Degenerative processes of the intervertebral disc (IVD) and cartilaginous endplate lead to chronic spine pathologies. Several studies speculated on the intrinsic regenerative capacity of degenerated IVD related to the presence of local mesenchymal progenitors. However, a complete characterisation of the resident IVD cell populations, particularly that isolated from the endplate, is lacking. The purpose of the present study was to characterise the gene expression profiles of human nucleus pulposus (NPCs), annulus fibrosus (AFCs) and endplate (EPCs) cells, setting the basis for future studies aimed at identifying the most promising cells for regenerative purposes.

Cells isolated from NP, AF and EP were analysed after in vitro expansion for their stemness ability, immunophenotype and gene profiles by large-scale microarray analysis. The three cell populations shared a similar clonogenic, adipogenic and osteogenic potential, as well as an immunophenotype with a pattern resembling that of mesenchymal stem cells. NPCs maintained the greatest chondrogenic potential and shared with EPCs the loss of proliferation capability during expansion. The largest number of selectively highly expressed stemness, chondrogenic/tissue-specific and surface genes was found in AFCs, thus representing the most promising source of tissue-specific expanded cells for the treatment of IVD degeneration.

Keywords: Intervertebral disc, nucleus pulposus, annulus fibrosus, endplate, phenotype, microarray.
The IVD is a complex avascular fibrocartilaginous structure composed of the gelatinous NP encapsulated in the AF (Colombini et al., 2008). Between the disc and upper and lower vertebral bodies there is the interposition of the cartilaginous EP (Nguyen et al., 2012). Each of these anatomical compartments has a peculiar matrix structure and cell populations with exclusive phenotypes and functions (Pattappa et al., 2012). The degenerative processes of IVD and EP lead to chronic spine pathologies causing low-back pain and patient’s disability. In particular, the nutrient supply to disc cells occurs by diffusion from the EPs; therefore, their calcification and/or subchondral bone sclerosis contribute to IVD degeneration (Urban et al., 2004). The loss of disc height, mainly caused by cell death and proteoglycan degradation, results in decreased fluid-binding ability and reduction of IVD mechanical properties (Colombini et al., 2008).

Several studies have speculated on the intrinsic regenerative capacity of degenerated IVD related to the presence of local mesenchymal progenitors (Blanco et al., 2010; Brisby et al., 2013; Brown et al., 2018; Gruber et al., 2016; Huang et al., 2012) or NPCs with a notochordal-like phenotype (Rodrigues-Pinto et al., 2018). Nevertheless, the IVD degeneration is difficult to counteract for many reasons – including the small number of resident cells and their inability to prevent efficiently the tissue damage produced by the chronic catabolic and inflammatory environment in which they reside. Regenerative medicine would provide different tools for the treatment of degenerative disc disease by means of tissue-specific cell therapy; therefore, a complete characterisation of the resident IVD cell populations would allow focusing on those with the highest regenerative capability. To achieve good results, cell expansion is required because only few cells are initially isolated from pathological tissues and because a range of 1-120 million cells (MSCs or NPCs) per disc has been used in clinical trials (Schol and Sakai, 2019). CFU ability has been also reported as a parameter required because only few cells are initially isolated from pathological tissues and because a range of 1-120 million cells (MSCs or NPCs) per disc has been used in clinical trials (Schol and Sakai, 2019). CFU ability has been also reported as a parameter.
be interesting to confirm these results also for NPCs, AFCs and EPCs.

Although several studies have characterised IVD cells, to date only preliminary studies have been conducted to fingerprint the molecular signature of IVD cell subtypes. However, there are still many controversial topics, especially about the influence of age and pathological status. A subset of notochord-specific markers was identified in human NPCs (Rodrigues-Pinto et al., 2018). Moreover, the expression of keratins, already identified in human embryonic and foetal spine as well as in human unexpanded, freshly isolated, adult NPCs obtained from degenerated IVDs, demonstrates that this population contains a notochord-derived cell subpopulation (Rodrigues-Pinto et al., 2016; Rutges et al., 2010). Particularly, higher levels of KRT19 – with an age-related decrease and detection only in donors younger than 54 years – as well as of NCAM1, A2M and DSC2 are noted in NPCs when compared to AFCs, whilst COMP and GPC3 expression is higher in AFCs (Rutges et al., 2010). Another marker of a progenitor population in human-expanded NPCs that decreases with age and IVD degeneration degree is TEK (Sakai et al., 2012). Nevertheless, since TEK is also expressed in AFCs, it is not specific for NPCs (Schubert et al., 2018). Indeed, a larger inter-subject similarity was found when comparing the transcriptome profile of AFCs and NPCs; a higher expression of only some surface markers was observed in NPCs with respect to AFCs (Power et al., 2011). Furthermore, 11 out of 47,000 transcribed genes analysed were identified as differentially expressed in NPCs or AFCs derived from cervical discs (Schubert et al., 2018).

