

## Effects of fish collagen peptides on collagen post-translational modifications and mineralization in an osteoblastic cell culture system

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Collagen is one of the most widely used biomaterials for tissue engineering and regenerative medicine. Fish collagen peptides (FCP) have been used as a dietary supplement, but their effects on the cellular function are still poorly understood. The objective of this study was to investigate the effects of FCP on collagen synthesis, quality and mineralization using an osteoblastic MC3T3-E1 cell culture system. Cells treated with FCP significantly upregulated the gene expression of several collagen modifying enzymes and more collagen was deposited in the cultures. Collagen in the treated group showed a greater extent of lysine hydroxylation, higher levels of hydroxylysine-aldehyde derived cross-links and accelerated cross-link maturation compared with the untreated group. Furthermore, the treated group showed accelerated matrix mineralization. These results indicate that FCP exerts a positive effect on osteoblastic cells in terms of collagen synthesis, quality and mineralization, thereby suggesting the potential utility of FCP for bone tissue engineering.

**Keywords:** Fish collagen peptides, Collagen post-translational modification, Collagen cross-link, Maturation of collagen, Mineralization

### INTRODUCTION

Collagens are a large family of structurally related extracellular matrix proteins accounting for about 30% of the total proteins in the human body. Collagen has been widely used as a biomaterial, *e.g.* scaffold of cell and growth factors, wound dressing, soft tissue augmentation, dietary supplement, *etc.*<sup>1-4</sup>, due to its biocompatibility, stability and bioactivity. One of the major commercial sources of collagen has been bovine tissues but other species have also been sought as an alternative source of collagen, because of the occurrence of Bovine Spongiform Encephalopathy (BSE). Considerable attention has been drawn to the collagen-rich fish solid waste that constitutes 50–70% of the original raw material for the effective utilization of resources<sup>5</sup>. Fish collagen or collagen peptides derived from the skin, bones, swim bladder and scales has been used as a functional food or dietary supplement<sup>6,7</sup>. However, their biological effects at the cellular and biochemical levels are still not clearly understood. The fibrillar type I collagen functions as a structural organizer of mineralization in bone, and its proper post-translational modifications such as cross-linking pattern are crucial for this function<sup>8-13</sup>. This study used an osteoblastic cell culture system to examine the effects of fish collagen peptides (FCP) on the gene expression of collagen modifying enzymes that play critical roles in covalent intermolecular cross-linking, collagen content, the extent of lysine (Lys) hydroxylation, cross-links and matrix mineralization.

### MATERIALS AND METHODS

#### *Partial characterization of fish collagen peptides*

The FCP powder used in this study was kindly provided by Yaizu Suisankagaku Industry (Shizuoka, Japan). This product has been used as a food additive for food companies and food processing manufacturers, and it is the product of enzymatic digestion of purified skin collagen mainly from Gadiformes and Pleuronectidae by *Bacillus* species-derived proteinase. Aliquots of FCP were subjected to amino acid, SDS-PAGE and mass spectrometric analyses to partially characterize FCP. Approximately 1 mg of FCP powder was hydrolyzed with 6 N HCl and an aliquot of the hydrolysate was subjected to amino acid analysis as described previously<sup>14</sup>. The analysis was performed in duplicate. An aliquot of FCP was also separated by 15–20% SDS-PAGE and then the gel was stained by Coomassie brilliant blue (CBB).

The molecular mass of FCP was evaluated by a Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) mass spectrometer (Voyager DE PRO, Applied Biosystems, CA, USA) in the linear positive ion mode with delayed extraction. The extraction and guide wire voltages were set at 25 kV and 0.15%, respectively. Three profiles containing 300 shots each from the same spot were acquired. Insulin was used as an external standard. Ionization was achieved with a nitrogen laser (377 nm) using sinapinic acid as a matrix.

#### *Preparation of fish collagen peptides solution*

A FCP solution (5% w/v) containing  $\alpha$ -modified essential medium ( $\alpha$ -MEM, Gibco, Carlsbad, CA, USA) was prepared, the pH was adjusted to 7.4 and filtered using

a 0.2  $\mu\text{m}$  filter membrane (Nalge Company, Rochester, NY, USA).

