Osteoarthritis and Cartilage

Functional consequences of glucose and oxygen deprivation on engineered mesenchymal stem cell-based cartilage constructs

M.J. Farrell † ‡ §, J.I. Shin †, L.J. Smith † § ||, R.L. Mauck † ‡ § *

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<td>Objective: Tissue engineering approaches for cartilage repair have focused on the use of mesenchymal stem cells (MSCs). For clinical success, MSCs must survive and produce extracellular matrix in the physiological context of the synovial joint, where low nutrient conditions engendered by avascularity, nutrient utilization, and waste production prevail. This study sought to delineate the role of microenvironmental stressors on MSC viability and functional capacity in three dimensional (3D) culture.</td>
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| Design: We evaluated the impact of glucose and oxygen deprivation on the functional maturation of 3D MSC-laden agarose constructs. Since MSC isolation procedures result in a heterogeneous cell population, we also utilized micro-pellet culture to investigate whether clonal subpopulations respond to these microenvironmental stressors in a distinct fashion. |

| Results: MSC health and the functional maturation of 3D constructs were compromised by both glucose and oxygen deprivation. Importantly, glucose deprivation severely limited viability, and so compromised the functional maturation of 3D constructs to the greatest extent. The observation that not all cells died suggested there exists heterogeneity in the response of MSC populations to metabolic stressors. Population heterogeneity was confirmed through a series of studies utilizing clonally derived subpopulations, with a spectrum of matrix production and cell survival observed under conditions of metabolic stress. |

| Conclusions: Our findings show that glucose deprivation has a significant impact on functional maturation, and that some MSC subpopulations are more resilient to metabolic challenge than others. These findings suggest that pre-selection of subpopulations that are resilient to metabolic challenge may improve in vivo outcomes. |

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Introduction

Given the high prevalence of chronic arthritic conditions and the limited healing capacity of cartilage, tissue-engineering strategies for the treatment of cartilage damage are widely investigated. Advances in tissue culture techniques have resulted in the production of three dimensional (3D) chondrocyte-laden tissues with mechanical properties on the order of native tissue, and these constructs are beginning to transition through pre-clinical models. While promising, these approaches are limited by the need for healthy autologous tissue, low cell yield, and dedifferentiation of chondrocytes during expansion. As such, alternative cell sources are being explored for cartilage repair applications.

Bone marrow-derived MSCs were first described by Friedenstein in the 1970s as colony forming units that adhere to and expand upon tissue culture plastic. Since then, the multipotential differentiation capacity of these cells has been widely established. In the presence of chondrogenic factors, MSCs are capable of producing a cartilage-like matrix with high glycosaminoglycan (GAG) content in vitro. However, when cultured in the same conditions, chondrocytes outperform MSCs, with higher viability, increased matrix production, and increased compressive modulus. A recent study from our group, using a 3D agarose hydrogel model and local analysis of mechanical properties (compressive equilibrium modulus), showed that the properties of MSC-based constructs are higher at the construct periphery compared to the...
same region of constructs based on chondrocytes that were cultured identically\textsuperscript{26}. The marked disparity in overall (bulk) construct properties arose from deficiencies in the central regions, where local mechanical properties in MSC-based constructs were significantly lower than those of chondrocyte-based constructs. This deficit in mechanical function in the central region was associated with a loss of cell viability and lower GAG content relative to chondrocyte-based constructs. Since MSCs perform well in areas of maximal nutrient supply (at the construct periphery), but poorly within central regions (where nutrient supply is lower), these data suggested that MSCs might be more sensitive than chondrocytes to deprivation of nutrients and other metabolic factors.

One confounding factor that likely contributes to the disparity found between chondrocytes and MSCs is the inherent heterogeneity in these populations that arise from multiple, distinct adherent colonies\textsuperscript{9,16}. Within a single donor, individual MSC colonies display noticeable differences in morphology, proliferation, and differentiation potential (along adipogenic, osteogenic, and chondrogenic lineages)\textsuperscript{9,16–22}. Thus, MSC populations utilized in most tissue engineering (TE) applications are comprised of cells with varying chondrogenic potential. While some recent studies have attempted to tie stem cell differentiation potential to cell mechanical properties\textsuperscript{22}, \textsuperscript{23}, no readily available markers exist to identify colonies (or clones) of optimal performance, and so heterogeneous populations are commonly used\textsuperscript{24,25}. In our previous studies, we noted that while central regions were associated with poor viability in MSC-laden constructs, a subset of the population remained viable (\textasciitilde20\%–40\%)\textsuperscript{26}. These cells may represent MSC clonal subpopulations that can not only undergo chondrogenesis, but also survive and thrive in a demanding, nutrient-poor, and hypoxic environment.

