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METHODS ARTICLE

Growth Factor Stimulation Regimes to Support the Development and Fusion of Cartilage Microtissues

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Scaffold-free tissue engineering strategies using cellular aggregates, microtissues, or organoids as "biological building blocks" could potentially be used for the engineering of scaled-up articular cartilage or endochondral bone-forming grafts. Such approaches require large numbers of cells; however, little is known about how different chondrogenic growth factor stimulation regimes during cellular expansion and differentiation influence the capacity of cellular aggregates or microtissues to fuse and generate hyaline cartilage. In this study, human bone marrow mesenchymal stem/stromal cells (MSCs) were additionally stimulated with bone morphogenetic protein 2 (BMP-2) and/or transforming growth factor (TGF)-β1 during both monolayer expansion and subsequent chondrogenic differentiation in a
AQ: 3 microtissue format. MSCs displayed a higher proliferative potential when expanded in the presence of TGF-β1 or TGF-β1 and BMP-2. Next, the chondrogenic potential of these human MSCs was explored in a medium–high throughput microtissue system. After 3 weeks of culture, MSCs stimulated with BMP-2 during expansion and differentiation deposited higher levels of glycosaminoglycans (GAGs) and collagen, while staining negative for calcium deposits. The fusion capacity of the microtissues was not impacted by these different growth factor stimulation regimes. After 3 weeks of fusion, it was observed that MSCs stimulated with TGF-β1 during expansion and additionally with BMP-2 during chondrogenic differentiation deposited the highest levels of sulfated GAGs. No increase in type X collagen deposition was observed with additional growth factor stimulation. This study demonstrates the importance of carefully optimizing MSC expansion and differentiation conditions when developing modular tissue engineering strategies (e.g., cellular aggregates and microtissues) for cartilage tissue engineering applications.

Keywords: hyaline cartilage, osteoarthritis, spheroids, scaffold-free, growth factors, chondrogenesis

Impact Statement

Mesenchymal stem/stromal cells (MSCs) represent the most commonly used cell source for the fabrication of microtissues for modular cartilage and endochondral bone tissue engineering strategies. However, little is known about how different chondrogenic growth factor stimulation regimes during cellular expansion and differentiation influence their capacity to fuse and generate specific types of cartilage. This study identifies specific combinations of growth factors that can not only be used to increase the yield of human bone marrow-derived MSCs during monolayer expansion but also support the generation of more hyaline cartilage microtissues. These microtissues maintain robust fusion potential, pointing to the benefit of using such growth factor simulation regimes when developing modular cartilage tissue engineering strategies.

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Introduction

Cellular microtissues and organoids are finding increased use in the fields of tissue engineering and regenerative medicine. They are typically generated from cellular aggregates via a *self-assembly* process and mimic many of the cell-to-cell and cell-to-extracellular matrix interactions observed during embryogenesis.[1] Microtissues are derived from clusters of cells that adopt a specific phenotype when exposed to certain combinations of growth factors, leading to the production of tissue-specific extracellular matrix components.[2] Organoids are multicellular 3D structures, commonly derived from embryonic stem cells or induced pluripotent stem cells, which mimic the functions of a target tissue or organ.[3] Microtissues and organoids possess an intrinsic fusion capacity that allows them to be used as biological building blocks in the biofabrication or 3D bioprinting of engineered grafts.[4–6] This has motivated the use of cartilage microtissues for the engineering of scaled-up grafts for biological joint resurfacing.[7–10] Furthermore, microtissues have been integrated into extrusion-based bioprinting platforms to enable the 3D bioprinting of more complex cartilage grafts.[11–13] However, those biofabrication strategies require large numbers of cells to generate sufficient quantities of microtissues. Bone marrow-derived mesenchymal stem/stromal cells (MSCs) represent the most commonly used cell source for the fabrication of microtissues for musculoskeletal applications. In the context of cartilage tissue engineering, it is well known that the chondrogenic potential of these cells is strongly influenced by the specific growth factors they are exposed to during monolayer expansion and subsequent differentiation[14,15]; however, it remains unclear how such stimuli will influence the development of cartilage microtissues generated using MSCs.

Numerous studies have explored the impact of different growth factor stimulation regimes on stem/progenitor cell proliferation and subsequent chondrogenic differentiation.[16–22] Stewart and collaborators (2007) and Morito and collaborators (2009) observed that fibroblast growth factor-2 (FGF-2) increased the proliferation of MSCs during monolayer expansion and improved subsequent chondrogenic differentiation in a pellet culture system. In addition, it is well established that members of the transforming growth factor-beta (TGF-β) family of growth factors, and in particular TGF-β1 and TGF-β3, promote chondrogenesis of MSCs.[23–32] While the majority of studies typically use either TGF-β1 or TGF-β3 in isolation to induce chondrogenesis of MSCs, additional stimulation with factors such as bone morphogenetic protein 2 (BMP-2)[33] and BMP-7[34] has been shown to synergistically enhance cartilage matrix deposition. The growth factors BMP-6, human insulin growth-like factor 1, and BMP-2 have also been shown to be potent inducers of chondrogenesis in adipose-derived stem cells.[35,36] However, there is limited understanding of how various chondrogenic growth factor stimulation protocols during MSC expansion and differentiation affect the ability of cellular aggregates or microtissues to fuse and form functional cartilage. While numerous studies point to the potential of cartilage microtissues for cartilage[12,37,38] and endochondral bone tissue engineering,[39,40] the culture conditions to rapidly expand MSCs and generate large numbers of microtissues remain unclear.

Therefore, in this study, human bone marrow MSCs were additionally stimulated with TGF-β1 ± BMP-2 during both monolayer expansion and differentiation, and their capacity to undergo chondrogenesis in microtissue format was assessed over 3 weeks of culture. In this study, we demonstrated the impact of these growth factor stimulation regimes on the proliferation of human MSCs and on the development of hyaline cartilage microtissues generated using a medium–high-throughput approach. We further demonstrate the assembly of a large number of microtissues into a cohesive hyaline cartilage tissue construct. Overall, this study demonstrates the importance of carefully optimizing MSC expansion and differentiation conditions when developing modular tissue engineering strategies using cellular microtissues.

