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# MicroRNA-targeting nanomedicines for the treatment of intervertebral disc degeneration

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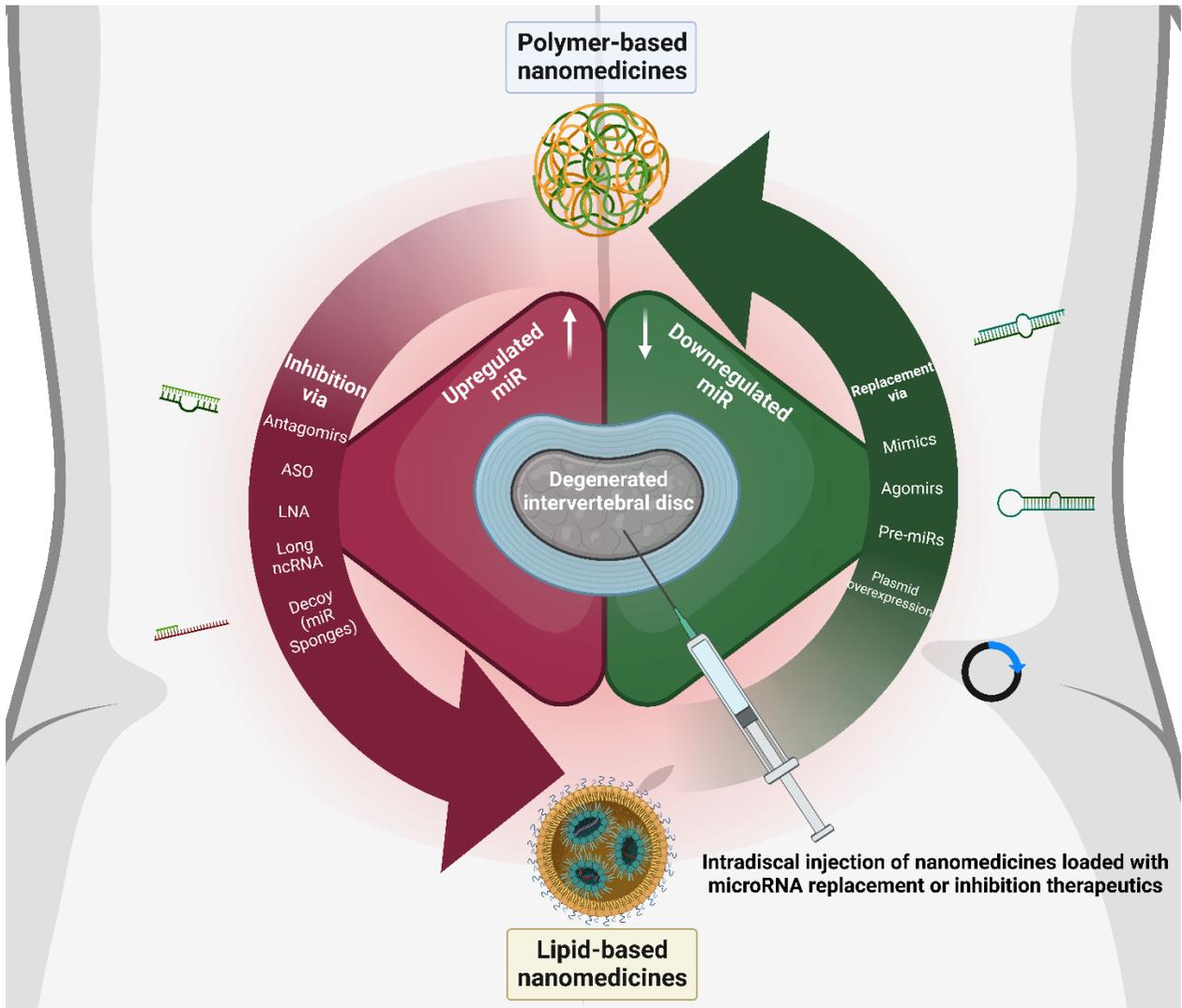
## Abstract

Low back pain stands as a pervasive global health concern, afflicting almost 80% of adults at some point in their lives with nearly 40% attributable to intervertebral disc degeneration (IVDD). As only symptomatic relief can be offered to patients there is a dire need for innovative treatments. Given the accumulating evidence that multiple microRNAs (miRs) are dysregulated during IVDD, they could have a huge potential against this debilitating condition. The way miRs can profoundly modulate signaling pathways and influence several cellular processes at once is particularly exciting to tackle this multifaceted disorder. However, miR delivery encounters extracellular and intracellular biological barriers. A promising technology to address this challenge is the vectorization of miRs within nanoparticles, providing both protection and enhancing their uptake within the scarce target cells of the degenerated IVD. This comprehensive review presents the diverse spectrum of miRs' connection with IVDD and demonstrates their therapeutic potential when vectorized in nanomedicines.

## Keywords

Intervertebral disc degeneration (IVDD), *nucleus pulposus* cells (NP cells), microRNA (miR), nanomedicine

# Graphical abstract



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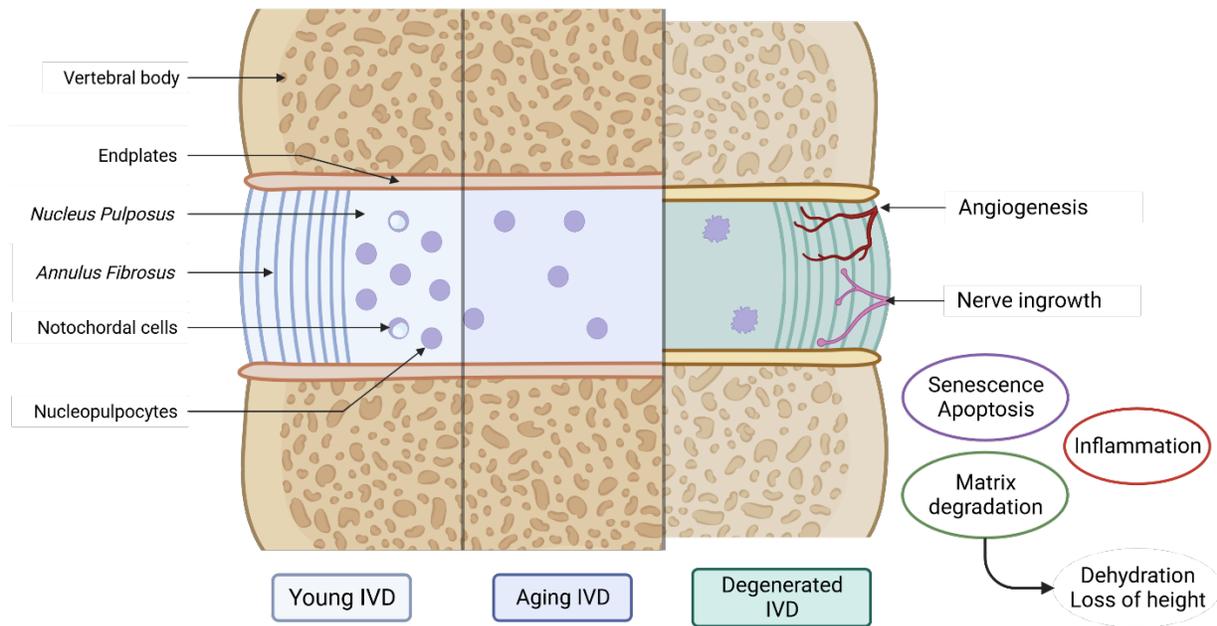
## Abbreviations

AF	<i>Annulus fibrosus</i>
ASO	Antisense oligonucleotide
CEP	Cartilaginous endplates
circRNA	Circular RNAs
ECM	Extracellular matrix
EPR	Enhanced permeation and retention effect
EV	Extracellular vesicle
FADD	Fas-associated death domain-containing protein
IL	Interleukins
IVD	Intervertebral disc
IVDD	Intervertebral disc degeneration
LBP	Low back pain
LNA	Locked nucleic acid
LNC	Lipid nanocapsules
LncRNA	Long non-coding RNA
LNP	Lipid nanoparticles
miR	microRNA
MMP	Matrix metalloprotease
MOFs	Metal-organic framework nanoparticles
MSC	Mesenchymal stromal cell
ncRNA	Non-coding RNA
NP	<i>Nucleus pulposus</i>
PEG	Poly(ethylene glycol)
PEI	Poly(ethyleneimine)
piRNA	Piwi-interacting RNAs
PLGA	Poly(lactic-co-glycolic acid)
PRP	Platelet-rich plasma
RES	Reticuloendothelial system
rRNA	Ribosomal RNAs (rRNAs),
SNALPs	Stable nucleic acid-lipid nanoparticles
sncRNA	Small non-coding RNAs
tRNA	Transfer RNAs
ZP	Zeta potential

## 1. Introduction

Low back pain (LBP) is one of the most frequent chronic conditions and a leading cause for years lived with disability [1,2]. It encompasses an array of underlying disorders, with one of the most common being intervertebral disc degeneration (IVDD) [3]. The intervertebral disc (IVD) is the avascular and aneural fibrocartilaginous structure linking vertebrae and providing flexibility to the spine. It is composed of an inner highly hydrated *nucleus pulposus* (NP) and a surrounding *annulus fibrosus* (AF) constrained between two cartilaginous endplates (CEP) ensuring nutrient diffusion from the vertebrae. From a developmental point of view, cells of the AF and CEP derive from the sclerotome, while all cells in the NP are from the notochord [4]. At birth, two cell types cohabit in the NP. Nucleopulpcytes are the chondrocyte-like differentiated cells responsible for extracellular matrix (ECM) production in the NP, while the remaining notochordal cells have a regulatory role. In humans, these notochordal cells are progressively lost during growth, thereby initiating the aging of the IVD. Genetic and environmental factors, such as abnormal mechanical loading, can accelerate this process, eventually leading to IVDD [5]. Based on the severity and progression of LBP, the treatment typically involves a combination of non-invasive symptomatic and invasive strategies [6]. Physical therapy, pain management, and lifestyle modifications can help strengthen the spine-supporting muscles, improve flexibility and range of motion, and help alleviate pain [7–10]. More severe cases may need the more invasive option of epidural steroid injections to temporarily decrease the pain and inflammation [6]. In cases where IVDD is the main issue, surgical interventions may be required as a last resort; i.e., spinal fusion or disc replacement [6]. Hence, IVDD is only treated symptomatically, which necessitates a new strategy that can provide less invasive etiological therapeutics. Emerging potential treatments, based on mesenchymal stromal cells for example, are yielding promising results in terms of pain management, although they still have considerable limitations that need to be overcome [11].

Although the exact mechanisms leading to IVDD are not fully understood, some aspects have been well-documented (**Figure 1**), and the ways by which a regenerative therapy could be successful are widely accepted [3,11,12]. IVDD is characterized by a decrease in cellularity and ECM quality in the NP. This leads to dehydration and thus deterioration of the IVD biomechanical characteristics. These changes can be accompanied by nerve and vessel ingrowth from the outer AF, as well as inflammation, participating further to the pain and the imbalance between ECM anabolism and catabolism. Targeting the NP to prevent apoptosis or senescence of the remaining cells is thus a major objective for any therapeutic strategy. Quiescent progenitor cells may still be present, and stimulating their proliferation and differentiation may be another way to compensate for the degenerating nucleopulpcytes. The cells that are present could then be reprogrammed to re-equilibrate or even reverse the anabolism/catabolism imbalance that has taken hold. To ensure mechanical stability, substantial synthesis of ECM would be needed alongside inhibition of metalloproteases (MMPs), which are the main ECM-degrading enzymes. Lastly, modulating the inflammatory response may also help break the vicious cycle of cell death and ECM degradation, resulting in stabilization of the IVD microenvironment to make it suitable for regeneration. Overall, senescence, apoptosis, ECM degradation, and inflammation are all intertwined processes and influence one another.

