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Lysyl oxidase mediates regeneration of chondrocytes and extracellular matrix in the construction of tissue-engineered cartilage in vitro

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Cartilage tissue engineering affords great promise for cartilage tissue regeneration in clinical practice. Unfortunately, in vitro engineered cartilage falls short of mechanical properties compared to natural cartilage, which has not yet become the mainstream of cartilage defect repair. Notably, the collagen fiber structure in the extracellular matrix (ECM) of chondrocytes accounts for the mechanical properties of engineered cartilage. Specifically, massive collagen fibers in ECM are covalently cross-linked by lysyl oxidase (LOX) to form a rigid cross-linked structure, contributing to certain mechanical properties. Accordingly, LOX may be a key enzyme for improving the mechanical properties of tissue-engineered cartilage in vitro. As reported, transforming growth factor-β1 (TGF-β1) can activate LOX via the SMAD-independent MAPK pathway, and β-aminopropionitrile (BAPN), an irreversible inhibitor of LOX, directly represses the cross-linking between macromolecules in the ECM of chondrocytes. In the present study, we intervened in cartilage regeneration in vitro using the aforementioned activator and inhibitor of LOX, thereby evaluating the quality and mechanical properties of tissue-engineered cartilage and further investigating the role of LOX in cartilage regeneration in vitro. Our results demonstrated that TGF-β1 improved the mechanical properties of cartilage in vitro at both two-dimensional and three-dimensional constructs by activating LOX. Therefore, further experimental studies are warranted to probe the activators of LOX and their mechanisms in cartilage regeneration in vitro.

Keywords JNK inhibitor, Chondrogenic medium, *In vivo*, Cartilage regeneration

Articular cartilage is an avascular tissue with limited capacities for self-regeneration[1]. Available surgical or conservative techniques fail to generate tissues that adequately repair damaged cartilage[2]. Accordingly, cartilage defect repair remains a salient clinical challenge. In recent years, cartilage tissue engineering has emerged as a perspective technique for cartilage tissue regeneration. Although in vitro cartilage-like constructs are critical for cartilage tissue engineering, significant hurdles remain in achieving the same mechanical strength as natural cartilage for in vitro engineered cartilage. Cartilage tissues are composed of chondrocytes, chondrocyte-derived extracellular matrix (ECM), and collagen fibers[3]. According to our previous research, the insufficient mechanical properties of cultured cartilage in vitro are attributed to the deficiency of collagen cross-linking, which is closely linked to pyridinoline (PYR). Specifically, PYR is positively correlated with collagen cross-linking levels, but not with ECM contents[4]. Another study also unveiled that the mechanical strength of cartilage was governed by the arrangement and spatial structure of collagen fibers in ECM[4]. Moreover, the existing cartilage engineering system alone is inadequate for enhancing the mechanical strength of in vitro constructed cartilage. Hence, the

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addition of some exogenous factors into the culture system may be a novel approach to improve the spatial structure and arrangement of collagen in ECM, thereby modifying the in vitro construction of cartilage.

High-density collagen fibers are cross-linked and relatively uniformly distributed in ECM, which increases the mechanical strength of cartilage under pressure loading[5]. Therefore, the cross-linking and 3-dimensional (3D) spatial distribution of collagen are considered the most key influencing factors for the mechanical strength of cartilage tissues[6]. Reportedly, PYR is the most crucial molecule in collagen cross-linking formed by covalent bonds between collagen molecules and assumes a key role in stabilizing collagen chains[7]. As an ECM synthetase, lysyl oxidase (LOX) can encourage PYR synthesis and is involved in balancing ECM synthesis and degradation when expressed at a low level in vitro[8]. Recent studies on LOX and PYR have focused mainly on tumors, cardiovascular diseases, and pelvic connective tissue diseases[9–11], but rarely on cartilage regeneration in vitro. As a consequence, it is unestablished whether the regulation of LOX can affect the mechanical strength of in vitro regenerated cartilage. Transforming growth factor-β1 (TGF-β1) can up-regulate LOX in the kidneys of hereditary nephrotic mice with chronic renal fibrosis[12] and human lung fibroblasts[13]. TGF-β1 also controls the expression and enzymatic activity of LOX in many pathological fibrotic situations[14]. A prior study reported that TGF-β1 increased LOX expression in vascular smooth muscle cells through the SMAD-independent mitogen-activated protein kinase (MAPK) pathway[15]. Additionally, β-aminopropionitrile (BAPN), an irreversible inhibitor of LOX, has been revealed to directly suppress the cross-linking of macromolecules in ECM of chondrocytes[16].

On this basis, we intervened in cartilage regeneration in vitro using TGF-β1 and BAPN to assess the quality and mechanical strength of in vitro regenerated cartilage and further clarify the molecular mechanism of LOX in cartilage regeneration in vitro. Firstly, the optimal concentrations of TGF-β1 and BAPN that do not affect the number and proliferation of chondrocytes and the regeneration of cartilage tissues in vitro were determined by respectively screening the minimum effective activation and inhibitory concentrations of TGF-β1 and BAPN in cells. Meanwhile, the effects of BAPN at different concentrations on LOX secretion and chondrocyte regeneration were measured to choose an appropriate concentration of BAPN that can inhibit LOX without affecting chondrocyte proliferation *in vitro*. Next, TGF-β1 at the optimal concentration was added into the in vitro cartilage construction system alone or in combination with BAPN for 12 weeks of culture. Finally, the study systematically analyzed the effects of the LOX activator or inhibitor on cartilage regeneration.

The hypothesis of the study is that enhancement of collagen crosslinking through activation of LOX can significantly improve the mechanical properties of tissue-engineered cartilage in vitro, while inhibition of LOX activity can destroy the collagen crosslinking network and weaken its mechanical function. TGF-β1 may promote collagen crosslinking by activating LOX, significantly improving the Young's modulus and compressive strength of constructed cartilage in vitro, while maintaining the uniformity and stability of cartilage matrix. BAPN may reduce collagen crosslinking by inhibiting LOX activity, resulting in decreased mechanical properties and matrix relaxation of constructed cartilage in vitro, and this effect is independent of cell proliferation and matrix secretion. The combination of TGF-β1 and BAPN partially counteracts the effect of LOX activation, suggesting that LOX is a key target for TGF-β1 to improve the mechanical properties of cartilage.

Based on the above hypothesis, the core research aim of this study is to solve the question: "Does lysyl oxidase (LOX) influence the mechanical properties of tissue-engineered cartilage in vitro by regulating the degree of cross-linking and spatial distribution of collagen fibers?"

Materials and methods

Isolation and culture of articular chondrocytes

Articular chondrocytes were isolated from the joint surface of newborn swine (Shanghai Jia Gan Experimental Animal Raising Farm; Shanghai, China). All animal experiments were reviewed and ratified by the Animal Care and Use Committee of Shanghai Jiao Tong University School of Medicine. Chondrocytes were isolated and harvested with the method in our laboratory[17] and then seeded into 10 cm culture dishes (the final concentration of 1.5×10^6 cells/cm^2) containing Dulbecco's Modified Eagle Medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone) and 1% penicillin-streptomycin (regular medium). Chondrocytes of the second passage (P2) were utilized for in vitro cartilage construction.

