Gene Therapy: Future Therapies in Osteoarthritis

David D. Frisbie, DVM, PhD, Diplomate ACVS, and C. Wayne McIlwraith, BVSc, PhD, FRCVS, Diplomate ACVS

1. Introduction
The field of equine veterinary practice is in an ever-evolving state, requiring current technologies to be constantly evaluated for new applications. The field of molecular biology is already integrated into equine practice for use in blood typing, identification of Salmonella species from fecal samples, detecting bovine papillomavirus types 1 and 2 in sarcoids, specific strains of equine herpesviruses, and virulence factors associated with Rhodococcus equi, to cite a few examples. The specific use of gene therapy in the horse is a novel application. The purpose of this review article is to help familiarize the equine practitioner with the concept of gene therapy, and introduce its utility as well as potential future benefits for the equine industry in the treatment of osteoarthritis.

2. Background to Gene Therapy
In 1944, DNA was first reported to carry genetic information. Since this time great attention has been devoted to unlocking the information encoded within DNA molecules, given the realization that DNA contained a genetic blueprint for each species and could hold key information for normal function as well as disease processes.

The functional unit of DNA is the gene, which can be defined as the set of DNA sequences that are required to produce a single polypeptide. The gene sequence codes for a specific messenger RNA (mRNA) molecule that, in turn, carries the genetic information from the nucleus to the cytoplasm for translation into an amino acid sequence (i.e., a protein). Many recognized disease states relate to a lack of, a defect in, or an imbalance of a particular protein(s). Since the gene is the basal unit ultimately responsible for protein production, it is also a logical therapeutic target. Currently, most gene therapy protocols are directed towards increasing levels of selected therapeutic proteins in an attempt to alter specific disease processes. Depending on the natural function of the particular protein, we may be able to enhance or repress certain direct effects on specific cellular processes.

An example of an application of gene therapy being explored is the correction of abnormal proliferation of smooth muscle cells in coronary arteries causing narrowing and obliteration of coronary vessels following angioplasty. This holds great promise for patients with coronary heart disease.

The technique uses the expression of the DNA encoding for thymidine kinase. This enzyme acti-
vates a prodrug (drug administered in an inactive form) capable of blocking cell proliferation when activated. Preliminary research involving pigs, whose arteries closely resemble humans showed that local arterial administration of the DNA coding for thymidine kinase concurrent with prodrug infusion followed by angioplasty procedure reduced proliferation of the vascular smooth muscle by 50–90% in the vascular group compared to control animals.26

To more clearly understand the utility of gene therapy the following sections will define its components and briefly explore the advantages and disadvantages of each component.

3. Gene Therapy Defined

The term gene therapy is commonly understood to mean the use of molecular methods to replace defective or absent genes, or to counteract those that are over-expressed. The key technologies needed for gene therapy are the methods by which genes are isolated (cloned), manipulated (engineered), and transferred (gene transfer) into host cells.

Isolation of Genes

The “classical” isolation of genes often starts with the identification of a protein of interest, and then determination of the amino acid sequence coding for the protein. This amino acid sequence information is in turn used to deduce the DNA sequence ultimately encoding for the protein of interest. However, equine research genes (DNA sequences) are often deduced based on known human gene sequences where much of the protein and amino acid identification has already been completed. Because gene sequences of important genes are highly conserved across species, isolation of equine genes based on the known human DNA sequence is often very rewarding without the need for protein identification/purification or amino acid sequencing.

Gene Manipulation

Once the gene sequence has been isolated and characterized, the level of gene expression can be manipulated in the laboratory through the addition of regulatory elements to the genes’ DNA sequence. Regulatory elements are DNA sequences surrounding the DNA coding region of a gene (the specific region of DNA coding for the amino acid sequence unique to a specific protein) and these supply information dictating the level of mRNA production. These regulatory sequences may produce high or low levels of mRNA specific to certain cell types, and in some cases the level of mRNA production may be dictated by local environmental factors. Therefore, through the selection of an appropriate regulatory sequence the level of protein production from a given gene can be controlled.

