

***In Vitro* and *Ex Vivo* Screening of microRNA Combinations with Enhanced Cell Penetrating Peptides to Stimulate Intervertebral Disc Regeneration**

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23 **Abstract**

24 **Background:** Low back pain (LBP) is predominantly caused by degeneration of the
25 intervertebral disc (IVD) and central nucleus pulposus (NP) region. Conservative treatments
26 fail to restore disc function, motivating the exploration of nucleic acid therapies, such as the
27 use of microRNAs (miRNAs). miRNAs have the potential to modulate expression of
28 discogenic factors, while silencing the catabolic cascade associated with degeneration. To
29 deliver these miRNAs, non-viral cell penetrating peptides (CPPs) are gaining favour given their
30 low immunogenicity and strong targeting ability. Single miRNA therapies have been
31 investigated for IVD repair, however dual-miRNA delivery strategies have not been commonly
32 examined and may augment regeneration.

33 **Materials and methods:** Transfection of four pro-discogenic miRNAs (miRNA mimics: 140-
34 5p; 149-5p and inhibitors: 141-3p; 221-3p) and dual delivery of six miRNA pairings was
35 performed using two CPPs, RALA and GET peptide (FLR), in primary rat NP monolayer
36 culture, and in an *ex vivo* organ culture model of rat caudal discs. Protein expression of
37 discogenic (aggrecan, collagen type II, and SOX9) and catabolic markers (ADAMTS5 and
38 MMP13) were assessed.

39 **Results:** Monolayer investigations signified enhanced discogenic marker expression following
40 dual miRNA delivery, signifying a synergistic effect when compared to single miRNA
41 transfection. Utilisation of an appropriate model was emphasised in our *ex vivo* organ culture
42 experiment, revealing the establishment of a regenerative microenvironment characterised by
43 reduced catabolic enzyme activity and enhanced matrix deposition, particularly following
44 concurrent delivery of FLR-miRNA-149-5p mimic and miRNA-221-3p inhibitor.
45 Bioinformatics analysis of miRNA-149-5p mimic and miRNA-221-3p inhibitor identified
46 distinct targets, pathways, and interactions, suggesting a mode of action for this amplified
47 response.

Conclusion: Our findings suggest the potential of FLR-miRNA-149-5p + miRNA-221-3p inhibitor to create an anti-catabolic niche within the disc to foster regeneration in moderate cases of disc degeneration, which could be utilised in further studies with the overarching aim of developing treatments for LBP.

1. Introduction

The incidence of low back pain (LBP) is on the rise, affecting an estimated 600 million people worldwide creating a growing socioeconomic burden, particularly in ageing populations.¹ Though the causes are pleiotropic in nature, there is consensus that degeneration of the nucleus pulposus (NP) region of the intervertebral disc (IVD) through degradation of the glycosaminoglycan (GAG)-rich extracellular matrix (ECM) and its key components is a primary cause. This is exemplified by a disruption to the catabolic : anabolic homeostasis, with a loss of matrix-related transcription factors (TFs) such as Sry-Box TF 9 (SOX9), and proteins, for example, aggrecan and collagen type II.^{2,3} Concurrently there is an escalation in levels of matrix degrading enzymes, namely members of the matrix metalloproteinases (MMP) and a disintegrin and metalloprotease with thrombospondin motifs (ADAMTS) families.⁴⁻⁸ Treatments for LBP are primarily prophylactic in nature, involving physiotherapy and pain management, progressing to spinal surgeries when degeneration increases in severity.⁹ Surgeries such as discectomy and spinal fusion offer short term relief however this effect is not sustained,¹⁰ indicating a need for more effective therapeutic options.

With this in mind, the introduction of regenerative nucleic acids has gained ground over the last decade, with microRNAs (miRNAs) offering the potential to treat a range of disorders.¹¹⁻¹⁴ miRNAs function in the post-transcriptional regulation of target genes, of which for each miRNA hundreds of targets are applicable,^{15,16} and this activity results in the stimulation or

dampening of protein expression.¹⁷⁻¹⁹ From a regenerative standpoint, we have selected 4 pro-discogenic miRNAs that have demonstrated therapeutic potential, both in terms of predicted targets and those experimentally validated in the literature, namely: miRNA-140-5p mimic, miRNA-141-3p inhibitor, miRNA-149-5p mimic, and miRNA-221-3p inhibitor.

miRNA-140-5p has been shown to have a positive impact on driving chondrogenesis in models of osteoarthritis (OA), with similar key markers as those observed in discogenesis; SOX9, aggrecan, and collagen type II,^{20, 21} and in catabolic enzyme expression, specifically ADAMTS5 and MMP13,^{22, 23} indicating its potential usage in a regenerative capacity in the disc. The presence of miRNA-141 has been implicated as a driver of catabolic degeneration in human NP cells, stimulating ADAMTS5 and MMP13 expression.^{24, 25} Xu *et al* observed that by inhibiting miRNA-141 there was a return towards healthy levels of collagen II and aggrecan deposition, with decreases in gene expression of ADAMTS and MMP family members.²⁶ The transduction of rat NP cells cultured in cytokine-stimulated media with miRNA-149-5p restored gene and protein expression of aggrecan and collagen type II towards non-stimulated, healthy levels.²⁷ In chondrocytes, a study by Wang *et al* found evidence indicating an anti-apoptotic role and silencing of MMP13,²⁸ which could aid in the stimulation of NP cells towards a regenerative behaviour. Finally, the silencing of miRNA-221 has been linked to enhancement of a discogenic-like phenotype for human NP cells *in vitro*.^{29, 30} Dubbed chondroprotective, the inhibition of this miRNA may function in driving the deposition of ECM-related markers. Taken together, there is experimental evidence to suggest the applicability of these miRNAs for IVD regeneration.

To exploit this regenerative potential of miRNAs, a method of delivery must be chosen to taxi these miRNAs into the cells. Though efficacious, the viral delivery of nucleic acids has decreased in popularity due to safety concerns and immunogenicity.³¹⁻³³ To this end, non-viral cell penetrating peptides (CPPs) that retain internalisation capabilities while proving less

stimulatory of the immune system are being explored. In this study, two CPPs were identified for investigation, cationic 30mer amphipathic peptide (RALA) and GAG-binding enhanced transduction (GET) peptide FGF2B-LK12-8R (FLR), both of which function by complexing nucleic acids through electrostatic interactions. RALA, an amphipathic cationic peptide, has arginine residues incorporated throughout the structure to enhance the cationic charge and improve membrane transduction and internalisation.³⁴⁻³⁶ A modified GAG-binding enhanced transduction (GET) peptide, FLR contains a fibroblast growth factor 2 heparin-binding domain that facilitates homing to the cell membrane conjugated to an amphipathic and cationic peptide sequence.^{37,38}

The overall objective of this work was to explore the regenerative utility of six combined miRNA pairings *in vitro* and in an *ex vivo* organ culture model, while simultaneously assessing the suitability of CPPs for miRNA delivery to the NP region.

2. Materials and methods

2.1 Primary nucleus pulposus cell isolation and culture

Rat tissue was obtained from discarded tissue of animals that underwent procedures in the Comparative Medicine Unit (CMU) of Trinity College Dublin following approval by the animal research ethics committee (AREC) of Trinity College Dublin and the Health Products Regulatory Authority (HPRA) in Ireland (Approval - AE19136/P149). NP cells were harvested under sterile conditions from adult, skeletally mature, female Wistar rats (10-20 weeks) immediately following euthanasia, with 3 tails pooled to represent a donor. Due to the ease of access and high prevalence of use in IVD degeneration studies³⁹, caudal discs were employed for all cell culture studies. NP tissue was enzymatically digested simultaneously with pronase (70 U/mL, Millipore, Merck Life Science Ltd., U.K.) and collagenase type II (400 U/mL

Molecular Probes, Bio-Sciences, Ireland) for 6 hours at 37°C at 10 r.p.m.. Once cells were digested to a single cell level, they were plated in flasks for expansion at a density of 5×10^3 cells/cm³. Cells were expanded in expansion media (XPAN) comprised of low glucose Dulbecco's Modified Eagle Medium (LG DMEM, Sigma-Aldrich, Merck Life Science Ltd., U.K.), 2% penicillin/streptomycin (Pen/Strep), and 10% Foetal Bovine Serum (FBS, all Invitrogen, Bio-Sciences Ltd., Ireland). XPAN culture media was measured within a range of 300-325 mOsm, which correlates to that of the degenerative IVD.⁴⁰ For ease of culture, all initial screenings were carried out under normoxic (20% O₂) conditions in a humidified atmosphere at 37°C. NP cells were used between passage 1 and 3, as no morphological changes were visually observed.

2.2 2D miRNA-vector complex formation

miRNA complexes were generated through electrostatic interactions of FLR (Dixon Laboratory, University of Nottingham, U.K.) at predefined Nitrogen : Phosphate (N:P) charge ratios of 5,^{37, 41} and RALA (GenScript, Netherlands) at an N:P ratio of 10,^{42, 43} for miRNA mimics-140-5p & 149-5p, and 141-3p & 221-3p inhibitors (all miRNAs were purchased from Horizon Discovery, U.K.). For assessment of dual miRNA delivery, the following miRNA pairings were formed: miRNA-140-5p mimic + 141-3p inhibitor, 140-5p + 149-5p mimics, 140-5p mimic + 221-3p inhibitor, 141-3p inhibitor + 149-5p mimic, 141-3p + 221-3p inhibitors, and 149-5p mimic + 221-3p inhibitor. For all monolayer experiments an miRNA concentration of 10 ng/μl was used, which was previously optimised in our lab.

2.3 Cell transfections in monolayer

For immunofluorescence of protein markers cells were seeded at a density of 1×10^4 in 18-well μ -well slides (Ibidi GmbH, Germany) and allowed to attach for 24 hours prior to transfection. For biochemical analysis of aggrecan and collagen production, NP cells were seeded at 1×10^5 in 6 well plates, again allowing 24 hours for attachment. In all cases, complexes were formed for 30 minutes at room temperature (RT) as described above in serum free OptiMEM (Invitrogen, Bio-Sciences Ltd., Ireland). Complexes were added to cells following a phosphate buffered saline (PBS) wash and transfection allowed to proceed for 5 to 6 hours at 37°C . At the end of incubation, complexes were removed, and cells cultured for 3 days for immunofluorescence analysis, and 14 days for biochemical analysis with media changes performed biweekly where appropriate. For all transfection experiments a non-transfected (NT) control receiving OptiMEM alone was utilised. Preliminary transfection efficiency had previously been shown in our lab by flow cytometry as being above 90% for RALA and FLR at a dose of $10 \text{ ng}/\mu\text{l}$, with viability 24 hours post-transfection above 80% as assessed by Calcein staining. To ensure transfection was not impacting on ECM protein production, a negative mimic control (Horizon Discovery, U.K.) was employed as per manufacturer's instructions. For monolayer cultures, between 3 and 5 independent biological replicates were performed, as indicated in the Figure legends. Table 1 outlines the specific cell culture experimental set-up for each experiment.

Table 1. Experimental outline of nucleus pulposus cell monolayer experiments.

Assay	Single/Dual miRNA	Time Point	Plate Size	Seeding Density	Cytokine Insult	Output
Immunofluorescence	Single	3 days	18- well μ -well slide (Ibidi)	1×10^4	No	Protein: aggrecan, collagen type II
Immunofluorescence	Dual	3 days	18- well	1×10^4	Yes	Protein: aggrecan,

			μ -well slide (Ibidi)			collagen type II
Immunofluorescence	Dual	3 days	18- well μ -well slide (Ibidi)	1×10^4	Yes	Protein: ADAMTS5, MMP13
Biochemical Quantification	Dual	14 days	6-well	1×10^5	Yes	DNA, sGAGs, collagen

164

165 2.4 Proinflammatory cytokine challenge

166 To better represent physiological conditions a cytokine challenge was employed for assessing
167 dual miRNA transfections. 24 hours post-seeding, cells were starved of serum for 24 hours and
168 subsequently insulted with TNF- α (50 ng/mL) and IL-1 β (10 ng/mL) for a further 24 hours as
169 previously described.^{44, 45} After 24 hours of incubation, cytokine media was removed, cells
170 were washed with PBS and miRNA-vector complexes were incubated as previously described.

