OXFORD

Precision Clinical Medicine, 2023, **6**: pbad014

DOI: 10.1093/pcmedi/pbad014
Review

New treatment for osteoarthr: pbad014itis: Gene therapy

Xinyu Li[1], Leyao Shen[2], Zhenghan Deng[3,*] and Zeyu Huang[1,*]

[1]Department of Orthopaedic Surgery, West China Hospital, West China Medical School, Sichuan University, Chengdu 610041, China
[2]School of Dentistry, University of Michigan, Ann Arbor, MI 48109-1078, USA
[3]Department of Sports Medicine, the First Affiliated Hospital of Shenzhen University, Shenzhen Second People's Hospital, Shenzhen 518035, China
*__Correspondence:__ Zeyu Huang, zey.huang@gmail.com; Zhenhan Deng, dengzhenhan@email.szu.edu.cn

Abstract

Osteoarthritis is a complex degenerative disease that affects the entire joint tissue. Currently, non-surgical treatments for osteoarthritis focus on relieving pain. While end-stage osteoarthritis can be treated with arthroplasty, the health and financial costs associated with surgery have forced the search for alternative non-surgical treatments to delay the progression of osteoarthritis and promote cartilage repair. Unlike traditional treatment, the gene therapy approach allows for long-lasting expression of therapeutic proteins at specific sites. In this review, we summarize the history of gene therapy in osteoarthritis, outlining the common expression vectors (non-viral, viral), the genes delivered (transcription factors, growth factors, inflammation-associated cytokines, non-coding RNAs) and the mode of gene delivery (direct delivery, indirect delivery). We highlight the application and development prospects of the gene editing technology CRISPR/Cas9 in osteoarthritis. Finally, we identify the current problems and possible solutions in the clinical translation of gene therapy for osteoarthritis.

Keywords: osteoarthritis, gene therapy, expression vectors, CRISPR/Cas9

Introduction

Osteoarthritis (OA) is a degenerative joint disease that causes pain and disability in 250 million people.[1,2] Rather than defined as a single mechanically induced disease as before, OA has been increasingly considered to be a complex inflammatory and metabolic syndrome involving the entire joint tissue.[3] The most significant change of OA is degeneration in articular cartilage. Components derived from degraded cartilage trigger foreign body reaction in synoviocytes to induce synovitis. Synovitis leads to the production of metalloproteinases and inflammatory factors and synovial angiogenesis, which further aggravates cartilage destruction.[4] Current treatment approaches, which focus on alleviating pain and restoring motor function, include pharmacotherapy, non-pharmacotherapy, patient education and self-management.[5] Pharmacotherapy is indicated for moderate and severe OA patients to alleviate pain, improve joint function and quality of life. Non-pharmacological treatments mainly refer to arthroplasty, which is the most effective treatment for patients with end-stage OA.[6] The disadvantage of drug therapy is that patients need to take long-term drugs that pose a high risk to the gastrointestinal and cardiovascular systems such as non-steroidal anti-inflammatory drugs (NSAIDs) and cyclooxygenase 2 (COX2).[7] Although surgical treatment can significantly improve patients' motor function, postoperative bleeding and infection as well as traumatic damage caused by surgery are still of concern. Due to the change in perception of OA, the therapeutic principle of OA has shifted from support to regeneration or prevention. In order to slow down or reverse the process of cartilage degeneration, disease-modifying osteoarthritis drugs (DMOADs) have been proposed due to their effect of alleviating structural exacerbation of OA while relieving symptoms.[8]

As a result of advances in biological sciences, genetic engineering has recently evolved into an effective method for cartilage regeneration. Compared with traditional treatment methods, gene therapy has advantages such as effective targeting, prolonged half-life and minor side effects. In addition, gene therapy, either alone or in combination with cells and scaffolds, can alleviate local inflammation and metabolism in OA[9] and even promote cartilage repair.[10] Therefore, the study of gene therapy in OA will contribute to the development of DMOADs and application of long-term clinical translation. In this review, we summarize the current applications of gene therapy in OA, and discuss the choice of vector, gene, and mode of delivery for gene therapy. Finally, we highlight the development and application of CRISPR/Cas9 in OA therapy and indicate the difficulties and solutions for extending OA gene therapy to clinical applications.

History of gene therapy and its development in OA

Gene therapy is described by the US Food and Drug Administration (FDA) as "items that mediate their effects through transcriptional and/or translational transfer of genetic material and/or integration into the host genome, including nucleic acids, viruses, or genetically modified microbes". These items can be used to modify cells *in vivo* directly or *ex vivo* cells before delivering cells to recipients.[11] In 1989, Rosenberg et al. used lentiviruses to genetically modify tumor-infiltrating lymphocytes (TILs) and thereafter transfused transgenic TILs back into patients. It was the first ever approved trial to transfer the gene into a human, demonstrating the feasibility of retroviral gene transfer in human gene therapy.[12,13] In 1990, the FDA approved the first clinical trial of

Received: March 31, 2023. **Accepted:** May 24, 2023. **Published:** 30 May 2023

therapeutic gene transfer in humans. Blaese *et al.* used retroviruses to transfer genes mediating adenosine deaminase (ADA) expression into T cells from two children with severe combined immunodeficiency (ADA-SCID).[14] The study has been a driving force in the application of gene therapy in clinical trials.

Interleukin-1 (IL-1), which is induced in OA-associated cartilage and synovial tissue, is one of the main factors in the disease. IL-1 enhances cartilage catabolism by up-regulating extracellular proteolytic enzymes, accelerates the inflammatory response of OA, and inhibits cartilage anabolism by down-regulating collagen and proteoglycan production.[15,16] The endogenous IL-1 receptor antagonist (IL-1Ra), which competes with IL-1 for IL-1 receptor binding, is one of the most potent inhibitors of the IL-1 pathway as it blocks the initiation of the signal transduction.[17] The earliest gene therapy for OA was focused on inhibiting the IL-1 pathway in articular cartilage and synovium. The initial study was to transduce IL-1Ra cDNA into rabbit synovial fibroblasts using retroviruses, and then transgenic synoviocytes were transplanted into rabbit knees by intra-articular injection. It was shown that the transgenic synovial cells could protect against damage triggered by exogenous IL-1β injections .[18] Baragi *et al.* transfected human articular cartilage monolayer culture with adenovirus vector carrying human IL-1Ra gene, and found that the genetically modified chondrocytes were resistant to IL-1β-induced degradation of the extracellular matrix (ECM).[19] Pelletier *et al.* demonstrated for the first time *in vivo* that transplantation of synoviocytes transduced with the IL-1Ra gene following intra-articular injection delayed the progression of early anterior cruciate ligament transection (ACLT) model induced OA.[20] The results showed that human IL-1Ra gene could be effectively introduced into canine synoviocytes by retrovirus infection and produce high levels of IL-1Ra in transgenic knee joints under the experimental conditions. In summary, these early studies collectively indicated gene therapy as a promising treatment option for OA, setting the stage for a large number of subsequent studies.

Targets

Unlike gene therapy for inherited diseases, which is dedicated to replacing defective genes with normal ones, gene therapy for OA is aimed at overexpressing therapeutic genes or downregulating the expression of deleterious genes by direct or indirect means. Currently, the function of genes used in the treatment of OA typically includes (1) promotion of chondrocyte proliferation and growth; (2) inhibition of chondrocyte senescence and apoptosis; (3) elevation of anabolism resulting in more ECM production; (4) prevention of inflammation, catabolism, and synthesis of matrix degrading enzymes.

