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ORIGINAL ARTICLE

Tissue Engineering of Cartilage on Ground-Based Facilities

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Abstract Investigations under simulated microgravity offer the opportunity for a better understanding of the influence of altered gravity on cells and the scaffold-free three-dimensional (3D) tissue formation. To investigate the short-term influence, human chondrocytes were cultivated for 2 h, 4 h, 16 h, and 24 h on a 2D Fast-Rotating Clinostat (FRC) in DMEM/F-12 medium supplemented with 10 % FCS. We detected holes in the vimentin network, perinuclear accumulations of vimentin after 2 h, and changes in the chondrocytes shape visualised by F-actin staining after 4 h of FRC-exposure. Scaffold-free cultivation of chondrocytes for 7 d on the Random Positioning Machine (RPM), the FRC and the Rotating Wall Vessel (RWV) resulted in spheroid formation, a phenomenon already known from spaceflight experiments with chondrocytes (MIR Space Station) and thyroid cancer cells (SimBox/Shenzhou-8 space mission). The experiments enabled by the ESA-CORA-GBF programme gave us an optimal opportunity to study gravity-related cellular processes, validate ground-based facilities for our chosen cell system, and prepare long-term experiments under real microgravity conditions in space

Keywords Microgravity · Random Positioning Machine · Clinostat · Rotating Wall Vessel · Chondrocytes · Cartilage · Cytoskeleton · Tissue engineering

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Introduction

Cartilage is a skeletal tissue consisting of cartilage cells (chondrocytes), originating from mesenchymal stem cells undergoing differentiation (Aubin et al. 1995; Johnstone et al. 1998; Kuznetsov et al. 2001; Ohgushi and Caplan 1999; Wakitani et al. 1994). Their extracellular matrix consists of water (75 %), collagen type II, and proteoglycan. Thus, chondrocytes sparsely contribute to the composition of adult human articular cartilage representing only about 1 % of the cartilage volume (Temenoff and Mikos 2000; Stockwell 1967) and changing their functions and activity

during the skeletal development (Zanetti and Solursh 1984). During their formation, maturation and ageing, the activity of the chondrocytes declines (Buckwalter and Mankin 1998) resulting in cartilage degradation. Moreover, the exposure to higher loads over the physiological limit may lead to cartilage injuries as osteoarthritis (Nakamura et al. 2006).

To replace the degraded cartilage tissue, new cartilage can be engineered *in vitro* from chondrocytes under laboratory conditions. However, the culture of chondrocytes growing in a monolayer is problematic, as these cells tend to dedifferentiate to a more fibroblast-like phenotype over time and show only low proliferation activity in general.

3D-rotation enables chondrocytes to redifferentiate and to develop a cartilage-specific extracellular matrix (Marlovits et al. 2003a). Numerous rotating systems exist for the high-density 3D cultivation of chondrocytes (Koch and Gorti 2002). Three kinds of ground-based facilities are available for tissue engineering under simulated microgravity. Using different physical principles they aim to achieve functional weightlessness (simulated microgravity) for the exposed system, which has finally to be verified in space (Herranz et al. 2013). The Random Positioning Machine (RPM) is a ground-based facility, enabling the position of a biological experiment in 3D space to be randomly reoriented by changing speed and direction of rotation according to the corresponding software (van Loon 2007). On the 2D Fast-Rotating Clinostat (FRC), the cells are kept in a constant state of free fall by preventing their sedimentation by means of a fast and steady rotation of the culture vessel around the horizontal axis (Briegleb 1992). The Rotating Wall Vessel (RWV) developed by NASA is a culture system that utilizes a circular vessel with a semipermeable membrane also rotating around one horizontal axis to provide culture medium flow and counteracting sedimentation (Schwarz et al. 1992; Mitteregger et al. 1999).