171

172 2.5 Immunohistochemistry of monolayer cultures

173 3 days post-transfection, cells were washed with PBS and fixed in 4% paraformaldehyde (PFA)
174 for 12 minutes, cell membranes were permeabilised in 0.1% Triton X-100 for 20 minutes, and
175 non-specific binding was blocked with 1% bovine serum albumin in PBS for 30 minutes (BSA,
176 all Sigma-Aldrich, Merck Life Science Ltd., U.K.). Primary antibody solutions: rabbit-anti-
177 collagen II (PA599159), rabbit-anti-aggrecan (MA532695), rabbit-anti-ADAMTS5
178 (PA532142), and mouse-anti-MMP13 (MA514247, all Invitrogen, Bio-Sciences Ltd., Ireland),
179 were incubated overnight at 4°C. A concentration-matched IgG control was used for all
180 staining. Species-matched AlexaFluor secondary antibodies: goat anti-rabbit 488 (ab150077,
181 Abcam, U.K.) and goat anti-mouse 488 (A11001, Invitrogen, Bio-Sciences Ltd., Ireland) were
182 incubated for 1.5 hours, with nuclear counterstaining with Hoechst Bisbenzimidazole 33258

(Sigma-Aldrich, Merck Life Sciences Ltd., U.K.). Images for each independent biological replicate were captured in triplicate on a Leica SP8 Scanning Confocal Microscope (Leica Microsystems, Germany), with analysis of protein expression quantified using a custom CellProfiler pipeline (CellProfiler 4.2.1), which measures the signal intensity and normalises per cell number. Fluorescence intensity was normalised to NT control to determine the degree of fold change between groups, with 3-5 independent biological replicates performed.

2.6 Quantitative biochemical analysis

Cells were digested for 18 hours at 60°C in 350 µL 100mM Sodium Phosphate/5 mM Na₂EDTA Buffer, with 3.88 U/mL papain enzyme and 5mM L-Cysteine (both Sigma-Aldrich, Merck Life Science Ltd., Ireland). Media samples were pooled and stored at -80°C prior to lyophilisation following a standard freeze-drying protocol (500 mTorr, -10°C, 16 – 18h, Harvest Right™, USA) and incubated in activated papain solution as above. DNA content was assessed using Hoechst Bisbenzimidazole 33258 dye assay (DNA QF Kit, Sigma-Aldrich, Merck Life Science Ltd., Ireland.) with a calf thymus DNA standard. Determination of sulphated glycosaminoglycans (sGAGs) was carried out using the dimethylmethylene blue binding assay (DMMB) (Blyscan, Biocolor Ltd., Northern Ireland) with a chondroitin sulphate (CS) standard. Total collagen content was assessed through hydroxyproline measurement, with samples hydrolysed in concentrated hydrochloric acid (38% HCl) at 110°C for 18 hours. A chloramine-T assay was performed and collagen content was estimated using a hydroxyproline:collagen ratio of 1:7.69.⁴⁶ All biochemical measurements were normalised to the non-transfected (NT) control, with 5 independent biological replicates performed.

2.7 Rat ex vivo organ culture

The top three caudal discs were isolated from rat tails, removing the skin and tendons to allow for easier access to the disc section. To avoid swelling of the NP, the CEP and vertebral elements were left intact. Following successive washes in povidone iodine (Duggan Veterinary Supplies Ltd., Ireland) and 70% industrial methylated spirit (IMS) the discs were incubated in LG DMEM, supplemented with 2% Pen/Strep, 0.2% Primocin (InvivoGen), and 0.25µg/mL Amphotericin B (Sigma-Aldrich, Merck Life Sciences Ltd., Ireland) for 24 hours, before culture for 7 days in XPAN at physioxia (5% O₂) to more accurately simulate the native IVD. At day 7, discs were injected with 2µL of 0.025U chondroitinase ABC (cABC, Sigma-Aldrich, Merck Life Sciences Ltd., Ireland) using a 30G needle and degeneration allowed to progress for 7 days. Complexes of the miRNA combinations were formed for 30 minutes at a concentration of 25ng/µL and 2µl was delivered directly into the NP region. 14 days post-transfection the discs were fixed overnight in 4% PFA and decalcified in decalcification solution-lite (Sigma-Aldrich, Merck Life Sciences, Ireland) until soft (~7 days). Each experiment was performed independently with 3 biological replicates. NP cell viability was assessed by live/dead staining with Calcein AM (Biotium) and Ethidium homodimer (Sigma-Aldrich, Merck Life Sciences Ltd., Ireland) for 4 independent biological replicates and imaged on a Leica SP8 Scanning Confocal Microscope (Leica Microsystems, Germany).

2.8 Histology and immunocytochemistry

Following fixation, discs were dehydrated sequentially in increasing ethanol solutions and wax embedded. Disc sections were sliced in the sagittal orientation to a 10µm thickness on a microtome (Leica Microsystems, Germany) with staining carried out for picrosirius red to demonstrate collagen deposition, and alcian blue to detect the presence of sGAGs. Imaging was performed on an Aperio Scanscope (Leica Microsystems, Germany). For immunocytochemistry, antigen retrieval was carried out using 35 U/mL pronase at 37°C for 30

minutes, followed by membrane permeabilisation with 0.1% Triton X-100 and blocking for 1 hour with 5% BSA in PBS. Primary antibody solutions for rabbit-anti-SOX9, rabbit-anti-ADAMTS5 (PA532142, Invitrogen, Bio-Sciences Ltd., Ireland), and mouse-anti-MMP13 (MA514247, Invitrogen, Bio-Sciences Ltd., Ireland) were prepared in 1% BSA in PBS, with a concentration-matched IgG control, and incubated overnight at 4°C. Species-matched AlexaFluor secondary antibodies: goat anti-rabbit 488 (ab150077, Abcam, U.K) and goat anti-mouse 488 (A11001, Invitrogen, Bio-Sciences Ltd., Ireland) were incubated for 1.5 hours at room temperature, with nuclear counterstaining using Hoechst Bisbenzimidazole 33258 (Sigma-Aldrich, Merck Life Sciences, Ireland). Samples were mounted with Fluoromount Aqueous Mounting Media (Sigma-Aldrich, Merck Life Sciences, Ireland) and imaged on a Leica SP8 Scanning Confocal Microscope. To assess protein expression, regions of interest (ROIs) of equal size were captured and fluorescence measured. The corrected total cell fluorescence was then determined as follows: Integrated Density – (Area of Selected Cell Mean * Mean Fluorescence of Background Readings). All read outs were normalised to the NT control to express fold changes of protein expression.

2.9 Bioinformatics analysis

To further investigate the roles of miRNA-149-5p mimic and miRNA-221-3p inhibitor in the degeneration of the IVD, strongly evidenced miRNA-gene interactions (those identified through reporter assays, Western blotting, or quantitative polymerase chain reaction (qPCR)) were determined using the online repositories [miRNATarBase](#),⁴⁷ and [MIENTURNET](#).⁴⁸ A miRNA-gene target list was achieved with a cut-off of $p < 0.05$. The lists for both miRNAs were visualised in Cytoscape, Version 3.10,⁴⁹ and enriched utilising the Gene Ontology Biological Process Tool, removing redundant results. Relevant pathways were isolated, and protein-protein interaction (PPI) networks were visualised through the Search Tool for the

Retrieval of Interacting Genes (STRING) database,^{50,51} employing a 0.8 confidence score cut-off. Nodes were displayed as being larger with higher levels of expression in the extracellular space, given the importance of ECM production in the IVD. The datasets obtained from miRTarBase and MIENTURNET are available in the Supplemental Information.

2.10 Statistical analysis

Statistical analysis was carried out using GraphPad Prism (version 9.4.1) with a minimum of three biological replicates analysed for each experimental group. For analysis between groups of three or more, a one-way ANOVA with Tukey's post-hoc test was performed. p-values below 0.05 were considered statistically significant. Data is presented as mean $\pm$ standard error of mean (SEM).

3. Results

3.1 Delivery of individual miRNAs demonstrated favourable influence on the modulation of extracellular matrix proteins in monolayer culture

As identified from literature, the four selected miRNAs, miRNA mimics 140-5p and 149-5p, and inhibitors of miRNAs 141-3p and 221-3p, were delivered individually to NP cells in monolayer by both RALA and FLR vectors, and their effect on regenerative ECM markers aggrecan and collagen II was assessed. Immunofluorescence quantification demonstrated transfection with miRNA-140 mimic significantly enhanced aggrecan deposition following transfection with FLR ($p=0.015$), and collagen II protein production was significantly increased by RALA delivery ($p=0.014$), with a positive trend for FLR ($p=0.081$) (Fig. 1A, B). Some increases were also exhibited for miRNA-149 mimic (Fig. 1C, D) and miRNA-221-inhibitor (Fig. 1E, F), although these were not found to be statistically significant. RALA-

miRNA-221-3p inhibitor stimulated significant aggrecan expression ($p=0.046$), with collagen II showing increases (Fig. 1G, H). Generally, improvements to matrix protein expression were approximately 1.5 to 2-fold range in comparison to the NT control.

