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The Presence of Local Mesenchymal Progenitor Cells in Human Degenerated Intervertebral Discs and Possibilities to Influence These In Vitro: A Descriptive Study in Humans

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Low back pain is common and degenerated discs (DDs) are believed to be a major cause. In non-degenerated intervertebral discs (IVDs) presence of stem/progenitor cells was recently reported in different mammals (rabbit, rat, pig). Understanding processes of disc degeneration and regenerative mechanisms within DDs is important. The aim of the study was to examine the presence of local stem/progenitor cells in human DDs and if these cell populations could respond to paracrine stimulation in vitro. Tissue biopsies from the IVD region (L3-S1) were collected from 15 patients, age 34-69 years, undergoing surgery (spinal fusion) and mesenchymal stem cells (MSCs) (iliac crest) from 2 donors. Non-DD cells were collected from 1 donor (scoliosis) and chordoma tissue was obtained from (positive control, stem cell markers) 2 donors. The IVD biopsies were investigated for gene and protein expression of: OCT3/4, CD105, CD90, STRO-1, and NOTCH1. DD cell cultures (pellet mass) were performed with conditioned media from MSCs and non-degenerated IVD cells. Pellets were investigated after 7, 14, 28 days for the same stem cell markers as above. Gene expression of *OCT3/4* and *STRO-1* was detected in 13/15 patient samples, *CD105* in 14/15 samples, and *CD90* and *NOTCH1* were detected 15/15 samples. Immunohistochemistry analysis supported findings on the protein level, in cells sparsely distributed in DDs tissues. DDs cell cultures displayed more undifferentiated appearance with increased expression of *CD105*, *CD90*, *STRO-1*, *OCT3/4*, *NOTCH1*, and *JAGGED1*, which was observed when cultured in conditioned cell culture media from MSCs compared to cell cultures cultured with conditioned media from non-DD cells. Expression of *OCT3/4* (multipotency marker) and *NOTCH1* (regulator of cell fate), MSC-markers, *CD105*, *CD90*, and *STRO-1*, indicate that primitive cell populations are present within DDs. Furthermore, the possibility to influence cells from DDs by paracrine signaling /soluble factors from MSCs and from nondegenerated IVD cells was observed in vitro indicating that repair processes within human DDs may be stimulated.

Introduction

LOW BACK PAIN IS ONE of the most common and costly musculoskeletal disorders in many countries today [1,2], both in terms of socioeconomic costs as well as suffering for the individual. Degenerated intervertebral discs (IVDs) are believed to be a major cause of this condition and more than half of the population will experience pronounced low back pain during their life time [3].

The degenerated disc (DD) is typically characterized by loss of disc height, fissure formation, cell clustering, and cell death, as well as a decrease in fluid binding ability. Hence, in DD disease, the flexibility properties of the IVD are drasti-

cally reduced. On the molecular level, a decrease in glycosaminoglycan production is seen as well as an increase in matrix degrading enzymes; for example, proteins of the metalloprotease family 1, 3, 9, and 13 [4-6] and cytokines, for example, interleukin 1 beta [7,8].

It has been suggested that dysfunctional cells may be one plausible cause of DD. Cell-based therapy and/or stimulation of local immature cells might offer a more biological approach to regenerate the disc or slow down disc degeneration [9,10] as a complement to the treatment methods of today.

In nondegenerated IVDs, the presence of stem and progenitor cells has recently been reported in mammals in different localizations [11] and progenitor cells have been sorted

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out from the total IVD cell population [12–14]. There is a gap of knowledge regarding these cell populations in human IVDs and their functions in DD. Therefore, to further understand the process of IVD degeneration, knowledge about regenerative mechanisms within DDs is of importance. Different types of cellular surface markers can be used to detect progenitor and stem cells within a tissue. OCT3/4 is a transcription factor and OCT3/4 expression is necessary for stem cells to sustain self-renewal and multipotent ability [15,16]. CD90 [thymocyte differentiation antigen 1 (THY-1)] is a glycoprotein expressed on mesenchymal stem cells (MSCs) [17,18]. Stromal cells antigen 1 (STRO-1) is a MSC marker associated with tissue growth and regeneration [19–22]. CD105 (endoglin) is a transmembrane cell surface protein associated with, for example, human bone marrow MSCs, endothelia cells, and smooth muscle cells [17,23,24]. Furthermore, NOTCH signaling is a primitive interspecies universal signaling pathway that decides cell fate; for example, proliferation or differentiation in embryogenesis as well as in adult tissues [25,26]. JAGGED1 is a ligand to NOTCH1 that participate in NOTCH signaling [27,28] and is associated with cell communication present in stem cell niches [28,29].

The aim of the study was to examine the presence of local stem/progenitor cells in human DDs and their eventual response to stimulation by soluble factors in an *in vitro* model.

