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REVIEW

Enhancing human nucleus pulposus cells for biological treatment approaches of degenerative intervertebral disc diseases: a systematic review

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Abstract

Intervertebral disc (IVD) degeneration has been described as an aberrant, cell-mediated, age- and genetics-dependent molecular degeneration process, which can be accelerated by nutritional, mechanical and toxic factors. Collective involvement of these factors can result in structural failures, which are often associated with pain. Current treatment approaches are restricted to symptomatic therapies, not addressing options of restoring structural or biological deterioration of the IVD as the underlying problem. Therapeutic potentials of IVD cell transplantation, biomaterials, inhibiting or activating bioactive factors, including gene-therapeutic approaches, have been shown *in vitro* or in small animal models. Since human degenerative IVD cells display distinctive features with regard to cell biology and regenerative potential, we attempted a systematic review, investigating the *in vitro* response of human nucleus pulposus cells to different stimuli. Therefore, we conducted an electronic database search on Medline through July 2011 to identify, compare and discuss publications concerning the effects of cell–cell stimulation, bioactive factors, biomaterials and combinations thereof in terms of cell isolation, proliferation, differentiation and matrix protein synthesis. This survey and discussion might serve as a source for designing future biological treatment strategies for the human IVD.

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1. Introduction

1.1. The intervertebral disc (IVD)

IVDs have a specific load-bearing organization that spreads loading evenly on the vertebral bodies, resists spinal compression and provides flexibility in the spine. IVD functions are often discussed in terms of: (a) the highly hydrated nucleus pulposus (NP), consisting mainly of proteoglycan, hyaluronan and type II collagen; and (b) the radially aligned type I collagen fibrils of the annulus fibrosus (AF) (Roughley, 2004). The swelling of a hydrated NP is resisted radially by the collagen fibrils of the AF and axially by the vertebral endplates, and provides the ability to withstand the compressive loads due to weight and bending. Additionally, the collagen fibrils of the AF offer the capacity to resist the tensile forces encountered during twisting and bending (Adams and Dolan, 2005).

1.2. IVD degeneration

A degenerated IVD is characterized by structural failure together with accelerated or advanced signs of ageing (Adams and Roughley, 2006), often accompanied by inflammatory and patho-immunological processes (Kaneyama *et al*., 2008; Hoyland *et al*., 2008). Painful IVD disc degeneration is referred to as 'degenerative disc disease'. When considering regenerative strategies to treat degenerative disc disease, however, we have to keep in mind that, ultimately, our main objective is the elimination of back pain

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for the patients. In the literature, three major causes for the development of pain are suggested: (a) segmental spinal instabilities and pathological loading patterns caused by proceeding degeneration and aggravated by many surgical interventions (Panjabi *et al.*, 1984; McNally *et al.*, 1996; Panjabi 2003); (b) pathological ingrowths of nerves into the inner layers of the torn annulus fibrosus, sometimes even penetrating the nucleus pulposus (Freemont *et al.*, 1997; Peng *et al.*, 2005); and (c) pain-mediating inflammatory cytokines, such as TNF-α and IL-1 secreted by IVD cells (Burke *et al.*, 2002; Mulleman *et al.*, 2006). Human IVDs, however, have only very limited self-repair capabilities. Therefore, the biological IVD cell manipulation techniques discussed in this review might prove to be valuable options or components of future treatments for degenerative disc disease.

1.3. Nucleus pulposus (NP) cells and matrix in ageing and degeneration

NP cell density declines considerably during childhood and adolescence and remains at a low level in adulthood (Maroudas *et al.*, 1975; Ishii *et al.*, 1991; Hastreiter *et al.*, 2001; Liebscher *et al.*, 2011). A recent comprehensive study reports cell densities of 2204 (SD636) cells/mm^3 in a middle-aged population and 2783(SD2,007) cells/mm^3 in an elderly population (Liebscher *et al.*, 2011). No correlations of cell density to adulthood age or histological grade of degeneration were found in this study. Some previous studies reported even increased cell densities in highly degenerated IVDs (Ishii *et al.*, 1991; Hastreiter *et al.*, 2001). These findings may indicate that degenerative changes are predominately due to a loss of NP cell functionality, rather than to a loss of NP cells.

With ageing, NP cells are subject to senescence and thereby lose their ability to proliferate and replace cells lost to necrosis or apoptosis (Roberts *et al.*, 2006; Gruber *et al.*, 2007). Moreover, decreased anabolism or increased catabolism of senescent cells may facilitate IVD degeneration (West *et al.*, 1989). Large proportions of IVD cells (up to 92%) were reported to be SA-β-Gal-positive for senescence in middle-aged patients undergoing IVD surgery (Gruber *et al.*, 2007). Beside an apparent correlation of degeneration with the prevalence of senescence (Gruber *et al.*, 2007), another study showed a distinct difference of prevalence between patients operated for disc herniations (13%) and patients operated for discogenic back pain (0.9%) (Roberts *et al.*, 2006).

Metabolite transport, among other factors important for nutrition, has been explored mostly in animal models. It is mainly achieved by diffusion through the vertebral endplates and the annulus fibrosus (Urban *et al.*, 1977) and can be significantly impaired by IVD degeneration (Benneker *et al.*, 2005). Due to the subsequent low oxygen tension, NP cells, by nature specialized in an anaerobic metabolism, produce elevated concentrations of lactic acid, resulting in a lowered pH (Urban *et al.*, 2004; Grunhagen *et al.*, 2006). Although NP cells are very resistant to low oxygen tension (Bibby and Urban, 2004; Bibby *et al.*, 2005), they are very sensitive to larger decreases of pH (Ohshima and Urban, 1992; Bibby *et al.*, 2005), and especially to decreases of glucose concentration, in terms of impaired matrix production and cell viability (Bibby *et al.*, 2002; Bibby and Urban, 2004; Neidlinger-Wilke *et al.*, 2012).