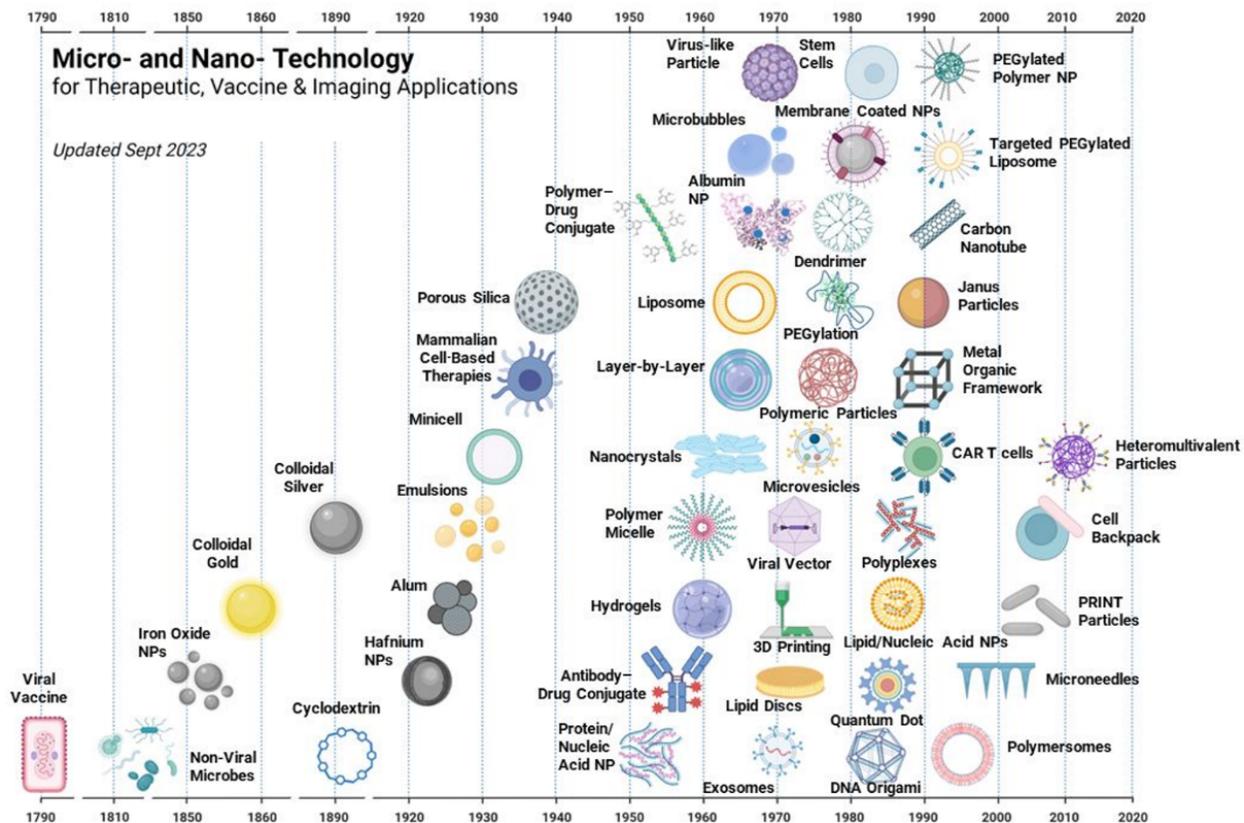


**Figure 1.** Process of human intervertebral disc (IVD) aging and degeneration, inspired by [11,13]. The IVD is composed of the inner *nucleus pulposus* (NP) and outer *annulus fibrosus* (AF) and is separated from the vertebral bodies by cartilaginous endplates. The young NP contains both notochordal cells and nucleopulpyocytes, the former being lost progressively with age. In degenerated IVDs, a vicious cycle involving nucleopulpyocyte senescence and apoptosis, matrix degradation, and inflammation takes hold (with potential ingrowth of nerves and vessels), eventually leading to NP dehydration and loss of IVD height.

Recent studies have found that microRNAs (miRs) can be involved in all these interconnected processes, which encourages to consider them as new targets and biomarkers as well as potential therapeutic agents [14,15]. Indeed, these small RNAs with regulatory functions can profoundly affect key signaling pathways, thus affecting cell fate in multiple ways. They may be the perfect candidate to profoundly modulate the NP environment as they have multiple targets, which can be involved in several mechanisms, and they can even be used in combination to achieve a synergistic effect. However, for miR delivery, they must be vectorized into nanomedicines to both protect them from rapid degradation by nucleases and to achieve cellular uptake in targeted cells. To this end, nanoparticles appear to be perfect carrier candidates to rapidly transfer the knowledge regarding miR biology in the IVD into effective new therapeutic options [16].

Nanomedicine has undergone remarkable progression in its relatively short history. Starting with the first generation of biodegradable nanomedicines introduced in the 1970s with a size range of 10–100 nm. Their applications were, however, limited due to their immunogenicity and rapid detection by the reticuloendothelial system owing to their positively charged unmodified surface [17,18]. The progression continued with the emergence of second-generation nanomedicines in the 1980s and 90s and the pivotal concept of modifying the surface of nanoparticles with a hydrophilic polymer called poly(ethylene glycol) (PEGylation)[19–21], providing the nanoparticles stealth properties that have facilitated the implementation of passive targeting and the subsequent exploitation of the enhanced permeation and retention effect (EPR) to achieve better localization in several diseases characterized by perturbed membranes (e.g., cancer, inflammatory diseases, and cardiovascular diseases), which is not the case with IVDD [22–26]. This transformative concept did not stop at merely making nanomedicines undetectable by the immune system, but it also opened the eyes of the scientific world to the potential of surface decoration, paving the way for a third generation of nanoparticles, in the late 90s, that utilizes active targeting to increase cellular uptake as well as to achieve enhanced localization in targeted cells by functionalization with homing ligands (i.e., molecules with the ability to specifically bind to certain

receptors expressed on the surface of targeted cells) [27–30]. Since then, a fourth generation of nanoparticles has been developed based on the concept of interaction of nanoparticles with their bioenvironment, resulting in the generation of stimuli-responsive or “smart” nanomedicines [31,32]. Over the course of 50 years of development, nanomedicine has evolved into a more distinct field, thus attracting more resources and offering a substantial potential to revolutionize therapeutics through a plethora of nano-objects, as illustrated in **Figure 2**.



**Figure 2.** Chronological overview of nanomedicine development. (Adapted from [33] licensed under a Creative Commons Attribution 4.0 International License)

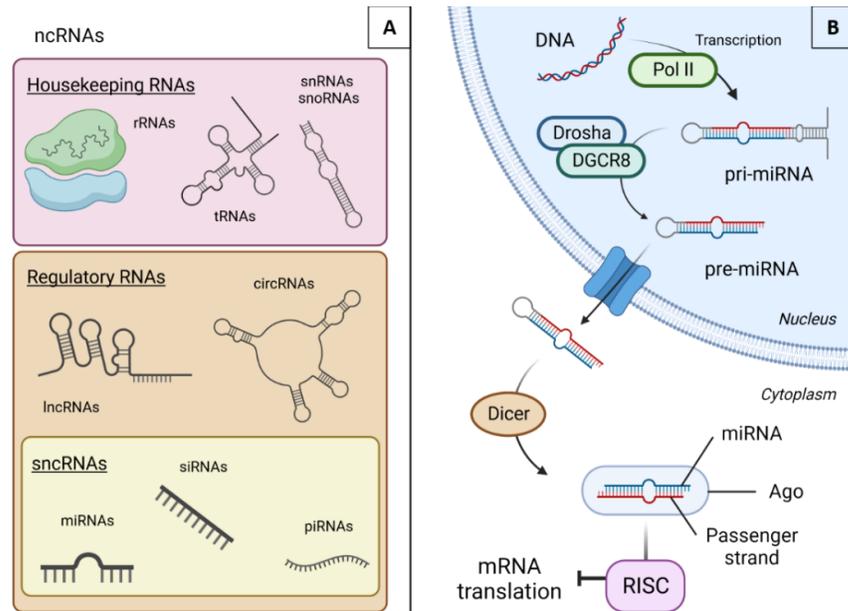
In the present review, a brief introduction of the diversity of non-coding RNAs and the biogenesis of miR will be provided before delving deeper into the description of miRs known to be involved in IVDD, and whether they have a positive or a negative impact on the progression of the disease. An extensive literature review and analysis have been performed and summarized, with highlights and examples provided to illustrate the diversity and complexity of the processes involved. Vectorization strategies to deliver promising miR-targeting molecules in the challenging IVD environment are then discussed based on the most advanced studies in the field.

## 2. miR biology and implications for intervertebral disc degeneration

### 2.1. miR biogenesis and regulation

In 1993, a study on *Caenorhabditis elegans* described for the first time a small non-coding RNA that was nevertheless essential for the development of the organism [34]. This first miR, called lin-4, was found to be complementary to a sequence in the 3' untranslated region (3'UTR) of the messenger RNA (mRNA) encoding the protein LIN-14. These observations led to the hypothesis of an mRNA translation inhibitory mechanism through “RNA-RNA antisense interaction”. Following the identification of other

small non-coding RNAs in this nematode, homologs of these genes were found in other organisms. Since then, a plethora of miRs have been discovered, their biogenesis pathways unveiled, and their regulatory functions described in diverse physiological or pathological contexts [35]. As of September 2023, the public repository miRBase [36] references 1,917 mature miRs in humans and 48,885 across all species. Other databases, such as miRTarBase [37] and ENCORI/starBase [38], are now referencing interactions between miRs and mRNAs or even other non-coding RNAs (ncRNAs), thus facilitating the spread of knowledge and its transfer to the study of other tissues or diseases.



**Figure 3.** MicroRNAs in the diversity of non-coding RNAs and their main biogenesis pathway. (A) The non-coding RNA classification includes, on the one hand, the housekeeping RNAs that can be ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), or small nuclear/nucleolar RNAs (snRNAs/snoRNAs) and, on the other hand, regulatory RNAs that can be circular RNAs (circRNAs), long non-coding RNAs (lncRNAs), or small non-coding RNAs (sncRNAs), the latter class comprising micro RNAs (miRs), small interfering RNAs (siRNAs), and piwi-interacting RNAs (piRNAs). (B) The miR canonical biogenesis pathway begins in the nucleus, where a first pri-miR is transcribed from the DNA. The Drosha/DGCR8 complex then crops the hairpin out, creating a pre-miR that can exit into the cytoplasm where the terminal loop is cleaved by Dicer. Lastly, an Ago protein binds the remaining miR duplex, the passenger strand is discarded, and the miR can inhibit its targets through the RNA-induced silencing complex (RISC).

Several ncRNAs (**Figure 3.A**) [39] can be of interest in the understanding of the mechanisms underlying IVDD or for developing new treatments. Apart from ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), and other small RNAs involved in messenger RNAs (mRNAs) processing and translation to proteins, ncRNAs that have a regulatory function can be circular (circRNAs) or linear. The linear ones are classified as either long (long non-coding RNAs, lncRNAs, including the subgroup of long intergenic non-coding RNAs, lincRNAs [40]) or small (small non-coding RNAs, sncRNAs). miRs are part of the latter group, alongside small interfering RNAs (siRNAs) and piwi-interacting RNAs (piRNAs). The use of RNA interference as a therapeutic tool can be achieved with either siRNA or miR, with the main difference residing in the complementarity to the mRNA target. While siRNAs are highly specific, the seed sequences of miRs are shorter, thereby allowing them to target multiple mRNAs. It can be beneficial to simultaneously affect several signaling pathways in complex diseases, but it can also be challenging as miRs effects are thus more complex to decipher [41].

miRs are 20 to 25 nucleotide-long RNAs originating from longer pri-miRs transcripts that can fold into a hairpin structure. Briefly, in the canonical biogenesis pathway (**Figure 3.B**), the Drosha (RNase III)–DGCR8 complex, or microprocessor complex, crops the hairpin structure out of the pri-miR to produce the

