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Applications of RNA interference in the treatment of arthritis

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Abstract

RNA interference (RNAi) is a cellular mechanism for post-transcriptional gene regulation mediated by small interfering RNA (siRNA) and microRNA. siRNA-based therapy holds significant promise for the treatment of a wide-range of arthritic diseases. siRNA selectively suppresses the expression of a gene product and can thus achieve the specificity that is lacking in small molecule inhibitors. The potential use of siRNA-based therapy in arthritis, however, has not progressed to clinical trials despite ample evidence for efficacy in pre-clinical studies. One of the main challenges to clinical translation is the lack of a suitable delivery vehicle to efficiently and safely access diverse pathologies. Moreover, the ideal targets in treatment of arthritides remain elusive given the complexity and heterogeneity of these disease pathogeneses. Herein, we review recent preclinical studies that use RNAi-based drug delivery systems to mitigate inflammation in models of rheumatoid arthritis and osteoarthritis. We discuss a self-assembling peptide-based nanostructure that demonstrates the potential of overcoming many of the critical barriers preventing the translation of this technology to the clinic.

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Keywords

RNA interference; drug delivery systems; nanoparticle; rheumatoid arthritis; osteoarthritis

Introduction

RNA interference (RNAi) is an intrinsic cellular mechanism for post-transcriptional control of protein expression in which messenger RNA (mRNA) is targeted for degradation by short double stranded RNA (Figure 1).[1] Tuschl *et al.* initially proposed that exogenous small interfering RNA (siRNA) could be delivered to exert RNAi.[2] siRNAs are short 21–23 base pair duplex oligonucleotides in which the “antisense” strand is complementary to a target mRNA, and the “sense” strand acts as a bystander. siRNA operates through the native RNAi machinery to assemble the RNA induced silencing complex (RISC). In the RISC, siRNA initiates cleavage of both the sense and antisense strands, based on sequence specificity.[3] This selective degradation of mRNA provides an avenue to decrease the expression of proteins involved in disease pathogenesis.

Although the promise of RNA silencing with exogenous siRNA has continued to excite scientists, engineers, and pharma companies since its introduction over two decades ago, only a single product has gained FDA approval: a lipidic complex targeted to the liver galactose receptor now marketed by Alnylam for the treatment of hereditary transthyretin-mediated amyloidosis (Onpattro®).[4–6] Sophisticated molecular modifications of the siRNA itself have both reduced off target effects, and enhanced efficacy.[7, 8] However, because negatively charged siRNA does not cross cell membranes freely, the main hurdle to widespread adoption remains the lack of a suitable delivery vehicle to safely access diverse cell populations after systemic injection.

Traditional classes of delivery agents such as polymers or lipidic nanostructures heretofore have resisted widespread clinical application because they are taken up mostly in the liver and the macrophage phagocytic system (MPS) despite efforts to render them stealthy. What is needed are new approaches for systemic siRNA delivery that avoid the MPS, which would allow sufficient penetration to other molecular targets. Moreover, the problem becomes more complex by the necessity to sequentially breach various physical barriers with sufficient numbers of siRNA to effect silencing. These barriers generally involve: vascular access, traversal of endothelium, cell membrane interactions, cellular uptake, endosomal escape, and cytoplasmic trafficking to the RISC complex. Once in the cytoplasmic compartment, longevity of the exogenous siRNA becomes important for sustained efficacy. If any of these sequential steps fails, the entire process fails.

Comprehensive review articles describing siRNA therapeutics have been published over the last two decades and readers are referred to these for general information on molecular mechanisms of RNAi.[9–11] A more recent review of clinical trials and commercial activity in the siRNA space by Tatiparti *et al.* also is available.[12] In this review, we highlight recent developments in RNAi applications for the treatment of arthritic conditions and provide updates on peptide-based delivery systems for RNAi.

RNAi applications in rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory arthritis affecting approximately 1% of the general population worldwide, ~1.5 million adults in the United States alone. RA is characterized by inflammation of the synovial lining of diarthrodial joints and an influx of leukocytes through leaky angiogenic blood vessels.[13–15] This synovial proliferation, termed pannus, and cellular influx contribute to the destruction of connective tissues, cartilage, and subchondral bone of the affected joints.

RA is a complex and heterogeneous disease influenced by genetics and environmental factors that shape the immune responses. Insights into these responses have led to the development of a number of “biologics” aimed at inhibiting the action of several inflammatory cytokines, including IL-1, IL-6, and TNF.[16] Despite their effectiveness, continuous systemic administration of biologics may cause significant side effects and heightens the risk of opportunistic infection.[17] Studies have shown that about half of the initial responders either stop responding to a biologic or have to discontinue therapy altogether due to side effects.[18]

siRNA nanotherapy in RA

The use of nanocarriers to deliver therapeutics specifically to the desired sites of inflammation represents a promising and attractive therapeutic approach to RA. In addition to targeted delivery, nanotherapeutics can theoretically lower the drug dose and dose frequency to avoid bystander effects. Many excellent reviews over the years have highlighted the potential of various nanocarriers for the treatment of RA.[19–21] In addition, a recent comprehensive review discussed advances in the applications of siRNA nanotherapeutics for rheumatic conditions, including RA.[22] Strategies highlighted included local application by intra-articular injection, without or with electroporation, hydrodynamic injection, and biocompatible systems that achieved safe and targeted *in vivo* delivery of siRNA. In this review we will cover studies published since, with a focus on systemic delivery only (Table 1). We will also discuss advances in intra-articular injection as a treatment modality for osteoarthritis (OA) (see below).

Targeting inflammatory pathways in RA

A number of inflammatory/catabolic molecules and pathways have been targeted to treat RA. Here we highlight some of the key molecules that have gained attention for therapy.