1.4. Risk factors for IVD degeneration

It is well considered that genetic factors play an important role in the pathogenesis of disc degeneration. Specifically, it may be enhanced in individuals with gene polymorphisms, particularly with genes encoding molecules linked to the properties of the IVD matrix (Battié *et al.*, 2009; Kalb *et al.*, 2012). Consequent impaired biomechanical strength of the IVD matrix may result in low tolerance to mechanical loading.

Although loading is a physiological stimulus for matrix turnover (Setton and Chen, 2006), excessive loading can be an important risk factor for IVD degeneration (Videman *et al.*, 1995). Unfavourable mechanical loading may accelerate the rate of structural deteriorations on a normal IVD or even with physiological loading on a genetically predisposed weak IVD.

1.5. Biological treatment strategies

Current treatment approaches are restricted to symptomatic therapies, not addressing options of restoring structural or biological deteriorations of the IVD as the underlying problem. Therapeutic potentials of IVD cell transplantation, biomaterials, inhibiting or activating bioactive factors, including gene-therapeutic approaches, have been shown *in vitro*, mostly with animal NP cells or in animal IVD models, and have been comprehensively reviewed elsewhere (Kandel *et al.*, 2008; Hegewald *et al.*, 2008; An *et al.*, 2011; Leung *et al.*, 2011). Human degenerative IVD cells, however, were shown to display distinctive features with regard to cell biology and regenerative potential, often differing considerably from animal data (Lotz, 2004; Alini *et al.*, 2008). Especially small animal models have shown differences with regard to disc cell density, metabolite transport and the existence of notochordal cells in the matured NP. These differences may display increased self-repair capabilities in comparison to human IVDs and their cells that are often predisposed by age, genetic dispositions and consecutive degenerative alterations. Moreover, we showed that the site of tissue harvest appears to have an impact on the regenerative potential of isolated IVD cells in the event of a lumbar disc herniation (Hegewald *et al.*, 2011a). When operating on lumbar disc herniations, it would be an elegant solution to remove only the herniated disc tissue for cell isolation and regenerative approaches. In this case, the NP compartment could be left untouched – a surgical consideration associated with better clinical outcomes as compared with performing a

nucleotomy (Barth *et al*., 2008). Our study results, however, suggested a very limited regenerative potential for IVD cells harvested from herniated disc tissue in comparison to cells harvested from tissue of the NP compartment (Hegewald *et al*., 2011a). This example illustrates why, in our opinion, the biological enhancement of human IVD cells will be of high relevance for successful clinical translation of cell-based approaches for the IVD.

We therefore attempted a systematic review, investigating the *in vitro* responses of human NP cells to different *in vitro* processes and biological stimuli. An electronic database search on MEDLINE was conducted through July 2011 to identify and discuss publications concerning the effects of cell to cell stimulation, bioactive factors, biomaterials and combinations thereof in terms of cell isolation, proliferation, differentiation and matrix protein synthesis. We included only studies in which the methods section directly or indirectly indicated the use of tissue from the nucleus compartment for cell isolation, and excluded works reporting explicitly on annulus fibrosus cells. This survey and discussion might serve as a source for designing future biological treatment strategies for the human IVD. The studies are summarized in Tables 1–5.

2. NP cell isolation and cell expansion procedures

The manual methods of NP cell isolation and expansion for basic research settings consist of the enzymatic isolation of cells from NP tissue and their cultivation with appropriate media on cell culture plates allowing cell attachment and proliferation. NP cells isolation from degenerative IVD is hampered by their limited abundance. For IVD cell expansion, monolayer culture systems are often used, because in this system the proliferative capacity of disc cells is substantially higher compared to non-adherent systems. The use of autologous serum and growth factors such as FGF-2 has been shown to improve NP cell expansion (Mauth *et al*., 2009; Hegewald *et al*., 2011b). Similar to previous experiences with cartilage tissue engineering (Kaps *et al*., 2006), NP cells cultured as monolayer undergo changes such as aberrant flattened morphology and gene expression in terms of dedifferentiation toward a more fibrotic expression profile, including decreased expression levels of type II collagen and proteoglycan, as well as increased type I collagen (Kluba *et al*., 2005; Preradovic *et al*., 2005; Mauth *et al*., 2009; Hegewald *et al*., 2011a, 2011b). Thus, after obtaining a suitable number of cells, the monolayer culture system can be changed to a three-dimensional (3D) system, promoting redifferentiation by inducing a characteristic NP gene expression profile resulting in NP matrix production.

For clinical settings, automated, reproducible and safe methods of cell isolation and expansion will be mandatory. A first step in this direction is an automated liquid handling cell-culture platform reported on recently, which was used to isolate, expand and characterize human IVD cells in monolayer culture (Franscini *et al*., 2011). All steps, from the enzymatic isolation of cells to the final quality control, while monitoring and controlling multiple parameters, were performed completely by the automated system with satisfactory results.

3. Enhancing matrix protein synthesis of NP cells

3.1. 3D cell culture

Two-dimensional (2D) surfaces for *in vitro* cell expansion create an environment in which cells are forced to adopt unnatural spatial characteristics, resulting in desired cell proliferation, but usually also in dedifferentiation, as outlined in Section 2. Biomaterials can provide structural support for NP cell attachment, subsequent differentiation and NP tissue development in a physiological 3D environment. This has been shown for animal (Zeiter *et al*., 2009) as well as for human (Stern *et al*., 2004; Mauth *et al*., 2009; Hegewald *et al*., 2011b) NP cells. A vast variety of biomaterials, such as porous scaffolds, hydrogels, absorbable and non-absorbable materials, are available (Nicodemus and Bryant, 2008; Chan and Leong, 2008). The choice of biomaterial is crucial to allow NP cells to produce tissues with desired composition, shape and size, as well as adequate biomechanical and immunological properties. It is conceivable that for the different facets and severities of degenerative disc disease, different biomaterials will have to be considered to meet individual requirements.

