

Supplemental Information for:

Breast cancer exosomes contribute to pre-metastatic niche formation and promote bone metastasis of tumor cells

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Supplementary Figure 1. The influence of primary tumor on osteoblasts before the occurrence of bone metastasis

A. Quantitative micro-CT analysis of BMD of recipient mice at different checkpoint from the indicated groups (n = 6 mice per group).

B. The values of micro-CT parameters (Tb.N, Tb.Sp) at the distal femur metaphysis from tumor-free and tumor-bearing mice. (n = 8 mice per group). Tb. N, trabecular number, Tb.Sp, trabecular separation.

C. Osteoblast surface/bone surface (Ob.S/BS) and osteoblast number/bone perimeter (N.Ob/B.Pm) of the proximal tibia of recipient mice from the indicated groups (n = 3 in each group).

D. Representative images of the osteocalcin (OCN) staining in tibia sections from tumor-free and tumor-bearing mice. Scale bar, 25 μ m.

E. ELISA analysis of the bone formation marker PINP levels in serum of tumor-free (n = 6) and tumor-bearing mice (n = 5).

F. Representative images of anti-GFP staining of tibia section from tumor-free, tumor-bearing mice and mice with bone metastasis of SCP28 cells as a positive control at checkpoint of 5 weeks post implantation. The brown areas represent tumor. Scale bar, 50 μ m.

G. The changes of body weight in recipient mice at different checkpoint from tumor-free (n = 8) and tumor-bearing mice (n = 7).

Cumulative data are means \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (unpaired Student's t test).

All data are from at least three independent experiments.

Supplementary Figure 2. The effect of SCP28 cell-secreted exosomes on osteoclasts and osteoblasts *in vivo*

- A.** Body weight of recipient mice treated with SCP28 exosomes or PBS (n = 6 in each group).
- B.** Analysis of the expression of osteoclast marker genes including *Acp5*, *Ctsk*, *Mmp9*, and *Nfatc1* in tibias and femurs from mice treated with exosomes (n = 5) or PBS as controls (n = 6), respectively. The relative expression of each target transcript (after normalization to the housekeeping *Gapdh* gene) in control mice was set as 1, and that in exosome-treated mice was normalized, accordingly.
- C.** Ob.S/BS and N.Ob/B.Pm of the proximal tibia from the indicated groups (n = 3 in each group).
- D.** Representative images of the OCN staining in tibia sections from recipient mice treated with SCP28 exosomes or PBS. Scale bar, 25 μ m.
- E.** ELISA analysis of the level of PINP in serum of recipient mice treated with SCP28 exosomes (n = 5) or PBS (n = 6).

Cumulative data are means \pm SEM. * $P < 0.05$ (unpaired Student's *t* test). All data are from at least three independent experiments.

Supplementary Figure 3. The effect of SCP28 cell-secreted exosomes on osteoclasts and osteoblasts *in vitro*

A. Western blot analysis of expression of NFATc1 in BMM cell induced osteoclasts treated with or without SCP28 cell-secreted exosomes.

B. *Rab27a* mRNA level was analyzed in SCP28 cells treated with *Rab27a* siRNA or NC. The relative expression of *Rab27a* (after normalization to the housekeeping *Gapdh* gene) in NC samples was set as 1, and that in *siRab27a* samples was normalized, accordingly. *** $P < 0.001$ by Student's *t* test.

C. Western blot analysis of expression of NFATc1 in SCP28 cells treated with *Rab27a* siRNA or NC.

D. The amounts of exosomes secreted by SCP28 cells treated with *Rab27a* siRNA or GW4869 was analyzed by NanoSight.

E. Western blot analysis of expression of RUNX2 in osteoblasts treated with or without SCP28 cell-secreted exosomes.

F. Representative images of ALP staining in osteoblasts treat with SCP28 exosomes or not. Scale bar, 3 mm.

G. qRT-PCR analysis of *Alp*, *Colla1*, and *Ocn* mRNA in osteoblasts treated with or without SCP28 cell-secreted exosomes. The relative expression was normalized to *Gapdh* gene which is set as 1. Cumulative data are means \pm SEM. *** $P < 0.001$ (unpaired Student's *t* test). All data are from at least three independent experiments.

Supplementary Figure 4. SCP28 cells-secreted exosomes enhance bone metastasis of MDA-MB-231 tumor cells

A. Flowchart of the experimental processes and scheme of SCP28 cell-secreted exosome education and metastasis

B. BLI quantitation of dynamic bone metastasis of breast cancer MDA-MB-231 cells in recipient mice educated by SCP28 cell-secreted exosomes (n = 6) or controls (n = 7); ** $P < 0.01$ by two-way ANOVA.

C. Kaplan-Meier curve showing bone metastasis of MDA-MB-231 cells in recipient mice educated by SCP28 cell-secreted exosomes (n = 6) or controls (n = 7); * $P < 0.05$ (log-rank test).