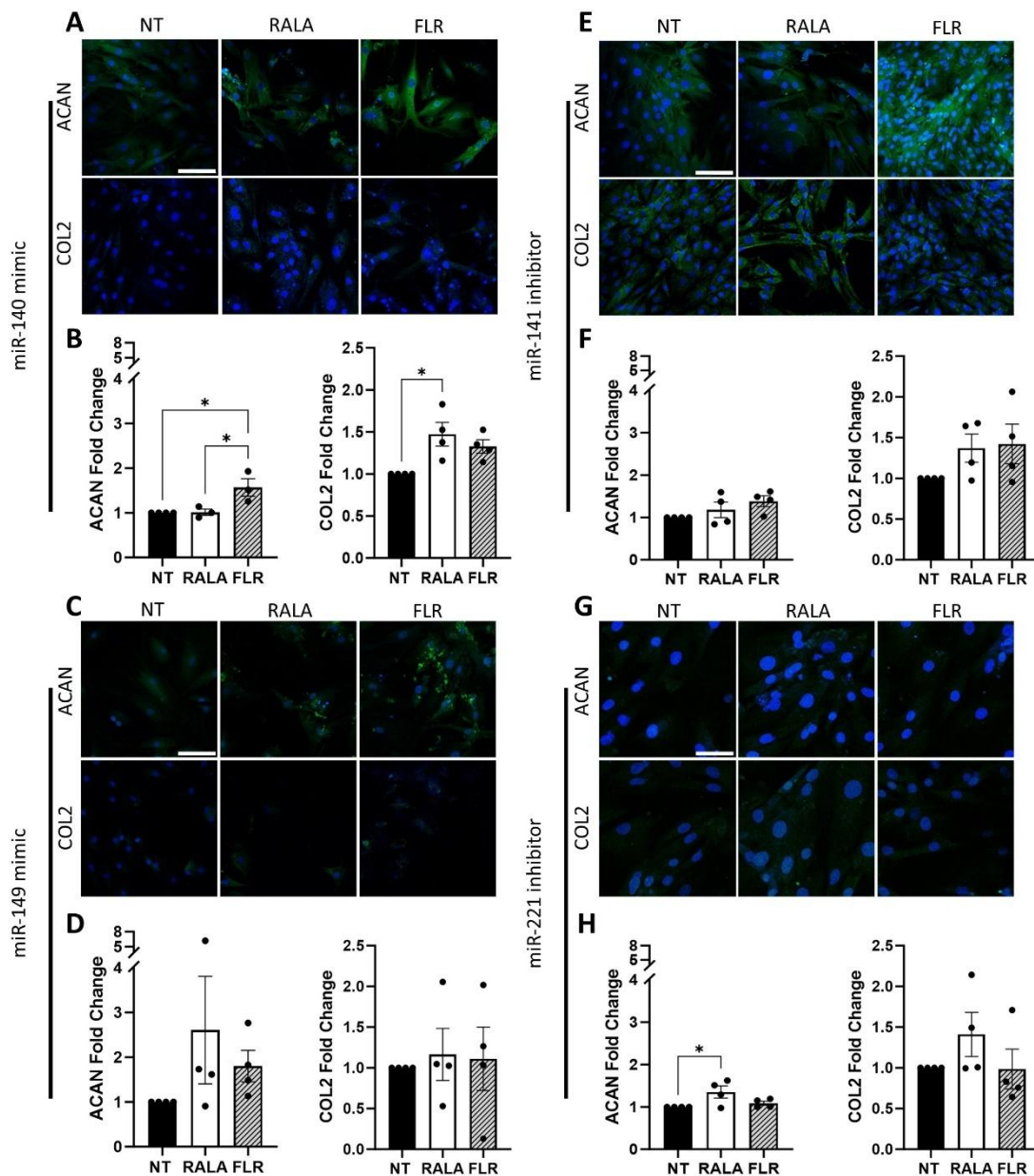


Figure 1. Transfection of rat nucleus pulposus cells with individual miRNA mimics and inhibitors with cell penetrating peptides demonstrated positive trends towards increased matrix production in monolayer culture. (A&B) miRNA 140 mimic transfection significantly increased aggrecan (ACAN) and collagen type II (COL2) protein expression, (C&D) 149 mimic, (E&F) 141 inhibitor and (G&H) 221 inhibitor demonstrated instances of increased protein expression of ACAN and COL2 three days post-transfection. * ($p<0.05$)

indicates statistical significance, N=3-4. Scale bars are 100 μ m.

3.2 Transfection of therapeutic miRNA pairings enhanced the deposition of matrix proteins in 2D monolayer cultures

While some upward trends were identified from individual miRNA delivery, we next explored if a combinatorial approach may amplify the regenerative response while also dampening the catabolic cascade associated with degeneration. Here, all groups, including NT controls were stimulated with pro-inflammatory cytokines to induce catabolic enzyme production in order to evaluate how miRNA pairings could influence the promotion of matrix proteins and the suppression of degradative catabolic factors. With regards to aggrecan production, miRNA-140 mimic + miRNA-141 inhibitor markedly improved protein expression (RALA-group $p=0.002$, FLR-group $p=0.045$), with RALA-miRNA inhibitors 141 + 221 demonstrating similar promise ($p=0.046$, Fig. 2A, B). FLR-miRNA mimics 140 + 149 approached significance ($p=0.051$) for aggrecan deposition. Amongst the remaining three combined groups, increases approaching a 4-fold increase were observed, in comparison to approaching 1.5 to 2-fold with single miRNA transfections, signifying the enhanced potential of dual miRNA delivery. Immunofluorescence analysis of collagen displayed less altered protein expression, though increases approached a mean 5-fold increase for a number of groups, such as miRNA mimics 140+149, miRNA-141 inhibitor+miRNA-149 mimic, miRNA inhibitors 141+221, and miRNA-149 mimic and 221 inhibitors (Fig. 2C, D), again this was enhanced compared to the delivery of individual miRNAs (Fig. 1A-H).

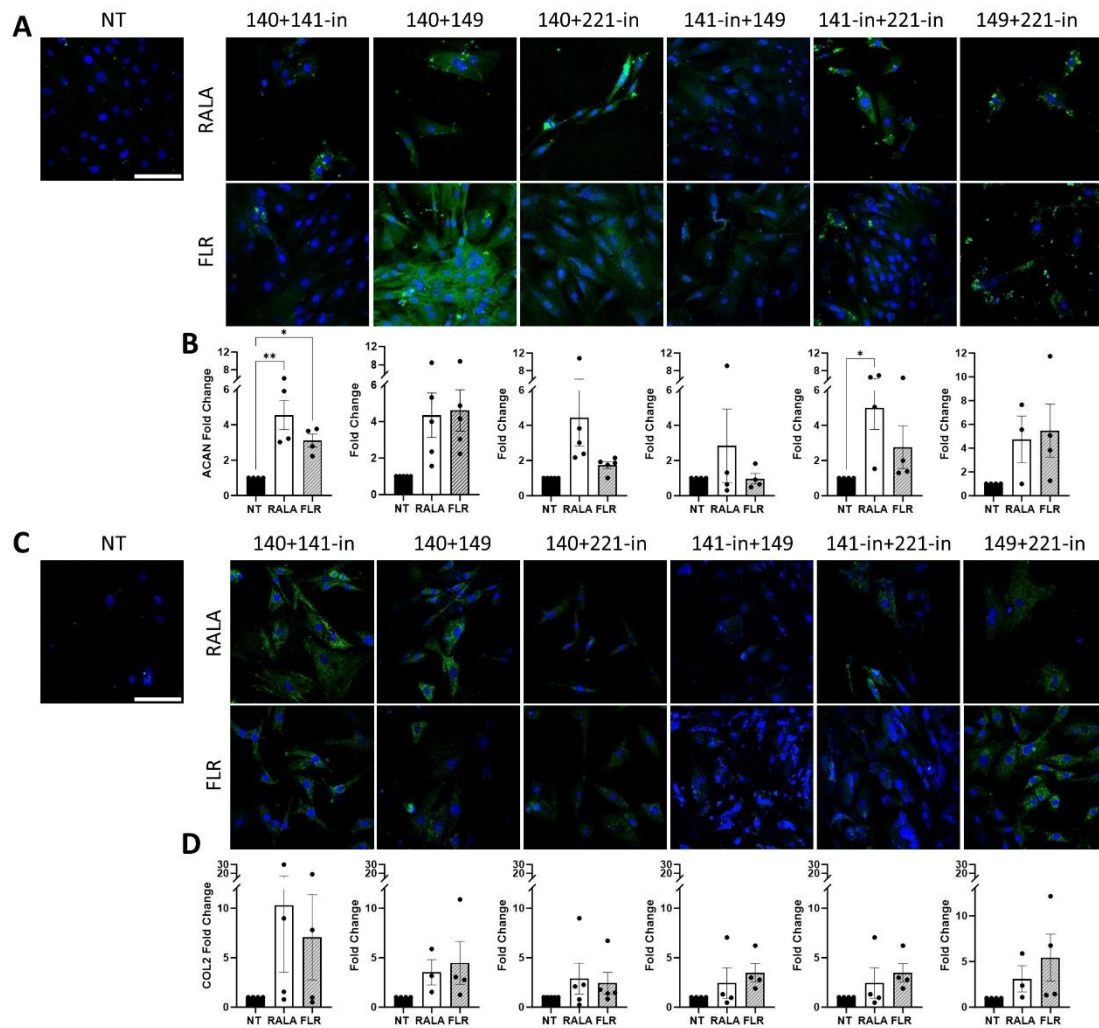


Figure 2. Delivery of miRNAs pairings with cell penetrating peptide mediated delivery increased extracellular matrix protein deposition in rat nucleus pulposus monolayer culture. (A&B) Immunofluorescence analysis 3 days post-transfection exhibited increases in aggrecan (ACAN) expression for miRNA-140-mimic + miRNA-141-inhibitor, and miRNA-141-inhibitor + miRNA-221-inhibitor. Deposition of aggrecan demonstrated some positive trends toward increased deposition after 14 days in monolayer culture. **(C&D)** Collagen type II (COL2) protein expression demonstrated similar non-significant increases in production at 3 days post-transfection. * (p<0.05), ** (p<0.01) indicates statistical significance, N=4-5. NT: Non-transfected. Scale bars are 100 μm.

Upon ascertaining that delivery of a negative control scramble miRNA did not significantly alter DNA, sGAG, or collagen content (Supplementary Figure 1), biochemical analysis was performed on each miRNA-pairing. No significant differences were found between the NT control and transfected groups (Supplementary Figure 2A). Collagen deposition in 2D culture

demonstrated limited changes in comparison to NT groups (Supplementary Figure 2B), potentially representing the unsuitability of monolayer culture systems for identification of ECM factors and their modulation. Values of the samples prior to normalisation are displayed in Supplementary Figure 3.

3.3 Impact of combined vector-miRNA delivery on catabolic enzyme expression in monolayer culture

As mentioned, cytokine stimulation was included for all groups to drive catabolic enzymes such as ADAMTS5 and MMP13 and screen the prospective downregulation of tandem miRNA delivery. RALA-mediated delivery substantially increased ADAMTS5 above that of the NT and/or FLR groups, in the case of miRNA groupings 140 + 149 mimics, miRNA-140 mimic + miRNA-221 inhibitor, miRNA inhibitors 141+221, and miRNA-149 mimic + 221 inhibitor (Fig. 4A, B). Similar increases were identified for MMP13 in particular for miRNA-140 mimic + miRNA-141 inhibitor, and miRNA mimics 140 + 149 ($p=0.069$), and to a greater extent in the RALA groups (Fig. 4C, D). Some non-significant subtle decreases for FLR groups miRNA-141 inhibitor + 149 mimic, and miRNA inhibitors 141 and miRNA-221 were also observed.

