Chapter 62: TISSUE ENGINEERING FOR REGENERATION AND REPLACEMENT OF THE INTERVERTEBRAL DISC


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Introduction

The intervertebral disc (IVD) is the fibrocartilaginous part of a “three-joint complex” that governs motion, flexibility and weight-bearing in the spine (Figure 1). As part of this complex, the disc undergoes a lifetime of “wear and tear” that contributes to multiple IVD disorders of enormous consequence for human disability and suffering. These IVD disorders are poorly understood musculoskeletal pathologies characterized by multiple anatomic features including internal disc disruption, IVD tears and herniated disc fragments (Andersson, 1996; Bodguk, 1988; Boos et al., 2002). These anatomic features are believed to associate with nerve root compression or irritation, spinal canal narrowing (stenosis or spondylolisthesis), or loss of disc height that contribute to symptoms of low back pain, neurological deficits and disability that affect between 4 to 33% of the US population annually (Hurri & Karppinen, 2004; Praemer, Furner & Rice, 1999; Woolf & Pfleger, 2003). Like most cartilaginous tissues, the IVD is an avascular and alymphatic structure that exhibits little to no capacity for repair following injury, and experiences aging-related cell density losses that may further limit biologically-mediated repair (Urban & Roberts, 2003). The extreme mechanical demands on the IVD may also contribute to tissue failure and degeneration, due to the high magnitudes of pressure, compressive, tensile and shear stresses and strains that result from joint loading, muscle activation and spinal flexibility. As a result, strategies to intervene in the progression of IVD disorders are met with significant biological and mechanical challenges that frustrate success.

Numerous surgical procedures have been developed to treat IVD disorders, largely focused on bony fusion across the disc space to restore stability and eliminate symptomatic motions and weight-bearing. The majority of these procedures have relied on fixation of devices to inhibit motion during the bony fusion process. In cases where the pathology permits, removal of extruded IVD fragments may be performed in a procedure termed discectomy. Together these procedures comprise more than 300,000 in-patient hospitalizations annually in the USA alone (DeFrances & Podgornik, 2006). More recently, intervertebral disc replacements have been approved as an alternative therapy to bony fusion for disc-related pathologies (McAfee, 2004). The concept for these “motion preservation devices” is that maintenance of load sharing across the IVD and a small range of motion are important to provide full range of spinal motion, to maintain IVD health, and to minimize IVD height loss, facet joint degeneration, stenosis and related symptoms that may occur in progressive IVD pathology. These devices present all the risks associated with conventional joint replacements, such as subsidence, wear and failure, and are indicated for only a small portion of symptomatic pathologies. There exists a very
compelling need to develop alternative strategies to not only treat the consequences of IVD disorders, but also to detect and limit the progression of symptomatic IVD pathology.

Success with cellular therapies for articular cartilage regeneration, gene therapy, and in vitro regeneration of cartilaginous tissue, has raised hope for tissue engineered treatments for IVD disorders. Tissue-engineered approaches to IVD regeneration have been focused around implantation of cell-supplemented or acellular biomaterials that may partially replace the IVD structure, as well as delivery of cells or bioactive factors designed to promote the natural repair process. In this chapter, a review of these tissue-engineering strategies will be provided along with evaluations of their adaptation and implementation for treatment of IVD disorders.

**IVD Structure and Function**

In all structures of the IVD, the extracellular matrix provides physical and biochemical cues that regulate cell-mediated repair or breakdown in mature or aging tissues (Oegema, 1990; Oegema, 2002). The native matrix organization and interaction with the local IVD cell population will be important considerations in the design of any tissue-engineered regeneration strategy. The IVD is composed of a centrally situated and gelatinous tissue, the nucleus pulposus, that differs substantially from the more fibrocartilaginous anulus fibrosus, on the radial periphery (Figure 2). On both superior and inferior faces is a cartilaginous endplate that provides an intimate mechanical and biophysical connection between the vascularized vertebral bone and the avascular IVD. Both the anulus fibrosus, with a vascularized periphery, and the cartilaginous endplates are believed to be important routes of nutrient transport to all cells of the IVD (Nachemson et al., 1970; Urban et al., 1977). Given the very low cell density of the IVD, maintenance of both cellularity and a generous nutrient supply are often held to be critical to a successful biologically-based regenerative strategy.

The immature nucleus pulposus is highly hydrated (> 80% water) with extracellular matrix components that include randomly organized type II collagen fibers and multiple forms of negatively charged proteoglycans (Table 1, (Roughley, 2004)). A population of large and highly vacuolated cells is present in the nucleus pulposus during development and growth, with a shift towards a more chondrocyte-like cell population by age seven (Meachim & Cornah, 1970; Taylor & Twomey, 1988; Trout et al., 1982). Like all IVD regions, the nucleus pulposus contains multiple collagenous and non-collagenous proteins including types III, V, VI, and IX collagens, elastin, fibronectin and laminin
The nucleus pulposus is largely loaded in compression (Figure 3) and experiences high interstitial swelling and fluid pressures, that arise from joint loading and a high density of osmotically active, proteoglycan-associated negative charges (Urban & McMullin, 1985; Urban & McMullin, 1988). Nachemson and co-workers showed, as early as the 1960s, that this interstitial fluid pressure is greater than 0.5MPa (or ~ 5x atmospheric pressure) in the nucleus pulposus region (Andersson et al., 1982; Nachemson, 1960; Nachemson, 1992). An early loss of hydration or tearing in the nucleus pulposus (Boos et al., 2002), often detected as a loss of MR signal (Yu et al., 1989), is believed to contribute to a loss of fluid-pressurization in the IVD that may lead to herniation or stenosis with aging (Buckwalter, 1995; McNally & Adams, 1992; Schultz et al., 1982). With loss of fluid pressurization, the load distribution to the anulus fibrosus will shift from a characteristic outward “bulging” of the anulus to one of inward displacements (Adams et al., 1996; Nachemson, 1992; Panjabi et al., 1988; Shirazi-Adl, 1992). Partial or complete removal of the nucleus pulposus, occurring in some discectomy procedures, may lead to a loss of disc pressurization and disc height that will transfer loads to facet joints of the spine, increase segmental range of motion and impact overall spinal stability. Restoration of this interstitial swelling pressure in the nucleus pulposus, or restoration of MR signal intensity, is an oft-cited criterion for restoration of a healthy functioning disc.

