4-phenylbutyric acid enhances the mineralization of osteogenesis imperfecta iPSC-derived osteoblasts

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Supporting information list

Table S1. The list of primer sequences used for real-time quantitative PCR

Gene		Primer sequence
COL1A1	Forward	5'- GGGATTCCCTGGACCTAAAG -3'
	Reverse	5'- GGAACACCTCGCTCTCCA -3'
COL1A2	Forward	5'- AGAGGACCACGTGGAGAAAG -3'
	Reverse	5'- GGCCTGTGGGACCATCTT -3'
ACTB	Forward	5'- TCAAGATCATTGCTCCTCCTGAG -3'
	Reverse	5'- ACATCTGCTGGAAGGTGGACA -3'
BIP	Forward	5'- TGTTCAACCAATTATCAGCAAACTC -3'
	Reverse	5'- TTCTGCTGTATCCTCTTCACCAGT -3'
CHOP	Forward	5'- AGAACCAGGAAACGGAAACAGA -3'
	Reverse	5'- TCTCCTTCATGCGCTGCTTT -3'
OCT4	Forward	5'- TGTACTCCTCGGTCCCTTTC -3'
	Reverse	5'- TCCAGGTTTTCTTTCCCTAGC -3'
NANOG	Forward	5'- CAGTCTGGACACTGGCTGAA -3'
	Reverse	5'- CTCGCTGATTAGGCTCCAAC -3'
CD44	Forward	5'- ATGGACAAGTTTTGGTGGCAC -3'
	Reverse	5'- AATACACCTGCAAAGCGGCA -3'
NT5E	Forward	5'- CACTATCTGGTTCACCGTGTACA -3'
	Reverse	5'- CGATCAGTCCTTCCACACCAT -3'
RUNX2	Forward	5'- TAGCAAGGTTCAACGATCTGAG -3'
	Reverse	5'- AGCTTCTGTCTGTGCCTTCTG -3'
ALPL	Forward	5'- CCTCGTTGACACCTGGAAGAG -3'
	Reverse	5'- TTCCGTGCGGTTCCAGA -3'
PARP1	Forward	5'- AGACAGTGTGCAGGCCAAG -3'
	Reverse	5'- ACTTCCTGATGATCTCGGCTTC -3'
PARP2	Forward	5'- GATGGTATGCCAGGAAGGTCA -3'
	Reverse	5'- TGTTGAACTGGAGATTGGTCTGA -3'
ENPP1	Forward	5'- CACTACAGCATTGTCACCGGAT -3'
	Reverse	5'- GGCCTTGATACTTAGCTGTGACC -3'
ANKH	Forward	5'- CTCTGTCACTCACGCTCTGT -3'
	Reverse	5'- TCTTCAGTGTCATCAGCCACC -3'

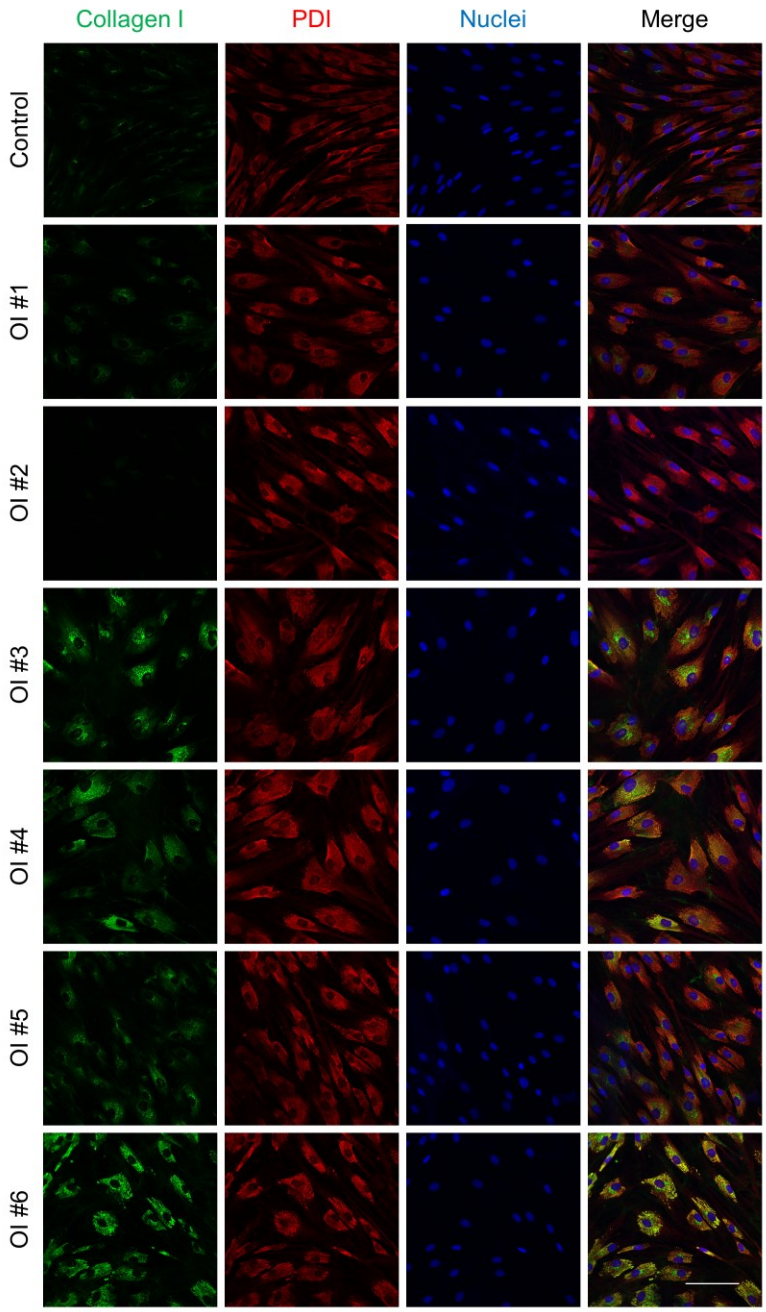

Figure S1. Immunofluorescence staining of dermal fibroblasts. Type I collagen is in green. Endoplasmic reticulum marker protein, disulfide isomerase (PDI), is in red. Nuclei are in blue. Image panels of Control, OI #2 and OI #3 are redisplays of image panels of Figure 1. Scale bar is 100 μm.