In order to understand the influence of gravity in general and also of altered gravity conditions like micro- or hypergravity on such cells as chondrocytes, fibroblasts or others, *ex-vivo* studies had been employed (Buravkova et al. 2005). To investigate the influence of clinorotation on human chondrocytes with respect to cytoskeletal changes, we performed experiments cultivating monolayer cultures of cryogenically preserved human articular chondrocytes in DMEM/F-12 medium on FRC for 2 h, 4 h, 16 h, and 24 h, and compared the results with the corresponding static 1*g* controls. We focused on vimentin and F-actin because these cytoskeletal components play a potential role in the signal transduction from the membrane to the nucleus under microgravity conditions (Cogoli-Greuter et al. 2004). Cytoskeletal changes induced by RPM-exposure have been previously investigated and published in Aleshcheva et al. (2013). However, in this study we focus on the comparison of the results achieved on the RPM and the FRC to find limitations and advantages of both devices. As cells are added to the RWV in suspension, it was not possible to investigate the short-term influence of wall vessel rotation on chondrocytes.

An enhanced spheroid formation, that means formation of cell aggregates, had been detected in space experiments lasting for 3 months on the MIR Space Station (Freed et al. 1997). To study whether spheroid formation can also be induced by ground-based facilities and to investigate the cell biology of 3D biological structures with respect to *in vivo* tissue formation, long-term cultivation (7 days) of human chondrocytes in DMEM/F-12 medium was performed on the RPM, FRC and the RWV.

Our findings illustrate the importance of the physical environment and cultivation conditions on cellular organization and tissue formation.

Materials and Methods

Cells and Cell Culture Procedures

Commercially available cryogenically preserved human chondrocytes (Provitro®, Berlin, Germany) were cultured in DMEM/F-12 medium (Life Technologies Europe®, NAERUM, Denmark) supplemented with 10 % fetal calf serum (Biochrom®, Berlin, Germany) and antibiotics – 100 IU penicillin/mL and 100 μg streptomycin/mL (Biochrom®, Berlin, Germany).

The cells from frozen stocks (passage 1) were grown in 2 T175 cell culture flasks (175 cm^2; Sarstedt, Nümbrecht, Germany) until subconfluent layers were obtained. Afterwards, the cells were subcultured (passage 2) in 10 T175 flasks: 6 T175 culture flasks for 7-day RWV experiments (3 T175 for each vessel); 1 T175 – for FRC experiments (2 h, 4 h, 16 h, 24 h, and 7 day-experiments), and 3 T175 – for 7-day RPM experiments.

2D FastRotating Clinostat (FRC)

The cells in one T175 culture flask were subcultured into 48 slide flasks (10^6 cells/each) (BD, Heidelberg, Germany) which, prior to the experiment, were completely filled with medium avoiding air bubbles, in order to assure a minimization of turbulences. The FRC (DLR, Cologne, Germany) was loaded with four slide flasks on each of the 6 parallel rotating axes. The device was placed inside an individual incubator at 37 °C and 5 % CO_2 and constantly rotated with 60 rpm. The 2D clinostat rotating constantly at this speed generates a maximum residual acceleration below 0.036*g* calculated at the border of the flasks (Eiermann et al. 2013).

The experiment lasted 2 h, 4 h, 16 h, 24 h, and 7 days with medium change every second day under 1g conditions.

3D Random Positioning Machine (RPM)

3D cultivation was performed using a Desktop Random Positioning Machine (RPM) manufactured by ADS (former Dutch Space, Leiden, Netherlands) (van Loon 2007).

Prior to the experiment, 3 T175 were subcultured in 30 T25 culture flasks (passage 3) that after reaching subconfluence were completely filled with DMEM/F-12 medium without air bubbles. 15 T25 were fixed on the RPM, as close as possible to the centre of the platform, which was then rotated at a speed of 60 °/s in real random mode, meaning that speed and direction of the two rotating frames changed randomly according to a software. Residual accelerations for RPM exposure have to be considered for all 3g-vector components that means in x, y and z directions. Exemplary measurements of a RPM running at 60–120 °/s showed accelerations due to gravity of -1 t o $+1g$ for each direction (Borst and van Loon 2009). The RPM was positioned in a commercially available incubator set at 37 °C and supplied with 5 % CO_2. 15 T25 culture flasks for 1g ground control cultures grown in parallel in identical equipment, were, however, kept statically in the same incubator as the RPM. The medium in all culture flasks was changed every third cultivation day. For this purpose, the device had to be stopped for about 30 min. After 7 days of continuous cultivation, we removed the flasks from the RPM to investigate the spheroid formation.

