

BME Department PhD Qualifying Examination

July, 2009

Minimally Invasive Delivery of Mesenchymal Stems to the Nucleus Pulposus Using an
In Situ Thermoresponsive Hydrogel

Matthew A. Engel
101339298
3 July 2009

PROJECT SUMMARY (See instructions):

This proposal aims to design an in situ curable hydrogel composed of PNIPAAm-PEG capable of delivering therapeutic cells and drugs to the degenerated nucleus pulposus (NP) of an intervertebral disc (IVD). The therapeutic cells will be human mesenchymal stem cells (hMSCs) differentiated into NP-like cells by exposure to hypoxic low oxygen conditions in the presence of transforming growth factor β 1 (TGF- β 1). The hydrogel is composed of a solution containing 10% PNIPAAm-PEG(8000) at a 1:1600 ratio of PNIPAAm:PEG which undergoes a sol-gel transition at physiological temperatures (32°C). Modifications to the PEG molecular weight or concentration can be used to alter the hydrogel stiffness or sol-gel transition temperature. These properties will allow users to prepare cell and drug loaded solutions at ambient temperatures and then inject them into the degenerated disc, at which point they solidify in situ. The hydrogel is capable of being blended with small molecule inhibitors of nitric oxide synthase (NOS). The inhibition of NOS has been shown to increase proteoglycan content which can restore disc height to the degenerated IVD.

RELEVANCE (See instructions):

Relevant to human patients with degenerative disc disease or prolapse. Stem cell delivery technology could also be applied to other degenerative or acute injuries to the spine.

Human Embryonic Stem Cells **NO**

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: <http://stemcells.nih.gov/research/registry/>. Use continuation pages as needed.

If a specific line cannot be referenced at this time, include a statement that one from the Registry will be used.

Cell Line

The name of the program director/principal investigator must be provided at the top of each printed page and each continuation page.

RESEARCH GRANT TABLE OF CONTENTS

| | <i>Page Numbers</i> |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------|
| Face Page | 1 |
| Description, Project/Performance Sites, Senior/Key Personnel, Other Significant Contributors, and Human Embryonic Stem Cells | 2 |
| Table of Contents | 3 |
| Detailed Budget for Initial Budget Period | _____ |
| Budget for Entire Proposed Period of Support | _____ |
| Budgets Pertaining to Consortium/Contractual Arrangements | _____ |
| Biographical Sketch – Program Director/Principal Investigator (<i>Not to exceed four pages each</i>) | _____ |
| Other Biographical Sketches (<i>Not to exceed four pages each – See instructions</i>)..... | _____ |
| Resources | 4 |
| Checklist | _____ |
| Research Plan | 5 |
| 1. Introduction to Resubmission Application, if applicable (<i>Not to exceed three pages.</i>), or Introduction to Revision Application, if applicable (<i>Not to exceed one page.</i>) | _____ |
| 2. Specific Aims..... | _____ |
| 3. Background and Significance | _____ |
| 4. Preliminary Studies/Progress Report | _____ |
| } (Items 2-5: not to exceed 25 pages)..... } | |
| 5. Research Design and Methods | 12 |
| 6. Inclusion Enrollment Report (Renewal or Revision applications only) | _____ |
| 7. Bibliography and References Cited/Progress Report Publication List | 29 |
| 8. Protection of Human Subjects | _____ |
| 9. Inclusion of Women and Minorities | _____ |
| 10. Targeted/Planned Enrollment Table | _____ |
| 11. Inclusion of Children..... | _____ |
| 12. Vertebrate Animals..... | _____ |
| 13. Select Agent Research..... | _____ |
| 14. Multiple PD/PI Leadership Plan | _____ |
| 15. Consortium/Contractual Arrangements..... | _____ |
| 16. Letters of Support (e.g., Consultants) | _____ |
| 17. Resource Sharing Plan (s) | _____ |
| Appendix (<i>Five identical CDs.</i>) | <input type="checkbox"/> Check if Appendix is Included |

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the project/performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. If research involving Select Agent(s) will occur at any performance site(s), the biocontainment resources available at each site should be described. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:

Significant laboratory space is required to conduct experiments for in vitro analysis of cells and materials.

Clinical:

None.

Animal:

None.

Computer:

Office:

Other:

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

- Hypoxia workstation (In vivo2, Ruskin, UK)
- 37°C incubator
- Differential scanning calorimeter Model #2010 (TA Instruments, Wilmington, DE)
- Instron mechanical testing system Model 4200 (Park Ridge, IL)

A. SPECIFIC AIMS

This proposal aims to design an in situ curable hydrogel composed of PNIPAAm-PEG capable of delivering therapeutic cells and drugs to the degenerated nucleus pulposus (NP) of an intervertebral disc (IVD). The therapeutic cells will be human mesenchymal stem cells (hMSCs) differentiated into NP-like cells by exposure to hypoxic low oxygen conditions in the presence of transforming growth factor $\beta 1$ (TGF- $\beta 1$). The hydrogel is composed of a solution containing 10% PNIPAAm-PEG(8000) at a 1:1600 ratio of PNIPAAm:PEG which undergoes a sol-gel transition at physiological temperatures (32°C). Modifications to the PEG molecular weight or concentration can be used to alter the hydrogel stiffness or sol-gel transition temperature. These properties will allow users to prepare cell and drug loaded solutions at ambient temperatures and then inject them into the degenerated disc, at which point they solidify in situ. The hydrogel is capable of being blended with small molecule inhibitors of nitric oxide synthase (NOS). The inhibition of NOS has been shown to increase proteoglycan content which can restore disc height to the degenerated IVD.

Hypothesis 1: Human mesenchymal stem cells isolated from the femur and grown in alginate gels differentiate into nucleus pulposus-like cells when exposed to TGF- $\beta 1$ under hypoxia.

Specific Aim 1: The differentiation of hMSCs will be determined by analyzing the gene expression, protein expression, and proteoglycan synthesis using RT-PCR, western blotting, and DMMB assay, respectively.

Hypothesis 2: Naked and cell-loaded PNIPAAm-PEG will have different lower critical solution temperature (LCST) values.

Specific Aim 2: The lower solution critical temperature of naked and cell loaded polymers will be found using differential scanning calorimetry which measures the amount of energy released as a function of temperature.

Hypothesis 3: NP-like cell-loaded PNIPAAm-PEG(8000) hydrogels cured in media with serum will have a lower compressive modulus compared to healthy nucleus pulposus tissue in serum at 37°C.

Specific Aim 3: The compressive modulus of cell loaded hydrogels and NP tissue will be found using an Instron mechanical testing system to produce stress-strain curves. The compressive modulus will be calculated from the slope of this curve at 15% strain.

Hypothesis 4: Naked and PNIPAAm-PEG embedded NP-like cells will show an increase in nitrite content and suppression of GAG synthesis when exposed to IL-1 β . That presence of L-NMA blended into the scaffold will cause a decrease in nitrite content and restore GAG synthesis in PNIPAAm-PEG embedded NP-like cells.

Specific Aim 4: The nitrite and proteoglycan content of naked and embedded cells in the hydrogel scaffold with and without the NOS inhibitor L-NMA will be measured by Griess and DMMB assay, respectively.

B. BACKGROUND AND SIGNIFICANCE

The intervertebral disc (IVD) is a pad-like structure that acts as a ‘shock absorber’ in the spine and provides flexibility while evenly distributing load throughout the vertebrae. The unique structure of the disc allows the spine to bend or twist in response to mechanical stimuli during rest, flexion or extension. Roughly one quarter of the height of the human spine is generated by the 23 intervertebral discs that secure the vertebral bodies together¹³. IVD degeneration is a common disorder which accounts for the majority of lower back pain cases. Autopsies have shown that 97% of individuals over 50 years of age show signs of disc degeneration; however the cause of disease progression is unknown. Some possible causes of degeneration include trauma to the cartilage endplates that join the discs together, vertebrae microfractures, and loss of specific cell types within the IVD. What is certain is that degeneration has a genetic component and is closely associated with aging⁵.

Back pain afflicts 80% of those with disc degeneration and disables approximately 1 in 50 Americans. 40% of all absences in the workplace are associated with back pain which causes a substantial loss in productivity, increase in medical expenses and worker’s compensation benefits. In the United States the annual cost of back pain related employee losses totaled \$50 billion in 1998²⁵. While most adults who develop back pain improve without medical direct treatment, a small amount of patients continue to experience chronic lower

back pains and progressive disc degeneration. Unfortunately, current treatments are symptomatic, and not curative.

