Continuous exposure to simulated hypergravity induced changes in proliferation, morphology and gene expression of human tendon cells

RUNNING TITLE: Effects of hypergravity on tendon cells

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2

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3

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4

Abstract

Gravity influences physical and biological processes, especially during development and homeostasis of several tissues in the human body. Studies under altered gravity have been receiving great attention towards a better understanding of microgravity, hypogravity ($<1g$) or hypergravity ($>1g$) induced alterations. In the present work, the influence of simulated hypergravity over human tendon-derived cells (hTDCs) was studied at 5, 10, 15 and $20g$ for 4 or 16 h, using a large diameter centrifuge (LDC). Main results showed that 16 h of simulated hypergravity limited cell proliferation. Cell area was higher in hTDCs cultured at 5, 10 and $15g$ for 16 h, in comparison to $1g$ control. Actin filaments were more pronounced in hTDCs cultured at 5 and $10g$ for 16 h. Focal adhesion kinase (FAK) was mainly expressed in focal adhesion sites upon hypergravity stimulation, in comparison to perinuclear localization in control cells after 16 h; and FAK number/cell increased with increasing g-levels. A tendency towards an up-regulation of tenogenic markers was observed; *SCX, TNC, COL3A1* and *DCN* were significantly up-regulated in hTDCs cultured at $15g$ and *COL3A1* and *DCN* were significantly up-regulated in hTDCs cultured at $20g$. Overall, simulated hypergravity affected the behavior of hTDCs, with more pronounced effects in the long-term period (16 h) of stimulation.

Keywords: Altered gravity; Cytoskeleton; F-actin; Large diameter centrifuge; Mechanosensing; Tenogenic markers

5

1. Introduction

Mechanical forces, like gravity, influence physical and biological processes and play a critical role during development and homeostasis of several tissues in the human body [1]. Particularly in the musculoskeletal system, which is composed of several mechano-responsive tissues, altered gravitational forces are known to affect bone mineral density and skeletal muscle mass [2-5]. Mechanical loading is critical for skeletal morphogenesis and maintenance of tissue homeostasis by preventing excessive extracellular matrix (ECM) degradation, thus, contributing for a delicate balance of cellular signals that mediate between health and degeneration [6,7]. Interestingly, the adage "use it or lose it" holds true for musculoskeletal tissues as not only astronauts but also hospital patients rapidly (within days) lose muscle mass once they are in a near-weightlessness (microgravity) environment, which is found during spaceflight and, not less importantly, during bed resting. In fact, major health deteriorations generated by prolonged microgravity exposure are analogous to bed rest and ageing-induced physiological degeneration [8].

Over the years, several studies have been conducted under simulated conditions of altered gravity using advanced ground-based facilities [9], such as bioreactors for microgravity / hypo-gravity (<1g) research [10] and centrifuges for hypergravity (>1g) studies [11,12]. Microgravity tools have been explored to generate three-dimensional (3D) microtissues by inducing cell aggregation and formation of spheroids [13]. In turn, exposing tissues to hypergravity constitutes a way of increased mechanical loading [14] to rescue cell phenotype after exposure to near-weightlessness conditions or towards the development of novel tissue engineered substitutes [15].

Interestingly, simulated hypergravity has been widely demonstrated to act as a force stimulus [14], having positive effects on osteoblasts and chondrocytes by inducing a faster deposition of ECM components [16,17] or promoting osteogenic differentiation of stem cells [18]. Besides bones, cartilage and muscles, tendons are critical musculoskeletal components, acting as mechanically active bridges by transmitting forces generated by muscles to bones, being subjected to distinct mechanical loads, from posture control (gravity) to daily body movements. Different studies have been focusing on bone, cartilage

6

and skeletal muscle, but effects on tendons (as well as ligaments) have been underappreciated. For instance, results from a 90-day study of bed-rest simulated microgravity showed a reduction in tendon mechanical properties, including tendon stiffness and Young's modulus (58 and 57% reduction, respectively) [19]. On the other hand, no studies have focused on the effect of simulated hypergravity conditions on tendon cells and their biology. Therefore, the main objective of this study was to evaluate the influence of increasing g-levels ($5g$, $10g$, $15g$ and $20g$) and different hypergravity exposure periods (4 and 16 h) on the behaviour of human tendon-derived cells (hTDCs), a mixed population of tenocytes and tendon stem/progenitor cells [20]. Overall, simulated hypergravity conditions tested herein affected cell proliferation, morphology and the expression of tenogenic markers at the gene level, with more pronounced effects in the long-term period (16 h) of stimulation.

2. Materials and methods

2.1. Isolation and culture of hTDCs

Tendon samples were collected from patients undergoing elective orthopedic reconstructive surgeries at Hospital da Prelada (Porto, Portugal) and obtained under informed consent, according to the Declaration of Helsinki and to the ethical committee of the hospital. Human tendon-derived cells (hTDCs) were isolated through collagenase digestion as previously described [20,21]. Human TDCs were isolated from healthy tendon (autograft) collected from the knee (Sartorius) of a male patient with 25 years. Cells were cultured in Minimum Essential Medium alpha (α-MEM, Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS, Alfagene) and 1% (v/v) antibiotic/antimycotic solution (AB/AM, Life Technologies). Cells were maintained in a humidified atmosphere with 5% CO_2 − 95% air at 37ºC and expanded until a sufficient cell number was obtained. Human TDCs were used at passages 2-3 for all experiments.

7

2.2. Hypergravity simulation using a large diameter centrifuge (LDC)

In order to study the influence of hypergravity on tendon cell behavior, hTDCs were cultured and exposed to hypergravity conditions (5g, 10g, 15g and 20g) for 4 or 16h using the LDC (Zeugma) at the European Space Research and Technology Center (ESTEC, European Space Agency, ESA, Noordwijk, The Netherlands). This centrifuge contains four arms with up to six free-swinging gondolas and can be used for experiments at hypergravity levels from 1g to 20g [22]. As the LDC arms rotate, gondolas swing out, with a resultant acceleration vector perpendicular to their bottom surface. The large dimension of the LDC ensures minimal inertial shear effects [23].

A standard cell culture incubator was placed inside each gondola and cells were cultured in 24-well plates inside the incubator at 37ºC in humidified air-locked chambers containing a 95% air, 5% CO_2 atmosphere (Figure 1). For all experiments, cells were seeded in 24-wells tissue culture polystyrene plates at a density of 5×10^4 cells/well and left to adhere for at least 1 h at 37 ºC, 95% air, 5% CO_2 prior to centrifugation. In addition, benchtop controls (1g) were performed for both time points studied (4 h and 16 h).

