Developing a Customized Perfusion Bioreactor Prototype with Controlled Positional Variability in Oxygen Partial Pressure for Bone and Cartilage Tissue Engineering

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Skeletal development is a multistep process that involves the complex interplay of multiple cell types at different stages of development. Besides biochemical and physical cues, oxygen tension also plays a pivotal role in influencing cell fate during skeletal development. At physiological conditions, bone cells generally reside in a relatively oxygenated environment whereas chondrocytes reside in a hypoxic environment. However, it is technically challenging to achieve such defined, yet diverse oxygen distribution on traditional in vitro cultivation platforms. Instead, engineered osteochondral constructs are commonly cultivated in a homogeneous, stable environment. In this study, we describe a customized perfusion bioreactor having stable positional variability in oxygen tension at defined regions. Further, engineered collagen constructs were coaxed into adopting the shape and dimensions of defined cultivation platforms that were precasted in 1.5% agarose bedding. After cultivating murine embryonic stem cells that were embedded in collagen constructs for 50 days, mineralized constructs of specific dimensions and a stable structural integrity were achieved. The end-products, specifically constructs cultivated without chondroitin sulfate A (CSA), showed a significant increase in mechanical stiffness compared with their initial gel-like constructs. More importantly, the localization of osteochondral cell types was specific and corresponded to the oxygen tension gradient generated in the bioreactor. In addition, CSA in complementary with low oxygen tension was also found to be a potent inducer of chondrogenesis in this system. In summary, we have demonstrated a customized perfusion bioreactor prototype that is capable of generating a more dynamic, yet specific cultivation environment that could support propagation of multiple osteochondral lineages within a single engineered construct in vitro. Our system opens up new possibilities for in vitro research on human skeletal development.

Keywords: 3D cell culture, bioreactors, bone, oxygen tension, tissue engineering

Introduction

The development of synthetic biological scaffolds for bone and cartilage tissue engineering holds great potential to improve patient outcomes.1–4 However, it is technically challenging to manufacture engineered products resembling the native tissue.5,6 One of the major hurdles is the incapability to cultivate complex osteochondral constructs in vitro. Although both osteogenic and chondrogenic lineages are derived from mesenchymal cells, each lineage requires different niche as they differentiate and mature. At normal physiological conditions, the long bone contains an extensive network of vasculatures and oxygen tension (partial pressure of oxygen [pO2]) in the bone marrow is...
Further, in vitro cultures of bone-derived cells are typically conducted in normoxic conditions (149.3 Torr/21%). Hence, osteogenic lineages generally favor and propagate in an oxygenated environment. In contrast, chondrocytes are typically exposed to a hypoxic environment as native cartilage tissue lacks vascular infiltration. Several studies had also shown a hypoxic in vitro environment to enhance chondrogenesis.10–13

The effects of pO2 and preference of each lineage are the most prominent in endochondral ossification. Long bone formation is initiated from a vascularized hyaline cartilage that is composed of chondrocytes, which remains in a hypoxic environment until vascular infiltration occurs. With the development of an elaborated vascular network, the chondrocytes undergo hypertrophy that marks the initiation of significant bone building and remodeling activities.14,15

Multiple cell types such as osteoprogenitors, osteoblasts, osteocytes, and osteoclasts are subsequently derived to modulate bone growth. Diverse interactions between these cells then play a pivotal role in maintaining homeostasis of native bone and cartilage tissues, as well as in healing of injuries.16–20

Unlike the dynamic physiological environment, traditional in vitro platforms are rather simplified. They often support only single or two cell types and it is challenging to generate diverse niches with a defined pO2 variation on a single platform. Such a simplified environment is insufficient for engineering functional artificial tissues in vitro, as complex cellular interactions among multiple cell types are necessary for gain of function. Taken together, it is essential to develop new in vitro platforms that are capable of generating a controlled and stable dynamic cultivation environment; to derive and support multiple osteochondral lineages simultaneously in vitro; and to achieve complex engineered constructs.

In this article, we describe a customized perfusion bioreactor system that contains cultivation platforms with specific positional variability in pO2. Extended from a previous study by Krawetz et al.,21 the chondrogenic potency of chondroitin sulfate A (CSA) in conjunction with pO2 was studied. Further, we applied a three-step differentiation protocol to investigate the persistence of chondrogenesis after removal of CSA for 20 days, as well as the efficacy to derive osteogenic lineages from chondrocytes. This was done as an initiative to recapitulate in vitro the early avascular stages of endochondral ossification.

