Differential Gene Regulation under Altered Gravity Conditions in Follicular Thyroid Cancer Cells: Relationship between the Extracellular Matrix and the Cytoskeleton

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Key Words

Thyroid cancer • Extracellular matrix • Apoptosis • Cytoskeleton • Weightlessness • Microgravity • Hypergravity • Vibration

Abstract

Extracellular matrix proteins, adhesion molecules, and cytoskeletal proteins form a dynamic network interacting with signalling molecules as an adaptive response to altered gravity. An important issue is the exact differentiation between real microgravity responses of the cells or cellular reactions to hypergravity and/or vibrations. To determine the effects of real microgravity on human cells, we used four DLR parabolic flight campaigns and focused on the effects of short-term microgravity (22 s), hypergravity (1.8 g), and vibrations on ML-1 thyroid cancer cells. No signs of apoptosis or necrosis were

detectable. Gene array analysis revealed 2430 significantly changed transcripts. After 22 s microgravity, the F-actin and cytokeratin cytoskeleton was altered, and *ACTB* and *KRT80* mRNAs were significantly upregulated after the first and thirty-first parabolas. The *COL4A5* mRNA was downregulated under microgravity, whereas *OPN* and *FN* were significantly upregulated. Hypergravity and vibrations did not change *ACTB*, *KRT-80* or *COL4A5* mRNA. *MTSS1* and *LIMA1* mRNAs were downregulated/ slightly upregulated under microgravity, upregulated in hypergravity and unchanged by vibrations. These data indicate that the graviresponse of ML-1 cells occurred very early, within the first few seconds. Downregulated *MTSS1* and upregulated *LIMA1* may be an adaptive mechanism of human cells for stabilizing the cytoskeleton under microgravity conditions.

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Introduction

Long-term microgravity can seriously affect the health of humans in space. A variety of diseases can occur such as osteoporosis, muscle and cardiac atrophy, immune problems and more [1].

It has been known for a long time that microgravity induces alterations in human cells [2]; however, the corresponding mechanisms are still unclear. The exposure of human follicular thyroid cancer cells to a random positioning machine (RPM) in order to simulate functional weightlessness induced apoptosis, altered the morphology, and changed the cellular differentiation [3]. In addition, endothelial cells, thyroid cells, chondrocytes and others grew in the form of three-dimensional assemblies when cultured on the RPM or the high-aspect ratio vessel (HARV) bioreactor [3-6]. However, little is known about the rapid gravity-dependent changes in thyroid cells. Experiments in an airplane flying a parabolic trajectory were used to investigate early changes in human cells within 22 s of microgravity, corresponding to the microgravity phase of the first of 31 parabolas.

In suspensions of purified tubulin, Moos et al. [7] identified a significant difference between microtubules assembled in the 30 s microgravity phase and the 2 g hypergravity phase of a parabolic flight on the KC-135 aircraft. These results showed that microtubule polymerization is obviously affected by changes in gravity. This finding was substantiated by sounding rocket [8] and shuttle flight [9] experiments.

Guignandon et al. [10] demonstrated that alteration of the gravitational environment between 1 g, 2 g, and microgravity during 15-30 parabolas caused a decrease in cell area and focal contact plaque reorganization in osteosarcoma ROS 17/2.8 cells, demonstrating the sensitivity of anchorage-dependent cells to transitions in gravity. The adaptation of these cells to changes in gravity appeared to depend on microtubule functioning.

Experiments on sounding rockets showed that the F-actin content increased in A431 epidermoid carcinoma cells after 7 min under microgravity [11], leading to the suggestion that the actin microfilament system is sensitive to changes in gravity and that remodelling of actin microfilaments may affect signal transduction.

In addition, the presence of the intermediate filament vimentin was significantly higher in T lymphocytes and Jurkat cells flown on MAXUS and MASER sounding rockets [12-14]. Vimentin filaments and the microtubule network in Jurkat cells were altered after 30 s under microgravity. Vimentin appeared as discontinuous points

of stained protein (probably depolymerized vimentin) in the cytoplasm. These cytoskeletal changes were not due to the acceleration at 13-15 g during the launch of the rocket. No changes were noted in the structure of actin or the inner side of the cell membrane after the binding of concanavalin A (Con A) and the co-localization of actin with Con A receptors.

Biochemical, mechanical, topographical, and geometrical stimuli can induce cells to reprogram their functions to dynamically adapt to their environmental conditions. Internal and external forces regulate cell shape and studies have shown that cell shape can control apoptosis, gene expression and protein synthesis, in addition to stem cell fate [15]. The cytoskeleton, membrane integrins, and extracellular matrix (ECM) have key roles in orchestrating multiple signalling pathways [16]. Changes in cytoskeletal geometry and mechanics, due to perturbations in cell-cell and cell-ECM interactions, may affect biochemical reactions and alter the gene activity [17].

The ECM interacts with cells to provide relevant microenvironmental information, and also biochemically through soluble and insoluble mediators and physically through the imposition of structural and mechanical constraints [18]. On the other hand, a variety of stimuli, including mechanical ones, strictly regulate the production of ECM proteins indirectly by stimulating the release of paracrine growth factors or directly by triggering intracellular signalling pathways, leading to the activation of genes involved in ECM turnover [19].

Recently, a key role of the ECM-membrane receptors-cytoskeleton system in cancer and cancer invasion has been recognized [20]. It has become increasingly clear that most transmembrane proteins (growth factor receptors, adhesion proteins and ion channels) are either permanently or transiently associated with the sub-membranous system of actin filaments and control their force generating capacity. Although there has been great progress in our understanding of the physiological importance of the microfilament system, many aspects of actin microfilament formation and its regulation are still unclear. Perturbations of the actin microfilament system in cell motility and migration and their role in cancer pathophysiology are an emerging field of research [20].

Therefore, the aim of this study was to investigate the early effects of hypergravity (20 s), microgravity (22 s) and vibrations on follicular thyroid cancer cells of the ML-1 cell line [21] using a parabolic flight. Ultra-short, initial and primary effects as well as their mechanisms

can be studied during and after the first parabola. The airbus A300 ZERO-G of the company NOVESPACE at Bordeaux-Mérignac Airport (France) is licensed to conduct parabolic flights. A parabolic flight campaign consists of three flight days with 31 parabolic flight manoeuvres each day. Our main focus was to examine the cytoskeletal organization, changes in protein content and gene expression levels, and alterations to ECM gene expression in response to altered gravity. Studying the ECM-membrane receptor-cytoskeleton relationship in thyroid cancer cells cultured under microgravity can provide new insights into the role of the interaction between the cell and its microenvironment in cancer progression. The principal aim of this study was to prepare for experiments in space under real microgravity conditions, such as the Shenzhou-8 mission. This experiment will be performed with thyroid carcinoma cells and will be flown in autumn 2011.

