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MicroRNA-146a reduces IL-1 dependent inflammatory responses in the intervertebral disc

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Abstract

Because miR-146a expression in articular chondrocytes is associated with osteoarthritis (OA), we assessed whether miR-146a is linked to cartilage degeneration in the spine. Monolayer cultures of nucleus pulposus (NP) cells from the intervertebral discs (IVD) of bovine tails were transfected with a miR-146a mimic. To provoke inflammatory responses and catabolic extracellular matrix (ECM) degradation, cells were co-treated with interleukin-1 (IL-1). Transfection of miR-146a decreases IL-1 induced mRNA levels of inflammatory genes and catabolic proteases in NP cells based on quantitative real-time reverse transcriptase PCR (qRT-PCR) analysis. Similarly, miR146a suppresses IL-1 induced protein levels of matrix metalloproteinases and aggrecanases as revealed by immunoblotting. Disc segments from wild type (WT) and miR-146a knockout (KO) mice were cultured ex vivo in the presence or absence of IL-1 for 3 days. Histological and immunohistochemical (IHC) analyses of disc organ cultures revealed that IL-1 mediates changes in proteoglycan (PG) content and in-situ levels of catabolic proteins (MMP-13 and ADAMTS-5) in the nucleus pulposus of the disc. However, these IL-1 effects are more pronounced in miR-146a

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Conflict of Interest

The authors declare that there are no conflicts of interest.

KO discs compared to WT discs. For example, absence of miR-146a increases the percentage of MMP-13 and ADAMTS-5 positive cells after treatment with IL-1. Thus, miR-146a appears to protect against IL-1 induced IVD degeneration and inflammation. Stimulation of endogenous miR-146a expression or exogenous delivery of miRNA-146a are viable therapeutic strategies that may decelerate disc degeneration and regain a normal homeostatic balance in extracellular matrix production and turn-over.

Keywords

Intervertebral disc degeneration; miR-146a; IL-1; MMP-13; ADAMTS-5; Nucleus pulposus

1. Introduction

The lifetime prevalence of back pain in the United States is 70–85%, with roughly 10–20% of the population experiencing chronic symptoms (Andersson, 1999). While the etiology of back pain is multifactorial, it has been associated with degeneration of the intervertebral disc (IVD) (Freemont et al., 2001; Mooney, 1989). Disc degeneration results from an imbalance between catabolic and anabolic genes expressed by chondrocytes residing in the nucleus pulposus (NP) (Nomura et al., 2001). Upregulation of pro-inflammatory cytokines and catabolic proteases can disrupt extracellular matrix (ECM) homeostasis of the disc and shift disc maintenance towards a degenerative and catabolic state. As a result, breakdown of ECM components, including collagen fibrils and aggregates of proteoglycans (PGs), occurs (Lee et al., 2009; Masuda et al., 2006).

MicroRNAs are key post-transcriptional regulators of gene expression that control proliferation, differentiation, inflammation, and a wide range of other biological processes (Bartel, 2004; Jovanovic and Hengartner, 2006; Selbach et al., 2008). miR-146a is one of the earliest microRNAs in cartilage that has been associated with osteoarthritis (Yamasaki et al., 2009). This miRNA acts as a negative feedback regulator of the inflammation response by targeting adapter protein, TRAF6 (TNF receptor-associated factor 6) and IL-1 receptor-associated kinase 1, which are crucial for proinflammatory signaling (Ahmad et al., 2007). Potential targets for miR-146a include genes involved in inflammation, oxidation, and apoptosis (Lu et al., 2010). The exact etiological mechanism by which miR-146a expression in chondrocytes contributes to OA is not clear. Li et al. (2012) proposed that expression of miR-146a in chondrocytes contributes to OA pathogenesis by diminishing the response to TGF- β by directly binding to the 3'UTR of Smad4. The resulting reduction in Smad4 levels diminishes the cellular responsiveness to TGF- β and increases chondrocyte apoptosis. However, our results support a model in which miR-146a plays a protective anti-inflammatory role in OA (Li et al., 2013, 2011). We have observed that miR-146a significantly suppresses extracellular matrix-associated pro-inflammatory mediators (TNF α , COX-2, iNOS, IL-6, IL-8, RANTS, TRPV1) in human knee joint chondrocytes and downregulates inflammatory cytokines in synovial cells. The apoptosis and inflammation related activities of miR-146a indicate that it can act as a potent regulator with diverse revealed biological functions in chondrocytes.

