

Human nucleus pulposus intervertebral disc cells becoming senescent using different treatments exhibit a similar transcriptional profile of catabolic and inflammatory genes

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Abstract

Purpose Chronic low back pain has been associated with intervertebral disc (IVD) degeneration, which is characterized by the accumulation of extracellular matrix (ECM)-degrading proteases and inflammatory molecules in the degenerate tissue. IVD degeneration could be the outcome of natural organismal ageing and/or of the exposure of the disc to cumulative stressful environmental stimuli and is accompanied by an increased population of senescent cells in the tissue. On the other hand, senescent cells are known to secrete proteolytic enzymes and inflammatory molecules, which can contribute to ECM catabolism. The aim of this study was to investigate the transcriptional profile of selected metalloproteinases (MMPs) and inflammatory mediators in human nucleus pulposus IVD cells that became senescent using three different approaches: serial subculturing, exposure to ionizing radiation and p16INK4a overexpression.

Methods Gene expression was assessed using quantitative RT-PCR and protein levels were determined by western blot analysis. The proliferative potential of the cells, as well as the percentage of senescent cells in the population were estimated by nuclear BrdU incorporation and by senescence-associated β galactosidase staining, respectively.

Results All senescent cells showed a similar regulation of MMP-1, -2, -3, -9, interleukin (IL) 6, IL8 and interferon γ at the level of transcription, with only some quantitative differentiations observed in p16INK4a-overexpressing cells.

Conclusions Data described here suggest that senescent cells may have similar functions in IVD homeostasis, irrespective of the origin of senescence induction.

Keywords Senescent intervertebral disc cells · p16^{INK4a} overexpression · SASP · MMPs · Interleukins

Introduction

Even though intervertebral disc (IVD) degeneration has been associated with the occurrence of low back pain, this widespread spinal pathology still remains nowadays a challenging condition to treat. Disc degeneration in vivo may be the result either of ageing or of exposure of the organism to repetitive external insults [1]. Nevertheless, whatever the origin of degeneration is, it is always accompanied by the accumulation of extracellular matrix (ECM)-degrading molecules and inflammatory mediators. For example, a number of matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinase (TIMPs) and disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSSs) have been identified in degenerate discs [2–7], while elevated levels of cytokines, such as interleukins (ILs), tumour necrosis factor α (TNF α) and interferon γ (IFN γ) have been described for lumbar disc herniation and other IVD disorders [8–10]. In addition, degenerate IVDs are characterized by the presence of a high number of senescent cells, as identified by their positive senescence-associated β galactosidase (SA- β Gal)

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staining and by the overexpression of p16^{INK4a}, a cell cycle inhibitor that is also a marker of replicative and stress-induced senescence [11–14]. On the other hand, it is known for several cell types that senescent cells express and secrete a common set of catabolic and pro-inflammatory proteins—collectively termed senescence-associated secretory phenotype (SASP) [15]—including several MMPs and inflammatory cytokines. Accordingly, it may be suggested that the accumulation of senescent cells may contribute to the ECM catabolism and inflammation observed during IVD degeneration.

The origin of senescent cells in the IVD is not thus far fully elucidated. In previous publications, their presence has been associated with a decrease of the mean telomere length of IVD cells, implying that cells have become senescent after serial replications (a process called replicative senescence) [16, 17]. However, given that IVD cells have a very low proliferation rate in the tissue [18], cellular senescence seems also to be stress-induced [19], since it has been well established that a series of genotoxic (i.e., DNA damaging) stresses can trigger premature cellular senescence. However, it has not been tested yet whether IVD cells that have become senescent by different means may have similar expression levels of catabolic factors, such as MMPs and inflammatory cytokines, and thus exert comparable changes on local tissue homeostasis. We thus compared early passage nucleus pulposus cells, replicative senescent cells and cells that became senescent after exposure to ionizing radiation—a classical genotoxic insult. In addition, we tested here for the first time, to the best of our knowledge, the effect of p16^{INK4a} overexpression on this cell model, as in all types of cellular senescence increased expression of this particular cell cycle inhibitor stabilizes the senescent state. For that reason, we used an artificial system (lentiviral overexpression of p16^{INK4a}) to solely investigate the role of p16^{INK4a} irrespective of the other parameters of senescence induction after serial subculturing or exposure to ionizing radiation. MMP-1, -2, -3, -9, IL6, IL8 and IFN γ mRNA levels were selected to be studied, since these molecules represent major SASP components at the same time that they have been detected to be upregulated in pathological conditions of the IVD [2, 3, 8, 9].