The anulus fibrosus is a lamellar, fibrocartilaginous structure that is highly organized into distinct lamellae (Coventry et al., 1945a; Coventry, Ghormley & Kernohan, 1945b) of highly oriented, and largely type I collagen containing fiber bundles (Cassidy et al., 1989; Hickey & Hukins, 1980). Type II collagen concentration increases towards the innermost region of the anulus fibrosus, as the concentration of type I collagen is diminished. As with the nucleus pulposus, the anulus fibrosus contains proteoglycans within the collagenous extracellular matrix, although at lesser concentrations that vary from outer to inner regions of the tissue. The collagen reinforcement within the anulus fibrosus resists the tensile loads that arise during physiological joint motions, and the swelling effects, that give rise to significant anular bulging and deformation. Consequently, the anulus fibrosus has a very high stiffness in tension, with moduli that vary with the angle of orientation along the principal collagen fiber direction (Table 1, (Ebara et al., 1996; Elliott & Setton, 2001; Fujita et al., 1997; Galante, 1967; Holzapfel et al., 2005; Skaggs et al., 1994)). Cells of the anulus fibrosus originate from the mesenchyme and exhibit many characteristics of fibroblasts and chondrocytes (Bayliss & Johnstone, 1992; Oegema, 2002; Postacchini et al., 1984; Rufai et al, 1995; Urban & Roberts, 1995). These cells
are sparsely distributed in the mature IVD and exhibit very little intrinsic ability for self-repair. Disorders of the IVD that involve displacement or herniation of an IVD fragment are believed to arise from tears in the anulus fibrosus region, and discectomy procedures frequently involve removing a portion of this anulus tissue. Some tissue engineering strategies are being developed around restoration of healthy anulus fibrosus function or composition, although the complexities of anulus structure and composition make this a very challenging goal.

The hyaline cartilage endplates of the IVD are important structures that transmit and distribute loads of the spinal column to the discs. Because of their direct contact with both the anulus fibrosus and the nucleus pulposus, the endplates are believed to be an important route of nutrient transport, particularly to cells of the nucleus pulposus (Antoniou et al., 1996; Benneker et al., 2005; Roberts et al., 1996; Selard et al, 2003; Urban et al., 1977). With aging, the cartilage endplate will thin, as it undergoes mineralization and eventual replacement by bone. This mineralization of the endplate is thought to impede diffusion and nutrient flow to the disc, principally the nucleus pulposus that is lacking in an alternate short diffusion pathway. Endplate changes, such as sclerosis, fracture or modified vascularity may be detected by MRI changes (Modic et al., 1984), and are believed to contribute to symptomatic IVD degeneration (Bodguk, 1988; Kokkonen et al., 2002; Weishaupt et al., 2001). Thus, tissue-engineering strategies that preserve the health of the endplate without inducing additional damage are believed to be critical to restoring IVD function.

Biomaterials for Nucleus Pulposus Replacement

In situ hydrating polymers

The development of biomaterials and cellular therapies for tissue-engineered IVD replacement has a long history, but has not often progressed past pre-clinical evaluations. The complexity of the IVD with its three distinct sub-structures and multiple pathologies, together with very harsh loading conditions and mechanical requirements, has led to challenges for engineering tissue replacements. The concept that nucleus pulposus changes are an important contributor to IVD disorders has led to an initial focus on use of acellular biomaterials for restoration of the nucleus pulposus tissue or function (Carl et al., 2004; Di Martino et al., 2005; Klara & Ray, 2002). In this section, attention will be given to strategies developed around the concept of using “in situ hydrating”, synthetic polymers to restore nucleus pulposus hydration and consequently, IVD disc pressure and disc height. The device with the
longest clinical history is based on a co-polymeric hydrogel encased in a polyethylene fiber jacket (polyacrylonitrile and polyacrylamide, PDN™, Raymedica Inc., Figure 4). When implanted in a dessicated state, the polymers absorb water while the polyethylene jacket restricts excessive swelling of the polymer. Similar concepts have been developed based on implantation of pre-formed devices constructed from semi-hydrated poly(vinyl) alcohol (Aquarelle™, Stryker Spine Inc.), a co-polymer of poly(vinyl alcohol) (PVA) and poly(vinyl pyrrolidone) (PVP) (Thomas et al. '03), or modified poly(acrylonitrile) reinforced by a Dacron mesh ((Bertagnoli et al., 2005) NeuDisc™, Replication Medical). As shown for the PVA/PVP co-polymeric implant (Figure 5), the design goal is to exploit implant swelling pressure to restore the high compressive stiffness of the IVD, that is lost upon dehydration or denucleation of the nucleus pulposus (Joshi et al. 2006). The relevant stiffness is that measured after placement of the implant, with stiffness values reflecting both the material behaviors of the implant as well as the integration with the containing anulus fibrosus and endplates. An additional concept that has promoted development of these devices is an ability to maintain disc height.