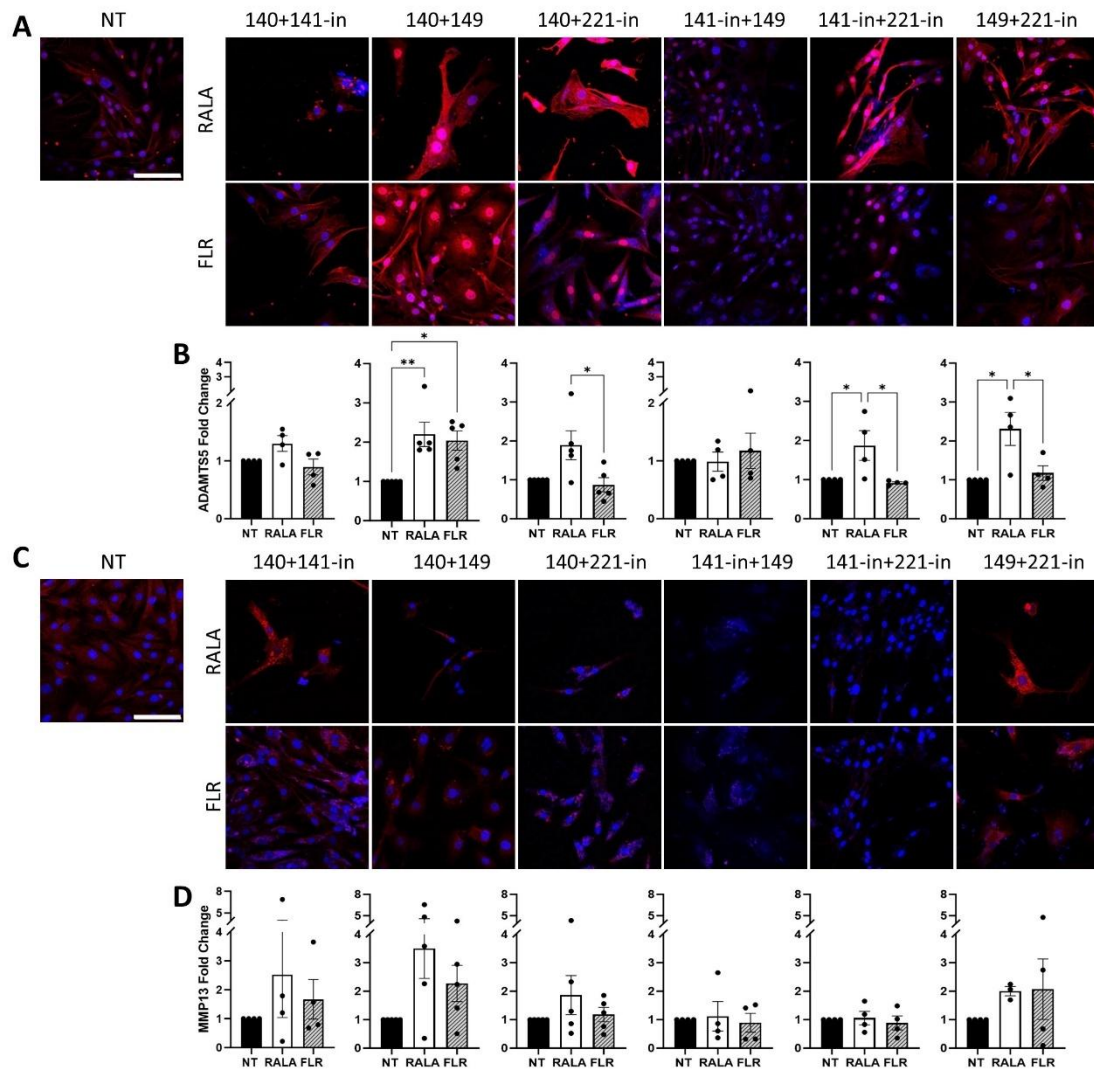


Figure 3. Effect of vector-miRNA pairings on the production of catabolic enzymes in rat nucleus pulposus monolayer culture. (A&B) ADAMTS5 expression three days post-transfection was significantly increased for miRNA-140 + miRNA-149 mimics, miRNA-140 mimic + miRNA-221-inhibitor, miRNA-141-inhibitor + miRNA-221-inhibitor, and miRNA-149 mimic + miRNA-221-inhibitor in comparison to non-transfected (NT) and FLR transfected cells. **(C&D)** MMP13 protein expression showed some instances of increases though none to a significant degree. * $p < 0.05$, ** $p < 0.01$ indicates statistical significance, $N = 4-5$. NT: Non-transfected. Scale bars are 100 μm .

3.4 Vector-miRNA pairings fostered a regenerative niche in rat ex vivo organ culture

Though useful in initial screenings, *in vitro* studies do not robustly represent a physiological environment. To this end, a mildly degenerated model was established in ex vivo rat caudal organ cultures to evaluate the effects of tandem vector-miRNA treatments. NP viability

360 following 21 days in culture was found to be 48.32% for the healthy control, 47.51% for the
361 NT control, and 49.16% for the vector-miRNA-pair treatment, with no significant differences
362 between groups (Supplemental Figure 4). Histological appraisal of sGAG presence revealed
363 marked increases in a number of the groups, such as RALA-miRNA-140 mimic + miRNA-221
364 inhibitor and miRNA-141 + miRNA-221 inhibitor. Transfection using FLR led to increased
365 sGAG deposition for miRNA-140 mimic + miRNA-141 inhibitor, and miRNA-140 + 149
366 mimics. Overall, the strongest staining was shown for miRNA-149 mimic + miRNA-221
367 inhibitor, with the FLR group supporting the largest increase in sGAG staining (Fig. 4A). A
368 significant increase in the key transcription factor SOX9 was observed following RALA-
369 miRNA-140 + 149 mimics delivery in comparison to both the healthy and NT control, with
370 evidence of increasing expression in the other groups for both vector-mediated transfections
371 (Fig. 4B, C).

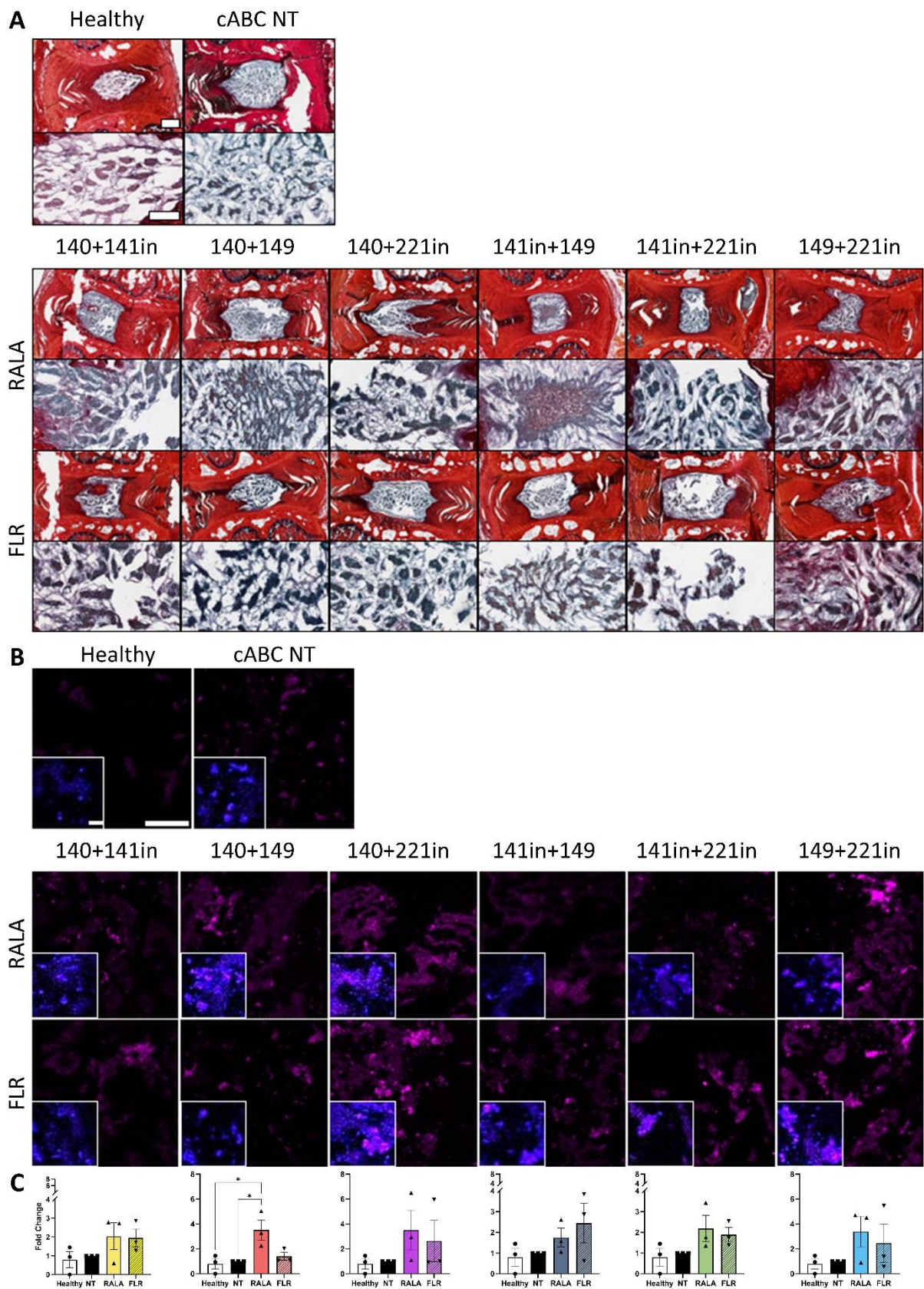


Figure 4. Histological staining of miRNA pairings delivered with cell penetrating peptides (RALA and FLR) in ex vivo rat caudal organ cultures 14 days post-transfection demonstrated instances of increases to glycosaminoglycan (GAG) and transcription

factor SOX9. (A) Collagen was detected with picrosirius red staining (red), and (GAG) presence with alcian blue (blue/purple). There is evidence of increased GAG deposition in comparison to the controls in groups 140+141-in, 140+149, and 141-in+149, with more intense GAG staining observed for FLR 149+221-in. (B) Antibody labelling of SOX9 (magenta) revealed elevated expression levels across the majority of groups with a significant upregulation after transfection with RALA-miRNA-140+149. (C) Semi-quantitative analysis of SOX9 expression. * ($p<0.05$) indicates statistical significance, N=3 independent biological replicates. Healthy controls did not receive cABC treatment, with non-transfected (NT) controls degenerated with cABC and injected with a sham PBS injection. Scale bar for histology images of whole discs is 500 μm , images of GAG staining are 150 μm . Scale bar for fluorescence images is 100 μm . Scale bar for insets with nuclear (blue) staining are 20 μm .

In addition to increases in sGAGs, an encouraging suppression of key ECM-degrading proteins was evidenced with immunofluorescence imaging in the FLR-miRNA-149 mimic + miRNA-221 inhibitor group A significant decrease in ADAMTS5 ($p=0.036$) protein expression was identified (Fig. 5A, B).

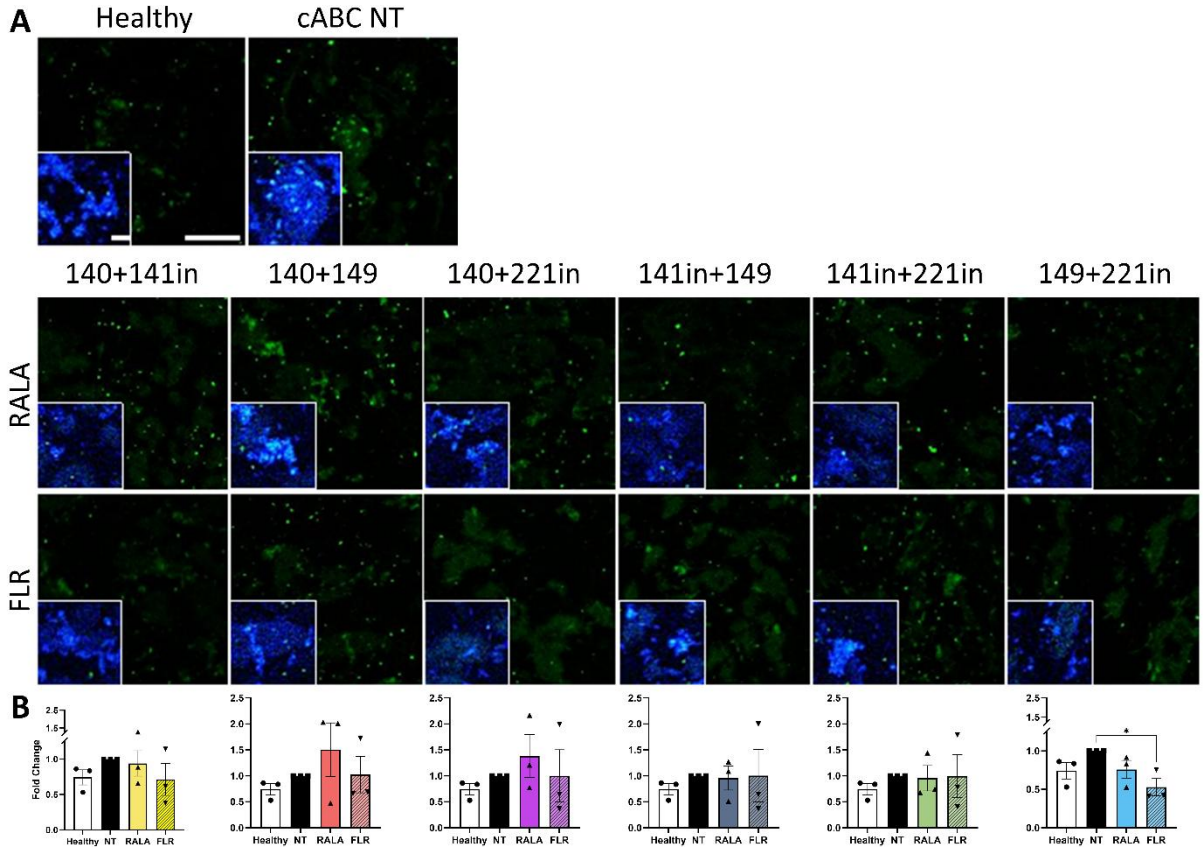


Figure 5. Combined delivery of FLR-miRNA-149 mimic and miRNA-221-inhibitor markedly decreased protein expression of the catabolic enzyme ADAMTS5 in rat caudal organ cultures 14 days post-transfection. (A) Immunolabelling for ADAMTS5 (green) demonstrated significantly reduced expression following transfection of miRNA-149 mimic and miRNA-221-inhibitor using FLR, lower than that of the non-transfected (NT) control. **(B)** * ($p<0.05$) indicates statistical significance, N=3 independent biological replicates. Scale bar is 100 μm . Scale bar for insets with nuclear (blue) staining are 20 μm .

