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14. ABSTRACT Disc injury through trauma, vibration loading, or mechanical overload, and the resulting disc degeneration in response to these insults over time are tremendous problems affecting the active and veteran military population. Current treatment options fail to restore disc structure and mechanical function. Our goal in this proposal is to develop methodologies for the engineering and implanting of a functional biologic disc replacement. Significant progress has been made in the last 12 months towards achieving this goal. For the in vitro component of the proposal, we have optimized HA hydrogels for NP tissue engineering, investigated the effects of physiologically accurate low-oxygen conditions on the biosynthetic properties of NP cells, optimized MSC seeding techniques to maximized cell infiltration and proliferation in DAPS AF constructs, fabricated complete DAPS comprised of a PCL nanofiber AF and a hyaluronic acid hydrogel NP, and designed and commencement of construction of a multi-axis bioreactor that will be used to enhance in vitro functional maturation. For the in vivo component of the proposal, we have undertaken extensive implantation of acellular AF DAPS constructs, and have successfully maintained disc height for several weeks. To overcome problems related to the construct migrating out of the disc space within a few days of implantation, we have designed and implemented a novel external fixator to stabilize the joint and facilitate long term integration with the adjacent vertebral bodies.					
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Introduction:

The focus of this project is on restoration of the intervertebral disc through tissue engineering methodologies. Disc injury through trauma, vibration loading, or mechanical overload, and the resulting disc degeneration in response to these insults over time are tremendous problems affecting the active and veteran military population. Neither conservative treatments, such as stretching and exercise, nor surgical options, such as fusion and arthroplasty, restore disc structure or mechanical function. Total disc arthroplasty is relatively new to clinical practice, but will suffer from the same problems as traditional implant materials – wear and the need for eventual replacement. An alternative to these methods involves implantation with a biologic tissue engineered replacement. Such a biologic tissue restoration method would not be subject to wear as occurs with prosthetic devices, and would restore flexibility and motion about that spinal segment. Because the function of the disc is mechanical, it is important to focus upon mechanics in the design of functional tissue engineered constructs (TECs) and to direct the biology (maintenance of phenotype and ECM deposition) towards these mechanical outcomes. Direct biologic restoration of the disc with a TEC that duplicates the mechanical properties of the native tissue and restores range of motion would be an ideal alternative. Our goal in this project is to develop methodologies for the engineering and implantation of a functional biologic disc replacement. **The objective of this proposal is to move to the translational space and towards clinical implementation by creating and implanting a tissue engineered disc-like angle ply structure (DAPS),** and throughout this development, remain mindful of the fundamental importance of the mechanical function.

During the second year of funding on this project, the PIs have made substantial progress in the Aims. Given the highly collaborative and interdisciplinary activities of this project, a single document detailing all progress is presented, but is uploaded under each of the PIs specific award numbers. The research team has been meeting on a weekly or bi-weekly basis over this time course, and have established major advances in the formation of disc-like angle-ply structures for disc tissue engineering applications, and their *in vivo* translation using a rat caudal model.

Body:

Drs. Elliott, Mauck, and Hebela have made marked progress on their project (OR090090) entitled: “Development and Translation of a Tissue-Engineered Disc in a Preclinical Rodent Model”. Below, we provide a listing of the original specific aims of our project, followed by detailed descriptions from several recent publications based on these efforts. Our work stems from the translation of the single and bi-layer constructs with tensile properties approaching that of the native annulus fibrosus tissue into full 3D Disc-like Angle Ply Structures (DAPS), inclusive of a hyaluronic acid hydrogel seeded with adult stem cells or annulus fibrosus (AF)/nucleus pulposus (NP) cells that can be used to replace the degenerate native disc.

Proposed Aim 1: Create a mesenchymal stem cell (MSC) seeded 3D structural TEC disc from concentric AF constructs surrounding an engineered nucleus pulposus (NP) composed of a hyaluronic acid (HA) hydrogel. Measure the disc structural mechanics in compression and torsion, and the isolated AF and NP substructures in compression following time in culture. Evaluate the molecular, histological, and biochemical properties of these TEC discs as a function of time in culture and with variations in media conditions.

A substantial portion of Aim 1 has been completed, including optimizing HA hydrogels for NP tissue engineering, investigating the effects of physiologically accurate low-oxygen conditions on the biosynthetic properties of NP cells, optimizing MSC seeding techniques to maximized cell infiltration and proliferation in DAPS AF constructs, fabrication of complete DAPS comprised of a PCL nanofiber AF and a hyaluronic acid hydrogel NP, and design and commencement of construction of a novel multi-axis bioreactor that will be used to enhance in

vitro functional maturation of DAPS using combined torsion and compression loading, prior to implantation (funded via additional external funds). Detailed results of these experiments are provided in the following sections.