Methods

Bone marrow MSC isolation and expansion

MSCs were isolated from fresh human bone marrow (Lonza, USA). Briefly, a total of 500,000 cells were seeded in 175 cm^2 culture flasks and maintained in expansion medium (X + F) made from the following: Dulbecco's modified Eagle's medium (DMEM) high glucose (4500 mg/L of glucose) (Gibco) supplemented with: 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco), 10% (w/v) fetal bovine serum (FBS), and 5 ng/mL basic FGF-2 under physioxic conditions (37°C in a humidified atmosphere with 5% CO_2 and 5% O_2). At 80% confluency, MSCs were trypsinized using 0.25% (w/v) trypsin ethylenediaminetetraacetic acid (EDTA) and frozen in liquid nitrogen using 10% (w/v) DMSO diluted in 90% (w/v) FBS solution. All the experiments were performed using one donor and three independent experiments for each assay. AQ: 4

MSC expansion under different growth factor stimulation regimes

MSCs at passage two were expanded up to passage three in T75 cm^2 flasks using one of three different growth factor regimes: (1) expansion media (defined below) containing 5 ng/mL of FGF-2 (termed "X + F") that was considered the control group of this study; (2) expansion media supplemented with 5 ng/mL of FGF-2 and 1 ng/mL of TGF-β1 (termed "X + FT"); and (3) expansion media supplemented with 5 ng/mL of FGF-2, 1 ng/mL of TGF-β1, and 1 ng/mL of BMP-2 (termed "X + FTB," Fig. 1 and Table 1). Expansion media was composed of DMEM high glucose (hgDMEM GlutaMAX) (Gibco) with 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco), and 10% (w/v) FBS. All growth factors were purchased from PeproTech (UK). After the cells reached 80% confluency, they were trypsinized to fabricate microtissues. F1, T1

In addition, MSCs were expanded using the three different regimes described in 6-well plates to obtain a cell proliferative curve. For this, MSCs at passage two were seeded at a density of 9×10^3 cm^2 per well, trypsinized, and counted every 2 days up to day 14.

Tripotentiality assay of human MSCs

For chondrogenic differentiation, MSCs expanded using the "X + F" expansion media described in the "MSC

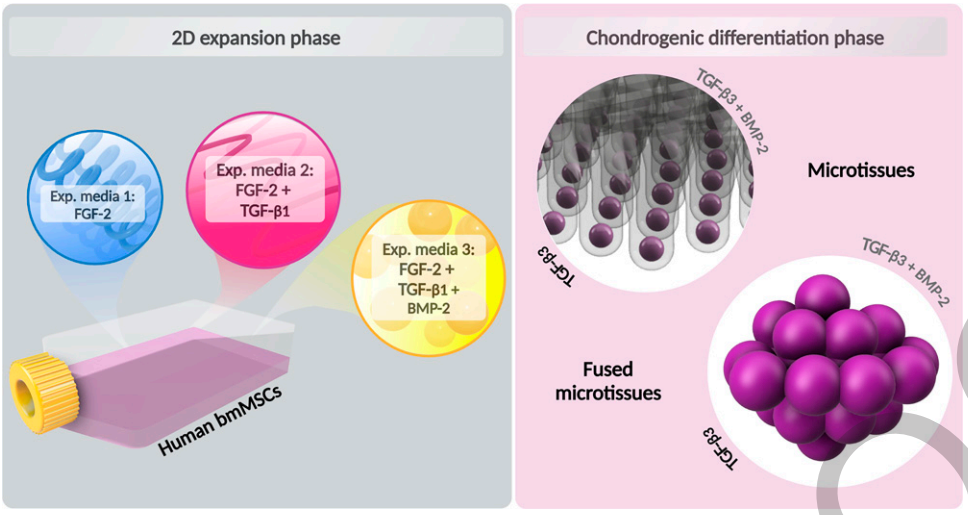

FIG. 1. Culture conditions during monolayer expansion and differentiation of MSCs. Human bone marrow-derived MSCs were expanded for up to 8 days in monolayer culture in one of three different media: (1) expansion media (X-Pan) supplemented with FGF-2 (termed "X + F"); (2) expansion media supplemented with FGF-2 and TGF-β1 (termed "X + FT"); and (3) expansion media supplemented with FGF-2, TGF-β1, and BMP-2 (termed "X + FTB"). Next, cells from each group were trypsinized and used to generate microtissues. These microtissues were then exposed to one of following two differentiation media: (1) chemically defined media (CDM) supplemented with TGF-β3 (termed "CDM + T"); and CDM supplemented with TGF-β3 and BMP-2 (termed "CDM + TB"). ("Image created using BioRender."). MSCs, mesenchymal stem/stromal cells; FGF, fibroblast growth factor; TGF, transforming growth factor; BMP, bone morphogenetic protein.

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expansion under different growth factor stimulation regimes" section were trypsinized at passage three, and 250,000 cells were centrifuged for 5 min at 650 *g* in *Eppendorfs* to fabricate pellets. After 24 h, pellets were chondrogenically induced using chemically defined media (CDM) with TGF-β3 (termed "CDM + T"). The CDM consisted of hgDMEM GlutaMAX, 100 U/mL penicillin, 100 μg/mL streptomycin (both Gibco), 100 μg/mL sodium pyruvate, 40 μg/mL L-proline, 50 μg/mL L-ascorbic acid-2-phosphate, 4.7 μg/mL linoleic acid, 1.5 mg/mL bovine serum albumin, 1 × insulin–transferrin–selenium, 100 nM dexamethasone, and 2.5 μg/mL amphotericin B (all from Sigma). This media was additionally supplemented with 10 ng/mL of human TGF-β3 (PeproTech, UK). Pellets were cultured under physioxic conditions (37°C in a humidified atmosphere with 5% CO_2 and 5% O_2) for 21 days.

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For osteogenic and adipogenic induction, a final density of 100,000 cells was seeded in a 6-well plate and cultured for 48 h in the "X + F" cell expansion media. After achieving 80% confluency, 100 nM of dexamethasone, 10 mM glycerol phosphate, and 0.05 mM ascorbic acid (all from Sigma) were added to the media for osteogenic differentiation. For adipogenic differentiation, 100 nM of dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 50 μM indomethacin were added to the cell culture media. After 21 days, the cell monolayers were washed twice with 1× phosphate buffer saline (PBS, Sigma-Aldrich, Wicklow, Ireland) before the addition of iced ethanol at room temperature for 10 min. After removing the ethanol, each well was washed first with 1× PBS and then with distilled water. Next, 2 mL of 1% of Alizarin Red (Sigma) was added to stain for calcium deposits in the osteogenic-induced cells, and cells that were adipogenic induced were stained with 1% Oil Red O (Sigma) for 1 min to detect lipid deposits. Next, samples were rinsed with distilled water and imaged using a phase contrast microscope (Zeiss).