So far, 3D culture of human IVD cells had been tested for biocompatibility and characteristic matrix synthesis *in vitro* in different biomaterials. Here, we have summarized their effects on human NP cells in terms of *in vitro* cell proliferation, differentiation and matrix protein synthesis (Table 1).

3.2. Interrelationships of human IVD cells with mesenchymal stem cells (MSCs)

3.2.1. Direct cell–cell co-culture system

Several studies have reported stimulatory effects on human NP cells using direct co-culture with MSCs (Le Visage *et al*., 2006a; Richardson *et al*., 2006; Watanabe *et al*., 2010; Strassburg *et al*., 2010). For instance, co-culture pellets formed by combining MSCs with NP cells promoted cell proliferation (Le Visage *et al*., 2006a). A positive effect on matrix production, however, was not observed. Other groups, on the other hand, demonstrated significantly enhanced matrix gene expression and synthesis after direct cell–cell contact culture (Richardson *et al*., 2006; Watanabe *et al*., 2010; Strassburg *et al*., 2010). The effect of MSCs on cell proliferation was greater in NP cells from less degenerated discs (Watanabe *et al*., 2010). More highly degenerated discs, however, turned out to be receptive to matrix gene expression enhancement by MSCs

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Table 1. Effects of cell culture systems and bioactive factors on human NP cells in terms of *in vitro* cell proliferation and characteristic matrix protein synthesis and matrix gene expression

Culture system	Bioactive factors	*In vitro* effects	Reference
Hyaluronic acid and fibrin, 3D culture 1, 2 and 3 weeks		Cells from NP compartment of scoliotic IVD: ↑ DNA, PG and HP synthesis Cells from NP compartment of osteochondrotic IVD: ↑ DNA and PG synthesis, no relevant changes in HP Cells from herniated IVD tissue: no relevant changes in DNA, PG and HP synthesis	(Stern *et al*., 2004)
Type I collagen 3D culture 4 weeks		Cells from NP tissue separated after discectomy: ↑ cell proliferation ↑ aggrecan expression ↑ type I collagen expression but decreasing over time Moderate and variable type II collagen expression	(Neidlinger-Wilke *et al*., 2005)
Gelatin, chondroitin-6-sulphate, 3D culture 6 and 12 weeks		Cells from NP compartment of scoliotic IVD: ↑ DNA and GAG synthesis	(Yang *et al*., 2005a)
Gelatin, chondroitin-6-sulphate/ hyaluronan, 3D culture 4 weeks		Cells from NP tissue of degenerated IVD: ↑ DNA and GAG synthesis ↑ aggrecan, type II collagen and SOX9 expression ↓ type I and X collagen expression	(Yang *et al*., 2005b)
Small intestinal submucosa, 3D culture *3 month*s		*Cells from NP tissue of degenerated IVD*: ↑ GAG synthesis ↑ aggrecan, type I, II collagens and SOX9 expression	Le Visage *et al*., 2006b
Fibrin clot in comparison to alginate, 3D culture *1 and 2 weeks*		*Cells from discectomy of degenerated IVD in comparison to 3D culture in alginate*: ↑ DNA and GAG synthesis ↑ aggrecan, type I, IV and X collagen expression ↓ SOX9 expression No change in type II collagen expression in both scaffolds	Yang *et al*., 2008a
Polyurethane and fibrin, 3D culture *3 days, 1 and 2 weeks* Pellet culture *2 weeks*		*Cells from discectomy of degenerated IVD*: ↑ DNA and GAG synthesis ↑ aggrecan, type I and II collagen and SOX9 expression ↑ aggrecan, type I and II collagen and SOX9 expression	Mauth *et al*., 2009
Polyglycolic acid, hyaluronic acid and fibrin, 3D culture *1 and 2 weeks*		*Cells from NP compartment of degenerative IVD*: ↑ aggrecan, type I, II and III collagen expression *Cells from herniated IVD tissue*: no change in aggrecan synthesis ↑ type I collagen expression ↑ (moderate) type II and III collagen expression	Hegewald *et al*., 2011b Hegewald *et al*., 2011a
Pellet culture, cell-to-cell contact co-culture *1, 2 and 3 weeks*	Allogenic MSCs	*Cells from NP compartment of scoliotic IVD*: ↑ cell proliferation ↑ DNA synthesis No change in GAG synthesis	Le Visage *et al*., 2006a
Culture plates and inserts, cell-to-cell contact co-culture, monolayer *7 days*	Allogenic MSCs	*Cells from NP compartment of non-degenerated IVD from young cadaver*: ↑ cell proliferation ↑ aggrecan, SOX9, type II and VI collagen expression	Richardson *et al*., 2006
Culture plates and inserts, non-contact co-culture, monolayer *7 days*	Allogenic MSCs	No change in aggrecan, SOX9, type II and VI collagen expression	

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Table 1. (Continued)