Optimizing HA Hydrogels for NP Tissue Engineering

The objective of this study was to investigate the maturation capacity of NP cells within methacrylated HA hydrogel and to determine the appropriate NP seeding density to produce a functional NP construct. 65 kDa HA (Lifecore) was methacrylated to a level of ~25%. NP cells (passage 2, isolated from adult bovine caudal discs) were suspended in 1% MeHA solutions at either 20 million cells/ml (20M) or 60 million cells/ml (60M). The diameter and thickness of constructs was 4mm and 1.5mm, respectively. Constructs were cultured for 8 weeks in a chemically defined media containing 10 ng/ml TGF- β 3. Unconfined compression tests were performed to determine construct mechanical properties as previously described. The equilibrium modulus was determined from the equilibrium stress and strain values normalized to the construct dimensions. After mechanical testing, samples were weighed and the DNA and GAG contents were assessed via the Picogreen (Molecular Probes) and DMMB assays respectively. DNA content was normalized per construct, and GAG was normalized to wet weight. GAG released to the culture medium was also measured. To test for viability, constructs were imaged using the Live/Dead staining kit (Molecular Probes). Constructs embedded in paraffin, and sectioned (8 μ m). Samples were stained with either Alcian blue or picosirius red to visualize proteoglycans and collagen, respectively. Real time RT-PCR was performed to determine gene expression levels normalized to GAPDH. Expression levels were compared to that of NP cells prior to encapsulation.

HA-based NP constructs formed using both 60M and 20M cell densities matured with time in culture. In general, both DNA content (not shown) and direct visualization (Fig. 1) showed higher cell numbers in the 60M constructs compared to the 20M constructs. Interestingly, and unlike chondrocytes and MSCs, NP cells

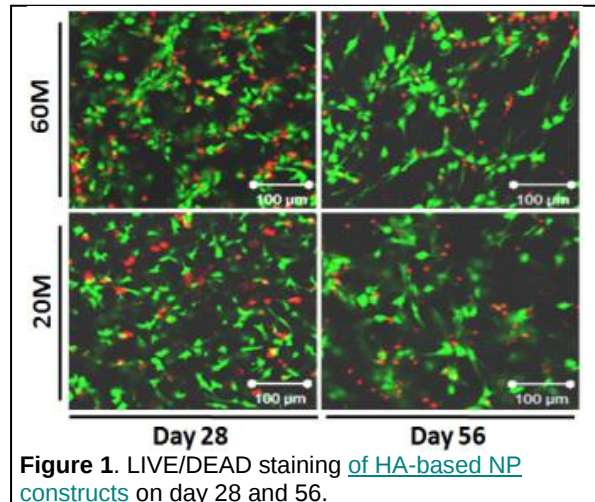


Figure 1. LIVE/DEAD staining of HA-based NP constructs on day 28 and 56.

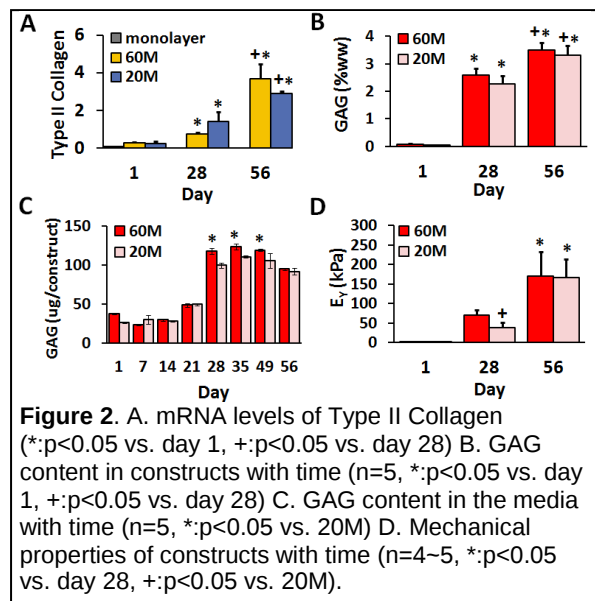


Figure 2. A. mRNA levels of Type II Collagen (*:p<0.05 vs. day 1, +:p<0.05 vs. day 28) B. GAG content in constructs with time (n=5, *:p<0.05 vs. day 1, +:p<0.05 vs. day 28) C. GAG content in the media with time (n=5, *:p<0.05 vs. 20M) D. Mechanical properties of constructs with time (n=4-5, *:p<0.05 vs. day 28, +:p<0.05 vs. 20M).

