Molecular and genetic advances in the regeneration of the intervertebral disc

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Abstract

Background: Owing to the debilitating nature of degenerative disc disease (DDD) and other spine pathologies, significant research has been performed with the goal of healing or regenerating the intervertebral disc (IVD). Structural complexity, coupled with low vascularity and cellularity, make IVD regeneration an extremely challenging task.

Methods: Tissue engineering-based strategies utilize three components to enhance tissue regeneration; scaffold materials to guide cell growth, biomolecules to enhance cell migration and differentiation, and cells (autologous, or allogeneic) to initiate the process of tissue formation. Significant advances in IVD regeneration have been made utilizing these tissue engineering strategies.

Results: The current literature demonstrates that members of the transforming growth factor beta (TGF-β) superfamily are efficacious in the regeneration of an anabolic response in the IVD and to facilitate chondrogenic differentiation. Gene therapy, though thwarted by safety concerns and the risk of ectopic transfection, has significant potential for a targeted and sustained regenerative response. Stem cells in combination with injectable, biocompatible, and biodegradable scaffolds in the form of hydrogels can differentiate into *de novo* IVD tissue and facilitate regeneration of the existing matrix. Therapies that address both anabolism and the inherent catabolic state of the IVD using either direct inhibitors or broad-spectrum inhibitors show extensive promise.

Conclusion: This review article summarizes the genetic and molecular advances that promise to play an integral role in the development of new strategies to combat DDD and promote healing of injured discs.

Key Words: Disc tissue engineering, growth factors, intervertebral disc regeneration, stem cells, scaffolds

INTRODUCTION

The limited regenerative response, biomechanical importance, and structural complexity of the intervertebral disc (IVD) pose significant challenges to both the clinician and researcher in the development of regenerative strategies. Degenerative disc disease (DDD) is the result of adverse loading, dehydration, cellular apoptosis, and an imbalance

in tissue anabolism and catabolism. In the setting of DDD, local metabolism favors tissue lysis as manifested by the increased expression of pro-inflammatory cytokines and proteolytic enzymes with a concomitant reduction in matrix anabolism. The presence of catabolic biomolecules that drive this degenerative process not only facilitate tissue degeneration, but they also hinder regenerative efforts.

Rapid advances in our understanding of the biology of the IVD from a genetic and molecular perspective have resulted in several promising methods to facilitate the translation of laboratory-based techniques to clinical disc regeneration. Tissue engineering strategies that employ biomimetic scaffold materials, differentiation-driving bioactive molecules, and multipotent cells to enhance disc regeneration are being developed at a rapid pace. This review compiles and summarizes high-quality basic science studies that employ molecular therapy and tissue engineering-based techniques to aid in regeneration of the IVD. All discussed techniques are summarized in Table 1.

DISC REGENERATION VIA MORPHOGENS AND MITOGENS

Delivery of exogenous proteins: Growth factors and cytokines

The delivery of exogenous proteins in the form of growth factors and/or cytokines is a promising therapy to address DDD. While a wide variety of bioactive proteins and the associated biologic responses they elicit within the IVD have been studied, the principle behind this therapy remains consistent: Altering cellular metabolism and/or phenotype to increase proliferation, extracellular matrix (ECM) synthesis, cell signaling, and/or to downregulate catabolic and inflammatory processes that perpetuate DDD.

Factors with biologic activity: Mitogens, morphogens, and intracellular mediators

Mitogens, morphogens, and intracellular mediators known to be present during neonatal development of the IVD and factors known to induce differentiation of stem cells toward the IVD phenotype have both been identified as candidate therapeutic factors with pleiotrophic biologic activity. Members of the transforming growth factor beta (TGF-β) superfamily, namely TGF-β_1 and TGF-β_3 in addition to bone morphogenetic proteins (BMPs), as well as insulin-like growth factor-1 (IGF-1), growth and differentiation factors (GDFs), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), basic fibroblastic growth factor (bFGF), and others, have all been studied in the context of IVD regeneration.

Transforming growth factor beta superfamily; morphogens and biologic mediators of intravertebral disc development

Members of the TGF-β superfamily are morphogens and biologic mediators during neonatal development of the IVD.[16] TGF-β is present in lower levels in herniated discs,[27] and its concentration in the IVD has been shown to decrease with age.[42,54] Early *in vitro* studies by Risbud *et al.* demonstrated that the stimulation of proteoglycan (PG) synthesis in IVDs by transforming growth factor-beta 3[72] (TGF-β_3) increases proliferation and ECM macromolecule synthesis via the extracellular signal-regulated kinase/mitogen activated protein (ERK/MAP) signaling pathway.[61] Human annulus fibrosus (AF) cells grown in 3D agarose or alginate systems have shown increased proliferation following treatment with TGF-β_1[14] Abbott *et al.* demonstrated that TGF-β_3 in combination with dexamethasone (Dex) induces a decreased expression of the catabolic genes MMP1 (encoding for matrix metalloproteinase-1) and ADAMTS5 (encoding for a disintegrin and metalloproteinase with thrombospondin motifs 5) and increased expression of ECM genes.[1]

In-vitro study of bone morphogenetic protein-2 for IVD regeneration

BMPs, named after the osteoinductive potential of some of its members, have also been extensively studied in the context of IVD regeneration. Bone morphogenetic protein-2 (BMP-2) and bone morphogenetic protein-7 (BMP-7 or OP-1) are the only human recombinant growth factors approved by the Federal Drug Administration (FDA). Although BMP-2 is an osteoinductive growth factor approved for use in lumbar spine fusion, off-label use is wide-spread, and has been implicated as a potential source of numerous complications.[80] *In vitro* treatment of IVD cells with BMP-2 upregulates genes for aggrecan, type II collagen, and sex determining region Y-Box9 (Sox9). It increases protein expression of proteoglycans and type II collagen.[35,73] without inducing an osteogenic phenotype and/or associated calcification or mineralization.[24,43]

In-vivo study of bone morphogenetic protein-2 for IVD regeneration

The regenerative potential of BMPs in the setting of disc regeneration has also been studied with animal models. In an *in vivo* model of an annular tear, Huang, *et al.*[20] demonstrated that administration of rhBMP-2 with or without coral, actually caused a greater degree of degenerative changes in the IVD compared with controls. Additionally, the study demonstrated that rather than regenerating the AF with new fibrous tissue, several animals exhibited bony fusions. The study also noted increased inflammation, vascularity, and fibroblastic proliferation in rhBMP-2-treated animals compared with saline-treated animals. Future *in vivo* studies are necessary to understand the apparent discrepancy between *in vitro* and *in vivo* findings.

BMP-7 (OP-1) induces similar in vitro effects as BMP-2 in IVD cells

BMP-7 or OP-1 induces similar *in vitro* effects as BMP-2

by stimulating proliferation, proteoglycan, and collagen synthesis in IVD cells.[25,41,71,90] Furthermore, BMP-7 exhibits antifibrotic, antiapoptotic, antiinflammatory, and proangiogneic properties.[86,87] Takegami, *et al.* demonstrated that primary rabbit AF and nucleus pulposus (NP) cells previously exposed to interleukin-1 beta (IL-1β) recovered their proteoglycan and collagen synthesis after BMP-7 treatment.[41] Im, *et al.* showed that BMP-7 decreased IL-1β-induced expression of matrix metalloproteinase-13 (MMP-13) in chondrocytes.[21] Masuda, *et al.* studied the *in vivo* effect of 10[72] μg/mL BMP-7 injection into rabbit nucleus pulposi following the induction of degeneration using the needle puncture model.[40] The study showed recovered disc height, improved magnetic resonance image (MRI) findings, and higher proteoglycan content in both the AF and NP in the BMP-7-treated animals compared with control (lactose-treated) animals. The same group performed a similar experiment on rabbit discs treated with chondroitinase-ABC to induce degeneration.[22] They observed a recovery of disc height and higher proteoglycan content, but no significant histological improvement.

As very high concentrations of BMP-7 were used in these studies, future experimentation is necessary to determine whether less protein/BMP-7 would achieve comparable positive results. Further research should also explore the variable efficacy of BMP-7 treatment in a model using annular puncture versus a model employing chemical digestion (e.g., chondroitinase-ABC injection). This research should also include an assessment of whether a proteinase inhibitor adds a synergistic effect by counteracting catabolic processes.