Cell viability assay

P2 chondrocytes were seeded into 96-well plates (2×10^3 cells/well). After 24 h of culture at 37℃, chondrocytes were maintained in 10% FBS-containing DMEM supplemented with different concentrations of BAPN (Tokyo Chemical Industry, Tokyo, Japan; 0, 0.05, 0.1, 0.2, and 0.5 mM) or TGF-β1 (R&D Systems, Minneapolis, MN, USA; 0, 5, 10, and 20 ng/mL). Cell viability was examined with the cell counting kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan) on days 1, 3, 5, 7, 9, and 11 as previously described[18].

Enzyme-linked immunosorbent assay (ELISA)

When chondrocyte confluence reached 70%, DMEM was replaced with serum- and phenol red-free DMEM containing 0.1% bovine serum albumin, 50 mg/mL vitamin C phosphate, and BAPN or TGF-β1 at the above concentrations. Following 24-h culture, the medium was obtained for the quantification of LOX with the swine LOX ELISA kit (R&D Systems)[19].

Cell morphology observation

P2 Chondrocytes were seeded into 6-well plates (1.5×10^6 cells/mL; 1 mL/well) and then cultured in a medium containing 0.1 mM BAPN or 10 ng/mL TGF-β1 at 37℃ for 24 h. The optimal concentrations of BAPN or TGF-β1 that do not affect chondrocyte proliferation were determined according to the results of Sect. 2.2 and 2.3. Cells were observed under a light microscope on days 1, 3, and 7.

Preparation and in vitro culture of cell-scaffold constructs

A silicone rubber mold was fabricated with a 9-mm-diameter cylindrical cavity measuring 2.5 mm in depth. Fifteen milligrams of polyglycolic acid (PGA) nonwoven fibers (Albany International Research, Albany, NY) were packed into the cavity, followed by the gradual addition of 0.5 mL of 0.3% polylactic acid (PLA, Sigma-Aldrich, St. Louis, MO) dissolved in dichloromethane to stabilize the scaffold structure, until the PLA contents reached 10% in the PGA/PLA scaffolds[20].

The scaffolds were disinfected with 75% ethanol solutions for 40 min, followed by two washes with phosphate-buffered saline (PBS). P2 chondrocytes were seeded into PGA/PLA scaffolds at a concentration of 6.0×10^7 cells/mL (80 μL in each scaffold), followed by 4-h culture in a regular medium as previously described[20].

Grouping

The above chondrocyte-scaffold complexes were randomly assigned into four groups: control, BAPN, TGF-β1, and TGF-β1 + BAPN. Cells in the other three groups except for the control group were cultured in a regular medium containing TGF-β1 and/or BAPN at the optimal concentration determined in Sect. 2.4. All constructs were cultured randomly with DMEM, BAPN, TGF-β1, and TGF-β1 + BAPN in vitro for 12 weeks after grouping.

Scanning electron microscope (SEM)

After in vitro culture for 12 h and 7 days, the microstructure of PGA/PLA scaffolds and the ECM deposition of the scaffold surface were examined with an SEM (Philips XL-30; Philips, Amsterdam, Netherlands). After being fixed overnight in 2.5% glutaraldehyde at 4℃, all samples were dehydrated with gradient ethanol and then observed under the SEM.

Histology and immunohistochemistry

Subsequent to 12 weeks of in vitro culture, the samples were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 5 μm. The sections were subjected to hematoxylin-eosin staining and Safranin-O staining to observe the tissue structure and ECM deposition of cartilage. For immunohistochemistry, type II collagen expression was detected with the use of mouse anti-human type II collagen monoclonal antibodies (1:100 in PBS; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and horseradish peroxidase-conjugated anti-mouse secondary antibodies (1:200 in PBS, Santa Cruz Biotechnology). Thereafter, the sections were developed with diaminobenzidine tetrahydrochloride (Santa Cruz Biotechnology) as described previously[20].

Wet weight

All samples were quantitatively transferred to tared vessels and measured using a precision electronic autobalance under ambient laboratory conditions (25 ± 1 °C, 50 ± 5% RH). Differences in wet weight among groups were analyzed with one-way analysis of variance (ANOVA).

Biomechanical test

Young's modulus was measured with a biomechanical analyzer (Instron-5542; Instron, Canton, MA, USA) for the biomechanical test[21]. For samples in each group ($n = 6$ in each group), a constant compressive strain rate of 0.5 mm/min was used up to 80% of maximal deformation. The Young's modulus of the samples was calculated according to the slope of the stress-strain curve.

Glycosaminoglycan (GAG) quantification

The GAG contents of in vitro engineered cartilage (vitro-EC) in each group ($n = 6$ in each group) were quantified with dimethyl methylene blue chloride (Sigma). Total GAG was precipitated by guanidinium chloride solution (0.98 mol/L). After the GAG precipitate was dissolved, the optical density (OD) was determined at 595 nm. A standard curve was established according to the OD values of chondroitin-4-sulfate with different concentrations. The total GAG amounts were determined on the basis of the OD value and the standard curve[22].

Total collagen quantification

Total collagen contents in each group ($n = 6$ in each group) were quantified with the hydroxyproline assay. The samples were prepared through alkaline hydrolysis, and free hydroxyproline hydrolysates were assayed as previously described[23]. Hydroxyproline contents were finally converted to total collagen contents according to the collagen-to-hydroxyproline mass ratio of 7.25.

ECM content quantification

Samples in each group ($n = 6$ in each group) were completely homogenized to release ECM proteins, and the supernatant was attained for ELISA[20]. LOX and PYR contents were quantified with swine LOX and PYR ELISA kits (R&D), respectively.

Scanning electron microscope (SEM) and transmission electron microscope (TEM)

Three days after cell seeding, PGA scaffolds and cell-scaffold complexes of the 3D control group were rinsed with PBS and fixed overnight with 0.05% glutaraldehyde at 4℃. After dehydration with gradient ethanol, the samples were subjected to critical-point drying, and the adhesion and distribution of chondrocytes on the scaffolds were directly observed under the SEM (JEOL-6380LV; JEOL, Kyoto, Japan).

As previously reported[24], the samples in different 3D culture groups were cut into 1.0 mm^3 and rinsed with PBS. Then all samples were fixed by immersion in a fixative consisting of 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH7.4) at 4℃ for 24 h, rinsed overnight in the phosphate buffer. Following a postfixation for 1 h with 1% osmium tetroxide in phosphate buffer. After that they were dehydrated and embedded in 618# resin. Ultrathin

sections were prepared using a Leica EM UC7 ultramicrotome, contrasted with uranyl acetate and lead citrate, examined under a Philips CM80 transmission electron microscope at 60kv.

Statistical analysis

Normality was assessed using the Shapiro-Wilk test, and all datasets met the normality assumption. One-way ANOVA was applied to compare group differences. Homogeneity of variances was verified by Bartlett's test; for equal variances, Tukey's post hoc test was used for pairwise comparisons. If variances were unequal (Bartlett's $p < 0.05$), Welch's ANOVA with Games-Howell post hoc test was performed.

Ethics statement

All experimental protocols were approved by the Experimental Animal Ethics Committee of Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine (approval number: SH9H-2023-A683-SB). All procedures were conducted in accordance with relevant guidelines and regulations, including the ARRIVE Guidelines (https://arriveguidelines.org).

