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OSTEOARTHRITIC HUMAN CHONDROCYTES PROLIFERATE IN 3D CO-CULTURE WITH MESENCHYMAL STEM CELLS IN SUSPENSION BIOREACTORS

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Abstract

Osteoarthritis (OA) is a painful disease, characterized by progressive surface erosion of articular cartilage. The use of human articular chondrocytes (hACs) sourced from OA patients has been proposed as a potential therapy for cartilage repair, but this approach is limited by the lack of scalable methods to produce clinically relevant quantities of cartilage-generating cells. Previous studies in static culture have shown that hACs co-cultured with human mesenchymal stem cells (hMSCs) as 3D pellets can upregulate proliferation and generate neocartilage with enhanced functional matrix formation relative to that produced from either cell type alone. However, since static culture flasks are not readily amenable to scale-up, scalable suspension bioreactors were investigated to determine if they could support the co-culture of hMSCs and OA hACs under serum-free conditions to facilitate clinical translation of this approach. When hACs and hMSCs (1:3 ratio) were inoculated at 20,000 cells/mL into 125 mL suspension bioreactors and fed weekly,

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7. ETHICAL CONSIDERATIONS

All studies were performed with cells taken from surgical discards of patients undergoing total knee arthroplasty or during routine orthopedic surgery. Approval for the use of human cells was obtained from the ethics review board of the University of Alberta, Edmonton, Canada and safety guidelines were followed. Consideration was taken to preserve the privacy of specimen donors and the need for written informed consent was waived considering tissues were intended for discard in the normal course of surgical procedure.

8. COMPETING INTERESTS

The authors report that they do not have any conflict of interest related to the work presented in this publication.

9. AUTHOR'S CONTRIBUTIONS

MK designed the bioprocess and studies, carried out the experiments and wrote the manuscript. In addition, MK performed the cell density and growth analyses, nutrient and metabolite analysis, and aggregate characterization, as well as the corresponding data analysis and statistics. AMS performed biochemical assays, histology and gene expression studies, as well as data analysis. AA conceived the experimental study, performed gene expression, data analysis, provided funding, participated in writing the manuscript, and co-supervised the study. AS conceptualized and designed the experiments, performed data analysis, participated in writing the manuscript, provided funding for the project, and co-supervised the study.

they spontaneously formed 3D aggregates and proliferated, resulting in a 4.75-fold increase over 16 days. Whereas the apparent growth rate was lower than that achieved during co-culture as a 2D monolayer in static culture flasks, bioreactor co-culture as 3D aggregates resulted in a significantly lower collagen I to II mRNA expression ratio, and more than double the GAG/DNA content (5.8 versus 2.5 μg/μg). The proliferation of hMSCs and hACs as 3D aggregates in serum-free suspension culture demonstrates that scalable bioreactors represent an accessible platform capable of supporting the generation of clinical quantities of cells for use in cell-based cartilage repair.

Keywords

chondrocytes; mesenchymal stem cells; cartilage tissue engineering; bioprocess development; scale-up; three-dimensional culture; suspension bioreactors; aggregates

2. INTRODUCTION

Articulating joint surfaces enable near-frictionless movement due to the presence of hyaline cartilage, a complex, three-dimensional (3D) extracellular matrix of collagen II and proteoglycans, which houses articular chondrocytes (Csaki et al., 2008; Dhinsa & Adesida, 2012; Huselstein et al., 2012; Vinatier et al., 2009). Defects in articular (hyaline) cartilage, as a result of injury or wear and tear, can initiate a progressive, degenerative process that can eventually lead to osteoarthritis (OA), a chronic and debilitating medical condition that affects 25% of adults over 50 years of age (Schulz & Bader, 2007). Since articular cartilage is avascular and has a very limited capacity for spontaneous self-repair, clinical interventions, such as microfracture and cartilage grafting, have been developed to facilitate repair. However, microfracture results in the formation of fibrocartilage, which has significantly inferior biomechanical properties compared to articular cartilage, and grafts are limited by poor integration with the native tissue (Dhinsa & Adesida, 2012; Huselstein et al., 2012; Vinatier et al., 2009). Thus, alternative approaches have emerged.

Cell-based therapies have been examined to determine their potential in resurfacing cartilage defects (Brittberg et al., 1994; Csaki et al., 2008; Dhinsa & Adesida, 2012; Vinatier et al., 2009). Autologous chondrocyte implantation/transplantation (ACI) is a procedure in which a cartilage biopsy is taken from a non-weight bearing site and processed to isolate chondrocytes (Brittberg et al., 1994). Due to the paucity of articular chondrocytes (ACs) in articular cartilage (12–16 million chondrocytes/g tissue (Jakob et al., 2003)) and low post-isolation yields (<22% (Jakob et al., 2003)), chondrocytes are typically expanded in culture to obtain clinically relevant cell numbers (2.6–5 million cells for typical focal defect sizes of 1.6–6.5 cm^2) (Brittberg et al., 1994). Following monolayer (2D) cell culture, the expanded chondrocytes are then reintroduced into the defect site, either in a scaffold or with a membrane, in an attempt to regenerate hyaline cartilage (Csaki et al., 2008; Dhinsa & Adesida, 2012; Huselstein et al., 2012). In contrast to treating focal defects, autologous chondrocyte treatment of OA cartilage necessitates restoration of larger surfaces of “at least 25 cm^2” (Tallheden et al., 2005). This requires large numbers of OA-derived ACs that are difficult to obtain using current culture techniques, since OA-derived ACs have low proliferation and matrix production rates (Tallheden et al., 2005).

Whereas ACI has shown some clinical success (Huselstein et al., 2012), it suffers from a number of problems. The chondrocytes serially expanded in monolayer undergo replicative senescence and de-differentiate, exhibiting decreased production of collagen II and aggrecan, and increased production of collagen I (Csaki et al., 2008; Hardingham et al., 2002; Huselstein et al., 2012; Vinatier et al., 2009). Furthermore, certain long-term complications of ACI, such as graft hypertrophy, have been linked to prior cell culture methods (Studer et al., 2012). Moreover, implantation of de-differentiated human articular chondrocytes (hACs) into a defect produces fibrocartilage. Thus, improved culture methodologies are required.

As an alternative to hACs, human mesenchymal stem cells (hMSCs) are being studied extensively for cartilage repair applications (Csaki et al., 2008; Dhinsa & Adesida, 2012; Huselstein et al., 2012; Vinatier et al., 2009). hMSCs can be isolated from a number of adult tissues, including bone marrow, and can undergo chondrogenic differentiation (Pittenger et al., 1999). However, when serially passaged on a 2D surface in serum-containing medium (SCM) for an extended period of time, mesenchymal stem cells (MSCs) can lose their ability to proliferate and to differentiate along a chondrogenic lineage (Banfi et al., 2000; Banfi et al., 2002; Sethe et al., 2006). Additionally, cultured MSCs can produce hypertrophic cartilage (Csaki et al., 2008).

It has been suggested that issues related to expanding articular chondrocytes (ACs) and MSCs in 2D culture can be overcome by utilizing 3D culture methods (Bonaventure et al., 1994; Csaki et al., 2008; Frith et al., 2010; Gigout et al., 2009; Johnstone et al., 1998; Malda et al., 2003; Ronga et al., 2004; Vinatier et al., 2009). Recently, the 3D co-culture of ACs and MSCs has been shown to generate higher quality cartilage tissues than the monoculture of either of these cell types (Acharya et al., 2012; Cooke et al., 2011; Hubka et al., 2014). Co-culturing ACs and hMSCs in a 1:3 ratio as 3D pellets in static culture has been shown to result in significantly greater glycosaminoglycan (GAG)/deoxyribonucleic acid (DNA) content than expected from the proportional contribution of each cell type (Acharya et al., 2012). Furthermore, co-culture has been shown to decrease hypertrophy in the generated tissues (Studer et al., 2012).