In vitro and in vivo studies of BMP-14 (GDF-5): Morbidity and questions regarding impact of dosing

Similar to BMP-2 and -7, bone morphogenetic protein-14 (BMP-14), also known as growth and differentiation factor-5 (GDF-5), or cartilage-derived morphogenetic protein-1 (CDMP-1), was initially investigated as an osteoinductive protein for use in spine fusion. BMP-14 is present during precartilaginous mesenchymal condensation and localized in the cartilaginous cores of long bones during embryogenesis and skeletal development.[4] While its precise role in IVD development, metabolism, degeneration, and regeneration is not fully known, GDF-5 deficiency causes a variety of ECM abnormalities and defects in the IVD.[36]

In vitro, BMP-14 induces cellular activation and proliferation in primary IVD cells.[78] When compared with BMP-2 and BMP-7, BMP-14 appears more effective at promoting collagen synthesis, whereas BMP-2 and BMP-7 stimulate proteoglycan synthesis.[91] In a murine model of disc degeneration, a single 1 μg/mL injection

of GDF-5 caused increased disc height compared with saline-injected controls.[77] Multiple 1 μg/mL injections of GDF-5 (once per week for 4 weeks), however, caused inflammatory reactions in the adjacent vertebrae and connective tissue that infiltrated the nucleus. These data indicated that the positive effect of GDF-5 may depend on dosing, and that multiple treatments can cause catabolic and/or inflammatory processes.

Chujo, *et al.* demonstrated that primary bovine AF and NP cells cultured in alginate beads and treated with rhGDF-5 exhibited significantly increased DNA content, proteoglycan synthesis, and collagen synthesis.[6] Following their *in vitro* experimentation, injections of rhGDF-5 were assessed in an annular puncture model of disc degeneration in rabbits. Results demonstrated increased disc height, improved scores on MRI grading, and improved histologic scores. Improved healing as measured by disc height, MRI grade, and histologic score was, however, not exhibited with increasing doses.

More work is needed to ascertain effects of lesser known bone morphogenetic proteins family members

BMP-4

The BMP family has numerous members, which have yet to be investigated in terms of their *in vitro*, or *in vivo* regenerative potential for disc regeneration. Although BMP-4 is a stimulator of chondrogenesis, little work has been done to study the specific effect of BMP-4 on the IVD.[44] Many studies have assessed the regenerative effect of BMP-4 on articular cartilage,[29,37] articular cartilage chondrocytes,[57] and on the differentiating effect on MSCs toward chondroprogenitor cells.[44] Similar to BMP-4, little high-quality work has been done to study the regenerative effect of BMP-13 (also termed GDF-6 or CDMP-2) on DDD.

BMP-13

BMP-13, a member of the BMP family with only 50% homology to BMP-2, is highly evolutionarily conserved across mammals (95% +), which suggests an important biologic function. It has been detected in hypertrophic chondrocytes in ossifying long bone centers, but its exact role in the IVD remains unknown.[38] Li, *et al.* showed that murine chondrocytes treated with BMP-13 increased total proteoglycan production and aggrecan mRNA in a dose-dependent manner, but that this effect was not synergized by the addition of BMP-2.[34] Future work is necessary to study the efficacy of BMP-13 on IVD regeneration.

Regenerative capacity of mitogenic factors

IGF-1, PDGF, EGF, and bFGF are mitogenic factors of the IVD. While they do not act as chondrogenic factors like the morphogenic molecules previously outlined, they act as "growth factors" by increasing the rate of

mitosis. The earliest work with mitogenic molecules in the context of IVD regeneration was performed by Thompson *et al.* in 1991.[66] This study showed that mitosis is affected by mitogenic factors in canine IVD cells.[72] After Okuda, *et al.* demonstrated an age-related, IGF-1-dependent decline in proteoglycan synthesis in rat IVDs,[53] they suggested that IGF-1 therapy may be beneficial in reversing age-related degeneration.

Although most authors have used mitogenic factors in combination with morphogens in regenerative therapeutic approaches, some studies have used them in isolation. Walsh, *et al.*[77] showed that a single injection of IGF-1 into a degenerate murine disc increased cell density and proliferation at 1 week, but this effect was not sustained at later time points. Furthermore, multiple IGF-1 injections caused inflammatory reactions in adjacent vertebrae, indicating a dose-dependent response. When administered in combination with BMP-7, IGF-1 caused a synergistic effect on anabolic BMP-7. This was attributed to signaling by activating the Smad2 pathway and, consequently, causing increased proteoglycan synthesis. IGF-1 and PDGF both possess *in vitro* antiapoptotic action,[15] but this effect has not been shown *in vivo*. *In vitro*, EGF was found to increase activation and proliferation,[72] but bFGF by itself did not elicit a significant proliferative or anabolic effect when injected into a degenerate disc *in vivo*.[77]

CELL-BASED REGENERATIVE STRATEGIES

Cell-based strategies for IVD regeneration represent an important family of techniques that are rooted in the hypothesis that scaffolds and growth factors alone are insufficient for the regeneration of tissues. Delivery of exogenous cells to injured tissues has been shown to be effective in a variety of clinical scenarios. In the setting of the IVD, these techniques can be classified by the cell types utilized to effect regeneration, including chondrocytes and fibrochondrocytes from the IVD, notochordal cells (NCs), and mesenchymal stem cells (MSCs).

Mesenchymal stem cells

MSCs are multipotent cells that are capable of migration and homing, as well as differentiation toward chondrocytic, osteoblastic, adipocytic, myofibroblastic, and tenofibroblastic lineages.[8] MSCs are found in numerous anatomic locations, including the bone marrow where they associate with stromal cells in a stromal cell-derived factor-1/C-X-C chemokine receptor type 4 (SDF-1/CXCR4)-dependent fashion until mobilized by the inflammatory cascade initiated by tissue injury.[69] They maintain an inherent stemness and are capable of self-renewal by expressing specific self-renewal genes such as Oct-4, Sox-2, and Rex-1.[23] The International Society for Cellular

Therapy (ISCT) published criteria by which to identify MSCs, which include positive expression of cluster of differentiation (CD) 73, CD90, and CD105 and negative expression of CD45, CD34, CD14 or CD11b, CD79α, or CD19 and human leukocyte antigen-DR (HLA-DR).[8]

Marrow-derived MSCs differentiate toward a nucleus pulposus-like phenotype

Risbud, *et al.* demonstrated that marrow-derived MSCs are capable of differentiating toward a NP-like phenotype under the *in vitro* conditions of hypoxia and exogenous TGF-β$_1$.[60] Marrow-derived MSCs from rats also exhibit the ability to differentiate into NP-like cells when co-cultured with intact IVDs.[82] Using an organ culture model, Le Maitre, *et al.* showed that intradiscal injection of human, marrow-derived MSCs were not only capable of surviving, but also induced expression of SOX-9, aggrecan and type II collagen indicative of differentiation toward a NP-like phenotype.[31] Richardson *et al.* found that direct contact between MSCs and NP chondrocytes stimulated the differentiation of the MSCs toward an NP-like lineage, as illustrated by upregulation of SOX-9, type I, II, and VI collagen, as well as aggrecan, versican, and elastin.[59]

In vivo experimentation demonstrates the promise of marrow-derived mesenchymal stem cells

In vivo experimentation has also demonstrated the promise of marrow-derived MSCs. Transplantation of marrow-derived MSCs into rabbit degenerative discs enhanced glycosaminoglycan (GAG) content and gene-level expression of aggrecan and type II collagen. Hiyama *et al.* transplanted marrow-derived MSCs into previously nucleotomized discs.[19] They demonstrated not only histologic and reverse transcription polymerase chain reaction (RT-PCR) evidence of disc regeneration, but that the transplanted cells could survive 8 weeks posttransplantation.[19]