Results

Effects of the inhibitor or activator of LOX on cell viability and LOX expression

Chondrocyte viability was increased after culture with different BAPN concentrations, except for 1.0 mM BAPN, which exerted a mild inhibitory effect on cell viability (Fig. 1A). Different TGF-β1 concentrations all significantly decreased chondrocyte viability (Fig. 1B). LOX expression was not changed significantly in 0.05

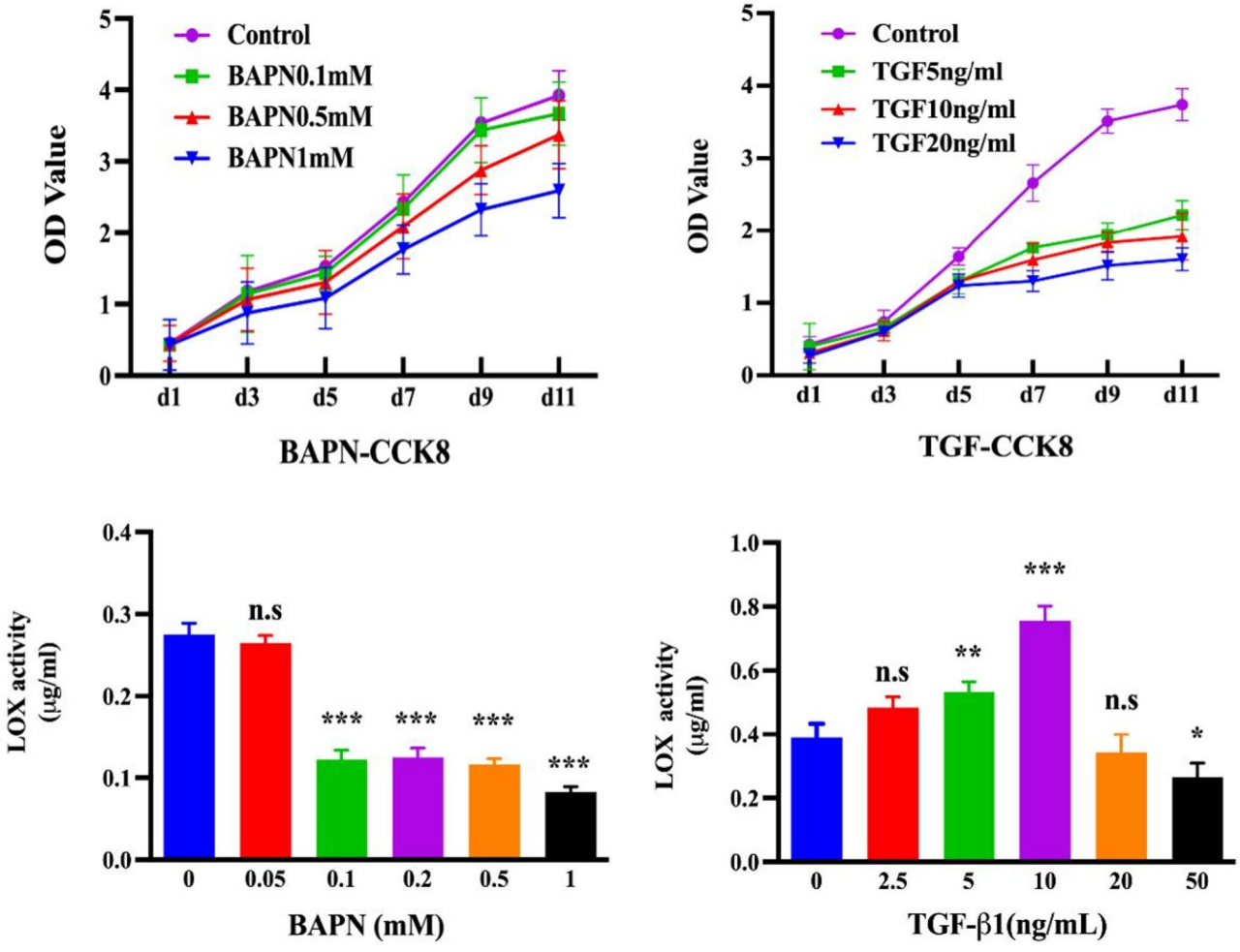

Fig. 1. Cell viability and LOX activation in two-dimensional chondrocytes cultured with different concentrations of BAPN or TGF-β1. (**A**-**D**) According to CCK-8 assay results, there were no significant differences in cell viability after culture with different concentrations of BAPN except for 1 mM BAPN ($p < 0.05$); different concentrations of TGF-β1 all decreased cell viability ($p < 0.05$); BAPN at a concentration of above 0.1 mM decreased LOX expression ($p < 0.05$); 5ng/mL TGF-β1 shows higher LOX activity ($p < 0.01$), LOX expression peaked at 10 ng/mL TGF-β1 ($p < 0.001$). (n.s means no significant difference compared to control, * means $p < 0.05$ compared to control, ** means $p < 0.01$ compared to control, *** means $p < 0.001$ compared to control).

mM BAPN group compared with control, but significantly declined in groups with BAPN at 0.1 mM or higher. (Fig. 1C). Additionally, LOX expression was substantially reduced when the TGF-β1 concentration reached 20 ng/mL (Fig. 1D), TGF-β1 at low concentration (0-10ng/ml) can promote the generation of functional cartilage[25], and with the gradual increase of concentration, it will increase the proportion of hypertrophic chondrocytes, cause calcification and ossification, destroy the mechanical properties of cartilage, and induce some in vitro chondropathic changes. Therefore, this may also be the reason why the expression of LOX decreased when TGF-β1 concentration exceeded 20ng/ml or higher. According to the above results, we determined 0.1 mM as the optimal concentration of BAPN for subsequent experiments and 10 ng/mL as the optimal concentrations of LOX activators for engineered cartilage regeneration in vitro.

Effects of the inhibitor and activator of LOX on cellular morphology in two-dimensional (2D) cell culture

According to the aforementioned results, 0.1 mM BAPN and 10 ng/mL TGF-β1 were used for 2D culture. Under the light microscope, the morphology and distribution of chondrocytes showed no significant differences among the control, 0.1 mM BAPN, and 10 ng/mL TGF-β1 on day 1 after seeding on 2D culture dishes. However, on day 3, chondrocytes in the 10 ng/mL TGF-β1 group tended to cluster, while the other two groups presented with no prominent differences in cell morphology and distribution. On day 7, chondrocyte clustering was more obvious in the 10 ng/mL TGF-β1 group, accompanied by decreases in the total number of chondrocytes. Conversely, no obvious differences were found in cell morphology and number among the other two groups (Fig. 2). Overall, 10ng/ml TGF-β1 group represented the largest biocompatible survival signal of chondrocytes but accompanied

