miR-34a blocks osteoporosis and bone metastasis by inhibiting osteoclastogenesis and Tgif2

Jing Y. Krzeszinski1, Wei Wei1, HoangDinh Huynh1, Zixue Jin1, Xunde Wang1, Tsung-Cheng Chang2, Xian-Jin Xie3,4, Lin He5, Lingegowda S. Mangala6,7, Gabriel Lopez-Berestein7,8, Anil K. Sood6,7,9, Joshua T. Mendell2,3 & Yihong Wan1,3

Bone-resorbing osteoclasts significantly contribute to osteoporosis and bone metastases of cancers1–3. MicroRNAs play important roles in physiology and disease4–6, and present tremendous therapeutic potential7. Nonetheless, how microRNAs regulate skeletal biology is underexplored. Here we identify miR-34a as a novel and critical suppressor of osteoclastogenesis, bone resorption and the bone metastatic niche. miR-34a is downregulated during osteoclast differentiation. Osteoclastic miR-34a-overexpressing transgenic mice exhibited lower bone resorption and higher bone mass. Conversely, miR-34a knockout and heterozygous mice exhibit elevated bone resorption and reduced bone mass. Consequently, ovariectomy-induced osteoporosis, as well as bone metastasis of breast and skin cancers, are diminished in osteoclastic miR-34a transgenic mice, and can be effectively attenuated by miR-34a nanoparticles. Mechanistically, we identify transforming growth factor-β-induced factor 2 (Tgif2) as an essential direct miR-34a target that is pro-osteoclastogenic. Tgif2 deletion reduces bone resorption and abolishes miR-34a regulation. Together, using mouse genetic, pharmacological and disease models, we reveal miR-34a as a key osteoclast suppressor and a potential therapeutic strategy to confer skeletal protection and ameliorate bone metastasis of cancers.

We examined the levels of several cancer-related microRNAs (miRNAs) during a time course of bone marrow osteoclastogenesis assay (Fig. 1a). While the expression of an osteoclast marker tartrate-resistant acid phosphatase (TRAP) was rapidly increased by RANKL and further elevated by rosiglitazone (Fig. 1b), miR-34a was rapidly downregulated by RANKL and further diminished by rosiglitazone (Fig. 1c). The levels of miR-34b/c, two other members in the miR-34 family, were unaffected and expressed at much lower levels than miR-34a (Fig. 1d).

The sequence of miR-34a is evolutionarily conserved and identical in mice and humans. Osteoclast differentiation both from mouse bone-marrow precursors (Fig. 1e, f) and from human peripheral blood mononuclear cells (hPBMCN) (Fig. 1g–i) was inhibited by a miR-34a mimic (pre-miR-34a) but enhanced by an antisense miR-34a inhibitor (anti-miR-34a), indicating that miR-34a regulation of bone resorption in mice will probably translate to human pathophysiology.

We generated osteoclastic miR-34a transgenic mice using CAG34a mice (Fig. 2a) and Tie2-cre mice8. Fluorescence-activated cell sorting (FACS) and imaging showed that osteoclast progenitors from the 34a-Tie2-Tg (CAG34a Cre+) mice were converted to GFP LacZ whereas the controls (CAG34a cre−) remained GFP LacZ (Extended Data Fig. 1a, b). Northern blot confirmed the overexpression of mature miR-34a in the bone marrow of 34a-Tie2-Tg mice (Extended Data Fig. 1c).

Osteoclast differentiation assay reveals that the higher levels of mature miR-34a in the 34a-Tie2-Tg cultures resulted in a lower induction of osteoclast markers, diminished number/size of mature osteoclasts and reduced resorptive activity, whereas precursor proliferation or survival was unaltered (Fig. 2b and Extended Data Fig. 1d–g). Consequently, serum bone resorption marker CTX-1 (carboxy-terminal telopeptides of type I collagen) and osteoclast number were decreased, whereas osteoblast number, bone formation rate and mineral apposition rate were unaltered (Fig. 2c and Extended Data Fig. 1h, i).

Micro-computed tomography of the proximal tibiae showed that 34a-Tie2-Tg mice had increased bone mass and decreased structure model index (SMI), which quantifies the relative amount of plates (SMI = 0, strong) and rods (SMI = 3, fragile) (Fig. 2d, e). Cortical BV/TV was also higher (Fig. 2f). Moreover, miR-34a transgenic mice generated by three other osteoclast-targeting cre drivers also exhibited a similar phenotype (Extended Data Figs 2 and 3). Thus, miR-34a in the osteoclast lineage augments bone mass by suppressing osteoclastogenesis and bone resorption.

