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An in Vitro Microenvironment for Nucleus Pulposus Regeneration

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ABSTRACT

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Intervertebral disc degeneration begins early in life, increases with age, and can ultimately result in disc failure. At the center of the disc, the nucleus pulposus (NP) resides in a hypoxic environment where degeneration is thought to begin. Initially, nucleus pulposus cells (NPCs) display altered morphology and function, along with increased rates of cellular senescence and apoptosis. Though no widely accepted treatment exists, autologous NP implantation techniques have shown promise. However, the process is flawed as a result of the long in vitro expansion required to obtain sufficient numbers of cells. Prolonged expansion on plastic produces negative changes such as loss of the NP phenotype and reduced redifferentiation ability. Because cell based tissue engineering techniques require a metabolically active population of cells, stem cells have attracted attention for regenerative NP strategies due to their self-renewal ability and multipotent differentiation capacity. Specifically, synovium derived stem cells (SDSCs) have a higher chondrogenic capacity compared to stem cells derived from other tissues. Another advantage and potential use of SDSCs is that they can produce decellularized extracellular matrix (DECM); expansion of SDSCs or NPCs on this substrate can dramatically increase the rates of proliferation, delay senescence associated changes, and support chondrogenic activity upon induction. In this study, it was determined that NPCs alone and NPCs in co-culture with SDSCs can produce their own unique DECM. It was determined that DECM properties may be modulated by varying oxygen tension during DECM deposition. While the DECM deposited by NPCs along with SDSCs improved proliferation and guided SDSC differentiation towards the NP lineage, the effect was greater than NPC derived DECM and comparable to SDSC derived DECM. Normoxic conditions during DECM preparation were more beneficial to cell proliferation, but hypoxic conditions promoted differentiation towards the NP lineage. Finally, hypoxic conditions during pellet culture promoted NPC viability and redifferentiation. Low oxygen combined with DECM can facilitate cell-based NP regeneration.

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Table of Contents

List of Figures

List of Abbreviations

αMem	alpha modified Eagle's medium
AF	Annulus Fibrosus
BMSCs	Bone Marrow Stem Cells
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
ECM	Extracellular Matrix
DECM	Decellularized Extracellular Matrix
FBS	Fetal Bovine Serum
GAG	Glycosaminoglycan
MSCs	Mesenchymal Stem Cells
NP	Nucleus Pulposus
NPCs	Nucleus Pulposus Cells
SDSCs	Synovium Derived Stem Cells

Chapter 1. Introduction

The intervertebral disk (IVD) is a composite tissue consisting of three distinct regions. Found interspaced between the bony vertebrae of the spine, the function of discs is mechanical; it transmits loads, dissipates energy, and facilitates spinal motion. The disc undergoes profound degenerative changes with age, the first of which occurs very early in life and can be observed with changes in nucleus pulposus cells (NPCs)[13]. Disc degeneration is a downward cascade that increases with age until almost everyone over 50 years of age is affected to some extent. Despite often times being asymptomatic[11], degeneration is often thought to be the underlying cause of low back pain[12, 26, 37, 81] and can result in more serious pathologies such as sciatica, disc herniation [153], and prolapse[146]. The high frequency and increasing severity with age result in a staggering annual cost of £12 in the United Kingdom[89] and up to $200 billion in the United States[68].

Currently, there is no way to regenerate damaged disc tissue. The first stage of treatment begins in the early phases of degeneration as pain presents. The focus is to alleviate pain and mitigate further damage. This is accomplished pharmacologically using non-steroidal anti-inflammatory drugs and through other interventions such as weight loss or physical therapy. If degeneration progresses to a point where it is no longer manageable, surgery becomes the only option. Spinal fusion is considered the standard of care and aims to prevent further degeneration and eliminate pain by bridging adjacent vertebrae. However, it has been shown that pain often persists and patient quality of life does not reasonably improve. Newer options such as disc arthroplasty are still under investigation, though current results do not indicate better long term outcomes.

Cell based tissue engineering approaches are an area of great interest for the potential to provide a method to repair damaged tissue. However, changes the NP undergoes with aging and degeneration present a number of hurdles to repair strategies. These include changes in cell phenotype, an increase in cellular senescence as well as death of the cells in the disc, and a hostile disc environment. Consequently, autologous implantation techniques using NPCs are at a disadvantage because the cell population is already negatively altered. To help address these shortcomings, stem cell supplementation has shown favorable results. Additionally, the in vitro environment represents a valuable opportunity to revitalize cells during the prolonged culture period and to prepare them for their in vivo environment.

The aim of this study is to investigate more suitable in vitro microenvironments for NP regeneration. The extracellular matrix (ECM) is as a potent regulator of cellular behavior and provides many cues missing with plastic substrates. In addition to dramatically increasing proliferation, DECM can support chondrogenic activities upon induction[112]. This is in agreement with a recent hypothesis clarifying that treatment during in vitro culture is a crucial step where essential cues are given by the microenvironment to both immediately and in the future impact cell behavior[47]. The current standard microenvironment both lacks the appropriate cues and additionally gives abnormal signals originating from the rigid plastic substrates. Because the quality of cells used is strongly associated with outcomes in tissue engineering techniques, developing more appropriate in vitro microenvironments is an important step for improving outcomes.

The **first** hypothesis is that nucleus pulposus cells (NPCs) alone and a 50%-50% coculture of SDSCs-NPCs can deposit DECMs to facilitate SDSC proliferation and guide differentiation towards the NP lineage. Additionally, it was hypothesized that DECMs deposited

under hypoxic versus normoxic conditions would facilitate chondrogenic differentiation in a hypoxic environment. In experiment 1, DECM was produced by either NPCs, SDSCs, or a coculture of SDSCs & NPCs. Each type of DECM was produced in both hypoxic (5%) and normoxic (21%) conditions. Subsequently, SDSCs were expanded for one passage on each type of DECM or plastic as a control, used to form pellets, and cultured in serum-free chondrogenic medium for 14 days to assess differentiation.

The **second** hypothesis in this study is that SDSC derived DECM deposited under hypoxia will promote NPC differentiation. In experiment 2, SDSC produced DECM under either hypoxic or normoxic conditions and NPCs were expanded for one passage on each substrate. Pellets from each group were formed and cultured in serum-free chondrogenic medium for 14 days in culture to assess differentiation. A summary of the study design is shown in figure 1.

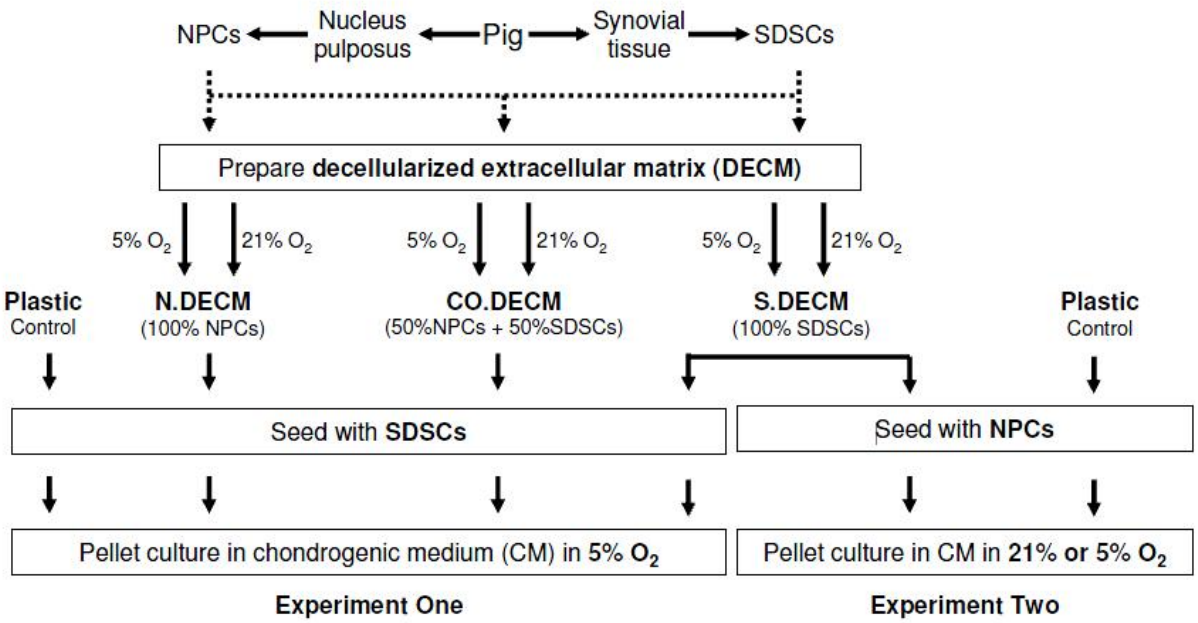

Figure 1. Research design.

Chapter 2 Intervertebral Disc Physiology

2.1 Intervertebral Disc Physiology

Found between adjacent vertebral bodies in the spine, the intervertebral disc is a composite fibrocartilaginous tissue that acts to dissipate energy, support spinal loads, and facilitate spinal movement. In humans, there are a total of 24 discs that are all similar in composition and structure, but increase in size from the cervical to lumbar region[74]. Each disc is formed by the NP at the center, surrounding annulus fibrosus (AF), and cartilaginous endplates (CE) that form a boundary at the top and bottom between the disc and adjacent bony vertebral bodies. Nutrients to the avascular NP and inner AF are transported primarily via diffusion from vasculature originating in the vertebral bodies that terminates at the bone-disc boundary[53, 55].

