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Major traits of the senescent phenotype of nucleus pulposus intervertebral disc cells persist under the specific microenvironmental conditions of the tissue

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ABSTRACT

Intervertebral discs (IVDs) are the joints of the spine, mainly consisting of extracellular matrix (ECM) with a low number of cells embedded therein. Low cellularity stems from nutrient deprivation due to the lack of blood supply, as well as from the hypoxic and hyperosmotic conditions prevailing in the tissue. Intervertebral disc degeneration (IDD) has been firmly connected with low back pain, a major age-related disease, whereas degenerated discs have been characterized by increased proteolytic activity and accumulation of senescent cells. While the catabolic phenotype of senescent IVD cells has been documented, whether this phenotype is preserved under the harsh conditions met in the IVD milieu has never been investigated. Here we showed that a combination of low glucose, hypoxia, high osmolality and absence of serum is anti-proliferative for young disc cells. In addition, we demonstrated for the first time that classical senescence markers, namely $p16^{INK4a}$, $p21^{WAF1}$ and ICAM-1, remain up-regulated in senescent cells under these conditions. Finally, up-regulation of the main senescence-associated ECM degrading enzymes, i.e. MMP-1, -2 and -3 was maintained in this strict environment. Conservation of IVD cells' senescent phenotype under the actual conditions these cells are confronted with *in vivo* further supports their possible implication in IDD.

1. Introduction

Low back pain (LBP) is a major age-related disease ranked first among the leading causes of chronic disability in adults globally, thus imposing to the health care systems of modern societies a great financial burden (Hong et al., 2013; Katz, 2006; Vos et al., 2012; Wong et al., 2017). Even though numerous risk factors have been assessed for their implication in LBP (Wong et al., 2017), intervertebral disc degeneration (IDD) has been thus far considered a concrete etiologic factor for the prevalence of this multi-parametric spinal disorder (Luoma et al., 2000; Urban and Roberts, 2003).

Intervertebral discs (IVDs) lie between the vertebral bodies constituting the joints of the spine, as they are fully responsible for its posture, motion and flexibility. They consist of two distinct regions, the outer annulus fibrosus (AF) containing fibroblast-like cells and an inner gelatinous core (the nucleus pulposus, NP) with cells resembling chondrocytes (Urban and Roberts, 2003). IVDs are mainly composed of extracellular matrix (ECM) and more specifically of collagen type I produced by AF cells and of collagen type II and aggrecan predominately produced by NP cells (Urban and Roberts, 2003). ECM homeostasis is preserved by a low number of cells; these maintain the subtle dynamic equilibrium between synthesis and degradation of ECM components indispensable for a healthy tissue. IVD cells are kept to low numbers due to the avascular nature of the tissue that imposes nutritional restraints. Nutrition of the NP is achieved only via diffusion through the cartilaginous endplate of the upper and lower vertebrae (Urban, 2002). More specifically, the absence of blood supply results in low glucose concentrations and limited oxygen availability, even though metabolism is glycolytic. In addition, osmolality of the IVD is higher than that prevailing in most tissues of the body due to the negatively charged proteoglycans of the ECM that attract cations from the extracellular environment, while the mechanical forces the tissue is subjected to lead to water loss. This further increases osmolality and provokes its diurnal fluctuations (Urban, 2002).

Besides low cellularity that characterizes IVDs, cellular senescence has been also described in aged or degenerated discs. Senescence of IVD

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cells was first reported by Roberts et al. in herniated discs *in vivo* based on their positive senescence-associated β galactosidase (SA-β-Gal) staining (Roberts et al., 2006) and has been confirmed thereafter (Feng et al., 2016; Gruber et al., 2009; Gruber et al., 2007; Jeong et al., 2014; Kim et al., 2009; Le Maitre et al., 2007).

It is well established that there are two types of senescence: replicative senescence being the result of the telomere attrition occurring after consecutive cell divisions and stress-induced premature senescence (SIPS), which is the outcome of the exposure of the cells to subcytotoxic doses of environmental insults (Campisi and d'Adda di Fagagna, 2007; Toussaint et al., 2000). Both replicative senescence and SIPS programs have been linked to the activation of a DNA damage response, since on one hand telomere attrition is perceived by the cells as a disturbance of their chromosomal integrity and on the other hand stresses leading to SIPS are mainly genotoxic (Bartkova et al., 2006; d'Adda di Fagagna et al., 2003; Herbig et al., 2004; Serrano et al., 1997). Even though IVD cells' senescence has been attributed to telomere erosion due to replicative exhaustion by some groups (Jeong et al., 2014; Kim et al., 2009), SIPS seems to be more plausible given the very low proliferation rate of IVD cells *in vivo* combined with the harsh conditions and often genotoxic stresses they constantly face (Kletsas, 2009).

Senescent cells in general are characterized by a pro-inflammatory, catabolic phenotype - called senescence associated secretory phenotype (SASP) - and it has been hypothesized that through this phenotype they may affect tissue homeostasis locally and contribute to the development of age-related diseases (Acosta et al., 2008; Coppe et al., 2008; Nelson et al., 2018; Rodier and Campisi, 2011; Rodier et al., 2009). In favor of this hypothesis, removal of senescent cells by chemical means, including senolytic drugs, has been shown to ameliorate tissue homeostasis by delaying several age-associated pathologies (Baker et al., 2011; Zhu et al., 2015). Senescent IVD cells also display an inflammatory and catabolic phenotype (Dimozi et al., 2015; Hiyama et al., 2010; Le Maitre et al., 2007), while we have recently shown that they share a common transcriptional profile of SASP factors irrespective of the stimulus used to trigger senescence (Vamvakas et al., 2017).