#### Cell culture

The mouse calvaria-derived MC3T3-E1 cells (subclone 4) purchased from American Type Culture Collection (CRL-2593) were used in this study. The cells were maintained in  $\alpha$ -MEM supplemented with 10% fetal bovine serum (FBS, Atlanta, Lawrenceville, GA, USA), 100 U/mL penicillin G sodium and 100  $\mu\text{g}/\text{mL}$  streptomycin sulfate in a humidified atmosphere of 5%  $\text{CO}_2$  at 37°C. The medium was changed every 3 days.

#### Quantitative real-time polymerase chain reaction analysis

MC3T3-E1 cells were seeded in a 6-well plate at a density of  $2 \times 10^5$  cells/well and cultured under the aforementioned conditions until confluent. The medium was then replaced with those containing 0.05, 0.1, 0.2 and 0.5% (w/v) of FCP prepared by the addition of appropriate volumes of 5% FCP solution based on the findings of a previous report<sup>15</sup>. The cells cultured with  $\alpha$ -MEM without FCP served as a control.

Total RNA was isolated after 48 h of culture using Trizol reagent (Invitrogen, Carlsbad, CA, USA)<sup>16</sup> and first-strand cDNA was synthesized using an Omniscript Reverse Transcriptase Kit (Qiagen, Valencia, CA, USA). The mRNA expression levels of type-I collagen  $\alpha 2$  chain (COL1A2) and collagen modifying enzyme genes, *i.e.* lysyl hydroxylase (LH)1–3, lysyl oxidase (LOX), LOX-like protein (LOXL)1–4 and glycosyltransferase 25 domain containing 1 (GLT25D1), were quantitatively analyzed by real-time polymerase chain reaction (PCR). The expression of GLT25D1 in MC3T3-E1 cell line was recently reported<sup>17</sup>. PCR was performed by the ABI Prism 7000 Sequence detection system (Applied Biosystems, Foster City, CA, USA) using the specific primer-probes: COL1A2 (Applied Biosystems, ABI assay number: Mm00483888\_m1), GLT25D1 (Mm00600638\_m1), LH1 (Mm00599925\_m1), LH2 (Mm00478767\_m1), LH3 (Mm00478798\_m1), LOX (Mm00495386\_m1), LOXL1 (Mm01145738\_m1), LOXL2 (Mm00804740\_m1), LOXL3 (Mm00442953\_m1) and LOXL4 (Mm00446385\_m1) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH, ABI assay number: 4308313). The fold changes in gene expression relative to GAPDH were calculated using the values obtained from the control group as a calibrator by using the  $2^{-\Delta\Delta C_T}$  method<sup>18</sup>. All measurements were performed in triplicate and confirmed by three independent experiments. The concentration of FCP for the following experiments was determined based on the gene expression pattern.

#### Cell proliferation study

The cells were seeded in a 24-well plate at a density of  $2 \times 10^4$  cells/well and cultured overnight. The medium was then replaced with that containing 0.2% FCP, the concentration determined by the real-time PCR analysis (see above). The cells were cultured with  $\alpha$ -MEM with or without FCP, and then the cell number was counted at 2, 5, 7, 10 and 14 days, using a hemocytometer under

a phase contrast microscope. All measurements were confirmed by three independent experiments.

#### Amino acid and collagen cross-link analyses

The cells were seeded in a 6-well plate at a density of  $2 \times 10^5$  cells/well and cultured. The cells grew to confluence, and the medium was replaced with that containing 50  $\mu\text{g}/\text{mL}$  of ascorbic acid with or without 0.2% FCP. The cells were cultured for 14 days. The cell/matrix layers were scraped, washed with phosphate buffered saline (PBS) and deionized distilled water, and lyophilized. Aliquots of dried samples (about 2 mg) were reduced with standardized  $\text{NaB}^3\text{H}_4$  as reported previously<sup>19</sup>. After flushing with  $\text{N}_2$ , the reduced samples were hydrolyzed with 6N HCl *in vacuo* at 110°C for 22 h. The hydrolysates were then dried by a speed vacuum concentrator (Savant, Phoenix, AR, USA), dissolved in distilled water and filtered using a 0.22  $\mu\text{m}$  membrane. An aliquot of each hydrolysate was then subjected to amino acid and cross-link analyses on a Varian high-performance liquid chromatography (HPLC) system as described previously<sup>14</sup>. Collagen derived from the MC3T3-E1 cells was calculated as  $\mu\text{g}$  of collagen/mg of dried cell matrix based on a value of 300 residues of Hyp per collagen and the molecular weight of collagen. The extent of Lys hydroxylation (Hyl) was calculated as moles of Hyl/mole of collagen based on a value of 300 residues of Hyp per collagen molecule. The analyses were performed in duplicate in two independent experiments.

