HHS Public Access

Author manuscript

Biochemistry. Author manuscript; available in PMC 2021 February 17.

Published in final edited form as:

Biochemistry. 2019 December 17; 58(50): 5040–5051. doi:10.1021/acs.biochem.8b00984.

Role of Glycosyltransferase 25 Domain 1 in Type I Collagen Glycosylation and Molecular Phenotypes

Masahiko Terajima[†], **Yuki Taga**[‡], **Marnisa Sricholpech**[§], **Yukako Kayashima**[‖], **Noriko Sumida**[†], **Nobuyo Maeda**[‖], **Shunji Hattori**[†], **Mitsuo Yamauchi**[*,†]

[†]Oral and Craniofacial Health Sciences, School of Dentistry, The University of North Carolina, Chapel Hill, North Carolina 27599, United States [‡]Nippi Research Institute of Biomatrix, Ibaraki 302-0017, Japan [§]Department of Oral Surgery and Oral Medicine, Faculty of Dentistry, Srinakharinwirot University, Bangkok 10110, Thailand [‖]Department of Pathology and Laboratory Medicine, The University of North Carolina, Chapel Hill, North Carolina 27599, United States

Abstract

Glycosylation in type I collagen occurs as O-linked galactosyl- (G-) lesser and glucosylgalactosyl-hydrox-ylysine (GG-Hyl); however, its biological significance is still not well understood. To investigate the function of this modification in bone, we have generated preosteoblast MC3T3-E1 (MC)-derived clones, short hairpin (Sh) clones, in which *Glt25d1* gene expression was stably suppressed. In Sh clones, the GLT25D1 protein levels were markedly diminished in comparison to controls (MC and those transfected with the empty vector). In Sh collagen, levels of both G- and GG-Hyl were significantly diminished with a concomitant increase in the level of free-Hyl. In addition, the level of immature divalent cross-links significantly diminished while the level of the mature trivalent cross-link increased. As determined by mass spectrometric analysis, seven glycosylation sites were identified in type I collagen and the most predominant site was at the helical cross-linking site, α1–87. At all of the glycosylation sites, the relative levels of G- and GG-Hyl were markedly diminished, i.e., by ~50–75%, in Sh collagen, and at five of these sites, the level of Lys hydroxylation was significantly increased. The collagen fibrils in Sh clones were larger, and mineralization was impaired. These results indicate that GLT25D1 catalyzes galactosylation of Hyl throughout the type I collagen molecule and that this modification may regulate maturation of collagen cross-linking, fibrillogenesis, and mineralization.

[*]**Corresponding Author**: Division of Oral and Craniofacial Health Sciences, School of Dentistry, The University of North Carolina, Chapel Hill, NC 27599. mitsuo_yamauchi@unc.edu. Telephone: 919-537-3217.

Author Contributions

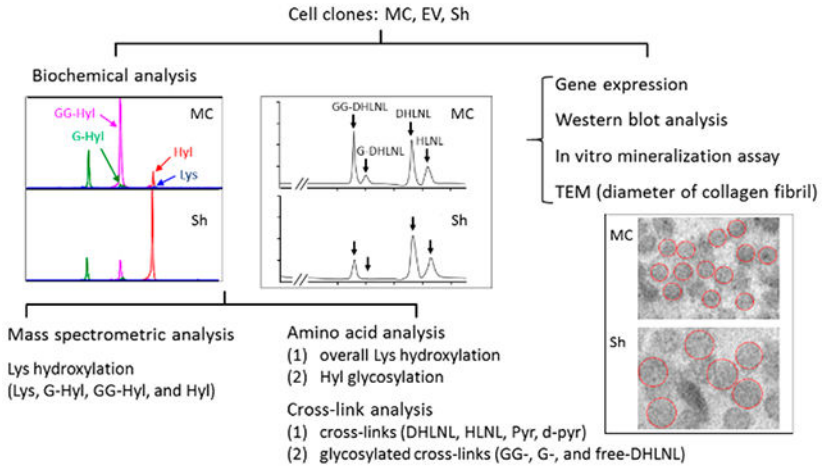
M.T. and Y.T. contributed equally to this work. M.Y. conceived and designed the experiments. M.T., Y.T., M.S., Y.K., and N.S. performed the experiments. M.T. and Y.T. analyzed the data. N.M. and S.H. contributed reagents and helped with the interpretation of data. M.T., Y.T., and M.Y. wrote the paper. All authors read and approved the manuscript.

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.8b00984.

Gene expression of *Glt2Sd1* from transient transfection of short hairpin constructs targeting *Glt2Sd1* (Figure S1), GLT25D1 levels produced by Sh clones with and without overexpression of Glt25d1 cDNA (Figure S2), mass spectrometric analysis of hydroxylysine glycosylation at specific molecular sites in type I collagen isolated from Sh clones with and without overexpression of Glt25d1 cDNA (Figure S3), ratios of α chain and collagen type (Table S1), summary of site-specific modifications by mass spectrometry of non-cross-linked, hydroxylated, and glycosylated residues in type I collagen from controls (MC and EV) and Sh clones (Table S2), and collagen cross-links and glycosylation in controls (MC and EV) and Sh clones (Table S3) (PDF)

The authors declare no competing financial interest.

Graphical Abstract



Collagens are the most abundant protein in vertebrates.[1] Among different types of collagen, fibrillar type I collagen is the predominant type that provides tissues and organs with form and mechanical strength. Type I collagen biosynthesis is a highly complex process involving a number of post-translational modifications, association of three pro-α chains, folding into a triple-helical procollagen molecule, secretion to the extracellular space, processing of procollagen, self-assembly into a fibril, and stabilization via covalent intermolecular cross-linking.[1,2]

The post-translational modifications of type I collagen that include hydroxylation of lysine (Lys) and proline (Pro) residues, O-glycosylation of hydroxylysine (Hyl)[3] residues, and covalent intra- and intermolecular cross-linking are important for its structural functions.[2] Inside the cell, a series of Lys modifications in the nascent proα1(I) and 2(I) chains take place in the rough endoplasmic reticulum (ER) prior to the formation of a triple-helical procollagen molecule. Hydroxylation of Lys is catalyzed by lysyl hydroxylases 1–3 (LH1–3)[4,5] encoded by three respective genes (*PLOD1–3*). LH1 likely catalyzes this modification in the helical domain of fibrillar collagens[6] and LH2 in the telopeptides.[7] In the helical domain of the molecule, Hyl residues in the sequence of Gly-X-Hyl can be further modified by O-linked glycosylation.[6,8] For this modification, two steps are involved. The first step is to attach galactose to the 5-hydroxyl group of Hyl by hydroxylysyl galactosyltransferase forming galactosyl-Hyl (G-Hyl)[9] (EC 2.4.1.50), and the second step is to attach glucose to G-Hyl by galactosylhydroxylysyl glucosyltransferase (GGT) (EC 2.4.1.66) forming glucosylgalactosyl-Hyl (GG-Hyl).[10] It has been reported that LH3 is a multifunctional enzyme possessing all LH, GT, and GGT activities.[10,11] However, in type I collagen, it mainly functions as GGT and the resulting modification may modulate collagen cross-linking, fibrillo-genesis, and mineralization.[12,13]

As for the initial glycosylation step, i.e., galactosylation of Hyl, it has been reported that glycosyltransferase 25 domains 1 and 2 (GLT25D1/2) encoded by *Glt25d1* and *Glt25d2* genes, respectively, likely catalyze this process.[14] Of the two isoforms, the *Glt25d1* gene is highly expressed in many tissues while *Glt25d2* is expressed at low levels in a few cell

types.[14] We have also shown that *Glt25d1* was highly expressed in the mouse osteoblast cell line, whereas *Glt25d2* was undetectable.[12]

To further investigate the function of collagen glycosylation in bone, we employed a loss-of-function approach *in vitro*[12] and characterized collagen produced by MC3T3-E1 (MC)-derived clones in which *Glt25d1* expression was stably suppressed.

EXPERIMENTAL PROCEDURES

Cell Cultures.

MC3T3-E1 subclone 4, a mouse calvaria-derived osteoblastic cell line,[15] was purchased from American Type Culture Collection (CRL-2593) and grown as reported previously.[12]

Generation of the Short Hairpin RNA-Expressing Plasmid.

The target sequences for suppressing *Glt25d1* were designed on the siDesign Center Web site (Dharmacon RNAi Technologies). The three highest-ranked targets were chosen [si 1, GGAATAATGAGCAGGTTAA (start position 1891); si 2, AGGCAGAGGTGCAAATGTA (985); si 3, TGTCAGAATACAAGAGTCA (1762)], converted to short hairpin oligonucleotide sequences, annealed with their complementary sequences, and cloned into the pSilencer2.1-U6/neo vector (Ambion) as reported previously.[12] After bacterial transformation of the short hairpin plasmids, the clones with 100% nucleotide sequence accuracy for each target (Sh1–Sh3) were evaluated for their *Glt25d1* suppression efficiency by transient transfection into MC cells.[12] The nontransfected MC cells and those transfected with the empty vector (EV) served as controls. Forty-eight hours following transfection, total RNA was extracted and converted into cDNA and the mRNA expression levels of *Glt25d1* were assessed by quantitative real time polymerase chain reaction (PCR) with ABI Prism 7000 (Applied Biosystems, Foster City, CA)[12] using specific probes for Glt25d1 (*Glt25d1*; Applied Biosystems, ABI assay Mm06600638_m1) and type I collagen α2 chain (*Col1A2*) Mm00483888_m1). The mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ABI assay 4308313).[16]

MC Single-Cell-Derived Clones with Stable Suppression of *Glt25d1*.

The pSilencer2.1-U6/neo-*Glt25d1* plasmid containing the Sh target with the most suppression of *Glt25d1*, from transient tranfection described above, was transfected into MC cells using the FuGENE 6 transfection reagent (Roche Applied Science). Single-cell-derived short hairpin (Sh) clones were then generated with constant selection using 400 μg/mL G418 sulfate (Mediatech, Inc., Manassas, VA), and several Sh clones were isolated. Similarly, single-cell-derived clones with stable transfection of EV were also generated, as previously reported.[12] The isolated Sh clones were characterized by comparing the *Glt25d1* expression levels among the Sh, EV clones, and MC cells using quantitative real time PCR.[12] Three to six Sh clones with various *Glt25d1* suppression levels were further characterized for their effects on the mRNA expression of LH1 (*Plod1*; Mm00599925_m1), LH2 (*Plod2*; Mm00478767_m1), LH3 (*Plod3*; Mm00478798_m1), lysyl oxidase (LOX) (*Lox*; Mm00495386_m1), LOX-like 1 (LOXL1) (*Loxl1*; Mm01145738_m1), LOXL2 (*Loxl2*;

Mm00804740_m1), LOXL3 (*Loxl3*; Mm00442953_m1), and LOXL4 (*Loxl4*; Mm00446385_m1).

Western Blot Analysis.

Sh clones and control cells were cultured for 7 days, and the cell lysates were subjected to Western blot analysis as previously reported.[12,17] The following primary antibodies were used: rabbit polyclonal GLT25D1 antibody (1:500, catalog no. 16768-1-AP, Proteintech), rabbit monoclonal PLOD1 antibody (1:100, catalog no. sc-271640, Santa Cruz Biotechnology), rabbit polyclonal PLOD2 antibody (1:100, catalog no. 21214-1-AP, Proteintech), rabbit polyclonal PLOD3 antibody (1:200, catalog no. 11027-1-AP, Proteintech), and anti-GAPDH rabbit monoclonal antibody (1:1000, catalog no. 14C10, Cell Signaling Technology).

Collagen Preparation for Biochemical Analysis.

Cells were cultured for 2 weeks, and cell/matrix layers were washed and lyophilized for collagen analysis as reported previously.[12]

Quantification of Collagen α Chains and Type I and III Collagens by Mass Spectrometry.

