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THERAPY

Targeting Extracellular miR-21-TLR7 Signaling Provides Long-Lasting Analgesia in Osteoarthritis

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Osteoarthritis (OA) is the most prevalent joint disorder associated with severe chronic pain. Although synovial inflammation is well correlated with pain severity, the molecular mechanism responsible for OA pain remains unclear. Here, we show that extracellular miR-21 released from synovial tissue mediates knee OA pain in surgical OA model rats. miR-21 was the most abundant among increased microRNAs (miRNAs) in the synovial tissue. miR-21 was released into extracellular space from the synovial tissue and increased in the synovial fluid. A single intra-articular injection of miR-21 inhibitor exerted long-term analgesia of knee OA pain, whereas miR-21 injection in naive rats caused knee joint pain. miR-21 mutant, which lacks the Toll-like receptor (TLR) binding motif, but not in the seed sequence, did not cause joint pain, suggesting a non-canonical mode of action different from translational repression. Consistent with this, the algesic effect of miR-21 was blocked by antagonizing TLR7. The TLR7 antagonist also exerted a long-lasting analgesic effect on knee OA pain. Therefore, extracellular miR-21 released from synovial tissue mediates knee OA pain through TLR7 activation in surgical OA model rats. Extracellular miRNA in the joint may be a plausible target for pain therapy, providing a novel analgesic strategy for OA.

INTRODUCTION

The prevalence of knee osteoarthritis (OA) pain is very high, ranging from 7% to 19%.[1] Pain relief strongly reduces distress even if the radiographic OA persists.[2] However, current drug therapies have a low treatment effect associated with life-threatening adverse effects,[3,4] thereby necessitating joint replacement. How joint pain is induced in OA is poorly understood. Recently, inflammation of the synovial tissue lining the joint cavity,[5] in addition to bone marrow lesions and denuded subchondral bone, was revealed to correlate with OA pain.[3,6,7] It is reported that knee OA pain increased even if radiographic change did not increase.[2,3] The molecular mechanisms underlying synovial inflammation have been well characterized,[3] although involvement of synovial tissue in OA pain is poorly understood. Therefore, it is important to investigate the molecular mechanisms of OA pain, with a special focus on the synovial tissue.

microRNA (miRNA) is an approximately 22-nt-long non-coding RNA, which post-transcriptionally regulates the expression of multiple specific genes.[8] Previous studies have shown that miRNAs are involved in cartilage degeneration[9] and synovitis in the OA.[10] miRNAs are also shown to be an important regulator of a variety of pain disorders through inhibiting the translation of multiple specific genes.[11-13] However, the roles of miRNAs in OA pain remain unknown,[14] although changes in the levels of several miRNAs were reported in neural systems in a OA model rat.[15]

Here, we show that OA pain is mediated by extracellular miR-21 released from synovial tissue. miR-21-induced knee pain was mediated through a non-canonical function that extracellular miR-21 activated Toll-like receptor (TLR) 7 as a ligand, consistent with a potential of miRNAs containing a GU-rich motif that is essential for miRNA recognition by TLR.[16] Interestingly, a single intra-articular injection of a miR-21 inhibitor or TLR7-9 antagonist provided the long-term relief of pain and therefore may be a novel therapeutic approach for OA.

RESULTS

Anterior cruciate ligament transection (ACLT) was performed on 7- to 8-week-old male rats to develop a knee arthralgia model, a commonly used OA model.[17] Joint line tenderness was measured because of its relevance even in early OA patients.[18] After ACLT, knee line tenderness developed and was maintained for at least 56 days (Figure 1A). We also assessed mechanical allodynia in the plantar surface, which is commonly examined in the OA model rat[19-21] because the primary sensory neurons innervate both the knee joint and foot skin.[22] Mechanical allodynia was observed after ACLT (Figure 1B). To investigate changes in miRNA expression in the synovial tissue, we performed miRNA microarray at day 14 after ACLT and revealed that several miRNA expressions were changed
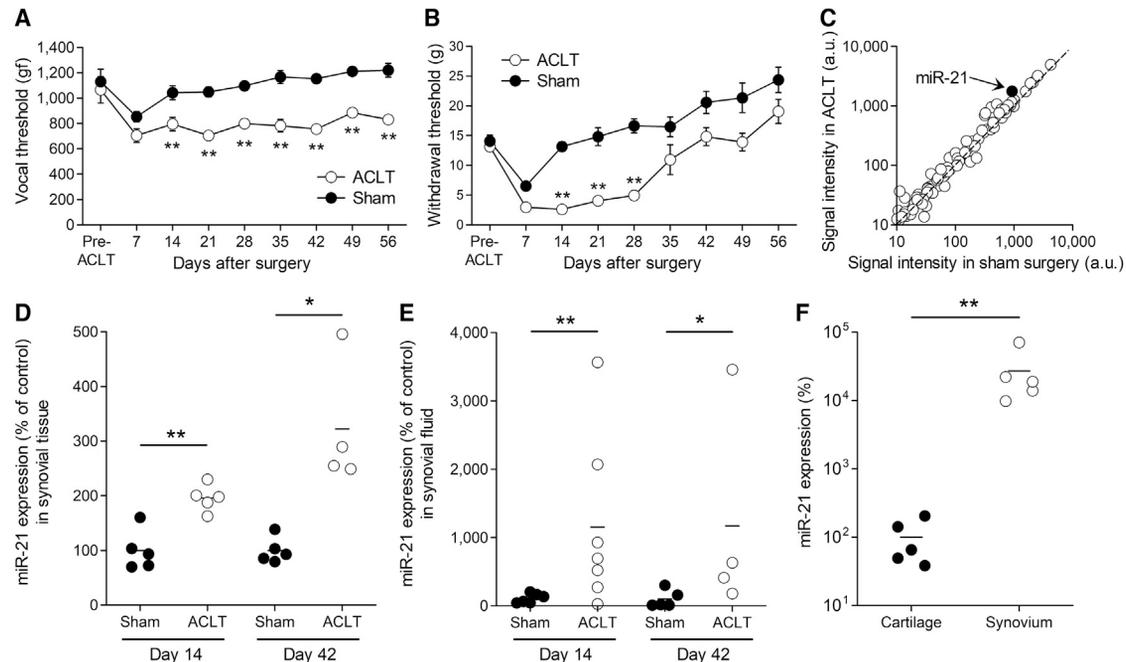
Figure 1. miR-21 Is Increased in the Synovial Tissue and Fluid of Knee OA

