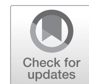

7 Gene Delivery to Chondrocytes

Christopher V. Nagelli, Christopher H. Evans, and Rodolfo E. De la Vega

Abstract

Delivering genes to chondrocytes offers new possibilities both clinically, for treating conditions that affect cartilage, and in the laboratory, for studying the biology of chondrocytes. Advances in gene therapy have created a number of different viral and non-viral vectors for this purpose. These vectors may be deployed in an ex vivo fashion, where chondrocytes are genetically modified outside the body, or by in vivo delivery where the vector is introduced directly into the body; in the case of articular and meniscal cartilage in vivo delivery is typically by intra-articular injection. Ex vivo delivery is favored in strategies for enhancing cartilage repair as these can be piggy-backed on existing cell-based technologies, such as autologous chondrocyte implantation, or used in conjunction with marrow-stimulating techniques such as microfracture. In vivo delivery to articular chondrocytes has proved more difficult, because the dense, anionic, extra-cellular matrix of cartilage limits access to the chondrocytes embedded within it. As Grodzinsky and colleagues have shown, the matrix imposes strict limits on the size and charge of particles able to diffuse through the entire depth of articular cartilage. Empirical observations suggest that the larger viral vectors, such as adenovirus (~100 nm), are unable to transduce chondrocytes in situ following intra-articular injection. However, adeno-associated virus (AAV; ~25 nm) is able to do so in horse joints. AAV is presently in clinical trials for arthritis gene therapy, and it will be interesting to see whether human chondrocytes are also transduced throughout the depth of cartilage by AAV following a single intra-articular injection. Viral vectors have been used to deliver genes to the intervertebral disk but there has been little research on gene transfer to chondrocytes in other cartilaginous tissues such as nasal, auricular or tracheal cartilage.

Keywords

Chondrocyte · Gene therapy · Cartilage · Osteoarthritis

7.1 Introduction: Why Transfer Genes to Chondrocytes?

Gene transfer has emerged as a valuable technology serving both as a therapeutic modality and as a research tool. In the context of diseases that

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affect cartilage, genetic modification of chondrocytes promises to improve the treatment of osteoarthritis (OA) and other arthritides, as well as to promote the regeneration of damaged cartilage. As a research tool, gene transfer enables the biology of chondrocytes to be interrogated in new and unique ways. To exploit this potential, it is necessary to develop technologies allowing the efficient transfer of genes to chondrocytes and the expression of those transgenes in controlled, predictable ways. This chapter summarizes progress made in these endeavors.

7.2 A Gene Transfer Primer

7.2.1 Viral Vectors

Genes do not spontaneously enter cells in a fashion that allows their meaningful expression. Instead genes or, more usually, their complementary (c)DNA equivalents, are purposefully transferred to cells by vectors that cross the cell membrane and deliver their genetic payloads to the nucleus of the cell where the transcriptional machinery resides. The most powerful vectors for gene transfer take advantage of the natural ability of viruses to enter cells and deliver their own genomes in a manner where the virally encoded genes are expressed efficiently. Gene transfer using viruses is known as transduction.

Vectors for gene delivery have been extensively reviewed in a number of recent publications [1–3]. Although several different viruses are in pre-clinical development as a basis for gene therapy vectors [4], the main viruses that have been successfully modified for gene therapy in human clinical trials are retrovirus, adenovirus and adeno-associated virus (AAV). Two different types of retrovirus have been employed in this fashion, γ-retrovirus and lentivirus. The main relevant properties of the major viral vector groups are summarized in Table 7.1.

Retroviruses were the first viruses to be developed usefully for human gene therapy. On entering cells, their RNA genomes are reverse transcribed into DNA (hence the word retrovirus) which then integrates into genomic DNA within the host nucleus where the transferred coding sequences (transgenes) are expressed. Because integration occurs at unpredictable sites there is a finite possibility of insertional mutagenesis leading to malignant transformation. Although the likelihood of this is low, it has been observed in clinical trials [5]. Of practical concern, γ-retroviruses require host cell division for transduction to occur whereas lentiviruses transduce both dividing and non-dividing cells. Because of the safety concerns raised by insertional mutagenesis, retroviruses are unlikely to be used clinically to treat diseases affecting cartilage but they remain powerful research tools.