Materials and Methods

Cell culture and maintenance

ES-D3 (ATCC®, CRL-11632™) murine embryonic stem cells (mESC) were cultured in ESC medium comprising high-glucose Dulbecco’s modified Eagle’s medium (hgDMEM), 15% fetal bovine serum (FBS), 1% nonessential amino acids (NEAA), 1% antibiotic-antimycotic (Anti-Anti), 0.1 mM 2-mercaptoethanol (all Gibco), and 1000 U/mL leukemia inhibitory factor (LIF). For cell expansion, mESC were co-cultured on mitomycin-deactivated murine embryonic fibroblasts (MEFs) at 37°C, 5% CO2. Before experimentation, culture flasks were coated with 0.1% w/v gelatin in Dulbecco’s phosphate-buffered saline (DPBS) and mESCs were expanded for two passage phases (PP) to deplete the MEFs. mESCs between PP6 and PP15 were used.

Setting up and specifications of customized perfusion bioreactor

Descriptions to set up the bioreactor are included in Supplementary Data (Supplementary Data are available online at www.liebertpub.com/tec). Briefly, S-50-HL medical tubing (No. AAX00062; Tygon) was filled with 1.5% w/v UltraPure™ agarose (Invitrogen) in hgDMEM to attain the agarose bedding. Two cultivation platforms were casted symmetrically within the agarose by using Grade316 stainless-steel molds, to allow comparative analyses, with one construct used for genetic analysis and the other used for mechanical and histologic analysis. In addition, four hollow channels for medium perfusion were casted as shown in Figure 1a with Grade316 stainless steel rods (3 mm diameter). The individual platform was subsequently coated with 100 mg/mL murine collagen type 4 (Col4) in 0.05 M hydrochloric acid (HCl) for 1 h at room temperature (RT), and it was washed with DPBS to remove residual HCl. Next, 5 × 105 mESCs were resuspended in 500 μL collagen scaffold (seeding density: 107 mESC per cm2) to derive a collagen construct. Each construct was inoculated into an individual platform with a glass pipette, before cooled agarose (~42°C) was added slowly on top to complete encapsulation in agarose. The agarose was allowed to set at RT for 15–20 min, before all stainless steel rods were removed and the bioreactor was sealed. The medium reservoir was positioned 15 cm above the bioreactor to generate sufficient back pressure required to fill the bioreactor (Fig. 1b). The medium was perfused through the bioreactor at 5.5 mL/min for a maximum of 50 days. Subsequent medium changes were performed by draining all old medium into a waste bottle and replacing with a new flask containing fresh medium.

The collagen scaffolds comprise 40% v/v bovine fibrillar collagen 1 (Advance Biomatrix), 40% v/v CSA (Sigma) in serum-free hgDMEM, and 20% v/v 5× hgDMEM with 50 mM β-glycerolphosphatase (BGP). The 5× hgDMEM consisted of powdered mix (Gibco) dissolved in a 1/5 volume of water. Bovine trachea CSA comprising 60% chondroitin 4-sulfate and 40% chondroitin 6-sulfate was used to initiate chondrogenesis. Collagen constructs supplemented with CSA are depicted as C+. Corresponding negative controls had replaced CSA with serum-free hgDMEM and are depicted as CS−. This was done to validate the potency of CSA to initiate chondrogenic differentiation of mESC in conjunction with varying pO2 in this bioreactor. A three-step differentiation protocol was applied, with the intention to induce chondrogenesis in the first two phases and osteogenesis in the final phase (Table 1).

Determining pO2 across the cultivation platform

pO2 was measured by using the OXYLITE 2000E multi-channel modular monitoring system (Oxford Optronix) at a 12 h interval over 36 h. Fiber-optic probes were inserted into the bioreactor via 18G intravenous catheters, with the tip positioned at the medial region of the top- and bottom section of the cultivation platform, respectively. pO2 measurements were conducted in the medium reservoir, with cultivation platforms containing cell-free collagen constructs or constructs with mESC. Twenty readings were recorded in a 5 s interval at 0, 12, 24, and 36 h and the mean values were tabulated. Additional measurements at the mid-section containing
constructs with mESC were recorded at 36 h to validate whether an oxygen gradient was, indeed, achieved. All parameters were set up as per actual experiments and the first set of measurements at 0 h was performed after stable flow was established, which requires 2 h on connecting the bioreactor to the peristaltic pump. Measurements were performed on separate bioreactors with perfusion rates at 1.5 and 5.5 mL/min, respectively, to determine the impact of medium perfusion rate on pO2 distribution.