Collagen was extracted from the lyophilized samples with 5 mg/mL pepsin (Sigma-Aldrich, St. Louis, MO) in 0.5 N acetic acid for 24 h at 4 °C and subsequently purified by salt precipitation with 0.7 M NaCl for 1 h on ice.[12] The purified collagen samples were mixed with stable isotope-labeled collagen (SI-collagen).[8] After denaturation at 60 °C for 30 min, the samples were digested with sequencing grade modified trypsin (Promega, Madison, WI) (1:50 enzyme:substrate ratio) in 100 mM Tris-HCl buffer containing 1 mM $CaCl_2$ (pH 7.6) at 37 °C for 16 h. The digests were then analyzed by electrospray LC–triple-quadrupole (QqQ)–MS on a hybrid QqQ/linear ion trap 3200 QTRAP mass spectrometer (AB Sciex, Foster City, CA) with an Agilent 1200 Series HPLC system (Agilent Technologies, Palo Alto, CA) using a C18 HPLC column (5 μm particle size, 2.1 mm × 150 mm; Supelco, Bellefonte, PA).[18] The specific peptides derived from α1(I), α2(I), and α1(III) chains (two peptides for each chain) were analyzed by multiple-reaction monitoring mode as described previously.[8,18,19] The α1(I):α2-(I) ratio was determined by the molar concentrations of α1(I) and α2(I) chains based on the ratio of their marker peptides to stable isotopically heavy ones derived from SI-collagen in which the absolute concentrations of the α chains were predetermined.[20] The concentrations of type I and III collagens were calculated on the basis of the light to heavy peak area ratio of α1(I)/α2(I) and α1(III) marker peptides, respectively.[8]

Site-Specific Lysine Modifications in Type I Collagen.

The purified collagen samples were treated with trypsin as described above and subjected to electrospray LC–MS using an ultrahigh resolution (UHR) quadrupole time-of-flight (QTOF) mass spectrometer (maXis II, Bruker Daltonics, Bremen, Germany) with a Shimadzu Prominence UFLC-XR system (Shimadzu, Kyoto, Japan). The samples were applied to an Ascentis Express C18 HPLC column (5 μm particle size, 2.1 mm × 150 mm; Supelco) with a binary gradient of 0.1% formic acid and acetonitrile as described previously.[19] The MS

scan was obtained over the *m/z* range of 50–2500 with a frequency of 2 Hz in positive ion mode. The relative extent of Lys hydroxylation and Hyl glycosylation (Lys, Hyl, G-Hyl, and GG-Hyl) at the specific sites in type I collagen was calculated as reported previously.[19,21]

Reduction with NaB^3H_4.

Lyophilized cell/matrix samples (~2.0 mg) were reduced with standardized NaB^3H_4, washed, and lyophilized.[22]

Analysis of Hyl Glycosylation by High-Performance Liquid Chromatography (HPLC).

Reduced samples were hydrolyzed with 6 N HCl and subjected to amino acid analysis,[23] and the total number of Hyl residues (i.e., a sum of Hyl, G-Hyl, and GG-Hyl) per collagen molecule was calculated by normalizing the values to 300 residues of hydroxyproline (Hyp). Samples were also hydrolyzed with 2 N NaOH, and nonglycosylated[3] and glycosylated Hyl (G- and GG-Hyl) were analyzed as described previously.[12]

Collagen Cross-Link Analysis.

Reduced samples hydrolyzed with 6 N HCl or 2 N NaOH were subjected to cross-link analysis.[23] Upon reduction, dehydrodihydroxylysinonorleucine (dehydro-DHLNL)/its ketoamine and dehydrohydroxylysinonorleucine (dehydro-HLNL)/its ketoamine are reduced to stable secondary amines, DHLNL and HLNL, respectively. These cross-links were analyzed as their reduced forms, and the terms DHLNL and HLNL will be used for both unreduced and reduced forms hereafter. The quantities of these reducible cross-links and a mature nonreducible cross-link, pyridinoline (Pyr), were determined using the acid hydrolysates and calculated as moles per mole of collagen.[23,24] The total number of aldehydes involved in these cross-links was calculated as DHLNL + HLNL +; 2 × Pyr.[2]

The glycosylated reducible cross-links were analyzed using the base hydrolysates, and the nonglycosylated and glycosylated (G- and GG-) cross-links were quantified as moles per mole of collagen as reported previously.[13] Because Pyr is labile in base hydrolysis,[25] its glycosylation forms were not analyzed.

Characterization of Collagen Fibrils.

Cells were cultured for 2 weeks; cell/matrix layers were fixed, embedded, sectioned, and stained,[26] and collagen fibrils were observed under a LEO EM-910 transmission electron microscope. The images were obtained at 25000× magnification, and the diameters of collagen fibrils were measured (1200 fibrils/group) using ImageJ 1.44p software as described previously.[27]

Mineralization Assay.

Cells were plated at a density of 2×10^5 cells/35 mm dish, cultured in mineralization medium[28] for ≥4 weeks. At the end of each week, the cell/matrix layer was stained with 1% Alizarin Red S (Sigma-Aldrich) to assess mineralization.[28,29] At the end of 4 weeks when mineralization is apparent in control groups, the extent of mineralization in each group was quantified by the triplicate measurements and compared.

Generation and Characterization of GLT25D1 Sh Clones with Overexpression of *Glt25d1* cDNA.

The pPM-C-His vector containing mouse *Glt25d1* cDNA (anti-bodies-online Inc., catalog no. ABIN4411012) was transfected into Sh clones to restore GLT25D1 using FuGENE 6 transfection reagent (Roche Applied Science). The transfected cells were selected with G418 sulfate (400 μ/mL) for 7 days. Expression of GLT25D1 protein in the isolated clones was confirmed by Western blot analysis. Then, the stably transfected cells were cultured for 2 weeks, and type I collagen was prepared and subjected to mass spectrometric analysis to assess the extent of Hyl glycosylation as described above.

Statistical Analyses.

Data were statistically evaluated with Jmp8.0 software (SAS Institute Inc., Cary, NC). Statistical differences were calculated by Kruskal–Wallis one-way analysis of variance and means comparison by a Student's *t* test. The results are shown as means ± the standard deviation (SD), and a p value of <0.05 was interpreted as being statistically significant.

RESULTS

Suppression of *Glt25d1* by Sh RNA.

Following the transient transfection of pSilencer2.1-U6/Neo plasmids encoding hairpin oligonucleotides targeting three different sites in *Glt25d1* (Sh1–Sh3) into MC cells, the levels of *Glt25d1* and *Col1A2* expression were evaluated by real time PCR. Relative to *Gapdh*, all three Sh targets exhibited significant suppression of *Glt25d1* expression. The expression levels of *Glt25d1* in Sh1–Sh3 were 37.7%, 39.5%, and 29.4%, respectively, while it was ~89.5% in EV, when compared to that of the MC nontransfected cells (100%) (Figure S1A). Suppression of *Col1A2* expression relative to *Gapdh* was also evaluated in the cells transfected with either Sh1, Sh2, or Sh3 constructs, and the results showed 27.9%, 91.8%, and 82.1% downregulation of *Col1A2*, respectively (Figure S1B). The relative expression of *Glt25d1* to *Col1A2* is an important factor for determining the gene manipulation effect on the levels of collagen post-translational modification.[12] In the study presented here, transfection with the Sh1 construct led to a lower level of *Glt25d1* relative to *Col1A2* ($p < 0.05$ compared to those of MC and EV), while the Sh2 and Sh3 constructs indicated significant off-target effects on *Col1A2* expression (Figure S1C). On the basis of these findings, short hairpin target sequence 1 (Sh1) was used to create single-cell-derived clones with stable suppression of *Glt25d1*.

Sh1-Derived Clones with Stable Suppression of *Glt25d1*.

Three representative clones (Sh1-1, -2, and -3) exhibiting cell morphology and growth patterns similar to those of the controls (MC and EV) were chosen for further characterization. The expression levels of *Glt25d1* were significantly suppressed in all Sh clones in comparison to EV and MC ($p < 0.01$) (Figure 1). The possible effects of *Glt25d1* suppression on the expression of *Plod1*–3 genes were further explored. Relative to *Gapdh,* the expression levels of *Plod1* were slightly but significantly higher in Sh1-2 and -3 clones ($p < 0.05$), while that in Sh1-1 was not different from that of MC (Figure 1). Levels of *Plod3*

expression in Sh1-1 and -2 were also slightly but significantly higher than those of controls (MC and EV) ($p < 0.05$), whereas in Sh1–3, it was not significantly different from that of EV. Levels of *Plod2* expression were not significantly different between Sh clones and controls ($p > 0.05$). Possible consequences of *Glt25d1* suppression on the expression of *Lox and Loxl1–4* were also examined. The results demonstrated that the levels of *Lox* and *Loxl2–4* in the Sh clones were comparable to those of controls (MC and EV), whereas the level of expression of *Loxl1* in Sh clones was slightly lower compared to those of controls (Figure 1).

The GLT25D1 protein levels in cell lysates were assessed by Western blot analysis (Figure 2). An immunopositive band at ~72 kDa, the expected molecular weight of GLT25D1, was observed in the controls and Sh clones, but the immunor-eactivities were significantly decreased in all Sh clones (Figure 2A). When normalized to the protein levels of GAPDH, GLT25D1 levels in Sh clones were 2.5% (Sh1-2), 4.7% (Sh1-1), and 35% (Sh1-3) of that of MC (Figure 2B). Thus, GLT25D1 protein levels in Sh clones were effectively suppressed.

Protein Levels of Lysine-Modifying Enzymes by Western Blot Analysis.

Because Lys modifications of Sh type I collagen were significantly altered (see Figures 3 and 5 and Tables S2 and S3), we examined the protein levels of LH1–3. When normalized to GAPDH, the level of LH1 was significantly increased in Sh clones, except for Sh1-1. However, levels of both LH2 and −3 were decreased in all Sh clones (Figure 2).

Lysine Modifications of Collagen.

The extent of Lys modifications in collagen was also analyzed by HPLC[12] (Figure 3). In Sh clones, the level of Lys hydroxylation, including glycosylated and free Hyl, was slightly but significantly increased compared with those from MC and EV (Figure 3A). A significant decrease in the level of GG-Hyl was observed in all Sh clones compared to those of controls ($p < 0.05$), while the level of free Hyl tended to be higher than controls but did not reach significant levels (Figure 3B). G-Hyl was not detected in Sh clones (Figure 3B). When calculated as percentages of total Hyl (Figure 3C), levels of GG-Hyl in Sh clones (8.2% Sh1-1, 6.4% Sh1-2, and 8.5% Sh1-3) were significantly lower compared to those of controls (19.3% for MC and 17.3% for EV) ($p < 0.05$). The relative levels of free-Hyl in Sh clones (91.8% Sh1-1, $p < 0.01$; 93.6% Sh1-2, $p < 0.01$; and 91.5% Sh1-3, $p < 0.01$) were significantly higher than those of controls (76.2% for MC and 78.8% for EV) (Figure 3C). When the extent of Hyl glycosylation (both G- and GG-) is calculated, it is 65–75% lower in Sh collagens than those of controls.

α Chain and Collagen Type Ratios.

The ratio of type I collagen α chains and the relative abundance of type I and III collagens were determined by LC–QqQ–MS analysis.[8] For all samples, the α1(I):α2(I) ratio was 2.2–2.3, which is consistent with the value for the type I collagen $[\alpha 1(I)]_2\alpha 2(I)$ heterotrimer (Table S1). The $[\alpha 1(I)]_3$ homotrimer was estimated as less than 10% of total type I collagen in all groups, and there were no differences between controls (MC and EV) and Sh clones (Table S1). This demonstrates that the severely suppressed level of *Glt25d1* (Figures 1 and 2) or slightly suppressed level of *Col1A2* (Figure S1B) observed in Sh clones did not affect

the α chain ratio. Also, the ratio of type I to III collagen did not change with *Glt25d1* suppression (Table S1). The results showing 95–98% type I collagen and 2–5% type III collagen in MC cells are consistent with previously published data.[30]

Effect of Glt25d1 Suppression on Lysine Modifications of Type I Collagen.

