CSF (20 ng/mL) to RAW 264.7 cells. After 7 days of incubation, TRAP assay findings revealed mature osteoclasts (red arrow) (scale bars: 200 μm). **O** Model showing how melatonin inhibits RANKL expression in prostate and lung cancers via the p38 pathway, which in turn downregulates cancer-related osteoclast differentiation via the ERK and p65 pathways. All data are expressed as the mean ± the S.D. of triplicate samples. $*p < 0.05$ compared with the control group; $\#p < 0.05$ compared with the melatonin-treated group.

secretion in bone marrow, which program the migration and differentiation of HSPCs, as well as blood replenishment, whereas darkness-induced melatonin secretes locally in the bone marrow and metabolically programs the self-renewal of HSPCs and their homing to bone marrow [15, 16]. Moreover, melatonin suppresses circadian *clock* genes, the regulation of circadian rhythm in bone metabolism, and modulates proinflammatory cytokine secretion in joints



[22]. Rheumatoid arthritis is the prototypic autoimmune joint disease [47]. However, the role of melatonin in models of inflammation-induced arthritis is controversial. While some evidence suggests that melatonin attenuates *clock* gene *Cryptochrome1*, which in turn provokes mouse anti-type II collagen antibody-induced arthritis [48], other investigations including our research indicate that melatonin has anti-inflammatory effects in models of adjuvant-induced arthritis and collagen-induced arthritis [49, 50]. Finally, melatonin also participates in regulating bone density through various mechanisms including the reduction of oxidative stress in osteoclasts, the promotion of cell differentiation and activity in osteoblasts, and an increase in osteoprotegerin expression from the osteoblast, preventing

◀ **Fig. 5 Melatonin affects in vivo tumor growth and bone erosion.** **A** A flowchart showing an in vivo model of bone metastasis. Luciferase-positive human prostate tumor cells PC-3 (PC-3-Luc) and A549-Luc cells expressing luciferase were injected into the right tibias of nude mice at a dose of 1×10^5 cells. Starting 1 week after tumor stabilization, the mice received twice-weekly melatonin (20 or 60 mg/kg) for 4 weeks. **B**, **C** Fluorescent images using IVIS spectrum were performed weekly, and luminescent intensity of photons emitted from each tumor in the images was quantified. **D**, **E** The size of the tumor was measured manually and quantified. **F**, **G** After 4 weeks, all mice were humanely sacrificed and X-rayed for evidence of bone erosion (white arrows) (scale bars: 5 mm). The resorption areas were measured by ImageJ software. **H** H&E staining was used to perform bone resorption areas (B bone, T Tumor, M bone marrow). **I**, **J** IHC staining determined RANKL expression in murine tumor tissues. **K**, **L** TRAP staining was used to examine osteoclast numbers and activity in murine tissue with tibia bone. In all sets of images, the upper row depicts images photographed at ×200 magnification, while images in the bottom row are at ×400 magnification. All data are expressed as the mean ± the S.D. of triplicate samples. $*p < 0.05$ compared with the A549 and PC-3 groups.

the differentiation of precursors to osteoclasts and bone resorption [46]. Our study findings concur with these effects, by showing that melatonin can significantly reduce the lifespans of mature osteoclasts by promoting apoptosis and also directly suppress osteoclastic bone resorption. Thus, melatonin appears to protect against bone resorption, suggesting that this compound has important therapeutic potential in bone-wasting diseases such as osteoporosis and cancer-related osteolysis.

