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Embryonically inspired scaffolds regulate tenogenically differentiating cells

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ABSTRACT

Tendon injuries heal as scar tissue with significant dysfunction and propensity to re-injure, motivating efforts to develop stem cell-based therapies for tendon regeneration. For these therapies to succeed, effective cues to guide tenogenesis are needed. Our aim is to identify these cues within the embryonic tendon microenvironment. We recently demonstrated embryonic tendon elastic modulus increases during development and is substantially lower than in adult. Here, we examined how these embryonic mechanical properties influence tenogenically differentiating cells, by culturing embryonic tendon progenitor cells (TPCs) within alginate gel scaffolds fabricated with embryonic tendon mechanical properties. We showed that nano- and microscale moduli of RGD-functionalized alginate gels can be tailored to that of embryonic tendons by adjusting polymer concentration and crosslink density. These gels differentially regulated morphology of encapsulated TPCs as a function of initial elastic modulus. Additionally, higher initial elastic moduli elicited higher mRNA levels of scleraxis and collagen type XII but lower levels of collagen type I, whereas late tendon markers tenomodulin and collagen type III were unaffected. Our results demonstrate the potential to engineer scaffolds with embryonic mechanical properties and to use these scaffolds to regulate the behavior of tenogenically differentiating cells.

1. Introduction

Tendons are highly collagenous tissues that transmit forces from muscle to bone to enable skeletal motion. Unfortunately, tendon injuries are common and their incidence is increasing (AAOS, 2008; Butler et al., 2004; Lantto et al., 2015). There was a nearly 10-fold increase in the incidence of Achilles tendon ruptures between 1979 and 2011 (Lantto et al., 2015), and more than 135,000 annual Achilles, patellar and rotator cuff tendon surgeries in the USA reported in 2004 (Butler et al., 2004). This is problematic, as even with surgical intervention, injured tendons heal as scar tissues that possess aberrant mechanical and biochemical properties, and are associated with long-term pain and compromised function (Lin et al., 2004). These significant drawbacks are motivating efforts to engineer replacement tendon tissues from stem cells seeded in 3-dimensional (3D) scaffolds. Tendon tissue engineering approaches commonly utilize scaffolds fabricated with the unique characteristics of adult tendon, including high collagen type (Col) I content, highly aligned fibers, and high elastic modulus and tensile strength (Chainani et al., 2013; Chokalingam et al., 2009; Kuo et al., 2010; Kuo and Tuan, 2008; Nirmalanandhan et al., 2008; Qiu et al., 2014; Subramony et al., 2013; Xie et al., 2010; Zhang et al., 2012). However, engineered tissues with normal tendon structure and mechanical properties have not been achieved. One possibility is that tissue engineering strategies based on adult tendon properties are presenting cells with the cues that promote aberrant and dysfunctional tissue formation during healing.

We recently demonstrated that, in contrast to adult tendon, embryonic tendon possesses high cell density, low collagen content, less organized matrix, and low elastic modulus (Marturano et al., 2013; Schiele et al., 2013; Schiele et al., 2015). These findings suggest *embryonic tendon progenitor cells* (TPCs) experience a significantly different microenvironment in embryonic tendon than mature tenocytes do in adult tendon. However, examining the effects of tissue mechanical properties on TPC function during embryonic development is challenged by a multitude of confounding physical and biochemical cues in vivo. This study aimed to develop 3D scaffolds with embryonic tendon mechanical

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properties, and to examine TPC behavior in these microenvironments. Previous studies have shown that TPCs harvested from embryonic tendons are an excellent model system to study stem cell tenogenesis, and that adult mesenchymal stem cells (MSCs) respond similarly as TPCs to embryonic developmental cues (Brown et al., 2014, 2015).

