

Electro-transfer of small interfering RNA ameliorated arthritis in rats

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Abstract

RNA interference provides the powerful means of sequence-specific gene silencing. Particularly, small interfering RNA (siRNA) duplexes may be potentially useful for therapeutic molecular targeting of human diseases, although novel delivery systems should be devised to achieve efficient and organ-specific transduction of siRNA. In the present study, we demonstrated that electro-transfer of a siRNA–polyamine complex made efficient and specific gene knockdown possible in the articular synovium. Targeted suppression of the tumor necrosis factor- α gene through this procedure significantly ameliorated collagen-induced arthritis in rats. Our results suggest the potential feasibility of therapeutic intervention with RNA medicines for treatment of rheumatoid and other locomotor diseases. © 2005 Elsevier Inc. All rights reserved.

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RNA interference (RNAi) was first reported in 1998 by Fire et al. [1] who demonstrated that double-strand RNA (dsRNA) induced sequence-specific silencing of gene expression in nematode cells. Molecular mechanisms of RNAi have been intensively investigated in a variety of organisms including fungi, hydra, plants, and amphibians, which indicates that RNAi provides the quite useful means of functional analyses of genes as well as epigenetic engineering of cells of various species [2,3]. Although it was initially difficult to induce this system into the mammalian somatic cells, in which dsRNA induces interferon response

leading to non-specific protein synthesis, Elbashir et al. [4] demonstrated that RNAi can be achieved in the mammalian cells by using oligoribonucleotide duplex 21 or 22 bases in length (small interfering RNA; siRNA). The siRNA-mediated genetic knockdown was also demonstrated in vivo in animal organs including the liver [5,6] and skeletal muscle [7]. More recently, several studies applied RNAi to the treatment of various disorders in animal models [8–20] and showed that RNAi may provide promising strategies to treat human diseases by suppressing disease-responsible genes in vivo.

Rheumatoid arthritis (RA) is a systemic inflammatory disease characterized by inflammation of synovial membranes as well as destruction of the cartilage and bone.

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Upon progression, RA patients suffer from functional impairment of the joints which results in ankylosis or deformation of the joints. Although the etiology of RA still remains unclear, the disease is associated with immunological abnormalities, and a variety of inflammatory cytokines are critically involved in the pathogenesis. In particular, Tumor necrosis factor- α (TNF- α) produced by the synovial tissue is located upstream of the cytokine cascade and is playing responsible roles in the induction of the synovitis, as revealed in previous *in vivo* [21] and *in vitro* [22] studies. TNF- α -neutralizing agents have been attracting attention as novel antirheumatic drugs. A TNF- α receptor-IgG Fc fusion protein (Etanercept: Enbrel) [23] and a chimeric monoclonal anti-TNF α antibody (Infliximab: Remicade) [24] have already been applied clinically, demonstrating considerable efficacies in controlling RA. TNF- α -neutralizing agents also demonstrate considerable inhibitory effects on the bone destruction that develops along with the progression of RA, which cannot be achieved by conventional antirheumatics. The anti-TNF- α therapies, however, are associated with high incidences of serious adverse events such as tuberculosis and *Pneumocystis carinii* pneumonia due to immunosuppression. In addition, these drugs should be systemically administered to patients. The treatment is expensive and imposes enormous economic burden on patients. If TNF- α can be locally inhibited at the diseased joints, it would alleviate the burden on such patients who suffer from inflammation at relatively small number of joints. So far previously reported, however, intra-articular administration of TNF- α agonists failed to show successful therapeutic outcomes against the local arthritis lesions [25].

We previously established an *in vivo* electroporation technique, in which synoviocytes were successfully transfected by transcutaneously applying a voltage load to rat knee joint after an injection with plasmid DNA [26]. We also reported that RNAi was achieved in murine skeletal muscle by transducing siRNA duplexes *in vivo* through transcutaneous electroporation [7]. In this study, we attempted to deliver siRNA-polyamine complex into a joint by electroporation and investigated therapeutic potential of RNAi against experimental arthritis by silencing TNF- α in a sequence-specific and organ-specific fashion.