The hydrolysates with known amounts of Hyp were then subjected to cross-link analysis with the system described previously<sup>14</sup>. The cross-link precursor aldehydes (*i.e.* Hyl<sup>ald</sup> and Lys<sup>ald</sup>) and the major reducible cross-links (*i.e.* dehydrodihydroxylysinoonorleucine/its ketoamine [deH-DHLNL], dehydrohydroxylysinoonorleucine/its ketoamine [deH-HLNL]) were analyzed as reduced forms, *i.e.* dihydroxynorleucine (DHNL), hydroxynorleucine (HNL), DHLNL and HLNL, respectively. The non-reducible cross-links, *i.e.* Pyr and deoxyypyridinoline (d-Pyr) were also analyzed simultaneously<sup>14</sup>. The contents of these compounds were quantified as moles/mole of collagen. The total number of aldehydes (free and those involved in cross-links) was calculated as a sum of DHNL, HNL, DHLNL, HLNL and  $2 \times (\text{Pyr} + \text{d-Pyr})$ . The cross-link maturation was calculated as  $(\text{Pyr}/\text{DHLNL}) \times 100$ . The analyses were performed in duplicate in two independent experiments.

#### In vitro mineralization assay

The cells were seeded in a 6-well plate at a density of  $2 \times 10^5$  cells/well and cultured until confluence. The medium was then replaced with that containing 50  $\mu\text{g}/\text{mL}$  of ascorbic acid and 2 mM  $\beta$ -glycerophosphate (Sigma Chemical, St. Louis, MO, USA) with or without FCP. The medium was changed every 3 days. The cells were cultured for 21 days. The cells were washed with PBS, fixed with 100% methanol and stained with 1% alizarin red S (Sigma Chemical, St. Louis, MO, USA). Alizarin red S in a stained cell monolayer was semiquantified by the method described previously<sup>20,21</sup>. Briefly, 10%

(v/v) acetic acid was added to each well and incubated at room temperature for 30 min. The monolayer was then scraped, transferred into a microcentrifuge tube, heated at 85°C for 10 min and cooled in ice for 5 min. The slurry was then centrifuged at 20,000 g for 15 min and 10%(v/v) ammonium hydroxide was added to the supernatant. Aliquots of the supernatant were read in triplicate at 405 nm using Powerwave X340 microplate spectrophotometer (BioTek Instruments, Tigan Street Winooski, VT, USA). The analyses were performed in triplicate in three independent experiments.

#### Statistical analysis

The data regarding the mRNA expression were statistically evaluated using the one-way analysis of variance (ANOVA) and Fisher's PLSD test as a post-hoc test.

Statistical analyses for the other studies were performed using Student's *t*-test. All data were expressed as the means±standard error and a *p* value less than 0.05 was considered to be statistically significant.

## RESULTS

#### Amino acid, SDS-PAGE and MALDI-TOFMS analyses

The hydroxyproline content was ~5% of the total amino acids and glycine ~39% (Fig. 1a). The relatively low hydroxyproline (Hyp) content in this fish collagen was consistent with the findings of a previous report<sup>22</sup>. The SDS-PAGE analysis showed a smear band migrating from ~5 k to 13 kDa (Fig. 1b). The TOF-MS data identified many peptides with varied molecular sizes ranging from ~2 k to ~8 kDa (Fig. 1c).

#### Gene expression of type I collagen, LH isoforms, LOX, LOXL isoforms and GLT25D1

The mRNA expression patterns are shown in Fig. 2. The expression of COL1A2, all LHs, LOXL2-4 and GLT25D1 mRNAs were significantly upregulated by the FCP treatment. LH2 mRNA expression in the 0.2% (w/v) FCP treated group showed the highest increase reaching to 3.4-fold that of the controls ( $p < 0.01$ ), followed by COL1A2, LOXL2, LOXL4, GLT25D1, LH1, LOXL3 and LH3 (3.0, 2.4, 1.8, 1.7, 1.4, 1.3 and 1.2-folds, respectively:  $p < 0.01$  except  $p < 0.05$  in LH3). The LOX and LOXL1 mRNA expression tended to increase (1.3 and 1.2-fold increases, respectively) but did not reach significant levels. 0.2% (w/v) FCP was used for the subsequent studies, based on these results.

