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Mechanical culture conditions effect gene expression: gravity-induced changes on the space shuttle

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Hammond, T. G., E. Benes, K. C. O'Reilly, D. A. Wolf, R. M. Linnehan, J. H. Kaysen, P. L. Allen, and T. J. Goodwin. Mechanical culture conditions effect gene expression: gravity-induced changes on the space shuttle. *Physiol Genomics* 3: 163–173, 2000.—Three-dimensional suspension culture is a gravity-limited phenomenon. The balancing forces necessary to keep the aggregates in suspension increase directly with aggregate size. This leads to a self-propagating cycle of cell damage by balancing forces. Cell culture in microgravity avoids this trade-off. We determined which genes mediate three-dimensional culture of cell and tissue aggregates in the low-shear stress, low-turbulent environment of actual microgravity. Primary cultures of human renal cortical cells were flown on the space shuttle. Cells grown in microgravity and ground-based controls were grown for 6 days and fixed. RNA was extracted, and automated gene array analysis of the expression of 10,000 genes was performed. A select group of genes were regulated in microgravity. These 1,632 genes were independent of known shear stress response element-dependent genes and heat shock proteins. Specific transcription factors underwent large changes in microgravity including the Wilms' tumor zinc finger protein, and the vitamin D receptor. A specific group of genes, under the control of defined transcription factors, mediate three-dimensional suspension culture under microgravity conditions.

renal cells; cell culture; gene array; flow cytometry; vitamin D receptor

ALTHOUGH CELL CULTURE has become a standard technique in laboratories pursuing cell biology and numerous other disciplines, the dedifferentiation of cells in culture continues to represent a major impediment to suspension culture and tissue engineering applications (3, 6, 17). We hypothesize that the differentiation of cells in culture depends on the provision of three conditions: three-dimensionality, low shear and turbulence, and cospatial relation of dissimilar cell types (3, 18). A seemingly endless variety of suspension culture vessels have been designed to attempt to provide these culture conditions. As a group, these vessels suffer from the problem that defeating gravity in a suspension culture can only by achieved at the cost of other forces that balance gravity, forces that are associated with shear stress and turbulence (3, 6, 18, 19).

Recognition that shear and turbulence in suspension cultures is derived from movement of cell aggregates against the vessel wall and stirring by the impeller allows engineering optimization of culture parameters (6, 18, 19). Engineers embodied the optimal ground-based stirred fermenter in the rotating wall vessel, a horizontal cylinder rotating along its long axis with a coaxial oxygenator (17–19). In this vessel, the vessel wall rotates synchronously with aggregates in culture, minimizing shear and turbulence from this source. This rotation induces sufficient mixing that the impeller can be removed, further reducing shear and turbulence (18, 19).

Unfortunately, even under the optimized conditions of the rotating wall vessel, suspension culture is still gravity limited: as cell aggregates increase in size, the shear stress on them rises (6, 17, 19). Eventually the aggregates are ripped apart by differential shear stresses induced by their size (6, 17, 19). Cell aggregates in the rotating wall vessel reach a terminal velocity, which is directly proportional to gravity, the radius of the particle squared, and the difference in density between the particle and the culture media, as well as being inversely proportional to the media viscosity (15, 18). This provides a *1*) formal mathematical basis with which to investigate effects of mechanical culture conditions and *2*) intuitive insight into the role of gravity in mechanical culture conditions (15, 18).

The rotating wall vessel was originally developed to simulate conditions of cell growth in the microgravity of space (3, 15, 17–19). This represented optimization of the mechanical culture conditions of suspension culture, which has found extensive ground-based application (3, 17) in culture of numerous cell lines for academic (17) and industrial (15) applications. Space allows extension of mechanical culture conditions into otherwise unobtainable ranges (15, 18). The motiva-

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tion for these studies is to understand the molecular mechanisms underlying suspension culture to enhance ground-based applications.

The intuitively obvious scientific extension of gravity-limited suspension culture systems is to culture cells in microgravity. Microgravity conditions release us from this trade-off of aggregate size and forces balancing gravity. Indeed, during microgravity culture, shear and turbulence approximate zero, while cospatial relations of cells and three-dimensionality are near perfect. We hypothesize that culture of cells in microgravity conditions, such as are provided during space shuttle flight, optimizes mechanical culture conditions (6, 17). Cell culture in true microgravity will both define the mechanisms of cell differentiation and show how thoroughly ground-based rotating wall vessel cultures approach conditions of actual microgravity. As a negative control, the paradigm would predict that culturing cells in a centrifuge with increased gravity, changes none of these parameters, and therefore there would be scant induced change in gene expression (6, 17, 19).

We chose renal cells for these studies of differentiation, as they are the clinically important source of two of the biggest selling pharmaceuticals in the world: erythropoietin (2) and the active 1,25-dihydroxy form of vitamin D_3 (5). Stable differentiation of a renal cell line may lead to implantable sources of these hormones. These cells have readily identifiable differentiated features and have growth kinetics perfect for multiple-day cultures best suited to current flight incubators (6, 17).

Recently developed methods of gene expression analysis using microarray technology allow detailed quantitative observations to detect the role of culture conditions on steady-state genetic expression (6, 11, 12, 20). Overall differential display of gene expression has been most powerful at steady state (20). This allows for quantitative comparison of the nature, grouping, and extent of genetic expression change between different culture conditions. Gene array also allows dissection of specific gene groups of mechanistic interest such as the identity of transcription factors, which mediate these effects (6, 11, 12).

MATERIALS AND METHODS

Reagents and Quenching Antibodies

All reagents were from Sigma Chemical (St. Louis, MO) unless otherwise stated. All fluorescence measurements were made in the presence of 7 μl/3 ml anti-fluorescein antibodies to quench extravesicular fluorescein fluorescence (6, 20).

Cells

Human. Human renal cells were used for experiments on the space shuttle and all the associated controls. Clonetics (San Diego, CA) isolated human renal cortical cells from kidneys unsuitable for transplantation due to multiple renal arteries (4, 6). Cell fractions consisted of the natural mixture of cells in the renal cortex. To initiate cell culture, culture vessels were filled with medium and seeded by addition of cell suspension (1×10^6 cells/ml). DMEM-F12 was supplemented with 10% fetal calf serum and an antibiotic cocktail (ciprofloxacin and Fungizone) (4, 6). Concomitant with cells, microcarrier Cytodex-3 beads were added at 5 mg/ml to promote aggregate formation. All cultures were derived from aliquots of the same stock of cells. All experiments were done with identical culture conditions of feeding, seeding, and duration, designed to match the flight experiment (see below). As primary human renal cells grow relatively slowly, it is possible to perform a 6-day experiment without refeeding. For flight and short arm centrifuge experiments, bags impermeable to CO_2 but freely permeable to O_2 were utilized (American Fluoroseal, Urbana, IL). In this manner, CO_2 was derived in the cultures by trapping the CO_2 produced by the cultures themselves while maintaining free O_2 exchange with ambient sources.

