POTENTIAL OF MESENCHYMAL STEM CELLS TO TREAT LOW BACK PAIN IN ANIMAL MODEL

by

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A dissertation submitted in partial fulfillment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY IN BIOLOGICAL AND BIOMEDICAL SCIENCES

2026

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Dedicated to parents, wife, and children for all their love and support.

ACKNOWLEDGMENTS

I sincerely thank my mentor, Dr. Rasul Chaudhry, for welcoming me into his lab and for his continued support and encouragement. This opportunity has brought me great joy and given me skills I will cherish forever. I am also very grateful to my dissertation committee members, who helped turn what initially seemed daunting into an incredible learning experience. I also want to thank the OU-WB Institute for Stem Cell and Regenerative Medicine, Oakland University's Department of Biological Sciences, and King Khalid University for their generous financial support. Their investment in my research truly makes me feel honored and privileged. To my lab mates, who have become more than just colleagues and feel like family, thank you for the shared laughter and the late-night struggles that made even the toughest days easier. I couldn't have done it without you all by my side.

Finally, I want to express my heartfelt gratitude to my family. Thank you for your patience, strength, and unconditional support, which have given me a haven. That is why I am standing here today, and I am truly thankful!

Ali Alamri

PREFACE

The field of stem cell research is one of the most exciting yet challenging areas of modern science. It offers great promise: the ability to use the body's own power to heal conditions once thought impossible to treat. However, the path from basic laboratory discoveries to actual patient treatments is long and complex. It involves moving from understanding fundamental principles to developing safe and effective therapies suitable for clinical use. I entered this complex world thanks to my advisor, Dr. Rasul Chaudhry. I started in his lab as a student, but with his mentorship, I grew into a scientist. Dr. Chaudhry's innovative ideas and visionary approach to regenerative medicine sparked my passion for this field. He did more than just teach me techniques; he expanded my understanding of the deep importance of stem cell biology and encouraged me to think critically about its potential to change lives. His guidance has been the foundation of my academic success.

Through his influence, I have not only developed a versatile skill set but also cultivated a deep passion for research that I will carry into my future career. This dissertation reflects the intellectual curiosity he fostered in me and demonstrates our shared commitment to bridging the gap between laboratory research and patient care.

ABSTRACT

POTENTIAL OF MESENCHYMAL STEM CELLS TO TREAT LOW BACK PAIN IN ANIMAL MODELS

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Degenerative disc disease (DDD) involves the breakdown of intervertebral discs (IVDs), which can cause chronic neck and lower back pain. The IVD consists of a gel-like nucleus pulposus (NP) at its center, encased by a tough outer layer called the annulus fibrosus (AF). Current treatments mainly aim to relieve symptoms rather than repair the disc's structure and function. Recently, research has begun to focus on cell therapy for DDD, particularly using adult mesenchymal stem cells (MSCs)

We have isolated mesenchymal stem cells (MSCs) from perinatal tissues, specifically the umbilical cord, using a xenofree medium developed in our lab. We call these cells primitive (p) MSCs because they not only express typical MSC markers, such as CD29, CD44, CD73, CD90, and CD105—but also several self-renewal markers, including CD49f, OCT4, NANOG, SOX2, and KLF4. pMSCs expanded in xenofree medium are suitable for therapeutic applications.

We have successfully differentiated pMSCs into nucleus pulposus-like cells (NPCs) that express characteristic markers, including SOX9, ACAN, COL2, TGFβ1, TGFβ3, VCAN, and GDF10. Additionally, we have developed three-dimensional (3-D)

scaffolds that mimic the native environment of the IVD. We hypothesize that pMSC-derived NPCs can regenerate the nucleus pulposus of the IVD.

The aims of this study are as follows:

1. To isolate mesenchymal stem cells (MSCs) using a xenofree medium. 2. To differentiate MSCs into nucleus pulposus-like cells (NPCs). 3. To develop three-dimensional (3-D) scaffolds that mimic the in vivo microenvironment of the intervertebral disc (IVD). 4. To investigate the effectiveness of combining cells and scaffolds transplanted in a rat model of lower back pain (LBP). These objectives will be achieved using behavioral assays, MRI analysis, and various biochemical, immunological, and molecular biology techniques. Overall, this research establishes a foundation for clinical studies aimed at regenerating the IVD and alleviating pain and suffering in patients with DDD.

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LIST OF ABBREVIATIONS

3D	Three-dimensional
ABL1	Abelson murine leukemia viral oncogene homolog 1
AD	Alzheimer's disease
AF	Amniotic fluid
AT	Adipose tissue
BM	Bone marrow
BRTX	BioRestorative Therapies
C	Caudal
CD	Cluster of Differentiation
CL	Cord lining
cLDD	Chronic Lumbar Disc Disease
COL1	Collagen type 1
COL2	Collagen type 2
CPJ	Cord-placenta junction
DDD	Degenerative disc disease
EGF	Epidermal growth factor

LIST OF ABBREVIATIONS - Continued

ECP	Cartilaginous Endplates
ESCs	Embryonic stem cells
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGF2	Fibroblast growth factor 2
GAG	Glycosaminoglycans
GMP	Good Manufacturing Practices
GvHD	Graft-vs-host disease
h	Human
H&E	Hematoxylin and eosin
HSCs	Hematopoietic stem cells
IACUC	Institutional Animal Care and Use Committee
IBC	Institutional Biosafety Committee
iPSC	Induced pluripotent stem cell
ISCT	International Society for Cellular Therapy
IVD	Intervertebral disc
IVDD	Intervertebral Disc Degeneration

LIST OF ABBREVIATIONS - Continued

LBP	Lower Back Pain
MSCs	Mesenchymal stem cells
NP	Nucleus pulpous
NPCs	Nucleus pulposus-like cells
PBS	Phosphate-buffered saline
PC	Placenta
PSC	Pluripotent stem cells
qRT-PCR	Quantitative reverse transcriptase-polymerase chain reaction
sGAG	Sulfated Glycosaminoglycans
SOX9	Sex determining region Y-box 9
TGFβ1	Transforming growth factor-beta 1
UC	Umbilical cord tissue
UCB	Cord blood
WJ	Wharton's jelly

CHAPTER ONE

INTRODUCTION

Lower Back Pain

Low back pain (LBP) caused by disc degeneration disease (DDD) affects a significant portion of the population (Chantal S. et al., 2021). The overall societal burden of individuals with LBP is expected to increase as the population ages. Therefore, this issue remains important in both research and clinical practice. The goal is to better understand and help reduce the shared pain and suffering associated with LBP (Hartvigsen et al., 2018). In 2015, it was reported that lower back pain limited the activities of 7.3 percent of the global population, which was about 540 million people. The burden of this condition has increased especially in low- and middle-income countries, where inadequate healthcare systems often struggle to manage it (Henschke et al., 2017).

LBP has several associated risk factors. Mechanical causes make up the majority of cases, often resulting from improper lifting of heavy objects, sudden twisting movements, or repetitive activities (Hartvigsen et al., 2018). Additionally, obesity is strongly linked to chronic low back pain due to its effects on the spine and systemic inflammation (Mafuyai et al., 2014).

The intervertebral discs (IVD) between the vertebrae of the spine provide mobility and shock absorption. Each disc consists of three main components: the nucleus pulposus (NP), the annulus fibrosus (AF), and thin cartilaginous endplates (ECP) (Clouet et al., 2011). IVD degeneration results from known factors that induce structural or biochemical changes

in these components. The most common factors are mechanical stress, aging, and genetic influences (Ciapetti et al., 2012). When each component degenerates, its function is impaired, leading to discomfort and pain. For example, the NP acts as a shock absorber because of its gel-like structure, which contains proteoglycans, sulfated glycosaminoglycans (sGAG), and water (Ciapetti et al., 2012). The AF, made of fibrous tissue, surrounds and contains the NP. Degeneration or herniation of the NP can lead to degenerative disease, and unfortunately, there is currently no cure for this condition (Kirnaz et al., 2022).

Physical therapies and painkillers often serve as the initial treatment for patients, especially when chronic pain persists, and irritation of the lumbar nerve roots causes pain to radiate below the knee. These short-term treatments are widely accessible and generally have a low risk of adverse effects (A. Shipton, 2018).

Various surgical techniques can be used to relieve pain. The most common procedures include discectomy (removal of herniated disc material), laminectomy (removal of the bony rear part of the spine), and fusion (joining two or more bones permanently). Other options, like artificial disc replacement, may be available for patients who are good surgical candidates (H Kim et al., 2018). However, these surgical techniques often fail because they focus on treating symptoms rather than addressing the root cause (Ampat et al., 2022; Wu et al., 2023). In this context, stem cell treatment aimed at regenerating the NP and alleviating LBP offers promising therapy for patients with DDD.

Stem Cell and Hypothesis

Stem cells are found in various organs and tissues throughout the body, and they have an exceptional ability to regenerate, playing a vital role in repairing damaged tissues throughout our lives. This regenerative ability is why they are used in cell therapy. However, understanding their therapeutic effectiveness is essential for treating DDD. Stem cells are categorized into three main types: embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and adult stem cells (ASCs) (see Figure 1). ESCs possess the unique ability to self-renew indefinitely and can differentiate into the three primary germ layers: ectoderm, mesoderm, and endoderm (Keller, G., 2005; Chaudhry, G., et al., 2009; Moon, S.Y., et al., 2005). Despite their potential for cell-based therapies, ESCs pose ethical and technical challenges, as well as a risk of teratoma formation when used in vivo (Zhang, W.Y., et al., 2008; Knoepfler, P.S., 2009).

iPSCs are created by reprogramming differentiated animal and human cells by introducing specific genes. However, concerns about tumor formation related to iPSCs arose from studies involving mice generated using the tetraploid complementation assay, which indicated a higher risk of cancer development (Kim, Jean J., 2015). As a result, adult stem cells, such as mesenchymal stem cells (MSCs) sourced from various tissues, have gained interest due to fewer ethical, moral, and safety concerns. MSCs can be isolated from bone marrow (BM), adipose tissue, and peripheral blood, and they have the potential to differentiate into multiple cell types, including adipogenic, chondrogenic, osteogenic, and neural lineages (Beeravolu, N., et al., 2016).

Although BM-MSCs are commonly used in research because of their autologous origin, harvesting them is invasive and can cause discomfort for patients. Additionally, BM-MSCs have limited capacity for growth and differentiation (Beeravolu, N., et al., 2016). Researchers have identified MSCs in perinatal sources, including umbilical cord blood (CB), umbilical cord tissue (UC), and the placenta. These perinatal sources, such as CB, UC, placenta (PC), amniotic fluid (AF), and fetal tissue—which is often considered biological waste - offer excellent alternatives for MSCs. They provide several benefits, including non-invasive collection, enhanced MSC expansion, and lower immunogenicity compared to BM-MSCs.

In our laboratory, we successfully isolated cells from these perinatal tissues, particularly from different niches within the UC and PC (Beeravolu, N., et al., 2017). All MSCs derived from segments of umbilical cord tissue, such as the cord lining (CL), Wharton's jelly (WJ), cord-placenta junction (CPJ), chorion (CH), and PC, were cultured as plastic-adherent cells. These cells expressed specific surface markers (CD73, CD90, and CD105) and tested negative for lineage markers (CD14, CD19, CD34, and CD45). Additionally, MSCs demonstrated multipotency by successfully differentiating in vitro into mesenchymal lineages, including adipocytes, osteocytes, and chondrocytes (Beeravolu, N., et al., 2016). Notably, MSCs derived from the CPJ showed the highest proliferation and differentiation capacities among the segments, suggesting they may be a more effective source for therapeutic applications.

It is crucial to avoid using undefined animal-derived components, such as fetal bovine serum (FBS), in stem cell cultures intended for human clinical trials. FBS is frequently

used as a growth supplement in in vitro human cell culture, but its use introduces variability and complicates therapeutic applications, raising concerns about xenogeneic contamination that could limit human transplantation. Therefore, developing animal-free (xenofree) media and culture conditions offers significant benefits for translational research and clinical trials (Aghayan, H., et al., 2022). Ongoing efforts aim to replace FBS in culture media with human platelet lysates and human serum (Subbiahanadar, C., et al., 2021; Zhang, Y., et al., 2025). In our lab, we have successfully isolated and expanded MSCs under defined xenofree conditions, and these MSCs exhibited similar immunophenotypes and multilineage differentiation capacity, with higher expansion rates compared to FBS cultures (Mazzella, M., et al., 2023).

Our research aims to develop a treatment to regenerate the nucleus pulposus (NP) using MSCs and their derivatives, namely nucleus pulposus cells (NPCs), in a rat model of DDD.

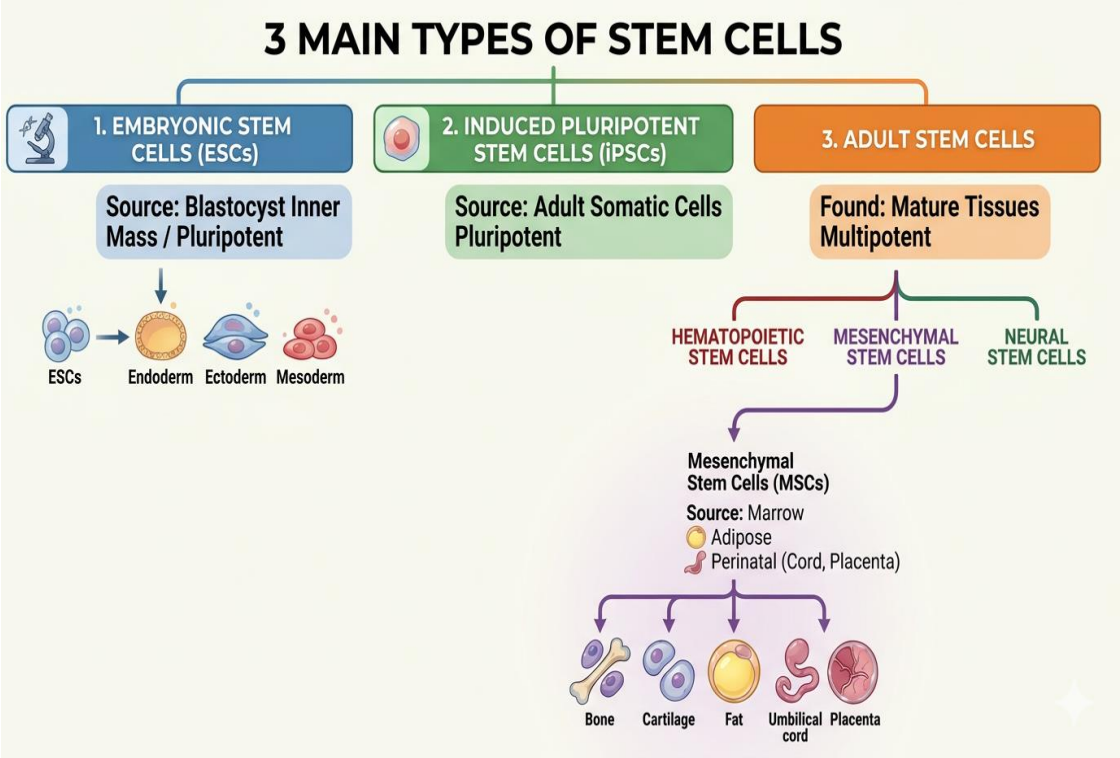

Figure 1: Types and sources of Stem Cells.