In earlier work, we demonstrated the ability to fabricate structurally uniform 3D alginate gels with uniform cell distribution and controllable mechanical properties as a function of Ca^{2+} concentration (i.e. crosslink density) and polymer concentration (Kuo and Ma, 2001). These alginate gels, favored for their highly controllable bulk-level mechanical properties, are now commonly utilized for tissue engineering (Jang et al., 2014; Korecki et al., 2009; Nunamaker et al., 2011). Here, we characterized the ability to control the cell length-scale mechanical properties of these gels, and to mimic the cell length-scale elastic moduli of embryonic tendon.

We hypothesized that TPCs are responsive to the cell length-scale mechanical properties of developing embryonic tendon. We engineered 3D alginate gel scaffolds with chick embryonic tendon cell length-scale elastic moduli, and investigated the effects of these gels on chick embryo TPCs. The nano- and microscale elastic moduli of embryonic tendon were successfully achieved in ionically crosslinked alginate gels as a function of polymer concentration, crosslink density, arginyl-glycyl-aspartic acid (RGD)-peptide functionalization, and cell density. TPCs encapsulated within these alginate gels varied in cell morphology and tendon marker gene expression with differing initial nanoscale elastic moduli. Our results demonstrate scaffolds that recapitulate mechanical properties of embryonic tendon can regulate the behavior of tenogenically differentiating cells and potentially be remodeled during this process. These findings suggest developmentally inspired scaffolds may be useful for stem cell-based tendon tissue regeneration approaches.

2. Materials and methods

2.1. Reagents

Unless otherwise stated, all reagents were from Sigma-Aldrich Co. (St. Louis, MO).

2.2. Primary TPC isolation

All animal procedures received approval from the Institutional Animal Care and Use Committee at Tufts University. Fertilized white leghorn chick embryos (UConn Poultry Farm, Storrs, CT) were cultured in a humidified rocking incubator at 37.5 °C and sacrificed at Hamburger–Hamilton (HH) (Hamburger and Hamilton, 1951) stages 37 and 40. Calcaneus tendons were dissected from both limbs of ten HH 37 and six HH 40 chick embryos, and pooled for each stage. Tendons were digested in collagenase type II (1 mg/mL; Gibco, Grand Island, NY) with shaking at 200 RPM and 37 °C, and then quenched with complete medium (CM) consisting of Dulbecco's Modified Eagle Medium (DMEM; Life Technologies), 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA), and 100 U/mL penicillin and 100 μg/mL streptomycin. The digested suspension was filtered through a 40-μm cell strainer. TPCs were expanded and used at passage 1–2.

2.3. Alginate functionalization

Purified low viscosity, high guluronate (60–70%) content alginate was obtained from FMC Biopolymer (Sandvika, Norway). The same lot of alginate was used for all experiments. We functionalized alginate with RGD peptides based on the protocol kindly provided by Dr. David Mooney (Drury et al., 2005; Rowley et al., 1999). Alginate was suspended at 1% (w/v) in 0.3 M 4-morpholineethanesulfonic acid (MES) and 0.1 M NaCl, adjusted to pH 6.5 with NaOH, and functionalized with GGGGRGDSP peptides (Peptides International, Louisville, KY) using carbodiimide coupling. *N*-hydroxysulfosuccinimide (NHS), *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), and RGD peptides were dissolved sequentially in mass ratios of 0.82, 1.64, and 0.03 relative to alginate, respectively. This reaction proceeded for 20 h at 22 °C with stirring until termination by addition of hydroxylamine hydrochloride (HA) at a 0.043 mass ratio relative to alginate. Alginate solutions were dialyzed (3500 Da cut-off) for 72 h in distilled water, treated with activated charcoal, passed through a 0.2-μm filter, lyophilized, and re-suspended at 5% (w/v) in Hanks' Balanced Salt Solution without Ca^{2+} or Mg^{2+} (HBSS; Life Technologies, Grand Island, NY) to produce RGD-functionalized alginate (RGD-alg). This process was replicated for "non-functionalized" alginate, which we designated as control (CTRL-alg), except that NHS, EDC, RGD, and HA were not included.