A limited number of studies (Wang et al., 2011; Ohrt-Nissen et al., 2013; Song et al., 2013; Liu et al., 2014; Yu et al., 2013; Zhao et al., 2014) have begun to address the roles of miRNAs in the pathogenesis of IVD, but there are no reports yet on the functional activity of miR-146a in spine tissue. Based on our previous studies on the effects of miR-146a in articular cartilage (Li et al., 2013, 2011; Ellman et al., 2012; Li et al., 2011), we hypothesize that miR-146a may have a protective effect in the pathogenesis of intervertebral disc degeneration. In this study, we investigated whether miR-146a controls production of IL-1 responsive inflammatory cytokines and/or catabolic proteinases and inflammatory cytokines in primary NP cells in vitro. We also examined miR-146a in disc organ cultures ex vivo in which the combined integrity of the nucleus pulposus and annulus fibrosus continues to support physiological mechanisms based on their native chemical and physical boundaries (Korecki et al., 2007; An and Masuda, 2006; Risbud et al., 2003). Ex vivo disc organ cultures permit evaluation of IL-1 in the absence of far more complex and confounding inflammatory responses that are operative in vivo. Our main finding is that miR-146a inhibits sthe would cause a higher response to IL-1. These studies will help us determine if inducing the expression of miR-146a or providing it exogenously may serve as a potential treatment strategy to slow disc degeneration and shift homeostasis away from catabolism.

2. Material and methods

2.1. IVD cell isolation and culture and transfection

After dissection of the NP tissues of the discs from bovine tails, NP cells were released by subsequent enzymatic digestion with 0.2% pronase and 0.05% collagenase P, as previously described (Kim et al., 2010). Isolated NP cells from bovine tails were counted and plated onto 12-well plates at 8×10^5 cells/cm² and cultured as monolayers as previously described (Im et al., 2003). Cells were transiently transfected with miR-146a mimic (pre-microRNA mimics, Applied Biosystems) using Lipofectamine plus (Invitrogen, Carlsbad, CA), and control transfections lacking miR-146a (Li et al., 2011). After 24 h, cells were stimulated with IL-1 (10 ng/mL) in serum-free medium for 24 h at 37 °C under 5% CO₂. Supernatants were collected 24 h after the initiation of each treatment and subjected to western blotting analyses of secreted matrix-degrading enzymes. Cells were harvested and prepared for either cell lysates or total RNA extraction followed by real-time qPCR analyses, as previously described (Li et al., 2008).

2.2. Real-time qPCR (quantitative polymerase chain reaction)

Total RNA was isolated from cells using Trizol reagent (Invitrogen, Carlsbad, CA) following the instructions provided by the manufacturer. Reverse transcription was carried out with 1 µg total RNA using the ThermoScript™ RT-PCR system (Invitrogen, Carlsbad, CA) for first strand cDNA synthesis. For real-time qPCR, cDNA was amplified using the MyiQ Real-Time qPCR Detection System (Bio-Rad Hercules, CA). Synthesized cDNAs were subjected to real-time qPCR utilizing the Bio-Rad iQTM SYBR Green supermix (Bio-Rad Hercules, CA). A threshold cycle (C_T value) was obtained from each amplification curve using iQ5 Optical System Software provided by the manufacturer (Bio-Rad Hercules, CA). Relative mRNA expression was determined using the C_T and calculated as follows: $C_T = C_T(\text{gene of interest}) - C_T(\text{endogenous control})$. Gene quantification was

conducted for each treatment group and expressed as fold change relative to untreated control levels. Expression of 18 s rRNA was used as the internal control. The standard deviation of gene expression values is derived from 3 different sets of bovine tails in 3 separate experiments. The primer sequences are summarized in Table 1.