As with many tissue replacements, there is a long list of requirements that must be satisfied for biomaterials to be used in this application, including the need to achieve: (1) “favorable mechanical stiffness” or mechanical properties matched to that of the native structure; in particular, the compressive stresses generated must not exceed the failure strength of the adjacent endplate, in order to avoid endplate fracture or subsidence of the device, IVD height loss, and associated problems; (2) integration with adjacent structures in order to promote load transfer, minimize device migration or extrusion, and restore stability for the motion segment; (3) durability, or an ability to maintain physical support over millions of cycles of loading; (4) minimal generation of wear debris, if appropriate; (5) standards of biocompatibility and must not elicit systemic, cellular or immuno-toxicity. Some of the polymeric devices for nucleus pulposus replacement have experienced device extrusion, endplate failure and endplate sclerosis after implantation. These observations are thought to relate to a mismatch in mechanical stiffness that leads to excessive endplate loading, and poor integration associated with device migration (Boyd et al., 2004; Huang et al., 2005). Controlling interstitial hydration of the polymer is a desirable feature for this class of polymers in general, as excessive swelling can cause implant stiffness and endplate overloading.

*In situ forming polymers*
Injectable polymer systems, such as polymers that will undergo a physical transition to a gel-like or solid-like form via crosslinking or thermal or pH-induced transitioning, have been evaluated for placement into a vacant nucleus pulposus space, (Bao & Yuan, 2002; Boyd et al., 2004; Temenoff & Mikos, 2000; Thomas et al, 2003). Many of the requirements for success of this strategy are similar to that described above, with additional requirements that the polymers must exhibit minimal leaching during the \textit{in situ} forming procedure, and must provide the benefit of minimally invasive insertion into the disc space. \textit{In situ} curing polyurethane is one widely studied \textit{in situ} curing polymer that has been delivered to the disc space through an inflatable polyurethane “balloon” in order to contain excessive swelling.

Two alternative injectable polymers evaluated for nucleus pulposus replacement have been developed from crosslinkable biopolymers. The BioDisc™ (Cryolife) is composed of bovine serum albumin that is crosslinked via glutaraldehyde at the time of injection to form a mechanically stiff implant. Similarly, Nucore™ (SpineWave Inc.) is a protein hydrogel developed from a silk and elastin peptide containing sequence that is crosslinked via di-isocyanate at the time of injection (Boyd et al., 2004). The crosslinking confers an extra stiffness to protein polymers that is necessary to achieve satisfactory stiffness values for a disc implant. Both implant systems have been able to maintain disc stiffness and to restore disc height when implanted, providing evidence that successful integration is being achieved upon injection. Furthermore, systems composed of native IVD polymers, such as the elastin peptide sequences, may confer some additional benefit as recognized components of the body. These approaches are promising as they move through clinical trials of implant feasibility in the current period.

**Cell-Biomaterial Constructs for IVD Regeneration**

A persistent limitation of materials-based replacements of the IVD is their biologically-mediated, or mechanically-induced failure due to the harsh loading conditions and cellular responses within the disc space. These challenges are rooted in the fact that the materials used for such applications have no capacity for self-renewal nor self-repair. This has led to increasing interest in tissue engineering methods to regenerate new IVD \textit{in situ} or to transplant IVD tissue that has been generated \textit{ex vivo}. Such strategies have been employed to augment repair of other types of cartilage, most notably articular cartilage, and meniscus, which share some features of the harsh biologic and mechanical loading environment within the IVD.
Scaffolds for Cell-Based Tissue Engineering in the IVD

As in other cartilage tissue engineering applications, a main strategy for IVD regeneration has been the inclusion of cells with biomaterials to enable production and long term maintenance of newly generated tissue. Biomaterials that enable appropriate cellular phenotypes and matrix biosynthesis, and that sometimes enable polymeric degradation or resorption, have been proposed as alternative implantable biomaterials and have been studied largely in vitro. The goals for use of these scaffolds are similar to that for other biomaterial implants, with the added requirements that the biomaterial must generate no cytotoxic or immunogenic degradation or breakdown fragments and that new matrix formation is enabled. Studies of cell-biomaterial constructs cultured in vitro have demonstrated potential for many materials (Table 2), including thermosensitive gels such as chitosan, modified chitosans and elastin-like polypeptides (Au et al., 2003; Betre et al., 2002; Mwale et al., 2005; Roughley et al., 2006). Self-associating gels composed of agarose, collagen and fibrin (Gruber et al., 2004; Peretti et al., 2006) or modified forms of these same materials, crosslinkable alginites, polyethylene glycol, poly(glycolic acids) and more (Baer et al., 2001; Burkoth & Anseth, 2000; Elisseff, 2004; Masuda et al., 2003; Mercier et al., 2004; Sontjens et al., 2006). In vitro studies with these materials are based on evaluating new matrix formation and sometimes degradation characteristics, through culturing native disc or other cell types within these matrices. Hydrogels, such as alginate and gelatin, have been used most commonly for engineering nucleus pulposus tissue, likely due to the fact that such materials reasonably approximate the gel-like properties of the native tissue. Cells of different origin, including native IVD cells, stem cells and chondrocytes, are capable of synthesizing and depositing collagen and glycosaminoglycans within these hydrogels although there is little agreement upon the targeted composition necessary to achieve a satisfactory tissue construct. This is a particularly challenging determination for the intervertebral disc as the matrix contains varying amounts of both types I and II collagen, so that the exclusive presence of type II collagen does not serve as a phenotypic matrix marker as is the case for articular cartilage.

