Minireview *Highlight Article*

Animal models of regenerative medicine for biological treatment approaches of degenerative disc diseases

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Impact statement

Degenerative disc disease (DDD) in human is a complex and multifactorial process involving for example cellular senescence, mechanical stresses, nutritional deprivation, and genetic predisposition. The complex and multifactorial process has been studied in different animal models. Little progress has been achieved from biological treatment approaches in animal models to clinical trials. Nevertheless, animal models play increasingly significant roles in treatment approaches of DDD. Therefore, we provide here an up-to-date overview of biological treatment strategies in degenerative DDD using small and large animal models from mouse to non-human primate. This review may help the specification of our knowledge about the pathophysiology and treatment modalities of DDD, in order to develop new and hopeful therapy approaches for future clinical applications.

Abstract

Degenerative disc disease (DDD) is a painful, chronic and progressive disease, which is characterized by inflammation, structural and biological deterioration of the intervertebral disc (IVD) tissues. DDD is specified as cell-, age-, and genetic-dependent degenerative process that can be accelerated by environmental factors. It is one of the major causes of chronic back pain and disability affecting millions of people globally. Current treatment options, such as physical rehabilitation, pain management, and surgical intervention, can provide only temporary pain relief. Different animal models have been used to study the process of IVD degeneration and develop therapeutic options that may restore the structure and function of degenerative discs. Several research works have depicted considerable progress in understanding the biological basis of disc degeneration and the therapeutic potentials of cell transplantation, gene therapy, applications of supporting biomaterials and bioactive factors, or a combination thereof. Since animal models play increasingly significant roles in treatment approaches of DDD, we conducted an electronic database search on Medline through June 2020 to identify, compare, and discuss publications regarding biological therapeutic approaches of DDD that based on intradiscal treatment strategies. We provide an up-to-date overview of biological treatment strategies in animal models including mouse, rat, rabbit, porcine, bovine, ovine, caprine, canine, and primate models. Although no animal model could profoundly reproduce the clinical conditions in humans; animal models have played important roles in specifying our knowledge about the pathophysiology of DDD. They are crucial for developing new therapy approaches for clinical applications.

Keywords: Degenerative disc disorders, chronic back pain, animal models of disc degeneration, biological treatment approaches, intradiscal regenerative strategies, biological restoration of intervertebral disc tissues

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Introduction

Structure and function of intervertebral discs

Intervertebral discs (IVDs) separate adjacent vertebra in the spinal column that provides the base body support and protects the spinal cord. IVDs are fibrocartilaginous articulations that are made up of the soft jelly-like inner core, the nucleus pulposus (NP), and the outer fibrous ring, the anulus fibrosus (AF), which are interfaced with the vertebra by the vertebral end plates (VEPs).[1–3] The highly hydrated NP is mainly composed of hyaluronan and proteoglycan that are entangled in a network of fine type II collagen; whereas the AF is mainly composed of the radially aligned type I collagen fibers. The VEP is made of the cartilaginous layer, which is fused with the IVD as well as with the bony endplate layer attached to the vertebra. The permeability of the VEP allows the diffusion of water, nutrition, and oxygen to the predominantly avascular IVDs.[1] IVDs have, due to their particular load-bearing organization, the ability to withstand biomechanical stress and to provide tensile strength, stability, and flexibility of the spine by spreading load evenly on the vertebral bodies and resisting spinal

compression. During loading the swelling of the hydrated NP exerts a radial and axial hydrostatic pressure on AF and VEP, while AF and VEP resist and distribute the compressive loading on the adjacent vertebral bodies.[2,3] IVDs have aneural environment, as the sinovertebral nerves (the neural supply) emerge from the dorsal root ganglion and extend only to the outer layer of anulus. The avascular environment of IVDs with low supply of nutrient and oxygen promotes degeneration and impairs the regeneration process of degenerative IVDs, especially in the center of the IVDs.[4–9] The structure of human vertebra is illustrated in Figure 1.

Degenerative intervertebral disc disease

IVD degeneration is a normal process of aging that can be accelerated by different environmental and biological factors. These include for example (a) unfavorable mechanical stresses that damage the structure of the extracellular matrix (ECM), (b) genetic risk factors that contribute to IVD degeneration by affecting the biomechanical strength of the ECM and lead to low tolerance for mechanical stress, and (c) calcification of the vertebral endplates (VEPs) along with atherosclerotic obstruction of the lumbar arteries that impair nutrient supply to IVDs and affect the intradiscal cell viability and ECM synthesis.[10–15]

IVD degeneration associated with pain, referred to as degenerative disc disease (DDD), can considerably affect patients' quality of life with a substantial socioeconomic impact. It can impel disabilities, social isolations, and cause huge socioeconomic cost in terms of medication, disability benefits, and lost productivity.[16–18] IVD degeneration leading to backpain is caused by structural failure of the disc affecting the biomechanics of the spine and it can be aggravated by inflammatory ingrowth of blood vessels and nerves into the inner layers of the torn IVDs[19,20] (Figure 2). Current treatment modalities of DDD are based on: (a) conservative measures as bed rest, non-steroidal anti-inflammatory medication, analgesia, and physical therapy, (b) interventional measures as epidural steroid injections and ablation techniques, and (c) surgical procedures as discectomy, fusion, and artificial disc replacement. Those treatment modalities are restricted on or put the emphasis on the management of DDD symptoms. They do not address options of restoring the structural or biological deterioration of the discs as the underlying problem.[21,22] Therefore, biological treatment approaches of DDD that may decelerate disc degradation or restore the structure and function of IVDs have gained increasingly greater consideration over the last years.

Biological treatment approaches of degenerative disc disease

Various *in vivo* studies have demonstrated promising biological strategies that may decelerate disc degeneration or restore the structure and function of the discs. The different biological strategies include cell transplantation, gene therapy, applications of bioactive factors and bioscaffolds, or a combination thereof.[23–27] Imbalances between proinflammatory (catabolic) cytokines and anabolic factors within IVDs are the major aggravating factors of IVD degeneration. The imbalances could lead to loss of proteoglycan content, loss of structural organization, and loss of hydration and affect the load-bearing ability of the IVDs. Enhanced expression of inflammatory cytokines, such as tumor necrosis factor alfa (TNFα), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-17, and cell-surface heparan sulfate, can be induced by initiating factors of IVD degeneration. The inflammatory cytokines have been shown to enhance the expression levels of catabolic enzymes, such as disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS4, ADAMTS5), matrix metalloproteinases (MMP1, MMP3), and metalloproteinase inhibitors (TIMP1, TIMP3), which in turn substantially affect the matrix turnover within the IVDs. Loss of matrix turnover can result in reduced disc height, anulus tears,

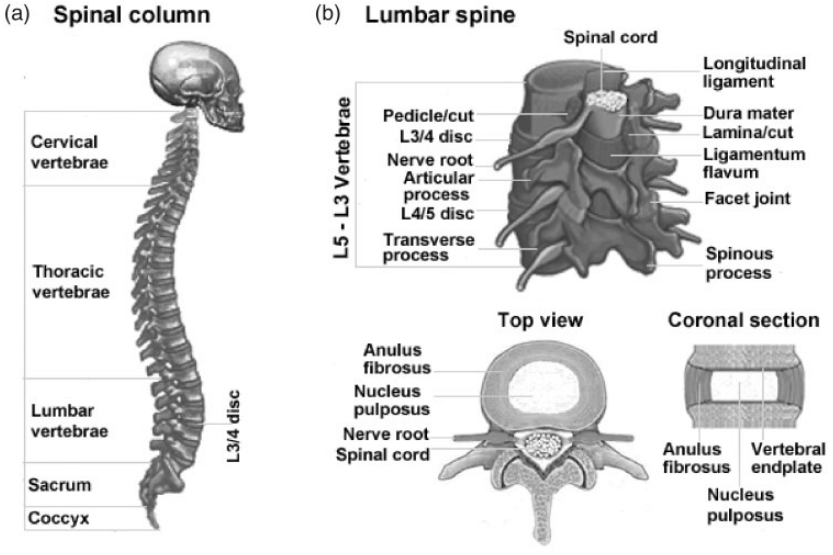

Figure 1. Structure of human vertebra. (a) Spinal column illustrating the vertebral bodies separated by intervertebral discs. (b) Lumbar spine (L3–L5) with L3/L4 and L4/L5 discs along with nerve roots and the spinal canal enclosing the spinal cord (© Mern DS).

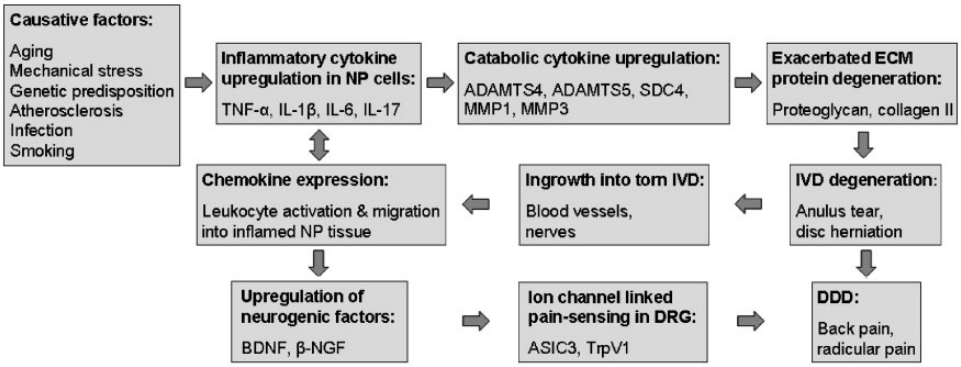

Figure 2. The course of degenerative disc disease. Causative factors such as aging, mechanical stress, genetic, etc. upregulate the expression of inflammatory cytokines in NPCs. The inflammatory environment leads to the overexpression of catabolic genes that degrade ECM proteins in NP tissue. Continuous degradation of the ECM proteins results in mechanical instability, disintegration of the disc tissue, annular tears, and disc herniation. Ingrowth of blood vessels and nerves into the inner layers of the torn disc releasing chemokines can promote activation and infiltration of leukocytes. These in turn amplify the inflammatory response and reinforce degeneration with back and radicular pain causing DDD (© Mern DS).

and collapsed disc space that may sensitize the spinal nerves and trigger radicular pain[28–34] (Figure 2). Several studies have attempted to deregulate the imbalances by downregulating the inflammatory and catabolic factors or by upregulating the anti-catabolic and anabolic factors that inhibit the degradation of ECM or boost its synthesis within IVDs. Moreover, in various *in vivo* studies intradiscal upregulation of matrix anabolism has been attempted using cell transplantation combined with growth factors, such as transforming growth factor beta (TGF-β1, TGF-β3), insulin-like growth factor 1 (IGF-1), and bone morphogenetic proteins (BMP2, BMP4, BMP6, BMP7).[27,35–39]

Different animal models of disc degeneration have been used for regenerative approaches of cell transplantation and gene therapy with applications of bioactive factors and bioscaffolds.[40–156] Autologous or allogeneic transplantations of stem cells or chondrocytes with or without genetic modifications of the cells have been often combined with the use of biodegradable scaffolds.[40–52,68–82,107–114,124–127,136–140] In addition, transplantations of human stem cells or human chondrocytes have been performed in some animal models of disc degeneration.[40,41,44,52,77,82,112] Stem cells and chondrocytes can be harvested, *in vitro* expanded, genetically modified and injected intradiscally for regeneration attempts of degenerated discs. The genetic modification of the cells can include inhibition of inflammatory and catabolic genes or overexpression of anti-catabolic and anabolic genes, or a combination thereof. The combination of both approaches can be more important to achieve additive outcomes, namely inhibition of ECM degradation along with its restoration. However, gene therapeutic approaches depend not only on the identification of the relevant target genes but also on the optimal selection and application of gene delivery systems. *In vivo* gene therapeutic approaches have been presented in different animal models of disc degeneration by using retrovirus, lentivirus (LV), adenovirus, and adeno-associated virus (AAV) gene delivery vectors.[53–58,83–89,107,110,136,139,148] Viral vector-based gene therapeutic treatments of DDD can be performed: (I) by direct injection of viral vectors into degenerative IVDs leading to the expression of the therapeutic gene by disc cells transduced within the IVDs; (II) by injection of genetically modified cells into degenerative IVDs that are able to express the therapeutic gene *in vivo*; and (III) by transplanting bioscaffolds that are seeded with genetically modified cells enabling the *in vivo* expression of the therapeutic gene. Biological treatment approaches of DDD are schematically illustrated in Figure 3.

Analyses of animal models in biological treatment approaches of DDD

Various animal models belonging to different species, which have been found to reproduce the degenerative changes in human IVDs, have been used for biological treatment approaches of DDD. Therefore, we conducted an electronic database search on Medline through May 2020 to identify, compare, and discuss findings associated with intradiscal biological treatment approaches of DDD in various animal models of different species, including mouse, rat, rabbit, porcine, bovine, ovine, caprine, canine, and non-human primate models. Inclusion criteria for selection of the articles were studies that based on intradiscal applications of biological factors in an attempt to achieve IVD regeneration in animal models. Exclusion criteria were the use of non-biological factors or non-intradiscal application routes of biological factors, such as intravenous, subcutaneous, intraperitoneal, or oral routes. Searches were performed in MEDLINE by all authors of the manuscript for *in vivo* biological treatment approaches of IVD by using multiple search terms such as: intradiscal treatment and IVD regeneration, IVD degeneration and cell transplantation, gene therapy and IVD regeneration, biomaterials and IVD repair, etc.

Rodent models for biological treatment approaches of DDD

Primarily rats and rarely mice have been used for biological treatment approaches of DDD. They are considered to be desirable models, since these models have the advantages of giving advanced options for genetic manipulation as well as easiness of use and husbandry with low costs. In addition, they provide readily accessible tail discs for easy induction of mechanical trauma, asymmetrical

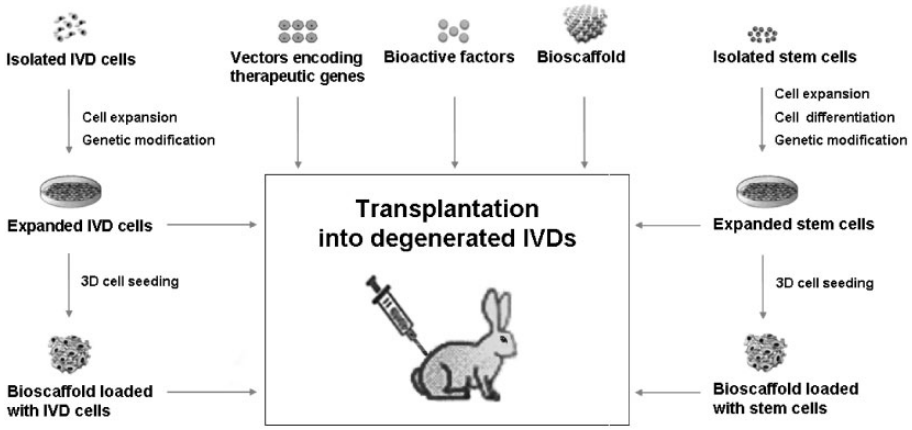

Figure 3. Schematic illustration of procedures for biological treatment approaches of degenerative disc disease. Tissue isolated and expanded stem cells and chondrocytes can be used for autologous or allogeneic transplantations with or without genetical modification or loaded on bioscaffolds. Beyond that, therapeutic vectors, bioactive factors, and bioscaffolds can be injected into degenerative IVDs for inhibition of disc degeneration or boosting of its regeneration (© Mern DS).

Table 1. Cell transplantation approaches in rodent models of disc degeneration.

