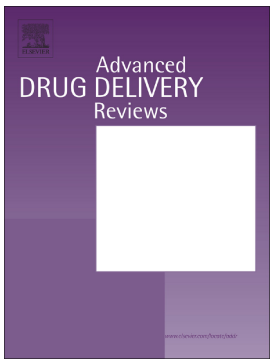

Lessons learned from intervertebral disc pathophysiology to guide rational design of sequential delivery systems for therapeutic biological factors

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Lessons learned from intervertebral disc pathophysiology to guide rational design of sequential delivery systems for therapeutic biological factors

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Abstract

Intervertebral disc (IVD) degeneration has been associated with low back pain, which is a major musculoskeletal disorder and socio-economic problem that affects as many as 600 million patients worldwide. Here, we first review the current knowledge of IVD physiology and physiopathological processes in terms of homeostasis regulation and consecutive events that lead to tissue degeneration. Recent progress with IVD restoration by anti-catabolic or pro-anabolic approaches are then analyzed, as are the design of macro-, micro-, and nano-platforms to control the delivery of such therapeutic agents. Finally, we hypothesize that a sequential delivery strategy that i) firstly targets the inflammatory, pro-catabolic microenvironment with release of anti-inflammatory or anti-catabolic cytokines; ii) secondly increases cell density in the less hostile microenvironment by endogenous cell recruitment or exogenous cell injection, and finally iii) enhances cellular synthesis of extracellular matrix with release of pro-anabolic factors, would constitute an innovative yet challenging approach to IVD regeneration.

Keywords: Drug delivery systems; Degenerative Disc Disease; Anti-catabolic factors; Pro-anabolic factors; Cell supplementation; Endogenous repair

1. Introduction

Low back pain (LBP) is a particularly common affliction in the 21st century, with 80 % of the world population experiencing it at some point in their life [1,2]. It is the second most common reason for a medical consultation in industrialized countries and one of the costliest diseases for the healthcare system [3–5]. LBP consists of pain in the lumbar part of the spine that can also extend to the legs, with patients suffering from impaired mobility in the most severe cases. Thus, management of LBP patients, estimated to be 600 million worldwide, presently constitutes a major socio-economic problem [6]. It is now well established that 40 % of the chronic LBP cases are due to degeneration of the intervertebral disc (IVD) [7,8], which is the fibrocartilaginous joint situated between two vertebrae. The IVD is a complex tissue composed of three distinct components that assume a unique structural organization essential for its function as a shock absorber. A healthy IVD, which is a key requirement for spine movements, exhibits mechanical properties such as high compressive and tensile strength that are tightly linked with its complex structural organization. For reasons that are not yet fully understood, very early after skeletal maturity, the IVD can undergo a degenerative process that manifests as cell death, extracellular matrix (ECM) changes and dehydration, culminating in failure of its biomechanical properties, thereby leading to pain and disability [9]. This progressive natural aging process can be accelerated and amplified by the occurrence of various traumatic, environmental or genetic factors. Conventional treatments for degenerative disc disease (DDD) are focused only on pain or inflammatory relief, with pharmacological approaches as the first intention, followed by invasive surgical procedures as the last option for treatment of resistant and severe disease. Ultimately, none of these treatments restore disc function. Consequently, the development of innovative therapeutic strategies has become a high-priority issue in the scientific community, with the main view being that these strategies should be based on therapeutic agents that allow reversal of the degenerative processes so as to fully restore the healthy tissue physiology in terms of its structural and functional complexity. Thus, therapeutic strategies are now focused on the mechanisms of IVD degeneration, primarily with the aim of correcting the anabolic/catabolic balance, albeit only one at a time. However, IVD degeneration occurs at different levels, with the occurrence of three main events that are intertwined and that work in a vicious circle: i) inflammation and catabolic cascades, ii) progressive loss of cells, and iii) decline in cellular functions and anabolic activities. Thus, although strategies based on rectifying only one of these

events, by delivering either anti-inflammatory cytokines, growth factors, or cells, have shown promising results, they have so far not been sufficiently robust to allow for full IVD restoration.

In this review, we formulate and support the notion that all three of the main events of IVD degeneration need to be addressed in order to achieve complete reversal of DDD. Thus, we hypothesize that a combination of strategies that may have already been assessed individually is likely to stimulate the degenerated IVD microenvironment to the point where full regeneration of disc tissue may occur. Local delivery of therapeutic agents of various natures (e.g., cytokines, growth factors, nucleic acids, and cells) into a tissue constitutes a challenge, as does the need to avoid an invasive surgical procedure, as the spine is a particularly delicate entity. Thus, one of the crucial parameters for designing a drug delivery system (DDS) specifically for IVD regeneration is the requirement for an injectable biomaterial that can both protect the therapeutic agents and allow for their sustained delivery, while avoiding repeated injections as these are deleterious to the structure of the IVD. Being able to act on the different stages of disc degeneration in a consecutive and controlled manner by use of a single injection would therefore be optimal. Such a sequential DDS must allow effects on i) firstly, the inflammatory, pro-catabolic, and hostile microenvironment by the release of anti-apoptotic and anti-catabolic or anti-inflammatory cytokines; ii) secondly, on the cell density by increasing the number of cells in this healed microenvironment; and then iii) thirdly, the cellular functions, mostly the synthesis of adequate ECM, by the release of pro-anabolic factors. Nevertheless, before considering the clinical application of such sequential strategies, many design issues need to be addressed. Indeed, the optimal timing and profiles of release, as well as adequate doses of the various therapeutic agents still need to be properly determined. Further studies are needed to define these various issues before the establishment of such sequential DDS for full IVD reparation.

Here, we first review the current knowledge of IVD physiology and physiopathological processes in terms of homeostasis regulation and consecutive events that lead to tissue degeneration. Recent advances to restore the IVD by anti-catabolic or pro-anabolic approaches are then analyzed, as are the design of smart macro-, micro- and nano-platform biomaterial to control the delivery of such therapeutic agents. Finally, a single-injection-based sequential regeneration strategy that aims to act in a time-dependent manner on all three of the main events of IVD degeneration is discussed.

2. IVD physiology

2.1 IVD structure

The IVD is a fibrocartilaginous tissue located between two adjacent vertebral bodies, in a ventral position with respect to the spinal cord [10]. By maintaining the stability and dynamics of the spine, this amphiarthrosis plays an important role in spinal kinematics, as it absorbs the shocks produced during movement of the entire body and it distributes mechanical loads along the spinal column [11–13]. Each IVD is composed of three distinct regions that ensure a unique internal organization while also providing elasticity and strength. At the center of the IVD, the nucleus pulposus (NP) is a gelatinous and highly-hydrated tissue; at the IVD periphery, surrounding the NP, there is the annulus fibrosus (AF), which is a highly-organized fibrous structure, while cartilage endplates (CEP) are present at the top and bottom of the vertebral bodies and they interface the IVDs with the adjacent vertebrae. Each region represents a structural and biological entity that participates jointly in disc homeostasis.

The NP has a low cell density of approximately 3,000 cells/mm^3 [14,15]. Young and healthy human IVDs have been reported to have two types of cells: notochordal cells, which are large vacuolated cells that originate from the embryonic notochord, and nucleopulpocytes (NPCy), which are small spherical cells with a singular phenotype resembling that of articular chondrocytes but that express specific markers (OVOS2, CA12, CD24, HIF-α, and cytokeratin 8/18/19, amongst others) [16–18]. NPCy synthesize the ECM, which is rich in aggrecan, type II collagen (with a 27:1 ratio vs. 2:1 for articular chondrocytes) and hyaluronic acid (HA) that retains water and contributes to the osmotic pressure responsible for NP biomechanical properties [19]. These two cell types coexist only during the first few years of life due to the gradual post-natal disappearance of notochordal cells that ultimately disappear permanently prior to skeletal maturity in humans [20]. This short-lived cohabitation of notochordal cells and NPCy appears to be fundamental for disc homeostasis and the synthesis of a functional ECM [21,22] (**Fig. 1**). A cellular dialogue between notochordal cells and NPCy occurs as a result of the secretion of growth factors: connective tissue growth factor (CTGF), transforming growth factor (TGF-β), and sonic hedgehog (Shh), which together limit enzymatic degradation of the ECM by both inhibition of matrix metalloproteinases (MMPs) production and stimulation tissue inhibitors of metalloproteinases (TIMPs) production [23,24]. This cellular dialogue also protects the NPCy

from apoptosis and it prevents tissue neovascularization through inhibition of the expression of vascular endothelium growth factor (VEGF), interleukins 6 and 8 (IL-6 and IL-8). Aside from these anti-catabolic actions, notochordal cells, primarily as a result of CTGF and Shh production, stimulate the synthesis of ECM components by NPCy, which in turn secrete TGF-β1 to stimulate CTGF synthesis by notochordal cells [23,25–27]. This molecular exchange between notochordal cells and NPCy, which is responsible for all of these anti-catabolic and pro-anabolic effects, is therefore strongly involved in the metabolic balance and matrix integrity of the tissue and is not limited to NP cells. Indeed, it has been reported that notochordal cells, through Shh, also have a beneficial effect on the proliferation and anabolic activities of AF cells and endplate chondrocytes [27–29].

While the NP is a network of randomly oriented type II collagen fibers within an amorphous matrix of glycosaminoglycans (GAG), the AF is organized into 15-25 concentric lamellae composed essentially of type I collagen fibers [12,30]. Within the same lamella, the collagen fibers are arranged at an angle to each other and have a specific orientation of 30° relative to the transverse plane [31]. This structurally organized ECM is also composed to a lesser extent of type II collagen, proteoglycans (PGs), and elastin [32]. The AF can be divided into two parts: the outer AF, which is mainly composed of organized type I collagen fibers and exhibits a high level of resistance to tensile strength; and the inner AF, which is a transitional zone between the outer AF and the NP and is less dense and less organized [33]. The entire AF tissue contains fibroblast-like cells at a density of approximately 9,000 cells/mm^3 [14].

On both sides of the IVD, CEPs, which interface with the adjacent vertebral bodies, are composed of hyaline cartilage cells and chondrocytes that synthesize an ECM rich in type II collagen and proteoglycans [14]. The diffusion of oxygen and glucose in the IVD, which is necessary for growth and nutrition of the tissue as well as for metabolite excretion, takes place through the CEPs [34], via their network of fine blood vessels [35]. This diffusion route is particularly important since the IVD is the most avascular tissue in the human body [12,36,37]. IVD cells consequently have to adapt to a hypoxic environment and thus preferentially engage in anaerobic metabolism. This adaptation of disc cells is partially due to the stabilization of the hypoxia-inducible factor-α (HIFα), which is constantly expressed by NP cells irrespective of the oxygen content [38,39]. Furthermore, it has been reported that the IVD environment is acidic,

due to the anaerobic lactic acid metabolic pathway used by cells [40]. Interestingly, NP is immune privileged, like brain, retina, testicles, and fetus. This means that the NP environment should be amenable to the introduction of foreign antigens without this necessarily causing an inflammatory immune response.

2.2 IVD extracellular matrix homeostasis

Under physiological conditions, the IVD dynamic structure is characterized by an anabolic/catabolic balance that ensures slow physiological turnover of the ECM by synthesis and degradation [41]. As mentioned above, this homeostasis is particularly dependent on a cellular dialogue between notochordal cells and the other disc cells that involves the following growths factors: CTGF, TGF-β, and Shh, as well as by other molecules such as growth factors that belong to the bone morphogenetic protein (BMP) family: BMP-2, osteogenic protein-1 (OP-1, also called BMP-7), and growth differentiation factor 5 (GDF5, also called BMP14), which promote the synthesis of ECM components and which regulate MMPs production by NPCy. Catabolic factors, including MMPs and aggrecanases, a type of A Disintegrin And Metalloproteinase with Thrombospondin (ADAMTS) motif enzyme, participate in metabolic regulation of the tissue, along with TIMPs that regulate the action of these matrix-degrading enzymes [42].

2.3 IVD function

Physiological compressive loads applied to the spine are first distributed over the vertebral bodies and then transmitted to the IVDs along the spine. Indeed, in a healthy lumbar disc, NP pressure vary between 91 kPa to 1300 kPa, depending on the lying, seated, or standing position [43]. As their biological and physical characteristics are highly related, the three specific regions of the IVD have complementary mechanical properties. In addition to its gelatinous aspect and hydrated nature, the NP is a highly deformable tissue that acts as a viscoelastic liquid that can absorb compression forces by variation of the osmotic pressure. This osmoregulation depends particularly on proteoglycans in the ECM and the water content that can both alter the height of the IVD [44,45]. The primary function of the AF is to contain and maintain the osmotic pressure imposed by the NP with its tensile strength. The structure of the concentric collagen lamellae of the AF provides natural resistance to flexion, torsion, and shearing [33,40]. Finally, as a result of their cartilaginous structure, the endplates provide a flexible entity that can support substantial loads and distribute the intradiscal pressure towards the adjacent vertebrae [46,47].