2.3. Immunoblotting

Cell lysates were prepared using a modified RIPA buffer, as previously described (Li et al., 2008). Total protein concentrations of cell lysates and culture-media were determined by a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). Equal amounts of protein (30 µg protein per lane) were resolved in 10% SDS-polyacrylamide gels (SDS-PAGE) and transferred to a nitrocellulose membrane for immunoblot analyses, as described previously (Jovanovic and Hengartner, 2006). Immunoreactivity of antibodies against TRAF6 (Thermo Scientific, Rockford, IL, USA), ADAMTS-4 (Affinity BioReagents, Golden, CO, USA), ADAMTS-5 (Millipore, Billerica, MA, USA), and MMP-13 (provided by Dr. Gillian Murphy, Cambridge University, Cambridge, UK) was visualized using the ECL system (Amersham Biosciences, Piscataway, NJ, USA) and the Signal Visual Enhancer system (Pierce) to magnify signal intensity.

2.4. Ex vivo analysis using miR-146a KO mice lumbar disc organ culture model

We obtained miR-146a KO mice from Jackson Laboratories (Bar Harbor, ME, USA). Lumbar spine segments from deceased mice (7 months old, eight WT mice and eight miR-146a KO mice) were dissected under sterile conditions within 12 h after sacrifice at the Rush University animal facility for ex vivo organ culture as previously described (An and Masuda, 2006; Ellman et al., 2012). Lumbar discs were then separated and incubated in DMEM/Ham's F-12 medium supplemented with 1% mini-ITS. After 24 h, discs were cultured in the absence or presence of IL-1 (100 ng/mL) for 3 days.

2.5. Histological analysis

Harvested discs were fixed in 4% paraformaldehyde and decalcified in EDTA solution, which was changed every 5 days. The decalcified discs were paraffin embedded. Serial disc sections of exactly 5 µm thickness were obtained to prepare slides. Alcian blue hematoxylin/orange G staining was used to assess general morphology and the loss of PG in the discs as previously described (Flick et al., 2003). Briefly, deparaffinized, rehydrated sections were immersed in 1% acid alcohol for 1 min. Slides were then stained in Mayer's hematoxylin solution containing 1% alcian blue (Sigma) for 30 min at room temperature followed by differentiation in acid alcohol. The slides were immersed briefly in 0.5% ammonia water, followed by extensive washes in distilled water and one rinse in 95% ethanol. Counterstaining was performed with eosin Y solution containing 0.67% phloxine B and 0.58% orange G for 1 min and 30 s, followed by three rinses in 95% ethanol. Specimens were then dehydrated, cleared in xylene, and coverslips were added. To assess tissue degeneration, each disc level was evaluated in 3 nonadjacent zones per spine (200 µm separation) (Boyd et al., 2008), and relative histological analysis was evaluated by two blinded independent investigators as previously reported (Boos et al., 2002).

Immunohistological analyses of catabolic factors (MMP-13 and ADAMTS-5) were performed using the streptavidin–biotin peroxidase complex method. Briefly, paraffin was removed from the sections using xylene and rehydrated through alcohol gradient and distilled water. Antigen retrieval was performed by microwaving in 0.01 M sodium citrate buffer (pH 6.0) at 96–100 °C for 15 min. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol for 20 min, and endogenous biotin was blocked with 10% non-immune normal goat serum for 30 min. The sections were then incubated with rabbit polyclonal antibody against MMP-13 (1:1,000 dilution, Oncogene, Pittsfield, MA, USA), or rabbit polyclonal antibody against ADAMTS-5 (1:1,000 dilution, Millipore, Billerica, MA, USA), overnight at 4 °C. Using Vectastain Elite ABC Kit (Vector laboratories, Burlingame, CA, USA), secondary antibody and other reagents were applied according to the manufacturer's protocol. Sections in which the primary antibody was omitted were used as negative controls. Two blinded investigators were responsible for counting the immunostaining-positive cells in the samples, with a Nikon TE200 inverted microscope (Nikon Co, Japan). The positive cells were assessed by point counting through a microscope using a 40× objective lens and a 10 × subjective lens. Numbers of positive cells were separately counted in two randomly chosen slides for each disc. The percentage of positively stained cells was calculated by dividing the number of stained cells with the total number of cells and multiplied by 100% as previously described (Zhao et al., 2008).

