Bone & Joint Research

Supplementary Material

10.1302/2046-3758.116.BJR-2021-0596.R1

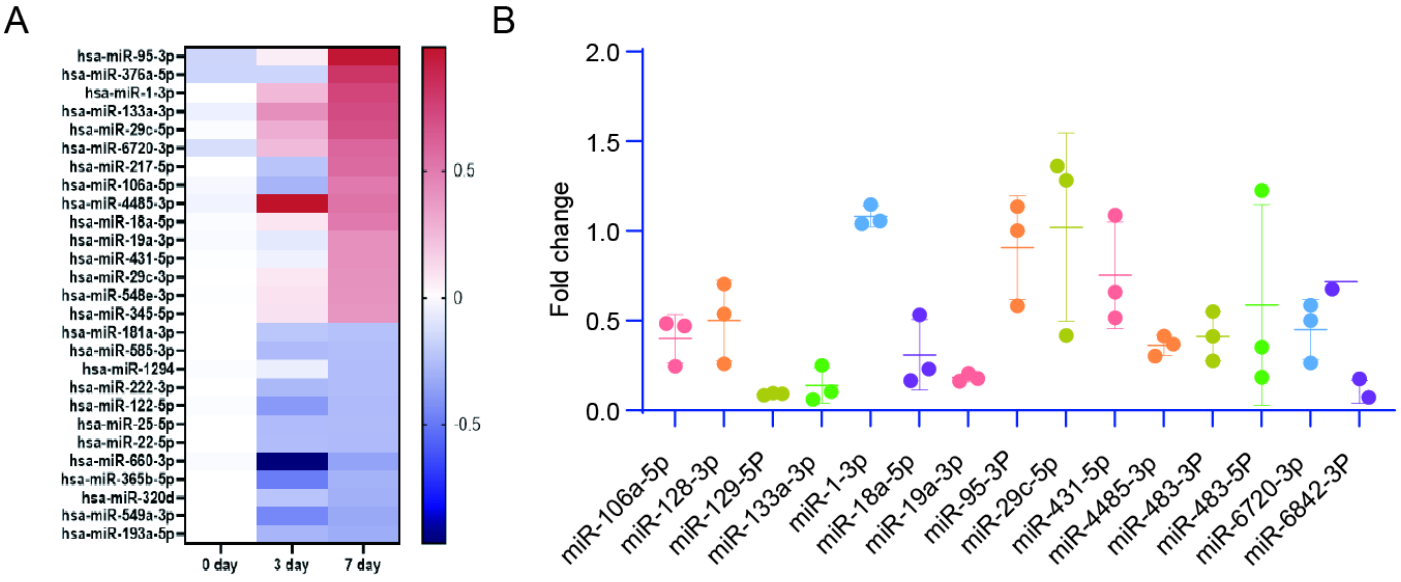

Fig a. a) Representative differential microRNAs (miRNAs) were revealed by miR sequencing (downregulation, fold change < 0.5; upregulation, fold change > 2; $p < 0.05$, one-way analysis of variance; n = 3). Bone mesenchymal stem cells (BMSCs) were incubated in osteogenic differentiation medium for either three and seven days, or not at all (control group). b) Expression of representative differential miRNAs in ethanol-treated BMSCs ($p < 0.05$, one-way analysis of variance; n = 3).

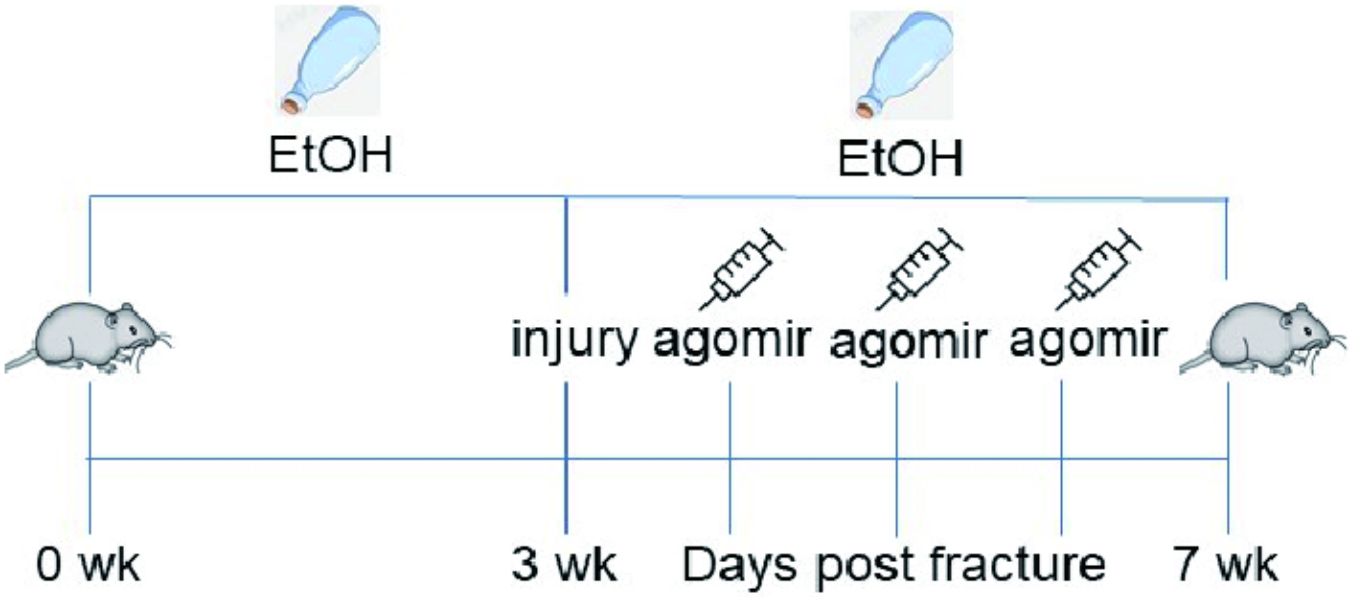

Fig b. Schematic of the experiment design. EtOH, ethanol.