IVDs are composed of several types of fibrocartilage which are functionally and morphologically distinct in healthy discs; yet highly dependent on each other in so that they function in unison and provide vertebrates with mechanical support. A nondegenerate IVD is composed of three distinct anatomic zones: the annulus fibrosus, nucleus pulposus and cartilage endplate. During disc degeneration, the highly hydrated nucleus pulposus becomes desiccated and the annulus fibrosus (AF) develops tears radiating outwards³. During aging, the cellular environment of the nucleus pulposus dramatically changes with a near total loss of notochordal cells, and an increase in chondrocyte-like cells in within the central portion of the disc. When degeneration occurs and notochordal cells disappear, the NP becomes less gelatinous and more fibrous as remaining NP cells are not able to synthesize the proper quantity of extracellular matrix (ECM); particularly the proteoglycans which are responsible for water absorbing properties of the nucleus matrix. Pathologies include reduced disc hydration, decreased disc height, and loss of cell density. One of the most pressing pathological features is the loss and change of proteoglycan content. This causes a drop in swelling pressure critical to homeostatic IVD function⁴⁴.

The annulus is a highly organized fibrous outer ring composed of thin layers of lamellae which can withstand significant tensile load during movement. Individual layers of lamella contain alternating strips of collagen type I fiber bundles oriented roughly 30° from the transverse spinal plane. The material behavior is highly anisotropic, as tensile stiffness differs by several orders of magnitude between the

circumferential, axial and radial directions. This is the only region of the disc to entertain blood vessels,

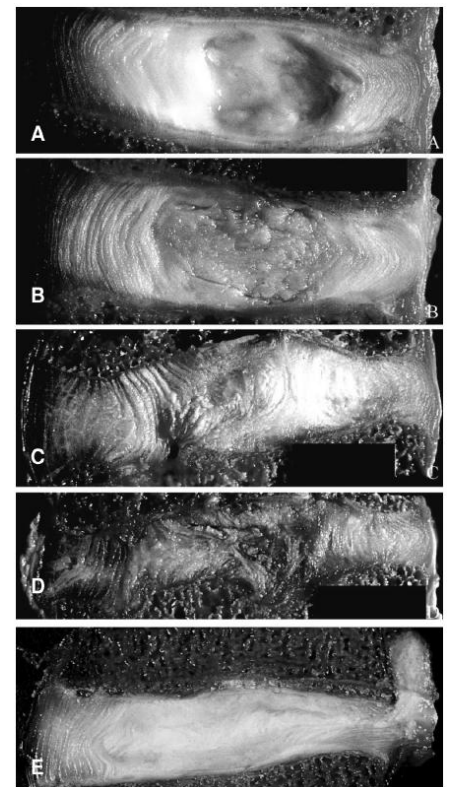


Figure 1. Cadaver IVD (anterior on the left). (A) Young disc (male, 35 years old). (B) Mature disc (male, 47 years old). (C) Disrupted young disc (male, 31 years old). (D) Collapsed young disc (male, 31 years old). (E) Disc induced to prolapsed in lab (male, 40 years old). NP has herniated through a posterior radial fissure in the AF².

especially in young adulthood. Vascular channels run from the cartilage end plate and into the lamellae of the AF, where nerve endings are often found. Within the first decade of life, many of these vascular channels fill up with ECM isolating the disc tissue, particularly the nucleus, from the inflow of nutrients and outflow of metabolites. Adult IVD is the largest avascular tissue in humans⁴¹.

Nucleus degeneration and radial fissures in the annulus leads to lumbar disc prolapse (herniation). Prolapse is the most common cause of nerve root pain (sciatica) and surgical interventions aim to relieve nerve root irritation or compression caused by herniated disc material¹⁶. During aging, cell density of the IVD decreases and defects in the annulus increase. Internal nuclear pressure is reduced, allowing the NP to bulge outward into the vertebral bodies under compression. As humans age, the NP clumps into fibrous nodes. At this stage the NP materials may migrate from the center of the disc into its periphery resulting in protrusion, extrusion or sequestration of the NP material. Prolapse can be simulated in cadaveric discs when bending or loading is taken beyond the physiological limit. It can also be mechanically induced as a result of intense repetitive loading. In the lab, severely degenerated discs do not prolapse themselves due to the lack of any hydrostatic pressure exerted by the NP².

Proteoglycans in the NP are composed of a core protein from which chains of GAGs containing aggrecan, keratin and chondroitin sulphate radiate. Multiple proteoglycans are linked together by hyaluronic acid to form large aggregates, which are themselves surrounded by type II collagen. These aggregates carry a large net negative charge and are primarily responsible for the NP's hydroscopic properties¹⁸. Hydration properties of the GAG chains cause NP tissue to swell until equilibrium is reached, balanced by

the tensile forces of the surrounding collagen network. Figure 2 shows the interior of an IVD under compression. As the spine undergoes load, water escapes from the disc increasing aggrecan concentration and

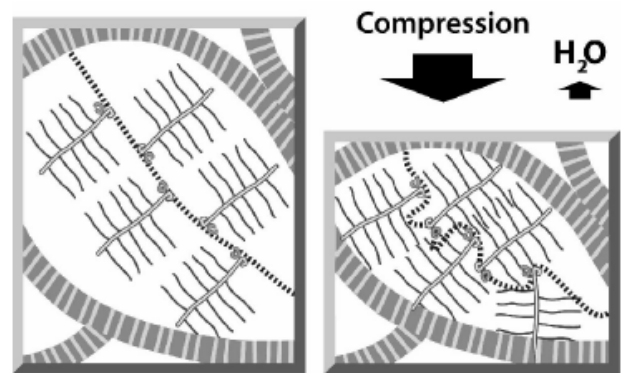


Figure 2. Hyaluronan (thin dashed line) substituted with aggrecan proteoglycans surrounded by type II collagen (thick striped line) in the NP. Aggrecan is the central protein (open line) displaying many GAG side chains (solid lines). As compressive forces are applied to the spine, water is forced from the disc.

resisting further compression. Conditions which lower proteoglycan concentration are pathological to disc function.

ECM of the gel-like nucleus is a unique combination of Type I and II collagen, proteoglycans such as aggrecan, and the glycosaminoglycans (GAGs) chondroitin and keratin sulfate surrounded by a fluid bath^{31,45}. Aggrecan contributes 5-15% of the wet tissue weight and consists of a large core protein (>250kDa) which is highly glycosylated by ~100 chondroitin sulfate chains and a lesser amount of keratin sulfate chains. Each of these GAGs carry a high fixed negative charge due to their sulfation, attracting counter ions. Their high local concentration of ions alters the osmotic balance and draws in water from the surrounding area¹⁹. It is known that the NP contains even greater amounts of aggrecan than cartilage, though it is degraded faster, since a lower proportion of the aggrecan is bound to hyaluronan. Roughly 95% of disc ECM is collagen, proteoglycans and water. The concentration of collagen decreases towards the center of the disc. As cells die by apoptosis in the NP, they are unable to continue producing large quantities of matrix molecules, resulting in a dramatic loss of proteoglycans and shift in balance between type I and II collagen. The major collagen of the inner disc, type II collagen, is heavily down regulated while type I collagen that is not usually present in significant quantities becomes over expressed. The high density of negatively charged glycosaminoglycans, collagens and noncollagenous proteins allows the NP to swell with water, maintain the appropriate interstitial pressure and disc height while providing load support⁴⁴. A loss of these proteoglycans will cause a reduction in water and hence disc height causing stiffness to increase. These processes occurs naturally during natural aging and are some of the first changes to be seen in disc degeneration⁷. It is clear that IVD degeneration is closely associated with pathological changes in the structure and biochemistry of the ECM of the NP.