Significances of Stem Cells in Lower Back Pain Treatment

Persistent low back pain in older adults significantly affects their daily lives, influencing their social roles, coping strategies, and social settings (Wong et al., 2018). Currently, common treatments like physical therapy and pain medications mainly focus on controlling pain and delaying surgery for patients with DDD (Shi et al., 2020).

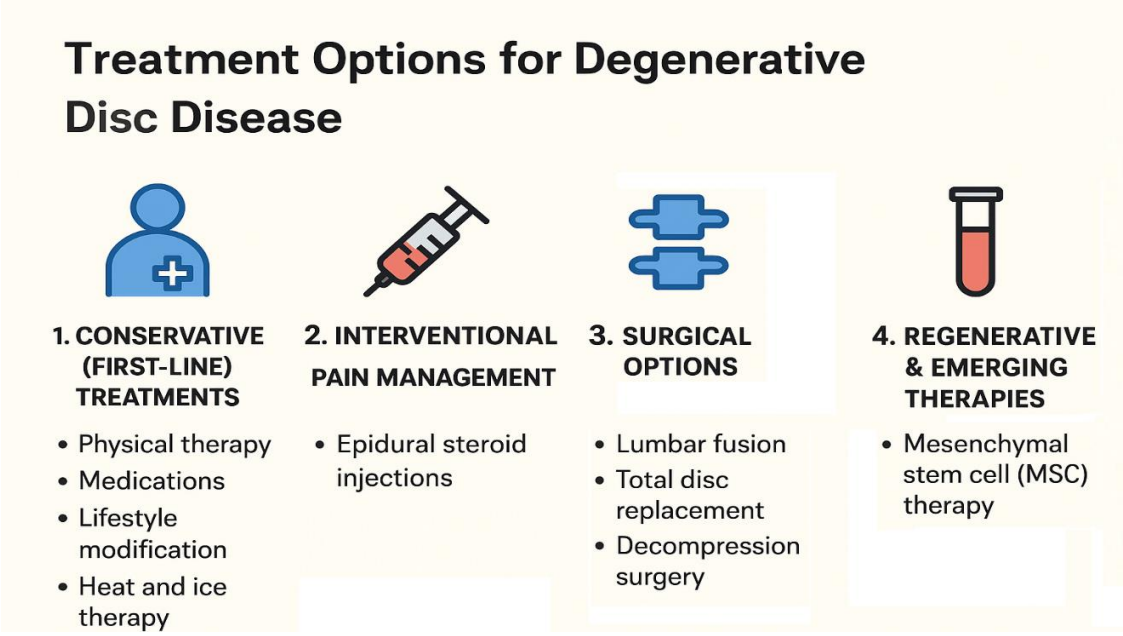

Figure 2: Current treatment options for degenerative disc disease (DDD)

Understanding the biology of the intervertebral disc at the genetic and molecular levels has advanced goals of restoration and tissue regeneration through cell-based approaches (Maerz et al., 2013). Early research highlights the role of pro-inflammatory cytokines and proteolytic enzymes in driving degeneration. However, multipotent stem cells have been shown to stimulate the regeneration of the disc's complex structure (Maerz et al., 2013). The success of cell therapy for disc repair depends on both the cell dose and the method of delivery (Sakai and Jordy, 2017).

Human-induced pluripotent stem cells (iPSCs) have the potential to generate notochordal-like cells that could address disc degeneration in large animal models (Nukaga et al., 2016). Although iPSCs carry a risk of teratoma formation, mesenchymal stem cells (MSCs) are considered safer and offer better immune privilege, making them suitable for allogeneic use (Zomer et al., 2015). MSCs are widely used in ongoing preclinical and clinical studies for the treatment of degenerative disc disease (DDD), with evidence indicating that they can alleviate pain and improve disc height (Song et al., 2020; Wang et al., 2015).

Animal models are essential for understanding intervertebral disc degeneration (IVDD) and evaluating regenerative methods. Puncture-induced injury models have been used in small animals such as rats, rabbits, and mice, resulting in disc degeneration in the lumbar region or in the tail. These small animal models offer effective platforms for studying injury responses and testing treatments (Daly et al., 2016; Li et al., 2014). Larger animals, such as dogs and pigs, share greater similarities with human disc size, loading, and biology, which is important for translational research (Wang et al., 2015).

Modeling disc degeneration is often done through annulus needle puncture under C-arm fluoroscopy or using a stereotaxic frame for precise targeting (Beeravolu et al., 2018; Li et al., 2014; Navone et al., 2021). Additionally, static bending and compression can also cause disc degeneration in animal models (Ji et al., 2021). These models replicate human-like degenerative processes and clinical outcomes, thereby reducing translational barriers.

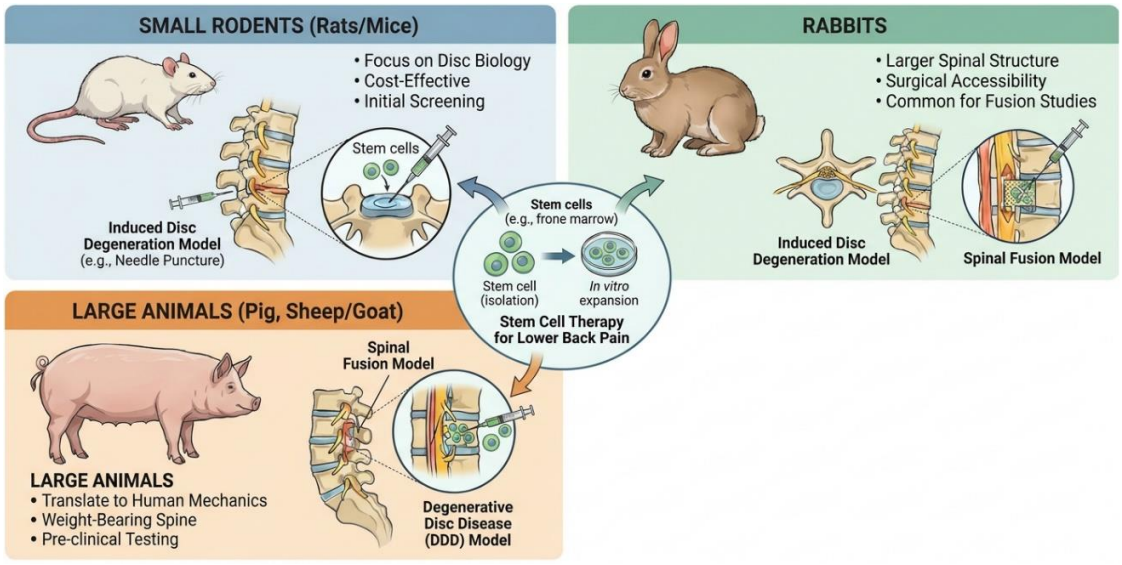

Figure 3: Animal models for investigating stem cell treatment in lower back pain

Specific Aims

Aim 1. To isolate and characterize mesenchymal stem cells (MSCs) using xenofree medium.

Hypothesis: MSCs can be isolated from ideal perinatal tissues, such as the umbilical cord, and cultured in a xenofree environment, where they grow similarly to MSCs cultured in animal-derived chemicals.

a) Isolation of MSCs using xenofree medium

Rationale: The current gold standard for culturing MSCs typically involves the use of animal-based products, such as fetal bovine serum (FBS). For a culture medium to be suitable for human clinical trials, it must be free of any animal-based components, including FBS. We aim to optimize xenofree media and identify the most commercially viable growth media for our MSCs. We will evaluate the ideal media based on factors such as cell morphology, population doubling times, and growth assays, and compare these results with those from routine FBS cultures.

b) Characterization of MSCs cultured in xenofree medium

Rationale: MSCs are characterized by their ability to adhere to plastic surfaces and their fibroblast-like morphology. They can be identified by the expression of specific surface markers, including CD105, CD90, and CD73, while they notably lack CD14, CD19, CD34, and CD45. Additionally, MSCs can differentiate into three distinct lineages: adipogenic, chondrogenic, and osteogenic. For analysis, MSCs will be examined using several methods, including light microscopy, flow cytometry, quantitative reverse transcription

polymerase chain reaction (qRT-PCR), and various immunohistochemical and histological stains.

Aim 2. To differentiate and characterize MSCs into nucleus pulposus (NP) like cells (NPCs)

Hypothesis: MSCs have the potential to differentiate into various cell lineages, including chondrogenic, osteogenic, and adipogenic progenitors. Specifically, we hypothesize that MSCs can differentiate into NPCs and regenerate the IVD in a DDD animal model.

a) Differentiation of MSCs into NPCs

Rationale: MSCs will be examined for their potential to differentiate into chondrogenic lineages such as notochordal cells or glycosaminoglycan (GAG)-producing cells that can be used to regenerate the NP and AF in the IVD.

b) Characterization of MSCs-NPCs

Rationale: This will be characterized by NP-specific markers (SOX9, ACAN, and COL2) using qRT-PCR and immunocytochemistry.

Aim 3 To develop three-dimensional (3-D) scaffolds that mimic the in vivo microenvironment of the intervertebral disc (IVD).

Hypothesis: Self-assembling scaffolds support the long-term growth and differentiation of stem cells. Specifically, we hypothesize that self-assembling scaffold MSCs have greater potential to regenerate the intervertebral discs in a degenerative disc disease animal model.

a) Selection and Composition of Scaffolds for 3-D Culture of MSCs and NPCs

Rationale: The scaffolds were tested in different compositions to identify the optimal concentration, molar ratio, and degree of modification that best support the growth of MSCs and NPCs.

b) Transplantation of MSCs-NPCs

Rationale: MSCs and NPCs were be investigated for their potential to regenerate the NP and AF in the IVD.

Aim 4. To investigate the effectiveness of a combination of cells and scaffold transplanted in a rat model of LBP

Hypothesis: It is suggested that pre-differentiation of MSCs is a necessary first step in the field of regenerative medicine. We hypothesize that MSC-derived NPCs would be more effective in regenerating the NP and AF in the IVD, respectively. This aim will focus on conducting preclinical studies to repair damaged IVDs using a rat tail model.

a) Determination the effects of transplanted cells in a rat model of LBP

Rationale: NPCs have the potential to restore the degenerated NP and AF in vivo. Therefore, NPCs can be used to regenerate damaged IVDs and treat degenerative disc disease in a rat model of LBP. Since there is no suitable animal model available to determine the feasibility and safety of therapeutic use of MSCs derivatives, we plan to use a degenerated rat tail model for IVD regeneration following the approach illustrated in the flowchart (Figure 4).

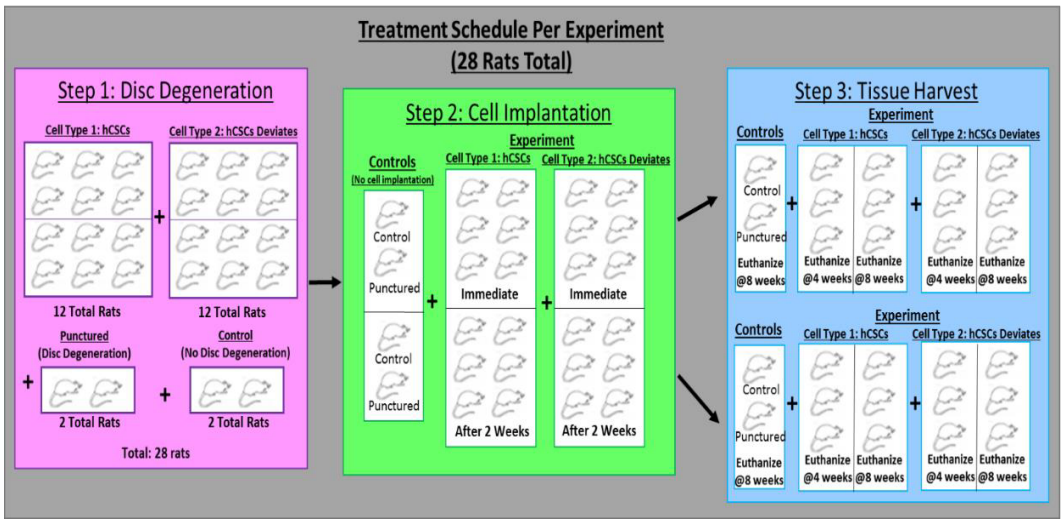

Figure 4: Experimental scheme of rat used for the regeneration of the IVD in a rat model

We propose an experimental approach to assess the feasibility and safety of transplanted MSCs and their derivatives in a rat intervertebral disc (IVD) model. In this experiment, we will inject two human-derived cell types into damaged rat tail IVDs to study tissue regeneration. A total of 84 rats will be required for this study.

To reduce the number of rats needed, we will harvest experimental discs (punctured disc with cell injection), sham discs (punctured disc), and control discs (intact disc) from the same rat. Additionally, we will need two control rats (each with all intact discs) and two rats with punctured IVDs (without cell transplantation) for behavioral assays in each replicate.

This experiment will follow the outlined scheme for both cell types and will be performed in triplicate to ensure statistically significant and reproducible results.

b) Optimization of the rat IVD degeneration model for low back pain

Rationale: To establish a tail-puncture model of intervertebral disc degeneration (IVDD), the rat tail will be cleaned with 70% ethanol. The coccygeal disc will then be punctured using a sterile 21-G × 1-inch needle to induce disc degeneration.

c) Post-implantation analyses of transplanted cells into Degenerated Intervertebral Discs

Rationale: To further analyze the molecular mechanisms involved in IVD regeneration, we will perform biochemical, molecular, and immunohistochemical analyses. This will help evaluate the transcriptional and translational levels of gene expression in transplanted cells in more detail.

Figure 5: Conventional x ray image for normal rat tail caudal C3-4 experimental (punctured disc with cell injection), C4-5 sham (punctured disc), and C5-6 control (intact disc).