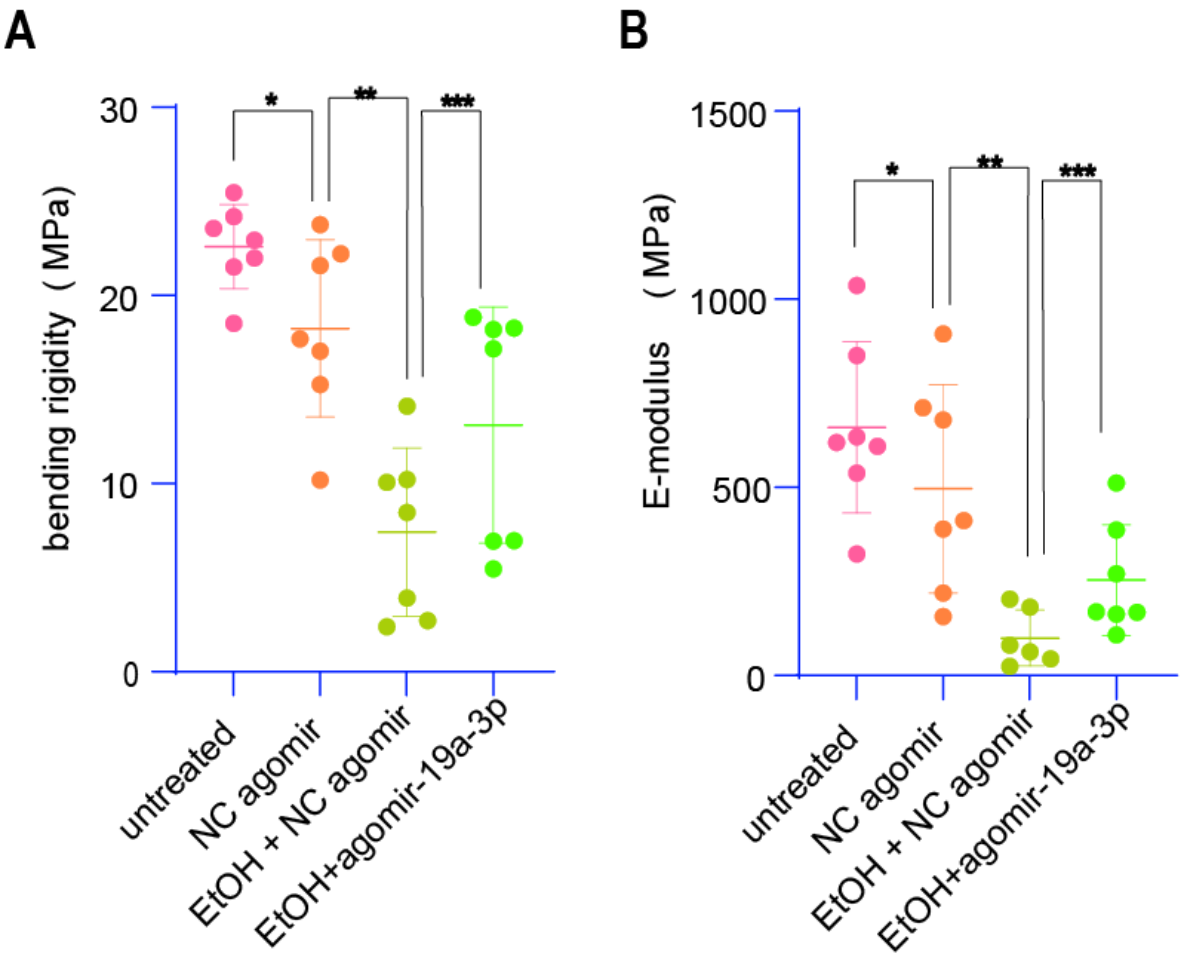

Fig c. Biomechanical testing of femurs. a) Bending rigidity (n = 7 for each group, *p = 0.0547, **p < 0.001, ***p = 0.108, one-way analysis of variance). b) E-modulus (n = 7 for each group, *p = 0.039, **p = 0.006, ***p = 0.040, one-way analysis of variance).

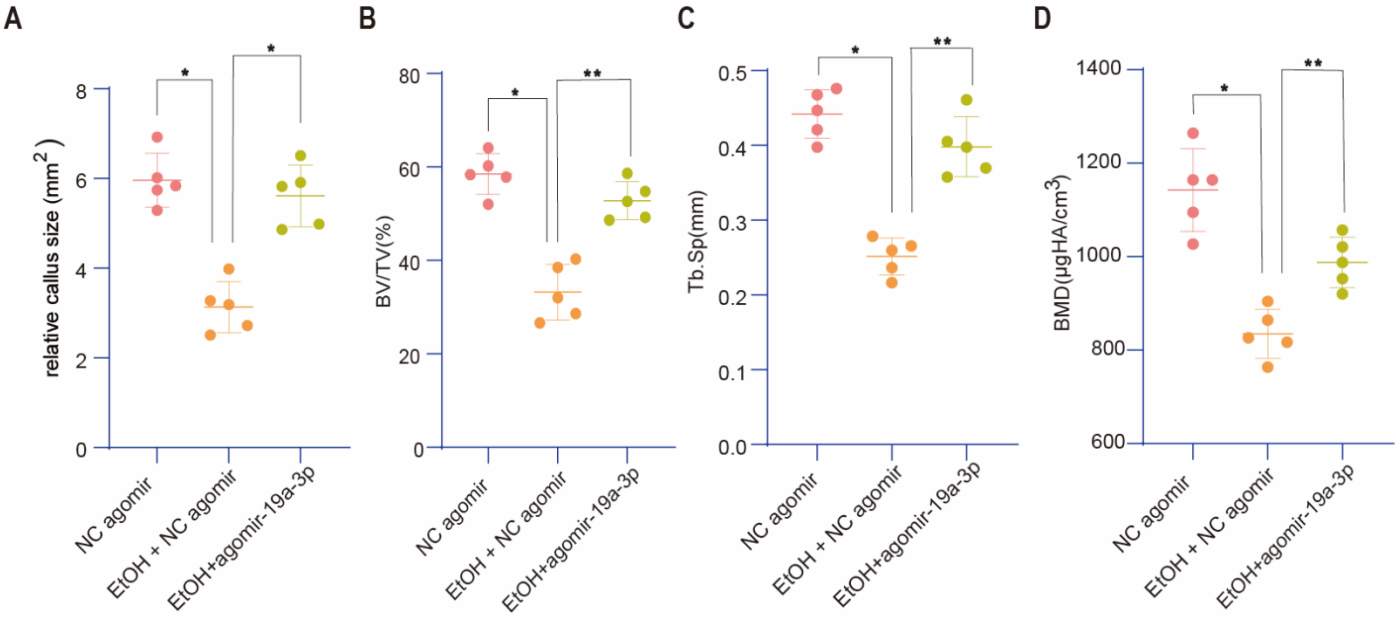

Fig d. Micro-CT analysis of femurs. a)The relative callus size (*p < 0.001), b) trabecular bone volume fraction (BV/TV)(*p < 0.001, **p = 0.003), c) trabecular spacing (Tb.Sp) (*p < 0.001, **p = 0.003) and d) bone mineral density (BMD) (*p < 0.001, **p = 0.002) were measured from the reconstructed images (n = 3 for each group, one-way analysis of variance).