Efforts to regenerate anulus fibrosus have also involved gels such as alginate, agarose, gelatin and collagen as well as fibers or sponges made from materials such as poly(glycolic) acid, collagen, hyaluronic acid and/or glycosaminoglycans. Oftentimes, the same scaffolds evaluated for nucleus pulposus cells are also studied with cells of the anulus fibrosus, with findings that generally illustrate the importance of cell origin in determining the resultant extracellular matrix synthesis. A common observation, however, is that cells of either origin that are maintained in a rounded morphology tend to
generate more type II collagen, characteristic of hyaline cartilage, whereas those that are cultured in an elongated morphology generate more type I collagen (Figure 6). A main challenge has been reproducing the intricate lamellar arrangement of collagen fibers that give the anulus fibrosus its unique mechanical properties and the cells their unique morphology. For this reason, the majority of anulus tissue engineered materials have been generated from scaffolds that lack any apparent lamellar microstructure. Some investigators have developed polymeric scaffolds with anisotropic features such as an oriented honeycomb structure, demonstrating production of multiple collagen types as well as proteoglycan (Sato et al., 2003). These results are indeed suggestive of the potential to engineer anisotropic collagenous tissues although no results for generating new, functional lamellar anulus tissue are known to exist to date.

Assessment of the success of IVD tissue engineering efforts is critical to moving this technology toward clinical application. Most studies have focused on generating new IVD in vitro, with few documenting tissue formation and integration in pre-clinical evaluations in vivo. To date, the most common tool for assessment of newly generated IVD tissue has been histology as a method to evaluate cell and tissue morphology. Given the structural complexity of the tissue, analysis of gene expression and extracellular matrix composition has been commonly employed to confirm the appropriate phenotypic behavior in engineered IVD. Relatively few studies have documented mechanical analysis of engineered IVD tissue, but this will undoubtedly be critical as efforts to engineer functional tissue continue. Nevertheless, these in vitro studies begin to lay the foundation for necessary and/or sufficient characteristics of a successful scaffold for nucleus pulposus replacement. From these studies, for example, it is evident that a high starting cell density, and a high degree of initial matrix stability is essential for promoting long-term construct stability and matrix accumulation to eventually restore mechanical function and swelling pressure (Wilson et al, 2002).

**Composite Cell-Biomaterial IVD Implants**

Cell-based regeneration of the IVD ex vivo is complicated by the inherent multi-component structure of the IVD, which includes two distinct regions, the anulus fibrosus and the nucleus pulposus. Given this added complication, it is not surprising that there are fewer examples of efforts to engineer in integration of the multiple components of the IVD ex vivo. This can prove to be a critical limitation as integration is needed to insure proper load transfer and to limit damaging motions during disc loading. In studies conducted by Bonassar and co-workers, IVD regeneration was attempted with a fully
integrated scaffold combining poly(glycolic co-lactic acid) as a scaffold for anulus fibrosus, and a
crosslinked alginate hydrogel as a scaffold for nucleus pulposus tissue (Figure 7) (Mizuno et al., 2004;
Mizuno et al., 2006)). Primary cells for culture within each scaffold region were derived from the
corresponding native IVD tissues, and the resultant cell-laden scaffolds were implanted subcutaneously
into athymic mice for a period of 12 or 16 weeks. Results illustrate spatially-directed matrix
regeneration with extracellular matrix that exhibited distinct morphologies and contained both collagen
and glycosaminoglycans (Figure 8). In biomechanical tests, the composite tissue engineered disc was
found to have a compressive modulus about one order of magnitude lower than that of the native tissue,
with a permeability to fluid flow that fell between values for the nucleus pulposus and anulus fibrosus.
Thus, this approach illustrated an ability for cell-laden scaffolds to regenerate extracellular matrix with
some of the functional and compositional features of the native tissue.

In another integrative tissue engineering study of note, investigators Kandel and co-workers
generated nucleus pulposus tissue *in vitro* by culturing primary bovine nucleus pulposus cells at high
density upon a calcium polyphosphate substrate, in order to mimic the natural integration of the nucleus
pulposus against the vertebral endplate (Figure 9, (Seguin et al., 2004)). A similar strategy was
employed to generate a calcified tissue-cartilaginous endplate-nucleus pulposus construct by first
generating a hyaline cartilage tissue layer upon the calcium polyphosphate substrate prior to seeding
with nucleus pulposus cells (Figure 9) (Hamilton et al., 2006)). The nucleus pulposus cells formed
tissue with a proteoglycan, but not collagen content matched to that of the native nucleus pulposus.
Importantly, functional properties in some testing configurations approached that of the native tissue.
Additional work will be required in adapting these integrative tissue engineering approaches to insure
that mechanical integration with adjacent tissues is adequate, but these studies focused on generating
integrated nucleus-endplate or nucleus-anulus are an important step in illustrating feasibility for this
approach.

**Cellular Engineering for Intervertebral Disc Regeneration**

Given the relatively small numbers of studies in the area of IVD tissue engineering, there is a
surprising amount of breadth to not only the biomaterials, but also the cell sources utilized for
regeneration. The question of cell source is particularly of note for IVD tissue engineering, given that
the availability of autologous disc cells is extremely low in the adult, and that the phenotype of cells
varies so substantially with both spatial position and with age. In animals studied for IVD tissue engineering *ex vivo*, the origin of cells in the nucleus pulposus may be partly notochordal or mesenchymal, depending on the age of the animal in question. As such, the choice of species used as a source of cells may be quite important. Due to the ease of availability, porcine and bovine cells are the most commonly used, with other efforts reporting the use of cells of canine, lapine, and ovine origin as well as human. However, cells derived from bovine tissues may be exclusively mesenchymal in origin while those derived from porcine, lapine or ovine may be largely notochordal. These phenotypic differences add an additional and unique complicating factor for investigators studying preclinical models for IVD tissue regeneration.

