HHS Public Access

Author manuscript

Ann N Y Acad Sci. Author manuscript; available in PMC 2020 April 01.

Published in final edited form as:

Ann N Y Acad Sci. 2019 April ; 1442(1): 61–78. doi:10.1111/nyas.13964.

Annulus fibrosus cell phenotypes in homeostasis and injury: implications for regenerative strategies

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Abstract

Despite considerable efforts to develop cellular, molecular, and structural repair strategies and restore intervertebral disc function after injury, the basic biology underlying intervertebral disc healing remains poorly understood. Remarkably little is known about the origins of cell populations residing within the annulus fibrosus, or their phenotypes, heterogeneity, and roles during healing. This review focuses on recent literature highlighting the intrinsic and extrinsic cell types of the annulus fibrosus in the context of the injury and healing environment. Spatial, morphological, functional, and transcriptional signatures of annulus fibrosus cells are reviewed, including inner and outer annulus fibrosus cells, which we propose to be referred to as annulocytes. The annulus also contains peripheral cells, interlamellar cells, and potential resident stem/progenitor cells, as well as macrophages, T lymphocytes, and mast cells following injury. Phases of annulus fibrosus healing include inflammation and recruitment of immune cells, cell proliferation, granulation tissue formation, and matrix remodeling. However, annulus fibrosus healing commonly involves limited remodeling, with granulation tissues remaining, and the development of chronic inflammatory states. Identifying AF cell phenotypes during health, injury, and degeneration will inform reparative regeneration strategies aimed at improving annulus fibrosus healing.

Graphical abstract

Despite considerable efforts to develop cellular, molecular, and structural repair strategies and restore intervertebral disc function after injury, the basic biology underlying intervertebral disc healing remains poorly understood. This review focuses on recent literature highlighting the intrinsic and extrinsic cell types of the annulus fibrosus in the context of the injury and healing environment, and describes the.spatial, morphological, functional, and transcriptional signatures of annulus fibrosus cells.

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Competing Interests

The authors declare no competing interests.

Keywords

annulus fibrosus; intervertebral disc; cell phenotype; intervertebral disc injury models; healing; regeneration

Introduction

Acute and chronic injuries of the annulus fibrosus (AF) of the intervertebral disc (IVD) result in spinal pathologies—including herniation and degeneration—that are known causes of back pain, one of the leading causes of global disability.[1] AF repair is challenging due to the tissue's structural complexity, which is key to its biomechanical function. Consequently, injuries such as IVD herniation have a relatively high rate of re-herniation.[2] Many studies are developing repair strategies designed to deliver cells, drugs, and biomaterials (alone or in combination) to the IVD, although these strategies remain far from clinical translation. Cell delivery approaches have been especially challenging due to the inexact definition of AF cell phenotypes and the absence of robust differentiation strategies for deriving AF cells.[3,4] Designing informed biologic AF repair strategies that aim to restore IVD structure and function requires an improved understanding of the distinct morphological and functional characteristics of the heterogeneous cell types residing in the AF and how they change with injury and degeneration. However, there is relatively little known about AF cell phenotypes.

To date, the majority of IVD cell studies have focused on nucleus pulposus (NP) cell and tissue responses to aging and degeneration, since the NP is regarded as an important signaling center of the IVD. In addition, many of the earliest age- and degeneration-related changes are most prominently displayed in the NP region, suggesting it is an important source of IVD pathologies.[5–7] In contrast, there are relatively few studies that have focused on endogenous AF repair capacity even though AF disruption is more commonly associated with pain and disability. Thus, the success of IVD repair strategies relies on the development of effective AF repair and/or regeneration techniques to promote the restoration of IVD function as a whole composite tissue.

Many experimental strategies under development for AF repair consist of combinatorial biomaterial approaches of cells and/or drug delivery that commonly attempt to emulate mature AF structure with relatively little information available on the cell and developmental processes involved in creating the native AF structure. Despite the wide array of experimental approaches, a recent review highlighted that only a small number of investigations focusing on biologic repair strategies to promote AF regeneration have reached the preclinical stage.[8] Furthermore, current AF repair design criteria guidelines are often centered primarily on safety, biocompatibility, and biomechanical behaviors, with a focus on preventing reherniation by achieving adhesion and integration with native tissue and/or restoring biomechanical function.[9,10] Repair or regenerative potential is often assessed with extracellular matrix protein measurements, with less focus on structure. Most experimental repairs do not match the complex angle-ply lamellar structure of native AF, or its structural or cellular heterogeneity by region. An improved understanding of naive AF cell phenotypes and cellular injury responses may help advance the development of AF

repair strategies more rapidly than extensive, iterative screenings of hydrogels, cells, factors, and combinations thereof.

Designing robust AF repair strategies would require improved understanding of the complex and coordinated processes involved in normal AF development as well as the pathological changes occurring with aging, injury, and degeneration. Tissue engineering of biologic laminates demonstrated that cells both develop and respond to the unique cross-ply fiber form of the AF.[11] There is also a base knowledge on AF mechanical interlamellar interactions.[12–16] Even with these important advances and knowledge, there remains little information on the characteristic phenotypes of different AF cell populations throughout development, maturity, and aging. Identifying cell phenotypes is challenging since very few markers exist and consequently distinction of AF cell phenotypes is currently based on anatomic separation of cells and tissues. Elucidating AF cell responses to injury in both non-regenerative and regenerative contexts is also an important area for research since it may inspire logical design approaches that promote regeneration or reverse injury effects and scarring associated with poor healing in the adult IVD. This literature review therefore specifically focuses on identifying cells of the native AF and synthesizes the known cellular responses to injury using *in vivo* model systems. We believe this characterization of the cells of the native, healthy AF is an important step in identifying the most promising cell targets for AF repair strategies. This point is highlighted since even nomenclature for AF cells often lacks consistency. Furthermore, the characterization of AF cellular responses to various models of AF injury is intended to help inform the development of repair strategies with potential to advance towards AF regeneration.

Clinical significance

Back and spine pathologies are among the most common sources of pain and disability, and they affect approximately 7.6 million people in the United States.[1,17,18] Clinically significant lower back pain has an incidence of 1.39 per 1000 person-years.[19] This disability comes at a significant economic cost of approximately $100–200 billion spent on back pain annually, two-thirds of which is a result of lost wages and decreased productivity.[20,21] Furthermore, most patients with lower back pain will experience recurrent symptoms, with estimates ranging from 42–80%.[22] It is therefore essential to develop successful and lasting treatments for back pain in order to allow patients to return to the workplace and to live without pain and disability.