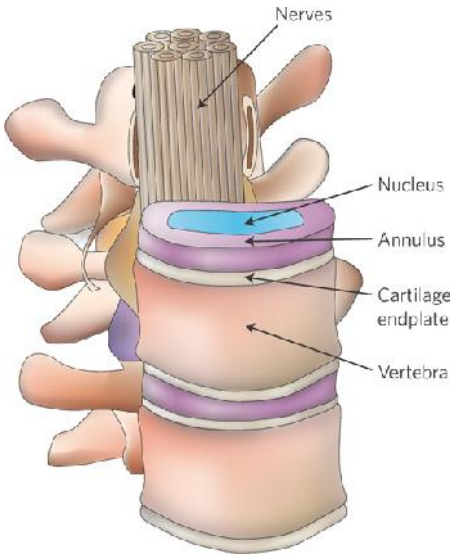

Figure 2. Intervertebral discs and relation to adjacent vertebrae[63].

The AF surrounds and encloses the NP. It is composed of 70% collagen[33], and formed by 15-25 concentric lamellae consisting of bundles of obliquely oriented collagen I fibers that

alternate direction between each layer[83, 21]. In the outer AF, the lamellae are anchored into the adjacent vertebrae and resist excessive vertebral movement. Upon simple compression of the IVD, the AF experiences radial bulging and tensile hoop stresses due to force transferred through the NP's high osmotic pressure[103]. The outer annulus contains the only innervations found in the IVD[85], and small vascular beds found at the dorsal and ventral AF help supply the central disc with nutrients[49, 98]. While there is no distinct boundary between the AF and NP[62], there is a transition zone where proteoglycan content decreases and the predominant type of collagen shifts from type II transition to type I[33]. Additionally, cells in the outer AF are elongated fibroblast-like cells and at the center they are more rounded[15].

Responsible for over one-third the total height of the spinal column[143], the NP is a highly hydrated, gelatinous structure. The NP is composed primarily of large aggregating proteoglycans in a predominantly type II collagen network, with sparsely distributed cells responsible for maintaining the ECM. The high water content has been recorded as composing up to 86% of tissue mass[64], and is crucial for proper tissue function since this gives the tissue its ability to resist loading. Proteoglycans are composed of negatively charged glycosaminoglycan (GAG) chains attached to a protein core which attract water; GAG concentration is correlated to osmotic pressure and high osmotic pressure is essential for proper disc function[115]. Similar to cartilage, the main proteoglycan is aggrecan, which is formed by the GAGs keratin and chondroitin sulfate attached to a protein core[139]. Unlike similar tissues, the NP has a remarkably high ratio of proteoglycans to collagen II of 27:1 glycosaminoglycans to hydroxyproline[96]. Though there are other components present, they are only in small quantities. These include various other collagens including types III, V, VI, IX, X, XI, XII, and XIV[127] as well as other small proteoglycans including lumican, biglycan, decorin, and fibromodulin[87]. Additionally, there is a

small amount of other glycoproteins including fibronectin and amyloid. While their role is not well understood, the minor collagens may mediate collagen type I and II fibrillogenesis[127] in addition to interactions between collagen fibrils and proteoglycans[97]. The adult disc is populated and maintained by a low density (around 4,000 cells/mm^3) of cells referred to as chondrocyte-like[84] or Nucleus Pulposus cells (NPCs).

2.2 Intervertebral Disc Aging and Degeneration

It is currently thought that the degenerative process is a pathological case of maturation where normal aging associated changes take place at an accelerated rate[1, 77]. With both aging and degeneration, the disc undergoes many changes in matrix structure and composition as well as in the resident population of cells. Because of the complex mechanical interactions through which force is transmitted between tissues in the disc, an initial disturbance in disc function trigger a downward spiral that may culminate in total disc failure. Since these changes originate in the cell population of the NP, the embryological origins and alterations from development to degeneration can provide insight about which factors are important in an NP-specific culture environment.

During development, the notochord gives rise to the immature NP which remains populated by large, vacuolated notochordal cells[142]. It was initially thought that this population of active cells disappeared or only remained at undetectable levels by the end of the first decade of life[108]. However, recent evidence suggests that while changes in the population occur through life, both populations are present well into adulthood[150]. The alterations in phenotype during adolescence and adulthood have been implicated as a key instigator in the degenerative process[16], in part because notochordal cells are capable of higher rates of proteoglycans synthesis[20]. Additionally, the first signs of degeneration can be detected around the same time as

the identifiable variations in NPCs, supporting the current thought that degeneration originates in the NP[13]. Though not fully understood, the disappearance of the original embryonically derived notochordal cell phenotype may occur through autocrine or paracrine induced Fas-mediated apoptosis[70]. The contribution of programmed cell death in degeneration is the result of numerous signal transduction pathways triggered through many different stimuli[156].

The in vivo disc microenvironment is considered inhospitable due to the lack of direct vasculature. The small capillary beds that originate in the vertebral bodies and extend superficially over the endplates[55, 61] are the predominant source of nutrients and oxygen, which must diffuse through the endplate before reaching the NP[105]. The only other vasculature found is at the dorsal and ventral annulus regions, but it never extends to the nucleus pulposus[49, 98]. Consequently, even in healthy disks, there are large gradients in nutrient concentration as a result of higher rates of consumption versus diffusion from surrounding tissues[29, 133]. One major problem with aging is calcification of the endplates, which places additional hurdle diffusive transport[104]. The decrease in capillary buds is correlated to proteoglycan content, age, and degeneration grade[9]. Finite element studies on solute transport in the disc suggest that neither elastic modulus nor proteoglycan concentration greatly influence solute distribution, but endplate properties including porosity, diffusion coefficient, and diffusion area are important[82]. Therefore, it may be important for cell based tissue engineering strategies to be performed before endplate calcification to optimize outcomes. The consequences of ECM degradation[6, 7, 141] include fibrosis of the NP[65] and disorganization of annular lamellae[80].

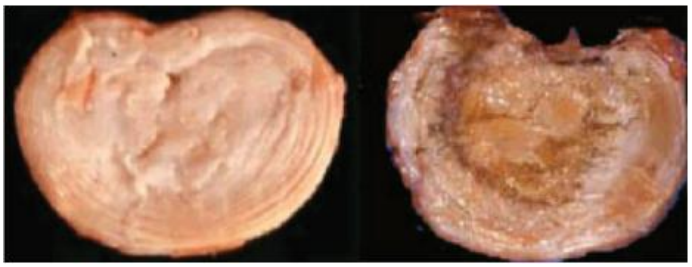

Figure 3. Healthy versus degenerate intervertebral discs[153].

Another important change is reduced proliferation rates in cells harvested from aged or degenerate discs[52]. Increased rates of markers associated with replicative senescence[18] in NPCs include senescence associated β-galactosidase biomarker and decreased mean telomere length[51, 126]. Cells from degenerate discs also express various matrix degrading enzymes, a metabolic change also characteristic of senescent cells[28, 78, 151].

2.3 Current Treatment

Because many cases of IVD degeneration are asymptomatic despite some degree of degeneration, conservative treatments to address symptoms are first used. These include medication, physical therapy, and exercise. Should conservative treatment fails and symptoms persist, surgical intervention becomes the only option. Spinal fusion is a widely performed procedure that involves using an autograft to link adjacent vertebrae and consequently stabilize the disk with the goal of eliminating pain at the cost of motion. Though it has long been the standard treatment, its efficacy in treating discogenic low back pain has been debated and results conflicting[38, 41, 91]. Total disc replacement is a newer procedure for which a number of implants have been developed. While this procedure has shown promise, evidence regarding long term safety and efficacy versus fusion in degenerative discs is still lacking; this procedure is generally limited to scientific studies[36, 145]. Minimally invasive NP replacement is another new technique

employing synthetic biomaterials that has shown some potential, but investigations are still underway[30]. Specifically, one promising option by Klara et al. found the device to be effective, but 38% of patients required additional surgery due to device migration[72]. As a consequence of their shortcomings and general lack of efficacy, biological therapeutic techniques aiming to restore damaged disc tissue is a topic of great interest and promise[131] as either a standalone treatment or to supplement existing surgical procedures.

2.4 Autologous Intervertebral Disc Repair

First investigated in 1968[25], autologous chondrocyte implantation for cartilage defect repair displayed positive results that were translated to clinical investigation in 1994[14]. A recent analysis of long-term (6-10 years) outcomes indicated sustained improvement in 69% of patients[93]. Due to similarities between the NP and cartilage, investigators later began studying autologous cell implantation techniques for IVD regeneration. One key factor in determining therapeutic outcome is the source of cells, which can be problematic since some harvest procedures may cause further damage, and using cells from a damaged tissue is not ideal. Cell types investigated for autologous NP implantation include chondrocytes from articular cartilage[46, 155], NPCs[39, 101], and adult stem cells.

One of the major drawbacks of using autologous NPCs is that cells derived from the degenerate disc are not as suited for rebuilding tissue as healthy counterparts, even after expanded to large numbers. With degeneratation, cells undergo negative morphological, phenotypical, and metabolic alterations. These include increased numbers of cells in the process of dying or already dead through either necrosis or apoptosis, as well as a higher frequency of cells displaying the senescent phenotype[13, 50, 126, 142]. These cells display altered genotypic and

phenotypic behavior in vitro, such as lower gene expression of critical matrix proteins and increased catabolic matrix enzyme expression[66]. Suspected shortcomings of degenerate cells for in vivo repair were verified when Hegewald et al. used cells from patients with herniated discs to form 3D constructs and found significant signs of dedifferentiation that increased further with in vitro expansion and a decreased ability to redifferentiate[57]. Therefore, for the patient population demonstrating the greatest need, autologous NPCs may not be a viable option.