Microtissue formation and chondrogenic induction

Human bone marrow MSC microtissues were fabricated using an in-house high-throughput nonadherent agarose hydrogel microwell system,[8–10] in which it is possible to fabricate up to 1889 microtissues. For the fabrication of the microtissues, human bone marrow MSCs at passage three were seeded into each mold at a density of 4×10^3 cells/microtissue in X + F media. After 24 h to allow for cell aggregation, the microtissues were induced under one of two different regimes: (1) CDM + T (as described above) and (2) CDM supplemented with 10 ng/mL of TGF-β3 and 100 ng/mL of BMP-2 (termed "CDM + TB," Fig. 1 and Table 2). The microtissues were cultured under physioxic conditions

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Table 1. Details of Media Compositions for MSC Expansion (with Abbreviations)

Medium composition	*Abbreviation*
Expansion media with FBS, PS, and 5 ng/mL FGF-2	**X + F**
Expansion media with FBS, PS, 5 ng/mL FGF-2, and 1 ng/mL TGF-β1	**X + FT**
Expansion media with FBS, PS, 5 ng/mL FGF-2, 1 ng/mL TGF-β1, and 1 ng/mL BMP-2	X + FTB

FBS, fetal bovine serum; MSC, mesenchymal stem/stromal cell; FGF, fibroblast growth factor; TGF, transforming growth factor; BMP, bone morphogenetic protein.

Table 2. List of Media Compositions and Concentrations During Single and Fused Microtissues Differentiation Stage (with Abbreviations)

Medium composition	*Abbreviation*
Chondrogenic defined media (100 μg/mL sodium pyruvate, 40 μg/mL L-proline, 1 × ITS, 50 μg/mL ascorbic acid, 4.7 μg/mL linoleic acid, 100 nM dexamethasone) with 10 ng/mL TGF-β3	**CDM + T**
Chondrogenic defined media (100 μg/mL sodium pyruvate, 40 μg/mL L-proline, 1 × ITS, 50 μg/mL ascorbic acid, 4.7 μg/mL linoleic acid, 100 nM dexamethasone) with 10 ng/mL TGF-β3 and 100 ng/mL BMP-2	CDM + TB

ITS, insulin–transferrin–selenium.

(37°C in a humidified atmosphere with 5% CO_2 and 5% O_2) for 21 days.

Microtissue diameter and sphericity measurements

MSC microtissues were monitored every 2 days over the culture period, and phase contrast images were acquired using the 4× objective of an optical inverted microscope (Primovert, Zeiss, USA) equipped with a digital camera. The width and length of each single microtissue were measured using the ImageJ 1.53e software (Wayne Rasband and Contributors, USA). Next, a diameter ratio of each microtissue (sphericity) was obtained by dividing width by length. The analysis was performed using a total of 15 microtissues obtained from one cell donor, and three independent experiments were performed.

Microtissues fusion assay

Microtissues generated using MSCs expanded in X + F, X + FT, and X + FTB were manually harvested at day 2, and an average of 25 ± 5 microtissues were assembled in a 2% (w/v) agarose (Sigma)-coated well of a 48-well plate. After 10 min, either the CDM + T or the CDM + TB differentiation media was added in each well, and microtissues were allowed to fuse under physioxic conditions (37°C in a humidified atmosphere with 5% CO_2 and 5% O_2) for 21 days. Phase contrast images were taken to monitor the fusion process at 6 h and 21 days. Of note, we observed that the microtissues started to fuse to one another after 2 h (data not shown). Images were acquired using the 4× and 10× objectives of an optical inverted microscope (Primovert, Zeiss, USA) equipped with a digital camera.

Histological analysis

Initially, all samples were fixed using 4% (w/v) paraformaldehyde solution (Sigma) overnight at 4°C. Next, samples were dehydrated in graded ethanol solution series (70–100%), cleared in xylene, and embedded in paraffin wax (all from Sigma). Tissue sections of 5 μm were obtained at the central region of each sample in the transverse plane using a manual microtome (Leica) and rehydrated before staining in the tissue processor (Leica). Sections were then stained with hematoxylin and eosin, 1% (w/v) alcian blue to visualize sulfated glycosaminoglycan (sGAG), 0.1% (w/v) picrosirius red for collagen deposition, and 1% (w/v) alizarin red (pH 4.1) to identify mineral deposition (all from Sigma). Stained sections were then finally imaged using an Aperio ScanScope slide scanner (Leica).

Immunohistochemistry analysis

Immunohistochemistry assay was performed in the samples at the end of the culture period (21 days) to assess chondrogenesis efficiency. The analyses were performed for collagen I (Abcam ab90395 1:400), collagen II (Santa Cruz sc52658 1:400), and collagen X (Abcam ab49945 1:200) as previously described.[41]

Biochemical analysis

After harvesting, samples were washed twice in 1× (v/v) PBS and manually dried using the pipette. Next, 3.88 U/mL of papain enzyme in 100 mM sodium phosphate buffer/5 mM Na2 EDTA/10 mM L-cysteine, pH 6.5 (all from Sigma Aldrich) was used to digest the samples at 60°C for a maximum period of 18 h using a rotator at 10 RPM. The completely digested samples were then vortexed for 1 min and centrifuged for 5 min at 650 *g*. The DNA content, the total amount of sGAG, and collagen were obtained as previously described.[8]

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software, CA, USA). A *two-way* analysis of variance (ANOVA) was performed followed by Tukey's multiple comparison posttest to assess the influence of the culture media composition on the proliferation, diameter, and sphericity of the microtissues with time in culture. A *one-way* ANOVA was performed followed by Kruskal–Wallis multiple comparisons posttest to assess the effect of media composition on the biochemical content of the tissues. Numerical and graphical results are presented as mean ± standard deviation. Significance was determined when $p < 0.05$.

Experiment

MSCs have a higher proliferative potential during monolayer expansion when additionally stimulated with BMP-2 and/or TGF-β1

Initially, we investigated the proliferative potential of human bone marrow-derived MSCs during monolayer expansion under different growth factor stimulation regimes (Fig. 2). MSCs maintained in expansion media containing FGF-2 and TGF-β1 (here termed "*X + FT*") or media containing FGF-2, TGF-β1, and BMP-2 (here termed "*X + FTB*") had a more elongated morphology and a higher level of confluency after 8 and 14 days of monolayer culture compared with cells grown in expansion media that was only additionally supplemented with FGF-2 (here termed "*X + F*") (Fig. 2A). When MSCs were cultured in standard *X + F* expansion media, approximately 100,000 cells were retrieved