Problems and Challenges

In our lab, mesenchymal stem cells (MSCs) have demonstrated promising results in both preclinical and clinical studies for various treatments (Beeravolu et al.). Research suggests that MSCs can be differentiated into notochordal precursor cells (NPCs) to support the regeneration of the nucleus pulposus in intervertebral discs (IVD) (Beeravolu et al., 2018; Perez-Cruet et al., 2018). Despite advancements and enthusiasm for using MSCs in therapy, several challenges remain. One notable benefit of MSCs is their ability to proliferate in vitro for a limited number of passages before entering senescence (Liu et al., 2020).

MSCs were exposed to various culture conditions, including 3-D cultures and hypoxic environments, to help maintain their undifferentiated state, promote self-renewal, and preserve their therapeutic potential by simulating their natural niche (Ohori-Morita, Y. et al., 2025). Additionally, isolating and culturing MSCs in xenofree conditions presents a significant challenge, as traditional fetal bovine serum (FBS) carries risks of immunogenic reactions (Patrikoski, M. et al., 2013). This study aims to address these challenges by isolating MSCs in xenofree conditions, thereby facilitating their use in future therapeutic applications.

CHAPTER TWO

MATERIAL AND METHODS

Aim 1. To isolate mesenchymal stem cells (MSCs) using xenofree medium

Background

MSCs are typically cultured in media enriched with fetal bovine serum (FBS), raising safety concerns that limit their application in cell therapy (Rakian et al., 2015). To resolve this issue, we propose developing xenofree media and culture conditions for our stem cells. Several studies have reported that human blood-derived alternatives, such as human serum and human platelet lysate, can replace animal serum when cultivating human cells in commercially available xenofree media (Subbiahanadar et al., 2021; Zhang et al., 2025).

In our lab, we have successfully optimized a xenofree basal medium and a serum supplement to replace FBS (Mazzella et al., 2023). Xenofree conditions were employed from the dissection of the umbilical cord (UC), with the manually minced tissue plated as explants in culture flasks without FBS. Once the cells grew out from the tissue pieces and reached 70% confluency, they were dissociated from the tissue flasks and classified as passage 1 (P1) cells.

Notably, cells maintained in xenofree media exhibited greater proliferation and shorter doubling times compared to those grown in FBS. Overall, we plan to analyze multiple UC samples to compare the reproducible outgrowth and proliferation of these cells in both FBS and xenofree media. Additionally, we have optimized FBS-free cryopreservation of umbilical cord tissue and cells using human serum, which successfully revived the

cryopreserved minced tissues and cells (Beeravolu et al., 2016; Beeravolu et al., 2018; Sono et al., 2024; Mazzella et al., 2023).

Aim 1 Method

a. Isolate MSCs Using Xenofree Medium

Umbilical cord samples were collected immediately after birth from Providence Hospital following informed consent and approved protocols in a sterile bottle basal medium supplied with antibiotics (penicillin and streptomycin) (IRB# 820662 and IBC# 2858). In a biosafety cabinet, the cord sample was placed in a sterile petri plate on ice with the help of forceps and rinsed thoroughly with cold autoclaved phosphate-buffered saline (PBS) to eliminate debris and blood clots (Beeravolu N., et al., 2017). The tissue was then dissected into five segments: CT, WJ, CPJ, CH, and PC, and minced into small pieces of 1–2 mm (Figure 6). Each cord tissue segment piece was plated in medium containing DMEM nutrient mix F12 medium (DMEM/F12; Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (VWR, Radnor, PA, USA), and 5.6% of antibiotic solution (0.1% gentamicin, 0.2% streptomycin, and 0.12% penicillin) (Sigma, St Louis, MO, USA)., under standard incubation conditions (37 °C, 5% CO2) (Mazzella, M., et al., 2023). The tissue is small pieces plated in 75 cm2 culture flasks for 2-3 days to allow for the adherence of tissue pieces. Fresh medium was changed with 9 ml after 3 days. Once the outgrowth of cells from the explants was observed under a light microscope, they were dissociated using 1 ml of commercial TrypLE solution per 75 cm2 culture flask and sub-cultured. The trypsinized cells were neutralized with 1-2 ml of medium and centrifuged at 3000 RPM for 3 minutes. The supernatant was removed, and the cells were resuspended in

2 ml of a selected culturing medium. Cells were then amplified, characterized, cryopreserved, and considered to be P1. Cells at passages 2–4 will be used in all subsequent experiments and maintained in xenofree medium. Medium was comprised of MSC NutriStem® XF Medium supplemented with MSC NutriStem® XF Supplement Mix (Sartorius, Goettingen, Germany) and the 5.6% of antibiotic solution. Light microscopy images were acquired daily to monitor growth and morphological changes. Once the cells reached 70% confluency, they were dissociated using a commercial TrypLE solution at each passage.

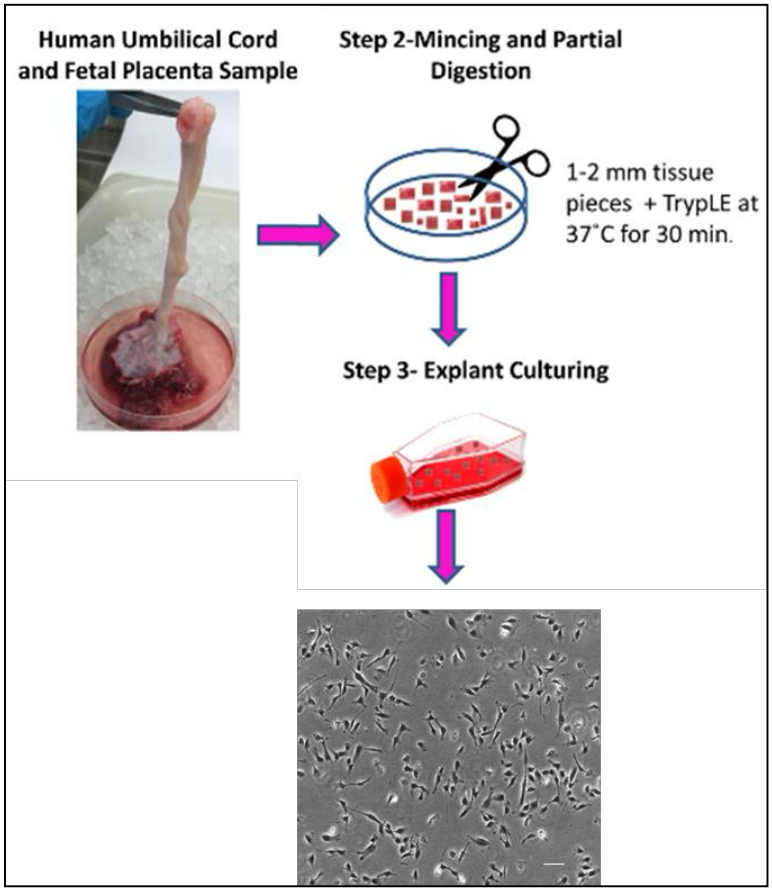

Figure 6: Isolation and characterization of mesenchymal stem cells (MSCs) using Xeno-free medium (Beeravolu, N., et al., 2016).

b. Characterize MSCs Cultured in Xenofree medium

To identify and quantify the specific surface marker profile defined by the International Society for Cell & Gene Therapy (ISCT), cells were grown to 70% confluency and harvested for analysis. The harvested cells were treated with TryplE and neutralized with an equal volume of culture medium. They were then centrifuged at 3000 RPM for 3 minutes. After removing the supernatants, the cell pellets were re-suspended in 2 ml of PBS for a wash to remove any residual media. The cells were centrifuged again to remove the supernatant and then re-suspended in FACS buffer (0.1% sodium azide in PBS containing 2% FBS).

A total of 100,000 cells were suspended in 100 µl of FACS buffer and stained with FITC-conjugated antibodies against CD44 and CD90, as well as APC-conjugated antibodies against CD73, CD29, and CD105 (Becton Dickinson, Franklin Lakes, NJ, USA). The cells were then incubated for 30 minutes at 4ºC in the dark. Each 100,000-cell sample was centrifuged to remove the supernatant and washed with 200 µl of FACS buffer. The cells were immediately analyzed on a FACS Canto II (Becton Dickinson) using Diva Software (Becton Dickinson). The percentages of positive and negative cells, which represent the cell population expressing the selected MSC surface markers, were analyzed.

Aim 2. To differentiate MSCs into nucleus pulposus (NP) like cells (NPCs)

Background

Lower back pain is the leading cause of disability worldwide, affecting millions of individuals (GBD 2021 Low Back Pain Collaborators, 2023). The healthcare costs and consequences associated with this condition have become a significant socioeconomic burden (Lin, Chung-Wei et al., 2018). Intervertebral disc (IVD) degeneration is strongly linked to lower back pain (Kirnaz, S. et al., 2022). The IVD, located between the vertebrae, provides mobility and shock absorption. It consists of three main components: the nucleus pulposus (NP), the anulus fibrosus (AF), and the thin cartilaginous endplates (ECP) (Mohd Isa, I. et al., 2022). IVD degeneration is caused by various known contributors that lead to structural or biochemical changes in these components.

Mesenchymal stem cells (MSCs) derived from cord blood (CB) have the potential to differentiate into chondrogenic, osteogenic, and adipogenic progenitors. Thus, MSC therapy represents a promising approach for treating degenerated intervertebral discs (IVD) (Beeravolu, N. et al., 2018). Additionally, the pre-differentiation of MSCs is a crucial step in the field of regenerative medicine (Han, Yu et al., 2019). In this study, MSCs will be differentiated into glycosaminoglycan (GAG)-producing cells or nucleus pulposus cell progenitors of notochordal lineage (Nair, D. et al., 2014). This will be accomplished through the addition of growth factors. Notochordal progenitors will be characterized using biochemical, immunological, and molecular biology techniques.

The MSCs and the differentiated cells will be transplanted into an animal model of lower back pain to repair and regenerate the degenerated IVD. We will also investigate the

molecular mechanisms underlying IVD regeneration using various techniques, including quantitative reverse transcription PCR (qRT-PCR), confocal microscopy, and immunohistochemistry.

Aim 2 Method

Differentiation of MSCs Into NPCs

We will differentiate MSCs to NPCs that were isolated in Aim 1. MSCs will be maintained in xeno-free media. In this study, we aim to differentiate MSCs into notochordal cells that may be more efficient for the regeneration of damaged NP tissue. Cells were plated at a concentration of 105 cells/6-well in 2 ml of culture medium and grown overnight. The following day, MSCs will be differentiated by culturing in a cocktail of growth factors including 2% FBS, 20 ng/mL TGFβ (PeproTech, Rocky Hill, NJ, USA), 100 nM dexamethasone (Thermo Fisher Scientific), 20 ng/mL insulin (Peprotech), 50 μg/mL ascorbic acid (Thermo Fisher Scientific) and 20 ng/mL BMP7 at 37ºC in a humidified incubator with 5% CO_2 for 3 weeks. Growth factors will help in MSCs induction toward their respective lineages. We will confirm notochordal cell differentiation using various techniques, including morphological changes, FACS, qRT-PCR, and immunohistochemistry, with positive staining for NP cells and their ECM-related gene expression. The expression of SOX9, aggrecan (ACAN), and collagen II (COL II) is a chondrogenic-specific extracellular matrix marker, while KRT19, COMP, and FOXF1 are more specific for NP. Differentiated cells will also be analyzed by flow cytometry based on decreased expression of mesenchymal surface markers (CD29, CD44, CD73, CD90, and CD105).

Characterization of NPCs

Flow Cytometry analyses

After cells were grown in differentiation medium for 2 weeks, TryplE was applied to the collected cells, and an equivalent volume of growth media was used to neutralize them. They were then centrifuged for three minutes at 3000 RPM. Similar to flow cytometry procedure of MCSs, Cells will be washed and re-suspended in FACS buffer. Each 100,000 cells will be stained with FITC-conjugated antibodies against CD44 and CD90, as well as APC-conjugated antibodies against CD73, CD29, and CD105 (Becton Dickinson, Franklin Lakes, NJ, USA). After cells incubation in dark, cells were immediately analyzed on a FACS Canto II (Becton Dickinson) using Diva Software (Becton Dickinson). The depleted percentages of positive and negative cells, which represent MSCs cell population expressing the selected MSC surface markers, were analyzed to confirm MSCs differentiation.

Immunocytochemical analyses

Immunocytochemical analyses of MSCs cultured in differentiation medium will be done using specific markers SOX9, ACAN and Collagen II by confocal images. Cells will be cultured on coverslips, then washed with PBS and fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature (RT). Cells will be permeabilized with 0.2% Triton X-100 in PBS for 10 min, then rinsed three times with PBS for 5 min each. Non-specific binding sites will be blocked by treating the cells with blocking solution (2% bovine serum albumin in PBS) for 1 h at 37 °C. The cells will be stained with specific primary antibodies diluted in the blocking solution at the recommended dilutions and incubated overnight at 4 °C. The

next day, cells were washed three times for 5 min each with PBS and then stained with secondary antibodies at 1:200 dilution for 2 hours at room temperature. Fluorescent images were captured using a confocal microscope (NIKON Instruments Inc.). For proteoglycan analysis, differentiated MSCs were washed 3 times with PBS, then fixed in 4% paraformaldehyde for 10 minutes and washed with PBS 1x. Extracellular matrix produced by the NPCs will be assessed by staining the cells with toluidine blue solution (Sigma) for 1 hour at room temperature. After washing cells with distilled water, cells were visualized under a light microscope to observe blue 42 staining which indicates synthesis of proteoglycans

Immunocytochemical staining for ECM production

Cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature. Cells are then washed twice with PBS. Alcian blue staining: pH was adjusted to 2.5, and a 1% Toluidine Blue O solution was prepared. Cells were incubated in Alcian Blue solution (pH 2.5) for 30 minutes at room temperature, or in Toluidine Blue O solution for 10 minutes. The plate was then rinsed in 3% Acetic Acid to remove excess stains or in distilled water. Cells were covered with PBS and observed under a light microscope to analyze the ECM production.

qRT-PCR analyses

qRT-PCR analyses of differentiated cells will be performed to detect chondro-specific differentiation markers in the harvested Cells. RNA will be isolated using the SV Total RNA isolation GeneJET RNA purification kit (Fisher Scientific) according to the manufacturer's protocol. The total RNA will be purified by incubating with DNase at 37

°C for 30 minutes using a thermocycler (Bio-Rad, Hercules, CA, USA). First-strand cDNA will be synthesized by using the iScript kit (Bio-Rad). Amplification reactions will be performed in three biological replicates in 96-well plates using Sso-Advanced Universal SYBR Green Supermix Kit (Bio-Rad) on CFX96 Real-Time System (Bio-Rad). A 10 µl reaction will be used, containing 5 µl of SYBR Green, 3 µl of distilled H2O, 0.5 µl of forward primer, 0.5 µl of reverse primer, and 1 µl of 1:10-diluted cDNA. Each reaction will be subjected to the following conditions: 98°C for 10 min, followed by 44 cycles of 98°C for 30 s, 60°C for 20 s, and 72°C for 30 s in 96-well optical reaction plates (Bio-Rad). GAPDH and ACTIN will be used as reference genes to normalize the target gene. qPCR analysis of specific differentiation markers will be performed using specific primers of chondrogenic (SOX9), and NP specific markers (FOXF1, CA12, COMP, and KRT19).