Materials and Methods

Human tissues and cells

Human DD tissue samples (2–3 on the Thompson scale, level LIV–SI) were collected from patients ($n=15$), (age range 33–69 years) undergoing spinal fusion surgery. All DD samples were divided for the following 2 analyses: (1) for RNA analysis, samples were placed in RNA later solution (Qiagen) and, thereafter, frozen in -80°C until analysis and (2) for immunohistochemical analysis, samples were placed in 4% formaldehyde (Histofix[®]), embedded in paraffin, and sectioned (5–7 μm). For cell cultures, DD cells were isolated from 4 of the donors; female age 32 (donor 1), male age 61 (donor 2), male age 41 (donor 3), male age 34 (donor 4), and non-DD cells were isolated from 1 patient with scoliosis undergoing anterior corrective surgery, age 19. MSCs were taken (iliac crest), from 2 donors; male age 50 [used for a conditioned medium in 1 of the pellet cultures (donor 1)] and from female, age 38 [used for a conditioned medium in 3 of the cell cultures (donors 2–4)]. Chordoma tissue (from the peripheral region of tumor) was taken from 2 donors; male age 69 and female age 70 during surgery. Different panels of cell surface markers, for example, the stem cell marker, C-KIT (CD117), are currently used (among laboratory methods) as part of diagnosis and classification of different cancer types [30,31]. Hence, chordoma tissues were hypothesized to serve as positive controls and as a comparison tissue type for the stem cell markers used in this study.

The study was approved by the local human ethics committee and the samples were collected with informed consent from all patients.

Histology

All tissues were treated with 4% formaldehyde before paraffin embedment. Paraffin sections (5–7 μm) were depar-

affinized with xylene 2×10 min and rehydrated in 99%, 95%, and 70% ethanol for 5 min in each solution. Sections of DDs tissues, chordoma control tissues, and cell pellets were stained for morphology and proteoglycans with the Alcian Blue van Gieson method using standards protocols [32,33].

Isolation of RNA

The tissues were placed in RNA-later (Qiagen) and, thereafter, frozen in -80°C until analysis. Homogenization was performed in Trizol (Qiagen) using a Tissue lyser instrument (Qiagen). The homogenized tissue was centrifuged, the supernatant extracted with chloroform, RNA, and washed with ethanol. Thereafter, a clean-up step was done following the standard protocol for the RNeasy Mini Kit (Qiagen). cDNA was prepared from a minimum of 62.5 ng RNA using the High-Capacity cDNA Reverse Transcriptase kit with random hexamers. In 7/12 samples, preamplifications were performed using TaqMan PreAmp Master Mix (Applied Biosystems).

Quantitative real-time polymerase chain reaction analysis

All instruments, software and reagents were purchased from Applied Biosystems. Quantitative real-time polymerase chain reaction (PCR) analysis was performed using the instrument, 7900HT. The following TaqMan gene Expression Assays were used: OCT3/4 (Hs01895061_u1), CD105 (Hs00923996_m1), THY-1 (CD90) (Hs00174816_m1), BST1 (bone marrow stromal antigen 1, STRO1 (Hs00174709_m1), NOTCH1 (Hs00413187_m1), and Cyclophilin A (Hs99999904_m1) and CREBBP was used as reference genes. Real-time PCR was performed in 20 μL volume with 10 μL of $2\times$ TaqMan[®] Gene Expression Master Mix and 1 μL of $20\times$ gene expression assays, with standard temperature programs. Data were analyzed with the relative comparative method (the $\Delta\Delta\text{Ct}$ method) and the gene expression results are presented in relative units according to User Bulletin 2 (Applied Biosystems).

Immunohistochemistry analysis

Sections were deparaffinized as described above. Sections thereafter treated with an antigen retrieval step which were used for all samples with a 10 mM citrate buffer, pH 6.0 in 90°C for 20 min. Sections stained for OCT3/4 (R&D systems) were blocked with 2% bovine serum albumin (BSA) (Sigma) and 0.5% Triton-X 100 diluted in the phosphate saline buffer and NOTCH1 samples with 3% BSA for 10 min in room temperature (RT). Secondary antibodies were applied to sections: goat anti-mouse horseradish peroxidase (HRP) (Jackson Laboratory) in dilution 1:150 or donkey anti-goat HRP (Santa Cruz Biotechnology). The sections were thereafter incubated in RT in a dark container for 3 h and visualized by using the TSA-Direct Cy-3 kit (Perkin Elmer) according to the manufacturer's instructions.

Sections stained for CD105, STRO1, NOTCH1, and CD90 were blocked with 3% BSA for 5 min, incubated over night in 4°C with primary antibodies: goat anti-CD105 (R&D Systems); dilution 1:100, mouse anti-STRO1 (R&D Systems); dilution 1:100, and mouse anti-CD90 (Abcam); dilution 1:500, NOTCH1 (Santa Cruz Biotechnology); dilution 1×100

in a 3% BSA blocking solution. Secondary antibodies; donkey anti-goat Alexa Fluor 546 and goat anti-mouse Alexa Fluor 568 (Invitrogen) dilution 1:250 were added and sections were incubated for 3 h in RT. Nuclear staining was performed using 4,6 diamino-2 phenylindole 0.05 $\mu\text{g}/\text{mL}$ (Sigma-Aldrich) and sections were mounted with the Prolong Gold mounting medium (Invitrogen). Results were visualized by using NIKON eclipse80i (NIKON, Tokyo, Japan) and NIS-elements software (NIKON, Tokyo, Japan). Negative controls were sections with isotype controls and/or sections with the primary antibody were omitted.