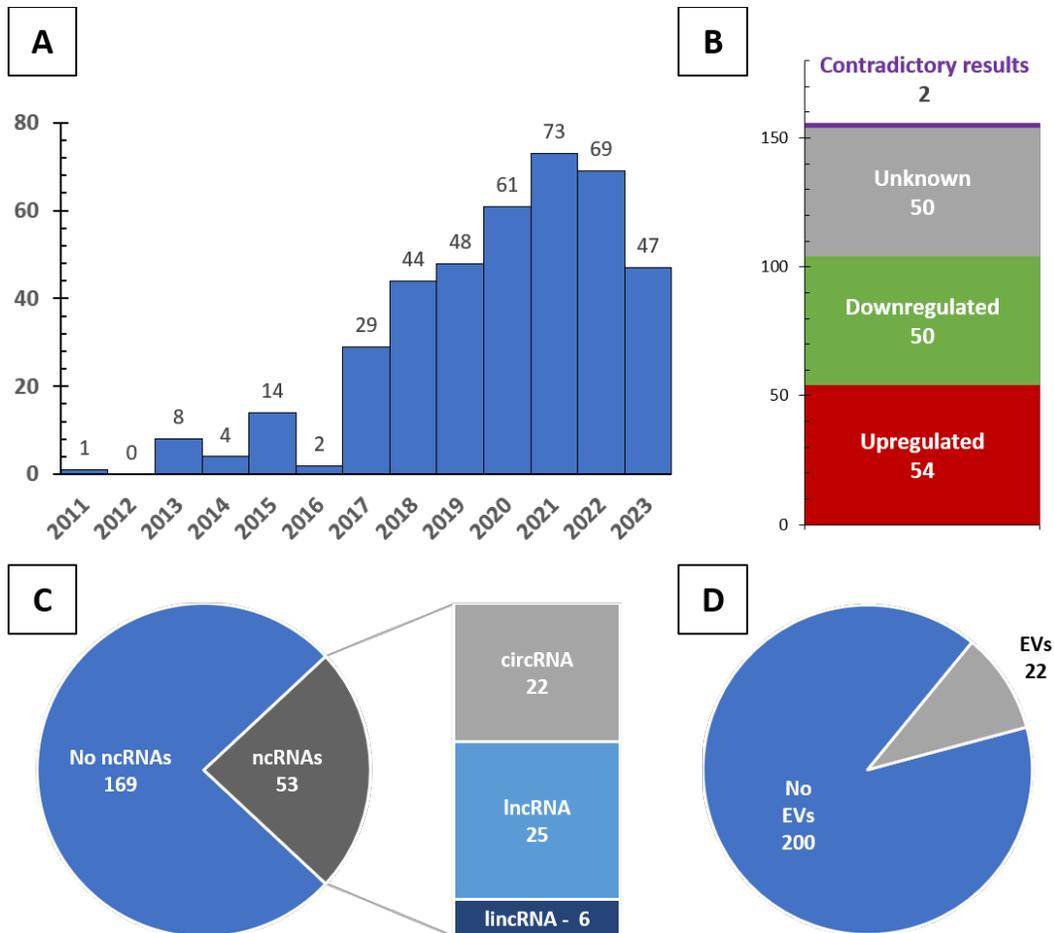
pre-miR. This pre-miR is then exported out of the nucleus and the terminal loop is cleaved by another RNase III, Dicer. The remaining miR duplex is captured by an Argonaute (Ago) protein, thereby forming the RNA-induced silencing complex (RISC) with other proteins. The passenger strand is discarded, and the RISC can bind with mRNA targets, typically to the 3'-untranslated region (3'-UTR), complementary to the mature miR (guide strand), thereby degrading them or repressing their translation. Other non-canonical pathways that are Drosha- or Dicer-independent exist, as do complex regulations of miR synthesis and turnover, as thoroughly reviewed by Shang and colleagues [42].

Additionally, a wide variety of RNA-binding proteins can also modulate miR activity [43] and be involved in the degeneration of the IVD [44]. Altogether, the complexity of miR regulatory networks makes them challenging to study, but accumulating evidence of their involvement in aging and diseases, notably in IVDD, indicates underexploited therapeutic opportunities. To fully utilize these recently generated data, a thorough inventory and analysis of the literature on the topic is required.

## 2.2. Selection and analysis of the literature on miRs in the intervertebral disc

To summarize the literature on miR involvement in IVDD, all original articles on PubMed mentioning “microRNA” and “intervertebral disc” until November 2023 were analyzed, filtering out reviews and purely bioinformatic papers without functional validation. **Table S1** illustrates this work, created from 222 articles studying 156 different miRs in cultures of IVD cells (NP, AF, or CEP) and *in vivo* models of IVDD. For each study, details regarding the target mRNAs or pathways identified, the main mechanisms related to IVDD impacted, and the models used were added and can be efficiently filtered. It is worth noting that miRs reported to be involved in cartilage development [45,46] or other joint pathologies such as osteoarthritis [47] or rheumatoid arthritis [48] were not explored, although they could also be implicated in IVDD.

While gathering the literature, more than a dozen potentially relevant articles were retracted. Journals with relatively high impact factors are not immune to accepting falsified manuscripts or inaccurate findings for publication [49], but lower impact factor journals, in which most of the studies referenced in this first section were published, are even more susceptible to failure to identify articles that should be retracted [50]. In addition, as shown in **Table S1**, most of the miRs in the scope of this review were studied in only one or two papers. On the one hand, the field of miR appears to be particularly prone to instances of plagiarism and paper mill articles [51]. On the other hand, studies on IVDD are challenging due to the tissue itself. Notably, the weak relevance of the most common animal models [52], as well as the difficulty with accessing usable patient samples makes any attempt at merely reproducing other researchers' data usually not worth the effort. Regardless, it is unfortunately possible that some articles cited here will end up being retracted after the publication of this review. A most concerted effort was made to highlight novel or well-designed studies and to focus on several miRs for which a number of different teams have provided complementary evidence of their relevance in IVDD, but readers should nonetheless maintain a degree of caution and always meticulously and independently assess the studies they will rely on most heavily.



**Figure 4.** Bibliographical data from literature analysis. (A) All PubMed entries for “microRNA” and “intervertebral disc” for the indicated years. After filtering, 222 articles studying 156 miRs in IVDD were analyzed, and the common topics that stood out were (B) miR dysregulation in human samples, (C) involvement of other ncRNAs in the regulation of miRs, and (D) delivery by extracellular vesicles.

As can be seen from the time repartition of the articles of interest (**Figure 4.A**), it is clear that the involvement of miRs in the development and treatment of IVDD is a trending topic. The expression levels of miRs in degenerated IVDs are a first point of interest that was notably studied by high-throughput transcriptomic analyses (**Table S2**). From **Table S1**, dysregulated miRs from individual studies can also be extracted (**Figure 4.B**). Additionally, other subtopics emerged from this bibliography analysis and will be discussed further below: first, the regulation of miRs by lincRNA and circRNA (**Figure 4.C**) and then, the identification of miRs delivered by extracellular vesicles (**Figure 4.D**). Finally, and as previously mentioned, the most studied miRs in IVDD will be succinctly described.

### 2.3. Dysregulated miRs in IVDD

Using microarray or sequencing, several studies aimed at generating an exhaustive list of all miR modulated in human IVDD, resulting in the production of five freely available datasets (**GSE63492** [53,54], **GSE116726** [55], **GSE167199** [56], **GSE19943** [57], and **GSE45856** [58], **Table S2**). For example, 50 out of the 2059 miRs detected in IVD samples were found to be dysregulated during IVDD by Lan *et al.* [54]; 31 of which were found upregulated and 19 downregulated. Interestingly, this study also contained mRNA, lincRNA, and circRNA expression profiles (**GSE67567**) from the same samples, allowing a more in-depth bioinformatic analysis and the prediction of regulatory networks. Similarly, Li *et al.* [56] also included

lncRNA and mRNA sequencing. Unfortunately, few overlaps have been found between dysregulated miRs identified in the datasets from different experiments, probably due to the different techniques, the low number of samples, and the variability between patients and the degenerative stage of the IVDs used. However, in all studies, qRT-PCR validation with greater sample sizes was performed on RNAs of interest to confirm the predicted changes in a larger population of human patients. A final limitation is the use of samples from discectomy and, therefore, ruptured IVDs, which could contain contaminants from surrounding tissues and a higher degree of immune infiltration. Some miRs identified as dysregulated would, therefore, be irrelevant for therapeutic application, as a regenerative therapy would most likely target mildly degenerated IVDs rather than completely dehydrated or herniated ones.

From the bibliographical analysis (**Figure 4.B, Table S1**), 54 of the miRs studied in detail were reported to be upregulated and 50 downregulated in human IVDD tissues (or cells extracted from them), mostly from NP samples and some from CEP samples. Only two miRs had contradictory reports on their expression during IVDD. Interestingly, Dong *et al.* [59] were able to separate tissues from AF versus NP, and they found upregulation of miR-640 only in the NP. Some instances of dysregulation reported for human samples have also been found in small animal models of other studies. For example, the expression of miR-494 was found to be increased with IVDD and correlated with the severity of the disease [60], and it was also increased in a rat puncture model [61]. This is also the case for the upregulation of miR-431 in humans [62] and in a mouse puncture model [63]. By contrast, the upregulation of miR-143-5p [64] and downregulation of miR-140-3p [65] have only been reported in rat models of induced IVDD and should thus be confirmed with patient samples. Even further from the clinic, mimicking some aspects of the degenerative process *in vitro*, such as inflammation with lipopolysaccharide or cytokines (primarily TNF and IL1 $\beta$ ) and oxidative stress by H<sub>2</sub>O<sub>2</sub> treatment, can modulate some miR expression profiles and allow their dysregulation in response to these specific mechanisms to be studied. A few novel examples include miR-20a, which was found to be upregulated in CEP chondrocytes on a stiffer matrix, mimicking advanced degeneration, and it participated in the *in vitro* mineralization process [66]. Other authors have used advanced glycation end products on human NP cells *in vitro* to induce a degenerative profile, and they observed downregulation of miR-195a in this model [67].

While the array of dysregulated miRs in degenerated IVDs remains incomplete, these data are still great entry points to select potentially relevant miRs and further analyze their role in IVDD. As one might expect, most upregulated miRs participate in the degenerative process by promoting cell apoptosis or by enhancing ECM degradation. The expression level of these miRs should ideally be brought down using an antisense sequence (an antagomir or anti-miR) so as to diminish their deleterious effect by derepressing the targeted mRNA. By contrast, miRs that are downregulated with disease progression are most likely involved in the physiological functioning of the IVD and protect it against the establishment of the degenerative vicious cycle. Therapeutic approaches would, therefore, seek to compensate for their shortage by delivering miR mimics (or agomirs).

Whether the causes or consequences of IVDD, the expression of miRs can be modulated by several factors. Among these, researchers have recently become interested in lncRNAs and circRNAs, as these are important regulators of miR expression in many physiological and pathological mechanisms [68].

#### 2.4. miR regulation by lncRNAs and circRNAs in IVDD

Involvement of other ncRNAs in the regulation of miRs in IVDD was reported in 53 articles, 22 of which concerned circRNAs, 25 lncRNAs, and 6 lincRNAs (**Figure 4.C**). lncRNAs, including the subgroup of lincRNAs, are complex regulators of transcription, translation, and more [69]. In the cell nuclei, they were shown to affect chromatin organization as well as splicing and enhancer activity. In the cytoplasm, they can impact mRNA stability or even protein post-transcriptional modifications. Regarding the regulation of

miRs, they can also act as decoy sequences, binding miRs instead of the intended targets, thus dampening mRNA degradation. Similarly, among the many functions of circRNAs is their ability to act as sponges for miRs [70]. In aging and disease development, notably in the IVD [68], both lncRNAs [71] and circRNAs [72] can be dysregulated and contribute to the degenerative process, and they can hence be seen as potential therapeutic targets or tools. Following are some examples of ncRNAs referred to twice in the literature as regulating miRs in IVDD.

#### 2.4.1. lncRNA-HOTAIR

Two studies focusing on lncRNA-HOTAIR showed its reduced expression in IVDD samples [73], and even a negative correlation between its expression level and the Pfirrmann grade, the most widely used classification system for IVDD based on magnetic resonance imaging (MRI) [74]. It exerted an anti-apoptotic effect in NP cells through inhibition of miR-34a-5p [73]. This miR was previously known to target Notch1, and this interaction appears to be relevant in NP cells as well. Surprisingly, overexpression of lncRNA-HOTAIR has been shown to inhibit cell proliferation [74]. The relevant target in this case was miR-130b and its interaction with the mRNA of PTEN.

#### 2.4.2. lncRNA-H19

Based on previously produced microarray data [54], studies have sought to elucidate the role of lncRNA-H19 in IVDD, as it was among the most upregulated lncRNAs. This overexpression in degenerative tissues has been confirmed by PCR in human samples [75] and rat IVDs that had undergone puncture surgery [76]. The first study [75] revealed that knocking down lncRNA-H19 could temper the effect of H<sub>2</sub>O<sub>2</sub> treatment on human NP cell senescence and ECM production. The detrimental effect of lncRNA-H19 appeared to take place by indirect activation of Wnt/ $\beta$ -catenin. Indeed, lncRNA-H19 can sponge miR-22-3p, allowing expression of LEF1, which is a transcriptional activator of Wnt signaling. The second study [76] confirmed that silencing of lncRNA-H19 reduced the degeneration of punctured rat IVDs. The authors identified the miR-139-3p/CXCR4/ NF- $\kappa$ B axis as a target of lncRNA-H19 involved in cell autophagy and apoptosis.