Nitric oxide (NO) is free radical and highly permeable gaseous cellular messenger produced by the enzyme nitric oxide synthase (NOS). NO is a potent inflammatory mediator involved in the biochemical pathways of lumbar disc herniation²⁷, rheumatoid arthritis⁵¹, and osteoarthritis¹. It plays an important role in vasoregulation and neurotransmission and is implicated in neuropathic pain. As a vasodilator, this small molecule functions to maintain blood pressure and increase vascular permeability. In situ hybridization analysis

of herniated IVD found several NOS isoforms present in disc tissue- particularly the inducible and endothelial types clustered in the small rounded cells and fibroblasts of inflammatory granulation tissue²⁰. In addition, herniated lumbar disc specimens in culture have been found to produce increased levels of matrix metalloproteinases (MMPs), interleukin-1 (IL-1), and NO compared to control²⁶. MMPs are enzymes known to be continually secreted by chondrocytes in order to degrade ECM. IL-1 is a catabolic cytokine which can inhibit the formation of matrix and promote the production of MMPs¹⁸. Natural matrix degradation occurs when these proteases act on the ECM, and in healthy discs ECM is replenished by newly-synthesized materials. These biomolecules are all known to play an important role in articular cartilage degeneration and inflammation; however their exact biochemical pathway in the disc degeneration process is not precisely defined.

In culture, herniated disc cells have been shown to make increased amounts of NO which biochemically affect the spinal nerve root. More recently, the inducible form of nitric oxide synthase (iNOS) was found in nerve roots exposed to nucleus pulposus, but not animals with sham surgery. This up regulation of iNOS is in contrast to the neuronal and endothelial isoforms which are present in both NP-exposed nerve roots and control. These findings show that only the inducible isoform of nitric oxide synthase is up-regulated in nerve roots exposed to NP. Importantly, no iNOS activity was found in healthy NP tissue alone, indicating that iNOS activation is only induced after exposure to NP which occurs during herniation⁸.

One of the most direct effects of NO on the IVD is its inhibitory effect on proteoglycan and collagen synthesis. An early study showed that articular chondrocytes grown in alginate beads strongly up-regulated expression of iNOS and produced large amounts of NO in response to cytokine IL-1²¹. Further studies found that exposure of cartilage cultures to IL-1 induced a major increase in NO synthesis accompanied by a 60-80% drop in GAG synthesis. Addition of L-NMA, a competitive inhibitor of NOS, significantly reduced the production of NO and relieved the suppression of GAG synthesis⁴⁸. This was independently verified by Liu et al. who showed that NO suppressed proteoglycan synthesis in the IVD, and that an increase in hydrostatic pressure could also affect these results³⁰. Therefore any treatment for IVD degeneration should account for the effect of NO on cells and their newly synthesized matrix.

Importantly, the local oxygen content has an effect on NP cells during differentiation in vitro. Tissue hypoxia is considered a critical factor for maintaining NP phenotype during cell culture and differentiation. Due to the limited vascular supply of the disc, cells of the NP remain in hypoxia in vivo. Therefore it is thought that the optimal cells for treatment should also be exposed to hypoxia prior to implantation as a method of preconditioning and differentiation⁴⁰. Since hypoxia up-regulates the expression of matrix genes, it is possible that the presence of NO could induce vasodilation increasing the local oxygen content and preventing progenitor cells from obtaining the correct phenotype.

Current operative treatments for patients with lumbar disc herniation include open discectomy, laminectomy and spinal fusion. Once a degenerated disc has undergone prolapse it may begin to cause pain by allowing NP tissue to enter the spinal canal and interact with the nerve root. Therefore surgical procedures aim to alleviate this pressure and eliminate pain in various ways. However, none of these methods restore disc height or its original load bearing capacity. While lumbar discectomy is a well-established procedure, it may result in disc space narrowing which can result in discogenic pain or cause overloading in other structures. Laminectomy requires removal of a portion of the vertebrae, and both of these procedures are used to achieve only indirect decompression of the nerve root. Success can be variable, and is highly dependent on the skill of the surgeon.

Since surgical treatments are designed primarily to relieve pain instead of actually treating or preventing further disease progression, there is a serious need for new therapies. Biological repair of degenerated IVDs using cell transplantation therapy is viewed as a promising method to hinder disease progression by replacing or supplementing cells of the NP. The tissue engineering approach suggests autologous cells could be extracted from the patient, cultured in vitro and prepared with a scaffold for minimally invasive implantation. In this proposal, we outline a method for culturing and differentiating mesenchymal stem cells into the nucleus pulposus-like phenotype which can produce proteoglycans for ECM matrix repair. In addition, these cells can be encapsulated in a curable polymer which only gels in situ.

C. RESEARCH DESIGN AND METHODS

Human Mesenchymal Stem Cell Differentiation

Hypothesis 1: Human mesenchymal stem cells isolated from the femur and grown in alginate gels differentiate into nucleus pulposus-like cells when exposed to TGF- β 1 under hypoxia.

Normal, healthy adult IVD is made of nearly cell-free connective tissue. Compared to the large proportion of ECM in the disc, there are only a very small number of cells dispersed throughout the region comprising only 1% of disc volume⁴¹. When these cells die via apoptosis as happens during aging, there is little in way of replacement cells to repopulate a degenerate nucleus. Because of the disc's limited intrinsic capacity for regeneration, there is a rapidly growing body of work which proposes the use of cell-based therapies to initiate disc repair⁴². The current question being explored questions what cell type is best to use for implantation into the deserted NP. The primary cells being considered are 1) native adult differentiated NP cells and 2) mesenchymal stem cells (MSCs). Studies have found that implantation of both cell types can arrest degeneration or partially reverse it. In general, MSCs appear to have several advantages over existing chondrocytes-like NP cells. The primary advantage is that MSCs are readily available from many tissues such as adipose tissue or bone marrow, while there are a limited amount of NP cells available from the patient's IVD⁴⁰. Therefore the major limiting factor of autologous disc cell transplantation in the clinic is obtaining sufficient numbers of target cells from IVD tissue. Obtaining NP cells from a patient requires opening the AF and causing unnecessary damage to the annulus. The very low cell density in degenerated discs would complicate the process of securing ample cells for successful in vitro culture, and removing cells from a healthy disc increases the risk of inflicting donor site morbidity and inducing degeneration in neighboring discs. In addition degenerate NP cells in culture appear senescent and show a decreased rate of matrix synthesis and

increased production of MMPs and aggrecanases³⁶. Therefore we propose a MSC based tissue engineering approach.

The primary question regarding transplantation of MSCs is whether one should implant undifferentiated MSCs or preconditioned MSCs which appear to be NP-like. It has been shown that MSC differentiation in vivo depends primarily on the environment in which they are placed. There are few published studies which have applied unconditioned MSCs directly to the degenerated NP²². Hohaus et al. were able to successfully extract adipose derived cells, combine them with a hyaluronic acid carrier and implant them in the canine IVD via fluoroscopy²³. Disc cells up-regulated gene and protein expression of aggrecan, type 1 and 2 collagen demonstrating some improvements in the ECM. In vitro experiments of NP and MSC co-cultures have shown that cell-cell contacts between MSCs and normal human NP cells produce MSCs with NP-like phenotype demonstrated by increased expression of collagen and aggrecan genes³⁷. However, there is no way of measuring the phenotypical outcome of implanted MSCs without in vivo experimentation. Currently, the majority of work is focusing on various strategies of conditioning MSCs to produce optimal ECM components^{38,43}.

NP cells and articular chondrocytes share many phenotypic markers such as transcription factor SOX-9, aggrecan and type 2 collagen. Both NP cells and chondrocytes tend to revert to the undifferentiated state when cultured in monolayer. Cell morphology becomes more fibroblastic and spindle-shaped, type II collagen expression decreases and type I increases. This has been overcome by the use of 3-dimensional matrices for cell culture that have been well established. Gels such as sodium alginate can maintain chondrocytic morphology, and the addition of TGF- β 1 improves differentiation towards the NP phenotype. Risbud et al. have successfully used this system in conjunction with a hypoxic environment to differentiate MSCs into NP-like cells. The use of sodium alginate 3D matrices to maintain chondrocyte morphology and phenotype has been in practice for over 25 years⁶. This method will be used to culture, differentiate and expand cells before seeding them into an injectable carrier for implantation. Many of the gels used for differentiation, such as alginate, are not appropriate for tissue engineering due to their inability to withstand mechanical load³⁶. The following cell culture approach uses a modified procedure from Risbud et al. which will be applied to human MSCs.

