SHORT COMMUNICATION

EXPERIMENTAL ONCOLOGY

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MiR-519d-3p REDUCES OSTEOCLASTOGENESIS BY LOWERING *MMP2* AND SHIFTING OSTEOBLAST SIGNALING TOWARD OSTEOPROTEGERIN

Background. Paracrine crosstalk between tumor cells and bone-resident cells drives osteoclastogenesis in the metastatic bone niche. miR-519d-3p has been linked to suppression of matrix metalloproteinases. **Aim.** To test whether osteoblastic miR-519d-3p restrains tumor-conditioned osteoclastogenesis by lowering *MMP2* expression and shifting signaling toward osteoprotegerin (OPG). **Materials and Methods.** Mouse osteoblasts (MC3T3-E1) were transfected with miR-519d-3p mimic, inhibitor, or corresponding negative controls. Osteoblast-conditioned medium (OB-CM) was mixed 1:1 with MDA-MB-231 tumor-conditioned medium (231-CM) and applied to bone-marrow-derived macrophages for tartrate-resistant acid phosphatase (TRAP) staining. Osteoblastic *MMP2* and *OPG* mRNAs were quantified by RT-qPCR, and a dual-luciferase system compared the wild-type vs seed-mutant *MMP2* 3′UTR reporters. **Results.** Relative to their controls, miR-519d-3p mimic OB-CM produced fewer TRAP-positive multinucleated osteoclasts, whereas inhibitor OB-CM produced more. In osteoblasts, the mimic decreased *MMP2* mRNA, whereas the inhibitor increased it, and the changes in *OPG* mRNA were modest. Dual-luciferase assays did not show a reproducible wild-type–over-mutant selective repression by miR-519d-3p, indicating context-dependent or indirect regulation of *MMP2*. **Conclusion.** Elevating osteoblastic miR-519d-3p in vitro associated with lower *MMP2* and an anti-osteoclastogenic paracrine output, suggesting a tractable microRNA-based approach to modulate the metastatic bone niche and warranting further validation in vivo.

Keywords: miR-519d-3p, *MMP2*, osteoclastogenesis, osteoblast, conditioned medium, TRAP.

The metastatic bone disease is sustained by a crosstalk between tumor cells and bone-resident cells. Osteoclast formation is mainly controlled by the balance between RANKL (receptor activator of NF-κB ligand) and OPG (osteoprotegerin) provided by osteoblast-lineage cells; a shift toward high RANKL or low OPG drives osteolysis, and tumor progression [1, 2]. MicroRNAs (miRNAs) act as post-transcriptional switches in this niche. Among them, miR-519d-3p — part of the primate-specific C19MC cluster — has been linked to reduced invasion via matrix metalloproteinases

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(MMPs) in several cancers. MMP-2, a gelatinase implicated in osteolytic metastasis, can also depress osteoblastic OPG, enhancing osteoclastogenesis [3]. We hypothesized that osteoblastic miR-519d-3p restrains tumor-induced osteoclastogenesis by lowering MMP-2 and tilting signaling toward OPG. Here, we combine osteoblast transfection, 3′UTR reporter tests, and TRAP staining of bone marrow-derived macrophages exposed to osteoblast-conditioned medium (OB-CM) to examine this axis.

Materials and Methods

Cell lines. Human breast cancer MDA-MB-231 cells (ATCC HTB-26) and mouse osteoblastic MC3T3-E1 cells (subclone 4, ATCC CRL-2593) were obtained from the American Type Culture Collection (ATCC, USA). MDA-MB-231 cells were cultured in DMEM (Gibco, Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin–streptomycin, whereas MC3T3-E1 cells were maintained in α-MEM (Gibco) containing 10% FBS and 1% penicillin–streptomycin. All cells were kept at 37 °C in a humidified atmosphere of 5% CO_2. Primary mouse bone-marrow-derived macrophages (BMMs) were purchased from Lonza (Switzerland) and cultured in α-MEM with 10% FBS supplemented with recombinant M-CSF (30 ng/mL; PeproTech, USA). No human subjects or live-animal procedures were involved in this study.