To determine whether miR-34a is a physiologically relevant regulator of bone resorption, we next examined miR-34a knockout (34a-KO) and heterozygous (34a-Het) mice (Fig. 2g). Northern blot confirmed the diminished levels of miR-34a in 34a-KO (Extended Data Fig. 4a). Consistent with prior reports9,10, miR-34a deletion had no overt effect on mouse development. Osteoclast differentiation was augmented in 34a-Het and 34a-KO cultures, whereas precursor proliferation or survival was unaltered (Fig. 2h and Extended Data Fig. 4b–c). As a result, serum CTX-1 and osteoclast number were elevated (Fig. 2i and Extended Data Fig. 4g, h). Micro-computed tomography revealed that 34a-KO and 34a-Het mice exhibited a low bone mass with decreased connectivity density and increased SMI (Fig. 2j–l). Global miR-34a deletion also decreased bone formation as the serum marker PINP (amino-terminal propeptide of type I procollagen), osteoblast number, bone formation rate and mineral apposition rate were reduced (Fig. 2m and Extended Data Fig. 4g, h). The increased resorption in 34a-Het indicates that miR-34a function is haploinsufficient and sensitive to dosage reduction. The recently published miR-34abc triple knockout (34abc-TKO)10 and full miR-34a KO10 also showed a similar phenotype (Extended Data Fig. 5a–h), which validates our miR-34a gene trap mice and strengthens the finding that miR-34a loss-of-function elevates bone resorption.

Bone marrow transplantation showed that wild-type (WT) mice receiving 34a-KO marrow also exhibited higher CTX-1 (Extended Data Fig. 4i) compared with WT mice receiving WT marrow. Furthermore, osteoclastic miR-34a conditional knockout mice (34a-Tie2-KO) also exhibited elevated osteoclast differentiation and bone resorption, but unaltered bone formation, leading to a decreased bone mass (Extended Data Fig. 5i–n). Thus, miR-34a deletion in the osteoclast lineage elevates bone resorption.

Our genetic findings prompted us to investigate whether pharmacological administration of a miR-34a mimic can attenuate postmenopausal osteoporosis using an ovariectomy (OVX) mouse model and a chitosan (CH) nanoparticle vehicle. Reduction of uterine weight in all ovariectomized mice indicated effective oestrogen depletion (Fig. 3a). Unaltered body weight indicated the absence of obvious toxicity from CH nanoparticles.
Figure 1 | miR-34a suppresses osteoclastogenesis ex vivo. a. Diagram of bone marrow osteoclast differentiation assay; rosi, rosiglitazone. b–d, TRAP expression (b) and mature miRNA levels (c, d) (n = 3). e, f, Osteoclast differentiation was decreased by pre-miR-34a (e) but increased by anti-miR-34a (f) (n = 3). Left, mature miR-34a levels; right, TRAP expression; bottom, images of TRAP-stained cultures; mature osteoclast numbers (black) and resorptive activity (blue). Scale bar, 25 μm. V, vehicle; R, RANKL.

Figure 2 | miR-34a inhibits bone resorption and increases bone mass in vivo. a. Diagram of the conditional miR-34a transgene (CAG-34a). b, 34a-Tie2-Tg cultures showed decreased osteoclast differentiation (n = 3). Left, miR-34a levels; middle, TRAP expression; right, TRAP staining, osteoclast numbers (black) and resorptive activity (blue). Scale bar, 25 μm. c, hPBMN + hRANKL cultures showed decreased osteoclast differentiation (n = 4). g, Mature miR-34a levels. h, TRAP expression. i, Mature osteoclast numbers. j, TRAP staining and resorptive activity. Scale bar, 25 μm. Error bars, s.d. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001; NS, non-significant.
Figure 3 | miR-34a attenuates osteoporosis and cancer bone metastases. a–f, OVX or sham operation was performed on 10-week-old female mice. Three days after surgery, the OVX mice were treated with miR-34a-CH (34a) or miR-Ctrl-CH (Ctrl) at 5 μg per mouse twice a week for 5 weeks (n = 5). a, Uterine weight. b, Body weight. c, Serum CTX-1. d, Serum P1NP. e, Micro-computed tomography. f, Trabecular bone parameters. g, miR-34a levels in each tissue from miR-34a-CH- vs. miR-Ctrl-CH-treated mice 72 h after a single injection (n = 3). Top, mature miR-34a levels; bottom, fold induction. BM, bone marrow. h–k, 34a-Tie2-Tg mice or controls (3-month-old, female, n = 7) were subjected to OVX and analysed 5 weeks after surgery. h, Uterine weight. i, Serum CTX-1. j, Serum P1NP. k, BV/TV by micro-computed tomography. l, Xenograft of MDA231-BoM-1833 cells into 34a-Tie2-Tg (n = 8) or control (n = 9). m, Allograft of B16-F10 cells into 34a-Tie2-Tg (n = 4) or control (n = 6). n, MDA231-BoM-1833 cells in 34a-KO (n = 6), 34a-Het (n = 6) or control (n = 6). p, Bone metastasis of MDA231-BoM-1833 cells was attenuated by miR-34a-CH delivered 3 days after xenograft at 10 μg per mouse twice a week for 5 weeks (n = 5). p–r, Bone metastasis of MDA231-BoM-1833 cells was attenuated by miR-34a-CH delivered 3 days after xenograft at 10 μg per mouse twice a week for 5 weeks (n = 5). p, Bioluminescence imaging (BLI) signal. q, Left, BLI images; right, number and size of bone metastases (met). r, X-ray images and histology images for TRAP and ALP (alkaline phosphatase) staining. Arrows, osteolytic lesions. s, Bone metastasis of B16-F10 cells was attenuated by miR-34a-CH delivered at 5 μg per mouse twice a week for 4 weeks starting 1 week before cancer cell injection (n = 8). t–u, Statistical analyses used a Mann–Whitney U test and are shown as mean ± s.d. with P values illustrated. a, c, d, f, h–l, n–p, P < 0.05 by analysis of variance. **P < 0.01, ***P < 0.001, ****P < 0.0001, NS, non-significant.

