Chondrogenic potential of physically treated bovine cartilage matrix derived porous scaffolds on human dermal fibroblast cells

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Abstract: Extracellular matrices have drawn attention in tissue engineering as potential biomaterials for scaffold fabrication because of their bioactive components. Noninvasive techniques of scaffold fabrication and cross-linking treatments are believed to maintain the integrity of bioactive molecules while providing proper architectural and mechanical properties. Cartilage matrix derived scaffolds are designed to support the maintenance of chondrocytes and provide proper signals for differentiation of chondroinducible cells. Chondroinductive potential of bovine articular cartilage matrix derived porous scaffolds on human dermal fibroblasts and the effect of scaffold shrinkage on chondrogenesis were investigated. An increase in sulfated glycosaminoglycans production along with upregulation of chondrogenic genes confirmed that physically treated cartilage matrix derived scaffolds have chondrogenic potential on human dermal fibroblasts. © 2015 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 104A: 245–256, 2016.

Key Words: cartilage matrix, scaffold, human dermal fibroblast, chondrogenic, shrinkage

INTRODUCTION

Cell-based therapies in cartilage tissue engineering take advantage of highly interconnected porous scaffolds capable of providing enough surface area and space for cell attachment, proliferation, nutrient, and waste exchange as well as extracellular matrix (ECM) production and deposition. The material from which the scaffold is fabricated plays a key role in chondroinduction. Various types of hydrogels, polymers, and composites of different materials that can support cartilage matrix production have been investigated.1–4 Modified native ECM may contain bioactive factors that can contribute to cell growth, migration, and chondrogenic differentiation. Human5 and porcine6 articular cartilage ECM derived scaffolds have shown promising results with human adipose derived adult stem cells and canine bone marrow stem cells respectively, supporting the hypothesis that a scaffold derived absolutely from cartilage ECM can induce chondrogenesis. The source from which the matrix is derived is a primary concern. There are limitations regarding the volume of human tissue available for this application. Porcine derived products also have limitations because of religious ethical considerations. Animal sources with high abundance but without ethical issues (e.g. bovine tissue) can be the solutions of choice. Preserving the integrity of native cartilage ECM components is a key factor in taking advantage of the total capacity of bioactive factors responsible for chondrogenesis. For cartilage tissue engineering purposes, the scaffold is expected to support the maintenance of chondrocytes or differentiation of other cells with chondrogenic potential.

In our previous work, we reported fabrication, characterization, and optimization of porous scaffolds composed of decellularized bovine articular cartilage (BAC) ECM.7 Physical treatments were applied to the cartilage matrix derived (CMD) scaffolds to enhance the cross-linking and hence to improve their mechanical properties. We showed that Ultra-Violet irradiation (UV) and Ultra-Violet+Dehydrothermal (UVDHT) treatments yield highly interconnected porous scaffolds with the best architecture and mechanical properties.8 The high porosity of the scaffolds, measured through our highly precise and accurate microvolumetric method7 provided easy cell penetration. UV and UVDHT treated 15% CMD scaffolds showed desired cell attachment and proliferation results.9

Three-dimensional culture of cells for tissue engineering necessitates the seeding of high densities of cells.9–11 This high

number of cells must either be isolated in large quantities or subcultured to reach these numbers. Therefore the cells with easy isolation and rapid proliferation would be the source of choice. While harvesting stem cells from bone marrow or through liposuction are said to be minimally invasive, other more readily available and much easier to extract cells are presently available. Fibroblast cells which can be cultivated from embryonic and adult tissues can differentiate into chondrocytes that are cells of mesenchymal origin as well. The skin with an average surface area of 1.5–2 m² in adult is one such source. High-yield of cell isolation is the other advantage of using fibroblasts over Human Mesenchymal Stem Cells (HMSCs).

The chondroinductive capabilities of physically treated CMD scaffolds on human dermal fibroblasts (HDFs) and the effects of scaffold shrinkage on chondrogenesis are investigated in the present study.