Given the very limited availability of native IVD cells that can be effectively harvested for tissue engineering, there has been interest in using other cells as sources for these efforts. The primary target for other sources has been mesenchymal stem cells (MSC) derived from sources such as bone marrow (Richardson et al., 2006a) and adipose tissue (Li et al., 2005). A major challenge in this approach has been the development of methods to guide the development of MSC toward phenotypes found in the IVD (see next section). This has been attempted through manipulation of the culture medium and gas conditions (Risbud et al., 2004), as well as co-culture with primary cells from the IVD (Richardson et al., 2006b). In comparison to use of adult primary disc cells derived from often pathological or degenerated IVDs, the use of autologous or other MSCs or progenitor cells may be most promising to the future of *ex vivo* tissue engineering strategies that rely upon cell supplementation.

In addition to origin, cell density is also known to have a profound effect on the efficacy of the tissue engineering process. Here there has been a great deal of variability in protocols, with studies reporting densities of delivered cells ranging from 0.2-50 x 10^6 cells/ml. While the lower end of this scale is likely more reflective of the actual cell density in nucleus pulposus tissue, the densities at the higher end of the scale are more in line with those known to be effective in generating other types of cartilage (Puelacher et al., 1994). A critical concern for disc tissue regeneration, particularly in the case of strategies that employ high cell densities, is the issue of nutrient and gas supply necessary to maintain cell viability and health. The IVD is both avascular and alymphatic, meaning that the transport of nutrients and oxygen is driven largely by diffusion from the vascularized periphery and through the vertebral endplates (Holm et al., 1981; Maroudas et al., 1975; Nachemson et al., 1970; Stairmand, Holm & Urban, 1991; Urban et al., 1977). It is noteworthy that calcification and endplate changes in the degenerating IVD can led to impaired nutrient transport which is presumably linked to
decreased cell viability (Benneker et al., 2005; Roberts et al., 1996). Thus, supplementation of scaffolds with very high cell densities may not be optimized for long-term survival in the largely hypoxic and glucose-poor, lactate-rich environment within the IVD. This concern has been expressed for cell-laden IVD scaffolds, but has not been directly addressed nor investigated as an issue in IVD tissue regeneration.

**Cellular Supplementation in the IVD**

If the local environment within the IVD is conducive to the survival of cells, direct cell supplementation without biomaterial scaffolds may hold promise for IVD repair. This strategy has been pursued by several groups, using either IVD cells, chondrocyte-like cells, or progenitor cells. In the first reported work, Nishimura and Mochida (Nishimura & Mochida, 1998) inserted nucleus pulposus cells from the rabbit following removal of nucleus tissue, and showed some beneficial effects in inhibiting the degenerative IVD changes of nucleotomy. Similar procedures have also shown the effectiveness of autologous disc cell implantation in both a sand rat model of spontaneous disc degeneration (Gruber et al., 2002) as well as a canine model of disc degeneration (Ganey et al., 2003). Furthermore, work by Nomura and co-workers has shown that supplementation with allogeneic nucleus pulposus cells did not induce any appreciable host-versus-graft rejection response, and also retarded disc degeneration in a rabbit nucleotomy model (Nomura et al., 2001). It is noteworthy that nucleus pulposus cell insertion resulted in a slightly poorer outcome than did insertion of the allograft nucleus pulposus tissue itself, indicating that inclusion of the extracellular matrix present in the allograft may be as or more important than the absolute number of cells inserted (Nomura et al., 2001).

Limitations will always exist in obtaining sufficient numbers of autologous or allogeneic disc cells from a single site, as well as concerns about impaired cellular activity for the native cells. Some studies have thus focused on using co-culture of nucleus pulposus and anulus fibrosus cells to stimulate cell metabolism prior to re-insertion (Okuma et al., 2000). These approaches were shown to be effective in delaying some degenerative features such as the loss of disc architecture following reinsertion of the “activated” cells in a rabbit model. Still, other studies have focused on delivery of cells through allograft tissues based on the concept that preservation of extracellular matrix is an equally important criteria for regeneration (Matsumoto et al., 2001a; Sato et al., 2003; Seguin et al., 2004; Yung Lee et al., 2001).
Since 2002, a prospective, controlled, multicenter study has been performed to compare autologous disc cell transplantation plus discectomy against discectomy alone (Meisel et al., 2006). The interim analysis of the first 28 patients at two years showed a clinically significant reduction of low back pain in the transplantation group compared to the discectomy group, suggesting a potential benefit of the cell transplantation strategies described above. Little is understood about the mechanism by which the cell supplementation provides this benefit, although disc hydration but not disc height was found to be higher in the patients receiving the cell transplantation compared to the discectomy group. This clinical study underscores the role of autologous cell-mediated biological factors in regulating symptoms with IVD pathology, and illustrates a potentially important role for sustaining cell viability of the IVD in inhibiting this pathology.

Clinically, the autologous reinsertion of the nucleus pulposus cells into the degenerative disc remains challenging (Evans, 2006). As a source of cells for transplantation, MSCs that can be harvested from a patient’s own bone marrow are a possible candidate (Leung et al., 2006; Risbud et al., 2004; Sakai et al., 2005). In vitro, the differentiation of MSCs into nucleus pulposus-like or chondrocyte-like cells has been shown under hypoxic and high osmotic conditions, (Risbud et al., 2004) and also with TGF-β stimulation (Steck et al., 2005). Studies have followed injection of autologous MSCs embedded in atellocollagen gel as well as direct injection of MSCs into rabbit or rat models of IVD degeneration, and observed an ability for these cells to differentiate or regenerate matrix (Crevensten et al., 2004; Sakai et al., 2005; Sakai et al., 2003). In one set of studies, transplantation of MSCs with an atellocollagen carrier into the rabbit discs effectively maintained disc height, MR signal intensity and the histological appearance of the nucleus pulposus and anulus fibrosus regions at 24 weeks after transplantation (Figure 10) (Sakai et al. 2003). Some of the positive outcomes observed for tissue regeneration in the animal models may arise from factors released from MSCs, or direct contact with MSCs, that may enhance metabolism of native disc cells (Yamamoto et al., 2004) (Richardson et al., 2006b).

