Bone & Joint Research

Supplementary Material

10.1302/2046-3758.132.BJR-2023-0146.R2

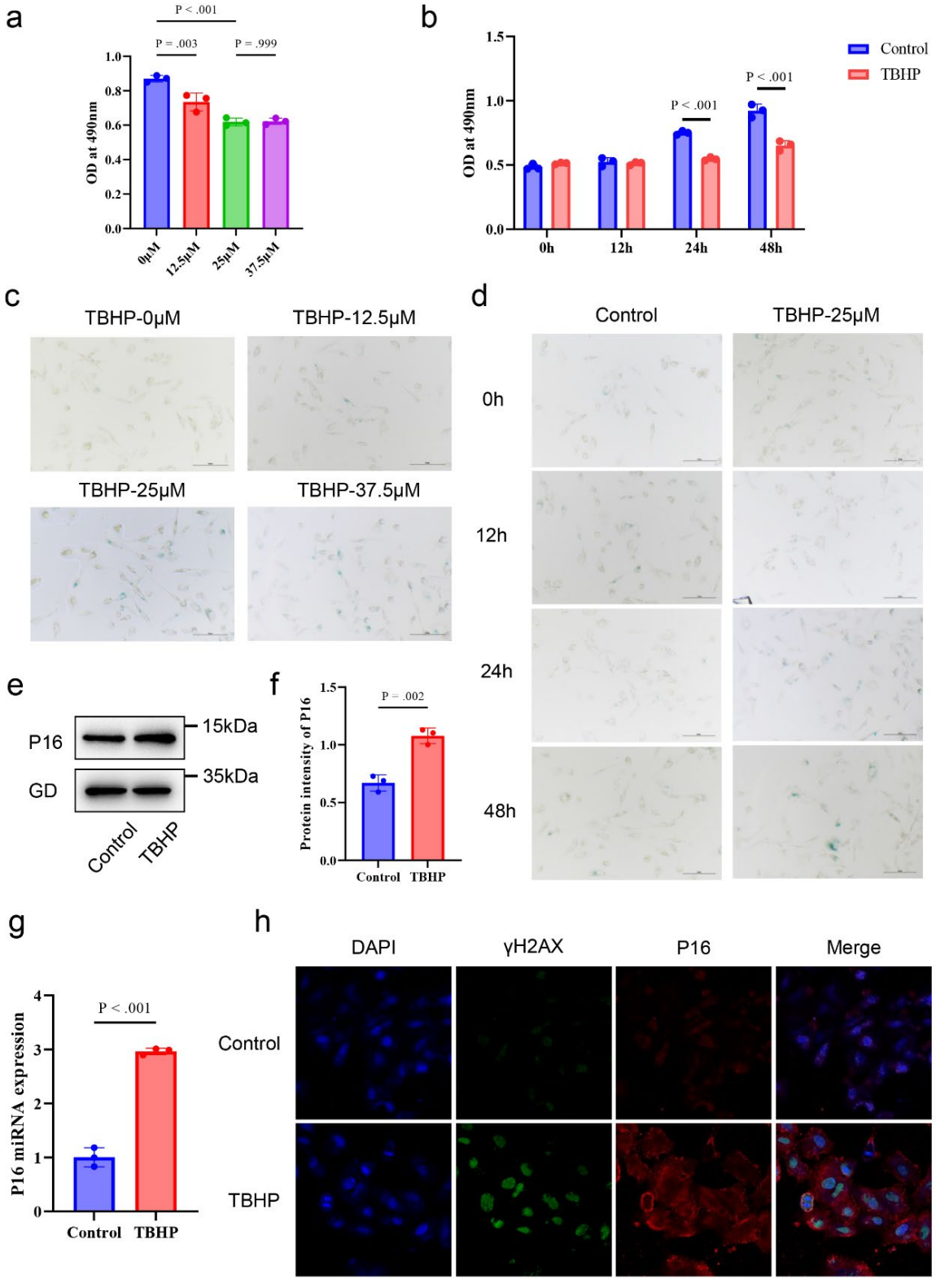


Fig a. Senescence induction in MLO-Y4 cells. a) and c) Cell Counting Kit 8 (CCK-8) assay and senescence-associated β-galactosidase (SA-β-Gal) staining of MLO-Y4 cells treated with different concentrations of tert-Butyl hydroperoxide (TBHP) (0, 12.5, 25, or 37.5 μM) for 24 h. b) and d) CCK-8 assay and SA-β-Gal staining of MLO-Y4 cells treated with 25 μM TBHP for 0, 12, 24, or 48 hours. e) and f) Western blotting and grayscale analysis of P16 expression in MLO-Y4 cells treated with 25 μM TBHP for 24 hours. g) Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

analysis of P16 expression in MLO-Y4 cells treated with 25 μM TBHP for 24 hours. h) Fluorescence staining of P16 and γH2AX in MLO-Y4 cells treated with 25 μM TBHP for 24 hours. The magnification in c), d), and h) is 10×. Data are shown as column charts and the p-values were calculated by the independent-samples *t*-test except those in Figure aa, which were calculated by one-way analysis of variance with Sidak's multiple comparison tests. The p-values were specified only when $p < 0.05$ (statistically significant). DAPI, 4 ,6-diamidino-2-phenylindole; GD, glyceraldehyde 3-phosphate dehydrogenase; miRNA, microRNA; OD, optical density.