Adenoviruses are non-integrating DNA viruses that are relatively straightforward to construct and propagate. They transduce a wide range of dividing and non-dividing cells. Depending on the promoter used in the vector, transgene expression can be very high. Because the viral DNA remains episomal it is rapidly lost from dividing cells and adenoviral vectors tend to provide high levels of transgene expression for a limited period time. The ability of adenovirus to activate both the innate and adaptive immune systems is a disadvantage for *in vivo* applications. The innate immune system is triggered because infection of cells with adenovirus stimulates mitogen-activated protein (MAP) kinases, leading to the activation of nuclear factor kappa-B (NF-κB), a pro-inflammatory transcription factor. Adaptive immunity occurs in response to highly antigenic adenoviral capsid proteins. Cells infected with early generation adenovirus vectors express low levels of these proteins and are killed by the resulting CD8+ T-cell response. Later generation vectors have addressed this issue by removing additional viral DNA leading to the construction of high-capacity vectors (also known variously as "gutted", "gutless" or "helper-dependent" adenovirus) that lack all adenovirus coding sequences. These vectors can accommodate a DNA cargo as large as 36 kb but are difficult to manufacture.

AAV is a small parvovirus with a single-stranded DNA genome. It is attractive for human gene therapy because the wild-type virus is endemic in human populations yet causes no known disease. However, the single-stranded genome presents a limitation for gene therapy

Table 7.1 Salient properties of commonly used viral vectors

Viral vector	Advantages	Disadvantages	Other properties
Adenovirus	Easy to produce in high titers Transduces both dividing and non-dividing cells Relatively good freeze-thaw stability Easy to procure and produce (first and second generation vectors)	Immunogenic Difficult to procure and produce (third generation) Does not transduce chondrocytes in situ	~1 in every 50–100 viral particles is infectious Non-integrating Carrying capacity 8–30 kb Transient transduction of dividing cells
Adeno-associated virus (AAV)	Transduces both dividing and non-dividing cells Relatively good freeze-thaw and thermal stability Capable of transducing chondrocytes in vivo No human disease associated with AAV Multiple serotypes allow for directed tropism	Difficult to procure and produce Gene carrying capacity is small Large number of the human population have pre-existing neutralizing antibodies to certain serotypes Expensive	Depending on serotype ~1 in 50 particles is infectious Transducing capacity varies widely between serotypes, cells, species and different preparations Non-integrating
Retrovirus (Moloney murine leukemia virus derived)	Easy to produce Selection of transduced cells straightforward	Modest titers Does not transduce non-dividing cells Risk of insertional mutagenesis Does not transduce chondrocytes in vivo	~1 in every 100–1000 viral particles is infectious ~8 kb of packaging capacity Integrating
Lentivirus	Transduces both dividing and non-dividing cells Selection of transduced cells straightforward	Risk of insertional mutagenesis Does not transduce chondrocytes in vivo	~1 in every 100–1000 viral particles is infectious ~8 kb of packaging capacity Integrating

because second-strand synthesis is required within the nucleus of the host cell before gene expression can occur. In certain types of cells and in certain species second strand synthesis is very inefficient. The development of self-complementing AAV genomes comprising double-stranded DNA has overcome this problem at the expense of reducing the packaging capacity of AAV from an already modest 5 kb to 2.5 kb DNA. However, this capacity is ample for the small cytokine molecules and growth factors relevant to many aspects of chondrocyte biology. The genomes of recombinant AAV vectors are non-integrating but exist as stable, concatemeric episomes which provide the basis for long-term expression in non-dividing cells. Multiple years of transgene expression in liver and eye have been noted in human clinical trials [6]. AAV has a number of distinct serotypes, both natural and synthetic, which display different tropisms.

Practical aspects of chondrocyte transduction have been described recently by Nagelli et al. [3].

7.2.2 Non-viral Vectors

Although viral vectors are very powerful and dominate clinical application, there is also interest in non-viral vectors. Non-viral vectors promise to be simpler, less expensive and possibly safer than viral vectors; they are also less likely to have packaging constraints. Gene transfer with non-viral vectors is known as transfection.

