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Gene Therapy for Intervertebral Disc Degeneration

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Introduction

Intervertebral disc degeneration (IDD) is a very common and potentially debilitating disease process that affects millions in the United States and worldwide. IDD has a variable presentation from relatively benign to completely disabling and can be associated with disc herniation, spinal stenosis, radiculopathy, myelopathy, instability, and low back pain. The socioeconomic impact of musculoskeletal disorders of the spine such as IDD and low back pain cannot be overstated as these conditions are the leading cause of disability in people 45 years and younger resulting in national economic losses over 90 billion annually¹. Despite the prevalence of IDD and its enormous socioeconomic impact, operative and non operative treatment options are suboptimal and associated with unpredictable outcomes. Current treatment options for IDD and the pathology associated with it include decompression, spinal fusion, discectomy, electrothermal therapy, and arthroplasty all address the clinical symptoms associated with this disease process (pain, mechanical instability) and not the underlying pathophysiology²⁻⁵. With advances in molecular as well as cellular biology researchers have begun to characterize the pathophysiological pathways associated with disc degeneration and thus provided targets for potential biological treatments to augment or reverse the course of IDD. This chapter will briefly discuss the pathophysiology of disc degeneration, the premise behind gene therapy for the treatment of IDD, strategy for delivery, transfer of therapeutic genes to the disc and safety concerns associated with its application.

Pathophysiology of Intervertebral Disc Degeneration

The intervertebral disc is the largest avascular structure in the body and is composed of three morphologically distinct regions. The central nucleus pulposus is a gelatinous matrix rich in large aggregating proteoglycans which imbibe water and may assist in the diffusion of

nutrients from the periphery through maintenance of an osmotic gradient. The peripheral annulus fibrosis encases the nucleus pulposus and is a dense fibrotic tissue composed of concentric lamellae rich in type I collagen. The cartilaginous endplates are present at the cranial and caudal aspect of each disc and contain the peripheral vasculature which nourishes the disc. In concert these structures absorb transmit and transmit mechanical stress to the vertebrae and surrounding musculoligamentous structures of the spine. Maintenance of the morphological distinctions between the nucleus pulposus and annulus fibrosis is essential for normal biomechanical function of the disc during loading⁶.

The exact pathophysiology of degenerative disc disease has not yet been completely delineated, however it is known to be influenced by the interaction between various genetic, biologic and biomechanical factors⁷⁻¹⁰. In addition the morphologic, biochemical and radiographic changes which occur with progressive degeneration of the intervertebral disc have been well characterized. The hallmark of disc degeneration is the progress loss of proteoglycans which coincides with decreases in oxygen tension, free radical accumulation, decreased pH, and the increased activity of aberrant proteolytic enzymes¹¹⁻¹³. With the loss of proteoglycans the nucleus pulposus cannot maintain normal physiologic hydrostatic pressure which results in the dehydration of the disc¹⁴. In addition there is a progressive fibrosis of the nucleus pulposus as the ratio between type I to type II collagen increases¹⁵. As degeneration progresses the nucleus pulposus and annulus fibrosis lose their morphological distinction which ultimately disrupts the finely balance biomechanics of the disc and spine as whole^{14, 16}. **(Insert Figure 1)**

Multiple studies have indicated that the loss of proteoglycans within the disc is due to an imbalance between anabolism and catabolism within the disc, as proteolytic enzymes (MMPs, ADAMTs) are upregulated, while collagen and proteoglycan synthesis is diminished¹⁷⁻²⁰. Over

the past few years key biological factors which regulate disc extracellular matrix production, nutrition, cellular proliferation, signaling, and cell death have been identified.

The development of novel biologic treatments such as gene therapy have the potential to treat disc degeneration on a molecular level by correcting the biochemical imbalance within degenerating discs and thus potentially changing the natural history of this disease without the morbidity associated with surgery.

Gene Therapy

The idea of gene therapy originated as a means to treat heritable genetic disorders by replacing defective genes with functional copies thus curing the underlying disorder. The current concept of gene therapy has expanded to include the transfer of exogenous genes encoding therapeutic proteins into cells to treat disease. Upon transduction of this genetic material into the genome of the target cell the host transcribes the transgene into messenger ribonucleic acid (mRNA) which is then translated by ribosome in the cytoplasm into the desired protein product. These protein products not only affect the metabolism of the host cell but also that of adjacent cells via a paracrine effect. The direct application of therapeutic exogenous proteins is inadequate as their short half life within the cell precludes any long term disease modifying treatment. Gene therapy alters host cell DNA and thus providing a mechanism for the sustained production of the desired therapeutic product.

The packaging of exogenous genes is a technically demanding and labor intensive process. First the enzyme reverse transcriptase is utilized to construct the complimentary DNA (cDNA) of the gene of interest from messenger RNA (mRNA). Next the newly constructed cDNA is cloned in an expression plasmid under the control of an appropriate promoter which drives the expression of the transgene. Finally the completed expression plasmid (with the

cDNA of the therapeutic gene) is integrated into a vector which facilitates the entry of the exogenous gene into the host cell.

Vectors

The success of gene therapy is not only dependent on sustained expression of the therapeutic gene but also efficient transfer of the genetic material to the host cell. With very few exceptions naked plasmid DNA alone is not an effective means of gene transfer. Therefore the use of vectors is necessary to facilitate the transfer of genetic information to host cell. There are several types of vectors most of which fall into one of two distinguishing classes, viral and non viral.