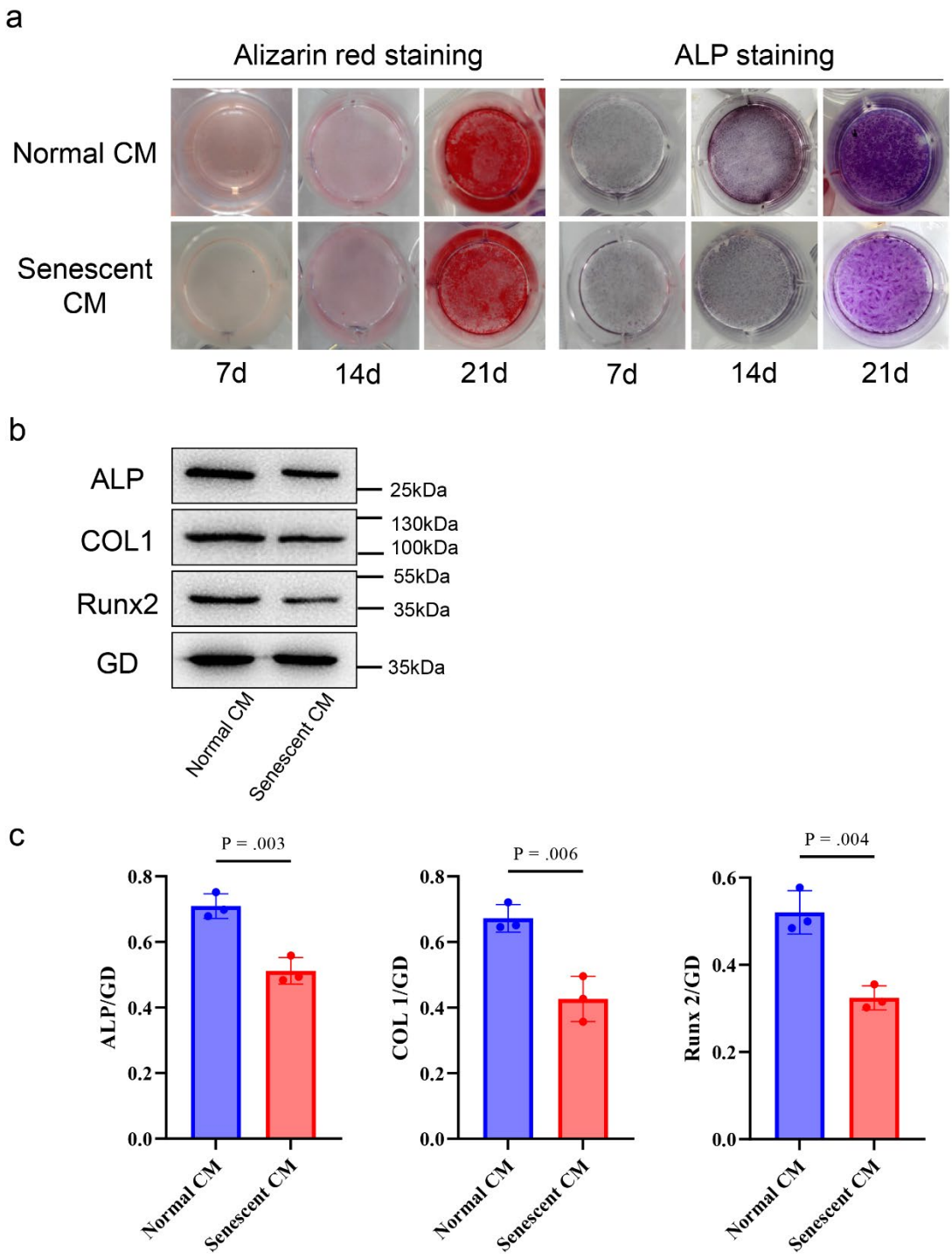


Fig b. Effects of conditioned medium (CM) from normal or senescent MLO-Y4 cells on MC3T3-E1 cells. a) Alizarin Red S and alkaline phosphatase (ALP) staining of MC3T3-E1 cells on days 7, 14, and 21 in culture with indicated CM. b) and c) Western blotting and grayscale analysis of ALP, Runt-related transcription factor 2 (Runx2), and collagen type I α1 (Col-I) expression in MC3T3-E1 cells exposed to indicated CM. Data are shown as column charts and the p-values were calculated by two-tailed independent-samples *t*-test. The p-values were specified only when $p < 0.05$ (statistically significant). GD, glyceraldehyde 3-phosphate dehydrogenase.

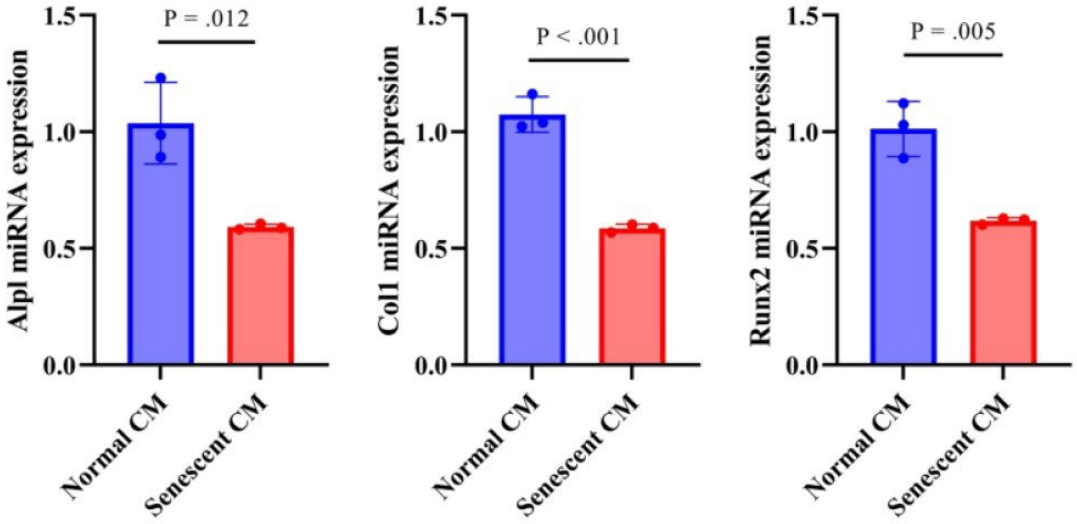


Fig c. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of alkaline phosphatase (*Alpl*), collagen type I α1 (*Col-I*), and Runt-related transcription factor 2 (*Runx2*) expression in MC3T3-E1 cells exposed to indicated conditioned medium (CM). Data are shown as column charts and the p-values were calculated by two-tailed independent-samples *t*-tests. The p-values were specified only when $p < 0.05$ (statistically significant). miRNA, microRNA.

