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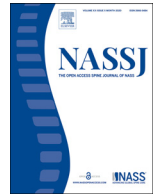
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Advances in Spinal Regenerative Therapies

Intervertebral disc cell fate during aging and degeneration: apoptosis, senescence, and autophagy



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ABSTRACT

Background: Degenerative disc disease, a major cause of low back pain and associated neurological symptoms, is a global health problem with the high morbidity, workforce loss, and socioeconomic burden. The present surgical strategy of disc resection and/or spinal fusion results in the functional loss of load, shock absorption, and movement; therefore, the development of new biological therapies is demanded. This achievement requires the understanding of intervertebral disc cell fate during aging and degeneration.

Methods: Literature review was performed to clarify the current concepts and future perspectives of disc cell fate, focused on apoptosis, senescence, and autophagy.

Results: The intervertebral disc has a complex structure with the nucleus pulposus (NP), annulus fibrosus (AF), and cartilage endplates. While the AF arises from the mesenchyme, the NP originates from the notochord. Human disc NP notochordal phenotype disappears in adolescence, accompanied with cell death induction and chondrocyte proliferation. Discs morphologically and biochemically degenerate from early childhood as well, thereby suggesting a possible involvement of cell fate including age-related phenotypic changes in the disease process. As the disc is the largest avascular organ in the body, nutrient deprivation is a suspected contributor to degeneration. During aging and degeneration, disc cells undergo senescence, irreversible growth arrest, producing proinflammatory cytokines and matrix-degradative enzymes. Excessive stress ultimately leads to programmed cell death including apoptosis, necroptosis, pyroptosis, and ferroptosis. Autophagy, the intracellular degradation and recycling system, plays a role in maintaining cell homeostasis. While the incidence of apoptosis and senescence increases with age and degeneration severity, autophagy can be activated earlier, in response to limited nutrition and inflammation, but impaired in aged, degenerated discs. The phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) is a signal integrator to determine disc cell fate.

Conclusions: Cell fate and microenvironmental regulation by modulating PI3K/Akt/mTOR signaling is a potential biological treatment for degenerative disc disease.

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Background

Epidemiological and clinical intervertebral disc aging and degeneration

Low back pain (LBP) is a global health problem with an enormous lifetime prevalence of 70% to 85% [1] and a huge socioeconomic burden up to \$102.0 billion/year in the US [2], as the most common reason for the worker's disability [3]. The cause of LBP is largely nonspecific; however, intervertebral disc degeneration (IDD) has been identified as the independent, main risk factor for disabling LBP [4]. Magnetic resonance imaging has shown a high prevalence of lumbar IDD with age in approximately 40% of people under 30 years and over 90% of those by 50 to 55 years [5], which further increases with age and facilitates impaired daily activities of the elderly [6].

In addition to severe LBP, IDD can also cause neurological disorders, including numbness, radicular pain, muscle weakness, paralysis, intermittent claudication, and even bladder and bowel dysfunction [7]. Despite clinical successful outcomes of nonoperative management for degenerative disc disease (DDD) [6,8], nonresponders require surgery [9]. The current primary surgical treatment is symptomatic disc excision and/or spinal fusion, resulting in the functional loss of load, shock absorption, and movement [10]. Therefore, the development of new biological therapies with maintaining the physiological function is an urgent issue to delay, prevent, and treat DDD.

Anatomical, biochemical, and biological intervertebral disc aging and degeneration

The spinal intervertebral disc anatomically has a complex structure, consisting of the central nucleus pulposus (NP) encapsulated by the peripheral annulus fibrosus (AF) and sandwiching cartilage endplates [7]. The collagenous, laminar AF maintains the pressurization of the gelatinous, oval NP, providing support during compressive loading and enabling multidimensional spinal movement [11]. In the disc development, the NP originates from the notochord [12] while the AF comes from the mesenchyme [13,14]. In human life, disc NP notochordal cells only exist during the first 10 years, which are subsequently replaced by non-notochordal, chondrocyte-like cells of unknown provenance [13,14]. More recent evidence has identified that chondrocyte-like cells arise from NP cells, based on the phenotype representing a terminal differentiation stage during IDD [15]. Notochordal cells produce larger amounts of matrix proteoglycans than non-notochordal cells [16], stimulate non-notochordal cells to produce proteoglycans [17], and protect non-notochordal cells from inflammation and apoptosis [18], thereby facilitating the healthy disc development.

Morphologically, IDD begins during early childhood [19,20], which is more severe in the NP than in the AF [20]. Disc NP cleft and radial tear formation can occur between ages 11 and 16 years [20]. Then, biochemically, inner AF and NP cells both have chondrocytic phenotypes [21], promoting anabolic activities through the production of extracellular matrix components, for example, proteoglycans (predominantly aggrecan) and collagens (principally types I in the AF and II in the NP) [7], whereas outer AF cells are mainly fibroblast-like [22]. Therefore, matrix degradation is the primary hallmark of IDD [7]. While aggrecan biosynthesis and procollagen type-II content peak at ages ≤ 5 years and diminish between ages 5 and 15 years, denatured collagen type II increases after 5 years of age [19]. This matrix catabolism is regulated by the balance between catabolic enzymes, matrix metalloproteinases (MMPs) and disintegrins and metalloproteinases with thrombospondin motifs (ADAMTSs), and anticatabolic factors, tissue inhibitors of metalloproteinases (TIMPs) [23]. Decreased matrix accumulation with increased MMP and ADAMTS levels relative to TIMP levels has actually been observed in human [24–26] and rodent experimental IDD [27–29]. However, what drives this imbalance in matrix metabolism still remains unclear.

It is a potential biological trait for cell-fate modulation that the intervertebral disc is the largest immune-privileged, low-nutrient, avascular organ in the human body, characterized by the low-glucose, low-oxygen, low-pH, high-osmolality, and load-fluctuating environment which cells are exposed to [30]. Compared to peripheral disc AF cells, central NP cells depend on the diffusion from blood vessels at the disc margins to obtain nutrients [31]. Therefore, decreased blood supply, subchondral bone sclerosis, and endplate calcification, which all occur during mechanical stress, injury, smoking, and aging, can easily reduce nutrient transport to the disc [31]. Actually, NP cells are susceptible against nutrient deprivation, hypoxia, and inflammation [32]. This additional loss of nutrient supply is a suspected contributor to the progression of IDD.

