

Original Paper

Simulated Microgravity Modulates Differentiation Processes of Embryonic Stem Cells

Vaibhav Shinde^a Sonja Brungs^b Margit Henry^a Lucia Wegener^b Harshal Nemade^a
Tamara Rotshteyn^a Aviseka Acharya^a Christa Baumstark-Khan^c
Christine E. Hellweg^c Jürgen Hescheler^a Ruth Hemmersbach^b Agapios Sachinidis^a

^aInstitute of Neurophysiology, Center for Molecular Medicine Cologne (CMMC), University of Cologne,

^bGerman Aerospace Center, Institute of Aerospace Medicine, Gravitational Biology and ^cGerman Aerospace Center, Institute of Aerospace Medicine, Radiation Biology, Linder Hoehe, Cologne, Germany

Key Words

Microgravity • Embryonic stem cells • Differentiation • Transcriptomics • Signal transduction pathways • Clinostat • Cardiomyogenesis

Abstract

Background/Aims: Embryonic developmental studies under microgravity conditions in space are very limited. To study the effects of altered gravity on the embryonic development processes we established an *in vitro* methodology allowing differentiation of mouse embryonic stem cells (mESCs) under simulated microgravity within a fast-rotating clinostat (clinorotation) and capture of microarray-based gene signatures. **Methods:** The differentiating mESCs were cultured in a 2D pipette clinostat. The microarray and bioinformatics tools were used to capture genes that are deregulated by simulated microgravity and their impact on developmental biological processes. **Results:** The data analysis demonstrated that differentiation of mESCs in pipettes for 3 days resulted to early germ layer differentiation and then to the different somatic cell types after further 7 days of differentiation in the Petri dishes. Clinorotation influences differentiation as well as non-differentiation related biological processes like cytoskeleton related 19 genes were modulated. Notably, simulated microgravity deregulated genes *Cyr61*, *Thbs1*, *Parva*, *Dhrs3*, *Jun*, *Tpm1*, *Fzd2* and *Dll1* are involved in heart morphogenesis as an acute response on day 3. If the stem cells were further cultivated under normal gravity conditions (1 g) after clinorotation, the expression of cardiomyocytes specific genes such as *Tnnt2*, *Rbp4*, *Tnni1*, *Csrp3*, *Nppb* and *Mybpc3* on day 10 was inhibited. This correlated well with a decreasing beating activity of the 10-days old embryoid bodies (EBs). Finally, we captured *Gadd45g*, *Jun*, *Thbs1*, *Cyr61* and *Dll1* genes whose expressions were modulated by simulated microgravity and by real microgravity in various reported studies. Simulated microgravity also deregulated genes belonging to the MAP kinase and focal

V. Shinde and S. Brungs contributed equally.

Agapios Sachinidis

Institute of Neurophysiology, Center for Molecular Medicine Cologne (CMMC),
University of Cologne, 50931 Cologne (Germany)
Tel. +49 (0) 221 478 7373, Fax +49 (0) 221 478 6965, E-Mail A.Sachinidis@uni-koeln.de

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adhesion signal transduction pathways. **Conclusion:** One of the most prominent biological processes affected by simulated microgravity was the process of cardiomyogenesis. The most significant simulated microgravity-affected genes, signal transduction pathways, and biological processes which are relevant for mESCs differentiation have been identified and discussed below.

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Introduction

Life on Earth has developed under the constant force of gravity. In a more general perspective, the future vision of space colonization and long-term space flights includes the embryonic development under space-conditions [1]. The embryonic development in space is profoundly hampered and this has been attributed to the effect of radiation and microgravity (compensation and reduction of the influence of gravity) [2]. The developmental studies, which have been conducted in space so far, showed that the different space conditions played a crucial role in developmental abnormalities in the various species.

Experiments on the amphibian *Pleurodeles waltl* (Urodele) showed neural tube defects in developing embryos [3] along with the hampered cortical cytoplasm movements, a decrease of cell adhesion and a loss of cells [4]. The space studies on rats at different stages of pregnancy also revealed developmental neuronal defects [5, 6], whereas fertilized mice embryos were not developed on a space shuttle flight (Columbia, STS-80) [2]. Horneck discussed that microgravity alone played a role in developmental problems in *Drosophila melanogaster* [7]. So far, studies with mammals under simulated microgravity conditions are rare and results demonstrated that space conditions have adverse effects on mammalian embryonic development.

However, embryonic developmental studies under space conditions are very limited due to extensive logistics and high costs as well as restricted spaceflight opportunities. Moreover, till now experiments have been performed with animal species which very often do not represent optimally the human situation. Therefore, various challenges to study the effects of microgravity on embryonic development forced the development of ground-based facilities and other *in vitro* cellular models which are more relevant for the in human situation.

Different types of ground-based facilities are in use such as a 2D clinostat, Random Positioning Machine (RPM) and Rotating Wall Vessel aiming to simulate microgravity [8]. These technologies allowed monitoring of developmental defects as well as effect on signal transduction in primary cells induced due to microgravity exposure [9-11]. We decided to use for our studies the principle of clinorotation for simulation of microgravity that means fast rotation of a sample around one axis perpendicular to the direction of the gravity vector resulting in neutralization of sedimentation. By using this method for cell culture similar results as in real microgravity in space have been obtained as shown for the oxidative burst reaction in macrophages [12, 13].

The randomly differentiated embryonic stem cells (ESCs) isolated from the inner cell mass (ICM) of a blastocyst resembles early and late *in vivo* embryonic development [14-16]. Applying extensive transcriptome studies we have recently demonstrated that day 3 and day 10 of differentiation is very critical for the differentiation of murine ESCs (mESCs) to three germ layers (ectoderm, endoderm, and mesoderm) and to various somatic cells, respectively. More recently, we have developed the so-called “pipette-based” methodology which is suitable for mESCs differentiation into embryoid bodies (EBs) within commercial plastic pipettes under a 2D clinorotation [17]. This study has been designed for the first time, to identify early and late biological processes as well as signal transduction pathways affected by simulated microgravity in differentiating mESCs. In this context, mESCs were differentiated by the pipette-method for 3 days under normal 1 *g* in comparison to the 2D

clinostat exposure. In another series of experiments, the 3-days old 1 *g* EBs and the 3-days old clinorotated EBs were further differentiated under normal 1 *g* conditions for 7 days. After isolation of total RNA, whole genome microarray analysis has been performed. In parallel cell cycle progression was studied with respect to the proliferative behaviour of the differentiating mESCs under the different gravity conditions. Here, we report that the simulated microgravity deregulated genes were belonging to the cytoskeleton and anatomical structure development process. The two signal transduction pathways (MAP kinase and focal adhesion) have been identified as prominently affected pathways by simulated microgravity.

Materials and Methods

Cell Culture

CGR8 (ECACC No.95011018), a mouse embryonic stem cell line was maintained on a gelatin (0.2%) coated flasks in a culture medium which contains Glasgow's minimum essential medium (Life Technologies, Darmstadt, Germany) with 2 mmol/L glutamine (Life Technologies, Darmstadt, Germany), 50 µmol/L β-mercaptoethanol (Life Technologies, Darmstadt, Germany), 1000 units/mL leukemia inhibitory factor (LIF) (Merck Chemicals, Darmstadt, Germany), penicillin (100 units/mL)/streptomycin (100 µg/mL) (Life Technologies, Darmstadt, Germany) and 10% fetal bovine serum (GIBCO, Life Technologies, Darmstadt, Germany). The differentiation of CGR8 cells in 1 mL pipettes to form embryoid bodies (EBs) was performed with the medium containing Iscoves Modified Dulbeccos Medium (Life Technologies, Darmstadt, Germany) with 2 mmol/L -glutamine (Life Technologies, Darmstadt, Germany), 100 µmol/L β-mercaptoethanol (Life Technologies, Darmstadt, Germany), 1% non-essential amino acids, penicillin (100 units/mL)/streptomycin (100 µg/mL) (Life Technologies, Darmstadt, Germany) and 20% fetal bovine serum (GIBCO).