2.3. DNA quantification by PicoGreen assay

The amount of DNA of hTDCS was quantified by Quant-iT TM PicoGreen® dsDNA assay (ThermoFisher Scientific), according to the manufacturer's instructions. For that, cells were washed twice with PBS, each sample was collected in 1 mL of sterile ultra-pure water and stored at -80 ˚C until further quantification. Samples were thawed to room temperature, sonicated for 15 minutes and then incubated for 5 minutes protected from light with fluorescent dye PicoGreen®, which has affinity for dsDNA. The fluorescence intensity was measured using a microplate reader (Synergy HT, BIO-TEK) with excitation and emission wavelengths of 485/20 and 530/25.

8

2.4. Immunofluorescence analysis of FAK

Immunocytochemical analysis was performed following 4 h and 16 h exposure to hypergravity of 1g, 5g, 10g, 15g and 20g. Cells were fixed with 10% (v/v) neutral buffered formalin (Richard-Allan Scientific^{TM}, ThermoFisher Scientific) for 20 minutes, permeabilised with 0.25% (v/v) Triton X-100 (Sigma-Aldrich) for 30 minutes and unspecific reactions were blocked with 1% (w/v) bovine serum albumin (BSA, Sigma-Aldrich) for 30 minutes. For immunostaining, cells were incubated with primary antibody against FAK (rabbit polyclonal, 5 μg/mL, ab39967, Abcam) at 4˚C overnight and then incubated with secondary antibody Alexa Fluor® 488 donkey anti-rabbit IgG (H+L) (1:1000, A21206, Molecular Probes®) for 1 h. Actin filaments were stained with Phalloidin (1:200, P1951, Sigma-Aldrich) for 30 minutes and nuclei were counterstained with DAPI (1:1000, D9542, Sigma-Aldrich) for 5 minutes. All antibodies were diluted in 1% (v/v) BSA; phalloidin and DAPI were prepared in PBS. Cells were washed at least three times between incubation steps and following DAPI staining. Stained cells were observed by fluorescence microscope (Transmitted and Reflected Light Microscope with Apoptome 2, Zeiss Group, Oberkochen, Germany).

2.5. Quantitative analysis of fluorescence images

Fluorescence images were analysed using ImageJ 1.50i software. Changes in cell morphology were determined in the red channel (F-actin signal) by randomly outlining 30 isolated cells followed by quantification of the selected area (cell area) and the elongation index (aspect ratio, major axis/minus axis). For actin cytoskeleton analysis, selected cells were counted and segregated into two categories according to the amount of stress fibers. The extent of stress fiber formation was then determined by plotting the relative percentages of each cell category for the different conditions. Stress fibers were further studied using the ImageJ plug-in FibrilTool, which measured the anisotropy of actin fibers [24]. For each selection created, an anisotropy score was given, which indicated how parallel stress fibers were within cells (0 for no order and 1 for perfectly parallel ordered fibers).

9

Finally, the number of FAK phosphorylated in each of the immunostained cells for every condition tested was performed using the function Analyze Particles. A minimum of 20 cells were analysed and results are expressed as mean ± SEM.

2.6. Real time reverse transcription polymerase chain reaction (RT-PCR)

After culture, hTDCs were collected in RNA*later*® RNA Stabilization Solution (TermoFisher Scientific). Total mRNA was extracted using TRI Reagent® RNA Isolation Reagent (Sigma-Aldrich), according to the manufacturer's instructions. Then, RNA was quantified on a Nanodrop® ND-1000 spectrophotometer (ThermoFisher Scientific) and cDNA was synthesized from 50 ng of mRNA using qScript^{TM} cDNA Synthesis Kit (Quanta BioSciences). RT-PCR was performed using PerfeCTA® SYBR Green FastMix (Quanta BioSciences), following manufacturer's instructions, on RT-PCR Mastercycler ep realplex gradient S machine (Realplex, Eppendorf). Primer sequences (Eurofins Genomics, UK) were designed using Primer-BLAST tool (Table 1). The relative gene expression was quantified using Livak's method ($2^{-\Delta\Delta Ct}$) [25,26]. For this purpose, transcript expression of target genes was first normalised to the average expression of multiple internal control genes (glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*); β-actin (*ACTB*) and Ribosomal 18S RNA (*RNA18S*)) [27] and then to a calibrator sample (1g control). Five samples of each condition were considered and results are represented as fold change.

2.7. Statistical analysis

Statistical analysis was conducted using GraphPad Prism v6.0 software. Results are expressed as mean ± SEM. Statistical analyses were performed using One-way ANOVA test with Tukey posthoc test. Statistical significances were established for p values < 0.05.

10

3. Results

3.1. Hypergravity exposure for 16 h reduced DNA content

DNA content was assessed to evaluate the influence of hypergravity on the viability/proliferation of hTDCs. Different exposure times resulted in a differential cell response. After a 4 h period of hypergravity exposure, no differences were observed in DNA content, independently of g-level tested (Figure 2). Nevertheless, culturing hTDCs under hypergravity for 16 h resulted in diminished DNA content (Figure 2). A significant reduction in DNA content was observed at 5g ($p<0.0001$), 10g ($p<0.0001$), 15g ($p<0.0001$) and 20g ($p<0.005$), in comparison to normogravity (1g) control, but no significant reduction was observed in comparison to the "initial" (4 h, 1g) DNA concentration.

3.2. Cytoskeleton is dynamically affected by hypergravity in a time and g-level dependent manner

To study the effects of simulated hypergravity in hTDCs morphology, cell surface area and the extent of elongation were determined in cells stained for filamentous actin (F-actin) expression (Figure 3). As shown in Figure 3k and 3l, 4 h exposure to hypergravity revealed no major changes in cell area and morphology. hTDCs exhibited their characteristic fibroblast-like shape (Figure 3a-j and 4a-j), which did not change under hypergravity conditions, including for longer exposure times (16 h) (Figure 4l). Yet, a significant increase ($p<0.001$) in surface area was observed for cells cultured in hypergravity for 16 h (5, 10 and 15g), when compared to 1g controls (Figure 4k).

Next, actin cytoskeleton organization was analyzed by phalloidin staining (Figure 3, 4a-j). In general, F-actin expressed by hTDCs was mainly organized as parallel stress fibers, stretching along the major axis (yellow arrows), with some cells exhibiting protrusions in the form of filopodia (magenta arrows). Few peripheral actin bundles (green arrows) and lamellipodia were detected in both 1g and hyper-g samples. The extent of stress fiber formation was further assessed by categorizing cells according to their content in stress fibers. For 4 h-period experiment, the relative number of hTDCs with pronounced and

11

well-defined stress fibers was found to decrease as g levels were incremented up to 10g (Figure 3b, d, f, n). However, exposure to higher g forces (15g and 20g) seemed to elicit a reverse effect, in which stress fibers inside hTDCs became more evident again (Figure 3h, j, n). Interestingly, the opposite response was observed for hTDCs exposed to hypergravity for 16 h (Figure 4 b, d, f, h, j, n).

