European Spine Journal (2018) 27:2621–2630
https://doi.org/10.1007/s00586-018-5688-8

ORIGINAL ARTICLE

TRPC6 in simulated microgravity of intervertebral disc cells

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Received: 19 November 2017 / Accepted: 25 June 2018 / Published online: 2 July 2018

Abstract

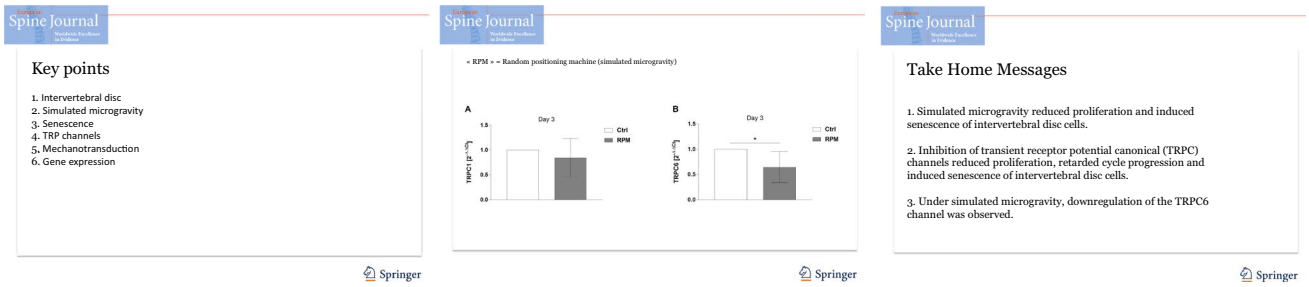
Purpose Prolonged bed rest and microgravity in space cause intervertebral disc (IVD) degeneration. However, the underlying molecular mechanisms are not completely understood. Transient receptor potential canonical (TRPC) channels are implicated in mechanosensing of several tissues, but are poorly explored in IVDs.

Methods Primary human IVD cells from surgical biopsies composed of both annulus fibrosus and nucleus pulposus (passage 1–2) were exposed to simulated microgravity and to the TRPC channel inhibitor SKF-96365 (SKF) for up to 5 days. Proliferative capacity, cell cycle distribution, senescence and TRPC channel expression were analyzed.

Results Both simulated microgravity and TRPC channel antagonism reduced the proliferative capacity of IVD cells and induced senescence. While significant changes in cell cycle distributions (reduction in G1 and accumulation in G2/M) were observed upon SKF treatment, the effect was small upon 3 days of simulated microgravity. Finally, downregulation of TRPC6 was shown under simulated microgravity.

Conclusions Simulated microgravity and TRPC channel inhibition both led to reduced proliferation and increased senescence. Furthermore, simulated microgravity reduced TRPC6 expression. IVD cell senescence and mechanotransduction may hence potentially be regulated by TRPC6 expression. This study thus reveals promising targets for future studies.

Graphical abstract These slides can be retrieved under Electronic Supplementary Material.



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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00586-018-5688-8) contains supplementary material, which is available to authorized users.

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Keywords Intervertebral disc · Simulated microgravity · Senescence · TRP channels · Mechanotransduction · Gene expression

Introduction

Intervertebral discs (IVDs) show signs of degeneration as early as the second decade of life [1]. As normal physiological ageing and pathological degeneration recruit similar signalling cascades, pathological IVD degeneration can be described as accelerated tissue ageing, contributed to by multimodal stress-induced changes with ultimate tissue failure [2]. Although genetics plays an essential role in the onset of ageing and the progression of degeneration [3], mechanical loading and damage, particularly of the lumbar spine, have been identified as contributing factors [4]. Whereas physiological levels of mechanical stress support the metabolic balance of the IVD [5], hyper-physiological mechanical stresses or mechanical unloading biases the IVD towards catabolism, inflammation and reduced viability, hence promoting degeneration [6]. Mechanical unloading, occurring, e.g. during prolonged bed rest, causes long-lasting changes in IVDs [7, 8]. In fact, the changes in IVD morphology measured by magnetic resonance imaging after 21 days of bed rest in 7 healthy subjects persisted after 5 months [7]. The recovery of the IVDs was still incomplete 2 years after 60 days of bed rest [8]. Moreover, astronauts, who are exposed to microgravity during space flights, exhibit spinal lengthening [9], low back pain [10, 11] and an elevated risk of IVD herniation [12]. In vitro experiments [13] and animal studies conducted during space flight [14, 15] have shown degenerative biomechanical or biochemical effects of microgravity. To study microgravity on Earth, random positioning machines (RPMs) are commonly used [16]. Signs of IVD degeneration were observed in mice discs cultured in a rotary wall vessel bioreactor [17], although this was not the case with rat IVDs [18].