However, characterization of the senescent IVD phenotype has been performed so far in cells cultured under classical culture conditions that do not simulate closely the conditions of the intact disc and especially of the NP. Given that several studies have provided evidence suggesting that the harsh IVD environment may affect the function of resident cells (Bibby et al., 2005; Bibby and Urban, 2004; Grunhagen et al., 2006; Haschtmann et al., 2006; Holm et al., 1981; Razaq et al., 2003; Urban, 2002; Wuertz et al., 2008, 2007), the question that arises is whether senescent IVD cells retain their characteristics under the specific conditions routinely met in the non-degenerate tissue *in vivo* mentioned above. Accordingly, aim of the current study was to explore the role of the IVD environment in the preservation of IVD cells' senescent phenotype. For that reason, early-passage and senescent bovine NP IVD cells were cultured under typical conditions (normal osmolality, hyperoxia, high glucose concentration and in the presence of serum, thereafter termed "classic" conditions), as well as under conditions that prevail in the disc (hyperosmolality, low oxygen and glucose concentration and in the absence of serum, thereafter termed "IVD" conditions) to assess if the senescent phenotype is affected by the specific environment of this tissue.

2. Materials and methods

2.1. Cell culture conditions

Our study was conducted using NP IVD cells from bovine caudal discs. NP cells were selected over AF cells, since IVD conditions become more extreme in the gelatinous core of the disc due to its anatomy (e.g. the NP is more distant from blood supply than the outer AF) and biochemical composition (NP ECM is rich in negatively charged proteoglycans) (Urban, 2002). Furthermore, bovine cells were selected given their easy acquisition and their high proliferation rate and extensive *in vitro* lifespan in comparison to human cells isolated from surgical specimens which are characterized by a very low proliferative potential and limited *in vitro* lifespan (our unpublished observations), possibly because they usually derive from pathological cases. Still, bovine caudal discs have been recognized as an appropriate biological and biomechanical model for the study of the human lumbar disc (Alini et al., 2008), as it has been well established that the swelling pressure of bovine coccygeal discs is similar to that of the discs of a person in the resting position and that *in vitro* proteoglycan and matrix synthesis in response to hydrostatic pressure occurs with similar rates in human and bovine discs (Alini et al., 2008; Ishihara et al., 1996; Ohshima et al., 1995; Oshima et al., 1993).

Establishment of bovine caudal NP cell cultures has been described previously (Pratsinis and Kletsas, 2007). Routine culture was implemented in complete Dulbecco's modified Eagle's medium (DMEM) with osmolality of 300 mOsm/kg H_2O, supplemented with penicillin (100 U/ml), streptomycin (100 mg/ml) and 10% (v/v) FBS (all media from Gibco, Life Technologies Europe BV, Thessaloniki, Greece) in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were subcultured when confluent by using a trypsin/citrate (0.25%/0.30% w/v) solution. The two distinct culture conditions tested throughout this work are termed "classic" (10% FBS, 4.5 mg/ml glucose, 300 mOsm/kg H_2O, 20% O_2) and "IVD" (serum-free, 0.9 mg/ml glucose, 400 mOsm/kg H_2O, 2% O_2). Exceptionally for conditioned media collections (see below), serum was not included in classic conditions, retaining the other parameters unchanged. Osmolality of the culture medium was adjusted by adding a solution containing 5 M NaCl and 0.4 M KCl to the standard iso-osmotic medium and measured as previously described using a cryoscopic osmometer (Osmomat 030, Gonotec, Berlin, Germany) (Mavrogonatou and Kletsas, 2009, 2012; Mavrogonatou et al., 2015). Hypoxic conditions were implemented through culture of the cells inside an InVivo$_2$ 400 hypoxia workstation (Ruskinn Technology LTD, Bridgend, UK).

2.2. Cellular senescence

In order to induce SIPS, NP cell cultures were exposed to γ-irradiation in a ^{60}Co gamma source (Gamma Chamber 4000 A, Isotope Group, Bhadha Atomic Research Company, Trombay, Bombay, India) at a rate of 2.5 Gy/min. Cells were then serially subcultured until cultures became senescent (Liakou et al., 2016; Papadopoulou and Kletsas, 2011; Vamvakas et al., 2017).

2.3. Bromodeoxyuridine (BrdU) incorporation assay

Incorporation of 5-bromo-2′-deoxyuridine (BrdU) was estimated as previously described (Dimozi et al., 2015; Vamvakas et al., 2017). In detail, cells were seeded on glass coverslips at a density of 2×10^4 cells/cm^2 and cultured as indicated. BrdU was added to the culture medium at 50 μM for 48 h. After fixation with 4% (v/v) formaldehyde in PBS for 10 min, permeabilization with 0.2% Triton X-100 in PBS for 10 min, and DNA hydrolysis with 2 N HCl for 30 min, samples were incubated overnight with a mouse monoclonal FITC-conjugated anti-BrdU antibody (clone BMC9318, Roche/Sigma, St. Louis, MO, USA) at 4 °C, and finally counter-stained with 1 μg/ml 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) solution for 5 min. Washes with phosphate buffered saline (PBS) were applied between each step. Coverslips were mounted on microscopy slides and observed on an upright Zeiss Axioplan 2 UV/visible microscope (Zeiss, Jena, Germany) with a 40x objective. Quantification of the results was made at fields containing approximately 200 cells.

2.4. Senescence-associated β-galactosidase (SA-β-Gal) staining

SA-β-Gal staining was performed as reported before (Dimozi et al., 2015; Vamvakas et al., 2017). Cells seeded on glass coverslips were washed in PBS and fixed with 3% (v/v) formaldehyde for 5 min. After washing with PBS, a fresh SA-β-Gal solution (1 mg/ml X-Gal in a buffer containing 40 mM citric acid/sodium phosphate pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM sodium chloride and 2 mM magnesium chloride) was added and samples were incubated at 37 °C (in the absence of CO_2). Coverslips were mounted on microscopy slides and observed under a Zeiss Axioplan 2 UV/visible microscope. Quantification of the results was made at fields containing approximately 200 cells.