The etiologies of back pain are diverse and involve the IVD, vertebrae, facet joints, neural elements, as well as surrounding musculature and fascia, or from a combination of these structures.[23] One of the most common and best-studied, identifiable sources of back pain is the IVD, which consists of the central NP, surrounded by the AF, and cartilaginous endplates. When the IVD functions properly, it provides flexibility and load transmission to the spine.[24–27] After damage through the accumulation of degenerative changes or the acute disruption of AF structure, IVD pathology may be associated with increased pain in patients due to greater rotational motion, [28] instability, loss of IVD height, or NP herniation, with potential to impinge on neural elements, resulting in radiculopathy. Furthermore, the damaged IVD structure is thought to enable neurovascular growth into the normally aneural

and avascular regions deep within the IVD, which can be irritated by increased pro-inflammatory signals that enhance nociception and cause pain.[29–33] While degenerative changes to the spine and IVD are associated with back pain, the specific phenotype of degenerative changes to the IVD is often difficult to identify and this challenge is confounded since many patients with back pain do not have positive MRI findings for IVD degeneration and since non-painful control subjects often exhibit degenerative changes to their IVDs.[34–37] Consequently, it is clear that back pain is a multifactorial condition and that it involves structural injury and degeneration of spinal tissues in addition to multiple competing psychological, social, and economic factors that all require additional research to identify new ways of addressing this global healthcare challenge.[18]

Annulus fibrosus development

The general development of IVD structures and the key signaling pathways identified to date have been previously reviewed, so here we briefly summarize the main events specific to AF development.[38–41] The NP, AF, and vertebral bodies are all mesodermal in origin,[6,38,40] although the NP is formed from the notochord (a cartilaginous axis ventral to the neural tube) while the AF is formed from the sclerotome compartment of the somites (repeating paired structures formed on either side of the neural tube).[42–46] The patterning of the distinct structures of each vertebral body starts at cranially and proceeds caudally, so that cervical IVDs develop before thoracic and lumbar levels.[47] However, within each level, the components develop concurrently, resulting in tightly bound but structurally distinct elements.[48] All cells of the AF derive from a population of $Scx^{+}/Sox9^{+}$ progenitors.[49] While AF progenitors are initially disorganized, they subsequently align in the characteristic angle-ply lamellar pattern, forming a network of actin stress fibers coupled via adherens junctions, which may also serve as guides for cell alignment.[50,51] Importantly, this cellular alignment precedes the appearance of oriented collagen matrix and is likely critical for the subsequent organization of AF lamellae structure.[51] Mechanical loading also plays an important role in IVD development, as a study in chick embryos suggests that fetal movements influence spinal curvature and segmentation.[52] These intriguing findings highlight the need to better understand the coordinated formation of neuronal, muscular, and skeletal structures and their interdependencies during development. Although the developmental processes distinguishing inner and outer AF differentiation may inform their postnatal response to injury and healing potential, these mechanisms have yet to be identified.[40]

Annulus fibrosus structure and function

The AF is a highly fibrous and well-organized tissue surrounding the outer region of the IVD. It is composed of multiple layers of concentric lamellae in an angle-ply fiber orientation that serves to constrain mobility of the IVD and contain the inner NP. This characteristic fibrous organization of the AF confers its ability to withstand circumferential loads and to limit the amount of torsional rotation and bending motions.[25,53]

The complex AF structure is often separated into two distinct regions: the inner AF containing primarily type II collagen produced by rounded, fibrocartilage cells, and the outer AF containing primarily type I collagen produced by elongated fibroblast-like cells.[24,48,51,54–56] The transition between the inner to the outer AF is complex and has been

further distinguished at the structural and cellular levels, with regional differences in extracellular matrix composition,[12,57,58] cytoskeletal organization,[59] and cell types [12,24] that are required to produce matrix and molecules specific to different regions. From the outer to inner AF regions, the ratio of type I collagen to type II collagen decreases.[54,55,60] The angle-ply fiber orientation also changes from 65° in the outer AF to 30–45° in the inner AF.[61,62] This angle-ply structure contributes to the non-linear and anisotropic mechanical properties of the AF.[63] Adding to this structural complexity, individual lamellae are separated by a disorganized interlamellar tissue containing proteoglycan-rich matrix, elastic fibers, and cells,[13] and are connected by networks of interlamellar cross-bridges consisting of elastin and type VI collagen, which are thought to be important for maintaining healthy AF function.[13,64,65] Elastic fibers are also known to bridge layers, and these have been speculated to be functional tie fibers and/or remnants of vascular channels that regress following IVD development.[64,66] The organization of this complex fiber-reinforced material also exhibits excellent mechanical performance, inhibiting crack progression and maintaining excellent stiffness behaviors even after damage has accumulated. [67,68] The distribution and organization of the various components that constitute the native and healthy AF accumulate over decades of life and pose a challenge for the design of repair strategies that aim to mimic this complex and hierarchical tissue.

Cells of the Annulus Fibrosus

In the human adult, reported AF cell density ranges from approximately 3000 cells/mm^3 to 9000 cells/mm^3.[69–71] This range is markedly lower than that of mature hyaline cartilage, which ranges from 16,000 cells/mm^3 to 60,000 cells/mm^3.[71,72] Despite low AF cell density, there is significant heterogeneity of cell types residing within the AF under healthy conditions. While a few studies have identified possible cell phenotypes within the AF, there are few unique or definitive markers to distinguish intrinsic AF cell populations. Functional definitions of the heterogeneous cell types residing in the AF remain unclear. A recent study of primary and immortalized human AF cells revealed 1161 genes showing higher expression in AF than in NP tissue, and 125 AF-specific genes that encode membrane-associated proteins, providing a substantial set of novel AF membrane-associated markers.[73] Thus, these cells are often defined based on regional location, morphology, and/or function (Table 1). We propose that the term “annulocyte” be used to refer to the native, heterogeneous population of sclerotome-derived cells expressing the markers *Scx* and *Tnmd*, but should not specifically refer to a single cell type within. Additional cell types that reside within or near the AF, but whose origins are unknown, include peripheral cells, interlamellar cells, and stem/progenitor cells (Fig. 1).