Data Fig. 6e). These results indicate that osteoclastic miR-34a overexpression is sufficient to impede osteoporosis, and the osteoclast is a key site for miR-34a therapeutic benefit.

To determine whether osteoclastic miR-34a confers protection from bone metastases, we employed two cancer-cell–cardiac-injection models. First, a human breast cancer cell line (MDA231-BoM-1833) was xenografted into female nude mice. This model allowed us to assess cancer cells from human. Second, a mouse melanoma cell line (B16-F10) was allografted into immune-competent male mice. This model took consideration of adaptive immunity. In both models, bone metastases were attenuated in 34a-Tie2-Tg and 34a-PT-Tg mice but exacerbated in 34a-KO and 34a-Het mice (Fig. 3l–o and Extended Data Fig. 7a–f). Because miR-34a remained intact in the exogenous cancer cells, the altered bone metastases resulted from the altered miR-34a in the bone microenvironment of the host.
Pharmacologically, we tested both a treatment protocol using the human breast cancer model and a prevention protocol using the mouse melanoma model. In both cases, bone metastases were diminished by miR-34a-CH (Fig. 3p–q). Systemic miR-34a-CH delivery affected neither tumour growth nor metastasis to other organs such as lung (Extended Data Fig. 8a, b). Moreover, treating only the cancer cells with miR-34a-CH before injection had no effect (Extended Data Fig. 8c, d). Consistent with the published finding that miR-34abc deletion does not increase tumorigenesis18, our 34a-KO mice also showed unaltered cancer susceptibility (Extended Data Fig. 8e).

Since systemic miR-34a-CH treatment not only decreases bone resorption but also increases bone formation, we examined the effects of miR-34a overexpression in osteoblasts. We bred the CAG34a mice with Osterix-CreER mice to generate 34a-Osx-Tg mice. Osteoblast differentiation was reduced for 34a-KO and 34a-Het mice, but increased for 34a-Osx-Tg mice (Extended Data Fig. 9a–d). Consequently, 34a-Osx-Tg mice exhibited a higher bone formation but unaltered bone resorption, leading to an increased bone mass (Extended Data Fig. 9e–g). Importantly, however, the elevated bone formation alone in the 34a-Osx-Tg mice was insufficient to attenuate either OVX-induced bone loss or cancer bone metastases (Extended Data Fig. 9h). Together, our conditional miR-34a transgenic mouse models pinpointed the mechanisms underlying the therapeutic benefits of miR-34a by revealing that osteoclast, rather than cancer cell or osteoblast, is the critical and essential player.

To elucidate the mechanisms, we identified Tgif2 as a novel direct miR-34a target in the osteoclast lineage (Extended Data Fig. 10a–c). Tgif2 expression was suppressed by miR-34a gain-of-function, but increased by miR-34a loss-of-function, both in mouse and human osteoclast cultures (Fig. 4a, b and Extended Data Fig. 10d, e). The miR-34a seed region in Tgif2 3′ untranslated region (UTR) is evolutionarily conserved in mammals (Fig. 4c). Luciferase reporter assay showed that Tgif2 3′ UTR is sufficient to confer miR-34a regulation (Fig. 4d, e). Importantly, when the miR-34a seed region in the Tgif2 3′ UTR was mutated, miR-34a regulation was abolished (Fig. 4d, e).