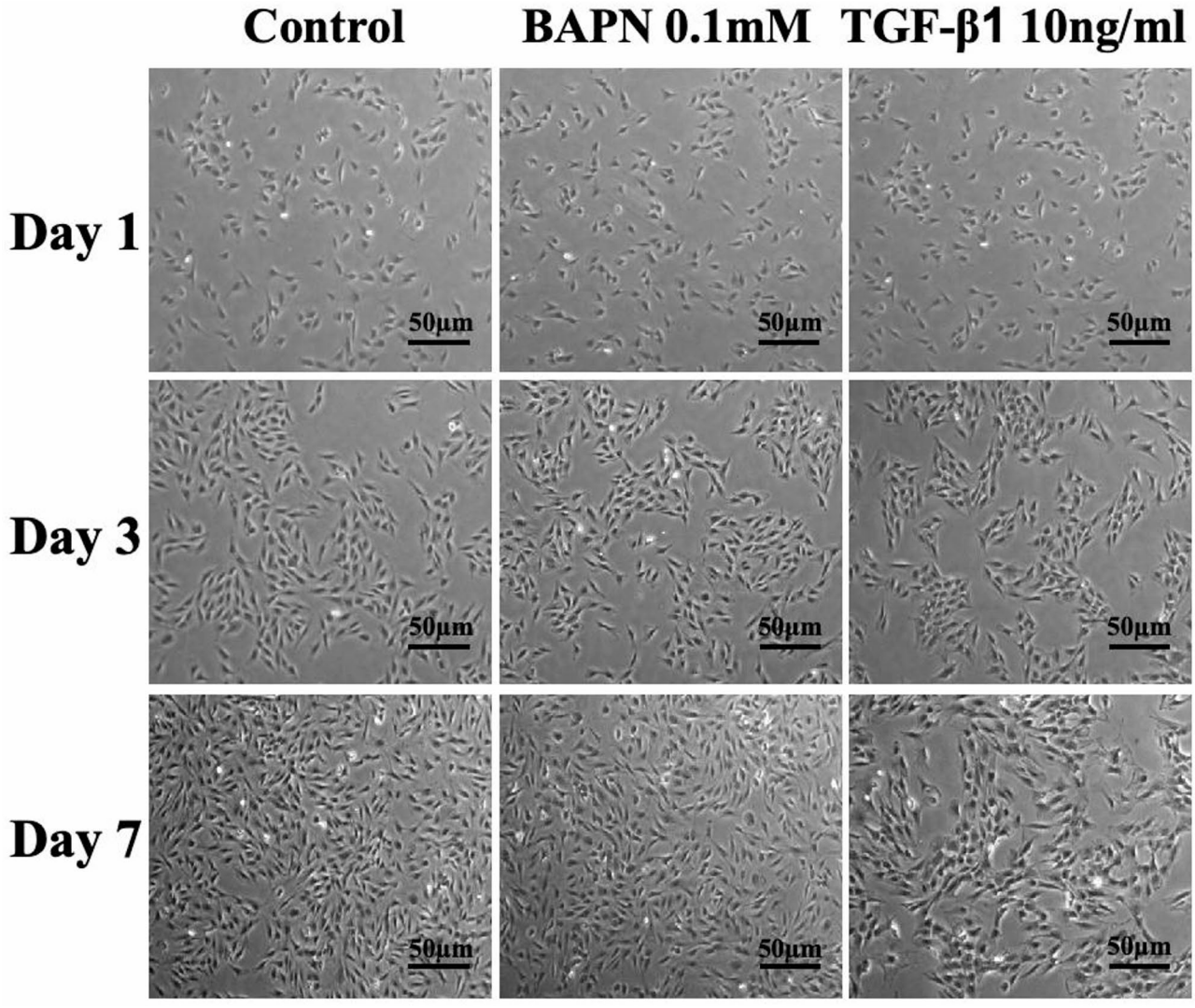

Fig. 2. Morphology of P2 chondrocytes cultured with a regular medium, 0.1 mM BAPN, or 10 ng/mL TGF-β1 on days 1, 3, and 7. On days 1 and 3, there was no significant difference in cell morphology and number among the three groups under the light microscope. On day 7, chondrocytes in the TGF-β1 group clustered, with a reduction in the number of cells under the visual field.

by decreases in the total number of chondrocytes in 2D culture, consistent with CCK-8 assay results, whereas BAPN failed to intervene in cell proliferation.

Tissue-engineered cartilage regeneration in vitro with LOX inhibitor or activator culture

Macroscopic and histological evaluation of in vitro cartilage regeneration

After PLA coating, nonwoven PGA fibers (Fig. 3A) could be readily prepared into cylindrical scaffolds with a diameter of 9 mm and a thickness of 2.5 mm (Fig. 3B). Under the SEM, the ultrastructure of the interlaced PGA fibers and PLA coating was observed (Fig. 3C). P2 chondrocytes could easily adhere to PGA fibers, forming a cell-scaffold construct (Fig. 3D). After 3 days of culture in vitro, observation under the light microscope and SEM demonstrated that chondrocytes could secret sufficient ECM to cover PGA fibers (Fig. 3E-F).

After 12 weeks of in vitro culture with TGF-β1 and/or BAPN, all samples formed cartilage-like tissues with basic retention of their original shape, and scaffold materials on the surface were fully degraded and replaced with new cartilage-like tissues. However, the color and texture of the samples were different in different groups. Samples in the control and TGF-β1 groups were relatively smooth on the surface and faint yellow, whilst samples in the BAPN and TGF-β1 + BAPN groups were lighter yellow with a looser tissue texture. These results indicated that LOX inhibition might influence the secretion and deposition of ECM in cartilage.

Histological examinations further supported the results of macroscopic examinations. Generally, all samples presented with cartilage-specific ECM deposition. Relatively thick cartilage was formed in the TGF-β1 and TGF-β1 + BAPN groups. The tissue structure was the most homogeneous in the TGF-β1 group, accompanied by abundant lacuna and strong cartilage-specific ECM staining. In the TGF-β1 + BAPN group, the samples exhibited relatively strong cartilage-specific ECM staining but non-homogeneous tissue structures with looser and haphazardly arranged cartilaginous lacunae. For the other three groups, only a thin layer of cartilage-like tissues with weak cartilage-specific ECM staining was observed in the outer region of samples (Fig. 4). In conclusion, TGF-β1 activated engineered cartilage formation in vitro, and BAPN as a LOX inhibitor disturbed cartilage formation.

Effect of the inhibitor and activator of LOX on the mechanical properties and collagen cross-linking of engineered cartilage in vitro

It is the most concerned issue in our study whether LOX activators can improve the mechanical properties of engineered cartilage in vitro. As depicted in Fig. 5, wet weight was the highest in the BAPN and TGF-β1 + BAPN groups ($p < 0.001$) (Fig. 5A), indicating that the addition of LOX inhibitors provoked ECM fluffiness and disorganization in cartilage, thus increasing the water content of the matrix. The TGF-β1 group displayed the highest Young's modulus with marked differences ($p < 0.001$) (Fig. 5B). The TGF-β1 group and the TGF-β1