#### Cell growth

The number of cells in both groups was counted up to 14 days and is shown in Fig. 3. Cell morphology was not changed by the addition of FCP (data not shown). The cell numbers were essentially the same between the FCP treated and control groups during the course of the study except at day 5 when the former showed a lower number of cells in comparison to the control ( $p < 0.01$ ).

#### Collagen content, lysine hydroxylation of collagen and collagen cross-links

The collagen contents of MC3T3-E1 cells and Hyl are shown in Table 1. The collagen contents significantly increased (14.8%) by FCP treatment in comparison to the controls ( $p < 0.05$ ).

The Hyl content per collagen derived from MC3T3-E1 cells in the FCP treated group was also

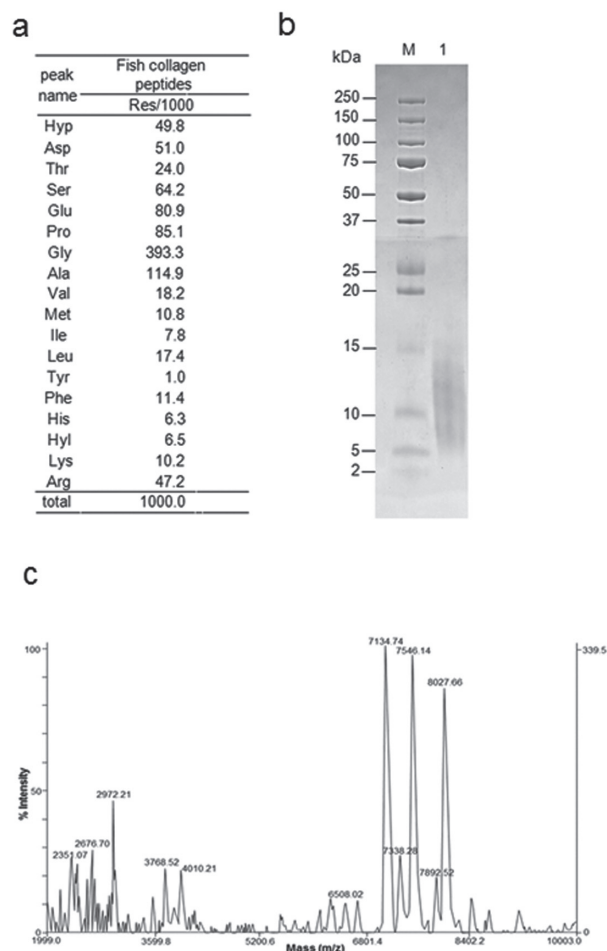


Fig. 1 Biochemical characterization of FCP. (a) Amino acid composition of FCP expressed as residues per 1,000 total amino acid residues. Hyp, hydroxyproline; Asp, aspartic acid; Thr, threonine; Ser, serine; Glu, glutamic acid; Pro, proline; Gly, glycine; Ala, alanine; Val, valine; Met, methionine; Ile, isoleucine; Leu, leucine; Tyr, tyrosine; Phe, phenylalanine; His, histidine; Hyl, hydroxylysine; Lys, lysine; Arg, arginine. (b) SDS-PAGE profile of FCP separated on a 15–20% polyacrylamide gel and stained with CBB. Lane M, broad range molecular weight standard. Lane 1, FCP. (c) MALDI-TOF mass spectrometry obtained from FCP analyzed in the positive mode. The analysis was performed in the linear mode using insulin as an external standard. The effective peak patterns are shown in the values.

