Functional Role of Toll-like Receptor Associated MicroRNAs in Intervertebral Disc Pathophysiology

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Functional Role of Toll-like Receptor Associated MicroRNAs in Intervertebral Disc Pathophysiology

by

Petra Cazzanelli

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctorate of Philosophy in Biomedical and Chemical Engineering

Biomedical and Chemical Engineering PhD Program
Kate Gleason College of Engineering

Rochester Institute of Technology
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Functional Role of Toll-like Receptor Associated MicroRNAs in Intervertebral Disc Pathophysiology

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Abstract

Intervertebral disc (IVD) degeneration is one of the main contributing factors to low back pain (LBP), the leading cause of years lived in disability worldwide. The degenerative processes in the IVD tissue are characterized by the metabolic dysregulation of IVD cells and aberrant mechanical loading. The major hallmarks of this multifactorial pathology are the degradation of the extracellular matrix (ECM), inflammation, and cell loss. Inflammation has been specifically linked to the development of discogenic LBP, as increased secretion of pro-inflammatory cytokines can cause the infiltration of immune cells and nerve fibers. Toll-like receptors (TLR) are key regulators of inflammation, amongst which TLR-2 is known for its increased expression in degenerated IVD cells and its contribution to IVD degeneration upon activation by affecting gene expression and intracellular signaling. As potent post-transcriptional regulators, microRNAs can modulate numerous cellular mechanisms including inflammation, catabolism, apoptosis, and senescence. Most importantly, the dysregulation of microRNAs has been linked to numerous pathologies, including multiple degenerative diseases.

This project aims to study miRNAs in the context of TLR-2 activation, inflammation, and mechanosensing for a better understanding of TLR-2 dysregulation and IVD mechano-immunosensing. As a first step, TLR-2-associated microRNAs were identified by activation of the receptor followed by small RNA sequencing. The impact of TLR-2 activation on miRNA dysregulation was investigated in degenerated and non-degenerated IVD cells. Thereafter, the functional role of miRNA-155-5p in inflammation and degeneration was studied. To that end, the effect of up/downregulation of this miRNA on ECM-degrading proteins, pro-inflammatory cytokines, intracellular signaling, and neurotrophins was analyzed. Furthermore, the role of miRNA-155-5p in mechanosensing was determined by subjecting IVD cells to cyclic stretching. The miR-155-5p was consistently upregulated in degenerated and non-degenerated IVD cells following TLR-2 activation and its expression contributed to the expression of pro-inflammatory cytokines, neurotrophins and the activation of inflammatory signaling pathways. Overall, the project identified TLR-2-associated miRNAs and investigated their role in IVD degeneration, inflammation and mechanosensing, for a better understanding of underlying disease mechanisms and contributing to the identification of new therapeutic targets for IVD degeneration and LBP.
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Chapter 1  Introduction
1.1 Thesis Motivation

Low back pain (LBP) is a widespread condition that affects over 80% of the population worldwide at some point during their life and is hence the leading cause of years lived in disability [1, 2]. This pathology poses a major socio-economic burden worldwide with an estimated yearly cost of $100 billion in the United States alone, which includes direct costs (healthcare) and indirect costs (productivity loss and absence from work) connected to the disease [3]. LBP can present as an acute episode or persist over an extended period, lasting at least 12 weeks or longer and is then termed chronic LBP. Chronic LBP has a lifetime prevalence of 23% and significantly impacts the quality of life of the affected patients, causing disability in 11-12% of the population [1]. Current treatments range from physical therapy, patient education, and cognitive behavioral therapy to pharmacological treatments against pain and inflammation (non-steroidal anti-inflammatory drugs, opioids) [4]. However, these treatments are often connected to modest success rates with marginal effectiveness in reducing pain and improving function [4]. Furthermore, the long-term benefits of high-risk and cost-intensive surgical treatments like epidural injections (corticosteroids or anesthetics), microdiscectomy, spinal fusion or total disc replacement have also been challenged recently[5]. A major contributor to low back pain is degenerative disc disease (DDD), accounting for at least 40% of LBP cases [4]. DDD is characterized by intervertebral disc degeneration, inflammation and nociception [6, 7].

Intervertebral disc (IVD) degeneration is a multifactorial pathology causing the deterioration of IVD tissue and the dysregulation of IVD resident cells [8]. The key
pathological processes occurring during IVD degeneration include the degradation of the extracellular matrix (ECM), aberrant intracellular signaling, inflammation, innervation, and cell loss [6]. While the etiology of DDD and IVD degeneration is not completely understood yet, several factors have been found to contribute to the disease including age, lifestyle (diabetes, obesity, smoking), genetic predisposition, and non-physiological mechanical loading [9]. Despite an improved understanding of the involved cellular processes and their effects on the disease progression, the exact mechanisms initiating and driving IVD degeneration are still poorly understood. Therefore, research efforts into the underlying mechanisms of DDD are crucial for the development of novel treatment solutions against discogenic LBP.

Chronic inflammation is a known key element of IVD degeneration and is highly associated with the development of pain [7]. Secretion of pro-inflammatory cytokines and chemokines by IVD native cells can lead to their accumulation in the tissue and the subsequent recruitment of host immune cells [10]. Furthermore, pro-inflammatory cytokines are known to activate and dysregulate inflammatory signaling pathways and enhance the catabolic shift by increasing the secretion of ECM-degrading proteins and decreasing the expression of ECM structural molecules [11]. Upon the deterioration of the ECM, inflammation in conjunction with innervation can lead to nociception and pain [10]. Toll-like receptors (TLR) are key regulators of inflammation and have become of increasing interest in the study of IVD degeneration. Amongst the TLRs present in the IVD, TLR-2 is of specific relevance, since it has been shown that TLR-2 expression increases with progressive degrees of degeneration [12]. Furthermore, the activation of TLR-2 by
pathogen- or damage-associated-molecular-patterns was shown to contribute to IVD degeneration by increasing the expression of cytokines and chemokines [13] and by enhancing cellular senescence [14]. The results of these studies clearly indicate that TLR-2 might be an important factor in IVD inflammation and the development of pain during degeneration, but the molecular mechanisms driving TLR-2 signaling and inflammation are still not completely understood.

MicroRNAs (miRNA) are small non-coding RNAs essential for post-transcriptional regulation of gene expression, due to their capability to regulate multiple genes and signaling pathways simultaneously [15]. Dysregulation of miRNAs has been linked to multiple pathologies, including cancer [16] and osteoarthritis [17]. Recent studies in other tissues have suggested a link between miRNAs and TLR-2 signaling through the miRNAs' ability to target important players in TLR-2 and other inflammatory signaling pathways [18, 19]. However, miRNAs associated with TLR-2 have not been studied yet in the context of inflammation and IVD degeneration.

Therefore, the goal of this project is to study the role of TLR2-associated miRNAs in DDD and to provide a better understanding of the underlying disease mechanisms, as well as eventually identify new therapeutic targets for the development of miRNA-based therapies for IVD degeneration.
1.2 Thesis Aims

The overall objective of this study is to identify miRNAs that are associated with TLR-2 signaling in human IVD cells, followed by establishing their pathophysiological role in DDD with a specific focus on inflammation, catabolism, and mechanosignaling. The project is divided into three main aims:

**Aim 1: Identification of miRNAs dysregulated by TLR-2 activation**

Due to the role of TLRs in inflammation as one of the key regulators, the study of TLR signaling in IVD degeneration and DDD has become of increasing interest. Specifically, TLR-2 signaling and the downstream effects on the progression of the disease have been the focus of recent studies [13, 14]. This pattern-recognition receptor can be activated through pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) such as fragmented ECM proteins like fibronectin or hyaluronic acid [13, 20]. Activation can lead to changes in gene expression of pro-inflammatory cytokines and other catabolic factors. However, the interplay of miRNAs and TLR-2 signaling has not been studied so far. We aimed to identify miRNAs that are dysregulated by PAMP-induced TLR-2 activation with next-generation sequencing, followed by studying their expression profile in degenerated and non-degenerated IVD cells and the selection of a prime candidate for subsequent functional studies (Figure 1.1).

To that end, human IVD cells were sequenced following TLR-2 activation, whereby TLR-2 knockdown cells (siRNA) were used as controls. Differentially expressed miRNAs were studied in degenerated and non-degenerated human Nucleus pulposus (NP, i.e. the inner
zone of the IVD) and Annulus fibrosus (AF, i.e. the outer zone of the IVD) cells. Furthermore, their expression was investigated following the activation of TLR-2 with the DAMP fragmented fibronectin. Lastly, target and pathway prediction were performed before the selection of the most promising candidate for functional studies. We hypothesized that the activation of TLR2 and subsequent downstream signaling in human NP and AF cells will change the expression profile of miRNAs and lead to the dysregulation of specific TLR2-associated miRNAs.

Figure 1.1 Overview and experimental plan for the identification of TLR-2-associated miRNAs in Aim 1 (created with biorender.com)
**Aim 2: Investigation of the functional role of the selected miRNA in DDD**

The dysregulation of miRNAs is known to contribute to pathologies due to their multifaceted regulation of gene expression and cell signaling [15, 21]. Based on the results obtained in Aim 1 the most consistently dysregulated miRNA following TLR-2 activation was miR-155-5p, which was hence selected for further investigation in Aim 2. For a better understanding of the role of miR-155-5p in IVD degeneration, we aimed to study its function in the degenerative process with a specific focus on inflammation (Figure 1.2). To that end, we transfected human degenerated AF and NP cells with miRNA mimics or inhibitors for gain- and loss-of-function studies. Thereafter we analyzed the inflammatory cell response by determining the secretion of cytokines and chemokines, as well as the activation of key enzymes associated with the mitogen-activated protein kinases (MAPK) pathway. Due to the importance of ECM degradation and innervation in DDD and the interconnectivity between these processes, we further analyzed the effect of the selected miRNA on matrix-degrading enzymes like matrix metalloproteinases (MMPs) and neurotrophic factors. We hypothesized that the gain- and loss-of-function of the selected miRNA (miR-155-5p) in human NP and AF cells will impact the cell’s response to the inflammatory environment by dysregulating intracellular signaling as well as the secretion of catabolic and pro-inflammatory factors.
**Figure 1.2** Overview and experimental plan for studying the functional role of miRNAs in DDD and inflammation in Aim 2 (created with biorender.com)

### Aim 3: Investigation of the functional role of the selected miRNA in mechanosensing

Mechanical loading is an important factor of disc degeneration and a contributor to IVD pathophysiology [22]. Furthermore, recent efforts in the study of mechanobiology have identified the presence of mechanosensitive miRNAs in cartilage [23]. Based on the results obtained in Aim 1 the most consistently dysregulated miRNA, miR-155-5p, was selected for further investigation in Aim 3. The goal was to study the role of miR-155-5p in mechanosensing (Figure 1.3). To that end, *in vitro* experiments were used to study changes in the response of human AF cells to cyclic stretching after the miRNAs gain- and loss-of-function. We first fabricated and characterized PDMS stretching chambers with the appropriate stiffness corresponding to the degenerated AF tissue. After the optimization of the cyclic stretching conditions, cells were transfected with miRNA mimics and inhibitors followed by cyclic stretching. Gene and protein expression of
mechanosensitive and DDD-related targets were studied. We hypothesized that the gain- and loss-of-function of the selected miRNA (miR-155-5p) in AF cells will impact the cell's response to mechanical stretching by dysregulating the expression and secretion of catabolic and pro-inflammatory factors.

![Diagram](image)

**Figure 1.3** Overview and experimental plan for studying the functional role of miRNAs in mechanosensing in Aim 3 (created with biorender.com)

### 1.3 Thesis Outline

This thesis is structured into 5 main chapters followed by a section dedicated to the references and an appendix. The content of each section is described below.

**Chapter 1** provides an introduction to the clinical and economic motivation behind the study and identifies the gap in the current literature. Furthermore, it describes the objectives and aims as well as the structure of the thesis.
Chapter 2 gives an overview of relevant literature and provides background information on the anatomy of the IVD, the degenerative processes, current therapeutic strategies and the role of miRNAs in IVD degeneration.

Chapter 3 addresses the first aim of the thesis. We showed that TLR-2 activation in human IVD cells leads to the dysregulation of 10 miRNAs and subsequent studies of the expression profile of these miRNAs show characteristic changes dependent on the activation with PAMPs or DAMPs. Moreover, differences in the expression of these miRNA in degenerated vs non-degenerated IVD cells were identified. The consistently upregulated miR-155-5p was used for target prediction, demonstrating its involvement in multiple pathways relevant to IVD degeneration, as well as the possible regulation of the transcription factor c-FOS.

Chapter 4 addresses the second and third aims of the thesis. We studied the functional role of miR-155-5p in inflammation and mechanosensing and showed that it acts as a pro-inflammatory mediator in IVD degeneration. Increased expression of miR-155-5p leads to the increased secretion of interleukins and MMPs, and upregulates phosphorylation of MAPK pathways. Furthermore, we showed that miR-155-5p enhances the catabolic shift during mechanical loading.

Chapter 5 presents the conclusion of the thesis, giving an overall summary, listing major contributions, the novelty and significance. Furthermore, this chapter provides suggestions for future directions.

The appendix contains the supplementary material for the chapters 3 and 4.
Chapter 2  Literature


2.1 Intervertebral Disc

The Intervertebral disc (IVD) is the fibrocartilaginous tissue located between the vertebrae in the cervical, thoracic and lumbar regions of the human spine. The IVD plays a crucial role in spinal kinematics, facilitating flexibility and stability of the spine and absorbing mechanical loads. The IVD consists of 3 distinct regions: the outer, highly structured annulus fibrosus (AF); the inner, gelatinous nucleus pulposus (NP), and the cartilaginous endplates at the superior and inferior faces of the IVD (Figure 2.1) [24].

![Schematic representation of an IVD](image)

**Figure 2.1** Schematic representation of an IVD. The cartilaginous endplate (CEP) and the adjacent vertebrae (VB) are visualized in the sagittal section of the disc (A). The AF is formed by structured lamellae which encapsulate the central NP (B). Adapted from Pattappa, et al [24].

The AF consists of 15 to 25 concentric lamellae made of collagen fiber bundles and hosts fibroblast-like cells. The collagen bundles follow a distinct alignment at angles from 55° to 20° and alternate directions at every layer to provide stability and confine the gelatinous NP [25]. The composition of AF tissue varies between the inner and outer AF, consisting of water (70-78% inner AF, 55-65% outer AF), collagen (25-40% inner AF,
60-70% outer AF dry weight), and proteoglycans (11-20% inner AF, 5-8% outer AF). Furthermore, collagen composition transitions in a radial gradient from 100% type I collagen in the outer lamellae to 100% type II in the inner lamellae [25].

The center of the IVD is composed of the NP region, which is highly hydrated and essential for the joint to sustain compressive loads, as well as hydrostatic and osmotic pressures [22]. The resident cells are chondrocyte-like cells that are essential for the tissue homeostasis. Their capability of synthesizing extracellular matrix (ECM) molecules and secreting ECM-degrading enzymes results in a healthy turnover of ECM components. The NP is characterized by its main component water (80-90%) and a high amount of proteoglycans (50% dry weight). Additionally, it consists of 15-20% collagen II fibers (dry weight) [25]. The negatively charged proteoglycans provide the osmotic and swelling properties of the NP. Furthermore, the region is defined by the harsh microenvironment for cells due to low oxygen, low pH and low concentration of nutrients [26].

The cartilaginous endplates (CEP) are the porous structures separating the IVD from its adjacent vertebrae. Importantly, CEPs are vascularized, hence providing nutrient and fluid transport through diffusion. The CEPs are composed of hyaline cartilage, which consists of a thin collagen network, proteoglycans and chondrocytes [25].

The interaction between these three distinct regions is essential to the functioning of the IVD and largely depends on the maintenance of their composition and ultrastructure. The healthy response of resident cells to physical, biological, and chemical cues is transmitted through the ECM and ensures the balance between anabolism and catabolism, securing the homeostasis of the tissue [25].
2.2 Biomechanics of the Intervertebral Disc

The intervertebral discs are essential for the articulation of the spine, allowing for flexion, extension and torsion motions. The highly hydrated NP allows for the bearing of compressive loads by sustaining hydrostatic and osmotic pressures during movements (Figure 2.2) [22]. The axial compression and subsequent swelling of the NP leads to radial and circumferential tension in the AF, which withstands the tensile stress through organized collagen bundles [22]. Importantly, the structure of the lamellae in the AF provides stability and prevents the expansion of the IVD in the transverse plane [22]. Native IVD cells are therefore subjected to combinations of mechanical loading like compressive, hydrostatic, shear or tensile stress, depending on their location within the IVD [27].

*Biomaterials*

**Figure 2.2** Schematic representation of the mechanical deformation of the IVD. Axial compression can be either catabolic or anabolic depending on mode (static, dynamic), magnitude, frequency, and duration. Loading of the hydrated NP matrix results in hydrostatic and osmotic pressures. When the disc is axially loaded, the NP region becomes compressed and the AF undergoes radial and circumferential tension to limit overall disc expansion in the transverse plane. Adapted from Fearing, et al [22].
2.3 **Intervertebral Disc Degeneration**

Intervertebral disc degeneration is characterized by the deterioration of the IVD on the tissue, cellular and molecular level due to mechanical loading and the catabolic shift of IVD resident cells [26]. The disruption of tissue homeostasis is caused by the increased degradation of the ECM and cell loss due to apoptosis and senescence, accompanied by inflammation and innervation (Figure 2.3) [8, 11].

![Figure 2.3](image)

**Figure 2.3** Schematic diagram of the different factors contributing to the metabolic shift from anabolism to catabolism in IDD, including genetics and epigenetics, biomechanics, microenvironment, presence of bacteria and other factors. Adapted from Bermudez-Lekerika, et al [11].

Increased inflammation in the tissue can lead to the secretion and accumulation of pro-inflammatory cytokines and dysregulation of inflammatory signaling pathways [7, 11]. Furthermore, the inflammatory environment is known to contribute to the catabolic shift by increasing the secretion of ECM-degrading proteins and reducing the capabilities of the native cells to produce ECM components [11]. These effects are aggravated by the decrease of functional cells in the tissue due to apoptosis and senescence [28, 29]. With
progressive degrees of degeneration, ECM breakdown can lead to the loss of the structural boundary between AF and NP, widening of the intralamellar space and loss in hydration, leading to aberrant mechanical loading (Figure 2.4) [26, 30].

Figure 2.4 Various stages of IVD degeneration observed in sagittal sections of human lumbar IVDs. Adapted from Galbusera, et al [30].

These structural changes in the tissue allow for the recruitment of host immune cells and the ingrowth of blood vessels and nerves. These processes are accompanied by the accumulation of noxious inflammatory stimuli and the mechanical stimulation of nociceptors, leading to nerve sensitization and discogenic LBP [31].