Plasmids are the simplest of non-viral vectors. Although they do not provoke adaptive immunity in the same way as viral vectors, unmethylated cytosine-phosphate-guanine (CpG) dinucleotide motifs in DNA activate innate immunity by interacting with toll-like receptors. Moreover, transfection efficiency is inversely proportional to the

size of the construct; plasmid uptake and expression is very low with constructs >3 kb in length; it is most efficient with mini-circles of 650 bp or less [7, 8]. Transfection is very inefficient in non-dividing cells.

The negative charge of DNA impedes cell uptake because the surfaces of cells also have a net negative charge. Various cationic agents may be added to mask the repulsive electrostatic charges and may additionally facilitate uptake by condensing the DNA. Physical methods to improve uptake include electroporation, hydrodynamic injection, ultrasound, and "magnetofection". In general, transfection provides low and transient transgene expression, especially in primary cells. Non-viral gene delivery is reviewed in references [9, 10].

There is much recent interest in the use of RNA as a therapy and a research tool [11]. Delivery of mRNA serves to enhance expression of the encoded protein, albeit transiently, while RNA inhibition suppresses expression of specific transcripts. Transfection with chemically modified mRNA encoding bone morphogenetic protein-2 (BMP-2) has recently been shown to promote the formation of cartilage within an osseous defect in the rat femur [12].

7.2.3 Gene Activated Matrices

Gene activated matrices (GAMs), combining vectors with scaffolds, are of interest in the context of tissue regeneration. For most envisaged applications the GAM is implanted into a defect where host cells infiltrate the matrix during which process they become genetically modified by the associated vectors. Genes encoding regenerative products are thus expressed locally by host cells within the defect where they stimulate a reparative response. First introduced in for bone healing [13], GAMs have also been explored in the context of cartilage repair and regeneration [14]. The original formulations combined plasmid DNA with a collagen sponge, but later iterations include viral vectors, RNA and more elaborate scaffolds [15].

7.3 Gene Delivery to Chondrocytes

For *in vitro* genetic modification, chondrocytes in monolayer culture need only be incubated with the vectors of choice using techniques of the type described by Nagelli et al. [3]. A sizeable literature dating back 25 years confirms that cultures of chondrocytes can be transduced efficiently with viral vectors [16–18].

For *in vivo* genetic modification, in which genes are transferred to the articular cartilage within a joint, there is the choice of *ex vivo* or *in vivo* delivery (Fig. 7.1). For *ex vivo* delivery the cells are transduced *in vitro* and then implanted into the cartilage. For *in vivo* delivery, the vector

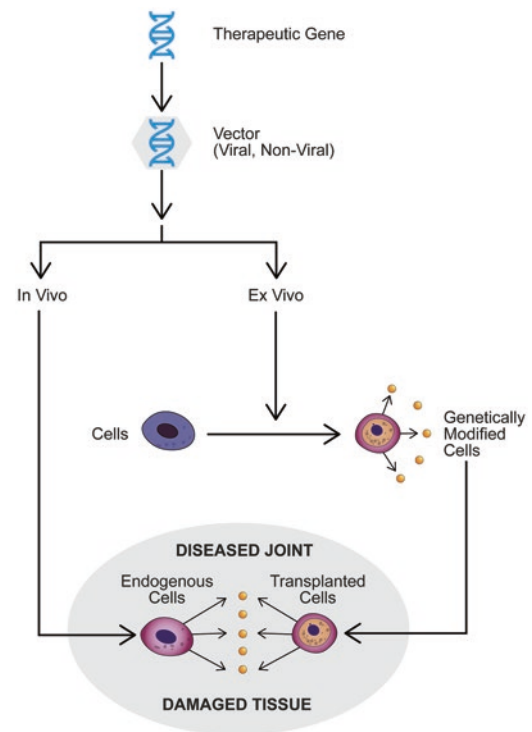

Fig. 7.1 Principles of local gene therapy to chondrocytes and cartilage. The therapeutic gene, usually in its cDNA form, is incorporated into a viral or non-viral vector and delivered to the site of cartilage disease or damage in an in vivo or ex vivo fashion. For in vivo delivery, the vector is administered directly to the relevant site. For ex vivo delivery, the vector transfers genes to cells outside the body, and the genetically modified cells are then administered to the relevant site. (Reproduced from [1])

