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Functional Properties of MSC-Based Engineered Cartilage are Unstable with Very Long Term In Vitro Culture

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Abstract

The success of stem cell-based cartilage repair requires that the regenerate tissue reach a stable state. To investigate the long term stability of tissue engineered cartilage constructs, we assessed the development of compressive mechanical properties of chondrocyte and mesenchymal stem cell (MSC)-laden three dimensional agarose constructs cultured in a well defined chondrogenic *in vitro* environment through 112 days. Consistent with previous reports, in the presence of TGF- β , chondrocytes outperformed MSCs through day 56, under both free swelling and dynamic culture conditions, with MSC-laden constructs reaching a plateau in mechanical properties between days 28 and 56. Extending cultures through day 112 revealed that MSCs did not simply experience a lag in chondrogenesis, but rather that construct mechanical properties never matched those of chondrocyte-laden constructs. After 56 days, MSC-laden constructs underwent a marked reversal in their growth trajectory, with significant declines in glycosaminoglycan content and mechanical properties. Quantification of viability showed marked differences in cell health between chondrocytes and MSCs throughout the culture period, with MSC-laden construct cell viability falling to very low levels at these extended time points. These results were not dependent on the material environment, as similar findings were observed in a photocrosslinkable hyaluronic acid (HA) hydrogel system that is highly supportive of MSC chondrogenesis. These data suggest that, even within a controlled *in vitro* environment that is conducive to chondrogenesis, there may be an innate instability in the MSC phenotype that is independent of scaffold composition, and may ultimately limit their application in functional cartilage repair.

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Keywords

Mesenchymal Stem Cells; Chondrogenesis; Three-Dimensional Culture; Cartilage; Mechanical Properties

1. Introduction

The use of adult derived progenitor or stem cells for the clinical repair of cartilage defects has been investigated since the early 1990s. Purified isolations of bone marrow derived mesenchymal stem cells (MSCs) were first described by Friedenstein in the 1970s as colony forming fibroblast-like cells (Friedenstein et al., 1970). Since then, both the self-renewing and multipotent nature of these cells has been demonstrated (Pittenger et al., 1999). Importantly, these cells can undergo chondrogenic differentiation in defined culture conditions, suggesting that they may serve as a suitable alternate cell source for cartilage repair techniques (Johnstone et al., 1998; Mauck et al., 2006; Pittenger et al., 1999), overcoming the limitation of insufficient chondrocyte numbers needed for such repair strategies (Johnstone et al., 2013). Indeed, the chondrogenic potential of marrow derived MSCs motivates the widespread use of microfracture, wherein progenitor cells are mobilized from the underlying marrow to fill cartilage defects with regenerate tissue (Steadman et al., 2001).

Although popular, microfracture techniques have yet to achieve large scale, long-term clinical success, especially in large defects, as the initial clot remodels into a disorganized fibrocartilaginous scar tissue rather than a hyaline-like cartilage (LaPrade et al., 2008; Mithoefer et al., 2009). In some instances, hypertrophic overgrowth of the underlying subchondral bone has been noted (Brown et al., 2004; Mithoefer et al., 2005). The formation of disorganized tissue suggests the need for additional physical and chemical cues to guide MSC chondrogenesis and improve matrix production. Much of the work in the field of cartilage tissue engineering over the last decade has focused on the development of better materials to support the initial chondrogenic event and the rate and distribution of tissue formation (Bian et al., 2013; Connelly et al., 2011; Huang et al., 2010b; Rowland et al., 2013; Tuan et al., 2013; Unterman et al., 2012; Wang et al., 2005). With advances in growth factor treatments and three-dimensional culture techniques, stem cell based cartilage tissue engineering strategies have progressed to the point where constructs with hyaline-like cartilage constituents and mechanics can be formed *in vitro* with culture over the course of 2–3 months (Erickson et al., 2012). However, while these engineered constructs approach native cartilage properties, MSC-laden constructs produce tissue that is inferior to that produced by chondrocyte-laden constructs cultured identically, suggesting a sustained difference between chondrocytes and chondrogenically induced MSCs (Farrell et al., 2012; Mauck et al., 2006; Vinardell et al., 2009).