IVD is therefore a complex tissue with specific biological, physicochemical and mechanical properties, all possibly affected by degeneration mechanisms, making it particularly difficult to fully regenerate."

3. The pathophysiology of IVD degeneration

IVD degeneration or degenerative disc disease (DDD) is a multifactorial condition caused by aging, genetics, biomechanics, and environmental factors [48–54]. In all cases, a degenerated IVD exhibits dysregulation of its anabolic/catabolic balance and a decline in NP cell density [55,56]. Clinically, DDD is a progressive and chronic affliction, manifesting as low back and leg pains that result in a substantial degree of disability [57]. In severe cases, the DDD results in radiculopathies, stenosis, or hernia [45]. These painful symptoms are due to molecular and cellular changes in the tissue that compromise the mechanical properties of the IVD.

3.1 Cellular and molecular changes

With aging, the physiological processes of the IVD change naturally and the ability of the IVD to withstand mechanical stresses is reduced. Nevertheless, some factors accelerate these pathophysiological processes towards degeneration and they drive aberrant responses in IVD cells [45].

On the one hand, several studies have highlighted the pivotal role of the disappearance of notochordal cells and the breakdown of their dialogue with NPCy in the establishment of a degenerative process, which correlates particularly with a low level of NPCy survival [23,58,59]. The NPCy undergo phenotypic changes, leading to an increase in factors that degrade the ECM and the production of pro-inflammatory cytokines, while, in parallel, there is a decrease in the level of anabolic factors and the synthesis of healthy ECM (**Fig.2**) [60–62]. NPCy synthesize MMP-1, MMP-3, and ADAMTS, which inhibit aggrecan and type II collagen synthesis, thus leading to alteration of the integrity of the ECM [42,63,64]. Furthermore, an increase in IL-6, IL-8, and prostaglandin E2 (PGE2) synthesis by NPCy results in the stimulation of nerve growth factor (NGF) production. Combined with upregulation of VEGF in the degenerated NP, this generates abnormal nerve ingrowth and vascularization in this formerly avascular and non-innervated tissue that can lead to the generation of pain [65]. The induction of vascularization and the increase in chemokine synthesis by NP cells, particularly CCL2, 3, 4, 5, 7, and 13, CXCL10,

and IL-8, stimulate the recruitment of immune cells that can populate the NP and produce IL-1β and tumor necrosis factor α (TNFα) [65]. Together, these two major pro-inflammatory cytokines mediate catabolism and anti-anabolic metabolism inside the NP and they participate greatly to the establishment and progression of the degenerative state of disc tissue [66,67]. IL-1β and TNFα, which are synthesized by immune and IVD cells under degenerative conditions, are responsible for several metabolic impairments [68–70]. Indeed, IL-1β and TNFα stimulate the production of MMPs and ADAMTS by NPCy and they suppress the expression of TIMPs (reviewed in [65]). In addition to these pro-catabolic actions, the alteration of matrix integrity is amplified by down-regulation of GDF-5 by IL-1β and TNFα, thus reducing the synthesis of essential ECM components such as aggrecan and type II collagen. The decline in aggrecan and type II collagen content is associated with concomitant enhancement of the expression of type I collagen. The NP gradually loses its gelatinous and highly-hydrated aspect to become more fibrous and less elastic, thereby compromising its ability to exert its role as a hydraulic shock absorber [9,45,71,72].

On the other hand, calcification of CEPs (**Fig.2**) has been observed with hypertrophy of chondrocytes, the occlusion of marrow spaces, and sclerosis, which alter the energy supply/demand balance and reduce the rate of nutrient and metabolite diffusion [73–75]. The nutrient supply decreases whereas the demand increases with the recruitment of immune cells. This imbalance between demand and supply decreases the availability of nutrients to disc cells, thereby leading to adverse effects on cellular activity and viability (reviewed in [76], [77]). In addition, a reduction of metabolite excretion and acidification of the IVD microenvironment occurs with the calcification of CEPs, thus resulting in cellular stress that stimulates the apoptosis and senescence of NPCy [78]. The accumulation of metabolites in the NP boosts the production of MMPs and ADAMTS, thereby further increasing the degradation of ECM components.

Eventually, the ECM of the AF also becomes weakened as collagen fibers become irregular and thinner, and disorganization of its concentric structure occurs with the possible formation of fissures. Finally, these matrix changes lead to disruption of the mechanical forces sensed by the surface mechanoreceptors of AF cells, thereby leading to their apoptosis [79,80].

3.2 Structural and mechanical changes

IVD degeneration involves cellular, molecular, and inflammatory mechanisms that lead to pronounced structural, physical, and mechanical changes that give rise to the patient's pain.

Dehydration of the NP results in loss of its mechanical properties and an increase in the tensile forces exerted on the AF [81]. Consequently, the AF can tear, while in severe cases expulsion of the NP (disc herniation) can occur, which is often accompanied by significant low back and leg pain due to compression of the adjacent nerve root [78,82]. The inner AF can also suffer damage, followed by nerve ingrowth and secretion of inflammatory factors that amplify the degenerative cascade and the patient's pain. Finally, calcification of CEPs alters their mechanical characteristics and makes them less flexible [74].

3.3 Diagnosis and current treatments of discogenic LBP

The diagnosis of DDD is mainly based on the occurrence of low back pain, and is then confirmed by complementary imaging analysis (e.g., X-rays, CT scan, or magnetic resonance imaging (MRI)) to evaluate the severity of the degeneration by assignment of a score [83–85]. This scoring is based on the hydration state of the NP and the IVD height (Pfirrmann) or on the CEP aspect (Modic) [86–88]. The currently available treatments for IVD degeneration are conservative and focused on reduction of the pain by physical therapy, analgesia, or anti-inflammatory drugs and, in severe cases, by invasive surgical procedures such as spinal fusion or arthroplasty [89–94]. Despite positive effects on the patient's pain, the long-term efficacy of these pharmaceutical and surgical therapeutic strategies remains moderate and unreliable, with a high risk of vertebral body fractures and degeneration of adjacent levels [95,96]. Hence, based on recent improvements in the understanding of disc pathology, innovative therapeutic approaches have been devised that are aimed at prevention, reduction of the rate, or even reversal of the IVD degenerative cascade.

4. Innovative bioinspired therapeutic strategies

IVD degeneration is the result of complex pathological processes involving cell, molecular, inflammatory, structural, and mechanical mechanisms that cause dysregulation of the tissue homeostasis, as evidenced by alteration of ECM components, abnormal enzymatic activity, the production of pro-inflammatory factors and a dramatic decrease in cell density. The ECM profoundly affects the phenotype and behavior of cells through various signaling pathways. Unhealthy cells and unhealthy ECM are interconnected, leading to a vicious circle of degeneration (reviewed in [97,98]). Thus, restoring a healthy cell-ECM dialogue appears to be paramount for reversal or reducing the rate of IVD degeneration. The main common hypothesis

for innovative therapeutic strategies for regenerating IVDs is based on restoration of the homeostatic mechanism by readjustment of anabolic/catabolic events in the NP [14,99]. With this aim in mind, the regenerative potential of various mini-invasive approaches based on the injection of specific therapeutic agents in the NP microenvironment to counteract the degeneration have been evaluated. Almost all studies have been performed using the trans-annular route to directly inject therapeutics into the NP, with reported issues of material leakage after needle removal, permanent AF lesions, osteophyte formation and accelerated degeneration. The transpedicular approach has been recently described as an alternative route, but long term effect on the endplate integrity has not been evaluated yet [100,101]. Thus, anti-catabolic and/or anti-inflammatory strategies have been investigated, such as halting or minimizing the inflammation and the degradation of the ECM. Conversely, pro-anabolic treatments to repopulate the NP with specific cells and/or stimulation of the production of healthy ECM by cells have been tested, with the aim of re-establishing a healthy cell/ECM synergy.

4.1 Anti-catabolic and anti-inflammatory strategies

Inhibition of both the catabolic and the inflammatory cascades could be a promising therapeutic approach to preventing degradation of the ECM and alteration of resident IVD cells, thus attenuating the adverse effect of the degenerated IVD microenvironment. The regenerative effect derived from the injection of anti-catabolic or anti-inflammatory factors has been investigated by in vitro, ex vivo and in vivo studies, as summarized in **Table 1**. Inhibition of catabolic enzymes (i.e., MMPs and ADAMTs) has been carried out either by direct suppression of their activities or by regulation of their inflammatory mediators (i.e., TNF-α and IL-1β). The use of short non-coding sequences of nucleotides that control ribonucleic acid (RNA) silencing, translation, or degradation appears to be an attractive option for IVD regeneration as the dysregulation of many processes during IVD degeneration includes micro-RNAs (miRNAs) involved in MMPs overexpression, pro-inflammatory cytokine upregulation, and other catabolic events. However, the potential of such therapeutic small interfering RNAs (siRNAs) and miRNAs (reviewed in [102]) may be limited due to their in vivo degradation by endonucleases. On the other hand, suppression of the ADAMTS5 gene by injection of anti-ADAMTS5 siRNA oligonucleotide in the lumbar IVDs of a rabbit annular needle-puncture model revealed improvement of both the Thompson and histological grade scores at two months [103]. Direct injection, in a rabbit

anulotomy model, of adeno-associated virus serotype 2 (AAV2) carrying the gene for TIMP-1, which is an MMPs inhibitor that is downregulated in disc disease, resulted in less MRI and histological evidence of degeneration, albeit without statistical significance, compared to the puncture group at three months, combined with a significant decrease in the degradation of type II collagen [104]. Other strategies that are focused on the inflammatory pathway have sought to reduce the expression and activity of inflammation mediators such as TNF and interleukins. Clinical studies have been performed on LBP patients with or without sciatica, radiculopathy, or IVD herniation to evaluate the role of anti-TNFα intradiscal injections, which are used clinically in polyarthritis rheumatoid treatments. Etanercept (Enbrel®, a recombinant TNF receptor), adalimumab (Humira®, a human monoclonal antibody directed against TNFα) and infliximab (Remicade®, a chimeric monoclonal antibody directed against TNFα) can neutralize the biological activity of TNFα and they have been shown to reduce pain based on a reduction on visual analog scales (VAS) of intensity and Oswestry disability indexes (ODI) [105–110]. Nevertheless, no significant reduction of the need for surgical procedures has been shown to date. The antagonist of IL-1 receptor, IL-1Ra, has also been investigated due to its ability to inhibit IL-1β inflammatory activity, and an attenuation of the degradative effects of MMPs was observed in vitro and by ex vivo analysis performed on human degenerated IVD. [111,112].

IL-6, IL-8, and PGE2 pathways are also promising targets for anti-catabolic treatment of IVD degeneration. Firstly, in vitro studies performed on human IVD cells cultured with anti-TNFα found that there was a decrease in IL-6, IL-8, and PGE2 expression levels, hence suggesting that therapeutic effects of anti-TNFα could be mediated by these cytokines [113,114]. In LBP patients, intradiscal injection of tocilizumab, which is a humanized monoclonal antibody directed against the IL-6 receptor, has been shown to result in similar effects as those observed with anti-TNFα injections, with a reduction in pain without any adverse effects. On the other hand, blocking p38 mitogen-activated protein kinases (MAPKs) that are involved in IVD cell apoptosis appears to have potential to help reduce the vicious catabolic circle in human degenerated NP cells, including a decrease in PGE2 levels and IL-6 induction by IL-1β and TNFα [115]. Of note, some molecular entities, such as triptolide from traditional Chinese medicines [116], lovastatin [117], and the synthetic NSAID diclofenac [118] have exhibited encouraging effects on catabolism, with downregulation of pro-inflammatory cytokines and proteolytic enzymes, combined with a pro-anabolic action on the NP microenvironment [117,118]. Moreover,

inhibition of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which is involved in the control of cellular responses to stress and cell survival, has been reported to lead to an increase in cell density and proteoglycan synthesis after intraperitoneal injection in a mouse model of accelerated aging [119].