Despite their involvement in the pathogenesis of IVD degeneration (Jackson et al., 2011; Urban et al., 2004), few studies have characterised EPCs. In this population, after progenitor cell selection, the stem-cell-related genes OCT4, NANOG and SOX2 are expressed (Huang et al., 2012). The purpose of the present study was to characterise the human-expanded NPC, AFC and EPC stemness potential, immunophenotype and gene expression profiles by large-scale microarray analysis, with particular emphasis on the expression of stemness, chondrogenic, surface and senescence markers. The identification of the cells with the greatest stemness and trophic potential as well as of markers characteristic of a single cell population will allow the selection of the most promising tissue-specific cells for the treatment of IVD degeneration. Finally, differently from the great interest in the restoration of NP, few studies have focused on the repair of the AF (Sakai and Grad, 2015) or EP (Bendtsen et al., 2011) although their integrity is fundamental for disc health (Bendtsen et al., 2016). For this reason, thoroughly assessing the features and therapeutic potential of the cells derived from each of these anatomical tissues will be necessary.

Materials and Methods

Human tissue harvesting
The present study was approved by the Institutional Review Board (GenVDisc Version 1, 20 November 2015 protocol for use of waste biological material) and specimens were collected after receiving patient informed consent. NP, AF and EP from lumbar IVD of 8 patients (mean age 51.9 ± 6.9 years; 4 males and 4 females) affected by discopathy, Pfirrmann grade III-IV, were harvested during discectomy through an accurate macroscopic dissection performed by an experienced surgeon.

Cell isolation and expansion
NPCs, AFCs and EPCs were isolated by enzymatic digestion as previously described (Colombini et al., 2015; Lopa et al., 2016). NPCs, AFCs and EPCs were cultured in a control medium consisting of 4.5 mg/mL HG-DMEM (Life Technologies) supplemented with 10 % FBS (Lonzà), 0.29 mg/mL L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 10 mM HEPES and 1 mM sodium pyruvate (all from Life Technologies). For all cell types, the seeding density was 5 × 10³ cells/cm².

Cell DT
Disc-cell DT was calculated from passages 1 to 3. Cells were plated at a density of 5 × 10³ cells/cm² in control medium. Fresh medium was supplied every 3 d and cells were split at 80-90 % confluence using trypsin/EDTA (0.5 % trypsin/0.2 % EDTA; Sigma-Aldrich). DT was calculated using the equation

\[ DT = CT / \ln(N_f - N_i) / \ln(2) \]

where CT is the cell culture time (h), N_f is the final number of cells and N_i is the initial number of cells.

Clonogenic ability
A CFU-F assay was performed to assess the cell clonogenic ability (Lopa et al., 2014) at P1 and P3. Cells were plated at different low densities (range: 48-12 cells/cm²) and cultured in control medium with 20 % FBS. After 14 d, cells were fixed in 10 % neutral-buffered formalin and stained using Gram’s crystal violet (Sigma-Aldrich). CFU-F frequency was established by expressing the number of colonies as a percentage relative to the number of seeded cells.

Multi-lineage differentiation
Previously validated adipogenic or osteogenic differentiation protocols (de Girolamo et al., 2009) were used on cells at P1 and P3. Lipid vacuoles were quantified by oil red O staining and absorbance was read at 490 nm (Perkin Elmer Victor X3 microplate reader). Alizarin red S staining was performed to assess calcified matrix deposition (Sigma-Aldrich) and absorbance was read at 570 nm.
Chondrogenic differentiation was obtained by centrifugation of $4 \times 10^5$ cells (2 min at 232 × g) at P1 and P3. Then, the pellets were maintained for 28 d in chondrogenic medium as already reported (Colombini et al., 2012). Pellets were fixed in 10% neutral-buffered formalin, embedded in paraffin-wax, sectioned at 4 µm thickness and stained using alcian blue (Sigma-Aldrich) to evaluate GAG deposition. GAG quantification was performed using dimethylmethylene blue (Sigma-Aldrich) and absorbance was read at 500 nm.