Cell fate during intervertebral disc aging and degeneration

The reduction in cell number is another major characteristic of IDD [7]. During development, intervertebral disc cell death substantially increases between ages 11 and 16 years, accompanied with the disappearance of notochordal phenotype and chondrocyte proliferation [20]. This concurrence suggests a possible involvement of cell fate including age-related phenotypic changes in the pathogenesis of IDD [13]. During aging and degeneration, disc cells undergo the irreversible growth arrest of senescence [33], consequently producing proinflammatory cytokines and matrix-degradative enzymes [34,35]. Excessive stress ultimately leads to programmed cell death including apoptosis [36], necroptosis [37], pyroptosis [38], and ferroptosis [39]. Autophagy, the intracellular degradation and recycling system, also plays a role in maintaining cell homeostasis [40–43]. Taken together, disc cell phenotype and matrix metabolism appear to be closely related to the fate including apoptosis, senescence, and autophagy.

Hypothesis

The pathophysiological understanding of the intervertebral disc's unique surroundings is essential to develop biological treatment strategies for age-related, inflammatory, degenerative disease. Harsh disc conditions should significantly affect cell fate. We thus hypothesized that resident disc cells would utilize apoptosis, senescence, and autophagy to cope with the low-nutrient, stressful situations [31]. However, available evidence is still limited regarding the involvement and roles of these protective mechanisms in disc cell and tissue homeostasis. Here we present a brief literature review, discussing the current concepts of apoptosis, senescence, and autophagy and future perspectives of biological cell fate-modulating therapies for DDD.

Methods

Literature search of scientific articles published between 1966 and 2022 was performed in PubMed (<https://pubmed.ncbi.nlm.nih.gov/>, accessed on December 31, 2022). Three keywords of “apoptosis” (524,558), “senescence” (553,818), and “autophagy” (74,425) were examined with the combination of “intervertebral disc” (39,621), “disc aging” (2257), “disc degeneration” (13,794), “aging” (588,399), “degeneration” (220,250), “phenotype” (848,468), and “spine” (282,171), as numbers in the parenthesis showed in-relevant articles. Specifically, “apoptosis”, “senescence”, and “autophagy” with “intervertebral disc” were 1098, 1460, and 252, respectively. Important articles regarding clinical and basic science aspects of the intervertebral disc were additionally obtained by hand search. The abstract was evaluated and discussed by 3 authors (T.Y., Y.T., and Y.K.). Finally, 193 articles were selected and reviewed in this paper.

Results

Programmed cell death including apoptosis, necroptosis, pyroptosis, and ferroptosis: mechanisms

The loss of intervertebral disc cells, in particular NP cells, is an early key event during aging and degeneration, primarily consisting of cell death [20] and notochordal cell disappearance [44]. During this process, multiple mechanisms of programmed cell death are involved, among which apoptosis is the most well-known [45–47]. Apoptotic cells stop growing and enter the process of controlled death, including blebbing, shrinkage, nuclear fragmentation, chromatin condensation, DNA fragmentation, and messenger RNA (mRNA) decay [48]. Subsequently, apoptosis results in releasing cell fragments, called “apoptotic bodies” [48]. Then, apoptotic bodies containing dead cell components can be engulfed by phagocytes without the spillage of cellular contents into the surrounding environment [49].

Apoptosis has 2 signaling cascades: the intrinsic and extrinsic pathways [48,49]. The intrinsic pathway is induced in response to mitochondrial damage [50], endoplasmic reticulum (ER) stress [51], oxidative stress [52,53], and DNA damage [54]. When these damages reach to be irreparable, nuclear transcription and also mitochondrial translocation of p53, while repressing antiapoptotic genes, for example, B cell leukemia/lymphoma 2 (Bcl-2), activate proapoptotic genes, for example, Bcl-2-associated X (Bax) and Bcl-2 homologous antagonist/killer (Bak) [55]. This imbalance induces mitochondrial outer membrane permeabilization (MOMP), possibly through the opening of the mitochondrial permeability transition pore (MPTP) [56]. The MOMP releases proapoptotic proteins including the cytochrome c, second mitochondria-derived activator of caspases (Smac/Diablo), high temperature requirement protein A2 (HtrA2)/Omi, apoptosis-inducing factor (AIF), and endonuclease from the mitochondria into the cytoplasm [56]. The cytochrome c binds to the WD domain of apoptotic protease activating factor 1 (Apaf-1) monomers, which results in the exposure of the nucleotide-binding oligomerization domain of Apaf-1 to bind the deoxyadenosine triphosphate (deoxy-ATP) [57]. This binding drives additional conformational changes in Apaf-1, exposing both the caspase recruitment domain and oligomerization domain, which allows cytosolic Apaf-1 to assemble into a protein complex, known as the apoptosome [57]. The apoptosome recruits and activates the initiator caspase 9, further triggers the effector caspase 3, and results in the induction of apoptosis [58]. Caspase 3 cleaves the poly (ADP-ribose) polymerase (PARP) and suppresses the ATP depletion causing necrosis [38]. The Smac/Diablo and HtrA2/Omi both help initiate apoptosis by suppressing the inhibitors of apoptosis proteins (IAPs) [59]. Then, AIF facilitates nuclear condensation and disrupts chromatin function [60]. Furthermore, endonuclease activation promotes chromosomal DNA degradation, leading to the formation of apoptotic bodies [61] (Fig. 1A).

The extrinsic pathway, also known as the death receptor pathway, is initiated by natural killer cells or macrophages [62]. These immune cells produce death ligands, including Fas ligand (FasL), tumor necrosis factor (TNF), and TNF-related apoptosis-inducing ligand (TRAIL) [63]. Death ligands bind with death receptors, including Fas, TNF receptors (TNFRs), and TRAIL receptors (TRAILRs) [64] on the target cell membrane, recruit adaptor proteins of Fas-associated death domain (FADD) and TNFR-associated death domain (TRADD), form the death-inducing signaling complex (DISC), and activate the initiator caspase 8 [62]. Subsequently, caspase-8 activation directly cleaves caspase 3 [65]. Caspase 8 also cleaves the BH3-interacting domain death agonist (Bid) into the truncated Bid which translocates to the mitochondria to initiate the intrinsic apoptosis, leading to the release of cytochrome c and Smac/Diablo from the mitochondria via antagonizing Bcl-2 and heterodimerizing with Bax and Bak [65] (Fig. 1A).