**Figure 4** | Tgif2 is an essential miR-34a direct target and a pro-osteoclastogenic factor. a, Tgif2 expression was inhibited by pre-miR-34a in osteoclast cultures (n = 3). b, Tgif2 expression in WT and 34a-PT-Tg osteoclast cultures (n = 3). c, Sequence alignment of the Tgif2 3′ UTR. d, A diagram of Tgf2 3′ UTR reporters. e, Luciferase readout from WT or mutant Tgf2 3′ UTR reporter co-transfected in HEK293 cells with pre-miR-34a or anti-miR-34a (n = 3). f–h, Comparison of Tgfl2-KO, Tgf2-Het and WT control mice (1.5-month-old, male, n = 7). f, Serum CTX-1. g, h, Micro-computed tomography of tibiae. i, j, Images of the trabecular bone of the tibial metaphysis (scale bar, 10 μm). k, Decreased osteoclast differentiation in Tgf2-KO and Tgf2-Het cultures (n = 3). l, Tgf2-KO cultures were resistant to the anti-osteoclastogenic effects of pre-miR-34a (n = 3). m–o, Top, TRAP expression; bottom, TRAP staining; osteoclast number (black) and resorptive activity (blue). L–p, Tgif2/34a double knockout (DKO) mice were compared with WT, Tgf2-KO or 34a-KO (2-month-old, male, n = 4). k, Osteoclast differentiation. I, Serum CTX-1. m, Tgf2 mRNA in RAW264.7 cells after transfection of transcription factors (n = 3). n, Chromatin immunoprecipitation of transcription factor binding and H3K4me3 levels at the endogenous Tgf2 promoter in RAW264.7 cells 3 days after RANKL treatment (n = 6). o, Luciferase reporter assay showed that Tgf2 3′ UTR was transfected into WT, Tgf2-KO or 34a-KO osteoclast cultures (n = 6). q, r, Nfat1 mRNA (q, n = 3). c-Jun phosphorylation and IκBα degradation (p) in WT, Tgf2-KO or 34a-KO osteoclast cultures. Ratios of c-Jun/total-c-Jun and IκBα/β-actin are shown. s, A model for how miR-34a suppresses osteoclastogenesis. Error bars, s.d. **P < 0.05, ***P < 0.01, ****P < 0.001, NS, non-significant.
miR-34a, miR-34b and miR-34c are commonly deleted in human cancers. In vitro studies suggest that they may be critical mediators of p53 function and potential tumour suppressors. Specifically, in vitro studies reveal that miR-34abc triple knockout mice exhibit intact p53 function without increased tumorigenesis. Nonetheless, systemic miR-34a administration can indeed attenuate cancer malignancy. This raises the intriguing possibility that its anti-cancer effects may reside in other cells that constitute the tumour microenvironment such as the osteoclasts in the bone metastatic niche. Indeed, our findings illustrate that bone metastases are effectively blocked by miR-34a in osteoclasts, thus providing the first in vivo genetic evidence that miR-34a opposes malignant progression of cancer by disarming the metastatic niche.

**METHODS SUMMARY**

Conditional miR-34a transgenic mice were generated using the CAG-Z-EGFP vector. miR-34a knockout mice were generated using a gene trap embryonic stem cell line.

**Online Content**

Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper. References unique to these sections appear only in the online paper.

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**Author Contributions** J.Y.K. and Y.W. conceived the project and designed the experiments. All experiments, except those listed below, were performed by J.Y.K. W.W. assisted with micro-computed tomography, enzyme-linked immunosorbent assay and histomorphometry analyses. H.D.H. assisted with bone marrow transplantation and injection. Z.J. assisted with FACS analyses. X.W. assisted with western blot analyses. T.C.C. assisted with northern blot analyses and lifespan experiments. X.J.X. assisted with statistical analyses. L.H. provided the miR-34a knockout mice. L.S.M., G.L.B. and A.K.S. assisted with nanoparticle packaging. J.T.M. provided the miR-34a gene trap embryonic stem cell.

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METHODS

Mice. To generate cre-flox controlled conditional miR-34a transgenic mice (CAG34a), a 431-base-pair (bp) genomic sequence containing 168 bp 5′ and 161 bp 3′ of the pre-miR-34a sequence was inserted into the CAG-Z-EGFP vector23. Transgenic founders on pure C57BL/6J background were established by pronuclear injection at the University of Texas Southwestern transgenic core. From 14 founders that carried the LacZ and GFP transgenes, we selected six that had the highest tail LacZ expression, and bred them to cre transgenic mice. Representative results from at least two independent founders are reported here. To establish osteoclastic miR-34a transgenic mice, CAG34a mice were bred with the previously described Tie2cre mice24, PPARγ-TAT;TRE-cre (PT-cre) mice25, lysosome-cre (Lys-cre) mice26 or Ctsk-cre mice27. To establish osteoblastic miR-34a transgenic mice, miR-34a mice were bred with the previously described Ocsx-creER mice28. All conditional miR-34a transgenic mice were on pure C57BL/6J background, and compared with littermate controls that carry only the transgene allele or only the Cre allele; representative results for ‘transgene only’ group is shown as ‘Ctrl’ group; consistent with previous studies, these Cre lines alone do not exhibit bone phenotype. miR-34a knockout mice in a C57BL/6-129P2 mixed genetic background were generated using a mouse embryonic stem cell line (International Gene Trap Consortium done YH3AS) harbouring a gene-trap integration in the miR-34a transcription unit, and backcrossed to C57BL/6J mice for at least five generations. In this gene-trap allele, a splice-acceptor cassette (fusion of β-galactosidase and neomycin transferase) was inserted between exon 2 and 3 of the mouse miR-34a gene, leading to a truncated and non-functional pri-miR-34a transcript, miR-34abc triple knockout mice and WT controls in a C57BL/6-129sva/bd mixed background were provided by A. Ventura29.