**Immunophenotype**
Flow cytometry analyses were conducted on $2.5 \times 10^5$ expanded cells (P3) incubated with anti-human primary monoclonal antibodies: CD34-biotinylated, CD44-FITC, CD105-biotinylated, CD166-FITC (all from Ancell Corporation, Bayport, MN, USA), CD90-FITC and CD73-PE (Miltenyi Biotec) and CD151 (R&D Systems) (De Luca et al., 2019). Cells stained with biotinylated antibodies were incubated with streptavidin-PE (Ancell Corporation), whereas samples stained with anti-CD151 primary antibody were incubated with a FITC-conjugated goat anti-mouse secondary antibody (Ancell Corporation). Background fluorescence was established by unstained controls. A minimum of 10,000 events were acquired using a FACSCalibur flow cytometer (BD Biosciences) and analysed using CellQuest software (BD Biosciences).

**RNA isolation and quality assessment**
RNA was isolated from lysates of cell at P3 using the RNeasy Plus Mini Kit (Qiagen). RNase-Free DNase Set (Qiagen) was used for residual genomic DNA digestion and the isolated RNA was quantified and its purity checked spectrophotometrically (Nanodrop, Thermo Fisher Scientific). Agilent RNA ScreenTape System (Agilent Technologies) was used to evaluate the RIN, where values range from 10 (intact RNA) to 1 (totally degraded RNA) (Schroeder et al., 2006) (Fig. 1).

**Gene expression microarray**
All reagents, instruments and software when not specified were purchased from Agilent Technologies. The One-Color RNA Spike-In Kit was used to prepare a spike mix to add to the samples, useful to assess the correct annealing of 10 optimised positive control transcripts to the complementary probes of the array, evaluating also the auto- and cross-hybridisation.

RNA (100 ng) of 4 pooled donors for each cell type at P3 was labelled and amplified using a Low Input Quick Amp Labeling Kit, one-colour, to obtain cRNA. The obtained cRNA was purified using an RNeasy Plus Mini Kit (Qiagen) and used with the Gene Expression Hybridization Kit for the microarray hybridisation. To evaluate the gene expression profile of interest, a suitable Agilent Technologies algorithm was used (Web ref. 1). The custom gene expression microarray allowed to analyse a maximum of 3,000 genes considering a minimum of 5 replicates for each gene. Gene symbol and NM of the analysed genes, divided into housekeeping, chondrogenic/IVD/growth factors, surface, stemness and senescence, are reported in Supplementary Table 1. After washing, the microarray slide was scanned using SureScan Microarray Scanner and, to obtain a high-resolution image of the fluorescence values for each probe, the data were extracted by Feature Extraction v.12.0 software. Data analysis was performed by means of Genespring GX software and an Fc over or under an arbitrary cut-off of 2 was considered of interest.

Only up- or down-regulated genes (Fc ≥ +2 or ≤ −2) in the different cell populations were described. Gene ontology analyses were performed using Panther, PubMed, QuickGO and GeneCards databases.

**Gene expression analysis and microarray validation**
The expression of the pluripotent stem cell markers \textit{NANOG} (Hs04260366_g1) and \textit{POU5F1} (Hs04260367_g1), already extensively used to characterise IVD cells (Lyu et al., 2019), was evaluated at P1 and P3.
Table 1. Gene symbols and TaqMan® probe codes of validated genes.

| Gene   | Assay               |
|--------|---------------------|
| TBP    | Hs00427620_m1       |
| YWHAZ  | Hs01122445_g1       |
| ANKRDI | Hs00173317_m1       |
| CLDN11 | Hs00194440_m1       |
| FGF18  | Hs00818572_m1       |
| IGBP5  | Hs00181213_m1       |
| KRT14  | Hs00265033_m1       |
| KRT19  | Hs00761767_s1       |
| SYT4   | Hs01086433_m1       |
| C5AR1  | Hs00383718_m1       |
| RETN   | Hs00220767_m1       |
| CXCR2  | Hs00174304_m1       |
| ERG    | Hs01554629_m1       |
| FAM20A | Hs01034073_m1       |
| HHIP   | Hs00368450_m1       |

RNA was isolated from disc cells using the RNeasy Plus Mini Kit, quantified spectrophotometrically (NanoDrop, Thermo Fisher Scientific), reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) and gene expression was evaluated by real-time PCR (StepOne Plus, Life Technologies) using TaqMan® Gene Expression Master Mix and TaqMan® Gene Expression Assays (Life Technologies).