Other programmed cell death mechanisms are necroptosis [47,66], pyroptosis [47,67,68], and ferroptosis [47,69]. Necroptosis has characteristics of necrosis, which is however tightly regulated in the apoptosis-

deficient environment by the receptor-interacting proteins 1 (RIP1) and 3 (RIP3) [70,71]. This pathway is also largely mediated by death receptors [72]. Death ligand TNF binds to TNFR1, recruiting the prosurvival complex I, consisting of TRADD, RIP1, and ubiquitin E3 ligases [49]. Then, deubiquitinated RIP1 results in the formation of the prosurvival complex IIa or IIb [49]. The prosurvival complex IIa promotes apoptosis through caspase-8 activation whereas the prosurvival complex IIb enhances necroptosis [49]. The necrosome is formed during necroptosis, a protein complex of FADD, caspase 8, RIP1, and phosphorylated RIP3 [49]. The RIP3 further phosphorylates the mixed lineage kinase domain-like pseudokinase (MLKL), which oligomerizes, translocates from the cytoplasm to the cytoplasmic membrane to perforate pores, and cause necroptotic cell lysis [49] (Fig. 1B).

Pyroptosis is an inflammatory form of cell death [38,73]. Following the activation of caspase 1 by pathogens, interleukin (IL)-1 beta (IL-1 β) and IL-18 induction results in cell death with the concurrent release of inflammatory cytokines into the surrounding environment [74]. Caspase-1 cleavage facilitates the formation of plasma membrane pores, which leads to excessive extracellular IL secretion and intracellular water flux, followed by cell swelling and lysis [75] (Fig. 1B).

Ferroptosis is characterized by lipid peroxidation and free iron-dependent Fenton reactions [39], accompanied by the redox imbalance [76]. The iron-induced generation of reactive oxygen species (ROS) in the mitochondria leads to elevated potential and oxidative stress-induced ferroptosis [77]. Meanwhile, glutathione peroxidase 4 (Gpx4), tetrahydrobiopterin (BH4), and coenzyme Q10 (CoQ10) play a suppressive role in lipid peroxidation and ferroptosis [78] (Fig. 1B).

Programmed cell death during intervertebral disc aging and degeneration

During aging and degeneration, the intervertebral disc experiences multiple types of programmed cell death in response to diverse stresses [45–47,66–69]. In fact, the incidence of apoptosis is notably high in discs during human clinical [79] and rodent experimental [29,44] IDD. The involved intrinsic and extrinsic pathways of apoptosis are both observed in human disc tissues [80], rabbit disc NP cells [50], and animal models of IDD [44,81]. While human disc NP cells primarily underwent the intrinsic pathway-mediated apoptosis, the extrinsic pathway was also involved to a lesser extent [80]. However, Fas and FasL expression was elevated by annular puncture in rabbit lumbar spine discs [82]. Increased expression of proinflammatory cytokines, including TNF, IL-6, and IL-1 β , was also observed in a rat tail disc puncture model [83]. Thus, the observed primary involvement of mitochondrial apoptosis compared to death signal-induced apoptosis in a rat tail IDD model by mechanical overload would be due to no distinct annular violation [44]. Necroptosis is observed in human herniated disc NP cells and tissues [84] and rat disc NP cells under compression [85]. Pyroptosis is involved in IDD, accelerating extracellular matrix destruction [86,87]. As the direct product of pyroptosis, IL-1 β is a key cytokine associated with discogenic LBP [88]. Ferroptosis induction occurs in human degenerated disc tissues, confirmed by the reduced GPX4 and ferritin heavy chain expression [89]. Then, annular puncture-induced IDD was attenuated by a ferroptosis inhibitor of deferoxamine in rat tails [89].

Programmed cell death-modulating therapies during intervertebral disc aging and degeneration

Upon modulating cell death, the effector caspase 3 [90–92] and antiapoptotic Bcl-2 [93] are a likely candidate. Caspase-3 knockdown or Bcl-2 overexpression suppressed disc cell apoptosis in vitro [90,91,93]. Caspase-3 knockdown or knockout delayed IDD in injury-induced and mechanical stress-induced animal models in vivo [90–92]. More recently, noncoding RNAs (ncRNAs), including microRNA, long ncRNA, and circular RNA, have gained increasing attention in the pathomechanism of IDD [47]. Interventions to apoptosis and other types of programmed cell death should have a therapeutic potential for DDD.