Rotating Wall Vessel (RWV)

The RWV bioreactor is a cell culture technology designed by NASA and the first bioreactor system to simultaneously co-culture cells.

For our tissue engineering experiments we used the RCCS-2D system with 50-mL disposable vessels (Synthecon Incorporated, Houston, TX, USA). The cells in suspension from 6 T175 were added under sterile conditions to each 50-ml HARV culture vessel of 12.7-cm-diameter. The RWV was placed in a commercially available incubator set at 37 °C and supplied with 5 % CO_2. The initial rotation speed was manually adjusted with tachometer on 10 rpm and increased to 20 rpm to maintain cells in suspension (Marlovits et al. 2003a). The rotary culture was performed for 7 days with medium change every third day under 1g conditions.

Due to operational constrains - cells were added to the RWV in suspension - investigations of the short-term influence of altered gravity with respect to cytoskeletal changes were not performed.

Indirect Immunofluorescence Staining (IIF)

For the investigation of cytoskeletal alterations visualized by immunofluorescence staining, cells (1 x 10^6 cells/cm^2) of passage 2 were seeded into several slide flasks (BD, Heidelberg, Germany) and placed in the incubator (37 °C, 5 % CO_2) overnight, until they attached to the slides. The next day, the slide flasks were completely filled with medium avoiding air bubbles, sealed with paraffin, and placed on the FRC for the 2 h, 4 h, 16 h, and 24 h runs.

As the clinostat was stopped, the chondrocytes were washed twice with DPBS and fixed for 30 min with 4 % paraformaldehyde (4 °C), and permeabilized with Triton X-100 (Sigma, Taufkirchen, Germany). After washing with DPBS the cells were incubated with primary antibody to investigate the morphology of intermediate filaments (vimentin, 1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA) for 24 h at room temperature. Afterwards, the chondrocytes were washed twice with DPBS and incubated for 2 h with the secondary FITC-tagged antibody, used at a dilution of 1:500 (Cell Signaling Technology, Inc., Danvers, MA, USA). For nuclear staining, the cells were further stained with propidium iodide (1:100; Invitrogen, Carlsbad, CA, USA) for 10 min, mounted with Vectashield immunofluorescence mounting medium (Vector, Burlingame, CA, USA), and analysed microscopically.

F-actin Staining

F-actin was visualised by means of rhodamine-phalloidin staining (Molecular Probes®, Eugene, OR, USA) (Grimm et al. 2009; Infanger et al. 2007). For this purpose, seeded cells were fixed for 30 min with 4% PFA (in DPBS), washed twice with DPBS, incubated with 5 μg/ml fluorescent phalloidin conjugate solution (in DPBS with 1 % BSA) for at least 20 min at room temperature and then washed several times with DPBS to remove unbound phalloidin conjugate. Afterwards, the nuclei were stained with Hoechst 33342 (Molecular Probes® , Eugene, OR, USA) for 5 min and washed twice with DPBS. For evaluation, the samples were mounted with Vectashield® (Vector, Burlingame, CA, USA) and analysed microscopically.

Microscopy

The occurrence of the spheroids was examined by phase-contrast microscopy (Olympus, Hamburg, Germany) immediately after the 7th day of RPM, FRC or RWV exposure. Immunofluorescence staining and F-actin were analysed with a Zeiss 510 META inverted confocal laser scanning microscope (Zeiss, Germany), equipped with a

Plan-Apochromat 63 x1.4 objective. Excitation and emission wavelengths were: $\lambda_{exc} = 488$ nm and $\lambda_{em} = \geq 505$ nm for FITC.