Transcription factors

The main transcription factors involved in the progression of OA include Runt–related transcription factor (Runx1,2,3), Sex-determining region Y (Sry)-box-containing family (Sox5,6,9), CCAAT/enhancer binding protein (C/EBPβ) and Hypoxia-inducible factor (HIF-1α,2α).[21] Runx2 is a transcription factor essential for chondrocyte maturation and bone formation in normal cartilage. It promotes the expression of catabolic factors into the extracellular matrix of cartilage and is upregulated in human osteoarthritic cartilage and in mouse articular cartilage after joint injury.[22] Runx3 acts similarly to Runx2 and also regulates chondrocyte hypertrophy, although Runx2 has a more pronounced effect.[23] In contrast, upregulation of Runx1 mRNA by intra-articular injection ameliorates the progression of OA in mice, including osteophyte formation and cartilage destruction.[24] Runx2 and C/EBP work closely together physically and functionally to stimulate the production of matrix metallopeptidase-13 (MMP13), the main collagenase of type 2 collagen, leading to the progress of OA.[25] Sox9 is a transcription factor responsible for chondrogenesis and promotes the expression of Coll II. Delivery of the Sox9 gene via the recombinant adeno-associated virus (rAAV) vector durably enhances the repair process at sites of osteochondral damage in sheep and counteracts the progression of focal OA in adjacent joints.[26] HIF-1α promotes cartilage formation through upregulation of Sox9 expression. In addition, HIF-1α protects articular cartilage by promoting chondrocyte differentiation, maintaining chondrocyte viability, and supporting metabolic adaptation to hypoxic environments. In contrast to HIF-1α, HIF-2α directly induces chondrocyte catabolism, mediates chondrocyte apoptosis and regulates mature chondrocyte autophagy.[27,28]

Growth factors

Insulin-like growth factor 1(IGF-1) is closely involved in cartilage formation and maintenance. IGF-1 promotes chondrocyte proliferation, enhances ECM production, and inhibits ECM degradation and chondrocyte apoptosis. However, under OA condition, the chondrocytes produce excessive IGF receptor binding protein. In addition, the arthritis related inflammation environment accelerates IGF-1 degradation. Thus, direct injection of IGF-1 into the joint cannot achieve the expected effect in most cases.[29] Implantation of chondrocytes overexpressing IGF-1 at the site of injury has shown to improve predominance and type 2 collagen production in articular cartilage.[30] The transforming growth factor-β (TGFβ) is also implicated in OA and cartilage biology. TGFβ superfamily consists of nearly forty ligands, and in cartilage, the main representatives of this superfamily are TGF-β, bone morphogenetic protein (BMP) and growth/differentiation factor-5 (GDF-5). All of them are essential for normal joint development and homeostasis, and have been implicated in the pathogenesis of OA.[31] TGF-β is beneficial to cartilage because it stimulates chondrocytes *in vitro* to induce an increase in proteoglycan and type 2 collagen production. In addition, TGF-β can also resist IL-1β and TNF-α induced catabolism.[32] BMP is generally thought to have a protective effect on articular cartilage, but it has also shown to be involved in chondrocyte hypertrophy and ECM degradation. Steinert *et al.* transferred genes for BMP-2 and BMP-4 into human bone marrow-derived mesenchymal stem cells (MSCs) and found that BMP promoted upregulation of various cartilage hypertrophy and maturation indicators. This suggests that the therapeutic use of BMPs for OA should be used cautiously.[33] GDF-5 can stimulate the expression of aggrecan (ACAN) and Sox9 in human chondrocytes, and thus promote anabolism. In addition, GDF-5 also inhibits the expression of ECM degrading enzyme MMP-13 and disintegrin and metalloproteinase with thrombospondin motifs-4 (ADAMTS4).[34] Fibroblast growth factor 2(FGF-2) and FGF-18 of the fibroblast growth factor family also have regulatory effects on cartilage. FGF-18 is an anabolic factor that promotes articular cartilage repair and cartilage formation. The role of FGF-2 in cartilage metabolism is currently controversial. Some reports suggest that FGF-2 is involved in catabolism, while others show that it inhibits ADAMTS-5 and delays cartilage degradation in murine OA.[35,36] Indeed, co-transfection of multiple growth factor genes into cells has a better therapeutic effect on OA than single gene transfer into cells. For example, transfer of IGF-1 combined with FGF into rabbit knee joint via adenoviral vector induced more type 2 collagen expression and reduced ECM enzyme expression.[37]

Inflammation-related cytokines

The disruption of cytokine homeostasis is one of the most prominent contributors in the pathogenesis of OA. This is primarily because the process favors the generation of pro-inflammatory cytokines that degrade cartilage and other intra-articular structures by activating catabolic enzymes. Among all the cytokines, IL-1β, TNF-α, and IL-6 are the most significant inflammatory mediators in the etiology of OA.[38] IL-4 and IL-10 are major anti-inflammatory factors in the pathology of OA and have significant chondroprotective effects, alleviating chondrocyte apoptosis either alone or in combination.[39] Therefore, down-regulation of pro-inflammatory factors or up-regulation of anti-inflammatory factors by gene therapy is a promising treatment for OA. Intra-articular overexpression of IL-1β and TNF-α antagonists via viral vectors has shown to reduce ECM degradation, attenuate chondrocyte apoptosis, and slow the progression of OA. In addition, completed or ongoing clinical trials support the effectiveness of this approach for the treatment of OA.[16,40] MSCs spheroids with the expression of IL-4 gene have also been reported to alleviate painful symptoms of OA in rats and provide durable protection against cartilage.[41] Intra-articular administration of AAV5-IL-10 in horses achieved sustained local IL-10 expression in the joint and resulted in a significant decrease in IL-1β and IL-6 expression.[42] It is worth mentioning that the combination of anti-inflammatory factors is more helpful in terms of therapeutic effect than the single use.[43]

Non-coding RNAs

With the rising number of high-throughput profiling studies and mechanistic studies of microRNAs (miRNAs), long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs) in joint tissues and biological fluids, non-coding RNAs (ncRNAs) have been shown to possess a significant regulatory role in the pathogenesis of OA. ncRNAs regulate a wide range of physiological processes (inflammation, senescence, oxidative stress, chondrogenic differentiation, autophagy, methylation), signaling molecules (TGF-β, NFκB, Wnt–β-catenin) and mediators (FGF18, Sox9, Sox5, Hedgehog). These regulatory roles of ncRNAs directly or indirectly influence the inflammatory response, cell death and extracellular matrix production in OA pathology.[44] A variety of ncRNAs can inhibit IL-1β and TNF-α-induced inflammatory responses in chondrocytes by acting upstream and downstream of the NFκB pathway or on pathway-related molecules. Inhibition of IL-1β-mediated cartilage inflammatory responses is executed by targeting connexin 43 (CX43) miR-382–3p via TLR4/MyD88/NF-κB signaling pathway.[45] LncRNA SNHG1 attenuates IL-1β-induced OA by inhibiting miR-16–5p-mediated NF-κB signaling pathway.[46] The effect of ncRNAs on apoptosis and proliferation of chondrocytes usually occurs via the phosphoinositide 3-kinase (PI3K)-AKT signaling pathway. For example, overexpression of miR-455–3p in chondrocytes ameliorates apoptosis and mitigates OA progression by modulating the PI3K/AKT pathway.[47] In addition, miR-20 blocks chondrocyte proliferation and autophagy by targeting ATG10 via the PI3K/AKT/mTOR signaling pathway.[48] Furthermore, ncRNAs in most cases affect the progression of OA by regulating ECM enzymes. For example, both circCDR1as and circTMBIM6 can up-regulate MMP13 expression through the mechanism of competing endogenous RNAs (ceRNAs).[49,50] CircRNA has been studied as a biomarker for various diseases due to its higher stability compared to other ncRNAs. For example, circ_016901, circ_0032131, circ_0044235 and circ0000175 in peripheral blood have been suggested as markers for the early diagnosis of arthritis.[51,52]