Animal model	Transplanted cell/ Gene modification	Implanted biomaterial	Disc regeneration effect	Ref.
Rat: Caudal disc puncture injury	hMSC/AdV-GFP	Hyaluronan gel (15%)	Increased disc height, maintained cell viability, and proliferation	40
Rat: Caudal disc puncture injury	BCP-hMSC-bNPCs	FS carrier	High cell retention, increased disc height, and proteoglycan content	41
Rat: Caudal disc puncture injury	Allogeneic hMSC/AdV-GFP		Improved cell population, disc high, collagen II, and aggrecan levels	42
Rat: Caudal disc puncture injury	hADSC	PLGA-TGFβ3	Enhanced disc height, MRI signal intensity, and proteoglycan content	43
Rat: Caudal disc puncture injury	hADSCs-NP-lC	Heparin-PEAD- GDF5	Improved disc height, NP cell proliferation, and ECM synthesis	44
Rat: Caudal disc puncture injury	Autologous iPSC-NP-lC	Polymeric GM-GDF5	Enhanced disc height, NP cell proliferation, and ECM formation	45
Rat: Lumbar disc puncture injury	Allogeneic stem cell from the olfactory mucosa / RV-GFP		Successful engraftment of donor cells expressing collagen II and chondroitin sulfate proteoglycan	46
Rat: Caudal disc puncture injury	Allogeneic NPMSCs / LV-mCherry	RGD-peptide-hydrogel	Decreased cell apoptosis rate with restored ECM content and structure	47
Rat: Caudal disc puncture injury	Ovine AF cells	Riboflavin-HDC gel	Accelerated AF reparative sealing, better disc height, and NP hydration	48–50
Rat: Lumbar disc puncture injury	Allogeneic NPC/AdV-Luc Allogeneic ADSC/AdV-Luc Allogeneic MSC/AdV-Luc	Alginate bead Alginate bead Alginate bead	Highest noninvasive track of AdV-Luc gene expression within IVDs after injection with NPCs	51
Mouse: Biglycan knockout ($Bgn^{-/0}$)	hADSCs		High survival of hADSCs able to express biglycan with improved MRI index and aggrecan level	52

hMSC: human mesenchymal stem cell; AdV: adenoviral vector; GFP: green fluorescent protein; BCP: bilaminar cocultured pellets; bNPC: bovine nucleus pulposus cell; hADSCs: human adipose-derived stem cells; NP-lC: nucleus pulposus-like cell; iPSCs: induced pluripotent stem cells; RV: retroviral vector; NPMSCs: nucleus pulposus-derived mesenchymal stem cells; LV: lentiviral vector; NP: nucleus pulposus; AF: anulus fibrosus; Luc: luciferase; MRI: magnetic resonance imaging; ECM: extracellular matrix; IVD: intervertebral disc.
Stem cell- or chondrocyte-based treatments of degenerative discs with or without gene modification or in combination with biomaterials have been attempted.

compression, and easy administration of bioactive agents. However, the small size and geometry of their discs that reduces the nutritional challenge, the different mechanical loading, and the persistent notochordal cells encompass the disadvantages of those models to mimic the degenerative or regenerative processes in human IVDs. Nonetheless, different regenerative treatment approaches have been developed using rodent models of disc degeneration.

Cell-based *in vivo* therapy approaches in rodent models of disc degeneration. Different rat models have been used for stem cell or chondrocyte-based treatments of degenerative discs with or without gene modification and mostly in combination with appropriate biomaterials that may maintain the viability and function of the transplanted cells[40–52] (Table 1). Human mesenchymal stem cells (hMSCs) harvested from bone marrow specimens of iliac crests have been tested as

cell-based therapies in a rat tail models of IVD degeneration.[40–42] To explore the feasibility of MSC delivery, their retention, and survival in NP, rat coccygeal discs were injected with 15% hyaluronan gel that was seeded with MSCs expressing green fluorescent protein (GFP). After injection discs were weekly assessed for 28 days using radiography and histology. Injected MSCs could maintain viability and proliferate within the rat IVDs with 100% viability over 28 days. Moreover, a trend of increased disc height was observed as compared to blank gel injection.[40] In another study, the direct treatment of denucleated rat coccygeal discs was tested with bilaminar cocultured pellets (BCPs) of human MSCs and bovine NP cells (bNPCs) that were loaded in fibrin sealant (FS) carrier. Between two and five weeks after injury, BCP treatment increased disc height over time with high cell retention as compared to control discs that were treated only with MSCs, NPCs, or FS.[41] Since hypoxic and hypertonic microenvironment in IVDs can decrease the treatment effects of cell transplantation, hypoxic-preconditioned rat MSCs that express GFP (H-MSC-GFP) were tested in needle-punctured coccygeal discs (Co5/Co6 and Co6/Co7). Hypoxic-precondition was generated by culturing of MSCs in a complete medium with cobalt chloride ($CoCl_2$) at the concentration up to 0.3 mmol/L for 48 h. Cell transplantation (2×10^4 H-MSC-GFP dissolved in 2.0 μL PBS) was performed at two weeks after initial surgery, and radiographs were taken every two weeks till six weeks. The survival, migration, and differentiation of transplanted MSCs as well as matrix protein expression were directly evaluated in freshly harvested discs. Treatments with H-MSC-GFP showed extensively increased disc height, higher cell population in NP and AF regions, and enhanced collagen II and aggrecan expressions as compared to discs treated with cells without hypoxic-preconditioning (MSC-GFP). The results indicated that hypoxic-preconditioning enhances the capacity of MSCs to repair degenerated discs.[42]

Human adipose-derived stem cells (hADSCs), induced pluripotent stem cells (iPSCs), and multipotent stem cells from the olfactory mucosa have been used for therapeutic approaches in rat models of disc degeneration.[43–46] Human ADSCs that were seeded in nano-structured three-dimensional poly-lactide-co-glycolide (PLGA) microspheres, which could dually release dexamethasone and TGF-β3, were used in needle-punctured rat coccygeal discs (Co7/Co8 and Co8/Co9). Discs were injected with 2 μL of PLGA microspheres (5 mg/mL) containing ADSCs (1×10^6 cells/mL; PMA group) at two weeks after puncture injury, and weekly analyzed by magnetic resonance imaging (MRI), radiographical, histological, immunohistochemical, and biochemical methods till 24 weeks after injection. The PMA group showed improved disc height values and MRI signal intensities with proteoglycan accumulation as compared to punctured discs treated only with 2 μL PLGA microspheres (PM groups) or untreated punctured discs (DC group).[43] Furthermore, ADSCs, which were seeded on a polymer heparin-PEAD (heparin-poly(ethylene arginylaspartate diglyceride)) and differentiated into an NP-like phenotype using growth and differentiation factor-5 (GDF5), were therapeutically tested in the same manner as above in needle-punctured rat coccygeal discs. Sustainable release of GDF5, enhanced disc height, increased water content, and improved NPC proliferation were obtained with enhanced ECM synthesis.[44] In the same manner, the regenerative potentials of autologous iPSCs, which were seeded on a polymeric gelatin microsphere (GM) and differentiated into NP-like cells (NP-LCs) using GDF5, were examined in needle-punctured rat coccygeal discs. Similar results with sustainable GDF5 release, enhanced disc height, increased water content, and improved NP cell (NPC) proliferation with enhanced ECM formation were demonstrated.[45] In addition, the therapeutic potentials of human multipotent stem cells from the olfactory mucosa were investigated in view of disc repair after needle aspiration of the NP in lumbar discs of rats. Two weeks after injury discs were injected with undifferentiated olfactory neurosphere-derived cells (1×10^4 cells/μL) that were transduced with retroviral vectors encoding for GFP (RV-GFP). Immunocytochemical and microscopical analyses of the disc tissues at three weeks after injection showed the survival of GFP-positive donor-derived cells within the NP of transplanted discs. They were able to express NP distinctive markers such as collagen II and chondroitin sulfate proteoglycan, indicating the potential of stem cells from the olfactory mucosa to repair injured IVDs.[46]

Chondrocytes from NP and annulus fibrosus (AF) have been used usually in combination with biomaterials to restore the proteoglycan content in the NP or to repair anular defects that could prevent re-herniation and further degeneration.[47–51] NPCs from rats expressing mCherry that were cultivated on RGD-peptide modified polysaccharide hydrogel in culture medium of MSCs (referred to as nucleus pulposus-derived mesenchymal stem cells: NPMSCs) were used for regeneration approaches in needle-punctured coccygeal discs (Co5/Co6). Two weeks after puncture, the hydrogel-loaded NPMSCs were injected into the discs and these were analyzed by MRI, radiographical, and histological analyses at one, two, four, and eight-weeks after injection. Significantly higher ECM content with partly restored ECM structure and decrease cell apoptosis rate were determined in hydrogel-loaded NPMSC discs until eight weeks after injection as compared with discs that obtained only hydrogel or NPMSC injection.[47] The utilities of AF cells that were loaded in riboflavin-crosslinked high-density collagen gel (HDC gel) were examined to repair anular defects and prevent re-herniation in rat tail discs. Degeneration was induced by needle puncture of coccygeal discs (Co5/Co6) that was subsequently followed by the injection HDC gel loaded with ovine AF cells. X-rays, MRI, and histological assessments conducted over five weeks showed significantly better retention of disc height, NP size, and hydration with accelerated reparative sealing after treatment with HDC gel loaded with AF cells as compared to discs with acellular treatment (HDC gel alone) or without treatment.[48–50] Furthermore, genetically modified rat NPCs, ADSCs, and MSCs that were transduced with adenovirus vectors expressing luciferase reporter gene (AdV-Luc) were used to investigate the feasibility and usefulness of *in vivo* bioluminescent imaging of transplanted cells in

degenerative rat lumbar discs. Lumbar discs were needle punctured and directly injected with transduced ADSCs, MSCs or NPCs (1×10^5 cells in 10 μL alginate bead). *In vivo* bioluminescent imaging and gene expression in lumbar IVDs were assessed at several time points up to 14 days after surgery. Although luciferase expression was detected in all ADSCs-, MSCs-, or NPCs-injected discs at all imaging time points, the highest luciferase expression was found in discs injected with NPCs. Moreover, higher expression of luciferase was found with the transplanted cells than with the direct *in situ* injection of the adenovirus vectors expressing the luciferase gene (3×10^7 AdV-luc pfu in 10 μL solution). The results presented the feasibility to noninvasive monitoring of therapeutic gene expression within IVDs of small animals.[51]

Moreover, knockout mice of biglycan gene ($Bgn^{-/0}$) that undergo spontaneous disc degeneration with aging have been used to examine the regenerative potential of hADSCs. $Bgn^{-/0}$ mice of 16 months age that exhibited severe disc degeneration on MRI were intradiscally injected with human ADSCs (8×10^4 cells) at lumbar level L1/L2. MRI, histological, and immunohistochemical evaluations until 12 weeks after transplantation displayed in hADSCs-treated discs increased MRI signal intensity and surviving ADSCs with expression of biglycan that led to enhanced level of aggrecan as compared to untreated or PBS-treated discs.[52]

In situ gene therapy approaches in rodent models of disc degeneration. *In situ* gene therapy approaches have been attempted by direct injection of recombinant viral vectors into degenerative IVDs of rats. Recombinant LV, adenoviral (AdV), or AAV vectors were injected into degenerative lumbar or coccygeal discs to examine the therapeutic relevance of target genes in DDD[53–58] (Table 2).

Lentiviral vectors encoding the transcription factor EB (TFEB), which is known as a master regulator of autophagic flux, have been used to investigate the therapeutic potential of the transcription factor. Three microliters of recombinant LV vectors (1×10^6 pfu) expressing TFEB (LV-TFEB) or normal control (LV-NC) were injected into the NP of coccygeal discs directly after needle puncture of the discs. The MRI, radiographical, and histological analyses performed at four or eight weeks after surgery showed that LV-mediated overexpression of TFEB could decelerate the loss of disc height, improve MRI signal intensity, and delay histopathological changes as compared to injured control discs.[53] Similarly, 10 μL of recombinant LV vectors (1×10^6 pfu) expressing the insulin-like growth factor binding protein 5 (LV-IGFBP5) or normal control (LV-NC) were injected into the NP of coccygeal discs at seven days after needle puncture. Histological and biochemical evaluations of the discs at eight weeks after initial surgery displayed that LV-IGFBP5 injection significantly inhibited the progression of IVD degeneration by promoting NPC proliferation and inhibiting apoptosis.[54] These results suggest that TFEB and IGFBP5 could be seen as potential therapeutic targets in the pathogenesis of IVD degeneration.[53,54] Furthermore, the therapeutic potential of LV-mediated inhibition of the pro-apoptotic transcription factor CHOP was investigated in degenerative lumbar discs using anti-CHOP shRNA. A 2 μL injection of lentiviral vectors (1×10^6 pfu) carrying a CHOP inhibitory shRNA (LV-CHOP-shRNA) or normal control (LV-NC) was carried out at one week after mechanical induction of disc degeneration. Lumbar disc degeneration was induced by applying unbalanced dynamic and static forces on the lumbar spine for a week. MRI, radiological, histological, and TUNEL staining evaluations of the lumbar spines at one, three, five, and seven weeks after injection revealed that injection of LV-CHOP-shRNA could decelerate disc degeneration by inhibiting NPC apoptosis and by improving the MRI and histologic scores as compared to control discs. However, no significant change in disc height was observed until seven weeks after injection.[55]

Adenoviral vectors expressing mitofusin 2 (AdV-Mfn2), a dynamin-like GTPase playing a central role in regulating mitochondrial fusion and cell metabolism, were applied in degenerative lumbar discs. The aim was to investigate, whether the low abandoned of Mfn2 in degenerative NPCs is implicated in the pathogenesis of IVD

Table 2. *In situ* gene therapy approaches in rodent models of disc degeneration.

Animal model	*In situ* gene delivery	Disc regeneration effect	Ref.
Rat: Caudal disc puncture injury	LV-TFEB	Delayed loss of disc height with delayed MRI and histopathological changes	53
Rat: Caudal disc puncture injury	LV-IGFBP5	Increased NP cell proliferation and decreased NP cell apoptosis	54
Rat: Lumbar disc loading injury	LV-CHOP-shRNA	Improved MRI and histologic disc scores and inhibited apoptosis of NP cells	55
Rat: Lumbar disc puncture injury	AdV-Mfn2	Impaired NP cell apoptosis with impaired autophagic flux and mitochondrial dysfunction	56
Rat: Caudal disc puncture injury	AAV2-BMP2	Improved disc height, inhibited cell apoptosis with upregulated collagen II, aggrecan, SOX9 and downregulated MMP13 and CTX II	57
Mouse: Lumbar disc puncture injury	AdV-GDF5	Increased disc height, improved MRI index with enhanced GAG level, and DNA content	58

LV: lentiviral vector; TFEB: transcription factor EB; LV-IGFBP5: insulin-like growth factor binding protein 5; shRNA: short hairpin RNA; AdV: adenoviral vector; Mfn2: mitofusin 2; AAV-2: adeno-associated virus vector serotype 2; BMP2: bone morphogenetic protein 2; GDF5: differentiation factor 5; MRI: magnetic resonance imaging; MMP: matrix metalloproteinase; GAG: glycosaminoglycan.
In situ gene therapies have been attempted using lentiviral, adenoviral, and adeno-associated viral vectors carrying therapeutically relevant genes.

degeneration. A 3 μL of AdV-Mfn2 (1×10^6 pfu) or normal control (AdV-NC) was directly injected after induction of needle-punctured lumbar disc degeneration (L4–L6). At eight weeks after surgery, discs were evaluated using MRI, histology, immunofluorescence, transmission electron microscopy, TUNEL staining, and immunoblotting. In discs injected with AdV-Mfn2, the development of IVD degeneration was ameliorated by improving the impairment of autophagic flux, mitochondrial dysfunction, and cellular apoptosis in NP. The results indicated that Mfn2 repression is deeply involved in the pathogenesis of IVD degeneration and its overexpression could be a promising therapeutic target.[56] Moreover, AAV vectors of serotype 2 (AAV2) expressing the BMP2 were tested in degenerative coccygeal discs. A 5 μL of AAV2 expressing BMP2 (AAV2-BMP2) or normal control (AAV2-NC) at different concentrations (1×10^6, 1×10^8 or 1×10^{10} pfu) was injected two weeks after needle puncture of the discs Co6/Co7 and Co8/Co9. At eight weeks after surgery, the discs assessed by MRI, histological, and biochemical methods showed that AAV2-BMP2 treatment could significantly decelerate loss of disc height, inhibit NPC apoptosis, upregulate the expression levels of collagen II, aggrecan, and SOX9 and downregulate the levels of MMP-13 and CTX II in a dose-dependent manner.[57]

Furthermore, a mouse model of disc degeneration has been used to examine the regenerative effect of AdV vectors carrying the GDF5 (AdV-GDF5). Needle-punctured lumbar discs were directly injected with a 5 μL of AdV-GDF5 (1.8×10^{11} pfu/mL) or 5 μL of AdV-Luc (2×10^{11} pfu/mL) vectors that had been engineered to express the luciferase gene. Bioluminescent imaging, MRI scanning, histological, and biochemical analyses performed at one, two, four, or eight weeks after injection showed that discs treated with AdV-GDF5 could significantly increase the disc height index, the glycosaminoglycan level (GAG), the DNA content, and improve the MRI signal intensity as compared with injured discs that were treated with AdV-Luc or left untreated.[58]

Intradiscal application of bioactive factors in rodent models of disc degeneration. Bioactive factors, such as cytokines, antibodies, kinases, shRNAs, exosomes, lignans, and growth factors have been applied in degenerative rat discs to explore their therapeutic effects[59–65] (Table 3). To investigate the regenerative potential of the anti-inflammatory cytokine, interleukin-10 (IL-10), 20 μL (10 ng/mL) of exogenous IL-10 peptide was injected into degenerative coccygeal discs (Co7/8-Co9/10) one week after needle puncture injury. Discs were evaluated using MRI, histological, and immunohistochemical methods at one, two, and four weeks after injection. In discs treated with IL-10 peptide improved MRI signal intensity, increased expression levels of sox-9, collagen II, and aggrecan with decreased expression levels of IL-1β, TNFα, and collagen X and reduced phosphorylation level of p38 MAPK were determined. The data indicated that IL-10 could have the potential to be used for anti-inflammation therapy of DDD.[59] Moreover, an inhibition of the inflammatory cytokine, TNFα, was assessed in needle-punctured lumbar discs (L3–L6) using anti-TNFα monoclonal antibody. Punctured discs were directly injected with 2.5 μL anti-TNFα monoclonal antibody (0.5 mg/kg) or 2.5 μL TNFα peptide (0.25 ng) and pain behavior of the rats was measured at one and six weeks after surgery using hind-paw mechanical hyperalgesia test. In addition, disc height, disc morphology, and expression levels of pro-inflammatory cytokine were evaluated at six weeks after

Table 3. Intradiscal applications of bioactive factors in rodent models of disc degeneration.