Culture system	Bioactive factors	*In vitro* effects	Reference
Alginate, 3D co-culture *2 weeks*	Allogenic MSCs	Cells from discectomy of degenerated IVD: ↑ SOX9 No change in aggrecan, type I and II collagen expression	Vadalà *et al*., 2008
Culture plates and inserts, non-contact co-culture, monolayer *3 days*	Allogenic MSCs	*Cells from discectomy of degenerated IVD*: ↑ cell proliferation ↑ aggrecan No change in SOX9, type I and II collagen expression	Yang *et al*., 2008b
Culture plates and inserts, cell–cell contact co-culture, monolayer 3, 5 and 10 days	Autologous MSCs	*Cells from NP compartment of degenerative and traumatic IVD*: ↑ cell proliferation ↑ DNA and PG synthesis *6 month subcutaneously in nude mice* No chromosomal abnormality	Watanabe *et al*., 2010
Culture plates and inserts, cell–cell contact co-culture, monolayer *7 days*	Allogenic MSCs	*Cells from NP compartment of nondegenerated cadaver IVD*: higher baseline levels for aggrecan and SOX9 No change in aggrecan and SOX9 expression *Cells from NP separated after surgery of degenerated IVD*: lower baseline levels for aggrecan and SOX9 ↑ aggrecan expression No change in SOX9 expression	Strassburg *et al*., 2010
Pellet culture or alginate, 3D culture *3 weeks*	AdTGF-β1	*Cells from degenerated IVD*: ↑ PG and type II collagen synthesis	Lee *et al*., 2001
Monolayer culture *3 weeks*	AdTGF-β1	↑ (moderate) PG and type II collagen synthesis	
Monolayer culture *9 days*	PRP(TGF-β1)	*Cells from NP of healthy IVD*: ↑ cell proliferation ↑ GAG synthesis ↑ aggrecan, collagen II and SOX9 expression	Chen *et al*., 2006
Collagen type I, 3D culture *4 weeks*	PRP(TGF-β1)	↑ PG and type II collagen synthesis	
Collagen type I and II, 3D culture *4 weeks*	PRP(TGF-β1)		
Monolayer culture *3 days*	TGF-β1	*Cells from discectomy of degenerated IVD*: ↑ aggrecan and type I collagen expression ↓ SOX9 expression No change in cell proliferation and type II collagen expression	Yang *et al*., 2008b
Pellet culture *1 week*	TGF-β1	*Cells from NP compartment of adolescent scoliotic IVD*: ↑ GAG and HP synthesis ↑ type I and II collagen expression ↓ aggrecan and SOX9 expression *Cells from dis*cectomy of adult degenerated IVD: ↑ GAG and HP synthesis ↑ type I collagen expression ↓ aggrecan, SOX9 and type II collagen expression	Yang *et al*., 2010
Alginate, 3D culture *3 days*	BMP-2	*Cells from NP compartment of degenerative IVD*: ↑ PG synthesis ↑ aggrecan, type I and II expression No change in osteocalcin expression after 3 weeks	Kim *et al*., 2003
Pellet culture *2 days*	*Ad-BMP-2*	*Cells from degenerated IVD*: ↑ DNA synthesis ↑ PG synthesis	Wallach *et al*., 2003

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Table 1. (Continued)

Culture system	Bioactive factors	*In vitro* effects	Reference
Alginate, 3D culture *21 days*	BMP-7	*Cells from NP compartment of degenerated cadaver IVD and from NP compartment of a young scoliotic IVD*: ↑ DNA and PG synthesis	Imai *et al*., 2007
Monolayer culture *2 days*	BMP-2 or BMP-12	*Cells from NP compartment of degenerated IVD*: ↑ PG, collagen and non-collagen protein synthesis	Gilbertson *et al*., 2008
Pellet culture *6 days*	*Ad-BMP-12*	*Cells from NP compartment of degenerated IVD*: ↑ DNA, PG, collagen and non-collagen protein synthesis	
Alginate, 3D culture *2 days*	*Ad/TGF-β1* *Ad-IGF-1* *Ad-BMP-12* *Ad/TGF-β1 + Ad-IGF-1* *Ad/TGF-β1 + Ad-BMP-12* *Ad-IGF-1 + Ad-BMP-12* *Ad/TGF-β1 + Ad-IGF-1 + Ad-BMP-12*	*Cells from NP compartment of degenerative IVD*: ↑ PG synthesis (+) ↑ PG synthesis (+) ↑ PG synthesis (+) ↑ PG synthesis (++) ↑ PG synthesis (++) ↑ PG synthesis (++) ↑ PG synthesis (+++)	Moon *et al*., 2008
Alginate, 3D culture *3 weeks*	BMP-14	*Cells from NP compartment of degenerative IVD from surgical and cadaver specimens*: No change in DNA synthesis ↑ GAG synthesis ↑ aggrecan and type II collagen expression	Le Maitre *et al*., 2009
Polyglycolic acid, hyaluronic acid and fibrin, monolayer *1 week*	FGF-2 + human serum	*Cells from NP compartment of degenerative IVD*: ↑ cell proliferation	Hegewald *et al*., 2011b
Pellet culture *2 days*	*Ad-TIMP-1*	*Cells from degenerated IVD*: ↑ DNA synthesis ↑ PG synthesis	Wallach *et al*., 2003

3D, three-dimensional; DNA, deoxyribonucleic acid; GAG, glycosaminoglycans; HP, hydroxyproline; PG, proteoglycan; PRP, platelet-rich plasma.

Table 2. Comparison of proteoglycan synthesis after 3 weeks in adTGF-β1-transduced NP cells in monolayer and in 3D culture systems (Lee *et al*., 2001)

Culture system	***Proteoglycan*** (%)
Monolayer	150–180
Alginate beads	250–315
Pellet culture	375–425

(Strassburg *et al*., 2010). Chromosome abnormalities and tumourigenesis were not observed when MSC-activated human NP cells were transplanted into nude mice for 6 months (Watanabe *et al*., 2010).

Co-culture of IVD cells and MSCs could be a suitable method for generating large and highly differentiated populations of IVD cells and MSCs for cell-based regenerative treatment strategies.