Pipette clinostat

By continuously rotating 1 mL pipettes (diameter 3.5 mm) along their horizontal axis, the clinostat can mimic a microgravity environment: the influence of the gravity vector is randomized and sedimentation of the samples (cells) in liquid is neutralized [12]. The pipette clinostat is equipped with 10 in parallel rotating 1 mL pipettes. During a 60 rpm rotation a maximum residual centrifugal force of $7 \times 10^{-3} g$ is applied. We have recently established a method to adapt differentiation of mESCs in the 1 mL pipettes within the 2D clinostat [17]. Shortly, the details of the experiments are diagrammatically represented in Fig. 1A.

Embryoid bodies morphology and beating pattern analysis

After the 3 day exposure to clinorotation and continued cultivation at 1 *g* on a shaker until day 10, the EBs were photographed (stereomicroscope Nikon SZM 1500, Nikon Instruments Europe B.V., Germany; Zeiss Axiovert 10 Inverted Microscope, Carl Zeiss, Oberkochen, Germany). ImageJ (imagej.nih.gov/ij/) was used to determine the area, perimeter, diameter and beating of each EB. For morphological analysis area and perimeter of EBs were calculated. Circularity, an indicator for the roundness of EBs, has been calculated according to the formula:

$$\text{Circularity} = \frac{4 \pi \text{Area}}{\text{Perimeter}^2} \quad (\text{circularity factor of 1.0 means perfectly round shape}).$$

At day 10, videos of beating EBs were taken for at least one min with the stereomicroscope and TSview 6.2.3.3 TUCSEN Imaging Technology Co. software. The numbers of beating EBs were counted from a single video frame with the aid of multiple point detection tool provided in ImageJ.

Cell cycle analysis

Immediately after the clinostat experiment, the EBs were trypsinized twice with 0.05% Trypsin/EDTA (Sigma Aldrich, Munich, Germany) for 5 min at 37 °C and single cells were obtained by using 40 µm cell strainer (Greiner Bio-one, Solingen, Germany). The pellets were re-suspended in 1.5 mL PBS and fixed in 4.5 mL of ice cold 100% ethanol (Merck Chemicals, Darmstadt, Germany) to generate a final concentration of 70% ethanol. The cells were stored at -20°C for at least 24 h. Then the cells were diluted with PBS 1:1 and centrifuged at 500 *g* for 5 min at room temperature. The pellet was re-suspended in 1 mL staining

solution in PBS containing 50 µg/mL RNase (VWR Chemicals, Darmstadt, Germany), 0.1 % Triton X-100 (Sigma Aldrich, Munich, Germany) and 20 µg/mL propidium iodide (Calbiochem part of Merck, Darmstadt, Germany). Cells were incubated at 37 °C for 1 h. Flow cytometry was performed with a FACScan using the CellQuest software (BD Biosciences, Heidelberg, Germany). For cell doublet discrimination, width and area of the fluorescence signal in channel FL-2 (propidium iodide) were measured and displayed in a dot plot using Flowing Software 2.5.1 (Perttu Terho, Turku Centre for Biotechnology, Finland, www.flowingsoftware.com). The propidium iodide fluorescence histogram thereby contained only data from single cells. The percentage of cells in the different cell cycle phases (G1, S, G2), of cells with a sub-G1 DNA content (apoptotic cells) and of polyploid cells was determined by step-wise identification of the cell populations implying the PI histogram to represent the sum of the 5 respective Gaussian distributions for the subpopulations. This is achieved by using the generalized reduced gradient (GRG) non-linear algorithm with the Solver function of a Microsoft Excel Macro Sheet developed by Prof. Dr. Christa Baumstark-Khan (DLR, Cologne, Germany). Based on an user-defined estimated value for G1 peak position and peak maximum starting values for determining the positions and peak maxima of the other subpopulations are iteratively determined and the Gaussian distributions are calculated until the minimum for the least squares of the errors for the sum of the 5 distributions is found to be minimized.

Microarray experimental details

RNA isolation from the samples was performed as per the reported method. Briefly, total RNA was isolated using TRIzol and chloroform (Sigma, Steinheim, Germany) and purified with miRNeasy mini kit (Qiagen, Hilden, Germany). The quantification was done using NanoDrop (ND-1000, Thermo-Fisher, Langenselbold, Germany). For microarray labeling 100 ng total RNA was taken as a starting material and after amplification 12.5 µg amplified RNA was hybridized on Mouse Genome 430 version 2.0 arrays (Affymetrix, Santa Clara, CA, USA) for 16 h at 45°C. The arrays were washed and stained in Affymetrix Fluidics Station-450 according to the manufacturer's instructions. After staining arrays were scanned with Affymetrix Gene-Chip Scanner-3000-7G and Affymetrix GCOS software has been used for quality control analysis.

Quantitative real-time-polymerase chain reaction analysis

Validation of the Affymetrix data was performed by quantitative real-time-polymerase chain reaction (qRT-PCR) analysis with ABI 7500 FAST Detection System (Applied Biosystems). One microgram of RNA from each sample was reverse transcribed using SuperScript VILO cDNA synthesis kit (Invitrogen GmbH). qRT-PCR was performed for minimum of three biological replicates using SYBR-Green PCR Master Mix (QuantiFast qPCR Master Mix; Qiagen). The gene expression of target genes was normalized to reference gene GAPDH. The Fold-expression changes were calculated by rising to the power of the negative value of delta-delta Ct value ($2^{-\Delta\Delta Ct}$) at base 2. The resulting mRNA expression values were plotted as a fold change relative to respective control. The nucleotide sequences of the primers used are provided in Table 1.

Statistical Analysis

Cell cycle data were analyzed using Graph Pad Prism 5 (GraphPad Software, Inc, USA): Kolmogorov Smirnov test followed by a one-way ANOVA and Bonferroni post hoc test were used to determine the level of statistical significance. A paired t-test was used to analyze the statistical significance for differences in morphological parameters and in the number of beating EBs between 1 g and simulated microgravity

Table 1. Nucleotide sequences of the primers used for qRT-PCR

Gene Symbol	Forward	Reverse
<i>Gapdh</i>	GAGGGATGCTGCCCTTACC	TGATGGCATGGACTGTGGTC
<i>Cyr61</i>	GGA TGA ATG GTG CCT TGC	GTC CAC ATC AGC CCC TTG
<i>Gadd45g</i>	CTGCTGTGAGAACGACATTGA	CTCTCCTCGCAGAACAACTG
<i>Dll1</i>	GCAGGACCTTCTTTTCGCGTAT	AAGGGGAATCGGATGGGGTT
<i>Flnb</i>	GCTCATCTTGGGCTGGTATG	CTGAATCCATCCCAGTAGCCT
<i>Ltb</i>	TGGCAGGAGCTACTTCCCT	TCCAGTCTTTTCTGAGCCTGT
<i>β-Actin</i>	AGGGAAATCGTGCGTGACAT	CGCAGCTCAGTAACAGTCCG
<i>Afp</i>	AGCTTCCACGTTAGATTCTCC	ACAAACTGGGTAAAGGTGATGG
<i>Thbs1</i>	CTCAGGAACAAAGGCTGCTC	CAGGAGAGGCCACAGATAGC
<i>Fzd2</i>	ATTAGTGACATGCAGCGATTTC	AGCAGGAAGGATGTACCGATGAA
<i>Tpm1</i>	TGGAAGATGAGCTGGTGTAC	TCAATGACTTCATGCCTCT
<i>Hoxa9</i>	GGCAACTACTACGTGGACTCG	CCAGTTGGCTGCTGGGTTAT
<i>Tnnt2</i>	GCTACAGACTCTGATCGAGGCT	GCTCATTGCGAATACGCTGCTG
<i>Tbx4</i>	CCAAGGCTGCACATCGTTAAG	TGGCAAAAGGGTGTGTCTCAAT
<i>Tcf21</i>	CCCCTAAGAAAAGCCCGCTC	CCGTTCTCGTACTTGTGCTTG
<i>Frzb</i>	CACAGCACCCAGGCTAACG	TGCGTACATTGCACAGAGGAA