Materials and methods

Cells and cell culture conditions

Human nucleus pulposus IVD cells were isolated from the discs of consenting orthopaedic patients (Supplementary Table 1), as reported previously [20]. In brief, the nucleus pulposus was aseptically separated from the inner and outer

annulus fibrosus regions based on morphology, cut into small pieces and subjected to collagenase digestion. The quality of nucleus pulposus-annulus fibrosus separation was assessed by RT-PCR for the gene collagen I (used as a marker for annulus fibrosus cells) and the genes aggrecan and collagen II (used as markers for nucleus pulposus cells) (data not shown). Nucleus pulposus cells were retrieved by centrifugation and seeded into culture dishes in DMEM supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin (Biochrom AG, Berlin, Germany) and 20% (v/v) FBS (Gibco BRL, Invitrogen, Paisley, UK) until they became confluent. Human embryonic kidney 293 cells containing the SV40 T-antigen (HEK293T), an established cell line for efficient viral growth, were routinely cultured in DMEM containing 10% (v/v) FBS. Primary nucleus pulposus IVD cell cultures and HEK293T cells were maintained at 37 °C and 5% CO₂ and subcultured using a trypsin/citrate (0.25%/0.30%, w/v) solution.

Human nucleus pulposus IVD cells of passage 3–6 isolated from at least two different donors were used for all experimental procedures with no significant differences in cellular responses. Cells were plated and left to proliferate until confluence and (a) were serially subcultured to reach replicative senescence (approximately at passage 30), (b) exposed to a ⁶⁰Co gamma source (Gamma Chamber 4000A, Isotope Group, Bhabha Atomic Research Company, Trombay, Bombay, India) as described earlier [21, 22] and subcultured after 16 h for three times to reach stress-induced premature senescence, and (c) were infected with viruses carrying an empty vector or a vector for p16^{INK4a} overexpression. Early passage cells plated under the same conditions at the beginning of treatments and left untreated throughout the experiment (except for routine medium change and subculturing whenever necessary) served as the untreated control (young cells). Given the similarity of results among different donors, averages of at least three experiments with cells deriving from one representative donor are presented.

Construction of lentiviral vectors, viruses' production in HEK293T cells and infection of nucleus pulposus cells

For stable overexpression of p16^{INK4a} the lentiviral expression vector pLenti7.3/V5-GW/lacZverA from Invitrogen (Paisley, UK) (gift from Dr. Lesley Probert) was modified. The plasmid was digested by the restriction endonuclease EcoRV and religated using T4 DNA Ligase (New England Biolabs, Beverly, MA, USA). This modification resulted in the elimination of the *lacZ* gene and the attB1 and attB2 sequences designated as pLenti7.3/V5 EFGP NO attB. Plasmid pLenti6gw u6laminshrna verB (also gift from Dr. Lesley Probert) was digested by the

EcoRV/KpnI restriction endonucleases for the isolation of a DNA fragment including early SV40 promoter, EM7 promoter and the blasticidin resistance gene. This fragment was inserted in the EcoRV/KpnI digested pLenti7.3/V5 EFGP NO attB plasmid. The resulting plasmid pLenti7 Bsd(R) contained the blasticidin resistance gene as the selection marker under the control of SV40 and EM7 promoters (Fig. 1a). Subsequently, p16^{INK4a} open reading frame was amplified using primers (5'-CGGGATCCAG-CAGCATGGAGCCGGC-3') and (5'-CGGAATTCT-CAATCGGGGATGTCTGAG-3') and was then subcloned into the aforementioned plasmid at BamHI/EcoRI sites, resulting in pLenti7 Bsd(R) p16 plasmid (Fig. 1b). pLenti7 Bsd(R) and pLenti7 Bsd(R) p16 plasmids were used for the production of lentiviruses in HEK293T cells, using the ViraPower LentiViral Expression System (Invitrogen). Supernatants were collected and used for the infection of human nucleus pulposus IVD cells. Infected cells were selected with 3 µg/ml blasticidin for a total time period of 15 days. RNA and protein were extracted 3 days after the antibiotic selection period.