Gene Transfer

The final key component in the efficient transfer and expression of therapeutic genes within a cell population is by inserting the manipulated gene sequence into a vector. Vectors are vehicles that facilitate a therapeutic genes transport into the nucleus of the target cell, where the gene can be decoded (expressed) and in some cases produce a therapeutic protein without harming the cell. Vectors are most often based on viruses because their life-cycle has evolved based on the ability to transfer genes into a host cells in an efficient manner. To better understand vectors a brief discussion on the methods by which vectors are delivered to cells is needed.

Method of Gene Transfer

Two basic methodologies are utilized to transfer vectors into target tissues. In the first method, ex vivo gene transfer, cells are collected from the patient or host and grown in the laboratory (Fig. 1). During a finite culture period the therapeutic genes are transferred into the cells often using a viral vector. Once tested for the correct behavior or, in many cases, protein production, the transduced cells are re-implanted in the patient. This methodology is currently quite prevalent in human gene therapy trials, partially because the administrator has the ability to control and test the cells prior to re-introduction into the patient, thus giving the utmost consideration to safety issues.

The second method, in vivo, refers to the direct transfer of the vector to the target tissues in situ (Fig. 2). Although this method does not allow for extensive safety testing, its utility and ease of application are very attractive, and for these reasons many planned gene therapy protocols are using this methodology in both human and veterinary applications. Having defined the methods available for transferring vectors to the target cells, further discussion on the specific types of available vectors is needed.

Vector Classification

Vectors are generally either non-viral (synthetic) or viral. Non-viral vectors typically refer to synthetic molecules that facilitate the uptake of DNA into cells by condensing the DNA with lipids, peptides, proteins, inactivated virus particles, crystals of calcium phosphate, or coated microprojectiles. Viral vectors are viruses from which the viral genes have been removed to allow insertion of the therapeutic gene(s), and the viral vector has usually been rendered incapable of replicative spread. Viral vectors typically produce a greater efficiency of gene delivery than non-viral vectors. Many well-characterized viruses have been explored for use as vectors; however, to date retroviral and adenoviral vectors have proven the most useful.

Retroviral vectors integrate their DNA (including a gene sequence of interest) into the chromosomal DNA of the target cells, ensuring gene transfer to the target cell progeny. In theory, this would provide long-term transgene expression (protein expression from the gene of interest). However, the
integration of DNA coding for a gene of interest still does not guarantee mRNA or protein expression, or even prolonged transgene expression. In fact, in some cases inactivation of transgene expression has been shown to limit protein production even when the transgene DNA is still detectable.4 Further limitation of the retroviral vector is its ability to only transduce dividing cells, making it useful in certain situations but less useful in others. If, for example, the target population of cells were neuronal (non-dividing), and the retroviral vector carried a gene causing cell death, this vector could be quite useful in a gene transfer protocols for rapidly dividing neoplastic cells invading brain tissues. On the other hand, if the retroviral vector carried a transgene coding for an anti-arthritic gene and the target cells were chondrocytes, gene transfer to the chondrocytes would be very inefficient due to their low mitotic rate.

Adenoviral vectors are the other major class of viral vectors being proposed for clinical use.24 Similar to retroviruses, they can be converted into powerful non-pathogenic vectors, through recombinant techniques, for use in gene transfer protocols. One advantage of adenoviral vectors are their ability to transduce both dividing and non-dividing cells. This also makes the use of adenoviral vectors very amenable to in vivo gene transfer simplifying the transduction of the target population of cells and the clinical application of gene transfer. Although integration of adenovirus into chromosomal DNA can occur, it is not part of the normal viral life cycle and is rare. Rather, the adenoviral DNA is maintained as an episomal element (extrachromosomal) in the nucleus of the host cell.14 One limitation with current adenoviral vectors, in part due to their episomal location, is the duration of transgene expression which in first generation vectors is approximately 7–10 days.37 Second generation vectors, engineered to address obstacles related short transgene expression periods as previously discussed and factors such as cellular and humoral responses directed at the vector and vector associated proteins, have yielded transgene expression in the range of 21–40 days.8,40 In another improvement, gutless adenoviral vectors leaving only sequences defining the beginning and end of the adenoviral genome have shown transgene expression for up to 84 days although the assembly of this type of vector remains technically challenging.6,41 Other methodologies to prolong gene expression periods by suppressing the immune response and have also been successful. The use of oral adenoviral antigens including cyclophosphamide, cyclosporine A, and specific anti-T cell molecules have led to extended transgene expression for up to 300 days.18,36,41 Repeat administra-