Materials and methods

siRNA duplexes. siRNA duplexes targeting rat TNF- α gene were synthesized (A: 5'-GCCCGUAGCCCACGUCGUAd(TT)-3' and 5'-UACGACGUGGUCUACGGGcd(TT)-3'; B: 5'-UGGGCUCCUCUCAUCAGUd(TT)-3' and 5'-ACUGAUGAGAGGGAGCCAd(TT)-3'; C: 5'-GGAGGAGAAGUCCCAAUd(TT)-3', and 5'-AUUUGGGAACUUCUCCUCCd(TT)-3'; and D: 5'-AGACAACCAACUGGUGGUAd(TT), and 5'-UACCACCAGUUGGUUGUCUd(TT)-3'). Two nucleotide mismatches were introduced into the TNF- α -siRNA-A to generate the mismatched TNF- α -siRNA (5'-GCCCGUAGAACACGUCGUAd(TT)-3' and 5'-UACGACGUGUUCUACGGGcd(TT)-3'). GAPDH gene-specific and negative control siRNAs were purchased from Ambion (Austin, Texas, USA), while GFP-specific siRNA was purchased from Dharmacon (Lafayette, CO, USA). FAM labeling of GAPDH-specific siRNA was performed using the Silencer siRNA Labeling Kit (Ambion).

Animals. Dark Agouti (DA) rats were purchased from Shimizu Laboratory Suppliers (Kyoto, Japan). The SD-Tg (Act-EGFP) Cz-004sb rats were kindly provided by Professor Masaru Okabe (Genome Information Research Center, Osaka University, Osaka, Japan) [27]. Animal experiments were conducted according to the Guidelines regarding Animal Research of the Kyoto Prefectural University of Medicine.

Electroporation-assisted siRNA transduction *in vivo*. Rats were anesthetized by an intraperitoneal injection of 1 μ l/g of sodium pentobarbital, and 50 μ l of the siRNA/siPORT Amine (Ambion) complex containing 800 pmol siRNA and 10 μ l siPORT Amine was administered into the left knee joint using a 27-gauge needle. Immediately after the injection, keratin cream (Fukuda Denshi, Tokyo, Japan) was coated around the knee joint, onto which a pair of electrode pads (1.0 cm in diameter) was placed. Using a CUY21 electric pulse generator (NeppaGene, Tokyo, Japan), three square-wave pulses with a pulse length of 100 ms were loaded at 150 V/cm at a frequency of 1 s⁻¹ followed by two other pulses with the opposite polarity [26].

Observation of GFP expression. EGFP rats were sacrificed 1 ($n = 3$) and 3 ($n = 3$) days after delivery of GFP siRNA (800 pmol siRNA) into the left knee joint. Synovial membrane was removed from the region surrounding the patella of left knee joint of EGFP rats. After 72 h of fixation in 3.7% formaldehyde, the synovium was dehydrated in 25% sucrose solution for 24 h. The specimens were cryosectioned into 14 μ m slices, which were stained with H&E and observed under fluorescence microscope (SZX12).

Induction and evaluation of Collagen-induced arthritis (CIA rats). To induce CIA, collagen type II (Collagen Research Center, Tokyo, Japan) was dissolved in 0.01 M acetic acid (2 mg/ml) and emulsified 1:1 in Freund's incomplete adjuvant (Sigma) on ice (CII/FIA). DA rats weighing 200–250 g were intradermally injected with 200 μ l of the CII/FIA solution at the base of the tail [28]. After electro-transduction of siRNA, foot volume was measured using a water replacement plethysmometer (Uicom Japan, Tokyo, Japan) [29]. The left knee and ankle joints were excised 28 days after immunization and fixed in 3.7% formaldehyde. Following decalcification with formic acid, sagittal sections 6 μ m in thickness were prepared from the center of the lateral condyle of the femur, as well as from the center of the foot joint. The sections were then stained with H&E or Safranin O. Arthritic changes, such as infiltration of inflammatory cells, synovial proliferation, destruction of articular cartilage, and bone erosion, were evaluated 28 days after immunization using the histological scores as described [30].

RT-PCR. To analyze TNF- α mRNA expression *in vivo*, the synovium was removed from the region surrounding the left patella of CIA rats 16 days after immunization. Total RNA was extracted and subjected to RT-PCR using a pair of primers for GAPDH (sense: 5'-TACAGCAACCAGGGTGGTGA and antisense: 5'-ACCACAGTCCATGCCATCAC), or TNF- α (sense: 5'-CGCTCTTCTGTCTACTGAAC-3' and antisense 5'-TTC TCCAGCTGGAAGACTCC-3') genes. PCR amplification of GAPDH sequence was performed using the following conditions: denaturing at 94 °C for 60 s, annealing at 57 °C for 60 s, and extension at 72 °C for 120 s, for a total of 30 cycles. PCR amplification of TNF- α sequence was performed as follows: denaturing at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C, for a total of 36 cycles. The PCR products were electrophoresed through a 2% agarose gel, and DNA fragments were visualized by ethidium bromide staining.

