Gene Expression Profile Analysis of Human Mesenchymal Stem Cells from Herniated and Degenerated Intervertebral Discs Reveals Different Expression of Osteopontin

Article *in* Stem Cells and Development · September 2014

DOI: 10.1089/scd.2014.0282 · Source: PubMed

CITATIONS
8

READS
119

15 authors, including:

Giovanni Marfia
University of Milan
83 PUBLICATIONS **1,382** CITATIONS
SEE PROFILE

Stefania Elena Navone
Fondazione IRCCS Ca' Granda - Ospedale Maggiore Policlinico
69 PUBLICATIONS **1,356** CITATIONS
SEE PROFILE

Clara Di Vito
University of Milan
21 PUBLICATIONS **618** CITATIONS
SEE PROFILE

Silvia Tabano
University of Milan
134 PUBLICATIONS **1,771** CITATIONS
SEE PROFILE

Gene expression profile analysis of human mesenchymal stem cells from herniated and degenerated intervertebral discs reveals different expression of Osteopontin

Giovanni Marfia[1∞], Stefania Elena Navone[1,∞], Clara Di Vito[2], Silvia Tabano[3], Lorenzo Gianmattei[1], Andrea Di Cristofori[1], Roberta Gualtierotti[4], Monica Miozzo[3], Paolo Rampini[1], Manuela Caroli[1], Laura Riboni[2], Rolando Campanella[5]

∞ Co-First authors

[1]Laboratory of Experimental Neurosurgery and Cell Therapy, Neurosurgery Unit, Fondazione IRCCS Ca'Granda Ospedale Maggiore Policlinico, Milan, Italy;

[2]Department of Medical Biotechnology and Translational Medicine, LITA-Segrate, University of Milan, Italy;

[3]Division of Pathology; Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, University of Milan, Milan, Italy;

[4]Division of Rheumatology, Istituto Gaetano Pini, University of Milan, 20122 Milan, Italy

[5] Division of Neurosurgery, San Carlo Borromeo Hospital, Milan, Italy

Corresponding author

Dr. Giovanni Marfia

[1]Laboratory of Experimental Neurosurgery and Cell Therapy, Neurosurgery Unit, Fondazione IRCCS Ca'Granda Ospedale Maggiore Policlinico, Milano, Italy

Abstract

Gene expression analysis provides an effective methodology to identify clinically relevant genes implicated in intervertebral disc (IVD) pathology. The analysis of gene profile in mesenchymal stem cells (MSCs) of human herniated and degenerated IVD has not yet been investigated. We hypothesize that MSCs could be different in terms of extracellular matrix deposition and inflammatory cytokines depending on disc pathological condition.

Therefore, we perform a characterization of MSCs isolated from clinically categorized herniated (H-IVD) (n=10) and degenerated (D-IVD) (n=11) samples. H-IVD-MSCs and D-IVD-MSCs showed multipotent mesenchymal differentiation ability, expressing positivity for adipogenic, osteogenic, and chondrogenic markers with an immunophenotypical profile representative of MSCs. FACS analysis revealed higher expression for CD44, in D-IVD-MSCs compared to H-IVD-MSCs. Gene expression profile reveals that most genes under investigation displayed large variations and were not significantly different in D-IVD-MSCs versus H-IVD-MSCs. Conversely, the gene expression of Osteopontin (OPN), a protein involved in mineralization bone matrix and extracellular matrix destruction, was found 466 folds increased in D-IVD-MSCs versus H-IVD-MSCs and its protein expression was detectable only in D-IVD-MSCs. Moreover OPN protein levels were correlated with D-IVD severity. These findings suggest that abnormal expression of OPN, detected in D-IVD-MSCs, may play a pivotal role in the human pathogenic process. Moreover, the regulation of the OPN pathway could be a therapeutic target to counteract disc degeneration.