During degeneration, IVD cells acquire numerous pathobiological features, including increased levels of cellular senescence [19, 20]. Affected cells possess a so-called senescence-associated secretory phenotype (SASP), characterized by proliferative dysfunction, unresponsiveness to mitogenic stimulation (and hence hampered cell cycle progression), as well as catabolic and inflammatory behaviour [20]. The molecular mechanisms leading to IVD degeneration and ageing are not yet completely understood. However, high levels of free calcium in the cartilaginous endplates are associated with IVD degeneration [21]. Furthermore, free calcium seems to contribute to IVD degeneration through the calcium-sensing receptor (CaSR) [22]. Recently, the expression of transient receptor potential (TRP) channels, a superfamily of ion channel with relevance in calcium regulation [23], has been demonstrated in the IVD [24–26]. The members of the canonical family (TRPC) are especially interesting. A direct correlation between TRPC6 expression and the degree of IVD degeneration has been recently shown [25], highlighting TRPC6 as a candidate for further studies on IVD pathobiology. Aside from TRPC6, the isoform TRPC1 may also be of specific relevance. TRPC1 and TRPC6 are both known to affect cell cycle progression in various cell types [27–29], and TRPC6 was shown to modulate cellular senescence [30]. Therefore, their modulation and (dys-)regulation may play a crucial role in IVD health, but also degeneration. Importantly, TRPC1 and TRPC6 have both been described as mechanosensitive [26]. TRP channels are therefore interesting candidates when aiming to link IVD mechanobiology with IVD ageing and degeneration. Interestingly, simulated microgravity was shown to depress TRPC1 expression associated with an accumulation of cells into G2/M, effectively stalling proliferation of mouse myoblasts [28, 31].

The aim of this study was to investigate the effects of simulated microgravity and TRPC channel pharmacological inhibition on human IVD cell proliferation, cell cycle progression and senescence (as key features of IVD degeneration).

Materials and methods

Cell isolation and culture

Primary cells were isolated from human IVD biopsies from patients undergoing spinal surgery (approved by the ethical committees in Zurich and Lucerne, Switzerland, and after informed consent), as previously described [32]. For patient demographics, see Table 1. Biopsies were composed of both annulus fibrosus and nucleus pulposus (with different ratio), as it is difficult to distinguish these tissues in degenerated samples. The *n* number in each experiment (between 4 and 6) represents the number of donors used per experimental condition.

Cells were cultured in DMEM/F-12 (Gibco, 31330-038) with 10% FCS (Sigma-Aldrich, F7524) and 1% Anti-Anti (= 50 units/mL penicillin, 50 µg/mL streptomycin and 125 ng/mL ampicillin) (Gibco, 15240-062) at 37 °C and 5% CO_2 up to passage 1–2. For the experimental phase, cells were cultured in reduced-serum DMEM/F12 (5% FCS) without antibiotics to avoid any possible interaction of aminoglycoside antibiotics with TRP channel activity [28].

Table 1 Patient demographics

Patient number	Patient age	Sex	Disc level	Type of disease	Pfirmann grade
1	62	F	L4/5	Spondylolisthesis	V
2	53	M	L5/S1	Herniation	IV
3	71	F	L4/5	DDD	II
4	42	F	L4/5	Herniation	IV
5	uk	uk	uk	uk	uk
6	46	M	L5/S1	DDD	V
7	43	F	L4/5	Herniation	IV
8	uk	uk	uk	uk	uk
9	41	F	L5/S1	Herniation	IV
10	47	F	L4/L5	Herniation	III
11	56	F	L3/4	Herniation	IV
12	57	M	L5/S1	DDD	IV
13	68	M	L4/5	Herniation	III
14	uk	uk	uk	uk	uk
15	40	F	C6/7	Herniation	III
16	44	M	L4/5	Herniation	IV