The hallmark of a successful tissue-engineered, stem cell-based cartilage construct is the formation of a stable, viable tissue with functional properties that approximate native cartilage. Not only must these constructs achieve a stable state through in vitro culture, but once implanted into the joint space, cells within engineered cartilage must survive and function within low oxygen (\textasciitilde1–7\%)\textsuperscript{27,28} and low nutrient conditions (\textasciitilde0.7–1.0 g/L glucose in the fasting state\textsuperscript{29,30}). Our previous studies demonstrated regional dependency with respect to viability and matrix production in MSC-laden constructs of an anatomically relevant thickness (2.25 mm), with fewer differences apparent in chondrocyte laden constructs. As a number of factors may contribute to the performance and health of MSCs, the objective of this study was to first investigate the consequence of decreased nutrient and metabolite availability (glucose and oxygen) on the functional properties of MSC-laden constructs. Further, to clarify the roles that distinct clonal subpopulations may play in overall tissue maturation, our second objective was to explore the MSC clone-dependent response to these same stressors using a micro-pellet assay. Findings from this study may aid in the optimization of culture methods for growing engineered cartilage using MSCs, and in the development of new tools for the selection of MSC subpopulations that are particularly suited for cartilage tissue engineering applications.

Materials and methods

**MSC isolation and hydrogel culture**

Bone marrow derived mesenchymal stem cells (MSCs) were isolated from two donor calves (3–6 months old; Research 87, Boylston, MA, USA) as previously described\textsuperscript{12}. Cells were expanded through passage 2 (plating density of \textasciitilde5,000 cells/cm\textsuperscript{2}) in a high glucose basal media (BM) (Dulbecco’s Modified Eagles Medium (DMEM; Gibco, Invitrogen Life Technologies, Carlsbad, CA), 10% fetal bovine serum (FBS, Gibco), and 1% penicillin, streptomycin, and fungizone (PSF; Gibco)]. Passage 2 cells (the two donors combined for one biological replicate) were trypsinized, resuspended in chemically defined media\textsuperscript{12} at a density of 40 \times 10\textsuperscript{6} cells/mL, and mixed with 4% w/v molten Type VII agarose (49\% Sigma–Aldrich, St. Louis, MO, in PBS) at a 1:1 ratio. The agarose/cell solution (2\% agarose, 20 \times 10\textsuperscript{6} cells/mL) was cast between two parallel glass plates separated by either a 2.25 mm spacer (‘thick’; the same thickness as in work previously published by our group\textsuperscript{12,14,15}) or 0.75 mm spacer (‘thin’; to reduce diffusion distances), and constructs (4 mm diameter) were formed using a biopsy punch. For each hydrogel assay to follow, technical replicates are denoted with number of constructs or samples ‘n’.

Constructs were cultured through 28 days in chemically defined media with varying concentrations of glucose, oxygen, and transforming growth factor-beta 3 (TGF–β3). DMEM (Gibco) glucose concentrations were 1 g/L (low, –5.5 mM) or 4.5 g/L (high, –25 mM), and media was either supplemented with 10 ng/mL (+) TGF–β3 (R&D Systems, Minneapolis, MN) or contained no TGF–β3 (–). To control oxygen levels, constructs were cultured either in a humidified incubator at 37\°C with 5% carbon dioxide in ambient air (oxygen level of \textasciitilde21\% (normoxic)) or continuously within a humidified glovebox chamber (HypOxystation; HypOxygen, Frederick, MD) at 37\°C, 5\% carbon dioxide, and 2\% oxygen. A summary of culture conditions and abbreviations are provided in Table I. Media was changed twice weekly and volume scaled to construct size (0.3 and 1.0 mL/construct for thin and thick constructs, respectively). Media was sampled each week (thin constructs), 3 days after the previous feeding, and glucose concentration measured using the Amplex Red Glucose Assay (Molecular Probes, Invitrogen Life Technologies, Carlsbad, CA).