#### 2.4.3. circRNA-GRB10

The circular RNA GRB10 has been shown to be downregulated in NP tissues from IVDD patients [77]. The authors identified miR-328-5p as a target of circRNA-GRB10, thus becoming upregulated in IVDD, that could participate in the development of the disease by promoting NP cell apoptosis through repression of human epidermal growth factor receptor 2 (HER2 or ERBB2). In a follow-up study [78], they showed that ERBB2 could also enhance ECM production and confirmed the protective effect of GRB10 on IVDs in an *in vivo* model. They also identified the RNA-binding protein FUS and miR-141-3p as new players in the complex regulation of circ-GRB10. It would appear that FUS enhances the production of circRNA-GRB10 by binding its pre-mRNA. Moreover, FUS expression is downregulated by miR-141-3p, itself inhibited indirectly by ERBB2 through ERK1/2 phosphorylation. Altogether, a vicious cycle occurs in the degenerative IVD, whereby downregulation of circRNA-GRB10 results in higher levels of miR-328-5p, thus inhibiting ERBB2 [77]. Lowering the level of ERBB2 removes the inhibition of miR-141-3p, which can then target FUS, thereby reducing its enhancing effect on the biogenesis of circRNA-GRB10 [78].

Given these examples, innovative strategies could take advantage of these preexisting endogenous regulations of miR by other ncRNAs. Treatments using or targeting one or the other could be designed to affect the entire regulatory network. Both miRs and their regulatory lncRNAs and circRNAs can be delivered intercellularly as cargos of extracellular vesicles.

## 2.5. Identification of miRs from extracellular vesicles

The use of extracellular vesicles (EVs) is a growing research prospect in tissue regeneration, and the treatment of IVDD is in line with this trend [79,80]. EVs regroup all cell-secreted particles with a lipid bilayer, ranging from small exosomes released from multivesicular bodies to larger microvesicles budding directly at the plasma membrane and apoptotic bodies [81]. They can contain virtually any intracellular and membrane-bound cell components, extending the diversity of potential intercellular communication molecules from proteins that are only secreted to intracellular ones, metabolites, and even nucleic acids such as miRs [82]. Given the importance of paracrine signaling in the efficacy of mesenchymal stem cells (MSCs), notably their immunomodulatory properties, their secreted vesicles appear to play an important role and could ultimately be used on their own to circumvent the technical and regulatory limitations of cell therapy [83].

In 22 of the 222 articles retrieved, miRs were identified as being involved in EV-associated communication (**Figure 4.D**), whether they were directly shuttled in EVs or a target of an EV-delivered circRNA. In keeping with the notion of considering the use of EVs as an alternative to cell therapy, most studies investigated MSC-derived EVs and found them to be rather favorable in terms of mitigating IVDD. Among these, we can cite Hao *et al.* [84], who used both human and rat bone marrow MSCs (BMSCs) on cultured human NP cells and in an IVDD rat model, respectively. Through various gain- and loss-of-function experiments, they showed that these BMSCs secreted miR-217-loaded EVs that could reverse the deleterious effect on cell viability and ECM degradation of TNF treatment *in vitro* and diminish the *in vivo* severity of IVDD. Quite similarly, miR-129-5p was also detected as a cargo of human BMSCs that could inhibit apoptosis and ECM degradation in IL-1 $\beta$ -treated human NP cells and reduce inflammation in a rat IVDD model [85]. Other authors have suggested that pre-treatment of the BMSCs by exposure to hypoxic conditions could enhance the therapeutic properties of the produced EVs [86]. They identified miR-17-5p as a key cargo of these hypoxic BMSC-EVs. Other types of stem cells have also been used to produce EVs with therapeutic potential for the treatment of IVDD with other miRs associated with the observed effect; miR-105-5p from MSCs derived from IPSCs [87], miR-302c from embryonic stem cells [88], and miR-26a-5p from umbilical cord stem cells [89].

Highly relevant in the initiation of IVDD, notochordal cells have been shown to deliver miR-140-5p through EVs [90]. The authors were able to isolate notochordal cells from rat IVDs and stimulate EV production in a compression culture chamber. The EVs produced had an increased miR-140-5p content and could more efficiently inhibit angiogenesis, both *in vitro* on HUVECs and *in vivo* in a mouse IVDD model. These findings support the notion that notochordal cells have an essential role in the maintenance of IVD homeostasis. Identifying other signals from notochordal cells could help elucidate how to offset their disappearance and slow down the irrepressible aging of the IVD.

Only two studies examined miRs implicated in EV-related mechanisms responsible for the development of IVDD. The first study identified miR-141-5p as a target of circRNA\_0000253, carried by exosomes produced by human degenerative NP cells [91]. The circRNA-carrying exosomes promoted apoptosis of human NP cells and *in vitro* expression of ECM degrading enzymes. The authors were also able to confirm the relevance of this circRNA/miR couple in a rat IVD puncture model, whereby injection of circRNA\_0000253 or a miR-141-5p-inhibitor exacerbated the IVDD, while a circRNA\_0000253-siRNA alleviated it. A second recent article explored the difference in miR content between exosomes from normal and degenerated human NP cells [92]. They demonstrated *in vitro* that miR-27a-3p was enriched in exosomes from degenerated NP cells and that it could induce macrophage polarization towards an inflammatory phenotype using a monocytic cell line (THP1) via silencing of PPAR $\gamma$ . In a rat IVDD model,

they validated that injection of a miR-27a-3p-mimic worsened the IVDD and enhanced inflammatory macrophage infiltration, while an inhibitor had the opposite effects.

## 2.6. The most described miRs in the intervertebral disc

Finally, a number of miRs were examined by several studies in the context of IVDD. Their pertinence in the field is thus strengthened by the use of complementary models and techniques to demonstrate their role. A few identified examples are detailed in the following section.

### 2.6.1. miR-21

miR-21 is potentially involved in the development of human IVDD, as its expression in the tissue positively correlates with the severity of the disease measured with the Pfirrmann grading system [93–95]. The mRNA of the phosphatase and tensin homolog (PTEN) protein was identified as a target of miR-21 in two different articles [94,96]. Inhibition of PTEN by miR-21 activates cell proliferation [96] through the AKT/mTOR signaling pathway, suggesting a role of the miR in the development of NP cell clusters during IVDD but also in the imbalanced turnover of the ECM [94]. Programmed cell death 4 (PDCD4) is another target of miR-21, reinforcing its effects towards increased cell proliferation and expression of MMPs [95]. Based on these findings, strategies to inhibit miR-21 could be developed to reduce its detrimental effect on the condition of IVDs. The only *in vivo* study using an antagomiR-21 to date demonstrated a protective effect against neo-angiogenesis in the AF and against apoptosis of NP cells in a rat model [97].

### 2.6.2. miR-25-3p

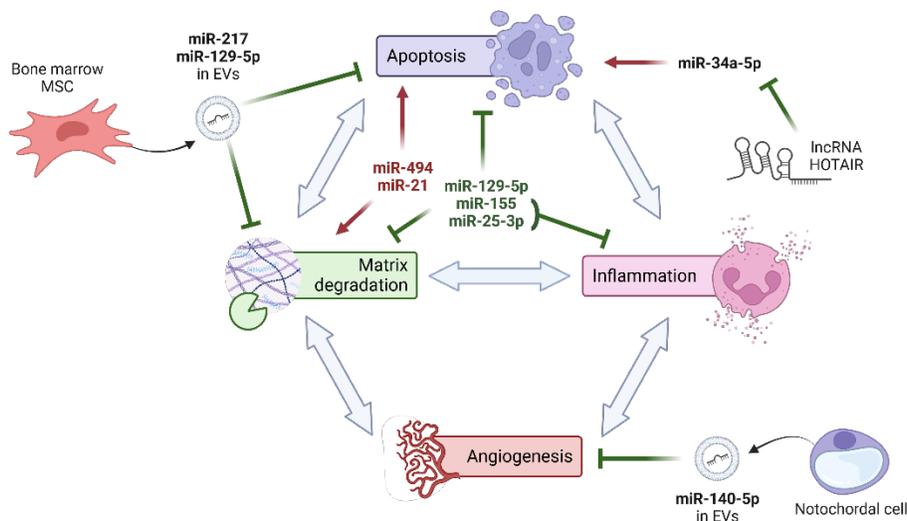
Expression of miR-25-3p was found to be downregulated in human patient samples [98,99] and in IL-1 $\beta$ -treated NP cells from mice [100] or rats [101]. Its expression could even be inversely correlated with the Pfirrmann grading and the expression of lncRNA OIP5-AS1, which appears to be responsible for this downregulation [98]. *In vitro*, miR-25-3p could promote proliferation and inhibit apoptosis of human NP cells by targeting the transcript of small ubiquitin-related modifier 2 (SUMO2) [99]. Its overexpression also diminished the production of ECM-degrading enzymes following IL-1 $\beta$  treatment via inhibition of the transcription factor MTF1 [102]. In keeping with this, after IL-1 $\beta$  treatment of rat NP cells, miR-25-3p reduced the apoptosis rate by targeting the Bcl-2 interacting mediator of cell death (Bim) [101]. EVs from mouse platelet-rich plasma (PRP) were also shown to carry miR-25-3p and compensate for its downregulation in mouse NP cells treated with IL-1 $\beta$ , restoring cell viability and lowering the production of inflammatory cytokines through direct targeting of SOX-4 [100]. In a rat puncture model, delivery of a miR-25-3p mimic with a thermo-responsive vector confirmed its therapeutic potential, as micro-computed tomography showed an enhanced disc height and histological scoring demonstrated an improvement in the matrix quality [102].

### 2.6.3. miR-129-5p

In all articles exploring the involvement of miR-129-5p in IVDD [85,103–105], downregulation of its expression was found in pathological human tissues. One of the targets of miR-129-5p is Beclin-1 [105]. This interaction was proven to reduce autophagy in human NP cells. BMP-2 was also identified as a target of the miR, and its inhibition reduced apoptosis in NP cells [103]. As previously mentioned, miR-129-5p was also shown to be a potential therapeutic cargo if enriched in BMSC-derived EVs [85]. In addition to its anti-apoptotic effect, delivery of this miR to human NP cells could modulate their secretome to reduce ECM degradation and promote anti-inflammatory macrophage polarization. The target identified by this study was the leucine-rich  $\alpha$ 2-glycoprotein 1 (LRG1). The authors were able to confirm, in a rat model of IVDD induced by puncture, that the therapeutic effect of the EVs could be further enhanced by overexpression of miR-129-5p in the producing cells. Lastly, it was also demonstrated that miR-129-5p could silence the expression of mitogen-activated protein kinase 1 (MAPK1), thereby favoring cell proliferation and avoiding the deleterious senescent phenotype [104]. These results obtained *in vitro* with human NP cells stimulated with lipopolysaccharides (LPS) to mimic a degenerative environment were confirmed *in vivo* in a rat puncture model.

### 2.6.4. miR-155

miR-155 is the most studied miR in IVD, with 8 articles investigating its mechanism of action and its potential as a therapeutic molecule. Its expression was shown to be downregulated in both the IVD tissue [57] and the serum [106] of human patients exhibiting advanced IVDD. Similar observations were made in a cohort of patients exhibiting IVDD following spinal tuberculosis [107]. This reduced expression of miR-155 during IVDD was connected with the activation of several cellular mechanisms associated with the development of the disease. For example, both mRNAs of Fas-associated death domain-containing protein (FADD) and caspase-3, essential links in the apoptosis pathways, were identified as targets of miR-155 [57]. Other targets of miR-155 include MMP-13 [107] and MMP-16 [108], two important metalloproteases involved in ECM degradation during IVDD, were also confirmed. The transcription factor TCF7L2 was also identified as a target of miR-155 implicated in the regulation of ECM degradation. Previously known to induce MMP expression and activity in chondrocytes, it was demonstrated that its knockdown inhibits ECM degradation in cultures of rat and human NP cells, suggesting this new path of action for miR-155 [109]. The same team also showed a protective role of miR-155 on ECM during inflammation, mediated by its targeting of CEBP $\beta$  mRNA. This protein was upregulated in rat NP cells by TNF or IL-1 $\beta$  stimulation, and its knockdown reduced the expression of ECM-degrading enzymes (MMP-3, MMP-13, ADAMTS-4, and ADAMTS-5) [110]. In the same article, the authors also reported use of a miR-155 knockout mouse model to confirm that the loss of this miR induced lumbar disc degeneration. By contrast, an overexpression model had a phenotype similar to the wild-type animal at one year. More recently, again by Wei Ye's group, miR-155 was shown to counteract the deleterious effect of cholesterol on IVD in various models [111]. First, mice overexpressing miR-155 were shown to be resistant to high-fat diet-induced IVDD. *In vitro*, a miR-155 mimic could reduce ECM degradation and apoptosis of rat NP cells after cholesterol treatment. The authors demonstrated that the miR-155 target of interest in their models was ROR $\alpha$ , and they confirmed its overexpression in patient samples as well as in a rat puncture model. Inhibiting ROR $\alpha$  with its inverse agonist SR3335 decreased the severity of cholesterol-induced degeneration in an *ex vivo* culture model of rat IVD and counteracted the damaging effect of cholesterol on rat NP cells, similar to the miR-155 mimic.