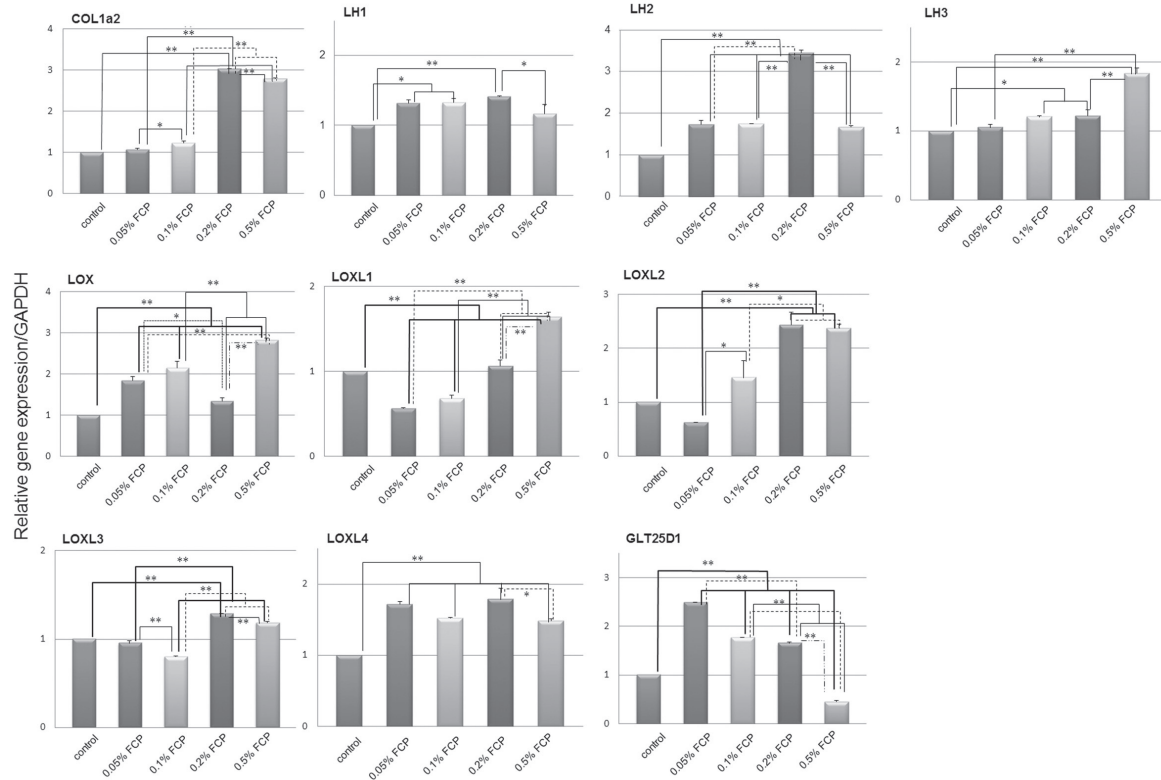


Fig. 2 mRNA expression levels of type I collagen and collagen modifying enzymes with or without FCP treatment in MC3T3-E1 cells analyzed by real-time PCR. The expression levels were normalized to GAPDH. The values are shown as the mean $\pm$ SE from triplicate assays. \* $p$ <0.05, \*\* $p$ <0.01. LH: lysyl hydroxylase, LOX: lysyl oxidase, FCP: fish collagen peptide.

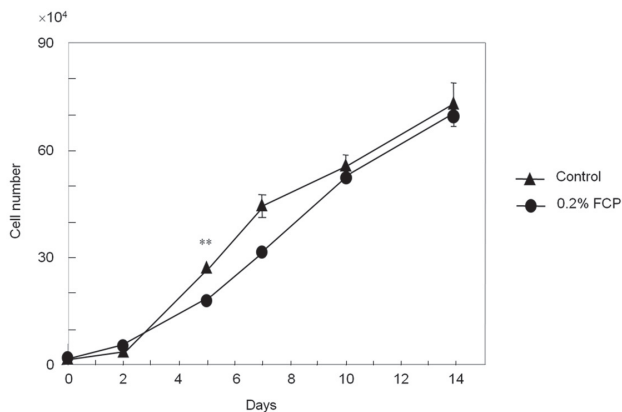


Fig. 3 Effect of FCP on cell growth. The values are shown as the mean $\pm$ SE from triplicate assays. \*\* $p$ <0.01.

significantly higher in comparison to that in the control group ( $p$ <0.05). These findings indicate that FCP treatment not only increased collagen synthesis and deposition in the cultures but also elevated the extent of Lys hydroxylation of collagen.