Differences between MSC- and chondrocyte-based engineered constructs have been investigated on the molecular, microscopic tissue, and macroscopic tissue level (Boeuf et al., 2008; Farrell et al., 2012; Huang et al., 2010c). Multiple studies have noted that MSC-based constructs increase in content and properties for a period of time, before reaching a plateau in cartilage-like matrix content and macroscopic (whole tissue level) equilibrium mechanical

properties (Huang et al., 2010a; Mauck et al., 2006; Vinardell et al., 2012). Our previous studies showed that this plateau and the resultant lower properties of MSC-laden constructs (in comparison to chondrocyte-laden constructs) were due in part to the lack of tissue elaboration and compromised stem cell health in central regions of constructs that were deprived of nutrients (Farrell et al., 2012). This deficit could be partially rescued by increasing nutrient supply via exposure to dynamic culture systems (i.e. orbital shaking) that improved nutrient access. However, even with this modification, the mechanical properties of MSC-laden constructs remained significantly lower than chondrocyte-laden constructs cultured similarly (Farrell et al., 2012).

One potential reason for the lack of mechanical equivalence between engineered cartilage constructs formed from MSCs and chondrocytes may simply be that a lag exists during which MSCs differentiate to the chondrogenic state. Chondrocytes, and the tissue they produce, are exposed to a number of soluble and mechanical factors through development, which culminate over a period of years in a tissue with refined properties (Koyama et al., 2008; Williamson et al., 2001). Conversely, engineered tissues based on MSCs are forced to undergo both differentiation and maturation within an abbreviated time scale. Notably, MSC-based constructs appear to respond negatively to dynamic loading early in culture (Thorpe et al., 2008), but respond in a positive fashion after a brief period (1–3 weeks) of differentiation (Huang et al., 2010a; Mouw et al., 2007). Supporting this notion, whole genome profiling revealed that many genes remain differentially regulated between MSCs and chondrocytes cultured in agarose after 28 days (Huang et al., 2010c). However, gene expression remained dynamic through day 56, suggesting that MSCs may have the capacity to continue towards a more chondrogenic state with prolonged culture. Thus the disparity in mechanical properties might be a function of insufficient time to achieve the chondrogenic state, rather than an innate limitation in cartilage-forming potential by MSCs.

An alternative explanation for the disjunction between chondrocyte and MSC-based engineered cartilage may lie in the completeness of phenotypic conversion. It may well be that the best conditions for chondrogenesis of MSCs *in vitro* simply prolong their residence in that state, but does not eliminate the possibility of differentiation towards alternative lineages. For example, it has been shown that MSCs committed to one lineage (e.g., adipogenesis) can be recovered and forced down another lineage (e.g. osteogenesis), suggesting a somewhat tenuous hold on the differentiated phenotype (Song and Tuan, 2004). Our recent studies have shown that transient application of pro-chondrogenic factors, including transforming growth factor beta (TGF- β), in a defined serum free medium, is sufficient to induce and sustain the chondrogenic state, without evidence of type X collagen or mineral deposition (Huang et al., 2009; Kim et al., 2012). However, a number of other studies have reported transition from a chondrogenic to a hypertrophic phenotype (with expression of type X collagen, bone markers, and eventual mineralization) when constructs were transferred to environments that presented conflicting signals (Studer et al., 2012). For example subcutaneous implantation of chondrogenic pellets and hydrogels commonly results in formation of a mineralized tissue (Bian et al., 2011b; Pelttari et al., 2006; Vinardell et al., 2012), and challenge with pro-hypertrophic conditions (i.e., removal of TGF and addition of thyroid hormone T3) can result in *in vitro* mineralization.

Collectively, these data suggest that assessment of cartilage tissue development over a longer period, within a highly controlled chemical environment, will be required to fully appreciate both the potential of these engineered tissues, and to further their *in vivo* efficacy. The purpose of this study was therefore to evaluate the long-term time course of cartilage development and phenotypic stability in MSC- and chondrocyte-laden three-dimensional agarose hydrogel constructs. We evaluated the cartilage-like properties of these constructs in both free-swelling and dynamic culture (to increase nutrient supply) over a long *in vitro* culture period (4 months). Furthermore, to investigate material dependency, we assessed whether the long-term chondrogenic tissue development and phenotypic stability differed in an alternative 3D hydrogel system (photocrosslinkable hyaluronic acid (HA) (Burdick et al., 2005)). We hypothesized that a lack of inherent potential, rather than simply a lag phase in tissue production, governs the long-term maturation of MSC-laden constructs. We further hypothesized that MSC-based constructs would achieve a stable equilibrium state (in terms of mechanics and biochemical content) that was lower than chondrocyte-based constructs similarly maintained.

