

miR-122 and the WNT/ β -catenin pathway inhibit effects of both interleukin-1 β and tumor necrosis factor- α in articular chondrocytes in vitro

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Abstract

Interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), and WNT/ β -catenin signaling cause dysregulation of rat primary articular chondrocytes (rArCs), resulting in cartilage extracellular matrix destruction and osteoarthritis (OA) progression. microRNA (miR) miR-122 represses these effects whereas miR-451 exacerbates IL-1 β -stimulated matrix metalloproteinase-13 (MMP-13) and prostaglandin E2 (PGE2) production. The goals of this study were to evaluate crosstalk between these signaling pathways and determine if miR-122 and miR-451 exert their protective/destructive effects through these pathways in an in vitro model of OA. Primary rArCs were treated with IL-1 β or TNF- α for 24 h and total DNA, MMP-13, and PGE2, as well as expression levels of miR-122 and miR-451 were measured. After 24-h transfection with miR-122, miR-451, miR-122-inhibitor, or miR-451-inhibitor, rArCs were treated with or without TNF- α for 24 h; total DNA, MMP-13, and PGE2 were measured. Similarly, cells were treated with WNT-agonist lithium chloride (LiCl), WNT-antagonist XAV-939 (XAV), or PKF-118-310 (PKF) with and without IL-1 β or TNF- α stimulation. Both IL-1 β and TNF- α -stimulation increased MMP-13 and PGE2 production. Transfection with miR-122 prevented TNF- α -stimulated increases in MMP-13 and PGE2 whereas transfection with miR-451 did not change these levels. No differences were found in MMP-13 or PGE2 production with miR-122 or miR-451 inhibitors. LiCl treatment decreased PGE2 production in cultures treated with TNF- α , but not MMP-13. XAV increased TNF- α -stimulated increases in PGE2 but not MMP-13. LiCl reduced IL-1 β -stimulated increases in MMP-13 and PGE2. XAV and PKF increased IL-1 β -stimulated increases in MMP-13 and PGE2. In this in vitro OA model, miR-122 protects against both IL-1 β and TNF- α stimulated increases in MMP-13 and PGE2 production. miR-451 does not act through the TNF- α pathway. The WNT/ β -catenin pathway regulates the effects of IL-1 β and TNF- α

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stimulation. This study suggests that miR-122 can be used as a treatment or prevention for OA.

KEYWORDS

chondrocytes, interleukin-1 β , miR-122, miR-451, osteoarthritis, tumor Necrosis Factor- α , WNT/ β -catenin

1 | INTRODUCTION

Osteoarthritis (OA) is estimated to affect 30.8 million adults in the United States alone and 300 million worldwide.^{1,2} This disease is characterized by articular cartilage damage, bone spurs, eburnation, and pain.^{1,3} To date, there are no disease-modifying therapeutics, only strategies to slow OA progression through pain management, regenerative medicine approaches, and ultimately joint replacement.²

On a microscale, a phenotypic shift of the normally quiescent articular chondrocytes leads to the aberrant expression of pro-inflammatory and catabolic pathways,^{4,5} which are hypothesized to contribute to OA progression. Of note, interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) are key players disrupting cartilage homeostasis and driving inflammatory pathway activation.^{6,7} Both cytokines are elevated in the synovial fluid of OA patients,^{5,8,9} and can remain chronically elevated more than 1 year after an injury to the anterior cruciate ligament (ACL).⁵ These pathways become dysregulated and form a positive feedback loop, increasing their own production and that of other inflammatory cytokines such as prostaglandin E2 (PGE₂); matrix-degrading proteases, including a disintegrin and metalloproteinase with thrombospondin motifs-4 and -5 (ADAMTS-4, ADAMTS-4) and matrix metalloproteinase-1, -3, and -13 (MMP-1, MMP-3, MMP-13).

As both IL-1 β and TNF- α signaling pathways converge on the inflammatory nuclear factor κ B (NF- κ B) signaling pathway, it is not surprising that increases in many of the same inflammatory and catabolic markers are seen. Their involvement in OA progression has been well documented. Interestingly, TNF- α stimulation has been positively linked to an increase in canonical WNT-3a and WNT-7a and IL-1 β stimulation positively increases β -catenin,^{10,11} which also signals through the NF- κ B signaling pathway during inflammation.¹² Evidence suggests that the WNT/ β -catenin signaling pathway plays a pivotal role in OA progression as well. Both mediators and downstream targets of WNT/ β -catenin signaling are elevated in OA.¹³ Canonical and non-canonical WNT signaling is linked to the expression of MMP-1, MMP-3, MMP-13, MMP-14, ADAMTS-4, and

ADAMTS-5, and induces cartilage degradation.¹⁴ Additionally, inhibiting WNT/ β -catenin using PKF115-584, and PKF118-310 block IL-1 β and TNF- α induced cartilage degradation,¹⁵ and inhibition of WNT/ β -catenin using XAV-939 in a destabilization of the medial meniscus model of OA in mice reduced cartilage degradation.¹⁶

Increasing evidence suggests that disease pathologies can be enhanced or inhibited through microRNA (miR) regulation,^{17,18} and specific microRNAs such as miR-17-5p, miR-21-5p, miR-101, and miR-451a are elevated in OA.^{19–21} Recent findings determined that miR-122-5p prevents the associated increase in MMP-13, PGE₂, and other cytokines implicated in OA in primary rat articular chondrocytes (rArCs) upon stimulation with IL-1 β .²⁰ Conversely, miR-451-5p exacerbated the increase of MMP-13, PGE₂, and numerous other cytokines, but only in the presence of IL-1 β .²⁰ These microRNAs were found elevated 10 weeks following an ACL transection model of OA in Sprague Dawley rats, suggesting they are playing a role in the disease pathology. To date, these microRNAs have only been examined after IL-1 β stimulation, and how they may be interacting with the TNF- α and WNT/ β -catenin signaling pathways has yet to be elucidated.