Table 1
Ca content (X) and corresponding Ca-to-COOH molar ratio used in calculations for $CaCO_3$ and alginate.

Ca content (X)	Ca/COOH (mol/mol)
1.5X	0.27
2X	0.36
3X	0.54
4X	0.72

2.4. Fabrication of gels and culture of encapsulated TPCs

We fabricated alginate gels as previously described (Korecki et al., 2009; Kuo and Ma, 2001, 2008; Schiele et al., 2015). Briefly, alginate was mixed with $CaCO_3$ in HBSS to yield between a 1.5 and 4X Ca content, where "X Ca content" represents a molar ratio between added $CaCO_3$ and COOH groups on the alginate (Table 1) (Kuo and Ma, 2001). Trypsinized HH 40 TPCs were re-suspended in HBSS to yield either a final cell density of 1×10^6 (1 M/mL) or 10×10^6 cells/mL (10 M/mL). A fresh 21.4% (w/v) D-glucono-δ-lactone (GDL) solution in HBSS was then added in a 1:2 M ratio of $CaCO_3$ to GDL. Final alginate concentrations were 1.5% or 3% (w/v). A 40-μL volume of alginate-cell solution was pipetted into custom 6-mm diameter polydimethylsiloxane molds (Dow Corning) and allowed to crosslink for 2 h at 37 °C. RGD-alg gels and CTRL-alg gels encapsulating TPCs were transferred into 24-well plates and cultured at 37 °C in CM, which was replaced every 48 h.

2.5. FV-AFM testing of gels

After 48 h of culture, gels were immersed in HBSS and immediately mechanically tested with FV-AFM, as we previously described for embryonic chick tendons and agarose gels (Marturano et al., 2013). Cantilevers with 0.06 N/m spring constants (Bruker, Camarillo, CA) and either 20 nm or 5 μm tip radius were employed for nano- and microscale measurements, respectively. Indentation force curves were measured over $10 \times 10\ \mu m^2$ areas, with 256 indentations per area, at two different locations near the gel center. The linear regions of force curves were converted to elastic moduli using an empirically derived calibration curve developed with agarose gel standards, as previously described (Marturano et al., 2013). In previous work, we derived moduli of embryonic tendon from AFM measurements using either agarose gel standards or Hertzian theory calculations, and found that the two methods yielded similar values of embryonic tendon modulus over the entire range of development (Marturano et al., 2013). Three different gels were tested for each experimental condition.

2.6. Quantitative polymerase chain reaction (qPCR)

RGD-alg gels encapsulating 10 M/mL HH 37 TPCs were homogenized in TRIzol LS (Invitrogen) after 7 days of culture. Total RNA was extracted and quantified using spectrophotometry (Nanodrop ND-2000, Thermo Scientific, Wilmington, DE), and reverse-transcribed into cDNA using the Superscript III First-Strand Synthesis System (Invitrogen). QPCR was performed with Brilliant II SYBR Green qPCR Master Mix (Applied Biosystems, Foster City, CA) and the MX3000p qPCR System (Agilent Technologies, Santa Clara, CA). Chick-specific primer pairs were designed for tendon marker (scleraxis, tenomodulin, Col I, III, and XII) and housekeeping (18S) genes (Table 2). The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in gene expression. The data are presented as the fold change in target gene expression normalized to the housekeeping gene (18S), relative to HH 37 TPCs cultured in RGD-alg gels with an elastic modulus of 3.4 kPa. QPCR was performed on HH 37 TPCs encapsulated in six RGD-alg gels for each elastic modulus condition.

2.7. Statistical analysis

All data are shown as mean ± standard deviation. To determine the statistical significance, Student's *t*-test or one-way ANOVA with Tukey's post-hoc test were performed using $p < 0.05$. All statistical calculations were performed with Graphpad (GraphPad Software Inc., San Diego, CA).

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