Spheroid-Based Cell Therapies for Degenerative Disc Disease

Subjects: Cell & Tissue Engineering

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Degenerative disc disease, a painful pathology of the intervertebral disc (IVD), often causes disability and reduces quality of life. A long-lasting episode of low back pain (LBP) affects 80% of people at least once in their lifetime. The major cause of LBP is degenerative disc disease (DDD), an age-related pathology of the intervertebral disc (IVD). IVD contains distinct anatomical regions, namely the nucleus pulposus (NP), annulus fibrosus (AF), and cartilaginous endplates, which are all substantially different and unique structurally, mechanically, and biochemically, and present challenges for IVD tissue engineering. Ideally, an engineered construct should closely resemble the ECM architecture of the target tissue and rapidly integrate within a defect. Spheroids are three-dimensional multicellular aggregates with architecture that enables the cells to differentiate and synthesize endogenous ECM, promotes cell-ECM interactions, enhances adhesion, and protects cells from harsh conditions. Spheroids could be applied in the IVD both in scaffold-free and scaffold-based configurations, possibly providing advantages over cell suspensions.

regenerative medicine | intervertebral disc degeneration | spheroids | tissue engineering | nucleus pulposus | annulus fibrosus

1. Nucleus Pulposus

The NP is a hydrated structure predominantly composed of a loose network of highly hydrated proteoglycans (PG) and collagen type II, with PG/collagen ratio 26:1 in healthy IVD [1]. Notably, the microenvironment of degenerated NP contains low levels of oxygen and glucose, acidic pH, high osmolarity (relative to other tissues), and complex loading [2][3]. These harsh conditions were shown to induce a cellular catabolic shift that accelerates the degradation of ECM and negatively influences the function of therapeutic cells [4][5]. The catabolic shift is characterized by upregulation of pro-inflammatory cytokines and ECM degrading enzymes, as well as downregulation of inflammation antagonists and inhibitors [6][7]. The survival rate of therapeutic cells in the NP is also affected by reduced nutrient supply due to large IVD size and endplate calcification [8]. Altogether, these conditions limit the numbers of therapeutic cells to be used. Strategies to regenerate NP should consider the specific anatomy, limited diffusion rate, and harsh microenvironment while providing resistance to the compressional and torsional stresses within the spinal column [9]. An ideal therapy for NP regeneration would be liquid before application (injectable) and rapidly solidify and/or integrate upon injection to ensure correct distribution and retention in the NP [10][11].

The intrinsic ability of spheroids to rapidly fuse with target tissue is believed to be crucial for regeneration [12]. In order to prevent their extrusion from NP, the adhesion of spheroids and migration of surface cells into NP, followed by spheroid remodeling, must take place. Consequently, spheroids are expected to secrete an NP-like matrix into the defect cavity, leading to restoration of IVD height, gap filling, and biochemical integration of spheroid cells into the surrounding NP tissue [13][14].

In scaffold-free conditions, a supportive material is not used, thus there is no need to consider long-term effects of an implanted scaffold [15]. 3D configuration and increased paracrine effects of spheroids (compared to 2D) are thought to enhance the differentiation potential of therapeutic cells. The ability of spheroids to synthesize their own ECM results in the encapsulation of cells in native ECM, the composition of which is driven by the original cell type and culture conditions [16][12]. In the clinical repair of cartilage defects in the knee, AC-based spheroids (Spherox) generated a hyaline-like structure and showed the potential to synthesize an articular cartilage-specific matrix [13][14]. We have recently demonstrated that ECM and biomechanical properties of spheroids derived from human NC are tuneable by cell culture supplements, possibly to match properties of target tissue (NP) and that spheroids of less than 600 µm are injectable into an (bovine) IVD through a spinal needle, without their mechanical damage [16]. A self-produced ECM of spheroids is also believed to retain growth and trophic factors and constitute a physical barrier between harsh target tissue and therapeutic cells [17]. However, in scaffold-free tissue repair, the cell numbers required to maintain the same 3D architecture as constructs that are scaffold-based, are higher. Importantly, native NP tissue contains, proportionally, very low cell numbers compared to the amount of ECM; thus the use of a supportive biomaterial might be warranted to maintain the graft volume.