Multiple niches of mesenchymal stem cells display regenerative properties

There are several other niches of MSCs that display regenerative properties. Utilizing a pellet co-culture of synovium-derived MSCs and NP chondrocytes, Chen, *et al.* demonstated NP-like differentiation.[5] He and Pei showed that culturing NP chondrocytes in plastic flasks coated with ECM produced by synovium-derived MSCs resulted in increased NP cell proliferation and redifferentiation capacity.[17] Transplanting synovium-derived MSCs immediately after nucleotomy in a rabbit model has also been shown to prevent radiographic, MRI, and histologic evidence of disc degeneration. The transplanted cells suppressed the expression of genes, which encode for MMPs, while also enhancing matrix synthesis.[47] Murrell, *et al.* transplanted olfactory neurosphere-dervied stem cells into nucleotomized rat discs, and observed increased

type II collagen and chondroitin sulfate proteoglycans within the disc tissue.[50] In a canine nucleotomized disc model with transplanted adipose-derived MSCs with a hyaluronic acid carrier, Ganey, *et al.* demonstrated radiographic, MRI, histologic, and biochemical evidence of disc regeneration.[10]

INTERVERTEBRAL DISC-DERIVED CELLS

Cells derived from intervertebral disc respond may halt degeneration and rejuvenate endogenous cells

Cells derived from the IVD have been studied extensively in terms of their response to soluble factors, variable culture conditions and paracrine signaling. In contrast to MSCs, IVD-derived cell therapy focuses on utilizing cells of a committed phenotype to halt the process of degeneration and, in some instances, rejuvenating endogenous cells that have been damaged by aging, or pathology. In 2003, Watanabe, *et al.* examined a novel technique of "activating" autologous NP cells via co-culture with AF cells and subsequently reinserting them into the degenerate disc.[79] Degenerative changes in discs that were reinserted with activated NP cells were less severe than in sham-treated discs. However, the results from this study must be interpreted with care, as the model used was a rabbit, which is known to retain NCs in the disc much longer than other animals. Xin, *et al.* injected unmodified NP cells and NP cells transduced with telomerase reverse transcriptase (hTERT) into explanted canine whole IVDs, which were subsequently transplanted into donor canines.[84] NP cell modification of the allograft discs resulted in significantly less degeneration in the recipients, with hTERT-transduced NP cells offering the greatest protection. Ganey, *et al.* demonstrated that reimplantation of *ex vivo* cultures of autologous disc chondroctyes resulted in the production of proteoglycans and a matrix similar to the native disc, while maintaining disc height.[11] The study also highlighted that the disc chondrocytes maintained viability at all examined end points.

Notochordal cells represent a promising cell population for regeneration

NCs represent a promising cell population for regenerative approaches for the IVD. NCs are involved in the formation and development of the IVDs in early life. Early work by Aguiar, *et al.* demonstrated that coculture of NCs with NP cells resulted in significant increases in proteoglycan content in both a juxtacrine and paracrine fashion.[2] Conditioned media from NCs was shown to be more effective than traditional chondrogenic media (containing Dex and TGF-β_3) in terms of inducing NP-like differentiation in MSCs.[28] Erwin, *et al.* found that NP cells cultured with NC conditioned media displayed reduced apoptosis via inhibition of caspase-3/7

and -9.[9] The NP cells also showed reduced expression of matrix catabolic factors, including MMP-3, while showing upregulation of genes associated with matrix anabolism, including aggrecan, collagen type II, and tissue inhibitor of MMP-1.

ADVANCES IN GENE THERAPY AND DELIVERY

Gene therapy aims to alter the genome of a cell population to elicit a desired molecular effect – generally the increased or decreased production of a gene product to increase/decrease the production of a protein of interest. While the direct administration of recombinant proteins has been shown to be successful, the short half-life of a protein, unknown protein–protein interactions, localized and undesirable thermodynamic phenomena, and cost are all considered disadvantages of this type of therapy.

Altering genotype of cell population, transcription/translation sustains protein synthesis

By altering the genotype of a cell population of interest, gene transcription and translation can be "reprogrammed" to invoke *sustained* protein synthesis. Diffusion properties of most delivery vehicles containing proteins (e.g., collagen sponge containing BMP-2) cause a burst release followed by a rapid decrease in protein concentration. This burst release, often times over 90% of the total protein in the first 24 hours, can cause protein concentrations to exceed the upper therapeutic threshold, rendering the therapy inefficient. Gene therapy induces a sustained, biomimetic production and release of a protein and can be tailored to adjust the magnitude of total release.

Direct in vivo gene therapy: Direct administration of infecting agent(s) to host

Direct, or *in vivo*, gene therapy involves the direct administration of the infecting agent into the host.[68] While this modality is less invasive and less costly, the efficiency and location of infection is highly variable, and the desired effect may be difficult to achieve with a single injection. Some authors have demonstrated active infection of rabbit disc cells for up to 2 years, but infection rate and duration depend on cell type and environment. Indirect, or *ex vivo*, gene therapy necessitates the isolation of host cells, subsequent *in vitro* infection of these cells, followed by surgical reimplantation of gene-modified cells. While this therapy is costly and requires multiple surgical procedures, the efficiency of infection is high and ectopic infection in undesirable anatomic locations can be avoided. However, cell viability can be lower, and cells may need to be reimplanted in a bioconductive scaffold to ensure cellular incorporation. Cells do not uptake DNA molecules by themselves, and, as such, delivery vehicles must be utilized to transfer the gene of interest into the

cell to cause efficient infection. Recombinant adenoviral vectors, adeno-associated virus (AAV), or nonviral vectors can be utilized to deliver a gene of interest.[68] The multiplicity of infection (MOI) describes the ratio of infecting agents (i.e., the vector containing the gene) to host agents (i.e., the cell) and is optimally minimized to reduce cost and avoid ectopic infection.

Safety concerns and positive results with gene therapy

There are significant safety concerns regarding the clinical *in vivo* use of gene therapy. Major hurdles to the "safe" *in vivo* use of gene therapy include ectopic transfection, patient-specific dose responses, immune reactions to viral proteins, and unknown side effects. Following the death of a seemingly healthy patient in a clinical trial of gene therapy for liver disease in 1999,[39] extensive concern triggered research studies assessing the potential side effects of gene therapy. While such studies related to IVD regeneration are limited, some authors have drawn conclusions regarding the safety of gene therapy in the spine. In an animal model, Wallach, *et al.* performed intradural injections of adenoviral TGF-β_1 to study how the spinal cord would react to ectopic adenoviral therapy.[75] They found no adverse clinical symptoms or histologic abnormalities in animals that received control (saline injections), $10^{[61]}$ plaque-forming units (PFU) of Ad-TGF-β_1, $10^{[1]}$ PFU of Ad-BMP-2, or injections of rhTGF-β_1. However, animals that received supra-therapeutic doses of Ad-TGF-β_1 developed bilateral lower extremity paralysis on postoperative day 10. Other evidence confirming the potential risks of supra-therapeutic gene therapy in the spine included the documentation of: Dural fibrosis, inflammation and fibroblastic infiltration of dural tissue.[75]

Numerous other studies, however, document the safety and efficacy of *in vivo* gene therapy. Wehling, *et al.* were the first to employ gene therapy to address IVD degeneration.[81] In their study, primary bovine chondrocytes isolated from vertebral end plates were infected with retroviruses containing bacterial β-galactosidase (LacZ) and the complementary DNA (cDNA) of human interleukin-1 receptor antagonist (IL-1Ra), the direct inhibitor of IL-1 receptors. They demonstrated that sustained release of a therapeutic protein was feasible in IVD cells; X-gal staining indicated only 1% of the total cell population was infected with the LacZ gene, and 24 ng/mL/10$^{[61]}$ cells of IL-1Ra was secreted in 48 hours.