The reduction in ADAMTS5 protein release was concomitant with a marked reduction in MMP13 ($p=0.001$) (Fig. 6A, B). In both cases, the fold change were equivalent to expression in the healthy, non-degenerated discs. Interestingly, mirroring somewhat what was observed in monolayer, RALA-miRNA-140 mimic + miRNA-221 inhibitor elevated MMP13 expression and was found to be significant ($p=0.022$).

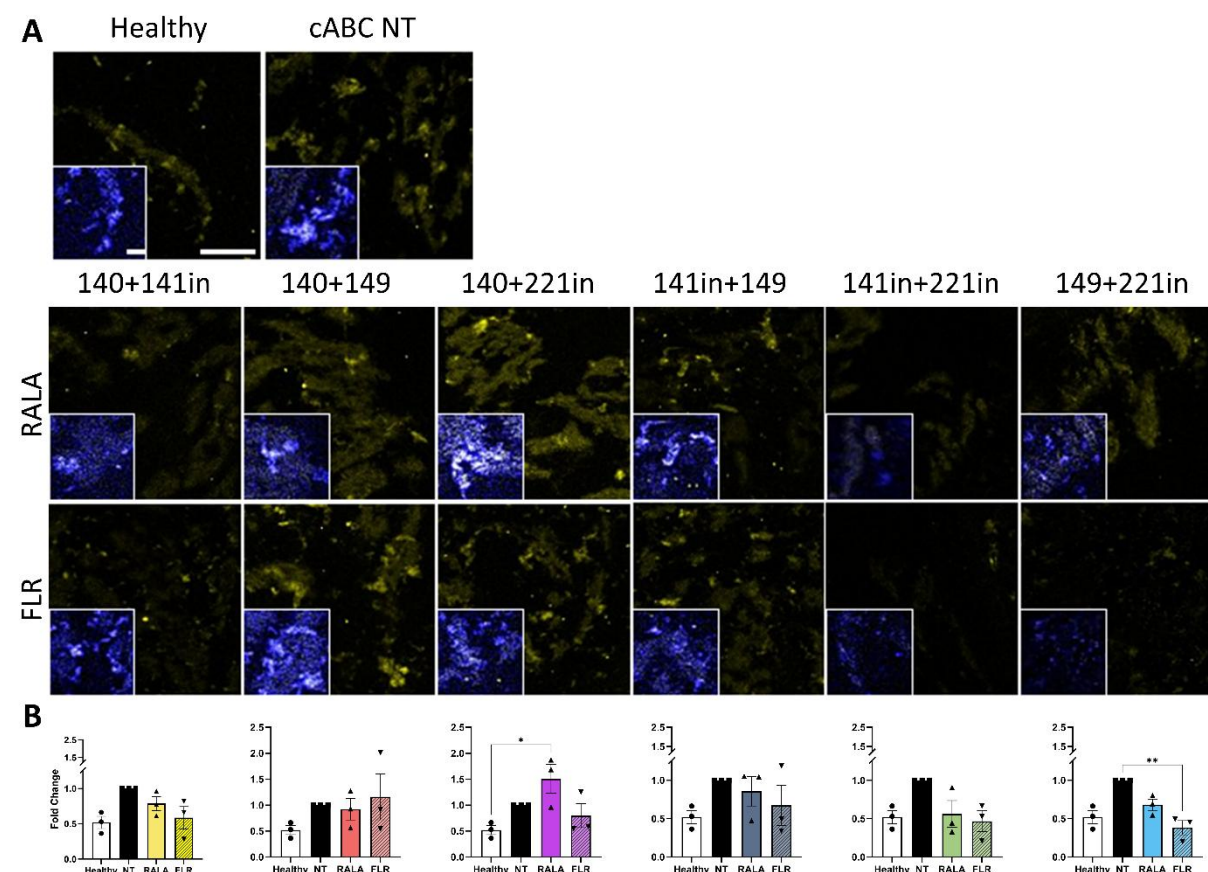


Figure 6. FLR-miRNA-149 mimic and miRNA-221-inhibitor combined delivery significantly reduced protein expression of the catabolic enzyme MMP13 in rat caudal ex vivo organ culture explants 14 days post-transfection. (A) Immunofluorescence detected significantly reduced MMP13 (yellow) expression following transfection of FLR-miRNA-149

mimic and miRNA-221-inhibitor in comparison to the non-transfected (NT) control. **(B)** RALA-mediated miRNA-140 mimic and miRNA-221-inhibitor transfection significantly increased MMP13 production. * ($p<0.05$) ** ($p<0.01$) indicates statistical significance, N=3 independent biological replicates. Scale bar is 100 μm . Scale bar for insets with nuclear (blue) staining are 20 μm .

3.5 Enrichment analysis identified distinct pathways and targets that interact with miRNA-149-5p mimic and miRNA-221-3p inhibitor

To further investigate the mechanism behind miRNA-149-5p mimic and miRNA-221-3p inhibitor in the downregulation of catabolic factors and stimulation of anabolic factors in the IVD, bioinformatic tools were utilised. A total of 54 strongly validated enriched targets were identified for the two miRNAs. miRNA-149-5p mimic was significantly implicated in a number of relevant pathways, including Response to Lipopolysaccharide (LPS), Execution Phase of Apoptosis, Response to Peptidoglycan, and Regulation of Signal Transduction (Fig. 7A-D). A table of all gene name abbreviations is provided in Supplementary Table 1.

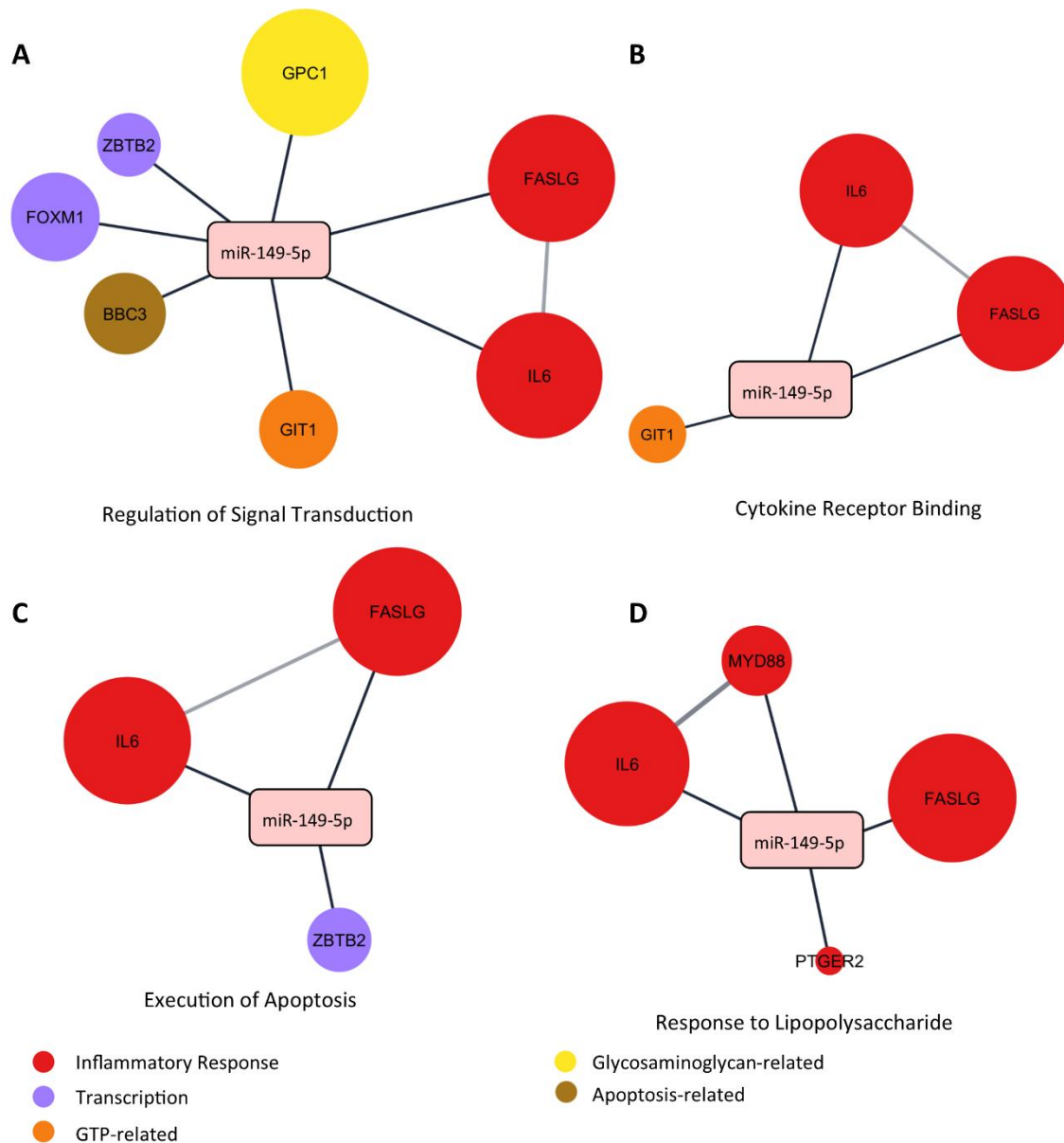


Figure 7. Gene ontology (GO) pathway enrichment analysis of miRNA-149-5p and experimentally validated protein targets. **A.** Regulation of Signal Transduction. **B.** Cytokine Receptor Binding. **C.** Execution of Apoptosis. **D.** Response to Lipopolysaccharide. Expression levels within the extracellular compartment are visually presented, where larger nodes correspond to greater degree of expression within the extracellular space. Nodes are connected if an interaction is known to occur. Network constructed in Cytoscape Version 3.10. GTP: Guanosine triphosphate.

Conversely, miRNA-221-3p was enriched for pathways that relate to IDD, including Response to Hypoxia, Signal Transduction, Response to Cytokine, and Regulation of Protein Serine/Threonine Kinase Activity (Fig. 8A-D). Interestingly, except for BCL2 Binding

Component 3 (BBC3), there was no overlap in the target protein interactions, with both miRNAs having distinct targets and pathways with which they interact. A full list of abbreviated gene terms is provided in Supplementary Table 2.