D. Representative BLI imaging showing the MDA-MB-231 cells localization on day 42 in recipient mice educated by SCP28 cell-secreted exosomes or controls.

E. Representative images showing three-dimensional architecture after micro-CT reconstruction of the distal femurs from recipient mice. Scale bars, up 1 mm; bottom 300 μm .

F. Quantitative micro-CT analysis of distal femurs from recipient mice educated by SCP28 cell-secreted exosomes (n = 5) or controls (n = 7), including BMD, SMI, Tb.N, Tb.Th, Tb.Sp and C.Th. * $P < 0.05$; ** $P < 0.01$ by Student's *t* test.

G. Representative X-ray images (up) and quantification of osteolytic lesions (bottom) in MDA-MB-231 cell-implanted mice educated by SCP28 cell-secreted exosomes or controls (n = 7 per group). Arrows indicate osteolytic bone areas. ** $P < 0.01$ by Student's *t* test.

H. Representative images of H&E, TRAP, and OCN staining from MDA-MB-231 cell-implanted mice educated by SCP28 cell-secreted exosomes or controls. T, tumor; M, bone marrow. Scale bar, 25 μm .

Cumulative data are means \pm SEM. The statistical method indicated relatively. All data are from at least three independent experiments.

Supplementary Figure 5. Bisphosphonate treatment attenuates pro-metastatic effect of breast cancer cell-derived exosomes in the bone

A. Representative images showing three-dimensional architecture after micro-CT reconstruction of the distal femurs from recipient mice. Scale bars, up 1 mm; bottom 300 μm .

B. Quantitative micro-CT analysis of distal femurs from control recipient mice with different treatment: Control, $n = 5$; BP, $n = 4$; exosomes, $n = 5$; exosomes + BP, $n = 5$, including BMD, SMI, Tb.N, Tb.Th, Tb.Sp and C.Th. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ by one-way ANOVA.

Cumulative data are means \pm SEM. All data are from at least three independent experiments.

Supplementary Figure 6. SCP28 cell-derived exosomal miR-21 promotes osteoclastogenesis

A. Heatmap globally showing enriched miRNAs in SCP28 cell-secreted exosomes. Normalized expression level of miRNAs which are higher in the top 200 miRNAs was labeled on the top and the scale bar on the right.

B. Normalized miRNA expression levels for the top 17 miRNAs except *miR-21-5p* in SCP28 cell-secreted exosomes. The relative expression was normalized to *U6* gene which is set as 1. * $P < 0.05$; ** $P < 0.01$; ***, $P < 0.001$ by Student's *t* test.

C. qRT-PCR analysis of *miR-21* level in exosomes secreted by MCF10A, MDA-MB-231, SCP28 and parental MDA-MB-231-LM2 cells. The relative expression was normalized to *miR-16* gene which are set as 1, respectively. * $P < 0.05$; ** $P < 0.01$ by one-way ANOVA.

D. The expression of *miR-21* in SCP28 cells and exosomes was measured by qRT-PCR. The relative expression of *miR-21* in cell or exosomes was normalized to *U6* or *miR-16* gene which are set as 1, respectively. * $P < 0.05$; ** $P < 0.01$ by one-way ANOVA

E. qRT-PCR analysis of *miR-21* level in SCP28 cells treated with miR-21 ShRNA or NC. The relative expression of *miR-21* was normalized to *U6* gene which is set as 1. ** $P < 0.01$ by Student's *t* test.

F. Representative images showing three-dimensional trabecular architecture after micro-CT reconstruction of the distal femoral metaphysis of mice. Scale bar, 300 μm .

G. Quantitative micro-CT analysis of distal femurs from mice in each experimental group, including BMD, SMI, Tb.Th, Tb.Sp and BV/TV. Control, $n = 7$; SCP28/NC, $n = 6$; SCP28/Sh-miR-21, $n = 6$. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ by one-way ANOVA.