**Quantification of cell viability**

Constructs were stained with a Live/Dead cell viability kit (Molecular Probes, Invitrogen Life Technologies). Thick constructs were halved through the median plane and imaged at 2 \times on Day 28 with an inverted fluorescence microscope (TE2000U; Nikon, Tokyo, Japan). For thin constructs, images of both axial surfaces (construct top and bottom) were acquired at 2 \times and 10 \times magnification on Days 7, 14, 21, and 28. Percent viability (thin constructs) was calculated by counting the number of dead cells (ethidium homodimer-1, red) and live cells (calcein, green) in the 10 \times images\textsuperscript{27}. Since viability differed greatly between the two surfaces, the sides of minimum and maximum viability were grouped for each condition.

**Construct mechanical properties and biochemical content**

Thick constructs (n = 4) were tested via unconfined, uniaxial compression as in\textsuperscript{11}. Testing consisted of a 2 g creep load for 300 s followed by a stress relaxation test (10\% strain applied at 0.05\% per second, 1000 s relaxation phase), from which equilibrium load was recorded and equilibrium modulus calculated. Dynamic modulus was calculated from a subsequent dynamic test, with 1\% sinusoidal strain applied at 1 Hz. Tested constructs (n = 4) were digested with papain for 24 h at 60\°C\textsuperscript{27}. Sulfated GAG was measured via the ortho-hydroxyproline (OHP) assay\textsuperscript{31} with an OHP:collagen factor of 7.14\textsuperscript{32}. Data are presented as percent of construct wet weight (% ww).

**Histology**

Constructs (n = 3) were fixed, paraffin embedded, and sectioned to 8 \mum thickness. Sections were stained for proteoglycans with...
Alician Blue (pH 1.0; Rowley Biochemical Inc, Danvers, MA, USA). Additional sections underwent immunohistochemical detection of type II collagen (5 μg/mL; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), following the manufacturer’s instructions for the Millipore Immunoperoxidase Secondary Detection System (EMD Millipore Corporation, Billerica, MA).

**Isolation of clonal MSC subpopulations**

Clonal MSC subpopulations from a single donor were isolated using the trypsin droplet technique (adapted from35). Briefly, two marrow isolates from the same donor were plated and cultured for 10–11 days until clearly demarcated colonies were present. One plate was passaged resulting in a mixed population. Colonies of the second plate were identified at 4× magnification and their positions were marked. The plate was then washed with phosphate buffered saline (PBS), and a cell scraper used to remove cells outside of the identified colonies. A surgical spear was used to outline the outer rim of each colony, drying the plate to allow for sufficient surface tension to hold a droplet of trypsin in place. Colonies with surface tension to hold a droplet of trypsin in place. Colonies with hypoxic conditions. (A) Schematic outlining culture conditions and their combinations. Dark gray boxes indicate control conditions. Biomechanical properties: (B) equilibrium modulus and (C) dynamic modulus. Biochemical constituents: (D) GAG content, and (E) collagen content reported as a percent wet weight (% ww). * indicates significant difference of Norm vs Hyp in same TGF-β and glucose condition. # indicates significant difference of LG vs HG in same TGF-β and oxygen condition. Ø indicates significant difference of (-) vs (+) in same glucose and oxygen condition. n = 4 constructs for all groups. P-value for comparison is P < 0.001 unless otherwise denoted in brackets. Gray box underlay displays 95% confidence interval of data set.

**Table 1**

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<td>Oxygen</td>
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<td>Norm</td>
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<tr>
<td></td>
<td>Hypoxia</td>
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<td>Without TGF</td>
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<td>(−)</td>
</tr>
<tr>
<td></td>
<td>With TGF</td>
<td>10 ng/mL</td>
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Cells from each clonal subpopulation were pelleted (20,000 cells per pellet) in a 96-well conical well plate and cultured in low glucose or high glucose chemically defined media under normoxic or hypoxic conditions, with all media containing TGF-β3. Pellets were cultured for 14 days and fed twice weekly with 100 μL of media per pellet (approximately five times the media volume per cell compared to hydrogel studies). Sample number varied for each assay (n = 1–3 with n denoting number of pellets assayed as technical replicates), depending on colony cell yield. On Day 14, pellets were stained with the Live/Dead kit. Confocal stacks were acquired from the edge of the pellet to a depth of 100 μm using an Olympus Fluoview FV1000 confocal microscope (Olympus America Inc, Center Valley, PA) with a 10× UPlanFL objective and 2× zoom. Velocity 3D Image Analysis Software (PerkinElmer, Waltham, MA) was used to reconstruct pellet volume (green/live channel) and count objects (nuclei of dead cells; red/dead channel) within that volume [Fig. 6(C)]. Data are presented as number of dead cells counted/pellet volume. Additional pellets were digested with papain and GAG content quantified. DNA content was measured via the Quant-IT PicoGreen dsDNA Kit (Molecular Probes, Invitrogen Life Technologies). Matrix production is presented as both μg GAG per pellet and μg GAG per μg DNA.