miR-34a full knockout mice and WT controls on a pure C57BL/6J background were provided by L. He30. Tg2iKO mice on a C57BL/6J-129 mixed background were provided by D. Wotton31, miR-34a flox mice on a pure C57BL/6J background were from Jackson Laboratory. Osteoclastic miR-34a conditional knockout mice (34a-Tie2KO) were generated by breeding miR-34a flox mice with Tie2-cre mice. Bone marrow transplantation was performed as described32. Briefly, bone marrow cells from 2-month-old male donor (WT or 34aKO) were intravenously transplanted into five 2-month-old male C57BL/6J recipients that were irradiated at lethal dose (1,000 rontgens); the mice were analysed 3 months after transplantation. Ovariectomy or sham operation was performed on 10- to 20-week-old female mice. miRNA-carrying chitosan (CH) nanoparticles33 were delivered by intravenous injections at 5 μg per mouse or 10 μg per mouse twice a week for 4–5 weeks. Sample size estimate was based on power analyses performed using the SAS 9.3 TS X64_7PRQ platform. With the observed group differences, which were of great biological value, and the relatively small variation of the in vivo measurements, a sample size of four per group (n = 4) provided higher than 90% power at type 1 error rate of 0.08 (two-sided test), and a sample size of three per group (n = 3) provided higher than 80% power at type 1 error rate of 0.05 (two-sided test). For example, on BV/TV measures with a mean difference of 0.12 between the WT and mutant groups (s.d. 0.035 and 0.03 for each of the two groups), four mice per group yielded 98% power and three mice per group yielded 83% power using two sample t-test. Samples were randomly allocated to each group. Analyses were conducted in a blind fashion to the operator. All experiments were conducted using littermates. All protocols for mouse experiments were approved by the Institutional Animal Care and Use Committee of University of Texas Southwestern Medical Center.

Reagents. Mouse Tg2iKO short interfering RNA (siRNA) or control siRNA were from Santa Cruz Biotechnology. miR-34a precursor (pre-miR-34a) and negative control (pre-control), miR-34a inhibitor (anti-miR-34a) and negative control (anti-control) were from Life Technologies. All mRNA expression was normalized by L19. For mature miRNA expression, miRNA was reverse transcribed into cDNA using NCCode VILO miRNA cDNA Synthesis Kit (Life Technologies) then analysed using real-time PCR (SYBR Green) and a primer specific for the mature miRNA. All miRNA expression was normalized by snor251.

Identification of miR-34a targets in the osteoclast lineage. To elucidate the molecular mechanisms for miR-34a inhibition of osteostegnosis and bone resorption, we identified key direct miR-34a target genes that are pro-osteoclastogenic. First, we used the TargetScan bioinformatic tool to predict all the miR-34a targets by searching for conserved eight- or seven-base oligonucleotide sites that match the miR-34a seed region. Second, we searched databases such as BioGPS to select secondary targets that are expressed in the macrophage–osteoclast lineage. Third, we performed reverse transcription quantitative PCR to select tertiary targets that can be inhibited by miR-34a during osteoclast differentiation. Fourth, we performed luciferase reporter assay to test if the 3′ UTR of each target could directly suppress gene expression in response to miR-34a. To generate a CMV-Luc-3′ UTR reporter, an approximatively 300 bp Tg2iKO region centring the miR-34a target sequence cloned into the pMiyREPORT vector (Life Technologies) downstream of the luciferase open reading frame. To generate a mutant reporter with miss-matched miR-34a binding site, the 3′-miR-34a target sequence was altered using QuikChange XL site-directed mutagenesis kit (Stratagene). The reporters were co-transfected with CMV-β-galactosidase (as an internal transfection control), together with pre-miR-34a or pre-miR-control, anti-miR-34a or anti-miR-control using FuGENE HD reagent (Roche). The transfection assay was conducted in human embryonic kidney 293 cells and CV-1 monkey kidney cells to assess the intrinsic properties of the 3′ UTR in different cellular context, and representative results for 293 cells are shown. Luciferase activity was normalized by β-galactosidase activity. Bone metastasis analyses. Using a VisualSonics Vevo770 small animal ultrasound device, luciferase-labelled cancer cells were injected into the left cardiac ventricle so that they could bypass the lung and efficiently migrate to the bone34. Bone metastases were detected and quantified weekly after injection by BLI using a Caliper Xenogen Spectrum instrument at University of Texas Southwestern small animal imaging core facility. The osteolytic metastatic lesions were imaged by radiography using a CT-35 instrument (SCANCO Medical) and the structural resolution) and the structural characteristics of the trabecular and cortical bone (7 μm resolution). Trabecular bone parameters were calculated using Scanco software to analyse the bone scans from the trabecular region directly distal to the proximal tibial growth plate. An osteoclast marker gene, serum CTX-1 was measured with a RatLaps EIA kit (Immunodiagnostik Systems)35. As a bone formation marker, serum amino-terminal propeptide of type I collagen (PINP) was measured with a Rat/Mouse PINP enzyme immunoassay kit (Immunodiagnostic Systems)36. Static and dynamic histomorphometry used femurs and vertebrae as described37. Calcein (20 mg kg−1) was injected into 2-month-old mice 2 and 10 days before bone collection.