Following trypsin digestion, Lys modifications at specific molecular sites of type I collagen isolated from MC, EV, and Sh clones were analyzed by LC–MS on the maXis II UHR-QTOF system. Figure 4 presents the representative extracted ion chromatograms (EICs) of tryptic peptides containing the major glycosylation site, α1–87.[13,19] The heterogeneity of peptide peaks in LC–MS was observed due to its charge state, partial prolyl 4-hydroxylation, and partial miscleavage by trypsin at Hyl and glycosylated Hyl;[13] thus, the most intense peaks were depicted to analyze and compare each modification form. In both MC and EV, the Lys residue at α1–87 was almost fully hydroxylated and the Hyl residue was predominantly diglycosylated (GG-Hyl). The monoglycosylated form, G-Hyl, and free (nonglycosylated) Hyl were both detected but at low levels. In contrast, in Sh clones in which *Glt25d1* gene expression was stably suppressed, the nonglycosylated Hyl was the major form at this site and levels of GG- and G-Hyl were both markedly decreased.

The site occupancy of modified Lys residues at specific molecular sites was semiquantitatively calculated using the peak area ratio of all detected Lys-, Hyl-, G-Hyl-, and GG-Hyl-containing peptide peaks (Figure 5A; also see Table S2). As shown in Figure 4, G-Hyl, a very minor form of glycosylation, was decreased by *Glt25d1* suppression (3.3–3.5% for MC and EV and 1.5–1.7% for Sh clones) at α1–87. In addition, the concomitant decrease in 83.7% for MC and EV and 19.5–21.9% for Sh clones) and marked increases in the level of free Hyl (12.1–12.6% for MC and EV and 76.3–78.7% for Sh clones) were observed at this site. Similarly, the level of G-Hyl was decreased in Sh collagens at α1–99 (7.4–7.8% for MC and EV and 2.8–2.9% for Sh clones), α1–174 (3.0–3.5% for MC and EV and 0.9–1.0% for Sh clones), α1–564 (4.5—4.7% for MC and EV and 1.4–1.5% for Sh clones), α1–603 (0.9–1.4% for MC and EV and 0.4–0.6% for Sh clones), α2–174 (55.0–62.1% for MC and EV and 25.6–27.8% for Sh clones), and α2–219 (2.2–3.4% for MC and EV and 0.8–0.9% for Sh clones). Underglucosylation and overhydroxylation were also observed at all of these sites. Glycopeptide containing α2–87 was not detected, though trace amounts of G-Hyl and/or GG-Hyl could be present as previously reported.[13,21] Also, glycosylation at α1–219 was not detected while that on an α2 chain was detectable (Figure 5A; also see Table S2). The alterations in Lys hydroxylation/glycosylation were more apparent when shown as percentages of GG-, G-, and nonglycosylated forms in total Hyl (Figure 5B; also see values in parentheses in Table S2). Seven glycosylation sites, i.e., α1–87, α1–99, α1-/α2-174, α1–564, α1–603, and α2–219, were identified, among which the predominant glycosylation site was the helical cross-linking site, α1–87. At this site, the relative levels of G- (3.4–3.7% for MC and EV and 1.5–1.7% for Sh clones) and GG-Hyl (83.1–84.4% for MC and EV and 19.6–21.9% for Sh clones) were markedly diminished in Sh collagens. Another major glycosylation site, α2–174, also showed significant decreases in the levels of G- (71.5–72.3% for MC and EV and 27.7–30.2% for Sh clones) and GG-Hyl (16.8–17.9% for MC and EV and 3.5–4.0% for Sh clones) with a concomitant increase in the level of free-Hyl (10.6–10.8% for MC and EV and 65.9–68.8% for Sh clones). Due to

the decreases in the levels of G- and GG-Hyl and the increase in the level of free Hyl, the nonglycosylated form became the most dominant molecular species at all of the glycosylation sites in Sh collagens.

Collagen Cross-Links.

In all samples, two immature reducible cross-links, DHLNL and HLNL, and a mature nonreducible cross-link, Pyr, were detected. The levels of cross-link precursors, Hyl- and Lys-aldehydes, were minimal (<0.01 mol/mol of collagen), and another mature cross-link, deoxy-Pyr, was not detected in any of the samples analyzed. The amounts of DHLNL, HLNL, Pyr, and total aldehydes involved in these cross-links are shown in Figure 6A and Table S3A. The levels of DHLNL and HLNL cross-links were both significantly lower in Sh clones than in controls (MC and EV) ($p < 0.05$). In Sh clones, the level of Pyr cross-link was significantly higher than those of the controls ($p < 0.05$). The total number of aldehydes was also lower in Sh clones in comparison to both controls ($p < 0.05$).

The HLNL cross-link did not significantly change between acid and base hydrolysates, indicating that the majority of this cross-link was not glycosylated. The GG-, G-, and non-glycosylated forms of DHLNL were quantified, and their relative abundance was calculated as a percentage of total DHLNL (Figure 6B and Table S3B). In MC and EV, ~50% of total DHLNL was glycosylated (40–41% GG- and 5–6% G-forms) and ~50% nonglycosylated. In all Sh clones, however, >80% was free DHLNL (~81.6%) with significantly lower levels of GG-DHLNL (~23.0%) ($p < 0.05$ compared to controls). G-DHLNL was undetectable in Sh clones. This is consistent with the MS analysis described above. Namely, the residue at α1–87 (note that α2–87 of mouse type I collagen is not glycosylated),[12] the major glycosylation site (Figure 5; also see Table S2), is also the major cross-linking site for the formation of DHLNL (α1–16^{C} × α1–87);[31] thus, the results indicate that the level of free Hyl at this site was increased with concomitant decreases in the levels of GG-Hyl in Sh clones (Figure 5; also see Table S2). When cross-link maturation was assessed as a ratio of tri- to bivalent cross-links, Pyr/DHLNL (i.e., the former is a maturational product of the latter),[25] it was significantly higher in all Sh clones (0.045–0.069) than in controls (0.013–0.018) ($p < 0.05$). Thus, in Sh collagen, crosslink maturation was accelerated.

Characterization of Collagen Fibrils.

Typical images of collagen fibrils and the diameter distribution in Sh clones and controls are presented in Figure 7. From these cross-sectional views, the shapes of the fibrils were circular in general and did not differ among the groups. Within the control groups, EV showed slightly but significantly smaller fibril diameters in comparison to MC ($p < 0.05$). The mean and range of the fibril diameters were significantly larger in all Sh clones in comparison to MC and EV controls ($p < 0.0001$). Among Sh clones, Sh1–3 that showed the lowest suppression levels of both the *Glt25d1* gene (Figure 1) and the GLT25D1 protein (Figure 2) revealed the smallest difference in the fibril diameter in comparison to controls. The means of fibril diameters increased in the following order: EV < MC < Sh1–3 < Sh1–1/Sh1–2 clones. Therefore, the fibril diameter was inversely correlated with the *Glt25d1* gene/protein expression levels, suggesting that the glycosylation regulates growth of collagen fibrils.

In Vitro Mineralization.

To assess the ability to form mineralized nodules *in vitro*, Sh clones and control cells were cultured for up to 4 weeks and subjected to Alizarin Red S staining (Figure 8). The results showed that, in the control groups (MC and EV), mineralized nodules were formed at 3 weeks, and the level was significantly increased at 4 weeks. However, no nodules were observed in Sh clones for ≤4 weeks (Figure 8A). This was confirmed by the quantitative measurement of Alizarin Red S content at week 4 showing that mineralization in Sh clones was markedly diminished in comparison to MC and EV ($p < 0.01$) (Figure 8B). Thus, a reduced level of glycosylation of Hyl affects matrix mineralization.

Overexpression of GLT25D1 in Sh Clones and Characterization of Hyl Glycosylation.

To validate the function of GLT25D1 observed above, we performed rescue experiments by overexpressing the *Glt25d1* gene in Sh clones and analyzing the collagen glycosylation. In these transfected clones, the GLT25D1 protein levels were increased (Figure S2) and the extents of Hyl glycosylation (G- and GG-Hyl) at the major glycosylation sites were all significantly increased compared to those of Sh clones (Figure S3), thus confirming the GLT25D1 function as glycosyltransferase.

DISCUSSION

It has been reported that O-linked glycosylation of collagen is catalyzed mainly by GLT25D1 and LH3 for the initial galactosylation of Hyl[14] and following glucosylation of G-Hyl,[12,32] respectively. Though these modifications have been implicated in collagen fibrillogenesis,[12,19,33–37] intermolecular cross-linking,[19,22,38–41] collagen–cell interaction,[42,43] remodeling,[35] and induction of vessel-like structures,[44] the specific effects of GLT25D1-catalyzed galactosylation have not been well-defined.

To investigate the role of collagen glycosylation, we employed a loss-of-function approach by generating MC-derived clones stably suppressing the *Glt25d1* gene encoding GLT25D1 protein (Sh clones), a putative GT, and characterized its collagen phenotypes. Here we demonstrated that a decrease in the level of GLT25D1 results in significantly decreased levels of both G- and GG-Hyl at all glycosylation sites analyzed, and this appeared to affect collagen crosslinking, fibril formation, and matrix mineralization. The glycosylation phenotype seen in Sh clones was rescued when the *Glt25d1* gene was overexpressed in the Sh clones.

Baumann and Hennet have recently reported that inactivation of *GLT25D1* decreased the level of diglycosylation of type I collagen by 42–60% in osteosarcoma cells, SaOS-2 cells, and led to the accumulation of type I collagen in the ER.[32] Though they took a loss-of-function approach similar to that used in the study presented here, there are distinct differences. First, in our study, we quantified the glycosylated (both GG- and G-) and nonglycosylated forms of Hyl per collagen and then quantitatively analyzed the changes in glycosylation pattern at the specific sites of the type I collagen molecule. This is important because the glycosylation pattern varies in a site- and chain-specific manner in type I collagen.[13] Second, because the predominant glycosylation site in type I collagen, α1(I)-87,

is also one of the major helical cross-linking sites,[13] we investigated the impact on collagen cross-linking. Third, we also characterized the effects of GLT25D1 suppression on collagen fibrillogenesis and matrix mineralization using a nontransformed osteoblastic cell line, MC cells.

The data from quantitative analysis of each form of glycosylation clearly indicated that Glt25d1 functions as GT in osteoblastic MC cells. By utilizing UHR-QTOF-MS, seven glycosylation sites were identified in type I collagen synthesized by osteoblastic MC cells, α1–87, α1–99, α1-/α2–174, α1–564, α1–603, and α2–219 (Table S2). Three glycosylation sites, α1–99, α1–564, and α1–603, were newly identified in the MC culture system, though the latter two sites were recently reported in type I collagen from mouse primary osteoblast cultures and bone.[21] Glycosylation of Hyl residue at α2–87 that was previously reported as a very minor glycosylation site[13] was not detected in the study presented here. These minor differences could be due to the cell culture conditions. Among the non-cross-linked peptides in control groups, the highest degree of glycosylation was seen at the helical cross-linking site, α1–87 (~87.0% of total Lys), followed by α2–174 (~76.6% of total Lys). The rest of the residues were glycosylated to a much lesser extent (<12%), especially, in α1–603 (Table S2). The reason why G-Hyl was not detected in Sh collagen by biochemical analysis (Figure 3B,C) is due likely to its very low levels as it was analyzed in the whole protein hydrolysate, not in the specific peptides as in the case of MS analysis. Of the two major glycosylation sites at α1–87 and α2–174, GG-Hyl is the predominant form in the former while G-Hyl is in the latter. This pattern is consistent with our previous report[13] and primary osteoblast cultures[21] as well as with mouse bone tissues.[21] The reason for this distinct glycosylation pattern between these two major glycosylation sites is not clear at present. However, it is notable that there is a significant difference in the distribution of basic and acidic amino acids adjacent to the respective Hyl residues, i.e., in the former PGMK(87)GHR and in the latter VGAK(174)GEA (α1 chain, Uniprot accession number P11087; α2 chain, Uniprot accession number Q01149). Considering the fact that the poly-aspartate sequence is critical for the GGT activity of LH3,[45,46] the basic environment adjacent to the Hyl residue could be important for the functionality of LH3 in MC cells.