(A and B) The vocal threshold for knee pressure (A) and withdrawal threshold in response to mechanical stimuli (B) were measured in rats after ACLT (n = 5 rats per group). Data are represented as mean ± SEM. (C) miRNA microarray was performed in the synovial tissue 14 days after ACLT. Each symbol represents the mean signal intensity for individual miRNAs (n = 4 rats per group). A closed circle represents miR-21. (D and E) miR-21 expression was measured using qPCR in the synovial tissue (D; n = 5 rats per each group at day 14, n = 5 rats for sham surgery group and n = 4 rats for ACLT group at day 42) and synovial fluid (E; n = 6 rats for sham surgery group and n = 7 rats for ACLT group at day 14, n = 5 rats for sham surgery group and n = 4 rats for ACLT group at day 42) 14 and 42 days after ACLT and was shown as a percentage of the mean expression after sham surgery. (F) miR-21 expression was measured using qPCR in the culture medium of synovial tissue and was shown as a percentage of the mean expression in the culture medium of cartilage (n = 5 rats per group). Horizontal line represents the mean in each group. *p < 0.05, **p < 0.01.

more than 1.5-fold (Figure 1C; Table S1). We chose to analyze the role of miR-21 in joint pain after ACLT because it had the highest signal intensity among the increased miRNAs. Interestingly, miR-21 in primary sensory neurons is involved in neuropathic pain caused by traumatic nerve injury.[11,23] qPCR confirmed that miR-21 expression in synovial tissue was significantly increased 14 and 42 days after ACLT (Figure 1D). Because miR-21 is released extracellularly,[24] we also investigated the miR-21 content in the synovial fluid, where the synovial tissue is the main source of miRNA.[25] miR-21 content in the synovial fluid was increased 14 and 42 days after ACLT as well (Figure 1E). Consistent with this increase *in vivo*, extracellular miR-21 was abundantly detected in culture media from synovial tissue rather than cartilage (Figure 1F), suggesting miR-21 is mainly released from synovial tissue compared with cartilage.

To examine whether extracellular miR-21 plays a role in knee line tenderness and mechanical allodynia of OA model rats, we injected a miR-21 inhibitor without any cell-penetrating vehicle into the articular cavity of the left knee 14 days after ACLT. After a single injection of the nuclease-resistant miR-21 inhibitor, knee joint line tenderness was strongly and persistently alleviated at least for 3 weeks (Figure 2A). Mechanical allodynia was also suppressed after the injection of the miR-21 inhibitor (Figure 2B).

