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Translating the application of transforming growth factor-β1, chondroitinase-ABC, and lysyl oxidase-like 2 for mechanically robust tissue-engineered human neocartilage

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Abstract

Strategies to overcome the limited availability of human articular chondrocytes and their tendency to dedifferentiate during expansion are required to advance their clinical use and to engineer functional cartilage on par with native articular cartilage. This work sought to determine whether a biochemical factor (transforming growth factor-β1 [T]), a biophysical agent (chondroitinase-ABC [C]), and a collagen crosslinking enzyme (lysyl oxidase-like 2 [L]) are efficacious in forming three-dimensional human neocartilage from expanded human articular chondrocytes. Among the treatment regimens, the combination of the three stimuli (TCL treatment) led to the most robust glycosaminoglycan content, total collagen content, and type II collagen production. In particular, TCL treatment synergistically increased tensile stiffness and strength of human neocartilage by 3.5-fold and 3-fold, respectively, over controls. Applied to two additional donors, the beneficial effects of TCL treatment appear to be donor independent; tensile stiffness and strength were increased by up to 8.5-fold and 3-fold, respectively, over controls. The maturation of human neocartilage in response to TCL treatment was examined following 5 and 8 weeks of culture, demonstrating maintenance or further enhancement of functional properties. The present study identifies a novel strategy for engineering human articular cartilage using serially passaged chondrocytes.

KEYWORDS

cartilage tissue engineering, chondrocyte expansion, chondroitinase-ABC, human articular chondrocytes, lysyl oxidase-like 2, neocartilage maturation

1 | INTRODUCTION

Current surgical treatments to repair cartilage lesions involve microfracture, chondroplasty, osteochondral autograft/allograft (e.g., mosaicplasty), and autologous chondrocyte implantation (Huang, Hu, & Athanasiou, 2016; McCormick et al., 2014; Montgomery et al., 2014). However, formation of fibrocartilaginous tissue, inadequate integration with adjacent host tissue, donor site morbidity, lesion size restrictions, and poor long-term outcomes signify the need for further improvement of techniques for effective cartilage repair (Makris, Gomoll, Malizos, Hu, & Athanasiou, 2015; Richter, Schenck, Wascher,

& Treme, 2016). As a promising early disease-modifying treatment for cartilage degeneration, tissue engineering aims to replace cartilage lesions with in vitro-generated neocartilage possessing functional properties akin to native tissue (Huey, Hu, & Athanasiou, 2012).

Human articular chondrocytes (hACs) as an autologous cell source are used for cell-based and tissue-engineered cartilage products for clinical use in knee cartilage defect (Huang et al., 2016). Chondrocyte expansion in monolayer is known to dedifferentiate cells, elevating type I collagen expression and diminishing chondrogenic potential (Cournil-Henrionnet et al., 2008; Schulze-Tanzil, 2009; von der Mark, Gauss, von der Mark, & Muller, 1977). As a result, tissue-engineered

cartilage using expanded hACs is generally not sufficiently biomimetic. Previously, an effective regimen by a combined treatment of transforming growth factor-β1 (TGF-β1), growth differentiation factor-5 (GDF-5), and bone morphogenetic protein-2 (BMP-2) during postexpansion in aggregate culture has been shown to revert hACs into a chondrogenic phenotype with reproduced type II collagen and glycosaminoglycan (GAG)-rich matrix (Murphy, Huey, Hu, & Athanasiou, 2015). Scaffold-free neocartilage constructs derived from the redifferentiated hACs generated robust cartilaginous tissue, though functional properties of the resulting neocartilage were insufficient and significantly lower when compared with the properties of native tissue.

To improve the functionality of engineered cartilage, various exogenous stimuli including signalling molecules and matrix-modifying agents have been investigated (Kwon, Paschos, Hu, & Athanasiou, 2016). Particularly, direct modification of the engineered tissues with a biophysical agent (chondroitinase-ABC [c-ABC]) and a collagen crosslinking enzyme (lysyl oxidase-like 2 [LOXL2]), in modulating biochemical content and enhancing collagen crosslinks, respectively, has led to improvements in the mechanical properties of engineered cartilage (Makris, Responte, Paschos, Hu, & Athanasiou, 2014; Natoli, Responte, Lu, & Athanasiou, 2009; Natoli, Revell, & Athanasiou, 2009). Temporal suppression of GAG in tissue-engineered cartilage by c-ABC, an enzyme that degrades chondroitin and dermatan sulfates (Prabhakar et al., 2006), has been shown to significantly enhance collagen production and increase tensile mechanical properties (Bian et al., 2009; Natoli, Responte, et al., 2009; Natoli, Revell, et al., 2009). Interestingly, a combined treatment of c-ABC and TGF-β1 synergistically increased collagen content in neocartilage and significantly improved tensile stiffness when compared with application of each factor alone (Responte, Arzi, Natoli, Hu, & Athanasiou, 2012). LOXL2 forms covalent pyridinoline (PYR) crosslinks between collagen fibres, which play an important role in controlling swelling properties of proteoglycan to provide appropriate tensile and shear properties of cartilage (Asanbaeva, Masuda, Thonar, Klisch, & Sah, 2007). Exogenous LOXL2 has been shown to significantly improve tensile properties of engineered musculoskeletal tissues, resulting in increased amount of PYR crosslinking (Makris, Responte, et al., 2014). Applying TGF-β1, c-ABC, and LOXL2 simultaneously to bovine fibrocartilaginous tissues enhanced tensile properties by increasing collagen density and fibril diameter (Makris, MacBarb, Paschos, Hu, & Athanasiou, 2014), though it is still unclear if the combined effects will likewise be seen for articular cartilage or for tissues generated using human cells.

The application of exogenous stimuli and the resulting effect can vary with different species and cell passage (Akens & Hurtig, 2005; Guerne, Sublet, & Lotz, 1994). Interestingly, hACs isolated from donors of different ages have been shown to reveal distinctive responses to different growth factors (Guerne, Blanco, Kaelin, Desgeorges, & Lotz, 1995). Outcome discrepancies resulting from differences in species, passage number, and donor source motivate the present work for translating regimens verified in animal cells to human cells. Evaluating the responsiveness of human passaged cells from multiple donors to the exogenous stimuli (TGF-β1, c-ABC, and LOXL2)

not only will confirm the utility of developing regimens first using animal cells but also will lead to effective strategies for engineering functional human cartilage tissue.

The scaffold-free, self-assembling process has been successful in generating mechanically robust neocartilage (Athanasiou, Eswaramoorthy, Hadidi, & Hu, 2013). Matrix development and mechanical properties in self-assembly of articular cartilage over 8 weeks of culture have been shown to recapitulate the developmental process of native articular cartilage (Ofek et al., 2008). In this prior report, during the phase of neocartilage development (1–3 weeks), following cadherin-mediated cell interactions, chondrocytes produced immature matrices consisting primarily of type VI collagen and chondroitin-6-sulfate (CS-6). During the maturation phase at 4 weeks of culture and beyond, the formed construct contained mature matrices consisting primarily of type II collagen with pericellularly localized type VI collagen and decreased level of CS-6/CS-4 ratio, mimicking the developmental process of native tissue.