Annulocytes and other resident cells

The outer AF contains elongated, fusiform, fibroblast-like cells that have previously been referred to as “outer AF cells”, “fibroblast-like AF cells”, “AF-like cells”, “AF fibrochondrocytes”, or simply “AF cells”.[12,74,75] We propose that cells residing in the outer portion of the AF be referred to as “outer annulocytes” (Table 1). Vimentin staining for cytoskeletal elements in the bovine AF demonstrated that these elongated cells reside in the outer 20% of the AF and run parallel to oriented collagen fibres with extended lateral

processes perpendicular to the lamellae.[12] Outer annulocytes have been identified in several species, and a handful of genes and proteins have been proposed as distinct phenotypic markers that can differentiate these outer annulocytes from inner annulocytes and other cells of the IVD. Classically, the extracellular matrix surrounding outer annulocytes is composed of a higher ratio of type I to type II collagen, as demonstrated by immunostaining studies in humans across a range of ages.[74] Unsurprisingly, expression of *COL5A1*, a gene encoding type V collagen, which is thought to regulate type I collagen assembly, is distinctive to the outer AF of rabbit IVDs relative to NP and articular cartilage.[76] The glycosaminoglycan keratan sulfate was identified in the inner and outer AF of the developing rat IVD, and has also been identified in human AF tissue as a specific matrix protein of annulocytes.[57,77] A transcriptional signature of *COL1A1, COL5A1, COL12A1*, and *SFRP2* was proposed for a population of annulocytes isolated from young human tissue after culture.[78] The association of type XII collagen with type I collagen is well established and is thought to modify the interactions between collagen I fibrils and surrounding matrix. *SFRP2*, a modulator of Wnt signaling, is a putative AF marker specific to outer annulocytes in human and bovine tissue.[78,79] Outer annulocytes share similar markers to other dense, fibrous connective tissue cells, such as tenocytes and ligamentocytes, including the genes *Tnmd, Mkx*, and *Scx* in human, bovine, and murine AF.[79–82]

From the outer AF region towards the inner AF and NP, there is a marked change in cell morphology and phenotype from that of the classically defined outer annulocyte to a more rounded cell shape. These cells have been variously termed “chondrocyte-like cell”, “inner-AF cell”, or “discoidal chondrocytes”.[83] Like chondrocytes, inner annulocytes produce primarily type II collagen.[74,84] *Fmod*, a gene encoding for fibromodulin, which is a proteoglycan important in collagen assembly, is expressed at high levels in inner annulocytes at embryonic stages and is known to be an AF-specific marker in rodents.[85] While these cells are also sometimes described as “NP-like”, we will avoid this terminology since the AF and NP are derived from distinct embryonic progenitors and to date there is no evidence for transdifferentiation of these cells types *in vivo*. We propose the use of “inner annulocyte” to refer to these rounded, chondrocyte-like cells residing within the inner AF (Table 1). The transition from the fibrous phenotype of outer annulocytes to the chondrogenic phenotype of inner annulocytes may be due to differences in mechanical loading (tensile versus compressive, respectively). The close proximity to the highly pressurized and outward-bulging NP may also provide an additional mechanical cue, as well as direct molecular signals from NP cells. Interestingly, there may be additional heterogeneity within the inner annulocyte population, as one study reported that ~2% of cells isolated from the inner AF also expressed α-smooth muscle actin.[86] Staining for cytoskeletal vimentin also showed that inner annulocytes at the NP/inner AF border exhibit longer cellular processes.[12] Whether these features are indicative of specific functional activity within the inner annulocyte population is currently unexplored.

The lamellae comprising the AF are separated by interlamellar matrix that functions to maintain AF integrity by integrating the lamellae and providing some interface lubrication between sliding AF structural features.[13,14,87] The interlamellar matrix is highly distinct from AF lamellae, with complex elastic fiber and cross-bridge arrangements. This unique structure suggests the existence of a population of specific interlamellar cells that are distinct

from inner or outer annulocytes. These interlamellar cells are likely subjected to unique physical cues (such as shearing) that may guide matrix synthesis and homeostasis. To date, interlamellar cells remain poorly characterized but have been described as forming a lace-like network between AF lamellae (Table 1).[12]

A single layer of cells expressing the markers CD146 and transgelin (SM22α) was identified in the AF periphery in both mouse and human IVD (Table 1).[88] While there is speculation that these CD146$^+$ cells may be a population of resident stem cells, their function is still not clear, and they may also serve important functions in wound healing responses and contractility.[88]

Stem and progenitor cells

Stem and/or progenitor cells have been identified in young, adult, and aged AF tissue (degenerative and non-degenerative); however, there is currently no clear consensus on AF-specific stem/progenitor markers (Table 1). These cells are therefore typically distinguished using surface markers associated with mesenchymal stem cells (MSCs) or pluripotency markers.[89,90] IVD stem/progenitor cells reside in a niche at the AF/cartilaginous endplate border and in the nearby perichondrium, and are speculated to migrate into the IVD during homeostasis.[89,91–95] With aging, immunohistochemical detection of mesenchymal and pluripotent markers decreases,[95,96] suggesting that loss of stem/progenitor cells may be a possible contributor to impaired healing in adults. However, this has not been directly tested. In addition to AF stem/progenitors, putative neural stem cells (expressing the markers nestin and neuron-specific enolase) were identified in young human AF tissue and these progenitors were capable of differentiating into neural cells *in vitro*; however, their *in vivo* function remains undetermined.[90,97]

Cellular responses to injury

The established phases of wound healing include: initial inflammation and extrinsic/inflammatory cell recruitment; cell proliferation; formation of collagenous and fibrotic granulation tissue; and remodeling.[98] A thorough characterization of cellular responses to AF injury throughout these wound healing phases without therapeutic intervention is lacking, particularly with age and across various species. It is broadly known that aged and degenerated human IVDs have particularly poor healing capacity due to low cellularity,[70] accumulation of structural defects, and chronic inflammation,[31,32,99] but a comparison with young human IVD injury responses is difficult because it is uncommon for young human IVDs to be injured and available for study. Injury to the AF leads to a number of pathological changes, including decreased IVD cellularity, upregulated matrix-degrading enzymes, innervation, inflammation, increased growth factor production, and formation of granulation tissue.[3,100,101] Delamination of the AF due to excessive loading increased apoptosis of outer annulocytes and it has been suggested that annulocyte apoptotic responses may be associated with excessive cell stretching of annulocytes with limited capacity to reorient and thereby minimize the strain from loading.[102,103] Furthermore, aging of IVD tissues is known to induce aberrant cellular responses to injury, including cellular senescence and dysregulated signaling, and lead to the loss of biological structure and

function,[104] which may affect healing processes. Young and old mice express similar patterns of anabolic cytokines, although differences in the degree of expression suggests different innate responsiveness as a function of age.[105] A more thorough characterization of the cellular injury responses and the time course of wound healing in the AF requires further elucidation.