The potential interaction among IL-1 β , TNF- α , and WNT/ β -catenin signaling pathways, as well as the overwhelming evidence of their roles in driving OA progression make them attractive options to explore the mechanism of action of miR-122 and miR-451. In the present study, we used an *in vitro* model of OA to evaluate the ability of miR-122 and miR-451 to modulate the effects of IL-1 β and TNF- α on OA markers in rArCs. In addition, we assessed the crosstalk between IL-1 β or TNF- α with WNT/ β -catenin on these markers to better understand the interaction of these signaling pathways in the development and progression of the disease.

2 | MATERIALS AND METHODS

2.1 | Cell isolation and culture

rArCs were isolated using a previously established protocol.^{20,22,23} All animal procedures were approved

by Virginia Commonwealth University's Institutional Animal Care and Use Committee. Briefly, rArCs were isolated from femoral condyles and tibial plateaus from 100 to 125 g male Sprague Dawley rats. Cartilage was sterilely sliced from the articular surfaces using a surgical blade and washed in Dulbecco's modification of Eagle's medium with 1 g/L glucose, 50 U/ml penicillin and streptomycin (pen-strep) (DMEM), incubated with 0.05% trypsin (20 min, 37°C), rinsed with DMEM (50 U/ml pen-strep, 20 min, 37°C), and then digested with sterile-filtered 0.03% collagenase type II solution (Worthington Biochemical) in Hank's balanced salt solution (16 h, 37°C). Digestion was quenched with an equal volume of DMEM containing 10% fetal bovine serum (FBS) and 50 U/ml pen-strep, strained through a 40- μ m strainer (Greiner Bio-One), spun down at 500g for 10 min, and plated at 15 000 cells/cm². These cells are at passage 0. Primary cells harvested for all experiments were collected from eight animals and pooled together. All experiments used Passage 1 cells.

2.2 | Cell culture

rArCs were plated at 15 000 cells/cm² and fed with DMEM supplemented with 10% FBS and 50 U/ml pen-strep. Media were changed 24 h after plating and then every 48 h.

After treatments described below, conditioned media were collected, centrifuged at 1000g for 10 min to remove cellular debris, aliquoted, and stored at -80°C until assayed. Cell monolayers were rinsed twice with 1 ml of 1 \times phosphate-buffered saline (PBS), lysed in 500 μ l of 0.05% Triton X-100, and frozen for later DNA analysis. MMP-13 (ANASpec) and PGE2 (R&D Systems) were measured in the conditioned media using enzyme-linked immunosorbent assays in accordance with the manufacturer's directions. Cell layers were thawed and mechanically lysed by ultrasonication (40 V, 10 s/well) (VCX-120; Vibra-cell), and DNA was measured using the QuantiFluor dsDNA system kit (Promega) and quantified on a fluorescence plate reader at ex/em 504 nm/531 nm. This kit contains a double-stranded DNA-binding fluorescent dye that does not bind to single-stranded DNA, RNA, or protein and therefore allows for quantitation of small amounts of double-stranded DNA. Data were normalized to the DNA content of the cell monolayer.

2.3 | Mimic and inhibitor transfections

The concentration of the miRNAs, transfection efficiency, and cytotoxicity studies were all performed and

previously established. Cell transfection was performed using this previously established protocol.²⁰ Briefly, at 60% confluence, rArCs were transfected for 24 h with 14.5 nM of mirVana miRNA mimic rno-miR-122-5p or rno-miR-451-5p (Thermo Fisher Scientific) using 0.2% lipofectamine RNAiMAX (Lipo; Life Technologies) in antibiotic-free DMEM supplemented with 10% FBS.²⁴ Lipo groups served as a negative control and contained 0.2% Lipo. LNA Inhibitor transfection was performed with rno-miR-122-5p miRCURY or rno-miR-451-5p miRCURY LNA miRNA-inhibitors (Qiagen). Cells were transfected at 60% confluence using 0.2% Lipo and 30 nM inhibitor for 24 h. Following mimic or inhibitor transfection, fresh media with or without 10 ng/ml TNF- α (Peprotech) were added for 24 h and then harvested as described above.

2.4 | WNT agonist experiments

The canonical WNT agonist lithium chloride (LiCl) was obtained from Millipore Sigma. Dosing concentrations were determined from a previously published protocol.²⁵ rArCs were treated at 80% confluency with 0, 5, or 10 mM LiCl for 24 h. Following treatment, media were replaced with fresh media with or without 10 ng/ml of IL-1 β (Peprotech) or 10 ng/ml TNF- α for 24 h and then harvested as described above.

2.5 | WNT inhibitor experiments

XAV-939 (XAV) and PKF-118-310 (PKF) were obtained from Millipore Sigma. XAV is a tankyrase 1 and 2 inhibitor that works by stabilizing AXIN2 in the cytosol, while PKF selectively inhibits β -catenin from complexing with TCF-4 in the nucleus.²⁶ Concentrations for XAV^{27,28} and PKF²⁹ were chosen based on previously published studies. rArCs were treated at 80% confluence with 0, 0.5, or 1 μ M of XAV or 0, 0.1, 0.5, or 1 of PKF, or with or without 10 ng/ml of IL-1 β or 10 ng/ml TNF- α for 24 h and then harvested as described above. IL-1 β and TNF- α were added to culture media at the same time as WNT inhibitors to assess the interactions of their pathways.