Rat renal cortical cells. Rat renal cells were used for culture experiments on the Mir Space Station, and associated parallel ground controls. Rat renal cells were isolated from renal cortex harvested from euthanized Sprague-Dawley rats (Harlan Sprague-Dawley, Cleveland, OH) as previously described (4). In brief, renal cortex was dissected out with scissors, minced finely in a renal cell buffer 137 mmol NaCl, 5.4 mmol KCl, 2.8 mmol $CaCl_2$, 1.2 mmol $MgCl_2$, and 10 mmol HEPES-Tris, pH 7.4. The minced tissue was placed in 10 ml of a solution of 0.1% type IV collagenase and 0.1% trypsin in normal saline. The solution was incubated in a 37°C shaking waterbath for 45 min with intermittent titubation. The cells were spun gently (800 rpm for 5 min), the supernatant was aspirated, and the cells were resuspended in 5 ml renal cell buffer with 0.1% bovine serum, then passed through a fine (70 μm) mesh. The fraction passing through the mesh was layered over a discontinuous gradient of 5% bovine serum albumin and spun gently. The supernatant was again discarded. The cells were resuspended in DMEM-F12 medium (ciprofloxacin and Fungizone treated) and placed into culture in various culture vessels in a 5% CO_2-95% O_2 incubator, or flight bags as above.

Culture Techniques

Rotating wall vessels. Renal cells were cultured in rotating wall vessels known as 55-ml slow turning lateral vessels (STLV) (3, 6, 17, 19). Residual air was removed through a syringe port, and vessel rotation was initiated at 10 rotations per minute.

Short arm centrifuge. Fifty-five-milliliter gas-permeable bags of cells were spun at 3 *g* in the short arm centrifuge (**http://ccf.arc.nasa.gov/dx/archives/life_sciences/artificial_gravity/centrifuge1.html**) at NASA Ames Research Center in Mountain View, CA. This centrifuge is custom engineered with gears to allow steady narrow tolerance application of low *g* forces to cell cultures. The gas supply is shared with an adjacent attached incubator that houses 1 *g* control cultures.

Static controls. Gas-permeable Fluoroseal bags (American Fluoroseal) in 55-ml size were selected as conventional static controls.

Flight Experiment

To optimize three-dimensionality, minimize shear, and turbulence and provide opportunity for near perfect cospatial relationships, human cell cultures were grown in Space Transportation System flight 90 (space shuttle flight STS-90) "Neurolab" (**http://www.ksc.nasa.gov/shuttle/missions/sts-90/mission-sts-90.html**). Cells were cultured in six separate 25-ml bags incubated in the Biological Specimen Tem-

perature Controller (**http://microgravity.msfc.nasa.gov/cs.html**). As proof of concept, rat renal cells were previously flown in the same hardware on the Mir Space station during an engineering shakedown flight (**http://www.ksc.nasa.gov/shuttle/missions/sts-86/mission-sts-86.html**).

Gene Array

Human cell samples were fixed with Omnifix and stored at 7°C at the end of 6 days of cultures. mRNA was selected with oligo(dT) cellulose [MicroPoly(A)Pure; Ambion, Austin, TX]. The poly(A) RNA from flight, centrifuge, rotating wall vessel, and ground samples were reverse-transcribed with fluorescent bases tagged with green/cyanine-3 or red/cyanine-5. A binding analysis was performed by annealing the fluorescent probes competitively to 10,000 cDNAs immobilized in a grid on a glass microscope slide. The bound fluorescent DNA was quantitated with a fluorescent reader. Incyte Pharmaceuticals (Palo Alto, CA) performed this analysis, including post-array analysis using Incyte's GemTools software (**http://www.incyte.com**). The fluorescence of each pixel in each cDNA spot was measured, and the average value was calculated. An annular ring of equal area surrounding each cDNA spot was pixelated, and fluorescence intensity was measured as background. To reach threshold and be included in the analysis, each cDNA had to reach 2.5 times the average background fluorescence in 35% of the pixels.

Flow Cytometry Analysis of Cells and Membranes

Flow cytometry analysis was performed on a Becton-Dickinson FACSVantage flow cytometer (6). Excitation was at 488 nm using a Coherent 5-W argon-ion laser. For each particle, emission was measured using photomultipliers at 530 ± 30 nm and 585 ± 26 nm. Data were collected as 2,000 event list mode files and were analyzed using LYSYS software. The vitamin D receptor antibody we used is a mouse anti-human vitamin D_3 receptor monoclonal (Diagnostic Systems Lab, Webster, TX).

Western Blot Analysis of AT_{1a} Receptor

The angiotensin AT_{1a} receptor antibody was a rabbit polyclonal antibody raised to two 16-amino acid peptides, one derived from the cytosolic tail, and the other derived from the exofacial domain. Sodium channel antibody was a rabbit polyclonal raised to a 16-amino acid cytosolic domain. SDS-PAGE was performed on a 10% gel using 4 μg protein per lane of total membranes, a 1:1,000 dilution of primary antiserum, and revealed with the peroxidase-based ECL system (ECL, Springfield, IL). Protein content of membranes was determined using bicinchoninic acid reagent (Pierce, Rockford, IL) to allow equal total protein loading on each gel lane.

Detection of Gene Expression in Cell Cultures by Semi-Quantitative RT-PCR

Cell aggregates from the rotating wall vessel or bag cultures were washed once in ice-cold PBS and snap frozen at −70°C until RNA was isolated. Total RNA was isolated using Trizol (GIBCO BRL). First-strand cDNA was reverse transcribed from 2 μg of total RNA using random primers and Superscript II RT (GIBCO BRL). Before cDNAs were subjected to semi-quantitative RT-PCR, they were normalized by PCR using 18S rRNA primers/copetimers from the QuantumRNA Quantitative RT-PCR Module (Ambion) and primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Twenty percent of the PCR reaction was electrophoresed on agarose/ethidium bromide gels and visualized under ultraviolet light. Electrophoresis results were recorded and quantitated using the Kodak Digital Science 1D Image Analysis Software. Semi-quantitative PCR for each gene of interest was performed at two concentrations of cDNA and 28 and 32 cycles of amplification to ensure we made measurements on the initial