2.5. RNA extraction and quantitative RT-PCR analysis

RNA extraction from young and senescent cells cultured in 100-mm tissue culture dishes was performed using Trizol (Invitrogen, Paisley, UK) following the manufacturer's instructions, as described before (Dimozi et al., 2015; Vamvakas et al., 2017). First-strand cDNA synthesis was performed with the PrimeScript RT Reagent Kit (Takara, Tokyo, Japan) in 20-μl reactions using 1 μg RNA as template. Real-time PCR experiments were performed in 20 μl using 100 times diluted cDNA and the qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems Ltd, London, UK) in a MX3000 P cycler (Stratagene, La Jolla, CA, USA). Relative gene expression of senescent cells compared to that of young ones was estimated as previously reported (Dimozi et al., 2015; Vamvakas et al., 2017) using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene. Primers used in this study were designed with Beacon Designer 7.0 (PREMIER Biosoft International, Palo Alto, CA) and are shown in Table 1.

2.6. Conditioned media and MMP content assessment

Confluent cultures were washed repeatedly with serum-free medium and then incubated for 48 h with serum-free (classic or IVD) medium (see 2.1). Conditioned media (CMs) were harvested in pre-chilled containers, centrifuged (1000 × *g*, 10 min, 4 °C), aliquoted and frozen (−70 °C) until further use, as previously described (Pratsinis et al., 2013), while total cell number and viability of the cultures were recorded.

2.6.1. Assessment of MMP content using fluorescent substrates

CMs were mixed at a 1:1 ratio with MMP incubation buffer [50 mM Tris (pH: 7.6), 150 mM NaCl, 10 mM $CaCl_2$, 1 μM $ZnCl_2$] containing the fluorogenic substrate Dabcyl-Gaba-Pro-Gln-Gly-Leu-Glu-(EDANS)-AIa-Lys-NH2 (TNO211, Anaspec, Fremont, CA, USA) at a final concentration of 10 μM (Liakou et al., 2016). TNO211 is a fluorescence resonance energy transfer (FRET) substrate for MMP-1, -2, -3, and -9, i.e. cleavage of the Gly-Leu bond by these MMPs abrogates EDANS fluorescence quenching by Dabcyl. After incubation at 37 °C in the dark, fluorescence was monitored at 480 nm after excitation at 340 nm, using a Fluostar Optima microplate reader (BMG Labtechnologies, Offenburg, Germany). CM from HT1080 cells was included as positive control. Total MMP content of CMs (i.e. both latent and active forms) was assessed after their incubation at 37 °C for 3 h with 1 mM 4-aminophenylmercuric acetate (APMA).

A more specific approach was followed for assessing MMP-1, i.e. the SensoLyte® Plus 520 MMP-1 Assay Kit (Anaspec), involving a first step of separation of MMP-1 from each CM by means of a microplate coated with monoclonal anti MMP-1 antibody, followed by incubation with the FRET peptide 5-FAM/QXL™520. In this case, fluorescence was monitored at 520 nm after excitation at 480 nm, using a Fluostar Optima microplate reader.

2.6.2. Gelatin zymography

MMP-2 and -9 content of the CMs was assessed by gelatin zymography, essentially as described (Pratsinis et al., 2013). Briefly, following separation of CMs under non-reducing conditions on 10% SDS-polyacrylamide gels containing 0.05% (w/v) gelatin (Sigma), elution of SDS from the gels by shaking in substrate buffer (5 mM $CaCl_2$, 50 mM Tris-HCl [pH 7.4]) containing 2.5% Triton X-100, incubation for 48 h at 37 °C in substrate buffer without Triton X-100, and staining with Coomasie Brilliant Blue R250 (Sigma), gelatin-degrading enzymes were identified as clear bands against a blue background. The intensity of the bands was quantified after capture with a CCD camera connected to a computer, using the Advanced Image Data Analyzer (AIDA 4.22.034, raytest Isotopenmessgeraete GmbH, Straubenhardt, Germany) software. CM derived by HT1080 human fibrosarcoma cells served as positive control.

2.7. Statistical analysis

Data presented in the graphs are means ± standard deviations. Differences were considered statistically significant when $p < 0.05$ (Student's t test).

3. Results

3.1. Exposure to γ-irradiation renders bovine NP IVD cells senescent

Ionizing radiation, a principal DNA damaging agent, was selected to trigger senescence of bovine NP cells in this study. To induce senescence, bovine NP IVD cells were exposed to γ-irradiation followed by serial subcultures. Ionizing radiation-induced senescent NP cells showed distinct morphological characteristics in comparison to their untreated counterparts (enlarged and flattened shape, cytoplasm filled with vacuoles, etc.), accompanied by a great reduction in their proliferative potential and an increase in the percentage of SA-β-Gal-positive cells in the culture (Fig. 1). In detail, senescent NP cells after exposure to γ-irradiation exhibited 1.9 ± 0.1% BrdU incorporation, while young cells showed 91.4 ± 3.2% BrdU incorporation. At the

Table 1
Primers used for qPCR analysis.