2.6 | Gene expression

To determine if TNF- α stimulation increases miR-122 or miR-451 expression, rArCs were grown to 80% confluence and treated with or without 10 ng/ml TNF- α for 12 h. RNA was isolated from cells using the Qiagen

TABLE 1 Primer sequences used in real-time qPCR analyses.

Gene name	Accession number	Forward primer	Reverse primer
Rno-miR-122-5p		TGGAGTGTGACAATGGTGT	NA
Rno-miR-451-5p		AAACCGTTACCATTACTGAGTT	NA
RNU6-1	NR_004394.1	GUGCUCGCUUCGGCAGCACAUAUACUAAAAUU GGAACGAUACAGAGAAGAUUAGCAUGGCC CUGCGCAAGGAUGACACGCAAUUCGUGAA GCGUCCAUAUUUU	NA

RNeasy mini kit, quantified (Tank3 Micro-Volume Plate; Biotek), and cDNA was generated using the miScript II RT Kit (Qiagen). Quantitative PCR was performed using miScript SYBR® Green PCR Kit (Qiagen) with miR-specific primers (Table 1) and normalized to U6 small nuclear 1 (RNU-6, Mysticq® microRNA qPCR Control Primer; Sigma-Aldrich).

2.7 | Statistical analysis

Data presented in the figures are from single representative experiments, and are represented as mean \pm standard error ($n = 6$ independent cultures in the well plate per group). Each experiment was repeated independently a minimum of two times to ensure the validity of the results. A one-way analysis of variance was performed followed by a two-tailed Tukey correction using an α equal to 0.05. All statistical analyses were performed using GraphPad Prism version 5.04 or JMP Pro 14.

3 | RESULTS

3.1 | Dose-dependent treatment with TNF- α

To investigate the effects of TNF- α on MMP-13 and PGE2 production, we performed a dose-dependent treatment with TNF- α . There were no differences in DNA between the control and treatment groups (Figure 1A). An increase was seen in MMP-13 after IL-1 β treatment (Figure 1B). Treatment with both 5 and 10 ng/ml of TNF- α caused a significant increase in MMP-13 production compared to both IL-1 β treated and control groups (Figure 1B). Similarly, IL-1 β , as well as both 5 and 10 ng/ml of TNF- α treatment significantly increased PGE2 production compared to control levels, with the greatest increase in the 10 ng/ml TNF- α treated group (Figure 1C). A concentration of 10 ng/ml TNF- α was used for later experiments. No differences were seen in miR-122 or

miR-451 expression after TNF- α or IL-1 β stimulation (Figure 1D,E).

3.2 | miR-122 and miR-451 regulation of TNF- α -stimulated effects

Previous data showed that transfection with miR-122 was able to inhibit the IL-1 β -stimulated increase in MMP-13 and PGE2 while transfection with miR-451 exacerbated the production of these two molecules.²⁰ However the effects of these microRNA on TNF- α -dependent MMP-13 or PGE2 have not been tested. To examine the effects of these microRNAs on the inflammatory TNF- α response, cell cultures were transfected with microRNAs and then stimulated with TNF- α . miR-122 transfection increased total DNA while miR-451 transfection reduced total DNA compared to all groups (Figure 2A). Treatment with TNF- α decreased total DNA in the lipo control and miR-122 treatment groups but did not change total DNA in miR-451-transfected cells (Figure 2A). TNF- α stimulation increased both MMP-13 and PGE2 compared to its respective controls (Figure 2B,C). Transfection with miR-122 prevented the TNF- α -stimulated increase in MMP-13 production (Figure 2B); interestingly while transfection with miR-122 decreased PGE2 production compared to the TNF- α stimulated control, it did not rescue it completely back to non-TNF- α stimulated control levels (Figure 2C). Conversely, transfection with miR-451, with or without TNF- α stimulation, did not change MMP-13 or PGE2 levels compared to control (Figure 2B,C).

Next, we evaluated inhibiting miR-122 and miR-451 with and without TNF- α stimulation. There were no changes in total DNA in any of the treatment groups (Figure 2D). TNF- α stimulation increased both MMP-13 and PGE2 compared to its respective controls (Figure 2E,F). Inhibition of both miR-122 and miR-451 did not change MMP-13 or PGE2 production (Figure 2E,F). Because the microRNAs had differing effects in the IL-1 β -stimulated cultures compared to the TNF- α -stimulated cultures, and it is known that IL-1 β , TNF- α , and the WNT/ β -catenin pathways play predominant