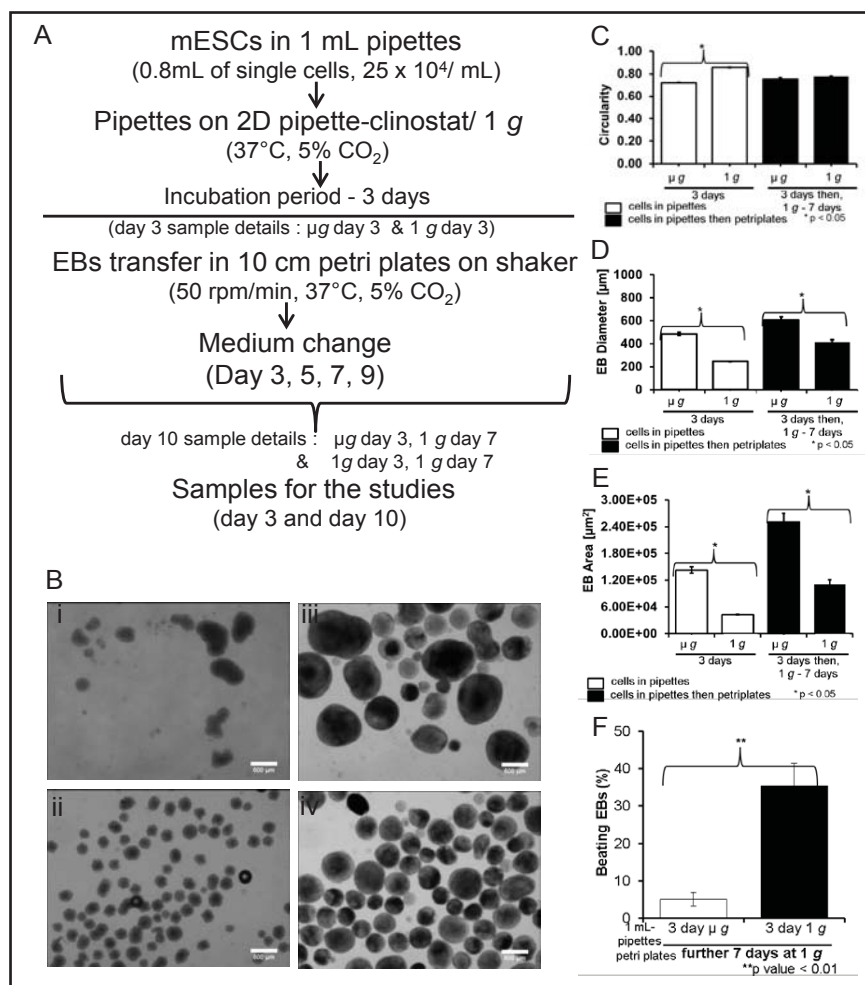
samples. For microarrays, the statistical data analysis and visualization have been performed by uploading CEL files in Partek Genomics suite (PGS) version 6.6 (Partek, St Louis, MO, USA). The PS intensity values were generated by RMA background correction, quantile normalization, log2 transformation and median polished probe sets (PS) summarization. The normalized PS were used for the generation of principal component analysis (PCA) and one-way ANOVA model has been used to generate the differentially regulated transcripts with at least a 2 fold change ($p \leq 0.05$). The signals of differentially regulated transcripts were normalized by Z score and clustered using hierarchical cluster analysis (unsupervised, PGS). The online free software Database for Annotation, Visualization and Integrated Discovery (DAVID) was used for functional annotation and gene ontology categories (GOs) of differentially expressed transcripts.

Results

Microscopic observations and morphological data analysis

The microscopic observations revealed that the morphology of the EBs formed under clinorotation on day 3 was less round and bigger in size (Fig. 1Bi) as compared to the regular round morphology of control EBs formed at static 1 g conditions (Fig. 1Bii). During the continued cultivation at 1 g for 7 additional days, clinorotated EBs readapted to 1 g conditions

Fig. 1. Experimental setup and morphology of embryoid bodies (EBs). (A) Experimental setup representing that the mESCs were differentiated in 1 mL pipettes at 1 g (1 g day 3, group 1) or clinorotation (μ g day 3, group 2) for 3 days and then transferred on Petri dishes at 1 g for further 7 days and designated names 1 g day3, 1 g day7 (group 3) and μ g day 3, 1 g day7 (group 4) respectively. Samples were collected for microarray analysis on day 3 and at day 10. (B) EBs morphology generated from mESCs after 3 days and 10 days (i) EBs after a 3 days exposure to simulated microgravity, (ii) EBs after 3 days at 1 g, (iii) EBs after a 3 days exposure to simulated microgravity followed by 7 days at 1 g, (iv) EBs after 10 days at 1 g (the scale bars in B represent 500 μ m). The data represent mean \pm SEM of four independent experiments. For each group of 42 EBs were considered for statistical analyses of (C) Circularity, (D) Diameter, (E) Area, and (F) Number of beating EBs.



and developed a normal round morphology. However, as shown in Fig. 1Biii a fraction of EBs had a bigger size than the other fraction in the same group which had a similar and homologue size as the corresponding control EBs (Fig. 1Biv).

Circularity represents the overall shape (roundness) of the EBs. The analysis revealed that there is a significant difference between the shapes of EBs cultivated under simulated microgravity for 3 days which shows less circularity as compared to the EBs cultivated at 1 *g* (Fig. 1C). A significant difference has been observed between the diameters as well as area of the EBs cultivated under simulated microgravity for 3 days as compared to the EBs cultivated at 1 *g* (Figure 1D and 1E). The number of beating EBs at day 10 was significantly decreased in the clinorotated ESCs as compared to the corresponding control samples (Fig. 1F). Furthermore, the total number of the cells was decreased after clinorotation, as shown in the number of the cells at day 3 which has been determined by flow cytometry (Fig. 2).

Cell cycle analysis

To determine whether simulated microgravity (μg) influenced the cell cycle of the proliferating cells, cell cycle profile was obtained for the EBs generated under clinorotation compared to 1 *g* on day 3. The cell cycle analysis indicated that there was no significant difference in the distribution of cells in different phases of cell cycle between the 1 *g* and simulated microgravity groups (Fig. 2A, 2B). The cell cycle pattern obtained for ESCs under clinorotation on day 3 captures 9.0 ± 1.0 % of cells in subG1, 30.8 ± 2.1 % in G1, 33.8 ± 0.6 % cell in G2 phase and 24.4 ± 1.2 % cells in S phase (Fig. 2B) whereas the differentiated cells at 1 *g* for 3 days showed 8.8 ± 0.6 % cells in subG1, 35 ± 3.0 % in G1, 32.3 ± 2.2 % cell in G2 phase and 23.0 ± 2.4 % cells in S phase (Fig. 2A). The undifferentiated cells at day 0 captured more than 70% cells in the S (30.7 ± 0.9) and G2 (41.8 ± 1.5) phase together (Fig. 2C), whereas less than 60% cells were present in these phases of differentiated cells on day 3.