is introduced directly into the body and transduction occurs *in situ*. *In vivo* gene delivery to articular chondrocytes has several barriers to overcome. Particles, such as vectors, delivered systemically barely enter joints and, in any case, articular cartilage is avascular. Direct injection of vectors into the joint by-passes the systemic circulation but there are two further barriers to the genetic modification of chondrocytes. The first is rapid efflux via lymphatic drainage which removes particles, including vectors, from joints [19]. The second is the dense, extra-cellular matrix (ECM) of cartilage that prevents the vectors from gaining access to the chondrocytes embedded within it (Fig. 7.2) [20].

Much of what we know about diffusion through the ECM of cartilage comes from the work of Grodzinsky and colleagues at MIT who have studied the ability of molecules to diffuse into articular cartilage from both empirical and theoretical perspectives [21–23]. The dominating parameters are the size and charge of the diffusate; shape may also be a factor. The high, fixed, negative charge of glycosaminoglycans (GAGs) within the cartilaginous ECM excludes anionic materials electrostatically, while the dense packing of proteoglycans sterically excludes particles with a Stoke's radius larger than about 15 nm. Although a positive surface charge neutralizes the electrostatic exclusion of a particle from cartilage, an excessive positive charge is counterproductive because the affinity of the particles for cartilage GAGs will be too high, in which case particles will accumulate at the surface and fail to diffuse through the full thickness of cartilage. To enter cartilage in a useful way, it is thus necessary for a vector to have a net positive charge, but one that is not too high, and an affinity for GAGs whose off-rate permits progress through the matrix. In this context, Bajpayee *et al* studied the

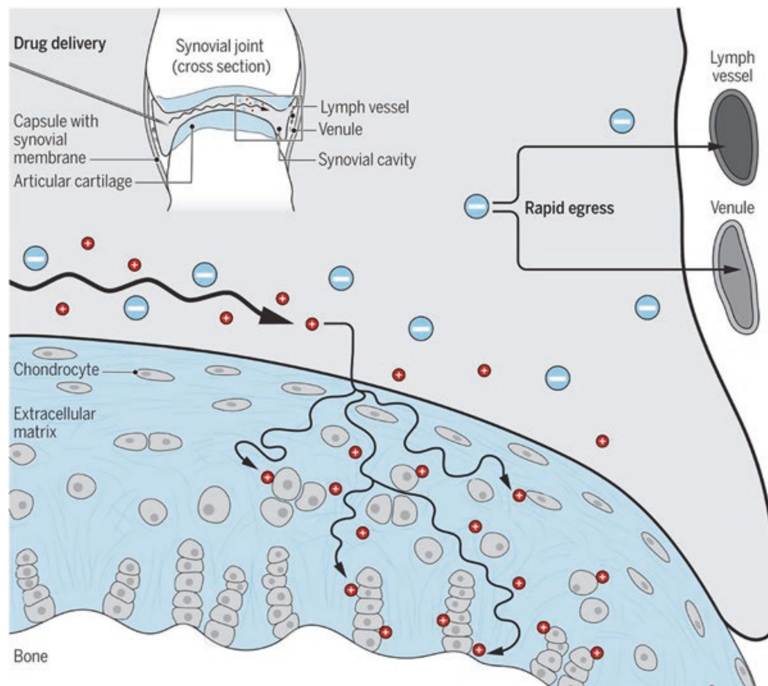

Fig. 7.2 Delivering drugs to chondrocytes in situ by intra-articular injection. Although drugs can be easily injected into joints, most materials within the joint space are rapidly removed by lymphatic drainage or by diffusion into the subsynovial capillaries. Penetration of the articular cartilage, where the chondrocytes reside, is restrained sterically and electrostatically by the high concentration of anionic (−) glycosaminoglycans (GAGs). Particles with an appropriate positive (+) surface charge bind reversibly to the anionic GAG chains enabling transport through the cartilage to the chondrocytes, where vectors can deliver their genetic payload. (Reproduced from [20])

diffusion of the cationic protein avidin through the ECM of bovine articular cartilage, noting that its weak and reversible binding to cartilage GAGs (K_D ~ 150 μM) allowed it to diffuse through the entire thickness of the matrix as it underwent sequential binding and release (Fig. 7.2) [22]. Under these circumstances, the higher concentration of GAGs in the deeper zones of the cartilage may have helped diffusion through the entire depth of the tissue. Thus a vector must satisfy strict biophysical requirements to be able to transduce chondrocytes *in situ* throughout the full thickness of cartilage.