**Figure 5.** A multitude of miRs can be involved in the regulation of processes associated with IVDD. Apoptosis, matrix degradation, inflammation, and angiogenesis are all interconnected processes driving one another to worsen IVDD. The most studied downregulated (in green) and upregulated (in red) miRs in IVDD can influence both cell apoptosis and extracellular matrix degradation; miR-155 and miR-25-3p have also been reported to affect inflammation. In addition, some miRs, such as miR-140-5p, miR-217, and miR-129-5p, could have therapeutic potential as cargos of extracellular vesicles (EVs) produced by bone marrow mesenchymal stromal cells (MSCs) or notochordal cells. CircRNA and lncRNA can regulate the activity of miRs, for example, the lncRNA HOTAIR has been shown to inhibit the pro-apoptotic effect of miR-34a-5p.

Overall, miRs can influence all processes associated with IVDD (**Figure 5**), from regulating cell survival and ECM homeostasis to promoting or inhibiting angiogenesis and immune infiltration. Knowledge of these regulations and identification of the main miRs in IVDD could help the development of new therapeutic strategies. These new approaches would be based on the delivery of a single or a combination of pro-regenerative miRs or antagomirs of upregulated deleterious ones. Given the anatomical specificities of the IVD, a local administration route appears to be the most appropriate way to target its degeneration. However, the nature of the delivery system remains to be ascertained, to properly protect the miR of choice considering the physicochemical nature of this type of cargo as well as the specificities of the targeted organ (low pH, high osmolarity, hypoxia, etc.).

### 3. MicroRNA therapeutic strategies for IVDD; a therapeutic agent or a target?

As stated earlier in this review, miRs can either be upregulated or downregulated in IVDD, which renders the therapeutic strategy plausibly quite mechanical. In simple terms, when the miR is downregulated, then it needs to be balanced by the delivery of that miR's mimic as a therapeutic agent. On the other hand, when a miR is upregulated in IVDD, then it needs to be balanced by the delivery of an inhibitor, making the miR a therapeutic target, as shown in **Figure 6**.

#### 3.1. MicroRNA as a therapeutic target: miR inhibition approaches

Downregulation of specific upregulated miRs or inhibition of their activity have emerged as potential therapeutic strategies for the treatment of IVDD. Here, we discuss several ways to achieve such inhibition.

### 3.1.1. Synthetic oligonucleotides for miR downregulation

Antagomirs, antisense oligonucleotides (ASO)/anti-miRs, and locked nucleic acid (LNA) are all synthetic chemically modified oligonucleotides designed to either downregulate the miR of interest or to cause it to lose its function. An antagomir is a chemically modified RNA molecule that acts by binding the miR, thereby preventing it from interacting with its targets [112]. On the other hand, an ASO can be composed of DNA, RNA, or a combination of both to bind complementary RNA targets, thereby inducing their degradation or blocking their translation [113]. Lastly, an LNA is a modified RNA that binds to its complementary sequence of RNA with high specificity and affinity, thereby silencing the endogenous miR through liaison base pairing [114]. More specifically, the furanose rings are locked in the monomer unit in a spatial structure of endo/N-type C3 comparable to RNA. Its sugar ring is complexed with a methylene unit between O-2' and C-4', thereby enhancing the pairing capacity of the LNA, as a 2'-O, 4'-C-methylene- $\beta$ -d-ribofuranosyl [115]. LNAs are known to be very stable *in vivo* due to their composition, and they possess high binding specificities with their miR targets [116].

LNAs, which can be vectorized by nanomedicines to enhance their cellular uptake, have been investigated for gene-regulation therapeutics in light of their stability, non-toxicity, aqueous solubility, and antisense performance [117]. Recent developments have also provided a new perspective for the regulation of gene expression by another class of RNA, lncRNAs [118].

### 3.1.2. Long non-coding RNAs (lncRNAs)

lncRNAs are known to interact with proteins and chromatin and to regulate their methylation [119]. This type of sequence interacts with RNA and DNA by complementary base pairing to form hetero- or homo-duplexes. lncRNAs can form DNA-RNA-DNA triplexes through their unique conformation properties, and they can also recognize chromatin conformation and RNA special binding sites [118]. Moreover, they can contain multiple binding sites, thereby allowing multiple binding to different structures, and they are naturally present in two forms, circular or linear, as generated by different pathways [120,121]. The goal is to choose the lncRNAs corresponding to the number of binding sites of the targeted miR to downregulate its biological activity. The emergence of lncRNAs as inhibitors of miRs was first supported by the discovery of IPS1 in *Arabidopsis thaliana* [122,123]. Numerous approaches have since been developed for a broad variety of diseases to inhibit miR action, especially in cancer therapy, and some scientists have sought to synthesize lncRNAs with specific binding sites to target specific miRs acting as decoy vectors [124].

### 3.1.3. Decoy vectors (also known as miR sponges)

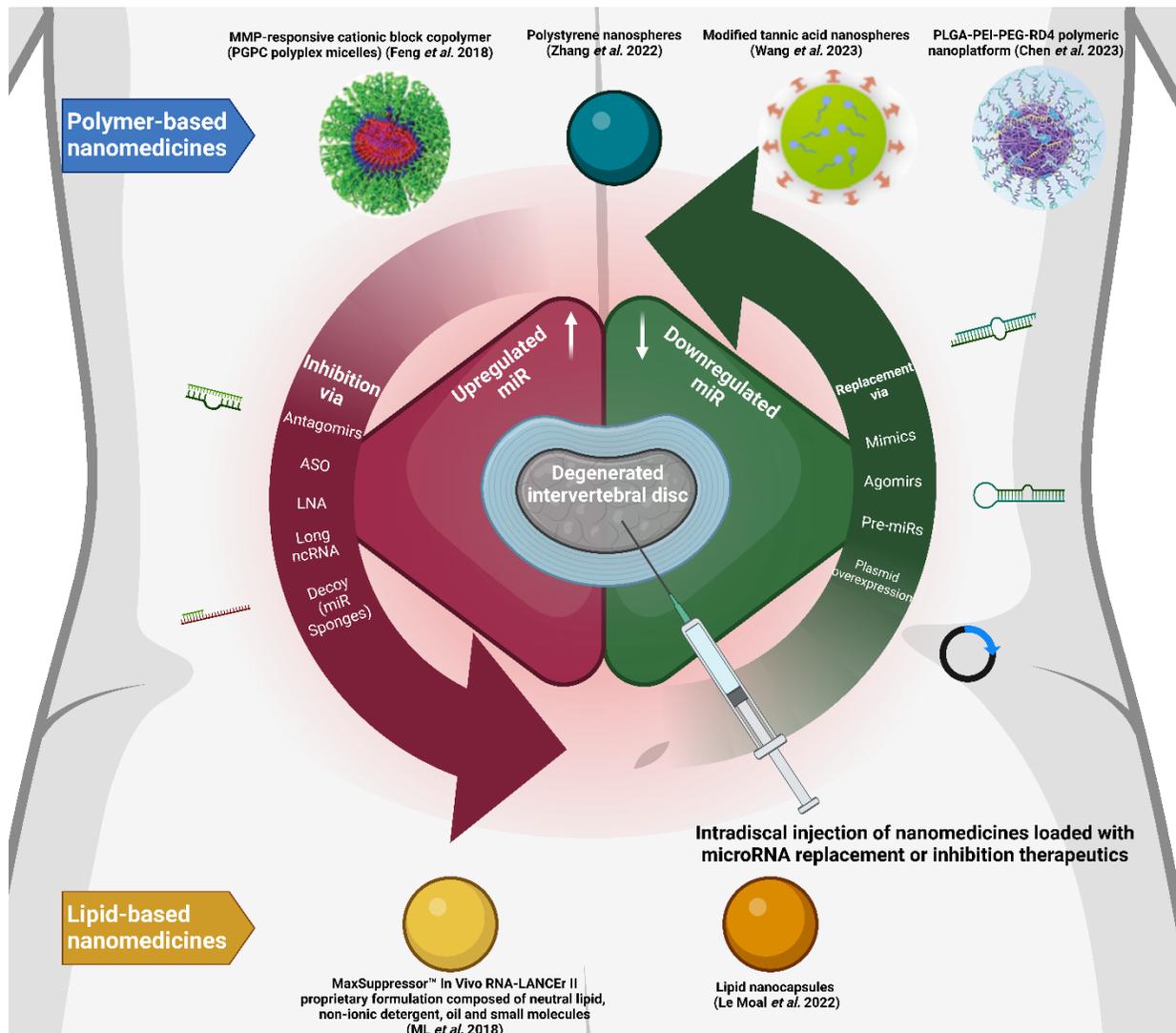
MicroRNA sponges are the most well-known miR inhibitors as they are RNA molecules that contain target sequences to bind the intended miR [125,126]. Their mechanism is based on having an excess of exposed miR recognition sites, thereby enhancing the binding of the target miR and decreasing the binding of the miR to its natural target mRNA. Based on the intended use, two decoy-type inhibitors (RNA Pol III- and RNA Pol II-transcribed) can be used to inhibit miR function [127].

## 3.2. MicroRNAs as therapeutic agents: miR replacement therapy

MicroRNA replacement therapy has emerged as a potential therapeutic strategy to enhance the level of miRs that are pathologically downregulated in IVDD. Here, we discuss several candidates for this enhancement.

Replacement therapy with mimics and agomirs is another miR therapeutic approach, primarily investigated in diseases involving reduced miR expression [128]. Synthetic miR mimics have the ability to

perform the natural regulatory roles of miRs. While miR mimics are double-stranded miRs that mimic the original miR, agomirs are very similar yet with more chemical modifications to enhance their stability [129]. Cancer is a perfect example of pathologies involving miR downregulation, as tumor-inhibiting miRs are downregulated, and hence require miR mimics to restore the miR balance and suppress the cancer [130]. Another approach for miR replacement is plasmid-based over-expression. Construction of a miR-expression plasmid involves cloning the sequence of interest into a plasmid with a suitable promoter, which once transfected will promote sustained expression of the miR [131,132]. Plasmids can also be successfully delivered with the help of nanoparticles until the miR loaded within is overexpressed [133,134]. Furthermore, precursor microRNAs (pre-miRs) can be also used for replacement therapy, as pre-miRs are chemically modified ssRNAs that are transformed into mature miRs after their cellular uptake [129,135]. Loading these pre-miRs within a nanoparticle can enhance their cellular uptake so that they can reach the cytoplasm and transform into the dysregulated miR of interest [136,137].



**Figure 6.** Nanomedicines investigated for IVDD treatment, administered locally, delivering either miR replacement therapeutics (via mimics, agomirs, pre-miRs, or plasmid overexpression) or miR-inhibiting therapeutics (antagomirs, antisense oligonucleotides (ASO), locked nucleic acids (LNAs), long non-coding RNAs (lncRNAs), or decoy miR sponges). PEG: poly(ethylene glycol), PEI: poly(ethyleneimine), PLGA: poly(lactic-co-glycolic acid), RD4: route to disk aptamer, and MMP: matrix metalloproteases

## 4. Translating the success of nanomedicine in nucleic acid delivery to the treatment of IVDD

As mentioned above, there is accumulating evidence that various miRs are dysregulated during IVDD. Hence, they could have huge potential as biomarkers, targets, or active principles against this debilitating condition. The way miRs can profoundly modulate signaling pathways and influence several cellular processes at once is a particularly promising approach to tackling this multifaceted disorder [12,15,138–140]. However, miR delivery encounters extracellular and intracellular barriers [141,142]. Extracellularly, they would be rapidly degraded by endogenous nucleases, and intracellularly, their uptake would be hindered as a result of their relatively high molecular weight, hydrophilicity, and negative charge [141,143–145]. A promising technique to address this challenge is based on exploitation of the potential of nanomedicines; to successfully load nano-vectors with miRs, whether encapsulated inside or bound to the nanoparticles, thereby providing both protection from degradation and enhancing their uptake by the few targeted cells of the degenerated IVD [146–148].