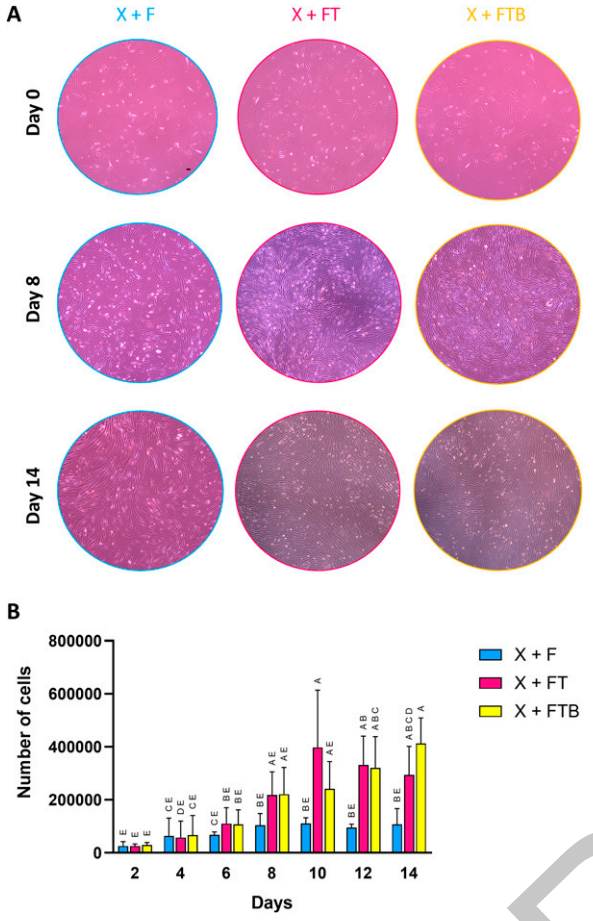


FIG. 2. MSCs have a higher proliferative potential when expanded *in vitro* in the presence of BMP-2 and/or TGF-β1. **(A)** Phase contrast images of MSCs in monolayer culture and maintained in X + F (control), X + FT, and X + FTB at days 0, 8, and 14 of culture. **(B)** Cell proliferative curve of MSCs expanded in the presence of X + F (control), X + FT, and X + FTB over 14 days of culture. The data are expressed as mean ± SD ($n = 3$). The presence of different letters (A, B, C, D, and E) between the experimental groups in the bar's charts indicates significantly different values ($p < 0.05$) obtained by nonpaired *two-way* ANOVA followed by Tukey's multiple comparisons posttest. X-Pan: Expansion media; X + F: X-Pan + FGF-2; X + FT: X-Pan + FGF-2 + TGF-β1; X + FTB: X-Pan + FGF-2 + TGF-β1 + BMP-2. ANOVA, analysis of variance; SD, standard deviation. Scale bar: 50 μm.

from each 6-well plate after 8 days of culture (Fig. 2B). In contrast, when cultured in X + FT and X + FTB, approximately 250,000 cells were retrieved from each plate at day 8 (Fig. 2B). After 14 days of culture, significantly higher numbers of cells were retrieved from plates additionally supplemented with BMP-2 and/or TGF-β1 compared with X + F (Fig. 2B). No significant differences were observed in later time points (from day 6 onwards) between groups X + FT and X + FTB (Fig. 2B).

Spherical microtissues are generated under all growth factor stimulation regimes

Next, we explored the fabrication of microtissues using the MSCs previously expanded in 2D under the different growth factor stimulation regimes (Fig. 3). MSCs from each group were seeded into microtissue forming microwells and differentiated using two different chemically defined media, supplemented with either TGF-β3 (here termed "*CDM + T*") or TGF-β3 and BMP-2 (here termed "*CDM + TB*") (Fig. 3A). Larger microtissues were generated using MSCs expanded in TGF-β1, reaching values of 450 μm after 21 days of culture (Fig. 3B). Microtissues of similar diameter (approximately 300 μm at day 21) were generated under the other stimulation regimes (Fig. 3B). The microtissues from all the groups were relatively homogeneous in shape after 21 days of culture, with sphericity values of ∼0.9 (Fig. 3C). These results suggest that the growth factors used for the expansion and differentiation of MSCs can impact the growth of microtissues without impairing their shape homogeneity.

Additional stimulation with BMP-2 during MSC expansion and differentiation supports sGAG and collagen deposition in microtissues

All microtissues stained positive for sGAG and collagen deposition, and weakly for calcium accumulation, irrespective of the growth factors that MSCs were exposed to during their expansion and differentiation (Fig. 4A). Immunohistochemical analyses showed that when MSCs were expanded in X + F and microtissues cultured in CDM + T (our control group), there was weak staining for collagen types I and X and positive staining for collagen type II (Fig. 4B). If additionally stimulated with BMP-2 during differentiation (CDM + TB), microtissues stained more intensely for collagen types I and II, but still weakly for collagen type X (Fig. 4B). Greater deposition of collagen type I was observed when MSCs were additionally stimulated with TGF-β1 during expansion (X + FT), irrespective of the growth factors used during chondrogenic differentiation (either CDM + T or CDM + TB; Fig. 4B). Again, only weak staining for collagen type X deposition was observed in these groups (Fig. 4B). When MSCs were expanded and differentiated in the presence of BMP-2, microtissues stained intensely for collagen type II and weakly for collagen types I and X (Fig. 4B). Somewhat unexpectedly, MSCs expanded in the presence of BMP-2, but not differentiated in the presence of this growth factor, stained weakly for collagen type II, and positive for collagen type X deposition (Fig. 4B).

Biochemical quantification of the microtissues showed no differences between the groups regarding DNA and sGAG/DNA content (Fig. 4C), although it appeared that additional growth factor stimulation continued to support a more proliferative phenotype during microtissue development. Total sGAG and collagen levels within the microtissues were highest for MSCs expanded and differentiated in the presence of BMP-2, with significant differences observed between this group [termed "X + FTB (CDM + TB)"] and MSCs maintained in control conditions [termed "X +F (CDM + T)"], see Figure 4C.

Additional stimulation with BMP-2 during microtissue fusion supports type II collagen deposition in scaled-up grafts

Finally, we performed a fusion assay to investigate the impact of the different growth factor stimulation regimes on