Materials and Methods

Cell cultures

ML-1 cell line. The ML-1 cell line was established from a recurring tumour of a follicular thyroid carcinoma (stage pT4) of a 50-year-old woman [21] and added to the histological type of poorly differentiated thyroid carcinoma. The doubling time was 4 days. The cells were able to take up glucose and secrete thyroglobulin, chondroitin sulphate, fibronectin, thyroxine, and triiodothyronine. They were tumourigenic in nude mice [21].

Tissue culture media. Cells were grown at 37 °C in RPMI 1640 medium (Invitrogen, Darmstadt, Germany) supplemented with 10% foetal calf serum (FCS) (Biochrom, Berlin, Germany), 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen) [21].

Cell culture procedure. For the flight experiment, each T75 (75 cm²) cell culture flask (Sarstedt, Nümbrecht, Germany) with a subconfluent layer of about 10⁶ cells was filled with 10 ml of medium and the Slide Flasks (10⁵ cells, Nunc, Langenselbold, Germany) with 2 ml medium. Syringes were fixed to the T75 cell culture flasks and Slide Flasks, connected by a flexible tube via a three-way valve. The syringes contained the fixative, which differed according to the future investigation procedure (Fig. 1). For the GeneArray group, we used RNAlater (Applied Biosystems, Darmstadt, Germany) as the fixative at a ratio of 4:1, for the Western blot group we used 100% EtOH (Carl Roth, Karlsruhe, Germany), adjusted to a final concentration of 70% mixed with medium, and for the Slide Flasks we used paraformaldehyde (Carl Roth, Karlsruhe, Germany) at a final concentration of 3%. The Slide Flasks for acridine orange/ethidium bromide and Hoechst 33342 staining remained unfixed and were used directly after the flight.

On the morning of each flight day, the cell culture flasks were transported to the airplane in transportable Cell Trans 4016 incubators and placed into similar incubators (Labotect,

Fig. 1. Parabolic flight overview and experimental setup. Schematic illustration of a single parabola (A) flown with the Novespace A300 ZERO-G Airbus (B, Novespace). Overview of the experimental rack (C), detailed views of the assembled Slide Flasks (D), placed inside the incubator (E), and T75 cell culture flasks with attached syringes containing fixative (F).

Göttingen, Germany) fixed to a working rack and heated up to 37 °C (Fig. 1C, E). The 1 g samples were placed in the 1 g-in-flight control centrifuge. In addition, corresponding static 1 g samples were cultured in the laboratory (ground controls).

Fixation occurred after the first parabola (P1) and the last parabola (P31) for the Gene-Array and PCR samples, and after P31 for the Western blot samples. Moreover, fixation was performed at four time points for the Slide Flasks: 1. in the plane just before P1; 2. after the first hypergravity phase; 3. after the first microgravity phase; 4. after the second hypergravity phase.

After the airplane landed the cells were transported back to the laboratory for further investigations.

For both parabolic flight samples (microgravity) and 1 g control groups (in-flight 1 g centrifuge), we collected N=6 T75 cell culture flasks for Western blot analyses (P31), quantitative polymerase chain reaction (PCR) (P1, P31), and Gene-arrays (P1, P31) (Fig. 1F), as well as N=6 NUNC™ Slide Flasks for histology (Hoechst 33342, acridine orange, and immunofluorescence staining) (Fig. 1D).

Parabolic flight

The parabolic flight experiments were performed on the Airbus A300 ZERO-G (Fig. 1B). A parabolic flight campaign day encompasses 31 parabolas and lasts about 3 h, including take-off and landing. Each parabola started from a steady, normal horizontal flight. A parabola included two hypergravity periods of 20 s, which were separated, on average, by a 22 s-microgravity period (Fig. 1A). After the first test parabolas, six series of five parabolas followed, separated by breaks of 4 and 8 min, respectively. The microgravity level achieved by the parabolic flights was 0±0.05 g. The data presented emerged from the 12th, 13th, 14th and 16th parabolic flight campaigns of the German Space Agency (DLR).

Hypergravity experiments

Parabolas were simulated by exposing the cells to two 20 s-long 1.8 g phases interrupted by a 22 s pause. Hypergravity was achieved by centrifugation on a Multi Sample Incubator

Centrifuge (MuSIC, DLR, Cologne, Germany). For both 1 g controls and 1.8 g samples, we collected N=6 samples for Western blot analyses and N=6 samples for quantitative PCR, and N=6 NUNC™ Slide Flasks for histology.

Vibration experiments

The Vibraplex vibration platform (frequency range 0.2 Hz-14 kHz) was used to create vibrations like the ones that occur during parabolic flights. The device is driven by an amplified wave signal. The injected wave signal is equal to a 1/f² noise (red noise). The signal was generated with the WaveLab 4.0 software from Steinberg.

The parabolic flight manoeuvre comprised three phases: pull up (1.8 g), free fall (microgravity), and pull out (1.8 g). The corresponding vibrations were recorded and analysed by Schmidt [22]. These data were then used for our simulation experiments with the Vibraplex.

The vibration treatment of the samples during the free fall phase was alleviated by about -6 dB. This is half of the energy found during the simulated horizontal flight. During the simulated pull up and pull out phases, the vibrations were amplified by about +6 dB.

The intensity of the noise applied was equivalent to 100 W/m². The Vibraplex uses 2W with an experimental area of about 0.02 m².

For both quantitative PCR analyses and histology we collected N=6 samples from the 1 g groups and the 31 parabolas of vibration groups for both quantitative PCR and histology.

Viability staining

After the flight, control and microgravity-exposed cells were stained with acridine orange/ethidium bromide (Molecular Probes, Darmstadt, Germany) according to the method of Zhou et al. [23] and Hoechst 33342 (Molecular Probes)-containing medium. Stained nuclei were investigated by fluorescence microscopy [3, 4].