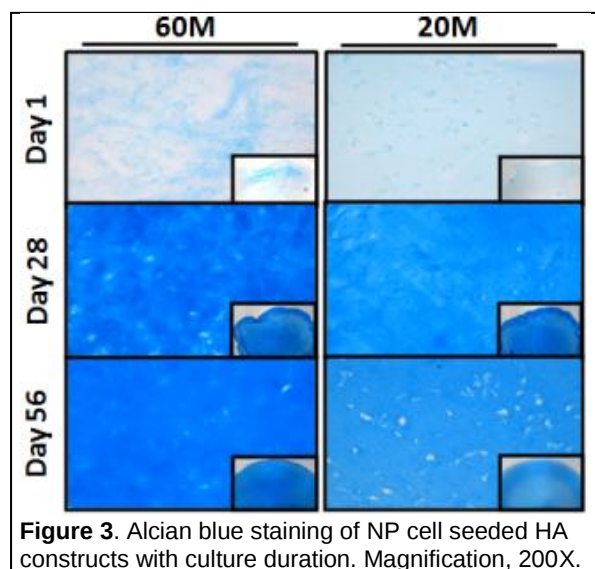


Figure 3. Alcian blue staining of NP cell seeded HA constructs with culture duration. Magnification, 200X.

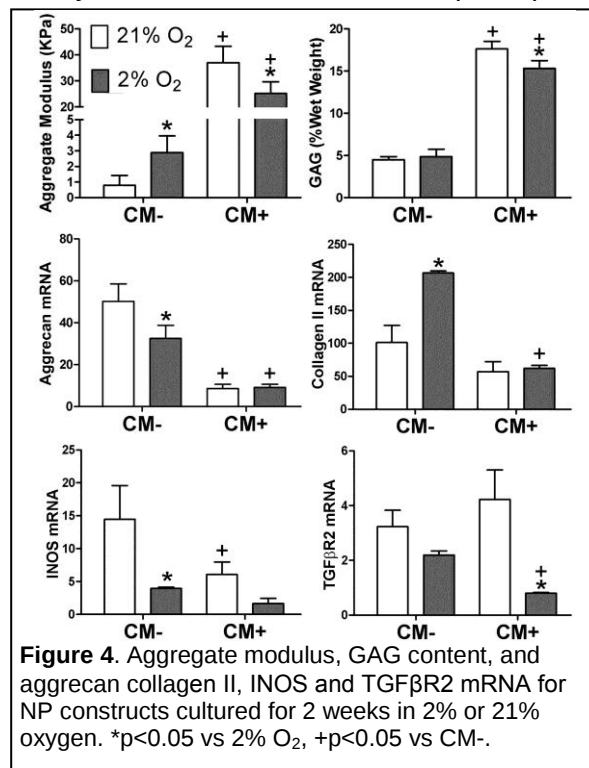
adopted a stellate morphology in HA hydrogels, forming small clusters with time, especially at the higher seeding density. Both type II collagen gene expression (Fig. 2A) and GAG content (Fig. 2B) increased over the 56 day culture period. While there were some small differences between groups with time, GAG content was not significantly different at day 56 (Fig. 2B). GAG released into the media also increased significantly with culture time, plateauing at a similar level after day 28 in both groups (Fig. 2C). The mechanical properties of the 60M constructs were higher than those of the 20M constructs on day 28, but were no longer different on day 56 (Fig. 2D), with both groups achieving a modulus in excess of 150 kPa. Glycosaminoglycan staining was more intense in the 60M constructs compared to the 20M constructs (Fig. 3), especially at early time points.

Engineering a functional and clinically translatable NP replacement will require a material that can promote matrix accumulation and maintain NP cell phenotype. In this study, we demonstrated that NP cells generate a proteoglycan-rich matrix in photo-crosslinkable HA hydrogels. As a consequence of matrix production, constructs increased in mechanical properties with time in culture, matching or surpassing native tissue levels, and developed high expression levels of key ECM markers (i.e., type II collagen). In comparing low and high cell seeding densities, we noted early differences in cellular morphology within the material, and some differences in GAG accumulation and mechanics at day 28. However, by day 56, these differences were no longer apparent. DNA data revealed a >3-fold increase in cell number during the culture period, which may have enabled the lower seeding density gels to catch up to the higher seeding density gels. Further, there appeared to be a large fraction of dead cells (Fig. 1) at each time point, suggesting that only a sub-population of the originally seeded NP cells mediates the growth response. Taken together, these results suggest that a lower (20M) cell density may be sufficient for engineering NP tissues using HA hydrogels. This has clinical relevance, as the supply of healthy autologous NP cells is limited, and culture expansion of such cells may limit retention of phenotype. Having established that this cross-linkable HA material is supportive of the formation of an NP-like tissue, current work is focused on implementing other gelation mechanisms that would enable injectable delivery of this material to the disc space (i.e., redox-mediated reactions).