Nanomedicine employs nanoparticles, i.e., particles for which one of their external dimensions is less than a micrometer, as carriers for nucleic acids or other intended therapeutic cargos. These nanoparticles act as protective vehicles, shielding the nucleic acids from degradation and facilitating their entry into cells [149]. Various types of nanoparticles have been explored for non-viral nucleic acid delivery, including organic (polymeric or lipidic) [145–148,150,151], inorganic [152,153], and metal-organic framework nanoparticles (MOFs) [154]. More specifically for RNAs, many of these particle types have proven to be successful both *in vitro* and *in vivo*, paving the way for the first FDA approval of the first RNA-interference-based nanoparticles; patisiran [155,156]. Patisiran, sold under the brand name Onpattro®, is made of siRNA-loaded liposomes for the treatment of polyneuropathy, a fatal rare disease that affects approximately fifty thousand people worldwide [155,156]. Another leap forward for RNA-based nanomedicine was the FDA approval of lipid nanoparticle mRNA vaccines, which are now in clinical use against COVID-19, highlighting a cornerstone for both the fields of nanomedicine and RNA therapeutics [157,158]. Moreover, for these successful examples to be approved, they had to overcome the challenges facing all non-viral gene delivery systems.

### 4.1. Nanomedicine general challenges

Despite its promise, nanomedicine for nucleic acid delivery still faces significant challenges [141,143,159–161]. These include (i) the extracellular environment: nanoparticle materials and surfaces, if not well designed, can be rapidly tagged by the reticuloendothelial system (RES), (ii) cellular uptake and intracellular trafficking: nanoparticles must have an optimal size, surface charge, and corona to be effectively taken up by the cells, and if the uptake pathway is endosomal-dependent then (iii) endosomal escape is necessary to deliver their nucleic acid cargo to the desired target site, namely the cytoplasm [141,143,159–161], and (iv) off-target effects: as nanoparticles can accumulate in non-target tissues, potentially causing unintended side effects, especially if they belong to earlier generations of nanomedicines or if the cargo is a material that affects multiple targets simultaneously (such as miRs) [16,18]. However, both RES detection and off-target effects apply primarily to systemic administration of nanoparticles and do not apply to local administration of nanoparticles into their intended site of action, unless leakage to the systemic circulation is involved.

These challenges are critical for the ultimate safety and efficacy of nanoparticles, and although these require experience and knowledge to be solved, these hurdles can be overcome using the right techniques [143,146,162]. Using biocompatible materials and formulations, and decoration of their surfaces based on their intended delivery site and administration route helps eliminate nanoparticle

immunogenicity and decreases unintended off-target effects. Moreover, tuning the size, dispersity, surface charge, and surface functionalization of nanomedicines improves their cellular internalization. Regarding endosomal escape, many polymeric and lipidic nanoparticles have proven their ability to achieve successful endosomal escape, either by the proton sponge effect or the umbrella hypothesis for polymers or by lipid nanoparticles topology and the ability to destabilize the endosomal membranes in numerous ways [163–170]. Finally, to decrease their off-target effects, again, surface functionalization can increase the uptake into the targeted cells [171,172]. However, in some cases, local injection at the intended site of action is indeed necessary, as is the case with some tumors and intervertebral disc degeneration (IVDD) [173–175].

#### 4.2. IVDD microenvironment, specific challenges, and opportunities with nanomedicine

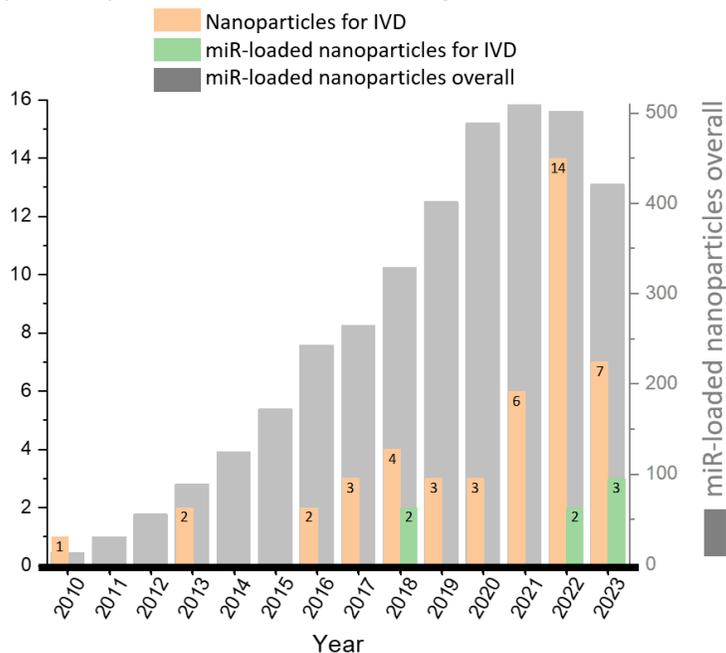
The IVD has a harsh microenvironment that impedes drug, gene, and even nanoparticle delivery. Moreover, pathologically, IVDD exhibits features such as increased degrading enzymes (e.g., MMPs), interleukins, phenotype changes, and apoptosis of *nucleus pulposus* (NP) cells, along with a decrease in ECM integrity [171,173,176,177]. This challenging microenvironment is characterized by (i) avascularity: degenerated IVD lacks direct blood supply to NP cells, leading to an acidic hypoxic nutrient-depleted environment that compromises the distribution and therapeutic efficacy of systemic interventions, although this avascularity can also be an advantage if local delivery is intended, as it helps maintain the dose in the injected region [171,173,176,177] and (ii) endplate barriers, which hinder the penetration and distribution of systemic nanoparticles into and within the disc, thereby compromising their effectiveness [47,178–180]. Additionally, the specific location and nature of IVDD also imposes challenges such as (i) inflammation and damage risk, as frequent injections are often required to maintain therapeutic levels of drugs at the IVD, which can damage the disk and can even cause rupture of the AF, resulting in safety concerns and affecting patient compliance [180–183] and (ii) central nervous system side effects, due to a tendency for leakage of drugs after direct injection [180,184,185]. Moreover, there are some general challenges that affect all cells, including the NP cells of the IVD, such as (i) clearance, compared to non-vectorized molecules that will indeed be rapidly cleared, nanoparticles injected *in situ* can still be cleared, thereby reducing the time they are available to be effectively taken up by the target cells, thus limiting their potential therapeutic effect [176,179,181] and (ii) cellular uptake, compared to free nucleic acids, which are hydrophilic, negatively charged, and with a relatively high molar mass, which greatly reduces their uptake [138,142], nanoparticles have more potential for uptake. However, a degree of morphological optimization and surface decoration can greatly increase uptake compared to other nanoparticles that are not functionalized [28,171,172].

Regarding the avascularity and the endplates, these challenges can be overcome by a local administration of the nanomedicines directly into the NP tissue, thereby avoiding the obstacles encountered with systemic administration discussed above [173,177,180]. Moreover, the acidic pH can provide an opportunity for smart formulations that can be pH-responsive, with release kinetics dependent on pH [186]. Surface-optimized nanoparticles can both protect the nucleic acid delivered and increase its homing to the targeted NP cells, via aptamers, peptides, or antibodies that have been proven to increase uptake in NP cells [171,187–190], or by exploitation of the pathophysiology of IVDD by targeting MMP or ferroptosis (iron-dependent programmed cell death), or by utilizing external-facilitated targeting such as ultrasound [191]. This can increase the uptake either directly by promoting interaction with cell receptors or indirectly by increasing the available interaction time between nanomedicines and NP cells. Nanoparticles can also be optimized to achieve sustained release of therapeutic moieties, thereby avoiding the need for frequent injections [182,192,193]. Additionally, the gradual release of these nanomedicines from a scaffold or a hydrogel can increase their residency time in the affected area and

can even help regain some of the mechanical properties that the IVD has lost [182,186,192,194–196]. Actively targeted nanoparticles and hydrogels can be the most suitable approaches to prevent leakage in the injection site.

To summarize, nanomedicine offers a huge potential for IVDD therapeutics. Most nanomedicines already investigated for IVDD are nontoxic, biocompatible, and biodegradable, and some can even induce microenvironment regeneration [145,146,186]. Moreover, nanoparticles have the ability to bypass cellular barriers, thereby increasing their intracellular load and enhancing their retention. Last but not least, many nanomedicines have the capacity for sustained release of their load, which is not necessarily limited to one miR or drug [197,198], thereby extending the duration of the effective drug concentration and decreasing or completely negating the need for frequent administration, which eventually leads to effect enhancement per dose, fewer side effects, and better patient compliance. The natural materials currently used for nanomedicine formulations have been shown to provide a more favorable cellular microenvironment for IVDD repair, symptom relief, functional improvement, and subsequent treatment. This research can provide safer, more effective, and less invasive treatment for IVDD than conventional surgical intervention.

The use of nanomedicine for drug delivery to treat IVDD is still in its infancy. To the best of our knowledge, 90% of the relevant studies were only published after 2017, as can be seen in **Figure 7**. Many nanoparticles have been investigated for the treatment of IVDD, including: polymeric nanoparticles [171–173,175,186,191,192,199–205], lipid-based nanoparticles [55,197,206,207], inorganic nanoparticles [177,208–217], and injectable hydrogels or scaffolds that can incorporate these nanoparticles [182,192–196,202,218–220], many of which have been covered in the recent work of Liu *et al.* [180]. A huge gap exists, however, between the advancement of miR-loaded nanoparticles in IVDD compared to other diseases, notably cancer. This can be attributed to the specificity of IVDD as the primary cause of low back pain, versus cancer, which comprises a multitude of different tumor types, locations, and complexities. Hence, this comparison should be seen in a positive sense, as the huge success and potential of miR-based nanomedicines therapeutically is now in dire need of being translated for IVDD treatment.



**Figure 7.** Number of publications, based on PubMed, of nanoparticles (i) intended for IVDD irrespective of their cargo, (ii) miR-loaded nanoparticles intended for IVDD, and (iii) miR-loaded nanoparticles irrespective of their use. These numbers include work on inorganic nanoparticles and nanohydrogels.

### 4.3. Therapeutic potential of microRNA-loaded nanoparticles investigated for IVDD

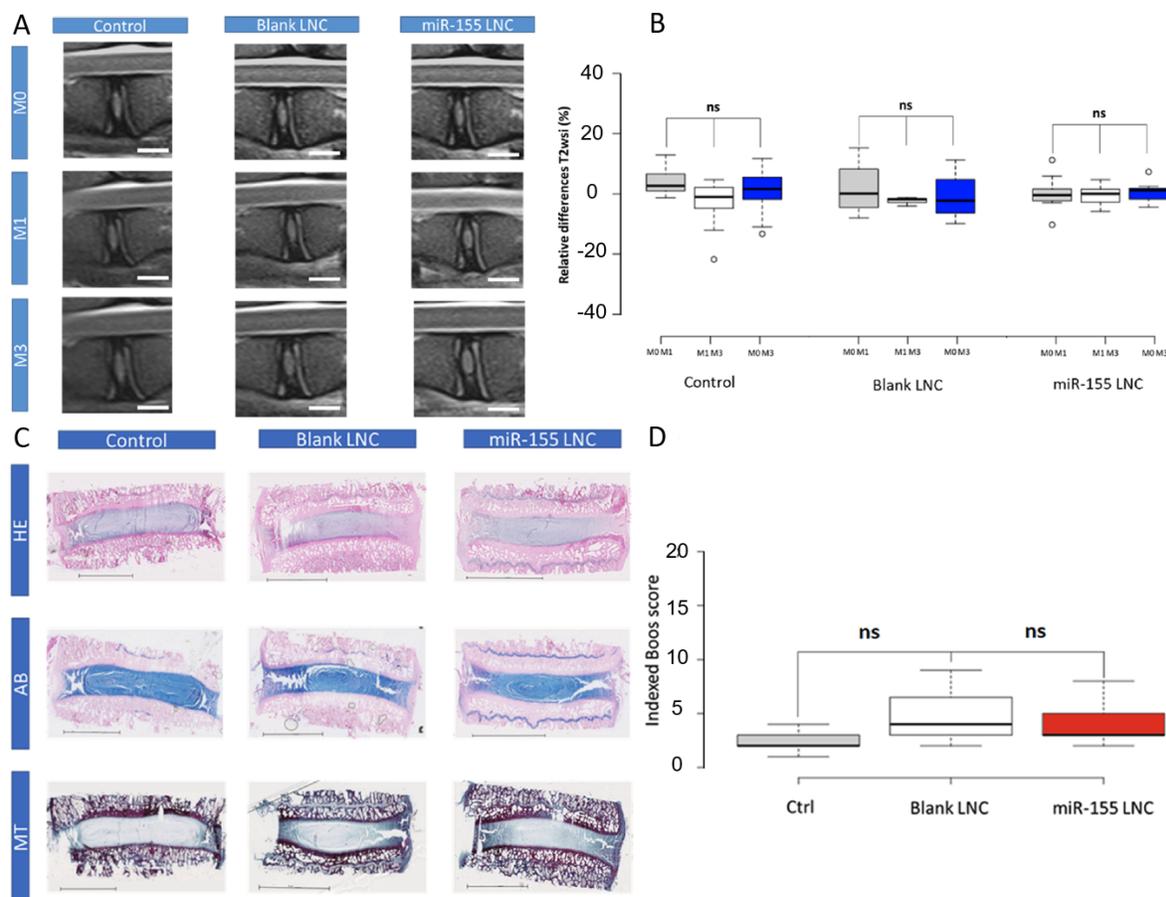
As shown in **Figure 7** and **Table 1**, since the beginning of nanomedicine in the 1970s [17] and RNA interference in the 1990s [221], only seven publications have investigated synthetic organic nanoparticles, three of which were incorporated within scaffolds or hydrogels, to deliver miR therapeutics for IVDD treatment. Two of these nano-objects were formulated using lipid-based vectors, four were polymeric, and only one of the seven can be considered to be a hybrid organic/inorganic formulation given their use of both poly(ethyleneimine) (PEI) and iron oxide nanoparticles, **Figure 6**. Two of these nanomedicines were incorporated into injectable hydrogels and one was incorporated into a polymeric scaffold.