Results

Short-term Exposure of Chondrocytes on the FRC

To learn more about the early steps of spheroid formation monolayer cultures of chondrocytes were cultivated for 2 h, 4 h, 16 h, and 24 h in DMEM/F-12 medium on the FRC and the achieved results were compared with the corresponding static 1*g* controls. Here, we focused on two cytoskeletal elements – vimentin and F-actin. Already after a 2h-exposure on the FRC, larger meshes in the vimentin network and perinuclear accumulation of vimentin were observed (Fig. 1e compared to Fig. 1a). However, cells cultivated on the FRC, did not reveal more meshes in the vimentin network after 4 h, 16 h and 24 h (Fig. 1f–h) as compared to 2h, indicating differences in adaptation of different cell types to the exposure to the FRC (Tables 1 and 2).

As visualised by F-actin staining, the shape of the chondrocytes cultivated on the FRC changed: Cortically localized stress fibres were visible after the 4-hour-exposure (Fig. 2b, Table 2) in contrast to the 1*g* control cells (Fig. 2a Table 2). The fibres were reduced in length and thickness during further incubation until 24 h on the FRC compared to the control cells (Fig. 2d Table 2), which revealed nearly no F-actin fibres even up to 24 h under static 1*g* conditions (Fig. 2c, Table 2).

Long-Term Exposure of Chondrocytes on Ground-Based Facilities

The main result of this study is that human articular chondrocytes form spheroids without addition of scaffolds or other artificial materials after a 7-day exposure on all three ground-based facilities – 3D RPM, 2D FRC and RWV No spheroids were observed in the corresponding static 1*g* controls.

On the RPM and FRC the cell population is separated in two different groups - adherent cells growing on the bottom of the cultivation flask and detached cells forming multicellular spheroids. However, the adherent layer serves as a starting point for a transition from a 2- to a 3-dimensional growth that takes about 5-7 days (Fig. 3a and b). With respect to the RWV experiments, chondrocytes are added already as cell suspension resulting in a faster spheroid formation. We observed spheroids of larger sizes compared to those produced on the FRC and the RPM (Fig. 3c).

All three ground-based facilities lead to spheroid formation, even though the experimental approaches strongly differ with respect to culture volume and vessel geometry