Overall, these results indicate that while anti-catabolic and anti-inflammatory therapeutic approaches appear to have ample potential, they are not yet sufficiently robust to result in full regeneration of the IVD. Indeed, the reduction of catabolic events did not restore the anabolic function of the tissue, nor has rehydration or restoration of IVD height been observed. Despite the improvement in the IVD microenvironment and the decrease in tissue inflammation, resident cells failed to fully restart the disc biological machinery and synthetic activities.

4.2 Pro-anabolic strategies

4.2.1 Pro-anabolic molecules

The induction of extracellular matrix biosynthesis may also prove to be an effective strategy, as shown by studies in which the disc was supplemented with anabolic agents, mostly growth factors that regulate the survival, proliferation, and fate of cells (**Table 2**). Numerous in vitro studies have focused on the effect of OP-1 (BMP-7) on degenerated rabbit or human IVD cells, and they have reported a consistent increase in proteoglycans, aggrecan and type II collagen gene expression levels [120–124]. These in vitro results have been confirmed by in vivo investigations over periods of up to 6 months in various preclinical models, including induced IVD degeneration models in rabbits and rats, as indicated in Table 2 [125–130]. Partial improvement in the disc height index (DHI) has often been noted, suggesting that rehydration of the NP is probably related to the presence of more proteoglycan molecules in the tissue. However, after intradiscal injection of OP-1, extradiscal bone formation has also been reported to occur, notably in a spontaneous canine early IVD degeneration model [131]. Beneficial results on in vitro ECM synthesis by rat AF cells [132,133] and improvement of MRI scores in an in vivo rabbit IVD degeneration model have also been observed with bone morphogenetic protein 2 (BMP-2) [104]. Interestingly, the biomechanical properties also sometimes improved after these pro-anabolic GF injections, with significant increases in the viscous, stiffness, and elastic properties of the treated IVDs [104,127]. GDF-5, which belongs to the same BMP family, has also undergone extensive

assessment for use in disc treatments. Indeed, it has been shown that GDF-5 is essential for disc development, and it occurs naturally in IVD tissue [134], which makes it a highly suitable potential candidate for boosting a regenerative effect. In vitro treatment of IVD cells from various mammals with GDF-5 has been reported to increase the protein and gene expression levels of aggrecan and type II collagen as well as cell proliferation and the DNA content [135–138]. In vivo studies in rabbit, mice, or rat IVD degeneration models using GDF-5 injections have shown that there is a partial restoration of IVD height with significant improvement of MRI and histological grading scores as early as 16 weeks post-injection [138–140]. Therefore, four clinical trials have taken place with patients who had at least one symptomatic lumbar level (L3/L4 to L5/S1) confirmed and who were suffering from persistent LBP with at least three months of non-surgical therapy. These clinical trials lasted 12 months, with follow-up at 36 months to evaluate the safety, tolerability, and preliminary effectiveness of a single injection of GDF-5 (0.25 mg to 2.0 mg) (NCT01158924, NCT00813813, NCT01182337, and NCT01124006 on https://clinicaltrials.gov). In three of these four trials, no major adverse events directly related to GDF-5 injection were observed. The patients reported moderate improvement of their pain and disability. To our knowledge, the results of these clinical trials have not yet been published. TGF-β is another essential factor for IVD development and physiology that has been assessed as a potential therapeutic factor for restoring disc biological functions. In vitro and ex vivo studies have demonstrated a similar impact of TGF-β treatment compared to GDF-5, with an increase in PGs and collagen synthesis [141–144]. In particular, TGF-β has been shown to increase CTGF expression along with an enhancement of cellular activity via SMAD-3 and AP1 (activator protein) pathways [145]. Growth factors such as insulin-like growth factor (IGF-1) [146–149], platelet-derived growth factor (PDGF) [149,150], fibroblast growth factor (FGF) [149], and the transcription factor Sox9 [151,152] have also been investigated, and anabolic effects on ECM synthesis have been noted, along with cell proliferation and reduced apoptotic activity. In particular, Sox 9 and IGF-1 lead to an increase in proteoglycan and collagen synthesis in the NP [146,151–153].

Partially beneficial outcomes on IVD matrix biosynthesis after injection of a single growth factor has led to the notion of using a concentrated form of these growth factors, such as platelet-rich plasma (PRP). PRP activates the proliferation of human and porcine NP cells and it upregulates the synthesis of aggrecan and type II collagen [154–156]. Injection of autologous PRP in an

induced-IVD degeneration model in rabbit delayed the IVD degeneration and partially restored the disc height by stimulation of the synthesis of PGs, type II collagen, as well as cell proliferation [157,158]. Following these encouraging results in rabbits, clinical trials have been carried out with patients affected by moderate to severe discogenic LBP refractory to conventional treatments. Intradiscal injection of autologous PRP, with or without stromal vascular fraction (SVF), resulted in an improvement in pain relief and mobility as well as a reduction of disability [159,160].

Link-N peptide, which is an alternative bioactive entity that is less expensive as it is produced synthetically, has been shown to exert growth factor-like effects. Link-N naturally stabilizes PG aggregates in cartilaginous tissues, such as IVD, and it is expressed in vivo during tissue turnover [161,162]. Human and rabbit IVD cells treated with Link-N produced more PGs and type II collagen, and they underwent downregulation of MMP and ADAMT expression compared to non-treated cells [163–165]. These encouraging results appear to confirm that combined therapies are superior to single-target approaches to achieve a higher level of restoration of disc homeostasis. Some studies have already combined different biological factors in order to simultaneously operate on different biological targets (**Table 2**). Thus, the aim is to enhance the therapeutic effects of these distinct biological factors by delivering them concomitantly. Of the cocktails assessed to date, BMP-2 and TGF-β1 have undergone testing in several in vitro studies. Their synergistic effects have been shown to stimulate the synthesis of PGs by human, rabbit, and pig degenerated IVD cells, enhance gene expression of aggrecan and collagen, and increase DNA synthesis [166–168]. Moreover, association with a third growth factor, IGF-1, further enhanced the anabolic effect on PG synthesis [166]. Interestingly, BMP-2 coupled with TGF-β1 exhibited an additional anti-catabolic effect on porcine AF cells cultured with pro-inflammatory cytokines, which was probably due to a decrease in IL-1β and MMP-1 expression [168]. Other growth factor cocktails comprising TGF-β1 and FGF2 [169] or GDF-5 and CTGF [170], have been shown to result in a similar in vitro enhancement of ECM synthesis by degenerated human IVD cells. An in vivo gene therapy study performed in a rabbit IVD degeneration model indicated that intradiscal injection of AAV-mediated OP1 and SOX9 double gene co-transfection had a positive therapeutic effect, with partial restoration of IVD height and a greater T2-signal intensity due to increased aggrecan and type II collagen gene and protein expression [171]. Interestingly, a synergistic action of TGF-β1 and GDF-5, which have been shown to drive robust differentiation

of human adipose stromal cells into NPCy cells [59], has been reported to result in a beneficial effect on cell proliferation, specific gene expression, and partial restoration of IVD height following their intradiscal injection in degenerated murine caudal discs [172].

In order to achieve an ad integrum biological repair of IVD, the presence of cells able to respond to pro-anabolic factors is an essential requirement. Indeed, since most approaches are based on the stimulation of cellular biological functions, the presence of healthy cells in the diseased microenvironment appears to be paramount to ensure that these pro-anabolic strategies have a sufficient degree of efficacy. However, as previously described, during IVD degeneration there is a decline in the cell density in the NP, which becomes more pronounced as the degeneration increases in severity. Therefore, the few cells that remain are in an unfavorable environment and subject to constant biological stresses, with consequent impairment of their synthetic capacities. These pro-anabolic approaches to restoration of IVDs are hence greatly limited by the lack of healthy cells in the NP during IVD degeneration. In light of these shortcomings, NP supplementation with functional cells has been widely recognized by the scientific community as a particularly relevant approach for restoration of disc homeostasis.

4.2.2 Cell supplementation

4.2.2.1 Endogenous cell recruitment followed by mobilization

Interestingly, stem/progenitor cells have recently been described in healthy and degenerated IVD tissues in different mammals, including humans, rabbits, mini-pigs, and rats [173–177]. There is still no consensus on the phenotypic profiles of these progenitor cells, although Tie-2 and NOTCH-1 have been reported by several groups [174,178,179]. Resident stem cells are localized in several anatomical regions of the IVD (the CEP, AF, and NP), they express mesenchymal stem cell (MSC) markers, such as CD44, CD90, CD105, CD73 (reviewed in [102]), and they have been shown to resemble bone marrow MSC (BM-MSCs) [173,175–177,180–182]. Stem cells from the CEP, AF, and NP have been isolated and their phenotypic profiles, capacities for differentiation into the mesenchymal lineage, and abilities to migrate have been compared [183–185]. The chondrogenic differentiation properties of CEP-MSCs were better than those of NP- or AF-derived MSCs, and they were even superior to BM-MSCs [184,186]. CEP-MSCs also exhibited greater migration and invasive properties [183]. Thus, CEP-MSCs are likely to

represent a potential source of endogenous reparative cells. Endogenous repair, as an alternative to cell-based therapies, has already been reported for the regeneration of other tissues, such as cartilage, tendon, and heart [183,187–189], while it has only recently been considered for degenerated discs [190]. With the proximity of stem cell niches around the peripheral IVD (the perichondrium region and the AF border to the ligament zone) and the decreasing gradient of stem/progenitor cells from the CEP to the NP, potential migration patterns towards the degenerated NP have been suggested [173,191,192]. In order to recruit the stem/progenitor cells towards the NP tissue, the specific chemokines regulated and normal T cell expressed and secreted (RANTES), also called C-C motif ligand 5 (CCL5), and stromal cell-derived factor-1 (SDF-1), also called C-X-C motif chemokine ligand 12 (CXCL12), have been investigated. CCL5 is overexpressed by degenerative bovine [193] and human IVD cells [194], and it enhances the migration of BM-MSCs. In parallel, MSCs have been reported to express chemokine receptors and, more especially, human discal stem cells have been shown to express these chemokines receptors, with an increase of CXCR4 expression, which is the CXCL12 receptor, in MSCs isolated from endplates [183]. In light of the results demonstrating a superior response in terms of CEP-MSCs migration compared to AF-MSCs and NP-MSCs, recruitment followed by mobilization of MSCs from the CEP toward the NP has been evaluated, as well as the regenerative and pro-anabolic impact. Ex vivo studies performed in a bovine nucleotomized IVD degeneration model have shown that it is possible to stimulate the migration of MSCs from the CEP toward the NP, thereby boosting ECM neosynthesis [195,196]. In summary, these resident disc stem/progenitor cells constitute a reservoir of potential reparative cells that could be an alternative to the injection of exogenous cells. They could be recruited from the vicinity of the disc towards the center of the degenerated IVD by the injection of chemokines into the NP tissue. However, their number and regenerative potency have been shown to decrease with age and DDD [178], thus limiting the use of these endogenous cells to repopulate the NP in severe cases of DDD and in elderly patients. Hence, in these cases of late IVD degeneration, recourse to exogenous cell injections appears to be a better option.