Statistical analysis. Fisher's exact test and non-parametric Mann-Whitney *U* test were used to evaluate the statistical significance of differences in the incidence of paw swelling, and in paw volume and histological score, respectively.

Results

Electro-transfer of siRNA into the synovium of the rat knee joint

GAPDH-specific siRNA duplex was labeled with 6-carboxyfluorescein (FAM) and injected into the left knee joint

of DA rats, to which electric field was applied subsequently at the joint. Twenty-four hours later, the labeled siRNA was present at the intra-articular synovial tissue as revealed by fluorescence stereomicroscopic observation (data not shown). The siRNA was more effectively transduced when it was conjugated with a polyamine (siPORT Amine) before electro-transfer (Figs. 1A–D). The labeled siRNA localized at synovium but not cartilage.

GFP silencing in synovium of the EGFP rat knee joint

To investigate whether siRNA was capable of inducing sequence-specific gene silencing in the synovial tissue, GFP-specific siRNA was delivered into the knee joint of the EGFP transgenic rats. The intensity of green fluorescence at the synovial tissue surrounding the patella decreased remarkably within 24 h after the transduction (Figs. 1E and F), while the silencing effect did not persist for more than 72 h (data not shown). On the basis of these findings, *in vivo* electroporation was found to be an effective method for delivering siRNA–polyamine complex into the articular synovium.

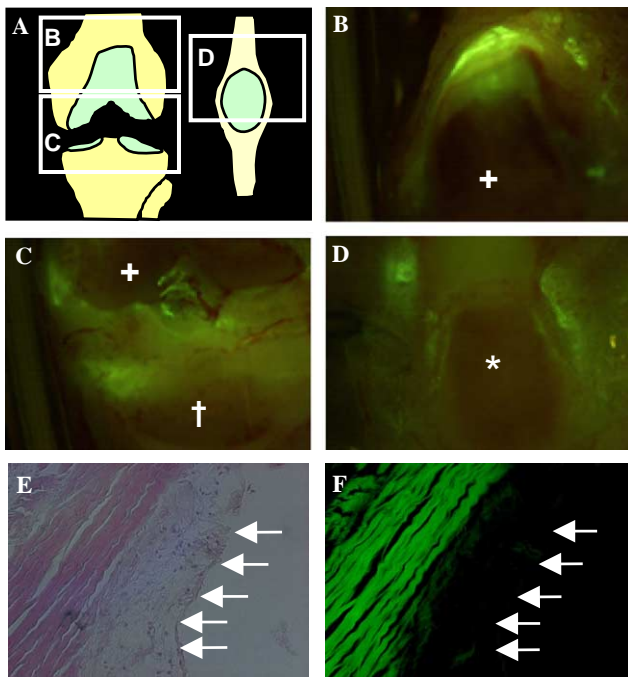


Fig. 1. Electro-transfer of siRNA into the knee joint. (A–D) GAPDH-specific siRNA was labeled with FAM and coupled with polyamine (siPORT Amine). The complex was transduced into the knee joint of DA rats by *in vivo* electroporation. The articular tissue was observed under fluorescence stereomicroscope 24 h after the transduction. Scheme of a joint (A), as well as representative fluorescent images at distal site of femur (B), joint space of knee (C) and surrounding area of patella (D) are shown. +, femur; †, tibia; and *, patella. Original magnification was 15 \times . (E,F) GFP-specific siRNA was transduced into the knee joint of EGFP rats as above. One day later, the cryosections of articular tissue were stained with H&E and observed under fluorescence microscope. Representative bright field (E) and fluorescent (F) images are shown. Arrows indicate the margin of synovial membrane. Original magnification was 100 \times .

Therapeutic effects of TNF- α -specific siRNA in CIA rats

Four rat TNF- α -specific siRNA duplexes (designated as A, B, C, and D) were synthesized. Although significant silencing of TNF- α was induced by any of the four siRNAs, the most potent effects were observed with TNF- α -specific siRNA-A, which successfully reduced the cytokine production in the supernatant of cultured peritoneal macrophages (data not shown). TNF- α -specific siRNA-A was used for the following examinations.

