Eur Spine J (2012) 21 (Suppl 1):S10–S19
DOI 10.1007/s00586-012-2234-y

ORIGINAL ARTICLE

Ex vivo observation of human intervertebral disc tissue and cells isolated from degenerated intervertebral discs

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Received: 13 February 2012 / Accepted: 19 February 2012 / Published online: 7 March 2012
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Abstract
Purpose Disc degeneration, and associated low back pain, are a primary cause of disability. Disc degeneration is characterized by dysfunctional cells and loss of proteoglycans: since intervertebral tissue has a limited capacity to regenerate, this process is at present considered irreversible. Recently, cell therapy has been suggested to provide more successful treatment of IVD degeneration. To understand the potential of cells to restore IVD structure/function, tissue samples from degenerated IVD versus healthy discs have been compared.
Methods Discal tissue from 27 patients (40.17 ± 11 years) undergoing surgery for degenerative disc disease (DDD), DDD + herniation and congenital scoliosis, as controls, was investigated. Cells and matrix in the nucleus pulposus (NP) and annulus fibrosus (AF) were characterized by histology. AF- and NP-derived cells were isolated, expanded and characterized for senescence and gene expression. Three-dimensional NP pellets were cultured and stained for glycosaminoglycan formation.
Results Phenotypical markers of degeneration, such as cell clusters, chondrons, and collagen disorganization were seen in the degenerate samples. In severe degeneration, granulation tissue and peripheral vascularization were observed. No correlation was found between the Pfirrmann clinical score and the extent of degeneration.
Conclusion The tissue disorganization in degenerate discs and the paucity of cells out of cluster/chondron association, make the IVD-derived cells an unreliable option for disc regeneration.

Keywords Intervertebral disc · Degeneration · Annulus fibrosus · Nucleus pulposus · Cells

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Introduction

Degeneration of the disc, naturally occurring during aging and accelerated by environmental or genetic factors, is one of the major causes of low back pain (LBP). Indeed, the degenerative process of the disc begins as early as the second decade of life, with most of the lumbar discs showing some evidence of degeneration by the fifth decade [1, 2].

Conservative therapies are current treatments to relieve of pain. When such therapies are not effective, with persistent pain and dysfunction, or in case of further degeneration, an emerging approach is the cell-based regeneration of the intervertebral disc [3, 4].

Embryogenetic origin and structure of the intervertebral disc (IVD) are well known: it is macroscopically made of a fibrous coat (annulus fibrosus, AF) surrounding a gel-like

matrix with cells (nucleus pulposus, NP). Two thin end-plates of hyaline cartilage extend superiorly and inferiorly to interface with the vertebral bodies, and regulate nutrient diffusion. The fibrillar matrix is made up of tension-resistant collagen type-I, in outer annulus, and compression-resistant collagen type-II, in inner annulus, nucleus and end-plate [5].

Intervertebral disc is a relatively acellular environment, with $\sim$5,000 cells/mm^3 in the NP region and 9,000 cells/mm^3 in the AF region.

Nucleus pulposus cells are rounded with a chondrocyte-like phenotype, while AF cells are thin, elongated and aligned with the collagen fibers [6].

It has been shown that phenotype and morphology of both cell types is interchangeable, dependent on culture conditions: NP cells in monolayer become fibroblastic, decrease type-II collagen and increase type-I collagen production. Conversely, AF cells cultured in alginate increase type-II collagen production and chondrocyte differentiation factor SOX-9 [7].

The disc is the largest avascular tissue in the body, and the cells are nourished by diffusion through the matrix. Therefore, the NP matrix is rich in glycosaminoglycans (GAGs) and highly hydrated to support cell viability and to resist mechanical loads transferred through the AF. The rare NP cells are located within a glycan-rich niche, like cartilage cells, express chondrocyte markers, including SOX-9, collagen type-II and aggrecan, and are able to withstand severe conditions, such as hypoxia. Unfortunately, due to its avascular nature, the disc has a very low self-repair ability. The cell population changes dramatically with age, with a progressive loss by apoptosis of the large notochordal cells in the NP, and a shift to small sparse chondrocyte-like cells; since the composition of the matrix is determined by the cells within it, there is a net loss of large matrix proteoglycans, and a change in collagen secretion.

Severe disc degeneration is associated with increased vascularization and innervation of peripheral tissue, changes of extracellular matrix (ECM) composition, appearance of cell clusters, and chondrons.

Summarizing, changes in ECM composition, swelling, and paucity of NP cells are mutually influencing processes, which progressively cause disc degeneration.

Thus, the characterization of the cells and matrix in the degenerate IVD tissue, in comparison with healthy discs, is useful in order to understand/predict the feasibility of a cell therapy to restore IVD structure/function. Several tissue engineering (TE) protocols of the disc have been developed, and the use of mesenchymal cells (MSC) has been shown to be promising, but MSC introduction in the degenerate tissue should be accurately designed using TE strategies, because some adverse effects have been reported [8, 9].