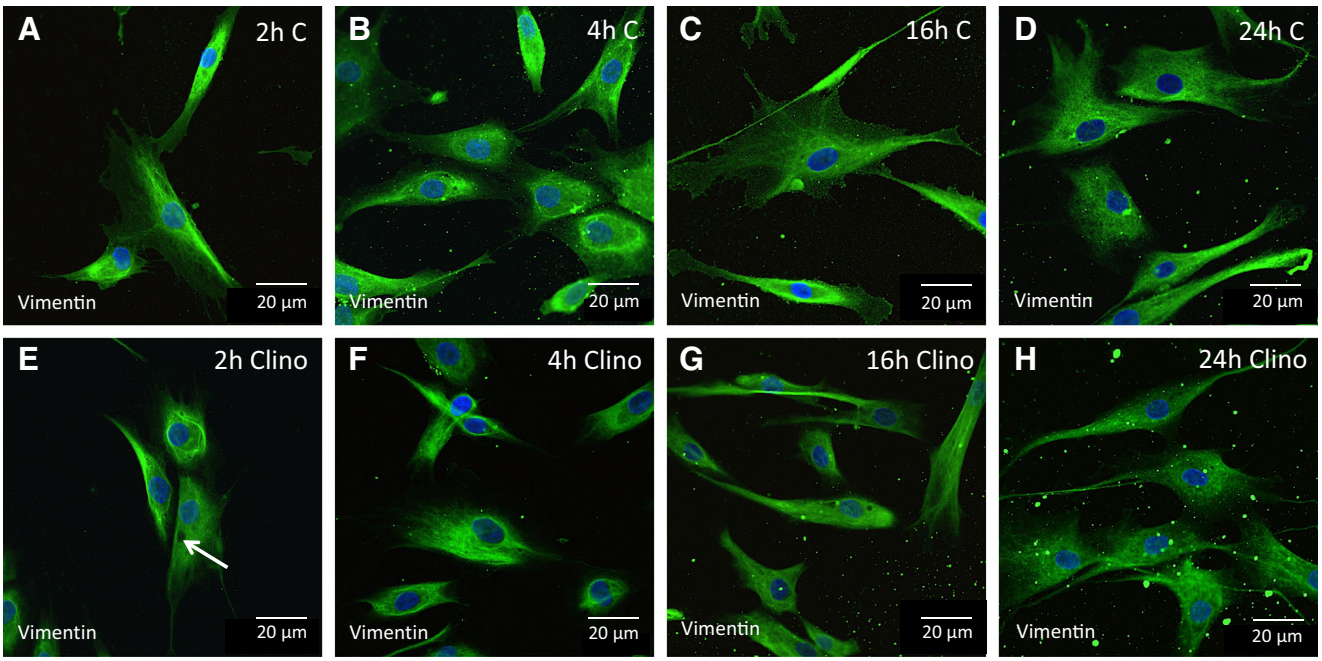

Fig. 1 Immunofluorescence staining of chondrocytes cultured under conditions of normal gravity 1*g* (**a**–**d**) and of cartilage cells grown in the 2D Fast Rotating Clinostat (**e**–**h**) for 2 h (**a**, **e**), 4 h (**b**, **f**), 16 h (**c**, **g**), and 24 h (**d**, **h**). Perinuclear vimentin deposits are visible in clinorotated samples. Vimentin accumulated in the perinuclear region. In addition, small mesh-like irregularities in the vimentin network (white arrows) appeared, in contrast to the rather even distribution in 1*g* control cells

Table 1 Differences in handling with chondrocytes during the experiments under clinorotation, wall vessel rotation, and real microgravity conditions

Parameter	RPM 7 days and short-term (Aleshcheva et al. 2013)	FRC	RWV	Space (MIR Space Station) (Freed et al. 1997)
Cells	Cryogenically preserved human chondrocytes isolated from articular cartilage (Provitro, Berlin, Germany)			Chondrocytes isolated from articular cartilage (femoropatellar grooves of 2- to 3-week-old bovine calves)
Scaffolds	No scaffolds	No scaffolds	No scaffolds	Discs made of polyglycolic acid (PGA) of 5-mm diameter and 2-mm thickness
Cultivation vessel	T25 culture flasks	Slide flasks	HARV	Custom-made
Cultivation medium	DMEM/F-12 medium + 10 % fetal calf serum + 100 IU penicillin/ml + 100 μg streptomycin/ml			DMEM + 4.5 g/l glucose +10 % fetal bovine serum + 10 mM N-2-hydroxy-ethyl piperazine N'-2-ethane sulfonic acid + 0.1 mM non-essential amino acids + 0.4 mM proline + 50 mg/l ascorbic acid + 100 units/ml penicillin + 100 μg/ml streptomycin + 0.5 μg/ml fungizone
Cultivation volume	64 ml Completely filled	10 ml Completely filled	50 ml Completely filled	110 ml Completely filled
Cultivation time	Short-term or 7 days	7 days	7 days	4 month on Earth and 3 months on MIR
Medium change	50% every 3 days	50% every 2 days	50% every 3 days	50% every 3–4 days
Operation mode of the culturing device	60° (10 rpm) Real random mode	(60 rpm) Continuously	(20 rpm) Continuously	15–28 rpm over 3 months

(Table 1). This in turn results in different treatments of the cultured cells: While the medium in the flasks on the RPM (60 ml) and in the RWV (50 ml) had to be changed to 50 % every 3rd day, the medium in the slide flasks on the clinostat (12 ml) was exchanged every 2nd day (Table 1). Due to the small cultivation area in the slide flasks – in order to keep residual accelerations as minimal as possible - the handling has to be performed carefully in order to avoid loss of spheroids during medium exchange.

Scaffold-Free Spheroid Formation

RPM, FRC and RWV enabled the 3D formation of spheroids without any addition of scaffolds or other artificial materials. After 7 days of cultivation, we detected an enhanced spheroid formation in the T25 culture flasks on the RPM and in the slide flasks on the FRC (Fig. 3a and b). The cells in the RWV started to form visible spheroids after 3 days of continuous cultivation reaching about 3 mm in size on the 7th day (Fig. 3c). As already mentioned, the size of the spheroids differed comparing FRC, RPM to RVW due to the different geometry of the culture vessels (Table 1), cells separation (adherent cells and MCS) and possibly different operational modes of the devices.