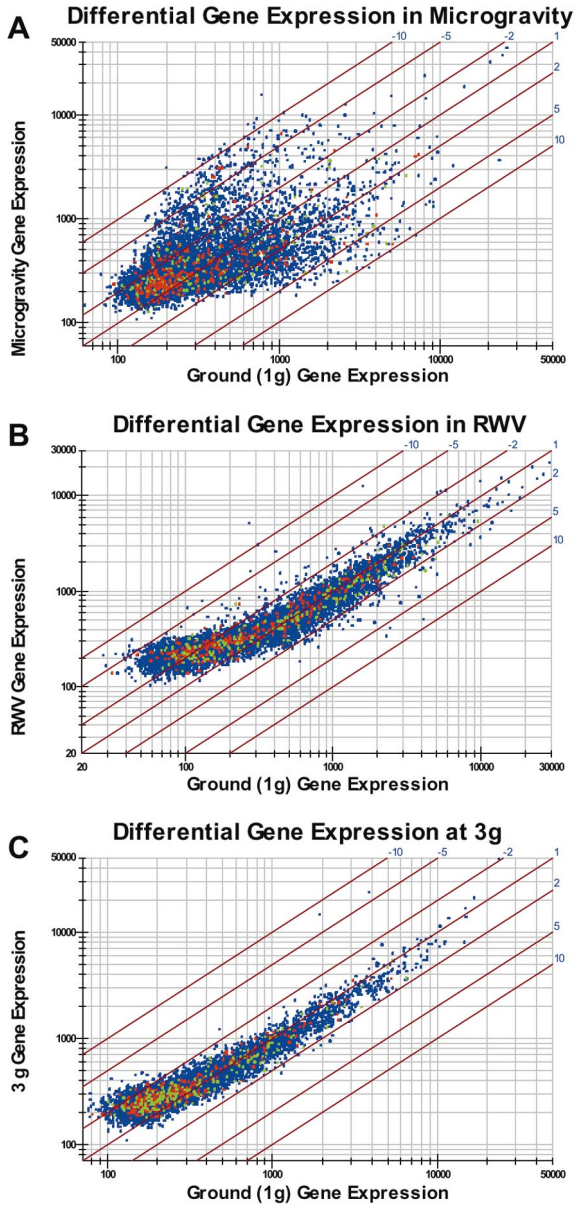

Fig. 1. Gene array. Each cell culture condition including microgravity (*A*), rotating wall vessel (RWV) (*B*), and 3-*g* centrifuge (*C*) is compared with a static nonadherent bag culture. In each panel, 10,000 individual genes are represented by individual dots. Shear stress proteins and heat shock proteins are shown in green, and transcription factors are in red. Gene expression is displayed on a log scale to the base 10. Changes greater than threefold are outside the region of background noise. For each gene, the distance from the origin denotes level of gene expression, and movement on the *x*- and *y*-axes reports change in gene expression compared with the static bag control culture. More than 1,600 genes (1,632) change more than the specific threshold of threefold up and down in the flight (microgravity) culture (*A*); only a few genes (5) changed in the centrifuge culture (*C*), and more than 900 genes (914) changed in the RWV culture (*B*).

Table 1. *Genes whose expression changed >3.0 in space with comparative results in the rotating wall vessel*

Gene Groups	IMAGE No.	Space	RWV
Adhesion			
Human laminin β2-chain (LAMB2)	359741	−3	2.1
Human platelet/endothelial cell adhesion	489123	−3.5	2
Human mRNA for integrin α6-subunit	159512	−4	2.6
Human carcino-embryonic antigen mRNA	510405	−6.4	2.3
Apoptosis			
Human tumor necrosis factor receptor 2	470493	−11.1	−1.4
Human cysteine protease Mch2 isoform	323500	−6.6	1.2
Human B94 protein mRNA; complete	487045	−5.8	−1.2
Human mRNA for mutated p53 transformation	236338	3	−1.3
Cytoskeletal proteins			
Human skeletal muscle α-tropomyosin	488479	−3.8	1.3
Human non-lens βγ-crystallin like	297589	−6	1.2
Human mRNA for plasma gelsolin	359675	8.2	1
Human mRNA for kinesin-related protein	327575	3.9	−1.7
Human mRNA for integrin α6-subunit	159512	−4	1.2
Human mRNA for cytokeratin	509980	−7.1	1.3
Human mRNA for coronin	487988	8.6	1.2
Human mRNA for β-centractin	381596	5.1	1.2
Human laminin B2 (LAMB2) mRNA; partial	485332	−3.5	1.4
Human keratin type II (58 kDa) mRNA; complete	345925	−5	−1
Human I-plastin mRNA; complete	381819	−3.1	1.2
Human dystrophin gene	294950	−3	−1.4
Human cytokeratin 18 mRNA; 3′ end	114872	−4.6	1
Human cardiac myosin heavy chain mRNA; 3	300069	3.5	1
Human β-tubulin pseudogene; complete	296731	−7.5	−1.1
Human α-spectrin gene; exon 52	427750	4.9	−1.1
Human α-cardiac actin gene; exon 6	485743	4.6	1.5
Human actin-related protein Arp3 (ARP3)	359910	5	1.6
Human (clone PWHLC2-24) myosin light chain	417479	−3.9	1
Differentiation			
vav; VAV	199381	−3.2	0
Rat developmentally regulated protein mRNA	360210	−3.6	1.3
Rat clone C101 intestinal epithelium protein	363123	−3.1	2.1
Human zinc finger/leucine zipper protein	113307	3	−1.1
Human Wilms' tumor (WT33) protein mRNA	470470	9.1	1.1
Human ubiquitin protease (Unph) proto-oncogene	486665	2.9	−1.2
Human triiodothyronine receptor (THRA1)	364080	−6.7	1.4
Human shorter form basic fibroblast growth factor	21955	−6.9	−1.3
Human set gene; complete	510260	−3.7	1
Human novel growth factor receptor mRNA	180447	−4.2	−1
Human nerve growth factor-β (β-NGF)	72869	−4.4	−1.4
Human mRNA for p0071 protein	52476	3.3	−1
Human mRNA for myoblast cell surface antigen	363658	4.6	1.1
Human membrane-associated protein (HEM-1)	322627	6	−1.1
Human JNK2 α1 protein kinase (JNK2A1)	322029	3.4	−1.5
Human IEF 9306 mRNA	429361	3.2	−1.1
Human homolog of *Drosophila* enhancer	469370	−3	−1.4
Human GM-CSF receptor mRNA; complete	140352	−7.7	−1.3
Human glycogen synthase kinase 3 mRNA	22047	4.8	1.2
Human dek mRNA	486647	−3	−1.7
Human CD34 mRNA; complete	213635	3	1
Human calcineurin B mRNA; complete	489081	3.2	1.1
Human c-syn proto-oncogene; complete	323555	5.1	−1.2
Human BTG1 mRNA	291035	4.2	−1
FMS; growth factor receptor; CSF-1 receptor	204653	6.7	1.1
C33 antigen; type III integral membrane protein	488596	−3	−1
Electron transport			
Thioredoxin	415014	3.4	1
NADH:ubiquinone oxidoreductase (428 AA)	509804	7	1.4
Human mRNA for electron transfer flavoprotein	308745	3	1.2
Human gene for very-long-chain acyl-CoA	428227	3.7	1.1
Human electron transfer flavoprotein alp	488221	3	1.2
Human cytochrome b561 gene; exon 6	376146	−3.6	−2
Human cytochrome b5 mRNA; complete	415153	5.2	1.2
ATR1; NADPH-ferrihemoprotein reductase	309770	−3	−1
Adhesion			
Intracellular signaling proteins			
Human tyrosine kinase-type receptor	365147	−5	−1
Human shorter form basic fibroblast growth factor	21955	−6.9	−1.3