Human Primary Mesenchymal Stem Cell Isolation and Culture. Human mesenchymal stem cells will be obtained from the bone marrow of the proximal femur from samples removed during hip replacement surgery. Only patients who voluntarily give informed consent and are approved by the local Ethical Committee for use in research will be selected³⁵. Mononucleated cells will be separated from the tissue using a Hitopaque-1077 (sigma) density gradient. Cells will be placed in Dulbecco Modified Eagles Medium (DMEM), 10% fetal bovine serum (FBS), antibiotics (50µg/ml streptomycin, 50 IU penicillin/ml) supplemented with heparin (10,000 U/ml). The marrow suspension is transferred to a 15 ml tube and spun at 2000 rpm for 5 minutes to sediment cells and resuspended in the same culture medium in a flask. After 24 hours later, nonadherent cells are removed by washing with PBS and media changes. The adherent cells should form colonies and expand in monolayer culture. After confluence, the cells will be trypsinized with 0.25% trypsin and replated. All cultures will be kept at 5% CO₂ at 37°C and subcultured twice to allow for cell expansion over the course of 7 days.

In Vitro Differentiation of Human Mesenchymal Stem Cells in Alginate Beads. Mesenchymal stem cells suspended in culture medium will be counted and resuspended in a solution of 1.2% low viscosity sodium alginate (Pronova, Norway) in 0.9% NaCl at a concentration of 2×10^6 cells/ml. The alginate-cell suspension will be expelled through a 26-gauge needle into a solution of 100mM CaCl₂, causing instantaneous formation of semi-solid microspheric beads. The beads are cured for 10 minutes in the CaCl₂ solution. To induce differentiation, the cultures are maintained in differentiating medium containing DMEM supplemented with 10ng/ml transforming growth factor β 1 (TGF- β 1; R&D Systems, MN), 100 nM dexamethasone, 50 µg/ml ascorbate 2-phosphate, 100 µg/ml sodium pyruvate, 40 µg/ml proline, and ITS-plus (Collaborative Biomedical Products, MA). Alginate beads will be cultured in a hypoxia workstation (In vivo2, Ruskin, UK) at 2% oxygen, 5% CO₂ and 93% N₂. Two additional groups of cells will be used as controls for this experiment: (1) human MSC in alginate beads in differentiating medium at 20% oxygen (normoxia) and (2) human MSC in alginate beads without any differentiating media supplements also at normoxia.

It is not possible to answer with certainty whether newly differentiated cells have achieved a particular differentiated phenotype; however there are several methods to examine if these cells are similar to the NP-like phenotype. In this case, it is considered standard practice to analyze the gene expression, protein expression and proteoglycan synthesis of the MSCs in order to assess if they have differentiated into the NP-like phenotype under hypoxia or normoxia in the presence of TGF- β 1.

Sub-hypothesis 1(a): Human mesenchymal stem cells isolated from the femur and grown in alginate gels will show increased gene expression of type II collagen, aggrecan and matrix metalloproteinase-2 when exposed to TGF- β 1 under hypoxia.

Reverse Transcription Polymerase Chain Reaction Analysis. After 7 days in culture, cells are released from alginate beads by Na-citrate and EDTA treatment (in 0.15M NaCl). Retrieved cells are washed in PBS to prepare for RNA isolation. Total RNA will be extracted from MSCs grown in hypoxia, normoxia and untreated cultures using Trizol (Invitrogen, CA) according to the manufacturer. RNA will be reverse transcribed into

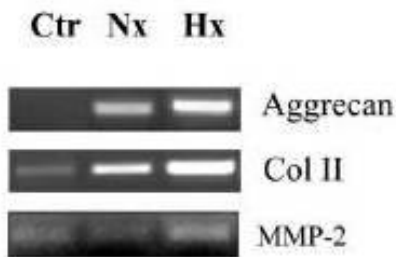


Figure 3. Reverse transcription PCR analysis of hypoxia responsive genes expressed by human MSCs. After 1 week in culture cells increased their expression of aggrecan, collagen II and MMP-2 under hypoxia in the presence of TGF- β 1.

cDNA using Superscript II RT (Invitrogen) and oligo primers. Primers for aggrecan and collagen type II from human bone marrow MSCs were described by Richardson et al. ³⁵ and primers for human matrix metalloprotein-2 (MMP-2) will be based on sequence data found in GeneBank. For polymerase chain reaction (PCR) 1 μ l of cDNA template was used for each reaction and sequences were amplified using Taq DNA polymerase (Invitrogen). Primers will be synthesized by the Stony Brook DNA Sequencing Facility. The PCR product will be visualized using a digital imaging station.

Experimental Design, Data Analysis and Statistics. Risbud et al. reported a significant increase in aggrecan, collagen type II, and MMP-2 gene expression; however no values were given for the groups³⁸. Independent analysis of the data supplied in their publication using ImageJ found that aggrecan and collagen were expressed ~1.5-1.9 times higher in cells exposed to hypoxia over normoxia, meaning these cells were probably closer to the actual ideal NP phenotype. Since this experiment was only repeated once, the data is not normally distributed and would require non-parametric tests. In our experiment, we propose reproducing multiple independent cell culture experiments (n>6) from the same marrow in order to use parametric testing methods. Cells will be analyzed independently for gene expression from each experiment. Assuming a 1.5-fold difference in means and 10% standard deviation between Nx and Hx MSC exposed gene expression, we require at least ten unique experiments to produce a well powered study ($\alpha=0.05$). Results between groups will be analyzed with ANOVA followed by the Bonferoni post-hoc test on SPSS.

Hypothesis 1(a) will be true if MSCs exposed to hypoxia and TGF- β 1 show a significant increase in gene expression under hypoxia (Hx) as per Risbud et al. Cells exposed to normoxia and TGF- β 1 should show lower levels of gene expression (Nx). Cells without any differentiating medium (Ctr) should show very low levels of collagen II and MMP-2, and no aggrecan gene expression.

Sub-hypothesis 1(b): Human mesenchymal stem cells isolated from the femur and grown in alginate gels will show increased protein expression of transcription factor HIF and matrix metalloproteinase-2 when exposed to TGF- β 1 under hypoxia.

MMP-2 and HIF-1 α are both known to be selective markers for the differentiated NP phenotype. Disc cells harvested directly from the spine express these proteins, and they can be used as specific markers to assess the NP phenotype^{38,39}. Both native NP cells and those cultured in vivo show elevated levels of HIF-1 α protein expression. HIF is a transcription factor which controls the expression of MMP-2. Up-regulation of these proteins are a characteristic signature of NP cells³³.

Western Blot Analysis. Hypoxic and normoxic cells will be washed with ice-cold PBS and harvested in 100 μ l of Mammalian Protein Extraction Reagent buffer (MPER, IL) containing NaF (5 mM) and Na₃VO₄ (200 μ M). Lysates will be centrifuged for 2 minutes at 14,000g and separated by SDS-PAGE with a 12% gradient. Proteins are transferred by electroblotting to nitrocellulose membranes (Bio-Rad). Membranes will be blocked with 5% dry milk and incubated overnight with the desired antibody. After washing and incubation with HRP-conjugated secondary antibodies, immunolabeling will be detected with chemiluminescence (Amersham Biosciences).

Experimental Design, Data Analysis and Statistics. Risbud et al. report a significant increase in HIF-1 α and MMP-2 protein expression; however no values are given for these groups³⁸. Independent analysis of the data

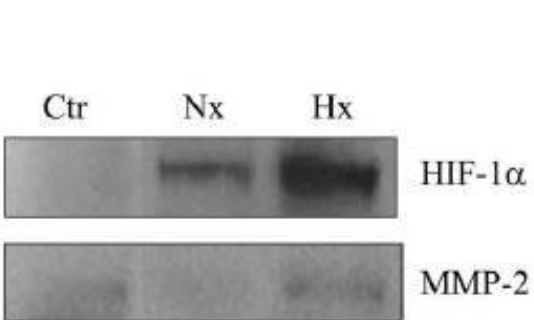


Figure 4. Western blot analysis of hypoxia responsive gene products expressed by MSC. Cells exposed to hypoxia had significantly increased HIF-1 α protein expression. MMP-2 protein expression moderately increased compared to control.

supplied in their publication using ImageJ found that HIF-1 α was expressed 30 times more in cells exposed to hypoxia with TGF- β 1 over the control without TGF- β 1, implying these cells had differentiated into the NP-like phenotype. ImageJ analysis of this gel also shows that the cells exposed to normoxia produced less MMP-protein than those under hypoxia. Hypoxic and normoxic cells both produced roughly twice as much MMP-2 compared to the control, indicating were differentiating. Therefore, both of these markers will be used in our experiment to evaluate differentiation of MSCs exposed to TGF- β 1. We propose repeating the experiment

with distinct cell cultures from the same marrow. Cells will be analyzed independently for protein expression from each experiment. Assuming a 30x difference in means and 10% standard deviation for HIF-1 α at least two repetitions (n=2) from unique cell culture experiments would be necessary to produce well a powered study ($\alpha=0.05$). Assuming a 200% difference in MMP expression between HX and Ctr, also only two samples would

be needed for a well powered study ($\alpha=0.05$). The non-parametric data will be displayed as a box plot. Results will be analyzed using the Kruskal Wallis one-way analysis of variance by ranks on SPSS.