Given the remarkable role of LOXL2 and c-ABC, together with TGF-β1, in generating neocartilage using animal cells (Makris, MacBarb, et al., 2014; Makris, Responte, et al., 2014; Natoli, Revell, et al., 2009), it is of interest to apply them towards engineering human articular cartilage. The objectives of this study were (a) to investigate the translatability of a biochemical factor, a biophysical agent, and a collagen crosslinking enzyme, applied on expanded hACs, on improving the biochemical and mechanical properties of engineered human neocartilage; (b) to evaluate the robustness of the response using different donors; and (c) to investigate neocartilage maturation. In Study I, TGF-β1, c-ABC, and LOXL2 were applied alone or in combination to test the hypothesis that efficacies shown using animal cells are also applicable to human cells. Furthermore, it was hypothesized that a combined treatment of the three stimuli results in the greatest enhancement of neocartilage functional properties. In Study II, the most effective combination of stimuli from Study I was carried forward to test the hypothesis that the influence of treatment on improving hAC neocartilage properties is donor independent. Just as the stimuli were effective during the developmental phase of the engineered neocartilage, it was also hypothesized that the functional properties are further enhanced during the maturation phase.

2 | MATERIALS AND METHODS

2.1 | Isolation and expansion of chondrocytes

Human articular cartilage was obtained from the Musculoskeletal Transplant Foundation (Kansas City, MO), a tissue bank. This research is exempt from institutional review board approval because it does not constitute as human subject research. The demographic information of the donors are as follows: Donor 1: 43 years of age, male, and Caucasian; Donor 2: 19 years of age, male, and Caucasian; and Donor 3: 21 years of age, male, and Caucasian. Cartilage was minced, washed in washing medium composed of Dulbecco's modified Eagle's medium with high glucose/GlutaMAX™ (Life Technologies, Grand Island, NY) and 1% penicillin–streptomycin–fungizone (P/S/F; Lonza, Basel,

Switzerland), and followed by digestion in 0.2% collagenase type II (Worthington, Lakewood, NJ) and 3% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) for 18 hr at 37°C in washing medium. Isolated chondrocytes were passed through a 70-μm strainer to remove any undigested cartilage. The cells were counted and stored in liquid nitrogen until use. hACs were expanded in chemically defined chondrogenic culture medium (CHG; Dulbecco's modified Eagle's medium with high glucose/GlutaMAX, 1% P/S/F, 1% nonessential amino acids [Life Technologies], 1% ITS+ Premix [BD Biosciences], 50 μg/ml ascorbate-2-phosphate [Sigma-Aldrich, St. Louis, MO], 40 μg/ml L-proline [Sigma], 100 μg/ml sodium pyruvate [Sigma], and 100 nM dexamethasone [Sigma]), supplemented with 2% fetal bovine serum, 1 ng/ml TGF-β1 (PeproTech, Rocky Hills, NJ), 10 ng/ml platelet-derived growth factor (PeproTech), and 5 ng/ml basic

fibroblast growth factor (PeproTech). Cells were passaged three times using 0.05% Trypsin–EDTA (Life Technologies), followed by 0.2% collagenase type II solution (Figure 1a).
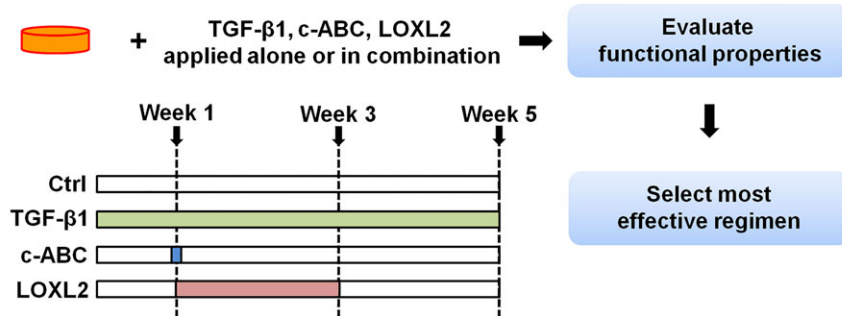
2.2 | Aggregate culture for postexpansion chondrogenic differentiation

Passaged hACs were seeded on 1% agarose-coated plates at a density of 750,000 cells per ml and maintained in aggregate culture for 7 days in CHG supplemented with 10 ng/ml TGF-β1, 100 ng/ml GDF-5, and 100 ng/ml BMP-2. After 7 days of culture, aggregates were treated with 0.05% Trypsin–EDTA and digested in 0.2% collagenase type II
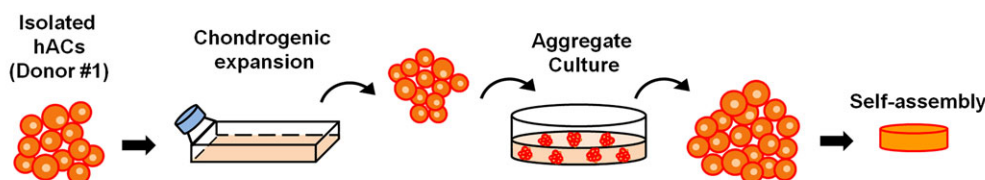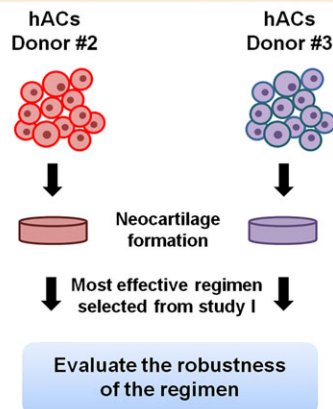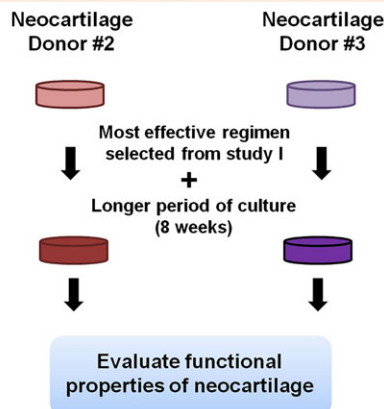

FIGURE 1 Schematic diagram of the study. (a) Isolation and expansion of human articular chondrocytes (hACs). hACs underwent chondrogenic expansion and aggregate culture before self-assembly of neocartilage. (b) Study I: Neocartilage derived from Donor 1 treated with transforming growth factor-β1 (TGF-β1), chondroitinase-ABC (c-ABC), and lysyl oxidase-like 2 (LOXL2), separately or in combination, was evaluated to determine the most effective regimen to improve functional properties. (c) Study II: The most effective regimen selected from Study I was carried forward and applied to two additional donors to examine the robustness of neocartilage and maturation using the TGF-β1 + c-ABC + LOXL2 regimen. Ctrl: control [Colour figure can be viewed at wileyonlinelibrary.com]

solution. Digested cells were filtered through a 70-μm strainer and used in neocartilage formation (hAC neocartilage) (Figure 1a).