Animal model	Intradiscal injected bioactive factor	Disc regeneration effect	Ref.
Rat: Caudal disc puncture injury	IL-10 peptide	Improved MRI signal intensity with enhanced levels of aggrecan, collagen II, and sox-9 plus decreased levels of IL-1β, TNFα, and collagen X	59
Rat: Lumbar disc puncture injury	Anti-TNFα monoclonal antibody	Alleviated pain, delayed loss of disc height, and decreased levels of TNFα and IL-1β	60
Rat: Lumbar disc puncture injury	JAG2 peptide	Enhanced MRI signal intensity with raised NP cell proliferation and Notch2 expression level	61
Rat: Caudal disc puncture injury	hMSC-exosomes	Retarded progression of disc degeneration with decreased apoptotic rate of NP cells	62
Rat: Caudal disc puncture injury	Simvastatin loaded in PEG-PLGA-PEG gel	Recovered NP weight, MRI signal intensity, and raised sGAG, BMP2, aggrecan, collagen II levels	63
Rat: Caudal disc puncture injury	Sesamin	Improved MRI signal, aggrecan, and collagen II levels and reduced ADAMTS5 and MMP3 levels	64
Mouse: Caudal disc loading injury	GDF-5 IGF-1 bFGF TGF-β1	Improved cell density with enhanced levels of aggrecan and collagen II levels in each growth factor treatment, but increased disc height only in GDF-5 treatments	65

IL-10: interleukin 10; TNFα: tumor necrosis factor alfa; JAG2: jagged-2; hMSC: human mesenchymal stem cell; PEG-PLGA-PEG: poly(ethyleneglycol)-poly(lactic acid-co-glycolic acid)-poly(ethylene glycol); GDF-5: differentiation factor-5; IGF1: insulin-like growth factor-1; bFGF: basic fibroblast growth factor; TGF-β1: transforming growth factor-beta1; MRI: magnetic resonance imaging; NP: nucleus pulposus; GAG: glycosaminoglycan; ADAMTS: metalloproteinase with thrombospondin motifs; MMP: matrix metalloproteinase.

Intradiscal injections of bioactive factors, such as cytokines, antibodies, kinases, shRNAs, exosomes, lignans, and growth factors have been applied aiming at regenerative effects.

surgery using radiographical, histological, and biochemical methods. As expected, the intradiscal injection of TNFα peptide increased pain and aggravated disc degeneration; whereas pain threshold was linearly associated with the loss of disc height and enhanced levels of TNFα and IL-1β. In contrast, the injection of anti-TNFα antibody mitigated disc degeneration and alleviated pain to sham level.[60] Likewise, the therapeutic effect of the peptide jagged-2 (JAG2), a ligand that activate notch signaling pathway, was investigated in needle-punctured lumbar discs (L4/5). Punctured discs were injected with 2 μL of JAG2 peptide (1.6 ng) and evaluated by MRI, histological, and biochemical methods at three and nine weeks after surgery. Intradiscal injection of JAG2 peptide increased the MRI signal intensity, induced Notch2 expression, and enhanced proliferation of NPC by inhibiting TNFα-induced apoptosis. Quite contrary to that a 2 μL injection of anti-Notch2-shRNA (1×10^6 pfu) decreased the MRI signal intensity and downregulated Notch2 expression with induction of NPC cycle arrest.[61]

It is apparently suggested that the therapeutic effects of mesenchymal stem cells (MSCs) are largely mediated by paracrine factors such as exosomes. Exosomes are very small membrane-bound vesicles functioning as paracrine factors as mediators of cell-to-cell communication. The therapeutic effect of exosomes that were isolated from hMSCs (hMSC-exosomes) and loaded in AGEs (advanced glycation end products) was examined in needle-punctured coccygeal discs (Co7/8, Co8/9, and Co9/10). After surgery the punctured discs were injected every two weeks for eight weeks with 2 μL mixture of AGEs plus exosomes (200 μg/mL plus 100 μg/mL) or 2 μL of AGEs alone (200 μg/mL) and 2 μL of PBS, respectively. Histological and biochemical analyses showed retarded progression disc degeneration and decreased apoptotic rate of NPC after two months of treatment with MSC-exosomes and not with AGEs or PBS alone.[62] Furthermore, the regenerative potential of lignans, such as simvastatin and sesamin, has been assessed in rat models of disc degeneration. To investigate the therapeutic role for simvastatin, a cholesterol-lowering medicament, injured coccygeal discs (Co5/Co6, Co7/Co8) were injected four weeks after needle puncture either with PEG-PLGA-PEG (poly(ethylene glycol)-poly(lactic acid-co-glycolic acid)-poly(ethylene glycol)) gel that was loaded with 2 μL of simvastatin (5 mg/mL) or with a gel alone. MRI, histological, and biochemical evaluations of the discs performed at two weeks after injection showed significantly retarded progression of disc degeneration by injection of simvastatin. It reverted the decreased NP weight, delayed histological changes, and improved the MRI signal intensity as well as the levels of aggrecan, sGAG, BMP-2, and collagen type II.[63] Similarly, a 2 μL injection of the major lignan extracted from sesame seed (sesamin; 0.1 M) into needle-punctured coccygeal discs (Co4/Co5, Co6/Co7) resulted in retarded progression of disc degeneration. Discs treated with sesamin showed enhanced MRI signal intensity and increased levels of aggrecan and collagen II along with decreased levels of MMP-3 and ADAMTS-5.[64] Moreover, regenerative effects of various growth factors, such as human GDF5, IGF-1, basic fibroblast growth factor (bFGF), and TGF-β1, have been examined in a mouse model of disc degeneration. After induction of caudal disc degeneration using static compression for seven days and a three-week period of no compression, degenerative discs were injected with 8 μL of GDF-5 (1 μg/mL), or IGF-1(1 μg/mL), or bFGF (1 μg/mL), or TGF-β1 (200 ng/mL). *In situ* hybridization, histological, and immunohistochemical assessments of the discs performed up to four weeks after injection showed improved cell density with increased levels of aggrecan and collagen II in all discs treated with each growth factor. However, only treatments with GDF-5 showed significantly increased disc height at four weeks after injection.[65]

Rabbit models for biological treatment approaches of DDD

Rabbits have been frequently used to study the process of IVD degeneration and the regenerative effects of intradiscal therapeutical approaches. Compared to the rodent models, rabbit models exhibit higher degree of structural and size homology to human spine. Due to the appearance of facet joints, paravertebral muscles, ligaments, and closed growth plates of the vertebral bodies in the rabbit spine, the biomechanical behavior is more comparable with the human spine contrary to mouse and rat tails. Moreover, in contrast to discs of mouse and rat tails, rabbit discs contain much less notochordal cells after their maturation age of 10 months.[66,67] Beyond that the use of rabbit models is more cost-effective than that of larger animal models such as porcine, ovine, caprine, bovine, canine, and non-human primate models.

Cell-based *in vivo* therapy approaches in rabbit models of disc degeneration. To retard the process IVD degeneration or maintain the structure as well as the function of degenerative discs, different rabbit models of disc degeneration have been used with applications of autologous, allogeneic or human derived stem cells as well as chondrocytes with or without genetic modification of the cells or in combination with bioscaffolds[68–82] (Table 4). Bone marrow-derived MSCs, synovial MSCs, umbilical cord tissue-derived MSCs, adipose-derived stem cells (ADSCs), and embryonic stem cells (ESCs) have been used with or without *in vitro* differentiation toward a chondrocyte lineage.[68–78] The regenerative effect of bone morphogenic protein 2 (BMP2), which is known to have biological effects on the differentiation of MSCs into chondrocyte-like cells in platelet-rich plasma (PRP) gel culture, was investigated in a rabbit model of disc degeneration. Autologous bone marrow-derived MSCs transduced with AdV vectors encoding for BMP2 (AdV-BMP2) and loaded in PRP gel (MSC-AdV-BMP2-PRP; 1×10^6 cells/20 μL) were directly injected into anular-punctured lumbar discs. MRI, histological, and immunohistochemical evaluations at 6 and 12 weeks after transplantation showed that discs treated with MSC-AdV-BMP2-PRP could retain relatively well-preserved NP structure, significantly higher MRI signal intensity, and a greater amount of ECM proteins as

Table 4. Approaches of cell transplantation in rabbit models of disc degeneration.

Animal model	Transplanted cell/Gene modification	Implanted biomaterial	Disc regeneration effect	Ref.
Rabbit: Lumbar disc puncture injury	Autologous MSC /AdV-BMP2	Platelet-rich plasma gel	Improved MRI signal intensity, NP structure, ECM content	68
Rabbit: Lumbar disc puncture injury	Autologous MSC /AdV-Sox9	Chitosanglycero-phosphate gel	Improved MRI signal intensity, NP structure, ECM content	69
Rabbit: Lumbar disc puncture injury	Autologous MSC /AdV-TIMP1		Improved disc space with increased ECM content	70
Rabbit: Lumbar disc NP aspiration	Autologous MSC /LV-BMP7	Thiol-modified hyaluronic acid hydrogel	Stimulated cell differentiation, cell proliferation and survival with ECM homeostasis	71
Rabbit: Lumbar disc NP aspiration	Autologous MSC /RV-GFP	Atelocollagen gel	Promoted cell differentiation, survival and ECM homeostasis	72
Rabbit: Lumbar disc puncture injury	Allogeneic hypoxic-conditioned MSC	Photopolymerizable gel: methacrylated gelatin plus LAP	Improved disc space, cellularity, histological scores and ECM content with enhanced collagen II/XI, CD105, and BMP7 levels	73,74
Rabbit: Lumbar disc puncture injury	Allogeneic ADSC	NPCS: Bioscaffold from decellularized fresh porcine NP	NP-like differentiation, repaired NP/ECM structure with restored disc height, MRI signal intensity	75
Rabbit: Lumbar disc NP aspiration	Allogeneic synovial MSC/AdV-GFP		Improved disc height, level of collagen II, MRI signal intensity	76
Rabbit: Lumbar disc puncture injury	Human umbilical tissue-derived MSC	Hydrogel carrier	Enhanced disc cellularity, ECM architecture, and viscoelasticity	77
Rabbit: Lumbar disc puncture injury	Murine ESC /AdV-GFP		Growth of new notochordal cell populations in degenerated NP	78
Rabbit: Lumbar disc nucleotomy	Allogeneic NP cells	Tri-copolymer scaffold: CII/HyA/CS	Increased cell population, ECM production with improved disc space and MRI signal intensity	79
Rabbit: Lumbar disc nucleotomy	Autologous NP cells activated by AF cells		Improved cell population, ECM production, restored disc space	80
Rabbit: Lumbar disc puncture injury	Allogeneic NP cells /AdV-BMP2		No effect on disc height and disc degeneration grade	81
Rabbit: Lumbar disc puncture injury	Human NP cells	Sodium hyaluronate gel (1%)	Restored disc height and ECM architecture	82

MSC: mesenchymal stem cell; AdV: adenoviral vector; BMP2: bone morphogenetic protein 2; TIMP1: metalloproteinase inhibitor 1; RV: retroviral vector; GFP: green fluorescent protein; ADSC: adipose-derived stem cell; ESC: embryonic stem cells; NP: nucleus pulposus; AF: anulus fibrosus; MRI: magnetic resonance imaging; ECM: extracellular matrix.
Regenerative treatments of degenerative discs were based on stem cell and NP cell transplantation with or without genetic modification or in combination with biomaterials.

compared with control discs treated with MSC-PRP or PRP alone.[68] Likewise, autologous MSCs transduced with AdV vectors encoding for the transcription factor Sox9 (AdV-Sox9), which is also known to have biological effects on the differentiation of MSC into chondrocyte-like cells in chitosan-glycerophosphate (C/Gp) gel culture, were used in a rabbit model of disc degeneration. Following anulus puncture, lumbar discs were injected with MSCs transduced with Sox9 and loaded in C/Gp gel (MSC-AdV-Sox9-C/Gp). Treatment with MSC-AdV-Sox9-C/Gp showed similar effects as above at 6 and 12 weeks after injection as shown by the MRI, CT, histological, immunohistochemical, and biochemical evaluations.[69] Furthermore, the application of autologous MSCs transduced with AdV vectors encoding for tissue inhibitor of metalloproteinase 1 (AdV-TIMP1) also showed regenerative effect in a rabbit model disc degeneration as determined by radiographical, immunohistochemical, and biochemical analyses. Anular-punctured lumbar discs injected with 30 μL MSC-AdV-TIMP1 (1×10^6 cells) showed significantly improved disc space and ECM content at 12 weeks after surgery as compared to discs treated with unmodified MSCs.[70] Moreover, autologous MSCs transduced with lentiviral vectors encoding for bone morphogenetic protein-7 (LV-BMP7) and loaded in thiol-modified hyaluronic acid (TMHA) hydrogel were used to investigate its therapeutic efficacy and feasibility in a rabbit model disc degeneration. For that MSC-LV-BMP7-TMHA (1×10^6 cells/20 μL) was transplanted in injured discs at two weeks after needle aspiration of the NP and discs were analyzed using histological and immunohistological methods at 6 and 12 weeks after transplantation. Stimulated cell differentiation, enhanced cell proliferation, and cell survival with greater ECM homeostasis were confirmed in discs treated with MSC-AdV-BMP7-TMHA, indicating its potential for the treatment of DDD.[71] Similarly, transplantation autologous MSCs (1×10^6 cells/20 μL) transduced with retroviral vectors encoding for GFP (MSC-RV-GFP) and embedded in 20 μL atelocollagen gel was performed two weeks after needle aspiration of lumbar NP. Immunohistochemical and biochemical evaluations of the lumbar discs at 2, 4, 8, 16, 24, and 48 weeks after transplantation displayed detectable GFP positive cells up to 48 weeks, which were able to express matrix molecules such as collagen II, keratan sulfate, chondroitin sulfate, aggrecan, hypoxia inducible factor 1 alpha, glutamine transporter 1, and MMP2; while the MSCs did not show significant expression of these molecules before transplantation.[72] Moreover, hypoxic (1% O_2) and normoxic (air) expanded allogeneic MSCs were examined to compare their *in vivo* therapeutic effects in anular-punctured lumbar discs of rabbits. At three to six weeks after puncture injury, discs were injected either with MSCs (1×10^6 cells) embedded in photopolymerizable biogel scaffold (25 μL), 10% w/v methacrylated gelatin biogel plus 0.15% w/v lithium phenyl 2,4,6-trimethylbenzoylphosphinate (LAP)[73] or with MSCs (1×10^6 cells) suspended in 0.1 mL PBS or PBS alone.[74] Discs were assessed up to 12 weeks after initial surgery using MRI, radiographical, histological, and immunohistochemical methods. As compared with controls and with discs treated with normoxic MSCs, the discs treated with hypoxic MSCs, especially if combined with gel, showed less disc space narrowing, significantly better histological scores, enhanced ECM deposition of collagen II and collagen XI, and increased CD105 and BMP7 expressions.[73,74]