RNA isolation

In the laboratory, the fixative was removed and replaced by 10 ml of RNeasy Lysis Buffer. The flasks were cooled down to 4 °C and transported to our home laboratories in Berlin. The next day, RNeasy Lysis Buffer was replaced by PBS (Invitrogen, Darmstadt, Germany). The cell layer was scraped off using cell scrapers (Sarstedt, Nümbrecht, Germany) and transferred to tubes. Total RNA was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions [24]. The isolated RNA had an A260/280 ratio of >1.5. The RNA concentrations were spectrophotometrically determined at 260 nm.

The cDNA was generated with the First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) using 1 µg of total RNA in a 20 µl RT-mixture.

Gene-Array technique

The Illumina Human WG-6_V2_0_R3_11223189_A arrays were normalized using the Illumina BeadStudio 1.0.2.20706 Platform and quantile normalization over probes without

Gene	Primer Name	Sequence
<i>18S rRNA</i>	18S-F	GGAGCCTGCGGCTTAATTT
	18S-R	CAACTAAGAACGGCCATGCA
<i>ACTB</i>	Actb-F	TGCCGACAGGATGCAGAAG
	Actb-R	GCCGATCCACACGGAGTACT
<i>KRT80</i>	Krt80-F	GAGAGCTTCGTGGAGTTGATGA
	Krt80-R	GCCGCTCAGGTCGATGTG
<i>BCL2L1</i>	Bcl2l1-F	TTTGCCCTAAGGCGGATTT GA
	Bcl2l1-R	GAGTCTCGTCTCTGGTTAGTGATTCTC
<i>Col4A5</i>	Col4A5-F	ATTGGTTATTCTTCATGATGCATAC
	Col4A5-R	TGAAGGGAGCTGAACGAACTC
<i>LIMA1</i>	Lima1-F	TGGTAGCAGCGCAGAGGAA
	Lima1-R	CTCAATGATAGTGAGGTCCATTGC
<i>MTSS1</i>	Mtss1-F	CCGTCATCTCAGATCCCTAGCT
	Mtss1-R	GGTCCGGTTCTCGCTTCTC
<i>TSHR</i>	Tshr-F	CATAGAAATTCGGAATACAGGAACTT
	Tshr-R	CAGTGTGAAAATGCCAAGGAA
<i>FN</i>	Fn-F	TGAGGAGCATGGTTTATAGGAGAA
	Fn-R	TCCTCATTTACATTCGGCGTATAC
<i>OPN</i>	Opn-F	GGACACGGACTCCGAGGAA
	Opn-R	GTGACCAGCTCATCGGATTCA
<i>ITGB1</i>	Itgb1-F	GAAAAACAGCGCATATCTGGAATTT
	Itgb1-R	CAGCCAATCAGTGATCCACAA
<i>CD44</i>	CD44-F	ACCCTCCCCTCATTACCAT
	CD44-R	GTTGTACTACTAGGAGTTGCCTGGATT

Table 1. Primers used for quantitative real-time PCR. All sequences are given in 5’-3’ direction.

background correction.

After normalization, an offset addition and the log(2) transformation outlier were removed according to a Nalimov test p-value threshold of 10⁻³ [25]. Probes with a consistently low-level expression were removed (transformed signal < 7; 12,542 probes left). The general expression profile was characterized using principal component analysis (PCA), with correlation as dispersion matrix and normalized eigenvector scaling. Differential expression over the three experimental conditions with respect to the five replicates was ascertained using ANOVA statistics followed by a false discovery rate (FDR) correction using the Benjamini-Hochberg procedure [26]. The resulting probe list was condensed by fold change (FC) cut-offs and according to the existence of a Gene Ontology (GO) biological process annotation. Hierarchical average linkage clustering was performed using a Euclidean distance function, and ANOVA, PCA and hierarchical clustering was performed using the Partek Genomic Suite version 6.3.

Quantitative real-time PCR

We employed the real-time quantitative RT-PCR to quantify the expression levels of the genes of interest. Appropriate primers with a Tm of about 60 °C were designed with the Primer Express® software (Table 1). The primers were synthesized by TIB Molbiol (Berlin, Germany). The StepOnePlus Real-Time PCR System in conjunction with the Power SYBR® Green PCR Master Mix (Applied Biosystems, Darmstadt, Germany) was employed to perform the assays. The reaction volume was 25 µl with a final concentration of 500 nM for the primers. The PCR conditions were as follows: 10 min at 95 °C, 40 cycles of 30 s at 95 °C and 1 min at 60 °C, followed by a melting curve analysis step (temperature gradient from 60 °C to 95 °C with +0.3 °C per cycle).

The PCR reactions were considered specific if all amplicons showed one single T_m similar to the one predicted by the Primer Express software. Relative quantification was effected by means of the comparative C_T ($\Delta\Delta C_T$) method. Each sample was measured in triplicate. In order to normalize our expression data, we utilized 18S rRNA as a housekeeping gene.

Western Blot analysis

Cells were fixed with ethanol adjusted to 70% for Western blot analysis after the last parabola. SDS-PAGE, immunoblotting and densitometry were performed out on six replicates following routine protocols [27–29]. Antibodies against the following antigens were used: beta-tubulin (Sigma, Taufkirchen, Germany), cytokeratin 8, vimentin, pan-actin (all Santa Cruz, Santa Cruz, CA, USA).

For densitometric quantification of the bands, the stained membranes were analysed on a Personal Densitometer 50301 (Molecular Dynamics, Krefeld, Germany) [3].

F-actin staining

The F-actin filaments were stained with rhodamine-phalloidin (Molecular Probes, Eugene, OR, USA) as described by the manufacturer. For evaluation, cover slips were mounted in Moviol and examined on a Zeiss Axiovert wide-field microscope. Images were taken using a CCD camera (Hamamatsu, Solothurn, Switzerland) and a standard FITC filter [3].

Immunofluorescence of cytokeratin

For immunofluorescence staining [3, 4], the fixed cells were washed twice in PBS and then incubated with a cytokeratin antibody (dilution: 1:100; Sigma, Taufkirchen, Germany) for 24 h at room temperature (RT). The distribution of the intermediate filament cytokeratin was evaluated by the indirect immunofluorescence method and analysed with a Zeiss 510 META inverted confocal laser scanning microscope equipped with a Plan-Apochromat 63×1.4 objective. The excitation and emission wavelengths for FITC were: $\lambda_{exc} = 488$ nm and $\lambda_{em} \geq 505$ nm, respectively.