Small nucleolar RNA (snoRNA) is a class of ncRNAs ranging from 60 to 300 nt in length and is closely related to the splicing and processing of ribosomal RNA (rRNA) precursors, post-transcriptional modification processes and ribosome biosynthesis.[53] Studies have shown that snoRNAs can also be used as diagnostic biomarkers and therapeutic targets for joint aging and OA. The results of SnoRNASeq showed 27 differential snoRNAs in serum from young and aged mice and 18 differential snoRNAs in serum from sham-operated groups and experimental OA group.[54] The results of snoRNA matrix analysis of cartilage from young, old and OA patients showed that SNORD26 and SNORD96A were associated with aging while SNORD26 and SNORD116 were associated with the development of OA.[55]

Gene delivery vectors

A gene must be put into a vector for proper expression in a target cell. The ideal vector is chosen based on two criteria: (1) effective gene transfer with simple access to target cells and steady gene transcription after entering the nucleus; and (2) safety with minimal consequences of infection, mutation, or immunogenicity.[56] Currently, two types of vectors are commonly used, viral vectors and non-viral vectors, which have different advantages and disadvantages.

Viral vectors

The transmission of particular cell types with extreme specificity and dependability is the major benefit of viral vectors.[57] On the other hand, viral vectors also have drawbacks, including limited carrying capacity of therapeutic genetic material, invasive administration, and the possibility of unfavorable immune reactions or subsequent genetic impacts.[58,59] Typically, three recombinant viral vectors are used as appropriate means of delivering therapeutic genes directly and specifically into cells, including retroviruses, adenoviruses and AAV.[60]

Retrovirus

The vectors of the retroviral family are spherical enclosed viruses with a diameter of about 100 nm. Their genomes are made up of two copies of sense (+) strand RNA. Retrovirus is often identified by the ability of reverse transcribing its RNA genome into cDNA, and permanently integrates these cDNA copies into the genome of the host cell. This allows maintenance of gene expression over a longer time span.[61] The most often used retroviral vectors are gamma retroviral vectors and lentiviral vectors. These vectors are characterized by their efficient gene transfer capabilities and ability to integrate into the cellular genome so as to achieve stable genetic modification of the target cells.[62] Among retroviruses, lentiviral vectors are more suitable for transfection of non-dividing cells. Studies showed that the efficiency of transfection of chondrocytes using lentivirus was up to 95% and that of MSCs was 70%.[63,64] The biggest drawback of retroviral applications is the possibility of insertional mutations and oncogene formation.[65,66] Therefore, the use of retroviruses for gene therapy in OA patients requires further investigation.

Adenovirus

At present, the most commonly used adenoviral vectors are based on human adenovirus type 5 (Ad5), with 36 kb long genome and linear double-stranded DNA structure. Adenoviruses exist in the form of loose bodies in cells.[67] The advantages of adenoviral vectors include a wide range of tropism, effective

transduction of dividing and non-dividing cells, high transduction efficiency and rapid onset of action and relatively large packaging capacity.[68] Adenoviruses have been used to deliver genes encoding growth factors,[37,69] transcription factors,[28,70–75] enzymes,[76–80] and cytokines[81,82] to chondrocytes, synovial fibroblasts, and MSCs.

Because of the strong host humoral and cellular immune responses to adenoviral gene products, the use of adenoviral vectors in clinical settings has received serious attention. Using vectors that only contain genes, packaging sequences and flanking viral end duplications can help reduce immune responses, which is one approach to addressing this issue. However, this strategy requires the use of another helper virus to achieve cell transduction.[40]

Helper-dependent adenovirus (HDV) vectors lack the coding sequences of the main components of adenoviruses and contain only about 500 bp cis-acting elements for adenoviral DNA replication and packaging. Therefore, in addition to helper viruses, the process also requires all proteins required for adenoviral vector packaging. Compared with adenoviral vectors, HDV vectors have advantages such as long transgene expression time, large transgene capacity, excellent safety and less side effects.[83] Previous studies have shown that HDV can transduce chondrocytes and synoviocytes more efficiently compared to AAV. Additionally, HDV-transduced genes might continue to express for more than a year after being injected into the knee joints of normal mice.[84] FX201, constructed based on the HDV vector of human serotype 5, is an innovative intra-articular therapeutic agent for IL-1Ra. Using FX201, the gene is delivered to joint cells and produces IL-1Ra under the control of an inflammation-sensitive promoter.[84] Senter *et al.* demonstrated that a single intra-articular injection of FX201 7 days after ACLT model relieved osteoarthritic changes in cartilage, bone, and synovium 12 weeks after surgery.[85] Moreover, FX201 persisted in the joint for at least 3 months without any adverse effects. Currently, clinical trials investigating the safety and tolerability of FX201 in patients with knee OA are ongoing.

Adeno-associated virus

AAV is a single-stranded DNA parvovirus that is non-pathogenic and non-enveloped. The primary benefits of AAV vectors include their small size, low immunogenicity, and inability of viral infection. Therefore, AAV is currently the most commonly used viral vector.[86] AAV can be modified to remove viral genetic material so that it can produce targeted gene transcription without generating viral proteins.[87] The most prevalent AAV serotype is AAV-2, which has a wide tissue tropism and a respectable cell infection effectiveness. Different serotypes of AAV exhibit different transduction efficiencies in chondrocytes and MSCs. Serotypes of AAV2, AAV5, AAV6 and AAV6.2 are appropriate for human chondrocyte *in vitro* culture systems. Different AAV serotypes also differ in tissue specificity. For example, AAV2 and AAV6.2 are better choices for hard cartilage tissue as they have a higher transduction efficiency on chondrocytes than other AAV serotypes. The high transduction efficacy of AAV2 and AAV6.2 in human chondrocytes and MSCs suggests that these viruses have stronger chondrocyte selectivity both *in vitro* and *in vivo*.[88]

In the current field of gene therapy, the most used AAV vectors are the rAAV vector family or self-complementary AAV (ScAAV). The rAAV cannot be integrated into genome; thus, it can be used to infect both dividing and non-dividing cells. The rAAV is thought to be the least harmful viral system because it causes relatively little innate and adaptive immune reactions to transduced cells.[89] Even though rAAV's genome is still an episome, it can support *in vivo* long-term transgene expression in dormant cells.[90] In multiple clinical studies, rAAV is the vector system of choice for human protocols involving *in vivo* gene delivery due to the safty.[68] ScAAV complementary vectors package an inverted repeat genome that can fold into dsDNA without DNA synthesis or base pairing between multiple vector genomes, thus improving transduction efficiency.[91] When testing the ability of scAAV vectors to deliver genes in joints, researchers found that in the presence of the same number of viral genomes in infected cells, the transgene expression of scAAV vectors was earlier and the gene product was approximately 25-fold higher than traditional AAV.[92] The drawback of scAAV in comparison to standard AAV is its inability of carrying larger genes. Numerous studies of OA have focused on the use of rAAV and scAAV in the studies of mammals, including humans,[93,94] horses,[95,96] dogs,[97] rabbits,[92] sheep[26,98] and pigs.[99,100]

For gene therapy, the efficiency of vector delivery has always been one of the most important concerns of researchers. Studies have shown that synovial tissue has better delivery efficiency than cartilage when adenovirus is injected directly into the joint as a vector. To assess the dose-response and cellular distribution of transduction, Ruan *et al.* evaluated the knee joints of mice injected with 10^9 or 10^8 HDV viral particles.[84] These doses were much less than the maximum tolerated systemic dose in humans. At the higher doses, HDV transduced superficial chondrocytes and synovial cells, whereas at the lower doses only synovial cells were transduced.