TBP and YWHAZ were evaluated as housekeeping genes. TBP was selected for its higher stability, as observed in a previously published validation (Lopa et al., 2016) and in the present study. Data are expressed according to the ΔΔCt method. TaqMan® Gene Expression Assays used for validation are reported in Table 1.

Table 2. Immunophenotype of IVD cells. Mean of percentage ± SD of cells positive for typical mesenchymal (green) and hematopoietic (red) markers; n = 6 donors.

|       | NPCs                | AFCs                | EPCs                |
|-------|---------------------|---------------------|---------------------|
| CD34  | 2.7 ± 2.4           | 2.7 ± 2.0           | 3.3 ± 2.3           |
| CD45  | 2.3 ± 1.9           | 2.7 ± 1.4           | 3.9 ± 2.1           |
| CD146 | 5.8 ± 2.9           | 3.3 ± 1.4           | 5.2 ± 2.4           |
| CD44  | 98.3 ± 3.0          | 98.5 ± 1.6          | 96.4 ± 4.9          |
| CD73  | 99.6 ± 0.4          | 99.5 ± 0.7          | 99.7 ± 0.2          |
| CD90  | 94.4 ± 2.9          | 94.2 ± 5.7          | 96.4 ± 3.5          |
| CD105 | 87.0 ± 14.4         | 94.0 ± 7.3          | 91.4 ± 5.3          |
| CD151 | 95.0 ± 4            | 87.9 ± 16.4         | 88.9 ± 8.8          |
| CD166 | 65.1 ± 20.9         | 73.0 ± 19.1         | 68.8 ± 26.6         |

Results

NPCs, AFCs and EPCs shared clonogenic behaviour and immunophenotype, while showed different proliferative and chondrogenic potential.

NPCs and EPCs shared an increase in DT during culture expansion (P2 vs. P3 p < 0.05 and p < 0.01, respectively, and P1 vs. P3 p < 0.05 for both cell types), whereas AFCs did not show any change (Fig. 2a). The clonogenic ability was similar for all the IVD cells at P1 and maintained until P3 (Fig. 2b). All three cell populations displayed a similar immunophenotype. They were negative for CD34, CD45, CD146 and positive for CD44, CD73, CD90, CD105, CD151 and CD166 (Table 2).

All disc cells displayed osteogenic (Fig. 3a) and chondrogenic (Fig. 3b) capability. From P1 to P3
AFCs and EPCs showed a loss of their chondrogenic differentiation potential, whereas NPCs showed a stable chondrogenic capability with passages. No adipogenic differentiation was observed. *POU5F1* and *NANOG* were expressed by all the analysed disc cells, whereas AFCs and EPCs showed a decrease of their expression from P1 to P3 (data not shown).

**Gene expression microarray in expanded cells**

*Stemness genes*

AFCs showed the largest number of highly expressed stemness genes, followed by EPCs and NPCs (Fig. 4a, Supplementary Table 2). In particular *AN02, BST2, CCL26, CEBPA, CHST6, CNMD, ERG, FAM20A, GALNT3, GLDC, Hhip, NPR1, PDGFRα, PTGIS, RA12, ROR2, SFRP2, SORBS1 and TEK* were more expressed in AFCs in comparison to both NPCs and EPCs. Moreover, validation of gene expression confirmed the higher expression of *ERG, FAM20A* and *HHIP* in AFCs in comparison to the other two cell populations (Fig. 5). No gene was more expressed selectively in NPCs and only *CXCR2* was more expressed in EPCs with respect to the other two populations, although after validation this gene was undetected in all samples. Table 3 reports the molecular functions of these genes, which mainly are related to catalytic and binding activity.

Concerning the similarities between cell populations, AFCs and EPCs shared 53 genes more highly expressed than in NPCs, whereas only 9 genes had a higher expression in NPCs and EPCs than in AFCs. NPCs and AFCs shared no genes with a higher expression than in EPCs. In Fig. 4b, genes with a higher expression in one disc-cell population in comparison to another are shown. AFCs showed upregulation of 47 and 5 stemness genes in comparison to NPCs and EPCs, respectively; NPCs presented 22 and 8 genes with higher expression than AFCs and EPCs, respectively; EPCs had 18 and 2 genes with higher expression than AFCs and NPCs, respectively.

**Surface marker genes**

AFCs and EPCs shared 22 surface-related genes with a higher expression in comparison to NPCs, while EPCs shared only 2 genes with NPCs with a higher expression than in AFCs (Fig. 6a, Supplementary Table 2). Among these markers, *CD74* and *HLA-DRA* were more expressed in AFCs, *C5AR1* in NPCs and *VCAM1 and RETN* in EPCs than in the other two cell populations. After validation, *C5AR1* and *RETN* resulted undetected. The molecular functions of these genes are shown in Table 4. Genes with higher expression in one disc-cell population in comparison to another are shown in Fig. 6b.