Keywords: intervertebral disc, degenerated, herniated, mesenchymal stem cells, osteopontin,

Introduction

Intervertebral disc (IVD) degeneration and IVD herniation are common spine disorders characterized by low back pain (LBP),[1] a debilitating and economically important condition.[2] Both herniation and degeneration processes are thought to involve sequential events that lead to the loss of cellular disc matrix, and to altered biomechanics.[3] Despite growing evidence suggest that, not only lifestyle, but also genetic predisposition plays a role as risk factor,[4] up to now the classification of herniated (H-)IVDs and degenerated (D-)IVDs is merely based on clinical observation and Magnetic Resonance Imaging (MRI) findings. Therefore, a better comprehension of the molecular events essential in the pathogenesis of IVD disease, could represent a critical step in the development of new diagnostic tools, avoiding an unambiguous diagnosis. In this regard, previous studies, focusing on molecular events and gene expression pattern in D-IVDs, revealed that the expression of metalloproteinases (MMPs), ADAMTS-7 and ADAMTS-12, were significantly higher in endplate cells of degenerative discs compared with those in non-degenerative discs.[5] In addition, the development of IVD degeneration seems to be associated to genetic polymorphisms in collagen I, collagen IX, collagen XI, aggrecan, extracellular matrix- degrading enzymes, inflammatory cytokines, such as interleukin (IL)-1, IL-6, and TNFα, Fas/FasL, and vitamin D receptors.[6] A recent population-based study also provided evidence for a heritable predisposition to developing symptomatic lumbar disc disease.[7] Despite this evidence, how such genetic predisposition and pathological changes will affect therapeutic outcomes are still unknown. The molecular characterization of both H-IVD and D-IVD human tissues, could increase our knowledge on these pathologies, allowing a correct and univocal diagnosis. However, evidence on a different molecular background in H-IVD and D-IVD is scarce. Recent studies identified different inflammatory cytokine expression profile in human tissues obtained from H-IVD and D-IVD. The authors

demonstrated that immunoreactivity for IL-4, IL-6, IL-12 and interferon-γ (INFγ) were modest in D-IVDs, although the expression was higher in H-IVDs, suggesting an involvement of these cytokines in the pathophysiological mechanisms of disc herniation.[8] Moreover, recently, in support of a putative role of inflammatory processes in IVD disease onset, we demonstrated that in D-IVDs a subpopulation of cells, named mesenchymal stem cells (MSCs), expressed neuro-inflammatory markers during differentiation.[9] Therefore, the possibility to isolate MSCs from both human H-IVDs and D-IVDs could elucidate the molecular pathological mechanisms involved in the alteration of the extracellular matrix. On these premises, the objective of this study was to compare the gene expression profile of human inflammatory cytokines and receptors in human MSCs isolated from D-IVDs (D-IVD-MSCs) and H-IVDs (H-IVD-MSCs).

Methods

Participant and specimens

The study protocol was approved by institutional ethics committee and patients' informed consents to the procedure were obtained. Specimens were collected from the lumbar IVDs (L4-L5 or L5-S1 regions) of 21 patients: 10 with H-IVD, and 11 with diagnoses of D-IVDs including spondylolisthesis and spinal instability, but without herniation. An anonymous clinical data sheet was obtained for each patient and the Thompson grade of each disc was recorded from MRI scan.[10] Donors with trauma history, neoplastic disease, or previous lumbar surgery were excluded from the study design (Table 1).

Establishment of H-IVD-MSCs and D-IVD-MSCs population in culture condition

After surgery, disc samples were collected and placed in a tube with Stem Cells Medium (SCM), supplemented with 10% of foetal bovine serum (FBS, Gibco, Grand Island, NY) and 1% penicillin and streptomycin solution (Sigma-Aldrich, Basel, Switzerland) as previously

described.[9] Subsequently, tissue sample was enzymatically digested for 6 hours in Dulbecco's phosphate buffered saline (D-PBS, Euroclone, Milan, Italy) supplemented with 0.25% Liberase Blendzyme2 (2.5 mg/ml, Roche Diagnostics, Indianapolis, IA), passed through a 100 µm cell strainer (Falcon, Becton Dickinson, Allschwil, Switzerland), to remove tissue debris, and centrifuged at 300g for 10 min. Human disc cells were seeded in 75cm^2 flask (2x10^4 cells/cm^2) and weekly detached from the plates using Tryple Select (Gibco). Cell cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. Cells were observed with an inverted phase-contrast microscope (Nikon Eclipse TE300, Nikon, Shinjuku, Tokyo, Japan), and images were acquired with a digital camera (Carl Zeiss Microscopy GmbH, München Germany).

Proliferation assays

Cells were seeded in 25 cm^2 flask with 3.5 mL of SCM (2x10^4 cells/cm^2). After 7 days of culture, cells were harvested, counted and seeded at the same initial density for next passage count. The total number of dead and viable cells was counted at each passage by Trypan Blue dye-exclusion assay in a Fuchs-Rosenthal chamber and the percentage of viability was assessed by the formula: (n viable cells/ n viable cells + n dead cells) x 100. Growth curves were obtained by counting the number of viable cells at each passage and by calculating the cumulative population at each passage.