We assessed whether TNF- α -specific siRNA inhibited clinical manifestations of arthritis in a rat model. When the siRNA was delivered into the knee joint 7 and 10 days after immunization, significant therapeutic effect was not observed, although the onset of arthritis of the paw and distal joints tended to be delayed (data not shown). Based on the aforementioned results of experiments involving intra-articular delivery of GFP-specific siRNA, the absence of therapeutic outcome might be due to the short duration of the silencing effect of TNF- α -specific siRNA. Consequently, siRNA was then delivered into the joints more frequently. The siRNA was transduced every 3 days (i.e., 7, 10, 13, and 16 days after immunization), which resulted in significant suppression of paw swelling, as well as remarkable delay of the onset time of the symptoms (Figs. 2A–C). These effects were not observed in the group given mismatched siRNA. Arthritis inhibitory effects were observed in the hind limb on the delivered side, but not on the opposite side. According to the histological examinations of the paw performed 28 days after immunization, the control animals, which were untreated or treated with mismatched siRNAs, showed typical signs of arthritis including massive infiltration of inflammatory cells into the articular cavity, prominent hypertrophy of the synovium, degeneration of the articular cartilage caused by pannus, and signs of bone erosion (Fig. 3A, left panel). In marked contrast, the paws of TNF- α -specific siRNA-treated animals were virtually normal in histological appearance, and the cartilage and bone remained almost unaffected (Fig. 3A, right panel). Histological arthritis scoring also indicated that manifestations of arthritis were significantly milder in the TNF- α -specific siRNA-treated group than in other groups (Fig. 3B). Unlike the paws, knee joints showed only mild arthritis in all groups 28 days after immunization. This was not surprising because knee arthritis occurred temporarily in CIA rats and spontaneously dissipated by 28 days after immunization. However, Safranin O staining showed that the cartilage matrix was comparatively destroyed in the knee joints of control animals, which was not evident in TNF- α -siRNA-treated group (Fig. 3C).

To assess silencing effect of the siRNA transfer, RNA was extracted from the synovial tissue of knee joint 16 days after immunization, when arthritis emerged in the untreated and mismatched siRNA groups. RT-PCR analysis strongly suggested that the expression of the cytokine