RNA extraction and qPCR analysis

RNA extraction was performed with Trizol (Invitrogen) according to the manufacturer's instructions. First-strand cDNA synthesis was carried out with the PrimeScript RT

Reagent Kit (Takara, Tokyo, Japan), using 500 ng RNA as template after spectrophotometric determination of the concentration of each sample in a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). qPCR experiments were executed in a MX3000P cyclor (Stratagene, La Jolla, CA, USA) using qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems Ltd, London, UK). Relative gene expression to that of young cells was calculated as described before [20] with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serving as the reference gene. Gene expression in pLenti7 Bsd(R) p16-infected cells was calculated as a ratio to that of pLenti7 Bsd(R)-infected cells. Primer sequences are shown in Table 1.

Western blot analysis for p16^{INK4a} protein expression

Protein extracts were collected, electrophoretically separated and transferred to PVDF membranes, as reported previously [23]. Western blot analysis was performed against p16^{INK4a} and GAPDH (antibodies were from BD Pharmingen, San Diego, CA, USA and Santa Cruz Biotechnology, Santa Cruz, CA, USA, respectively) using a secondary anti-mouse horseradish peroxidase-conjugated antibody purchased from Sigma (St. Louis, MO, USA). Immunoreactive products were detected by an ECL reagent (Amersham Biosciences, Buckinghamshire, UK).

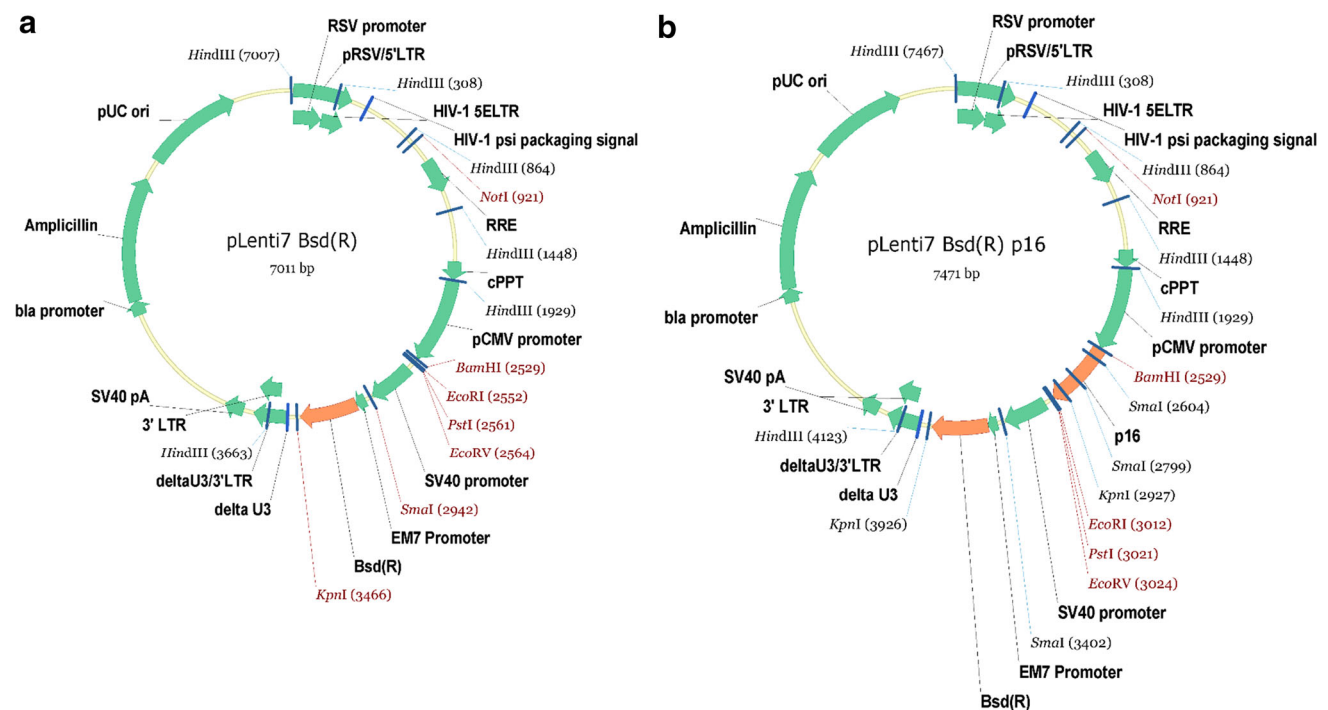


Fig. 1 Plasmid maps of the modified overexpression vector carrying the blasticidin resistance gene [Bsd(R)] (a) and of p16^{INK4a} cloned into the overexpression vector (b). p16^{INK4a} gene is under the control

of the strong CMV promoter. The expression of the blasticidin resistance gene is under the control of SV40 and EM7 promoters