Multipotent differentiation ability of H-IVD-MSCs and D-IVD-MSCs

H-IVD and D-IVD-MSCs were tested for their capacity to differentiate into the three mesodermal lineages, according to the minimal criteria suggested by Dominici *et al*.[11] Human Mesenchymal Stem Cell Functional Identification Kit (R&D Systems, Minneapolis, MN) was used to induce adipogenic, osteogenic, and chondrogenic differentiation according to the manufacturers' instruction, as yet previously done.[9] Briefly, cells were cultured in SCM in

presence of adipogenic or osteogenic supplements. To test *in vitro* chondrogenic differentiation ability a micromass pellet culture was performed in SCM in presence of chondrogenic supplement. After 21 days of culture, cells were fixed and stained with FABP-4, Osteocalcin, or aggrecan antibodies (R&D systems), to determine adipogenic, osteogenic, and chondrogenic differentiation ability, respectively. Moreover, to assess the expression of glycosaminoglycans (GAG), H-IVD and D-IVD MSCs differentiated toward chondrocytes were stained with Safranin O and Fast Green Solutions (Sigma-Aldrich). Nuclei were then counterstained with DAPI (1 mg/mL) (Chemicon, Millipore, Billerica, MA). Cells were viewed with a Zeiss Axiophot-2 microscope. Images were acquired by Axion Vision Software (Zeiss).

Immunophenotipic analyses

For each sample, $5x10^4$ cells were incubated with appropriate phycoerytrine (PE) or fluorescein isothiocyanate (FITC) conjugated antibodies to test the expression of a pattern of mesenchymal, hematopoietic, endothelial and immunological markers: CD14, CD34, CD45, CD44, CD73, (BD Pharmingen, San Jose, CA, USA), CD105 (AbDSerotec, Raleigh, NC, USA), CD90 (Millipore Temecula, CA, USA) and CD19 (Beckman Coulter Cassina de' Pecchi, Milano, Italy), as previously reported.[9]

RT^2 profiler analysis

H-IVD-MSCs (n=3) and D-IVD-MSCs (n=3) between passages 6-8 were used for molecular analysis. Briefly, total RNA was extracted by RNeasy kit (Qiagen, Valencia, CA, USA) and reverse-transcribed using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA), following manufactures' instructions. cDNA was then added to the RT2 SYBR Green qPCR Master Mix (SABiosciences, Frederick, MD, USA) and each sample was aliquoted in triplicate on the Human Inflammatory Cytokines & Receptors RT^2 Profiler PCR Array

(SABioscience). Real-time polymerase chain reaction was carried out on an ABI 7300 thermal cycler (Applied Biosystems, Foster City, CA 94404, USA) PCR-array data were analysed using the Free PCR Array Data Analysis Software provided by manufacturers (http://www.sabiosciences.com/pcrarraydataanalysis.php). Data normalisation was based on correcting all Ct values for the average Ct values of several constantly expressed housekeeping genes present on the array.

Western blotting

H-IVD and D-IVD MSCs passages 6-8 were lysed with RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris pH 8) in presence of a protease inhibitor cocktail (Sigma-Aldrich). Cell proteins were resolved by SDS-PAGE on 12% polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked in TBS-Tween20 0.1%/5% BSA using for 1 hour. After washing, membranes were incubated prior for 1 hour at room temperature with the anti-osteopontin antibody (Abcam, Cambridge, UK) diluted in TBS-Tween20 0.1%/1% BSA and then with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody for 30 min at room temperature. β-actin was used as loading control. Bound antibodies were visualized by ECL (SuperSignal West Pico Sensitivity Chemiluminescent Substrate), and membranes were exposed to Kodak Biomax films.

Statistical analysis

The results were expressed as mean ± SD of at least three replicates. One way Anova two-tailed test was used for all statistical analyses and performed with GraphPad Prism software, version 4.0. P values of less than 0.05 were considered to be significant.

RESULTS

Categorization of surgical specimens

Surgical IVD tissues from 21 patients were analysed for this study and categorized as representing H-IVD or D-IVD disease (Table 1). Each specimens of H-IVD was fully extruded through the posterior annulus at operation. Specimens of D-IVD were removed from discs that were neither herniated nor bulging, and so in structural continuity with the rest of the disc.