2.3 | Neocartilage self-assembly and treatment

hAC neocartilage was formed through the self-assembling process as described previously (Hu & Athanasiou, 2006). Briefly, a custom stainless steel mould, structured with 5-mm diameter cylindrical prongs, was immersed in 2% agarose solution in a 48-well plate and allowed to solidify. Solidified agarose 5-mm well plates were washed with washing medium at least twice prior to cell seeding. A total of 2×10^6 hACs in 100 μl of CHG supplemented with 200 unit/ml hyaluronidase type I-S from bovine testes (Sigma) and 2 μM cytochalasin D (Enzo Life Sciences, Farmingdale, NY) were added to each well. For treatment of TGF-β1, c-ABC, and LOXL2, self-assembled neocartilage was maintained in CHG supplemented with (a) 10 ng/ml TGF-β1, (b) 2 unit/ml c-ABC, or (c) 0.15 μg/ml LOXL2. On the basis of previously identified regimen of combined treatment of TGF-β1, c-ABC, and LOXL2 (Makris, MacBarb, et al., 2014), in Study I, neocartilage was treated with c-ABC at Day 7, LOXL2 from Day 7 to Day 21, and TGF-β1 throughout the culture period (Figure 1b). The robustness of the response using different donors and neocartilage maturation were examined in Study II using the regimen determined in Study I (Figure 1c). At Day 7, c-ABC was applied for 4 hr at 37°C, and 1 mM zinc sulfate was used to stop the reaction for 10 min at 37°C. From Day 7 to Day 21, LOXL2 was added continuously in CHG with 0.146 mg/ml hydroxylysine and 1.6 μg/ml copper sulfate. CHG was added 4 hr after seeding and exchanged every 24 hr. For the first 72 hr, 2 μM cytochalasin D was added in CHG. After neocartilage was unconfined from the agarose well plate and transferred to an uncoated 24-well plate, medium was exchanged every other day.

2.4 | Histological evaluation

Neocartilage constructs were fixed in 10% neutral-buffered formalin for histological evaluation. Samples were embedded in paraffin and sectioned at a thickness of 5 μm. Sections were subsequently processed and stained with hematoxylin and eosin (H&E), Safranin O, and Picrosirius Red using standard protocols. Immunohistochemistry was used to detect type I and II collagen in samples. The type I and II collagen expression was developed using Vectastain ABC and DAB substrate kits.

2.5 | Mechanical and biochemical evaluation

After 5 or 8 weeks of culture, hAC neocartilage was collected for mechanical and biochemical assessment (n = 4–8 per donor). For mechanical properties, compressive and tensile properties of neocartilage were evaluated. Unconfined compressive testing was performed using an Instron 5565. Samples were submerged in phosphate buffered saline and preconditioned by applying 15 cycles of 5% compressive strain followed by sequential stress-relaxation tests at 10% and 20%. The standard linear solid model was used to determine instantaneous modulus (E_i), relaxation modulus (E_r), and coefficient of viscosity (μ; Mow, Kuei, Lai, & Armstrong, 1980). Tensile testing was performed using a TestResources 840L. Samples were tested at a constant strain rate of 1% per second. Young's modulus (E_Y) and ultimate tensile strength (UTS) were analysed using a custom MATLAB program.

For biochemical evaluation, the GAG and total collagen content in each sample was assessed. Weights of samples were measured before and after samples were frozen and lyophilized. Samples were digested in 125 μg/ml papain (Sigma) in phosphate buffer for 18 hr at 60°C. GAG content was measured using a Blyscan Glycosaminoglycan Assay kit (Biocolor, Newtownabbey, Northern Ireland). Total collagen content was measured using a chloramine-T hydroxyproline assay and SIRCOL collagen standard (Accurate Chemical and Scientific Corp., Westbury, NY).

2.6 | Statistical analysis

All data are presented as mean ± standard deviation. The statistical differences of mechanical properties and biochemical contents among conditions were analysed in a one-way analysis of variance with Tukey's post hoc test using the JMP v12 statistical software package. Groups with different letters indicate that there are statistically significant differences ($p < 0.05$).

3 | RESULTS

3.1 | Study I

3.1.1 | Gross morphology and histology of hAC neocartilage

In Study I, hAC neocartilage was subjected to exogenous stimuli TGF-β1, c-ABC, and LOXL2 alone or in combination to investigate their effects on improving the functional properties. Gross morphological images of self-assembled neocartilage are shown in Figure 2a. In general, neocartilage thickness increased when treated with TGF-β1 compared with groups without TGF-β1. The surface also appeared uneven following treatment with TGF-β1 or with TGF-β1 + c-ABC (TC) and appeared curved following treatment with TGF-β1 + LOXL2 (TL). Neocartilage treated with all three stimuli (TGF-β1 + c-ABC + LOXL2 or TCL) was flatter and more opaque than all other groups, possibly due to increased matrix deposition.

For histological evaluation, hAC neocartilage was assessed using H&E, Safranin O, and Picrosirius Red staining (Figures 2b and S1). Cross-sectional images of the entire neocartilage in all conditions are shown in Figure S2. Neocartilage in all conditions displayed chondrocytes in lacunae, as demonstrated by H&E staining (Figure S3). In the native cartilage, pericellular matrix exhibited more intense staining. Due to the high cell density, the pericellular matrix in neocartilage is not as easily discerned. Although all neocartilage stained positively for Safranin O, considerably more intense staining was observed in TGF-β1-treated groups (Figure 2b). Similarly, Picrosirius Red staining was observed in all neocartilages, and the
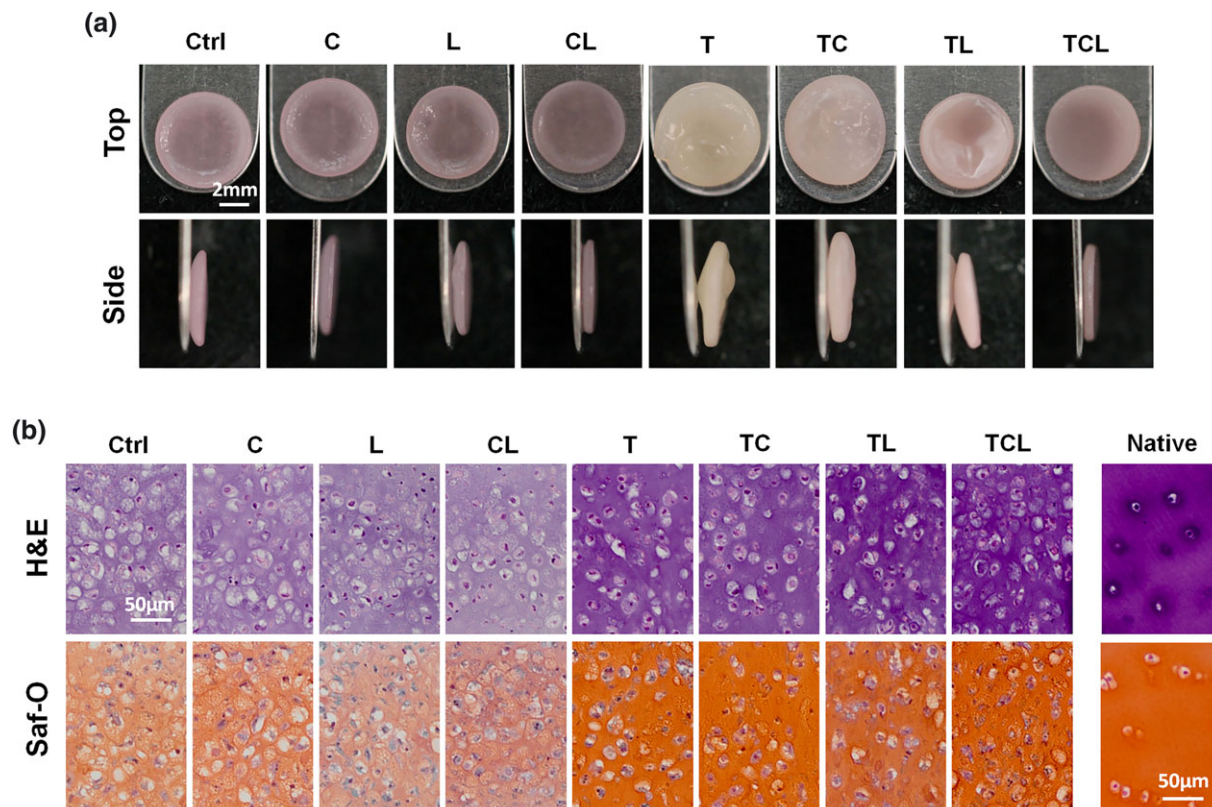
FIGURE 2 Gross morphology and histology of self-assembled human neocartilage at 5 weeks. (a) Top and side views of human neocartilage derived from Donor 1. (b) Hematoxylin and eosin (H&E) and Safranin O (Saf-O) staining of human neocartilage derived from Donor 1. Ctrl: control; C: chondroitinase-ABC; L: lysyl oxidase-like 2; CL: chondroitinase-ABC + lysyl oxidase-like 2; T: transforming growth factor-β1; TC: transforming growth factor-β1 + chondroitinase-ABC; TL: transforming growth factor-β1 + lysyl oxidase-like 2; TCL: transforming growth factor-β1 + chondroitinase-ABC + lysyl oxidase-like 2 [Colour figure can be viewed at wileyonlinelibrary.com]