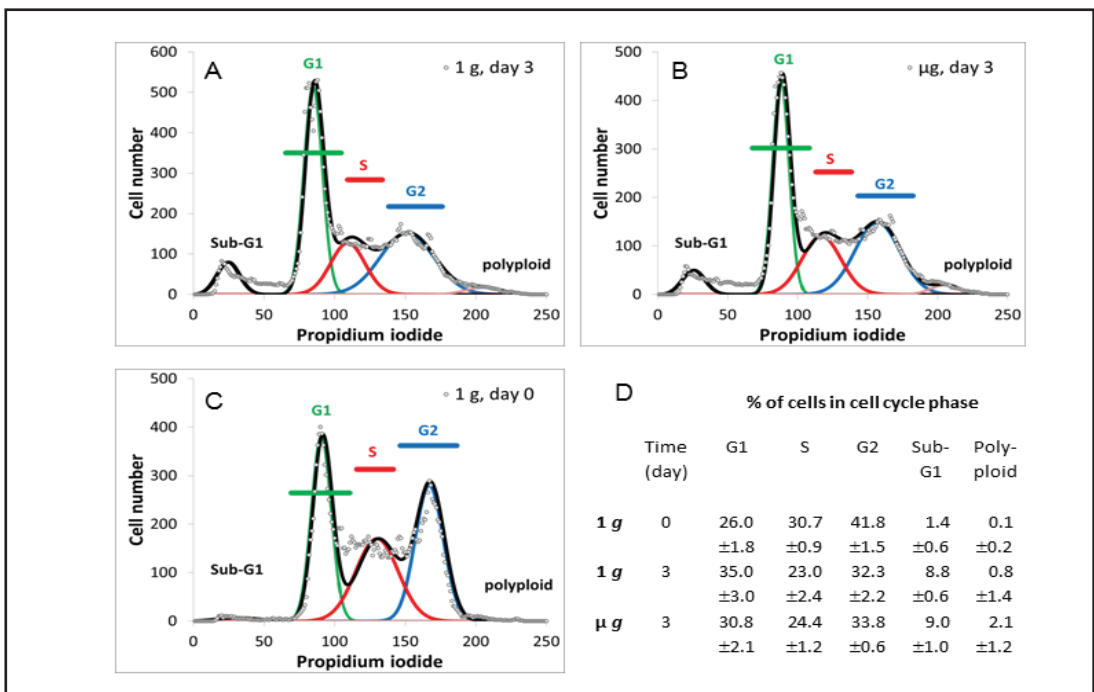


Fig. 2. Cell cycle distribution of differentiated mESCs. Flow cytometry analysis of cells obtained from trypsinized EBs or mESCs and stained with PI. The data is a representative of each group from single experiment. (A) 1 *g* group on day 3, (B) Simulated microgravity exposed EBs on day 3, (C) mESCs at day 0. (D) The distribution of cells in various cell cycle phases represented as percentages. Data are means \pm SEM from three independent experiments.

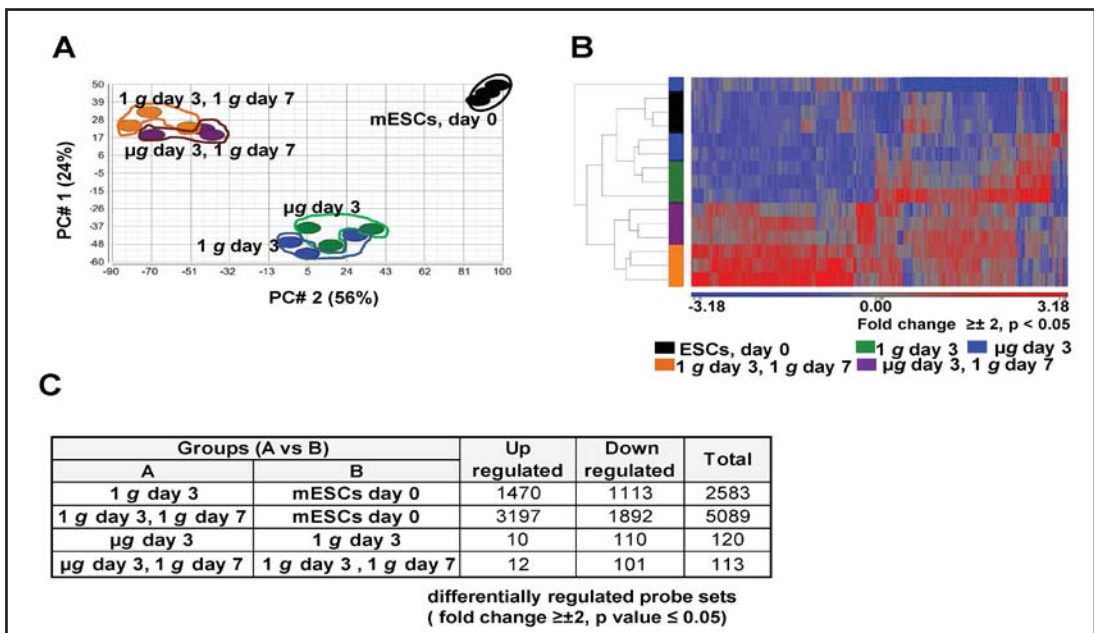


Fig. 3. Data structure of gene expression profile influenced by simulated microgravity during differentiation of mouse embryonic stem cells (mESCs). The data represents three independent experiments. (A) The data structure of transcriptome data sets (deregulated during differentiation and by μg) was dimensionality-reduced and presented in the form of a 2D-Principle Component Analysis (PCA) diagram. PCA illustrates a relatively large distance between mESCs and the cells after 3 days and 10 days of differentiation, small distance between the microgravity and the 1 g group indicates relatively less number of transcripts was influenced by μg , (B) The signals of first 40 most deregulated transcripts during differentiation and microgravity were normalized by Z score and clustered using hierarchical cluster analysis (unsupervised, PGS). The results are represented as heatmap with each column representing one transcript, and the color of each square indicating the absolute gene expression level (blue low; red: high), (C) The number of up-regulated and down-regulated probe sets were represented with various comparisons.

Visualization of the differentiation and microgravity induced influence on transcriptome

The principal component analysis (PCA) was performed for visualization of the differences between the transcriptomes of the different cell populations by analyzing the gene expression variability levels and presented as Figure 3A. PCA mapping of principal component (PC) 2, independently of the 3-days different gravity conditions, shows a marked gene expression variance of 56%, demonstrating significant deregulation between the transcriptomes of the undifferentiated mESCs (ESC, day 0) and in total the 10-days differentiated mESCs. Significant deregulation of transcriptomes between undifferentiated and 3 or 10 days of differentiated mESCs was expected. This reflects the different somatic cell populations derived from undifferentiated mESCs at day 3 or day 10 of differentiation. The differences between the transcriptomes of the 3-days differentiated ESCs and the other cell populations were captured by PC1 and PC2. It reflects difference between early differentiated cells, undifferentiated ESCs and 10-days late differentiation to distinct somatic cell types. In general the transcriptomes among the different cell populations were similar, independently on the gravity conditions (Fig. 3A). The transcriptomes of the differentiated microgravity exposed ESCs are closer to the 1 g control groups and clearly separated from the undifferentiated ESCs. These findings demonstrate that only a relative small number of genes were affected by exposure to simulated microgravity. For further visualization of the gravity affected genes, a heatmap was prepared with the differentially regulated PS showing at least 2-fold up or down-regulation (p value ≤ 0.05 , fold change $\geq \pm 2$) (Fig. 3B). The color change from red (high expression level) to blue (low expression level) designates the