This study is the first part of an investigation on the tissue features of IVD retrievals, and ability of disc cell to expand in vitro. Human IVD tissues were taken at surgery from patients suffering from LBP (degenerative disc disease, DDD) or DDD plus herniation following informed consent of the donors.

Disc tissue from patients undergoing surgery for scoliosis was used as control tissue [10]. The histology of degenerate and 'healthy' discs was investigated to describe cellularity and matrix organization, whereas the disc-derived cells were cultured and assayed, with a view to using one or other of these cells in future experiments.

Materials and methods

Following institutional review board approval and patient informed consent, tissue specimens of human IVD were obtained from 27 patients (40.17 $\pm$ 11 years) undergoing surgery at the Orthopedic-Trauma Spine Surgery Dept. of the Istituto Ortopedico Rizzoli, due to DDD, or DDD plus herniation of the disc or congenital scoliosis (Table 1).

Tissue collection

Separated AF and NP tissues, collected in Dulbecco's modified minimum essential medium (D-MEM), were dissected, and a $\sim$50 mg-fragment of each tissue fixed and stained for histology, while another part was finely minced and seeded in separate 75 cm^2 flasks for cell expansion.

Histology

After AF and NP fixation and embedding, 5 μm-thick sections were cut. Alcian Blue, 1% in 0.1 M HCl, pH 1.0, for 1 h, and Toluidine blue, 0.04% in 0.1 M sodium acetate buffer, pH 4.0, for 10 min, were used for the glycosaminoglycan-rich matrix. Alcian blue–Sirius red–hematoxylin (ASH) were combined: after nuclear staining (Weigert-haematoxylin, 10 min), proteoglycans were stained with Alcian blue (0.5% in 1% acetic acid, 5 min), followed by molybdophosphoric acid (1%, 20 min) and Sirius-red F3B (0.1% in 30% picric-acid, 1 h) for collagen.

AF and NP cells

AF and NP fragments were cultured with D-MEM containing 10% FBS, and 1% antibiotic/antimycotic. Cells were expanded for 3–4 weeks: at confluence, 3 $\times$ 10^5 NP cells were centrifuged at low speed and pellets were cultured in loosely capped tubes at 37°C, 5% CO_2, with Alamar-Blue assay used for cell proliferation. At the end of cell proliferation, pellets were fixed and stained with toluidine blue for the matrix production by 3D-arranged cells.

Table 1 Age and gender of the donors, diagnosis, Pfirrmann score and type of surgical intervention

code	Diagnosis	Surgery	Age	Sex	Pfirrmann
3319	DDD + herniation L5–S1	Discectomy	68	F	4
3330	DDD + herniation L4–L5	Post lumbar fusion	57	F	4
3332	DDD + herniation L5–S1	Discectomy	52	M	4
3335	DDD L5–S1	Ant lumbar fusion + disc prosthesis L5–S1	37	M	4
3340	DDD L5–S1	ant lumbar fusion + disc prosthesis L5–S1	33	F	5
3356	DDD + herniation L5–S1	Post lumbar fusion	26	M	4
3358	DDD + herniation L5–S1	Discectomy	45	M	4
3365	DDD L4–L5	Discectomy	39	F	4
3387	DDD L5–S1	Disc prosthesis L4–L5	45	F	5
3392	Congenital scoliosis	Disc prosthesis L5–S1	10	M	–
3421	DDD L5–S1	Ant lumbar fusion	29	M	4
3431	DDD L5–S1	Ant lumbar–sacral fusion	39	F	5
3462	DDD L4–L5	Disc prosthesis L5–S1	30	F	4
3535	DDD L4–L5/L5–S1	Disc prosthesis L5–S1	30	F	4/5
3539	DDD + herniation L4–L5	Disc prosthesis L4–L5	45	F	4
3541	DDD + herniation L4–L5	Disc prosthesis L4–L/L5–S1	24	M	4
3659	DDD L4-L5/L5–S1	herniectomy	30	F	4/5
3751	DDD L4-L5/L5–S1	Discectomy	43	F	4
3776	DDD L4-L5/L5–S1	Disc prosthesis L4–L5/L5–S1	54	F	4
3952	Congenital scoliosis	Ant thoraco-lumbar fusion	12	F	–
3979	DDD + herniation L4–L5	Discectomy	40	M	4
3990	DDD + herniation L4–L5	Discectomy	45	M	4
4024	Congenital scoliosis	Post thoraco-lumbar fusion	13	F	–
4029	Congenital scoliosis	Post lumbar fusion	10	F	–
4081	DDD L5–S1	Disc prosthesis L5–S1	32	F	4
4111	DDD L4–L5/L5–S1	Disc prosthesis L5–S1	40	F	4
4124	DDD L4–L5	Disc prosthesis L4–L5	41	F	3/4

DDD degenerative disc disease, *post* posterior, *ant* anterior

AF and NP monolayers were stained for β-galactosidase, i.e. an intracellular green-blue, insoluble precipitate, using the Senescence Detection Kit β-Gal (Calbiochem, QIA117) [11].