The clinical translation of these promising co-culture results will require efficient and reproducible methods of cell expansion. Most of the studies cited above were carried out in static culture vessels, which represent an inefficient means to scale up cell production compared to suspension bioreactors (Sen et al., 2001, 2002a, 2002b). Bioreactors are scalable vessels, which have been used successfully to rapidly generate large quantities of cells using microcarriers (Frauenschuh et al., 2007; Malda et al., 2004; Schrobback et al., 2011; Dry, 2010; Jorgenson, 2012; Yuan et al., 2014) and as 3D aggregates (Frith et al., 2010; Gigout et al., 2009; Lee et al., 2011). The culture environment in bioreactors can be monitored and controlled, which can promote the generation of reproducible cell populations. This is an important consideration when the goal is to generate cell populations for application in a clinical setting (Thirumala et al., 2013). The co-expansion of hACs and hMSCs in suspension bioreactors is novel and has yet to be reported.

In addition, the aforementioned co-culture studies utilized fetal bovine serum (FBS), which represents a regulatory hurdle. FBS is undesirable as it poses a risk of transmission of infectious zoonotic agents, and suffers from batch-to-batch variability, which can result in irreproducible cell growth (Jung et al., 2012; Sen & Behie, 1999). In the present study, the feasibility of a suspension bioreactor process for the co-culture of hMSCs and hACs, as 3D aggregates under serum free conditions, was evaluated. Additionally, serial passaging and feeding were investigated as a means of prolonging the co-culture period in bioreactors.

3. MATERIALS AND METHODS

3.1 Cell harvest and expansion

Human articular chondrocytes were isolated from articular cartilage samples of OA donors. This particular cell source was chosen since this study was focused on evaluating the utility of using bioreactor based bioprocess to expand cell populations considered to have potential in an autologous treatment for OA. hMSCs were isolated from human bone marrow as previously described (Acharya et al., 2012; Adesida et al., 2012) . Primary hACs were cryopreserved at passage level zero. When needed for experiments, these cells were thawed and expanded in monolayer culture in T-flasks (Nunc, Penfield, USA) using a chondrocyte SCM [10% (v/v) FBS (Lonza Group, Basel, Switzerland) and 90% (v/v) Dulbecco's modified Eagle's medium (DMEM) (VWR International, Radnor, USA), supplemented with Gentamicin-Amphotericin (Lonza), 0.292 mg/mL L-Glutamine (Lonza) and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Sigma-Aldrich)]. Expansion of hACs in monolayer culture was limited to a passage level of 2 before the cells were used to inoculate bioreactors. hMSCs were cryopreserved at passage 2 (p2). When required for experiments, these cells were thawed and expanded in PPRF-msc6 (Jung et al., 2010, 2012), supplemented with Penicillin and Streptomycin (PenStrep) (Life Technologies). The cells were not expanded in tissue-culture flasks beyond passage level 6 before being used to inoculate bioreactors.

3.2 Media screening

Initial studies focused on evaluating two SFM for their ability to support the co-culture of hMSCs and hACs; a commercially-available medium called TheraPEAK (Lonza) and an in-house formulation called PPRF-msc6 (Jung et al., 2010, 2012). A SCM [10% (v/v) FBS–22.5% (v/v) DMEM and 67.5% (v/v) modified Eagle's medium α (αMEM) with Gentamicin-Amphotericin, 0.292 mg/mL L-Glutamine, 0.75% (v/v) sodium pyruvate and 10 mM HEPES] was used as a control. Cell populations, previously expanded in SCM, were propagated in each medium for three consecutive passages in T-flasks. The cells were inoculated into 4 mL of medium at a density of 5 000 cells/cm^2 at every passage and at a ratio of 1 hAC to 3 hMSCs (Acharya et al., 2012; Dahlin et al., 2014; Meretoja et al., 2012; Saliken et al., 2012) in the first passage. Cell densities and viabilities were determined using a haemocytometer (Hausser Scientific, Horsham, PA) and the trypan blue exclusion assay (Sigma-Aldrich) (Sen et al., 2001).

3.3 Bioreactor and static co-culture

For the initial study on bioreactor co-culture, hACs and hMSCs were stained with the fluorescent dyes, PKH26 (red) and PKH67 (green) respectively. Cells were inoculated at a ratio of 1 hAC to 3 hMSCs and a viable cell density of 19 000 cells/mL in 60 mL of SCM. Bioreactors were operated at 60 rpm, and medium changes were carried out once a week.

Cells were inoculated at a density of 20 000 cells/mL into 125 mL suspension bioreactors (NDS Technologies, Vineland, USA) in 125 mL of PPRF-msc6 supplemented with PenStrep, at a ratio of 1 hAC to 3 hMSCs. Based on preliminary experiments, the agitation rate was maintained at 60 rpm for the first two days, and increased to 80 rpm to support greater cell expansion. For comparison, hACs and hMSCs were also inoculated at a 1:3 ratio (20 000 cells/mL) in gelatin-coated T-flasks containing 4 mL of PPRF-msc6. The T-flask surface was coated prior to culture by adding a 0.1% gelatin in water solution at a rate of 0.1 mL/cm^2. The T-flasks were rocked to allow the solution to coat the entire surface, and after 30 minutes, any excess solution was aspirated out, and the T-flasks left to dry for 1–2 hours. Following culture in T-flasks, representative 4 mL samples of aggregates were removed, resuspended in 4 mL of trypsin-ethylenediaminetetraacetic acid (EDTA) (Life Technologies) and incubated at 37°C for 10–30 min (depending on the aggregate size) with mechanical trituration every 10 min. Cell densities and viabilities were evaluated using the Vi-CELL Cell Viability Analyzer (Beckman Coulter Canada, Mississauga, Canada). For nutrient and metabolite analysis, samples were taken and analyzed using established protocols (Yuan et al., 2014). The glutamine and ammonia values were adjusted for spontaneous glutamine degradation and ammonia formation respectively.

In batch cultures, where the medium is not changed during the culture process, mammalian cell populations can cease to expand due to the exhaustion of key nutrients or the build-up of toxic metabolites (Schop et al., 2009; Sen & Behie, 1999; Sen et al., 2002b). To address this issue, fresh medium can be used to replace part or all of the spent medium in a culture. To determine if exposure to fresh medium (i.e. feeding) could prolong the period during which co-cultured hAC and hMSC populations are able to expand, two approaches were evaluated. In approach 1 (serial passaging condition), the cultures were passaged every 10 days. In this approach, aggregates were dissociated by being exposed to two cycles of 0.25% trypsin-EDTA at 37°C for 10 minutes followed by mechanical trituration. The resulting single cell suspension was used to inoculate a new culture vessel containing fresh medium at 20 000 cells/mL. In approach 2 (fed-batch condition), 50% of the spent medium in a culture vessel was replaced with fresh medium on days 8, 12 and 16 of culture without dissociating the aggregates. Rather, in this approach, during each feed, the top half of the medium in a bioreactor was removed and replaced by an equal volume of fresh medium. To ensure no aggregates were lost during the medium replacement process, the spent medium was centrifuged at 400 x g for 10 minutes to separate out any aggregates, and these aggregates were returned to the bioreactor.