Furthermore, allogeneic ADSCs, allogeneic synovial MSCs, human umbilical cord tissue-derived MSCs, and murine ESCs that were expanded and differentiation toward a chondrocyte lineage have been therapeutically analyzed in rabbit models of disc degeneration.[75–78] Allogeneic ADSCs were applied in combination with an injectable bioscaffold (NPCS), which was made-up by decellularization of fresh porcine NP. NPCS is able to retain the biochemical composition, the microstructure, and the mechanical properties of the native NP that could have stimulatory effects on the NP-like differentiation of ADSCs. A 30 μL ADSCs (2×10^6 cells) loaded in the NPCS or medium (DMEM) alone was injection of lumbar discs at four weeks after anular needle puncture. Evaluation of the discs was performed up to 16 weeks after injection using MRI, radiographical, histological, biochemical, and immunohistochemical methods. Higher NP-LC differentiation, restoration of NP/ECM structure with improved disc height, and MRI signal intensity could be detected in discs treated with ADSCs loaded in NPCS.[75] In addition, synovial MSCs expressing GFP were directly transplanted into degenerative lumbar discs of rabbits after needle aspiration of the NP. The postoperative disc evaluations at 2, 4, 6, 8, 16, and 24 weeks were performed using MRI, radiographical and histological methods. As compared to untreated injured discs, synovial MSCs-treated discs showed improved disc height, MRI signal intensity, and level of type II collagen with 24 weeks intradiscal existence of the transplanted GFP positive cells.[76] Moreover, human umbilical cord tissue-derived MSCs embedded in a hydrogel carrier, which were transplanted in needle-punctured rabbit lumbar discs could also show regenerative effects with improved values of disc cellularity, ECM architecture, and viscoelastic property as determined by MRI, histological, and uniaxial compression analyses at 3, 6, and 12 weeks after transplantation.[77] Additionally, anular needle-punctured rabbit lumbar discs were directly treated with 20 μL of murine ESCs (1×10^6 ESCs) that were transduced with AdV vectors encoding for GFP (ESC-AdV-GFP) and cultured with cis-retinoic acid, TGF-β1, ascorbic acid, and IGF-1 for inducing differentiation toward a chondrocyte lineage. Evaluations of the discs were completed at two, six, and eight weeks after transplantation using MRI, confocal fluorescent microscopy, histochemical, and immunohistochemical methods. The results revealed the existence of notochordal-type cells and islands of notochordal cell growth in discs treated with ESCs; while untreated punctured discs and non-punctured discs were negative for notochordal cells indicating that new notochordal cell populations can be generated in degenerated discs using ESCs. However, immune response to a xenograft of mouse cells was lacking in an immunocompetent rabbit model.[78]

In addition to stem cells autologous, allogeneic, or xenogeneic (human origin) NPC have been used in rabbit

models of disc degeneration to evaluate their therapeutic potential of retarding disc degeneration or maintaining the structure and function of degenerative discs.[79–82] Allogeneic NPC (1×10^5 cells), which were seeded in tri-copolymer scaffold-construct, consisting of collagen II, hyaluronan, and chondroitin-6-sulfate (CII/HyA/CS scaffold: 6 mm in diameter, 1.5 mm thick) and cultured for one week, were transplanted in lumbar discs directly after nucleotomy. Assessments of the discs were performed up to 24 weeks after nucleotomy using MRI, radiographical, gross morphological, and histological methods plus cell viability assays. Higher T2-weighted signal intensity, retarded narrowing of the disc space, and higher number of viable NPCs with higher ECM production were detected in discs transplanted with cell-seeded scaffold than in discs transplanted with cell-free scaffold or untransplanted nucleotomy.[79] Likewise, the direct reinsertion of autologous NPCs that were activated by coculture with AF cells showed comparable results as stated above.[80] Contrary to that, transplantation of allogeneic NPCs that were transduced with AdV vectors encoding for bone morphogenetic protein-2 (AdV-BMP2) could not show any regeneration effect, since similar disc height and disc degeneration grade were determined using MRI and histology in all injured discs with or without cell transplantation.[81] On the other hand, anular-punctured lumbar discs that were transplanted with adult human NPCs loaded in sodium hyaluronate (1%) gel showed restored disc height and disc tissue architecture as compared to injured discs with only gel treatment or without treatment.[82]

In situ gene therapy approaches in rabbit models of disc degeneration. In view of restoring IVD structure and function, intradiscal injection of recombinant LV, AdV, or AAV vectors encoding for target genes have been used for *in situ* gene therapy approaches in rabbit models of disc degeneration[83–89] (Table 5).

Anular-punctured lumbar discs of rabbits were injected at four weeks after puncture surgery with recombinant LV vectors encoding for three genes: TGFβ3, the connective tissue growth factor (CTGF), and the tissue inhibitor of metalloproteinase 1 (TIMP1) that are separated by 2A-self-cleaving-peptides (LV-TGFβ3-P2A-CTGF-T2A-TIMP1). Treatments of degenerated discs with 20 μL of LV-TGFβ3-P2A-CTGF-T2A-TIMP1 (2×10^8 pfu) showed improved MRI signal intensity and enhanced levels of aggrecan and collagen II as determined by MRI and biochemical analyses at 16 and 20 weeks after initial surgery.[83] Similarly, recombinant LV vectors encoding for the anti-apoptosis gene Survivin, the anti-catabolic gene TIMP1, and the anabolic gene TGF-β3 (LV-Survivin-P2A-TGFβ3-T2A-TIMP1) were injected into anular-punctured lumbar discs of rabbits three weeks after puncture injury. Caspase-3 activity assays as well as MRI, histological, and biochemical assessments of the discs were performed at 3 and 12 weeks after injection. As above, treatments of degenerated discs with 20 μL of LV-survivin-P2A-TGFβ3-T2A-TIMP1 (2×10^8 pfu) could enhance the levels of aggrecan and collagen II with improved MRI signal intensity as well as inhibited apoptosis of NPCs.[84]

Furthermore, 20 μL of recombinant AdV vectors (6×10^6 pfu) encoding for the human TGFβ1 (AdV-TGFβ1) gene or luciferase reporter (AdV-LUC) were injected directly after anular puncture of rabbit lumbar discs. Immunohistochemical and gene expression assays performed at one week after injection displayed a 30-fold increase in active TGFβ1 production and a 100% increase in proteoglycan synthesis in AdV-TGFβ1-treated discs as compared to AdV-LUC treated or untreated punctured discs.[85] Likewise, anular-punctured rabbit lumbar discs injected with 20 μL of AdV-TGFβ1 (10×10^6 pfu) or AdV-LacZ at eight weeks after surgery showed improved MRI and histopathological indexes at three weeks when injected with AdV-TGFβ1 as determined by MRI, histological, and immunohistochemical evaluations.[86] Also, a 10 μL injection of AdV vectors (1×10^9 pfu) encoding for a chondrocyte-specific transcription factor Sox9 (AdV-Sox9) directly after anular puncture of lumbar discs resulted in disc regeneration at five weeks after injection. Histological and immunohistochemical assessments displayed maintained chondrocytic phenotype and preserved architecture of the

Table 5. *In situ* gene therapy approaches in rabbit models of disc degeneration.

Animal model	*In situ* gene delivery	Disc regeneration effect	Ref.
Rabbit: Lumbar disc puncture injury	LV-TGFβ3-P2A-CTGF-T2A-TIMP1	Enhanced MRI signal intensity with increased levels of aggrecan and collagen II	83
Rabbit: Lumbar disc puncture injury	LV-Survivin-P2A-TGF β3-T2A-TIMP1	Inhibited cell apoptosis with improved MRI signal intensity, collagen II, and aggrecan levels	84
Rabbit: Lumbar disc puncture injury	AdV-TGFβ1	Increased TGFβ1 and proteoglycan synthesis with improved MRI and histological indexes	85,86
Rabbit: Lumbar disc puncture injury	AdV-Sox9	Maintained chondrocytic phenotype and preserved architecture of NP tissue	87
Rabbit: Lumbar disc Fn-f injection injury	AAV2-TGFβ1	Rapid and prolonged TGFβ1 expressions with increased proteoglycan and collagen II synthesis	88
Rabbit: Lumbar disc puncture injury	AAV2-BMP2 or AAV2-TMP1	Increased collagen II content with improved viscoelasticity, MRI, and histological indexes	89

LV: lentiviral vector; TGFβ3: transforming growth factor beta 3; CTGF: connective tissue growth factor; TIMP1: metalloproteinase inhibitor 1; AdV: adenoviral vector; AAV2: adeno-associated virus vector serotype 2; BMP2: bone morphogenetic protein 2; NP: nucleus pulposus; MRI: magnetic resonance imaging.
Recombinant lentiviral, adenoviral, and adeno-associated viral constructs encoding for therapeutic genes have been used for intradiscal injections.

NP as compared with discs treated with AdV-GFP (1×10^9 pfu/10 μL) or untreated injured discs.[87]

Moreover, the serotype 2 of AAV2 vectors encoding for TGF (AAV2-TGFβ1), bone morphogenetic protein-2 (AAV2-BMP2), or tissue inhibitor of metalloproteinase 1 (AAV2-TIMP1) were examined in rabbit models disc degeneration.[88,89] After stimulation of lumbar disc degeneration by injection the fibronectin fragment (Fn-f), lumbar discs were instantly treated with 10 μL injection of AAV2-TGFβ1 or AAV2-GFP (1×10^9 pfu) or PBS alone as control. The efficiency and effect of AAV2-mediated gene transfer was assessed at 1, 4, 8, and 12-week time points after injection using immunohistochemical staining, fluorescence observation, immunoblotting, and 35S-sulfate incorporation assay. Treatment with AAV2-TGFβ1 exhibited rapid and prolonged TGFβ1 expression and increased ECM production with significantly enhanced levels of proteoglycan and collagen II, as compared to the control discs that were treated with AAV2-GFP or PBS.[88] Similarly, a 10 μL injection of AAV2-BMP2 or AAV2-TIMP1 (1×10^9 pfu) into anular-punctured lumbar discs directly after surgery led to increased level collagen II and enhanced viscoelastic properties at 12 weeks after surgery as determined by MRI, uniaxial load-normalized displacement tests, and histology.[89]

Intradiscal applications of bioactive factors in rabbit models of disc degeneration. Bioactive factors, such as PRP, platelet-derived growth factor (PDGF), serum immersed hydrogels, growth factor analogs, small interfering RNAs (siRNAs), short peptides, antibodies, and hormones have been intradiscally applied in rabbit models of disc degeneration to examine their *in vivo* therapeutic potentials in DDDs[90–106] (Table 6).

PRP, PDGF, and serum have been described to stimulate the metabolism in IVD cells.[90–94] To study the regenerative effect of PRP (a fraction of plasma in which several growth factors are concentrated at high levels) and platelet-poor plasma (PPP), a 20 μL of autologous PRP or PPP was injected into anular-punctured rabbit lumbar discs at four weeks after the puncture injury. Restoration of disc height, improvement MRI signal intensity, and growing number of chondrocyte-like cells were verified in PRP-treated discs related to PPP- or PBS-treated punctured discs as

Table 6. Intradiscal application of bioactive factors as regenerative therapy approaches in rabbit models of disc degeneration.

Animal model	Intradiscal injected bioactive factor	Disc regeneration effect	Ref.
Rabbit: Lumbar disc puncture injury	Autologous platelet-rich plasma (PRP)	Restoration of disc height with better MRI signal intensity and number of chondrocyte-like cells	90
Rabbit: Lumbar disc NP aspiration	PRP-GHMs: PRP-impregnated gelatin hydrogel microspheres	Improved disc height, preserved water content, higher levels of proteoglycan, and collagen II with lower NP cell apoptosis	91
Rabbit: Lumbar disc puncture injury	Platelet-derived growth factor BB (PDGF-BB) in TMHA gel	Improved compressive strength with improved disc area, MRI index, and deposition of ECM as well as lower disc cell apoptosis	92
Rabbit: Lumbar disc microdiscectomy	Allogeneic serum immersed PGA implant	Enhanced cell migration into the defect area with improved MRI signal intensity and disc height	93
Rabbit: Lumbar disc microdiscectomy	Allogeneic serum immersed PVA implant	Enhanced cell migration into the defect area with improved MRI signal intensity and disc height	94
Rabbit: Lumbar disc puncture injury	Osteogenic protein-1 (OP-1)	Improved disc height, MRI signal intensity, proteoglycan content, and elastoviscous property	95–97
Rabbit: Lumbar disc puncture injury	Growth differentiation factor-5 (GDF-5)	Dose-dependent restoration of disc height with improved MRI and histological grading scores	98
Rabbit: Lumbar disc puncture injury	Anti-caspase3 siRNA	Reduced level of caspase 3 and apoptotic NP cells with improved MRI and histological scores	99
Rabbit: Lumbar disc puncture injury	Anti-ADAMTS5 siRNA	Reduced level of ADAMTS5 with improved MRI and histological scores, but no disc height change	100
Rabbit: Lumbar disc puncture injury	Anti-Caspase3 / anti-ADAMTS5 siRNAs	Synergistic effect with improved disc cellularity, ECM formation, histological, and MRI scores	101
Rabbit: Lumbar disc puncture injury	Short link N peptide	Increased disc height, GAG, and aggrecan levels with reduced levels of ADAMTS4/5 and MMP3	102,103
Rabbit: Lumbar disc puncture injury	Biglycan-derived peptide (P2K)	Increased disc height, MRI signal intensity, PG content with improved disc tissue architecture	104
Rabbit: Lumbar disc puncture injury	CDH2 antibody/RES embedded PLGA-NBs	Retarded disc degeneration with increased anti-catabolic activity and accelerated RES release	105
Rabbit: Lumbar disc puncture injury	Melatonin	Improved MRI signal intensity and NP cell viability with enhanced levels of aggrecan and collagen II as well as restrained cell arrest	106

PRP: platelet-rich plasma; GHM: gelatin hydrogel microspheres; PDGF: platelet-derived growth factor; TMHA: thiol-modified hyaluronic acid; PGA: poly(glycolic acid); PVA: polyvinyl alcohol; OP-1: osteogenic protein-1; GDF-5: differentiation factor-5; siRNA: small interfering RNA, ADAMTS5: disintegrin and metalloproteinase with thrombospondin motifs 5; CDH2: cadherin-2; RES: resveratrol; PLGA: poly L-lactic-co-glycolic acid; NB: nanobubble; MRI: magnetic resonance imaging; ECM: extracellular matrix; GAG: glycosaminoglycan; MMP: matrix metalloproteinase; PG: proteoglycan; NP: nucleus pulposus.
Bioactive factors, such as platelet-rich plasma, platelet-derived growth factor, serum immersed hydrogels, growth factor analogs, siRNAs, short peptides, antibodies, and hormones have been intradiscally applied.

determined by MRI, histological, and biochemical analyses.[90] Moreover, a 20 μL injection of PRB-impregnated gelatin hydrogel microspheres (PRP-GHMs) in degenerative lumbar discs at two weeks after partial aspiration of the NP showed significantly restored disc height, preserved water content, higher expression of proteoglycan, and collagen II with lower NPC apoptose. The results determined by MRI, immunohistochemistry, TUNEL staining, and gene expression assays at two, four, and eight weeks after injection showed the greater potential of PRP-GHMs treatment as compared to control discs that were treated with PBS-GHMs or PRP without impregnation of GHMs.[91] Furthermore, recombinant human platelet-derived growth factor BB (PDGF-BB), a primary component of PDGF, was intradiscally injected in combination with TMHA gel into rabbit lumbar discs after four weeks of anular puncture. Injured lumbar discs were injected with either 20 μL of PDGF-BB (1 ng/mL) in TMHA gel (PDGF-TMHA-treated group), PDGF-BB (1 ng/mL) without TMHA gel (PDGF-treated control group), TMHA without PDGF (TMHA-treated control group), or PBS alone. Treatment efficacies were determined using x-ray, MRI, histology, and biomechanical testing at four and eight weeks after injection. Lower cell apoptosis, improved deposition of ECM in NP and AF, restored compressive strength with improved disc area and MRI index were found in PDGF-TMHA-treated or PDGF-treated groups, suggesting that PDGF-BB significantly decreases disc degeneration.[92] In addition, serum-immersed polyglycolic/hyaluronic acid (PGA) hydrogel implant and serum immersed rod-shaped polyvinyl alcohol (PVA) hydrogel implant were examined for stimulations of IVD tissue repair directly after microdiscectomy of lumbar discs. Allogeneic serum-immersed PGA implant[93] or PVA implant[94] was inserted into injured discs, and transplanted discs were assessed using MRI and histology between 1 and 24 weeks after surgery. Improved MRI signal intensity, disc height, and cell migration into the defect area were confirmed in PGA or PVA implanted discs as compared to injured discs without implant.[93,94]