intensity of staining was increased in TGF-β1-treated groups (Figure S1). Type I collagen expression was not detected in either control or TCL-treated neocartilage (Figure 6a). Type II collagen expression was richer in TCL-treated neocartilage when compared with control (Figure 6b).

3.1.2 | Biochemical and mechanical properties of hAC neocartilage

The amount of GAG and total collagen content of hAC neocartilage at 5 weeks was evaluated per wet weight (Figure 3). Treating the neocartilage with c-ABC alone did not significantly affect GAG content (Figure 3a). The GAG content in c-ABC-treated neocartilage was significantly increased by 25% when combined with TGF-β1. hAC neocartilage treated with LOXL2 alone or a combination of LOXL2 and c-ABC (CL) contained significantly lower GAG content compared with control. This effect was countered by TGF-β1 addition; TCL treatment increased GAG content by 75% compared with CL treatment (Figure 3a) and by 52% compared with control. All treatments, with the exception of c-ABC treatment alone, significantly increased collagen content when compared with control (Figure 3b). In addition, TGF-β1 treatment significantly enhanced the effect of LOXL2 as well as c-ABC with respect to collagen content. Among the treatment regimens, TL and TCL treatments resulted in significantly larger collagen contents, exhibiting 71% increases over control.

The mechanical properties of engineered hAC neocartilage were assessed in both compression and tension (Figure 3). hAC neocartilage treated with TCL exhibited the highest average instantaneous modulus of 331 kPa, which represents an 87% increase over control (Figure 3c). The relaxation modulus in hAC neocartilage, treated with each factor either alone or in combination, was not significantly different when compared with that of control (Figure 3d). Tensile stiffness was significantly increased with TL and TCL treatment by 230% and 350%, respectively, when compared with control (Figure 3e). These increases were greater than the sum of the effects of each factor applied alone, indicating synergistic effects of the combined treatment. LOXL2 treatment significantly increased UTS compared with control (Figure 3f). TGF-β1, c-ABC, or TC treatment did not enhance tensile strength. TCL treatment had the largest effect on the tensile strength, resulting in 0.8 ± 0.1 MPa. There was also synergism on the effects of TL and TCL, which increased tensile strength by 150% and 300%, respectively, over control.

3.2 | Study II

3.2.1 | The robustness of TCL treatment in forming hAC neocartilage examined using different donors

The TCL treatment was carried forward to Study II, and hAC neocartilage derived from two additional donors was evaluated for
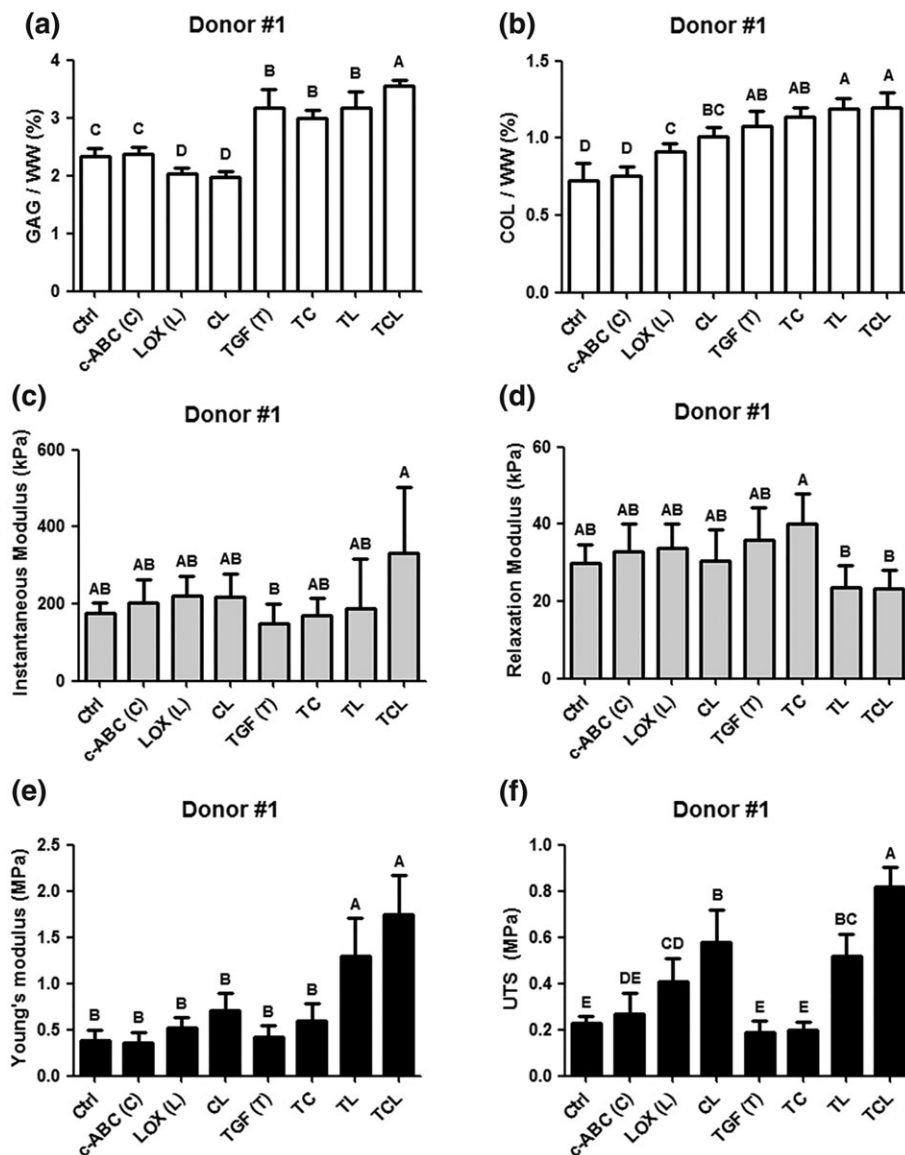
FIGURE 3 Biochemical and mechanical properties of self-assembled human neocartilage at 5 weeks. (a) Glycosaminoglycan (GAG) content in human neocartilage derived from Donor 1 normalized by wet weight (WW). (b) Total collagen (COL) content in human neocartilage derived from Donor 1 normalized by WW. (c) Instantaneous modulus and (d) relaxation modulus were assessed for compressive properties of human neocartilage derived from Donor 1. (e) Young's modulus and (f) ultimate tensile strength (UTS) were assessed for tensile properties of human neocartilage derived from Donor 1. All data presented as mean ± standard deviation. Groups with different letters indicate statistical difference ($p < 0.05$). Ctrl: control; c-ABC: chondroitinase-ABC; LOX: lysyl oxidase-like 2; TGF: transforming growth factor-β1; CL: chondroitinase-ABC + lysyl oxidase-like 2; TC: transforming growth factor-β1 + chondroitinase-ABC; TL: transforming growth factor-β1 + lysyl oxidase-like 2; TCL: transforming growth factor-β1 + chondroitinase-ABC + lysyl oxidase-like 2