Although there are still no customized strategies for cartilage and synovial gene therapy, what has been reported can provide us with some reference. For cartilage, HDV with the same titer has a higher transduction efficiency than many popular AAVs (including AAV2, AAV2.5 and AAV6).[84] Combining HDV with an alpha-10 integrin monoclonal antibody (a10mab) will reposition chondrocytes, and this modified HDV can reduce the effective treatment dosage by 10-fold compared to normal HDV.[101] For synovium, AAV serotype DJ (AAV-DJ) is a recombinant virus of AAV2 and AAV5, which has a better ability to evade immune neutralization and is suitable for *in vitro* and *in vivo* DNA therapy, with a higher transfection efficiency for synovial cells and a higher expression of the transfected protein in the joint fluid.[102] In addition to using a different delivery vehicle, changing the timing of gene delivery can also have an impact on the site of treatment. When AAV was injected before injury, AAV transgene expression was localized to the soft tissues of uninjured and injured joints; whereas when injected after injury, AAV was able to transduce articular chondrocytes within the cartilage matrix.[103]

Non-viral vectors

There are two categories of design of non-viral gene delivery vectors: the delivery of DNA or RNA packaged in a vector, and bare-leakage delivery of DNA or RNA.[104] The former normally utilizes physical methods (gene gun, laser, ultrasound, electroporation, microinjection) to physically deliver the target gene into the target cell. The latter involves use of chemical methods (lipofection, lipoplexes, exosomes, vesicles, nanoparticles) to penetrate cell membranes by packaging target genes into components such as lipophilic particles.[105] The design of non-viral vectors has received increasing attention because they are inexpensive, easy to handle, less toxic and minor immunogenic than viral vectors.

Electroporation is a physical transfection method that promotes cellular uptake of nucleic acids or drugs by applying a high-voltage pulse to the cell membrane for a short period of time. Earlier reports suggested that gene transfer into articular cartilage and synovial cells can be performed using electroporation.[106,107] Grossin *et al.* delivered a plasmid containing the heat shock

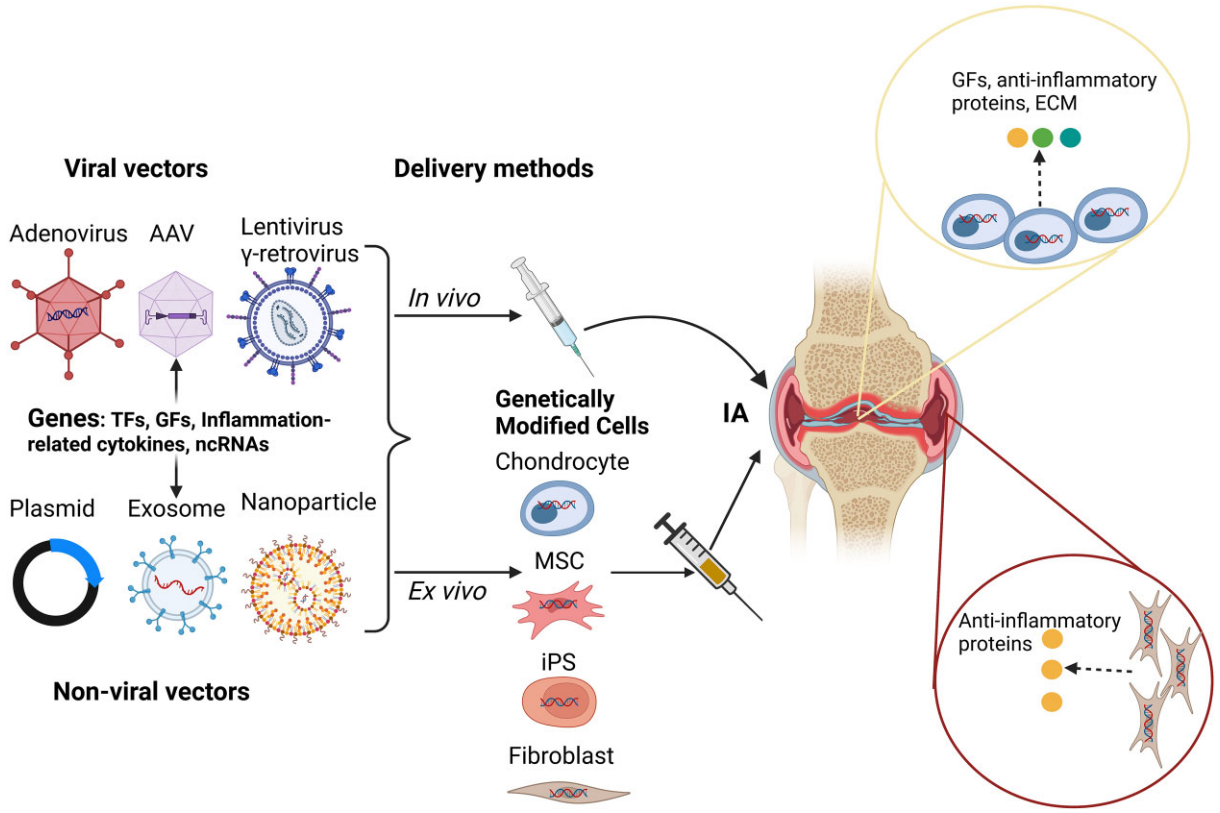

Figure 1. Overview of osteoarthritis gene therapy targets, vectors, delivery methods and treatment processes. Commonly used genes include transcription factors, growth factors, inflammation-associated cytokines, and non-coding RNAs. Delivery vectors consist of viral vectors (including adenovirus, adeno-associated virus, γ-retrovirus and lentivirus) and non-viral vectors (including plasmids, exosomes, nanoparticles). Direct delivery (*in vivo*) refers to the direct delivery of the vector carrying the gene to the target region. Indirect delivery (*ex vivo*) is the genetic modification of cells (including chondrocytes, mesenchymal stem cells, induced pluripotent stem cells and fibroblasts) before delivery to the target site. TFs: transcription factors, GFs: growth factors, ncRNAs: non-coding RNAs, MSC: mesenchymal stem cell, iPS: induced pluripotent stem cell, IA: intra-articular.