Tumor necrosis factor alpha (TNF-α)—TNF inhibitors (TNFis), developed as monoclonal antibodies, are currently the best-selling biologics for the treatment of RA. The major adverse effect of TNFis is increased risk of opportunistic infections, especially tuberculosis.[23] With their promise of specificity and potentially lower toxicity, RNAi delivery systems targeting TNF-α have been extensively explored in animal models of RA. Ye *et al.* used a small peptide, RVG-9R, a 29-amino acid peptide derived from rabies virus glycoprotein fused to 9 arginine residues to silence TNF-α in the collagen antibody-induced arthritis (CAIA) model.[24] When administered systemically, the treatment led to approximately 60% reduction in inflammation when compared to dexamethasone, a steroid that has general immunosuppressive effect. The treatment is preventative, given prior to

arthritis development and no toxicity or immune responses (i.e. antibody production against the peptide) were examined. Due to its biocompatibility and low immunogenicity, chitosan (CH) and its derivatives have also been developed to deliver siRNA *in vivo.* One drawback to CH is poor solubility at physiological pH. A soluble derivative containing diethyl ethylamine (DEAE) was synthesized, conjugated to folic acid for enhanced cellular uptake by folate receptor and used to deliver TNF-α siRNA to mice with CAIA prior to disease development and shown to modestly reduce disease activity while preserving bone structure, as evidenced by decreased bone erosions scores and bone metabolism markers.[25] In addition, self-polymerized thiol-modified siRNA (poly-siRNA) can form stable complexes with biocompatible thiolated glycol CH (tGC) polymers that are readily degraded under reductive conditions in the cell cytosol to monomeric siRNA.[26] These complexes accumulate in inflamed joints and suppresses collagen-induced arthritis (CIA) as efficiently as methotrexate.[26] Strong cationic polymers such as polyethyleneimine (PEI) have been used to deliver siRNA; however, PEI is harder to condense and may be unstable, resulting in early release and degradation of siRNA in serum. Cytotoxicity also limits its application.[27, 28] Low molecular weight PEI (<20 kDa), cross-linked by degradable linkers, shows lower toxicity, self-degrades in an acidic environment (i.e. inflammatory milieu), and significantly suppresses CIA.[29] Complexing TNF-α siRNA with biocompatible cationic lipids such as lecithin, cholesterol and a previously reported acid-sensitive sheddable polyethylene glycol (PEG)[30] led to the development of nanoparticle formulation with minimum burst release (5% of siRNA was released in one-month release study *in vitro).*[31] The formulation mitigated CAIA by approximately 33% when given prior to disease onset.[31]

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) ––This is a signaling pathway that controls gene products closely linked to inflammation.[32] The NF-κB family consists of five members: p105 (constitutively processed to p50), p100 (processed to p52 under regulated conditions), p65 (also known as RelA), RelB, and c-Rel. [33] These members form homo- and heterodimers that, in the resting cell, are normally held inactive in the cytoplasm by the association with inhibitors, the IκB proteins. Activation of NF-κB is controlled by the IκB kinase (IKK) complex that phosphorylates IκB proteins and targets them for degradation, releasing the NF-κB subunits for nuclear translocation and transactivation of a multitude of responsive genes, including several inflammatory cytokines. Thus NF-κB pathway plays a crucial role in the inflammatory response of macrophages and lymphocytes in RA. Using the CAIA model and the p5RHH peptide-based nanosystem (described in detail in the section below) that delivered p65 siRNA systemically, we showed that the nanoparticles penetrated through inflamed and leaky vasculature, much like the endothelial permeability and retention (EPR) effect proposed for nanoparticle localization in tumor, and potently suppressed ongoing inflammation in the robust model of K/BxN serum transfer arthritis.[34] This self-assembling, largely non-toxic siRNA delivery platform has promising translational potential for the treatment of RA (and other chronic inflammatory diseases) where repeated dosing may be required. Since this publication, a number of studies have confirmed the utility of targeting NF-κB in inflammatory arthritis models. Delivery of NF-κB siRNA targeting p65 was achieved using block polymers, polymeric micelles, or hybrid nanocarrier.[35–37] Many of these included additional drugs such as dexamethasone

or methotrexate loaded onto the particles. Other NF-κB targets have also been explored such as c-Rel and the non-canonical NF-κB-induced kinase (NIK) signaling pathway.[38, 39]

Complement system ––—The complement system is an effector arm of the innate immune response and plays a central role in RA development. The complement system comprises three pathways: the classical pathway, the lectin pathway, and the alternative pathway. Activation of all three pathways converges with the cleavage of C3 and C5, generating the anaphylatoxins C3a and C5a. The importance of C5a and its receptor C5aR in preclinical models of arthritis is well delineated.[40–42] However, antagonism of complement C5a-C5aR axis in patients with RA has met with disappointing results in the clinic.[43] In a more recent study, the investigators conjugated protamine to a monoclonal antibody (Ab) directed against C5a receptor 1 (C5aR1) to generate anti-C5aR1 Ab-protamine-C5 siRNA conjugates, taking advantage of the charge-charge interaction between protamine (positively charged) and siRNA (negatively charged).[44] Injection of C5aR1 Ab-protamine-C5 siRNA conjugate *in vivo* in mice with CAIA led to 83% reduction in CAIA when injected three times, 5 days prior to disease induction (days −5, 0, and 3, in a preventative treatment) while injection with unconjugated components (anti-C5aR1 Ab and C5 siRNA) only mitigated disease by 19%. The same group also targeted mannan-binding lectin-associated serine proteases 3 (MASP-3), a component of the lectin pathway.[45] The investigators hypothesized that silencing liver-derived MASP-3 synthesis would modulate complement activation and attenuate arthritis. Triantennary *N*-acetylgalactosamine (GalNAc) was conjugated to MASP-3 siRNA (GalNAc-MASP-3-siRNA) to enhance liver uptake and injected into mice with CAIA. GalNAc-MASP-3-siRNA administration did not completely inhibit MASP-3 expression and only delayed onset of arthritis by one day and suppressed disease activity by about 50% if given 10 days prior to arthritis induction. Whether MASP-3 depletion in an established disease has effect remains to be seen.

Miscellaneous targets ––—Two separate studies used either PEI/siRNA complex[46] or PEI-superparamagnetic iron oxide (SPIO) nanoparticles[47] to target IL-2/IL-15 receptor beta chain Both approaches reduced the severity of adjuvant arthritis in rats but the effect was augmented by the application of a magnetic field to SPIO-containing nanoparticles.[47] Other targets include a disintegrin and metalloproteinase 15 (ADAM15),[48] heterogeneous nuclear RNP A2/B1,[49] Notch 1,[50] Connexin 43,[51] CCR5,[52] the pore-forming subunit of calcium release-activated calcium (CRAC) channels,[53] and transforming growth factor beta-activated kinase-1 (TAK-1).[54]