#### 4.3.1. Lipid-based nanoparticles

Lipid-based nanoparticles, including liposomes, lipid nanocapsules (LNCs), and stable nucleic acid–lipid nanoparticles (SNALPs), are promising candidates for nucleic acid delivery in light of their general profile regarding safety and efficacy proven in the field [16,147,151,197,222]. They increase the safety profile of their cargo and they are generally considered safe for gene delivery, as lipids constitute the main components of cell membranes, making them able to promote membrane interactions and successful cellular uptake of their components [223,224]. Another critical aspect for the success of these delivery systems in transfection is their ability to escape endosomes and have their cargo released in the cytoplasm, where it can then exert its therapeutic action [163,164].

For IVDD, our team has formulated lipid nanocapsules for miR-155 delivery to the NP cells by *in situ* transannular injection in healthy sheep, proving the safety and feasibility of such an approach, **Figure 8**. As discussed above, miR-155 is the most studied miR in IVDD, with documented downregulation in IVDD, making it an excellent candidate for this study. With sustained release over 48 hours, nearly 100% transfection efficiency, effective miR-protection, minimal cytotoxicity, and target gene downregulation, this formulation holds ample promise for IVDD treatment, although the *in vivo* efficacy is yet to be proven [206].

Another team has used a commercially available formulation (composed of neutral lipids, non-ionic detergent, oil, and small molecules) to deliver and investigate the role of miR-141 in IVDD. Comprehensively, both miR-141 mimics and inhibitors were used in this study, confirming their mechanisms of action while also showing efficacy. As blocking miR-141 *in vivo* may serve as a potential therapeutic approach for IVDD treatment, this work showed that miR-141 is a therapeutic target rather than a therapeutic agent [55]. This perfectly highlights the miR-equilibrium restoration strategy discussed above. Moreover, this practice not only proves the therapeutic efficacy but also provides a more in-depth understanding of the miR's role in IVDD.



**Figure 8.** Safety of LNC transannular injection in sheep IVD by (1) MRI and analysis of lumbar spines in T2wsi. MRI scans with T2wsi signal analysis were performed once a month for three months (M0 corresponding to MRI before injection). A total of 25 sheep IVDs were analyzed: 14 for the control, 5 for blank LNC treatment, and 9 for miR-155 LNC. (a) Each month, a magnetic resonance imaging (MRI) scan of the spine was performed. Representative MRI scans are shown. Bar = 3 cm. (b) Representative graph of the relative difference in T2wsi signal, as a percentage, after treatment. At each time point, there was no significant difference between the treated IVDs and the control. (2) Histological staining and Boos' scoring of sheep IVDs, three months after injection. (c) Representative sections of endplates three months after the injection of blank LNC or miR-155 LNC (70.3 mg/mL). A total of 25 IVDs were collected three months after injection under miR-155 LNC or control conditions. Hematoxylin-eosin (HE), Alcian blue (AB), and Masson's trichrome (MT) staining were performed according to the Materials and Method section. No signs of degeneration were observed compared to the control for both the blank and miR-155 LNC conditions. (Scale bar = 10 mm), (d) Indexed Boos' scores on the NP and AF were determined for the histological staining by three readers. Blank LNC and miR-155 LNC injections had no significant impact on the Boos' scoring. Adapted with permission from [206].

#### 4.3.2. Polymer-based nanoparticles

Many polymers have been investigated in the literature for nucleic acid delivery, and many have proven to offer characteristics that are favorable for gene delivery, such as biocompatibility, biodegradability, stimuli-responsiveness, and the ability to induce endosomal rupture or swelling for successful endosomal escape [145,148,166].

One of the most investigated polymers for gene delivery is poly(lactic-co-glycolic acid), or PLGA, which is an FDA-approved polymer composed of glycolic and lactic acid building blocks. PLGA is safe and can be hydrolyzed and broken down into nontoxic monomers that can then be metabolized by the body without causing any side effects [225]. Furthermore, PLGA nanoparticles can escape from the endo-lysosomal compartment to the cytoplasm, thereby resulting in sustained intracellular release of encapsulated agents and prolonged therapeutic efficacy [226]. Another extensively used polymer in the

practice of gene delivery is polyethyleneimine (PEI) which is often used as a transfection standard for comparison, as its very characteristic proton sponge effect that leads to successful endosomal escape is well recognized [166,168], although its toxicity due to cationic charges is a main drawback.

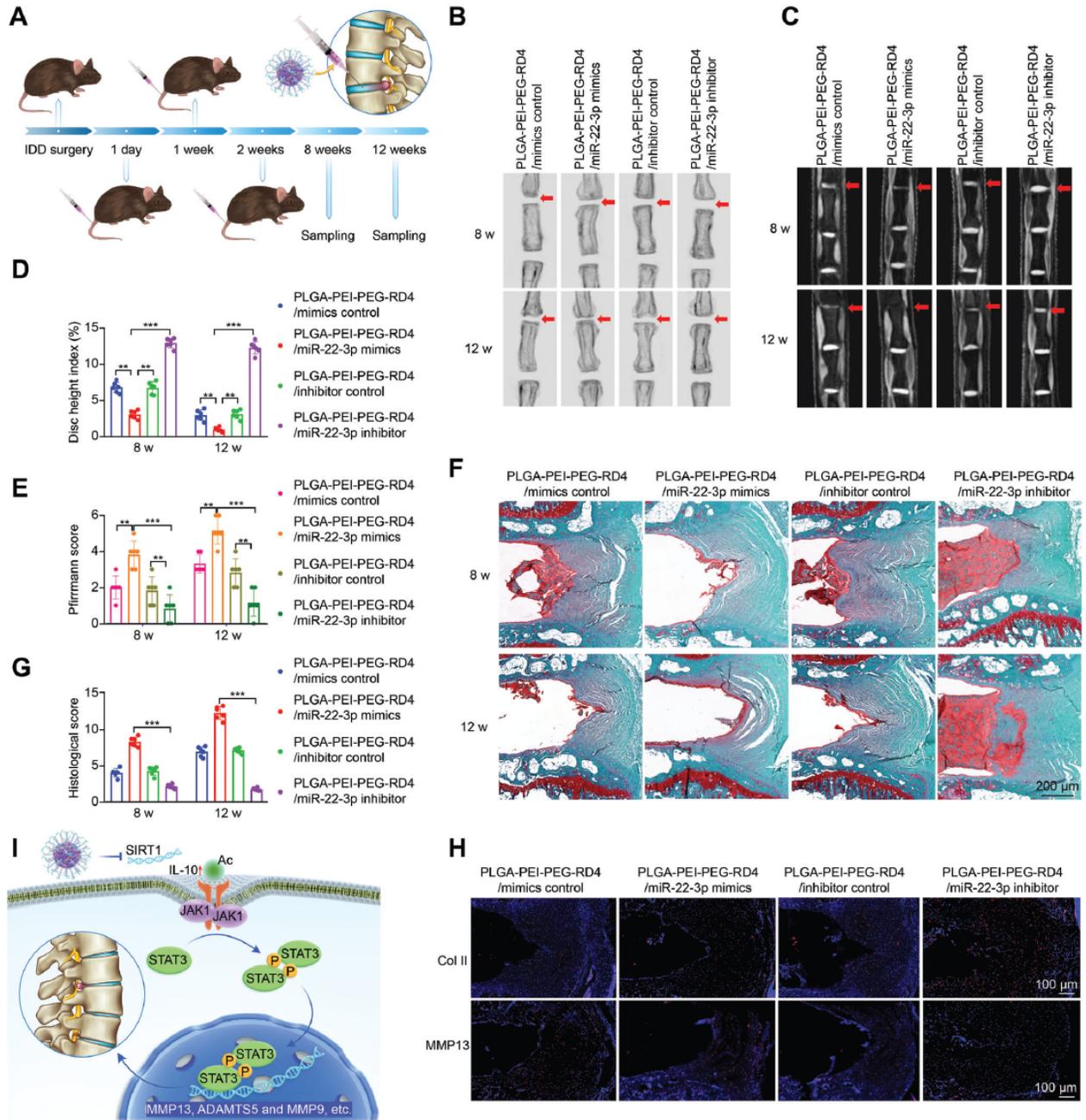
In light of these characteristics, one team has utilized both PLGA, PEI, and PEG to formulate a nanoplatform decorated with their designed IVD-targeted aptamer called RD4 to deliver miR-22-3p inhibitors and mimics for IVDD treatment. PLGA was used in this work for its known safe biodegradable biocompatible characteristics. On the other hand, PEI, which can be cytotoxic, was added to enhance the stability and uptake of PLGA nanoparticles. Hence, PEG was added not only to make stealthy nanoparticles but also to decrease their cytotoxicity. Furthermore, the aptamer used was shown to have an enhanced binding affinity compared to other candidates. Interestingly, in this same study, miR-22-3p was also confirmed to be a therapeutic target rather than a therapeutic agent, as its downregulation was shown to induce the proliferation and synthesis of NP cell matrix and its overexpression exacerbated IVDD by acting on the SIRT1/Interleukin-10/JAK1/STAT3 pathway (**Figure 9** and **Table 1**) [171].

Another article demonstrated the ability of polyphenolic nanoparticles to deliver antagomir-21 for IVDD treatment. Tannic acid (TA) nanospheres were incorporated into an injectable pH-responsive hydrogel (glycidyl methacrylate-modified carboxymethyl-chitosan (CMC-GMA)), taking advantage of the acidic microenvironment of IVDD, which has proven to be an effective strategy for sustained release of the nanoparticles from the hydrogels as well as the release of the antagomir itself from the nanoparticles. Aside from the ability of TA as a crosslinking agent for hydrogel synthesis, its well-known antioxidant anti-inflammatory properties have proven to be highly beneficial in terms of providing a suitable environment for the proliferation of degenerated NP cells by effectively scavenging reactive oxygen species (ROS). Furthermore, the antagomir restored the ECM metabolic balance, inhibited MMP, and promoted ECM regeneration [186].

In another attempt to exploit the harsh microenvironment of IDD, a team designed a two-stage matrix metalloprotease (MMP)-responsive delivery system. For this purpose, they utilized MMP-responsive cationic block copolymers, poly(ethylene glycol)-GPLGVRG-poly{N'-[N-(2-aminoethyl)-2-aminoethyl]aspartamide}-cholesteryl (PEGGPLGVRG-PAsp(DET)-Chole) (PGPC), to make polyplexes with miR-29a, which when injected *in situ* with CGPLGVRGC peptide and an 8-arm maleimide-terminated-star-PEG formed complex-encapsulated hydrogels *in situ*. Interestingly, this design allowed MMP-responsive dePEGylation to enhance cellular internalization. These systems owe their successful endosomal escape to the PAsp(DET) moieties capable of inducing the "proton sponge" effect, and also to hydrophobic interactions between the cholesterol moieties and endosomal membranes. Finally, this system was able to inhibit fibrosis and slow down or even reverse chronic IVDD[192].

Trying to take advantage of the favorable properties of polymers for gene delivery, some teams working with inorganic nanoparticles have started adding polymers to their systems to attain characteristics that their systems initially lacked in terms of surface modifiability and endosomal-escape capabilities. Thus, one research team used PEI to coat their miR-loaded iron oxide nanoparticles (IONPs) to enhance their intracellular uptake and exploit its ability to activate the "proton sponge" effect, facilitating endosomal escape. These systems also utilized polycaprolactone/hyaluronic acid (PCL/HA) scaffolds to obtain a tissue-engineered bone that successfully achieved interbody fusion [208].