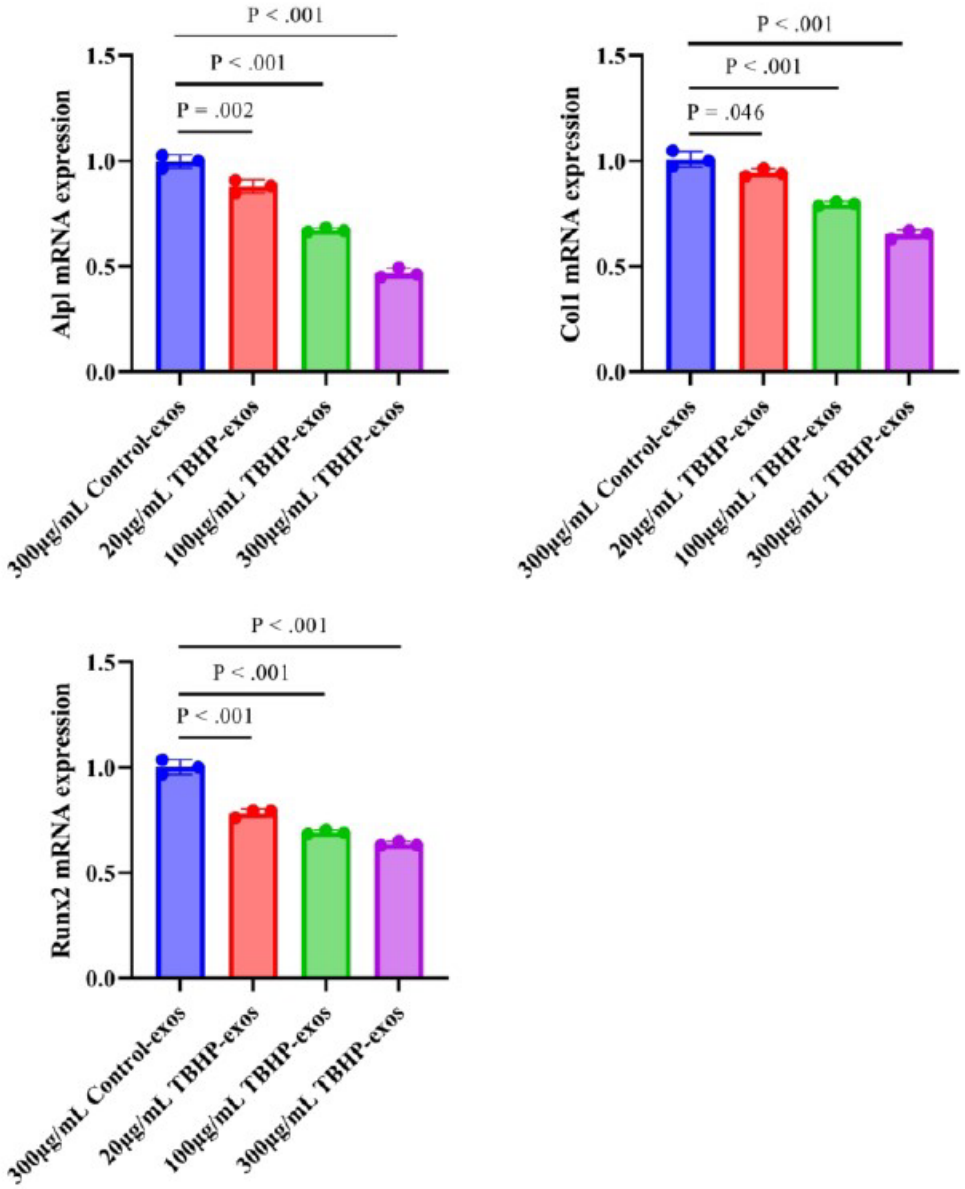
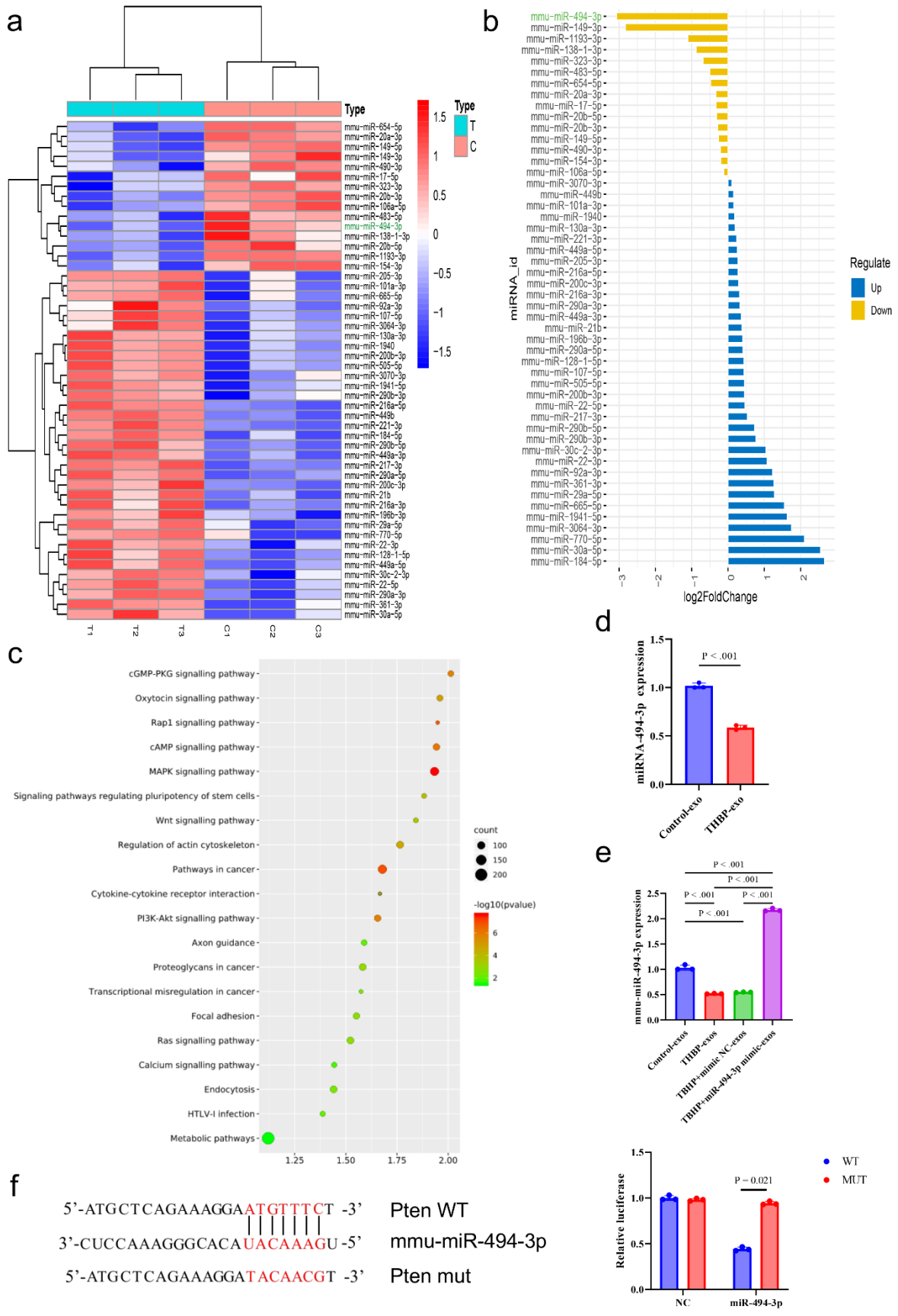