Melatonin receptors, MT_1 and MT_2, are considered to be very important for the relationship of melatonin with sleep disorders such as insomnia, rheumatoid arthritis, the immune system, and cancer [25, 50]. MT_1 and MT_2 receptors form monomeric, homo- and hetero-oligomers between themselves, altering the pharmacological properties of the individual receptors [51]. Targeting to the MT_1 and MT_2 receptors has been proposed as potential therapy for the treatment of learning and memory deficits, neurodegeneration, and drug addiction [25]. Melatonin can directly bind to its nuclear receptors, RZR/RORα and RZRβ, and exert its effects in altering gene transcription, such as 5-LOX, p21, and BSP [52, 53]. Melatonin also acts through non-receptor-mediated mechanisms [54]. A previous study has reported that MT_1/MT_2 levels are downregulated during osteoclastogenesis [30]. The fact that the silencing of MT_1/MT_2 receptors does not prevent the anti-osteoclastogenic effects of melatonin suggests that melatonin inhibits osteoclast differentiation independently of the MT_1/MT_2 receptors [30]. Our findings in this study demonstrate that the MT_1, but not MT_2 receptors are involved in melatonin-mediated downregulation of RANKL expression in cancers and cancer-related osteoclastogenesis, although confirmation is needed as to whether non-receptors affect melatonin-induced inhibition upon RANKL expression in cancer cells.

Several investigations have revealed oncostatic properties of melatonin in a variety of tumors, as well as the inhibition of cancer cell proliferation and induction of apoptosis [25, 55]. Melatonin is rapidly metabolized in peripheral tissues and its metabolite, N^1-acetyl-N^2-formyl-5-methoxykynuramine, also contributes to many physiological functions [56, 57], such as the enhancement of chemosensitivity in cancer cells [58]. This evidence indicates that the actions of melatonin can be direct or indirect via its metabolites. In this study, we did not evaluate whether or not melatonin metabolites are involved in regulating cancer-related osteoclastogenesis. This aspect deserves further investigation. Since 1992, Lissoni et al. have conducted clinical trials using melatonin in patients with non-small cell lung cancer, metastatic breast cancer, melanoma, and other types of tumors [59–61]. Compared with chemotherapy, the melatonin-containing regimen prolonged survival and was better tolerated [60]. Promising preclinical evidence suggests that melatonin has potential in the treatment of cancer [23, 62]. A disadvantage of most clinical trials involving melatonin treatment in cancers is that melatonin has been used as an adjuvant to improve radiochemotherapy-related side effects including neurotoxicity, thrombocytopenia, sleep disturbances, and depressive symptoms [63, 64]. It is highly recommended that the antitumor therapeutic effects of melatonin are explored in multiple clinical trials to strengthen the evidence base.

Prostate, breast, and lung cancers have a high likelihood of metastasizing to the bone [65]. It has been suggested that the rate of skeletal complications in bone metastasis may be related to the level of bone resorption [66]. Our study presented here presents new information supporting the contention that melatonin is a potential treatment that may prevent bone-lytic metastasis in lung and prostate cancer. When we conducted further tests with additional cell lines from each cancer type, we observed the same results (Supplementary Fig. 1), confirming that melatonin can be used to treat bone metastases from lung and prostate cancer. In women, breast cancers with osteolytic lesions are found in 80% of patients with stage IV metastatic disease [67]. Second-generation bisphosphonates, such as alendronate, are currently used for the treatment of bone metastases in postmenopausal women with early breast cancer, as these agents significantly improve the overall survival rate and slow cancer-related osteolysis [68]. However, in this study, we did not explore whether melatonin has the same effects as bisphosphonates in the treatment of bone erosion in breast cancer. Preclinical research has shown that disrupting circadian melatonin signaling through exposure to dim light at night promotes osteolytic metastases in breast cancer [69]. The study researchers found that chronotherapeutic use of doxorubicin in circadian alignment with melatonin reduced tumor growth and bone erosion. The data indicate