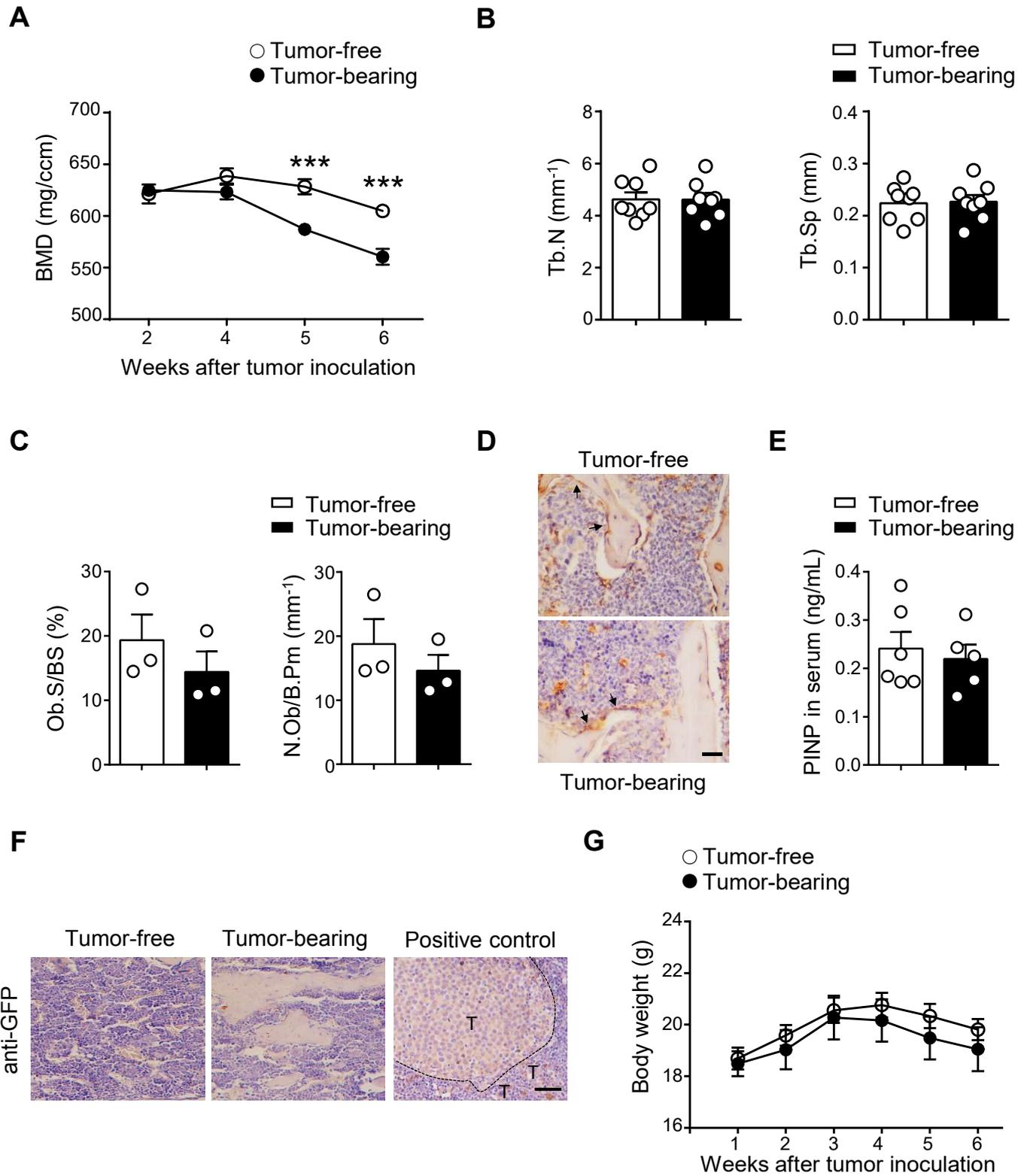
H. ELISA analysis of serum CTX-1 (ng/mL) in control ($n = 7$), SCP28/NC ($n = 6$) and SCP28/Sh-miR-21 ($n = 7$) mice. ** $P < 0.01$ by one-way ANOVA.

I. qRT-PCR analysis of the expression of osteoclast marker genes including *Acp5*, *Ctsk*, *Mmp9*, and *Nfatc1* in tibiae and femurs from control ($n = 6$), SCP28/NC ($n = 5$) and SCP28/Sh-miR-21 ($n = 5$) mice. The relative expression of each target transcript (after normalization to the housekeeping *Gapdh* gene) in tumor-free mice was set as 1, and that in tumor-bearing mice was normalized, accordingly. *