Another approach to supplement cells without donor morbidity is the use of established cell lines, as shown recently following transplantation of a human nucleus pulposus cell line (Sakai et al., 2005; Sakai et al., 2004) into degenerated discs of the rabbit nucleotomy model (Iwashina et al., 2006). Despite evidence that the cell supplementation was beneficial in retarding the progression of IVD degeneration, concerns about tumorigenesis and/or carcinogenesis associated with cell lines and the consequences of the use of a recombinant SV40 adenoviral vector need to be clarified before this
strategy could be widely adopted. Given the increasing importance of MSC and other progenitor cell therapies in articular cartilage, meniscus and other cartilage regeneration strategies, expanded research on use of MSCs can be expected to comprise a significant effort in the future of IVD regeneration.

**Growth Factors and Biologics for Intervertebral Disc Regeneration**

Disc cells modulate their activity by a variety of substances, including cytokines, growth factors, enzymes and enzyme inhibitors in a paracrine and/or autocrine fashion (Masuda & An, 2004). Tissue engineering approaches to disc regeneration have been based on attempts to up-regulate important matrix proteins (e.g., aggrecan), or to down-regulate pro-inflammatory cytokines (e.g., interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α)) (Ahn et al., 2002; Burke et al., 2003; Igarashi et al., 2000; Kang et al., 1996; Le Maitre et al., 2005; Olmarker & Larsson, 1998; Seguin et al., 2005; Weiler et al., 2005) and matrix-degrading enzymes (e.g., metalloproteinases and aggrecanases) (Evans, 2006; Le Maitre et al., 2004; Roberts et al., 2000; Sztrolovics et al., 1997). Delivery of these modulating biologic agents, with and without scaffolds and/or through cell transplantation, has been the subject of many years of efforts in tissue engineering. *In vitro* studies have shown that the rate of matrix synthesis or gene expression for matrix proteins, principally proteoglycan or collagen, can be increased several-fold in IVD cells in the presence of supplemental transforming growth factor-β (TGF-β), osteogenic protein-1 (OP-1) (Imai et al., 2002; Masuda et al., 2003), bone morphogenetic proteins (e.g., BMP-2) (Kim et al., 2003; Tim Yoon et al., 2003), growth and differentiation factor-5 (GDF-5) (Chujo et al., in print; Li et al., 2004), epidermal growth factor (EGF) (Gruber et al., 1997; Thompson et al., 1991), or insulin-like growth factor-1 (IGF-1) (Osada et al., 1996). Other studies have demonstrated the potential of these growth factors, as well as platelet-derived growth factor (PDGF) to reduce cell apoptosis and to promote cell proliferation (Gruber et al., 1997; Gruber et al., 2000). Autologous platelet-rich plasma, which contains a variable mixture of growth factors, has also been shown to be an effective stimulator of cell proliferation, proteoglycan and collagen synthesis, as well as proteoglycan accumulation, when added to IVD cell cultures *in vitro* (Akeda et al., 2006). In a different approach, supplementation of IVD cell cultures with a naturally-occurring anti-inflammatory molecule, interleukin-1 receptor antagonist (rhIL-1Ra) has been shown to inhibit the downregulation of biosynthesis induced by the pro-inflammatory cytokine, IL-1 (Akeda et al., 2006, Pichika et al. 2006). This illustrates that both stimulatory factors, as well as anti-inflammatory or anti-catabolic factors, may be considered for therapeutic purposes in IVD regeneration. Overall, these *in vitro* studies have illustrated the potential
for biologics to assist in matrix regeneration through controlling both cell metabolism and cell number, and have paved the way for more recent studies evaluating these biologics \textit{in vivo}.

Protein injection into the disc space is relatively simple and practical and has been the most widely studied of all approaches for delivery of biologics for IVD regeneration. Walsh and co-workers reported the \textit{in vivo} effect of a single injection of several growth factors, including basic fibroblast growth factor (bFGF), GDF-5, IGF-1 or TGF-β, in mouse caudal discs with degeneration induced by static compression (Walsh et al., 2001). An increase in neo-matrix was observed following the injection of GDF-5, while increases in the anulus fibrosus cell population were found under the influence of IGF-1 (Walsh et al., 2002). In separate studies by Masuda and co-workers, a single intradiscal administration of rhOP-1 in normal rabbit discs \textit{in vivo} was shown to result in increased disc height and proteoglycan content in the nucleus pulposus regions in comparison to a saline injection control group (An et al., 2005). In an animal model of disc degeneration caused by needle puncture of the anulus fibrosus, an injection of rhOP-1 (100 µg/disc) restored disc height, structural change, and mechanical properties (Figure 11) (Masuda et al., 2006; Miyamoto et al., in print). The effectiveness of direct protein delivery was also confirmed in experiments using rhGDF-5, where a single injection of rhGDF-5 resulted in a restoration of disc height, and improvements in MRI and histological grading scores, in this same animal model of disc degeneration (Chujo et al., in print). These works are important for demonstrating that biologic manipulation of IVD cells \textit{in vitro} may translate to an observed effect \textit{in vivo}, and that direct protein delivery may be useful for promoting new matrix formation in the absence of cell delivery. It is noteworthy that the latter studies using rhOP-1 and rhGDF-5 also provide documentation of pre-clinical measures such as disc height, disc mechanics and MRI appearance that may be important for translating these technologies to non- or minimally-invasive clinical outcomes for patient treatment. Indeed, data for the \textit{in vitro} studies have led to an investigational new drug study that has been initiated to test the safety and effect of injections of rhOP-1 into the disc space.