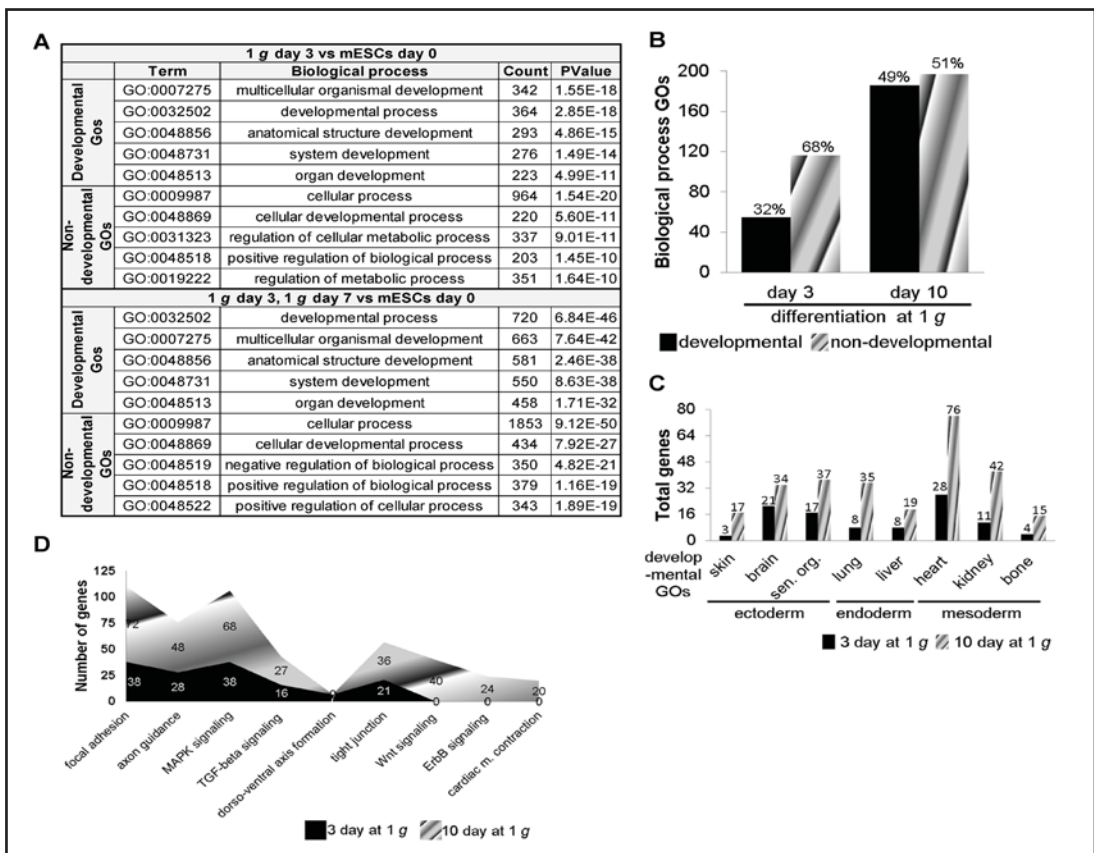


Fig. 4. Characterization of mESCs differentiation on day 3 and day 10 at 1 *g*. The transcriptome profiles were obtained from mESCs differentiated in 1 mL pipette at 1 *g* for 3 days and then transferred in the Petri dishes for subsequent 7 days of differentiation at 1 *g* (day 10). The differentially regulated PS on day of differentiation with respect to mESCs were uploaded on online tool DAVID for further characterization of the differentiation. (A) The gene ontology categories (GOs) “biological processes” were further divided into the developmental and non-developmental GOs and most significant first five were represented in the figure. (B) The developmental GOs were captured on day 3 of differentiation in the 1 mL pipettes at 1 *g* and the percentage of them were increased with further differentiation for 7 days at 1 *g* (total number of GOs are indicated on y-axis). (C) Few genes belonging to the specific differentiation were captured on day 3 and the number increased with further differentiation for 7 days. (D) The differentiation relevant significant KEGG pathways were captured and represented in the figure (the number on top represents total genes involved). The data represent three independent experiments.

magnitude of expression levels. Depending on expression pattern, the simulated microgravity treatment for 3 days was seen to be clustered with mESCs whereas microgravity exposed cells differentiated for further 7 days clustered with 1 *g* group.

Differentially regulated probe sets (PS)

The list of differentially regulated significant PS (p value ≤ 0.05 , fold change $\geq \pm 2$) was obtained and the numbers of up-regulated and down-regulated PS are presented in Figure 3C. The random differentiation of mESCs in 1 mL pipette (absence of LIF) resulted in total 2583 differentially regulated PS on day 3 (1470 up, 1113 down) and 5089 PS on day 10 (3197 up, 1892 down). Three days simulated microgravity exposure resulted in deregulation of total 120 PS on day 3 (10 up, 110 down) and 133 PS on day 10 (12 up, 101 down) as compared to respective controls on day 3 and day 10. The probe sets from the most crucial comparisons were considered for the further analysis.

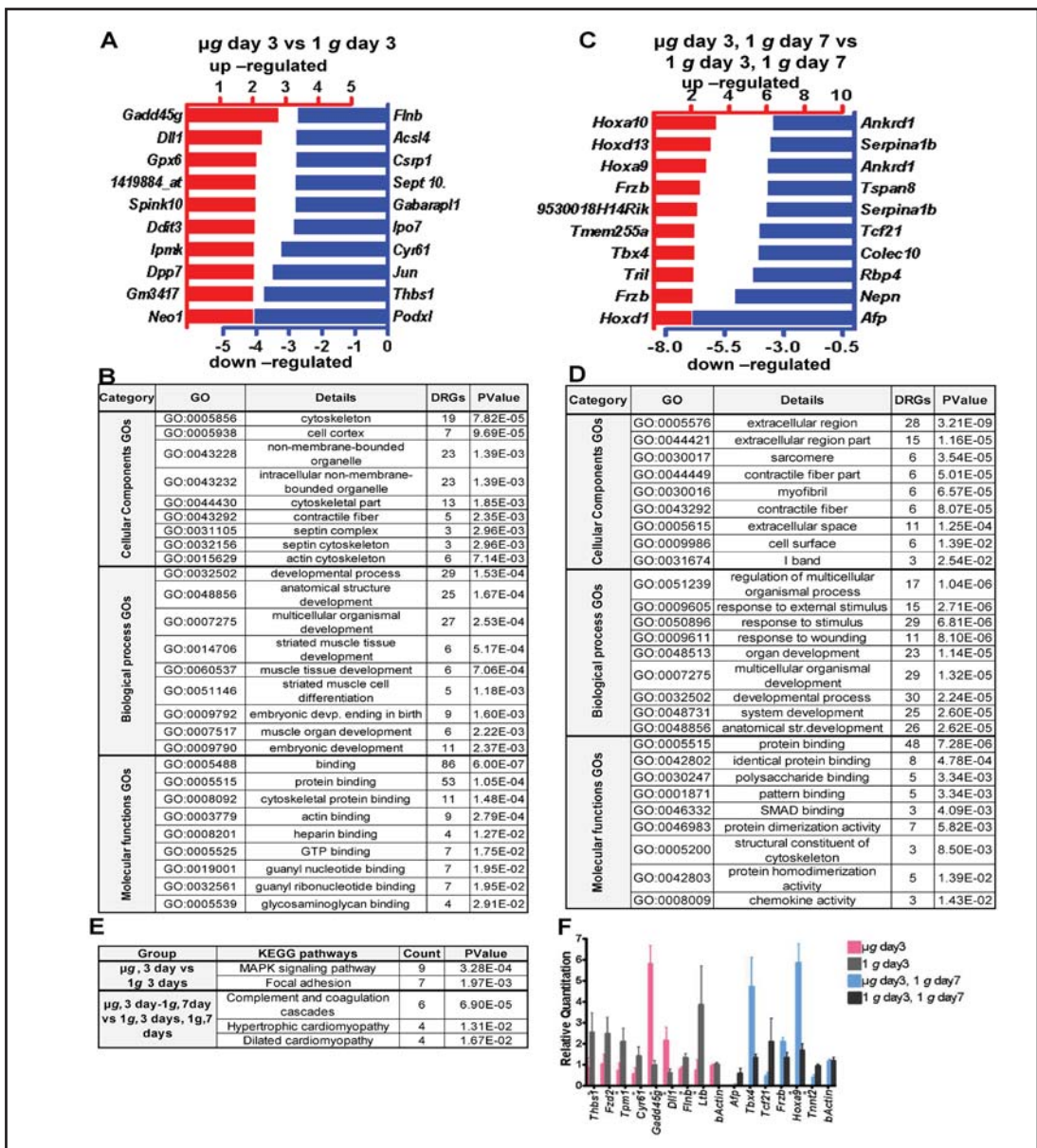


Fig. 5. Influence of simulated microgravity on mESCs differentiation on day 3 and day 10. The direct comparison of transcriptome profiles were performed from the samples obtained from the pipettes kept on a 2D clinostat with the samples from the pipettes kept at 1 g for 3 days as well as the samples obtained from further 7 days of differentiation at 1 g in the Petri dishes. (A) First 10 most differentially up-regulated and down-regulated genes by simulated microgravity on day 3, (B) The most significant GO categories captured by simulated microgravity deregulated genes on day 3, (C) First 10 most differentially up-regulated and down-regulated genes by 3 days simulated microgravity on day 10, (D) The most significant GO categories captured by microgravity deregulated genes on day 10 as withdrawal effect, (E) The most significant KEGG pathways influenced by simulated microgravity deregulated genes on day 3 and day 10 of differentiation, (F) The validation of microarray results was performed with qRT-PCR for 14 genes significantly deregulated by simulated microgravity and is represented by the relative expression. Data are means \pm SEM from minimum of three independent experiments.