Table 1.—*Continued*

Gene Groups	IMAGE No.	Space	RWV
Human set gene; complete	510260	−3.7	1
Human phosphatidylinositol 4,5-bisphosphatase	29804	4	−1.6
Human mRNA for rab 13	366489	−3.7	1.2
Human mRNA for putative serine/threonine	472138	6.2	1.4
Human mRNA for KIAA0118 gene; partial	151066	−3.5	−1.1
Human GTP binding protein (ARL3) mRNA	360902	−3	1.1
Human glycogen synthase kinase 3 mRNA	22047	4.8	1.2
Human cyclin E (CCNE) gene; exon B	357807	−3.9	1.6
Human calcineurin B mRNA; complete	489081	3.2	1.1
Nucleic acid synthesis and modification			
Human RNA polymerase II subunit hsRPB4	302293	−3.5	1.5
Human mRNA for myoblast cell surface antigen	363658	4.6	1.1
Receptors			
OB-R gene related protein (OB-RGRP)	323210	−3.2	1.1
Human vitamin D receptor mRNA; complete	365566	−7.4	1.1
Human tyrosine kinase-type receptor	365147	−7.4	1.1
Human tumor necrosis factor receptor-2	470493	−5	−1
Human triiodothyronine receptor (THRA1)	364080	−11.1	−1.4
Human transforming growth factor-β	209655	−6.7	1.4
Human shorter form basic fibroblast growth factor	21955	−6.6	−1
Human putative G-protein-coupled receptor	345973	−6.9	−1.3
Human OB-RGRP gene	265571	−4.7	−1.4
Human novel growth factor receptor mRNA	180447	−5.6	1.2
Human mRNA for T-cell receptor β-chain	302157	−4.2	−1
Human mRNA for interferon α/β receptor	123950	−3.6	−1.1
Human mRNA for growth hormone receptor	295389	3.6	−1.1
Human lectin-like type II integral membrane protein	415086	−4.7	1
Human GM-CSF receptor mRNA; complete	140352	−3.2	−1.6
Human endothelial cell protein C/APC receptor	376268	−7.7	−1.3
Human DNA for human P2XM; complete	60566	−6.3	1.6
Human 180-kDa transmembrane PLA_2 receptor	320355	−3.7	1.4
FMS; growth factor receptor; CSF-1 receptor	204653	−3.4	1.3
Stress			
ESTs	509962	6.7	1.1
Superoxide dismutase	417829	4.1	1.2
Transcription factors			
Pzf; zinc finger protein	297856	−6.2	−1.1
Human ZNF81 gene	229467	−6.1	1
Human zinc finger/leucine zipper protein	113307	3	−1.1
Human zinc finger protein ZNF136	343096	−4.1	1
Human zinc finger protein mRNA; complete	61531	−3.4	1.1
Human zinc finger protein (LD5-1) mRNA	417295	−3	1
Human Wilms' tumor (WT33) protein mRNA	470470	9.1	1.1
Human vitamin D receptor mRNA; complete	365566	−7.4	1.1
Human triiodothyronine receptor (THRA1)	364080	−6.7	1.4
Human transcription factor TFIIA small subunit	485192	3	−1.8
Human transcription factor (ITF-2) mRNA	380738	5.6	0
Human TFII-I protein (TFII-I) mRNA; complete	376973	−3.6	−1.4
Human sequence-specific DNA-binding protein	293032	−7.1	1
Human mRNA for RNA polymerase II association	323480	−6.5	1
Human mRNA for mutated p53 transformation	236338	3	−1.3
Human mRNA for KIAA0262 gene	485008	4.5	1
Human interferon regulatory factor 3	203017	3	1.1
Human IEF 9306 mRNA	429361	3.2	−1.1
Human EWS-E1A-F chimeric protein mRNA	363805	3.8	1.5
Human dek mRNA	486647	−3	−1.7
Human aryl hydrocarbon receptor nuclear	324799	6.3	1.1
Drug metabolism			
Human P-glycoprotein (MDR1) gene; exon 1	39920	−3.5	1.1
Human glutathione *S*-transferase (GSTM5)	377731	3.4	−1.5
Human glutathione *S*-transferase (GST)	365434	−4.3	1.6
Heat shock			
Human tra1 mRNA	242829	−5.5	−1.2
Human heart mRNA for heat shock protein	510320	3.1	1.1

Gene expression changes in space. Genes whose expression changes more than 3-fold in space are listed according to gene groups. For comparison, changes during rotating wall vessel (RWV) culture in the same genes are also listed. The values shown represent balanced differential gene expression. Gene expression is reported as the difference between the cyanine-3 fluorescent signal in the control cDNA sample and cyanine-5 signal on the experimental cDNA sample. The experimental cDNA sample is corrected for differences in total signal strength based on internal controls on the gene chip. See Ref. 8a for description of IMAGE nos. (see also **http://image.llnl.gov/**).

linear portion of the response curve. A control PCR with GAPDH was also carried out with each cDNA to assure that the input of RNA and reaction efficiencies were all similar. The PCR reactions were electrophoresed and quantitated using NIH Image software.

Scanning Electron Microscopy

Scanning electron micrographs were performed on cell aggregates from the rotating wall vessels, conventional monolayers, 3 *g* centrifuge samples, and flight experiments.

RESULTS

Metabolic data suggests that each group of cell cultures grew at a similar rate: glucose fell 30 $mg \cdot ml^{-1} \cdot day^{-1}$, BUN rose 0.5 $mg \cdot ml^{-1} \cdot day^{-1}$, and pH was maintained between 7.31 and 7.34. Oxygenation was excellent in all cultures with P_{O_2} of 155–167 mmHg for 99% saturation, P_{CO_2} of 12–13 mmHg, and HCO_3^- of 6–6.6 mmol/l. As the results were similar in each group, they are not tabulated here (full gene array data set at **http://www.tmc.tulane.edu/astrobiology/microarray**).