The study presented here demonstrates that, though the extent of glycosylation varies at different molecular sites (see above), the relative contents of GG- and G-Hyl were both decreased at all of these sites by 50–75% with a concomitant increase in the level of nonglycosylated (free) Hyl in Sh type I collagen. These results clearly indicate that (1) suppression of GLT25D1 affects all glycosylation sites without significant site preference in type I collagen and (2) because the effect did not show a significant difference in the ratio of GG- to G-, GLT25D1 most likely functions as galactosyltransferase for all of these sites.

Collagen intermolecular cross-linking is initiated by the conversion of telopeptidyl Lys or Hyl residues to the respective aldehydes (Lys^{ald} and Hyl^{ald}, respectively) through the action of LOX/LOXL enzymes. The aldehyde can then condense with the ϵ-amino group of the juxtaposed helical Lys or Hyl on a neighboring molecule to form divalent cross-links that can mature into trivalent cross-links.[2] Though the amounts of cross-links were diminished in Sh collagen due possibly to the impaired LOX/LOXL activity[12] (Figure 1), cross-link maturation, i.e., from di- to trivalent cross-links (DHLNL to Pyr), was significantly

enhanced (Figure 6B and Table S3B). These data indicate a possible role of GLT25D1-mediated galactosylation in collagen cross-link formation and maturation. Previously, we reported that suppression of LH3-catalyzed glucosylation resulted in a decrease in the level of GG-Hyl with a concomitant increase in the level of G-Hyl, whereas the level of nonglycosylated Hyl was unchanged.[12,13] Thus, the effect of glucosylation was investigated in these studies. Our study showed that suppressed Glt25d1 resulted in decreases in the levels of both GG- and G-Hyl with a concomitant increase in the level of free Hyl. Interestingly, in both cases, cross-link maturation from di- to trivalent crosslinks was significantly accelerated. It is, thus, possible that the non- or monoglycosylation form of divalent cross-links does not affect the maturation process but the diglycosylation form does by steric hindrance in the condensation reaction. Further studies such as *in vitro* incubation studies[13,47] using high- resolution MS analysis may provide insights into the fate of diglycosylated divalent cross-links.

Collagen fibrillogenesis can be controlled by many factors, e.g., the presence of minor fibrillar collagens such as type V[48,49] and III collagen,[50] fibril-associated collagens with interrupted triple helices (FACITs),[51] small leucine-rich proteoglycans (SLRPs),[52] and the extent of the post-translational modifications within the major fibrillar collagens.[12,13,53] The study presented here indicates that the decreased level of GLT25D1-mediated galactosylation also affects collagen fibrillogenesis, i.e., diameter and shape. At this point, we cannot conclude that this effect [and that on mineralization (see below)] is exerted directly by the suppressed collagen galactosylation or indirectly by altered interaction with other noncollagenous proteins/SLRPs that controls fibrillogenesis or by other effects such as increased type I collagen retention within the cells[32] that may affect extracellular collagen assembly.

The collagen fibril diameter of Sh clones was significantly larger than that of controls, and the diameter appeared to be inversely correlated to the *Glt25d1* gene and GLT25D1 protein levels. It is possible that the collagen molecules with a low extent of glycosylation could self-assemble faster, forming thicker fibrils because of the lower level of steric hindrance. This is consistent with our previous reports[12,13] indicating that the extent of LH3-catalyzed collagen glucosylation is inversely correlated with collagen fibril growth *in vitro*.

The results presented here also showed that mineralization is significantly affected in Sh clones. This could be due to the abnormal fibrils caused by low glycosylation levels and consequently altered collagen cross-linking, which then could impair collagen mineralization. Those fibrils may not be suitable as a functional template to support mineralization. It is also possible that an interaction between collagen and noncollagenous proteins (e.g., biglycan[28] or decorin[54]) is altered resulting in impaired mineralization. Overglycosylation of collagen in bone disorders, e.g., osteogenesis imperfecta[55,56] and osteoporosis,[57,58] is well-documented; however, the study presented here indicates that underglycosylation of collagen may also affect bone mineralization, which is consistent with our previous report.[13] During the long process of collagen biosynthesis, the subtle changes in collagen glycosylation may result in aberrant consequences by affecting cross-linking, fibrillogenesis, and ultimately mineralization.

In the study presented here, we found that the levels of *Plod1* and −3 gene expression were significantly increased by *Glt2Sdl* suppression while that of *Plod2* was unchanged (Figure 1). The protein level of LH1 (encoded by *Plod1*) was also increased; however, those of LH2 (encoded by *Plod2*) and LH3 (*Plod3*) were found to be decreased (Figure 2). A significant increase in the total level of Lys hydroxylation of Sh collagen (Figure 3) is consistent with higher LH1 activity as helical LH. Upregulation of *Plod3* expression in Sh clones could be viewed as a compensatory activity as LH3 could also function as GT,[59] though this activity of LH3 is very low under normal conditions. ' At this point, it is not clear why the LH3 protein level was decreased in Sh clones. A possible explanation is that, because it forms a complex with GLT25D1 in ER,[60] loss of GLT25D1 protein may lead to destabilization and degradation of LH3. However, the fact that GG-Hyl is, though very low, present at all of the analyzed sites in Sh type I collagen (Table S2) suggests that the activity of LH3 as GGT[12,14] remained in Sh clones. For the decrease of LH2, though its interaction with GLT25D1 is not known, a similar process may occur because it has also several binding partners within the ER,[19,61,62] some of which may interact with GLT25D1. However, the activity of LH2 as telopeptidyl LH[63,64] appeared to be maintained in Sh clones as the LH2-mediated cross-links (DHLNL and Pyr) were still formed in Sh collagen (Figure 6).

In conclusion, our loss-of-function study showed that the main function of GLT25D1 in osteoblasts is to attach galactose to Hyl residues in type I collagen. The suppression of *Glt2Sd1* resulted in significant decreases in the level of collagen glycosylation at all glycosylation sites analyzed that led to alterations in the formation and maturation of cross-links, collagen fibrillogenesis, and mineralization. These findings indicate the critical role of this modification in the formation and function of fibrillar collagens in bone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

This work was supported in part by National Institutes of Health Grants NIAMS R21AR060978 and R01HL042630.

ABBREVIATIONS

Lys	lysine
Hyl	hydroxylysine
ER	endoplasmic reticulum
LH	lysyl hydroxylase
TEM	transmission electron microscopy
PBS	phosphate-buffered saline

G-	galactosyl-
GG-	glucosylgalactosyl-
Pro	proline
Hyp	hydroxyproline
Lox	lysyl oxidase
Plod	procollagen-lysine, 2-oxoglutarate 5-dioxygenase
Loxl	lysyl oxidase-like
MC	MC3T3-E1
GLT25D1	glycosyltransferase 25 domain 1
GLT25D2	glycosyltransferase 25 domain 2
Sh	short hairpin
EV	empty vector
GT	hydroxylysyl galactosyltransferase
GGT	galactosylhydroxylysyl glucosyltransferase
SI-collagen	stable isotope-labeled collagen
QqQ	triple quadrupole
UHR	ultrahigh resolution
QTOF	quadrupole time-of-flight
HPLC	high-performance liquid chromatography
EICs	extracted ion chromatograms
DHLNL	dihydroxylysinonor-leucine
HLNL	hydroxylysinonorleucine
Pyr	pyridinoline
d-Pyr	deoxy-pyridinoline
ald	aldehyde

REFERENCES

(1). Ricard-Blum S (2011) The collagen family. Cold Spring Harbor Perspect. Biol 3, a004978.

(2). Yamauchi M, and Sricholpech M (2012) Lysine post-translational modifications of collagen. Essays Biochem. 52, 113–133. [PubMed: 22708567]

(3). Mercer DK, Nicol PF, Kimbembe C, and Robins SP (2003) Identification, expression, and tissue distribution of the three rat lysyl hydroxylase isoforms. Biochem. Biophys. Res. Commun 307, 803–809. [PubMed: 12878181]

(4). Valtavaara M, Papponen H, Pirttila AM, Hiltunen K, Helander H, and Myllyla R (1997) Cloning and Characterization of a Novel Human Lysyl Hydroxylase Isoform Highly Expressed in Pancreas and Muscle. J. Biol. Chem 272, 6831–6834. [PubMed: 9054364]

(5). Valtavaara M, Szpirer C, Szpirer J, and Myllyla R (1998) Primary Structure, Tissue Distribution, and Chromosomal Localization of a Novel Isoform of Lysyl Hydroxylase (Lysyl Hydroxylase 3). J. Biol. Chem 273, 12881–12886. [PubMed: 9582318]

(6). Kivirikko KI, and Myllylä R (1982) Posttranslational enzymes in the biosynthesis of collagen: intracellular enzymes. Methods Enzymol. 82, 245–304. [PubMed: 6210830]

(7). Uzawa K, Grzesik W, Nishiura T, Kuznetsov S, Robey P, Brenner D, and Yamauchi M (1999) Differential expression of human lysyl hydroxylase genes, lysine hydroxylation, and cross-linking of type I collagen during osteoblastic differentiation in vitro. J. Bone Miner. Res 14, 1272–1280. [PubMed: 10457259]

(8). Taga Y, Kusubata M, Ogawa-Goto K, and Hattori S (2014) Stable isotope-labeled collagen: a novel and versatile tool for quantitative collagen analyses using mass spectrometry. J. Proteome Res 13, 3671–3678. [PubMed: 24953783]

(9). Banushi B, Forneris F, Straatman-Iwanowska A, Strange A, Lyne AM, Rogerson C, Burden JJ, Heywood WE, Hanley J, Doykov I, Straatman KR, Smith H, Bem D, Kriston-Vizi J, Ariceta G, Risteli M, Wang C, Ardill RE, Zaniew M, Latka-Grot J, Waddington SN, Howe SJ, Ferraro F, Gjinovci A, Lawrence S, Marsh M, Girolami M, Bozec L, Mills K, and Gissen P (2016) Regulation of post-Golgi LH3 trafficking is essential for collagen homeostasis. Nat. Commun 7, 12111. [PubMed: 27435297]

(10). Heikkinen J, Risteli M, Wang C, Latvala J, Rossi M, Valtavaara M, and Myllyla R (2000) Lysyl hydroxylase 3 is a multifunctional protein possessing collagen glucosyltransferase activity. J. Biol. Chem 275, 36158–36163. [PubMed: 10934207]

(11). Wang C, Luosujarvi H, Heikkinen J, Risteli M, Uitto L, and Myllyla R (2002) The third activity for lysyl hydroxylase 3: galactosylation of hydroxylysyl residues in collagens in vitro. Matrix Biol. 21, 559–566. [PubMed: 12475640]

(12). Sricholpech M, Perdivara I, Nagaoka H, Yokoyama M, Tomer KB, and Yamauchi M (2011) Lysyl hydroxylase 3 glucosylates galactosylhydroxylysine residues in type I collagen in osteoblast culture. J. Biol. Chem 286, 8846–8856. [PubMed: 21220425]

(13). Sricholpech M, Perdivara I, Yokoyama M, Nagaoka H, Terajima M, Tomer KB, and Yamauchi M (2012) Lysyl hydroxylase 3-mediated glucosylation in type I collagen: molecular loci and biological significance. J. Biol. Chem 287, 22998–23009. [PubMed: 22573318]

(14). Schegg B, Hulsmeier AJ, Rutschmann C, Maag C, and Hennet T (2009) Core glycosylation of collagen is initiated by two beta(1-O)galactosyltransferases. Mol. Cell. Biol 29, 943–952. [PubMed: 19075007]

(15). Wang D, Christensen K, Chawla K, Xiao G, Krebsbach P, and Franceschi R (1999) Isolation and Characterization of MC3T3-E1 Preosteoblast Subclones with Distinct In Vitro and In Vivo Differentiation/Mineralization Potential. J. Bone Miner. Res 14, 893–903. [PubMed: 10352097]

(16). Livak KJ, and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408. [PubMed: 11846609]

(17). Terajima M, Taga Y, Cabral WA, Liu Y, Nagasawa M, Sumida N, Kayashima Y, Chandrasekaran P, Han L, Maeda N, Perdivara I, Hattori S, Marini JC, and Yamauchi M (2019) Cyclophilin B control of lysine post-translational modifications of skin type I collagen. PLoS Genet. 15, No e1008196. [PubMed: 31173582]

(18). Taga Y, Kusubata M, Ogawa-Goto K, Hattori S, and Funato N (2018) Collagen-derived X-Hyp-Gly-type tripeptides promote differentiation of MC3T3-E1 pre-osteoblasts. J. Funct. Foods 46, 456–462.