Ex vivo osteoclast and osteoblast differentiation. Osteoclasts were differentiated from bone marrow cells as described38. Briefly, haematopoietic bone marrow cells were purified with a 40 μm cell strainer, and differentiated with 40 ng m1 of mouse M-CSF (R&D Systems) in α-MEM containing 10% FBS for 3 days, then with 40 ng m1 of mouse M-CSF and 100 ng ml of mouse RANKL (R&D Systems) for 3–9 days, in the presence or absence of rosiglitazone (1 μM). Mature osteoclasts were identified as multinucleated (more than three nuclei) TRAP+ cells. Osteoclast differentiation was quantified by the RNA expression of osteoclast marker genes using reverse-transcription quantitative PCR, as well as number and size of mature osteoclasts. For osteoclast resorptive function analyses, bone marrow osteoclast differentiation was conducted in OsteoAssay bone plate (Lanza), and osteoclast activity was quantified as calcium release from bone into culture medium using CalciFluo ELISA assay (Lanza). Osteoclast precursor proliferation was quantified using a bromodeoxyuridine (BrdU) cell proliferation assay kit (GE Healthcare) as described39. Osteoclast apoptosis was quantified using Annexin V: PE Apoptosis Detection Kit 1 (BD Biosciences). Human PBMCs cells (RexBio) were differentiated into osteoclasts in α-MEM containing 10% FBS, 25 ng ml−1 M-CSF, 50 ng ml−1 hRANKL, 1 μM Dexamethasome and 1 μM rosiglitazone for 14 days; pre-miR or anti-miR were transfected on day 0 and day 6. BM-derived RANKL was added on day 7. Osteoblasts were differentiated from bone marrow cells as described40.

Gene expression analyses. For miRNA expression, RNA was reverse transcribed into complementary DNA (cDNA) using an ABI High Capacity cDNA RT Kit (Life Technologies) and then analysed using real-time PCR (SYBR Green) in triplicate. All miRNA expression was normalised by L19. For mature miRNA expression, RNA was reverse transcribed into cDNA using NCCode VILO miRNA cDNA Synthesis Kit (Life Technologies) then analysed in triplicate using real-time PCR (SYBR Green) and a primer specific for the mature miRNA. All miRNA expression was normalised by snor251.

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Extended Data Figure 1 | Additional analyses of 34a-Tie2-Tg mice. a–c, Further characterization of the transgene expression in 34a-Tie2-Tg mice. a, FACS analysis of the percentage of GFP<sup>+</sup> bone marrow osteoclast progenitors (c-Fms<sup>+</sup>RANK<sup>+</sup>) in 34a-Tie2-Tg mice and ‘transgene only, no cre’ control (n = 3). b, GFP and LacZ expression in osteoclast progenitors from 34a-Tie2-Tg mice (GFP<sup>+</sup>LacZ<sup>+</sup>) and ‘transgene only, no cre’ control mice (GFP<sup>+</sup>LacZ<sup>-</sup>). Scale bar, 100 μm. c, Northern blot analysis confirmed miR-34a overexpression in the haematopoietic bone marrow cells of 34a-Tie2-Tg mice. Ct, control; Tg, 34a-Tie2-Tg; EtBr, ethidium bromide. d, Quantitative PCR of mRNA expression of additional osteoclast marker genes (n = 3). e, Osteoclast function analysis. Bone marrow osteoclast differentiation was conducted in OsteoAssay bone plates (Lonza), and osteoclast activity was quantified as calcium release using CalciFluo ELISA assay (Lonza) (n = 8, mean ± s.e.m.). f, Osteoclast proliferation was not affected, quantified by BrdU incorporation (n = 6). g, Osteoclast apoptosis was not affected, quantified by FACS analysis of AnnexinV<sup>+</sup>7-AAD<sup>+</sup> cells (n = 6). h, i, Static and dynamic histomorphometry. h, Representative images of distal femur sections (2-month-old, male). Scale bars, 1 mm for Von Kossa images; 10 μm for TRAP, ALP and calcein images. i, Quantification of parameters at distal femur and vertebrae in 2-month-old male and female mice. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001; n.s., non-significant.
Extended Data Figure 2 | Effects of miR-34a overexpression using additional cre driver targeting osteoclast progenitors. 34a-PT-Tg mice were generated using PPAR-γ-TTA-TRE-cre driver. a, Bone marrow osteoclast differentiation assays. Left, mature miR-34a level (n = 3); middle, TRAP mRNA expression (n = 3); right, TRAP staining of differentiation cultures, quantification of mature osteoclast numbers per well in 24-well plates (black, n = 3), and quantification of bone resorptive activity by calcium release from bone plate into culture medium (μM) (blue, n = 6). b, Serum CTX-1 bone resorption marker (2-month-old males, n = 10). c, Micro-computed tomography analysis of the trabecular bone in proximal tibiae (2-month-old males, n = 4). d, Histomorphometry of the distal femur and vertebrae in 2-month-old mice. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001.
Extended Data Figure 3 | Effects of miR-34a overexpression using additional osteoclastic cre drivers. a–d, 34a-Lys-Tg mice were generated using Lysozyme-cre driver. e–h, 34a-Ctsk-Tg mice were generated using Ctsk-cre driver. a, e, Bone marrow osteoclast differentiation assays. Left, mature miR-34a level (n = 3); middle, TRAP mRNA expression (n = 3); right, TRAP staining of differentiation cultures, quantification of mature osteoclast numbers per well in 24-well plates (black, n = 3), and quantification of bone resorptive activity by calcium release from bone plate into culture medium (μM) (blue, n = 6). b, f, Serum CTX-1 (2-month-old males; b, n = 5; f, n = 8). c, g, Trabecular BV/TV of proximal tibiae by micro-computed tomography (2-month-old males; c, n = 4; g, n = 4). d, h, Histomorphometry of the distal femur and vertebrae in 2-month-old mice. *P < 0.05, **P < 0.01, ***P < 0.005.
Extended Data Figure 4 | Additional analyses of gene-trap miR-34a knockout mice. a, Northern blot analysis confirmed decreased miR-34a expression in the miR-34a gene trap knockout mice. Six-week-old female mice with corresponding genotypes were irradiated with a dose of 6 Gy, and 4 h later the spleen was collected for RNA extraction. Northern blotting for miR-34a was performed as described31. b, Quantitative PCR of mRNA expression of additional osteoclast marker genes (n = 3). c, Osteoclast function analysis. Bone marrow osteoclast differentiation was conducted in OsteoAssay bone plates (Lonza), and osteoclast activity was quantified as calcium release using CalciFluo ELISA assay (Lonza) (n = 8, mean ± s.e.m.). d, Osteoclast proliferation was not affected, quantified by BrdU incorporation (n = 6). e, Osteoclast apoptosis was not affected, quantified by FACS analysis of AnnexinV−7-AAD− cells (n = 6). f, WT mice transplanted with 34a-KO bone marrow cells exhibited higher serum CTX-1 levels than WT mice transplanted with WT bone marrow cells (n = 5 recipients per group). g, h, Static and dynamic histomorphometry. g, Representative images of distal femur sections (2-month-old, male). Scale bars, 1 mm for Von Kossa images; 10 μm for TRAP, ALP and Calcein images. h, Quantification of parameters at distal femur and vertebrae in 2-month-old male and female mice. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001; n.s., non-significant.
osteoclasts were increased. Expression of osteoclast markers was increased.