\textbf{Gene Therapy for Intervertebral Disc Regeneration}

While the studies described above illustrate a range of proteins considered as possible therapies for IVD regeneration, it is important to consider the unavoidable limitations of protein delivery to the disc space. Issues such as protein half-life or solubility, the need for a proper carrier, need to preserve
mechanical environment or cell numbers, and/or the presence of inhibitors are all factors that can be expected to affect the therapeutic efficacy of protein delivery in vivo. A consideration for the use of recombinant protein therapies is also cost, as some disc pathologies and the need to inhibit disc degeneration may be chronic in nature or require multiple treatments. Gene therapy has been advocated as a therapeutic alternative for the delivery of biologics in disc regeneration (Nishida et al., 1999; Wehling et al., 1997; Yoon, 2005; Yoon et al., 2004). DNAs that encode specific proteins may be delivered into the cells by viral or non-viral transfection, with the result that these cells produce proteins to, theoretically, prolong the duration of action. The first successful attempt for *in vitro* gene transfer was reported for chondrocyte-like cells from the endplate of the IVD using a retro-viral mediated technique (Wehling et al., 1997). An *ex vivo* approach was used based on harvesting host cells, infecting these host cells *in vitro*, selecting and enriching infected cells, and finally returning these cells to the host. This approach avoids problems associated with low cell numbers and transfection efficiencies, but is both challenging and costly to perform. Nevertheless, because a decrease of cells by apoptosis or necrosis is considered to be associated with advanced disc degeneration, cell supplementation with genetically manipulated cells will continue to hold promise for disc regeneration.

Adenoviral vectors often possess high titers and infectivity and are able to infect non-dividing cells such as IVD cells. Adenoviral-mediated gene transfer to human IVD cells has been shown to be efficient and to produce transcripts across non-degenerative to degenerative cell types, using adenovirus carrying lacZ (Ad/CMV-lacZ) or luciferase “marker” genes (Ad/CMV-luciferase), as well as Sox9 (Ad/Sox9-GFP), GDF-5 and TGF-β1 adenoviral constructs (Moon et al., 2000; Wang et al., 2004; Paul et al., 2003). Yoon and co-workers also used adenoviral vector to transfer LIM mineralization protein-1 (LMP-1) to rat IVD cells *in vitro* and observed an increase of BMP-2 and BMP-7 gene expression and protein production, and proteoglycan synthesis (Yoon et al., 2004). The feasibility of using direct *in vivo* adenoviral-mediated gene transfer to disc cells has also been demonstrated using the lacZ, TGF-β, LMP-1 and Sox9 genes (Moon et al., 2000; Nishida et al., 1998; Yoon et al., 2004), finding transgene expression to be present or to exert a biological effect on biosynthesis, often for several weeks.

In addition to upregulation of anabolic factors, inhibition of catabolic processes has also been studied using gene therapy for IVD regeneration. Wallach and co-workers reported that gene transfer of the tissue inhibitor of metalloproteinase-1 (TIMP-1), an inhibitor of catabolic enzymes, can increase proteoglycan accumulation within pellet cultures of human IVD cells (Wallach et al., 2003). LeMaitre
recently reported that human disc cells infected with Ad-IL-1 receptor antagonist (Ad/IL-1Ra) were resistant to IL-1 (Le Maitre et al., 2006). When *in vitro* infected cells were injected into disc explants *in vitro*, IL-1 receptor antagonist protein expression was also increased and maintained for the two-week time period investigated.

There are significant concerns about adenoviral vector use clinically, however, that may include significant toxicity when used in spinal applications (Driesse et al., 2000; Wallach et al., 2006). These concerns have led investigators to begin consideration of adeno-associated viral vectors (Lattermann et al., 2005) and baculoviral vectors, the latter which may be non-toxic (Liu et al., 2006). Both approaches may provide safe alternatives for future disc therapies, although much work remains. Also to avoid safety concerns found with viral gene transfer, several non-viral methods for direct gene transfer to cells have been proposed. Preliminary reports using microbubble-enhanced ultrasound gene therapy (Nishida et al., 2006) and a “gene-gun” method (Chang et al., 2000; Matsumoto et al., 2001b) have shown that introduction of a marker or growth factor gene could be accomplished and provide sustained gene expression without need for viral vectors. Transfection efficacy with non-viral means is lower, however, than that in viral transfection and further investigation will be needed to apply these in a clinical setting. Nevertheless, both viral and non-viral transfection methods have pros and cons.

Safety and immunological reactions, as well as the control of expression in viral-mediated gene therapy, are potential problems. The comparatively low immunologic exposure of the healthy disc and its low cellularity seem to suggest that safety with gene delivery of therapeutic agents is a lesser concern in the IVD. Cells are needed to transduce the biological effect, however, so that transplantation of *ex vivo* transfected cells may be an important part of the future potential for gene therapy in the IVD. During this time, work continues on identification of broader and diverse molecular targets that can be useful for gene delivery in the treatment of IVD regeneration.