2.5 Mesenchymal stem cells in Nucleus Pulposus Regenerative Techniques

Mesenchymal stem cells possess the capacity to differentiate into various cell types capable of creating a number of tissues[19]. While a variety of different cell sources have been proposed to overcome the challenges of using degenerate NP cells, MSCs are thought to offer the greatest potential for successful clinical application[121]. Most notably, MSCs were recently successfully identified in degenerate NP and possess a capacity to differentiate towards an NP-like lineage[121]. Stem cells isolated from the disc display morphological, immunophenotypical, and differentiation criteria[10, 124] typical of stem cells. However, while NP derived MSCs may play a role in regenerative strategies in the future, their extremely limited availability and extensive harvesting procedures make them a poor option for regenerative techniques. Their presence does however help support the trend to use cocultures of MSCs and NPCs as a method to specify MSC lineage in a population of MSCs isolated from various adult tissues.

Stem cells derived from the bone marrow are the most frequently used for stem cell based NP regeneration. The newest data supports their use in a coculture environment; stem cells exert a positive effect on overall proteoglycan production, and NPCs may help guide MSCs towards the NP lineage. When cultured with bone marrow derived stem cells (BMSCs), coculture with

direct cell-to-cell contact as observed in most in vivo stem cell niches enhances the properties of human derived BMSCs and NPCs[149]. Studies on cells of the NP suggests there may be a three cell types present: notochordal cells, chondrocyte-like cells, and an NP derived stem cell population. While their interactions are still poorly understood, in vitro experiments using notochordal cell conditioned medium (NCCM) identified the precursor of the multifunctional connective tissue growth factor; an increase in proteoglycan expression in NPCs occurs when treated with NCCM. Conditioned medium additionally can support in vitro MSC differentiation towards the NP lineage[117]. This is thought to occur due to the secretion of growth factors and soluble proteins which promote anabolic metabolism in cells as opposed to the catabolic behavior observed during degeneration[86]. This evidence may additionally suggest that notochordal cells act as regulators of NPCs.

One frequently used technique to guide MSC differentiation to the NPC lineage is through coculture. Recently, Allon et al. developed a novel bilaminar coculture pellet system that more closely resembles the embryonic development of the IVD[4,5]. When tested in vivo, implanted bilaminar coculture cells improved both disc height and grade over time[4]. Their most recent study simulating the degenerate disc environment verified the benefits of bilaminar coculture, but did not compare the results to traditional coculture to determine whether the bilaminar system is superior[5]. The BMSC-NPC coculture system has also been verified by others to support differentiation toward an NP-like lineage[102, 140].

Though BMSCs remain the most widely investigated type of adult stem cell for NP regeneration, they may not represent the ideal source. Synovium derived stem cells (SDSCs) have been proposed as another important candidate for NP-regeneration. The argument for SDSCs in NP tissue engineering is similar to that for their successful use in cartilage tissue

engineering; both the synovium and cartilage originate from a common pool of precursor cells[106] and express similar link protein[35] and GAG profiles[54]. Additionally, some comparative studies to BMSCs have also shown SDSCs to possess a higher capacity towards chondrogenesis[130, 136, 154]. All of these traits suggest they may better be suited to rebuild the degenerate cartilage-like NP.

The coculture technique has also been successfully demonstrated in SDSC-NP cultures that produce high aggrecan and collagen type II tissue similar to that of found in the NP[24]. During culture, human serum supplemented SDSCs also expand more rapidly than BMSCs[100], suggesting they may offer advantages over BMSCs for regeneration. Similar results to in vivo BMSC studies were achieved when SDSCs were implanted in degenerate discs and found to increase type II collagen production and suppress matrix degrading enzymes implicated in the degenerative process[92]. The inhibition of catabolic enzymes and cytokines may provide a good therapeutic target because of their association with disc degeneration[151]. Thus, SDSCs may offer another good supplement of regenerative NP strategies.

2.6 The Extracellular Matrix as an In Vitro Substrate

Both in vitro and in vivo, the extracellular matrix to which cells are attached is a major component of the microenvironment that provides signals to determine cell behavior through both physical and biochemical interactions. The extracellular matrix is a dynamic structure that remains active throughout life, facilitating the transmission of physiological signals though it's mechanical structure and chemical composition[75]. A primary method of cell-ECM communications occurs via integrins, a family of transmembrane proteins operating as cell surface receptors which connect the cytoskeleton to the surrounding extracellular matrix[40]. Functioning as heterodimers, integrins are composed of an α and a β subunit; each unique

subunit combination determines binding to specific certain ECM components. Importantly, integrin expression is dependant on the cell type and can serve to help identify downstream cellular responses from cell-matrix or cell-cell interactions.

Work by Nettles et al. initially identified functional integrin subunits in NP and AF, and found discrepancies in the prevalence of subunits in each[99]. The primary difference was higher expression of the α6 and β4 subunits in the NP[99]. The α6 subunit exists in two forms, and has been suggested as a requirement for MSC chondrogenic differentiation[132]. Using flow cytometry to quantify integrin expression levels first upon isolation and subsequently after monolayer culture, it was found that the α6 expression was significantly higher in NPCs and that type II collagen adhesion occurred through the α1β1 receptor, but type I collagen adhesion was only partially mediated through this receptor. Finally, fibronectin attachment was found to occur through the α5β1 integrin[42].

In combination with β1 or β4, the α6 subunit is a primary mediator of cellular adhesion to the extracellular matrix protein laminin, a heterotrimeric protein composed of individual α, β, and γ chains. Currently, at least 15 identified isoforms have been identified which are expressed differently in various tissues[27, 90]. In NPCs, higher expression of the α5 laminin chain, the laminin receptors integrin subunits α3, α6, and β4 along with CD239, and CD151 were found compared to cells from the AF[23]; these suggest that Laminin may be a good match for NPCs. Gilchrist et al. examined cell-matrix interactions and found faster cell spreading and higher risistance to detachment on laminin isoforms LM-511 and LM-332, in contrast to LM-111, fibronectin, and collagen II[44]. Substrates coated with laminin softer than 720 Pa were also found to promote in vitro proteoglycan production, cell-cell interactions, and the NP morphology[43]. Unsurprisingly, the notochord from which the NP is believed to drive is a laminin rich

structure[120] and laminin coatings may one alternative to plastic substrates and promote the NP lineage. However, these single component ECMs may not capture the needed complexity of in vivo ECMs for regulating cell behavior. A promising alternative method that has been shown to produce very strong positive changes in proliferation and differentiation for cartilage tissue engineering utilizes cell produced ECM.

2.7 Hypoxia in the disc

The avascular NP[129] relies on diffusion through long distances for nutrient transport; cells must therefore be adapted for a hypoxic and nutrient deprived environment. Blood vessels from the vertebral bodies extend across the superficial regions of endplates[55]. Additionally, small, sparsely distributed vascular beds extend to the dorsal and ventral surfaces of the annulus fibrosus but never penetrate the NP, even after major disc disruption[49, 98]. In agreement, oxygen concentrations are lowest and lactate concentrations highest in the NP[8]. The lack of vasculature causes an accumulation of anerobic metabolism byproducts which produce a low pH[144] and high osmolarity[71]. The NP can therefore be concluded to exist in a hypoxic niche where cells express a unique phenotype that differentiates them from those of surrounding tissues[118]; this phenotype also facilitates survival and function in a hypoxic environment. In the experiments performed for this study, we investigate the interplay between DECM and the hypoxic environment on proliferation and differentiation toward the NP lineage.

Chapter 3. Materials and Methods

3.1 Isolation and culture of SDSCs and NPCs

Two three month old pigs were obtained from a local slaughterhouse and sacrificed for synovial tissue from both knees and NP from each IVD between the lumbar vertebrae.

Synovial tissue digestion

Random tissue samples were harvested aseptically from the intimal layer of synovial tissue from the knees of each of the three month old pigs and immediately placed in complete medium at 4°C. The tissue was finely minced with scalpels and digested on an x-y-z shaker (Clay Adams Nutator, BD) initially in a solution of phosphate buffered saline (PBS) from Invitrogen containing 0.1% trypsin (Roche) for 30 minutes at 37 ºC, and subsequently in a 0.1% collagenase P (Roche) solution with 10 U/mL hyaluronidase (Sigma) in DMEM/10% FBS, 100 U/ml penicillin, 100µg/ml streptomycin for 2 hours. To eliminate any remaining undigested tissue, the suspension was filtered through a 70µm nylon filter. Cells were plated in complete medium [αMEM containing 10% fetal bovine serum (FBS), 100 U/ML penicllin, 100 µg/mL streptomycin, and 0.25 µg/mL fungizone (Invitrogen)]. Medium changes were performed every two days to remove nonadherent cells.