#### Collagen cross-link analysis

The contents of cross-link precursor aldehydes and collagen cross-links are shown in Table 1. Neither cross-link precursor aldehydes (DHLNL and HNL) nor a non-reducible/mature cross-link, d-Pyr, were detected in any of the groups examined. Two major reducible cross-links (DHLNL and HLNL) were identified in both groups, and the levels were comparable between the two groups, although there was a trend toward a higher DHLNL in the FCP treated group. In contrast, the Pyr content in the FCP treated group was significantly higher than that in the control group ( $p$ <0.05). The ratio of DHLNL to HLNL, total number of aldehydes and the maturation of collagen cross-links are shown in Fig. 4. The ratio of DHLNL to HLNL in the FCP treated group was significantly higher than that in the control group ( $2.988\pm 0.067$  vs.  $2.701\pm 0.078$ ) ( $p$ <0.05). The total number of aldehydes was not significantly different between the two groups ( $1.165\pm 0.084$  vs.  $1.076\pm 0.058$  in control). The cross-link maturation (Pyr/DHLNL  $\times 100$ ) in the FCP treated group was significantly higher than that in the control group ( $2.702\pm 0.175$  vs.  $1.690\pm 0.046$ ) ( $p$ <0.01).

#### In vitro mineralization assay

Alizarin red S staining was carried out to assess the extent of matrix mineralization. The representative

Table 1 Contents of collagen, hydroxylysine, cross-link precursor aldehydes and cross-links of collagen

|                  | control                | 0.2% FCP                |
|------------------|------------------------|-------------------------|
| Collagen content | 265.783 ( $\pm$ 8.488) | 305.220 ( $\pm$ 8.550)* |
| Hyl              | 42.3 ( $\pm$ 0.4)      | 52.8 ( $\pm$ 3.3)*      |
| DHNL             | ND                     | ND                      |
| HNL              | ND                     | ND                      |
| DHLNL            | 0.766 ( $\pm$ 0.026)   | 0.839 ( $\pm$ 0.037)    |
| HLNL             | 0.284 ( $\pm$ 0.009)   | 0.281 ( $\pm$ 0.006)    |
| Pyr              | 0.013 ( $\pm$ 0.001)   | 0.023 ( $\pm$ 0.002)*   |
| d-Pyr            | ND                     | ND                      |

Collagen content is expressed in  $\mu$ g of collagen/mg of cell matrix. The other values are expressed in moles/mole of collagen. Hyl, hydroxylysine; DHNL, dihydroxynorleusine; HNL, hydroxynorleucine; DHLNL, dihydroxylysinylnorleucine; HLNL, hydroxylysinylnorleucine; Pyr, pyridinoline; d-Pyr, deoxypyridinoline; ND, not detected. The values are shown as the mean $\pm$ SE. \* $p$ <0.05 ( $n$ =4).