A biomaterial could be present after spheroid fabrication or already at the stage of spheroid assembly. Hydrogels have several advantages for NP repair, such as a 3D structure that generates volume and promotes cell adhesion, migration, and integration. Natural materials, such as collagen, hyaluronan, chitosan, or fibrin, mimic an in vivo environment, as they bear similarities with the native ECM. For example, injectable colloidal gelatine hydrogels with encapsulated MSCs support the NP-like differentiation, reduce cell leakage, and improve the survival of therapeutic cells in a rabbit model [18]. On the other hand, synthetic polymers, such as poly(lactide) (PLA), poly(glycolide) (PGA), and poly(ε-caprolactone) (PCL), offer easier processing, tuneability of mechanical properties and degradation patterns, and low immunogenicity [19][20].

While the combination of spheroids with hydrogels has yet to be investigated in the IVD field, spheroid-based constructs have already been tested in cartilage repair. As an example, an alginate/hyaluronic acid (HA) hydrogel was used to embed MSC spheroids in bi-layered osteochondral implants that supported the functional regeneration of articular cartilage in sheep [21]. An encapsulation of spheroids in an injectable biomaterial might help to hold them in place, protect them further from unfavorable microenvironments, and instruct them towards differentiation [22][23]. It would be expected that a biomaterial will not impair the ability of spheroids to spread and fuse with NP tissue but rather modulate these functions. In cartilage repair, it was suggested that delayed spheroid spreading, achieved by the use of PLGA/chitosan (CS)-containing constructs, can provide superior chondrogenic effects in vitro and in vivo due to the fact that spheroid 3D architecture is preserved longer [24]. Whether delaying spheroid spreading/fusion by the use of a biomaterial would produce beneficial effects in NP repair has yet to be

investigated. It should be noted that spheroids rely exclusively on diffusion to transport nutrients and eliminate waste, so their interior might start suffering from a lack of nutrients, oxygen, and excess waste products, if spreading is inhibited for longer periods. Nevertheless, these negative effects and consequent onset of necrosis could be partially regulated by spheroid size and the total number of therapeutic cells in the NP.

Recent developments expanded the possibilities for modulating spheroid spreading (and other parameters) by generating composite spheroids, with a biomaterial included already during spheroid fabrication. In adipose tissue engineering, composite multicellular spheroids formed by MSCs and synthetic biodegradable nanofilaments showed enhanced adipogenic potential compared to homotypic spheroids. It was also demonstrated that the size of these spheroids could be readily controlled with the integrated amount of nanofilaments. Moreover, the material part of the spheroids could be used to sustainably release bioactive drugs (e.g., GFs) in order to fine-tune the properties of target tissue [25]. Including biomaterials during spheroid fabrication process was also shown to influence spheroid roundness in ligament tissue engineering [26].

Combining spheroids with an injectable instructive biomaterial is an attractive possibility for the regeneration of the NP. In the future, it will be necessary to define the best biomaterial for spheroid encapsulation with regard to their fusion kinetics (with target NP tissue and with each other) and biomechanical stability.

2. Annulus Fibrosus

Approaches to regenerate NP are likely to have limited success without sufficient repair of the AF, i.e., the outer ring of the IVD. The AF is composed of circumferential layers of lamellae formed by closely arranged fibers of collagen type I. AF provides load-bearing function, tensile resistance, and adequate support to maintain NP pressure [27]. During IVD degeneration, non-physiological loading and catabolic shift reduce ECM turnover, leading to the development of microdamage, clefts, and tears in the AF [28]. Due to the loss of PG and inflammation-associated upregulation of specific growth factors (NGF, VEGF), nerves and vessels from adjacent tissues grow deeper into the IVD [29], which causes nerve irritations and aggravates pain [29][30]. Strategies to regenerate AF thus focus on filling structural defects and rapidly restoring physiological ECM structure and function (collagen lamellae) to support AF's tensile resistance and prevent excess nerve ingrowth. Persistent AF defects increase the risk of recurrent IVD herniations, which then require reoperations [31].