Non-viral vectors include liposomes, gene guns, DNA-ligand complexes, and microbubble enhanced ultrasound. Liposomes are phospholipid vesicles which deliver the genetic material into the cell by fusing with the host's cellular membrane. DNA-ligand complexes and gene gun are other non pathogenic vectors which are inexpensive and easy to construct. Nishida et al. illustrated that ultrasound transfection with microbubbles enhances the efficiency of plasmid DNA uptake into the nucleus pulposus of rats *in vivo*²¹. The main issue with non viral vectors is transient transgene expression due to low transfection efficiency into the host genome, thus making these vectors suboptimal for the treatment of chronic diseases in which the sustained production of the desire product is required for disease modification.

Viral vectors utilize the natural capability of viruses to infect host cells and thus transfer the viral genetic information into the host. Viral vectors are very efficient at transducing the desired genetic material to the host cell, even into slowly dividing senescent cellular populations like those of the intervertebral disc. However they are also associated with greater risks than non viral vectors such as cytotoxicity and immune mediated response. Viral vectors utilized for gene

therapy applications include adenovirus, adeno-associated virus, herpes simplex virus, lentivirus, retrovirus, and pox virus. Each viral vector is associated with specific advantages and disadvantages. Therefore proper selection of vector is critical to successful gene therapy. The most commonly employed viral vectors for disc degeneration are adeno and adeno-associated viral vectors.

Adenoviruses are dsDNA viruses which have the ability to infect many cell types. There are 47 known human serotypes of which serotypes 2 and 5 are most commonly used for gene therapy applications. Adenovirus vectors are especially efficient at the transfer of genetic material to host cells, are relatively easy to construct at high titers, and can transduce non dividing quiescent cellular populations. These vectors are easily modifiable for gene therapy applications by the removal of the envelop E1 gene, which is essential for viral gene replication and expression²². In addition, the adenovirus genome does not integrate into that of the host cell and remains as an episome within the host cell's nucleus. Therefore, adenoviruses for gene therapy applications are thought to be associated with low rates of insertional mutagenesis during transduction in comparison to other vectors. The major limitation associated with the use of adenovirus vectors is the relatively short duration of transgene expression in most tissues. This transient expression is attributed to the production of adenoviral antigens by the host cell, with the resulting immune response degrading the adenoviral episome within the nucleus thus halting therapeutic protein production^{23,24}. In addition as the adenovirus is not integrated into the host cell's genome during cellular division the episome is not replicated. Finally wild type adenoviral infections can cause upper respiratory and gastrointestinal illnesses. These issues although concerning, likely have less clinical relevance due to the avascular encapsulated nature of the

disc. Currently ways to minimize host adenoviral antigen production and viral protein expression are being investigated.

Unlike adenovirus, adeno-associated virus (AAV) is a parvovirus with a single stranded DNA genome. AAV also has the ability to transfect multiple different cell types, however much less efficiency than the adenovirus. There are several distinct differences between adenovirus and adeno-associated virus. First the wild type AAV is not associated with any human disease and thus there are less safety concerns with its application. The AAV vector integrates into a specific site on the 19th chromosome without damaging the intrinsic genetic material present. Another beneficial feature is that AAV has only two genes (Rep & Cap) which cannot self replicate, and require the presence of a helper virus. Therefore in the absence of helper virus there is no expression of intrinsic AAV gene products after transduction limiting the cell mediated immune response. The difficulty with utilizing AAV vectors is that they can carry much less foreign (therapeutic) DNA than the adenovirus vector, and the purification of AAV is very challenging as the helper virus must be isolated and removed. Despite these shortcomings AAV has shown real promise as the conduit for successfully gene therapy into musculoskeletal tissues. Currently improvement in vector immunogenicity as well as inducible and tissue specific promoters are being developed. The selection of the appropriate vector is critical for successful gene therapy and depends on disease pathophysiology, therapeutic gene utilized, and strategy for delivery.

Gene Delivery Strategy

In addition to the selection of the appropriate gene and vector, another important consideration with gene therapy applications is the delivery strategy utilized. There are currently two basic strategies for the delivery of exogenous therapeutic genes into target cells. The *in vivo* strategy involves the direct transfer of the gene-vector complex to the targeted cellular population

within the living host. The *ex vivo* strategy differs significantly as the targeted cells are isolated and removed from the living host. These cells are then cultured with transduction of the therapeutic gene occurring *in vitro*. The final step includes the reimplantation of the genetically altered cells back into the host. **(Insert Figure 2)**

Theoretically the *ex vivo* method may be a safer approach to gene therapy as the genetically altered cells are observed *in vitro*, therefore the cells which show abnormal responses to the transfer of the therapeutic gene can be identified and isolated from the target cell population prior to reimplantation. However there are several disadvantages with the utilization of an *ex vivo* strategy for clinical gene therapy applications. First there may be significant morbidity associated with the harvesting as well as the reimplantation of the targeted host cells. Second, often the *in vivo* environment from which the host cells are removed cannot be replicated *in vitro*. *In vitro* the cells themselves may be irreversibly altered and not making them suitable to reimplantation. This is of particular concern when considering gene therapy for intervertebral disc degeneration, as the harsh conditions the cells of the nucleus pulposus experience (low oxygen tension, nutrients, and pH) cannot easily be replicated. Additionally *in vitro* cells will not be subjected to biomechanical stimuli which may be important to cellular signalling and cytokine production. Due to these reasons, most gene therapy applications for disc degeneration employ an *in vivo* strategy for gene delivery.

