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Functional properties of bone marrow-derived 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.

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 et al. (1970) as colony forming fibroblast-like cells. 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 newly formed 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;

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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 h @ 37 °C, Calbiochem/EMD Chemicals, Gibbstown, NJ) followed by collagenase digestion (0.5 mg/mL, 6 h @ 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, 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.

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