Gene expression

AF and NP gene expression was measured by real time PCR (Table 2). RNA was extracted from 1st and 2nd pass cells using RNeasy mini kit (Qiagen, GmbH, Germany), and cDNA obtained by MuLV Reverse Transcriptase (Applied Biosystems, CA, USA). 1 µg of cDNA was amplified with Light Cycler and Universal ProbeLibrary system (Roche Applied Science, Italy) [12].

Probes and primers were selected using a web-based assay software (ProbeFinder https://www.roche-applied-science.com) (Table 2). Housekeeping gene GAPDH was used as reference to normalize PCR data, expressed as a gene of interest/GAPDH ratio. A 'variation index' was calculated for each experiment and for each 'gene of interest/GAPDH' by relating the result of degenerate disc cells with that of healthy discs.

Results

Histology

Morphology of non-degenerate discs was observed in young scoliotic patients: the cells were smaller than in adults, cell clusters were absent and cartilage was hypertrophic. Regularly arrayed collagen-rich lamellae with fibro-chondrocytes were seen in the AF (Fig. 1).

In the AF/NP transition zone, type-I collagen fibers crossed diagonally each other, and cells were included within GAG-rich niches (Fig. 2). In the NP area single cells or islets of few chondrocyte-like cells surrounded by a capsule were seen among sparse collagen fibers. The cartilage endplate showed the typical amorphous matrix with sparse single and grouped chondrocytes (Fig. 3).

Table 2 List of primers and probes selected for real time PCR analyses

Gene, gene symbol, NCBI reference number		Primer sequence (5′-3′)	Probe	Detection limit (µg)
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (NM_002046.3)	Sense	agccacatcgctcagacac	#60	3e−12
	Antisense	gcccaatacgaccaaatcc		
Type 1 collagen, alpha 1 chain (COL1A1) (NM_000088.3)	Sense	cccctggaaagaatggagat	#60	3.7e−13
	Antisense	aatcctcgagcaccctga		
Type 2 collagen, alpha 1 chain (COL2A1) (NM_033150.2)	Sense	tctgggttccagggacttc	#79	2.2e−12
	Antisense	cagcttcaccgggaacac		
Aggrecan (ACAN) (NM_013227.2)	Sense	tcaccagtgaggacctcgt	#44	5.0e−11
	Antisense	ggcggtagtggaagacgac		
Homo sapiens SRY (sex determining region Y)-box 9 (SOX-9) (NM_000346.3)	Sense	gtacccgcacttgcacaac	#61	1.5e−12
	Antisense	tcgctctcgttcagaagtctc		

Vascularized granulation tissue was observed in severe DDD/disc herniation, with fibrotic NP, no NP/AF demarcation, and AF lamellar structure disrupted (Fig. 9)

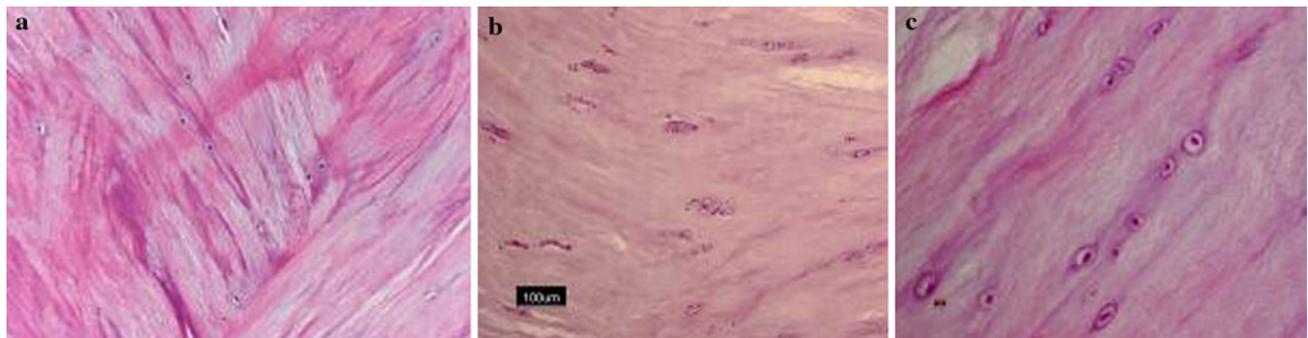

Fig. 1 IVD tissue from the control patients. **a** Inner annulus fibrosus with crossing fibers and rare cells (HE, ×4); **b** grouped chondrocyte-like cells (HE, ×10); **c** outer zone with single cells (HE, ×20)