Biological processes and signal transduction pathways enriched at day 3 and day 10 after differentiation of mESCs at normal 1 g gravity conditions

The significant gene ontology categories (GOs) and KEGG pathways were identified by the DAVID bioinformatics tool. The GOs belonging to the biological processes were further

Table 2. Significantly deregulated specific genes by simulated microgravity on day 3 and day 10

μg day 3 vs 1 g day 3						μg day 3, 1 g day 7 vs 1 g day 3, 1 g day 7					
anatomical structure development			cytoskeleton			anatomical structure development			extracellular regions		
symbol	fold	p-value	symbol	fold	p-value	symbol	fold	p-value	symbol	fold	p-value
<i>Jun</i>	-3.5	2.39E-03	<i>Flnb</i>	-2.7	3.44E-03	<i>Tgfb3</i>	-2.0	2.99E-03	<i>Ghr</i>	-2.4	3.70E-05
<i>Gadd45g</i>	2.8	3.10E-03	<i>Pja2</i>	-2.6	9.97E-03	<i>Tcf21</i>	-4.0	1.97E-05	<i>Ambp</i>	-3.1	3.18E-08
<i>Flnb</i>	-2.7	3.44E-03	<i>Parva</i>	-2.3	1.04E-02	<i>Nfib</i>	-2.0	9.77E-04	<i>Frzb</i>	2.1	2.80E-04
<i>Cyr61</i>	-3.2	4.59E-03	<i>Plekhh1</i>	-2.2	1.06E-02	<i>Nppb</i>	-2.2	1.06E-03	<i>Nppb</i>	-2.2	1.06E-03
<i>Ltb</i>	-2.5	4.70E-03	<i>Cotl1</i>	-2.2	1.07E-02	<i>Ednrb</i>	-2.4	1.91E-03	<i>Gc</i>	-2.1	1.26E-03
<i>Dll1</i>	2.3	5.58E-03	<i>Parp10</i>	-2.4	1.14E-02	<i>Rbp4</i>	-4.3	2.88E-03	<i>Tff3</i>	-3.2	1.52E-03
<i>Ddit3</i>	2.1	7.28E-03	<i>Utrn</i>	-2.6	1.85E-02	<i>Hoxd13</i>	3.0	3.79E-03	<i>Epha3</i>	-2.8	2.04E-03
<i>Ipmk</i>	2.1	1.00E-02	<i>Sept10</i>	-2.8	2.38E-02	<i>Tnni1</i>	-2.6	3.82E-03	<i>Fgb</i>	-2.1	2.35E-03
<i>Utrn</i>	-2.6	1.85E-02	<i>Csrp1</i>	-2.1	2.46E-02	<i>Srpk3</i>	-2.1	4.76E-03	<i>Rbp4</i>	-4.3	2.88E-03
<i>Top2b</i>	-2.1	2.07E-02	<i>Myl9</i>	-2.3	2.47E-02	<i>Hoxd10</i>	2.1	4.92E-03	<i>Tgfb3</i>	-2.0	2.99E-03
<i>Neo1</i>	2.0	2.11E-02	<i>Sept8</i>	-2.4	2.49E-02	<i>Afp</i>	-5.2	9.39E-03	<i>Pnoc</i>	-2.3	3.02E-03
<i>Syne2</i>	-2.4	2.49E-02	<i>Tpm1</i>	-2.2	2.54E-02	<i>Hoxa10</i>	3.3	9.72E-03	<i>Hpx</i>	-2.7	4.44E-03
<i>Nfatc4</i>	-2.2	2.51E-02	<i>App</i>	-2.1	2.78E-02	<i>Col5a2</i>	-2.3	1.02E-02	<i>Ccl9</i>	-2.9	5.99E-03
<i>Tpm1</i>	-2.2	2.54E-02	<i>Tcte3</i>	2.0	3.00E-02	<i>Enpp1</i>	-2.3	1.05E-02	<i>Serpina1b</i>	-3.5	7.48E-03
<i>Cdh1</i>	-3.5	2.64E-02	<i>Flna</i>	-2.1	3.32E-02	<i>Hnf4a</i>	-2.0	1.19E-02	<i>Colec10</i>	-4.0	8.96E-03
<i>Rhoc</i>	-2.5	2.77E-02	<i>Acta1</i>	-2.3	4.52E-02	<i>Hoxd1</i>	2.0	1.78E-02	<i>Afp</i>	-5.2	9.39E-03
<i>App</i>	-2.1	2.78E-02	<i>Gabarapl1</i>	-2.8	4.77E-02	<i>Ptpcr</i>	-2.1	1.93E-02	<i>C1qb</i>	-2.4	1.01E-02
<i>Etl4</i>	-2.1	3.31E-02	<i>Sept11</i>	-2.3	4.80E-02	<i>Clu</i>	-2.8	2.03E-02	<i>Col5a2</i>	-2.3	1.02E-02
<i>Pbx2</i>	-2.2	3.55E-02	<i>Jakmip1</i>	-2.2	4.86E-02	<i>Tbx4</i>	2.2	2.06E-02	<i>Enpp1</i>	-2.3	1.05E-02
<i>Fzd2</i>	-2.3	3.64E-02			<i>Apoa1</i>	-2.7	2.38E-02	<i>Nepn</i>	-5.0	1.19E-02	
<i>Itgb1</i>	-2.4	3.76E-02			<i>Cryab</i>	-2.1	2.41E-02	<i>Serpina1b</i>	-3.7	1.55E-02	
<i>Tug1</i>	-2.6	4.05E-02			<i>Mybpc3</i>	-2.5	2.53E-02	<i>Ccl3</i>	-2.3	1.56E-02	
<i>Gna12</i>	-2.1	4.10E-02			<i>Csrp3</i>	-2.6	2.92E-02	<i>Pf4</i>	-2.3	2.02E-02	
<i>Rpl22</i>	-2.2	4.13E-02			<i>Hoxa9</i>	2.8	3.01E-02	<i>Clu</i>	-2.8	2.03E-02	
<i>Acta1</i>	-2.3	4.52E-02			<i>Robo2</i>	-2.0	3.11E-02	<i>Apoa1</i>	-2.7	2.38E-02	
					<i>Tnnt2</i>	-2.7	4.17E-02	<i>Col1a2</i>	-2.4	2.48E-02	
								<i>C1qc</i>	-2.0	2.54E-02	
								<i>Vnn1</i>	-3.2	2.86E-02	

subcategorized into developmental GOs and non-developmental GOs to segregate out the genes involved in embryonic development process (Fig. 4A). It has been observed that during differentiation of mESCs in 1 mL pipettes, 32% of biological processes were related to the development at 3 days and at day 10 of differentiation these processes increased to 49% (Fig. 4B). The developmental GOs were further subcategorized into skin, brain, sensory organ, lung, liver, heart development. As shown in Figure 4C, development of germ layer cells and their somatic cell derivatives captured at day 3 of differentiation and the number of genes in these developments was increased at day 10 of differentiation (Fig. 4C). The significant KEGG pathways captured on day 3 were focal adhesion, axon guidance, MAPK signaling, TGF- β signaling, dorso-ventral axis formation and tight junction. On day 10 of differentiation, additionally, the Wnt, ErbB signaling and the cardiac muscle contraction pathways were captured (Fig. 4 D).

Biological processes and signal transduction pathways affected by simulated microgravity at day 3 of differentiation and after further 7 days differentiation of mESCs at normal 1 g

Three days exposure of the mESCs to the simulated microgravity resulted in dysregulation of 120 PS, and 113 PS found dysregulated at day 10 of differentiation as compared to their respective controls. The first 10 mostly up- and down-regulated genes on day 3 affected by simulated microgravity are presented in Figure 5A. The most significant GOs belonging to biological processes, cellular components and molecular functions are presented in Figure 5B. Simulated microgravity influenced the expression of 19 genes belonging to the cytoskeleton, 29 genes belonging to the developmental process and 86 genes having molecular function as a protein binding on day 3 as compared to 1 g. The deregulated genes belonging to the anatomical structure development and cytoskeleton GO categories are enlisted in Table 2. The first 10 most significant simulated microgravity-induced up- and down-regulated genes on day 10 are presented in the Fig. 5C. The most significant GOs belonging to biological process, cellular components and molecular functions are presented in Fig. 5D. Simulated microgravity had an impact on 28 genes belonging to the extracellular region, 26 genes belonging to the anatomical structure development process and 48 genes having molecular function as a “binding” related to protein on day 10 as compared to 1 g control conditions. The