**METHODS**

**Scaffold fabrication**

The details of fabrication, characterization, and optimization of BAC matrix derived porous scaffolds have been described in our previous work. Briefly, BAC was excised from metacarpalpalphalangeal joints of calf hooves, minced, shattered, and homogenized. The resulting slurry was permeabilized in 1% TritonX-100 in a 4°C for 12 h then decellularized in DNase I and RNase A solution for another 12 h at 37°C. After intensive washing with PBS, the slurry was mixed with ultrapure water at a concentration of 15% (w/v) and placed in cylindrical Teflon molds [3 mm (H) × 7 mm (Ø)]. The molded slurries were freeze-dried and post-treated by ultraviolet light (henceforth‘UV treated group’) the treatment in the second group was followed by an additional step of dehydrothermal treatment (heating at 120°C for 24 h) (‘UVDHT treated group’). All scaffolds were sterilized using ethylene oxide before use.

**Cell isolation and culture**

Primary HDFs were isolated from redundant skin (which otherwise would have been discarded) from cosmoelastic surgery using outgrowth method described elsewhere. Briefly, the dermis was minced into ~1 mm² pieces and placed in 25 cm² culture flasks pre-wetted with culture medium consisting of high-glucose Dulbecco's modified Eagle's medium (DMEM-HG) (Cellgro Mediatech, USA) supplemented with 20% (v/v) FBS, 1% Penicillin-Streptomycin, 1% HEPES, 1% nonessential amino-acids, 1% ITS+ Premix, 50 µg/mL L-ascorbic acid, 40 µg/mL l-Proline, 10 mM Dexamethasone, 10 mM TGF-b3, 8,12 UV and UVDHT treated CMD scaffolds (n = 17 for each group at every time point) were pre-wetted with 50 µL of chondrogenic medium and incubated in a 37°C, 5% CO₂, humidified incubator for 15 min. 50 µL of HDF cell suspension (5 × 10⁵ cells) was gently seeded on each scaffold. The scaffolds were then incubated in 37°C, 5% CO₂ humidified incubator for 3 h, followed by gently addition of 1 mL of pre-warmed fresh chondrogenic medium. Chondrogenic medium was changed every two days. Cell viability tests, scanning electron microscopy for cell–matrix interaction studies and biochemical and molecular analyses were performed at days 2, 8, 15, 22, 29, 35, and 42 time points.

**Cell characterization**

**Morphology.** The morphology of HDFs on tissue culture polystyrene was studied using an inverted phase microscope as well as Confocal and Furrier Emission Scanning Electron Microscope (FESEM) micrographs of HDFs seeded on collagen scaffolds.

**Tri-lineage differentiation.** The STEMPRO® adipogenic, osteogenic, and chondrogenic differentiation kits (GIBCO, USA) were used to assess the tri-lineage differentiation capabilities of HDF cells. Human bone marrow stem cells (HBMSCs) were used as positive control. Oil-red O, Alizarin-red, and Safranin-O Staining were performed respectively for evaluation of adipogenic, osteogenic, and chondrogenic differentiation.

**Flow cytometry.** Cells were subjected to antibody staining using CD90 (primary antibody) (Calbiochem®) and Cy5 conjugated goat anti-mouse IgG secondary antibody (Thermo Scientific) prior to flow cytometry assessment. The data were acquired using a flow cytometry system (BD FACS Canto II, Becton Dickinson).

**Cell seeding**

HDF cells at passage 4 were resuspended in chondrogenic medium at a final density of 10 × 10⁶ cells/mL. The chondrogenic medium consisted of DMEM-HG supplemented with 20% (v/v) FBS, 1% Penicillin-Streptomycin, 1% HEPES, 1% nonessential amino-acids, 1% ITS+ Premix, 50 µg/mL L-ascorbic acid, 40 µg/mL l-Proline, 10 mM Dexamethasone, 10 mM TGF-b3, 8,12 UV and UVDHT treated CMD scaffolds (n = 17 for each group at every time point) were pre-wetted with 50 µL of chondrogenic medium and incubated in a 37°C, 5% CO₂, humidified incubator for 15 min. 50 µL of HDF cell suspension (5 × 10⁵ cells) was gently seeded on each scaffold. The scaffolds were then incubated in 37°C, 5% CO₂, humidified incubator for 3 h, followed by gently addition of 1 mL of pre-warmed fresh chondrogenic medium. Chondrogenic medium was changed every two days. Cell viability tests, scanning electron microscopy for cell–matrix interaction studies and biochemical and molecular analyses were performed at days 2, 8, 15, 22, 29, 35, and 42 time points.
described elsewhere. Briefly, scaffolds were digested by incubating in 1 mL of papain digest buffer (0.01 M L-cysteine, 0.01 M Na2EDTA, and 0.125 mg/mL papain in 0.1 M sodium phosphate buffer) for overnight at 65°C with intermittent agitations. After precipitating the remaining debris, the digest solution was analyzed for DNA content using Hoechst 33258 dye (0.1 μL mL⁻¹ in TEN buffer) with calf thymus DNA (Sigma, USA) as the standard. Plate reading was performed at (ex/em: 355 nm/460 nm) in a microplate reader (FLUostar OPTIMA BMG LABTECH, Germany).