Discussion

The reduction of mechanical forces experienced by astronauts during their space missions has a direct negative impact on their skeletal system. Microgravity reduces bone and muscle mass and may also induce a deterioration of cartilage integrity (Akmal et al. 2006). Articular cartilage is the main load-bearing tissue of the synovial joint and mechanical forces are important for its maintenance. So far, it has been established that *in vitro* cultivated cells are affected by altered gravity conditions in multiple ways, on both the transcriptional and the translational level, as well as in their intracellular protein organization (Buravkova et al. 2005; Vorselen et al. 2014; Svejgaard et al. 2015; Warnke et al. 2014). Altered gravity conditions simulated on ground and in reality in space represent a unique environment to study the gravity-dependent response of cells (Vorselen et al. 2014; Grimm et al. 2009, 2010, 2014; Ma et al. 2014).

The cytoskeleton as initial gravity sensor has been subject to some recent studies before (Vorselen et al. 2014; Cogoli-Greuter et al. 2004). It is central in the binding and subsequent intracellular signal transduction of mitogens. This process may be affected by changes in the vimentin and tubulin networks, which have been observed in

Table 2 Tissue engineering of chondrocytes under microgravity conditions

Parameter		RPM - 7 days and short-term (Aleshcheva et al. 2013)		FRC		RWV		Space (Freed et al. 1997)
Spheroid formation (size/weight)		20 μm	On day 5–7	Yes 20 μm	On day 5–7	Yes 3 mm	On day 3	After 3 months 0.3−0.4 g
Cytoskeletal changes	Vimentin	Meshes in the network	After 2h	Holes in the network	After 2h			
		Perinuclear accumulation	After 2h	Perinuclear accumulation	After 2h			
	F-actin	Stress fibres	After 4h	Stress fibres	After 4h			Not determined
		Stress fibres are reduced, length and thickness; cortical localization	After 24h	Stress fibres are reduced thickness, cortical localization	After 24h	Not investigated		
Gene expression/ protein changes		Increased expression of *ACTA2*, *ACTB*, *VIM*, *TUBB6*, *ITGB1* and *TGFB1*		After 30 min	Not investigated			Proteoglycan and type II collagen