Aim 3. To develop three-dimensional (3-D) scaffolds that mimic the in vivo microenvironment of the intervertebral disc (IVD)

Background

Hydrogels are three-dimensional polymeric networks that can absorb and retain large amounts of water while preserving their structural integrity (Nair, D. et al., 2014). These materials are increasingly important in various applications, particularly in the biomedical field, including drug delivery, tissue engineering, and biosensing (Quazi, Z. et al., 2025; Wang, K. et al., 2019). Recently, self-assembling scaffolds have been developed to create structures that promote cell and tissue growth and regeneration (Nair, D. et al., 2014).

One notable scaffold design is the 8-arm polyethylene glycol (PEG), which has gained attention for tissue engineering applications involving mesenchymal stem cells (MSCs) (Ju, Y. et al., 2022; Su, X. et al., 2021). PEG is favored due to its hydrophilicity, low toxicity, biocompatibility, and, most importantly, its ability to form hydrophilic hydrogels (Nair, D. et al., 2014). Hydrogels are highly sought after in tissue engineering because they can mimic the natural extracellular matrix (ECM) found in human tissues, allowing MSCs to grow and differentiate into various cell types (Jung, P. et al., 2016). Consequently, scaffolding plays a crucial role in modulating MSC interactions.

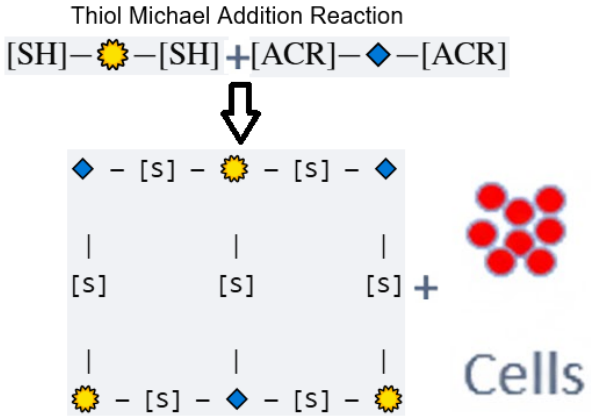

Figure 7: Development of three-dimensional (3-D) scaffolds that mimic the in vivo microenvironment of the (IVD)

Aim 3 Method

a) Selection and composition of scaffolds for 3-D culture of MSCs and NPCs

Thiol and acrylate functionalities are particularly appealing because they enable efficient network formation through thiol–Michael addition chemistry (McKee et al., 2019). The scaffolds were composed of two 8-arm polyethylene glycol (PEG) polymers, thiol-terminated (PEG-8-SH) and acrylate-terminated (PEG-8-Acr), that underwent self-assembly. Various scaffold formulations were tested to determine the optimal concentration, molar ratio, and level of modification to effectively promote mesenchymal stem cell (MSC) growth (Nair et al., 2014). The ideal concentration was found to be 2 w/v% (dry weight of polymer per volume of culture medium) at 4 °C, which polymerized in 19 minutes (Figure 8).

Percentage %	Time to polymerization
2.5% at room temp	6.33 min
2.5% on ice	18.13 min
2% at room temp	7.8 min
2% on ice	19.55 min
1% at room temp	18.22 min
1% at 37 °C	12.3 min

Figure 8: Polymerization Time of scaffold was made by mixing thiol (PEG-8-SH) and acrylate (PEG-8-Acr) in different temperature and various concentrations

b) Transplantation of MSCs-NPCs

After dissolving PEG-8-SH and PEG-8-Acr polymers at a concentration of 2 w/v%, they were combined at a 1:1 molar ratio and mixed with cells on ice. The resulting mixture was

then transferred using a sterile 21G needle syringe before polymerization, and it was immediately injected into the rat tail intervertebral disc (IVD).

Aim 4. To investigate the effectiveness of a combination of cells and scaffold transplanted in a rat model of LBP

Background

There is currently no treatment that can cure the underlying causes of DDD (Kirnaz, S., et al., 2021). Existing surgical options have many limitations, and current treatments primarily focus on pain relief rather than addressing the root of the problem. However, transplanted human stem cells show great promise as a potential cure for DDD.

This study aims to conduct preclinical research to regenerate the IVD using a rat model of LBP. To induce degeneration of the IVD, we will puncture the disc with a needle. After two weeks, we will transplant the cells and subsequently analyze disc regeneration through behavioral assays, histological evaluations, and biochemical and molecular biological techniques.

Aim 4 Method

Determination of the effects of transplanted cells in a rat model of LBP

NPCs have the potential to restore the degenerated NP and AF in vivo. Rat tail IVD was degenerated and served as a model for IVDD. This model serves as an effective, high-throughput in vivo models by mimicking human lumbar mechanics through controllable, minimally invasive techniques. Deu to anatomical similarity, a needle puncture to the NP would cause a progressive disc degeneration. This technique is easy to access and monitor

the progress of disease and treatment. Sprague Dawley rat tail is the most common animal use. We started the experiment at 6 weeks old to allow long-term investigations.

Optimization of rat IVD degeneration model for low back pain

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Oakland University in Rochester, Michigan (IACUC #20062), as well as the Institutional Biosafety Committee (IBC) at the same institution (IBC #2858). Sprague Dawley male rats were obtained directly from Charles River. Initially, these rats were anesthetized with isoflurane. Rimadyl (5 mg/kg, subcutaneously) was administered to the dorsal flank 3 hours prior to the procedure. The skin on the rat's tail was sterilized with 70% ethanol, and a 3 cm incision was made on the dorsal side of the tail to access the caudal (C3-4, C4-5, and C5-6) disc spaces. Two adjacent discs at C3-4 (experimental group, involving puncture and cell injection) and C4-5 (sham group, with only a puncture) were punctured to induce degeneration of the rat tail discs. The C5-6 disc served as the control and was not punctured. A sterile 21-G needle was used to pierce the tail from the ventral to the dorsal side, targeting the center of the nucleus pulposus (NP). Needle penetration depth was controlled using a stereotaxic device, with a target depth of 5 mm to reach the center of the NP. The incision was closed with a 5.0 Vicryl suture (Ethicon Inc., Somerville, NJ). The rats were allowed to recover for two weeks to enable the gradual degeneration of the IVD.

Labeling and transplantation of cells in degenerated IVD's

Following the supplier's instructions for the cell membrane-labeling dye PKH26, the cells were washed with PBS, harvested with TrypLE, and neutralized. They were then washed again in DMEM without FBS and centrifuged at 2000 RPM for 5 minutes. Meanwhile, a 2X cell suspension was prepared by adding 1 ml of Diluent C to the cell pellet. Immediately, the 2X dye solution was prepared in the same Diluent C by adding 4-5 µl of the dye to 1 ml of Diluent C in a centrifuge tube and mixing thoroughly. The cell suspension and dye solution were then combined by pipetting and incubated at 37°C for 5 minutes. An equal volume of FBS was added, and the mixture was incubated at 37°C for an additional minute. The cells were centrifuged and washed with 10 ml of medium and then centrifuged at 2000 RPM for 10 minutes twice. Confocal microscopy was used to confirm the efficiency of cell fluorescence labeling prior to transplantation. PKH labeling of the cells was also assessed by flow cytometry.

The rats were anesthetized using an isoflurane gas induction chamber, and the tails were sterilized with 70% ethanol. A 3 cm incision was made on the dorsal side of the tail to access the C3-4 disc space. We differentiated our mesenchymal stem cells (MSCs) into nucleus pulposus cells (NPCs) and labeled them with the cell membrane stain PKH26 for cell transplantation and tracking purposes in short- and long-term analyses.

A total of 1 x 10^6 labeled cells in 15 µl of PBS solution were injected into the C4-5 degenerated disc segment using a Hamilton syringe equipped with a 27-gauge needle. No cells were injected into the C4-5 degenerated control disc, while C5-6 served as a normal control. Following the procedure, rats were returned to their respective cages and

monitored for 1 hour post-surgery. Additionally, the rats were monitored daily for any signs of distress or behavioral changes until the harvesting endpoints were reached. The animals were regularly observed over the experimental timeline (as shown in Figure 9) for 4 to 8 weeks following cell transplantation.

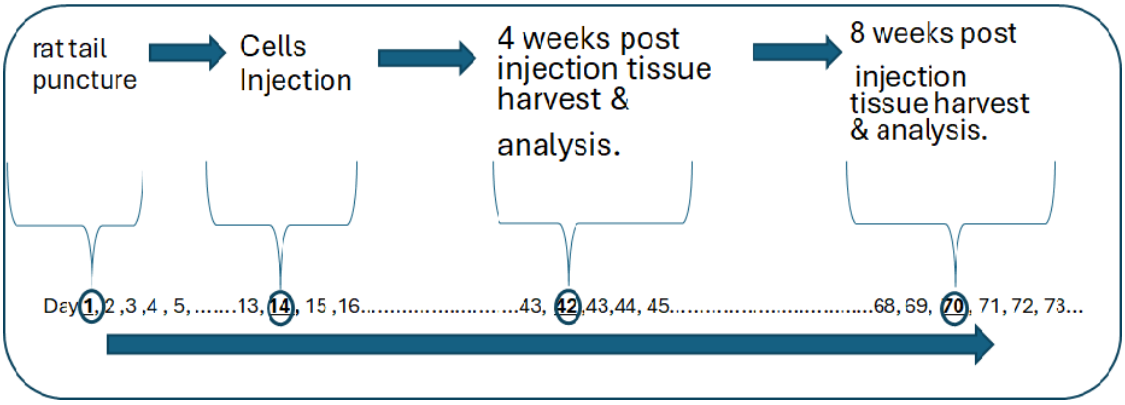

Figure 9: Groups of animals and treatment plan, with two main rat groups: MSCs treated IVD with and without scaffold, and NPCs treated IVD with and without scaffold.

Behavioral Analysis

The two behavioral assays to be used are the mechanical sensitivity assay and the inclined balance beam test. The investigators conducting these assays will be blind to the rats' conditions to prevent any bias in the results. Mechanical sensitivity to varying pressures will be assessed using von Frey hairs (see Figure 10), which will be applied to the underside of the hind paw.

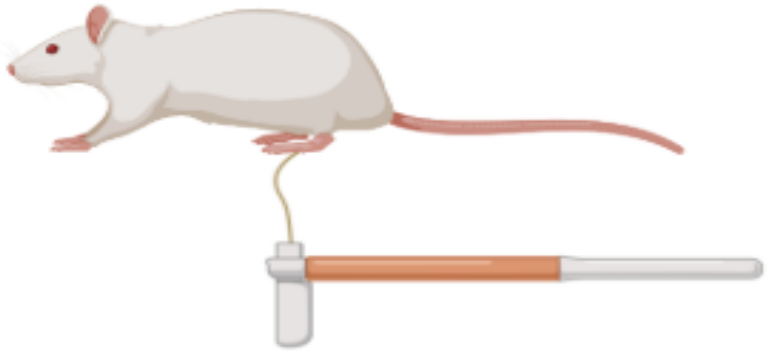

Figure 10: Von Frey hairs applied to the bottom of the hind paw

This study was conducted one week before disc degeneration, on the day of disc degeneration, and once a week thereafter until the rats were euthanized. Each rat was placed in an individual cage with a mesh bottom to allow access to their hind paws. To measure the pressure required for a rat to respond to pressure on its hind paw, a probe connected to a measuring device was used. The probe was held with both hands, and pressure was applied slowly to the middle of either the left or right hind paw. The pressure was increased until a behavioral response is observed, such as hind paw retraction, hind paw licking, or jumping. The amount of pressure at which the response occurs was then recorded. This assay was performed on each rat five times at every time point.

Control rats, which had all intact discs, and punctured-only rats (those with punctured intervertebral discs but without transplanted cells) were used as negative and positive controls, respectively, for the behavioral assays in each replicate.

The balance beam (Figure 11) was conducted one week before disc degeneration, on the day of disc degeneration, and once a week thereafter until the rats were euthanized. This was done in a procedure room in the animal facility.

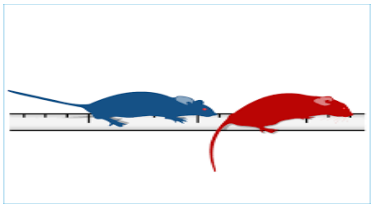

Figure 11: Balance beam test when rat tail is used for balance

The balance beam will be 6.5cm wide and 122cm in length. The balance beam will be 60cm above ground, and a net will be placed below the beam. The rats will be trained two days prior to reduce neophobia, the fear of anything new. A wider beam will be used, and gentle guiding can be done until the animal crosses the beam. Once the animals are trained, they will undergo trial runs and be analyzed. Each rat will be timed as they cross the beam. Each rat will run the balance beam for 3 trials at each time point. The behavioral assays will provide evidence (changes in behavior) for the functional recovery of IVDs upon cell treatment.

Post implantation analyses of transplanted cells into Degenerated Intervertebral Discs

Harvesting rat tail disc for cell tracking

The animals were sacrificed by CO2 overdose, and the tails were harvested and unskinned after 4 weeks or 8 weeks post-transplantation. After removing residual bone fragments, leaving only the disc, the tissue was fixed in 4% paraformaldehyde for 10 minutes, then placed in 10% sucrose for 30 minutes, and finally in 30% sucrose overnight at 4 °C. Then, tissue was embedded in OCT medium (Thermo Fisher Scientific) and sectioned (10 μm thick) using a cryostat.

The sections were observed under confocal microscopy for the presence of PKH26 red dye (Cy3)- labeled cells throughout the disc sections, using fluorescent images captured with a confocal microscope (NIKON Instruments Inc., Melville, NY, USA). To mount, the section was flattened on the slide, then fixed in 4% paraformaldehyde, and analyzed using a light microscope, with images captured.