Strategies to mechanically repair AF were developed (sutures, patches) but none of these techniques significantly altered annular healing in animal models nor demonstrated long-term benefits in clinical trials [32]. It is crucial that AF implants maintain adhesion to target tissue, especially under strain. Novel AF sealants have been generated and showed promising results [32][33][34][35]. Compared to acellular therapies, cell-based implants improve ECM deposition and organization in animal studies and show more successful AF remodeling in the long-term [35]. Nevertheless, neither biological/mechanical properties similar to AF tissue nor native-like ECM organization were fully reproduced to date [35]. Importantly, regeneration of inner AF has been a challenge, as current implants fail to fully bridge inner AF defects.

Due to their intrinsic ability to adhere, spheroids could serve as building blocks for a living AF patch that fuses to larger 3D structures in situ or before implantation. Spheroid-based architecture could achieve successful defect bridging and fix the implanted material in place [35], especially if combined with a biomaterial [17]. Recent advances make it possible for spheroids to be seeded into biomaterials during or after the fabrication process, immobilized on pre-fabricated scaffolds, or embedded between scaffold layers in a patterned manner, possibly achieving the typical lamellar structure of the AF [36]. Combining spheroids with injectable hydrogels could efficiently fill irregularly shaped defects in a minimally-invasive and rapid manner as well as instruct the behavior of therapeutic cells [37][38].

A sufficiently porous biomaterial is needed to seed spheroids randomly or into a specific structure. Although spheroids were not widely explored in AF tissue engineering, recent developments expanded the possibilities for spheroid-biomaterial seeding in related areas of musculoskeletal repair. In bone tissue engineering, foaming/freeze-drying techniques were used to produce scaffold microporosity that promoted spheroid penetration into the scaffold and fixed them in place [17][39]. Spheroids were also generated in situ in a novel porous PLGA/CS scaffold obtained after lyophilization. ASC spheroids formed in these scaffolds promoted hyaline cartilage-specific chondrogenesis in vitro and structural/functional regeneration in vivo (rabbit model). This method reproducibly yielded spheroids of smaller sizes (diameter less than 200 µm), which facilitated the penetration of oxygen and nutrients into spheroids [40]. In situ generation of spheroids directly within an implantable scaffold might reduce culture time and lab manipulation, supporting the applicability in clinical AF repair. However, the exact pore size and porosity of scaffolds produced by methods like foaming or lyophilization might be difficult to control.

3D printing can reproducibly control the internal pore size (50–800 µm), porosity, pore interconnectivity, and mechanical performance of tissue-engineered scaffolds. Huang (2013) used a solid freeform fabrication method to prepare PLGA-CS scaffolds that delayed spheroid spreading in cartilage repair. Their scaffold showed a fully interconnected macroporous structure and controlled geometry, maintained the 3D microenvironment of MSC spheroids, and a showed superior ability to regenerate chondral defects in a rabbit model when combined with spheroids (vs. single cells) [24]. 3D printing also holds promise to generate scaffolds that precisely fit the geometry of interest, allowing for guidance of the spheroid placement into specific shapes and geometries [36]. However, the automated seeding of spheroids onto 3D-printed scaffolds to produce a complex 3D construct has not yet been largely explored [17]. To precisely replicate AF structure using spheroids as building blocks, patterned micro- and nano-structures could be produced, e.g., by an innovative “lockyball” approach, where ASCs were immobilized into solid synthetic microscaffolds (lockyballs) fabricated by two-photon polymerization and designed with hooks and loops to enhance the retention and integration at the implantation site [41].

The generation of functional double-layered AF patches with one side promoting integration with inner AF and the other side sealing the defect from outside is an attractive proposition. In cartilage repair, Favreau (2020) developed compartmentalized, multi-layered implants seeded with spheroids to treat osteochondral defects. The first compartment was based on therapeutic collagen membranes associated with BMP-2 to provide structural support and promote subchondral bone regeneration, while the second compartment contained BMSC spheroids dispersed in alginate hydrogel to support the regeneration of the articular cartilage [21]. These implants showed promising

results in a sheep model. With modifications relevant for AF tissue, a spheroid-laden part could be used to bridge AF tears while a cell-free layer could possibly serve for AF sealing.