Limitations and future directions

Although most studies using siRNA-based therapeutic delivery systems show some efficacy in preclinical animal models of RA, translation to the clinic is far from reality. Several critical issues remain to be worked out, including identification of off-target effects of excess siRNA that can induce type I interferon response.[55] In addition to anti-inflammatory approaches, targeting cell lineages may provide beneficial outcomes. Aberrant T cell regulation is proposed as one of the mechanisms that promote RA.[56] Thus T-cell targeting strategies could offer therapeutic utility. T cell co-stimulation inhibition (Abatacept) is a biologic that is currently doing well in the clinic.[57] Other T cell subsets being explored as

targets include Th17 cells that produce IL-17 cytokines (IL-17A and IL-17F), which have been shown to play a critical role in many inflammatory arthritides, including RA and psoriasis/psoriatic arthritis.[58] Humanized monoclonal antibody against IL-17A, however, has mixed effects in RA [59–61] and may worsen inflammatory bowel disease in some instances.[62] Since Th17 cells produce many cytokines other than IL-17 cytokines, targeting Th17 development may lead to improved efficacy against autoimmune arthritides. Thus, silencing the retinoic acid-related orphan receptor gamma t (RORγT), the master transcriptional factor of Th17 lineage may prove superior to blocking a single cytokine.[63] This was recently accomplished *in vitro* using CD4 aptamer-based delivery of RORγT-siRNA to suppress Th17 differentiation.[64] Aptamers are nucleic acid-based ligands (single stranded DNA or RNA oligonucleotides) that are produced through a process known as systemic evolution of ligands by exponential enrichment (SELEX). [63] SELEX enables the generation of aptamers that bind specifically to a molecule, such as cell-surface receptor, gaining entrance to target cells through receptor-mediated endocytosis. Aptamer-siRNA complex targeting HIV-gp120 inhibits HIV replication *in vitro* and *in vivo*,[65, 66] suggesting that this system holds promise as a universal tool for siRNA delivery to specific targets.

RNAi applications in OA and post-traumatic OA

OA is a complex polygenic disease, which is now recognized as a clinical syndrome.[67] It is one of the most common causes of disability in the aging population and its incidence is becoming higher in younger population, especially in association with traumatic knee injuries. Moreover, once reserved for elderly, joint replacement surgeries are becoming more common in the young and active individuals. The true root cause and pathogenesis of primary age-related OA remains incompletely understood. Insights from large-scale genetic studies and information gained from injury-related post-traumatic OA have enabled us to capture some aspects of the disease process. However, a true picture of the pathogenetic pathways is yet far from reality. As the pathogenesis remains to be fully elucidated, treatment options for OA are also limited.

Currently there is no disease modifying OA drug (DMOAD). Despite numerous attempts to devise therapeutic strategies for OA, none has thus far made it to clinic.[68] OA is a chronic pain condition, often associated with structural changes. Although articular cartilage is not innervated, high expression of nerve growth factors in OA joints is associated with pain severity.[69] Hence, the development of therapeutic interventions based on antagonism of nerve growth factors is of great interest. Other treatment modalities include non-steroidal anti-inflammatory drugs and corticosteroids, which provide only a transient relief and can be associated with serious side-effects. In patients where the aforementioned medications are ineffective or contraindicated, opioids are often used.[70] It is estimated that the rate of prescribing opioids for knee OA is about 16%,[71] adding to the opioid epidemic in the USA. Joint replacement surgery is a last resort for patients in whom pain medication or other methods have failed. A number of complications associated with surgery such as chances for infection, osteolysis, need for second or third surgery, high costs as well as extended rehabilitation, make joint replacement a less welcome option.[72] However, with new

improvements in techniques and materials, total knee arthroplasty is able to extend the active life and offers a new joint with reduced hospital stays.[73]

Critical barriers to effective OA treatments

A number of other factors contribute to the challenges of developing effective OA therapies.

Genetic complexity ––—OA is a complex polygenic disease with multiple risk loci conferring small effects.[74] Moreover, environmental and genetic factors play a key role in disease pathogenesis,[67] with genetic factors accounting for significant variation in OA susceptibility. [75]

Multi-tissue disease ––—Mounting evidence suggests that OA is a disease of the whole joint and all tissues within the joint are involved.[76] For instance, in the knee OA, meniscus degeneration, subchondral bone sclerosis, and synovial proliferation and joint degeneration are all consequences of OA. Nonetheless, cartilage degeneration remains a hallmark of end-stage disease.

Incomplete understanding of pathogenesis ––—Despite rapid progress in the field of genomics and genetics and emergence of high throughput screening tools, and paradigm shift from the simple “wear and tear” process, there is a clear vacuum in the understanding of the OA pathogenesis. A number of pathways are implicated in OA pathogenesis including Wnt signaling, NF-κB, apoptosis, autophagy, cell cycling, TGF-β, Notch, among others.[77, 78]

Disease heterogeneity ––—Even when the spatiotemporal nature of injury is known, it is still unclear how these injuries move the joint in the direction of OA. For example, it was thought that anterior cruciate ligament (ACL) tear destabilizes the joint and mechanical nature of this injury results in OA.[79] However, ACL reconstruction does not improve the biology of the joint and is thought to even increase the risk for future OA.[80, 81] In addition, only about 50% of patients go on to develop post-traumatic OA.[82]

Multifactorial disease ––—A number of factors such as age, sex as well as genetic and environmental influences contribute to OA development.[83] Their interaction with the disease process is very complex and some are difficult, if not impossible, to modify. Modification of certain risk factors such as obesity and activity level has shown little effects on OA development.

Survivability of cell-based and cell-engineered biomaterials ––—A great deal of effort has been made to identify and test various cell types and biomaterials for an effective cell-based and/or tissue-engineered approach to treat focal cartilage as well as osteochondral defects.[84] However, these approaches have met with a number of challenges.[85] A common problem in tissue engineering strategies for OA is that while the natural tissue has degenerated the underlying cause (inflammation) is not resolved, leaving a slim chance for the engineered construct to survive in the hostile inflammatory or catabolic environment. Nevertheless, we must acknowledge the efforts in this exciting area of research. Biologists,

biomechanical engineers and material scientists remain committed to generate a product that is more sustainable, resembles the native tissue and can withstand the catabolic milieu.