Fig d. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of alkaline phosphatase (*Alpl*), collagen type I α1 (*Col-I*), and Runt-related transcription factor 2 (*Runx2*) expression in MC3T3-E1 cells in different treatment groups. Data are shown as column charts and the p values were calculated by one-way analysis of variance with Sidak's multiple comparison tests. The p-values were specified only when $p < 0.05$ (statistically significant). mRNA, messenger RNA; TBHP, tert-Butyl hydroperoxide.


a
Type
T
C
mmu-miR-654-5p
mmu-miR-20a-3p
mmu-miR-149-5p
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mmu-miR-490-3p
mmu-miR-17-5p
mmu-miR-323-3p
mmu-miR-20b-3p
mmu-miR-106a-5p
mmu-miR-483-5p
mmu-miR-494-3p
mmu-miR-138-1-3p
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mmu-miR-200c-3p
mmu-miR-21b
mmu-miR-216a-3p
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mmu-miR-30c-2-3p
mmu-miR-22-5p
mmu-miR-290a-3p
mmu-miR-381-3p
mmu-miR-30a-5p
T1
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b
miRNA_id
log2FoldChange
Regulate
Up
Down
c
cGMP-PKG signalling pathway
Oxytocin signalling pathway
Rap1 signalling pathway
cAMP signalling pathway
MAPK signalling pathway
Signaling pathways regulating pluripotency of stem cells
Wnt signalling pathway
Regulation of actin cytoskeleton
Pathways in cancer
Cytokine-cytokine receptor interaction
PI3K-Akt signalling pathway
Axon guidance
Proteoglycans in cancer
Transcriptional misregulation in cancer
Focal adhesion
Ras signalling pathway
Calcium signalling pathway
Endocytosis
HTLV-I infection
Metabolic pathways
count
-log10(pvalue)
d
miRNA-494-3p expression
P < .001
Control-exo
THBP-exo
e
mmu-miR-494-3p expression
P < .001
Control-exos
THBP-exos
THBP+mimic NC-exos
THBP+miR-494-3p mimic-exos
f
5'-ATGCTCAGAAAGGAATGTTTCT -3'
Pten WT
3'-CUCCAAAGGGCACAUACAAAGU-5'
mmu-miR-494-3p
5'-ATGCTCAGAAAGGATACAACGT -3'
Pten mut
Relative luciferase
P = 0.021
WT
MUT
NC
miR-494-3p

Fig e. RNA sequencing profiles of exosomes derived from normal and senescent MLO-Y4 cells and the target gene of exosomal miR-494-3p. a) Heat map of differentially expressed microRNAs (miRNAs) according to RNA sequencing. b) Difference degree of exosomal miRNA expression. c) Kyoto Encyclopedia of Gene and Genomes (KEGG) pathway analysis of miR-494-3p. d) Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) of miR-494-3p expression in normal and senescent MLO-Y4 cell-derived exosomes. e) RT-qPCR of miR-494-3p expression in exosomes derived from MLO-Y4 cells transfected by miR-494-3p mimics. f) Binding site of miR-494-3p in Pten and luciferase reporter assay. Data are shown as column charts and the p-values were calculated by two-tailed independent-samples t-tests except those in Figure 5e, which were calculated by one-way analysis of variance with Sidak's multiple comparison tests. The p-values were specified only when $p < 0.05$ (statistically significant). cAMP, cyclic adenosine monophosphate; cGMP-PKG, cyclic guanosine monophosphate-protein kinase G; HTLV-1, human T-lymphotropic virus 1; MAPK, mitogen-activated protein kinase; mut, mutant; NC, negative control; PTEN, phosphatase and tensin homolog; TBHP, tert-Butyl hydroperoxide; WT, wild-type.