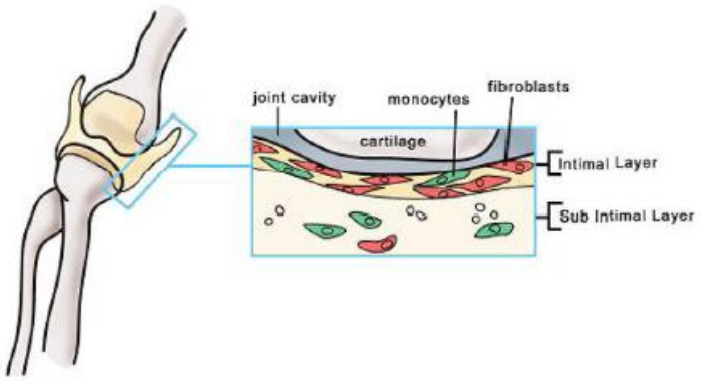

Figure 4. Synovial tissue layers in the knee joint[110]

Nucleus Pulposus digestion and NP isolation

The harvested NP tissue was digested using a solution of 0.1% collagenase A (Sigma, St. Lous, MO) and 10 U/mL hyaluronidase (Sigma) in DMEM containing 10% FBS for 4 hours at 37 ºC. Any remaining undigested tissue was removed by filtering through a 70µm nylon filter. Cells were plated in complete medium and medium changes were performed every two days to remove nonadherent cells.

3.2 *Negative Isolation and of SDSCs*

The SDSCs were negatively isolated from primary cultures of adherent synovial cells composed of both macrophages and fibroblasts[109, 113, 157] by first detaching cells by trypsinization (0.25% trypsin/0.2% EDTA) for no longer than 2 minutes. After washing, cells were suspended in PBS/2% FBS (10^7/ml) and incubated with $5x10^7$/mL Dynabeads® M-450 CD14 containing a monoclonal antibody specific for macrophages (Dynal Biotech, Oslo, Norway) at 4°C for 1 h. The conjugated cells and unbound Dynabeads® were collected using the Dynal Magnetic Particle Concentrator® (Dynal Biotech, Oslo, Norway). The now depleted supernatant with synovial fibroblasts (referred to as SDSCs[8]) was saved for further passaging.

3.3 Gelatin Surface Preparation

This procedure was performed for all DECM groups before seeding cells. Plastic flasks were first coated with 0.2% gelatin (Sigma, St. Louis, MO) and placed in 37ºC incubators for 1 hour. The gelatin solution was aspired and the surface washed with PBS. The flasks were then coated with a 1% glutaraldehyde solution and allowed to rest at room temperature for 30 minutes. Next, flasks were washed 3 times for 5 minutes each, coated with 1M ethanolamine solution, and allowed to rest at 37ºC for 30 minutes. The flasks were again washed 3 times for one hour.

3.4 DECM Production

Passage 3 SDSCs for S.DECM or passage 1 NPCs for N.DECM were plated at 6,000 cells/cm^2. Co-culture ECM was produced by seeding 3,000 cells/cm^2 SDSCs with 3,000 cells/cm^2 NPCs. Until reaching 90% confluence, all groups were placed in 21% O_2 incubators. Medium changes were performed every two days (complete medium). Upon reaching 90% confluence, the complete medium was supplemented with 50 μM of L-ascorbic acid (Wake Chemicals USA Inc., Richmond, VA) and one group was left in the 21% O_2 incubator while another was transferred into a 5% oxygen (hypoxia) incubator for 8 days during DECM deposition. Finally, each group was incubated with 0.5% Triton X-100 containing 20 mM ammonium hydroxide at 37ºC (in the same oxygen concentration incubator as during ECM production) for an average of 4 minutes to remove cells. The DECMs in each group were stored at 4ºC in PBS containing 100 u/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL fungizone.

Experiment 1 Part 1: SDSCs passaged on SDSC, NPC, or SDSC-NPC DECM in hypoxia

In this experiment, there were a total of 7 experimental groups. Passage 3 SDSCs were seeded at a density of 3,000 cells/cm^2 for one passage onto N.DECM, Co.DECM, or S.DECM, at either 21% or 5% oxygen. After 1 passage, cell counting was performed and pellets were created. Following overnight incubation, pellets were treated with condrogenic medium and maintained at 5% oxygen for the remaining 14 days in an incubator.

Experiment 2 Part 1: NPCs cultured on SDSC-ECM deposited under hypoxia

In this experiment, there were a total of 6 experimental groups. Passage 1 NPCs were seeded onto S.DECM deposited at either normal (21% O_2) or hypoxic (5% O_2) conditions. Subsequently, passage 1 NPCs were seeded at a density of 3,000 cells/cm^2 for one passage on each type of DECM. Finally, after pellets were created and following overnight incubation were treated with chondrogenic medium and maintained at either 21% or 5% oxygen for the remaining 14 days in an incubator.

Experiments 1&2 Part 2: Pellet Culture and Chondrogenic Differentiation

After *in vitro* expansion, 0.3x10^6 cells from each pretreatment were centrifuged at 500 g for 7 min in 15-mL polypropylene tubes to form cell pellets. Following overnight incubation, pellets were cultured in serum-free chondrogenic medium containing high-glucose DMEM, 40 μg/mL praline, 100 nM dexamethasone, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1 mM ascorbic acid-2-phosphate, and 1 x ITS^{TM} Premix (6.25 μg/mL insulin, 6.25 μg/mL transferring, 6.25 μg/mL selenous acid, 5.35 μg/mL linoleic acid and 1.25 μg/mL bovine serum albumin from BD Biosciences, Bedford, MA) with supplementation of 10 ng/mL transforming growth factor

beta3 (TGF-β3, PeproTech Inc., Rocky Hill, NJ) for 14 days. Pellets were collected at days 0, 7, and 14 for chondrogenic evaluation.

3.5 Histochemistry and immunochemistry

Randomly selected pellets (n=3) from each data point were first fixed in a 4% paraformaldehyde solution at 4ºC overnight. Samples were embedded in paraffin and 5μm sections were cut. Successive segments were stained with safranin O/fast green for sulfated GAG and immunostained with monoclonal antibodies against collagen I (Sigma) and collagen II [II-II6B3, Developmental Studies Hybridoma Bank (DSHB) Iowa City, IA], collagen I (Sigma). Immunohistochemical sections were hydrated, treated with 1% hydrogen peroxide to inhibit endogenous peroxidase, and incubated first for 30 minutes with 2 mg/mL testicular hyaluronidase in phosphate-buffered saline (pH 5) at 37ºC, another 30 minutes with 1.5% normal horse serum, and finally overnight at 4ºC with the primary antibody. A secondary antibody of biotinylated horse anti-mouse IgG (Vector, Burlingame, CA) was incubated on sections for 30 minutes). Immunoactivity was detected using the Vectastain ABC reagent (Vectastain ABC, Burlingame, CA) with 3-3'-diaminobenzidine (DAB, 0.05%) and hydrogen peroxide (H_2O_2, 0.015%) as a substrate with hematoxylin (Vector) as a counterstain.

3.6 Biochemical analysis for DNA and GAG content

The pellets (n=4) were digested for 4 hours at 60°C with 125 μg/mL papain in PBE buffer (100 mM phosphate, 10 mM EDTA, pH 6.5) containing 10 mM cysteine. Each sample was digested using 100 μL of enzyme. Quantification of cell density was accomplished by measuring the amount of DNA in the papain digestion using a QuantiT^{TM} PicoGreen$^{®}$ dsDNA

assay kit (Invitrogen, Carlsbad, CA) with a CytoFluor® Series 4000 (Applied Biosystems, Foster City, CA). The GAG content was measured using dimethylmethylene blue dye and a Spectronic™ BioMate™ 3 Spectrophotometer (Thermo Scientific, Milford, MA) with bovine chondroitin sulfate from Sigma as a standard.

3.7 TaqMan® Quantitative PCR

The total RNA was extracted from samples (n=4) using an RNase-free pestle in TRIzol® (Invitrogen). One µg of mRNA was used for reverse transcriptase (RT) with a High-Capacity cDNA Archive Kit at 37ºC for 120 min as recommended by the manufacturer (Applied Biosystems). Chondrogenic marker genes [*collagen II* (*Col II*) (Assay ID: AIJ9V6I)] and hypertrophic genes [*collagen X* (*Col X*) (Assay ID: AI382VX) and *alkaline phosphatase* (*ALP*) (Assay ID: AI1Q8RQ)] were customized by Applied Biosystems as part of the Custom Taqman® Gene Expression Assays. Eukaryotic *18S* RNA (Assay ID: HS99999901_s1 ABI) was carried out as the endogenous control gene. Real-time PCR was performed with the iCycler iQ™ Multi Color RT-PCR Detection System and calculated by computer software (Perkin-Elmer, Waltham, MA). Relative transcript levels were calculated as $\chi=2^{-\Delta\Delta Ct}$, in which $\Delta\Delta Ct=\Delta E-\Delta C$, $\Delta E=Ct_{exp}-Ct_{18s}$, and $\Delta C=Ct_{ct1}-Ct_{18s}$.

3.8 Statistics

The Kruskal-Wallis test was used to test for significant differences among all groups and the Mann-Whitney U test was used for pairwise comparison in biochemistry analysis and real-time PCR data. All statistical analyses were performed with SPSS 13.0 statistical software (SPSS Inc., Chicago, IL). *P* values less than 0.05 were considered statistically significant.