The complexity of IVD degeneration due to the multifactorial dysregulation of IVD cell activity and the numerous, highly interconnected processes simultaneously driving the disease pose a major challenge for the development of novel therapeutics. The focus
of current preclinical therapeutic strategies are cell therapy, molecular and gene therapy, as well as tissue engineering approaches [32]. IVD cell-based therapies mostly focus on the supplementation of NP with autologous NP cells, notochordal cells, articular chondrocytes, mesenchymal stromal cells (MSC) or induced pluripotent cells (iPSC) [33]. However, these strategies face major challenges due to the limited availability and low proliferation capability of autologous NP cells or articular chondrocytes and low survival of MSCs and iPSCs caused by the harsh microenvironment in the disc [32]. Molecular therapies currently tested include growth factors, anti-catabolic, anti-inflammatory or senolytic approaches [33, 34], with their major disadvantage being short half-lives and hence requiring multiple injections. Replacement of parts or the entire IVD is currently being studied in tissue engineering-focused approaches [32]. The replacement is mostly aimed to be achieved with scaffolds fabricated from natural or synthetic biomaterials with 3D bioprinting, electrospinning, and composite hydrogel fabrication. The major challenges for these approaches include the integration of the scaffold with native tissue, biomechanics and scalability [25]. Gene therapies has been of specific interest in recent years due to the promising technological advances and applications [32, 35]. Targeting of genes like sex-determining region Y box 9 (SOX9), transforming growth factor beta 1 (TGFβ1), bone morphogenetic protein 2 (BMP2), and interleukin 1 receptor antagonist (IL1Ra) has shown encouraging results in in vitro and in vivo studies [36-38]. Importantly, current research is exploring gene silencing through RNA interference with small interfering RNAs and miRNAs and has shown promising preclinical results [39, 40].
2.4 MicroRNAs in Intervertebral Disc Degeneration


**Abstract**

Intervertebral disc (IVD) degeneration is a multifactorial pathological process associated with low back pain, the leading cause of years lived in disability worldwide. Key characteristics of the pathological changes connected with degenerative disc disease (DDD) are the degradation of the extracellular matrix (ECM), apoptosis and senescence, as well as inflammation. The impact of nonphysiological mechanical stresses on IVD degeneration and inflammation, the mechanisms of mechanotransduction, and the role of mechanosensitive miRNAs are of increasing interest. As post-transcriptional regulators, miRNAs are known to affect the expression of 30% of protein-coding genes and numerous intracellular processes. The dysregulation of miRNAs is therefore associated with various pathologies, including degenerative diseases such as DDD. This review aims to give an overview of the current status of miRNA research in degenerative disc pathology, with a special focus on the involvement of miRNAs in ECM degradation, apoptosis, and inflammation, as well as mechanobiology.

**Introduction**

Disc degeneration is a pathological process that leads to the deterioration of intervertebral discs (IVDs), the connective tissue between vertebrae which plays a crucial
role in spinal kinematics. The degenerative processes occur on the tissue, cellular, and molecular level, leading to major changes in the morphology and physiology of the disc, and ultimately resulting in its decreased capability to bear compressive loads. Being a multifactorial disease, its etiology is as of yet still not completely understood. However, it is widely recognized that several factors, such as genetic predisposition, age, lifestyle (obesity, smoking, depressive symptoms), and nonphysiological mechanical loading contribute to its progression [6, 9, 41]. Beyond that, IVD degeneration is known to be associated with low back pain (LBP) [42]. This is of significance as LBP has been confirmed as the leading cause of years lived in disability worldwide in recent decades, affecting 80% of adults at some point in their lives [1]. Direct costs associated with LBP are estimated to run as high as $90 billion per year in the United States alone [43]. Taking into account the indirect costs caused by disability, such as the reduced productivity of patients, the overall socio-economic implications of LBP are posing a notable challenge for societies [3].

Several pathological changes in the IVD are connected with disc degeneration, amongst which degradation of the extracellular matrix, inflammation, and cell loss (apoptosis) are the most prevalent [41]. Focusing on the extracellular matrix (ECM), which plays an essential role in the mechanical functionality of the IVD, two main components are vital for its integrity: the type I and type II collagen network that provides tensile strength [44], and water-binding proteoglycans such as aggrecan [45]. However, metabolic dysregulation of Nucleus pulposus (NP) cells (i.e., cells in the central region of the IVD) results in their reduced ability to synthesize these ECM components while increasingly secreting ECM degradative molecules such as matrix metalloproteinases.
(MMP) and a disintegrin and metalloproteinases with thrombospondin motifs (ADAMTS) [46, 47]. In consequence, dysregulation combined with proteoglycan breakdown leads to a diminished water-binding capacity of the tissue and finally to its structural collapse. Although the degenerative processes first arise in the NP, it later involves the Annulus fibrosus (AF) (i.e., the outer zone of the IVD), with the boundary between both tissues ultimately being lost [6, 48-50]. ECM degradation is further enhanced by cell loss, a well-studied contributor to disc degeneration. Cell loss can be caused by programmed cell death (apoptosis) and is accompanied by cell senescence, with underlying factors such as mechanical stresses inducing both mechanisms [51-53].

Apart from ECM degradation and cell loss, inflammation also plays an important role and has emerged as a distinguishing factor between asymptomatic disc degeneration and symptomatic disc degeneration, often termed degenerative disc disease (DDD) [7, 31]. It is well known that NP cells increasingly release a number of proinflammatory cytokines with progressive degrees of degeneration and often pain development, with tumor necrosis factor α (TNFα), interleukin (IL)-1β, IL-6, and IL-17 being the most prominent. These cytokines have been proved to promote matrix degradation and to activate a host immune response, eventually leading to the infiltration of immune cells and nerve fibers. The latter is particularly relevant because nerve infiltration is the source of pain associated with DDD [10].

ECM degradation, apoptosis, and inflammation are termed as the hallmarks of DDD and are known to be interconnected and interdependent from each other [6]. Proinflammatory cytokines contribute to the dysregulation of the ECM metabolism by
upregulating the expression of ECM degradative enzymes and downregulating ECM structural components [7, 10]. The inherent degradation of ECM leads to an extracellular accumulation of ECM fragments, which further stimulate the inflammatory response of NP cells [20]. Additionally, higher rates of apoptosis and senescence in IVD tissue are connected with lower ECM production capabilities and inflammation, the latter caused by the senescent-associated secretory phenotype [51, 54, 55].

In recent years, the interplay of mechanics and biology, termed mechanobiology, as well as its role in IVD degeneration, has been of growing interest. Nonphysiological mechanical loading of the IVD has been shown to be tightly associated with matrix degradation and changes in cellular physiology [27, 56-59]. Several attempts have been made to explain the interdependency of mechanobiology, ECM degradation, cell loss, and inflammation, as well as possible feedback loops [48, 60, 61]. However, the observed connection between these mechanisms is not yet sufficiently understood and adds to the multifactorial nature of pathological changes associated with IVD degeneration.

Based on the current knowledge and understanding of IVD degeneration, standard therapy has been relying on pharmacological treatments, physiotherapy and, as a last resort, invasive surgical procedures, such as spine fusion or arthroplasty [62, 63]. Due to the major limitations of these treatments, including modest success rates, invasiveness, and high costs, there has been a high demand for novel, targeted treatments that counteract the degenerative processes and reduce pain. Several approaches are being studied and tested as possible treatment options, amongst which cell therapies, endogenous repair strategies by activation of IVD reparative cells, and
treatment based on biological factors such as microRNAs (miRNAs) are the most promising [32, 33].

As key regulators of gene expression, miRNAs, a type of small noncoding RNAs, inhibit the translational process by binding to the 3′-untranslated region (3′-UTR) of target mRNA molecules, often leading to their degradation. In general, primary miRNAs are generated in the nucleus and further processed to mature miRNAs in the cytoplasm by the enzyme Dicer. After incorporation into the RNA-induced silencing complex (RISC), they can target and inhibit the translation of multiple mRNAs, thereby regulating approximately 30% of human protein-coding genes and multiple intracellular processes, including cell proliferation, apoptosis, and cytokine release [64]. Furthermore, miRNAs are known to interact with other endogenous RNAs, such as long noncoding RNAs, circular RNAs, and mRNAs, forming an extensive network of gene regulation in IVD cells, which was recently described by Zhu et al. [65]. Dysregulation of miRNAs has been associated with several pathological conditions, including cancer [16], cardiovascular diseases [66], as well as osteoarthritis [67] and IVD degeneration [68]. This has fueled the interest in miRNAs, their role in DDD, and their potential as novel biomarkers and therapeutics.

This review aims to provide a comprehensive overview of the past five years of literature on miRNAs associated with degenerative disc pathology. Particular attention will be paid to the dysregulation of miRNAs and their role in regulating the hallmarks of IVD disease, namely ECM degradation, apoptosis, and inflammation, as well as mechanobiology as an overarching theme (Error! Reference source not found.5).
Figure 2.5 The role of microRNAs in the pathology of DDD. The miRNAs listed have been studied for their involvement in extracellular matrix (ECM) degradation, apoptosis, and inflammation, as well as mechanobiology as an overarching theme, respectively.

ECM Degradation

The composition of the ECM, as well as homeostasis between ECM degradation and rebuilding are crucial for the physiological function of IVDs. NP cells play a central role in the anabolism and catabolism of the ECM, maintaining this homeostasis and compositional integrity of the tissue. More specifically, the term NP cells comprises two specific cell types: nucleopulpocytes and notochordal cells. The presence of both nucleopulpocytes and notochordal cells seems to be essential for ECM homeostasis, and
the age-related loss of notochordal cells leads to an imbalance, which is connected to ECM degradation. In the pathological event of IVD degeneration, the ECM metabolism is also dysregulated due to changes in the gene expression and protein secretion of NP cells, leading to increased release of ECM degradative enzymes and decreased production of ECM structural molecules [50]. Proteolytic enzymes, such as MMPs and ADAMTS, are mainly responsible for the structural and content-related changes of the ECM[46, 47]. MiRNAs, being post-transcriptional regulators, have been shown to play a crucial role in the metabolic dysregulation by regulating the gene expression of NP cells (Table 2.1).

### Table 2.1 microRNAs associated with ECM degradation in DDD and their corresponding targets.

<table>
<thead>
<tr>
<th>microRNA</th>
<th>Target</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>miR-93</td>
<td>MMP-3</td>
<td>Jing et al. (2015) [69]</td>
</tr>
<tr>
<td>miR-193-3p</td>
<td>MMP-14</td>
<td>Ji et al. (2016) [70]</td>
</tr>
<tr>
<td>miR-27b</td>
<td>MMP-13</td>
<td>Li et al. (2016) [71]</td>
</tr>
<tr>
<td>miR-133a</td>
<td>MMP-9</td>
<td>Xu et al. (2016) [72]</td>
</tr>
<tr>
<td>miR-98</td>
<td>IL-6/STAT3</td>
<td>Ji et al. (2016) [73]</td>
</tr>
<tr>
<td>miR-132</td>
<td>GDF5</td>
<td>Liu et al. (2017) [74]</td>
</tr>
<tr>
<td>miR-7</td>
<td>GDF5</td>
<td>Liu et al. (2016) [75]</td>
</tr>
<tr>
<td>miR-494</td>
<td>SOX9</td>
<td>Kang et al. (2017) [76]</td>
</tr>
<tr>
<td>miR-21</td>
<td>PTEN</td>
<td>Liu et al. (2014) [77]</td>
</tr>
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</table>

Alterations in the expression of structural proteins such as type II collagen and aggregan by miRNAs seem to occur through two main routes: either by targeting enzymes that are directly involved in ECM degradation such as MMPs [69-72], or by targeting intermediate signaling enzymes such as phosphatase and tensin homolog protein (PTEN), the IL-6/signal transducer and activator of transcription 3 (IL-6/STAT3) signaling pathway or growth differentiation factor 5 (GDF5) [73-77]. Concentrating first on miRNAs directly targeting MMPs, it has been confirmed that miR-93 targets and regulates MMP-3, a
collagen and proteoglycan degrading enzyme. The downregulation of miR-93 observed in degenerative NP cells isolated from patients with DDD led to increased levels of MMP-3, ultimately resulting in type II collagen degradation [69].

Another study identified 28 differentially expressed miRNAs in NP tissue from patients suffering from DDD compared to patients with fresh lumbar fractures by next-generation sequencing (Illumina sequencing). Amongst the identified miRNAs, miR-193-3p was shown to be significantly downregulated in DDD tissues. This downregulation correlated with the grade of degeneration. Target prediction with several online prediction tools and dual luciferase reporter assay confirmed MMP-14 as a target of miR-193-3p. In vivo experiments, where DDD-related miR-193-3p downregulation was counteracted with the injection of miR-193-3p-expressing lentivirus in a DDD rat model, showed significantly increased type II collagen and aggrecan expression levels compared to the untreated control group [70].

Amongst other MMPs, the collagen-degrading enzyme MMP-13 is known to be overexpressed in DDD [46]. Li et al. provided evidence that this overexpression is partially mediated through miR-27b downregulation in NP cells isolated from DDD tissue. Functional characterization confirmed that MMP-13 is a target of miR-27b [71]. Furthermore, studies have shown that miR-133a downregulation, seen in both degenerative NPs and spinal tuberculosis, resulted in the loss of type II collagen. While miR-133a negatively regulates the intracellular gelatinase MMP-9, it has also been shown to be lower expressed in degenerated IVD tissue, thus limiting the tissues’ means to
control MMP-9 expression, ultimately resulting in increased MMP9-associated type II collagen degradation [72, 78].

Focusing on miRNAs targeting signaling enzymes involved in ECM metabolism, miR-98 was reported to target the IL-6/STAT3 signaling pathway, a potential regulator of DDD. The downregulation of miR-98 in DDD led to increased IL-6 levels in NP tissue. In addition, reduced miR-98 levels activated the STAT3 signaling pathway by increasing protein levels of STAT3, pSTAT3, and MMP-2, thereby promoting IVD degeneration [73].

Growth differentiation factor 5 (GDF5) is known to be involved in ECM anabolism [79]. Polymorphisms of GDF5 have been associated with susceptibility to degenerative diseases such as osteoarthritis [80]. The expression of GDF5 was shown to be negatively regulated by two miRNAs in degenerated IVDs: miR-132 [74] and miR-7 [75]. miR-132 promoted ECM degradation by directly targeting GDF5 and leading to increased MMP-13 and ADAMTS4 expression through the mitogen-activated protein kinase/extracellular signal-regulated kinases (MAPK/ERK) pathway. In vivo experiments confirmed that the inhibition of miR-132 attenuated ECM degradation, making it a promising therapeutic target [74]. The role of miR-7 targeting GDF5 was studied in the context of degenerated and IL-1β-stimulated NP cells. Results demonstrated that miR-7 overexpression enhanced ECM degradation, whereas the inhibition of miR-7 reduced this effect [75].

The decreased expression of SRY-box transcription factor 9 (SOX9), a regulator of chondrogenesis, is associated with aging and degeneration of IVDs [81]. With miR-494 being upregulated in DDD and directly targeting SOX9, gain-of-function experiments showed enhanced gene expression and protein levels of ECM degradative enzymes
MMP-3, MMP-13, and ADAMTS5. In parallel, loss of function experiments indicated increased type II collagen and aggrecan expression [76]. Finally, the upregulation of miR-21 in degenerated IVD tissue generates higher MMP-3 and MMP-9 expression levels by directly targeting PTEN involved in the Akt signaling pathway [82].

Taking these recent studies into account, it becomes evident that miRNAs play a key role in the metabolic dysregulation in IVD pathologies. Alterations in the miRNA expression profile of degenerative NP cells lead to the dysregulation of ECM metabolic enzymes, resulting in the degradation and compositional change of the ECM. This provides an opportunity to counteract the degenerative processes with miRNAs as therapeutic targets. For instance, a recent preclinical study evaluated the in vivo effects of injecting the inhibitor of miR-141, known to be involved in ECM degradation and apoptosis. The successful delivery of anti-miR-141 seemed to have a protective effect against DDD [39]. Another study investigated the use of high MMP levels in the ECM of degenerated IVDs for a novel two-stage delivery system of therapeutic miRNAs by encapsulating them in an outer MMP-degradable hydrogel and an inner MMP-responsive polyplex micelle for injection into IVD [83].

**Apoptosis**

The degenerative processes in IVDs are associated with high rates of apoptosis and senescence, leading to decreasing cell numbers in NP tissue. Along with metabolic dysregulation, this cell loss adds to the disruption of tissue homeostasis [51-53, 84]. Moreover, the avascular nature of the NP tissue contributes to the accumulation of cellular senescence, which is accompanied by inflammatory responses, low cell
proliferation, and a catabolic phenotype, all of which take part in IVD degeneration [85]. However, mechanisms behind the increased levels of apoptosis and senescence in degenerated IVDs are, as of now, not completely understood. Changes in the expression profile of miRNA were shown to play a role in the intracellular regulatory cascade leading to apoptosis in DDD (Figure ) and other degenerative diseases such as osteoarthritis [86]. Furthermore, miRNAs are known to be implicated in cellular senescence and senescent-associated secretory phenotype [87].

The most promising and thoroughly conducted studies investigating the role of miRNAs in IVD degeneration identified miR-185 and miR-143-5p to be associated with apoptosis. Both studies conducted in vivo experiments using DDD rat models established by needle puncture of the NP tissue[88, 89]. miR-185 was found to be targeting galectin 3, a β-galactosidase-binding protein involved in apoptosis and the Wnt/β-catenin pathway [90, 91]. Expression levels of galectin 3 were significantly elevated in rats with DDD compared to the healthy control group. These expression levels, as well as the activation of the Wnt/β-catenin signaling pathway, increased even more when miR-185 was inhibited. However, transfection of miR-185 in vitro and injection of miR-185 in vivo attenuated these effects, leading to decreased rates of apoptosis[88]. miR-143-5p, on the other hand, was shown to be upregulated in degenerated NP tissue of rats. Eukaryotic elongation factor 2 (eEF2), as one of the targets of miR-143-5p, is known to be involved in apoptosis and cell proliferation, and its activation is connected with the 5’ adenosine monophosphate-activated protein kinase (AMPK) signaling pathway [92]. The dysregulation of miR-143-5p in DDD was shown to lead to the decrease of eEF2 and
activation of AMPK signaling pathway, which consequently decreased type II collagen and aggrecan expression. The inhibition of miR-143-5p resulted in lower levels of apoptosis and senescence via the inactivation of AMPK in vitro [89]. In addition, the effect of miR-143 upregulation was also studied in human NP tissue isolated from patients suffering from DDD [93]. Overexpression of miR-143 was found to be proapoptotic by directly targeting and reducing intracellular B-cell lymphoma-2 (BCL2), an enzyme blocking apoptosis. Focusing also on epigenetic factors of miRNA regulation, it was shown that the promoter of miR-143 is hypo-methylated in DDD [93].

![Figure 2.6](image)

**Figure 2.6** Overview of miRNAs associated with increased rates of apoptosis and senescence in DDD. Targets of negative miRNA regulation are shown in ellipses. The dysregulation of miRNAs and subsequently of their targets influence the downstream signaling, ultimately promoting apoptosis and senescence.
Amongst the pathways involved in apoptosis, the phosphoinositide 3-kinase (PI3K)/Akt pathway is known to play an important role especially in degenerative diseases and cancer. Importantly, this pathway is negatively regulated by PTEN [94]. Two miRNAs were found to influence apoptosis by regulating PTEN/PI3K/Akt signaling, either by directly targeting PTEN (miR-21)[77, 82] or via sirtuin 1 (miR-138-3p) [95].

Interestingly, the upregulation of miR-494 in degenerative IVDs was reported to not only be involved in ECM degradation [76], but also in apoptosis [76, 96]. Two pathways of post-transcriptional regulation of miR-494 were studied, targeting either JunD [96] or SOX9 [76]. The first study showed that TNF-α-induced apoptosis led to miR-494 upregulation, whereas the knock-down of miR-494 resulted in lower apoptosis rates via JunD upregulation [96]. A more recent study proposed that miR-494 upregulation leads to lower intracellular SOX9 levels. SOX9 is known to protect against IL-1β-induced apoptosis in other degenerative diseases, such as osteoarthritis [97]. The downregulation of SOX9 by miR-494 seen in degenerative IVDs, therefore, increased apoptosis, shedding light on mechanisms underlying increased apoptotic rates [76].

Other studies have shown the dysregulation of miR-660 [98], miR-145 [99], and miR-34a [100] in DDD as well as their association with increased levels of apoptosis. miR-145, targeting Adam17, repressed NP cell apoptosis in vitro, both in the presence and absence of oxidative stress [99]. On the other hand, miR-660 was significantly upregulated in DDD and after TNFα-induced apoptosis. Inhibition of miR-660 led to lower apoptosis levels and seemingly downregulated c-caspase3 and c-caspase7, both involved in apoptosis [98]. Furthermore, miR-34 was also found to be upregulated in degenerated
cartilage endplate tissue, negatively regulating BCL2. Silencing of miR-34 resulted in reduced rates of apoptosis in vitro [100]. Similar results have been observed in a study investigating miR-34 in an in vitro rat osteoarthritis model [101]. However, these studies should be regarded with caution because the connection between miRNA targets and apoptosis was not addressed sufficiently and no substantial proof of the connection between these miRNAs and apoptosis was provided.