Target gene	Forward Primer	Reverse Primer
$p16^{INK4a}$	AATGCCAGTTTCCAGTGCCAATC	GGACTGTAGCCCGCCAAGAATAC
$p21^{WAF1}$	GGCTGTGGGTAGGATGTGCTAAG	CTTGAGAGGCTTCCAGTCTAGGTG
icam1	ACTGAGATGTGTCCGACTCTTGTG	TTCATGTGACCCTGTGGTGTAGC
mmp-1	ACACTCTCCGTGATGTCTTCTCTC	GTGGTGGTTGCTCATACATTCCTG
mmp-2	CTGGATGCTGTGGTGGACCTG	GCTTCCGAACTTCACGCTCTTC
mmp-3	ACAATGGACAAAGGATACATCAGG	TTCGGTTGAGTGCTGGAGAC
adamts-4	CAGGGTGGTGGTGATAGGTGTAC	AGGAAAGCTGGGCAGGATGAAC
adamts-5	GAGCCACAGGGACGACTTCTTG	TTGGAGCCGACTTCTTGGGAAC
acan	AGAAGAGAGCCAAACAGC	TTGTGGTTGCCTCTGAAT
col2a1	ATCAACGGTGGCTTCCACT	TTCGTGCAGCCATCCTTCAG
gapdh	GCCATCACTGCCACCCAGAA	GCGGCAGGTCAGATCCACAA

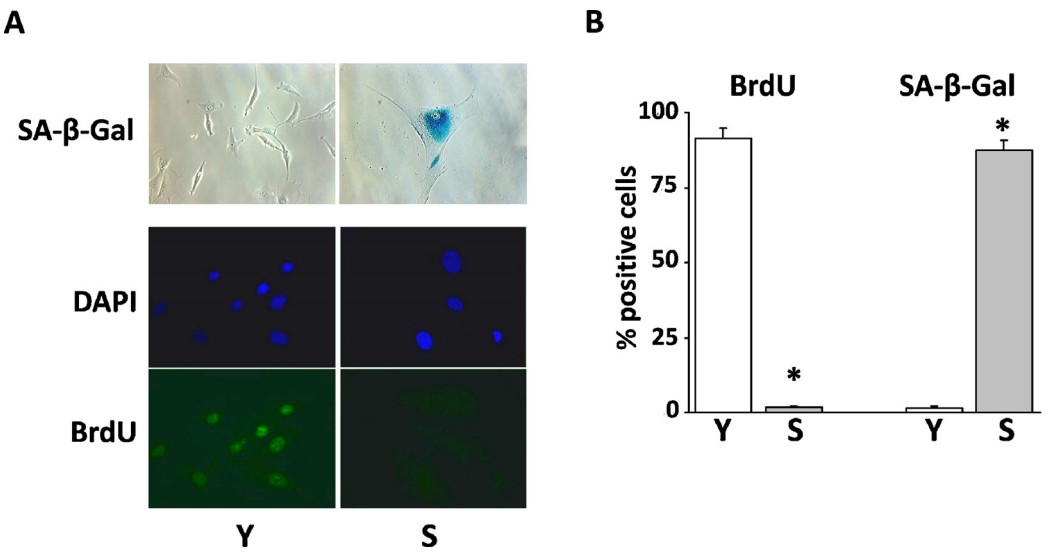

Fig. 1. Ionizing radiation provokes senescence of bovine nucleus pulposus intervertebral disc cells. (A) After exposure to γ-irradiation and serial subcultures, cells were plated on glass coverslips. For SA-β-Gal staining cells were fixed with 3% (v/v) formaldehyde, while for the estimation of BrdU incorporation, BrdU was added at a concentration of 50 μM for 48 h before fixation with 4% (v/v) formaldehyde and labeling with an anti-BrdU-FITC antibody. Samples were observed under a phase contrast and fluorescence microscope, respectively. (B) Graphical representation of means ± standard deviations of the positive for BrdU incorporation and SA-β-Gal staining cells. Asterisks represent statistically significant differences in comparison to young cells (Student's t-test, $p < 0.05$). Y: young cells, S: senescent cells.

same time, a percentage of 87.5 ± 3.3% and 1.59 ± 0.6% of SA-β-Gal positively stained cells was calculated for senescent and young NP cells, respectively. Accomplishment of senescence in NP IVD cells by ionizing radiation was further validated by the up-regulation of the known cyclin-dependent kinase inhibitor $p16^{INK4a}$ in comparison to young cells (see below, Fig. 3A).

Notably, when young NP IVD cells were exposed to IVD conditions for a short period (i.e. for 4 d), a significant reduction in their BrdU incorporation was observed (93.3 ± 2.7% and 17.9 ± 2.3% under classic and IVD conditions, respectively) (Fig. 2), which supports the anti-proliferative effect of this particular environment on IVD cells.

3.2. The senescent phenotype of NP IVD cells is generally retained under the conditions prevailing in the disc

We then assessed putative alterations in the transcriptional regulation of key genes implicated in IVD cell senescence (i.e. genes encoding cell cycle regulators, markers of senescence, ECM components and ECM degrading enzymes) under classic and IVD conditions in order to evaluate if the senescent phenotype of NP IVD cells is conserved in the IVD environment. Our findings demonstrate that classical markers of senescence were up-regulated in γ-irradiation-induced senescent NP cells even in the IVD environment. More specifically, increased $p16^{INK4a}$, $p21^{WAF1}$ and ICAM-1 mRNA levels were observed in senescent NP IVD cells under both classic and IVD conditions (Fig. 3A).

The loss of ECM components (especially of aggrecan and collagen type II) is a hallmark of NP degeneration due to ADAMTS' and especially MMPs' up-regulation (Vo et al., 2013). Accordingly, we assessed the expression levels of genes coding for the major ECM constituents of the NP, i.e. aggrecan and collagen type II, and for the major NP ECM degrading enzymes, i.e. ADAMTSs and MMPs. Regarding genes coding for aggrecan and collagen type II, senescent cells after exposure to ionizing radiation were found to up-regulate aggrecan and to down-regulate collagen type II (Fig. 3B). However, while senescence-associated aggrecan up-regulation was retained under the harsh conditions of the disc microenvironment, collagen type II down-regulation observed under classic conditions was reversed to up-regulation under IVD conditions (Fig. 3B).