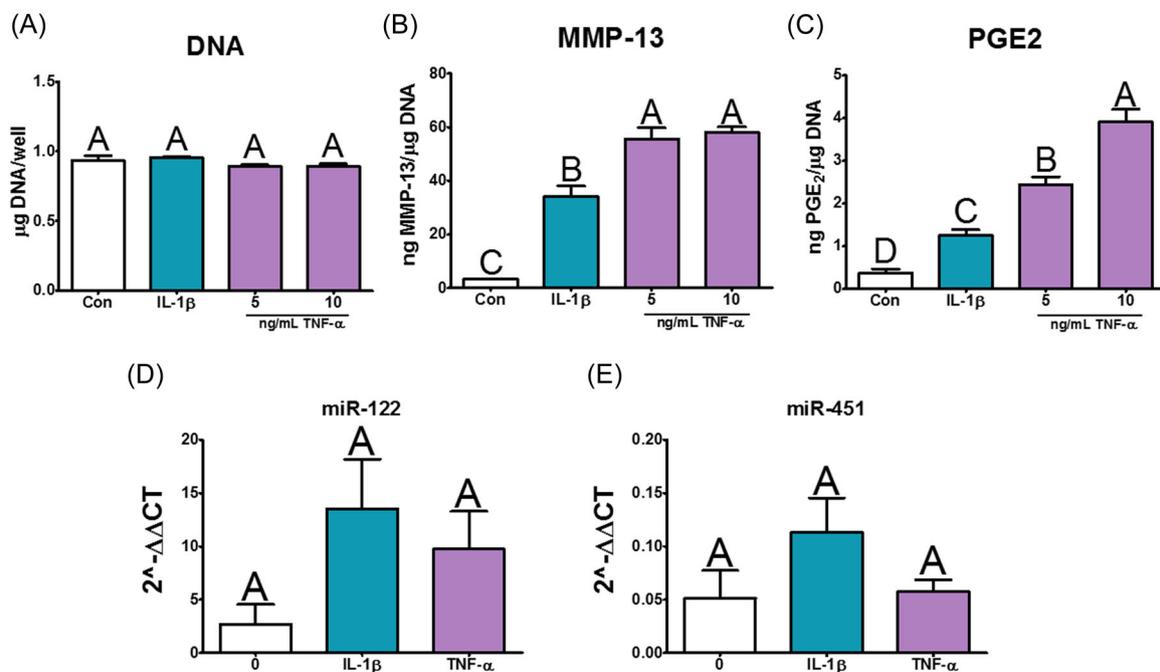


FIGURE 1 Stimulation with IL-1 β and TNF- α . rArCs were treated with 10 ng/ml IL-1 β , 5 or 10 ng/ml of TNF- α , or nothing (con) for 24 h. Total DNA (A) was measured from the cell monolayer and MMP-13 (B) and PGE2 (C) proteins were measured from the conditioned media. Gene expression of miR-122 (D) and miR-451 (E) were measured following IL-1 β and TNF- α stimulation. Groups not sharing a letter are statistically significant using a one-way analysis of variance with a Tukey's correction using an $\alpha = 0.05$. IL-1 β , interleukin-1 β ; MMP-13, matrix metalloproteinase-13; PGE2, prostaglandin E2; rArC, rat articular chondrocyte; TNF- α , tumor necrosis factor- α .

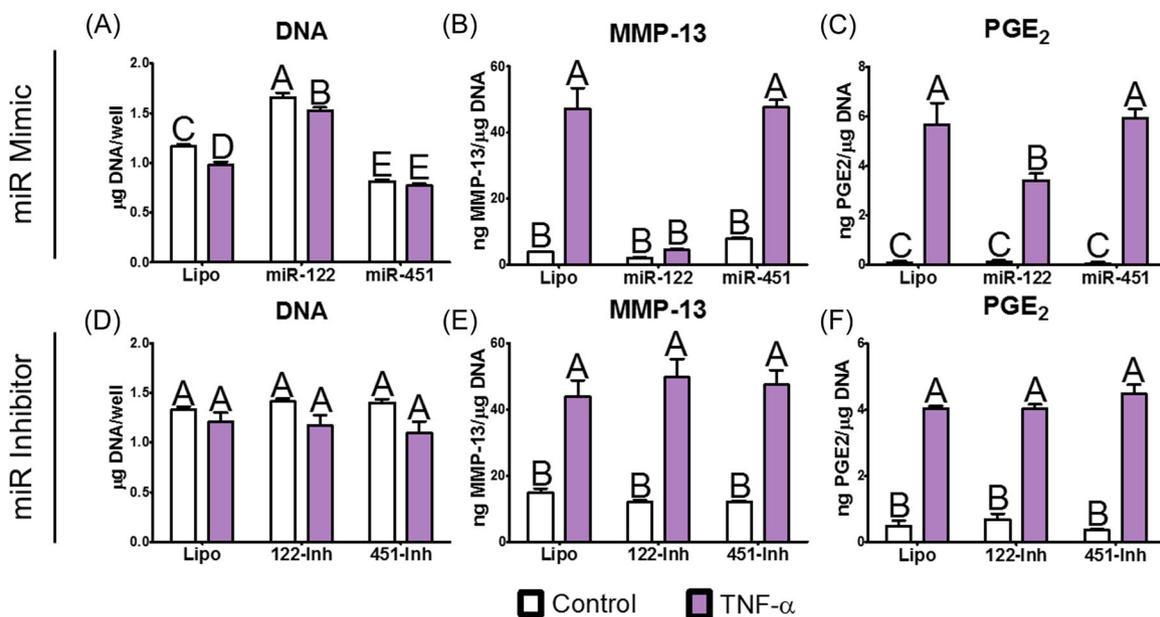


FIGURE 2 Assessment of miR-122 and miR-451 overexpression or inhibition on TNF- α stimulated rat articular chondrocytes. rArCs were transfected at 60% confluence with miR-122, miR-451, an miR-122-inhibitor (122-Inh), an miR-451 inhibitor (451-Inh), or an empty vehicle (Lipo) for 24 h followed by 24 h with or without 10 ng/ml TNF- α stimulation. Total DNA (A, D) was measured from the cell monolayer and MMP-13 (B, E) and PGE2 (C, F) proteins were measured from the conditioned media. Groups not sharing a letter are statistically significant using a one-way ANOVA with a Tukey's correction using an $\alpha = 0.05$. MMP-13, matrix metalloproteinase-13; PGE2, prostaglandin E2; rArC, rat articular chondrocyte; TNF- α , tumor necrosis factor- α .

roles in OA progression, the crosstalk between these pathways was then examined in subsequent experiments.