2. Materials and Methods

2.1 Study 1: Long-Term Culture of Cell-Seeded Agarose Hydrogels

2.1.1 Cell Isolation, 3D Encapsulation, and Agarose Culture—Juvenile bovine bone marrow derived mesenchymal stem cells (MSCs) were isolated from the femurs of three donor calves (3–6 months old; Research 87, Boylston, MA) (Mauck et al., 2006) and expanded through passage 2 in media consisting of high glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco, Invitrogen Life Sciences, Carlsbad, CA), 10% fetal bovine serum (FBS, Gibco), and 1% penicillin-streptomycin-fungizone (PSF; Gibco). Primary chondrocytes were isolated from the carpometacarpal joint of the three donors. Briefly, cartilage was diced and subjected to pronase digestion (2.5 mg/mL, 1 hr @ 37°C, Calbiochem/EMD Chemicals, Gibbstown, NJ) followed by collagenase digestion (0.5 mg/mL, 6 hrs @ 37°C, Sigma-Aldrich, St. Louis, MO) (Mauck et al., 2003). Expanded MSCs and freshly isolated chondrocytes were independently encapsulated in 2% agarose at a density of 20 million cells/mL. Specifically, a cell suspension (40 million cells/mL in a chemically defined media) was homogenously mixed with molten 4% w/v agarose (type VII (Sigma), 49°C) at a 1:1 ratio and cast between two parallel plates (Mauck et al., 2003; Mauck et al., 2006). Constructs 4 mm in diameter and 2.25 mm in depth were extracted from the hydrogel slab using a biopsy punch. Constructs were fed twice weekly with chemically defined media with (+) or without (–) supplementation with 10 ng/mL transforming growth factor-beta 3 (TGF-β3; R&D Systems, Minneapolis, MN). Chemically defined media consisted of high glucose DMEM, PSF, dexamethasone, ascorbate 2-phosphate, insulin, transferrin, selenous acid, bovine serum albumin, and linoleic acid as in (Farrell et al., 2012; Mauck et al., 2006). Constructs were cultured in free swelling (FS) or dynamic conditions (Dyn) (as in (Farrell et al., 2012)) through 112 days. For dynamic culture, constructs were exposed to continuous orbital shaking at 1.2 Hz (Bellco 115V Orbital Shaker, Bellco Glass, Inc., Vineland, NJ). Throughout the remainder of this work, FS(+) or Dyn(+) refers to constructs in free swelling or dynamic conditions with TGF-β, while FS(–) and Dyn(–) refers to constructs under those same conditions without TGF-β.

CM(−) and CM(+) denote groups cultured without or with TGF- β , regardless of free swelling or dynamic conditions.

2.1.2 Mechanical Analysis of Bulk Properties—Mechanical properties of constructs (n=5) were assessed via uniaxial unconfined compression (Mauck et al., 2000). First, constructs were equilibrated under creep (tare load for days 1–28=2 g; day 56=5 g; day 112=10 g) for 300 sec. Stress relaxation tests were carried out by applying 10% strain at a strain rate of 0.05%/sec followed by a 1000 sec relaxation phase. Stress relaxation data was curve fit with a double exponential decay function using a custom MATLAB script. Equilibrium modulus was calculated from equilibrium load and sample geometry. After stress relaxation, a 1% sinusoidal strain was applied at 1 Hz, and the dynamic modulus was calculated from the dynamic stress-strain response. Tested samples were frozen at −20°C for subsequent biochemical assessment.

2.1.3 Histological Analysis—Construct halves (n=2) were fixed in 4% paraformaldehyde (FD NeuroTechnologies, Inc, Ellicott City, MD), paraffin processed following dehydration with a series of ethanol solutions, and sectioned (8 μ m). After rehydration, sections were stained for proteoglycans (Alcian Blue, Rowley Biochemical, Inc, Danvers, MA) or collagens (stained after 1 hr hyaluronidase incubation [1 mg/ml] at 37°C (Melrose et al., 2004); Picrosirius Red [Sirius Red (Sigma), Picric Acid (Fisher Scientific)]). Alizarin Red (Rowley Biochemical) staining was performed to identify calcium deposits. Finally, immunohistochemistry was performed to discriminate between type I and type II collagen deposition. Specifically, deparaffinized sections were rehydrated and subjected to proteinase K antigen retrieval for 15 min at 37°C. Sections were then incubated with either a type II collagen antibody (5 μ g/mL; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) or a type I collagen antibody (10 μ g/mL; Anti-Collagen type I Antibody, clone 5D8-G9, Millipore) for 1 hr. After washing, signal was detected using the Millipore Immunoperoxidase Secondary Detection System (EMD Millipore Corporation, Billerica, MA) per the manufacturer's protocol.