F = female; *M* = male; *L4/5* = lumbar 4–5; *L5/S1* = lumbar 5-sacral 1; *C6/7* = cervical 6–7; *DDD* = degenerative disc disease; *uk* = unknown

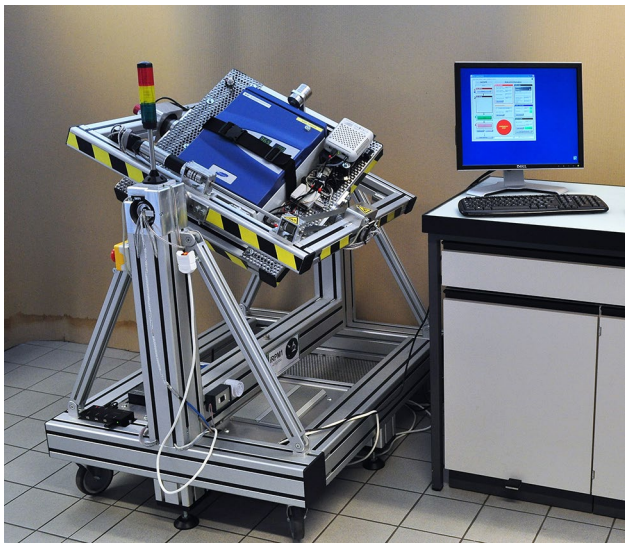

Fig. 1 Photograph of the random positioning machine (RPM) used in this study. The samples were mounted in the integrated miniaturized CO_2 incubator in the inner of the two rotating gimbal-mounted frames that are independently moved by motors

Cellular treatments

Simulated microgravity

To expose cells to simulated microgravity, a previously described RPM was used (Fig. 1) [28]. The RPM consists of two gimbal-mounted frames independently moved by motors. Samples are placed at the centre of the frames, where the generated motion pattern is designed to equally distribute the gravity vector spatially and temporally (the gravity mathematically averages zero) [33]. Because the constant reorientation of the gravity vector prevents biological systems to adjust to this force, their response is similar to the one achieved upon real microgravity exposure [16].

In our experiments, the rotation speed of the frames was set to 60° per second. IVD cells ($n = 4$–5, seeded at 6000 cells/cm^2 in T25 flasks (150,000 cells per flask)) were cultured for 24 h prior to simulated microgravity. Flasks were completely filled with culture medium to avoid air bubbles and closed airtight with a filter-less cap [28]. Flasks were mounted onto the RPM (integrated miniaturized CO_2 incubator), whereas corresponding control samples (1 g) were cultured in the same conditions and simultaneously kept in a standard CO_2 incubator [28]. Treated cells were analyzed for proliferation (up to 5 days of treatment), cell cycle progression (24 h of treatment), cellular senescence (72 h of treatment) and TRP channel expression (72 h of treatment) and compared to untreated controls.

Pharmacological TRPC channel inhibition

Twenty-four hours before pharmacological TRPC inhibition with the broad TRPC antagonist SKF-96365 [34] (Sigma-Aldrich, S7809), IVD cells were seeded at a density of ~8000 cells/cm^2 in either 24-well plates for subsequent analysis of proliferation ($n = 6$, 15,000 cells per well), 25 cm^2 flasks for cell cycle analysis ($n = 6$, 200,000 cells per flask), or 12-well plates for senescence analysis ($n = 530{,}000$ cells per well). For cell cycle analysis, cells were exposed to high doses of SKF-96365 (20 µM) for 48 h, whereas proliferation and senescence experiments were conducted for up to 5 days at lower concentrations (1.7 and 2.5 µM) to avoid cell death or complete growth arrest. Treated cells were analyzed as described below and compared to untreated controls.

Cell analysis

Cell proliferation

Manual counting of trypsinized cells was conducted to identify the effects of simulated microgravity and pharmacological TRP inhibition on cell proliferation. Proliferation was analyzed over 5 days (day 0 = seeding cell number, 1, 3 and 5) by counting each replicate twice using a hemocytometer.