3.4 Aggregate characterization

Representative aggregate samples were photographed using an Axio Observer light microscope (Carl Zeiss, Oberkochen, Germany). The mean aggregate diameter of >20

aggregates per sample was determined by averaging the length of the longest axis and the perpendicular diameter (ZEN 2011 microscope software), excluding cell clusters with diameters of <35 μm (Sen et al., 2001). The eccentricity of an aggregate was calculated as the square root of the difference between the squares of the semi-major and semi-minor axes, divided by the semi-major axis. For fluorescent imaging, representative aggregate samples were photographed with the LSM 700 Confocal Microscope (Carl Zeiss) using the brightfield, FITC and Rhodamine settings.

3.5 Biochemical assays

As an indication of matrix production, the normalized sulfated GAG levels (i.e. the GAG/DNA) was measured. Cell suspension samples (5–10 mL) were centrifuged at 433×g, and the resulting cell pellet/aggregate was washed with phosphate-buffered saline (PBS) (Sigma-Aldrich), and stored at −80°C until GAG and DNA assay, using previously used protocols (Adesida et al., 2012). Note that this assay was used to evaluate the GAG content in the aggregates only, and not the GAG released by the cells into the medium.

3.6 Histology

Cell aggregates were stained with Safranin O to detect the presence of sulfated cartilaginous proteoglycan matrix. Cell aggregates were obtained via centrifugation, immersed in 10% (v/v) neutral phosphate buffered formalin (Thermo Fisher Scientific) overnight at 4°C, resuspended in PBS and stored at 4°C. Samples were embedded in paraffin wax, sectioned at 5 μm, stained with 0.1% (w/v) Safranin O (Sigma-Aldrich) and counterstained with 0.01% (w/v) fast green (Sigma-Aldrich) to visualize collagenous matrix.

3.7 Gene expression

Total RNA was isolated by using Trizol (Life Technologies) for the monolayer cultures or in conjunction with molecular grinding resin (Geno Technology Inc. St. Louis, USA) when extracting RNA from the aggregates. The gene expression was measured using an established protocol (Adesida et al., 2012). The primer sequences used are shown in Table I. Gene (mRNA) expression levels were normalized to the expression level of human β-actin by the $2^{-\Delta ct}$ method (Livak & Schmittgen, 2001).

3.8 Statistics

Statistical analysis was carried out in Microsoft Excel 2013. Tukey's test was performed using MATLAB. Cell density, aggregate diameter and metabolism data were analyzed using two-way analysis of variance. GAG and DNA data were analyzed using a t-test. All data was reported with mean and standard error of the mean. Metabolism data was reported with mean and range of data. Significant differences were defined at $p<0.05$.

4. RESULTS AND DISCUSSION

The feasibility of co-expanding populations of hACs and hMSCs in suspension bioreactors was examined under serum-free conditions.

4.1 Serum-Free Media Screening in Static Co-culture

Both hAC and hMSC populations could be expanded separately in SCM, TheraPEAK and PPRF-msc6 cell culture media for multiple passages (data not shown). Thus, these three media were used to carry out the initial co-culture studies. As shown in

4.2 Co-culture in Suspension Bioreactors

As a first step, batch bioreactor co-culture was investigated in SCM, since this medium is commonly reported for the culture of hMSCs as well as hACs. The cells formed aggregates in culture. Fluorescent photomicrographs (Figure 2) showed the presence of both hACs and hMSCs within each sampled aggregate suggesting that these two cell populations can be co-expanded in suspension culture. The cell growth rate in SCM was slow (0.003 h^{-1}) and the maximum culture density was 74 000 cells/mL at day 25. However, the GAG/DNA was high (27.2 μg/μg), and the collagen I to II mRNA expression ratio in the bioreactor culture was low (0.14) at day 28. The expression levels (all relative to β-actin expression) of cartilage oligomeric matrix protein (COMP), aggrecan and collagen X were 0.11, 0.10 and 1.4×10^{-3} respectively. The chondrogenic characteristics of these bioreactor-cultured aggregates are notable, since the cells were not exposed to chondrogenic induction media. For comparison, literature reports of co-cultured cells that were intentionally exposed to chondrogenic induction media had 1–30 μg/μg of GAG/DNA and collagen I to II mRNA expression ratios of 0.01–1.0 (**Error! Reference source not found.**). Thus, simply co-culturing in suspension bioreactors resulted in cell populations which exhibited similar or better chondrogenic characteristics compared to cells that have been exposed to chondrogenic induction factors, thereby supporting the use of suspension bioreactors as a platform to generate cells with the potential to repair cartilage.

While the results from 3D bioreactor co-culture using SCM were positive, SCM was not able to support cell co-expansion in 2D co-culture using T-flasks (see previous section). By contrast, serum-free PPRF-msc6 medium supported co-expansion in T-flasks for three passages. As a result, PPRF-msc6 was tested as the growth medium in 3D bioreactor co-culture, and was able to successfully support cell growth in both 2D and 3D co-culture (data not shown). Thus, one SFM medium was able to support the entire cell expansion process from T-flasks to bioreactors. It is also important to note that SFM is the preferred choice for any future applications of this technology, due to concerns around the use of animal-derived serum. Thus, PPRF-msc6 medium was chosen for the remainder of the bioreactor experiments detailed in this manuscript. The cells generated in bioreactors utilizing this SFM still exhibited chondrogenic potential without exposure to chondrogenic induction media (i.e. up to 5.8 μg/μg of GAG/DNA and up to 0.58 of collagen I to II mRNA expression ratio), albeit not as strongly as observed in the presence of SCM. This is not surprising considering that serum is known to contain factors which can promote stem cell differentiation.

4.3 Passaging in Bioreactor Co-Culture

Passaging was studied as a means to extend the period of time that the cells could be co-expanded in suspension culture. In the first passage (which was run as a batch culture), cells inoculated at 20 000 cells/mL increased in density during culture to 47 000 viable cells/mL,

and maintained a cell viability of over 80% (Figure 3A). The aggregates in the first passage contained significant deposits of self-generated matrix (GAG/DNA = 2.0±0.5 μg/μg), but weak to no Safranin O staining was detected (Figure 3B). The GAG/DNA levels observed after the first culture period was maintained through to the end of the second passage (data not shown).

Cells isolated from the first passage were then inoculated into a new bioreactor vessel containing fresh medium (i.e. passage 2). A small sample was also inoculated into static T-flasks. The cell viability in the passage 2 bioreactor (also operated in batch mode) remained high, and similar to what was observed in the first passage, the cells spontaneously formed spherical aggregates (Figure 3C–D). However, the cell density in the passage 2 bioreactor did not measurably increase over the 10-day culture period (growth rate of −0.001/h in the second passage versus 0.009/h in the first passage). This was surprising, since the cells inoculated into the static T-flasks were able to proliferate (data not shown), suggesting that the bioreactor environment was no longer conducive to cell proliferation.