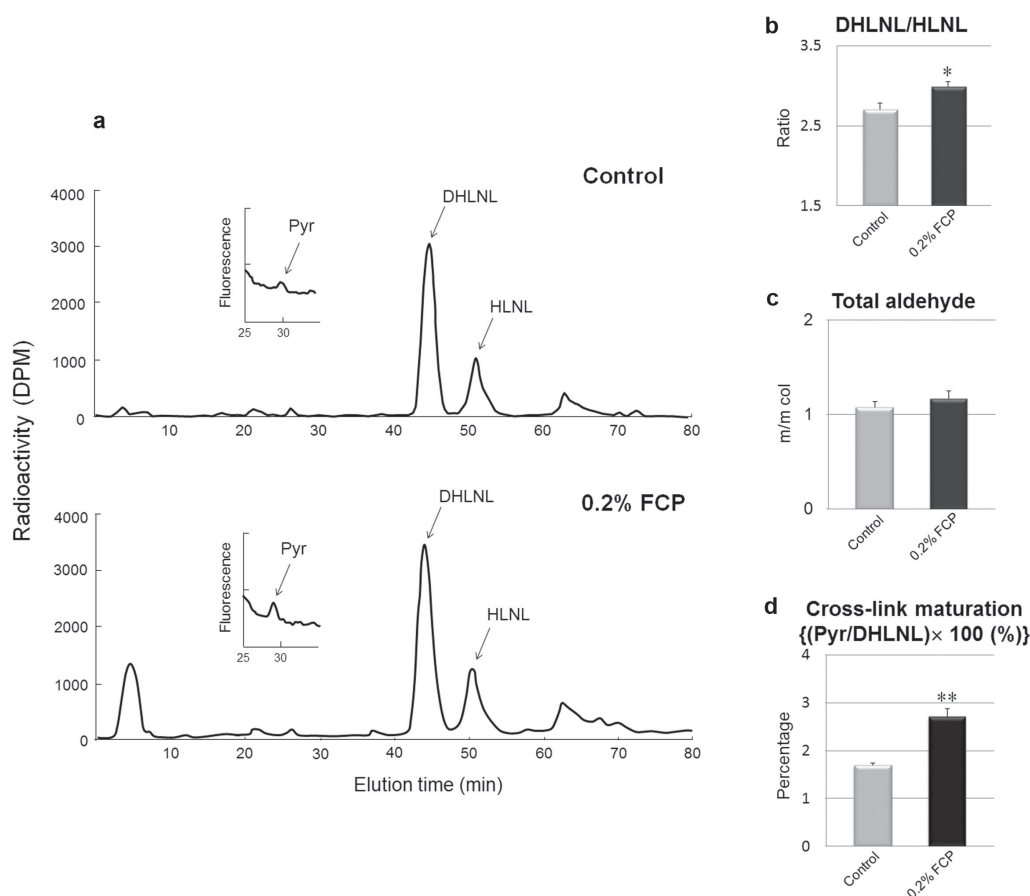


Fig. 4 Effect of FCP on collagen cross-linking in MC3T3-E1 cell/matrices at 2 weeks of cultures. (a) Representative chromatographs of collagen cross-links and their precursor aldehydes. (b) DHLNL/HLNL. (c) A total number of aldehydes calculated as a sum of DHLNL+HLNL+2 $\times$ Pyr. m/m col: moles/mole of collagen. (d) Collagen cross-link maturation evaluated as the ratio of the mature (Pyr) to the immature (DHLNL) cross-links. FCP: Fish collagen peptides, \* $p$ <0.05, \*\* $p$ <0.01 ( $n$ =4).

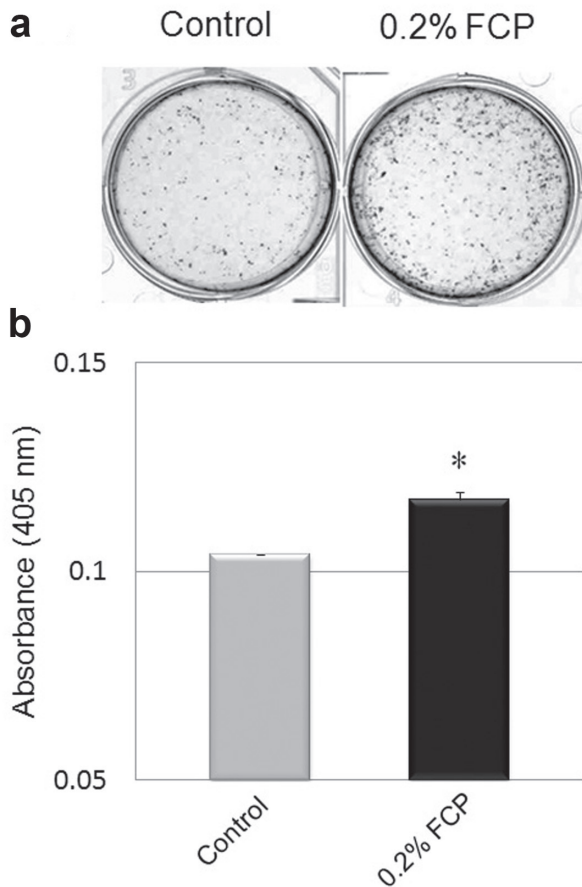


Fig. 5 Effect of FCP on matrix mineralization in MC3T3-E1 cell culture system at 21 day. (a) The cultures were stained with alizarin red S to visualize mineralized nodules. (b) Semi-quantification of the mineral content in the cultures shown in (a). The values are shown as the mean $\pm$ SE. \* $p$ <0.05 ( $n$ =3).