3.2.2. Non-contact co-culture system

Co-culture of human NP cells and MSCs using a non-contact system are discussed controversially in the literature. As a result of paracrine stimulation, one group reported a considerable increase in viability and proliferation of NP cells, together with a higher expression of aggrecan but unchanged SOX9 and type I and II collagen expressions (Yang *et al*., 2008b). Conversely, proliferation of MSCs and their differentiation toward NP-like cells, indicated by increased expression of type II collagen, was observed. In contrast, another group did not find any significant changes in NP marker gene expression in either NP cells or MSCs of a non-contact system, regardless of cell ratio (Richardson *et al*., 2006). In the same study, NP cells and MSCs showed significant increases in the expression of NP marker genes (aggrecan, SOX-9 and types II and VI collagen) when cells were co-cultured with direct cell–cell contact for 7 days. These changes were regulated by cell ratio.

3.3. Transforming growth factor-β1 (TGF-β1)

TGF-β plays a crucial role in the development, growth and maintenance of the IVD (Sohn *et al*., 2010; Jin *et al*., 2011) and is a major growth factor in the chondrogenic differentiation of MSCs (Pittenger *et al*., 1999).

One group investigated the role of TGF-β1 in the human IVD by studying degenerative adult NP cells retrieved from

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Table 3. Effects of BMPs on matrix protein synthesis of human NP cells in alginate, pellet and monolayer culture systems

BMPs	*Proteoglycan* (%)	*Collagen* (%)	*Non-collagen protein* (%)	Reference
Pellet culture ***(Ad-BMP-12, 6 days; AD-BMP-2, 2 days)***				
Ad-BMP-12 (50 MOI)	390	370	280	Gilbertson *et al*., 2008
Ad-BMP-2 (50 MOI)	171	nt	nt	Wallach *et al*., 2003
Ad-BMP-2 (100 MOI)	279	nt	nt	Wallach *et al*., 2003
Ad-BMP-2 (150 MOI)	419	nt	nt	Wallach *et al*., 2003
Alginate culture (*rhBMP-2*, 3 days; *rhBMP-7*, 21 days)				
rhBMP-2 (300 ng/ml)	67	nt	nt	Kim *et al*., 2003
rhBMP-2 (1500 ng/ml)	200	nt	nt	Kim *et al*., 2003
rhBMP-2 (2000 ng/ml)	152	nt	nt	Kim *et al*., 2003
rhBMP-7 (200 ng/ml)	257	nt	nt	Imai *et al*., 2007
Monolayer culture (2 days)				
rhBMP-2 (300 ng/ml)	355	388	234	Gilbertson *et al*., 2008
rhBMP-12 (300 ng/ml)	140	143	160	Gilbertson *et al*., 2008

nt, not tested.

Table 4. Adenoviral 'cocktail' with low-dose adenoviral mixtures (MOI of 75) of the TGF-β1 (*Ad/TGF-β1*), IGF-1 (*Ad/IGF-1*) and BMP-2 (*Ad/BMP-2*) genes in single-, double- and triple-gene approaches. Depending on the combination of genes applied, additive anabolic effects on proteoglycan synthesis of NP cells in 3D culture with alginate beads after 2 days were observed (Moon *et al*., 2008)

Single-gene approach	*Ad/TGF-β1*	*Ad/IGF-1*	*Ad/BMP-2*
Proteoglycan (%)	290	180	190
Double-**gene approach**	*Ad/TGF-β1 + Ad/IGF-1*	*Ad/TGF-β1 + Ad/BMP-2*	*Ad/IGF-1 + Ad/BMP-2*
Proteoglycan (%)	390	350	320
Triple-**gene approach**	*Ad/TGF-β1 + Ad/IGF-1 + Ad/BMP-2*		
Proteoglycan (%)	470		

Table 5. *Ad-TIMP-1* increased proteoglycan synthesis in 2 day pellet cultures of NP cells. An optimal response was observed at 100 MOI (Wallach *et al*., 2003)

Ad-TIMP-1	50 MOI	100 MOI	150 MOI
Proteoglycan (%)	139	388	302

disc herniation tissue of the lower lumber spine (mean patient age, 26 years) and from less degenerated adolescent NP cells from the upper lumbar spine of idiopathic scoliosis patients (mean age, 16 years) (Yang *et al*., 2010). Although the proliferation rate of adolescent NP cells was significantly higher after 1 week of cultivating in cell pellet cultures, adult degenerative NP cells expressed significantly higher aggrecan and type I collagen, but significantly lower type II collagen. Both adolescent and adult NP cells cultivated in cell pellets endogenously expressed several anabolic cytokines, including TGF-β1, BMP-2, BMP-4, BMP-6, BMP-7, IGF-1 and EGF, with similar expression levels, except for IGF-1, which was significantly more highly expressed by adult degenerative NP cells. Stimulation of human adolescent and adult NP culture pellets with 10 ng/ml TGF-β1 similarly enhanced characteristic matrix protein production, such as sGAG and hydroxyproline, but also an enhanced expression of type I collagen and a reduced expression of aggrecan and SOX9 was observed, indicating a more fibrous expression profile.

In another study of the same group, a monolayer culture instead of pellet culture was used (Yang *et al*., 2008b). Three days after treatment with 10 ng/ml TGF-β1, NP cell proliferation and type II collagen expression were not significantly different between untreated and TGF-β1-treated cells, whereas type I collagen expression was significantly enhanced in TGF-β1-treated cells.

With a gene-therapeutic approach, the transduction of NP cells with an adenovirus containing the TGF-β1 gene (*AdTGF-β1*) was attempted (Lee *et al*., 2001). In a pellet culture system, the transduced NP cells produced, on average, 1.1 ng/ml TGF-β1/million cells within the pellets, whereas the control pellets, not transduced with *AdTGF-β1*, showed no detectable TGF-β1 production. To further analyse the responsiveness of the NP cells to TGF-β1 in different culture systems, *AdTGF-β1*-transduced cells were cultured in a monolayer, in alginate beads or in pellets, for 3 weeks and the results of their proteoglycan synthesis were compared with the respective non-transduced controls, grown in an identical manner (Table 2). The three cultures with *AdTGF-β1* showed an increased production of proteoglycan. Of the three, the cells grown in the pellet system showed the highest increase of proteoglycan, by 375–425%. The cells in the monolayer and alginate bead system showed 150–180% and 250–315% increase, respectively, over their controls. Additionally, the cells grown in the pellet system displayed a more intense immunohistochemical staining for type II collagen. To test

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the pellets as a gene delivery vehicle *in vivo*, the cells were labelled with *LacZ* gene by adenoviral transduction. *In vivo* implantation of the pellets into the thigh muscles of SCID mice showed that the transduced pellets remained aggregated and produced large amounts of the marker gene after 2 weeks.