The lack of cell proliferation observed after being passaged from one bioreactor to the next may have been due to the vigorous enzymatic dissociation method that was required to break down the aggregates, which were strongly held by matrix, into single cells. The cleavage of the cell-to-cell attachments that formed in 3D aggregates may have had a negative impact on the cells from the first passage (Sen et al., 2004). The cells may have been able to recover from this damage in static culture conditions, but not in the shear environment of the bioreactors. Thus, the dissociation protocol for serial passaging may need to be improved by: decreasing the trypsin concentration, decreasing the period of exposure, using another enzyme such as collagenase (Gigout et al., 2009), TrypLE, or Accumax cell aggregate dissociation medium (Abbasalizadeh et al., 2012), or employing chemical and mechanical dissociation methods (Sen et al., 2002a, 2004; Baghbaderani et al., 2010).

4.4 Feeding in Bioreactor Co-Culture

As bioreactor passaging into fresh medium did not extend the cell expansion period, the effect of feeding fresh medium into an existing culture without passaging was examined. As shown in

Feeding was expected to result in greater cell densities and a greater culture period, since it replaces nutrients and lowers the concentration of harmful metabolites. Both batch and fed conditions had similar levels of glucose and glutamine throughout the culture period, but the fed condition had lower concentrations of lactic acid and ammonia. At day 8, both conditions had similar ammonia levels. In the batch condition, ammonia levels rose to 2.2 mM at day 10 and continued to climb past known inhibitory levels for hMSCs (2.4 mM ammonia) (Schop et al., 2009). At the same time, the cell densities in the batch condition ceased to increase. In contrast, the feeding strategy was able to maintain ammonia concentrations below at 2.0 mM and there was cell growth until day 16. Similarly, the maximum lactic acid concentration was lower in the fed condition (6.9 mM at day 16 versus 9.1 mM at day 19 in the batch condition). The levels in both conditions were below growth-inhibitory levels for hMSCs (35.4 mM lactic acid) (Schop et al., 2009). Low culture medium pH, which may be due to the generation of lactic acid, has been shown to reduce the

deposition of matrix by chondrocytes *in vitro* (Mobasheri et al., 2006; Suits, 2006). Thus, feeding is important for maintaining healthy co-culture in bioreactors.

Medium analyses revealed that the cumulative glutamine consumption and waste production were higher in the fed condition ($p<0.0005$), as shown in Both culture conditions resulted in similar amounts of GAG, and the GAG/DNA ratios were not significantly different (Figure 6A–C). Furthermore, both conditions were negative for Safranin O staining (Figure 6D–E). So, feeding had no impact on chondrogenic traits.

The average aggregate diameter (Figure 6F) increased over the culture period from approximately 50 μm to 150 μm in both conditions. For other cell types, it has been demonstrated that the aggregate diameters below 300 μm prevent dissolved gas and nutrient mass transfer limitations (Sen et al., 2001). The aggregate diameter distribution (Figure 6G) showed smaller aggregates in the fed condition (62% of aggregates were 50–150 μm) than the batch (45%) at day 16, which represents a narrow diameter distribution, resulting in more homogenous aggregates. The heterogeneity in aggregate size was the result of several factors of different magnitudes acting at different times. These factors were: cell proliferation, spontaneous cell aggregation, agglomeration of aggregates, the effects of shear and the formation of matrix, which limited the effect of shear. Most of these factors were similar in both conditions. However, the increased handling and agitation of the cells during feeding may have caused larger, loosely-held agglomerates to come apart, resulting in the decrease and homogeneity in aggregate size in the fed condition.

Feeding provided a means to extend the culture period, and obtain greater cell productivity out of a single culture vessel. Based on these results, the bioreactor cell co-expansion protocol was modified to incorporate feeding at days 8 and 12 during a 16 day culture period.

4.5 Comparison of Bioreactor and Static Co-culture Protocols

Due to the advantages bioreactors have over static vessels, the cell productivity of the suspension culture protocol was compared to the corresponding static culture protocol (i.e. under serum-free conditions and with feeding). The growth curve of the static condition (Figure 7A) is displayed in units of cells/cm^2, since it represents cell growth on a 2D surface, whereas the growth curve of the bioreactor condition (Figure 7B) is shown in units of cells/mL, since it represents growth as 3D aggregates in suspension. The cell density in static co-culture peaked at 113 000 cells/cm^2 (707 000 cells/mL in 4 mL medium) whereas in bioreactor co-culture, the peak density was 91 000 cells/mL. Even though the cell growth rates in bioreactors (0.010/h from days 4–14) were lower than in static culture flasks (0.021/h from days 0–6), the ability to increase bioreactor culture volume, while maintaining a controlled environment, can result in substantially greater numbers of cells.

In static culture, adherent cell populations typically grow as a monolayer and cease proliferation due to contact-inhibition, which is the ability of the cells to deplete the mitogens in the surrounding culture medium and the limit the growth of surrounding cells (Higuera et al., 2009; Schop et al., 2009). However, in this study, the cells formed an additional layer on top of the monolayer, as has previously been reported (Hendriks et al.,

2006). Whereas a near confluent monolayer was observed by day 4, feeding on day 8 induced a second layer of cells to grow on top of the existing monolayer (i.e. stratified monolayers) (Figure 7C), representing a second period of growth (Figure 7A). This linear growth period following an exponential growth phase resulted in a peak density of 113 000 cells/cm^2 by day 12. It is possible that the high levels of matrix produced by the cells within the first monolayer served as a substrate to which new cells could bind. Furthermore, the stratified layers formed after feeding, suggesting that the increased nutrient levels and/or decreased waste concentrations promoted additional growth.

In bioreactors, the co-cultured cells grew as aggregates (Figure 7D) with an average aggregate diameter of 75–125 μm (B), most aggregates were 50–100 μm on day 16, similar to the previous study. This demonstrates the reproducibility of the bioreactor platform in producing aggregates within this size range.

As expected, the total DNA and GAG content in static culture was greater than in the bioreactor condition (As one objective of this study was to generate cells capable of chondrogenic differentiation, it is noteworthy that the aggregates in this study were not exposed to a chondrogenic differentiation medium, as typically practiced in the art to induce differentiation (Acharya et al., 2012; Johnstone et al., 1998; Tallheden et al., 2005). Even so, the GAG/DNA values obtained in this study were similar to those reported in some 3D co-culture studies, some of which were conducted with differentiation factors (Diao et al., 2013; Saliken et al., 2012; Xu et al., 2013). Furthermore, the hACs used in this study were derived from patients suffering from OA, so high collagen X expression and decreased GAG were expected in favor of higher cell growth (Saliken et al., 2012; Studer et al., 2012; Tallheden et al., 2005; Yang et al., 2006). This data is encouraging, since it suggests that this bioreactor process intrinsically supports chondrogenic traits compared to static culture.

5. CONCLUSIONS

It has been demonstrated here, for the first time, that hMSC and hAC populations can be co-expanded as aggregates in suspension bioreactors under serum-free conditions. In the presence of SCM, the resulting cell populations exhibited chondrogenic characteristics similar to that reported for cells which had been exposed to forced chondrogenic induction. The co-culture of hMSCs and hACs was also found to be possible under serum-free conditions in suspension bioreactors, a key finding when considering clinical translation.

The serum-free bioprocess described here generated 6–11 million cells per bioreactor, which is sufficient for the treatment of a cartilage surface defect that typically requires around 1 million cells/cm^2 (Tallheden et al., 2005). Furthermore, OA chondrocytes were able to generate GAGs following bioreactor co-culture, demonstrating the potential for their use in autologous therapies for OA. This study lays the foundation for optimizing a bioreactor-based process for generating clinical quantities of chondrogenic cells, for use in therapies aimed at repairing cartilage or cartilage-like tissues.