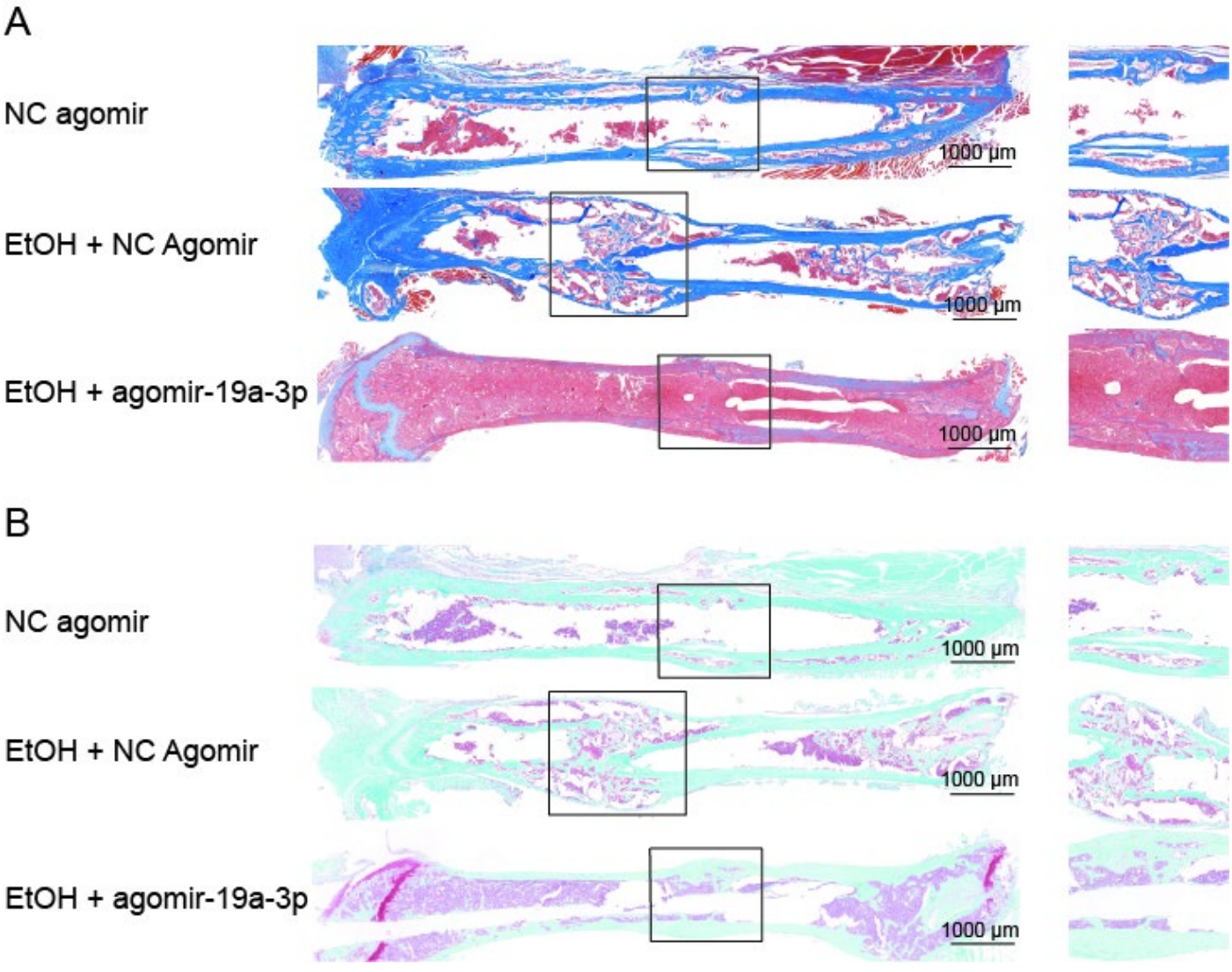

Fig e. Inhibition of microRNA (miR)-19a-3p can impair bone regeneration. a) Masson staining of fractures. b) Safranin-fixed green staining of fracture sites (left column magnification 20×; right column magnification 60×). EtOH, ethanol; NC, negative control.