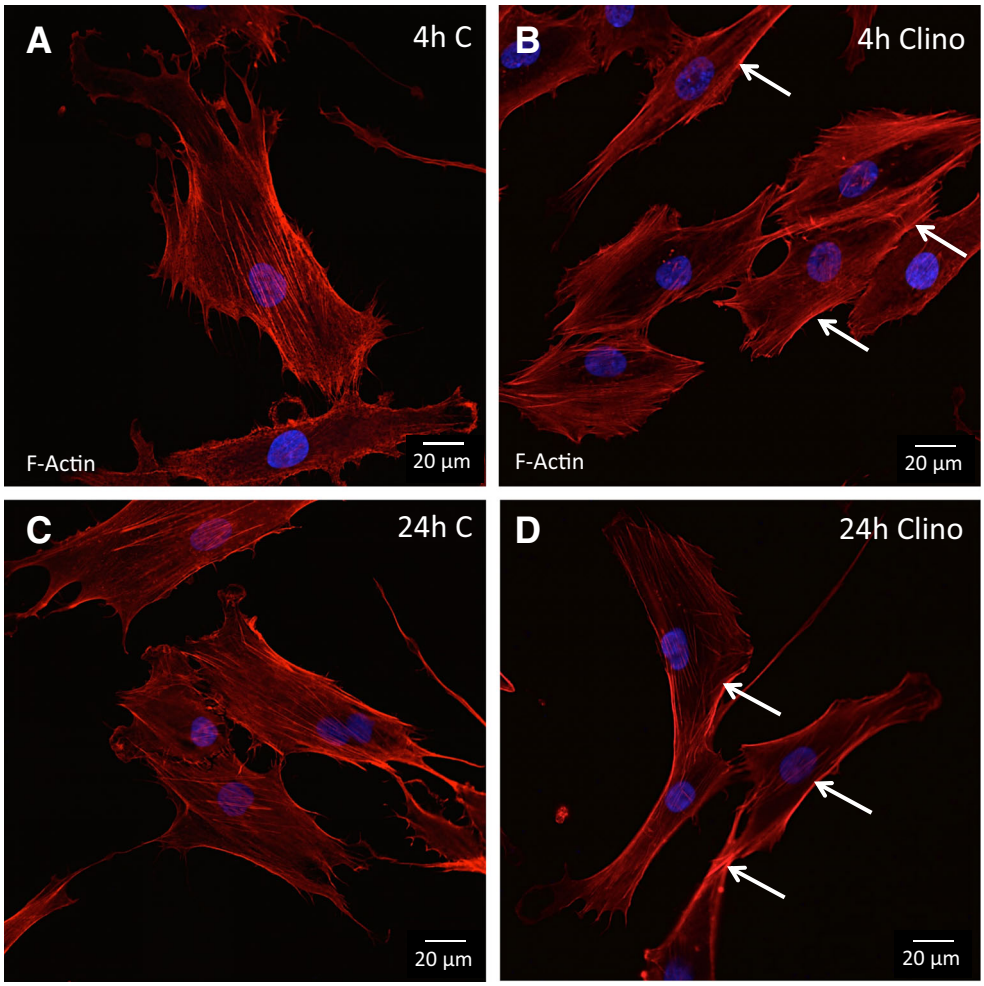

Fig. 2 F-actin staining of human chondrocytes cultivated on the FRC (Clino). **a** – F-actin staining of 1*g* control cells cultivated for 4 h in the incubator, **b** – F-actin staining of the cells cultivated for 4 h in the FRC. **c** – F-actin staining of 1*g* control cells cultivated for 24 h in the incubator, **d** – F-actin staining of the cells cultivated for 24 h in the FRC. After 4 h actin stress fibres are reduced in number, length and thickness. Moreover, the F-actin cytoskeleton showed a cortical localization of accumulations that persisted also after 24 h

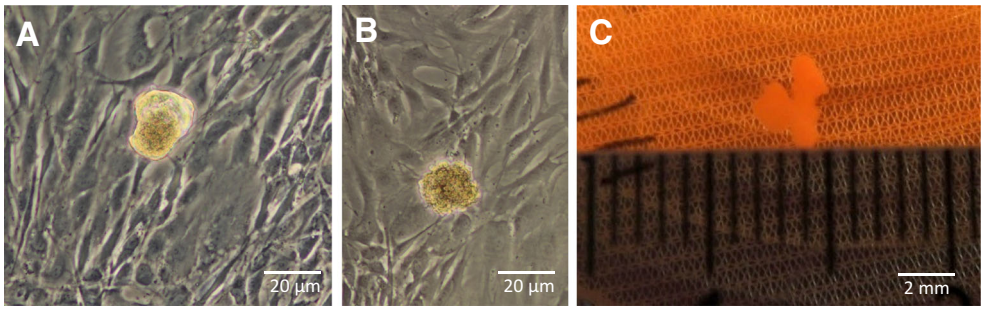

Fig. 3 Comparison of scaffold-free 3D-growth on ground–based facilities. 3D formation of chondrocyte cells after 7 days on the RPM (**a**), on the FRC (**b**) and the RWV (**c**)

cells grown under microgravity conditions (Cogoli-Greuter et al. 2004). Moreover, the F-actin cytoskeletal component has also been suggested to be involved in the observed microgravity-induced alterations of cellular shape, function, and signalling. Under standard (1*g*) conditions microtubules were organized in a radial shape in adherent cells, while intermediate filaments formed a loose network. Actin was found at the cell border and also in the form of stress fibres binding to the cell membrane, creating sites where the cell may attach to its extracellular environment via focal adhesion complexes (Vorselen et al. 2014).