Modulation of Disc Cell Biology

The foundation for the application of gene therapy to treat disc degeneration is rooted in the identification of exogenous proteins and cytokines which have shown potential therapeutic benefits albeit transient, when cultured with disc cells *in vitro* or directly into the disc *in vivo*²⁵. As stated several studies suggest that the loss of proteoglycan associated with disc degeneration

is due to an imbalance between catabolism and anabolism within the disc. Thus the goal of gene therapy for the treatment of disc degeneration is to transfer genes which will correct this imbalance therefore slowing or reversing the loss of proteoglycans within the disc. Correction of the biochemical imbalances within the disc would likely facilitate the recovery and maintenance of normal disc morphology thereby improving the biomechanical function of the disc and ultimately altering the natural course of this disease process. In addition, the intervertebral disc is a good target for gene therapy as it is encapsulated and avascular, thus acting as an “immune privileged” organ allowing for sustained periods of transgene expression due to the lack of immune response to the therapeutic gene product or the intrinsic proteins produced by the vector.

Thompson et al. was the first to demonstrate that *in vitro*, proteoglycan synthesis within the disc could be upregulated through the application of exogenous human transforming growth factor (TGF- β 1) in cultured canine disc tissue²⁶. There have been several subsequent studies which have identified other growth factors that have the ability to increase proteoglycan synthesis in intervertebral disc cells. Osada et al demonstrated increased proteoglycan synthesis in cultured bovine disc cells stimulated with exogenous Insulin Growth Factor-1²⁷. Takegami et al illustrated a similar increase in proteoglycan synthesis in cultured rabbit nucleus pulposus cells exposed to exogenous OP-1, in addition to the recovery of proteoglycan content lost in cells exposed to inflammatory cytokine interleukin-1²⁸. Li et al illustrated that exogenous BMP-2 not only increased aggrecan expression, but also stimulated OP-1 expression further boosting proteoglycan synthesis in cultured rat disc cells²⁹. Yoon et al performed an *in vitro* study on rat intervertebral disc stimulated with BMP-2 and found that this growth factor not only increased cell proliferation and proteoglycan synthesis, but also increased the mRNA of Type II collagen, aggrecan, and Sox9 genes which are all chondrocyte specific genes³⁰.

(Insert Figure 3)

In addition to the anabolic function of these growth factors, Gruber et al demonstrated that the exogenous application of IGF-I and Platelet Derived Growth Factor (PDGF) significantly decreased the number of apoptotic disc cells in culture³¹. Thus these growth factors may have some utility in treating disc degeneration by maintaining the disc cell population, phenotype, and proteoglycan content.

Gene Therapy to Intervertebral Discs

With the identification of potential therapeutic growth factors and cytokines, the idea of producing them endogenously using gene therapy arose. This idea was first expounded upon by Wehling et al who performed an *in vitro* study utilizing retroviral mediated transfer of the bacterial β -galactosidase marker gene (LacZ) and the cDNA of the human interleukin-1 receptor antagonist (IL-1Ra) to bovine endplate chondrocytes³². Despite only successfully transducing approximately 1% of the cultured bovine cells, the transgene expression of IL-1Ra was significantly increased over controls at 48 hours. From this data it was concluded that the *ex vivo* method of gene therapy may be a novel approach to the treatment of disc degeneration via the reimplantation of genetically altered disc cells. The first successful *in vivo* gene transfer was demonstrated by Nishida et al who reported sustained transgene expression of adenoviral mediated LacZ marker gene in skeletally mature New Zealand White rabbits³³. These rabbits showed no evidence of systemic illness which was supported by the lack of cellular immune response histologically up to 3 months post transduction, with evidence of transgene expression within the disc detected 1 year post transduction³⁴. **(Insert Figure 4)**

This study also demonstrated much more efficient transfer of genetic material than previously published, as *in vitro* transduction of LacZ in cultured rabbit cells was nearly 100% using the

adenovirus vector³³. In addition to these findings, Nishida et al also found no change in the intradiscal expression of a genetic marker luciferase in rabbits immunized subcutaneously with the transgene-vector construct weeks before delivery into the disc. In other tissues such as the testis and retina, their immune privilege characteristics have been attributed to the local expression of Fas ligand within their tissues which induces apoptosis to invading Fas positive T cells. Takada et al subsequently demonstrated the presence of Fas ligand in disc cells, specifically those of the nucleus pulposus providing a mechanism to support the theory of the immune privilege status of the disc³⁵. These pivotal studies not only illustrated the potential of *in vivo* gene therapy for disc degeneration, but also legitimized the claim that gene therapy into the intervertebral disc is feasible due to its immune privileged nature.

Genetic Transfer of Therapeutic Genes

The next step in the evolution of gene therapy for the treatment of disc degeneration was to replace the marker gene (LacZ) with ones having therapeutic potential. Using an adenovirus vector Nishida et al successfully transduced complementary DNA for TGF- β 1 into the nucleus pulposus of lumbar rabbit discs³⁶. This study illustrated a 30-fold increase in synthesis of active TGF- β 1 and a 5 fold increase in total production of this growth factor in discs injected with the adenovirus-gene complex. **(Insert Figure 5)**