Table 1 qPCR primer sequences

Target gene	Forward primer	Reverse primer
<i>p16^{INK4a}</i>	TAGTTACGGTTCGGAGGCCGAT	GCACGGGTCGGGTGAGAG
<i>mmp-1</i>	CCTTCTACCCGGAAGTTGAG	TCCGTGTAGCACATTCTGTC
<i>mmp-2</i>	AAGAACCAGATCACATACAGGATCA	GTATCCATCGCCATGCTCC
<i>mmp-3</i>	TTTTGGCCATCTCTCCTTCA	TGTGGATGCCTCTTGGGTATC
<i>mmp-9</i>	GCACGACGTCTTCCAGTACC	TCAACTACTCCGGGAACTC
<i>il6</i>	CTTTTGGAGTTTGAGGTATACCTAG	CGCAGAATGAGATGAGTTGTC
<i>il8</i>	TGCCAAGGAGTGCTAAAG	CTCCACAACCCTCTGCAC
<i>ifnγ</i>	GCATCCAAAAGAGTGTGGAGACCA	TTGCTTTGCGTTGGACATTCAAGTC
<i>gapdh</i>	GAGTCCACTGGCGTCTTC	GCATTGCTGATGATCTTGAGG

Cell counting

Cell number of human nucleus pulposus IVD cells 2 and 20 days post-selection of infected with the empty or the p16^{INK4a} overexpression (p16 O/E) vector cells was measured using a hemocytometer after trypsinization of the cells.

Estimation of BrdU incorporation

Immunofluorescence experiments for the estimation of 5-bromo-2'-deoxyuridine (BrdU) incorporation in the nuclei of human nucleus pulposus IVD cells were performed as described before [20]. Briefly, after fixation with 4% (w/v) formaldehyde and permeabilization with 0.2% (v/v) Triton X-100, cells were incubated with 2 N HCl and labelled with an anti-BrdU-FITC antibody (Roche Applied Science, Mannheim, Germany). BrdU-positive and negative nuclei counter-stained with 2 μ g/ml DAPI were detected in the cell population under a Zeiss AxioPlan 2 microscope (Carl Zeiss, Jena, Germany).

SA- β Gal staining

SA- β Gal staining was performed as previously reported [20]. Cells were seeded and cultured on glass coverslips before fixation with 3% (w/v) formaldehyde and incubation with the SA- β Gal staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mg/ml X-Gal in a 40 mM citric acid/sodium phosphate pH 6.0, 150 mM NaCl and 2 mM MgCl₂ solution) at 37 °C. SA- β Gal- positive cells were counted using a Zeiss AxioPlan 2 phase contrast microscope.

Statistical analysis

Values presented are the means \pm standard deviations. Differences in comparison to young cells were considered statistically significant when $p < 0.05$ (Student's t test).