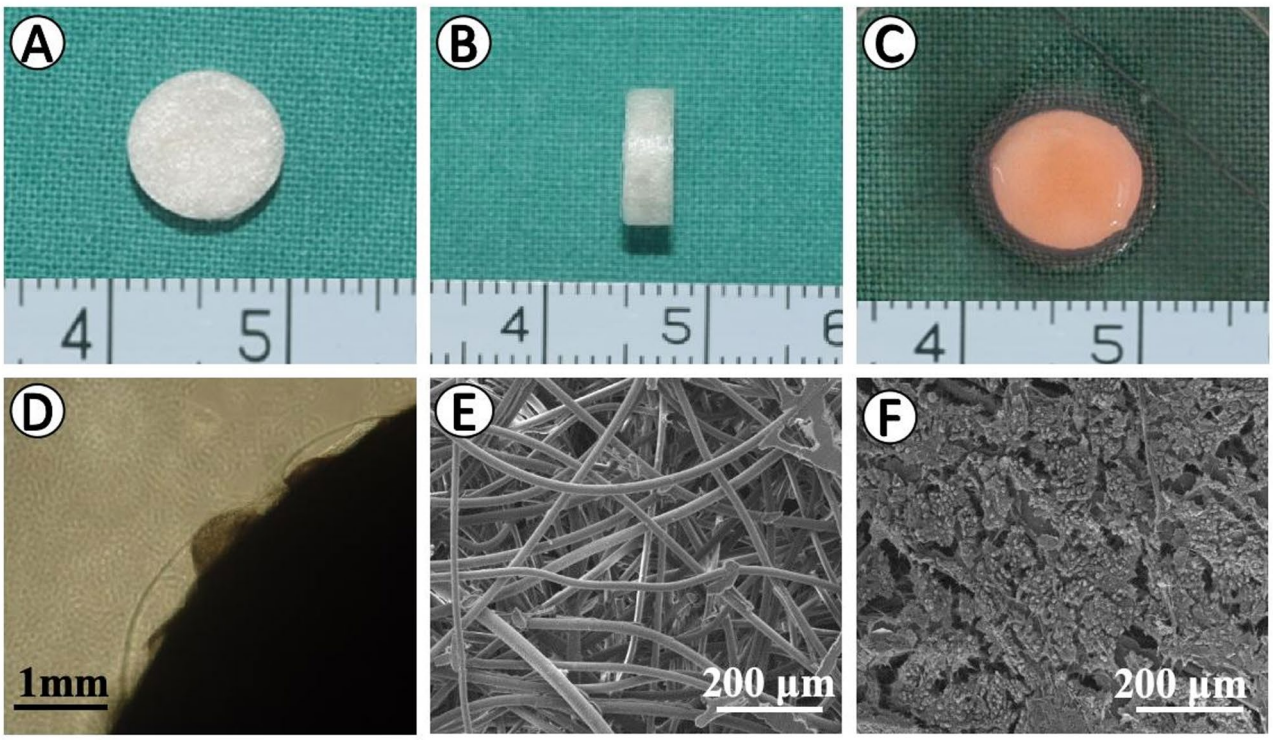

Fig. 3. Preparation of the in vitro cell-scaffold construct with front and side views. (**A**-**B**) Macroscopic observation. (**C**) Macroscopic view of the cell-scaffold construct after cell seeding. (**D**) ECM deposition at the edge of the cell-scaffold construct. (**E**) SEM image of the scaffold. (**F**) SEM image of the cell-scaffold construct in regular culture systems on day 7.

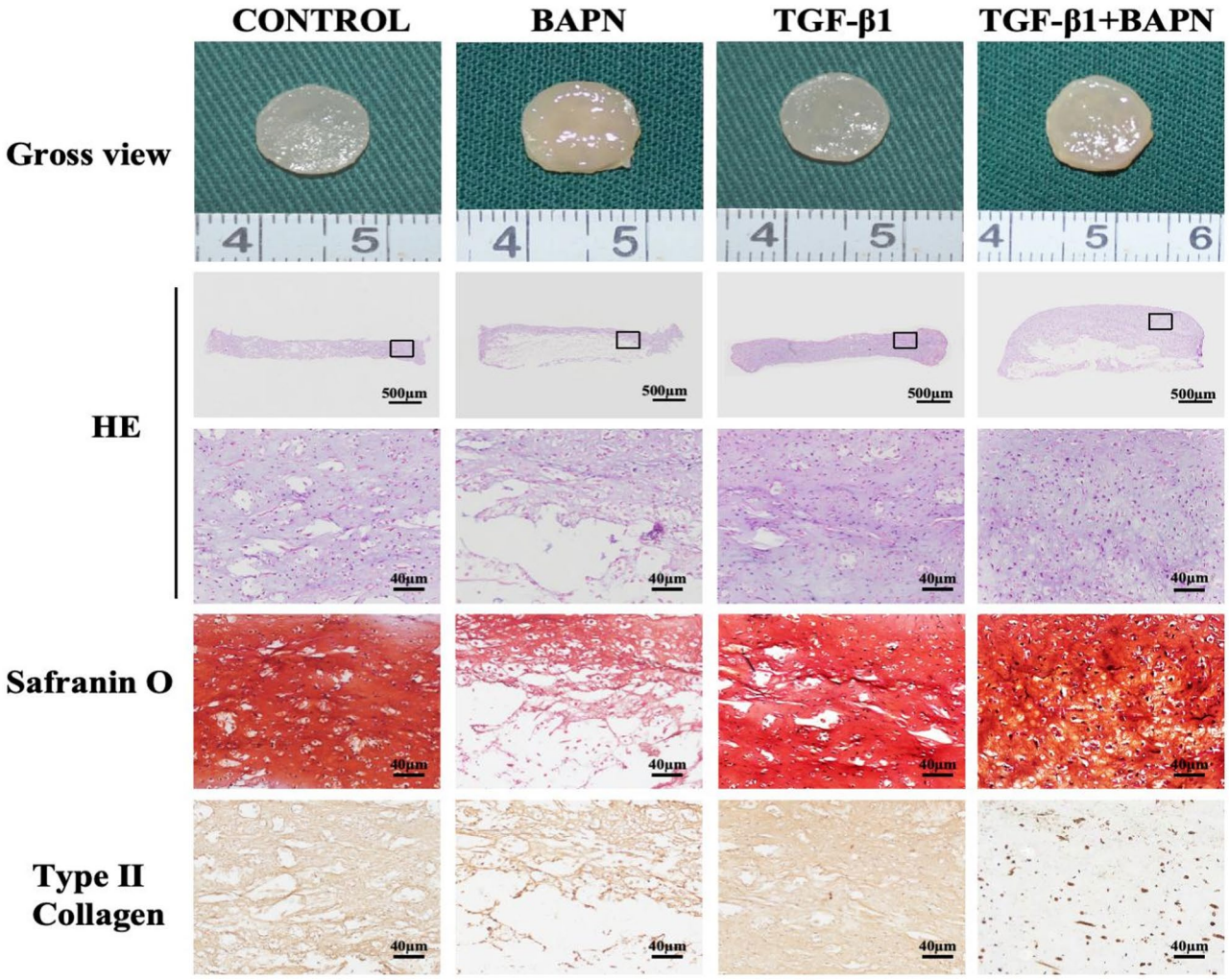

Fig. 4. Macroscopic view and histology of in vitro regenerated cartilage cultured with TGF-β1 and/or BAPN. Hematoxylin and eosin staining (HE), Safranin O staining and Type II Collagen staining of in vitro engineered cartilage. All samples presented with a cartilage-like appearance and cartilage-specific ECM deposition. Relatively thick cartilage was formed in the TGF-β1 and TGF-β1 + BAPN groups. The tissue structure was the most homogeneous in the TGF-β1 group, accompanied by abundant lacuna and strong cartilage-specific ECM staining. In the TGF-β1 + BAPN group, the samples exhibited relatively strong cartilage-specific ECM staining but non-homogeneous tissue structures with looser and haphazardly arranged cartilaginous lacunae. For the other two groups, there was a thinner layer of cartilage-like tissues with weak cartilage-specific ECM staining.