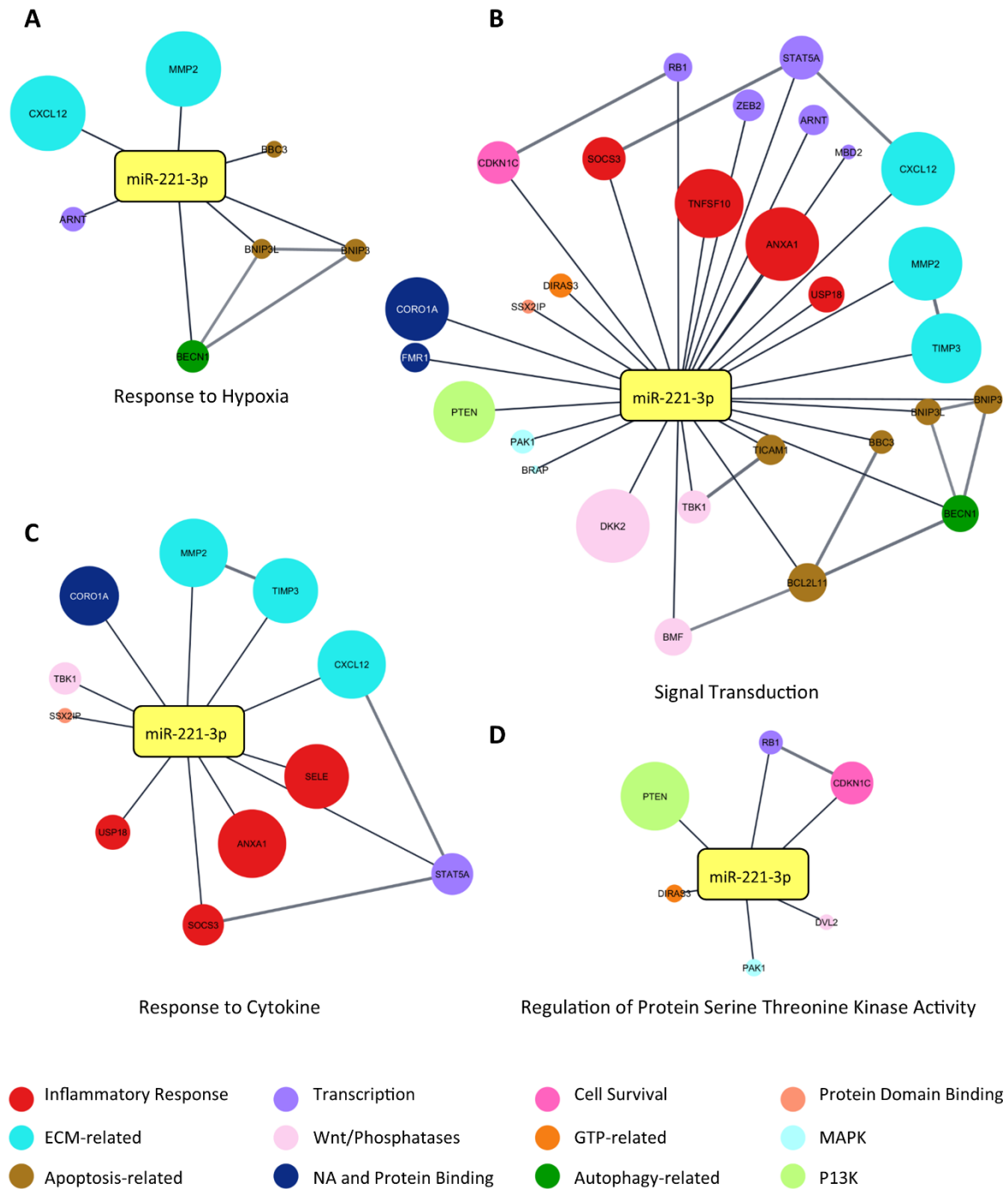


Figure 8. Gene ontology (GO) pathway enrichment analysis of miRNA-221-3p and experimentally validated protein targets. A. Response to Hypoxia. B. Signal Transduction. C. Response to Cytokine. D. Regulation of Protein Serine Threonine Kinase Activity. Expression levels within the extracellular compartment are visually presented, where larger

nodes correspond to greater degree of expression within the extracellular space. Nodes are connected if an interaction is known to occur. Network constructed in Cytoscape Version 3.10. NA: Nucleic acid, GTP: Guanosine triphosphate, MAPK: Mitogen-activated protein kinases, P13K: Phosphoinositide 3-kinase.

4. Discussion

The introduction of non-virally delivered nucleic acids is an attractive therapeutic avenue, whereby key proteins and pathways can be regulated in a manner that drives regeneration or suppresses catabolic factors. For IVD treatment, this work highlights the potential of dual miRNA delivery when combined with CPPs and demonstrated that FLR-miRNA-149-5p mimic + miRNA-221-3p inhibitor stimulated the production of sGAGs and ECM proteins, while encouraging the downregulation of matrix degrading enzymes (ADAMTS5 and MMP3). Although there were promising results observed in monolayer culture, the adoption of an ex vivo organ culture model underscored the significance of using biologically relevant models. In this model, a notable reduction in catabolic enzyme activity was observed, further emphasising its relevance and potential.

Within our monolayer investigations, we identified increases in aggrecan and type II collagen protein expression above 4-fold in our miRNA pair groupings, suggesting that dual-transfection is amplifying the regenerative response. In vitro protein expression increases following miR delivery above 1.5-fold of both aggrecan, and collagen type II have been linked to improvements to disc height index (DHI), GAG production, and restoration of the catabolic : anabolic balance in rodent models.^{25, 52} The potential and efficacy of dual miRNA pairings has also been observed in other fields. In the context of diabetic wounds, Wang and colleagues identified that while transfection using miRNAs-129 and -335 individually promoted healing, when delivered in tandem this effect was strengthened.⁵³ In a similar manner, it was identified that dual delivery of let-7b and miRNA-34a reduced the proliferation of non-small cell lung cancer lines to a greater degree than single delivery alone, subsequently reducing tumour size.

⁵⁴ Building on a 2009 study, ⁵⁵ Mariner *et al* differentiated human MSCs towards an osteogenic lineage with co-delivery of miRNA-148b mimic and miRNA-489 inhibitor, to a greater extent than individual delivery. ⁵⁶ Loading of collagen-nanohydroxyapatite scaffolds with a combination of miRNA-210 mimic and miRNA-16 inhibitor resulted in significantly increased vascularisation and bone density in a rat calvarial defect model, further indicating the potential of dual miRNA delivery. ⁵⁷ These examples offer insight into the potential strengthening of positive stimuli with the correct combination of miRNAs and their cooperative impact.

In relation to the expression of catabolic proteins in monolayer, ADAMTS5 expression was, for the majority of the miRNA-pair groups, enhanced in the RALA groups, with FLR-transfected combinations at times being significantly reduced in comparison. A similar, though not as pronounced effect was seen for MMP13. The FLR CPP was designed to include a GAG-binding peptide which fosters interaction with the lipid bilayer of the target cell and enhances cellular uptake of the given cargo. ⁵⁸ Potentially, the homing effect of FLR to GAG-rich domains, which is abundant in the NP, facilitated targeted delivery of the miRNA-pairings, resulting in more efficient downregulation of ADAMTS5 and MMP13. The monolayer parameters of these screening experiments should be taken into consideration however, and the inherent lack of a protective ECM. ⁵⁹ These conditions may have exacerbated the effect of the cytokine challenge, whereby any potential anabolic effect of the vector-miRNA-pairings may have been reduced. The variability identified may lie in the inherent inter-donor differences, with rats being from different donors and preparations. Added to this, cells used for in vitro studies often lose their circadian synchronicity, which may impact upon their responses to treatments, ^{60, 61} prompting further investigations in a more relevant *ex vivo* organ culture model. As a limitation to our 2D screenings, the implementation of normoxic culture conditions may have impacted upon matrix production and influenced the phenotype of NP cells, however, for our *ex vivo* organ culture experiments we adopted physioxenic oxygen levels.

Similar to our 2D experiments, when examining ex vivo organ cultures, we observed elevated deposition of ADAMTS5 and MMP13 in the RALA groups compared to the FLR groups. For most FLR-miRNA pairings, a decrease in enzyme production was observed, which was comparable to the levels seen in the non-degenerated, healthy control group. However, the strongest decreases for both ADAMTS5 and MMP3 were identified in the FLR-miRNA-149-5p mimic + miRNA-221-3p group. We also identified increases in the expression of the key transcription factor SOX9 in all groups, with the strongest degree of sGAG deposition observed histologically in the FLR-miRNA-149-5p mimic + miRNA-221-3p inhibitor group. Deemed an anti-chondrogenic miRNA, the inhibition of miRNA-221-3p has been shown to promote chondrogenesis through stimulation of aggrecan and collagen type II production.^{62,63} Recently, the application of acetone extracts from Violina pumpkin leaves to IVD cells demonstrated the suppression of miRNA-221 expression and promoted the restoration of a healthy disc phenotype. This was notably characterised by a significant increase in the levels of aggrecan, collagen type II, and SOX9, in conjunction with enhanced resilience to damage induced by reactive oxygen species (ROS).⁶⁴ miRNA-149-5p was identified as a promoter of discogenic behaviour under LPS-stimulated culture conditions.²⁷ Its role in positively regulating the inflammatory response has been extensively documented in disease models of OA.^{65, 66} Elevated levels of pro-inflammatory cytokines, specifically IL-1 β and TNF- α , are observed in degenerated discs, exacerbating the catabolic cascade.⁶⁷⁻⁷⁰ To further elucidate the potential of FLR-miRNA-149-5p mimic + miRNA-221-3p inhibitor, future investigations will integrate anti-inflammatory mechanisms and their role in IVD degeneration. While outside the scope of this work, future work may also benefit from the inclusion of NP-specific markers, such as members of the Cytokeratin family, Brachyury T, and CD24.

The enhanced modulation of regenerative outcomes with dual miRNA delivery may be explained by the targeting of distinct sets of pathways by both miRNA-149-5p mimic +

miRNA-221-3p inhibitor. Through the analysis of enriched and experimentally validated miRNA-target interactions, various pathways and targets of both miRNA-149-5p mimic and miRNA-221-3p inhibitor were identified. miRNA-149-5p was primarily associated with a role in inflammatory processes, such as validated interactions with interleukin-6 (IL-6), Fas Ligand (FASLG), and myeloid differentiation primary response protein 88 (MyD88), all of which were highly expressed in the extracellular compartment. A meta-analysis conducted by Deng *et al* demonstrated a significant increase in IL-6 protein expression in patients with IDD compared to controls.⁷¹ Additionally, a separate study established a correlation between IL-6 concentration and LBP.⁷² Clinically, the incidence of reported pain and higher levels of IL-6 has been identified,⁷³ suggesting the importance of this cytokine in the degenerative cascade involved in IDD and the associated clinical relevance. As a member of the TNF family, polymorphisms in FASLG have been associated to degeneration of the IVD,⁷⁴ with FASLG driving NP cell apoptosis through the binding of FAS and recruitment of pro-inflammatory cytokines.^{75, 76} The toll-like receptor MyD88 has previously been linked with miRNA-149 in LPS-stimulation, whereby its silencing facilitated a dampening of the inflammatory milieu and stimulated a return towards a healthy expression of aggrecan and collagen type II.²⁷ MyD88 expression has been identified as being driven by pro-inflammatory cytokines in the disc⁷⁷ along with the immune response in cardiovascular and neurological disorders.^{78, 79} Considering the role of inflammation in IDD, the potential of miRNA-149-5p to mitigate these effects holds significant promise. Future research endeavours will explore this potential while striving to more accurately replicate the intricate interactions that drive the degenerative cascade and impede regenerative efforts.

As a chondrogenesis-related miRNA, miRNA-221-3p was enriched for ECM-related pathways, with highly expressed targets in MMP2, Tissue Inhibitor of Metalloproteinase 3 (TIMP3), and C-X-C Motif Chemokine Ligand 12 (CXCL12) within the extracellular space.