Cell cycle

Cell cycle progression was analyzed by flow cytometry analysis as previously described [28]. Cells were fixed in

70% ice-cold EtOH, stored at − 20 °C overnight and then stained with PI/RNase staining buffer (BD Pharmingen, 550825). Cells were analyzed with a FACSCalibur system (BD Biosciences), outfitted with a FL1 channel for PI staining. Appropriate settings for forward- and side-scatter gates were applied to examine 10′000 events/sample. The cell cycle profile was quantified with FlowJo flow cytometry analysis software (TreeStar Inc.), applying the Watson model to fit the histograms of single-gated cells.

Senescence

The level of cellular senescence was assessed by SA-β-galactosidase staining. Cells were washed twice with PBS, fixed with 3% paraformaldehyde in PBS (pH 7.4), washed twice with PBS and incubated overnight at 37 °C with X-gal chromogenic substrate (1 mg/ml) at pH 6.0 [35]. After washing and dehydration with graded ethanol (75–95–99.9%, 1 min each), six images per sample were taken under bright-field illumination (10×). The percentage of senescent cells was determined by manually counting cells with ImageJ.

Gene expression

Gene expression analysis was conducted using a previously described protocol [36]. Briefly, RNA was extracted with TRIzol/chloroform (ThermoScientific, 15596018), the quality and quantity of RNA was determined with the NanodropLite (ThermoScientific) and 1 µg of RNA was reverse-transcribed into cDNA in a 30 µl volume using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4374966). For each qPCR reaction, 150 ng cDNA was mixed with TaqMan primers (*TRPC1: Hs00608195_m1; TRPC6: Hs00989190_m1; TBP: Hs00427620_m1*), TaqMan Fast Universal PCR Master Mix (2X) (Applied Biosystems, 4352042) and RNAse-free water to a total volume of 10 µl and gene expression was measured with the CFX96 Touch™ Detection System (Bio-Rad). TATA-binding protein (TBP) was used as a housekeeping gene. Results are shown as $2^{-\Delta\Delta Ct}$ values (i.e. relative to TBP and relative to the untreated control).

Statistical analysis

Data were analyzed for consistency, outliers and for normality. We used normal probability plots for visual inspection of data and Kolmogorov–Smirnov tests to test for normality. Because each donor was measured in the control as well as in the experimental group, samples were dependent (clustered). Data were not normally distributed in any subgroup, and to consider both circumstances, a generalized estimation equation model (GEE) was applied. In detail, we applied a full factorial model with a main, time and interaction effects and used the robust Huber–White sandwich estimator for the covariance matrix (unstructured approach). The lognormal distribution was used to model the underlying distribution. Least significance differences were computed for pairwise comparisons of means. Results are given in terms of means, standard deviations and 95% confidence intervals for means. A significance level of 5% was used. All tests were two-sided. All analyses were done by using STATISTICA 13 (Hill, T. & Lewicki, P. Statistics: Methods and Applications. StatSoft, Tulsa, OK) and PASW 24 (IBM SPSS Statistics for Windows, Version 24.0., Armonk, NY).

Results

Simulated microgravity and TRPC channel inhibition reduce cell proliferation

Exposing IVD cells ($n = 5$) to simulated microgravity significantly reduced proliferation at days 3 (189 573 cells ± 54 620) and 5 (337 325 cells ± 184 026) compared to cells maintained at 1 g (day 3: 365 600 cells ± 21 637, $p < 0.0001$; day 5: 620 463 cells ± 394 720, $p = 0.0041$) (Fig. 2a). Similarly, IVD cell proliferation rate ($n = 6$) was slowed at day 3 and day 5 by SKF-96365 (1.7 and 2.5 µM), a non-selective TRPC channel antagonist (Fig. 2b) [34]. Statistical significance was reached both when comparing each SKF concentration to the control group ($p < 0.0001$ for both concentration at both day 3 and 5) and when comparing the 1.7 µM to the 2.5 µM group ($p = 0.0007$ and $p = 0.002$ for day 3 and 5, respectively), thus proving dose-dependency.