Statistical analysis

Statistical analysis was performed using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA). All data are expressed as mean values $\pm$ standard deviation (SD). Statistical analysis was performed using one-way ANOVA or the Mann-Whitney U test where applicable. Differences were considered significant at the level of $p < 0.05$.

Results

Viability staining shows no apoptosis and no necrosis

In both the 1 g controls as well as in cells exposed to a parabola no apoptotic or dead cells were revealed by Hoechst 33342 (Fig. 2A and 2B) or acridine orange/ethidium bromide (Fig. 2C and 2D). Moreover, the cells

subjected to 31 cycles of vibration (Fig. 2F), one cycle of hypergravity (Fig. 2G), and 31 cycles of hypergravity (Fig. 2H) did not visibly differ from the 1 g control group (Fig. 2E) and did not show any apoptotic or necrotic cells.

Acridine orange/ethidium bromide staining, according to the method of Zhou et al. [23], indicated that the 1 g control cells and the cells exposed to microgravity, hypergravity or vibrations had remained viable and impermeable to the dye (green fluorescence). In the case of dead or necrotic cells, the cells would have taken up the dye and shown a red fluorescence (phase III, according to Zhou).

Parabolic flight-induced changes in the cytoskeleton

We investigated the proteins involved in the formation of the cytoskeleton: actin, cytokeratin 8, tubulin, and vimentin. During the parabolic flight actin allocation did not change after the first hypergravity phase (Fig. 3B) when compared to the 1 g control (Fig. 3A). After the first microgravity phase, however, we found a clear rearrangement of the actin network, with perinuclear clustering (Fig. 3C) in contrast to the corresponding 1 g control (Fig. 3D). Neither 31 hypergravity phases (Fig. 3E) nor vibrations (Fig. 3F) induced any changes in the actin cytoskeleton.

The Western blot analyses revealed significant increases in protein expression after 31 parabolas of microgravity for cytokeratin 8 (Fig. 3I), tubulin (Fig. 3J) and vimentin (Fig. 3K). As shown by immunofluorescence, microgravity induced a clustering of cytokeratin (Fig. 3H).

Microarray analysis of cells exposed to microgravity parabolas

At the general transcriptome level the difference between 1 g and P1 was rigorously stronger than the differences due to increasing numbers of parabolas. The portion of the variance described by principal component (PC) #1 (44.3%) was mainly driven by the parabola factor, whereas no clear discrimination was observed between the P1 and P31 samples (Fig. 4A). Probes with an ANOVA FDR $< 10^{-3}$ ($n = 2430$) were selected as being significantly differentially expressed and further condensed to a set of strongly differentially expressed probes using twofold change criteria (1 g vs. P1 $abs(FC) \geq 2.5$; 1 g vs. P31 $abs(FC) \geq 1.5$; $N = 276$). Four functional candidate genes that passed the FDR criterion but missed the FC criteria (*LIMA1*, *KRT80*, *BCL2L1* and *ACTB*) were manually added to the candidate list. Filtering by

Fig. 2. Analysis of cell viability under parabolic flight conditions. In order to assess the extent of apoptosis and necrosis, cells were stained with Hoechst 33342 (A: 1 g control, B: 1 μ g parabola) and acridine orange (C, E: 1 g controls, D: 1 μ g parabola, F: simulated vibration, G: one cycle of simulated hypergravity, H: 31 cycles of simulated hypergravity). All cells were viable: no apoptosis or necrosis was detected.

Fig. 3. F-Actin distribution and expression. The F-actin network was visualized by rhodamine-phalloidin at $\times 200$ magnification. A, D: 1 g controls, B: after first 1.8 g phase, C: after first microgravity phase, E: 31 cycles of simulated 1.8 g, F: one cycle of simulated vibrations, G: 1 g and H: microgravity cytokeratin immunofluorescence (magnification: $\times 200$). I: cytokeratin-8, J: tubulin and K: vimentin Western blot analyses and densitometric evaluation after 31 parabolas; # $p < 0.05$ vs. 1 g.

GO biological process annotations resulted in a list of 148 significantly regulated genes: 102 genes were downregulated, and 46 were upregulated after the parabolas (Fig. 4B). A String network analysis using a confidence score of 0.15 [30] resulted in 44 singletons and a network of 100 genes (Fig. 4C). The biological functions comprised, amongst others, cell adhesion, apoptosis, the cytoskeleton, G-protein signalling and metabolism. These results are summarized in Table 2.

Quantitative real-time PCR analysis of selected genes showed a strong influence of microgravity

We chose several genes of interest that were identified as being differentially expressed by the microarray analysis for further investigation by quantitative

real-time PCR. This group comprised genes involved in the cytoskeleton (*ACTB*, *LIMA1*, *MTSS1*, *KRT80*), extracellular matrix (*COL4A5*), apoptosis (*BCL2L1*), and G-protein signalling (*TSHR*). In addition, we also included the extracellular matrix genes osteopontin (*OPN*), fibronectin (*FN*), integrin $\beta 1$ (*ITGB1*), and *CD44*.

The *ACTB*, *BCL2L1*, and *KRT80* transcripts were significantly upregulated, whereas *COL4A5* and *MTSS1* were significantly downregulated after one microgravity parabola. After 31 parabolas, only *BCL2L1* and *KRT80* showed further significant changes, with *BCL2L1* decreasing to 1 g levels and *KRT80* increasing to more than the P1 levels. The *LIMA1* and *TSHR* transcripts remained unchanged throughout the experiment (Fig. 5A).

Fig. 4. Microarray analysis. A: PCA mapping over 12,542 expressed probes. The first (x-axis) and second principal components (y-axis) accounted for 44.3% and 21.4% of the dataset variance, respectively. The variability in expression was stronger in the set of parabola samples (red and blue) compared to the control samples (green). B and C: Hierarchical clustering (B) and String network connections (C) of 148 significantly regulated genes. B: The cluster dendrograms represent the cluster node similarity matrix of the samples (left) and probes (top). The heatmap represents the differences in expression between the samples on a colour scale ranging from red (upregulated) to blue (downregulated). Outliers ($>$ double standard deviation) are marked in orange (upregulated) or light blue (downregulated). C: The known and predicted protein interactions including direct (physical) and indirect (functional) associations are visualized in a network. The colours of the connecting lines represent the data source according to the list on the right-hand side.