Finally, the anisotropy of actin stress fibers was determined for hTDCs exposed to 1g and hypergravity as a measure of the degree of alignment and organization of actin stress fibers. The measured anisotropy score was not significantly altered with increasing g levels for hTDCs cultured for 4 h (Figure 3m). Yet, longer hypergravity exposure significantly increased the anisotropy score when compared to 1g controls (Figure 4m). Indeed, stress fibers inside hTDCs exposed to higher g levels showed more well defined and parallel actin fibers, while under 1g conditions, stress fibers formed inside hTDCs with more than one direction, which overall contributed for a lower anisotropy score.

3.3. Expression of activated FAK is affected under hypergravity

The expression of activated focal adhesion kinase (phosphorylated FAK), a protein of the focal adhesion complex, was studied by immunocytochemistry (Figures 5 and 6).

After a 4 h period of stimulation, FAK was found mainly localized at focal adhesion sites in cells cultured at 1g (Figure 5a), 5g (Figure 5b), 15g (Figure 5d) and 20g (Figure 5e), while in hTDCs cultured at 10g, FAK appeared mainly with a perinuclear location (Figure 5c). In addition, although smooth changes were registered in terms of FAK number/cell, particularly with an increase in hTDCs cultured at 5g, no significant changes were observed upon exposure to simulated hypergravity for this shorter period (p>0.05, Figure 5f).

After a 16 h period, in all hypergravity stimulation conditions, FAK appeared at focal adhesion sites (Figure 6b-6e), whereas in control cells, FAK was mainly localized in the perinuclear region (Figure 6a). Moreover, this period of stimulation seemed to result in a tendency for an increased FAK number/cell with increasing g-levels (Figure 6f). In fact, the

12

number of activated FAK points per cell was significantly higher in hTDCs exposed to 15g and 20g, in comparison to cells cultured at 1g control conditions ($p < 0.05$, Figure 6f).

3.4. Gene expression profile is altered under continuous hypergravity (16 h)

Gene expression analysis was performed to evaluate the effect of hypergravity in the regulation of tenogenic markers. Figure 7 shows the results from quantitative RT-PCR analysis of scleraxis (*SCX*), tenomodulin (*TNMD*), decorin (*DCN*), collagen types I (*COL1A1*) and III (*COL3A1*) and tenascin C (*TNC*) in hTDCs cultured under hypergravity for 16 h. In hTDCs cultured at 15g, *SCX* expression was significantly up-regulated by 4-fold ($p < 0.05$) in comparison to normogravity control (Figure 7). However, no differences were observed for the transcript levels of *TNMD*, a tenogenic differentiation marker regulated by SCX. Moreover, although no differences were found for *COL1A1*, increasing g-levels clearly resulted in an up-regulation of the expression of tendon ECM-associated genes *TNC, COL3A1, DCN* (Figure 7). Indeed, cells cultured at 15g exhibited 5.4-fold up-regulation of *TNC* ($p < 0.01$), 6.4-fold up-regulation of *COL3A1* ($p < 0.05$) and 7-fold up-regulation of *DCN* ($p < 0.05$). Similarly, hTDCs exposed to 20g presented 5.9-fold and 6.5-fold higher expression levels of *COL3A1* and *DCN* ($p < 0.05$), respectively.

Furthermore, the expression of mesenchymal stem cell (MSC)-related markers, *CD73*, *CD90* and *CD105* was studied as a preliminary assessment regarding the influence of hypergravity on the stemness phenotype of the mixed population of hTDCs (Figure 7). Herein, no differences on transcript levels of these markers were found between hypergravity-stimulated and control cells.

4. Discussion

Effects of altered gravity, in particular of hypergravity, on cell behavior are still far from being understood. Few studies exist and experimental results are even contradictory, due to the lack of standardized protocols, different experimental set-ups and equipment [28]. Nonetheless, novel insights into the influence of acceleration forces caused by

centrifugation can be helpful on several areas of biomedicine towards (i) understanding the potential of centrifugation as a countermeasure for microgravity-induced cellular alterations and (ii) using these force stimuli to manipulate cell fate in tissue engineered constructs. In this study, the behavior of human tendon-derived cells (hTDCs) cultured under hypergravity conditions was evaluated using different g-levels (5*g*, 10*g*, 15*g* and 20*g*) and different exposure times (4 h or 16 h), with particular focus on cell morphology, cytoskeleton organization and gene expression. Herein, hypergravity exposure periods selected were based on previous studies of the team, in which endothelial cells (also mechano-responsive) were used, and time-, as well as *g*-level dependent effects were reported [29]. To the best of our knowledge, this is the first report on the influence of hypergravity on hTDCs. The isolated hTDCs population has been described to exhibit MSC-related surface markers (positive ≥ 95% for CD73, CD90 and CD105; ≤ 2% for CD34 and CD45) [20] and to differentiate at least towards the osteogenic lineage (data not shown), supporting the existence of a mixed population containing mature tenocytes and tendon stem/progenitor cells. Previous reports under simulated microgravity have not only shown the maintenance of stemness properties of embryonic stem cells [30] and adipose-derived MSC [31]; but also the influence of exposure time on directing MSC differentiation toward soft tissue lineages or osteogenesis [32]. In turn, hypergravity can be applied as a tool to simulate loading [15], which has also been shown to direct osteogenic differentiation of MSC [33]. Therefore, we studied simulated hypergravity under the hypothesis that the behavior of hTDCs, as a heterogeneous population of mechano-sensitive cells, could be altered with potential beneficial effects for the pre-conditioning of cells in tissue engineering strategies.