Adenoviral vectors, which can efficiently infect dividing as well as non-dividing cells, could be used for a high-level and persistent expression of TGF-β1 to increase the synthesis of proteoglycan in NP cells. In contrast, the direct treatment of NP cells with TGF-β1 is, due to its short half-life, transient and less effective, which could limit its applications.

3.4. Platelet-rich plasma (PRP)

Platelet-rich plasma (PRP) extracts from total blood contain various growth factors, in which TGF-β1 is the highest (Everts *et al*., 2006). It was investigated in various concentrations, designated by the concentration of TGF-β1, for its effect as a growth factor cocktail on human NP cells (Chen *et al*., 2006). A time-course experiment of NP cell proliferation showed, on day 7 of monolayer culture, a substantial dose-dependent effect of TGF-β1. Of the different concentrations tested (99, 200 and 750 pg/ml, 1, 1.5 and 2 ng/ml), 1 ng/ml TGF-β1 in PRP was found to be the most effective concentration for NP cell proliferation. However, a significant inverse concentration effect was observed when TGF-β1 reached 2 ng/ml. The cell numbers showed no increase in the 7 days of culture, nearly similar to the controls cultured in 1% FBS medium without PRP. Moreover, an anti-apoptotic effect of PRP was shown with NP cells in monolayer culture, pretreated with IL-1β, which is known to induce cellular and matrix changes of IVD degeneration (Le Maitre *et al*., 2005). NP cells were treated with 10 ng/ml IL-1β for 24 h and followed by PRP (TGF-β1 1 ng/ml) treatment for 24 h (Chen *et al*., 2006). The results of flow cytometry at sub-G_1 stage of the cell cycle showed that apoptotic cell numbers were dramatically decreased in the PRP group compared with the control IL-1β group. Furthermore, the mRNA expression rate of *type II collagen*, *aggrecan* and *SOX9*, as well as the accumulation of GAGs, were significantly increased after 9 days of treatment with PRP containing 1 ng/ml TGF-β1. Treatment of NP cells with PRP also activated the phosphorylation of Smad2/3, which is supposed to be the key regulatory protein of chondrogenic genes specific to TGF-β1 activation. Moreover, 3D cultures were utilized to maintain NP cells in PRP + type I collagen, PRP + type I + type II collagen, as well as in the controls type I collagen and type I + type II collagen, respectively (Chen *et al*., 2006). Chondrocyte-like rounded and evenly distributed cells appeared in the matrix of all groups. In the PRP-treated cultures, more intense type II collagen expression with abundant proteoglycan matrix accumulation indicated the participation of PRP in enhanced chondrogenic differentiation of NP cells in 3D cultures.

It appears that the combination of TGF-β1 with other bioactive factors, as present in PRP, provides more consistent results than single TGF-β1 stimulation in terms of chondrogenic matrix expression. In contrast to commercial recombinant growth factors, PRP is a natural source of autologous growth factors that can be easily purified from total blood and could avoid adverse immune responses.

3.5. Bone morphogenetic proteins (BMPs)

BMPs are potential candidates for the treatment of disc degeneration, as they possess pleiotropic effects on the formation of cartilage, bone and the connective tissues associated with the skeleton (Chen *et al*., 2004). Recombinant human (rh) BMP-2, rhBMP-12 and adenoviral BMP-12 (Ad-BMP-12) were used to evaluate and compare the *in vitro* effects of BMPs on matrix protein synthesis of human NP cells (Gilbertson *et al*., 2008). Cells were maintained in monolayer for 2 days or incubated for 6 days in pellet culture. In monolayer culture, rhBMP-2 (300 ng/ml) significantly increased NP proteoglycan, collagen and non-collagen protein synthesis to 355%, 388% and 234%, respectively, whereas rhBMP-12 increased the same NP matrix proteins synthesis to only 140%, 143% and 160% (Table 3). Additionally, in pellet culture, Ad-BMP-12 significantly increased matrix protein synthesis and DNA content in NP cells 6 days after transduction. Fifty-fold multiplicity of infection (50 MOI) of Ad-BMP-12 increased the same NP matrix protein synthesis to 390%, 370% and 280% compared with sham-transduced cells (Table 3).

Another study with human cells from degenerated IVDs, which were subjected to adenoviral transduction with BMP-2, demonstrated a progressive increase in proteoglycan synthesis with an increasing viral MOI (Wallach *et al*., 2003). For 50 MOI, proteoglycan synthesis increased to 171%, for 100 MOI to 279% and for 150 MOI to 419% of the control (Table 3). The non-osteogenic action of BMP-12 combined with its stimulating effect on matrix production as well as NP cell proliferation makes gene therapy with BMP-12 interesting for regenerative treatment attempts.