**Chondrogenic, IVD- and growth-factor-related genes**

AFCs and EPCs shared 22 genes more expressed than in NPCs, while EPCs and NPCs shared only 7 genes in comparison to AFCs (Fig. 7a, Supplementary Fig. 3. Multi-lineage differentiation of IVD cells at P1 and P3. (a) Osteogenic and (b) chondrogenic cell differentiation. Scale bars: 200 µm. Scale bars of the bigger chondrogenic images: 100 µm. *p < 0.05, **p < 0.01 of NPCs, AFCs or EPCs differentiated vs. control; n = 8 donors. Ch = chondrogenic; O = osteogenic.
Among these genes, ANKRD1, BMPR1B, CSF1R, FGF18, IGFBP5, KRT14, KRT19, PENK, REEP1 and TMEM71 were more expressed in AFCs in comparison to the other two populations. After validation, these results were confirmed in 3 patients out of 4 for ANKRD1 and KRT14, whereas FGF18, IGFBP5 and KRT19 were confirmed in all patients (Fig. 5). Only CLDN11 and SYT4 were more expressed in NPCs in comparison to the other two cell populations and this result was confirmed in 3 patients out of 4. In Table 5 are shown the molecular functions of these genes.

In Fig. 7b genes with a higher expression in one disc-cell population in comparison to another are shown. In particular, NPCs showed a higher expression of 11 genes in comparison to AFCs. An upregulation of 5 genes in comparison to NPCs and of 3 genes in comparison to EPCs was observed in AFCs. EPCs showed higher expression of 1 and 11 genes in comparison to NPCs and AFCs, respectively.

| Genes | Protein class | Molecular function | AFCs vs. NPCs | AFCs vs. EPCs | EPCs vs. NPCs |
|-------|---------------|-------------------|---------------|---------------|---------------|
| ANO2  | Transmembrane protein 16B | n.d. | 3.4 | 3.4 | / |
| BST2  | n.d. | a, c, d | 5.1 | 4.4 | / |
| CCL26 | Chemokine | a, c, d, e | 2.5 | 3.6 | / |
| CEBPA | Basic leucine zipper transcription factor, nucleic acid binding | a, b | 2.4 | 2.4 | / |
| CHST6 | Sulphotransferase | c | 3.1 | 6.1 | / |
| CNMD  | Glycosylated transmembrane protein | f | 2.8 | 2.2 | / |
| CXC2R | C-X-C motif chemokine receptor 2 | c, e | 2.4 | −2.9 | 2.4 |
| ERG   | Nucleic acid binding, signaling molecule transcription factor | b | 2.9 | 8.0 | −2.7 |
| FAM20A| Allosteric activator of the Golgi Serine/threonine protein kinase FAM20C | c | 17.9 | 14.6 | / |
| GALT3 | Calcium ion binding and transferase activity | c | 2.2 | 3.0 | / |
| GLDC  | Dehydrogenase | a, c | 2.1 | 4.2 | / |
| HHIP  | Quinone binding and hedgehog family protein binding | a | 15.2 | 5.8 | / |
| NPR1  | Adenylate/guanylate cyclase | a, c, e | 2.2 | 2.2 | / |
| PDGFRL| Tyrosine protein kinase receptor | c | 2.4 | 2.7 | / |
| PTGIS | Endoplasmic reticulum membrane protein | a, c | 5.5 | 5.1 | / |
| RAI2  | n.d. | a | 4.0 | 3.6 | / |
| ROR2  | Transmembrane protein | a, c | 2.1 | 3.2 | / |
| SFRP2 | G-protein-coupled receptor, protease inhibitor, signaling molecule | a, e | 3.6 | 6.0 | / |
| SORBS1| Protein kinase binding, cytoskeletal protein binding | a | 2.2 | 2.8 | / |
| TEK   | Cell-surface receptor | c | 3.0 | 3.5 | 3.5 |

Table 2). Among these genes, ANKRD1, BMPR1B, CSF1R, FGF18, IGFBP5, KRT14, KRT19, PENK, REEP1 and TMEM71 were more expressed in AFCs in comparison to the other two populations. After validation, these results were confirmed in 3 patients out of 4 for ANKRD1 and KRT14, whereas FGF18, IGFBP5 and KRT19 were confirmed in all patients (Fig. 5). Only CLDN11 and SYT4 were more expressed in NPCs in comparison to the other two cell populations and this result was confirmed in 3 patients out of 4. In Table 5 are shown the molecular functions of these genes.