Alizarin red S staining pattern at day 21 is shown in Fig. 5a. The formation of mineralized nodules was evident in both groups. However, the number of mineralized nodules that formed in the FCP treated group appeared to be significantly higher than that of the controls. A semiquantitative analysis of Alizarin red S staining indeed confirmed that MC3T3-E1 cells treated with FCP produced significantly more stained mineral than the untreated cells ( $p$ <0.05; Fig. 5b).

## DISCUSSION

Previous studies have indicated that collagen peptides derived from fish skin might have a positive effect on bone by increasing bone mineral density and by exerting anti-inflammatory activity in Osteoarthritis<sup>23,24</sup>.

The present study explored the effects of FCP on the collagen synthesis, post-translational modifications and mineralization in an osteoblastic cell culture

system. The mRNA expression levels of COL1A2, LH1-3 (especially LH2), LOXL2-4 and GLT25D1 were all significantly upregulated in the FCP treated group. Recent studies have indicated that LH1 is the helical LH<sup>25</sup>, LH2 telopeptidyl LH<sup>26-28</sup>, and LH3 and GLT25D1 collagen glycosyltransferases<sup>29,30</sup>. Therefore, the biochemical findings of collagen synthesized by the FCP-treated cells, *i.e.* higher collagen content, higher extent of Lys hydroxylation in collagen, higher levels of Hyl<sup>ald</sup>-derived collagen cross-links (*i.e.* higher DHLNL/HLNL ratio, slightly higher DHLNL and significantly higher Pyr) in comparison to the control cells, are consistent with the gene expression pattern. The upregulation of the gene expression for LH3 and a recently identified glycosyltransferase, GLT25D1, in the FCP treated group indicate that the collagen synthesized in this group could be more glycosylated<sup>31</sup>. We have recently reported that LH3 primarily functions as a glucosyltransferase for type I collagen and possibly GLT25D1 as a galactosyltransferase in an osteoblastic cell culture system<sup>17</sup>. We have also suggested a regulatory role of LH3-mediated glucosylation in cross-link maturation when occurred at the specific molecular site, *i.e.* residue 87<sup>32</sup>. The current finding that the maturation of collagen cross-links was accelerated in the FCP treated group (Fig. 4) seems inconsistent with this notion. However, to address this, further studies are warranted to determine whether or not the extent of collagen glycosylation in the FCP-treated group is indeed increased at the protein level, and if so, what type of glycosylation (*i.e.* mono- and/or di-glycosylation) and where in the molecule the increases occur. These data will certainly provide more insight into the nature of collagen produced by FCP-treated cells and the role of glycosylation in cross-linking.

The expression levels of LOX and LOXL1 were unchanged by the FCP treatment, and this is likely the reason why the total number of aldehydes was also comparable between the FCP treated and control groups. The biological functions of LOXL2-4 in osteoblasts are unknown, the results obtained in this study suggest their contribution to the formation of collagen cross-links in this culture system may not be significant. Therefore, the major effect of FCP on collagen cross-linking in osteoblasts could be the change in the cross-linking pathway toward the Hyl<sup>ald</sup>-derived pathway<sup>14</sup>, but not the quantity of cross-links.

The increased collagen content and the modifications described above may favor the occurrence of matrix mineralization by providing a stable structural template, thus resulting in an increased formation of mineralized nodules.

The MS analysis identified several molecular weight species ranging from 2,351.07 to 8,027.66 (m/z). The apparent molecular weights based on the SDS-PAGE were ~5 k to 13 kDa. The larger values obtained from SDS-PAGE are likely due to the fact that the peptides with a collagenous sequence tend to migrate slower in the gel than other/non-collagenous peptides<sup>33,34</sup>.

At this point, it is not clear if the effect of FCP

observed was exerted by the single, fish collagen-derived peptide or a combination of several peptides. Identifying such peptide(s) will require fractionation of the peptide mixture of FCP and testing for the effects, which will be the natural next step of this study.

### CONCLUSIONS

The present study demonstrated that FCP exerts a positive effect on collagen synthesis, collagen quality and matrix mineralization in an osteoblastic cell culture system, likely by upregulating the gene expression of the respective collagen modifying enzymes. This suggests the potential utility of FCP as a biomaterial for bone healing and regeneration.

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