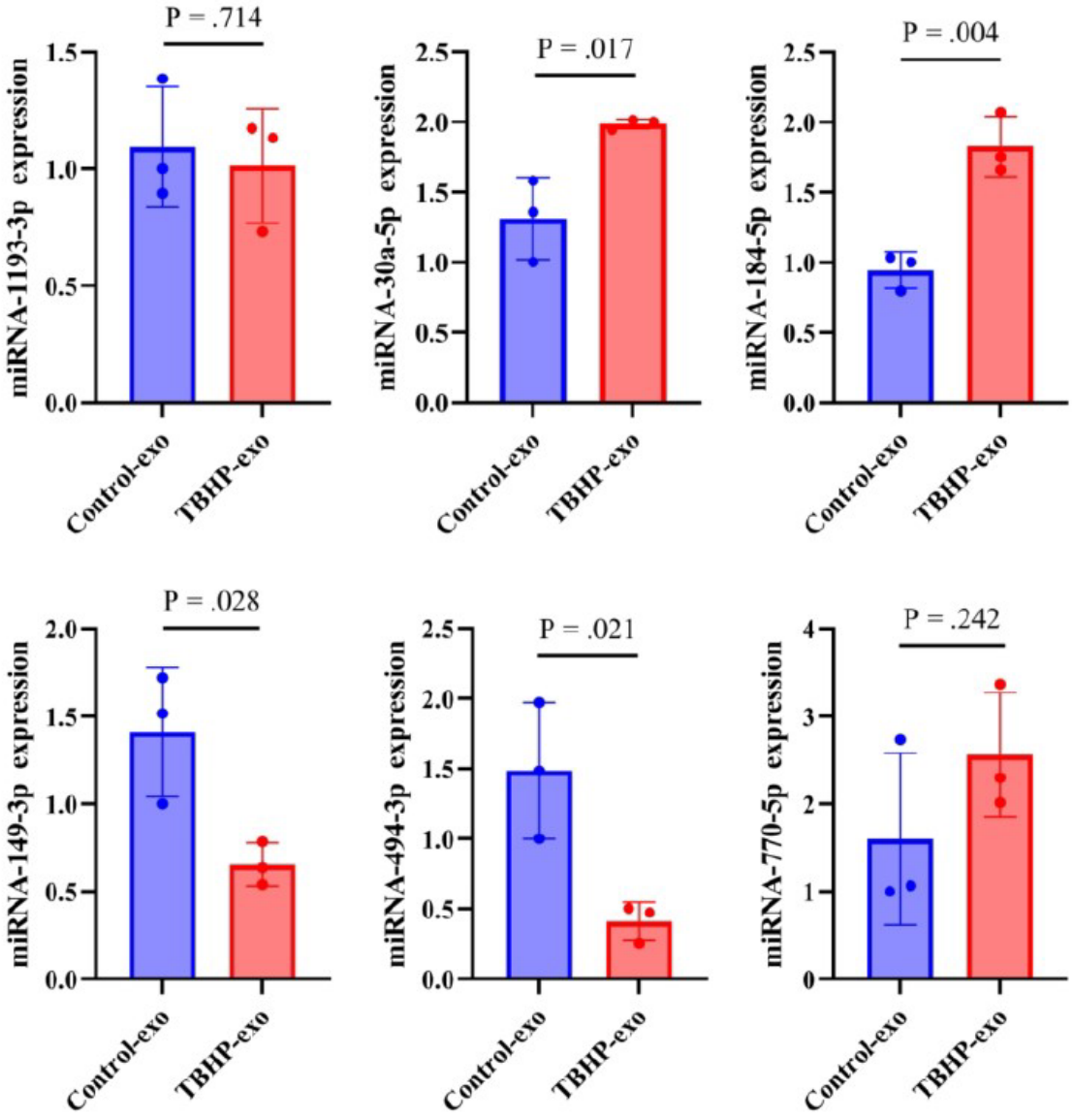

Fig f. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) of miR-184-5p, miR-30a-5p, miR-1193-3p, miR-149-3p, miR-494-3p, and miR-770-5p expression in normal and senescent MLO-Y4 cell-derived exosomes. Data are shown as column charts and the p-values were calculated by two-tailed independent-samples *t*-tests. The p-values were specified only when $p < 0.05$ (statistically significant). miRNA, microRNA; TBHP, tert-Butyl hydroperoxide.

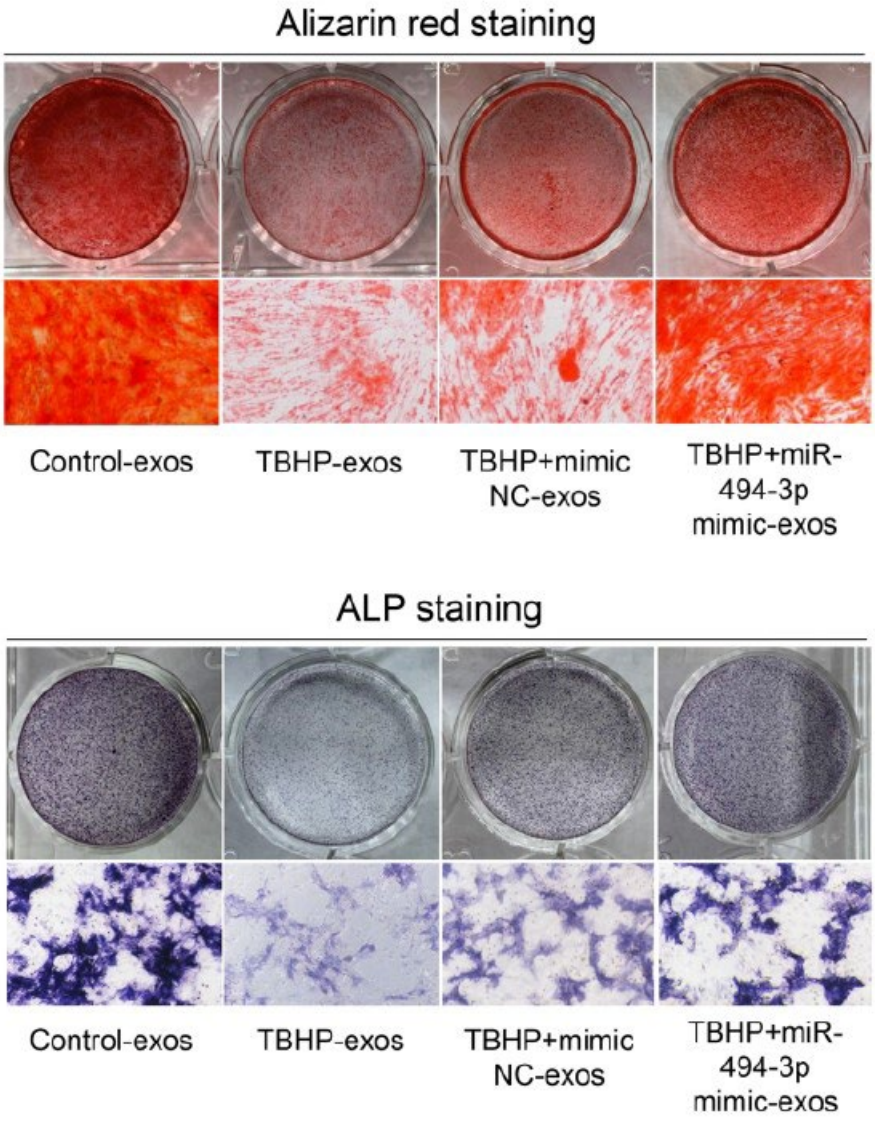

Fig g. Alkaline phosphatase (ALP) and Alizarin Red S staining of MC3T3-E1 cells treated with different exosomes for 21 days. miR, microRNA; NC, negative control; TBHP, tert-Butyl hydroperoxide.