4.2.2.2 Injection of exogenous cells

A variety of potential sources for replacement NP cells have been explored in the past two decades, although no consensus has been reached regarding the ideal cell population for

regeneration of IVDs [197]. Cell candidates that can replace the degenerated disc cells and act as metabolically active disc cells include IVD-derived cells (healthy NPCy and AF cells), cells with an NP-like phenotype that are able to synthesize cartilaginous ECM (e.g., chondrocytes from articular cartilage), and stem/progenitor cells with the ability to differentiate into NP cells (e.g., MSCs or induced pluripotent stem cells) [99,102,197,198]. The most rational approach involves the use of native disc cells to repopulate the degenerated IVD. Autologous NP cell transplantation has been assessed in several in vivo studies that were performed in a number of different types of animal models. Doing so delayed the degenerative progress, with sometimes the promotion of regeneration after injection, and the production of type II collagen (reviewed in [199]). Clinical trials have since been performed in patients undergoing spinal fusion in adjacent IVDs, and the safety and the effectiveness in terms of the decrease in the patient's pain after autologous cell injection have been described with a two-year follow-up [200–202]. Despite encouraging results, this strategy is nonetheless greatly limited by the low availability of such IVD-derived cells and the associated risk of donor site morbidity [200,202,203]. Human IVD cells have so far only been harvested from adjacent discs or from herniated discs, which were certainly degenerated and consequently compromised by a low metabolic activity [204]. To boost the biological activity of such cells, in vitro processing and/or co-culture with mesenchymal stem cells (MSCs) or AF cells prior to their injection have been suggested [205–207]. However, the low proliferative capacity of these cells and their tendency to undergo dedifferentiation during their in vitro expansion are major limitations of this strategy [204,208]. Others studies have sought to overcome the donor site morbidity by using xenogeneic IVD-derived cells, as they have been shown to survive for 6 months post-injection; however, these cells were ultimately not efficient enough to regenerate NP tissue [206,209,210]. As an alternative to IVD-derived cells, transplantation of chondrocytes has been investigated as a means to regenerate IVDs in rabbit and porcine models [211,212]. Even though their phenotypic similarities with NPCy make them potential candidates to replace degenerated NP cells, limited chondrocyte availability, a low capacity to proliferate, the risk of dedifferentiation after in vitro expansion, and donor site morbidity have thus far precluded their use in disc cell therapies. Finally, MSCs, which constitute the third source of cells, have been studied the most and they have been shown to have key properties for discal regeneration while allowing some of the previously mentioned limitations to be overcome. Indeed, MSCs are readily available, as they can be obtained from bone marrow, adipose tissue, skeletal muscles, synovial

membranes, and umbilical cord blood with less donor site morbidity. BM-MSCs have been the most studied MSCs for disc regeneration, closely followed by adipose stromal cells [213]. Their ability to differentiate along the mesenchymal pathway, notably into chondrocyte-like cells, as well as their capacity for in vitro expansion without alteration of their differentiation potential, make them promising candidates for IVD cell therapy. Consequently, several studies have investigated the differentiation of MSCs toward NPCy-like cells, with the aim of repopulating the degenerated NP following their intradiscal injection [59,214,215]. In vitro studies have assessed the use of a specific cocktail of growth factors to drive in vitro NP differentiation [216]. Growth factors of the TGF-β superfamily are very attractive candidates for initiating MSCs differentiation toward NPCy-like cells, particularly TGF-β and GDF-5 or GDF-6, which should act synergistically to stimulate the typical NP ECM synthesis and the gene expression of distinctive markers of NPCy [59,170,217–221]. Other strategies are focused on the cell-cell and paracrine interactions between NPCy and MSCs, which could alter the differentiation of MSCs toward the NPCy phenotype. Co-culturing MSCs derived from bone marrow, adipose tissue, and skeletal muscle with NP cells has been investigated extensively in order to study the relationship between these two cells types. This has revealed synergistic effects, with an increase in PGs and type II collagen gene expression and synthesis as well as upregulation of specific miRNAs [222–226]. In light of the crucial role of the microenvironment on the fate of MSCs, co-culture of degenerated NPCy has also been assessed [227,228]. Interestingly, enhancement of NP cell viability and proliferation has been observed, thus indicating that collaboration between these two cell types can occur [206,229]. In keeping with these encouraging achievements, undifferentiated MSCs have been injected into degenerated IVDs to evaluate their regenerative action as a result of communication with NPCy and their known immunomodulatory and anti-inflammatory properties [230,231]. A considerable number of such pre-clinical studies have been performed, in both small and large animal models (i.e., mice, rats, rabbits, dogs, pigs, goats, and sheep). In most cases, the restoration of IVD height, an increase in T2 signal intensity, the upregulation of ECM component synthesis, and specific gene expression by resident IVD cells and/or transplanted MSCs have been reported (reviewed in [199]). While some authors have reported a superior regenerative effect with MSCs following disc injection, or with disc-like cells [212], most studies indicate that MSCs and chondrocyte-like cells have similar efficiencies [232,233]. Moreover, the use of more readily available allogenic or xenogenic cells has also been assessed, with

comparable results and no immune reaction in light of the hypovascularity and immune privilege of the IVD [209,234–236]. Finally, a small number of clinical trials conducted in discogenic patients have confirmed the feasibility and the safety of intradiscal injection, as well as a significant decrease in pain [237] and improvements in IVD hydration and height have been observed [238–242].

While it could be more beneficial to associate simultaneous supplementation with cells and biological factors to allow for a greater degree of anabolic restoration of ECM synthesis, only a small number of studies along these lines have been reported to date (Table 2). Among these, combined intradiscal injection of Link-N peptide and mesenchymal stem cells in an ex vivo bovine induced IVD degeneration model resulted in anabolic effects on ECM synthesis and on MSC survival in the IVD microenvironment [165]. More recently, intradiscal injection of a PRP/BM-MSCs suspension in a rabbit IVD degeneration model has yielded similar results at 8 weeks post-injection, with an increase in T2 signal intensity observed after 1 week, and a continuous regenerative effect for the following 7 weeks [243].

Finally, the precise therapeutic mechanism of MSCs has not yet been elucidated. It may be mediated by direct cell-cell contact, the transfer of secreted molecules, or the transfer of extracellular vesicles (EVs) between cells. Indeed, EVs act as major mediators of intercellular communication [244] by transferring biologically active molecules such as proteins, lipids, mRNA, and miRNA from parental cells to target cells [245]. The term EVs encompasses exosomes, microvesicles, and apoptotic bodies, which are 40-500 nm-sized particles that are released by cells in a constitutive or inducible manner. As a cell-free strategy, EVs may provide an alternative to cell therapy, with the advantage of having an extended shelf life [246]. However, while the therapeutic benefits of EVs have been clearly shown with skin injuries [247] and inflammation-associated degenerative diseases [248,249] (e.g., osteoarthritis), their use in IVD regeneration is still in its infancy. Only two recent in vitro studies have shown that human NPCy-derived exosomes induce MSCs migration and differentiation toward an NP cell-like phenotype; that human MSCs-derived exosomes boost proliferation and healthy ECM synthesis by degenerated NPCy [250]; and that porcine MSC-derived EVs exert anabolic effects on canine and human NP cells [251].

5. Sequential delivery systems for IVD regeneration

A common denominator to all of these innovative strategies aimed at restoration of the catabolic/anabolic balance is that they rely on the injection of biological factors and/or cells into a degenerated and harsh NP microenvironment. Since anti-catabolic and pro-anabolic factors, which are known to have very short half-lives, are particularly prone to in vivo degradation, and cells are known to have very low in vivo post-injection survival, their protection within a biomaterial is highly relevant and could promote their tissue regenerative effects [252,253].

For over a decade, drug delivery systems (DDS) have been designed as macro-, micro-, and nano- platforms for specific applications and delivery routes (systemic and local). In the case of an avascular organ such as the IVD, systemic administration is not a viable option, and the recommended delivery route is minimally invasive injection directly into the damaged tissue. In light of this, IVD biomaterials should be injectable and allow local and controlled release of biological factors and/or cells. Macro-hydrogels, microparticles, or nanoparticles appear to be the most appropriate biomaterials (reviewed in [99]). Synthetic or natural polymers have been extensively studied in the context of tissue engineering approaches, with the aim of structurally replacing the IVD and restoring its functionality. Various scaffolds able to withstand physiological loads have been reviewed in terms of their specific advantages and drawbacks for IVD regeneration [254]. Due to the high number of reviews published on the topic, we have focused this paper on the delivery of biological factors and cells. In terms of natural polymers, hyaluronic acid (HA) and collagen are particularly relevant due to their natural presence in healthy IVD tissue, their potential bioactivity, and their biodegradability in vivo. Alginate and chitosan, although not naturally present in the human body, are also options, given their biodegradability properties and the anti-bacterial capacities of chitosan [255,256]. In parallel, synthetic and inert polymers, such as polyethylene glycol (PEG), polyurethane (PU), polylactic/glycolic acid (PLA and PGA) and derivates (e.g., PLGA), and poly-caprolactone (PCL) offer chemical versatility.

As detailed above, IVD degeneration involves multiple dysregulations at different levels, with co-involvement of various processes such as catabolic and inflammatory events that occur in parallel with impairment of ECM component synthesis as well as cell senescence and apoptosis. Thus, a combination of strategies may be needed for complete restoration of IVD tissue. Going one step further, we envision sequential delivery by a single intradiscal injection that would target

i) the inflammatory, pro-catabolic, and hostile microenvironment by the release of anti-catabolic or anti-inflammatory cytokines; followed by ii) the cell density, by increasing the number of cells in this healed microenvironment either by endogenous cell mobilization or by exogenous cell injections, and finally iii) the cellular functions, mostly the synthesis of adequate extracellular matrix, by the release of pro-anabolic factors. Overall, the regenerative process (as illustrated in Fig. 3A) would be based on the release of anti-catabolic factors, to induce a more favorable microenvironment by both reduction of inflammation and downregulation of degradative enzymes, followed by supplementation with cells and anabolic factors to encourage the neosynthesis of ECM that is essential for tissue restoration. It is worth noting that the sequence of effects needs to be given careful consideration since most of the anabolic cues undeniably require the presence of healthy cells to exert their therapeutic effect and that cell survival depends on the nature of the IVD microenvironment. The presence of cells in the damaged IVD tissue differs, along with their biological activities, according to the severity of the disease. Hence, when cells are non-functional or when the IVD cell number decreases, cell supplementation by the recruitment of endogenous cells or by exogenous cell injection is a fundamental pre-requisite for the subsequent efficacy of the pro-anabolic factors that are released. Thus, a hierarchical approach with sequential steps of therapeutic agent delivery would be most beneficial to counteract the various IVD degenerative processes.

Although multistage drug delivery systems that are triggered by specific tumor microenvironment stimuli have been used for cancer therapy and imaging purposes, their design is based on parameters that do not apply to IVD regeneration, such as stability during transport in the bloodstream, deep penetration into tumor tissues, and multidrug resistance issues (reviewed in [257]). In this context, a complex design is needed to be able to deliver the biological factors in a consecutive and optimal manner as a single injection. Controlled release strategies are usually based on diffusion throughout a polymeric network (reviewed in [258]), polymer cleavage (reviewed in [259]), or affinity binding [260].

5.1 Sequential delivery of biological factors

In terms of skeletal tissue regeneration, sequential delivery systems specifically designed for bone and cartilage have been extensively reviewed [261–264]. These systems are multiphasic platforms composed of various polymers and biomaterials, often associating nano- or micro-

particles (e.g., spheres, capsules) with an injectable macro-scale hydrogel (**Figure 3A**). Hydrogels designed at different scales can be combined to form a system for the delivery of multiple therapeutic agents (for a review on hydrogel features, see [265,266]). For example, IGF-1 has been loaded into gelatin microspheres that were then dispersed in a chitosan gel containing BMP-2. The in vitro release profiles showed that were was a slowdown of IGF-1 delivery when the loaded microspheres were embedded in a chitosan gel, compared to microspheres in suspension, and this temporally-controlled release of the two molecules was shown to enhance osteoblast differentiation in vitro [267]. Recently, the combination of three different biomaterials has also been tested as a dual delivery scaffold [268]. A calcium phosphate porous cement containing alginate microspheres loaded with BMP-2 was coated with an alginate hydrogel loaded with PDGF. Following the rapid release of the PDGF over a 5-day period, there was a burst of BMP-2, followed by sustained release of both PDGF and BMP-2 for 10 days. A recent attempt at generating a flare-responsive hydrogel, considered as a smart biomaterial, that could undergo disassembly and thereby release the encapsulated drug in response to the concentration of matrix metalloproteinase MMP-2, MMP-3, or MMP-9, has been reported [269]. This system for "on demand" drug release upon exposure to high levels of inflammation, which was designed for inflammatory arthritis treatment when arthritis-related enzyme concentration increases during flares, could be considered for stage 1 of IVD regeneration by the incorporation anti-catabolic factors.

Sequential release of IVD therapeutic factors, whether anti-catabolic and pro-anabolic entities, should be carefully designed and could be based on sustained delivery systems (**Table 3**). PLA pellets (approximately micro-scaled particles) provided sustained release of the anti-catabolic factor clonidine over a three-month period in a porcine model of IVD [270]. Moreover, poly-ϒ-glutamic acid (PGA) and chitosan nanoparticles loaded with diclofenac exhibited anti-catabolic effects for at least 14 days in an ex vivo bovine model of degenerated IVD [118]. Two chondrogenic factors, kartogenin (KGN) and pentosane polysulphate (PPS), combined with chitosan or PEG, respectively, have been studied in association with an HA-based hydrogel [271,272]. The results revealed sustained in vitro release and a prolonged impact of these chondrogenic factors on BM-MSCs in terms of GAG and collagen synthesis. Pro-anabolic TGF-β3 was released in vitro from photocrosslinked carboxymethylcellulose (CMC) hydrogels and was shown to have prolonged biological effects on GAG and collagen synthesis for 8 weeks

[273]. However, in this study, macro-scale hydrogels might not be effective when it comes to releasing chondrocyte-inducing factors or TGF-β3 during the last stage of the regeneration process, that is to say, long after the anti-catabolic factors and cells have been released (Fig. 3A). Lee et al. devised a combined system of PCL microspheres embedded in a pluronic F127/sodium hyaluronate hydrogel, and they confirmed that there was sustained release of this model drug in an ex vivo IVD model [274]. Similarly, PLGA microspheres have also been shown to allow sustained release of IL-1Ra for up to 35 days of culture of NP/agar constructs [111]. On the other hand, sustained in vitro IGF-1 release from chitosan or PLGA microparticles improved when PLGA microspheres were embedded in a silk fibroin porous scaffold [275,276]. Likewise, pullulan microparticles or nanofibrous silica can release TGF-β1 and GDF5 for at least 28 days in vitro, and their release was even slower when they were embedded in a hydroxypropyl methylcellulose silanized (HPMC-Si) hydrogel [277,278]. PRP, a concentrate of GFs, resulted in sustained released from gelatin microspheres for up to 8 weeks in a rabbit induced IVD degeneration model [279,280].