Since we have previously demonstrated that senescence of NP IVD cells is characterized by a catabolic phenotype which could probably be associated with IDD (Dimozi et al., 2015; Vamvakas et al., 2017), we further investigated the transcriptional profile of selected proteases and degrading enzymes of the ECM in ionizing radiation-induced senescent NP cells. As shown in Fig. 3C, MMP-1, -2 and -3 were all up-regulated in senescent IVD cells after exposure to γ-irradiation under classic and IVD conditions. On the other hand, ADAMTS-4 mRNA levels were found to be lower in ionizing radiation-induced senescent NP cells under classic conditions and were unaltered under IVD conditions compared to young cells. Finally, no changes in the transcription of ADAMTS-5 were observed between young and senescent cells under neither classic nor IVD conditions (Fig. 3C).

3.3. MMP activity is enhanced in senescent NP IVD cells under both classic and IVD conditions

Given the established major role of MMPs in IVD senescence

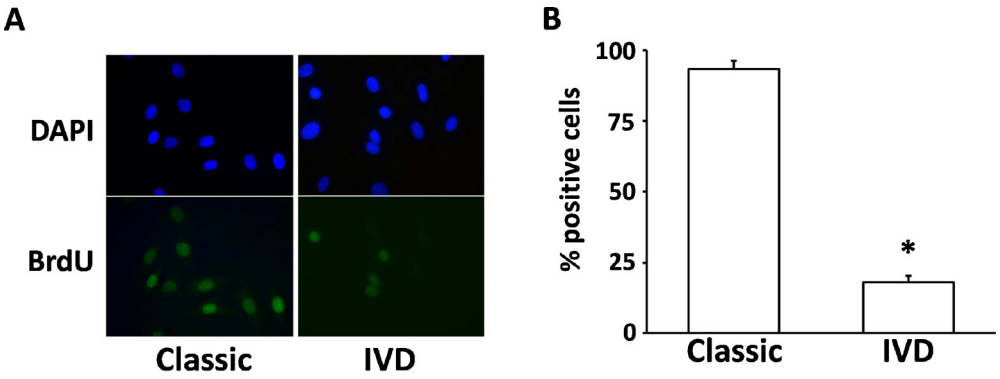

Fig. 2. IVD conditions restrain the proliferation of young nucleus pulposus intervertebral disc cells. (A) Cells were plated on glass coverslips and exposed to IVD conditions for 48 h before the addition of 50 μM BrdU for another 48 h. Subsequently, cells were fixed with 4% (v/v) formaldehyde and labeled with an anti-BrdU-FITC antibody. Samples were visualized under a fluorescence microscope. (B) Graphical representation of means ± standard deviations of the positively stained cells for BrdU incorporation. Statistically significant differences between classic and IVD conditions are marked by asterisks (Student's t-test, $p < 0.05$).