proteins70 (HSP70) gene into rat knee cartilage by electroporation.[108] Their study found that 50% of chondrocytes exhibited HSP70 positivity the day after *in vivo* use of electroporation; and after 3 months, long-term expression of the transgene was seen only in the deeper layers (20%–30% of positive cells). They isolated rat chondrocytes for testing at different time points of gene introduction and showed that electrical pulses do not affect ECM synthesis in articular cartilage, indicating that this method is safe for *in vivo* application. Another report suggests that intramuscular electroporation has a better therapeutic effect and duration for arthritis than intra-articular plasmid injection for electroporation.[109]

Cationic liposomes and other lipid-based systems are easy and safe to prepare. Moreover, the transport of this type of vector is not limited by the size of the delivered DNA.[110] However, lipid-based systems have several disadvantages compared to viral vectors, including the low transfection efficiency, which can be affected by the amount of lipid reagent per DNA.[111] FuGENE6 is a commercially available lipid transfection reagent that was used to transfect chondrocytes *in vitro*. The results showed that the efficiency of FuGENE6 is 41% in normal bovine articular cartilage, 20.7% in normal human articular cartilage, and 7.8% in cartilage of patients with OA.[112] The integration of plasmids, and biocompatible and better-targeted components into nanoparticles can produce nanovectors with higher transfection efficiency.[113] Chitosan (CS) has superior biocompatibility and biodegradability and can easily pass through cell membranes and bind to negatively-charged genes. The combination of CS, sodium hyaluronate (HA), chondroitin sulfate (CHS) and plasmid produces nanospheres, which have high stability, elevated transfection efficiency and low cytotoxicity.[114] Chen *et al.* used the above nanoparticle-packed GDF-5 plasmid to transfect rabbit chondrocytes *in vitro*, to achieve a transfection rate of 60%.[115]

Exosomes are membrane-bound extracellular vesicles surrounded by a phospholipid bilayer and released by cells in normal or pathological states into body fluids such as blood, saliva, urine and synovial fluid.[116] Cell-derived exosomes can be modified to carry exogenous genes as a non-viral delivery system.[117] Exosome-cell responses and interactions are based on recognition of membrane receptors. Activation upon receptors triggers certain signaling pathways, which in turn leads to the fusion of the exosome with the plasma membrane or the exocytosis of the exosome. Exosomes can release their cargo into the receiving cell through the internalization pathway mentioned above. Therefore, exosomes as gene carriers are characterized by high targeting efficacy, good safety and low xenobiotics.[118] Currently, a great number of studies have been focusing on the use of engineered exosomes for the treatment of OA.[119–121]

Gene delivery methods

At present, the most commonly used delivery strategies can be summarized into two categories: (1) direct delivery of the target gene to the lesion site by surgical incision or local injection (*in vivo*); (2) *in vitro* transfection or infection of the cells, followed by delivery of the cells to the target tissue (*ex vivo*) (Fig. 1). In

addition, protocols using natural or synthetic scaffolds to carry genes or cells prior to performing the above two delivery methods have increasingly gained popularity in the field.

In vivo methods

Direct delivery is convenient, relatively cheap and less time-consuming. However, the delivered gene via this method cannot be individually targeted to a particular cell, resulting in non-specific targeting in intra-articular cells spread, leading to potential off-target effects. Articular chondrocytes are surrounded by a dense ECM, and the rAAV vector is considered to be the most suitable option for *in vivo* transfection up to this point.[122] At present, direct delivery methods are more commonly used to deliver anti-inflammatory genes,[123] growth factors[124] and ncRNAs.[125]

Ex vivo methods

Ex vivo gene therapy has better safety and fewer off-target effects; however, it is involved with more technical steps and is less applicable because of the technical complexity and the more stringent environmental requirements for *in vitro* cell amplification. Indirect gene therapy strategies for OA typically employ cells such as chondrocytes[126,127] and MSCs.[128,129] Chondrocytes are the only cell type in articular cartilage that can produce ECM to maintain basic joint homeostasis, and autologous articular cells are used for autologous chondrocyte implantation in cartilage repair. The source used for gene therapy in OA is mostly allogeneic cartilage. Chondrocytes isolated from allogeneic cartilage are first modified *in vitro* with an anti-inflammatory gene (IL-1Ra) or growth factor (TGF-β), and then injected into the patient's joint cartilage.[130] TissueGene-C (TG-C), a novel gene therapy for OA, includes a mixture of human allogeneic chondrocytes and irradiated cells modified to overexpress TGF-β1. Currently, TG-C has been used in numerous animal studies and clinical trials. Transformed chondrocytes can adhere to cartilage and continuously secrete TGF-β1. Animal studies have also shown that transformed chondrocytes can promote M2 polarization in rat OA macrophages, which provides an anti-inflammatory microenvironment, thereby relieving pain and improving joint structure.[131,132] Several clinical studies have shown that TG-C improves the International Knee Documentation Committee (IKDC) and Visual Analog Scale (VAS) scores in patients with chronic degenerative knee OA.[126,133,134] Other sources like induced pluripotent stem cells (iPSCs) and MSCs have also been used in preclinical or clinical trials (NCT01448434, NCT01448434). Due to the chondrogenic potential, iPSCs can be an alternative if there is a shortage of autologous and allogeneic chondrocyte sources.[135] MSCs are self-renewing and directionally differentiated cells, which have chondrogenic differentiation potential and can secrete inflammatory regulators for immune regulation in OA. MSCs can exert a better therapeutic effect on OA after the treatment with growth factors, inflammatory regulators and transcription factors.[136] One study showed that a mixture of MSCs and chondrocytes in equal proportions promoted cartilage repair in destabilization of the medial meniscus (DMM)-induced OA compared to single cell types alone.[137] Therefore, the use of genetically modified MSCs and chondrocytes together for *ex vivo* gene therapy may be more promising for the treatment of OA. We summarize the current gene types, vectors, expression regulation types, species, model construction methods, delivery methods and treatment effects of OA gene therapy in Table 1.

Gene-editing for OA

The ability to precisely edit mammalian cell genomes allows us to gain greater insight into deeper mechanisms while minimizing off-target effects. The recently developed CRISPR/Cas9 technology provides a simple and efficient option for gene editing.[148,149] CRISPR/Cas consists of a single guide RNA (sgRNA), targeting ends (including base pairs complementary to the nucleic acid to be edited) and Cas proteins. The use of different target ends enables precise editing of specific genes. After a double-stranded break caused by a Cas proteolytic cleavage of a gene, the cell employs non-homologous end-joining (NHEJ) to repair the broken DNA, thus achieving a knockout. On the other hand, the introduction of exogenous DNA templates into cells can lead to the occurrence of homologous recombination repair (HDR), resulting in gene knock-in.[150] An earlier report transfected human articular chondrocytes with ribonucleoprotein complexes (RNP) containing Cas9 and crisprRNA targeting exon 2 of MMP13. The results showed that the editing efficiency of MMP13 was 63%–74% and the level and activity of secreted MMP13 protein was significantly reduced.[151] This suggests that gene editing within chondrocytes is feasible.