In conclusion, nanomedicine has been shown to have great potential for miR delivery for IVDD treatment, whether by protection of miR, increasing cellular uptake, decreasing side effects, releasing miR in a sustained manner to avoid frequent dosing, or even special characteristics such as decreasing inflammation, fibrosis, and ROS scavenging. However, what this work needs now is preclinical confirmation in large animal models and demonstration of clinical safety and efficacy in humans.



**Figure 9.** In situ injection of nanoparticles loaded with miR-22-3p inhibitor decreased the development of IVDD in an IDD model of male mice (C57BL/6, 12 weeks old). A) The experimental design. B,C) Disc degradation, evaluated by micro-CT and MRI. D,E) Disc height index and Pfirrmann score at 8 and 12 weeks with different nanoparticles. F) The group receiving PLGA-PEI-PEG-RD4/miR-22-3p inhibitor exhibited an increased number of NP cells, as assessed by Safranin O staining. G) Histology scores. H) Levels of MMP13 and Col II in an IVDD model administered nanoparticles, as illustrated by immunostaining. Scale bar = 100  $\mu$ m. I) Graphical summary of the mechanisms by which miR-22-3p promotes the development of IVDD. Adapted with permission from [171].

**Table 1.** MicroRNA-loaded nanoparticles investigated for IVDD treatment

miR loaded	Formulation	Particle size and ZP	EE %	Release	<i>In vitro</i> model	<i>In vitro</i> outcome	<i>In vivo</i> model	Administration method	Outcome	Ref.
miR-22-3p inhibitors and mimics	PLGA-PEI-PEG-RD4 polymeric nanoparticles  RD4: route to disk aptamer (surface receptor IL-6R)	150 nm, +ve ZP (ca. +10 mV)	-	-	Primary human <i>nucleus pulposus</i> (NP) cells	-RD4 showed higher binding affinity to NP cells than three other ligands. - enhanced delivery of miR into NP cells. -no cytotoxicity. -successful endosomal escape after 10 h of transfection -miR-inhibitor-induced decrease in miR-22-3p promoted NP cell proliferation, unlike mimics. -miR inhibitor-treated NP cells overexpressed aggrecan and Col II while the expression of ADAMTS-5 and MMP13 declined. -SIRT1 expression significantly decreased in the mimic group.	Surgically induced IDD model on wild-type (WT) and miR-22-3p knockout (KO) mice	<i>In situ</i>	-RD4 functionalized nanoparticles signal remained for 3 months vs. 1 month for the RD4-free group. -increased cellular uptake No pathological abnormalities miR-22-3p inhibitor nanoparticles alleviated IVDD: 1. protective impact on the surgery-induced IDD model compared to the control. 2. reduced the expression of MMP13 and enhanced the expression of Col II in contrast to mimics-loaded nanoparticles.	Chen <i>et al.</i> 2023 [171]
Antagomir-21 (cholesterol-modified miR21 inhibitor)	Modified tannic acid (TA) nanoparticles incorporated into an injectable pH-responsive hydrogel (glycidyl methacrylate-modified carboxymethyl-chitosan)	260 nm, -9.5 mV, changing to -19 mV and -30 mV after loading with antagomir and SH-PEG-BA coating	ca. 94%	Sustained release of both TA nanoparticles with miR and the hydrogel with TA nanoparticles	L929 cell viability, NP cells	- inhibition of the MAPK/ERK signaling pathway regulated the ECM metabolic balance. -TA nanoparticles-loaded hydrogel made a suitable microenvironment for the proliferation of degenerative NP cells. - promotion of ECM regeneration, inhibition of matrix proteases, and decrease of inflammation via intracellular ROS scavenging.	IDD rat model	<i>In situ</i>	- restored disc height. - hydrogel was critical for sustained release and providing mechanical support for spinal stability. - synergistic ECM regeneration and anti-inflammatory impact effectively induced IVDD treatment.	Wang <i>et al.</i> 2023 [186]
miR-375	Polystyrene nanoparticles	40–100 nm	-	-	NP cells	-miR PS nanoparticles can inhibit inflammation by blocking the NF- $\kappa$ B pathway. -decreased proliferation and promoted apoptosis of inflammatory cells in the NP. -miR downregulation had the opposite effects	-	-	-	Zhang <i>et al.</i> 2022 [199]

miR-155	Lipid nanocapsules LNCs	75 nm, +10 mV	75%	Sustained release: 50% after 1 day, 80% after 2 days	Human adipose stromal cells (hASCs) & ovine NP cells	-minimal cytotoxicity -protection of the miR by the LNC from enzymatic degradation -successful internalization after 24 h -downregulation of ERK1/2	Healthy sheep	<i>In situ</i> (transannular)	-feasibility and safety of miR-155-LNC <i>in situ</i> injection, no significant change was observed 90 days after the injection (based on MRI, T2wsi, Boos' scoring)	Le Moal <i>et al.</i> 2022 [206]
miR-29a	MMP responsive cationic block copolymer (PGPC) Polyplex Micelle-Loaded Injectable Hydrogels	72 nm, +5 increasing to +22 mV after de-PEGylation		Sustained ≈ 40% released within 18 days at a low MMP concentration of 0.1 μg mL <sup>-1</sup> . At 1 μg mL <sup>-1</sup> MMP, > 90% released at day 12.	Rabbit NP cells (fibrosis inhibition, uptake)	-successful internalization. -high-efficiency endosomal escape. -downregulation of fibrotic genes including α-SMA and stress fibers induced by TGF-β.	Needle puncture method on rats for <i>in vivo</i> miR delivery & rabbits for fibrosis inhibition	<i>In situ</i>	-hydrogel can prevent undesirable leakage (signal maintained for a month).  -miR-nanoparticles-hydrogel potentially inhibited fibrosis and showed disc regeneration by blocking the β-catenin translocation pathway, reversing IVDD	Feng <i>et al.</i> 2018 [192]
miR-141 inhibitor or mimics	Nanocarrier system (MaxSuppressor™ <i>In vivo</i> RNA-LANCER II Kit & Lipid Extruder, BIOO Scientific)	-	-	-	Primary human NP cells	-upregulation or downregulation of miR exacerbated or alleviated experimental IDD, respectively. -miR promoted IDD progression by interacting with the SIRT1NF-kappa B pathway. - miR-141 inhibitor decreased NP cell apoptosis. -miR-141 overexpression dominantly decreased cell proliferation while inhibitor increased proliferation	IDD model (AF needle puncture) in miR-141 KO and WT mice	<i>In situ</i>	-blocking miR-141 <i>in vivo</i> protected discs from destruction.	Ji <i>et al.</i> 2018 [55]

## 5. Conclusion

Intervertebral disc degeneration (IVDD) is one of the main etiologies for lower back pain (LBP), which is a pervasive disease affecting nearly the entire human population at one point or another, thus posing a substantial societal and economic burden. Unfortunately, although IVDD-related LBP can be severely disabling, there is still no definite cure. Innovative therapies based on intradiscal injection of stem cells [227], platelet-rich plasma [228], TNF inhibitor [229], or a monoclonal antibody targeting the IL6 receptor [229], although feasible and safe, have failed to result in genuine regenerative effects beyond analgesia. Our comprehensive literature analysis highlights that a diverse array of miRs is dysregulated during IVDD, making them a potential therapeutic agent or target for the treatment of this intricate disorder. A possible therapeutic strategy may involve restoration of the equilibrium of the miR of interest, either by inhibition in cases of upregulation or replacement in cases of downregulation.

Unfortunately, direct administration of miR mimics or inhibitors locally into the IVD, while theoretically plausible, is practically unfeasible because of the extracellular and intracellular barriers these nucleic acid moieties face upon their injection, resulting in poor therapeutic efficiency, if any, thus necessitating development of a carrier that can protect miR therapeutics as well as enhance their cellular uptake and trafficking while also ensuring safety and minimal side effects.

Nanomedicine offers a promising platform for miR delivery in IVDD, with a plethora of nanoparticles of various biocompatible and biodegradable materials that can readily be tailored for specific cellular targeting; particularly the *nucleus pulposus* NP in the case of IVDD. Non-coding-RNA loaded nanoparticles have already been quite well investigated, leading to the first FDA-approval of the liposomal siRNA formulation patisiran. Moreover, there are currently 167 clinical trials taking place that involve nanoparticles, 31 of which are dedicated to gene delivery, primarily for vaccination.

Although no nanomedicine formulation has been investigated clinically for IVDD to date, nanoparticles have proven their potential in IVDD for miR protection, enhanced NP cellular uptake and transfection, targeted localization decreasing adverse effects, sustained release (ensuring less frequent dosing, fewer side effects, and better compliance), and providing an anti-inflammatory, antioxidant, biocompatible microenvironment for the cells while exploiting the harsh acidic inflammatory environment for stimulus responsiveness to either pH or enzymes. Incorporating these nanoparticles into injectable biocompatible hydrogels has proven advantageous for ensuring sustained release and more therapeutic time in the damaged area while also retaining some of the mechanical attributes lost due to IVDD.

Determination of the optimal nanoparticle type for localized miR therapeutics delivery remains a topic of active discussion, which highlights the diverse array of options available in this actively growing field. Indeed, both polymer- and lipid-based nanoparticles have demonstrated great potential for IVDD treatment, and an opinion given in haste may be far from the truth. Our perspective is that within both categories of organic nanomedicines, there are various excipients, formulation processes, and potential candidates for miR delivery to NP cells. Hence, to juxtapose one candidate from one category with another, whether successful or not, fundamentally contradicts one of the main pillars of nanomedicine: which is to reject the notion of one-size-fits-all. To that end, lipid-based nanoparticles for RNA delivery have so far performed very well on the FDA approval front. On the other hand, polymer-based nanoparticles happen to have been investigated more for IVDD owing to their favorable characteristics of biocompatibility, biodegradability, controlled-release possibility, and surface functionalization potential, yet lipid-based nanoparticles also have these advantages. It could hence even be argued that a hybrid system of both lipid and polymer materials may offer “the best of both worlds”, although clinical proof is still pending.

Nevertheless, we believe the optimal approach for the treatment of IVDD is locally injectable hydrogels that further encapsulate lipid-based nanoparticles, surface functionalized with bioactive molecules, and loaded with miR mimics, inhibitors, or a combination thereof within the same formulation. This intricate strategy is based on both the existing literature and our view of the recent clinical advancements in lipid-based nanoparticles. On one hand, hydrogels offer 3D networks characterized by high biocompatibility and the ability to encapsulate various therapeutics including drugs, cells, bioactive molecules, nanoparticles, or even exosomes. Mimicking the ECM and exhibiting tunable permeability, these hydrogels enable the transfer of nutrients, oxygen, and bioactive molecules within the IVD. Moreover, their ability to swell within predefined dimensions and withstand certain levels of pressure helps maintain the mechanical integrity of the IVD, partially regaining some attributes lost due to IVDD. In addition, hydrogels can be tailored to respond to the microenvironment of IVDD allowing a tunable sustained release profile, a crucial feature in managing this pathology. However, hydrogel intradiscal injection still faces the challenge of potential damage to AF integrity upon injection and ensuring precise delivery [182,192–196,202,218–220,230,231]. On the other hand, lipid-based nanoparticles, particularly LNCs, were the only particles investigated and proven to be safe for intradiscal injection in the IVD of sheep, a large animal model that closely resembles human IVDD in terms of NP content and mechanics. Additionally, they were investigated for the treatment of IVDD in chondrodystrophic dog breeds [232]. Furthermore, the extensive research, regulation, and scalability of lipid-based nanoparticles for nucleic acid delivery, exemplified by the FDA approval of COVID-19 vaccines and Patisiran, highlight their translational potential. Attributes such as safety, colloidal stability, lyophilization feasibility, and scalability, widely validated through vaccine production, position lipid-based nanoparticles as a benchmark for clinical translation in intradiscal injection, whether for veterinary or human applications [206,222–224,233–241]. Finally, the selection of miR mimics and inhibitors as the primary cargo is most advantageous in addressing the complex multifaceted nature of IVDD. As discussed throughout the article, IVDD necessitates therapeutic molecules capable of targeting various pathophysiological pathways and mechanisms. To sum up, the integration of these intricate choices of miR as the therapeutic moiety encapsulated, lipid-based nanoparticles, their surface decorative bioactive molecules, and the responsive, injectable, and permeable nature of the hydrogel hosting this system, all together represent a synergistic approach to enhance therapeutic efficacy, providing safer and more reliable treatments of IVDD.

Collaborations among diverse scientific disciplines, whether academic or industrial, are crucial for achieving a high-throughput analysis of a selection of therapeutic miR agents (or targets) coupled with a systematic study of multiple formulation designs and their preclinical testing. Such cooperative efforts can be expected to lead to the design of an optimal miR-loaded nanoplatform for the treatment of IVDD.

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