As matrix degrading enzyme promoters and inhibitors, respectively, a dysregulation of members of the MMP and TIMP families exacerbates the catabolic imbalance at the crux of IDD.^{7, 80} MMP2 specifically acts through the degradation and remodelling of collagens,⁸¹ and polymorphisms in the gene have been associated with a statistically significant increase in the risk of developing IDD.⁸² TIMP3 is unique among the TIMP family as it can suppress all MMPs, and a range of ADAMTS, including -4 and -5 which are of particular relevance to IDD.^{8, 83, 84} A recent study identified a suppression of the discogenic pain marker Substance P following stimulation with TIMP3 in a rodent model, possibly through a reduction in vascularisation.⁸⁵ The upregulation of TIMP3 could therefore serve a therapeutic role when seeking regenerative therapies. Serum levels of the chemokine CXCL12, also referred to as stromal cell-derived factor 1 (SDF-1), have been found to be elevated with more severe levels of IVD degeneration and associated pain.⁸⁶ A connection has been identified between a dose-dependent effect of CXCL12 upon MMP2 protein expression in cartilage endplate (CEP) cells, however, when delivered directly to the NP region in a rat tail model facilitated an increase in proteoglycan deposition, possibly via a stem cell homing mechanism.⁸⁷ Contrary to this amelioration of degeneration in the NP, CXCL12 has predominantly been linked to inflammation-induced apoptosis and matrix destruction.⁸⁸⁻⁹⁰ Given the potential relationships between TIMP3, MMP2, and CXCL12, their interactions with miRNA-221-3p may prove advantageous in restoring regenerative function to the damaged IVD.

Finally, this work highlights the importance of adopting an appropriate and relevant study model, particularly when working within the three-dimensional constraints of the native ECM. While our monolayer investigations evidenced some trends, more meaningful results were demonstrated in the *ex vivo* organ culture model. In the context of the IVD, NP cells readily lose their native phenotype when cultured in monolayer.⁹¹ Furthermore, rats belong to the subsection of species that retain an NP progenitor notochordal cell population throughout

adulthood. However, previous work has identified the reduction of notochordal porcine cells upon passaging, whereby NP and notochordal cells were indistinguishable from one another⁹². In the rat *ex vivo* organ culture model, notochordal cells will be present and constitute up to 30% of the NP cell population. However, injury in the form of degeneration diminishes this number.⁹³ Notochordal cells have been linked to regenerative potential^{94,95}, and their presence may have aided in some of the positive changes identified following miRNA delivery. Future works will explore miRNA pairs in animal models experience a loss in their notochordal cell population throughout development, thereby providing a more accurate representation of humans. Nonetheless, utilising the entire organ imparts a more physiologically relevant niche upon which to assess therapeutics, with the native ECM providing paracrine signalling to better replicate those reported *in vivo*.⁹⁶ Furthermore, *ex vivo* culture systems better reconstitute the metabolic microenvironment, offering more realistic systems to explore potential therapeutics.⁹⁷ The inclusion of a whole organ also offers the potential of employing loading pressure that more closely resembles the native mechanical stimuli experienced,⁹⁸ and warrants further investigation.

The reduction shown in pro-catabolic enzymes possibly signifies the beginning of the creation of a microenvironmental niche with reduced catabolic activity that would facilitate regeneration. The salient biochemical characteristics of the degenerated NP are defined by a destabilisation of homeostasis causing reductions in vital discogenic proteins such as aggrecan and type II collagen, stimulation of degradative enzymes, including members of the ADAMTS and MMP families, and an increased inflammatory response.⁹⁹⁻¹⁰¹ The importance of ameliorating this degradative cascade has been well documented.^{80, 102-105} There could potentially be an inflammatory response to the carrier; however, such a reaction has not previously been reported for RALA or FLR.^{36, 106} Additionally, inflammation at the site of the injection could occur, as has been documented in *in vivo* rodent models.^{107, 108} The marked

reduction in ADAMTST5 and MMP13 identified in the *ex vivo* organ culture for FLR-miRNA-149-5p mimic + 221-3p inhibitor may signify a calming of this catabolic environment, creating a niche whereby regenerative effects can proceed, as observed with the increased sGAG deposition. This promising result demonstrates how the delivery of exogenous therapeutic vector-miRNA pairings can influence and engineer a suitable microenvironment to facilitate the return towards a homeostatic balance.

5. Conclusion

Through our screening of 6 potentially regenerative miRNA pairings delivered by employing CPPs, we identified FLR-miRNA-149-5p mimic + miRNA-221-3p inhibitor pairing as being the most suitable for the deposition of sGAGs, and simultaneous dampening of pro-catabolic factors. A bioinformatics approach to analyse the experimentally validated targets of these two miRNAs demonstrated distinct targets and pathways that may be beneficial for IVD regeneration, with dual delivery amplifying those expected when delivered in isolation. Overall, this work suggests a therapeutic vector-miRNA pairing that may foster regeneration by the creation of an anti-catabolic niche within the IVD.

6. Author contributions

Tara Ní Néill and Conor T. Buckley contributed substantially to the conception and design of the work. Tara Ní Néill performed the acquisition and interpretation of literature, data analysis, presentation and interpretation of results, drafting of the article, revising it critically, and final approval. Marcos N. Barcellona and Niamh Wilson contributed to acquisition of laboratory data. Fergal J. O'Brien, James E. Dixon, Caroline M. and Curtin provided contributions to study design, technical support and interpretation of data. Conor T. Buckley, as the overall

project funding holder, takes responsibility for the integrity of the work from inception to finalised article, provided substantial contribution to data interpretation and presentation. Tara Ní Néill and Conor T. Buckley drafted the manuscript. All authors critically revised the manuscript and approved the final version.

7. Supplementary Information

Supplementary datasets associated with this article can be found in an online repository at DOI: 10.5281/zenodo.10079400

8. Conflicts of Interest

Conor T. Buckley is an Editorial Board member of JOR Spine and co-author of this article. They were excluded from editorial decision-making related to the acceptance of this article for publication in the journal.

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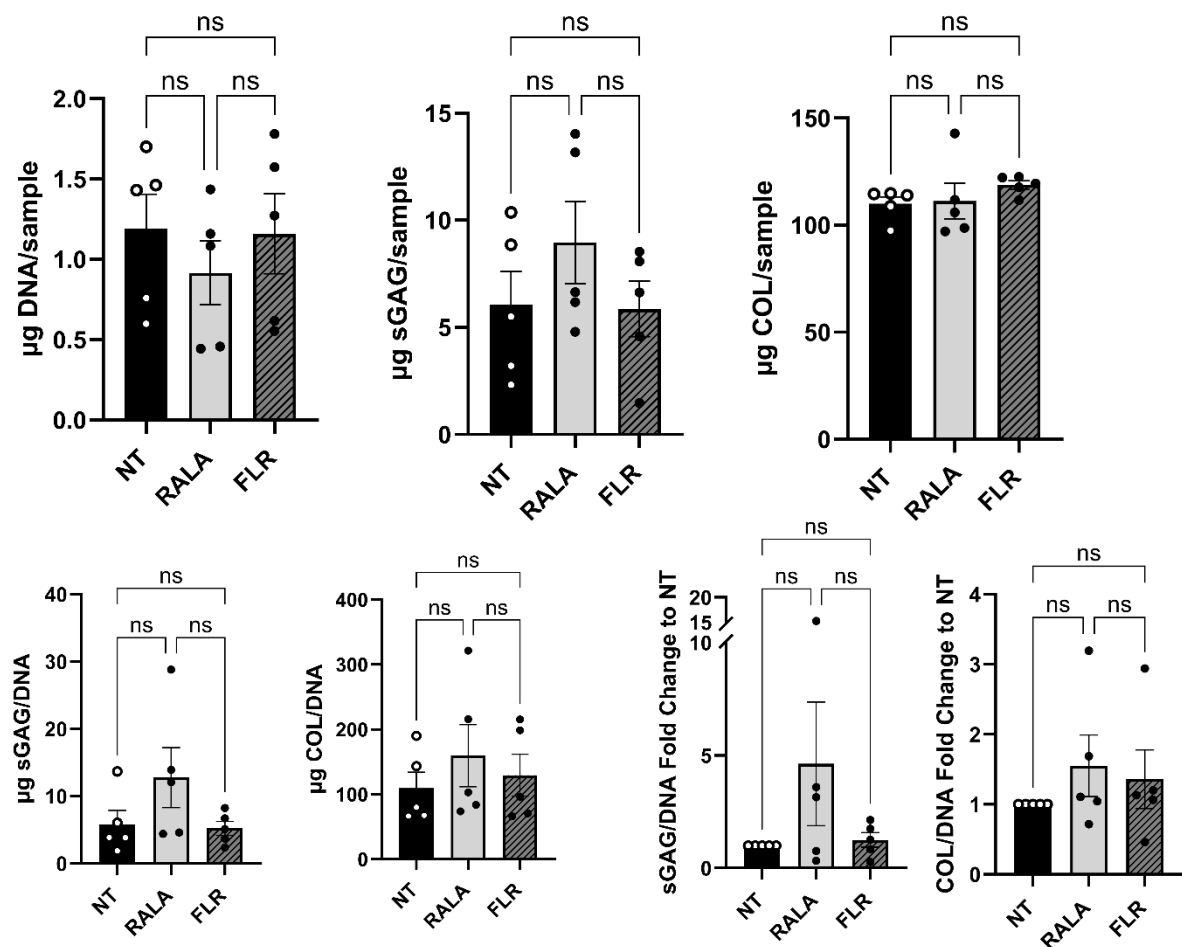
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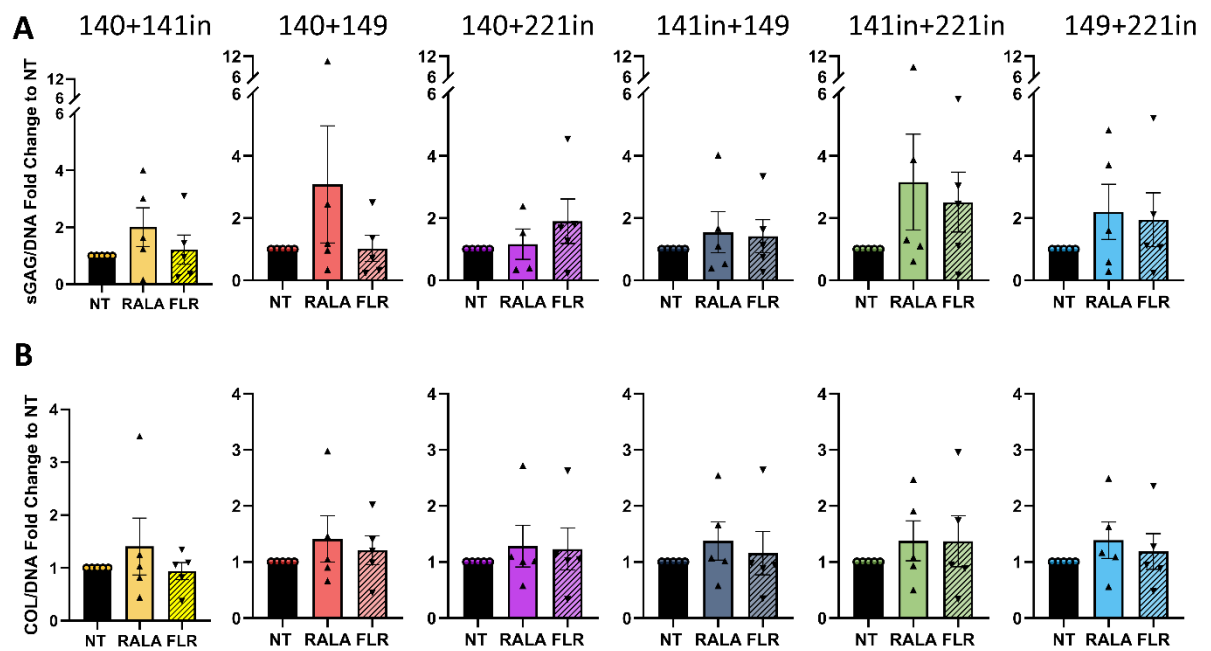
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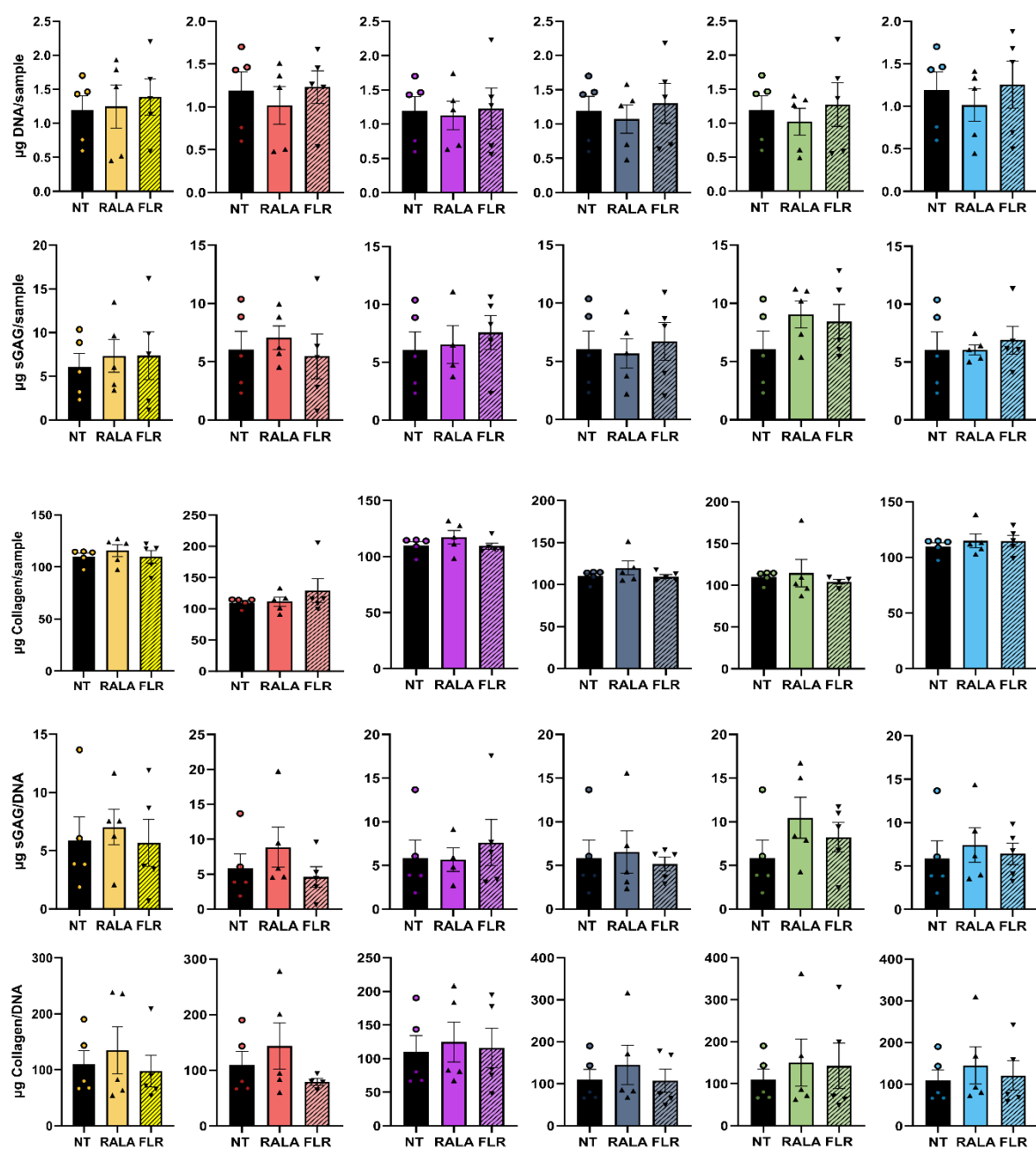
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Supplementary Figure 1. Transfection of scramble negative control miR did not significantly alter the production of DNA, sulphated glycosaminoglycans (sGAGs) or collagen in monolayer. Delivery of a negative control miR showed no significant changes to biochemical quantification 14 days post-transfection. N = 5, ns: non-significant.