In order to recapitulate natural ECM structure and facilitate interactions between living AF patches and resident cells, electrospinning could be the fabrication method of choice. Electrospinning and its modifications can generate randomly organized or aligned fibers that mimic the natural ECM and provide wide cell adhesion surfaces and adjustable porosity. This allows spheroid immobilization and modulation of spreading as well as cell migration and differentiation. An alignment of electrospun fibers was shown to regulate ASC spheroid functions. Non-aligned nanofibrillar structures demonstrated a heterogeneous dispersion of ASC spheroids, preventing efficient cell colonization of the nanofibers' surface [42]. On the other hand, ASC spheroids seeded on aligned nanofibrillar structures (produced by jet-spraying) showed rapid and homogeneous cell dispersion, high viability, chondrogenic differentiation, and fused with each other, increasing the cell contact of the surface of the nanofibers. Therefore, fiber alignment that mimics the lamellar AF structure could produce a patch that integrates with target tissue more rapidly.

A combination of the above-mentioned fabrication methods may enhance the desired properties of (hybrid) living AF patches and/or immobilized spheroids in the scaffold. In skin tissue engineering, Lee (2020) seeded ASC spheroids onto a 3D-printed alginate-based mesh, which was followed by electrospinning of alginate/polyethylene oxide fibers directly onto the spheroids. The alginate scaffolding structure clearly retained the characteristics of the spheroids and maintained their superior regenerative capacity over scaffolds without the mesh [43].

The main function of spheroid-based living AF sealants would be to sustain tension generated by the NP and thus prevent NP extrusion until the defect is healed. Mechanical properties of AF sealants can be increased by crosslinking agents (genipin, glutaraldehyde, riboflavin), which also promote their attachment to native tissue. Genipin crosslinked (cell-free) hydrogels achieve biomechanical properties of AF tissue [44][45][46], even possibly outperforming FDA-approved materials under loading [46]. However, their failure to adhere to AF tissue at higher strains of 15–30% (typical for degenerative overloading) was also reported [44]. As some crosslinking agents might negatively influence cell viability, adhesion, and spreading, preliminary tests should be performed to select an appropriate agent/concentration for each material and application [44][45]. Nevertheless, recent advances in other fields of tissue engineering clearly demonstrate the potential of the synergistic scaffold-based and scaffold-free strategies for AF repair.

3. Enhancement of Spheroid Functions

Besides degradation of ECM, DDD is characterized by sterile tissue injury and unresolved inflammation. The evidence suggests that therapeutic cells can mediate tissue repair not only by differentiation towards target structures but also via the secretion of soluble factors that enhance tissue repair. Directing therapeutic spheroids towards paracrine trophic, anabolic, and anti-inflammatory functions is an exciting strategy to potentiate their performance and resistance. It is known that spheroids already release higher amounts of growth factors and anti-inflammatory factors, compared to single cells [47][48][49]. The secreted biomolecules are entrapped in ECM and

readily control a range of biological processes, becoming a source of relevant regenerative cues. Thanks to recent developments, the secretion of beneficial factors from spheroids can be further enhanced by specific 3D-culture conditions, providing superior functions even without the use of stimulative growth factors [47][50].

In addition to secretome, therapeutic properties of spheroids are mediated (at least partially) via exosomes, the nanometer-size type of extracellular vesicles (EVs) that carry RNAs, proteins and lipids from the parent cell [51][52]. Recently it was shown that inhalation treatment of lung spheroid-derived exosomes (as well as secretome) provided anti-inflammatory properties and improved lung regeneration in two animal models of pulmonary fibrosis [53]. The therapeutic potential of EVs in IVD regeneration was recently reviewed [54]. For example, MSC-derived EVs are believed to promote regeneration and proliferation and reduce inflammation and apoptosis in the IVD, possibly via miRNAs and other (yet unknown) mechanisms. The use of spheroids could improve the quality and possibly increase the yield of therapeutic vesicles. The application of spheroid conditioned medium containing therapeutic secretome and/or EVs could be considered as a cell-free alternative for the treatment of DDD, with a lower regulatory burden [48][49].