Targets in OA

In light of the above discussion, one could perceive that the search for Holy Grail of OA therapy has been disappointing. However, this is not true. We now know a lot more about OA process than before. A number of elegant studies have made significant breakthroughs toward identification of genes and pathways to discern the disease mechanisms. These pathways inform each other and take us one step forward toward better understanding of the disease pathogenesis. Research on epigenetic mechanisms is also on the rise. A variety of highly relevant animal models of OA have been developed. A great deal of work has been done to knockout or mutate individual genes using cutting edge genomic engineering technologies such as the use of CRISPR/Cas9 system to unravel disease pathogenic mechanism and develop novel treatment approaches.[86, 87] In a recent study, investigators explored CRISPR/Cas9-mediated gene editing to treat OA, by targeting Ngf, Il1b, and Mmp13 genes in a mouse model of PTOA. It was demonstrated that Il1b and Mmp13 reduced PTOA progression, while Ngf ablation significantly palliated PTOA-related pain. These findings suggest that CRISPER/Cas9-based gene editing is a useful technology for the identification of promising drug targets and the development of feasible therapeutic strategies for OA treatment.[88]

The focus of OA from a disease of cartilage has changed to a concept of whole joint disease and the concept of orthoregeneration is on the horizon. Available genetic and epigenetic tools and availability of high throughput screening methods, as well as a variety of new materials for tissue engineering has made it all possible to better handle some or most of aspects of the disease. The significance of restoring joint homeostasis has emerged as a conceptually appealing approach and sets new directions for effective OA therapy. Last but not least, therapeutic applications may need to be applied early in the disease process, especially in the case of post-traumatic OA, where molecular changes take place much earlier than clinical manifestation of disease.[89] Table 2 lists a number of key pathways that have been targeted using siRNA.

siRNA nanotherapy in OA: anti-catabolic and anti-inflammatory

According to clincialtrial.gov there is no ongoing clinical trial for the use of siRNA in OA treatment, although a number of siRNA candidates have reached various stages of clinical trials for the treatment of other conditions.[90] For local delivery of siRNA for OA treatment, a great deal of work still needs to be accomplished before a targeted, sustained-release system that enables spatiotemporal control of gene silencing becomes a reality.[91] RNAi-based therapies for OA have been described elsewhere up to 2012.[22] Here we highlight the studies from 2013-2019 (Table 3).

Indian hedgehog (Ihh) ——has been implicated in OA progression.[92, 93] While Ihh deletion in mice is lethal, siRNA-mediated ablation in rats has been shown not only to have chondroprotective effects but could ameliorate cartilage degeneration.[94]

NF-κB ––This pathway is most prominent among gene signatures in human OA and rodent OA and plays a key regulatory role for inflammatory signaling and is therefore an important therapeutic target.[95] We have recently demonstrated that siRNA targeting NF-κB improved joint homeostasis, suppressed synovitis and inhibited cartilage degeneration in a mouse model of joint injury.[96]

Yes-associated protein (YAP) ––The role of YAP in OA has just begun to emerge. Levels of YAP are increased in human OA and rodent model of experimental OA.[97] siRNA-mediated knockdown of YAP mitigated OA development by reducing bone formation and preventing cartilage degradation.[97]

Hypoxia induced factor 2 a (Hif2a) ––This molecule acts as a catabolic factor and its overexpression is associated with OA.[98] Inhibition of Hif2a with the use of siRNA nanoparticle complex resulted in mitigation of OA, maintenance of cartilage integrity, and reduction in cartilage degeneration and synovitis.[99]

Matrix degrading enzymes ––Matrix degrading enzymes such as matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs) are well-established contributors of cartilage degeneration.[100] In particular, MMP-13 and ADAMTS-5 have emerged as candidate targets for development of OA therapies.[101–103]

siRNA nanotherapy in OA: anabolic and regenerative

siRNA not only can be used to inhibit catabolic genes to reduce the inflammatory milieu in the joint, it can also be used to silence a protein that inhibits tissue regeneration or that results in defective tissue formation in order to improve tissue regeneration process.[104] Here we provide a list of siRNAs that have improved cell function, tissue homeostasis or regeneration of bone and cartilage in vivo (Table 4). The identification of processes and pathways or a set of pathways is an important first step towards a targeted therapy to circumvent the disease progression.

siRNA nanotherapy in OA: microRNAs

Like siRNAs, microRNAs are also noncoding RNAs with important role in gene regulation. MicroRNAs are endogenous, small (18-24 nucleotide), single-stranded RNAs that regulate gene expression at post-transcriptional level by binding to 3’ untranslated region of target mRNAs.[105] Mounting evidence suggests that microRNAs are implicated in a variety of cell functions such as cell cycle, apoptosis, migration and proliferation.[106] Both microRNAs and siRNAs share a number of similarities. However, their mechanism of action as well as clinical applications are different. For instance, siRNA is highly specific with only one mRNA target, while microRNA has multiple targets. Therefore, the therapeutic applications of siRNA and microRNAs are very distinct.[90] Recent evidence in microRNA research suggests that they play a pivotal role in OA.[107]) The emerging role of microRNAs is evident from studies that compare microRNA expression in both healthy and diseased (OA) cartilage. Depending on the expression pattern of a given microRNA, it is either used as an agomir or antagomir to treat OA. An agomir is a chemically engineered

double stranded miRNA that is used to mimic upregulation. In contrast, an antagomir is a chemically modified single stranded miRNA inhibitor that prevents other molecules from binding to desired site on an mRNA molecule. Antagomir is used to silence endogenous miRNAs. It is perfectly complementary to the specific miRNA target that mispairs at the cleavage site of Ago2 (argonaute RNA induced silencing complex catalytic component 2) to inhibit Ago2 cleavage. Here, we have highlighted some of the important studies that utilized microRNAs to mitigate OA (Table 5).

Summary, limitations and future directions

Available evidence from literature suggests that siRNA-based OA therapies appear to work effectively in treating some aspects of the disease. Moreover, most nanoplatforms are effective in delivering the drug cargo when injected intra-articularly, but they lack specificity for a cell or tissue. So, there is an unmet need for the development of nanoplatforms that will target specific tissue(s) in the joint. In addition to specificity, multiplexing is a characteristic that is not universally available in all available platforms. Multiplexing could increase many fold the effectiveness of treatment as more than one pathway can be targeted simultaneously.

Peptide-based delivery systems for RNAi

A natural starting point for considering peptide nanostructures as oligonucleotide delivery agents begins with the general class of cell penetrating peptides.[108–113] In particular, the HIV-derived Tat peptides and Antennapedia-derived “Pentratin” peptides from Drosophila were among the first described to translocate across cell membranes. The structures and membrane penetrating mechanisms for many of these peptides entail interactions of cationic basic amino acid-rich moieties allowing either direct energy independent membrane translocations or energy requiring uptake by macro- or micro-pinocytosis, or other endocytic mechanisms. Some of these agents have been developed as chemically conjugated peptide-nucleic acid structures that may be susceptible *in vivo* to proteolysis or toxicity due to high arginine content. Other peptides exert electrostatic interactions with negatively charged siRNA that self-assemble into 50-250 nm particles when mixed in appropriate charge and molar ratios.