3.3 | WNT/ β -catenin crosstalk with the TNF- α pathway

To evaluate the crosstalk between the TNF- α and WNT/ β -catenin pathways, cultures were treated with the WNT/ β -catenin agonist, LiCl, or an antagonist, XAV, with or without TNF- α treatment (Figure 3). Treatment with TNF- α did not change total DNA in the control group (Figure 3A). Both the 5 and 10 mM treatment with LiCl decreased total DNA to similar levels (Figure 3A). The expected increases in MMP-13 and PGE2 production were seen after stimulation with TNF- α in the control groups (Figure 3B,C,E,F). There were no differences in MMP-13 in either the 5 or 10 mM LiCl group with or without TNF- α treatment compared to their respective controls. There was a decreasing trend in MMP-13 production with increased concentrations of LiCl in the absence of TNF- α ; however, it was not significantly different due to the variability in the TNF- α treated groups. When analyzed using only the control groups, there is a significant difference between both 5 and 10 mM LiCl treated versus 0 control. Treatment with 5 or

10 mM LiCl alone did not change PGE2 production; however, a dose-dependent decrease was seen in the presence of TNF- α (Figure 3C). Treatment with the WNT inhibitor XAV caused a decrease in total DNA in the presence of TNF- α stimulation (Figure 3D). Treatment with XAV alone did not change MMP-13 compared to control levels. In all, 0.5 μ M XAV decreased the TNF- α -stimulated increase in MMP-13, but this effect was not present in the 1 μ M XAV + TNF- α group (Figure 3E). Conversely, XAV had a greater stimulatory effect on PGE2 production on its own and caused increases in both the 0.5 and 1 μ M XAV groups (Figure 3F). This stimulatory effect acted synergistically in the presence of TNF- α stimulation (Figure 3F).

3.4 | WNT/ β -catenin crosstalk with the IL-1 β pathway

To evaluate the crosstalk between the IL-1 β and WNT/ β -catenin pathways, cultures were treated with the WNT/ β -catenin agonist, LiCl, or with two different antagonists, XAV or PKF with or without IL-1 β treatment (Figure 4). Both the 5 and 10 mM LiCl treatment decreased total DNA as previously seen (Figure 4A). Stimulation with IL-1 β increased both MMP-13 and PGE2 as expected

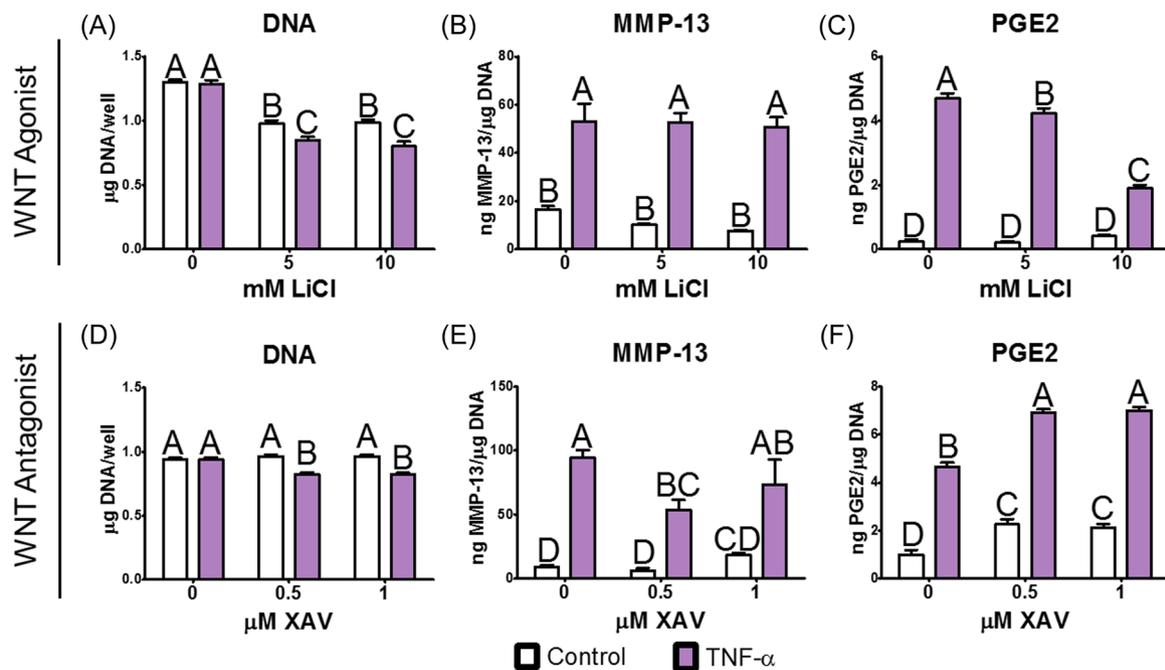


FIGURE 3 Assessment of crosstalk between WNT/ β -catenin and TNF- α pathway. rArCs were treated at 80% confluence with WNT/ β -catenin agonist lithium chloride (LiCl) or WNT/ β -catenin antagonist XAV-939 (XAV) for 24 h followed by 24 h with or without 10 ng/ml TNF- α stimulation. Total DNA (A, D) was measured from the cell monolayer and MMP-13 (B, E) and PGE2 (C, F) proteins were measured from the conditioned media. Groups not sharing a letter are statistically significant using a one-way ANOVA with a Tukey's correction using an $\alpha = 0.05$. MMP-13, matrix metalloproteinase-13; PGE2, prostaglandin E2; rArC, rat articular chondrocyte; TNF- α , tumor necrosis factor- α .