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10. ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
αMEM	Modified Eagle's medium α
AC	Articular chondrocyte
COMP	Cartilage oligomeric matrix protein
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
GAG	Glycosaminoglycan
hAC	Human articular chondrocyte
hMSC	Human mesenchymal stem cell
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
MSC	Mesenchymal stem cell
OA	Osteoarthritis
p(number)	Passage (number)
PBS	Phosphate-buffered saline
PenStrep	Penicillin and streptomycin solution
RNA	Ribonucleic acid
SCM	Serum-containing medium
SFM	Serum-free medium
T-flask	Tissue culture flask
TGF-β	Transforming growth factor β

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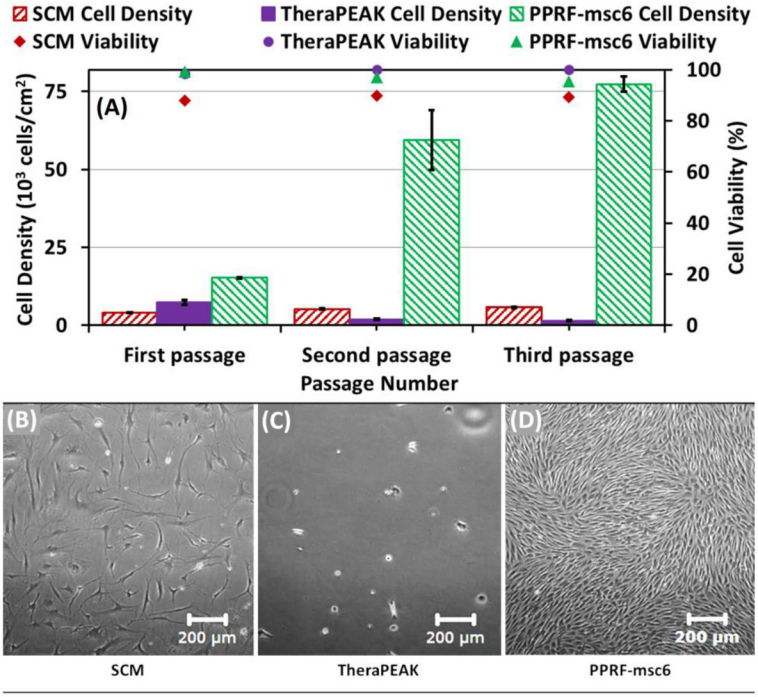

Figure 1. Media screening

Three media (SCM, TheraPEAK and PPRF-msc6) were screened for their ability to support the proliferation of hACs and hMSCs in static co-culture. All experiments were conducted in duplicate. Cells were passaged three times for 6 days each without medium changes. The first passage was inoculated at a ratio of 1 hAC to 3 hMSC and each passage was inoculated at 20 000 cells/mL in 4 mL of medium. A) Cell densities at day 6 in the three media are shown over three passages. Error bars show standard error of the mean of duplicate cell counts. Photomicrographs of cells in static co-culture at the third passage on day 6 are shown at 5x magnification for B) SCM, C) TheraPEAK and D) PPRF-msc6.

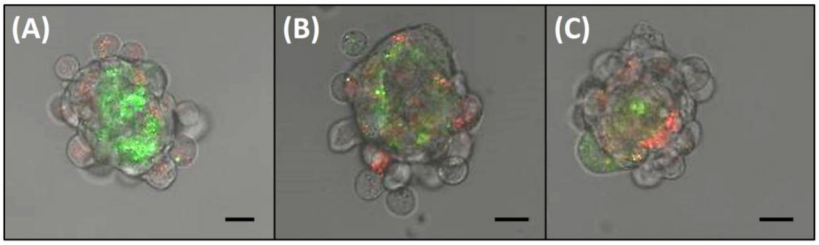

Figure 2. Photomicrographs of co-cultured aggregates

hMSCs were stained with PKH67 (green) and hACs were stained with PKH26 (red) prior to inoculation in bioreactors in a ratio of 1 hAC to 3 MSCs. Bioreactors were inoculated with 60 mL of SCM and agitated at 60 rpm. Representative samples of aggregates were imaged A) 4, B) 7 and C) 10 days after inoculation.

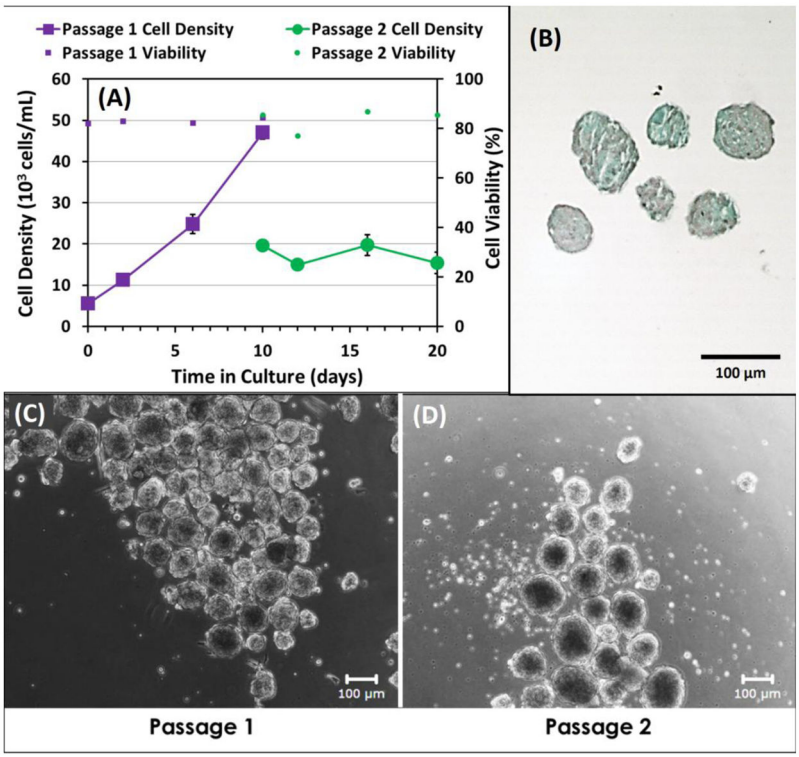

Figure 3. Serial passaging of cells in bioreactor co-culture

Bioreactors were operated at 60 rpm for the first two days followed by 80 rpm. Cells were cultured for 10 days without medium changes for each of two passages. Aggregates harvested from the first bioreactor were dissociated with trypsin-EDTA and inoculated (20 000 cells/mL) into new bioreactor vessels as single cells in fresh medium. A) Cell densities in bioreactor co-culture in two consecutive passages are shown. Error bars show standard error of the mean of duplicate cell counts from duplicate flasks. B) Safranin O staining of cells co-cultured in the first passage is shown. C–D) Photomicrographs of bioreactor co-cultures for each of two passages are shown at 10x magnification after 10 days in culture.