In a later study, Le Maitre, *et al.* also utilized IL-1Ra gene therapy in attempts to inhibit molecular processes of DDD *in vitro*.[32] Degenerate human IVD cells were infected with adenoviral vectors containing the IL-1Ra gene (Ad-IL-1Ra) and showed sustained release of IL-1Ra. Infected cells were demonstrated to be IL-1-resistant, as measured by IL-1-mediated MMP-3 and MMP-13 gene expression. The same group later studied IL-1Ra gene therapy on explants of degenerate human IVDs and found that this approach inhibits IL-mediated MMP production and, consequently, matrix degradation.[33]

In early work, Nishida, *et al.* delivered adenoviral TGF-β_1 into lumbar IVDs of rabbits.[52] Culturing isolated cells after 1 postoperative week, revealed significantly more *in vitro* TGF-β_1. Immunohistochemical staining of whole IVDs also showed significantly more tissue staining positive for TGF-β_1 than control tissue. Recently, Leckie, *et al.* treated rabbits with adeno-associated virus sero-type 2 (AAV2) vectors carrying either BMP-2 or TIMP-1 following annulotomy. Serial quantitative T2 mapping, histologic analysis, and biomechanical testing were performed to compare healthy controls, defect-only, and gene therapy-treated animals. In addition, the study quantified circulating concentrations of C-telopeptide of collagen type II (CTX-II), a serum biomarker previously shown to correlate to DDD.[12,58,70] AAV2-TIMP2-treated animals exhibited a significant difference in MRI index and quantitative T2 values compared with defect-only animals at 6 weeks. Biomechanical testing demonstrated that AAV2-BMP2 and AAV2-TIMP2-treated groups had mechanical properties that were more representative of healthy animals, although these findings were not indicated to be significant. Qualitative histologic analysis showed preservation of NP area, architecture, and cellularity in treated groups compared with defect-only animals. Significantly lower concentrations of CTX-II were detected in treated animals at 12 weeks compared with defect-only animals.

Adenoviral vectors expressing numerous bone morphogenetic proteins

Zhang, *et al.* undertook a study to assess the effects of adenoviral vectors expressing numerous BMPs and the transcription factor Sox9 on ECM production of bovine NP cells.[91] Proteoglycan production, collagen production, and cellular proliferation were measured 6 days following infection. BMP-2, -3, -4, -5, -7, -8, -10, -13, -15-infected and Sox9-infected cells all exhibited significantly more proteoglycan accumulation compared with controls. BMP-2, -4, -5, -7, -8, -10, -14, -15-infected and Sox9-infected cells exhibited significantly more collagen accumulation compared with controls. Only BMP-2 and -8 increased cellular proliferation. One of the study's most significant findings was that BMP-2 and -7-transduced cells exhibited similar proteoglycan production as cells treated with 100 ng/mL rhBMP-7 for one day.

Sox9 gene delivery in human IVD cells

Sox9 gene delivery has also been studied in human IVD cells. Paul *et al.* demonstrated that Sox9 gene delivery increased Type II collagen production in primary cells from degenerated IVDs; this finding was duplicated utilizing intradiscal injections of Ad-Sox9 in rabbits.[56]

Primary human IVD cells respond favorably to adenoviral infection with growth factors

Primary human IVD cells have also been shown to respond

favorably to adenoviral infections with TGF-β_1, IGF-1, and BMP-2 in another study.[48] The study demonstrated increased proteoglycan production due to treatment with each single adenovirus. However, and more interestingly, they demonstrated the largest proteoglycan production due to infection with the triple combination adenovirus containing all three genes. This suggested that gene therapy with both morphogens and mitogens combined could exhibit synergistic effects.

Treatment of IVD cells with lim mineralization protein-1 causes increased production of proteoglycans both in vitro and in vivo

Yoon *et al.* demonstrated that adenoviral treatment of IVD cells with LIM Mineralization Protein-1 (LMP-1), an intracellular regulator known to induce the production of BMPs in osteoblasts,[46] causes increased production of proteoglycans both *in vitro* and *in vivo*.[85] The study showed that rat disc cells respond to Ad-LMP-1 in a dose-dependent manner. In addition, this process was BMP-dependent, as BMP inhibition with noggin diminished the production of sGAGs. *In vivo*, rabbit IVDs were treated with an optimized MOI of Ad-LMP-1, and qPCR of harvested NP at 3 weeks demonstrated upregulation of aggrecan, BMP-2, and BMP-7 mRNA.

MULTI-FACETED APPROACHES TO DISC REGENERATION

The three vertices of the tissue engineering triangle include scaffolds, cells, and growth factors. This section of the review will focus on summarizing *in vivo* studies that describe strategies that are dependent on two or more of the vertices to effect IVD regeneration.

In vivo performance of cell-seeded scaffolds

Scaffolds represent a multi-functional tool in tissue engineering. The scaffold structure provides mechanical support to resist biomechanical loading, while guiding new tissue growth by providing compositional and structural cues to cells. Sato, *et al.* seeded allogenic AF cells on an atelocollagen scaffold, with a honeycomb morphology.[66] Cells remained viable throughout the *in vitro* culture period, and were also able to prevent disc space collapse when transplanted into the nuclear space of the rabbit IVDs. Sakai, *et al.* also used an atelocollagen scaffold, but transplanted marrow-derived MSCs, rather than AF cells into the discs of rabbits.[65] The study demonstrated radiographic, MRI, and histologic evidence that MSC-seeded ateolocollagen scaffolds were able to restore disc height, promote proteoglycan synthesis, and preserve normal disc morphology. Huang, *et al.* utilized a tri-copolymer consisting of collagen type II, hyaluronan, and chondroitin-6-sulfate to enhance NP tissue regeneration following nucleotomy in a rabbit model.[20] Disc regeneration was markedly improved when

the tri-copolymer constructs were cultured with allogenic NP cells for one week prior to implantation. Using a canine model, Ruan, *et al.* investigated the effects of NP cell-seeded poly(lactic-co-glycolic acid) (PLGA) scaffolds on nucleotomized discs.[63] They found that the segmental stability, and disc height were preserved in the animals receiving the cell-seeded scaffolds, while the scaffold alone was insufficient to promote these "regenerative" changes.

HYDROGEL-BASED SCAFFOLDS: A PROMISING METHOD FOR NUCLEUS REGENERATION

Hydrogel-based scaffolds also represent a promising method for nucleus regeneration. These polymers can be synthesized to be responsive to environmental stimuli, and to promote cell attachment. The gel-like consistency also mimics the gelatinous NP. Henriksson *et al.* showed that human MSC's were more effective in contributing to IVD tissue regeneration when suspended in a peptidic hydrogel (PuraMatrix, BD Biosciences), versus cell culture media.[18] Cells transplanted into degenerate porcine IVDs with the peptide-based hydrogel exhibited greater *in vivo* viability, and enhanced production of type II collagen, aggrecan, and type I collagen.

In vivo growth factor delivery from scaffolds

In addition to providing a synthetic microenvironment to facilitate cell delivery to the IVD, scaffolds can also be implemented as growth factor delivery systems. Most often, the release kinetics of the growth factor are dependent on the degradation profile of the carrier system. Incorporation of growth factors within polymeric carriers delays proteolysis and also facilitates the sustained and localized release of growth factors, or drugs. Zhang, *et al.* utilized a polyethylene glycol - poly(lactic-co-glycolic acid) - polyethylene (PEG-PLGA-PEG) thermosensitive gel to deliver simvastatin intradiscally in a rat disc stab injury model.[89] They demonstrated that the slow release of simvastatin from the thermosensitive and resorbable PEG-PLGA-PEG gel "regenerated" the degenerative disc, and "slowed" the degenerative process. Nagae, *et al.* described using gelatin hydrogel microspheres for the sustained, intradiscal delivery of platelet rich-plasma, an autologous depot of growth factors and cytokines.[51] Implanted gelatin microspheres could not be discerned at 8 weeks postimplantation, but increased immunohistochemical evidence of proteoglycan production was observed. In 2009, the group used similar gelatin microsphere technology to again deliver platelet rich plasma in a degenerative rabbit disc model, but studied the effects in greater detail.[67] They demonstrated a lower number of apoptotic cells in the nucleus, and higher mRNA levels of proteoglycans and collagen type II.