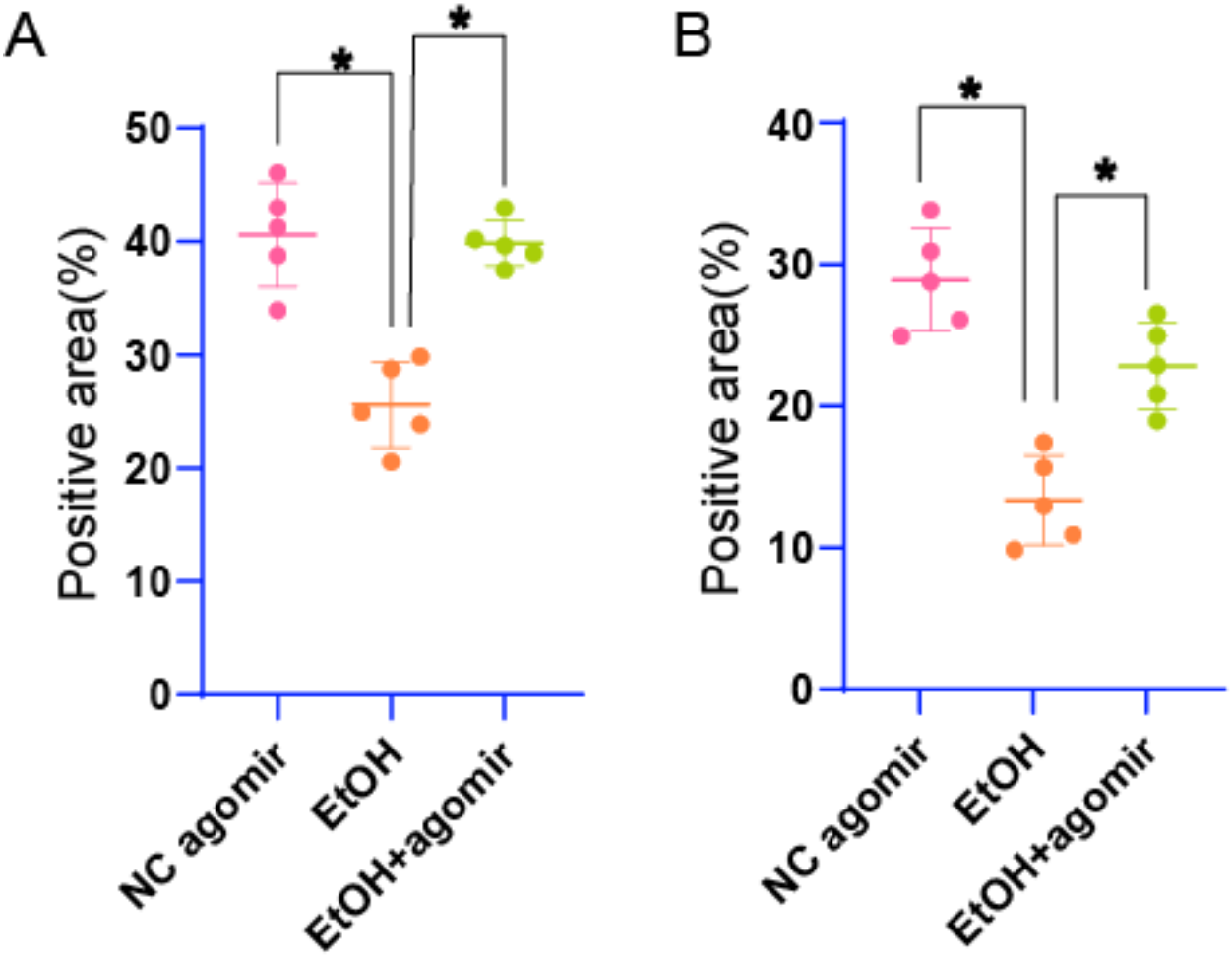

Fig f. Quantification of immunohistochemical staining with a) Collagen-1 (COL-1) and b) matrix metalloproteinase 13 (MMP13) (n = 5 for each group, *p < 0.001, one-way analysis of variance). EtOH, ethanol; NC, negative control.

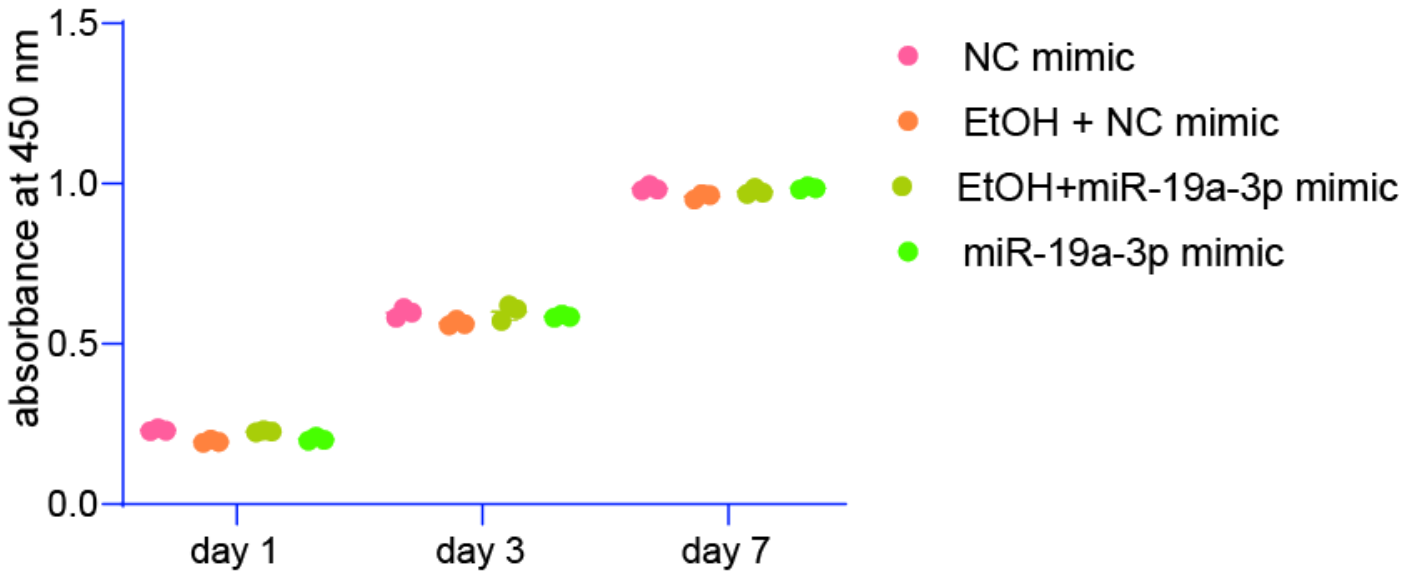

Fig g. Cell Counting Kit-8 assay of bone mesenchymal stem cells treated with the indicated conditions. EtOH, ethanol; NC, negative control.