Chapter 4 Results

(Partially adapted from Pei M, Shoukry M, et al.[114])

4.1 Experiment 1: SDSC expansion on DECM (Figure 5A & B)

To determine the feasibility of varying oxygen tension as a method of modulating DECM properties, S.DECM, N.DECM, and Co.DECM were prepared under either normoxic or hypoxic conditions. For one passage, SDSCs were cultured on each substrate and plastic to serve as a control. After 5 days, cell numbers were used to assess the effect of each DECM on SDSC proliferation (Figure 5A). It was found that the effect of hypoxia or normoxia produced DECM on SDSC numbers after 1 passage was not significant, but all DECMs greatly enhanced proliferation compared to plastic controls. The highest cell numbers were recorded by S.DECMs. Pronounced morphological changes in cell shape and organization were observed in DECM groups relative to plastic (Figure 5). Namely, plastic expanded cells became large and flattened while DECM expanded cells became small and spindle shaped. Additionally, cell density remained similar between days 3 and 5 on plastic while dramatic increases were observed in DECM groups. In DECM groups, cells were also observed to propagate along DECM fibers and can be seen to above and below other cells, while propagation in plastic appeared limited to the 2D substrate.

4.2 Experiment 1: Histology and Immunostaining (Figure 5C)

Differentiation potential was assessed after one passage on each type of DECM using pellet culture for fourteen days in serum-free chondrogenic medium supplemented with TGF-ß3. The results of staining for collagen type I, type II, and GAGs are displayed in Figure 5 below.

After 14 days, DECM expanded SDSCs produced larger pellet sizes than plastic. In all DECM expanded groups, intense staining was observed for sulfated GAGs by both Alcian blue and Safranin O staining. Immunostaining for collagen II was stronger and more homogenous in hypoxic versus normoxic cultures, most notably in the center of pellets. Only minimal staining for collagen, either type I or II, was observed in the plastic group. Finally, immunostaining for collagen I was also stronger in DECM groups than plastic, though 5% S.DECM appeared to have the lowest levels among DECM groups, with the exception of the 5% Co-culture group that was similar.

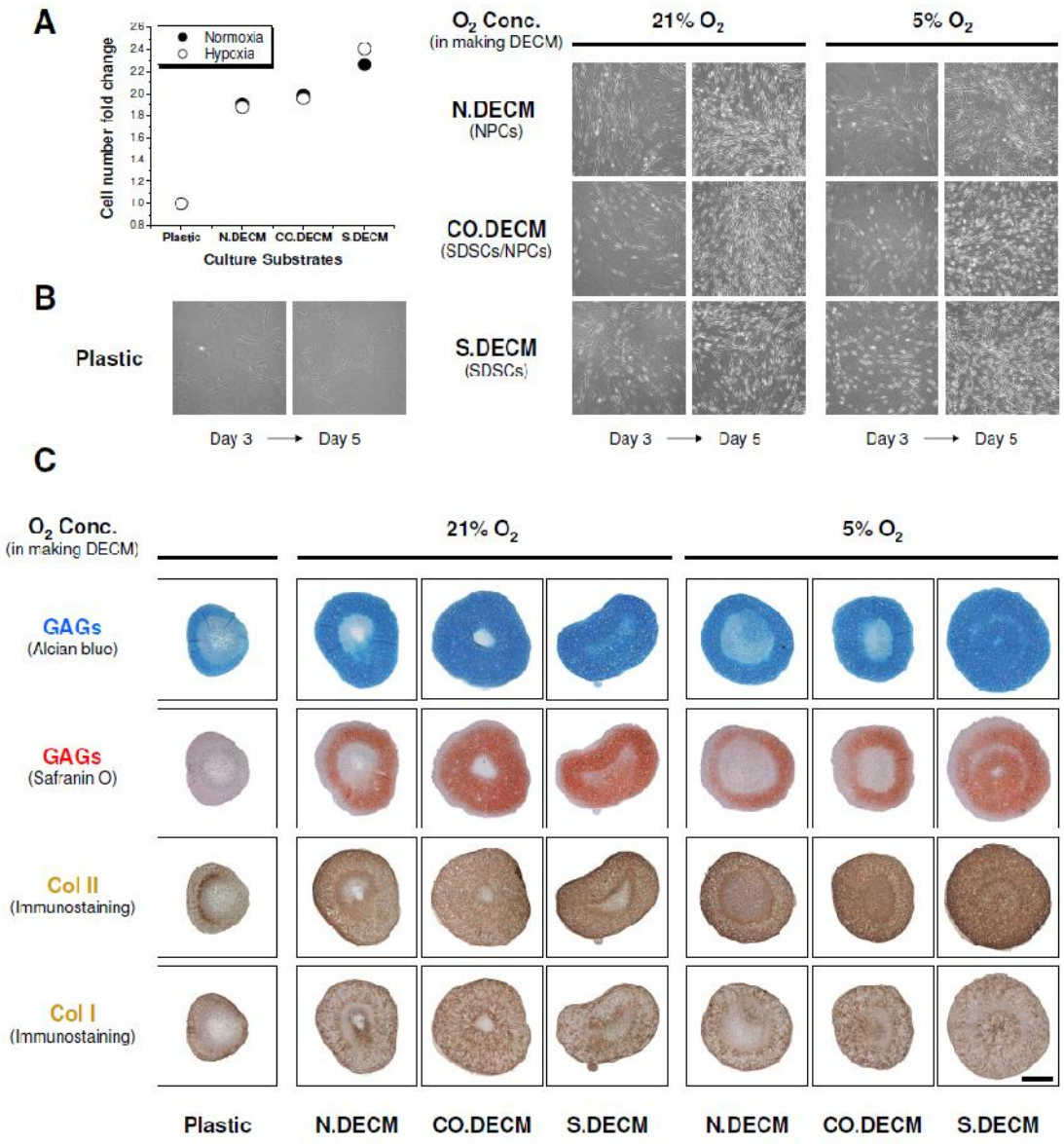

Figure 5. The proliferation rates and chondrogenic potential were improved after DECM expansion. (A) Passage 3 SDSCs were expanded on seven substrates for one passage. Cell number was quantified using a counting hemocytometer from four 175 cm^2 plastic flasks in each group; (B) Cell morphology at days 3 and 5 during expansion; and (C) Expanded cells were cultured in a serum-free chondrogenic medium in a pellet system for 14 days. Alcian blue and Safranin O staining were used to detect sulfated GAGs. Immunostaining was used for collagens I and II. The scale bar is 800 μm.

4.3 Experiment 1: Biochemical Assessment (Figure 6A)

Relative DNA contents were calculated by dividing the DNA content at a given time point (i.e. days 7 and 14) by that of day 0 to facilitate comparison between groups. At day 7, S.DECM, CO.DECM, and plastic all remained at similar levels. Both N.DECM groups were also at a similar level, with the hypoxic the N.DECM group at a slightly (statistically significant) lower level. Ratios dropped by day 14 and any significant inter-group variance was eliminated, showing that viability was maintained well in all groups regardless of DECM pretreatment.

Relative GAG content (GAG index) was adjusted by dividing the GAG content at a time point by the DNA content at that time point, providing a comparable measure for variation in GAG content between groups after adjusting for differences in DNA content. At day 0, all groups were at a minimal level. By day 7, all DECM groups had a higher total GAG content and GAG index than the plastic control. The highest total GAG content at this time point was in the normoxic CO.ECM and S.ECM groups, but after adjusting for DNA the difference became minimal. The trend continued at day 14 where absolute GAG content was again highest in the normoxic CO.DECM and S.DECM groups, but after adjusting for DNA, variation became minimal. The plastic group remained at a very low level compared to DECM groups.

4.4 Experiment 1: Messenger RNA Analysis (Figure 6B)

Since the major type of collagen in the disc is type I, variability in the properties of DECMs created as a result of either the cell type that produced them or environment in which they were produced, the effect on this gene was examined. All DECM groups exhibited collagen II mRNA levels many times higher than plastic. No statistical difference was observed between

both N.DECM, S.DECM, and CO.DECM groups. However, both hypoxic and normoxic N.DECM groups were at the lowest level among DECM groups.

The hypertrophic markers used were collagen X since it signifies late-stage chondrocyte hypertrophy [119, 95] and alkaline phosphatase for key roles in mineralization[60, 45]. Plastic yielded significantly higher mRNA levels of collagen X than either of the CO.DECM and S.DECM groups, but similar levels to both N.DECM groups. In contrast, plastic produced the lowest ALP mRNA levels. Among DECM groups, the lowest significant levels were in S.DECMs and the highest level was found in the normoxic N.DECM group.

