**SPECIAL FOCUS: STRATEGIC DIRECTIONS
IN MUSCULOSKELETAL TISSUE ENGINEERING***

Optimization of Preculture Conditions to Maximize the *In Vivo* Performance of Cell-Seeded Engineered Intervertebral Discs

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The development of engineered tissues has progressed over the past 20 years from *in vitro* characterization to *in vivo* implementation. For musculoskeletal tissue engineering in particular, the emphasis of many of these studies was to select conditions that maximized functional and compositional gains *in vitro*. However, the transition from the favorable *in vitro* culture environment to a less favorable *in vivo* environment has proven difficult, and, in many cases, engineered tissues do not retain their preimplantation phenotype after even short periods *in vivo*. Our laboratory recently developed disc-like angle-ply structures (DAPS), an engineered intervertebral disc for total disc replacement. In this study, we tested six different preculture media formulations (three serum-containing and three chemically defined, with varying doses of transforming growth factor β 3 [TGF- β 3] and varying strategies to introduce serum) for their ability to preserve DAPS composition and metabolic activity during the transition from *in vitro* culture to *in vivo* implantation in a subcutaneous athymic rat model. We assayed implants before and after implantation to determine collagen content, glycosaminoglycan (GAG) content, metabolic activity, and magnetic resonance imaging (MRI) characteristics. A chemically defined media condition that incorporated TGF- β 3 promoted the deposition of GAG and collagen in DAPS *in vitro*, the maintenance of accumulated matrix *in vivo*, and minimal changes in the metabolic activity of cells within the construct. Preculture in serum-containing media (with or without TGF- β 3) was not compatible with DAPS maturation, particularly in the nucleus pulposus (NP) region. All groups showed increased collagen production after implantation. These findings define a favorable preculture strategy for the translation of engineered discs seeded with disc cells.

Keywords: animal model, electrospun scaffold, hydrogel, intervertebral disc, tissue engineering, transforming growth factor beta 3

Introduction

THE DEVELOPMENT of engineered tissues has progressed over the past 20 years from *in vitro* characterization to *in vivo* implementation, particularly for the musculoskeletal system, where *in vitro* performance metrics for engineered bone,^{1,2} cartilage,^{1,3,4} and fibrocartilage⁵⁻⁷ meet or exceed native tissue benchmarks. The emphasis of many of these

studies was to select conditions that maximized functional and compositional gains *in vitro*. However, the transition from the favorable *in vitro* culture environment to a less favorable *in vivo* environment has proven difficult, and, in many cases, engineered tissues do not retain their preimplantation phenotype after even short periods *in vivo*.⁸⁻¹¹ Developing a successful preculture strategy that allows for functional extracellular matrix production within constructs

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in vitro and continues this progression after *in vivo* implantation is critical to clinical translation.

Tissue engineering strategies for the replacement of diseased intervertebral discs are under active development, advancing from *in vitro* research to preclinical animal models.^{12,13} We have developed disc-like angle-ply structures (DAPS) for total disc replacement that incorporate a nanofibrous annulus fibrosus (AF) region, which matches the structural hierarchy of the native AF, along with a nucleus pulposus (NP) region based on a photopolymerizable hydrogel. These constructs achieve compositional and functional parity with native tissue after *in vitro* culture.¹⁴ Consistent with other studies in the field, we found that, after implantation of DAPS in an *in vivo* rat tail disc replacement model, proteoglycans that had accumulated in the AF and NP regions during preculture diminished markedly postimplantation.¹⁵ The culture medium used in these studies was designed for mesenchymal stem cell (MSC) chondrogenesis,¹⁶ and is a chemically defined (serum-free) formulation that includes potent factors for extracellular matrix production (transforming growth factor β 3 or TGF- β 3, ascorbate-2-phosphate, proline, and dexamethasone) and has proven useful for developing many engineered musculoskeletal tissues, including cartilage,^{17,18} meniscus,^{19,20} and the intervertebral disc.¹⁴ It remains unclear, however, whether preculture in chemically defined media (CM) with TGF- β 3 will support the *in vivo* transition of engineered tissues. For example, MSCs differentiated using this formulation do not match the gene expression profiles of articular chondrocytes,²¹ and the phenotype imprinted on MSCs following culture in this medium is altered when media components are selectively removed.²²

A modified media formulation may better prepare engineered implants for the *in vivo* environment. For example, preculture in serum-containing media (SM) may prime cells for exposure to factors in the *in vivo* environment. This has been demonstrated for AF cells, NP cells, and chondrocytes in implantation sites that include the subcutaneous space, the disc space, and articular cartilage defects.^{13,23–28} Furthermore, transient TGF- β 3 administration *in vitro*, which models the rapid formation of tissue during development, improved the chondrogenesis of MSCs and chondrocytes, imprinting a phenotype that is sustained over long-term culture.^{29–32} This strategy of “weaning” cells from TGF- β 3 before implantation may promote a chondrogenic phenotype *in vitro* that is sustained after the *in vivo* transition. Combining the benefits of preculture in media with chondrogenic factors and those of SM may improve extracellular matrix deposition *in vitro* while promoting phenotype and matrix retention *in vivo*.