Results

p16^{INK4a} overexpression leads nucleus pulposus cells to senescence

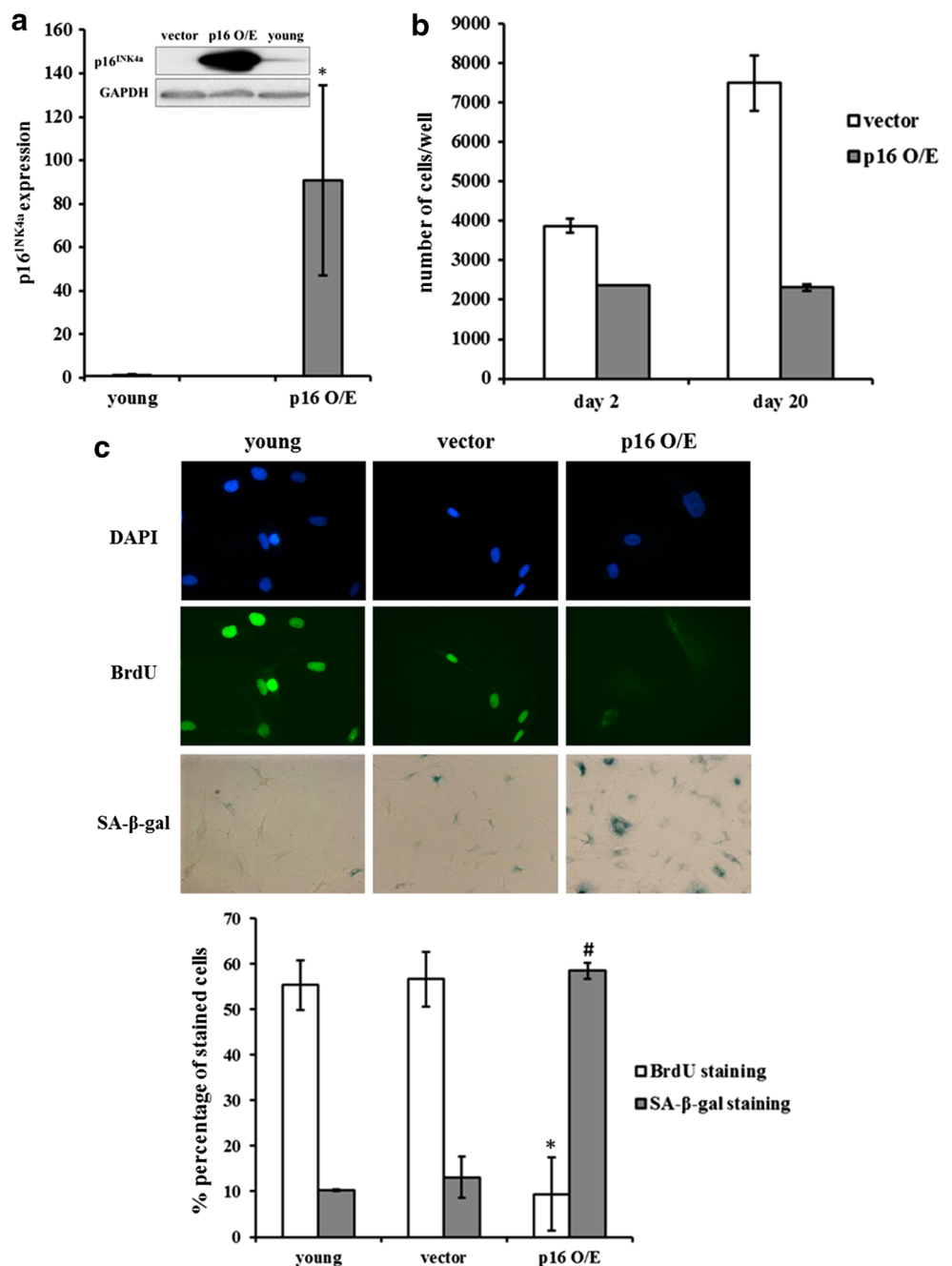
Infected human nucleus pulposus IVD cells with lentiviruses carrying pLenti7 Bsd(R) p16 plasmid showed a significantly higher p16 expression both at the mRNA and protein levels compared to young cells and cells infected with lentiviruses carrying the empty vector (Fig. 2a). p16^{INK4a} overexpression decreased the proliferative capacity of the cells, as shown by the direct cell counting of empty vector- and pLenti7 Bsd(R) p16-infected cells (Fig. 2b). Cell number of p16^{INK4a}-overexpressing cells remained constant for 2 and 20 days post-selection—suggesting an arrest of proliferation during this time period—while cells infected with the virus containing the empty vector demonstrated a progressive increase of their cell number. Reduction of proliferation in p16^{INK4a}-overexpressing cells was confirmed by their diminished ability to incorporate BrdU in the nuclei, evidencing the decrease of novel DNA synthesis (Fig. 2c). At the same time, an increased percentage of SA- β Gal-positive staining was observed for cells infected with lentiviruses containing pLenti7 Bsd(R) p16 plasmid (Fig. 2c), evidencing that the aforementioned lower proliferation rate did not result from a transient cell cycle delay, but rather from the establishment of senescence. Human nucleus pulposus cells after serial subculturing and exposure to ionizing radiation also exhibited increased p16^{INK4a} mRNA levels (data not shown) and were characterized by a percentage of BrdU incorporation and SA- β Gal staining that was always <5 and $>60\%$, respectively, confirming the induction of senescence.