Transfection and qPCR. MC3T3-E1 osteoblasts were seeded in 6-well plates and transfected with miR-519d-3p mimic, miR-519d-3p inhibitor, or the corresponding non-targeting negative control oligonucleotides (all from RiboBio, China) using Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The final concentration of each oligonucleotide was 50 nM in all groups. At 48 h after transfection, the total RNA was isolated using a TRIzol reagent (Invitrogen), and 1 μg of RNA was reverse-transcribed with a PrimeScript RT reagent kit (Takara, Japan). RT-qPCR was performed with SYBR Green Master Mix (Takara) on a CFX96 real-time PCR system (Bio-Rad, USA). Expression of *MMP2* and *TNFRSF11B* (*OPG*) was normalized to *GAPDH*, and relative mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Each experimental condition included at least three biological replicates, each measured in technical triplicate.

Dual-luciferase assay. For reporter assays, MC3T3-E1 osteoblasts were seeded in 24-well plates and co-transfected with Firefly Luciferase reporter plasmids carrying either the wild-type (WT) or a miR-519d-3p seed-mutant (MUT) human *MMP2* 3′UTR sequence (pGL3-based constructs), together with miR-519d-3p mimic or mimic negative control, plus a Renilla luciferase plasmid (pRL-TK, Promega, USA) for normalization. Transfections were performed using Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific, USA) according to the manufacturer's protocol. At 24 h after transfection, Firefly and Renilla activities were measured using the Dual-Luciferase Reporter Assay System (Promega) on a GloMax luminometer (Promega). The relative luciferase activity was calculated as the Firefly/Renilla ratio and then normalized to the mimic negative control group.

Conditioned media and TRAP osteoclast assay. OB-CM and tumor-conditioned medium from MDA-MB-231 cells (231-CM) were prepared. To prepare OB-CM, MC3T3-E1 osteoblasts were transfected as described above and, 24 h later, switched to serum-free α-MEM for an additional 24 h before supernatants were collected and centrifuged to remove debris. For 231-CM, near-confluent MDA-MB-231 cultures were incubated in serum-free DMEM for 24 h, and supernatants were collected in the same way. For osteoclastogenesis assays, BMMs were seeded in 96-well plates and induced with RANKL (50 ng/mL; PeproTech, USA) in the presence of a 1:1 mixture of 231-CM and OB-CM. Control wells received 231-CM mixed with OB-CM from negative control–transfected osteoblasts. After 5—7 days, the cultures were fixed in 4% paraformaldehyde and stained for tartrate-resistant acid phosphatase (TRAP) using a commercial TRAP staining kit according to the manufacturer's instructions. TRAP-positive multinucleated cells (≥ 3 nuclei, counted as osteoclasts) were counted in 10 random fields per well by two independent observers blinded to treatment.

An overview of the in vitro workflow, including osteoblast transfection, conditioned medium preparation, and TRAP osteoclast assays, is summarized in Fig. 1.