Current applications of CRISPR-Cas9 technology in OA therapy focus on reducing the inflammatory response. Cell transplantation therapy is effective in the treatment of OA. However, inflammatory factors can lead to failure of cartilage differentiation of transplanted cells. Karlsen *et al.* used CRISPR/Cas9 to knock out the IL-1β receptor (IL-1R1) in human chondrocytes and inserted a puromycin resistance gene to screen the cells.[152] The results showed that deletion or knockdown of IL-1R1 improved cell therapy outcomes in OA patients as well as cytokine stimulation by TNF-α independent of IL-1β signaling. The implanted chondrocytes secrete a large amount of cytokines, which in turn leads to the invasion of M1 macrophages in the grafted cartilage, thus reducing the therapeutic effect. To address this situation, knockdown of the TGF-β-activated kinase 1 (TAK1) gene in chondrocytes by CRISPR-Cas9 technology significantly reduced M1 macrophage infiltration and inflammatory factor secretion in transplanted cartilage.[153] CRISPR-Cas9 system is also applied to target growth factor in the treatment to OA. Nerve growth factor (NGF) is upregulated in OA and associated with the onset of pain. However, reduced NGF can also lead to bone destruction.[154] Thus, gene therapy of NGF alone cannot achieve the best therapeutic effect. In this case, simultaneous deletion of NGF, MMP13 and IL-1β genes by intra-articular injection of AAV expressing CRISPR/Cas9 components alleviates pain and structural damage in a post-traumatic OA (PTOA) model.[155]

Although numerous scaffolds can prolong the expression of the target gene, neither the delivery method nor the delivery of any gene can achieve self-regulated, dynamic and feedback release of the target gene. The pathological changes in OA are progressive, and exogenous gene expression usually begins to decline after reaching the peak, which does not meet the subsequent regulatory requirements. Guilak's team has come up with a promising solution to this situation.[156,157] One of their studies packaged a synthetic promoter consisting of NFκB recognition gene sequences (including INF-β1, IL-6, CCL2, Adamts5, and Cxcl10), along with the IL-1Ra gene, into a lentiviral vector. Thereafter they transfected lentiviruses into iPSCs and induced iPSCs into chondrocytes. Such chondrocytes respond in a time- and dose-dependent manner to IL-1α stimulation, rapidly producing therapeutic levels of IL-1Ra to counteract the effects of inflammation. This method may be broadly relevant to the creation of

Table 1. Genes, vectors, animal models, and outcomes in OA gene therapy.

Genes	Vectors	Expression	Species	Model of OA	Delivery methods	Outcome	References
TFs							
Sox9	rAAV	Up	Sheep	Osteochondral defects	*In vivo*	Reduced histological signs of perifocal OA changes	26
Sox5,6,9	Retrovirus	Up	Rat	ACLT	*Ex vivo* (ASCs)	Prevented the progression of degenerative changes	138
KLF2/KLF4	AAV	Up	Mouse	DMM	*In vivo*	Reduced severity of OA-associated changes in cartilage, meniscus and synovium, and improved pain behaviors	70
Mohawk	Adenovirus	Up	Mouse	DMM	*In vivo*	Suppressed meniscus and cartilage damage, reduced OA severity	139
BATF	Adenovirus	Up	Mouse	DMM	*In vivo*	Caused cartilage destruction and synovial inflammation	140
GFs							
IGF-1	Plasmid	Up	Mouse	DMM	*Ex vivo* (ADSCs)	Reduced histological OARSI score and decreased loss of cartilage ECM	141
IGF-1 + bFGF	Adenovirus	Up	Rabbit	ACLT	*In vivo*	Enhanced chondrocyte proliferation, proteoglycan synthesis and type 2 collagen expression	37
TGF-β	Plasmid	UP	Mouse	DMM	*Ex vivo* (MSCs)	Enhanced the repair of damaged cartilage	113
GDF-5	NMPs	Up	Rabbit	DMM + ACLT	*In vivo*	Increased the expression of ECM proteins in chondrocytes	115
VEGF	Lentivirus	Down	Rat	DMM	*In vivo*	Enhanced chondrogenesis and prevented OA progression	142
Inflammation-related cytokines							
IL-1Ra	AAV	Up	Equine	OCF	*In vivo*	Reduced joint effusion and synovitis, and improved repair of the osteochondral lesion	143

Table 1. (*Continued*)

Genes	Vectors	Expression	Species	Model of OA	Delivery methods	Outcome	References
	HDAd	Up	Mouse/horse	ACLT/OCF	*In vivo*	Prevented development of cartilage damage, osteophytes, and synovitis. Improved lameness parameters.	86
IL-10	Adenovirus	Up	Mouse	CIA	*Ex vivo* (MSCs)	Induced long-term reduction in activated T cells	81
IL-1Ra + IL-10	Retrovirus	Up	Rabbit	Excision of the medial collateral ligament and medial meniscectomy.	*Ex vivo* (synoviocytes)	Inhibition of cartilage breakdown	144
IL-4	Plasmid	Up	Rat	ACLT	*Ex vivo* (MSCs)	Improved cartilage protection and pain relief function	41
ncRNAs							
miR-140	Exosomes	Up	Rat	DMM	*In vivo*	Inhibited cartilage-degrading proteases, and alleviated OA progression	119
miR-101	Adenovirus	Down	Rat	MIA	*In vivo*	Inhibition of cartilage degradation	145
circRNA.33 186	Lentivirus	Down	Mouse	DMM	*In vivo*	Increased type 2 collagen expression and decreased MMP-13 expression	146
circPDE4D	AAV	Up	Mouse	DMM	*In vivo*	Suppressed ECM catabolism and alleviated OA progression.	125
lncRNA PILA	Adenovirus	Up	Mouse	DMM	*In vivo*	Triggered spontaneous cartilage loss and exacerbated OA	147

OA: osteoarthritis, TFs: transcription factors, KLF2/4: Krüppel-like factor 2/4, AAV: Adeno-Associated virus, GFs: growth factors, IGF-1: Insulin-like growth factor 1, TGF-β: Transforming growth factor-β, GDF-5: Growth/differentiation factor-5, NMPs: nano-and microplastic particles, HDAd: Helper-dependent adenovirus, ACLT: anterior cruciate ligament transection ASCs: adipose stem cells, ADSCs: adipose derived stem cells, DMM: destabilization of the medial meniscus, VEGF: vascular endothelial growth factor, OCF: osteochondral fragmentation, CIA: collagenase-induced osteoarthritis, MIA: Mono-iodoacetate-induced arthritis