---

**Fig. 1.** Schematic drawing representing ex vivo gene transfer, using gene transfer to the synovium as an example. (A) The construction of vector DNA containing a gene of interest (blue) and a gene allowing selection of infected cells (Red). (B) Packaging the vector DNA into an infectious particle that can be stored in the freezer. (C) Harvesting synoviocytes and culturing them in vitro. (D) After adding the vector to the culture medium. (E) Culturing the synoviocytes in the presence of the G418 antibiotic that kills cells not expressing the Red (Neo) gene sequence. (F) Represents harvesting the cells and transferring the cells back to the joint where some subpopulation can take-up permanent residence in the joint tissue.
tion of adenoviral vectors with immunosuppression, a previous obstacle, has been possible without either evoking deleterious immune responses or shortened transgene expression.

Once the appropriate route, method, and vector have been chosen, a gene sequence of interest must be identified for expression. This ability to choose a gene sequence of interest provides the power of gene transfer. Because only a small fraction of the genetic code has been assigned a specific function and the role or interaction of multiple genes in the pathophysiology of disease processes is largely not understood, the power of manipulating the genome through gene transfer is largely unexplored.

4. Gene Therapy for Equine Joint Disease

The potential value of gene therapy in the treatment of joint disease is in part based on limitations of traditional therapy, which has been partially hindered by the relative inability to target therapeutic agents directly to the joint when they are not administered intraarticularly (IA). Also, the half-life of most commonly used agents administered IA is short dictating frequent IA injections to sustain biologic activity for prolonged treatment periods, which may be avoided through the use of gene therapy. In addition, many drugs cannot be given by direct IA injection. Gene transfer provides an excellent alternative to conventional therapy whereby a single IA injection can result in local production of a specific therapeutic protein within diseased joint(s) for a prolonged period of time.

It has been shown that cytokines such as interleukin-1 and tumor necrosis factor modulate the synthesis of metalloproteinases by both chondrocytes and synovial cells, and are largely responsible for the mediation of joint disease. This provides an indication for the therapeutic use of cytokine inhibitors or modulating agents. Clinical trials in using the protein forms of cytokine modulating agents such as interleukin-1 receptor antagonist (IL-1Ra), soluble forms of receptors, and monoclonal antibodies against cytokines have shown significant therapeutic benefits. Furthermore, using both ex vivo and in vivo gene transfer, the human IL-1RA gene sequence has shown beneficial effects in joint disease models using both dogs and laboratory animals. The duration of protein (IL-1RA) expression in these studies, however, was limited and the authors cite one potential cause of this problem as expression of a human gene sequence in a non-human species. This problem has been circumvented in current equine work with the use of the equine IL-1RA gene sequence.

A collaborative effort between the University of Pittsburgh School of Medicine Departments of Orthopaedic Surgery and Molecular Genetics, and Colorado State University (CSU) Equine Orthopaedic Research Laboratory have been successful in treating equine joint disease using gene therapy. Specifically, the first step was the identification and sequencing of the gene encoding equine IL-1RA and this was done in the new laboratory in 1998. Proof of principal experiments began with in vitro expression of an active equine IL-1RA protein following gene transfer of the equine IL-1Ra gene sequence to cultured equine synoviocytes using an adenoviral vector. Following confirmation that the adenoviral vector could infect equine synoviocytes and produce a biologically active IL-1Ra protein, an in vivo dose titration was performed. Using the same adenoviral vector carrying the equine IL-1RA gene (Ad-EqIL-1Ra), the optimal vector concentration to provide peak concentration and duration of IL-1RA protein expression was determined. This dose was also determined to be free of any significant measured side effects.