In this study, we tested six different preculture strategies for their ability to preserve DAPS composition and metabolic activity during the transition from *in vitro* culture to *in vivo* implantation in a subcutaneous athymic rat model. We tested three hypotheses: (1) that preculture in SM with TGF- β 3 would improve *in vivo* performance, (2) that a transient high dose of TGF- β 3 would improve phenotype retention *in vivo* and (3) that, just before implantation, a gradual transition from CM to SM would facilitate a stable *in vivo* transition and preserve the elaborated matrix. To do so, we evaluated DAPS histological characteristics, composition, and metabolic activity before and after implantation. We also performed magnetic resonance imaging (MRI) T2 mapping, which correlates the spatial distribution of

extracellular matrix within tissue,³³ to establish a noninvasive methodology to evaluate DAPS composition *in vivo*.

Materials and Methods

DAPS fabrication and cell seeding

DAPS were fabricated to match the hierarchical features of the native intervertebral disc as described previously.^{12,14} An electrospun nanofibrous AF region was fabricated with aligned fibers that altered in apposed layers $\pm 30^\circ$ to the transverse plane. Specifically, AF regions were composed of poly(ϵ -caprolactone) (PCL; Shenzhen Bright China Industrial Co., Hong Kong, China) as the stable polymer, and poly(ethylene oxide) (PEO; 200 kDa; Polysciences, Inc., Warrington, PA) as the sacrificial soluble polymer. PCL was dissolved in a 1:1 mixture of tetrahydrofuran (THF) and N,N-dimethylformamide (DMF) (Fisher Chemical, Fairlawn, NJ) to form a 14.3% w/v solution. PCL was electrospun onto a grounded rotating mandrel to produce a sheet of aligned fibers; PEO was dissolved in 90% ethanol at 10% w/v and electrospun atop the PCL layer. The PCL/PEO layers were then cut to produce strips with a 30° fiber angle, and two strips with opposing alignment were rolled concentrically about a post and spot welded with a soldering pencil to form the AF region. The final construct had two 125 μ m layers of PCL for every one 250 μ m layer of PEO, a diameter of 5 mm diameter and a height of 2 mm. Before cell seeding, constructs were washed to remove PEO layers and then coated in 20 μ g/mL fibronectin (Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline (PBS). Next, a photopolymerizable NP region was formed from hyaluronic acid (HA), an essential component of the disc extracellular matrix.³⁴ Sodium hyaluronate (65 kDa; Lifecore, Chaska, MN) and methacrylic anhydride (Sigma-Aldrich) were reacted to produce a methacrylated HA, which was dissolved in PBS at 1% w/v, sterilized by ultraviolet (UV) light, and combined with 0.05% photoinitiator [Irgacure 2959, 2-methyl-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone; Ciba-Geigy, Tarrytown, NY].

To harvest AF and NP cells, caudal intervertebral discs were dissected from adult bovine tails obtained from a local abattoir with institutional approval. The disc tissue was isolated in aseptic conditions and the AF and NP regions were separated. Each region was digested, first with pronase at a concentration of 2.5 mg/mL for 1 h, and subsequently with collagenase at 0.5 mg/mL (4 h for NP tissue, 8 h for AF tissue). AF and NP cells were then expanded in a standard serum-containing expansion medium (Table 1). At passage 2–3, cells were trypsinized and then seeded, AF cells onto the top and bottom of the electrospun AF region (1×10^6 cells per side) and NP cells in HA solution (20×10^6 cells/mL or $\sim 6 \times 10^5$ cells/NP), which was subsequently photopolymerized with UV light exposure for 10 min, and then punched to 2 mm diameter. For all aspects of these studies, cell culture was performed in standard conditions (21% O₂, 5% CO₂, 37°C) and the culture medium was changed every third day.