In addition to the successful transduction of TGF- β 1, proteoglycan synthesis increased 100% from baseline values. As in previous studies with vector-marker gene constructs, there was no evidence of a systemic or local immune response. Interestingly cells that received adenovirus TGF- β 1 had increased synthesis of matrix components (proteoglycans, collagen) in comparison with those receiving the exogenous protein alone, which is likely due to the sustained expression produced by gene therapy. The transduced cells also exerted a paracrine effect on adjacent non

treated cells, as the viral load required to increase proteoglycan synthesis was much lower than that required to transduce the entire cell population of the disc. Assays for TGF- β 1 and proteoglycan synthesis were also performed on discs transduced with a viral vector control gene construct (Adeno-Luciferase marker gene). There was no increase in TGF- β 1 or proteoglycan synthesis detected in these discs indicating that the observed response in the treatment group was due to the presence of TGF- β 1 and not a non specific response to the adenovirus vector. This study demonstrated that intervertebral disc cells could not only be transduced successfully with therapeutic genes, but also capable of producing enough growth factor to significantly modulate the biologic activity of the treated cell population. These results have been replicated in other studies using growth factors (BMP-2, IGF-1) vector complexes which increase proteoglycan synthesis in a dose dependant manner^{37,38}. However it should be noted that tissue inhibitor of metalloproteinase 1 (TIMP-1) displayed the same ability when transduced into nucleus pulposus cells. TIMP-1 is an inhibitor of matrix metalloproteinases which breaks down the extracellular matrix components, and is thus one of many regulators of the catabolic activity of the disc. Upregulation of this anti catabolic cytokine was matrix protective by inhibiting the breakdown of existing proteoglycans within the disc. Wallach et al. demonstrated increased proteoglycan synthesis in human disc cells cultured in 3-D pellets exposed to adenovirus TIMP-1³⁸. **(Insert**

Figure 6)

This finding provided credence to the hypothesis that the imbalance between catabolism and anabolism results in proteoglycan loss, and that biologic modification could target increasing anabolism or decreasing catabolism in the disc.

Two other potentially disease altering genes being investigated include LIM mineralization protein (LMP-1) and Sox 9. LMP-1 is a regulatory protein that upregulates the

anabolic activity of bone morphogenic proteins (BMP) in the disc. Yoon et al found significantly increased expression of BMP-2 and 7 mRNA following the *in vitro* transduction of adenovirus LMP-1³⁹. This finding was correlated with an increase in proteoglycan synthesis both *in vivo* and *in vitro* after transduction of rat intervertebral disc cells. Sox9 is a transcription factor which has many responsibilities including type II collagen expression, an essential component of the nucleus pulposus ECM. Human intervertebral disc cells treated with adenoviral vector Sox9 complex *in vitro* showed increase type II collagen production compared with controls⁴⁰. As the pathophysiology of disc degeneration is better delineated, the use of certain specific therapeutic genes or a combination of those may be better suited for the biological augmentation of diseased disc cells. Continued research is geared toward identifying more potentially therapeutic genes and improving the efficiency and safety of their delivery.

Regulation of Pathologic Gene Expression

RNA interference (RNAi) has emerged as another strategy for altering genetic information within diseased disc cells. RNAi reduces the overall production of a targeted gene product by the use of small interfering RNA (siRNA) which binds to the targeted mRNA in a sequence specific manner leading to either suppressed translation or enhanced degradation of the message. Thus catabolic genes within the disc tissue can be targeted and silenced through siRNA mediated destruction preventing translation of those genes. As with therapeutic exogenous genes, siRNA has a short half life. Kakutani et al has demonstrated sustained down regulation of specific targeted genes using DNA vector-siRNA complexes⁴¹. In this study expression of *Firefly* Luciferase was inhibited 94.7% in rat and 93.7% in human nucleus pulposus cells cultured *in vitro* that were treated with siRNA targeting this gene. However this inhibitory effect was gone 3 weeks after initial treatment. RNAi may provide another

mechanism to address the resultant imbalance between catabolism and anabolism in intervertebral disc cells, however sustained down regulation of specific target genes is required in order to treat chronic progressive disorders such as IDD.

Safety Considerations

Due to the potential adverse effects associated with the use of viral vectors for gene therapy applications current areas of investigation include strategies to reduced viral load required to produce significant therapeutic end product production, inducible on off mechanisms, tissue specific promoters, and adeno-associated viral vector constructs. One strategy to amplify transgene expression while limiting viral load is to transduce combinations of anabolic genes on one viral vector. Experiment with various combinations of growth factors (TGF- β 1,BMP-2, IGF-1) show a synergistic effect amplifying the production of extracellular matrix components³⁷. In this study human nucleus pulposus cells were isolated and cultured in vitro. Upon confluence cells were subsequently transduced with one of the growth factors, a combination of two growth factors (TGF- β 1 + IGF-1, TGF- β 1 + BMP-2, IGF-1 + BMP-2), or all three growth factors using an adenovirus vector. Proteoglycan synthesis was significantly increased from control (180-295%) when treated with one factor, and a synergistic effect was observed when cells were treated with a combination of two factors as increases in proteoglycan synthesis ranged from 322 to 398%. Cell cultures that received all three factors had a 471% increase in new proteoglycan synthesis. Future studies will determine if combinations of these anabolic growth factors in addition to anti-catabolic factors such as TIMP-1 have even greater effects on the biologic activity within the disc.

Another concern with the application of gene therapy is the inability to regulate transgene expression once successfully transduced into the host cell. Wallach et al demonstrated

that accidental intradural injection of vector-therapeutic gene constructs (Ad-TGF- β 1) can result in severe histological changes and paralysis in New Zealand White rabbits⁴². Therefore the development of inducible systems to regulate transgene expression are being developed. A number of inducible gene expression systems have been investigated including systems utilizing heat shock proteins, metallothionine, steroid regulatory promoters, tetracycline, and most recently, the EcR insect receptor. Most of these inducible systems work by linking a ligand activated promoter region to the potential therapeutic gene within the vector construct. There are two basic strategies for regulation which depend on whether the exogenously applied ligand turns transgene expression “On” or “Off”. The “Tet-on” system is one in which transgene expression is activated by administration of a tetracycline derivative, and this inducible system has been incorporated into AAV vector constructs⁴³. These “Tet-on” systems have demonstrated successful regulation of a marker gene in chondrocytes from new Zealand white rabbit knees⁴⁴. Current research is investigating ways to increase efficiency of “Tet-on” systems and evaluating their control of therapeutic transgene expression in intervertebral disc cells. Additionally regulation systems with tissue-specific promoters are also being developed and would further optimize efficacy and minimize treatment side effects.