Gene array analysis of gene expression under the various culture conditions is displayed in Fig. 1. Each panel depicts the individual expression of 10,000 separate genes comparing flight samples (Fig. 1*A*), rotating wall vessel (Fig. 1*B*), and centrifuge at 3 *g* (Fig. 1*C*). In each case, the experimental sample is compared with a control bag culture grown in parallel. Each dot represents expression of an individual gene. A gene that does not change would lie on a line from the origin to the top right corner (labeled 1) with expression level reported by distance from the origin. The scale on each axis is log to the base 2. A change greater than threefold is regarded as above background (**http://www.incyte.com**).

The genes whose expression changed the most are tabulated in Table 1, and include adhesion molecules, apoptosis genes, cytoskeletal proteins, differentiation mediators, drug metabolizing proteins, select heat shock proteins, intracellular signaling proteins, receptors, transcriptions factors, and elements of the electron transport chain. Figure 1 depicts the flight data in pictorial format and shows an amazingly large degree of change in gene expression at steady state. A select but substantial group of more than 1,600 genes changed in each direction in microgravity (1,632 genes >3-fold change). The genes that changed in microgravity include many cytoskeletal proteins (shown as green dots). Specific transcription factors (shown in red) underwent large changes in microgravity including the Wilms' tumor zinc finger protein and the vitamin D receptor (full data set at **http://www.tmc.tulane.edu/astrobiology/microarray**).

Rotating Wall Vessel

The number of genes with demonstrable changes in expression during rotating wall vessel culture is between flight and the centrifuge (Fig. 1*B*). Unlike the flight array, which shows an amazing degree of steady-state change in gene expression, gene expression changes in the rotating wall vessel were either less marked or more transient, returning to baseline by 6 days in culture.

Centrifuge

In contrast, we analyzed the genes that change in hypergravity (3 *g*) during a centrifuge experiment. In this experiment an exact replica of the flight experiment was performed, except in a centrifuge at 3 *g*. Figure 1*C* shows that only a very few genes (~15) changed more than threefold under these circumstances. These genes, detailed in Table 2, are diverse, ranging from an oncogene and a steroid dehydrogenase to a *P*-450 cytochrome and a G protein binding protein.

Pattern of Changes in Gene Expression

If the genes changing the most in microgravity are selected and mapped on the rotating wall vessel array, then the genes selected on the microgravity array are randomly spread throughout the rotating wall vessel array (data not shown). The same is true in reverse, whereby the genes that changed in the rotating wall vessel assay are randomly distributed on the microgravity array. Hence, the gene expression changes observed in microgravity are unique and not just an extension of the rotating wall vessel.

Table 2. *Relative gene expression in short arm centrifuge*

Genes	IMAGE No.	SAC	RWV	Space
V-raf murine sarcoma 3611 viral oncogene homolog 1	360531	−5.8	1.6	1.8
11β-Hydroxysteroid dehydrogenase 2	70843	2.8	2.1	0
Human cytochrome *P*-450-IIB (hIIB3) mRNA, complete cds	84275	2.6	5.7	1
Homo sapiens Cyr61 mRNA, complete cds	486700	3.1	5.5	−1.2
Homo sapiens CAGH3 mRNA, complete cds	487986	2.6	6.3	0
Homo sapiens (clone p5-23-3) mRNA	296903	2.7	2.7	−2.1
Guanine nucleotide binding protein (G protein), β polypeptide 1	380288	3.1	5.5	−2.1
Eukaryotic translation initiation factor 4E	254041	3	2.3	1
ESTs, highly similar to calcyphosine (*Canis familiaris*)	284264	−2	2.6	2.2

Genes whose expression changes more than 2-fold during short arm centrifuge (SAC) culture at 3 *g*. Comparison is made to changes in the same genes during culture in a rotating wall vessel (RWV) and space. Values represent balanced differential gene expression; cds, complementary DNA sequence.

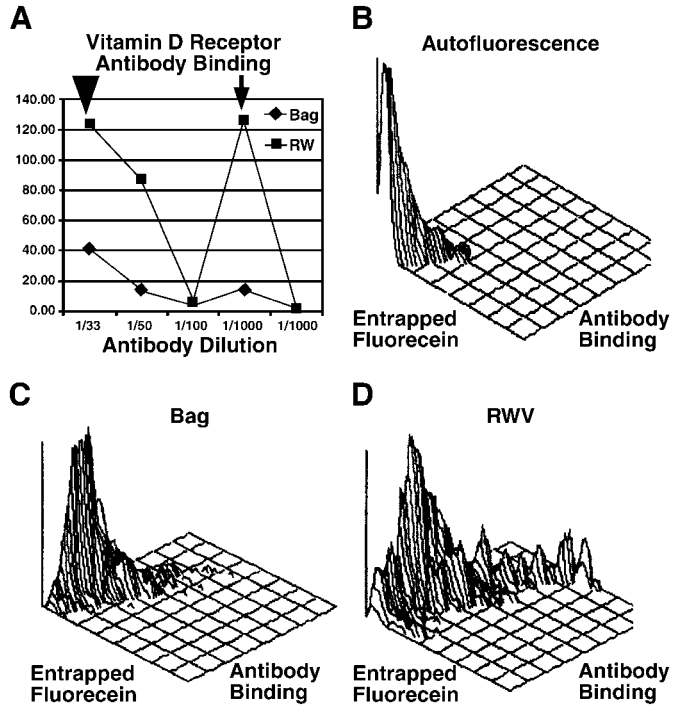

Fig. 2. Confirmation of changes observed on gene array: protein by flow cytometry and Western blot analysis. *A–D*: flow cytometry analysis of vitamin D receptor binding on membrane vesicles. The binding curves of anti-vitamin D receptor antibody in control static and RWV culture demonstrate classic nonspecific binding at high antibody dilutions (bold arrowhead), with an increase in binding and a decrease in antibody titer pathognomonic of specific antibody binding (small arrow). Specific vitamin D receptor antibody binding is an order of magnitude greater in cells cultured in the rotating wall vessel than in static culture controls. To test whether the vitamin D receptor resides in a select group of membranes, flow cytometry colocalization of entrapped fluorescein dextran and antibody binding was performed. In *B–D*, horizontal axes depict fluorescence of entrapped fluorescein-dextran and vitamin D receptor antibody binding, and vertical axis depicts number of endosomes per channel. *B*: control. Human renal cells were exposed to nonfluorescent dextran and labeled with preimmune serum, rather than anti-vitamin D receptor antiserum, before addition of phycoerythrin-conjugated secondary antibody; this demonstrates low autofluorescence and scant nonspecific antibody binding. *C*: following static cell culture, there is detectable vitamin D receptor antibody binding in a minor component of membranes that colocalize with entrapped fluorescein dextran. *D*: following RWV culture, not only is vitamin D receptor expression increased at the protein level, but it also colocalizes with entrapped fluorescein dextran. Representative of $n = 4$.