Because *in vivo* delivery of genes to chondrocytes seemed extremely difficult, *ex vivo* gene delivery to cartilage was the early strategy of choice [16, 24]. There was initial optimism that genetically-modified chondrocytes would adhere to the surface of cartilage following intra-articular injection, especially to sites of damage, thus providing a new strategy for cartilage repair. However, subsequent research has confirmed that the injected cells do not adhere to cartilage but are rapidly cleared from the joint [25–27]. A more promising approach has been to implant genetically modified chondrocytes, or chondroprogenitors, surgically. To do this effectively it is necessary to use an appropriate scaffold, discussion of which lies beyond the scope of this chapter (see Ref. [28] for a recent review).

The prospect of *in vivo* delivery of genes to chondrocytes has been recently revisited on the basis of experiments in which AAV encoding green fluorescent protein (GFP) was injected into the joints of horses [29]. An unexpectedly high proportion of articular chondrocytes throughout the full thickness of the cartilage became GFP+ (Fig. 7.3). This had not been seen in earlier experiments using rats and rabbits, suggesting that the pharmacokinetics of the larger joints differ from those of smaller animals. The greater thickness of the equine cartilage was probably an additional major factor because the kinetics of diffusion-reaction transport through cartilage depend on the square of its thickness. In agreement with this, Bajpayee *et al* showed 5–6 times longer half-lives of avidin in rabbit cartilage than in rat cartilage following intra-articular injection [30].

AAV is an icosahedron, about 20–25 nm in size. This is larger than the 15 nm cut-off determined by Grodzinsky and colleagues [21–23], but entry into the cartilage of horse joints may be facilitated by the pumping action occurring as the horse moves and the articular cartilage intermittently bears weight; the apparatus used at MIT

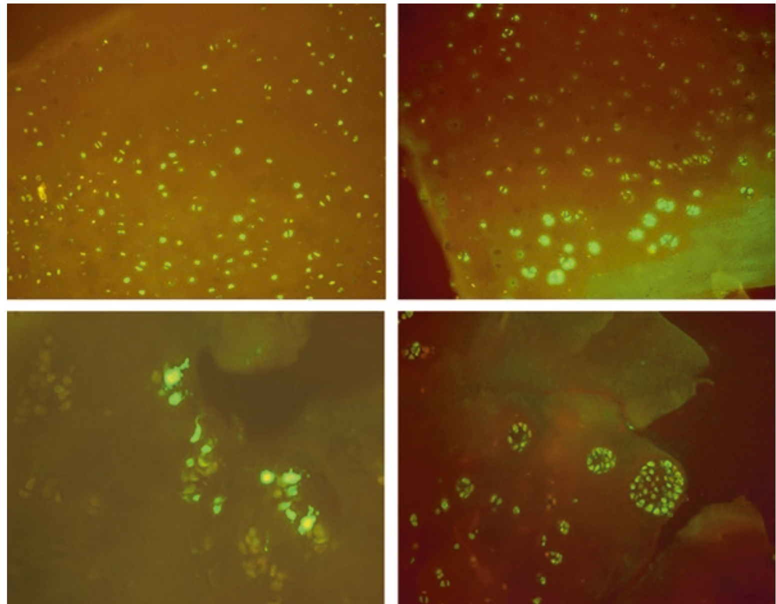

Fig. 7.3 Expression of GFP in chondrocytes of cartilage 2 weeks after the intra-articular injection of 5 × 10^{12} viral genomes of AAV. GFP into the intercarpal joint of the horse

provides a static system which does not subject the cartilage to loading. Age may be a second factor. The studies by Grodzinsky's group used calf cartilage in which the proteoglycan chains are very long and the fixed charge density very high. In adult horses the fixed charge density is likely to be lower and the matrix of the cartilage subjected to some degree of degradation as happens during natural aging. In a disease such as osteoarthritis (OA) the matrix is further degraded, allowing greater access to AAV and possibly other, larger vectors such as adenovirus as the disease progresses.