3.6. Combination of growth factors (Ad/TGF-β1, Ad/IGF-1 and Ad/BMP-2)

To examine the biological effect of 'cocktail' adenovirus-therapeutic gene transfer of anabolic genes, human IVD cells isolated from NP were transduced by recombinant adenovirus constructs bearing the TGF-β1 gene (*Ad/TGF-β1*), the IGF-1 gene (*Ad/IGF-1*) and the BMP-2 gene (*Ad/BMP-2*) in single, double and triple combinations (Moon *et al*., 2008). In 3D culture with alginate beads, the 'cocktail' gene therapy has shown, depending on the combination of genes applied, additive anabolic effects on matrix synthesis with low doses of adenoviral mixtures. Compared to the controls, cultures with single *Ad/TGF-β1*, *Ad/IGF-1* and *Ad/BMP-2* (75 MOI) showed 290%, 180% and 190% increases in proteoglycan synthesis, respectively (Table 4). Cultures with double gene combinations *Ad/TGF-β1 + Ad/IGF-1*, *Ad/TGF-β1 + Ad/BMP-2* and *Ad/IGF-1 + Ad/BMP-2* (MOI of 75) showed 390%, 350% and 320% increases, respectively (Table 4). Cultures with a triple gene combination

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(75 MOI) demonstrated 470% increases in proteoglycan synthesis (Table 4). However, virus-induced toxicity is considered to be critical in the clinical application of gene therapy, especially in adenovirus-mediated gene delivery (Mazzolini *et al*., 2001; Nemunaitis *et al*., 2001; Lozier *et al*., 2002). The low dose utilized in this study could minimize or avoid virus-induced toxicity, immune response and the possible occurrence of replicating virus. The mechanisms underlying the enhanced additive biological effect of cocktail gene therapy have not yet been investigated. Although very promising, the *in vitro* results obtained with the 'cocktail' gene therapy approach in cultured human IVD cells require additional validation in animal models.

3.7. Catabolic enzyme inhibition

The progressive decline of aggrecan, the primary proteoglycan of the NP, is known to be a major characteristic of disc degeneration (Buckwalter, 1995), as it alters the normal hydrostatic and biomechanical properties of the disc (Urban and McMullin, 1988). The reduction in proteoglycan content reflects an imbalance in the normal anabolic and catabolic functions of the NP cells, resulting from decreased anabolism, increased catabolism or a combination of these two processes (Masuda *et al*., 2004). As discussed in previous sections, various growth factors have considerable anabolic capacities with regard to proteoglycan synthesis in human IVD cells. Inhibition of degenerative catabolic processes, on the other hand, may provide an alternative or additional regenerative approach. Several structurally similar proteolytic enzymes, capable of degrading collagen, proteoglycan and other structural components of the extracellular matrix, have been described in the degenerative process of the IVD (Kanemoto *et al*., 1996; Handa *et al*., 1997; Roberts *et al*., 2000).

To investigate the potential role of the endogenous inhibitor of MMP-3 in IVD regeneration, the tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) was delivered to degenerated human NP cells *in vitro* (Wallach *et al*., 2003). Cells were cultured in monolayer and transduced with different concentrations of adenoviral TIMP-1 (*Ad-TIMP-1*). Successful delivery of the anti-catabolic gene, *TIMP-1*, resulted in increased proteoglycan synthesis in pellet cultures of degenerated IVD cells. Increased proteoglycan synthesis was demonstrated in different transduction rates with increasing MOI. IVD cells treated with *Ad-TIMP-1* demonstrated an optimal response at 100 MOI (Table 5). Measured proteoglycan synthesis increased to 139% for 50 MOI, to 388% for 100 MOI and to 302% for 150 MOI. Pellets treated at 100 MOI were consistently larger than the control group or other MOI-assessed groups.

4. Outlook

4.1. Opportunities and open questions

Progressive characterization of the course of IVD degeneration has identified several targets with significant impact on matrix synthesis and catabolism. This provides the opportunity to develop novel therapeutic approaches at a cellular and molecular level.

One focus is the highly specialized NP cells. The available data suggest that in most cases the problem is not a declining number of NP cells, but a considerable functional impairment due to factors such as genetic disposition, cell senescence and nutritional impairment. It is therefore evident that enhancing the functionality of NP cells will be a crucial component in designing future regenerative treatment strategies.

The design of regenerative treatment strategies is an extremely complex subject that has to take into consideration the variety of degenerative disc diseases and their implications for different biological approaches. These biological approaches, on the other hand, offer many potential possibilities to proceed, leading to many unanswered questions: Should NP cells be treated *in situ* by injecting bioactive factors into the IVD? When is it better to decide for autologous or allogenic NP cell transplantation? What is the role of stem cells in that context? Is it enough to aim for augmentation of the NP? When is it necessary to remove the diseased NP before attempting biological treatment? Is a single application of bioactive factors sufficient to induce IVD repair, or are gene-therapeutic methods or other releasing systems necessary for meaningful results? Is it possible to improve the impaired nutritional environment? Although many interesting concepts had been formulated in the literature, the clinical validity of these concepts needs to be verified.

4.2. Autologous and allogeneic possibilities

In light of the current knowledge, we think that in many cases meaningful and lasting clinical results will involve gene-therapeutic techniques with autologous or allogeneic NP and/or stem cell transplantation. Autologous NP cell transplantation therapy has been shown to decelerate IVD degeneration in animal models such as rabbit (Okuma *et al*., 2000), sand rat (Gruber *et al*., 2002) and dog (Ganey *et al*., 2003), as well as safety of application in humans (Meisel *et al*., 2006). Although there is a report of allogenic NP cell transplantation that showed successful deceleration of IVD degeneration in a rabbit (Nomura *et al*., 2001), its clinical applicability remains to be seen with regard to adverse immunological reactions. The immunologically privileged character (Kaneyama *et al*., 2008) of the IVD and its avascularity, however, are promising preconditions for allogenic approaches. This would allow a one-procedure treatment, whereas autologous approaches are always based on two invasive procedures. Moreover, NP cell banks would offer the possibility of preselection of healthier and more potent NP cells.