2.6. Statistical analysis

Analysis of variance was performed using StatView 5.0 software (SAS Institute, Cary, NC). All results are expressed as mean values $\pm$ SD. Data were analyzed by one-way ANOVA with a post-hoc Tukey's test using 2nd values of each sample. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Effect of transfection of miR-146a in bovine NP cells

To assess the biological role of miR-146a in the intervertebral disc, bovine NP cells were cultured in monolayer and transfected with synthetic miR-146a (or sham as a negative control). The cells were then treated with IL-1 (10 ng/mL) for 24 h to provoke a catabolic response (Li et al., 2011). The presence of IL-1 (10 ng/ml) markedly increased the mRNA levels of collagenases and aggrecanases, such as ADAMTS-4, ADAMTS-5, MMP-3, and MMP-13, as has been observed previously (Eger et al., 2002). Transfection of miR-146a (10 nM) suppressed the up-regulation of IL-1 induced collagenases and aggrecanases ($p < 0.05$) (Fig. 1A–D). Immunoblot analyses revealed that protein levels of aggrecanases (ADAMTS-4 and ADAMTS-5) and collagenase (MMP-13) also decreased after transfection of miR-146a and treatment with IL-1. (Fig. 2).

Next, we evaluated the biological effects of miR-146a on inflammatory related genes. The presence of IL-1 significantly stimulated cyclooxygenase-2 (COX2) and IL-6, as previously reported in articular chondrocytes (Bartel, 2004). Upon transfection of miR-146a at a concentration of 10 nM, the expression levels of inducible nitric oxide synthase (iNos), IL-6,

tumor necrosis factors- α (TNF- α), and COX2 were significantly suppressed by miR-146a (Fig. 1E–H).

Other studies have shown that miR-146a down-regulates TNF receptor-associated factor 6 (TRAF6), an essential factor that mediates receptor signaling in response to ligands of the TNF α superfamily (Pauley et al., 2008). This miR-146a mediated down-regulation of TRAF6 protein levels may decrease cytokine signaling through a negative feedback regulation loop (Taganov et al., 2006). Although we did not observe any significant differences in the level of TRAF6 mRNA in miR-146a transfected cells compared to control cells after IL-1 treatment (Fig. 1I), western blot analysis showed that TRAF6 protein level was significantly suppressed by miR-146a (Figs. 2,3). These experiments indicate that miR-146a can suppress IL-1-induced production of catabolic proteinases and inflammatory cytokines in vitro.

3.2. Histological analysis using an ex vivo organ cultures of miR-146a KO mice

Given the in vitro results obtained above with cultured cells, we sought to elucidate potential physiological effects of the absence of miR-146a in an organ culture model. Considering the complexity of microRNA functions (Yekta et al., 2008; Bartel, 2009), which have been shown to depend on stage of development, disease state and age, we performed studies with discs from mice in which the function of miR-146a is genetically ablated (Skarnes et al., 2011) to provide definitive evidence for the specific physiological roles of miR-146a in spine.

Lumbar spine discs of WT and miR-146a KO mouse were dissected and cultured ex vivo for 3 days in the presence or absence of IL-1 (100 ng/mL), as described previously (Ellman et al., 2008, 2013). To assess general morphology and tissue generation, we performed alcian blue hematoxylin/orange G staining to monitor loss of PG in the discs. The results show that in the absence of external stimuli, GAG content was not significantly different between discs from wild type (WT) and miR-146a knockout (KO) mice. In contrast, when treated with IL-1, GAG content was significantly decreased in miR-146a KO disc compared to WT (Fig. 4). These results suggest that miR-146a expression in the spinal disc genetically mitigates PG loss by preventing the degenerative effects of the inflammatory cytokine IL-1.

Using immunohistochemistry, we monitored protein expression of MMP-13 and ADAMTS-5 in disc organ cultures. After IL-1 treatment, we observed significantly higher expression of both MMP-13 and ADAMTS-5 in discs from miR-146a KO mice than those from WT mice (Fig. 5). This result suggests that loss of miR-146a function renders mice more vulnerable to degeneration by IL-1 treatment. We also calculated the percentage of MMP-13 and ADAMTS-5 positive cells in both groups. After IL-1 treatment, discs from mice with the miR-146a null mutation had significantly higher number of positive cells than those from WT mice. Yet, in untreated discs, there were no noticeable differences in expression of either MMP-13 or ADAMTS-5 between discs from miR-146a KO and WT mice (Fig. 6). We conclude that miR-146a protects mice from inflammation-mediated disc degeneration.