Simulated microgravity and TRPC channel inhibition affect cell cycle

Although 24 h of simulated microgravity slightly reduced the mean percentage of cells in the G1 phase of the cell cycle from 64.6% ± 8.5 in the controls to 59.0% ± 6.1 and caused an accumulation within the G2/M phase from 11.6 ± 1.9 to 15.2% ± 3.6, these differences were not significant ($p = 0.062$ and $p = 0.076$, respectively) (Fig. 3a). However, short-term pharmacological blocking of TRPC-mediated calcium entry with SKF-96365 (20 µM, 48 h) resulted in a significant accumulation of IVD cells within the G2/M phase, concomitant with a reduction of cells within the G1 phase ($p < 0.0001$ for both phases) (Fig. 3b). Specifically, G2/M accumulation increased from 11.4% ± 6.0 (untreated control) to 30.3% ± 8.5, and G1 distribution decreased from 79.7% ± 8.5 to 56.7% ± 11.9.

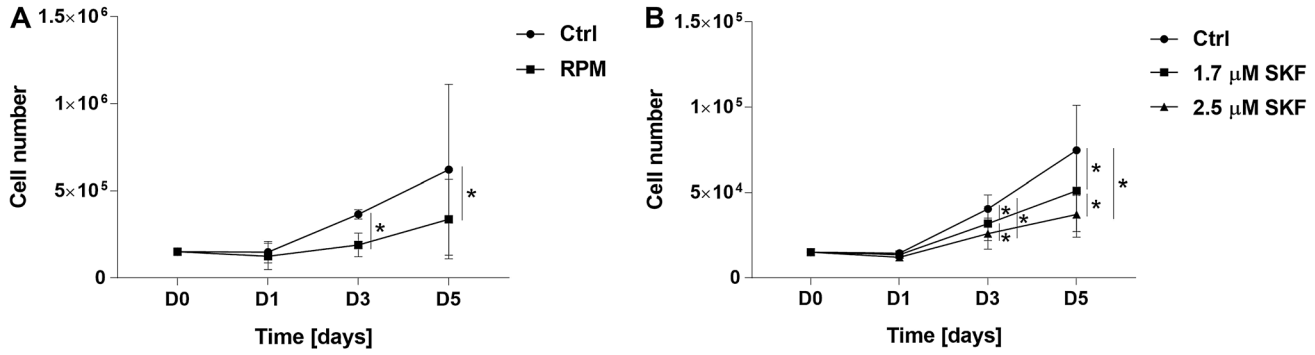

Fig. 2 Effects of simulated microgravity and TRPC channel inhibition on proliferation. **a** Proliferation (total cell number) of human IVD cells at terrestrial gravity (Ctrl) or exposed to simulated microgravity (RPM) up to 5 days ($n=5$). **b** Proliferation (total cell number) of human IVD cells with or without TRPC channel inhibition with 1.7 or 2.5 μM SKF-96365 for up to 5 days ($n=6$). Data are shown as mean with 95% confidence interval, $*p<0.05$