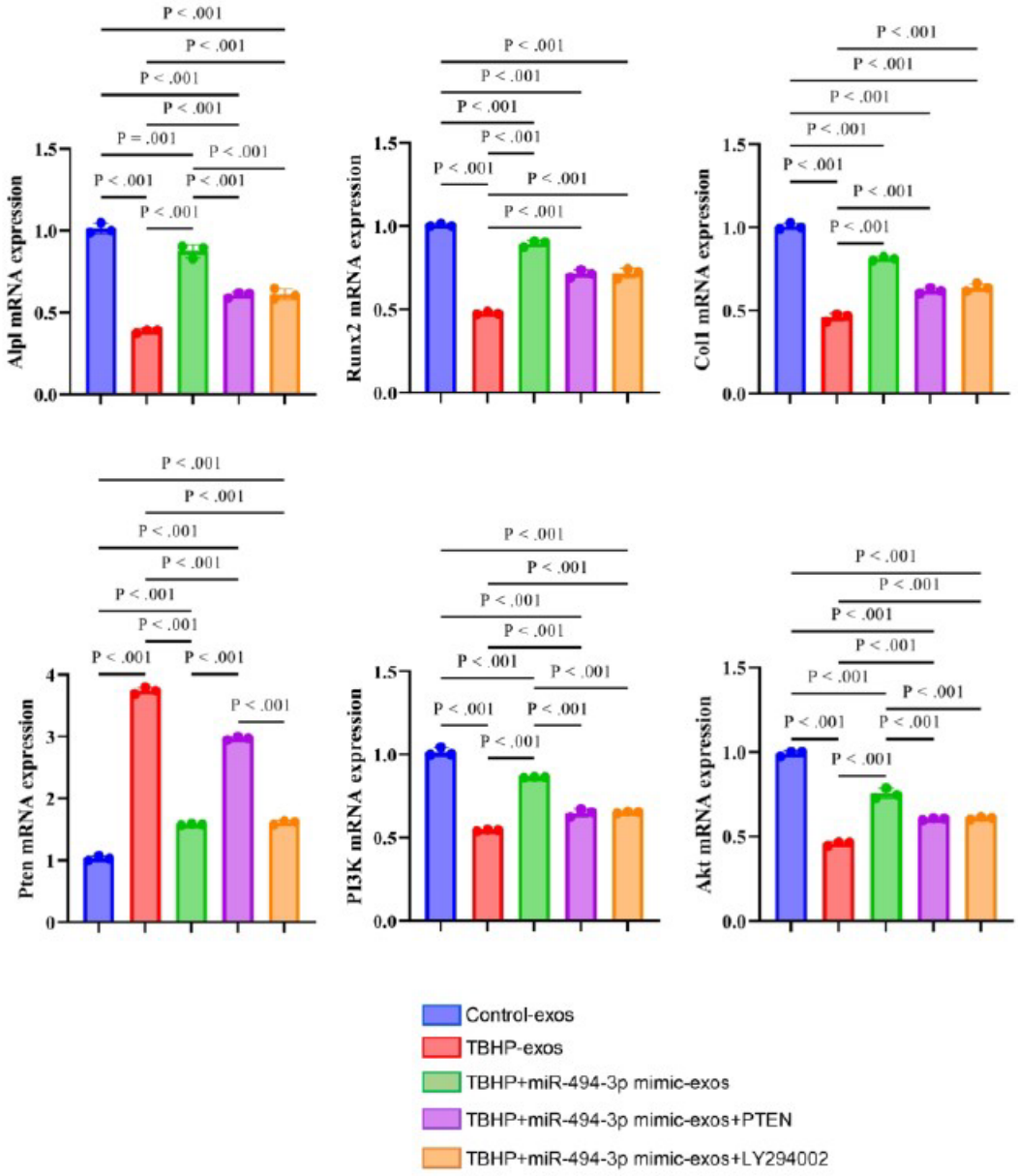

Fig h. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of alkaline phosphatase (*Alpl*), Runt-related transcription factor 2 (*Runx2*), collagen type I α1 (*Col-I*), phosphatase and tensin homolog (*Pten*), phosphoinositide 3-kinase (*PI3K*), and *Akt* expression in MC3T3-E1 cells in different treatment groups. Data are shown as column charts and the p-values were calculated by one-way analysis of variance with Sidak's multiple comparison tests. The p-values were specified only when $p < 0.05$ (statistically significant). TBHP, tert-Butyl hydroperoxide.