Study design

AF and NP regions were cultured separately for 2 weeks in one of six media conditions. After 2 weeks, AF and NP regions were combined to form DAPS, cultured for an

TABLE 1. FORMULATION OF BASE CULTURE MEDIA

Media component	Manufacturer	Expansion medium	SM ^{35,36}	CM ^{4,16}
Dulbecco's modified Eagle's medium	Gibco (Invitrogen Life Sciences, Carlsbad, CA)	Base	Base	Base
Fetal bovine serum	Gibco (lot No. 1495557; reserve No. 25218706)	10%	10%	—
Penicillin	Gibco	100 U/mL	100 U/mL	100 U/mL
Streptomycin	Gibco	100 µg/mL	100 µg/mL	100 µg/mL
Amphotericin B	Gibco	0.25 µg/mL	2.5 µg/mL	0.25 µg/mL
MEM vitamin solution	Gibco	—	1%	—
HEPES buffer	Gibco	—	25 mM	—
Ascorbate 2-phosphate	Sigma-Aldrich (St. Louis, MO)	—	50 µg/mL	50 µg/mL
Dexamethasone	Sigma-Aldrich	—	—	40 ng/mL
L-proline	Sigma-Aldrich	—	—	40 µg/mL
Bovine serum albumin	Sigma-Aldrich	—	—	1.25 mg/mL
Linoleic acid	Sigma-Aldrich	—	—	5.35 µg/mL
Sodium pyruvate	Corning Life Sciences (Corning, NY)	—	—	100 µg/mL
ITS Premix Universal Culture Supplement	Corning Life Sciences	—	—	0.10%

SM and CM were supplemented with TGF-β3 in various doses and concentrations as described in the text. CM, chemically defined medium; SM, serum-containing medium; TGF-β3, transforming growth factor β3.

additional 3 weeks in the same media, and then either implanted into dorsal subcutaneous pockets of athymic rats for 5 weeks, or cultured for an additional 5 weeks *in vitro* (Fig. 1A and Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea). At 2.5, 5, 6, and 10 weeks during *in vitro* culture, AF and NP region composition (glycosaminoglycan, GAG; hydroxyproline, OHP; MRI; $n=3$ /group/time point), metabolic activity [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT; $n=3$ /group/time point], and histological appearance (Picrosirius Red, Alcian Blue; $n=1-2$ /group/time point) were analyzed (total $n=7-8$ group/time point) (Fig. 1B). These same features were measured 1 and 5 weeks after *in vivo* implantation (corresponding to *in vitro* weeks 6 and 10).

DAPS maturation and *in vivo* phenotype were evaluated after culture in one of three SM^{35,36} conditions or one of three CM^{4,16} conditions. The base formulations of these media are listed in Table 1. Two groups (SM+, CM+) were supplemented with 10 ng/mL of TGF-β3 (R&D Systems, Minneapolis, MN), the standard dose used during *in vitro* culture. Two groups (SM++, CM++) received a high dose of TGF-β3 (100 ng/mL) for 1 week followed by culture in SM or CM. Two additional groups (SM+T, CM+T) were cultured in SM+ or CM+ and then transitioned over 10 days (four media changes) to SM, just before week 5. Specifically, the transitioning strategy for the SM+T and CM+T groups was: day 24 (100% SM+ or CM+; 10 ng/mL TGF-β3), Media Change 1 at day 25 (75% SM+ or CM+, 25% SM; 7.5 ng/mL TGF-β3), Media Change 2 at day 28 (50% SM+ or CM+, 50% SM; 5 ng/mL TGF-β3), Media Change 3 at day 31 (25% SM+ or CM+, 75% SM; 2.5 ng/mL TGF-β3), and Media Change 4 at day 34 (100% SM; 0 ng/mL TGF-β3).

Implantation surgery

DAPS were implanted into the dorsal subcutaneous space of athymic retired breeder rats ($n=12$, Hsd:RH-Foxn1^{tmu}, male, 9–10 months, 463 ± 45 g; Harlan Laboratories, Indianapolis, IN) with local institutional approval. Rats were

anesthetized with isoflurane and dorsal pockets were opened at seven positions 1 cm lateral to the spine (four left, three right). One DAPS was inserted into each pocket (seven DAPS total per rat), which were then closed with suture. Rats were returned to normal cage activity after the procedure.

Compositional evaluation of DAPS

Following *in vitro* culture or subcutaneous implantation, DAPS were assayed for collagen and GAG content ($n=3$ /group/time point). To do so, DAPS were divided into AF and NP regions, which were individually digested in proteinase K at 60°C. GAG was then determined through the DMMB assay³⁷ and collagen through the p-diaminobenzaldehyde/chloramine-T assay for OHP.³⁸ Composition is reported as total GAG or OHP content (µg) per AF or NP region.