**Statistics**

Data are presented as mean and standard deviation. Statistical analysis was carried out using SYSTAT (Systat Software, Inc., Chicago, IL, USA) to determine significance (P < 0.05). For statistical
comparisons, 2-way analysis of variance (ANOVA) tests were conducted, with media type (HG+, HG−, LG+, LG−) and oxygen level (normoxic, hypoxic) as the independent variables. Tukey’s post-hoc tests were used to make pairwise comparisons between groups. Due to limited sample number, no statistical analyses were performed for colony studies.

Results

Impact of oxygen and glucose on construct mechanics and matrix content

Under standard culture conditions (Norm, HG), and with the addition of TGF-β3 (HG+ vs HG−), construct equilibrium (142 vs 20 kPa) and dynamic moduli (1.0 vs 0.2 MPa) increased markedly by Day 28 (P < 0.001 for both; Fig. 1(B) and (C)). Culture in low oxygen (Hyp) in HG+ conditions resulted in lower equilibrium and dynamic moduli (vs Norm HG), reaching values of 77 kPa (P < 0.001) and 0.5 MPa (P < 0.001), respectively. Hyp HG− did not differ from Norm HG− conditions, with constructs reaching an equilibrium modulus of 19 kPa and a dynamic modulus of 0.2 MPa (P = 1.000 for both). While lower mechanical properties were achieved under hypoxic conditions, the most marked differences were found in (+) constructs cultured in low glucose conditions (LG) compared to HG of the same oxygen tension. Equilibrium moduli of low glucose constructs reached lows of 8 kPa in Norm conditions and <1 kPa in Hyp conditions, with dynamic modulus following a similar pattern, reaching ~0.2 and <0.1 MPa, respectively.
GAG content of Norm HG(+) constructs was ~2-fold higher than (-) conditions (2.7% ww vs 1.2% ww, P < 0.001; Fig. 1(D)). Similar to trends in mechanical properties, hypoxic culture reduced GAG by ~30% (1.9% ww in Hyp HG+ conditions, P = 0.004 vs Norm HG+). Under LG conditions, GAG content was lower by 67% (P < 0.001) and 63% (P < 0.001) compared to respective HG+ conditions, reaching 0.9% ww in Norm LG+ conditions and 0.7% ww in Hyp LG+ conditions. A similar result was apparent for collagen content [Fig. 1(E)], where Norm HG+ conditions had the greatest collagen content at 0.7% ww, Hyp HG+ was 44% lower (P < 0.001), and LG+ conditions were 61% (Norm, P < 0.001) and 57% (Hyp, P = 0.001) lower compared to respective HG+ controls of similar oxygen tension.

Matrix distribution and cell viability in thick constructs

Staining for type II collagen and proteoglycans in Norm HG conditions showed light, punctate staining, homogeneously distributed in (-) conditions, and more intense staining in (+) conditions, with the greatest intensity towards the construct periphery [Fig. 2(A) and (B)]. Little difference was discernible in staining between Norm and Hyp constructs. In LG+ conditions, matrix staining was almost completely restricted to the construct periphery. Cell viability in cross sections of thick constructs showed similar patterns, where viable cells were restricted to the periphery of LG+ constructs, with little difference between the Norm HG+ and Hyp HG+ conditions [Fig. 3(A)].