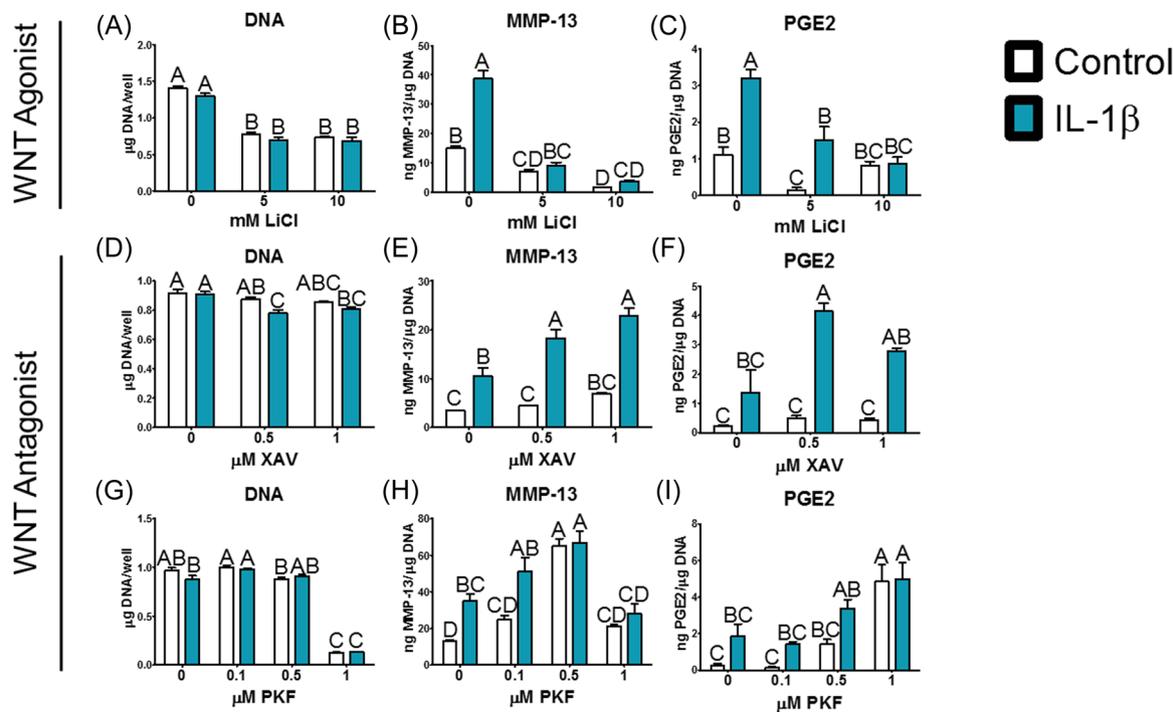


FIGURE 4 Assessment of crosstalk between WNT/ β -catenin and IL-1 β pathway. rArCs were treated at 80% confluence with WNT/ β -catenin agonist lithium chloride (LiCl) or WNT/ β -catenin antagonists XAV-939 (XAV) or PKF-118 (PKF) for 24 h followed by 24 h with or without 10 ng/ml IL-1 β stimulation. Total DNA (A, D, G) was measured from the cell monolayer and MMP-13 (B, E, H) and PGE2 (C, F, I) proteins were measured from the conditioned media. Groups not sharing a letter are statistically significant using a one-way ANOVA with a Tukey's correction using an $\alpha = 0.05$. IL-1 β , interleukin-1 β ; MMP-13, matrix metalloproteinase-13; PGE2, prostaglandin E2; rArC, rat articular chondrocyte.

(Figure 4B,C). Increasing concentrations of LiCl resulted in a decrease in MMP-13 production both in the presence and absence of IL-1 β (Figure 4B). The lowest production of PGE2 was observed at 5 mM LiCl in the control group, and the lowest production of PGE2 was observed at 10 mM LiCl in the IL-1 β group (Figure 4C). There was a significant decrease in total DNA with treatment of both XAV at 0.5 and 1 μ M when compared to IL-1 β -treated controls; only cells treated with 0.5 μ M XAV and IL-1 β were significantly decreased compared to cells treated with 0.5 μ M XAV alone (Figure 4D). There were no differences in MMP-13 or PGE2 with increasing concentrations of XAV alone; however, XAV treatment with IL-1 β -stimulation showed synergistic increases in both MMP-13 and PGE2, but this was not found in PGE2 at 1 μ M XAV (Figure 4E,F). Concentrations of 1 μ M PKF, but not 0.1 μ M or 0.5 μ M PKF, drastically decreased total DNA (Figure 4G). There was a dose-dependent increase in MMP-13 with increasing concentrations of PKF alone with the exception of the 1 μ M group (Figure 4H). An expected increase was seen in MMP-13 with IL-1 β -stimulation in the 0.1 μ M PKF, but this was not present in the 0.5 μ M and 1 μ M PKF IL-1 β -stimulation groups (Figure 4H). There was no difference in the 1 μ M PKF

cultures both with and without IL-1 β -stimulation compared to control cultures (Figure 4H). A dose-dependent increase in PGE2 production was seen with increasing concentrations of PKF (Figure 4I), with the largest increase in the 1 μ M PKF treated cultures, both with and without IL-1 β -stimulation (Figure 4I). The IL-1 β -stimulated increase in PGE2 was not seen in these cultures (Figure 4I).