4. Discussion

Results from this study indicate that miR-146a suppresses IVD degeneration by down-regulating the inflammatory cytokine response. Evidence supporting this conclusion is based on studies using cultured bovine tail NP cells in which we elevated miR-146a levels by transfecting a miR-146a mimic, as well as ex vivo organ culture studies comparing discs derived from mice in which the miR-146a gene is constitutively ablated with those from WT mice. Regarding the mechanism of action by which miR-146a prevents IVD degeneration, our results show that miR-146a significantly decreases the IL-1-mediated induction of ECM catabolic and inflammatory genes/proteins in bovine tail disc NP cells. Furthermore, genetic loss of miR146a function results in a higher response to IL-1 in ex vivo disc organ cultures, concomitant with a greater loss in PG content and higher expression of catabolic proteins in discs. These results are corroborated with in situ immunohistochemistry results showing that the MMP-13 and ADAMTS-5 positive cells are each significantly higher in miR-146a KO mice compared to WT mice after IL-1 stimulation. Taken together, these findings suggest that miR-146a may protect discs from PG loss that can be induced by inflammatory cytokines such as IL-1, and that miR146a is critical for maintaining fidelity of disc matrix homeostasis.

The major new finding of this study that miR-146a has anti-inflammatory and anti-catabolic effects on IVD suggests that enhancing of miR-146a expression in degenerative disc may have potential therapeutic benefits. The anti-catabolic role is revealed by the result that transfection of miR-146a suppresses IL-1-mediated induction of ECM degrading enzymes, including MMP-13, ADAMTS-4, and ADAMTS-5 in bovine NP cells. We also observed that miR-146a significantly suppresses the induction of TRAF6 protein levels by IL-1. Because miR146a did not reduce TRAF6 mRNA levels, it appears that miR-146a may control TRAF6 protein translation and/or degradation. Experimentally elevating miR-146a levels also suppresses the IL-1 upregulation of COX2, IL-6, iNOS and TNF- α , and these results are further indicative of its anti-inflammatory and anti-oxidative effects in the intervertebral disc. Finally, IL-1-mediated PG loss and expression of MMP-13 and ADAMTS-5 was more intense in miR-146a KO mouse discs than in WT discs. Because our studies with both bovine and murine discs (as biological sources for cells and organ cultures) yield converging results, it appears that disc-protective effects of miR-146a expression in discs are conserved across mammalian species, and thus our findings may translate to human intervertebral discs.

Our findings complement previous studies that address the biological effect of microRNAs in spine tissues (Wang et al., 2011; Ohrt-Nissen et al., 2013; Song et al., 2013; Liu et al., 2014; Yu et al., 2013; Zhao et al., 2014; Sun et al., 2013). Several of these prior studies have compared microRNA expression in degenerative disc (Wang et al., 2011; Ohrt-Nissen et al., 2013; Zhao et al., 2014) with normal discs isolated by discectomy from scoliosis patients or during vertebral fusion in spine trauma patients. To date, these analyses have not yielded consistent results reflecting the difficulties of analyzing spine tissue at the molecular level. Studies by Wang and colleagues suggest that deregulation of miR-155, which targets caspase-3 and FADD, contributes to human intervertebral disc degeneration by promoting Fas-mediated apoptosis (Wang et al., 2011). Zhao and co-workers examined specimens from

three patients with IVD and three with spinal cord injury by microarray analysis (Zhao et al., 2014). Their results showed changes in microRNAs linked to several signaling pathways including phosphoinositide 3-kinase (PI3K)-Akt, mitogen-activated protein kinase (MAPK), epidermal growth factor receptor (EGFR; ErbB) and Wnt signaling. More recently, Yu and colleagues compared nucleus pulposus tissues from 50 degenerative discs with 4 idiopathic scoliosis discs (Yu et al., 2013). This comparison showed that miR-10b levels correlate with the pathological grade of disc degeneration. In their model, aberrant elevation of miR-10b may contribute to abnormal nucleus pulposus cell proliferation by suppressing HOXD10 and indirectly stimulating the RhoC-Akt pathway. Liu and others extended these findings by confirming that miR-10b levels are associated with disc degeneration grade, and that miR-21 is significantly upregulated in degenerative nucleus pulposus tissues compared with idiopathic scoliosis disc (Liu et al., 2014). The up-regulation of miR-21, which targets PTEN, may also contribute to intervertebral disc degeneration by stimulating the Akt pathway and increasing abnormal proliferation of nucleus pulposus cells. The identification of different microRNAs in several studies reflects the complexities of in the biological functions of microRNAs at different stages of musculoskeletal development or in distinct degenerative disease states (Miyaki and Asahara, 2012).