An IVD-specific platform based on injectable MMP-responsive hydrogel containing MMP-responsive polyplex-micelles loaded with a specific miRNA known to suppress fibrosis was recently developed by Feng and collaborators [281]. miR-29a was complexed with 70-nm MMP-responsive polyplex micelles and then encapsulated in an MMP-responsive poly(ethylene glycol) (PEG) hydrogel. Two-stage delivery of miR-29a was demonstrated in vitro, in the presence of MMP-2, and significantly attenuated fibrosis progression in IVD was obtained in two needle puncture models (rat tails and rabbit lumbar spines). However, this could not be considered to be a sequential delivery system since the same factor was released in each stage. Of note, the efficacy of any miRNA strategy undeniably requires the presence of either exogenous or endogenous healthy cells. To our knowledge, no study to date has reported an IVD-specific sequential delivery system.

5.2 Cell and biological factors combined strategies

In terms of the cell supplementation stage, biocompatible natural or synthetic biomaterials that provide mechanical properties and that enable specific ECM synthesis have been extensively reviewed for cell delivery aiming to restore the cell density in degenerated IVDs [198,254,282]. Among these, hyaluronic acid, which is a natural component of the NP matrix, has been widely

considered for cartilage and spine applications [283,284], and as an IVD cell supporting hydrogel in particular [285]. In vitro studies have shown maintenance of the phenotype of human, bovine, and rabbit NP and AF cells for one week when cultured in an HA hydrogel [286–289], as well as mechanical properties close to those of the of native tissue, which is a significant advantage for maintaining the NPC phenotype [287,289]. In some cases, an increase in the production of specific ECM components, as well as the expression of specific IVD markers have been reported [287,288]. Interestingly, alginate and chitosan hydrogels have yielded similar results in terms of maintenance of the cellular phenotype and ECM synthesis by IVD cells from rabbits [290], pigs [291], sheep [292], cows [252,293,294]. and humans [295,296]. Multiple pre-clinical studies have assessed the regenerative effects of cell supplementation strategies, combined with a macro-scaled hydrogel, after intradiscal injections in various animal models of IVD degeneration (reviewed in [199,297]).

The combined injection of exogenous cells and biological factors has also recently been investigated with the development of biphasic or even triphasic delivery systems that can be administered as a single injection. For example, PLGA nanoparticles were used to encapsulate TGF-β3 and these nanoparticles were then loaded into a dextran/gelatin hydrogel with MSCs. The in vitro results confirmed sustained release of TGF-β3 and induction of NP-like cell differentiation of the MSCs in this biphasic system, notably by the synthesis of suitable ECM [298]. In another study, basic fibroblast growth factor (bFGF) [299] or TGF-β3 [300] were encapsulated in heparin/poly-L-lysine nanoparticles that were loaded into dexamethasone/PLGA microspheres, and the resulting carrier was used to intradiscally deliver allogenic BM-MSCs or allogenic adipose-derived MSCs, respectively. This led to a significantly higher expression of matrix-specific genes and enhanced ECM synthesis in vitro, an improvement in the DHI score, and an accumulation of PGs in a rat IVD degeneration model. This strategy fits the requirements for a sequential delivery system, as illustrated in Fig. 3A. Indeed, anti-catabolic dexamethasone (stage 1, microparticles), exogenous cells (stage 2, micro-scaled carriers), and pro-anabolic TGF-β3 (stage 3, nanoparticles) are generated to ultimately allow maximal restoration of IVD tissue. However, the sequence of events may not be optimal, as the cells are released first from the microcarriers into a harsh environment in which they may not survive. Hence, when finally released from the nanoparticles, the TGF-β3 target, i.e., healthy cells, will not be present and there will be no beneficial anabolic effect. Incorporation of this combined micro- and nano-

particle systems within a macro-scale hydrogel (Fig. 3A) may provide a degree of protection to the cells, and delay their release into what by then has become a less inflammatory microenvironment.

Recent cell therapy strategies have relied on the recruitment of endogenous reparative cells, such as resident stem/progenitor cells, by injection of specific chemokines embedded in either micro-scaled particles or macro-scaled hydrogels (Fig. 3A). CXCL-12, also called SDF-1, can be released over a 120 h period from chitosan/ ϒ-PGA polyelectrolyte complexes and thus enhance migration of BM-MSCs in vitro after its release [301]. CXCL-12 released by a hyaluronic acid-based hydrogel in an ex vivo bovine IVD model after intradiscal injection has been shown to result in an increase in cell migration from the CEP to the NP in terms of both cell number and migration distance [195]. A recent in vivo study performed in a rat IVD degeneration model has shown enhanced IVD regeneration 8 weeks after intradiscal injection of CXCL12 loaded on albumin/heparin nanoparticles [302]. There was an increase in Sox9, aggrecan, and type II collagen mRNA and protein levels, which could be due to either stimulation of the resident NP cells or the recruitment of stem/progenitor cells from the vicinity of the IVD towards the NP. These recruited cells could then synthesize these specific ECM components in the NP microenvironment or stimulate resident NP cells [302].

5.3 On-demand release strategies

IVD Although a sequential release system can be a powerful tool for restoration of degenerated IVDs, its rational design is much more complex than that of a single-compound delivery system. Such a strategy requires the separate control of different drug releases, in space, time, and dose. This is particularly difficult to achieve in the harsh environment of a degenerated tissue, and even more so when combined with the high pressure and osmolarity of IVDs. Furthermore, while examples of DDS with simultaneous multi-release rates (i.e., fast vs. slow) have been reported [303], the feasibility of an actual sequential release in vivo, where one compound is released after another, remains to be shown. To date, most stimuli-responsive strategies for on-demand release are based on sudden changes in pH, temperature, redox potential, chemical environment, or light exposure [304]. These are mostly not compatible with single injection approaches, and they are often not practical for treatment, especially for IVDs, which are not readily accessible. Thus, being able to independently set intervals between release phases is a major roadblock that cannot

be overcome with the currently available technologies; and the development of innovative in situ triggers will be required in order to achieve this. In addition, it is worth noting that protein therapeutics are often prone to rapid unfolding and degradation, with their efficacy limited to days or weeks, thus calling for advanced stabilization techniques in protein design for further development of sequential release methods. Finally, when considering the co-delivery or in situ activation of cells, the secretion of biologically active factors must occur as a second stage mechanism, thus implying transient ways of maintaining cells in a dormant state or of blocking secretion for a certain amount of time, which adds to the complexity. Notwithstanding these challenges, tremendous progress in drug delivery and conceptual release strategies has nonetheless been made over the past 20 years, hence giving rise to innovative and promising approaches. Polymer-drug conjugates and tunable nanocarriers are becoming common tools for the controlled release of small-molecule drugs [305–307]; libraries of cleavable linkers and conjugation kits have become commercially available; and materials with programmable degradation, including self-immolating, stimuli-responsive, and supramolecular approaches, are being developed [308,309], thus paving the way for more achievable sequential release strategies. All things considered, it seems likely that new generations of DDS that are based on composite materials whereby bio-physico-chemical phenomena are controlled from the nano- to the macroscopic scale will eventually become available. This will presumably involve the convergence of innovations in drug design, biological targets, macromolecular design, and biomaterial engineering, with the cutting-edge technologies of today becoming the building blocks of tomorrow.

5.4 Sequential release strategies as a function of disease progression

Finally, in terms of the severity of disc disease, different release sequences can be considered, in association with the use of macro-, micro- and nanoplatforms (Fig. 3B). For discs at an early stage of degeneration, when not all of the local disc cells have already died, anti-catabolic factors followed by pro-anabolic strategies may be a suitable approach. For discs at an intermediate stage of degeneration, an additional step involving endogenous cell recruitment is necessary to provide healthy cell targets for the pro-anabolic factors. Finally, tissues at a late stage of degeneration, and thus when the endogenous cells may no longer be available, would require either autologous or allogenic exogenous cells being added to the anti-catabolic and pro-anabolic factors. The difficulties in implementing these strategies are, however, multiple. Clinically, assessment of

early DDD, which is mostly asymptomatic, and the consequent establishment of preventive treatments to reverse the IVD degeneration processes before the appearance of pain and disability, constitute a real challenge. An imaging technique such as T1ρ-weighted MRI, which correlates with the PG concentration [310], appears to be a promising way to detect early asymptomatic DDD that is superior to the T2-weighted MRI in athletes [311] and in an ex vivo model of lumbar caprine IVD [312]. Indeed, the quantitative T1ρ values of weightlifters were significantly lower than those of sedentary matched control patients [311] and they displayed a strong positive correlation with the disc mechanical behavior, IVD histology, and water and GAG content of an ex vivo early IVD degeneration caprine model [312], with higher differences than with T2-weighted images compared to IVDs that have not undergone degeneration. Loss of diffusion routes is another parameter that needs to be considered when implementing these strategies based on the severity of the disease. Indeed, calcification of CEPs during IVD degeneration decreases oxygen, nutrient, and waste metabolite diffusion, which places stress on the IVD microenvironment. Restoration of transport thus appears to be paramount for the effectiveness of biological approaches. This is particularly the case for restoration of the nutrient supply, which is an essential prerequisite for the therapeutic effect of endogenous or exogenous cells [76].

6. Conclusion

The process of IVD degeneration is complex and multifactorial, with dysregulation of multiple processes, including the overexpression of degradative enzyme, pro-inflammatory cytokine upregulation, the loss of healthy cells, and decreased matrix synthesis. While approaches based on biological factors or cell therapy, sometimes in association with biomaterials, have yielded promising results, it seems reasonable to speculate that effective strategies for full biological and mechanical healing of the tissue will likely require advanced strategies based on multiscale delivery systems of biological factors. We hypothesize that sequential delivery systems, aimed at targeting the three main stages of IVD degeneration, can restore the IVD machinery and regenerate all of the tissue components, both structurally and functionally. It should be stressed, however, that the sequence of biological factor release needs to be given careful consideration. To our knowledge, no IVD-specific sequential delivery system has been reported to date. Although a sequential delivery system could constitute a powerful tool for restoring degenerated IVDs, the rational design of such a system is much more difficult than for a single-component

system. Among the limitations that could stymie such development, the precise control of temporal release, both in terms of the timing and the degree of the release, is difficult to engineer, in light of the harsh environment of a degenerated IVD, combined with the high local pressure and osmolarity. There is nonetheless ample reason to believe that recent developments with complex delivery systems, based on macro, micro-, and nano-objects will pave the way to innovative and effective regenerative strategies.

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Figures legends

Fig. 1: Internal structure and the network of biological factors involved in homeostasis of young and healthy intervertebral discs (IVDs)

The IVD is a hypovascular tissue that has three distinct components: the nucleus pulposus (NP), which is a gelatinous and hydrated structure surrounded by the annulus fibrosus (AF), which is a lamellar collagenic region, and finally two cartilaginous endplates (CEPs) that interface with the vertebral bodies. All of these components play a significant role in maintaining disc homeostasis. CEPs contain chondrocytes and enable oxygen, nutrient, and waste metabolite diffusion. The AF contains fibroblasts that are organized into a complex multilayer structure that constrains the NP and that provides the IVD with elastic properties. In the early years of human life, notochordal cells and nucleopulpocytes (NPCy) communicate by secreting specific growth factors: CTGF, Shh, and TGF-β1, which regulate the anabolic/catabolic balance. Notochordal cells secrete CTGF and Shh to stimulate cell proliferation and the synthesis of ECM components such as aggrecan

and type II collagen fibers. The TGF-β1 synthetized by NPCy acts in a self-regulatory loop that enhances CTGF expression by notochordal cells. Others biological factors synthesized by NPCy also play crucial roles in IVD physiology. Among them, GDF-5 boosts the proliferation and function of NPCy and ensures the balance of anabolic/catabolic events.