Upon internalization into endosomes in particular, the task of release of oligonucleotide cargo and escape from endosomes becomes a critical and time dependent task to avoid persistent sequestration and siRNA degradation. Strategies to achieve endosomolysis have traditionally been based on osmotic agents, fusogenic lipids, and fusogenic peptides.[108] Addition of osmotic endosomolytic agents such as chloroquine create proton buffering effects that induce swelling and rupture of endosomes. Indeed, the use of chloroquine *in vitro* after cell loading with siRNA constructs is useful for understanding the capacity of novel agents to escape the endosome as full release is achieved after chloroquine.

Self-assembling peptide-based siRNA delivery system

Our own efforts have concentrated on an emerging approach to siRNA delivery: a self-assembling nanostructure comprising of a peptide with intrinsic membrane-disrupting activity that offers an alternative for endosomolysis and siRNA release. Melittin, a 26 amino acid, cationic amphipathic component of bee venom represents an example that has found

use in several types of oligonucleotide delivery constructs.[114–117] It has been used in certain types of stable perfluorocarbon nanoparticle (PFC NP) formulations as a potent anti-cancer therapeutic by inducing either necrosis or apoptosis. [118, 119] Melittin assumes a random coil secondary configuration in solution and interacts rapidly with negatively charged cell membranes initially through electrostatic interactions. Subsequently it undergoes a change in secondary structure to alpha helical that facilitates hydrophobic interactions with phospholipid tail moieties in an exothermic process that results in stable membrane insertion. In fluid cell membranes, oligomerization and pore formation can occur, followed by cell lysis that overwhelms native cell membrane repair mechanisms.[120] Although melittin rapidly destroys red blood cells and liposomes,[118, 119, 121] it can be carried safely in PFC NP structures within the surfactant-lipid monolayer surrounding the perfluorocarbon nanostructures because the PFC core is both hydrophobic and lipophobic and remains unaffected by melittin's pore forming behaviors.[122]

Because melittin acts like a cationic cell penetrating peptide, it was thought to be a potentially interesting candidate for drug and oligonucleotide delivery. However, for *in vivo* applications, its cell lysis properties need to be controlled until it reaches the target cell where it then might exert endosomolytic behaviors. Initial efforts to block the lytic activity of melittin by several authors utilized acid labile protecting groups in polyconjugated polymeric nanostructures that prevented melittin activation and membrane insertion until released in an acidic endosomal environmental [123–125] This strategy for endosomal escape was adopted for clinical trials against Hepatitis B for siRNA delivery, but proved to cause liver toxicity in related preclinical studies resulting in study abandonment. The platform was modified and subsequently has been restarted by Arrowhead Pharmaceuticals in several trials (e.g., hepatitis, anti-trypsin deficiency) with the use of a subcutaneous delivery approach.

In contrast to the use of masked native melittin for endosomal escape, others have taken advantage of its ability to bind negatively charged nucleotides via electrostatic interactions. A polymerized form of lysine modified melittin and plasmid DNA was described by Chen *et al.* that operated via thiol oxidation of incorporated cysteine residues to depolymerize and release the melittin in order to effect endosomal escape.[126] In this construct, the binding of DNA itself masked melittin's lytic activity. However, attempts to tailor the lytic potential of melittin by certain N- or C-terminus truncations abrogated its membrane disruptive and transfective potencies.

A safe and potent alternative format for polymerizing melittin and siRNA has been engineered by Hou *et al.*[108, 127, 128] Initiation of these efforts began with trials of native melittin as an anti-cancer agents carried in a protective perfluorocarbon nanostructure that sequestered melittin in the outer lipid monolayer until interacting with melanoma cancer cells. [118, 119] The fusogenic potential of melittin proved useful to deliver the peptide to the cell membrane via formation of a hemifusion complex with the cancer cell.[129] This allowed the lipids and associated melittin surrounding the perfluorocarbon core to flow into the membrane leaflet of the cancer cell and enter the cytoplasmic compartment to induce apoptosis and/or necrosis depending on conditions.

Subsequent modifications to melittin along the lines of Chen *et al.* discussed above were tested to reduce intrinsic lytic capacity by selected truncations.[130, 131] A specific N-terminal truncation of 7 amino acids by Pan *et al.* resulted in greater than 2 orders of magnitude minimization of cell necrosis such that doses that might be used in vivo should not be hemolytic. This format (so called “peptide 5,” or p5) ultimately was developed as a linker molecule capable of carrying conjugated molecular cargo across cell membranes without cell disruption. However, neither p5 nor native melittin was capable of condensing siRNA into a transfective nanostructure.

Further modifications to p5 were proposed by Hou *et al.* by adding histidine and arginine moieties to form peptide “p5RHH”, which maintains the original 7 amino acid N-terminus truncation.[127, 128] The added arginines enhance electrostatic interactions between siRNA and the peptide. The uncharged histidines (at neutral pH) permit formation of noncovalent hydrogen bonds between siRNA and the peptide in initial exothermic reactions to enhance the stability, silencing activity, and transfection efficacy of the peptide polyplexes.[132] After uptake by micro-pinocytosis, protenation of the imidazole group of histidines in late endosomal structures upon acidification results in disassembly of the polyplex as pH drops below the pKa of the imidazole group (~6.1) (Figure 2). Coordinated release of the siRNA then permits free p5RHH to interact with the endosomal membrane. The free p5RHH, now in high concentrations in the endosome, elicits endosomolysis without perturbing cell viability according to Hou *et al.*[127, 128] The overall model accords with that proposed by Chou *et al.* where a peptide:siRNA polyplex is formed by electrostatic interaction and hydrogen bonding, and disassembly of the nanostructure follows protenation of histidines by overcharging thereby allowing interaction of the peptide with the endosomal membranes. In the case of the p5RHH complex, the modified melittin now greatly facilitates endosomal membrane permeabilization. Subsequent dilution of p5RHH in the cytoplasm and protease activity restrains its intracellular lytic potential.