Proof of principal experiments began with in vitro expression of an active equine IL-1RA protein following gene transfer of the equine IL-1Ra gene sequence to cultured equine synoviocytes using an adenoviral vector. Following confirmation that the adenoviral vector could infect equine synoviocytes and produce a biologically active IL-1Ra protein, an in vivo dose titration was performed. Using the same adenoviral vector carrying the equine IL-1RA gene (Ad-EqIL-1Ra), the optimal vector concentration to provide peak concentration and duration of IL-1RA protein expression was determined. This dose was also determined to be free of any significant measured side effects.

Next using an established experimental model of equine OA the in vivo anti-arthritic potential of this therapy was evaluated. This model uses creation of an osteochondral fragment in one intercarpal joint followed by treatment, in this case with IA administration of Ad-EqIL-1Ra, 14 days after fragment creation. Starting the day after treatment, exercise simulating race training began on a high-speed treadmill and continued for...
56 days. The results of testing the Ad-EqIL-1Ra gene therapy treatment in this model showed horses receiving treatment were significantly less lame and had significant less synovial effusion in the arthritic/fragmented joints. Horses receiving gene therapy treatment also had significantly less pathologic change noted on gross examination of joints treated with gene therapy compared to placebo treated arthritic/fragmented joints (Fig. 3). Also, both microscopic and biochemical assessment of articular cartilage samples showed significant improvement after gene therapy treatment when compared to the placebo treatment. Lastly, no significant long-term side effects were demonstrated with the gene transfer treatment in arthritic joints.

In an attempt to put the therapeutic benefit of the described gene therapy treatment in perspective with currently utilized medications, gene therapy treatment had better improvement in reducing the progression of experimentally created equine arthritis compared to Vetalog®, Depo-Medrol®, Celestone Soluspan® and Legend®,9–11 tested in a similar fashion. Further work is still needed to increase the effectiveness of this treatment on repeat administration and confirm similar improvement occurs when used in clinical cases of equine arthritis; however, gene therapy may revolutionize the treatment of equine arthritis in the near future. This work also represents novel advances in the field of gene therapy for treatment of human arthritis in that it was the first evidence of clinical improvement associated with gene therapy for treatment of a musculoskeletal disease in any species. Based on these promising results presented here, clinical trials have been proposed to test this novel therapy in clinical case of acute traumatic equine joint disease.

5. Future Directions of Equine Gene Therapy

Ongoing gene therapy research being conducted at CSU’s Equine Orthopaedic Research Laboratory includes the investigation of other gene sequences with anti-arthritic potential, as well as the potential to augment fracture healing in both routine and cases of delayed fracture healing. Studies to evaluate gene transfer of insulin-like growth factor-1 (IGF-1) and its ability to augment cartilage healing are underway at Cornell University’s Orthopaedic Research Laboratory under the direction of Dr. Alan Nixon. Although the work is in the early stages, research has been able to demonstrate biological activity referable to IGF-1 that could be beneficial in healing full thickness equine cartilage defects in an in vivo setting.

One of the biggest challenges facing gene therapy today is the improvement of current vectors and this area continues to be the major limiting factor in gene therapy applications. Although the current adenoviral vectors are suitable for long-term transgene expression when administered in conjunction with immunomodulating agents, obvious problems exist with this approach. Researchers in the field of vector engineering have realized these problems and have focused significant effort into a solution. The future of gene therapy as a viable medical application can be nicely summarized by a quote from a skeptic of the technology: “Despite our present lack of knowledge, gene therapy will almost certainly revolutionize the practice of medicine over the next 25 years. In every field of medicine, the ability to give the patient therapeutic genes offers extraordinary opportunities to treat, cure and ultimately prevent a vast range of disease that now plague mankind.”

Fig. 3. Photographs of the third carpal bones from arthritic/fragmented joints of (A) a horse treated with the placebo and (B) a horse treated with gene therapy. More extensive full-thickness articular cartilage erosions can be seen in the joint from the horse treated with the placebo, especially in areas of the third carpal bone not adjacent to the fragment. The fragment (1), area of full-thickness cartilage erosion on the third carpal bone in the placebo and not in the gene therapy treated joint (2), and an iatragenic harvest site of cartilage from the intermediate carpal bone post-mortem (3) are demonstrated numerically.
The authors would like to acknowledge Dr. Gary M. Baxter for his comment on this article.

References