Sub-hypothesis 1(c): Human mesenchymal stem cells isolated from the femur and grown in alginate gels will show increased proteoglycan synthesis over time when exposed to TGF- β 1 under hypoxia.

Tsai et al. found that primary NP cells cultured in sodium alginate beads in the presence of TGF- β 1 (10ng/ml) for one week produced significantly more (50%) proteoglycans than undifferentiated cells grown in monolayer using dimethylmethylene blue (DMMB) assay⁴⁹. In monolayer alone, primary disc cells produced 400% more GAGs when exposed to TGF- β 1. Compared to cells not exposed to TGF- β 1, primary disc cells cultured in pellets produced significantly more (20%) proteoglycans as per the DMMB assay with roughly 2-8% standard deviation²⁹. Bai et al. found that when bone marrow MSCs were cultured in pellets and differentiated into chondrocytes, GAG synthesis increased 500% when exposed to 30ng/ml TGF- β 1 compared to control⁴. Clearly, there is a relevant increase in GAG synthesis in MSCS exposed to TGF- β 1. However, none of these experiments were done under hypoxic conditions, though Risbud et al. found GAG synthesis to qualitatively increase via cell staining. Ibusuki et al. found that primary chondrocytes cultures in PNIPAAm-gelatin produced over ten times as much GAGs at the 12-week time point compared to the 1-week time point²⁴. We project that MSCs exposed to TGF- β 1 cultured in sodium alginate beads under hypoxia will show a significant increase (50-500%) in GAG synthesis over time, compared to the normoxic and control conditions.

Dimethylmethylene Blue (DMMB) Assay. The proteoglycan content will be measured from three groups of cells: (1) human MSCs grown in alginate gels under hypoxia in the presence of TGF- β 1; (2) human MSCs grown in alginate gels under normoxia in the presence of TGF- β 1; and (3) human MSCs grown in alginate gels under normoxia. The proteoglycan content of the cells in culture will be measured as sulfated GAGs by

colorimetric assay with 1-9-DMMB (Blyscan, Biocolor Ltd. UK) with chondroitin-4-sulfate as a standard (0-2 $\mu\text{g/ml}$) according to manufacturer's instructions. Briefly, cells are digested by papain, and GAGs are precipitated and stained with DMMB which is quantified by measuring UV absorbance at 656 nm. Data will be calculated in $\mu\text{g/ml}$ and expressed as an arbitrary ratio.

Experimental Design, Data Analysis and Statistics. The three groups (Hx, Nx, Ctr) will each be analyzed for GAG content at the 1,2,4,6,8,10 and 12-week time points by DMMB assay. This experiment should produce large expected differences in GAG content between the differentiated (Hx) and undifferentiated groups (Ctr) which should increase over time. However, previous quantitative differences in GAG content between the normoxia and hypoxia exposed groups is unknown - though qualitative staining experiments show promising results. Assuming a 50% difference between Hx and Ctr groups with a 2-8% standard deviation, a well powered study would require less than 6 unique cell culture samples (each sample will be a separate experiments). In order use parametric data analysis, the assay will be run 9 times in parallel. This will not significantly increase the cost or length of the experiment. Therefore, at each time point there will be 9 data points per group. GAG content will be expressed as mean $\mu\text{g/ml}$ with standard deviation. Results between Hx, Nx, and Ctr groups at a specific time point will be analyzed with ANOVA followed by the Tukey post-hoc test on SPSS. Results from each group between time points will be analyzed with ANOVA followed by the Bonferoni post-hoc test on SPSS, as these should show a more pronounced difference.

Thermo-responsive Scaffold Engineering

The most commonly proposed minimally invasive treatment for IVD degeneration is to inject cells capable of synthesizing appropriate ECM into the NP. However, a major inadequacy of this approach is that the high IVD pressure and aqueous nature of a cell suspension cause the payload to be evacuated. Therefore, injectable carriers capable of effectively delivering and mechanically retaining cells in the degenerative NP space are

necessary. A promising method for accomplishing this is to use self-assembling hydrogels as a scaffold to deliver cells in solution via injection¹⁰. The scaffold should solidify after injection and provide a suitable mechanical support for cells to attach and survive. One problem in this treatment is the immediate loss of cells due to back-flow through the injection path, which could be overcome with gels of higher viscosity and stiffness. Crevensten et al. show that MSCs embedded in a 15% hyaluronan gel carrier implanted in the NP caused rat IVD disc height to increase, implying increased matrix synthesis¹⁵. Sakai et al. found that MSCs from rabbit bone marrow embedded in an atelocollagen gel injected via 27-gauge needle produced a 91% increase in disc height⁴³. However, due to the long healing time (7-14 days) of needle puncture to the AF and low viscosity of the carrier, cells escaped the delivery site. This may be prevented by an in situ curable polymer scaffold¹⁵.

Scaffolds used to deliver cells should be biocompatible with the spine and provide initial mechanical stability while ensuring a homogeneous cell distribution post-implantation. There are many scaffolding materials to choose from including gels composed of poly(lactic acid), hyaluronic acid, collagen, and chitosan. Each of these materials provide a three-dimensional scaffold for cell proliferation and can propagate a stable NP-like phenotype³. These materials are designed to provide only temporary mechanical support due to degradation over time.

Other types of hydrogel materials could be designed to polymerize in situ for “custom-made” implantable therapeutics that can fill the injury site. This is commonly accomplished via gelation (cross-linking) such as photopolymerization or chemical crosslinking in vivo which can lead to complications from unreacted monomers or excess reactants. Recently thermo-responsive hydrogels which form in situ due to local temperature stimuli have been used for biomedical applications. Thermal sensitive hydrogels can gel when injected into the body where temperature increases from ambient to physiological levels. This stimulus produces a phase transition called the sol-gel transition. The temperature at which the gel solidifies is known as the lower critical solution temperature (LCST). Below the LCST a polymer is soluble. As one approaches the LCST, the solution becomes more hydrophobic and insoluble resulting in gel formation²⁸.

LCST Characterization

Poly(N-isopropyl acrylamide), or PNIPAAm is a thermo-responsive polymer which has several important properties for tissue engineering: (1) its phase transition occurs between the ambient and physiological temperatures (29-32°C) and (2) its material properties can be altered by copolymerization with other monomers¹⁴. For example, PNIPAAm has been grafted to chitosan and used to encapsulate human MSCs which were injected in the rabbit and found to stimulate cartilage growth¹¹. PNIPAAm-PEG copolymer blends have been used to successfully culture MSCs. In addition, the in situ formation should not result in excess reactants since it is not biodegradable, and can mold to the site of injection.

Therefore, we are proposing the use of PNIPAAm-PEG thermo-responsive scaffolds for in situ tissue engineering. Here we propose testing and comparing some of the physical parameters between naked PNIPAAm-PEG and NP-like cell-loaded scaffolds.

Hypothesis 2: Naked and cell-loaded PNIPAAm-PEG will have different lower critical solution temperature (LCST) values.

Polymer Synthesis. *Methacrylation of PEG:* Poly(ethylene glycol) dimethylacrylate (PEGDM, ME=8000) will be synthesized by reacting poly(ethylene glycol) (PEG, MW=8000) diol with excess methylacryloyl chloride under anhydrous conditions as described by Bryant⁹. *Copolymer synthesis:* PNIPAAm-PEG copolymer will be synthesized as per Vernengo et al⁵⁰. Briefly, NIPAAm monomer (Sigma) will be mixed with PEGDM 8000 at a 1600:1 monomer ratio in methanol, and polymerization will be thermally initiated by azobisisobutyronitrile 98% (Sigma) at 65°C and allowed to equilibrate for 48 hours. Excess methanol will be removed through evaporation. The copolymer is ground and purified by washing with hexane and dried. Solutions containing 15% (w/v) polymer will be prepared in deionized water.