the robustness of this treatment. Morphological difference of hAC neocartilage derived from different donors is shown in Figure 4. The effects of TCL treatment for Donors 2 and 3 were consistent with those seen in Study I; the treatment increased thickness of neocartilage and showed more intense histological staining (Figures 4 and S4). TCL treatment significantly increased GAG (increase by 170% and 250% for Donors 2 and 3, respectively) and collagen contents (increase by 80% and 120% for Donors 2 and 3, respectively) compared with controls (Figure 5a,b). Both control and TCL-treated neocartilage were negative for type I collagen staining (Figure 6a). Type II collagen staining was more intense in TCL-treated neocartilage when compared with control (Figure 6b).

Mechanical properties, assessed in compression and tension, were also evaluated. Similar to Donor 1, hAC neocartilage from Donors 2 and 3 was found to have an increased instantaneous modulus following TCL treatment compared with controls (Figure 5c,d). Although, no statistically significant difference was detected for the relaxation modulus of Donor 2's neocartilage after TCL treatment, an increase of 63% was observed for Donor 3's neocartilage (Figure 5c,d). Similar to neocartilage from Donor 1, the tensile stiffness and strength, following TCL treatment, increased for both Donor 2 and Donor 3, when compared with controls (Figure 5e,f). The effect was observed to be greatest for Donor 3; the tensile stiffness and strength of Donor 2 neocartilage increased by 750% and 130%, respectively, whereas
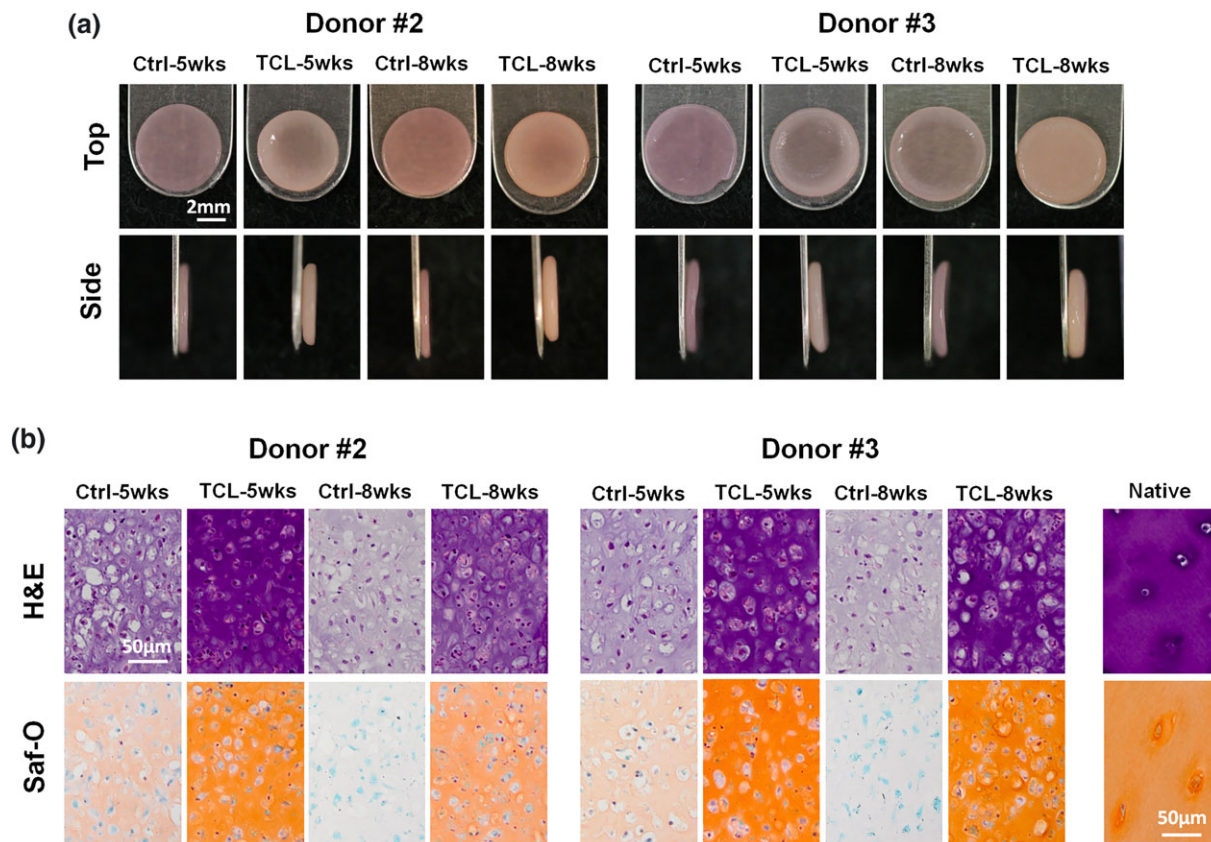
FIGURE 4 Gross morphology and histology of self-assembled human neocartilage at 5 and 8 weeks. (a) Top and side views of human neocartilage derived from Donor 2 (left panel) and Donor 3 (right panel) at 5 and 8 weeks. (b) Hematoxylin and eosin (H&E) and Safranin O (Saf-O) staining of human neocartilage derived from Donor 2 (left panel) and Donor 3 (right panel) at 5 and 8 weeks. Ctrl: control; TCL: transforming growth factor-β1 + chondroitinase-ABC + lysyl oxidase-like 2 [Colour figure can be viewed at wileyonlinelibrary.com]

Donor 3 neocartilage experienced increases of 850% and 300%, respectively.

3.2.2 | The effect of TCL treatment on hAC neocartilage maturation

Study I investigated effects up to 5 weeks in culture. To investigate TCL treatment's effect on neocartilage maturation, hAC neocartilage from Donors 2 and 3 was evaluated at 5 and 8 weeks in this study. After 8 weeks of culture, control neocartilage from both Donors 2 and 3 exhibited no apparent gross morphological difference, whereas neocartilage treated with TCL had a more opaque appearance, possibly due to cartilage matrix deposition (Figure 4). For all examined time points, Safranin O, Picrosirius Red, and type II collagen staining were more intense in TCL-treated neocartilage versus controls (Figures 4, S1, and 6b). Type I collagen production was not detected in either control or TCL-treated neocartilage (Figure 6a).

GAG content in control neocartilage from both donors remained unchanged at 8 weeks when compared with 5 weeks (Figure 5). Interestingly, GAG content following TCL treatment in Donor 3's neocartilage further increased by 16% between 5 and 8 weeks (Figure 5b). Collagen content in TCL-treated neocartilage from both donors significantly increased from 5 to 8 weeks by 27% (Figure 5a, b). Compression and tensile properties of control neocartilage did not change from 5 to 8 weeks (Figure 5). At all time points, mechanical

properties of control neocartilage were inferior to those treated with TCL. With TCL treatment, instantaneous modulus increased from 5 to 8 weeks by 40% and 53% for Donors 2 and 3, respectively (Figure 5c,d). For all engineered human neocartilage examined here, tensile stiffness and strength remained stable over 8 weeks of culture (Figure 5e,f).