Field emission scanning electron microscopy (FESEM). The samples were preserved in 2.5% glutaraldehyde and underwent dehydration process in a graded ethanol series (25%, 40%, 60%, 80%, and 100%, each for 10 min), followed by freeze-drying. The samples were then cross-section cut and coated with gold-palladium (Quorum, Q150R s) and scanned using a Quanta™ 250 FEG – FEI microscope for cell-scaffold interaction studies.

Chondrogenesis

GAGs quantification. The sulfated glycosaminoglycan (GAGs) content in the medium and the papain digested samples was assessed using a Dimethyl-methylene blue (16 mg mL⁻¹) assay with chondroitin-4-sulfate standard as described elsewhere. Absorbance reading was performed at ex–595 nm in a microplate reader.

Gene expression. RNA isolation (n = 7 for each group) was performed using Tri-Reagent® solution (Lifetechnologies™, USA) and according to the instructions from the manufacturer. RNA quantification was performed using Nanodrop ND2000 (Thermo Scientific, USA). cDNA synthesis was performed using high-capacity RNA-to-cDNA™ Kit (Applied Biosystems, USA). Subsequently, real time PCR was carried out with TaqMan® gene expression assays (Applied Biosystems, USA), using a StepOnePlus™ Real-Time PCR system (Applied Biosystem, USA). Table I shows the list of genes used in this study.

GAPDH (Hs02758991_g1) was used as the housekeeping gene for normalization. Following the normalization, data were expressed as fold change as compared to the gene expression of Day 2.

Shrinkage and contraction

A separate 48-well plate with seeded and nonseeded UV and UVDHT treated scaffolds (n = 7 for seeded and n = 5 for nonseeded) was used for shrinkage testing. Sterile plastic discs with known constant diameter of 9 mm were used as the scales (Fig. 7A). All scaffolds were treated with chondrogenic medium. The media were removed every other day for GAGs quantification. Digital images of top views of the plate were captured after discarding the medium and were analyzed with ImageJ® software for cross-sectional surface area changes. The plastic discs were used for setting the scales in each picture in ImageJ®.

Total shrinkage was calculated by dividing the cross-sectional surface area at each time point to that of the original scaffold using the following equation:

\[
\text{Total Shrinkage} \% (\text{TS}) = 100 - \left( \frac{\text{top surface area}}{\text{original top surface area}} \times 100 \right)
\]

To determine the cell-mediated contraction (CMC) using the following equation, the contraction of seeded scaffolds was deducted from that of nonseeded controls:

\[
\text{CMC} = \left( \frac{\text{original top surface area-top surface area}}{\text{original top surface area}} \right)_{\text{Seeded}} - \left( \frac{\text{original top surface area-top surface area}}{\text{original top surface area}} \right)_{\text{Non-seeded}}
\]

Statistical analysis

Two-way ANOVA was performed to find the effect of time and treatment on chondrogenic effects of CMD scaffolds at different time points. The significance value for Levene’s test of homogeneity of variances was assigned as \( P > 0.05 \), indicating that the assumption of homogeneity of variance has not been violated. Post-hoc comparisons using the
Tukey HSD test were used to define the significant difference between the scaffolds with different concentration/treatments. A value of $P < 0.05$ was defined as significant.