There is extensive literature detailing impaired IVD healing after adult injury, and animal models of IVD injury have been investigated for several decades. The earliest studies of IVD injuries *in vivo* determined that annular injury produced similar characteristics to human spondylosis deformans, and gross morphological examination demonstrated the first evidence of AF fibrosis following injury.[106,107] Models of adult AF injury by using needle puncture of varying sizes result in an impaired, fibrotic healing response to the acute injury that can contribute to slow, progressive degeneration and altered IVD mechanical properties.[25,108–114] Such needle puncture injuries are known to result in permanent IVD defects, leading to loss of NP pressurization and accumulation of fibrotic tissue in the NP region, as well as disorganization of AF lamellae and deposition of fibrotic matrix in the puncture tract (Fig. 2). Puncture injuries are also speculated to be at least partly responsible for the accelerated IVD degeneration known to occur in humans following discography.[112,115]

Inflammation and recruitment of immune cells

After injury, activation of immune cells initiates a phagocytic and pro-inflammatory response to remove cell and tissue debris. Although inflammation is necessary to initiate healing, resolution of initial inflammation is required to initiate fibrosis and remodeling. Annular injury results in the recruitment of irregular cells (which are potentially immune cells) to the wound area that is sustained over time, prolonging the pro-inflammatory environment and promoting degenerative changes (Fig. 3). Multiple studies have identified immune cells after IVD injury and degeneration, including macrophages, T lymphocytes, and mast cells (Table 2), as well as important pro-inflammatory cytokine responses.[32,116,117] While some studies demonstrate a weak immune response in herniated tissue [118] and with induced AF injury in animal models,[119] repeated injury is known to increase that pro-inflammatory condition.[110,113,114,120,121] T lymphocyte and macrophage infiltration is not widely observed in AF injury models with herniation,[119] although this may be due to limited reporting since IVD cells are considered immunoprivileged in their healthy state, with few resident inflammatory cells. Similar injury models have demonstrated a poor ability of the AF to heal,[122,123] indicating that the weak immune cell response to AF injury without herniation may result in poor healing outcomes. Furthermore, *in vivo* studies have demonstrated that exposure of NP tissue to AF and to DRG increases macrophage recruitment,[124,125] indicating that NP tissue from herniation may be important but not necessarily required for immune cell recruitment following AF injury.

In humans, macrophages and mast cells have been shown to infiltrate into IVDs with age and degeneration, as well as with herniation.[118,126,127] Host IVD cells can be sensitized by chronic inflammatory signals and by the increased presence of macrophages that remain in pro-inflammatory and remodeling states and do not revert to anti-inflammatory healing states.[126] Clinical evidence also supports the hypothesis that bacterial infection and low-

grade discitis is a potential contributor to IVD degeneration.[128–130] Interestingly, endplate changes, recorded as modic changes on MRI, are commonly thought to involve fibrogenic and pro-inflammatory cross-talk between bone marrow and adjacent IVDs.[131] Regardless of what the initiator of IVD degeneration is, this inflammatory response, which is required for healing, can become chronic in the IVD, resulting in a frustrated healing state that may predispose the tissue to degeneration and painful conditions.[27,32,132]

Cell proliferation and granulation tissue formation

In the AF, granulation tissue forms at the outermost layers.[123,133,134] Following annular stab in canine IVDs, NP herniation results in the formation of a thin fibrous layer on the outermost layers, with no repair of the interior injury site observed at 20 weeks, while larger block defects resulted in the formation of a solid fibrotic plug throughout the defect.[122,134] Similar findings were reported in rabbit, goat, and porcine IVDs, where the most superficial aspect of the AF healed by rapid fibrosis following stab injury, whereas interior regions remained unrepaired even after long time periods.[114,133,135,136] In sheep, discrete annular tears or rim lesions without NP herniation resulted in peripheral granulation tissue 18 months after injury, with no bridging of the middle and inner portions of the lesion.[123] The presence of granulation tissue staining strongly for TNFα and advanced glycation end products has also been observed, suggesting that granulation tissue can accumulate as a result of disease as well as acute injury and that it is highly pro-inflammatory.[137] Furthermore, granulation tissue in human IVDs has been further characterized to contain macrophages as well as other cells that exhibit increased pro-inflammatory and hypertrophic markers.[126,138] The source of fibrotic cells in granulation tissue has not been identified.

The early dynamics of cell proliferation/apoptosis, and the contribution of neighboring tissues to AF repair and granulation tissue formation have not been fully defined for most of these injury models. However, poor repair of interior tissues suggest that intrinsic AF cell proliferation is likely minimal following injury. In mice, IVD cells experience a broad decline in proliferative capacity after 9 weeks of age.[139] Long-term, end-stage time points at 12 months in ovine IVDs also indicate there is little spontaneous cell recruitment to injured, untreated annular injury sites,[140] although definitive lineage tracing experiments have not been carried out.

Matrix remodeling

The remodeling phase of wound healing can occur over a period of years in humans and involves the deposition of type I collagen to replace type III collagen formed during initial wound healing responses.[98] Evidence from animal models of annular injury suggest that little matrix remodeling occurs once fibrotic tissue is formed. Indeed, fibrotic matrix can persist from 12 weeks to 25 months post-injury.[122,133] In sheep, annular lesions failed to heal and remained void of tissue up to 18 months post-injury, which may be more consistent with human pathology.[123] In addition to persistent scar formation within the defect site, the IVD also experiences slow and progressive degeneration after injury. In all animal models, regeneration does not occur, despite prolonged healing times.[108,122,123,141] Structural and histological assessments demonstrate evidence of fibrotic and incomplete healing for small injuries, and progressive fibrosis and degeneration in larger injuries.[25,109,113,114]

Lessons From Models of Mammalian Regeneration

Regenerative medicine is an evolving field that investigates the extent of innate healing capacity of tissues with the goal of understanding the cellular and molecular drivers of cellular, structural, and functional regeneration. Using this knowledge, tissues damaged due to injury, degeneration, or aging may potentially be restored to healthy, regenerated states. This tissue damage may result in the formation of fibrotic scarring, of which the origin and underlying signaling pathways remain largely unknown. Further research on this topic will be required to develop targeted therapies that prevent and/or reverse fibrosis. Regenerative mechanisms vary widely among different mammalian tissues,[142] thus indicating the importance of investigating tissue-specific healing and regeneration potential. In particular, the cellular mechanisms involved are highly tissue- and context-specific.[143]