H-IVD and D-IVD MSC cultures show multipotent differentiation ability

MSCs were isolated from tissue samples obtained from H-IVDs and D-IVDs. When seeded, both cell types adhered on plastic and exhibited a fibroblastic-like morphology (Figure 1a and b). The number of viable cells, after each passage, was higher in H-IVD-MSCs (82.13±4.06%) with respect to D-IVD-MSCs (71.78±4.67%). Moreover, growth curves indicate that in both cell models the number of cells gradually increased (Figure 1d). Of note, H-IVD-MSCs showed a higher ability to proliferate than D-IVD-MSCs. To investigate the multilineage differentiation ability of expanded H-IVD and D-IVD MSCs, we performed *in vitro* in adipogenic, osteogenic, and chondrogenic differentiation. Our data reveal that after 3 weeks of adipogenic induction both cell types were positive to the intracellular lipid transport protein FABP4 (Figure 2a). Regarding osteogenic differentiation, H-IVD-MSCs and D-IVD-MSCs positive to osteocalcin staining, after 21 days in the appropriate culture medium (Figure 2b). Moreover, both MSCs were able to differentiate toward chondrogenic phenotype, as revealed by aggrecan staining (Figure 2c) and positivity to Safranin O and Fast Green staining, indicating GAG deposition (Figure 2d).

H-IVD and D-IVD MSCs express stem cell markers

To better characterize our cultures we investigated the antigenic phenotype by flow cytometric analysis in H-IVD-MSCs (Figure 3a) and in D-IVD-MSCs (Figure 3b). Our data demonstrate that both cell cultures showed a similar positivity to many markers common to

MSCs, including CD105, CD73, CD90, and CD44. Moreover, the H-IVD and D-IVD MSCs were negative for the pan-monocytic antigen CD14, the hematopoietic markers CD34 and CD45, and the pan-B-cell marker CD19. Noteworthy, the percentages of CD44 positive cells was higher in the D-IVD-MSCs than in the H-IVD-MSCs (Figure 3).

H-IVD and D-IVD MSCs differ in gene profile and OPN expression

Growing evidence suggests an important role of inflammatory processes in the pathophysiology of both H-IVD and D-IVD.[8] To date, up to now, the role of the different molecules involved in inflammation is not fully understood in IVD-MSCs. Therefore, we evaluated the expression profile of 84 key genes involved in mediating immune cascade reactions during inflammation. In our current analysis, only four Housekeeping genes (HPRT1, RPL13A, GAPDH and ACTB) were used for normalisation. Beta-2-microglobulin was not included, since it was significantly altered on the PCR-array. Applying the flagging criteria, the measurements obtained by the PCR-array were consistent between cell cultures and showed very small variation (Figure 4a and b). The results obtained reveal that the expression of most genes under investigation was not significantly different in both cell types. Nevertheless, some genes showed a different expression (Figure 4a). In particular, the chemokine genes CCL2, CCL7, CXCL12, and CXCL6 were down-regulated in D-IVD-MSCs compared to H-IVD-MSCs. On the other hand, the chemokine gene CCL25, the chemokine receptor genes CCR1 and CCR9, and the cytokines IL-37 and OPN were upregulated. Of note OPN expression was 466 times more expressed in D-IVD-MSCs than in H-IVD-MSCs (Figure 4b). Prompted by this finding, we evaluated the expression of OPN protein by immunoblotting in 10 MSCs isolated from 5 H-IVD and 5 D-IVD patients, showing different Thomson grades. Our data demonstrated that in all H-IVD-MSCs analysed, the expression of OPN protein was undetectable. On the other hand, all D-IVD-MSCs expressed OPN.

Furthermore, the expression of OPN were correlated with the Thomson grade (Tg) in D-IVD-MSCs (Figure 4c).