(19). Terajima M, Taga Y, Chen Y, Cabral WA, Hou-Fu G, Srisawasdi S, Nagasawa M, Sumida N, Hattori S, Kurie JM, Marini JC, and Yamauchi M (2016) Cyclophilin-B modulates collagen

cross-linking by differentially affecting lysine hydroxylation in the helical and telopeptidyl domains of tendon type I collagen. J. Biol. Chem 291, 9501–9512. [PubMed: 26934917]

(20). Fujii KK, Taga Y, Sakai T, Ito S, Hattori S, Nagata K, and Koide T (2019) Lowering the culture temperature corrects collagen abnormalities caused by HSP47 gene knockout. Sci. Rep 9, 17433. [PubMed: 31758055]

(21). Cabral WA, Perdivara I, Weis M, Terajima M, Blissett AR, Chang W, Perosky JE, Makareeva EN, Mertz EL, Leikin S, Tomer KB, Kozloff KM, Eyre DR, Yamauchi M, and Marini JC (2014) Abnormal type I collagen post-translational modification and crosslinking in a cyclophilin B KO mouse model of recessive osteogenesis imperfecta. PLoS Genet. 10, No. e1004465. [PubMed: 24968150]

(22). Yamauchi M, Katz EP, and Mechanic GL (1986) Intermolecular cross-linking and stereospecific molecular packing in type I collagen fibrils of the periodontal ligament. Biochemistry 25, 4907–4913. [PubMed: 3768322]

(23). Yamauchi M, and Shiiba M (2008) Lysine hydroxylationand cross-linking of collagen. Methods Mol. Biol 446, 95–108. [PubMed: 18373252]

(24). Yamauchi M, and Katz E (1993) The post-translational chemistry and molecular packing of mineralizing tendon collagens. Connect. Tissue Res 29, 81–98. [PubMed: 8403898]

(25). Eyre D (1987) Collagen cross-linking amino acids In Methods in Enzymology (Colowick SP, and Kaplan NO., Eds.) pp 115–139, Academic Press, Inc., Orlando, FL.

(26). Reynolds E (1963) The use of lead citrate at high pH as an electronopaque stain in electron microscopy. J. Cell Biol 17, 208–212. [PubMed: 13986422]

(27). Sricholpech M, Perdivara I, Yokoyama M, Nagaoka H, Terajima M, Tomer KB, and Yamauchi M (2012) Lysyl Hydroxylase 3-mediated Glucosylation in Type I Collagen: MOLECULAR LOCI AND BIOLOGICAL SIGNIFICANCE. J. Biol. Chem 287, 22998–23009. [PubMed: 22573318]

(28). Parisuthiman D, Mochida Y, Duarte WR, and Yamauchi M (2005) Biglycan modulates osteoblast differentiation and matrix mineralization. J. Bone Miner. Res 20, 1878–1886. [PubMed: 16160746]

(29). Gregory CA, Gunn WG, Peister A, and Prockop DJ (2004) An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction. Anal. Biochem 329, 77–84. [PubMed: 15136169]

(30). Hata R, Hori H, Nagai Y, Tanaka S, Kondo M, Hiramatsu M, Utsumi N, and Kumegawa M (1984) Selective inhibition of type I collagen synthesis in osteoblastic cells by epidermal growth factor. Endocrinology 115, 867–876. [PubMed: 6086289]

(31). Terajima M, Perdivara I, Sricholpech M, Deguchi Y, Pleshko N, Tomer KB, and Yamauchi M (2014) Glycosylation and cross-linking in bone type I collagen. J. Biol. Chem 289, 22636–22647. [PubMed: 24958722]

(32). Baumann S, and Hennet T (2016) Collagen Accumulation in Osteosarcoma Cells lacking GLT25D1 Collagen Galactosyltransferase. J. Biol. Chem 291, 18514–18524. [PubMed: 27402836]

(33). Amudeswari S, Liang JN, and Chakrabarti B (1987) Polar-Apolar Characteristics and Fibrillogenesis of Glycosylated Collagen. Collagen Relat. Res 7, 215–223.

(34). Torre-Blanco A, Adachi E, Hojim Y, Wootton JAM, Minor RR, and Prockop DJ (1992) Temperature-induced Post-translational Over-modification of Type I Procollagen. J. Biol. Chem 267, 2650–2655. [PubMed: 1733961]

(35). Yang LC, Rui H, Mosler S, Notbohm H, Sawaryn A, and Müller KP (1993) Collagen II from articular cartilage and annulus fibrosus Structural and functional implication of tissue specific posttranslational modifications of collagen molecules. Eur. J. Biochem 213, 1297–1302. [PubMed: 8504821]

(36). Notbohm H, Nokelainen M, Myllyharju J, Fietzek PP, Müller PK, and Kivirikko KI (1999) Recombinant Human Type II Collagens with Low and High Levels of Hydroxylysine and Its Glycosylated Forms Show Marked Differences in Fibrillogenesis in Vitro. J. Biol. Chem 274, 8988–8992. [PubMed: 10085145]

(37). Bätge B, Winter C, Notbohm H, Acil Y, Brinckmann J, and Müller PK (1997) Glycosylation of Human Bone Collagen I in Relation to Lysylhydroxylation and Fibril Diameter. J. Biochem 122, 109–115. [PubMed: 9276678]

(38). Eyre DR, and Glimcher MJ (1973) Analysis of a crosslinked peptide from calf bone collagen: evidence that hydroxylysyl glycoside participates in the crosslink. Biochem. Biophys. Res. Commun 52, 663–671. [PubMed: 4351144]

(39). Hanson AD, and Eyre DR (1996) Molecular Site Specificity of Pyridinoline and Pyrrole Cross-links in Type I Collagen of Human Bone. J. Biol. Chem 271, 26508–26516. [PubMed: 8900119]

(40). Robins SP (1983) Cross-linking of collagen. Isolation, structural characterization and glycosylation of pyridinoline. Biochem. J 215, 167–173. [PubMed: 6626172]

(41). Yamauchi M, Noyes C, Kuboki Y, and Mechanic GL (1982) Collagen structural microheterogeneity and a possible role for glycosylated hydroxylysine in type I collagen. Proc. Natl. Acad. Sci. U. S. A 79, 7684–7688. [PubMed: 6961443]

(42). Vogel W, Gish DG, Alves F, and Pawson T (1997) The Discoidin Domain Receptor Tyrosine Kinases Are Activated by Collagen. Mol. Cell 1, 13–23. [PubMed: 9659899]

(43). Jurgensen HJ, Madsen DH, Ingvarsen S, Melander MC, Gardsvoll H, Patthy L, Engelholm LH, and Behrendt N (2011) A novel functional role of collagen glycosylation: interaction with the endocytic collagen receptor uparap/ENDO180. J. Biol. Chem 286, 32736–32748. [PubMed: 21768090]

(44). Palmieri D, Valli M, Viglio S, Ferrari N, Ledda B, Volta C , and Manduca P (2010) Osteoblasts extracellular matrix induces vessel like structures through glycosylated collagen I. Exp. Cell Res 316, 789–799. [PubMed: 20006603]

(45). Wang C, Risteli M, Heikkinen J, Hussa AK, Uitto L, and Myllyla R (2002) Identification of amino acids important for the catalytic activity of the collagen glucosyltransferase associated with the multifunctional lysyl hydroxylase 3 (LH3). J. Biol. Chem 277, 18568–18573. [PubMed: 11896059]

(46). Scietti L, Chiapparino A, De Giorgi F, Fumagalli M, Khoriauli L, Nergadze S, Basu S, Olieric V, Cucca L, Banushi B, Profumo A, Giulotto E, Gissen P, and Forneris F (2018) Molecular architecture of the multifunctional collagen lysyl hydroxylase and glycosyltransferase LH3. Nat. Commun 9, 3163. [PubMed: 30089812]

(47). Veis A (1981) The Chemistry and Biology of Mineralized Connective Tissues, Developments in Biochemistry, Vol. 22, Elsevier Noth Holland.

(48). Birk D, Fitch J, Babiarz J, Doane K, and Linsenmayer T (1990) Collagen fibrillogenesis in vitro: Interaction of type I and V collagen regulates fibril diameter. J. Cell Sci 95, 649–657. [PubMed: 2384532]

(49). Doane K, Babiarz J, Fitch J, Linsenmayer T, and Birk D (1992) Collagen fibril assembly by corneal fibroblasts in three-dimensional collagen gel cultures: Small-diameter heterotypic fibrils are deposited in the absence of keratin sulfate proteoglycan. Exp. Cell Res 202, 113–124. [PubMed: 1511726]

(50). Romanic A, Adachi E, Kadler K, Hojima Y, and Prockop D (1991) Copolymerization of pNcollagen III and collagen I. pNcollagen III decreases the rate of incorporation of collagen I into fibrils, the amount of collagen I incorporated, and the diameter of the fibrils formed. J. Biol. Chem 266, 12703–12709. [PubMed: 2061335]

(51). Shaw L, and Olsen B (1991) FACIT collagens: Diverse molecular bridges in extracellular matrices. Trends Biochem. Sci 16, 191–194. [PubMed: 1882421]

(52). Hoshi K, Kemmotsu S, Takeuchi Y, Amizuka N, and Ozawa H (1999) The primary calcification in bones follows removal of decorin and fusion of collagen fibrils. J. Bone Miner. Res 14, 273–280. [PubMed: 9933482]

(53). Weis MA, Hudson DM, Kim L, Scott M, Wu JJ, and Eyre DR (2010) Location of 3-hydroxyproline residues in collagen types I, II, III, and V/XI implies a role in fibril supramolecular assembly. J. Biol. Chem 285, 2580–2590. [PubMed: 19940144]

(54). Mochida Y, Parisuthiman D, Pornprasertsuk-Damrongsri S, Atsawasuwan P, Sricholpech M, Boskey AL, and Yamauchi M (2009) Decorin modulates collagen matrix assembly and mineralization. Matrix Biol. 28, 44–52. [PubMed: 19049867]

(55). Cabral WA, Chang W, Barnes AM, Weis M, Scott MA, Leikin S, Makareeva E, Kuznetsova NV, Rosenbaum KN, Tifft CJ, Bulas DI, Kozma C, Smith PA, Eyre DR, and Marini JC (2007) Prolyl 3-hydroxylase 1 deficiency causes a recessive metabolic bone disorder resembling lethal/severe osteogenesis imperfecta. Nat. Genet 39, 359–365. [PubMed: 17277775]

(56). Taga Y, Kusubata M, Ogawa-Goto K, and Hattori S (2013) Site-specific quantitative analysis of overglycosylation of collagen in osteogenesis imperfecta using hydrazide chemistry and SILAC. J. Proteome Res 12, 2225–2232. [PubMed: 23581850]

(57). Moro L, Bettica P, Romanello M, and Noris Suarez K (1997) 17 β-Estradiol and tamoxifen prevent the overglycosylation of rat trabecular bone collagen induced by ovariectomy. Clin. Chem. Lab. Med 35, 29–33.