Harvesting of tissue/cells for cell cultures

Human MSC isolation and monolayer cell cultures. Human MSCs were aspirated from iliac crest, centrifugated in cell preparation tubes (Ficoll; Becton Dicksson) according to the manufacturer's protocol. The MSCs were seeded at a concentration 200×10^3 cells/ cm^2 and cultured in the Dulbecco's modified Eagle medium, with low-glucose, (DMEM-LG) medium (Invitrogen). The medium was supplemented with 2 mM L-glutamine, 100 U/mL/0.1 mg/mL penicillin/streptomycin (PAA Laboratories), 10 ng/mL β -fibroblast growth factor (Invitrogen), and 10% human serum. At 90% confluence, the MSCs were detached using the trypsin/ethylenediaminetetraacetic acid (EDTA) solution (Invitrogen), re-seeded in a concentration 15×10^3 cells/ cm^2 and thereafter cultured to passage 3 or 5.

Human IVD cell isolation and monolayer cell cultures. IVD cells were isolated from 4 patients with disc degeneration undergoing spinal fusion surgery and from 1 patient with scoliosis undergoing anterior corrective surgery. The IVD tissues were manually cut to small pieces with a scalpel. Tissue pieces were then treated with 0.8 mg/mL collagenase type II (Worthington) and incubated 24 h in an incubator (37°C, 7% CO_2 , 93% air). Single cells were seeded at the concentration 4.0×10^5 cells/ cm^2 in the DMEM/F12 (Invitrogen) supplemented with ascorbic acid 0.1 mg/mL (Apoteket), L-glutamine (2 mM; Gibco), penicillin/streptomycin (100 U/mL/0.1 mg/mL), 10% human serum in cell culture flasks. At 90% confluence, the NP cells were detached using the trypsin/EDTA solution (Gibco), harvested, and reseeded again at a concentration 7×10^3 cells/ cm^2 and cultured to passage 2.

Flow cytometry analysis

The MSCs (2 donors) were characterized for a set of accepted mesenchymal cell surface markers [34], such as CD105, CD166, CD34, and CD45 by flow cytometry (passage 0 and 3–5). Fluorescein-, phycoerythrin-, or peridinin-conjugated antibodies (Ancell) were used in flow cytometry analysis using a FACSaria flowcytometer instrument (Becton Dickson).

Preparation of conditioned medium

For studies of differentiation and proliferation of DD cells in a three-dimensional (3D) culture (pellet mass system (DD pellets) potentially influenced by soluble factors, the conditioned medium was prepared from MSCs (a conditioned MSC medium) and from non-DD cells (a conditioned NDDC medium). The cells were radiated at 25 Gy, and then seeded at $6 \times 10^3/\text{cm}^2$ in cell culture flasks coated with 0.1% gelatin. The MSCs were cultured in the DMEM/F12-LG and the non-DD cells were cultured in the DMEM/F12 for 5 days. The

medium was changed twice and collected and pooled during this period. The 2 types of conditioned medium were sterile filtered and stored at -20°C until usage.

3D DD cell cultures (pellet mass)

After expansion, DD cells (passage 2–3) from 4 donors were placed in a 3D cell culture system (pellet mass). About 200,000 cells were applied in polypropylene conical tubes and 0.5 mL of the conditioned medium/tube was added. The collected conditioned medium was supplemented with 5.0 $\mu\text{g}/\text{mL}$ linoleic acid (Sigma-Aldrich), insulin, transferrin, and selenium (concentrate no. 41400-045; Life Technologies), 1.0 mg/mL human serum albumin (Equitech-Bio), 10 ng/mL transforming growth factor $-\beta 1$ (R&D Systems), 10^{-7} M dexamethasone (Sigma-Aldrich), 14 $\mu\text{g}/\text{mL}$ ascorbic acid (Sigma-Aldrich), and 1% penicillin–streptomycin (PAA Laboratories). These are additives commonly used in the chondrogenic medium [35,36]. The cells were thereafter centrifuged at 500 g for 5 min. 0.5 mL of the conditioned medium from MSC or non-DD cells (scoliosis) was added to the cultures. The medium was changed twice a week and the pellets harvested after 7, 14, and 28 days (except in one case were pellets were cultured only to day 7 and 14 due to shortage of cells).

Results

Histology of DD tissues

All the human IVD samples displayed morphological signs characteristic of degeneration, such as disorganized irregular extracellular matrix appearance and cell clusters (cell foci) (Fig. 1).

Stem cell/progenitor cells—gene expression in human DD tissue

Gene expression of *OCT3/4* and *STRO-1* was detected in 13/15 patient samples, *CD105* in 14/15 samples, and *CD90* and *NOTCH1* was detected 15/15 samples (Fig. 2).

Stem cell/progenitor cells—protein expression in human DD tissue

CD105+, *STRO-1+*, *OCT3/4+*, and *NOTCH1+* cells were found in 12/15 samples. *CD90+* cells were detected in 9/15 samples. The positive cells were detected relatively sparsely throughout the tissue as solitary cells or in cell-foci. In the positive control tissues, chordoma ($n=2$) *CD105+*, *CD90+*, *STRO1+*, *OCT3/4+*, and *NOTCH1+* cells was detected in higher numbers compared to the degenerative disc tissue samples. Negative controls showed no staining (Fig. 3A, B; Tables 1 and 2).