For histological analysis, DAPS were fixed in formalin, embedded in paraffin, and sectioned axially to 10 µm thickness ($n=1-2$ /group/time point). Sections were stained with either Alcian Blue or Picrosirius Red to identify proteoglycans and collagens, respectively, and then imaged in brightfield (Eclipse 90i; Nikon, Tokyo, Japan).

Metabolic activity

Metabolic activity was assayed using the MTT colorimetric assay ($n=3$ /group/time point). To do so, DAPS were halved (to improve diffusion) and submerged in a solution of Dulbecco's modified Eagle's medium (DMEM) and MTT at 0.5 mg/mL, and incubated for 5 h at 37°C and 5% CO₂. AF and NP regions were then separated and minced, and then the formazan product was solubilized in dimethyl sulfoxide and absorbance was measured on a microplate reader at 540 nm.

Magnetic resonance imaging

The T2 relaxation time calculated from MRI correlates to the water-binding properties and tissue anisotropy³⁴ and is a noninvasive, clinically relevant method for assessing

FIG. 1. Study design. (A) DAPS were cultured in one of six preculture conditions and implanted into the subcutaneous space of athymic rats. There were three SM preculture conditions: (1) SM+: serum-containing media plus TGF- β 3; (2) SM++: serum-containing media plus a 1 week high dose of TGF- β 3; (3) SM+T: serum-containing media plus TGF- β 3 and then a transition to SM without TGF- β 3 over 10 days. There were three CM preculture conditions: (4) CM+: chemically defined media plus TGF- β 3; (5) CM++: chemically defined media plus a 1 week high dose of TGF- β 3; (6) CM+T: chemically defined media plus TGF- β 3 and then a transition to SM without TGF- β 3 over 10 days. (B) DAPS metabolic activity [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT], composition (GAG;OHP; MRI), and histological appearance (Picrosirius Red, Alcian Blue) were analyzed after 2.5, 5, 6, and 10 weeks of *in vitro* culture. The same assays were also performed 1 and 5 weeks after *in vivo* implantation (corresponding to *in vitro* weeks 6 and 10). CM, chemically defined medium; DAPS, disc-like angle-ply structures; GAG, glycosaminoglycan; MRI, magnetic resonance imaging; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OHP, hydroxyproline; SM, serum-containing medium; TGF- β 3, transforming growth factor β 3. Color images available online at www.liebertpub.com/tea

intervertebral disc health; T2 has been demonstrated to correlate with proteoglycan content, water content, and biomechanical properties.³⁴ A 4.7T MRI spectrometer (MagneX Scientific Limited, Abington, United Kingdom) and custom software³⁹ was used to calculate T2 maps of DAPS using a multislice, multiecho acquisition (three 500 μ m thick slices, 16 echoes, echo time/repetition time = 7.84 ms/2,000 ms, field of view = 15 $\times$ 15 mm², matrix = 128 $\times$ 128, 4 averages). AF and NP regions were manually segmented from T2 maps and the mean T2 signal was calculated for each region ($n = 3$ /group/time point).

Statistical analysis

For OHP, GAG, MTT, and T2 MRI measurements, results after 5-week subcutaneous implantation were normalized to the preimplantation 5-week CM+ measurements, as a percent difference (Eq. 1).

$$\frac{\sum_{i=1}^n \frac{W10_i - W5CM + avg}{W5CM + avg}}{n} \quad (1)$$

These data were then analyzed using a one-way analysis of variance with media condition as the main factor. Bonferroni's method was used for *post hoc* comparisons (significance as $p < 0.05$, trend as $p < 0.10$). All data are displayed as mean $\pm$ standard deviation. To evaluate correlations between variables, OHP, GAG, MTT, and T2 results were split into

FIG. 2. Picrosirius Red (collagen) and Alcian Blue (proteoglycan) staining. DAPS were sectioned after 5 and 10 weeks of *in vitro* culture (W5, W10) or after 5 weeks *in vitro* culture and an additional 5 weeks of subcutaneous implantation (SQ, W10). One half-section is displayed for each condition. In terms of staining intensity and physical growth of the constructs, CM groups outperformed SM groups. Color images available online at www.liebertpub.com/tea

four groups: measurements of AF regions *in vitro*, AF regions *in vivo*, NP regions *in vitro*, and NP regions *in vivo*. Within each group, Pearson correlation coefficients were determined for each combination of variables and significance was defined when $p < 0.05$. All statistical analyses were carried out in Prism (GraphPad Software, Inc., La Jolla, CA).