In Fig. 7b genes with a higher expression in one disc-cell population in comparison to another are shown. In particular, NPCs showed a higher expression of 11 genes in comparison to AFCs. An upregulation of 5 genes in comparison to NPCs and of 3 genes in comparison to EPCs was observed in AFCs. EPCs showed higher expression of 1 and 11 genes in comparison to NPCs and AFCs, respectively.

Senescence/telomere-related genes
Analysing the genes related to senescence and telomere markers, AFCs showed the highest value of TP63 when compared to both NPCs and EPCs. More in general, NPCs and EPCs showed a higher expression of Tbx2 and TERT in comparison to AFCs. SPARC upregulation was observed in EPCs in comparison to AFCs. An upregulation of NOX4 and SERPINB2 was observed in AFCs and EPCs in comparison to NPCs. ALDH1A3 upregulation was observed in AFCs in comparison to EPCs. In Supplementary Table 2, fold change of up- or down-regulated senescence/telomere-related genes are shown.

Discussion
The present study allowed the deep characterisation of the different cell populations of the human IVD.
Fig. 4a. Stemness genes expression in IVD cells. Gene array showing only the differentially expressed genes between cell populations. Heat map was created using a two-colour scale from red (highest value) to yellow (lowest value), n = pool of 4 donors.
Fig. 4b. Stemness genes expression in IVD cells. Higher expressed genes in one single disc-cell population respect to another one. NPCs (red), AFCs (green) and EPCs (blue); n = pool of 4 donors.
The validated markers obtained could be used to identify AFCs in the presence of a mixed biological material consisting of tissue contaminated by the NP, if considering surgical waste samples from discectomy as a potential reservoir of cells to be exploited for cell-based treatment of IVD degeneration. In fact, the higher expression of stemness and chondrogenic genes in expanded AFCs with respect to the other two cell populations would suggest that these cells could be the preferential focus of future approaches of tissue-specific cell therapies for IVD degeneration. In addition, as far as it can be ascertained, the present was the first study in which EPCs, highly involved in IVD homeostasis, were compared with the other two most studied IVD cell types.

Despite the morphological differences in the structural organisation of the different anatomical portions of the IVD to which they belong, NPCs, AFCs and EPCs showed common proliferative, clonogenic, multi-differentiation characteristics and immunophenotype, which were maintained during culture. Differences were observed only in terms of loss of proliferative potential by NPCs and EPCs and of chondrogenic potential and pluripotent stem cell gene expression (POU5F1 and NANOG) by AFCs and EPCs at P3. The surface marker layout after expansion indicated a typical MSC-like immunophenotype (Dominici et al., 2006) of IVD cells and the positivity for CD44, CD73, CD90, CD105 and CD166 was consistent with what has already been observed in IVD cells (Blanco et al., 2010; Brisby et al., 2013; Liu et al., 2011; Wang et al., 2014). Furthermore, although they have an MSC-like immunophenotype, IVD cells do not share a perivascular origin given the lack of CD146 expression. To reinforce the MSC-like phenotype of the analysed disc cells, CD151 also showed a high expression already previously observed in articular chondroprogenitor cells (De Luca et al., 2019) and MSCs (Lee et al., 2009).

A group of stemness, surface and chondrogenic genes was found as selectively upregulated in expanded single-cell populations. After validation, the stemness genes ERG, FAM20A, HHIP and the chondrogenic FGF18, IGFBP5 and KRT19 were confirmed as being selective for AFCs in all patients, suggesting that these genes can be exploited as specific cell population markers. Differently, the chondrogenic genes ANKRD1 and KRT14 for AFCs and CLDN11 and SYT4 for NPCs after validation were confirmed in 3 out of 4 patients, suggesting a more donor-dependent result. Thus, they cannot be considered reliable as single disc-cell population markers.