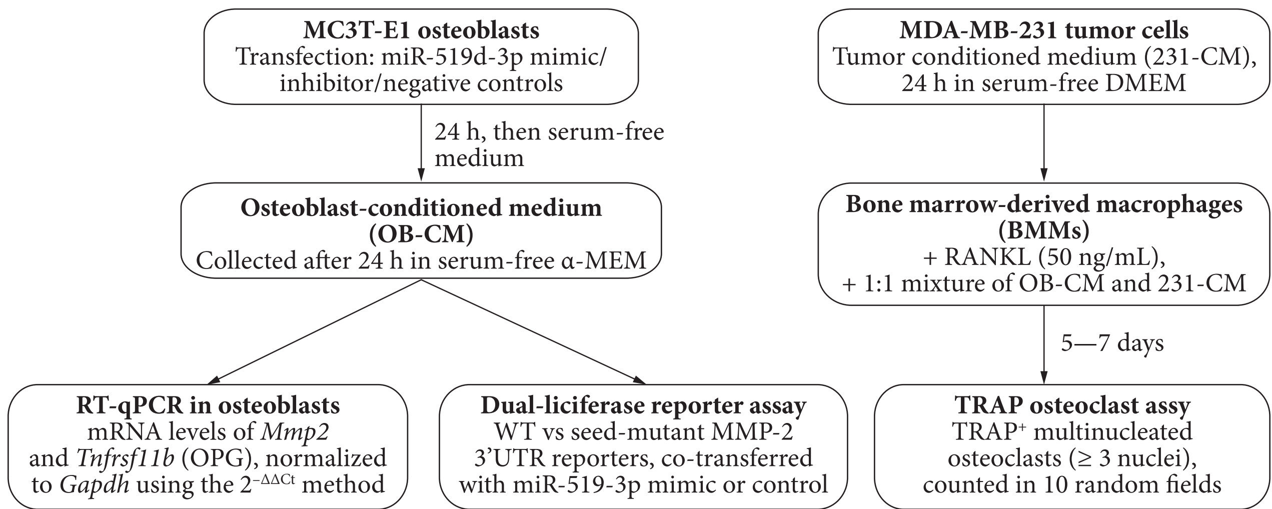

Fig. 1. Experimental workflow of the miR-519d-3p–MMP-2–OPG study. MC3T3-E1 osteoblasts were transfected with miR-519d-3p mimic, inhibitor, or negative controls. Osteoblast-conditioned medium (OB-CM) was collected after 24 h in serum-free α-MEM and mixed 1:1 with MDA-MB-231 tumor-conditioned medium (231-CM). The OB-CM/231-CM mixture was applied to bone marrow–derived macrophages (BMMs) in the presence of RANKL to induce osteoclastogenesis for TRAP assays. In parallel, osteoblasts were analyzed by RT-qPCR for *MMP2* and *TNFRSF11B* (*OPG*) and by dual-luciferase reporter assays using WT and seed-mutant MMP-2 3′UTR constructs