As a first approach, DNA content was quantified to infer on cell proliferation under hypergravity. Previous studies have reported an increased proliferation of adipose-derived stem cells (discontinuous exposure to 10*g*, 20*g*, 40*g* and 60*g* – 3 times 20 minutes – with intervals of 40 minutes at 1*g*) [34], HeLa and JTC-12 cells (continuous exposure to 40*g* up to 3 days) [35] and endothelial cells (HUVEC, continuous exposure to 3.5*g* for 48 h and 96 h) [36]. In addition, by studying the time-dependent effect of hypergravity (5*g*) on the proliferation of MC3T3-E1 cells, Miwa *et al.* demonstrated an increase in DNA synthesis

14

after 15 and 30 minutes of stimulation, but proliferation seemed to reach a plateau since no differences could be observed between 30 and 60 minutes of exposure [37]. In contrast, a slight reduction on the proliferation of human osteoblasts cultured for 24 h at 13g has been described [38]; whereas Hirasaka *et al.* reported no differences in the proliferation of rat myoblasts exposed to a short-term period (10 minutes) of hypergravity (3g and 100g) [39]. Similar results have been observed for rat mesenchymal stem cells continuously cultured for 1 day at 10g using the LDC [40]. Moreover, after culturing C2C12 mouse myoblasts for 2 h in the LDC at 5g, 10g or 20g, an increase in DNA content has been described for 10 and 20 g-levels, in comparison to controls and to 5g [41]. In our study, no differences in DNA synthesis were observed after 4 h of exposure to 5g, 10g, 15g or 20g; however, DNA content was reduced for all g-levels after 16 h, in comparison to control. Altogether, these results suggest that the influence of hypergravity on cell proliferation might be orchestrated in a time, g-level and cell type dependent manner.

Besides cell proliferation, the morphology and cytoskeleton organization of hTDCs were evaluated upon hypergravity exposure. Although no studies have been conducted using tendon cells, reports in the literature with other cell types have demonstrated distinct results. For instance, no morphological alterations were detected on human osteoblasts cultured at 13g for 24 h [38], neither on endothelial cells cultured at 3g or 10g for 4 h and 16 h [29]. On the other hand, an increase in cell aspect ratio (major cell axis/minor cell axis) has been described in adipose-derived stem cells cultured following a discontinuous exposure to 10g, 20g, 40g and 60g (3 times 20 minutes – with intervals of 40 minutes at 1g) [34]. Also, an increased cell area and cell perimeter were found in osteoblasts cultured at 3.3g and 4g for 3 h or 2 days [42]. Herein, changes in cell area were only observed for 16 h stimulation period. Indeed, hTDCs cultured at 5g, 10g and 15g for 16 h were significantly bigger but no differences were detected in cell ratio for any condition in comparison to control, suggesting a time- and g-level dependent effect on the morphology of hTDCs.

Furthermore, the cytoskeleton is the first responder to mechanical stimuli, allowing the cell to sense the surrounding environment and translate the input signals in alterations at cellular, molecular and genetic levels. Overall, changes in cytoskeleton organization occur in response to altered gravity [43]. In particular, F-actin is the main protein of the

15

microfilaments, which compose the cytoskeleton. Major alterations under hypergravity have been reported to include an increased thickness of actin stress fibers, as well as a higher number of fibers/cell [42,44,45]. Strikingly, F-actin anisotropy in hTDCs exposed to hypergravity was changed at 5g in a time-dependent manner: after 4 h, actin filaments were less aligned than in control cells; while, after 16 h of hypergravity actin filaments were more aligned; no changes were detected for higher g-levels (>5g) in any of the time points studied. Accordingly, more pronounced stress fibers were also observed at 5g after 16 h. These results suggest that cytoskeleton is dynamically affected in response to hypergravity, being again dependent on the time of exposure and on different g-levels.

In matter of response to mechanical stimuli, cytoskeleton organization is strongly influenced by integrin mediated focal adhesion complex integrating protein including focal adhesion kinase, mostly activated via an auto-phosphorylation process at the tyrosine residue Tyr-397, acting therefore as an important integrator to control cell adhesion and motility [46]. Upon mechanical input via simulated hypergravity, the expression of FAK was enhanced in hTDCs exposed for the longer period (16 h) both at 15g and 20g, leading to higher FAK number/cell in comparison to 1g, while no changes were observed at 4 h exposure. Although no studies have been reported concerning the activation of FAK under hypergravity in tendon cells, a particular study demonstrated that MC3T3-E1 osteoblasts did not change the expression profile of FAK when stimulated either at 1g and 12g for 15 minutes [47]. However, longer periods of stimulation were not reported. On the other hand, cyclic stretched (1 Hz) neonatal rat ventricular myocytes (NRVMs) between 5% to 20% for 10 minutes increased FAK phosphorylation at Tyr-397 from 1.5- to 2.8-fold, and such phosphorylation escalated from 2.5- to 3.5-fold when NRVMs were stretched at 15% from 10 to 120 minutes [48]. In agreement with the present study, phosphorylated FAK Tyr-397 were localized towards the periphery of the cell rather than the nuclear region. These findings suggest that phosphorylation of FAK is similar in localization whether cells are mechanically stimulated with hypergravity or cyclic stretching; however its expression is highly encouraged by cell type and time of exposure.

Finally, the influence of hypergravity on the expression of tenogenic genes was also assessed after stimulating hTDCs for 16 h. Although there are no specific markers for

16

characterizing tendon-derived cells, a panel of markers is commonly accepted, including scleraxis (*SCX*), tenomodulin (*TNMD*), decorin (*DCN*), collagen types I (*COL1A1*) and III (*COL3A1*) and tenascin C (*TNC*). Scleraxis is a basic loop-helix-loop transcription factor that has been identified in developing and mature tendons [49] and is involved in the regulation of collagen type I expression and production [50,51]. Mechanical forces have been previously described to regulate *SCX* expression [52,53], but the effect of hypergravity is yet to unveil. Herein, a trend for *SCX* up-regulation was evidenced after 16 h of hypergravity stimulation. In particular, hTDCs cultured at 15*g* exhibited significantly higher *SCX* transcript levels (4-fold increase), in comparison to normogravity controls at 1*g*. In addition, scleraxis also regulates the expression of tenomodulin, a type II transmembrane glycoprotein found in tendons [54,55]. Although no significant changes in *TNMD* transcript levels were found in hypergravity-stimulated hTDCs, it is worth to mention that *TNMD* expression is temporally regulated, lagging behind *SCX* induction [56]. Thus, other regimens of stimulation (e.g., longer periods or discontinuous exposure) should be further tested in order to gain deeper insights into the regulation of relevant tenogenic genes by hypergravity.