CTGF: Connective tissue growth factor; **Shh**: Sonic hedgehog; **TGF-β**: Transforming growth factor β; **ECM**: Extracellular matrix; **CCL5**: C-C motif ligand 5, also called Regulated And Normal T cell Expressed and Secreted (RANTES); **CXCL12**: C-X-C motif chemokine Ligand 12, also called, stromal cell-derived factor-1 (SDF-1); **VEGF**: Vascular endothelium growth factor; **IL-6 and -8**: Interleukin 6 and 8, **TIMPS**: Tissue inhibitors of metalloproteinases; **MMPs**: Metalloproteinases; **GDF5**: Growth differentiation factor-5.

Fig. 2: Pathophysiological cascade in IVD degeneration

The dysregulation of homeostasis is a key element of IVD degeneration, with a significant decrease in the nucleopulpocyte (NPCy) number combined with qualitative and quantitative changes in ECM components. Endplate calcification impedes diffusion paths, decreasing the nutrient supply to IVD cells and increasing the accumulation of metabolic waste within the tissue and it leads to NPCy phenotypic changes and ultimately senescence and apoptosis. Meanwhile, NPCy produce pro-inflammatory cytokines such as IL-1β and TNFα that boost the synthesis of MMPs and ADAMTSs, which in turn inhibit the production of ECM components and drive changes in the ECM integrity. In parallel, the overexpression of IL-6, IL-8, and PGE2 by NPCy stimulates innervation and vascularization by NGF and VEGF, thereby generating IVD pain. This neo-innervation and vascularization favor the recruitment of immune cells, which amplifies degeneration by stimulating the pro-inflammatory and anti-catabolic effects of TNFα and IL-1β. The IVD degeneration processes thus amount to a self-perpetuating vicious circle that amplifies degeneration.

ECM: Extracellular matrix; **IL-6 and IL-8**: Interleukin 6 and 8; **PGE2**: Prostaglandin 2; **MMP-1 and -3**: Metalloproteinases 1 and 3; **ADAMTS**: A Disintegrin And Metalloproteinase with Thrombospondin Motifs; **NGF**: Nerve growth factor; **TIMPS**: Tissue inhibitors of metalloproteinases; **TNF-α**: Tumor necrosis factor α; **IL-1β**: Interleukin 1β; **GDF5**: Growth differentiation factor 5; **VEGF**: Vascular endothelium growth factor; **NPCy**: Nucleopulpocytes.

Fig. 3: Possible sequential biological agent release strategies for a maximal IVD regeneration

We hypothesize that a sequential delivery that would i) target the inflammatory, pro-catabolic, and hostile microenvironment by the release of anti-inflammatory or anti-catabolic cytokines; then ii) increase the cell density in this microenvironment, and finally iii) enhance the cellular synthesis of extracellular matrix by the release of pro-anabolic factors, constitutes an innovative yet challenging approach to IVD degeneration. It is worth noting that the sequence of events needs to be given careful consideration, as most of the anabolic cues undeniably require the presence of healthy cells to exert their therapeutic effect.

A. Anti-catabolic factors can be incorporated into macro-scaled hydrogels, micro-scaled (matrix, porous, or capsules) particles, and nano-scaled particles (micelles, nanotubes, or matrix), with the aim of obtaining a sustained initial release in the hostile microenvironment. Due to the size of cells, the use of nanoparticles is not suitable for cell supplementation, and they are hence not presented in the diagrams (although nanoparticles could be used to deliver chemoattractant molecules to recruit endogenous cells). Moreover, macro-scaled hydrogels, which usually have quite fast delivery profiles for soluble molecules, are not suitable for the delivery of anabolic factors, which should occur in the final stage of regeneration, and they are hence not represented in this figure.

B. The disease progression has to be taken into account when selecting a sequential delivery treatment. Indeed, to reverse the complex degenerative IVD process, treatment strategies should be applied that have an appropriate timing based on the degenerative cascade. According to the severity of the disease, different sequential release patterns can be envisioned, involving the use of macro-, micro-, and nano-scaled biomaterials. For discs at an early stage of degeneration, when local disc cells are still healthy and available, anti-catabolic entities followed by pro-anabolic strategies are likely to be suitable. For discs at an intermediate stage of degeneration, an additional step of endogenous cell recruitment is necessary to provide healthy targets for the pro-anabolic factors. Finally, tissue at the late stage of degeneration, whereby endogenous cells may no longer be available, would require supplementation with either autologous or allogenic exogenous cells.

TNF-α: Tumor necrosis factor α; **IL-1β**: Interleukin 1β; **IL-6**: Interleukin 6; **NP**: Nucleus pulposus; **TGF-β**: Transforming growth factor β; **PRP**: Platelet-rich plasma; **NPCy**: Nucleopulpocytes.

Table 1: Anti-catabolic / anti-inflammatory strategies for the regeneration of IVD

	Factor	Model	Study design	Effects	Ref
Anti-cytokines	anti-TNFα: Infliximab	Human IVD cells cultured with TNFα [114] Induced IVD degeneration rat model [313]	In vitro study [114] In vivo study of 6 weeks – by intradiscal injection [313]	Reduction of Il-6, Il-8, Il-1β expression levels [114] Decrease of pain and semi-quantitative IVD degeneration grading scale [313]	[114,313]
	anti-TNFα: Infliximab [110] Etanercept [53,105,109] Adalimumab [106,107]	Chronic discogenic low back pain patients with severe sciatic [105,110], or radicular pain [53,106,107]	Clinical study from few months [53,105,107,109,110] to 3 years [106] follow-up about 20, 50, 143 or 265 patients – by single intravenous [110], 2 or 3 subcutaneous [105–107], 1 perispinal [109] or 2 transforaminal epidural injections [53]	Reduction of patient's pain and VAS intensity [105,109] Small decrease [106,107] or no reduction of the need of surgical procedures [110]	[53,105–107,109,110]
	Anti-IL-6 receptor: Tocilizumab	Patients with discogenic LBP [314,315] and spinal stenosis [314]	Clinical study of few month follow-up about 60 patients – by epidural infiltration onto the spinal nerve [314] or intradiscal injection [315]	Reduction of LBP, leg pain and leg numbness without adverse event [314] Improvement of ODI and NRS [315]	[314,315]
Anti-proteases	anti-ADAMTS5 oligonucleotide	Induced IVD degeneration rabbit model	In vivo study of 8 weeks - delivered by intradiscal injection	Improvement of MRI and histologic grades	[103]
	TIMP-1	Induced IVD degeneration rabbit model	In vivo study of 12 weeks - delivered by AAV vector intradiscally injected	Improvement of MRI grades, histological observations, serum biochemical analysis and biomechanical properties	[104]
Inhibitors of signaling pathways	anti-TNFα : soluble TNF receptor type II	Human IVD cells cultured with TNFα	In vitro study	Attenuation of the inflammation Reduction of TNFα, NO and PGE2 levels	[113]
	IL-1 receptor antagonist : Il-1Ra	Degenerate human IVDs from surgical patients	Ex vivo study of 2 weeks - delivered directly or by AAV intradiscally injected	Attenuation of the matrix degradation	[112]
	Inhibition of p38 MAPK activity	Human degenerated NP cells cultured with IL-1β or TNFα	In vitro study with alginate beads	Reduction of PGE2 and Il-6 Increase the TIMP-1 / MMP3 ratio	[316]
	Triptolide [116] Bovine lactotransferrin [317]	Human [116,317] or bovine [317] degenerated IVD cells pre-treated by IL-1β or rabbit IVD organ model cultured with Il-1 [317]	In vitro studies [116,317] – cultured with cells un alginate beads [317] or ex vivo study [317] – by intradiscal injection	Inhibition of Il-6 [116,317], Il-8 [116], PGE2 [116], MMPs [116,317], ADAMTs [317], toll-like receptors -2 and -4 expression [116,317] Increase of PGs [317], aggrecan and collagen II [116] expression	[116,317]
	Lovastatin	Induced IVD degeneration rat model	In vivo study of 4 weeks - by intradiscal injection	Up-regulation of GAG, aggrecan, collagen type II, Sox9 and BMP-2 expression Decrease of collagen type I	[117]
	Wogonin	Rat NP cells treated with IL-1β Induced IVD degeneration rat model	In vitro study In vivo study of 8 weeks	Suppression of iNOS, IL-6, COX2, MMPs and ADAMTSs expression Up-regulation of collagen type II	[318]

	Inhibitors of NF-κB : Nemo Binding Domain (8K-NBD) peptide	DNA repair-deficient mouse model of accelerated aging (Ercc1-/Δ mice)	In vivo study of 25 months-by 3 intraperitoneal injections per week	Increase disc cellularity and PGs synthesis	[119]

TNFα: Tumor necrosis factor α, **IL-6**: Interleukin 6, **VAS**: Visual analogue scale, **ODI**: Oswestry disability index, **NRS**: Numeric rating scale, **ADAMTS5**: A Disintegrin And Metalloproteinase with Thrombospondin Motifs 5, **MRI**: Magnetic resonance imaging, **TIMP-1**: Tissue Inhibitor of Metalloproteinases, **NO**: nitric oxide, **PGE2**: Prostaglandin E2, **IL-1Ra**: Interleukin 1 receptor antagonist, **AAV**: Adenoviral vector, **MAPK**: Mitogen-activated protein kinases, **COX2**: Cyclooxygenase 2, **PGs**: Proteoglycans

Table 2: Pro-anabolic strategies for the regeneration of IVD

	Factor	Model	Study design	Effects	Ref
Growth Factors from TGFβ superfamily	BMP-2	Rat AF cells	In vitro studies	Increase of aggrecan [132,133], collagen type II [132,133], mRNA levels [132,133], cell number [132], Sox9 [132], osteocalcin [132], TGF-β1 [133], OP-1 [133] and sGAG production [132]	[132,133]
	BMP-2	Induced IVD degeneration rabbit model	In vivo study of 2 months - delivered by AAV vector	Improvement of histological grades, MRI indices, serum biochemical analysis and biomechanical properties	[104]
	OP-1	Rabbit [120–122] or human [123,124] IVD cells	In vitro studies – cultured in alginate beads with IL-1 [120], chondroitinase ABC [121] or TNFα [124]	Increase of PGs and collagen production [120–124] and accumulation [123] Increase cell number, aggrecan and collagen type II mRNA levels [121] Blocking of the TNFα effects on activation of NFκB, ADAMTS-4 and -5 and ECM degradation [124]	[120–124]
	OP-1	Healthy [125] and induced IVD degeneration rabbit model [126–128,130]	In vivo studies of 2 [127,128], 3 [125,130] or 6 [126] months about 10, 20 [125,127,128] or 90 [126] rabbits - by intradiscal injection	Improvement of disc height [125,126,128,130] Increase of PGs [125–128] and collagen [127] production in NP Increase of DNA content in AF [128] Suppression of Il-6, 1β and TNF-α expression in AF and NP [130]	[125–128,130]
	OP-1	Induced IVD degeneration rat model	In vivo study on 34 rats - intradiscal injection	Reduction of aggrecanases, MMP-13 and substance P	[129]
	OP-1	Spontaneous IVD degeneration canine model	In vivo study of 6 months- by intradiscal injection	No regenerative effect and extensive extradiscal bone formation	[131]
	GDF-5	GDF-5 deficient mice IVD cells [135] rabbit [136], human [136], mouse [137] and bovine [138] IVD cells	In vitro studies – directly delivered in medium[135,137,138] or by cells transfection via AAV[136,137]	Up-regulation of aggrecan and collagen type II protein[136,138] and gene[135,137] expression Stimulation of IVD cells growth[136–138] Decrease MMP-3 gene expression[137]	[135–138]
	GDF-5	Spontaneous [140] or induced [138,139] IVD degeneration rabbit [138,140] or mice [139] model	In vivo studies of 2 [139], 3 [140] and 4 [138] months - by directly [138,140] or AAV [139] intradiscal injection	Restoration of disc height [138–140], histologic grading [138–140] and MRI [138–140]	[138–140]
	GDF-5	Patients with early lumbar disc degeneration	Clinical trials of 36 months about 30 [319–321] or 40 patients [322] - by single intradiscal injection	Safety, tolerability and efficacy studies	[319–322]
	TGF-β1 [141,145] or 3 [142]	Rat [145], bovine [141] or porcine [142] IVD cells	In vitro studies – cultured with cells on microfibrous PLLA scaffold [141] or porous alginate scaffold [142]	Increase of CTGF expression and cell activity via SMAD and AP1 pathways signaling [145] Sustained stimulation of ECM synthesis GAG- and collagen rich [141,142]	[141,142,145]