**Inflammation**

As one of the hallmarks of DDD, inflammation of the degenerated IVD tissue accompanied by increased release of proinflammatory cytokines is one of the key factors leading to discogenic pain. The secretion of cytokines, with TNF-α, IL-1β, and IL-6 being the most prominent ones, results in the recruitment of host immune cells (macrophages, neutrophils, and T-cells) if structural defects exist. As the inflammatory response progresses, immune cells and nociceptive nerve fibers from the dorsal root ganglion start to infiltrate the damaged IVD tissue. Once NP and immune cells start releasing neurotrophins, the nociceptive nerve fibers get stimulated and start transducing the pain sensation. Furthermore, inflammatory cytokines enhance the degenerative process by activating the expression of ECM degradative proteins and inhibiting the expression of ECM structural molecules. Additionally, these cytokines are known to contribute to cellular senescence and apoptosis [7, 10, 31]. The close association between miRNA and inflammation, either by being part of the cellular response to the inflammatory environment or by contributing to inflammation through dysregulating cytokines, has been described in numerous diseases [102].
A study by Dong et al. investigated the role of miR-640 in DDD and inflammation. After confirming the upregulation of miR-640 in both, DDD tissue and cells, they found that this upregulation could be caused by the inflammatory environment. Stimulation of cells with TNF-α and IL-1β led to a significant increase in miR-640 mediated through the nuclear factor-κB (NF-κB) signaling pathway. The target of miR-640 was predicted and confirmed to be the low-density lipoprotein receptor-related protein 1 (LRP1), an indirect inhibitor of NF-κB. Furthermore, they provided evidence that miR-640 is involved in the degenerative process by inducing senescence and apoptosis in NP cells, increasing expression of MMP-3 and MMP-9, and decreasing aggrecan and type II collagen [103].

miR-625-5p was also found to be induced by the NF-κB signaling pathway through the toll-like receptor 4 (TLR4), after stimulation with lipopolysaccharide (LPS). The target of miR-625-5p was confirmed to be the ECM structural protein type I collagen [104].

Evidence of the involvement of miR-194 in the inflammatory response was provided in two studies investigating two different targets. Both studies showed that miR-194 was downregulated in an inflammatory environment after treatment with LPS [105, 106]. This downregulation was connected with the overexpression of the two cullin proteins CUL4A and CUL4B. The increased content of CUL4A and CUL4B was confirmed in degenerated IVD tissue, positively correlating with the degree of degeneration[106]. Furthermore, it was shown that miR-194 also targets TNF receptor-associated factor 6 (TRAF6) and that the transfection of healthy rat NP cells with miR-194 could decrease the expression of inflammatory cytokines, even after LPS treatment [105]. Interestingly, a thoroughly conducted third study showed that miR-194 together with miR-515 was
upregulated in DDD tissue by inflammation [107]. These contradictory findings could arise from the fact that the two previously mentioned studies evaluated miR-194 levels by using rat NP cells [105] or human NP cell lines [106], while this study used primary NP cells isolated from human degenerated IVD tissue. Cell lines and animal models are known to show phenotypic differences compared to primary tissue, the latter being generally preferred, but only available in limited amounts. The upregulation of miR-194 and miR-515 led to the degradation of chondroitin sulfate synthase CHSY-1/2/3 in human degenerated NP cells [107]. These glycosyltransferases are responsible for the synthesis of chondroitin sulfate, a water-binding molecule which is an essential component of aggrecan [108-111]. Their results provided evidence that miRNAs act as mediators between inflammatory cytokines and ECM synthesis [107].

Other studies showed the inflammation-dependent dysregulation of miRNAs such as miR-149 [112], miR-181a [113], miR-146a [114], and miR-155 [115] and their involvement in intracellular signaling by targeting myeloid differentiation primary response 88 (MyD88) [112], tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [113], or transcription factor 7-like 2 [115]. Even though these studies failed to provide sufficient proof of the underlying mechanisms, the above-mentioned miRNAs might serve as potential biomarker candidates due to their strong dysregulation. For instance, preliminary screening of DDD-associated miRNAs in patient serum showed that miR-155-5p was significantly downregulated when comparing a small cohort of patients with DDD to healthy patients (n = 3 each) [116]. However, variations in serum miRNA content could be influenced by numerous other diseases such as obesity or diabetes,
which, together with limited extracellular stability of miRNAs, poses a major limitation and challenge in finding DDD-specific circulating miRNAs.

**Mechanobiology**

IVDs are crucial for the flexibility of the spine, as well as for the compensation and distribution of mechanical loads. Being embedded between two vertebrae, the inner NP and outer AF tissue are connected with the vertebral bodies through cartilaginous endplates (CEP) [24]. Nonphysiological mechanical loads are one of the known factors contributing to DDD and LBP [56-58]. In recent decades, the impact of mechanical forces on cells and their gene expression has become of growing interest. Research clearly indicates that this connection between mechanics and biology is of complex nature, affecting several cellular processes including inflammation, ECM degradation, and apoptosis [48]. The regulation of these processes might be mediated through mechanosensitive miRNAs, which in the context of IVDs have mainly been studied in the CEP[117-120]. Degeneration of the CEP results in its calcification [121] and a decrease of thickness [122], limiting the supply of nutrients to NP tissue and thereby contributing to DDD [123].

A study by Liu et al. showed that matrix stiffness of the CEP positively correlates with the degree of DDD. Structural changes in the collagen network and increased matrix stiffness, indicating severe degeneration of CEP, promoted inorganic phosphate-induced calcification. The microRNA expression profile of CEP chondrocytes cultured on stiff matrices showed significant upregulation of miR-20a, which in turn downregulated ankyloses protein homolog (ANKH). This dysregulation of ANKH is associated with
inorganic phosphate-induced calcification and with the degree of DDD in clinical samples [117].

Focusing on the effects of mechanical tension on miRNAs, intermittent cyclic mechanical tension (ICMT) of isolated CEP chondrocytes resulted in the significant upregulation of 21 and downregulation of 62 miRNAs. These results were validated with RT-qPCR, generating the most promising upregulated (let-7a, miR-29c, miR-142, miR-181a) and downregulated candidates (miR-H14, miR-637). Gene target prediction showed that most of these miRNAs are mainly targeting the MAPK and Wnt signaling pathways [120]. Another study identified miR-365 to be mechanosensitive after ICMT activation of CEP chondrocytes. The target of miR-365 was predicted to be histone deacetylase 4 (HDAC4) and the downregulation of miR-365 after mechanical stress led to the downregulation of type II collagen and aggrecan. This downregulation and loss of ECM structural molecules was attenuated after transfecting CEP chondrocytes with miR-365 mimics. Furthermore, the dysregulation of HDAC4 caused by the mechanosensitive miR-365 resulted in the activation of the Wnt/β-catenin signaling pathway [119]. Mechanical tension also affected the expression of miR-455-5p in endplate chondrocytes, accompanied by the dysregulation of RUNX2 [118].

In conclusion, it is evident that we currently only have very limited insight into the effects of mechanical stresses on miRNA expression and regulation. In particular, the implications of mechanosensitive miRNAs on DDD in NP and AF tissues have not yet been studied. The role of the intralamellar matrix, located between the lamellae of the AF tissue and densely packed with elastic fibers, has recently become evident in the context
of micromechanical properties of the IVD [124]. Studying miRNAs in the context of disrupted intralamellar matrices and subsequent changes in the mechanical properties of the tissue would provide an interesting new direction for future research. Furthermore, focusing on the differences of beneficial and nonbeneficial mechanical loads on miRNA expression, which have already been studied in other degenerative diseases such as osteoarthritis [23], would be of big importance. This provides an opportunity for future studies to gain a better understanding of the overreaching effects of nonphysiological mechanical loads on the degenerative process.

**Conclusion**

Based on the current knowledge provided herein, it is evident that pathological changes in cells of DDD tissues are associated with the dysregulation of miRNAs and their targets. Their involvement in multiple cellular processes appears to contribute to the three hallmarks of DDD: ECM degradation, apoptosis, and inflammation. A small number of studies have also provided evidence that miRNAs might be involved in the process of mechanosensing in IVD cells. However, a better understanding of the mechanisms behind the dysregulation of miRNAs and their role in DDD progression is still needed. Additionally, future studies should consider investigating the broader miRNA–mRNA network in order to gain a deeper knowledge of the regulatory pathways in DDD pathology.

A better understanding of miRNAs in DDD provides a considerable opportunity for their use as (a) biomarkers or (b) drug targets and future therapeutics: (a) As in many other diseases, circulating miRNAs have been discussed and studied as potential
biomarkers in blood for early noninvasive detection of IVD degeneration. Challenges connected with the selection of circulating miRNAs as biomarkers are the need for extensive screenings and the selection of DDD-specific dysregulated miRNAs that are not associated with other pathologies [125], the latter being one of the major limitations due to the fact that miRNAs are often linked to multiple diseases. Therefore, changes in serum miRNA levels might not only be connected to IVD degeneration, possibly making miRNA serum levels a biased reflection of the degree of degeneration. Extensive preliminary screenings have already been done in similar pathologies, such as osteoarthritis [126] or ossification of the posterior longitudinal ligament [127]. (b) As the search for novel noninvasive treatment options for DDD regeneration continues, cell therapies are amongst the most studied, ranging from the use of NP cells, chondrocytes, and mesenchymal stem cells to in vitro differentiated nucleopulpocytes and notochordal cells [32, 33]. On the other hand, biological factors, such as miRNAs, provide a huge therapeutic potential in counteracting dysregulated cellular metabolism. Of course, several challenges need to be faced in the process of developing miRNA-based therapeutics. Methods of delivery, including the challenges of direct injection and miRNA vectorization, are being studied; for example, the use of injectable MMP-degradable hydrogels containing MMP-responsive polyplex micelles as a two-stage delivery system for miRNAs [83]. Other challenges that should be addressed in the future are the appropriate dosing, recognition of target cells, and timing of miRNA-based therapeutics. Moreover, potential miRNAs, as opposed to small-interfering RNAs, have to be carefully
selected and meticulously studied in vitro and in vivo with regard to their nature of targeting multiple genes and their interaction network with other endogenous RNAs.

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Chapter 3
Exploring the Impact of TLR-2 Signaling on miRNA Dysregulation in Intervertebral Disc Degeneration
Exploring the Impact of TLR-2 Signaling on miRNA Dysregulation in Intervertebral Disc Degeneration

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**Abstract**

Toll-like receptors (TLRs) are key mediators of inflammation in intervertebral disc (IVD) degeneration. TLR-2 expression and activation contributes to degeneration by increasing the expression of ECM-degrading enzymes, pro-inflammatory cytokines, and neurotrophins. As potent post-transcriptional regulators, microRNAs can modulate numerous intracellular mechanisms and signaling pathways including TLR signaling, downstream inflammation, and catabolism. More importantly, the dysregulation of miRNAs is known to contribute to numerous pathologies. This study aims to investigate the impact of TLR-2 signaling on miRNA dysregulation and to identify miRNAs specifically associated with TLR-2 signaling in the context of IVD degeneration. TLR-2-associated miRNAs were identified by small-RNA sequencing in degenerated human IVD cells and further studied in both, degenerated and non-degenerated IVD cells. We identified 10 dysregulated miRNAs following TLR-2 activation by PAM2CSK4 (TLR-2/6 agonist). The miR-155-5p was significantly upregulated by TLR-2 signaling in degenerated and non-degenerated annulus fibrosus (AF) and nucleus pulposus (NP) cells. Sequence-based target and pathway prediction showed its involvement in inflammation- and cell fate-related pathways. Downregulation of the miR-155-5p target c-FOS was confirmed following the TLR-2-induced miR-155-5p expression. Changes specific to the activation of TLR-2 through fragmented fibronectin (fFN) compared to PAM2CSK were seen in miR-484 and miR-487. Furthermore, a comparison of the miRNAs expression profile in degenerated vs. non-degenerated IVD cells showed degeneration-dependent changes in miR-100-3p, miR-320b, and miR-181a-3p. These results show that TLR-2 signaling leads
to the dysregulation of miRNAs in IVD cells as well as their possible downstream effects on inflammation and degeneration. The identified miRNAs provide important opportunities as potential therapeutic targets and biomarkers of IVD degeneration and low back pain.

**Introduction**

Inflammation is one of the key pathological processes involved in the degeneration of the intervertebral disc (IVD), the fibrocartilaginous tissue located between vertebrae [6, 7, 11]. During IVD degeneration, morphological and physiological changes affect the tissue on a cellular and structural level [6, 48]. Inflammation, in conjunction with a catabolic shift causing the degradation of the extracellular matrix (ECM) and cell loss due to apoptosis and senescence, are the key elements of these pathological changes and more importantly, are highly interconnected [6, 8, 48]. Increased secretion and accumulation of pro-inflammatory cytokines during IVD degeneration enhances ECM degradation by inducing the expression of matrix-degrading enzymes and reducing the synthesis of ECM proteins [10, 128, 129]. The presence of cytokines together with the catabolic shift in cell activity has also been connected to apoptosis in NP and AF cells [130]. Furthermore, continuous inflammation in degenerated IVD tissue can result in the activation of the host immune response leading to the infiltration of immune cells and nociceptive nerve fibers. With progressive degrees of degeneration, the inter-connected processes of chronic inflammation, innervation, and nociception can lead to discogenic low back pain (often termed degenerative disc disease, DDD), contributing to the leading cause of years lived in disability worldwide [1, 7, 42].
Toll-like receptors (TLRs) are regulators of inflammation in the innate immune response and have been shown to play an essential role in IVD inflammation and senescence [14, 131]. Activation of TLRs is caused by pathogen-associated (PAMPs) and damage-associated molecular patterns (DAMPs) [132]. As IVD degeneration is characterized by ECM degradation, certain DAMPs, such as fragmented hyaluronic acid (fHA) or fragmented fibronectin (fFN), are of particular relevance in DDD [133, 134]. Our group has previously shown that the expression of TLR-1, -2, -4, and -6 increases in human IVD cells with progressive degrees of degeneration [12]. Amongst these, TLR-2 has been shown to be particularly relevant in IVD degeneration [12, 13]. The activation of TLR-2 in human IVD cells leads to downstream signaling involving pathways associated with DDD, such as NF-κB, p38, and ERK, as well as to elevated expression and secretion of pro-inflammatory cytokines, ECM degrading proteinases and neurotrophins (IL-6, TNFα, IL-1β, NGF, MMP-1, MMP-3, etc) [13, 20, 135]. The contribution of TLR-2 to pro-inflammatory cytokine expression and macrophage infiltration has also been shown in spinal cord glial and dorsal root ganglia [136, 137]. More recently TLR-2 signaling was also linked to senescence in IVD cells [14]. Taking this evidence together, there is a clear indication of TLR-2 dysregulation being an important factor in IDD and the development of discogenic LBP/DDD.

Major contributors to the dysregulation of gene expression and intracellular signaling are microRNAs (miRNAs) [64]. These small non-coding RNAs are strong post-transcriptional regulators that have been connected to numerous degenerative diseases including osteoarthritis [67] and DDD [138]. Their involvement in cellular
processes includes apoptosis, senescence, cell cycle, tumorigenesis, and inflammation [15, 16, 139]. In recent years, a connection between TLRs and miRNAs has been suggested in immune cells [18, 19, 140]. However, this interaction between miRNAs and TLRs in IVD tissue and its role in the degenerative process has not been studied yet.

This study aims to identify microRNAs that are associated with TLR-2 signaling in order to enhance our understanding of the underlying mechanisms of inflammation in IVD degeneration. We hypothesized that increased TLR-2 activation leads to dysregulation of miRNA expression in both degenerated and non-degenerated human IVD cells, possibly contributing to IVD degeneration. Our findings show that activation of TLR-2 leads to the differential expression of 10 miRNAs, out of which miR-155-5p was most prominently and consistently upregulated.

**Results**

**TLR-2 activation leads to dysregulation of miRNA expression in degenerated IVD cells**

In earlier studies, we and other groups showed that PAMP-induced activation of the TLR-2 signaling pathway in human IVD cells promotes a catabolic phenotype and induces disc degeneration. The activation of this pathway occurs through the binding of PAMPs to the TLR 2 heterodimers, such as the TLR-2/6 specific agonist PAM2CSK4 [10-12]. To identify microRNAs associated with the TLR-2 signaling pathway, cells isolated from degenerated human IVDs were treated with PAM2CSK4 (100 ng/mL for 6h), followed by Illumina small RNA sequencing (Figure 3.1 A). As controls, TLR-2 knockdown cells were included (Figure 3.1 B-C). The pathway activation was confirmed by detecting a significant increase in IL-6 (30.5 ± 8.1 pg/mL, p = 0.0002) and IL-8 secretion (28.9 ± 5.4 pg/mL,
Small RNA sequencing showed significant changes in the miRNA profile of IVD cells in response to TLR-2 activation, detecting 168 miRNAs of which 10 were differentially expressed (log fold change > ± 0.5 and p < 0.05, Figure 3.1 E-F). These 10 miRNAs were selected for further investigation. Overall, this data shows significant changes in the miRNA expression profile following the activation of TLR-2 signaling in degenerated IVD cells.

**Figure 3.1** Identification of TLR-2-associated miRNAs through next-generation sequencing (NGS). a Schematic of the experimental setup using human degenerated IVD cells. b Immunocytochemistry of TLR-2 (yellow) in IVD cells (nucleus: blue). c Gene and protein expression of TLR-2 in IVD cells transfected with non-targeting control (NT siRNA) and TLR-2 siRNA (n = 5). d Protein expression of TLR-2 and β-Actin in IVD cells following siRNA transfection (n = 1). e Secretion of the cytokines IL-6 and IL-8 after PAM2CSK4 treatment (n = 5). f Volcano plot and G heat map of miRNAs identified by Illumina small RNA sequencing (n = 5). Mean ± SD, * p < 0.05, *** p < 0.001, **** p < 0.0001
miR-155-5p is upregulated in AF and NP cells following TLR-2 activation

In a next step, the 10 miRNAs that were identified in IVD cells without zonal separation (Figure 3.1) were validated in degenerated annuls fibrosus (AF) and nucleus pulposus (NP) cells after TLR-2 activation. Accordingly, AF (Figure 3.2 A) and NP cells (Figure 3.2 B) were treated with PAM2CSK4 (100 ng/ml for 6h) followed by miRNA isolation and miRNA RT-qPCR. TLR-2 activation and knockdown were confirmed in the respective samples (Appendix, Suppl. Fig. 1). Of the 10 tested miRNAs, miR-155-5p was significantly upregulated in AF cells (1.3 fold, p = 0.034, Figure 3.2 C) and NP cells (1.8 fold, p = 0.035, Figure 3.2 D). This upregulation was partially reversed in TLR-2 knockdown cells. Furthermore, miR-30c-5p and miR-100-5p showed slight downregulation following TLR-2 activation in AF cells, while NP cells showed a minor (but non-significant) upregulation. The other miRNAs showed non-significant changes following TLR-2 activation. These experiments confirm the TLR-2-dependent upregulation of miR-155-5p previously detected by next-generation sequencing (NGS) in both AF and NP cells of degenerated IVDs.