ENHANCING DISC REGENERATION BY SLOWING THE DEGENERATIVE PROCESS

Catabolism and anabolism are clearly not in balance in

the setting of DDD. Promising therapeutic approaches for IVD regeneration, therefore, include downregulating the expression or directly inhibiting inflammatory and/or catabolic moieties are. *In vitro*, Kuroki, *et al.*

Table 1: Summary table of therapy techniques used in intervertebral disc regeneration

Section	Summary	Advantages	Disadvantages
Morphogen therapy	Morphogens mediate tissue development by affecting cell signaling, transcription/translation, and ultimately ECM production. Examples: Members of the TGF-β family such as TGF-β$_1$, TGF-β$_3$, BMPs, GDFs, CDMPs, etc.	• Affect stem cell fate (chondrogenic) • Cause increased production of ECM macromolecules • Positive feedback to self, other cells • Injectable • Soluble in some polymers; able to be delivered with hydrogels	• Short half-life • Cost • FDA status • Adverse dose-dependent effects • Poor control over localized concentration • Little direct effect on mitosis
Mitogen therapy	Mitogens cause intracellular signaling for increased mitosis. Mitogens are generally delivered in combination with morphogens to address both proliferation and ECM production. Examples: IGF-I, PDGF, EGF, FGF, etc.	• Cause increased cell proliferation (mitosis) • Can cause synergistic effects when combined with morphogenic therapy • Positive feedback to self, other cells • Injectable • Soluble in some polymers; able to be delivered with hydrogels	• Short half-life • Cost • FDA status • Poor control over localized concentration • Little direct effect on chondrogenesis
Cell delivery	Cell-based strategies aim to delivery healthy, proliferative, anabolic cells into a degenerative tissue. Delivered cells produce ECM molecules, morphogens, and mitogens to shift native cell phenotype towards anabolism. Examples: Mesenchymal stem cells, AF cells, NP cells, notochordal cells, articular chondrocytes, etc.	• Produce ECM • Produce morphogens, mitogens • Able to be engineered *in vitro* • Control over cell number • Purity • Can be autologous • Injectable	• Rapid decrease in viability if directly injected • Large cell # needed • Unknown fate (if stem cell) • Immune rejection (if not autologous) • Expensive • FDA status
Gene therapy	Gene therapy alters the genome of a cell, generally to increase or decrease the production of a protein. This is done by inserting a genetic sequence of interest into the existing genome with recombinant adenoviral vectors, adeno-associated virus (AAV), or nonviral vectors	• Sustained production of proteins • Production of autologous proteins • Potentially less invasive • Higher relative protein production • Tailorable kinetics	• Low infection efficiency • Risk of ectopic transfection • transfection is expensive • FDA Status
Cell-seeded scaffolds	In order to provide a bioconductive or bioinductive environment for cells, a scaffold that mimics native tissue geometry can be utilized. Scaffolds should be biocompatible, biodegradable, able to bear *in vivo* loading and produce no cytotoxic degradation products. Scaffolds can be injectable hydrogels or solid matrices requiring implantation.	• Excellent localization • Increased cellular viability • Restore load-bearing capacity • Can be injectable • Provide conduit for native ECM depositio	• Manufacturing cost • Imperfect sterilization • Cytotoxic degradation products • Large cell # needed • Potentially invasive • FDA Status
Growth-factor loaded scaffolds	Growth-factor loaded scaffolds can provide a biomimetic architecture while providing bioactive molecules such as morphogens, mitogens, or even gene therapy-related molecules. This therapy relies on the infiltration of endogenous cells rather than introducing cells as part of the therapy.	• Excellent localization • Tailorable biomechanical properties • Tailorable degradation rates • Can be injectable • Provide more sustained delivery of proteins via chemical bonds • Restore load-bearing capacity	• Manufacturing cost • Imperfect sterilization • Cytotoxic degradation products • Non-permanent protein delivery • Potentially invasive • Limited *in vivo* infiltration of cells
Anticatabolic therapy	Anti-catabolic therapy addresses the degenerative state of the IVD. By delivering molecules that directly inhibit catabolic molecules such as MMPs or by inhibiting the intracellular production of those molecules, the anabolic state of the cell is thought to rebound, shifting total tissue homeostasis.	• Some MMP inhibitors already FDA approved in other applications • Broad-spectrum inhibitors can target numerous molecules • Injectable • Can cause synergistic effects when combined with morphogens or mitogens	• FDA status • Short half-life • Adverse dose-dependent effects • Poor control over localized concentration • Numerous inhibitors may be necessary

demonstrated that canine chondrocytes cultured in 3D produced decreased concentrations of MMP-1 and MMP-3 when treated with TIMP-1 and TIMP-2. However, IL-1β-associated matrix degradation was not significantly thwarted, indicating the need to address pathways of degradation not inhibited by TIMP-1 and TIMP-2.[30]

Proteinase inhibitors utilized in gene therapy-based approaches

Proteinase inhibitors have also been employed in gene therapy-based approaches. Wallach *et al.* delivered adenovirus TIMP-1 to human primary cells isolated from degenerate IVDs.[76] They demonstrated the *in vitro* benefit of downregulating catabolic processes to allow anabolism to predominate when they showed that gene delivery of TIMP-1 increased proteoglycan production in 3D pellet cultures.

Statins act as intracellular inhibitors of MMP production and enhance BMP-2 mRNA expression

Statins are a class of drugs approved for lowering cholesterol, but their activity as intracellular inhibitors of MMP production make them an attractive therapeutic option in orthopedics. By inhibiting the enzyme 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase), MMP transcription is inhibited by downregulating the RhoA/ROCK pathway necessary for MMP production.[64] Barter, *et al.* showed that statins can reverse the production of MMP-1 and MMP-13 induced by interleukin-1 and oncostatin-M (OSM) in articular chondrocytes and articular cartilage tissue.[3] Simvastatin also specifically inhibits MMP-9 production by saphenous vein smooth muscle cells.[74]

Interestingly, statins have also been shown to increase BMP-2 mRNA in osteoblasts *in vitro* and cause mature bone formation *in vivo*.[49] Zhang, *et al.* has studied the effect of simvastatin both *in vitro*[88] and *in vivo*[89] in the context of IVD regeneration. In their *in vitro* work, they showed that simvastatin upregulated BMP-2 mRNA expression in rat IVD cells and consequently caused increased expression of type II collagen mRNA and proteoglycan. The expression of type II collagen and proteoglycan were dependent upon the expression of BMP-2, as shown by the inhibition of BMP-2 by 500 ng/mL noggin, which reduced the expression of collagen and proteoglycans. Furthermore, the study demonstrated that simvastatin directly facilitates the BMP-2 pathway by inhibiting HMG-CoA reductase; this process can be reversed by the addition of melavonate.[88] *In vivo*, the same group demonstrated the clinical applicability of the direct administration of simvastatin. They showed that the intradiscal injection of a synthetic hydrogel loaded with 5 mg/mL simvastatin following annular puncture in rats significantly upregulated BMP-2 and type II collagen mRNA while also reversing degenerative changes attributed to the annular puncture.[89]

Lactoferricin causes increased PG accumulation and downregulated catabolic processes

In a recent study, Kim and coworkers treated bovine NP cells with lactoferricin, an amphiphatic heparin-binding glycoprotein known for its antiviral, antioxidant, antioncologic, and analgesic properties,[13] and measured the expression of MMPs, ADAMTSs, TIMPs,[26] and ECM macromolecules. Results show that lactoferricin treatment caused increased PG accumulation and downregulated catabolic processes by increasing the expression of several TIMPs in a dose-dependent manner via the ERK and p38 protein kinase pathways.

TNFα-stimulated gene product TSG-6 with inter-α-inhibitor downregulate MMP activation

TNFα-stimulated gene product (TSG-6) is a 35 kD glycoprotein known to be involved in the inflammatory cascade. It has been implicated as an early marker of osteoarthritis in mice,[45] and shown to have antiinflammatory effects *in vivo*.[83] When TSG-6 forms a complex with inter-alpha-inhibitor (IαI), it is hypothesized to downregulate MMP activation, therefore thwarting the catabolic processes active in disease states such as osteoarthritis or DDD.[45] Roberts, *et al.* collected 58 herniated discs from 54 patients, and found TSG-6 and its binding protein, IαI, to be present in nearly all discs.[62] While the precise function of TSG-6, IαI, and their interaction is not fully known, future research is merited to study how these molecules can be exploited in anticatabolic treatment approaches.

Other pharmacologics being studied for their impact on IVD regeneration and/or discogenic back pain

Other pharmacologics currently utilized in other diseases have been studied in the context of IVD regeneration and/or discogenic back pain. Etanercept is an approved TNF-α inhibitor used primarily for the treatment of rheumatoid arthritis. Direct injections of TNF-α inhibitor into perispinal tissue constitute a potential therapeutic approach for treating chronic discogenic pain.[7] The oral administration of CPA-926 an esculetin prodrug, has shown efficacy in delaying the onset of disc height loss following annular puncture in rabbits.[55] The precise molecular mechanisms of these results are, however, not fully outlined and need to be studied in greater detail.