Furthermore, recombinant human growth factors, such as osteogenic protein-1 and growth and differentiation factor-5 (rhGDF-5) that are known to stimulate proteoglycans and collagen synthesis in cells, have been intradiscally applied to study their ability of restoring IVD tissues.[95–98] A 10 μL injection of OP-1 solution with different concentrations (100 μg OP-1 in 10 μL 5% lactose)[95–96] or (2 μg OP-1 in 10 μL PBS)[97] have been performed into anular-punctured lumbar discs four weeks after puncture injury. The MRI, radiographical, histological, biochemical, and biomechanical analyses that were made between 2 and 24 weeks after injection revealed that the injection of OP-1 in each concentration could induce restoration of disc height, improvement of MRI signal intensity, enhancement of proteoglycan content for the entire experimental period up to 24 weeks. The dynamic viscoelastic property test (0.05 to 2 Hz) showed significantly higher elastic and viscous moduli after eight weeks in all discs that were treated with OP-1.[95–97] Similarly, a 10 μL injection of rhGDF-5 solution at different dosages (10 ng, 1 μg or 100 μg in 10 μL PBS) into anular-punctured lumbar discs also showed that a single injection of rhGDF-5 at all tested dosages could have a reparative capacity through noticeable restoration of disc height, improvement of MRI, and histological grading scores with statistical significance $P < 0.05$ (10 ng and 1 μg rhGDF-5 injection) and $P < 0.01$ (100 μg rhGDF-5 injection).[98]

Additionally, siRNAs were tested to inhibit one of the major caspases in cell apoptosis (caspase 3) and one of the major aggrecanases responsible for disc degradation (ADAMTS5).[99–101] Anular-punctured lumbar discs were injected one week after puncture surgery either with 100 μg anti-caspase3 siRNAs labeled with red fluorescence Alexa Fluor 555,[99] anti-ADAMTS5 siRNAs,[100] or dual siRNAs (anti-caspase3 plus anti-ADAMTS5 siRNAs).[101] Discs were evaluated using MRI, histological, immunohistochemical, fluorescence staining, gene expression assay, and TUNEL staining techniques until eight weeks after the injection. Significantly improved MRI and histological scores, lower number apoptotic NPCs as well as lower expression of caspase 3 at mRNA and protein levels were verified in discs treated with the anti-caspase 3 siRNA as compared to control siRNA or PBS-treated discs.[99] The anti-ADAMTS5 siRNA-treated discs also showed improved MRI and histological scores, but no significantly changed disc height.[100] Synergistic effect was observed in discs treated with the dual siRNA formulation (anti-caspase 3 plus anti-ADAMTS5 siRNAs) with improved MRI and histopathological scores, disc cellularity as well as ECM formation at eight weeks after injection.[101] Moreover, to evaluate the restoration in degenerative discs the short Link N (sLN) was used, the fragment of the link N peptide consisting of 16 amino acid sequence released from the N-terminus as a proteolysis product, which is supposed to play a role in matrix homeostasis and stabilization of the proteoglycan aggregates in cartilage and discs.[102,103] A 10 μL injection of sLN solution (25 μg in PBS) or 10 μL PBS alone was performed in anular-punctured lumbar discs at two weeks after puncture surgery. Evaluations of the discs were carried out using radiographical, histological, and biochemical methods up to 12 weeks after injection. The results exhibited in sLN-treated discs decelerated loss of disc height, increased levels of GAG, and aggrecan with decreased levels of proteinases such as ADAMTS4, ADAMTS5, and MMP3 as compared to PBS alone-treated discs.[102,103] In a similar way the biglycan-derived peptide (Peniel 2000 or P2K), which is known as a regulator of TGF-β1 signaling on ECM metabolism, was tested in anular-punctured lumbar discs at four weeks after surgery (10 μg P2K in 15 μL of 5% lactose or 15 μL 5% lactose). At 12 weeks after treatment MRI, radiographical, biochemical, and histological evaluations of the discs showed improved disc height, MRI signal intensity, proteoglycan content, and disc architecture in discs treated with P2K as compared to discs treated with lactose alone.[104] Besides, the application of ultrasound (US) has been suggested as an effective means to advance gene and drug delivery by using echogenic nanoparticles, such as nanoparticles made from poly lactic-co-glycolic acid (PLGA), echogenic PLGA-nanobubbles (PLGA-NBs) combined with US. In order to

enhance the intradiscal gene delivery in NPCs *in vivo*, PLGA-NBs embedded with resveratrol (RES) and conjugated with NP-cell-targeting-biomarker CDH2-antibody (AbCDH2) were tested in rabbit models of disc degeneration (US-mediated RES/AbCDH2 PLGA-NBs). Anular-punctured lumbar discs were injected with (US-mediated RES/AbCDH2 PLGA-NBs) four weeks after puncture surgery, where the therapeutic US was irradiated on the surface of injected discs. US-mediated RES/AbCDH2 PLGA-NBs treatment resulted in retarded disc degeneration with accelerated RES release and increased anti-catabolic activity as determined by histological and biochemical methods up to 12 weeks after injection. The results suggest that the combination of US irradiation and drug delivery through RES/AbCDH2 PLGA-NBs can be considered as a therapeutic option for DDD.[105] In addition to that, melatonin, a neuroendocrine hormone secreted by the pineal body, is supposed to activate the ERK signaling pathway and promote the proliferation of NPC and the expression of aggrecan and collagen II. Thus melatonin was examined in anular-punctured lumbar discs to prove its *in vivo* regenerative potential. Discs were injected at four weeks after injury with a 20 μL of melatonin (2 mg/mL) or melatonin plus U0126 as ERK inhibitor (2 mg/mL plus 0.4 mg/kg) or PBS alone. MRI, histological, and biochemical evaluations of the discs at four and eight weeks after injection demonstrated that treatment with melatonin alone could improve MRI signal intensity, promote NPC viability, and inhibit cell arrest as well as upregulate aggrecan and collagen II with down regulation of collagen X as compared with discs treated melatonin plus U0126 or with PBS alone. Moreover, inhibition of the ERK1/2 signals by U0126 that reversed the role of melatonin in the regulation of NPC proliferation indicated that melatonin affected disc degeneration by activating ERK1/2 signaling pathway.[106]

Porcine models for biological treatment approaches of DDD

In relation to small animal models like rodents and rabbits, porcine models of disc degeneration have been used for intradiscal therapeutic approaches due to their IVD size and shape, which are more similar to that of humans. However, the abundant of notochordal cells in porcine IVDs and the higher costs related to small animal models should be taken into consideration.

Intradiscal biological therapy approaches in porcine models of disc degeneration. Cell transplantation, gene therapy, application of bioactive factors, and bioscaffolds have been used for *in vivo* therapy approaches in porcine models of disc degeneration. Bone marrow-derived autologous, allogeneic or xenogenic (human derived) MSCs, ADSCs, iPSCs, and chondrocytes have been implanted with or without gene modification or in combination with bioscaffolds[107–117] (Table 7). To achieve anterior intradiscal fusion in porcine model of disc degeneration using minimally invasive techniques, autologous MSCs that were transduced with AdV-BMP2 were used. After removal of 1 cm tissue from each disc, four thoracic disc spaces in each porcine were injected thoracoscopically with autologous MSCs as followed: two of the discs were injected with MSCs transduced with AdV-BMP2 (MSC-AdV-BMP2), the third disc was injected with MSCs transduced with

Table 7. Biological treatment approaches of degenerative discs in porcine models of disc degeneration.

Animal model	Transplanted cell/ Gene modification	Implanted biomaterial	Disc regeneration effect	Ref.
Pig: Thoracic disc discectomy	Autologous MSC/AdV-BMP2		Anterior spinal fusion, bridging bone from end plate to end plate	107
Pig: Lumbar disc laser vaporization	Autologous MSC	HyStem hydrogel	Improved MRI signal intensity and damage site ECM formation	108
Pig: Lumbar disc partial nucleotomy	Autologous MSC	Fibrin matrix	Enhanced collagen I in AF and BMP2 in NP with lesser IL-1β	109
Pig: Lumbar disc partial nucleotomy	Autologous MSC /RV-LUC	Albumin-hyaluron-hydrogel	Implant retainment with *in vivo* activity of the implanted cells	110
Pig: Lumbar disc partial nucleotomy	Allogeneic MSC or Allogeneic JC	Fibrin matrix Fibrin matrix	JC enhanced ECM and cartilage formation with vigorous viability	111
Pig: Lumbar disc partial nucleotomy	Human MSC or Human chondrocytes	Durolane gel Durolane gel	Not suitable for use *in vivo* due to endplate bone mineralization	112
Pig: Lumbar disc puncture injury	Autologous ADSC within MFAT		Enhanced disc height, NP water content and ECM structure	113
Pig: Lumbar disc puncture injury	Hypoxic iPSC cocultured with MSC	Geltrex hydrogel	Improved levels of notochordal markers with greater MRI index	114
Pig: Lumbar disc CABC inject injury		TMHA/EP matrix composite	Good disc integrity, but without long-term disc recovery	115
Pig: Lumbar disc partial nucleotomy		Collagen-hyaluronan matrix composite	Good disc integrity, but without long-term disc recovery	116
Pig: Lumbar disc puncture injury		Gelfoam	Good disc integrity, but without long-term disc recovery	117

MSC: mesenchymal stem cell; AdV: adenoviral vector; BMP2: bone morphogenetic protein 2; Luc: luciferase; JC: juvenile articular chondrocyte; ADSC: adipose-derived stem cell; MFAT: micro fragmented adipose tissue; iPSC: induced pluripotent stem cell; MRI: magnetic resonance imaging; ECM: extracellular matrix; NP: nucleus pulposus; AF: anulus fibrosus.
Intradiscal treatments include cell transplantation with or without gene modification as well as cell-based or non-cell-based implantation of biomaterials.

AdV-betagal as control, and the fourth disc served as the sham-operated control. At six weeks after the implantation, animals were euthanized and the spines were evaluated using CT and histology. Anterior spinal fusion and bridging bone from end-plate to end-plate were found in disc spaces treated with MSC-AdV-BMP2, while control disc spaces had shown no intervening or bridging bone. The results indicated that MSCs transduced with AdV-BMP2 could differentiate into osteoblasts and induce anterior spinal fusion in porcine model.[107] In addition, autologous MSCs transduced with AdV-BMP2 (MSC-AdV-BMP2) and loaded in hydrogel were used in porcine model of disc degeneration to investigate their integration level and repair efficacy. After induction of lumbar disc degeneration by percutaneous delivery of vaporizing laser light at four lumbar discs L2–L5 (dehydration of NP using laser vaporization of the disc with an energy pulse of 1000 J over 1 min), MSC-AdV-BMP2 (1×10^6 cells) loaded in HyStem hydrogel were delivered into the disc space using the percutaneous approach. L5/L6 discs were left as non-transplantation controls and L1/L2 discs were the non-injury controls. Assessment of the discs at four and eight weeks after transplantation using MRI and histological methods showed deceleration of disc degeneration with improved MRI signal intensity and ECM architecture at the damage site as compared to non-transplantation controls.[108] Also, four lumbar discs were implanted with autologous MSCs loaded in fibrin matrix at 12 weeks after partial nucleotomy. Two discs were injected with MSCs loaded in fibrin matrix (2×10^6 cells in 150 μL fibrin) and remaining two discs with acellular fibrin matrix, and discs were evaluated by micro-CT, histology, and gene expression analyses at 1 and 12 weeks after injection. Discs treated with MSCs loaded in fibrin matrix showed retarded disc degeneration with lower expression levels of IL-1ß, higher expression level of BMP2 in NP, and collagen I in AF as compared to discs treated with acellular fibrin matrix. However, regenerative changes, such as restoration of disc height, were not significant.[109] Similarly, autologous MSCs transduced with retroviral vectors encoding for the reporter luciferase gene (MSC-RV-LUC) and loaded in albumin-hyaluronan hydrogel were applied in a porcine nucleotomy model. Directly after partial nucleotomy of two lumbar discs (~10% of total nucleus volume), MSC-RV-LUC (3.3×10^6 cells) that were seeded in albumin-hyaluronan hydrogel and cultured under low levels of oxygen and glucose were injected (1 mL cell suspension) into injured discs. After three days of postoperative survival time, a short-term follow-up of intradiscal cell therapy, results of micro-CT, and biochemical evaluations demonstrated an *in vivo* evidence for hydrogel compression with implant volume shrank to 61% and luciferase expression of the implanted MSCs, emphasizing implant retainment inside the disc and persistence of metabolically active MSCs.[110] Furthermore, allogeneic MSCs and allogeneic juvenile articular chondrocytes (JCs) seeded in fibrin matrix have been examined in a porcine model disc degeneration to compare their restoration capacities in injured IVDs. After induction of lumbar disc degeneration by nucleotomy at three randomized levels between L1 and L5 discs (one level left uninjured), denucleated discs were transplanted either with allogeneic MSCs in fibrin matrix or with allogeneic JCs in fibrin matrix at a volume of 0.5–0.7 mL cell suspension (1×10^7 cells) or with fibrin matrix alone. Radiological, histological, immunohistochemical, and biochemical analyses of the lumbar spines were performed at 3, 6, and 12 months after surgery. Discs treated with JCs exhibited abundant cartilage formation with collagen II rich ECM at 3 months and to a lesser extent at 6 and 12 months. Viable JCs were observed at all time points, whereas no evidence of viable MSCs was found. These results support the premise that committed chondrocytes are more appropriate for use in disc repair, as they are uniquely suited for survival in the ischemic disc microenvironment.[111] Likewise, xenotransplantation of hMSCs or human-derived chondrocytes (hCs) in combination with hyaluronan gel (Durolane®) (Smith & Nephew, Inc. Mississauge, Ontario, Canada) were performed in porcine model of disc degeneration. Two weeks after partial nucleotomy of four lumbar discs (L1–L5), discs were injected either with hMSCs/gel or hCs/gel mix (0.5×10^6 cells), where the third disc was injected with only Durolane® gel and the fourth disc was left untreated. At 12 and 24 weeks after implantation assessments of the discs were completed using MRI, histology and immunohistochemistry, where xenotransplanted cells were traced with anti-human antibodies. Although xenotransplanted hMSCs and hCs could survive for 24 weeks and produce collagen II in all animals, more pronounced endplate changes (bone mineralization) and greater degenerative changes at MRI were observed in MSCs/gel, hCs/gel or gel alone-transplanted discs than in untransplanted injured discs. The results indicate that hyaluronan gel (Durolane®) is not suitable for use in cell therapy of injured IVDs. The high cell proliferation observed *in vitro* in the gel (Durolane®) could have been a negative factor *in vivo*.[112] Since ADSCs within micro fragmented adipose tissue (MFAT) are assumed to be a promising agent for a wide range of applications in regenerative medicine, autologous MFAT that were co-cultured with nucleus pulposus cells (NPCs) were examined in porcine model of disc degeneration. At four weeks after needle puncture injury of lumbar discs L2–L5, the L4/L5 discs were injected with 200 μL of autologous ADSCs (2×105), the L3/L4 discs with 200 μL of autologous MFAT, the L2/L3 discs with 200 μL of DMEM and the L1/L2 discs were left untreated as stab control. Radiographical and MRI analyses performed at 0, 4, 8, and 16 weeks and followed by histological, immunohistochemical, and biochemical analyses at 16 weeks after injection showed that implantation of autologous MFAT co-cultured with NPCs led to significantly improved disc height, disc water content, and ECM structure in NP as compared to untreated, DMEM-treated or ADSCs-treated discs. The results verified that MFAT could promote the function of NPCs and NPCs could stimulate the NP-like differentiation of ADSCs.[113] Furthermore, iPSCs generated from normal human dermal fibroblasts and co-cultured with MSCs in hypoxic condition (2% O_2) and defined growth media for differentiation towards notochordal-like cells (NCs) were used in a porcine model of disc degeneration. The co-cultured iPSCs or MSCs alone (1×10^6 cells)

suspended in 100 μL hydrogel (Geltrex™) or hydrogel alone were injected into lumbar discs at four weeks after anular puncture injury. MRI, histological, gene expression, and quantitative chemical exchange saturation transfer analyses at 12 weeks after injection displayed higher expression levels of the notochordal markers such as keratin 8, keratin 18, keratin 19, CD24, Gal3, and CTGF as well as improved MRI and histological indexes with minor change of pH level in discs treated with the co-cultured iPSCs. The results confirm that iPSCs can be differentiated into NCs and able to retard IVD degeneration.[114] Moreover, non-cell-based implantation of biomaterials such as thiol-modified hyaluronan elastin-like polypeptide composite (TMHA/EP),[115] hyaluronan collagen gel matrix composite,[116] or gelfoam[117] have been used in porcine model of disc degeneration as replacement agent for NP or to prevent recurrent disc herniation. Although the disc integrity of the implanted discs was better than that of bone-cement, tissue-glue, or platinum-coil implanted discs, in all cases disc injuries could not be recovered between 8 and 24 weeks after transplantation. The mechanical behavior of implanted discs was restored only in early-stage of degeneration, indicating the importance of cell or bioactive factors in transplantation for long-term and effective recovery of IVD injury.[115–117]