Flowcytometry results—human MSCs

Cells isolated from human iliac crest were characterized by staining for a set of accepted markers for MSCs; *CD105* and *CD166* (MSC markers), *CD34* (expressed on hematopoietic stem cells), and *CD45* (expressed on leukocytes) [34]. The flowcytometry analysis showed that MSCs markers increased during culturing time and with passages. Cell surface markers of MSCs were as follows (both donors passage 3 and 5): >92% of the cells were positive for *CD105*, >90%

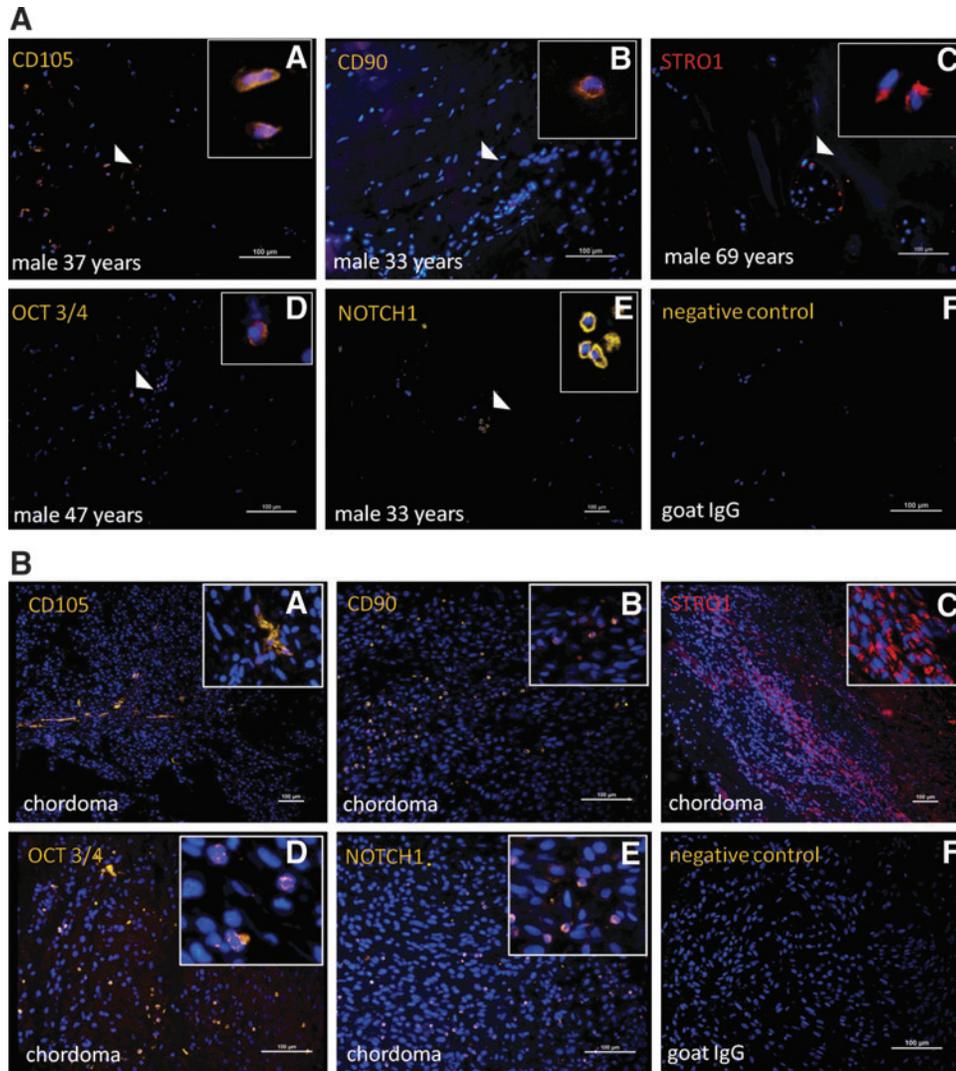


FIG. 3. (A) Immunohistochemical stainings of cells expressing mesenchymal stem/progenitor markers and NOTCH1 in human degenerated discs: (A-A) CD105+ cells, mesenchymal stem cell (MSC) marker (yellow color), (A-B) CD90+ cells, MSC marker (yellow color), (A-C) STRO1+ cells, MSC marker (red color), (A-D) OCT3/4+ cells, marker of multipotency (yellow color), (A-E) NOTCH1 marker involved in cell fate proliferation/differentiation (yellow color) with enlarged image in upper right corner and (A-F) negative control, total goat immunoglobulin G (IgG). Nuclei stained with 4,6 diamino-2 phenylindole (DAPI) (blue color). (B) Positive control: human chordoma from male donor, age 69 displaying positive staining for the stem/progenitor markers and NOTCH1 used in immunohistochemistry analyses (B-A) CD105+ cells (yellow color), (B-B) CD90+ cells (yellow color), (B-C) STRO1+ cells (red color), (B-D) OCT3/4+ cells (yellow color), (B-E) NOTCH1+ cells (yellow color) with enlarged image in upper right corner and (B-F) negative control, total goat IgG. Nuclei stained with DAPI (blue color). When sparsely distributed, positive cells are indicated by white arrowheads in images.

culture systems; however, the expression was more pronounced in pellets cultured in the conditioned medium from MSCs.

At the time point 7 days, NOTCH1+ and JAGGED1+ cells were present in the DD pellets cultivated in the conditioned MSC medium in 4/4 donors and in 3/4 donors when cultured in the conditioned NDDC medium. At the time point 14 days, NOTCH1+ cells were found in DD pellets from 3/4 donors cultured in either type of medium and JAGGED1+ cells were detected in DD pellets from 3/4 donors cultured in the conditioned MSC medium and in 2/4 donors in the conditioned NDDC medium.

At the time point 28 days, NOTCH1+ cells were found in DD pellets cultured in 2/3 donors for the con-

ditioned MSC medium and in 1/3 donors for the conditioned NDDC medium. JAGGED1+ expression was detected in 3/3 donors in the conditioned MSC medium, while no expression was found in the pellets cultivated in the conditioned NDDC medium at the time point 28 days.