As previously detected, a 30 min-exposure of cultured human chondrocytes to altered gravity conditions on the RPM was sufficient to change their morphology, to induce a rearrangement of the cytoskeletal proteins, or to affect gene expression (Aleshcheva et al. 2013; Wehland et al. 2015). Moreover, it was demonstrated, that a 30 min-exposure to the RPM induced a severe disorganization of the microfilaments and intermediate filaments, a loss of radial arrangement of microtubules, and a compromised shape of rat glial cells. In addition, altered chromatin condensation and DNA fragmentation in the nuclei indicated apoptotic processes (Uva et al. 2002).

Our study also showed larger meshes in the vimentin network and perinuclear accumulations of vimentin, after 2 h, but not after 30 min of clinorotation on FRC (Fig. 1). However, after a 24 h-incubation of human chondrocytes on the FRC these meshes disappeared but still persisted in corresponding RPM-samples (Aleshcheva et al. 2013). This indicates the capacity of chondrocytes to adapt to an altered mechanical environment as it has been previously detected in glial cells after a 32 h-incubation on the RPM (Uva et al. 2002). Our results also indicate that RPM and FRC due to their different physical principles might provide differences in the distribution of cytoskeletal components of chondrocytes. However, cultivation of chondrocytes in DMEM/F-12 medium on the RPM and the FRC resulted in comparable changes with respect to alteration of the cytoskeleton: formation of meshes in the vimentin network and of F-actin stress fibres (Figs. 2 and 3).

Microgravity can be regarded as a tool to induce 3D growth of human cells resulting in spheroids or even tissues by changing their morphology, phenotype and metabolic activity (Cerwinka et al. 2012). As investigated by Marlovits et al. (2003a), dedifferentiated chondrocyte cells exposed to RWV for 12 weeks, showed spontaneous aggregation and formation of solid tissue. The RWV proved to be a useful tool for providing an environment that enabled de-differentiated chondrocytes to redifferentiate and produce a cartilage-specific extracellular matrix (Marlovits et al. 2003a).

Tissue engineering of cartilage, i.e. the *in vitro* cultivation of cell-polymer constructs consisting of bovine articular chondrocytes on polyglycolic acid scaffolds, was studied first for three months on Earth and then for an additional four months on either MIR or Earth in the bioreactor yielding cartilaginous constructs, each weighing between 0.3-0.4 *g* and consisting of viable, differentiated cells that synthesized proteoglycan and type II collagen (Freed et al. 1997). Compared with control cells grown on Earth, constructs arising from experiments on board the MIR exhibited a more spherical shape and were smaller (Freed et al. 1997). Morphological changes of spheroids could be also detected during a flight on board the Shenzhou-8 spacecraft, where human follicular thyroid cancer cells (FTC-133) were exposed to real microgravity for 10 days and revealed scaffold-free formation of multicellular tumor cell spheroids (MCTS) (Pietsch et al. 2013; Ma et al. 2014). The spheroids obtained from the spaceflight experiment were larger, than those growing on an RPM on Earth. However, both types of spheroids shared a similar overall shape (Grimm et al. 2002, 2014, Pietsch et al. 2011a, Pietsch et al. 2013, Ma et al. 2014). Thus, we conclude that the development of 3D aggregates is triggered by microgravity as one of the most important factors. Moreover, 3D formation in microgravity is dependent on the confluence of the cells in space and on the RPM (Riwaldt et al. 2015). As the identification and characterization of molecules and signalling pathways involved in this process are still in their infancy, further experiments in both real and simulated microgravity are

necessary (Grimm et al. 2011; Pietsch et al. 2011b; Warnke et al. 2014; Wehland et al. 2015).

Our data show that some similar results could be achieved on ground-based facilities for microgravity simulation and under real microgravity conditions (Fig. 3, Table 1). However, as each cell or microorganism type reacts differently to such a challenge, comparative studies need to be undertaken for each investigated biological system (Herranz et al. 2013). To facilitate the interpretation of the resulting data and to eliminate possible confounding factors caused by too many variables, standardized hardware and experimental procedures should be used in space and ground-based studies.

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