Investigating the effects of physiologically accurate low-oxygen conditions on the biosynthetic properties of NP cells

Previous studies aimed at functional NP tissue engineering have largely been undertaken in normoxic (21% oxygen) conditions; however, due to the avascular nature of the native NP tissue, NP cells reside in a hypoxic tissue niche. The objective of this study, therefore, was to investigate the effects of a physiologically appropriate low oxygen tension on the functional and biosynthetic response of NP cells using an established 3D agarose culture model.

NP cells were isolated from adult bovine caudal discs and expanded in monolayer. Passage 2 cells were suspended in 2% agarose at $20 \times 10^6/\text{ml}$. Constructs 4 mm diam. x 2.25 mm thick were cultured for 2 weeks in chemically defined media with (CM+) or without (CM-) 10 ng/ml TGF- β 3, in either 21% oxygen or 2% oxygen in an environmental workstation



(HypOxygen, Frederick, MD). For histology, samples (n=3) were processed into paraffin and sections were stained with alcian blue or picosirius red to evaluate GAG and collagen deposition. Constructs (n=5) were tested in confined compression as described previously [3]. Constructs were subjected to a 0.02 N preload for 500 sec, followed by a stress relaxation test of 10% strain applied at 0.05%/sec followed by relaxation to equilibrium for 10 min. Aggregate modulus was calculated from the equilibrium stress/applied strain. After mechanical testing, constructs (n=5) were digested in papain, and GAG content determined using the DMMB assay and normalized to wet weight. DNA content was determined using the PicoGreen assay. RNA was isolated from the constructs (n=3), and quantitative PCR performed to determine mRNA levels of matrix proteins: aggrecan, collagen I and collagen II; the transcription factor SOX9; TGF signaling genes TGF- β 1, TGF- β R1 and TGF- β R2; and cell stress markers inducible nitric oxide synthase (iNOS), p53 and Bcl-2-associated X protein (BAX), with expression normalized to GAPDH and presented as a ratio to initial (day 0 of culture). Viability and cell number was assessed using the Live/Dead staining kit (n=3). Cells from 10X fluorescent images from three regions on each sample were analyzed using a custom Matlab program [4]. Effects of oxygen tension (21% or 2%) and media (CM+ or CM-) for were established using 2-way ANOVAs with Bonferroni post hoc tests ($p < 0.05$).

After 2 weeks of culture NP cells deposited both GAG and collagen in a uniform manner throughout the construct. Matrix deposition was more robust for the CM+ groups. As expected, modulus and GAG content were both significantly higher for CM+ than CM- for both oxemic states (Fig. 4). Constructs cultured in CM- had greater modulus (not significant) in 2% O₂ than in 21% O₂; however, for constructs cultured in CM+, the 21% O₂ group had a significantly higher modulus. These same trends were reflected in the GAG content. Neither the media formulation nor oxemic state had a significant effect on cell proliferation or cell viability. mRNA levels of BAX, p53, iNOS, TGF- β 1, TGF- β R1, TGF- β R2, collagen I, and SOX9 for constructs cultured in 2% O₂ were lower than those cultured in 21% O₂ for both media conditions. In CM-, mRNA levels for aggrecan and collagen II were significantly lower and higher, respectively, in 2% O₂ than 21% O₂, and not significantly different between 2% and 21% O₂ in CM+.

In this study we examined the effects of low oxygen tension on the biomechanical properties, biochemical composition, and mRNA expression of engineered nucleus pulposus constructs. Our results suggest that low oxygen tension attenuates the biosynthetic response of NP cells in the presence of TGF- β 3 (CM+), potentially due to reduced expression of TGF receptors. This has important implications for NP tissue engineering and growth factor-based therapies, as it suggests that under physiologically appropriate oxemic conditions, NP cells are less responsive to anabolic stimulation. Importantly our results demonstrate that low oxygen tension does not have a negative effect on NP cell viability, nor increase cell stress as indicated by markers of apoptosis and autophagy. Ongoing work will further examine the associated molecular pathways and examine the effect of low oxygen tension on other important anabolic and catabolic mediators of disc development and function.