The expression of NOTCH and JAGGED was overall more pronounced in the DD pellets cultured in the conditioned MSC medium compared to the conditioned NDDC medium.

When summarizing the scores for all donors for one time point, the DD pellets cultivated in the conditioned MSC medium displayed a higher expression of all markers compared to DD pellets cultivated in the conditioned NDDC medium (Fig. 4; Table 3).

TABLE 1. SUMMARY OVER RESULTS OF EXPRESSION FROM THE INVESTIGATED MARKERS IN THE HUMAN DEGENERATED DISC TISSUE BIOPSIES (N=15)

Marker	Donor 1 Age 35 Male	Donor 2 Age 69 Male	Donor 3 Age 37 Female	Donor 4 Age 40 Male	Donor 5 Age 43 Female	Donor 6 Age 50 Male	Donor 7 Age 56 Female	Donor 8 Age 41 Female	Donor 9 Age 33 Male	Donor 10 Age 37 Male	Donor 11 Age 65 Female	Donor 12 Age 47 Male	Donor 13 Age 61 Male	Donor 14 Age 52 Female	Donor 15 Age 34 Male
CD105	1	3	2	1	1	2	2	1	1	2	1	2	2	2	2
CD90	0	1	2	1	2	1	2	0	1	1	0	1	0	1	1
STRO1	2	3	2	3	2	2	2	2	2	2	2	3	1	0	2
OCT3/4	3	1	3	3	1	2	1	3	1	1	1	1	1	2	0
NOTCH1	3	3	2	4	2	3	2	3	2	3	2	2	1	1	1

Score for tissue biopsies: 0 = no detectable expression of marker, 1 = < 2% positive cells/section, 2 = 2%–5% positive cells/section, 3 = 5%–10% positive cells/section, and 4 = > 10% positive cells/section.

TABLE 2. SUMMARY OVER RESULTS OF EXPRESSION FROM THE INVESTIGATED MARKERS IN HUMAN CHORDOMA TISSUE BIOPSIES (N=2)

Marker	Donor 1 Age 69 Male	Donor 2 Age 70 Female
CD105	4	3
CD90	4	2
STRO1	4	4
OCT3/4	4	2
NOTCH1	4	3

The chordoma tissue was used as a positive control for the stem/progenitor markers used in the analyzes.

Score for tissue biopsies: 2 = 2%–5% positive cells/section, 3 = 5%–10% positive cells/section, and 4 = > 10% positive cells/section.

Histology of DD cell pellets stimulated with conditioned medium

The DD cells formed cell pellets with various rounded shapes in the 3D cell cultures with the conditioned NDDC medium. Matrix accumulation was seen in pellets at the time points day 14 and 28. In the cultures with the conditioned MSC medium, the DD cells formed cell pellets with rounded or irregular shapes in the 3D cell cultures. No distinct matrix accumulation was observed in DD pellets cultured in the conditioned MSC medium at any time point (Fig. 5).

Discussion

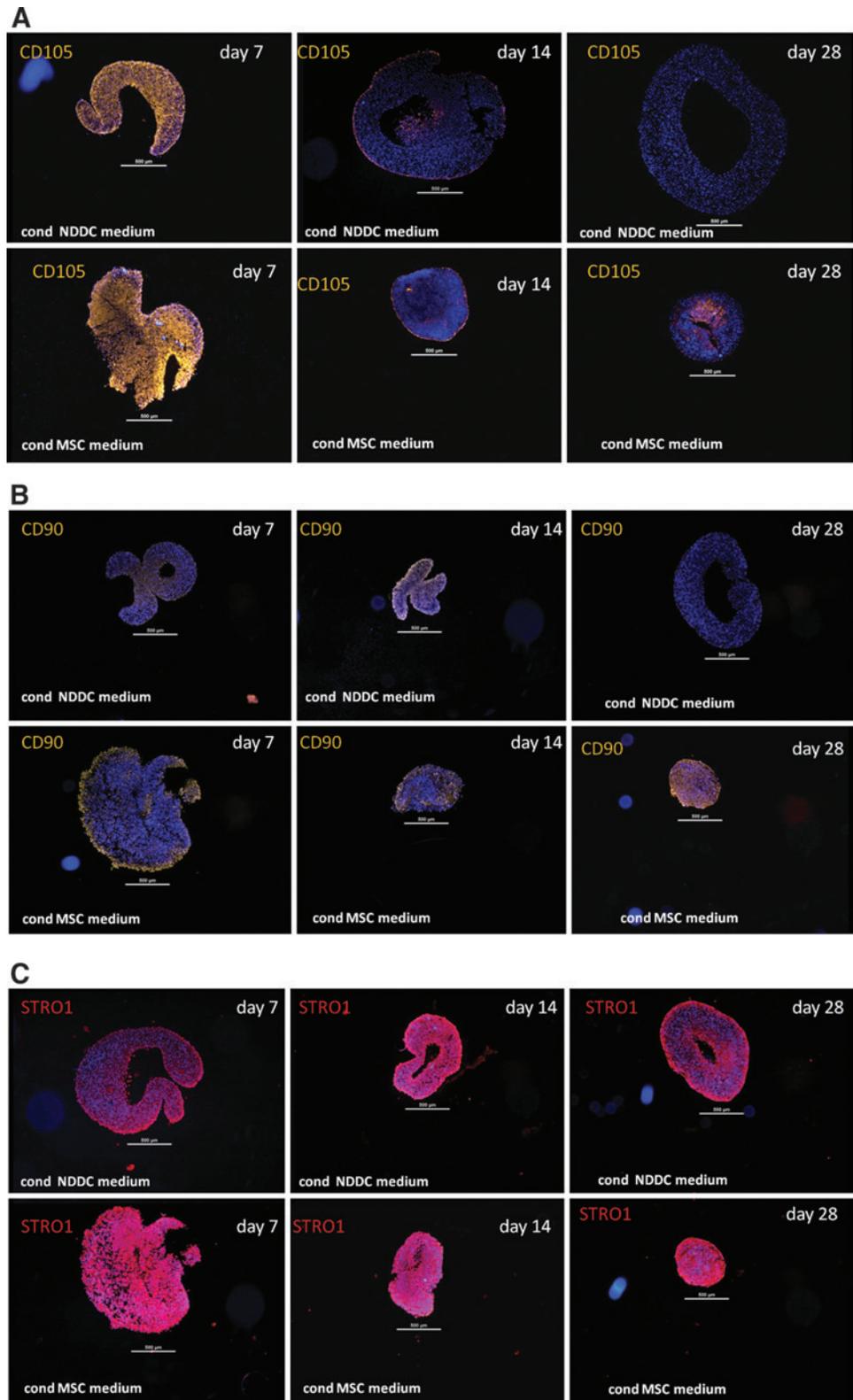
The results from the present study shows the presence of cells with expression on both gene and protein level of OCT3/4, marker of multipotency and NOTCH1, regulator of cell fate, together with the classical MSC markers CD105, CD90, and STRO-1 are present in human degenerated IVDs. However, a relatively low expression of these markers was found in the IVD tissue which is to be expected. This indicates that primitive minor MSC populations are present within DDs. The results from this study is in alignment with a few other studies that has identified the presence of skeletal progenitor cells and stem/progenitor cells within human DDs [13,14], as well as in normal IVDs of other mammal species [11,12].