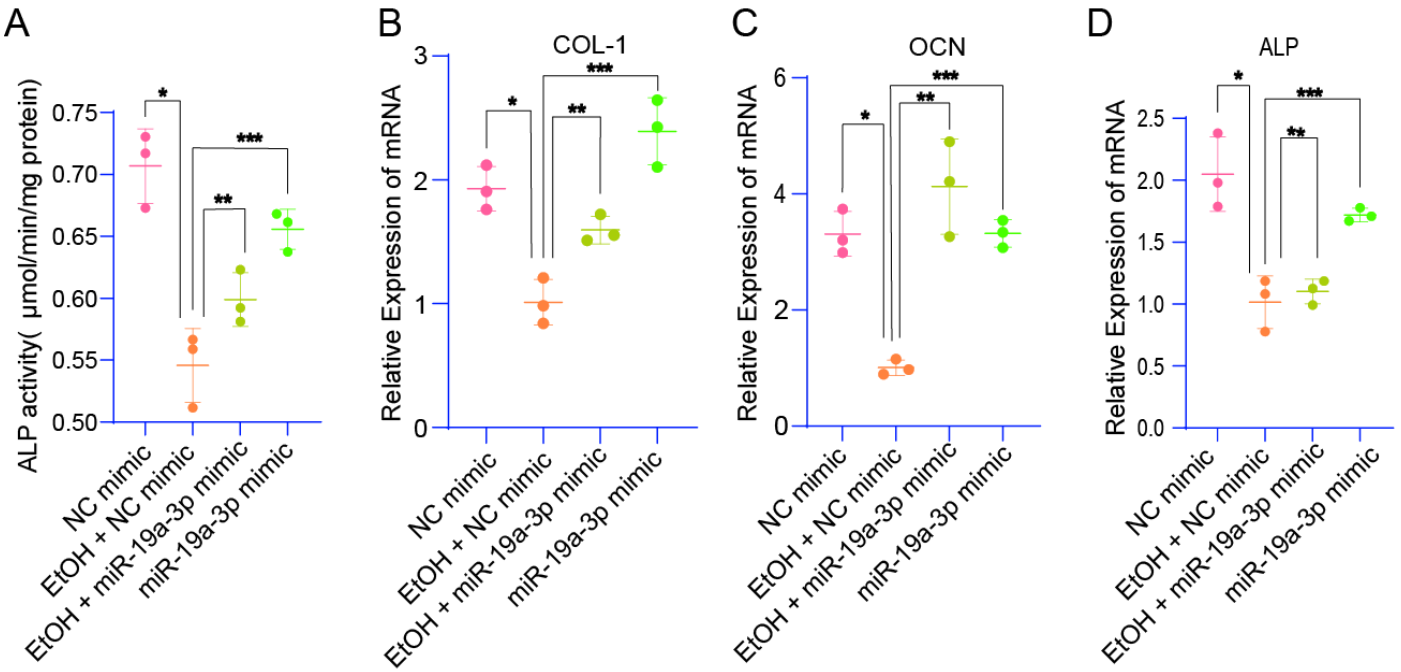

Fig h. MicroRNA (miR)-19a-3p can alleviate the impaired osteogenic differentiation caused by ethanol (EtOH). a) Quantification of alkaline phosphatase (ALP) activity (*p = 0.003, **p = 0.046, ***p = 0.005). b) to d) Messenger RNA (mRNA) expression in bone mesenchymal stem cells (BMSCs) was measured using quantitative real-time polymerase chain reaction after the indicated treatment in osteogenic differentiation (OB) medium for three days. b) COL-1 (*p = 0.003, **p = 0.009, ***p = 0.001). c) Osteocalcin (OCN) (*p < 0.001, **p = 0.003, ***p < 0.001). d) ALP (*p = 0.008, **p = 0.048, ***p = 0.005) (n = 3 for each group; *p < 0.001, one-way analysis of variance). NC, negative control.

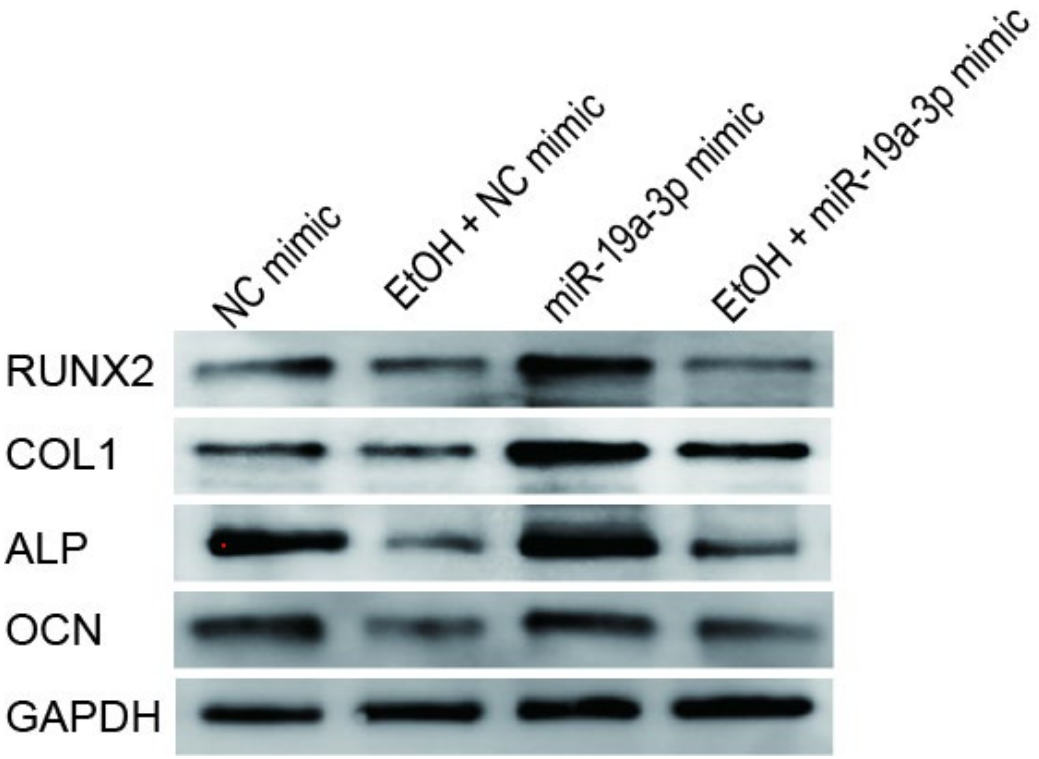

Fig i. Western blots of osteogenic-related proteins in bone mesenchymal stem cells incubated in OB medium containing the indicated treatments for three days. ALP, alkaline phosphatase; EtOH, ethanol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; miR, microRNA; NC, negative control; OCN, osteocalcin.

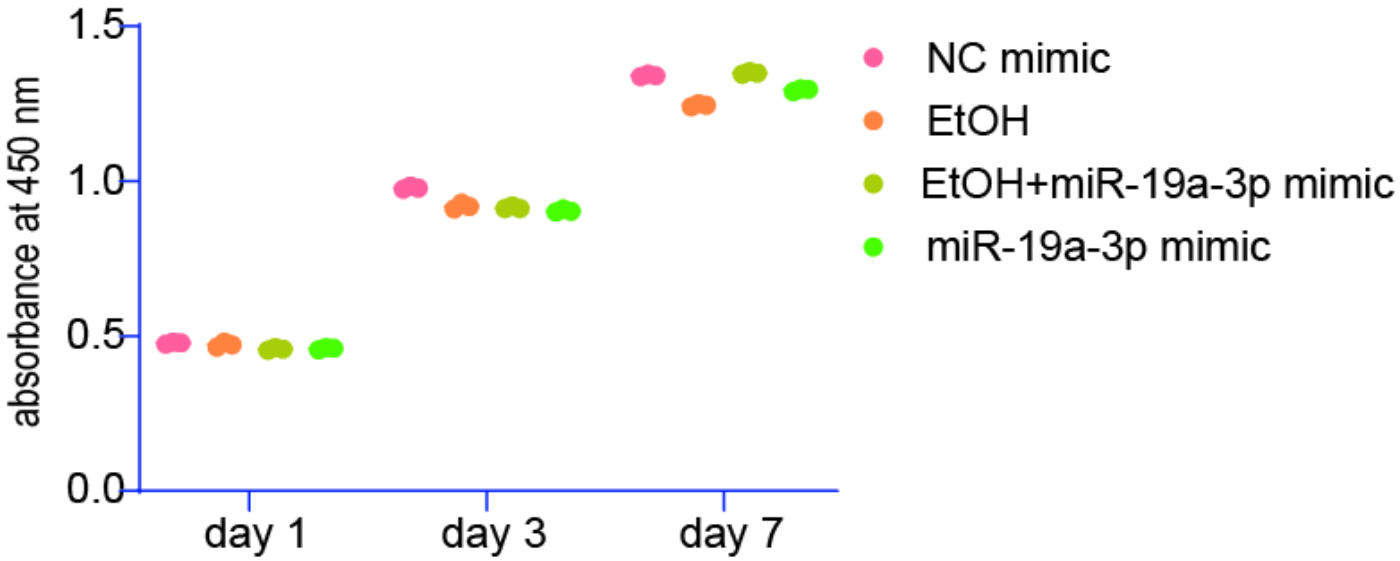

Fig j. Cell Counting Kit-8 assay of human umbilical vein endothelial cells treated under the indicated conditions. EtOH, ethanol; NC, negative control.