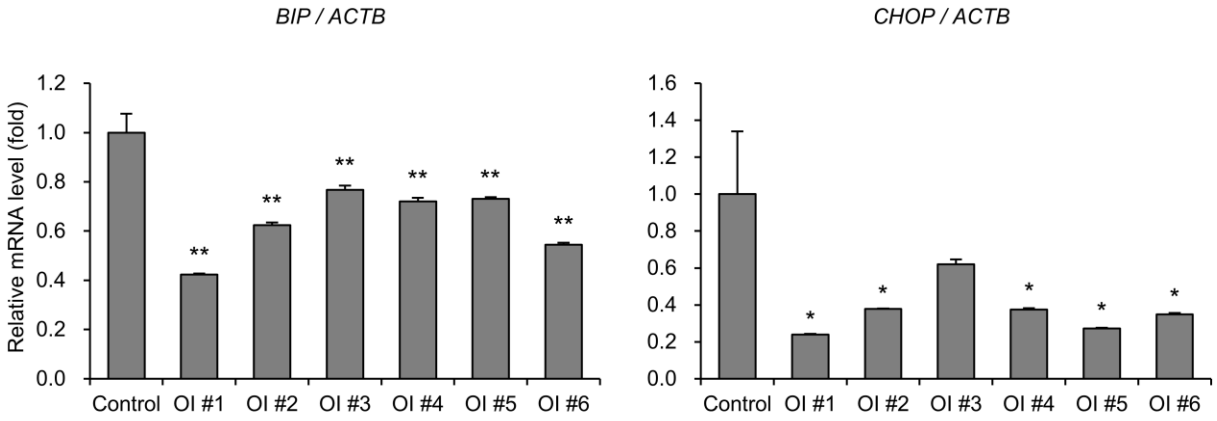

Figure S2. Real-time quantitative PCR of *BIP* and *CHOP*. Data are mean ± SEM. Differences were tested by ANOVA followed by Tukey's HSD post hoc test. **P* < 0.05 vs. control, ***P* < 0.01 vs. control.

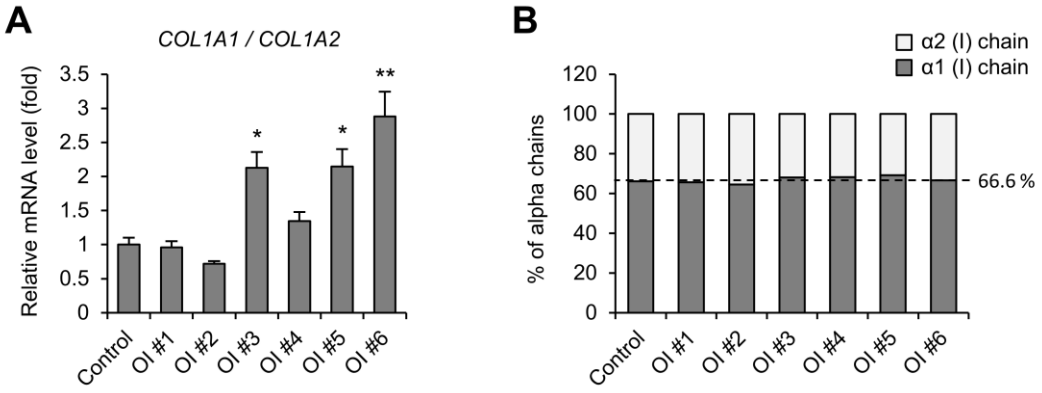

Figure S3. Comparison of α1 and 2 chains of type I collagen. **A.** The ratio of the gene expression of *COL1A1* to *COL1A2*. **B.** The percentage of α1 and 2 chains of type I collagen in conditioned medium. Data are mean ± SEM. Differences were tested by ANOVA followed by Tukey's HSD post hoc test. **P* < 0.05 vs. control, ***P* < 0.01 vs. control.

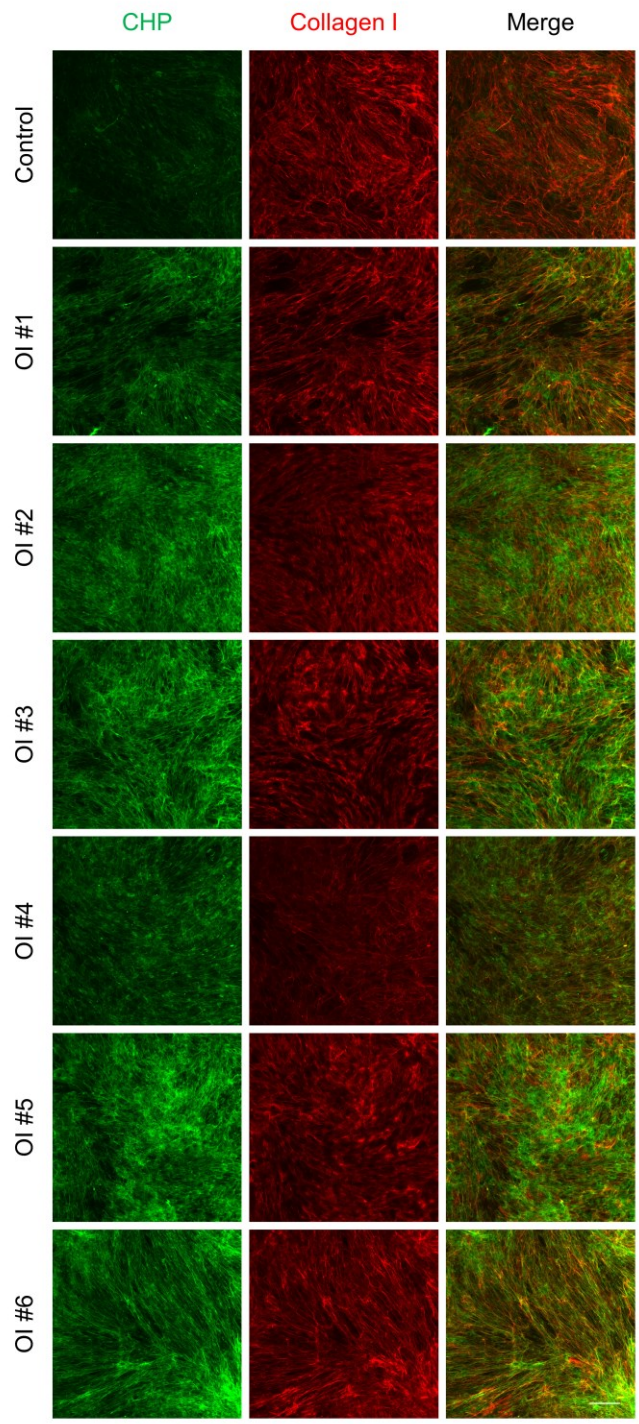

Figure S4. Fluorescence staining of deposited collagen. Unfolded triple-helical chains are stained in green by collagen hybridizing peptide (CHP). Type I collagen is stained in red. Image panels of Control and OI #3 are redisplays of image panels of Figure 4. Scale bar is 200 μm.