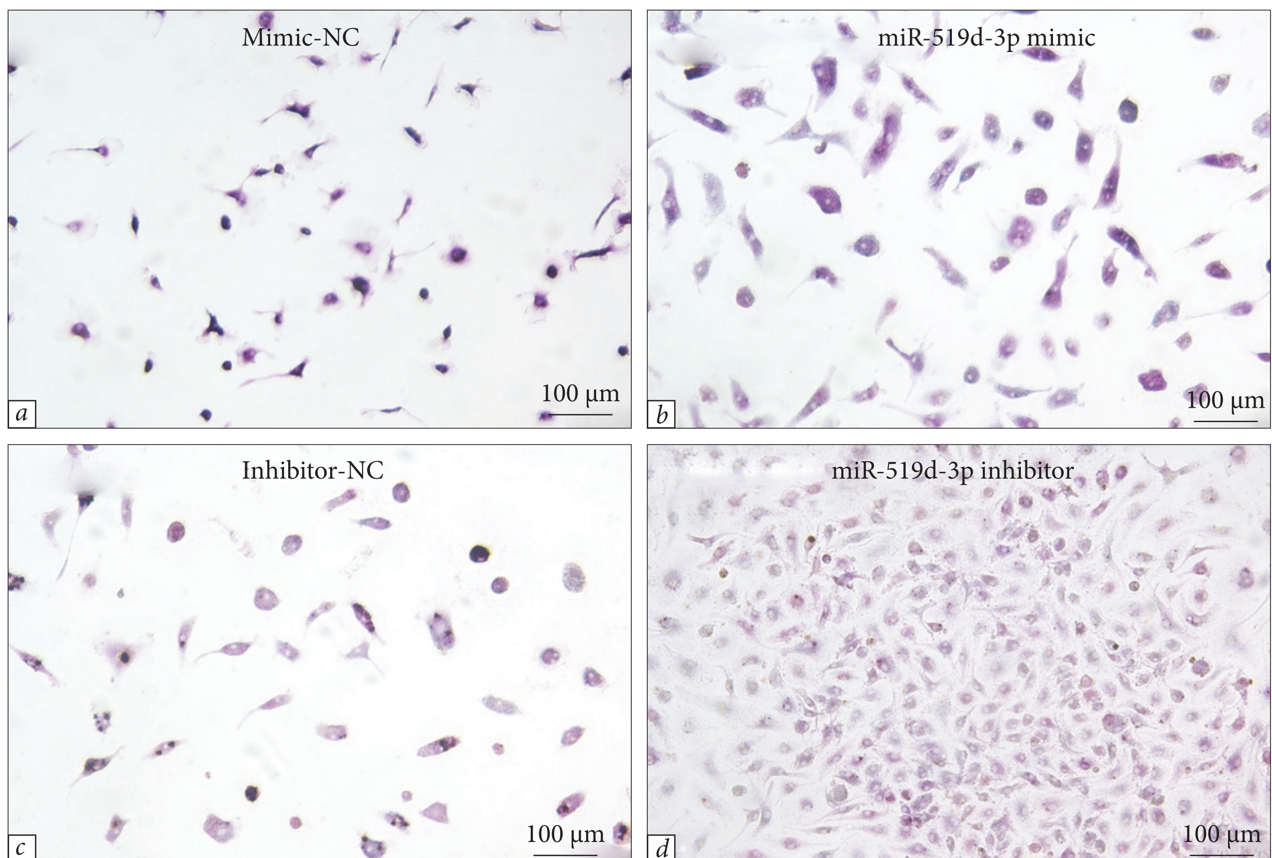

Fig. 2. TRAP-stained cells demonstrating osteoclastogenesis under different osteoblast-conditioned media (OB-CM). (*a*) OB-CM from miR-519d-3p mimic–transfected osteoblasts (mimic OB-CM); (b) OB-CM from mimic negative control–transfected osteoblasts (mimic NC OB-CM); (*c*) OB-CM from inhibitor negative control-transfected osteoblasts (inhibitor NC OB-CM); (*d*) OB-CM from miR-519d-3p inhibitor–transfected osteoblasts (inhibitor OB-CM). Images were acquired at the same magnification; scale bars = 100 μm. Representative fields from three independent experiments