Strikingly, increasing g-levels led to an up-regulation of tendon ECM-associated genes. The expression of main collagens of tendon ECM, namely *COL1A1* and *COL3A1*, as well as the expression of *TNC*, that encodes a glycoprotein of tendon ECM, and *DCN,* which encodes a proteoglycan involved in collagen fibrillogenesis in tendon ECM, were evaluated as indicators of matrix synthesis. Although tendon-derived cells have not been the focus of research under altered gravity, results on fibroblast behaviour are contradictory. Indeed, reports from Spacelab D2 mission in 1993 showed an increase of collagen synthesis under microgravity conditions and a decrease of collagen synthesis with increasing g, being down to 15% at 10*g* [57,58]. Contrarily, hypergravity has been reported to stimulate collagen synthesis by osteoblasts, reaching a significant increase of 42% at 13*g* [38]. Herein, no significant differences were detected in *COL1A1* transcript levels. Nonetheless, hTDCs cultured for 16 h at 15*g* and 20*g* expressed significantly higher levels of *COL3A1.* Similarly, *DCN* transcript levels were significantly increased in the same conditions and *TNC* was highly expressed in cells at 15*g.* Although no significant differences were found for lower

17

g-levels tested (5*g* and 10*g*), transcript levels of the studied tendon ECM-associated genes tended to be higher than in cells cultured at 1*g* control condition. Similarly, previous studies applying mechanical loading have shown effects on the expression of tendon-related genes and tenogenic differentiation of tendon stem cells (TSCs). In particular, murine TSCs isolated from a treadmill running model have shown increased proliferation and up-regulation of tenocyte-related genes [59]; subjecting tendons to mechanical loading has been demonstrated to result in an up-regulation of *COL3A1* and *TNC* [60]; and different stretching magnitudes could balance between tenogenic differentiation (low mechanical stretching forces, 4%) and the phenotypic drift of TSCs toward adipogenic, chondrogenic and osteogenic lineages (large stretching, 8%) [61]. Furthermore, mechano-magnetic actuation of hTDCs has also been reported to result in an up-regulation of tenogenic genes, including *SCX, COL1A1, COL3A1, TNC* and *DCN* [21].

Although the effect of simulated hypergravity as a mechanical stimulus needs to be further elucidated at the protein level, overall, our results showed that hypergravity might act on hTDCs to increase the expression of tenogenic genes, particularly the ones related to tendon ECM, potentially directing tenogenesis.

This study, being the first to investigate hTDCs under simulated hypergravity opens new avenues for more fundamental investigations regarding the influence of this mechanical stimulus on their differentiation potential and the mechanisms underlying cellular responses. Therefore, future studies will generate new insights on the prospective use of simulated hypergravity loading as a countermeasure to rescue tendon properties and regenerate tendons upon near-weightlessness exposure (either microgravity or bed-resting).

In summary, hypergravity induced g-level and time-dependent changes in hTDCs in terms of proliferation, morphology, cytoskeleton organization and tenogenic genes expression. Therefore, the behavior of hTDCs under hypergravity should be further explored using different regimens (i.e., g-levels, periods of stimulation, continuous *versus* discontinuous exposure), as well as 3D models in order to modulate cell function as a prospective tool towards enhancing the development of tendon constructs in tissue engineering strategies.

18

5. Conclusion

Our results showed for the first time that simulated hypergravity impacts the behavior of human tendon-derived cells. The longer exposure period (16 h) limited cell proliferation, independently of the *g*-level tested. Alterations in cell morphology were observed, which seemed to be dependent on *g*-level and time of stimulation. Similarly, changes were found in cytoskeleton organization, in particular the presence and distribution of actin filaments, which were more pronounced in lower *g*-levels (5*g* and 10*g*) after 16 h. The expression of FAK shifted from the perinuclear region in control (1*g*) cells to focal adhesion sites in hypergravity stimulated hTDCs and FAK number/cell increased with increasing *g*-levels. Increasing g-levels resulted in an up-regulation of tenogenic markers, including *SCX, COL3A1, TNC* and *DCN*.

Overall, alterations on the behavior of hTDCs under simulated hypergravity were more pronounced in the long-term period (16 h) of stimulation. Nevertheless, both time- and *g*-level dependent effects were observed. Altogether, these results suggest that simulated hypergravity might be a useful tool for conditioning human tendon-derived cells aiming at tendon tissue engineering strategies.

Acknowledgements

19

Disclosure Statement

We have no competing interests.

20

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28

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29

Table captions

Table 1 - Primer sequences for RT-PCR analysis.

Target gene	Primer Sequence	NCBI reference
Housekeeping genes		
Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	F: TGTACCACCAACTGCTTAGC	NM_002046.4
	R: GGCATGGACTGTGGTCATGAG	
β-actin (*ACTB*)	F: CTGGAACGGTGAAGGTGACA	NM_001101.4
	R: AAGGGACTTCCTGTAACAA	
Ribosomal 18S RNA (*RNA18S*)	F: GAAACCTTCCGACCCCTCTC	NR_046235.3
	R: TACGAGGTCGATTTGGCGAG	
Mesenchymal stem cell related genes		
Cluster of differentiation 73 (*CD73*)	F: CCCCTTTTCTCTCAAATCCA	NM_002526.3
	R: CGTCCACACCCCTCACTTTCT	
Cluster of differentiation 90 (*CD90*)	F: TGACCCGTGAGACAAAGAAG	NM_006288.3
	R: CTGATGCCCTCACACTTGAC	
Cluster of differentiation 105 (*CD105*)	F: GTCTCAAGACCAGGAAGTCCA	NM_001114753.2
	R: TGTACCAGAGTGCAGCAGTG	
Tendon-related genes		
Scleraxis (*SCX*)	F: AGAACACCCAGCCCAAACAGAT	NM_001080514

30

	R: TCGCGGTCCTTGCTCAACTTT	.2
Tenomodulin (*TNMD*)	F: AGCCTATGACATGGAGCACAC	NM_022144.2
	R: AGATGCCAGTGTATCCGTTTT	
Decorin (*DCN*)	F: CTAGTCACAGAGCAGCACCTAC	NM_001920.4
	R: CCAGGGAACCTTTTAATCCGGGAA	
Collagen type I (*COL1A1*)	F: CCCCAGCCACAAAGAGTCTAC	NM_000088.3
	R: TTGGTGGGATGTCTTCGTCT	
Collagen type III (*COL3A1*)	F: CCTGAAGCTGATGGGGGTCAA	NM_000090.3
	R: CAGTGTGTTTCGTGCAACCAT	
Tenascin C (*TNC*)	F: ACTGCCAAGTTCACAACAGACC	NM_002160.3
	R: CCCACAATGACTTCCTTGACTG	