Histological analysis

Paraffin sectioning

The animals were sacrificed by CO2 overdose, and the tails were harvested and unskinned after 4 weeks or 8 weeks post-transplantation. After removing remnant bone pieces, leaving only the disc, tissue was fixed in in 4% paraformaldehyde for 10 minutes at 4 °C. Tissue was then dehydrated using increasing concentrations of alcohol (50% to 70 % to 100 % ethanol) for 2-3 minutes per step, then 100% ethanol for 15 minutes, and then transferred

to xylene for 15 minutes. Discs were then embedded in paraffin and sectioned (10 μm thick) using a microtome.

Hematoxylin and Eosin (H&E) staining

Disc tissue sections were dehydrated with xylene for 2 minutes, followed by 100% ethanol, 95% ethanol, 70% ethanol, 50% ethanol for 2 minutes each. The sections were then washed with distilled water for 1 minute, then stained with hematoxylin (Sigma) for 45 seconds. The sections were then rinsed with 95% ethanol for 1 minute and counterstained with eosin (Sigma) for 1 minute. The sections were then washed with 95% ethanol, then 100% ethanol for 1 minute each. The sections were then cleaned with xylene for 2 minutes. The sections were then mounted with Permount (Thermo Fisher Scientific).

Immunohistochemical staining

Tissue sections were deparaffinized using xylene for 5 min, then hydrated through graded alcohols and washed in distilled water. Alcian blue staining PH was adjusted to 2.5. The section was incubated in Alcian Blue solution (pH 2.5) for 30 minutes at room temperature. The slide was then rinsed in 3% Acetic Acid to remove excess stain, then in distilled water. The slide was then dehydrated through graded alcohols, cleared in xylene, and mounted with Permount (Thermo Fisher Scientific).

Immunostaining Staining

To detect expression of human notochordal markers in disc sections, samples were stained with primary antibodies at a 1:100 dilution at 4 °C overnight, followed by staining with secondary antibodies at a 1:200 dilution (Col2 and human-specific antibody K19) at room

temperature for 2 hours. They were counterstained with DAPI at a 1:100 dilution for 5 minutes at room temperature. Representative fluorescent images/sections were captured using a confocal microscope (NIKON Instruments Inc.).

Magnetic resonance imaging (MRI) evaluations

An MRI of the IVD was performed before the puncture and two weeks afterwards to confirm degeneration. Additionally, MRI scans were performed at 4 and 8 weeks post-cell transplantation to assess regeneration. After euthanizing the rats, a localized T2-weighted image was obtained to examine the experimental group (punctured disc with cell injection), the sham group (punctured disc), and the control group (intact disc). The discs, specifically the caudal regions (C3-4, C4-5, and C5-6), were harvested from the same rat. T2-weighted sections were obtained in both sagittal and axial planes. IVD degeneration and regeneration was assessed based on changes in signal intensity. The nucleus pulposus (NP) content was analyzed by measuring the mean signal intensity using Philips DICOM viewer software (Philips Healthcare).

CHAPTER THREE

HUMAN PRIMITIVE MESENCHYMAL STEM CELL-DERIVED NOTOCHORDAL PROGENITOR CELLS AND SCAFFOLD PROMOTED IVD REGENERATION IN LBP RAT MODEL.

Generation of Xenofree primitive MSCs

For isolation and amplification, MSCs are often cultivated with growth hormones and supplements derived from animal FBS. However, using certain animal products in cell culture, including FBS, presents restrictions and safety concerns. Additionally, the components of FBS are unknown and may carry risks of zoonotic infections that could be transmitted to humans after cell transplantation (Bieback K., 2013). We have explored alternative xenofree serums in our lab and standardized MSCs isolation, amplification, and differentiation in xenofree medium (Brown C., 2022). In our lab, tissue culture flasks with xenofree culturing media were plated. We monitored the tissue daily for cell outgrowth that emerged after approximately one week. The isolated cells grown in xenofree medium were then subjected to further analysis to characterize the cells and assess their differentiation potential. We standardized perinatal MSCs to differentiate into mesenchymal lineages (chondrogenic, osteogenic, and adipogenic cells) in vitro using selective differentiation media. This approach may enable the use of these cells in human clinical trials.

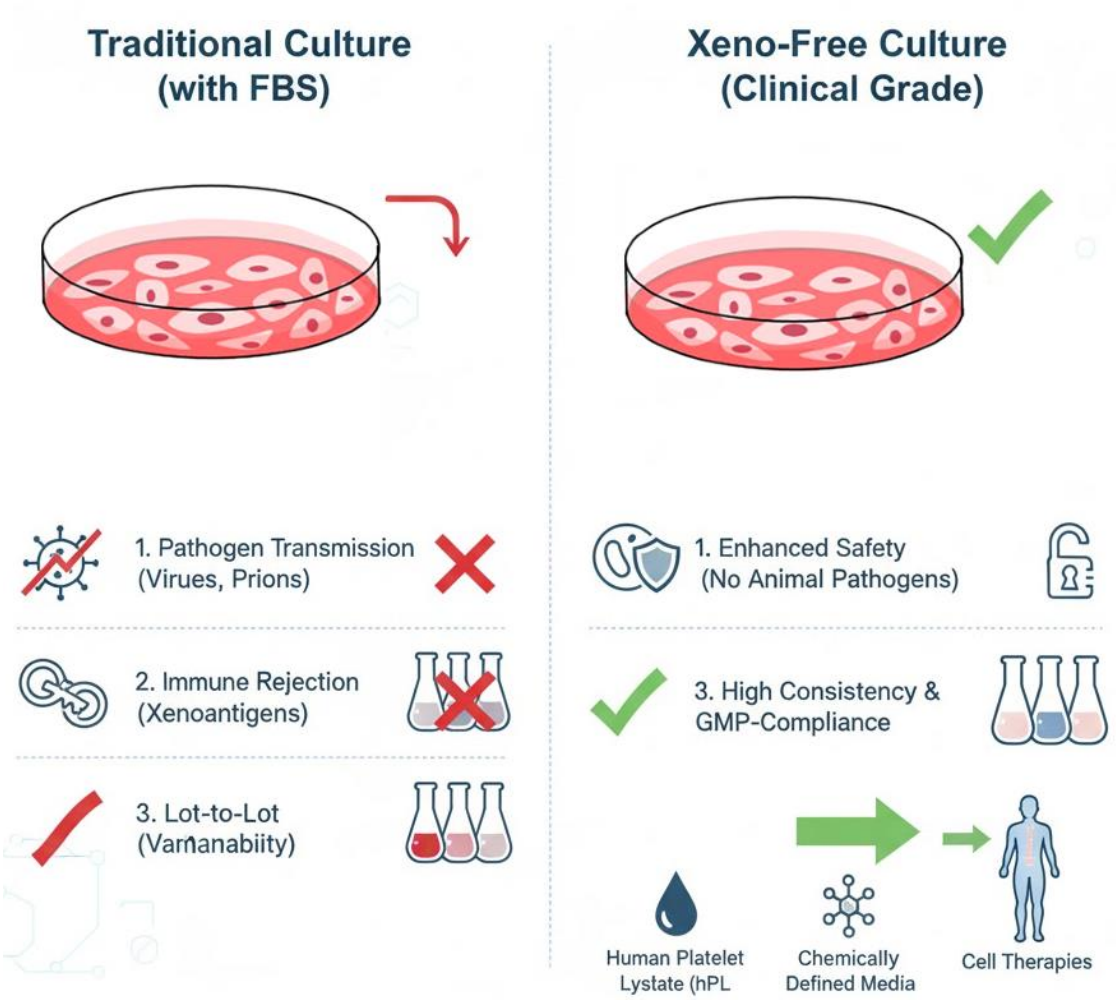

Figure 12: The adoption of xenofree media is driven primarily by the need to increase safety, regulatory, and consistency for cell therapies intended for patients.

Surface marker analysis of primitive MSCs

MSCs were further characterized by FACS analysis for expression of MSC-specific surface markers, including CD29, CD44, CD73, CD90, and CD105, using xenofree medium. The FACS analysis (Figure 13) shows that all markers represent more than 90% of the MSC population.

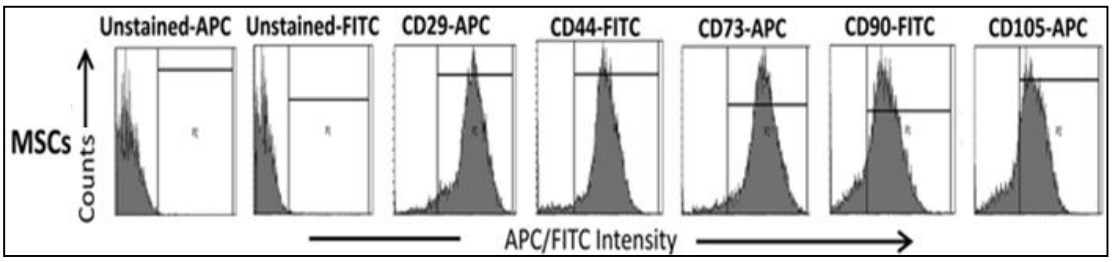

Figure 13: Histogram's representation of expression of mesenchymal markers in MSCs by flow cytometry

MSCs generation into nucleus pulposus (NP) like cells (NPCs)

We first differentiated the primitive MSCs into NPCs. Cell morphology showed some changes from the typical MSCs morphology (Figure 14). Histograms and graphical representations of the expression of MSC markers, as determined by flow cytometry, indicated that the expression of markers such as CD29, CD44, CD73, CD90, and CD105 was significantly reduced in NPCs (Figure 15). The differentiation was further validated by the expression of notochordal genes, as determined by qRT-PCR. The expression of the notochordal genes SOX9, ACAN, and COL2 was demonstrated by NSCs (Figure 16).

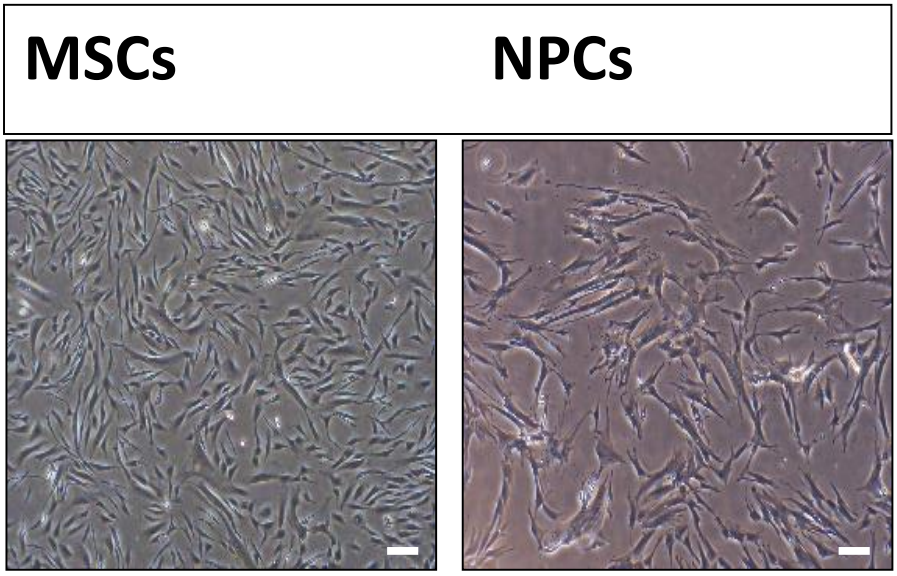

Figure 14: Phase contrast image of MSCs and differentiated NPCs. Scale bars represent 100 µm (Magnification: 4x).

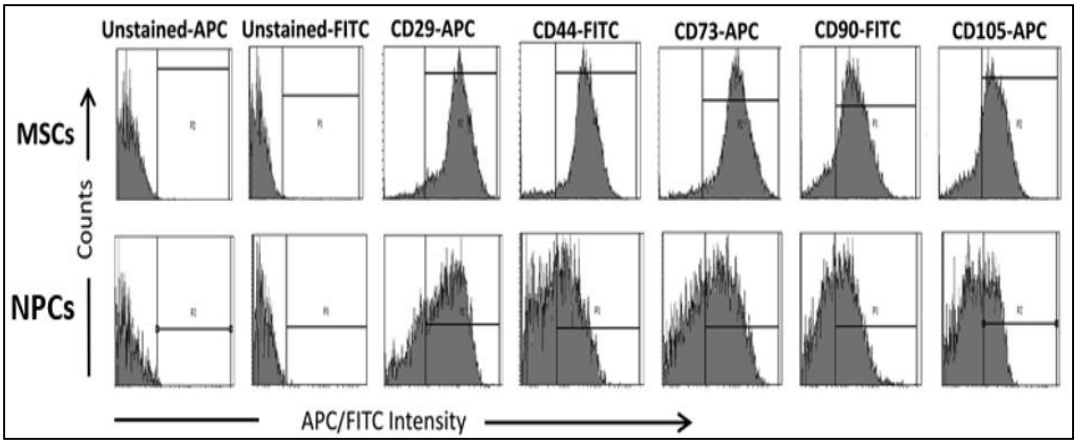

Figure 15: Histograms showing the expression of mesenchymal markers in MSCs and NPCs as determined by flow cytometry. NPCs showed a decrease in all MSC surface markers.

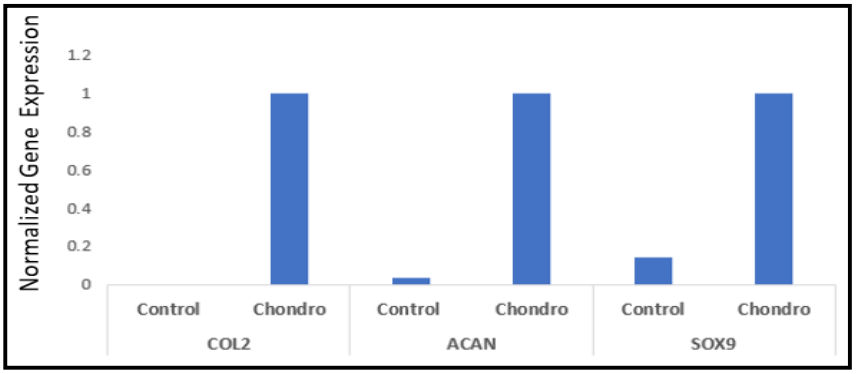

Figure 16: Expression of chondrogenic progenitor markers (COL2, ACAN, SOX9) was significantly higher in NPCs compared to MSCs, as determined by qRT-PCR. Gene expression was normalized to GAPDH and ACTIN.