However, the presence of OCT3/4+ cells in the human IVD detected in this study is a novel finding that has not been described before. Further, the *chordoma* tissues displayed the presence of all the markers and illustrate that MSCs are present in this tumor type, including that *chordoma* tissue can function as a positive control on the protein level for the investigated markers. *Chordomas* are rare malignant bone tumors that in most cases develop at the sacrococcygeal region or the skull base, but in rare cases also in the mobile spine. They are believed to derive from notochordal cell remnants in the IVD and/or vertebrae and usually have a locally invasive growth pattern and metastasize late [37–40].

Furthermore, in recent years, cancer also has come to be believed as a disease which at least in some cases, originates from abnormal hyperactive stem cells [14,41–43] and it has been observed that *chordomas* can develop from the IVD region [39].

In addition, in vitro 3D cultures supported the existence of cells expressing stem/progenitor markers and indicated that DD cells can be influenced by paracrine stimulation with soluble factors from MSC as seen in the pellet cultures. This

FIG. 4. (A–F) of selected immunohistochemical stainings of degenerated disc cell pellets expressing mesenchymal stem/progenitor cell markers at time points day 7, 14, and 28 days cultured in conditioned medium from nondegenerated discs (cond NDDC medium) (*upper row*) or conditioned medium from bone marrow-derived MSCs (cond MSC medium) (*lower row*): (A) CD105+ cells, MSC marker (*yellow color*) (male, age 40), (B) CD90+ cells, MSC marker (*yellow color*) (male, age 34), (C) STRO1+ cells, MSC marker (*red color*) (male, age 34), (D) OCT3/4+ cells, marker of multipotency (*yellow color*) (male, age 34), (E) NOTCH1+ cells, marker of cell fate proliferation/differentiation (*yellow color*) (male, age 40), and (F) JAGGED+ cells (ligand to NOTCH1), (*yellow color*) (male, age 34). Nuclei stained with DAPI (*blue color*).



was demonstrated by the higher cellular expression of the markers OCT3/4, STRO1, CD90 and CD105, NOTCH and JAGGED1 (see the summed score for all markers in Table 3) in the DD cell pellets from all donors when cultured with the conditioned medium from MSCs compared to the medium from non-DDs cells.

During embryonic development of cartilage, mesenchymal progenitor cells undergo extensive proliferation followed by condensation. The pellet mass culture is a 3D cell culture system that mimics embryonic chondrogenesis and has been demonstrated to be a good model for studies involving processes, such as tissue induction and cellular