Figure captions

(a) (b) (c)
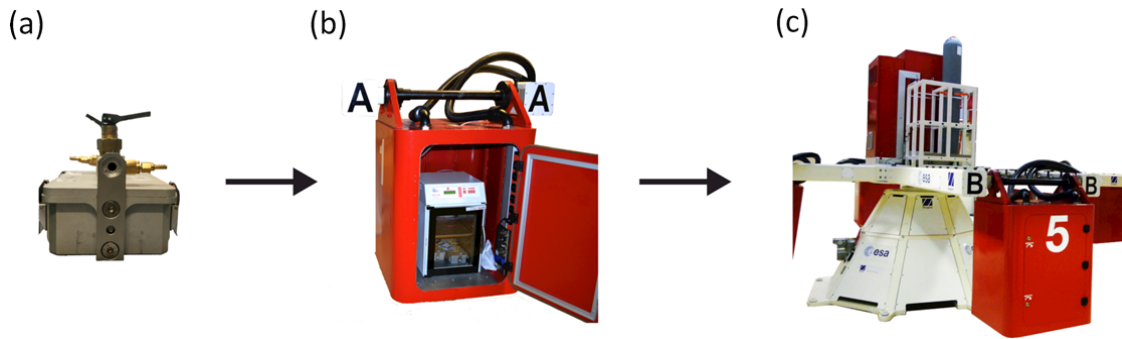
Figure 1. Experimental payload. (a) Air-locked chamber used to maintain suitable culture conditions; (b) Inside view of a gondola with a cell culture incubator; (c) The Large Diameter Centrifuge.
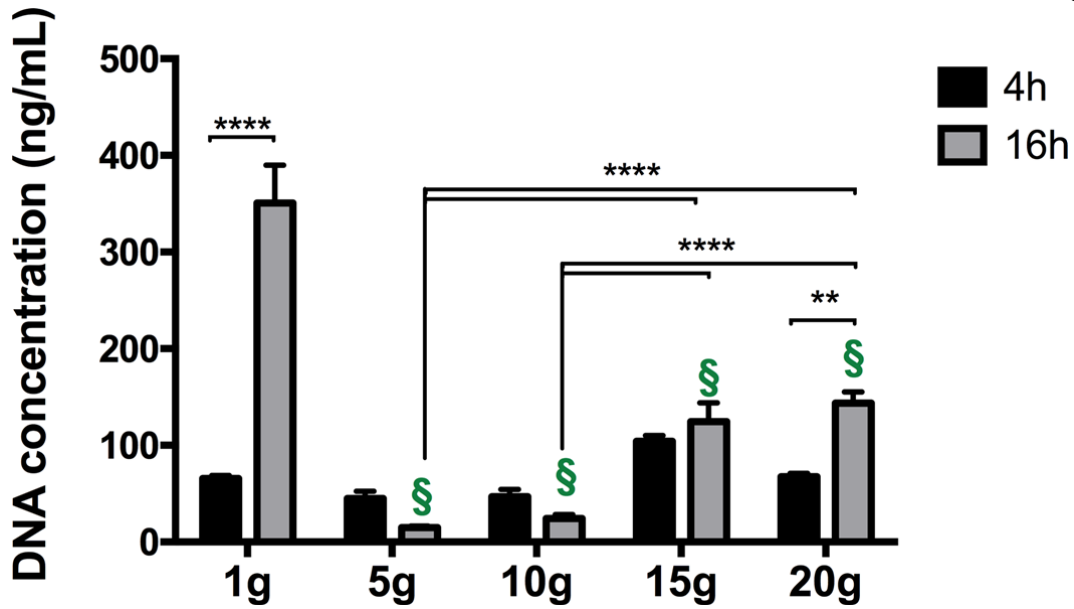
Figure 2. DNA content of hTDCs cultured at different *g*-levels. Results are presented as mean±SEM (n=5). Statistically significant differences are shown as **, p<0.05, ****, p<0.0001. § represents statistical difference in comparison to 1*g* 16h control with p<0.0001.
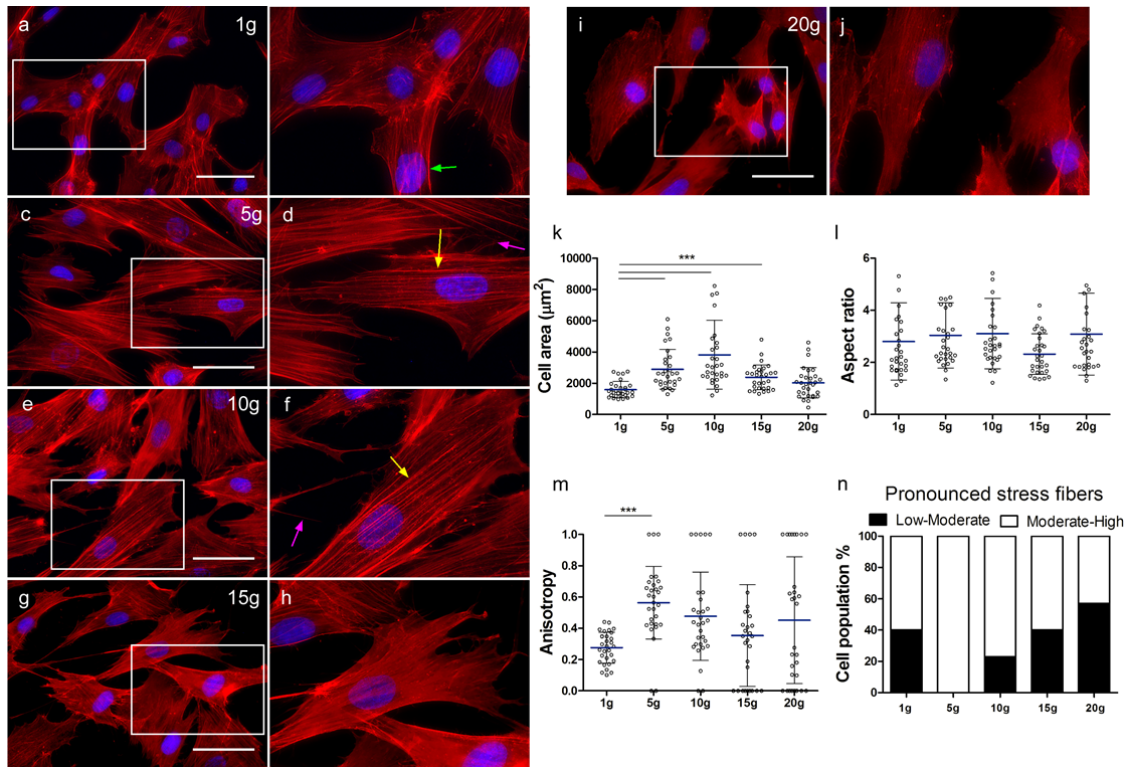
33
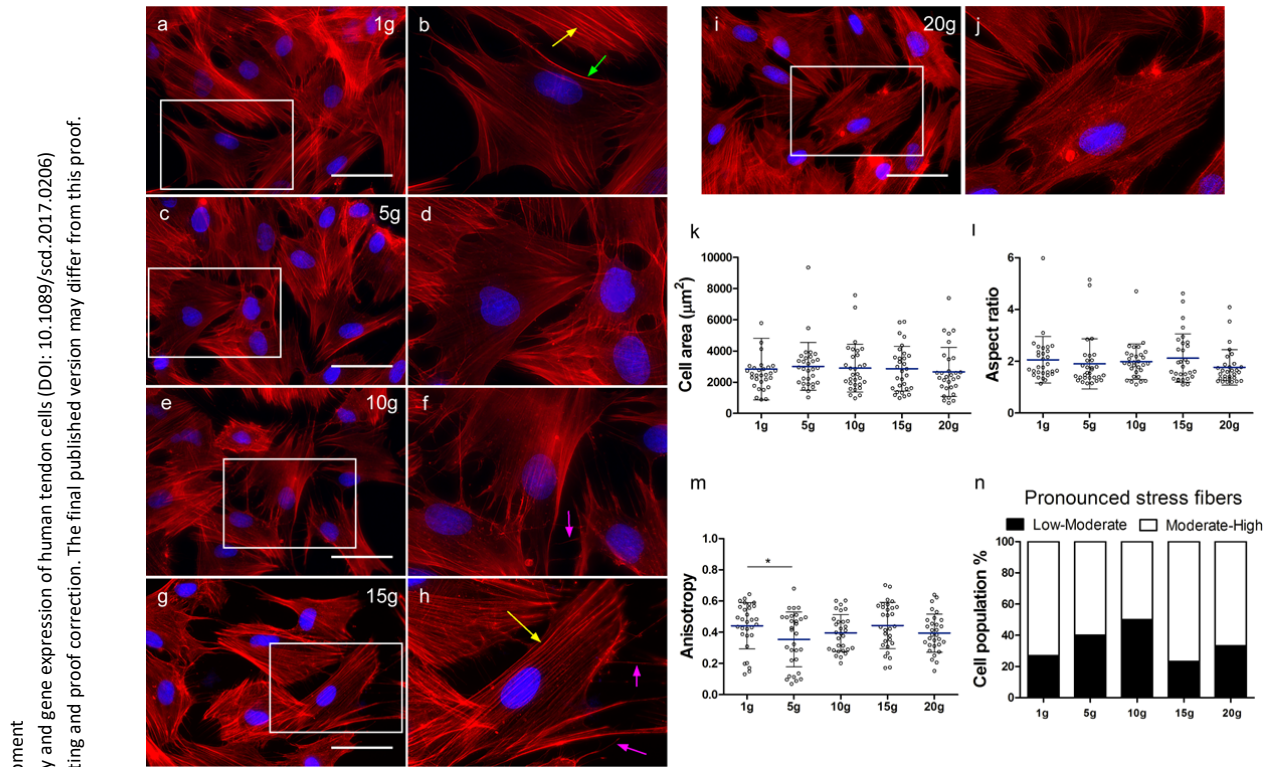
Figure 3. Effect of 4 h exposure to hypergravity on hTDCs morphology and F-actin distribution. (a-j) Organization of F-actin (red) of hTDCs cultured in normal and hypergravity conditions; scale bar 50 μm. Yellow arrows, parallel stress fibers; magenta arrows, filopodia; green arrows, peripheral actin bundles. (k,l) Quantification of the average cell surface area and aspect ratio (major cell axis/minor cell axis) of 30 individual cells using ImageJ software. (m) Measurement of the anisotropy of actin stress fibers inside hTDCs. The anisotropy score was obtained using the ImageJ plug-in FibrilTool. **(n)** Quantification of the relative number of hTDCs with stress fibers. Statistically significant differences are shown as *, $p<0.05$. Results are presented as mean±SEM (n=30).