Expression of miR-34a was diminished in osteoclast precursors on day 3. Serum CTX-1 was increased.

miR-34a knockout mice by Tie2-cre (34a-Tie2-KO) were compared with littermate miR-34a/b/c triple knockout (34abc-TKO) mice were compared with WT control mice (2-month-old females, n = 4). Bone marrow osteoclast differentiation assay. a–c, Bone marrow osteoclast differentiation assay. a, Expression of miR-34a was diminished whereas expression of miR-34b and miR-34c remained absent/low in osteoclast precursors on d3. b, Expression of osteoclast markers was increased. c, Number, size and resorptive activity of mature osteoclasts were increased. d, Serum CTX-1 was increased. e–h, Targeted full miR-34a knockout (34a-full-KO) mice were compared with WT control mice (2-month-old females, n = 3). e–g, Bone marrow osteoclast differentiation assay. e, Expression of miR-34a was diminished in osteoclast precursors on day 3. f, Expression of osteoclast markers was increased. g, Number, size and resorptive activity of mature osteoclasts were increased. h, Serum CTX-1 was increased. i–n, Conditional miR-34a knockout mice by Tie2-cre (34a-Tie2-KO) were compared with littermate miR-34a/b/c triple knockout (34abc-TKO) mice were compared with WT control mice (2-month-old females, n = 4).
Extended Data Figure 6 | Anti-osteoporosis effects of miR-34a.

a, Histomorphometry of the distal femur and vertebrae in OVX mice treated with miR-34a-CH nanoparticles. OVX or sham operation was performed on 10-week-old WT female C57BL/6J mice. Three days after surgery, the OVX mice were intravenously injected with miR-34a-CH (34a) or miR-Ctrl-CH (Ctrl) at 5 μg per mouse twice a week for 5 weeks (n = 5).

b–d, Osteoprotective effects of miR-34a-CH in sham control mice. WT female C57B/6J mice (n = 5, 10 weeks old) were subjected to sham operation and then treated with miR-34a-CH or miR-34a-Ctrl at 5 μg per mouse twice a week for 5 weeks. b, Serum CTX-1. c, Serum P1NP. d, BV/TV of proximal tibiae by micro-computed tomography.

e, Histomorphometry of the distal femur and vertebrae in WT and 34a-Tie2-Tg mice after OVX. 34a-Tie2-Tg mice or controls (3-month-old females, n = 7) were subjected to OVX or sham operation and analysed 5 weeks after surgery. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001.
Extended Data Figure 7 | Additional characterization of bone metastases. a, Representative BLI images. b, Quantification of the number of metastasis. c, Quantification of the size of metastasis. For a–c, n = 9 for control, n = 8 for 34a-Tie2-Tg, n = 6 for WT and 34a-KO; results are shown as mean ± s.e.m. d, Xenograft of MDA231-BoM-1833 human breast cancer cells into 34a-Tie2-Tg nude mice (n = 8) or littermate control nude mice (n = 9). Results from each week are shown separately to visualize the difference better. e, Xenograft of MDA231-BoM-1833 human breast cancer cells into 34a-PT-Tg nude mice (n = 8) or littermate control nude mice (n = 8). Results from each week are shown separately to visualize the difference better. f, Allograft of B16-F10 mouse melanoma cells into 34a-PT-Tg (n = 7) or littermate control mice (n = 7). *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001; n.s., non-significant.
Extended Data Figure 8 | Effects of miR-34a on cancer cells.  

