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

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SUMMARY

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.
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.

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[1,2], and these constructs are beginning to transition through pre-clinical models[3]. While promising, these approaches are limited by the need for healthy autologous tissue, low cell yield, and dedifferentiation of chondrocytes during expansion[4,5]. 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[6–8]. 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*[9–12]. However, when cultured in the same conditions, chondrocytes outperform MSCs, with higher viability, increased matrix production, and increased compressive moduli[12–14]. 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

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same region of constructs based on chondrocytes that were cultured identically[15]. 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[9,16–18]. Within a single donor, individual MSC colonies display noticeable differences in morphology, proliferation, and differentiation potential (along adipogenic, osteogenic, and chondrogenic lineages)[9,19–22]. 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[23], no readily available markers exist to identify colonies (or clones) of optimal performance, and so heterogeneous populations are commonly used[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 (~20–40%)[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* preculture, but once implanted into the joint space, cells within engineered cartilage must survive and function within low oxygen (~1–7%)[27,28] and low nutrient conditions (~0.7–1.0 g/L glucose in the fasting state[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[12]. Cells were expanded through passage 2 (plating density of ~5,000 cells/cm^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[12] at a density of 40×10^6 cells/mL, and mixed with 4% w/v molten Type VII agarose (49°C; Sigma–Aldrich, St. Louis, MO, in PBS) at a 1:1 ratio. The agarose/cell solution (2% agarose, 20×10^6 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[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 ~21% (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 [Fig. 1(A) and 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× 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× and 10× 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× images[15]. 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[31]. 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[26]. Sulfated GAG was measured via the 1,9-dimethylmethylene blue dye-binding assay[32] and collagen content with the orthohydroxyproline (OHP) assay[33], with an OHP:collagen factor of 7.14[34]. Data are presented as percent of construct wet weight (% ww).

Histology

Constructs ($n = 3$) were fixed, paraffin embedded, and sectioned to 8 μm thickness. Sections were stained for proteoglycans with

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