Introduction

It is estimated that lower back pain (LBP) has a global lifetime prevalence of 38.9% and is the single leading cause of disability worldwide [1, 2]. Degenerative disc disease (DDD) and associated pathologies are considered major contributors to LBP and the progression of DDD has been associated with an inflammatory environment that includes the presence of inflammatory cytokines (e.g. TNF-α, IL-1β, IL-6, IL-8) and invading immune cells. This environment is associated with the breakdown of the extracellular matrix (ECM) and more recently with direct links to the sensitization of pain sensing nociceptive neurons in the degenerated disc providing a direct link to discogenic back pain. It is important to note that these inflammatory cytokines have significant redundancy in their action limiting the potential of single cytokine targeting therapeutic strategies [3]. Multiplex strategies capable of simultaneously altering the progression of multiple components of the inflammatory environment have great potential to impact both the catabolic ECM changes observed in the disc and painful symptom development.

Recently, Clustered Regularly Interspaced Short Palindromic Repeats interference (CRISPRi) has been developed to induce gene knockdown in a sequence specific manner. The CRISPRi system achieves this through the expression of a deactivated Cas9 (dCas9) nuclease fused to the Krüppel-associated box (KRAB) and the expression of short genomic site-specific guide RNAs (gRNAs), complementary to the promoter of the gene. CRISPRi has been shown to be effective in performing multiplex gene knockdown and it has been shown to work in mammalian cells [4, 5]. CRISPRi provides a controlled method to regulate genes in a multiplex fashion to synergistically reduce inflammation in the degenerating disc.

Proposed Research Plan

Inflammatory cytokines present in the degenerating disc exert their function through interactions with their respective receptors. For example, elevated levels of TNF receptors have been identified in the degenerating disc, and have been associated with elevated pain levels in patients [6]. Specifically, pro-inflammatory signaling for TNF-α, IL-1β, IL-6 and IL-8 are regulated through their respective receptors TNFR1, TNFR2, IL1R1, IL1R2, IL6R, CXCR1 and CXCR2. Using CRISPRi methods, one may investigate the regulation of these receptors to determine efficacy of regulating receptor expression to control inflammation in DDD. Regulation of these receptors through multiplex CRISPRi knockdown can provide a powerful method to directly regulate inflammation in the degenerating disc and/or counteract the influence of the inflammatory environment on therapeutic cells and tissue engineering strategies being developed for delivery to the disc.

Hypothesis: Multiplex knockdown of cytokine receptors TNFR1, IL1R1, IL6R, and CXCR1 will inhibit over expression of matrix metalloproteases, inflammation and apoptosis, therefore preventing structural breakdown of intervertebral discs (IVDs) and painful DDD.

To test this hypothesis, CRISPRi knockdown of TNFR1, IL1R1, IL6R, and CXCR1 will be performed *in vitro*. The initial focus will be on TNFR1 and IL1R1 as studies show they have prominent roles in inflammation and ECM degradation in the IVD [6, 7]. To investigate the impact of multiplex CRISPRi regulated knockdown, this study will follow the two aims described below.

Aim One: In order to study the effects of knockdown of these cytokine receptors on degenerative IVDs, each knockdown must be performed on applicable cells. Cytokine receptor knockdown will be performed using the previously described CRISPRi system in human mesenchymal stem cells (hMSCs) and degenerative human nucleus pulposus (hNP) cells. Human NP cells will be obtained from surgical tissue from a collaborating surgeon. Receptor down regulation will be performed for

individual receptors in each cell type using gRNA and dCas9-KRAB expressing lentivirus that targets the promoter regions of these genes. The engineered cells will then be screened for expression of key inflammatory cytokines and matrix degrading proteases within an inflammatory environment using qPCR (i.e. Qiagen RT² Profiler™ Human Inflammatory Cytokines & Receptors). An inflammatory environment will be modeled by culturing engineered cells in the presence of media supplemented with their respective receptor regulated cytokine (i.e. TNF-α, IL-1β, IL-6, or IL-8). Following the verification of an altered response to an inflammatory environment, engineered cells will be seeded and cultured on an alginate matrix in the presence of the same inflammatory cytokine supplemented media to form a surrogate NP tissue. The media will be chondrogenic for the hMSCs. This experiment will allow investigation of IVD tissue formation from the engineered cells in an inflammatory environment and examine the effect each knockdown has on tissue development and mechanical properties. Surrogate NP tissue will be characterized via histology, biochemistry, and mechanical testing. Mechanical tests will focus on the compressive viscoelastic properties of the NP construct, as these are the primary properties associated with NP mechanics.

Aim Two: Once successful knockdown of the receptors mentioned above is achieved, the effects of knocking down multiple receptors at once will be tested. In order to do this, multiplex knockdown of the receptors will be performed with specific emphasis on positive findings from aim one. Once multiplex knockdown is achieved, previously described surrogate NP tissue containing IVD cells will be cultured in the presence of supernatant of *in vitro* cultured surgical human IVD tissue. The tissue supernatant will contain many of the cytokines that cells are exposed to in the degenerating IVD and will simulate the degenerative environment to test multiplex knockdown. Post culture, surrogate NP tissue will be analyzed for gene expression, tissue development (histology/biochemistry), and mechanical properties (similar to aim 1).

Intellectual Merit and Broader Impacts

With much to still be discovered in the field of DDD, this proposed study will elucidate novel tools that may be utilized for further understanding the mechanisms of DDD and chronic back pain or for development of DDD therapeutics. The preliminary results regarding this study have shown promising results with effective gene and receptor signaling regulation of TNFR1 and/or IL1R1 in hMSCs and hNP cells [8, 9]. Further research of these CRISPRi tools via *in vitro* studies will set the stage for future *in vivo* studies (beyond the scope of this proposal). Back pain is both understudied and of great significance to human health, making it an exciting field to begin my academic career in. My experience with this project over the past year has allowed me to gain a cutting edge tissue/cell engineering skill set. With these tools at my fingertips, I have the means of developing innovative research ideas as I move forward through my academic career. During my journey, I plan to continue interacting with my community, by performing outreach activities that highlight the tissue and cell engineering aspect of bioengineering. For example I can demonstrate how alginate gels are made and how they have applications in biomedicine. Through these activities my main goal is to be a role model to aspiring female engineers, letting them know that there are opportunities for them to develop careers as STEM innovators.

References:

[1] [redacted]. [2] [redacted] [3] [redacted] [4] [redacted] [5] [redacted] [6] [redacted] [7] [redacted] [8] [redacted] [9] [redacted]