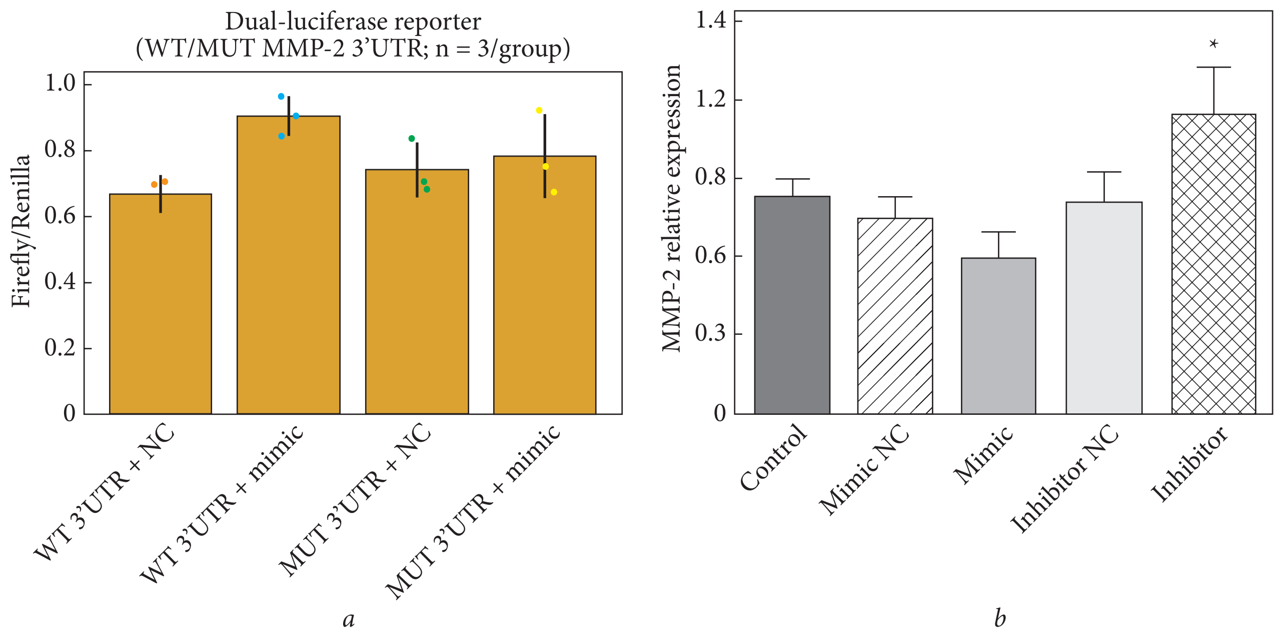

Fig. 3. Reporter and mRNA readouts of the miR-519d-3p–*MMP2* axis. (*a*) Dual-luciferase assays using wild-type (WT) and seed-mutant (MUT) *MMP2* 3′UTR reporters in the presence or absence of miR-519d-3p mimic (n = 3 per group). Bars indicate mean ± SD with individual data points. Across replicates, no reproducible WT-vs-MUT selective repression was observed. (*b*) RT-qPCR in osteoblasts shows decreased *MMP2* mRNA after miR-519d-3p mimic and increased levels after miR-519d-3p inhibitor relative to their respective negative controls, whereas *TNFRSF11B* (*OPG*) changes were modest. $p < 0.05$ as compared to control

Statistics. Data were presented as mean ± standard deviation (SD). Comparisons between two groups were made using a two-tailed Student's *t*-test, and comparisons among more than two groups were performed using a one-way ANOVA followed by Tukey's post hoc test. A *P* value < 0.05 was considered statistically significant. All statistical analyses were conducted using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA).

Results

Morphology (TRAP staining). BBMs exposed to OB-CM displayed treatment-dependent osteoclastogenesis. Compared to their respective negative controls, OB-CM from miR-519d-3p mimic-transfected osteoblasts (mimic OB-CM) yielded fewer TRAP-positive multinucleated osteoclasts, whereas OB-CM from miR-519d-3p inhibitor–transfected osteoblasts (inhibitor OB-CM) produced more (Fig. 2, *a*–*d*). Across three independent experiments, mimic OB-CM consistently showed the lowest osteoclast counts and inhibitor OB-CM — the highest.

Reporter and mRNA readouts. In osteoblasts, RT-qPCR showed a decrease in *MMP2* mRNA after miR-519d-3p mimic and an increase after the inhibitor, whereas *TNFRSF11B* (*OPG*) mRNA changed only modestly (Fig. 3, *b*). In dual-luciferase assays using wild-type (WT) and seed-mutant (MUT) *MMP2* 3′UTR reporters, the mimic did not reproducibly lower WT reporter activity relative to MUT across replicates (Fig. 3, *a*), suggesting that *MMP2* repression in osteoblasts is context-dependent or indirect. Taken together, elevating miR-519d-3p in osteoblasts is associated with lower *MMP2* transcript levels and an anti-osteoclastogenic paracrine output, whereas its inhibition shows the opposite effect.

Discussion

These in vitro data support a model in which osteoblastic miR-519d-3p tempers the osteoclastogenic milieu created by tumor factors [1]. The most parsimonious explanation links the observed drop in osteoblastic MMP-2 to a relative preservation of OPG and, consequently, a lower RANKL/OPG drive for osteoclastogenesis. While prior work on non-bone cells showed direct miR-519d-3p targeting of *MMP2* or *MMP3*, our luciferase results in osteoblasts were not robust, implying context-dependent or upstream regulation [4,

5]. Functionally, across both OB-CM and 231-CM settings, elevating osteoblastic miR-519d-3p reduced TRAP-positive multinucleated osteoclasts, and vice versa.