**RESULTS**

**Cell characterization**

The typical spindle-shaped morphology of fibroblasts is shown in inverted-phase, confocal, and FESEM micrographs [Fig. 1-i(A–C)].

Oil-red-O, Alizarin-red, and Safranin-O staining of HDF cells cultured in monolayer and treated respectively with adipogenic, osteogenic, and chondrogenic differentiation induction media for 3 weeks showed no evidences of tri-lineage differentiation and hence lack of stem cells in HDF cell population (Fig. 1-ii).

Flow cytometry graphs of HDF cells show a single cell population stained for CD90 as the fibroblast-specific marker. Cells positive for CD90 only were located in the Q4 quarter. The histogram shows that the majorities of events accumulated in the Cy5 channel and were positive for CD90 (Fig. 1-iii).

**Cell viability and proliferation**

**Confocal laser scanning microscopy.** Serial Confocal laser scanning microscopy overlay micrographs of HDF cells seeded on both groups of CMD scaffolds illustrated the cell attachment, progressive proliferation, and infiltration of the growing cells into and filling the pores of the scaffolds. Both groups kept showing the same constant dense features in confocal images after the third week of culture. Cell morphology remained fibroblastic during the 6 weeks of culture (Fig. 2).

**Cell proliferation.** DNA quantification showed a sharp upward trend during the first three weeks after culture indicating an increasing proliferation of cells on both UV and UVDHT treated CMD scaffolds. The steep slope collapsed after the third week to a mild decreasing trend (Fig. 3). No significant differences were seen in the DNA content between the UV and UVDHT groups at different time points ($P > 0.05$).

**FESEM.** Serial FESEM micrographs of cross-sectional areas of HDF seeded UV and UVDHT treated CMD scaffolds at different time points are shown in Figure 4. The first couple of images (A and A') are representative for the unseeded UV and UVDHT treated scaffolds, showing the wrinkled morphology of surfaces and filamentous structures of interconnected pores. Cell penetration, attachment, and proliferation on all over the surfaces and inside the pores of both types of CMD scaffolds showed the gradual growth and spreading.
of the cells, progressively filling all the spaces and covering the surfaces, resulting in entirely consistent cell-coated surfaces all over the constructs at Day22. FESEM images of the samples at any time point after Day22 showed surfaces covered with intertwined visually inseparable cells.

**Chondrogenic differentiation**

**GAGs content.** An upward trend of GAGs accumulation within the cells from day 2 to 15 was followed by a reduction in the third week and the subsequent plateau. There was no statistically significant difference in the GAGs production between the two groups of scaffolds ($P > 0.05$) (Fig. 5).

**qPCR.** Although an initial down regulation of COL1A1 gene was seen in day 2 compared to the negative control HDF cells, the overall trend was increasing in both UV and UVDHT groups during the culture period with a significant higher expression ($P < 0.05$) in UV group and up to 3–4 folds compared to the negative control [Fig. 6(A)]. An increase in the expression of COL1 expression has been
FIGURE 3. Total DNA by Hoechst method for cell seeded UV and UVDHT treated CMD scaffolds (n = 5 for each group) at different time-points after subtraction of average DNA in unseeded (blank) samples.

FIGURE 4. FESEM micrographs of cross-sectional areas of unseeded (A & A') and cell seeded UV and UVDHT treated CMD scaffolds at weekly time points from day 2 (B & B') to day 42 (H & H'). The scale-bars for image A and A' represent 100 μm while for the rest of the images they represent 50 μm.

FIGURE 5. Sulfated Glycosaminoglycan content in cell seeded UV and UVDHT treated CMD scaffolds (n = 5 in each group) at different time points after subtraction of the average GAGs in unseeded samples measured through DMMB method.
reported to be linked with early stages of chondrogenesis and during the condensation of cells.\textsuperscript{19}

COL1A2 gene showed an upward trend in both UV and UVDHT groups during the first 3 weeks of culture, followed by a drop and the consequent plateau in both groups during the rest of the culture period. The expression of Col1A2 was significantly higher ($P < 0.05$) in UVDHT group in all time points [Fig. 6(B)].