Changes in the transcriptional profile of MMP-1, -2, -3 and -9 in senescent nucleus pulposus IVD cells arising from three different treatments

We then assessed the transcriptional profile of selected MMP-encoding genes in senescent due to p16^{INK4a}-

Fig. 2 p16^{INK4a} overexpression leads human nucleus pulposus IVD cells to senescence.

a Quantitative RT-PCR and western blot analysis for the estimation of p16^{INK4a} mRNA and protein levels, respectively. *Asterisk* denotes a statistically significant difference (Student's *t* test, $p < 0.05$) from young cells. **b** Direct counting of the final number of cells infected with lentiviruses containing the empty and the p16^{INK4a} overexpressing vectors 2 and 20 days post-selection with blasticidin. **c** Determination of proliferating cells by nuclear BrdU incorporation and of SA-β Gal-positive cells after p16^{INK4a} overexpression. In the graph means ± standard deviations are presented. *Asterisk* and *number* sign represent statistically significant differences from the respective young and empty vector-containing cells for BrdU incorporation and SA-β Gal staining, respectively. p16 O/E: cells infected with the pLenti7 Bsd(R) p16 plasmid



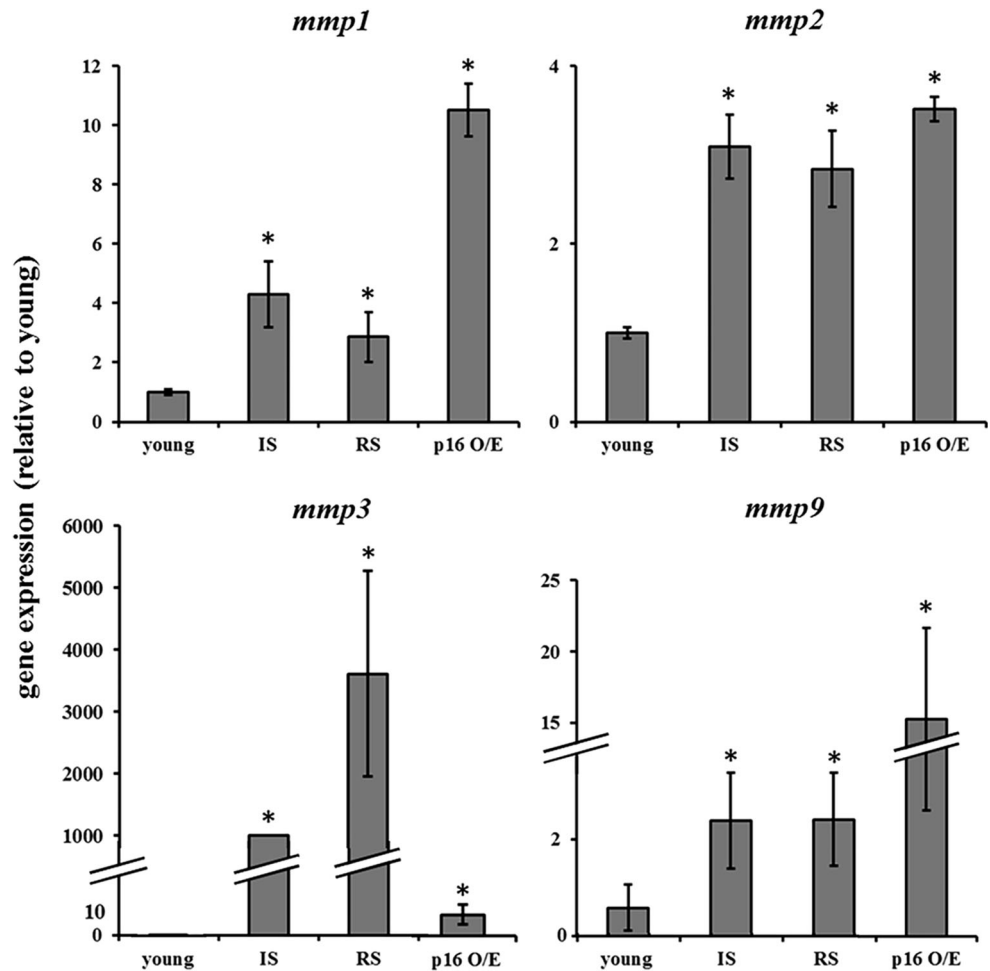
overexpression cells in comparison with that of two well established senescent phenotypes, the replicative senescent cells and the γ -irradiation-induced senescent cells (Fig. 3). The general trend was similar, that is, all MMP-encoding genes investigated showed increased mRNA levels in senescent cells compared to young ones, irrespective of the means originally used to induce senescence. The order of the upregulation's magnitude was comparable for replicative and γ -irradiation-induced senescent cells, while it differed in the case of cells infected with lentiviruses carrying pLenti7 Bsd(R) p16 plasmid (with the exception of MMP-2 that presented the same transcriptional profile in

all senescent cells). More specifically, p16^{INK4a}-overexpressing cells were characterized by higher MMP-1 and -9 mRNA levels and lower MMP-3 upregulation in comparison to senescent cells resulting from the other two treatments.