7.4 Progress in the Clinical Application of Gene Transfer to Chondrocytes

7.4.1 Osteoarthritis

Osteoarthritis (OA) is the one application that has advanced to clinical trials [1]. The first approach used *ex vivo* gene delivery and built on the earlier success of delivering genes in this fashion to human metacarpophalangeal joints affected by rheumatoid arthritis [31]. Almost all examples of *ex vivo* gene therapy use autologous cells as the vehicle for gene transfer. The method of Ha *et al* [32] broke new ground in using allogeneic cells, derived from the finger joints of an infant with polydactyly, to deliver a gene to human joints. Cultures of the donor chondrocytes obtained from the amputated finger were divided into two lots, one of which was retrovirally transduced to express large amounts of transforming growth factor-beta (TGF-β). Because of the potential for insertional mutagenesis, as described earlier in this paper, the transduced cells were irradiated at a dose that prevented cell division but maintained TGF-β production. Before intra-articular injection into joints of patients with knee OA, the transduced cells were mixed with untransduced chondrocytes from the same allogeneic donor finger joints.

Phase I, II and III clinical trials of this product in South Korea met their primary end points and the gene therapeutic was approved in 2017 by the Korean authorities as the drug Invossa [33]. This was the first gene therapy approved in Korea. In 2019 this approval was revoked [34]. The genetically modified cells were identified as HEK293 cells, a line of human embryonic kidney cells, not chondrocytes. The initial preclinical work had been performed with chondrocytes. HEK293 cells are used as a producer line for generating retrovirus, so it is possible that some of these cells were inadvertently introduced into the chondrocyte cultures during retroviral transduction. The high growth rate of HEK293 cells would enable them to out-compete the chondrocytes, which have a slower growth rate. At the time of writing, the fate of Invossa in Korea is uncertain. However, the FDA has allowed a Phase III trial of Invossa in knee OA (National Clinical Trial Identifier (NCT) 03291470) and a Phase I/II trial in hip OA (NCT 05276011) to proceed in the USA.

The second approach in clinical trials uses *in vivo* gene delivery by intra-articular injection into knee joints with OA. Three such trials are underway. NCT 03477487 uses a plasmid to deliver a variant of interleukin- (IL-) 10. For reasons discussed earlier in this chapter, it is unlikely to transduce chondrocytes. NCT 03477487 uses a high-capacity adenovirus to deliver the IL-1 receptor antagonist (IL-1Ra) and it is not known whether gene transfer to chondrocytes occurs. Adenoviral transduction of chondrocytes *in situ* following intra-articular injection has not be reliably observed in pre-clinical models. In particular, a detailed study by Goossens *et al* [35] using rhesus monkeys failed to observe transgene expression in cartilage following intra-articular injection of adenovirus vectors even though the adjacent synovium was transduced efficiently. Clinical trial NCT 04119687 also uses IL-1Ra as the transgene product, but with AAV2.5 as the vector. This is the same serotype vector shown to transduce chondrocytes after injection into equine joints (Fig. 7.3), so there is the expectation that human chondrocytes will be similarly transduced. This possibility is enhanced by the similar thickness of human and equine cartilage

in large joints (1.5–2 mm). Both of the trials with viral vectors are in Phase I, whereas evaluation of the plasmid vector has progressed to Phase II.

7.4.2 Cartilage Regeneration

There is considerable interest in promoting cartilage regeneration using genetically modified chondrocytes or chondroprogenitor cells [36, 37]. Data from *in vitro* experiments and preliminary studies in small animals are encouraging, but there has been limited progress towards the large animal studies that are a necessary prelude to human clinical trials.