DAMP-based TLR-2 activation leads to changes in miRNA expression

One characteristic of IVD degeneration is the degradation of the ECM, resulting in the accumulation of fragmented ECM proteins in the tissue due to its avascular nature and poor waste exchange. Amongst these fragmented ECM proteins, fragmented fibronectin and hyaluronic acid are known activators of the TLR-2 signaling pathways[13, 20, 141].
Figure 3.2 TLR-2-associated miRNA expression in degenerated AF and NP cells following treatment with PAM2CSK4. TLR-2 knockdown (KD) cells served as an additional control. a-b MiRNA expression in AF and NP cells was detected with RT-qPCR. c-d Expression of miR-30c-5p, miR-100-5p, and miR-155-5p in AF and NP cells. n = 5, mean ± SD, * p < 0.05, ** p < 0.01

To assess the effect of DAMP-induced TLR-2 activation, we studied the expression of the identified miRNAs following treatment with fragmented fibronectin (fFN). Therefore, AF and NP cells were treated with 30 kDa fFN (1 µM) for 6h. Analysis of the miRNA expression in degenerated AF (Figure 3.3 A) and NP (Figure 3.3 B) cells showed a significant increase in miR-484 (1.2 ± 0.35, p = 0.047) and miR-487 (1.2 ± 0.16, p = 0.04) expression in AF cells (Figure 3.3 C). Moreover, an increase in miR-181a-3p was detected in both AF and NP cells. However, this increase was highly donor-dependent and therefore not significant. Lastly, a donor-dependent increase in miR-155-5p was also evident in AF cells, while NP cells showed no changes in miR-155-5p expression. These results show that TLR-2 activation by DAMPs leads to unique changes in the miRNA expression profile, different from those induced by PAMPs.
Figure 3.3 TLR-2-associated miRNA expression in degenerated AF and NP cells following treatment with fFN. TLR-2 knockdown (KD) cells served as an additional control. a-b MiRNA expression in AF and NP cells was detected with RT-qPCR. c-d Expression of miR-155-5p, miR-181a-3p, miR-484, and miR-487 in AF and NP cells. n = 5, mean ± SD, * p < 0.05

**TLR-2 dependent miRNA expression in non-degenerated vs. degenerated IVDs**

To get a better understanding of the role of TLR-2-based miRNA regulation in the development of IVD degeneration, the expression of the identified miRNA was studied in cells from non-degenerate human IVDs. The baseline expression of the 10 miRNAs identified by NGS was analyzed in non-degenerated and degenerated IVD cells (shown as $2^{-\Delta Ct}$ values, Figure 3.4 A-B). The expression of miR-484 was significantly higher in AF and NP cells isolated from non-degenerated IVDs compared to degenerated IVDs (AF: 0.12 ± 0.01 vs 0.08 ± 0.01, p = 0.0001, Figure 3.4 A) (NP: 0.12 ± 0.01 vs 0.07 ± 0.03, p = 0.03, Figure 3.4B). Furthermore, miR-320b expression was significantly higher in non-degenerated NP cells (0.33 ± 0.07 vs 0.27 ± 0.04, p = 0.046, Figure 3.4B). In order to study
changes in the baseline miRNA expression following TLR-2 activation, both non-degenerated and degenerated AF and NP cells were treated with PAM2CSK4 (100 ng/ml for 6h). Interestingly, while the miR-155-5p baseline expression showed no significant differences between non-degenerated and degenerated IVD cells, activation of TLR-2 resulted in a significantly higher expression in non-degenerated AF cells compared to degenerated ones (0.58 ± 0.15 vs 0.28 ± 0.12, p = 0.0001, Figure 3.4 A). Similar effects were observed in non-degenerated NP cells, even though the increase in miR-155-5p expression was not significant (0.59 ± 0.21 vs 0.26 ± 0.12, p = 0.21, Figure 3.4B). TLR-2 activation also caused a significantly higher expression in miR-100-3p in non-degenerated AF cells (0.03 ± 0.01 vs 0.02 ± 0.01, p = 0.0001, Figure 3.4A). Other previously identified miRNAs showed no significant differences in baseline expression in non-degenerated and degenerated IVD cells (Appendix, Suppl. Fig. 2).

After assessing the baseline expression of the 10 identified miRNAs, we tested the effect of PAM2CSK4 treatment (100 ng/ml for 6h) on these miRNAs in non-degenerated NP and AF cells, with inclusion of TLR-2 knockdown control cells (Figure 3.5 A-B). The activation and knockdown of TLR-2 were confirmed by ELISA and RT-qPCR (Suppl. Fig. 3). In non-degenerated NP cells, miR-100-3p and miR-155-5p were significantly increased 2.3 ± 0.5 fold (p < 0.0001) and 3.3 ± 0.5 fold (p < 0.0001), respectively (Figure 3.5 D). Interestingly, we also saw increases of miR-100-3p and miR-155-5p in non-degenerated AF cells, even though these results were only significant in TLR-2 KD cells and not in TLR-2 expressing cells due to high donor-variability (2.8 ± 1.27, p = 0.1310, Figure 3.5 C).
Furthermore, non-degenerated AF cells showed a 2.1 ± 0.4 fold increase in miR-181a-3p (p < 0.0001) (Figure 3.5 C).

Figure 3.4 MiRNA baseline expression in non-degenerated and degenerated human NP and AF cells. a Expression of miRNAs in AF cells from non-degenerated (non-deg) and degenerated (deg) IVDs in the absence (w/o) or presence (+) of PAM2CSK4 treatment. b Expression of miRNAs in NP cells from non-degenerated (non-deg) and degenerated (deg) IVDs in the absence (w/o) or presence (+) of PAM2CSK4 treatment. n = 3, mean ± SD, * p < 0.05, *** p < 0.001

Other miRNAs showed minor significant downregulations, such as miR-484 in non-degenerated AF cells (p < 0.0001) and miR-30c-5p and miR-100-5p in
non-degenerated NP cells (p < 0.0001) (Figure 3.5 C-D). These results show that TLR-2 signaling leads to significant changes in the miRNA expression profile in non-degenerated IVD cells. In accordance with the results of degenerated IVD cells, miR-155-5p shows the highest increase in expression in non-degenerated AF and NP cells following TLR-2 activation.

**Figure 3.5** TLR-2-associated miRNA expression in non-degenerated AF and NP cells following treatment with PAM2CSK4. TLR-2 knockdown (KD) cells served as an additional control. a-b MiRNA expression in AF and NP cells was detected with RT-qPCR. c Expression of miR-100-3p, miR-155-5p, miR-181a-3p and miR-484 in AF cells. d Expression of miR-100-3p, miR-155-5p, miR-30c-5p, miR-100-5p in AF and NP cells. n = 3, mean ± SD, * p < 0.05 , *** p <0.001, **** p < 0.0001

**TLR-2 dependent downregulation of the miR-155-5p target c-FOS**

Based on our findings that miR-155-5p was most consistently and highly upregulated through TLR-2 signaling, we studied downstream targets that are relevant in the IVD degeneration process. Target and pathway predictions of miR-155-5p were
performed with the miRabel target prediction tool, which ranks the results of four main prediction software (miRanda, PITA, SVMicrO, and TargetScan). Prediction of miR-155-5p targets in the human genome based on sequence resulted in 7,194 possible target genes and 304 targeted pathways. We analyzed the top 100 ranked pathways and filtered out non-relevant pathways, such as pathways specific to cancer or other pathologies. The resulting IVD-relevant pathway prediction showed a possible miR-155-5p-associated regulation of the inflammatory pathways TLR, TNF, TGF-β, and NF-κB (Figure 3.6 A-B). Furthermore, numerous pathways involved in cell fate, proliferation, and apoptosis are targeted by miR-155-5p: PI3K-Akt, mTOR, Hippo, Wnt, Jak-STAT, and p53 signaling (Figure 3.6 A-B). More specifically, miR-155-5p targets 20% of the genes of the mTOR pathway, 13.6% of the TNF pathway, and 12% of the TLR signaling pathway (Figure 3.6 B). In terms of overall highest number of genes targeted by miR-155-5p are the pathways PI3K-Akt (31 genes), focal adhesions (17 genes) and Hippo (17 genes) (Figure 3.6 A). Finally we analyzed the gene expression of 4 known, highly ranked targets of miR-155-5p in degenerated IVD cell samples that showed significant miR-155-5p increase following TLR-2 activation (Figure 3.6 C-D). The transcription factor c-Fos was significantly downregulated in degenerated NP cells when miR-155-5p expression was increased through TLR-2 activation (0.49 ± 0.06 fold, p = 0.0002, Figure 3.6 D). This downregulation was reversed in TLR-2 knockdown cells. On the other hand, AF cells showed a downregulation in TLR-2 expressing and TLR-2 knockdown cells (0.69 ± 0.29 fold and 0.47 ± 0.22 fold, respectively, Figure 3.6 C). Additionally, a slight but significant increase in CEBPB expression was detected (1.23 ± 0.01 fold, p = 0.0090). No significant changes in the expression of MYD88
and PI3KCA were seen in AF and NP cells (Figure 3.6 C-D). In summary, this data together with the target and pathway prediction suggest a broad downstream regulation of inflammatory pathways and cell proliferation/apoptosis through miR-155-5p.

Figure 3.6 miR-155-5p target and pathway prediction. A Pathways targeted based on miR-155-5p sequence in the number of targets per pathway. B Pathways targeted based on miR-155-5p sequence in % of genes targeted per pathway. C-D Gene expression of miR-155-5p targets c-FOS, MYD88, CEBPB, and PIK3CA in degenerated AF (C) and NP (D) cells. n = 3, mean ± SD, * p < 0.05, ** p < 0.01, *** p < 0.001, ns: not significant.

Discussion

Toll-like receptors are key regulators of inflammation, a known hallmark of IVD degeneration. In earlier studies, we showed that with progressive degrees of
degeneration, the expression of TLR-2 increases in human IVD cells [12]. This increased expression and activation of TLR-2 is known to contribute to the degenerative process by inducing the expression and secretion of ECM-degrading proteins, pro-inflammatory cytokines, and neurotrophins [13, 20, 135]. Furthermore, recent studies have also pointed out the connection between TLR-2 signaling and senescence, where its activation led to increased senescence in both degenerated and non-degenerated IVD cells [14]. As regulators of cellular signaling and gene expression, miRNAs and their dysregulation have been associated with numerous pathologies and have also been studied in the context of osteoarthritis and DDD [67, 138]. However, the interplay of the TLR-2 signaling pathway and miRNA expression, as well as the possible role of TLR-2 in miRNA dysregulation has not been studied yet in IVD pathophysiology. Therefore, this work sought to analyze global changes in miRNA expression caused by TLR-2 signaling in degenerated and non-degenerated IVD cells and to identify specific TLR-2-associated miRNAs.

We were able to identify 10 miRNAs that were differentially expressed following TLR-2 activation through small-RNA sequencing. Further examination of the expression patterns of these miRNAs in response to PAMP-induced TLR-2 signaling showed a clear association between TLR-2 and miR-155-5p upregulation. Expression of miR-155-5p was increased significantly in both, degenerated AF and NP cells. This increase in miR-155-5p expression was even more pronounced in non-degenerated NP cells, while non-degenerated AF cells showed an evident, though not significant increase. The effects were reduced in TLR-2 knockdown cells. As a next step, we compared the baseline expression of miR-155-5p in degenerated and non-degenerated IVD cells. On the other hand, DAMP-
induced TLR-2 activation with fFN showed only donor-specific increases, with high donor-to-donor variability in AF cells and no changes in NP cells. This could be due to the fact, that DAMPs lead to a more moderate activation of TLR-2 compared to PAMPs [13]. Overall, these results are in concordance with studies performed with cells of the immune system, including macrophages and dendritic cells, where miR-155-5p was upregulated in response to TLR-2 and TLR-4 signaling, and its role in enhancing their inflammatory response was shown in vitro and in vivo [142-144]. The pro-inflammatory role of miR-155-5p in innate immune cells and microglia was also studied under neuroinflammatory conditions and its inhibition was shown to protect retinal degeneration by reducing inflammation [145, 146]. The overexpression of miR-155-5p was furthermore found in human osteoarthritis knee cartilage, leading to the suppression of autophagy in chondrocytes [147].

Apart from miR-155-5p, our data showed significant changes in the miR-484 and miR-487 expression patterns. Activation of TLR-2 resulted in the downregulation of miR-484 in non-degenerated AF cells. This downregulation of miR-484 was also detected in degenerated IVD cells through small-RNA sequencing, while validation experiments did not yield significant changes. Furthermore, degenerated cells showed significantly lower miR-484 baseline expression compared to non-degenerated AF and NP cells. In contrast, the treatment of cells with fibronectin fragments results in a slight increase of miR-484. Similarly, miR-487 which also showed a decrease in expression after PAMP-induced TLR-2 activation in the NGS data, shows an increase in expression after treatment with fFN. These effects were not seen in TLR-2 knockdown cells, hence pointing to the conclusion
that these miRNAs might play a role downstream of TLR-2 signaling specific to activation with DAMPs. Such differences in downstream effects of TLR-2 activation with DAMPs compared to PAMPs were also seen in the gene expression of cytokines and chemokines in other studies [13, 148-150], but have never been shown in miRNA expression so far. While miR-484 and miR-487 have only been studied to a small extent so far and their functional role is mostly still unknown, a few studies point out their possible involvement in apoptosis and proliferation [151-153].

Three miRNAs showed changes in expression that were specific to non-degenerated IVD cells, namely miR-100-3p, miR-320b, and miR-181a-3p. A TLR-2-dependent upregulation of miR-100-3p was seen in non-degenerated AF and NP cells following activation. A much lower increase was detected with NGS in degenerated IVD cells. Furthermore, baseline miR-100-3p expression was significantly higher in non-degenerated compared to degenerated cells when treated with PAM2CSK4. This confirms that TLR-2 induced miR-100-3p upregulation is stronger in non-degenerated IVD cells and this regulation seems to be lost during degeneration. The expression of miR-320b was also significantly higher in non-degenerated NP cells, while the expression was clearly downregulated in degenerated NPs. This difference in expression was increased even more following TLR-2 activation. Since miR-100-3p and miR-320b have been mostly studied as biomarkers in various pathologies, their functional role and hence their possible role in IVD degeneration is still mostly unknown and would require further investigation. Another evident degeneration-dependent change in expression showed miR-181a-3p. Small-RNA sequencing of degenerated IVD cells showed a downregulation
of miR-181a-3p after the activation of TLR-2. In contrast, non-degenerated AF cells showed a clear increase in miR-181a-3p expression. Interestingly, validation of miR-expression in degenerated IVD cells showed no significant changes dependent on DAMP or PAMP-induced TLR-2 signaling, but a slight trend of upregulation was noticeable. Hence, further studies of miR-181a-3p and its involvement in TLR-2 signaling and IVD degeneration are necessary. Changes in miR-181a-3p would be of particular interest since previous studies have shown its involvement in inflammation in other cell types [154, 155]. Furthermore, the miR-181 family is known to be highly involved in multiple pathologies including osteoarthritis [156].

As a last step, we chose miR-155-5p for target and pathway analysis, since it was highly upregulated in both degenerated and non-degenerated cells. Sequenced-based target predictions showed that miR-155-5p targets a total of 12% of the genes in the TLR-2 signaling pathway. The expression of a selection of the targeted genes was analyzed in samples where miR-155-5p was upregulated. Most strikingly, the transcription factor c-Fos was significantly downregulated in degenerated AF and NP cells, indicating a negative regulation through miR-155-5p and TLR-2. The targeting and downregulation of c-Fos by miR-155-5p was also shown by a previous study in vascular smooth muscle cells with downstream effects on cell viability and apoptosis, however not in the context of TLR-2 signaling [157]. Apart from TLR-2, miR-155-5p was predicted to target numerous pathways that are involved in inflammation in the IVD (TNF, TGF-β, NF-κB) as well as cell fate/proliferation pathways (PI3K-Akt, mTOR, Hippo, Wnt, Jak-STAT, and p53 signaling). Several in vitro studies have experimentally validated the regulation of some of these
pathways in various cell types and an inhibitor of miR-155-5p has been tested as a therapeutic agent against cutaneous T-cell lymphomas in phase 2 clinical trials [147, 158-161]. This indicates that miR-155-5p could be a potential mediator between TLR-2 signaling and the induction of IVD degeneration. Hence, the functional role of miR-155-5p in degeneration, inflammation, and proliferation should be further investigated comprehensively.

In summary, this study gives a comprehensive overview of the effects of TLR-2 signaling on the miRNA expression profile in IVD cells, as well as their possible roles as mediators of TLR-2-induced IVD degeneration. We identified miR-155-5p being highly upregulated by TLR-2 activation in degenerated and non-degenerated IVD cells, acting as a pro-inflammatory miRNA by targeting multiple degeneration-relevant pathways and genes. Furthermore, we were able to show that the dysregulation of miR-484 and miR-487 is dependent on whether TLR-2 is activated through PAMPs or DAMPs. Lastly, miR-100-3p, miR-320b, and miR-181a-3p showed degeneration-dependent changes in expression when comparing degenerated with non-degenerated cells. Future studies will focus on the functional role of these miRNAs in the context of IVD degeneration and inflammation, in order to identify possible disease-modifying targets for the reduction of inflammation and discogenic low back pain.

Materials and Methods

Human IVD cell isolation and culture

Human degenerated IVD cells were isolated as described previously from biopsies obtained from patients undergoing spinal surgery due to disc herniation or DDD [162].
Briefly, degenerated AF, NP, or mixed IVD tissue was excised intraoperatively, mechanically diced, and enzymatically digested with 0.2% collagenase NB4 (Nordmark) and 0.3% dispase II (Sigma-Aldrich) in 1x Dulbecco’s Phosphate Buffered Saline (DPBS, Cytiva) with 5% antibiotic-antimycotic (anti-anti, Gibco) overnight at 37 °C, 5% CO₂. Following isolation, cells were cultured up to passages 1-2 in growth medium (Dulbecco’s Modified Eagle’s Medium/Ham’s F-12 (DMEM/F-12 Cytiva) supplemented with 1% anti-anti and 10% fetal bovine serum (FBS, Cytiva)). Experiments involving non-degenerated cells were performed at McGill University, where cells were isolated and cultured in growth medium as described previously [34]. An overview of the patients and donor characteristics can be found in Appendix, Supplementary Table 1-2.

**TLR-2 knockdown and activation**

Knockdown of TLR-2 was achieved by reverse transfection of IVD cells (10000 cells/cm²) with 1 µM self-delivering Accell™ TLR-2 siRNA Smartpool (Horizon Discovery, E-005120-00-0050) or Accell™ non-targeting siRNA Pool (Horizon Discovery, D-001910-10-20) in no-serum media (DMEM/F12 with 0.1% anti-anti). After 48h the medium was changed to growth medium in order to let the cells recover. The efficiency of the knockdown was analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot (WB) after 72h.

For the analysis of TLR-2-associated miRNAs, IVD cells were first transfected as described above and medium was changed after 48h to growth medium. After 16h of recovery in growth medium, the cells were starved for 2h in no-serum media followed by 6h treatment with 100 ng/mL PAM2CSK4 (TLR-2/6 agonist, Invivogen, tlrl-pm2s-1), 1 µM
30 kDa fibronectin proteolytic fragments (fFN, Sigma-Aldrich, F9911) or vehicle. After a total time interval of 72h, cells were lysed for miRNA and mRNA extraction and conditioned media was used for protein analysis by enzyme-linked immunosorbent assay (ELISA).

**MiRNA extraction and RT-qPCR**

Extraction of miRNA for small-RNA sequencing and miRNA expression analysis by RT-qPCR was achieved with the miRNeasy Tissue/Cells Advanced Mini Kit (Qiagen, 217604). To that end, cells were rinsed twice with ice-cold PBS immediately after treatment, lysed with 260 uL lysis buffer, and processed according to the manufacturer’s instructions. The concentration and quality of miRNA were measured either with the 42000 TapeStation (Agilent Technologies) for small-RNA sequencing or NanoPhotometer N50 (Implen) for RT-qPCR.