Three-dimensional cell culture in PNIPAAm-PEG gels. After 7 days in culture, differentiated NP-like cells will be released from alginate beads by Na-citrate and EDTA treatment (in 0.15M NaCl). PNIPAAm-PEG will be dissolved in the culture medium to give a final concentration of 10% PNIPAAm-PEG. The cells will be collected from the monolayer by centrifugation (300 x g, 4°C, 5 min), washed 3 times in culture medium at room temperature and diluted with culture medium at room temperature to 3×10^7 cells ml⁻¹ (2x concentration). This suspension will next be mixed with an equal volume of 20% (w/v) PNIPAAm-PEG solution at 4°C to produce a liquid thermo-responsive scaffold containing 1.5×10^7 cells ml⁻¹ in 10% (w/v) PNIPAAm-PEG solution²⁴. The mixture will be placed in a 12-well cell culture dish or a mold and incubated at 37°C to be cured for further studies. Some mixture will be placed aside before gelation for LCST analysis.

Lower Critical Solution Temperature Determination. The LCST of the PNIPAAm-PEG solutions will be found using a differential scanning calorimeter (DSC) from TA Instruments (Model #2010, Wilmington, DE). 10mg of cell-loaded and naked PNIPAAm-PEG samples will be placed in an anodized aluminum pan which is hermetically sealed and heated from 10 to 45°C at 10°C min⁻¹. The onset temperature of the endothermic phase transition on the DSC thermograph will be taken as the LCST.

Experimental Design, Data Analysis and Statistics. Multiple samples of freshly made PNIPAAm-PEG will be prepared loaded with and without cells. Our null-hypothesis is that there will be no difference between the LCSTs of naked and cell-loaded scaffolds. We will set this threshold to 10%, meaning that the null-hypothesis is true if there are changes in LCST <10%. Assuming a 10% difference in means between the lower critical solution temperature (~3°C) of naked and cell-loaded scaffold with a 5% standard deviation, 5 samples would be required for a well powered study ($\alpha=0.05$). To use parametric data analysis, nine samples of each group will be used, justified by the minimal increase in cost and time. The expected value for both cell loaded and naked samples is between 31 and 32 °C. The LCST of each group will be expressed as mean and standard deviation

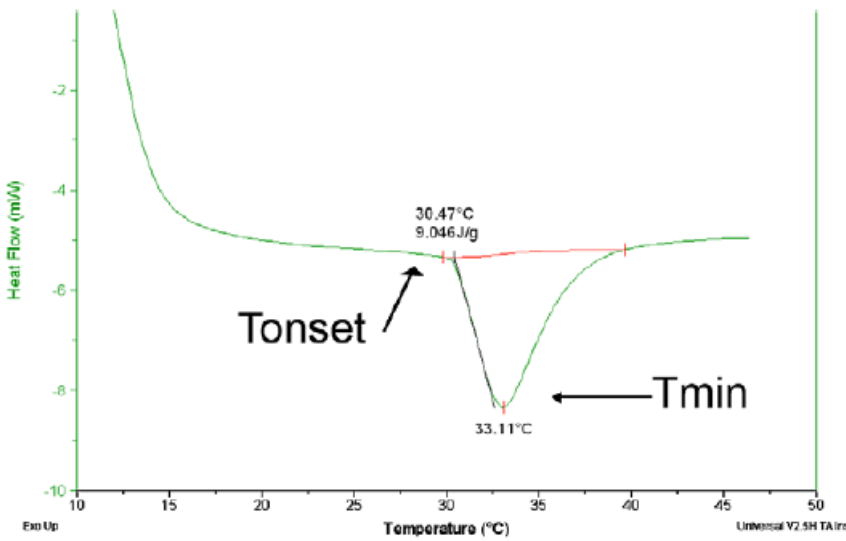


Figure 5. A typical PNIPAAm-PEG DSC thermogram¹⁴.

PEG. T_{onset} occurs at the beginning of the endotherm and indicates the initial phase change from liquid to solid. T_{min} is the minimum transition temperature that will occur, while T_{onset} is the LCST, as this is the temperature the solid gel begins to form.

Mechanical Properties

When designing a nucleus pulposus implant, there are many mechanical factors to consider due to the constant compressive forces being applied to the spine. The ideal implant should partially mimic the function of a healthy disc, be biologically compatible and fatigue resistant. The average human takes 2 million strides per year and if an implant is to survive 40 years, it should withstand approximately 100 million load cycles. The elastic modulus of the implant material should show resistance to wear and prevent formation of particulate byproducts. The modulus should also be compatible to the vertebral end plates, as implants with modular mismatches result in pathological load distributions that can deteriorate the endplate. To obtain a modulus match, the NP replacement biomechanics should resemble native healthy tissue. Polymers are constantly being

($T^{\circ}C$). To test for differences between the naked and cell-loaded groups, an unpaired 2-tailed t-test will be used to find significance. A p-value > 0.05 would imply there were no significant differences between the naked and cell-loaded groups and that the null-hypothesis was true.

A typical DSC thermogram is shown in figure 5 below demonstrating heat flow (mW) over temperature ($^{\circ}C$) for PNIPAAm-

engineered to replicate viscoelastic properties of the IVD, and NP stiffness is viewed as a critical factor for preventing implant extrusion¹⁷.

Hydrogels have the capability to absorb water in the presence of fluid and release water when loaded, much like a healthy NP. In the future this parameter can be engineered to most effectively mimic tissue by modifying the hydrogel's chemical composition. Methods of measuring the biomechanical property of tissue engineered gels include compressive and stress relation studies.

Hypothesis 3: NP-like cell-loaded PNIPAAm-PEG(8000) hydrogels cured in media with serum will have a lower compressive modulus compared to healthy nucleus pulposus tissue in serum at 37°C.

Sample Preparation. Samples will include both cell-loaded PNIPAAm-PEG cured hydrogels and human NP tissue both tested in media with serum at 37°C. Human tissue samples will be prepared as per Cloyd et al.¹². In brief, human lumbar spines will be obtained from IRB approved tissue sources and the IVD will be removed by dissection and assessed for degeneration by morphological grading. 12.7mm plugs will be removed using a Miltex dermal biopsy punch (Fischer Scientific, PA) from the NP and cut in uniform 5mm slices via microtome (Model S42400, Leica Microsystems, Germany). A smaller 7.1mm sample will be removed using a Miltrex dermal biopsy punch and stored at -20°C until testing. Samples of cured hydrogels containing 1.5×10^7 cells ml⁻¹ in 10% PNIPAAm-PEG will be retrieved from a 7.1 mm diameter mold for compressive studies.

Compressive Studies. Unconfined uniaxial compression tests will be performed on gel and human samples immersed in differentiating medium at 37°C. The compressive modulus of all samples will be evaluated using an Instron mechanical testing system (Model 4200, Park Ridge, IL) and compressed at a strain rate of 100% min⁻¹ while immersed in the warm aqueous medium. The Instron will feature a clear acrylate tank used to house the solution and will be warmed by an electric immersion heating coil (Nordic 559, Norpro). Directly beneath the tank will be a calibration grid aligned with a mounted digital camera to carefully measure the lateral strain

before and after compression as per Cloyd et al.

Load and displacement data will be recorded with

Bluehill software and converted to stress and strain

values. The compressive modulus will be calculated

as the slope of the linear region of the stress-strain

curve (from 0% to 15% strain) as per Vernengo et

| Immersion media | Compressive modulus (kPa) | LCST (°C) |
|------------------|---------------------------|-----------|
| PBS | 2.2 ± 0.6 | 31 ± 0.5 |
| Media | 3.8 ± 0.7 | 31 ± 1.3 |
| Media with serum | 1.4 ± 0.7 | 30 ± 1.8 |

Table 1. The compressive modulus and LCST of naked PNIPAAm-PEG(8000) scaffolds in varying media conditions at 37°C.

al.⁵⁰.