Analytical modeling for oxygen distribution in bioreactor

A finite-element approach was used to model the perfusion bioreactor. The application COMSOL Multiphysics was chosen to carry out the calculations. The incompressible Navier-Stokes equations describe the fluid flow through the bioreactor. Water inflow was equally distributed over the top surface, which reduces the size of the model to 1/4 due to symmetry effects. The pressure at the opposing outlet was set to zero. The convective flux is calculated from the stationary velocity field. The diffusion coefficient of oxygen in water at 37°C is assumed as $D_{O2} = 1.71 \times 10^{-10}$ m²/s. The same value is also chosen for the rest of the bioreactor given the similarity in diffusion characteristics of agarose and water. To match the experiment, the starting pO2 in the bioreactor and inflowing water is assigned as 100 Torr.

The consumption rate of oxygen per cell ($R_{O2}$) is described by the Michaelis-Menten equation:

$$R_{O2} = \frac{V_m \cdot P}{(K_{0.5} + P)}$$  \hspace{1cm} (1)

This equation limits the maximum consumption rate ($V_m$), at low partial oxygen pressure ($P$), through a half-consumption parameter ($K_{0.5}$). These parameters are unknown for mESC. Hence, experimental values for human bone marrow stromal cells were applied:

$$V_C = 7.91 \cdot 10^{-18} \text{mol/(cell} \cdot \text{s); } K_{0.5} = 5.6 \text{ Torr}$$  \hspace{1cm} (2)

Combination of this consumption rate per cell with the cell concentration yields the oxygen consumption rate per volume that was applied to the cultivation platform dimensions. The cell concentration in the upper and lower section could be altered independently.

FIG. 1. General specifications and overall setup of perfusion bioreactor. (A) The general dimensions and shape of cultivation platforms are illustrated in longitudinal plane. The alignment of cultivation platforms with hollow channels for medium perfusion are illustrated and presented in transverse plane. (B) The actual setup of the prototype was placed in an incubator, with a media reservoir 150 mm above the bioreactors and the bioreactor slightly tilted to ensure it was completely filled with media. Color images available online at www.liebertpub.com/tec
Complementary DNA (cDNA) was synthesized from 500 ng according to the manufacturer’s protocol (Invitrogen). 24G needle several times. The samples were subsequently incubated at RT for 5 min, and total RNA was isolated using a High-Capacity cDNA reverse transcription reaction mix (Applied Biosystems) as per the manufacturer’s protocol.25 Total RNA was used by using a High-Capacity cDNA reverse transcription reaction mix (Applied Biosystems) as per the manufacturer’s protocol.25

**Table 1. Composites of Differentiation Media with the Duration of Application**

<table>
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<tr>
<th>Phases</th>
<th>Time points</th>
<th>Media composition</th>
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<tr>
<td>Phase 1</td>
<td>Day 1–15</td>
<td>DMEM high glucose (No. 11965; Gibco) 15% Fetal bovine serum (No. 26140095; Gibco) 1% MEM nonessential amino acid (No. 11140076; Gibco) 1% Antibiotic-antimycotic (No. 15240062; Gibco) 1% MycoZap® Prophylactic (No. 8482; Lonza) 0.18% 2-Mercaptoethanol (21985–023; Gibco) 50 µg/mL L-Ascorbic acid (D4902; Sigma) 10−7 M Dexamethasone (D4902; Sigma) 1 mg/mL Chondroitin sulfate A (C9819; Sigma)</td>
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<tr>
<td>Phase 2</td>
<td>Day 16–30</td>
<td>DMEM:F12, HEPES (No. 11330; Gibco) 10% Fetal bovine serum (No. 26140095; Gibco) 1% Antibiotic-antimycotic (No. 15240062; Gibco) 50 µg/mL L-Ascorbic acid (D4902; Sigma) 10−7 M Dexamethasone (D4902; Sigma) 1 mg/mL Chondroitin sulfate A (C9819; Sigma)</td>
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<tr>
<td>Phase 3</td>
<td>Day 31–50</td>
<td>DMEM:F12, HEPES (No. 11330; Gibco) 10% Fetal bovine serum (No. 26140095; Gibco) 1% Antibiotic-antimycotic (No. 15240062; Gibco) 50 µg/mL L-Ascorbic acid (D4902; Sigma) 1 µM Dexamethasone (D4902; Sigma) 10 mM β-glycerolphosphate (A5422; Sigma)</td>
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The differentiation protocol was divided into three phases. Phase 1 was involved in the induction of chondrogenesis to derive prechondrocytes. Phase 2 would allow further expansion of prechondrocytes. Phase 3 would induce osteogenesis and mineralization of engineered constructs.