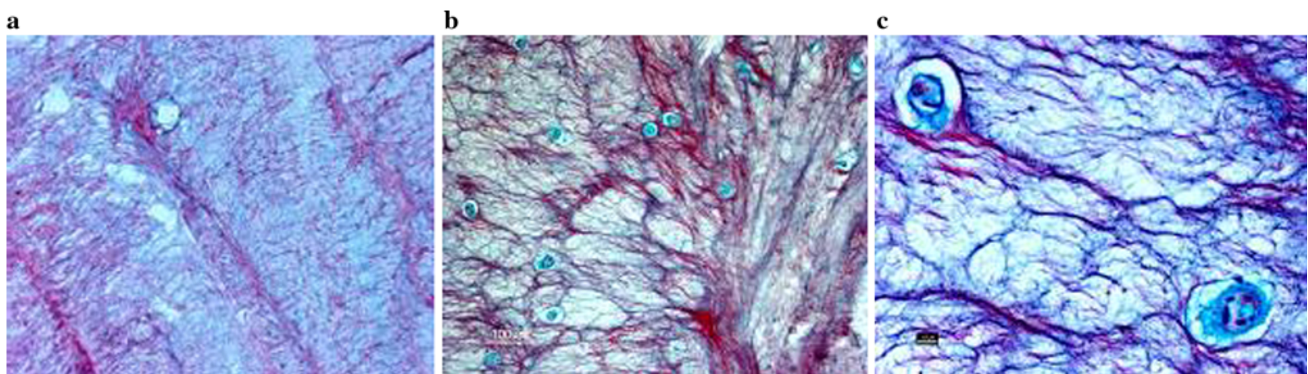

Fig. 2 IVD tissue from the scoliotic patients: **a** layers of collagen fibers in the AF zone (HE, ×4); **b** transition area (ASE, ×20); **c** in the NP area collagen fibers are stained *red* and cells are contained within a GAG-rich (*light blue*) niche (ASH, ×40)

Degenerate discs showed highly fibrotic lamellae and interlamellar cells with a flattened “pancake” morphology in the AF. Layers become more irregularly distributed with increased interbundle spaces (Fig. 4): the loss of distinct layers carries with it the inability for a sustained response to loads.

Chondrons, i.e. aggregates of three or more cells within a common territorial matrix whose frequency seems to be related to the grade of degeneration [13], were seen in AF and NP tissue of degenerate discs (Figs. 5, 6, 7, 8, 9).

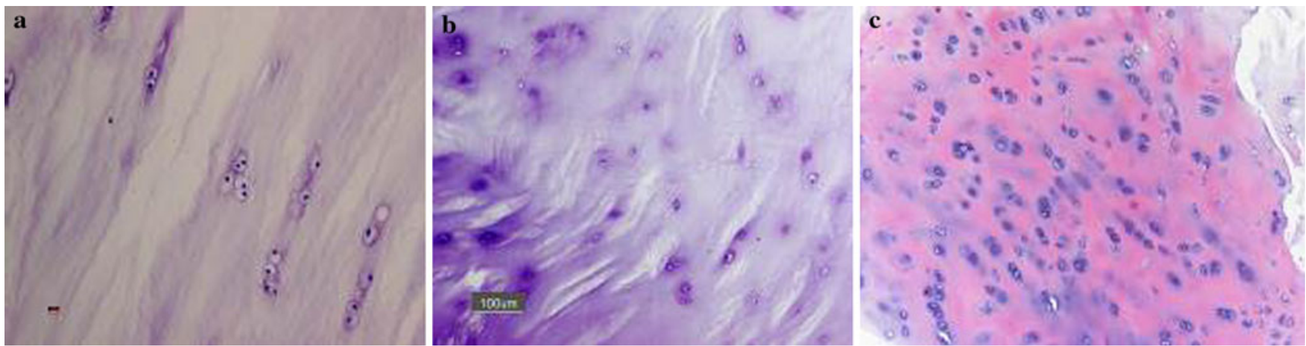

Fig. 3 IVD tissue from a scoliotic 10 year-patient: **a** NP cells are small in size (toluidine, ×20); **b** transition area between NP and AF with no cell cluster in a GAG-positive matrix (toluidine, ×10); **c** hyaline cartilage matrix with chondrocytes (HE, ×10)

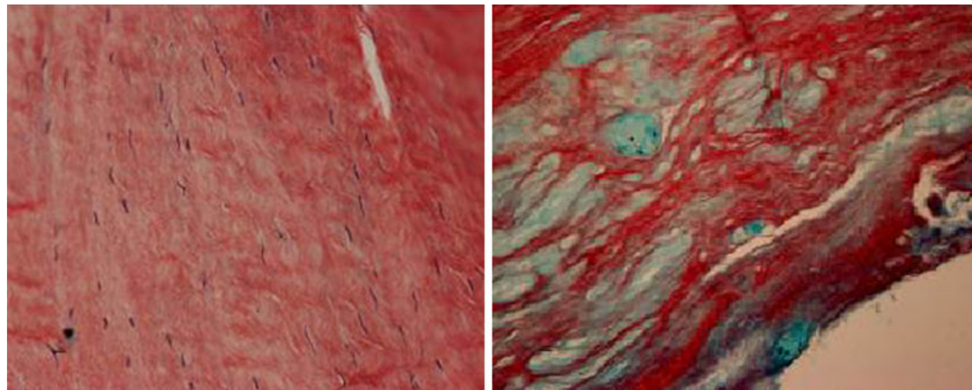

Fig. 4 Highly fibrous AF with fibroblast-like cells and a tear (*left* ASH, ×20), and with disorganized fibers, interlamellar GAG matrix (*light blue*) and chondrons (*right* ASH, ×40)