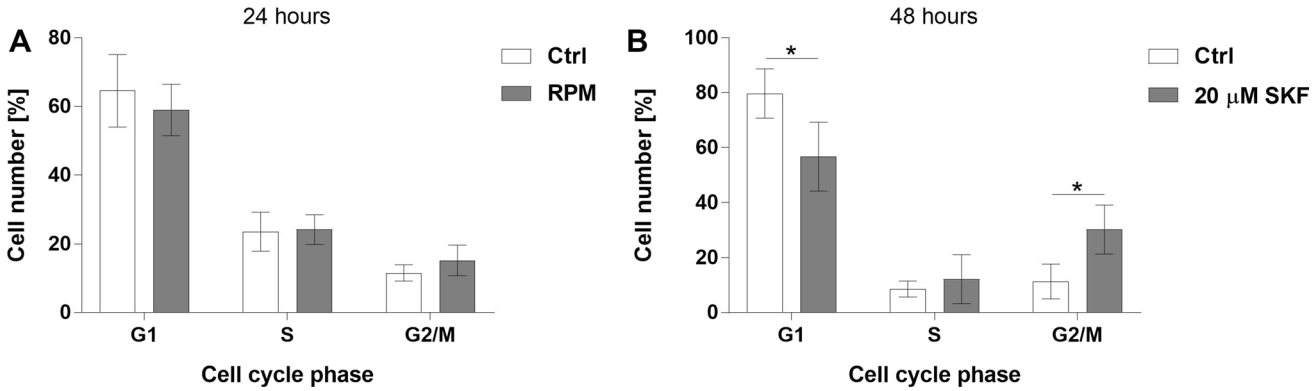

Fig. 3 Effects of simulated microgravity and TRPC channel inhibition on cell cycle. **a** Cell cycle distribution (% of cells in each phase) of human IVD cells at 1 g (Ctrl) or exposed to simulated microgravity (RPM) for 24 h ($n=5$). **b** Cell cycle distribution (% of cells in each phase) of human IVD cells with or without TRPC channel inhibition with 20 μM SKF-96365 for 48 h ($n=6$). Data are shown as mean with 95% confidence interval, $*p<0.05$

Simulated microgravity and TRPC channel inhibition increase cellular senescence

We found that exposing IVD cells to simulated microgravity for 3 days increased senescence (positive SA-β-galactosidase staining) from 40.0% ± 3.6 (control group) to 50.0% ± 6.9 ($p<0.0001$) (Fig. 4a). Similarly, SKF-96365 treatment also augmented the percentage of SA-β-galactosidase-positive cells relative to untreated controls (Fig. 4b). A significant difference was already observed between the 2.5 μM SKF group and controls at day 1 ($p<0.0001$), which was maintained at both days 3 and 5 ($p=0.012$ and $p<0.0001$, respectively). At these later time points, statistical significance was also reached for the 1.7 μM SKF group ($p=0.0018$ and $p<0.0001$) (Fig. 4b).

Simulated microgravity downregulates TRPC6 expression

Motivated by the fact that TRPC channel inhibition had similar effects on proliferation, cell cycle progression and senescence to simulated microgravity, we analyzed the gene expression of the TRPC channel isoforms TRPC1 and TRPC6 under simulated microgravity. While TRPC1 mRNA levels were not affected ($p=0.14$) (Fig. 5a), TRPC6 expression was significantly reduced (mean $2^{-\Delta\Delta Ct}$ value = 0.64 ± 0.19, $p<0.0001$) (Fig. 5b).

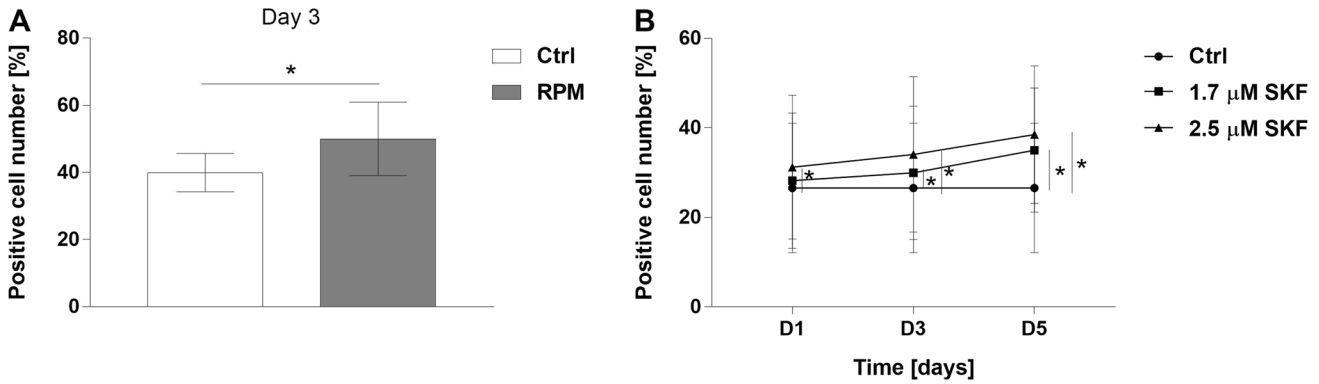

Fig. 4 Effects of simulated microgravity and TRPC channel inhibition on senescence. **a** Percentage of SA-β-galactosidase-positive human IVD cells at 1 g (Ctrl) or exposed to simulated microgravity (RPM) for 3 days ($n=4$) **b** Percentage of SA-β-galactosidase-positive human IVD cells with or without TRPC channel inhibition with 1.7 or 2.5 μM SKF-96365 for up to 5 days ($n=5$). Data are shown as mean with 95% confidence interval, $^*p<0.05$