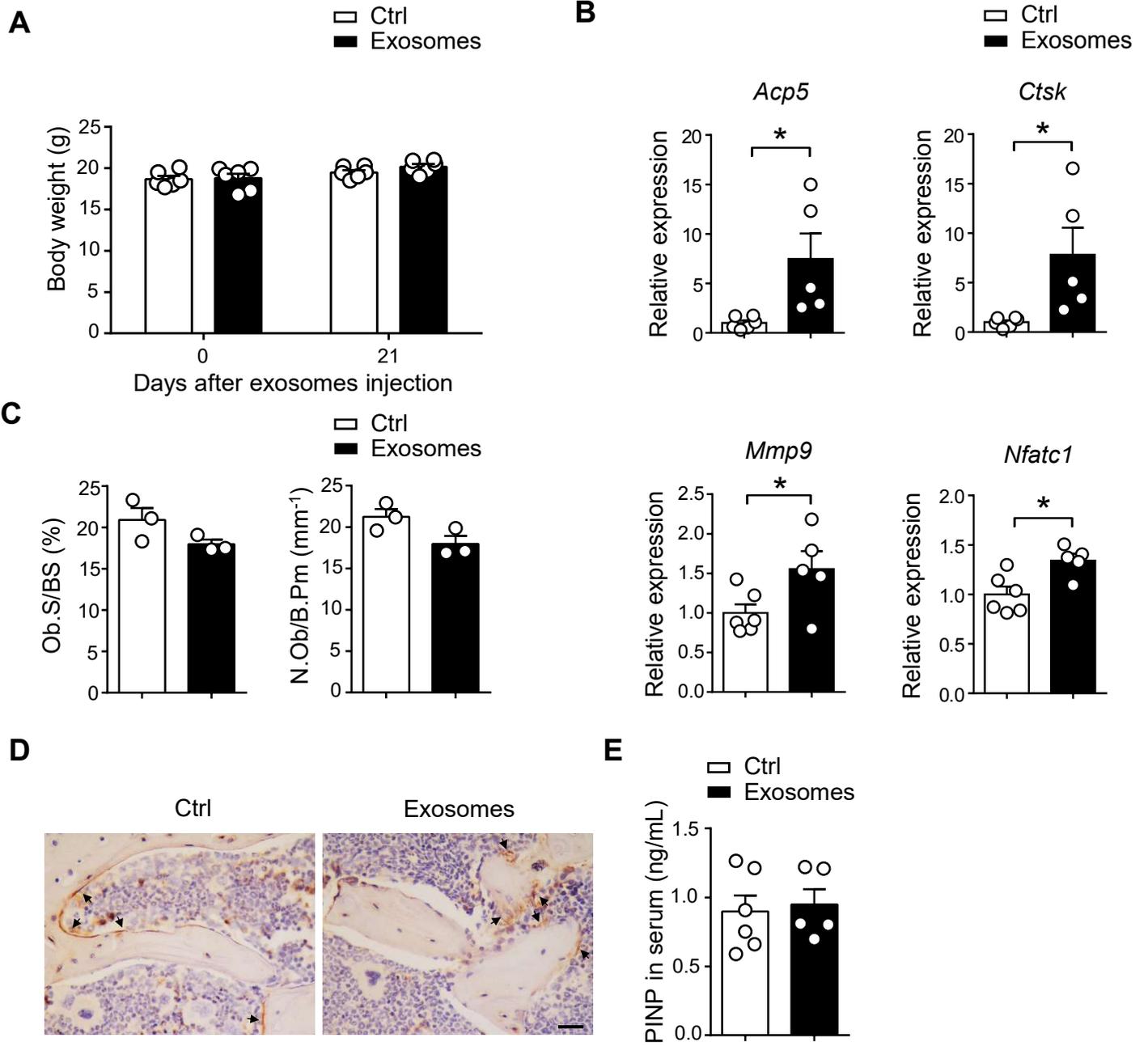
$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ by one-way ANOVA.

Cumulative data are means \pm SEM. All data are from at least three independent experiments.