4.3. Gene-therapeutic strategies

Small-interfering RNAs (siRNAs)-mediated RNA interference (RNAi), resulting in degradation of target

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mRNAs, has opened the door to the therapeutic use of siRNAs. The therapeutic potential of siRNAs in gene therapy of human degenerative IVDs, however, has barely been investigated. siRNAs could be applied to silence genes involved in the pathogenesis of disc degeneration. It has been shown, for example, that aggrecanase-1 (ADAM-TS4) and aggrecanase-2 (ADAM-TS5), proteolytic enzymes reported to cause aggrecan degradation in human osteoarthritic cartilage (Malfait *et al.*, 2002; Naito *et al.*, 2007), are also expressed in the human IVD. siRNA-mediated *in vitro* inhibition of aggrecanase-1 and aggrecanase-2 significantly increased the aggrecan and type II collagen content of rat chondrocytes and facilitated chondrocyte differentiation and cartilage formation *in vitro* (Wang *et al.*, 2008, 2010). *In vitro* application of anti-aggrecanase-2 siRNA suppressed *aggrecanase-2* gene expression by 70% in both monolayer and alginate bead culture of rabbit NP cells (Seki *et al.*, 2009). Moreover, the *in vivo* suppressive effects of siRNA injections on IVD degeneration have been tested in the rabbit annular needle-puncture model (Seki *et al.*, 2009). The injection of anti-aggrecanase-2 siRNA *in vivo* resulted in improved MRI scores with increased signal intensity and improved histological grade scores. These results suggest that aggrecanases (ADAM-TSs) could represent potential targets for the treatment of human IVD degeneration with modern gene-therapeutic techniques. To achieve a significant change in the treatment of advanced chronic IVD degeneration, the prolonged application of growth factors and/or catabolic enzyme inhibitors might be necessary. Appropriate genes can be delivered directly into the IVD, or NP target cells can be isolated, genetically enhanced *in vitro* and re-implanted into the IVD. Genome-incorporating viral vectors (retrovirus and lentivirus), non-genome-incorporating viral vectors (herpesvirus, adenovirus and adeno-associated virus) or non-viral vectors have been used. Although gene transfer using retrovirus and lentivirus allows long-term transgene expression, a random insertion of the exogenous target gene in the host cell genome could knock out a crucial housekeeping or tumour suppressor gene (Robbins and Ghivizzani, 1998; Hacein-Bey-Abina *et al.*, 2003). As a result, adenoviral vectors are mainly used, which can efficiently infect dividing as well as non-dividing cells and permit a high level of transient gene expression. The downturn of transgene expression with time, due to immune reactions to viral proteins, however, is the main limitation of this system (Yang *et al.*, 1994). In spite of the potential efficiency of adenoviral vectors in IVD gene therapy, there are concerns about safety in clinical applications (Nishida *et al.*, 1999; Driesse *et al.*, 2000; Wallach *et al.*, 2006). Their use in the IVD near to sensitive neural structures could have potential toxic and immunological side-effects, which could result in unacceptable symptoms, such as neurological deficits or serious pain. One group investigated the worst-case scenario of an errant injection that placed recombinant viral constructs in both the intradural and epidural space in a rabbit model (Wallach *et al.*, 2006). Intradural injection of an incorrect dose of *Ad/TGF-β1* in rabbits resulted in bilateral lower extremity paralysis with pathological changes of the spinal cord in histological analysis. Serious side-effects, such as paraesthesia, systemic illness and death, were seen in the majority of rabbits with epidural injection of *Ad/TGF-β1* and *Ad/BMP-2*.

Virus-induced toxicity is considered to be critical in the application of gene therapy, especially in adenovirus-mediated gene delivery (Mazzolini *et al.*, 2001; Nemunaitis *et al.*, 2001; Lozier *et al.*, 2002). To improve safety in IVD gene therapy, the regulation of transgene expression after gene transfer could be essential. After cells become capable of producing increased levels of the desired proteins, using an inducible system could exogenously control the amount of synthesized proteins. Inducible systems of transgene expression have been developed by linking ligand-activated promoter regions to a particular gene of interest within a single vector construct. The two basic strategies for gene regulation are termed 'off' and 'on' systems, depending on the effect of an exogenously supplied ligand on gene expression. The application of a tetracycline (tet)-inducible system for improved safety of IVD gene therapy has been described (Vadalà *et al.*, 2007). In this study, human NP cells were transduced with an adenoviral vector that expresses Fas ligand (FasL) and green fluorescent protein (GFP) under the control of a tetracycline-regulated gene expression system. The transgene expression was efficiently regulated by inclusion of tetracycline in the culture medium, demonstrating the ability to control transgene expression in NP cells.

An alternative to the adenoviral systems of gene therapy is the adeno-associated virus (AAV) system, which is potentially less immunogenic and possibly safer. It integrates the target therapeutic gene into a specific site on chromosome 19 in a stable and non-pathogenic manner. AAV has high transduction efficiency in dividing and non-dividing cells and permits prolonged transgene expression. It does not express any viral gene and, as yet, it has not been linked with any known disease in humans (Afione *et al.*, 1999). Nevertheless, the maximum *in vivo* gene expression using the AAV–luciferase vector in rabbit IVD has reached only about 50% of the maximum gene expression obtained after transduction with an adenoviral vector (Lattermann *et al.*, 2005).

Another alternative would be the use of non-pathogenic, non-viral vectors, which could abolish potential risks linked with viral gene transfer. Recently, it has been shown that the ultrasound transfection method with microbubbles significantly enhanced the transfection efficiency of non-viral vector into NP cells *in vivo*, resulting in 24 weeks-long transgene expression (Nishida *et al.*, 2006).

The use of an inducible non-viral gene therapy of specific anabolic and/or anti-catabolic factors, combined with biocompatible IVD stabilizing biomaterials, could be a promising biological approach for treating degenerative disc diseases.

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