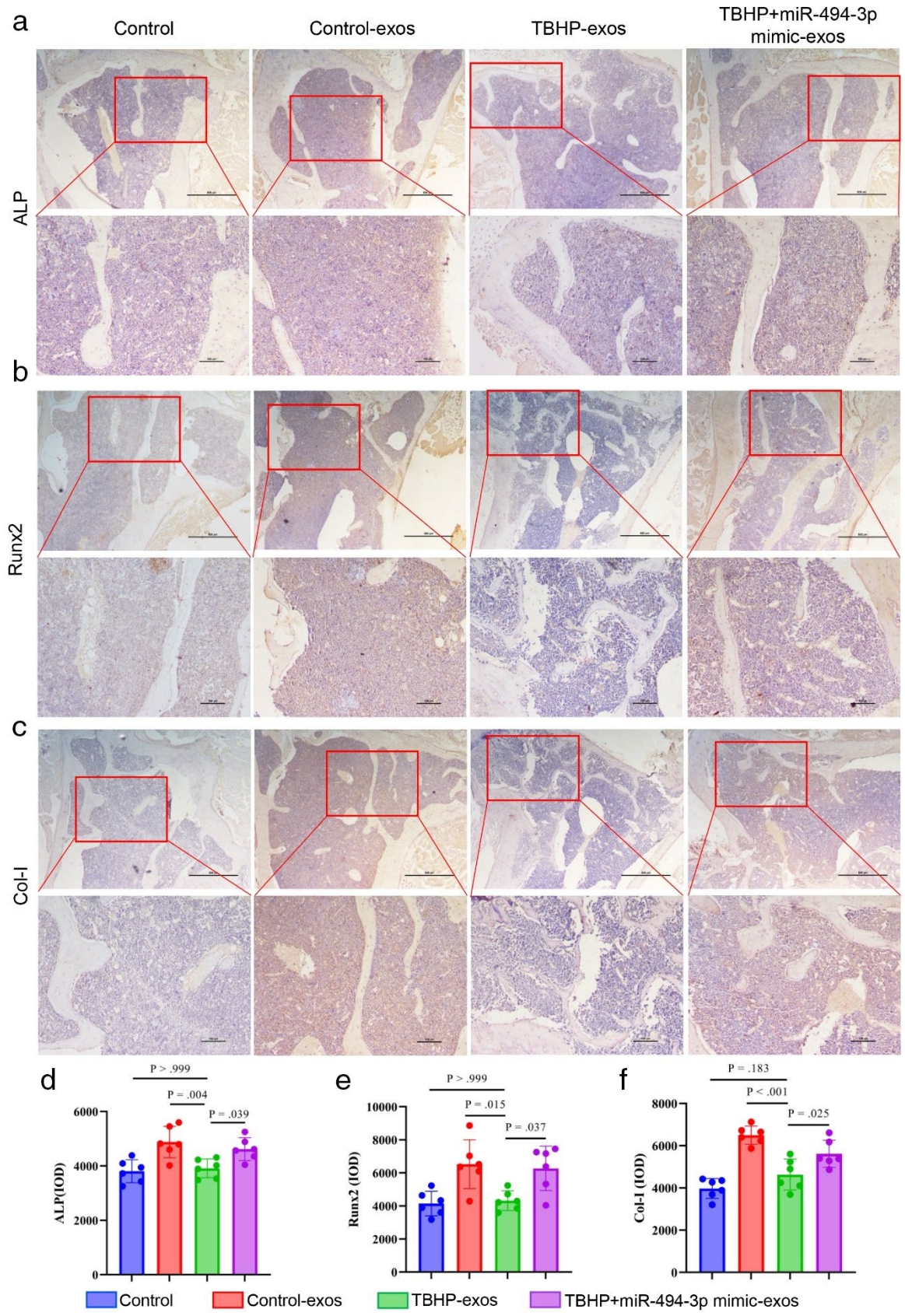

Fig i. Immunohistochemical staining and quantitative analysis for: a) and d) alkaline phosphatase (ALP); b) and e) Runt-related transcription factor 2 (Runx2); and c) and f) collagen type I α1 (Col-I) of distal femoral metaphysis from SAMP6 mice treated with different exosomes. Scale bar = 500 μm in

4× objective lens. Scale bar = 100 μm in 10× objective lens. Data are shown as column charts and the p-values were calculated by one-way analysis of variance with Sidak's multiple comparison tests. The p-values were specified only when $p < 0.05$ (statistically significant). IOD, integrated optical density; miR, microRNA; TBHP, tert-Butyl hydroperoxide.

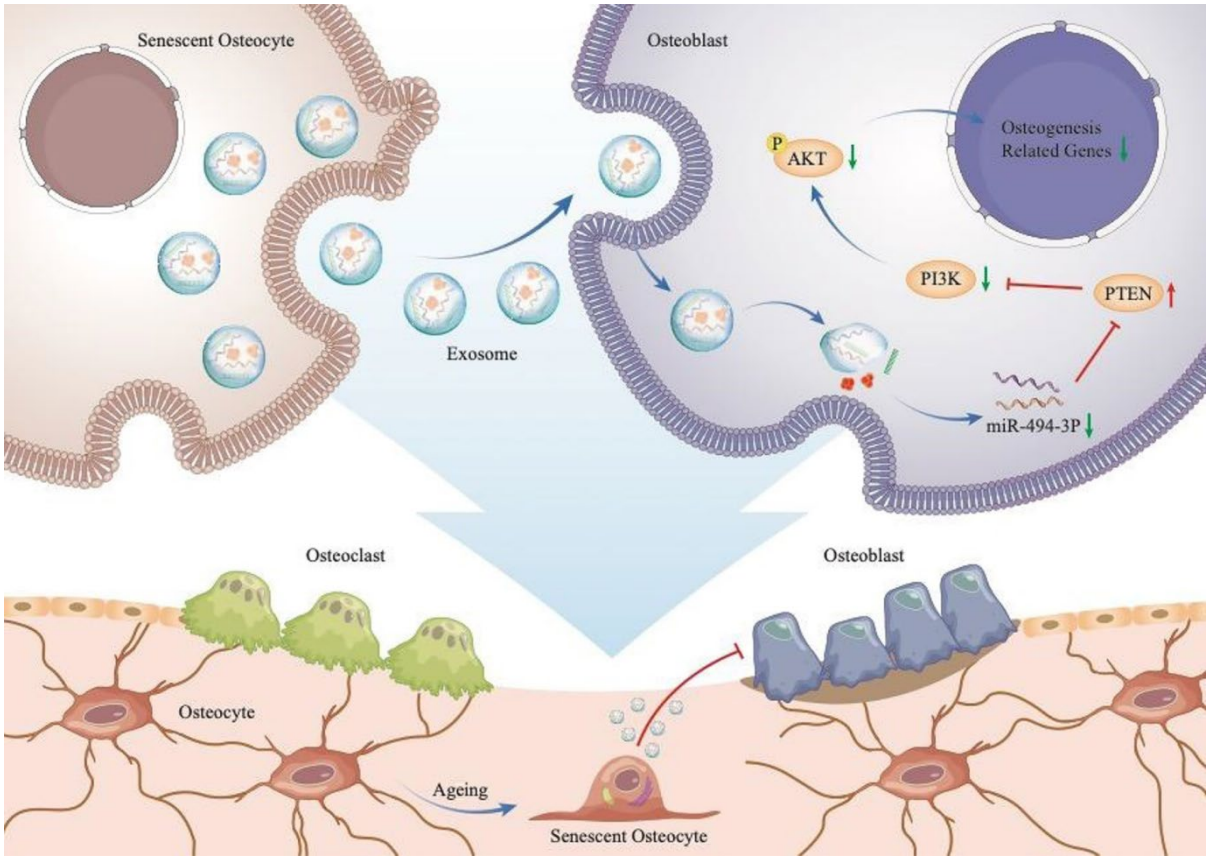

Fig j. Reduced expression of miR-494-3p in exosomes derived from senescent osteocytes inhibits osteogenesis of osteoblasts by targeting the phosphatase and tensin homolog (PTEN)/phosphoinositide 3-kinase (PI3K)/AKT pathway. miR, microRNA.