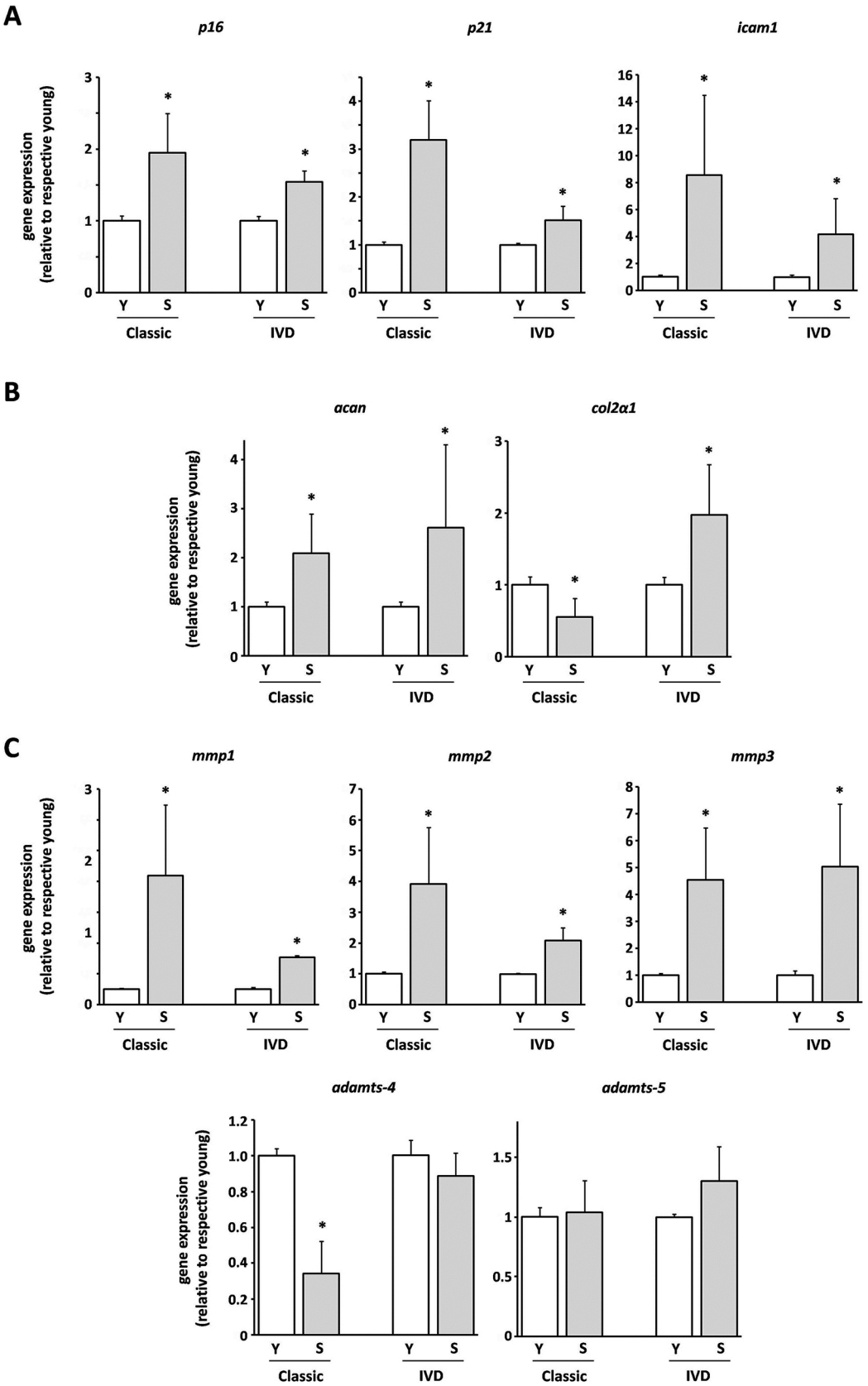

Fig. 3. Gene expression analysis of classical senescence markers (A), extracellular matrix components (B) and extracellular matrix degrading enzymes (C) in senescent nucleus pulposus intervertebral disc cells by quantitative RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase served as the reference gene. mRNA levels in the senescent cells are presented as ratios of those in young cells. Means ± standard deviations from at least three independent experiments are depicted. Asterisks pinpoint statistically significant differences compared to the respective young (Student's t test, $p < 0.05$). Y: young cells, S: senescent cells.

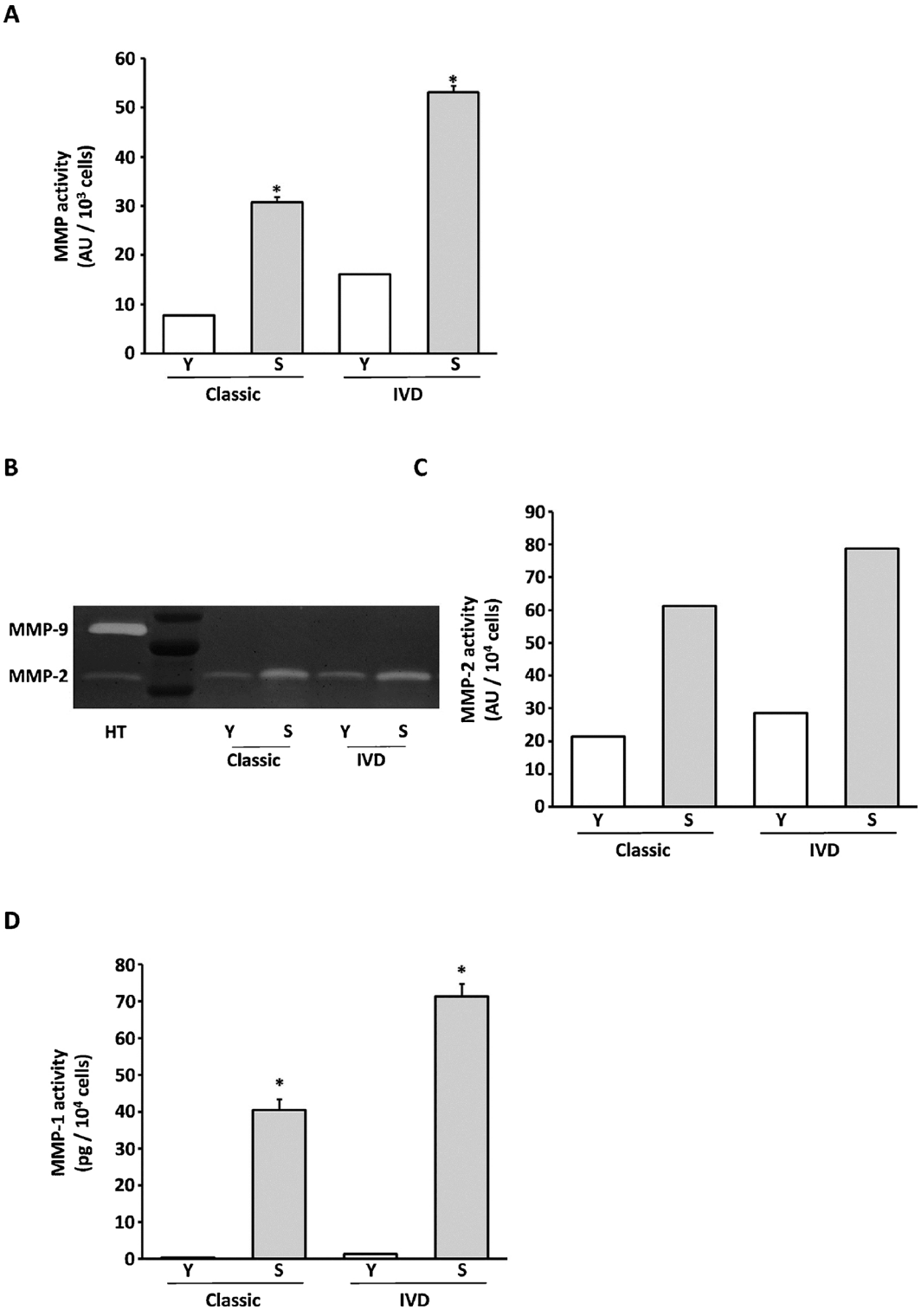

Fig. 4. MMP activity secreted by senescent nucleus pulposus intervertebral disc cells. Media conditioned by young (Y) and senescent (S) cells were tested for their capacity to digest the FRET peptide TNO211 (A) or gelatin (B) as described in Materials and methods. In (C) densitometric analysis of the zymogram is presented, while in (D) the MMP-1 activity of the conditioned media using the SensoLyte® Plus 520 Kit is presented. Medium conditioned by HT1080 (HT) served as positive control in gelatin zymography. A representative zymogram is presented in (B) and (C), while in (A) and (D) means ± standard deviations are presented. Asterisks pinpoint statistically significant differences compared to the respective young (Student's t test, $p < 0.05$).