2.1.4 Biochemical Analysis—Matrix components were solubilized via papain digestion at 60°C for 24 hours (20 μ l papain per 1 mL buffer [0.1 M sodium acetate, 10 M cysteine HCl, 0.05 M ethylenediaminetetraacetic acid, pH 6.0]). Glycosaminoglycan (GAG) content was quantified using the 1,9-dimethylmethylene blue dye-binding assay (Farndale et al., 1986), and collagen content quantified using the orthohydroxyproline assay (Stegemann and Stalder, 1967). An OHP:collagen ratio correction factor of 7.14 was used to convert μ g of OHP to μ g of collagen (Neuman and Logan, 1950).

2.1.5 Quantification of Viability—Using the LIVE/DEAD Cell Viability Assay Kit for mammalian cells (Molecular Probes, Invitrogen, Life Sciences), construct halves (n=3) were stained for 30 min in PBS. Calcein-AM and ethidium-homodimer-1 signal were imaged in the central region of the construct under 10X magnification. A custom MATLAB script (The MathWorks Inc, Natick, MA) was used to count the number of cells within each channel, from which percent viability was determined (Farrell et al., 2012).

2.2 Study 2: Long-Term Culture of Cell-Seeded Hyaluronic Acid Hydrogels

2.2.1 Hyaluronic Acid Hydrogel Formation and Cell Encapsulation—MSCs (2 donors) were isolated and expanded through passage 2 as in Study 1. Cells were suspended at a density of 60 million cells/mL in a 1% (w/v) methacrylated hyaluronic acid (HA) solution that was subsequently crosslinked into a hydrogel via a UV light initiated addition reaction. The HA hydrogel methacrylation process and UV cell encapsulation process has previously been described in (Burdick et al., 2005; Erickson et al., 2009b). Constructs were fed thrice weekly with 1 mL/construct of CM+ through 126 days of culture in free swelling conditions.

2.2.2 Mechanical, Biochemical, and Histological Analyses—Using a cryotome, the top and bottom of each construct was leveled. Compressive equilibrium modulus was evaluated (n=4) via unconfined compression as described in Study 1 (creep tare load = 2 g for all time points assessed). Following testing, samples were papain digested and assessed for glycosaminoglycan and collagen content as described in Study 1. Paraffin processed sections were stained for collagens (Picrosirius Red) and proteoglycans (Alican Blue) and imaged under 10x magnification.

2.2.3 Statistical Analysis—The statistical software SYSTAT (Systat Software, Inc., Chicago, IL) was used to conduct pair-wise comparisons between groups. For Study 1 (agarose hydrogel), significance ($p < 0.05$) was established with 1-way or 2-way ANOVA and Tukey's post-hoc correction (the independent variable for viability was day; independent variables for equilibrium modulus, dynamic modulus, GAG content, and collagen content were day and culture condition). For Study 2 (HA hydrogel), significance ($p < 0.05$) was established with 1-way ANOVA and Tukey's post-hoc correction with day as the independent variable for equilibrium modulus, dynamic modulus, GAG content, and collagen content. Data are presented as mean and standard deviation.

3. Results

3.1 Study 1: Long-Term Culture of Cell-Seeded Agarose Hydrogels

3.1.1 Biomechanical and Biochemical Analyses—In order to determine the stability of cell-seeded agarose constructs over long-term culture, we evaluated functional outcomes at defined time points through 112 days of culture. Consistent with previous findings, the equilibrium modulus of constructs increased with time (D14 to D112; $p < 0.001$) for chondrocyte-laden constructs in CM(+) conditions (Figure 1A). Peak modulus was achieved on day 112, and was either stable from day 56 to day 112 in free swelling conditions (FS+; 341 to 434 kPa) or increased in dynamic conditions (Dyn+; 538 kPa to 707 kPa). Dyn(+) construct modulus was markedly higher than FS(+) constructs at both time points. Dynamic modulus increased with time (D14 to D112; $p < 0.001$) in all culture conditions for chondrocyte-laden constructs, and either increased from day 56 to day 112 for FS(−) (2.01 to 4.90 MPa), Dyn(−) (2.00 to 3.21 MPa), and FS(+) (7.75 to 11.1 MPa) conditions or was stable for Dyn(+) (8.61 to 8.98 MPa) conditions (Figure 1B).