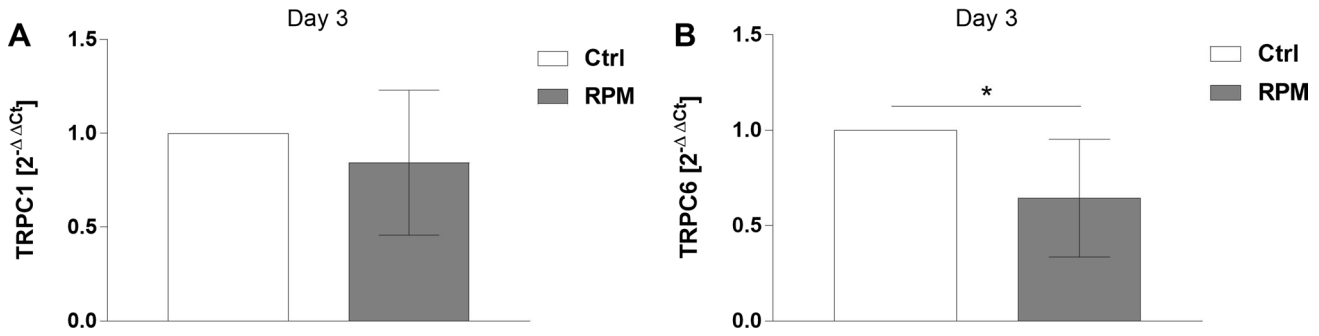

Fig. 5 Gene expression of TRPC1 and TRPC6 under simulated microgravity. **a** Relative gene expression of TRPC1 and **b** TPRC6 in human IVD cells at 1 g (Ctrl) or exposed to simulated microgravity (RPM) for 3 days ($n=4$). Results are shown as $2^{-\Delta\Delta Ct}$ values (i.e. relative to TBP and relative to the untreated control) ($n=4$). Data are shown as mean with 95% confidence interval, $^*p<0.05$

Discussion

In this study, we found that (1) simulated microgravity and pharmacological antagonism of TRPC channels reduced cell proliferation, retarded cell cycle progression and induced cellular senescence, and that (2) simulated microgravity concomitantly inhibited TRPC6 expression (while not affecting TRPC1 expression). TRPC6 may hence potentially play a role in mediating the effects on proliferation and cell cycle distribution that were observed upon simulated microgravity. Further studies including modulation of TRPC6 (with, e.g. activation with CRISPRa or knockout with CRISPRi [37]) are needed to reveal the possible involvement of TRPC6 in IVD mechanotransduction.

Mechanical unloading, as observed in astronauts, para-/tetraplegic patients, bed rest studies [7, 8] or animals undergoing hindquarter suspension, negatively affects IVD metabolism and matrix composition. Here, we simulated microgravity by using an RPM [28] and similarly observed detrimental, degeneration-associated changes. These changes are likely due to the integrated signalling network that couples mechanosensitive receptors and pathways to gene and transcription modulation [38], and may furthermore be associated with structural adjustments on the cell level (e.g. actin cytoskeleton reorganization) [39].

Although constituting a well-accepted method to simulate microgravity, the use of an RPM with its constant reorientation of the samples induces additional forces (e.g. shear stresses) that may create artefacts. These forces present in the culture flasks on the RPM and their effects on the cultured cells were recently thoroughly studied by numerical analysis [40]. Parameters such as rotational velocity of the RPM play a role in the appearance of shear stress [40]. Therefore, additional stressors and artefacts can be prevented by taking the necessary measures for an accurate operation of the RPM [40]. IVD cell culture in flasks on the RPM does not recapitulate the complex three-dimensional disc matrix environment. However, signs of degeneration were similarly observed in whole mouse discs cultured under microgravity

in a rotating wall vessel bioreactor [17]. Compared to studies in the entire human (astronauts and bed rest patients), the RPM represents a simplified in vitro model system, which is nevertheless useful to uncover the underlying molecular mechanisms related to IVD degeneration.