Experimental Design and Statistical Analysis. Vernengo et al. show that the stiffness of a PNIPAAm-PEG hydrogel is strongly dependent on the PEG content, PEG molecular weight, and time spent curing. In previous experiments measuring the compressive modulus of various PNIPAAm-PEG mixtures, the modulus significantly increased over time until day 30 at which point it stabilized. Importantly, the compressive modulus of lower MW PEGs was significantly higher and is thought to be caused by lower affinity for water. Higher MW PEGs attract more water, and the water trapped in hydrogels is responsible for increasing its flexibility. Here the reported modulus values range from 20-100 kPa after 7 days depending on the PEG conditions⁵⁰. These experiments are in contrast with different findings from the same lab which report much lower values for the compressive modulus. By using a higher MW PEG (8000) lower PEG concentration (1:1600), and curing the samples in media with serum, Comolli et al. found a compressive modulus of ~2-4 kPa with a 0.7 kPa standard deviation¹⁴. Cloyd et al. report a value of ~2kPa for the compressive modulus of healthy NP tissue. It is hypothesized that the inclusion of cells in the polymer mixture will result in a lower compressive modulus than the modulus of the polymer mixture only. The cell-loaded PNIPAAm-PEG compressive modulus may be closer to 1.5 kPa – the modular value of PNIPAAm-PEG(8000) in media with serum.

Assuming a 30% difference between the modulus of healthy NP tissue and cell-loaded scaffolds with a 20% standard deviation, one would required 8 samples to produce a well powered study ($\alpha=0.05$). The results will be expressed as mean and standard deviation in kPa. The values for the modulus are calculated from the stress-

strain diagrams at 15% strain. Results between the two groups will be compared using an unpaired two-tailed t-test on SPSS.

Effect of Nitric Oxide on Differentiated NP-like Cells Embedded in Cured PNIPAAm-PEG Scaffolds

Interleukin-1 (IL-1) is known to up-regulate the expression of iNOS leading to the synthesis of large amounts of NO in cells. IL-1 is found in arthritic joints and suppresses the generation of ECM in human chondrocytes. Cartilage cultures not exposed to IL-1 used as a control produce very little NO and can synthesize normal levels of GAGs. Identical cultures exposed to 10 ng ml⁻¹ of IL-1 β produced approximately 6 times as much NO and 75% less GAGs^{21,48}. Human disc samples have shown a direct increase in nitrite production as a function of age corresponding to lowered proteoglycan synthesis. In addition, since patients with degenerative lumbar disease are known to have elevated levels of NO in the CSF⁵¹, it is critical to understand the effects of this molecule on any therapeutic cell implant.

In this study we have elected to observe the effects of NO on human MSC derived NP-like cells embedded in a PNIPAAm-PEG tissue engineering scaffold. In addition, PNIPAAm-PEG(8000) scaffolds have been found capable of releasing bioactive agents such as proteins¹⁴. Here we propose the use of a PNIPAAm-PEG as a joint cell and drug delivery device capable of simultaneously delivering NP-like cells and NOS inhibitors via minimally invasive approach.

Several NOS inhibitors exist which allow investigators to probe the effects of NO on matrix degradation and gene expression. L-NMA (L-N-methyl-arginine) is one of the most commonly used non-specific NOS inhibitors³² and has successfully been used to prevent the production of NO and restore proteoglycan synthesis in IVD cells^{34,46,47}. Our goal is to assess if L-NMA is released from PNIPAAm-PEG scaffolds and determine its effect on the NO production and GAG synthesis of differentiated NP-like cells embedded in the scaffold. In addition, we will be able to observe the effect of IL-1 on NO production in both naked and scaffold embedded cells. To determine if L-NMA is released from the cell-loaded scaffold, we will measure the amount of nitrite produced from both cells exposed to the inhibitor and a control. We will also measure the amount of nitrite

produced by cells exposed to IL-1 and a control in order to understand if IL-1 can stimulate NO production in differentiated MSCs embedded in a PNIPAAm-PEG scaffold.

Hypothesis 4: Naked and PNIPAAm-PEG embedded NP-like cells will show an increase in nitrite content and suppression of GAG synthesis when exposed to IL-1 β . That presence of L-NMA blended into the scaffold will cause a decrease in nitrite content and restore GAG synthesis in PNIPAAm-PEG embedded NP-like cells.

Assembly of PNIPAAm-PEG Gels Containing NP-like Cells and L-NMA. Polymer scaffolds composed of 10% PNIPAAm-PEG solution containing 1.5×10^7 cells ml^{-1} will be made as previously described with the exception that some cultures will be treated with media containing 10 ng ml^{-1} of IL-1 β over the course of 7 days (+IL-1). The control group will not have any IL-1 β added to the media (-IL-1). To prepare scaffolds containing L-NMA, L-NMA (1.25 mM; Sigma, MI) will be added to the cell/polymer mixture before curing at 37°C. Both Taskiran and Stevens et al. show that 1.25 mM NMA is sufficient to significantly reduce NO content and restore proteoglycan synthesis.

Experimental Design and Statistical Analysis: The proteoglycan synthesis and nitrite content will be measured for six different groups. The six groups will be organized as shown in figure 5. Naked chondrocytes

| | Naked NP-like cells | NP cell loaded PNIPAAm-PEG scaffolds | NP cell loaded PNIPAAm-PEG scaffolds |
|--------|---------------------|--------------------------------------|--------------------------------------|
| + IL-1 | - NMA ↑ NO ↓ PG | - NMA ↑ NO ↓ PG | + NMA ↓ NO ↑ PG |
| - IL-1 | - NMA ↓ NO ↑ PG | - NMA ↓ NO ↑ PG | + NMA ↓ NO ↑ PG |

Figure 5. Schematic of experimental design and projected results. Cells will be exposed to IL-1 β in order to stimulate NO synthesis in naked cells and cells embedded in hydrogels. The presence of the NOS inhibitor L-NMA in the hydrogel should lower NO content and restore proteoglycan synthesis if it is successfully released.

exposed to 10 ng ml^{-1} of IL-1 β (+IL-1) were found to produce 6-times more nitrite than control (-IL-1) with ~16% standard deviation (nmol/mg)⁴⁷. Therefore to produce a well powered study, only two samples are required. Chondrocytes cultured in alginate beads showed a 65% reduction in GAG synthesis when exposed to 1 ng ml^{-1} of IL-1 β ²¹. Assuming a 10% standard deviation, only two samples are required to produce a well powered study. Since NP cells will be exposed to IL-1 β before being embedded in PNIPAAm-PEG gel.

Hauselmann et al. show that naked human chondrocytes exposed to 500 pg ml^{-1} of IL-1 β and 1 mM L-NMA (+IL-1/+NMA) demonstrate a ~3-fold increase in GAG synthesis compared to cells exposed only to IL-1 β (+IL-1/-NMA). Assuming a 10% standard deviation, only two samples are required to produce a well powered study. However, to use parametric testing methods we will produce nine unique culture experiments and measure the nitrite concentration using a Griess Assay as described by Stevens et al. and the DMMB assay as previously described to measure GAG content. Results for proteoglycan and nitrite content will be displayed as means and standard deviation. Results will be analyzed with ANOVA followed by the Tukey post-hoc test on SPSS to find significant differences between groups.

1. Abramson SB. Osteoarthritis and nitric oxide. *Osteoarthritis Cartilage* 2008;16 Suppl 2:S15-20.
2. Adams MA, Roughley PJ. What is intervertebral disc degeneration, and what causes it? *Spine* 2006;31:2151-61.
3. Anderson DG, Risbud MV, Shapiro IM, et al. Cell-based therapy for disc repair. *Spine J* 2005;5:297S-303S.
4. Bai X, Xiao Z, Pan Y, et al. Cartilage-derived morphogenetic protein-1 promotes the differentiation of mesenchymal stem cells into chondrocytes. *Biochem Biophys Res Commun* 2004;325:453-60.
5. Battie MC, Videman T. Lumbar disc degeneration: epidemiology and genetics. *J Bone Joint Surg Am* 2006;88 Suppl 2:3-9.
6. Benya PD, Shaffer JD. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell* 1982;30:215-24.
7. Bibby SR, Jones DA, Lee RB, et al. The pathophysiology of the intervertebral disc. *Joint Bone Spine* 2001;68:537-42.
8. Brisby H, Byrod G, Olmarker K, et al. Nitric oxide as a mediator of nucleus pulposus-induced effects on spinal nerve roots. *J Orthop Res* 2000;18:815-20.
9. Bryant SJ, Chowdhury TT, Lee DA, et al. Crosslinking density influences chondrocyte metabolism in dynamically loaded photocrosslinked poly(ethylene glycol) hydrogels. *Ann Biomed Eng* 2004;32:407-17.
10. Chan BP, Leong KW. Scaffolding in tissue engineering: general approaches and tissue-specific considerations. *Eur Spine J* 2008;17 Suppl 4:467-79.
11. Cho JH, Kim SH, Park KD, et al. Chondrogenic differentiation of human mesenchymal stem cells using a thermosensitive poly(N-isopropylacrylamide) and water-soluble chitosan copolymer. *Biomaterials* 2004;25:5743-51.
12. Cloyd JM, Malhotra NR, Weng L, et al. Material properties in unconfined compression of human nucleus pulposus, injectable hyaluronic acid-based hydrogels and tissue engineering scaffolds. *Eur Spine J* 2007;16:1892-8.