DISCUSSION

Lumbar disc degeneration is a multifaceted condition, in which aging,[12] genetic,[13] systemic, and toxic factors[14] play an pivotal role. Furthermore, IVD is often associated to LBP,[15] rendering this condition a invalidating and clinically relevant problem. Therefore, a better understanding of disc degeneration biology is critical to determine preventative and therapeutic measures for LBP. In this context, a large body of literature is focused on pathological IVD, however molecular studies comparing degenerated and herniated IVD are scarce. Although most studies primly compare IVD tissues, growing evidence suggests that in both H-IVDs and D-IVD are present MSCs. Therefore the study of MSC biology of H-IVDs and D-IVDs, could be of relevance to better elucidate tissue arrangements and the pathophysiological processes involved in IVD diseases. Risbud and colleagues first identified a proliferating population of skeletal progenitors within the nucleus pulposus (NP) and annulus fibrosus (AF) of moderately degenerated human IVDs. These progenitor cells expressed general stem cell markers, such as CD90 and CD105, and were able to differentiate *in vitro* into the three mesodermal lineages.[16] Consistent with these findings, our H-IVD-MSCs and D-IVD-MSCs showed a MSC immunophenotipic profile and, upon appropriate stimuli, expressed adipogenic, chondrogenic, and osteogenic markers, with similar expression in both cell types. These results are in accordance with our previous data on MSCs isolated from NP of human D-IVD.[9]

In the present study, we performed RT^2 Profiler PCR Array analysis of human D-IVD-MSCs compared to H-IVD-MSCs and we focused on human inflammatory cytokines and their receptors. Indeed, a large body of literature indicate that several cytokines are locally

increased in IVD diseases. [8,17] These molecules may contribute to the biochemical alterations, that exacerbate pathological differentiation of herniated or degenerated indwelling MSCs. Our data suggest for the first time that D-IVD-MSCs and H-IVD-MSCs display a similar expression profile of inflammatory cytokines and their receptors. However, D-IVD-MSCs, compared to H-IVD-MSCs, showed a significant over-expression of OPN, a protein involved in cell attachment[18] and calcification of mineralized tissue.[19] Since IVD is primarily avascular, the inflammatory cytokines and chemokines, produced by disc cells themselves, are probably not removed from the tissue. Therefore, we speculate that MSCs encounter a pro-inflammatory cytokine-rich milieu which may induce the synthesis of OPN exacerbating the unbalanced equilibrium of IVD. The overexpression of OPN in D-IVD-MSCs may accelerate degenerative changes via mineralization-inducing conditions, as previously recognized in annulus fibrosus cells.[20] Moreover, our findings reveal that at protein level, the expression of OPN appears to be correlated with the Thomson grade in D-IVD-MSCs, hence representing a potential marker of IVD degeneration. It is well known that OPN acts interacting with CD44, an important mediator in chondrocyte cell-matrix interactions that involve proteoglycan-hyaluronan-link protein aggregates.[21] Noteworthy, our results show a higher expression of CD44 in D-IVD-MSCs compared to H-IVD-MSCs. Therefore, we hypothesize that higher levels of OPN and CD44 can stimulate different signal transduction pathways exacerbating ECM degeneration or their mineralization process. Indeed, it has been reported that OPN induces tissue mineralization through CD44 interaction and, alternatively, after integrin binding may prompt disc matrix degradation via MMPs activation, like MMP-13.[22] Thus, our results demonstrate for the first time that the expression of OPN from indwelling MSCs, may reflect responses to the IVD microenvironment during degeneration process, suggesting that OPN could function as a biochemical marker to determine IVD

disease severity. Further research are needed to clarify the mechanisms through OPN acts in promoting tissue mineralization or alternatively ECM degradation in human IVD. A better understanding of OPN pathway could allow to sub-categorized IVD disease and to identify new target approaches in disc degeneration therapy.

ACKNOWLEDGMENT

This work was supported by IRCCS Foundation Ca'Granda Ospedale Policlinico Milan, Italy and University of Milan, Italy. The authors declare that they have no conflict of interest.

References

1. Luoma K, Riihimaki H, Luukkonen R, et al. 2000. Low back pain in relation to lumbar disc degeneration. Spine 25: 487-449.

2. Krismer M, van Tulder M. 2007. Low back pain (non-specific). Best Pract Res Clin Rheumatol 21: 77-91.

3. Buckwalter JA. 1995. Aging and degeneration of the human intervertebral disc. Spine (Phila Pa 1976) 20: 1307-1314.

4. Wu N, Chen J, Liu H, et al. 2014. The involvement of ADAMTS-5 genetic polymorphisms in predisposition and diffusion tensor imaging alterations of lumbar disc degeneration. J Orthop Res 32: 686-694.

5. Zhang Q, Huang M, Wang X, et al. 2012. Negative effects of ADAMTS-7 and ADAMTS-12 on endplate cartilage differentiation. J Orthop Res 30: 1238-1243.