To investigate the causal involvement of miR-21 in knee joint pain, we injected miR-21 into the knee articular cavity of naive rats. Previous studies reported that extracellular miR-21 activated TLR7 as a ligand,[26–28] although its pathophysiological significance remains elusive. TLR7 recognizes extracellular guanosine- and uridine-rich single-stranded RNA including miR-21.[26,29] Therefore, we used mutant miR-21 that contains a 1-nt substitution in the GUUG motif outside of seed sequence[16] (Figure 2C), thus reducing its ability to activate TLR,[26] as a control. Transient mechanical allodynia with early onset developed after the intra-articular injection of miR-21 in naive rats, but not mutant miR-21 (Figures 2D and 2E). This acute effect also supports the idea that miR-21-induced knee joint pain is mediated by a noncanonical mode of miRNA action, because miRNA actions generally occur in a delayed fashion because of the nature of translational repression. Interestingly, a single injection of miR-21 caused only transient pain, in contrast with nuclease-resistant miR-21 inhibitor, suggesting that miR-21 was continuously released to cause pain in the OA consistent with the persistent increase in the miR-21 levels (Figures 1D and 1E). Then, to determine whether TLR inhibition also alleviates knee joint pain, we injected a TLR7–9 antagonist into the knee articular cavity 14 days after ACLT. Knee joint line tenderness was persistently alleviated after the TLR7–9 antagonist injection (Figure 3A), similar to that for the miR-21
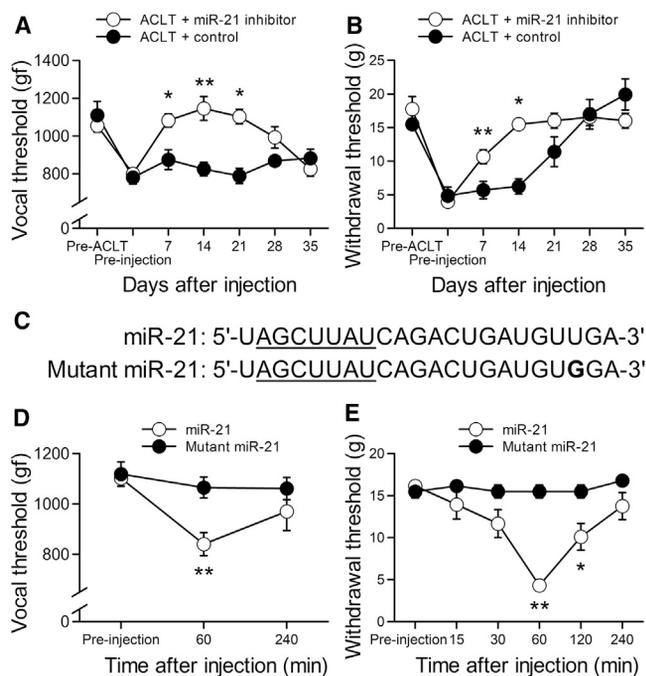
Figure 2. Intra-articular Injection of a miR-21 Inhibitor Exerts Long-Lasting Analgesia in OA Rats

(A and B) A miR-21 inhibitor or its control was intra-articularly injected 14 days after ACLT (n = 7 rats per group). The vocal threshold in response to knee pressure (A) and withdrawal threshold in response to mechanical stimuli (B) were measured (n = 7 rats per group). (C) Sequences of miR-21 and mutant miR-21, which contains one nucleotide substitution, were shown. Seed sequence, which is critical for mRNA target recognition to inhibit translation, is underlined. (D and E) Vocal threshold in response to knee pressure (D; n = 8 rats per group) and mechanical allodynia (E; n = 7 rats per group) were measured after miR-21 or its mutant control injection into the knee articular cavity of naive rats. Data are represented as mean ± SEM. $*p < 0.05$, $**p < 0.01$.

inhibitor. Mechanical allodynia was also suppressed after the TLR7–9 antagonist injection (Figure 3B). Next, we investigated whether TLR7–9 antagonist alleviates pain caused by intra-articular injection of miR-21 in naive rats. Both knee joint line tenderness and mechanical allodynia induced by miR-21 were reversed by intraarticular injection of TLR7–9 antagonist (Figures 3C and 3D). Furthermore, to examine the specific involvement of TLR7 in miR-21-induced knee joint pain, we injected a TLR7-specific antagonist or its control concomitantly with miR-21 into the knee articular cavity of naive rats. The TLR7-specific antagonist strongly suppressed the miR-21-induced knee joint line tenderness (Figure 3E) and mechanical allodynia (Figure 3F).