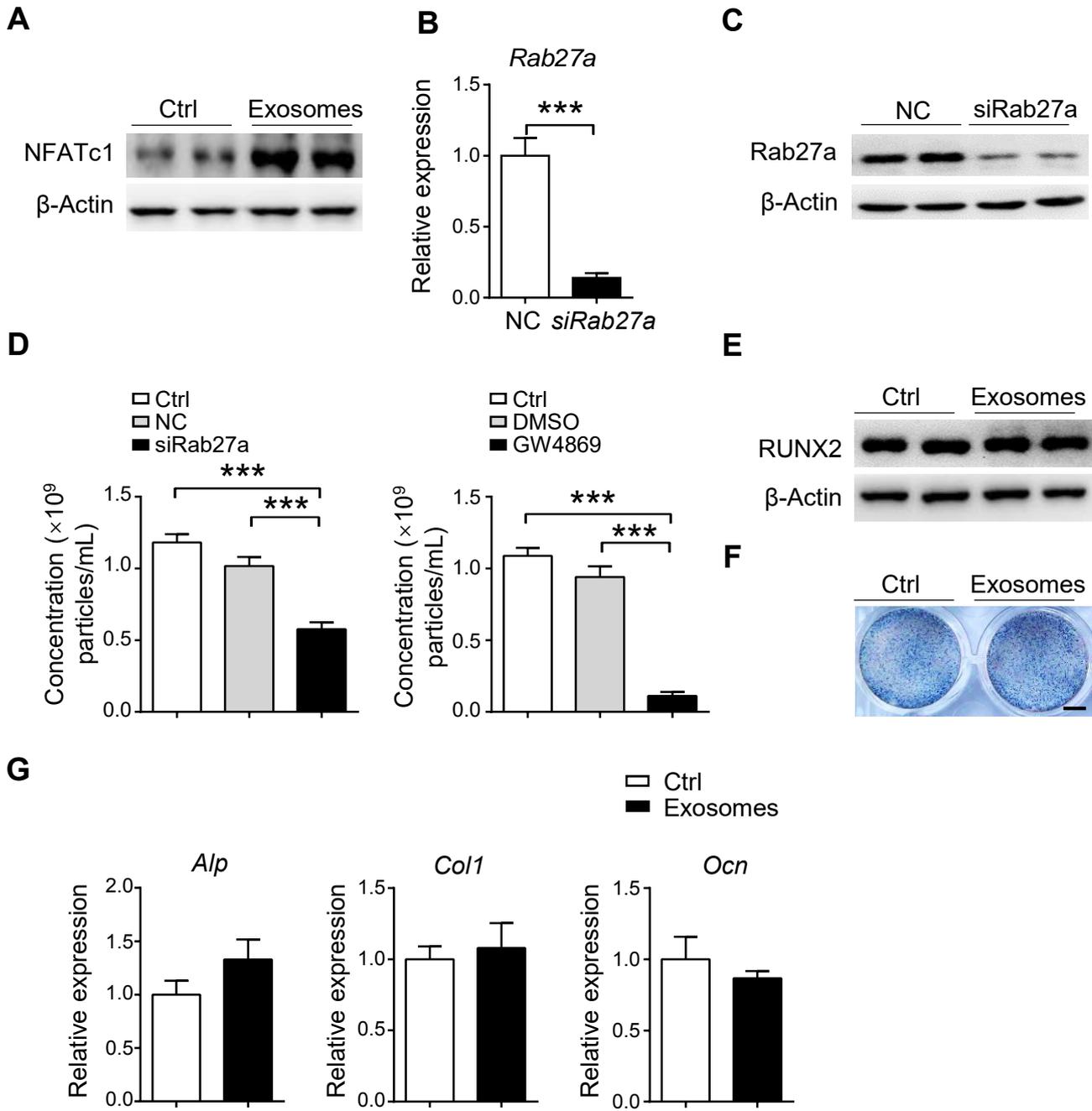
Yuan et al Supplementary Figure 1



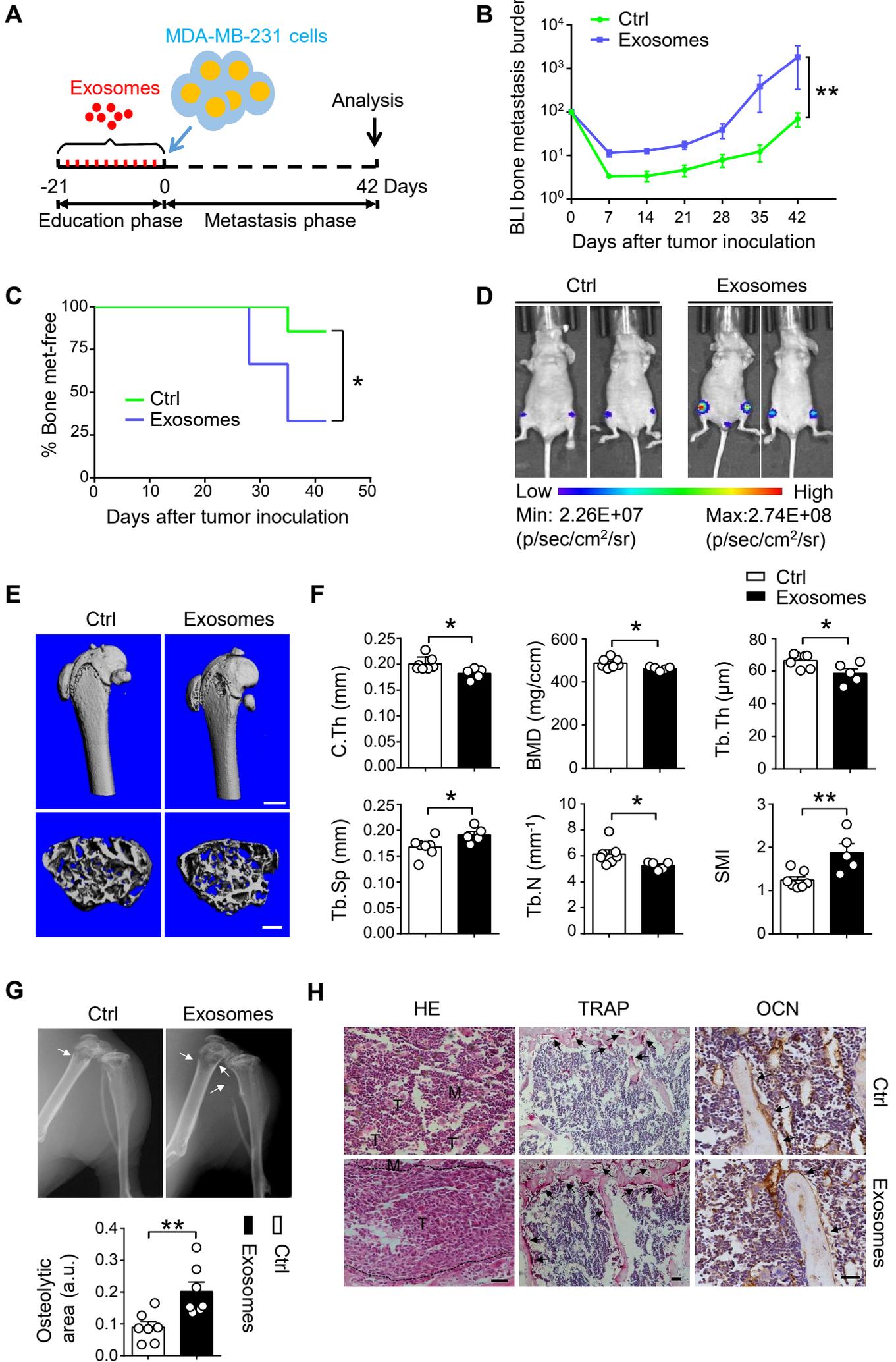
Yuan et al Supplementary Figure 2



Yuan et al Supplementary Figure 3

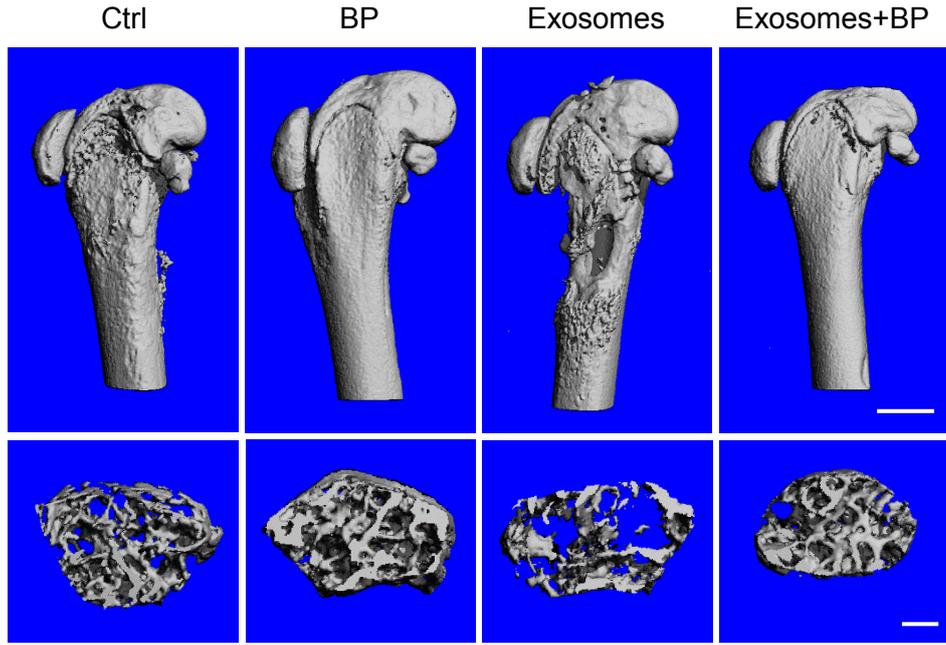


Yuan et al Supplementary Figure 4

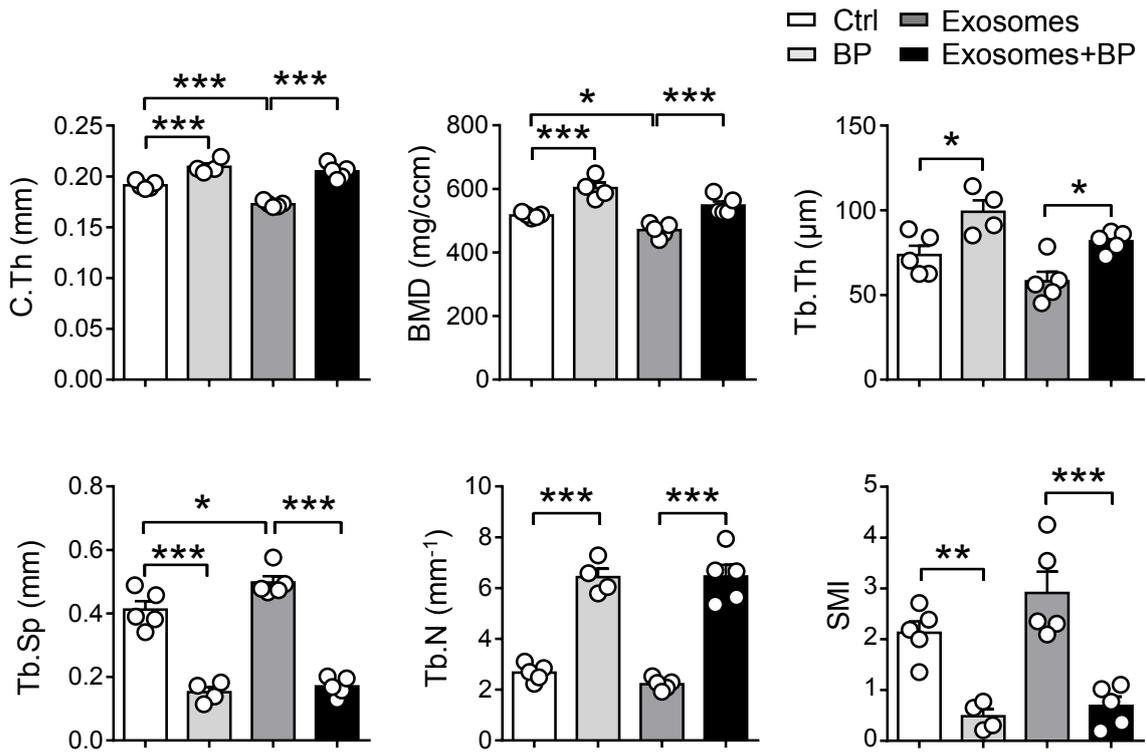


Yuan et al Supplementary Figure 5

A



B



Yuan et al Supplementary Figure 6

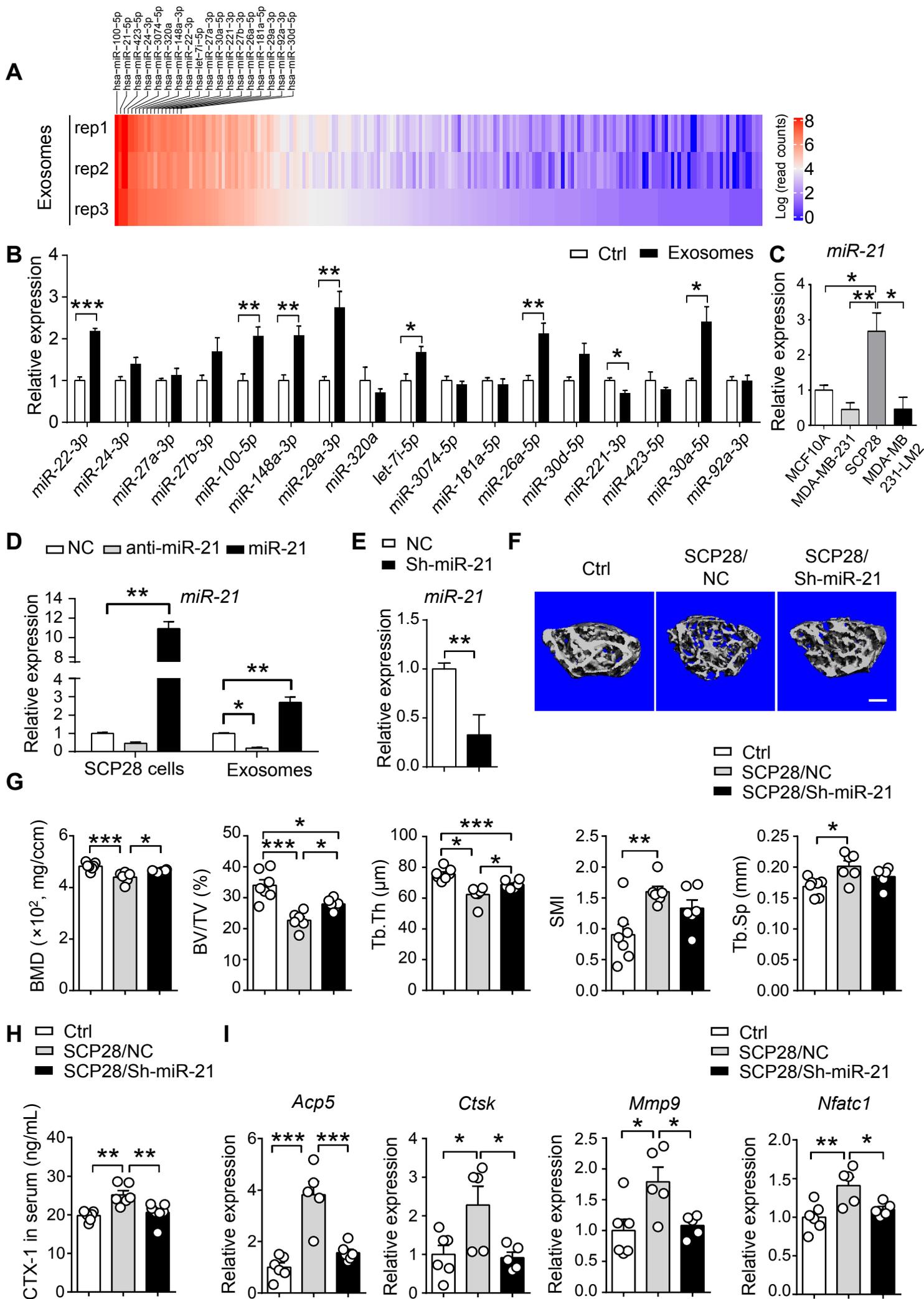


Table S1. The primer list for qRT-PCR

Name	sense primer (5'-3')	anti-sense primer (5'-3')
<i>mmu-Gapdh</i>	ATGGTGAAGGTCGGTGTGAA	GTCGTTGATGGCAACAATCTCC
<i>mmu-Nfatc1</i>	ACGCTACAGCTGTTCATTGG	CTTTGGTGTGGACAGGATG