Hypergravity had a much lower effect on the expression of these genes. Only *MTSS1* and *TSHR* showed strong reactions with significant increases after one cycle of hypergravity, while *COL4A5* and *LIMA1* were only moderately upregulated, but not until after 31

cycles of hypergravity. Vibrations did not show any effect on gene expression (Fig. 5B).

Both *OPN* and *FN* were upregulated under microgravity conditions. In the case of *OPN*, the increase was significant after just 1 microgravity

Table 2. Differentially expressed transcripts detected by microarray analysis. Transcripts highlighted in bold were validated by quantitative real-time PCR, duplicates indicate multiple probesets for one transcript

GO Biological Process	Gene symbol	FOC 1 P vs. 1 g	FOC 30 P vs. 1 g	Gene bank accession
adenylate cyclase activation	<i>ADCY6</i>	-2.994	-2.288	NM_020983
	<i>ADCY6</i>	-2.588	-1.957	NM_020983
	<i>ADCY9</i>	-2.600	-2.148	NM_001116
amino acid metabolism	<i>SLC6A6</i>	4.145	4.842	NM_003043
apoptosis	<i>BAG4</i>	2.531	1.712	NM_004874
	<i>CARD8</i>	-2.905	-2.006	NM_014959
	<i>AXUD1</i>	3.303	2.479	NM_033027
	<i>PPP1R15A</i>	2.679	3.042	NM_014330
	<i>TNFRSF11B</i>	-3.040	-2.454	NM_002546
	<i>TIA1</i>	-3.299	-2.657	NM_022037
	<i>TIA1</i>	-2.711	-2.829	NM_022173
	<i>BCL2L1</i>	2.093	1.851	NM_138578
	<i>TNFRSF25</i>	-2.622	-2.438	NM_148973
blood coagulation, proteolysis and peptidolysis	<i>MST1</i>	-3.093	-2.607	NM_020998
brain development	<i>CDK5RAP3</i>	-2.531	-2.566	NM_025197
	<i>SPG11</i>	-3.931	-3.997	NM_025137
	<i>MAN1C1</i>	-2.786	-2.764	NM_020379
carbohydrate transport	<i>SLC35B3</i>	-3.403	-2.351	NM_015948
catecholamine metabolism	<i>INMT</i>	-3.140	-3.127	NM_006774
cell adhesion	<i>COL4A5</i>	-4.242	-4.039	NM_000495
	<i>TSPAN12</i>	-3.531	-3.031	NM_012338
	<i>PILRB</i>	-4.642	-4.277	NM_013440
	<i>PILRB</i>	-3.118	-2.811	NM_013440
	<i>HSPC159</i>	3.629	2.518	NM_014181
	<i>COL24A1</i>	-3.672	-3.448	NM_152890
	<i>NCAM1</i>	-2.582	-2.439	NM_000615
	<i>ATRN</i>	-2.936	-2.240	NM_139321
	<i>PCDHA3</i>	2.530	2.720	NM_018906
cell differentiation	<i>PAPPA</i>	-3.801	-3.588	NM_002581
cell growth and/or maintenance	<i>BEGAIN</i>	2.581	1.755	NM_020836
	<i>DTNA</i>	2.653	2.240	NM_001392
	<i>MLLT6</i>	-3.373	-2.872	NM_005937
	<i>MAF</i>	2.604	2.156	NM_005360
	<i>RRAS2</i>	2.886	2.734	NM_012250
cell motility	<i>MARCKSL1</i>	2.590	2.327	NM_023009
cell proliferation	<i>EVI2A</i>	2.708	2.909	NM_014210
	<i>RAPGEF3</i>	-3.543	-3.106	NM_006105
cell-cell signaling	<i>FJX1</i>	2.689	2.807	NM_014344
chloride transport	<i>CLCNKA</i>	-3.852	-4.310	NM_004070
chromatin assembly/disassembly	<i>CBX7</i>	-2.825	-2.858	NM_175709
chromatin remodeling	<i>KAT2A</i>	-2.832	-3.058	NM_021078
collagen fibril organization	<i>NSFL1C</i>	2.821	2.556	NM_182483
cytoskeleton	<i>MTSS1</i>	-3.231	-2.718	NM_014751
	<i>FSCN1</i>	2.863	3.008	NM_003088
	<i>ACTB</i>	1.673	1.637	NM_001101
	<i>KRT80</i>	1.960	2.247	NM_182507
	<i>LIMA1</i>	2.059	2.657	NM_016357
	<i>KRT8</i>	2.568	2.396	NM_002273
de novo IMP biosynthesis	<i>GART</i>	-2.613	-2.387	NM_000819
DNA damage response	<i>ABL1</i>	3.615	2.310	NM_007313
DNA packaging	<i>HMGB1</i>	2.798	2.546	NM_002128
DNA repair	<i>GADD45A</i>	2.514	3.024	NM_001924
	<i>GADD45A</i>	2.703	3.614	NM_001924
	<i>CRY1</i>	2.592	3.008	NM_004075
	<i>ATM</i>	-3.918	-3.186	NM_000051
	<i>ATM</i>	-3.384	-2.919	NM_000051
electron transport	<i>CYP24A1</i>	-2.839	-2.243	NM_000782
	<i>LIME1</i>	-2.711	-2.296	NM_017806
	<i>ACADVL</i>	-2.591	-2.386	NM_000018
endocytosis	<i>CAV2</i>	2.901	3.201	NM_001233
	<i>CAV2</i>	2.677	3.208	NM_001233
	<i>CAV1</i>	2.715	2.796	NM_001753
extracellular matrix	<i>MMP23B</i>	-3.208	-2.768	NM_006983
fatty acid biosynthesis	<i>ACACB</i>	-2.503	-2.794	NM_001093
fatty acid metabolism	<i>ACSL3</i>	-3.810	-2.735	NM_004457
gametogenesis	<i>COX4NB</i>	3.226	2.852	NM_006067
gamma-aminobutyric acid signaling pathway	<i>GABRE</i>	-2.926	-2.511	NM_004961
	<i>GABRE</i>	-3.361	-2.896	NM_004961
	<i>GABBR1</i>	-2.524	-2.109	NM_021905
glycine catabolism	<i>GLDC</i>	-2.762	-2.478	NM_000170
	<i>AMT</i>	-2.975	-3.218	NM_000481
glycosaminoglycan metabolism	<i>IDS</i>	2.729	2.242	NM_006123
	<i>IDS</i>	4.384	3.109	NM_006123
Golgi organization and biogenesis	<i>ZNF800</i>	2.633	2.748	NM_176814
G-protein coupled receptor protein signaling pathway	<i>EBI2</i>	4.398	6.380	NM_004951
	<i>RGS16</i>	-2.534	-3.631	NM_002928
G-protein signaling	<i>TSHR</i>	-3.060	-3.029	NM_000369
	<i>LTB4R</i>	-3.192	-2.879	NM_181657
immune response	<i>IL18R1</i>	-2.904	-2.705	NM_003855
intracellular protein transport	<i>NUP43</i>	-2.601	-2.296	NM_024647
	<i>USO1</i>	-2.919	-2.117	NM_003715
intracellular signaling cascade	<i>RGS11</i>	-4.457	-4.726	NM_183337
	<i>STAT6</i>	-2.821	-2.501	NM_003153
L-fucose catabolism	<i>FUT5</i>	3.604	3.045	NM_002034
L-glutamate transport	<i>SLC1A3</i>	-2.542	-2.677	NM_004172