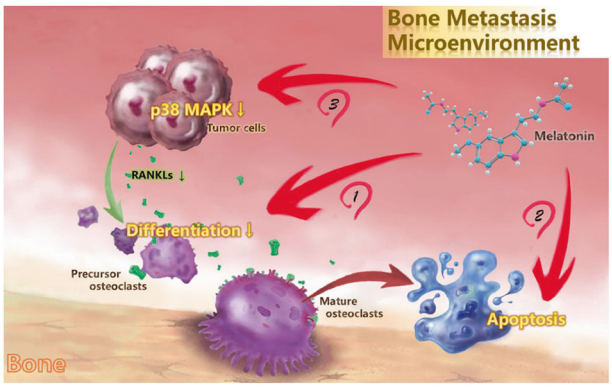


Fig. 6 Schema depicting the effects of melatonin in osteolytic bone disease. In the bone metastatic microenvironment, melatonin primarily affects the functioning of osteoclast lineage and metastatic cancer cells. When applied to osteoclast lineage cells, melatonin inhibits the differentiation of osteoclast precursors into mature osteoclasts (1). Melatonin also regulates mature osteoclast functioning by promoting apoptosis and inhibiting bone resorption activity (2). In metastatic cancer cells, melatonin suppresses cancer-related bone erosion by reducing levels of RANKL expression, which interrupts cancer-induced osteoclastogenesis and bone resorption (3).

that melatonin may have a similar action in treating osteolytic metastases of breast cancer.

Osteoblastic lesions are caused by tumor-derived osteoblastic factors such as platelet-derived growth factor, insulin-like growth factor, and endothelin-1, which are capable of acting on osteoblasts and promoting abnormal bone formation [70]. Prostate cancer has been classified as osteoblastic, based on the radiographic appearance of the lesion, although other clinical evidence indicates that both bone resorption and formation are observed in men with prostate cancer bone metastases [71]. Interestingly, 24 weeks of combination treatment with atrasentan (an endothelin receptor antagonist that inhibits osteoblast activity) and a bisphosphonate (zoledronic acid, which inhibits osteoclast activity) in mice bearing prostate cancers was associated with a greater osteoblastic response than either treatment alone [71], which indicates that osteoclastic bone resorption also contributes to the pathophysiology of osteoblastic metastases. Thus, combination treatment targeting both osteoblasts and osteoclasts should be an effective therapy for osteoblastic bone metastases. Up until now, there are no reports detailing whether melatonin can treat pathological osteogenesis during cancer-induced bone metastasis. The impact of melatonin on osteoblasts is controversial. Melatonin reportedly increases osteoblast differentiation and bone formation by enhancing osterix expression [72]. In contrast, high concentrations of melatonin may inhibit miR-590-3p expression, leading to the promotion of target genes that induce osteoblastic apoptosis [73]. Cellular and preclinical studies are needed to determine the mechanism(s) by which melatonin regulates osteoblastic bone metastases.

When cancer metastasizes into the bone microenvironment, the release of osteolytic factors promotes osteoclast activity and increases bone resorption, causing a vicious cycle of bone metastasis. Our study results show that melatonin intervention affects this cycle in three ways: (1) by directly inhibiting the differentiation of osteoclast precursors into mature osteoclasts; (2) by promoting cell apoptosis in mature osteoclasts and decreasing osteoclastic bone resorption; and (3) by reducing the release of the osteolytic factor sRANKL from tumors and thus inhibiting cancer-induced osteoclast differentiation in vitro and bone erosion in vivo (Fig. 6). Our findings are the first to show that melatonin slows the progression of bone metastases and inhibits cancer-related bone erosion via the downregulation of RANKL expression in prostate and lung cancer. Thus, melatonin has antitumor and anti-osteoclastogenic effects in bone metastases.

Materials and methods

Cell culture

Human A549 and PC-3 cell lines were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA); the murine RAW 264.7 cell line was obtained from the Bioresource Collection and Research Center (BCRC; Hsinchu, Taiwan) and used as osteoclast precursor cells. A549 cells and RAW 264.7 cells were propagated in DMEM medium (Gibco, USA) containing 10% fetal bovine serum (FBS; Gibco, USA); PC-3 cells were grown in RPMI-1640 medium (Gibco, USA) supplemented with 10% FBS. All cells were maintained in a humidified incubator at 37 °C, 5% CO_2 and subcultured at ~80% of cell confluence.

Human A549 and PC-3 cells (2×10^6) were grown to confluence in 100-mm culture dishes. The cells were incubated with different concentrations of melatonin in 1% FBS culture medium for 72 h prior to collection of the cancer-cell-derived conditioned medium (CC-CM).

MTT assay

Cells were seeded onto 96-well culture plates then treated with varying concentrations of melatonin (Sigma-Aldrich, USA) for 24 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, USA) was then added to the plates to assess cell viability, as according to our previous research [74, 75].