NPCs GAGs production analyses

MSCs were differentiated into GAG-producing cells or nucleus pulposus cell progenitors over two weeks by adding growth factors. This growth-factor-based differentiation (using TGFβ, insulin, dexamethasone, ascorbic acid, GDF5, and BMP7) aims to direct signaling pathways toward either the chondrogenic-like or the notochordal lineage. When the differentiated cells reach 100% confluency, ECM production accumulates, as observed in the monolayer culture. The expression of chondrogenic-specific extracellular matrix proteins, such as GAG (glycosaminoglycan), was upregulated, as shown in Figure 17. Toluidine blue and Alcian Blue effectively stain GAG and the cartilage matrix within the ECM, aiding visualization of cartilage quality and cell density.

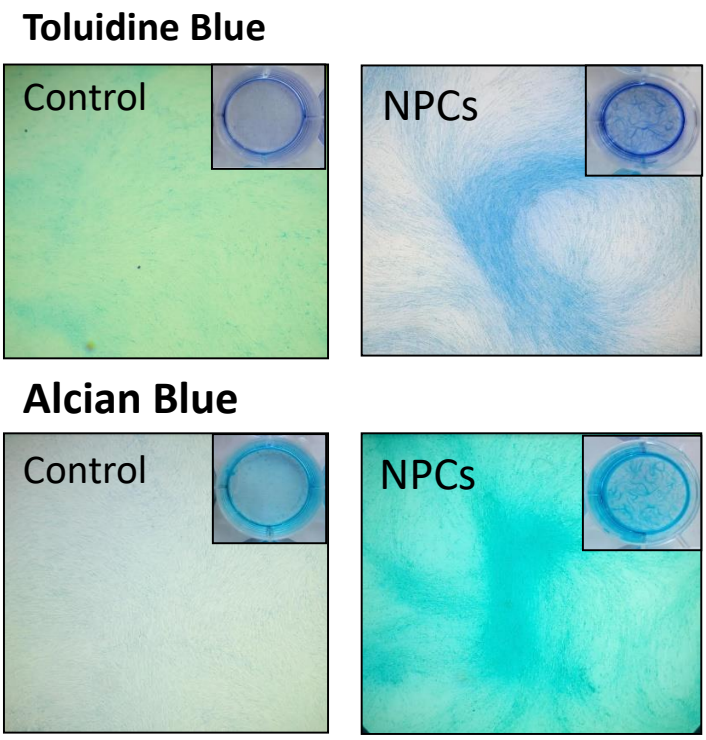

Figure 17: Analysis of alcian blue and toluidine blue staining of plate cultures, along with a contrast image (Magnification: 4x) of differentiated NPCs, for the presence of glycosaminoglycans (GAG).

Develop three-dimensional (3-D) scaffolds that mimic the in vivo microenvironment of the IVD

Since self-assembling scaffolds supported the differentiation of MSCs into NPCs in vitro (Mckee C., et al., 2019), we investigated the ability of self-assembling scaffolds to promote NPC regeneration in a rat model of LBP. PEG-4-SH and PEG-4-Acr polymers self-assemble and are mixed to form a hydrogel scaffold. An equal volume (Figure 18) of cells and scaffolds is mixed and tested for monolayer cell culture. NPCs were grown on culture plates and encapsulated in PEG-4-SH/PEG-4-Acr scaffolds for three days, showing greater growth and proliferation under a light microscope (Figure 19). We ensure cell growth before transplantation into a damaged IVD. The scaffold will help prevent cell leakage after injection and provide an optimal environment for tissue regeneration.

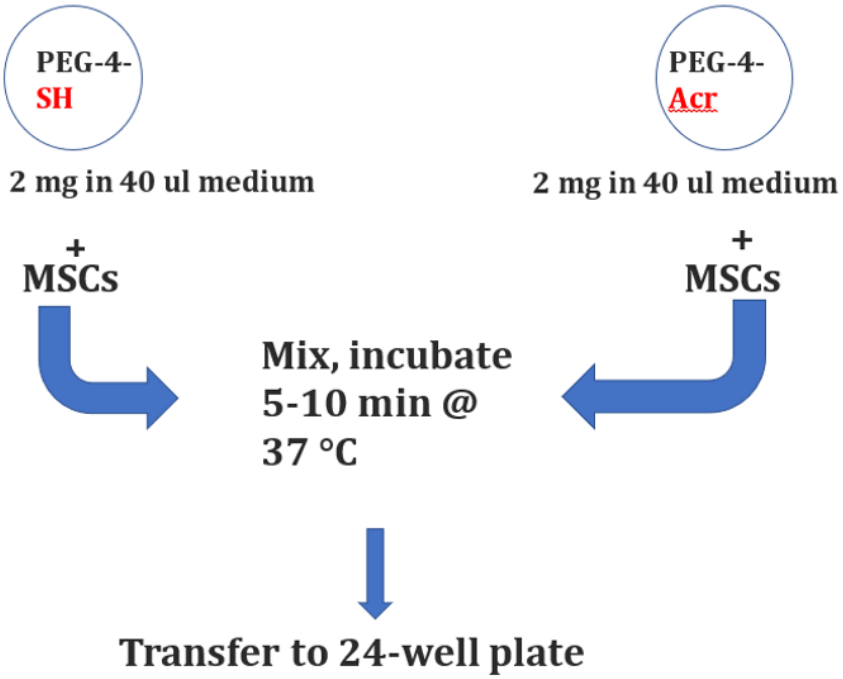

Figure 18: An equal volume of cells and scaffolds will be mixed and tested for monolayer cell culture.

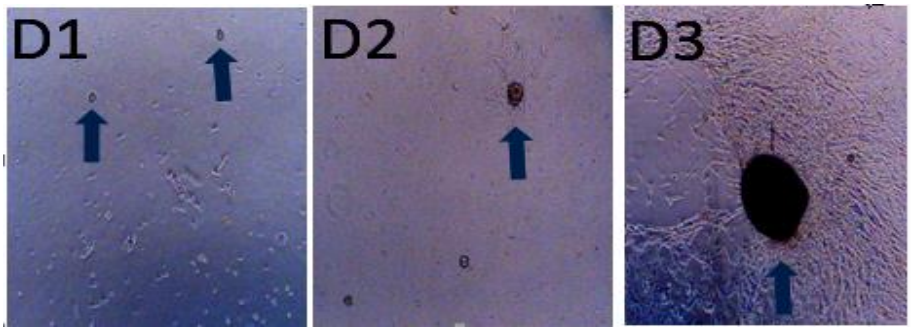

Figure 19: Phase contrast images of MSCs. Cells were encapsulated in self-assembling scaffolds for 3-D culture for 3 days (D). Scale bars represent 100 µm (Magnification: 4x).

Cells and scaffolds labeled with a Cy3 fluorescent PKH26 membrane dye

PKH cell labeling kits (Sigma) provide stable, intense, and non-cytotoxic fluorescent labeling of live cell membranes for long-term tracking up to 3 months. It has been successfully used to track cells in vivo for more than 8 weeks, as reported in our previous studies (Beeravolu et al., 2018). It is an ideal label for long-term studies. Cells and scaffolds were labeled following the kit protocol as described above. Before transplanting cells and scaffolds, we monitored the fluorescence intensity using flow cytometry (Figure 20) and confocal microscopy (Figure 21) to confirm the efficiency of fluorescent labeling.

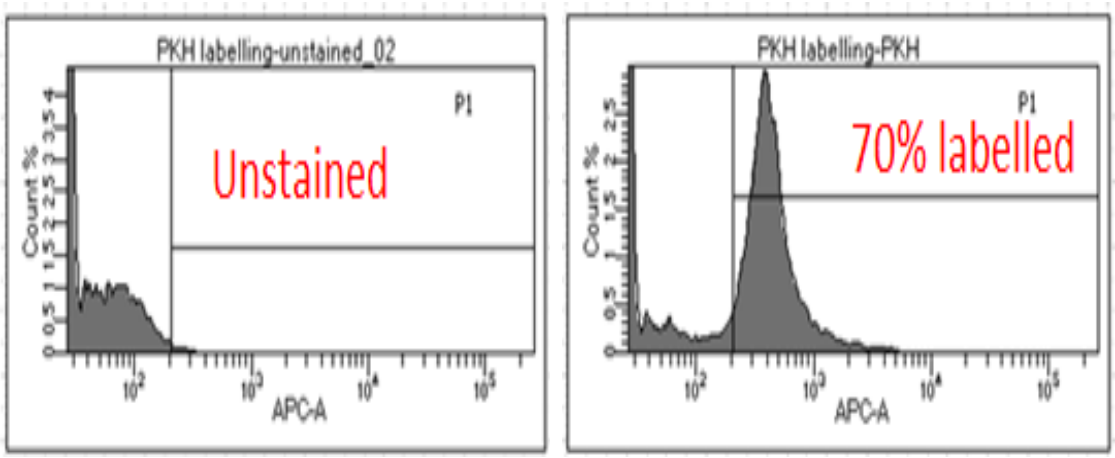

Figure 8: Histograms representation of the PKH labeling of cells as determined by flow cytometry. Cells were stained with directly (APC) conjugated antibodies.

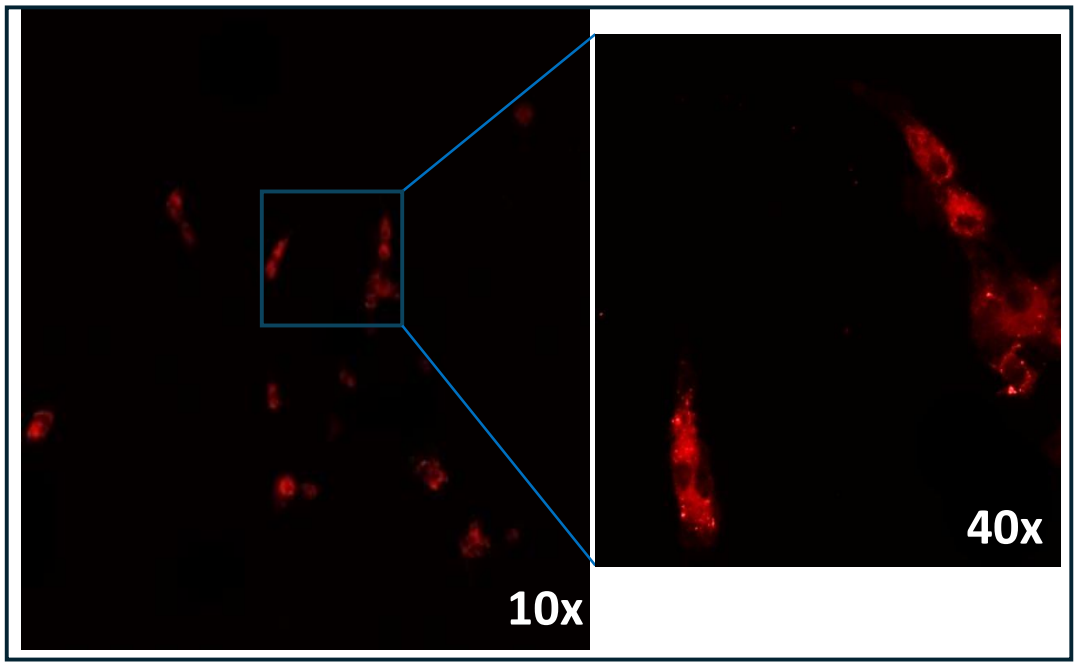

Figure 9: PKH26-labeled MSCs as shown by confocal images at 10x and 40x.

Post transplantation analyses and Tissue Regeneration evaluations

Disc Degeneration Procedure evaluation

Sprague Dawley male rats (6 weeks old) will be initially anesthetized with isoflurane gas. The rat tail skin will be sterilized with 70% ethanol, and a 3 cm incision will be made on the dorsal side of the tail to access the C3-4, C4-5, and C5-6 disc spaces. The needle penetration depth will be controlled using a stereotaxic device at 5 mm to reach the center of the NP (Figure 22). Needle punctures of two adjacent discs will be performed to induce rat tail disc degeneration (Figure 23). The incision will be closed with a 5.0 Vicryl suture (Ethicon Inc., Somerville NJ).

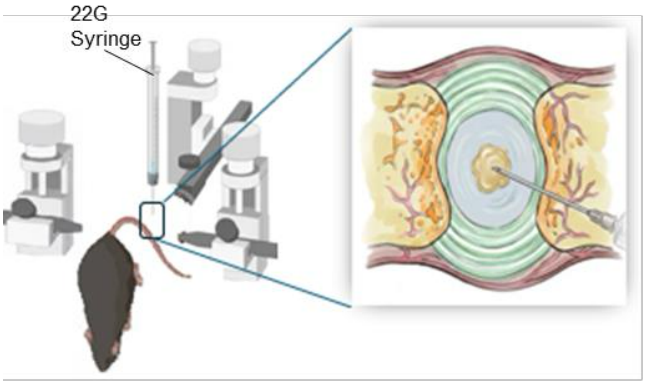

Figure 22: 21G needle will be used to pierce the tail IVD through the center of the NP

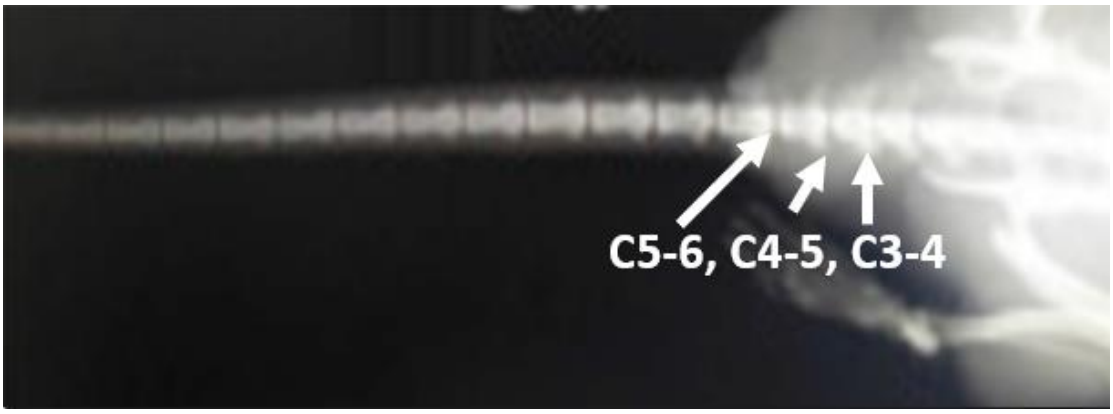

Figure 23: Conventional X-ray image for normal rat tail caudal C3-4 experimental (punctured disc with cell injection), C4-5 sham (punctured disc), and C5-6 control (intact disc).

After two weeks of disc puncture, the animal was euthanized, and the tail was harvested to confirm degeneration through H&E, MRI, and immunohistochemistry analysis. For MRI images, we evaluated intervertebral disc (IVD) degeneration and structure, typically by assessing the hydration level of the nucleus pulposus, which appears bright on T2 images. During the one week after puncture, the disc showed less degeneration compared to the control. Two weeks after puncture, we observed complete degeneration and loss of NP (Figure 24).