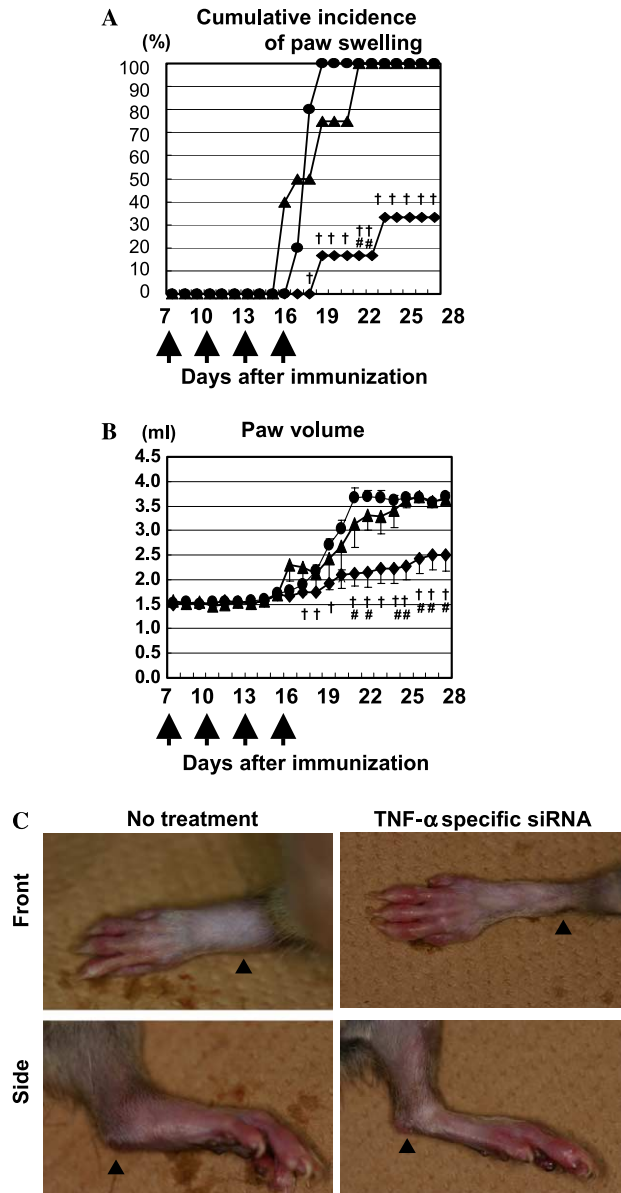


Fig. 2. Therapeutic effects of TNF- α -specific siRNA in CIA rats. (A,B) Rats were immunized with type II collagen (day 0), and 7, 10, 13, and 16 days later (arrows) TNF- α -specific (diamonds) ($n = 6$) or mismatched (triangles) ($n = 4$) siRNA duplexes were electro-transduced into the knee joint as in Fig. 1. A group of rats were left untreated (circles) ($n = 5$). Cumulative incidence of paw swelling (A) and kinetic change of paw volume (means \pm SE) (B) are plotted. $^{\#}P < 0.05$, versus the mismatched siRNA group; $^{\dagger}P < 0.05$, versus the no treatment group. (C) CIA rats were treated with TNF- α -specific siRNA as above. Representative macroscopic views of the lower limbs on day 28 are shown. Arrowheads indicate the ankles of CIA rats.

was significantly inhibited in the synovium of the TNF- α -specific siRNA-treated animals (Fig. 4).

Discussion

Therapeutic application of RNAi has been performed against a variety of disorders including hepatic [8], respiratory [10,11], ocular [12], neuronal [13,14], renal [15], and

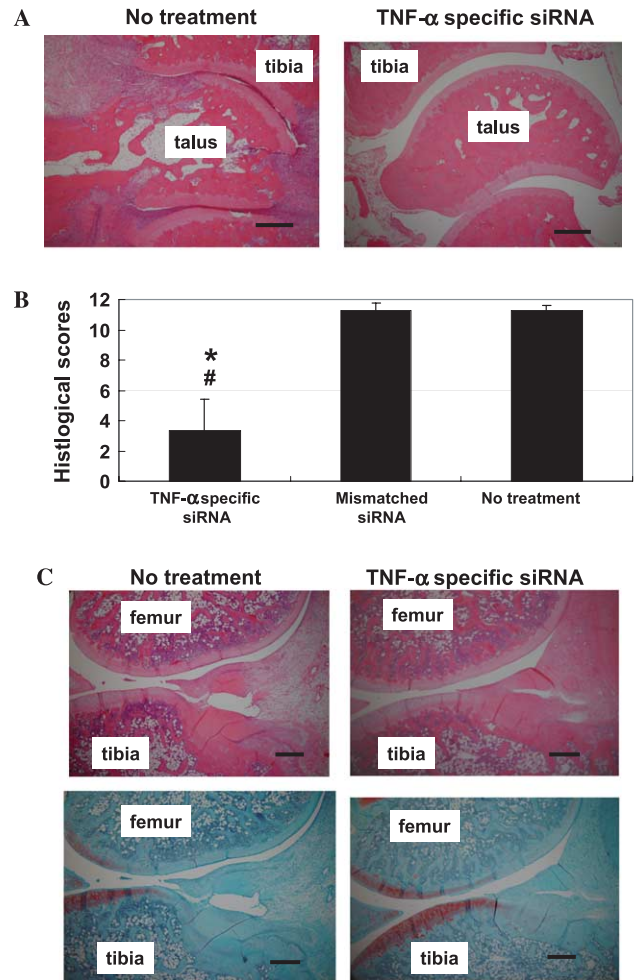


Fig. 3. Histological demonstration of therapeutic outcome of siRNA therapy. CIA rats were treated with TNF- α -specific siRNA as in Fig. 2. (A) Ankle joints were sectioned on day 28. Representative microscopic images of H&E-stained sagittal sections are shown. Scale bars represent 500 μ m. (B) Histological scores (means \pm SE) of the TNF- α -specific siRNA ($n = 6$), mismatched siRNA ($n = 4$), and no treatment ($n = 5$) groups on day 28 are plotted. $^{\#}P < 0.01$, versus the no treatment group; $^*P < 0.05$, versus the mismatched siRNA group. (C) Representative microscopic images of sagittal sections stained with H&E (upper panels) or Safranin O (lower panels) on day 28 are shown. Scale bars represent 500 μ m.