Confirmation of Changes Observed on Gene Array: Protein by Flow Cytometry

To determine whether changes in rotating wall vessel gene expression correlated at the protein level, we performed small particle flow cytometry antibody binding analysis of vitamin D receptor, parathyroid hormone receptor, and focal adhesion kinase-2, as well as Western blot analysis of the AT_{1a} receptor (6). Figure 2*A* demonstrates a classic dilution curve for vitamin D antibody binding to renal cell membranes derived from human cells grown in the rotating wall vessel or bag controls. The nonspecific binding at high antibody concentrations (open arrow) is followed by an increase in binding with a decrease in antibody dilution, pathognomonic of specific antibody binding (6) (solid arrow). To confirm that the vitamin D receptor antibody binding was only in a select population of membranes, we colocalized entrapped dextran with antibody binding. Figure 2*B* defines the level of autofluorescence and nonspecific binding by examining the fluorescent emission of cells exposed to nonfluorescent dextran and labeled with preimmune serum. In Fig. 2, *B–D*, each depicts 2,000 membrane vesicles with fluorescein fluorescence and phycoerythrin goat anti-rabbit secondary antibody fluorescence on the horizontal axes, with the number of particles per channel on the axis coming up out of the page. When cells are incubated in fluorescein dextran and labeled with a mouse monoclonal anti-vitamin D receptor antiserum, there is colocalization of entrapped dextran and vitamin D receptor antibody binding with far greater receptor expression in the rotating wall vessel samples (Fig. 2*D*) than in the bag controls (Fig. 2*C*). A similar pattern exists for the PTH receptor and focal adhesion kinase-2 (Table 3) (flow cytometry graphs not shown), as well as Western analysis of AT_{1a} receptor (Table 3) consistent with a 50% reduction in this protein reported by gene array.

Confirmation of Changes Observed on Gene Array: Semi-Quantitative RT-PCR

To confirm some of the changes in specific genes observed on the gene array analysis we examined the expression of selected genes by RT-PCR (Fig. 3). Analysis of human renal cells grown in the rotating wall

Table 3. *Confirmation of gene array findings by other techniques*

Gene	Analysis Method	Culture Conditions	Gene Array Changes, total binding signal		Confirmatory Method Changes, direction/change
			Bag	RWV or Space	
Vitamin D receptor	Flow cytometry antibody binding	RWV	5.1	18.7	+110 FU (3) [15 → 125]
Sodium channel β-subunit	Western blot	RWV	5.6	16.8	+220% binding (1)
AT_{1a} receptor	Western blot	RWV	3.0	2.1	−50% binding (2)
Mn-dependent SOD	RT-PCR	RWV	61.0	12.4	−43% product (3)
Villin	RT-PCR	RWV	2.4	1.9	−30% product (2)
Villin	RT-PCR	Space	2.0	2.5	+230% product (2)

Correlation of changes in gene expression reported by gene array with RT-PCR, flow cytometry antibody binding and Western blot analysis. RWV, rotating wall vessel; FU, fluorescence units; SOD, superoxide dismutase. For "Confirmatory Method Changes," the number in parentheses is *n*.

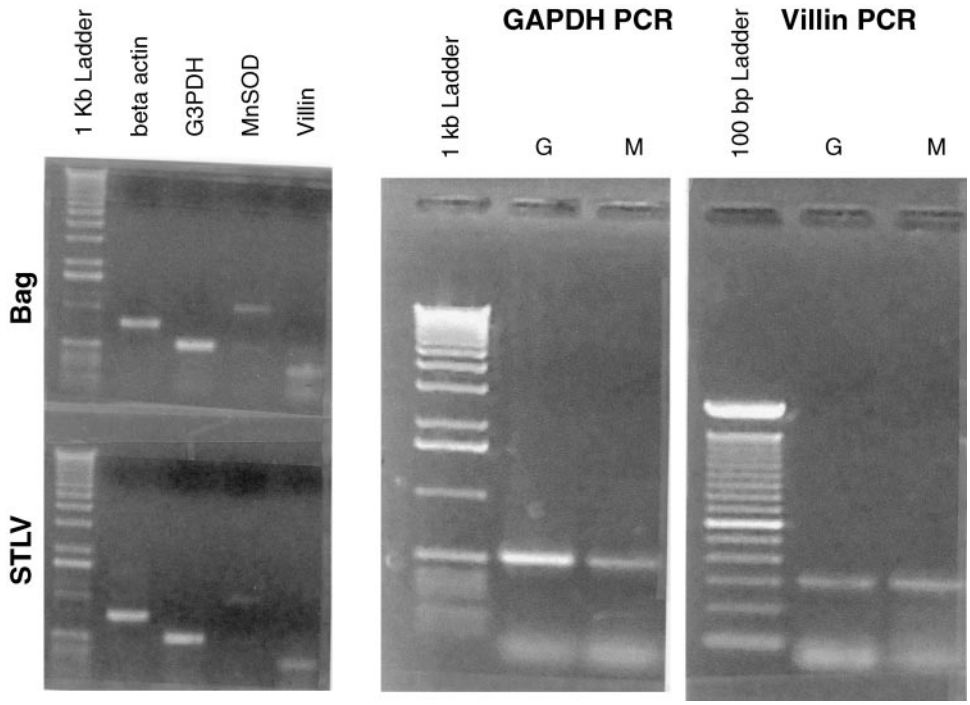

Fig. 3. Confirmation of changes observed on gene array: semi-quantitative RT-PCR (representative of $n = 2$). To confirm some of the changes in specific genes observed on the gene array analysis, we examined the expression of selected genes by RT-PCR. Analysis of human renal cells grown in the RWV shows that although there was no change in β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), changes in Mn-dependent superoxide dismutase (MnSOD) and villin were within 20% of each other by gene array and RT-PCR (*left*; Table 3). Villin decreased in the RWV gene array analysis but increased in the space culture gene array. Villin increased from 2.0 to 2.5 balanced differential expression on the human cell space gene array, and RT-PCR for rat kidney cells samples grown on the Mir Space Station (M) compared with ground controls (G) (*right*) confirms a modest increase, more if ratioed for changes in GAPDH (*middle*).

vessel shows that although there was no change in β-actin or GAPDH, changes in Mn-dependent superoxide dismutase (MnSOD) and villin were within 20% of each other by gene array and RT-PCR (Fig. 3 *left*; Table 3). Villin is especially interesting as it decreased in the rotating wall vessel gene array analysis and increased in the space culture gene array. Although we had no residual human RNA, we were able to perform semi-quantitative RT-PCR on villin for rat kidney cells samples grown on the Mir Space Station (Fig. 3, *right*). Villin increased from 2.0 to 2.5 balanced differential expression on the gene array, and the RT-PCR confirms a modest increase, more if ratioed for changes in GAPDH.