In the context of designing strategies to restore AF structure and function following injury, understanding the limited capacity of the AF to regenerate has been a topic of many studies. The healing process may result in tissue regeneration, but may also result in non-regenerative, scar-mediated healing. Perfect regeneration of a tissue is generally defined as complete restoration of structure, composition, and function relative to the native, healthy condition. However, regeneration may occur to varying degrees of completeness. To overcome the limited capacity of the adult AF to heal via perfect regeneration, many repair strategies that aim to seal AF defects and deliver cells and factors to promote regeneration are under investigation.[3,4,8,100,144] Despite recent advances and approaches designed to overcome the adult AF's limited healing response and to promote regeneration, achieving perfect regeneration of structure, composition, and function remains difficult. Additionally, the gap in knowledge regarding the molecular pathways that regulate distinct AF cell phenotypes and the paucity of AF regeneration models presents a challenge for the development of cell-based strategies that improve AF healing and promote regeneration.

MSCs reside within the proteoglycan and non-collagenous protein-rich matrix of the IVD, surrounded by the fibrous and cartilaginous outer AF region.[140] The presence of MSCs within the IVD may implicate these cells in IVD healing, regeneration, and repair.[145] In clinical applications, MSCs injected directly into the IVD space resulted in improvements in IVD hydration and collagenous tissue formation.[146] In parallel, decreased structural degeneration-related parameters and patient-reported lumbar pain scores were also observed.[140,146] While these results are promising, it is important to note that the functional role of MSCs in healing and repair is not currently known. For example, MSCs may directly differentiate along IVD-specific lineages to replace damaged tissues or MSCs may indirectly modulate the local immune environment. Future investigation is therefore required to elucidate their direct and/or indirect roles in healing. Although true AF regeneration has yet to be achieved, the native AF has the potential for improved healing within certain contexts, such as in the neonatal mouse where IVDs heal via restored function following herniation injury.[81] Delivery of cells and factors to promote regeneration show promise in animal models, such as delivery of MSCs overexpressing Mohawk, a transcription factor important in regulating outer AF growth and homeostasis.[82] However, several challenges regarding clinically translatable and efficacious delivery remain unresolved. Despite the poor self-healing capacity of adult IVD, the presence of resident cells with stem/progenitor potential

poses an attractive target for regenerative therapies. Whether these cells can be activated to promote healing or regeneration remains an unanswered question. Additionally, the paucity of regenerative models presents a challenge for developing cell-based strategies that promote AF healing and restoration of function. Using such a model of intrinsic AF regeneration, key cells and signaling pathways associated with regeneration can provide a roadmap toward engineering successful repair strategies that mimic these biologic cues to improve healing of the human IVD.

Although there are many mammalian models for the repair of complex tissues (such as the IVD), the ability of these models to recapitulate the human condition is a constant challenge and limitation in terms of clinical relevance. The smaller size of some animal models limits the ability to directly compare healing responses that occur in the human anatomical environments; important considerations such as nutrient supply and metabolic activity may be highly dependent on tissue size. It is also worth noting that the IVDs of many animals do not naturally degenerate. This may be due to differing cellular compositions (such as presence of stem/progenitor pools that are absent in humans), biomechanical loading, size, and weight distributions as compared to humans.

Nonetheless, the neonatal mouse is an exciting mammalian model for regeneration of diverse tissues, including the heart,[147–151] cochlear hair cells,[152,153] and digit tip.[154–156] In the context of musculoskeletal tissues, the neonatal IVD [81] and neonatal tendon [157] show improved regenerative capacity relative to adult models. Cell mechanisms underlying neonatal regeneration appear to be tissue- and species-specific; for example, heart and tendon regeneration is driven by mitotically active, differentiated cardiomyocytes and tenocytes, respectively, while cochlear hair cell regeneration is driven by transdifferentiation of supporting cells, and the digit tip is driven by dedifferentiation and redifferentiation of lineage-restricted cells. Collectively, these neonatal murine models identify potential cellular mechanisms and provide a roadmap toward adult mammalian regeneration. In the context of the IVD, neonates demonstrated excellent healing of severe IVD injuries involving large AF defects and complete loss of NP.[81] After 8 weeks of healing, neonatal mouse IVDs restored full functional biomechanics and improved collagen organization surrounding the puncture tract. Nevertheless, even in this excellent healing model, there was incomplete repair and persistent collagen disruption. As a result, tissue engineering and other systems that emulate native AF structure are important to both enhance repair and to learn about cell behavior within these defined conditions.[11] Furthermore, it remains notable that even with the evolving knowledge about IVD healing processes and enhanced characterization of functional biomechanical and matrix changes following injury, there remains limited knowledge of the identity and function of annulocytes during the injury, repair and healing processes which represents an important open area of future investigation.

Summary

Understanding of AF cell biology during homeostasis and injury has advanced in recent years; however, there is a clear need for further work to validate previously published findings that are highlighted in this review. Understanding the phenotypes and functions of cells residing in the normal, healthy AF, as well as their behaviors following injury, is crucial

for developing cellular strategies to repair AF defects and treat IVD degeneration. Developmental processes may inspire reparative regeneration strategies, but developmental and regenerative mechanisms can be distinct, as developmental processes are not always recapitulated in reparative regeneration.[142] An improved understanding of development, injury responses, and healing processes can be used to inform repair strategies. Approaches that may be feasibly used to induce reparative regeneration include using knowledge of developmental stages to inform annulus repair, manipulating fibrotic scar formation (which typically interferes with regenerative outcomes), or designing biomimetic tissue-engineering constructs to replace injured tissues. Attempts at *in vitro* models to better understand developmental processes and develop repair strategies have been made and such models may be key in furthering knowledge of AF development and biology.[11]

Understanding AF injury and healing is particularly important because AF disruption permits neurovascular invasion, NP herniation, and/or biomechanical instability, which are commonly considered pain generators. The complex structure and composition of the AF, which provides it with excellent mechanical properties and high fracture resistance,[68] also makes it a challenging tissue to regenerate. Furthermore, multiple aspects of the microenvironment, including nutrition, oxygenation, acidity, and mechanics are known to influence AF cell behaviors and have been manipulated as part of attempts to develop AF repair strategies.[100] The microenvironment and the need to resist high spinal loads are critical factors that must be overcome to achieve successful regeneration and warrant further investigation.[100,158,159] To date, there is little information on basic AF cell biology, in particular regarding the cell types residing within the AF and their functional roles in maintaining healthy homeostasis, and the phenotypic shifts after injury or disease. Compared with classical wound-healing phases, the IVD injury response is characterized by unresolved inflammation and formation of granulation tissue that does not remodel over time, a condition that has been referred to as “frustrated healing.”[27] The initial inflammatory response of the IVD is limited compared to vascularized tissue, but increases and is maintained over time. This delayed and increased inflammatory response is a likely contributor to the formation of persistent, fibrotic granulation tissue and degenerative changes associated with limited matrix remodeling. In addition to inducing reparative regeneration processes, modulating the inflammatory response may be a potential strategy to prevent fibrosis and promote regeneration in the AF, and it warrants further investigation.