The most successful media condition was determined based on a simple scoring system. Scoring categories were based on each assay (OHP, GAG, MTT) and comparisons were made to the week 5 CM+ group. Points were assigned based on a statistically significant difference: “+1” for an increase in OHP, GAG, or MTT absorbance postimplantation, “-1” for a decrease in these measurements postimplantation, “0” for no difference between pre- and postimplantation. The scores were tallied for both the AF and NP regions to develop an aggregate score.

Results

Histological appearance of DAPS before and after implantation

Histologically, there were qualitative differences between SM groups and CM groups with *in vitro* culture (Fig. 2). Based

on construct size and staining intensity, CM conditions appeared conducive to DAPS *in vitro* growth and maintained preimplantation characteristics *in vivo*, whereas SM conditions performed comparatively poorly, both *in vitro* and *in vivo*. DAPS from CM conditions were larger and had more intense Picrosirius Red and Alcian Blue staining in the AF and NP region at every time point. For SM conditions, there was no apparent change in DAPS size and minimal evidence of extracellular matrix accumulation *in vitro*. After implantation, strong collagen staining in the AF was apparent for all conditions.

Analysis of composition before and after implantation

All media conditions demonstrated an increase in collagen after implantation (Fig. 3). In SM conditions, cells produced minimal collagen *in vitro* in both the AF and NP regions. However, after implantation, collagen increased in all SM groups in both the AF and NP regions. In CM conditions, collagen production varied by media type. In the CM+ condition, seeded cells produced collagen in both the AF and NP regions, whereas in the CM++ and CM+T conditions, cells produced substantial collagen in the AF

FIG. 3. Time course of composition changes (OHP) in long-term culture and after implantation. **(A)** Measurements of total AF region (left) and NP region (right) OHP content for each of the six preculture media conditions. Solid lines represent measurements from *in vitro* culture, dotted lines represent after implantation. **(B)** OHP content presented as percent difference from the week 5 CM+ group. Light blue and red bars indicate the standard deviation of the week 5 CM+ group. In all cases: * $p < 0.05$ versus week 5 CM+. Note: -100% line is drawn to indicate a value 0 μg of OHP per construct, or -100% difference (the maximum possible difference) from the control group. Results from the one-way analysis of variance evaluating the effect of media condition: AF, $p < 0.01$; NP, $p < 0.05$. Missing values for SM+ NP group at week 2.5 is due to technical error. AF, annulus fibrosus; NP, nucleus pulposus; SM, serum-containing medium. Color images available online at www.liebertpub.com/tea

FIG. 4. Time course of composition changes (GAG) in long-term culture and after implantation. **(A)** Measurements total AF and NP region GAG content for each of the six preculture media conditions. *Solid lines* represent measurements from *in vitro* culture, *dotted lines* represent after implantation. Note: the y-axis for the CM+ group is different than all other conditions. **(B)** GAG content presented as percent difference from the week 5 CM+ group. *Light blue* and *red bars* indicate the standard deviation of the week 5 CM+ group. In all cases: * $p < 0.05$ and + $p < 0.1$ versus week 5 CM+. Results from the one-way analysis of variance evaluating the effect of media condition: AF, $p < 0.05$; NP, $p < 0.01$. Missing values for SM+ NP group at week 2.5 is due to technical error. Color images available online at www.liebertpub.com/tea

region, but not in the NP region. In all CM groups, after implantation, collagen increased in the AF region, whereas in the NP region, collagen increased in all groups, but only substantially in the CM+ group. In comparison to the week 5 CM+ control group (Fig. 3B), collagen was significantly higher after 5 weeks of implantation in the AF regions of the SM++, SM+T, CM+, CM++, and CM+T groups. In the NP region, the only significant difference was higher collagen content in the CM+ group.

GAG content was affected by the presence of serum in the preculture media (with or without TGF- β 3) (Fig. 4). In all SM groups, GAG produced *in vitro* and *in vivo* was minimal in comparison to the CM conditions. In all CM conditions there was significant GAG produced *in vitro*, however, in the CM++ and CM+T groups, GAG decreased after implantation. Furthermore, during *in vitro* culture, GAG accumulation for the CM+T group sharply decreased from 5 to 10 weeks (after the transition to serum-containing media and removal of TGF- β 3), whereas CM+ and CM++ groups either increased or maintained GAG levels over the same span. In comparison to the week 5 CM+ control group (Fig. 4B), GAG was significantly lower after 5 weeks implantation in all SM conditions in the AF and NP regions (trend SM+ NP group), there was a trend toward decreased GAG in the CM++ AF region and significantly less GAG in

the CM+T group. The CM+ group was the only group that had statistically similar GAG content to the week 5 CM+ group in the AF and NP regions.