+ BAPN group also demonstrated higher total collagen and GAG than other groups(Fig. 5C-D). Meanwhile, Young's modulus in the TGF-β1 + BAPN group was higher than that in other groups, although slightly lower than that in the TGF-β1 group (Fig. 5B). These results were highly concordant with collagen cross-linking levels. Specifically, the highest PYR and LOX contents were observed in the TGF-β1 group (Fig. 5E-F), illustrating that TGF-β1 as a LOX activator might foster collagen cross-linking and thus enhance the mechanical properties of engineered cartilage in vitro. Under TEM, high-density collagen fibers with relatively uniform distribution were found only in TGF-β1 groups. The density of collagen fibers and the number of long collagen fibers, particularly collagen fibers of above 1000 nm in length, were higher in TGF-β1 groups than those in the other three groups, which further supported mechanical analysis results (Fig. 6).

Effect of the inhibitor and activator of LOX on ECM contents in vitro-EC

The quantitative analysis of ECM contents revealed a regular change in the wet weight of samples, further validating the results of the above macroscopic and histological examination results. The contents of total GAG and total collagen were the highest in the TGF-β1 and TGF-β1 + BAPN groups with remarkable differences ($P < 0.001$). Altogether, TGF-β1 might stimulate ECM secretion of tissue-engineered cartilage in vitro via multiple pathways, whereas BAPN might only partially block the role of TGF-β1 in activating LOX to facilitate collagen cross-linking.

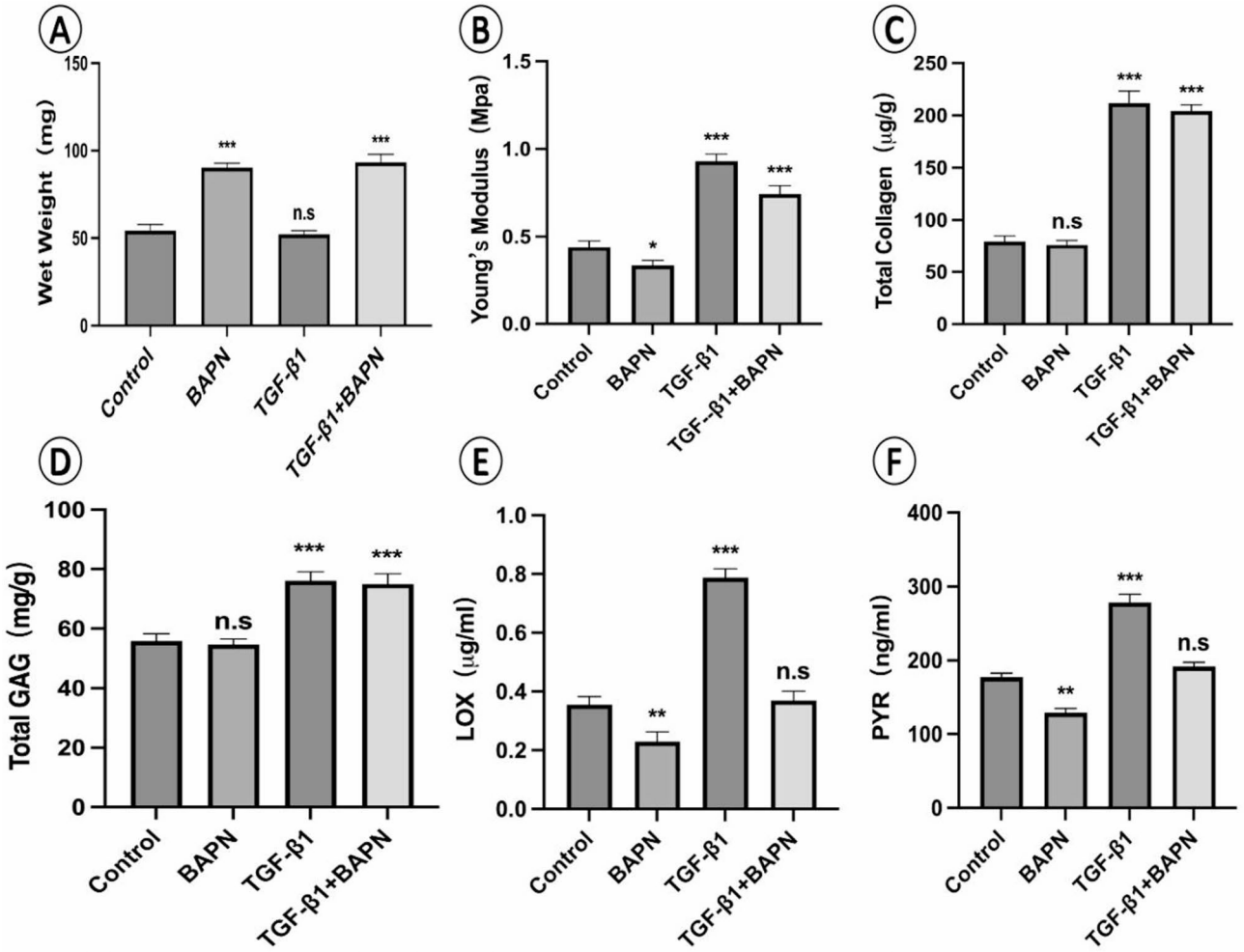

Fig. 5. Quantitative evaluation and LOX expression of in vitro regenerated cartilage cultured with TGF-β1 and/or BAPN. Wet weight was higher in the BAPN and TGF-β1 groups ($P < 0.001$), while the contents of total GAG and total collagen were the highest in the TGF-β1 and TGF-β1 + BAPN groups with significant differences ($P < 0.001$). Young's modulus reached the peak value in the TGF-β1 and TGF-β1 + BAPN groups ($P < 0.001$). LOX and PYR contents were reduced to the lowest value under BAPN treatment ($P < 0.01$) but were the highest in the TGF-β1 group ($P < 0.001$). (n.s means no significance, * means $P < 0.05$ compared to control, ** means $P < 0.01$ compared to control, *** means $P < 0.001$ compared to control).

Discussion

Tissue engineering has emerged as a promising approach for the repair of articular cartilage defects. However, engineered cartilage constructed in vitro is being confronted with challenges in clinical transformation due to its insufficient mechanical properties. As reported, the ordered spatial arrangement of macromolecules and the cross-linking between collagen in ECM provide reliable mechanical properties for in vitro regenerated cartilage[26]. LOX and PYD are key enzymes implicated in the cross-linking between collagen and elastin lysine residues in ECM, which contribute to the transformation of collagen and elastin from soluble monomers to insoluble fibers[27]. Therefore, facilitating collagen cross-linking through the addition of exogenous factors may be a worthwhile method to be explored for improving the mechanical properties of tissue-engineered cartilage in vitro. In the present study, TGF-β1 or BAPN, an activator or inhibitor of LOX, was used to intervene in 2D chondrocyte culture, and then regenerated 3D cartilage was cultured with TGF-β1 or BAPN at appropriate concentrations. The results demonstrated that TGF-β1 stimulated ECM secretion and improved the mechanical properties of cartilage in vitro, which might be partially achieved by activating LOX and increasing collagen cross-linking. On the contrary, the addition of BAPN disrupted the lacunae structure of ECM in cartilage in vitro and resulted in relatively fluffy and loose regenerated cartilage, implicating the impact of collagen cross-linking suppression on the mechanical properties of in vitro engineered cartilage.