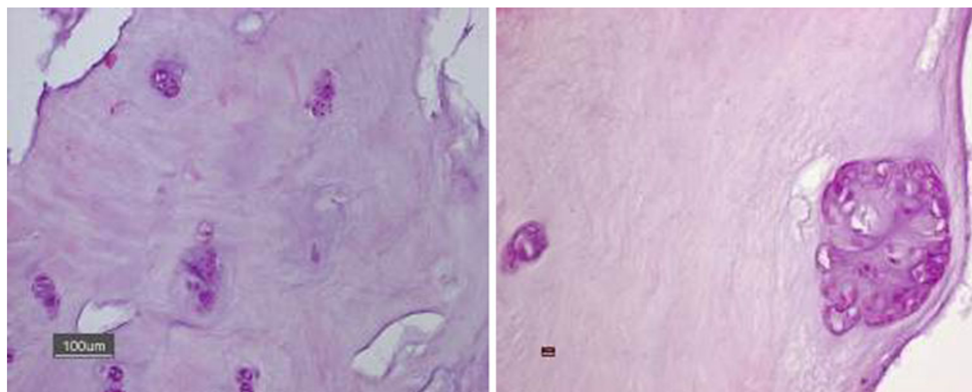

Fig. 5 Clusters of cells and tears (*left* HE, ×10) and a large chondron within NP tissue in a degenerate disc (*right* HE, ×20)

IVD cell culture

Cell outgrew after 3–4 days from AF and NP seeding in culture flask (Fig. 10).

After a lag phase (7–14 days) before proliferation, the mean time to confluence in 75 cm^2-flasks was 40.9 days for AF and 44.9 days for NP cells, independent from the donor age or sex.

Cells from DDD-patients reached the first confluence later than cells from young patients (NP: 42 ± 3 and 28 days, $p = 0.05$; AF: 35 ± 5 and 28 days, respectively). The two cell types were fibroblast-like, likely due to the 2D-culture (Fig. 10). Interestingly, cytochemical ALP was negative for AF cells, while ALP-positive cells were found in NP monolayers, confirming that some osteoprogenitors can be found in the NP tissue.

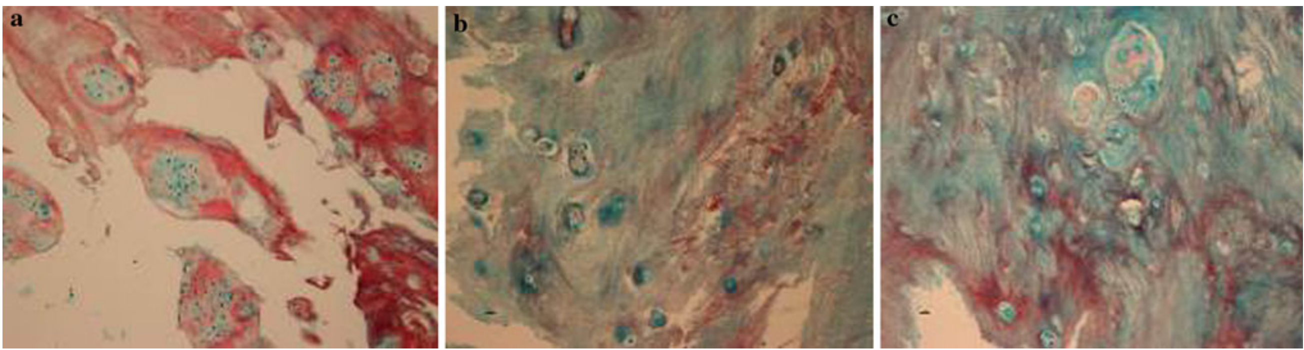

Fig. 6 Chondrons within AF (**a**, ASH, ×20) and NP tissue (**b**, **c**; ASH, ×10) in severely degenerate discs (45-year patient with DDD L4–L5, Pfirrmann score 5)