Direct identification of cells involved in AF injury and healing using more precise markers, as well as their cell proliferation responses following injury, remain important areas for future investigation. A major challenge for understanding IVD injury responses is that investigations probe injury responses at varied time points and across multiple species, making direct comparisons difficult. The use of murine models allows for genetic modifications and mechanistic probing of signaling pathways. There is wide availability of antibodies to detect phenotypic markers, which has been an important tool in the identification of the key cellular players of AF biology; however, the relevance of findings from murine models and the relevance of their biomechanical environment to the human condition often comes into question. When normalized for geometry, mechanical parameters such as the torsional stiffness of murine IVDs are comparable to human lumbar IVDs.[160] The mouse and rat lumbar and mouse tail IVDs have been shown to be the closest

representation of human lumbar IVD geometry in terms of IVD height, anterior-posterior width, and NP area.[161] However, small animal models have different nutrient and waste transport dynamics compared to humans, a distinct structural difference (more pronounced NP/AF border), and increased matrix water content.[162] The major differences in human and animal cells of the IVD is a potential limiting factor for the development of AF repair strategies; as our understanding of animal AF cell biology increases, the relevance to the human condition should be appropriately considered. However, an improved understanding of these responses may provide clues and targets to mitigate non-regenerative pathways or promote regenerative ones, which can then be incorporated into repair strategies to treat painful conditions associated with IVD herniation.

This review described multiple cell types in the IVD as well as several key areas that are open for further investigation. Four distinct types of AF cells reside in the normal, healthy AF: peripheral cells, outer annulocytes, inner annulocytes, and interlamellar cells. AF stem/progenitor cells and neuronal stem cells in healthy AF tissue have also been identified as being present in the IVD, yet their specific identity and roles during aging, injury, and degeneration are still undetermined. A limited number of investigations reported macrophage, T lymphocyte, and mast cell recruitment after injury and in surgical herniation tissue and IVD degeneration, with the suggestion that they may remain at the site or even promote pro-inflammatory activity of native IVD cells, yet the responses of intrinsic annulocytes and AF stem/progenitor cells to these extrinsic cells are unclear. The cellular responses to IVD injury and healing involve inflammation and recruitment of immune cells, cell proliferation and granulation tissue formation, and matrix remodeling. The very few studies investigating normal AF cellular phenotypes and their responses to injury suggest granulation tissue remains, with limited matrix remodeling and the development of chronic inflammatory states. Neonatal mice demonstrate excellent regeneration in various tissues, and recent research suggests that neonatal AF healing may provide a potential roadmap to promote IVD regeneration with cell-based repair strategies. Consequently, further studies are required, including investigations of AF cell types and using tissue engineering and other regenerative medicine techniques to develop strategies that promote improved healing following AF injury. It remains an open question whether any model or regenerative medicine technique can overcome the high loads and harsh IVD microenvironment to enable reparative regeneration of the AF.

Acknowledgements

This work was supported by U. S. National Institutes of Health, National Institute of Arthritis and Musculoskeletal and Skin Diseases Grants R01 AR064157 (to JCI), and R01 AR069537 (to AHH).

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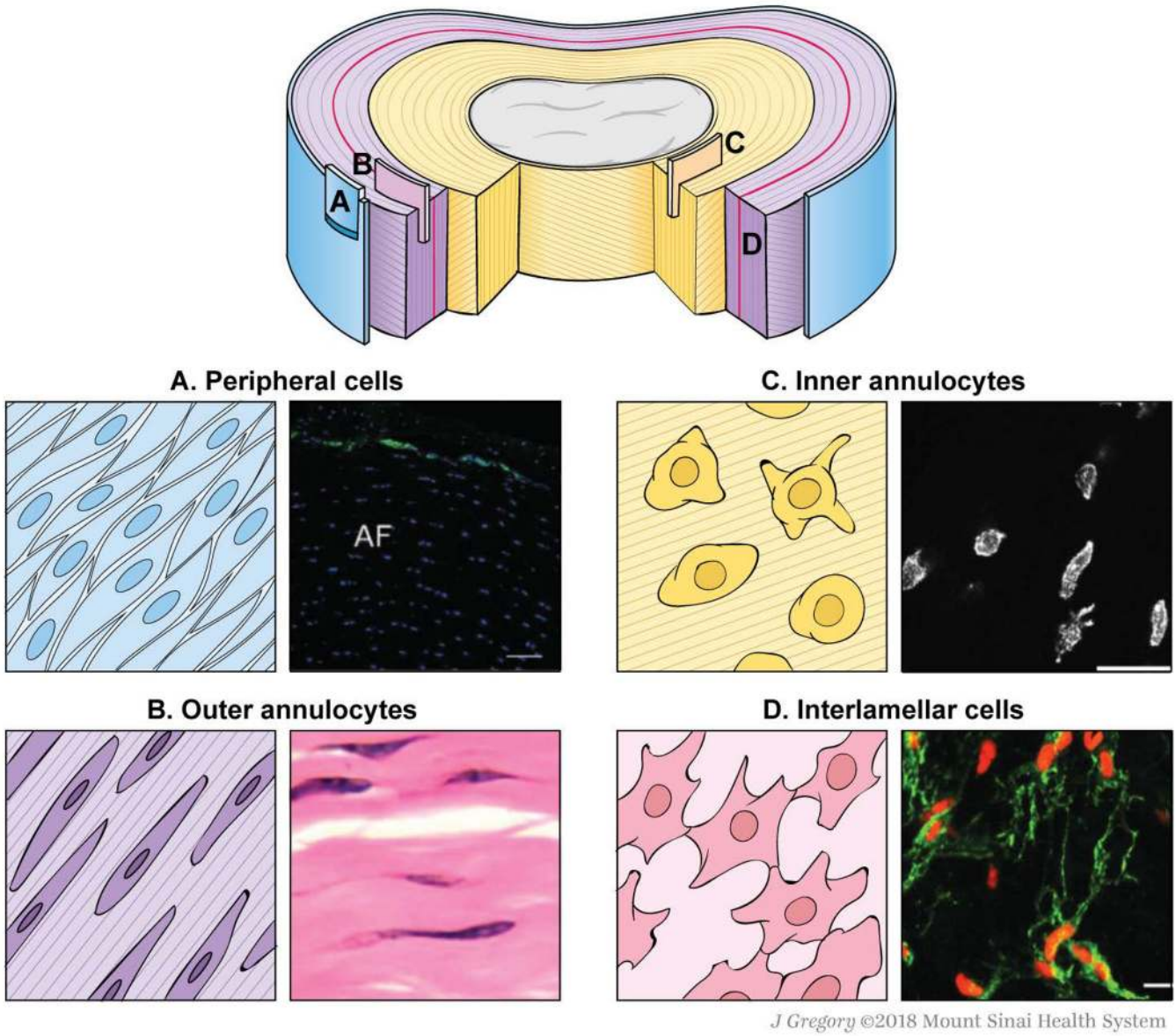