**Concluding Remarks**

Efforts to regenerate and replace the tissues of the intervertebral disc have virtually exploded over the last two decades, although the field remains in its infancy. The complexity of the diverse degenerative and pathological processes that affect the IVD, as well as the intrinsic complexity of the heterogeneous disc structures, demands that multiple strategies be developed for treatment of the IVD. Partial IVD replacements using acellular, pre-formed or *in situ* formed biomaterials have the longest history of development and are important for defining procedural outcomes that will be relevant to
long-term functional success. Development of strategies use cells, biologics, or gene therapy is often focused upon restoration of a single tissue source, such as nucleus pulposus or anulus fibrosus, and with or without biomaterial scaffolds. Only a few tissue engineering solutions have been proposed to integrate two dissimilar tissues in the repair process, and additional work to promote integration amongst native, neo-generated and implanted tissues will be critical to restoring IVD function. Many of the identified strategies derive largely from knowledge gained in cartilage tissue engineering, although the differing cellular, functional and structural requirements of the IVD suggest that custom approaches are needed. Advances in IVD cell biology are needed to enable the identification of novel therapeutic targets, to select for classes of biomaterials, and to suggest appropriate drug delivery strategies, as disc cell phenotype, cell-biomaterial interactions, and the biology of aging for these cells, are still poorly understood. While a diverse array of molecules, cell sources and materials are suggested as appropriate for IVD regeneration, additional work is needed to reveal some common and unique themes in human IVD cell responses that focus research on IVD specific strategies. Currently underway clinical trials of autologous cell therapies or autologous protein products will pave the way for later generations of cellular and biologic-based therapies, as they are expected to illustrate the unique challenges of treating the pathologic and aged human IVD. The next decade promises great advances in the translation of basic and applied sciences to the clinical treatment of IVD regeneration and replacement.
Table 1. Ranges reported for compositional features and mechanical properties for nucleus pulposus and anulus fibrosus tissue regions of the non-degenerate intervertebral disc. Both composition and mechanical properties of the disc vary substantially with region and with degeneration. Additional mechanical features important to tissue function, such as failure strength, are not shown here.

reported also as peak hydrostatic pressures, or swelling pressures.

<table>
<thead>
<tr>
<th></th>
<th>water (% wt)</th>
<th>collagen (% dry wt)</th>
<th>proteoglycan (% dry wt)</th>
<th>other proteins (% dry wt)</th>
<th>compressive modulus (MPa)</th>
<th>shear modulus (MPa)</th>
<th>tensile modulus (MPa)</th>
<th>interstitial pressure (MPa)</th>
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<tr>
<td>nucleus pulposus</td>
<td>70-90</td>
<td>15-35</td>
<td>25-60</td>
<td>20-45</td>
<td>0.5-1.5</td>
<td>0.005-0.01</td>
<td>NA</td>
<td>0.5-3.0</td>
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<tr>
<td>anulus fibrosus</td>
<td>65-80</td>
<td>10-65</td>
<td>10-35</td>
<td>15-40</td>
<td>0.5-1.5</td>
<td>0.08-0.40</td>
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- types I, II, VI, IX, XI
- aggrecan, decorin, biglycan, fibromodulin, versican and more

- reported also as peak hydrostatic pressures, or swelling pressures.
<table>
<thead>
<tr>
<th>material</th>
<th>cell type</th>
<th>cell source</th>
<th>cell density</th>
<th>in vitro/in vivo</th>
<th>assessment</th>
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<tr>
<td>PCL (1)</td>
<td>AF, NP</td>
<td>bovine</td>
<td>$5 \times 10^3$/cm$^2$</td>
<td>in vitro</td>
<td>Histology, SEM, gene expression</td>
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<td>Alginate (2)</td>
<td>AF, NP</td>
<td>porcine</td>
<td>$4 \times 10^6$/ml</td>
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<td>DNA, ECM analysis</td>
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<td>Gelatin/C6S/HA (3,4)</td>
<td>NP</td>
<td>human</td>
<td>$20 \times 10^6$/ml</td>
<td>in vitro</td>
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<td>CPP (5,6)</td>
<td>NP</td>
<td>bovine</td>
<td>$16 \times 10^6$/cm$^2$</td>
<td>in vitro</td>
<td>Histology, mechanical analysis</td>
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<td>Agarose, collagen (7)</td>
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<td>human</td>
<td>$0.2 \times 10^6$/ml</td>
<td>in vitro</td>
<td>Histology, ECM analysis</td>
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<td>Gelatin, PLA (8)</td>
<td>NP</td>
<td>porcine</td>
<td>$5 \times 10^6$/ml</td>
<td>in vitro</td>
<td>Histology, ECM analysis, gene expression</td>
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<tr>
<td>Collagen/GAG (9,10)</td>
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<td>canine</td>
<td>$40 \times 10^6$/ml</td>
<td>in vitro</td>
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<td>Collagen/HA (11)</td>
<td>AF, NP</td>
<td>bovine</td>
<td>$13 \times 10^6$/ml</td>
<td>in vitro</td>
<td>Histology, DNA, ECM analysis, gene expression</td>
</tr>
<tr>
<td>Alginate (12,13,14)</td>
<td>AF, NP</td>
<td>porcine</td>
<td>$1-10 \times 10^6$/ml</td>
<td>in vitro</td>
<td>Histology, gene expression, mechanical analysis</td>
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<tr>
<td>Collagen (15)</td>
<td>AF</td>
<td>lapine</td>
<td>$10 \times 10^6$/ml</td>
<td>in vivo</td>
<td>Histology</td>
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<tr>
<td>PGA, alginate (16,17)</td>
<td>AF, NP</td>
<td>ovine</td>
<td>$25-50 \times 10^6$/ml</td>
<td>in vivo</td>
<td>Histology, DNA, ECM analysis, mechanical analysis</td>
</tr>
</tbody>
</table>

Table 2: Representative overview of studies involving cell-scaffold based tissue engineering of IVD using cells obtained from native tissues only. Abbreviations: PCL (polycaprolactone); C6S (chondroitin-6-sulfate); HA (hyaluronan); CPP (calcium polyphosphate); PLA (polylactic acid); GAG (glycosaminoglycan); PGA (polyglycolic acid); AF (anulus fibrosus); NP (nucleus pulposus); SEM (scanning electron microscopy); DNA (deoxyribonucleic acid); ECM (extracellular matrix).

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