Transcriptional alterations in the inflammatory mediators IL6, IL8 and IFN γ in senescent nucleus pulposus IVD cells

qPCR analysis revealed increased IL6, IL8 and IFN γ mRNA levels in all types of senescence (Fig. 4). Cells

Fig. 3 Real-time PCR analysis for the estimation of MMP-1, -2, -3 and -9 mRNA levels of senescent cells due to ionizing radiation (IS), serial subculturing (RS) and infection with lentiviruses carrying pLenti7 Bsd(R) p16 plasmid (p16 O/E). Graphs present mean values \pm standard deviations. Statistically significant differences in comparison to young cells (Student's *t* test, $p < 0.05$) are marked by asterisks



becoming senescent after consecutive replications and prematurely senescent cells after exposure to ionizing radiation had both an approx. 20-fold IL6 upregulation in comparison to young cells, while p16^{INK4a}-overexpressing cells showed an approx. 120-fold increase. On the other hand, there was an around 200-fold increase of IL8 mRNA levels in replicative and γ -irradiation-senescent cells and a sixfold increase in pLenti7 Bsd(R) p16-infected cells. Upregulation of IFN γ was comparable for all senescent cells (approx. fivefold induction).

Discussion

The population of senescent cells has been shown to increase in the IVD with ageing or with progression of degeneration [11–14], as has also been reported for several other tissues [15] and age-related pathologies [24]. Senescent cells are characterized by altered expression of genes encoding, amongst others, cytokines, growth factors and proteases (namely the SASP) that may in turn influence the structure of the extracellular microenvironment [25]. The

manifestation of the SASP has been attributed to the accumulation of DNA damage and not to senescence itself and it has not always been consistent and firm in all cell models investigated [15]. DNA damage triggers a DNA repair response involving a p53-mediated cell cycle delay [26] that becomes an irreversible growth arrest by the expression of the cell cycle inhibitor p16^{INK4a} [24]. Given that cellular senescence could promote IVD degeneration through the secretion of catabolic enzymes and inflammatory cytokines, here we tested the transcriptional alterations of major SASP factors in human nucleus pulposus IVD cells after inducing senescence with three different modes, i.e., replicative exhaustion and exposure to ionizing radiation (both provoking a DNA damage response) and overexpression of p16^{INK4a}, a key molecule securing the establishment of senescence even in the absence of a functional p53 [27].

We showed that senescent IVD cells after consecutive replications, exposure to ionizing radiation and overexpression of p16^{INK4a} had a similar profile of upregulated genes encoding the secreted molecules MMP-1, -2, -3, -9, IL6, IL8 and IFN γ . In the same vein, a catabolic phenotype