(58). Bailey AJ, and Knott L (1999) Molecular changes in bone collagen in osteoporosis and osteoarthritis in the elderly. Exp. Gerontol 34, 337–351. [PubMed: 10433388]

(59). Myllylä R, Wang C, Heikkinen J, Juffer A, Lampela O, Risteli M, Ruotsalainen H, Salo A, and Sipilä L (2007) Expanding the Lysyl Hydroxylase Toolbox: New Insights Into the Localization and Activities of Lysyl Hydroxylase 3 (LH3). J. Cell. Physiol 212, 323–329. [PubMed: 17516569]

(60). Liefhebber JM, Punt S, Spaan WJ, and van Leeuwen HC (2010) The human collagen beta(1-O)galactosyltransferase, GLT25D1, is a soluble endoplasmic reticulum localized protein. BMC Cell Biol. 11, 33. [PubMed: 20470363]

(61). Gjaltema RA, van der Stoel MM, Boersema M, and Bank RA (2016) Disentangling mechanisms involved in collagen pyridinoline cross-linking: The immunophilin FKBP65 is critical for dimerization of lysyl hydroxylase 2. Proc. Natl. Acad. Sci. U. S. A 113, 7142–7147. [PubMed: 27298363]

(62). Duran I, Martin JH, Weis MA, Krejci P, Konik P, Li B, Alanay Y, Lietman C, Lee B, Eyre D, Cohn DH, and Krakow D (2017) A Chaperone Complex Formed by HSP47, FKBP65, and BiP Modulates Telopeptide Lysyl Hydroxylation of Type I Procollagen. J. Bone Miner. Res 32, 1309–1319. [PubMed: 28177155]

(63). van der Slot AJ, Zuurmond AM, Bardoel AF, Wijmenga C, Pruijs HE, Sillence DO, Brinckmann J, Abraham DJ, Black CM, Verzijl N, DeGroot J, Hanemaaijer R, TeKoppele JM, Huizinga TW, and Bank RA (2003) Identification of PLOD2 as telopeptide lysyl hydroxylase, an important enzyme in fibrosis. J. Biol. Chem 278, 40967–40972. [PubMed: 12881513]

(64). Pornprasertsuk S, Duarte WR, Mochida Y, and Yamauchi M (2004) Lysyl hydroxylase-2b directs collagen cross-linking pathways in MC3T3-E1 cells. J. Bone Miner. Res 19, 1349–1355. [PubMed: 15231023]

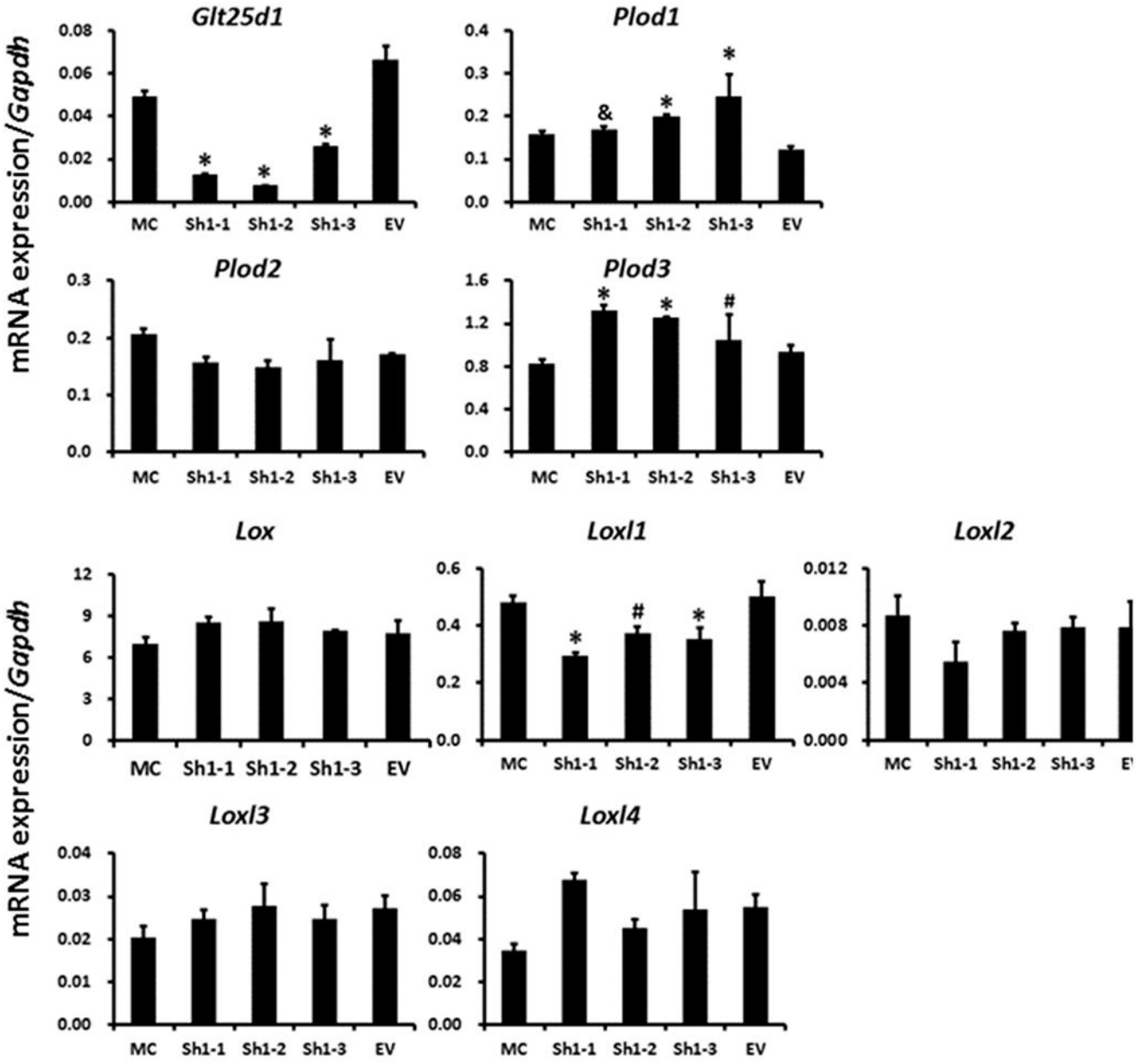


Figure 1.

Gene expression of *Glt25d1*, *Plod1–3,* and *Lox/Loxl1–4* in Sh1 single-cell-derived clones and controls. Following 48 h of culture, real time PCR was performed and their mRNA levels relative to the internal control (*Gapdh*) were calculated. Values represent means ± SD from three independent experiments. $^{*}p < 0.05$ compared to MC and EV. $^{\#}p < 0.05$ compared to MC. $^{\&}p < 0.05$ compared to EV. Abbreviations: *Lox*, lysyl oxidase; *Glt25d1*, glycosyltransferase 25 domain 1; Sh, short hairpin; *Plod*, procollagen-lysine, 2-oxoglutarate 5-dioxygenase; *Loxl*, Lox-like.

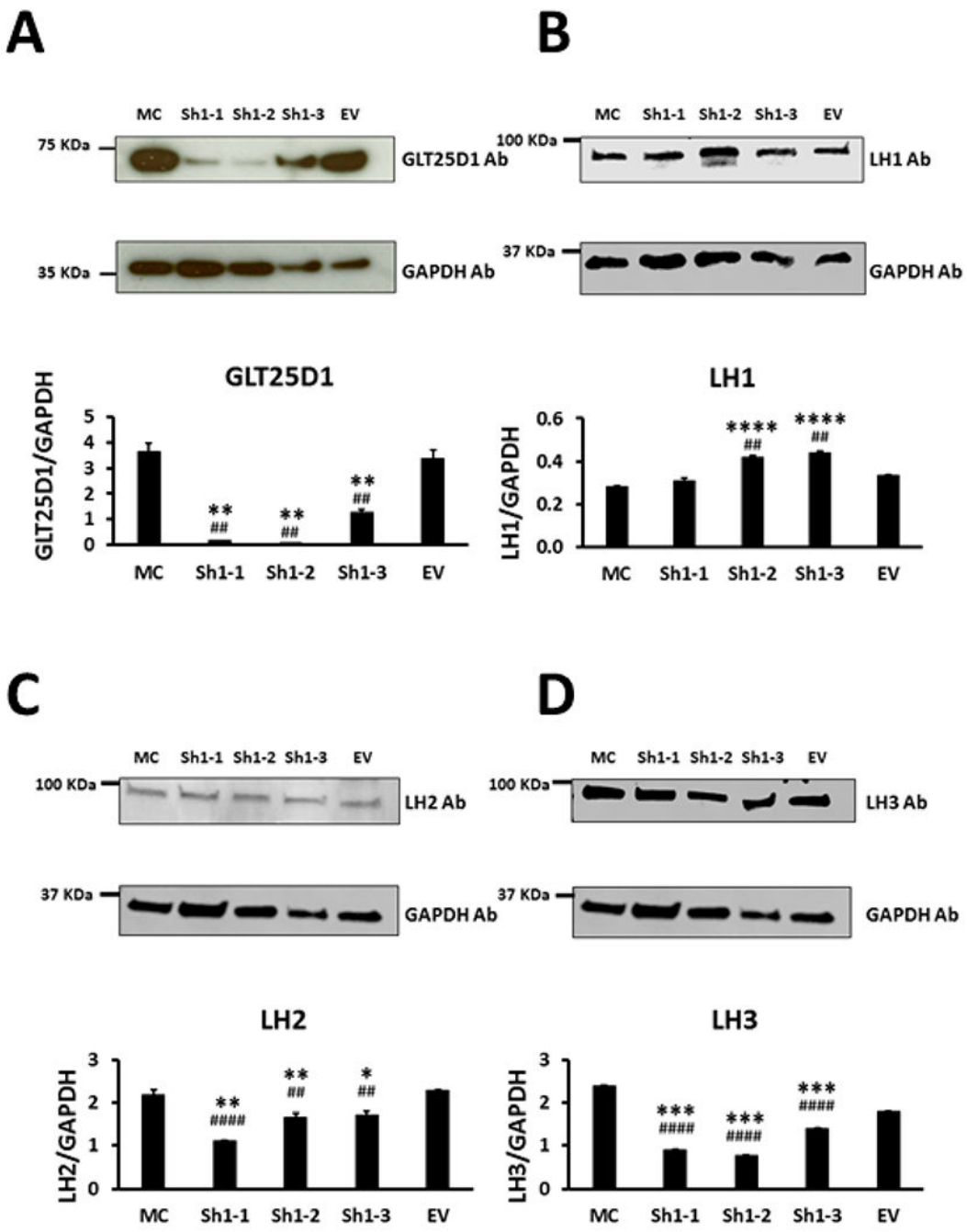


Figure 2.

GLT25D1 and LH1–3 protein levels in Sh1 single-cell-derived clones and controls. Western blot analyses were performed with respective antibodies and the anti-GAPDH antibody. Seven micrograms of protein per group was loaded, and the protein level was calculated by the immunoreactivity relative to GAPDH. (A) GLT25D1, (B) LH1, (C) LH2, and (D) LH3. Abbreviations: GLT25D1, glycosyltransferase 25 domain 1; LH, lysyl hydroxylase; Sh, short hairpin; Ab, antibody. Compared to MC, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.Compared to EV, $^{\#\#}p < 0.01$ and $^{\#\#\#\#}p < 0.0001$.