To identify the target cells of extracellular miR-21, we assessed TLR7 distribution using immunofluorescence. Because synovial tissue receives a rich nerve supply from primary sensory neurons,[5,30] primary sensory neurons innervating knee joint were labeled by the intra-articular injection of a retrograde tracer (1,10-dioctadecyl-3,3,30,30-tetramethylindocarbocyanine perchlorate [DiI]) in naive rats. TLR7
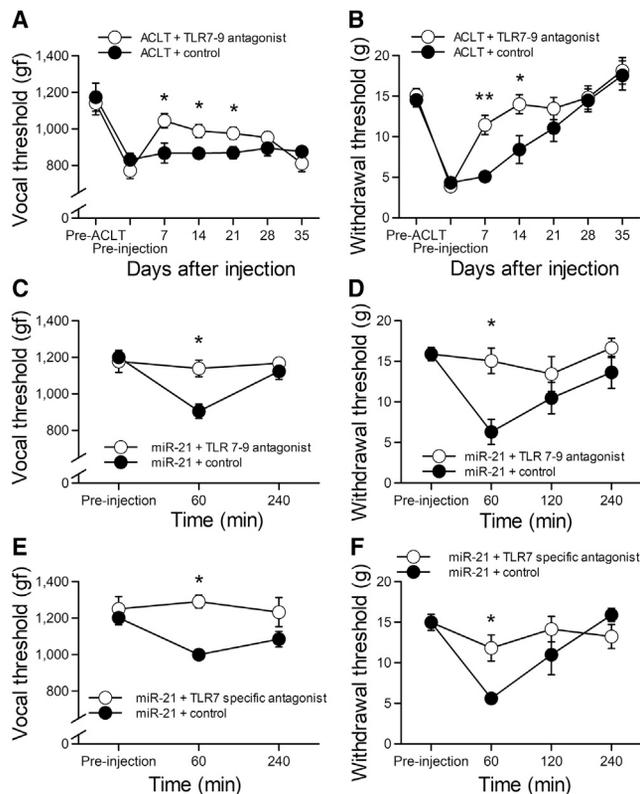
Figure 3. miR-21-Induced Knee Joint Pain Is Mediated by TLR7 Activation

(A and B) A TLR7–9 antagonist or its control was intra-articularly injected 14 days after ACLT (n = 5 rats per group). The vocal threshold in response to knee pressure (A) and withdrawal threshold in response to mechanical stimuli (B) were measured (n = 8 rats per group). (C–F) miR-21 was intra-articularly injected to naive rats concomitantly with either TLR7–9 antagonist (C and D; n = 7 rats per group) or TLR7-specific antagonist (E and F; n = 5 rats per group). Vocal threshold in response to knee pressure (C and E) and mechanical allodynia (D and F) were measured. Data are represented as mean ± SEM. $*p < 0.05$, $**p < 0.01$.

was expressed in 26.9% of DiI-positive primary sensory neurons in the fourth lumbar (L4) dorsal root ganglion (DRG) (Figure 4A). TLR7 expression was observed in small-to-large primary sensory neurons (Figure 4B). In the synovial tissue, TLR7 expression was detected in CD11b-positive macrophage-like (type A) and Thy-1-positive fibroblast-like (type B) synoviocytes[31] in the intimal layer of naive rats (Figures 4C and 4D).

To investigate whether miR-21 directly affects primary sensory neurons, we treated isolated primary sensory neurons with miR-21, and mRNA expressions of well-known TLR7-induced cytokines, interleukin (IL)-1β, IL-6, and tumor necrosis factor alpha (TNF-α),[32] were measured by qPCR. miR-21 significantly increased IL-1β and IL-6, but not TNF-α, mRNA expressions (Figure 5).

DISCUSSION

In summary, we have shown that extracellular miR-21 causes knee joint pain through TLR7 activation in OA model rats. miR-21 was
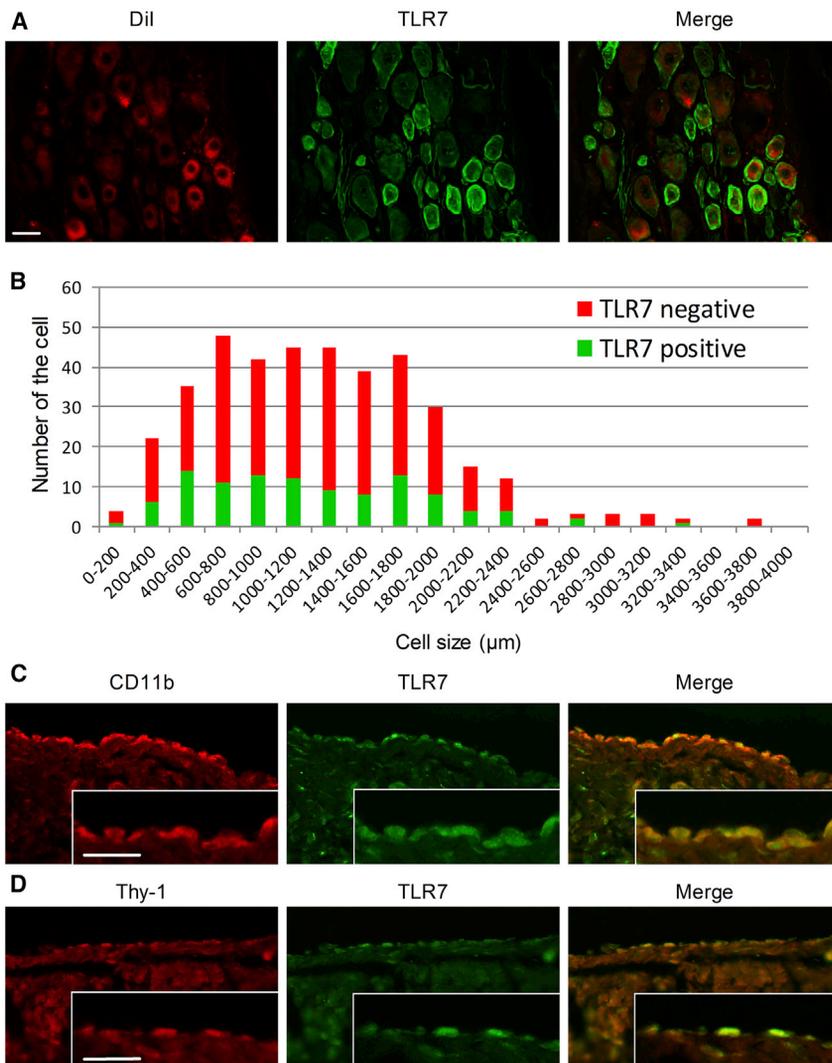
Figure 4. TLR7 Expression in Primary Sensory Neurons and Synoviocytes