Analysis of metabolic activity before and after implantation

With the transition to the *in vivo* environment, cells within DAPS were affected in terms of metabolic activity (Fig. 5). In all groups except SM+ and CM+, there was a decrease in metabolic activity between weeks 5 and 6. However, in most cases, levels either rebounded or were maintained after week 6. Conversely, in both the SM+ and CM+ groups, the effect of transitioning *in vivo* was not detrimental, as the metabolic activity of the SM+ group improved in the AF and was maintained in the NP region, whereas the CM+ group maintained preimplantation levels in both the AF and NP. The negative impact of transitioning may have actually been due to withdrawal from TGF- β 3, as DAPS in the SM++, SM+T, CM++, and CM+T conditions cultured *in vitro* in parallel to the subcutaneous implantations (which had TGF- β 3 removed from media before implantation) also decreased between weeks 5 and 6. In comparison to the week 5 CM+ control group (Fig. 5B), after 5 weeks of implantation, there were no differences in

FIG. 5. Time course of metabolic activity changes (MTT) in long-term culture and after implantation. **(A)** Measurements total AF and NP region MTT absorbance for each of the six preculture media conditions. *Solid lines* represent measurements from *in vitro* culture, *dotted lines* represent after implantation. **(B)** MTT absorbance values presented as percent difference from the week 5 CM+ group. *Light blue* and *red bars* indicate the standard deviation of the week 5 CM+ group. In all cases: * $p < 0.05$ and + $p < 0.1$ versus week 5 CM+. Results from the one-way analysis of variance evaluating the effect of media condition: AF, $p = 0.71$; NP, $p < 0.01$. Missing values for AF/NP SM+ and CM+ groups at week 2.5 is due to technical error. Color images available online at www.liebertpub.com/tea

metabolic activity in the AF for all groups, whereas in the NP region, there were significant differences in every group, except CM+ and CM++.

Analysis of T2 relaxation time before and after implantation

The T2 relaxation time of DAPS decreased as extracellular matrix was deposited (Fig. 6). This relationship was particularly strong in the AF region, where decreased AF T2 after implantation corresponded with concurrent increases in collagen content and collagen staining (Fig. 6B). A summary of the T2 results with statistical analysis (Fig. 6C) shows that T2 decreased in the AF region of all groups after implantation, inversely matching OHP content measurements in this region in all groups (Fig. 3B). In the NP region, there was a decrease in T2 in the SM+ and SM++ groups, and a trend toward a decrease in the CM+ group after implantation. Preimplantation levels were maintained in the SM+T, CM++, and CM+T groups.

Correlations between GAG, OHP, MTT, and T2 measurements

Correlations between measured variables revealed relationships between OHP/GAG and OHP/T2 in the NP and

AF region, and metabolic activity/collagen and metabolic activity/GAG in the AF region (Table 2). The inverse relationship between OHP and T2 was particularly strong in the AF region. Furthermore, categorizing groups by measurements made after *in vitro* culture or *in vivo* implantation revealed that OHP and GAG were correlated in all cases, except for NP regions after *in vivo* implantation. There was a strong positive relationship between GAG and MTT in NP regions after *in vivo* implantation.

Analysis of results using simple scoring system

In terms of aggregate score, CM+ preculture outperformed all other strategies, with increased collagen, maintenance of GAG, and stable metabolic activity in both the AF and NP regions after implantation (Table 3). All preculture formulations were conducive to growth in the AF region after implantation, but only the CM+ and CM++ formulations were conducive to a healthy NP phenotype.