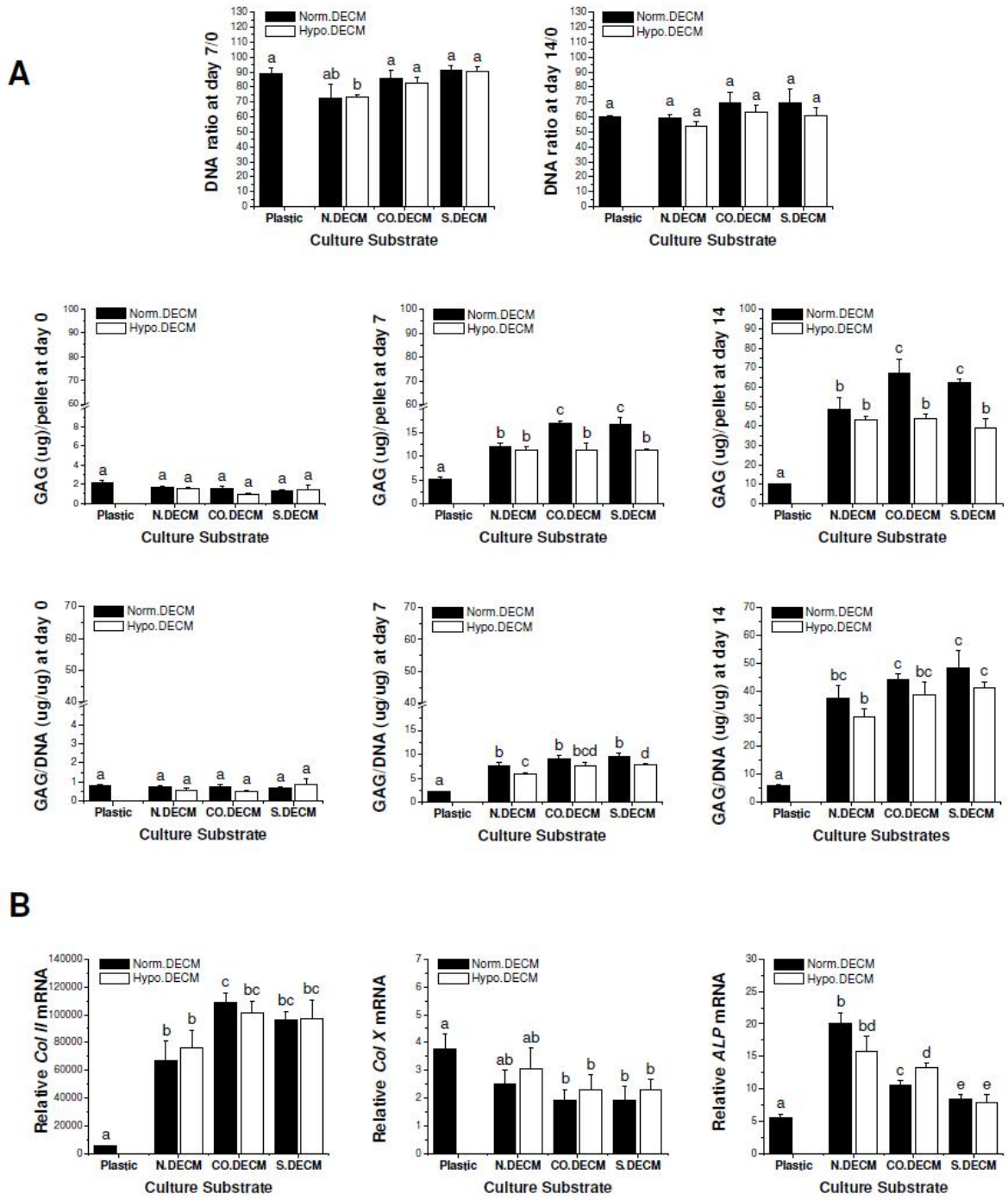

Figure 6. Expansion on DECM improved both SDSC viability and chondrogenic capacity in a pellet culture system. (A) Biochemical analysis for DNA and GAG contents in a pellet. Cell viability is shown with either day 7 or day 14 DNA ratios adjusted by day 0. Chondrogenic index assessed using the ratio of GAG to DNA. (B) Real-time PCR was used to assess chondrogenic

differentiation at the mRNA level. *Collagen II* (*Col II*) mRNA was a marker for chondrogenic differentiation; *collagen X* (*Col X*) and *ALP* mRNAs were used as markers for hypertrophy. Lower case letters (i.e., a, b, and c) indicate the results of one-way analysis of variance (ANOVA) at $p < 0.05$. Groups with a letter in common are not statistically different from one another. Error bars represent the mean ± SD for n=4.

4.5 Experiment 2: NPC expansion on DECM (Figure 7A)

In this experiment, NPCs were expanded for 1 passage on SDSC produced DECM. To determine if DECM can be customized during the deposition phase, DECMs were produced in either a hypoxic (5% O_2) or normoxic (21% O_2) culture environment. Finally, pellets were additionally cultured during chondrogenic differentiation in either 21% or 5% oxygen to assess the effects on matrix production. Gross observation in Figure 7A shows cell density to be drastically higher in DECM groups and a change in morphology to a smaller, thin shape. Additionally, cells propagated along DECM fibers and in a 3D arrangement while plastic cultured cells only propagated along the 2D substrate and remained widely distributed.

4.6 Experiment 2: Histology and Immunostaining (Figure 7B)

Gross pellet characteristics were similar to those observed in the previous experiment, showing much larger pellet sizes in all DECM groups. Surprisingly, collagen II immunostaining showed that plastic treated cells produced pellets with collagen II content comparable to DECM groups. Also, the hypoxic S.DECM differentiated in hypoxia produced the lowest observable levels of collagen II. Collagen type I immunostaining was similarly low in all groups. Staining for GAGs indicated similar levels among groups.

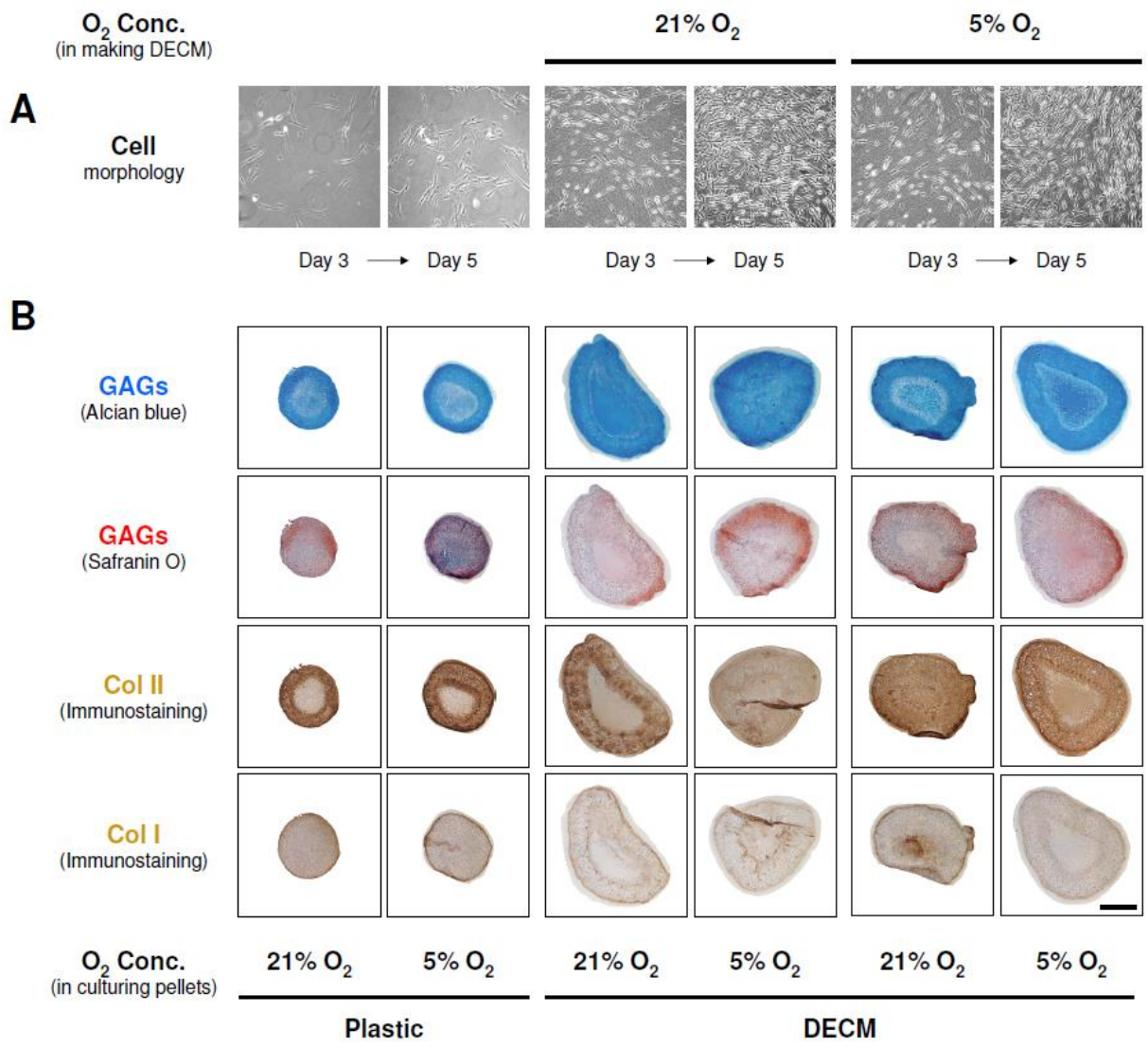

Figure 7. DECM expansion enhanced NPC proliferation and redifferentiation capacity. (A) Passage 2 NPCs were expanded on three substrates for one passage. Cell morphology was photographed at day 3 and day 5 during expansion. (B) Expanded cells were cultured in a serum-free chondrogenic medium in a pellet system for 14 days. Alcian blue and Safranin O staining were used to detect sulfated GAGs. Immunostaining was for collagens I and II. The scale bar is 800 μm.

4.7 Quantitative Biochemical Assessment (Figure 8A)

At day 7, all DECMs produced higher cell numbers and a significant difference between Hypoxic and Normoxic DECMs was observed. Among groups, 5% Hypo.DECM produced the highest numbers. By day 14, this difference between the Norm. and Hypo.DECMs became negligent, but cells differentiated in 5% oxygen maintained the highest numbers assessed by DNA content.

The total GAG content followed a similar trend to that of DNA. Specifically, the 5% O_2 differentiation environment produced the highest GAG content at day 14, but after accounting for DNA content changes, both Norm. and Hypo.DECM produced similar higher levels than other groups. While all DECM groups produced significantly higher levels than plastic, 21% Norm. and Hypo. DECM groups produced lower levels than 5% Norm. and Hypo.DECMs.