(A) TLR7 was immunostained in the L4 DRG 7 days after injection of the retrograde tracer DiI into the knee joint cavity of naive rats. Scale bar, 50 μm. (B) Size distribution of TLR7-positive (green) and -negative (red) neurons positive for DiI in the L4 DRG (n = 5). (C and D) TLR7 and either a macrophage-like (type A) synoviocyte marker CD11b (C) or a fibroblast-like (type B) synoviocyte marker Thy-1 (D) were immunostained in the synovial tissue of three separate naive rats. Scale bars, 25 μm.

or viral vectors, which are potentially harmful, to penetrate the cytoplasm to allow them to suppress gene translation.[36] However, targeting extracellular miR-21 is expected to be safer because the miR-21 inhibitor and TLR 7–9 antagonist did not require a drug carrier in this study. In addition, miRNA-related drugs should be effectively delivered to affected tissues.[36] In this regard, intra-articular injection, which is routinely performed in a clinical setting, can directly deliver therapeutic nucleic acids into the joint cavity. Therefore, the direct intra-articular injection of a miR-21 inhibitor may be an attractive option for OA pain treatment.

Synovial tissue appeared to be an important source of extracellular miR-21 in the synovial fluid because miR-21 was mainly released from synovial tissue rather than cartilage. However, miR-21 expression in the synovium and synovial fluid has not been examined in OA patients and therefore needs to be addressed for the development of miR-21-related analgesics in humans. Nevertheless, miR-21 was reported to be differentially expressed in the cartilage of OA patients and to control cartilage degeneration. Zhang et al.[37] reported that the miR-21 expression level in the cartilage of OA patients was higher compared with that in traumatic amputees, whereas Song et al.[38] showed that miR-21 expression was lower in OA cartilage compared with normal cartilage that underwent biopsy. Recently, the intra-articular injection of antagomiR-21 was reported to attenuate cartilage degeneration in OA model mice.[39] Thus, miR-21 inhibition in the OA joint may also be beneficial for cartilage degeneration.

the most abundant among miRNAs upregulated in the synovial tissue of OA model rats. miR-21 increase in the synovial fluid, which was probably released from synovial tissue, caused arthralgia. Notably, the single intra-articular injection of either a miR-21 inhibitor or TLR7–9 antagonist exerted long-term analgesia in OA model rats.

Extracellular miR-21 might be a plausible target for OA pain treatment. The single injection of either a miR-21 inhibitor or TLR7–9 antagonist was sufficient for long-term analgesia of knee joint pain. The miR-21 inhibitor and TLR7 antagonist used in the present study were modified RNA and DNA, respectively, which are highly stable.[33] The half-life of a modified oligonucleotide intrathecally injected was reported to be 107 days,[34] and steroids that were intra-articularly injected are also known to persist long term.[35] In general, there are obstacles for the clinical development of nucleic acid medicine, although miRNAs are attractive candidates for novel therapeutic approaches. Typical miRNA-related drugs require a vehicle, such as liposomes

Detailed molecular mechanisms underlying miR-21-mediated knee joint pain remain elusive. Extracellular miR-21 released from synovial tissue is speculated to cause the knee joint pain through TLR7 activation in the OA model. Consistent with this idea, synovial tissue plays a role in maintenance of the synovial fluid composition.[3,5] Synovial tissue might be the main source of miRNAs in the synovial fluid because the miRNA profile in the culture medium of synovial tissue is similar

Figure 5. miR-21 Increases IL-1β and IL-6 Expression in Primary Sensory Neurons

Acutely isolated primary sensory neurons were treated with 0.6 mM miR-21 (n = 5) or mutant miR-21 (n = 8) for 1 h. (A–C) IL-1β (A), IL-6 (B), and TNF-α (C) mRNA expression levels were examined by qPCR and shown as a percentage of the mean expression in mutant miR-21. Horizontal line represents the mean in each group. *p < 0.05.