The oxygen development was studied in three parts: (1) half an hour without flow, (2) 38 h with flow, and (3) a stationary solution.

**Mechanical stiffness of collagen constructs**

The mechanical stiffness of constructs was determined on the CellScale™ biomaterial testing platform after 50 days in culture, and cylindrical samples (3 mm diameter) were retrieved with a biopsy punch. Each sample was submerged in DPBS at RT and compressed for three cyclic periods at 50% magnitude to derive a stress-strain plot. Mechanical stiffness of each construct was calculated by determining the mean gradient of respective plots and tabulated as a bar chart. Corresponding cell-free collagen scaffolds kept in static conditions for 50 days were used as the baseline. Cell-free scaffolds kept in perfusing medium and the initial constructs were not tested, as they lack structural stability and will collapse after the first compression period. Further, only the top section of each construct was analyzed, as the bottom section was too small to attain uniform samples.

**Total RNA isolation and complementary DNA synthesis**

The top and bottom sections of engineered constructs were dissected and handled separately. Then, 1 mL Trizol™ (Invitrogen) was added to each sample and passed through a 23G needle several times. The samples were subsequently incubated at RT for 5 min, and total RNA was isolated according to the manufacturer’s protocol (Invitrogen).24 Complementary DNA (cDNA) was synthesized from 500 µg total RNA by using a High-Capacity cDNA reverse transcription reaction mix (Applied Biosystems) as per the manufacturer’s protocol.25

**Quantitative polymerase chain reaction**

cDNA was probed by using validated murine Taqman® gene expression assay primers (Applied Biosystems). Representative markers for cell linages that were present at the initial stages of endochondral ossification were studied. These include: (1) stem cells: Oct3/4, Sox2; (2) chondrocytes: Sox9, Col2a1; (3) hypertrophic chondrocytes: Col10a1; (4) osteoprogenitors/osteoblasts: Runx2, Col1a, Sp7 (osterix); and (5) endogenous control: 18S rRNA. The reaction was done in triplicate as per the manufacturer’s protocol26 for Taqman Universal PCR Master Mix (Applied Biosystem) on ABI 7900HT. Relative gene expression against undifferentiated mESC was calculated by using the 2−ΔACT method.27

**Histologic analysis**

Each sample was fixed overnight in cold 4% v/v paraformaldehyde in DPBS. Subsequently, the top and bottom sections were separated and embedded in Optimum Cutting Temperature compound (O.C.T.; Tissue-Tek), followed by quick freezing in 2-methylbutane (Sigma-Aldrich). Twelve-micrometer cryosections were prepared and stained with either alcin blue or alizarin red to identify glycosaminoglycans or calcium accumulation. Additional CS+ samples cultured without media perfusion for 50 days were used to study the influence of medium perfusion over mineralization of constructs. Also, to ensure positive alcin blue, staining was not
entirely resulted from initial CSA supplementation, but accumulated by viable cells over time. Corresponding CS-static culture is not presented, as it disintegrates on fixation.

**Statistical analysis**

The results were tabulated from two independent experiments, and values of at least three samples were presented as mean ± standard deviation. The results were evaluated by t-test or analysis of variance (ANOVA), and p-value < 0.05 was considered statistically significant. The statistical analyses were performed by using GraphPad Prism 6.