Collagen cross-linking is dictated by the activity of LOX. There are no reports as to whether the mechanical properties of engineered cartilage can be improved by adjusting the activity of LOX. LOX activity inhibition is associated with reduced collagen cross-linking in ECM of regenerated cartilage and damaged mechanical properties of engineered cartilage, underscoring the critical role of LOX activities in in vitro cartilage regeneration. Therefore, it is imperative to observe the effect of LOX inhibitors on cartilage regeneration by

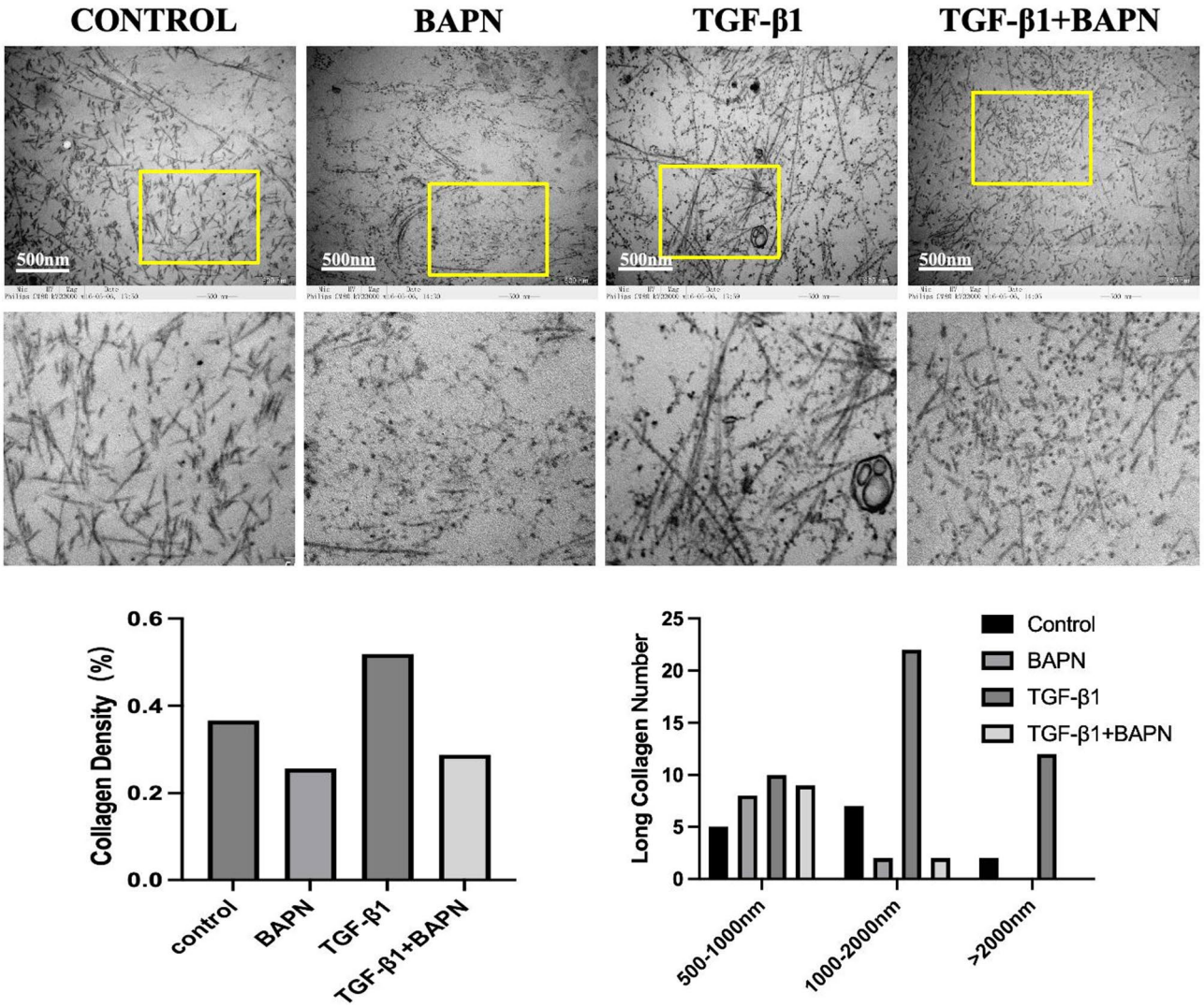

Fig. 6. TEM examination of in vitro engineered cartilage. Collagen was relatively uniformly distributed in the control and TGF-β1 groups, with the highest collagen deposition in the TGF-β1 group. Collagen fibers were arranged sparsely and disordered in the BAPN and TGF-β1 + BAPN groups.

using an appropriate LOX inhibitor to intervene in cartilage regeneration at 2D and 3D levels. Reportedly, BAPN as an irreversible inhibitor of LOX can directly repress collagen cross-linking in ECM of in vitro engineered cartilage. In the present study, BAPN was therefore utilized as an exogenous factor to inhibit the LOX activity during cartilage regeneration in vitro[28]. Meanwhile, a suitable concentration of BAPN was selected to inhibit collagen cross-linking in cartilage without affecting the regeneration of tissue-engineered cartilage in vitro. Our experimental results unraveled that the addition of 0.1 mM BAPN failed to obviously alter the macroscopic view and content of ECM but reduced the mechanical properties of cartilage in vitro, implicating the pivotal role of LOX inhibition in cartilage regeneration in vitro.

After testifying the efficiency of LOX inhibitor, our study shifted to verify the effect of LOX activation to enhance collagen cross-linking in ECM and then improve the mechanical properties of tissue-engineered cartilage in vitro. Accordingly, the activator TGF-β1 was selected to intervene in the regeneration of tissue-engineered cartilage in vitro in 2D and 3D culture. It was observed that TGF-β1 increased the activity of LOX in 2D chondrocyte culture. Next, the optimal concentration of TGF-β1 (10 ng/mL) was identified to maximize LOX activation without influencing the proliferation and regeneration of chondrocytes. In 3D culture, TGF-β1 facilitated cartilage regeneration and ECM secretion, ultimately enhancing the mechanical strength of cartilage in vitro.

TGF-β1, an essential member of the growth factor family[29], can drive cartilage formation in vitro through the MAPK pathway[30] and promote ECM deposition of chondrocytes[31]. Additionally, TGF-β1 can diminish the activity of ECM degradation-related proteins such as matrix metalloproteinases[32]. Combined with our results of cartilage regeneration in 3D culture, it can be confirmed that TGF-β1 induces cartilage regeneration through multiple pathways, including the activation of LOX and the promotion of collagen cross-linking in ECM. Consequently, additional experiments are warranted to screen the optimal concentration of LOX activators to

promote 3D cartilage construction. Overall, TGF-β1 activates LOX via diverse mechanisms, illustrating that LOX activation partially contributes to collagen cross-linking of ECM in cartilage. In the future, it is necessary to further explore the activator of LOX and its mechanism in cartilage regeneration.

Conclusion

In summary, the current study unravels that LOX activation can enhance the collagen cross-linking of ECM in cartilage at 2D and 3D levels. Through the intervention of cartilage culture with the inhibitor and activator of LOX, it can be concluded that LOX activation elevates the mechanical strength of 3D cartilage in vitro. The underlying mechanisms of LOX are also analyzed at tissue, cellular, and molecular levels. Nevertheless, further research is required to identify the optimal concentration of LOX activators, seek more effective LOX activators, and explore the exact mechanisms of LOX. The current study sheds new light on the optimization of culture conditions for tissue-engineered cartilage in vitro.