to that in the synovial fluid of OA patients.[25] In addition, knee joint pain induced by miR-21 had an acute onset and was blocked by a TLR7 antagonist, implicating a cell non-autonomous noncanonical action of miR-21. Failure to induce knee pain by intra-articular injection of mutant miR-21 that lacked TLR7-recognizing GUUG sequence also suggests the involvement of TLR7 in knee pain caused by miR-21. Consistently, TLR7 was expressed in putative nociceptive primary sensory neurons with small cell bodies that innervate the knee joint. Both macrophage-like and fibroblast-like synovial cells also expressed TLR7. TLR7 expression was also reported in human synovial tissue.[40,41] Thus, extracellular miR-21 may cause OA pain through TLR7 activation in nociceptive neurons and/or synoviocytes. TLR7 is localized in the endolysosomes to distinguish internalized GU-rich ligands from a cell's own nucleotides.[42] Activation of TLR7 induces multiple intracellular signaling pathway molecules such as mitogen-activated protein kinases (MAPKs). MAPK activation in the peripheral terminals of primary sensory neurons induced acute pain through the post-translational modification of proteins involved in nociception.[43] Interestingly, TLR7 also exists in the cell surface plasma membrane in primary sensory neurons.[44] Therefore, miR-21 released from synovial tissues may cause knee joint pain through TLR7 on the surface and/or in the cytoplasm of sensory nerve endings. However, miR-21-mediated TLR7 activation in synoviocytes might also contribute to knee joint pain. Synovial TLR7 activation by imiquimod led to nuclear factor κB (NF-κB), c-Jun, and c-Fos activation.[40] The miRNA-induced activation of TLR7 signaling pathways promoted the production of TNF-α and IL-6,[45] which sensitize nociceptive neurons.[46,47] In fact, miR-21 directly upregulated IL-1β and IL-6, which are well-characterized pro-nociceptive cytokines downstream of the TLR signaling pathway,[32] in isolated primary sensory neurons. In support of the direct nociceptive effects of miR-21, our preliminary results and previous reports[48] suggest only mild joint damage occurs around day 14. This miR-21-induced sensitization of primary sensory neurons may partially underlie the allodynia in the hind paws because some primary sensory neurons innervate both the knee joint and foot skin. Furthermore, joint damage may also have a significant role in miR-21-induced joint pain because a recent study reported that miR-21 inhibition protected against joint damage in OA mice.[39] In fact, a 32-mer fragment of cartilage proteoglycan aggrecan, generated during the cartilage degeneration, was reported to cause knee hyperalgesia in OA mice through another TLR family member, TLR2.[49] Similarly, damage-associated molecular patterns, S100A8 and α2-macroglobulin, present in the OA joint caused mechanical allodynia through TLR4.[50]

In conclusion, the extracellular miRNA miR-21 might function as a cell-to-cell communication molecule in joints, and targeting the intercellular communication mediated by miR-21 might provide an effective analgesic target for knee joint pain in OA.

MATERIALS AND METHODS

OA Model

All of the experimental procedures in this study were approved by the Animal Experiments Ethical Review Committee of Nippon Medical School (approval number 27-037) and were performed in accordance with the guidelines of the International Association for the Study of Pain.[51] Male Sprague-Dawley rats (7–8 weeks old) were used for all experiments. The animals were singly housed on a 14-h/10-h light/dark cycle and allowed free cage activity and food and water *ad libitum*.

Rats were anesthetized by inhalation of 2% isoflurane (Pfizer Japan, Tokyo, Japan) during all of the surgical procedures. ACLT procedure for producing an OA model was slightly modified from the protocol described previously.[19,52] It is shown that the development of knee OA at a relatively young age is an important consequence of ACL tear in humans.[53] In brief, the left medial parapatellar skin was incised to perform medial arthrotomy. The ACL was exposed by dislocating the patella laterally and then transected. Adequacy of the section was confirmed by positive anterior draw sign. After relocation of the patella, the capsule and skin were closed with 4-0 silk thread (Matsudaika Kogyo, Tokyo, Japan). For the sham operation, the ACL was exposed, but not transected. Due to possible surgical invasion, sham-operated rats showed transient hypersensitivity by the PAM (pressure applied measurement) and von Frey test 7 days after surgery, but the hypersensitivity had dissipated by at least 14 days after surgery.