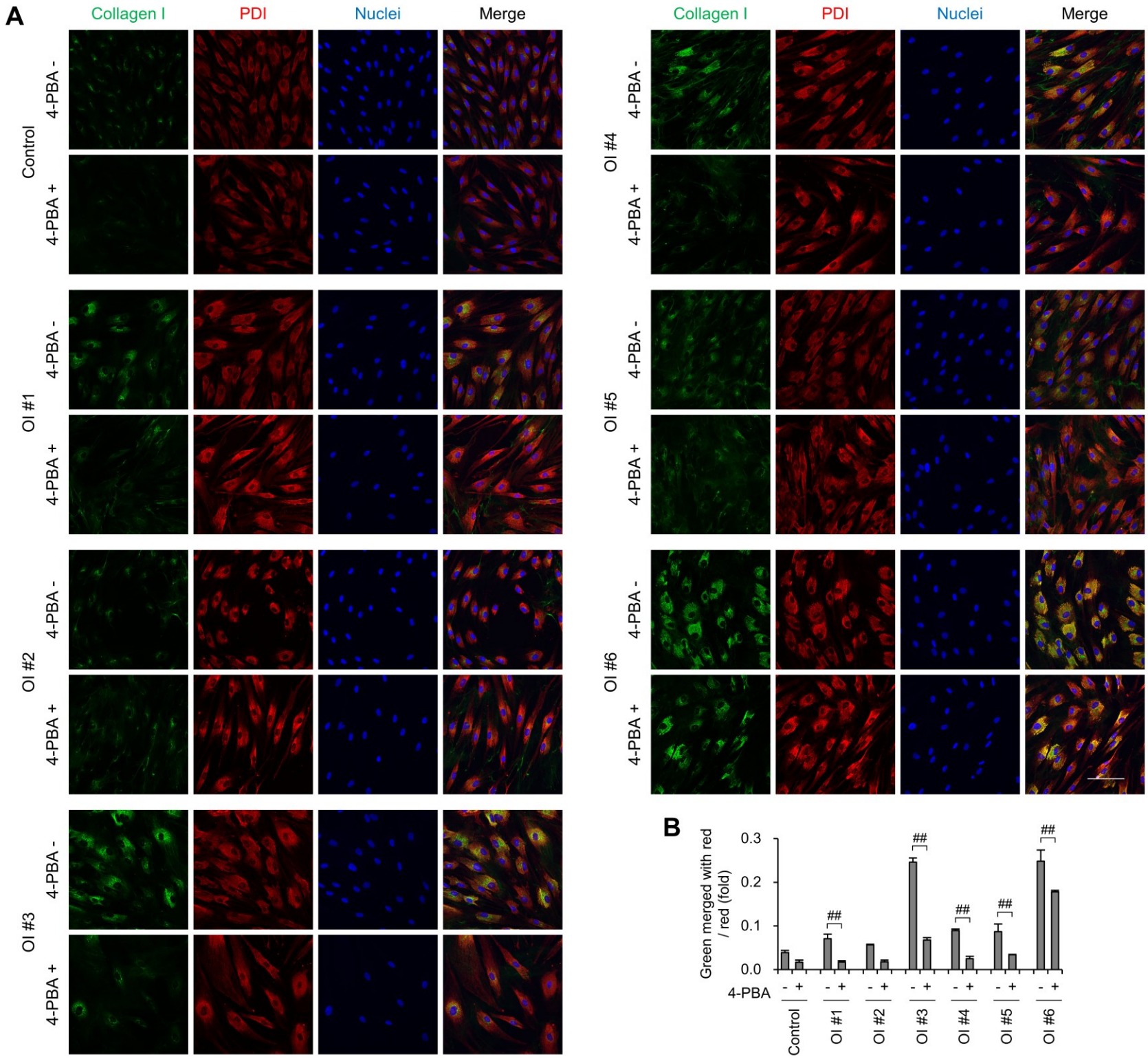

Figure S5. Immunofluorescence staining of dermal fibroblasts treated with or without 5 mM 4-phenylbutyric acid (4-PBA). **A.** Immunofluorescence staining of dermal fibroblasts. Type I collagen is in green. The endoplasmic reticulum marker protein, disulfide isomerase (PDI), is in red. Nuclei are in blue. Image panels of OI #3 are redisplays of image panels of Figure 5. Scale bar is 100 μm. **B.** Calculated values of green area merged with red area divided by red area of immunofluorescence staining. Data are mean ± SEM. Differences were tested by ANOVA followed by Tukey's HSD post hoc test. ##$P < 0.01$.