(Dimozi et al., 2015; Le Maitre et al., 2007; Vamvakas et al., 2017) and the increased mRNA levels of MMPs in γ-irradiation-induced senescent bovine NP cells shown in Fig. 3C, we proceeded with a functional analysis in order to assess the actual MMP protein levels released by young and senescent NP cells under classic and IVD conditions. Estimation of the total MMP activity in CMs deriving from young and ionizing radiation-induced senescent NP IVD cells using the FRET substrate TNO211 - a general MMP substrate with high catalytic efficiencies for MMP-2, −9, −3, and −1 in this order (Beekman et al., 1996) – showed a greater enzymatic activity in senescent cells under

both classic and IVD conditions (Fig. 4A). Furthermore, when exploring the gelatinolytic activity secreted in IVD cells' CMs through zymography, we showed a higher MMP-2 activity in senescent NP cells when compared to young ones under classic and IVD conditions, while MMP-9 content in both young and senescent cells' CMs was below the detection limit of this method (Fig. 4B). Finally, using a highly specific assay, including a binding step of solely MMP-1 among all released molecules in the CMs of young and senescent NP cells using a monoclonal antibody before FRET analysis, revealed enhanced MMP-1 activity in senescent NP cells under classic, as well as under IVD conditions (Fig. 4C).

4. Discussion

Degeneration of the IVD has been firmly associated with LBP, which is a major age-related disease affecting a great percentage of the adult population worldwide (Luoma et al., 2000; Urban and Roberts, 2003; Wong et al., 2017). Intervertebral disc degeneration (IDD) is characterized by a shift of the equilibrium between ECM synthesis and degradation towards degradation due to the up-regulation of catabolic enzymes (Mavrogonatou et al., 2017; Roberts et al., 2000; Vo et al., 2013; Xu et al., 2014; Zhao et al., 2011). In addition, hallmark of the aged and/or degenerated disc is the increased percentage of senescent IVD cells *in vivo* (Feng et al., 2016; Gruber et al., 2007; Le Maitre et al., 2007; Roberts et al., 2006), which in turn have been connected to the manifestation of tissue degeneration based on their secretome, rich in ECM degrading molecules (Dimozi et al., 2015; Hiyama et al., 2010; Hiyama et al., 2013; Le Maitre et al., 2007; Purmessur et al., 2013; Vamvakas et al., 2017; Ye et al., 2011). However, even though IVD cells' senescent phenotype has been studied thus far, it has never been investigated under the harsh conditions prevailing in the tissue (i.e. high osmolality, nutrient deprivation, low oxygen concentration, absence of serum). It is worth mentioning that acidic pH - also characterizing the IVD microenvironment as a result of the glycolytic metabolism of the cells - was intentionally excluded from our experimental setup, since it has been well established that NP IVD cells are capable of easily adapting to an acidic environment by expressing acid-sensing ion channel 3 (ASIC3) (Uchiyama et al., 2007). Thus, aim of the current study was to explore for the first time whether markers of senescence, as well as senescence-associated alterations in major ECM components' and degrading molecules' expression and function are preserved under conditions simulating the *in vivo* IVD extracellular environment.

Taking into consideration that IVD senescence is most probably stress-induced, as a result of the exogenous and mainly genotoxic stresses IVD cells are regularly confronted with in their microenvironment *in vivo* (Kletsas, 2009), senescence of bovine NP cells in this study was triggered using a principal DNA damaging agent, that is ionizing radiation. Exposure to ionizing radiation led bovine NP IVD cells to senescence after consecutive subcultures, as confirmed by the increased percentage of SA-β-Gal-positive cells, the reduction of BrdU incorporation in the nuclei of the cells and the overexpression of the known cell cycle regulator $p16^{INK4a}$. Our findings are in accordance with previous observations showing that ionizing radiation provokes senescence in various cell types (Konstantonis et al., 2013; Lafargue et al., 2017; Liakou et al., 2016; Liao et al., 2014; Meng et al., 2003; Papadopoulou and Kletsas, 2011; Wang et al., 2016). In favor of our data, it has been shown that articular chondrocytes - which share common characteristics with the chondrocyte-like NP IVD cells - undergo senescence after exposure to ionizing radiation (Hong et al., 2010) and that cellular senescence is induced in the discs of ionizing radiation-treated mice (Nasto et al., 2013). Furthermore, it should also be noted that combined IVD conditions had a clear growth inhibitory effect on young IVD cells, since even a short-term residence of the cells in this environment resulted in decreased BrdU incorporation and in elevated $p21^{WAF1}$ mRNA levels (data not shown). This finding is in accordance to a previous study showing a negative effect on the proliferation of mesenchymal stem cells exerted by the combination of low glucose, acidic pH and high osmolality (Wuertz et al., 2008).

We then assessed if the senescent phenotype of NP IVD cells is retained under the unusual physicochemical conditions of the IVD environment. For that purpose, all IVD conditions (high osmolality, low glucose and low oxygen concentration and absence of serum) were applied simultaneously in young and senescent IVD cells. $p16^{INK4a}$ up-regulation in senescent IVD cells was observed under both classic and IVD conditions. This finding alone supports that NP cells under IVD conditions certainly remain in their state of irreversible growth arrest, since $p16^{INK4a}$ up-regulation has been considered a required convention for the establishment of senescence (Stein et al., 1999). In addition, if taken apart, conditions constituting the IVD environment have been shown to be anti-proliferative and to promote cellular senescence in other cell types; intermittent high glucose has been shown to be more efficient in promoting vascular endothelial senescence than constant high glucose levels (Maeda et al., 2015). Additionally, high NaCl concentration has been reported to accelerate cellular senescence in HeLa cells, primary mouse embryonic fibroblasts, mouse renal medullary cells and *C. elegans* (Dmitrieva and Burg, 2007). When $p21^{WAF1}$ mRNA levels were investigated, it was shown that senescence-induced $p21^{WAF1}$ up-regulation was preserved under IVD conditions. Besides, it has been shown that high osmolality increases $p21^{WAF1}$ expression in bovine NP cells (Mavrogonatou and Kletsas, 2009) and hypoxia results in a HIF-1α-dependent overexpression of $p21^{WAF1}$ and $p27^{Kip1}$ (Cho et al., 2008; Goda et al., 2003). In addition, it has been shown that low glucose concentration does not generally affect the proliferative potential and gene expression of several cell types, including IVD cells (Bibby and Urban, 2004; Stolzing et al., 2006; Wuertz et al., 2008). The transcriptional profile of ICAM-1 was also investigated in senescent NP IVD cells, as an additional marker of senescence (Gorgoulis et al., 2005) and was found to be similar under classic and IVD conditions. Although previous studies have reported that ICAM-1 up-regulation is induced by high glucose, lack of glucose under IVD conditions in our experimental setup could be compensated by high osmolality (400 mOsm/kg H_2O) in the regulation of ICAM-1 (Kado et al., 2001; Park et al., 2000). Moreover, hypoxia has been reported to stimulate the expression of ICAM-1 through NF-kB, and not HIF, activation (Mojsilovic-Petrovic et al., 2007; Winning et al., 2010).