Scanning electron microscopy assessed the ultrastructure of renal cortical cells of human or rat kidney origin. Compared with control ground-based cultures (Fig. 4*A*; bar = 1.0 μm), rat renal cells grown on the Mir Space Station for 21 days showed far more microvilli on larger aggregates (Fig. 4*B*; bar = 0.6 μm). Human cells grown in a rotating wall vessel for 6 days have previously been shown to have many microvilli (Fig. 4: *C*, bar = 2.5 μm; and *D*, bar = 1.25 μm), whereas human cells grown in bag controls (Fig. 4*E*; bar = 20 μm) or at 3 *g* in the short arm centrifuge have few microvilli (Fig. 4*F*; bar = 20 μm) at any magnification.

DISCUSSION

Suspension culture modulates mechanical culture conditions to induce tissue-specific expression of differentiated features (6, 17, 19). Our hypothesis that tissue differentiation depends on provision of three-dimensionality, a low turbulence and low shear environment, and cospatial relation of dissimilar cell types (6, 19) makes several testable predictions. These optimal culture conditions for tissue-specific change should be near perfect in the true microgravity of space flight. Ground-based rotating wall vessel culture optimizes these conditions on earth, but balancing forces (such as shear) changing the character of the culture must offset gravity. This predicts that a rotating wall vessel culture will show a different pattern of responses than a flight culture. Finally, as a negative control, cells grown in a centrifuge change none of these parameters and should show little tissue-specific change.

One of the difficulties of suspension culture studies has been finding defined outcomes to assay that give a valid index of the true extent and specificity of tissue changes in various culture conditions (6, 17). Recent advances in gene array analysis of gene expression provide a powerful new method to monitor genetic expression both on an overall basis, as well as on specific groups of genes such as transcription factors,

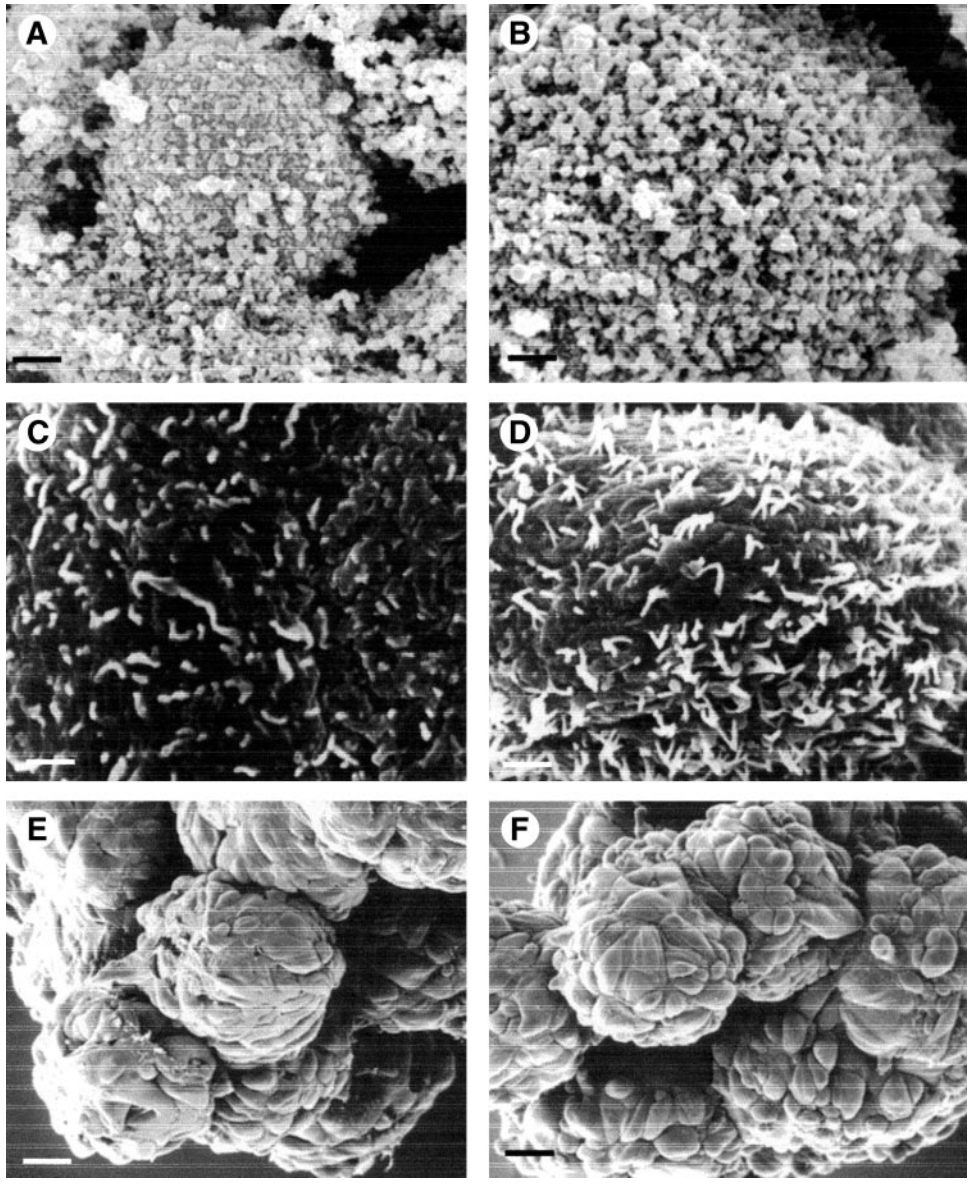

Fig. 4. Scanning electron micrographs of renal epithelial cells in culture (representative of multiple images from $n = 2$ samples). Scanning electron microscope images show some microvilli on rat renal cells in static bag culture (*A*; bar = 1.0 μm) but far more microvilli in the culture grown on Mir Space Station (*B*; bar = 0.6 μm). Human renal cells retain some microvilli in bag culture (*C*; bar = 2.5 μm) and many microvilli in rotating wall vessel culture (*D*; bar = 1.25 μm). Human renal cells have no observable microvilli either in static bags (*E*; bar = 20 μm) or the 3-*g* centrifuge (*F*; bar = 20 μm).

cytoskeletal proteins, and shear stress and heat shock proteins (11, 12). Hence, we applied state-of-the-art gene array analysis to assay gene expression in aliquots of human renal cortical cells grown in space flight, in a centrifuge, and in the rotating wall vessel, with each culture indexed to a parallel conventional culture grown in a nonadherent bag.