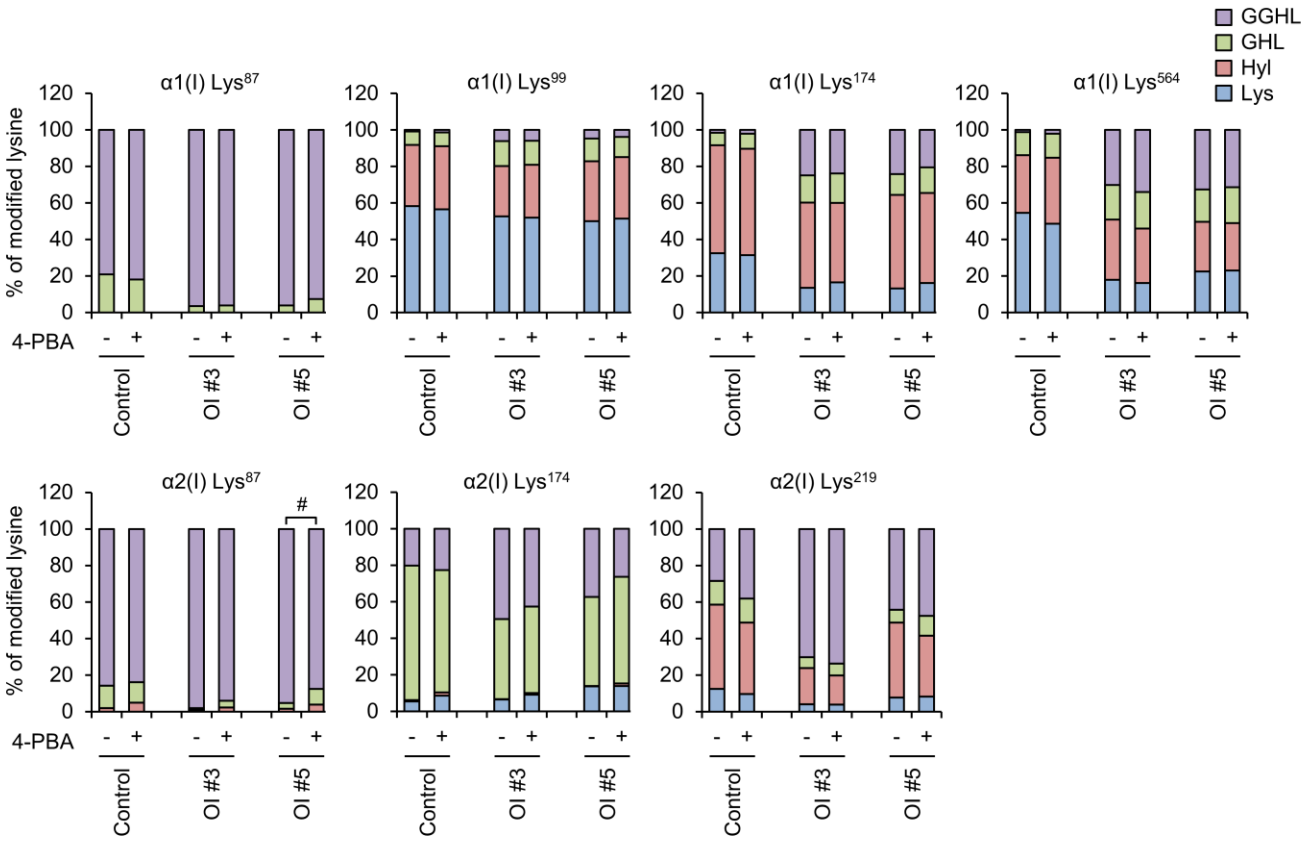

Figure S6. The effect of 4-phenylbutyric acid (4-PBA) on post-translational modification of type I collagen. Each cell line was treated with or without 5 mM 4-PBA. The percentage of post-translational modifications at Lys^{87}, Lys^{99}, Lys^{174}, and Lys^{564} of α1 and Lys^{87}, Lys^{174}, and Lys^{219} of α2 chain of type I collagen analyzed by LC-MS. Control is the average of 2 cell lines. Hyl, hydroxylysine; GHL, galactosyl-hydroxylysine; GGHL, glucosyl-galactosyl-hydroxylysine. Differences in the ratio of GGHL to non-GGHL were tested by Pearson's chi-square test. #$P < 0.05$, ##$P < 0.01$.

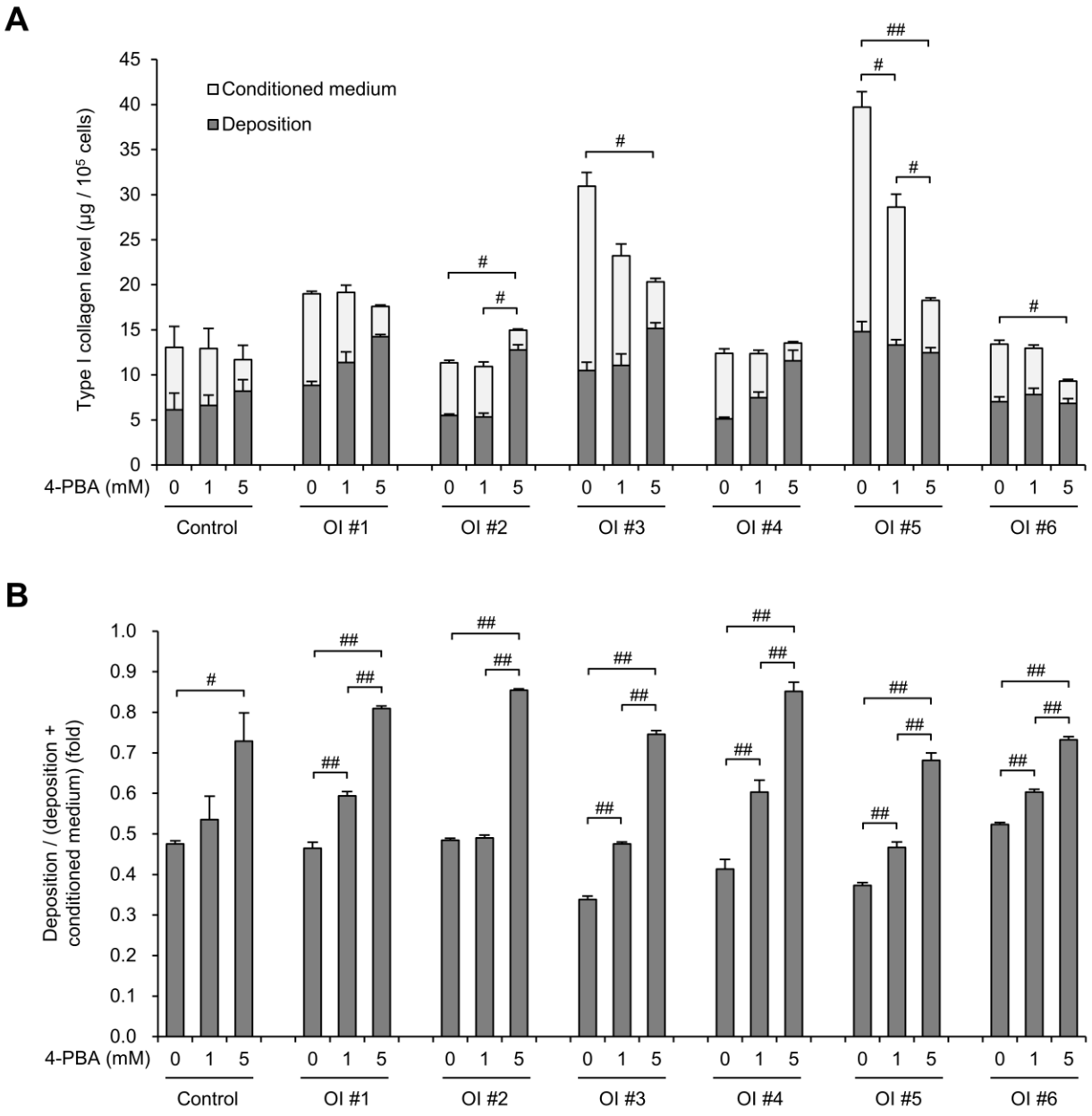

Figure S7. The effect of 4-phenylbutyric acid (4-PBA) on the production of type I collagen. **A.** Protein levels of type I collagen in conditioned medium and deposited on the dish measured by ELISA. **B.** Calculated values of protein levels of type I collagen. The formula for calculation is as follows: protein levels deposited on the dish/(protein levels deposited on the dish + protein levels in conditioned medium). All data are mean ± SEM. Differences were tested by ANOVA followed by Tukey's HSD post hoc test. #$P < 0.05$, ##$P < 0.01$.