Previous studies showed that miR-146a is significantly upregulated in peripheral OA knee joint tissues (Yamasaki et al., 2009; Li et al., 2011; Gibson and Asahara, 2013; Jones et al., 2009). However, studies on the function and expression of miR-146a in chondrocytes have not yet yielded unequivocal results. Li et al. (2012) showed in a rat OA model that forced expression of miR-146a contributes to OA pathogenesis, and may be involved in a series of IL-1 β -induced features of OA including reduced cellular response to TGF- β , elevated VEGF expression, and increased chondrocyte apoptosis. However, our group (Li et al., 2011) found that miR-146a may protect against cartilage degeneration and pain symptoms caused by OA. Our results revealed that mir-146a significantly reduces TRAF6 and multiple pro-inflammatory pain mediators (TNF α , COX-2, iNOs, IL-6, IL8, RANTS, TRPV1) in peripheral human joint tissues (Li et al., 2011). In this study, we find that miR-146a functions in an anti-catabolic manner in intervertebral disc by antagonizing the IL-1 induced expression of cartilage-degrading enzymes MMPs and ADAMTS. Transfection of bovine tail NP cells with miR-146a rescues IL-1-dependent upregulation of inflammatory gene expression, indicating that compromised miR-146a expression in disc correlates with an imbalance in anabolic-catabolic responses. Thus, simultaneously antagonizing proteinase-induced breakdown of ECM components such as PGs provides further evidence that miR-146a can suppress the effects of inflammatory cytokines.

Elevated expression of MMPs and ADAMTS is clearly associated with progressive degenerative conditions such as joint osteoarthritis and disc degeneration (Le Maitre et al., 2007). Studies from our laboratory have demonstrated catabolic and/or anti-anabolic effects of IL-1 on human articular cartilage and spine tissue, particularly via upregulation of MMP-13 (Im et al., 2008, 2007). Here, we demonstrated the ability of transfected miR-146a in NP cells to inhibit the IL-1-mediated upregulation of matrix-degrading enzyme gene expression and protein expression. In bovine NP cells and mouse discs, miR-146a had similar anti-inflammatory effects as previously seen in chondrocytes.

There are several recognized limitations of this study that must be taken into account. For example, the findings reported here relate only to in vitro and ex vivo culture models, which fail to adequately represent the complex variety of factors that may influence disc homeostasis in vivo. Indeed, some in vitro results may not be translated to the complex interplay of factors found in vivo. Also, the majority of our current studies is focused on NP cells and may not capture the effects of miR-146a on the entire disc, including the annulus fibrosus and neighboring endplates. Finally, we have reported the various effects on IL-1-mediated activity, but the specific cell signaling pathways and detailed cellular mechanisms mediating these effects remain unknown. Further studies are warranted to elucidate these pathways.

In summary, based on our finding from both in vitro experiment of transfection of miR-146a in bovine NP cells and ex vivo organ culture study on miR-146a KO mice, we thought that miR-146a may be involved in the preventing IVD degeneration by suppressing the effects of inflammatory factors. The relative importance of miR-146a as compared with other microRNA or pro-inflammatory factors in the process of IVD degeneration, however, needs further investigation.

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Abbreviations

ADAMTS	a disintergrin and metalloproteinase with thrombospondin motifs
ANOVA	analysis of variance
COX-2	cyclooxygenase-2
Ct	thresholdcycle
DAB	3,3'-diaminobenzidine
DMEM	Dulbecco's modified eagle medium
IHC	immunohistochemical
EGFR	epidermal growth factor receptor
GAG	glycosaminoglycan
HOXD10	homebox D10
IL-1	interleukin-1
IL-6	interleukin-6
IL-8	interleukin-8
iNOS	inducible nitricoxide synthase
IVD	intervertebral disc

KO	knockout
mini-ITS	mini-insulin, transferrin–selenium
MAPK	mitogen activated protein kinase
miR	microRNA gene
MMP	matrix metalloproteinase
mRNA	messenger RNA
NP	nucleus pulposus
PG	proteoglycan
PI3K	phosphoinositide 3-kinase
PTEN	phosphatase and tensin homologue
qRT-PCR	quantitative real-time polymerase chain reaction(qRT-PCR)
RIPA	radioimmunoprecipitation assay
rRNA	ribosomal RNA
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TGF-β	transforming growth factor-beta
TNFα	tumor necrosis factor-alpha
TRAF-6	TNF receptor associated–associated factor 6
TRPV1	transient receptor potential cation channel, subfamily V, member 1
UTR	untranslated region
WT	wild type
VEGF	vascular endothelial growth factor

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Fig. 1.