GO Biological Process	Gene symbol	FOC 1 P vs. 1 g	FOC 30 P vs. 1 g	Gene bank accession
lipid metabolism	<i>UBAP2L</i>	-2.601	-2.038	NM_014847
	<i>GSDMB</i>	-3.575	-2.982	NM_018530
MAPKKK cascade	<i>MAP2K3</i>	2.449	2.530	NM_002756
metabolism	<i>GSTM2</i>	-3.444	-3.855	NM_000848
	<i>DBT</i>	-2.698	-2.487	NM_001918
	<i>INSIG2</i>	-4.264	-3.416	NM_016133
	<i>C5orf4</i>	-3.079	-3.167	NM_032385
	<i>ACSS1</i>	-2.533	-2.070	NM_032501
	<i>CBR4</i>	-2.795	-2.336	NM_032783
microtubule-based movement	<i>DYNC1H1</i>	-2.893	-2.621	NM_001376
microtubule-based process	<i>DYNLL2</i>	4.231	3.624	NM_080677
mitotic checkpoint	<i>KNTC1</i>	-2.537	-2.134	NM_014708
mRNA catabolism	<i>ATP2B4</i>	-3.878	-3.296	NM_001698
mRNA splicing	<i>LUC7L</i>	-3.219	-2.552	NM_018032
muscle contraction	<i>SCN4A</i>	-2.963	-2.686	NM_000334
muscle development	<i>TAGLN</i>	-2.607	-2.735	NM_003186
	<i>CAPN3</i>	-4.055	-3.769	NM_024344
N-acetylglucosamine metabolism	<i>NAGK</i>	-2.746	-2.702	NM_017567
negative regulation of cell cycle	<i>RBL2</i>	-3.275	-3.075	NM_005611
negative regulation of cell proliferation	<i>CDK10</i>	-2.903	-3.013	NM_052988
neurogenesis	<i>AUH</i>	-2.512	-2.602	NM_001709
nucleotide-excision repair	<i>RAD23B</i>	3.991	3.532	NM_002874
phospholipid biosynthesis	<i>CEPT1</i>	-2.957	-2.677	NM_006090
	<i>AGPAT4</i>	-3.076	-2.535	NM_020133
potassium ion transport	<i>KCNJ15</i>	-3.257	-2.249	NM_170736
protein amino acid glycosylation	<i>ST6GALNAC3</i>	-3.130	-3.776	NM_152996
	<i>PRKX</i>	-2.583	-2.928	NM_005044
	<i>PRKX</i>	-2.711	-2.801	NM_005044
	<i>STK38</i>	-3.632	-2.711	NM_007271
	<i>NEK6</i>	2.841	2.663	NM_014397
	<i>NRBP2</i>	-2.857	-2.875	NM_178564
protein arginylation	<i>ATE1</i>	2.812	2.561	NM_007041
protein biosynthesis	<i>FAM57A</i>	2.574	2.447	NM_024792
protein complex assembly;signal transduction	<i>SKAP2</i>	-2.569	-2.682	NM_003930
protein-lipoylation	<i>NMT2</i>	2.617	2.151	NM_004808
proteolysis and peptidolysis	<i>ARHGEF10</i>	-3.408	-2.659	NM_014629
	<i>C1orf112</i>	-2.542	-2.241	NM_018186
	<i>OMA1</i>	-3.606	-3.316	NM_145243
regulation of cell cycle	<i>SKP2</i>	4.516	2.757	NM_005983
regulation of transcription	<i>STAT2</i>	-2.614	-2.900	NM_005419
	<i>TP53BP1</i>	-2.575	-2.368	NM_005657
	<i>HSF4</i>	-2.516	-2.704	NM_001538
	<i>ZNF337</i>	-2.562	-2.667	NM_015655
	<i>ZNF589</i>	-2.784	-2.094	NM_016089
	<i>KIAA1618</i>	-3.823	-4.511	NM_020954
	<i>ZNF248</i>	-2.815	-2.096	NM_021045
	<i>MZF1</i>	-3.901	-3.093	NM_198055
	<i>BAPX1</i>	-5.521	-4.245	NM_001189
regulation of translational initiation	<i>EIF2C3</i>	2.578	2.315	NM_024852
response to stress	<i>ATHL1</i>	-2.615	-2.786	NM_025092
RNA processing	<i>DDX17</i>	-3.838	-3.077	NM_006386
	<i>DDX17</i>	-3.817	-2.921	NM_030881
	<i>SNRP70</i>	-2.651	-2.772	NM_003089
	<i>SNRP70</i>	-2.842	-2.619	NM_003089
signal transduction	<i>CRHBP</i>	-2.553	-2.727	NM_001882
	<i>PDE7A</i>	-4.285	-3.223	NM_002604
	<i>PDE7B</i>	-4.345	-3.928	NM_018945
sodium ion transport	<i>SCNN1D</i>	-3.277	-3.671	NM_002978
synaptonemal complex formation	<i>STAG3L1</i>	-7.247	-4.490	NM_018991
TGFbeta receptor signaling pathway	<i>KLF10</i>	2.600	2.594	NM_005655
transcription	<i>PLAS2</i>	4.230	3.868	NM_004671
transport	<i>ABCC3</i>	-3.407	-2.692	NM_003786
	<i>ABCC5</i>	-2.857	-2.276	NM_005688
	<i>ABCA8</i>	-2.942	-2.846	NM_007168
	<i>TBC1D8B</i>	-3.256	-2.573	NM_017752
	<i>THADA</i>	-2.821	-2.682	NM_022065
	<i>C19orf44</i>	-2.881	-2.224	NM_032207
ubiquitin-dependent protein catabolism	<i>UBE2L3</i>	5.006	4.452	NM_198157
	<i>PSMD9</i>	3.362	2.635	NM_002813
	<i>PAN2</i>	-2.822	-2.597	NM_014871
Wnt receptor signaling pathway	<i>GSK3B</i>	2.717	2.467	NM_002093
	<i>DKK1</i>	2.970	6.192	NM_012242
zinc ion transport	<i>SLC30A1</i>	2.945	3.288	NM_021194