Intra-articular Injection

Drugs were injected into the articular cavity of the left knee through the infrapatellar ligament using a microsyringe with a 30G needle. All drugs were dissolved in 5 μL of sterile saline before injection. miR-21 (5′-uagcuuaucagacugauguuga-3′) and mutant miR-21 (5′-uagcuuaucagacugauguGga-3′) that contains one nucleotide substitution (G) were synthesized by Sigma-Aldrich Japan (Tokyo, Japan) and injected at a dose of 3 nmol based on preliminary results in which miR-21 was injected at various doses. The miR-21 inhibitor, synthetic tough decoy antisense RNA (Gene Design, Osaka, Tokyo), specifically and potently binds to miRNA.[54] The negative control was a synthetic

tough decoy RNA that does not have a complementary sequence to known miRNAs. Based on results of preliminary experiments in which synthetic tough decoy was administered to ACLT rats at various amounts, synthetic tough decoy RNAs were injected at a dose of 3 nmol. Phosphorothioated TLR7-specific antagonist (5′-TGCTTGCAAGCTTGCAAGCA-3′) and its control (5′-TCCT GGCGGAAAAGT-3′) were synthesized by Sigma-Aldrich Japan.[45,55] The TLR7–9 antagonist (5′-CTATCTGUC*G*TTCTCTGU-3′; C* is 5-methyl-dC, G* is 7-deaza-dG, underlined nucleotides are 2′-O-methyl-ribonucleotides, and all other nucleotides are 2′-deoxyribonu-cleotides)[56,57] and its control oligonucleotide (5′-CTATCTCACC TTCTCTGT-3′) were synthesized by Eurofins Genomics (Tokyo, Japan). A TLR7 antagonist or TLR7–9 antagonist was administered into the articular cavity at a dose of 1 nmol.

Behavioral Tests

Knee joint line tenderness was evaluated using PAM (Ugo Basile, Varese, Italy) as previously described.[58] The PAM device has a force transducer and automatically measures the peak force (gf). The transducer was worn on the examiner's thumb. Each rat was placed into a cotton glove, and the left knee was picked out softly. The examiner placed the thumb with transducer on the rat's knee joint and forefinger on the popliteal fossa. Then, gradually increasing force was applied on the lateral joint line at a rate of approximately 100 gf/s until the rat vocalized. The maximum pressure force was adopted as a vocal threshold. Mechanical allodynia was measured using a set of von Frey filaments (Muromachi Kikai, Tokyo, Japan). Each rat was placed on a metallic mesh floor covered with a plastic box, and a von Frey filament was applied from underneath the mesh floor to the plantar surface of the hind paw. The weakest force (g) inducing withdrawal of the stimulated paw at least three times in five trials was referred to as the paw withdrawal threshold. Only one rat that did not show reduction in the withdrawal threshold (<8 g) 2 weeks after ACLT was excluded from further analysis of miR-21 expression in the synovium. PAM and von Frey testing in naive rats with intra-articular miR-21 injection and in ACLT rats with intra-articular miR-21 inhibitor injection were undergone by an investigator blinded to experimental conditions.

miR-21 Quantification in the Culture Medium

Synovial tissue (2 mm × 3 mm) and cartilage (1 mm × 1.5 mm) were collected from the left knee of naive rats and cultured in DMEM supplemented with streptomycin and penicillin for 24 h. Then, culture medium was centrifuged at 4,700 × g for 30 min at 4°C, and the supernatant was filtered by Ultrafree-MC GV 0.22 μm (Merck, Darmstadt, Germany) to remove cell debris. The flow-through was incubated with Total Exosome Isolation (from cell culture media) (Thermo Fisher Scientific, Waltham, MA, USA) at 4°C overnight. After centrifugation at 10,500 × g for 60 min at 4°C, precipitated exosomes were subjected to qPCR.

Microarray

Synovial tissue was collected from the lateral side opposite to the incision made during the ACLT procedure to minimize the influence of

medial parapatellar incision during ACLT. Synovial tissue was collected from left knee of rat, which was not subjected to behavioral test, 2 weeks after ACLT or sham operation. Total RNA was isolated using an RNAiso plus kit (Takara Bio, Shiga, Japan). Cy3-labeled cRNA was prepared from total RNA (50 ng) using the Low Input Quick Amp Labeling Kit according to the manufacturer's protocol (Agilent Technologies, CA, USA). After purification, cRNA was hybridized overnight to a rat microarray slide (Rat miRNA Microarray, Release 21.0, 8 × 15K; Agilent Technologies) at 20 rpm at 55°C. Fluorescent images of the microarray slide were scanned using a DNA Microarray Scanner (Agilent Technologies). The fluorescent intensity of each spot was quantified using Feature Extraction software (Agilent Technologies). Signal intensities >10 were considered positive expression. Data were analyzed using GeneSpring GX software (Agilent Technologies). miRNAs that do not exist in humans were excluded from the analysis. Microarray data have been deposited in GEO: GSE139532.