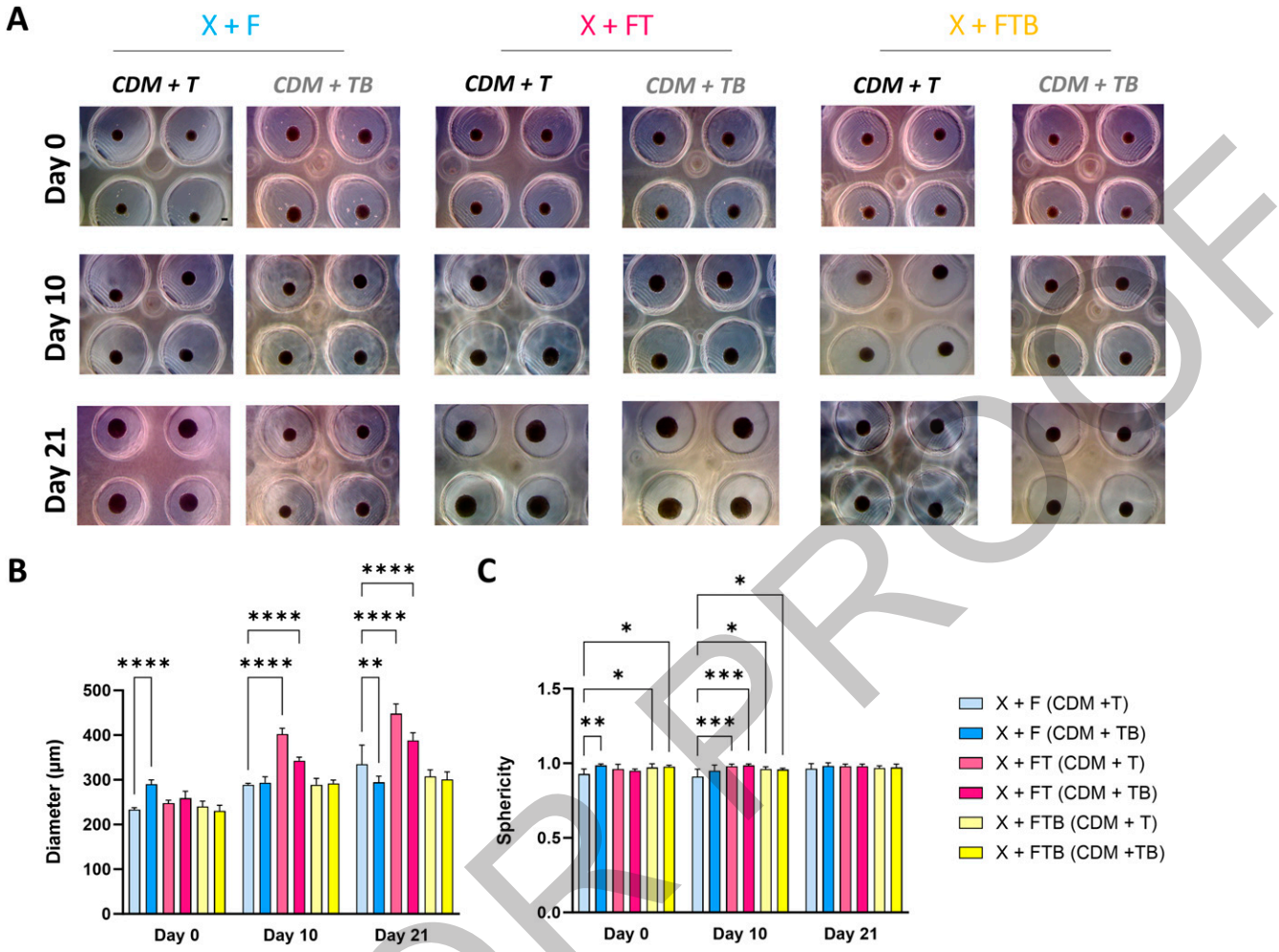
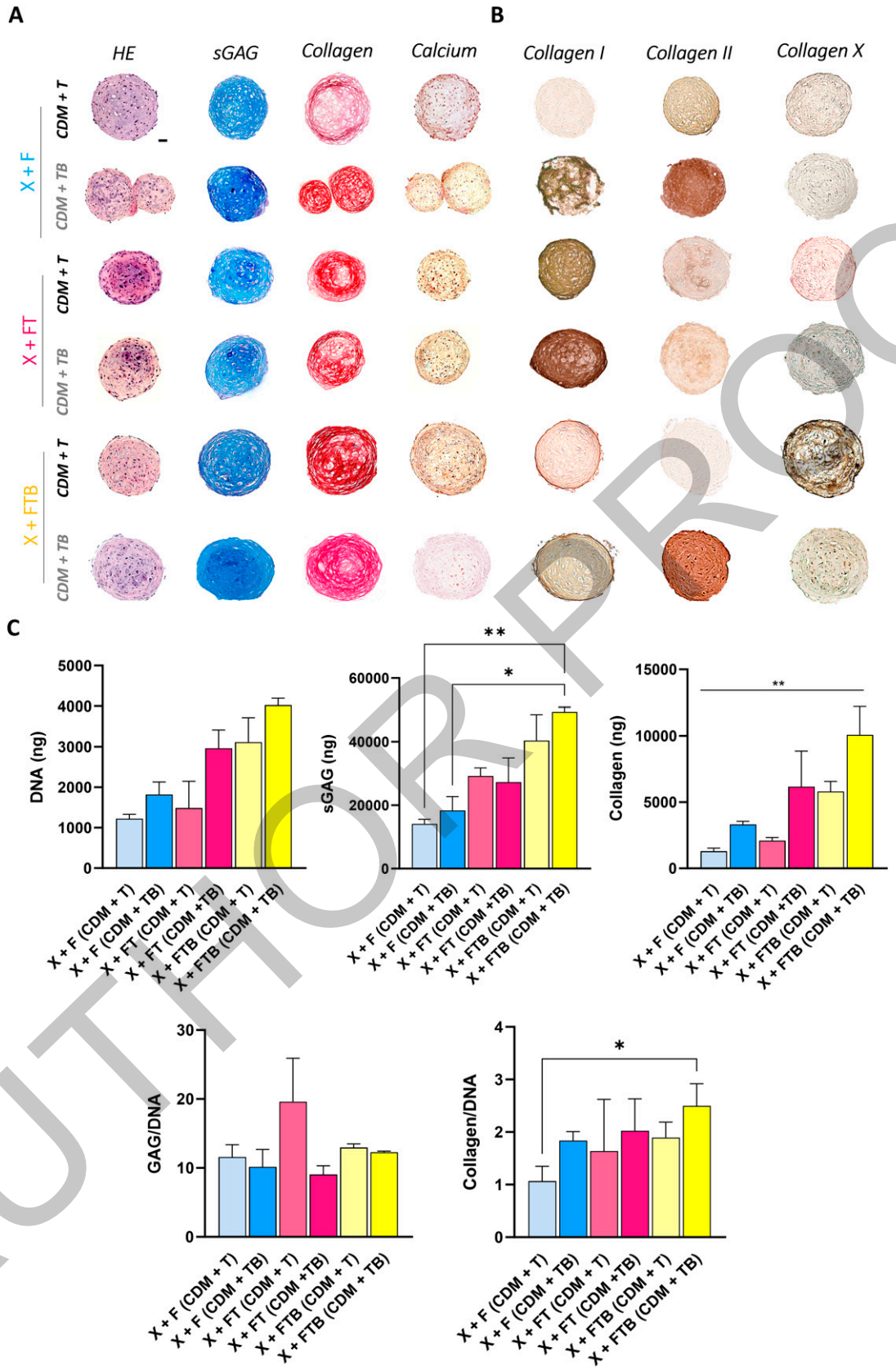