Contrary to these generally stable or increasing properties in chondrocyte-based constructs, mechanical properties of MSC-laden constructs were unstable over long-term culture in all conditions. In the absence of TGF- β [FS(–) and Dyn(–)], MSC-laden constructs failed to develop tissue with appreciable equilibrium modulus by day 56; however, there was an increase in dynamic modulus at day 112 for both FS(–) and Dyn(–) constructs (Figure 1A). When cultured in the presence of TGF- β [FS(+) and Dyn(+)], equilibrium modulus increased for MSC-laden constructs through day 28, with Dyn(+) constructs reaching a higher equilibrium modulus compared to FS(+) conditions (124 and 220 kPa, respectively). Dynamic modulus followed similar trends, reaching 1.09 MPa in FS(+) conditions and 1.74 MPa in Dyn(+) conditions at day 28. At day 112, there was a decline in equilibrium modulus from day 56 values. While this decline was substantial in FS(+) conditions (138 to 82 kPa), it was even more marked in Dyn(+) conditions (217 to 2 kPa). Similarly, there was a decline in dynamic modulus at day 112 in Dyn(+) conditions; however, this decline was less dramatic (1.95 to 0.89 MPa).

Analysis of glycosaminoglycan (GAG) and collagen content illustrated that the loss of construct mechanical properties was due to a loss of tissue constituents in MSC-laden constructs. For chondrocyte-laden constructs cultured in the presence of TGF- β , GAG content increased from D56 to D112 in both FS(+) (1.20 to 2.34 mg/construct) and Dyn(+) (1.73 to 4.31 mg/construct) constructs (Figure 2A). Conversely, for MSC-laden constructs, GAG levels dropped from 1.30 to 1.03 mg/construct in FS(+) conditions and 1.22 to 0.28 mg/construct in Dyn(+) conditions. Collagen content increased for chondrocyte-laden constructs from day 56 to day 112 in FS(+) (~0.08 to 0.15 mg/construct) and Dyn(+) (0.16 to 0.18 mg/construct) conditions (Figure 2B). Similarly, collagen content of MSC-laden FS(+) constructs increased from day 56 to day 112 (0.06 to 0.09 mg/construct). However, there was a decline in MSC-laden Dyn(+) constructs (0.07 to 0.04 mg/construct) over this same time period.

3.1.2 Histology and Immunohistochemistry—Staining for proteoglycans showed no evidence of tissue instability from day 56 to day 112 for chondrocyte-laden constructs (Figure 3A). Conversely, lighter and more diffuse staining of proteoglycans was apparent in MSC-laden FS(+) and Dyn(+) constructs when comparing day 112 to day 56. There were no apparent changes in collagen staining at these same time points (Figure 3B).

To determine whether the loss in mechanics represented a shift in phenotype, we next stained for collagen type on days 56 and 112. The presence of type II collagen would be indicative of positive and sustained chondrogenesis, while the presence of type I collagen would be indicative of a shift towards a fibrocartilage phenotype or potential hypertrophic differentiation. All groups stained heavily for type II collagen throughout the construct, and this was consistent over time (Figure 4A). Positive type I collagen staining was only apparent in the central regions of MSC-laden constructs cultured in the absence of TGF- β (Figure 4B). This staining was particularly evident in higher magnification images of MSC FS(–) constructs, with pockets of intense type I collagen staining along with more dispersed staining in inter-territorial regions (Figure 5A).

The presence of type I collagen and sudden increase in mechanical properties of MSC FS(–) and Dyn(–) constructs at the final time point suggested the emergence of a hypertrophic phenotype. To test for overt hypertrophy, Alizarin Red staining for calcium deposits was performed (Figure 5B). In both FS(–) and Dyn(–) MSC-laden constructs, there was a large amount of positive mineral staining in the center of constructs, whereas the remaining groups were negative for mineral deposition.

3.1.3 Cell Viability—Instability of MSC-laden constructs in long-term culture could potentially be precipitated by deficits in cell health and viability. To that end, we quantified cell viability over time in culture (Figure 6A). While there was some initial decline in viability in the center of chondrocyte-laden constructs in CM(–) conditions by day 14 (an ~10% decline), there was little further deviation through day 56, with values ranging from 75–85% for all conditions at days 28 and 56. At day 112, there was a small additional decline in FS(+) and Dyn(+) conditions with viability reaching 65% and 70%, respectively.