COL2A1 as the cartilage specific gene showed two phases of expression: an almost constant expression with even some mild down regulation during the first 2–3 weeks, followed by the second phase with a sharp upregulation in both groups, with a significantly higher trend in UVDHT group ($P < 0.05$). An overall increase up to over 3 and 4 folds in expression of COL2A1 was seen during the 7 weeks of culture in both UV and UVDHT groups [Fig. 6(C)].

The expression of ACAN, the gene encoding the cartilage proteoglycan molecule, Aggrecan, showed a drastically increasing trend in both groups with a significantly higher trend in UVDHT group compared to the negative control [Fig. 6(D)].

SOX9, the gene which contains the data for production of the transcription factor for the high mobility group domain expressed in cartilage cells, and COMP gene which is responsible for matrix assembly enhancement during chondrogenesis, both showed sharp upward slopes during the first three weeks of culture, followed by a sharp drop in SOX9 to a level even lower that negative control, while an almost steady state for COMP but still much higher than the negative control [Fig. 6(E,F)].

**Scaffold shrinkage/contraction**

Serial digital top images of representative scaffolds captured at different time points are shown in Figure 7(B). Decreases in the scaffold sizes during the time, especially in the seeded ones are visible. Surface area measurements using ImageJ\textsuperscript{6} was applied to conduct a quantitative study.

Figure 7(D) shows the average cross-sectional (top) surface area of dry, unseeded and seeded wet UV and UVDHT treated 15% CMD scaffolds ($n = 7$ for seeded and $n = 5$ for unseeded scaffolds) at seeding time and sequential weekly
time points. All wetted scaffolds either with cell suspension or equivalent volume of culture medium showed a slight non-significant ($P > 0.05$) increase in the cross-sectional surface area immediately after wetting, which is expected because of the hydrophilic spongy nature of the scaffolds and the surface tension of the medium compressing the scaffold, resulting in a light decrease in the height and a simultaneous slight increase in the top surface area as demonstrated in Figure 7(D).

As the cells attached on the surfaces and inside the pores of the scaffolds, shrinkage increased and the top surface area showed a rapid fall during the first three weeks concurrently with increasing proliferation of the cells as previously shown and confirmed in DNA content, confocal laser microscopy, and FESEM images. However, the sharp decrease in the surface area was slowed down after Day22 of culture. Unseeded scaffolds showed a downward trend of surface area during the first week of immersion in the culture medium which slowed down and showed a mild slope after Day8. This suggests that not all the shrinkage is cell induced and at least part of scaffold shrinkage results from scaffold contraction and degradation processes. Although the difference between the cross-sectional surface area of seeded UV and UVDHT scaffolds was not significant at different time points ($P > 0.05$), however a significant difference between unseeded UV and UVDHT scaffolds started

FIGURE 7. (A) Scaffold shrinkage test after HDF cell seeding at different time points was tested by measuring the cross-sectional surface area of the scaffolds. Plastic white discs with known constant diameter were used for setting the scale in each picture. (B) Serial digital images of top view of representative blank (unseeded) and HDF seeded UV and UVDHT treated CMD scaffolds at seeding time and consecutive weekly time points. The white circular plastic discs were used as the constant fixed-dimension scales. (C) The initial effect of wetting on the dimensions of porous spongy scaffolds. (D) Measurements of cross-sectional surface area (top view) of the seeded and unseeded UV and UVDHT treated CMD scaffolds ($n = 7$ for seeded and $n = 5$ for unseeded scaffolds). Significant and nonsignificant differences are shown by (*$P < 0.05$) and ($‡P > 0.0$). (E) Percentage of total shrinkage in HDF seeded UV and UVDHT treated CMD scaffolds ($n = 7$ for seeded scaffolds and $n = 5$ for unseeded scaffolds) at different weekly time-points. Part of this shrinkage is because of cell mediated contraction (CMC). Asterix stands for $P < 0.05$. 
from Day22 and onwards, showing a significantly bigger drop in the cross-sectional surface area of unseeded UVDHT scaffolds compared to unseeded UV treated scaffolds, reconfimring the higher stiffness of UV treated scaffolds. Although 15% UV and UVDHT treated CMD scaffolds have been shown to possess higher cross-linking among other CMD scaffolds,8 CMC of the scaffolds is still shrinking the scaffolds in a significant scale.