The use of less pathologic viral vector constructs such as adeno-associated virus have also been investigated as alternatives to adenovirus vectors. The efficacy of adeno-associated virus vector transduction in comparison to adenovirus was explored on nucleus pulposus *cells in vitro* and *in vivo*⁴⁵. Despite approximately half the transgene expression seen with adenovirus *in vitro* and *in vivo* there was sustained production of the luciferase marker at all time points up to 6 weeks. Additionally the overall transduction achieved with AAV vector LacZ marker was high setting the precedent for the use of AAV vector for gene therapy applications to treat disc

degeneration. The use of adeno-associated vector to transduce therapeutic anabolic and anti-catabolic genes (BMP-2, TIMP-1) is currently being investigated.

Conclusion

Intervertebral disc degeneration is a common and potentially debilitating disease process affecting millions of Americans each year. Current treatments address resultant symptoms and not the underlying pathophysiology of disease. This has spawned the development of biological treatments such as gene therapy which attempt to correct the imbalance between catabolism and anabolism within degenerating disc cells. The identification of therapeutic genes and development of successful delivery systems has resulted in significant advances in this novel treatment. However continued investigation of the pathophysiology of disc degeneration as well as safety mechanisms for the application of gene therapy are required for clinical translation.

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Figure 1

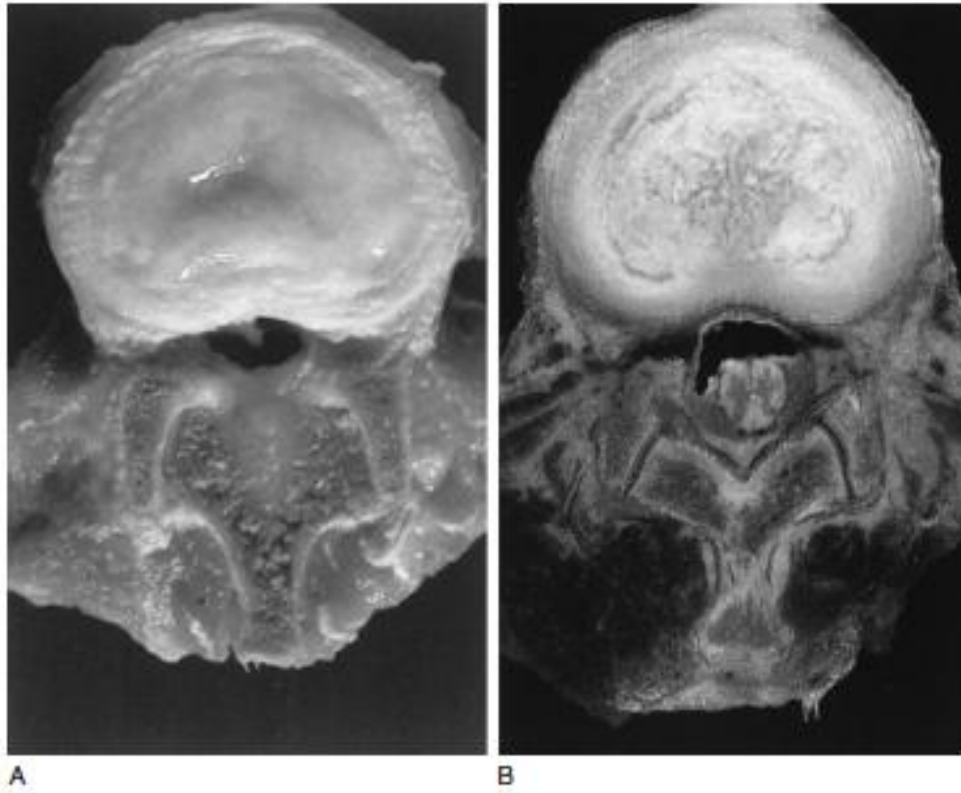


FIGURE 1: Disc Degeneration results in loss of proteoglycan content, morphologic distinction of the disc components, structural failure, and facet arthrosis. The loss of demarcation is clear in contrasting these two pictures as the type I/II collagen ratio in the NP increases and becomes progressively more fibrotic. Loss of hydration causes decreased osmotic pressure, increased tensile forces, annulus fissures and loading of the posterior elements. **A**, Healthy disc. **B**, DDD in 28 year old. Reprinted with Permission⁴⁶.