Using equine models, Nixon and colleagues evaluated the effects of *ex vivo* gene transfer on the repair of chondral defects using a variation of the autologous chondrocyte implantation approach. Allogeneic chondrocytes were transduced *in vitro* with adenovirus vectors expressing insulin-like growth factor-1 (IGF-1) [38] or bone morphogenetic protein-7 (BMP-7) [39]. The cells were encapsulated in a fibrin gel and arthroscopically introduced into experimental chondral lesions. In both cases, early healing was greatly accelerated by gene transfer but at later time points healing by the control chondrocytes had caught up. A subsequent study in which AAV was used to deliver IGF-1 to autologous chondrocytes provided longer lasting improvement, but it is unknown whether this was due to the choice of vector or the use of autologous cells [40].

Pascher *et al* [41] developed an abbreviated *ex vivo* gene transfer method based on the technique of microfracture that is frequently used to repair damaged cartilage. Microfracture and similar marrow-activating techniques allow chondroprogenitor cells from the underlying bone marrow to enter the lesion where they produce an inferior, but often serviceable, cartilagenous repair tissue that degenerates with time. Knowing that gene transfer can enhance the chondrogenic differentiation of mesenchymal stromal cells (MSCs) derived from bone marrow [42, 43], a technique was developed whereby bone marrow is aspirated and mixed with adenovirus vectors while it clots [41]. The clotted marrow, containing transduced marrow cells as well as free virus, is then press-fit into the lesion. Sieker *et al* obtained promising results when using BMP-2 and Indian hedgehog as the transgenes [44] in an osteochondral defect model in rabbits, but similar experiments with a TGF-β transgene gave equivocal results [45]. Use of a similar TGF-β construct in a chondral defect in sheep also gave equivocal results [46].

An alternative approach to improving the microfracture technique has been pioneered by Madry and Cucchiarini [47–51]. In this method, AAV vectors are directly applied to the osteochondral lesion as the marrow enters the defect. A number of different chondrogenic genes have been applied in a rabbit model with promising results [48, 49]. Similar studies delivering fibroblast growth factor-2 (FGF-2) [51] or Sox 9 [50] in a sheep osteochondral defect, and TGF-β in a minipig have also given promising early results [47]. In a refinement of this technique, this group has developed GAMs for the delivery of these vectors to osteochondral lesions [52].

Invossa has been applied in human patients with cartilage damage (NCT 01825811). The genetically modified cells were embedded in a fibrin gel and implanted in cartilage lesions present in joints of patients with knee OA. It is not known whether the genetically modified cells were chondrocytes or HEK293 cells. The promising results from this study have been presented, but not published.

7.5 Additional Considerations

Space does not permit discussion of additional matters related to gene transfer to chondrocytes. For example, the choice of promoters that drive transgene expression is important and chondrocytes express several genes, such as COL2A1, that permit tissue specific gene expression. Sub-sets of chondrocytes may also be targeted in this way. Expression of superficial zone proteoglycan, for example, is restricted to the superficial zone chondrocytes in cartilage,

although there is also expression by synovial fibroblasts. Various constitutive and inducible promoters are also available. The repertoire of interesting gene products continues to expand and includes various types of non-coding RNA as well as the machinery of gene-editing. Payloads such as these that operate intra-cellularly will usually need to be delivered by vectors able to reach chondrocytes throughout the entire cartilage, which may be challenging.

Gene transfer to additional cartilagenous tissues such as meniscus and the intervertebral disc has also been achieved using the same sorts of approaches as discussed in this chapter. There is considerable interest in using gene transfer to treat intervertebral disc degeneration. Pre-clinical experiments have confirmed the immune privilege of the nucleus pulposus, with expression of β-galactosidase, a highly antigenic bacterial protein, for over a year in the rabbit following delivery by intra-discal injection of a first-generation adenovirus vector, itself highly antigenic [53].

7.6 Conclusions

Transfer of genes to chondrocytes promises to advance the clinical management of OA and other forms of arthritis which destroy cartilage. Several clinical trials have been initiated in the field of OA. Gene therapy also has the potential to promote cartilage regeneration; one clinical study has taken place, but the data have not been published. As a laboratory tool, the ability to manipulate the genetics of chondrocytes offer many opportunities to learn more of their biology. The tools for both *ex vivo* and, more recently, *in vivo* delivery to chondrocytes both in culture and in articular cartilage are available to further these endeavors. Other cartilaginous tissues are also amenable to these approaches.

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