Reverse transcription and quantitative PCR of miRNAs were performed with the miRCURY LNA RT Kit (Qiagen, 339340) and miRCURY LNA SYBR Green PCR Kit (Qiagen, 339347). The respective miRCURY LNA miRNA PCR Assays were used for the specific miRNAs (YP00204783 - hsa-miR-30c-5p, YP00204535 - hsa-miR-100-3p, YP00205689 - hsa-miR-100-5p, YP00204308 - hsa-miR-155-5p, YP00204110 - hsa-miR-181a-3p, YP00204400 - hsa-miR-125b-1-3p, YP02119299 - hsa-miR-320b, YP00205613 - hsa-miR-335-3p, YP00205636 - hsa-miR-484, YP00204489 - hsa-miR-487b-3p, YP00204063 - hsa-miR-103a-3p, Qiagen, 339306). Furthermore, spike-ins were used to monitor the consistency of the extraction process and reverse transcription (RNA Spike-In Kit, Qiagen,
The results are shown as $2^{-\Delta\Delta Ct}$ values relative to the housekeeping miRNA miR-103a-3p and control conditions.

**MiRNA sequencing and bioinformatics analysis**

Illumina small RNA sequencing and data analysis were performed at the Functional Genomics Center in Zurich (FGCZ). The quality and quantity of miRNA samples were analyzed with the Qubit® (1.0) Fluorometer (Life Technologies) and a Bioanalyzer 2100 (Agilent Technologies). Samples with a 260 nm/280 nm ratio between 1.8–2.1 and a 28S/18S ratio within 1.5–2 were chosen for library construction using TruSeq small RNA Sample Prep Kit v2 (Illumina). Briefly, 3’ and 5’ RNA adapters were ligated to total RNA samples (1 μg) and ligated samples were reverse-transcribed into double-stranded cDNA. Fragments containing TruSeq adapters on both ends were selectively enriched with PCR. The small RNA fraction was selected based on size selection of 145-160 bp (including adapters) on polyacrylamide gels. The quality and quantity of the enriched libraries were validated using Qubit® (1.0) Fluorometer and the Agilent TapeStation (Agilent Technologies). Cluster generation was performed with the TruSeq SR Cluster Kit v4-cBot-HS (Illumina) using 10 pM of poled normalized libraries on the cBOT. Finally, the samples were sequenced on the Illumina HiSeq 2500 single end 70 bp using the TruSeq SBS Kit v4-HS (Illumina).

For the small RNA sequencing data analysis, input preprocessing, read mapping, read annotation, annotation analysis, and enrichment analysis were done via the ncPRO v.1.6.5 using the human genome build hg19 and mirBase v21. Differentially expressed miRNAs were then identified with the Bioconductor software package EdgeR.
Bioinformatic prediction of miRNA target genes and pathways was performed with miRabel software (http://bioinfo.univ-rouen.fr/mirabel/index.php). The software ranks targets of miRNAs based on the aggregated results of four miRNA databases (miRanda, PITA, SVmicrO, and TargetScan) and experimentally validated interactions (annotated using miRTarBase v.6.0 and miRecords), as well as 5’UTR and CDS predictions (identified with mirWalk database)[163]. The top 100 ranked targets and pathways were screened for relevance in IVD degeneration.

**RNA extraction and gene expression analysis (RT-qPCR)**

Gene expression was analyzed by extracting mRNA with the RNeasy Mini Kit (Qiagen, 74104) according to the manufacturer’s recommendations. Following RNA quantification with the NanoPhotometer N50 (Implen), 500-1000 ng RNA was reverse transcribed into cDNA with the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher Scientific, 4374967). Expression was quantified by qPCR with TaqMan™ Fast Advanced Master Mix (Thermo Fisher Scientific, 4444963) and the respective Taqman™ Gene Expression Assay (Hs00152932_m1 TLR-2, Hs00174131_m1 IL-6, Hs00174103_m1 IL-8, Hs99999140_m1 c-FOS, Hs00270923_s1 CEBPB, Hs00182082_m1 MyD88, Hs00907957_m1 PIK3CA, Hs00427620_m1 TBP, Thermo Fisher Scientific, 4331182) using the QuantStudio™ 3 (Thermo Fisher Scientific). The results are shown as $2^{-\Delta\Delta Ct}$ values relative to TBP and control conditions.

**Immunocytochemistry**

For immunocytochemistry, degenerated IVD cells were seeded in a four-well chambered coverglass (Thermo Fisher Scientific, 155383PK) at a cell density of 10000 cells
cells/cm². After 24h of cell attachment, cells were rinsed with PBS, fixed and permeabilized for 25 min with methanol (Thermo Fisher Scientific, A412) tempered at -20°C. Next, cells were blocked for 30 min with 5% goat serum in PBS (Sigma-Aldrich, G9023) and incubated overnight at 4°C with the primary antibody (TLR2 Recombinant Rabbit Monoclonal Antibody, Invitrogen, MA5-32787) diluted 1:100 in 5% goat serum in PBS. The following day, cells were incubated for 1h with the secondary antibody diluted 1:400 in PBS (Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488, Invitrogen, A-11008). Finally, the cells were incubated for 5 min with a 1:3000 dilution of 20 mM Hoechst 33342 Solution (Thermo Fisher Scientific, 62249) for the staining of nuclei. Samples were washed 3 times for 5 min with PBS between each step of the process. Imaging was performed with the Leica SP5 laser scanning confocal microscope.

**Protein analysis (WB and ELISA)**

In order to analyze TLR-2 protein levels, cells were rinsed twice with ice-cold PBS and lysed on ice with 60 µL radioimmunoprecipitation assay (RIPA) buffer supplemented with 100x phosphatase and protease inhibitor cocktail, which was diluted to a final concentration of 1x in RIPA buffer (Thermo Fisher Scientific, 89900 and 78440). The lysate was centrifuged at 14000 g for 15 minutes at 4°C and protein content was quantified with a BCA protein assay (Thermo Fisher Scientific, 23225). For the electrophoresis, 10 µg protein of each sample was mixed with 4X Lamelli buffer (Bio-Rad, 1610747) containing 10% β-mercaptoethanol (Sigma Aldrich, 444203) and heated for 5 min at 95°C. Samples and the protein ladder (Thermo Fisher Scientific, 26617) were loaded onto a 7.5% protein
gel (Bio-Rad, 4568024). Separation of proteins according to their size was achieved by electrophoresis in a Mini-PROTEAN Tetra Cell (Bio-Rad) and followed by the semi-dry transfer onto a PVDF membrane (Bio-Rad, 1704156). Membranes were blocked with 5% non-fat milk (Research Products International, M17200-500.0) in Tris-buffered saline with 0.05% TWEEN 20 (TBS-T) for 1h at room temperature and then incubated with primary antibodies (TLR-2 Rabbit monoclonal, Cell Signaling Technology, 12276S; β-Actin Rabbit mAb, Cell Signaling Technology, 8457L) diluted 1:1000 in 5% bovine serum albumin in TBS-T overnight at 4°C. Secondary antibodies (Anti-rabbit IgG HRP-linked Antibody, Cell Signaling Technology, 7074S) were diluted 1:2000 in TBS-T and applied for 1h at room temperature. Proteins were detected with a chemiluminescence substrate (Thermo Fisher Scientific, 34095) and imaged on a ChemiDocTouch Imaging System (Bio-Rad).

Conditioned media was used for the analysis of secreted proteins. To that end, media was collected immediately after treatment and centrifuged for 5 min at 500 g to remove cell debris. The resulting supernatant was used undiluted to quantify IL-6 and IL-8 (Human IL-6 DuoSet ELISA, R&D Systems, DY206-05 and Human IL-8/CXCL8 DuoSet ELISA, R&D Systems, DY208-05) according to the manufacturer’s instructions.

**Statistical Analysis**

Data were checked for consistency and normality. One-sample and two-sample tests based on dependent Student t-tests as well as dependent bootstrap t-tests were used to test means. All reported tests were two-sided, and p-values < 0.05 were considered as statistically significant. Whisker plots with standard deviation were used to illustrate results. All statistical analyses in this report were performed by use of
Declarations

Author contributions Petra Cazzanelli conceived the project, designed and conducted experiments, analyzed data, and wrote the manuscript. Mikkael Lamoca assisted with the miRNA expression experiments. Oliver Nic Hausmann, Addisu Mesfin, and Varun Puvanesarajah provided human degenerated IVD biopsies and provided input on clinical relevance. Wolfgang Hitzl performed the statistical analysis. Lisbet Haglund provided non-degenerated IVD cells and supported data interpretation. Karin Wuertz-Kozak provided funding, supervised the project, and supported data interpretation. All authors have contributed and reviewed the manuscript.

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Data availability The datasets generated during this study are available from the corresponding author upon request.

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study with degenerated IVD cells was approved by Swissethics, KEK Zurich, Switzerland (2019-00736), and the IRB Committee of the University of Rochester, NY United States (STUDY00005200). All procedures performed with non-degenerated IVD cells were approved by the ethical review board at McGill University (IRB 2019-4896).

Consent to participate Not applicable

Consent to publish Not applicable
Chapter 4
The Role of miR-155 in Inflammation and Mechanosensing during Intervertebral Disc Degeneration
The Role of miR-155 in Inflammation and Mechanosensing during Intervertebral Disc Degeneration

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Abstract

Intervertebral disc (IVD) degeneration is a multifactorial pathological process resulting in the dysregulation of IVD cell activity. The catabolic shift observed in IVD cells during degeneration leads to increased inflammation, extracellular matrix (ECM) degradation, aberrant intracellular signaling and cell loss. Importantly, these pathological processes are known to be interconnected and to collectively contribute to the progression of the disease. MicroRNAs (miRNAs) are known as strong post-transcriptional regulators, targeting multiple genes simultaneously and regulating numerous intracellular pathways. Specifically, miR-155-5p has been of particular interest, since it is known as a pro-inflammatory mediator and contributing factor to diseases like cancer and osteoarthritis. This study investigated the role of miR-155-5p in IVD degeneration with a specific focus on inflammation and mechanosensing. Increased expression of miR-155-5p facilitated through the transfection of miRNA mimics in AF cells resulted in significant upregulation of IL-8 cytokine secretion during cyclic stretching and a similar trend in IL-6 secretion during inflammation. Furthermore, miR-155-5p mimics increased the expression of the neurotrophic factor BDNF in AF cells undergoing cyclic stretching. In NP cells, miR-155-5p gain-of-function resulted in the activation of the MAPK signaling pathway through increased phosphorylation of p38 and p53. Lastly, miR-155-5p inhibition caused a significant increase in the anti-inflammatory cytokine IL-10 and the MMP inhibitor TIMP2, in AF and NP cells respectively. Overall, these results show that miR-155-5p contributes to IVD degeneration by enhancing inflammation through pro-inflammatory cytokines and MAPK signaling, as well as by promoting the catabolic
shift of AF cells during mechanical loading. The inhibition of miR-155-5p may constitute a potential therapeutic approach for IVD degeneration and low back pain.

Introduction

Intervertebral disc (IVD) degeneration causes the deterioration of the connective tissue located between vertebrae. One of its key characteristics is the multifactorial dysregulation of IVD cell activity through a catabolic shift resulting in extracellular matrix (ECM) degradation, inflammation, apoptosis, and senescence, as well as nerve and blood vessel ingrowth [6, 26, 50]. These processes are known to be highly interconnected, each of them contributing to the enhancement of the other degenerative processes, creating feedforward loops that collectively drive the progression of the disease [8].

Focusing on the deterioration of the IVD tissue, a disruption of metabolic homeostasis is observed, where native cells switch from ECM synthesis (anabolism) to ECM degradation (catabolism). Specifically, the secretion of matrix metalloproteinases (MMPs) is increased and the expression of ECM components like collagen and proteoglycans is reduced [47, 164]. Furthermore, the number of functional cells in the tissue decreases during degeneration due to the above-mentioned apoptosis and senescence which contributes to reduced capabilities of the tissue to produce ECM [28, 29, 165]. This disruption of tissue homeostasis changes the IVD’s composition and ultrastructure and can cause loss of disc height, dehydration of the nucleus pulposus (NP), fissures in the annulus fibrosus (AF), endplate defects, and in worst-case scenarios, disc bulging and herniation [26, 166, 167]. Apart from these macroscopic structural changes caused by ECM degradation, which also contribute to aberrant mechanical loading and
altered biomechanics, the avascular nature of the tissue and the catabolic shift of IVD cells lead to the accumulation of fragmented, degraded ECM molecules [6, 8]. These ECM fragments are known to induce changes in the expression profile of IVD cells through pattern recognition receptors such as toll-like receptor (TLR)-2 and TLR-4 [131]. Upon activation and downstream signaling, these receptors lead to the increased secretion of pro-inflammatory cytokines, chemokines, and proteases, hence establishing a link between inflammation and ECM degradation [13, 135].

Inflammation is one of the key processes driving IVD degeneration from the onset of the pathology to its advanced stages. The changes in cell activity of native cells lead to increased secretion of pro-inflammatory and pro-catabolic cytokines, such as interleukins (IL-1β, IL-6, IL-8, IL-17), tumor necrosis factor (TNF), and chemokine (C-C) ligands (CCL-2) [7, 11]. Furthermore, the inflammatory environment activates intracellular signaling pathways MAPK and NF-κB through phosphorylation of their key enzymes ERK, p38, and JNK [168]. Downstream effects of MAPK and NF-κB signaling are apoptosis [169], senescence [170], enhanced MMP expression [171], and secretion of pro-inflammatory cytokines [172, 173]. The increased accumulation of pro-inflammatory cytokines and neurotrophic factors, accompanied by the disruption of tissue integrity, can lead to the infiltration of immune cells and innervation. Recruited immune cells contribute to degeneration and the inflammatory environment by increasing chemokine and cytokine release and leading to sensitization of nerve roots, resulting in nociception and discogenic pain [10, 31, 128]. This is one of the major distinguishing factors between asymptomatic and symptomatic IVD degeneration [10, 31]. Symptomatic IVD degeneration, commonly
termed degenerative disc disease (DDD), is one of the major contributing factors of low back pain (LBP), the leading cause of years lived in disability worldwide that poses a significant socioeconomic burden [2, 42].

Due to the important role of the IVD tissue in spinal kinematics and the structural and biomechanical changes occurring during degeneration, an understanding of the impact of mechanical loads on cell activity is crucial. Abnormal mechanical stress is known to induce catabolic and inflammatory cascades, matrix degradation, and senescence [22, 48, 58]. Multiple cell receptors, channels, and signaling pathways are involved in the transduction of aberrant mechanical loading, including MAPK, ERK, and transient receptor potential channels [162, 174]. Hyperphysiological mechanical loading has been shown to further IVD degeneration. Cyclic stretching of AF cells at high strain of 8-20% induces downregulation of anabolic factors (ACAN, COL2) [175] and upregulation of catabolic and inflammatory factors (MMP1, MMP3, MMP9, MMP13, IL-1β, IL-6, IL-8, TLR-2, TNF, NGF) [175-177]. Therefore, mechanical stress and its impact on IVD cell activity is an essential factor in the degenerative process and its multifactorial nature.

As strong post-transcriptional regulators, miRNAs are known for targeting numerous genes and cell mechanisms and are hence of specific importance in multifactorial pathologies. These small non-coding RNAs regulate gene expression by binding to the 3’-untranslated region (3’-UTR) of target mRNAs leading to their reduced expression or degradation [64]. It is well known that miRNAs are pleiotropic and can affect cell behavior through the modulation of signaling pathways, especially during miRNA dysregulation in pathologies [16, 17, 138]. Amongst miRNAs characterized in IVD
degeneration [138], miR-155-5p is of specific interest. Previous research of our group linked increased TLR-2 signaling to upregulation of miR-155-5p in degenerated and non-degenerated human IVD cells [178]. Furthermore, multiple studies in different tissues suggest that miR-155 plays an important pro-inflammatory role in pathologies like cancer [179], arthritis [180], and neuroinflammatory disorders [181]. In studies conducted with cells of the immune system (macrophages and dendritic cells), upregulation of miR-155-5p through TLR-2 and TLR-4 enhanced the cell’s inflammatory response in vitro and in vivo [142-144, 182]. Furthermore, miR-155-5p was upregulated in innate immune cells and microglia under neuroinflammatory conditions [145] and its inhibition offered mild protection against retinal degeneration by reducing inflammation [146]. Additionally, miR-155-5p overexpression in human osteoarthritis knee cartilage was shown to suppress autophagy in chondrocytes [147]. MiRNAs have also been extensively investigated as biomarkers in numerous pathologies and a recent study showed that miR-155-5p levels in patient serum might be a possible biomarker for lumbar DDD [116]. However, the effects of miR-155-5p have so far not been studied in the broader context of IVD degeneration.

In this study, we provide a comprehensive overview of the role of miR-155-5p in intervertebral disc degeneration with a specific focus on its effects on IVD cell activity during inflammation and mechanosensing. To that end, we investigate changes in the expression and secretion of the key catabolic factors (matrix-degrading enzymes, innervation factors, pro-inflammatory cytokines and chemokines) and intracellular
signaling following miR-155 gain- or loss-of-function in an inflammatory environment and during mechanical loading.

**Material and Methods**

**Human IVD cell isolation and culture**

Biopsies from patients undergoing spinal surgery due to disc herniation or DDD were obtained for *in vitro* experiments with human degenerated IVD cells. As described previously, NP, AF or mixed IVD tissue was excised intraoperatively, diced and digested enzymatically overnight with 0.2% collagenase NB4 (Nordmark) and 0.3% dispase II (Sigma-Aldrich) in 1× Dulbecco's Phosphate Buffered Saline (DPBS, Cytiva) with 5% antibiotic-antimycotic (anti-anti, Gibco) at 37 °C, 5% CO₂[162]. After enzymatic tissue digestion, cells were kept in culture up to passages 1-2 in growth medium consisting of Dulbecco’s Modified Eagle’s Medium/Ham’s F-12 medium (DMEM/F-12, Cytiva) supplemented with 10% fetal bovine serum (FBS, Cytiva) and 1% anti-anti. An overview of the patients characteristics can be found in Appendix, Supplementary Table 1.

**Transfection of miRNA mimics/inhibitors**

In order to transfect human IVD cells with miRNAs, 3 uL HiPerFect Transfection Reagent (Qiagen, 301705) were mixed and incubated with 50 nM miRNA mimics/inhibitors or their corresponding non-targeting controls for 10 min at room temperature to allow for the formation of transfection complexes (hsa-miR-155-5p miRCURY LNA miRNA Mimic YM00472490-ADA, Qiagen, 339173; hsa-miR-155-5p miRCURY LNA miRNA Power Inhibitor YI04101510-DDA, Qiagen, 339131; negative control
miRCURY LNA miRNA Mimic YM00479902-ADB, Qiagen, 339173; negative control B miRCURY LNA miRNA Power Inhibitor YI00199007-DDA, Qiagen, 339136). Human IVD cells were then reverse-transfected at a density of 20000 cells/cm² with miR-155-5p mimic/inhibitor or non-targeting complexes in no-serum media (DMEM/F12 with 0.1% anti-anti). After 24 h the medium was changed to growth medium in order to let the cells recover. The efficiency of the miRNA mimic/inhibitor transfection was analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) after 72h.

**Induction of inflammation in IVD cells**

Effects of miRNA gain- and loss-of-function on the inflammatory cell response were studied by first transfecting human degenerated IVD cells with miRNA mimics or inhibitors as described above. After the recovery in growth medium, cells were starved for 2 h in no-serum medium followed by treatment with 5 ng/mL recombinant human IL-1β (PeproTech, 200-01B). For the analysis of protein phosphorylation, cells were treated with 5 ng/mL IL-1β for 30 min before being lysed for protein extraction. Secretion of cytokines and catabolic factors as well as gene expression were analyzed after treating AF and NP cells for 24h with 5 ng/mL IL-1β. To that end, cell culture supernatants were collected for cytokine/MMP arrays and IVD cells were lysed for miRNA and mRNA extraction.

**Fabrication and characterization of stretching chambers**

Polydimethylsiloxane (PDMS) stretching chambers were fabricated using a 1:1 mixture of Sylgard 184 (Dow, 2646340) and Sylgard 527 Silicone Dielectric Gel Clear 0.9 kg Kit (Dow, 1696742), cast using an aluminum mold with dimensions fitting the automated
cell stretching system and cured at room temperature overnight, at 80 °C for 40 min or at 140 °C for 15 min. After demolding, the stretching chambers were cleaned in 70% ethanol for 5 minutes in an ultrasound bath, rinsed with deionized water, dried and plasma-treated with the plasma cleaner PDC-001 (Harrick Plasma) before being used for characterization or cyclic stretching.