Figure 1.

Schematics and representative microscopic images of the different cell types and their locations in the healthy adult AF. (A) Peripheral cells.[88] (B) Outer annulocytes.[163] (C) Inner annulocytes.[12] (D) Interlamellar cells.[12]

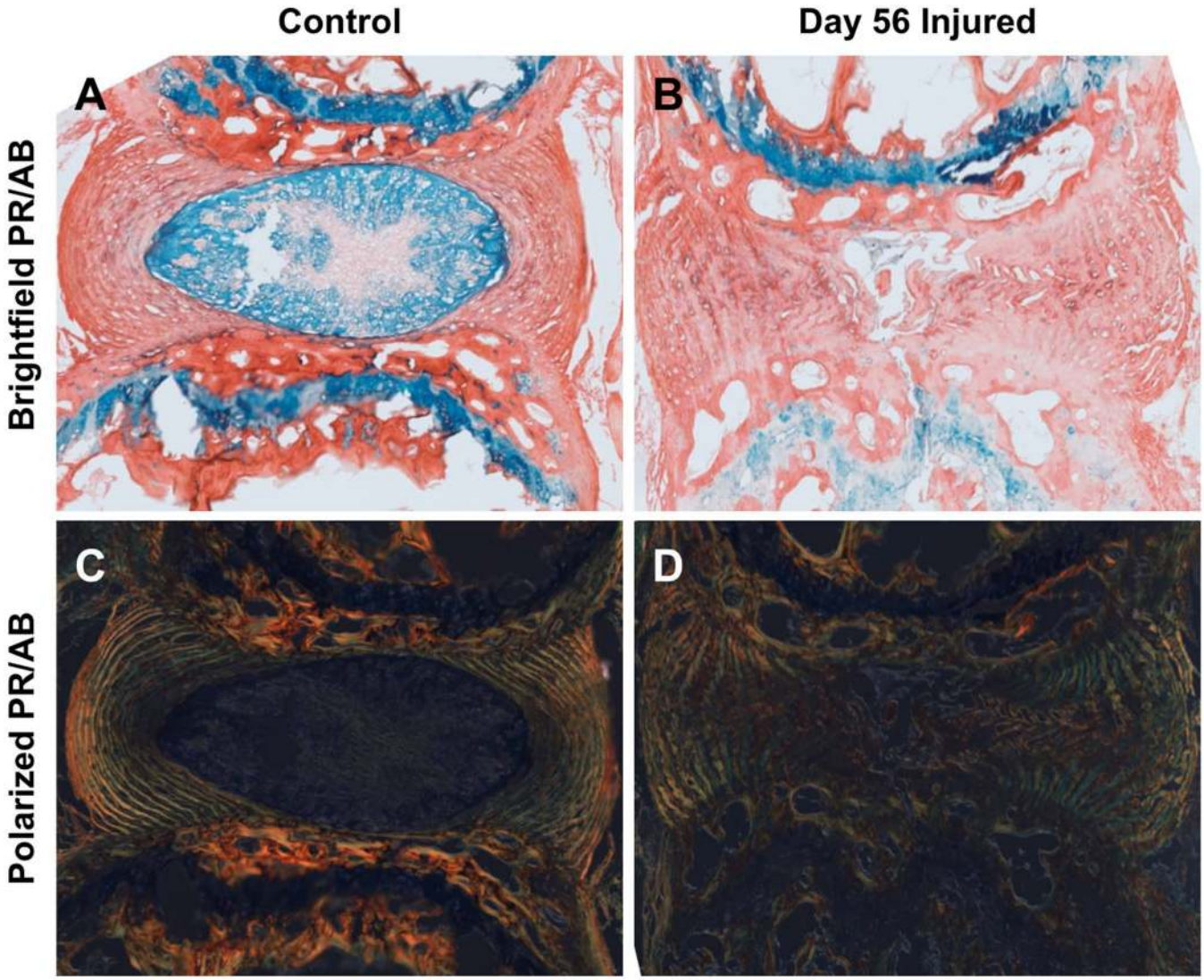

Figure 2.

The AF injury response in a mouse caudal IVD eight weeks following injury. Picrosirius red/alcian blue staining reveals uninjured control IVDs have an intact and proteoglycan-rich NP (A) and highly organized, collagen-rich AF as visualized under polarized light (C). Injury response 8 weeks after puncture consists of NP fibrosis and incomplete healing of puncture tract, with evidence of AF buckling, delamination, and fissures throughout the IVD, as shown by picrosirius red/alcian blue staining (B). Loss of AF lamellae organization is shown by decreased birefringence in polarized light imaging (D). 5 μm thick paraffin sections, 10X magnification.