Discussion

For successful clinical translation, engineered tissues must survive the transition from an optimal *in vitro* culture environment to a low-oxygen, nutrient-poor *in vivo* environment. In this study, six strategies were evaluated to

FIG. 6. Time course of MRI characteristics (T2) in long-term culture and after implantation. (A) Representative T2 MRI maps after 5 weeks of *in vitro* culture (W5) and after 5 weeks *in vitro* and then an additional 5 weeks of subcutaneous implantation (SQ, W10). (B) Measurements the mean AF and NP region T2 relaxation time for each of the six preculture media conditions. Solid lines represent measurements from *in vitro* culture, dotted lines represent after implantation. (C) T2 relaxation times presented as percent difference from the week 5 CM+ group. Light blue and red bars indicate the standard deviation of the week 5 CM+ group. In all cases: * $p < 0.05$ and $^+p < 0.1$ versus week 5 CM+. Results from the one-way analysis of variance evaluating the effect of media condition: AF, $p < 0.001$; NP, $p < 0.01$. Color images available online at www.liebertpub.com/tea

identify preculture conditions conducive to maturation *in vitro* and phenotype maintenance *in vivo* using an engineered intervertebral disc and a subcutaneous implantation model. We found that a chemically defined media (CM) that incorporated TGF- β 3 (CM+ group) best promoted the de-

position of GAG and collagen in DAPS *in vitro*, the maintenance of accumulated matrix *in vivo*, and minimal changes in the metabolic activity of cells within the construct. In addition, MRI T2 relaxation time correlated to collagen both in the AF and NP regions (similar to MRI studies of

TABLE 2. PEARSON CORRELATION COEFFICIENTS

	OHP	GAG	MTT	T2		OHP	GAG	MTT	T2
AF region					NP region				
OHP	1	0.25	-0.22	-0.65	OHP	1	0.09	-0.17	-0.23
GAG		1	0.27	-0.02	GAG		1	0.21	0.10
MTT			1	0.07	MTT			1	0.07
T2				1	T2				1
AF region— <i>in vitro</i>					AF region— <i>in vivo</i>				
OHP	1	0.79	0.16	-0.08	OHP	1	0.60	-0.26	-0.16
GAG		1	0.33	-0.13	GAG		1	-0.06	0.03
MTT			1	-0.16	MTT			1	-0.30
T2				1	T2				1
NP region— <i>in vitro</i>					NP region— <i>in vivo</i>				
OHP	1	0.69	0.00	-0.25	OHP	1	0.21	-0.03	-0.05
GAG		1	0.07	-0.08	GAG		1	0.67	0.19
MTT			1	-0.19	MTT			1	0.13
T2				1	T2				1

Bold values represent a significant correlation ($p < 0.05$).

AF, annulus fibrosus; GAG, glycosaminoglycan; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NP, nucleus pulposus; OHP, hydroxyproline.

TABLE 3. SIMPLE SCORING SYSTEM FOR EVALUATING PRECULTURE STRATEGIES

	<i>OHP</i> , <i>W10</i> in vivo vs. <i>W5 CM+</i> in vitro	<i>GAG</i> , <i>W10</i> in vivo vs. <i>W5 CM+</i> in vitro	<i>MTT</i> , <i>W10</i> in vivo vs. <i>W5 CM+</i> in vitro	Score	Total score
AF					
SM+	0	-1	0	-1	
SM++	+1	-1	0	0	
SM+T	+1	-1	0	0	
CM+	+1	0	0	1	
CM++	+1	-1	0	0	
CM+T	+1	0	0	1	
NP					
SM+	0	-1	-1	-2	-3
SM++	0	-1	-1	-2	-2
SM+T	0	-1	-1	-2	-2
CM+	+1	0	0	1	2
CM++	0	0	0	0	0
CM+T	0	-1	-1	-2	-1

The CM+ group achieved the highest total score (represented by bold font).

cartilage⁴⁰), where decreasing T2 values correlated to maturation of the construct, confirming this measure as a non-invasive means to quantify DAPS composition *in vivo*.

Subcutaneous implantation was conducive to collagen formation in all implants, whereas GAG production and retention was dependent on the formulation of the preculture media. After implantation, there was an increase in AF collagen in all conditions, and an increase in NP collagen in the CM+ group only. In terms of GAG production, it is likely that inducing and maintaining a chondrogenic phenotype *in vitro* is necessary to promote phenotype retention *in vivo*, and that including serum in the preculture media hinders this. Our finding that CM+ preculture is a potential strategy for translation of an engineered disc containing NP and AF cells is in contrast to the work published on chondrocytes. For example, chondrocytes encapsulated in hydrogels and precultured in CM with TGF- β 3, showed a depletion of GAG after implantation.⁹ As this study had a preculture duration of 3 weeks (compared with 5 weeks in the current study), it may be that preculture duration is also a factor, where longer durations allow for a more mature phenotype to develop and stabilize the engineered tissue for the *in vivo* transition. Furthermore, our results with AF/NP cells are in contrast to results with MSCs predifferentiated in CM with TGF- β 1 for 3 weeks and implanted subcutaneously as a pellet. In those studies, after implantation, there was a decrease in Toluidine Blue staining (GAG) and a decrease in cell viability.¹⁰ Similarly, MSCs predifferentiated in CM with TGF- β 3 and BMP-6 lost their phenotype after subcutaneous implantation, with a reduction in GAG and type II collagen, and an increase in Alizarin Red staining, indicative of hypertrophy.¹¹ It may be that disc cells are particularly well suited to survive the *in vitro* to *in vivo* transition, given that their normal physiological environment is nutrient- and oxygen-poor. As there is much interest in using stem cells for tissue engineering, it may be necessary to alter preculture methods to promote acquisition of features of the NP/AF phenotype to further successful translation of stem cell technologies.