The p5RHH nanostructures actually have proven to be more stable in serum, due most likely to coating with albumin which acts as a dis-integrin, helping to avoid uptake by liver.[127, 128] In fact, albumin was demonstrated to be a stabilizer with respect to both particle size and transfection efficiency. In lab-based procedures, a simple mixing procedure at selected ratios of p5RHH and siRNA, followed by albumin coating, creates a transfective 55 nm nanostructure in under 40 minutes. Interestingly, clearance is by the kidney not liver and spleen as the system avoids MPS uptake.[34, 96, 133] The primary mode of deposition is passive “endothelial permeability and retention” in inflamed tissues with leaky vasculature. This approach has proven useful and efficient for delivering the peptide-siRNA nanostructures to other pathologies without the need for molecular targeting. [34, 96, 133, 134]

Summary and future directions

The challenge of delivering siRNA in effective doses to selected pathologies *in vivo* is well known. Various nanostructures have been used in preclinical studies as siRNA carriers. Careful nanocarrier design is aimed at achieving: 1) high endosomal escapability, 2) specific cell or tissue recognition / homing, and 3) enhanced stability and release (Table 6). We have

shown that a variety of peptide-oligonucleotide nanostructures can be formed with the use of our cationic membrane-active melittin-derived peptides that include natural pH sensitive endosomal release mechanisms. The self-assembling nanostructures both prevent destruction of the siRNA in circulation and in endosomes and allow coordinated release of siRNA and endosomal escape. [127, 128] In the RA model we confirmed entry of the nanostructures into the desired compartment (synovial tissue) and subsequent uptake by macrophages following intravenous administration.[34] Due to their size (~55 nm) the peptide-siRNA nanocomplex penetrates through inflamed and leaky vasculature, much like the endothelial permeability and retention (EPR) effect proposed for nanoparticle localization in tumor. A critical barrier to the successful development of OA treatment is the ineffective delivery of therapeutic agents to the resident chondrocytes in cartilage, which is avascular. We show that our peptide-siRNA nanocomplex deeply penetrates human cartilage, suggesting that our approach promises to overcome the obstacles of drug delivery to the highly inaccessible chondrocytes.[96] One thrust of future work is to engineer molecularly targeted peptide-siRNA nanostructures that could confer even more selectively to certain cell types than the conventional endothelial permeability delivery mechanism. The hope is that a broader range of clinical applications will emerge beyond that of liver targeting with polymeric and lipid nanostructures.

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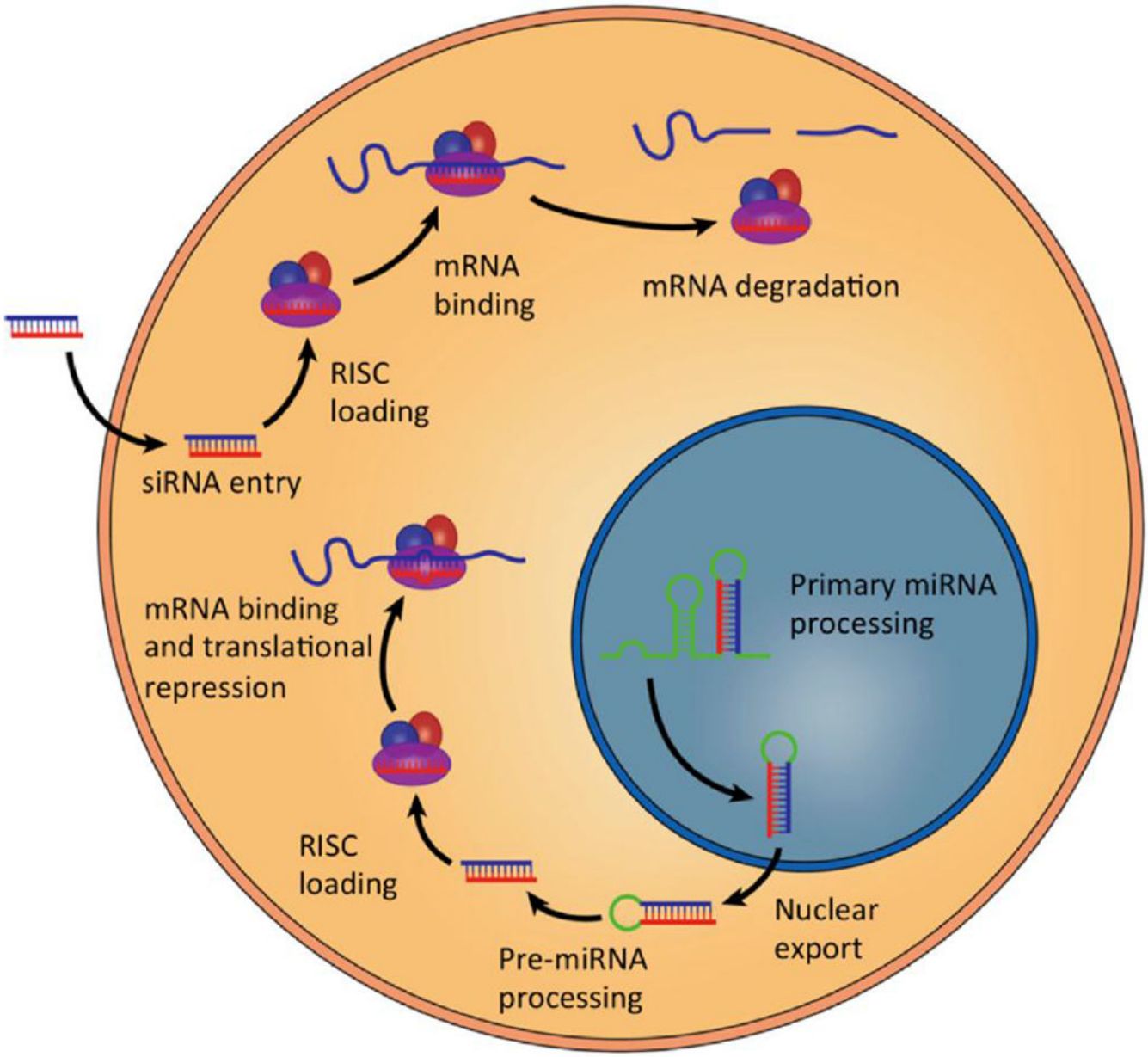

Figure 1.
Exogenous siRNA can induce mRNA degradation and gene silencing if delivered into the cytoplasm. (Reproduced with permission from Hou *et al.* Biotechnology Advances 2015; 33:931-940).