In a stark contrast, there was an immediate decline in viability in FS(–) and FS(+) MSC-laden constructs (~15–20% decline by day 4). This decline in viability was slightly delayed with dynamic culture. In FS(+), viability stabilized at ~40–45% between days 14 and 56. Differences in viability over the peak growth period (28 days) for MSC-laden constructs are shown in Figure 6B. By day 112, MSC viability had fallen further to very low levels in all conditions: 6% in FS(–), 25% in FS(+), 18% in Dyn(–), and 8% in Dyn(+).

3.2 Study 2: Long-Term Culture of MSC-Seeded Hyaluronic Acid Hydrogels

3.2.1 Biomechanical, Biochemical, and Histological Analyses—To determine if the instability in MSC-laden constructs was a function of the hydrogel culture system employed, we next carried out a similar long-term study investigating MSCs in FS(+) conditions through 126 days in a photocrosslinked HA hydrogel. Equilibrium modulus (Figure 7A), dynamic modulus (Figure 7B), and glycosaminoglycan content (Figure 7C) increased from day 14 values ($p < 0.001$), peaking at day 56 at 203 kPa, 2.19 MPa, and 1.28 mg/construct, respectively. Following these peaks, all three metrics declined substantially at 126 days to 4 kPa, 0.51 MPa, and 0.27 mg/construct, respectively. Collagen content continued to increase through day 84 to 0.11 mg/construct with no decline at day 126 (Figure 7D). Histological staining confirmed a dramatic loss in proteoglycans and an increase in collagen at day 126 (Figure 7E).

Discussion

For a cell-based biologic cartilage repair method to be successful, the neo-tissue formed must reach a stable equilibrium state with sufficient mechanical function. Ideally, this function would match that of native tissue and persist over the lifetime of the patient. Previous reports have noted that at time scales of approximately 8 weeks, MSC-laden hydrogels cultured *in vitro* under pro-chondrogenic conditions plateau in their functional maturation, with a lower equilibrium modulus compared to chondrocyte-based constructs cultured identically (Erickson et al., 2009a; Farrell et al., 2012; Mauck et al., 2006). Furthermore, while adult derived stem cell-based treatments are alluring, their phenotypic instability upon implantation remains a perplexing issue. Although there has been much

progress in engineering a mechanically robust cartilage tissue with these cells, stem cell-based cartilage properties can deteriorate as the tissue undergoes mineralization when presented with an *in vivo* subcutaneous environment (Pelttari et al., 2006), or when challenged with hypertrophic factors *in vitro* (Mueller and Tuan, 2008). These findings, coupled with the recognized limitations of microfracture, which produces a repair tissue that is unstable (Mithoefer et al., 2009), might suggest that bone marrow derived stem cells simply lack the capacity to produce a stable cartilaginous tissue.

To address this issue, this study evaluated the potential of MSC-based cartilage constructs (relative to chondrocyte-based constructs) over long-term culture in a well defined, stable *in vitro* environment. In doing so, we attempted to ask and answer two questions. First, we sought to determine whether the plateau in mechanical properties with time (through 56 days) simply represents a lag phase (during which MSCs undergo an initial round of chondrogenesis), and from which they might continue to produce matrix and increase in mechanical properties to match cartilage. Second, we attempted to clarify whether MSC-laden tissue engineered constructs remain stable in their chondrogenic phenotype over the long term, or whether they deteriorate towards a hypertrophic state with prolonged cultivation. To answer these questions, we investigated the development of mechanically viable chondrocyte- and MSC-laden tissue engineered constructs in a popular three-dimensional agarose hydrogel system with long-term culture (112 days). To assess cell health and stability, we analyzed the time progression of cell viability. Additionally, we evaluated the presence of cartilaginous (proteoglycans and type II collagen) and hypertrophic (type I collagen and calcium) matrix constituents via histological and immunohistochemical staining.

Through 56 days of culture, our results were consistent with previous reports (Erickson et al., 2009a; Farrell et al., 2012; Mauck et al., 2006). Namely, provision of TGF- β in chondrogenic culture medium resulted in an increase in GAG and collagen content and equilibrium modulus with time for both MSC- and chondrocyte-based constructs. Likewise, the absence of TGF- β resulted in less maturation in chondrocyte-based constructs and very little maturation of MSC-based constructs. Dynamic culture improved equilibrium modulus over free swelling controls for both cell types; however, chondrocytes continued to outperform MSCs. When we cultured these constructs for an additional 56 days (through day 112), however, MSC-based constructs not only failed to match properties of chondrocyte-based constructs, but rather evinced a marked decline in mechanics from day 56 to day 112. This decline in properties was exacerbated with dynamic culture.