Western blot analysis

Total cell lysates were prepared from A549, PC-3, and RAW 264.7 cells collected after 24 h of melatonin treatment. Protein concentrations for each cell lysate were determined using

the BCA Protein Assay Kit (Thermo Scientific, USA). Electrophoresis and transfer methods have been described in our previous study [76, 77]. Membranes were probed with their respective antibodies; anti-phospho-extracellular signal-regulated kinase (ERK)/ERK2 (SC-13073/SC-154; Santa Cruz, USA), anti-phospho-c-Jun N-terminal kinase (JNK)/JNK2 (SC-6254/SC-7345; Santa Cruz, USA), anti-phospho-p38/p38 (SC-166182/SC-271120; Santa Cruz, USA), anti-RANKL (GTX32834; GeneTex, USA), anti-phospho-p65/p65 (3033/SC-8008; Cell Signaling, USA/Santa Cruz, USA), and anti-α-tubulin (Santa Cruz, USA). Immunoblot images were visualized with the ImageQuant™ LAS 4000 biomolecular imager (GE Healthcare Life Sciences, USA).

Quantitative real-time PCR (qPCR)

Total RNA was extracted from A549 and PC-3 cells in six-well plates. An M-MLV RT kit (Invitrogen, Thermo Fisher Scientific, USA) was used to reverse transcribe total RNA to complementary DNA (100 ng). The qPCR method was conducted according to an established protocol [78].

Osteoclast differentiation

RAW 264.7 cells were incubated with RANKL (50 ng/mL) and different concentrations of melatonin. After 7 days, multinucleated (N3 nuclei) tartrate-resistant acid phosphatase (TRAP)-positive cells were identified as the mature osteoclasts, according to the results of our previous study [79].

Bone resorption assay

RAW 264.7 cells were seeded onto Corning Osteo Assay Surface 24-well plates (Corning Life Sciences, USA) and supplemented with RANKL (50 ng/mL; PeproTech, USA) for 5 days, followed by melatonin (0–0.7 mM) or no such treatment for another 2 days. Bone resorption areas were quantified by ImageJ v6.0 software.

Immunofluorescence staining

RAW 264.7 cells were prepared on 12-mm coverslips in 24-well plates then treated with RANKL and melatonin, as described previously [80]. The cells were stained with F-actin stain 488 Fluorescent Phalloidin (Cytoskeleton, USA) or 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, USA) for 1 h. Images were obtained using a fluorescence microscope (Zeiss; Berlin, Germany).

In vivo bone metastasis model

The animal study was approved by the Institutional Animal Care and Use Committee of China Medical University. A459 and PC-3 cell lines stably expressing luciferase (1×10^5) were mixed with Matrigel (100 µL; Corning Life Sciences, USA) and injected into the right tibias of 4-week-old nude mice (ten animals per group). Starting 1 week after tumor stabilization, the mice received twice-weekly melatonin (20 or 60 mg/kg) by intraperitoneal injection for 4 weeks. The IVIS® Spectrum imaging system was used to determine in vivo tumor mass and weekly tumor volume was calculated using the formula (length × width^2)/2. After 4 weeks, all mice were humanely sacrificed and tumor-induced bone erosion was evaluated by X-ray radiography. Tumor tissues were stained with hematoxylin and eosin (H&E; Rapid Science Co. Ltd, Taiwan). TRAP staining was performed to visualize osteoclast activity in tibia specimens [81].

Immunohistochemistry

Tumor tissues were obtained from the in vivo bone metastasis model then stained with anti-RANKL antibody (ab9679; Abcam, USA) and quantified according to the method described in our previous study [82].

Analysis of Oncomine database

Datasets (35107_at) from the Oncomine database (https://www.oncomine.org) were used to verify the levels of RANKL expression in prostate carcinoma or lung adenocarcinoma compared with normal tissues.

Statistics

All values are expressed as the mean ± standard deviation. All differences between the experimental groups and controls were assessed for significance using the Student's *t*-test. Between-group differences were considered to be significant if the p value was <0.05.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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