**Results**

**Medium perfusion at 5.5 mL/min generated stable pO2 gradient**

The medium reservoir had a mean pO2 of 80.20 ± 1.46 Torr at 0 h and 99.65 ± 1.11 Torr at 36 h (Fig. 2). pO2 across the entire cell-free construct was homogeneous and stable when the medium was perfused at 5.5 mL/min. The initial pO2 was 82.48 ± 2.91 Torr and was maintained at 83.98 ± 2.00 Torr at 36 h (data not shown). In cultivation platforms containing constructs with mESC and medium perfused at 5.5 mL/min, the mean pO2 started at 26.89 ± 0.37 Torr in the top section and 4.57 ± 0.13 Torr in the bottom section, which reached a steady state after 12 h. At 36 h, the top section had a mean pO2 of 83.26 ± 1.41 Torr, whereas the bottom section was 0.64 ± 0.09 Torr. Further, pO2 at the mid-section was 39.63 ± 2.14 Torr at 36 h, indicating a longitudinal pO2 gradient across the cultivation platform. In contrast, similar constructs with medium perfused at 1.5 mL/min had started with a lower pO2 of 1.72 ± 0.26 Torr at the top section and a higher pO2 of 10.78 ± 1.02 Torr at the bottom section. Subsequently, pO2 declined after 12 h and a homogenous hypoxic environment between 0.41 ± 0.23 Torr and 0.07 ± 0.08 Torr (top to bottom) was achieved at 36 h (Fig. 2).

**Simulation of spatial oxygen distribution in bioreactor**

The simulation shows that a flow rate higher than 1.5 mL/min has no influence on the oxygen distribution in the reactor. The entire bioreactor would be well supplied with oxygen, and oxygen depletion is solely caused by local cellular oxygen consumption (Fig. 3). Based on the experimentally measured oxygen concentration, concentration evolutions of similar trends were derived (Fig. 4A) by fitting cell concentration evolutions. The curves (Fig. 4B) were constructed by parametric sweeps over the cell concentration at selected time points. The different points were connected with piece-wise cubic interpolations. As shown in Figure 4B, strong variations in cell concentrations occurred when the medium was perfused at 1.5 mL/min. In contrast, changes in the fitted cell concentrations were more gradual when the medium was perfused at 5.5 mL/min. Further, the simulation indicated that the high pO2 region is only achievable when the cell concentration is ≤ 10^5 cells/cm^3.

**FIG. 2.** pO2 measurements across the cultivation platform and medium reservoir over 36 h. pO2 in medium reservoir and in bioreactors with 1.5 mL/min medium perfusion are represented by dotted lines. Bioreactors with 5.5 mL/min medium perfusion are represented by solid lines. The top section is presented in dark color, whereas the bottom section is indicated in light color. *Two-way ANOVA p < 0.001, F-test p < 0.001.

ANOVA, analysis of variance; pO2, partial pressure of oxygen.
Mechanically stiffer and mineralized constructs after 50 days

Both CS− and CS+ end-products showed no distinct differences in physical appearance. Both started as translucent gel, but they possessed dense ivory coloration with a stiffer structure after 50 days. Unlike the initial gel-like constructs that disintegrated easily (Fig. 5A), the end-products remained structurally intact even after removal from the bioreactor (Fig. 5B, C). The mean dimensions of both constructs were 47.6 ± 3.2 mm in length, with a diameter of 4.7 ± 0.5 mm at the top section and 2.5 ± 0.3 mm at the bottom section. It was also
noted that precoating of the cultivation platform with murine Col4 was critical for attaining end-products of such dimensions. Without Col4 coating, the collagen constructs condensed within the first 15 days of cultivation, resulting in severe shrinkage and deformed end-products after 50 days (Fig. 5D). Although condensation still occurred after 25 days with Col4 coating, it was less severe.

Despite similarities in appearance, the mechanical stiffness between the end-products was significantly different. The CS− constructs had a mean stiffness of $792.0 \pm 105.8$ Pa, with corresponding cell-free constructs being $29.71 \pm 1.39$ Pa. In contrast, the CS+ constructs had a mean stiffness of $144.6 \pm 4.08$ Pa, with corresponding cell-free constructs being $21.5 \pm 2.84$ Pa (Fig. 6). The CS− and CS+ constructs were subsequently stained with alizarin red to identify calcium accumulation, which was positive throughout both constructs. Conversely, negative staining was observed in CS+ static culture (Fig. 7a). In addition, the alizarin red staining had highlighted significant morphological and structural differences between the end-products. The CS− constructs were homogenously stained, with rather uniform cell distribution throughout the entire section. The cells were mainly encapsulated in an extracellular matrix (ECM), and a lesion was observed only in the medial region of the bottom section. In contrast, the CS+ constructs appeared more porous and the cells were in aggregates. Alizarin red had concentrated around these aggregates, and lesser ECM was observed. Although the bottom section of CS+ constructs possessed no lesion, it was significantly smaller than corresponding CS− constructs after freezing. The constructs were also stained with alcian blue to identify glycosaminoglycans accumulation, an indicator for chondrogenesis. Overall, alcian blue staining was positive at the bottom section of CS− constructs, but it was negative at the top section. In contrast, the entire CS+ constructs were positively stained. Specifically, at the bottom section, homogenous staining was observed whereas the top section was distinctly stained at the peripheral region. In comparison, corresponding CS+ constructs cultured in static condition were not stained (Fig. 7b).