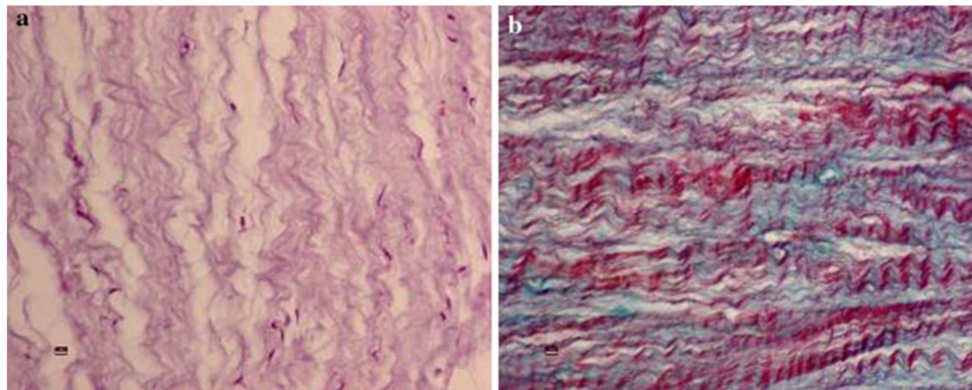

Fig. 7 IVD tissue from a 45 year patient with DDD + herniated disc. 'Waving' collagen bundles with elongated cells (*left* HE, ×20). Type-I collagen fibers are stained *red*, GAG matrix is *blue* and few round cells are *light blue* (*right* Alcian–Sirius–Hematoxylin, ×20)

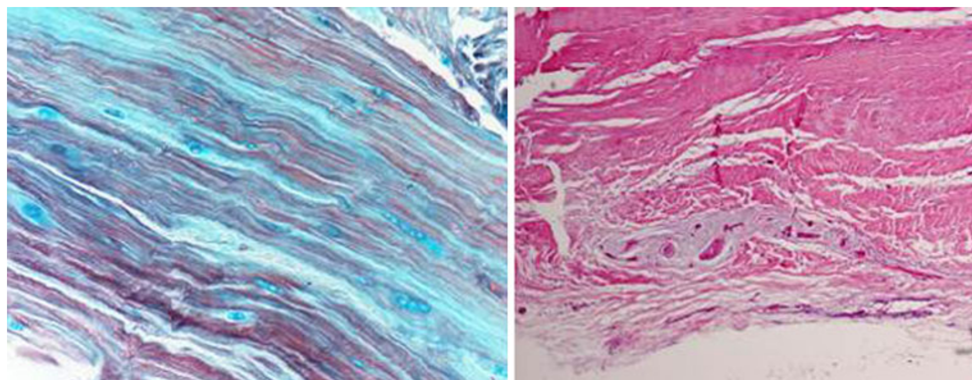

Fig. 8 AF tissue in degenerate discs. GAG-rich layers interpenetrate the lamellar structure of AF (*left* ASH, ×10); fibers derangement, tears and vessels in the outer annulus (*right* HE, ×4)

Beta-galactosidase is a biomarker of cell senescence, as senescent cells produce increased amounts of SA-β-Gal at pH 6.0. By L.M. no correlation was found between the frequency of positive cells and the severity of the score at diagnosis or the histological degeneration; the apparently increased number of senescent cells from pass 1 to pass 2/3 cells is due to the increased confluency (Fig. 11).

Cell viability in 3D pellet was measured by Alamar Blue assay: after 15–18 days cells stop proliferating, due to inability of oxygen and nutrients to reach the inner cells. With toluidine blue, cells in the 3D arrangement were shown to produce GAGs (Fig. 12).

Five NP and five AF from degenerate discs and 1 NP-1 AF from a young patient were tested for gene expression: a slight decrease of all transcripts was observed in AF cells of degenerate discs, but the changes were not significant (Fig. 13). Major changes were observed in NP: SOX-9 and ACAN were significantly decreased in degenerate disc

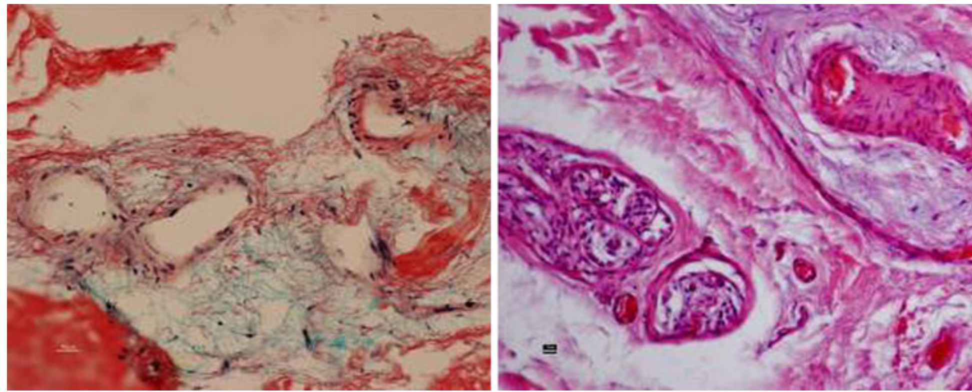

Fig. 9 IVD tissue from a 39 year-patient with grade 5-DDD. *Left* sparse cells in the NP zone (HE, ×20); *right* fibers disorganization and vascularization of the disc periphery (HE, ×20)