In agreement with our results obtained with human IVD cells, murine myoblasts exposed to simulated microgravity or SKF-96365 exhibited reduced proliferation and cell cycle arrest within the G2/M phase [28, 31]. This reflects the fact that proliferative capacity may concurrently correlate with mechanosensitivity and TRPC function [31]. However, myoblasts seem to rely on modulation of TRPC1 rather than TRPC6, indicating that the exact role of these TRP channels appears to be tissue and/or species dependent. To confirm the specific relevance of TRPC6 for IVD mechanosensing and mechanotransduction, future experiments should not only apply SKF-96365 [34, 41] (which may potentially also affect voltage-dependent calcium channels [42]), but should also target TRPC6 in a more specific manner.

Apart from analyzing cell proliferation and cell cycle progression, we also measured changes in cellular senescence (as assessed by SA-β galactosidase staining), a hallmark of IVD degeneration [43, 44]. TRPC channel inhibition and simulated microgravity both increased cellular senescence in IVD cells. However, variability in the basal levels of senescence (and proliferation capacity) between donors was present, as these differed with respect to age, pathology, degeneration grade and mixture of NP and AF tissue. Senescence in vivo is not only associated with the degree of disc degeneration [45], but also varies between NP and AF [20]. Furthermore, pro-inflammatory cytokines may trigger cell senescence [45], and these may not only be secreted by resident IVD cells, but also by invading immune cells found predominantly in IVD herniation samples [46]. Despite the differences in basal levels of senescence and proliferation capacity stemming from disparities in patient characteristics, we were able to show that microgravity and TRPC channel inhibition both increased cell senescence and decreased cell proliferation. The subsequent gene expression analysis of the TRPC channel isoforms TRPC1 and TRPC6 revealed that TRPC6 was downregulated under microgravity (while TRPC1 expression was not affected). Therefore, TRPC6 could potentially be of importance in the progression of IVD degeneration linked to cell senescence, but further studies are needed. In fact, the role of TRP channels in modulating senescence is also known for other cell types, such as endothelial cells (TRPC5) [47], pancreatic adenocarcinoma cells (TRPM7, TRPM8) [48, 49] and lung fibroblasts (TRPC6) [30].

Mechanistic studies modulating TRPC6 expression/activity are needed to determine whether TRPC6 restricts or reverses progression of a SASP in IVD cells and mitigates the loss of regenerative capacity. This could possibly offer a means to treat IVD degeneration. So far, TRPC6 modulation has been investigated for focal segmental glomerulosclerosis [50], pulmonary hypertension [51] and ischaemia reperfusion-induced lung oedema [52]. TRP channel modulation could be achieved by gene editing, using CRIPSR/Cas9 [37, 53, 54]. However, these approaches are still in an early experimental phase.

Conclusions

In conclusion, we show that human IVD cells subjected to RPM-simulated microgravity or to TRPC channel inhibition display reduced proliferation, retarded cell cycle progression and increased cell senescence. Furthermore, simulated microgravity reduces TRPC6 gene expression. The TRPC6 ion channel may hence be involved in mechanotransduction and in the regulation of cell proliferation and senescence in the IVD. These findings are in agreement with other studies where simulated microgravity induced signs of degeneration in whole mouse IVDs [17] and reduced proliferation in mouse myoblasts by inducing cell cycle arrest [28]. The fact that the effects caused by simulated microgravity (reduced proliferation, retarded cell cycle progression and increased senescence) were recapitulated by TRPC channel inhibition and were accompanied by downregulation of TRPC6 expression suggests that TRPC6 may potentially play a role in the underlying mechanisms. In fact, TRPC6 has also been associated with cell senescence in lung fibroblasts [30], a contributor to pulmonary hypertension [55], consequentially motivating first studies on the therapeutic potential of TRPC6 modulation [55]. This study thus reveals TRPC6 as a potential target for further studies aiming to investigate IVD degeneration.

Acknowledgements Open access funding provided by Paracelsus Medical University. The authors are thankful to Dr. Ermioni Touli (Hirslanden Klinik St. Anna, Switzerland) for support in biopsy collection.

Funding This study was funded by the Swiss National Science Foundation (SNF PP00P2_163678/1) and the Spine Society of Europe (Eurospine 2016_4).

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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