In vivo *treatment approaches of DDD in a bovine model*

Bovines are mostly used as large animal IVD organ donors for *ex vivo* study of disc biomechanical behavior. They are used to test the effect of stress under different loading conditions and the regenerative effect of different materials in an IVD-organ culture. Nevertheless, preliminary results involving the feasibility of the runt cow for *in vivo* testing of a spinal interbody prosthesis have been published. Transosseous implantations of a human-sized disc prosthesis were performed in lumbar spines (L4/L5) of mature runt cows (Corrientes breed). At 16 and 24 weeks after implantation surgery, the lumbar spines were harvested and evaluated based on healing process, motion segment mobility, and ability to remove the implant nondestructively. The results showed that all animals had successful implantation with surgical healing and intended device motion. Moreover, bone ingrowth into the device's porous plates was confirmed with trabecular bone increase adjacent to the implants. The results of this study have specified the possibility of using mature runt cow for *in vivo* testing of human-sized lumbar interbody implants and the ability of performing common analyses of explants.[118]

Ovine and caprine models for biological treatment approaches of DDD

Ovine and caprine have been used as large animal models in translational research, especially for testing the functionality of implants in regenerative therapy approaches. Ovine and caprine belong to animal species that lose their notochordal cells in early adulthood, predisposing their IVDs to degeneration similar to humans.[67] Moreover, mature ovine and cappine have roughly similar weight to humans and their discs have anatomical similarities regarding size and shape to human IVDs. Despite their quadrupedal stature, their IVDs are exposed to comparable mechanical stresses and show qualitatively similar craniocaudal variation in range of motion (ROM) in all load directions as human IVDs. They are resilient animals to endure invasive surgical interventions.[119–123]

Intradiscal biological therapy in ovine and caprine models of disc degeneration. Cell transplantation and implantation of supporting bioscaffolds have been used to repair disc injuries in ovine and caprin models of disc degeneration. Allogeneic bone marrow-derived MSCs, allogeneic bone marrow-derived mesenchymal progenitor cells (MPCs), autologous bone marrow-derived mononuclear cells (BMCs); as well as bioscaffolds such as riboflavin-crosslinked HDC gel, gelatin/fibrin scaffold (SCAF), dodecyl-amide of hyaluronic acid-based hydrogel (DDAHA), polyglycolic acid/polyvinylidene fluoride (PVDF), and small intestinal submucosa (SIS)-based scaffold (SIS-based patch and plug scaffold) have been used in ovine models of annulotomy or nucleotomy[124–130] (Table 8). Caprine models of disc degeneration have been used to investigate the regenerative potential of growth factors, such as BMP2, BMP7, IGFI, and TGFβ1 in combination with bioresorbable scaffolds or cages in monosegmental spinal fusion approaches[131–133] (Table 8).

To investigate their healing capacity in postero-lateral anular lesions, allogeneic bone marrow-derived MSCs were used in ovine model of disc degeneration. Scalpel blade postero-lateral anulotomy was performed in three lumbar discs (L3–L6) to induce disc injury. At 28 weeks after anulotomy, two discs were injected with allogeneic MSCs (1×10^6 in 200 mL PBS) either into the previously incised AF (AFI) or into the NP (NPI) and one disc was left uninjected as a negative control (Neg. C); whereas the NP of the superior adjacent uninjured disc (L2/L3) was injected with 200 mL PBS as a positive control (PC). Radiographs and MRI scans were obtained at baseline, 6, 9, and 12 months after injection and lumbar spines were harvested at 12 months for biochemical and histological analyses. Disc height, MRI, and histopathological grades were improved in AFI and NPI treatment groups with enhanced GAG content after six months. Both AFI and NPI groups showed repair of the postero-lateral anular lesion, while consistent disc degeneration was observed in control groups without MSC injection.[124] In addition, allogeneic bone marrow-derived MSCs have been used in combination with riboflavin-crosslinked HDC gel in an attempt to improve the *in vivo* anular repair capacity in an ovine model of annular injury. Scalpel blade lumbar disc injury was made (3 mm × 1 cm anulotomy plus 100 mg discectomy) at 12 discs from L1 to L6 in three sheeps, whereby 3 of the 15 randomized discs left intact without injury as controls. Directly after anular injury four discs were treated with HDC gel seeded with MSCs (1×10^6 cell/mL), four discs with acellular HDC gel, and four discs left untreated. Animals were euthanized at six weeks after surgery and lumbar discs were assessed

Table 8. Biological approaches of disc repair in ovine and caprine models of disc degeneration.

Animal model	Transplanted cell/ Gene modification	Implanted biomaterial	Disc regeneration effect	Ref.
Sheep: Lumbar disc anulotomy	Allogeneic MSC		Restored disc height, GAG level, and MRI / histological grade	124
Sheep: Lumbar disc anulotomy	Allogeneic MSC	HDC gel/riboflavin crosslinked	Improved disc height and MRI index with restored AF tissue	125
Sheep: Lumbar disc anulotomy	Allogeneic MPC	SCAF scaffold mixed with PPS	Restored disc morphology, disc height, and proteoglycan content	126
Sheep: Lumbar disc nucleotomy	Autologous BMC	iGG-MA/DDAHA/ antiangiogenic PEP	Only slight NP restoration with DDAHA gel loaded with BMC	127
Sheep: Lumbar disc anulotomy		PVDF scaffold	Repaired AF providing effective barrier for herniating NP	128
Sheep: Lumbar disc anulotomy		SIS-based patch and plug scaffold	Improved MRI index with ECM building and functional recovery	129
Sheep: Lumbar disc puncture injury		Recombinant BMP13 peptide	Enhanced NP hydration, NP cell population, and ECM formation	130
Sheep: Lumbar disc CABC injection injury		BMP2 and BMP2/7 conjugated FB/HA hydrogel/TGmoiety	Conjugated BMP2 and BMP2/7 appeared safe, but without any observed regeneration effect	131
Goat: Cervical disc discectomy		IGF-I, TGF-β1, and hydroxyapatite coated HCIF-Cage	Improved stiffness in flexion, extension, and lateral bending with higher DSH, IVA, and LA	132
Goat: Lumbar disc discectomy		Bioresorbable PLLA cage	More complete and faster fusion with unchanged grafting height, trabecular bone replaced PLLA	133

MSC: mesenchymal stem cell; MPC: mesenchymal progenitor cell; BMC: bone marrow derived mononuclear cell; HDC: high-density collagen; SCAF: gelatin/fibrin scaffold; PPS: polyurethane porous scaffolds; iGG-MA: hydrogels made of ionic crosslinked methacrylated gellan gum; DDAHA: dodecyl-amide of hyaluronic acid; PEP: anti-angiogenic peptide; PVDF: polyglycolic acid/polyvinylidene fluoride; SIS: small intestinal submucosa; BMP: bone morphogenetic protein; FB/HA: fibrin/hyaluronic acid; IGF-I: insulin-like growth factor 1; TGF-β1: transforming growth factor beta 1; HCIFC: hat-type cervical intervertebral fusion cage; PLLA: poly(L-lactide); GAG: glycosaminoglycan; MRI: magnetic resonance imaging; ECM: extracellular matrix; DSH: disc space height; IVA: intervertebral angle; LA: lordosis angle.

Disc repair approaches include intradiscal applications of cells, growth factors, and bioscaffolds or cages.

by MRI, radiographical, and histological methods. Although all treated discs retained gel plugs on gross assessment, discs treated with HDC gel seeded with MSCs showed significantly improved disc height and MRI index as compared with discs treated with acellular HDC gel or left untreated. The treatment of anular injury with HDC gel seeded with MSCs could improve the disc tissue (AF/NP) reconstitution and organization.[125] Furthermore, formulations of allogeneic MPCs and pentosan polysulfate (PPS) were used in combination with SCAF at the time of microdiscectomy to improve the capacity of disc restoration in ovine model. Scalpel blade lumbar disc injury was made (5 mm × 3 cm anulotomy plus 200 mg discectomy) at lumbar discs (L2–L5) and injured discs levels were randomized. Randomized discs were treated either with MPC + PPS + SCAF (100 µL containing 5×10^6 MPCs + 125 µg PPS loaded in SCAF piece [5 × 5 × 3 mm]) or treated with SCAF piece alone (5 × 5 × 3 mm) as positive control or left untreated as negative control. Animals were euthanized at six months after surgery for radiological, biochemical, and histological analyses of the lumbar spines. The capacity of disc repair was improved by restoring disc height, disc morphology, and proteoglycan content in discs treated with MPC + PPS + SCAF as compared with that in both positive and negative controls.[126] In addition, two different hydrogels as NP replacement were tested with joint use of an anti-angiogenic peptide or autologous BMCs to restore the biofunctionality of nucleotomized IVDs in an ovine model. For the anti-angiogenic peptide (PEP) approach a hydrogel made of ionic crosslinked methacrylated gellan gum (iGG-MA) was functionalized with non-cytotoxic polylysine-based VEGF-blockers (iGG-MA + PEP), and for the BMC approach a hydrogel made of DDAHA was used (DDAHA + BMC). The harvesting of bone marrow from the iliac crest as well as the isolation of BMCs and the orthotopic implantation of DDAHA + BMCs were combined in a one-step surgical procedure. Directly after scalpel blade neuclotomy of lumbar discs (L1–L6) with removal of 200 mg NP, discs were treated in an alternating sequence with iGG-MA, iGG-MA + PEP, DDAHA or DDAHA + BMC, or left untreated as neuclotomy control. Animals were euthanized after 6 weeks for only histological analysis and after 12 weeks for histological and biomechanical analyses. The six-week group for biomechanics was omitted, because anulur healing was not to be expected at this time period. For biomechanical and histological comparison with intact discs native lumbar spines and lumbar segments from independent comparable sheep were used. Albeit not significant, the DDAHA + BMC treatment slightly tended to decelerate degeneration between 6 and 12 weeks. Apart from that no marked differences in degeneration and biomechanical properties were observed between any of implanted or nucleotomized discs.[127]

Furthermore, cell free biointegrative anulus implants such as polyglycolic acid/PVDF and SIS were used for

anular defect closure in ovine models of anulotomy.[128,129] To investigate the efficacy of PVDF implant standardized box-shaped scalpel blade anular defects (3.5 × 3.5 mm) were made in lumbar discs (L3–L5) and PVDF implant was randomly inserted.[128] Animals were euthanized at 2, 6, and 12 weeks after surgery and explanted lumbar spines were directly analyzed using biomechanical and histological methods. A provocative pressure testing in implanted discs at a mean pressure of 0.53 MPa showed a contrast media leakage between different groups or within each group without any significant differences at 2, 6, and 12 weeks after implantation. However, the intact control discs did not show any contrast media leakage up to the pressure limit of 4.8 MPa. Nevertheless, the PVDF implant consistently provided an effective barrier for herniating NP without any implant dislocation at all time-points. Repair tissue thickness was significantly stronger with the PVDF implant as compared to defect control without implant.[128] To investigate the efficacy of a SIS-based patch and plug scaffold, lumbar discs (L2–L5) were randomly assigned to one of the three treatments after anulotomy (4 × 8 mm lesion with removal of AF to the level of NP): (a) exposure-only (no additional procedure), (b) box anulotomy with screw placement only (anulotomy-only), and (c) box anulotomy with placement of SIS (25 × 14 mm) and titanium screw fixation.[129] At 26 weeks after surgery, pressure-volume testing, MRI, and undecalcified histological evaluation were performed with randomized motion segments. As compared to the anulotomy-only group, the SIS-treated group showed significantly improved MRI signal intensity with new tissue formation that was integrated well with the native AF. The SIS patch and plug reduced the cascade of functional degeneration with substantial functional recovery (66% of its capacity to develop internal pressure).[129] In addition to that, a recombinant BMP13 was used in an ovine model to test its therapeutic effect on injured IVDs.[130] Lumbar discs were directly injected after annular puncture either with BMP-13 (300 μg/70 μL PBS) or 70 μL PBS alone. Discs were analyzed by X-ray at baseline, 2, and 16 weeks after surgery that was followed by evaluations by CT, MRI, and histology immediately after euthanasia at 16 weeks after surgery. Discs injected with BMP-13 showed greater retention of hydration and retarded histological changes with enhanced NPC population and ECM formation as compared with PBS-injected control discs.[130]

Furthermore BMP-2 and BMP-7 that were conjugated to a fibrin/hyaluronic acid (FB/HA) hydrogel allowing a slow release of the BMPs were used in a caprine model of disc degeneration. Mild IVD degeneration in lumbar discs (L1–L6) was induced by injection chondroitinase ABC (CABC; 0.25 unit/mL: CABC dissolved in PBS), whereas the disc (T13/L1) was left as a healthy control. At 12 weeks after CABC injection, injured discs were treated with injection of the FB/HA hydrogels supplemented with either BMP-2 monomer or BMP-2/BMP-7 dimer in two concentrations (1 or 5 μg/mL) or with injection of the FB/HA hydrogel alone as control. At 12 weeks after treatment spines (T13–L6) were harvested for radiographical, MRI, biochemical, and histological analyses. Radiographs showed significant disc height loss upon induction of mild degeneration that correlated with MRI, biochemical, and histological results. Surprisingly, no difference could be confirmed in any parameter between intervention groups. The conjugated BMP-2 and BMP-2/BMP-/7 appeared safe, but no disc regeneration was observed. Possible explanations could be too low dosages and/or insufficient release of the conjugated BMPs.[131] In addition to that of IGF-I and TGF-β1 in combination with a hydroxyapatite (HA)-coated cage (HCIFC: hat-type cervical intervertebral fusion cage) were used in a treatment approach of goat cervical discectomy model. Goats underwent C3/C4 discectomy and intervertebral fusion was performed using: (a) HCIFC coated with HA plus IGF-I and TGF-β1, (b) HCIFC coated with HA, (c) HCIFC only, or (d) autologous tricortical iliac crest bone graft. Radiography and measurements of disc space height (DSH), intervertebral angle (IVA), and lordosis angle (LA) were performed before surgery and at 0, 1, 2, 4, 8, and 12 weeks after surgery. Fused segments were harvested at 12 weeks after surgery to characterize the biomechanics in flexion, extension, axial rotation, and lateral bending with a nondestructive stiffness method. The stiffness of HCIFC coated with HA plus IGF-I and TGF-β1 in flexion, extension, and lateral bending was significantly greater than that of the other groups, and the stiffness of HCIFC coated with HA in extension, axial rotation, and lateral bending was significantly greater than that of fusion with the bone graft and cage (HCIFC) only. All three cage-treated groups showed significantly higher values for DSH, IVA, and LA as well as better fusion compared with the autologous bone graft group. The data indicate that HA plus IGF-I and TGF-β1 coating can improve the fusion effect of the cervical intervertebral cage by enhancing bone fusion.[132] Furthermore, two bioresorbable poly(L-lactic acid) (PLLA) cages of the same size (10 × 10 × 18 mm) with the respective axial compression stiffness of 2 kN/mm (wall thickness of 0.75 mm) or 4 kN/mm (wall thickness of 1.5 mm) were applied in goat model of lumbar discectomy for monosegmental spinal fusion. Their applicabilities were compared with titanium cages with axial compression stiffness of 700 kN/mm, but with the same dimension and wall thickness as the stiff PLLA cages, respectively. PLLA and titanium cages were packed with autogenous iliac bone graft and randomly inserted directly after discectomy of the lumbar disc L3/L4. To estimate interbody fusion within the cages, radiographs were taken immediately after surgery and at the time of euthanasia in a three-year follow-up *in vivo* study. Before euthanasia bones were labeled with tetracycline and calcein to analyze bone remodeling and bone formation within the cages followed by micro-CT and histological analyses of the spinal segments. The three-year follow-up study demonstrated a significantly faster and more complete fusion using PLLA cages as compared to titanium cages of the same dimensions. The PLLA group showed unchanged intervertebral grafting height and progressive replacement of PLLA by trabecular bone. A complete bone remodeling within the cage was achieved after two years and degradation of PLLA with mild tissue reaction was completed at three years after implantation.[133]

Canine models for biological treatment approaches of DDD

IVD degeneration in canines is a prevalent disorder. Diseases in canines that are associated with disc degeneration are among the major reasons for euthanasia of canines younger than 10 years old. Disc degeneration-associated diseases in canines include disc herniation, degenerative lumbosacral stenosis (DLSS), and cervical spondylomyelopathy. Several similarities have been shown between IVD degenerations in humans and in chondrodystrophoid breed canines: Similar to that in humans, degeneration of IVD with or without clinical signs of disease is prevalent in chondrodystrophoid canines, and it is more common in older canines than in younger onces; chondrodystrophoid canines exhibit loss of notochordal disc cells at the early young age and suffer spontaneous disc degeneration with reduction in proteoglycan content, gross pathology, and chronic low back pain as humans. Thus, typically chondrodystrophoid canines have been used to study the processes of IVD degeneration and to prove regenerative effects of therapeutic approaches.[134,135]

Intradiscal biological therapy approaches in canine models of disc degeneration. Autologous or allogeneic cells isolated from bone marrow (MSCs) and from NP tissue (NPCs), or from human umbilical cord matrix (WJCs: Wharton's jelly cells) were applied in degenerative discs of canines. Moreover, supporting biomaterials without any supplement or in combination with cells or bioactive factors have been intradiscally applied[136–147] (Table 9).