Results of total shrinkage (%) and CMC (%) at different weekly time points are shown in Figure 7(E). HDF seeded UV treated CMD constructs show a sharp decrease in the size during the first three weeks after seeding but then reach a steady state with a gentle increase in shrinkage and contraction during the next four weeks. UVDHT treated CMD scaffolds continue their sharp rise in shrinkage until the fourth week of seeding. While CMC has a considerably weaker effect on UVDHT treated scaffolds they exert the same degree of total shrinkage in the size as UV treated scaffolds.

Also having considered the equal porosity among both groups of scaffolds, there was no significant difference in total shrinkage between the UV and UVDHT groups.

The interrelation between the shrinkage, cell proliferation, and chondrogenesis
The correlation between COL2A1 and ACAN gene expression and the DNA content [Fig. 8(A,B)] showed two distinct phases of gene expression during the 42 days of culture. The first phase was determined by an increase in the DNA content with a plateau state of COL2A1 and ACAN genes expression within the first 2-3 weeks of culture. The second phase was defined by a cessation in the DNA content and a simultaneous increase in the expression of COL2A1 and ACAN genes. The asynchronicity of cell proliferation with differentiation has already been addressed in other works.20,21 Also, chondrogenic differentiation has been shown to be dependent on high cell density.9-11 During the first phase, cell proliferation
provides the high density as a prerequisite for the second phase of differentiation.

Plotting the DNA content as a function of cell proliferation against the shrinkage rate also showed a biphasic trend indicating that as the cells proliferate progressively, they contract and shrink the scaffolds accordingly until a certain critical point when a scarcity in the surface area and space leaves no sufficient room for cell proliferation [Fig. 8(C)]. Beyond this critical point any further increases in the shrinkage was contemporaneous with a slump in cell proliferation. This finding was in agreement with the previous studies.17,18

The interrelation between DNA production and the shrinkage rate is presented in Figure 8(D). The HDFs showed a tendency to produce GAGs when seeded on CMD scaffolds. GAGs production was decreased coincidentally with the scaffold shrinkage and contraction pattern. This trend suggests that scaffold shrinkage and contraction is a potential cause of the reduction in GAGs production. As addressed in other works increasing the proportional cell density favors chondrogenesis in terms of cartilage matrix production, but at a certain critical point when the cells face lack of enough space, their growth and ECM producibility diminishes.9,10

The interrelation between the expression of COL2A1 and ACAN genes and the scaffold shrinkage reconfirmed the biphasic trend of cell proliferation-differentiation and revealed that the cell proliferation phase and the increasing shrinkage were concurrent with steady state of slow chondrogenic gene expression, but during the second phase the fast increasing expression of COL2A1 and ACAN genes was simultaneous with an invariant shrinkage.

DISCUSSION

The UV and UVDHT treated CMD scaffolds with pore diameters of 215 ± 40 μm and 235 ± 50 μm respectively as well as a porosity of almost 98% have a highly interconnected highly porous structure8 which allows the cells, particularly HDFs, to penetrate deep into the core of the scaffolds. The overlay images as the result of stacking multiple images from different layers of the constructs, confirm good cell penetration into the scaffolds. Although cells seeded on UVDHT scaffolds showed a slower growth during the first 2 weeks, they reached the same confluent state at the third week as those seeded on UV treated CMD scaffolds.

The results of confocal imaging show that the decreases in DNA and gene expression are not a consequence of cell death.

In agreement with previous studies, our present findings confirmed that UV and UVDHT treated 15% bovine CMD scaffolds are suitable biocompatible constructs for HDF cells to attach and proliferate as the CMD scaffolds are mainly composed of collagen.8,22

While maintenance of chondrogenesis and production of cartilage proteins and upregulation in the expression of chondrogenic genes have been reported with stem cells,5,6,14,23 our findings documented the possibility of chondroinduction in HDF cells. Although synthesis of greater amounts of cartilaginous matrix in human MSCs cultured on UV and DHT treated porcine articular CMD scaffolds has been reported in a former study,23 however, our findings proved that properly treated CMD scaffolds can provide chondroinductive signals for HDFs as partially differentiated cells to synthesize increased levels of GAGs production and show upregulation of cartilage specific genes.