Gene array assay of the steady-state gene expression of human renal cortical cells after 6 days in microgravity culture on the space shuttle shows an amazing plethora of steady-state changes (Ref. 11 and **http://www.incyte.com**). Interestingly, there was scant steady-state change in known shear stress or heat shock proteins but large changes in select transcription factors such as the vitamin D receptor (5), which showed a large increase in microgravity, and the Wilms' zinc finger protein, which showed a large decrease in microgravity culture.

Gene array analysis allowed us to identify the transcription factors that undergo steady-state changes in expression (6, 11, 12). Although transcription factor translocation is the most frequently studied and cited example of transcriptional regulation (1, 6), steady-state changes in transcription factor gene expression give an index of some other likely nuclear regulatory mechanisms. The transcription factor undergoing the greatest upward change is the vitamin D receptor (5). This is a classic steroid receptor, resident on both

surface membranes and in the cytosol, becoming a translocatable transcription factor on binding the 1–25-dihydroxy active form of vitamin D_3. This extends our knowledge of the role of vitamin D in tissue differentiation. Initially identified for its role in calcium and phosphate metabolism, vitamin D has recently been recognized to have broad influence on immunity, cancer surveillance and the maintenance and development of tissue-specific cellular attributes (5). The transcription factor that decreased the most at steady state was the Wilms' zinc finger protein. This provides another line of evidence that this transcription factor plays a central role in renal cell proliferation and differentiation, from pediatric tumors to cell cycle regulation (1).

The steady-state changes in gene groups necessary to effect major structural and functional remodeling of the renal cell cultures during space culture are all quantitated in the microarray analysis (Table 1). The gene expression changes range from adhesion molecules, to apoptotic mediators, cytoskeletal proteins, differentiation markers, drug metabolism genes, intracellular signaling proteins, and many diverse receptor types, including transcription factors and electron transport chain elements. A select group of changes was confirmed by other methods. Given the plethora of posttranslational modifications and modulation of protein levels by changes in turnover rates, it is likely that a more extensive analysis would identify changes that fail to correlate as well. Microarrays continue to improve, with reconfirmation of the identity of clones on the array and quantitation of specificity and sensitivity (11, 12). None of these limitations detract from the observation that the rotating wall vessel gene array pattern and space flight are very different and show as many changes in reciprocal direction as the same direction: space provides unique mechanical culture conditions.

Spinning renal cell cultures in a centrifuge acted as a negative control for gravity-induced effects in our experiment (**http://ccf.arc.nasa.gov/dx/archives/life_sciences/artificial_gravity/centrifuge1.html**). Cultures were spun at 3 *g*, which for most cell types is just below the *g* force threshold to induce cell death through apoptotic and necrotic pathways. As predicted from the lack of change in three-dimensionality, shear, turbulence, or cospatial cellular relations, there was almost no measurable change in gene expression. Given the number of genes assayed and the potential for vibration, acceleration/deceleration, or other random errors, it is remarkable that so few changed. This is a powerful argument that the flight and rotating wall vessel samples report true physiological changes.

The important role shear stress can play in the induction of gene expression was initially documented in the remodeling of damaged blood vessel endothelium (1, 9, 10). The induction of gene changes in vascular endothelial cells establishes several important precedents for the current work. First, physical forces can induce changes in gene expression, not via a physical sensor, but by the changes they induce in the mechanical culture environment. Second, each induced gene has different kinetics of induction and return to baseline, many of which are measured in minutes or hours, not days. Our study at six days of culture reports steady-state changes only, but this has often been a successful approach to differential display technologies (6). Last, as shear stress increases during rotating wall vessel culture but not in centrifuge or flight cultures, it may play a role in the differences observed in gene expression patterns. Indeed, we have previously shown shear stress response elements to be active in renal epithelial cells (6). Rotating wall culture of renal cells changes expression of select gene products, including the giant glycoprotein scavenger receptors cubulin and megalin, the structural microvillar protein villin, and classic shear stress response genes ICAM, VCAM, and MnSOD (6). Using a putative endothelial cell shear stress response element-binding site as a decoy, we demonstrated the role of this sequence in the regulation of selected genes in renal epithelial cells (6).

This set of observations demonstrates several important principles of space science (6, 17, 19). First, although experiments are limited by current constraints on available flight equipment and crew time, state-of-the-art science is achievable on the space shuttle. This should be greatly improved with the facilities of the International Space Station, the first elements of which were launched this year. Studies of tissue-specific differentiation on the Space Station laboratory will act as a factory for state-of-the-art molecular analysis on mechanisms of gene expression in optimized suspension culture experiments. State-of-the-art space science is not only attainable, but most readily allows the study of genes regulated by the variables of cospatial interaction, three-dimensionality, and low shear/turbulence, which can be achieved in microgravity.

The current data are a dramatic demonstration that there are very large differences in gene expression at steady state in three different sets of mechanical culture conditions. The contribution of various factors and the mechanisms involved remain to be investigated. The current study established that there is a large dynamic range to dissect, so we can now make a systematic approach to optimizing the suspension culture parameters in the rotating wall vessel. Effects of rotating wall vessel culture have been compared with a plethora of control conditions, most commonly conventional two-dimensional cultures or gas-permeable nonadherence bags (17). Systematic comparisons of sets of rotating walls vessels run in parallel with a single parameter modified may be a more pragmatic and easily interpreted approach for the future.

Rotating wall vessel technology has already brought clinical benefits, as pancreatic islets are maintained differentiated in rotating wall vessels, maintaining specialized cellular features including production and regulation of insulin secretion (13). The intraperitoneal implantation of pancreatic islets grown in rotating wall vessels has maintained normoglycemia for 18 mo in diabetic patients and progressed to phase III clinical trials (13). Implants for other hormonal systems such

as erythropoietin and vitamin D are likely to be rapidly developed.

Suspension culture is used for industrial fermentation reactions (7), antibiotic production (8), interferon manufacture, and other bioproducts from hormones to hybridomas (7, 8, 14–16). If "space" biotechnology can be useful in the optimization of ground-based industrial and academic suspension cultures, then it should be vigorously pursued in a timely manner. If not, then biotechnology application of the academic and commercial use of space needs to be carefully reviewed and defined.

In conclusion, this suspension culture study shows that there is a group of genes responsive to cospatial cell relationships of mechanical culture conditions, including three-dimensionality and the degree of shear and turbulence in the culture environment.

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