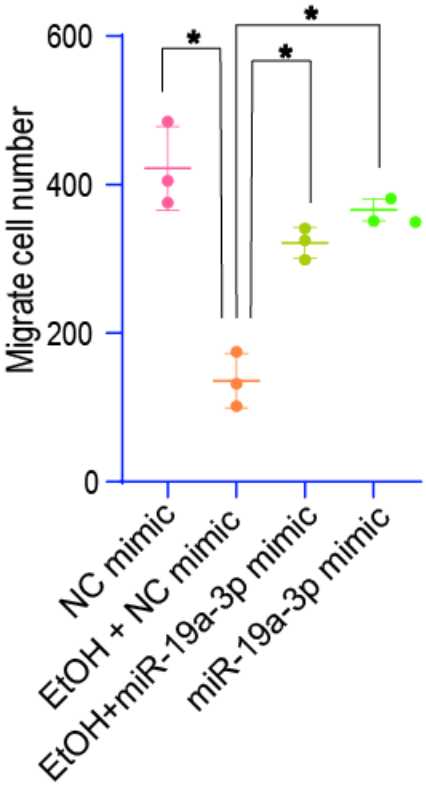

Fig k. Quantitative results of the scratch wound assay (n = 3 for each group; *p < 0.001, one-way analysis of variance). EtOH, ethanol; NC, negative control.

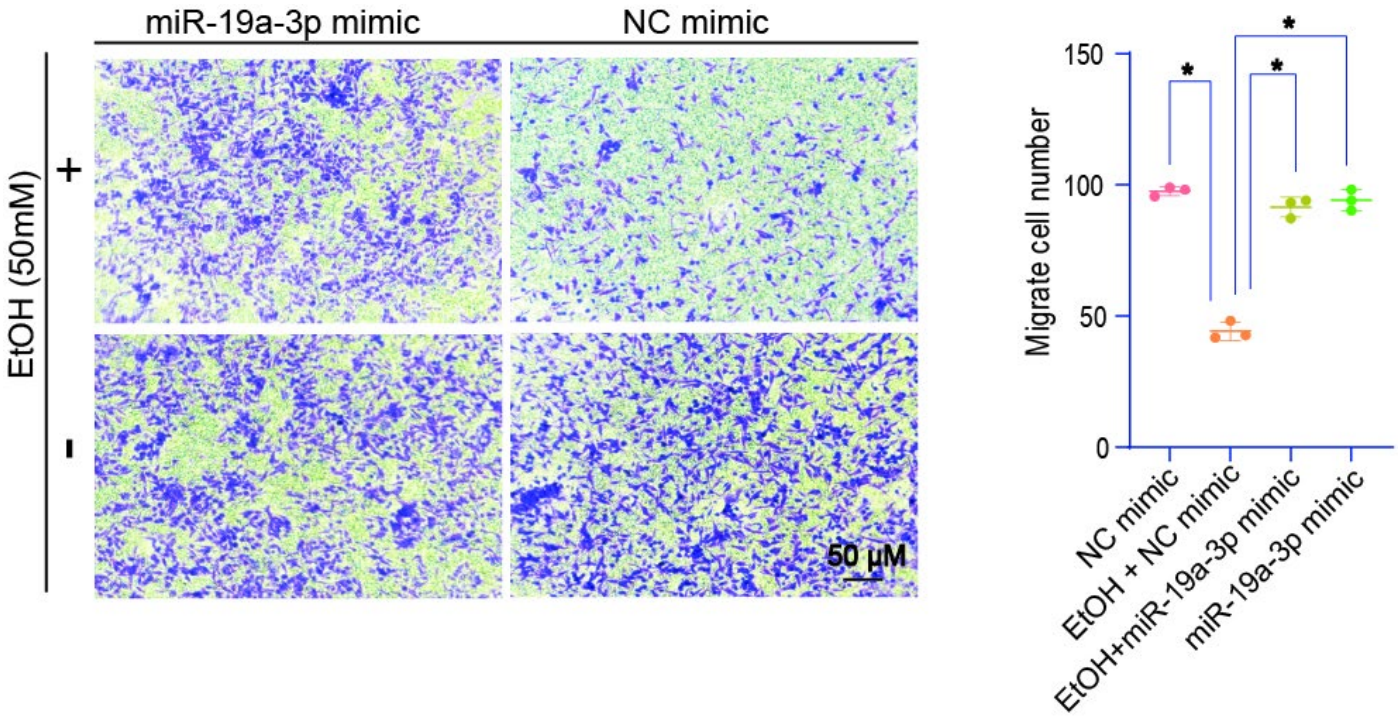

Fig l. Transwell migration of human umbilical vein epithelial cells treated under the indicated conditions (n = 3 for each group; *p < 0.001, one-way analysis of variance).

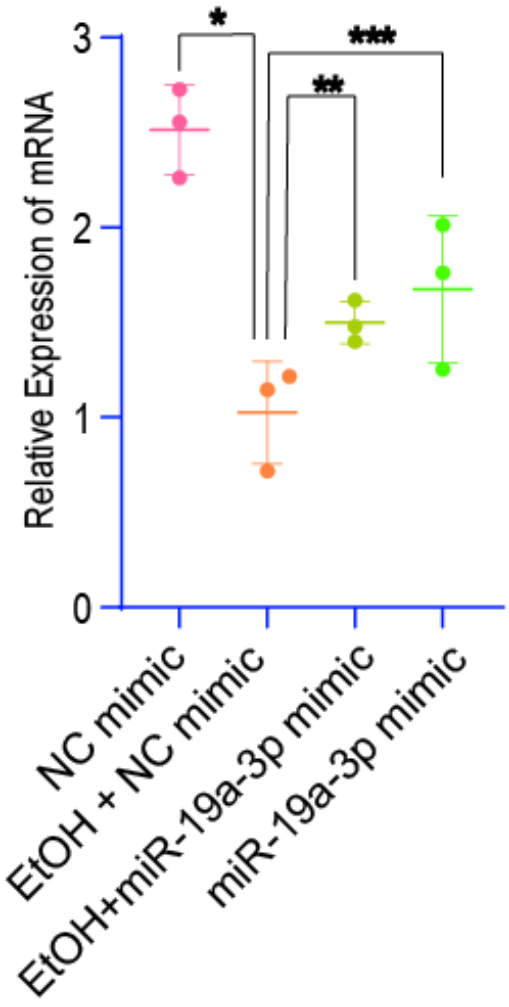

Fig m. Messenger RNA (mRNA) expression of vascular endothelial growth factor in human umbilical vein epithelial cells was measured using real-time polymerase chain reaction after the indicated

treatment for two days (n = 3 for each group; *p < 0.001, *p = 0.002, **p = 0.045, ***p = 0.001, one-way analysis of variance).