Figure 2

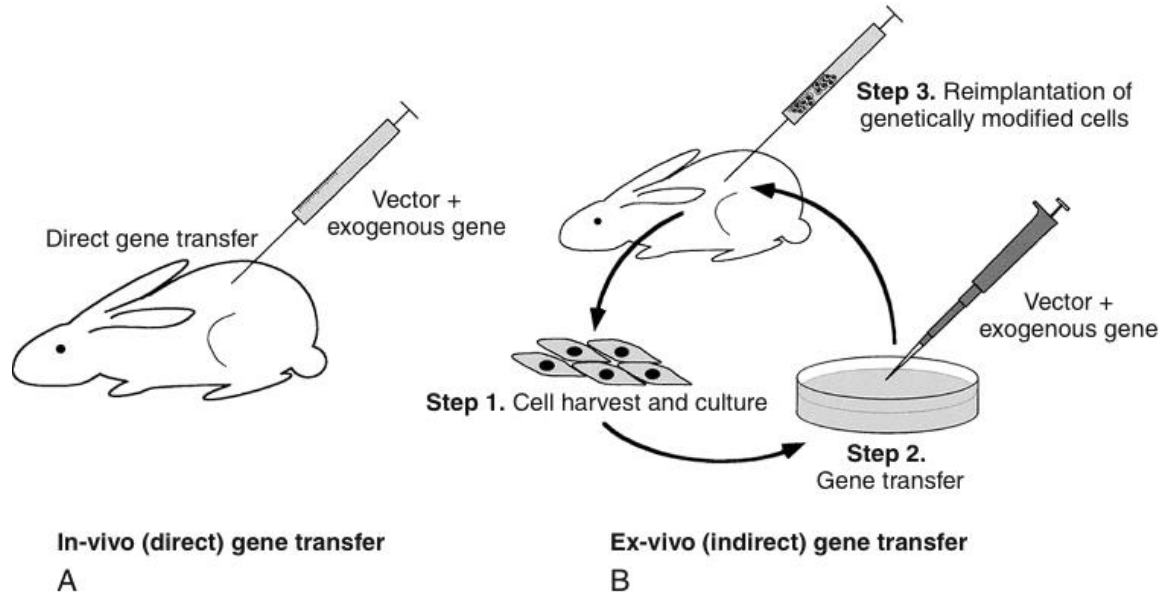


FIGURE 2: A, *In vivo* gene therapy involves the direct injection of vector-gene constructs into target tissues within the host. B, In the *ex vivo* method target cells are harvested from the host, which are then transduced, expanded, and propagated in culture before reimplantation. Reprinted with Permission⁴⁷.

Figure 3

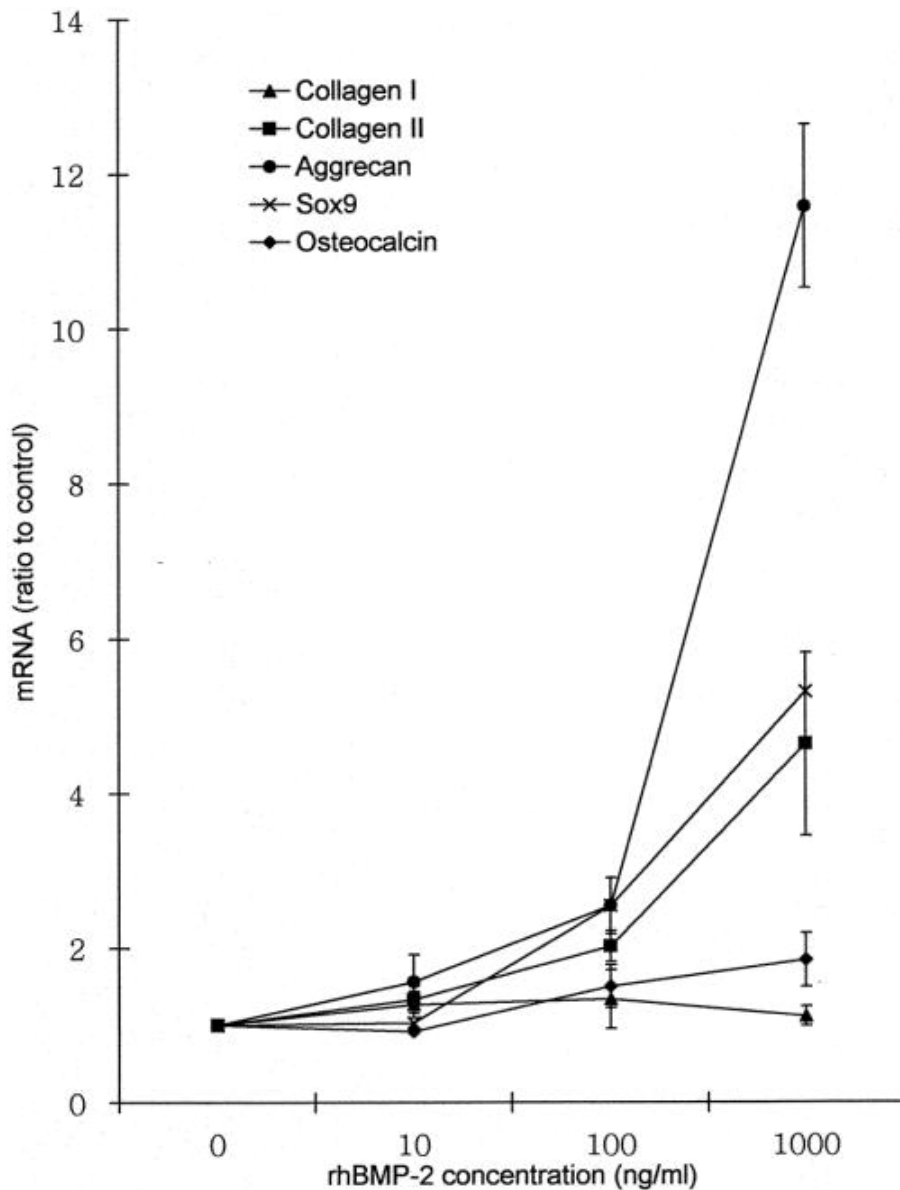


Figure 3: Quantitation of mRNA levels using real-time PCR 7 days after application of rhBMP-2 to rat annulus cells *in vitro*. Normalization was performed with internal control (GAPDH) and a standard curve for each primer. Aggrecan, Sox9, and Type II collagen were significantly increased at rhBMP-2 concentrations of 100 and 1000 ng/ml. Type I collagen levels did not change regardless of rhBMP-2 concentration. Reprinted with Permission³⁰