**a**, Systemic miR-34a-CH delivery did not affect the growth of B16-F10 melanoma cells injected subcutaneously (*n* = 5, 8-week-old males). Tumours were collected 18 days after cell injection; the result is shown as mean ± s.e.m.  

**b**, Systemic miR-34a-CH delivery did not affect cancer metastasis to other organs such as lung (*n* = 5, 8-week-old males). B16-F10 cells were intravenously injected retro-orbitally, BLI signals were quantified 2 weeks later and the result is shown as mean ± s.e.m.  

**c**, **d**, MiR-34a-CH treatment of cancer cell alone was not sufficient to inhibit bone metastasis. BoM-1833 cells were treated with miR-34a-CH or miR-Ctrl-CH in cultures for 24 h before cardiac injection (*n* = 5, 6-week-old males), and the mice were not treated with nanoparticles.  

**c**, Quantification of bone metastasis BLI signal 5 weeks after injection, shown as mean ± s.e.m.  

**d**, MiR-34a overexpression in BoM-1833 cells persisted for 5 weeks in cultures.  

**e**, Loss-of-function in 34a-KO and 34a-Het mice did not result in significantly increased susceptibility of cancer and mortality. Left, Kaplan–Meier survival curve for WT (*n* = 29), 34a-Het (*n* = 35) and 34a-KO (*n* = 29); *P* = 0.223 by log-rank (Mantel–Cox) test. Right, the 34a-KO allele was transmitted at normal Mendelian frequency. ****P < 0.001; n.s., non-significant.
Extended Data Figure 9 | Osteoblastic miR-34a overexpression is not sufficient to inhibit osteoporosis or bone metastases. a, Schematic diagram of the ex vivo bone marrow osteoblast differentiation assay. MSC GFs, mesenchymal stem cell growth factors; GP, β-glycerophosphate; AA, ascorbic acid. b, Osteoblast differentiation was decreased for bone marrow from 34a-KO and 34a-Het mice compared with WT controls, quantified by osteoblast marker genes osteocalcin and Col1a1 on day 13 (n = 6). c-h, Characterization of osteoblastic miR-34a transgenic mice. CAG34a mice were bred with Osterix-CreER mice to generate miR34a-Osx-transgenic (34a-Osx-Tg) mice or littermate control mice that carry only CAG34a transgene; all mice (1-month-old, male) received tamoxifen injection on two consecutive days and analysed 2 months later.

c, Elevated levels of mature miR-34a in 34a-Osx-Tg osteoblast differentiation cultures on day 13 (n = 6).

d, Osteoblast differentiation was increased for bone marrow from 34a-Osx-Tg mice compared with control mice, quantified by osteoblast marker genes osteocalcin and Colla1 on day 13 (n = 6). e, Serum P1NP was increased in 34a-Osx-Tg mice (n = 6). f, Serum CTX-1 was unaltered in 34a-Osx-Tg mice (n = 6). g, Histomorphometry of distal femur and vertebrae in 34a-Osx-Tg and control mice. h, OVX-induced bone resorption and bone loss was unaltered in 34a-Osx-Tg mice. 34a-Osx-Tg mice or controls (3-month-old females, n = 5) were subjected to OVX or sham operation and analysed 5 weeks after surgery.

i, Cancer bone metastasis was unaltered in 34a-Osx-Tg mice (n = 8). Statistical analyses in i were performed with a Mann–Whitney U Test and are shown as mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001; n.s., non-significant.
were normalized by internal control co-transfected in HEK293 cells with pre-miR-34a or pre-control. The results from 3'-UTR-Luc readout from 34a-KO and 34a-Het mice compared with WT control mice (right).

d. Western blot analysis showing that Tgif2 protein expression is decreased in the bone marrow osteoclast progenitors 34a-Tie2-Tg transgenic mice compared with control mice (left), but increased in the bone marrow osteoclast progenitors from 34a-KO and 34a-Het mice compared with WT control mice (right).

e. Human Tgif2 expression in hPBMN osteoclast differentiation cultures was suppressed by pre-miR-34a but enhanced by anti-miR-34a via transfection (n = 4).

Extended Data Figure 10 Additional characterization of Tgif2 as a key miR-34a direct target gene. a. A list of potential miR-34a target genes in the osteoclast lineage and characterization of miR-34a regulation. N.D., not determined. b. Fold changes in the expression of each candidate target gene after transfection with pre-miR-34a compared with pre-miR-ctrl in WT bone marrow osteoclast differentiation culture (n = 3).

c. Fold changes in the luciferase readout from 3'-UTR-Luc down-regulated by pre-miR-34a.

d. Representative western blot of Tgif2 protein expression. Ctrl, 5-month-old WT control mice; 34a-Tg, 1.5-month-old Tgif2-KO, Tgif2-Het and WT control mice. **P < 0.01, ***P < 0.005, ****P < 0.001; n.s., non-significant.