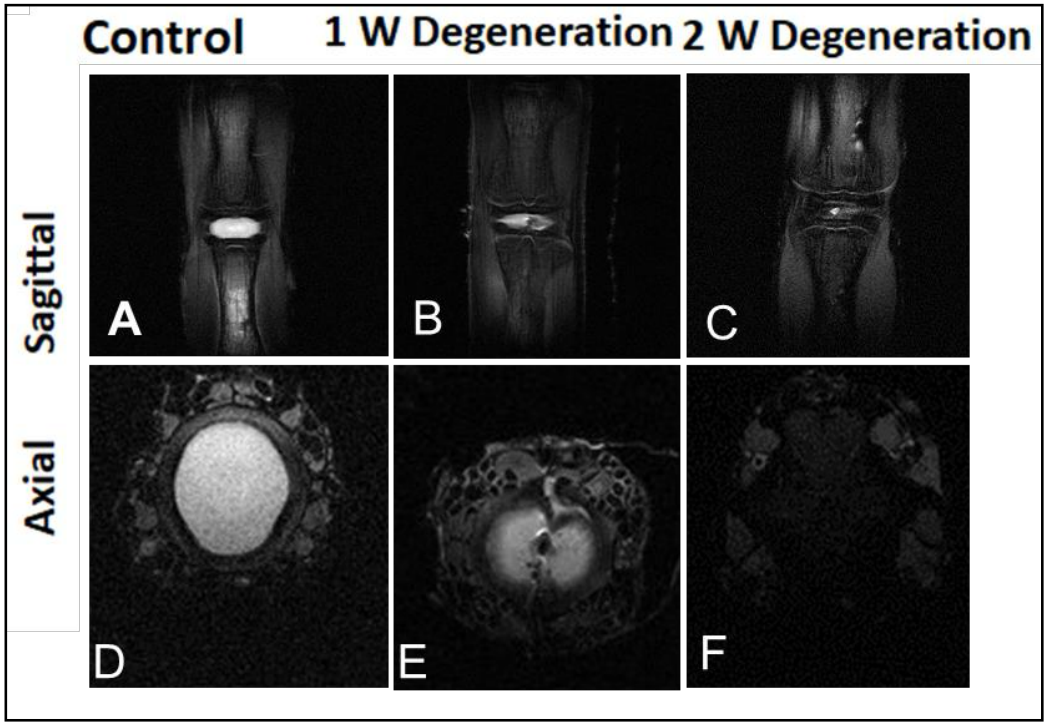

Figure 24: T2-weighted MRI images of rat tails indicate 1-2 weeks until full degeneration. A, B, C show sagittal T2-weighted MRI for control, 1 week, and 2 weeks post-puncture, respectively. D, E, F show axial T2-weighted MRI for control, 1 week, and 2 weeks post-puncture, respectively.

For H&E evaluation, the rat tail was harvested and skinned (Figure 25). The tissue was then decalcified to prepare it for sectioning (Figure 26). H&E is a useful technique for evaluating tissue structure and assessing degeneration or regeneration.

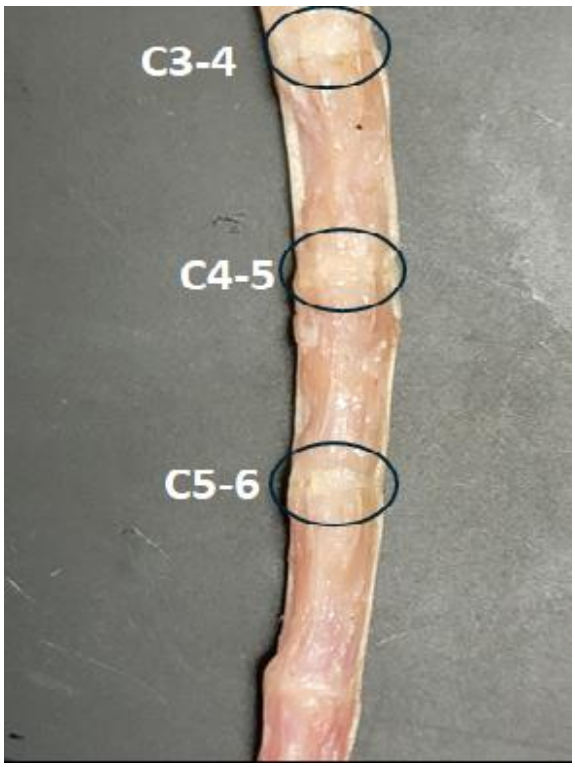

Figure 25: Unskinned rat tail showing the IVD used in the experiment

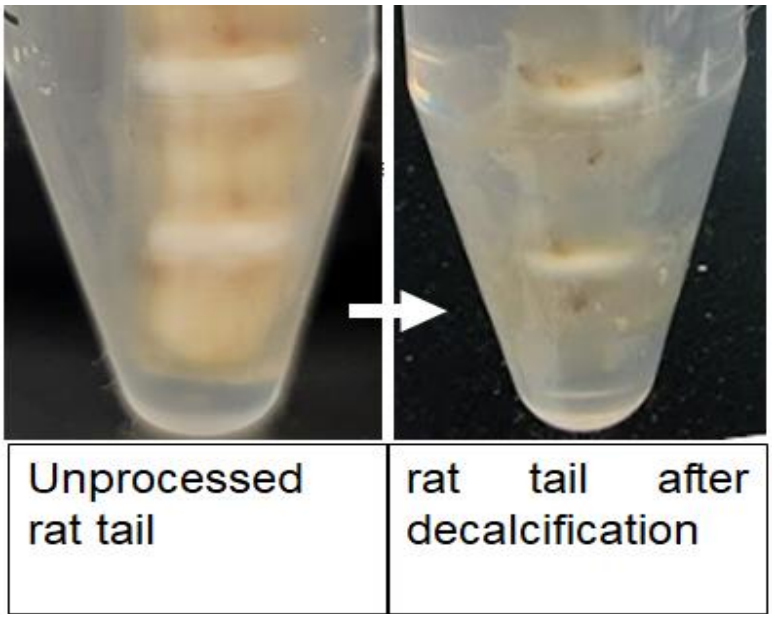

Figure 26: Decalcified rat tail using 11% formic acid for 10 days.

Regeneration of the nucleus pulposus in the degenerated rat IVD model is observed following NPC therapy. GAG production is higher at 8 weeks compared to 4 weeks (Figure 27). Alcian Blue staining showed the highest GAG content at 8 weeks in the NPCs- and scaffolds-treated group.

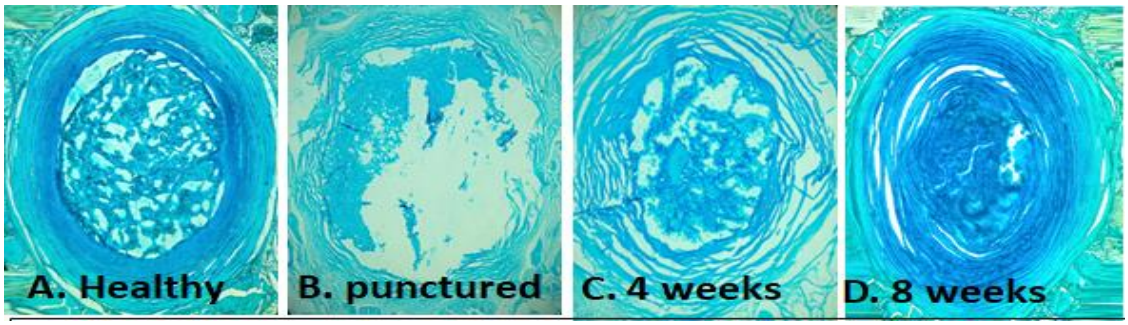

Figure 27: Immunohistochemistry analysis of the IVD stained with Alcian blue shows regeneration with NPCs+scaffold. A. Section of control disc. B. Section of degenerated disc. C., D. Sections of treated discs with NPCs after 4 and 8 weeks of injection, respectively (magnification: 4×).

Analysis of NP in punctured IVDs using H&E staining showed NP depletion and decreased cellularity, possibly due to loss of extracellular matrix content. The NP had shrunken morphology and a loss of fibrous connection in the AF region (Figure 28).

Disc Regeneration evaluation

NP of IVDs transplanted with NPCs showed a different cellular pattern than NP of punctured IVDs. Particularly in NPC-treated IVDs, the cellular content appeared more abundant and denser at 4 weeks and 8 weeks post-transplantation (Figure 28). The 8-week cell therapy period appeared to be the optimal time for regeneration.

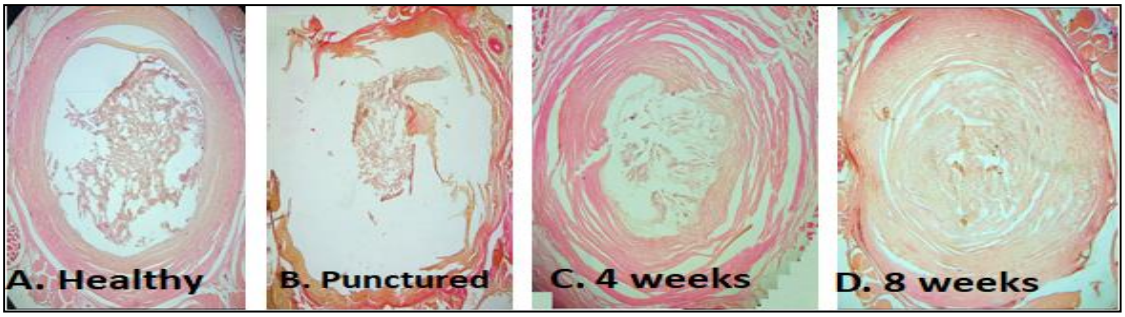

Figure 28: Histological analysis of the IVD stained with H&E shows regeneration with NPCs plus scaffold. A. Section of control disc. B. Section of degenerated disc. C, D. Sections of treated discs with NPCs after 4 and 8 weeks of injection, respectively (magnification: 4×).

The cells appeared larger in IVDs injected with human NPCs (Figure 29), indicating that the cells survived, integrated, and were distinct from host cells, as evidenced by larger human nuclei, suggesting that NPCs had greater potential to regenerate NP. These results imply that transplanted cells are homing to IVDs.

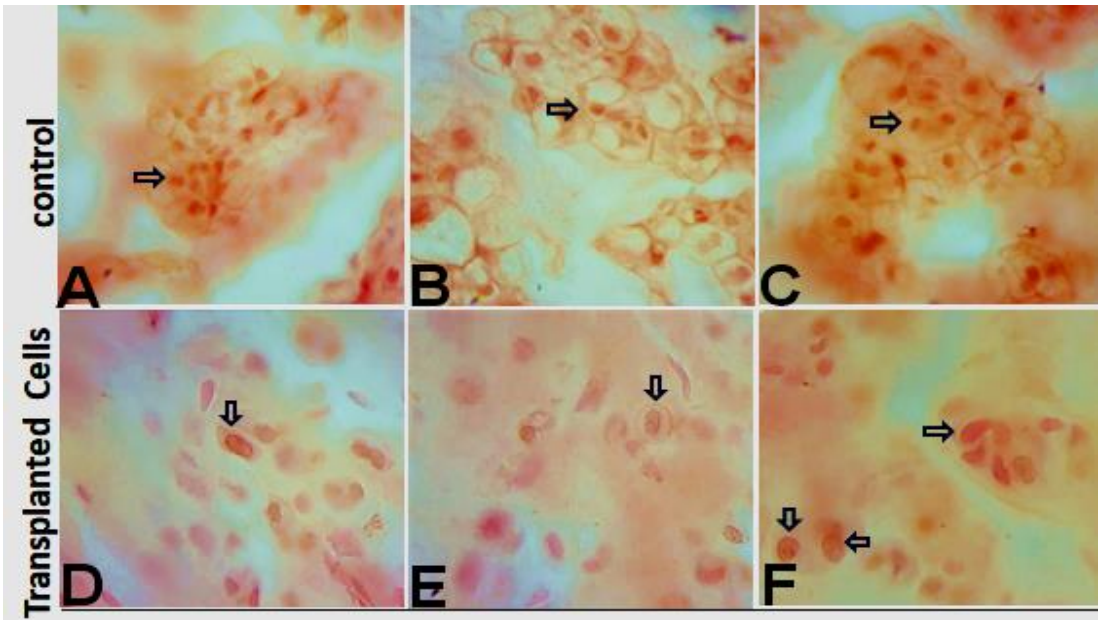

Figure 29: Histological analysis of the IVD stained with H&E. Arrows indicate larger transplanted human NPC nuclei in sections D, E, F compared to rat IVD cells in sections A, B, C (magnification: 100×).

Immunohistochemical Analyses of Treated IVDs

Similar to the H&E analysis, immunostaining studies also indicate that animals transplanted with cells expressed human NP marker (Col2) proteins at 4 weeks after transplantation (Figure 30). However, after 8 weeks, Col2-positive cells were more numerous and showed further integration into the IVD. Overall, these results suggest that transplanted cells integrate into various IVD cells.