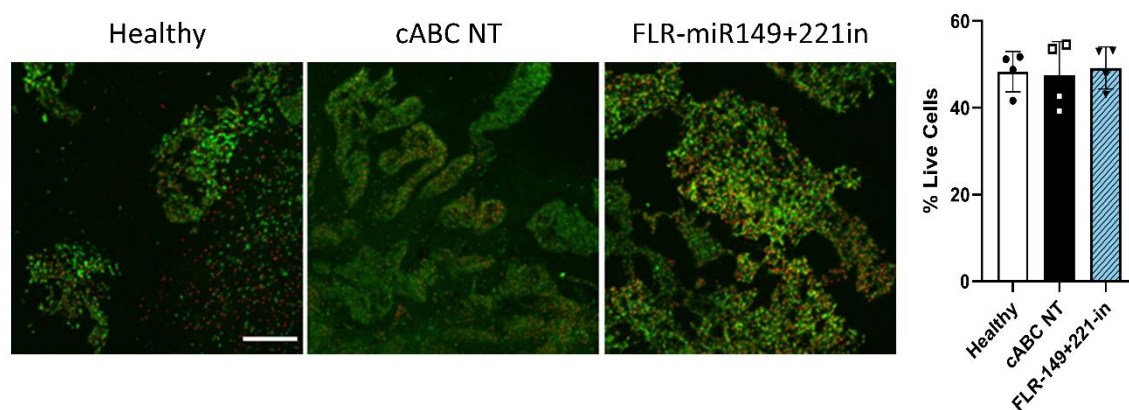


Supplementary Figure 2. Biochemical analysis of rat nucleus pulposus monolayer cultures following vector-miR pairing delivery demonstrated some positive increases 14 days post-transfection. (A) Sulphated glycosaminoglycans (sGAG) and (B) collagen (COL). Data is displayed as sGAG or COL normalised to DNA content and the non-transfected (NT) control. N=5.



Supplementary Figure 3. Biochemical Quantification of DNA, sulphated Glycosaminoglycan (sGAG), and Collagen (COL) levels 14 days post-transfection with RALA and FLR-delivered miR Combinations. N = 5.

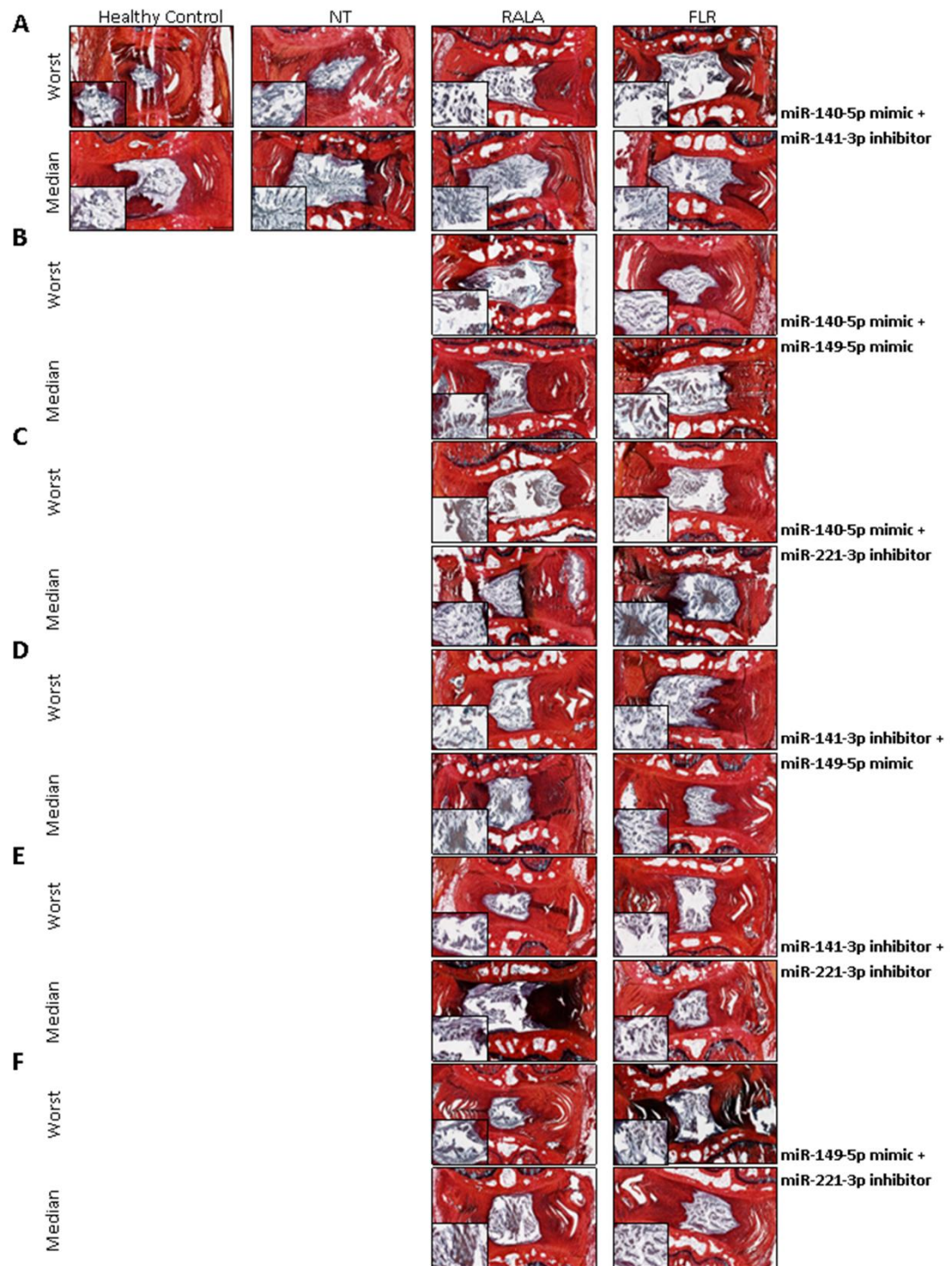
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955 **Supplementary Figure 4. Cell viability of rat caudal NP tissue following 21 days of ex**
 956 **vivo organ culture. A.** Representative images of maximum projection of z-stacks of
 957 Calcein AM (green, live) and Ethidium homodimer (red, dead) staining. **B.** Viability
 958 assessment of live and dead cells. Healthy discs were non-degenerated. Chondroitinase
 959 ABC (cABC) NT and FLR-miRNA-149+221-in groups were mildly degenerated with
 960 cABC for 7 days prior to Sham or miRNA-pair treatment. Scale bar is 150 μ m. N=4.

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Supplementary Figure 5. Median and Worst Representative Histological Images of rat motion segments 14 days following transfection with miRs A. 140-5p + 141-3p inhibitor, B. miR-140-5p mimic + miR-149-5p mimic, C. miR-140-5p mimic + miR-221-3p inhibitor, D. miRs-141-3p inhibitor + 149-5p mimic, E. miR-141-3p inhibitor

+ miR-221-3p inhibitor, and F. miR-149-5p + miR-221-3p inhibitor following 14 days
in culture post-transfection. Alcian blue for glycosaminoglycan staining, Picrosirius red
for collagen staining. Main image scale bar 500 μm , inset 150 μm . N=3