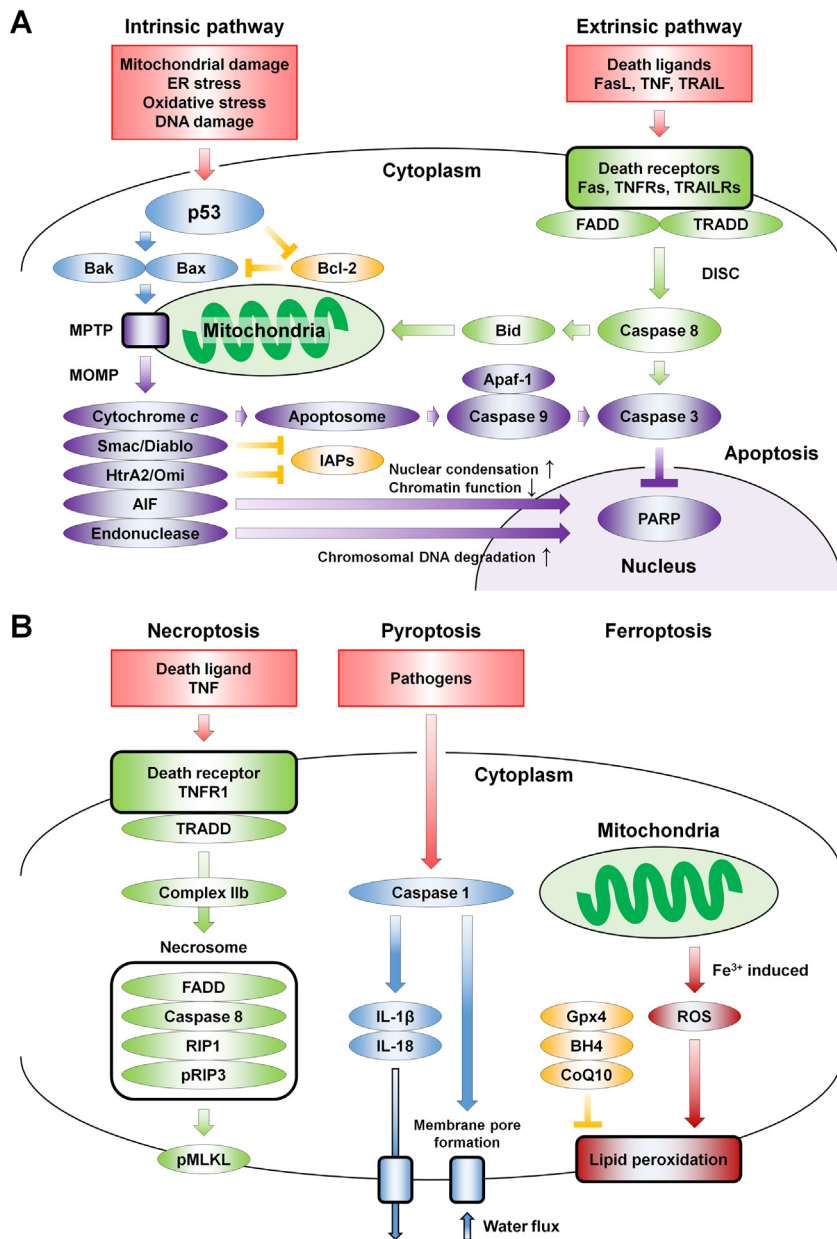


Fig. 1. (A) Schematic of apoptosis signaling pathways. AIF, apoptosis-inducing factor; Apaf-1, apoptotic protease activating factor 1; Bak, Bcl-2 homologous antagonist/killer; Bax, Bcl-2-associated X; Bid, BH3-interacting domain death agonist; Bcl-2, B cell leukemia/lymphoma 2; DISC, death-inducing signaling complex; ER, endoplasmic reticulum; FADD, Fas-associated death domain; FasL, Fas ligand; HtrA2/Omi, high temperature requirement protein A2; IAP, inhibitor of apoptosis proteins; MOMP, mitochondrial outer membrane permeabilization; MPTP, mitochondrial permeability transition pore; PARP, poly (ADP-ribose) polymerase; Smac/Diablo, second mitochondria-derived activator of caspases; TNF, tumor necrosis factor; TNFR, TNF receptor; TRADD, TNFR-associated death domain; TRAIL, TNF-related apoptosis-inducing ligand; TRAILR, TRAIL receptor. (B) Schematic of necroptosis, pyroptosis, and ferroptosis signaling pathways. BH4, tetrahydrobiopterin; CoQ10, coenzyme Q10; Gpx4, glutathione peroxidase 4; IL, interleukin; IL-1 β , IL-1 beta; MLKL, mixed lineage kinase domain-like pseudokinase; pMLKL, phosphorylated MLKL; RIP, receptor-interacting protein; pRIP3, phosphorylated RIP 3; ROS, reactive oxygen species.

Senescence: mechanisms

Cellular senescence has initially been discovered as an irreversible cell-cycle arrest mechanism that halts the spread of cancer cells after extensive serial passaging [94]. This particular type of senescence is known as replicative senescence, accompanied by the critical shortening of the telomere length [95]. Another type is stress-induced premature senescence (SIPS) without the shortening of telomeres, which occurs after the accumulation of genomic and mitochondrial DNA damage in response to various stresses [35]. Cells with the experience of SIPS acquire the senescence-associated secretory phenotype (SASP) [34], releasing high amounts of proinflammatory cytokines and proteases to exert catabolic effects on neighboring cells and extracellular matrix [34,35]. Therefore, cellular senescence and the SASP are strongly associated with aging alteration and chronic degenerative diseases [95,96]. Under stress, DNA damage stimulates diverse molecular signaling pathways but ultimately activates p53 and p16/inhibitor of cyclin-dependent kinase (Cdk) 4a (Ink4a) [95,96]. The ataxia telangiectasia-mutated (ATM)

and ataxia telangiectasia and Rad3-related (ATR) kinases block the cell-cycle progression through the stabilization of p53 and transcriptional activation of a Cdk inhibitor, p21/Cdk-interacting protein 1 (Cip1) [95]. The p16/Ink4a also inhibits the cell-cycle progression by targeting the cyclin D-Cdk4 and cyclin D-Cdk6 complexes [95,96]. Both p21/Cip1 and p16/Ink4a prevent the inactivation of the retinoblastoma tumor-suppressor protein (Rb), resulting in the cell-cycle arrest though the repression of E2F target genes required for S-phase onset [95]. Under severe stress, temporally arrested cells make the transition into senescence cells, leading to the acquisition of the SASP, while stress relief allows cells in the early stage to reenter the cell cycle [95]. Many other intracellular signaling pathways, including the silent information regulator 2 ortholog 1 (Sirt1) [97–100], Wnt/beta-catenin (β -catenin) [101], p38 mitogen-activated protein kinases (MAPKs) [102,103], nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) [103], mammalian target of rapamycin (mTOR) [104], insulin-like growth factor-1 (IGF-1) [105], and transforming growth factor-beta (TGF- β) [106], are also involved in cellular senescence (Fig. 2).