Data availability

The original contributions presented in this study are included in the article material. Further inquiries can be directed to the corresponding authors.

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References

1. Chen, S., Fu, P., Wu, H. & Pei, M. Meniscus, articular cartilage and nucleus pulposus: a comparative review of cartilage-like tissues in anatomy, development and function. *Cell. Tissue Res.* **370** (1), 53–70 (2017).
2. Dajiang Du, P., Hsu, Z., Zhu, C. & Zhang Current surgical options and innovation for repairing articular cartilage defects in the femoral head. *J. Orthop. Translation* (21):121–128. (2020).
3. Zhi et al. The regulation of cartilage extracellular matrix homeostasis in joint cartilage degeneration and regeneration. *Biomaterials* (268): 120555. (2021).
4. Mikic, B., Isenstein, A. L. & Chhabra, A. Mechanical modulation of cartilage structure and function during embryogenesis in the chick. *Ann. Biomed. Eng.* **32** (1), 18–25 (2004).
5. Chen, J. et al. Improvement of in vitro three-dimensional cartilage regeneration by a novel hydrostatic pressure bioreactor. *Stem Cells Translational Med.* **6** (3), 982–991 (2017).
6. Jonathan, W., Bourne, Peter, A. & Torzilli Molecular simulations predict novel collagen conformations during cross-link loading. *Matrix Biol.* **30** (5–6), 356–360 (2011).
7. Uebelhart, D. & Delmas, P. D. The dosage of collagen cross- links in the study of bone and cartilage degradation. *Pathol. Biol. (Paris).* **41** (10), 951–961 (1993).
8. Joni, M. et al. Lysyl oxidase is essential for normal development and function of the respiratory system and for the integrity of elastic and collagen fibers in various tissues. *Am. J. Pathol.* **167** (4), 927–936 (2005).
9. Wei Wang, X., Wang, F. & Yao Chao Huang. Lysyl oxidase family proteins: prospective therapeutic targets in Cancer. *Int. J. Mol. Sci.* **23**, 12270 (2022).
10. Maki, J. M. et al. Inactivation of the Lysyl oxidase gen lox leads to aortic aneurysms, cardiovascular dysfunction, and perinatal death in mice.2002;(**106**)19:2503–2509 .
11. May Alarab, Maria, A. T., Bortolini, H., Drutz, S., Lye, O. & Shynlova LOX family enzymes expression in vaginal tissue of premenopausal women with severe pelvic organ prolapse. *Int. Urogynecol. J.* (21):1397–1404. (2010).
12. Goto, Y. et al. Transforming growth factor-beta1 mediated up-regulation of Lysyl oxidase in the kidneys of hereditary nephrotic mouse with chronic renal fibrosis. *Virchows Arch.* **447**(5):859–868 .
13. Roy, R., Polgar, P. & Wang, Y. Regulation of Lysyl oxidase and cyclooxygenase expression in human lung fibroblasts: interactions among TGF-beta, IL-1 beta, and prostaglandin E. *J. Cell. Biochem.* **62** (3), 411–417 (1996).
14. Yoshida, M. & Fujii, K. Differences in cellular properties and responses to growth factors between human ACL and MCL cells. *J. Orthop. Sci.* **4** (4), 293–298 (1999).
15. Kothapalli, C. R. & Ramamurthi, A. Lysyl oxidase enhances Elastin synthesis and matrix formation by vascular smooth muscle cells. *J. Tissue Eng. Regen Med.* **3** (8), 655–661 (2009).
16. Silvia, P. & Canelón, J. M. Wallace. Substrate strain mitigates effects of β-aminopropionitrile induced reduction in enzymatic crosslinking. *Calcif Tissue Int.* **105** (6), 660–669 (2019).
17. Li, Y. et al. Three-Dimensional porous scaffolds with biomimetic microarchitecture and bioactivity for cartilage tissue engineering. *ACS Appl. Mater. Interfaces*. **11** (40), 36359–36370 (2019).
18. Zhang, L. et al. Regeneration of human-ear-shaped cartilage by co-culturing human microtia chondrocytes with BMSCs. *Biomaterials* **35**, 4878–4887 (2014).
19. Robins, S. P. An enzyme-linked immunoassay for the collagen cross-link pyridinoline. *Biochem. J.* **207**, 617–620 (1982).
20. Ding, C. et al. Regeneration of a goat femoral head using a tissue-specific, biphasic scaffold fabricated with CAD/CAM technology. *Biomaterials* **34** (28), 6706–6716 (2013).
21. Chen, W. et al. Superabsorbent 3D scaffold based on electrospun nanofibers for cartilage tissue engineering. *ACS Appl. Mater. Interfaces*. **8** (37), 24415–24425 (2016).
22. Enobakhare, B. O., Bader, D. L. & Lee, D. A. Quantification of sulfated glycosaminoglycans in chondrocyte/alginate cultures, by use of 1,9-dimethylmethylene blue. *Anal. Biochem.* **243**, 189–191 (1996).
23. GK & Enwemeka, R. A simplified method for the analysis of hydroxyproline in biological tissues. *Clin. Biochem.* **29**, 5 (1996).
24. Wu, C. et al. Defective autophagy in chondrocytes with Kashin-Beck disease but higher than osteoarthritis. *Osteoarthr. Cartil.* **22**, 1936–1946 (2014).
25. Anika Jonitz, K., Lochner, T., Tischer, D., Hansmann, R. & Bader TGF-β1 and IGF-1 influence the re-differentiation capacity of human chondrocytes in 3D pellet cultures in relation to different oxygen concentrations. *Int. J. Mol. Med.* **30**, 666–672 (2012).
26. Morglin, M., Paulsson, M., Heinegard, D., Aebi, U. & Engel, J. Evidence of a defined Spatial arrangement of hyaluronate in the central filament of cartilage proteoglycan aggregates. *Biochem. J.* **307**, 595–601 (1995).
27. Makris, E. A., Responte, D. J., Paschos, N. K., Hu, J. C. & Athanasiou, K. A. Developing functional musculoskeletal tissues through hypoxia and Lysyl oxidase-induced collagen cross-linking. **111**(45): e4832–4841. (2014).
28. Asanbaeva, A. et al. Cartilage growth and remodeling: modulation of balance between proteoglycan and collagen network in vitro with b-aminopropionitrile. *Osteoarthr. Cartil.* **16**, 1–11 (2008).
29. Ahmed et al. Role of transforming growth Factor-β in skeletal muscle fibrosis: A review. *Int. J. Mol. Sci.* **20**, 2446 (2019).
30. Weiss, A. & Attisano, L. The TGF beta superfamily signaling pathway. *Wiley Interdiscip Rev. Dev. Biol.* **2** (1), 47–63 (2013).

31. Usui, T. et al. Extracellular matrix production regulation by TGF-beta in corneal endothelial cells. *Invest. Ophthalmol. Visual Sci.* **39** (11), 1981–1989 (1998).
32. Biernacka, A., Dobaczewski, M. & Frangogiannis, N. G. TGF-β signaling in fibrosis. *Growth Factors*. **29**, 196–202 (2011).

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Declarations

Competing interests
The authors declare no competing interests.

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