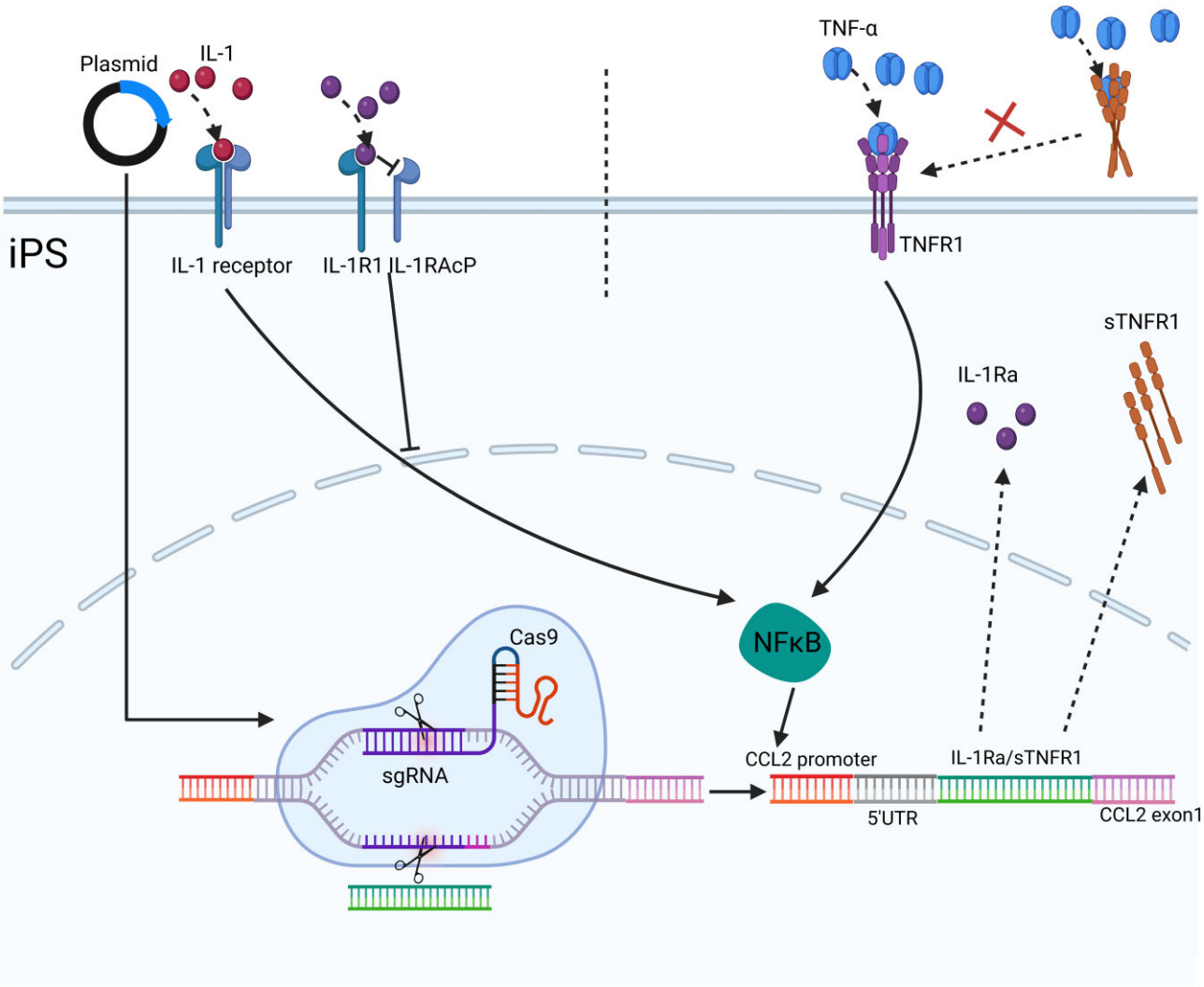

Figure 2. CRIPSR/Cas9-mediated expression of IL-1Ra or sTNFR1 genes downstream of the CCL2 promoter. Introduction of CRISPR/Cas9 into iPSCs by plasmid allows insertion of IL-1Ra or sTNFR1 genes into CCL2 after the promoter. Binding of exogenous IL-1 and IL-1 receptor induces intracellular NFκB pathway activation, which in turn induces CCL2 promoter activation. Similarly, binding of exogenous TNF-α and TNFR1 activates NFκB pathway, subsequently leading the binding of transcription factor NFκB to the CCL2 promoter. Activation of the CCL2 gene leads to the translation of IL-1Ra and sTNFR1 proteins. IL-1Ra binds IL-1R1 subunit with higher affinity than IL-1α or IL-1β, but does not recruit IL-1RAcP. sTNFR1 binds TNF-α without generating downstream intracellular signaling. The gene circuit formed in this way is capable of feedback release of anti-inflammatory factors. sTNFR1: soluble tumour necrosis factor receptor-1, IL-1RAcP: IL-1 receptor accessory protein, CCL2: C-C Motif Chemokine Ligand 2.

auto-regulatory biological systems for the delivery of gene and tissue engineering in response to a variety of disease.[158] The chemokine (C-C) ligand 2 (CCl2) gene product attracts a variety of inflammatory cells, leading to increased inflammation. The activation of CCL2 depends on the continuous expression of IL-1 and TNF-α, and the expression of CCL2 gene decreases along with alleviated inflammation.[159] Based on this property, Choi *et al.* used CRISPR-Cas9 technology to insert the IL-1Ra gene after the CCL2 promoter in iPSCs (Fig. 2).[160] This synthetic gene circuit can sense changes in endogenous inflammatory cytokine levels, thereby triggering a proportional treatment response and blocking IL-1Ra production when inflammation resolves. Gene-edited iPSCs delivered to OA cartilage significantly reduced joint pain, structural damage, and systemic and local inflammation in the mouse model with inflammatory arthritis.

CRISPRa system is a modified CRISPR-Cas9 system for upregulation of genes. It utilizes nuclease-inactivated Streptococcus pyogenes Cas9 (dCas9), fused to transcriptional activation domains (VP64, p65 and Rta). When paired with a guide RNA targeting a gene near the promoter region, the natural transcriptional start site of the gene is activated.[161] Farhang *et al.* used this system to successfully upregulate the gene transcription of ACAN and COL2A1 in pellet-cultured human adipose stem cells (hADSC), which mediated the deposition of sulfated glycosaminoglycan (sGAG) and type II collagen.[162] Compared to the use of growth factors to induce differentiation of stem cells into natural cell types, this approach overcomes the effect of the musculoskeletal microenvironment on growth factors *in vivo* and allows for more precise and controlled production of matrix proteins.

Among the factors associated with the development of OA, genetic factors account for approximately 50% of the total risk of disease in individuals. Currently, more than 100 polymorphic DNA variants have been associated with OA. Although data from OA genetics studies have not yet led directly to new treatments, a

Table 2. Completed, in-recruit and out-recruit clinical trials for OA gene therapy.

Intervention	Identifier	Target	Method of delivery	Conditions	Study	Trial status	Evaluations
TissueGene-C	NCT02341378	TGF-β1	Retrovirus, *ex vivo*	Degenerative arthritis	Randomized, Multicenter, Single-blind, Phase 2A	Completed	Efficacy and safety
	NCT00599248	TGF-β1	Retrovirus, *ex vivo*	OA	Randomized, Single-blind, Phase1	Completed	Safety and activity
	NCT02341391	TGF-β1	Retrovirus, *ex vivo*	Degenerative arthritis	Randomized, Dose-escalating, Single-center, Phase 1	Completed	Safety and efficacy
	NCT02072070	TGF-β1	Retrovirus, *ex vivo*	Degenerative arthritis	Randomized, Placebo Controlled, Double-blind, Multi-center Phase 3	Completed	Efficacy and safety
	NCT01671072	TGF-β1	Retrovirus, *ex vivo*	Degenerative arthritis	Randomized, Placebo Controlled, Single-blind, Multi-center Phase 2B	Completed	Safety and efficacy
	NCT01825811	TGF-β1	Retrovirus, *ex vivo*	Degenerative arthritis	Single-blind, Randomized, Parallel-group, Multi-center Phase 2	Completed	Efficacy and safety
Invossa K	NCT03412864	TGF-β1	Retrovirus, *ex vivo*	OA	Observational	Not recruiting	Efficacy and safety
FX201	NCT04119687	IL-1Ra	HDAd, *in vivo*	OA	Non-Randomized, Open-Label, Single Ascending Dose, Phase 1	Not recruiting	Safety and tolerability
ICM-203	NCT04875754	Nkx3.2	AAV, *in vivo*	OA	Randomized, Double-Blind, Placebo-Controlled Single Dose Escalation, Phase 1/2a	Recruiting	Safety, tolerability, and range
	NCT05454566	Nkx3.2	AAV, *in vivo*	OA	Randomized, Single Dose Escalation, Phase1/2	Not recruiting	Safety, tolerability, and activity
XT-150	NCT03477487	IL-10	Plasmid, *in vivo*	OA	Non-Randomized, Ascending dose-ranging, Phase 1	Completed	Safety, tolerability, and efficacy
	NCT04124042	IL-10	Plasmid, *in vivo*	OA	Randomized, Double-Blind, Placebo-Controlled, Phase 2	Completed	Efficacy and safety