Optimizing MSC seeding techniques to maximized cell infiltration and proliferation in DAPS AF constructs

A major challenge when it comes to seeded multi-laminate DAPS constructs with cells is to obtain sufficient infiltration between the construct layers. Initial studies in which cells were seeded on top of pre-fabricated constructs were unsuccessful in achieving sufficient infiltration. We developed a new construction

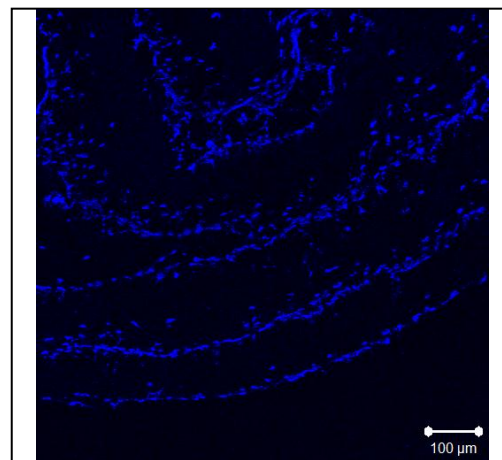


Figure 5. DAPI imaging showing excellent MSC infiltration between the layers of a completed DAPS construct.

algorithm in which MSCs were pre-seeded onto 1mm thick bi-lamellar strips, before those strips were rolled to produce complete DAPS constructs. DAPI staining (Fig. 5) demonstrated excellent infiltration using this revised technique.

Fabrication of complete DAPS comprised of a PCL nanofiber AF and a hyaluronic acid hydrogel NP

We have commenced fabrication and in vitro pre-culture of composite DAPS constructs. An AF region, comprised of rolled bi-layers of electrospun PCL and seed with rat AF cells, was combined with an NP region comprised of HA hydrogel seeded with rat NP cells (Fig. 6). This was achieved by coring the center of the AF construct with a 2mm biopsy punch and then press-fitting the pre-cast NP gel into place. Composite DAPS were then cultured in vitro for up to 4 weeks. Results, including histological and biochemical analyses of matrix deposition, biomechanical properties, and cell viability assays, are pending.



Figure 6. Composite DAPS comprised of multi-lamellar AF seed with rat AF cells and HA hydrogel NP seeded with rat NP cells, after 4 weeks of in vitro culture.

Design and construction of a multi-axis bioreactor to enhance in vitro functional maturation of DAPS

To further enhance the functional properties of our DAPS constructs during in vitro culture and prior to implantation, we have designed a novel multi-axis bioreactor (Fig. 7) that will be capable of simultaneously applying compression and torsional dynamic loading. Our team and others have previously shown that compressive and tensile dynamic loading improves the functional properties of a variety of engineered musculoskeletal tissues. In vivo, the intervertebral disc cells experience complex multi-directional loading. We hypothesize that this multi-directional loading is required to properly stimulate functionally appropriate extracellular matrix biosynthesis. The bioreactor is in the advanced stages of construction, with experiments anticipated to commence in early 2013. Additional funding for this bioreactor was obtained from a Force and Motion scholarship awarded to a graduate student in Dr Mauck's laboratory.

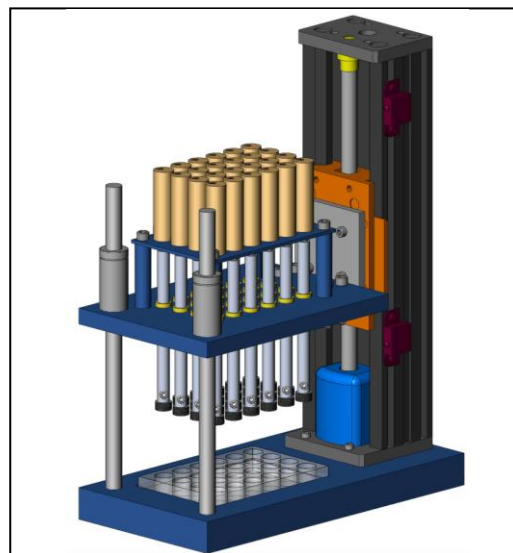


Figure 7. 3D representation of multi-axis bioreactor

Original Aim 2: Implant the tissue engineered DAPS in a subcutaneous environment after varying period of in vitro pre-culture and evaluate maturation using the same assays and evaluation criteria as in Aim 1.

In preparation for the initiation of work related to Aim 2, we have submitted ACORP protocols regarding DAPS implantation in the subcutaneous space. We have also (using other funds), fully developed this in vivo evaluation method at the VA, with a particular focus on improving scaffolds for the AF region (improving cell infiltration, providing drug delivering capacity). An amendment to our ongoing ACORP is under consideration for the implantation of our MSC-

based DAPS, and the long term portions of these studies have been initiated in culture. We anticipate that significant progress will be made on this Aim in the coming year.