6. Kalb S, Martirosyan NL, Kalani MY, et al. 2012. Genetics of the degenerated intervertebral disc. World Neurosurg 77: 491-501.

7. Patel AA, Spiker WR, Daubs M, et al. 2011. Evidence for an Inherited Predisposition to Lumbar Disc Disease. J Bone Joint Surg Am 93: 225-229.

8. Shamji MF, Setton LA, Jarvis W, et al. 2010 Proinflammatory cytokine expression profile in degenerated and herniated human intervertebral disc tissues. Arthritis Rheum 62: 1974-1982.

9. Navone SE, Marfia G, Canzi L, et al. 2012. Expression of neural and neurotrophic markers in nucleus pulposus cells isolated from degenerated intervertebral disc. J Orthop Res 30: 1470-1477.

10. Thompson JP, Pearce RH, Schechter MT, et al. 1990. Preliminary evaluation of a scheme for grading the gross morphology of the human intervertebral disc. Spine (Phila Pa 1976) 15: 411-415.

11. Dominici, M, Le Blanc K, Mueller I, et al. 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8: 315-317.

12. Videman T, Gibbons LE, Battie MC. 2008. Age- and pathology-specific measures of disc degeneration. Spine (Phila Pa 1976) 33: 2781-2788.

13. Battie MC, Videman T, Levalahti E, et al. 2008. Genetic and environmental effects on disc degeneration by phenotype and spinal level: A multivariate twin study. Spine (Phila Pa 1976) 33: 2801-2808.

14. Hadjipavlou AG, Tzermiadianos MN, Bogduk N, Zindrick MR. 2008. The pathophysiology of disc degeneration: A critical review. J Bone Joint Surg Br 90: 1261-1270.

15. de Schepper EIT, Damen J, van Meurs JBJ, et al. 2010. The association between lumbar disc degeneration and low back pain: The influence of age, gender, and individual radiographic features. Spine (Phila Pa 1976) 35: 531-536.

16. Risbud MV, Guttapalli A, Tsai TT, et al. 2007. Evidence for skeletal progenitor cells in the degenerate human intervertebral disc. Spine (Phila Pa 1976) 32: 2537-2544.

17. Specchia N, Pagnotta A, Toesca A, et al. 2002. Cytokines and growth factors in the protruded intervertebral disc of the lumbar spine. Eur Spine J 11: 145-151.

18. Azari F, Valli H, Guerquin-Kern J, et al. 2008. Intracellular precipitation of hydroxyaptite mineral and implications for pathological calcification. J Struct Biol 162: 468-479.

19. Van Dijk S, D' Errico JA, Somemen MJ, et al. 1993. Evidence that a non-RGD domain in rat osteopontin is involved in cell attachment. J Bone Miner Res 8: 1499-1506.

20. Nosikova Y, Santerre JP, Grynpas MD, Kandel RA. 2013. Annulus fibrosus cells can induce mineralization: an in vitro study. Spine J 13: 443-453.

21. Knudson W1, Loeser RF. 2002. CD44 and integrin matrix receptors participate in cartilage homeostasis. Cell Mol Life Sci 59: 36-44.

22. Lund SA, Giachelli CM, Scatena M. 2009. The role of osteopontin in inflammatory processes. J Cell Commun Signal 3: 311-322.

Figure Legends

Figure 1. H-IVD-MSCs and D-IVD-MSCs proliferative characterization

Phase contrast representative images of H-IVD-MSCs (a) and D-IVD-MSCs (b) after 6 passages of culture. (c) H-IVD and D-IVD MSCs, were plated at the same density ($2x10^4$ cells/cm^2) and after 7 days of culture the number of viable cells was evaluated. Similar data were obtained at different cell passages. (d) Cells were plated as above, counted at each passage, and cumulative population was calculated. Data are mean±SD **, $p < 0.01$, D-IVD-MSCs versus H-IVD-MSCs.

Figure 2. H-IVD-MSCs and D-IVD-MSCSs multipotentiality

MSCs were maintained under adipogenic (a) or osteogenic (b) inducing media for 21 days. After this period, they were fixed and stained with FABP-4 to identify the lipid droplets (a),

and with Osteocalcin to reveal extracellular calcium deposits (b). Pellet cultures formed by MSCs under media with chondrogenic inducing factors for 21 days showed positive expression for (c) Aggrecan and (d) Safranin-O Fast Green staining.