Localization of distinct cell populations in relation to pO$_2$

Relative gene expression is illustrated in Figure 8, and each plot presents single-gene expression in all samples. For easier interpretation, the exact fold increase of each gene is not presented. Instead, gene$^+$ indicates gene upregulation and gene$^-$ indicates gene downregulation or no expression, relative to undifferentiated mESC. Further, the representative genes that defined the cell types that resided in each sample were underlined. Similarly, in all samples, Col10a1 and Sp7 were not detected and excluded from Figure 8. In summary, the relative gene expression pattern of each sample is as follows: (1) CS− top section: Oct3/4$^-$, Sox2$^+$, Sox9$^-$, Col2a1$^-$, Runx2$^+$.
**Discussion**

In this study, we designed a prototype containing three-dimensional cultivation platforms with specific positional pO₂ variability. A more complex yet controlled *in vitro* cultivation niche that derives and supports long-term propagation of multiple osteochondral cell lineages concurrently on a single construct was derived. Further, it is intriguing to identify specific cell types propagating in a defined region of a single construct, in correspondence to cell concentrations, pO₂ distribution, and CSA supplementation.

**Rationale of perfusion bioreactor design**

Presently, cell cultures on traditional vessels can grow to a maximum of 100–150 μm in thickness before necrosis is initiated. Nonetheless, excessive fluid shear stress is destructive to biological scaffolds (i.e., hydrogels, collagen scaffolds) and is also lethal to cells. In this study, constructs were encapsulated within an agarose bedding to negate detrimental effects from rapid medium perfusion. Culture medium was instead perfused through channels casted along an individual cultivation platform, and mass transfer was accomplished via diffusion (Fig. 1). Consequently, the range of perfusion rate applicable in this bioreactor was significantly broadened. Furthermore, 1.5% w/v agarose in hgDMEM was tested most desirable, as melted agarose at this concentration kept the constructs compact within the cultivation platforms and would polymerize within 20 min at RT. At higher concentrations, a warmer temperature is required to retain melted agarose and is lethal to the cells. At lower agarose concentrations, a maximum of 1 h was required for complete agarose polymerization at RT. In addition, the channels casted in 1.5% agarose bedding could withstand fluid shear generated by rapid medium perfusion and do not collapse or degenerate over 50 days.

These studies demonstrated that direct exposure of constructs to fluid shear stress could enhance mineralization. Nonetheless, excessive fluid shear stress is destructive to biological scaffolds (i.e., hydrogels, collagen scaffolds) and is also lethal to cells. In this study, constructs were encapsulated within an agarose bedding to negate detrimental effects from rapid medium perfusion. Culture medium was instead perfused through channels casted along an individual cultivation platform, and mass transfer was accomplished via diffusion (Fig. 1). Consequently, the range of perfusion rate applicable in this bioreactor was significantly broadened. Furthermore, 1.5% w/v agarose in hgDMEM was tested most desirable, as melted agarose at this concentration kept the constructs compact within the cultivation platforms and would polymerize within 20 min at RT. At higher concentrations, a warmer temperature is required to retain melted agarose and is lethal to the cells. At lower agarose concentrations, a maximum of 1 h was required for complete agarose polymerization at RT. In addition, the channels casted in 1.5% agarose bedding could withstand fluid shear generated by rapid medium perfusion and do not collapse or degenerate over 50 days.
Other than being an intermediate against excessive fluid shear stress, the agarose bedding was used as a female mold to derive engineered constructs of customized dimensions (Fig. 1). We showed that tailored constructs could be achieved by coaxing the cells to adopt the dimensions of a predesigned cultivation platform. Interestingly, the constructs did not adopt the predesigned dimensions, unless the cultivation platforms were coated with Col4. Otherwise, rapid condensation of constructs would occur at the initial phase of cultivation to produce end-products of smaller and irregular dimensions (Fig. 5D). The exact principle was not investigated, but Col4 generally tends to form a collagen sheet in vivo, as the fibers connect end to end via the C4 domain on the N- and C-terminus. It also lacks glycine at every third residue, which is essential for collagen helix formation.\textsuperscript{33,34} Hence, the Col4 could have enveloped the collagen construct and defined boundaries for cellular propagation during initial cultivation. Since Col4 was not detected on the end-products, the effect was temporary and Col4 was likely degraded as the constructs propagated. Nevertheless, it was sufficient for the constructs to adopt the predesigned dimensions and to gain structural stability.