<i>mmu-Acp5</i>	GCGACCATTGTTAGCCACATACG	CGTTGATGTGCGCACAGAGGGAT
<i>mmu-Mmp9</i>	GCGGCCCTCAAAGATGAACGG	GCTGACTACGATAAAGGACGGCA
<i>mmu-Ctsk</i>	GCGTTGTTCTTATTCCGAGC	CAGCAGAGGTGTGTACTATG
<i>mmu-Alp</i>	ATCTTTGGTCTGGCTCCCATG	TTTCCCGTTCACCGTCCAC
<i>mmu-Ocn</i>	CCAAGCAGGAGGGCAATA	TCGTCACAAGCAGGGTCA
<i>mmu-Coll1a1</i>	GGGACCAGGAGGACCAGGAAGT	GGAGGGCGAGTGCTGTGCTTT
<i>mmu-Pdcd4</i>	AGCGGTTAGAAGTGGAGTTGCTGT	ACAAGGTGATTGACAGGCTGTTGC
<i>hsa-Gapdh</i>	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
<i>hsa-Rab27a</i>	ACAACAGTGGGCATTGATTTCA	AAGCTACGAAACCTCTCCTGC
<i>hsa-Pre-miR-21</i>	CTTTAGGAGCATTATGAGC	ACTATCCCCATTTCTCCA
<i>hsa-miR-21-5p</i>	TAGCTTATCAGACTGATGTTGA	GAATCGAGCACCAGTTACGC