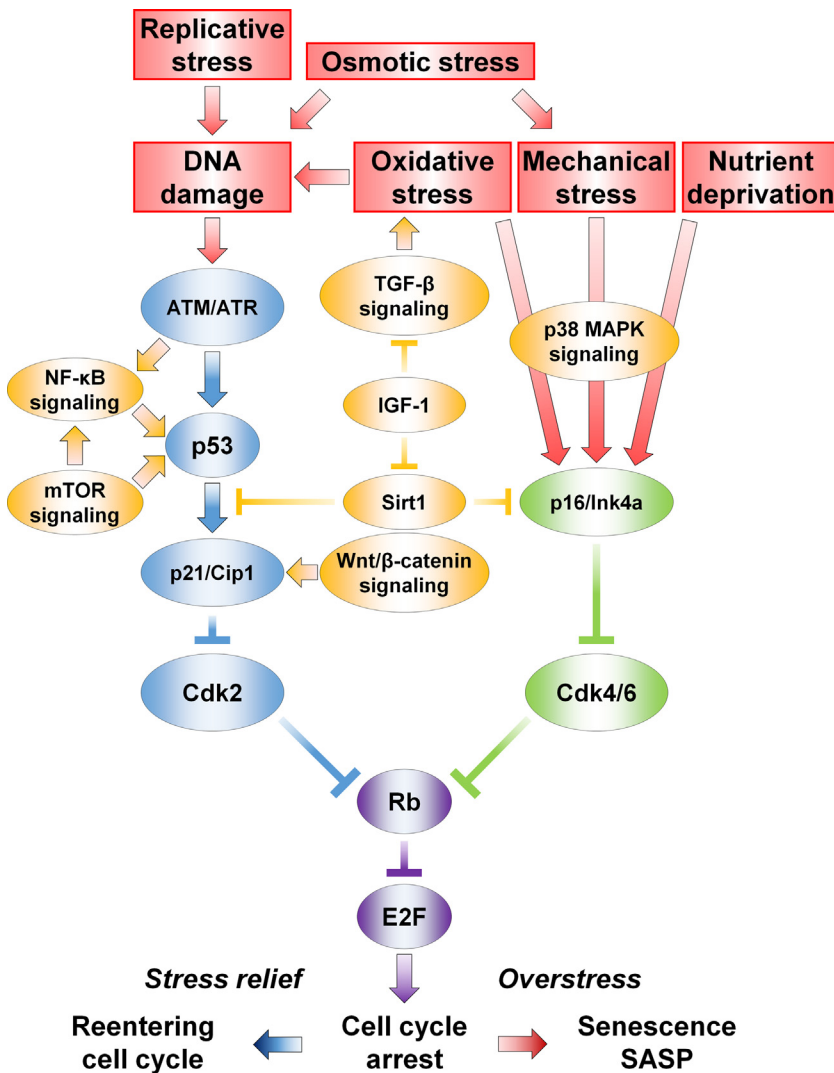


Fig. 2. Schematic of senescence signaling pathways. ATM, ataxia telangiectasia-mutated; ATR, ataxia telangiectasia and Rad3-related; β -catenin, beta-catenin; Cdk, cyclin-dependent kinase; Cip1, Cdk-interacting protein 1; IGF-1, insulin-like growth factor-1; Ink4a, inhibitor of Cdk4a; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; Rb, retinoblastoma tumour-suppressor protein; SASP, senescence-associated secretory phenotype; Sirt1, silent information regulator 2 ortholog 1; TGF- β , transforming growth factor-beta.

Senescence during intervertebral disc aging and degeneration

The incidence of senescence increases in human clinical IDD [107–112]. The senescence-associated beta-galactosidase (SA- β -gal) [108,109] and p16/INK4A expression [109] positively correlated with the severity of IDD. As p16/INK4A expression further had a positive correlation with the expression of catabolic MMP-13 and ADAMTS-5 enzymes [109], senescent disc cells could initiate matrix degradation by the SASP. The number of SA- β -gal-positive AF cells also negatively correlated to the expression of Ki-67, a proliferation marker [110]. In addition, while the percentage of SA- β -gal-positive NP cells increased with age, the telomere length and telomerase activity decreased [111], indicating the involvement of both replicative senescence and SIPS in aged IDD. Animal model studies also presented elevated disc cellular senescence in DNA repair-deficient *ERCC excision repair 1, endonuclease noncatalytic subunit*^{-/-} mice [113,114] and genotoxin-exposed mice [115,116], suggesting as a driver of degeneration.

Cellular senescence is often induced in response to a variety of stimuli including telomere attrition, oncogenes, and stress [95]. Accumulating evidence suggests that oxidative stress [105,117–121], mechanical overload [100,103,122–125], hyperosmolality [121,126,127], and nutrient deprivation [128] due to the specific harsh microenvironment are triggers of senescence during IDD. In vitro, H₂O₂-induced oxidative stress enhanced SIPS and catabolic changes in human [118] and rat [129] disc NP cells. High glucose-induced oxidative stress also accelerated SIPS in young rat disc NP [130] and AF [131] cells. In vivo,

chronic tobacco smoking, genotoxic agent administration, and ionizing radiation, which all can induce oxidative stress and DNA damage, accelerated mouse IDD with cellular senescence [115,116,132]. Ionizing radiation further induced rat disc cellular senescence in vitro [132]. Although hyperosmolality contributes to the disc homeostasis maintenance [133,134], NaCl or sorbitol supplementation to mimic hyperosmolality initiated responses to DNA damage in NP cells [126], arresting the cell-cycle transition [127]. Hyperosmolality with sucrose induced oxidative injury in rat disc NP cells [121]. Excessive mechanical loading is of course a contributor to IDD [27–29,44,135], as shown by the acceleration of senescence in overload compression models [100,103,122–125]. Dynamic compression increased SA- β -gal positivity and arrested the cell cycle in vitro [100,103,123] as well as increased p16/Ink4a expression and altered the cellular phenotype in ex-vivo organ cultures [123,124]. In-vivo forelimbs-amputated rats experienced overloading due to the prolonged upright position, leading to elevated p16/Ink4a expression [122]. Serum deprivation also increased disc cellular SA- β -gal and p16/Ink4a expression [128]. The interaction between multiple stress triggers causing DNA damage could lead to IDD with cellular senescence.

Senescence-modulating therapies during intervertebral disc aging and degeneration

Despite the irreversible nature of cellular senescence [94], emerging evidence suggests that cells in the early stage can reenter the cell-cycle

transition following the relief of senescence-inducing damages [95]. Antisenescence therapy thus has the potential to delay, stop, and favorably reverse IDD. Antioxidant supplementation [119–121], quitting smoking [115], minimizing genotoxic agent administration [116] and radiation exposure [116,132], avoiding overloading with keeping a reasonable body weight [136], and a good control of blood glucose in diabetic patients [130,131,137], could retard IDD by suppressing senescence-inducing triggers. Molecular interventions to senescence-related intracellular signaling pathways by gene therapy [138], Sirt1-activating resveratrol [99], inhibition of mechanoreceptors [124], supplementation of growth factors including IGF-1 [105] and TGF- β 1 [106], and mTOR inhibitors including rapamycin and temsirolimus [139–141], might also be effective in IDD. Then, senolytic therapy selectively killing senescent cells has recently gathered much attention [142]. Systemic clearance by ganciclovir of p16/Ink4a-positive senescent cells mitigated age-related IDD in p16-3MR transgenic mice [143]. Senolytic drug treatment, including dasatinib [144], quercetin [144,145], navitoclax (ABT263) [146], and o-vanillin [147,148], could ameliorate IDD. Further studies are warranted to develop clinically promising antisenescence approaches.