SUMMARY

Many basic science studies, utilizing molecular therapies and tissue-engineering-based strategies, are attempting to regenerate IVDs. Their aim is to treat/reverse DDD, and thereby, devise methods for treating low back pain. Although members of the TGF-β superfamily appear to

be the most promising molecules to address the thwarted anabolic processes in DDD, future research should continue to address the catabolic and inflammatory environment of the IVD. The evolution of gene therapy, namely a more extensive knowledge of ectopic transfection, optimizing transfection efficiency, and developing strategies for more localized delivery will play an important role in the future development of IVD regeneration. The continual development of cheap, easily manufactured, biocompatible, and biodegradable scaffolds able to be injected or implanted in the IVD should also be at the forefront of IVD regeneration research. Last, the rapid, intraoperative isolation of MSCs, autologous disc cells, or cartilage progenitor cells to provide cheap and biocompatible cell sources for reimplantation in combination with scaffolds and/or signaling molecules will likely be the most important area of future engineering-based research therapies.

REFERENCES

1. Abbott RD, Purmessur D, Monsey RD, Iatridis JC. Regenerative potential of TGFbeta3 + Dex and notochordal cell conditioned media on degenerated human intervertebral disc cells. J Orthop Res 2012;30:482-8.

2. Aguiar DJ, Johnson SL, Oegema TR. Notochordal cells interact with nucleus pulposus cells: Regulation of proteoglycan synthesis. Exp Cell Res 1999;246:129-37.

3. Barter MJ, Hui W, Lakey RL, Catterall JB, Cawston TE, Young DA. Lipophilic statins prevent matrix metalloproteinase-mediated cartilage collagen breakdown by inhibiting protein geranylgeranylation. Ann Rheum Dis 2010;69:2189-98.

4. Chang SC, Hoang B, Thomas J, Vukicevic S, Luyten FP, Ryba N, et al. Cartilage-derived morphogenetic proteins. New members of the transforming growth factor-beta superfamily predominantly expressed in long bones during human embryonic development. J Biol Chem 1994;269:28227-34.

5. Chen S, Emery SE, Pei M. Coculture of Synovium-Derived Stem Cells and Nucleus Pulposus Cells in Serum-Free Defined Medium With Supplementation of Transforming Growth Factor-β1: A Potential Application of Tissue-Specific Stem Cells in Disc Regeneration. Spine (Phila Pa 1976) 2009;34:1272-80.

6. Chujo T, An HS, Akeda K, Miyamoto K, Muehleman C, Attawia M, et al. Effects of Growth Differentiation Factor-5 on the Intervertebral Disc – In vitro Bovine Study and In vivo Rabbit Disc Degeneration Model Study. Spine (Phila Pa 1976) 2006;31:2909-17.

7. Cohen SP, Wenzell D, Hurley RW, Kurihara C, Buckenmaier III CC, Griffith S, et al. A double-blind, placebo-controlled, dose-response pilot study evaluating intradiscal etanercept in patients with chronic discogenic low back pain or lumbosacral radiculopathy. Anesthesiology 2007;107:99-105.

8. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8:315-7.

9. Erwin WM, Islam D, Inman RD, Fehlings MG, Tsui FW. Notochordal cells protect nucleus pulposus cells from degradation and apoptosis: Implications for the mechanisms of intervertebral disc degeneration. Arthritis Res Ther 2011;13:R215.

10. Ganey T, Hutton WC, Moseley T, Hedrick M, Meisel HJ. Intervertebral disc repair using adipose tissue-derived stem and regenerative cells: Experiments in a canine model. Spine (Phila Pa 1976) 2009;34:2297-304.

11. Ganey T, Libera J, Moos V, Alasevic O, Fritsch KG, Joerg Meisel H, et al. Disc chondrocyte transplantation in a canine model: A treatment for degenerated or damaged intervertebral disc. Spine (Phila Pa 1976) 2003;28:2609-20.

12. Garnero P, Charni N, Juillet F, Conrozier T, Vignon E. Increased urinary type II collagen helical and C telopeptide levels are independently associated with a rapidly destructive hip osteoarthritis. Ann Rheum Dis 2006;65:1639-44.

13. Gifford J, Hunter H, Vogel H. Lactoferricin. Cellular and molecular life sciences 2005;62:2588-98.

14. Gruber HE, Fisher EC, Jr., Desai B, Stasky AA, Hoelscher G, Hanley EN, Jr. Human intervertebral disc cells from the annulus: Three-dimensional culture in agarose or alginate and responsiveness to TGF-beta1. Exp Cell Res 1997;235:13-21.

15. Gruber HE, Norton HJ, Hanley Jr EN. Anti-apoptotic effects of IGF-1 and PDGF on human intervertebral disc cells in vitro. Spine (Phila Pa 1976) 2000;25:2153-7.

16. Hayes AJ, Ralphs JR. The response of foetal annulus fibrosus cells to growth factors: Modulation of matrix synthesis by TGF-beta1 and IGF-1. Histochem Cell Biol 2011;136:163-75.

17. He F, Pei M. Rejuvenation of Nucleus Pulposus Cells Using Extracellular Matrix Deposited by Synovium-Derived Stem Cells. Spine (Phila Pa 1976) 2012;37:459-69.

18. Henriksson HB, Svanvik T, Jonsson M, Hagman M, Horn M, Lindahl A, et al. Transplantation of human mesenchymal stems cells into intervertebral discs in a xenogeneic porcine model. Spine (Phila Pa 1976) 2009;34:141-8.

19. Hiyama A, Mochida J, Iwashina T, Omi H, Watanabe T, Serigano K, et al. Transplantation of mesenchymal stem cells in a canine disc degeneration model. J Orthop Res 2008;26:589-600.

20. Huang B, Zhuang Y, Li CQ, Liu LT, Zhou Y. Regeneration of the intervertebral disc with nucleus pulposus cell-seeded collagen II/hyaluronan/chondroitin-6-sulfate tri-copolymer constructs in a rabbit disc degeneration model. Spine (Phila Pa 1976) 2011;36:2252-9.

21. Im HJ, Pacione C, Chubinskaya S, van Wijnen AJ, Sun Y, Loeser RF. Inhibitory effects of insulin-like growth factor-1 and osteogenic protein-1 on fibronectin fragment-and interleukin-1β-stimulated matrix metalloproteinase-13 expression in human chondrocytes. J Biol Chem 2003;278:25386-94.

22. Imai Y, Okuma M, An HS, Nakagawa K, Yamada M, Muehleman C, et al. Restoration of disc height loss by recombinant human osteogenic protein-1 injection into intervertebral discs undergoing degeneration induced by an intradiscal injection of chondroitinase ABC. Spine (Phila Pa 1976) 2007;32:1197-205.

23. Izadpanah R, Trygg C, Patel B, Kriedt C, Dufour J, Gimble JM, et al. Biologic properties of mesenchymal stem cells derived from bone marrow and adipose tissue. J Cell Biochem 2006;99:1285-97.

24. Kim DJ, Moon SH, Kim H, Kwon UH, Park MS, Han KJ, et al. Bone morphogenetic protein-2 facilitates expression of chondrogenic, not osteogenic, phenotype of human intervertebral disc cells. Spine (Phila Pa 1976) 2003;28:2679-84.

25. Kim J, Ellman MB, An HS, van Wijnen AJ, Borgia JA, Im HJ, et al. Insulin-like growth factor 1 Synergizes with BMP7-Mediated Anabolism In Bovine Intervertebral Disc Cells. Arthritis Rheum 2010;62:3706-15.

26. Kim JS, Ellman MB, An HS, Yan D, van Wijnen AJ, Murphy G, et al. Lactoferricin mediates anabolic and anti-catabolic effects in the intervertebral disc. J Cell Physiol 2012;227:1512-20.

27. Konttinen YT, Kemppinen P, Li TF, Waris E, Pihlajamaki H, Sorsa T, et al. Transforming and epidermal growth factors in degenerated intervertebral discs. J Bone Joint Surg Br 1999;81:1058-63.