Fig. 5. Gene expression profiles of differentially expressed genes identified by microarray analysis. In order to validate the microarray results, selected genes were analysed using quantitative real-time PCR. (A) Impact of microgravity on gene expression, (B) Impact of hypergravity/vibrations on gene expression; # $p < 0.05$ vs. 1 g.

Fig. 6. Quantitative real-time PCR analysis of OPN, fibronectin, and integrin- β_1 . Expression profiles of the three genes were generated by quantitative real-time PCR for cells subjected to microgravity (A) and simulated hypergravity/vibrations (B); # $p < 0.05$ vs. 1 g.

parabola and it progressed after 31 parabolas. Although *FN* expression levels after both 1 and 31 parabolas were comparable, only the changes after 31 parabolas were statistically significant due to large variations in the P1 group. No significant changes were observed for *ITGB1*, while the expression of *CD44* was below the detection limit (Fig. 6A, *CD44* data not shown).

Hypergravity tended to induce small changes in the gene expression patterns of *OPN*, *FN*, and *ITGB1*, but none were significant (Fig. 6B).

Discussion

Exposure to a random positioning machine induced apoptosis changed the differentiation and growth

behaviour in follicular thyroid cancer cells [3] and other cells such as lymphocytes, glial cells and endothelial cells [31-33]. In addition, an increase in extracellular matrix proteins was observed in the three-dimensional multicellular tumour spheroids of thyroid cancer cells and also in prostate and breast cancer spheroids cultured on a high-aspect ratio vessel bioreactor (HARV) developed by NASA [5]. Moreover, alterations have been reported for a variety of human cells cultured under conditions of “modelled microgravity” [34]. Ground-based approaches aim to achieve the status of functional weightlessness. Real microgravity can verify the quality of the simulation by the technique applied. Here, we investigated the early phases of weightlessness. The principal aim of this study was to detect early changes or the adaptation processes of thyroid cancer cells induced by short-term microgravity.

Short-term microgravity alters the distribution and amount of intermediate filaments

The actin cytoskeleton is connected to various membrane proteins, influencing polarity, cell adhesion, migration and the response to extracellular signals. Cytoskeletal changes occur in a variety of cell types including lymphocytes [35], thyrocytes [3], glial cells [33], chondrocytes [6], endothelial cells [4] and osteoblasts [36, 37] during real microgravity and various simulation approaches. The external signals (altered gravity conditions) might be transduced into intracellular signals through the extracellular matrix, adhesion molecules, and the cytoskeletal structure. Although perturbations from structures within the cell itself could not be ruled out [38], these signals regulate differentiation, cellular motility, migration, and apoptosis, or survival. Rijken and de Groot were the first to demonstrate changes in cell shape in response to altered gravity in sounding rocket experiments [39]. Changes were also reported by Guignandon et al., who examined ROS/17/2.8 cells after a flight in an unmanned Foton mission, where they found a disorganization of the actin cytoskeleton [40].

Here, we demonstrated that the expression of actin and *KRT80* mRNA was significantly increased in ML-1 samples after 1 and 31 parabolas, whereas no changes were found with respect to vibration, hypergravity or the 1 g control samples. In parallel, the amounts of vimentin and cytokeratin-8 proteins were both significantly elevated after 31 parabolas, whereas the amount of tubulin protein did not change. In addition, rearrangements in the actin microfilament system were detectable. Alterations in the actin system were also found by Rijken et al. [41] in human A431 carcinoma cells on a sounding rocket. These sounding rocket experimental conditions included a real microgravity period of 6 minutes that may have influenced the steady state of the F-actin content of these cells. Real microgravity for 6 minutes showed that the stress fibres were less well organized. Furthermore, real microgravity increased the formation of filamentous actin in untreated A431 cells.

Moreover, early on during a sounding rocket flight marked alterations in the structure of vimentin and the microtubule networks in T lymphocytes and Jurkat cells were observed [14, 42, 43]. Our findings fit with the data about microgravity-dependent cytoskeletal alterations published by different authors for several cell types, such as glial cells [33], testicular cells [44], lymphocytes [35, 42], Jurkat cells [14, 35], breast cancer cells [45], and osteoblasts [46].

Short-term microgravity alters gene expression of extracellular matrix proteins and cell adhesion molecules