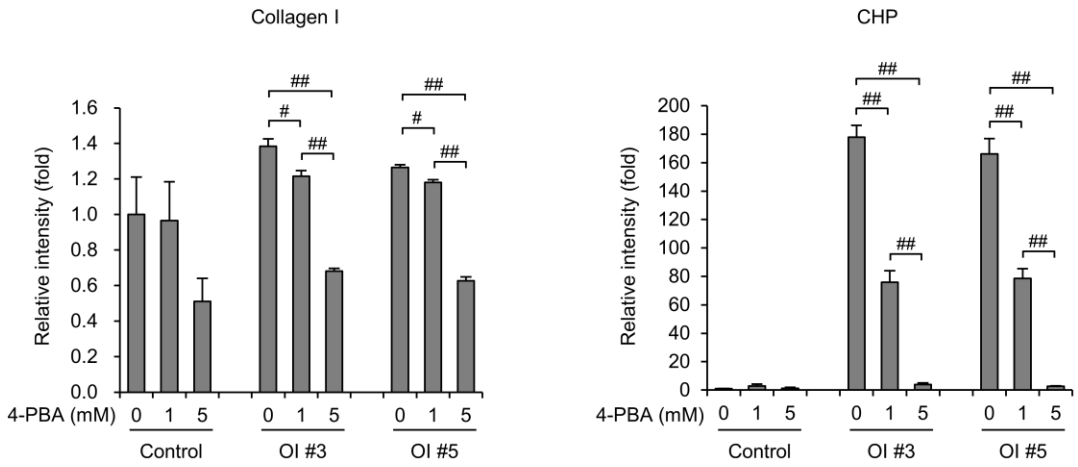

Figure S8. Calculated values of fluorescence staining. The formula for calculation is as follows: red area × red fluorescence density for type I collagen and green area × green fluorescence density for CHP. Data are mean ± SEM. Differences were tested by ANOVA followed by Tukey's HSD post hoc test. #$P < 0.05$, ##$P < 0.01$.

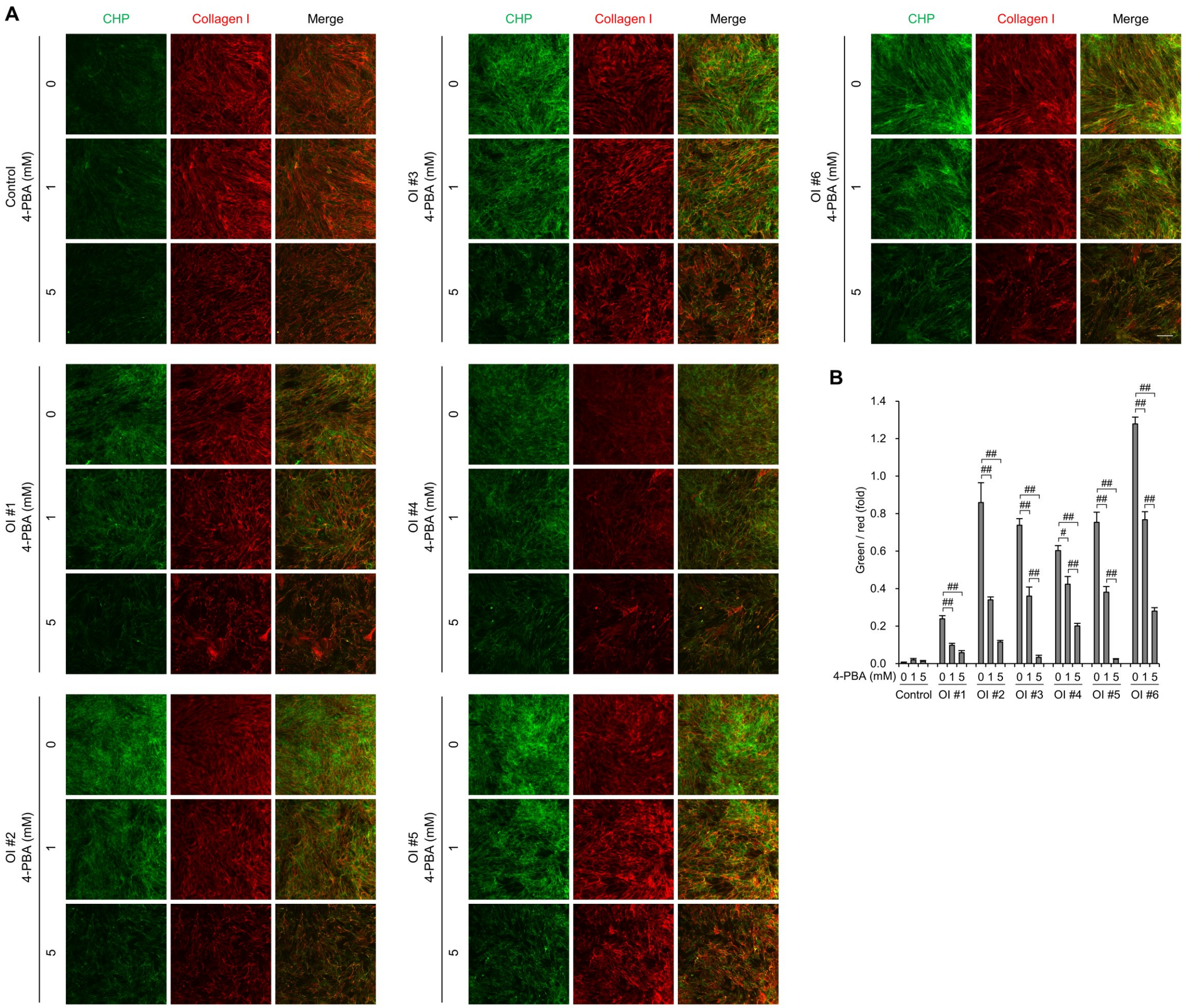

Figure S9. The effect of 4-phenylbutyric acid (4-PBA) on type I collagen in the extracellular matrix. **A.** Fluorescence staining of deposited collagen. Misfolded triple-helical chains are stained in green by collagen hybridizing peptide (CHP). Type I collagen is stained red. Image panels of OI #3 are redisplays of image panels of Figure 7. Scale bar is 200 μm. **B.** Calculated values of fluorescence staining. The formula for calculation is as follows: (green area × green fluorescence density)/(red area × red fluorescence density). Data are mean ± SEM. Differences were tested by ANOVA followed by Tukey's HSD post hoc test. #$P < 0.05$, ##$P < 0.01$.