4 | DISCUSSION

Although hACs represent a promising cell source for tissue-engineered cartilage products, the need for cell expansion results in loss of chondrogenic potential and, ultimately, tissue-engineered cartilage lacking sufficient chondrogenic properties. Towards engineering functional articular cartilage using human cells, this study sought to translate the application of exogenous stimuli, TGF-β1, c-ABC, and LOXL2, to generate mechanically robust tissue-engineered neocartilage. In Study I, it was hypothesized that treatment by TGF-β1, c-ABC, or LOXL2, alone or in combination, would yield efficacy similar to those seen previously with animal cells when applied during self-assembly of human neocartilage. The results indicated that LOXL2 alone and a combined treatment of TGF-β1, c-ABC, and LOXL2 (TCL) were efficacious as seen with animal cells in enhancing the properties of hAC neocartilage. As anticipated, the combined TCL treatment was most effective for enriching GAG and collagen content and for improving compressive
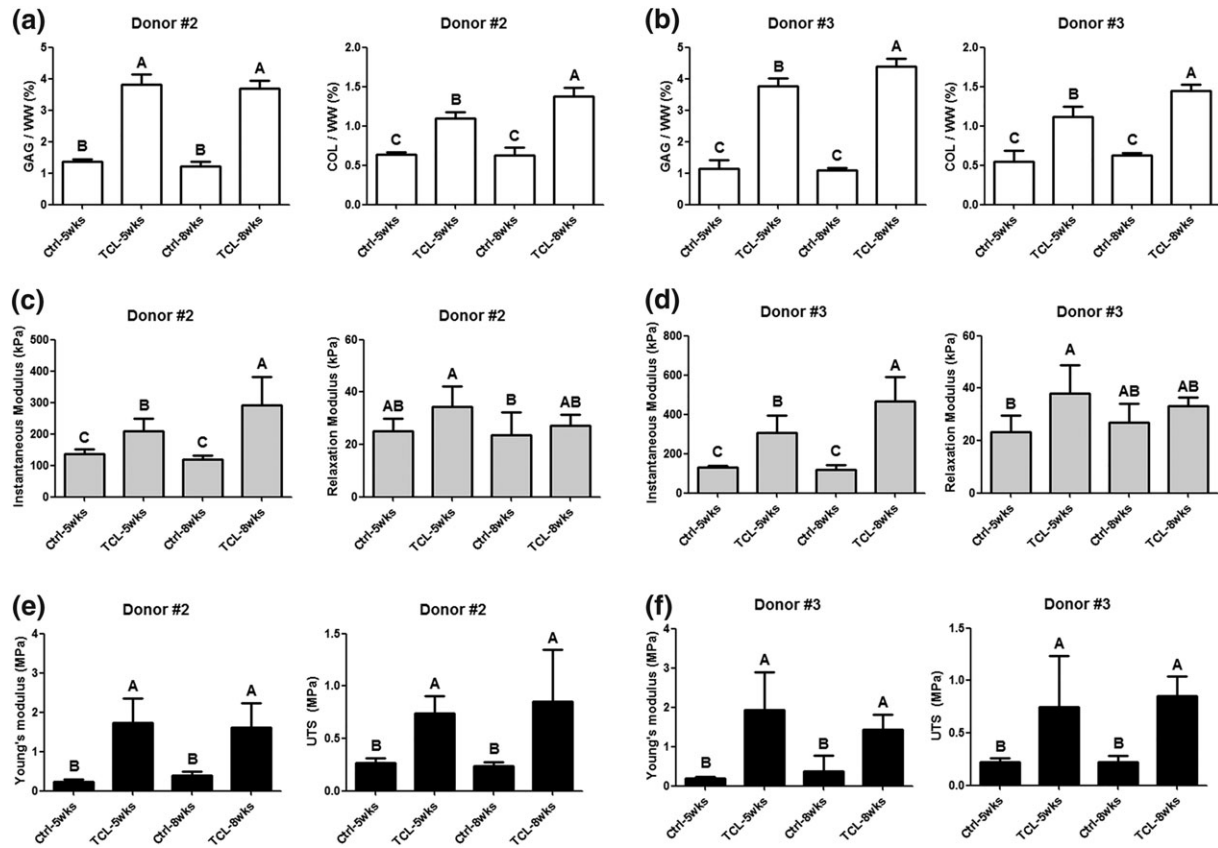
FIGURE 5 Biochemical and mechanical properties of self-assembled human neocartilage at 5 and 8 weeks. (a,b) Glycosaminoglycan (GAG) and total collagen (COL) contents per wet weight (WW) in human neocartilage derived from Donors 2 and 3. (c,d) Instantaneous modulus and relaxation modulus of human neocartilage derived from Donors 2 and 3. (e,f) Young's modulus and ultimate tensile strength (UTS) of human neocartilage derived from Donors 2 and 3. All data presented as mean ± standard deviation. Groups with different letters indicate statistical difference ($p < 0.05$)
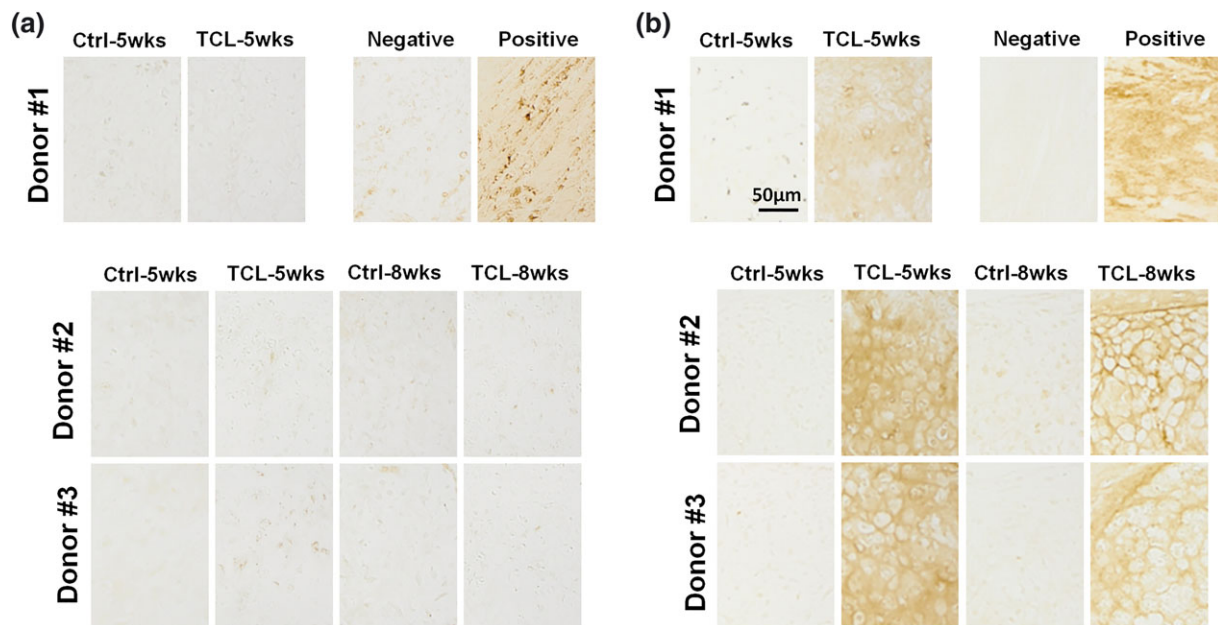