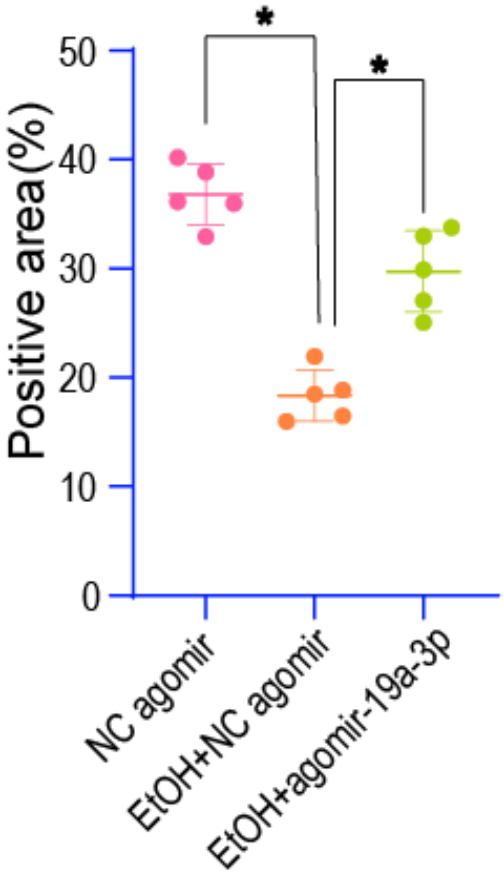

Fig n. Quantitative results of CD31 staining (n = 3 for each group; *p < 0.001, one-way analysis of variance). EtOH, ethanol; NC, negative control.

A

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context++ score	Context++ score percentile	Weighted context++ score	Conserved branch length
Position 662-668 of FOXF2 3' UTR hsa-miR-19a-3p	5' ...UAAAAGGAGGAUAUAUUUGCACU... 3' AGUCAAAACGUAUCUAAACGUGU	7mer-m8	-0.33	96	-0.33	4.142

B

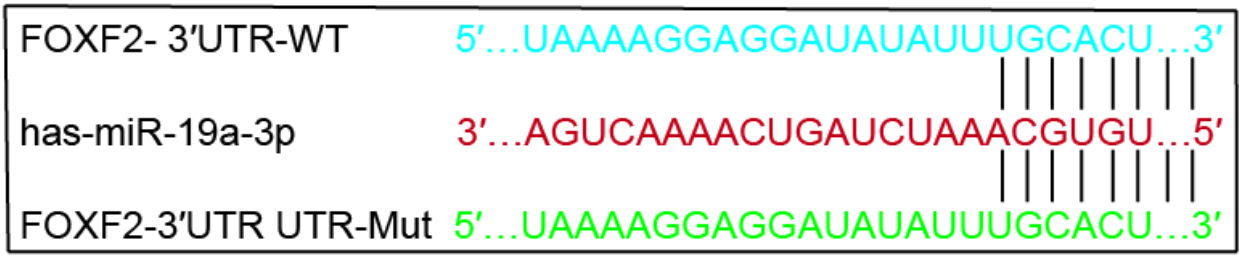

Fig o. a) Biological prediction of microRNA (miR)-19a-3p. b) Binding site between miR-19a-3p and Forkhead Box F2 (FOXF2). 3′-UTR, 3′-untranslated region.

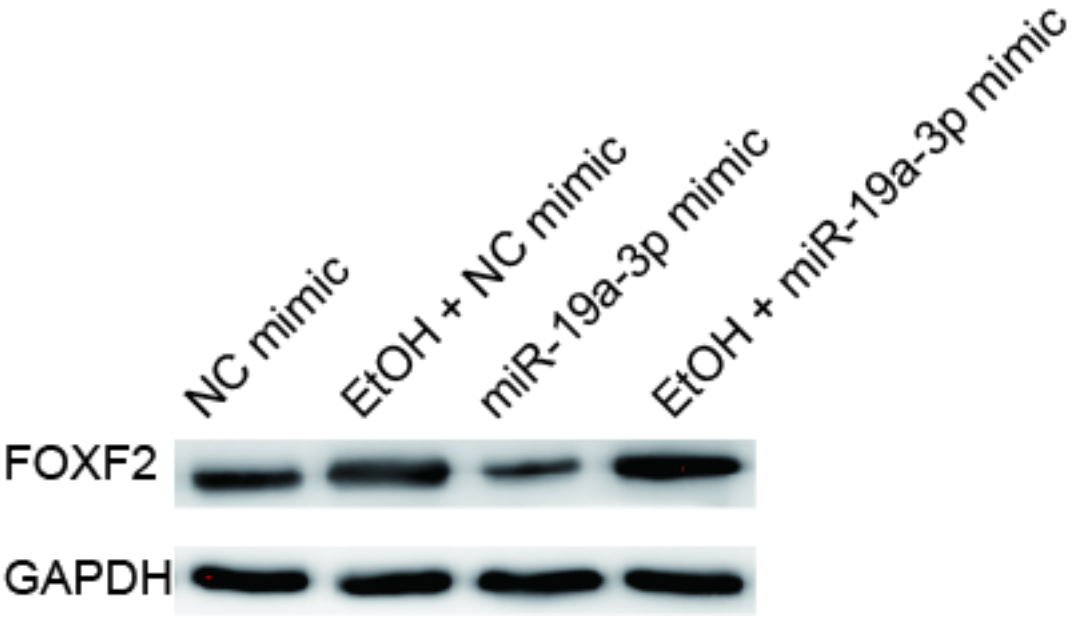

Fig p. Protein level of Forkhead Box F2 (FOXF2) in bone mesenchymal stem cells. EtOH, ethanol; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; miR, microRNA; NC, negative control.