Subsequently, we examined other characteristics of γ-irradiation-induced senescent NP IVD cells. Given that the catabolic phenotype of senescent IVD cells has been implicated in IDD (Dimozi et al., 2015; Hiyama et al., 2010; Hiyama et al., 2013; Le Maitre et al., 2007; Purmessur et al., 2013; Vamvakas et al., 2017; Ye et al., 2011), we focused among all SASP factors on the transcriptional regulation of genes encoding molecules comprising the ECM and proteolytic enzymes. Surprisingly, in contrast to previous studies showing aggrecan down-regulation and ADAMTS-5 up-regulation during senescence (Dimozi et al., 2015; Le Maitre et al., 2007; Purmessur et al., 2013), aggrecan mRNA levels were found to be elevated in γ-irradiation-induced senescent NP IVD cells under both classic and IVD conditions, which coincided with the unaltered or even lower mRNA levels of the two aggrecanases tested, i.e. ADAMTS-4 and ADAMTS-5, mainly responsible for aggrecan primary cleavage (Little et al., 1999). This discrepancy may be the result of the different species from which IVD cells were isolated (human in Dimozi et al., 2015 vs. bovine in this study), of the different experimental setup (whole IVDs in organ culture in Purmessur et al., 2013 vs. IVD cell culture monolayer in this study) or of the different stimulus used to trigger senescence (H_2O_2 in Dimozi et al., 2015, TNF-α in Purmessur et al., 2013 and ionizing radiation here). Nevertheless, in accordance to our findings, ADAMTS-4 levels have been shown to mainly increase in the degenerated AF rather than the NP (Pockert et al., 2009) and ADAMTS-5 protein expression along with ADAMTS-generated proteolytic aggrecan fragments have been found to be unchanged with degeneration in cadaveric human discs (Patel et al., 2007). Additionally, expression levels of ADAMTSs have been shown to

be controlled by numerous factors (i.e. mechanical, inflammatory, and oxidative stress), while it has been reported that aggrecanase action is not that essential in IVD as it is in articular cartilage (Sztrolovics et al., 1997; Vo et al., 2013). Furthermore, an aggrecanase with different substrate specificity from ADAMTS-4 and ADAMTS-5 has been shown to be expressed in the mouse cartilage (Rogerson et al., 2008). In contrast to aggrecan, collagen type II was found to be down-regulated in senescent NP IVD cells, in agreement with previous studies (Chen et al., 2016; Jiang et al., 2013; Purmessur et al., 2013).

Unlike the ambiguity concerning ADAMTSs, MMP up-regulation and activity has been more clearly associated with IVD ageing and degeneration (Bachmeier et al., 2009; Crean et al., 1997; Le Maitre et al., 2004; Richardson et al., 2009; Roberts et al., 2000; Weiler et al., 2002). Among all MMPs investigated so far in the literature, MMP−1, −2 and −3 have been more than any other member of this family connected with IDD and ageing (Roberts et al., 2000). For that reason, we selected these three key proteolytic enzymes in order to examine if their levels and activity are retained during ionizing radiation-induced senescence of NP cells under classic and IVD conditions. In agreement with their established role during IVD senescence mentioned above, senescence-induced up-regulation of MMP−1, −2 and −3 in NP IVD cells was maintained under the strict IVD conditions, suggesting that the catabolic phenotype of senescent IVD cells mainly determined by these proteolytic enzymes is well-kept in the IVD environment. We further examined proteolytic activity focusing on total MMP, MMP-1 and MMP-2. In accordance to transcriptional up-regulation, MMP activity levels were also found to remain increased during senescence irrespective of the extracellular conditions. Overall, the senescent phenotype of NP IVD cells is preserved in the IVD environment. It seems that even though each IVD condition - when applied separately - may differentially affect cell physiology and behavior, when combined together, one condition may counterbalance the negative effects of another one or they may even present cumulative action. Additionally, given that IVD cells may possess an inherent ability to counteract the stressful conditions met in their niche *in vivo*, preservation of the senescent phenotype under IVD conditions *in vitro* shown here may result from a prompt adaptation of these cells to an extracellular environment already familiar to them (Risbud et al., 2006; Uchiyama et al., 2007).

In conclusion, in this study we first demonstrated that classical senescent markers, as well as the catabolic characteristics of IVD senescent cells, are generally conserved under the very peculiar conditions that prevail in the disc *in vivo*, indicating their putative role in IDD. It should be noted that experiments of the current study were performed in monolayers. However, the role of another important parameter, i.e. of the three-dimensional cell culture (in alginate beads) in the preservation of the specific functions and the differential capacity of IVD cells (Wang et al., 2001) is currently under investigation in our laboratory.

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