The basis for the mechanical instability observed in MSC-laden constructs was further investigated at the cellular level. We found that while chondrocyte viability was relatively high and stable through long term culture, MSC viability progressively declined for all conditions. In free swelling conditions in the presence of TGF- β , there were two phases of decline. The first decline occurred very soon after encapsulation, with viability stabilizing at ~40% between days 14 and 56. The second reduction in viability, to lows around ~25%, occurred between days 56 and 112. We hypothesize that the immediate decline in viability may be a consequence of metabolic stress, as dynamic culture mildly delayed the decline from day 1 levels. However, the low MSC viability in all conditions at day 112 suggested

that the *in vitro* culture conditions, even with increased nutrient supply, are not suitable for long-term MSC stability.

One interesting caveat to these findings was that although there was a decline in mechanics and viability of MSC-laden constructs in the CM(+) conditions, these constructs remained negative for indicators of hypertrophic differentiation (including type I collagen and calcium). However, the progressive loss of Alcian Blue staining and GAG content suggested these constructs were in a catabolic state, losing key matrix constituents over this time period. It is not yet clear whether this response is a natural consequence of the time course of chondrogenesis, or whether this represents a catabolic response on the part of the MSCs in response to nutrient deprivation; this mechanism is currently being explored. In the absence of TGF- β , not only was there a dramatic decline in MSC viability, but constructs also stained heavily for calcium deposits and moderately for type I collagen. Along with this observation, we noted a moderate increase in dynamic modulus in free swelling conditions, which might be attributed to calcification of the tissue. In additional replicates (not shown), we found that once this calcification traversed the entire thickness of the construct, there was a significant spike in equilibrium modulus as well. Spontaneous calcification without the addition of hypertrophic medium supplements, including a phosphate source, is alarming and suggests that the continued presence of TGF- β may be required to prevent this unwanted phenotypic transition. Such a finding would suggest that *in vivo* application of MSCs for cartilage repair will require prolonged provision of TGF- to prevent unwanted phenotypic transitions.

In our original studies, we employed a simple agarose hydrogel to encapsulate cells. Agarose is a relatively inert biomaterial, offering no cell adhesion or other interactions (Buschmann et al., 1992). To determine if the instability in our MSC cultures was a function of the hydrogel used, a hyaluronic acid hydrogel supportive of MSC chondrogenesis was utilized in a follow-up study. This material provides cell-material interactions via both CD44 and CD168 surface receptors (Bian et al., 2013) and is more supportive of the chondrogenic phenotype than inert gels such as unmodified polyethylene glycol and agarose (Chung and Burdick, 2009). This hydrogel is also clinically relevant because it can be crosslinked *in situ* and can be readily remodeled as the tissue matures. Supporting our findings in agarose hydrogels, a similar time scale of matrix elaboration and mechanical property increases, peaking at day 56, was followed by catabolic declines by day 126 in this HA hydrogel. These findings suggest that the natural time course of MSC chondrogenesis and subsequent functional declines are not dependent on the material employed.

Taken together, our results show that, in a defined *in vitro* culture system where conditions are regulated to promote and preserve the chondrogenic state, MSC instability may be an innate characteristic of the cell type, involving both loss of viability and phenotypic conversion. These data have significant implications for *in vivo* application of MSC-based engineered constructs. Our data suggest that if such constructs are implanted at a point of peak mechanics, and ultimately fail *in vivo* long term, this failure may be the natural progression of the cell phenotype rather than a reaction to the *in vivo* environment. The expansion and chondrogenic culture conditions used for these studies are amongst the most popular for MSC based cartilage tissue engineering; however, methods to prevent MSC

hypertrophy, including mechanical loading (Bian et al., 2012), application of soluble factors such as parathyroid hormone-related protein (Bian et al., 2011b; Kim et al., 2008; Mueller et al., 2013; Mwale et al., 2010), or co-culture with chondrocytes (Bian et al., 2011a; Cooke et al., 2011; Fischer et al., 2010) should be further explored. Further, efforts should be focused on maintaining MSC viability after encapsulation by limiting metabolic stress, either through the provision of anabolic factors with sustained release from the material, by pre-conditioning MSCs to this environment before implantation, or by pre-selecting MSC subpopulations that are particularly resistant to loss of viability under the taxing *in vivo* conditions. Such steps are critical, as clinical success of stem cell based cartilage tissue will require not only that these cells achieve a high anabolic state, but more importantly, that cell health, phenotypic stability, and functional properties are retained over the long term and post-implantation.