Autophagy: mechanisms

Autophagy, the intracellular degradation pathway by which cells eliminate and recycle misfolded proteins and damaged organelles, should be considered as an important cell survival mechanism against stress, in particular nutrient deprivation and inflammation [40–43]. Autophagy involves autophagy-related (Atg) genes and proteins [149,150]. Under typical physiological conditions, Atg protein expression is relatively low; however, basal autophagy serves as the quality-control mechanism for cellular renovation and homeostasis [40,43]. Under stress conditions, Atg activation results in the formation and maturation of the autophagosome, which captures damaged organelles, misfolded proteins, and invading microorganisms as induced autophagy [40–42]. Then, the completed autophagosome fuses with the lysosome to form the autolysosome, which degrades the enclosed cargo and releases its constituents for reuse [40–42]. The microtubule-associated protein 1 light chain 3 (LC3) (mammalian Atg8 homolog) is a ubiquitin-like protein with the cytosolic (LC3-I) and phosphatidylethanolamine-conjugated (LC3-II) forms [151]. The LC3-II is the only protein that remains attached to the autophagosome membrane even after the completion [151,152]. The high mobility group box 1 (HMGB1) is a nuclear DNA-binding protein and an extracellular damage-associated molecular pattern molecule [153]. In response to stress, HMGB1 translocates from the nucleus to the cytoplasm, subsequently experiencing the extracellular release [153]. Cytoplasmic HMGB1 directly interacts with beclin1 (mammalian Atg6 homolog), resulting in the autophagosome formation by developing the phagophore through the beclin1–class-III phosphatidylinositol 3-kinase (PI3K) complex [152,153]. The p62/sequestosome 1 (p62/Sqstm1) is a ubiquitin-binding protein that acts as a link between LC3 and ubiquitinated substrates [154]. The p62/Sqstm1 and p62/Sqstm1-bound polyubiquitinated proteins are selectively incorporated into the completed autophagosome and degraded in the autolysosome [152,154]. Then, Atg5 is a key protein involved in the expansion of the phagophore, essential for the autophagosome formation [155]. The Atg5 is activated by Atg7 and forms the conjugation with Atg12 [155]. The Atg12–Atg5 conjugation acts as an E3-like enzyme required for LC3 lipidation [152,155]. Monitoring this dynamic, sequential process, called “autophagic flux”, is essential to understand autophagy (Fig. 3A).

In intracellular signaling, autophagy is under the tight negative control of mTOR [156]. The mTOR is a serine/threonine kinase that integrates nutrients, growth factors, energy, and stress signals to promote cell growth and division [156]. The mTOR exists in the 2 forms: mTOR complexes 1 (mTORC1) comprising the regulatory-associated protein of mTOR (Raptor) and 2 (mTORC2) consisting of the rapamycin-insensitive companion of mTOR (Rictor) [156]. Then, a downstream

mTORC1 effector, p70/ribosomal protein S6 kinase (p70/S6K), regulates cell proliferation, mRNA translation, and protein synthesis [156]. Meanwhile, mTORC1 is regulated by upstream Akt, a prosurvival mediator by suppressing apoptosis [157] and also a senescence inducer through mTORC1 and p53 [158]. The activation of Akt is further associated with the class-I PI3K and mTORC2 [156,157]. The PI3K/Akt/mTOR is a signal integrator of diverse stimuli to determine cell fate (Fig. 3B).

Autophagy during intervertebral disc aging and degeneration

Autophagy has received increasing attention in the intervertebral disc research field, based on the limited nutrient availability suggesting the conceivable involvement. In vitro, autophagy has thus been tested by diverse stress stimuli, such as nutrient deprivation [159–162], oxidative stress [163–165], compression overload [166], inflammation [167], hyperlactatemia [168], hyperosmolality [169], and hypoxia [170]. Meanwhile, only limited evidence regarding mTOR signaling in IDD is available [169–171].

In vivo, prior studies found the involvement and activation of autophagy by electron microscopy in human [172] and rat [137,173] discs. In rat disc NP tissues, Atg protein expression increased with natural aging [173]. A rat model of type-I diabetes induced by streptozotocin, toxic to the insulin-producing pancreatic beta cells, developed mild spontaneous lumbar IDD with stress-response apoptosis, senescence, and autophagy induction [137]. These age and disease-dependent induction of autophagy would be associated with the presence of NP notochordal cells in rodents [13,14], because of the slow proliferation and prolonged maintenance [174] possibly requiring autophagy. Subsequently, severe rat tail IDD under unphysiological sustained static compression clarified the loading duration-dependent reduction in autophagy marker and substrate expression [135].

In human IDD, compared to discs with the grades I–III, assessed by the Pfirrmann degeneration system [175], ATG gene expression was upregulated in discs with the grades IV–V [172]. However, in protein expression, autophagy levels increased in discs with the Pfirrmann grade III but decreased in discs with the grades IV–V [176]. Furthermore, the similar trend toward age-dependent decrease in ATG protein expression was observed in discs with the grades III and IV [177]. Therefore, downregulation of ATG protein expression would be degeneration severity-dependent [176] and age-dependent [177], whereas upregulation of ATG gene expression in discs with the grades IV–V could be compensative [172]. Autophagy would be activated transiently but reduced at least in aged, severely degenerated discs, suggesting the self-renewal concept in stress-response to DDD.

Autophagy-modulating therapies during intervertebral disc aging and degeneration

Prior studies did not mechanistically examine autophagic flux in intervertebral disc cells. Then, findings of autophagy regulation largely included confounders of PI3K/Akt/mTOR-signaling modulation. In addition, many in-vitro studies were conducted using conventional cell-culture conditions of 10% to 20% serum supplementation and normoxia, considerably different from the in-vivo disc environment [31]. Therefore, we decided to study the potential of autophagy and PI3K/Akt/mTOR signaling as a therapeutic target for DDD.