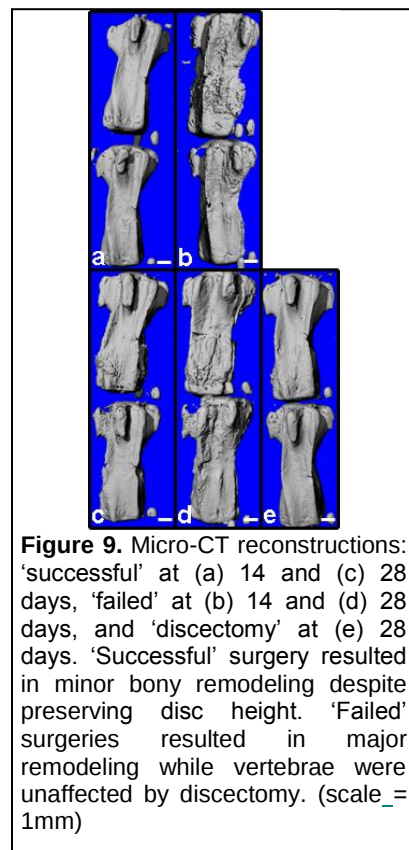
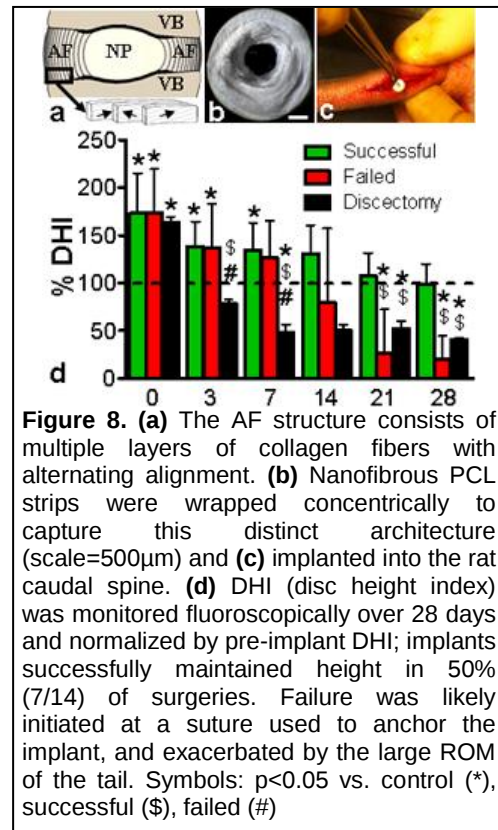
Original Aim 3: Implant the tissue engineered DAPS in situ using a rat tail disc replacement model.

This Aim represents the culmination of our project, and is by far the most challenging aspect. None the less we have made impressive progress on a number of fronts. Specifically, we have undertaken extensive implantation of acellular AF DAPS constructs, and have successfully maintained disc height for several weeks. To overcome problems related to the construct migrating out of the disc space within a few days of implantation, we have designed and implemented a novel external fixator to stabilize the joint and facilitate long term integration with the adjacent vertebral bodies.

In Vivo Implantation of Acellular DAPS Constructs

Electrospun PCL was collected on a rotating mandrel as aligned fibrous sheets 250 μ m thick. Strips were excised 30° to the fiber direction and two strips with alternating $\pm$ 30° alignment were wrapped concentrically, replicating the AF (Fig. 8B). Top and bottom surfaces were planed to control height. Final dimensions enabled a press fit (OD=2mm, ID=1.25mm, ht.=1.25mm).

AF-only DAPS (Fig. 8C) were implanted into the tails of mature Sprague Dawley rats. A dorsal incision spanning the C7 and C8 vertebral bodies was made. Dorsal tendons were detached from their bony insertions adjacent to the disc which was then removed with a scalpel. Double-armed suture (3-0 or 5-0) was passed through the DAPS lumen, fed through the disc space and tied exterior to the ventral skin to anchor the implant (Fig. 8C) and the incision was closed. Rats were euthanized at either 14 (n=3) or 28 (n=11) days. Additional rats designated for discectomy only (n=2) were euthanized at 28 days. Disc height index (DHI) was monitored via weekly fluoroscopic imaging. Surgery was deemed 'successful' if 75% of the pre-operative DHI was maintained and 'failed' if not. DHI was statistically assessed by first grouping 'successful', 'failed', and discectomy surgeries and implementing an ANOVA comparing groups within individual time points and a group that consisted of pooled pre-operative measurements. Tukey's test was used for post-hoc comparisons (p<0.05). Following euthanasia, tail segments scanned by micro-CT at 20.5 μ m resolution. Reconstructions were generated to assess vertebral body quality. Segments from 'successful' surgeries were fixed in formalin and decalcified. Sagittal sections were



viewed under polarized light. DAPS from 'failed' surgeries were removed and imaged for gross inspection. These were then fixed and transverse sections were stained with H&E.