The stiffness of the stretching chambers was determined by performing tensile testing with the UniVert tensile tester (CellScale Biomaterials Testing) at a crosshead speed of 10 mm/s until the point of failure using a 1-10 N and 10-100 N load cell. The Young’s Modulus was calculated using the stress/strain curve.

Digital image correlation (DIC) was used to determine the difference between engineering strain and true strain of the fabricated PDMS chambers during cyclic sinusoidal uniaxial loading with the automated cell stretching system (STB-140-10, STREX). To that end, fluorescent ink was incorporated into the PDMS mixture before casting and curing the chambers at 140 °C for 15 min. The stretching chambers were then imaged during cyclic sinusoidal uniaxial loading with the StrainMaster Portable System (LaVision) at an image sampling rate of 100 Hz using blue LED illumination. Calibration, data collection, image processing, and data analysis were performed using DaVis Software 10.2.1 (LaVision).

Cyclic stretching

Sterile PDMS stretching chambers were coated overnight with 50 μg/mL fibronectin (EMD Millipore, FC010) at 37 °C. The following day, human IVD cells, with or without simultaneous reverse-transfection of miRNA mimics/inhibitors, were seeded in
the stretching chambers (20,000 cells/cm², 10 cm² cell culture surface). After the transfection and recovery phase of 46 h, cells were starved in no-serum media for 2 h and then subjected to 8% cyclic sinusoidal uniaxial engineering strain for 24 h at a frequency of 1 Hz at 37 °C and 5% CO₂. Control chambers were kept in identical conditions without stretching. Cell lysates and conditioned media were harvested for expression analysis, cytokine and MMP arrays after a total time period of 72 h (experimental timeline depicted in Supplementary Figure 1).

**miRNA extraction and RT-qPCR**

MiRNA extraction for the expression analysis was performed with the miRNeasy Tissue/Cells Advanced Mini Kit (Qiagen, 217604). Following IL-1β treatment or mechanical loading, cells were rinsed twice with ice-cold 1x DPBS, lysed with 260 µL lysis buffer, and the samples were further processed according to the manufacturer’s instructions. The concentration and quality of miRNA were analyzed with the UV-Vis spectrophotometer NanoPhotometer N50 (Implen). Reverse transcription of miRNAs was achieved with the miRCURY LNA RT Kit (Qiagen, 339340) followed by quantitative PCR with the miRCURY LNA SYBR Green PCR Kit (Qiagen, 339347). The respective miRCURY LNA miRNA PCR Assays were used for the analysis of the expression of specific miRNAs (YP00204308 - hsa-miR-155-5p, YP00204063 - hsa-miR-103a-3p, Qiagen, 339306). Furthermore, spike-ins were used to monitor the consistency of the extraction process and reverse transcription (RNA Spike-In Kit, Qiagen, 339390). The results are shown as $2^{-\Delta\Delta Ct}$ values relative to the housekeeping miRNA miR-103a-3p and control conditions.
Cytokine and MMP array

In order to analyze the secretion of cytokines and MMPs, conditioned media was collected immediately after treatment and centrifuged at 500 g for 5 min for the removal of cell debris. The resulting supernatants were used undiluted for the Human Cytokine Array GS1 (RayBiotech Life, GSH-CYT-1-4) and Human MMP Array GS1 (RayBiotech Life, GSH-MMP-1-4), which were performed according to the manufacturer’s instructions. The concentration range of the cytokine and MMP array proteins was detected at a range of 1 pg/mL – 1 ng/mL. The median fluorescent signal was normalized to the plate background and array’s positive control. Fold changes of fluorescence were calculated relative to the non-targeting control conditions.

Phosphorylation array

The phosphorylation of proteins associated with the MAPK pathway was determined with the Human/Mouse MAPK Phosphorylation Array (RayBiotech Life, AAH-MAPK-1-8). To that end, cells were rinsed with ice-cold 1x DPBS and lysed on ice with 60 µL radioimmunoprecipitation assay (RIPA) buffer supplemented with 100x phosphatase and protease inhibitor cocktail, which was diluted to a final concentration of 1x in RIPA buffer (Thermo Fisher Scientific, 89900 and 78440). The lysate was incubated for 30 min at 4°C with gently shaking followed by centrifugation at 14000 g for 10 minutes at 4°C. Protein concentrations were quantified with the BCA protein assay (Thermo Fisher Scientific, 23225). The samples were then diluted to a final concentration of 75 µg/mL in RIPA buffer containing phosphatase and protease inhibitor cocktail and 1 mL of the diluted samples was used for the MAPK Phosphorylation Array, according to the
manufacturer’s instructions. The membranes were scanned with the chemiluminescence imaging system Odyssey XF imaging system (LI-COR Biosciences) and analyzed with the Empiria Studio Software (LI-COR Biosciences). Signal intensities were normalized to the positive control and membrane background. The results are shown as fold changes relative to the non-targeting controls.

**RNA extraction and gene expression analysis (RT-qPCR)**

Analysis of gene expression was performed by co-extracting mRNA with the miRNeasy Tissue/Cells Advanced Mini Kit (Qiagen, 217604) according to the manufacturer’s recommendations. After determining the quantity and quality of RNA with the NanoPhotometer N50 (Implen), 500-1000 ng RNA were used for reverse transcription into cDNA with the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher Scientific, 4374967). Finally, gene expression was quantified by qPCR with the TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, 4444963) and the respective Taqman Gene Expression Assay (Hs00153936_m1 ACAN, Hs00171458_m1 NGF, Hs02718934_s1 BDNF, Hs00174103_m1 CXCL8/IL-8, Hs00153133_m1 PTGS2/COX2, Hs00427620_m1 TBP, Thermo Fisher Scientific, 4331182) using the QuantStudio™ 3 (Thermo Fisher Scientific). The results were calculated as $2^{-\Delta\Delta Ct}$ values relative to the housekeeping gene TBP and control conditions.

**Cell viability assay**

The effect of PDMS on cell viability was determined with the alamar blue assay. Cells were seeded on PDMS stretching chambers and kept in culture for 72h. Thereafter, cells were incubated with a 1:10 dilution of alamarBlue Cell Viability Reagent (Thermo
Fisher Scientific, DAL1025) for 4h at 37 °C. Cells seeded in standard 6-well tissue culture plates were used as a positive control and cells lysed in a 6-well tissue culture plate were used as a negative control. Furthermore, the cytotoxicity of cyclic stretching was tested using the CyQUANT™ LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific, C20301) following the manufacturer’s instructions. Briefly, conditioned media of cells undergoing cyclic stretching for 24h was collected and 50 µL of each sample was used for the LDH cytotoxicity assay. Non-stretched cells were used as a positive control and lysed cells were used as a negative control.

**Statistical Analysis**

Bootstrap-t tests with and without the assumption of variance homogeneity were used to test means. 95% bias-corrected and accelerated (BCa) confidence intervals were computed to estimate the difference of means. All reported tests were two-sided, and p-values < 0.05 were considered statistically significant. All statistical analyses in this report were performed by use of NCSS (NCSS 10, NCSS, LLC. Kaysville, UT), and Wolfram Research, Inc., Mathematica, Version 13.1, Champaign, IL (2022).

**Results**

**Effects of miR-155-5p Expression on Cytokine Secretion during Inflammation**

Pro-inflammatory cytokines and chemokines are known key mediators that drive multiple pathological processes during IVD degeneration, including ECM degradation, inflammation and apoptosis [7]. The most prominent cytokines secreted by IVD cells are IL-1β, TNF, IL-6, IL-8 and IL-17. As their secretion increases with progressive degrees of
degeneration, these cytokines and chemokines accumulate in the tissue and enhance the catabolic shift in IVD cell metabolism and the secretion of ECM-degrading proteins [128]. Furthermore, higher levels of cytokines like TNF, IL-6 and IL-8 are linked to painful degenerative disk disease [10] and can cause the infiltration of host immune cells as well as increased sensitization of nerve fibers upon AF and cartilage endplate ruptures [183]. Since miR-155-5p is largely attributed to being a pro-inflammatory miRNA [180, 184], the effect of miR-155-5p gain- and loss-of-function on the secretion of cytokines was studied. To that end, AF and NP cells were transfected with miR-155-5p mimics or inhibitors and then exposed to an inflammatory environment by treatment with IL-1β (Figure 4.1a). Changes in miR-155-5p expression were confirmed in AF and NP cells, with increases of 466.5 ± 80.6 fold change (p < 0.0001, AF cells) and 588.9 ± 179.9 fold change (p < 0.0001, NP cells) following transfection with miRNA mimics and decreases of 0.01 ± 0.006 fold change (p < 0.0001, AF cells) and 0.03 ± 0.025 (p < 0.0001, NP cells) following transfection with miRNA inhibitors (Figure 4.1b-c). Quantification of cytokine secretion of AF cells (Figure 4.1d-e) showed that the inhibition of miR-155-5p resulted in a significantly increased secretion of anti-inflammatory cytokine IL-10 (1.6 ± 0.1 relative fluorescent signal, p < 0.001), while miR-155-5p under IL-1β treatment let to a decrease of CCL5 (0.7 ± 0.1 relative fluorescent signal, p < 0.001) and to a recognizable though not significant increase of IL-8 (4.1 ± 2.8 relative fluorescent signal, p = 0.129) due to high donor-donor variability (Figure 4.1f). The vascular endothelial growth factor (VEGF) was significantly increased upon miR-155-5p inhibition (1.6 ± 0.2 relative fluorescent signal, p < 0.001) and interestingly miR-155-5p mimics also showed an increase, even though not significant.
Figure 4.1 The effect of miR-155-5p gain-/loss-of-function on cytokine secretion. a Schematic of the experimental setup using human degenerated AF and NP cells. b–c Expression analysis of miR-155-5p in AF (b) and NP (c) cells following transfection with mimics, inhibitors or non-targeting (NT) controls. d–e Human cytokine secretion array performed with AF cells transfected with miR-155-5p mimics or inhibitors, untreated (w/o IL-1β) or being subjected to IL-1β treatment (+IL-1β); heatmap of the fluorescent signal relative to non-targeting controls (d) and arrays scanned with a fluorescent laser scanner (e). f Changes in secretion of IL-10, VEGF, CCL5 and IL-8 in AF cells. g–h Human cytokine secretion array performed with AF cells transfected with miR-155-5p mimics or inhibitors, untreated (w/o IL-1β) or being subjected to IL-1β treatment (+IL-1β); heatmap of the fluorescent signal relative to non-targeting controls (g) and arrays scanned with a fluorescent laser scanner (h). i Changes in secretion of IL-1β in NP cells. j Secretion of IL-6 following treatment with IL-1β compared to untreated AF and NP cells. (n =3), mean ± SD, ns = not significant, * p < 0.05, *** p < 0.001, **** p < 0.0001
The transfection of NP cells with miR-155-5p mimics resulted in a clear increase of IL-1β secretion (3.4 ± 2.3 relative fluorescent signal, p = 0.242) even though not significant (Figure 1g-i). The induction of inflammation in AF and NP cells after treatment with IL-1β was confirmed by detecting a significant increase in IL-6 secretion compared to untreated cells, though high variability in inflammatory response was observed between donors (Figure 4.1j). Overall these results suggest that miR-155-5p might play a pro-inflammatory role in IVD degeneration (Table 4.1).

Role of miR-155-5p in ECM Degradation and Innervation

Degradation of the ECM in IVD tissue occurs due to the increased secretion of ECM-degrading enzymes, such as MMP-1, -3, -9, -13 and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTs-4, -5) [46, 47]. The decreased capability of IVD cells to produce ECM components like aggrecan and collagen during degeneration further enhances the deterioration of the tissue. This leads to structural and molecular changes in the ECM as well as to the accumulation of ECM fragments and the disruption of tissue integrity and biomechanics [164]. As a consequence, immune cells and nerve fibers are able to infiltrate the tissue. The ingrowth of nerve fibers is enhanced further by the secretion of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) by IVD cells [185]. Since we observed increases in some pro-inflammatory cytokines caused by miR-155-5p expression and due to the close link between inflammation and ECM degradation, the effect of miR-155 expression on MMPs and their inhibitors (tissue inhibitor of metalloproteinases, TIMPs) was studied (Figure 4.2a and Figure 4.3a). AF cells showed significant increases in MMP-2 (1.28 ± 0.17 relative
fluorescent signal, \(p<0.0001\)) and MMP-9 (1.33 \(\pm\) 0.10 relative fluorescent signal, \(p<0.0001\)) when transfected with miR inhibitors without IL-1\(\beta\) treatment and a similar trend in MMP-3 (2.55 \(\pm\) 0.78 relative fluorescent signal, \(p<0.0001\)). In contrast, mimics of miR-155-5p decreased MMP-3 secretion (0.5 \(\pm\) 0.3 relative fluorescent signal, \(p<0.0001\)) under an inflammatory environment (Figure 4.2b). In NP cells, inhibition of miR-155-5p caused a significant increase in the MMP inhibitor TIMP4 (1.5 \(\pm\) 0.2 relative fluorescent signal, \(p<0.0001\)) (Figure 4.3b).

**Figure 4.2** ECM degradation and innervation under miR-155-5p gain-/loss-of-function. a Human MMP secretion array performed with AF cells transfected with miR-155-5p mimics or inhibitors, untreated (w/o IL-1\(\beta\)) or being subjected to IL-1\(\beta\) treatment (+IL-1\(\beta\)); heatmap of the fluorescent signal relative to non-targeting controls and array scanned with a fluorescent laser scanner. b Changes in the secretion of MMP-2, -3, and -9. c Gene expression of NGF and BDNF. (n =3), mean \(\pm\) SD, ns = not significant, **** \(p < 0.0001\)

Furthermore, the expression of NGF, BDNF and aggrecan were analyzed following the gain- or loss-of-function of miR-155-5p. While no significant changes in aggrecan (ACAN) were observed (Appendix, Supplementary Figure 2), miR-155-5p mimics caused a
significant increase in BDNF expression (1.51 ± 0.23 fold change, p<0.0001) and a similar trend in NGF expression under non-inflammatory conditions (1.77 ± 0.38 fold change, p = 0.141) in AF cells (Figure 4.2c). Treatment with IL-1β led to a significant increase in BDNF expression following miR-155-5p inhibition (1.58 ± 0.21 fold change, p<0.0001)(Figure 2c). In contrast, miR-155-5p mimics caused a significant downregulation of NGF (0.55 ± 0.19 fold change, p<0.0001) and BDNF (0.61 ± 0.10 fold change, p<0.0001) under inflammatory conditions and a similar trend in untreated NP cells (Figure 4.3c).

Figure 4.3 ECM degradation and innervation under miR-155-5p gain-/loss-of-function. a Human MMP secretion array performed with NP cells transfected with miR-155-5p mimics or inhibitors, untreated (w/o IL-1β) or being subjected to IL-1β treatment (+IL-1β); heatmap of the fluorescent signal relative to non-targeting controls and array scanned with a fluorescent laser scanner. b Changes in the secretion of MMP-2, -3, and -9. c Gene expression of NGF and BDNF. (n =3), mean ± SD, ns = not significant, **** p < 0.0001
These results show that the effect of miR-155-5p on ECM degradation and innervation is distinctively different in AF cells compared to NP cells. While miR-155-5p leads to a general increase in MMPs and neurotrophic factors in AF cells, downregulation of neurotrophic factors is observed in NP cells (Table 4.1). Furthermore, some of these effects are reversed when inflammation is induced by IL-1β.

Table 4.1 Inflammation, ECM degradation and innervation under miR-155-5p gain-/loss-of-function. Changes in gene expression in AF and NP cells transfected with miR-155-5p mimics or inhibitors, untreated (w/o IL-1β) or being subjected to IL-1β treatment (+IL-1β). Significant results are marked with *, while evident though not statistically significant trends are marked with #.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>MiRNA Transfection</th>
<th>Inflammation</th>
<th>Gene Expression</th>
</tr>
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<tbody>
<tr>
<td>AF cells</td>
<td>miR-155-5p mimics</td>
<td>w/o IL-1β</td>
<td></td>
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<td></td>
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<td>miR-155-5p inhibitor</td>
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</tr>
<tr>
<td>NP cells</td>
<td>miR-155-5p mimics</td>
<td>w/o IL-1β</td>
<td>IL-1β* ↑ NGF* ↓</td>
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<td></td>
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<td>+IL-1β</td>
<td>NGF* ↓ BDNF* ↓</td>
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<td>miR-155-5p inhibitor</td>
<td>w/o IL-1β</td>
<td>TIMP4* ↑</td>
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<td>+IL-1β</td>
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Regulation of MAPK signaling pathway through miR-155-5p

Cellular stresses like inflammation, hyperosmotic stress or mechanical loading are transmitted through the intracellular signaling pathways such as MAPK, NF-κB, and PI3/Akt, which are known to be dysregulated in IVD degeneration [8]. The MAPK pathway is a highly conserved signal transduction pathway, which is known to be activated in the IVD by inflammatory cytokines, hypoxia and hyperosmolarity [168]. Three major subfamilies of the MAPK pathway are prevalent in IVD degeneration: ERK, JNK and p38.
These subfamilies can be activated through different stimuli and lead to distinctive cell responses. Furthermore, extensive crosstalk between MAPK and other intracellular pathways like NF-κB, AKT, and mTOR can occur[8]. In order to determine whether the above-seen increases in pro-inflammatory cytokines and other catabolic factors are mediated through MAPK, we studied the effect of miR-155 expression on MAPK pathway phosphorylation in AF (Figure 4.4a) and NP cells (Figure 4.4d).

Figure 4.4 The effect of miR-155-5p gain-/loss-of-function on MAPK signaling. a-d Heatmap of MAPK phosphorylation array performed with AF (a) and NP (d) cells transfected with miR-155-5p mimics or inhibitors, untreated (w/o IL-1β) or being subjected to IL-1β treatment (+IL-1β). b-e MAPK arrays of AF (b) and NP (e) cells scanned with a chemiluminescent imaging system (e). c Changes in phosphorylation of mTOR and ERK1/2 in AF cells. f Changes in phosphorylation of p38 and p53 in NP cells. (n =3), mean ± SD, ns = not significant, *** p < 0.001
Moreover, inflammation was induced to study changes in MAPK signaling upon its activation through IL-1β (Figure 4.4b,e). AF cells showed an apparent though not significant increase in mTOR phosphorylation (1.60 ± 0.73 relative signal intensity, p = 0.25) upon transfection with miR-155-5p mimics and a similar trend under inflammation in ERK1/2 phosphorylation (1.54 ± 1.07 relative signal intensity, p = 0.36) (Figure 4.4c). In NP cells, miR-155-5p expression led to a significant increase of p38 phosphorylation (1.49 ± 0.23 relative signal intensity, p<0.001) and miR-155-5p inhibition decreased p53 phosphorylation (0.73 ± 0.01 relative signal intensity, p<0.001) (Figure 4.4f). This indicates that miR-155-5p enhances MAPK signaling, possibly regulating cell activity through different MAPK subfamilies in AF and NP cells and hence leading to distinct downstream cell responses.

**Fabrication and characterization of stretching chambers for uniaxial cyclic stretching**

Mechanical stimuli are known to be beneficial for IVD homeostasis at physiological levels. However, non-physiological mechanical loading can contribute to apoptosis, catabolism and inflammation [186]. Furthermore, structural changes during degeneration such as reduction in disc height, increased tissue stiffness and loss of water-binding proteoglycans result in aberrant mechanical loading and reduced capability of the tissue to bear compressive loads [26, 30, 48]. In order to study the effects of miR-155-5p expression and inhibition on mechanosensing, PDMS stretching chambers were fabricated for uniaxial cyclic stretching using an aluminum mold fitting the dimensions of the STREX automated cell stretching system (Figure 4.5a). To simulate the stiffness of
degenerated AF tissue, which has previously been reported to be $567 \pm 7$ kPa[187], different curing conditions were tested, followed by tensile testing for the investigation of the Young’s modulus. While lower curing temperatures and longer curing intervals produced chambers with lower stiffnesses (Figure 4.5b), curing PDMS at 140°C for 15 min resulted in a Young’s modulus coherent with the one of degraded AF tissue[187].