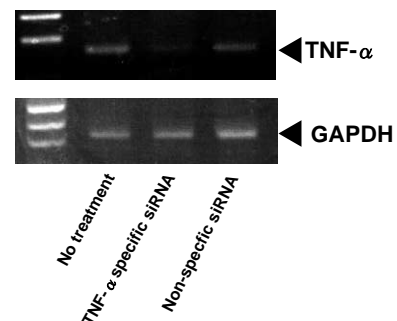


Fig. 4. Suppression of TNF- α in the synovial tissue of CIA rats. CIA rats were treated with TNF- α -specific siRNA as in Fig. 2. RNA was extracted from the synovial tissue on day 16 and subjected to RT-PCR using indicated primers. The leftmost lane in each panel indicates molecular weight markers.

malignant [16–19] diseases. To obtain therapeutic gene silencing in the liver, synthetic siRNA was intravenously administered to the animals [8], while RNAi in other organs was induced by siRNA [11,12,14,16,17,19,20] or a siRNA-expression plasmid [10,18] that were administered into the target organs (intranasal, intraocular, intraventricular, etc.) alone or in combination with some non-viral vehicle. In other studies, an adeno-associated virus vector [13] and the electroporation procedure [15] were employed to achieve effective RNAi in the brain and kidney, respectively. In this manner, delivery methods should be devised and optimized for each target disease based on the anatomical and pathophysiological characteristics of the organs.

The present study suggests a feasible method of accomplishing gene silencing therapy against joint diseases. The joint is the tissue of which genetic engineering cannot easily be succeeded in vivo at a significant efficacy. In this study, a combination of the polyamine and electric pulsation remarkably promoted transduction of siRNA duplex into the synovium. The electroporation may enable targeted delivery of therapeutic siRNA into specific sites of diseased tissues, because introduction of the molecules can be limited to the region charged with an electric field. Other advantages of the electroporation are that the procedure is easy and very rapid, requiring no special skill, and repetitive treatment may be possible [26].

The polyamine, as well as siRNA duplex, is a synthetic product that is suitable for the use as a medicine. The cationic charge of the polyamine may induce condensation of the nucleic acid, and the RNA–polyamine complex may show elevated accessibility to the cell surface. This may explain why polyamine significantly increased the efficacy of electro-transfer of siRNA into the joint tissue.

On the other hand, the potential of gene therapy to treat RA has been investigated over the past 10 years. Some strategies involved the ex vivo and in vivo transduction of genes for soluble receptors that antagonized inflammatory cytokines, while others attempted to inhibit inflammation with Th2-derived cytokines [31,32]. Other procedures aimed at inducing apoptosis in synovial cells through manipulation of signaling pathway or inhibition of angiogenesis [31]. Nevertheless, gene therapy for RA has not encountered a breakthrough in clinical aspects. Ensuring of safety, simplicity, and non-invasiveness is important for the clinical application of gene therapy to non-fatal diseases such as RA. To transduce a gene into the joint tissue at a considerable efficiency, it is necessary to use a virus vector [33], which may have crucial safety problems such as antigenicity, tumorigenicity, and cytotoxicity [34]. RNAi technology may expand the possibility of molecular therapeutics for locomotor disorders by silencing endogenous genes of pathophysiological importance rather than adding exogenous genes for therapeutic purposes.

As a local treatment of RA, an intra-articular administration of corticosteroid to the inflammatory joints is most popularly performed. The procedure is temporarily effective, but repeated administrations of corticosteroid cause

the steroid arthropathy, which is a critical obstacle of this treatment [35]. Therapeutic molecular targeting of RA by means of siRNAs may not cause such serious adverse effects due to the high specificity of RNAi machinery, although this issue should be examined further.

It is noteworthy that local siRNA transduction into the knee joint effectively suppressed inflammation at the paw (Figs. 2–4). This is consistent with the previous reports by Joosten et al. [36] who demonstrated that the inflammatory lesion at the ankle joint was drastically inhibited by an intra-articular administration of anti-TNF- α -neutralizing antibody into the knee of CIA mice.

Therefore, it is strongly suggested that the TNF- α produced from the knee joint may crucially affect progression of the paw arthritis, and the gene silencing at a large joint might have therapeutic effects on arthritis at the nearby small joints.

In conclusion, the present study not only reports a method for delivering synthetic siRNA into joints, but also indicates that the siRNA has great potential for use as a therapeutic tool to treat joint disorders. Our system may also be used for analyzing molecular pathogenesis of joint diseases including RA.

Acknowledgments

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