Autologous MSCs of different concentrations (1×10^5, 10^6, or 10^7 per disc) were transplanted into degenerative discs of beagle dogs to determine the optimal donor cell number for maximum benefit of cell transplantation. At four weeks after induction of lumbar disc degeneration (L3–L6) by aspiration of NP tissue (15 mg NP), the different amount of MSCs that were transduced with RV-GFP were transplanted into the injured discs in a randomized

Table 9. Biological approaches of disc repair in canine models of disc degeneration.

Animal Model	Transplanted cell/ Gene modification	Implanted biomaterial	Disc regeneration effect	Ref.
Dog: Lumbar disc NP aspiration	Autologous MSC/RV-GFP (1×10^5, 10^6, 10^7 cell/disc)		Well preserved disc height and ECM structure, but highest cell survival rate at 1×10^6 MSCs	136
Dog: Lumbar disc natural degenerated	Autologous MSC	TGF-β1 crosslinked collagen μ-carriers	Marked clinical improvement, without restoration of disc tissue	137
Dog: Lumbar disc nucleotomy or natural degenerated	Allogeneic MSC	NCM: Notochordal cell-derived matrix of healthy porcine	NCM improved disc height, MRI index, collagen II level, and ameliorated local inflammation	138
Dog: Lumbar disc NP aspiration	Autologous normal or cryopreserved NPC /AdV-GFP, activated with MSCs		Improved MRI signal intensity, disc height, enhanced levels of proteoglycan, collagen II with normal, or cryopreserved NPCs	139
Dog: Lumbar disc nucleotomy	Allogeneic NPC, PHK-26 marked	PLGA scaffold	Improved disc height, MRI index, and segmental stability	140
Dog: Lumbar disc NP aspiration	Wharton's jelly cells (WJCs/AAV2-GFP)		Improved disc tissue structure, disc height, stability, MRI grade	141
Dog: Lumbar disc natural degenerated		PEAMs: Polyester amide microspheres	Good compatibility for safe and sustained release of therapeutics	142
Dog: Lumbar disc NP aspiration		PEAMs loaded with celecoxib (CXB)	Decreased PGE2 level and NGF immunopositivity with boosted proteoglycan level in NP	143
Dog: Lumbar disc nucleotomy		PEAMs loaded with triamcinolone-acetonide (TAA)	Lower NGF immunopositivity, but absence of beneficial effects on disc tissue regeneration	144
Dog: Lumbar disc natural degenerated		PCLA-PEG-PCLA hydrogel loaded by celecoxib (CXB)	Clinical improvement with recurred or reduced back pain, but no disc tissue regeneration	145
Dog: Lumbar disc natural degenerated		rhBMP-7: dose/disc 2.5, 25, and 250 μg	No regenerative effect at all and wide extradiscal bone formation	146
Dog: Lumbar disc discectomy		γ-irradiated disc allografts (18, 25 or 50 kGy)	Dose-dependent allografts decellularization with increased segmental degeneration	147

MSC: mesenchymal stem cell; RV: retroviral vector; GFP: green fluorescent protein; NPC: nucleus pulposus cell; AdV: adenoviral vector; WJC: Wharton's jelly cell; AAV2: adeno-associated virus vector serotype 2; PLGA: poly L-lactic-co-glycolic acid; PEAMs: polyester amide microspheres; TAA: triamcinolone-acetonide; PCLA-PEG-PCLA: poly(ε-caprolactone-co-lactide)-b-poly(ethyleneglycol)-b-poly(ε-caprolactone-co-lactide); CXB: celecoxib; ECM: extracellular matrix; MRI: magnetic resonance imaging; PGE2: prostaglandin E2; NGF: nerve growth factor.
Treatment approaches include intradiscal application of cells, bioactive factors, biomaterial, or a combination thereof.

sequence. Radiographs and MRIs were taken at baseline, 4, 8, and 12 weeks after initial surgery that was followed by gross anatomical, histological, and apoptotical assessments at 12 weeks. As compared with injured discs without cell transplantation, discs transplanted with MSCs exhibited better preservation of the disc height and anular structure. However, higher number of remaining transplanted MSC cells and higher survival rate of NPCs with ECM maintenance were found in discs transplanted with 1×10^6 MSCs. Less viable cells were detected in discs transplanted with 1×10^5 MSCs, and more apoptotic cells in discs transplanted with 1×10^7 MSCs.[136] Furthermore, the suitability of intradiscal application of autologous MSCs was proved in a canine model of disc degeneration that recapitulates the spontaneous natural IVD degeneration occurring in humans. The study involved client-owned dogs with clinical signs of low back pain and MRI findings that confirmed the diagnosis of DLSS caused by a degenerated and centrally protruded L7–S1 disc. Dog breeds included: Belgian Malinois, Labrador Retriever, German Shepherd, Berger Blanc Suisse, Rhodesian Ridgeback, German Longhair, and Dalmatian. The dogs underwent decompressive spinal surgery and received an intradiscal injection of autologous bone marrow-derived MSCs that were attached on collagen microcarriers crosslinked with TGF-β1 (3×10^6 MSCs/250 mg collagen microcarriers plus100 ng/mL of TGF-β1) or without TGF-β1 crosslinking in 500 μL PBS suspension, along with a control suspension of microcarriers alone. Clinical performance and Pfirrmann grading of MRI were completed at 10 months after injection. Although marked clinical improvement was observed in all groups with clinical functioning restored back to normality, neither microcarriers nor MSCs could restore the structure of degenerated discs. Postoperative Pfirrmann grade remained equivalent in all dogs, and formation of Schmorl's nodes was detected in 11% of dogs.[137] In addition, allogeneic MSCs in combination with notochordal cell-derived matrix (NCM) of healthy porcine were used in two beagle dog models of disc degeneration: (a) a spontaneous natural degeneration model (mild degeneration) and (b) a neuclotomy induced degeneration model (moderate degeneration). Five lumbar discs (L1–L6) were injected with NCM, MSCs, NCM-MSCs, or left uninjected at six weeks after neuclotomy; whereas two NCM-treated IVDs per dog received an additional NCM injection at three months after the first injection. Radiological, macroscopical, histological, and biochemical analyses were performed at six months after the first injection to evaluate the effects of treatments on mildly and moderately degenerated discs. Injection of NCM in moderately (induced) degenerated discs stimulated collagen type II rich ECM production, improved the disc height, MRI signal intensity, and ameliorated local inflammation. But injection of MSCs exerted no (additive) effects.[138] Moreover, activated normal NPCs and cryopreserved NPCs have been used in a dog model of disc degeneration to compare their disc regeneration effect after transplantation. If NPCs that are cryopreserved show regeneration ability, then autologous cell transplantation could be offered to patients as and when required. Activated NPCs were obtained by coculturing of NPCs and bone marrow MSCs using the indirect cell-to-cell contact method. Disc degeneration (L2–L5) was induced by NP aspiration (15 mg NP) and bone marrow blood was aspirated at the same time from the iliac crest to isolate MSCs. Isolated NPCs that were cryopreserved for two weeks and normal NPCs were activated by coculturing with MSCs and transduced with AdV vectors encoding for GFP (AdV-GFP). At three weeks after the first surgery, cells were transplanted into one of the injured discs (L2–L5; 1×10^6 cells/100 μL of medium), where L5/L6 disc served as uninjured control. Radiological, MRI, gross anatomical, histological, and immunohistochemical examinations performed up to 12 weeks after initial surgery showed a similar attenuation of IVD degeneration in discs transplanted with cryopreserved or normal NPCs. Disc height, MRI signal intensity, and contents of proteoglycan and collagen II were significantly improved with improved histological findings in both cell transplantation groups as compared with injured untreated group.[139] Additionally, expanded and PHK-26 marked allogeneic NPCs were transplanted in combination with a three-dimensional porous PLGA scaffold into lumbar discs of beagle dogs after nucleotomy. Directly following nucleotomy injured discs were randomly injected either with NPC-PLGA composite (1×10^6 cells/100 μL) or with PLGA scaffold alone (100 μL) or left untreated. The regenerative effect of the composite was evaluated up to eight weeks after injection using X-rays and MRIs. Discs treated with the NPC-PLGA composite showed well-preserved disc height, segmental stability, and improved MRI signal intensity as compared with discs treated with PLGA alone or left untreated. Moreover, PHK-26-marked NPCs were found within the area of the NP up to eight weeks after treatment.[140] Furthermore, the survival and regenerative ability of WJCs were examined after intradiscal transplantation in beagles. At four weeks after injury operation (aspiration of NP) of the lumbar discs (L4–L7), the L6/L7 discs were injected with WJCs (1×10^6 cells/100 μL PBS) that were transduced with AAV2 vectors encoding for GFP (AAV2-GFP), while L5/L6 discs were injected with 100 μL PBS, L4/L5 discs were left untreated and L3/L4 intact discs served as control. Radiological and MRI analyses were performed at baseline, 4, 8, 12, and 24 weeks after the initial surgery that were followed by biomechanical, histological, immunohistochemical, macroscopical, and cell survival analyses at the 24th week. As compared with untreated or PBS-treated discs, WJCs-treated discs showed upregulation of aggrecan, collagen II, and SOX-9 with significantly smaller reduction of disc height and MRI signal intensity. Improved spinal segmental stability, preserved disc tissue structure, and enhanced survival of transplanted cells were confirmed up to 24 weeks.[141]

Biomaterials that have a good cytocompatibility and biocompatibility are considered as safe and sustained release systems for intradiscal delivery of bioactive factors that support disc regeneration or pain relief. Polyester amide microspheres (PEAMs), that were tested for safety of intradiscal injection and biocompatibility at long-term (24 weeks) follow-up in a canine model predisposed to spontaneous natural disc degeneration, did not induce

degenerative changes in T12–L5 discs of beagle dogs after injection of 40 μL PEAMs. The results determined by MRI, macroscopical, histopathological, and biochemical methods showed a good cytocompatibility and biocompatibility of PEAMs, suggesting them as safe and sustained release system for intradiscal delivery of bioactive factors.[142] Accordingly, PEAMs were used for intradiscal delivery and controlled release of celecoxib (CXB), the nonsteroidal anti-inflammatory cyclooxygenase-2 (COX-2) inhibitor. At four weeks after needle aspiration of the NP at five alternating levels (T12–T13, L1–L2, L3–L4, L5–L6, and L7–S1), CXB-loaded PEAMs or unloaded PEAMs were injected in a random fashion: 40 μL of low dose CXB-PEAMs (0.72 mg/mL particles plus 8.4 μg CXB) or 40 μL high dose CXB-PEAMs (72 mg/mL particles plus 280 μg CXB) both as duplicates, or 40 μL of PEAMs without CXB (70 mg/mL in PBS). At 12 weeks after treatment MRI, CT, macroscopical, histopathological, immunohistochemical, and biochemical evaluations were performed. The results showed no evidence of adverse effects by intradiscal and sustained delivery of CXB. Quite the contrary, the local and sustained delivery of CXB prevented progression of IVD degeneration by enhancing the proteoglycan content and decreasing the PGE2 level and NGF immunopositivity in NP. The results provided evidence that intradiscal delivery of CXB could address the pain-related IVD degeneration.[143] Likewise, PEAMs were used for intradiscal delivery and controlled release of triamcinolone acetonide (TAA), an anti-inflammatory corticosteroid. The purpose was to achieve a prolonged release of TAA with pain relief and to circumvent adverse effects usually caused by high dosages of corticosteroids, which are used to relieve symptoms of chronic low back pain. At four weeks after nucleotomy that was performed at five levels (T12–T13, L1–L2, L3–L4, L5–L6, and L7–S1), randomized lumbar discs were injected with 40 μL of low dose TAA-PEAMs (0.72 mg/mL particles; 8.4 μg TAA) or 40 μL of high dose TAA-PEAMs (72 mg/mL particles; 0.84 mg TAA) both as duplicates, or 40 μL of PEAMs alone (70 mg/mL in PBS). All adjacent non-nucleotomized noninjected lumbar discs were included as controls. At 12 weeks after treatment, discs were evaluated using MRI, CT, histopathological, immunohistochemical, and biochemical methods. The results showed that the low dosage of TAA-PEAMs with sustained intradiscal release of TAA significantly reduced NGF immunopositivity in degenerated NP tissue, suggesting its potential applicability for pain relief. However, discs treated with TAA-PEAMs did not show any beneficial effect on tissue regeneration. They showed no effect on disc height, MRI index, DNA content, and levels of prostaglandin E2 (PGE2), collagen, and GAG.[144] In addition, the COX-2 inhibitor (CXB) was also used in combination with a thermoresponsive PCLA-PEG-PCLA hydrogel (poly(ε-caprolactone-co-lactide)-b-poly(ethylene glycol)-b-poly(ε-caprolactone-co-lactide hydrogel) in a canine model of spontaneous natural IVD degeneration with chronic low back pain. Since previously IVD samples that were surgically obtained from canine patients showed increased COX-2 expression that specified the role of COX-2 in clinical IVD disease, patient dogs were selected to receive an intradiscal injection of CXB-loaded hydrogel (PCLA-PEG-PCLA). The long-term follow-up (24 weeks) after intradiscal injection showed no adverse reactions and no influence on MRI signal intensity. However, clinical examinations and owner questionnaires discovered that the back pain was recurred after three months in 3 of 10 dogs, and a reduction of back pain was achieved with clinical improvement in 9 of 10 dogs.[145] In another long-term follow-up (24 weeks) study, the regenerative effect of human bone morphogenetic protein-7 (rhBMP-7) was examined in a canine model of spontaneous natural disc degeneration (T13–L6) at three different dosages (2.5, 25, and 250 μg administered in a 40 μL injection volume per disc). MRI scans were completed in the course of the 24 weeks that were followed by postmortem evaluations of IVD tissues using radiographical, histological, and biochemical methods. The results exhibited no regenerative effect in all discs treated with different dosages of rhBMP-7. On the contrary, treatments with rhBMP-7 resulted in extensive extradiscal bone formation, suggesting that injection of rhBMP-7 should not be used for treatment of IVD degeneration.[146]

Moreover, a canine model of spontaneous IVD degeneration was used to explore the regenerative potential of decellularized disc allografts treated by 6°Co gamma irradiation for biological treatment of DDD. Beagle dogs received single-level allografted disc treated with different doses of gamma irradiation (irradiated with 18, 25 or 50-kGy doses and 0-kGy control group with no irradiation), where the cell viability and biomechanical properties of the allograft discs were previously *in vitro* determined among the dosage groups. After random removal of a disc between L2 and L6 levels, a disc allograft of the most compatible size was selected, trimmed according to the transplanted segmental space and positioned into the slot of the excised disc. Radiologic assessments were performed before the transplantation and at baseline, one, three, and six months after transplantation that were followed by biomechanical testing, macroscopical, and morphological evaluations at six months after operation. There were significant differences of cell viabilities among the various dosage groups, but there were no obvious differences of the biomechanical properties among the various dosage groups. The disc height and ROM decreased significantly in all groups as time went on. The disc height index, ROM, hydration status as well as segmental regeneration in irradiated groups were inferior to those in the control group, but they were better in 18-kGy group than those in 25 and 50-kGy groups.[147]