Collagen type IV (*COL4A5*) gene expression was significantly reduced under short-term real microgravity in thyroid cancer cells. Vibration and hypergravity did not alter *COL4A5* gene expression, but after 31 parabolas *COL4A5* expression increased. Moreover, we investigated osteopontin (*OPN*) and fibronectin (*FN*) gene expression. Both were significantly elevated after 31 parabolas. Fibronectin is a glycoprotein that links the extracellular matrix to the cytoskeleton through integrin surface receptors and it also binds to collagens and proteoglycans. Fibronectin plays a major role in cell adhesion, migration, differentiation, growth and wound repair [27]. Fibronectin and *OPN* proteins were upregulated under long-term exposure on a random positioning machine [3], mainly in the three-dimensional multicellular tumour spheroids. Interestingly, we detected an early upregulation of *OPN* mRNA. The *OPN* protein interacts with multiple cell-surface receptors that are ubiquitously expressed and is an active player in many physiological and pathological processes, including wound healing, bone turnover, tumourigenesis, inflammation, ischaemia/reperfusion, immune responses and weightlessness. It has also been shown to covalently bind to fibronectin via transglutaminase-catalysed cross-linking. As an adhesion protein, it has also been proposed as binding to calcium and collagen. This interaction with fibronectin and collagen indicates its importance in ECM organization [47]. In addition, soluble *OPN* inhibits apoptosis in endothelial cells [48], and *OPN* also reduces the formation of apoptotic bodies and suppresses DNA fragmentation. These data suggest that one function of *OPN* in homeostatic processes is to facilitate the survival of stressed endothelial cells, possibly by occupying unligated integrins and suppressing integrin-mediated death [48, 49]. The rise in *OPN* gene expression may be a self-protection mechanism of the ML-1 cells against apoptosis. Acridine orange/ethidium bromide staining and Hoechst 33342 staining revealed no increase in apoptosis or necrosis after 31 parabolas. In contrast, Kumei et al. [36] reported a decrease in *OPN* gene expression, but they also found an increase in fibronectin gene expression in rat osteoblasts when cultured for a longer period of 4 or 5 days aboard the Space Shuttle [36]. The mRNA levels of actin, vimentin, or β_1 -integrin (*ITGB1*) did not change under space flight conditions. We investigated the mRNA level of *ITGB1* after the first and

31 parabolas. It was slightly, but not significantly, elevated. This difference could be explained by its adaptation to long-term microgravity, which was also reported by other authors [33].

Metastasis suppressor 1 (MTSS1), also known as MIM or MIM-B (missing-in-metastasis), was originally identified as a potential metastasis suppressor gene that was present in non-metastatic bladder cancer cell lines, but it was not expressed in a metastatic bladder cancer cell line [50]. It was suggested that MTSS1 could be a potential metastasis suppressor. It was also found that it promoted the disassembly of actin stress fibres. Actin filament assembly is associated with cytoskeletal structure organization and cell motility. This alteration in actin dynamics could have serious *in vivo* consequences on the metastatic ability of cancer cells [51, 52]. Here, we report for the first time the expression of *MTSS1* mRNA in follicular thyroid cancer cells of the ML-1 cell line. The *MTSS1* transcript was downregulated under microgravity after the first and last parabola. As *MTSS1* was unaltered by vibration and clearly upregulated under hypergravity, the downregulation was clearly induced by microgravity conditions. This mechanism may be one of the factors that influence the actin cytoskeleton of ML-1 cells exposed to microgravity conditions. The MTSS1 protein may be important in regulating cytoskeletal dynamics and the finding that it is downregulated under microgravity may be an adaptive mechanism of the thyroid cancer cells.

The TSH receptor (TSHR) plays a pivotal role in thyroid gland functioning, growth, and differentiation. Exposure of ML-1 cells to microgravity induced a moderate downregulation of the *TSHR* mRNA expression. In contrast, vibrations did not change the expression of the *TSHR* gene, although it was clearly upregulated by hypergravity. Culturing ML-1 cells on a RPM over a period of 3 days revealed an upregulation of TSHR [3], which was accompanied by a reduced synthesis of thyroid hormones. The reasons for the differences found between the effects of short-term (22 s) micro-gravity and RPM exposure should be investigated in future studies with a focus on membrane proteins and the synthesis behaviour of ML-1 cells.

Interestingly, expression of the *LIMA1* gene was only slightly elevated after 31 parabolas. Vibrations had no effect, but hypergravity significantly elevated the gene expression of *LIMA1*, also known as the epithelial protein lost in neoplasm (EPLIN) [53]. The LIMA1 protein is a cytoskeletal-associated protein involved in the regulation of actin dynamics and cell motility. Its expression is

frequently reduced in a variety of cancer cells and tissues and this loss may account for increasingly invasive traits in cancer cells. The LIMA1 protein is known to suppress actin depolymerization and to stabilize F-actin fibres, resulting in establishment of the adhesion belt [54]. The upregulation of *LIMA1* mRNA may indicate a mechanism of thyroid cancer cells stabilizing the actin cytoskeleton.

In summary, the microarray analysis delivered 2430 differentially expressed transcripts. In this paper, we focused on the cytoskeletal genes, which were altered early on. We verified these data by quantitative PCR and examined the corresponding cytoskeletal proteins. Moreover, we investigated *OPN* and fibronectin mRNA expression, which was up-regulated after 31 parabolas, indicating a functional role in the regulation of growth, cell migration and cell adhesion of thyroid cancer cells grown under conditions of altered gravity.

Generally, both the microarray and quantitative real-time PCR data were in very good accordance (Table 2, Fig. 5a). With the exception of *LIMA1*, both methods showed comparable results, indicating that our techniques ensured high reproducibility between the different parabolic flights. Table 2 demonstrates that differential expressed genes of a variety of biological processes (e.g. apoptosis, cell adhesion, cell differentiation, e. c.) are influenced by hypergravity and microgravity after the first and last parabola. Here, we focus on cytoskeletal genes and on ECM genes.

Taken altogether, these data suggest that microgravity severely affects the cytoskeleton. Changes of the cytoskeleton were also reported by several other authors [55, 56]. This process starts very early on. Several gravisensitive signalling elements are involved in the reaction of follicular thyroid cancer cells to the altered gravity conditions created by a parabolic flight manoeuvre.

The effects of a longer period of real microgravity on thyroid carcinoma cells are to be the objective of further studies. The parabolic flight turned out to be feasible method for preparing for such experiments in space; for example, the Chinese Shenzhou-8 mission.

Abbreviations

µg (Microgravity); 1 g (ground control); 1.8 g (Hypergravity); ACTB (Actin b); BCL2L1 (Bcl-2 like (Bclx)); COL4A5 (Collagen, type IV, alpha 5); Con A (Concanavalin A); ECM (Extracellular matrix); FN (Fibronectin); HARV (High-aspect ratio vessel); ITGB1 (Integrin-β 1); KRT80 (Keratin 80);

LIMA1 (LIM domain and actin-binding protein 1); MTSS 1 (Metastasis suppressor 1); OPN (Osteopontin); P1 (After 1st parabola or equivalent period of time of 1.8 g); P31 (After 31st parabola or equivalent period of time of 1.8 g); RPM (Random positioning machine); TSHR (Thyroid-stimulating hormone receptor); VIB (Vibration).

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