The stemness marker CXCR2 and the surface markers C5AR1 and RETN were not confirmed after validation by real-time PCR. It is possible to ascribe this result to their lower expression and consequent inefficacious detection. For this reason, these markers should not be considered as being specific for a single IVD cell population. Consistently with the study’s findings, other studies have shown that NCAM1 and A2M are more expressed in NPCs in comparison to

| Gene | Protein class | Molecular function | Fc AFC vs. NPC | Fc AFC vs. EPC | Fc EPC vs. NPC |
|------|---------------|-------------------|--------------|--------------|--------------|
| C5AR1 | Protease inhibitor | a, c | −3.6 | / | −2.2 |
| CD74 | Major histocompatibility complex, class II, DR alpha | c, d | 2.9 | 2.3 | / |
| HLA-DRA | Major histocompatibility complex, class II, DR alpha | c | 7.5 | 3.8 | / |
| RETN | Cysteine-rich secreted protein | e | / | −4.75 | 3.9 |
| VCAM1 | Type I membrane protein | a | / | −2.2 | 2.2 |

Table 4. Protein class and molecular function of cell-surface-related genes. Molecular function legend: a = binding function, c = molecular function regulator, d = molecular transducer activity, e = hormone activity, / = no differences between cell populations. Higher expressed genes in NPCs (green), AFCs (red) and EPCs (blue). n = pool of 4 donors.

Fig. 5. Fc of genes validated by means of real-time PCR in cells obtained from the 3 single donors used for microarray gene expression analysis and from another patient of the 8 enrolled for the study. Floating bars show line at mean, minimum and maximum Fc.
Table 5. Protein class and molecular function of chondrogenic, IVD- and growth-factor-related genes.
Molecular function legend: a = binding function, b = catalytic activity, c = molecular function regulator,
d = molecular transducer activity and e = developmental function, n.d. = not defined, / = no differences
between cell populations. Higher expressed genes in NPCs (green) and AFCs (red). n = pool of 4 donors.

| Gene     | Protein class                                | Molecular function | Fc AFC vs. NPC | Fc AFC vs. EPC | Fc EPC vs. NPC |
|----------|----------------------------------------------|--------------------|----------------|----------------|----------------|
| ANKRD1   | RNA polymerase II transcription factor       | a                  | 11.4           | 4              | /              |
| BMPR1B   | Growth factor binding, transmembrane receptor protein | a, b             | 5.3            | 5.5            | /              |
| CLDN11   | Transmembrane protein                        | d                  | −4.2           | /              | −3.5           |
| CSF1R    | Transmembrane receptor protein               | b                  | 2.2            | 4              | /              |
| FGF18    | Growth factor                                | c                  | 5.4            | 9.7            | /              |
| IGFBP5   | Protease inhibitor                           | a                  | 2.5            | 3.8            | /              |
| KRT14    | Structural protein                           | a                  | 8.7            | 11.2           | /              |
| KRT19    | Structural protein                           | a                  | 2.4            | 3              | /              |
| PENK     | Neuropeptide                                 | d                  | 5              | 5.8            | /              |
| REEP1    | Receptor, transporter                        | a, e               | 2.8            | 4              | /              |
| SYT4     | Membrane trafficking regulatory protein       | a                  | −2.2           | /              | −3             |
| TMEM71   | Transmembrane protein                        | n.d.               | 2.4            | 3.1            | /              |
Fig. 7. Chondrogenic, trophic and growth-factor-related genes in IVD cells. (a) Gene array showing only the differently expressed genes between cell populations. Heat map was created using a two-colour scale from dark green (highest value) to white (lowest value). (b) Higher expressed genes in one single disc-cell population respect to another. NPCs (red), AFCs (green) and EPCs (blue); $n$ = pool of 4 donors.
AFCs (Rutges et al., 2010), while GPC3 (Rutges et al., 2010) and TEK (Schubert et al., 2018) are more expressed in AFCs. In contrast, in the present study, COMP was more expressed in NPCs and not in AFCs (Rutges et al., 2010) while the notochordal KRT19 was more expressed in AFCs and not in NPCs, as previously reported (Rodrigues-Pinto et al., 2016; Rutges et al., 2010). These discrepancies can be ascribed to the use of expanded cells undergoing dedifferentiation in the present study, while in previously published works the expression of these genes was evaluated directly in tissue explants (Rutges et al., 2010) or in embryonal discs (Rodrigues-Pinto et al., 2016).

AFCs and EPCs shared a larger number of highly expressed stemness, surface-related, chondrogenic, trophic and growth-factor-related genes, mainly having catalytic and binding activity. In particular, AFCs showed the largest number of highly expressed genes related to stemness ability, followed by EPCs and NPCs. Another indicator of the possible capability of AFCs in terms of their therapeutic use was the higher expression of TP63, a key regulator of ageing and senescence (Keyes and Mills, 2006), in these cells in comparison to both NPCs and EPCs. At the same time, NPCs and EPCs showed a higher expression of TBX2 and TERT than AFCs. Since the other analysed genes in the senescence pathway did not allow the establishment of whether there was a prevalence of pro- or anti-senescence processes in these IVD populations, further studies will be needed to better clarify if one of these populations is less prone to senescence.