28. Korecki CL, Taboas JM, Tuan RS, Iatridis JC. Research Notochordal cell conditioned medium stimulates mesenchymal stem cell differentiation toward a young nucleus pulposus phenotype. Stem Cell Res Ther 2010;1:18.

29. Kuroda R, Usas A, Kubo S, Corsi K, Peng H, Rose T, et al. Cartilage repair using bone morphogenetic protein 4 and muscle-derived stem cells. Arthritis Rheum 2006;54:433-42.

30. Kuroki K, Cook J, Kreeger J, Tomlinson J. The effects of TIMP-1 and-2 on canine chondrocytes cultured in three-dimensional agarose culture system. Osteoarthritis Cartilage 2003;11:625-35.

31. Le Maitre CL, Baird P, Freemont AJ, Hoyland JA. An in vitro study investigating the survival and phenotype of mesenchymal stem cells following injection into nucleus pulposus tissue. Arthritis Res Ther 2010;11:20.

32. Le Maitre CL, Freemont AJ, Hoyland JA. A preliminary in vitro study into the use of IL-1Ra gene therapy for the inhibition of intervertebral disc degeneration. Int J Exp Pathol 2006;87:17-28.

33. Le Maitre CL, Hoyland JA, Freemont AJ. Interleukin-1 receptor antagonist

delivered directly and by gene therapy inhibits matrix degradation in the intact degenerate human intervertebral disc: An *in situ* zymographic and gene therapy study. Arthritis Res Ther 2007;9:83.

34. Li J, Kim KS, Park JS, Elmer WA, Hutton WC, Yoon ST. BMP-2 and CDMP-2: Stimulation of chondrocyte production of proteoglycan. J Orthop Sci 2003;8:829-35.

35. Li J, Yoon ST, Hutton WC. Effect of bone morphogenetic protein-2 (BMP-2) on matrix production, other BMPs, and BMP receptors in rat intervertebral disc cells. J Spinal Disord Tech 2004;17:423-8.

36. Li X, Leo BM, Beck G, Balian G, Anderson GD. Collagen and proteoglycan abnormalities in the GDF-5-deficient mice and molecular changes when treating disk cells with recombinant growth factor. Spine (Phila Pa 1976) 2004;29:2229-34.

37. Lin L, Zhou C, Wei X, Hou Y, Zhao L, Fu X, *et al*. Articular cartilage repair using dedifferentiated articular chondrocytes and bone morphogenetic protein 4 in a rabbit model of articular cartilage defects. Arthritis Rheum 2008;58:1067-75.

38. Lin Z, Willers C, Xu J, Zheng MH. The chondrocyte: Biology and clinical application. Tissue Eng 2006;12:1971-84.

39. Marshall E. Gene therapy death prompts review of adenovirus vector. Science 1999;286:2244-5.

40. Masuda K, Imai Y, Okuma M, Muehleman C, Nakagawa K, Akeda K, *et al*. Osteogenic protein-1 injection into a degenerated disc induces the restoration of disc height and structural changes in the rabbit anular puncture model. Spine (Phila Pa 1976) 2006;31:742-54.

41. Masuda K, Takegami K, An H, Kumano F, Chiba K, Andersson G, *et al*. Recombinant osteogenic protein-1 upregulates extracellular matrix metabolism by rabbit annulus fibrosus and nucleus pulposus cells cultured in alginate beads. J Orthop Res 2003;21:922-30.

42. Matsunaga S, Nagano S, Onishi T, Morimoto N, Suzuki S, Komiya S. Age-related changes in expression of transforming growth factor-beta and receptors in cells of intervertebral discs. J Neurosurg 2003;98:63-7.

43. McCanless JD, Cole JA, Slack SM, Bumgardner JD, Zamora PO, Haggard WO. Modeling Nucleus Pulposus Regeneration *In vitro*: Mesenchymal Stem Cells, Alginate Beads, Hypoxia, Bone Morphogenetic Protein-2, and Synthetic Peptide B2A. Spine (Phila Pa 1976) 2011;36:2275-85.

44. Miljkovic ND, Cooper GM, Marra KG. Chondrogenesis, bone morphogenetic protein-4 and mesenchymal stem cells. Osteoarthritis Cartilage 2008;16:1121-30.

45. Milner CM, Day AJ. TSG-6: A multifunctional protein associated with inflammation. J Cell Sci 2003;116:1863-73.

46. Minamide A, Boden SD, Viggeswarapu M, Hair GA, Oliver C, Titus L. Mechanism of bone formation with gene transfer of the cDNA encoding for the intracellular protein LMP-1. J Bone Joint Surg Am 2003;85:1030-9.

47. Miyamoto T, Muneta T, Tabuchi T, Matsumoto K, Saito H, Tsuji K, *et al*. Intradiscal transplantation of synovial mesenchymal stem cells prevents intervertebral disc degeneration through suppression of matrix metalloproteinase-related genes in nucleus pulposus cells in rabbits. Arthritis Res Ther 2010;12:R206.

48. Moon SH, Nishida K, Gilbertson LG, Lee HM, Kim H, Hall RA, *et al*. Biologic response of human intervertebral disc cells to gene therapy cocktail. Spine (Phila Pa 1976) 2008;33:1850-5.

49. Mundy G, Garrett R, Harris S, Chan J, Chen D, Rossini G, *et al*. Stimulation of bone formation *in vitro* and in rodents by statins. Science 1999;286:1946-9.

50. Murrell W, Sanford E, Anderberg L, Cavanagh B, Mackay-Sim A. Olfactory stem cells can be induced to express chondrogenic phenotype in a rat intervertebral disc injury model. Spine J 2009;9:585-94.

51. Nagae M, Ikeda T, Mikami Y, Hase H, Ozawa H, Matsuda KI, *et al*. Intervertebral disc regeneration using platelet-rich plasma and biodegradable gelatin hydrogel microspheres. Tissue Eng 2007;13:147-58.

52. Nishida K, Kang JD, Gilbertson L, Moon S, Suh J, Vogt M, *et al*. Modulation of the biologic activity of the rabbit intervertebral disc by gene therapy: An *in vivo* study of adenovirus-mediated transfer of the human transforming growth factor beta 1 encoding gene. Spine (Phila Pa 1976) 1999;24:2419-25.

53. Okuda S, Myoui A, Ariga K, Nakase T, Yonenobu K, Yoshikawa H. Mechanisms of age-related decline in insulin-like growth factor-I dependent proteoglycan synthesis in rat intervertebral disc cells. Spine (Phila Pa 1976) 2001;26:2421-6.

54. Okuda Sy, Nakase T, Yonenobu K, Ariga K, Meng W, Ochi T, *et al*. Age-dependent expression of transforming growth factor β1 (TGF-β1) and its receptors, and age-related stimulatory effect of TGF-β1 on proteoglycan synthesis in rat intervertebral discs (Redo typing). J Musculoskelet Res 2000;4:151-9.

55. Okuma M, An H, Nakagawa K, Akeda K, Muehleman C, Masuda K. Oral administration of esculetin prodrug inhibits intervertebral disc degeneration in the rabbit annular needle puncture model. Orthop Res Soc 2005:370.

56. Paul R, Haydon RC, Cheng H, Ishikawa A, Nenadovich N, Jiang W, *et al*. Potential use of Sox9 gene therapy for intervertebral degenerative disc disease. Spine (Phila Pa 1976) 2003;28:755-63.

57. Pifer M, Kibuule L, Maerz T, Studzinski D, Baker K, Herkowitz H. *In vitro* response of human chondrocytes to a combination of growth factors and a proteinase inhibitor. Orthopedics 2012;35:35-42.

58. Poole AR. Biologic markers and disc degeneration. J Bone Joint Surg Am 2006;88:72-5.

59. Richardson SM, Walker RV, Parker S, Rhodes NP, Hunt JA, Freemont AJ, *et al*. Intervertebral disc cell–mediated mesenchymal stem cell differentiation. Stem Cells 2006;24:707-16.

60. Risbud MV, Albert TJ, Guttapalli A, Vresilovic EJ, Hillibrand AS, Vaccaro AR, *et al*. Differentiation of mesenchymal stem cells towards a nucleus pulposus-like phenotype *in vitro*: Implications for cell-based transplantation therapy. Spine (Phila Pa 1976) 2004;29:2627-32.