FIG. 3. Microtissues size is impacted by MSC expansion in the presence of TGF-β1. **(A)** Phase contrast images of microtissues where MSCs were expanded under the regimes of X + F (control), X + FT, and X + FTB and differentiated using CDM + T and CDM + TB media at days 0 and 21 of culture. **(B)** Diameter and **(C)** sphericity measurements of microtissues under differentiation regimes. X + F (control), X + FT, and X + FTB up to 21 days of culture. The data are expressed as mean ± SD ($n = 15$). The asterisks indicate p-values obtained by nonpaired *two-way* ANOVA followed by Tukey's multiple comparisons posttest (*$p < 0.05$; **$p < 0.001$; ***$p < 0.0005$; ****$p < 0.0001$). X-Pan: Expansion media; X + F: X-Pan + FGF-2; X + FT: X-Pan + FGF-2 + TGF-β1; X + FTB: X-Pan + FGF-2 + TGF-β1 + BMP-2. Scale bar: 100 µm.

the capacity of microtissues to fuse and generated a scaled-up tissue (Fig. 5A). Microtissue fusion was not impaired by the addition of the different growth factors during either the MSC expansion or differentiation phases (Fig. 5B). Histological assessment revealed similar cell morphology and positive staining for sGAG and collagen across all groups. In addition, none of the groups showed mineral deposition (Fig. 5C). sGAG/DNA levels were significantly higher than control conditions for MSCs expanded and differentiated in the presence of BMP-2 ["X + FTB (CDM + TB)"; Fig. 5D]. Similar trends were observed for collagen/DNA levels.

Immunohistochemistry analysis (Fig. 6) revealed that additional BMP-2 stimulation during MSC differentiation supported greater type II collagen deposition (Fig. 6B). No large differences in the intensity of collagen type I were observed across the groups (Fig. 6A). Moreover, all fused

FIG. 4. Histological, immunohistochemistry, and biochemical validation of chondrogenesis in microtissues. **(A)** Hematoxylin and eosin (HE), Alcian blue (sGAG), picrosirius red (collagen), and alizarin red (calcium) stains of microtissues at 21 days of culture. **(B)** Immunohistochemistry of collagens I, II, and X of microtissues at 21 days of culture. **(C)** Biochemical quantification of total DNA, sGAG, collagen, sGAG/DNA, and collagen/DNA of microtissues at 21 days of culture. The data are expressed as mean ± SD ($n = 3$). The asterisks indicate p-values obtained by nonpaired *one-way* ANOVA followed by Kruskal–Wallis multiple comparisons (*$p < 0.05$; **$p < 0.001$). X-Pan: Expansion media; X + F: X-Pan + FGF-2; X + FT: X-Pan + FGF-2 + TGF-β1; X + FTB: X-Pan + FGF-2 + TGF- β1+ BMP-2; CDM + T: chondrogenic defined media + TGF-β3; CDM + TB: chondrogenic defined media + TGF-B3 + BMP-2. sGAG, sulfated glycosaminoglycan. Scale bar: 50 µm.



constructs stained weakly for collagen type X deposition (Fig. 6C). These data suggest that BMP-2 has a positive impact on chondrogenesis of MSCs within fusing microtissues, appearing to support the development of a phenotypically stable hyaline-like cartilage tissue.

Discussion

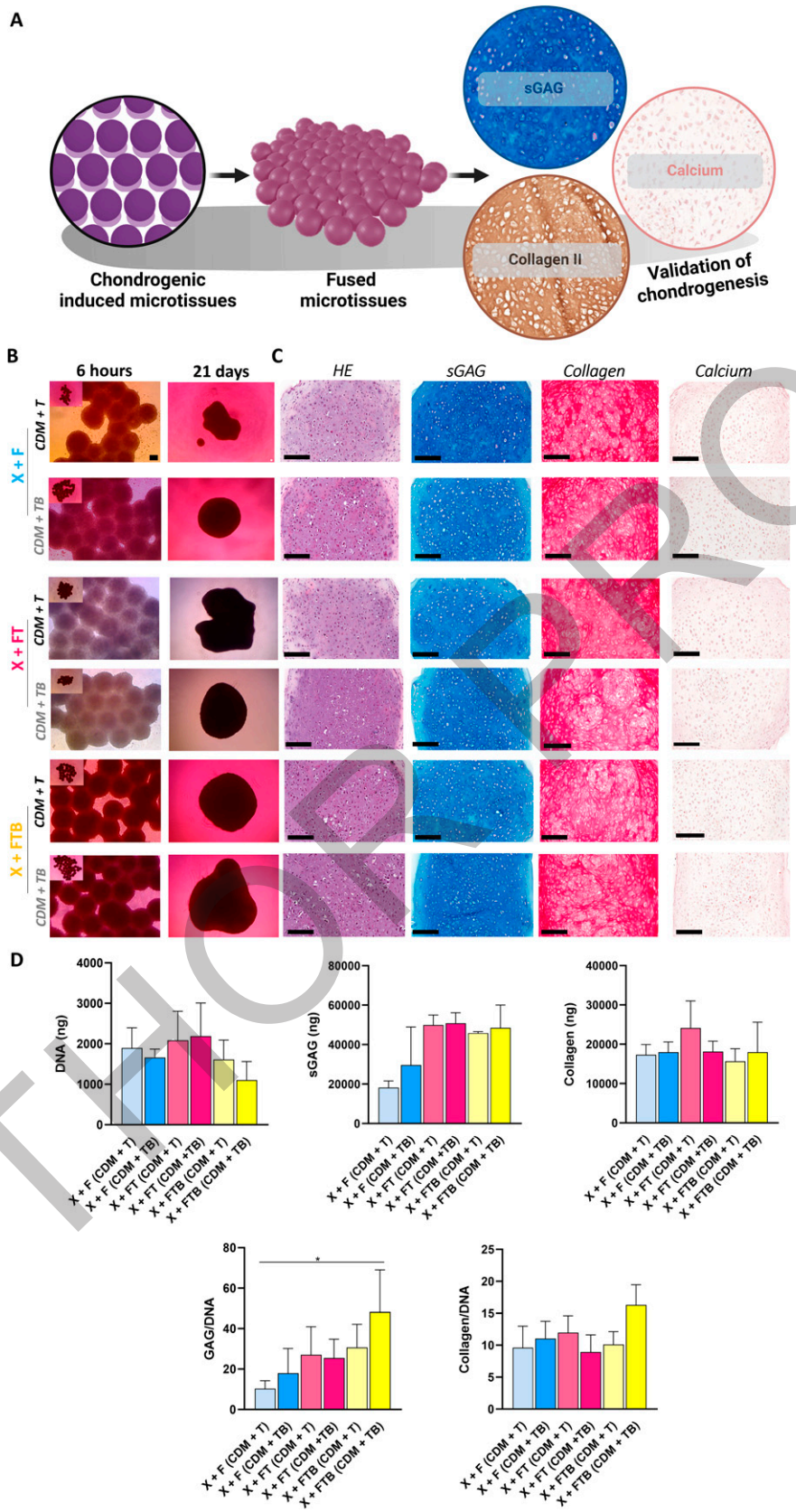
In this study, we investigated the chondrogenic differentiation potential of human bone marrow-derived MSCs in a microtissue format under different growth factor expansion and differentiation regimes. Since human bone marrow MSCs are widely utilized in tissue engineering and regenerative medicine applications, it is crucial to identify high-throughput methods capable of generating large numbers of cells with robust differentiation potential. The chondrogenic growth factor stimulation regimes explored in this study, in particular the additional use of BMP2, during both MSC expansion and differentiation, support enhanced cellular expansion and the development of superior cartilage grafts, which in the future may lead to better articular cartilage regeneration outcomes.