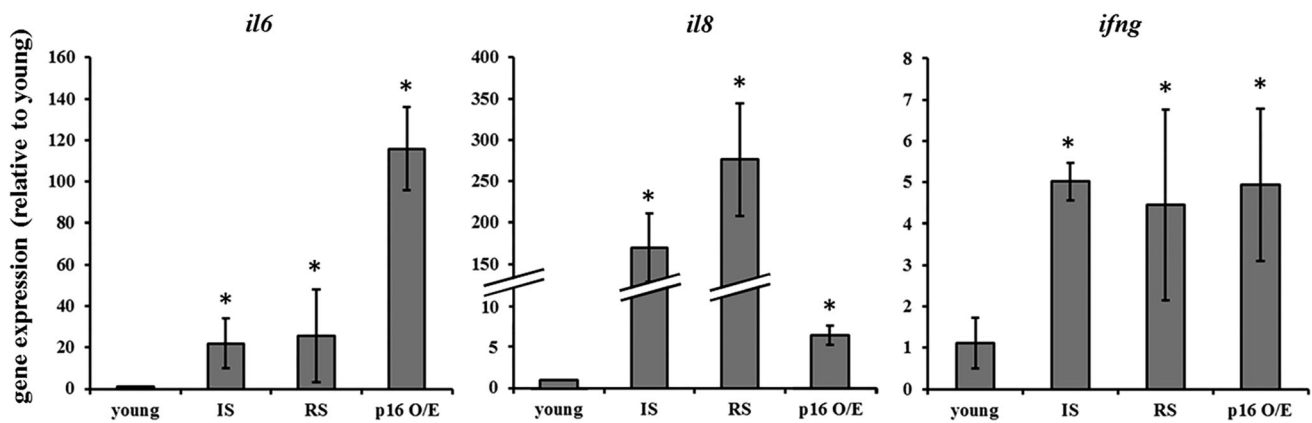


Fig. 4 Quantitative PCR analysis for the estimation of IL6, IL8 and IFN γ transcriptional alterations in senescent cells after exposure to γ -irradiation (IS), serial subculturing (RS) and infection with the virus containing pLenti7 Bsd(R) p16 plasmid (p16 O/E). In the graphs

means \pm standard deviations are depicted. Asterisks represent statistically significant differences in comparison to young cells (Student's *t* test, $p < 0.05$)

has been also shown to characterize human nucleus pulposus cells that senesced prematurely after exposure to a different stimulus, i.e., oxidative stress [20]. MMPs may provoke the catabolism of the tissue's ECM, while inflammatory cytokines—known to also have a degenerative effect by increasing the expression of catabolic enzymes—may in addition reinforce senescence [26], thus perpetuating the degenerative process. In favour of our findings, MMP-1, -2, -3 and -9 expression levels and/or activity have been reported to be increased in degenerate and herniated lumbar discs [2, 5, 8, 28]. The different tendency of MMP-3 upregulation in senescent cells deriving from the three treatments in comparison to the other MMPs investigated here may be attributed to the inherent variations of MMP expression in IVD tissues [29]. In addition, IL6 has been detected in cases of herniation, radiculopathy or spinal fusion [8–10], IL8 has been identified in surgical disc specimens removed to relieve pain and arthrodesis patients [8] and IFN γ has been found in degenerated discs and low back pain patients [8, 10]. IL6 and IL8 have been considered as major markers of the SASP, as they have been shown to be elevated in many types of senescence [30]. Furthermore, IL6 also enhances the action of other inflammatory molecules in the nucleus pulposus, such as IL1 and TNF α [10]. In contrast to our findings, it has been reported that ectopic expression of p16^{INK4a} in human and mouse fibroblasts leads to senescence that is not accompanied by a SASP at the mRNA or the protein level [24]. Moreover, it has been demonstrated that p16^{INK4a}-overexpressing chondrocytes retain the ability to increase MMP-1 and MMP-13 protein levels, however, without significantly upregulating IL6 and IL8 [31]. This discrepancy may arise from the different cell model used in each study or may reflect differences in the experimental design. Nevertheless, a positive correlation

between the increased p16^{INK4a} expression in the degenerate IVDs and that of matrix-degrading enzymes has been reported in cells isolated from control and degenerated discs [12]. Of note, in our study, senescent cells after serial subculture or exposure to ionizing radiation (both inducing a DNA damage response) exhibited quantitatively similar expression levels of the investigated genes, while in senescent cells after p16^{INK4a} induction alone an overexpression of all these genes was observed albeit with different potencies in some cases. However, to characterize in detail the SASP of these cells, other factors associated with IVD degeneration (e.g., ADAMTSs) need to be analyzed as well, which is a part of an ongoing research in our laboratory.

Conclusion

Low back pain that concerns the majority of the population worldwide has been connected to IVD degeneration, a cumulative result of ageing and external degenerative stimuli. Degenerated IVD is characterized by the presence of a high percentage of senescent cells, as a result of the continuous exposure to several external insults characteristic of the harsh environment of this tissue [32]. Findings of this, as well as of previous studies [20], generally indicate that nucleus pulposus IVD cells express a similar transcriptional profile of many catabolic and inflammatory genes known to be linked with IVD degeneration, irrespective of the mechanism triggering senescence. Accordingly, all these types of senescent cells may contribute to the disturbance of the structural integrity of the disc. Thus, understanding the mechanism of induction of cellular senescence, as well as the physiology and behaviour of these cells is of utmost importance for the design

of therapies targeting anti-senescence and for cell replacement strategies for the prevention or treatment of disc degeneration.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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