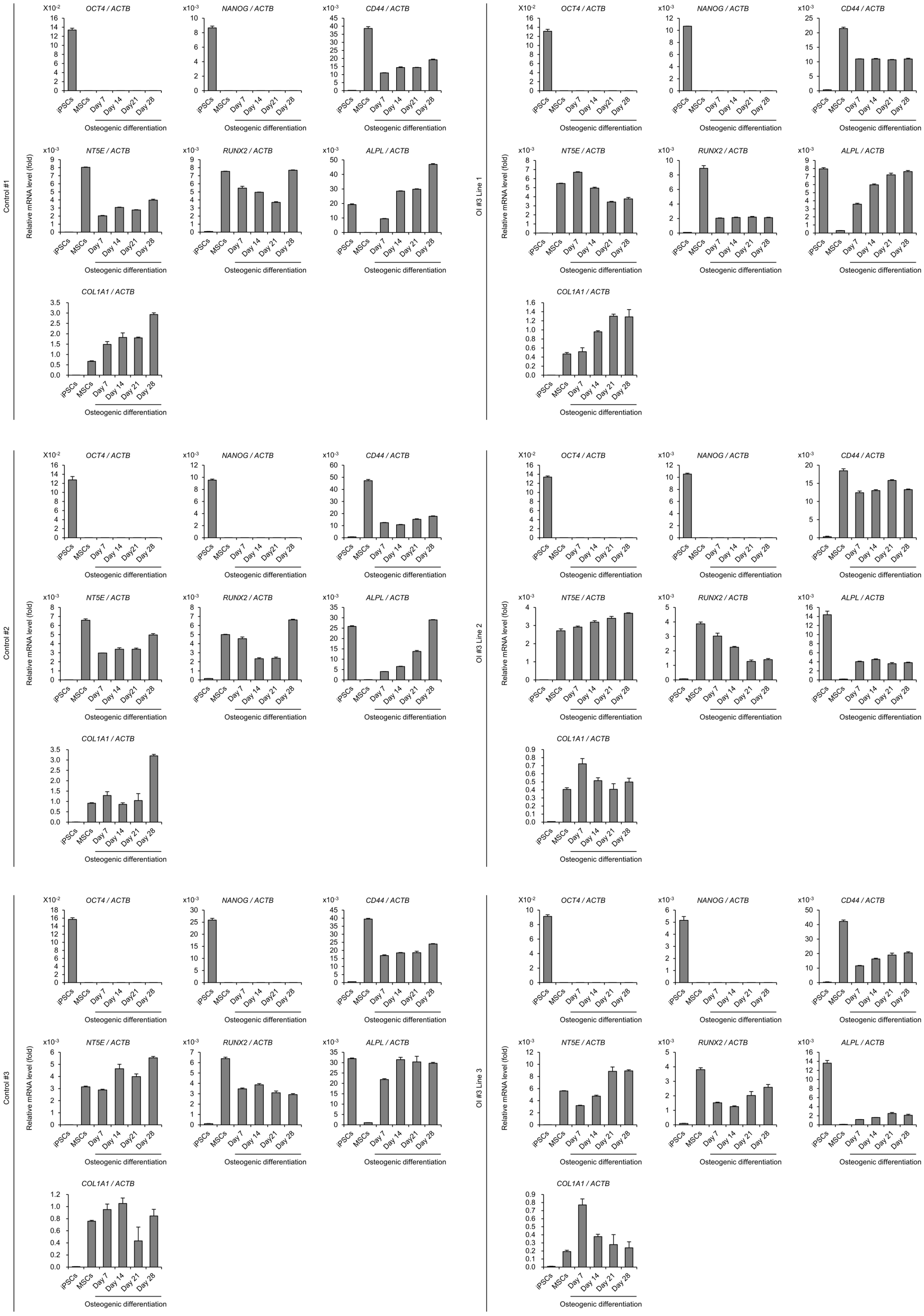

Figure S10. Gene expression of control and OI #3 cell lines. Pluripotency markers, *OCT4* and *NANOG,* mesenchymal stromal cell (MSC) markers, *CD44* and *NT5E,* and osteogenic markers, *RUNX2, ALPL,* and *COL1A1* were checked in induced pluripotent stem cells (iPSCs) and MSCs and after osteogenic differentiation for 7, 14, 21, and 28 d by real-time quantitative PCR. The values are indicated as relative gene expression using *ACTB* as a reference gene. All data are mean ± SEM.