Non-human primate models for biological treatment approaches of DDD

Non-human primates are the closest animal models to humans regarding anatomy, genetics, physiology, and behavior, but due to ethical restriction they have been more rarely used for therapeutic studies of DDDs. Although they are quadrupedal for locomotion, they spend plenty of time in semi-upright or upright positions and their discs are more exposed to mechanical stresses similarly to human discs. Although the methods to

accelerate progressions of disc degeneration are established in non-human primate models such as rhesus macaques and baboons, regenerative approaches of DDD have been sparsely attempted.[148–156]

In vivo regenerative approaches in non-human primate models of disc degeneration. Intradiscal gene therapeutic approach mediated by AAV2 encoding for CTGF and TIMP1 as well as applications of biocompatible or biodegradable scaffold, such as fresh frozen disc allograft, PVA hydrogels, and HA-coated porous ingrowth surfaces, have been tried in degenerative discs of rhesus macaques and baboons[148–152] (Table 10). A gene therapeutic approach of degenerative discs was attempted in rhesus macaques by intradiscal injection AAV2 vectors encoding for CTGF and TIMP1 genes (AAV2-CTGF-TIMP1). At 12 weeks after induction of lumbar disc degeneration (L1–L6) by annular needle puncture, where the success degeneration was proved by MRI at 4, 8, and 12 weeks after surgery, the L1–L4 discs were injected with 50 μL of AAV2-CTGF-TIMP1 (1×10^{12} pfu/L) as gene therapy group, while the L4–L6 discs were assigned as negative control group and injected with 50 μL PBS alone. The L6–L7 and L7–S1 discs that received neither needle puncture nor treatment were assigned as blank control group. At 8, 16, and 24 weeks after injection animals were euthanized to acquire the lumbar discs (L1–S1) and evaluate them using histological, immunohistochemical, and biochemical methods. An intradiscal injection of AAV2-CTGF-TIMP1 could enhance the mRNA expression levels of CTGF and TIMP1with higher synthesis of collagen II and aggrecan in NP tissue, indicating the regenerative effects of AAV2-CTGF-TIMP1 in degenerated lumbar discs of rhesus macaques.[148] Furthermore, rhesus macaques were used for examining the possibility of using fresh frozen IVD allograft in disc transplantation. Examinations were associated with the allograft survival, its maintenance of disc tissue metabolism, segmental mobility, and problems of rejection in short- and long-term follow-up after transplantation. Lumbar discs of donor monkeys were isolated and directly fresh deep-frozen. The fresh frozen allografts were randomly divided into a short-term group (followed up for 2, 4, 6, and 8 weeks, respectively), a midterm group (6 months) and a long-term group (24 months). Lumbar disc (L5/L6) replacement in recipient monkeys with a complete discectomy was performed, and after disc replacement the changes in fresh frozen disc allografts were evaluated using radiographical, histological, biochemical, and biomechanical methods. The allografts were able to survive through a deep freezing protocol and maintain cell viability after transplantation without significant immunoreaction. The biomechanical properties of stability and mobility of the transplanted allografts were similar to those of the controls. However, the radiographical and histological examinations showed severe disc degeneration at 24 months of follow-up with disc height decrease mainly in the early postoperative stage. Similarly, decrease in hydration as well as in proteoglycan and hydroxyproline contents of the allografts were confirmed at 6 and 24 months of follow-up. Taken together, the allografts were able to maintain stability and mobility of the functional spinal unit, but degeneration of the allograft was inevitable.[149] Moreover, baboons have been used for preclinical evaluation of a PVA hydrogel implant as a replacement for a diseased NP. A single-level discectomy was performed at lumbar discs (L3/L4 or L4/L5) with either cup forceps (2.5 mm tip diameter) or IVD rongeurs with a rectangular cross section (maximum dimension 6.0 mm) and the nuclear cavity was left empty. A hydrogel implant was injected into the nuclear cavity by means of a custom insertion device (PVA treatment group), and then animals were housed individually in indoor cages for the first month (4 weeks) and returned to gang cages for the rest of the study period (23 months). Follow-up evaluations over 24 months after surgery included: radiographical analyses (before surgery, immediately after surgery and at the time of euthanasia), MRI (at 2 weeks after surgery and at 1–3 days before euthanasia) as well as postmortem gross pathological and histopathological analyses of both local and remote tissues. The PVA implants were well tolerated over 24 months with no evidence of implant-related pathology in the adjacent disc tissue, spinal cord, or remote

Table 10. Intradiscal regenerative approaches in non-human primate models.

Animal Model	Implanted material	Disc regeneration effect	Ref.
Rhesus macaque: Lumbar disc puncture injury	AAV2-CTGF-TIMP1	Enhanced expression levels of CTGF and TIMP1 with increased synthesis of collagen II and aggrecan in NP tissue	148
Rhesus macaque: Lumbar disc complete discectomy	Fresh frozen IVD allograft	Maintained stability and mobility of the spinal unit with high cell viability, but severe allograft degeneration was certain in long-term follow-up	149
Baboon: Lumbar disc complete nucleotomy	PVA hydrogel implant	No implant-related pathology in the adjacent disc tissue, spinal cord, or remote tissues, but the ability as prosthetic NP replacement not proved	150
Baboon: Lumbar disc complete discectomy with EP decortication	AcroFlex and SB Charitè implants with HA-coated porous ingrowth surfaces	Excellent ingrowth at the level of the implant-bone interface, with no accumulation of wear debris or inflammatory cytokines	151,152

AAV2: adeno-associated virus vector serotype 2; CTGF: connective tissue growth factor; TIMP1: metalloproteinase inhibitor 1; IVD: intervertebral disc; PVA: polyvinyl alcohol; HA: hydroxyapatite; NP: nucleus pulposus.
Treatment approaches include applications of recombinant AAV2, fresh frozen disc allograft, PVA hydrogel implant, and hydroxyapatite coated porous ingrowth surfaces on disc prostheses.

tissues. However, additional studies are needed to verify the efficacy of the implant in its intended application as a prosthetic replacement for the diseased NP.[150] In addition, baboons were used for an *in vivo* comparison of two different types of unconstrained disc prostheses owing alternate in-growth surfaces for total disc replacement arthroplasty: (a) the AcroFlex device (consisted of sintered titanium beaded HA-coated ingrowth surfaces, bound together by a hexene-based polyolefin rubber core) and (b) the SB Charitè device (consisted of prosthetic VEPs from cobalt chrome, covered by two layers of thin titanium with an electrochemically bonded HA coating and an ultra-high molecular weight polyethylene core). After a complete discectomy of L5/L6 lumbar discs (AF/NP) and burr decortication of the VEPs, one of the implant devices per animal was inserted using a customized distractor and implant position was verified with anteroposterior and lateral fluoroscopic images. Following six-month survival periods the implants were evaluated using multidirectional flexibility testing, radiographical, histological, and immunohistological methods. Compared to the AcroFlex treatments, the SB Charitè treatments showed greater ROM under axial compression, flexion-extension, and lateral bending, but it exhibited similar ROM to the intact non-surgical controls. No loosening of any metallic prosthetic VEP was seen and excellent ingrowth at the level of the implant–bone interface was demonstrated on both AcroFlex and SB Charité prosthesis. Moreover, no local and systemic accumulation of particulate wear debris (titanium, polyethylene or cobalt chrome) or cytokines (TNFα, PGE2, IL-1, IL-2 or IL-6) were detected. The porous ingrowth calculations showed the mean ingrowth (linear apposition) ranging from $47.9 \pm 9.12\%$ for the SB Charitè device and $54.59 \pm 13.24\%$ for the AcroFlex device, which is more favorable for total disc replacement as compared to that affirmed for cementless total joint components in the appendicular skeleton (range 10–30%).[151,152] Above and beyond, baboons were used for *in vivo* testing of a technique that was able to sensitively monitor dynamic changes in strain and load during extreme activity. The technique was suggested to provide key biomechanical information that could be essential for designing of suitable implants. For that an intradiscal implant-load-cell connected to a telemetry transmitter was surgically placed in L4/L5 disc space of baboons to measure real-time *in vivo* loads that were imposed on the lumbar spine. Implants placed into the disc space and the transmitter in the flank allowed the collection of load data from the animals during activities.[153]

Discussions

For choosing appropriate animal models of disc degeneration and regeneration important points should be considered that might reflect the clinical facts of DDD in humans. Considerations for choosing an appropriate animal model include for example: the intradiscal deficiency of notochordal cells, the size of the animal and its IVD dimension relative to humans, the biomechanical stress exerting on the IVDs, and ethical concerns. Moreover, the significance of spontaneous IVD degeneration (example: aging or genetic predisposition) in comparison to mechanistical induced IVD degeneration (chemical, mechanical or surgical induced injuries) must be well thought out during the design of research projects involving animal models.

Notochordal cell loss in IVD is thought to precede the onset of IVD degeneration in humans, although the vital role of notochordal cells in initiating IVD degeneration it is still uncertain and their function in healthy discs during childhood are not yet clearly understood. Nonetheless, IVD degeneration that is characterized by changes in the mechanical integrity and biochemical composition in discs, especially in NP, is thought to be associated with the disappearance of notochordal cells in adult IVDs. In some animal species, notochordal cells steadily disappear during aging alike in humans, while in other species they persist for a long period of time after maturity or throughout their matured life. Different animal models of IVD degeneration, which differ significantly in the status of notochordal cell disappearance, have been used for regenerative approaches of DDD. Based on the literature, matured chondrodystrophic dogs, goats, sheeps, and non-human primates are the most suitable species regarding the cell populations mimicking those found in adult humans. Nevertheless, animal models that retain their notochordal cells could also be beneficial to understand the cell biology during development, maturation, degeneration, and maintenance of IVDs.[67,157–158]

Initiations of IVD degeneration in most animal models have been achieved using chemical, mechanical, or surgical interventions, whereas in some animals it occurs spontaneously. The success of regenerative treatment approaches could significantly depend on the standardization and replicability of the degeneration (intervention) mechanisms along with the severity and duration of the degeneration. Regarding spontaneous IVD degeneration in animals, the chondrodystrophoid canine,[134,159] non-human primates[160,161] along with genetically altered rabbits,[162,163] mice[52,164,165], and sand rats[166,167] are well described. However, the use of spontaneous models of disc degeneration is often limited by the long time course of degeneration and unanticipated progression of degeneration, which is combined with the high ethical consideration of using non-human primates and canine.

Different chemicals, for example chymopapain, collagenase, CABC, and Fn-f, have been used to induce the pathophysiological process of disc degeneration. Chemical agents can induce IVD degeneration by promoting the breakdown of GAG chains of proteoglycans that impair structural integrity of the IVDs. However, some chemical agents like CABC can affect the viability of disc cells, which in turn limits the restoration of the disc ECM.[88,168–171]

Mechanical models of disc degeneration are more suitable to ensure the benefit of introducing highly replicable degenerative cascade at a defined time point. Mechanical models of disc degeneration include the applications of static and cyclical compression or bending. They are mostly applied on rodent tails by using external fixation device (Ilizarov-type apparatus), which allows the application of mechanical force across the tail IVDs, or by attachment of an external loading device that allows the

application of compressive force on the lumbar spine of rats or rabbits.[55,65,172–176]

The most commonly used and well-established methods for initiation of IVD degeneration are surgical interventions that introduce injuries to AF or NP. Partial thickness anular injuries are mainly consisting of a smaller incision depth ($\leq$5 mm) or a smaller size anular injury that do not damage the inner AF and NP at the time of injury, and they persuade slower degenerative processes. Conversely, full thickness anular injuries are consisting of annular incisions accompanied by partial removal of AF and NP tissues, and they induce NP avulsion with hastier degeneration. However, great care must be taken to create a highly replicable disc injury.[121,177–180]

Initiation and progression IVD degeneration in animal models that can be confirmed by radiological, histological, biochemical, and biomechanical findings are essential to comprehend the process of disc degeneration on the bases of particular provocations. Although such findings are crucial markers of disc degeneration, their relation to pain is not yet well established in most animal models, which may affect their relevance for clinical translation. Pain assessments have been performed mainly in rodent models of disc degeneration. These include for example an intensified grooming, wet-dog-shake, declined locomotor capability, declined rotarod performance, sensitivity to thermal or mechanical stimuli (hind paw mechanical sensitivity) and declined general condition of the animals.[177,181–184] Pain assessments, especially in large animal models of disc degeneration, could significantly improve our current knowledge about the progression and therapy of DDD.

Intradiscal biological treatment approaches have been tried in different animal models of disc degeneration. The animal models used to test new biological therapeutic approaches include mice, rats, rabbits, porcine, ovine, caprine, canine, rhesus macaques, and baboons, which had developed disc degeneration either spontaneously or after chemical, mechanical, or surgical interventions. The animal experiments included: cell transplantation approaches with or without gene modification, *in situ* gene therapeutical approaches, intradiscal applications of bioactive factors and bioscaffolds or a combination thereof. These preclinical treatment approaches and their effects of disc regeneration in different animal models are promising (Tables 1 to 10) and may help to formulate regenerative therapy strategies for clinical applications. Different types of autologous, allogeneic, or human derived cells were intradiscally tested. These included: chondrocytes (NP and AF cells), bone marrow-derived MSCs, bone marrow-derived mesenchymal precursor cells, ADSCs, iPSCs, ESCs, multipotent stem cell from the olfactory mucosa, and umbilical cord tissue-derived MSCs. The intradiscal applications of bioactive factors included: cytokines, antibodies, kinases, shRNAs, exosomes, lignans, and growth factors. Regenerative *in situ* gene therapy approaches included: retroviral, LV, AdV, and AAV vectors carrying candidate therapeutic genes. However, safety assessments, especially during *in situ* gene therapy approaches with certain viral constructs, are essential, because misdirected injection, incorrect dose, and immune reactions near to sensitive neural structure could provoke toxicity and immunological side-effects with neurological deficits.

IVD degeneration in human is a complex and multifactorial process involving for example cellular senescence, mechanical stresses, nutritional deprivation, and genetical predisposition. The complex and multifactorial process indicates that no animal model is able to profoundly reproduce the clinical condition in humans. Nevertheless, progresses have been achieved from biological treatment approaches in animal models to clinical trials.[185–188] For example, transplantation of chondrocytes, harvested from damaged cartilaginous tissues and expanded in culture under controlled and defined conditions, was performed in a controlled, randomized and multicenter study (EuroDISC), which compared the safety and efficacy of autologous disc chondrocyte transplantation in patients undergoing single level discectomy. The interim analysis of 28 patients that was performed after two years could show improved long-term pain relief, maintained disc height, and enhanced hydration, as compared to discs that had undergone discectomy without cell transplantation.[185] Furthermore, in two small noncontrolled clinical trials expanded autologous bone marrow-derived MSCs were percutaneously injected in 12 patients, which had low back pain of DDD and unsuccessful conservative treatment. At two years after transplantation, the clinical studies showed similar results: improved long-term pain relief, maintained disc height, and enhanced hydration.[186,187] Moreover, a multicenter, randomized, and controlled trial of a phase II study was performed with allogeneic mesenchymal precursor cells (MPCs). The trial included 100 patients with moderate to severe low back pain that persisted for more than six months and caused by early disc degeneration. The results after 12 months showed that intradiscal injection of allogeneic MPCs was well tolerated and reduced back pain with improved disc functions. In addition, it reduced opioid use and the need for additional interventions.[188]

Conclusions

This review article provides a detailed and an up-to-date overview of biological treatment strategies in all small and large animal models used thus far in regenerative research of degenerative discs. Biological treatment strategies include applications of cell transplantation, gene therapy, bioscaffolds, and bioactive factors, or a combination thereof, which may able to address options of restoring the structural or biological deterioration of degenerative discs. However, it is limited to *in vivo* and intradiscal biological treatment strategies in research animal models and does not include studies based on non-intradiscal, non-biological, or *in vitro* strategies. In conclusion, although no animal model is able to profoundly reproduce the clinical conditions in humans, the various animal models have played important roles in specifying our knowledge about the pathophysiology of DDD. They are crucial for developing new and hopeful therapy approaches for clinical applications.

AUTHORS' CONTRIBUTIONS

All authors participated in the design, interpretation of the studies, and analysis of the data and review of the manuscript. DSM did the conception and design of the article, DSM, TW, and AB performed the acquisition of data, and DSM, TW, AB, and CT completed the analysis and interpretation of data, revision, and final approval of the article.

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