**Stable pO\textsubscript{2} gradient in the bioreactor**

Defined positional pO\textsubscript{2} variability was introduced to achieve complex cultivation niches that support different cell types simultaneously. Two contributing factors were defined:

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**FIG. 8.** Gene expression profile from qPCR analysis on CS– and CS+ end-products after 50 days. All gene expressions were normalized against undifferentiated ES-D3. *Paired t test \( p < 0.005 \), **paired t test \( p < 0.005 \), *un-paired t test \( p < 0.01 \), ##un-paired t test \( p < 0.01 \). qPCR, quantitative polymerase chain reaction.
(1) cellular oxygen consumption rate in relation to cell concentration, and (2) medium perfusion rate. Generally, each cultivation platform was sandwiched between two channels. The distance of the top section and bottom section of the cultivation platform from each channel was about 5 and 8 mm, respectively, to facilitate mass transfer along the longitudinal plane. Further, the bottom section was specifically designed smaller in diameter to minimize necrosis at the medial region.

Through the simulation, cell concentration in the individual section was identified as the primary causal factor for the oxygen concentration. Oxygen depletion occurs only locally within the cultivation platforms, leaving the other parts of the bioreactor nearly saturated with oxygen (Fig. 3). Although the simulation showed no direct influence of medium perfusion rate over pO2 distribution in the bioreactor, it is likely that the higher perfusion rate had contributed indirectly. As shown in Figure 4, the higher perfusion rate had resulted in less intense variation in the fitted cell concentration and pO2 in the first 24 h, suggesting the establishment of a more stable niche within a shorter timeframe when a higher medium perfusion rate was applied. Further, the differential pO2 distribution is a result of higher cell concentration in the bottom section than the top section, when medium was perfused at 5.5 mL/min (Fig. 4B). Since a rapid decrease in cell concentration was not observed in both sections, the effects of excessive fluid shear to cause cell death are negligible. Further, the constructs had continued to propagate in such an environment to produce mineralized constructs after 50 days. Instead, the distance of the top section and bottom section of the cultivation platform from each channel was about 5 and 8 mm, respectively, to facilitate mass transfer along the longitudinal plane. Further, the bottom section was specifically designed smaller in diameter to minimize necrosis at the medial region.

Effects of pO2 and CSA

Taking a step further, we attempted to recapitulate initial processes of endochondral ossification by applying a three-phase differentiation protocol. However, it is not economically sustainable to use traditional growth factors such as transforming growth factor beta-1 (TGF-β1) and bone morphogenetic protein-2 or -4 (BMP-2 or -4) to induce chondrogenesis, since a large volume of medium is required. Hence the differentiation protocol from Krawetz et al.\textsuperscript{21} using CSA as a chondrogenic inducer, was tested in this study.

Despite similarities in physical appearance, detailed analysis from mechanical stiffness measurements, histology, and gene expression profiles suggested otherwise. The top section of CS− constructs was significantly stiffer than corresponding CS+ constructs (Fig. 6), despite no differences between the cell-free constructs. In Figure 7a, it is shown that the added stiffness of CS− constructs was attributed by compact, homogeneous calcium and ECM distribution throughout the construct. In contrast, CS+ constructs were relatively porous and composed mainly of cell aggregates. Further, results from the quantitative polymerase chain reaction indicated that progenitors and preosteoblasts resided in the top section of the CS− constructs (Sox2\textsuperscript{+}, Sox9\textsuperscript{+}, Runx2\textsuperscript{+}, Col1a\textsuperscript{+}), but they were absent in the CS+ constructs (Sox9\textsuperscript{−}, Runx2\textsuperscript{−}). With the capability to remodel and deposit mineralized matrix for bone-building processes in vivo,\textsuperscript{35} these cells are responsible for the homogenous calcium and matrix distribution in CS− constructs. In the top section of CS+ constructs, the gene expression profile indicated the presence of pluripotent stem cells (Oct3/4\textsuperscript{−}, Sox2\textsuperscript{−}) and prechondrocytes (Sox9\textsuperscript{+}, Col2a1\textsuperscript{+}). This was further supported by cell aggregates in the construct (Fig. 5A), since both cell types favor and tend to proliferate as dense aggregates.\textsuperscript{36} The cell mixture is likely a result of varying exposure of cell aggregates to CSA. mESCs at the peripheral region of aggregates were in direct contact with CSA, which initiated chondrogenic differentiation to derive prechondrocytes. In contrast, a homophilic E-cadherin interaction between mESCs within the aggregate would have initiated the self-renewal mechanism.\textsuperscript{37} Although the presence of pluripotent stem cells in mature tissues is undesirable, as tumorigenesis could occur, it is, nonetheless, favorable to this study. Instead, the stem cells provided a stable pool of naive cells that are necessary for further maturation of the constructs.