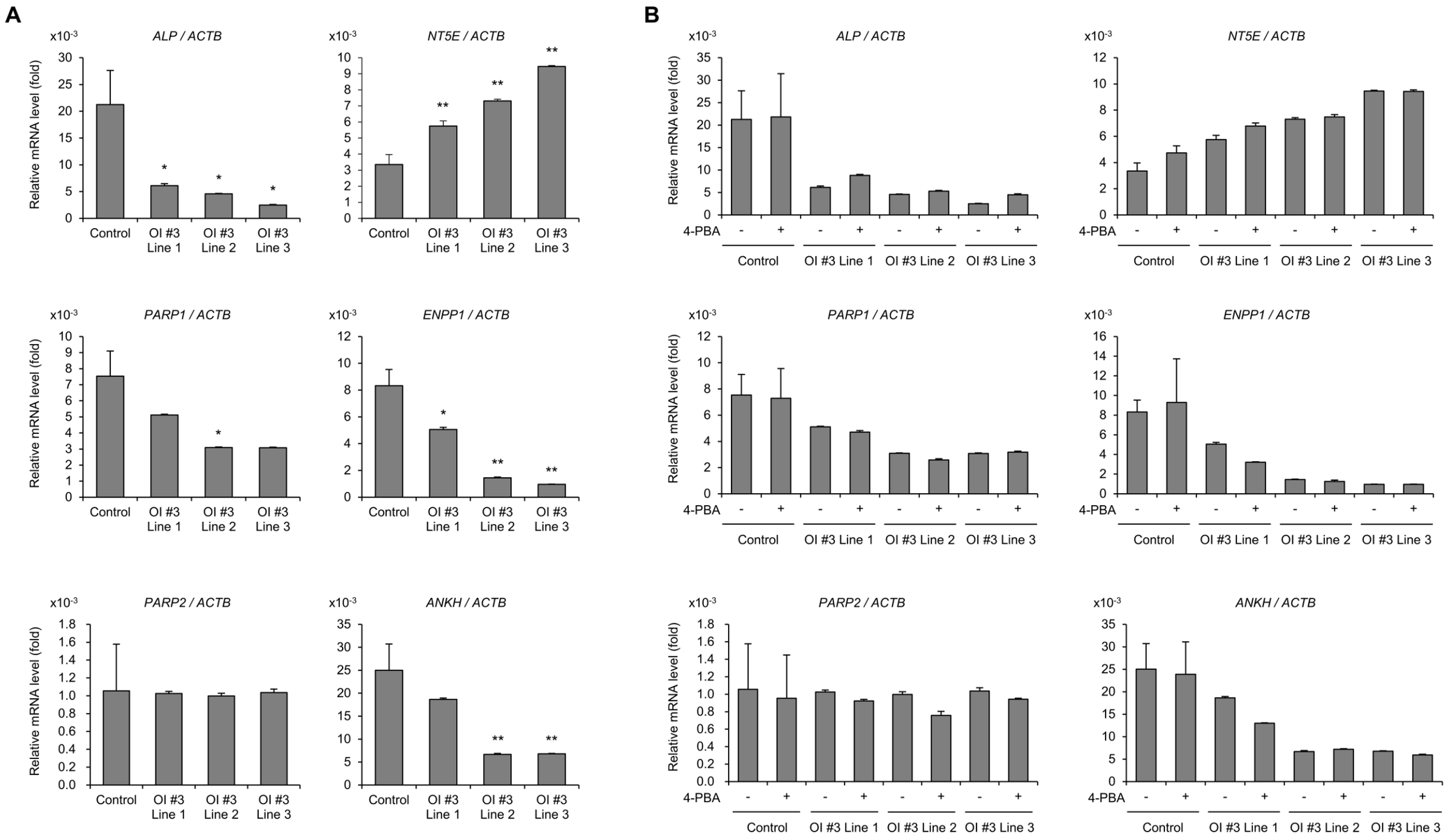

Figure S11. Gene expression of control and OI #3 cell lines. Pro-calcification genes, *ALPL, PARP1,* and *PARP2,* and anti-calcification genes, *NT5E, ENPP1,* and *ANKH,* were checked at day 14 following osteoblast differentiation with no 4-phenylbutyric acid (4-PBA) **(A)** and with or without 4-PBA **(B)** by real-time quantitative PCR. The values are indicated as relative gene expression using *ACTB* as a reference gene. All data are mean ± SEM. Differences were tested by ANOVA followed by Tukey's HSD post hoc test. **P* < 0.05 vs. control, ***P* < 0.01 vs. control.

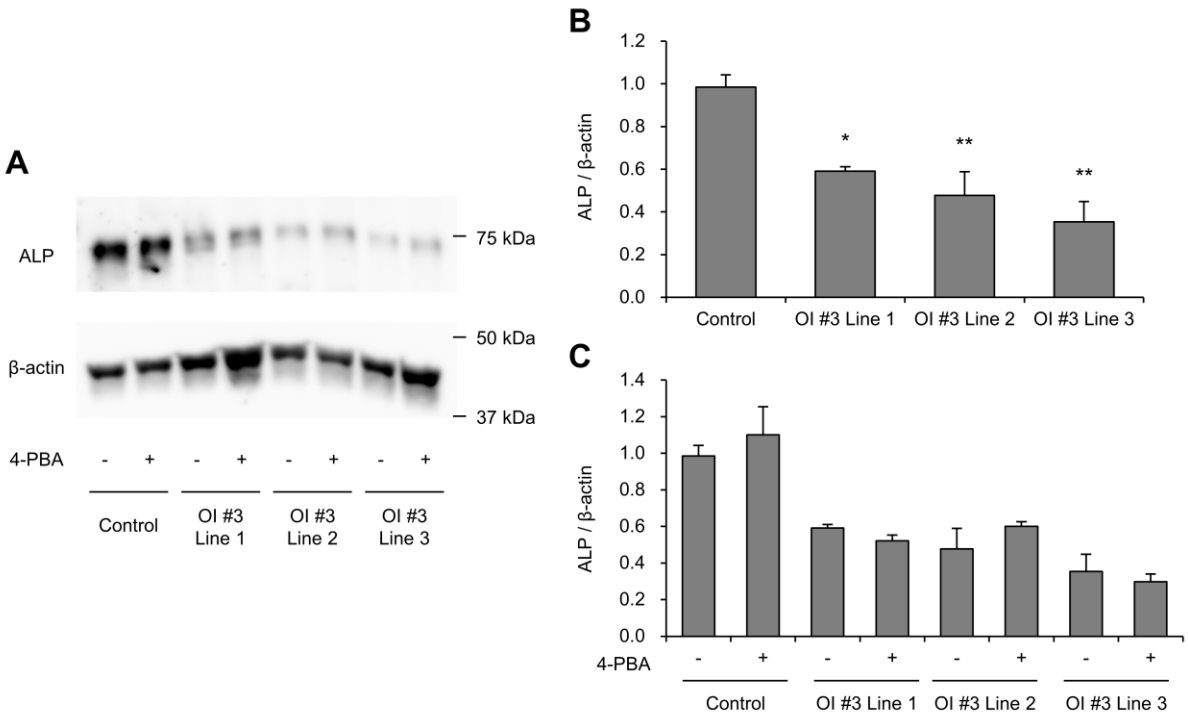

Figure S12. Protein level of alkaline phosphatase (ALP) in control and OI #3 cell lines. **A.** Western blotting of alkaline phosphatase and β-actin at day 14 following osteoblast differentiation with or without 4-phenylbutyric acid (4-PBA). **B and C.** ALP levels are indicated as the relative levels of β-actin quantified by densitometry. All data are mean ± SEM. Differences were tested by ANOVA followed by Tukey's HSD post hoc test. *$P < 0.05$ vs. control, **$P < 0.01$ vs. control.