Figure 3. Immunophenotypic characterization

MSCs isolated from H-IVD (a) and D-IVD (b) were investigated for surface marker expression using flow cytometry. Cells were stained with monoclonal antibodies conjugated with fluorescent dies: CD14, CD19, CD34, CD45, CD73, CD90 and CD105. For each profile, 10^4 events were acquired. Black lines on histograms graphs represents isotype controls.

Figure 4. H-IVD and D-IVD MSCs molecular characterization

Human Inflammatory Cytokines & Receptors RT² Profiler PCR Array was performed on cDNA of 3 H-IVD-MSCs and 3 D-IVD MSCs between passage 6-8, according to manufacturers' instructions. Panel (a) shows the expression profile of the 84 genes analysed. Spots in grey identify the genes with a similar expression, spots in red those upregulated, and spots in green those downregulated in D-IVD-MSCs versus H-IVD-MSCs. Upper panel (b) represents the genes upregulated (red), lower panel (b) represents those downregulated (green). Data are expressed as fold expression of D-IVD-MSC genes with respect to H-IVD-MSC genes. (c) Cell lysates (40 μg of proteins) of 5 H-IVD-MSCs and 5 D-IVD-MSCs showing a different Thompson grade (Tg) were analyzed by immunoblotting with anti-OPN and anti-β-actin antibodies. The immunoblottings are representative of one out of three.

Table 1. Clinical details of the two groups of IVD patients

	Herniated discs (H-IVDs)	**Degenerated discs (D-IVDs)**
Disc samples (n)	10	11
Mean age (yrs) (range)	39 (25 to 56)	43 (26 to 79)
Gender		
Male (n)	7	4
Female (n)	3	7
Spinal level		
L4/L5 (n)	2	3
L5/S1 (n)	8	8
Thompson grade		
Grade 3 (n)	7	4
Grade 4 (n)	2	6
Grade 5 (n)	1	1