4.8 Messenger RNA Analysis (Figure 8B)

The highest levels of relative collagen II mRNA were found in the 5% pellets/ Hypo.DECM. While 5% pellets had similar levels for both Norm.DECM and Hypo.DECM, there was a large significant difference between the two DECMs when pellets were cultured under 21% O_2. The lowest values were found in the 21% plastic group.

The highest levels of both hypertrophic markers were found in the 5% S.DECM groups. Both plastic groups produced the lowest relative mRNA levels of Col X mRNA and the 21% plastic group produced the lowest relative mRNA levels of ALP. Both 5% DECM groups had the highest collagen X and ALP levels.

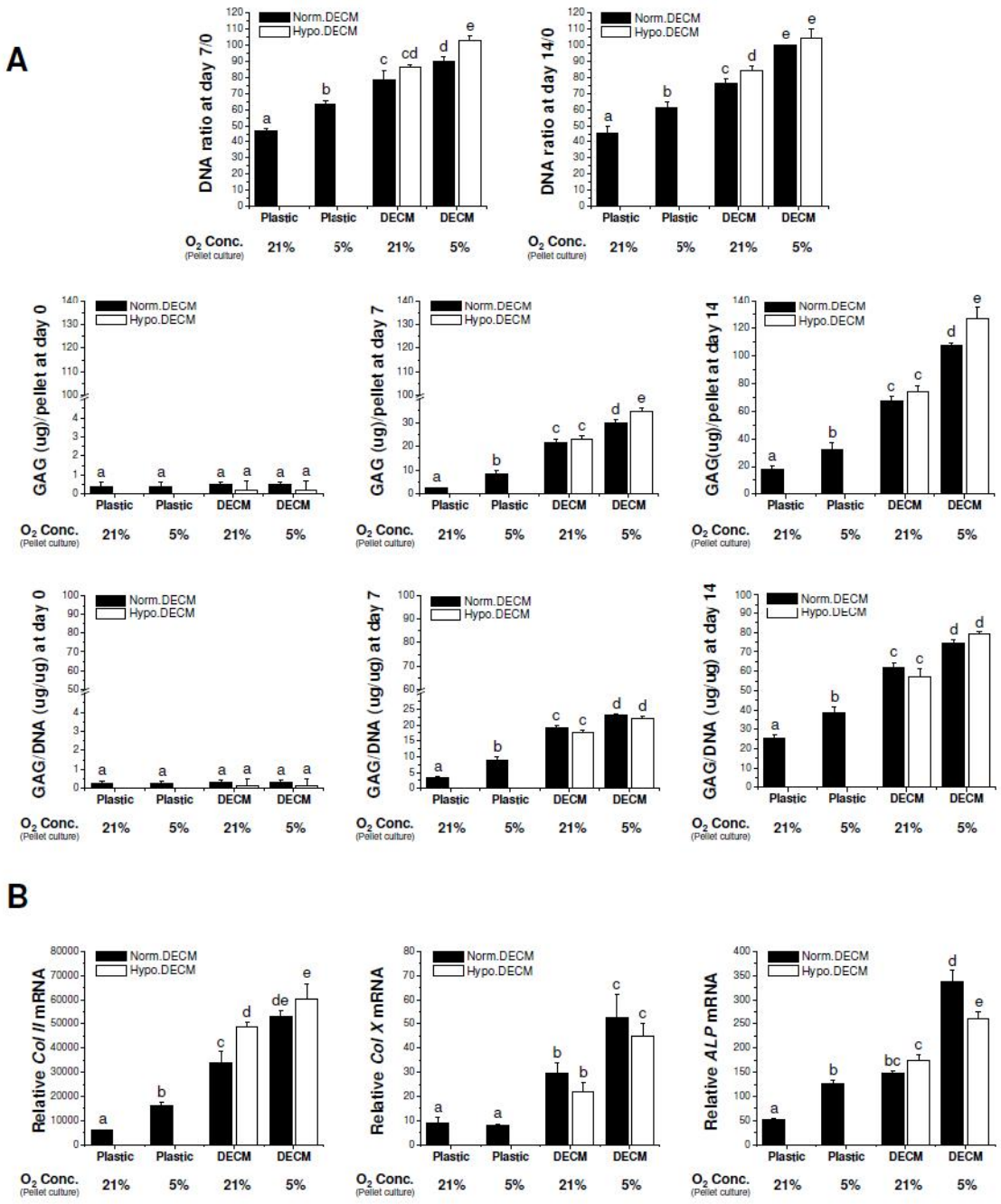

Figure 8 DECM expansion enhanced NPC viability and redifferentiation capacity in a pellet system. (A) Biochemical analysis was used to measure DNA and GAG contents in a pellet. Cell viability was reflected by DNA ratio at day 7 or day 14 adjusted by day 0. Chondrogenic index was evaluated by ratio of GAG to DNA. (B) Real-time PCR was used to assess NPC redifferentiation at the mRNA level. *Collagen II* (*Col II*) mRNA was a marker for chondrogenic

differentiation; *collagen X* (*Col X*) and *ALP* mRNAs were used as markers for hypertrophy. Lower case letters (i.e., a, b, and c) indicate the results of one-way analysis of variance (ANOVA) at $p < 0.05$. Groups with a letter in common are not statistically different from one another. Error bars represent the mean ± SD for n=4.

Chapter 5: Discussion

(Partially adapted from Pei M, Shoukry M, et al. and Shoukry M, Pei M, et al. [submitted])

5.1 Overview

Current autologous nucleus pulposus implantation techniques have failed to achieve success rates and levels necessary for clinical use. These limitations are thought to be the result of the cell population; inadequate monolayer culture environment tends to negatively impact cells with increasing expansion time. There is increasing evidence that suggests pretreatment with the correct conditions during expansion can produce a cell population with greater in vivo success[34,58,88]; this is of particular importance in the IVD due to the hostile microenvironment. When passaged in monolayer on plastic, NPCs are known to proliferate slowly[147] and undergo increasing dedifferentiation[57]. Cells from the degenerate NP also become increasingly senescent, which makes them minimally responsive to growth factor or cytokine stimulation. They also display a catabolic metabolism characterized by the increased production of matrix degrading enzymes[126, 69, 28]. Similarly, adult stem cells undergo analogous negative changes with time in two-dimensional (2D) monolayer culture. In this investigation, we explored the use of various DECMs to facilitate both SDSC differentiation and NP redifferentiation toward the NP lineage.

The avascular NP[129] relies on diffusion through long distances for nutrient transport; cells must therefore be adapted for a hypoxic and nutrient deprived environment. Blood vessels from the vertebral bodies extend across the superficial regions of endplates[55]. Additionally, small, sparsely distributed vascular beds extend to the dorsal and ventral surfaces of the annulus fibrosus but never penetrate the NP, even after major disc disruption[49, 98]. In agreement, oxygen concentrations are lowest and lactate concentrations highest in the NP[8]. The lack of vasculature

produces the accumulation of anerobic metabolism byproducts which produce a low pH[144] and high osmolarity[71]. The NP can therefore be concluded to exist in a hypoxic niche where cells express a unique phenotype that differentiates them from those of surrounding tissues[118]. Because the hypoxia is also associated with inhibition of cellular senescence[152], it is of unique importance for cell based NP regeneration techniques, especially those using stem cells.

Sufficient and high-quality cells, either tissue-specific SDSCs or NPCs, are critical for the success of cell-based NP tissue engineering and regeneration. Our current study demonstrated that DECM deposited by SDSCs, NPCs, or a combination could provide a robust cell expansion system for SDSC proliferation. However, S. DECM provided the highest mitotic effect. Compared to cells expanded on plastic, DECM expanded SDSCs also exhibited a strong chondrogenic differentiation capacity, particularly for S.DECM and CO.DECM. Intriguingly, DECM prepared under normoxia yielded SDSCs with higher cell viability and chondrogenic index but it was not a significant difference compared to hypoxia. DECM deposited by SDSCs was chosen for Experiment 2 due to its robust cell expansion and chondrogenic capacity. We found that DECM prepared under hypoxia favored NPC viability and GAG production as well as *collagen II* mRNA expression and decreased NPC hypertrophy, as evidenced by lower mRNA levels of *collagen X* and *ALP*. Moreover, low oxygen in a pellet culture system dramatically enhanced NPC viability and redifferentiation capacity.

5.2 Experiment 1 Discussion

In experiment 1, SDSCs were expanded for one passage on S.DECM, N.DECM, or Co.DECM, or plastic; each DECM was produced by cells under either normoxic or hypoxic conditions. After one passage, cell counting demonstrated that the highest SDSC numbers were

produced by the normoxia and hypoxia produced S.DECMs, with the hypoxia produced S.DECM producing slightly higher numbers. After 14 days of pellet culture under hypoxic conditions in serum free chondrogenic medium, RT-PCR analysis showed the highest total GAG production for normoxic CO. and S.DECMS, but variability was diminished after accounting for DNA content. Because DNA content provides a measure of cell numbers, day 14 results indicate that the higher absolute GAG contents are a result of higher cell numbers. Both N.DECM groups produced the lowest levels of collagen type II and GAG mRNA at day 14, suggesting that N.DECM may not be a good option as part of the in vitro environment for SDSCs. The relative mRNA contents of collagen type X, a marker of undesirable terminal differentiation was at the lowest levels for both CO.DECM and S.DECM groups. Additionally, ALP levels indicative of undesirable mineralization were lowest at the same level for both S.DECM groups. In this experiment, only 5% oxygen during SDSC differentiation in pellet culture was applied since this reflects the environment in vivo and is in agreement with experiment 2 where NPC redifferentiation occurs best under hypoxic conditions.