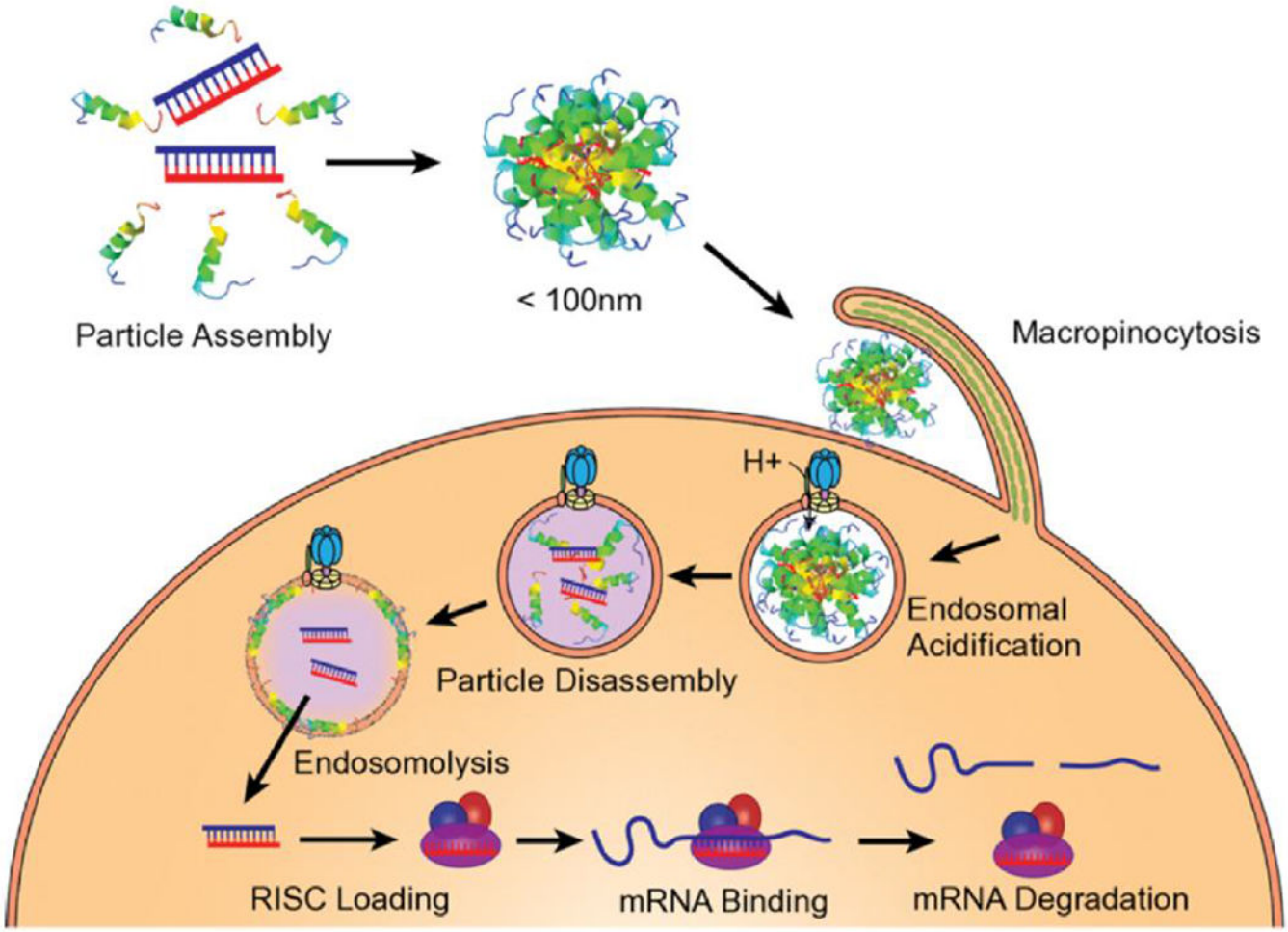

Figure 2.

Melittin-derived peptides promote endosomal escape after particle disassembly triggered by endosomal acidification (Reproduced with permission from Hou *et al.* ACS Nano 2013, 7:8605-15).

Table 1:

Recent siRNA applications for drug delivery in rodent models of RA

Target	Delivery route	Carrier	Analysis time point	Outcome	Model	Reference
TNF-α	Systemic	Peptide (RVG-9R)	10 days	Inhibited inflammation; inhibited cartilage and bone erosions	Mouse CAIA	[24]
TNF-α	Systemic	Thiolated glycol chitosan polymer	7 weeks	Abrogated inflammatory cytokine; protected from bone erosion; suppressed early inflammatory arthritis	Mouse CIA	[26]
TNF-α	Systemic	Degradable cationic polymer (PDAPEI)	5 weeks	Reduce severity of inflammation; lessened cartilage damage; inhibited TNF expression	Mouse CIA	[29]
TNF-α	Systemic	Folate-PEG-chitosan-DEAE nanoparticle	10 days	Decreased disease activity; decreased bone erosions and bone metabolism markers	Mouse CAIA	[25]
TNF-α	Systemic	Shedd able PEGylated solid-lipid nanoparticle	8 days	Decreased disease activity; decreased bone loss	Mouse CAIA	[31]
NF-κB (p65)	Systemic	Peptide (p5RHH)	10 days	Decreased disease activity; decreased bone erosions; reduced cartilage damage	Mouse CAIA	[34]
NF-κB (p65)	Systemic	Oligopeptide modified micelle	15 days	Decreased disease activity; decreased inflammatory cytokines	Mouse CIA	[35]
NF-κB (p65) + Dexamethasone	Systemic	PCL-PEI/PCL-PEG hybrid polymeric micelle	7 weeks	Repressed arthritis; preserved cartilage integrity	Mouse CIA	[36]
NF-κB (p65) + Methotrexate	Systemic	Folate conjugated liposome-based hybrid carrier	7 weeks	Suppressed arthritis; reduced expression of cytokines	Mouse CIA	[37]
NF-κB (c-Rel)	Systemic	PEG-PLL-PLLeu nanoparticle	22 days from onset of arthritis	Decreased disease activity; suppressed inflammatory cytokines	Mouse CIA	[38]
C5	Systemic	C5aR1 Ab-protamine	10 days	Decreased disease activity; decreased inflammation, pannus formation, cartilage and bone damage	Mouse CAIA	[44]
MAS P-3	Systemic	GalNAc-MASP-3 duplex	10 days	Decreased expression of MASP-3 in the liver; decreased clinical score	Mouse CAIA	[45]
ADAM15	Systemic	Atelocollagen-siRNA complex	21 days	Decreased arthritis score; reduced histological damage	Mouse CIA	[48]
IL-2/IL-15 receptor beta chain	Systemic	PEI or PEI-SPIO nanoparticle	30 days	Mitigated arthritis manifestation; effect augmented with the incorporation of SPIO	Rat AA	[46], [47]
hnRNP A2/B1	Systemic	Liposome	60 days (CIA) 10 days (STA)	Decreased incidence and severity of arthritis; decreased production of inflammatory cytokines	Mouse CIA and STA	[49]
Notch 1	Systemic	Thiolated glycol chitosan polymer	42 days	Slowed down progression of arthritis; mitigated cartilage and bone damage	Mouse CIA	[50]