The main limitation of the study was that the microarray analysis was conducted on 4 pooled donors for each cell type. Nevertheless, the identified genes were validated in cells obtained from the 3 single donors used for microarray gene expression analysis and from another patient of the 8 enrolled in the study. Moreover, the study did not include a functional analysis, which could have further helped to identify AFCs as the most suitable disc cell population for regenerative purposes. Finally, since cell expansion may cause alterations of the phenotype and surface markers related to in vitro dedifferentiation, especially in cells having a more chondrogenic phenotype such as NPCs and EPCs, the present findings should be considered applicable only to monolayer-expanded cells. It would be interesting to compare the outcome of the present study to results obtained from cells directly isolated from the respective tissue region to study possible influences of sub-culturing the cells in monolayer.

Conclusions

The study provided a whole gene expression characterisation of expanded AFCs, NPCs and EPCs identifying also a panel of genes useful to detect AFCs in the presence of a mixed material consisting of an AF contaminated by an NP. In fact, due to a stemness and chondrogenic profile that resembles that of regenerative cells such as MSCs, AFCs appeared as the most promising source of tissue-specific expanded cells to be used in cell-based approaches for the treatment of IVD degeneration.

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Discussion with Reviewers

Cornelia Neidlinger-Wilke: In the present study, cells isolated from different tissue regions were characterised after in vitro expansion in monolayer. Do you think that this had an influence on the outcome of the study? Could you please comment on this possible drawback of the study? Could this influence the conclusion that AF cells might be the best suitable cell population for regenerative purposes? The monolayer culture system is certainly better suitable for AFCs than for NPCs or EPCs. Could you please comment on this?

Authors: Cellular phenotype and surface markers might be altered by in vitro dedifferentiation in monolayer, especially if the cells have a more chondrogenic phenotype, such as EPCs and NPCs. The maintenance of cells in monolayer culture for several passages allows for the expansion of the cell population to have a sufficient amount for cell therapy. IVD, as well as cartilaginous EP from adult pathological subjects are low cellularised tissues and, to consider them as sources for cell therapy of degenerative disc disease, few passages in culture of the isolated cells are mandatory. Being not a physiological condition, cell expansion in monolayer may cause alterations to the cell phenotype, especially in NPCs and EPCs. Nevertheless, in this condition, AFCs showed a MSCs-like profile and appeared as the most promising source of tissue-specific cells to be used for the treatment of IVD degeneration.

Sibylle Grad: The authors concluded that expanded AFCs may be a promising source for IVD degeneration cell therapy. Thereby, the expansion conditions may also influence the cellular phenotype. Can the authors comment on the present expansion conditions, potential modifications and their applicability in a (pre-) clinical setting?

Authors: AFCs appeared as the ones having a more promising phenotype after a small number of passages in monolayer. In general, when cultured in monolayer, disc cells tend to lose their tissue-specific phenotype, especially the peculiar matrix proteins and collagen types. To help the cells maintaining their physiological features, 3D cultures, such as in alginate beads, could be considered, although associated with a loss of proliferation. Since a large number of cells is needed for cell therapy, cultivating cells on coated 2D surfaces or addition of growth factors or platelet lysate in the culture media could help to obtain a desirable number of exploitable cells in pre-clinical setting. In the present study, standard culture conditions have been used without addition of any previously mentioned supplement or culture plastic surface coating. Consequently, all the potential strategies above suggested to improve cell products to be exploited for cell therapy will need to be thoroughly tested and validated in vitro.

Sibylle Grad: Would the envisaged cell therapy particularly be applicable for AF rupture repair such as after IVD herniation?

Authors: The AF presents a structural complexity and a peculiar biomechanical function that are challenging to restore after rupture. In case of a complete IVD herniation, the anatomical tissue is probably too compromised to allow for the success of a cell therapy approach, even if associated with engineered materials aimed to seal defects in the IVD and, thus, avoid the recurrence of herniation as well as promote cell engraftment. Therefore, the envisaged cell therapy could be applicable for early phase IVD degeneration, in absence of a definitive AF rupture with extrusion of nuclear material and in association with materials able to withstand the mechanical load experienced by the IVD.

Editor’s note: The Scientific Editor responsible for this paper was Mauro Alini.