Especially for MSCs which may not express traits necessary for survival and proper function in a low oxygen environment, hypoxic preconditioning may provide a good strategy. When treated with hypoxia, MSC viability is increased and rates of apoptosis are decreased post-implantation[116]. One mechanism this occurs is through HIF-1α[22] expression which is continually expressed in NP cells and may therefore provide a good method to both enhance post implantation survival and prime cells for differentiation towards an NP like lineage. Because gene expression and metabolic activity of cells cultured in hypoxia is different compared to cells under normal oxygen tension, we hypothesized in this experiment that hypoxic DECM would favor NPC redifferentiation. Our results indicated that the two types of DECM impacted NPCs

differently with respect to GAG mRNA expression. This suggests that hypoxic DECM varies in either composition or structure.

Another other interesting finding in this experiment was that DECM deposited by a coculture of NPCs and SDSCs produced almost the same results as DECM deposited by SDSCs alone. In contrast, while NPC deposited DECM provided a much improved substrate compared to plastic, it ranked lowest among other DECM groups. A reasonable assumption is that all cells during culture are active producers of DECM. Therefore, a reasonable conclusion is that SDSCs were able to favorably and significantly impact NPC behavior to produce a DECM with almost the same properties as SDSC only produced DECM. These results suggest that NPCs and SDSCs are able to work together well in a culture environment, and more importantly, that SDSCs may play an important role in reprogramming NPCs. These results support analogous findings where transplanted SDSCs suppressed MMP gene activity NP cells[92] and taken together suggest that SDSCs coculture may be a useful tool in influencing NPC behavior while creating a tissue specific microenvironment. However, further studies are needed to uncover the underlying mechanism for SDSC regulation of NPC behavior.

5.3 Experiment 2 Discussion

In experiment 2, NPCs were expanded for one passage on S.DECM produced under either hypoxic or normoxic conditions, then cultured in serum free chondrogenic medium under either hypoxic or normoxic conditions. At day 14, the highest absolute GAG content was observed in the Hypo.DECM and 5% during pellet culture. After accounting for DNA content, the difference was maintained, though only by a slight margin after accounting for error. This group also displayed the highest levels of relative collagen II mRNA. While the levels of

collagen type X mRNA at day 14 were similar for both 5% pellet culture groups, ALP mRNA was lower for ALP mRNA. Importantly, ALP provides a measure of mineralization which is undesirable for the NP. Therefore, for NPCs, hypoxia during SDSC depositition of DECM additionally functions with hypoxia during redifferentiation. This is an important finding because it indicates better overall tissue quality and cell viability under hypoxic conditions, similar to those found in the native NP and may produce higher quality tissue in vivo.

Together, these results have important implications for autologous NP implantation techniques. If NPCs remain as the chosen cell source for autolgous techniques, then it is suggested that the required in vitro expansion should incorporate DECM as a key component of the in vitro expansion microenvironment. In agreement with a recently published concurrent study from our lab, SDSC deposited DECM has a very strong beneficial impact on NPCs. This includes dramatically increased proliferation ability for at least 6 passages in vitro and upregulation of key gene expression including collagen type II, aggrecan, and Sox 9[56]. Additionally, this study by He et al. found an increase in CD90 expression. As a stem cell surface marker positively related to immunosuppressive capacity[117], higher CD90 expression suggests DECM may also promote the reprogramming of cells with immunosuppressive traits.

In this experiment we additionally demonstrated that the DECM properties may further be attenuated using hypoxia to increase GAG production. Additionally, DECM deposited under hypoxia resulted in lower ALP expression that DECM deposited under normoxia, suggesting an additional impact on undesirable mineral deposition during differentiation. Unfortunately, NPC expansion on NPC deposited DECM could not be evaluated due to study size. However, a similar recent study in chondrocytes comparing chondrocyte deposited DECM with SDSC deposited DECM on chondrocyte expansion and redifferentiation indicated that SDSCs can

produce the best substrate[111]. Similarities between chondrocytes and NPCs might suggest that the results would be similar in NPCs, but this has yet to be investigated. Additionally, DECM in this study was deposited under normoxia, so the beneficial effects of hypoxia during DECM deposition have yet to be verified. Additionally, while speculation can be made regarding the mechanisms underlying hypoxia induced DECM differences; further study is needed to reveal them.

5.4 Mechanisms Underlying Hypoxia

The effects of hypoxia are regulated by the hypoxia inducible factor (HIF) group of transcription factors, whose family of HIF target genes regulates key cellular functions, including energy metabolism, angiogenesis, cell survival, apoptosis, matrix synthesis, proliferation, self-renewal, and differentiation[123]. Serving as the key regulator of energy metabolism and survival, HIF-1 is a heterodimer consisting of an α and a β subunit, with the former degrading quickly under normoxic conditions[134, 148]. Another key isoform, HIF-2α, has some common action with HIF-1 but also serves as a regulator for genes unresponsive to HIF-1[79]. The response to HIF-1 and HIF-2 are cell specific. For example, only HIF-2 was found to be a regulator of Sox9 in chondrocytes[76], but the hypoxic response of BMSCs includes HIF-1α regulation of the same gene[67,28]. Therefore, the combined action of HIF-1 and HIF-2 is considered to be dependant on cell type [138].

A central function of HIF-1α is in promoting transcription of glycolytic enzymes essential for cells to switch from aerobic to anerobic metabolism[134, 135] in part by inhibiting ATP production through oxidative phosphorylation[107]. One unique trait of NPCs is the continued expression of HIF-1α under normoxic conditions, whereas in other cell types this isoform is

quickly degraded[125]. Known to be involved controlling mineralization in various tissues including bone, the calcified zone of the growth plate, and the degenerate NP, ANK expression is negatively regulated by both HIF-1 and HIF-2 in the NP and is thought to prevent mineralization under physiological conditions [137]. A decrease in aggrecan expression is also observed upon HIF-1 inhibition, suggesting the unique oxygen-independent stabilization of HIF-1α is an important metabolic adaptation for both glycolysis and aggrecan expression[3]. This finding further supports the recent report that NPC metabolism is not reduced at O_2 concentrations as low as 1% and that the hypoxic environment positively impacts aggrecan production while promoting a healthy gelatinous as opposed to fibrous matrix[94].

Additionally, HIF-2 also was found to regulate the p300 binding protein cited2 which regulates both HIF-1 and HIF-2 dependent vascular endothelial growth factor (VEGF) expression in NPCs[2]. Another key consequence of the low oxygen environment in NP cells is promoting the expression of proteins responsible for Notch signaling, an important regulator of proliferation[59]. Hypoxic activation of the PI3K/Akt and MAPK pathways is thought to underly the downregulation of apoptosis in healthy NP cells in a hypoxic, serum deprived environment[122]. Hypoxia also induces expression of the Notch gene related to maintaining an undifferentiated state and cell fate determination[73, 48]. These mechanisms may have played a role in producing the variation in DECM properties observed in this study, but further studies are needed to determine the specific response. The significant positive results suggest that SDSC DECM may have important utility as a biomaterial for NP tissue engineering. It is suggested that the first step in developing this technology for future use is characterizing the composition and stiffness of this substrate, since this would yield important clues for understanding the cellular response.

5.5 Conclusion

As a substrate for in vitro expansion, DECM provides a highly superior surface that drastically increases proliferation and strengthens both stem cell differentiation towards the NP lineage and NP redifferentiation. Additionally, these beneficial effects can be mediated by hypoxia. This suggests that in the future, growth factors might be used in an analogous fashion to further customize DECMs. The requirement for high numbers of metabolically active cells for autologous techniques can be met by the inclusion of DECM as part of the in vitro microenvironment.

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Appendix A. Publications

Peer Reviewed Journal Publications

1. **Shoukry M**, Pei M. Targeting an *in vitro* "niche" to facilitate cell-based nucleus pulposus regeneration. (Accepted)

2. Pei M, **Shoukry M**, Li J, Daffner SD, France JC, Emery SE. "Modulation of In Vitro Microenvironment Facilitates Synovium-Derived Stem Cell-Based Nucleus Pulposus Tissue Regeneration." Spine 2012;37(18):1-10

3. William G, **Shoukry M**, Prucz J. "Analysis of Lightweighting Design Alternatives for Automotive Components ." SAE Technical Papers 2011: doi:10.4271/2011-01-2287

4. Pei M, Li JT, **Shoukry M**, Zhang Y. "A review of decellularized stem cell matrix: a novel cell expansion system for cartilage tissue engineering." European Cells and Materials 2011;22:333-343

5. Pei M, Yan Z, **Shoukry M**, Boyce BM. "Failure of xenoimplantation using procine synovium-derived stem cell based cartilage tissue constructs for the repair of osteochondral defects." Journal of Orthopaedic Research 2010;28(8):1064-1070 (*Journal Cover)*

Peer Reviewed Paper Presentations

William G, **Shoukry M**, Prucz J. "Analysis of Lightweighting Design Alternatives for Automotive Components." Commercial Vehicle Engineering Congress, September 2011

Poster Presentations

Pei M, **Shoukry M**, Li J. "Modulation of an in vitro tissue-specific microenvironment to facilitate nucleus pulposus tissue regeneration." Poster presented as part of the Orthopoaedic Research Society Meeting, San Francisco, Calif., 4-7 February 4-7 2012