<i>hsa-miR-22-3p</i>	AAGCTGCCAGTTGAAGAACTGT	GAATCGAGCACCAGTTACGC
<i>hsa-miR-24-3p</i>	TGGCTCAGTTCAGCAGGAACAG	GAATCGAGCACCAGTTACGC
<i>hsa-miR-27a-3p</i>	TTCACAGTGGCTAAGTTCCGC	GAATCGAGCACCAGTTACGC
<i>hsa-miR-27b-3p</i>	TTCACAGTGGCTAAGTTCTGC	GAATCGAGCACCAGTTACGC
<i>hsa-miR-100-5p</i>	AACCCGTAGATCCGAACCTTGTG	GAATCGAGCACCAGTTACGC
<i>hsa-miR-148a-3p</i>	TCAGTGC ACTACAGAACTTTGT	GAATCGAGCACCAGTTACGC
<i>hsa-miR-29a-3p</i>	TAGCACCATCTGAAATCGGTTA	GAATCGAGCACCAGTTACGC
<i>hsa-miR-320a</i>	AAAAGCTGGGTTGAGAGGGCGA	GAATCGAGCACCAGTTACGC
<i>hsa-let-7i-5p</i>	TGAGGTAGTAGTTTGTGCTGTT	GAATCGAGCACCAGTTACGC
<i>hsa-miR-3074-5p</i>	GTTCCCTGCTGAACTGAGCCAG	GAATCGAGCACCAGTTACGC