	TGFβ1	IVD degeneration canine [143] or rabbit [144] model	Ex vivo study of 4 days In vivo study of 1 week on 22 rabbits - by AAV/TGF-β1 intradiscal injection	Increase PGs synthesis by NP cells [143,144] Production of TGF-β1 in IVD in vivo [144]	[143,144]
	IGF-1	Bovine coccygeal [146,147,149] or degenerated human [147] IVD cells Degenerated caudal IVD bovine culture organ model [148]	In vitro studies [146,147,149] Ex vivo study of 14 days - by AAV transfection of BMSCs deposed onto IVD [148]	Increase of PGs synthesis by NP cells [146,148] and DNA synthesis in quiescent IVD cells [149] Reduction of cell apoptosis [147] Stimulation of the IVD cell proliferation via the ERK and Akt signaling pathways [149]	[146–149]
	PDGF-BB	Human degenerated IVD cells	In vitro study - cultured with cells in monolayer and 3D pellet	Inhibition of cell apoptosis and increase of cell proliferation and matrix production Maintains of ECM genes mRNA expression	[150]
	CTGF	Human degenerated NP cells	In vitro study - cultured with Il-1β or TNFα	Suppression of the inductive effect of Il-1β on catabolic genes (MMP-3, ADAMTS-5)	[323]
Trasncription factor	Sox 9	Human [151] or bovine [152] degenerated IVD cells IVD degeneration rabbit model [151]	In vitro studies [151,152] In vivo study [151] of 5 weeks on 4 rabbits - by AAV cell transfection	Increase of PGs[152] and collagen type II [151,152] synthesis and accumulation Increase cell proliferation and DNA content [152] Histological preservation of NP morphology and NP cell phenotypic appearance [151]	[151,152]
Natural molecule	Andrographolide	Human degenerated NP cells	In vitro study – cultured with LPS	Reversion of the inflammatory and catabolic effects, inhibition of the NF-κB pathway and increase of GAG secretion	[324]
Synthetic biological agent	Link N peptide	Human [164] or rabbit [163] IVD cells	In vitro studies – cultured with cells on peptide nanofiber [163] or alginate scaffold with IL-1 [164]	Increase of PGs [164], aggrecan [163] and collagen type II [163] synthesis and accumulation Decrease of MMPs and ADAMTs levels [164]	[163,164]
	Link N peptide	Induced IVD degeneration rabbit model	In vivo study of 12 weeks on 28 rabbits - by intradiscal injection	Stimulation of aggrecan gene expression and downregulation of MMPs expression	[161]
Combination of GFs	NTG-101 (TGF-β1 + CTGF)	Induced IVD degeneration rat and canine model	In vivo study of 6 or 10 weeks – by intradiscal injection	Reduction of IL-1β, -6, -8, MMP-13, Cox-2 and PGE2 expression Increase of aggrecan and collagen type II expression	[325]
	TGF-β3 + Kartogenin	Human ASCs	In vitro study - loaded with hASCs in chitosan/HA hydrogel	Stimulation of hASCs proliferation and differentiation into NP-like cells in hydrogel No synergic effect observed	[272]

	TGF-β1 [166–168] or 3 [63] + BMP-2 [166–168] + IGF-1 [166]	Rabbit[167], porcine[168] or human degenerated[166] IVD cells	In vitro studies – cultured with IVD cells on atelocollagen type II scaffold [167] with Il-1β and TNFα [168] or on alginate beads [166]	Synergistic effects [166–168] Increase of DNA[53], synthetized PGs [166–168], aggrecan, collagen type I and II genes expression [167] Decrease of IL-1β-mediated MMP-1 synthesis [168]	[166–168]
	TGFβ3 + FGF2	Human degenerated AF cells	In vitro study – cultured with cells in HA hydrogel	Formation of cartilaginous matrix and enhancement of MMP-13 expression	[169]
	TGF-β1 + GDF-5	Human ASCs[59] or induced IVD degeneration murine model[172]	In vitro study [59] – cultured on 3D In vivo study [172] – by intradiscal injection	Synergistic effects [59,172] Drive a robust and highly specific NP differentiation [59] Increase of protein and genic expression of NP markers and synthesis of NP-like ECM [59,172] Temporary increase of cell proliferation and DHI [172]	[59,172]
	GDF-5 + CTGF	Human degenerated NP cells	In vitro study	Increase of aggrecan and collagen type II gene expression and GAGs production	[326]
	OP-1 + SOX9	IVD degeneration rabbit model	In vivo study of 9 weeks - by AAV double gene co-transfection intradiscally injected	Improvement of DHI and T2-signal intensity Increase of aggrecan and collagen type II gene and protein expression	[171]
	PRP	Human [154,156] and porcine [155] NP cells	In vitro study – cultured with cells in alginate beads [155] or with Il-1β and TNFα [154,156]	Proliferation and aggregation of IVD cells [154,155] Upregulation of PGs and collagen synthesis [155] and GAG accumulation [154] Up-regulation of aggrecan [154,156], collagen type II [154,156] and Sox9 [154] mRNA expression levels Decrease of MMP-1 and COX-2 gene expression levels [156]	[154–156]
	PRP	Induced IVD degeneration rabbit model	In vivo studies of 6 [158] or 8 [157] weeks - by intradiscal injection	Increase of disc height and chondrocytes-like cells number[157] Delay of degeneration process and decrease of ECM lesions[158] Increase of collagen type II expression in NP and inner AF[158]	[157,158]
	PRP [160] + SVF [159]	Patients with chronic DD	Clinical trials of 12 months on 15 [159] or 47 patients [159] - by intradiscal injection	Improvement of pain, disability, quality of life and mobility of IVD No adverse effects and incidences of infection	[159,160]
Combination with cells	Link N peptide + exogenous MSCs	Induced IVD degeneration bovine model	Ex vivo study of 2 weeks - by intradiscal injection	Increase of sGAG, PGs, and collagen type II production Survive, integration and distribution throughout the NP of MSCs	[165]
	PRP + BM-MSCs	Induced IVD degeneration rabbit model	In vivo study of 8 weeks - by intradiscal injection	Rapid and stable improvement of MRI scores and T2 signal intensity Increase of cell density and ECM production with synthesis of collagen type II	[243]

BMP-2: Bone morphogenetic protein 2, **OP-1**: Osteogenic protein 1, **GDF5**: Growth differentiation factor 5, **TGF-β**: Transforming growth factor β, **IGF-1**: Insulin-like growth factor

1, **PDGF-BB**: Platelet-derived growth factor BB, **CTGF**: Connective tissue growth factor, **FGF-2**: Fibroblast growth factor 2, **PRP**: Platelet-rich plasma, **SVF**: Stromal vascular fraction, **MSCs**: Mesenchymal stem cells, **BM-MSCs**: Bone marrow mesenchymal stem cells, **AAV**: Adenovirus vector.

Table 3: Nano-, micro-, macro-scaled delivery platforms for IVD regeneration

	Biomaterial	Biological factors	Study design	Effects	Ref
Nano-scaled	Chitosan/ ϒ-PGA nanoparticles	Diclofenac	Ex vivo study of 14 days – induced IVD degeneration bovine model – by intradiscal injection	Down-regulation of Il-6, Il-8, MMP-1, -3 and PGE2 production Up-regulation of aggrecan and collagen type II	[118]
	Silica nanofibrous	TGF-β1 GDF-5	In vitro study – loaded on HPMC-Si hydrogel	Sustained release of bioactive TGF-β1 and GDF-5	[278]
	Albumin/heparin nanoparticles	CXCL12	In vivo study of 8 weeks – IVD degeneration rat model – by intradiscal injection	Improve of the regeneration of AF and NP with increase of histological grade score Increase of sox9, aggrecan and collagen type II mRNA and protein expression levels	[302]
Micro-scaled	PLA pellets	Clonidine	In vivo study of 3 months – IVD degeneration pig model – by postero-transforaminal placement	Detection of Clonidine up to 6 cm away from implantation zone Sustained local release	[270]
	PLGA microspheres	Il-1Ra [111] IGF-1 [275] GDF-5 [327]	In vitro studies – in bovine NP constructs (agarose) with IL-1β [111] or in silk fibrosin porous scaffold [275] In vivo study of 8 weeks – induced IVD degeneration rat model [327]	Release from PLGA microspheres for up 35 days [111] or 42 days [327] Increase of sustained release as compared to without silk-fibroin scaffolds [275] Restoration of disc height with increase of sGAG, DNA content and collagen type II mRNA level [327]	[111,275,327]
	Pullulan microbeads	TGF-β1 [277] GDF-5 [277] CCL5 [328]	In vitro study [277] – loaded on HPMC-Si hydrogel In vivo study [328] – injection in mouse ischemic hindlimb model	Sustained release of bioactive TGF-β1 [277], GDF-5 [277] and CCL5 [328]	[277,328]
	Gelatin microspheres	PRP	In vivo studies of 8 weeks[279,280] - intradiscal injection in induced IVD degeneration rabbit model	Stop the progression of DD [279] and decrease of cell apoptosis [280] Increase of disc height [280], PGs protein [39]and mRNA expression [280] levels	[279,280]
Macro-scaled	Elastin-like polypeptide hydrogel	Curcumin	In vivo study of 4 days – mice model - by intramuscular injection	Rapid delivery of active curcumin, with in situ depot formation of released curcumin and anti-neuro-inflammatory effects	[329]
	Chitosan/gelatin/ glycerol phosphate hydrogel	Ferulic acid	In vitro study – with NP cells cultured with H_2O_2	Sustained release for 48 hours Reduce the cellular stress, sGAGs accumulation, up-regulation of aggrecan and type II collagen and down-regulation of MMP-3 mRNA expression levels	[330]
	CMC hydrogel	TGF-β3	In vitro study - cultured with hMSCs	Increase of GAG and collagens type I and II accumulation with homogenous deposition Improvement of mechanical properties of scaffold overtime	[273]
	Chitosan/HA hydrogel	Kartogenin	In vitro study – with hASCs	In vitro sustained release for up 16 days Stimulation of hASCs proliferation and differentiation into NP-like cells in hydrogel	[272]
	PEG/HA hydrogel	PPS	In vitro study – with MSCs encapsulated in hydrogel	Increase the synthesis and deposition of sGAG, collagen	[271]

				Not affect the mechanical properties of hydrogel and cell viability	
	HA hydrogel	CXCL12	Ex vivo study - induced IVD degeneration bovine model with MSCs seed on one endplate – by intradiscal injection	Increase of MSCs migration number and distance from cartilage endplate to NP Enhancement of collagen type II expression	[195,331]
'Combined -scaled'	PCL microspheres in Pluronic F127/Sodium Hyaluronate hydrogel	Bupivacaine	In vitro and ex vivo studies – bovine lumbar IVD – by intradiscal injection	In vitro sustained release for 142 days Verification of delivery factors after injection in ex vivo IVD	[274]
	PGPC polyplex micelles in PEG hydrogel	miR-29a	In vivo studies of 30 days and 4 weeks – induced IVD degeneration rat and rabbit models – by intradiscal injection	Release of miR-29a/PGPC polyplex micelles from PEG hydrogel Attenuation of fibrosis progression Sustained inhibition of MMP-2	[281]
	Chitosan microparticles [276] Chitosan/ϒ-PGA complexes [301]	IGF-1 [276] CXCL12 [301]	In vitro studies – with BMSCs [301]	Sustained release of bioactive IGF-1 [276] Continuous release of CXCL12 during 120h [301] Increase of the cell migration up to 6 fold compared to without CXCL12 [301]	[276,301]
	PLGA nanoparticles in Dextran/Gelatin hydrogel	TGF-β3 + mouse BM-MSCs	In vitro study – loaded in PLGA nanoparticles seeded in Dextran/Gelatin hydrogel	Release of TGF and induction of NP-like cell differentiation of MSCs with specific ECM synthetisis	[298]
	Heparin/poly(L-lysine) nanoparticles loaded in PLGA microspheres	TGF-β3 [300] or FGF [299] + rat ASCs + Dexa	In vitro study [299] In vivo study of 24 weeks on 96 rats - induced IVD degeneration rat model [300] – by intradiscal injection	Increase of GAG/DNA ratio, aggrecan, collagen type II and versican genes expression levels [299] Improvement of DHI and PGs production and accumulation [300]	[299,300]

γ-PGA: Poly-γ-glutamic acid, **TGF-β**: Transforming growth factor β, **GDF-5**: Growth differentiation factor 5, **PLA**: Polylactic acid, **PLGA**: Poly(lactic-co-glycolic acid, **IL-1Ra**: Interleukin-1 receptor antagonist, **IGF-1**: Insulin-like growth factor 1 ,**PRP**: Platelet-rich plasma,**CMC**: Carboxymethyl-cellulose, **HA**: Hyaluronic acid, **PEG**: Poly(ethylene glycol), **PCL**: Polycaprolactone, **PGPC**: poly(ethylene glycol)-GPLGVRG-poly{N′-[N-(2-aminoethyl)-2-aminoehtyl]aspartamide}-cholesteryl (PEGGPLGVRG-PAsp(DET)-Chole), **BM-MSCs**: Bone marrow mesenchymal stem cells, **FGF**: Fibroblast growth factor, **ASCs**: Adipose stromal cells, **Dexa**: Dexamethasone.