34

Figure 4. Effect of 16 h exposure to hypergravity on hTDCs morphology and F-actin distribution. (a-j) Organization of F-actin (red) of hTDCs cultured in normal and hypergravity conditions; scale bar 50 μm. Yellow arrows, parallel stress fibers; magenta arrows, filopodia; green arrows, peripheral actin bundles. (k,l) Quantification of the average cell surface area and aspect ratio (major cell axis/minor cell axis) of 30 individual cells using ImageJ software. (m) Measurement of the anisotropy of actin stress fibers inside hTDCs. The anisotropy score was obtained using the ImageJ plug-in FibrilTool. **(n)** Quantification of the relative number of hTDCs with stress fibers. Statistically significant differences are shown as ***, $p < 0.001$. Results are presented as mean±SEM (n=30).
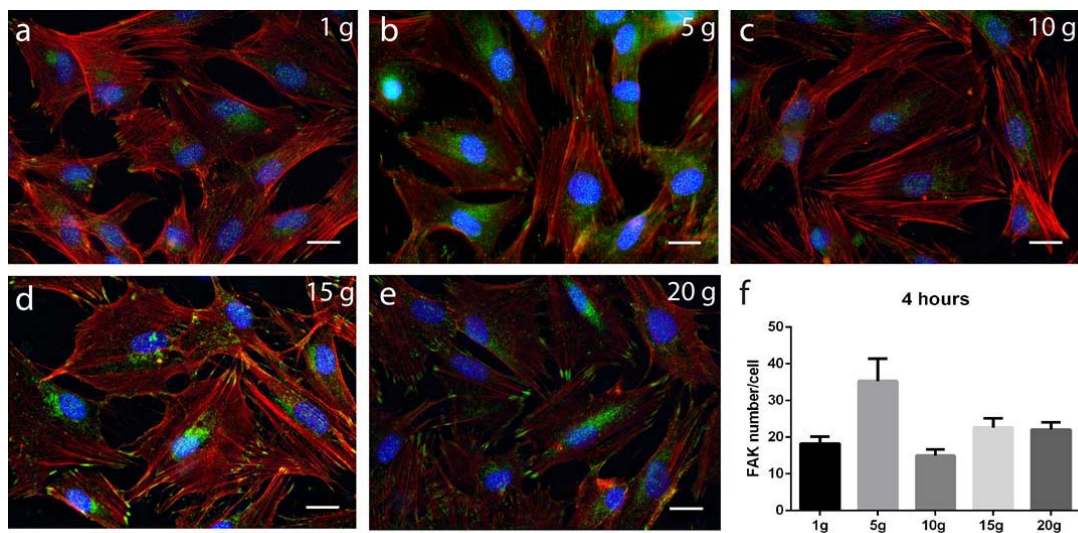
Figure 5. Expression of focal adhesion kinase (FAK Y397) under simulated hypergravity during 4 h. (a-e) Fluorescence microscope images of immunostained FAK Y397 (green). (f) Estimation of FAK Tyr-397 content *per* cell. F-actin and nuclei were counterstained with phalloidin (red) and DAPI (blue), respectively. Scale bars, 20 µm. Results are presented as mean±SEM (n=20).
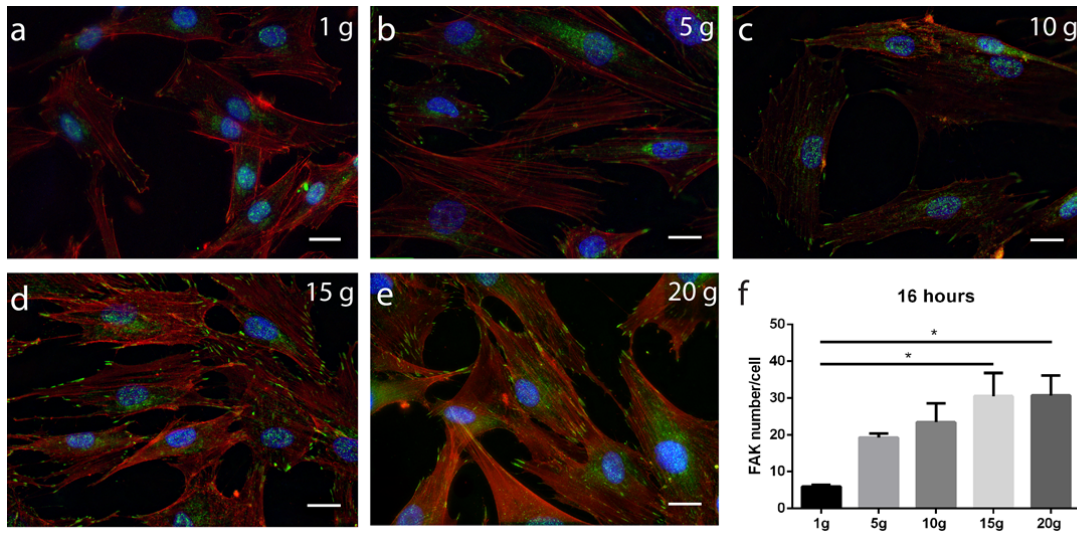
Figure 6. Expression of focal adhesion kinase (FAK Y397) under simulated hypergravity during 16 h. (a-e) Fluorescence microscope images of immunostained FAK Y397 (green). (f) Estimation of FAK Tyr-397 content *per* cell. F-actin and nuclei were counterstained with phalloidin (red) and DAPI (blue), respectively. Scale bars, 20 µm. Results are presented as mean±SEM (n=20). Statistically significant differences are shown as *, $p < 0.05$.
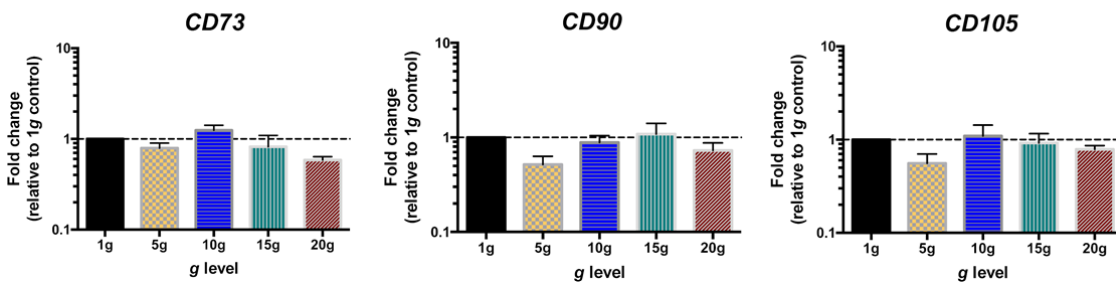
Tenogenic markers
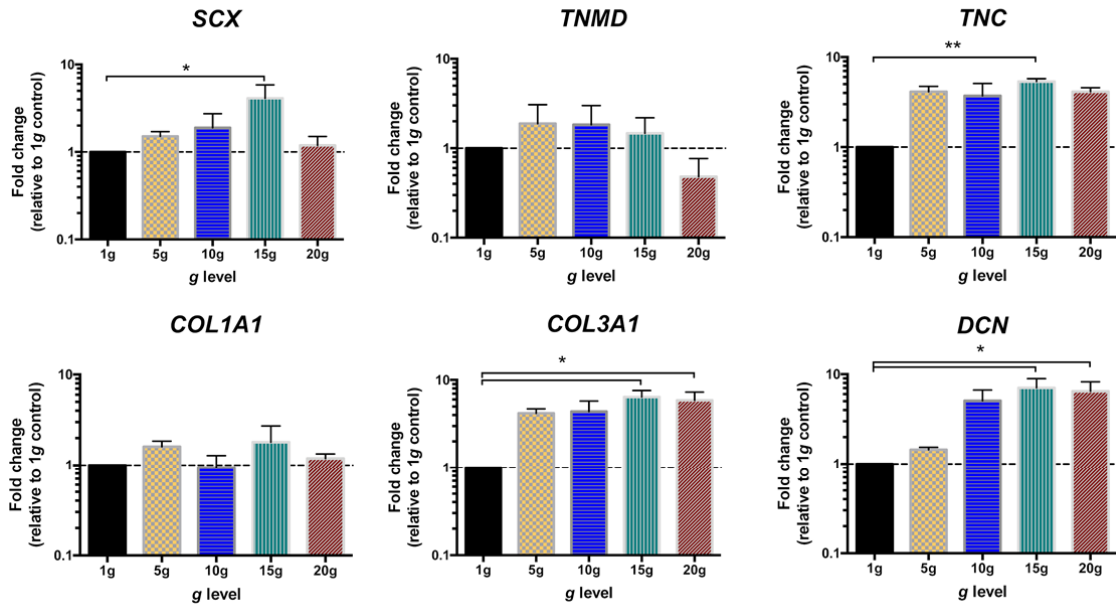
MSC-related markers

Figure 7. Expression of tendon-related genes and MSC-related markers in hTDCs cultured under hypergravity for 16h. Expression of target genes was normalized to average internal control genes expression (*GAPDH*, *ACTB* and *RNA18S*); 1*g* was used as calibrator sample. Results are presented as mean±SEM (n=5). Dashed lines represent the calibrator sample (considered as 1). Statistically significant differences are shown as *, p<0.05; **, p<0.01.