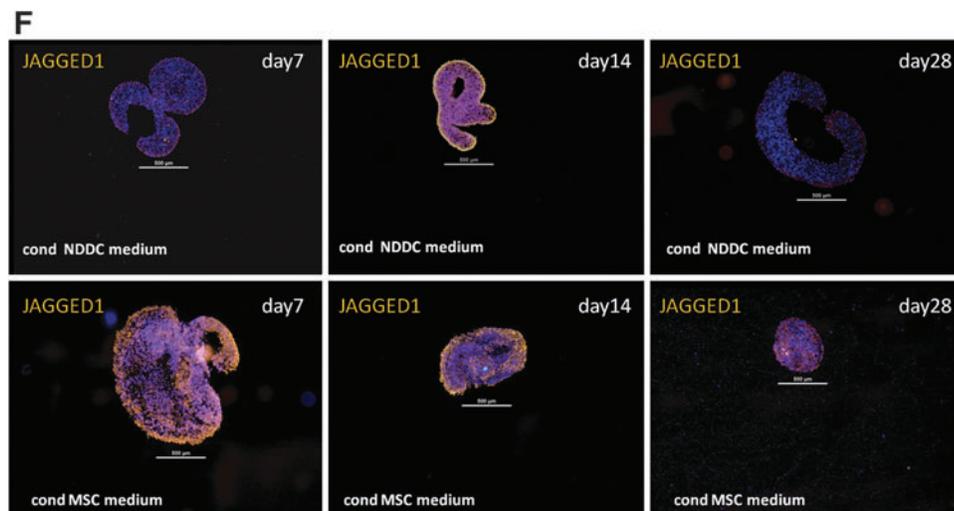
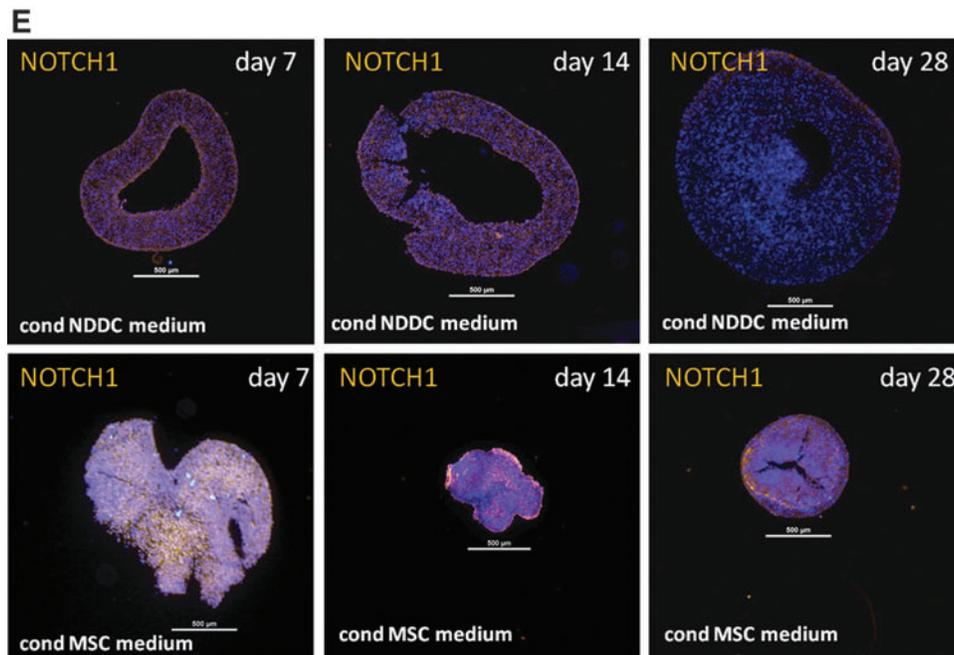
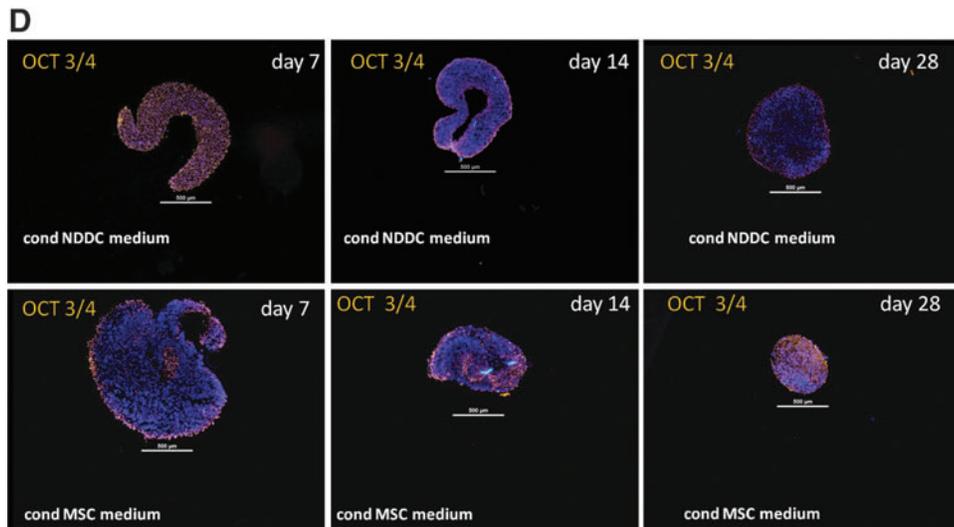


FIG. 4. (Continued).