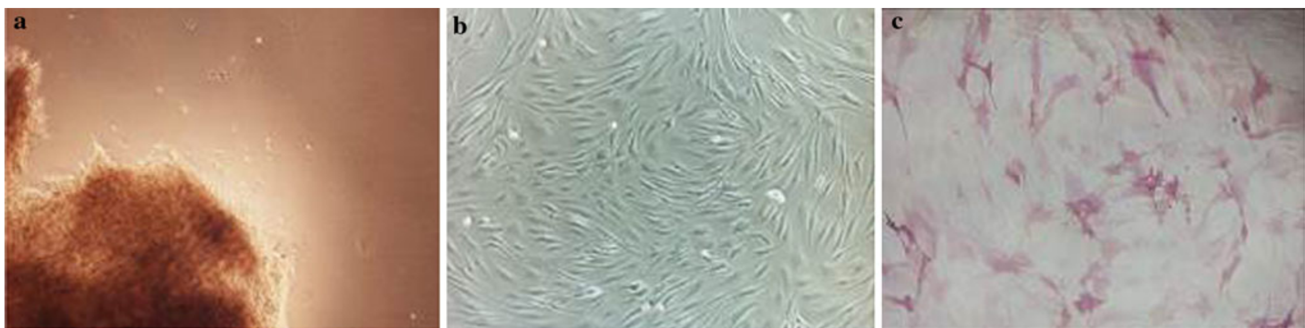

Fig. 10 IVD tissue in culture with D-MEM. **a** at 7 days from seeding cells spread from the tissue (×4); **b** AF cells at confluence (phase contrast, ×10); **c** NP cells at 14 days from seeding: alkaline phosphatase positive cells are *pink* (ALP, ×10)

Fig. 11 SA-β-gal senescence staining of AF cells from DDD + herniated disc (score 4) 1st pass (**a**), 2nd pass (**b**), and NP cells 2nd pass (**c**) (×10)

cells (unpaired t test: $p < 0.0001$, and $p = 0.005$, respectively), while COL2A1 expression was slightly diminished. On the contrary, COL1A1 was more expressed, but the increase was not statistically significant.

Discussion

Low back pain, a predominant cause of disability in adults, is associated with degeneration of the intervertebral disc. Among genetic, biologic and mechanical factors involved in the progress of disc degeneration, the loss of the gel-like consistency of the nucleus pulposus matrix, due to a decreased proteoglycan content, is recognized as a main factor [14]. This phenomenon progresses towards fibrosis of NP, altered transmission of the intervertebral forces, damage to the annulus fibrosus and various degenerative processes.

The purpose of this study was to understand the phenotypic features of a degenerative IVD tissue versus IVD tissue of young subjects. The more we understand the cell biology of discal tissue and discover the mechanisms

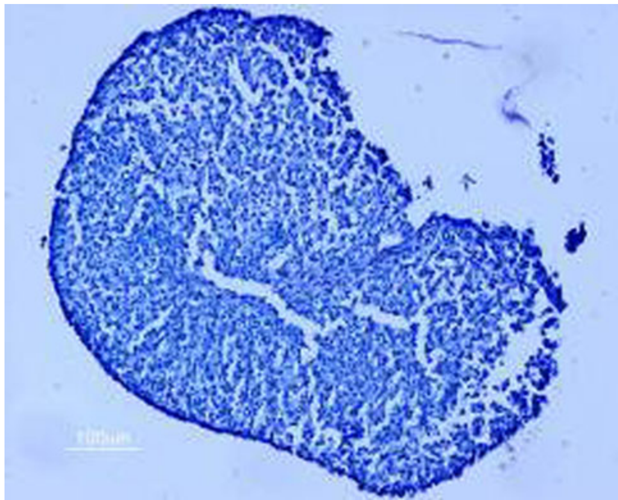

Fig. 12 Pelleted NP cells at 17 days: GAG matrix formation by 3D arranged cells (toluidine blue, ×10)

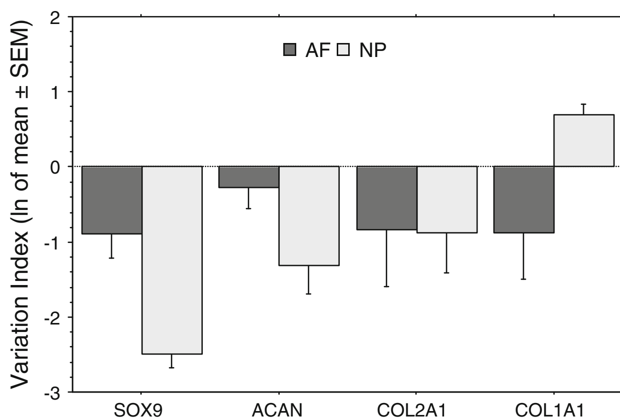

Fig. 13 Expression of genes encoding for markers of IVD. In the histogram the mean ± SEM of the variation indexes (logarithmic transformation), calculated for each experiment and for each 'gene of interest/GAPDH' by relating the result obtained in cells of degenerated disc with that of healthy disc

underlying degenerative processes, the greater is the potential to manipulate biological systems and intervene to prevent disease and pain in the future.