<i>hsa-miR-181a-5p</i>	AACATTCAACGCTGTCCGTGAGT	GAATCGAGCACCAGTTACGC
<i>hsa-miR-26a-5p</i>	TCAAGTAATCCAGGATAGGCT	GAATCGAGCACCAGTTACGC
<i>hsa-miR-30d-5p</i>	TGTAACATCCCCGACTGGAAG	GAATCGAGCACCAGTTACGC
<i>hsa-miR-221-3p</i>	AGCTACATTGTCTGCTGGGTTTC	GAATCGAGCACCAGTTACGC
<i>hsa-miR-423-5p</i>	TGAGGGGCAGAGAGCGAGACTTT	GAATCGAGCACCAGTTACGC
<i>hsa-miR-30a-5p</i>	TGTAACATCCTCGACTGGAAG	GAATCGAGCACCAGTTACGC
<i>hsa-miR-92a-3p</i>	TATTGCACTTGTCCCGCCTGT	GAATCGAGCACCAGTTACGC
<i>hsa-miR-16</i>	TAGCAGCACGTAAATATTGGCG	GAATCGAGCACCAGTTACGC
<i>cel-miR-39</i>	GGCGCTACCTGTATCAATGG	GTGGTCAGCCAACTCGTCA
<i>U6</i>	CGCTTCGGCAGCACATATA	TTCACGAATTTGCGTGTGTCAT