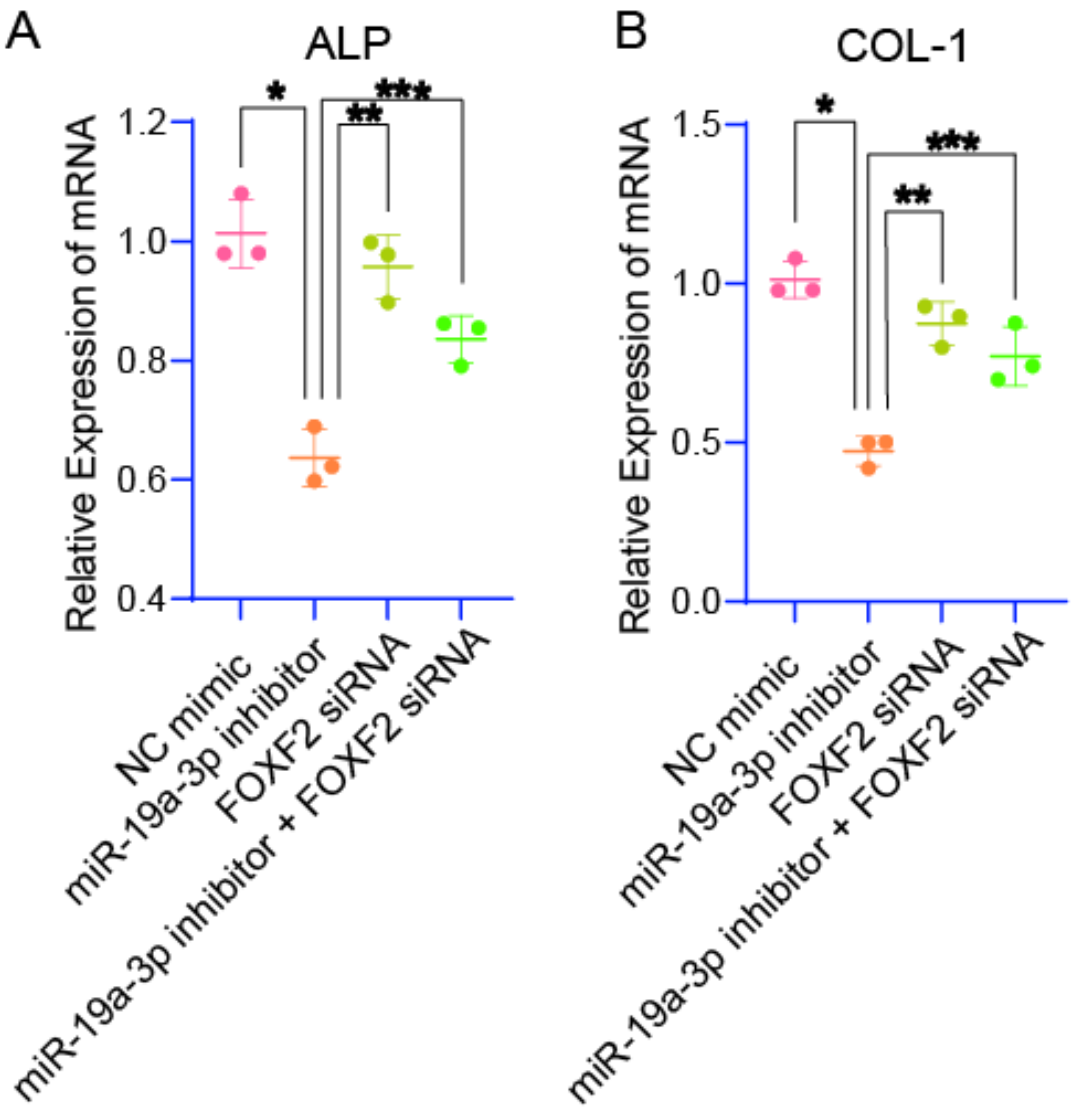

Fig q. Messenger RNA (mRNA) expression of Forkhead Box F2 (FOXF2) in bone mesenchymal stem cells was measured using real-time polymerase chain reaction after the indicated treatment. a) Alkaline phosphatase (ALP) (*p < 0.001, **p = 0.002, ***p = 0.005). b) COL-1 (*p < 0.001, **p = 0.001,

***p = 0.007) (n = 3 for each group; *p < 0.001, one-way analysis of variance). miR, microRNA; NC, negative control.

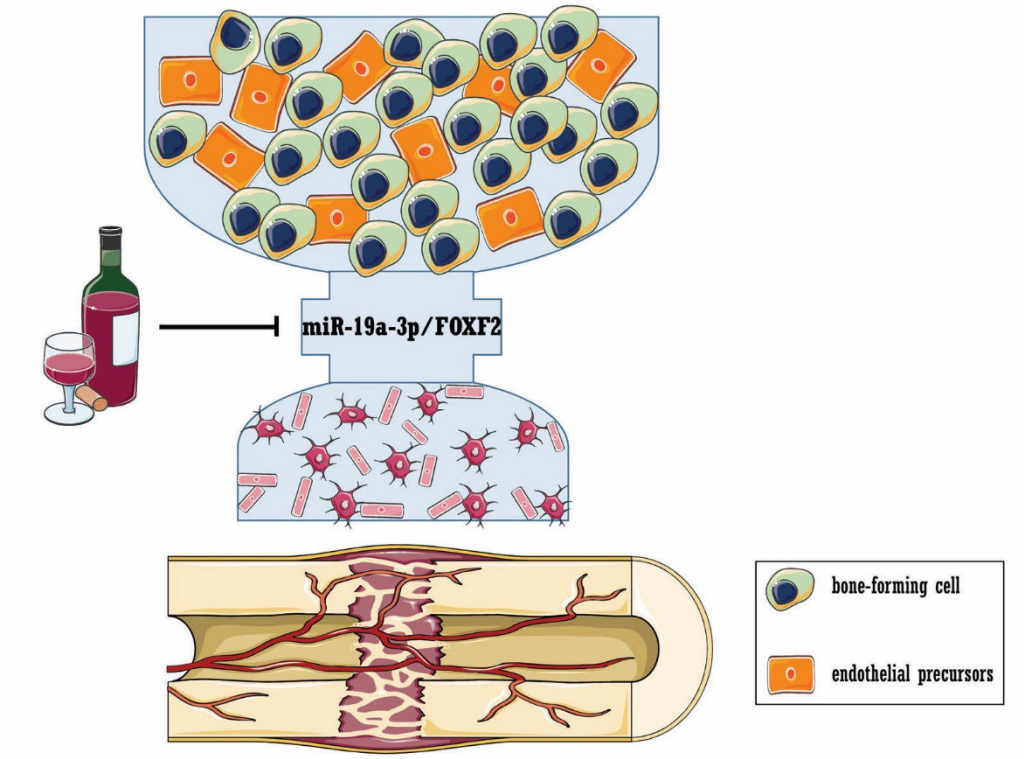

Fig r. Mechanical graph of alcohol-induced inhibition of bone formation and neovascularization, which contributes to the failure of fracture healing via the microRNA (miR)-19a-3p/Forkhead Box F2 (FOXF2) axis.

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).	Animal grouping and treatment Animal grouping and treatment
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.	Animal grouping and treatment Animal grouping and treatment
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.	Animal grouping and treatment No exclusion Methods and results
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.	Animal grouping and treatment Animal grouping and treatment
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).	Animal grouping and treatment
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.	Results Not Applicable
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.	Statistical analysis 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.	Animal grouping and treatment Animal grouping and treatment
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).	Animal grouping and treatment
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.	Results Not applicable