Target	Delivery route	Carrier	Analysis time point	Outcome	Model	Reference
Connexin 43	IA	Electroporation-assisted siRNA transduction	28 days	Suppressed arthritis in knee and ankle when siRNA injected into ipsilateral knee	Rat CIA	[51]
CCR5	IA	Electroporation-assisted siRNA transduction	28 days	Ameliorated arthritis in the knee and ankle when siRNA injected into ipsilateral knee	Rat AA	[52]

AA = adjuvant arthritis; CIA = collagen-induced arthritis; CAIA = collagen antibody–induced arthritis; IA = intra-articular; STA = serum transfer arthritis

Table 2:

Key pathways related to OA targeted by siRNAs

Pathway	Gene	Target cell	Reference
NFκB	*Nfkb*	Chondrocytes	[96], [135]
Wnt/β-catenin	*Lrp5, Dkk, Wnt*	Chondrocytes, synoviocytes, myocardiocytes	[136, 137], [96]
p38 MAPK	*Mkk2*	Chondrocytes	[138], [139]
TGF-β/ SMAD	*Ctgf*	Chondrocytes	[140]

Table 3:

Recent siRNA applications for drug delivery in rodent models of OA

Target	Delivery route	Carrier	Analysis time point	Outcome	Model	Reference
Ihh	IA	Lipid nanoparticle	10 weeks	Chondroprotective; attenuated cartilage degeneration	Rat ACLT	[93]
NF-κB	IA	Peptide (p5RHH)	2 weeks	Reduced synovitis; reduced chondrocyte apoptosis	Mouse joint loading	[96]
Yap	IA	None	8 weeks	Ameliorated OA development; reduced aberrant bone formation; prevented cartilage degradation	Mouse ACLT	[97]
Hif-2α	IA	Chondrocyte-homing peptide/PEI	7 weeks	Reduced cartilage degeneration; Alleviated synovitis	Mouse ACLT, MCLT	[99]
MMP-13 ADAMTS-5	IA	None	4, 8 weeks	Reduced cartilage degeneration; lowered OA score	DMM	[101]
MMP-13	IA	None	8 weeks	Reduced OARSI score; delayed cartilage degeneration	DMM	[102]
MMP-13	IA	None	2 weeks	Reduce cartilage degeneration; decreased OARSI score	DMM	[103]

ACLT = anterior cruciate ligament transection/tear; IA = intra-articular; MCLT = medial collateral ligament transection; DMM = destabilization of medial meniscus; OARSI = Osteoarthritis Research Society International

Table 4:

siRNA application for bone and cartilage regeneration

siRNA target	Tissue	Action	Regenerative effect	Model	Reference
PHD2	Bone	Block binding of PHD2 to HIF-1	Enhanced expression of angiogenic proteins	Sheep periosteal implant	[141]
GNAS1	Bone	Induce expression of transcription factor Cbfa1	Provoked the production of bone-differentiating proteins	Sheep periosteal implant	[141]
SOST	Bone	Silence the expression of SOST	Promoted bone formation	Female mice	[142]
HIF2A	Cartilage	Interfere with IL-1β and other catabolic signaling pathways	Restored cartilage homeostasis	Mouse ACLT, MCLT	[99]
NFkB	Cartilage	Suppress mTOR activity	Restored cartilage homeostasis	Mouse joint loading	[96]

ACLT = anterior cruciate ligament transection/tear; IA = intra-articular; MCLT = medial collateral ligament transection; DMM = destabilization of medial meniscus

Table 5:

MicroRNAs used to treat OA in rodents using intra-articular delivery

MicroRNA	Expression	Species	Model	Analysis time point	Outcome	Target gene	Reference
miR-140	Agomir	Rat	ACLT +MMx	4 weeks, 8 weeks, 12 weeks	Increased cartilage anabolism; reduced cartilage pathology; decreased *Mmp13* and Adamts4 expression	*Mmp13, Adamts4*	[143]
miR-181a-5p	Antisense oligonucleotide	Rat	DMM	10 weeks	Attenuated cartilage destruction; decreased expression of catabolic, hypertrophic and apoptotic genes; reduced collagen type II breakdown	*Mmp13, Col10, Parp, p85, Casp3*	[144]
miR-93	Agomir	Mouse	MMT	2 weeks	Inhibited levels of *Il1b, Tnfa*, and *Il6*; deceased chondrocyte apoptosis	*Tlr4, Nfkb*	[145]
miR-29a	Lentivirus	Mouse	CIA	8 weeks	Lessened the collagenase aggravation of excessive synovial remodeling reaction; lowered *Vegf* production and angiogenic activation	*Vegf*	[146]
miR-483-5p	Antagomir	Mouse	DMM	5 weeks	Decreased cartilage pathology score; marked reduction in *Runx2* positive chondrocytes	*Matn3*	[147]
miR-98	Antagomir	Rat	ACLT +MMT	2 weeks	Relieved cartilage degradation; prevented downregulation of *Bcl2* in cartilage	Bcl2	[148]
miR-222	Lentivirus	Mouse	DMM	8 weeks	Reduced cartilage destruction; decreased *Mmp13* levels	*Mmp13*	[149]

miR = microRNA; ACLT = anterior cruciate ligament tear; MMx, total medial meniscectomy; DMM = destabilization of medial meniscus; MMT = medial meniscus tear; CIA = collagen induced arthritis

Table 6:

Carrier design features to optimize siRNA delivery

Nanoparticle function	Carrier design	Reference
Endosomal escapability	Addition of polyethylenimine (PEI)	[29], [36], [46], [47]
	Polyarginines (RVG-9R)	[24]
	Peptides (p5RHH)	[34], [96]
Stability and release	Redox potential responsiveness (glutathione)	[26], [50]
	Acidic environment (pH) reponsiveness	[29], [32], [34], [96]
Cell and tissue specific recognition	Folate receptor	[25], [37]
	Monoclonal antibody to cell surface receptors	[44]
	Cell-specific ligands	[45]
	Tissue specific ligands	[48], [99]
	Aptamers	[66]