13. Colombini A, Lombardi G, Corsi MM, et al. Pathophysiology of the human intervertebral disc. *Int J Biochem Cell Biol* 2008;40:837-42.
14. Comolli N, Neuhuber B, Fischer I, et al. In vitro analysis of PNIPAAm-PEG, a novel, injectable scaffold for spinal cord repair. *Acta Biomater* 2009;5:1046-55.
15. Crevensten G, Walsh AJ, Ananthkrishnan D, et al. Intervertebral disc cell therapy for regeneration: mesenchymal stem cell implantation in rat intervertebral discs. *Ann Biomed Eng* 2004;32:430-4.
16. Gibson JN, Waddell G. Surgical interventions for lumbar disc prolapse. *Cochrane Database Syst Rev* 2007:CD001350.
17. Goins ML, Wimberley DW, Yuan PS, et al. Nucleus pulposus replacement: an emerging technology. *Spine J* 2005;5:317S-24S.
18. Hadjipavlou AG, Tzermiadianos MN, Bogduk N, et al. The pathophysiology of disc degeneration: a critical review. *J Bone Joint Surg Br* 2008;90:1261-70.
19. Hardingham TE, Fosang AJ. Proteoglycans: many forms and many functions. *FASEB J* 1992;6:861-70.
20. Hashizume H, Kawakami M, Nishi H, et al. Histochemical demonstration of nitric oxide in herniated lumbar discs. A clinical and animal model study. *Spine* 1997;22:1080-4.
21. Hauselmann HJ, Oppliger L, Michel BA, et al. Nitric oxide and proteoglycan biosynthesis by human articular chondrocytes in alginate culture. *FEBS Lett* 1994;352:361-4.
22. Ho G, Leung VY, Cheung KM, et al. Effect of severity of intervertebral disc injury on mesenchymal stem cell-based regeneration. *Connect Tissue Res* 2008;49:15-21.
23. Hohaus C, Ganey TM, Minkus Y, et al. Cell transplantation in lumbar spine disc degeneration disease. *Eur Spine J* 2008;17 Suppl 4:492-503.
24. Ibusuki S, Fujii Y, Iwamoto Y, et al. Tissue-engineered cartilage using an injectable and in situ gelable thermoresponsive gelatin: fabrication and in vitro performance. *Tissue Eng* 2003;9:371-84.
25. Kandel R, Roberts S, Urban JP. Tissue engineering and the intervertebral disc: the challenges. *Eur Spine J* 2008;17 Suppl 4:480-91.

26. Kang JD, Georgescu HI, McIntyre-Larkin L, et al. Herniated cervical intervertebral discs spontaneously produce matrix metalloproteinases, nitric oxide, interleukin-6, and prostaglandin E2. *Spine* 1995;20:2373-8.
27. Katsuno R, Hasegawa T, Iwashina T, et al. Age-related effects of cocultured rat nucleus pulposus cells and macrophages on nitric oxide production and cytokine imbalance. *Spine* 2008;33:845-9.
28. Klouda L, Mikos AG. Thermoresponsive hydrogels in biomedical applications. *Eur J Pharm Biopharm* 2008;68:34-45.
29. Lee YJ, Kong MH, Song KY, et al. The Relation Between Sox9, TGF-beta1, and Proteoglycan in Human Intervertebral Disc Cells. *J Korean Neurosurg Soc* 2008;43:149-54.
30. Liu GZ, Ishihara H, Osada R, et al. Nitric oxide mediates the change of proteoglycan synthesis in the human lumbar intervertebral disc in response to hydrostatic pressure. *Spine* 2001;26:134-41.
31. Nordin MaF, Victor Hirsch *Basic biomechanics of the musculoskeletal system*: Lippincott Williams & Wilkins, 2001.
32. Olken NM, Marletta MA. NG-methyl-L-arginine functions as an alternate substrate and mechanism-based inhibitor of nitric oxide synthase. *Biochemistry* 1993;32:9677-85.
33. Rajpurohit R, Risbud MV, Ducheyne P, et al. Phenotypic characteristics of the nucleus pulposus: expression of hypoxia inducing factor-1, glucose transporter-1 and MMP-2. *Cell Tissue Res* 2002;308:401-7.
34. Rannou F, Richette P, Benallaoua M, et al. Cyclic tensile stretch modulates proteoglycan production by intervertebral disc annulus fibrosus cells through production of nitrite oxide. *J Cell Biochem* 2003;90:148-57.
35. Richardson SM, Curran JM, Chen R, et al. The differentiation of bone marrow mesenchymal stem cells into chondrocyte-like cells on poly-L-lactic acid (PLLA) scaffolds. *Biomaterials* 2006;27:4069-78.
36. Richardson SM, Mobasher A, Freemont AJ, et al. Intervertebral disc biology, degeneration and novel tissue engineering and regenerative medicine therapies. *Histol Histopathol* 2007;22:1033-41.
37. Richardson SM, Walker RV, Parker S, et al. Intervertebral disc cell-mediated mesenchymal stem cell differentiation. *Stem Cells* 2006;24:707-16.

38. Risbud MV, Albert TJ, Guttapalli A, et al. Differentiation of mesenchymal stem cells towards a nucleus pulposus-like phenotype in vitro: implications for cell-based transplantation therapy. *Spine* 2004;29:2627-32.
39. Risbud MV, Izzo MW, Adams CS, et al. An organ culture system for the study of the nucleus pulposus: description of the system and evaluation of the cells. *Spine* 2003;28:2652-8; discussion 8-9.
40. Risbud MV, Shapiro IM, Vaccaro AR, et al. Stem cell regeneration of the nucleus pulposus. *Spine J* 2004;4:348S-53S.
41. Roberts S, Evans H, Trivedi J, et al. Histology and pathology of the human intervertebral disc. *J Bone Joint Surg Am* 2006;88 Suppl 2:10-4.
42. Sakai D. Future perspectives of cell-based therapy for intervertebral disc disease. *Eur Spine J* 2008;17 Suppl 4:452-8.
43. Sakai D, Mochida J, Iwashina T, et al. Regenerative effects of transplanting mesenchymal stem cells embedded in atelocollagen to the degenerated intervertebral disc. *Biomaterials* 2006;27:335-45.
44. Setton LA, Chen J. Cell mechanics and mechanobiology in the intervertebral disc. *Spine* 2004;29:2710-23.
45. Sieber AN, Kostuik JP. Concepts in nuclear replacement. *Spine J* 2004;4:322S-4S.
46. Stefanovic-Racic M, Morales TI, Taskiran D, et al. The role of nitric oxide in proteoglycan turnover by bovine articular cartilage organ cultures. *J Immunol* 1996;156:1213-20.
47. Stevens AL, Wheeler CA, Tannenbaum SR, et al. Nitric oxide enhances aggrecan degradation by aggrecanase in response to TNF-alpha but not IL-1beta treatment at a post-transcriptional level in bovine cartilage explants. *Osteoarthritis Cartilage* 2008;16:489-97.
48. Taskiran D, Stefanovic-Racic M, Georgescu H, et al. Nitric oxide mediates suppression of cartilage proteoglycan synthesis by interleukin-1. *Biochem Biophys Res Commun* 1994;200:142-8.
49. Tsai TT, Guttapalli A, Oguz E, et al. Fibroblast growth factor-2 maintains the differentiation potential of nucleus pulposus cells in vitro: implications for cell-based transplantation therapy. *Spine* 2007;32:495-502.
50. Vernengo J, Fussell GW, Smith NG, et al. Evaluation of novel injectable hydrogels for nucleus pulposus replacement. *J Biomed Mater Res B Appl Biomater* 2008;84:64-9.

Program Director/Principal Investigator (Engel,Matthew, A.):

51. Watanabe T, Kato S, Sato K, et al. Nitric oxide regulation system in degenerative lumbar disease. *Kurume Med J* 2005;52:39-47.