![Diagram of PDMS stretching chambers](image)

**Figure 4.5** Characterization of PDMS stretching chambers and cyclic stretching conditions. a Schematic of aluminum mold and PDMS stretching chambers. b Young’s modulus of stretching chambers undergoing different curing conditions (RT o/n: room temperature overnight) c Cell viability of AF cells cultured on PDMS chambers cured at 140°C for 15 min. d-e Axial strain distribution images (d) and distribution curve (e). f Gene expression of COX2 and IL-8. ($n =3$), mean ± SD, ns = not significant, * $p < 0.05$, ** $p < 0.01$
Following the determination of optimal curing conditions, cell viability was tested and no impact on cell viability was seen in cells cultured on PDMS chambers compared to standard tissue culture plates (87.3 ± 13.6 % cell viability) (Figure 4.5c). Furthermore, the true strain during cyclic stretching was determined through DIC, showing that while applying 8% engineering strain at 1 Hz frequency, PDMS stretching chambers were undergoing an axial deformation of 5.2 ± 0.2 % (Figure 4.5d-e) and a transverse deformation of 1.69 ± 0.1 % (Appendix, Supplementary Figure 3). The difference between engineering strain to true strain is caused by reduced deformation of PDMS chambers due to the selected higher stiffness. Lastly, cell responses to cyclic stretching with 8% engineering strain at 1 Hz were tested and significant increases in the expression of pro-inflammatory cytokines COX-2 (12.7 ± 5.2 fold change, p<0.05) and IL-8 (1.89 ± 0.5 fold change, p<0.05) were observed (Figure 4.5f).

The effect of miR-155-5p expression on cytokine secretion during cyclic stretching

Due to the known contributing effect of mechanical loading to degeneration through the downregulation of anabolic markers and upregulation of catabolic markers as well as pro-inflammatory mediators [176, 188], the effects of miR-155-5p gain-/loss-of-function on cytokine secretion during mechanical loading was studied. To that end, AF cells were seeded in PDMS stretching chambers and transfected with miR mimics and inhibitors (Figure 4.6a). The increase and decrease of miR-155-5p expression following transfection was confirmed by RT-qPCR (659.2 ± 245.6 fold change, p < 0.0001; 0.05 ± 0.03 fold change, p < 0.0001) (Figure 4.6b). The cytokine secretion array showed that inhibition of miR-155-5p reduced the secretion of IL-1β (0.6 ± 0.1 relative fluorescent
signal, p < 0.001) and miR-155-5p mimics caused a significant increase in IL-6 (1.5 ± 0.1 relative fluorescent signal, p < 0.001) (Figure 4.6d-e).

**Figure 4.6** The effect of miR-155-5p gain-/loss-of-function on cytokine secretion during cyclic stretching. **a** Schematic of the experimental setup. **b** Expression analysis of miR-155-5p following transfection with mimics, inhibitors or non-targeting (NT) controls. **c-d** Human cytokine secretion array performed with AF cells transfected with miR-155-5p mimics or inhibitors, being subjected to cyclic stretching (8% strain); heatmap of the fluorescent signal relative to non-targeting controls (c) and arrays scanned with a fluorescent laser scanner (d). **e** Changes in secretion of IL-1β, IL-6 and IL-8. (n =3), mean ± SD, ns = not significant, * p < 0.05, *** p < 0.001, **** p < 0.0001
During cyclic stretching, a significant increase was seen in IL-8 upon transfection with miR-155-5p mimic (1.4 ± 0.4 relative fluorescent signal, p < 0.05), while its inhibitor showed a similar but reduced increase (1.2 ± 0.2 relative fluorescent signal, p < 0.05). Furthermore, an apparent though not significant increase in IL-6 by miR-155-5p mimic was observed during cyclic stretching (1.9 ± 0.6 relative fluorescent signal, p = 0.1) (Figure 4.6e). This shows that miR-155-5p expression causes increases in the pro-inflammatory cytokines IL-6 and IL-8, while its inhibition can reduce the secretion of IL-1β.

**Role of miR-155 in catabolism during mechanical loading**

Lastly, the role of miR-155-5p expression on catabolic factors like ECM-degrading proteins (MMPs) and neurotrophic factors (NGF, BDNF) as well as anabolic factors (ACAN) was tested (Figure 4.7a). The inhibition of miR-155-5p caused the decreased secretion of MMP-1 (0.4 ± 0.3 relative fluorescent signal, p<0.0001), while the secretion of the MMP inhibitor TIMP2 was increased (1.4 ± 0.2 relative fluorescent signal, p<0.0001) (Figure 4.7b). While undergoing mechanical loading, inhibition of miR-155-5p furthermore caused significant downregulation of MMP-10 (0.6 ± 0.1 relative fluorescent signal, p<0.0001) and a similar, though not significant trend in MMP-1. When studying the expression of neurotrophic factors, miR-155-5p expression caused a significant increase of BDNF under cyclic stretching (1.28 ± 0.1 fold change, p<0.001), while inhibition showed an apparent though not significant trend in upregulating NGF. No significant changes in aggregcan expression were observed (Appendix, Supplementary Figure 4). Taking these
results together, miR-155-5p seems to promote the catabolic shift in AF cells during mechanical loading (Table 4.2).

Figure 4.7 ECM degradation and innervation under miR-155-5p gain-/loss-of-function and cyclic loading. a Human MMP secretion array performed with AF cells transfected with miR-155-5p mimics or inhibitors, being subjected to cyclic stretching (8% strain); heatmap of the fluorescent signal relative to non-targeting controls and array scanned with a fluorescent laser scanner. b Changes in the secretion of MMP-1, MMP-10, and TIMP2. c Gene expression of NGF and BDNF. (n =3), mean ± SD, ns = not significant, ** p < 0.01, **** p < 0.0001
Table 4.2 Inflammation, ECM degradation and innervation under miR-155-5p gain-/loss-of-function during mechanical loading. Changes in gene expression in AF cells transfected with miR-155-5p mimics or inhibitors, untreated (w/o strain) or being subjected to cyclic stretching (8% strain). Significant results are marked with *, while evident though not statistically significant trends are marked with #.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>MiRNA Transfection</th>
<th>Inflammation</th>
<th>Gene Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF cells</td>
<td>miR-155-5p mimics</td>
<td>w/o strain</td>
<td>IL-6* ↑</td>
</tr>
<tr>
<td></td>
<td>8% strain</td>
<td></td>
<td>IL-8* ↑ IL-6# ↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>BDNF* ↑</td>
</tr>
<tr>
<td></td>
<td>miR-155-5p inhibitor</td>
<td>w/o strain</td>
<td>IL-1β* ↓</td>
</tr>
<tr>
<td></td>
<td>8% strain</td>
<td></td>
<td>MMP-1* ↓ TIMP2* ↑ NGF# ↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MMP-10* ↓ MMP-1# ↓</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NGF# ↑</td>
</tr>
</tbody>
</table>

Discussion

The multifactorial dysregulation of cell activity during IVD degeneration poses a major challenge in understanding the underlying pathological processes and their etiology, but also in the development of therapeutics. The interplay between the key biological processes driving degeneration further amplifies the complexity of the pathology [8]. Therefore, the study of miRNAs has become of increasing interest in recent years, due to their capability to regulate intracellular processes and target multiple genes simultaneously [138]. Furthermore, miRNAs are known to be involved in numerous pathologies [179-181] and more recent research has also shown that miRNAs dysregulation can be affected by mechanical forces, hence being mechanosensitive [189]. The involvement of miR-155-5p in neoplastic and inflammatory diseases has been studied extensively in recent years, with its expression frequently being upregulated under pathological conditions [21]. Our group has previously reported that miR-155-5p is
upregulated in IVD cells following TLR-2 activation and miR-155-5p target prediction identified multiple targets in relevant pathways and transcription factors like cFOS [178]. The goal of the current study was to gather a comprehensive analysis of the role of miR-155 in IVD degeneration, with a specific focus on inflammation, ECM degradation, MAPK signaling, and mechanosensing.

The occurrence of chronic inflammation during IVD degeneration is often termed the distinguishing factor between asymptomatic IVD degeneration and symptomatic degenerative disc disease [7]. The secretion of pro-inflammatory cytokines and chemokines by IVD native cells and immune cells upon their infiltration into the tissue can enhance ECM degradation, senescence and nociception [10]. MiR-155-5p has been identified as a pro-inflammatory mediator in other tissues and pathologies such as neuroinflammation [181], fibrosis [190, 191], and arthritis [180]. Moreover, miR-155-5p is a known regulator of the immune system [182]. When studying the role of miR-155-5p in inflammation of the IVD, we found that the inhibition of miR-155-5p increased the secretion of the anti-inflammatory cytokine IL-10 as well as VEGF in AF cells. Furthermore, our data show apparent trends of miR-155 mimics increasing IL-8 secretion in AF cells under inflammatory conditions and IL-1β in NP cells under non-inflammatory conditions. Similarly, miR-155-5p inhibition in AF cells cultured in PDMS stretching chambers reduced IL-1β secretion and miR-155-5p mimics increased IL-6 and IL-8 secretion during cyclic stretching. These results indicate that miR-155-5p plays a pro-inflammatory role in IVD cells during inflammation and mechanosensing. This finding is in concordance with studies in arthritis, where miR-155 drives inflammatory activation of macrophages and
monocytes by targeting inhibitors of TLR and cytokine receptor pathways, thus resulting in increased production of the cytokines TNF, IL-6, IL-8, and IL-1β [192-194]. Regarding the role of miR-155-5p in mechanosensing, very few studies have investigated this interplay so far. The results of a study conducted with endothelial cells suggest that miR-155-5p might be involved in altering RhoA and the actin cytoskeleton organization during unidirectional shear stress [195].

The role of miR-155-5p in ECM degradation and innervation was studied with a specific focus on the secretion of MMPs and gene expression of the neurotrophic factors NGF and BDNF in AF and NP cells. These studies showed highly tissue-specific changes following miR-155-5p gain- or loss-of-function, with distinct differences between AF and NP cells. We found that NP cells show an increase in the MMP inhibitor TIMP4 following the inhibition of miR-155-5p. These results suggest that miR-155-5p inhibition in NP cells might reduce matrix degradation. In AF cells, miR-155-5p inhibition resulted in minor upregulation of MMPs under non-inflammatory conditions and in downregulation of MMPs during cyclic stretching. Under inflammatory conditions, miR-155-5p mimics reduced MMP-3 secretion. These results show not only tissue-specific changes but also distinct effects of miR-155-5p gain- or loss-of-function in AF cells depending on the type of external stressors such as inflammation or mechanical loading. However, the interaction of miR-155-5p and MMP secretion has not been thoroughly studied in IVD cells so far and future investigations should provide a better understanding of the effect of miR-155-5p on ECM degradation. This would be of particular interest since studies in chondrocytes have shown similar results, where upregulation of miR-155-5p led to the
increase of MMP-3 and MMP-13 during inflammation [159]. The effect of miR-155-5p on the expression pattern of NGF and BDNF showed also highly tissue-specific changes. In NP cells miR-155-5p downregulated both NGF and BDNF. On the other hand, AF cells showed an upregulation of BDNF with miR-155-5p mimics under non-inflammatory conditions and during cyclic stretching, while during IL-1β treatment upregulation of BDNF was facilitated by miR-155-5p inhibition. This shows a similar cell stress-dependent response to miR-155-5p gain- or loss-of-function in AF cells, as well as a general increase of neurotropic factors in AF cells. These results should be further investigated with in vitro and in vivo studies, since recent publications have shown that miR-155 promoted dorsal root ganglion neuron axon growth [196], while miR-155 inhibition reduced neuropathic pain [197] and promoted spinal cord repair [198].

Numerous intracellular pathways are involved in the transduction of external stressors to the downstream dysregulation of gene expression during IVD degeneration. The MAPK signaling pathway is known to be a key regulator of catabolism and inflammation in IVD tissue. The activation of ERK1/2 and p38 can lead to increases in catabolic activity, apoptosis and senescence [168]. Importantly, distinct genes are upregulated depending on which subfamily of MAPK signaling proteins are activated [8]. When studying the effect of miR-155-5p on the MAPK signaling pathway, NP cells showed increased phosphorylation of p38 by miR-155-5p mimics and a reduction in p53 phosphorylation by miR-155-5p inhibitors. Since it is known that activation of p38 results in increased expression of pro-inflammatory cytokines and ECM-degrading enzymes, these results suggest that increased miR-155-5p expression might contribute to IVD
degeneration by regulating p38 signaling in NP cells. Due to the detrimental role of p38 in NP cells during IVD degeneration, its inhibition has been discussed as a potential therapeutic strategy [199]. Furthermore, the downregulation of p53 by miR-155-5p inhibition could suggest a possible involvement of miR-155-5p in cell fate, which should be investigated in further studies. Gain- and loss-of-function studies of miR-155-5p in AF cells showed trends of increased mTOR phosphorylation by mimics under non-inflammatory conditions, while ERK1/2 was activated under inflammatory conditions. These results demonstrate the tissue-specific activation of distinct MAPK signaling enzymes, likely causing distinct downstream effects in the catabolic regulation of NP and AF cells. Moreover, the interaction between miR-155 and MAPK signaling regulation has been recently studied in vivo showing that miR-155 inhibition reduced pain through SOCS1 and p38 [197].

In conclusion, this study gives a comprehensive overview of the role of miR-155-5p in IVD pathophysiology. We demonstrate that miR-155-5p enhances MAPK signaling and inflammation in NP cells and contributes to the catabolic shift during cyclic stretching of AF cells. Future studies will focus on the effects of miR-155-5p in IVD degeneration in vivo, testing the therapeutical potential of its inhibition for the reduction of inflammation and discogenic low back pain.
Declarations

Author contributions PC conceived the project, designed and conducted experiments, analyzed data, and wrote the manuscript. JH and ML assisted with the fabrication and characterization of PDMS stretching chambers. ONH, AM, and VP provided human degenerated IVD biopsies and provided input on clinical relevance. WH performed the statistical analysis. KWK provided funding, supervised the project, and supported data interpretation. All authors have contributed and reviewed the manuscript.

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Competing Interest All authors declare no financial or non-financial competing interests

Data availability The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study with degenerated IVD cells was approved by Swissethics, KEK Zurich, Switzerland (2019-00736), and the IRB Committee of the University of Rochester, NY United States (STUDY00005200).
Chapter 5  Conclusion
5.1 Summary

Low back pain is the leading cause of years lived in disability worldwide with a lifetime prevalence of 80% and is hence posing a significant global socio-economic burden [1]. One of the key contributors of LBP is intervertebral disc (IVD) degeneration, causing the breakdown of the connective tissue between vertebrae [26]. The degeneration is characterized by the multifactorial dysregulation of cell activity and deterioration of the tissue, leading to changes in biomechanics and frequently to pain. The major elements driving this pathology include inflammation, ECM degradation, cell loss and innervation, as well as aberrant mechanical loading due to changes in the biomechanics of the tissue [26]. Inflammation has been of interest for the development of novel therapeutic approaches, as it is not only contributing to the catabolic shift but also leading to the recruitment of host immune cells and the sensitization of nerve fibers resulting in discogenic LBP [7, 10, 11]. Toll-like receptors (TLRs) are known key regulators of inflammation and the study of TLRs, including TLR-2 has been shown to be essential for the understanding of IVD degeneration [131]. The expression and activation of TLR-2 can contribute to disc degeneration by increasing the secretion of pro-inflammatory cytokines and catabolic factors, as well as by promoting senescence [12-14]. Another important factor in the regulation of gene expression and cell signaling are microRNAs. These small non-coding RNAs are post-transcriptional regulators with the capability to regulate multiple genes and pathways simultaneously and are known to be involved and dysregulated in numerous pathologies [138]. However, the important interplay between
miRNAs and TLR-2 signaling in the context of IVD degeneration, inflammation and mechanosensing has never been studied so far.

The objective of the study was to identify miRNAs associated with TLR-2 signaling in human IVD cells, followed by establishing their pathophysiological role in DDD with a specific focus on inflammation, catabolism, and mechanosignaling. The goal was to get a better understanding of underlying disease mechanisms and the possible identification of novel therapeutic targets. The project was comprised of three aims:

Aim 1: Identification of miRNAs dysregulated by TLR-2 activation
Aim 2: Investigation of the functional role of miRNA-155-5p in DDD
Aim 3: Investigation of the functional role of miR-155-5p in mechanosensing

We found that TLR-2 leads to the dysregulation of 10 miRNAs in degenerated IVD cells and saw distinct expression profiles of miRNAs depending on PAMP- or DAMP-induced TLR-2 signaling. In summary, miR-155-5p was consistently upregulated in degenerated and non-degenerated IVD cells upon activation of TLR-2 and target prediction showed its involvement in numerous IVD-relevant pathways and genes, such as the transcription factor cFOS. Functional studies of miR-155-5p demonstrated its pro-inflammatory role in IVD degeneration and mechanosensing by causing upregulation of cytokines, neurotrophic factors, and MAPK signaling. Furthermore, the inhibition of miR-155-5p in AF and NP cells increased the secretion of anti-inflammatory cytokines and MMP inhibitors. Overall, this project identified that TLR-2 signaling leads to miRNA
dysregulation in IVD cells and that miR-155-5p specifically contributes to IVD degeneration through its downstream effects on cell activity during inflammation and mechanosensing.

The limitations of this study are the sample size and high donor-donor variability, which both impact the conclusions of cell responses and the statistical analysis. However, the number of biological replicates was dictated by technical limitations such as low cell numbers and low proliferation rates of primary human IVD cells, as well as the goal to perform a comprehensive study to see the effect on multiple pathways and targets. Therefore, future studies into the effects of miR-155-5p on specific pathways or targets should be performed with higher numbers of biological replicates. Furthermore, mechanical stimuli affecting AF cells within the IVD are of a complex nature, involving a range of strain and shear stresses, which are altered during degeneration. Previous studies have used various strains and frequencies, as well as time intervals to study the effect of mechanical stimuli on AF cells, which made the choice of cyclic stretching conditions challenging. Therefore, a comprehensive study on the effects of varying strains, frequencies and rest phases on AF cell activity should be performed ideally in a 3D cell culture system. Moreover, the contribution of shear stress to the cell response in comparison to strain should be studied.