4 | DISCUSSION

IL-1 β , TNF- α , and WNT/ β -catenin signaling are dysregulated in OA and research has indicated they drive OA progression. Numerous studies examine how these pathways each individually contribute to OA. Inhibition of TNF- α decreases OA severity in both spontaneously and surgically induced OA models in mice³⁰ and in experimentally induced OA in rabbits.³¹ Additionally, TNF- α inhibition promotes the repair of osteochondral lesions and has chondroprotective effects in vivo.³² However, Zwerina et al.³³ found that articular cartilage changes caused by overexpression of TNF- α are not fully blocked by either TNF- α or IL-1 inhibition alone, but the

combined blockade of both TNF- α and IL-1 lead to almost complete remission of the disease.³³ These observations suggest that multiple aberrant pathways influence OA progression. Our data support this hypothesis and indicate that there is crosstalk between them.

We took advantage of the differential regulation of catabolic processes in rArCs using two microRNA found in OA cartilage, miR-122 and miR-451, to examine whether they play a major role in this disease. These results indicate that miR-122 transfection was able to prevent the TNF- α stimulated increase in MMP-13 and, to a lesser extent, in PGE2. Taken together with our previous data,²⁰ miR-122's ability to prevent both inflammatory and catabolic responses to IL-1 β and TNF- α signaling makes it a powerful tool to control the dysregulated signaling associated with OA. In contrast, miR-451 appears to exacerbate the MMP-13 and PGE2 production only in IL-1 β -stimulated cultures²⁰ and does not affect the TNF- α -stimulated increases in these markers. Interestingly, there were no changes in MMP-13 or PGE2 when miR-122 or miR-451 were inhibited. This may be due to low endogenous expression levels of miR-122 in our culture system. The delivery vehicle lipofectamine may have an effect on cells as we observed in some of our control groups, perhaps necessitating a better microRNA delivery vehicle in the future.

WNT/ β -catenin signaling plays a predominant role in OA; however, this pathway remains extremely complex. There is a delicate balance of WNT signaling needed to maintain cartilage homeostasis. Elevated levels of β -catenin, WNT-5B, WNT-7B, WNT-10B, WNT-11, and WNT-16 have been found in OA.^{14,34,35} Both repression and prolonged activation of β -catenin cause articular cartilage degradation.³⁴ Preventing β -catenin degradation (therefore, more β -catenin signaling) resulted in an OA-like phenotype with articular cartilage loss and osteophyte formation.¹³ Conversely, inhibiting β -catenin also caused articular cartilage destruction.³⁴

Activation of β -catenin in mature chondrocytes has been linked to increased apoptosis.³⁶ We saw a decrease in total DNA with both the 5 and 10 mM LiCl treatment indicating there may have been an increase in apoptotic events with our treatment; however, DNA alone is not a specific measurement for apoptosis. There are conflicting reports of LiCl treatment and resulting MMP-13 expression in the literature. The effect of IL-1 β was inhibited by the treatment of LiCl. This was found in our results confirming previous reports using different systems.^{36,37} However, in our control, the cells with no treatment were also inhibited by LiCl. In some papers, the control cells were stimulated by LiCl.³⁶ In others, like Hui et al.³⁷ no effect was observed. We do not have an explanation why it is different, other than the system. We saw a decrease

in MMP-13 and PGE2 in LiCl-treated cultures with IL-1 β -stimulation, and a decrease in PGE2 in the LiCl- and TNF- α -stimulated cultures, but not in MMP-13. This may indicate that TNF- α stimulation causes an increase in MMP-13 through a slightly different signaling mechanism that the protective effects of LiCl were not able to mitigate. It is well known in the literature that there is crosstalk between the TNF- α and IL-1 β signaling pathways through NF-KB; however, where these pathways diverge leads to different responses and has not been elucidated to date. Similar to our findings, multiple studies have examined LiCl treatment in human and bovine chondrocytes and found a decrease in both MMP-13 gene expression and protein production in IL-1 β -stimulated cultures.^{25,37} In contrast, another study shows that LiCl treatment with IL-1 β stimulation increases MMP-13 expression in rabbit chondrocytes,³⁸ while others found an increased MMP-13 expression with LiCl treatment alone.³⁶ These conflicting results may be explained by the fact that both too little and too much β -catenin signaling can cause OA-phenotypical changes.

When we used the cytosolic β -catenin inhibitor XAV in the TNF- α -stimulated culture we saw an increase in PGE2 production, which agrees with our LiCl data. The effect on MMP-13 was minimal, similar to our findings in the LiCl-treated cultures. A similar trend was seen when we inhibited with both the cytosolic and nuclear inhibitor XAV or PKF in the IL-1 β -stimulated cultures. We observed a significant decrease of DNA with cells treated with 1 μ M PKF and hypothesize that this concentration may be cytotoxic. However, cells were robust and attached at the end of the experiments as observed by microscopy, indicating no overt cytotoxicity. Production of both MMP-13 and PGE2 was exacerbated in groups treated with both the inhibitor and IL-1 β compared to IL-1 β alone. These data suggest that there is crosstalk between TNF- α , IL-1 β , and WNT/ β -catenin signaling pathways and that targeting one pathway alone may not totally negate the inflammatory signaling found in OA.