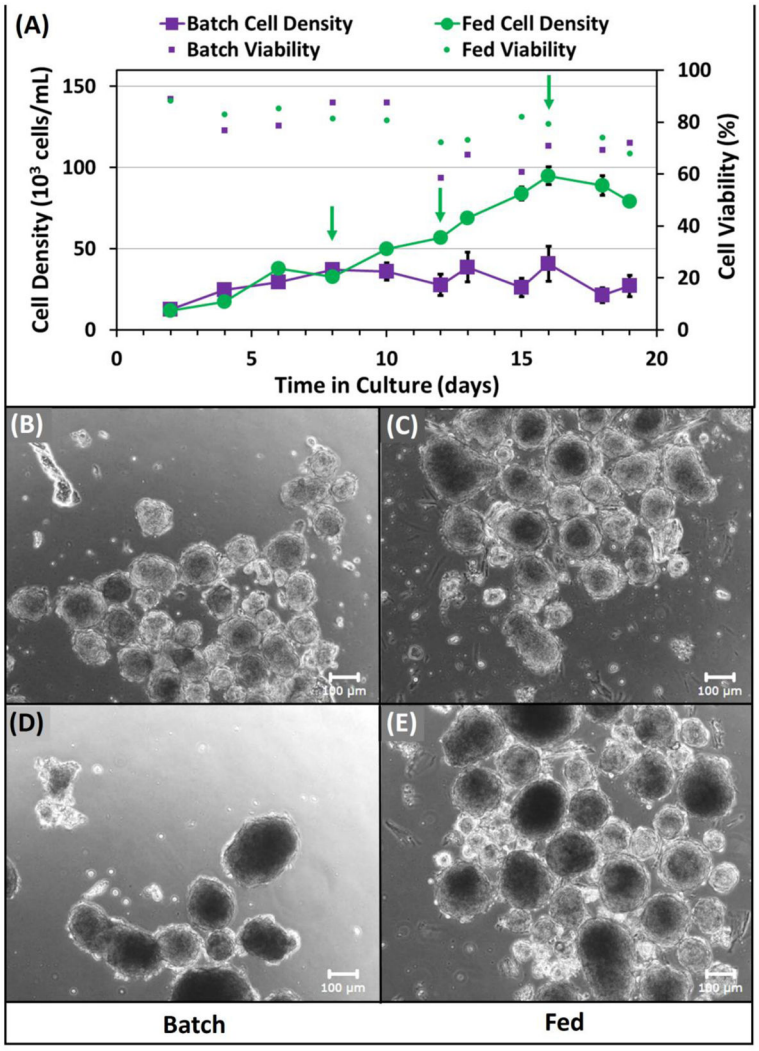

Figure 4. Feeding cells in bioreactor co-culture–cell density and cell morphology

Effect of feeding was tested in bioreactor co-culture with regards to cell density and morphology. A) Cell densities are shown in bioreactor co-culture in the batch and fed conditions. Error bars show standard error of the mean of duplicate cell counts from duplicate flasks. Green arrows indicate time points for 50% medium change for the fed condition. Photomicrographs of hMSC and hAC aggregates at B–C) 10 days and D–E) 16 days in culture are shown at 10x magnification.

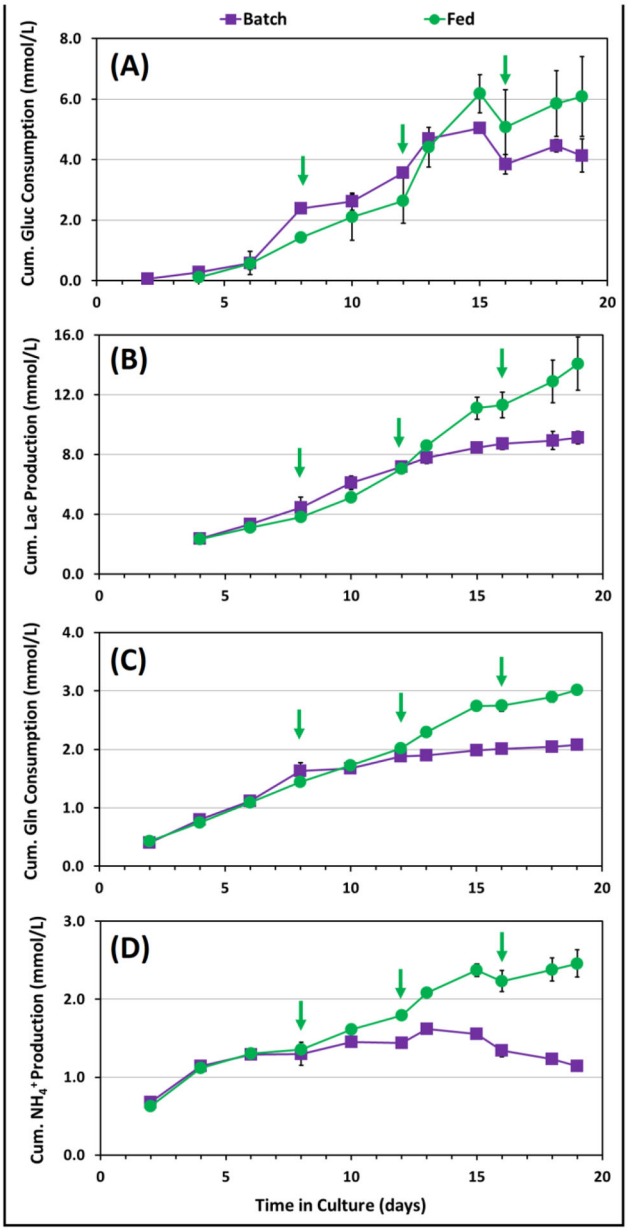

Figure 5. Feeding cells in bioreactor co-culture–nutrient consumption and waste production

The cumulative A) glucose consumption, B) lactic acid production, C) glutamine consumption and D) ammonia production are shown in both conditions. Error bars show range of data for duplicate samples from duplicate cultures. Green arrows indicate time points for 50% medium change in the fed condition.