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Graphical abstract

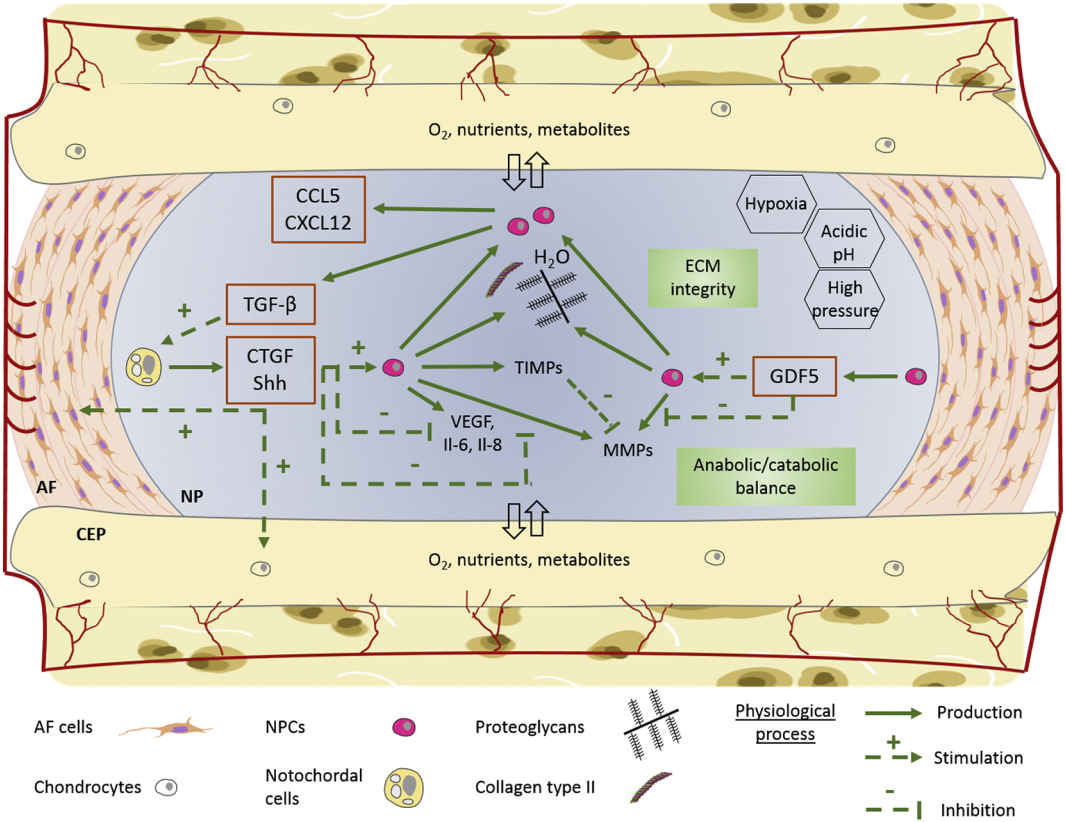
O2, nutrients, metabolites
CCL5
CXCL12
Hypoxia
Acidic pH
High pressure
H2O
ECM integrity
TGF-β
CTGF
Shh
TIMPs
GDF5
VEGF, Il-6, Il-8
MMPs
Anabolic/catabolic balance
AF
NP
CEP
O2, nutrients, metabolites
AF cells
NPCs
Proteoglycans
Physiological process
Production
Stimulation
Chondrocytes
Notochordal cells
Collagen type II
Inhibition

Figure 1

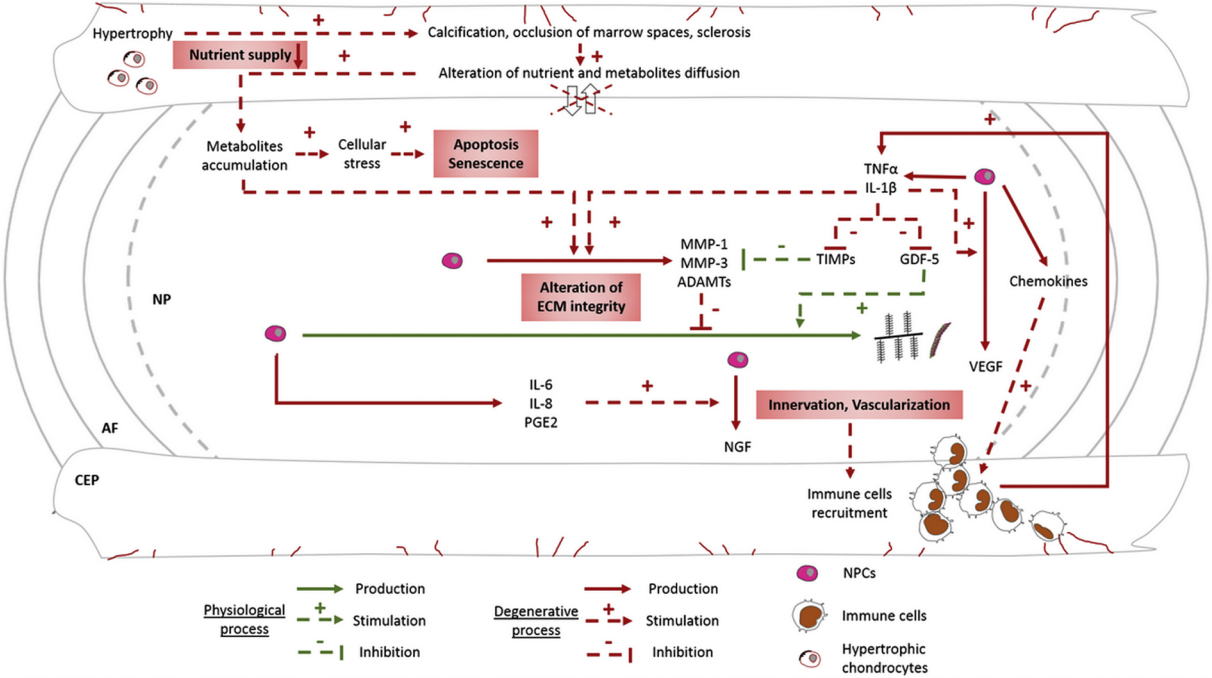
Hypertrophy
Nutrient supply
Calcification, occlusion of marrow spaces, sclerosis
Alteration of nutrient and metabolites diffusion
Metabolites accumulation
Cellular stress
Apoptosis Senescence
TNFα
IL-1β
MMP-1
MMP-3
ADAMTs
TIMPs
GDF-5
Chemokines
Alteration of ECM integrity
NP
VEGF
IL-6
IL-8
PGE2
Innervation, Vascularization
NGF
AF
CEP
Immune cells recruitment
Physiological process
Production
Stimulation
Inhibition
Degenerative process
Production
Stimulation
Inhibition
NPCs
Immune cells
Hypertrophic chondrocytes

Figure 2

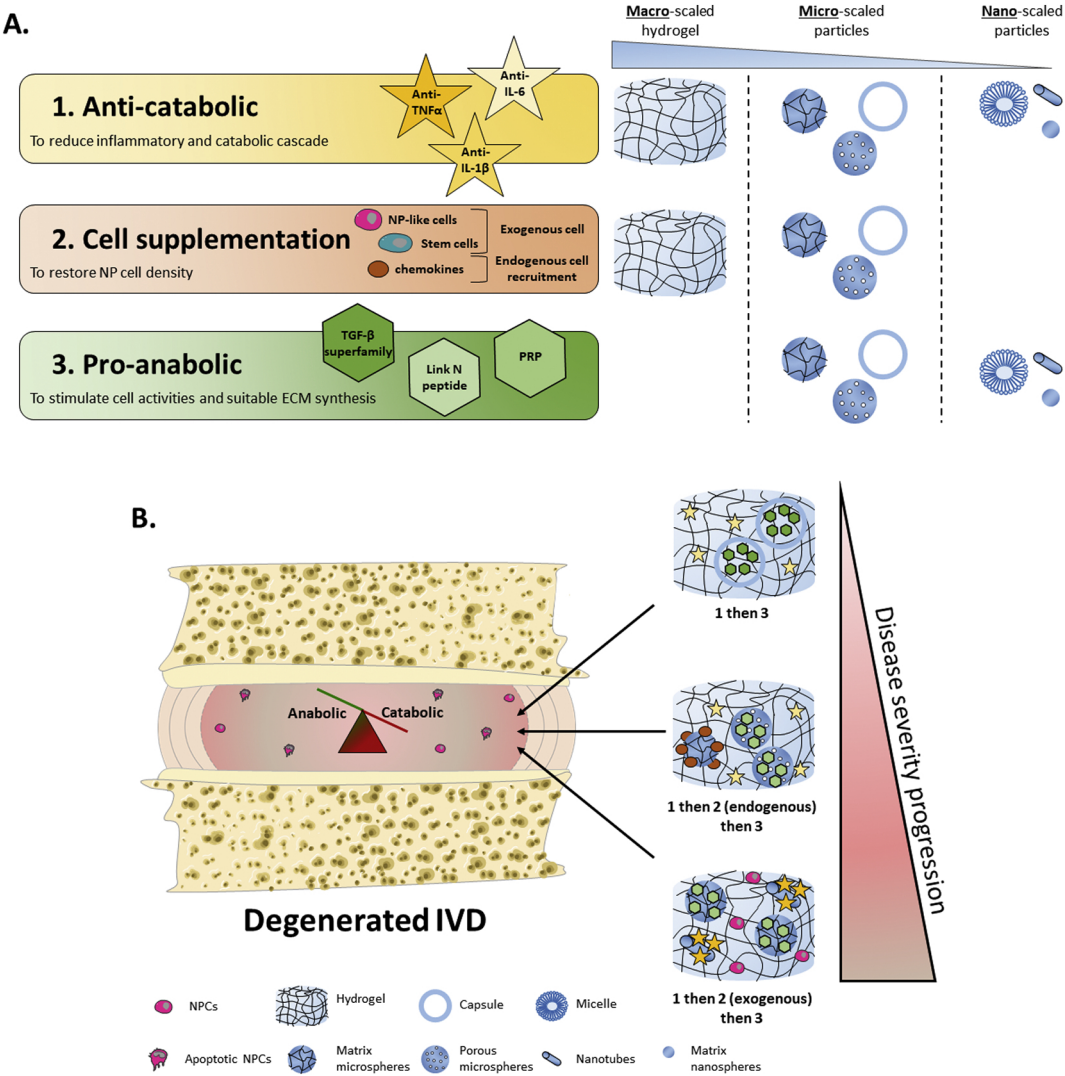
A.
Macro-scaled hydrogel
Micro-scaled particles
Nano-scaled particles
1. Anti-catabolic
To reduce inflammatory and catabolic cascade
Anti-TNFα
Anti-IL-6
Anti-IL-1β
2. Cell supplementation
To restore NP cell density
NP-like cells
Stem cells
chemokines
Exogenous cell
Endogenous cell recruitment
3. Pro-anabolic
To stimulate cell activities and suitable ECM synthesis
TGF-β superfamily
Link N peptide
PRP
B.
Anabolic
Catabolic
Degenerated IVD
1 then 3
1 then 2 (endogenous) then 3
1 then 2 (exogenous) then 3
Disease severity progression
NPCs
Hydrogel
Capsule
Micelle
Apoptotic NPCs
Matrix microspheres
Porous microspheres
Nanotubes
Matrix nanospheres

Figure 3