Initially, we investigated the impact of additional stimulation with BMP-2 and/or TGF-β1 (when combined with standard expansion media supplemented with FGF-2) on the proliferation of human MSCs in monolayer culture. We observed that additional stimulation with TGF-β1 alone, or BMP-2 and TGF-β1, supported greater proliferation compared with the control group (standard expansion media supplemented with FGF-2). This is in agreement with a number of studies reporting the positive impact of TGF-B1 supplementation on the proliferation of MSCs.[42–44] Although BMP-2 has been extensively described as a key regulator of osteogenesis and chondrogenesis, fewer studies have explored its effect on MSC expansion.[45,46] Our findings support the combined use of FGF-2, TGF-β1, and BMP-2 during the monolayer expansion of human MSCs to produce high yields of cells with high chondrogenic potential, which is beneficial for the development of tissue engineering strategies requiring large numbers of cells.

We observed that all growth factor stimulation regimes supported sGAG and collagen deposition by human MSCs in the microtissue format, although the extent of chondrogenesis did vary among the different groups. Microtissues generated using cells that were expanded in the presence of FGF-2, TGF-β1, and BMP-2 (X + FTB), and differentiated in the presence of TGF-β3 and BMP-2 (CDM + TB), stained more intensity for collagen type II and were negative for collagen type X. This is in agreement with previous studies, which observed that the combination of these two growth factors during MSC differentiation leads to a superior chondrogenic phenotype.[47–51] An interesting finding of this study is that MSC expansion and differentiation in the presence of BMP-2 supported the development of microtissues that were rich in type II collagen but generally lacking in type I collagen, indicative of a more hyaline-cartilage phenotype. This result is in agreement with previous findings, which reported a similar extracellular matrix composition in canine MSC spheroids induced with the same concentration of BMP-2 during the differentiation stage.[52] Furthermore, exposing MSCs to BMP-2 during expansion, but removing it during differentiation, would appear to support a fibrochondrogenic phenotype, as evident by the development of a tissue that was rich in sGAG and type I collagen. Together, these results suggest that the "priming" of MSCs with BMP-2 during monolayer expansion[53] influences their response to this growth factor during subsequent chondrogenic differentiation in the microtissue format. Therefore, the results of this study point to the benefit of continuous BMP-2 stimulation, during both monolayer expansion and subsequent differentiation, when seeking to engineer hyaline cartilage using MSCs.

Microtissues and organoids are attracting increased interest as biological building blocks in the biofabrication of regenerative grafts.[7,9,13,54] Such approaches require microtissues to cohesively fuse to one another and to produce the desired tissue-specific extracellular matrix components.[4,55] Several factors can influence the fusion of microtissues, including (1) the maturation level of the microtissues, as late-stage maturation has been shown to hinder cohesive fusion and self-organization[56]; (2) the use of hydrogels for encapsulating the microtissues[57–59] or polymeric scaffolds to direct the process of fusion[9]; and (3) the initial density of the microtissues, which can lead to nutrient limitations and the formation of necrotic cores.[60] In this context, we also explored if the different growth factor regimes would influence the capacity of the MSC microtissues to fuse and generate a larger hyaline cartilage graft. All growth factor stimulation regimes supported microtissue fusion and subsequent cartilage tissue formation. In particular, we observed that the addition of TGF-β1 (X + FT) and BMP-2 (X + FTB) during the expansion of MSCs, coupled with additional BMP-2 stimulation during the differentiation phase (CDM + TB), does not impair microtissue fusion, while supporting a higher cell yield during monolayer expansion and maintenance of robust chondrogenic potential. In addition, sGAG and collagen deposition in these scaled-up grafts were greatest when BMP-2 was used in both the expansion and differentiation phases. De Moor and collaborators (2019)[61] explored the fusion of human bone marrow MSC-derived microtissues for cartilage tissue engineering using TGF-β1 during the differentiation stage. Although the fused microtissues were positive for sGAG, the deposition of collagen II was relatively low, suggesting the need for further

FIG. 5. Microtissue fusion and chondrogenic potential under different growth factor stimulation regimes. **(A)** Graphical abstract of the fusion assay. Microtissues were manually harvested from the agarose microwells and fused in an agarose coated well of a 48-well plate. After 21 days, chondrogenesis was assessed. **(B)** Phase contrast images of microtissues at 0 h and 21 days of fusion under the different growth factor regimes. **(C)** HE, Alcian blue (sGAG), picrosirius red (collagen), and alizarin red (calcium) stains of fused microtissues. **(D)** Biochemical quantification of total, DNAs, sGAG, collagen, sGAG/DNA, and collagen/DNA in fused microtissues. The data are expressed as mean ± SD ($n = 3$). The asterisks indicate p-values obtained by nonpaired *one-way* ANOVA followed by Kruskal–Wallis multiple comparisons ($*p < 0.05$). ("Image created using BioRender"). X-Pan: Expansion media; X + F: X-Pan + FGF-2; X + FT: X-Pan + FGF-2 + TGF-β1; X + FTB: X-Pan + FGF-2 + TGF-β1 + BMP-2; CDM + T: chondrogenic defined media + TGF-B3; CDM + TB: chondrogenic defined media + TGF-β3 + BMP-2. Scale bar: **(B)** 100 μm; **(C)** 200 μm.