Overall, the results of this study contribute to a better understanding of the multifactorial regulation of cell activity in IVD degeneration and identify modulation of miR-155-5p as a novel potential therapeutic approach for discogenic LBP.
5.2 Key Contributions

As a first step, we studied the interplay between TLR-2 signaling and miRNA expression. We demonstrated that TLR-2 signaling leads to the dysregulation of 10 miRNAs in degenerated IVD cells. Specifically, we found that miR-155-5p is highly upregulated in degenerated and non-degenerated AF and NP cells upon TLR-2 activation. Target and pathway analysis based on the sequence of miR-155-5p showed its involvement in regulating inflammatory pathways and cell fate. Furthermore, we confirmed that the miR-155-5p target cFOS was downregulated following TLR-2-induced miR-155-5p expression. These results are of specific significance, as previous studies have shown that miR-155-5p is an important regulator of inflammation in cells of the immune system [200] and acts as a pro-inflammatory mediator in multiple pathologies including cancer [179], arthritis [180] and neuroinflammation [181]. However, the dysregulation of miR-155-5p in connection to TLR-2 signaling and IVD degeneration has not been studied so far and we were the first to show this interaction in IVD cells. Furthermore, distinct changes in the expression of miR-484 and miR-487 specific to the activation of TLR-2 through DAMPs compared to PAMPs were identified in degenerated IVD cells. The comparison of miRNA expression profiles in degenerated vs non-degenerated IVD cells showed degeneration-dependent changes in miR-100-3p, miR-320b, and miR-181a-3p. Overall, this comprehensive study of the dysregulation of miRNAs due to TLR-2 signaling provides opportunities for future studies on the identified miRNAs as biomarkers and potential therapeutic targets.
In a second step, the functional role of the most promising candidate identified in Aim 1, miR-155-5p, in IVD degeneration was investigated. The focus of the study was to determine the effect of the gain- and loss-of-function of miR-155-5p on the secretion of cytokines, chemokines, ECM degrading proteins and inhibitors, as well as neurotrophic factors. Furthermore, the effect of miR-155-5p on the MAPK signaling pathway was investigated. We showed that the increased expression of miR-155-5p in AF cells resulted in the upregulation of the pro-inflammatory cytokines interleukin (IL)-8 and IL-6 during cyclic stretching and inflammation, respectively. Furthermore, increases in miR-155-5p led to the expression of the brain-derived neurotrophic factor (BDNF). Gain-of-function of miR-155-5p in NP cells caused activation of the MAPK pathway by increasing the phosphorylation of p38 as well as the activation of p53, a regulator of cell fate and apoptosis. The inhibition of miR-155-5p resulted in increases of the anti-inflammatory cytokine IL-10 and the MMP inhibitor TIMP2. These results show that miR-155-5p contributes to IVD degeneration by enhancing catabolism and inflammation and by increasing the effects of mechanical loading on IVD cells. This is of specific significance since miR-155-5p has been recently connected to neuropathic pain and neuroinflammation in in vivo studies [181, 196, 198]. We were able to show the interplay between TLR-2 signaling, miR-155-5p dysregulation and its downstream effects on catabolism, inflammation and innervation as well as a possible link to cell fate. Therefore, we provided not only a deeper understanding of the multifactorial regulation of IVD cell activity but also identified a promising potential therapeutic target against inflammation and discogenic LBP.
In summary, we were the first to show that TLR-2 signaling dysregulates miRNA expression in degenerated and non-degenerated IVD cells and that miR-155-5p contributes to inflammation and catabolism in IVD degeneration and mechanosensing. These results are of specific significance as we were able to show the origin of miRNA dysregulation as well as its overall downstream effect on the pathology. In contrast, previous studies by other groups focused on the effect of miRNAs on a specific target or pathway, neglecting the nature of the miRNA being able to regulate multiple targets simultaneously. Furthermore, we were the first to investigate miR-155-5p in inflammation as well as mechanosensing, showing its role in IVD degeneration and identifying miR-155-5p inhibition as a potential therapeutic strategy against IVD degeneration.

5.3 Recommendations for Future Work

*In vivo* studies investigating the role of miR-155 in IVD degeneration

This thesis provides a comprehensive overview of the effects of miR-155-5p in inflammation, catabolism and mechanosensing in IVD cells through *in vitro* studies and shows its potential as a therapeutic target. As a next step, the role of miR-155-5p in IVD degeneration and the effects of its inhibition should be studied *in vivo*. Multiple *in vivo* models for IVD degeneration are available, each with specific advantages and disadvantages [201]. Depending on the availability of *in vivo* models and facilities, the SPARC (secrete protein, acidic, rich in cysteine)-null transgenic mice provide a good model for studying chronic low back pain and inflammation. The degenerative process can be studied by inducing IVD degeneration through surgical methods or chemical injections.
Thereafter, the effects of miR-155-5p could be determined by injection of miR-155-5p mimics and inhibitors, followed by evaluating changes in pain behavior and histological/radiographic evaluation of degeneration and inflammation.

The role of miR-155-5p in IVD cell fate

The sequence-based prediction of miR-155-5p targets showed its involvement in multiple cell fate pathways including mTOR and PI3K-Akt, which regulate proliferation, autophagy and apoptosis. Furthermore, miR-155-5p increased the phosphorylation of the cell-fate protein p53. Since it is known that the dysregulation of metabolic activity, proliferation and apoptosis are key factors of IVD degeneration, a comprehensive study of the role of miR-155-5p in cell fate would be of specific interest. These in vitro studies should investigate changes in proliferation (proliferation assays), apoptosis (caspase enzyme activity) and mitochondrial function (mitochondrial membrane potential assays) following the gain- and loss-of-function of miR-155-5p. Furthermore, miRNAs are known mediators of senescence, being part of the senescence-associated secretory phenotype and TLR-2 signaling was shown to promote senescence in IVD cells [14]. Therefore, the role of miR-155-5p in senescence should be studied with in vitro assays including β-galactosidase assays, flow cytometry and gene expression analysis of p16 and p21.

Investigation of TLR-2-dysregulated miRNAs in IVD degeneration

The study of dysregulation of miRNA expression caused by TLR-2 activation showed that - apart from miR-155-5p - multiple other miRNAs were dysregulated in degenerated and non-degenerated IVD cells. Specifically, miR-484 and miR-487 were upregulated by DAMP-induced TLR-2 signaling. Furthermore, miR-100-3p, miR-320b, and
miR-181a-3p showed specific changes in non-degenerated IVD cells. These miRNAs should be further studied in the context of inflammation and IVD degeneration. To that end, initial target and pathway prediction as well as extensive literature research should provide an overview of the possible downstream effects of these miRNAs on IVD degeneration. The functional analysis of these miRNAs should be tailored to the results found in the literature and target prediction.

The effects of different strains and rest phases during cyclic stretching

Lastly, the analysis of preliminary data of TLR-2 expression during cyclic stretching showed distinct changes depending on the strain (8%-20%) and on included rest phases. However, no comprehensive comparative study on different parameters of cyclic stretching and their effect on the cell response of IVD cells has been performed so far. To better understand mechanobiological processes in the IVD, the effects of different strains (4%-20%), different frequencies as well as varying time intervals of stretching and rest phases should be tested. The gene expression of key genes in IVD degeneration, like pro-inflammatory cytokines, ECM degrading enzymes, neurotrophic factors, and mechanosensitive signaling pathways should be analyzed. These studies should ideally be performed in a 3-dimensional cell culture system with similar characteristics to degenerated IVD tissue, which allows for the investigation of the complex mechanical stimuli affecting cells while they are enclosed in a matrix-like material and avoids the asymmetric forces affecting cells in a 2D system. However, major challenges are connected to 3-dimensional (3D) cell culture systems including the fabrication of a highly structured matrix simulating the AF tissue, transfection of cells with miRNAs within the
3D culture system and the downstream analysis of cell activity due to low protein and RNA yields. On the other hand, several aspects need to be taken into consideration when performing cyclic stretching studies in 2D PDMS stretching chambers. Due to the dimensions of the PDMS stretching chambers and the large amount of media (3-5 mL) contained within the chambers, the effects of shear stress on AF cells caused by the flow of cell culture media during cyclic stretching at higher frequencies should be investigated. To that end, the effects of shear and strain stresses should be studied by analyzing the reactions of hiVD cells (1) to shear stress when seeded on PDMS chambers and subjected solely to flow, (2) to the combination of strain and shear stress when seeded on PDMS chambers containing 3-5 mL of cell culture media undergoing cyclic stretching and (3) to no mechanical stress while growing under static conditions. This would allow for the study of cell responses to varying mechanical stimuli and determine the changes in cell activity depending on stretch or shear stress.

In Summary, future studies should concentrate on (i) in vivo studies for clinical translation, investigating the therapeutic potential of miR-155-5p inhibition and on (ii) in vitro studies for a better understanding of important fundamental questions in the field.


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S. AG, et al., "Cobomarsen, an oligonucleotide inhibitor of miR-155, co-ordinately regulates multiple survival pathways to reduce cellular proliferation and survival in


High mechanical strain of primary intervertebral disc cells promotes secretion of inflammatory factors associated with disc degeneration and pain, "Arthritis research & therapy," vol. 16, 01/23/2014 2014.


Title, unpublished.


Chapter 7  Appendix
7.1 Supplementary Material – Chapter 3

Exploring the Impact of TLR-2 Signaling on miRNA Dysregulation in Intervertebral Disc Degeneration

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Submitted and under review in the journal Advanced Biology

Supplementary Table 1: Patient characteristics of surgical degenerated IVD biopsies. f = female; m = male; DDD = degenerative disc disease; L = lumbar; C = cervical.

<table>
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<tr>
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<th>Disc Level</th>
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<td>m</td>
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<td>L5/S1</td>
<td>III</td>
</tr>
<tr>
<td>6</td>
<td>36</td>
<td>f</td>
<td>DDD</td>
<td>C4/5</td>
<td>III</td>
</tr>
<tr>
<td>7</td>
<td>23</td>
<td>m</td>
<td>Herniation</td>
<td>L5/S1</td>
<td>III</td>
</tr>
<tr>
<td>8</td>
<td>55</td>
<td>f</td>
<td>Herniation</td>
<td>C5/6</td>
<td>V</td>
</tr>
<tr>
<td>9</td>
<td>49</td>
<td>m</td>
<td>DDD</td>
<td>L4/L5</td>
<td>III</td>
</tr>
<tr>
<td>10</td>
<td>27</td>
<td>m</td>
<td>DDD</td>
<td>L5-S1</td>
<td>III</td>
</tr>
<tr>
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<td>41</td>
<td>f</td>
<td>Herniation</td>
<td>L3/L4</td>
<td>III</td>
</tr>
<tr>
<td>12</td>
<td>69</td>
<td>f</td>
<td>Herniation</td>
<td>L5/S1</td>
<td>IV</td>
</tr>
<tr>
<td>13</td>
<td>40</td>
<td>m</td>
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<td>III</td>
</tr>
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<td>24</td>
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<td>DDD</td>
<td>L5/S1</td>
<td>III</td>
</tr>
<tr>
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<td>47</td>
<td>m</td>
<td>DDD</td>
<td>L4/L5</td>
<td>V</td>
</tr>
</tbody>
</table>

Supplementary Table 2: Patient characteristics of non-degenerated IVD donors. f = female; m = male.

<table>
<thead>
<tr>
<th>N</th>
<th>Age</th>
<th>Sex</th>
<th>Cause of Death</th>
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<td>1</td>
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<td>Brain death</td>
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<tr>
<td>2</td>
<td>23</td>
<td>f</td>
<td>Medically induced suicide</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>m</td>
<td>Suicide</td>
</tr>
</tbody>
</table>
Supplementary Figure 1. TLR-2 activation in degenerated IVD cells. A-B Secretion of the cytokine IL-8 after PAM2CSK4 treatment in AF (A) and NP cells (B). n = 5, mean ± SD, ** p < 0.01, *** p < 0.001.

Supplementary Figure 2. MiRNA baseline expression in non-degenerated and degenerated IVD cells. A-B Expression of miRNAs in AF cells in the absence (A) or presence
(B) of PAM2CSK4 treatment. C-D Expression of miRNAs in NP cells in the absence (C) or presence (D) of PAM2CSK4 treatment. n = 3, mean ± SD, * p < 0.05, *** p < 0.001.

**Supplementary Figure 3.** TLR-2 knockdown and activation in non-degenerated IVD cells

A-B Gene expression of TLR-2 in AF (A) and NP cells (B) transfected with non-targeting control (NT siRNA) and TLR-2 siRNA. Secretion of the cytokine IL-8 after PAM2CSK4 treatment in AF (A) and NP cells (B). n = 3, mean ± SD, *** p < 0.001.
7.2 Supplementary Material – Chapter 4

The Role of miR-155 in Inflammation and Mechanosensing during Intervertebral Disc Degeneration

Petra Cazzanelli¹, Mikkael Lamoca¹, Johannes Hasler¹, Oliver Nic Hausmann²,³, Addisu Mesfin⁴, Varun Puvanesarajah⁵, Wolfgang Hitzl⁶,⁷,⁸ and Karin Wuertz-Kozak¹,⁹*

Manuscript in preparation for submission

**Supplementary Table 1**: Patient characteristics of surgical degenerated IVD biopsies. f = female; m = male; DDD = degenerative disc disease; L = lumbar; C = cervical.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Age</th>
<th>Sex</th>
<th>Pfirrman Grade</th>
<th>Disc Level</th>
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<td>f</td>
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<td>L5/S1</td>
<td>DDD</td>
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<tr>
<td>2</td>
<td>52</td>
<td>f</td>
<td>IV</td>
<td>C5/6</td>
<td>Herniation</td>
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<tr>
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<td>53</td>
<td>m</td>
<td>III</td>
<td>C6/7</td>
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<td>f</td>
<td>III</td>
<td>L5/S1</td>
<td>DDD</td>
</tr>
<tr>
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<td>68</td>
<td>f</td>
<td>IV</td>
<td>L5/S1</td>
<td>Herniation</td>
</tr>
<tr>
<td>6</td>
<td>60</td>
<td>m</td>
<td>IV</td>
<td>L4/5</td>
<td>Herniation</td>
</tr>
</tbody>
</table>

**Supplementary Figure 1** Experimental setup of *in vitro* cyclic stretching studies.

Timeline of miRNA transfection, recovery phase and cyclic stretching.
Supplementary Figure 2 Gene expression analysis of Aggrecan (ACAN) in a AF and b NP cells transfected with miR-155-5p mimics or inhibitors, untreated (w/o IL-1β) or being subjected to IL-1β treatment (+IL-1β). (n =3), mean ± SD, ns = not significant

Supplementary Figure 3 Characterization of PDMS stretching chambers during cyclic stretching conditions with DCI, showing the transverse strain distribution curve.
Supplementary Figure 4 Gene expression analysis of Aggrecan (ACAN) in AF cells transfected with miR-155-5p mimics or inhibitors, untreated or being subjected to 8% strain. (n =3), mean ± SD, ns = not significant
Curriculum Vitae

Petra Cazzanelli
Email: cazzanelli.petra@gmail.com
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SCIENTIFIC INTERESTS
Regenerative medicine, RNA biology, inflammation, mechanobiology, musculoskeletal diseases

SKILLS
- **Research Expertise:** RNA biology (specific focus on miRNA and siRNA), inflammation/immunology, mechanobiology, musculoskeletal diseases, molecular biology, cancer
- **Clinical experience:** Collaborations with clinicians for patient sample collection and patient data evaluation, GCP certification (in progress)
- **Data Analysis:** Statistical analysis and data visualization (GraphPad Prism – advanced proficiency, R – basic knowledge)
- **Interdisciplinary Collaboration and Communication:** Interdisciplinary and intercultural communication skills due to close collaborations with clinicians, engineers, biologists and bioinformaticians from diverse backgrounds
- **Publication and Presentation:** Experienced in scientific/technical writing and presenting complex concepts for audiences with different levels of expertise (see list of publications, conferences and teaching experience below)
- **Problem-solving:** Identifying and solving complex research problems through pragmatic and critical thinking, literature research and innovative, interdisciplinary approaches during my PhD project
- **Languages:** German (native), English (fluent), Italian (proficient), French (basic)

EMPLOYMENT AND RESEARCH EXPERIENCE

**PhD Candidate and Graduate Research Assistant**
Tissue Regeneration and Mechanobiology Lab
Rochester Institute of Technology, USA
December 2019 – Present

Institute for Biomechanics ETH Zurich, Switzerland
Prof. Karin Wuertz-Kozak
June 2019 – December 2019

Project: *Functional role of Toll-like receptor-associated microRNAs in intervertebral disc pathophysiology*
- Establishment of siRNA and miRNA transfection and mechatransduction experiments in IVD cells
- Identified TLR-2 associated microRNAs with next-generation sequencing and performed target analysis
- Studied microRNA gain- and loss-of-function and downstream effects in inflammation and mechanosensing

**Visiting Researcher**  
Orthopaedic Research Laboratory – McGill University, Canada  
Project: *Analysis of miRNA expression profile in non-degenerated intervertebral disc cells*  
- Performed siRNA knockdown of TLR-2 in non-degenerated IVD cells  
- Identified miRNAs associated with TLR-2 signaling in non-degenerated IVD cells

**Visiting Researcher**  
Medical Sociology and Psychobiology Lab – University of Potsdam, Germany  
Project: *Establishment of serum-derived extracellular vesicle isolation and miRNA expression analysis in stress-induced osteoporosis*  
- Developed extracellular vesicle isolation from the serum of patients with osteoporosis  
- Identified miRNAs enriched in extracellular vesicles of patients with stress-induced osteoporosis

**Research Assistant**  
Metabolic and Cell Engineering Lab – University of Natural Resources and Life Sciences Vienna, Austria  
In collaboration with MERCK KGaA  
Project: *Cloning, strain generation and characterization within the project of engineering Pichia pastoris for antibody surface display*

**Research Intern**  
Tissue Regeneration and Aging Lab –  
University of Natural Resources and Life Sciences Vienna, Austria  
Project: *Transfection and uptake analysis of extracellular vesicles isolated from human fibroblasts and keratinocytes*

**Visiting Graduate Researcher**  
Nanobiotechnology Research Group – Universitat Autonoma de Barcelona, Spain  
Project: *Cloning, production, purification and in vitro testing of nanoparticles*

---

**EDUCATIONAL BACKGROUND**

**Doctor of Philosophy – Biomedical and Chemical Engineering**  
Department of Biomedical Engineering – Rochester Institute of Technology, NY, USA  
Dissertation: *Functional role of Toll-like receptor-associated microRNAs in intervertebral disc pathophysiology*  
2019 – 2023

**Master of Science - Biotechnology**  
2016 - 2018
University of Natural Resources and Life Sciences Vienna, Austria
Thesis title: Transfer of therapy resistance in colorectal cancer cells via extracellular vesicles
Supervisors: Prof. Robert Mader, Prof. Johannes Grillari
Medical University of Vienna, Comprehensive Cancer Center

Bachelor of Science - Food Science and Biotechnology 2011 - 2016
University of Natural Resources and Life Sciences Vienna, Austria
Supervisors: Dr. DI Brigitte Gasser, Dr. DI Lina Heistinger

AWARDS AND INVITED TALKS
2022 – ORS PSRS International Spine Research Symposium Best Poster Award for Outstanding Scientific Research
2022 – Personalized Healthcare Technology Travel Award – Rochester Institute of Technology
2021 – Center for Musculoskeletal Research, University of Rochester – Invited Seminar Talk

MENTORSHIP AND TEACHING EXPERIENCE
Teaching 2020 – 2023
Practical Methods in Tissue Engineering – Graduate Lab Course (selected classes)
Tissue Engineering – Graduate Course (selected classes)

Undergraduate Students Mentoring 2020 – 2023
Iskender Mambetkadyrov – Project: MicroRNA normalization screening for miRNA expression analysis
Mikkael Lamoca – Project: MicroRNA validation and establishment of PDMS chambers fabrication for cell stretching
Nea Bergendahl – Project: Fabrication and testing of PDMS chambers for cell stretching
Gabbie Wagner – General supervision and teaching of cell culture and molecular biology techniques

PhD Students Mentoring 2021 – 2023
Janitri Babu – Project: Targeting TRPC6 for Chronic Discogenic Back Pain
Johannes Hasler – Project: Substrate Stiffness, Topography, and TRPV4 in Annulus fibrosus Mechanotransduction
Paola Bermudez-Lekerika – Project: Functional role of interleukin-4 receptor in intervertebral disc pathophysiology

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PUBLICATIONS


**Cazzanelli P.**, Lamoca M., Mesfin A., Puvanesarajah V., Hitzl W., Wuertz-Kozak K. “The Role of miR-155-5p in Inflammation and Mechanosensing during intervertebral disc degeneration” (Manuscript ready for submission)


SELECTED CONFERENCES


**Cazzanelli P.**, Hausmann O.N., Wuertz-Kozak K. “MicroRNAs Associated with TLR-2-induced Inflammation in Intervertebral Disc Pathophysiology” Eurospine, Vienna, Austria, 2021 **Podium**

**Cazzanelli P.**, Hausmann O.N., Wuertz-Kozak K. “MicroRNAs Associated with TLR-2-induced Inflammation in Intervertebral Disc Pathophysiology” Eurospine, Rome, Italy, 2021 **Podium** (presented by Prof. Wuertz-Kozak)