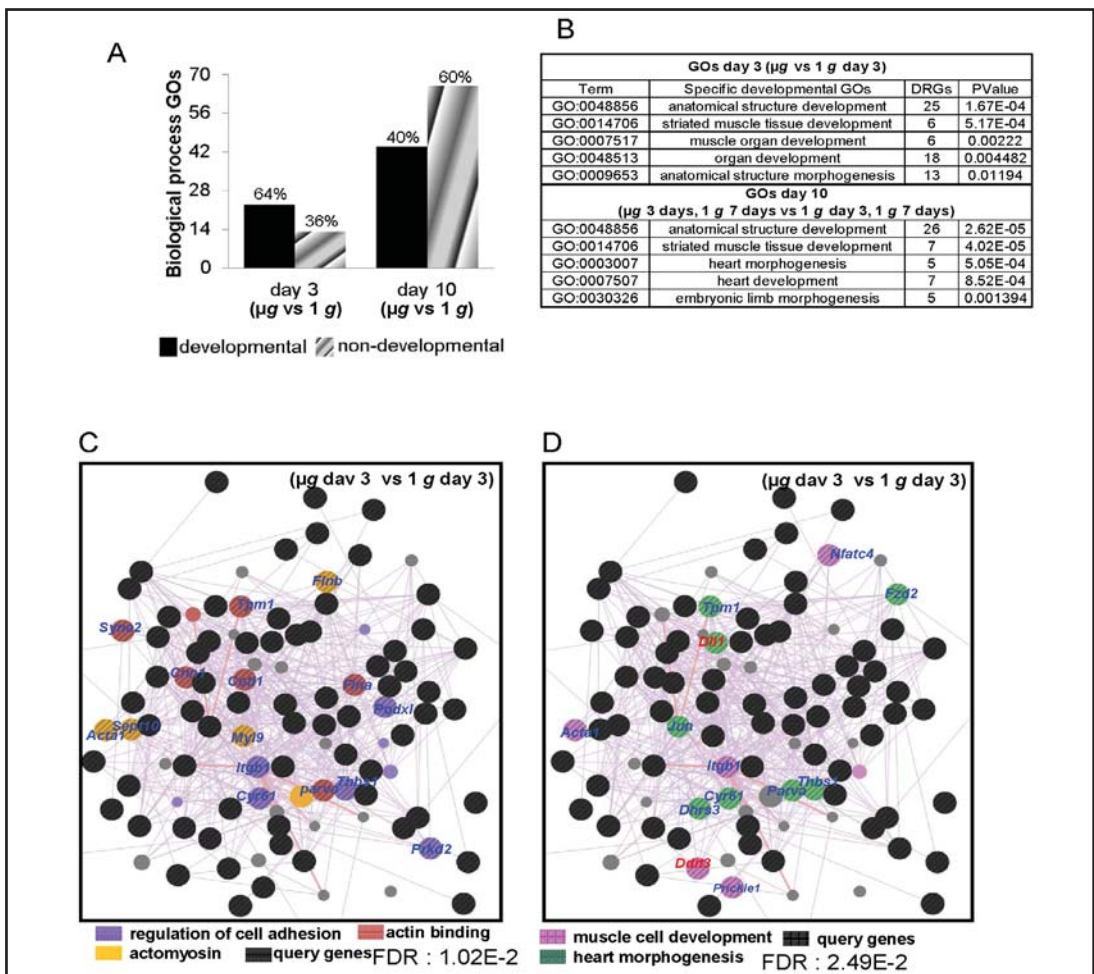


Fig. 6. Characterization of the influence of simulated microgravity on developmental biological processes. The gene ontologies (GOs) of biological processes influenced by simulated microgravity de-regulated genes on day 3 (μg day 3 vs 1 g day 3) and 10 (μg day 3, 1 g day 7 vs 1 g day 3, 1 g day 7) were further subdivided as developmental and non-developmental. (A) The percentages of developmental GOs influenced by simulated microgravity were higher on day 3 than day 10 (Total number of GOs are indicated on y-axis). (B) The first five most significantly captured developmental GOs on day 3 and days 10 are represented. (C, D) The simulated microgravity deregulated genes on day 3 were up-loaded on online tool GeneMANIA and gene regulatory network were linked with biological functions like regulation of cell adhesion, actin binding, actomyosin, muscle cell development and heart morphogenesis.

deregulated genes belonging to extracellular region and anatomical structure development GO categories are enlisted in the Table 2. The most significant KEGG pathways influenced by simulated microgravity on day 3 of differentiation were focal adhesion and MAP Kinase signaling (Fig. 5E). All genes belonging to the focal adhesion pathway like, *Itgb1*, *Myl9*, *Parva* and *Thbs1* are found to be down-regulated. In MAPK signaling pathway only *Gadd45g* and *Ddit3* are up-regulated while *Arrb1*, *Gna12*, *Map3k4* and *Nfatc4* were down-regulated. The genes involved in both pathways namely *Flna*, *Flnb* and *Jun* are found to be down-regulated.

To validate the microarray data, a few genes have been chosen and their gene expression has been validated by qRT-PCR. As indicated in Figure 5F, the gene expressions of the 14 genes reflect also the gene expression levels as observed by the microarray analysis. Another gene β -Actin was also tested as internal control and not shown significant difference between microgravity and control group on day 3 and day 10 of differentiation.

Subcategorization of simulated microgravity influenced biological process GOs

The total biological process related GOs were further divided as developmental and non-developmental GOs (Fig. 6A). These results showed that although very few GOs have been influenced by simulated microgravity on day 3, the deregulated genes belongs up to 64% of developmental GOs and 36% of other biological process GOs. Although less number of genes was influenced on day 10, the deregulated genes belong to only 40% of the developmental GOs. The developmental GOs were further evaluated to obtain deregulated genes belonging to specific developmental process. The five specific developmental processes influenced by simulated microgravity on day 3 and day 10 (3 days simulated microgravity, 7 days at 1 g) of differentiation have been presented in the Figure 6B. On day 3, genes mostly belong to the anatomical structure development (ASD), however different genes belonging to ASD were found to be deregulated on day 10. Interestingly, GO "heart development" was captured by significantly down-regulated genes (*Tnnt2*, *Rbp4*, *Tnni1*, *Csrp3*, *Nppb* and *Mybpc3*) on day 10. These results suggest that cell fate commitment to the cardiac lineage has been hampered by 3 day exposure to the simulated microgravity and need to be further explored with different approach.

Gene regulatory network based on biological functions influenced by simulated microgravity on day 3 of mESCs differentiation

To explore whether 3 days of simulated microgravity exposure during differentiation altered the cardiac cell fate commitment, another bioinformatics tool approach was followed. The list of simulated microgravity de-regulated genes on day 3 was uploaded on online available tool GeneMANIA (<http://www.genemania.org>) [18] and gene regulatory network was obtained based on genes co-expression related with the biological functions (Fig. 6C and 6D). As expected the significant number of genes was belonging to the biological processes "regulation of cell adhesion", "actin binding" and "actomyosin". The genes involved in these processes, *Cyr61*, *Itgb1*, *Thbs1*, *Prkd2*, *Flna*, *Flnb*, *Myl9*, *Tpm1*, *Cnn1*, *Acta1*, *Parva* are found to be significantly down-regulated. Furthermore, 8 genes belonging to the biological process heart morphogenesis were also captured. Amongst the genes the only up-regulated gene was *Dll1* whereas *Thbs1*, *Parva*, *Jun*, *Cyr61*, *Dhrs3*, *Tpm1* and *Fzd2* were down-regulated. Interestingly, few genes belonging to "cell adhesion", "actin binding" and "actomyosin" categories are also involved in the process of heart morphogenesis.

Discussion

The development of life beyond the planet was always the focus of space biologists and so far a few *in vivo* and *in vitro* studies have been conducted in space to evaluate the reproduction potentials. It has been reported that the embryonic development of mice in space was not successful to date. In this context, no two cell embryos development has been observed in the space shuttle Columbia (STS-80) [2]. *In vitro* fertilization studies conducted under simulated microgravity (horizontal clinostat/ three dimensional clinostat) conditions ruled out the adverse effect on fertilization. Authors concluded that simulated microgravity induced an increase in frequency of early embryonic lethality in the mouse [9, 11]. The early embryonic lethality may be attributed to the different factors such as disturbance of cell polarity and spatiotemporal regulation of gene expression involved in cell fate determination during pre and post implantation embryonic development. The disturbed gene expression pattern of genes such as *Pou5f1* may result in failure of ICM development whereas it is expected that the absence of *Taube nuss* (*Tbn*) will result in hampered blastocyst development. An absence of *Taf10* will result in normal blastocyst development but prenatally lethal [19-21].