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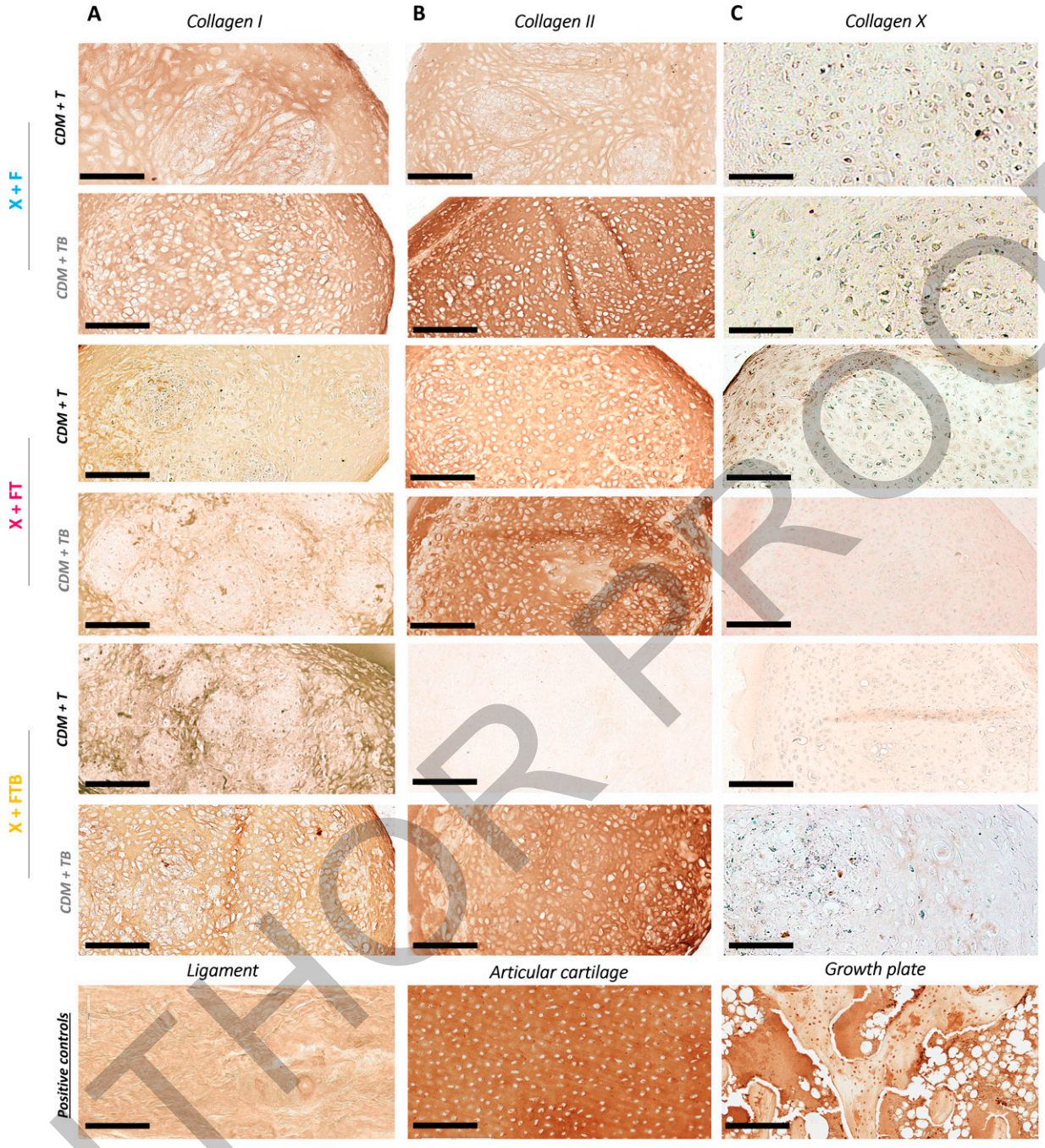

FIG. 6. Generation of phenotypically stable cartilage following microtissue fusion. Immunohistochemistry of collagen I **(A)**, collagen II **(B)**, and collagen X **(C)** for fused microtissues maintained under the different growth factor regimes after 21 days of culture and positive controls (ligament for collagen I, articular cartilage for collagen II, and growth plate for collagen X). X-Pan: Expansion media; X + F: X-Pan + FGF-2; X + FT: X-Pan + FGF-2 + TGF-β1; X + FTB: X-Pan + FGF-2 + TGF-β1 + BMP-2; CDM + T: chondrogenic defined media + TGF-B3; CDM + TB: chondrogenic defined media + TGF-β3 + BMP-2. Scale bar: 200 μm.

optimization when attempting to engineer hyaline cartilage using this cell source. In this study, we observed that the extent of chondrogenesis depended on the specifics of the MSC culture conditions, with microtissue-derived grafts cultured in the presence of both TGF-β3 and BMP-2 (CDM + TB) staining strongest for the deposition of collagen type II.

Although this work successfully demonstrated the positive impact of additional BMP-2 stimulation on human MSC expansion and cartilage microtissue development, a few limitations need to be highlighted. First, we used bone marrow-derived MSCs from a single human donor for the experiments. Since it is well-established in literature that the proliferation and differentiation of MSCs can vary depending on donor factors,[62–64] further work should explore how MSCs from different donors respond to each combination of growth factor stimulation, both during the proliferation and

differentiation stages. In addition, we did not explore the expression of key chondrogenic markers such as SOX-9, aggrecan, collagen type II, and hypertrophic markers such as collagen type X and alkaline phosphatase[47,65–67] in the single and fused microtissues at the gene level. Gene expression analyses would provide greater insight into phenotypic stability over time in culture,[68] particularly because we observed little type X collagen deposition (a marker of hypertrophy) during the differentiation stage for both single and fused microtissues in the presence of TGF-β3 and BMP-2. Some studies in the literature report the opposite effect,[69,70] whereas others find that this growth factor combination supports a more stable cartilage phenotype.[71,72] Furthermore, *in vivo* subcutaneous implantation into nude mice would provide greater insight into the long-term phenotypic stability of cartilage microtissues generated under altered growth factor stimulation regimes.[73,74] Those limitations will be addressed in future studies in our laboratory.

In conclusion, here we explored the impact of different growth factor stimulation regimes on the proliferation of human MSCs and their subsequent chondrogenic differentiation in a microtissue format. A key novelty of our approach is the priming of human MSCs using different combinations of growth factors during expansion and to immediately access the differentiation of these cells in a medium–high-throughput microtissue system. In addition, we also evaluated the fusion capacity of these microtissues and their subsequent chondrogenic potential. Interestingly, we found that the addition of TGF-β1 + BMP-2 (X + FTB) during the expansion of MSCs, followed by the continuous use of BMP-2 during their differentiation (CDM + TB), promoted the development of a stable cartilage phenotype in the single and fused microtissues, as well as the highest levels of sGAG and collagen accumulation. Our results support the future use of microtissues in modular strategies such as 3D bioprinting for the biofabrication of scaled-up human cartilage constructs that could ultimately be implanted in large animal models of articular cartilage defects to assess their regenerative potential, thereby advancing the field toward clinical translation.

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Figure 1 and schematic diagram of Figure 5 were created with BioRender.com and Fusion 360.

Author Disclosure Statement

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Supplementary Material

Supplementary Figure S1

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