FIGURE 6 Immunohistochemistry for types I and II collagen in self-assembled human neocartilage derived from all donors. Shown is type I collagen (a) and type II collagen (b) staining of human neocartilage derived from Donor 1 at 5 weeks and human neocartilage derived from Donors 2 and 3 at 5 and 8 weeks. Nucleus pulposus and annulus fibrosus from human native intervertebral disc were used for positive and negative controls. Ctrl: control; TCL: transforming growth factor-β1 + chondroitinase-ABC + lysyl oxidase-like 2 [Colour figure can be viewed at wileyonlinelibrary.com]

properties of self-assembled hAC neocartilage constructs. Excitingly, TCL treatment synergistically increased tensile stiffness and strength of human neocartilage. In Study II, it was hypothesized that the robustness of TCL treatment on functional properties of human neocartilage would be donor independent. Engineering neocartilage constructs using expanded hACs derived from two additional donors, in the presence of TCL treatment, exhibited improved biochemical and mechanical properties, supporting the hypothesis. Given that no data exist for the effect of TCL treatment on hAC neocartilage maturation, it was further hypothesized that TCL treatment would allow human neocartilage construct properties to be retained or enhanced during neocartilage maturation. TCL treatment, followed by a longer culture period, led to enhanced compressive properties while maintaining tensile properties. Biochemically, GAG and total collagen content and type II collagen staining were maintained or enhanced during the maturation phase. The results from this study demonstrate, for the first time, that among the examined regimens, a combination of TCL has the capacity to generate a rich cartilage matrix and to increase tensile mechanical properties of human neocartilage constructs derived from passaged hAC in a synergistic manner. Further, hAC neocartilage properties were maintained or enhanced during neocartilage maturation, regardless of donor source.

In Study I, the effects of c-ABC on hAC-derived self-assembled neocartilage were largely noncomparable with those seen with animal cells (Bian et al., 2009; Natoli, Responte, et al., 2009; Natoli, Revell, et al., 2009). Treatment of c-ABC alone did not have a significant impact on GAG and collagen content or on the compressive and tensile mechanical properties (Figure 3). These results appear contradictory to previous studies using bovine ACs, which demonstrated that the temporary depletion of GAG by c-ABC treatment increased collagen content and enhanced tensile properties of tissue-engineered cartilage (Bian et al., 2009; Natoli, Responte, et al., 2009; Natoli, Revell, et al., 2009). Self-assembled neocartilage derived from bovine ACs demonstrated approximately 6% of GAG per wet weight at 2 weeks and 10% at 4 weeks (Natoli, Revell, et al., 2009), whereas self-assembled hAC neocartilage in the present work contained only 2% of GAG per wet weight even after 5 weeks of culture. Thus, given that the matrix-modifying role of c-ABC in improving functional properties is predicated on GAG depletion, there may not be sufficient GAG to deplete in the hAC neocartilage at the treated time of $t = 7$ days, resulting in no noticeable effects. However, it should be noted that c-ABC in conjunction with the other two stimuli resulted in beneficial effects. Determining an appropriate time of c-ABC application and culture duration will be necessary for c-ABC to improve functional properties of hAC neocartilage.

In contrast to c-ABC alone, LOXL2 treatment by itself had a significant effect in enhancing tensile mechanical properties of hAC neocartilage, consistent with a previous study performed with bovine ACs (Makris, Responte, et al., 2014); tensile stiffness and strength were increased by 25% and 100%, respectively, over controls. The stimulatory effect of LOXL2 treatment by itself was further elevated in the presence of TGF-β1; this is a novel finding because a combined treatment of TGF-β1 with LOXL2 (TL) has not been previously examined. In this experiment, TL treatment significantly increased GAG and collagen contents by 60% and 33%, respectively, when compared with LOXL2 treatment only. A significant increase in tensile stiffness and

strength by 160% and 25%, respectively, was also shown when comparing TL-treated against LOXL2-treated groups. Compared with individually applied TGF-β1 and LOXL2, a combined treatment, TL, synergistically increased tensile stiffness and strength by 230% and 150%, respectively, over controls, suggesting potential crosstalk between the two stimuli to promote the functional properties of hAC neocartilage. In short, TL treatment generated mechanically and biochemically improved hAC neocartilage constructs due to their synergistic effect. The potential interactions between these two stimuli, as uncovered here, may lead to other alternative ways to combine them in enhancing neocartilage functional properties.

The combined treatment of the three stimuli, TGF-β1, c-ABC, and LOXL2 on human neocartilage was the most beneficial in significantly increasing the biochemical content and tensile properties. Previously, this effect was only demonstrated for bovine fibrocartilage (Makris, MacBarb, et al., 2014). In the previous study, TCL treatment exhibited significantly enhanced collagen content and tensile mechanical properties of neofibrocartilage over untreated control, LOXL2 treatment only, and TC treatment. Efficacy was shown to be resulting from a combined benefit of increased collagen content by TGF-β1, increased fibril diameters by c-ABC, and increased PYR crosslink content and maturation by LOXL2 (Makris, MacBarb, et al., 2014). Consistently, in the present study, the TCL combination yielded hAC neocartilage with increased tensile stiffness and strength by 350% and 300%, respectively, over controls; furthermore, these effects were synergistic in nature. The improved chondrogenic functional properties demonstrate successful translatability of the TCL treatment to human cells.

It is exciting to show that the synergistic response to TCL in Study I was also demonstrated in other donors. In Study II, carrying forward a combined TCL treatment, self-assembled neocartilage constructs derived from two additional donors were generated to evaluate the robustness of TCL treatment in forming human neocartilage. Consistent with outcomes derived from Donor 1, TCL treatment on hAC neocartilage derived from Donors 2 and 3 significantly increased GAG and collagen production, as well as type II collagen staining. Type I collagen staining was not detected throughout the 8 weeks of culture in either control or TCL-treated neocartilage derived from all donors, implying that type II collagen production in TCL-treated neocartilage was the major contributor to increased collagen content. These encouraging results bode well for the assessment of additional donors in future studies to fully assess donor variability.

Mechanically, both compressive and tensile properties were significantly enhanced. Although TCL treatment was consistently effective for all donors, there were variations in the magnitude of the effect. For example, TCL treatment was more efficacious in increasing GAG content and in enhancing Young's modulus in neocartilage derived from Donors 2 and 3 (170% and 250% increase for GAG; 750% and 850% increase for Young's modulus, respectively, over control) than Donor 1 (52% increase for GAG; 350% increase for Young's modulus, over control). UTS by TCL treatment was increased in Donor 2's neocartilage by 130%, whereas neocartilage from Donors 1 and 3 both demonstrated 300% increases in UTS. It is possible that donor variation with respect to age, race, and gender can result in differential responses to the stimuli, leading to varied outcomes. Although the mechanical properties of human native cartilage were not investigated

in this study due to lack of donor samples, it is important to note the large variability of mechanical properties of native tissue as a function of location on the joint and depth. For example, compressive aggregate modulus and tensile modulus of native articular cartilage vary from 0.5 to 1.8 MPa and from 5 to 25 MPa, respectively (Akizuki et al., 1986; Athanasiou, Agarwal, & Dzida, 1994; Athanasiou, Rosenwasser, Buckwalter, Malinin, & Mow, 1991; Kempson, Freeman, & Swanson, 1968; Woo, Akeson, & Jemmott, 1976). Nevertheless, this work shows that TCL treatment led to beneficial responses in hAC-derived neocartilage regardless of donor source, resulting in enriched biochemical contents and enhanced mechanical properties.