Together, these data indicate complex communication between these pathways; LiCl has a protective effect against IL-1 β stimulation, while it can only protect against PGE2 expression, and not MMP13, in TNF- α stimulated cultures. Interestingly, miR-122 appears to target FOXO3 which modulates the WNT/ β -catenin signaling pathway,³⁹⁻⁴² which may explain miR-122's protective role in IL-1 β - and TNF- α -stimulated cultures. miR-451 transfection exacerbates the production of MMP-13 and PGE2 in the presence of IL-1 β stimulation, but has no inflammatory signaling in the absence of IL-1 β .²⁰ Furthermore, miR-451 had no effect in TNF- α -stimulated cultures, indicating it is targeting a specific

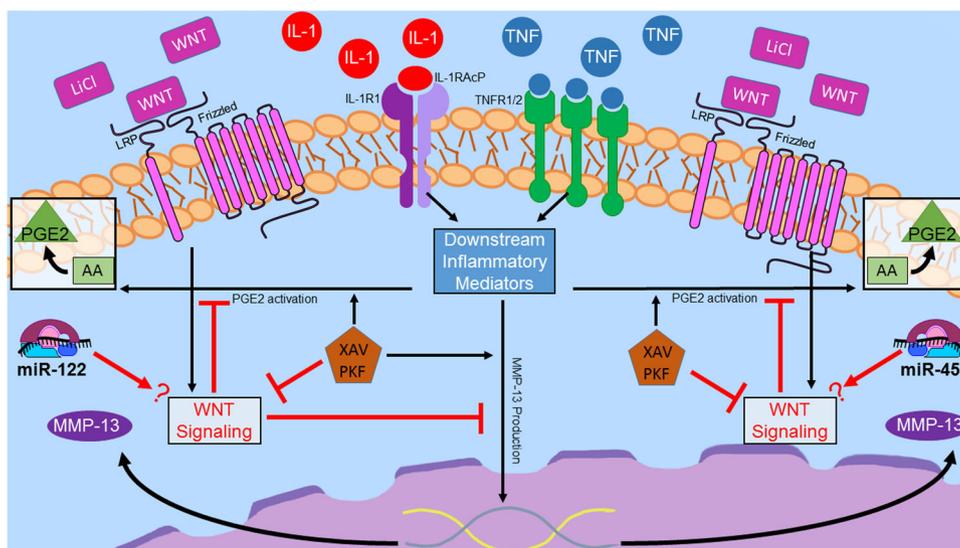


FIGURE 5 Proposed mechanism of WNT/ β -catenin signaling and inhibition in rat articular chondrocytes. WNT/ β -catenin agonist LiCl inhibits MMP-13 and PGE2 production when stimulated with IL-1 β , but only protects against the TNF- α -stimulated increases in PGE2 and not MMP-13. Inhibiting WNT/ β -catenin signaling using XAV or PKF exacerbated the production of MMP-13 and PGE2 in IL-1 β -stimulated cultures, but only PGE2 in TNF- α -stimulated cultures. How miR-122 and miR-451 are interacting with the WNT/ β -catenin signaling pathway remains to be elucidated. AA, arachidonic acid; IL-1 β , interleukin-1 β ; LiCl, lithium chloride; MMP-13, matrix metalloproteinase-13; PGE2, prostaglandin E2; TNF- α , tumor necrosis factor- α .

branch or inhibitor in the IL-1 β signaling pathway. It is not clear how the known targets of miR-122 and miR-451 are interacting with the IL-1 β and TNF- α pathways. We are currently using RISC complex pulldown experiments to determine the specific mRNA targets of the microRNA. The RISC complex is composed of proteins that allow the interaction of the microRNA with the mRNA.

A schematic that summarizes our study is presented in Figure 5. WNT/ β -catenin agonist LiCl inhibits MMP-13 and PGE2 production when stimulated with IL-1 β , but only protects against TNF- α -stimulated increases in PGE2 and not MMP-13. Inhibiting WNT/ β -catenin signaling using XAV or PKF exacerbated the production of MMP-13 and PGE2 in IL-1 β -stimulated cultures, but only PGE2 in TNF- α -stimulated cultures. How miR-122 and miR-451 are interacting with the WNT/ β -catenin pathway needs to be elucidated.

Future work will find the specific signaling mechanism that these two microRNAs are targeting to explain their protective and destructive effects in articular cartilage. This study examined two primary regulators of OA, IL-1 β and TNF- α , and how miRNAs or WNT signaling modulate their response. Future work will examine other mediators in the disease process, as well as examine how miRNAs directly interact with the WNT pathways in this process. Additionally, examining treatment with miR-122 and inhibition of miR-451 in an OA model in vivo would shed light on their roles in this complex disease pathology.

Transfection with miR-122 protects against both TNF- α and IL-1 β stimulation in vitro indicating treatment with miR-122 could be a viable therapeutic for reversing or preventing OA progression. Transfection with miR-451 only exacerbates IL-1 β and not TNF- α -stimulated signaling. Inhibition of miR-451 may still be a viable therapeutic for OA, but only by targeting the dysregulation in IL-1 β signaling and not TNF- α signaling. The effects of IL-1 β and TNF- α signaling is regulated by the crosstalk with WNT/ β -catenin signaling. Future studies will explore the clinical application of the use of miR-122 and the crosstalk between these microRNAs and the WNT/ β -catenin pathway.

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DATA AVAILABILITY STATEMENT

Not available.

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