Our findings extend the concept that miRNAs orchestrate tumor–bone communication [6]. In practical terms, the miR-519d-3p–*MMP2*/*OPG* axis could complement existing antiresorptives: local augmentation of miR-519d-3p in osteoblasts might suppress both matrix remodeling enzymes and osteoclastogenic bias, potentially slowing the "vicious cycle" of osteolysis [7]. Limitations of our study are the entirely in vitro design, a lack of direct RANKL/OPG protein quantification, and modest sample size. Future work should profile secretomes after miR-519d-3p manipulation and test in animal models of osteolytic metastasis.

To sum up, osteoblast-expressed miR-519d-3p attenuates tumor-induced osteoclastogenesis, accompanied by reduced *MMP2* and a shift toward OPG signaling. Although direct 3′UTR repression of *MMP2* in osteoblasts was not evident, the paracrine outcome is consistently anti-osteoclastogenic. This miRNA may offer a tractable starting point for modulating the metastatic bone niche.

Funding

None.

Conflict of interest

The author declares no conflict of interest.

Data availability

Available on reasonable request.

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MiR-519d-3p ГАЛЬМУЄ ОСТЕОКЛАСТОГЕНЕЗ ЧЕРЕЗ ЗНИЖЕННЯ ЕКСПРЕСІЇ *MMP2* ТА ЗСУВ СИГНАЛІНГУ В ОСТЕОБЛАСТАХ У БІК ЕКСПРЕСІЇ ОСТЕОПРОТЕГЕРИНУ

Стан питання. Перехресна паракринна взаємодія між пухлинними клітинами та резидентними кістковими клітинами є рушієм остеокластогенезу в передметастатичній ніші в кістковій тканині. МікроРНК miR-519d-3p пов'язують із супресією матриксних металопротеїназ. **Мета** роботи — з'ясувати, чи miR-519d-3p остеобластів гальмує остеокластогенез через зниження експресії *MMP-2* та зсув сигналінгу в остеобластах у бік експресії остеопротегерину (OPG). **Матеріали та методи.** Мишачі остеобласти лінії MC3T3-E1 трансфікували міметиком мікроРНК miR-519d-3p, її інгібітором або відповідними препаратами негативного контролю. Кондиційоване

середовище остеобластів (КС-ОБ) змішували в рівній пропорції з кондиційованим середовищем культури пухлинних клітин MDA-MB-231 (231-КС) та вносили до первинної культури макрофагів, що походять з кісткового мозку, для подальшого виявлення тартрат-резистентної кислої фосфатази (TRAP). мРНК *MMP-2* та OPG в остеобластах визначали кількісно за допомогою RT-qPCR, а також проводили дослідження за допомогою подвійного люциферазного репортерного аналізу з вектором, що містив 3′UTR ділянку мРНК MMP-2 для порівняння взаємодії між цією ділянкою мРНК та miR-519d-3p дикого та мутантного типів. **Результати**. КС-ОБ клітин, трансфікованих міметиком miR-519d-3p, спричинює зменшення утворення TRAP-позитивних багатоядерних остеокластів у порівнянні з відповідними контролями. Обробка макрофагів КС-ОБ клітин, трансфікованих інгібітором miR-519d-3p, приводить до утворення порівняно більшої кількості TRAP-позитивних клітин. В остеобластах трансфекція міметиком приводить до зниження експресії мРНК MMP-2, а трансфекція інгібітором — до збільшення експресії мРНК MMP-2. У подвійному люциферазному репортерному аналізі не виявлено відтворюваної переваги miR-519d-3p дикого типу над miR-519d-3p мутантного типу щодо репресії 3′UTR мРНК *MMP-2*, що свідчить або про залежну від контексту, або опосередковану регуляцію експресії *MMP-2*. **Висновок.** iR-519d-3p в остеобластах у системі in vitro асоціюється зі зниженням експресії *MMP-2*. Це свідчить про роль цієї мікроРНК як антиостеокластгенного паракринного фактора, що може бути використано як новий підхід для модулювання передметастатичній ніші в кістковій тканині після відповідної валідації в системі in vivo.

Ключові слова: miR-519d-3p, *MMP-2*, остеокластогенез, остеобласт, кондиційоване середовище, тартрат-резистентна кисла фосфатаза.