Although we previously showed that UV and UVDHT treatments can improve the mechanical properties of CMD scaffolds,9 however some shrinkage cannot be avoided as a result of CMC. The synchronous trend of cell proliferation documented in DNA quantification, and confocal microscopy and FESEM images with the shrinkage of constructs revealed that the cease and subsequent drop in cell proliferation within the constructs is a consequence of reductions in construct size. As long as the cells have sufficient room they continue their growth, secrete ECM, and contribute to cell transduction, but the exponential cell multiplication along with simultaneous proportional reduction in the growth space augmented by scaffold shrinkage and CMC will soon inhibit the cell growth and matrix production.

We showed that scaffold shrinkage may have controversial effects on chondrogenesis. Our findings revealed two distinct phases during the 6-week culture period. The initial proliferation phase and the secondary differentiation phase. The simultaneous cell proliferations and scaffold shrinkage during the proliferation phase sped up and facilitated the increments in cell density which has been reported to be critically essential for chondrogenesis.24 This phase was contemporaneous with sharp increases in SOX9 and COMP genes as major chondrogenesis enhancer genes.25–27 The chondrogenic differentiation phase started at the peak point of cell proliferation and scaffold shrinkage with a suspension in cell proliferation, and was defined by increases in the expression of Col2A1 and ACAN genes as major chondrogenic differentiation markers. The biphasic trend suggests the presence of a critical state of cell density and cell-scaffold interaction through which the chondrogenic mechanisms are triggered. This compulsory critical state was shown to be achievable partially through scaffold shrinkage which has so far been thought to have only limited effects on chondrogenesis.

CONCLUSION

In our previous work we described the process of fabrication, treatment, characterization, and optimization of BAC matrix derived porous scaffolds for cartilage tissue engineering application. The effects of different physical methods of cross-linking treatment (UV irradiation, heat treatment, and combinations of both methods) on architectural properties of CMD scaffolds were evaluated and compared. We showed that the 15% UV and UVDHT treated CMD scaffolds possessed higher compressive strength, pore diameter, porosity, cross-linking, and melting and decomposition temperature compared to all other tested different concentrations and physical treatments of CMD scaffolds in our study. Therefore, from the mechanical and architectural points of view, the 15% UV and UVDHT
treated CMD scaffolds were reported as the optimal choices.8

Although our previous results8 showed a higher degree of cross-linkage and hence improved mechanical properties in UV treated compared to UVDHT treated CMD scaffolds, no significant difference was seen in the total shrinkage, CMC, DNA content, and GAGs production between the two groups. However, UVDHT group showed significantly higher levels of Col2A1, ACAN, and COMP genes expression compared to the UV group, suggesting the UVDHT group as the CMD scaffolds of choice because of their chondroinductive potential.

Although an upregulation in gene expression does not necessarily mean that there is an increase in protein expression, however, our experimental setup showed the potential to disclose the mechanisms of gene activation during chondroinduction of HDF cells those behavior is certainly proving and reconfirming their ability to trespass lineage boundaries and differentiate into another cell type namely chondrocytes to some extent.

Improving the architecture of CMD scaffolds to enhance cross-linking and hence minimizing the total shrinkage as well as amendments in supplementary growth factors can be the alternative studies as future works.

Mechanical cues (stiffness and strain) play important role in directing stem cell differentiation, suggesting their potential impact on chondrogenesis of HDFs as well.26 Also chondrogenesis has been shown to be enhanced under hypoxic condition.29 The future works can be performed under different culture conditions, for example, with mechanical stimulation (stiffness and strain) and hypoxia. These conditions might enhance chondrogenesis of HDFs in the development of a tissue-engineered cartilage construct for effective cartilage repair and regeneration in future.

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AUTHOR CONTRIBUTIONS
A.M. performed all experiments and analyzed data and wrote the m. A. A. K. provided and pre-treated the skin samples. F.A. and K. S. helped in the experiments and proofread the article. P. P. C., S. P., T.K. and B.P-M. proof-read the article.

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