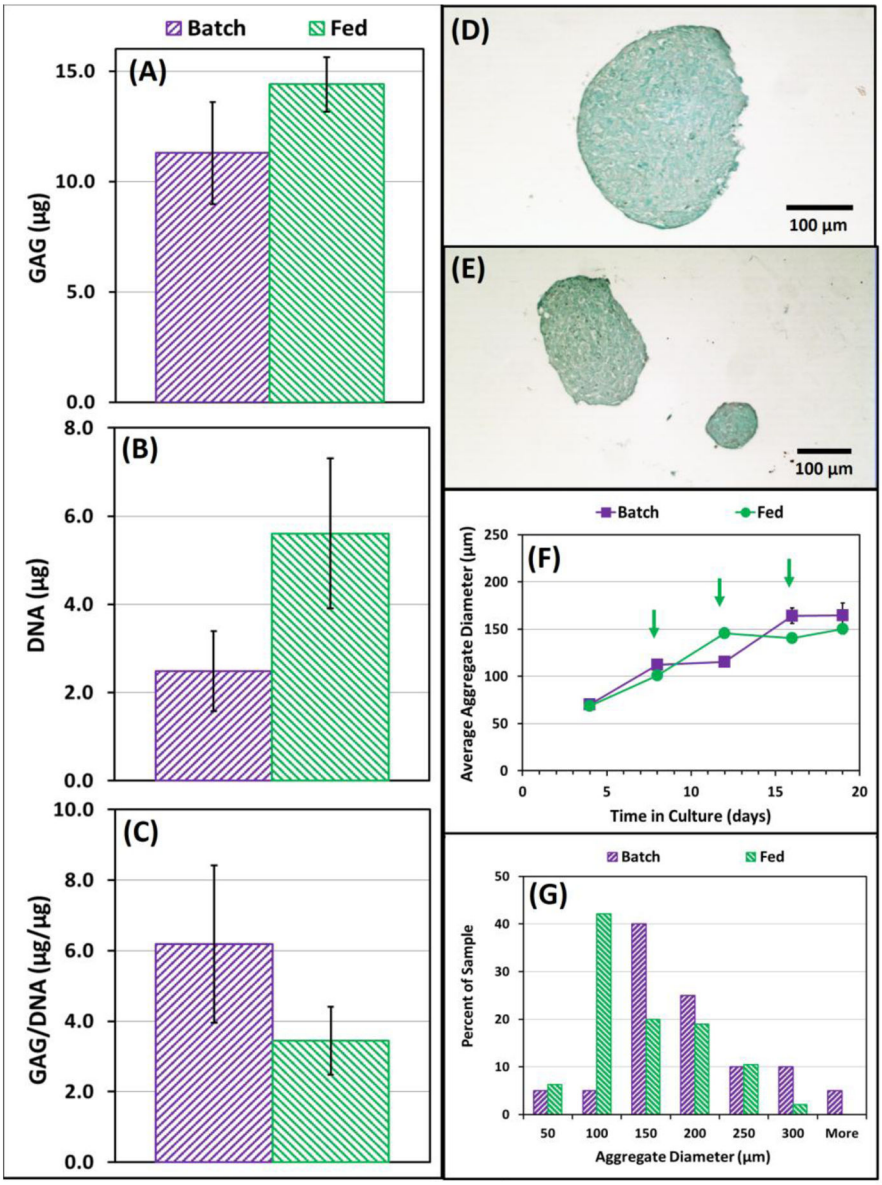

Figure 6. Feeding cells in bioreactor co-culture–GAG levels and aggregate morphology

A) GAG, B) DNA and C) GAG/DNA of the aggregates are shown in the batch and fed conditions after 19 days in culture. Error bars show standard error of the mean of duplicate samples. Safranin O staining of cells co-cultured in the D) batch and E) fed conditions are shown. F) Average aggregate diameter is shown over the culture period. Error bars show standard error of the mean of 20 aggregates from duplicate flasks. Green arrows indicate time points for 50% medium change for the fed condition. G) Aggregate diameter distribution after 16 days in culture is shown.

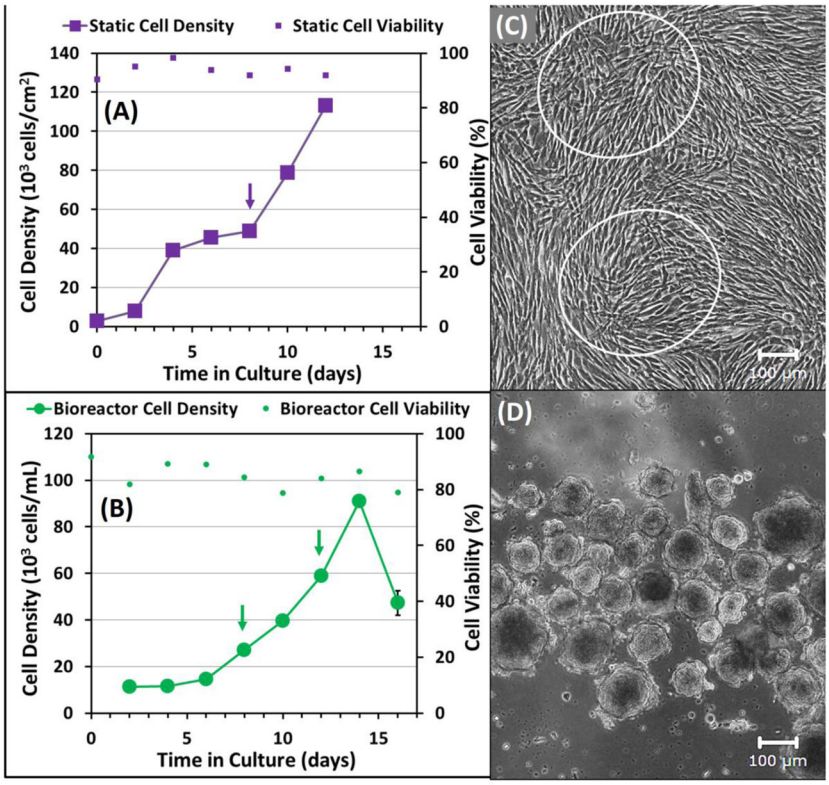

Figure 7. Comparison of bioreactor and static culture protocols–cell density and cell morphology

Cell densities in A) static co-culture and B) bioreactor co-culture are shown. Error bars show standard error of the mean of duplicate cell counts from duplicate flasks. Arrows indicate time points for 50% medium change. Photomicrographs of co-cultures of hMSCs and hACs are shown at 10x magnification after 10 days in culture in C) static culture flasks and D) bioreactors. White ovals highlight areas of stratified monolayers.

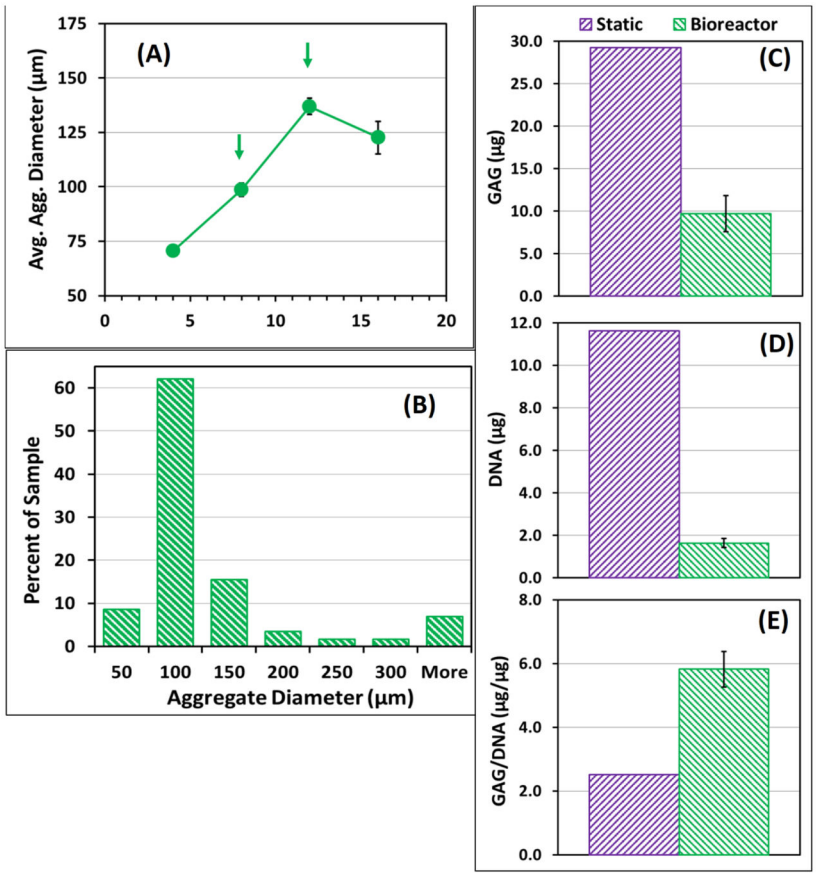

Figure 8. Comparison of bioreactor and static culture protocols–GAG levels and aggregate morphology

A) Average aggregate diameter is shown. Error bars show standard error of the mean of 20 aggregates from duplicate flasks. Green arrows indicate time points for 50% medium change. B) Aggregate diameter distribution after 16 days in culture is also shown. C) GAG, D) DNA and E) GAG/DNA in the bioreactor and static conditions are shown after 10 days in culture. Error bars show standard error of the mean of duplicate samples.