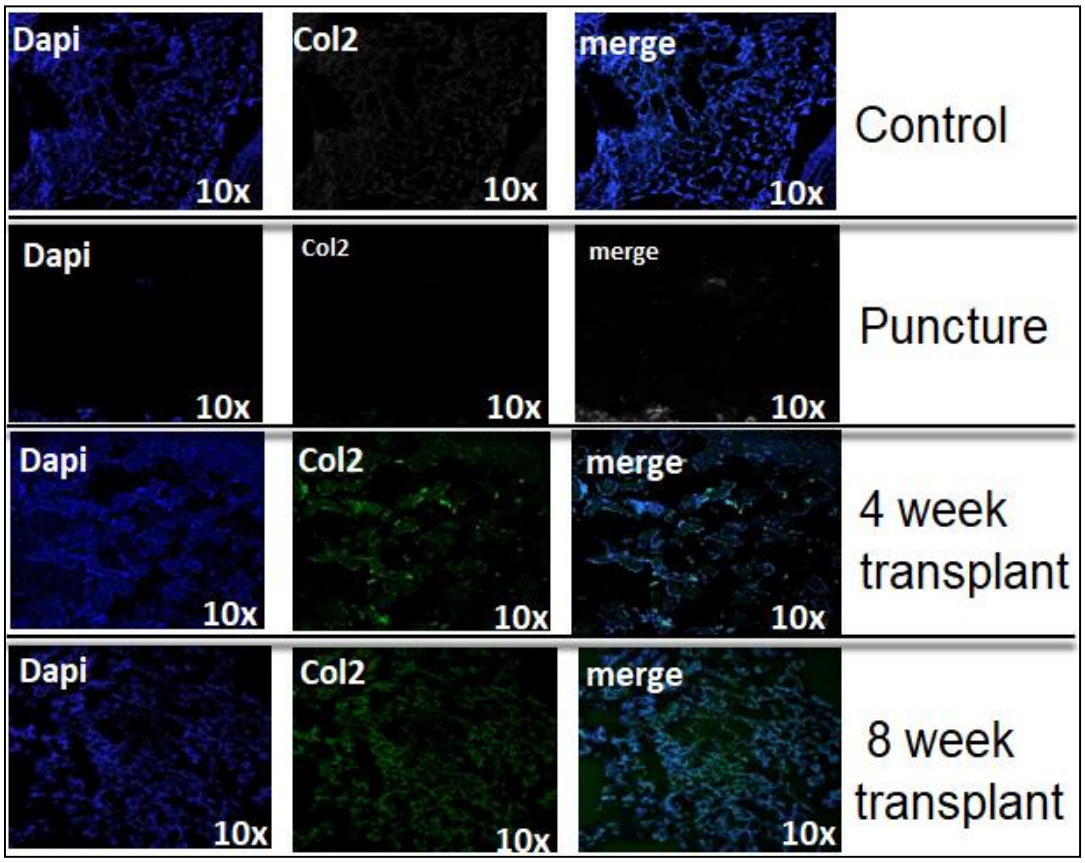

Figure 30: IVD treated with NPCs+scaffold showed expression of human specific notochordal proteins Col2 at 4 and 8 weeks post-transplantation, Blue and green colors represent the DAPI staining of the nuclei, and antibodies labeling human proteins, respectively.

Tracking Human Transplanted Cells in an Animal Model

Keratin 19 (K19) is used in animal model research as a marker to track specific human cell types. It is a protein marker used to identify and trace human cells. In xenotransplantation experiments (human cells in rats), K19 specifically detects human cells, distinguishing them from surrounding rat tissue. The immunostaining of the IVDs section showed positive results at 4 and 8 weeks post-transplantation (Figure 30). This indicates cell integration and viability.

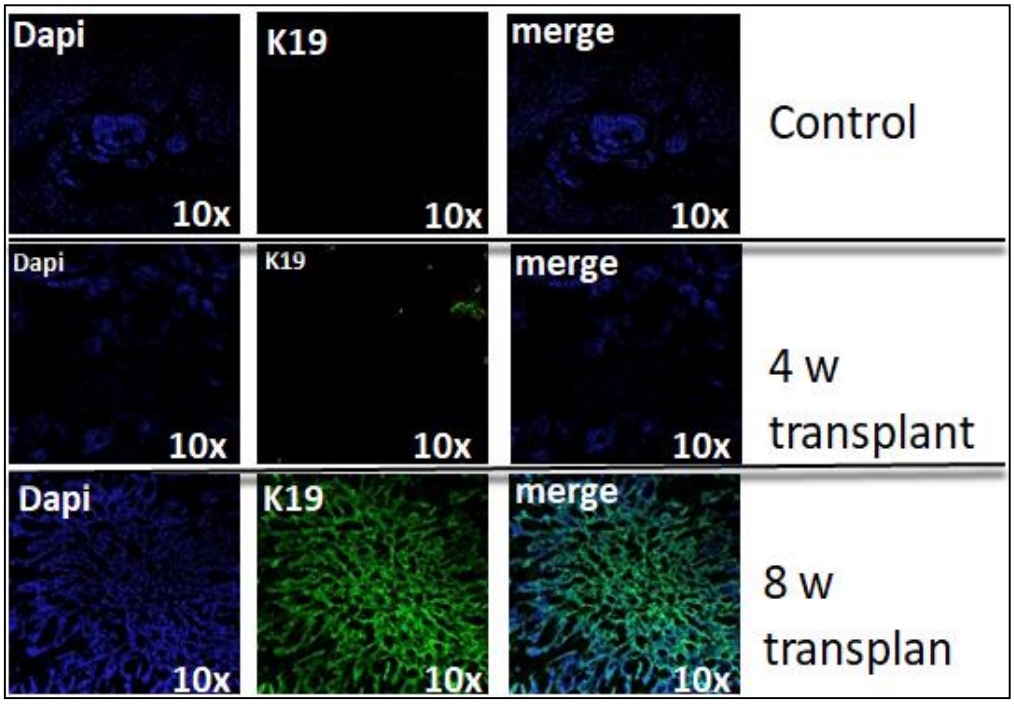

Figure 10: The IVD treated with NPCs showed expression of human specific proteins K19 at 4 and 8 weeks post-transplantation, Blue and green colors represent the DAPI staining of the nuclei, and antibodies labeling human proteins, respectively

MRI Evaluation for Treated IVD Using CNPs

The study evaluated seven experimental groups using MRI: intact healthy controls, discs injected with scaffold only, PBS, MSCs, MSCs with scaffold, NPCs, and NPCs with scaffold (Figure 31). At 4 weeks post-transplantation, MRI analysis in both sagittal and axial planes confirmed that scaffold or PBS injections caused disc degeneration. While MSC-treated discs showed a slower regenerative response than the PBS group, adding a scaffold significantly improved MSC effectiveness. Notably, NPCs alone performed better than the MSC-scaffold combination. The strongest regeneration was seen in the NPC-scaffold group, which proved to be the best treatment at just 4 weeks after transplantation. Section B shows cell tracking via two-photon microscopy (Figure 31), confirming the continued presence of transplanted MSCs and NPCs across all treated groups.

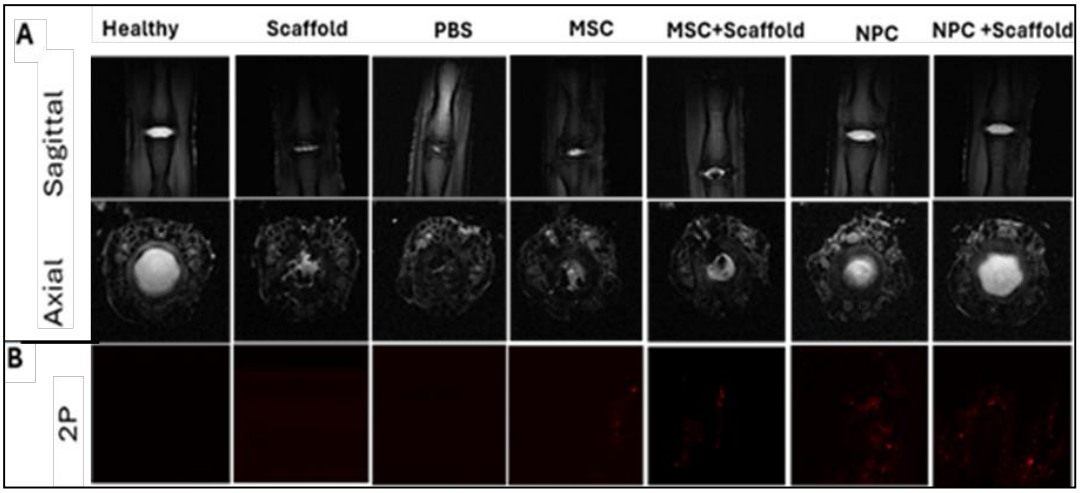

Figure 11: A. Sagittal and axial T2-weighted MRI images of the rat tails showing disc regeneration 4 weeks after degenerated disc was injected with scaffold, PBS, and cell (MSC and NPC) transplantation with and without scaffold compared to control disc B. Two-photon (2P) microscopy to track PKH labeled cells injection showing MSC and NPC present with and without scaffold.

At 8 weeks post-transplantation (Figure 32), both the MSC and MSC-scaffold groups showed increased regeneration; however, full restoration might require a longer follow-up. Sagittal and axial MRI analysis consistently indicated that the MSC-scaffold group achieved better regenerative outcomes compared to the MSC-only group. Discs treated with NPCs alone nearly fully regenerated compared to controls, while the NPC-scaffold combination showed the greatest overall regenerative capacity. These results suggest that a 3D microenvironment provided by the scaffold significantly boosts cellular regeneration. Additionally, differentiating MSCs into NPCs proved to be a more effective therapeutic strategy than using MSCs alone.

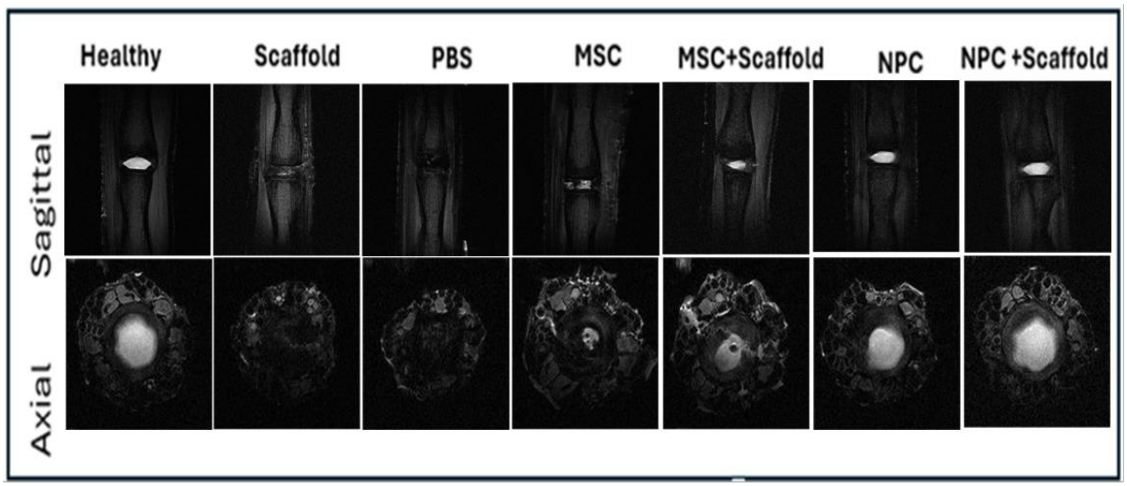

Figure 12: Sagittal and axial T2-weighted MRI images of the rat tails showing disc regeneration 8 weeks after degenerated disc was injected with scaffold, PBS, and cell (MSC and NPC) transplantation with and without scaffold compared to control disc.

CHAPTER FOUR

DISCUSSION AND CONCLUSION

The above discussed approaches in this study will help the scientific world provide a high yield of stem cells for therapeutic applications in the field of regenerative medicine. MSCs are considered the most primitive and promising source for cell therapy; the successful differentiation of these MSCs into NP-like cells will be a boon for repairing degenerated intervertebral discs. This will be helpful in addressing the most common cause of lower back pain, which is a major cause of discomfort and disability.

Our lab studies led to the isolation of MSCs from perinatal sources, which are more primitive, potent, and proliferative, and can be acceptable for allogeneic and xenogeneic transplantation studies (Beeravolu et al., 2016; Brown, C. et al., 2022). Our reports have indicated that perinatal MSCs have the potential to produce ECM, which is an integral component of NP of the IVD (Beeravolu, N. et al., 2018). We hypothesized that perinatal stem cells, such as UC-MSCs and their derivatives, can survive in the avascular NP environment and maintain long-term viability, thereby regenerating damaged IVDs in a clinical trial for DDD. In this study, tracking of labelled human cells showed that NPCs survived and integrated into damaged IVDs. Another measure to assess IVDs is histological analysis showing ECM content and cellularity in the NP of IVDs transplanted with human cells, which provides preliminary evidence of NP regeneration. The analysis of the immunohistochemical staining of NP retrieved from the transplanted IVDs indicated an increase in sGAG content. This study has used MRI analysis to confirm NP intensities and estimate water content, and the restoration of properties of the NP using human cells

has been reported in an in vivo study. Another measure to assess IVDs is the histological analysis showing ECM content and the presence of cellularity and glycoproteins. Our results indicated substantial improvements in histology and cellularity, as well as ECM regeneration, as evidenced by increases in glycoprotein and proteoglycan content in the transplanted IVDs.

MSCs are known to be involved in the generation of NP in vivo. We study MSCs transplanted directly into IVD compared to their derivatives. Moreover, we combined MSCs and NPCs with and without a scaffold to assess the most potent treatment for damaged IVD.

The 3-D scaffolds made of PEG-8-SH/PEG-8-Acr support the evidence for the best treatment. Our previous study (Mckee C., et al., 2019) showed that a 3-D scaffold improved the efficacy of cell therapy *ex vivo*. In this study, the combination of MSCs derivatives with 3-D scaffold showed their ability to repair or regenerate the degenerated NP tissue better compared to using MSCs or NPCs alone.

MSCs have been reported to be largely safe for IVD regeneration in xenograft models and are currently involved in many clinical trials (Song N. et al., 2020; Wang, Z., et al., 2015). Additionally, perinatal MSCs are considered highly promising for allogeneic transplantation because they possess lower immunogenicity compared to adult MSCs (Allouh, M., et al., 2025) and do not require tissue matching and have lower risk of graft-vs.-host disease (GvHD).

In conclusion, this study demonstrated that human MSCs are a promising cell source, but their differentiation into NPCs prior to transplantation can be more efficacious

for the treatment of DDD. Results from this study should also provide a basis for exploring the wider therapeutic use of human MSCs.

OAKLAND UNIVERSITY

Institutional Biosafety Committee

September 28, 2017

Professor G. Rasul Chaudhry
Department of Biological Sciences

Reference: Continuing Approval of IBC Application #2858, "Isolation and Characterization of Stem Cells Derived from Cord and Peripheral Blood and Tissues for Potential Therapeutic Applications"

Dear Professor Chaudhry:

The Institutional Biosafety Committee (IBC), responsible for the review of research involving materials of human origin, recombinant DNA, and biohazardous, infectious or toxic materials, has reviewed and approved the continuing review of the project referenced above.

The proposed activities will continue to be approved at a BSL-2 for another 5 years starting, September 28, 2017 and ending September 27, 2022. The approval number 2608/2858-12 remains the same.

Any unanticipated problems, changes in, or halting of the activities proposed in the project, as described, must be promptly reported to the IBC or me at luongo@oakland.edu.

In addition, all faculty, staff and students involved in the project must be trained and qualified to conduct the project in a responsible manner. Please note, in this regard, that you, the principal investigator, are fully responsible at all times for limiting or restricting access to laboratories and for assessing, in each circumstances, who may enter or work in a laboratory when biohazardous materials are in use.

Please let me know if you have any questions. Thank you.

Sincerely,

Domenic Luongo
Biosafety Officer

DL:bk

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