Figure 1

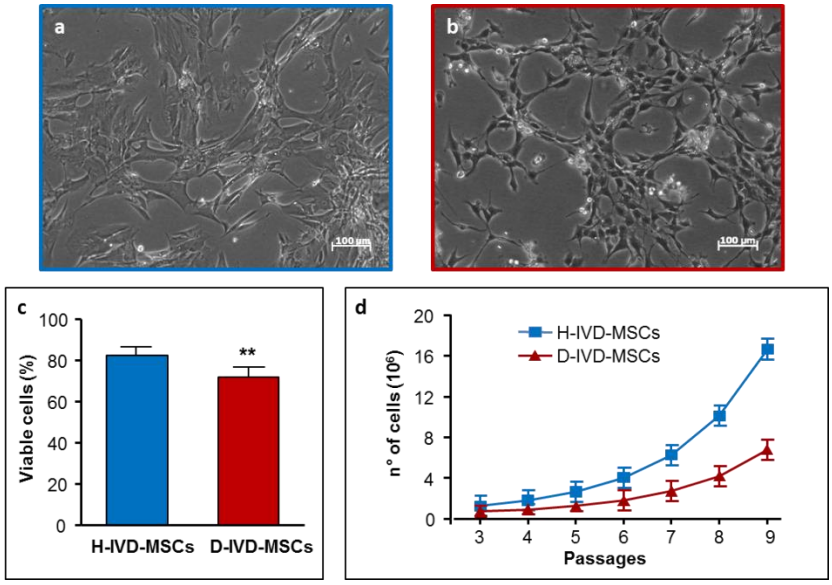

Figure 2

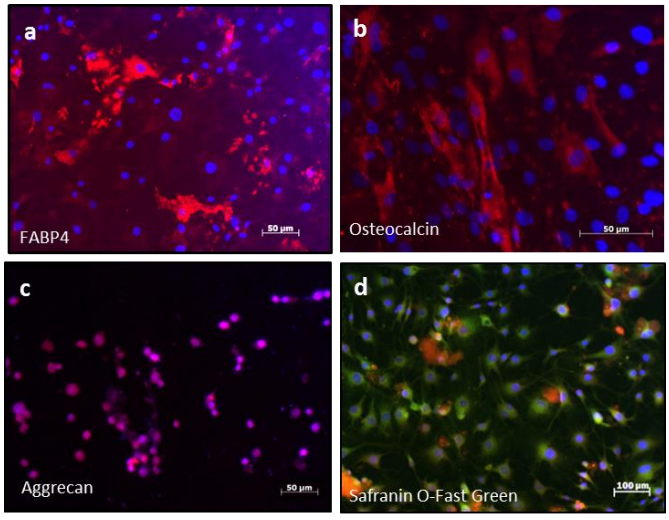

Figure 3

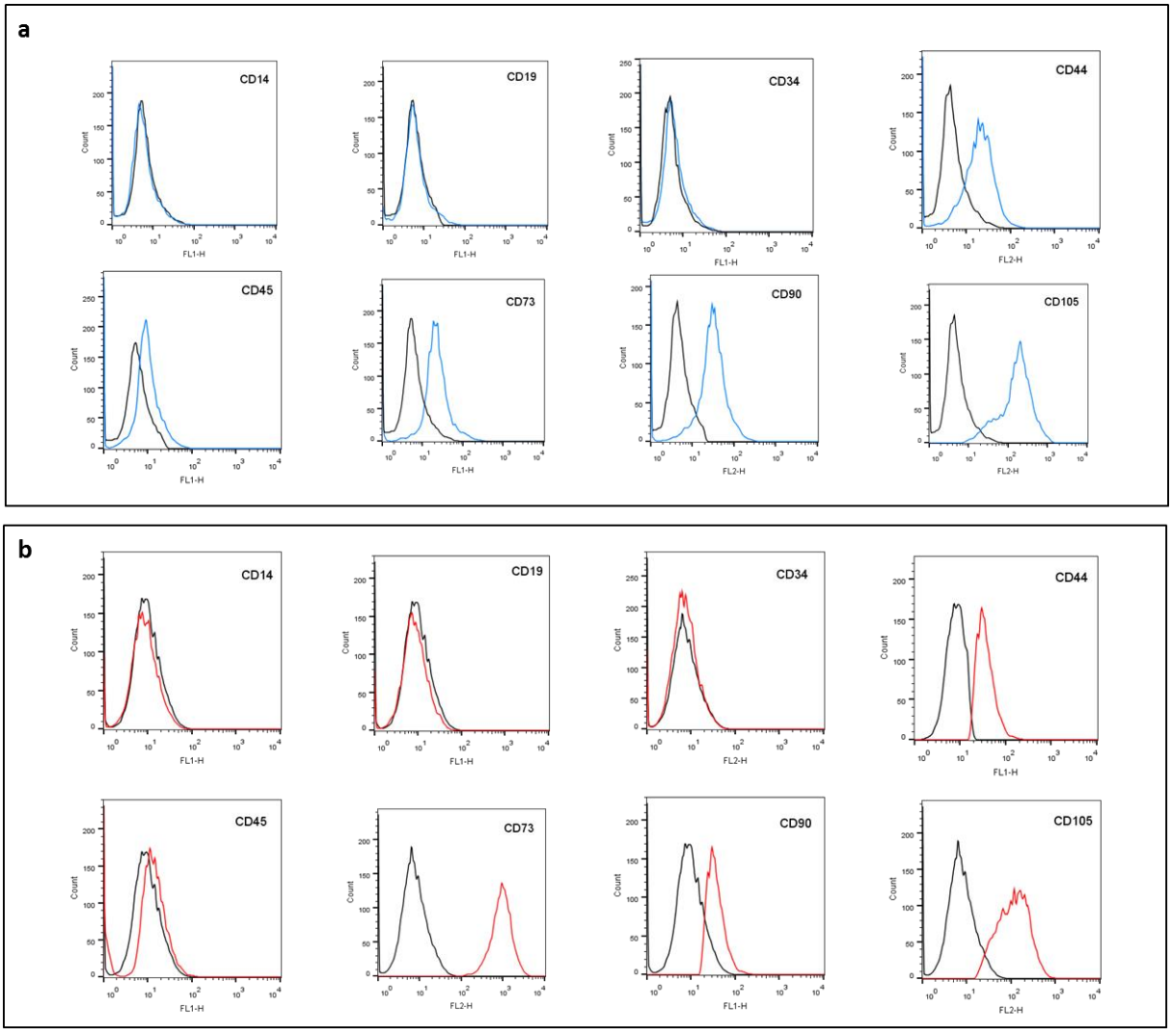
a
CD14
CD19
CD34
CD44
CD45
CD73
CD90
CD105
b
CD14
CD19
CD34
CD44
CD45
CD73
CD90
CD105

Figure 4