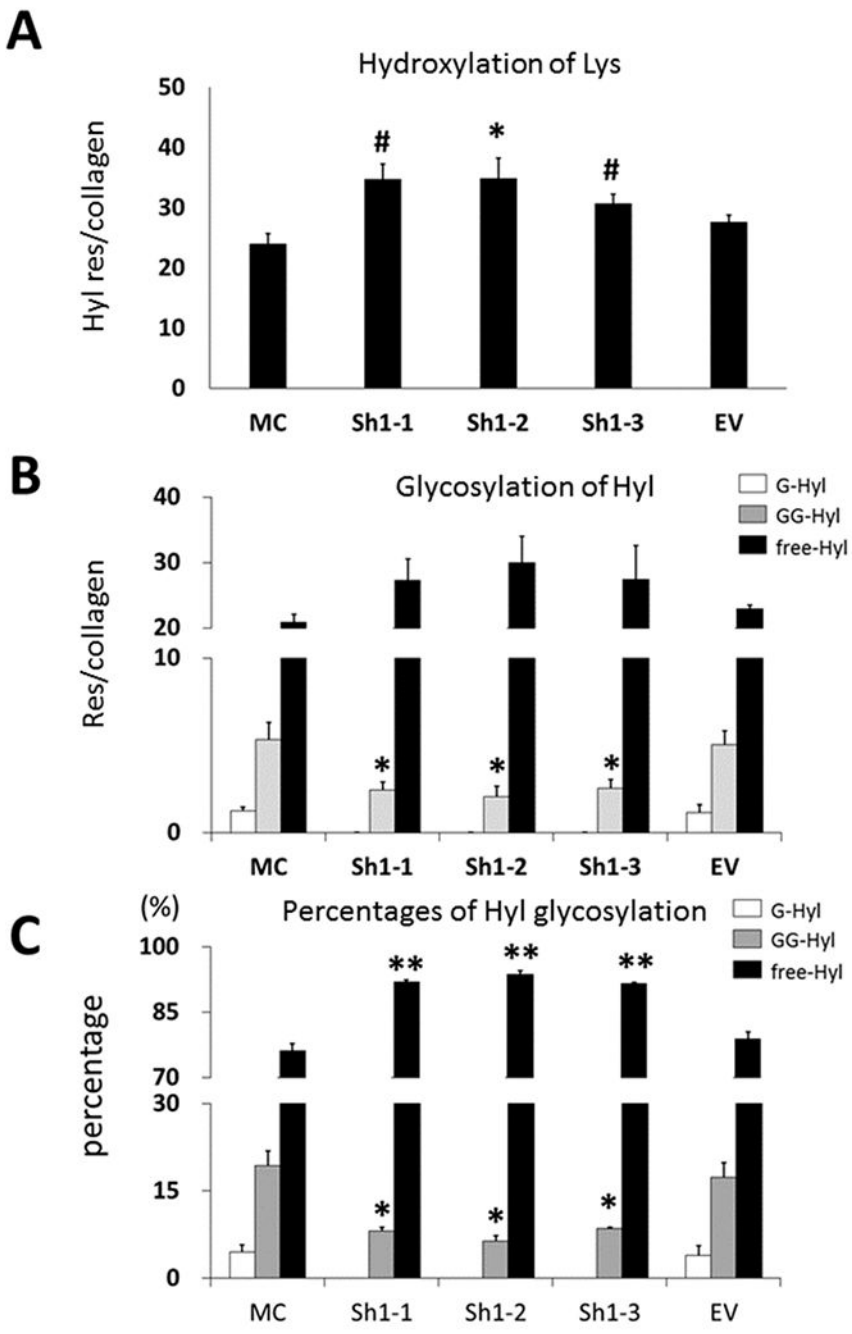


Figure 3.

Lysine hydroxylation and hydroxylysine glycosylation of collagen from Sh clones and controls. (A) Lys hydroxylation is shown as the number of residues of Hyl per collagen molecule. (B) Glycosylation of Hyl shown as the number of residues per collagen. (C) Relative abundance of Hyl glycosylation shown as percentages (G- + GG- + free-Hyl = 100%). Values represent means ± SD from three independent experiments. *Significantly different ($p < 0.05$) compared to MC and EV. **Significantly different ($p < 0.01$) compared

to MC and EV. Abbreviations: Lys, lysine; Hyl, hydroxylysine; res., residues; GG-, glucosylgalactosyl-; G-, galactosyl-; Sh, short hairpin.

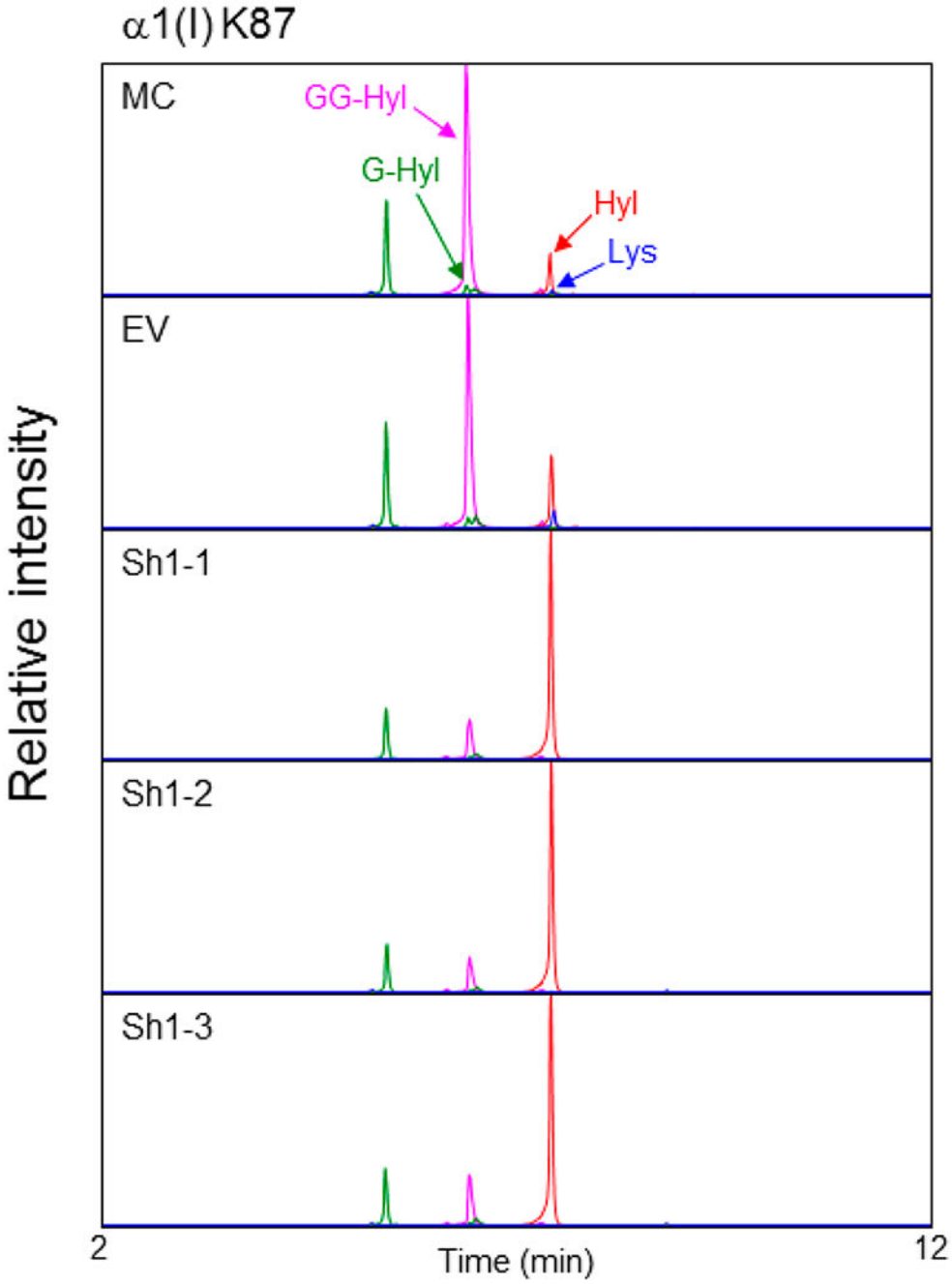


Figure 4.

Extracted ion chromatograms (EICs) of tryptic peptides that contained glycosylation sites in type I collagen isolated from MC, EV, and Sh clones. Monoisotopic EICs of Lys-containing (*m/z* 565.7901 ± 0.02; *z* = 2) and Hyl-containing (*m/z* 573.7876 ± 0.02; *z* = 2) peptides were extracted for α1(I) [76–87] GLOGTAGLOGMK (O indicates 4-Hyp). In addition, monoisotopic EICs of G-Hylcontaining (*m/z* 553.6030 ± 0.02; *z* = 3) and GG-Hyl-containing (*m/z* 607.6207 ± 0.02; *z* = 3) peptides were extracted for α1(I) [76–90]

GLOGTAGLOGMKGHR. Abbreviations: Lys, lysine; Hyl, hydroxylysine; GG-, glucosylgalactosyl-; G-, galactosyl-; Sh, short hairpin.

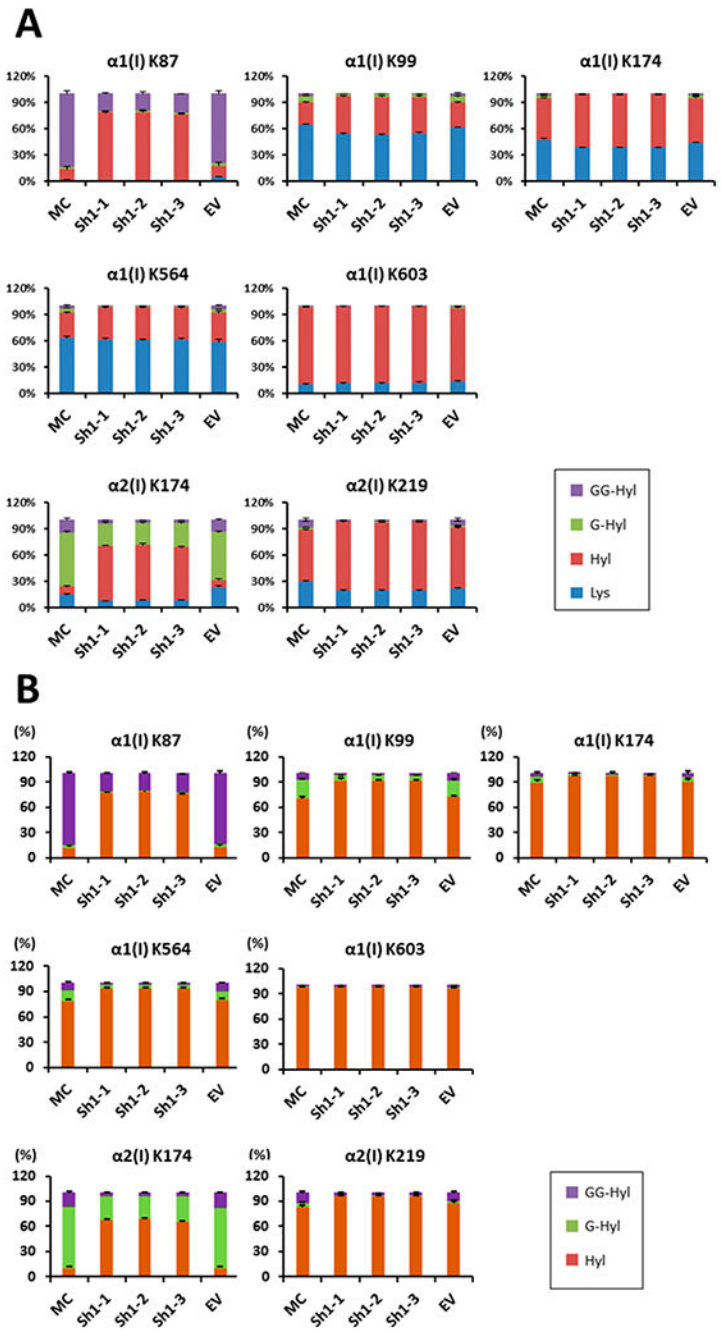


Figure 5.

Site-specific Lys modifications in type I collagen determined by mass spectrometry. (A) Lys + Hyl + G-Hyl + GG-Hyl = 100%. (B) Glycosylation of Hyl (Hyl + G-Hyl + GG-Hyl = 100%). Abbreviations: Lys, lysine; Hyl, hydroxylysine; GG-, glucosylgalactosyl-; G-, galactosyl-; Sh, short hairpin.