Tissue-specific functions were shown to be promoted by a biomimetic environment applied during spheroid generation in vitro. Biomimetic spheroid priming enhances their chondrogenic capacity and/or resistance in harsh conditions. In cartilage tissue engineering, scaffold-free chondrocyte spheroids generated under hypoxia, upregulated the expressions of collagen II and aggrecan at mRNA and protein levels, increased ECM deposition, and generated a higher quality of cartilage [55][56]. In IVD repair, preconditioning of MSCs with hypoxia is known to provide beneficial effects by activating a hypoxia-inducible factor (HIF) signaling pathway found to be involved in phenotype maintenance, metabolism, and homeostasis of the IVD [57]. It remains to be seen whether preconditioning of (MSC) spheroids with hypoxia (or other microenvironmental conditions) further augments their effects on IVD-specific phenotype and function.

Recently Muttigi et al. (2020) described the promising effect of spheroid priming with Matrilin-3, a noncollagenous ECM adaptor protein. Matrilin-3-primed ASC spheroids increased gene/protein expression of growth factors and reduced the secretion of hypertrophic ECM components. Furthermore, Matrilin-3-primed ASC spheroids induced the stable mRNA expression of SOX9, collagen type II, and aggrecan, and enhanced chondroitin sulphate accumulation in NP cells (indirect co-culture). Matrilin-3-primed ASC spheroids also facilitated IVD repair in a rabbit model with AF puncture-induced IVD degeneration [58], highlighting preconditioning as a useful approach to promote the regenerative capacity of spheroids for IVD repair.

Genetic modification could also enhance the chondrogenic capacity and resistance of spheroids. Genetic engineering to resist in a harsh IVD microenvironment has been widely considered [59][60]. Specifically, inflammation antagonists (e.g., IL1Ra) and IVD-related growth factors (e.g., GDF-5) appear to be promising targets [61]. Although not yet investigated in the IVD, genetically modified MSC spheroids with upregulated Runx2 were shown to overcome negative effects of a harsh microenvironment and promote regeneration in bone tissue engineering [62]. Major limitations of human genetic engineering are related to viral vectors and low (transient) expression of transgenes. Recently it was shown that the expression of non-viral transgenes could be maintained

much longer in spheroids transplanted in vivo versus single cells. In hepatic regeneration, such a genetically modified spheroid system contributed to significantly higher therapeutic effects of transplanted hepatocytes in the host tissue [63]. Genetically modified spheroid systems might thus contribute to the maintenance of non-viral transgenes in the IVD and enrich anti-inflammatory and/or anabolic functions.

Instructive biomaterials providing physical and chemical signals required to modulate cellular behavior and reinforce particular spheroid phenotypes were designed [64]. Materials, e.g., with/without RGD peptides, would modulate spheroid spreading, while encapsulation of growth factors promotes spheroid fusion [21]. While not yet applied to spheroids, cell/growth factor-loaded particles ensured sustained release and chondrogenic differentiation of encapsulated therapeutic cells, aiding IVD regeneration in animal models [65][66]. Similarly, cell-free siRNA complexes encapsulated in injectable HA hydrogels retained release/activity over a prolonged period of time in vitro and in vivo [67]. Continuous supply of bioactive material combined with 3D cell configurations might enhance differentiation into chondrocyte-like NP cells as well as rejuvenate resident IVD tissue [65]. An implant combining MSC spheroids and a biomaterial with slowly released growth factors showed promise in sheep osteochondral repair [21].

Although single spheroids might lack in ECM organization being mechanically inferior to native tissue, spheroids are mechanosensitive, potentially enhancing the right interaction between an implant and target tissue upon loading [68][69].

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