Results showed that DAPS maintained 75% of pre-operative DHI in 50% (7/14) of surgeries (Fig. 8D). DHI from rats with 'successful' implants was not different from pre-operative DHI at 28 days ($p>0.05$). Failure was likely a result of suture rupture/stretching and was exacerbated by the large ROM in the rat tail. There was wide variability in the timing of implant failure, evidenced by large standard deviations in DHI at 14 and 21 days. Following discectomy, the disc space was initially expanded, as a result of disc removal/detachment of the dorsal tendon bundles, but collapsed to 41% of preoperative DHI by 28 days. Micro-CT reconstructions confirmed the maintenance of disc height in 'successful' surgeries at 14 and 28 days (Figs. 9A and C). At 28 days, however, minor bony remodeling and misalignment was evident. In 'failed' surgeries, the implant space had completely collapsed and prominent remodeling persisted along the length of vertebrae (Figs. 9B and D). Remodeling was not present in the discectomy group, despite collapse (Fig. 9E). Polarized light revealed maintenance of a lamellar structure and alternating fiber pattern over the course of the experiment (Fig. 10A). 'Successful' DAPS were situated within the vacated disc space under aligned vertebrae at 14 days. At 28 days however, vertebral misalignment was present and implants appeared compressed and shifted despite preserving DHI. 'Failed' implants appeared to integrate with native tissue (Fig. 10D), but only peripheral cellular infiltration was apparent at 14 and 28 days (Figs. 10C and F).

To summarize these results, acellular engineered discs designed to reproduce the intervertebral disc fiber architecture were implanted in the rat caudal spine. Seven of 14 implants maintained disc height over the course of 28 days, despite vertebral misalignment and compaction of the implant structure. There was moderate vertebral remodeling in implant surgeries but not in discectomies, possibly due to increased loading with the compression fit and/or the presence of inflammatory cells, and there was little to no cellular infiltration at 28 days.

Joint Stabilization with a Novel External Fixator

As the "suture" technique adopted in the previously described experiment was only partially successful in preventing short-term migration of the DAPS implant from the disc space, a novel external fixator (Fig. 11, left) was designed to stabilize the intervertebral space and facilitate long-term integration of the implant with the adjacent vertebrae. This fixator consisted of concentric C-shaped rings which were placed axially of the two vertebrae adjacent to the intervertebral space where the implant was to be placed (Fig 11, right). These rings were then fixed to k-wires placed through those

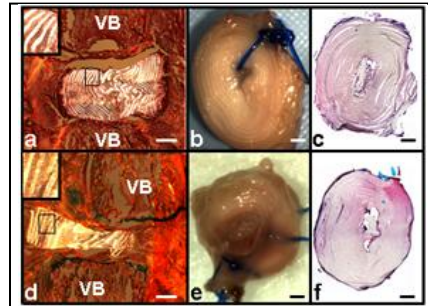


Figure 10. Polarized light images of 'successful' at (a) 14 and (c) 28 days (inset: 'lamellae' with alternating fiber alignment). Gross images of 'failed' at (b) 14 and (e) 28 days. H&E images of 'failed' at (c) 14 and (f) 28 days. 'Successful' surgery resulted in preserved height but a compacted implant with vertebral misalignment. 'Failed' discs appeared to integrate with native tissue but lacked cellular infiltration. (scale=500 μ m)