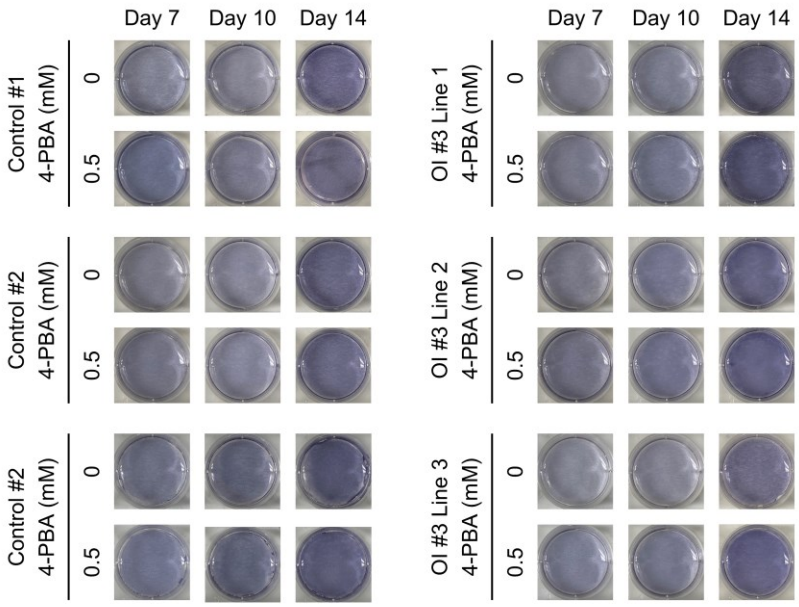

Figure S13. Alkaline phosphatase staining in induced osteoblasts. Induction of osteoblast differentiation from mesenchymal stromal cells was performed during the indicated day with or without 4-phenylbutyric acid (4-PBA).

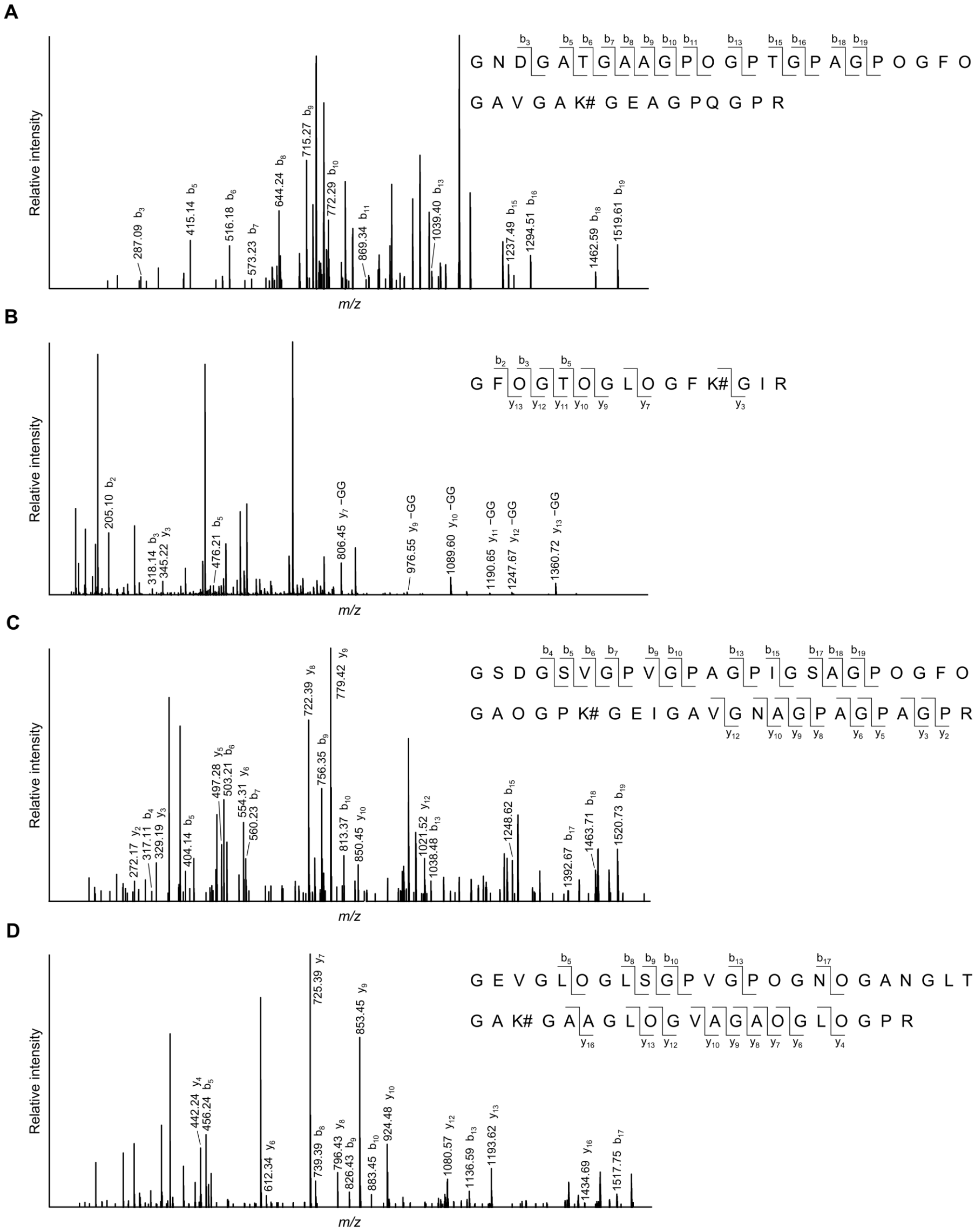

Figure S14. MS/MS spectra of GGHL-containing tryptic peptides in type I collagen. **A.** α1(I) [145–183] GNDGATGAAGPOGPTGPAGPOGFOGAVGAK#^{174}GEAGPQGPR (z = 4, m/z 935.1465; O indicates Hyp, and K# indicates GGHL). **B.** α2(I) [76–90] GFOGTOGLOGFK#^{87}GIR (z = 3, m/z 630.3103). **C.** α2(I) [145–192] GSDGSVGPVGPAGPIGSAGPOGFOGAOGPK#^{174}GEIGAVGNAGPAGPAGPR (z = 4, m/z 1115.5401). **D.** α2(I) [193–237] GEVGLOGLSGPVGPOGNOGANGLTGAK#^{219}GAAGLOGVAGAOGLOGPR (z = 4, m/z 1076.2862). −GG represents deglycosylated fragment ions.