qPCR

Synovial fluid was collected from left knee of rats that were not subjected to behavioral test, 14 and 42 days after ACLT. Synovial fluids at days 14 and 42 were obtained from a separate group of rats. Fifty microliters of saline was injected into the articular cavity through the infrapatellar ligament using a 1-mL syringe with a 28G needle. Then, the knee was flexed and extended 10 times, and the synovial fluid was aspirated as much as possible. The synovial fluid was centrifuged at 2,000 × g for 10 min at 4°C, and the supernatant was collected for analysis. All of the procedures were performed according to the manufacturer's protocols. For miR-21 quantification, total RNA obtained from synovial tissue or fluid was reverse transcribed with a mature miR-21-specific stem-loop primer using a TaqMan MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, Waltham, MA, USA). PCR mixtures were prepared with TaqMan Universal PCR Master Mix and TaqMan MicroRNA Assay, which includes a miR-21-specific TaqMan probe and primers (Thermo Fisher Scientific). PCR amplifications were performed at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. For quantification of mRNAs, total RNA (500 ng) obtained from isolated primary sensory neuron was reverse transcribed using iScript select cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) with a random primer. PCR amplifications were performed with TaqMan Gene Expression Master Mix using a premix of gene-specific TaqMan probe and primer pairs (Rn00580432 for IL-1β, Rn01410330 for IL-6, and Rn01525859 for TNF-α; Thermo Fisher Scientific) at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The amplification efficiency for one PCR cycle was obtained by assaying serially diluted samples (four points at dilutions of 1:5), and the relative expression was calculated for synovial tissue, synovial fluid, and culture medium of synovial tissue and cartilage.

Acute Isolation of Primary Sensory Neuron

L3, L4, and L5 DRGs were removed from naive rats, immersed in ice-cold Ham's F12 nutrient mixture (Thermo Fisher Scientific), and cut

into small pieces. DRGs were incubated in PBS containing collagenase A (5 mg/mL; Roche Diagnostics, Basel, Switzerland) and Dispase II (1 mg/mL; Roche Diagnostics) for 30 min at 37°C, followed by 0.05% Trypsin-1 mM EDTA (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for 15 min at 37°C. After addition of 15% fetal bovine serum in Ham's F12 nutrient mixture, the DRGs were dissociated by gentle pipetting and layered onto 30% Percoll followed by centrifugation at $180 \times g$ for 5 min at room temperature. After two washes with Ham's F12 nutrient mixture, the cell suspension was allowed to attach onto a 96-well plate for 2 h in Neurobasal Medium (Thermo Fisher Scientific) containing L-glutamine and B27 supplement (Thermo Fisher Scientific). Then, the cells were treated with miR-21 or mutant miR-21 (0.6 mM) for 1 h.

Immunofluorescence

Naive rats were transcardially perfused with PBS (pH 7.4) followed by fresh 4% paraformaldehyde in PBS. Synovial tissues were postfixed in the same fixative overnight and then immersed in 20% sucrose in PBS. Synovial tissues were rapidly frozen in dry ice/acetone and sectioned (10 μm) using a cryostat (Leica Microsystems, Wetzlar, Germany). The sections were blocked in PBS containing 0.2% Triton X-100 and 5% normal donkey serum for 30 min and then incubated with a rabbit anti-TLR7 antibody (1:1,000; NBP2-24906; Novus Biologicals), a mouse anti-Thy-1 antibody (1:200; SAB4200497; Merck), or a mouse anti-CD11b antibody (1:1,000, MCA275GA; Bio-Rad) at 4°C overnight. After washing with PBS, sections were incubated with a secondary antibody labeled with Alexa Fluor 488 (1:1,000, A21206; Thermo Fisher Scientific) or Alexa Fluor 594 (1:1,000, A21203; Thermo Fisher Scientific) at room temperature for 1 h. Antibodies were diluted by PBS containing 0.2% Triton X-100. Images were captured using a high-resolution digital camera equipped with a computer (Olympus, Tokyo, Japan). Five and three separate rats were used to examine the DRG and synovium, respectively. TLR7-positive neurons in the DRG were cumulatively counted in five separate rats by a blinded investigator.

Retrograde Tracing

The fluorescent neurotracer DiI (Thermo Fisher Scientific) was dissolved in N,N-dimethylformamide (50 mg/mL). Ten microliters of DiI solution was injected into the knee articular cavity of rats with a microsyringe and a 30G needle under anesthesia with 2% isoflurane. One week after DiI injection, rats were transcardially perfused with PBS (pH 7.4) followed by fresh 4% paraformaldehyde in PBS. The L4 DRG sections (10 μm) were immunostained as described above, except antibodies were diluted by PBS. Images were captured using a high-resolution digital camera equipped with a computer (Olympus, Tokyo, Japan), and the number of DiI-stained and TLR7-positive cells was counted by a blinded investigator.

Statistics

Values are expressed as the mean ± SEM. SPSS software (IBM) was used for statistical analyses. Sample sizes were not statistically estimated but were adopted to minimize the number of rats used.

The Mann-Whitney U test was used for all data. All tests were two-tailed, and p values <0.05 were considered statistically significant.

AUTHOR CONTRIBUTIONS
All authors designed the experiments. N.H., A.S., and H.S. analyzed the experiments and wrote the manuscript. N.H. performed the animal experiments, qPCR, retrograde tracing, and immunofluorescence. N.H. and A.S. performed the microarray analysis.

CONFLICTS OF INTEREST
The authors declare no competing interests.

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