The transcriptome data analysis revealed that differentiation of mESCs in the pipettes for 3 days and 10 days at 1 g monitor various early differentiation processes typically for all three germ layer lineages and their cell derivatives like skin, brain, sensory organ, lung, liver heart, kidney and bone respectively. The significant KEGG pathways known to be involved

in early embryonic development like TGF- β signaling, dorso-ventral axis formation, Wnt signaling were also captured [22, 23]. In correlation with the increased beating activity of the 10-days old EBs, characteristic genes involved in cardiac muscle contraction were maximally expressed on day 10. The differentiation of mESCs into beating cardiomyocytes has been validated as a parameter in *in vitro* embryo-toxicity assay to predict embryo-toxic potential of the chemicals [24], it was also reported that the cardiomyogenesis of the EBs obtained from mESCs can also be enhanced with the use of cytosolic Ca^{2+} modulating agents [25]. The electrophysiological parameters of various mESCs derived cardiomyocytes were also compared to investigate cardiac differentiation potential of mESCs [26]. These findings clearly proved the applicability of pipette methodology to capture early and late differentiation processes of mESCs and to detect sensitive dysregulation in differentiating mESCs under altered gravity conditions.

The deregulated genes between simulated microgravity exposed and 1 *g* control at 3 day of differentiation were captured. The most significant 19 genes deregulated by simulated microgravity were belonging to the cellular component GO 'cytoskeleton', 29 genes belonging to the GO 'developmental process' and 86 genes belonging to the GO 'protein binding' as a molecular function. The most of the *in vitro* studies on cells indicated that the simulated microgravity influenced the cell shape, cytoskeleton, cell migration and cell growth [27-30]. In this context, it has been reported that microgravity influences the cell cytoskeleton organization in osteoblasts (space shuttle, STS-56) [31], human mesenchymal stem cells (Random Positioning Machine) [32] and MCF7 (Foton-12 satellite) [33]. Interestingly, one of the prominent GOs in our study influenced by simulated microgravity at day 3 of differentiation was the GO 'Cytoskeleton'. From these results we may conclude that cytoskeletal genes predominantly are targeted by altered gravity. However, the role of the cytoskeletal genes affected by gravity for the differentiation processes of mESCs remains to be elucidated.

Further gene signaling network analysis based on biological functions predicted 8 genes involved in the heart morphogenesis process. Simulated microgravity inhibited the expression of 7 genes and up-regulated 1 gene suggesting disturbance of cell fate commitment towards cardiac lineage at early differentiation processes occurring at day 3 of differentiation. Disturbances in the differentiation fate were still observed on day 10, when the 3 days old EBs had been exposed to clinorotation followed by a further differentiation at 1 *g* for 7 days. In addition, the number of beating EBs at 10 days of differentiation was significantly reduced as compared to 10 days old EBs generated under 1 *g* control conditions. Accordingly, as compared to the 10 days old 1 *g* control EBs, differentiation of the 3-days old simulated microgravity EBs for further 7 days resulted in an inhibition of *Tnnt2*, *Rbp4*, *Tnni1*, *Csrp3*, *Nppb* and *Mybpc3* belonging to the heart development GO. This can be interpreted as a consequence of the 3-days exposure of the cells to simulated microgravity which induced inhibition of the differentiation of mESCs to mesodermal lineages like the cardiomyocytes.

Among the interesting simulated microgravity down-regulated genes we found *Podxl*, *Jun*, *Cyr61*, *Flnb*, *Tpm1*, and *Thbs1* which are reported to be crucial for the mouse embryonic development. In this context, Podocalyxin (*Podxl*) is a CD34 related sialomucin which is involved in blockage of cell adhesion during the embryonic development and *Podxl* knockout in mouse results in the lethality [34]. In Jun proto-oncogene (*Jun*) knockout mice, defects in liver and heart outflow tract development have been observed which resulted in lethality at embryonic day 12.5 [35]. Cysteine-rich, angiogenic inducer, 61 (*Cyr61*) secreted protein promotes cell adhesion, migration and proliferation of cells. *Cyr61* knockout was lethal to mice due to failure of chorioallantoic fusion or placental vasculature insufficiency [36]. In our study we found this gene down-regulated by simulated microgravity, in contrast, Allen et al. (2009) showed that *Cyr61* is up-regulated in mouse muscle cells during 11 days of spaceflight [37]. Filamins regulate extracellular signals to cellular cytoskeleton and guide migration of cells at appropriate anatomical position during embryonic development, out of three filamins, Filamin B (*Flnb*) was down-regulated by simulated microgravity. *Flnb* knockout mice were either prenatally lethal or born with severe skeletal malformation and

stunted growth [38]. Tropomyosin regulates actin-myosin interactions and encoded by four genes, out of four Tropomyosin alpha (*Tpm1*) was down-regulated by simulated microgravity, which is in line with the observation that *Tpm1* was found down-regulated in gastrocnemius muscles of mice exposed for 12 days to real microgravity in space shuttle Endeavour (STS-108) [37]. *Tpm1* homozygous knockout was reported to be prenatal lethal to the mice [39]. Among the interesting genes regulated by gravity we also found *Gadd45g*, *Dll1*, *Gpx6*, *Spink10*, *Ddit3*, *Ipmk*, and *Dpp7* which were up-regulated by simulated microgravity. It has been shown that the knockout of Growth arrest and DNA-damage-inducible, Gamma (*Gadd45g*) in mouse does not result in any embryonic abnormalities [40] except deregulation of male sex determination [41, 42]. *Gadd45g* is suggested to be involved in regulation of the methylation status of promoter regions [43]. Thrombospondin 1 (*Thbs1*) is an adhesive extracellular matrix glycoprotein and found to be down-regulated by simulated microgravity. Pardo et al. [44], reported an up-regulated expression in mouse preosteoblasts during 3 day exposure to simulated microgravity (Random Positioning Machine). However, differences in the expression level may reflect differences in the cellular models (mESCs vs preosteoblasts) and chosen facilities and thus different simulation techniques (clinostat vs. Random Positioning Machine).

We found that the deregulation of the two genes, namely *Jun* (down-regulated by simulated microgravity) and *Dll1* (up-regulated by simulated microgravity) is in line with a recently published study [45]. In this context, it has been shown that exposure of mESCs to real microgravity in spaceflight (STS 131) for 15 days inhibited the expression of germ layer and other somatic cells related genes [45]. The most of the microgravity deregulated genes investigated by classical qRT-PCR method mentioned [45] are not captured in our study using genome wide gene expression microarrays. The reasons can be attributed to different experimental variables e.g., 15 days exposure to real microgravity [45] vs 3 days exposure to the simulated microgravity and 7 days at 1 *g*, or furthermore variations in culturing conditions. Nevertheless, our transcriptome study gives a detailed insight in differentiation processes affected by altered gravity conditions. Interestingly, genes deregulated by simulated microgravity are relevant for the *in vivo* situation as has been demonstrated by several mice knockout experiments.

Besides the different gene expression pattern under simulated microgravity, we found also morphological changes in the EBs size and shape observed by clinorotation as compared to 1 *g* control EBs. The number of total EBs formed under clinorotation was less than 1 *g* conditions. Cell cycle analysis also revealed low number of cells obtained from simulated microgravity exposed EBs. However, the cell cycle analysis did not indicate significant differences. Both, differentiated mESCs exposed to 1 *g* and simulated microgravity showed similar percentages of cells in G1, G2 and S phase. Wang et al. [46] found a decrease in cell number after 3 days of 3D clinorotation of mESCs and they concluded from the cell cycle analysis that simulated microgravity does not impair cell proliferation of mESCs. The reduced number of the cells was attributed to the low adhesion rate of the cells on first day of clinorotation [46]. Taken together, from a technical perspective, contradictory results from our study in comparison with others can be attributed to the different study designs in terms of different microgravity simulation and cell culturing technologies (for reviews see Herranz et al. [47] and Brungs et al. [12]).

In conclusion, the most prominent biological process affected by simulated microgravity was the process of cardiomyogenesis which has to be confirmed with experiments under real microgravity in space.

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Disclosure Statement

All authors have contributed substantially to this work and declare no conflict of interest.

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