Evaluation of viability and glucose utilization in thin constructs

Given the differences between the edge of constructs and the center, we next fabricated ‘thin’ constructs (0.75 mm thick) to reduce the distance over which nutrients needed to travel. Since cross sections of these thin constructs were difficult to image, viability was calculated for the top and bottom surface of each construct [Fig. 3(B) and (C)], from which the maximum and minimum viabilities were determined [Fig. 3(D) and (E)]. For thin constructs on Day 28, viability was high and roughly equivalent on both surfaces, with minimum and maximum viabilities in Norm HG+
conditions of ~90%. This finding indicates a stable and viable cell population in these thin constructs through the depth. At this same time point, in Norm LG/C0 and Hyp LG+ conditions, the maximum viability was significantly lower than Norm HG+. The lowest maximum viability was observed in the Hyp LG+ group, which reached 45% (52% lower than Norm HG+ levels; \( P < 0.001 \)). Minimum viability was significantly lower for all Hyp conditions, and for both (− and +) Norm LG conditions. The lowest minimum viability was observed in LG+ constructs cultured under Norm and Hyp conditions, where minimum viabilities were 37% and 3%, respectively, on Day 28.

Since it was apparent that LG conditions evoked the poorest viability and matrix deposition, we next measured glucose in the media. Samples were taken at weekly intervals, with media sampled 3 days after the addition of fresh media. Fresh media glucose levels were ~25 mM for high glucose DMEM and ~5.5 mM for low glucose DMEM. Results from this analysis showed that glucose levels in ‘spent’ media were lower when constructs were cultured in the presence of TGF-β3 (Fig. 4). In both Norm and Hyp HG+ cultures, glucose concentrations fell to ~5 mM after 3 days, with no difference between the two groups at Day 28 (\( P = 1.000 \)). While a small fraction of the original glucose remained in Norm and Hyp LG− cultures (~1.0 mM), glucose concentration in LG+ cultures fell to very low levels (~0.05 mM) after 3 days of culture, with no difference between the Norm and Hyp groups (\( P = 1.000 \)).

Clone-dependent response to metabolic stressors

The above findings illustrated that MSCs are sensitive to metabolic stressors, including low oxygen and low glucose conditions. However, because not every cell under the most taxing situation (Hyp LG+) lost viability, and because the heterogeneous MSC population is comprised of cells of different clonal origin, we next evaluated the impact of these stressors on a clone-by-clone basis using micro-pellets. For this, we isolated a total of 15 clonal colonies (C#) and two heterogeneous parent populations (Het) from two different donors, and evaluated viability and GAG content over 14 days. Consistent with the hydrogel studies, the poorest performing groups were those cultured under Hyp LG+ conditions. However, within a single donor, there was marked variability in response between clonal populations. Notably, for the first donor [Fig. 5(A) and Fig. 6(A)], some clones (C3 and C6) performed poorly, with little matrix production in all culture conditions [Fig. 6(A)] and had a marked increase in the number of dead cells in Hyp LG+ compared to all other conditions. However, other poor performing clonal colonies such as C2 and C5 had a more consistent and slightly higher baseline in the number of dead cells per volume (Norm HG+). Clonal colonies (C1 and C4) and the heterogeneous parent population, each with high GAG per pellet, were still susceptible to low glucose culture, resulting in lower GAG/pellet and GAG/DNA compared to their Norm HG+ counterparts. Data from the second donor [Fig. 5(B) and Fig. 6(B)] showed a different response, indicative of donor-to-donor variability. Although, once again, the response was variable between clonal colonies, indicated by differences in viability [Fig. 5(B) and (D)] and matrix production [Fig. 6(B)], some colonies (C3 and C7) responded favorably to Hyp HG+ conditions, producing more GAG per pellet.

Discussion

Given the limited supply of healthy autologous chondrocytes, strategies to further regenerative medicine approaches for cartilage repair have focused on stem cells. For clinical success, these cells must survive and produce extracellular matrix in the hypoxic and
When cells experience nutrient deprivation, they can adjust their metabolic state to survive under hypoxic conditions. For example, in hypoxic culture conditions, MSCs maintain their viability and cellular functions, which is critical for tissue engineering applications. However, glucose is a significant metabolite that can affect cell function and survival. Low glucose conditions can lead to a decrease in cell viability and matrix production, which may be due to the cells' inability to maintain their metabolic state under these conditions.

In summary, while many factors can influence cell survival and function in hypoxic conditions, glucose availability appears to be a critical factor. Therefore, optimizing glucose supply to MSCs in vitro and in vivo can help enhance their ability to survive and function in hypoxic environments.
RX000700). The authors would like to thank Joseph Chiaro and Deborah Gorth for technical assistance with hypoxic culture. The authors have no conflicts of interest to report that relate to this work.

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