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Figure 1.

Mechanical properties of chondrocyte (CH) and mesenchymal stem cell (MSC)-laden agarose hydrogels cultured under free swelling (FS) or dynamic (Dyn) conditions in the absence or presence of TGF- β (-/+). (A) Equilibrium modulus through 112 days (D112) demonstrating a progressive increase and/or stability in properties in CH-laden constructs, and an overall lower and unstable mechanical growth trajectory for MSC-laden constructs in CM(+) conditions. (B) Dynamic modulus of constructs showing a similar growth trajectory (with a particular instability in this measure for MSC-laden Dyn(+) constructs). Significance established with $p < 0.05$. (*) vs. previous time point of same group; (#) Dyn(-) vs. FS(-) or Dyn(+) vs. FS(+) within cell type and time point. (Ø) FS(-) vs. FS(+) and Dyn(-) vs. Dyn(+) within cell type and time point.

Figure 2.

Biochemical content of CH and MSC-laden agarose constructs on day 56 (D56) and day 112 (D112). (A) Glycosaminoglycan (GAG) content from D56 to D112 differs with cell type and culture condition, with increases in GAG content apparent in CH-based constructs and loss of GAG content in MSC-based constructs. (B) Collagen content per construct similarly differs with cell type and culture condition. Significance established with $p < 0.05$. (*) vs. previous time point of same group; (#) Dyn(-) vs. FS(-) or Dyn(+) vs. FS(+) within cell type and time point. (Ø) FS(-) vs. FS(+) and Dyn(-) vs. Dyn(+) within cell type and time point.

Figure 3.

Histological analysis reveals differences in matrix formation and retention between groups on D56 and D112. (A) Alcian Blue staining showing differences in proteoglycan accumulation and distribution; most notably a lighter, more diffuse staining in MSC FS(+) and Dyn(+) groups in D112 compared to D56 constructs. Scale = 1mm. (B) No marked differences in collagen staining were apparent via Picrosirius Red staining. Scale = 1mm.

Figure 4.

Immunohistochemical analysis on D56 and D112. (A) Chondrocyte-laden hydrogels stain intensely for type II collagen, with little or no staining of type I collagen, indicative of a stable chondrogenic phenotype. Scale = 1mm. (B) Although MSC-laden hydrogels stain less intensely for type II than their chondrocyte counterparts, staining is relatively stable from D56 and D112. Positive type I collagen staining is apparent only in MSC FS(-) and Dyn(-) conditions, and increases from D56 to D112. Scale = 1 mm.

Figure 5.

(A) Higher magnification of type I collagen staining illustrates pockets of intense deposition in MSC FS(-) conditions at D112. Scale = 200 μ m. (B) Positive Alizarin Red staining for MSC FS(-) and Dyn(-) conditions at D112 indicates that these constructs are heavily calcified, despite the absence of specific pro-hypertrophic signals. Scale = 1mm.

Figure 6.

Short and long term viability in chondrocyte- and MSC-laden constructs. (A) Marked differences in viability between groups are observed as early as D28. MSC-based cultures continue to decline through D112. Nuclei of dead cells are labeled in red, cytoplasm of live cells are labeled in green. Scale = 100 μ m. (B) Quantification of percent viability in the center of constructs shows a marked decline in MSC viability at early time points, reaching an extremely low level by D112, compared with much smaller changes in CH-based construct viability over the same time course. Significance established with $p < 0.05$. Markers included in box above plots. (Line) vs. Day 1 with line style and color corresponding to respective group. (Circle and square) vs. previous time point with marker style and color corresponding to respective group.

Figure 7.

Mechanical, biochemical, and histological assessment of MSCs cultured in a hyaluronic acid (HA) hydrogel over long-term *in vitro* culture. (A) Equilibrium modulus and (B) dynamic modulus show instability in construct properties similar to that of agarose constructs, with marked declines occurring by D126. (C) Glycosaminoglycan (GAG) content per construct declines similarly while (D) collagen per construct is stable. Significance established with $p < 0.05$. (*) vs. previous time point. Histological staining of (E) proteoglycans (Alcian Blue) and (F) collagens (Picrosirius Red) confirm biochemical assays. Scale = 200 μm .