NOTE: Please save this file locally before filling in the table, DO NOT work on the file within your internet browser as changes will not be saved. Adobe Acrobat Reader (available free here) is recommended for completion.

The ARRIVE guidelines 2.0: author checklist

The ARRIVE Essential 10

These items are the basic minimum to include in a manuscript. Without this information, readers and reviewers cannot assess the reliability of the findings.

Item		Recommendation	Section/line number, or reason for not reporting
Study design	1	For each experiment, provide brief details of study design including: a. The groups being compared, including control groups. If no control group has been used, the rationale should be stated. b. The experimental unit (e.g. a single animal, litter, or cage of animals).	Methods-Mouse model a single animal
Sample size	2	a. Specify the exact number of experimental units allocated to each group, and the total number in each experiment. Also indicate the total number of animals used. b. Explain how the sample size was decided. Provide details of any *a priori* sample size calculation, if done.	Methods-Mouse model Methods-Mouse model
Inclusion and exclusion criteria	3	a. Describe any criteria used for including and excluding animals (or experimental units) during the experiment, and data points during the analysis. Specify if these criteria were established *a priori.* If no criteria were set, state this explicitly. b. For each experimental group, report any animals, experimental units or data points not included in the analysis and explain why. If there were no exclusions, state so. c. For each analysis, report the exact value of *n* in each experimental group.	N/A N/A Methods-Mouse model
Randomisation	4	a. State whether randomisation was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomisation sequence. b. Describe the strategy used to minimise potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly.	Methods-Mouse model The treatments and measurements of different groups of animals are carried out simultaneously.
Blinding	5	Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).	During the whole procedures of experiments and analysis, the specific group allocation of mice, cells and samples were conducted blind by researchers to reduce the bias of results.
Outcome measures	6	a. Clearly define all outcome measures assessed (e.g. cell death, molecular markers, or behavioural changes). b. For hypothesis-testing studies, specify the primary outcome measure, i.e. the outcome measure that was used to determine the sample size.	Methods-Micro-CT scanning and analysis Methods-Mouse model
Statistical methods	7	a. Provide details of the statistical methods used for each analysis, including software used. b. Describe any methods used to assess whether the data met the assumptions of the statistical approach, and what was done if the assumptions were not met.	Methods-Statistical analysis Methods-Statistical analysis
Experimental animals	8	a. Provide species-appropriate details of the animals used, including species, strain and substrain, sex, age or developmental stage, and, if relevant, weight. b. Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures.	Methods-Mouse model Methods-Mouse model
Experimental procedures	9	For each experimental group, including controls, describe the procedures in enough detail to allow others to replicate them, including: a. What was done, how it was done and what was used. b. When and how often. c. Where (including detail of any acclimatisation periods). d. Why (provide rationale for procedures).	Methods-Mouse model Methods-Mouse model Methods-Mouse model Results-Exosomal miR-494-3p regulates age-related bone loss in SAMP6 mice
Results	10	For each experiment conducted, including independent replications, report: a. Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g. mean and SD, or median and range). b. If applicable, the effect size with a confidence interval.	N/A Methods-Statistical analysis

The Recommended Set

These items complement the Essential 10 and add important context to the study. Reporting the items in both sets represents best practice.

Item		Recommendation	Section/line number, or reason for not reporting
Abstract	11	Provide an accurate summary of the research objectives, animal species, strain and sex, key methods, principal findings, and study conclusions.	Abstract
Background	12	a. Include sufficient scientific background to understand the rationale and context for the study, and explain the experimental approach. b. Explain how the animal species and model used address the scientific objectives and, where appropriate, the relevance to human biology.	Introduction Results-Exosomal miR-494-3p regulates age-related bone loss in SAMP6 mice
Objectives	13	Clearly describe the research question, research objectives and, where appropriate, specific hypotheses being tested.	Introduction
Ethical statement	14	Provide the name of the ethical review committee or equivalent that has approved the use of animals in this study, and any relevant licence or protocol numbers (if applicable). If ethical approval was not sought or granted, provide a justification.	Ethical review statement
Housing and husbandry	15	Provide details of housing and husbandry conditions, including any environmental enrichment.	Methods-Mouse model
Animal care and monitoring	16	a. Describe any interventions or steps taken in the experimental protocols to reduce pain, suffering and distress. b. Report any expected or unexpected adverse events. c. Describe the humane endpoints established for the study, the signs that were monitored and the frequency of monitoring. If the study did not have humane endpoints, state this.	The administration process was gentle and the rats could adapt to the experimental environment and operation. No obvious injury and other abnormalities were found in the animals. The rats were killed by cervical dislocation. Animal carcasses are sealed with white transparent self sealing bags and then sent to the Laboratory Animal Center for centralized harmless treatment.
Interpretation/ scientific implications	17	a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature. b. Comment on the study limitations including potential sources of bias, limitations of the animal model, and imprecision associated with the results.	Results-Exosomal miR-494-3p regulates age-related bone loss in SAMP6 mice Discussion
Generalisability/ translation	18	Comment on whether, and how, the findings of this study are likely to generalise to other species or experimental conditions, including any relevance to human biology (where appropriate).	Discussion
Protocol registration	19	Provide a statement indicating whether a protocol (including the research question, key design features, and analysis plan) was prepared before the study, and if and where this protocol was registered.	N/A
Data access	20	Provide a statement describing if and where study data are available.	Data sharing
Declaration of interests	21	a. Declare any potential conflicts of interest, including financial and non-financial. If none exist, this should be stated. b. List all funding sources (including grant identifier) and the role of the funder(s) in the design, analysis and reporting of the study.	ICMJE COI statement Funding information