Figure 4

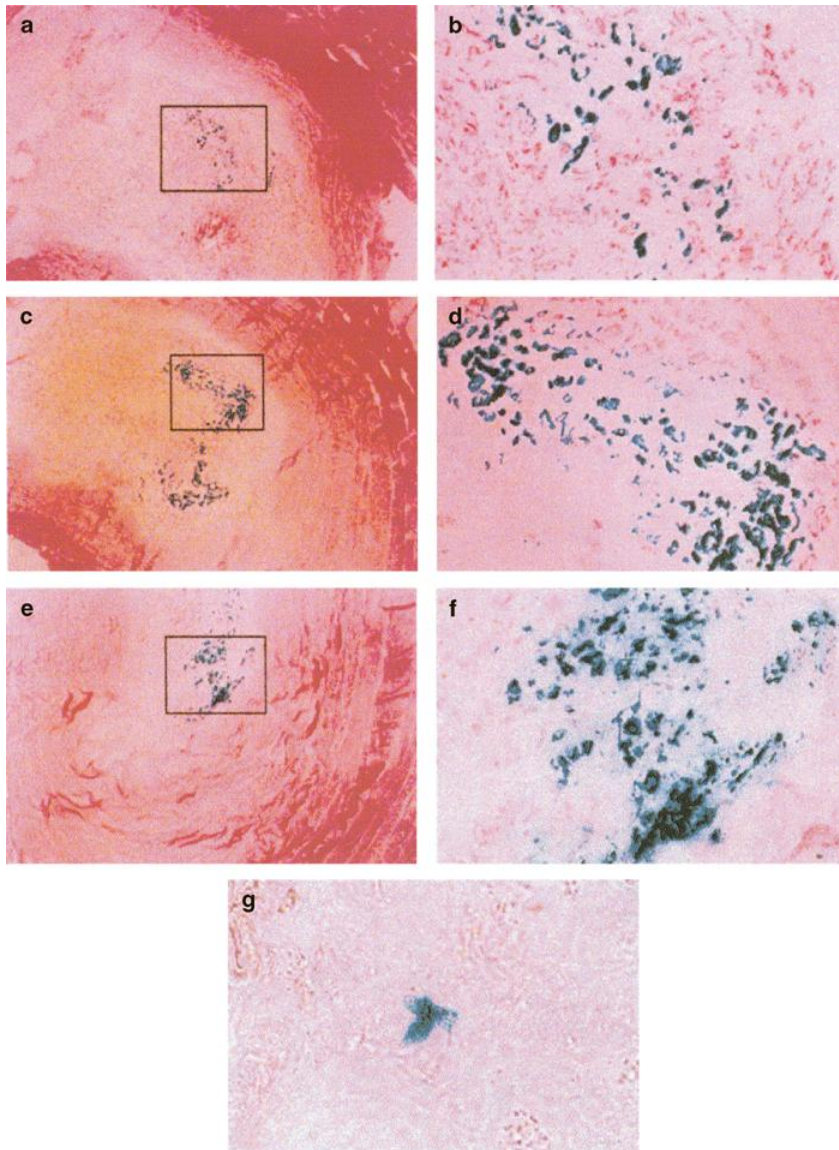


Figure 4: Histology stained with X-Gal and eosin representing transgene expression of Ad-lacZ in rabbit lumbar intervertebral discs at **(a,b)** 3 weeks, **(c,d)** 6 weeks, **(e,f)** 24 weeks, **(g)** and 52 weeks post transduction. At 52 weeks positive X-Gal staining was observed in the discs from two of the three rabbits, however the intensity of the positive stain was significantly diminished from previous time periods. (Original magnifications: a, c, e at 40X; b, d, f at 200X; g at 600X). Reprinted with Permission³³