TABLE 3. SUMMARY OVER RESULTS OF EXPRESSION FROM THE INVESTIGATED MARKERS IN THE HUMAN DEGENERATED DISC CELL PELLET (DD PELLETS) CULTURES (N=4)

Marker	Day	Donor 1		Donor 2		Donor 3		Donor 4		Total sum of score/time point (all patients)	
		Female age 32		Male age 61		Male age 41		Male age 34		Cond MSC	Cond NDDC
		Cond MSC medium	Cond NDDC medium								
CD105	7	3	0	2	2	3	3	2	2	10	7
	14	2	0	3	0	1	1	3	1	9	2
	28	2	1	No data	No data	2	0	3	0	7	1
CD90	7	0	0	2	2	1	2	2	0	5	4
	14	0	0	2	1	No data	1	3	1	5	3
	28	1	0	No data	No data	3	0	2	0	6	0
STRO1	7	3	2	2	2	3	1	3	1	11	6
	14	3	2	3	1	2	1	3	3	11	7
	28	3	2	No data	No data	3	1	3	2	9	5
OCT3/4	7	2	3	1	1	1	0	2	2	6	6
	14	1	0	2	0	0	1	3	0	6	1
	28	1	0	No data	No data	2	0	2	0	5	0
NOTCH1	7	2	2	2	1	3	0	2	1	9	4
	14	0	2	2	1	2	0	2	2	6	5
	28	0	0	No data	No data	1	0	1	1	2	1
JAGGED1	7	2	1	1	0	3	1	3	1	9	3
	14	0	0	2	1	1	0	2	2	5	3
	28	2	0	No data	No data	3	0	1	0	6	0
Total score/ donor (all time points)		27	15	24	12	34	12	42	19		

Score for pellets: 0=no detectable expression of marker, 1 = < 10% positive cells/section, score 2=10%-50% positive cells/section, and score 3 = > 50% positive cells/section.

The DD pellets cultivated in conditioned medium from mesenchymal stem cells (cond MSC medium) displayed a higher total sum of scores (all time points counted and summed) compared to degenerated disc cells pellets cultivated in conditioned medium from non-degenerated disc cells (conditioned NDDC medium).

differentiation and results in a differentiated, matrix-producing cell type when cultured in the chondrogenic medium [35,44,45]. Recently, an in vitro (pellet mass culture) study has shown that cell proliferation and chondrogenesis are tightly coupled processes when MSCs differentiate into chondrocytes [46,47]. In the same study, label retaining cells

(syn. slow cycling cells e.g., stem cells) [48] were observed in MSC pellets by labeling of DNA methods. This type of interactions between proliferation and differentiation has been observed during embryonic cartilage development [49,50].

Furthermore, positive effects on matrix formation by pellet cocultures of DD cells and MSCs have been reported [36,51].

Human degenerated disc cell pellets cultivated in two types of conditioned media

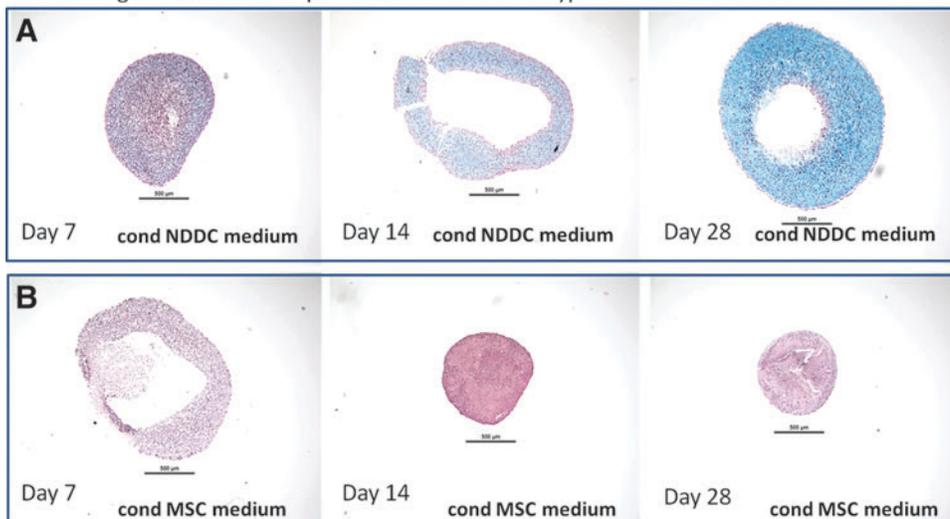


FIG. 5. Alcian blue Van Gieson histological stainings (proteoglycans appears blue and collagens red) of a set of representative human (male age 40) degenerated discs cell pellets cultured in: (A) cond NDDC medium. Matrix accumulation (blue color) was seen at time points 14 and 28 days. (B) Images of degenerated discs cell pellets cultured in cond MSC medium with no distinct visible matrix accumulation in any time point.

Observations from this study, where cell populations displayed ability to be stimulated *in vitro* to differentiate or to maintain a more undifferentiated state due to soluble factors secreted by different cell types suggest that DD cells may be possible to stimulate *in vivo*.

The limitations of the present study are that a relatively small sample size was used for the *in vitro* analyses. However, the soluble factors involved and their exact mechanisms in stimulation of DD cells remain to be identified and investigated.

In conclusion, this study showed that minor cell populations expressing stem cells and progenitor markers, including the marker of multipotency OCT3/4 are present in the adult human degenerated IVD. It was also observed that the chordoma biopsies displayed a higher expression of the stem cell/progenitor markers OCT3/4, NOTCH1, CD90 and CD105 compared to the DDs biopsies.

Possibly, these local immature cell populations present in the human degenerated IVD take part in repair attempts and may also be possible to stimulate with soluble factors as observed in the *in vitro* experiments performed in this study.

Further, this study shows that DDs cells can respond differently to stimuli through intercellular paracrine signaling; for example, soluble factors secreted from variant cell types *in vitro* and that the 3D-model used is a good model for such analyses. An increased knowledge about dynamic cell signaling processes can be a useful tool when developing future biological treatment options for patients with degenerated IVD disease.

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Author Disclosure Statement

No competing financial interests exist.

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