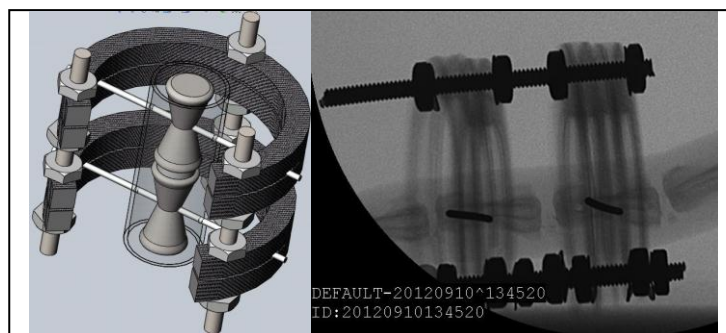
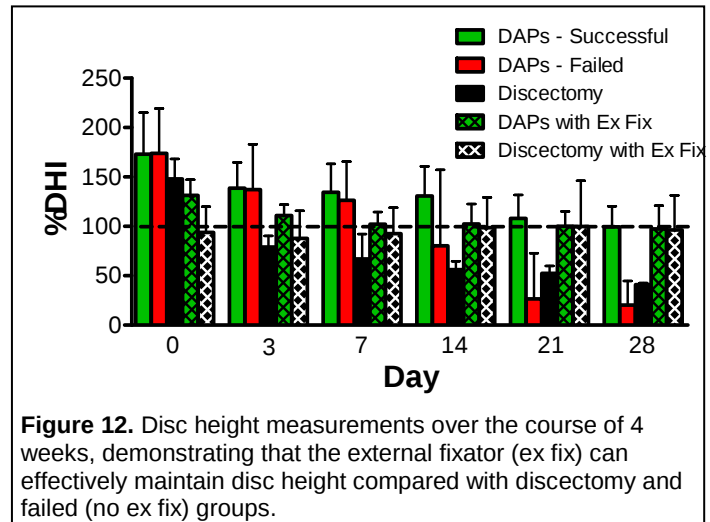


Figure 11. 3D schematic representation of external fixator (left) and fluoroscopic image (right) showing the fixator in place on the rat tail, with k-wires inserted through adjacent vertebrae to stabilize the intervening disc space.

vertebrae. Axial spacing of the C-rings was maintained via threaded rods and locator nuts. Key design criteria, in addition to joint stabilization, were that the device had to be radiolucent to facilitate x-ray imaging to assess disc height, and also be sufficiently resistant to chewing by the animals. The optimum material to achieve these criteria was found to be carbon fiber.

Additional implantation surgeries have been conducted using this fixator, and we have demonstrated that it can effectively maintain disc height for at least 4 weeks compared to discectomy and non-fixator controls (Figure 12).

This will allow us to proceed with our long term in vivo implantation studies in Aim 3, and evaluate the engineered rat cell-seeded IVD constructs developed in Aims 2 and 3.



Key Research Accomplishments:

According to the progress we have made on the Aims outlined above, we have achieved the following Key Research Accomplishments.

- We have demonstrated that nucleus pulposus cells seeded in hyaluronic acid hydrogels retain their native phenotype and synthesize a mechanically robust extracellular matrix. (Aim 1)
- We have shown that NP cells cultured under physiologically accurate low-oxygen conditions retain similar biosynthetic properties to those cultured under normoxic conditions. (Aim 1)
- We have optimized MSC seeding techniques to maximize cell infiltration and proliferation in DAPS AF constructs. (Aim 1)
- We have fabricated complete DAPS comprised of a PCL nanofiber AF and a hyaluronic acid hydrogel NP. (Aim 1)
- We have designed and commenced construction of a novel multi-axis bioreactor that will be used to enhance in vitro functional maturation of DAPS using combined torsion and compression loading, prior to implantation (Aim 1 + additional external funds received).
- We have perfected our techniques for subcutaneous implantation and submitted an IACUC (Aim 2).
- We have shown that cell-free DAPS AF constructs implanted into the rat caudal disc space maintain disc height for up to 4 weeks (Aim 3).
- We have developed and implemented novel instrumentation, namely an external fixator, that successfully stabilized the intervertebral joint and prevented premature ejection of the implant from the disc space (Aim 3).

Reportable Outcomes:

Our team has published 4 abstracts in the second year of this project, with 2 manuscripts in preparation. Further, portions of this work have been presented at the Philadelphia Spine Symposium. Funding through this award has supported one doctoral student in mechanical engineering at the University of Pennsylvania as well as several postdoctoral fellows.

Conclusions:

Our team has made marked progress in the formation of DAPS into angle ply structures and optimization of the in vitro growth of both the AF and NP regions, including optimization of the environmental conditions and materials to maximize functional maturation of composite constructs. We have performed extensive in vivo studies using acellular implants, including designing and implementing a novel external fixator to facilitate long term implant integration. All methods, materials, and approvals are in place for our team to continue to make important progress in disc tissue engineering over the next year. We are poised to commence the final and most exciting aspect of the study: implantation of cell-seeded composite (AF+NP) tissue engineered total disc replacements.

References/Publications:

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2. Kim DH, Gorth DG, Elliott DM, Smith LJ, Mauck RL (2013). *Functional Maturation of an Engineered Nucleus Pulposus in Photo-Crosslinked Hyaluronic Acid Hydrogels*, 59th Annual Meeting, Orthopaedic Research Society, San Antonio, USA (in review)
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Appendices:

None