Functional properties of bovine neocartilage were previously shown to decline after 4 weeks of self-assembly culture (Ofek et al., 2008). Total collagen content was observed to decrease by 36%, and the Young's modulus and UTS decreased by 92% and 71%, respectively, from 4 to 8 weeks of culture. This observation signified the need for exogenous stimuli to be applied after 4 weeks to enhance functional properties (Ofek et al., 2008). In contrast to this previous finding, the present study showed that human neocartilage maintained GAG and total collagen contents and retained both compressive and tensile properties (Figure 5). Aside from the difference in species, the passage number is different; primary bovine juvenile chondrocytes were used in this prior study (Ofek et al., 2008), whereas passage 3 hACs were used in this experiment. Importantly, because chondrocyte expansion in monolayer immediately brings forth dedifferentiation (Darling & Athanasiou, 2005), the hACs underwent an additional aggregate suspension culture step that has been shown to promote redifferentiation of expanded chondrocytes (Murphy et al., 2015; Wolf et al., 2008). Specifically, the present study involved a treatment consisting of culturing cell aggregates in TGF-β1, GDF-5, and BMP-2, which has been shown to lead hACs to express cartilage matrix markers including SOX9, ACAN, and COL2A1, resulting in mechanically robust neocartilage formation (Murphy et al., 2015). Thus, it is plausible that aggregate culture was responsible for the maintenance of hAC neocartilage properties during neocartilage formation and maturation, not only by enhancing chondrogenic potentials of expanded hACs but also by promoting the ability for hACs to retain their chondrogenic phenotype in self-assembly.

The beneficial effects of TCL treatment carried over 8 weeks of self-assembly culture. Excitingly, TCL treatment further enhanced compressive properties by 40% and 53% for Donors 2 and 3, respectively, without compromising tensile properties, in hAC neocartilage cultured for 8 weeks when compared with 5 weeks. Whereas GAG content was comparable or increased by 16% for Donors 2 and 3, respectively, collagen content was both significantly increased by 27% from 5 to 8 weeks. In the present study, TGF-β1 was continuously employed throughout the culture period, contributing to an increase in collagen content. Previously, it has been shown that a gradual increase in collagen content during development of bovine articular cartilage was associated with an increase in compressive properties (Williamson, Chen, & Sah, 2001). Thus, the enhanced compressive properties of hAC neocartilage at 8 weeks could be resulting from the increased collagen content, indicative of neocartilage maturation. Moreover, PYR crosslink concentration has been shown to increase in bovine cartilage from fetus to adult, correlating with

increased tensile properties (Williamson, Chen, Masuda, Thonar, & Sah, 2003). LOX-mediated PYR collagen crosslinks in cartilage explants grown in vitro have been shown to reach maturity after 7–30 days (Ahsan, Harwood, McGowan, Amiel, & Sah, 2005). With respect to engineered neotissue, neofibrocartilage treated with TCL has been shown to have significantly increased PYR crosslink content at 6 and 12 weeks, though no significant difference in the content between these two time points was observed. It was suggested that for neocartilage, longer time culture may be needed for formation of mature PYR crosslinks to influence tensile properties (Makris, MacBarb, et al., 2014). In the present work, although PYR crosslink content in hAC neocartilage was not examined, it is plausible that in light of the previous work with neofibrocartilage, a time point longer than 8 weeks may be necessary to reach mature PYR crosslinks, towards enhancing neocartilage tensile properties. Nevertheless, due to its efficacy in neocartilage development, as well as maturation, TCL treatment is emerging as a potent stimulation regimen in human cartilage repair and regeneration.

The limited amount of hACs available from donor sites hampers the use of autologous cells for tissue-engineered products. The cell number that can be obtained from cartilage can vary by donor, and extensive expansion may be necessary if the initially available cell number is low. Particularly, the self-assembling process uses a high initial seeding density of cells because it seeks to recapitulate the developmental process of native cartilage formation. The self-assembling process is analogous to mesenchymal condensation in which large numbers of cells in close proximity effect extensive cell-to-cell interactions, resulting in extensive cartilage matrix formation. Because high numbers of cells are needed for the self-assembling process to initiate, it is essential that cells be expanded in passage to achieve the high numbers needed. However, extensive cell expansion can result in loss of their ability to redifferentiate and fully revert into the chondrogenic phenotype (Schulze-Tanzil et al., 2002; Stokes et al., 2001). The gene expression of cartilage matrix proteins (i.e., aggrecan and type II collagen) has been shown to decrease as hAC passage number increased (Lin et al., 2008). Moreover, cell morphology and matrix components in tissue-engineered cartilage were largely affected by chondrocyte passage number (Kang, Yoo, & Kim, 2007). These phenotypic changes call for a new technique to effectively passage chondrocytes while maintaining their chondrogenic phenotype. A recent study showed that leporine ACs at passage 7, applied with postexpansion aggregate culture, generated self-assembled neocartilage with properties similar to or better than those derived from cells with lower passage numbers. Because the present study used hACs at passage 3, it would be of interest to examine whether the efficacy of aggregate culture, shown in the leporine model, can be applicable to hACs. Further, applying TCL to neocartilage formed from hACs of higher passage numbers should be examined to see if similar improvements can be obtained. In addition, because many growth factors are involved, clinical use of the engineered neocartilage would require determination of the residual levels of growth factors in the final product used for cartilage repair (U.S. Food & Drug Administration, 2008). Such studies will be valuable towards further enhancing the translational use of autologous cells for cartilage repair and restoration.

5 | CONCLUSION

The lack of sufficient functional properties of neocartilage derived from expanded hACs presents challenges towards clinical use. This study showed that exogenous stimuli TGF-β1, c-ABC, and LOXL2 enhanced the functional properties of neocartilage, derived from expanded hACs. When the three stimuli were combined, tensile stiffness and strength in human neocartilage were synergistically increased by 350% and 300%, respectively, over controls. The influence of TCL treatment was evaluated using two additional donors. For all donors, tensile stiffness and strength were increased by up to 850% and 300%, respectively, with TCL treatment. Treatment effects persisted in long-term culture, even after cessation of certain stimuli (of the three stimuli, only TGF-β1 was applied continuously). Compressive properties and total collagen content were further improved between 5 and 8 weeks without compromising tensile properties or GAG content. This suggests that TCL treatment improves mechanical maturation of constructs and long-term stability of cartilage matrix synthesis. Inasmuch as neocartilage treated with TCL exhibits robust functional properties and maturation, it is recommended that TCL treatment be considered as a new, effective strategy for tissue-engineering human neocartilage.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Picrosirius red staining in self-assembled human neocartilage. Shown is the staining of (A) human neocartilage derived from Donor 1 at 5 weeks and (B) human neocartilage derived from Donor 2 and 3 at 5 and 8 weeks.

Figure S2. Cross-sectional images of the entire self-assembled human neocartilage. Shown is the H&E staining of (A) human neocartilage derived from Donor 1 at 5 weeks and (B) human neocartilage derived from Donor 2 and 3 at 5 and 8 weeks.

Figure S3. H&E staining of self-assembled human neocartilage (scale-up of Figure 2B; Donor 1 at 5 weeks).

Figure S4. H&E staining of self-assembled human neocartilage (scale-up of Figure 4B; Donor 2 and 3 at 5 and 8 weeks).