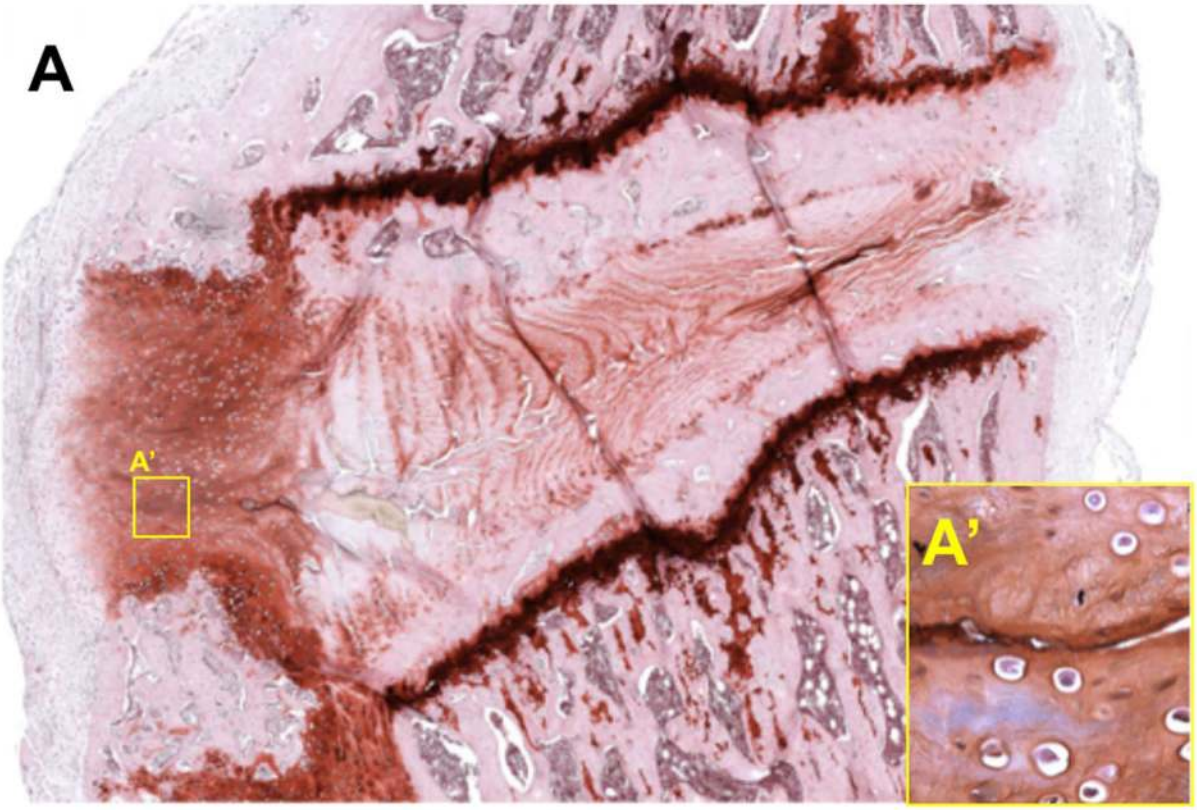

Figure 3.

Recruitment of unidentified and irregular cells in a punctured rat lumbar IVD six weeks following injury. Safranin-O staining reveals a dense, cellular cap surrounding the injury site on the dorsal aspect of a lumbar IVD (A). Rounded, unidentified cells that are potentially inflammatory cells remaining in the repair region over time are observed adjacent to the puncture tract (inset A' shows higher magnification of the corresponding region in panel A). 5 μm thick paraffin sections, 10X magnification.

Table 1.

Morphological, mRNA, and protein signatures characteristic of distinct AF cell populations residing within different AF regions.

Cell type		Morphology	Markers	Species	Location	References
Annulocyte	**Outer**	Elongated, fusiform, fibroblast-like	[*COL1A1*, *COL5A1*, *COL12A1*, *SFRP2*]	Human (14 years *)	Outer AF	78
			Collagen I > Collagen II	Human (27–52 years)	Outer AF	74
			-	Bovine (12–24 months)	Outer 20% of AF	12
		-	*COL5A1*	Rabbit	Outer AF	76
		-	*COL5A1, TNMD*	Human, bovine	Outer AF	79
		-	*Mkx*	Human, mouse	Outer AF	82
	Outer/Inner	-	*Scx*	Mouse	Outer AF (adult), entire AF (young)	80; 81
		-	Keratan sulfate	Human (69 years), rat (E15–21)	Outer and inner AF)	57; 77
	Inner	Rounded, chondrocyte-like	Collagen II	Human (27–52 years)	Inner AF	74
		Rounded, short processes	-	Bovine (12–24 months)	Inner AF	12
		Rounded, long processes	-	Bovine (12–24 months)	Inner AF/NP border	12
		Rounded	α-Smooth muscle actin	Canine (adult +)	Inner AF	86
		-	*Fmod*	Mouse (E15.5)	Inner AF	85
Interlamellar		Lace-like network	-	Bovine (12–24 months)	Interlamellar septae (outer AF)	12; 13
Peripheral		Elongated $	CD146, transgelin (SM22α)	Human, mouse (8–9 weeks)	Single-cell layer at outermost AF	88
AF stem/progenitor		-	Notch1, Delta4, Jagged1, C-KIT, Stro-1	Rabbit (3 months), rat (3 months), minipig (6 months)	AF border to ligament zone, perichondrium	91
		Small/spindle-shaped and polygonal	CD133, CD90, CD73, CD166, P75LNGFR	Human, rat	Unspecified AF	89
		-	CD29, CD49e, CD51, CD73, CD90, CD105, CD166, CD184, Stro-1	Human (adolescent)	Unspecified AF	90
		Varied (cobblestone or spindle-like)	Oct-4, nucleostemin, SSEA-4; CD29-, CD44-, CD166-positive; CD4-, CD8-, CD14-negative	Rabbit (6–8 weeks)	Unspecified AF	95
		-	CD166, C-KIT, Jagged	Rabbit (6 months)	Unspecified AF	96

[ ] collective mRNA signature;

* average of 2 donors;

+ age not specified;

$ from image

Table 2.

Inflammatory cells observed in the AF following injury include macrophages, T lymphocytes, and mast cells.

Cell type	Markers	Species	Injury type	Location	Time point	Reference
Macrophages	ED1	Rat	Herniation	Dorsal root ganglion	Day 3	124
	CD68	Pig	5 × 4 mm deep cuts in the center of the AF w/o herniation (L4/5, L5/6)	AF	1 month post injury	119
	CD68, Ber-MAC3	Human	Herniation (excess surgical tissue)	Herniated tissue	Early (time of operation)	118
	Morph-ology	Mice	Implantation of IVD tissue into peritoneum	AF and NP tissue	1, 3, 7 days (max at 7 days)	125
T lymphocytes	CD3	Pig	5 × 4 mm deep cuts in the center of the AF w/o herniation (L4/5, L5/6)	AF	1 week and 1 month after injury	119
Mast cells	Tryptase	Human	Herniation	Surgical tissue	Early (time of operation)	127