Figure 5

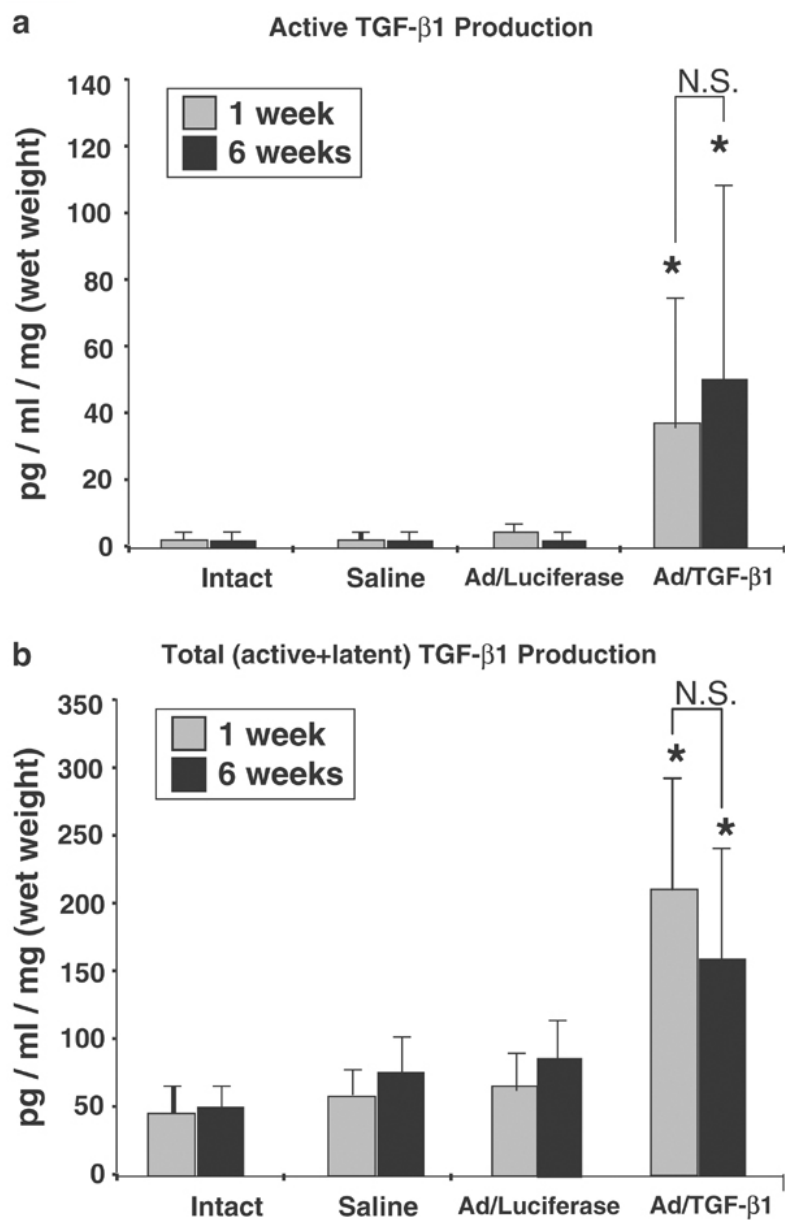


Figure 5: (a) Active TGF-β1, (b) Total active and latent TGF-β1 production in rabbit disc tissue 1 and 6 weeks after *in vivo* transduction. Sustained transgene expression was present at 6 weeks, as there was no significant difference in active or total TGF-β1 production between the 1 and 6 week time points. Asterisks denotes a significant increase in TGF-B1 production in treatment group over controls (P<0.05). Reprinted with Permission³⁶.

Figure 6

Active Proteoglycan Synthesis in Lumbar Disc Cells with Ad-TIMP-1 and Ad-BMP-2

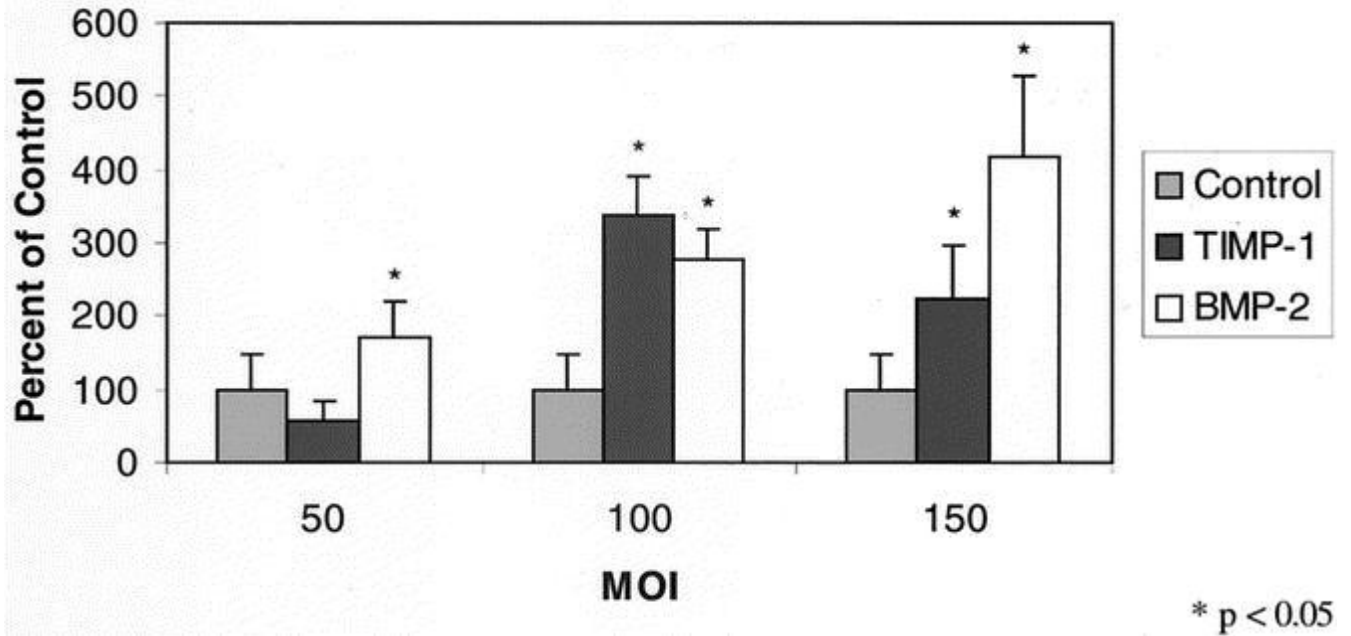


Figure 6: Measured proteoglycans in human lumbar discs cells cultured in 3-D pellet. Cells were transduced with Ad-TIMP-1 or Ad-BMP-2, and proteoglycans are expressed as percent measured in controls. An optimal response to Ad-TIMP-1 occurred at MOI of 100, and resulted in over a 300% increase in proteoglycan production from controls. Ad-BMP-2 application resulted in a dose dependant increase in proteoglycan content. Reprinted with Permission³⁸