First, we carefully observed rabbit disc AF-cell fate including autophagic flux under varying degrees of nutrient withdrawal under 5% O₂ [128], based on the peripheral bone marrow oxygen concentration of 4% to 7% [178]. In this study, cell proliferation was maintained in 1% serum-supplemented media while cell viability normalized to DNA amount was the highest in 5% serum-supplemented media, suggesting excessive serum concentrations to be detrimental. Time-course analysis identified increased autophagic flux in response to limited nutrition measured by LC3 puncta number, LC3-II expression, cytoplasmic

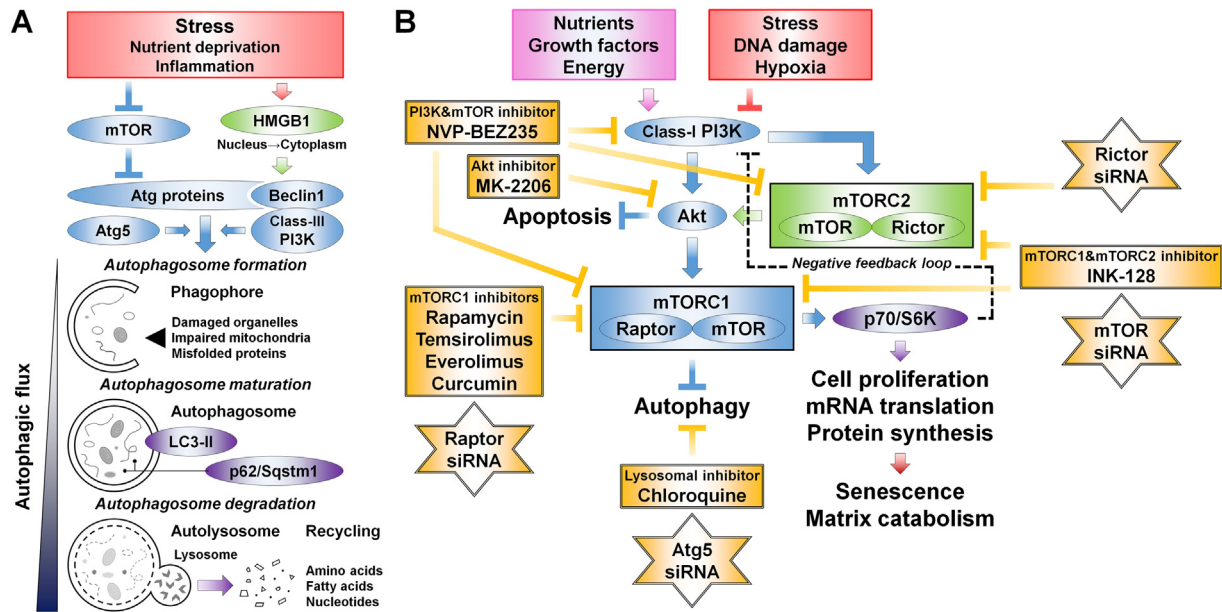


Fig. 3. (A) Schematic of autophagic flux. Atg, autophagy-related; HMGB1, high mobility group box 1; LC3, microtubule-associated protein 1 light chain 3; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; Sqstm1, sequestosome 1. (B) Schematic of PI3K/Akt/mTOR signaling pathways. mRNA, messenger RNA; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; Raptor, regulatory-associated protein of mTOR; Rictor, rapamycin-insensitive companion of mTOR; S6K, ribosomal protein S6 kinase; siRNA, small interfering RNA.

HMGB1 intensity, and p62/Sqstm1 expression. The incidence of disc cellular apoptosis and senescence also increased with the severity of nutrient withdrawal. Notably, autophagy was involved during both apoptosis and senescence, although the degree of autophagic response in apoptosis was greater than in senescence. This study provides the basic understanding of disc cell-fate determination (Fig. 3A).

We next utilized RNA interference (RNAi) to knockdown specific mTOR-signaling cascades, using small interfering RNAs (siRNAs) against mTOR, RAPTOR, and RICTOR, in human disc NP cells surgically collected [179]. Experiments were performed with IL-1 β to simulate inflammation [180] and under 2% O₂ to simulate hypoxia [31]. In this study, the specific downregulation of mTOR, RAPTOR, and RICTOR proteins was obtained by the corresponding siRNAs. While mTOR and RICTOR siRNAs decreased downstream p70/S6K and upstream Akt phosphorylation, RAPTOR siRNA decreased p70/S6K phosphorylation but increased Akt phosphorylation. Akt activation occurs through the negative feedback loop between p70/S6K and the class-I PI3K [181–183]. As the class-I PI3K is responsible for the activation of Akt [156], suppressed mTORC1 and p70/S6K by RAPTOR RNAi resulted in the class-I PI3K feedback loss and subsequent Akt activation [182]. In addition, mTOR, RAPTOR, and RICTOR siRNAs all presented enhanced autophagy by RNAi-mediated mTORC1 suppression. Cell proliferation and viability decreased with mTOR and RICTOR siRNAs but not RAPTOR siRNA, possibly resulting from mTORC1 and p70/S6K suppression in all treatments [156] and Akt induction in RAPTOR RNAi [157]. Then, IL-1 β -induced apoptosis was inhibited by RAPTOR RNAi only, possibly due to increased Akt phosphorylation [157]. Meanwhile, IL-1 β -induced senescence was blocked by all RNAi treatments, which would result from mTORC1 suppression-mediated impaired proliferation [156]. Furthermore, IL-1 β -induced catabolic MMP-3 and MMP-13 release diminished by all mTOR, RAPTOR, and RICTOR siRNAs; in particular, MMP-2 and MMP-9 activity declined by RAPTOR siRNA only. Expression and activation of MMPs might depend on mTORC1-mediated mRNA translation and protein synthesis. In summary, while the extensive suppression of mTORC1/mTORC2/mTOR and mTORC2/RICTOR is not always advantageous, the selective suppression of mTORC1/RAPTOR protects

against inflammation-induced disc cellular apoptosis, senescence, and matrix catabolism, possibly resulting from autophagy and Akt activation (Fig. 3B).