number of proteins encoded by genes associated with OA have potential for therapeutic applications.[163] Point mutations or exogenous knock-in using the CRISPR-Cas9 system can better explore the impact of risk variants in joint development on OA pathogenesis. Dicks *et al.* explains how functionally acquired mutations in transient receptor potential vanilloid 4 (TRPV4) can lead to skeletal dysplasia of varying severity by generating CRISPR-Cas9 gene edited hiPSC cell lines with V620I or T89I TRPV4 mutations.[164] Their results suggest that TRPV4 mutations alter BMP signaling in chondrocytes and prevent chondrocyte hypertrophy. Similarly, Klein *et al.* knocked the single nucleotide polymorphism (SNP) rs4730222 into the osteosarcoma cell line.[165] The results demonstrate that rs4730222 leads to increased expression of alternative transcripts of disease-associated alleles, followed by reduced expression of typical HMG box protein 1 (HBP1), leading to the development of OA.

Existing issues and future directions

The number of clinical trials of gene therapy for OA is relatively small compared to other diseases. A search on the website clinicaltrial.gov shows that 12 clinical trials (including TissueGene-C, FX201, ICM-203, XT-150, Invossa K) for OA and degenerative arthritis gene therapy are currently enrolled, ongoing, or completed. We categorized these clinical trials in Table 2 according to intervention name, identification number, target, delivery mode, disease type, trial type, and evaluated subjects. We believe that in addition to the high cost of gene therapy itself and the obstacle of commercializing therapeutic products, there are three major issues that need to be addressed.

The first and most important issue is the safety of gene therapy. In some cases, viral vector activation of the immune system led to death of patients due to multiple organ failure. These cases serve as a constant reminder of the awareness and caution of the safety of gene therapy.[166] Although there are no serious incidents in gene therapy for OA, the immune activation and tumorigenic side effects of viral vectors still demand a more rational use of gene therapy. The use of non-integrated and low-immunogenic rAAV or safer non-viral vectors may be a more promising direction for future research.

Second, the existing OA-related clinical trials all focus on *in vivo* suppression or overexpression of a single gene. However, the functions of genes are diverse and can lead to unpredictable harmful pathological processes. For example, the NF-κB family member RelA/p65 is a potent transcriptional activator of ADAMTS5 in chondrocytes, while it can also induce anti-apoptotic genes to protect chondrocytes from apoptosis during the development of OA.[167] In articular cartilage, heterozygous knockout of Rela caused a considerable acceleration of OA by increasing chondrocyte death, despite reduced expression of catabolic genes.[168] In this case, the use of multigene regulation instead of a single gene may be a more practical approach. Simultaneous regulation of NGF, MMP13 and IL-1β genes not only addressed the structural damage caused by deletion of NGF genes, but also alleviated the pain symptoms in OA.[155] In summary, the use of multiple gene regulation can effectively exploit the therapeutic effects of genes while avoiding harmful effects.

Lastly, gene expression requires dynamic homeostasis and should be maintained within an appropriate range. Otherwise, unintended adverse effects may occur. In the treatment of OA, it is not the continuous inhibition of inflammatory factor expression or the continuous promotion of growth factor expression that provides the best therapeutic effect. Excessive TGF-β in OA can also lead to synovial fibrosis, osteophyte formation and subchondral bone changes.[169] Potent overexpression of TGFβ1 in rabbit joints with experimental OA resulted in a significant reduction in cartilage ECM.[170] The above-mentioned self-feedback gene regulation circuit using CRISPR/Cas9 technology has an environment-dependent feature and can automatically regulate gene expression on demand. Future clinical trials should consider incorporating this technique, which may improve treatment efficacy and mitigate negative side effects.

Conclusions

The current dilemma in OA treatment is that there is no effective way to stop the continued destruction of joint structures and no plausible method to regenerate natural joint structures *in vivo*. Gene therapy offers a new strategy to alleviate symptoms and promote cartilage regeneration and joint function recovery in patients with OA. Meanwhile, gene therapy can lead to more sustained, targeted, site-specific expression of therapeutic proteins in a more physiologically relevant manner than traditional approaches. Future research should focus on safer, more convenient and less expensive gene delivery vectors, delivery methods and gene editing technologies. In addition, current gene therapy does not titrate the appropriate vector dosage to mimic the *in vivo* expression of delivered genes, which requires further investigation in the future. Deepening the understanding of OA pathophysiology and optimizing gene therapy approaches will shed light on the treatment of OA.

Abbreviations

OA: Osteoarthritis; DMOADs: Disease-modifying osteoarthritis drugs; TILs: Tumor-infiltrating lymphocytes; FDA: Food and Drug Administration; ADA: Adenosine deaminase; IL-1: Interleukin-1; IL-1Ra: IL-1 receptor antagonist; ECM: Extracellular matrix; ACLT: Anterior cruciate ligament transection; Runx: Runt–related transcription factor; Sox: Sex-determining Region Y-related HMG-box; C/EBPβ: CCAAT/enhancer binding protein; HIF: Hypoxia-inducible factor; MMP13: Matrix metallopeptidase-13; BMP: Bone morphogenetic protein; IGF-1: Insulin-like growth factor 1; AAV: Adeno-associated virus; ADAMTS: Thrombospondin Motif; MSCs: Mesenchymal stem cells; TGFβ: Transforming growth factor-β; GDF-5: Growth/differentiation factor-5; FGF: Fibroblast growth factor; HDAd: Helper-dependent adenovirus; HSP70: Heat shock proteins70; TG-C: TissueGene-C; iPSCs: Induced pluripotent stem cells; DMM: Destabilization of the medial meniscus; NGF: Nerve growth factor; PTOAPost-traumatic OA;CCL2: Post-traumatic OA;CCL2Chemokine (C-C) ligand 2

Authors' contributions

ZYH conceived of the work. XYL wrote the draft manuscript. LYS, ZHD, and ZYH performed critical revisions of the manuscript. XYL, LYS, ZHD, and ZYH take responsibility for the integrity of the work as a whole. All authors read and approved the final manuscript.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (NSFC) to ZYH (Grants No. 92049101, 81972097, and 81702185). This research was also supported by Sichuan Science and Technology Program to ZYH (Grant No. 2022YFH0101). This work was also supported by

Guangdong Basic and Applied Basic Research Foundation (Grant No. 2021A1515220030), Shenzhen Science and Technology Program (Grant No. RCYX20210609103902019). We would like to thank Biorender company for their schematic diagram in this review.

Conflict of interest

The authors declare that they have no conflict of interest.

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Received: March 31, 2023. **Accepted:** May 24, 2023. **Published:** 30 May 2023