In vitro culture with CM increased metabolic activity, enhanced matrix accumulation, and increased the physical

size of DAPS, whereas culture with SM (with or without TGF- β 3) was not conducive to DAPS maturation. These findings support previous work on NP cells in hydrogels.⁴¹ It may be that serum increases the turnover rate of extracellular matrix components, as high levels of cleaved aggrecan were measured after culture of NP cells in SM with TGF- β 3.⁴² FBS contains many growth factors that contribute to cell proliferation, differentiation, and matrix production, including those of the insulin-like growth factor family, fibroblast growth factor family, and the TGF family.⁴³ It may be that, in serum, finite resources within the cell respond to multiple signals simultaneously, interfering with the main action of TGF- β 3. Conversely, in defined media, TGF- β 3 may be able to drive matrix production without these confounding signals. Thus, these results suggest that the benefits of *in vitro* culture with media including TGF- β 3 are mitigated by the inclusion of serum. While we hypothesized that the removal of TGF- β 3 before implantation would better prime cells for the *in vivo* environment, we found that in every case, removal of TGF- β 3 lead to inferior *in vivo* performance. This finding suggests that a CM with TGF- β 3 (at a consistent concentration) is the gold standard for developing engineered disc tissues using this system.

The degradation of matrix in the NP region in this study may be a characteristic of culture in the HA hydrogel. In serum conditions, cells contracted or degraded the gel, as histological sections showed a decrease in NP dimensions during preculture (radius <1 mm in all cases) as compared with day 0 (1 mm). In other cases when this hydrogel was exposed to serum, for example, in the rat tail model¹⁵ and an *in vivo* pig osteochondral defect model,⁴⁴ HA was also degraded. In subcutaneous models, where less bleeding occurs during surgery,^{45,46} the hydrogel remains intact. In these *in vivo* systems, local inflammation, as communicated through the blood at the site of surgery, may induce the activation of hyaluronidases that degrade the hydrogel and release GAG (developed during preculture) from the implant. In this study, GAG content in the NP region correlated to metabolic activity after implantation. Hydrogel degradation may also impact the metabolism/viability of seeded cells.

It may be necessary to control inflammation or hyaluronidase activity when using HA hydrogels *in vivo* in conditions where HA is exposed to blood.

One limitation of this study was that the infiltration of endogenous cells, which may affect matrix accumulation after implantation, was not evaluated. DAPS cultured in SM formulations had poor matrix production *in vitro*, but had significant collagen accumulation *in vivo*, potentially as a result of local cell infiltration. Still, the inability of SM conditions to mature constructs *in vitro* precludes SM conditions from being useful for *in vivo* translation in this model. Cell-tracking methods are available to differentiate between local and implanted cells, and will be employed in the future to determine cell fate. Another limitation was that implants evaluated in a subcutaneous model do not replicate an orthotopic disc replacement environment. Future work will evaluate the best strategy, CM+, as a preculture method before *in situ* total disc replacement, and evaluate longer time points of *in vivo* implantation to determine if phenotype retention persists.

Conclusions

Chemically defined media with TGF- β 3 improved both *in vitro* maturation and *in vivo* phenotype retention of disc-cell-laden DAPS. Other strategies were not as effective; DAPS cultured in serum improved compositionally *in vivo*, but did not mature *in vitro*, particularly in the NP region. Additionally, transition strategies (SM+T and CM+T groups) reinforced the finding that serum was not compatible with maturation of the NP region. All groups showed increased collagen production after implantation, possibly in response to factors secreted by endogenous cells or as a result of infiltration by host fibroblasts. These findings define a favorable preculture strategy (CM+) for the translation of engineered discs seeded with disc cells.

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Disclosure Statement

No competing financial interests exist.

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