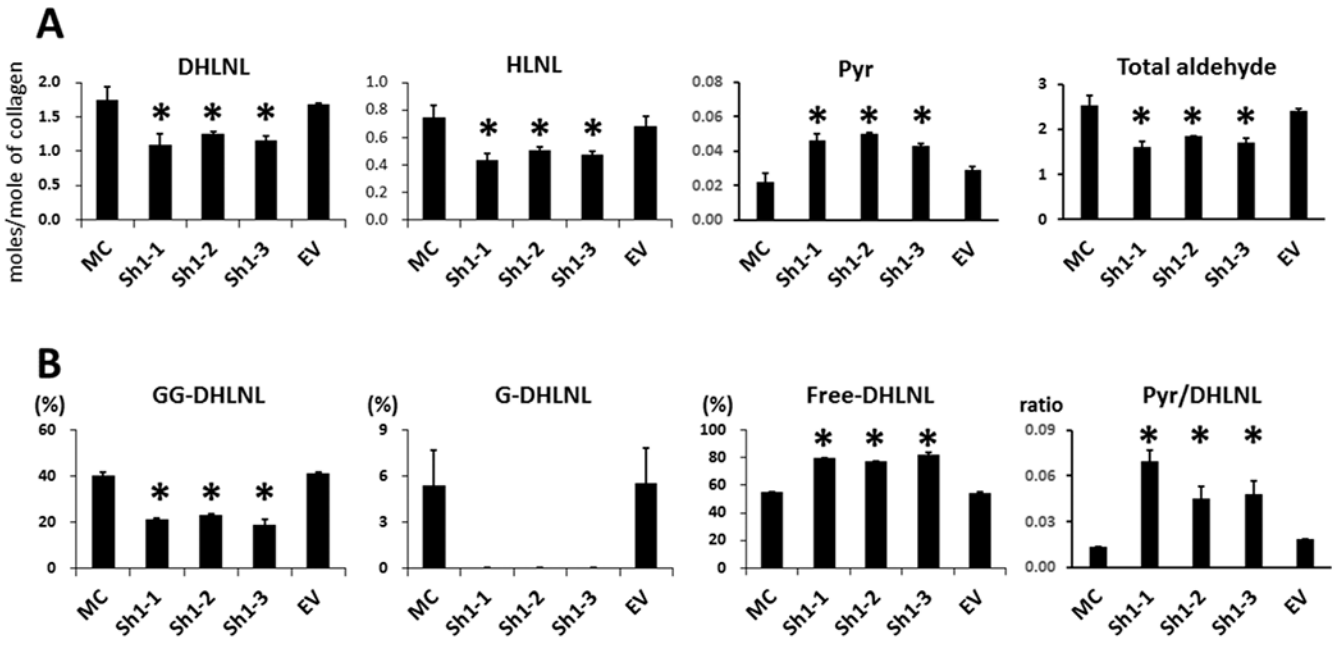


Figure 6.

Collagen cross-links and cross-link glycosylation. (A) Collagen cross-links. Values (moles per mole of collagen) represent means ± SD from three independent experiments. DHLNL (moles per mole of collagen) including GG- + G- + free-DHLNL, total aldehydes = total DHLNL + HLNL + 2 × Pyr. (B) Glycosylation and maturation of the DHLNL cross-link. Values represent means ± SD from three independent experiments. DHLNL (100%) = GG-(%) + G- (%) + free DHLNL (%). Abbreviations: DHLNL, dihydroxylysinonorleucine; HLNL, hydroxylysinonorleucine; Pyr, pyridinoline; GG-, glucosylgalactosyl-; G-, galactosyl-; Sh, short hairpin. * Significantly different ($p < 0.05$) compared to MC and EV.

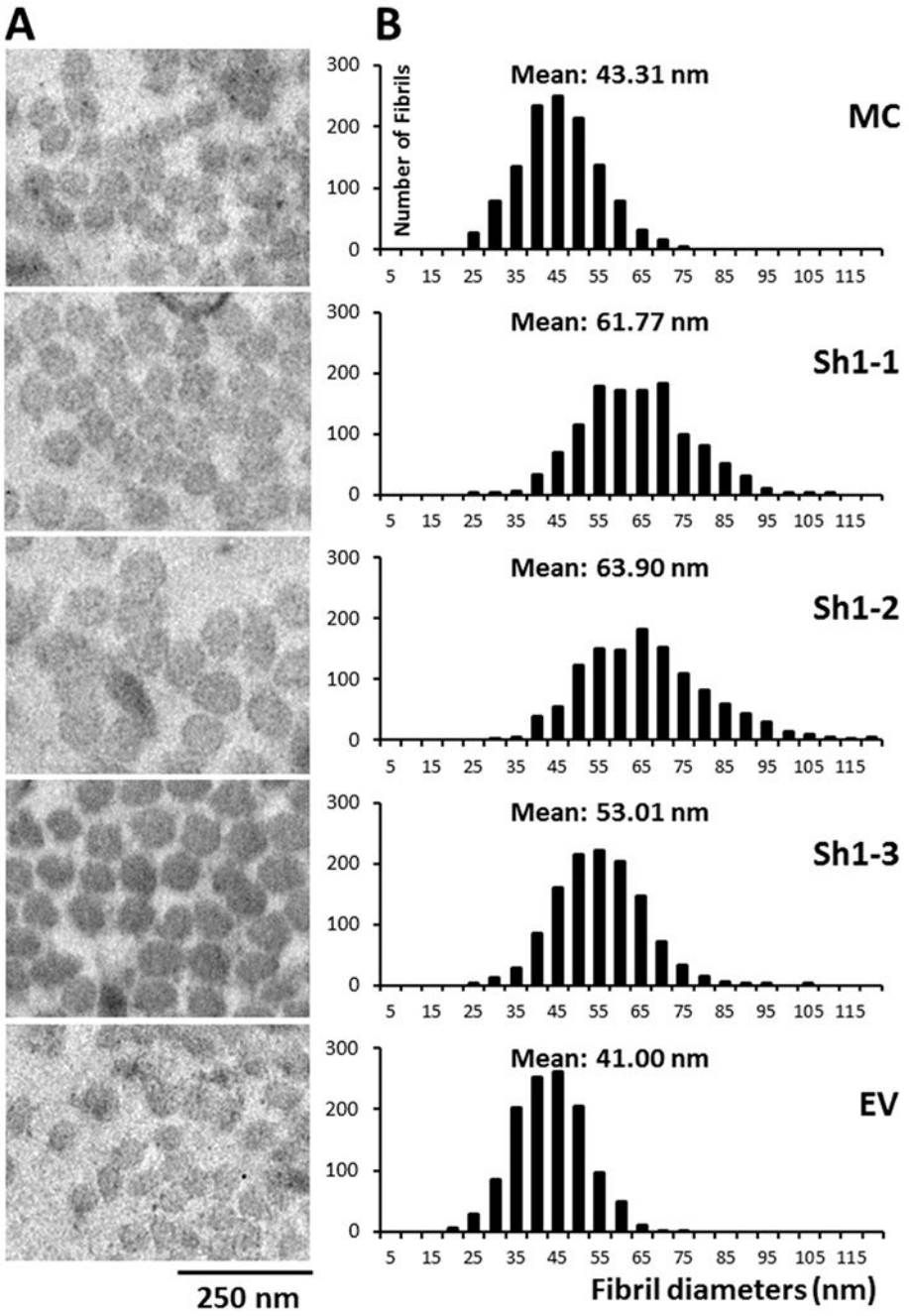


Figure 7.

Collagen fibrils in controls (MC and EV) and Sh clones. After 2 weeks of culture, cells and/or matrices were processed and observed under a transmission electron microscope. (A) Collagen fibrils at 25000× magnification. (B) Collagen fibril diameters were measured from 1200 fibrils per group, and the diameter distribution was plotted.

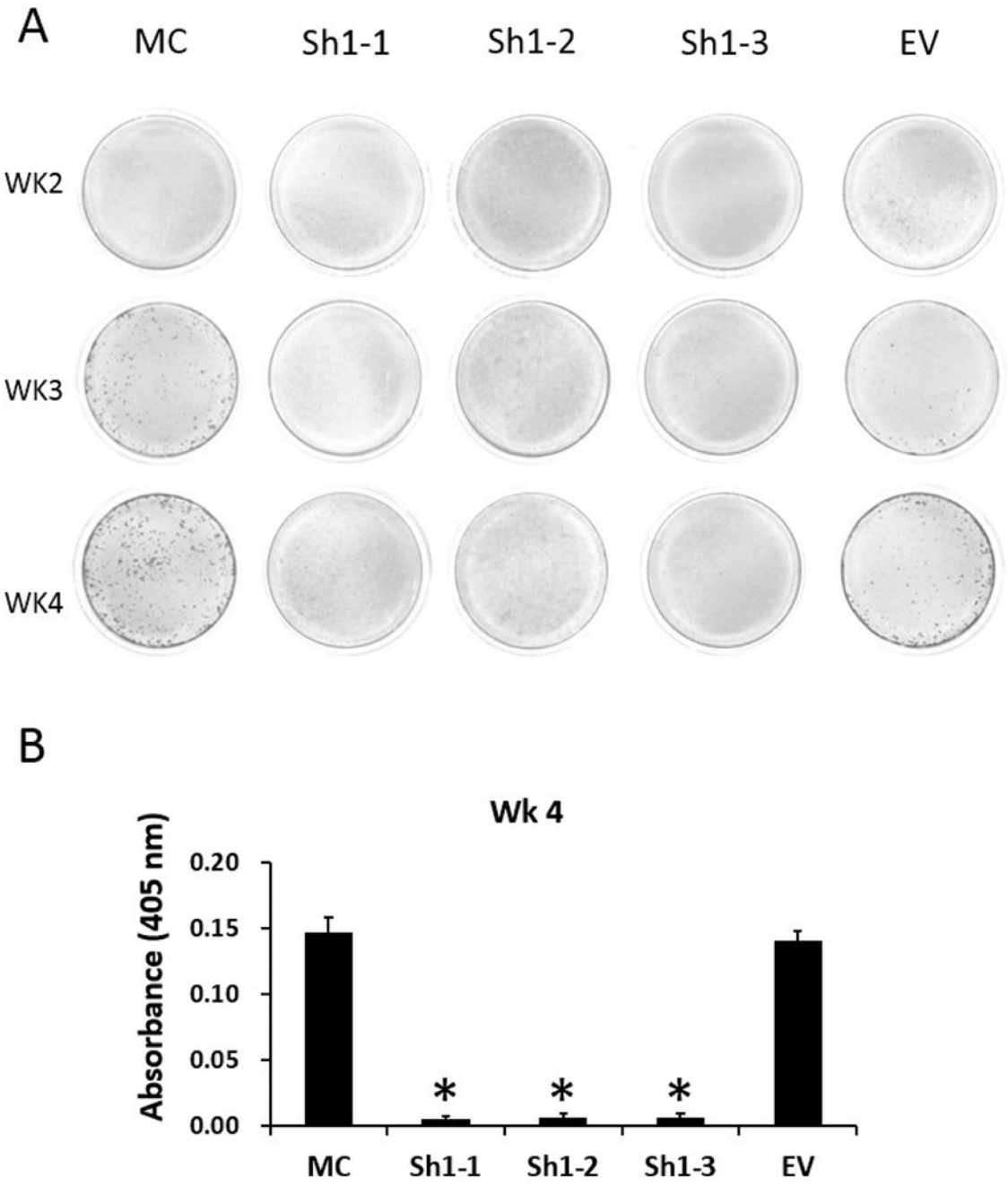


Figure 8.

In vitro mineralization. (A) Alizarin Red S staining of cells and/or matrices from controls (MC and EV), and Sh clones at weeks 2–4. (B) Quantification of Alizarin Red S contents at week 4. Error bars show the SD from triplicate measurements. *Significantly different ($p < 0.01$) compared to MC and EV. Abbreviations: Wk, week; Sh, short hairpin.