61. Risbud MV, Di Martino A, Guttapalli A, Seghatoleslami R, Denaro V, Vaccaro AR, *et al*. Toward an optimum system for intervertebral disc organ culture: TGF-beta 3 enhances nucleus pulposus and anulus fibrosus survival and function through modulation of TGF-beta-R expression and ERK signaling. Spine (Phila Pa 1976) 2006;31:884-90.

62. Roberts S, Evans H, Menage J, Urban J, Bayliss M, Eisenstein S, *et al*. TNFa-stimulated gene product (TSG-6) and its binding protein, IaI, in the human intervertebral disc: New molecules for the disc. Eur Spine J 2005;14:36-42.

63. Ruan DK, Xin H, Zhang C, Wang C, Xu C, Li C, *et al*. Experimental intervertebral disc regeneration with tissue-engineered composite in a canine model. Tissue Eng Part A 2010;16:2381-9.

64. Rupérez M, Rodrigues-Díez R, Blanco-Colio LM, Sánchez-López E, Rodríguez-Vita J, Esteban V, *et al*. HMG-CoA Reductase Inhibitors Decrease Angiotensin II–Induced Vascular Fibrosis. Hypertension 2007;50:377-83.

65. Sakai D, Mochida J, Iwashina T, Hiyama A, Omi H, Imai M, *et al*. Regenerative effects of transplanting mesenchymal stem cells embedded in atelocollagen to the degenerated intervertebral disc. Biomaterials 2006;27:335-45.

66. Sato M, Asazuma T, Ishihara M, Kikuchi T, Kikuchi M, Fujikawa K. An experimental study of the regeneration of the intervertebral disc with an allograft of cultured annulus fibrosus cells using a tissue-engineering method. Spine (Phila Pa 1976) 2003;28:548-53.

67. Sawamura K, Ikeda T, Nagae M, Okamoto S, Mikami Y, Hase H, *et al*. Characterization of *in vivo* effects of platelet-rich plasma and biodegradable gelatin hydrogel microspheres on degenerated intervertebral discs. Tissue Engineering Part A 2009;15:3719-27.

68. Sobajima S, Kim J, Gilbertson L, Kang J. Gene therapy for degenerative disc disease. Gene therapy 2004;11:390-401.

69. Son BR, Marquez-Curtis LA, Kucia M, Wysoczynski M, Turner AR, Ratajczak J, *et al*. Migration of bone marrow and cord blood mesenchymal stem cells *in vitro* is regulated by stromal-derived factor-1-CXCR4 and hepatocyte growth factor-c-met axes and involves matrix metalloproteinases. Stem Cells 2006;24:1254-64.

70. Sowa G, Westrick E, Pacek C, Coelho P, Patel D, Vadala G, *et al*. *In vitro* and *in vivo* testing of a novel regulatory system for gene therapy for intervertebral disc degeneration. Spine (Phila Pa 1976) 2011;36:E623-8.

71. Takegami K, An HS, Kumano F, Chiba K, Thonar EJ, Singh K, *et al*. Osteogenic protein-1 is most effective in stimulating nucleus pulposus and annulus fibrosus cells to repair their matrix after chondroitinase ABC-induced *in vitro* chemonucleolysis. Spine J 2005;5:231-8.

72. Thompson JP, Oegema TR, Jr., Bradford DS. Stimulation of mature canine intervertebral disc by growth factors. Spine (Phila Pa 1976) 1991;16:253-60.

73. Tim Yoon S, Su Kim K, Li J, Soo Park J, Akamaru T, Elmer WA, *et al*. The effect of bone morphogenetic protein-2 on rat intervertebral disc cells *in vitro*. Spine (Phila Pa 1976) 2003;28:1773-80.

74. Turner NA, O'Regan DJ, Ball SG, Porter KE. Simvastatin inhibits

MMP-9 secretion from human saphenous vein smooth muscle cells by inhibiting the RhoA/ROCK pathway and reducing MMP-9 mRNA levels. FASEB J 2005;19:804-6.

75. Wallach CJ, Kim JS, Sobajima S, Lattermann C, Oxner WM, McFadden K, Robbins PD, Gilbertson LG, Kang JD. Safety assessment of intradiscal gene transfer: A pilot study. Spine J 2006;6:107-12.

76. Wallach CJ, Sobajima S, Watanabe Y, Kim JS, Georgescu HI, Robbins P, *et al*. Gene transfer of the catabolic inhibitor TIMP-1 increases measured proteoglycans in cells from degenerated human intervertebral discs. Spine (Phila Pa 1976) 2003;28:2331-7.

77. Walsh AJL, Bradford DS, Lotz JC. *In vivo* growth factor treatment of degenerated intervertebral discs. Spine (Phila Pa 1976) 2004;29:156-63.

78. Wang H, Kroeber M, Hanke M, Ries R, Schmid C, Poller W, *et al*. Release of active and depot GDF-5 after adenovirus-mediated overexpression stimulates rabbit and human intervertebral disc cells. J Mol Med 2004;82:126-34.

79. Watanabe K, Mochida J, Nomura T, Okuma M, Sakabe K, Seiki K. Effect of reinsertion of activated nucleus pulposus on disc degeneration: An experimental study on various types of collagen in degenerative discs. Connect Tissue Res 2003;44:104-8.

80. Watts C. Off-label use of rhBMP-2. Surg Neurol Int 2011;2:40.

81. Wehling P, Schulitz KP, Robbins PD, Evans CH, Reinecke JA. Transfer of genes to chondrocytic cells of the lumbar spine: Proposal for a treatment strategy of spinal disorders by local gene therapy. Spine (Phila Pa 1976) 1997;22:1092-7.

82. Wei A, Chung SA, Tao H, Brisby H, Lin Z, Shen B, *et al*. Differentiation of rodent bone marrow mesenchymal stem cells into intervertebral disc-like cells following coculture with rat disc tissue. Tissue Engineering Part A 2009;15:2581-93.

83. Wisniewski HG, Hua J, Poppers DM, Naime D, Vilcek J, Cronstein BN. TNF/IL-1-inducible protein TSG-6 potentiates plasmin inhibition by inter-alpha-inhibitor and exerts a strong anti-inflammatory effect *in vivo*. J Immunol 1996;156:1609-15.

84. Xin H, Zhang C, Wang D, Shi Z, Gu T, Wang C, *et al*. Tissue-engineered allograft intervertebral disc transplantation for the treatment of degenerative disc disease: Experimental study in a beagle model. Tissue Eng 2013;19:143-51.

85. Yoon ST, Park JS, Kim KS, Li J, Attallah-Wasif ES, Hutton WC, *et al*. ISSLS prize winner: LMP-1 upregulates intervertebral disc cell production of proteoglycans and BMPs *in vitro* and *in vivo*. Spine (Phila Pa 1976) 2004;29:2603-11.

86. Zeisberg EM, Tarnavski O, Zeisberg M, Dorfman AL, McMullen JR, Gustafsson E, *et al*. Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. Nat Med 2007;13:952-61.

87. Zeisberg M, Hanai J, Sugimoto H, Mammoto T, Charytan D, Strutz F, *et al*. BMP-7 counteracts TGF-beta1-induced epithelial-to-mesenchymal transition and reverses chronic renal injury. Nature medicine 2003;9:964-8.

88. Zhang H, Lin CY. Simvastatin stimulates chondrogenic phenotype of intervertebral disc cells partially through BMP-2 pathway. Spine (Phila Pa 1976) 2008;33:E525-31.

89. Zhang H, Wang L, Park JB, Park P, Yang VC, Hollister SJ, *et al*. Intradiscal injection of simvastatin retards progression of intervertebral disc degeneration induced by stab injury. Arthritis Res Ther 2009;11:R172.

90. Zhang Y, An HS, Song S, Toofanfard M, Masuda K, Andersson GB, *et al*. Growth factor osteogenic protein-1: Differing effects on cells from three distinct zones in the bovine intervertebral disc. Am J Phys Med Rehabil 2004;83:515-21.

91. Zhang Y, An HS, Thonar EJ, Chubinskaya S, He TC, Phillips FM. Comparative effects of bone morphogenetic proteins and sox9 overexpression on extracellular matrix metabolism of bovine nucleus pulposus cells. Spine (Phila Pa 1976) 2006;31:2173-9.