The normal IVD is hypocellular, which amplifies the effect of increased senescence and declining matrix production associated with disc degeneration [15].

During degeneration, and aging too, intervertebral disc cells undergo multiple changes, including altered cell phenotype and senescence.

Actually, in early degeneration, cell proliferation is upregulated in an attempt to combat progressive ECM loss. This was observed in our samples, where cell clusters and chondrons try to restore matrix synthesis and mechanical function. In our study, the proportion of clustered cells was similar in AF and NP of DDD samples, as well as in herniated tissues.

Being intervertebral disc avascular, it is distinctly different from other tissues. When there is an annular tear or injury, repair response activates from the vascularized AF outer layer to inner layer along the tear/injury, but if it occurs in the inner layer of AF or NP, the process of wound healing cannot be initiated [16]. This may account for NP fibrosis and loss of delineation between AF and NP observed in the latter stages of degeneration.

The ECM constituents are not static: though continually degraded by matrix metalloproteases, they are promptly refreshed by newly-synthesized components produced by cells: while cells constitute only 1% of the adult disc tissue by volume, their role in matrix synthesis and metabolic turnover is vital.

Intervertebral disc tissue and cells undergo continuous adaptive changes during aging, regardless of any concurrent injury, which may stimulate degeneration; senescent cells cannot divide, and the disc ability to generate new cells to replace those lost to necrosis or apoptosis is reduced. Moreover, if senescent cells accumulate over time, their metabolic products may contribute to pathologic changes seen in degenerating discs [17].

Other authors found a greater proportion of senescent cells in herniated than non-herniated discs, and more senescent cells in NP compared to AF [5]: this was not observed in our study, where a slight increase of β-gal positive cells was seen with cell passaging, indicating a certain grade of replicative senescence.

Annulus cells have shown to lose their phenotype during two-dimensional (2D) culturing. Chou et al. showed that up to passage two, both inner and outer annulus cells are not different from freshly isolated cells. At later passages, however, both cell types became indistinguishable fibroblast-like with similar type-I collagen expression and protein elaboration [18].

An altered gene expression in senescent cells was recently reported [19]; therefore, the expression of transcripts coding for IVD cell phenotypic markers was assessed in degenerate discs in comparison to healthy discs. Currently, there are no specific markers for NP cells, and they have often been considered as chondrocyte-like cells due to ECM similarity. NP matrix mainly contains aggrecan and type-II collagen, while AF cells prevalently produce type-I collagen; SOX-9 is one of the key-regulators of MSC differentiation to chondrocyte-like cells [20]. The increase in COL1A1 expression with a concomitant decrease in COL2A1 and ACAN is consistent with the IVD aging, and suggest that NP cells undergo dedifferentiation [21].

We are aware of the different behavior of IVD-derived cells when cultured in a 3D-arrangement versus 2D culture.

Protein synthesis of the key molecules produced by NP cells, including collagen II and aggrecan, were shown to be

reduced during monolayer culture. But NP cells in 3D were found to have an increased matrix protein synthesis with only limited cell proliferation, and matrix protein synthesis was re-induced in monolayer-expanded cells by further culture as a pellet, i.e. expanded NP cells maintain the functional integrity [22].

Better understanding of the biology of IVD degeneration and discogenic low back pain will likely lead to the discovery of biomarkers that may help diagnose LBP more accurately, thereby leading to more biologic and mechanistic management strategies.

Undifferentiated precursors, including ALP-positive connective-tissue precursors and notochordal cells, have been found in young subjects. Unfortunately, such precursors are lost with aging, therefore a cell therapy relying on them to drive disc regeneration is unreliable in old subjects. 'Biologics' have been already described some years ago as the future in spinal surgery, and more recently cell-based therapies with mesenchymal stromal cells have been suggested. The efficacy of MSC in inducing IVD regeneration has been shown in several ex vivo systems, either by induction of the endogenous NP cells to regain a non-degenerate phenotype through interactions between MSC and degenerate NP cells or by direct injection [23, 24]. Unfortunately, repair mechanisms in animal studies may differ compared to patients with disc diseases, due to different pathophysiological changes within the IVD and different biomechanics.

Nonetheless, the feasibility of engineering a functional spinal motion segment using biological therapies for degenerative disc diseases is under extensive research, and clinical application is foreseen [25].

Acknowledgment Excellent technical assistance for histology was provided by Michela Greco.

Conflict of interest None.

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