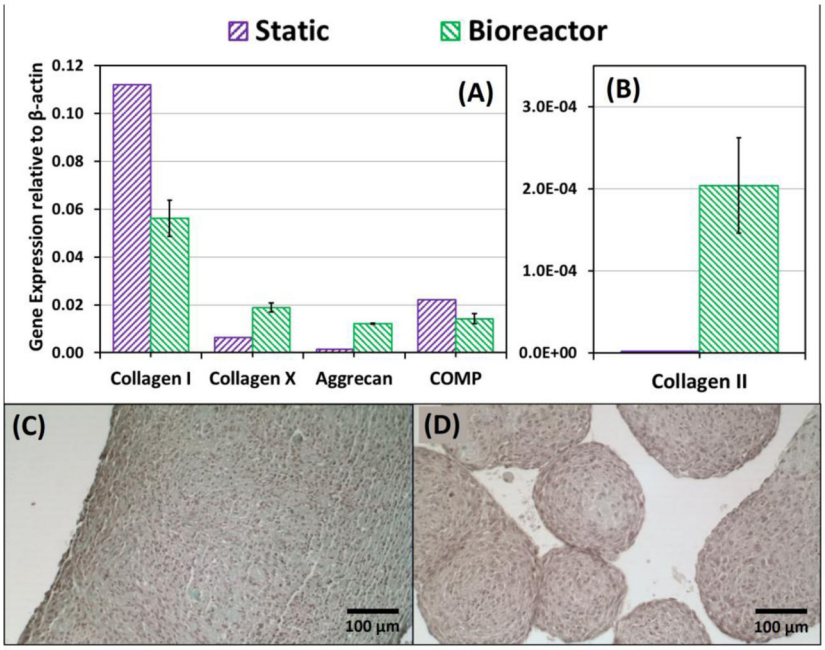

Figure 9. Comparison of bioreactor and static culture protocols–gene expression levels and GAG deposition

The gene expression, relative to β-actin, for the static and bioreactor conditions is shown after 10 and 16 days in culture respectively for C) collagen I, collagen X, aggrecan, COMP, and D) collagen II. Error bars show standard error of the mean of duplicate samples. Safranin O staining of cells co-cultured in the bioreactor condition is shown after C) 10 and D) 16 days in culture.

Table I

Primer sequences used in quantitative real-time polymerase chain reaction

All primers were purchased from Life Technologies.

Gene	Primer	Direction
β-Actin	5′-AAGCCACCCCACTTCTCTCTAA-3′	(Fwd)
Accession# NM_001101	5′-AATGCTATCACCTCCCCTGTGT-3′	(Rev)
Aggrecan	5′-AGGGCGAGTGGAATGATGTT-3′	(Fwd)
Accession# M55172	5′-GGTGGCTGTGCCCTTTTTAC-3′	(Rev)
Collagen I (*COL1A2*)	5′-TTGCCCAAAGTTGTCCTCTTCT-3′	(Fwd)
Accession# NM_000089	5′-AGCTTCTGTGGAACCATGGAA-3′	(Rev)
Collagen II (*COL2A1*)	5′-CTGCAAAATAAAATCTCGGTGTTCT-3′	(Fwd)
Accession# NM_033150	5′-GGGCATTTGACTCACACCAGT-3′	(Rev)
Collagen X	5′-CAAGGCACCATCTCCAGGAA-3′	(Fwd)
Accession# X_60382	5′-AAAGGGTATTTGTGGCAGCATATT-3′	(Rev)
COMP	5′-CCGACAGCAACGTGGTCTT-3′	(Fwd)
Accession# NM_000095	5′-CAGGTTGGCCCAGATGATG-3′	(Rev)

Table II

Comparison of the chondrogenic characteristics of cells co-cultured in bioreactors in the absence of chondrogenic media (the cells in the current study) to cells co-cultured in the presence of chondrogenic medium (published studies).

Cells	Medium	GAG/DNA	Collagen II/I Expression Ratio	Reference
OA hACs and hMSCs	SCM	27.2	7.1	This manuscript
OA hACs and hMSCs	SFM	5.8	0.58	This manuscript
Human meniscus cells and hMSCs	Chondrogenic	5	0.5–1	Saliken et al., 2012
hACs and hMSCs	Chondrogenic	13–14		Sabatino et al., 2012
OA hACs and hMSCs	Chondrogenic	7–15		Wu et al., 2012
Bovine ACs and Lapine MSCs	Chondrogenic without TGF-β3	20	0.01–0.1	Dahlin et al., 2014
Lapine ACs and hMSCs	Chondrogenic	1–30	<1	Xu et al., 2013
OA human chondrons and MSCs	Chondrogenic	2–8		Bekkers et al., 2013
hACs and hMSCs	Chondrogenic	18–23 (at 3 weeks)		Giovanni et al., 2010
OA hACs and hMSCs	Chondrogenic without TGF-β1	30 (at 14 days)	0.25–1 (at 3–21 days)	Aung et al., 2011

Table III

Kinetic parameters for hMSC and hAC bioreactor co-culture for batch and fed operation modes

Literature values for bovine ACs (Suits, 2006), hMSCs (Higuera et al., 2009; Pattappa et al., 2011; Schop et al., 2009) and murine MSCs (Deorosan & Nauman, 2011) in static culture are included for comparison. Note that literature values for hAC/hMSC co-culture in static conditions were not available, and literature values for bioreactor culture of hACs or hMSCs were also not available.

Parameter	Batch		Fed		Static Monoculture
	Value	R^2	Value	R^2	Literature Values
Maximum Viable Cell Density (cells/mL)	4.1E4	N/A	9.5E4	N/A	N/A
Maximum Apparent Growth Rate (h^{-1})	0.0072	N/A	0.0084	N/A	0.02 (Schop et al., 2009), 0.027–0.039 (Higuera et al., 2009)
Doubling Time (h)	96	N/A	83	N/A	35.5 (Schop et al., 2009), 17.7–25.5 (Higuera et al., 2009), 59.4–88.8 (Pattappa et al., 2011)
Glucose Uptake Rate (pmol/cell·day)	10	0.87	9.0	0.92	9.19 (Schop et al., 2009), 3.00–9.05 (Higuera et al., 2009), 6.54–9.08 (Pattappa et al., 2011)
Glutamine Uptake Rate (pmol/cell·day)	8.0	0.99	4.0	0.96	4.48 (Schop et al., 2009), 0.169–54.5 (Higuera et al., 2009)
Lactic Acid Production Rate (pmol/cell·day)	20	1.00	20	0.99	5.95–18.4 (Higuera et al., 2009), 15.3–23.0 (Pattappa et al., 2011)
Ammonia Production Rate (pmol/cell·day)	4.0	0.68	2.0	0.90	-
Yield of Lactic Acid over Glucose (mol/mol)	0.87	0.89	1.57	0.96	1.98 (Suits, 2006), 1.96 (Schop et al., 2009), 1.98–2.1 (Higuera et al., 2009), 2.32–2.69 (Deorosan & Nauman, 2011)
Yield of Ammonia over Glutamine (mol/mol)	0.49	0.74	0.67	0.97	1.63 (Schop et al., 2009)