Based on the human disc NP-cell protection by mTORC1/RAPTOR suppression [179], pharmacological treatment is more favorable than gene therapy in clinical practice due to the safety issue [138]. We further performed an in-vitro study to identify the most effective mTORC1 inhibitor for human DDD [140]. We tested the first-generation agents specifically inhibiting mTORC1: rapamycin, everolimus, and temsirolimus [184], the second-generation agents inhibiting multiple signaling pathways of mTORC1 and mTORC2: INK-128 and of PI3K and mTOR: NVP-BEZ235 [184], an allosteric Akt inhibitor: MK-2206 [185], and a natural polyphenol: curcumin [186]. In this study, mTORC1-inhibiting rapamycin, temsirolimus, and everolimus decreased mTOR and p70/S6K phosphorylation but increased Akt phosphorylation; however, curcumin did not affect phosphorylation. Rapamycin, temsirolimus, and everolimus demonstrated increased autophagic flux, showing the most prominent LC3-II accumulation by temsirolimus. Inhibitory effects of IL-1 β -induced apoptosis, senescence, and matrix catabolism were also remarkable by rapamycin, temsirolimus, and everolimus compared to by curcumin; notably, temsirolimus appeared to be the most effective, possibly resulting from the improved water solubility [184]. In contrast, curcumin was not toxic but less effective, possibly because of the poor aqueous solubility and low bioavailability [187]. Then, INK-128 and NVP-BEZ235 decreased mTOR, p70/S6K, and Akt phosphorylation, indicating extensive mTOR inhibition. However, INK-128 and NVP-BEZ235 did not induce autophagy, despite mTORC1 inhibition. Furthermore, INK-128 and NVP-BEZ235 did not decelerate but accelerated IL-1 β -induced apoptosis, senescence, and matrix catabolism. Thus, the second-generation mTOR inhibitors did not protect human disc NP cells against inflammation. Finally, MK-2206 decreased mTOR, p70/S6K, and Akt phosphorylation, indicating successful Akt inhibition, but did not enhance autophagy. Similarly, MK-2206 further exacerbated IL-1 β -induced apoptosis, senescence, and matrix catabolism. In summary, cell and matrix protective effects of mTORC1 inhibitors primarily depend on Akt activation in human disc NP cells.

Therefore, Akt is the key regulator of human disc cellular mTOR signaling. We propose temsirolimus as the optimal mTORC1 inhibitor to treat inflammation-associated DDD (Fig. 3B).

We have clarified the importance of Akt in human disc cell fate [140]; however, it is still unknown whether autophagy could truly protect disc cells. We thus designed an in-vitro study to elucidate specific roles of human disc cellular autophagy, by applying RNAi against ATG5 [177]. In human disc NP cells, RNAi showed the specific decrease in ATG5 protein expression, clearly suppressing autophagy with decreased LC3-II and increased p62/SQSTM1 but without affecting mTOR, p70/S6K, and Akt expression and phosphorylation. Cell viability was maintained by ATG5 RNAi in serum-supplemented media but reduced in serum-free media, particularly with IL-1 β . Then, ATG5 RNAi further promoted IL-1 β -induced apoptosis and senescence. Meanwhile, ATG5 RNAi unaffected IL-1 β -induced catabolic MMP release, downregulated anabolic gene expression, and MAPK pathway activation, a key downstream IL-1 β effector [23], essential for MMP activation [188]. Similar findings of autophagy inhibition were additionally confirmed by lysosomotropic chloroquine [152]. Hence, ATG5-dependent autophagy protects human disc NP cells against apoptosis and senescence rather than extracellular matrix catabolism (Fig. 3B).

Finally, an in-vivo RNAi study of Atg5 using rat tails was designed to further clarify the loss-of-function of disc cellular autophagy [189]. Time-course immunofluorescence detected positive signals for Alexa Fluor 555-labeled Atg5 siRNA even at 56 days after the intradiscal injection. Then, time-course immunoblotting found decreased autophagic flux with Atg5 knockdown for 56 days. Experimental IDD induced by 24-hour temporary static compression displayed radiographic height loss, histological disruption, and apoptosis and senescence induction in Atg5 siRNA-injected discs at 56 days. Autophagy thus contributes to the maintenance of disc cell and matrix homeostasis against apoptosis and senescence (Fig. 3B).

Discussion

Based on the narrative literature review, mTOR is a signal integrator for nutrients, growth factors, energy, stress, DNA damage, and hypoxia to determine intervertebral disc cell fate including apoptosis, senescence, and autophagy [156]. Therefore, in PI3K/Akt/mTOR signaling, the extensive interference of mTORC1/mTORC2/mTOR and mTORC2/RICTOR would be harmful. In fact, homozygous mTOR deletion results in embryonic lethality [190]. Identifying which subunit(s) of mTOR exerts effects on target cells is necessary. Our research experience could suggest the selective interference of mTORC1/RAPTOR as a future treatment target for DDD, by gene therapy targeting mTORC1/RAPTOR and pharmacological therapy using mTORC1-inhibiting temsirolimus. In gene therapy, safety is the primary focus; however, the disc NP is an encapsulated, isolated structure that would be suitable for local delivery [138,191]. Actually, effectiveness of intradiscal RNAi is prolonged for 24 weeks without adverse events [192]. In drug therapy, local administration to the disc is favorable. Systemic administration of mTORC1 inhibitors is potentially risky due to serious side effects, primarily immunosuppression [193]. Therefore, intradiscal gene and pharmacological therapies aiming the specific suppression of mTORC1/RAPTOR are potential biological approaches for DDD. These interventions could be applicable to prevent the long-term progression of IDD through stimulating autophagy and Akt by gene therapy and to treat inflammatory painful discs by pharmacological therapy.

Conclusions

This brief review describes the current understanding of cell fate, focused on apoptosis, senescence, and autophagy, during aging and degeneration in the intervertebral disc. The PI3K/Akt/mTOR is a suggested underlying intracellular network integrator for diverse stimuli to control the microenvironment, resulting in disc cell-fate determination. Further

mechanistic investigation of PI3K/Akt/mTOR signaling should prove useful for the development of biological therapies for age-related, inflammatory DDD.

Declarations of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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