Transfection of miR-146a counteracts IL-1 induced catabolic and inflammatory genes in bovine NP cells. The monolayer of bovine NP cells was transfected with miR-146a or sham transfection without miR-146a as control group; the monolayer was then treated with or without IL-1 (10 ng/mL). After stimulation for 24 h, cells were harvested and total RNA was extracted to perform real time qPCR, targeting key cartilage degrading enzymes (ADAMTS-4 (A), ADAMTS-5 (B), MMP-13 (C), MMP-3 (D)), proinflammatory factors (iNos(E), IL-6(F), TNF- α (G), Cox2 (H)), and the miR-146a target, TRAF6 (I). The means and standard deviations were calculated from 3 separate experiments. A value of $p < 0.05$ indicates a significant difference in ANOVA.

Proteins released into the media

Fig. 2.

Transfected miR-146a has anti-catabolic effects at the protein level. Proteins released into the media were analyzed for MMP-13, ADAMTS-4 and ADAMTS-5. TRAF6 protein was analyzed from cell lysates.

Fig. 3.

Band images from Western blots were digitally captured and the intensity of the bands (pixels/band) was obtained using the Image-J densitometry analysis software in arbitrary optical density units. Data shown are mean $\pm$ SD of triplicate determinations in three independent experiments. A value of $p < 0.05$ indicates a significant difference in ANOVA.

Fig. 4. Histological assessments (original magnification: $\times 20$). Discs were sectioned to 5- μ m thickness. Alcian Blue Hematoxylin/Orange G staining was used to assess general morphology and the loss of PGs in discs (n = 6 in each group).

Fig. 5. Immunohistological staining was performed to compare the total percentages of MMP-13 and ADAMTS-5 positive cells between WT and miR146 KO mouse discs.

Fig. 6. Percentages of MMP-13 (A) and ADAMTS-5 (B) positive cells in WT and miR146 KO disc organ cultures treated with IL-1. Values are the mean percentages of positive cells from 4 discs. A value of $p < 0.05$ indicates a significant difference in ANOVA.

Table 1

The primer sequences for qRT-PCR.

Primer	Forward sequences	Reverse sequences	Annealing Tm	NCBI accession no.
MMP-3	TCCTTGTGCTGCCCATGAACTTG	ACATCATCCTGAGAAAGGCGGAAC	55 °C	NM_001206637.1
MMP-13	GCTTCCTGATGATGATGTCCAAGGGA	AGGGTCACATTTGTCTGGCGTT	55 °C	NM_174389.2
ADAMTS-4	ACTGGGCTACTACTATGTGCTGGA	CACACACCATGCACTTGTCGAACT	55 °C	NM_181667.1
ADAMTS-5	ACGTGGTGTCTCTCCAAAG	CATACTGCAGCTTCGAGCCA	55 °C	NM_001166515.1
iNos	TGACATTGACCAGAAGTTGTCCCAGC	TTGCCGCTGACATCGAATGTCTCA	55 °C	NM_001076799
IL-6	TCCAATCTGGGTTCAATCAGGCGA	TTCCCTCATACTCGTTCTGGAGGT	55 °C	NM_173923.2
TNF- α	CACGTTGTAGCCGACATCAACTCT	GTTGTCTTCCAGCTTCACACCGTT	55 °C	NM_173966.3
Cox2	CCCATGGGTGTGAAAGGGAG	CAGATTTGTGCCCTGGGGAT	55 °C	NM_174445.2
TRAF6	GCGGCCTTCAAGTTAGGAGA	ATCAACTGCTCGTTCGGGTT	55 °C	NM_001034661
18S	GTAACCCGTTGAACCCATT	CCATCCAATCGGTAGTAGCG	55 °C	NR_003286