Taken together, higher pO2 was shown to initiate osteogenic differentiation, as indicated by negative alcian blue staining, positive calcium accumulation, and upregulation of osteogenic markers. Consequently, a stiffer end-product was derived. However, this effect can be negated with CSA supplementation to derive a mixture of pluripotent stem cells and prechondrocytes. Interestingly, further maturation of primary chondrocytes was not driven when cultured in the oxygenated niche, suggesting the inhibitory effect of oxygen for complete chondrogenic differentiation.

Looking at the effects of the hypoxic environment, many studies have shown pO2 between 1% and 5% (7.42–37.11 Torr) to enhance chondrogenic differentiation of various stem cells.\textsuperscript{38–40} In agreement, the hypoxic niche and high cell concentration in the bottom section had initiated chondrogenic differentiation of mESC in CS− constructs. This was supported by positive alcian blue staining and upregulation of Col2a1. Interestingly, chondrogenic differentiation was further enhanced in CS+ constructs, with higher upregulation of chondrogenic markers Sox9 and Col2a1 (Fig. 6) and the entire section was stained positive for alcian blue. The interplay of CSA and hypoxia for chondrogenic differentiation is not known, but the gene expression profile had identified CSA induction as an additive effect. Although positive alcian blue staining on CS+ constructs may be a result of initial CSA supplementation, negative staining of the corresponding static culture
suggested that initial CSA supplementation was either too diluted or depleted by cells. The accumulated glycosaminoglycans in end-products were likely synthesized by differentiated chondrocytes. Furthermore, CSA supplementation in medium was terminated after 30 days of cultivation and residual CSA should have been depleted by 50 days.

**Inhibitory effects of dexamethasone on osteogenic differentiation**

Although osteogenic differentiation medium was applied at phase 3, it was inadequate for inducing osteogenesis, specifically in CS+ constructs. In both CS− and CS+ constructs, Sp7, a marker for mature osteoblasts, was not detected. Further, ColⅡa1 was absent in CS+ constructs, which showed a lack of transition from prechondrocytes to hypertrophic chondrocytes. The absence of both makers was likely due to a high dexamethasone concentration. Although dexamethasone is an effective stimulator for ossification, the transition of primary chondrocytes to hypertrophic chondrocytes. Furthermore, CSA supplementation was either too diluted or depleted by cells. The accumulated glycosaminoglycans in end-products were likely synthesized by differentiated chondrocytes. Additionally, osteoclasts, as well as osteoblast maturation can be attenuated when more than 100 nM was applied. Hence, additional optimization of existing osteogenic differentiation protocol is necessary to achieve mature osteogenic lineages in future experiments.

In conclusion, we have shown the potential to generate stable dynamic in vitro niches and of using them to derive complex engineered constructs containing multiple naïve osteochondral lineages from mESC. Leveraging from this study, it will be intriguing to refine the differentiation protocol, in an attempt to recapitulate the early processes of endochondral ossification in vitro and drive further maturation of existing constructs.

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**Disclosure Statement**

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**References**

23. Streeter, I., and Cheema, U. Oxygen consumption rate of cells in 3D culture: the use of experiment and simulation to...
measure kinetic parameters and optimise culture conditions. Analyst 136, 4013, 2011.
42. Tangtrongsup, S., and Kisiday, J.D. Effects of dexamethasone concentration and timing of exposure on chondrogenesis of equine bone marrow-derived mesenchymal stem cells. Cartilage 7, 92, 2016.

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